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Distinguishing *Vaccinium* Species by Chemical Fingerprinting Based on NMR Spectra, Validated with Spectra Collected in Different Laboratories

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Abstract

A method was developed to distinguish *Vaccinium* species based on leaf extracts using nuclear magnetic resonance spectroscopy. Reference spectra were measured on leaf extracts from several species, including lowbush blueberry (*Vaccinium angustifolium*), oval leaf huckleberry (*Vaccinium ovalifolium*), and cranberry (*Vaccinium macrocarpon*). Using principal component analysis, these leaf extracts were resolved in the scores plot. Analysis of variance statistical tests demonstrated that the three groups differ significantly on PC2, establishing that the three species can be distinguished by nuclear magnetic resonance. Soft independent modeling of class analogies models for each species also showed discrimination between species. To demonstrate the robustness of nuclear magnetic resonance spectroscopy for botanical identification, spectra of a

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Conflict of Interest

The authors declare no conflict of interest.

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Supporting information

A description of the *Vaccinium* leaf extracts used, including voucher numbers, is available as Supporting Information.

sample of lowbush blueberry leaf extract were measured at five different sites, with different field strengths (600 versus 700 MHz), different probe types (cryogenic versus room temperature probes), different sample diameters (1.7 mm versus 5 mm), and different consoles (Avance I versus Avance III). Each laboratory independently demonstrated the linearity of their NMR measurements by acquiring a standard curve for chlorogenic acid ($R^2 = 0.9782$ to 0.9998). Spectra acquired on different spectrometers at different sites classified into the expected group for the *Vaccinium* spp., confirming the utility of the method to distinguish *Vaccinium* species and demonstrating nuclear magnetic resonance fingerprinting for material validation of a natural health product.

Keywords

NMR fingerprinting; *Vaccinium* spp; Ericaceae; nontargeted screening; multilab study; metabolomics

Introduction

Species from the genus *Vaccinium* L. (heath family, Ericaceae) have been used traditionally by indigenous cultures of the circumboreal floristic region (the largest floristic region in the world which includes most of Canada, Europe, and Russia) for food and for the treatment of a variety of disease conditions [1]. The genus includes plants that bear edible berries such as blueberry, bilberry, huckleberry, and cranberry. There are over 40 species in North America and about 450 species worldwide. Examples of medicinal preparations include blueberry leaf extract, which is a traditional Cree medicine shown to have antidiabetic properties [2,3]. The Lukomir Highlanders of Bosnia also use blueberry leaf extract for medicinal purposes [4]. As interest increases in traditional herbal medicines, these medicines are the subject of modern clinical trials to validate effectiveness [5].

For use as an herbal product, it is essential to verify the identity of the plant materials collected. Here we use ^1H nuclear magnetic resonance (NMR) fingerprinting in a multilaboratory setting to distinguish the taxonomic identity of blueberry leaf extracts supplied as powders, demonstrating the usefulness of NMR spectroscopy as a tool to validate crude botanical material. The NMR methodology employed is part of a broader effort to establish validated conditions for highly reproducible qualitative nontargeted and quantitative (qNMR) targeted NMR spectroscopy to be used for this product and to serve as a template for the analysis of other similar highly complex materials. Non-chromatographic botanical fingerprinting has previously been applied to discriminate between *Panax* species (Araliaceae), in particular *Panax ginseng*, *Panax quinquefolius*, and *Panax notoginseng* [6]. However, in that study, ultraviolet (UV) spectroscopy, near infrared spectroscopy, and mass spectrometry were used, rather than NMR. While NMR fingerprinting has been used for a variety of botanical materials [7,8], few multilab validations are reported in the literature. The reproducibility of NMR across sites has recently been demonstrated in a study including seven labs [9] using synthetic test mixtures and animal tissue (flounder liver extracts). Another interlaboratory study discussed the robustness of NMR for distinguishing between two cultivars of broccoli from the florets [10]. The five laboratory study reported here

demonstrates that an NMR metabolomics approach can be applied to the validation of wild harvested plant material used as traditional medicine with NMR spectrometers of significantly different configurations. Blueberry leaves contain a variety of phytochemicals, such as phenyl propanoids, flavonoids, and procyanidins, all well-known antioxidants, that may provide some of the leaves' numerous reported biological activities. A method to identify and quantify marker compounds in blueberry leaves using HPLC and mass spectrometry has previously been reported [11]. Subsequently it was shown that the chemical markers chlorogenic acid (COA) and hyperoside can be identified and quantitated by NMR in crude extract material without further separation [12]. Qualification of the crude extracts of blueberry leaf through the use of chemometric modeling for three *Vaccinium* species is reported here.

