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Perfil químico e bioprospecção de amêndoas de cacau analisadas por espectrometria de massas com ionização por *paper spray*

Chemical profile and bioprospecting of cocoa beans analyzed by paper spray mass spectrometry

Perfil químico y bioprospección de granos de cacao analizados por espectrometría de masas con ionización por paper spray

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Resumo

A qualidade dos produtos derivados do cacau tem cada vez mais recebido reconhecimento e relevância tanto pelos consumidores quanto produtores do fruto. As amêndoas são os principais componentes responsáveis por grande parte da cadeia agroindustrial sendo atualmente valorizadas pelas propriedades bioativas contidas nos subprodutos da espécie, gerando um grande interesse na exploração dos potenciais do cacau. Grande parte dos trabalhos que objetivam avaliar os compostos encontrados nas amêndoas do fruto empregam HPLC, UHPLC e LC-MS. Este trabalho objetivou empregar a técnica de Espectrometria de Massas com Ionização por *Paper Spray* (PS-MS) para o estudo do perfil químico e de bioprospecção de amêndoas de cacau (*Theobroma cacao*) da variedade forasteiro cultivados na região Transamazônica do Pará. Foram preparados extratos metanólicos de amostras de amêndoas de cacau e em seguida avaliadas nos modos de ionização negativo e positivo. Os resultados demonstram que no modo positivo foi possível identificar 11 compostos, constituintes das classes das metilxantinas (18,2%), fenilpropanoides (9,1%), esteróides (27,3%) e flavonóides (45,5%), já no modo de ionização negativa foi possível identificar 55 compostos entre ácidos hidroxibenzóicos (16,4%), fenilpropanoides (20,0%), flavonoides (52,7%), açúcares e glicosídeos (10,9%). A metodologia empregada de PS-MS se demonstrou eficaz para a avaliação das amostras, possibilitando identificar no total sessenta e seis compostos. As propriedades bioativas atribuídas ao cacau foram confirmadas nas amostras analisadas pelos compostos identificados por PS-MS indicando a qualidade da matéria prima e descrevendo o seu perfil químico e contribuindo para o maior entendimento dos seus atributos.

Palavras-chave: PS-MS; *Theobroma cacao*; Compostos bioativos; 20-Hidroxiecdisona; Clovamida.

Abstract

The quality cocoa derived products have increasingly received greater recognition and relevance both by consumers and producers. Cocoa beans are the main components responsible for much of the cocoa agro-industrial chain being currently valued for the bioactive properties found in the species' by-products, creating a great interest in exploring the potentials of cocoa. Much of the work that aims to evaluate the compounds found in the fruit's beans employ HPLC, UHPLC and LC-MS. In this work Paper Spray Mass Spectrometry (PS-MS) was employed as a method for characterizing and bioprospecting the chemical profile of cocoa beans (*Theobroma cacao*) of the *forrasteiro* variety grown in the Trans-Amazonian region of the Brazilian State of Pará. Methanolic extracts were prepared from samples of cocoa beans and evaluated in the negative and positive ionization modes. In the positive ionization mode it was possible to identify 11 compounds, comprising the classes of methylxanthines (18.2%), phenylpropanoids (9.1%), steroids (27.3%) and flavonoids (45.5%), while in the negative ionization mode, it was possible to identify 55 compounds among hydroxybenzoic acids (16.4%), phenylpropanoids (20.0%), flavonoids (52.7%), sugars and glycosides (10.9%). PS-MS proved to be an effective method for the evaluation of cocoa bean samples, being able to identify a total of sixty-six compounds. The bioactive properties attributed to cocoa were confirmed in the samples analyzed by the compounds identified through PS-MS whilst also indicating the quality of the raw material and describing its chemical profile, contributing to a greater understanding of its attributes.

Keywords: PS-MS; *Theobroma cacao*; Bioactive compounds; 20-Hydroxyecdysone; Clovamide.

Resumen

Productos derivados del cacao han recibido cada vez más reconocimiento y relevancia tanto por parte de los consumidores como de los productores. Los granos de cacao son los principales componentes responsables de gran parte de la cadena agroindustrial del cacao que actualmente se valora por las propiedades bioactivas que se encuentran en sus subproductos, lo que crea un gran interés en explorar los potenciales del cacao. Trabajos que objetivan evaluar los compuestos en semillas de la fruta emplean HPLC, UHPLC y LC-MS. Este trabajo empleó la espectrometría de masas por *paper spray* (PS-MS) como método para caracterizar y bioprospectar el perfil químico de granos de cacao (*Theobroma cacao*) de la variedad forrasteiro cultivada en el estado brasileño de Pará. Extractos metanólicos se prepararon con muestras de granos de cacao y se evaluaron en modos de ionización negativa y positiva. En el modo de ionización positiva, fue posible identificar 11 compuestos, que comprenden las clases metilxantinas (18.2%), fenilpropanoides (9.1%), esteroides (27.3%) y flavonoides (45.5%), mientras que en el modo de ionización negativa, fue posible identificar 55 compuestos entre ácidos hidroxibenzoicos (16.4%), fenilpropanoides (20.0%), flavonoides (52.7%), azúcares y glucósidos (10.9%). PS-MS demostró ser eficaz para la evaluación de muestras de granos de cacao, identificando un total de 66 compuestos. Las propiedades bioactivas atribuidas al cacao se confirmaron en muestras analizadas por los compuestos identificados a través de PS-MS al tiempo que indicaban la calidad de materia prima y describían su perfil químico, contribuyendo a una mayor comprensión de sus atributos.

