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Application of On-line C_{30} RP-HPLC-NMR for the Analysis of Flavonoids from Leaf Extract of *Maytenus aquifolium*

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The application of on-line C_{30}-reversed-phase high-pressure liquid chromatography–nuclear magnetic resonance spectroscopy is described for the analysis of tetraglycosylated flavonoids in aqueous and hydroalcoholic extracts of the leaves of *Maytenus aquifolium* (Celastraceae). Triacontyl stationary phases showed adequate separation for on-line ^{1}H-NMR measurements at 600 MHz and allowed the characterisation of these flavonoids by detection of both aromatic and anomeric proton signals. Copyright © 2000 John Wiley & Sons, Ltd.

Keywords: On-line HPLC-NMR; C_{30}-stationary phase; flavonoids; *Maytenus* (Celastraceae).

INTRODUCTION

The application of coupling techniques to the investigation of polar natural products from plant origin is becoming of increasing importance. The development of HPLC coupled with UV and diode array detection was the first step for the direct investigation of polar phytocompounds, including aqueous infusions, but it was obviously limited to UV-absorbing substances. More recently, several methods of coupling between HPLC and MS have evolved, allowing the detection and identification of a broader range of natural compounds in plant material and foods (Wolfender *et al.*, 1992, 1994; Verpoorte and Niessen, 1994; Careri *et al.*, 1998), including glycosides present in teas (Finger *et al.*, 1992). However, complete information about chemical composition is usually not possible by HPLC-MS owing to the possible occurrence of sugar and aglycone isomers that cannot be easily differentiated by their MS. Moreover, many natural products can undergo severe degradation when submitted to the elevated temperatures required for some MS interfaces, and sometimes the molecular ions are in low abundance or are even not detectable (Niessen and van der Greef, 1992).

High-resolution NMR is the most important method for the structural elucidation of complex compounds. In particular, for unstable compounds such as carotenoid stereoisomers, on-line HPLC-NMR is the method of choice since both light and oxygen may be excluded (Dachtler *et al.*, 1999), whereas LC-MS is not suitable for this kind of situation. The direct coupling of HPLC with NMR gives, in many cases, more complete structural information about complex plant compounds such as glycosides. One-dimensional (1D) and 2D NMR experiments can provide not only information concerning the aglycone structure, but can also give data for the identification of the sugars and for the determination of the sequence of the saccharide chain in the molecule. Direct HPLC-NMR coupling is one of the most promising tools for the fast analysis of plant extracts that have recently been developed (Wolfender *et al.*, 1998).

A crucial step to on-line HPLC-NMR experiments is the chromatographic separation prior to the introduction of the compound of interest into the NMR probe: spectra usually must be taken from the heart of chromatographic peaks, which corresponds to the maximum concentration of the available sample. However, usually HPLC columns must be overloaded in order to furnish a detectable amount of each desired compound, which must also be base-line separated. These requirements for on-line HPLC-NMR led to the search for new stationary phases with higher resolution and selectivity, such as triacontyl reversed phase (C_{30}-RP) in which the longer chain provides better resolution than conventional C_{18} columns in the separation of low polarity compounds (Pursch *et al.*, 1996). Furthermore, owing to their high loading capacity, C_{30} phases can be used with added advantage for on-line HPLC-NMR experiments. These new stationary phases have been utilised successfully for the HPLC-NMR analysis of a number of samples, including β-carotene isomers (Strohschein *et al.*, 1997), vitamin A derivatives (Albert *et al.*, 1996), tocopherol isomers (Strohschein *et al.*, 1998) and lutein and zeaxanthin isomers in bovine retina (Dachtler *et al.*, 1998). However, C_{30}-RP has not been evaluated for the analysis of polar compounds or for the analysis of infusions prepared from medicinal plants.

