

Dereplication of Phenolic Derivatives of *Qualea grandiflora* and *Qualea cordata* (Vochysiaceae) using Liquid Chromatography coupled with ESI-QToF-MS/MS

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Um método racional e seletivo de cromatografia de alta eficiência acoplado à espectrometria de massas (CLAE-EM) com ionização por *electrospray* e analisador do tipo quadrupolo em tempo-de-voe em sequência (ESI-QToF-MS/MS) foi desenvolvido para a desreplcação de derivados fenólicos presentes em *Qualea grandiflora* e *Qualea cordata*. Os extratos para a análise foram selecionados por meio dos resultados de atividade antioxidante revelados no ensaio *in vitro* com DPPH. A análise por HPLC-ESI-QToF-MS/MS foi realizada por detecção contínua das relações massa/carga em alta resolução e pela técnica MS/MS de colisão induzida para fragmentação dos íons moleculares selecionados. A desreplcação da fração AcOEt do extrato hidroalcoólico dos caules de *Q. grandiflora* permitiu a detecção dos flavonoides: 3',4',5',5,6,7-hexahidroxi-8-metilflavanona, 8-metil-naringenina e 3',7-dimetoxi-8-metil-4',5,7-trihidroxi-flavanona, assim como os derivados da benzofenona (bis(4,6-dimetoxi-2-hidroxi-3-metilfenil)-metanona, 3',4'-dimetoxi-8-metil-5,6,7-trihidroxi-flavanona, 7-metoxi-6-metil-3',4',5-trihidroxi-flavanona, 6,8-dimetil-3'-metoxi-4',5,7-trihidroxi-flavanona e 3',5'-dimetoxi-6,8-dimetil-4',5,7-trihidroxi-flavanona) foram detectados na fração AcOEt do extrato hidroalcoólico das folhas de *Q. cordata*.

A rational and selective method using on-line high-performance liquid chromatography (HPLC) coupled with *electrospray* quadrupole time-of-flight tandem mass spectrometry (ESI-QToF-MS/MS) was established for the dereplication of phenolic derivatives from *Qualea grandiflora* and *Qualea cordata*. The selection of the extracts was based on the antioxidant capacity measured by *in vitro* DPPH assay. The HPLC-ESI-QToF-MS/MS analysis was conducted by on-flow detection, using high-resolution mass/ratio ions as well as collision induced MS/MS experiments for selected protonated ions. The dereplication of the EtOAc fraction from the hydro-alcohol extract from the stem bark of *Q. grandiflora* allowed the detection of the flavonoids: 3',4',5',5,6,7-hexahydroxy-8-methylflavanone, 8-methyl-naringenine and 3',7-dimethoxy-8-methyl-4',5,7-trihydroxyflavanone, as well as a benzophenone derivatives: bis(4,6-dimethoxy-2-hydroxy-3-methylphenyl)-metanone, 3',4'-dimethoxy-8-methyl-5,6,7-trihydroxyflavanone, 7-methoxy-6-methyl-3',4',5-trihydroxyflavanone, 6,8-dimethyl-3'-methoxy-4',5,7-trihydroxyflavanone and 3',5'-dimethoxy-6,8-dimethyl-4',5,7-trihydroxyflavanone were detected in the EtOAc fraction from the hydro-alcohol extract from the leaves of *Q. cordata*.

Keywords: *Qualea grandiflora*, *Qualea cordata*, dereplication, HPLC-ESI-QToF-MS/MS, radical scavenging activity

Introduction

Vochysiaceae is composed of two tribes, eight genera and approximately 200 species of trees and shrubs, distributed in the tropical regions of Central and South America and Western Africa.¹⁻⁴

Many Vochysiaceae species, in special those belonging to *Qualea* and *Vochysia*, are used in traditional medicine to treat diarrhea, intestinal pain, gastric ulcer, inflammations and wounds.⁵⁻⁸

Previous phytochemical analysis of some species of this family led to the identification of lupane, oleanane and ursane triterpenes, steroids, flavonoids as well as ellagic acid derivatives (EAD).⁹