Palabras clave: PS-MS; *Theobroma cacao*; Compuestos bioactivos; 20-Hidroxiecdisona; Clovamida.

1 Introduction

The diversity of bioactive properties associated with cocoa beans has been a major stimulus for the exploration of chemical compounds in the fruit, directly correlating in some instances with the value of its subproducts and derivates, as well as providing novel insights on possible applications for its byproducts (Bliss, 2017). Such properties are attributed to the wealth of molecules found in the chemical constitution of cocoa beans, comprising but not restricted to, polyphenols, methylxanthines, and proanthocyanidins, whose biological activity is associated with health benefits ranging from the prevention of cardiovascular diseases, cancers, as well as a potent anti-inflammatory, bactericidal, and antioxidant activity (Barnaba, Nardin, Pierotti, Malacarne, & Larcher, 2017; Calderón, Wright, Hurst, & Van Breemen, 2009; Gallego et al., 2019; Oracz, Nebesny, & Żyżelewicz, 2015; Sánchez-Rabaneda et al., 2003)

Extensive work seeking to evaluate the chemical profile of cocoa beans has been carried out using several techniques. High-performance liquid chromatography (HPLC) has been observed to be most commonly found in the literature when it comes to research regarding secondary metabolites and characterizing the profile different aspects of cocoa beans, pulp, and husk (D'Souza et al., 2017; Gallego et al., 2019; Patras, Milev, Vrancken, & Kuhnert, 2014; Sánchez-Rabaneda et al., 2003).

Among recent and innovative methodologies developed for chemical profile analysis assays, paper spray mass spectrometry (PS-MS), developed by Wang, Liu, Cooks, & Ouyang (2010), uses an ambient ionization technique that has been increasingly employed in the study of complex substances such as drugs, secondary metabolites and pesticide residue due to its high versatility, simplicity and low cost, which has progressively contributed to broaden its use in research (Evard, Kruve, Lõhmus, & Leito, 2015; A. Li, Wei, Hsu, & Cooks, 2013; Oliveira et al., 2020; Ramos et al., 2020; Taverna et al., 2016). Paper spray mass spectrometry has been shown to compensate for limitations associated with other methodologies, overcoming disadvantages by allowing fast, wide range, spectra acquisition with minimal requirements of sample preparation (Guo, Zhang, Yannell, Dong, & Cooks, 2017; Riboni et al., 2019; M. R. Silva et al., 2019).

In this context, this work employed PS-MS as a novel way of exploring cocoa beans and their chemical profile properties.

2 Experimental

2.1 Cocoa bean samples

Naturally fermented, sun-dried, organic cocoa beans of the *Forasteiro* group were obtained in collaboration with the Federal University of Pará and the *Cooperativa Central de Produção Orgânica da Transamazônica e Xingu*, (CEPOTX), an organization which manages the distribution and processing of organic cocoa in the northern region of Brazil.

The organic processing of cocoa beans followed methodologies described by Bertorelli et al. (2009) and Rubén et al. (2012). The experiment was carried out using samples from the 2019 harvest year.

2.2 Methanol extract

The extracts were prepared to employ an adaptation of the methodology described by Rufino et al. (2010).

The plant material was homogenized, after which 1.0 mL of methanol/water solution (50:50, v/v) was added to 0.5 g samples. These samples were stirred for 20 s in a vortex mixer, then kept for 1 h for incubation at room temperature.

After incubation, the samples were centrifuged for 15 min at 4 ° C and a 15,000 x g rotation. The supernatant was then transferred to a 15 mL Falcon tube, to which 1 mL of acetone/water solution (70:30, v/v) was added, followed by another centrifugation step.

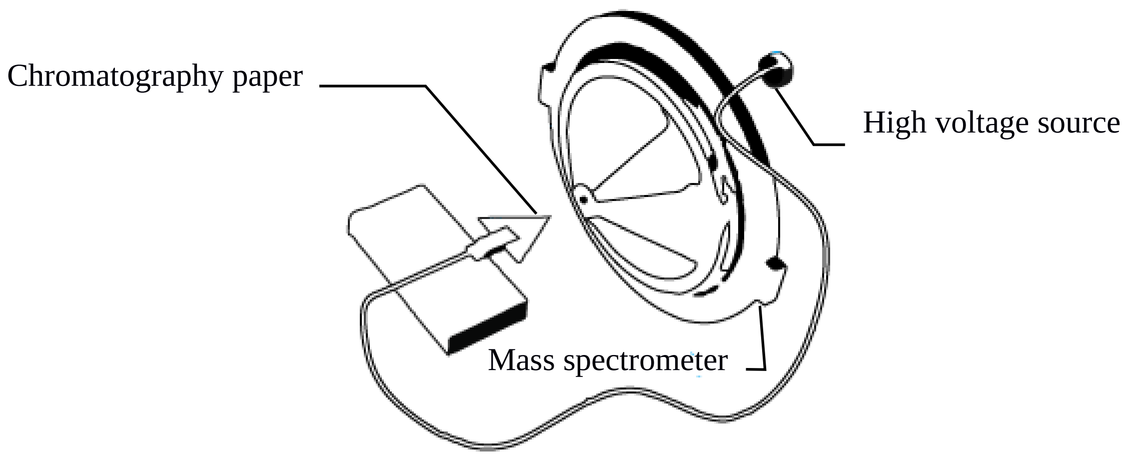
The supernatant from the second centrifugation was transferred to a 5 mL Eppendorf microtube, whose volume was completed with deionized water. The extracts were stored at low temperature and remained in this state until the analyzes were performed.