The infusion from the leaves of *Maytenus aquifolium*

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Martius (Celastraceae), a Brazilian species known as 'espinheira santa', is popularly used in the treatment of gastric ulcers and gastritis (Carlini, 1988). Previous phytochemical work with the infusion led to the isolation and structure elucidation of the two main flavonoid tetraglucosides: quercetin 3-*O*-α-L-rhamnopyranosyl (1→6)-*O*-[β-D-glucopyranosyl(1→3)-*O*-α-L-rhamnopyranosyl(1→2)-*O*-β-D-galactopyranoside] (**1**) and kaempferol 3-*O*-α-L-rhamnopyranosyl(1→6)-*O*-[β-D-glucopyranosyl(1→3)-*O*-α-L-rhamnopyranosyl(1→2)-*O*-β-D-galactopyranoside] (**2;** Sannomiya *et al.*, 1998; Vilegas *et al.*, 1999).

Flavonoids are an important class of compounds with a wide range of biological activities, including antioxidative, anti-mutagenic and anti-carcinogenic effects (Harborne, 1994). The chromatographic analysis (HRGC, HPTLC, HPLC, LC-MS, CE) of the leaves and of the infusion of *M. aquifolium* has been systematically performed in order to propose analytical procedures for the standardisation of 'espinheira santa' preparations (Vilegas *et al.*, 1994, 1995, 1998a, b), mainly using triterpenoids or flavonoids as chromatographic markers. The evaluation of such compounds in 'espinheira santa' is imperative since many drugs found in commerce are adulterated (Vilegas and Lanças, 1997). The present paper describes the HPLC-NMR analysis of an infusion from the leaves of *M. aquifolium* using a C_{30} stationary phase.

EXPERIMENTAL

Plant material and extraction. Authenticated leaves of *Maytenus aquifolium* Martius were collected in Ribeirão Preto by Dr Ana Maria Soares Pereira (UNAERP, Ribeirão Preto, SP, Brazil) from cultivated specimens. A voucher specimen is maintained in our laboratory. The leaves were dried at 40°C, powdered and sieved; only particles between 0.5 and 1.0 mm were used. A sample (1.0 g) of the plant material was boiled with 10 mL water for 10 min, after which the infusion was cooled, filtered and evaporated to dryness. The material was resuspended in 3.0 mL methanol, centrifuged and directly analysed by HPLC-NMR.

HPLC analysis. Chromatography was performed under ambient conditions using a Merck (Darmstadt, Germany) Lichrograph L-6200A gradient pump and a Merck Lichrograph L-4000/4200 UV–VIS absorbance detector. Separations were carried out on a stainless steel column (250 × 4.6 mm i.d.) containing C_{30}-reversed phase (particle size, 3 μm; pore diameter, 200 Å; Bischoff, Leonberg, Germany) eluted with an isocratic mixture of methanol:water (50:50, v/v). The flow-rate of the mobile phase was 0.8 mL/min, and elution was monitored at 370 nm. For the LC-NMR experiments 70 μL of a 0.1% solution (w/v) of the plant extract was injected onto the column.

On-line LC-NMR coupling. On-line experiments were conducted using a Bruker (Rheinstetten, Germany) model AMX 600 spectrometer equipped with an LC inverse probe with a detection volume of 120 μL. The chromatographic equipment and the peak sampling unit (BPSU-12, Bruker) necessary for stopped-flow experiments were controlled by Chromstar software (Bruker).

