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Artigo

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Isolamento de Flavonoides de *Dipteryx odorata* por Cromatografia Líquida de Alta Eficiência

Resumo: *Dipteryx odorata* (Aubl) Willd, família Fabaceae, é uma espécie nativa da floresta Amazônica, Brasil. O objetivo do trabalho foi isolar e identificar os flavonoides do endocarpo de *D. odorata* minimizando custos com solventes, tempo e resíduos. Seis flavonoides, 3',4',7-triidroxiflavona, 3',4',7-triidroxiflavanona, 3',4',6-triidroxiaurona, 3',4',5,7-tetraidroxiflavona, 2',3,4,4'-tetraidroxichalcona e 2',4,4'-triidroxichalcona foram isolados. Os flavonoides 3',4',7-triidroxiflavona e 2',3,4,4'-tetraidroxichalcona foram identificados pela primeira vez nesta espécie.

Palavras-chave: Cumaru; Fabaceae; CLAE.

Abstract

Dipteryx odorata (Aubl.) Willd, Fabaceae family, is a native species from the Amazon forest, Brazil. The goal of the work was to isolate and identify the flavonoids in the endocarp from *D. odorata* in order to minimize costs with solvents, time and residues. Six flavonoids, 3',4',7-trihydroxyflavone, 3',4',7-trihydroxyflavanone, 3',4',6-trihydroxyaurone, 3',4',5,7-tetrahydroxyflavone, 2',3,4,4'-tetrahydroxychalcone and 2',4,4'-trihydroxychalcone were isolated. The flavonoids 3',4',7-trihydroxyflavoneand 2',3,4,4'-tetrahydroxychalcone were identified for the first time in this species.

Keywords: Tonka bean; Fabaceae; HPLC.

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Isolation of Flavonoids from *Dipteryx odorata* by High Performance Liquid Chromatography

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1. Introduction

Dipteryx odorata (Aubl.) Willd. (syn. *Coumarouna odorata* Aubl); Fabacea family, commonly known as "tonka bean" tree or locally as "cumaru", is a high arboreal species native to the Amazon area.[1] Due to its high content of coumarin, the plant's seeds have significant commercial value and are widely used in the perfumery, cigarettes and cosmetics industries.[1,2]

Popularly, it is used to aid in the treatment of ulcers, ear infections, respiratory and cardiac disorders.[1] The seeds are characterized by acaricidal activity,[3] which revealed anti-carcinogenic effects, especially against breast cancer, potential phytotoxicity[4] and cancer chemopreventive.[5]

Previous phytochemical investigations of this plant resulted in the isolation of coumarins,[2,6] cassanediterpenoids,[5,7] isoflavonoids,[8-10] isoflavolignans,[5]fatty acids[4] and lupane triterpenoids.[11] Isoflavones, chalcones and aurones represents some flavonoids that have already been isolated from different parts of this species. From the endocarp, only diterpenoids were isolated but the presence of flavonoids has not been investigated in this plant structure.

The flavonoids are secondary metabolites, having a C_6-C_3-C_6 carbon framework. Variations in the basic carbon skeleton and the oxidation state lead to main classes of flavonoids: chalcones, aurones, flavanones, dihydroflavonols, flavones, flavonols, isoflavones, flavan-3-ols and anthocyanidins.[12] Flavonoids receive considerable attention in the literature, especially for being awarded several biological activities to this class, such as antioxidant activity, anti-inflammatory, antitumor and antiviral.[13-15]

The high performance liquid chromatography (HPLC) is the most wide spread technique and it is used for analytical scale separation of flavonoids. The major advantage over others chromatographic methods is that it provides high resolution and sensitive quantitative analysis in a single operation.[16] When the goal is to obtain the compounds individually isolated with sufficient purity and quantity for identification purposes, the preparative scale separation of flavonoids by classic liquid chromatography is most commonly used. However, it is a time consuming and laborious process[17] and uses large volume of solvents, causing an expressive environmental impact and economic demand.

An alternative involves the optimization of chromatographic methods to reduce these consequences. Thus, the objective of this work was the isolation and the identification of flavonoids from the endocarp minimizing costs, time and solvent's residues.

2. Material and Methods

2.1. Plant Material and Sample Preparation

Dipteryx odorata fruits were collected at Ducke Reserve – National Institute for Amazonian Research, Manaus, AM, Brazil. The voucher specimen (INPA00143012) is deposited in the Herbário INPA.

The fruits were dried at room temperature and broken up, separating the endocarp material from the seed. The endocarp (3000 g) was grinded in a ball mill and extracted with petroleum ether (5 L) by maceration for 6 days. The solid residue of the maceration was dried at room temperature and extracted by maceration for 6 days, this time with ethyl ether (5 L).

Dried ethyl ether extract (15 g) was fractionated by ultraturrax agitation and filtration under vacuum on sintered glass funnel plate, with *n*-hexane, dichloromethane, ethyl acetate and methanol in order of increasing polarity. From the dried fractions 1 mg was separated and re-suspended with 1 mL of methanol:water (1:1), and centrifuged. The supernatant was analyzed by HPLC-PDA.

2.2. Chromatographic conditions for flavonoid separation

The HPLC analysis was carried out on a Waters Alliance® 2695 system (Waters, MA, USA) equipped with an auto sampler, PDA detector (2996, Waters, MA, USA). The chromatographic separation was optimized

using reverse phase column (C_{18}, YMC 250 x 4.6 mm, with a particle size of 5 µm) at a temperature of 45 °C. The mobile phase consisted of water:acetic acid 98:2 (A) and acetonitrile:acetic acid 98:2 (B). The flow rate was 1.3 mL.min^{-1} and the gradient program was 0–6 min, 20–40 % B; 6–12 min, 40–50 % B; 12–14 min, 20–20 % B. The PDA detection wavelengths were set at 200 nm and 600 nm.