In the current study, 123 samples from three different species were analyzed by NMR. Principal component analysis (PCA) of the NMR spectra readily differentiated between these species. With the ability to differentiate species established, one sample of *Vaccinium angustifolium* was sent to five different laboratories for acquisition of NMR spectra. Spectra were acquired according to rigorously defined conditions on spectrometers whose performance had been validated. PCA shows that the spectra for this sample measured at different sites group with *V. angustifolium* and not the other species. Thus, the NMR fingerprinting confirms the species identification by comparison to reference spectra. In addition, it was shown that this identification is insensitive to details of NMR spectroscopy including probe type, spectrometer hardware and software, field strength, operator, and laboratory.

Results

The 1D ^1H NMR spectra of the set of samples representing each species (*V. angustifolium* Ait. – 82 samples; *Vaccinium ovalifolium* Sm. – 22 samples; *Vaccinium macrocarpon* L. – 19 samples) were collected at Bruker. All spectra were of good quality as judged by a linewidth at half maximum less than 1.25 Hz for the DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) reference signal. The average spectrum for each species is shown in Fig. 1. Although all the samples represent leaf extracts of plants from the same genus, visual inspection reveals noticeable variations between the species (as shown in Fig. 1B).

The spectra of the leaf extracts are a complex superposition of many components, represented at a high digital resolution to yield many potential variables. For this type of data, we chose PCA from the many powerful multivariate statistical tools to further explore the interspecies differences. The PCA plot comparing *V. angustifolium*, *V. ovalifolium*, and *V. macrocarpon* is shown in Fig. 2. Spectra for the three species clearly cluster into three distinct groups, with no overlap between their Hotelling ellipses at the 95% confidence limit. One-way ANOVA, followed by Tukey multiple comparisons, shows that in the PC1 dimension (horizontal axis in Fig. 2), *V. angustifolium* (black diamonds) is significantly different ($p < 0.05$) from *V. ovalifolium* (blue squares) and *V. macrocarpon* (green triangles, colors online only), while *V. ovalifolium* and *V. macrocarpon* are not significantly different from each other ($p = 0.364$). In PC2 (vertical axis in Fig. 2), all three species show statistically significant differences ($p < 0.05$). Thus, these three species can be distinguished

by PCA of the NMR spectra using the first two PCs. Comparison of the 1D loadings plots for PC1 and PC2 against a database of reference spectra in DMSO designed for natural products suggests that PC1 reflects COA and/or closely related compounds such as caffeic acid and quinic acid (Fig. 3). PC2 was not readily identified through comparison against the reference database (not shown).

Building on the observation that the *Vaccinium* species can be distinguished based on NMR spectra collected in one laboratory (Bruker), we tested how sensitive the PCA was to differences in the details of the data acquisition. For this purpose, an identical sample of *V. angustifolium* extract was sent to several different laboratories for measurement of the NMR spectrum, which was then used to compare the sample against the sets of reference spectra for the three species. Note that the original study design included 10 laboratories. However, upon examining the spectra from all sites, we realized there were changes over the time it took to ship the samples and run the spectra, as seen in other plant extract studies [13]. Preliminary assays exploring the activity of the blueberry extracts suggest the activity decreases with time dissolved in DMSO over a time frame similar to that observed in this study (J. Ferrier, personal observation). In addition, it is well established that DMSO, despite its universal application, does not necessarily represent an inert solvent for qNMR applications [14,15]. Therefore, the present study considered only the spectra that were recorded within the first month of dissolving the sample in DMSO- d_6 to yield five participating laboratories. These laboratories demonstrated their experimental proficiency by measuring a standard curve for COA, one of the compounds identified in blueberry leaf extract (Fig. 4). The integrated area of a COA peak in the NMR spectrum, scaled by the 90-degree pulse length according to the PULCON [16] and probe Q [17] principles, increases linearly with the concentration of COA. This linearity of the signal strength with the sample concentration is a basic property of NMR [18]. In practice, study participants observed a very tight correlation, with R^2 coefficients of 0.9782 and higher (Table 1).