2.3 Paper spray mass spectrometry

The chemical profile of the samples was analyzed using an LCQ Fleet mass spectrometer (Thermo Scientific, San Jose, CA, USA) coupled to an ambient paper spray ionization source. The analyzes were performed in triplicate on both the negative and positive ionization modes following the methodology described by Silva et al. (2019).

Two µL of the prepared methanol extract and 40 µL of methanol solvent were applied to chromatography papers cut into a triangular shape coupled to the spectrometer. For data acquisition, the high voltage source was connected to the instrument using the following operating conditions: ionization source voltage of +4 kV on positive ionization mode and -3 kV on negative ionization mode; capillary voltage of 40 V; transfer tube temperature at 275 ° C; tube lens voltage of 120 V; mass range from 100 to 1000 m/z in the negative ionization mode and from 100 to 2000 m/z in the positive ionization mode.

Figure 1. Paper spray ionization source set up.



Source: Adapted from Teodoro et al. (2017).

2.4 Instrumental data interpretation

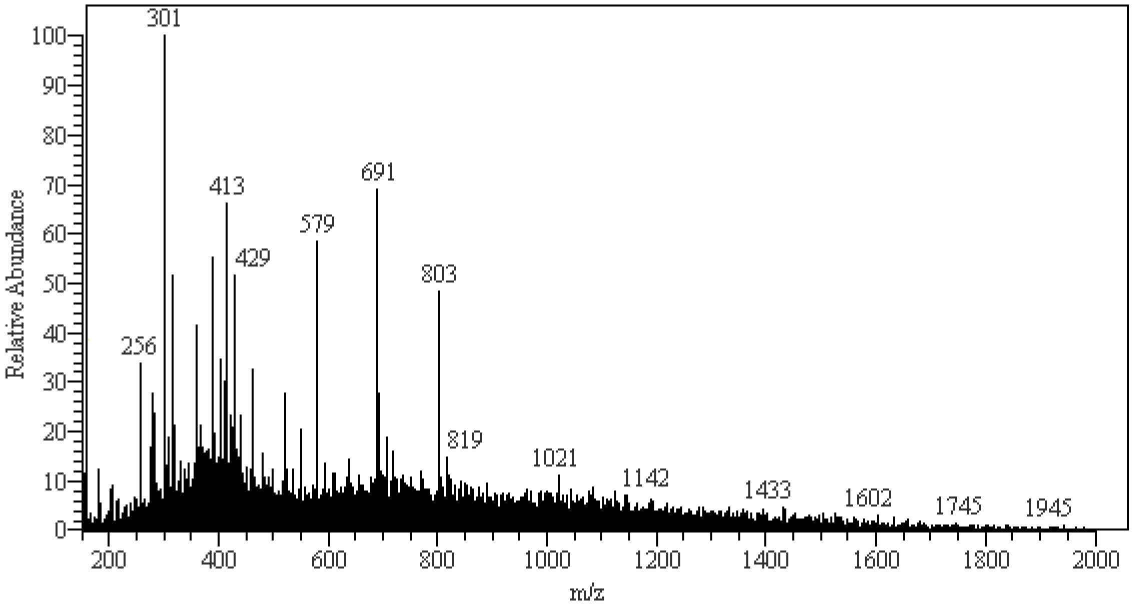
Tentative compounds identification from instrumental signals were made based on imported data from Thermo Scientific ™ FreeStyle ™ 1.6. Import files containing mass-to-charge ratios (m/z) were analyzed using automated spreadsheets developed in Microsoft® Excel® 2016 MSO which match input data with previously organized literature data.

3 Results and discussion

3.1 Positive ionization mode

Figure 2 shows the spectrum generated from samples of cocoa beans in the positive mode.

Figure 2. PS-MS spectrogram in the positive ionization mode of a cocoa bean sample.



Source: Authors (2020).

All identified ions on the positive ionization mode are shown in Table 1.

Table 1. PS(+)-MS analysis results.

N.	Compound	m/z	CAS	Reference
		Methylxanthines		
1	Theobromine	181	83-67-2	Ortega, 2008
2	Caffeine	195	58-08-2	Ortega, 2008
		Phenylpropanoids		
3	N-Feruloyltyramine	314	66648-43-9	Da Rosa, 2015
		Flavonoids		
4	Cyanidin 3-O-arabinoside	419	111613-04-8	Pereira-Caro, 2013
5	Cyanidin 3-O-galactoside	449	27214-71-7	Oracz, 2015
6	Quercetin	465	117-39-5	Türker, 2013

Table 1 (continued).

