1. PROJECT NO. 000800
– Confirming lady-beetle taint from 7-spot beetle and remediation of MALB and seven spot taint from juice using a protein-based fining method

Applicant Name: Debra Inglis, Final Report #2

Reporting Period: September 1, 2009 - Dec 2010.

Program coordinator: Debra Inglis

2. EXECUTIVE SUMMARY.

3-Alkyl-2-methoxypyrazines (MPs) represent an important and potent class of grape- and insect-derived odor-active compounds associated with wine quality. Specifically, 3-isobutyl-2-methoxypyrazine (IBMP), 3-isopropyl-2-methoxypyrazine (IPMP) and 3-*sec*-butyl-2-methoxypyrazine (SBMP) are 3 grape-derived volatile compounds that elicit green and vegetative aroma and flavour descriptions in wine. Recently, a further source of MPs has been identified in wine, of insect origin. Specifically, MPs from the Multicoloured Asian ladybeetle (MALB), *Harmonia axyridis* and the seven-spotted ladybeetle (C7), *Coccinella septempunctata* have been found in wine, concurrent with development of an off-flavor coined 'ladybug taint' (LBT). IPMP is the primary compound believed responsible for LBT although IBMP and SBMP have also been identified at lower concentrations in some affected wine. The difficulty in removing MPs from juice/wine manufacturing through traditional remediation methods has led us in search of alternative approaches in reducing the MP levels in juice/wine.

We investigated proteins with micromolar binding affinity for MPs for use as fining agents. Proteins from the lipocalin family, the piglet Odorant Binding Protein and the mouse Major Urinary Protein (pIOBP and mMUP) were cloned and over expressed in the methylotrophic yeast *Pichia pastoris*. Purified and concentrated proteins were added to chardonnay juice (pH 3.5) spiked at 300ng/L of IBMP or IPMP. The protein-MP complex was then removed from juice using one of two systems; bentonite fining at 3g/L or a 10 kDa MWCO membrane filtration system. The filtration system tested with both pIOBP and mMUP in buffer at pH 3 removed MPs to below the limit of quantitation of the HS-SPME-GC-MS system (<5 ng/L IBMP or <2 ng/L IPMP). When tested with chardonnay juice, greater than 99% of the MPs were removed. The bentonite protein-MP fining system was less effective, but still reduced MPs by 60% using the pIOBP and by 90% using the mMUP protein when tested using chardonnay juice.

Two differences with the intended outcomes in the original proposal versus the actual outcomes was the identification and testing of two proteins (murine and procine) as opposed to just one (porcine), and the development of the membrane system for removing the protein-MP complex as opposed to the MPs covalently bound to silica sand particles. The mouse protein proved to be more slightly effective thant he porcine protein so both were evaluated. In developing systems to remove the MP complex, we found the membrane system worked effectively in a relatively short period of time. This system was pursued over the silica sand system as the membrane was readily available and proved to reduce the MPs to a greater extent than the bentonite counter-fining system that the covalent binding of the MPs to silica sand was based upon.

3. DETAILED DESCRIPTION OF THE PROJECT

a) Objectives and Project Input.

The objective in this study was to test the ability of the porcine OBP purified from the growth media of the yeast *Pichia pastoris* to bind to IPMP (causal agent of MALB taint) and IBMP (causal agent of "green" character in under-ripe grapes) in a juice matrix. Two protein-MP removal systems were to be tested: bentonite fining followed by filtration to remove the protein-MP complex and a second system of covalently attaching the protein to silica sand and filtration for easier removal of the complex. The second silica sand system was not tested but instead, a membrane filtration system with a pore size that would not allow the protein-MP complex to pass through the sytem was studied in its place. Project Inputs have included the cost of reagents charged to OGWRI for the Sept/09 to Dec 10 period. Other granting sources allowed the concurrent study of the mouse Major Urinary Protein using the same system, the filing of a PCT patent for the MP removal systems based on both proteins and funding for a post-doctoral fellow that worked with both proteins (OPIC grant, $10,000 April 2009 to March 2010; MALB Strategic Grant $3200, grant completed; UBC Royalties donated by D. Inglis $6803.20). These inputs could all be considered as funding sources that contributed to the project outputs in addition to the funding supplied by OGWRI. In addition, CCOVI aquired funding from AAFC for a new GC MS system (Agilent) that would allow for Head Space SPME GC MS analysis of MPs. The total value of the equipment was $150,000.

b) Activities and Outputs

The Project milestone was to develop and compare systems where the pIOBP could bind to and remove MPs from grape juice. CCOVI acquired a new GC MS system that facilitated the direct measurements of MPs from samples using head space SPME analysis. With the new equipment and the new assay developed, we could now directly measure MP concentration in buffer or grape juice before addition of the MP-binding protein and after removal of the protein-MP complex. Upon developing the filtration system to remove the protein-MP complex, we determined that a 10 kDA molecular weight cutoff membrane was an easy way to remove the protein-MP complex from solution to determine the effective binding of the proteins to MPs. In studying this system, it was also found that the membrane material, made of Polyethersulfone, removed two thirds of the MPs in solution without any addition of protein. This system was further evaluated as opposed to binding the protein to silica sand as the potential to bind the protein directly to the membrane appeared to be a more direct solution than coupling the protein to silica. Based on the positive results reported below, a PCT patent was filed on April 14, 2010. PCT application based on US Provisional No. 61/169,121 D. Inglis, I. Brindle, G.

