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Quantification of rosmarinic acid levels by near infrared spectroscopy in laboratory culture grown spearmint plantlets

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A method for the rapid quantification of rosmarinic acid (RA) in tissues of spearmint using near infrared (NIR) spectroscopy was developed by correlating with the results of methanol extracts analysed on an HPLC photo-diode array (PDA) system. NIR and HPLC analyses performed on over 500 samples were used to develop an NIR analytical algorithm. Applying this algorithm resulted in very good calibration statistics for the prediction of RA concentrations in dried powdered samples prepared from the cultured mints (r^2=0.90, *SEP*=3.1 *RPD*=2.6). NIR can be used to rapidly and accurately quantitate the levels of this secondary metabolite in plant samples. High positive correlations occur between CO_2 levels and spearmint plantlet growth (fresh weight), morphogenetic responses (leaves, roots and shoots) and production of the secondary metabolite rosmarinic acid.

Keywords: spearmint, *mentha spicata,* rosmarinic acid, near infrared spectroscopy, carbon dioxide, plant culture

Introduction

Rosmarinic acid (RA) is caffeoyl ester phenolic from the phenylpropanoid pathway and is common among species of the *Boraginaceae* and *Lamiaceae* families.[1,2] RA has been shown to have health promotive effects and may be employed for gastrointestinal disorders and urinary anti-inflammation and also has anti-HIV activity.[1,3] In plant tissue culture, RA is constitutively expressed in cell suspensions and callus cultures,[1,4–9] micro-shoots[2,10] and cultured roots.[11–13]

In order to increase RA concentrations in sterile plantlets and micro-shoots a variety of nutritional and physical treatments have been employed.[14,15] Microshoots and plantlets readily express the same secondary metabolites as found in plants grown in the soil.[3,14–17] We have also examined the influence of CO_2 concentrations on RA production *in vitro* via this plant culture methodology.[18,19] This research generates a large number of samples which require time and labour to process and analyse. Consequently, they lead to delays in the experimental programme. As an alternative to conventional chromatographic analysis, we sought to employ NIR spectroscopy as a means for rapid RA analysis of dried samples to accelerate research efforts. NIR spectroscopy has been used to evaluate the general composition of a substance, detect impurities, determine differences in composition for substances and identifiy unknown substances.[20,21] This technology has been expanded to evaluate a number of general parameters in plant samples including protein, moisture, fibre and lipid content.[22] Currently, protocols exist to determine the levels of essential oils, carotinoids, alkaloids and total phenolics in

[a]Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the products, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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plant materials if the proper wet chemistry has been done for correlation.[20–23] Specific chemical constituents have been evaluated by NIR analysis including capsaicin, citric acid[22] and chicoric acid in purple coneflower roots.[24] It has also been used to evaluate a number of phenolic constituents including rosmarinic acid in rosemary, sage and other spice plant materials.[25–29] Rapid NIR spectroscopy has been used to determine essential oil composition in dried leaves of mint. While Fourier transform infrared (FT-IR) spectroscopy has been used to determine RA concentrations in suspension cells of *Lavandula officinalis*, the method is fairly complex.[25]

Once calibrated, NIR spectroscopy can be used to rapidly evaluate large numbers of samples quickly and efficiently.[20,22,23,30,31] Because of mini-spacial requirements and a high number of replicates, cultured plantlet systems are capable of producing hundreds of samples per experiment. Labour, time and equipment usage limit the evaluation of secondary metabolites. Literally, a dozen experiments can be conducted in a month which generates hundreds of samples to analyse. Once the sample is prepared, HPLC evaluation of these samples for RA content is limited to a few dozen samples per HPLC per day.

We have developed an efficient, reliable, rapid, non-destructive method to determine RA levels by NIR spectroscopy of dried mint leaves. This has been derived from a large number of plant culture samples analysed by conventional HPLC methods in order to more efficiently evaluate these samples.

Materials and methods

Plant tissue culture experiments

Mint shoot cultures from two spearmint (*Mentha spicata* L.) cultivars, "294099", "557793" and "557807" were used in a variety of plant culture conditions. These mint cultivars (cvs.) were originally obtained from the US Department of Agriculture, Agricultural Research Service, National Germplasm Repository, Corvallis, Oregon, USA.

