JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Use of NIRS for Quantification of Mangiferin and Hesperidin Contents of Dried Green Honeybush (*Cyclopia genistoides*) Plant Material

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Cyclopia genistoides, normally used for the preparation of an herbal tea, honeybush, is a good source of the bio-active compounds mangiferin and hesperidin and is in demand for the preparation of xanthone-enriched extracts. Near-infrared spectroscopy (NIRS) was used to develop calibration models to predict the mangiferin and hesperidin contents of the dried green plant material. NIRS measurements of plant material and pure compounds were performed in diffuse reflectance mode. The calibration sets for mangiferin and hesperidin contents ranged from 0.7 to 7.21 and 0.64−4.80 g/100 g, respectively. Using independent validation, it was shown that the NIRS calibration models for the prediction of mangiferin (SEP = 0.46 g/100 g; R^2 = 0.74; and RPD = 1.96) and hesperidin (SEP = 0.38 g/100 g; R^2 = 0.72; and RDP = 1.90) contents of the dried plant material are adequate for screening purposes, based on RPD values.

KEYWORDS: Near-infrared spectroscopy; honeybush; *Cyclopia genistoides*; mangiferin; hesperidin; xanthone; flavanone

INTRODUCTION

Natural antioxidant extracts, especially polyphenol extracts, are increasingly used as food additives and supplements as these products gain appeal among industry and consumers (*1*, *2*). Recently xanthone-enriched extracts joined the plethora of phenolic products on the market (*3*). *Cyclopia genistoides*, endemic to South Africa and better known as the herbal tea honeybush, which is consumed largely in a fermented (oxidized) form, contains relatively high quantities of the *C*-glucosyl xanthone, mangiferin, its major phenolic compound (*4*). It is also a good source of the flavanone hesperidin (*4*), which together with mangiferin could comprise as much as 11.5% of the dried plant material.

For production of extracts high in mangiferin and/or hesperidin content, their concentration in the plant material is a deciding factor. This is not determined as part of quality control. Rapid analysis or screening of plant material would be ideal to identify plant material with sufficient quantities of these compounds to ensure dried extracts with the desired phenolic content.

Near-infrared spectroscopy (NIRS) analyses have been used successfully to develop calibration models to quantify compounds in herbal and medicinal plant species (i.e., enchinacosides in *Enchinacea* roots (*5*), harpagoside, an iridoid glucoside in devil's claw (*Harpagophytum procumbens*) root (*6*), sennosides A and B in senna (*Cassia senna* L.) leaves (*7*), aspalathin, a dihydrochalcone in rooibos (*8*), and several *Camellia sinensis* flavanols (*9*, *10*). In addition, it has been used for the quantification of the total polyphenol content (*9*) and TAA of green tea (*10*, *11*). NIRS is versatile in that it is applicable to a wide variety of different types of samples with the additional benefit of simultaneous determinations of many parameters, making it an alternative to spectrophotometric and/or HPLC methods, especially if rapid analysis time with minimum sample preparation is required.

To our knowledge, no NIRS calibration models have been developed for mangiferin and hesperidin in plant material. This study evaluated the potential of using NIRS calibration models for the prediction of mangiferin and hesperidin contents of dried green *C. genistoides* plant material.

MATERIALS AND METHODS

Chemicals. Mangiferin, hesperidin (97%), dimethyl sulfoxide (DMSO) (99.5%), acetonitrile (R Chromasolv, Riedel-de Haën), and glacial acetic acid (min. 99.8%) (Riedel-de Haën) were obtained from Sigma-Aldrich (Cape Town, South Africa). Methanol (Analar) was purchased from Merck Chemicals (Pty.) Ltd. (Cape Town, South Africa). Water for HPLC analysis was treated with a Modulab Water Purification System (Separations, Cape Town, South Africa), followed by a Milli-Q 185 Académic Plus water purification system (Microsep (Pty.) Ltd., Bellville, South Africa).

Dried Green Plant Material. Fresh *C. genistoides* shoots, harvested during 2001, 2003, 2004, and 2005 (n = 240), were dried at 40 °C to ca. 8−10% moisture content in a temperature-controlled drying tunnel.

