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Simultaneous characterisation and quantitation of flavonol glycosides and aglycones in noni leaves using a validated HPLC-UV/MS method

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ABSTRACT

The leaves of *Morinda citrifolia* L. (noni) have been utilized in a variety of commercial products marketed for their health benefits. This paper reports on a rapid and selective HPLC method for simultaneous characterization and quantitation of four flavonols in an ethanolic extract of noni leaves by using dual detectors of UV (365 nm) and ESI-MS (negative mode). The limits of detection and quantitation were between 0.012 and 0.165 μg/mL. The intra- and inter-assay precisions, in terms of percent relative standard deviation, are less than 4.38% and 3.50%, respectively. The accuracy, in terms of recovery percentage, ranged from 96.66% to 100.03%. Good linearity (correlation coefficient >0.999) for each calibration curve of standards was achieved in the range investigated. The contents of four flavonoids in the noni leaves varied from 1.16 to 371.6 mg/100 g dry weight.

1. Introduction

Noni (*Morinda citrifolia* L.), belonging to the family Rubiaceae, is an indigenous tree of the tropical zones of South Asia, Australia, Hawaii, and the islands of French Polynesia. Its fruit, leaves, seeds, bark, and roots have been traditionally used for prevention or improvement of various diseases, including arthritis, infections, colds, cancers, diabetes, etc. (Mc Clatchey, 2002; Wang et al., 2002). In the past decade, noni fruit juice has emerged on the world-wide market as a safe and popular health product due to its phytochemicals and nutrients. Also, noni leaves are well-known for their strong antioxidant activity, and have been shown to be safe in acute, subacute, and subchronic oral toxicity tests on mice (West, Tani, Palu, Tolson, & Jensen, 2007). Sparked by ancient Polynesian legends, noni leaves have been developed into health teas. The leaves are also the source for a variety of other health-promoting commercial products. Commercial noni leaf products have been available in Japan and United States for more than seven years, mainly for use in making infusions. However, some manufacturers produce capsules containing powdered noni leaves. The major world-wide source of noni leaves is French Polynesia, with leaves from this nation having undergone a safety evaluation (West et al., 2007). Other sources include Panama, Fiji, and Hawaii.

Previous phytochemical studies reveal that noni leaves contain a variety of phytochemical constituents, including terpenoids (Ahmad & Bano, 1980; Saludes, Garson, Franzblau, & Aguinaldo, 2002; Takashima et al., 2007); phytosterols, fatty acids, and their glycosides (Takashima et al., 2007); iridoids and their glycosides (Sang et al., 2001a,b,c,d; Sang et al., 2003); flavonol glycosides (Sang et al., 2001a), among others.

Flavonol glycosides appear to predominate in noni leaves, rutin and other flavonol glycosides have previously been identified in raw noni leaves (Sang et al., 2001a). However, the presence of flavonol aglycones in noni leaves has not been previously reported. Flavonoids have been indicated to possess a variety of biological activities (Garcia-Mediavilla et al., 2006; Kampkotter et al., 2007), and may play an important role in noni leaves. To date, there has been no validated analytical method for determining the flavonol constituents of noni leaves. In this paper, we report a rapid, simple, and selective HPLC method to simultaneously characterize and quantitate the contents of flavonol aglycones and their glycosides in noni leaves. As shown

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below, the analytical method is validated for its LOD, LOQ, linearity, precision, and accuracy.

2. Experimental

2.1. Chemicals and reagents

Methanol (MeOH) and water (H_2O) of HPLC grade, and ethanol (EtOH) of analytical grade were obtained from Sigma–Aldrich (St. Louis, MO, USA). Acetic acid (analytical grade) was purchased from Acros Organics (Morris Plains, NJ, USA). Chemical standards of quercetin-3-*O*-α-l-rhamnopyranosyl-(1 → 6)-β-d-glucopyranoside (rutin, **1**), kaempferol-3-*O*-α-l-rhamnopyranosyl-(1 → 6)-β-d-glucopyranoside (**2**), quercetin (**3**), and kaempferol (**4**) were isolated from French Polynesian fruit and leaves in our laboratory (Deng et al., 2007), and their purities (>99%) and structures were determined by HPLC, MS, and NMR. Their structures are presented in Fig. 1.