Pickering, A. Beh, E. Humes. METHOD FOR REDUCING METHOXYPYRAZINES IN GRAPES AND GRAPE PRODUCTS.

Results in measurable and quantifiable terms:

Filtration Method

Protein removal using 10MWCO filter unit

The 10MWCO filtration unit was successful in removing the protein from the binding reaction as observed in Figures 1A-C for various preparations of pIOBP and mMUPII.

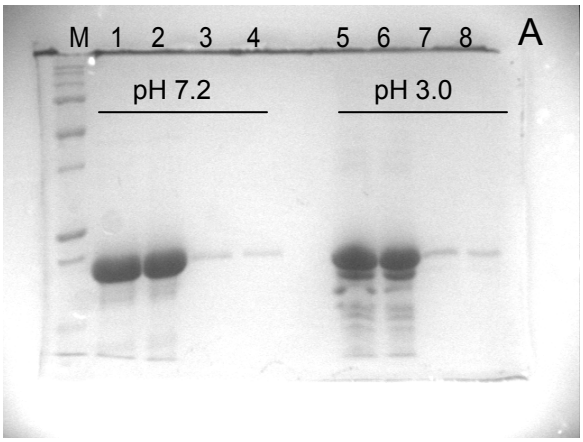

Figure 1A, Filtration of a pIOBP binding reaction at pH 7.2 and pH 3.0 through a 10MWCO filter unit, from Oct29 and Oct30 2009 assay; lane M, molecular weight standard, lanes 1, 2, 5, 6 before filtration; lanes 3,4,7,8 after filtration

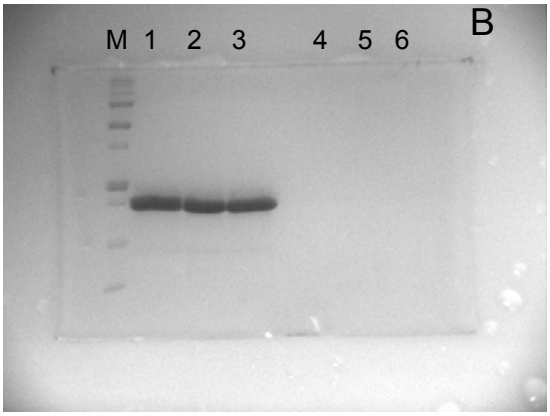

Figure 1B, Filtration of mMUPII binding reaction at pH 7.2 through a 10MWCO filter unit, from Jan 29 2010 assay; lane M, molecular weight standard, lanes 1-3, before filtration; lanes 4-6, after filtration

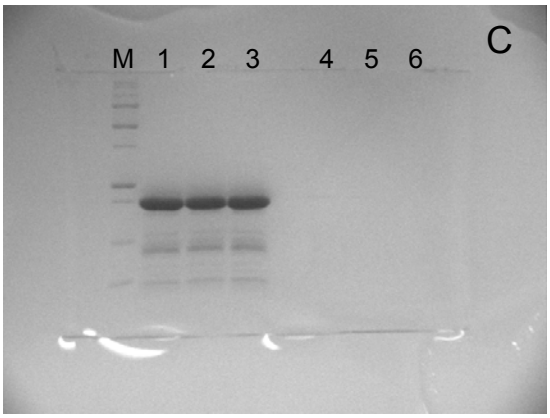

Figure 1C, Filtration of a mMUPII binding reaction at pH 3.0 through a 10MWCO filter unit, from Jan 29 2009 assay; lane M, molecular weight standard, lanes 1-3, before filtration; lanes 4-6, after filtration

Binding Results using plOPB and filtration removal of complex

The 10 MWCO filtration in the absence of protein removed approximately half of the methoxypyrazine in the binding reaction (Fig 2, 3). Further addition of pIOBP reduced IBMP to 10ng/L in pH 7.2 buffer and to the limit of quantification (5ng/L) in both pH 3.0 buffer and juice (Figure 2) . The compound IPMP was reduced to approx 7 ng/L in juice following treatment with plOBP (Figure 3). Bovine serum albumin (BSA) was tested as a negative control to show that the removal of IBMP and IPMP was specific to pIOBP and that the addition of a random protein to the binding reaction was not sufficient to remove the MPs to the limit of quantification for our system (Figure 4).