N.	Compound	m/z	CAS	Reference
7	Kaempferol-3-O-β-D-(600-E-p-cumaroyl)-	595	480-10-4	Da Rosa, 2015

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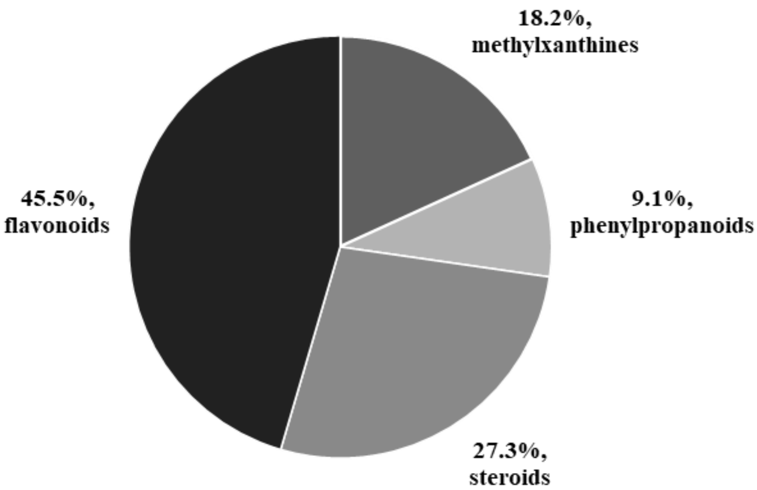
	glucopyranoside			
		Steroids		
9	20-Hydroxyecdysone	481	5289-74-7	Da Rosa, 2015
10	20-Hydroxyecdysone-3-O-β-D-xylose	613	–	Da Rosa, 2015
11	20-Hydroxyecdysone-3-O-β-D-glycopyranoside	643	–	Da Rosa, 2015

Source: Authors (2020).

Identification of compounds in the positive ionization mode distinguished four chemical classes, among flavonoids representing most of the compounds identified, followed by steroids, methylxanthines, and phenylpropanoids. The proportions of chemical classes found are shown in Figure 3.

Figure 3. The proportion of compound classes found in positive ionization mode analysis.



Source: Authors (2020).

3.1.1 Methylxanthines

Theobromine, identified by the mass-to-charge ratio of 181, is a metabolite of caffeine which according to Duke (1992) is commonly found in the beans and bark of cocoa fruit, mocambo fruit (*Theobroma bicolor*), and in the leaves of yerba mate (*Ilex paraguariensis*) and Indian tea (*Camellia sinensis*). Ortega et al. (2008) has previously described theobromine in cocoa beans using UPLC-MS/MS, whose biological activity ranges from a diuretic, stimulating, vasodilatory effects, as well as antioxidant and pro-oxidant activities, with recent studies pointing to theobromine as a cognitive modulator (Algharrawi, Summers, & Subramanian, 2017; Cova, Leta, Mariani, Pantoni, & Pomati, 2019; Stavric, 1988).

Caffeine (an ion with m/z 195) is another compound observed in previous analyzes using UPLC-MS/MS as one of the main methylxanthines found in cocoa beans (Ortega et al., 2008). According to Ágoston, Urbán, Rigó, Griffiths, & Demetrovics (2019) caffeine is the most widely consumed psychoactive natural stimulant in the world, with cognitive effects which reduce mental fatigue (burnout), reaction time improvement, higher alertness, and improvement in concentration and motor coordination (Snel & Lorist, 2011). Also used medicinally, caffeine is found in formulations that aim to prevent and treat lung problems, orthostatic hypotension, and apnea in premature newborns (Gibbons et al., 2017; Kugelman & Durand, 2011).

Theobromine and caffeine attest to the presence of methylxanthines in the cocoa samples, which are observed in the literature as ubiquitous compounds to the species, and largely contributing to the health-beneficial bioactive properties attributed to cocoa consumption, in particular of its fermented beans (Peláez, Guerra, & Contreras, 2016; Quelal-Vásconez et al., 2020).

3.1.2 Steroids

Steroid compounds such as 20-hydroxyecdysone, identified by the ion with m/z 481 and its metabolites with m/z 613, 20-hydroxyecdisone-3-O-β-D-xylose, and m/z 643, 20hydroxyecdisone-3-O-β-D-glycopyranoside, were previously found in the literature by Da Rosa et al. (2015) in samples of *guanxuma* (*Sida tuberculata* L., Malvaceae), this is the first description of these compounds in the composition of cocoa beans. Steroids are commonly present in plant species, such as 20-hydroxyecdysone, a phytoecdysteroid, found as the most significant compound in this class (Dinan, Mamadalieva, & Lafont, 2020).

Ecdysteroids or phytoecdysteroids are associated with the promotion of protein synthesis, maintenance of anabolic status, increase in muscle mass associated with the reduction of adipose tissue, also correlated with antidiabetic and antioxidant effects (Wilborn, 2006; Dinan et al., 2019).

In wheat seedlings (*Triticum aestivum*), Lamhamdi et al., (2016) described the effect of oxidative stress reduction caused by heavy metals, associated with the chelation of these elements and reduced absorption. Ajaha, Bouayad, Aarab, & Rharrabe (2019) observed the insecticidal effect of phytoecdysteroids, with potential for interference in molting processes, ecdysis, and alteration in the enzymatic activities of digestion and detoxification in coleoptera, demonstrating the versatility of the compounds found in cocoa beans, whose molecule diversity presents several possibilities for exploration.

3.1.3 Flavonoids

Flavonoids represent a group of polyphenolic compounds diverse in chemical structure and properties (Xiao et al., 2011). For plant species, more than nine thousand different compounds are described in this class of chemical compounds, with bioactivity including antioxidant protection and modulation of vascular homeostasis, as well as antidiabetic, anti-obesity and anticancer effects (Ballard & Maróstica, 2018; Steinberg, Bearden, & Keen, 2003).

