

**AGRICULTURAL RESEARCH FOUNDATION
FINAL REPORT
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ARF 8304A -- "Better berries through better metabolism" -

Metabolomics of bioactive compounds in berry

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SUMMARY

Oregon is one of the major producers of berries in the US. The farm gate value of berries produced by Oregon's agriculture approached \$200 million in 2012. Berries have been publicized as functional foods with health-promoting properties that ameliorate symptoms associated with chronic and degenerative diseases. Considering the increasing interest in plant phenolics as nutraceuticals, comprehensive profiling methods for plant extracts are in high demand.

OBJECTIVES: The primary objective of this grant was to screen the phenolic content of raspberry and blackberry to define the cultivars that produce berries with phenolic compositions, high bioactivity and high health-promoting potential. To obtain comprehensive profiles of the phenolic compounds in berries we conducted mass spectrometry-based metabolomics. Here we report our initial results of a study in which we compared the polyphenolic profiles of two red raspberry cultivars (Meeker and Vintage) and two black raspberry cultivars (Chester and Black Diamond). The berries were first subjected to acidic methanol extraction, followed by solid phase extraction (SPE) to remove highly water soluble components (sugars and citric acid). Extracts before and after SPE, were analyzed using liquid chromatography coupled to electrospray ionization time-of-flight mass spectrometry. We used an untargeted acquisition method to generate comprehensive profiles of berry metabolites. The sample groups were clearly separated in the principal component analysis (PCA) scores plot indicating that the berry extracts of the different cultivars have distinct metabolite profiles. For instance, the common polyphenolic, rutin, was found to be significantly upregulated in Black Diamond compared to the other groups. On the other hand, sanguin H6, was not significantly different between the two groups of raspberries. Work is in underway and will continue to establish workflows after data collection that will assist us in the identification of polyphenolics in order to build a mass spectral library for berry phenolics. The 2nd objective of this project was to attempt correlating metabolite profiles of phenolic extracts with the health promoting properties of berry extracts with focus on the potential anti-lipogenic activity of berry compounds. At this point in time, we have developed a MS-based assay to monitor the differentiation of mouse 3T3-L1 adipocytes, an accepted model for studying obesity. Future steps include testing to what extent berry extracts and phenolic compounds affect the lipid profiles during 3T3-L1 adipocyte differentiation.

The results of this grant will be presented as poster entitled "Polyphenolic profiling of Raspberry Cultivars from Western Oregon by Jeremiah Kelley, Brookelyn Long, Aaron Morrison, Bernadine Strik, and Claudia Maier at the upcoming 74th Annual Oregon Academy of Science Meeting (02/ 27/2016) at

Pacific University. Note: BL and AM are undergraduate students and JK is graduate student in the Department of Chemistry.

PROCEDURES

Experimental Workflow For Determining The Berry Metabolome

Research is currently ongoing and focuses on the optimization of the workflow after data collection to facilitate the identification/annotation of polyphenolics in berries. We used UPLC (ultraperformance liquid chromatography) in combination with high resolution mass spectrometry (HRMS). The extraction method, chromatographic and mass spectrometric parameters for profiling the semi-polar metabolites in the berry cultivars has been optimized, and consists of acidic methanol extraction followed by solid phase extraction to remove highly water soluble metabolites (e.g., citrate) prior to UPLC-HRMS. Metabolite annotation is based on accurate mass database searching (e.g., Metlin and HMDB) and manual inspections of fragment ion (MS/MS) spectra, and is followed by comparison to predicted fragmentation patterns when possible.

Figures 1 and 2 outline typical data that was used to annotate metabolites from berry extracts using UPLC-MS/MS. The spectra shown are that of Sanguin H6 and Procyanidin B2 illustrating the detection of singly and doubly charged polyphenolic ions from the berry extracts. As shown in Figure 1, the predicted fragmentation pattern is an 89% match with the experimentally acquired MS/MS spectrum for Sanguin H6.