The *V. angustifolium* spectra acquired at different sites were compared against the *V. angustifolium* spectra acquired at Bruker, as shown in Fig. 5A. The spectra collected at different sites (red circles) were well within the 95% confidence limits for the set of *V. angustifolium* spectra acquired at Bruker (black dotted line). The Student's t-test returned a low probability that the test spectra are from the same distribution as the *V. angustifolium* set, likely because the test spectra were acquired on one sample and clustered very tightly. Fig. 5B shows the comparison of the test *V. angustifolium* spectra against the *V. ovalifolium* spectra collected at Bruker. The test spectra form a distinct cluster, separate from the *V. ovalifolium* spectra, as clearly visualized by the non-overlapping Hotelling ellipses for the data sets. Due to the distribution of values for *V. ovalifolium*, this separation is statistically significant ($p < 0.05$) for both PC1 and PC2 in the Student's t-test. Fig. 5C shows the comparison of *V. angustifolium* against *V. macrocarpon*. Again, the scores plot of PC2 versus PC1 clearly suggests a distinction between the test sample and the *V. macrocarpon* samples acquired at Bruker. For *V. macrocarpon*, the distinction based on PC1 is statistically significant ($p < 0.05$), which corresponds to the observed separation in the horizontal axis in Fig. 5C.

The features of the spectra acquired in different laboratories, i.e., tight clustering in the (PC1, PC2) scores plots, grouping within *V. angustifolium* but not *V. ovalifolium* or *V. macrocarpon*, are all the more striking when the differences in the instrumentation at each site are considered (Table 1). Spectra were acquired at two different fields strengths (600 versus 700 MHz), with the accompanying difference in sensitivity attributable to field strength (26% higher at 700 MHz), resolution (17% higher at 700 MHz), and observed couplings (their fixed values in hertz show different values in ppm after scaling by field strength). The spectra were all acquired with NMR consoles of the Avance (AV) spectrometer series and represent spectrometers currently in use, manufactured within a 10-year time frame. The AVI console represents late stage analog technology whereas the AVIII consoles utilize state-of-the-art digital technology. The AVI console, albeit largely analog technology, contains digital quadrature detection and first generation digital signal generation technology. Most spectra were acquired with the AVIII generation which features an enhanced digital receiver and pulse programmer. A major difference in spectra resulting from the different console technologies is the baseline linearity, but this was compensated for by manual baseline correction of the spectra from the AVI console. The spectra were acquired with a variety of probes, some optimized for ^1H sensitivity and lineshape (inverse triple resonance probes: TCI and TXI) and some optimized for detection of multiple nuclei (observe dual and quad nucleus probes: DCH and QNP). Some of the probes were cryogenically cooled (CryoProbes at Bruker, DHMRI-1, DHMRI-2, NRC IMB, and UIC) to reduce noise and enhance sensitivity. Also, the probes were designed and optimized for two different diameter sample tubes, 5 mm and 1.7 mm, greatly affecting the quality factor (Q-factor) of the probe. The ^1H Q-factor range was well represented in this study with the highest Q-factor in the NRC IMB TCI CryoProbe at more than 30 times that of the smallest Q-factor found in the AAFC 1.7 mm TXI room temperature probe. Sensitivity, magnetic field homogeneity, and baseline are influenced by the probe configuration. Even the spectrometer operating software varied, in one case using the Xwin-nmr package compared to the various versions of the TopSpin software. This demonstrates that NMR produces the same robust result for the same sample across a variety of measuring conditions.

