Lady bugs and green bell peppers: Methoxypyrazine (MP) removal from grape juice using MP-binding proteins

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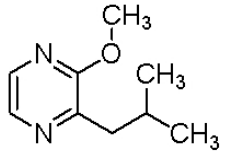
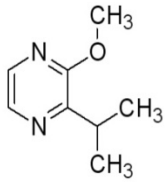
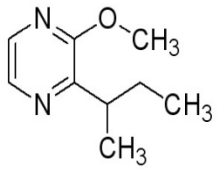
3-alkyl-2-methoxypyrazines (MPs)

- **MPs represent an important and potent class of grape and insect derived odor active compounds associated with wine quality**







3-isobutyl-2-methoxypyrazine (IBMP), 3-isopropyl-2-methoxypyrazine (IPMP), 3-secbutyl-2-methoxypyrazine (SBMP)

- **Elicit green and vegetative aroma and flavour descriptors in wine**

Impact of MPs on Wine Quality

Grape-Derived MPs

- Positive in Sauvignon blanc **(Parr et al., 2007)**
- Other varietals: unpleasant in wine at elevated levels - vegetal, green pepper aroma and flavor **(Allen et al., 1994)**
  - Related to suboptimal fruit ripeness & low wine quality (Roujou de Boubee, 2000)
  - IBMP most abundant
  - MPs Higher in cooler climate (Kotseridis et al., 1998)
- Very low sensory threshold for MPs – low ng/L (ppt)

Insect-derived MPs

Ladybug Taint (LBT)

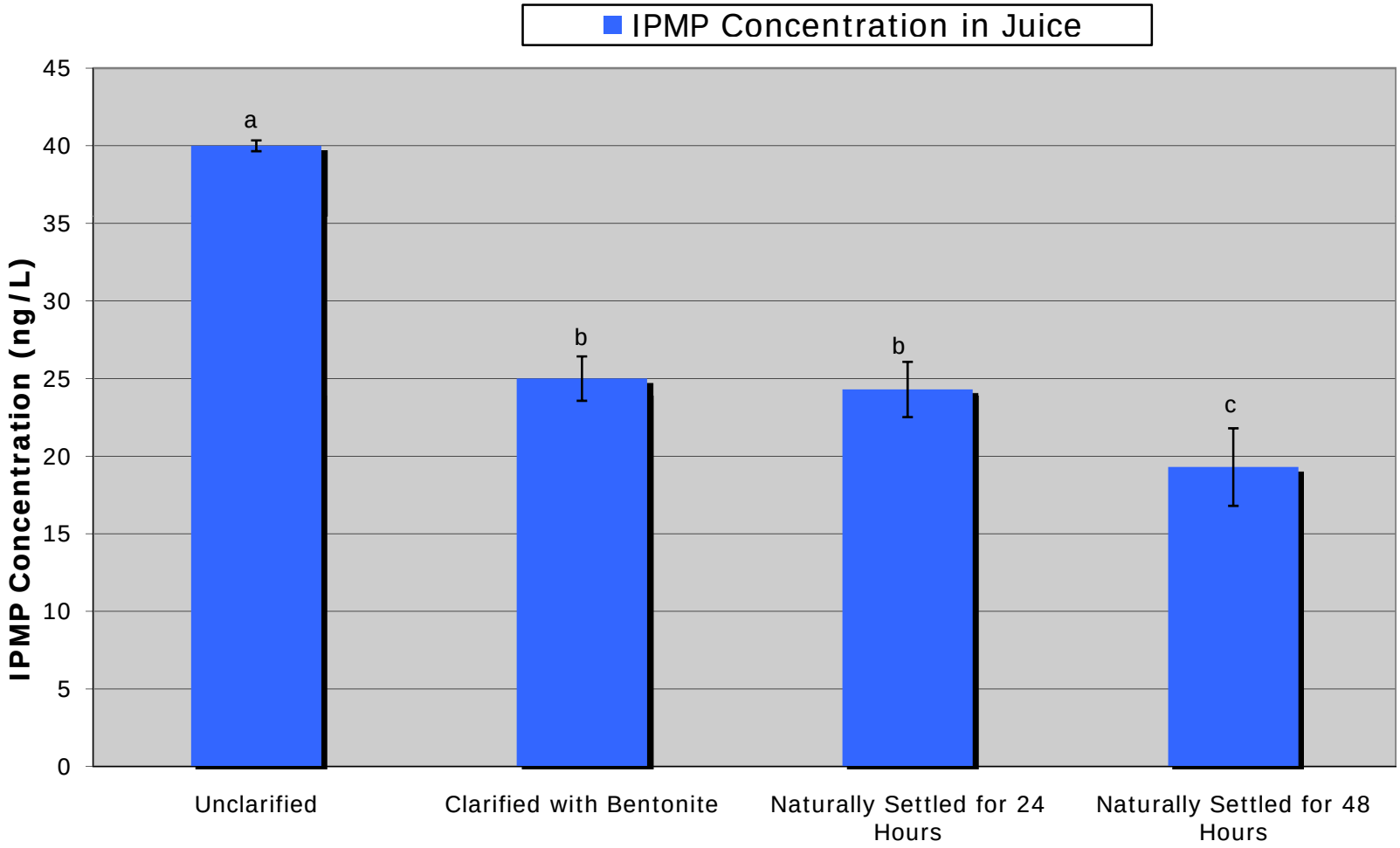
- Incorporation of *Harmonia axyridis* (MALB) or *Coccinella septempunctata* (C7-7spot) with grapes at harvest
- IPMP (haemolymph) causal compound (Pickering et al., '04, 05, 08)
- Sensory threshold of IPMP – as low as 320 pg/L (Pickering et al., '07)
- Atypical aroma and flavour: peanut, earthy, vegetal, green pepper
- Known problem in France, USA, Canada; likely in many other wine regions

Previous Research from Gary Pickering's research laboratory

- Can juice and wine processing and storage affect IPMP concentration?
  - Settling
  - Yeast choice
  - Common fining agents
  - Wine closures
  - Packaging
  - Light and temperature