2.3. Isolation and strutural characterization of the substances

The flavonoids separation was performed by HPLC analytical scale using a reverse phase column (C_{18}, YMC 250 x 4.6 mm, with 5 µm particle) and the isolation was performed through the automated collection at the detector output, using a selector valve channels (RV500-104/550-104, Rheodyne®) as a fraction collector. The valve was programmed to collect one flavonoid through each channel according to its retention time. In order to increase the amount of each isolated flavonoid, successive injections and collections were performed automatically.

Isolated flavonoids were dried by rotary evaporator and their structures were determined by interpretation of spectral data, mainly the one furnished by ^{13}C NMR and ^{1}H NMR (1D and 2D) and ESI-MS, including methyl derivatives prepared by reaction with diazomethane to confirm the structures. The purity of the isolated compounds was evaluated by HPLC-PDA.

2.4. Nuclear Magnetic Resonance Spectrometry Conditions

The spectral data was carried out on a Bruker AVANCE (operating in 499.80 MHz and 125.69 MHz to ^{1}H and ^{13}C, respectively). The chemical shifts (δ_H e δ_C in ppm) were referenced based on the residual values of the corresponding solvent MeOD used (δ_H 3.31 andδ_C 49.15 ppm). Coupling constants (*J*) were reported in Hz. Heteronuclear spectra 2D HSQC (^{1}H-^{13}C-COSY-$^{1}J_{CH}$) and HMBC (^{1}H-^{13}C-COSY-$^{n}J_{CH}$, n=2 and n=3) were acquired with 8 transitions/128 increments and 4 transitions/128 increments respectively. For homonuclear spectra 2D ^{1}H-^{1}H-COSY spectral widths of 5000 Hz in both dimensions and the normal number of transitions were used.

2.5. UPLC-Mass Spectrometry Conditions

MS spectra was carried out on a Waters Acquity UPLC system (Waters, MA, USA) equipped with an auto sampler and connected to the mass spectrometer Q-TOF Synapt detector (Waters, MA, USA) by electron spray ionization (ESI) or with direct infusion on mass spectrometer. The isolation was done on a reverse phase column (C_{18}, ACQUITY UPLC BEH 150 x 2.1 mm and 1.7 mM) at a temperature of 45 ºC. The mobile phase consisted of acetonitrile (A) and 0.1% formic acid in water (B). The flow rate was 0.35 mL.min^{-1} and the gradient program was: 0-5 min, 90-85 % B; 5-10.5 min, 85-81 % B; 10.5-11 min, 81-80 % B; 11-19 min, 80-30 % B; 19-25 min, 30-40 % B; 25-26 min, 40-90 % B; 26-30 min, 90-90 % B.

Mass spectrometer was operated in positive mode, analyzer mode V, with capillary voltage, cone voltage and extraction cone voltage set to 3000, 25 and 4000 V respectively. The desolvatation gas flow rate was set to 750 L/h at a temperature of 500 ºC and the source temperature was set to 120 °C.

2.6. Derivatization of isolated flavonoids

Flavonoids isolated from the *D. odorata* were subjected to reaction with diazomethane. The diazomethane was synthesized from the reaction of potassium hydroxide with nitrosomethylurea.[18]

3. Results

3.1. Fractionation of the extract

The crude extract of the *D. odorata* endocarp was fractionated with organic solvents of different polarities: *n*-hexane, dichloromethane, ethyl acetate and methanol. The four extracts were analyzed by high performance liquid chromatography in reversed phase column with photodiode array detector. The chromatogram was reported at λ 253 nm (Figure 1).

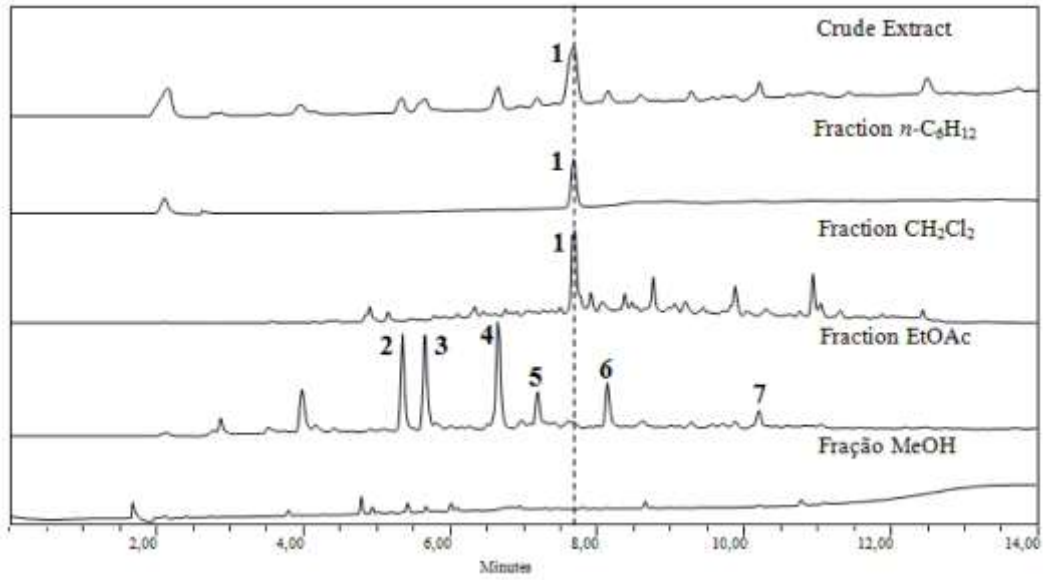

Figure 1. Chromatogram of the *D. odorata* endocarp crude extract, *n*-hexane (*n*-C_6H_{12}), dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc) and methanol (MeOH) fractions. Injection volume: 50 µL; concentration of the crude extract and fractions: 1 mg.mL^{-1}

The crude, *n*-hexane and dichloromethane extracts showed one major peak at 7.8 min, characterized as coumarin (**1**) by analysis of accurated mass spectrum. The ethyl acetate extract did not reveal the presence of coumarin (**1**) and showed a higher concentration of flavonoids than the other extracts. For that reason it was submitted to HPLC separation and purification/collection of these components.

