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Development and validation of an RP-HPLC method for the analysis of anthraquinones in noni fruits and leaves

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ABSTRACT

Noni fruits and leaves, which have been used traditionally for thousands of years to improve health, are increasingly attracting the interests of consumers and researchers. A selective and validated HPLC method for the analysis of anthraquinones in noni fruits and leaves has been developed and is reported for the first time. Four anthraquinones, 5,15-dimethylmorindol (5,15-DMM, **1**), lucidin (**2**), and alizarin (**3**), and rubiadin (**4**) are examined. The limits of detection of **1–4** were in the range of 1.0 and 20.0 ng. Intra- and inter-day precisions of **1** were determined to be less than 5.3%. The accuracy, expressed as the percent recovery of **1** after spiking at three concentrations ranged from 83.0% to 93.3%. Further, the linear correlation coefficient was >0.999, within the range of concentration investigated. The 5,15-DMM content of noni fruit puree and noni leaf infusion are between 0.186 to 0.202 μg/mL (ppm), and 5.82 to 20.93 ng/mL (ppb), respectively. Lucidin, rubiadin and alizarin were not detected in any of the noni samples. The presence of only trace amounts in the noni fruits and leaves may help to eliminate safety concerns regarding anthraquinone contents.

1. Introduction

Morinda citrifolia L., commonly known as noni, is a Rubiaceous plant widely distributed in many tropical areas, such as Asia, Hawaii, and the Tahitian Islands. Its fruits and leaves have been traditionally used as food and for prevention or improvement of diversified health problems, such as arthritis, infections, colds, cancers and diabetes (McClatchey, 2002; Wang et al., 2002). Following the commercialization of Tahitian Noni® juice in 1996, noni fruit and leaf based health products have increasingly attracted the interest of consumers and researchers. Recent biological studies have shown that noni fruits and leaves have strong antioxidant and immune-modulating activities (Palu et al., 2008; Yang, Paulino, Janke-Stedronsky, & Abawi, 2007). Furthermore, recent safety evaluations report no toxicity from noni consumption (West, Jensen, Westendorf, & White, 2006; West, Tani, Palu, Tolson, & Jensen, 2007; Westendorf, Effenberger, Iznaguen, & Basar, 2007). Noni fruit and leaves have been developed and marketed in many forms of commercial products.

Among the various phytochemicals in noni fruits and leaves, phenolic compounds, particularly flavonoids, are widely regarded as some of the major biologically active components, which may contribute to their reputed and diversified health benefits (Deng et al., 2007; Deng, West, & Jensen, 2008). Recently, some safety concerns have been raised and associated with the speculative presence of anthraquinones in noni products (Millonig, Stadlmann, & Vogel, 2005; Stadlbauer et al., 2005). Previous studies suggest that anthraquinones are mainly present in the roots (Deng, Chin, Chai, Keller, & Kinghorn, 2007; Ohsawa & Ohba, 1993; Rusia & Srivastava, 1989) and heartwood (Balakrishna, Seshadri, & Venkataramani, 1960; Srivastava & Singh, 1993), although the existence of trace amounts of anthraquinones in noni stems (Do et al., 1999), flower (Tiwari & Singh, 1977), fruits (Akihisa et al., 2007; Kamiya, Tanaka, Endang, Umar, & Satake, 2005; Pawlus, Su, Keller, & Kinghorn, 2005), and leaves (Takashima et al., 2007) have also been reported. An official re-assessment of the safety of noni fruit juice by the European Food Safety Authority concluded that "it is unlikely that consumption of noni juice, at the observed levels of intake, induces adverse human liver effects. This would also apply to the anthraquinones potentially present in the commercially produced noni juice" (European Food Safety Authority, 2006). Given that no quantitative analysis of anthraquinones in noni fruits and leaves has been reported so far, we have developed a validated reversed-phase HPLC method for analysing the contents of major anthraquinones in noni fruit puree, the raw material of Tahitian Noni® juice products, as well as the commercial noni leaf tea infusions. Referencing previous studies (Akihisa et al., 2007; Kamiya et al., 2005; Westendorf et al., 2007), four anthraquinones, including potentially genotoxic lucidin and rubiadin, and major