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Pharmacological studies focused on the gastroprotective and anti-inflammatory actions of some *Qualea* species indicated a relationship between the pharmacological effects and the presence of EAD and polyphenolic compounds.¹⁰

Since the therapeutic effects reported for *Qualea* are a result of their use as complex herbal preparations, *avant garde* analytical techniques are necessary to identify and detect the bioactive constituents of the crude mixture without performing any time consuming isolation. Dereplication therefore may be applied for that purpose since allows a fast *in situ* detection of compounds from complex matrixes, such as crude herbal extracts, in low-amounts, by the use of chromatographic and/or spectroscopic techniques.¹¹

In this work, *Qualea grandiflora* and *Qualea cordata*, two Brazilian Vochysiaceae species, were analyzed by means of HPLC-ESI-QToF-MS/MS guided by the *in vitro* DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging capacity aiming at the detection of bioactive polyphenolic molecules.

Experimental

Plant material

Leaves and stem bark of *Q. grandiflora* and *Q. cordata* were collected in 2006 in Assis City (São Paulo State, Brazil), and identified by Prof. Giselda Durigan. Voucher numbers (GD-2033 for *Q. grandiflora* and GD-2125 for *Q. cordata*) were deposited in the Herbarium of the Botanic Garden in São Paulo, Brazil.

Extraction

The leaves and stem bark of *Q. grandiflora* and *Q. cordata* were dried at 40 °C under forced ventilation, powdered in an analytical mill and extracted by maceration using an ultrasonic bath for 30 min with EtOH/H₂O (8:2, v/v). After performing the extract concentration under vacuum in a rotatory evaporator (Büchi, Falwil, Switzerland), they were fractionated by liquid-liquid extraction with hexane and EtOAc. All solvents used were of analytical grade (Mallinckrodt, Paris, Kentucky, USA).

Determination of the radical scavenging activity

DPPH was used as a stable radical in a methanol solution (200 µmol, 2 mL). Rutin was used as the standard compound at 100, 80, 40, 20, 10 and 5 µmol L⁻¹. Concentrations of 1.7, 2.7, 3.3, 6.7, 10.0, 33.3 and 66.7 µg mL⁻¹ were prepared using crude extracts and fractions from both species (leaves and

stem bark). A DPPH solution (2 mL) in methanol (1 mL) was set as the negative control. The samples and the standard (100 µL), previously dissolved in methanol, were added to 200 µL of the fresh prepared reagent. Each mixture was shaken and held for 30 min at room temperature, in the dark. The evaluation of the reduced form of DPPH, generated *in situ*, was performed measuring its absorbance at 517 nm in a spectrophotometer Synergy HT (Bio-Tek instruments, USA). The radical scavenging activity of the samples was expressed in terms of IC₅₀ (concentrations that induce 50% inhibition of cell growth).

Sample preparation

Prior to the reverse phase LC analyses, approximately 20.0 mg of the selected extracts were dissolved in 1.0 mL of methanol/H₂O (2:8, v/v) doped with 2% of acetic acid (pH 4). For removal of non-polar molecules, each solution was purified by solid phase extraction (SPE), using Strata C₁₈ cartridges (Phenomenex, Torrance, CA, USA), previously activated with 5.0 mL of methanol and equilibrated with 5.0 mL of methanol/H₂O (1:1, v/v). The resulted samples were dried under nitrogen and set to a final volume of 5.0 mL of methanol/H₂O (1:1, v/v), filtered through a 0.45 µm GHP filter, and aliquots of 20.0 µL were injected directly into the HPLC.

HPLC-ESI-QToF-MS/MS analyses

HPLC-MS/MS analyses were performed on a Shimadzu LC-20A HPLC apparatus controlled by the software CLASS-VP 6.14 using a manual injector (Rheodyne 6171; Cotati, CA, USA) coupled with a quadrupole time-of-flight instrument (UltrTOF-Q, Bruker Daltonics, Billerica, MA). An Onyx Monolithic C₁₈ column (4.6 × 100.0 mm; Torrance, CA, USA) was coupled with a guard column (4.6 × 5.0 mm) of equivalent material.