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10.1021/jf060617l CCC: $33.50
Published on Web 07/01/2006

The dried plant material was ground using a Retsch mill (1 mm sieve) and stored in airtight plastic containers at room temperature in the dark. Samples harvested during 2001, 2003, and 2004 represented the West Coast and Overberg types, which were both harvested from commercial plantations in the Pearly Beach area (Western Cape Province, South Africa). Harvesting took place approximately every 5−6 weeks from the end of March to mid-July 2001 and every 6−8 weeks from March 2003 to Jan 2004 to include variation that could occur during the growth cycle. Young regrowth of eight plants, of the West Coast type that adequately resprouted, was also harvested in January 2004. The 2005 samples represented the West Coast type harvested at Reins Farm, Albertinia, South Africa. Included were also samples (n = 10) comprising either leaves or stems to extend the range of the mangiferin and hesperidin contents.

Preparation of Extracts. Duplicate methanol extracts were prepared for the quantification of mangiferin and hesperidin in the plant material. The dried plant material (ca. 0.5 g) was extracted with 50 mL of methanol (boiling point 64.5 °C) in a 100 mL volumetric flask by heating in a water bath at 64 °C for 30 min, whereafter the extract was cooled to room temperature and filled to volume (100 mL) with methanol. The extracts were filtered through Whatman No. 4 filter paper, followed by 0.45 μm Millipore Milex-HV hydrophilic PVDF syringe filters (25 mm diameter) (Microsep (Pty.) Ltd., Bellville, South Africa) directly into HPLC autosampler vials for immediate HPLC analysis.

Quantification of Mangiferin and Hesperidin by HPLC. Quantification of mangiferin and hesperidin was conducted in duplicate by reversed-phase HPLC, using a LaChrom (Merck/Hitachi) HPLC system (Merck, Cape Town, South Africa) that was comprised of an L7000 interface, L7400 UV detector, L7100 pump, and L7200 autosampler. An L7450 DAD detector was used to confirm peak identity. Separation of a 10 μL sample was performed on a 150 × 4.5 mm i.d., 4 μm, Phenomenex Synergy MAX-RP C12 80 Å column with TMS endcapping (Separations, Johannesburg, South Africa) at 30 °C (*4*). The solvents used for separation were 2% acetic acid in water and acetonitrile at a flow rate of 1 mL/min. A Phenomenex Degasser Model DG-4400 (Separations, Cape Town, South Africa) was used to degas solvents in-line.

Measurements were made at 320 and 288 nm for mangiferin and hesperidin, respectively. Stock solutions of mangiferin and hesperidin were prepared in DMSO. Standard dilution series of mangiferin (concentration range 0.01−6.16 μg injected; $R^2 > 0.99$) and hesperidin (concentration range 0.01−4.77 μg injected; $R^2 > 0.99$) were analyzed weekly. Peak area integration was done with the LaChrom Multisystem Software D700. Results were expressed on an as-is basis (plant material).

NIRS Measurements of Plant Material, Mangiferin and Hesperidin. A Büchi NIRLab N-200 Fourier transform near-infrared (FT-NIR) spectrophotometer with NIRLabWare (version 3.0) near-infrared (NIR) measurement software was used to perform the measurements of the plant material in diffuse reflectance mode. The samples were presented to the instrument in rotating glass Petri dishes, and the NIR spectra were collected from 1100 to 2500 nm (9090−4000 cm^{-1}) at an optical resolution of 8 cm^{-1}, with a data point at every 3.8569 cm^{-1}, resulting in 1319 data points.

Spectra of the pure compounds, mangiferin and hesperidin, were recorded in diffuse reflectance mode, using a Perkin-Elmer IdentiCheck FT-NIR spectrophotometer and Spectrum IdentiCheck NIR measurement software (version 2.0). The measurements were performed from 1100 to 2500 nm (9090−4000 cm^{-1}) at an optical resolution of 16 cm^{-1}, with a data point at every 7.2605 cm^{-1}, resulting in 701 data points. The compounds were presented to the instrument in 4 mL Chromocol glass vials. For comparative purposes, one sample of dried green *C. genistoides* was also analyzed.