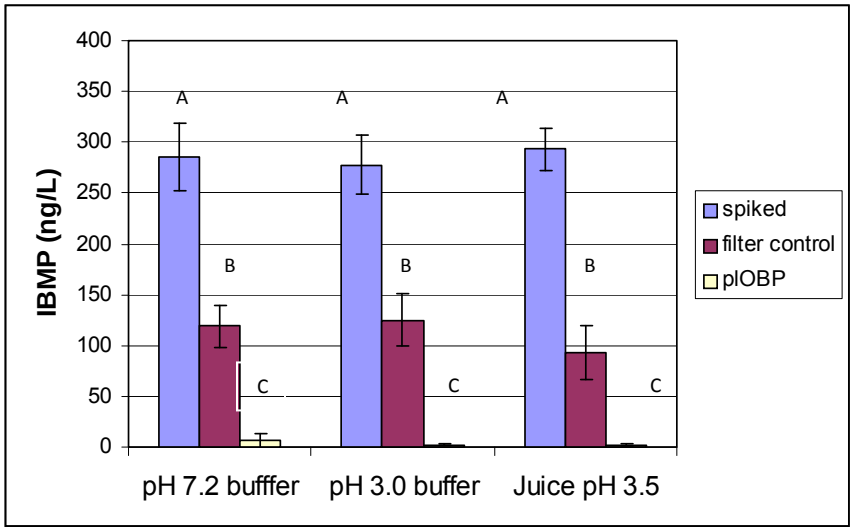

Figure 2. Reduction of IBMP from Phosphate Citrate Buffer pH 7.2 (n=9), Phosphate Citrate Buffer pH 3.0 (n=9) and Chardonnay Juice pH 3.5 (n=6) using plOBP protein and the filtration system. Treatments which are significantly different are represented by different letters. Data analysis was performed using ANOVA and mean separation by Fisher LSD ($P<0.05$)

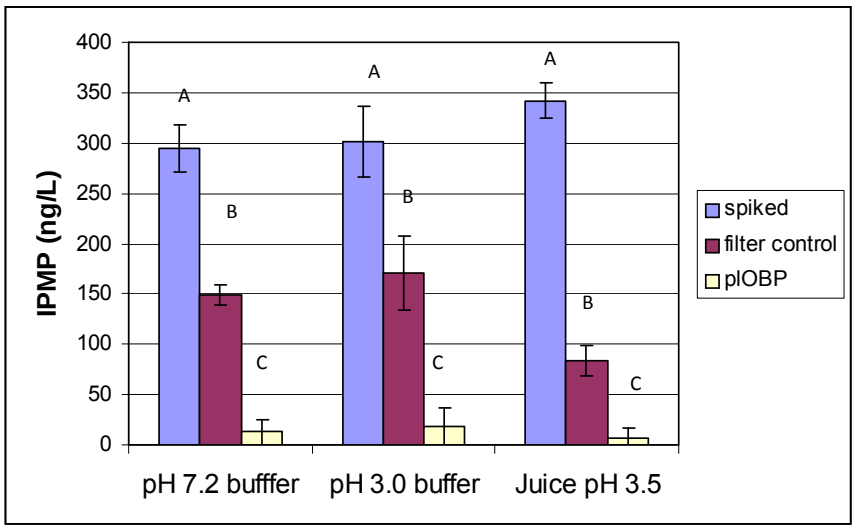

Figure 3. Reduction of IPMP from Phosphate Citrate Buffer pH 7.2(n=3), Phosphate Citrate Buffer pH 3.0 (n=3) and Chardonnay Juice pH 3.5 (n=3) using plOBP protein and the filtration system. Treatments which are significantly different are represented by different letters. Data analysis was performed using ANOVA and mean separation by Fisher LSD ($P<0.05$)

(a) (b)

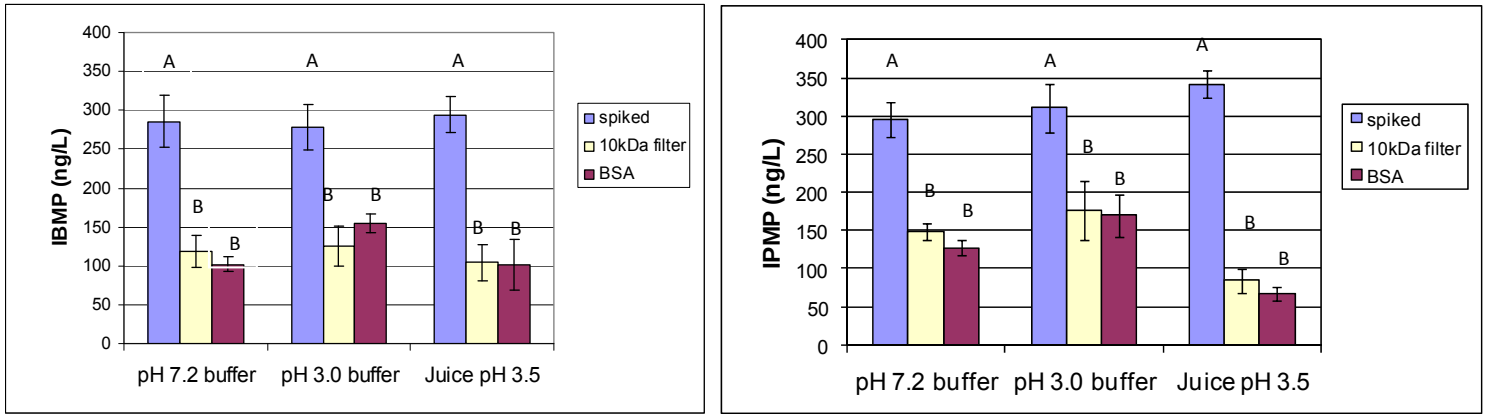

Figure 4. . Reduction of IBMP(a) or IPMP (b) from Phosphate Citrate Buffer pH 7.2(n=3), Phosphate Citrate Buffer pH 3.0 (n=3) and Chardonnay Juice pH 3.5 (n=3) using BSA and the filtration system. Treatments which are significantly different are represented by different letters.Data analysis was performed using ANOVA and mean separation by Fisher LSD ($P<0.05$)