The analyses were held using the following elution gradient: solvent A = aqueous acetic acid, 0.1% (v/v); solvent B = MeOH, acetic acid, 0.1% (v/v); elution profile = 0-30 min, 5-95% B (linear gradient), 30-35 min, 100% B (isocratic), 35-40 min, 95-5% B (return to initial conditions) and using a flow rate of 3.0 mL min⁻¹. ESI mass spectra, precursor as well as product ions were acquired in positive ion mode and recorded between *m/z* 50-1000. The mass spectrometer parameters were maintained constant for all analyses: 1000 scans *per s*; spectrum interval, 2 s; drying gas flow, 5.0 L min⁻¹; drying gas temperature, 180 °C; nebulizer gas pressure, 4 bar. For on-line MS/MS, *m/z* of the ion to be fragmented in the collision cell was used as the input for the mass spectrometer software. Collision induced

dissociation (CID) fragmentation was performed using N₂ (collision gas) on the isolated protonated molecules using collision energies between 10 and 40 eV.

Dereplication strategy

For dereplication of the plant extracts, a database was built containing all molecules and spectroscopic data reported for the Vochysiaceae.⁹ Thus, from the comparative analysis, the presence of the substances previously described¹¹ was detected. The scientific reports led to the creation of a database containing 92 molecules including 38 pentacyclic triterpenes, 10 ellagic acid derivatives, 36 flavonoids from the structural classes of flavanones, flavones, dihydroflavonols, flavanes and pyrrolidinoflavanes, 2 benzophenones, among others.⁹

For the on-line molecular identification, the high-resolution *m/z* ratio observed for the major chromatographic peaks were compared with the database in order to obtain the molecular formulas. From each formula, it was determined the relative error in ppm. Only molecular formulas bearing less than 10 ppm of error were considered for further MS/MS studies. Final molecules were proposed using collision induced MS/MS through the establishment of rational fragmentation patterns.

Determination of radical scavenging chromatographic profile

In order to evaluate the radical scavenging activity of the metabolites dereplicated by HPLC-ESI-QToF-MS/MS, the EtOAc fraction from the stem bark of *Q. grandiflora* and the EtOAc fraction from the leaves of *Q. cordata* were analyzed on a Shimadzu LC-20A HPLC apparatus controlled by the software CLASS-VP 6.14 coupled with a Gilson F203 fraction collector (Gilson, Middleton, WI, USA). The samples were injected (20 µL) at the same chromatographic conditions used on LC-MS experiments and the on-flow elution were collected in 96 well plates (2 mL well volume). For the scavenging activity evaluation, 100 µL of each collected well were transferred to 96 well microplates (350 µL well volume) and added 200 µL of 100 µmol L⁻¹ DPPH, prepared according to previous description. After gentle mixing and 30 min standing at room temperature, in the dark, the level of oxidation was determined using UV spectroscopy. Methanol (20 µL) was injected on LC, collected and tested as negative control.

Results and Discussion

Polyphenolic compounds usually show antioxidant properties associated with the capacity of delay or

prevent free radical mediated oxidation by forming a stable free radical.¹² Since polyphenolic compounds are associated with anti-inflammatory and anti-ulcer activity in some *Qualea* species,¹⁰ a bio-guided dereplication of *Q. grandiflora* and *Q. cordata* was devised based on *in vitro* radical scavenging assay. The results of the antioxidant activity showed a strong scavenging capacity for the hydro-alcohol extracts and fractions of *Q. grandiflora* and *Q. cordata*, especially for the EtOAc fraction from the stem bark of *Q. grandiflora* (IC₅₀: 10.0 µg mL⁻¹), final aqueous fraction from the stem bark (IC₅₀: 15.0 µg mL⁻¹) and crude extract from the leaves (IC₅₀: 22.0 µg mL⁻¹). EtOAc fraction from the leaves of *Q. cordata* also showed a strong scavenging activity (IC₅₀: 10.0 µg mL⁻¹), when compared with the positive control rutin (IC₅₀: 5.0 µg mL⁻¹).