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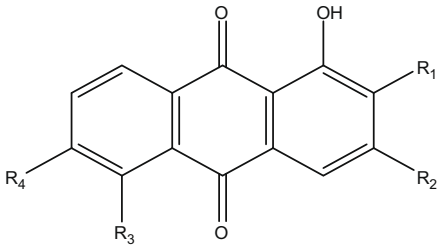

	R_1	R_2	R_3	R_4
5,15-dimethylmorindol (**1**):	$-CH_2OCH_3$	-H	$-OCH_3$	-OH
lucidin (**2**):	$-CH_2OH$	-OH	-H	-H
alizarin (**3**):	-OH	-H	-H	-H
rubiadin (**4**):	$-CH_3$	-OH	-H	-H

Fig. 1. The chemical structures of anthraquinone analytes **1**–**4**.

non-genotoxic anthraquinones, alizarin and 5,15-dimethylmorindol were examined in our experiments.

2. Experimental

2.1. Chemicals and standards

Acetonitrile (MeCN) and water (H_2O) of HPLC grade, and dichloromethane (CH_2Cl_2) and trifluoroacetic acid (TFA) of analytical grade were obtained from Sigma–Aldrich (St. Louis, MO, USA). The chemical standard 5,15-dimethylmorindol (5,15-DMM, **1**) was donated by Dr. Kohei Kamiya from Japan. 1,3-dihydroxy-2-(hydroxymethyl)-anthraquinone (lucidin, **2**) and 1,3-dihydroxy-2-methyl-anthraquinone (rubiadin, **4**) were obtained from the laboratory of Dr. Johannes Westendorf. A 1,2-dihydroxy-anthraquinone (alizarin, **3**) standard was purchased from Chromadex (Irvine, CA, USA). Their purities were determined by HPLC to be higher than 99%. The chemical structures of anthraquinones **1**–**4** are listed in Fig. 1. They were accurately weighed and then dissolved in an appropriate volume of MeCN to produce corresponding stock solutions. The working standard solution of **1** for the calibration curve was prepared by diluting the stock solution with MeCN in six concentration increments ranging from 0.0001 to 0.05 mg/mL. All stock and working solutions were maintained at 0 °C in a freezer. The calibration curve of standard **1** was plotted after linear regression of the peak areas *versus* concentrations.

2.2. Materials and sample preparation

The Tahitian Noni™ noni leaf tea (original) was made by Tahitian Noni International (Provo, Utah) via a roasting process of noni raw leaves. Following preparation instructions of the commercial noni leaf tea, 1 g of noni leaf tea was added into 240 mL of hot water (95 °C) for 10 min. to afford an aqueous solution. The solution was partitioned for three times with a total of 600 mL of CH_2Cl_2, evaporated to dryness under vacuum at 45 °C, and then dissolved in MeCN for HPLC analysis. Six batches of noni leaf tea were tested in the experiments. Tahitian noni fruit puree is the mashed whole fruits, excluding seeds and pericarp. The fruits were originally collected from the Tahitian Islands. 200 mL of the puree were combined with 1000 mL of H_2O, and mixed evenly. The aqueous solution was then partitioned with 1000 mL of CH_2Cl_2 for three times, and prepared for HPLC analysis as detailed above for the noni leaf tea. Five batches of noni puree were examined in the experiments. All sample solutions were filtered through a nylon microfilter (0.2-μm pore size) before HPLC injections. Voucher specimens of the noni leaf tea and fruit puree are deposited in our lab.