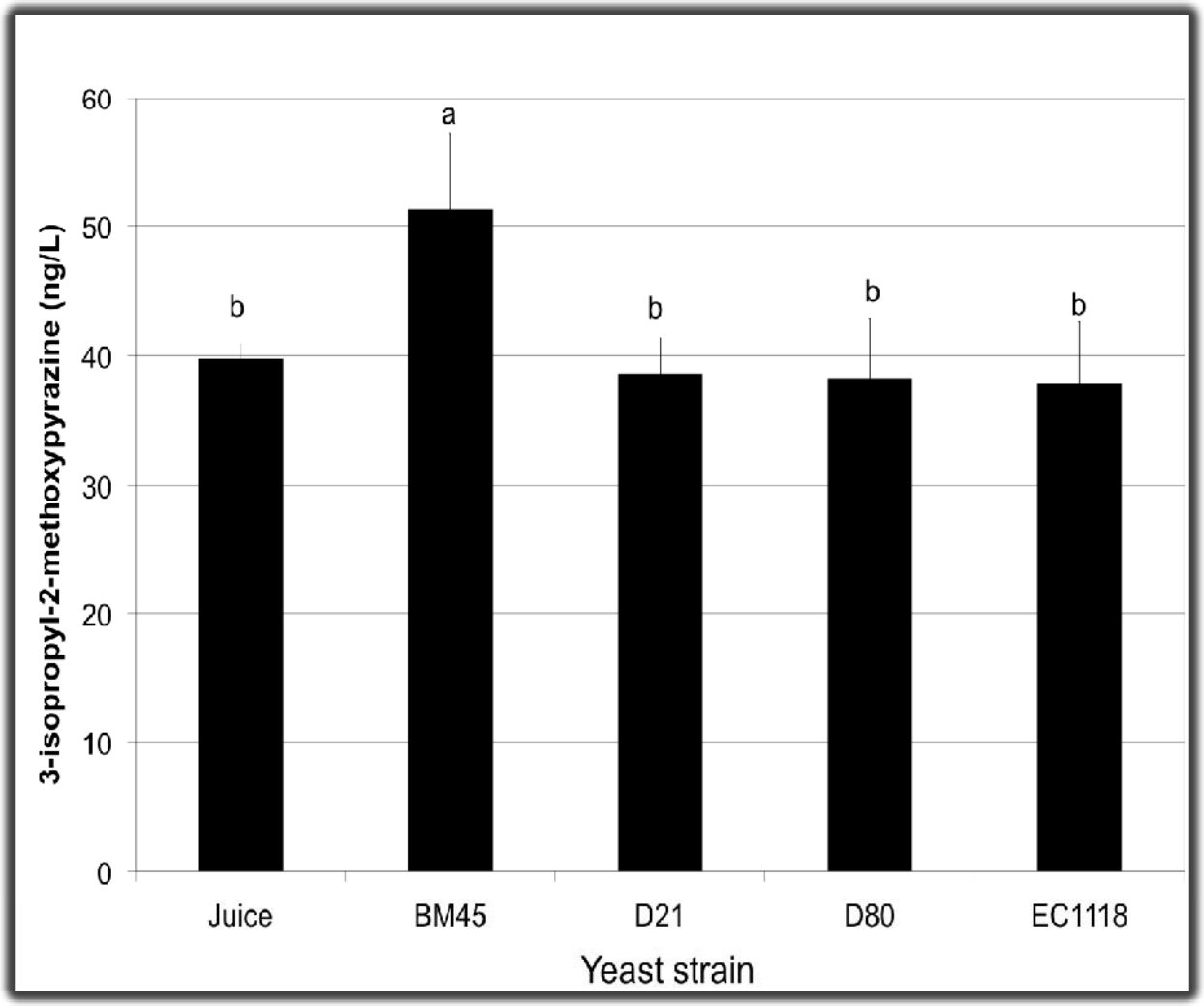
How does juice setting impact IPMP?

Chardonnay juice spiked with IPMP



(from Kotseridis et al '08 *J Chrom A*, 1190 (1/2))

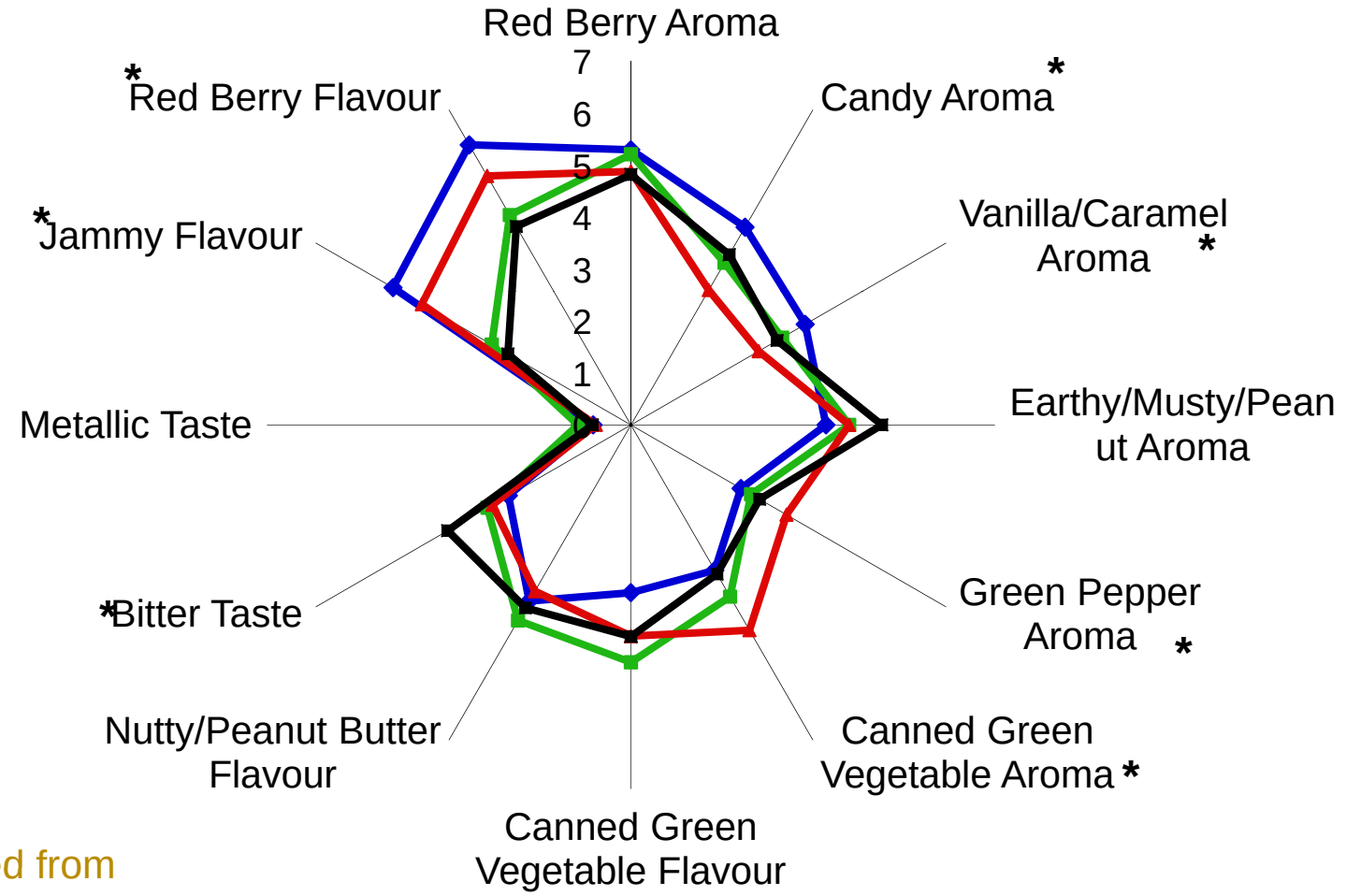
Can commercial *Saccharomyces* yeast strains reduce IPMP?



