Comparisons of Large (Vaccinium macrocarpon Ait.) and Small (Vaccinium oxycoccos L., Vaccinium vitis-idaea L.) Cranberry in British Columbia by Phytochemical Determination, Antioxidant Potential, and Metabolomic Profiling with Chemometric Analysis

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Abstract
There is a long history of use and modern commercial importance of large and small cranberries in North America. The central objective of the current research was to characterize and compare the chemical composition of 2 west coast small cranberry species traditionally used (Vaccinium oxycoccos and Vaccinium vitis-idaea) with the commercially cultivated large cranberry (Vaccinium macrocarpon Ait.) indigenous to the east coast of North America. Vaccinium oxycoccos and Vaccinium macrocarpon contained the 5 major anthocyanins known in cranberry; however, the ratio of glycosylated peonidins to cyanidins varied, and V. vitis-idaea did not contain measurable amounts of glycosylated peonidins. Extracts of all three berries were found to contain serotonin, melatonin, and ascorbic acid. Antioxidant activity was not found to correlate with indolamine levels while anthocyanin content showed a negative correlation, and vitamin C content positively correlated. From the metabolomics profiles, 4624 compounds were found conserved across V. macrocarpon, V. oxycoccos, and V. vitis-idaea with a total of approximately 8000–10000 phytochemicals detected in each species. From significance analysis, it was found that 2 compounds in V. macrocarpon, 3 in V. oxycoccos, and 5 in V. vitis-idaea were key to the characterization and differentiation of these cranberry metabolomes. Through multivariate modeling, differentiation of the species was observed, and univariate statistical analysis was employed to provide a quality assessment of the models developed for the metabolomics data.

Introduction
There are more than 450 species in the genus Vaccinium (Ericaceae) ranging across Europe, North America, Central America, Central and South East Africa, Madagascar, Japan and Asia [1]. In North America, 65 species of Vaccinium have been identified [1], and there are 18 identified species in the flora of British Columbia, Canada [2]. Vaccinium macrocarpon Aiton, commonly known as “large cranberry”, is a diploid (2n = 24) native of the northeastern USA, bred through agricultural selection and grown as a commercial crop in the lower mainland of British Columbia [3]. Vaccinium oxycoccos L., (2n = 24, 48, 72) commonly known as “small cranberry” or “bog cranberry”, and Vaccinium vitis-idaea L., (2n = 24) commonly known as “rock cranberry” or “northern mountain cranberry” in North America and “lingonberry” in Europe, are all found in native populations across North America and Europe including widespread natural populations in the coastal and mountainous regions of British Columbia [1, 2, 4–6]. Cranberries have had important roles in the traditional health and culture of indigenous people across North America as well as modern uses in the natural health products industry. Although Vaccinium vitis-idaea L. is taxonomically closer to the bilberry, Vaccinium myrtillus L., than it is to the cranberry species, in traditional practices, the natives essentially treated and used V. vitis-idaea in the same way as the V. oxycoccos and V. macrocarpon species [7–9]. Traditionally, V. macrocarpon berries were gathered from August through the fall, even when still unripe, allowed to ripen and then eaten either fresh or cooked [8]. Moerman reported the use of wild V. macrocarpon by the Algonquin, Chippewa, Ojibwa, and Iroquois in baked, dried, and raw foods, mixed with corn breads, and sold or traded for other commodities [10]. Several uses of V. oxycoccos by the indigenous people of northern Canada have been described including consumption of fresh berries, berries
stewed with fish, fish eggs or blubber, boiled fruit eaten with meat, fresh or dried fruit stored for winter, and fruit preserved in grease and stored in birch bark baskets underground [10]. Moerman also reports that the Galmad used leaves of the plant to make a tea and that the Ojibwa used an infusion of the plant as a treatment of mild nausea [10]. Turner reported that the people of Haida Gwaii know *V. oxyccoco* by the name ‘dah’ and that the name for the species also means “buying” indicating the importance of the crop for trade [6]. Patches of berry plants may have been considered the property of a family and passed through generations [6]. Likewise, there are many uses for *V. vitis-idaea*, known by the traditional names “sk’aagi chaay” which translates to “dog-salmon eggs” or “tlgaa gaanga” which translates to “ground/earth berries” [6]. Moerman reports that the Haida used *V. vitis-idaea* berries for food [10] and Turner reports that the practice continues and that people now make preserves with them [6]. Moerman reports that the berries were mixed with boiled fish eggs, livers, air bladders, and fat to make a winter meal for the Woodland Cree and that they also used the berries to color porcupine quills or strung on a string to make a necklace [10]. Further, Moerman reports that leaves of the plant were smoked as a tobacco alternative or substitute by the Inuitut [10].