2.3. Chromatographic conditions and instrumentation

Chromatographic separation was performed on a Waters 2690 separations module coupled with a 996 photodiode array detector, and equipped with an Atlantis C18 column (4.6 mm × 250 mm; 5 μm, Waters Corporation, Milford, MA, USA). The pump was connected to two mobile phases: A; 0.1% TFA in H_2O (v/v), and C; MeCN, and eluted at a flow rate of 1.0 mL/min. The mobile phase was programmed consecutively in a linear gradient as follows: 0–5 min, 80% A; 6–15 min, 80–55% A; 16–30 min, 55–0% A; 31–40 min, 0% A; 41–45 min, 80% A. The photodiode array detector was monitored in the range of 210–450 nm, and 410 nm was selected for quantitative analysis. The injection volume was 50 μL for each of the sample solutions. The column temperature was maintained at 25 °C. Data collection and integration were performed using Waters Millennium software revision 32. The Waters HPLC instrument is validated and calibrated regularly.

2.4. Method validation

The limits of detection (LOD) and quantitation (LOQ) were defined as the lowest concentrations of analytes in a sample that can be detected and quantified. These LOD and LOQ limits were determined on the basis of signal-to-noise ratios (S/N) of 3:1 and 10:1, respectively. The working solutions of standards **1**–**4** for LOD and LOQ were prepared by diluting them sequentially. The intra- and inter-day precision assays, as well as stability tests were performed by following the method applied to the sample analysis for three consecutive days. Accuracy of the method (recovery) was assessed by the recovery percentage of 5,15-DMM in the spiked samples. The samples were spiked with standard **1** at 3 different concentrations (0.10, 0.23, and 0.30 μg/mL for noni puree, and 4.2, 10.0, 22.0 ng/mL for noni leaf infusions). The recovery percentage was calculated using the ratio of concentration detected (actual) to those spiked (theoretical). The variations were evaluated using the relative standard deviation (RSD) of triplicate injections in the HPLC experiments.

3. Results and discussion

3.1. Method development

Noni fruits and leaves are natural complicated matrices of various classes of compounds. A preliminary purification of the samples is necessary before HPLC analysis. As anthraquinones are generally soluble in CH_2Cl_2, our experiments utilized a solvent partitioning of CH_2Cl_2 and H_2O to remove interfering water-soluble components, and to purify the target anthraquinones. In the HPLC experiments, mobile phases were optimized with different solvent combinations and at various proportions. Among the different options, the MeCN–H_2O gradient, selected in the experiments, afforded a good separation and symmetrical peak shapes of target analytes in the HPLC chromatograms. A photodiode array detector was used for monitoring the spectra over a range of 210–450 nm. Analytes **1–4** exhibited the UV maximum absorptions at three wavelength ranges, 224–248 nm, 265–279 nm, and 411–428 nm (Table 1). Sample peaks in the chromatograms derived from the photodiode array were integrated at 410 nm. No interference to the 5,15-DMM was observed at the selected wavelength.

3.2. Method validation

To validate the HPLC chromatographic method, a series of assays were performed, including the determination of LOD, LOQ, linearity, intra-day and inter-day precision, and accuracy. The

Table 1
Some characteristic profiles of anthraquinone reference compounds determined by HPLC-UV.

Reference compounds	UV λmax (nm)	Rt (Min)	LOD (ng)	LOQ (ng)	LOD in samples	
					Fruit puree (ng/mL)	Leaf infusion (ng/mL)
5,15-DMM (1)	224, 265, 415	24.71	1.0	3.3	2.5	0.4
Lucidin (**2**)	244, 279, 412	21.38	20.0	65.0	50.0	8.3
Alizarin (**3**)	248, 280, 428	22.73	2.5	8.3	6.3	1.0
Rubiadin (**4**)	244, 278, 412	26.66	25.0	82.0	62.5	10.4

measurement of LOD and LOQ values of standards **1**–**4** was conducted by diluting standard solutions of the corresponding compounds sequentially. The LODs (S/N = 3) and LOQs (S/N=10) for standards **1**–**4** range at 1.0–25 ng, and 3.3–65 ng on the column, respectively (Table 1). These values are equivalent to a range of 2.5–62.5 ng/mL for the noni fruit puree, and 0.42–10.42 ng/mL for the noni leaf tea infusion, respectively. A typical chromatographic profile of a mixed standard solution is shown in Fig. 2.

