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ORIGINAL ARTICLE

Analysis of phenolic compounds from cowpea (*Vigna unguiculata*) by HPLC-DAD-MS/MS[1]

Análise de compostos fenólicos presentes em feijão-caupi (Vigna unguiulata) por HPLC-DAD/MS/MS

Reinaldo A. Santos[1], Antonio Pedro S. Souza Filho[2], Antonio J. Cantanhede Filho[3]*, Giselle M. S. P. Guilhon[1], Lourivaldo S. Santos[1]

[1]Universidade Federal do Pará (UFPA), Programa de Pós-graduação em Química, Campus Universitário do Guamá, Belém/PA - Brasil
[2]Embrapa Amazônia Oriental, Laboratório de Agroindústria, Belém/PA - Brasil
[3]Instituto Federal de Educação Ciência do Maranhão (IFMA), Departamento Acadêmico de Química, São Luís/MA - Brasil

***Corresponding Author:** Antonio J. Cantanhede Filho, Instituto Federal de Educação Ciência do Maranhão (IFMA), Departamento Acadêmico de Química, Av. Getulio Vargas, 04, Monte Castelo, CEP: 65025-001, São Luís/MA - Brasil, e-mail: prof.antoniofilho@ifma.edu.br

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Abstract

Vigna unguiculata (L.) Walp (cowpea), Fabaceae family and also known as Leguminosae, is an important vegetable used as food in tropical regions, especially in Africa, South America and Asia countries. Phenolic compounds are associated with important biological properties and their occurrence in edible plants may result in a highly functional food. Chromatographic profiles of phenolic compounds were investigated in two cowpea cultivars, such as tracuateua (CT) and caldeirão (CC), and both were cultivated using High Performance Liquid Chromatography (HPLC) coupled to Mass Spectrometry (MS) (HPLC-DAD/MS/MS). The flavonols quercetin and kaempferol, the phenolic acids, *p*-coumaric and protocatechuic acid (PCA) were identified in cowpea (CC), while the phenolic acids, gallic and protocatechuic acids, were identified in the cowpea (CT). These phenolic compounds ratify cowpea as a functional and bioactive food, ensuring a healthy diet.

Keywords: Phenolic compounds; Beans, cowpea; *Vigna unguiculata*; Chromatography; Mass Spectrometry; Crude extract.

Resumo

Vigna unguiculata (L.) Walp (feijão-caupi), pertencente à família Fabaceae, é um dos mais importantes vegetais utilizados como alimento em regiões de clima tropical, especialmente em países da África, América do Sul e Ásia. Compostos fenólicos estão associados a importantes propriedades biológicas, e sua ocorrência em plantas

[1]Chemical compounds studied in this article: Quercetin (PubChem CID: 5280343); Kaempferol (PubChem CID: 5280863); *p*-Coumaric acid (PubChem CID: 637542); Gallic acid (PubChem CID: 46780424); Protocatechuic acid (PCA) (PubChem CID: 72).

comestíveis pode implicar em um alimento altamente funcional. Foi investigada a presença de compostos fenólicos nos extratos brutos metanólicos de duas cultivares de feijão-caupi, cultivar tracuateua (CT) e cultivar caldeirão (CC), utilizando a técnica HPLC-DAD/MS/MS. Os flavonóis quercetina e canferol, e os ácidos fenólicos *p*-cumárico e protocatecuico foram identificados no feijão-caupi (CC), enquanto no feijão-caupi (CT) foram identificados ácidos fenólicos gálico e protocatecuico. Esses fenólicos ratificam o feijão-caupi como um alimento funcional e bioativo, garantindo uma dieta saudável.

Palavras-chave: Compostos fenólicos; Feijão-caupi; *Vigna unguiculata*; Cromatografia; Espectroscopia de Massas; Extrato bruto.

1 Introduction

Vigna unguiculata (L.) Walp, included in the Fabaceae family, is known as cowpea and considered one of the most important vegetables used as food in tropical regions, especially in Africa, South America and Asia countries. Its world average production estimated from 2007 to 2012 was associated to 5.6 million ton and considering some countries, the major producers are related to Nigeria, Niger, Burkina Faso, Myanmar, Tanzania and Cameroon (Food and Agriculture Organization of the United Nations, 2014).