In view of the above results, the EtOAc fraction from the stem bark of *Q. grandiflora* and the EtOAc fraction from the leaves of *Q. cordata* were selected for dereplication analyses as described in the Experimental section in order to detect and elucidate molecular metabolites that may be responsible for the observed scavenging activity.

Dereplication and detection of bioactive compounds

The active compounds of the EtOAc fraction from the stem bark of *Q. grandiflora* and the EtOAc fraction from the leaves of *Q. cordata* were analyzed using a DPPH bioassay coupled with HPLC-ESI-QToF-MS/MS towards the dereplication process. Figures 1 and 2 show the total ion current chromatograms (TIC, positive ion mode) as well as the percentage of DPPH inhibition (superimposed). Retention time, observed *m/z* and calculated *m/z*, relative errors, molecular formulae and MS/MS fragments are outlined in Tables 1 and 2.

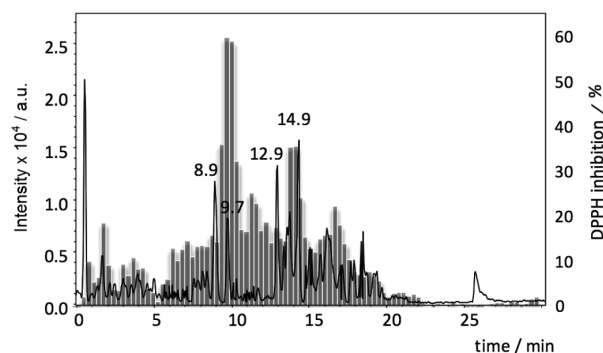


Figure 1. Total ion current (TIC) and percentage of DPPH inhibition (grey bars) of EtOAc fraction from the stem bark of *Q. grandiflora*.

Q. grandiflora analysis allowed the detection of three flavanones (3',4',5',5,6,7-hexahydroxy-8-methylflavanone (**1**), 8-methyl-naringenine (**2**) and 3',7-dimethoxy-

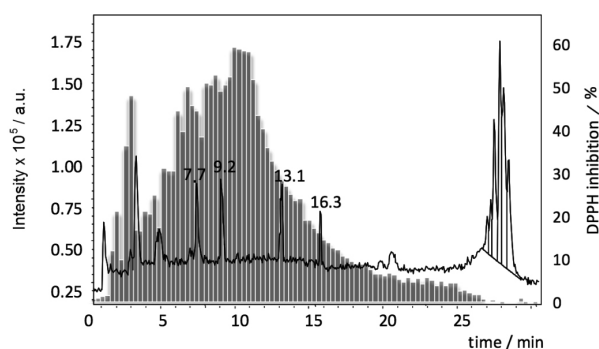


Figure 2. Total ion current (TIC) and percentage of DPPH inhibition (grey bars) of EtOAc fraction from the leaves of *Q. cordata*.

8-methyl-4',5,7-trihydroxyflavanone (**3**) and a benzophenone derivative (bis(4,6-dimethoxy-2-hydroxy-3-methylphenyl)-metanone (**4**)). Four flavanones (3',4'-dimethoxy-8-methyl-5,6,7-trihydroxyflavanone (**5**), 7-methoxy-6-methyl-3',4',5-trihydroxyflavanone (**6**), 6,8-dimethyl-3'-methoxy-4',5,7-trihydroxyflavanone (**7**) and 3',5'-dimethoxy-6,8-dimethyl-4',5,7-trihydroxyflavanone (**8**)) were as well detected for *Q. cordata* (Figure 3).

To validate the HPLC-MS results, rational mechanistic fragmentation proposals were established for all detected molecules using CID tandem mass spectrometry.

Fragmentation patterns for all detected flavonoids are depicted in Figure 4 following the proposed pathway: all

compounds showed abundant $[M + H]^+$ ions consistent with a retro-Diels-Alder (RDA) cleavage followed by elimination of carbon monoxide and/or neutral losses. Additionally, no dehydration previous to RDA was observed, suggesting that C-3 is not oxidized.¹³

For the compound **1**, a trihydroxylation on B ring due to RDA fragmentation (m/z 153.05), followed by a loss of water on the ion containing the B ring (m/z 135.04), was proposed.