In the modern marketplace, the large cranberry (*V. macrocarpon*) is one of the significant success stories of the functional foods industry. Large cranberries are perhaps best known for the treatment and prevention of urinary tract infections (UTIs) [11, 12]. Research suggests that phytochemical constituents of cranberry inhibit *Escherichia coli* from adhering to uroepithelial cells in the urinary tract or may reduce symptoms of UTI through broader anti-inflammatory effects [13, 14]. Chemical analyses of cranberries and cranberry products have identified multiple bioactive flavonoids including flavonols, flavan-3-ols (catechins), anthocyanins, anthocyanidins, and proanthocyanidins [15]. Many of these medicinal compounds are common among all 3 *Vaccinium* species. For *V. vitis-idaea*, Lehtonen et al. found that the species is a rich source of anthocyanins that are eliminated via methylation, oxidation, and excretion through urine, a common mechanism to the one proposed for *V. macrocarpon* [16, 17]. Polymeric proanthocyanin extracts of both *V. macrocarpon* and *V. vitis-idaea* have also shown antimicrobial activity against *Staphylococcus aureus* and inhibited hemagglutination of *E. coli* in *vitro* bioassays [18].

A broad spectrum of methods have been used for the assay of cranberry phenolic constituents, and in a recent review of the analytical literature for berries it was observed that methods can be interference prone, often over- or underestimating the polyphenol content [19]. The anthocyanin profile of cranberry is unique, making it a useful tool for evaluating the identity and quality of cranberry and its products [20–22]. A common approach for determining anthocyanin content in cranberry is high-performance liquid chromatography (HPLC) with ultraviolet detection, either with or without prior forced hydrolysis of the anthocyanins [23, 24]. While the hydrolysis method can be useful in cases where there is no doubt about the identity and purity of the test materials, it is a nonspecific approach and susceptible to interferences caused by adulteration with other anthocyanin-rich plants [19]. Berries of all 3 of the species have been found to have a strong potential for detoxification of reactive oxygen species or “antioxidant potential” [25, 26]. The high antioxidant activity observed in cranberry fruit and extracts is in part attributed to high levels of phenolic compounds [27–29]. It has been shown, however, that these compounds do not explain all of the antioxidant activity observed in extracts [30]. Studies in grape and other berries have shown that melatonin and its metabolites can act as signaling molecules that induce antioxidant responses and also directly act as reactive oxygen species (ROS) scavengers [31–33]. Synergism, from compounds such as vitamin C, has also been shown to have a significant effect on antioxidant response [30].

Unfortunately, the full nutritional and medicinal potential and activity of cranberries as well as their importance for maintaining health in traditional and modern diets is not fully understood. Another approach to determine the overall quality of a complex botanical material, such as cranberry, is metabolomics profiling. Described as untargeted quantitative chemical analysis of the whole array of small molecules contained within a cell or tissue [34–36], metabolomics analysis is quickly becoming an important tool for the characterization and assessment of commercial herbal products and plant extracts [35, 37, 38]. With the goal of gaining a better phytochemical understanding of *Vaccinium* species in British Columbia, the specific research objectives of this work were (a) to collect and investigate wild populations of native BC cranberries from the Haida Gwaii, (b) to compare the relative abundance of known cranberry phytochemicals such as anthocyanins and vitamin C in the species of large and small cranberries, (c) to discover previously undescribed phytochemicals in large and small cranberries such as indoleamine neuromodulators, and (d) to develop a model for metabolomics analysis to identify commonalities and differences between closely related species.