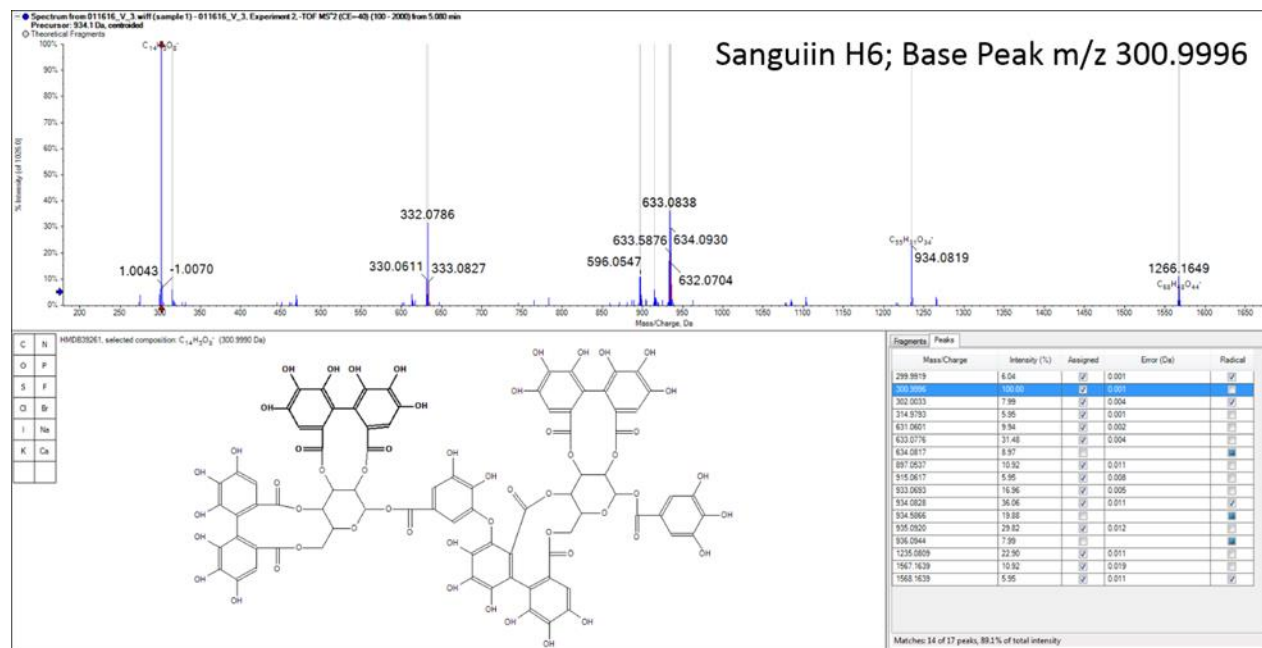


Figure 1: MSMS spectrum for the doubly charged sanguin H6 ion (top); Predicted fragments (bottom right) and structure (bottom left).

Figure 2 shows the MS/MS spectrum, structure, and predicted fragmentation pattern for procyanidin B2. The experimentally acquired fragmentation pattern matches 82% of the predicted fragments for procyanidin B2.