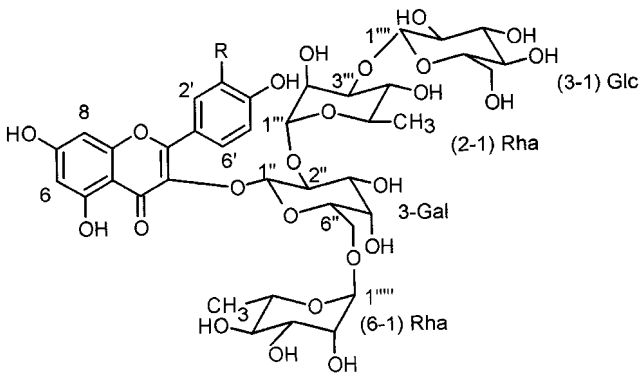
To record the ^{1}H-NMR spectra, solvent suppression was necessary. This was performed using shaped pulses (rectangle pulse with a length of 100 ms) for low-power presaturation of the two methanol signals (δ 3.3 and 4.7) for 1.6 s prior to the start of the acquisition. For the ^{1}H-NMR stopped-flow measurements, 2K transients were accumulated in a total acquisition time of about 1 h using a time domain of 16K and a sweep width of 9600 Hz. Processing was performed with 1D WINNMR software (Bruker). For all spectra, zero filling up to 32K data points and an exponential multiplication of the FID with a line broadening of 1 Hz were performed before Fourier transformation.

RESULTS AND DISCUSSION

Previous analytical studies, using C_8 and C_{18} columns, of the flavonoids from *Maytenus* indicated aqueous formic acid:acetonitrile mixtures as the best mobile phases for the separation of these compounds. However, the concentration of the buffer is often higher than the concentration of the analyte and a large number of solvent signals are visible in the NMR spectrum. Therefore, this buffer system is not suitable for on-line HPLC-NMR experiments. In the present work several methanol:deuterium oxide mixtures were tested, and the 1:1 (v/v) mixture gave satisfactory results considering chromatographic separation vs. HPLC-NMR parameters.

Methanol shows signals at δ 3.3 and 4.7, and can lead to distorted NMR peaks. A challenge in the on-line HPLC-NMR analysis of glycosylated compounds is to suppress the solvent signals without affecting the characterisation of the sugar signals, mainly those corresponding to the anomeric protons. The cost associated with the use of deuterium oxide in the mobile phase is acceptable; most other solvents commonly employed in LC are very expensive in deuterated form.

A typical extract of the leaves of *M. aquifolium* has a simple chromatographic profile with two main peaks corresponding to compounds **1** and **2** (Fig. 1). Under the



1 R = OH
2 R = H

Structures 1 and 2

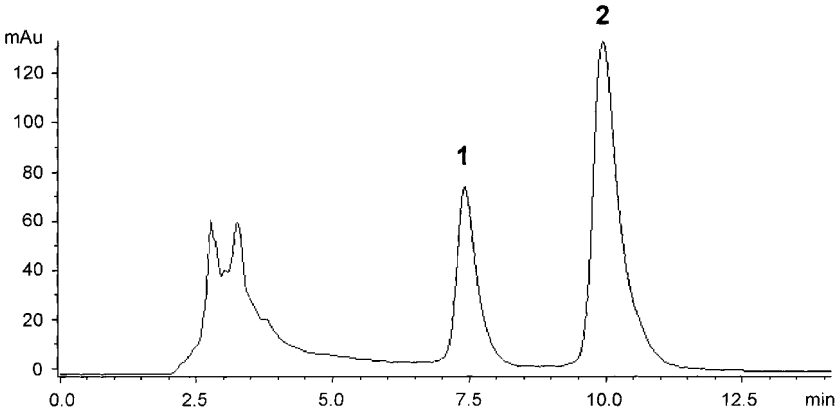


Figure 1. HPLC chromatogram with detection at 350 nm of the flavonoid fraction from the infusion of leaves of *Maytenus aquifolium*. Key to peak identity: **1**, quercetin 3-*O*-α-L-rhamnopyranosyl(1→6)-*O*-[β-D-glucopyranosyl(1→3)-*O*-α-L-rhamnopyranosyl(1→2)-*O*-β-D-galactopyranoside]; **2**, kaempferol 3-*O*-α-L-rhamnopyranosyl(1→6)-*O*-[β-D- glucopyranosyl(1→3)-*O*-α-L-rhamnopyranosyl(1→2)-*O*-β-D-galactopyranoside]. (For chromatographic protocol see the Experimental section.)

conditions employed here, **1** elutes at 7.5 min while the less polar **2** elutes at 10.0 min: thus, the chromatographic conditions afforded a baseline resolution and allowed the separation of these two tetraglucosylated flavonoids in a convenient time.