In the calibration experiments of standard **1**, a calibration curve was obtained with concentrations in six increments. The calibration curves were plotted after linear regression of the peak areas *versus* concentrations. The linear regression equation was calculated as: $y = 65639496.03013x - 3407.25805$, where x is the concentration, and y is the peak area of the standard. The result showed an acceptable linearity with correlation coefficient higher than 0.999 within the range of concentration investigated.

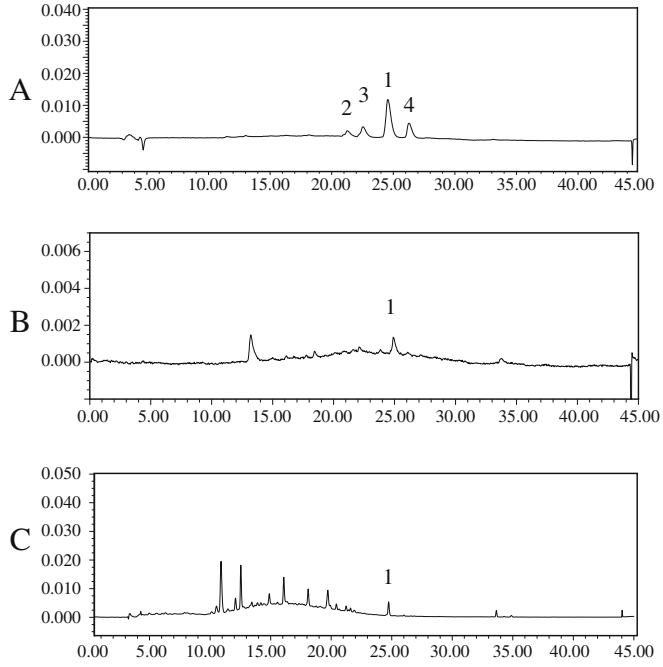

Fig. 2. Typical HPLC chromatographic profiles of anthraquinone reference compounds (A), noni fruit puree (B), and noni leaf tea infusion (C). The peaks in diagram A marked with **1**–**4** represent 5,15-DMM, lucidin, alizarin and rubiadin, respectively. The experimental conditions were described in the Experimental Section. The *x*- and *y*-axes represent the running time (min) and peak absorbance (au), respectively.

Repeatability is the degree of agreement of results when experimental conditions are maintained as constant as possible, and expressed as the RSD of replicates. In the repeatability study, both intra- and inter-day precisions of the proposed HPLC analytical method were investigated by triplicate injections of samples on three consecutive days. The results showed that the RSD's intra- and inter-day precisions were less than 5.3% for fruits and leaves (Table 2). As partial experiments needed to be conducted in 3 days, the stability of standard **1** in a working solution was also studied at the same time. The peak areas collected on each day were analysed. The data showed that 5,15-DMM in the working solution was stable within 3 days after preparation, with an RSD of ⩽3.0% (Table 2).

The accuracy, expressed as a recovery percentage, was determined by spiking **1** at three concentration ranges covering expected concentrations in the noni samples. Under the established experimental conditions, percent recoveries of analyte **1** were 83.0–90.2% for noni fruit, and 91.7–93.3% for noni leaves (Table 3).

All parameters achieved in the above-mentioned validation experiments lie within the tolerance ranges recommended in the guideline for dietary supplement issued by the Association of Analytical Communities (http://www.aoac.org/dietsupp6/Dietary-Supplement-web-site/slv_guidelines.pdf).