3.2. Optimization of separation and collection of compounds present in the fraction of ethyl acetate

The chromatographic separation was optimized in order to minimize the running time and get a good chromatographic resolution, minimizing the time and the solvent spent in the isolation without losing the quality and purity of the final product.

The isolation of the flavonoids from *D. odorata* involved the use of a HPLC analytical scale system connected to the selector valve channels Rheodyne® in the output of the detector (Figure 2). Successive injections and collections were performed to obtain the flavonoids isolated in quantity and purity appropriated to achieve good quality spectra and for the preparation of derivatives when necessary. The separation of flavonoids was monitored by a photodiode array detector (PDA) at λ 253 nm.

Each of the valve channels was programmed to open and close according to the retention time of each substance of interest. The valve has six channels, five

channels were used for flavonoid collection and one channel for waste, thus five flavonoids were collected at each injection. For instance, at the beginning of the injection the valve output is selected on channel 1, where is the waste. In 5.3 minutes the elution of flavonoid 2 starts, then the valve change the output to channel 2, where is the reservoir flavonoid 2. In 5.5 minutes when the flavonoid finished eluting, the valve returns to the channel 1, and so on until the end of the process. Each flavonoid was collected by a different channel reducing the contamination.

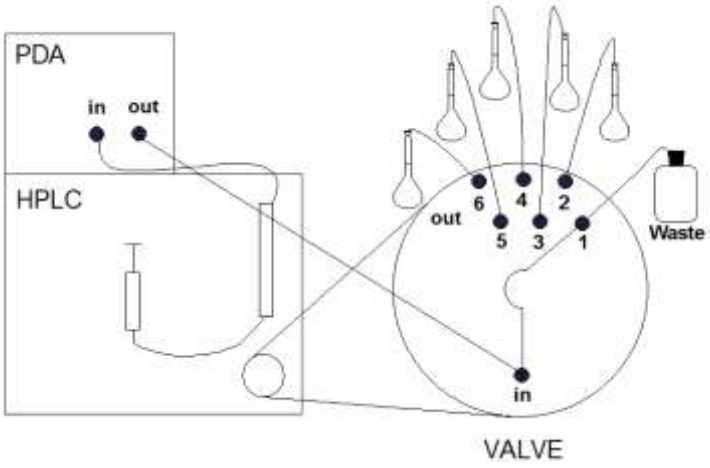

Figure 2. Flavonoids collection schema. Channel 1: waste reservoir; channel 2-5: flavonoid 2-5 reservoir; channel 6: flavonoid 6 or 7 (Source: own file)

From the ethyl acetate extract (40,6 mg) of the *D. odorata* were isolated the flavonoids **2** (1,2 mg, purity 93 %), **3** (1,3 mg, purity 90 %), **4** (1,5 mg, purity 96 %), **5** (1,0 mg, purity 94 %), **6** (0,9 mg, purity 96 %) and **7** (0,8 mg, purity 98 %) (Figure 3) with sufficient purity and quantity for structural elucidation. The flavonoids **2** and **6** were identified for the first time in this species. The methodology has proven effective in the isolation of flavonoids along with economic and environmental viability, therefore spending only 10 days using high performance liquid chromatograph and only 6.9 L of acetonitrile, 370 mL of acetic acid and 11 L of water. It is important to notice that automatic injection and collection were used, eliminating the full time work of the analyst and enabling the application of this methodology overnight, and for that not changing the laboratory routine.

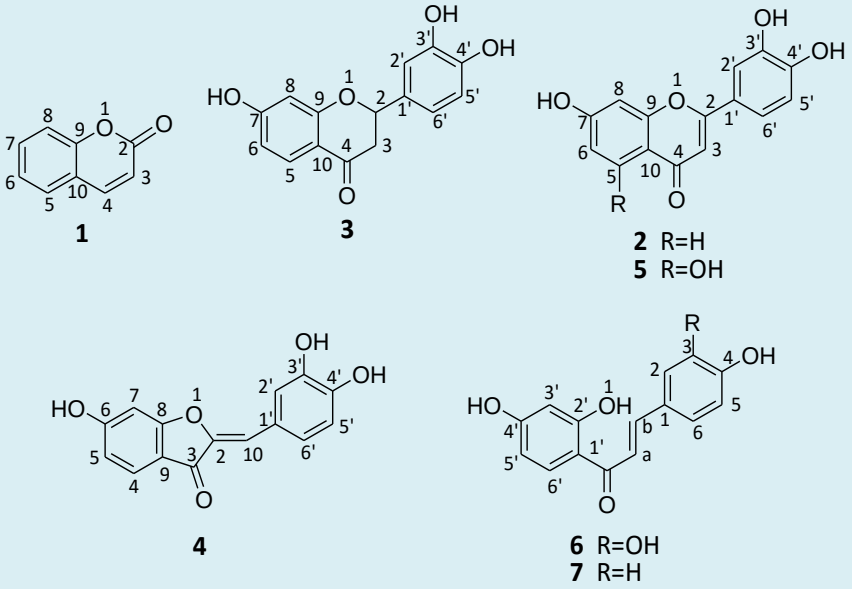

Figure 3. Structures of seven substances isolated from the *D. odorata*

3.3. Identification of the separated peaks

The structures of the isolated flavonoids were determined by interpretation of spectral data, specially the one furnished by ^{13}C NMR and ^{1}H NMR (1D and 2D), ESI-MS and UV, including of methyl derivatives prepared by reaction with diazomethane to confirm the structures.