Despite the large amount of sample injected onto the C_{30} column, which was necessary to produce sufficient sample concentration in the NMR flow cell, no overloading effects are visible. This high-loading capacity of the C_{30} phases makes them preferably usable for on-line HPLC-NMR experiments dealing with natural products, because it allows the separation and identification of minor compounds, which can be responsible for the biological activity. Moreover, the selectivity is quite high and almost no peak tailing is observed.

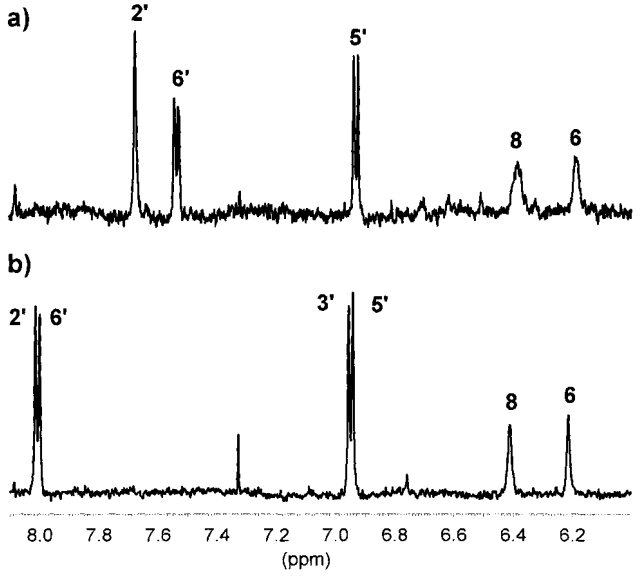


Figure 2. Stopped-flow ^{1}H-NMR (600 MHz) of the aglycone region of the spectra of (a) quercetin 3-*O*-α-L-rhamnopyranosyl(1→6)-*O*-[β-D-glucopyranosyl(1→3)-*O*-α-L-rhamnopyranosyl(1→2)-*O*-β-D-galactopyranoside] (**1**); and (b) kaempferol 3-*O*-α-L-rhamnopyranosyl(1→6)-*O*-[β-D-glucopyranosyl(1→3)-*O*-α-L-rhamnopyranosyl(1→2)-*O*-β-D-galactopyranoside] (**2**).

The stopped-flow ^{1}H-NMR spectral data for the peaks with retention times 7.5 and 10.0 min, corresponding to compounds **1** and **2**, respectively, fully correlate with those reported previously as obtained by traditional phytochemical studies (Sannomiya *et al.*, 1998; Vilegas *et al.*, 1999). The differences in the chemical shift values are due to the change of solvent necessary for LC-NMR and are in the range of 0.05 ppm for the aromatic protons and less than 0.2 ppm for the anomeric protons. The ^{1}H-NMR spectra (600 MHz; aromatic region) of the aglycones of **1** and **2** are shown in Fig. 2. The protons of the aromatic portion of the spectra of **1** [Fig. 2(a)] clearly show signals corresponding to three ABX systems at δ 7.67 (1H, d, J = 1.5 Hz), at δ 7.53 (1H, dd, J = 8.5, 1.5 Hz) and at δ 6.92 (1H, d, J = 8.5 Hz) due to the H-2′, H-6′ and H-5′, respectively, from the B-ring of the flavonoid nucleus, as well as signals for two AB systems at δ 6.38 (1H, d, J = 1.5 Hz) and at δ 6.19 (1H, d, J = 1.5 Hz), corresponding to the H-8 and H-6 from the A-ring. These signals allowed the unequivocal recognition of the aglycone of **1** as being quercetin (Harborne, 1994). Compound **2** [Fig. 2(b)] shows two doublets with J = 8.0 Hz, integrating for 2H each, at δ 8.00 and 6.94 and clearly corresponds to the H-2′/H-6′ and H-3′/H-5′, respectively, of the B-ring of a flavonoid nucleus, as well as two signals at δ 6.21 and 6.41 corresponding to H-6 and H-8 from a kaempferol derivative (Harborne, 1994).

