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Short communication

Use of on-line liquid chromatography–nuclear magnetic resonance spectroscopy for the rapid investigation of flavonoids from *Sorocea bomplandii*

Fabio D.P. Andrade[a], Lourdes C. Santos[a], Markus Datchler[b], Klaus Albert[b], Wagner Vilegas[a,*]

[a]*Instituto de Química de Araraquara, Universidade Estadual Paulista, CP 355, 14801-970, Araraquara, SP, Brazil*
[b]*Institute of Organic Chemistry, University of Tübingen, Auf der Morgenstelle 18, D-72076 Tübingen, Germany*

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Abstract

This paper reports the identification of di- and triglycosylated flavonoids from *Sorocea bomplandii* (Moraceae) by liquid chromatography coupled on-line to nuclear magnetic resonance (LC–NMR). These glycosylated flavonoids may be used as a taxonomic marker in future work.

Keywords: *Sorocea bomplandii*; Nuclear magnetic resonance spectroscopy; Flavonoids

1. Introduction

Hyphenated techniques are increasingly utilized in the field of natural products chemistry to analyze crude plant extracts [1]. The major advantages over the traditional off-line techniques are the rapidity of the analyses and the small amounts of material required. Column liquid chromatography coupled to nuclear magnetic resonance (LC–NMR) is a recent technique. It has already been used to study small amounts of many compounds including complex plant extracts [2–8].

In Brazil, the infusion of the leaves of *Maytenus aquifolium* and *M. ilicifolia* (Celastraceae, known as 'espinheira-santa'—holly spines) are used in folk medicine with proven biological activity against gastritis and ulcers [9]. *Sorocea bomplandii* Baillon (Moraceae) has morphological characteristics very similar to those of *Maytenus* species and has already been found in commerce as an adulteration [10]. The toxicological evaluation indicated that *S. bomplandii* does not show acute toxicity in rats [11]. However, the lack of the knowledge of its chemical composition represent a risk for its use as a medicinal plant.

Previous reports indicated that methanol extracts of the roots of *S. bomplandii* and *S. ilicifolia* afforded Diels–Alder adducts between chalcones and prenylated compounds [12–16]. The hexane and methylene chloride extracts of the leaves of *S. bomplandii* led to the isolation of pentacyclic triterpenes derived from oleanane, ursane and lupane skeleta [17]. Vilegas et al. [10] reported the detection of di- and triglycosylated flavonoids in the leaves of

*Corresponding author. Tel.: +55-16-201-6668; fax: +55-16-222-7932.

E-mail address: vilegasw@iq.unesp.br (W. Vilegas).

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S. bomplandii by high-performance thin-layer chromatography (HPTLC) as a way to distinguish between *Sorocea* and *Maytenus* plants, based on the fact that leaves from *Maytenus* species produces mainly quercetin and kaempferol tetraglycosides [18]. However, the structure of the flavonoids from *Sorocea* leaves were only suggested based on their R_F and on the monitoring of the plates with natural products–polyethylene glycol (NP–PEG) reagent [19].

Previous attempts to investigate the flavonoids using the traditional phytochemical approach (isolation by chromatographic techniques followed by identification by spectrometric techniques) was not successful due to the tiny amounts of these compounds in the leaves [17].

LC–mass spectrometry (MS) is a useful technique for the investigation of flavonoid glycosides in plant extracts [3]. However, differences between flavone and flavonol skeleta and also the substitution pattern of rings A, B and C are more easily established by LC–NMR experiments.

The aforementioned facts led us to investigate the glycosylated flavonoids present in the leaves of *S. bomplandii* using LC–NMR coupling.

2. Experimental

2.1. Plant material and sample preparation

Authentic samples of the leaves of *S. bomplandii* (Moraceae) were collected in Jaguariava, Paraná, Brazil in January 1992 and identified by Armando C. Cervi Voucher n° UPCB 20263 (Herbarium of the Universidade Federal do Paraná).

The leaves of *S. bomplandii* (88 g) were dried at 40 °C, powdered and boiled with 1 l water for 10 min. The infusion was cooled overnight and filtered. It was fractionated over an Amberlite XAD-2 column (25×3.5 cm I.D., flow-rate 1 ml/min) eluted first with 1 l water and then with 200 ml methanol. The methanolic fraction was evaporated to dryness under vacuum. Ten milligrams of the methanolic fraction were dissolved in 1 ml of methanol in an ultrasonic bath for 15 min and filtered in a 0.45 μm filter. Ten microliters of this solution were directly injected into the LC–NMR system.

2.2. Apparatus and chemicals

For LC separations, methanol (LiChrosolv gradient grade, Merck, Darmstadt, Germany) and D_2O 99.9% (Deutero, Herresbach, Germany) were used.