Compound 1 was identified by UV (λ_{max}=270 nm) and ESI-HRMS spectral data. The ESI-HRMS (positive mode) showed peaks at *m/z* 147.0834 and 103.0997, attributed to protonated molecular ion ($[M+H]^+$, calc *m/z* 147.0446) and elimination of CO_2 by protonated molecular ion ($[M+H-CO_2]^+$, *m/z* 103.0548). These spectral data allowed the identification of the compound **1** as coumarin.

Compound 2. UV λ_{max}: 253, 342 nm.1H-NMR (MeOH-d_4, 500 MHz) δ_H ppm: 6.67 (1H, s, H-3), 8.00 (1H, d, *J* = 8.8 Hz, H-5), 6.96 (1H, d, *J* = 8.8 Hz, H-6), 7.00 (1H,br s, H-8), 7.43 (1H, brs, H-2'), 6.94 (1H, d, *J* = 8.1 Hz, H-5'), 7.44 (1H, d, *J* = 8.1 Hz, H-6'). 13C-NMR (MeOH-d_4, 125 MHz) δ_C ppm: 164.67 (C-2), 103.76 (C-3), 178.89 (C-4), 126.35 (C-5), 115.40 (C-6), 163.46 (C-7), 102.08 (C-8), 158.24 (C-9), 115.81 (C-10), 122.56 (C-1'), 112.75 (C-2'), 145.63 (C-3'), 149.40 (C-4'), 114.89 (C-5'), 118.82 (C-6'). Comparison of these NMR spectral data with values described in the literature[19] allowed the identification of the compound **2** as 3',4',7-trihydroxyflavone. ESI-HRMS (positive mode) revealed peaks at *m/z* 271.0662 253.0787, 225.0811, 197.0809, 161.0441, 137.0380, 135.0627 (Figure 4).

Compound 3. UV λ_{max}: 277, 311 nm.1H-NMR (MeOH-d_4, 500 MHz) δ_H ppm: 5.34 (1H, dd, *J* = 12.9, 2.8 Hz, H-2 - axial position), 2.71 (1H, dd, *J* = 17.0, 2.8 Hz, H-3a – equatorial position), 3.03 (1H, dd, *J* = 17.0, 12.9 Hz, H-3b – axial position), 7.75 (1H, d, *J* = 8.8 Hz, H-5), 6.52 (1H, d, *J* = 8.8 Hz, H-6), 6.38 (1H, s, H-8), 6.95 (1H, s, H-2'), 6.81 (1H, d, *J* = 8.2 Hz, H-5'), 6.82 (1H, d, *J* = 8.2 Hz, H-6'). 13C-NMR (MeOH-d_4, 125 MHz) δ_C ppm: 79.68 (C-2), 43.63 (C-3), 192.14 (C-4), 128.45 (C-5), 110.33 (C-6), 165.41 (C-7), 102.40 (C-8), 164.14 (C-9), 113.56 (C-10), 130.62 (C-1'), 113.27 (C-2'), 145.09 (C-3'), 145.43 (C-4'), 114.82 (C-5'), 117.82 (C-6'). Comparison of these NMR spectral data with values described in the literature[20] allowed the identification of the compound **3** as 3',4',7-trihydroxyflavanone (butin). ESI-HRMS (positive mode) revealed peaks at *m/z* 273.0901, 255.0872, 227.0918, 163.0550, 137.0423 (Figure 5).

Compound 4. UV λ_{max}: 395 nm.1H-NMR (MeOH-d_4, 400 MHz) δ_H ppm: 7.63 (1H, d, *J* = 8.00 Hz, H-4), 6.73 (1H, d, *J* = 8.00 Hz, H-5), 6.73 (1H, H-7), 6.73 (1H, d, H-10), 7.55 (1H, s, H-2'), 6.87 (1H, d, *J* = 8.00 Hz, H-5'), 7.26 (1H, d, *J* = 8.00 Hz, H-6'). 13C-NMR (MeOH-d_4, 100 MHz) δ_C ppm: 146.29 (C-2), 183.07 (C-3), 125.45 (C-4), 113.29 (C-5), 168.40 (C-6), 97.97 (C-7), 166.98 (C-8), 113.44 (C-9), 112.71 (C-10), 124.11 (C-1'), 117.52 (C-2'), 145.34 (C-3'), 148.01 (C-4'), 115.27 (C-5'), 125.01 (CH-6'). Comparison of these NMR spectraldata with values described in the literature[21] allowed the identification of the compound **4** as 3',4',7-trihydroxyaurone (sulfuretin). ESI-HRMS (positive mode) revealed peaks at *m/z* 271.0783, 253.0670, 225.0701, 215.0865, 197.0757, 137.0380 (Figure 6).