2.2. Chromatographic conditions and instrumentation

Chromatographic separation was performed on an Agilent 1100 series LC/UV/ESI-MSD SL (single quadrupole) spectrometer equipped with multiple-mode sources (ESI or APCI), Chem Station software, an 1100 well plate autosampler, an 1100 binary pump (Agilent Technologies, Palo Alto, CA), and an XTerra MS C18 column (4.6 mm × 250 mm; 5 μm, Waters Corporation, Milford, MA, USA). The 1100 binary pump was connected to two mobile phases: [A: 0.3% acetic acid in MeOH (v/v), and B: 0.3% acetic acid in H_2O (v/v) that was eluted at a flow rate of 1.0 mL/min]. The mobile phases were programmed consecutively in linear gradients: A, 35%–50% (B: 65%–50%), 0–10 min; A, 50%–60% (B: 50%–40%), 10–15 min; A, 60%–65% (B: 40%–35%), 15–18 min; A, 65%–98% (B: 35%–2%), 18–20 min; and an isocratic elution of A 100%, 20–30 min. The injection volume was 20 μL each of the sample solutions. The column temperature was maintained at 25 °C. The UV spectra were monitored over a range of 210 and 400 nm. The scan measurements, using negative ESI mode in the mass spectrometer, were performed with the following settings: heater temperature of nitrogen gas, 350 °C; flow of heated dry nitrogen gas, 5.0 L/min; nebulizer gas pressure, 40 psi; capillary voltage, −2500 V for negative and 4,000 V for positive; and a scan range, 100–700 *m/z*. These parameters were optimized in preliminary experiments to get the highest abundance of the targeted molecular-related ions.

2.3. Plant materials and sample preparation

Noni leaves were collected from French Polynesia and air-dried. A voucher specimen is deposited in our lab. One-hundred grams of noni leaves were extracted with 6 L of EtOH using the percolation method. The filtrate was then evaporated to dryness by using a rotary evaporator in vacuum, which yielded 7.8 g of noni leaf EtOH extract. A sample of leaf EtOH extract was prepared with MeOH to a concentration of 50 mg/mL for the analysis of **1–4**. The solution prepared for HPLC analysis was passed through a 0.20 μm micropore membrane filter before use.

2.4. Preparation of standard solutions and calibration curves

Standards **1–4** were accurately weighed and then dissolved in an appropriate volume of MeOH to produce corresponding stock standard solutions (3 mg/mL for standard **1** and 1 mg/mL each for standards **2–4**). Working standard solutions for calibration curves were prepared by diluting the stock solutions with MeOH at five concentrations in the range of 0.0025–3 mg/mL for the four standards. All stock and working solutions were kept at 0 °C in a refrigerator. The calibration curves were plotted after linear regression of the peak areas versus concentrations.

2.5. HPLC method validation

The limits of detection (LOD) and limits of quantitation (LOQ) were defined as the lowest concentrations of analytes in a sample that can be detected and quantified. These limits were determined on the basis of the signal-to-noise ratios of 3:1 and 10:1, respectively. The standard solutions of compounds **1–4** for LOD and LOQ were prepared by diluting them sequentially. The intra- and inter-day precisions, together with accuracy (recovery) for the four analytes were assessed by spiking the EtOH extracts of noni leaves with standards **1–4** at similar concentrations. They were performed by following the method applied to the sample analysis for three consecutive days. The variations were evaluated using the relative standard deviation (RSD) of triplicate injections. The accuracy of the method was evaluated by analyzing the recovery percentage of compounds in EtOH extracts of noni leaves. The recovery percentage was calculated using the ratio of contents detected (actual) to those added (theoretical).

2.6. Identification and quantification

Identification of the different compounds was made by comparing the HPLC retention times, UV absorptions, and mass/charge ratios of target peaks with those of the standards. Quantification was performed on the basis of linear calibration plots of the UV absorption peak area at 365 nm against concentration.