Since the species can be distinguished based on PCA, we built soft independent modeling of class analogies (SIMCA) models for each species. SIMCA has the advantage that it is based on the same spectra used in the PCA without further assumptions. SIMCA has no implicit assumption of nonoverlapping classes, so in principle a test sample can belong to multiple classes [19]. The model for *V. angustifolium* included 14 PCs for an explained variance of 95.1%, the *V. ovalifolium* model included 8 PCs for 95.8% explained variance, and the *V. macrocarpon* model included 7 PCs for 95.5% explained variance. After building a SIMCA model for each species, the set of reference spectra for each species was tested against each model. The results are summarized in Table 2. For *V. ovalifolium* and *V. macrocarpon*, all the spectra used to build the model classify within the model (100%). For *V. angustifolium*, the model based on 82 spectra fits most of the spectra, with 77 (94%) classified as within the model. The five spectra that do not classify within the *V. angustifolium* model were collected in the same location, from the wild, over the same dates, and within the same habitat as other samples in the set. Therefore, with no clear rationale to exclude them, they

were retained when building the SIMCA model. Note that there is no confusion among the species using these models (0% for the off-diagonal elements in Table 2).

Discussion

This study shows that NMR is a valuable tool for identifying harvested wild plant material from crude extracts by spectroscopic fingerprinting. Specifically, it was possible to distinguish different *Vaccinium* species using PCA of the 1D ^1H spectra of samples of leaf extracts. Currently there are a sufficient number of samples to serve as inclusivity panels [20] for *V. angustifolium* (low-bush blueberry), *V. ovalifolium* (oval leaf huckleberry), and *V. macrocarpon* (cranberry), and build models. Preliminary assessment of spectra from additional species of blueberry and bilberry (such as *V. corymbosum*, *V. boreale*, and *V. caespitosum*) suggest that additional species can also be distinguished by the same NMR method. Further results on additional *Vaccinium* species will be reported in due course.

The NMR spectrum of a chemically complex sample is a characteristic feature of that sample, independent of the details of the measurement within the variation tested here. Spectra acquired at five different sites with differences in field strength, console, probe, sample diameter, and software clustered tightly together in the PCA. Comparison of these spectra against sets of reference spectra for three species acquired at yet another site allowed for the correct identification of the species of the test sample. Material validation of this natural health product is therefore possible using NMR spectroscopy on NMR spectrometers of various configurations and architecture. The ability to evaluate a botanical material with chemometric models generated from data acquired at a different site with an instrument of a different configuration demonstrates the power of NMR spectroscopy as a quality control and species identification tool.

Materials and Methods

Reagents

DMSO- d_6 (99.9% dimethyl sulfoxide- d_6) was purchased from Cambridge Isotopes (DLM-10–100 PSO#10K-221). DSS- d_6 was purchased from Isotec, a division of Sigma-Aldrich (613150 Lot TV0142). For the stock DMSO/DSS solvent solution, 157 μL of 300 mM DSS- d_6 (in DMSO- d_6) was added to 100 mL of DMSO- d_6 . COA, greater than 95% COA, was purchased from Sigma Aldrich (C3878, batch number 070M1331). The COA had a purity greater than 99% via HPLC (1100 series HPLC-DAD system, Agilent), consisting of an autosampler, a column thermostat, a quaternary pump, and a diode array detector DAD) [11], confirmed by NMR.

Plant material

Leaves from the *Vaccinium* spp. were harvested in the wild from reproductively isolated sites across Canada. Identification was made by Alain Cuerrier and Jonathan Ferrier using Scoggan's, "The Flora of Canada" [21], and herbarium vouchers were deposited at the University of Ottawa Herbarium (OTT) and the Marie-Victorin Herbarium (MT). A description of the samples, including the voucher numbers, is provided in Table 1S, Supporting Information.

Extraction

Leaves were dried overnight in a dehydrator. The dried leaves were ground in a Wiley mill through a size 40 mesh and extracted with 95% ethanol (10 mL per gram leaf material) with shaking at room temperature for 24 hours. After 24 hours, the solvent was decanted (phase 1) and the ground material was extracted again using 95% EtOH (5 mL per gram of leaf material) and shaken for another 24 hours. Subsequently, the solvent was decanted (phase 2) and phases 1 and 2 were pooled and centrifuged in an Eppendorf 5810 R at 1000 g for 5 minutes at room temperature. The solvent was decanted into a round bottom flask wrapped with aluminium foil, and all alcohol was removed in a Speed Vac (Thermo Scientific) at a water bath temperature of 37°C. To remove the water, the samples were transferred into pre-weighed 50-mL self-standing tubes and then lyophilized in a SuperModulyo freeze dryer (Thermo Electron Corporation) overnight. All extracts were stored at -20°C.