Binding Results using mMUPII and filtration removal of complex

The 10 MWCO filtration in the absence of protein removed approximately half of the methoxypyrazine in the binding reaction (Fig 5). Addition of mMUPII further reduced the IBMP concentrations to 11 ng/L in pH 7.2 buffer, and to the limit of quantification (5ng/L) in pH 3.0 buffer and Chardonnay juice (Figure 5). Treatment with mMUPII also reduced IPMP to near analytical limits of quantification in both buffered pH 7.2, pH 3.0, and in juice (3ng/L, 3ng/L and 4 ng/L respectively, Figure 6).

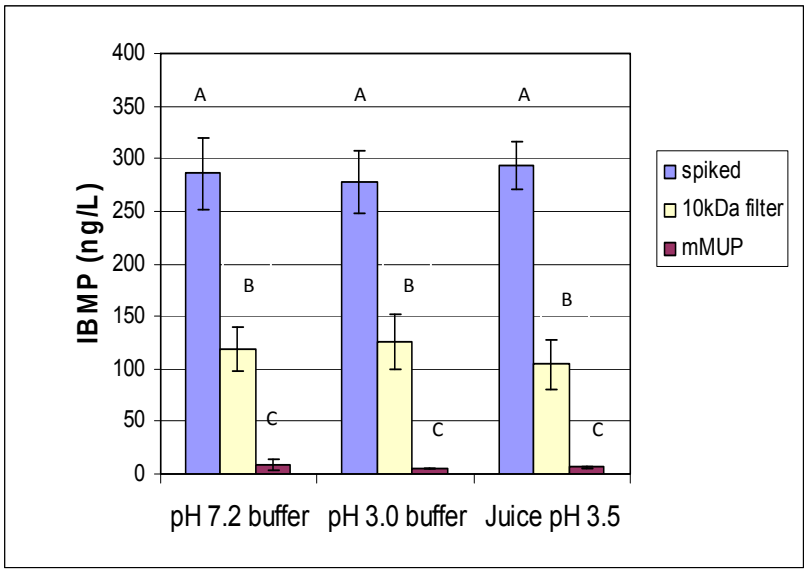

Figure 5. Reduction of IBMP from Phosphate Citrate Buffer pH 7.2(n=6), Phosphate Citrate Buffer pH 3.0 (n=6) and Chardonnay Juice pH 3.5 (n=3) using mMUPII protein and the filtration system. Treatments which are significantly different are represented by different letters. Data analysis was performed using ANOVA and mean separation by Fisher LSD (P<0.05)

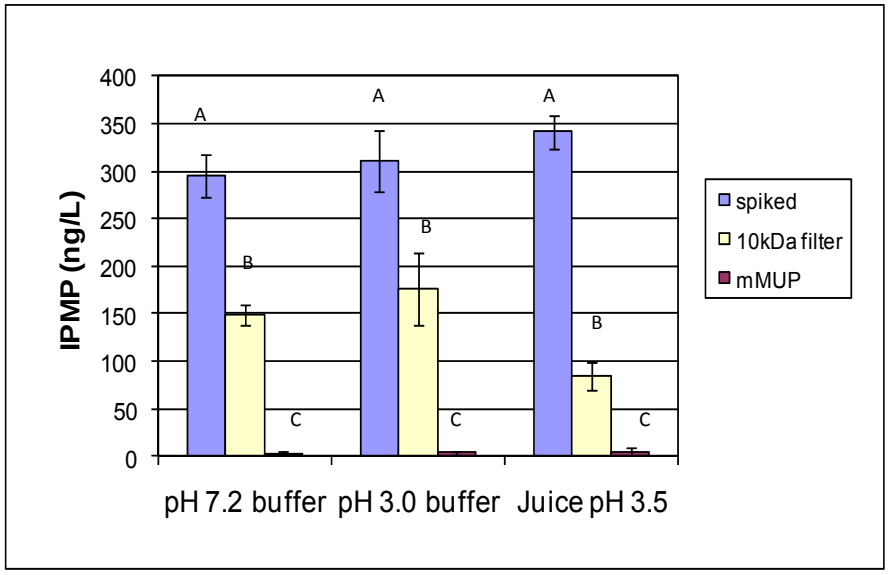

Figure 6. Reduction of IPMP from Phosphate Citrate Buffer pH 7.2 (n=3), Phosphate Citrate Buffer pH 3.0 (n=3) and Chardonnay Juice pH 3.5 (n=3) using mMUPII protein and the filtration system. Treatments which are significantly different are represented by different letters. Treatments which are significantly different are represented by different letters. Data analysis was performed using ANOVA and mean separation by Fisher LSD (P<0.05)

Bentonite Method

Protein removal by bentonite fining

Bentonite at 3g/L was successful in removing 45 µM pIOBP from the binding reaction at pH 3, whereas it was less effective at pH 4.0 and pH 5. It was also less effective at higher concentrations of pIOBP (Figure 7B)..