Cowpea has a great nutritional value as it has high amounts of proteins and minerals, as well as it is also associated with a healthy diet due to the occurrence of bioactive chemical compounds, such as the phenolic ones, which may contribute to the prevention of various illnesses like diabetes, cancer and cardiovascular disease. Vegetables such as cowpea are rich in fiber and slow digestion carbohydrates that produce desirable effects on the glycemic profile and promote gastrointestinal health (Ojwang et al., 2013).

Phytochemicals studies performed in grain and seed coats from several varieties of cowpea revealed chemical profiles of phenolic compounds as flavonoids and phenolic acids, not only related to their free forms but also to glycosylated forms (Dueñas et al., 2005; Gutiérrez-Uribe et al., 2011; Ojwang et al., 2012, 2013), which according to the authors, these compounds are associated with various biological activities, especially as antioxidant and anti-inflammatory agents.

This study aimed to investigate the chromatographic profile of phenolic compounds by High Performance Liquid Chromatography coupled (HPLC) to a Mass Spectrometry (MS) (HPLC-DAD-MS/MS), of the Crude Methanol Extracts (CME) of two cowpea cultivars, tracuateua (CT) and caldeirão (CC), that were cultivated in order to verify some differences in the composition of phenolic compounds of these two cultivars, as well as seeking information on these chemical compounds in cowpea cultivars planted in Brazil and ratifying the importance of their usage as a basis for a rich diet in chemical compounds.

2 Materials and methods

2.1 Collection of botanical material and preparation of crude extracts

The botanic material was collected in the state of Acre (AC) in Brazil, from Firm Ground (FG). The two cowpea cultivars of *V. unguiculata* (L.) Walp, cowpea CT (prostrate stem) and cowpea CC (erect stem), were identified by the botanists from Embrapa Amazônia Oriental.

The botanical material (aerial parts) was dried in an oven with forced air circulation at 42 °C and subsequently ground using knife mill. The 2 kg of the dried and milled botanical materials from each cultivar were extracted by maceration in cold organic solvents in a sequence extracted by hexane,

ethyl acetate and methanol. Each step of the extraction lasted 48 hours. After filtration through filter paper, the solvents were evaporated in a rotary vacuum evaporator, providing hexane, ethyl acetate and methanol crude extracts.

To obtain fractions without impurities and with a higher concentration of phenolic compounds, CME (10 g) of *caldeirão* (CC) cultivar was fractionated in silica-gel column chromatography, and fractions collected as following: hexane (aliquots 1-3); hexane/ethyl acetate 50% (aliquots 4-11); ethyl acetate (aliquots 12-20); ethyl acetate/methanol 25% (aliquots 21-32); ethyl acetate/methanol 50% (aliquots of 33-35); and methanol (aliquots 36-40). The CME of tracuateua (TC) cultivar have not been fractionated.

2.2 Chromatographic profiles of phenolic compounds by HPLC-DAD-MS/MS

Phenolic composition (chromatographic profiles) of the CME of the two cowpea cultivars was investigated, likewise the fractions 14 and 17 that were separated from the silica-gel column chromatography of cowpea CC CME.

The analysis of phenolic compounds of the CME was performed using a HPLC, and also a Thermo mark with autosampler, consisting of a sampling loop 20 µL and quaternary pump, coupled with a Diode Array Detector (DAD) and a mass spectrometer equipped with electrospray ionization source (ESI) and mass analyzer Ion-Trap. The equipment was operated at 25 ± 2 °C ambient temperature and chromatographic data were obtained and processed by the Xcalibur software. The chromatographic column used was a type of reverse phase column C18 (150 × 2.1 mm), and 1.9 µm particle size (Thermo, Hypersil Gold). The chromatographic method was based on Nováková et al. (2010), with minor modifications. The mobile phase consisted of acidified water (formic acid) 0.1% (A) and acetonitrile (B). The gradient elution started at 95:5 ratio, and at 0.32 mL min^{-1} flow. The "A" concentration decreased until reaching 5 min under 50:50 condition, and after 6 minutes it gradually returned to the initial condition 95:5 in 20 min and remained in this condition for 2 min so that the initial conditions could be restored. Detection of phenolic compounds was performed through the aid of the DAD (operating at 210, 260, 300 and 325 nm) and a mass spectrometer with an ESI operating in negative mode (the capillary temperature: 350 °C, capillary voltage: 2.5 kV, cone voltage of 5 kV). Helium gas (He) was used as the collision gas and nitrogen (N_2) was used as nebulizer gas 70 (arbitrary unit).