Compound 5. UV λ_{max}: 252, 348 nm.1H-NMR (MeOH-d_4, 500 MHz) δ_H ppm: 6.57 (1H, s, H-3), 6.23 (1H, s, H-6), 6.47 (1H, s, H-8), 7.41 (2H, sl, H-2'/H-6'), 6.93 (1H, sl, H-5'). 13C-NMR (MeOH-d_4, 125 MHz) δ_C ppm: 164.80 (C-2), 102.46 (CH-3), 182.47 (C-4), 161.80 (C-5), 98.81 (C-6), 93.66 (C-8), 158.03 (C-9), 103.86 (C-10), 122.27 (C-1'), 112.78 (C-2'), 145.67 (C-3'), 149.64 (C-4'), 115.41 (C-5'), 118.90 (C-6'). Comparison of these NMR spectraldata with values described in the literature[22] allowed the identification of compound **5** as 3',4',5,7-trihydroxyflavone (luteolin). ESI-HRMS (positive mode) revealed peaks at *m/z* 269.0679, 241.0630, 161.0394, 135.0584 (Figure 4).

Compound 6. UV λ_{max}: 229, 379 nm.1H-NMR (MeOH-d_4, 500 MHz) δ_H ppm: 7.59 (1H, d, *J* = 14.8 Hz, H-α), 7.76 (1H, d, *J* = 14.8 Hz, H-β), 7.22 (1H, s, H-2), 6.86 (1H, d, *J* = 7.9 Hz, H-5), 7.15 (1H, d, *J* = 7.9 Hz, H-6), 6.32 (1H, s, H-3'), 6.45 (1H, d, *J* = 8.8 Hz, H-5'), 7.99 (1H, d, *J* = 8.8 Hz, H-6'). 13C-NMR (MeOH-d_4, 125 MHz) δ_C ppm: 192.05 (C=O), 116.86 (C-α), 144.70 (C-β), 126.98 (C-1), 114.37 (C-2), 145.48 (C-3), 148.61 (C-4), 115.24 (C-5), 122.29 (C-6), 113.24 (C-1'), 165.17 (C-2'), 102.46 (C-3'), 166.12 (C-4'), 107.86 (C-5'), 131.92 (C-6'). Comparison of these NMR spectraldata with values described in the literature[20] allowed the identification of compound **6** as 2',3,4,4'-tetrahydroxychalcone (butein). ESI-HRMS (positive mode) revealed peaks at *m/z* 273.1023, 255.0872, 227.0973, 163.0550, 137.0423 (Figure 7).

Compound 7. UV λ_{max}: 229, 370 nm.1H-NMR (MeOH-d_4, 400 MHz) δ_H ppm: 7.65 (1H, d, *J* = 15.0 Hz, H-α), 7.81 (1H, d, *J* = 15.0 Hz, H-β), 7.66 (2H, d, *J* = 8.2 Hz, H-2/H-6), 6.87 (2H, d, *J* = 8.2 Hz, H-3/H-5), 6.31 (1H, s, H-3'), 6.44 (1H, d, *J* = 8.2 Hz, H-5'), 8.01 (1H, d, *J* = 8.2 Hz, H-6'). 13C-NMR (MeOH-*d*4, 100 MHz) δ_C ppm: 192.14 (C=O), 116.92 (C-α), 144.30 (C-β), 130.47 (C-2/C-6), 115.54 (C-3/C-5), 160.19 (C-4), 154.41 (C-2'), 102.41 (C-3'), 165.01 (C-4'), 107.78 (C-5'), 132.00 (C-6'). Comparison of these NMR spectraldata with values described in the literature[23] allowed the identification of compound **7** as 2',3,4'-tetrahydroxychalcone (isoliquiritigenin). ESI-HRMS (positive mode) revealed peaks at *m/z* 257.0919, 239.0935, 211.0967, 147.0587, 137.0380 (Figure 7).

The proposed fragmentation mechanisms to justify the principals peaks observed in the ESI-HRMS of all flavonoids were summarized in the Figures 4 (compounds **2** and **5**), 5 (compound **3**), 6 (compound **4**) and 7 (compounds **6** and **7**). The similarity revealed by the fragmentations reaction of these flavonoids, such as retro-Diels-Alder reaction, was also used to define the number of hydroxyl groups present on rings A and B (Figures 4 to 7). Through the 1D and 2D NMR spectral data involving comparison with the literature was possible to postulate the substitution pattern of each ring. Ultraviolet spectra were also useful in the structural classification of the compounds, contributing to the identification of the flavonoids class.

The compounds **3** and **6** showed very similar fragmentation and the same protonated molecular ion $[M+H]^+$. As expected, the UV absorption of compound **6** revealed expressive difference when compared with the compound **3**, this information suggested that these compounds are the isomers chalcone and flavanone, respectively. The ionization energy used in

the mass spectra can lead to interconversion between these isomers explaining the similarity between the mass spectra.

Compounds **2**, **5** (Figure 4) and **4** (Figure 6) revealed the fragments $[M+H-H_2O]^+$, $[M+H-H_2O-CO]^+$ and $[M+H-H_2O-2CO]^+$. Compounds **6** and **7** (Figure 7) revealed the fragments $[M+H-H_2O]^+$ and $[M+H-H_2O-CO]^+$ and compound **3** (Figure 5) revealed the fragments $[M+H-H_2O]^+$, $[M+H-CO]^+$.