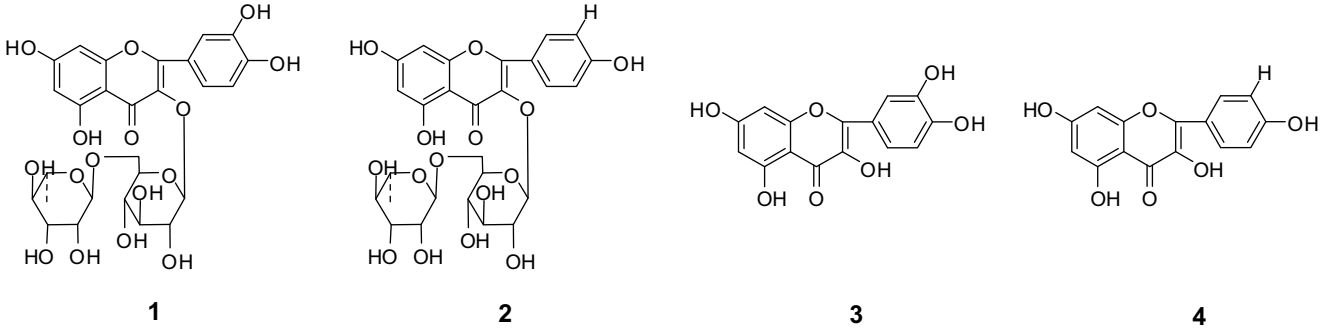


Fig. 1. The chemical structures of flavonol glycosides and aglycones found in the ethanol extract of noni leaves.

3. Results and discussion

3.1. Extraction

The exhaustive extraction of phytochemicals from noni leaves was achieved using a high-efficiency percolation method. The extraction was monitored by HPLC analysis, and the effluent was collected until negative detection of the target compounds.

3.2. Optimization of the HPLC chromatographic conditions

The HPLC solvent system applied in the experiment was optimized from different mobile phases, at various proportions, in order to obtain a good separation and resolution of target compounds in the chromatograms. Gradient elution is routinely applied to the separation of flavonoids in a complicated plant matrix during a short-period run time. In this experiment, a gradient solvent system composed of MeON–H_2O was selected as a mobile phase, since it afforded a good separation and resolution of target peaks from other constituents in the noni leaves. Additionally, 0.3% acetic acid was added into the mobile phase to adjust the pH value, reduce the peak tailing, and thus produce a symmetrical peak shape. The wavelength of 365 nm was selected for detection of target analytes since it showed maximum absorption and good sensitivity of the target peaks. The typical chromatographic profiles of a mixed standard solution and sample solution are shown in Fig. 2. No interference was observed for target compounds in the chromatograms of the samples.

3.3. HPLC method validation

The proposed chromatographic method was validated to determine the LOD, LOQ, linearity, intra-day and inter-day precisions, and accuracy. The determination of LOD and LOQ values of standard compounds **1–4** was carried out by diluting standard solutions of the corresponding compounds sequentially. The LOD (S/N = 3) and LOQ (S/N = 10) for compounds **1–4** were in the range of 0.012–0.055, and 0.04–0.165 μg/mL, respectively (Table 1). Calibration curves were obtained with concentrations in five increments for compounds **1–4**. The linear regression equations for compounds **1–4** were expressed as $y = mx + c$, where x is the concentration, y is the peak area of the standards, and m and c are constants (Table 1). A good linearity (correlation coefficient >0.999) is shown for each calibration curve.