Anthocyanidins observed in cocoa samples were identified by the ions of m/z 419 and 449, cyanidin 3-O-arabnoside and cyanidin 3-O-galactoside, also observed by Pereira-Caro et al. (2013) and Oracz, Zyzelewicz, & Nebesny (2015) in cocoa beans using the HPLC-MS/MS and RP-UHPLC-DAD/ESI-MS/MS respectively.

Cell culture studies, as well as animal models and clinical trials, demonstrate the antioxidant potential and antimicrobial activity of anthocyanidins and anthocyanins, also indicating benefits linked to neurological and visual health, as well as preventing the development of non-communicable diseases such as cancers and hyperglycemia (Khoo, Azlan, Tang, & Lim, 2017; Yousuf, Gul, Wani, & Singh, 2016). These effects are mainly attributed to the antioxidant property of these compounds associated with the cyclooxygenases and free radical elimination metabolic pathways, the mitogen-activated protein kinase metabolic pathway, and the inflammatory cytokine signaling system. (Chen et al., 2015; Roth et al., 2014; Saint-Cricq De Gaulejac, Glories, & Vivas, 1999; Seeram, Momin, Nair, & Bourquin, 2001; Tebayashi, Ishihara, Tsuda, & Iwamura, 2000).

The peaks with m/z 465 and 611 can be attributed to the flavonols quercetin, and the glycoside rutin (quercetin-3-O-rutinoside), respectively. Türker & Dalar (2013) identified these compounds in *Malva neglecta* Wallr., another species of the Malvaceae family, using HPLC-DAD, noting the antioxidant and inhibitory activity of enzymes of the phenolic compounds of cocoa *in vitro*. Quercetin is a flavonol with a wide distribution in nature, being one of the most abundant flavonoids in the human diet (Murakami, Ashida, & Terao, 2008).

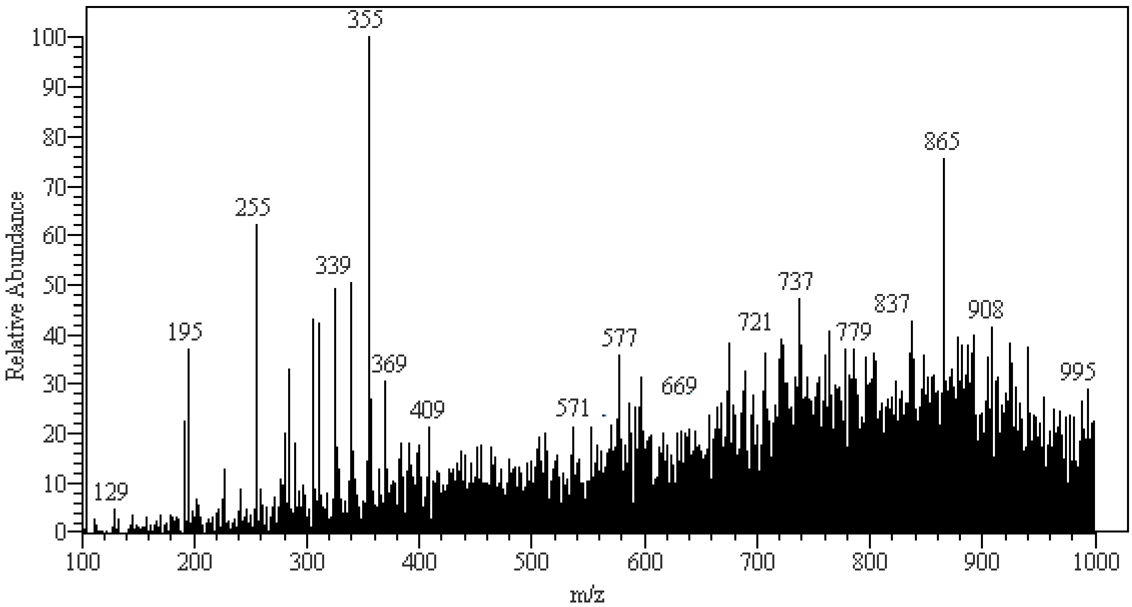
Quercetin is cited with the ability to inhibit the oxidation of other molecules due to its polyphenolic chemical substructure which prevents oxidation by capturing free radicals responsible for starting oxidative reaction chains. (Russo et al., 2014; Gupta et al., 2016).

Rutin, formed by the combination of quercetin and rutinose, has been explored for potential bioactive properties for the treatment and prevention of conditions related to the cardiovascular system such as reduction of post-thrombotic syndrome, venous insufficiency, and endothelial dysfunction, which values the byproducts of cocoa beans as sources of health-beneficial cardiovascular molecules (Martinez-Zapata et al., 2016; Morling, Yeoh, & Kolbach, 2015).

3.2 Negative ionization mode

Fifty-five distinct compounds were identified in the negative ionization mode, comprising the chemical classes of hydroxybenzoic acids, phenylpropanoids, flavonoids, among other compounds such as sugars, organic acids, and glycosides. An example of PS(-)-MS spectrum of cocoa samples analysis is shown in Figure 4.

Figure 4. PS-MS spectrogram in the negative ionization mode of a cocoa bean sample.



Source: Authors (2020).

Table 2 lists all identified molecules in the negative ionization mode.

Table 2. PS(-)-MS analysis results.