The basal medium (BM) consisted of Murashige and Skoog (M&S) salts,[32] plus: 0.5 mg L^{-1} thiamine.HCl, 100 mg L^{-1} myo-inositol, 30000 mg L^{-1} sucrose and 8000 mg L^{-1} agar (Difco Laboratories, Detroit, MI, USA). Medium pH was adjusted to 5.7 ± 0.1 with 0.1100 N HCl or NaOH before the addition of agar, then melted and dispensed in 25 mL aliquots into 25 × 150 mm borosilicate glass culture tubes. Tubes were capped with translucent polypropylene closures (Sigma Chemical Co., St Louis, MO, USA). Medium was autoclaved for 15 min at 1.05 kg cm^{-2} at 121°C. Stocks of shoots of spearmint (*Mentha spicata* L.) were maintained on BM under ambient air prior to testing. Cultures were transferred to fresh medium every eight weeks. For experiments, a single 6 cm long plant (four weeks old) was transferred to a double interlocking Magenta container (Chicago, IL, USA). Cultures were transferred to fresh medium every eight weeks.

To determine the influence of CO_2 on growth and RA production, shoots of spearmint cultivar "557807" were grown on BM under either 350 or 10,000 µmol mol^{-1} CO_2. The CO_2 test chambers and experimental conditions have been described elsewhere.[19,33] Cultures were grown in a culture room maintained at 25 ± 1°C and employed at a photoperiod of 16 h light/8 h dark. Light was supplied by a combination of cool white fluorescent tubes at a total PPFD of 100 µmol m^{-2} s^{-1} at the vessel periphery.

Sample preparation and extraction

Plants from the culture systems were packaged in paper bags and dried for 48 h in an oven at 40°C. They were stored in the dried state for up to three months in a zip lock plastic bag at room temperature. For analysis, samples were ground to fine powder with mortar and pestle, then passed through a number 30 mesh sieve to remove un-ground stem elements. The samples (typically 0.25 g) were placed in clear glass 1 dram vials (15 mm d × 45 mm with rubber lined caps) for reading in the NIR. For single step extraction analysis of RA by HPLC, 2 mL of methanol was added, the vials capped and wrapped with sealing tape, sonicated for 15 min at room temperature and allowed to stand at room temperature overnight. An aliquot was removed from the vial and filtered through a 0.45 µm nylon 66 filter for HPLC analysis.

During the past five years, well over 1000 cultured mint samples have been grown under various conditions and evaluated for their fresh weight yields, leaf size and morphology and other morphological characteristics. These plants were then placed in paper bags and oven dried as described above. If enough dried plant material was available, samples were prepared for NIR analysis and HPLC analysis as described above. Once the HPLC values were obtained, a reference set of 88 samples was selected. An additional set of 55 samples was selected from the remaining dried samples for a validation set. After NIR analysis, they were extracted and the RA concentrations were obtained by HPLC analysis.

Reference analysis

Analytical HPLC analysis

Samples were run on a stand-alone HPLC system (Shimadzu 10A with SCL-10A system controller, two LC-10A pumps, CTO-10A column oven and SIL-10A auto injector, Columbia, MD, USA). Peaks were monitored using a photodiode array detector (Hewlett-Packard 1040A , running under the HP Chemstation software version A.02.05 (Agilent Technologies, Santa Clara, CA, USA). The column used was an Inertsil ODS-3 reverse phase C-18 column (5 µm, 250 × 4.6 mm, with a Metaguard column, (Varian, Torrance, CA, USA). The initial conditions were 20% methanol and 80% 0.01 M phosphoric acid at a flow rate of 1 mL min^{-1}. The effluent was monitored at 285 nm. After injection (15 µL), the column was held at the initial conditions for 2 min, then developed to 100% methanol in a linear gradient over 55 min. A standard curve was prepared from pure RA (Chromadex, Santa Ana, CA, USA) based on absorbance versus nM concentration.