Reference standards of phenolic compounds used in this study were purchased from Sigma-Aldrich (Prague, Czech Republic) and consisted of: gallic acid; chlorogenic acid; 4-hydroxybenzoic acid; protocatechuic acid (PCA); catechin; epicatechin; syringic acid; vanillic acid; cinnamic acid; *p*-coumaric acid; ferulic acid; rutin; myricetin; quercetin; and kaempferol.

Retention time was used in order to identify the compounds present in the samples, the absorption spectra and co-chromatography compared to the standards and mass spectral analysis, which assisted in confirming the chemical structure of the compounds by comparing with molecular ion (*m/z*) and fragments of each compound with the respective standards.

3 Results and discussion

By using the HPLC-DAD-MS/MS system, it was possible to identify the profile of five phenolic compounds, as following: two flavonoids (quercetin and kaempferol); and three phenolic acids (*p*-coumaric acid, PCA and gallic acid) (Figure 1).

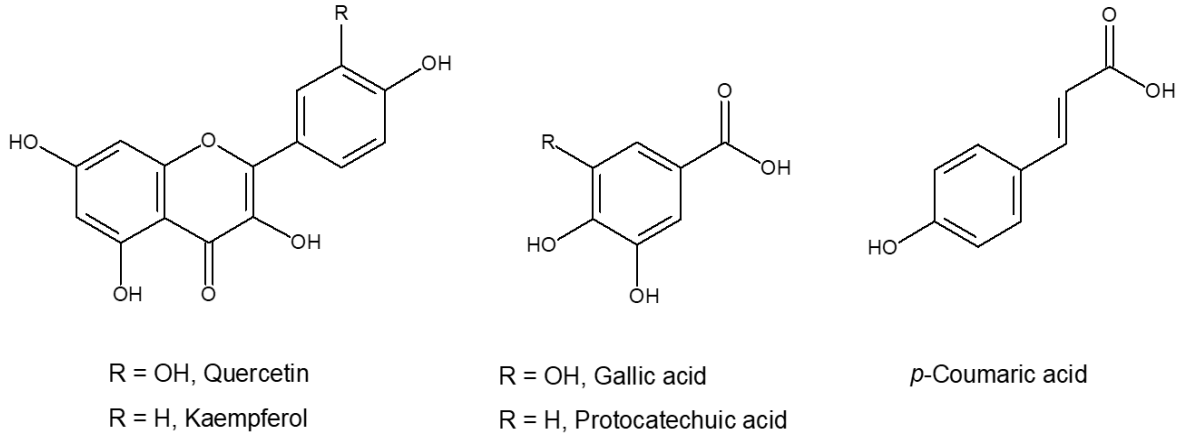

Figure 1. Phenolic compounds identified in cowpea cultivars.

3.1 Identification of phenolic compounds by HPLC-DAD/MS-MS

In Table 1, it is listed the compounds identified in the CME and fractions of cowpea CC and CT.

Table 1. Phenolic compounds identified in Crude Methanol Extracts (CME) of cowpea CC and CT based on mass spectrometry (MS) and fragments (MS^2).

Amount	Substance	$[M-H]^-$ *m/z*	MS^2 *m/z*
Cowpea CT	Gallic acid	[169]	[169] 125
	Protocatechuic acid	[153]	[153] 109
Cowpea CC	Quercetin	[301]	[301] 273, 193, 179, 151
Fraction 14	*p*-Coumaric acid	[163]	[163] 119
Fraction 17	Quercetin	[301]	[301] 273 257 179 151
	Protocatechuic acid	[153]	[153] 109
	Kaempferol	[285]	[285] 257 217 199 175 151

With respect to cowpea CC, the chromatogram (Figure 2a) showed many overlapping peaks, however, it was possible to identify the flavonol quercetin that was identified based on the peak with retention time of 16.51 min, whereas the mass spectrum corresponding to this peak exhibited the mass of its deprotonated molecular ion at *m/z* 301 $[M - H]^-$, in addition to the fragments at *m/z* 273, *m/z* 193, *m/z* 179 and *m/z* 151 which were compared with the pattern and literature data (Fabre et al., 2001), and then it could be confirmed the presence of flavonol quercetin. This phenolic compound has also been identified in other beans species, such as *Phaseolus vulgaris* L. and *Vigna faba* L. (Šibul et al., 2016).