N.	Compound	m/z	CAS	Reference
		Hydroxybenzoic acids		
1	Hydroxybenzoic acid	137	99-96-7	Ortega, 2008
2	Vanillin	151	121-33-5	Ortega, 2008
3	Protocatechuic acid	153	99-50-3	Sánchez-Rabaneda, 2003
4	Vanillic acid	167	121-34-6	Ortega, 2008
5	Gallic acid	169	149-91-7	Sánchez-Rabaneda, 2003
6	Syringic acid	197	530-57-4	Ortega, 2008
		Phenylpropanoids		
7	Cinnamic acid	147	140-10-3	Ortega, 2008
8	Coumaric acid	163	501-98-4	Sánchez-Rabaneda, 2003
9	Caffeic acid	179	331-39-5	Sánchez-Rabaneda, 2003
10	Ferulic acid	193	1135-24-6	Sánchez-Rabaneda, 2003
11	N-coumaroyl-L-aspartate	278	–	Pereira-Caro, 2013
12	p-Coumaroyl glutamine	291	–	D'Souza, 2017
13	N-caffeoyl-L-aspartate	294	–	Pereira-Caro, 2013
14	Hydroxybenzoic acid-O-hexoside	299	–	Türker, 2013
15	Chlorogenic acid	353	327-97-9	Türker, 2013
16	Caffeoyl tyrosine	342	124027-56-1	D'Souza, 2017
17	Clovamide	358	53755-03-6	Barros, 2016
		Flavonoids		
18	Apigenin	269	520-36-5	Sánchez-Rabaneda, 2003
19	Naringenin	271	67604-48-2	Sánchez-Rabaneda, 2003
20	Luteolin	285	491-70-3	Sánchez-Rabaneda, 2003
21	Dihydroxykaempferol	287	–	Ortega, 2008
22	Catechin	289	154-23-4	Sánchez-Rabaneda, 2003
23	Quercetin	301	117-39-5	Ortega, 2008
24	Taxifolin	303	480-18-2	Ortega, 2008

Table 2 (continued).

N.	**Compound**	**m/z**	**CAS**	**Reference**
25	Epigallocatechin	305	970-74-1	Ortega, 2008
26	Isorhamnetin	315	480-19-3	Sánchez-Rabaneda, 2003
27	Deoxyclovamide	326	–	Cádiz-Guerrea, 2014
28	Vitexin	431	3681-93-4	Sánchez-Rabaneda, 2003
29	Prunin	433	529-55-5	Sánchez-Rabaneda, 2003
30	Orientin	447	28608-75-5	Sánchez-Rabaneda, 2003
31	Kaempferol-3-O-hexoside	447	–	Sánchez-Rabaneda, 2003
32	Catechin-3-O-glucoside	451	–	Patras, 2014
33	isoscutellarein 8-O-β-D-glucuronide	461	–	Barros, 2016
34	Hyperoside	463	482-36-0	Sánchez-Rabaneda, 2003
35	Hypolaetin 8-O-β-D-glucuronide	477	–	Barros, 2016
36	Myricetin-glucoside	479	–	Ortega, 2008
37	Hypolaetin 3″-methyl ether 8-O-β-D-glucuronide	491	–	Pugliese, 2013
38	Amentoflavone	537	1617-53-4	Sánchez-Rabaneda, 2003
39	Isoscutellarein 8-O-β-D-glucuronide 3″-O-sulfate	541	–	Pugliese, 2013
40	Hypolaetin 8-O-β-D-glucuronide 3″-O-sulfate	557	–	Pugliese, 2013
41	Hypolaetin 3′-methyl ether 8-O-β-D-glucuronide 3″-O-sulfate	571	–	Pugliese, 2013
42	Isorhoifolin	577	552-57-8	Sánchez-Rabaneda, 2003
43	Naringin	579	10236-47-2	Sánchez-Rabaneda, 2003
44	Proanthocyanidin	591	18206-61-6	Cádiz-Guerrea, 2014
45	Nicotiflorin	593	17650-84-9	Sánchez-Rabaneda, 2003

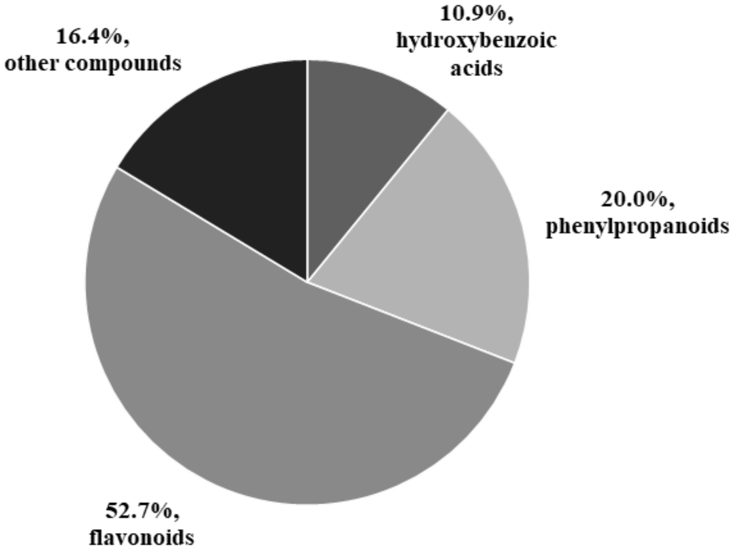
Table 2 (continued).