**Materials and Methods**

**Plant materials**

*Vaccinium oxyccocos* L. and *Vaccinium vitis-idaea* L. fruits were collected from wild populations in Haida Gwaii in September 2010. Berries, leaves, and stems for analytical samples and herbarium vouchers were collected for *Vaccinium oxyccocos* L. in the muskeg just off the Yellowhead Highway approximately 7 km south of Masset (53°55′33″N, 132°6′25″W) and for *Vaccinium vitis-idaea* L. a short distance south on the same highway just off the pavement where the vegetation is brushed by the highway crews (53°51′20″N, 132°5′20″W) (Fig. 1A, B). Species were identified based on local knowledge (Tim Wolthers) and comparison to published flora [2]. Berries were immediately frozen and shipped to the Okanagan campus of the University of British Columbia. Leaf and stem collections were pressed and dried with a field press, assembled and deposited as vouchers (V235344 and V235343) at the herbarium of the Beaty Biodiversity Center, University of British Columbia. Fresh fruit of the third species, *V. macrocarpon* Aiton, was grown in prepared peat beds and harvested by the standard wet-pick method in the Vancouver Greater Regional District under the standard commercial production conditions approved by Ocean Spray Canada Ltd., and immediately frozen prior to shipping.

**Reagents and standards**

HPLC grade methanol, acetonitrile, hydrochloric acid, and phosphoric acid were obtained from VWR International. Ascorbic acid (purity ≥ 99.0%), trolox [(±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; purity ≥ 97%], melatonin (N-acetyl-5-hydroxytryptamine; purity ≥ 99.5%), serotonin (5-hydroxytryptamine; purity ≥ 98%), and DPPH (1,1-diphenyl-2-picrylhydrazyl) were obtained from Sigma-Aldrich Canada Ltd. A mixed reference standard containing cyanidin-3-O-galactoside (C3Ga), cyanidin-
3-O-glucoside (C3Gl), cyanidin-3-O-arabinoside (C3Ar), peonidin-3-O-galactoside (P3Ga), and peonidin-3-O-arabinoside (P3Ar) at 250–500 µg/mL in methanol was obtained from Cerilliant Corp.

Anthocyanin determination by HPLC-DAD

The anthocyanin content was determined using a previously validated method [39] optimized for rapid resolution [3]. In brief, freeze-dried cranberries were weighed (0.250 ± 0.025 g) to 50-mL conical tubes and extracted with 20 mL of MeOH-concentrated HCl (98.2, v/v) by vortex mixing (Thermolyne Maxi Mix 1; Fisher Scientific Company), sonication for 15 min (Branson Model 3510R-MTH Ultrasonic Cleaner; VWR International), and shaking (Burrell Scientific model 57 040–82 Wrist Action Shaker) on an angle at 180 rpm for 30 min. The supernatant was decanted to a 25-mL glass volumetric flask, brought to volume with extraction solvent and mixed well. Approximately 1 mL of each sample solution was filtered (0.45 µm PTFE) to amber HPLC vials. Chromatographic separation was achieved on an Agilent 1100 Series HPLC System using a Zorbax SB C18 Rapid Resolution HT column (4.6 × 50 mm, 1.8 µm), mobile phase A: 0.5% water-phosphoric acid (99.5:0.5, v/v) and mobile phase B: 100% methanol; (a) 0% B (1.5 mL/min), (b) 15% B over 14 min: 0% B (0.5 mL/min), (c) 15–20% B over 20 min: 100% methanol; (d) 21.1–20 min: 0% B (1.5 mL/min), (e) 20–21.5 min: 0% B (0.5 mL/min). The analyte of interest was detected at 245 nm and the data collected and analyzed using Agilent ChemStation software, Rev. B.03.01.

Melatonin and serotonin determination

Melatonin and serotonin were analyzed following a previously published method [33]. Briefly, frozen berries were sectioned, weighed and homogenized in complete darkness with the extraction completed in less than 15 minutes for each individual sample. Cranberries, including seed flesh and skin (200 mg), were homogenized for 3 min in 200 µL methanol: water: formic acid (80:20:1 v/v) using a cordless motor Kontes Pellet Pestle™ grinder (Kimble Chase Life Science) and disposable pestles (Kontes). Samples were centrifuged for 3 min at 16000 g (Galaxy 16DH Microcentrifuge; VWR International), and the resulting supernatant was filtered (0.2 µm, Ultrafree-MC filtered centrifuge tubes; Millipore). Samples were kept at 4 °C in total darkness until analysis on a Waters Acquity UPLC System with separation achieved at 30°C on a Waters BEH C18 UPLC column (2.1 × 150 mm, 1.7 µm; Waters Corporation) with gradient elution using 1% aqueous formic acid: acetonitrile (0.0–4.0 min, 95–5:5:95 v/v, 4.0–4.5 min, 95–5:95:5 v/v, 4.5–5.0 min, 95:5:5 v/v) at 0.25 mL/min. Melatonin and serotonin were quantified by electrospray ionization in the positive mode with time-of-flight mass spectrometry (LCT Premier Micromass MS) using the “W” configuration and by comparison to authenticated standards and absolute mass as per the parameters for mass spectrometry [33].