Procyanidin B2 Base peak at m/z 407.0776

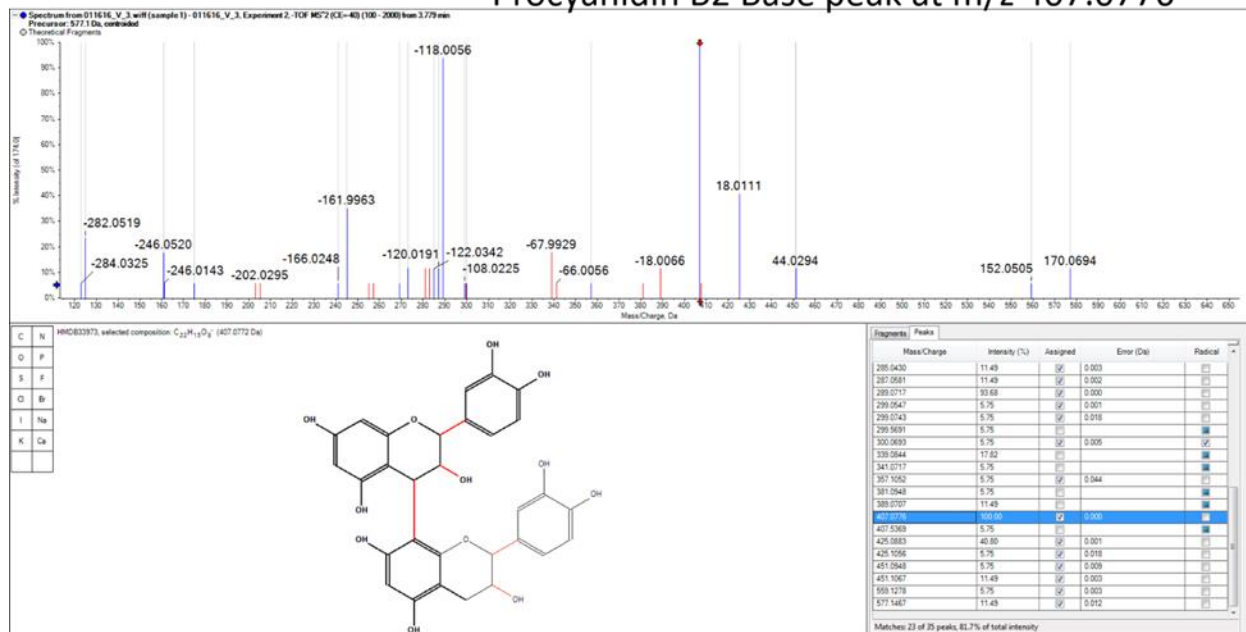


Figure 2: MSMS spectrum for the singly charged procyanidin B2 ion (top); Predicted fragments (bottom right) and structure (bottom left).

Polyphenolic Profiling Of Berry Extracts Of Raspberry Cultivars

The optimized protocol was then used to pursue a comparative study of the Raspberry cultivars from Western Oregon (Meeker, Vintage, Black Diamond, and Chester). A pooled technical replicate or quality control (QC) sample was used to demonstrate instrument performance during the UPLC-HRMS runs. As shown in Figure 2, the clustering of QC samples is tight compared to the biological replicates. The first step was to compare the polyphenolic profiles of Red raspberries (Meeker and Vintage) to that of Black berries (Black Diamond and Chester).

The common polyphenolic glycoside, rutin, was found to not be significantly different in black berries compared to red raspberries (p -value > 0.05). Upon further inspection however, it was found to be significantly upregulated in Black diamond compared to Chester (p -value < 0.001, and $\log_2(\text{fold change}) = 6.4$). When considering aglycones, quercetin was found to be significantly upregulated ($p < 0.01$, $\log_2(\text{fold change}) = 10$) when comparing blackberries to red raspberries, but not different when comparing cultivars of the same type. On the other hand, kaempferol was found to be upregulated in Chester compared to Black Diamond and Vintage compared to Meeker, but not significantly different when comparing red to black. Table 1 highlights differences in cultivars of the same type (i.e., Meeker versus Vintage and Chester versus Black Diamond) that have been annotated thus far. Structural assignments of phenolic compounds have been based on accurate mass (< 5 ppm), isotope pattern matching, and comparison to MSMS spectra when possible. An uncommon metabolite (see Table 1) was detected in the berry extracts that contains a hydroxymethylglutaryl moiety and was upregulated in Chester compared to the other berry samples. For further clarification, the information relating to the annotation of Quercetin 3-[6''-(3-hydroxy-3-methylglutaryl)galactoside] from berry extracts is shown in Figure 3.