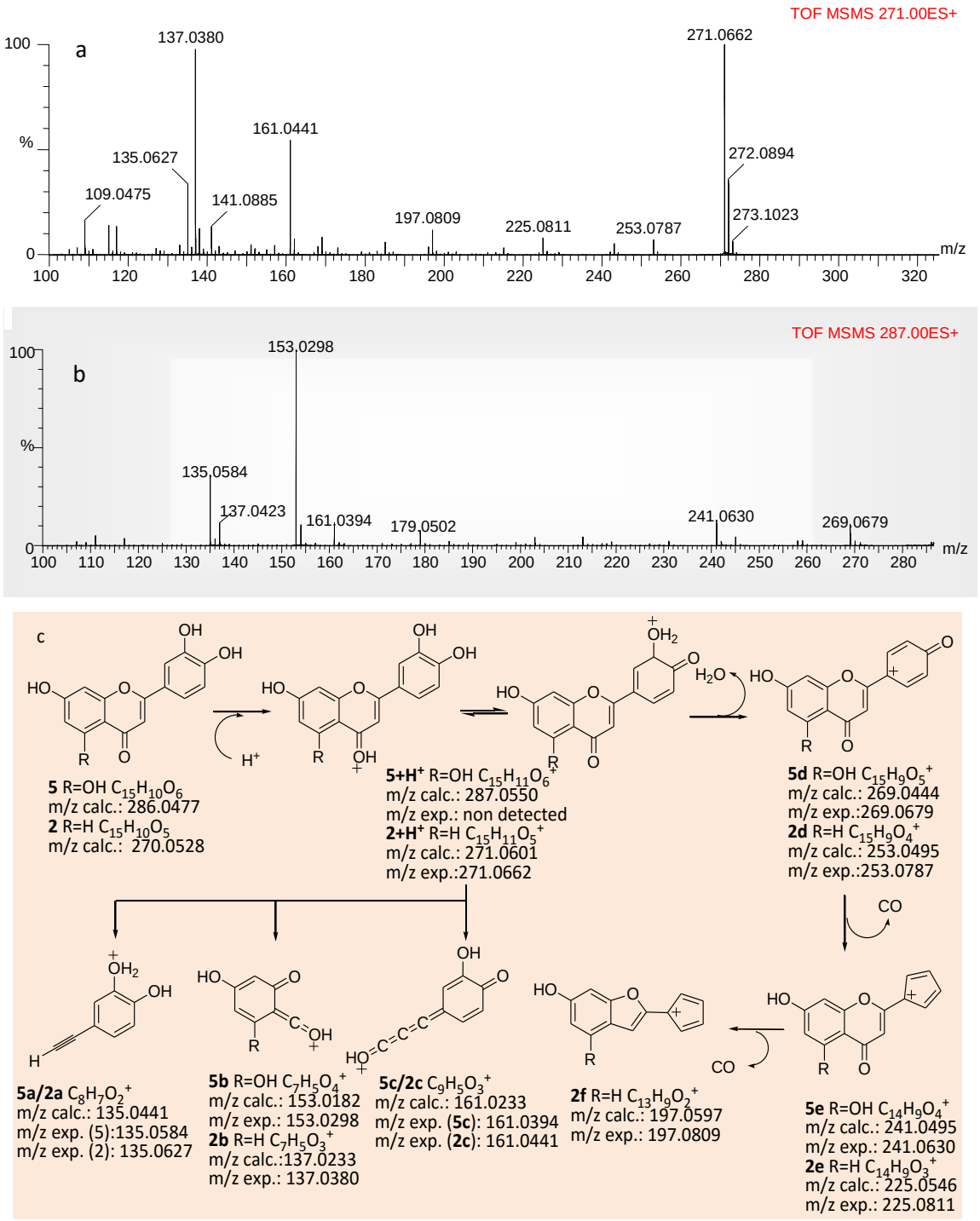

Figure 4. Mass spectra in positive mode of compound **2** (a) and **5** (b) and proposed MS fragmentation pathway(c)