Determination of free radical scavenging capacity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay employed to assess the free radical scavenging capacity of berries from the 3 Vaccinium species was adapted from published methods [40]. Briefly, approximately 0.3 g sections of fruit samples from each berry, prepared in triplicate, were homogenized in 500 µL of methanol (30 sec) and centrifuged (Galaxy 16DH Microcentrifuge; VWR International) for 3 min at 10000 rpm. The supernatant was collected and diluted to the following concentrations: 0.03, 0.02, 0.015, 0.01, 0.003, 0.0003, and 0.00003 (g/mL) and assayed immediately. Trolox standard solutions were prepared in methanol at 0, 1, 8, 16, 24, 32, 40, and 50 µM. For each 96-well plate, 100 µL of standards and diluted extracts were placed alongside 200 µL of methanol and extract blanks; all samples were randomly assigned to wells. To each standard and sample, 100 µL of 0.0994 mM DPPH was added, the plate was placed into a BioTek Synergy H multidetection microplate reader and slowly shaken for 2 sec prior to acquiring absorbance at 520 nm every 60 sec at 25°C. The DPPH radical scavenging activity was calculated at T = 15 min and expressed as fresh berry weight required to reduce free radicals (DPPH response) by 50%. Three replicates for each of the Vaccinium species were used in the determination.

Ascorbic acid determination

The ascorbic acid (vitamin C) assay was modified from previously reported methods with slight alterations [41]. Frozen cranberries were thawed, individually weighed to 15-mL conical tubes in triplicate and extracted in 1 mL of 5% aqueous o-phosphoric acid by mashing with a glass rod followed by sonication for 30 min in an ice bath. The test solutions were filtered (0.45 µm PTFE) to HPLC vials. Analysis was performed on an Agilent UFLC 1200 system with separation achieved with an Inertsil ODS-3 RP C18 column (4.6 × 50 mm, particle diameter 5 µm) (GL Science, Inc.) using a gradient elution of mobile phase A: 0.1% aqueous o-phosphoric acid and mobile phase B: 100% methanol; (a) 0–14 min: 0% B (0.5 mL/min), (b) 15–17: 100% B (1.5 mL/min), (c) 17.1–20 min: 0% B (1.5 mL/min), (d) 21.1–21.5 min: 0% B (0.5 mL/min). The analyte of interest was detected at 245 nm and the data collected and analyzed using Agilent ChemStation software, Rev. B.03.01.

Statistical analysis

All of the above determinations were performed using n = 5 for each Vaccinium sp. Correlations between the relative antioxidant potential of each species and the other variables analyzed above were determined by calculation of a Pearson product-moment correlation coefficient (r).
Metabolomic profiling by UFLC-TOF-MS
Experiments were performed as per a previously established protocol [3] with an ACQUITY™ series Ultra-Performance Liquid Chromatography System (Waters Corporation) coupled with a Micromass LCT Premier™ series ToF-MS (Waters Corporation) and controlled with a MassLynx V4.1 data analysis system (Waters Corporation). Phytochemicals were chromatographically separated at 30°C on a Waters BEH Acquity C18 column (2.1 x 150 mm, 1.7 µm) and eluted with a gradient of 1% aqueous formic acid:acetonitrile (0.0–10.0 min, 95:5 v/v, 10.0–15.0 min, 5:95 v/v, 15.0–20.0 min, 5:95–95:5 v/v, 20.0–25.0 min, 95:5 v/v) over 25 min at 0.25 mL/min. A Waters 1525 HPLC binary solvent manager provided a steady flow of 2 ng/mL leucine enkephalin at 10 µL/min.

