Annual Report
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Project Title: The Chemical Evolution and Preservation of Color in Red Wine Aging.

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Summary

During the last year we have been working on the production of labeled malvidin 3-glucoside. Using unlabeled material, we have been able to optimize the conditions of enzymatic synthesis as well as the purification of malvidin-3-glucoside from starting material and side-products so that the reaction will yield adequate material to continue the project. Labeled malvidin-3-glucoside is not commercially available and there is no published method to produce it, which made the synthesis particularly difficult. An alternative procedure based on chemical labeling, which was tried first, did not work due to the ionization of anthocyanins, but it has produced other anthocyanins not described in the literature, and so these products may be interesting to analyze at some point later on.

We have also advanced the understanding of red colored polymeric structures and the analytical procedures to measure them. We have found a poor agreement between two analytical procedures, the traditional method of SO_2 bleaching and the newly developed Normal Phase – HPLC methodology. It is necessary to resolve the causes of these differences and also to compare them with the Adams' assay (based on tannin precipitation). This wine study will unequivocally show the origin of the pigmented polymers and clarify the best method to measure them.

Objectives and Experiments Conducted to Meet Stated Objectives

Prepare tritium-labeled malvidin-3-glucoside.
- Type of co-factors and quantities for the enzymatic reaction
- Concentration of enzyme
- Time of reaction and pH
- Purification of products
- Analysis of products
- Alternative chemical labeling

Addition of labeled malvidin-3-glucoside to wines and allow aging reactions to occur.

Separate the different chemical fractions to determine the fate of the labeled malvidin
Analyze the effects of the different treatments, i.e. proportion of anthocyanin retained, losses to colorless products, polymeric color, transformed pigments, etc.
Analysis of wines produced with different techniques
Identify unknown labeled products
Analytical methods to calculate red colored polymeric structures (SO_2 bleaching, HPLC and Adams' assay).
Comparison of SO_2 bleaching versus HPLC

Summary of Major Research Accomplishments and Results (by Objective)

Prepare tritium-labeled malvidin-3-glucoside (Objective #1): The conditions for malvidin-3-glucoside labeling have been established. First of all, it was proved that enzymatic methylation of petunidin-3-glucoside using catechol-O-methyl transferase and S-adenosyl-L-methionine as methyl donor is a viable option to produce malvidin-3-glucoside. This procedure has been reported for other phenolic derivatives like caffeic acid but not for anthocyanins (1). After that, work has been focused on the best conditions for the labeling. The optimum concentration of the cofactors magnesium, S-adenosyl-L-methionine and DTT (Dithiothreitol) was found.

One of the major difficulties was to find the right amount of enzyme, the pH and the time of incubation. The reason for that is the quick degradation of malvidin-3-glucoside under the enzymatic conditions required. High pH and high temperatures are not good conditions for anthocyanin stability but are essential for the enzyme activity. A short incubation combined with a high concentration of enzyme was necessary for the highest production of malvidin-3-glucoside.

Another difficulty was the recovery of the product for later study. It is necessary to separate the anthocyanins from the cofactors and enzyme using a Solid Phase Extraction cartridge under acidic conditions. Additionally, a new HPLC method was necessary for the separation of isomeric anthocyanins. The retention time of the products and the detection with UV and Mass Spectrometry were used to identify and quantify the products.

Chemical labeling was also investigated to produce malvidin-3-glucoside. This mechanism has been proved successful for methylation of catechin and epicatechin. Unfortunately, anthocyanin ionization at high pH does not favor the incorporation of a methyl group in the 5' position that could generate malvidin from petunidin-3-glucoside. Although it did not produce the desire product, we obtained novel anthocyanins that have not been reported in the literature. It is possible that specific protection of hydroxyl groups could lead to the right methylation of petunidin. But because we achieved a successful enzymatic methylation, we will not investigate alternative chemical methylation pathways.

Analyze the effects of the different treatments, i.e. proportion of anthocyanin retained, losses to colorless products, polymeric color, transformed pigments, etc. (Objective #4): Wines produced during 1997, 1998 and 1999 have been analyzed to quantify the amount of red colored polymeric structures and monomers. These analyses do not involve radioactivity but provide useful initial observations that will help to analyze results with [^{3}H]-malvidin-3-glucoside additions. Some of the treatments appear to affect the proportions of small and large polymeric structures in some vineyards. At the same time it has been observed that the vineyards have

different levels of polymeric color and different proportions of small and large polymeric structures.

Analytical methods to calculate red colored polymeric structures (Objective #6): Comparison between SO_2 addition to bleach the monomers and quantify the polymers and the HPLC method indicated a poor correlation (Figure 1). The reason is that both methods provide different information about the polymeric pigments. SO_2 addition is based on the idea that the only monomers are bleached by the SO_2 whereas the polymers remain unbleached. The HPLC method is based on the retention time of the molecules. It remains unclear how SO_2 does not bleach polymers. It is also a matter of study the anthocyanin derivatives that do not incorporate into the larger polymers. The continuation of the proposal will elucidate the fate of anthocyanins and the relevance of the proposed structures. It will also determine the appropriate method to quantify polymeric color.

Outside Presentations of Research

Due to the early stage of the project no outside presentation of research has been done related to the radioactive labeling. Results obtained from additions to wines will be presented in the 2001 ASEV annual meeting in San Diego or the 2002 ASEV annual meeting in Portland.

Research Success Statements

This research is designed to establish a better understanding of the evolution of wine color and the production of pigmented molecules during winemaking and wine aging. This understanding will help winemakers decide how to affect wine color. It is also important to validate analytical methods that measure structures defined as polymeric color.

Fund Status

We have expended about $11,200 on salary, 1600 on benefits (tuition) and $11,000 on supplies and other expenses.