For 8-methyl-naringenine (**2**), a second RDA reaction to explain the ion m/z 121.06 and subsequent allene formation for m/z 95.05 were proposed.

For *O*-methyl flavanones, the substituent positions were proposed by RDA rearrangements, radical fragmentations and neutral eliminations.^{13,14}

According to literature, C-5 and C-8 *O*-methyl flavanones lead to prominent loss of methyl radical (m/z 15), not observed on compounds **3** and **6**, suggesting they are 7-*O*-methyl flavanones, already reported for Vochysiaceae species.^{9,14}

For the substances **3** and **7**, MS data suggested an *O*-methyl substituent at B or C rings due to an observed RDA fragment. Furthermore, the absence of characteristic *O*-methyl fragmentations ($[M - 15]^+$ and $[M - 16]^+$) indicated the substituent position at C-3'.^{13,14}

For the substance **5**, a neutral loss of methane indicated the presence of an *O*-methyl group at C-3' and C-4',

Table 1. HPLC-ESI-QToF-MS/MS data for ethyl acetate fraction from the stem bark of *Q. grandiflora*

Retention time / min	Substance	Observed m/z , $[M + H]^+$	Calculated m/z	Error / ppm	Emulated molecular formula	MS/MS data
8.9	3',4',5',5,6,7-hexahydroxy-8-methylflavanone (1)	335.0768	335.0767	-0.3	$C_{16}H_{15}O_8$	40 eV: 335 \rightarrow 183 ^{bp} ; 155; 153; 135
9.7	8-methyl-naringenine (2)	287.0917	287.0919	0.7	$C_{16}H_{15}O_5$	15 eV: 287 \rightarrow 167 ^{bp} ; 139; 121; 95
12.9	3',7-dimethoxy-8-methyl-4',5,7-trihydroxyflavanone (3)	347.1129	347.1131	0.6	$C_{18}H_{19}O_7$	40 eV: 347 \rightarrow 197; 169 ^{bp} ; 151
14.9	bis(4,6-dimethoxy-2-hydroxy-3-methylphenyl)-metanone (4)	363.1421	363.1444	6.3	$C_{19}H_{23}O_7$	40 eV: 363 \rightarrow 195 ^{bp} ; 167

bp: peak base.

Table 2. HPLC-ESI-QToF-MS/MS data for the ethyl acetate fraction from the leaves of *Q. cordata*

Retention time / min	Substance	Observed m/z , $[M + H]^+$	Calculated m/z	Error / ppm	Emulated molecular formula	MS/MS data
4.7	3',4'-dimethoxy-8-methyl-5,6,7-trihydroxyflavanone (5)	347.1119	347.1131	3.5	$C_{18}H_{19}O_7$	40 eV: 347 \rightarrow 183; 165; 155 ^{bp} ; 149
5.9	7-methoxy-6-methyl-3',4',5-trihydroxyflavanone (6)	317.1025	317.1025	0.0	$C_{17}H_{17}O_6$	15 eV: 317 \rightarrow 181 ^{bp} ; 137; 119
8.4	6,8-dimethyl-3'-methoxy-4',5,7-trihydroxyflavanone (7)	331.1165	331.1181	4.8	$C_{18}H_{19}O_6$	40 eV: 331 \rightarrow 181 ^{bp} ; 153; 151
10.3	3',5'-dimethoxy-6,8-dimethyl-4',5,7-trihydroxyflavanone (8)	361.1273	361.1287	3.9	$C_{19}H_{21}O_7$	40 eV: 361 \rightarrow 181 ^{bp} ; 163; 153

bp: peak base.