The chromatogram of the cowpea CT (CME) (Figure 2a) was quite similar to that shown in Figure 2b, indicating that both extracts had similarity in chemical compounds, in which gallic acid and PCA were identified. The identification (Figure 3) of gallic acid was performed based on the chromatogram peak with retention time of 4.26 min, whereas the mass spectrum corresponding to this peak resulted in a corresponding deprotonated molecular ion at *m/z* 169 $[M - H]^-$ mass and at *m/z* 125 fragment. For PCA, it could be observed in the chromatogram a peak with retention time of 4.33 min, which exhibited a deprotonated molecular ion peak at *m/z* 153 $[M - H]^-$ and the fragment at *m/z* 109. These data compared to the patterns could confirm the occurrence of these phenolic acids. These phenolic acids are also common to other populations of beans, such as in *P. vulgaris* L., *V. faba* L. and *C. gladiata* (Chen et al., 2015; Gan et al., 2016; Šibul et al., 2016).

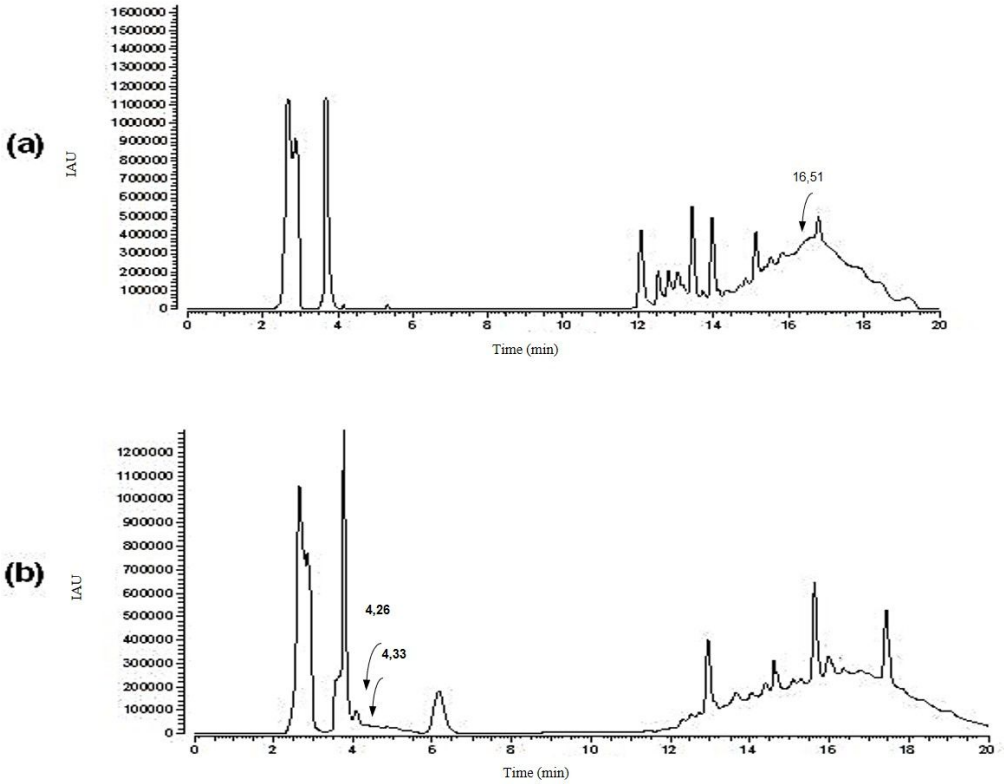

Figure 2. Chromatograms of cowpea CC **(a)** and CT **(b)**. Crude Methanol extracts (CME).