Analyses were performed under ambient conditions using a Merck Lichrograph L-6200A gradient pump and a Merck Lichrograph L-4000/4200 UV/VIS absorbance detector. Separations were carried out on a 250×4.6 mm stainless steel column (Bischoff, Leonberg, Germany) with a particle size of 3 μm and a pore diameter of 200 Å. Eluents were A=MeOH and B=D_2O. Separation was performed first with isocratic elution with MeOH–D_2O 40:60 for 20 min and then with linear gradient from 40% to 80% A within 40 min. The flow-rate was maintained at 0.7 ml/min and elution was monitored by absorbance detection at 370 nm.

All on-line LC–NMR experiments were conducted on a Bruker AMX 600 spectrometer (1H resonance frequency 600.13 MHz, Bruker, Rheinstetten, Germany) equipped with an LC inverse probe with a detection volume of 120 μl. The NMR spectra are referenced to the solvent signal of methanol at δ 3.3 and δ 4.7. The chromatographic equipment and the BPSU (Bruker Peak Sampling Unit) necessary for stopped-flow experiments were controlled by Chromstar Software (Bruker). For recording the ^{1}H-NMR spectra solvent suppression was necessary. This was done by employing shaped pulses (rectangle pulse with a length of 100 ms) of 1.6 s prior to the start of the acquisition for low power presaturation of the two methanol signals (δ 3.3 and δ 4.7). For the ^{1}H-NMR stopped-flow measurements 2 K transients were accumulated, which requires a total acquisition time of about 1 h. A time domain of 16 k and a sweep width of 9600 Hz was used for acquisition. Processing was performed with 1D WINNMR software (Bruker). For all spectra, zero filling up to 32 K data points and an exponential multiplication of the FID with a line broadening of 0.3 Hz was performed before Fourier transformation.

The LC–ES-MS analysis was performed in a Fisons VG Platform operating at 70 V, positive mode.

Chromatographic conditions were similar to those previously described.

3. Results and discussion

Fig. 1 shows the LC chromatogram of the compounds present in the leaves of *S. bomplandii* using a C_{30} column. After optimization, we have obtained base-line separation for the three main peaks (R_t= 31.0, 47.5 and 51.0 min) and good separation for the remaining peaks between 10 and 60 min. Separation of the compounds was performed within 60 min, with a simple linear gradient procedure using methanol–deuterium oxide, resulting in a very fast screening of the extract from *S. bomplandii*. The high selectivity of the C_{30}-phase led to a good separation of the *S. bomplandii* flavonoids.

Fig. 2 shows the ^{1}H-NMR spectra of the three major peaks. Minor peaks were not considered in this analyses because chromatographic resolution was not good enough, leading to a mixture of compounds in the same peak. Kaempferol and quercetin derivatives can be easily identified and differentiated by their ^{1}H-NMR spectra [20]. Kaempferol derivatives presents two A_2X_2 doublets near δ 8.0 and δ 6.9, with J=8 Hz, assigned to the *p*-substituted aromatic B ring of the flavonoid aglycon [20]. On the other hand, quercetin derivatives present three groups of characteristic ABX aromatic signals: a doublet at δ 7.6 (J=2 Hz, H2′), a double-doublet at δ 7.5 (J=8 Hz and 2 Hz, H6′) and a doublet at δ 6.9 (J=8 Hz, H5′). Therefore, the spectra shown in Fig. 2 allows to identify one quercetin derivative in the peak that elutes with R_t=47.5 and two different kaempferol derivatives in the peaks with R_t=31.0 and 51.0 min.

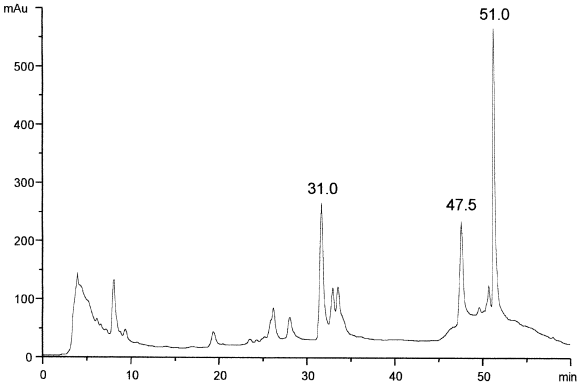

Fig. 1. LC chromatogram with detection at 370 nm of the flavonoid fraction of the leaves from *S bomplandii*.