The presence of RA was confirmed using liquid chromatography-mass spectrometry (LC-MS) by comparison of retention time and mass spectra. Samples were run on a ThermoFinnigan LCQ DECA XP Plus LC-MS system with a Surveyor HPLC system [autoinjector, pump, degasser and PDA detector with a nitrogen generator all running under the Xcaliber 1.3 software system (Thermo Scientific, San Jose, CA, USA)]. The MS was run with the ESI probe in the positive mode. The column was a 3 mm × 150 mm Inertsil reverse phase C-18, ODS 3, 3 µ column (Varian, Torrance, CA, USA) with a Metaguard guard column. The source inlet temperature was set at 250°C, the sheath gas rate was set at 70 arbitrary units and the sweep (auxiliary) gas rate was set at 20 arbitrary units. The MS was optimised for the detection of RA by using the autotune feature of the software while infusing a solution of RA standard in with the effluent of the column and tuning on *m/z* 361 $[M+H]^+$. The initial HPLC conditions were 20% methanol and 0.25% acetic acid in water, at a flow rate of 0.3 mL min^{-1}. The column was then developed to 100% methanol and 0.25% acetic acid over 50 min. The effluent was also monitored at 285 nm on the PDA. The spectra and retention time of the RA in the mint extracts was identical to that of the standard.

NIR measurements

The NIR spectrocopy methodology for measurement of rosmarinic acid in spearmint plants (oven-dried and ground to a powder) was performed on a scanning NIR spectrophotometer (NIRSystems model 6500, Foss North America, Inc, Eden Ridge, MN, USA) equipped with a rapid content analyser in reflectance mode. Vials (1 dram; 15 mm × 45 mm) containing plant samples (0.25 g) were used to gather the NIR spectral data, as it was determined that the 1 dram vials containing samples gave reliable data. Each sample was measured with 32 scans per analysis. NIR data, as log (1/*R*), were collected using ISIscan software (Foss North America, Inc.). NIR scan data were transferred to WINISI v.1.50 (Infrasoft International Inc., Port Matilda, PA, USA) for processing.

The wavelength region for all calibrations was 700 nm to 2498 nm; 1050 data points per spectrum. Spectral data were evaluated for reproducibility, scatter and interferences by varying sample sizes and introduction procedures. Several mathematical treatments (partial least squares, regression, second and third-derivative processing) were used. Several hundred scans of spearmint were collected and evaluated for their spectral integrity. WINISI II principle component analysis software was used to score and select 80 samples to give a robust calibration. Additional samples were added to expand the range of RA determination and to provide additional diversity to the standard sample set. After the HPLC evaluation of the samples was performed and further refinement of the selected samples for calibration were carried out, NIR spectral data and HPLC analytical data were combined for calibrations. A calibration model was developed using the full wavelength range using the modified partial least square (MPLS) algorithm. The software determined the optimum number of MPLS factors used for the prediction determined by cross-validation with the leave-one-sample-out method[34] Models were compared using the coefficient of determination between the calibration model and reference values (R^2) and the standard error of cross-validation (*SECV*). The calibration equations were validated with the independent set of 55 additional mint samples where the rosmerinic acid content had been determined by HPLC.

In developing procedures to perform NIR analysis, there was an attempt to minimise any additional sample preparation. The samples were analysed in the 1 dram vial they were received in. The variable spot size feature of the NIRSystem 6500 rapid content analyser allowed RA measurement of samples in the vials. To determine the affect of sample size to the resulting scans, several assays of the same samples were performed on 0.1, 0.2, 0.3 and 0.4 gram samples There were minor or insignificant differences in the analysis. Because of the high sample throughput, software and computer upgrades were necessary to maintain that level of performance during the course of this analysis.