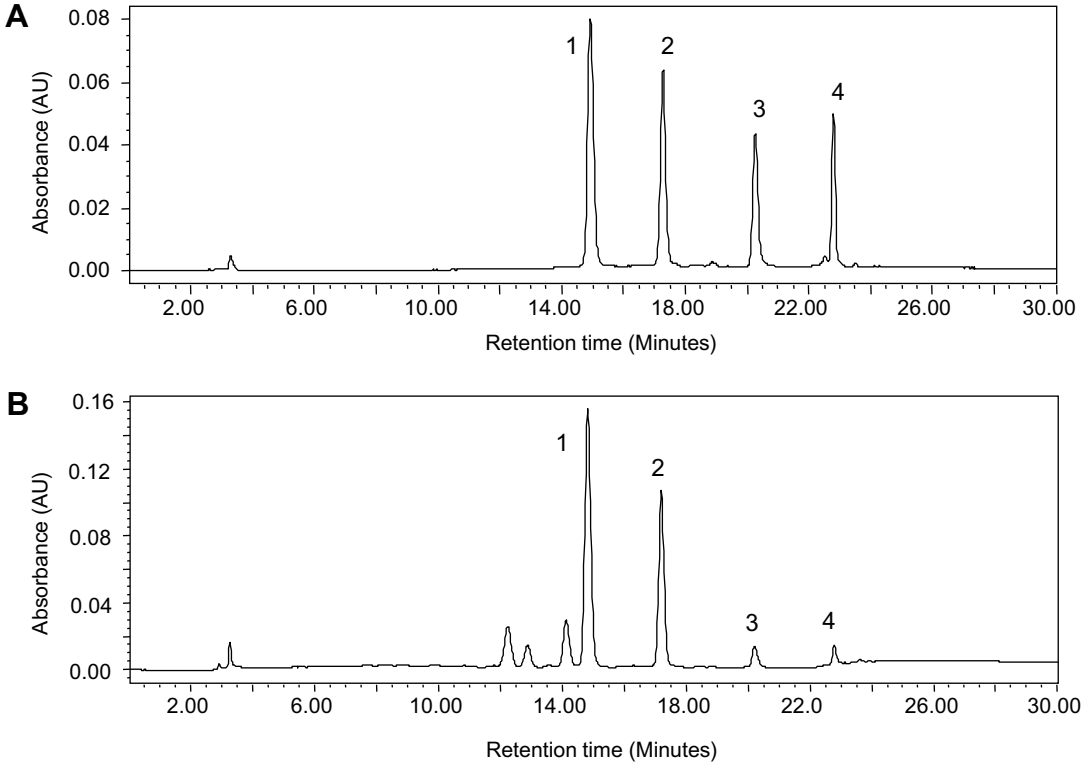
Intra-day precision (repeatability) and inter-day precision (intermediate) of the proposed HPLC analytical method, expressed as percent RSD, were investigated by triplicate injections of spiked samples on three consecutive days. Measurements of these intra-day and inter-day precisions yielded good results in the ranges of 0.34%–4.38% and 1.61%–3.50%, respectively (Table 2).

The accuracy, in terms of recovery, was performed by spiking the EtOH extracts of noni leaves with standards **1–4** at specific concentrations, and then, determined by the HPLC method. Under the

Table 1
Calibration curves, LOD and LOQ data of flavonols **1–4** determined by HPLC-UV/MS

Compound	Linearity range (mg/mL)	Calibration equation[a]	LOD (μg/mL)	LOQ (μg/mL)	Correlation coefficient
1	0.075–0.75	$y = 3.274 \times 10^4x + 754$	0.033	0.10	0.9996
2	0.25–3.00	$y = 6.215 \times 10^3x - 103$	0.012	0.04	0.9999
3	0.0025–0.1	$y = 9.538 \times 10^4x - 47$	0.025	0.08	0.9999
4	0.0025–0.1	$y = 5.610 \times 10^4x - 47$	0.055	0.165	0.9999

[a] Five data points ($n = 3$), x = concentration of compounds (mg/mL) y = peak area.



A: Mixed standard solution of compounds **1-4**;
B: Ethanolic extracts of noni leaves

Fig. 2. Typical HPLC-UV chromatograms of four flavonoids and ethanol extracts of noni leaves.