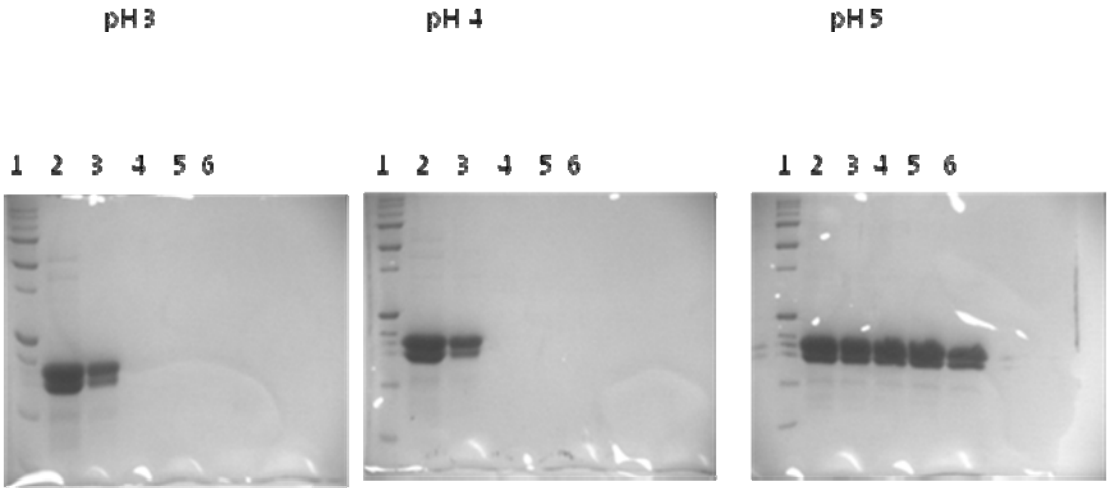

Figure 7A. Bentonite fining (3g/L) with 45 µM pIOBP in the binding reaction at pH 3, 4 and 5. lane 1, molecular weight standard, lanes 2,before bentonite treatment; lanes 3,4,5,6 after bentonite fining using 1,3,5,7 g/L bentonite

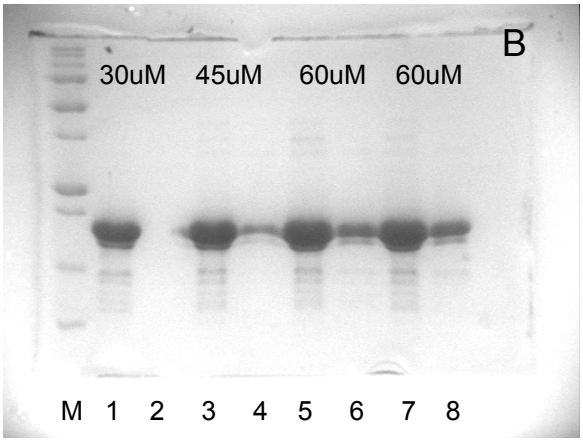

Figure 7B. Bentonite fining (3/gl) of binding reaction with increasing pIOBP concentrations, from Nov10 and Nov11 2009 assay; lane M, molecular weight standard; lanes 1,3,5,7, before bentonite treatment; lanes 2,4,6,8, after bentonite fining.

Protein binding saturation curve

To determine protein binding saturation, a binding curve was done with 300ng/l IBMP and increasing plOBP concentrations of plOBP up to 60 µM protein in reaction buffer at pH 4.0. 60 µM protein does not completely saturate binding of IBMP at 300 ng/L using this system (figure 8).