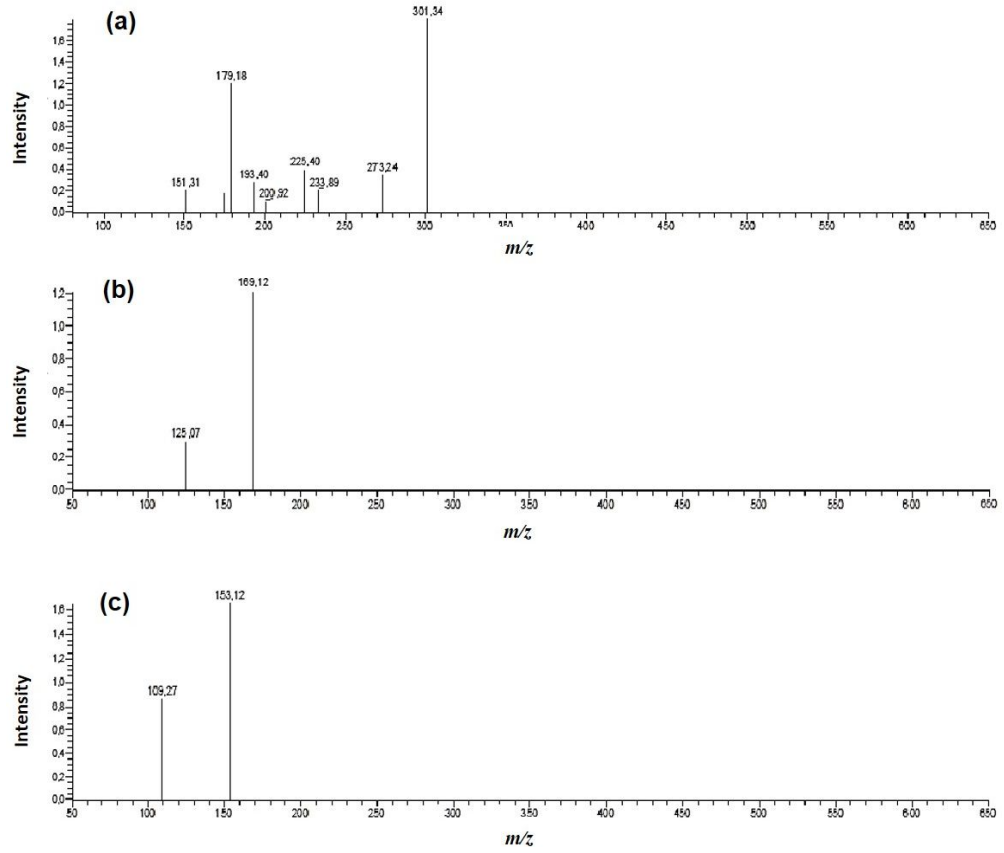

Figure 3. Mass spectra with molecular ion at *m/z* $[M - H]^-$ and fragments of phenolic compounds identified in cowpea **CC** and **CT**. (**a**) quercetin (**b**) gallic acid and (**c**) protocatechuic acid (PCA).

3.2 Aliquots 14 and 17 chromatographic profiles

The fractions 14 and 17 from the chromatographic fractionation of the cowpea CC (CME) exhibited significant number of chemical compounds when they were analyzed by Thin Layer Chromatography (TLC). Then, these extracts were selected to be analyzed by HPLC-DAD-MS/MS.

From the chromatogram analysis of the fraction 14 (Figure 4a), it was possible to identify the *p*-coumaric acid. The peak with retention time of 13.71 min exhibited a peak corresponding to the mass of its deprotonated molecular ion at *m/z* 163 $[M - H]^-$ and at *m/z* 119 fragment (Figure 5a), which compared with the standard it allowed the identification of *p*-coumaric acid. Regarding the chromatogram analysis of the fraction 17 (Figure 4b), it was possible to identify the flavonol quercetin (**5b**) and PCA (Figure 5c), which could be identified above, as well as the kaempferol, identified by comparison with standard, where the peak with a retention time of 18.12 min showed the molecular ion peak at *m/z* 285 $[M - H]^-$ and fragments at *m/z* 257, 217, 199, 175 and 151 (Figure 5d), corresponding to literature data (Fabre et al., 2001).