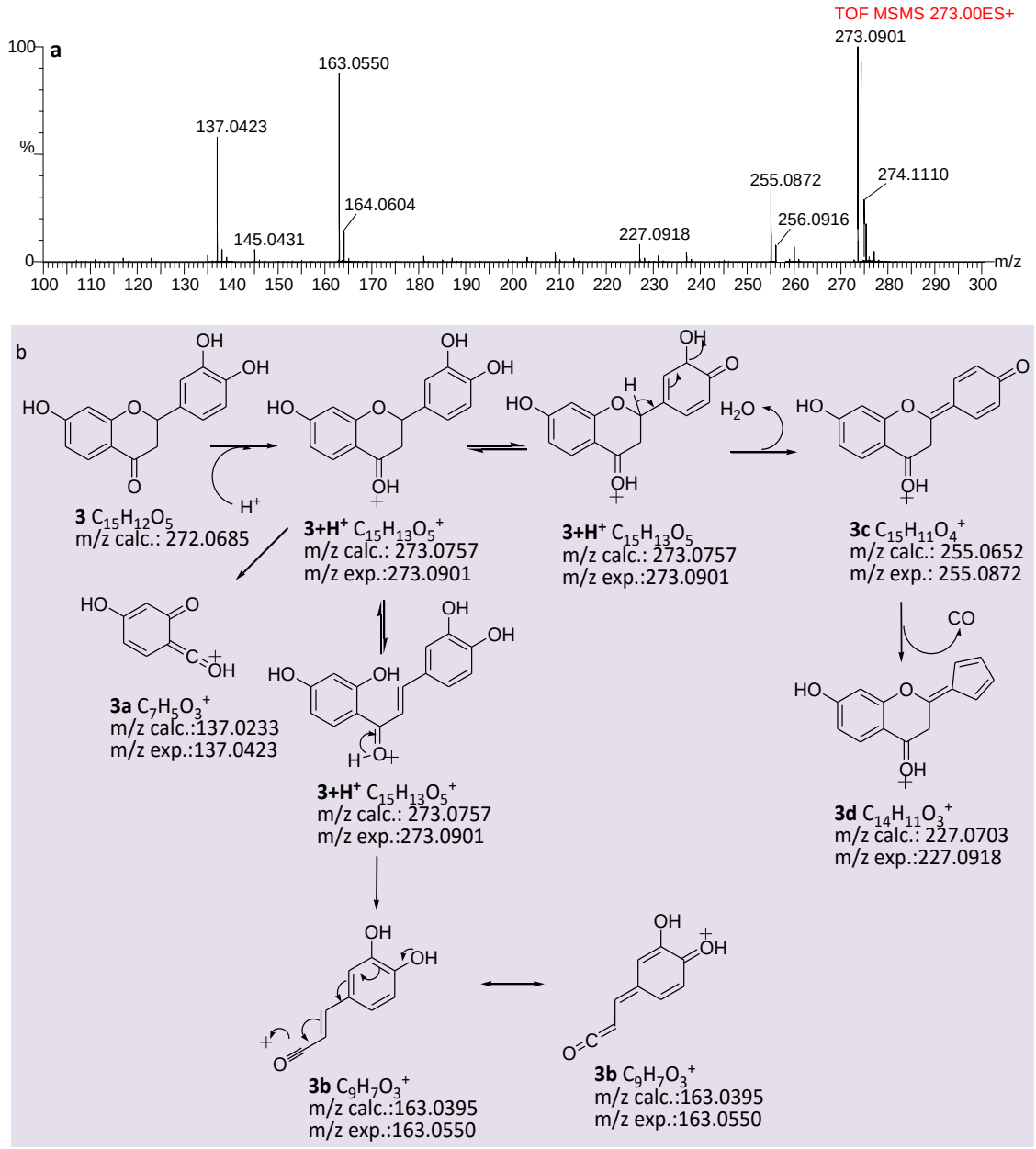

Figure 5. Mass spectra in positive mode of compound **3** (a) and proposed MS fragmentation pathway (b)

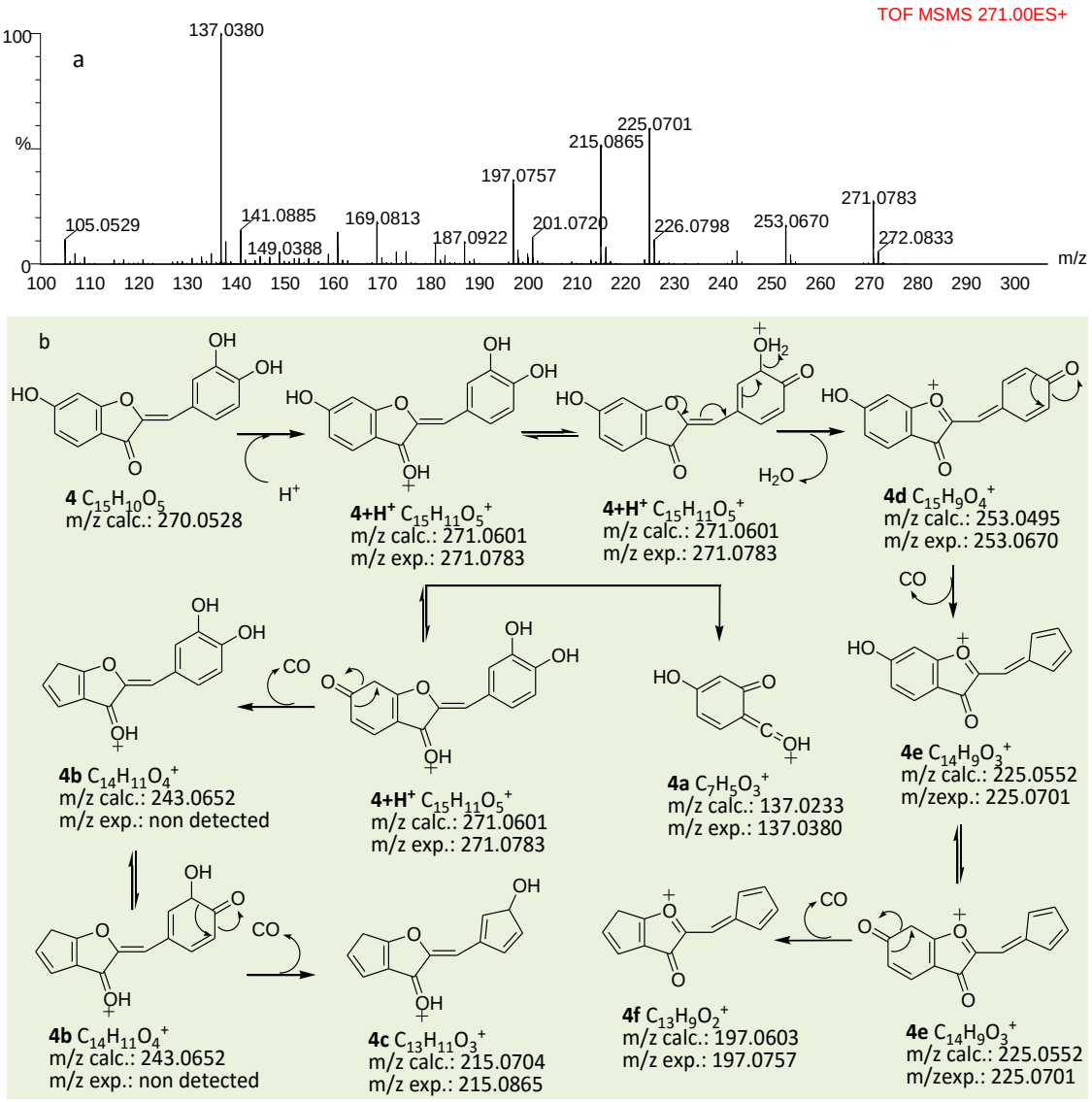

Figure 6. Mass spectra in positive mode of compound **4** (a) and proposed MS fragmentation pathway (b)