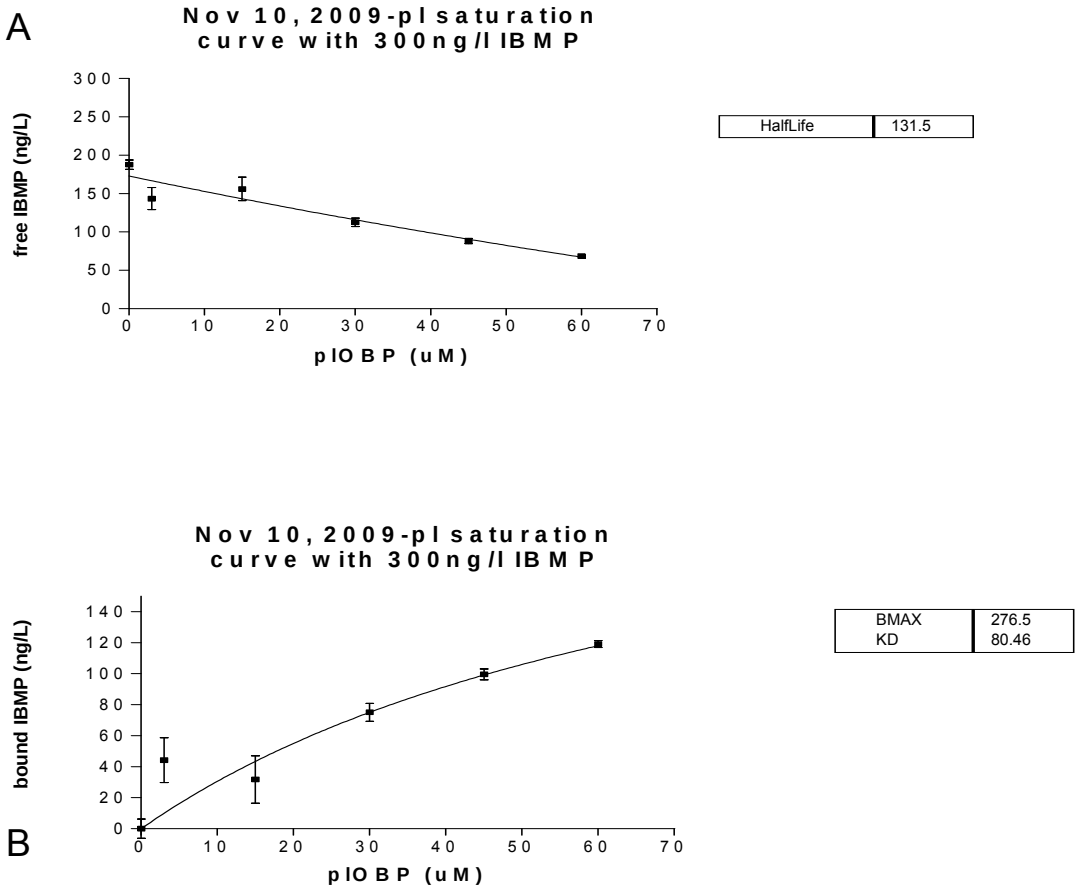

Figure 8. Binding of IBMP, 300ng/l with increasing plOBP concentrations.

(A) The free IBMP fraction was measured by GC/MS following separation from protein-bound complex by bentonite fining. The data was fit to a one phase exponential decay equation.

(B) Although not measured, the bound IBMP fraction was determined from the difference between the starting IBMP and the free IBMP after protein addition and removal by bentonite fining. The data was fit to a one-site saturation binding curve.

Binding Results using pIOPB and bentonite fining to remove complex

In the bentonite fining method, filtration through a 0.22µm Durapore filter is used to remove any final traces of bentonite in the reaction prior to the MP measurements. The filter itself removed approximately 15% of the IBMP from the reaction (Figure 9). The addition of bentonite and the use of the filter without any pIOBP protein reduced the IBMP by approx. 40%. The addition of pIOBP protein reduced the starting IBMP from approximately 300 ng/L to 100 ng/L in pH 4 buffer and 125 ng/L in buffer at pH 3.5. The protein-bentonite fining method was less effective in the juice matrix, reducing the IBMP concentration to approximately 150 ng/L. For IPMP reduction with pIOBP using the bentonite filtration system, the system was not very effective, not significantly reducing the values below the starting concentration of IPMP as illusted in Figure 10.

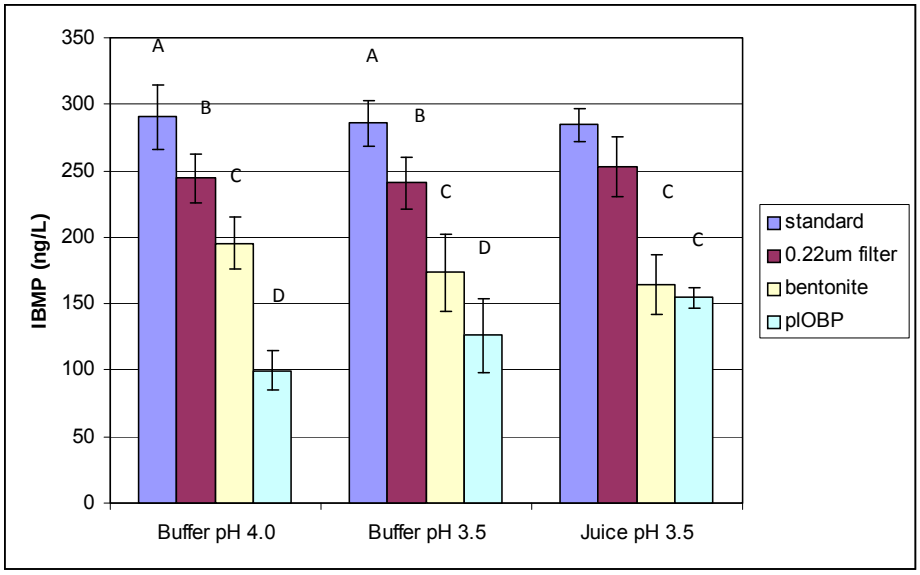

Figure 9 Reduction of IBMP from Phosphate Citrate Buffer pH 4(n=11), Phosphate Citrate Buffer pH 3.5 (n=12) and Chardonnay Juice pH 3.5 (n=6) using pIOBP protein and the bentonite system. Treatments which are significantly different are represented by different letters. Data analysis was performed using ANOVA and mean separation by Fisher LSD (P<0.05).