The aliphatic region of the stopped-flow ^{1}H-NMR spectra of **1** and **2** are almost identical, thus revealing a similar saccharide chain. As shown for the kaempferol tetraglycoside in Fig. 3, the four anomeric protons are clearly distinguished at δ 5.26 (1H, d, J = 7.5 Hz, gal-1″), at δ5.13 (1H, d, J = 1.5 Hz, rha-1‴), at δ4.59 (1H, d, J = 7.5 Hz, glc-1⁗) and at δ4.47 (1H, d, J = 1.5 Hz, rha-1⁗). The two doublets that integrate for 3H each at δ1.10 (d, J = 6.0 Hz, rha-6⁗) and at δ1.02 (d, J = 6.0 Hz, rha-6‴) were assigned to the two rhamnose moieties. The coupling constants of J = 7.5 Hz indicate that the galactose and the glucose moieties have β-configuration,

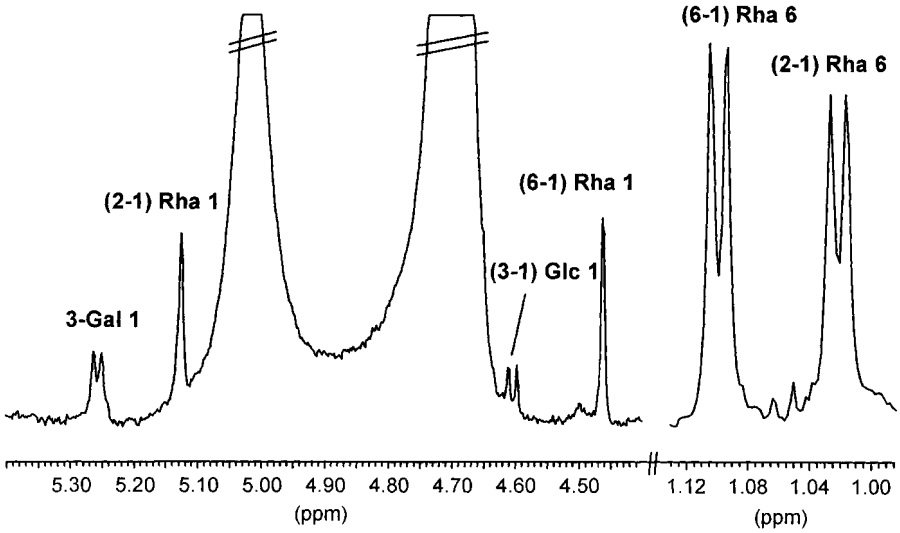


Figure 3. Stopped-flow ^{1}H-NMR (600 MHz) of the aliphatic region of the spectrum of kaempferol 3-*O*-α-L-rhamnopyranosyl(1→6)-*O*-[β-D-glucopyranosyl(1→3)-*O*-α-L-rhamnopyranosyl(1→2)-*O*-β-D-galactopyranoside] (**2**).

whereas the $J = 1.5$ Hz couplings indicate α-configuration for the rhamnose units. Despite the suppressed solvent signals, the anomeric protons were neither superimposed nor distorted. Thus, on-line LC-NMR measurements under the described conditions allowed the two complex flavonoid tetraglycosides from the infusion of *M. aquifolium* to be fully recognised.

On-line LC-NMR coupling using the newly developed C_{30} stationary phase is still an under-explored technique in the field of natural products chemistry. The present approach showed that LC-NMR coupling is one of the most promising techniques with which to investigate aqueous phytomedicines since it provides a quick and reliable method for the quality control of the complex constituents.

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