IPMP concentration for Cab Sauv wine made from juice spiked with 30 ng/L IPMP & fermented with 4 different yeast strains

(adapted from Pickering et al., '08 *Aust J Grape & Wine Res*, 14)

Sensory intensity scores for Cab Sauv wine made from juice spiked with 30 ng/L IPMP



(adapted from Pickering et al., '08 *Aust J Grape & Wine Res*, 14)

What about reducing IPMP using common fining agents/additives on IPMP

1. Activated charcoal @ 0.2 g/L;
2. Oak chips (French, medium toast) @ 4 g/L for 3 days;
3. De-odorised oak chips (as above, after ethanol extraction + water wash x3 + water boil + dry @ 60°C);
4. Light treatments:

   Red wine: UV - 254 nm, 18.3 W @ 100 m/min for 40 min in custom reactor

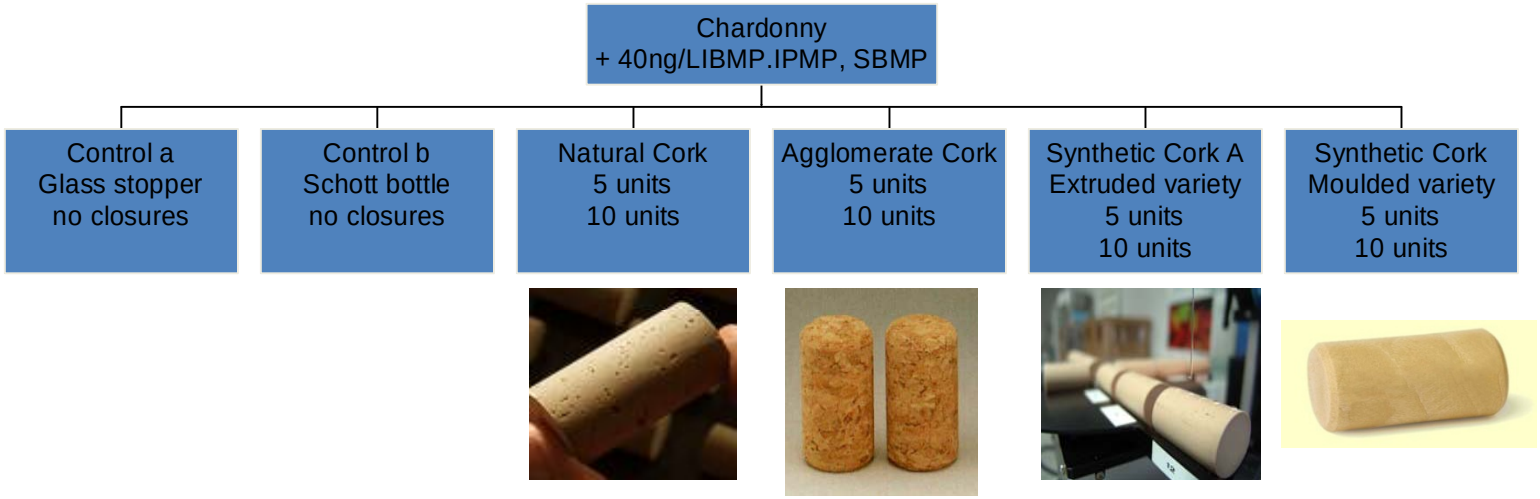
   White wine: visible light, halogen bulb, 120 W

Results (From: Pickering et al., '06, *Inter J Food Sci Tech*, 41)

- Only activated charcoal decreased IPMP concentration
- MP-associated sensory attributes only consistently reduced in oaked wine
  - masking effect

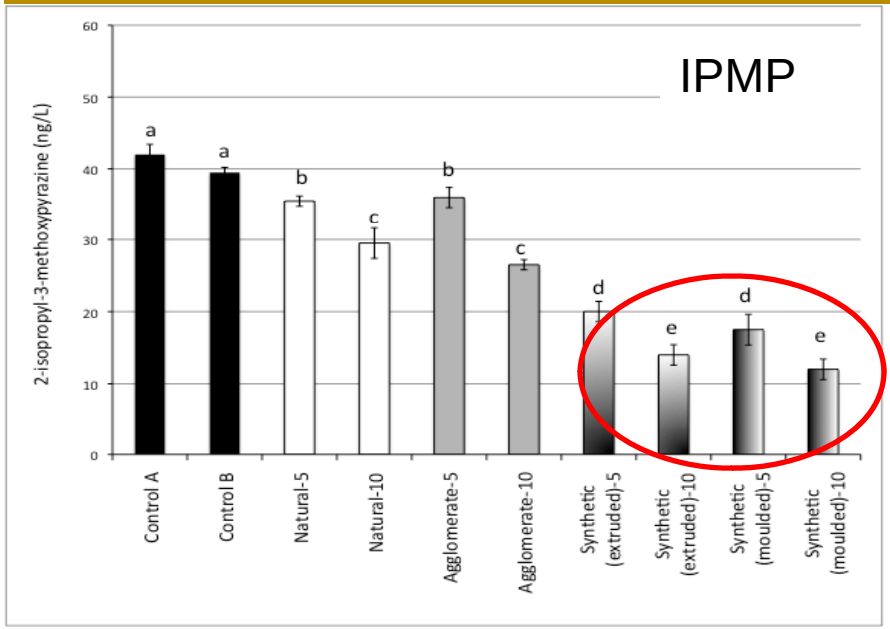
Can wine closure materials remove MPs?