Preparation of NMR samples

Twenty-five mg of crude leaf extract powder were dissolved in 1.0 mL of DMSO/DSS solvent solution. Specific sample preparation weights were measured to the digitization limits of the appropriate balance. Two analytical balances were used, one with a readability of 0.1 mg (4 decimal places) and the other with a readability of 0.01 mg (5 decimal places), both from Mettler Tole-do AG104. Samples were vortexed for 10 seconds and subsequently centrifuged for 10 seconds at 3800 g [Capsule Tomy HF-120 and Eppendorf 5810 R (Eppendorf AG)]. 600 µL of the resulting supernatant was transferred to a 5-mm NMR tube for spectroscopy. For the multilab study, *V. angustifolium* leaf extract was selected because we had many representative samples for this species to build a SIMCA model and we had a sufficient quantity of one sample to send to other laboratories. The leaf extract was sent to the first laboratory as a powder and dissolved in DMSO/DSS solvent solution there. This sample was sent on ice to the next laboratories.

NMR spectrometers

All spectrometers were manufactured by Bruker BioSpin. By site, the configurations were: **Bruker**: 600 MHz (14.1 tesla), AVIII, 5 mm TCI CryoProbe; **DHMRI-1**: 600 MHz (14.1 tesla), AVIII, 5 mm DCH CryoProbe; **DHMRI-2**: 700 MHz (16.4 tesla), AVIII, 5 mm QNP CryoProbe; **NRC IMB**: 700 MHz (16.4 tesla), AVIII, 1.7 mm TCI CryoProbe; **UIC**: 600 MHz (14.1 tesla), AVI, 5 mm TXI CryoProbe; **AAFC**: 600 MHz (14.1 tesla), AVIII, 1.7 mm TXI (room temperature) probe. Each spectrometer was equipped with a pre-cooling and temperature stabilization unit for the conditioning of gas feeding the probe. Note that AVI NMR spectrometer consoles are largely analog technology (with digital quadrature detection and first generation digital signal generation), whereas AVIII consoles are digital technology. Most spectrometers were equipped with TopSpin software for data acquisition and processing; the UIC instrument was equipped with an earlier software package, Xwin-nmr (Bruker BioSpin).

Supplies

Consumable supplies for the experiments included NMR tubes (Wilmad PP-535 and Bruker Z107374), 1.5-mL centrifuge tubes (VWR: 87003–294), pipettes (Eppendorf Research), and pipette tips (VWR sterile/aerosol tips).

System suitability

System suitability tests were used to validate the performance of the NMR spectrometer prior to acquiring data on the test samples. The system suitability tests used were: (1) ^1H sensitivity (which measures the signal-to-noise ratio, SINO) using 0.1% ethylbenzene in CDCl_3 ; (2) ^1H lineshape, using 1% CHCl_3 in acetone- d_6 (or 0.3% CHCl_3 in acetone- d_6 for CryoProbes); (3) the temperature was confirmed with a 99.8% MeOD temperature standard before instrument operation.

Standard curves

Solutions of known COA concentration were prepared by dissolving known weights of dried, solid powder in 600 μL of $\text{DMSO}-d_6$. For each spectrum, a signal associated with COA was integrated using AMIX 3.9.14 (Bruker BioSpin). The integrals were scaled by the calibrated 90-degree pulse length to account for the physical properties of the sample in accordance with the PULCON principle [16] and the Q correction method [17]. The scaled integrals as a function of COA concentration were plotted in Microsoft Excel 2010 and fitted to a line.

Data acquisition

All samples were run on Bruker NMR spectrometers with the temperature calibrated to 298 K. The 90-degree proton pulses were calibrated automatically using the nutation echo scheme [22] prior to acquiring the data, implemented as the “pulsecal” command in TopSpin. Proton NMR spectra were acquired using the one-dimensional proton nuclear Overhauser effect spectroscopy pulse sequence (noesy1d). For the standard procedure, 64 K data points were collected and the recycle delay was 4 seconds. A total of 64 scans were accumulated, with four dummy scans and a mixing time of 0.01 seconds, for an experiment time of 7:40 min.