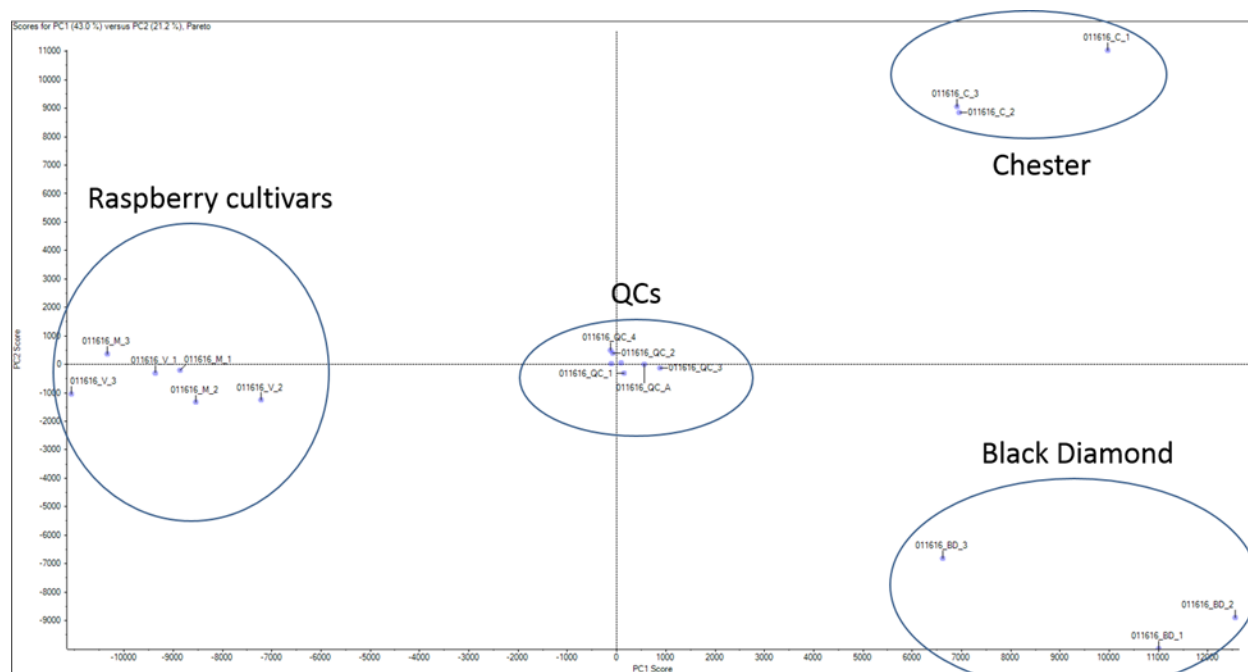


Figure 3: PCA scores plot for the analysis of berry extracts from blackberries (Black Diamond and Chester) and red raspberries (Meeker and Vintage). QCs, quality control, pooled samples used as technical replicates (injected after every 3 samples).