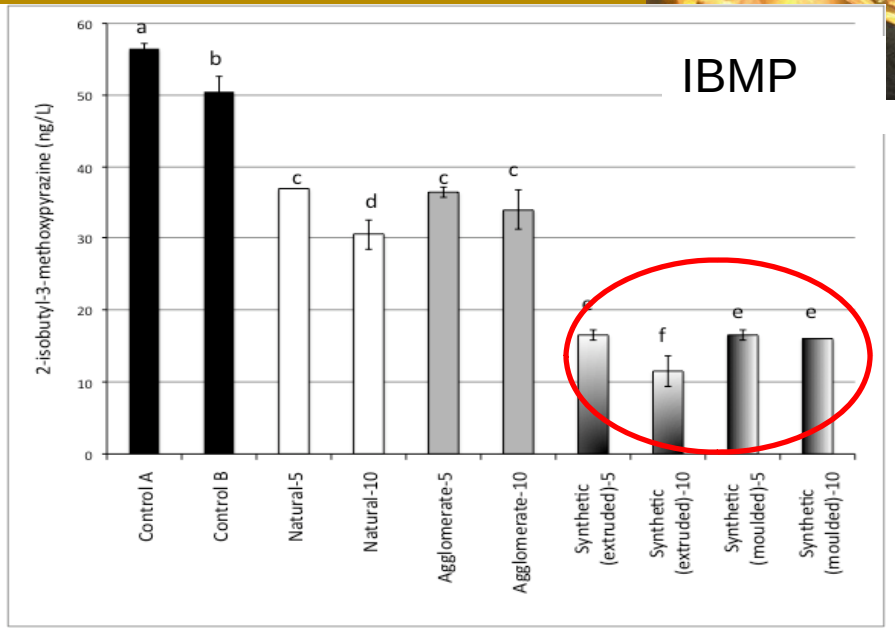
- **Sorptive processes ('flavour scavenging') previously identified for some volatiles (e.g. Capone et al., '03, 04)**

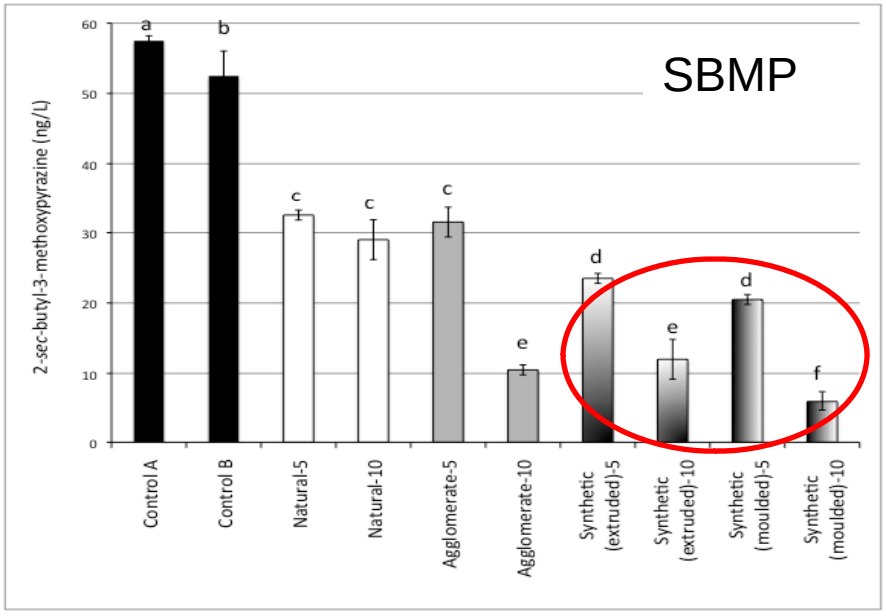


Analyzed for MP content (GC-MS, SPE) after 6d

Can wine closure materials remove MPs?







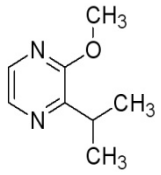
- **All MPs show a decrease with increasing closure units**
- **Greatest ↓ with synthetic corks**
- **70% - 89% reduction for SBMP**
- **? Impact on other wine odorants**

(from: Pickering et al., 2010. *JFAE 8(2)*)

(Selected) Results: Closure and Packaging Study

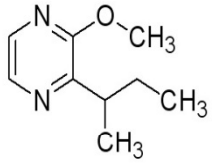
(from: Blake et al. 2009, *J Ag & Food Chem*, 57 (11))

IPMP



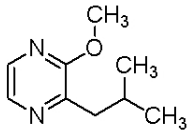
- Small changes from closures
- Tetrapak showed largest ↓ after 18 months (23% Riesling; 41% Cabernet Franc)
  - Polyethylene known to flavour scavenge in other foods (Sajilata et al., 2007)

SBMP



- ↑ in some closures
  - DMMP migrate into wine (Simpson et al., '04)
- Tetrapak showed largest ↓ after 18 months (17% average)

IBMP



- Steady ↓ with time for all closures & wines (33 - 46%)
- Most rapid (after 3 months) with Tetrapak

NOTE: Tetrapak performed worst for measures of oxidation

Light and Temperature Study

- **No MPs varied significantly with light or temp condition**
- **IPMP & SBMP relatively stable during aging**
- **IBMP decreased by approx 30%**

(Blake et al. 2009, *Food Chemistry*, 119 (3))

Summary for impact of juice/wine processing and storage on MPs

- Juice clarification prior to fermentation advantageous
- Yeast strain matters
- IPMP resilient to most traditional fining agents
- Synthetic cork material(s) show potential for sorption of MPs
- MP species behave differently during bottle aging
- Closure & packaging type can affect MP composition
- Light & temp during bottle aging minimal effect on MPs
- **Juice/wine interventions with high affinity and specificity for MPs needed**