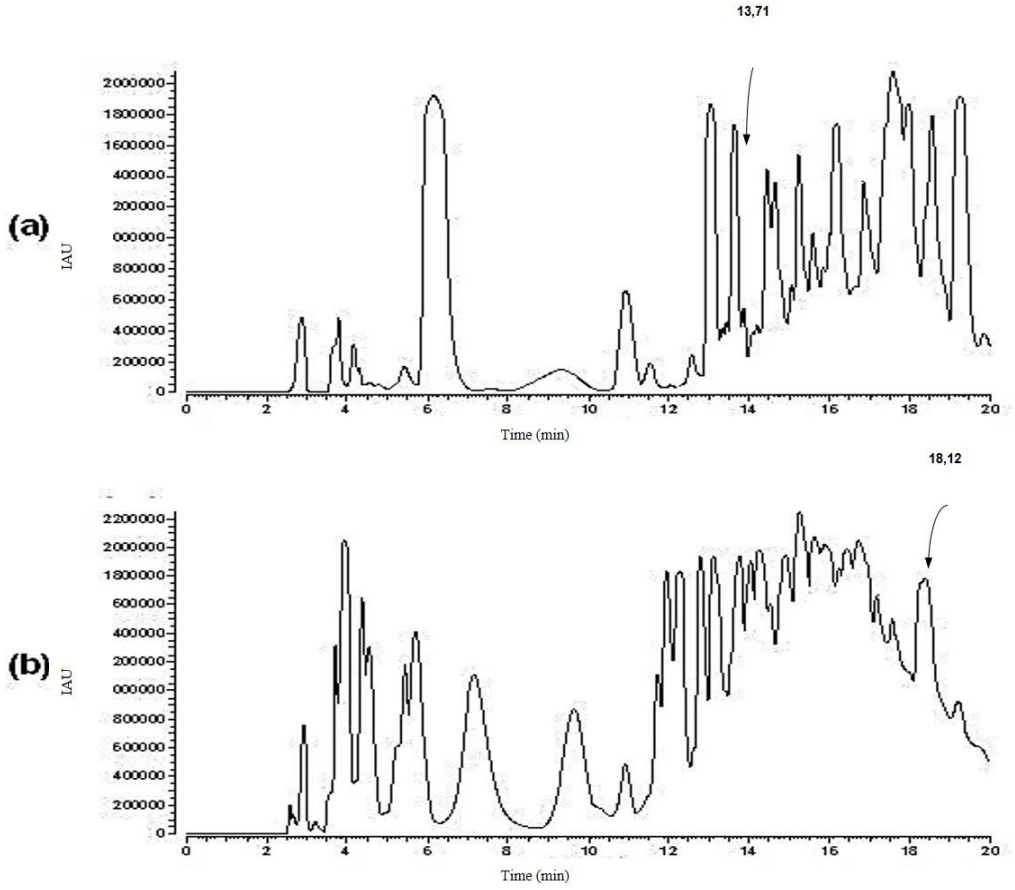

Figure 4. Chromatograms of the cowpea CC from fractions 14 **(a)** and 17 **(b)**.

The *p*-coumaric acid has been identified in bean species such as, *P. vulgaris* L. and L. *V. faba* (Chen et al., 2015; Šibul et al., 2016.). It has also been identified in *P. vulgaris* L., *V. faba* L. as well as in kaempferol (Chen et al., 2015; Šibul et al., 2016.).

Phenolic compounds are widely used as industrial chemicals, agrochemicals, pharmaceuticals and other consumer products, as they have various biological properties to combat free radicals, antioxidant activity, acting as metal chelators, modulating the activity of some enzymes, in atherosclerosis and also in antimutagenic actions (Palacios et al., 2011; Roby et al., 2013; Świsłocka et al., 2013). These compounds may have antimicrobial activity due to the fact that they are weak organic acids, and also its partially

lipophilic nature which allows them to pass through the lipid bilayer of the cell membrane, disrupting the cellular structure and acidifying the cytoplasm, and proteins can also be denatured (Campos et al., 2009). Phenolic acids have antioxidant properties both food and body, as there have been indication reports for cancer treatment and prevention, cardiovascular disease and other illnesses (Scalbert et al., 2005).