Results and discussion

Figure 1 shows the typical NIR reflectance spectrum of a representative RA sample using the rapid content analyser It was possible to derive an acceptable calibration model using less than the entire spectrum but the full spectrum was collected to enable us to predict other constituents in the future. The NIR reflectance measurements spectrum is similar to the expected forage or plant spectrum.[21] The distinct differ-

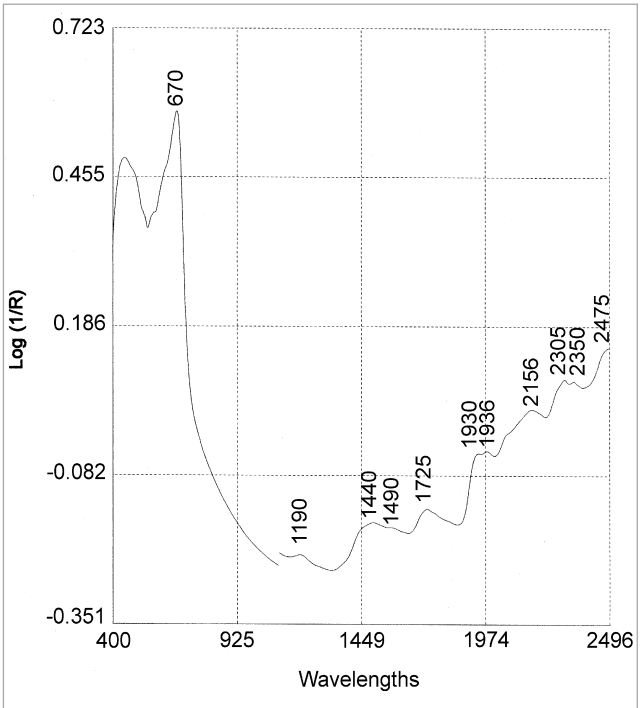

Figure 1. NIR reflectance spectrum obtained from oven-dried, ground spearmint leaf powder.

ences are the colour differences (688 nm) and water content (1488 nm). As with most forage samples, there are absorbances at 1188 nm (C–H 2nd overtone), 1722 (1st overtone S–H, O–H) 1936 nm (C=O 2nd overtone), 2058 (C=O 1st overtone) and combination bands at 2164 nm, 2270 nm and 2304 nm.

The statistical results of the calibration, Table 1, demonstrate that NIR non-destructive analysis of RA content of spearmint gives a reliable prediction based on the HPLC assay of similar samples. When the performance of the NIR model was tested with a validation data set, the results (Table 1 and Figure 2) indicated that the *SEP* was in the acceptable range of the laborious HPLC procedure. Similar results were achieved with an additional validation set (data not presented).

The R^2 value of 0.94 obtained for this calibration is well within the range of R^2 values of 0.80 to 0.96 reported for various individual essential oil (limonene, carvone, anthole, fenchone and estragole) concentrations in fennel, coriander, caraway and dill as reported by Shulz.[20] The r^2 value of 0.90 and the *RPD* value of 2.6 indicate that the calibration is at least suitable for routine screening purposes.[35]

Typical RA values obtained by HPLC and NIR analysis of samples of three cultivars which were grown with contrasting CO_2 and O_2 are shown in Table 2. The values obtained were, in general, in good agreement with each other. Oven-dried mint samples were used in these experiments due to the large number of samples generated and the time required for processing the samples for HPLC analysis. In general, the RA values obtained were similar to those obtained from the analysis of fresh samples (data not shown) but lower than that obtained by other research groups.

Under these experimental conditions, RA was the most abundant phenolic compound accumulated by these spearmint cultivars. While FT-IR spectroscopy has been used to determine RA levels *in situ*,[34] we obtained a good correlation using NIR. Regardless of the treatments administered, significant correlation was observed for HPLC and NIR analysis.

Rapid NIR analysis will allow us to quickly evaluate experimental results and determine which experimental samples will be used for further evaluation and allow quicker turn around times for planning new growth experiments. We plan on using NIR to rapidly assess large numbers of individual samples for RA (and possibly other phytochemicals) in order to circumvent the need for HPLC analysis of all samples. This will allow for a quicker turn around time in assessing hundreds of samples that can be obtained from field, greenhouse and plant culture experiments than can be obtained by submitting these samples to HPLC analysis. This also cuts down on the number of samples extracted for HPLC analysis and saves on solvents and other environmental waste that is generated by the classical chemical analysis. The aim at this laboratory is to obtain RA values following the influence of a large number of experimental variables (media composition,