Exploratory data analysis
For the metabolomic data for each Vaccinium species, the blanks were summed, subtracted against each sample, and any resultant negative values were reset to zero. The data was then assessed without scaling or further data transformation. A series of automated functions to create subtractive data sets were created in Excel (Microsoft Corp.), as previously described [3] using sequential algorithms and functions designated “subtractive metabolomics”. The data were then compiled to an ASCII text file with identifiers of samples in rows (objects) and retention time, m/z ratio, and abundance as columns (variables). Further statistical analyses were accomplished using Solo + MIA (Eigenvector Research Inc.). Principal component analysis (PCA) was applied to the data, creating covariance matrices and transforming them into a coordinate system, as a means to observe variance for the multivariate dataset. Auto scaling was selected for preprocessing before applying the PCA algorithm. Both PCA score and loading plots were generated for the entire dataset to visualize clustering by Vaccinium species. The data was also modelled by partial least squares discriminant analysis (PLS-DA) as a supervised approach to exploring clustering relationships.

Significance analysis
Within a binary comparison between each pair of Vaccinium species, receiver operating characteristic (ROC) curves for each model were generated using R statistical software (The R FOUNDATION for Statistical Computing). The area under the ROC curve (AUC) value for each metabolite in the binary model was computed using the “colAUC” algorithm within the caTools package in R [42]. The mean of the three calculated binary AUC values per metabolite (m/z value) was obtained and designated as “total AUC” [43]. For each metabolite the p value was derived from the nonparametric Kruskal-Wallis test using the “kruskal.test” algorithm in R. The SAM statistic [44] and associated false discovery rates (FDR) were calculated using the SAM algorithm from the siggenes package in R comparing across all three Vaccinium species being studied. Due to the limited number of replicates, n = 5, per species, permutations were used to set the expected d(i) as the null level of abundance which allows for the comparison of the observed d(i) based on actual data and the expected d(i). To consider which metabolites differ significantly in terms of abundance, an artificially selected threshold (Δ) was applied to flag metabolites beyond this threshold boundary and the associated false discovery rate (FDR) determined. The metabolites as identified by the SAM statistic were distinguished in the plot of p value vs. total AUC of each metabolite and examined for further interpretation.

Results
While the phytochemical composition of common cranberry (V. macrocarpon) has been relatively extensively studied, there have been very few studies of the native BC Vaccinium spp. including V. oxyccos and V. vitis-idaea. Our objective was to develop a qualitative and quantitative understanding of the phytochemical consistency and diversity among the wild and cultivated species of Vaccinium. To investigate the potential for the berries of the Vaccinium spp. to withstand environmental exposures that generate radical oxygen species, known metabolites with strong antioxidant potential were determined (Fig. 2), including anthocyanins, vitamin C (ascorbic acid), melatonin, and serotonin. V. macrocarpon had the highest total anthocyanin content, and all 5 of the major anthocyanins common to cranberry were quantified in the berries (Fig. 2A), with peonidin-3-O-galactoside present at significantly greater concentrations than other anthocyanins. The Vaccinium oxyccos fruit also contained all 5 of the major anthocyanins but the ratio of glycosylated peonidins to cyanidins was about 20:80, as compared to 60:40 in V. macrocarpon (Fig. 3). The V. vitis-idaea berries contained measurable amounts of only the glycosylated cyanidin anthocyanins and did not contain measurable amounts of glycosylated peonidins (Fig. 2A). Interestingly, there was a strong negative correlation (r = −0.92) between the anthocyanin content and the relative antioxidant potential (Fig. 2A, D).

Melatonin and serotonin (Fig. 4) are indoleamine neurohor mones found in plants, bacteria, fungi, and animals [45, 46] but have not previously been described in these Vaccinium species. We here report the first evidence of these indoleamine antioxidants in V. macrocarpon, V. oxyccos, and V. vitis-idaea and further find that there was no significant difference in serotonin content between the species but the melatonin content was significantly higher in the commercially cultivated cranberry (V. macrocarpon) as compared to the two species native to BC (Fig. 2B). Neither melatonin nor serotonin were strongly correlated with the relative antioxidant potential of the 3 Vaccinium spp. with correlation coefficients of r = −0.67 and r = −0.09, respectively (Fig. 2B, D). There were significant differences in the ascorbic acid contents of the 3 species (Fig. 2C) and a strong positive correlation (r = 0.84) between ascorbic acid content and the relative antioxidant potential (Fig. 2C, D).