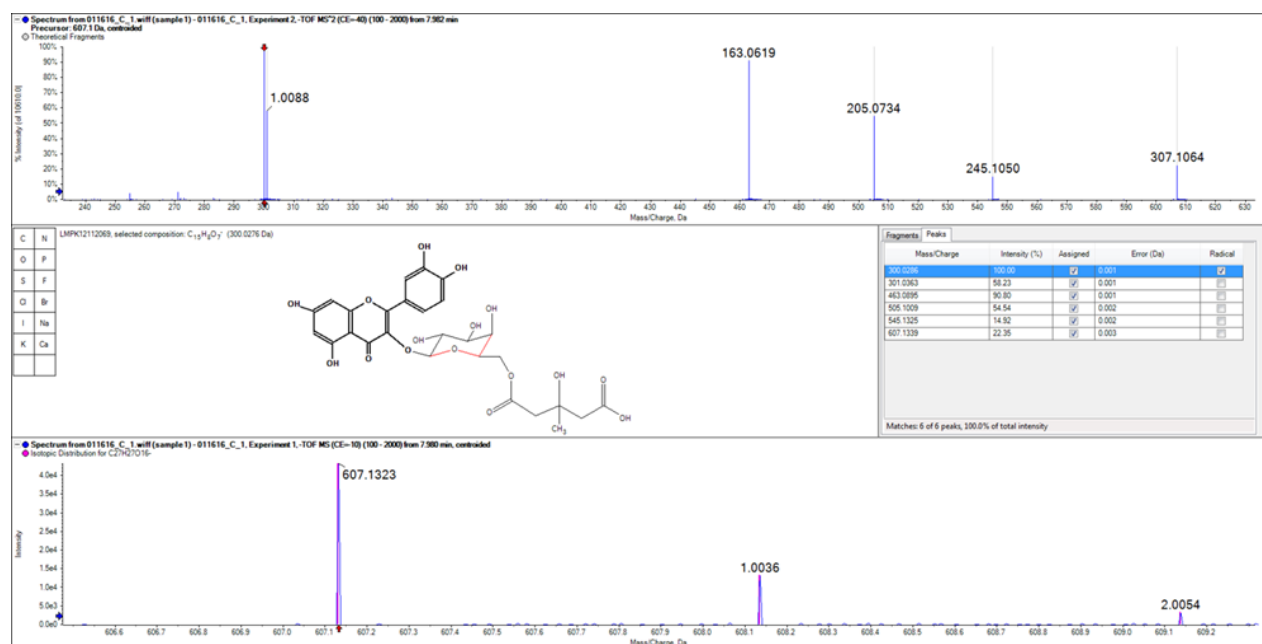


Figure 4: Process of annotation of Quercetin 3-[6''-(3-hydroxy-3-methylglutaryl)galactoside] using accurate mass (5 ppm error) and isotope matching (bottom pane). The experimental MSMS spectrum (top pane) was a 100% match to the predicted fragmentation pattern (middle right pane). The base peak in the MSMS spectrum is bolded in the structure and matches the [M-H]⁻ ion of the aglycone, quercetin.

Table 1: annotated features and detected differences found between black berries and red raspberries

Metabolite	m/z	ppm error	RT (min)	Black Diamond to Chester*	Meeker to Vintage
Sanguiin H6	934.079	7	5.08	logFC = 1.7 p<0.01	NSD; p>0.1
Procyanidin B2	577.13539	0.4	3.81	logFC = -1.1 p<0.01	NSD, p>0.05
Quercetin	301.036	2.1	9.68	NSD, p>0.1	NSD, p>0.05
Caffeic acid	179.03565	3.7	3.83	NSD, p>0.05	NSD, p>0.05
Ellagic acid	301.00041	4.7	7.23	NSD, p>0.05	NSD, p>0.05
Protocatechuic acid	153.0201	5	4.93	p<0.01 logFC = -2.6	NSD, p>0.05
Quercetin 3-[6''-(3-hydroxy-3-methylglutaryl) galactoside]	607.1335	5	7.98	logFC = -4.1 p<0.01	NSD, p>0.1

* logFC is log base 2 of the ratio of peak intensities of Black diamond to Chester. NSD: no significant difference

Ongoing experiments for determining the health promoting effects of phenolic extracts of berries

The 2nd objective of this project was to attempt correlating metabolite profiles of phenolic extracts with the health promoting properties of berry extracts with focus on the potential anti-lipogenic activity of berry compounds. At this point in time, we have developed a MS-based assay to monitor the differentiation of mouse 3T3-L1 adipocytes, an accepted model for studying obesity. Dr. Miranda and graduate student Fereshteh Zandkarimi have established the conditions for growing and reproducibly differentiating 3T3-L1 pre-adipocytes. Next steps include testing if berry extracts and phenolic compounds affect the lipid profiles during differentiation.

Conclusions

The method developed for the analysis of polyphenolics from berries can detect polyphenolics from a variety of flavonoid classes. The greatest bottleneck in any metabolomics assay is metabolite annotation, and future work will focus on providing a systematic way to annotate polyphenolics from berry extracts. This process has been demonstrated above in Figures 1-3. This preliminary work should set the framework for the generation of an HRMS based polyphenolic library in which the process of polyphenolic annotation will be more automated. The lipidomic profiling of mouse 3T3-L1 adipocytes differentiation seems a feasible assay for testing the anti-lipogenic activity of berry compound and fruit extracts.

BENEFITS & IMPACT: Raspberries contain a large collection of phytochemicals that may have health promoting effects. Methods are needed that screen the phenolic content of berry cultivars to assist breeding efforts to potentially select berries that show increased levels polyphenolics. This in turn would benefit the commercialization of berry varieties that show high and potent content of health promoting phenolic compounds as a high value commodity.

SIGNIFICANT ACCOMPLISHMENTS: We were successful in attracting funding for research on the health promoting properties of plant polyphenolic compounds (NIH R01 AT009168, Gombart/Maier/Stevens Multiple PIs).

FUTURE FUNDING POSSIBILITIES: We currently explore opportunities for attracting additional funds that would allow us to continue this line of research.