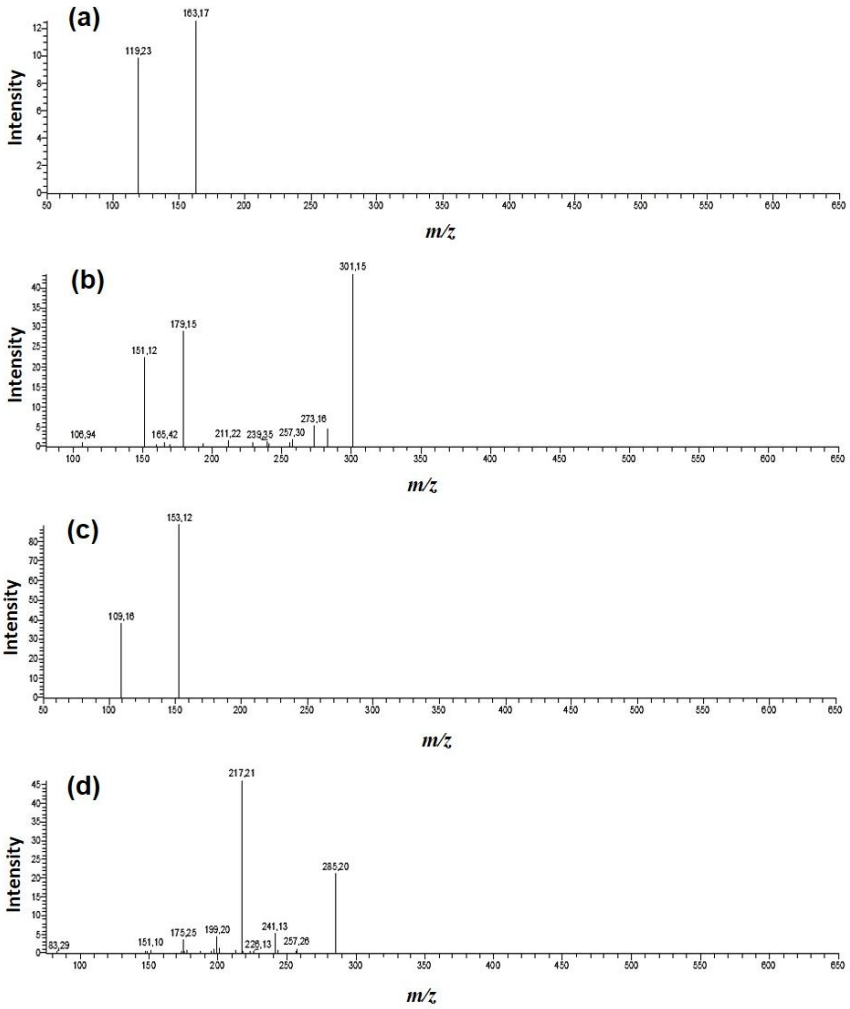

Figure 5. Mass spectra with molecular ion peak at *m/z* [M - H]$^-$ and fragments of *p*-coumaric acid **(a)**, quercetin **(b)**, protocatechuic acid (PCA) **(c)** and kaempferol **(d)** presented in the fractions 14 and 17.

The chemical compounds identified in this study have been widely studied; and many literature reviews have been reported a range of useful biological properties. The *p*-coumaric acid, for instance, has antioxidant activity as well as the ability to form complexes with transition metals, in addition to showing antimicrobial property (Estevinho et al., 2008; Kalinowska et al., 2013; Świsłocka et al., 2013).

Some researches have pointed out that the gallic acid has several biological properties, acting as antimutagenic, antitumoral and antioxidant agents, suggesting that this phenolic acid may be considered an effective compound to combat cancer cells (Gichner et al., 1987; Kim et al., 2002; Kroes et al., 1992; Locatelli et al., 2013).

The flavonoids are a group of natural substances and found in leguminous plants. They are also called natural pigments and they have a fundamental action in plant protection against oxidant agents such as ultraviolet ray and pollution. In medicine, these compounds are used as antimicrobials agents, anti-inflammatory and anti-ulcerogenic properties, as well as antioxidants (Lima-Saraiva et al., 2012; Oliveira-Júnior et al., 2013; Pereira & Cardoso, 2012; Santana et al., 2012).

According to Hanahan & Weinberg (2011, 2000), quercetin can be considered a development inhibitor of different types of cancer, since, according to the authors, this flavonoid can act as an anti-angiogenic agent, metastasis inhibitor, anti-proliferative and suppressant growth agents on malignant tumors, as well as being responsible for antioxidant and anti-inflammatory activities. The kaempferol is another important flavonoid, largely found in leguminous plants, which has been reported to have important biological properties such as anti-inflammatory, estrogenic activities as well as reducing risk of cancer and cardiovascular diseases (Mercader-Ros et al., 2013; Guo et al., 2012; Rho et al., 2011).

4 Conclusion

In this study, the phenolic compounds of great relevance and several biological properties could be identified. The chromatographic profiles obtained from CME of the two cowpea cultivars were very similar to each other. However, phenolic compound was only identified in one cultivar, comprising the flavonols quercetin and kaempferol (cowpea CC); while gallic acid was identified only in cowpea (CT). Nevertheless, the PCA was identified in both cultivars.

Phenolic compounds identified in cowpea cultivars may be considered common bean species, suggesting that there is a similarity in chemical composition between the species that have similar functional properties.

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