Data processing

Spectra were processed using TopSpin software, version 3.1. Free induction decays (fids) were multiplied with exponential line broadening (0.3 Hz) before Fourier transform. Spectra were phased to zero-order automatically using the apk0 command and referenced to DSS at 0.000 ppm. Baseline correction was performed manually using polynomial baseline correction (.basl command) on all spectra to give a zero vertical offset.

Data analysis

All spectra were analyzed using AMIXTM software, version 3.9.14 (Bruker BioSpin). The spectra were divided into buckets in the chemical shift region from 8.40 to 0.40 ppm with a bucket width of 0.01 ppm. Broad signals downfield of 8.40 ppm were excluded from the analysis because they are generally not well represented by narrow, uniform buckets.

Exclusions were made in the regions of peaks for DMSO (2.66–2.40 ppm), water (3.42–3.23 ppm), ethanol (1.23–0.93, 3.52–3.42, and 4.47–4.31 ppm), methanol (3.23–3.17 ppm), and DSS (0.125 to –0.125 ppm). SIMCA models were generated using tools in Assure-RMS™ (Bruker BioSpin).

Statistics

One-way analysis of variance (ANOVA) followed by post hoc Tukey multiple comparison tests of the data were performed on the PCA scores to indicate whether or not the species groups were significantly different from each other. All results were considered to be statistically significant if $p < 0.05$. ANOVA and Tukey multiple comparison tests were performed using SPSS 19.0 (IBM). For comparison of the spectra acquired in different laboratories to the reference set for a single species, the Student's t-test was used with two-tailed distribution and two-sample unequal variance (heteroscedasticity), as implemented in Microsoft Excel.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Moerman, DE. Native American ethnobotany. Portland: Timber Press Incorporated; 1998. p. 582-587.
2. Leduc C, Coonishish J, Haddad P, Cuerrier A. Plants used by the Cree Nation of Eeyou Istchee (Quebec, Canada) for the treatment of diabetes: A novel approach in quantitative ethnobotany. J Ethnopharmacol. 2006; 105:55–63. [PubMed: 16297584]
3. Ferrier J, Djeflal S, Morgan HP, Vander Kloet SP, Redži S, Cuerrier A, Balick MJ, Arnason JT. Antiglycation activity of *Vaccinium* spp. (Ericaceae) from the Sam Vander Kloet collection for the treatment of type II diabetes. Botany. 2012; 90:401–406.
4. Ferrier, J.; Ša iragi , L.; Redži , S.; Alikadi , E.; Chen, ECH.; Saleem, A.; Cuerrier, A.; Balick, MJ.; Arnason, JT. Ways the Lukomir Highlanders of Bosnia & Herzegovina treat diabetes: a cross cultural multidisciplinary approach. In: Pieroni, A.; Quave, CL., editors. Ethnobotany and biocultural diversities in the Balkans: Perspectives on sustainable rural development and reconciliation. Section: The herbal reservoir of Europe: from folk medical uses to the modern medicinal plant trade. Berlin, Heidelberg: Springer; 2014.
5. Ly C, Yockell-Lelievre J, Ferrier J, Ša iragi L, Saleem A, Arnason JT, Gruslin A. The influence of *Vaccinium angustifolium* var *laevifolium* house (Ericaceae) extracts on trophoblast biology. Reprod Sci. 2013; 20:265A.
6. Chen P, Luthria D, Harrington PB, Harnly JM. Discrimination among *Panax* species using spectral fingerprinting. J AOAC Int. 2011; 94:1411–1421. [PubMed: 22165005]
7. Krishnan P, Kruger NJ, Ratcliffe RG. Metabolite fingerprinting and profiling in plants using NMR. J Exp Bot. 2005; 56:255–265. [PubMed: 15520026]