The metabolite counts and results of subtractive metabolomics shown in Table 1 make direct comparisons across the 3 Vaccinium species. The total number of compounds observed in the metabolomics profiles was 10038 (V. macrocarpon), 8035 (V. oxyccos), and 9285 (V. vitis-idaea). A total of 4626 compounds were conserved across all species, and in binary comparisons, 2257 compounds were conserved between V. macrocarpon and V. vitis-idaea, significantly more than V. macrocarpon and V. oxyccos (1289) or V. vitis-idaea and V. oxyccos (1391). Of all compounds observed in the metabolome of V. macrocarpon, 18.6% were unique to that species, while only about 10% of the compounds for V. oxyccos and V. vitis-idaea were unique to those species. Comonalities and differences among the berries were determined by multivariate analysis using a PCA model. The score plot of the first (20.24%) and second (10.25%) principal component from the PCA model exhibited interesting clustering (Fig. 5A) with almost complete overlap of the 95% confidence boundaries around V. oxyccos and V. vitis-idaea and with the 95% confi-
dence boundary for *V. macrocarpon* encompassing both of the other species data sets.

With only 30.49% of the total variance (Q^2^) explained in the model of PC1 & PC2, a Kruskal-Wallis p value was applied to the data and all values associated with a p value < 0.05 were identified (Fig. 5B). The majority of the values in this loadings plot are clustered on the left side indicating the importance of those metabolite values to *V. oxycoccos* and *V. vitis-idaea* in the score plot (Fig. 5A) rather than for that of *V. macrocarpon*. After applying an inclusivity limit of p value < 0.05, the data was re-modeled and a loadings plot (Fig. 5C) and score plot (Fig. 5D) generated. In the score plot some differentiation of the *Vaccinium* species is now observed with 37.51% explained variance in PC1 and PC2.
18.84% in PC2, which suggests that this approach results in models that better represent the data in unsupervised analysis. Observing the metabolite value distribution in \textbf{Fig. 5C} shows three clearly visible clusters and is an indication of the importance of those values to the positioning of the 3 species in the score plot (\textbf{Fig. 5D}).

The Kruskal-Wallis p value is often plotted against the area under the ROC curve or \textit{AUC}, which has been designated as total AUC for our multiclass comparison, as shown in \textbf{Fig. 6}. Values that are plotted with a p-value of < 0.05 and a total AUC > 0.5 are assessed for their importance in the differentiation in a multiclass comparison, such as the metabolomics profiles of the 3 \textit{Vaccinium} species. The SAM statistic not only accounts for distribution across products but also the standard deviation of abundance in replicates and for the metabolomics data. For the comparison of the three species, a list of significant \textit{m/z} values, as identified by the SAM statistic at an FDR of 0.91%, is presented in \textbf{Table 2} and also highlighted in red in \textbf{Fig. 6}, so it can be seen how this univariate significance test compares to the AUC and p value. The \textit{m/z} values identified by the SAM statistic as significant had total AUC values ranging from 0.83 to 1.00, and although the SAM statistic ranks the values differently than the total AUC (\textbf{Table 2}), all values are concentrated in the region of the plot of AUC against a p value that indicates significant data (\textbf{Fig. 6}). The Kruskal-Wallis p values ranged from 0.0013–0.0079 for the values identified by the SAM statistic, well below the limit for statistical significance of p > 0.05.

Partial least squares discriminant analysis (PLS-DA), a supervised multivariate approach, was applied to the metabolomics data and resulted in a clustering pattern in LV1 and LV2 (\textbf{Fig. 7A}) very