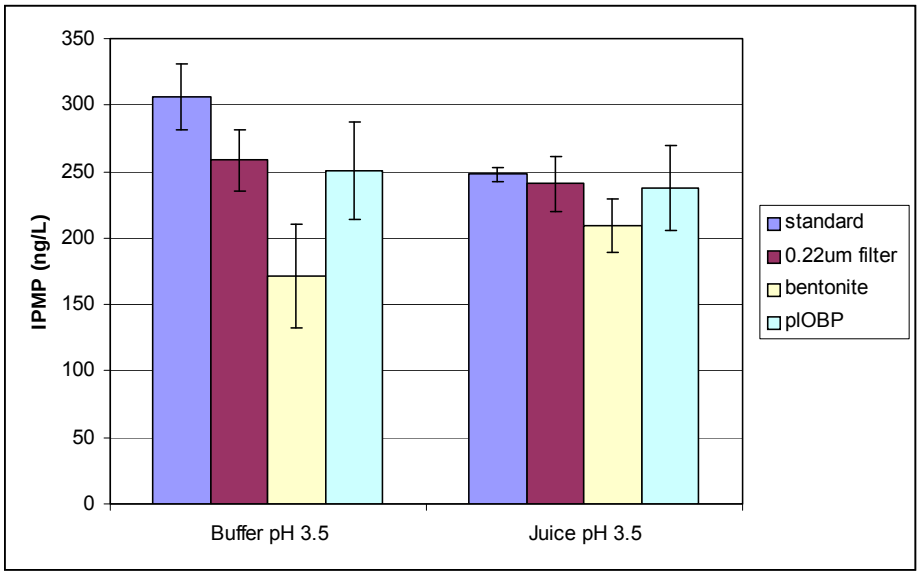

Figure 10. Reduction of IPMP from Phosphate Citrate Buffer pH 3.5 (n=3) and Chardonnay Juice pH 3.5 (n=3) using pIOBP protein and the bentonite system.Treatments which are significantly different are represented by different letters. Data analysis was performed using ANOVA and mean separation by Fisher LSD (P<0.05)

Binding Results using mMUPII and bentonite fining to remove complex

In the bentonite fining method, filtration through a 0.22µm Durapore filter is used to remove any final traces of bentonite in the reaction prior to the MP measurements. The filter itself removed approximately 15% of the IBMP from the reaction (Figure 11). The addition of bentonite and the use of the filter without any mMUPII protein reduced the IBMP by approx. 40-50%. The addition of mMUPII protein further reduced the IBMP in Chardonnay Juice at pH 3.5 by 95% of its starting concentration.

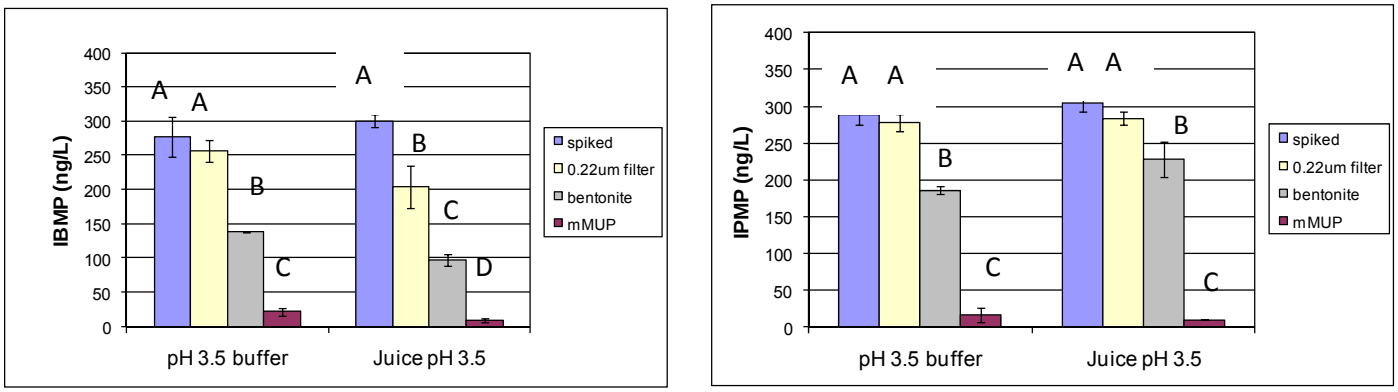

Figure 11. Reduction of IBMP and IPMP from Chardonnay Juice pH 3.5 (n=3) using mMUPII protein and the bentonite system.Treatments which are significantly different are represented by different letters. Data analysis was performed using ANOVA and mean separation by Fisher LSD (P<0.05)

The results to date are satisfactory. We have been able to show that the MP-binding proteins when used in conjuction with either a bentonite system or membrane filtration are able to reduce MP levels in buffer and juice. We need to check if this system would work in wine, and we need to determine if the proteins remove other beneficial flavour and aroma compounds from juice/wine that would detract from juice/wine quality.

c.) Reach and Communications.