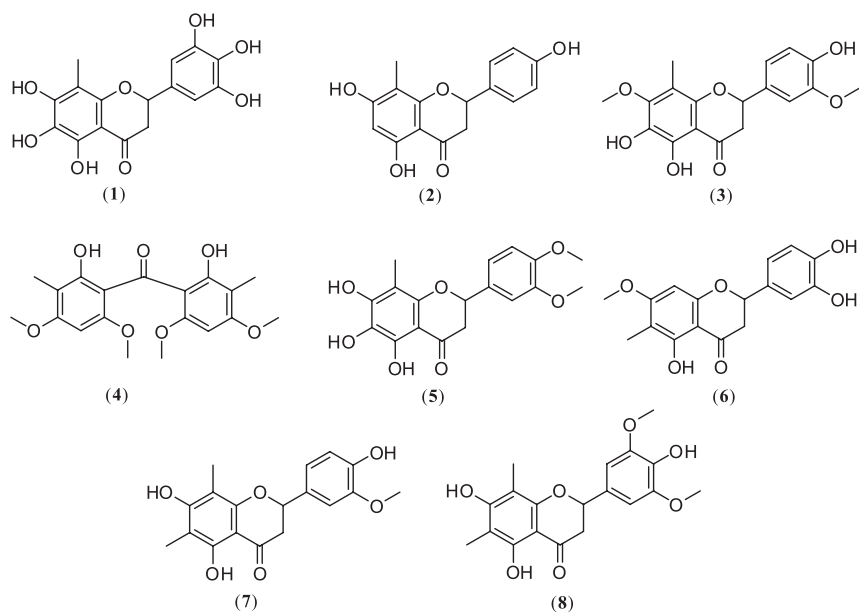


Figure 3. Molecules detected of *Q. grandiflora* and *Q. cordata* by HPLC-ESI-QToF-MS/MS.