Spectroscopic Characterization and NIRS Calibration Model Development. Spectroscopic characterization was performed on the raw spectra (no pretreatment). FT-NIR spectra, pretreated with multiplicative scatter correction (MSC), were investigated by means of principal component analysis (PCA) using Büchi NIRCal (version 4.21) software. Loading plots of principal components (PCs) 1−4 were constructed and used to describe the physical and chemical influences,

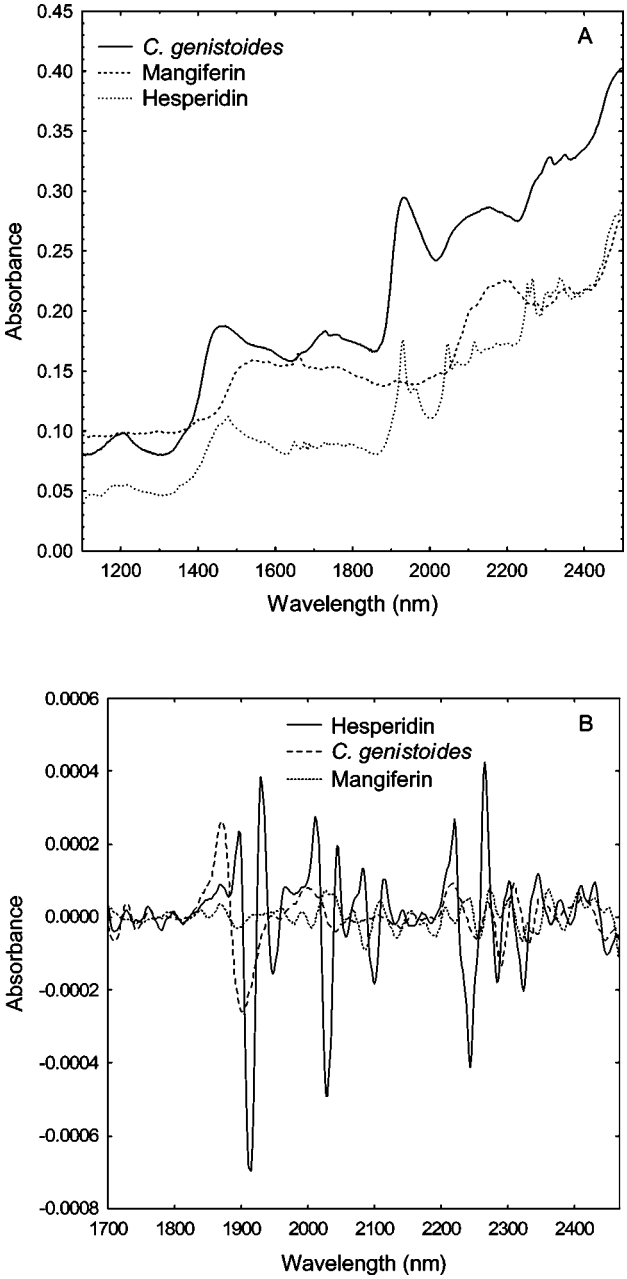

Figure 1. (**A**) Typical NIR spectra of mangiferin, hesperidin, and a ground dried green *C. genistoides* plant material sample and (**B**) their respective second derivative spectra.

causing spectroscopic variation. The same software was used for calibration model development. Spectra, pretreated with MSC and a first derivative, were used for the development of the partial least squares (PLS) calibration models for mangiferin and hesperidin contents, respectively. The number of PLS factors used was chosen according to the lowest estimated SEP value before the residual variance plot flattens or increases again. The number of factors should be chosen with caution to prevent over-fitting but still include the necessary factors to improve the accuracy of the calibration model. The calibration models were validated by means of independent validation, and no outliers were removed. For validation, every third sample (n = 80) was selected from a list of descending reference values. The remaining two-thirds of the sample set, including the lowest and highest values, comprised the calibration set (n = 160).