Primary target for this research are companies interested in commericalization of this process for the grape and wine industry. A PCT patent was filed on April 14, 2010 based on this research for both the mMUP protein (not directly funded by OGWRI) and the pIOBP protein (funded in part by OGWRI). PCT application based on US Provisional No. 61/169,121 D. Inglis, I. Brindle, G. Pickering, A. Beh, E. Humes. METHOD FOR REDUCING METHOXYPYRAZINES IN GRAPES AND GRAPE PRODUCTS, April 14, 2010.

The results of this work have been presented at the following conferences and public lectures, with OGWRI always acknowledged as a funding supporter for the research programs:

Inglis, D.L. Beh, A.L., Humes, E., Pickering, G. and Brindle, I. Lady bugs and green bell peppers: Methoxypyrazine (MP) removal from grape juice using MP-binding proteins. CCOVI lecture Series Jan. 19, 2011, Brock University.

Reach: 40 attending lecture online and in person, video posted to CCOVI website for download with 300 hits to site after first week of lecture posting (www.broclu.ca/ccovi/outreach-services/ccovi-lecture-series/debbie-inglis-video-2011)

Humes, E. Beh, A-L., Pickering, G., Brindle,I. and Inglis, D.L. (2010) Methoxypyrazine (MP) removal from grape juice using MP-binding proteins. 35th Annual Meeting of the American Society of Enology and Viticulture Eastern Section, Geneva, NY, USA. July 2010. Am.J. Enol. Vitic. 61(4):566A (2010)
Reach: 100 in Eastern Section of USA and Canada that attended conference

Pickering, G.J., Inglis, D.L., and Carter, N. (2010) Elicitation of Ladybug Taint in Wine Is Not Limited to Harmonia axyridis. Presented at the 7th International Symposium for Cool Climate Viticulture and Oenology, Seattle, WA July 2010.
Reach: International audience of approximately 200 attendees

Pickering, G.J., Blake, A., Soleas, G., Inglis, D.L. (2010) Managing Green Flavors after Harvest: Response of 3-Alkyl-2-Methoxypyrazines to Various Wine Treatments, Presented at the 7th International Symposium for Cool Climate Viticulture and Oenology, Seattle, WA July 2010.
Reach: International audience of approximately 200 attendees

4. PROJECT OUTCOMES (ACTUAL VS EXPECTED AT SHORT AND LONG-TERM

a) Short term
For the short-term outcomes of the project, all milestones were reached. In Milestone 4 for project 008, in the first year of the project, it was concluded that 7-spot lady-beetle can taint wine at similar levels as MALB, and therefore, a distinction at the scale-house between MALB and 7-spot is not warranted – any ladybeetle can cause a potential problem if at high enough numbers (200-400 beetles/tonne). For milestones 5 and 6 reported on in this final report, Porcine OBP (as well as murine MUP protein) was indentified as a conadidate protein for removal of MPs from juice or wine. Subsequently, both porcine and mouse proteins were found to remove both IPMP (lady beetle taint compound) and IBMP (causing green taint in cool climate wines). A new method of using a membrane to remove the protein-MP complex was discovered.

This research is at a pre-commercialization stage. Additional work required is to determine if the proteins, once covalently coupled to the membrane surface or silica sand, remove any additional flavour compounds from grape juice that might prove detrimental to final wine quality. This is unlikely as most flavour compounds are in a precursor form in grape juice, bound to sugars that may exclude them from the binding site of the proteins, and hence, they will not be removed. An NSERC Strategic Grant led by Gary Pickering, including Debbie Inglis and Rebecca Hallet, was applied for last April, 2010, but was not successful due to funding limits as announced in October, 2010. The next stages of research required for commercialziation have been included in the provincial ORF RE grant application, with results due in May of 2011.

The value of this research is not only in terms of tools to modulate the level of lady-beetle taint in wine, but this technology can also be applied to removing the “green” characteristic in wines produced in cool climate regions resulting from MPs in providing additional tools for winemakers.

b) Long term
This work is still at its precommercialization stage, so it is too early to talk about job creation, increased sales and impact on Ontario products.

5. Final Comments and Conclusions

There were minimal deviations from the work plan. One positive attribute was the identification of a second protein, the mouse protein, which also had high affinity for the MPs. This protein was studied alongside the pig protein in parallel. Due to this fact, a post-doctoral fellow was brought into this project to assist in managing the additional work. Therefore, an additional thousand dollars from supplies was re-allocated to offset the salary portion for the post-doctoral fellow, which was being paid out of other funds. Therefore, overall salaries charged to OGWRI were $11,000, and consumables were $11,500.

The researchers involved in this project would like to continue the research required to commercialize these research outputs for use in our indsutry. But in order for the research to continue, additional funding is required. Although D. Ingis and G.Pickering were not successful with the NSERC Strategic Grants proposal, the proposal did pass the scientific review. NSERC encouraged the researchers to apply to one of their partnership programs where funding is matched by industry. Subsequent to that grant competition, the researchers (D. Inglis and G. Pickering) have worked to incorporate the next stages of research for the MP-removal system into the provincial ORF RE grant, further leveraging the industry funding attained for the AAFC DIAP program. If unsuccessful in that competition, we would then look again at the NSERC partnership programs and come back to OGWRI for funding requests.