Table 2
Intra- and inter-day precision and accuracy for the quantitative determination of four flavonoids in the extract of noni leaves by HPLC-UV/MS ($n = 3$)

Compound	Spiked amount (mg/g)	Day 1		Day 2		Day 3		Inter-day		Recovery	
		Amount detected (mg/g)	RSD (%)	Amount detected (mg/g)	RSD (%)	Amount detected (mg/g)	RSD (%)	Amount detected (mg/g)	RSD (%)	Percentage	RSD (%)
1	8.00	7.97	1.26	7.63	1.24	7.60	0.48	7.73	2.61	96.66	2.30
2	18.00	18.08	2.09	17.24	2.86	17.21	1.50	17.51	2.84	97.27	2.94
3	1.50	1.44	1.87	1.47	0.89	1.48	0.34	1.46	1.61	97.47	1.51
4	1.30	1.27	0.63	1.30	3.56	1.33	4.38	1.30	3.50	100.03	3.30

Table 3
Characterization of standard compounds

Compounds	UV λ_{max} (nm)	m/z[a], $[M-H]^-$	Retention time (min)
1	254.5, 354.1	609	12.7
2	265.1, 347.0	593	14.7
3	254.5, 367.0	301	17.2
4	265.1, 363.6	285	19.3

[a] m/z = mass-to-charge ratio.

Table 4
Determination of compounds **1–4** in the ethanol extract of noni leaves

Samples	Content of analytes[a] (mg/100 g)			
	1	2	3	4
Raw leaf	94.3 ± 1.68	371.6 ± 2.47	1.16 ± 0.036	1.71 ± 0.066

[a] mean ± SD, $n = 3$.

established experimental conditions, recoveries of **1–4** were 96.66%, 97.27%, 97.45%, and 100.03%, respectively (Table 2).

3.4. HPLC-UV/ESI-MS characterization of flavonols *1–4* in noni leaves

After a complete chromatographic separation was achieved in the HPLC experiment, the individual peaks were characterized by using dual detectors consisting of mass and UV spectrometers. Although both positive and negative modes were attempted in the MS experiment, only the negative mode afforded a high sensitivity of the target compounds. Compounds **1–4** were used as reference standards. Their spectroscopic and chromatographic characteristics, including UV absorption, molecular ions, and LC retention times, were established under the experimental conditions for determining corresponding components in the samples (Table 3).

In the MS spectra, the most prominent mass-to-charge ratios of 609, 593, 301, and 285 corresponded to deprotonated molecular ions of compounds **1–4**, respectively. Additionally, the target peaks in the chromatograms of the samples exhibited consistency on UV absorptions and LC retention times to those of standards **1–4**. These results demonstrated the presence of quercetin-3-*O*-α-l-rhamnopyranosyl-(1 → 6)-β-d-glucopyranoside (rutin, **1**), kaempferol-3-*O*-α-l-rhamnopyranosyl-(1 → 6)-β-d-glucopyranoside (**2**), quercetin (**3**), and kaempferol (**4**) in the ethanol extracts of noni leaves.

3.5. HPLC-UV quantification of flavonols *1–4* in noni leaves

Flavonols **1–4** in the EtOH extracts of noni leaves were quantified using the developed and validated HPLC method. The content of each compound was subsequently determined by the corresponding regression equation shown in Table 1. Fig. 2 displays the HPLC chromatograms of standards and EtOH extracts of noni leaves. The content of flavonols **1–4** in dried noni leaves was shown to be 94.3, 371.6, 1.16, and 1.71 mg/100 g, respectively (Table 4).

4. Conclusions

A rapid and validated analytical method has been developed for the simultaneous characterization and quantitation of four flavonol glycosides and aglycones in noni leaves collected in French Polynesia. The experiment was accomplished using reverse-phase high-performance liquid chromatography, coupled with dual UV/ESI-MS detectors. Target flavonol compounds were successfully quantitated by using calibration curves with a quantitation limit of 0.04–0.165 μg/mL. The established method provides an accurate, simple reference, which could be used for both qualitative and quantitative analyses of noni raw leaves and their commercial products. Such a method could serve as a prerequisite for quality control and standardization of noni-leaf products.

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