The accuracy of the calibration models was expressed by means of the standard error of prediction (SEP) (*12*), the coefficient of determination (R^2), and the RPD, calculated by dividing the SD of the reference values of the validation set by the SEP (*12*), which is an indication of the efficiency of a calibration. The goal of model

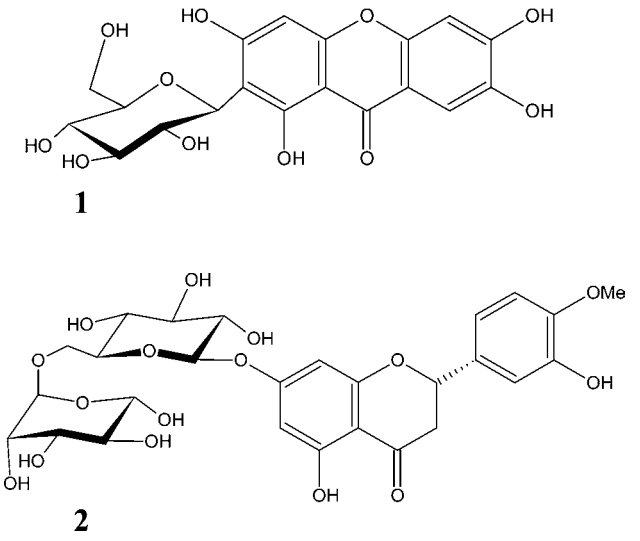

Figure 2. Molecular structures of mangiferin (**1**) and hesperidin (**2**).

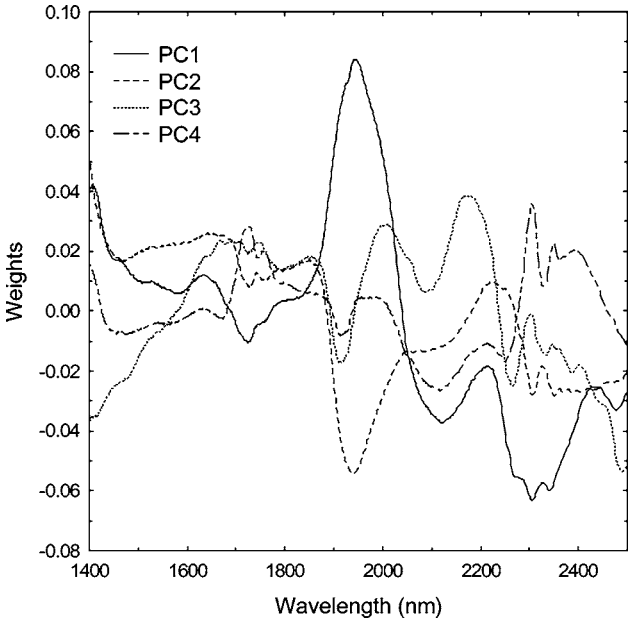

Figure 3. Loading plots of the first four principal components of the MSC pretreated spectra of dried green *C. genistoides* plant material.

development is to obtain a calibration model with a low SEP, a high R^2, preferably above 0.91, and a RPD higher than 5 (*12*). The SEP should also be as close as possible to the standard error of laboratory (SEL), calculated as the SD of differences between measurements on duplicate samples.

RESULTS AND DISCUSSION

Spectroscopic Characterization. Typical raw (no pretreatment) and second derivative NIR spectra of a ground dried green *C. genistoides* plant material sample, as well as that of mangiferin and hesperidin, are shown in **Figure 1A**,**B,** respectively. The correspondence in absorption peaks between *C. genistoides*, mangiferin, and hesperidin can be seen in the second derivative plots (**Figure 1B**), which confirms the important contribution of these two compounds to the composition of *C. genistoides*. The spectra of the plant material showed broad absorption bands at 1450 nm (O−H stretch first overtone, associated with water), 1940 nm (O−H stretch and O−H deformation, associated with water), and 2100 nm (C−O stretch and O−H stretch combination or O−H deformation second overtone) (*13*). These bonds (O−H and C−O) are abundant in the mangiferin and hesperidin molecular structures (**Figure 2**). The broad absorption band at 2100 nm could, however, also be due to C−H stretch and C═O stretch bond vibrations that would normally occur at ca. 2200 nm. The C−H and C═O bonds are found in the structures of mangiferin and hesperidin. The C═O stretch second overtone would have been expected to overlap with the O−H stretch first overtone bond at 1940 nm (*13*).