\begin{table}[h]
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\begin{tabular}{|l|l|l|}
\hline
\textbf{Description of occurrence in metabolomic profiles} & \textbf{Vaccinium macrocarpon} & \textbf{Vaccinium oxycoccos} & \textbf{Vaccinium vitis-idaea} \\
\hline
Total number of compounds detected & 10038 & 8035 & 9285 \\
Average number of compounds per biological replicate & 4477945 & 3415600 & 4090506 \\
\# of compounds found in all replicates & 252 & 296 & 646 \\
\# of compounds in \( \geq \) 50\% of all replicates & 3765 & 2656 & 3148 \\
\# of compounds unique to each species & 1868 & 731 & 1013 \\
\textit{V. macrocarpon} and \textit{V. vitis-idaea} only & 2257 &  &  \\
\textit{V. macrocarpon} and \textit{V. oxycoccos} only & 1289 &  &  \\
\textit{V. oxycoccos} and \textit{V. vitis-idaea} only & 1391 &  &  \\
\textit{V. macrocarpon}, \textit{V. vitis-idaea}, and \textit{V. oxycoccos} & 4624 &  &  \\
\hline
\end{tabular}
\caption{Summary of LC-MS-TOF metabolite counts and differences in metabolomic profiles of \textit{V. macrocarpon}, \textit{V. oxycoccos}, and \textit{V. vitis-idaea}.}
\end{table}

\begin{figure}[h]
\centering
\includegraphics[width=\linewidth]{figure5.png}
\caption{Multivariate analysis of the LC-MS-TOF metabolomic profiles of 3 \textit{Vaccinium} species. A PCA score plot of first two principal components and B the corresponding loadings plot with values having a calculated Kruskal-Wallis p value of < 0.05 identified in red. C The remodeled loadings plot with all values having a p value > 0.05 removed and D the remodeled PCA score plot.}
\end{figure}
similar to the remodeled PCA (Fig. 5D). The same process of identifying values with a p value < 0.05 (Fig. 7B), removing the values with p value > 0.05 and then remodeling LV1 and LV2 to generate a loadings plot (Fig. 7C) and score plot (Fig. 7D), resulted in a reduced overlap between the 95% confidence boundaries around each Vaccinium sp. The distribution in the loadings plot (Fig. 7B) and the number of values with p < 0.05 (Fig. 7C) should be related to the SAM statistic which contrasts the data sets by metabolite abundance but incorporates a measure of deviation across replicates within each data set. In

For LV1 and LV3, the PLS-DA score plot (Fig. 8A) shows a complete overlap of the 95% confidence boundaries of V. vitis-idaea and Vaccinium species with a confidence boundary of 95% in the score plot (Fig. 8D). Just as in the remodeled PLS-DA of LV1 and LV2 (Fig. 7C, D), the 5 metabolite values from V. vitis-idaea identified as significant in the SAM algorithm are within the clustered area of the loadings plot (Fig. 8C), and from the score plot (Fig. 8D), we can see that these values are important to the positioning of V. vitis-idaea and for this PLS-DA model, also influence the position of V. oxycoccos. For the PLS-DA model of LV2 and LV4, which shows complete differentiation of the three Vaccinium species with a confidence boundary of 95% in the score plot (Fig. 9A), an increase in differentiation is not observed in the remodeled score plot (Fig. 9D). Consistent with the remodeled loadings plots for LV1/LV2 (Fig. 7C) and LV1/LV3 (Fig. 8C), the 5 metabolite values identified as significant by the SAM algorithm are found to be positioned at the edge of the cluster associated with V. vitis-idaea in the loading plot (Fig. 9C). Again, this is an indication of the importance of these values to the position-

### Table 2

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ing of V. vitis-idaea in the score plot (Fig. 9D) and how the three species are differentiated in this PLS-DA model.

Discussion

Two approaches to phytochemical characterization and differentiation between three Vaccinium spp. were employed in this study; targeted quantitative determination of known analytes of interest or physical properties [3,45] and untargeted metabolomics profiling [3]. One of the most important aspects of the phytochemistry of plant tissues is the relative composition of phytochemicals with the potential to detoxify radical oxygen species, commonly known as antioxidant potential. For cranberry and other berries, it has been hypothesized that abundant anthocyanin composition provides evidence of strong antioxidant activity and has implications for human health [27,29,47,48]. Further, it has been also hypothesized that indoleamines such as melatonin are present in berries and other fruits to protect the genetic materials from oxidative damage due to environmental stresses [33,49]. Our data indicates that neither of these hypotheses can fully account for the phytochemical mechanisms in cranberry as the potential for detoxification of oxygen free radicals was more significantly correlated to ascorbic acid content than to the other antioxidants in the tissues. Previous researchers have also investigated the relationship between vitamin C and the total anthocyanin content of the commercial cranberry and found that the total anthocyanins but not ascorbic acid were significantly correlated with cytochrome c modulated oxidation of 6-hydroxydopamine, an important pathway for neurological health [26]. These results together with our results indicate 2 possibilities: (1) the total anthocyanin contents include antioxidant phytochemicals not detected with our targeted analysis of specific anthocyanins or (2) specific physiological mechanisms affected by individual dietary antioxidants may not be elucidated in a measure of the total antioxidant potential.