Current Project Goal: develop a fining agent specific for MPs

- **Remove LBT taint due to IPMP from grape juice and/or wine using a protein fining agent**
  - First: find a protein that can bind to IPMP in grape juice/wine
  - Second: remove the IPMP-protein complex from the juice/wine hence removing the taint from the juice/wine

Fining Agents and MP removal

- **Properties we are looking for in a protein fining agent to work in juice/wine**
  - High affinity for the compound to be removed
  - High specificity for the compound
  - Protein Stability
    - Over time of fining process
    - At low pH (3.0-4.0)
    - Stable in ethanol

What are Lipocalins and why are we interested in these proteins

- **Family of proteins which transport small hydrophobic molecules**
  - steroids, bilins, retinoids, lipids, aroma compounds
- **Subgroup of lipocalins known as odorant binding proteins and pheromone binding proteins**
  - transport odorants or pheromones
  - Known to have high affinity for methoxypyrazines

Two Proteins that bind MPs

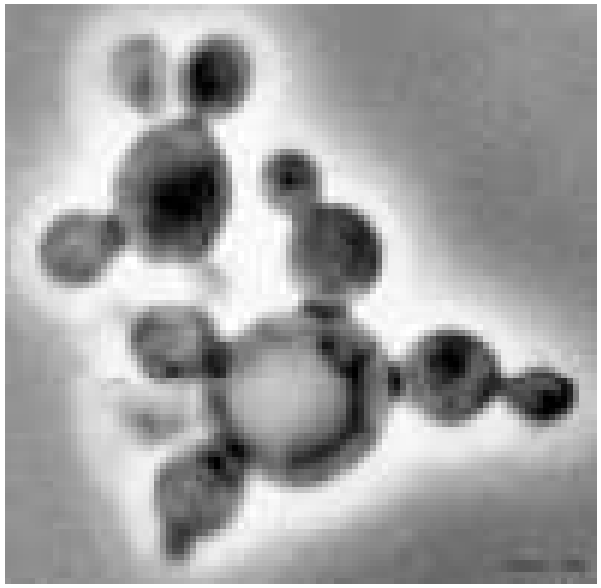
- **Two candidate lipocalin proteins that function as monomers and bind methoxypyrazines at lower pH according to the literature**
  - Mouse Major Urinary Protein 1(mMUP-1)

  - Porcine Odorant Binding Protein (plOBP)

Protein	Kd	Structure	Funtional pH
Rat OBP-1f	1.7 µm (IBMP	Dimer	7.5 (Briand, 2000)
pOBP	0.8 µm (IBMP)	Monomer	3.5 (Burova, 1999)
MUP	1.8 µm (IPMP)	Monomer	5.5 (Lucke, 1999)
hOBP	0.9 µm (IBMP)	Monomer	7.5 (Briand, 2002)

Cost effective source of protein

- **Expressed proteins in methylotrophic yeast *Pichia pastoris***
- **Purified these secreted protein from growth media, using anion exchange chromatography**



Two assays developed to test proteins as fining agents

Protein + MP
in solution

2 h incubation

Assay 1
Remove Protein-MP
complex by
10 kDa CutOff
membrane filtration
n = 9

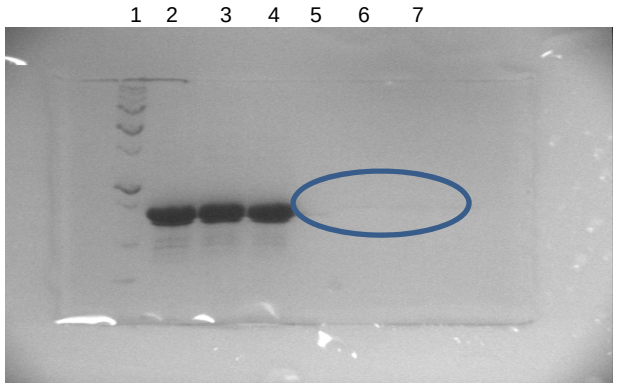
Assay 2
Remove Protein-MP
complex by
bentonite and
0.22 uM filtration
n = 6

Measure residual MP
HS-SPME-GC-MS
LOQ = 6 ng/L IBMP
LOQ = 2 ng/L IPMP

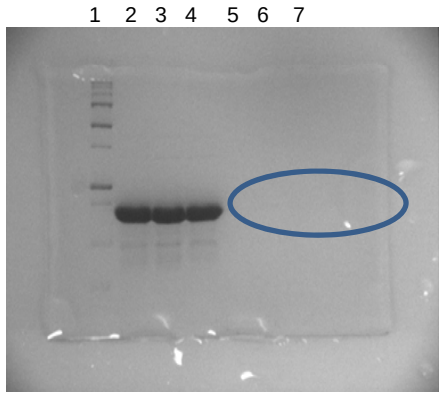
Protein Removal System
Assay 1: PES membrane

- **Polyethersulfone membrane**
- **10kDa MW cutoff (nothing bigger than 10kDa gets through)**

pH 3



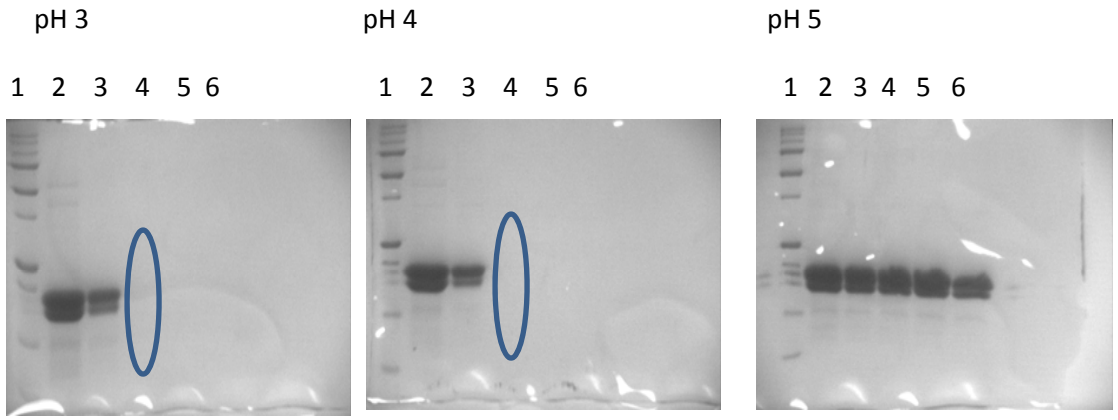
pH 7



Lane 1 Marker
Lane 2-4 Before Filtration
Lane 5-7 After Filtration

Figure 3: Membrane Fining trials at pH 3, and pH 7 2mL phosphate citrate buffer containing approximately 700 – 900 ug/mL of pI OBP