N.	Compound	m/z	CAS	Reference
46	Rutin	609	153-18-4	Sánchez-Rabaneda, 2003
	Other compounds			
47	Citric acid	191	77-92-9	D'Souza, 2017
48	Prolylhydroxyproline	227	–	D'Souza, 2017
49	Isopropyl citrate	233	39413-05-3	D'Souza, 2017
50	Leucine hexoside	292	–	D'Souza, 2017
51	L-aspartic acid	308	56-84-8	Cádiz-Guerrea, 2014
52	Sucrose	341	57-50-1	Cádiz-Guerrea, 2014
53	Sweroside	357	14215-86-2	Cádiz-Guerrea, 2014
54	Cis-3-hexenyl β-primeveroside	393	132278-37-6	Cádiz-Guerrea, 2014
55	Octen-3-yl primaveroside	421	209863-01-4	Patras, 2014

Source: Authors (2020).

The proportional representation of each chemical class composed of the identified compounds is illustrated in Figure 5.

Figure 5. The proportion of compound classes found in negative ionization mode analysis.



Source: Authors (2020).

3.2.1 Hydroxybenzoic acids

Hydroxybenzoic acid, identified by the ion with m/z 137, is a compound associated with salicylic acid and salicin, one the first compounds isolated with pharmacological activity (Norn et al., 2009). The bioactive potential of molecules derived from this compound includes the prevention and treatment of cardiovascular conditions such as hypertension, atherosclerosis, and dyslipidemia (Juurlink, Azouz, Aldalati, Altinawi, & Ganguly, 2014). Ortega et al. (2008) recorded the presence of this class of compounds in cocoa beans using UPLC-MS/MS in their work on the characterization of phenolic extracts from the cocoa of different sources.

Derivatives of hydroxybenzoic acid such as vanillic, syringic and protocatechuic acids are constituents of lignin, and therefore universal in angiosperm species. The origin of these compounds can be associated with the metabolic degradation of certain lignified regions of the fruit, such as tegument and husk (Clifford & Scalbert, 2000). The peak with m/z 153 is attributed to protocatechuic acid, whose presence was first observed in cocoa by Sánchez-Rabaneda et al., (2003) using LC-MS/MS. Winter et al. (2017) cite in their work neuroprotective and antioxidant effects in cell cultures associated with protocatechuic acid, anti-inflammatory properties and attenuation of amyloid deposits associated with the improvement of cognitive deficits are also observed in other researches (Song et al., 2014; Tsai & Yin, 2012).

Vanillin, identified by the peak with m/z 151, is a phenolic compound widely explored in the literature for its anti-inflammatory properties, recent studies explore its ability to reduce oxidative stress by deactivating molecules linked to various conditions such as neurodegenerative diseases, cardiovascular diseases, cancer and immune disorder (Scopioni et al., 2018). Silva et al., (2020) describe the chelating and cytoprotective potential of vanillin against the toxic activity of heavy metals in pro- and eukaryotic models as a complement to phytoremediation methodologies, pointing out, in addition to the physiological benefits, the plurivalent potential of these compounds in other systems.

3.2.2 Phenylpropanoids

Phenylpropanoids are secondary metabolites found in several plant species with biological antioxidant, anti-inflammatory, antiviral and antimicrobial activities (Gálvez, Martín-Cordero, & Ayuso, 2005; Kim, Seong, & Youn, 2011; Yazaki, Sasaki, & Tsurumaru, 2009). Lu et al. (2012) also propose anti-retroviral and cytotoxic potential for neoplastic cells activities of molecules within this class.

Clovamide can be attributed to the peak m/z 358, previously identified in cupuassu by Barros, García-Villalba, Tomás-Barberán, & Genovese (2016) employing UPLC-ESI-QTOF. The compound is generally found in small quantities in cocoa beans, it is most commonly found in species of the genus *Trifolium*, more specifically the red clover (*Trifolium pratense* L.) (Caballero et al., 2015; Tebayashi et al., 2000). Fallarini et al. (2009) describe in vitro tests that present the neuroprotective activity of clovamide while protecting neurons against injuries caused by oxidative stress, Ca^{2+} overload, and expression of the c-fos oncogene.

The peak with m/z 353 identifies chlorogenic acid, the most abundant compound among the isomers of caffeoylquinic acid. It is one of the most readily available acids among the phenolic compounds found naturally in coffee and tea extracts (Meng, Cao, Feng, Peng, & Hu, 2013; Venditti et al., 2015). Studies demonstrate its biological activity involving anti-inflammatory and antioxidant properties, with evidence pointing to the interference in the metabolism of lipids and glucose in hereditary metabolic disorders, acting in hepatoprotection, also inhibiting the concentration of glucose in the blood (Naveed et al., 2018; Wan et al., 2013; Yuan et al., 2017). Chlorogenic acid has been described by Türker & Dalar, (2013) in *Malva neglecta*, using HPLC-DAD while analyzing extracts of fruits of the species. In cocoa, this is the first register of the compound in the species' beans.

The peaks with m/z 179 and m/z 193 identify caffeic and ferulic acids, respectively, previously observed in cocoa bean extracts by Sánchez-Rabaneda et al. (2003) using LC/MS/MS. These acids are metabolites of chlorogenic acid, presenting positive impacts in the treatment of hypotension (Onakpoya, Spencer, Thompson, & Heneghan, 2015). The therapeutic effect of ferulic acid is mainly due to the anti-inflammatory and antioxidant properties, exhibiting biological activities such as anticarcinogenic, anti-apoptosis, and anti-diabetes, as well as cardioprotective and neuroprotective properties (Ghosh, Basak, Dutta, Chowdhury, & Sil, 2017).