The untargeted metabolomics approach to comparative phytochemistry is growing in popularity and application but the size and complexity of data sets can make experimental design and data interpretation difficult. The objectives of the comparative metabolomics described in this study were (1) to identify the degree of phytochemical commonality and difference among Vaccinium species and (2) to develop a model for experimental design and statistical analysis that could be applied to a range of other metabolomics profiling. One of the more interesting results of our metabolomics study is the discovery that 4624 compounds identified in the data set are common to all 3 of the Vaccinium species. Given that we are comparing different species within a
single genus and that 1 of the species was cultivated under commercial conditions while the other 2 were harvested from wild populations, the degree of phytochemical conservation was 46% of the total chemistry detected in *V. macrocarpon*, 57% of the total chemistry detected in *V. oxycoccos*, and 49% of the total chemistry detected in *V. vitis-idaea*. This measure of the conservation of chemistries across species indicates the importance of primary metabolism in the growth and survival of plants under diverse microenvironments.

It has become most common to analyze metabolomics data sets by multivariate analysis using untargeted algorithms like principle component analysis (PCA) [40, 41] but our data indicate that these simplified analyses may lead to false discovery of phytochemical differences. Typically, the influence of the metabolites (variables) in the loadings plots of multivariate analysis such as PCA and PLS-DA are within the edge regions, furthest away from the origin. While this is true, the observed clustering in the modeling of the *Vaccinium* spp. metabolomics indicates that each species has grouping of metabolites critical to the posting of the species in the score plot. Hence, using the p < 0.05 cut off ensures that the majority of the metabolites kept are significant in the model which exhibits species differentiation in the scores plot, although some that were excluded (with p value of > 0.05) were important for the original model in each case (© Fig. 9). This indicates that using a p value cut off can have an important impact on how metabolomics data is interpreted when modeled in multivariate analysis. The two algorithms, AUC and SAM, both capture the distribution of abundance in metabolomics profiles; however the approach is different. The ROC curve is a plot of sensitivity, defined as the true positive rate, and 1-specificity which corresponds to the false positive rate. The accuracy of this plot is determined by assessing the AUC, whereby an AUC of 1 would indicate 100% sensitivity at 0% false positive rate. This analysis becomes more meaningful when taken together with p values for significance, as in © Fig. 6, and is a way to prioritize metabolomics data and assess the quality of our regression models (PCA, PLS-DA). However, AUC is a binary comparison directly comparing only 2 species, so for the 3 species comparison all possible binary comparisons are made, and the AUC values are averaged to a “total” AUC to capture all possible comparisons [30]. In this way, AUC is unable to capture the interactions or relationships that may exist across the metabolites in the metabolomics profiles. The SAM statistic is a step towards capturing the more complex relationships as the degree of significance is compared to the null distribution of the data set by using permutations of all metabolite abundances and calculating an associated FDR which is not captured in AUC algorithms. With the use of the SAM statistic, it becomes possible to identify the most significant metabolites per species, as shown in © Table
When comparing the SAM values directly to the loadings plot generated in multivariate analysis (see Figs. 7C, 8C, and 9C), the distribution of the values and the number of significant values should be consistent if the MVA model is a good fit for the metabolomics data. The PLS-DA loadings plot of LV2 and LV4, after the p value < 0.05 limit was applied (Fig. 9C), shows that the tightest grouping of SAM significant values originate from V. vitis-idaea, whereas the SAM significant values from V. macrocarpon and V. oxycoccos are more evenly distributed. The results of the SAM algorithm support the positioning of the 3 species in the LV2/LV4 score plot (Fig. 8D) and indicates that the observed differentiation of the species is a reasonable interpretation of the metabolomics data.

Overall these data provide new information about the chemical diversity and chemotaxonomy of the Vaccinium species as well as a model for the development of experimental design and statistical tools for understanding complex plant metabolomes.

Acknowledgements

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Conflict of Interest

The authors report no conflict of interest.

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Vitis vinifera