8. Defernez M, Colquhoun IJ. Factors affecting the robustness of metabolite fingerprinting using ^1H NMR spectra. *Phytochemistry*. 2003; 62:1009–1017. [PubMed: 12590127]
9. Viant MR, Bearden DM, Bundy JG, Burton IW, Collette TW, Ekman DR, Ezernieks V, Karakach TK, Lin CY, Rochfort S, de Ropp JS, Teng Q, Tjeerdema RS, Walter JA, Wu H. International NMR-based environmental metabolomics intercomparison exercise. *Environ Sci Technol*. 2009; 43:219–225. [PubMed: 19209610]
10. Ward JL, Baker JM, Miller SJ, Deborde C, Maucourt M, Biais B, Rolin D, Moing A, Moco S, Vervoort J, Lommen A, Schäfer H, Humpfer E, Beale M. An inter-laboratory comparison demonstrates that [^1H]-NMR metabolite fingerprinting is a robust technique for collaborative plant metabolomics data collection. *Metabolomics*. 2010; 6:263–273. [PubMed: 20526352]
11. Harris CS, Burt AJ, Saleem A, Le PM, Martineau LC, Haddad PS, Bennett SA, Arnason JT. A single HPLC-PAD-APCI/MS method for the quantitative comparison of phenolic compounds found in leaf, stem, root, and fruit extracts of *Vaccinium angustifolium*. *Phytochem Anal*. 2007; 18:161–169. [PubMed: 17439018]
12. Hicks JM, Muhammad A, Ferrier J, Saleem A, Currier A, Arnason JT, Colson KL. Quantification of chlorogenic acid and hyperoside directly from crude blueberry (*Vaccinium angustifolium*) leaf extract by NMR spectroscopy analysis: single-laboratory validation. *J AOAC Int*. 2012; 95:1406–1411. [PubMed: 23175973]
13. Liu Y, Murphy PA. Alkamide stability in *Echinacea purpurea* extracts with and without phenolic acids in dry films and in solution. *J Agric Food Chem*. 2007; 55:120–126. [PubMed: 17199322]
14. Pauli GF, Jaki BU, Gödecke T, Lankin DC. Quantitative ^1H NMR. Development and potential of an analytical method: an update. *J Nat Prod*. 2012; 75:834–851. [PubMed: 22482996]
15. Pauli GF, Jaki BU, Lankin DC. Quantitative ^1H NMR: development and potential of a method for natural products analysis. *J Nat Prod*. 2005; 68:133–149. [PubMed: 15679337]
16. Wider G, Dreier L. Measuring protein concentrations by NMR spectroscopy. *J Amer Chem Soc*. 2006; 128:2571–2576. [PubMed: 16492040]
17. Burton IW, Quilliam MA, Walter JA. Quantitative ^1H NMR with external standards: use in preparation of calibration solutions for algal toxins and other natural products. *Anal Chem*. 2005; 77:3123–3131. [PubMed: 15889900]
18. Harris, RK. Nuclear magnetic resonance spectroscopy. Harlow: Longman Scientific and Technical; 1986. p. 9-11.
19. Esbensen, KH.; Guyot, D.; Westad, F.; Houmoller, L. Multivariate data analysis: in practice: an introduction to multivariate data analysis and experimental design. Trondheim: Camo ASA; 2002. p. 335-350.
20. Harnly J. Chemical fingerprinting for identification/authentication of botanical materials. *Planta Med*. 2012; 78:IL10.
21. Scoggan, HJ. The flora of Canada, publications in botany. Vol. 7. Ottawa: National Museums of Canada; 1979. p. 1208-1213.
22. Wu PSC, Otting G. Rapid pulse length determination in high-resolution NMR. *J Magn Reson*. 2005; 176:115–119. [PubMed: 15972263]

Fig. 1. Average spectrum over the reference set of spectra for each species – *V. angustifolium* (black line), *V. ovalifolium* (blue line), and *V. macrocarpon* (green line). **A** Full spectrum (10.5 to – 0.25 ppm); **B** expansion in the region containing the characteristic signals for chlorogenic acid (7.90 to 6.05 ppm). Note that all spectra have zero baseline offset; they are shifted here to facilitate comparison. (Color figure available online only.)

Fig. 2. Scores plot for all three species, *V. angustifolium* (black diamonds), *V. ovalifolium* (blue squares), and *V. macrocarpon* (green triangles). Hotelling ellipses, showing the region of the 95% confidence limit, are shown for each group by dashed lines of the matching color. Note that spectra for each species occupy a distinct region, with no overlap. (Color figure available online only.)