3.2.3 Flavonoids

Flavonoids present with varying structures between plant species, being found in cocoa as epicatechin (proanthocyanidin) monomers and oligomers, with strong evidence for its potential effects of reducing risk factors for development and cardiovascular diseases (Perez-Vizcaino & Fraga, 2018). Proanthocyanidin can be identified by the ion with m/z 591, also described by Cádiz-Gurrea et al. (2014) in cocoa extracts using HPLC-MSESI-QTOF, the compound has characteristics that promote health benefits mainly due to its antioxidant capacity (Odai, Terauchi, Kato, Hirose, & Miyasaka, 2019). The reduction of oxidative stress promoted by proanthocyanidin, for instance, is linked to reducing the impact of diabetes on the quality of life of patients with the disease (Ding et al., 2013).

As with proanthocyanidin, the peak with m/z 591 is identified as naringin, presenting a significant antioxidant potential, with studies pointing to its effectiveness in reducing DNA damage induced by hydrogen peroxide, this characteristic is also associated with reducing the risk of developing cancer due to its genoprotective effect (Bacanli, Başaran, & Başaran, 2015). Hepatoprotective properties are also known, specifically acting in protecting liver enzymes and subsequently preventing liver damage (Szczepaniak et al., 2020). The compound is already registered in the literature as occurring in cocoa by Sánchez-Rabaneda et al. (2003), identifying its presence using LC-MS/MS.

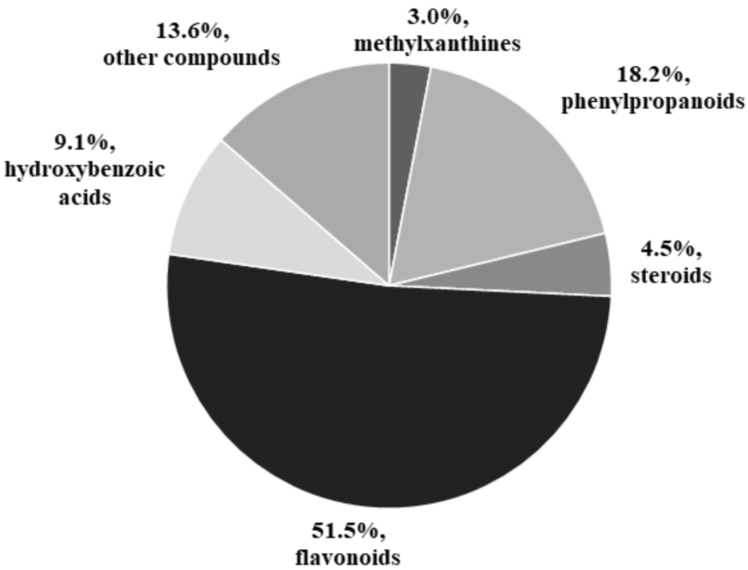
Nicotylflorine, attributed to the peak with m/z 593, is a glycoside commonly associated with neurological benefits. The compound has a protective potential for the nervous system, demonstrating significant improvement in cases of memory dysfunction, metabolic failure and oxidative stress associated with dementia related to stroke damage (Huang et al., 2007). According to Li, Guo, Zhang, Xu, & Li (2006) nicotylflorine reduces ischemic damage to the brain, improving protein synthesis in the blood vessel endothelium thus accelerating the recovery of endothelial cells. Orientin, identified by the peak with m/z 447, is another glycoside associated with neurological benefits, with effects of attenuation of damage caused by ischemia and reperfusion by acting on metabolic signaling pathways, also presenting neuroprotective mechanisms which prevent oxidative damage caused by reactive species in nerve cells (Lam et al., 2018; X. Wang, An, Wang, An, & Wang, 2017)

The ion with m/z 303 is identified as taxifolin, a flavonol commonly used in commercial products due to its biological activities and related molecular mechanisms. Taxifolin has anti-tumor, antioxidant, and cardiovascular protective effects, among these effects it's antineoplastic activity is one of the most noteworthy studied properties (Sunil & Xu, 2019).

3.3 Overview

PS-MS was able to identify sixty-six total compounds when combining the analyzes in positive and negative ionization modes, with compounds belonging to multiple chemical classes and several bioactive properties. The total composition of the chemical profile of the samples of cocoa beans analyzed is shown in Figure 6.

Figure 6. The proportion of all compounds found in the analyzes performed using paper spray mass spectrometry.



Source: Authors (2020).

4 Conclusion

By employing PS-MS, this study contributed to a better understanding of the complex chemical make-up of cocoa beans, further advancing the knowledge surrounding its bioactive compounds which present, among other effects, antioxidant, anti-inflammatory, neuroprotective and stimulating effects, providing a vast prospective analysis for future pharmacognosy research.

Paper spray mass spectrometry proved to be a powerful tool for the characterization of the profile and bioprospection of cocoa beans, is a relatively a simple, fast and extremely efficient technique for determining chemical profiles, it provided the identification of a large range of molecules distributed among several classes with distinct and synergistic properties.

The chemical profile of cocoa beans comprises mainly flavonoids, phenylpropanoids, hydroxybenzoic acids, steroids, methylxanthines, sugars, organic acids, and glycosides. Bioactive properties attributed to the consumption of cocoa are consistent with the chemical compound properties found in this work which characterize the profile of cocoa beans.

In addition to compounds cited in the literature, within the steroids, this work registered 20-hydroxyecdysterone for the first time in *Theobroma cacao* as well as clovamide, a bioactive phenylpropanoid of little literature exploration for this genus.

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