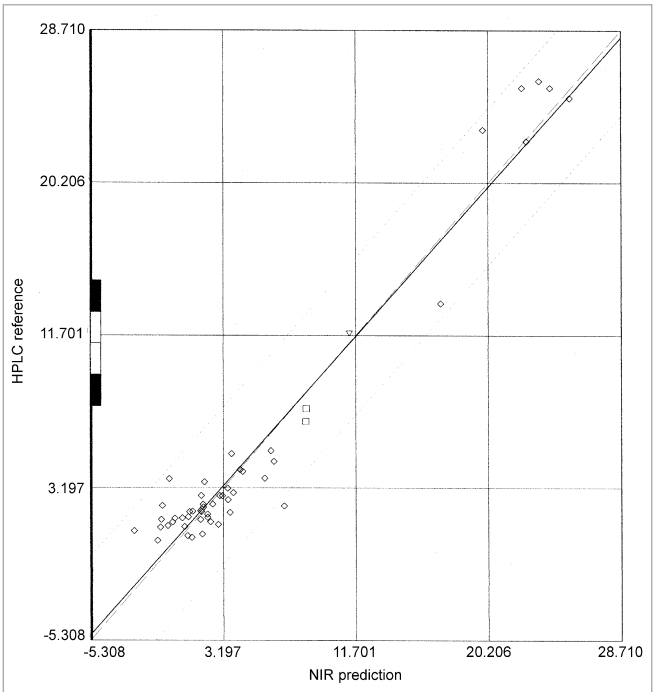

Figure 2. Reference determination versus NIR prediction (validation data set) of rosmarinic acid concentration in 0.25 g dried spearmint samples. Statistics are shown in Table 1.

Table 1. Summary of samples and calibration and validation statistics for NIR prediction vs HPLC evaluation of RA concentration in oven dried spearmint.

	Calibration set	Validation set
Samples	88	55
Range (mg g^{-1} DW)	0.2–26.8	0.2 –25.9
Mean (mg g^{-1} DW)	8.4	5.3
Spectra pre–treatments	None	
Number of PLS factors	7	
R^2	0.94	
SEC (mg g^{-1} DW)	1.94	
r^2		0.90
SEP (mg g^{-1} DW)		3.1
RPD		2.58

Table 2. Comparison of RA concentrations (mg g^{-1}) determined by HPLC and NIR in oven dried mint samples of three cultivars grown with contrasting CO_2 and O_2 conditions. Values are averages of three measurements.

Mint cv	CO_2 (ppm)	O_2 (%)	RA HPLC (mg g^{-1}) ± *SD*	RA NIR (mg g^{-1}) ± *SD*
294099	350	5	11.6 ± 0.1	9.8 ± 1.3
294099	350	10	8.5 ± 0.1	8.4 ± 1.1
294099	350	21	22.7 ± 1.1	19.4 ± 1.1
294099	350	32	21.5 ± 1.3	19.7 ± 0.3
294099	350	43	21.7 ± 3.1	19.7 ± 0.7
294099	10,000	5	8.7 ± 0.5	9.8 ± 1.0
294099	10,000	10	15.0 ± 1.3	19.3 ± 0.3
294099	10,000	21	22.7 ± 2.2	28.7 ± 1.0
294099	10,000	32	22.3 ± 1.0	24.2 ± 0.2
294099	10,000	43	5.5 ± 0.1	19.3 ± 0.9
557793	350	5	7.5 ± 0.4	10.4 ± 0.6
557793	1500	5	12.0 ± 1.9	13.3 ± 0.8
557793	3000	5	15.0 ± 1.0	18.1 ± 0.8
557793	10,000	5	16.8 ± 1.3	29.7 ± 1.1
557807	350	5	6.0 ± 0.5	8.1 ± 0.6
557807	1500	5	6.0 ± 0.4	9.3 ± 0.3
557807	3000	5	14.1 ± 1.6	14.9 ± 0.8
557807	10,000	5	14.6 ± 0.8	12.9 ± 1.0
557807	30,000	5	6.1 ± 0.1	9.8 ± 0.7
557807	50,000	5	10.6 ± 0.6	15.9 ± 1.1

CO_2 concentration, O_2 concentration, temperature, humidity etc.) on secondary metabolite production in plants.

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