Protein Removal System
Assay 2: Bentonite



Lane 1 Marker
Lane 2 Control
Lane 3 1g/L
Lane 4 3g/L
Lane 5 5g/L
Lane 6 7g/L

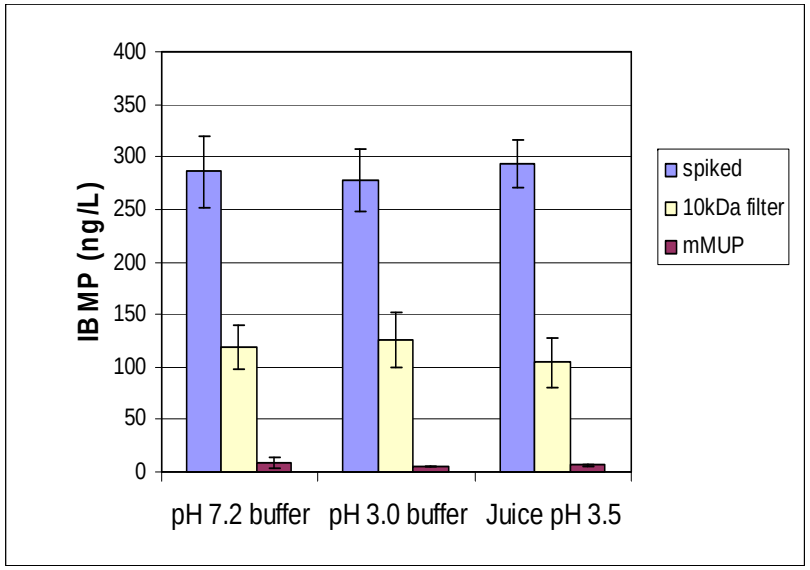
Figure 2: Bentonite Fining trials at pH 3, pH 4 and pH 5 using 1,3, 5 and 7 g/L of bentonite slurry in a 2mL phosphate citrate buffer containing approximately 700 – 900 ug/mL of pI OBP

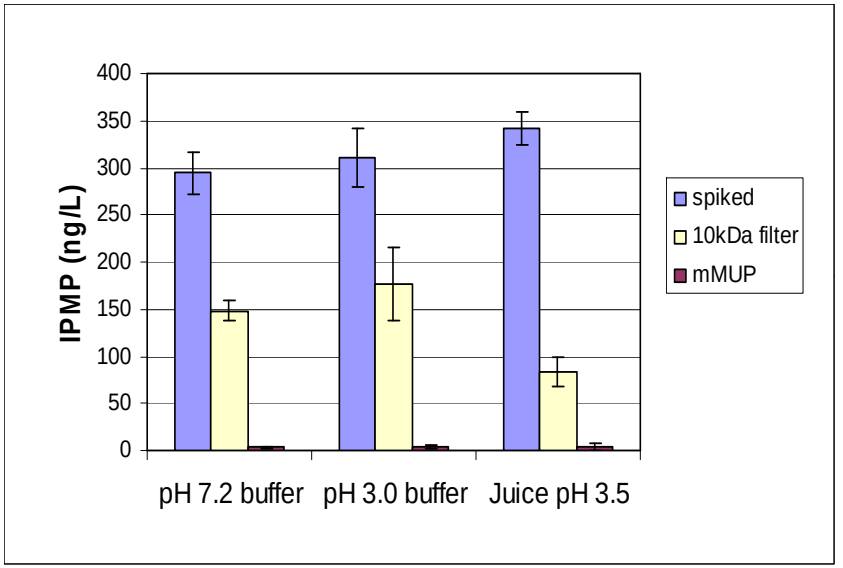
Can the proteins remove MPs from grape juice?

- ✓ Proteins can be removed either by 10 kDa membrane filtration or bentonite fining/filtration

- Question: If the proteins bound up MPs, and the membrane or the bentonite removed the MP-bound protein, would we see a reduction in MPs in the filtrate?