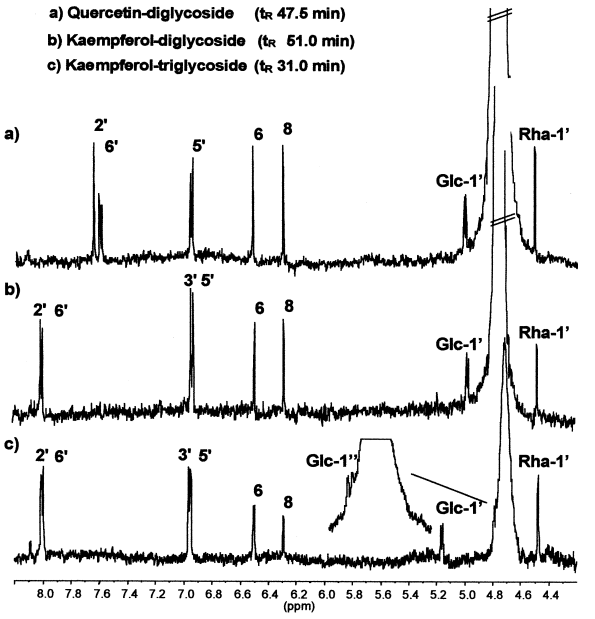

Fig. 2. Stopped-flow ^{1}H-NMR spectra of (a) peak at 47.5 min, (b) peak at 51.0 min and (c) peak at 31.0 min.

The number of sugar linked in each flavonoid aglycone could be determined observing the anomeric region of the spectra (between δ 4.0 and 5.5) [20]. Despite some interference of solvent signal between δ 3.3 and δ 4.7, the anomeric signals of the three flavonoids were clearly detected. A doublet (3H, J=6.0 Hz) at δ 1.00 refers to the CH_3 group of rhamnose moieties.

The ^{1}H-NMR spectrum of the kaempferol derivative with R_t=51.0 min (Fig. 2a) presents the signal of two anomeric protons at δ 5.0 (d, J=7.5 Hz) and δ 4.5 (d, J=1.5 Hz). These chemical shifts and coupling constants signals are consistent with one glucose moiety with β configuration directly bonded to the 3-OH of the aglycone and one rhamnose moiety with α configuration bonded to the 6′-OH of the glucose unity [20]. Therefore, the substance with R_t=44.9 min may be identified as being kaempferol-3-*O*-β-D-rhamnopyranosyl(1→6)glucopyranoside.

The ^{1}H-NMR spectrum of the quercetin derivative

with R_t=47.5 min (Fig. 2b) shows an anomeric region almost superimposable to that of the quercetin derivative. Thus, it could be identified as being quercetin-3-*O*-β-D-rhamnopyranosyl(1→6)glucopyranoside. This is also compatible with its lower R_t when compared to the kaempferol derivative, as quercetin has one more phenolic hydroxyl group than the kaempferol aglycone.

The ^{1}H-NMR spectrum of the kaempferol derivative with R_t=31.0 min (Fig. 2c), shows three anomeric proton signals. Two of them present chemical shifts at δ 5.2 (d, J=7.5 Hz) and at δ 4.8 (d, J=7.5 Hz). Their constant couplings suggests two glucose unities with β configuration. The third anomeric proton at δ 4.5 (d, J=1.5 Hz) indicates a rhamnose moiety with α configuration. These chemical shifts do not allow to unequivocally identify the interglycosidic linkages. However, this compound may be identified as a kaempferol triglycoside.

Triglycoside flavonoids have a lower retention time than diglycosides because of their higher polarity. Under the conditions employed, triglycosides eluted after 25–35 min while diglycosides eluted after 45–55 min. Quercetin derivatives eluted ca. 3.5 min earlier than the respective kaempferol derivative, probably because the extra 3′-OH makes these compounds more polar.

Additional evidence for the identification of the flavonoids were obtained from LC–ESI-MS experiments in the positive mode at 70 V. Table 1 presents the most important fragmentations of the flavonoid glycosides from *S. bomplandii* that confirm the identity of the analyzed compounds.These fragmentation patterns agree well with the data obtained from the LC–NMR experiments and provide further support for the structures proposed.

Table 1
Main MS fragmentations of the flavonoids from *S. bomplandii* leaves

R_t (min)	Fragmentations	m/z (u)
31.0	$[M+H]^+$	757
	$[M\text{-rha}+H]^+$	611
	$[M\text{-rha-glc}+H]^+$	449
	$[M\text{-rha-2glc}+H]^+$	287
47.5	$[M+H]^+$	611
	$[M\text{-rha}+H]^+$	465
	$[M\text{-rha-glc}+H]^+$	303
51.0	$[M+H]^+$	595
	$[M\text{-rha}+H]^+$	449
	$[M\text{-rha-glc}+H]^+$	287

4. Conclusions

On-line LC–NMR allowed the fast identification of three flavonol glycosides in the leaves of *S. bomplandii* using only small amount of extracts and little organic or deuterated solvents. Clean-up of the extract was kept to a single filtration through a porous membrane. The flavonol glycosides could be characterized as being quercetin and kaempferol glycosides containing two or three sugars bound to the flavonoidic aglycones.

These results are important not only for a future quality control of the Brazilian ‘espinheiras-santas’ but also for providing an alternative tool to study minor constituents in polar phytomedicines using only small amounts of crude extracts.

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