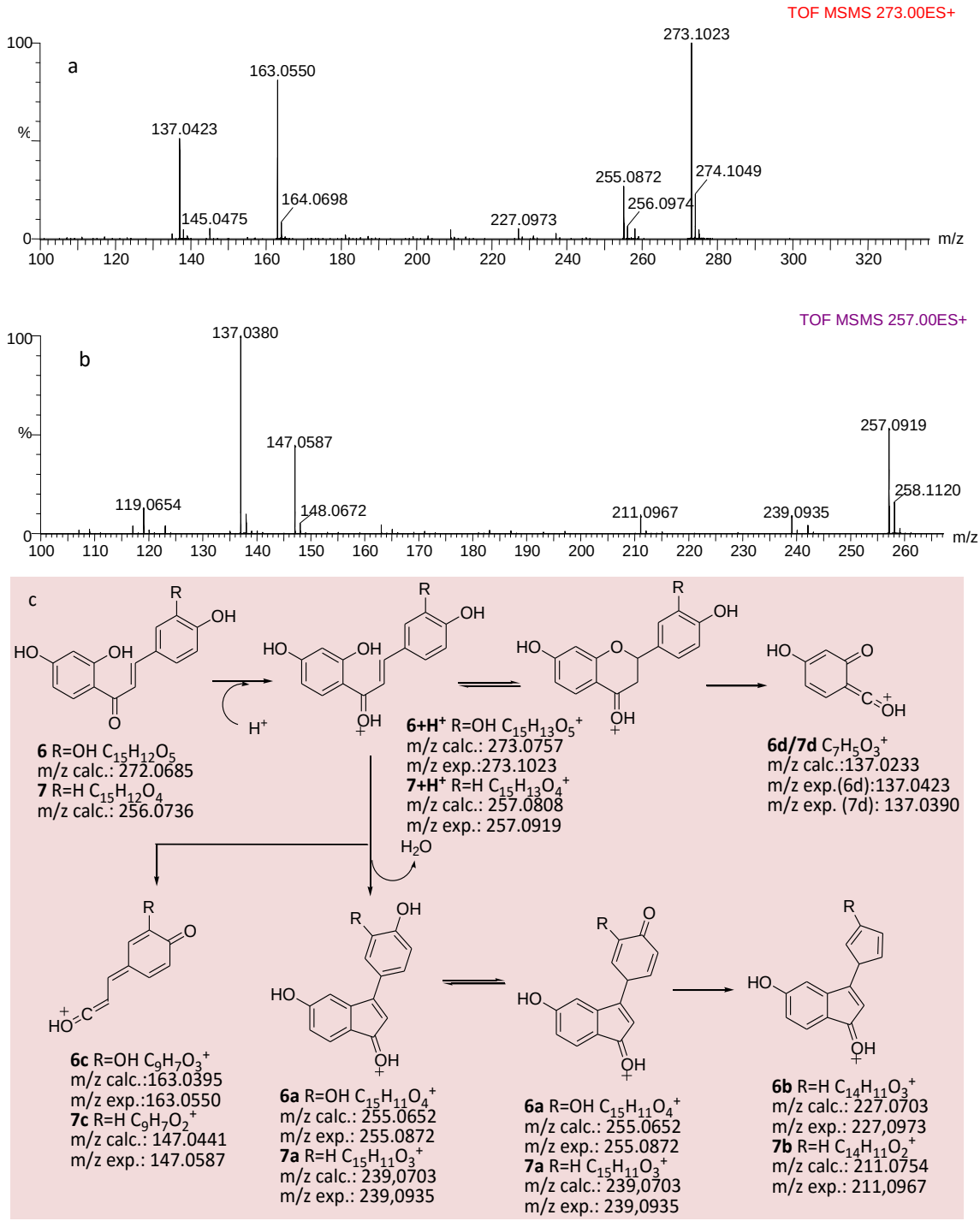

Figure 7. Mass spectra in positive mode of compound **6** (a) and **7** (b) and proposed MS fragmentation pathway (c)

3.4. Preparation of derivatives

The methylated flavonoids were analyzed by mass spectrometry. In the spectra of 3',4',7-trimethoxyflavone (**2M**), 3',4',7-trimethoxyflavanone (**3M**), 3',4',6-trimethoxyaurone (**4M**) was observed the addition of 42 mass units confirming the existence of three hydroxylated groups in the original flavonoid. In the spectrum of 3',4',7-

trimethoxy-5-hidroxyflavone (**5M**) and 3,4,4'-trimethoxy-2'-hidroxychalcone (**6M**) the addition of 42 mass units confirmed the existence of three hydroxyl groups located at positions compatibles with conditions to methylation and one involved in intramolecular hydrogen bond that do not react with diazomethane. Analogous result was observed in the spectrum of 4,4'-trimethoxy-2'-hidroxychalcone (**7M**) the addition of 28 mass units confirmed the existence of two hydroxyl groups at positions to methylation and one involved in intramolecular hydrogen bond. Spectra of derivatives confirmed unequivocally the structures of the isolated flavonoids.

4. Conclusion

Flavonoids **2**, **3**, **4**, **5**, **6** and **7** were isolated from *D. odorata* with sufficient purity and quantity for structural elucidation through interpretation of UV, NMR and MS spectra data. The flavonoids **2** and **6** were identified for the first time in this species. The methodology is effective in the isolation of flavonoids and viable both economically and environmentally.

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