Reduction of IBMP and IPMP by mMUP in filtration assay

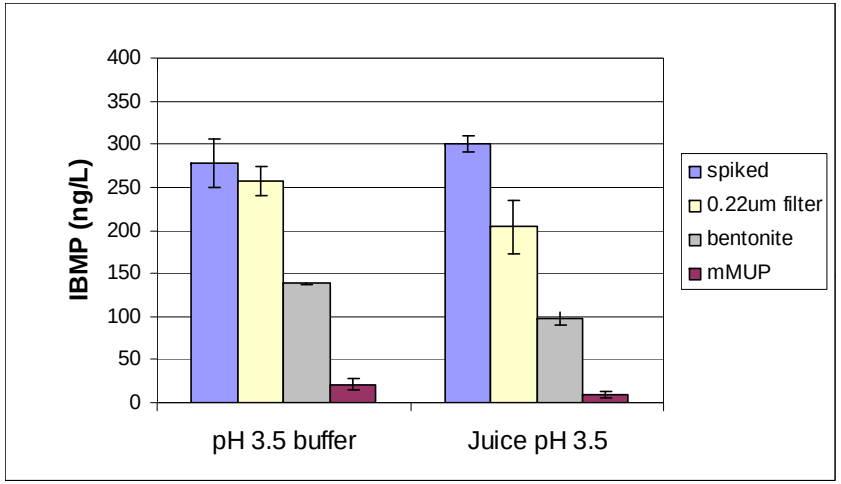


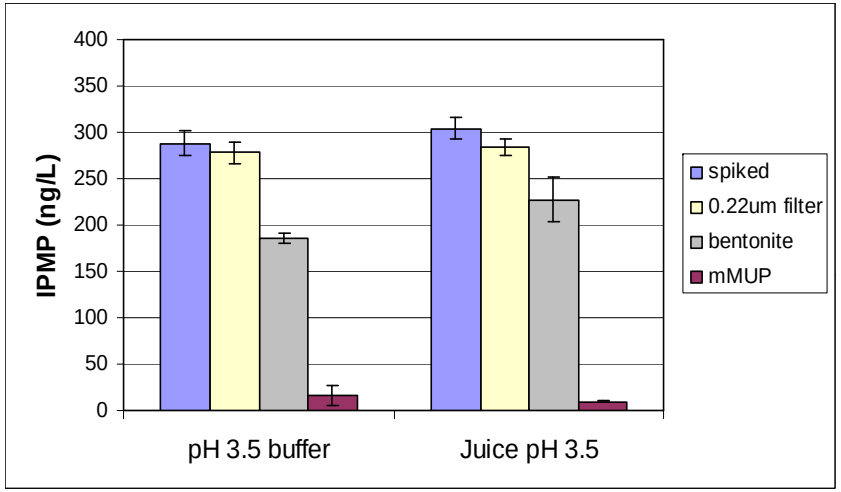


- **IBMP reduced from 300ng/L to less than 5ng/L (LOQ) in CHARDONNAY JUICE!**

- **IPMP reduced from 300ng/L to less than 2ng/L (LOQ) in CHARDONNAY JUICE!**

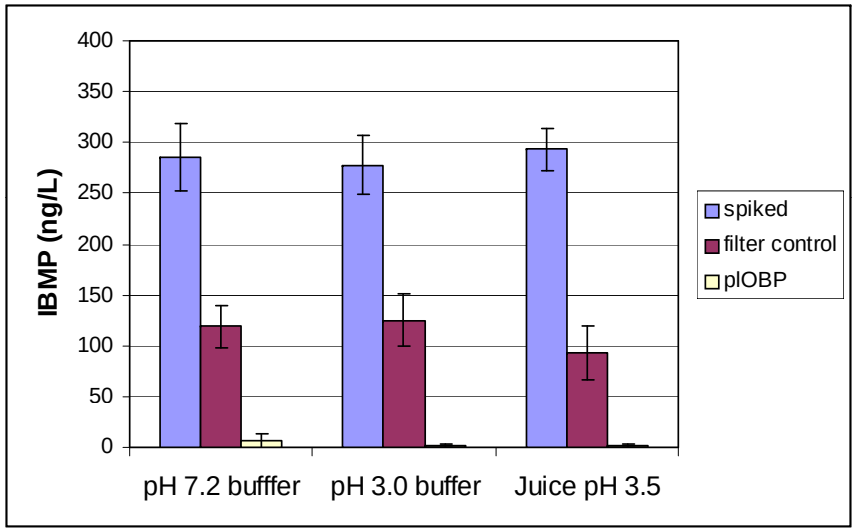
Reduction of IBMP and IPMP by mMUP in bentonite assay

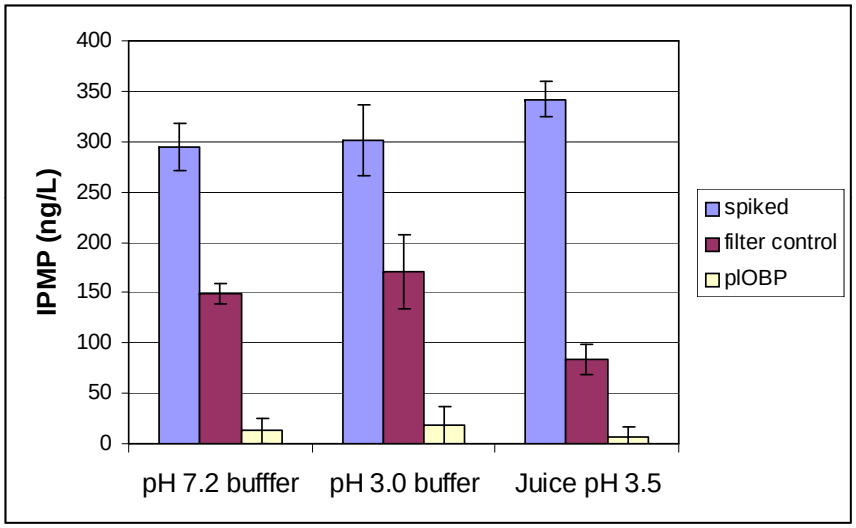




IBMP and IPMP both reduced by 95% using the mouse protein-bentonite system in JUICE!

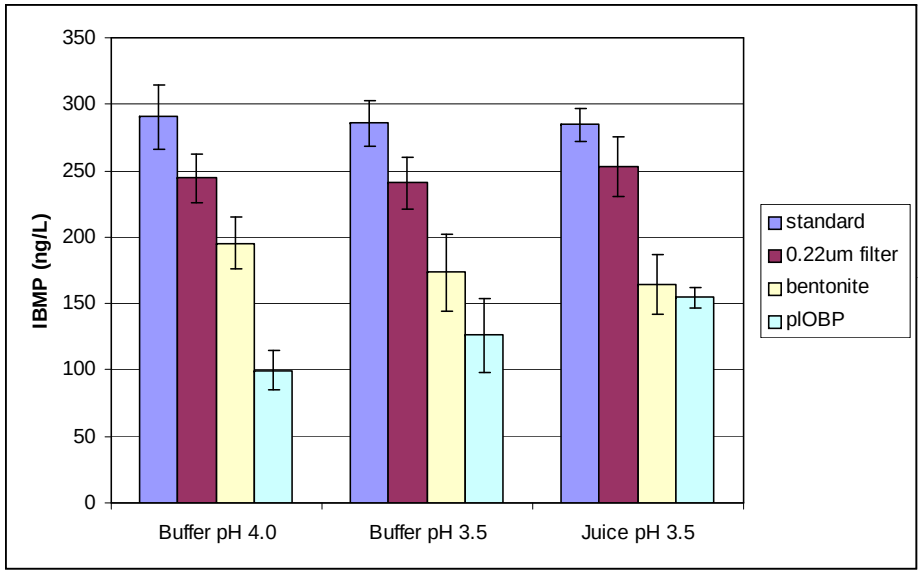
Reduction of IBMP and IPMP by plOBP in Filtration assay