Table 1. Reference Data and NIRS Validation Results for the Prediction of Mangiferin and Hesperidin Contents of Dried Green *C. genistoides* Plant Material

	mangiferin	hesperidin
Reference Data		
calibration set (*n*)	160	160
range (g/100 g)	0.70–7.21	0.60–4.80
mean (g/100 g)	3.64	2.04
validation set (*n*)	80	80
range (g/100 g)	1.17–7.18	0.70–3.81
mean (g/100 g)	3.68	1.98
SD[a]	0.90	0.72
SEL[b]	0.08	0.03
Validation Results		
SEP (g/100 g)	0.46	0.38
R^2	0.74	0.72
Bias	−0.04	0.02
PLS factors[c]	4	6
RPD	1.96	1.90

[a] Standard deviation of reference data of validation set. [b] Standard error of laboratory of reference data of complete data set. [c] Number of PLS factors used.

The mangiferin spectrum (**Figure 1A**) showed a broad band at ca. 2150 (C−O stretch and O−H stretch combination or O−H deformation second overtone), which was also present in the *C. genistoides* spectrum, but at a slightly shifted position toward 2100 nm. The hesperidin spectrum (**Figure 1A**) showed distinct absorption bands at 1450 (O−H stretch, associated with water), 1940 (O−H stretch and O−H deformation, associated with water), 2050 (O−H stretch and O−H deformation), and 2100 (2 × O−H deformation and 2 × C−O stretch), which are all bonds abundant in the hesperidin structure.

After exclusion of particle size variation by means of MSC, the largest remaining contributor to spectroscopic variation in the plant material was moisture content. The loading plots of the first two principal components (PCs) (**Figure 3**) showed a strong absorption at ca. 1940 nm (O−H stretch and O−H deformation). This wavelength region corresponds most strongly to the absorption of water (or sample moisture) (*13*). The relative contributions of each PC to the explained variation were, as expected, large for the first few factors, with 95% of the variation explained by the first four factors for *C. genistoides* (PC1 = 72%, PC2 = 15%, PC3 = 6%, and PC4 = 2%).

The loading plots of PC1 and PC2 (**Figure 3**) of the plant material showed, apart from the largest weight at 1940 nm, also weights at 2280 nm that could be related to the O−H stretch and C−C stretch, as well as the C−H stretch and C−H deformation bond vibrations present in the molecular structures of mangiferin and hesperidin (**Figure 2**). The loading plot of PC3 (**Figure 3**) had major absorption bands at ca. 2000 (2 × O−H deformation and C−O deformation), 2200 (C−H stretch and C═O stretch), 2280 (C−H stretch and C−H deformation), and 2360 nm (O−H deformation second overtone), which are all related to the most abundant structural groups present in the mangiferin and hesperidin molecular structures. The loading plot of PC4 showed similar weights to that of PC3.