Fig. 3. 1D loadings plot for PC1 (top, black) compared to the reference spectra for chlorogenic acid (red), caffeic acid (orange), and quinic acid (purple) in DMSO. The loadings plot shows strong negative values at positions corresponding to these acids, suggesting they may contribute to the discrimination between species in PC1 (40% explained variance). (Color figure available online only.)

Fig. 4. Representative calibration curve (from the David H. Murdock Research Institute's 600 MHz spectrometer). The plot shows integrated peak area, scaled by the 90-degree pulse length, versus the chlorogenic acid concentration. The data were fit to a line with a y-intercept of zero. The equation for the line and the square of the correlation coefficient (R^2) are shown. (Color figure available online only.)

Fig. 5.

Scores plots for representative spectra for each species, with the spectra acquired in different laboratories included. In all three panels, spectra of the identical *V. angustifolium* sample sent to different laboratories are represented by red circles. Hotelling ellipses, showing the region of the 95% confidence limit, are shown for each group by dashed lines of the matching color. **A** Scores plot for *V. angustifolium* with the spectra acquired in different laboratories included. Spectra of the *V. angustifolium* samples collected at Bruker are represented by black diamonds. Note that the ellipse for the spectra acquired in different laboratories is almost completely within the ellipse for the diverse *V. angustifolium* sample set. **B** Scores plot for *V. ovalifolium* with the spectra acquired in different laboratories included. Spectra of the *V. ovalifolium* samples collected at Bruker are represented by blue squares. Note that the ellipse for the spectra acquired in different laboratories is well separated from the ellipse for the *V. ovalifolium* samples. **C** Scores plot for *V. macrocarpon*, with the spectra acquired in different laboratories included. Spectra of the *V. macrocarpon* samples collected at Bruker are represented by green triangles. Note that the ellipse for the spectra acquired in different laboratories is well separated from the ellipse for the *V. macrocarpon* samples. (Color figure available online only.)

Table 1

Comparison of the experimental configuration at different laboratories in this study. Sites: **Bruker**: Bruker BioSpin Corporation, Billerica, MA; **DHMR1**: David H. Murdock Research Institute, Kannapolis, NC (there are two spectrometers at this site); **NRC IMB**: NRC Institute for Marine Biosciences, Halifax, Nova Scotia; **UIC**: University of Illinois at Chicago, Chicago, IL; **AAFC**: Agriculture and Agri-Foods Canada, Charlottetown, Prince Edward Island. Field strength, console, probe type, sample size, and software varied. Console technology ranged from nearly all analog (AVI) to state-of-the-art digital technology (AVIII). Inverse detection probes (TCI and TXI) and direct detection probes (QNP, DCH) were represented.

Feature	Bruker	DHMR1-1	DHMR1-2	NRC IMB	UIC	AAFC
R ² for standard curve	0.9999	0.9782	0.9992	0.9987	0.9990	0.9998
Signal/noise COA peak*	3620.3	3696.6	3689.1	1464.2	673.5	492.0
Field strength (MHz)	600	700	600	700	600	600
Console	AVIII	AVIII	AVIII	AVIII	AVI	AVIII
Probe	TCI	QNP	DCH	TCI	TXI	TXI
CryoProbe	yes	yes	yes	yes	yes	no
Sample diameter (mm)	5.0	5.0	5.0	1.7	5.0	1.7
Software	TopSpin 3.1	TopSpin 2.1	TopSpin 2.1	TopSpin 2.1	Xwin-nmr	TopSpin 2.1

* Doublet at 6.797 ppm in the same sample of *V. angustifolium*

Table 2

Classification of the blueberry species. A soft independent modeling of a class analogies model was generated for each species. Then, samples in the reference set for each species were tested against that model. The percentage of the sample set (rows) that tests within the model (columns) is reported.

Species	<i>V. angusti-folium</i>	<i>V. ovalifolium</i>	<i>V. macrocarpon</i>
<i>V. angustifolium</i>	94%	0%	0%
<i>V. ovalifolium</i>	0%	100%	0%
<i>V. macrocarpon</i>	0%	0%	100%