3.3. Sample analysis

3.3.1. Identification of the analytes

Characterisation of the anthraquinones was achieved by comparing the HPLC retention time and UV maximum absorptions of target peaks in the samples with those of the standards **1**–**4**. Their spectroscopic and chromatographic characteristics, including UV absorption and LC retention time, were summarized in Table 1. The typical HPLC chromatographic profiles of standards **1**–**4**, noni fruit puree, and noni leaf infusions are shown in Fig. 2. A chromatographic peak in the HPLC profiles of the samples exhibited consistency on UV absorptions and LC retention time to that of standard **1**, indicating the presence of 5,15-DMM in both noni fruits and leaves. However, no peaks in the chromatograms of samples were observed to display the same UV absorptions and retention time as those of standards **2**–**4**, demonstrating the absence of anthraqui-

Table 2
Intra- and inter-day precisions and stability assays for the quantitative determination of 5,15-DMM in noni fruits and leaves by HPLC-UV.

Samples	Day 1		Day2		Day3		Inter-day	
	Amount detected[a]	RSD (%)	Amount detected[a]	RSD (%)	Amount detected[a]	RSD (%)	Amount detected[a]	RSD (%)
Fruits (μg/mL)	0.188	3.0	0.191	3.8	0.177	4.3	0.188	5.3
Leaves (ng/mL)	3.85	2.6	3.81	1.8	3.82	1.4	3.83	1.8
5,15-DMM[b]	98914.7	1.0	98709.7	1.2	93188.0	1.4	96933.4	3.0

[a] $n = 3$.
[b] Absorption peak areas.

Table 3
Accuracy (recovery) assays for the quantitative determination of 5,15-DMM in noni fruits and leaves by HPLC-UV.

Samples	Concentration spiked	Concentration detected[a]	Recovery	
			Percentage (%)	RSD (%)
Fruits (μg/mL)	0.10	0.083 ± 0.0020	83.0	2.4
	0.23	0.19 ± 0.0059	84.6	3.0
	0.30	0.27 ± 0.0070	90.3	2.6
Leaves (ng/mL)	4.20	3.85 ± 0.10	91.7	2.6
	10.0	9.28 ± 0.07	92.8	0.8
	22.0	20.53 ± 0.35	93.3	1.7

[a] Mean ± SD; $n = 3$.

Table 4
HPLC-UV determination of 5,15-DMM (1) in noni fruit puree and leaf tea infusions.

Sample	Batch #	Contents[a]
Noni fruit puree (μg/mL)	14313	0.199 ± 0.0023
	14012	0.195 ± 0.0086
	13082	0.202 ± 0.0076
	14312	0.186 ± 0.0049
	14828	0.197 ± 0.0100
Leaf tea infusion (ng/mL)	20080519	6.28 ± 0.061
	20071226	6.87 ± 0.095
	20070920	20.93 ± 0.33
	20050825	19.73 ± 0.25
	20050824	16.99 ± 0.29
	20030909	5.82 ± 0.091

[a] Mean ± SD; $n = 3$.

nones lucidin, rubiadin, and alizarin in both noni fruits and leaves, within the detection limit of the method used in our experiment.

3.3.2. Quantification

Quantitation of 5,15-DMM (**1**) in noni fruit puree and leaf infusions was performed using the developed and validated HPLC method. Fig. 2 shows the HPLC chromatograms of the assayed standards and samples. Five batches of noni fruit puree and six batches of noni leaf infusions were tested. The amounts of 5,15-DMM are between 0.186 and 0.202 μg/mL (ppm) in the noni fruit puree, and range from 5.82 to 20.93 ng/mL (ppb) in the noni leaf infusions (Table 4).

In conclusion, a selective and validated analytical method has been developed for characterization and quantitation of anthraquinones in noni fruit and leaves. The established method provides an accurate and simple reference, which if applied to qualitative and quantitative analyses of noni and its commercial products, can be used for quality control.

Only trace amounts of 5,15-dimethylmorindol were found in the noni fruits and leaves. No other anthraquinones, such as lucidin, rubiadin, and alizarin were detected in the noni samples. The presence of only trace amounts of non-genotoxic anthraquinones in noni fruits and leaves should eliminate safety concerns relative to anthraquinone content.

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