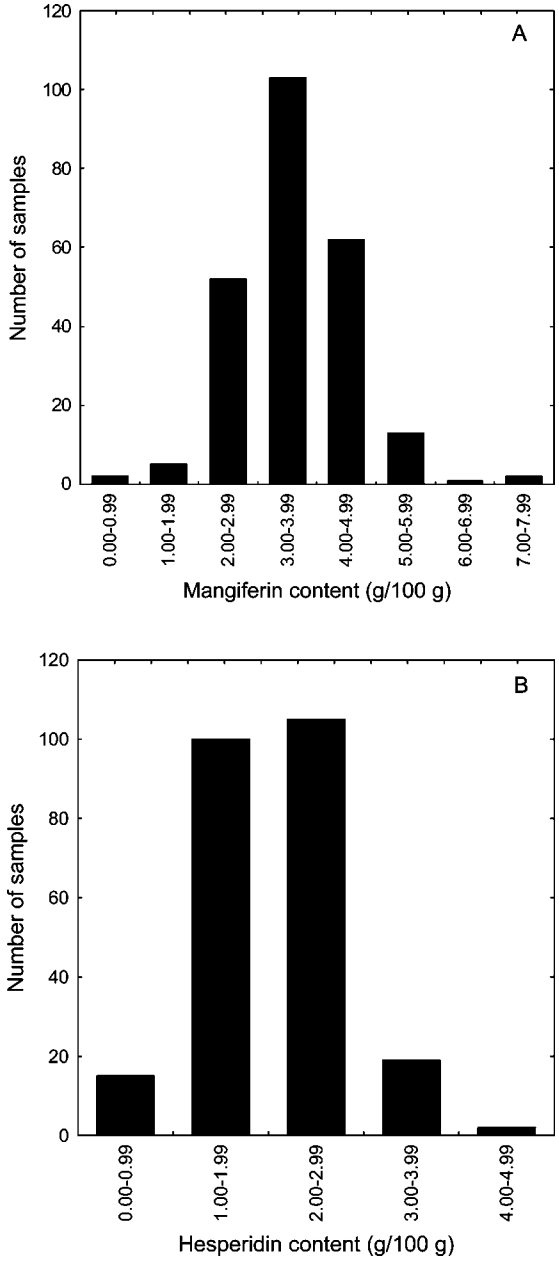

Figure 4. Histogram of the distribution of (**A**) mangiferin and (**B**) hesperidin contents of dried green *C. genistoides* plant material samples (calibration and validation sets combined).