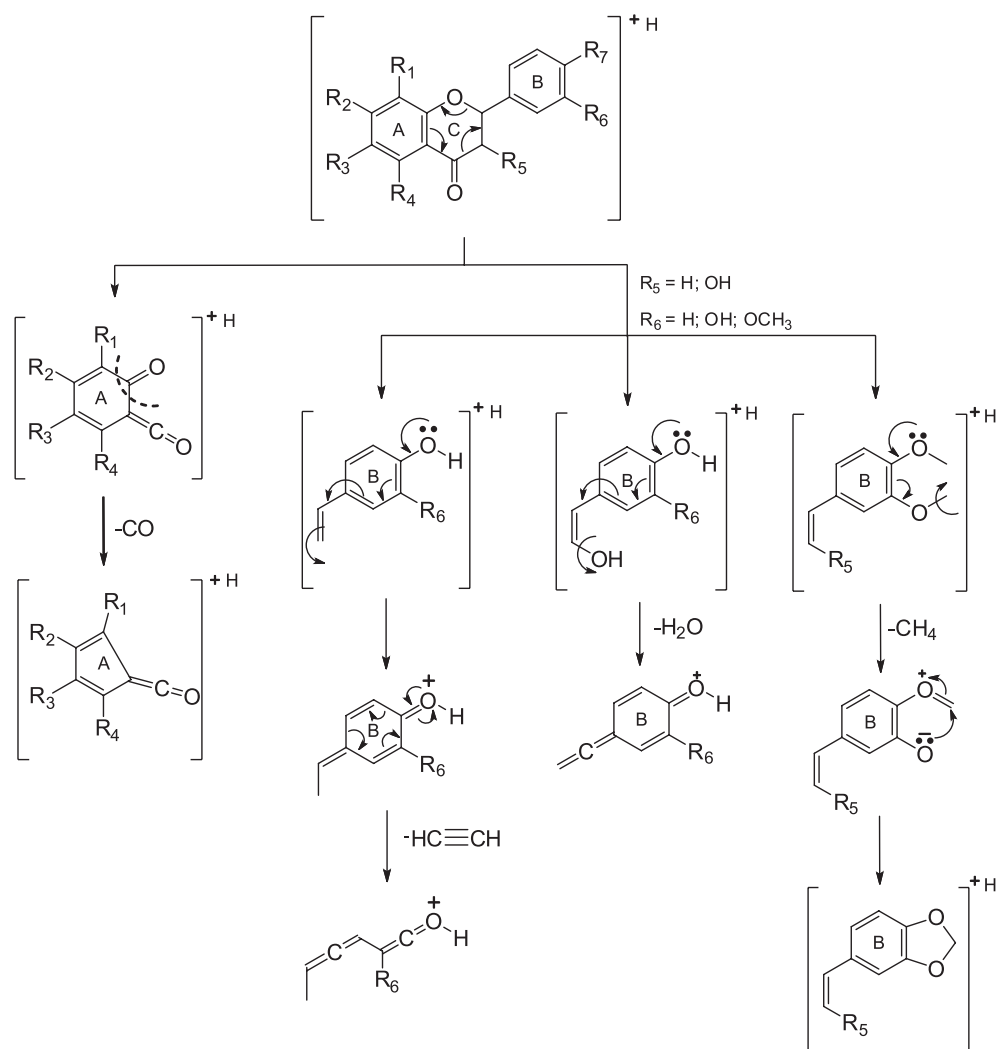


Figure 4. Proposed pathway fragmentation of the protonated flavonoids from *Qualea* species.

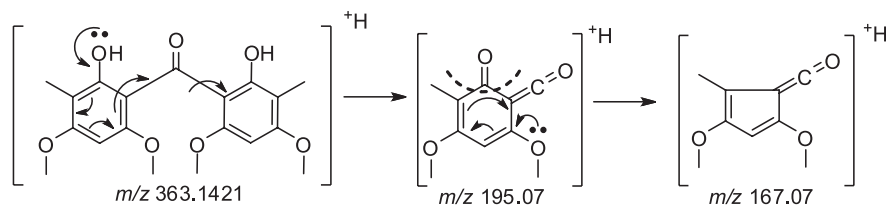


Figure 5. Proposed pathway fragmentation of the protonated benzophenone (**4**) from *Qualea grandiflora*.

in which the fragmentation can be rationalized by the formation of a C-3'/C-4' methylenedioxy intermediate. In the case of 3,3'-*O*-methyl substituents, two methane losses should be expected to be observed.¹³

Molecule **8** showed the absence of $[M - 15]^+$ and $[M - 16]^+$ indicating that the *O*-methyl groups may be at C-3' and C-5' (*meta* positions), restricting the formation of the methylenedioxy intermediate (3',4'-*O*-dimethyl) or C-2'/C-3 furan ring (3-*O*-methyl).^{13,14}

The detected benzophenone was proposed as bis(2-hydroxy-4,6-dimethoxy-3-methylphenyl)-metanone (substance **4**, Figure 5), justified by the cleavage of the carbonyl bond (m/z 195.07) followed by a loss of carbon monoxide (m/z 167.07). The observed ions are in agreement with MS data previously reported from the isolated benzophenone.¹⁵

The percentage of DPPH inhibition of the detected peaks showed the high radical scavenging activity, supporting the preliminary antioxidant results obtained for the fractions. This result suggests that the activity may be related to the dereplicated molecules since the observed phenolic compounds bear substituent arrangements that allow the delocalization of radicals.

The substitution pattern of the methyl groups proposed for carbons C-6 and C-8 is unusual in plants but already described in some Vochysiaceae species.⁹ All MS and MS/MS experiments carried out were unable to confirm the exact position of these substituents since there was no observed fragmentation capable to provide such information. Other analytical strategies such as derivatization followed by MS analysis or the use of nuclear magnetic resonance (NMR) may shed further information in order to conclude the *C*-methyl pattern for those flavanones.

Conclusions

The dereplication of *Qualea* species using HPLC-ESI-QToF-MS/MS was applied for the first time. LC-MS technique coupled with *in vitro* DPPH assay has proven to be rapid and sensitive, providing a great deal of preliminary information about the content and nature of radical scavenging properties from detected molecules without the need of isolation. In this study,

seven flavanones and a benzophenone were detected by retention times, accurate ESI mass spectra and further CID tandem experiments that allowed the proposal of rational fragmentation pathways for each compound.

Supplementary Information

All MS and MS/MS spectra of the detected molecules (Figures S1-S8) are available free of charge at <http://jbcs.sbq.org.br> as PDF file.

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