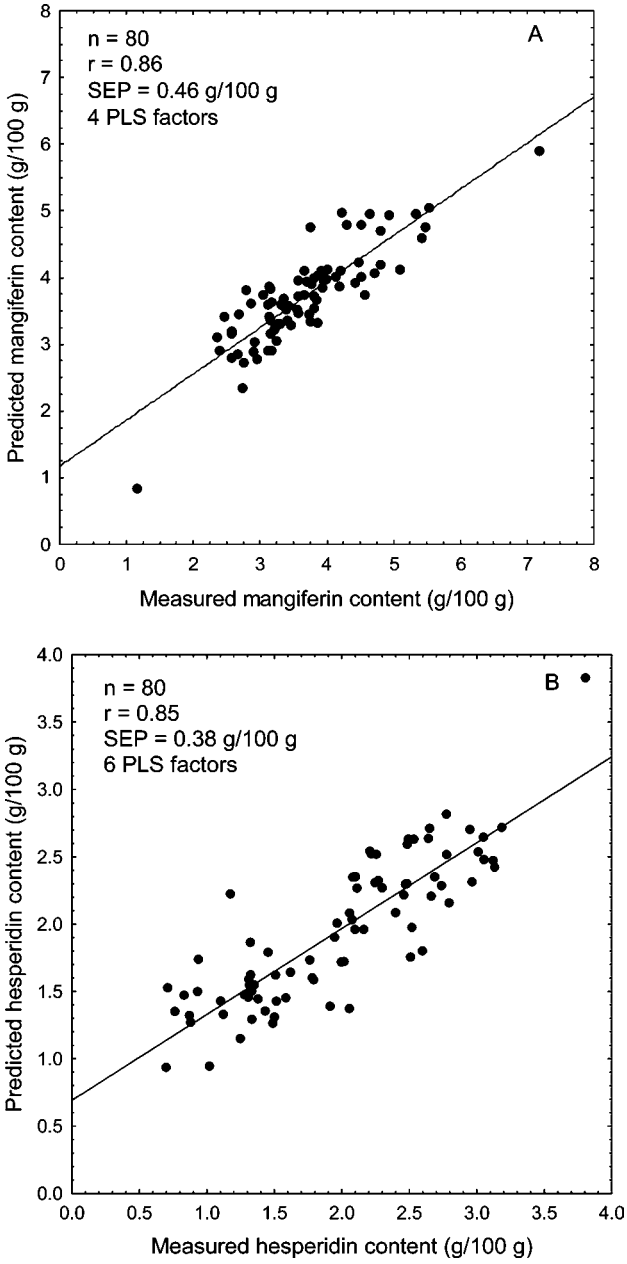

Figure 5. Validation plots of (**A**) predicted mangiferin content vs the measured (HPLC) mangiferin content and (**B**) predicted hesperidin content vs the measured (HPLC) hesperidin content for the calibration models for dried green *C. genistoides* plant material.

NIRS Calibration Model Development. A summary of the reference data for mangiferin and hesperidin contents of the dried plant material and NIRS validation results is given in **Table 1**. Histograms, showing the distributions of the mangiferin and hesperidin contents in the sample set, are depicted in **Figure 4A**,**B**. As expected from randomly collected samples, a Gaussian distribution was obtained for both compounds due to the difficulty of collecting an adequate number of samples with low and high contents of the active compounds. Most of the samples contained between 2 and 5 g of mangiferin and between 1 and 3 g of hesperidin per 100 g of dried plant material. It is likely that this type of distribution will produce more accurate predictions near the mean (3.68 and 1.98 g/100 g for mangiferin and hesperidin, respectively) and less accurate predictions for samples near the extremes of the range (*12*). The number of samples at the lower and higher end of the concentration range of both compounds could, however, still be increased to obtain a more even sample distribution. The availability of the calibration models would facilitate rapid selection of such samples, which could subsequently be analyzed by HPLC for accurate reference data to be included in the calibration model.

The validation results of the mangiferin calibration model (SEP = 0.46 g/100 g; RDP = 1.96; R^2 = 0.74; and SEL = 0.08 g/100 g) indicated, based on RPD values, that it could be used for screening and approximate predictions of the mangiferin content of dried green *C. genistoides* plant material (**Table 1** and **Figure 5A**) (*12*). The hesperidin calibration model gave similar results (SEP = 0.38 g/100 g; R^2 = 0.72; RPD = 1.90; and SEL = 0.03 g/100 g) (**Table 1** and **Figure 5B**).

Although RPD values of higher than 5 are recommended for calibration models to be suitable for quantification during quality control (*12*), the level of accuracy required by the industry and the range of mangiferin and hesperidin contents in the plant

material used for extraction should also be taken into account. The higher the mangiferin and hesperidin contents of the plant material, the less the effect of the prediction error would be due to the over- or under-estimation of their content. The calibration models in this study not only consist of a large number of samples but also cover the range of expected values in *C. genistoides* samples. The calibration set includes samples representative of seasonal variation, variation observed during the growth cycle, and samples containing predominantly leaves or stems. The inclusion of all these variations could have resulted in a slightly less accurate but more robust calibration model. The calibration models would therefore be suitable for use during quality control, especially as most samples expected to be analyzed are more likely to be at the higher end of the mangiferin and hesperidin content range.

ABBREVIATIONS USED

MSC, multiplicative scatter correction; NIRS, near-infrared spectroscopy; PC, principal component; PCA, principal component analysis; PLS, partial least squares; RPD, ratio of standard deviation of the validation set to the SEP; SEL, standard error of laboratory; SEP, standard error of prediction.

ACKNOWLEDGMENT

We acknowledge Büchi, Flawil, Switzerland for the loan of the Büchi NIRLab N-200 spectrophotometer and NIRCal (version 4.21) software. We thank Nico Malan of Reins Farm, Albertinia, Southern Cape and Fritz Joubert of Koksrivier, Pearly Beach, Overberg for providing honeybush plant material.

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Received for review March 3, 2006. Revised manuscript received May 5, 2006. Accepted May 23, 2006. Funding was supplied by the Raps Foundation, Germany, The Technology and Human Resource for Industry Program (THRIP), South Africa, and the National Research Foundation (NRF), South Africa. The NRF also supplied a student bursary for M.B.

JF060617L