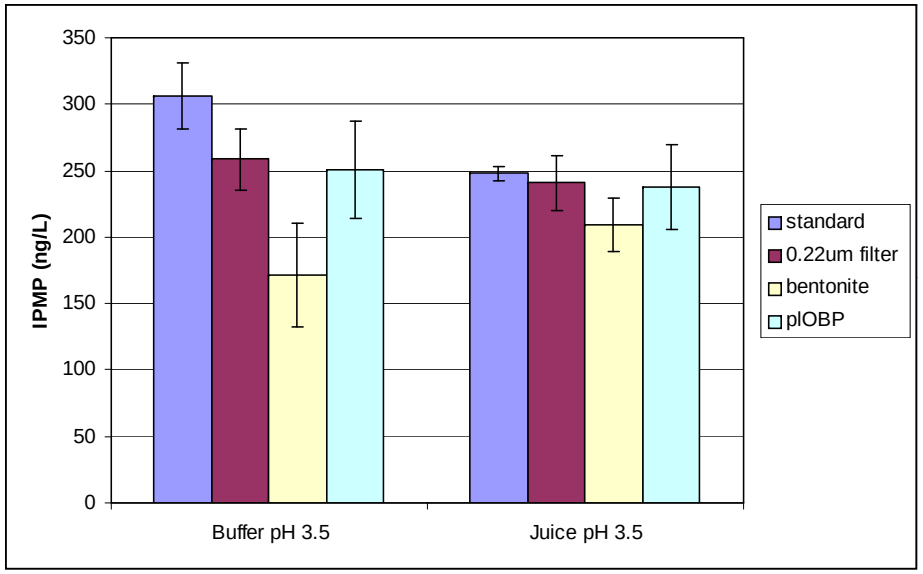




- **IBMP reduced from 300ng/L to less than 5ng/L (LOQ) in juice!**
- **IPMP reduced from 300ng/L to 7 ng/L in juice**

Reduction of IBMP and IPMP by plOBP in bentonite assay

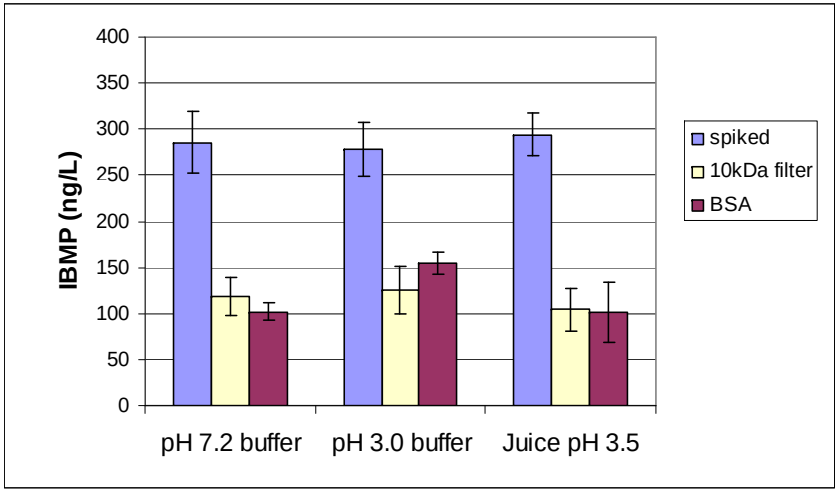


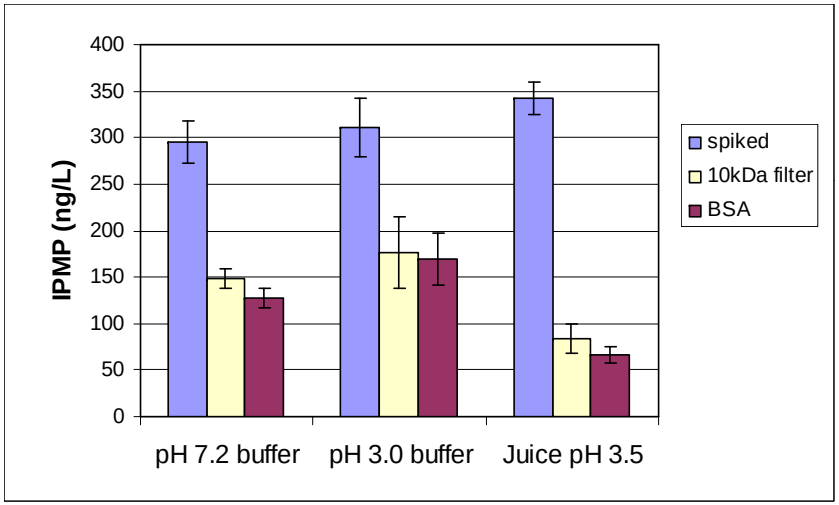


- **Pig protein-bentonite system not as effective in reducing IBMP and IPMP at low pH 3.5 buffer or juice**

Results are specific to proteins tested

BSA: Bovine Serum Albumin





BSA control protein shows no significant reduction of MPs in the 10kDa filtration system

Next Steps

- **What is the impact of the proteins on other juice/wine volatiles using current fining systems?**
  - Measure this chemically using GC, GC/MS, GCO
  - sensory impact of treatments
- **Will the proteins function in a wine matrix with ethanol or are they limited to juice fining?**
- **To look at the impact of proteins on other flavour compounds –Dr. George Kotseridis, CCOVI Flavour Chemist arriving January 24th**

Next Steps

- **Develop a commercial application for the technology**
  - Bind the protein to surface of PES membrane for juice processing, develop a protein-coated membrane that can be used multiple times
  - Bind the protein to silicon dioxide particles (ie like bentonite), either on surface or interior, develop multiple use system
  - Use MP binding proteins in conjunction with other sorptive materials
- **Has the potential to remove MPs that cause “green flavours” as well as LBT, application in cool climate wines**

Summary on using MP-binding proteins for MP removal

- **Both the mouse and the pig proteins (mMUP and pIOBP)are both able to bind to MPs at acidic pH, and remove MPs in JUICE!!!!**
  - Filtration system tested with mMUP and pIOBP removed at least 99% of MPs from juice to below LOQ of GC/MS
  - Bentonite system tested with mMUP reduced MPs by 95%, whereas tested with pIOBP reduced MPs by 60%
- **Now looking to immobilize the proteins to membrane or silica support for use in winery filtration operations**

Acknowledgements

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- Eric Humes (MSc student)
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- MALB Taskforce members
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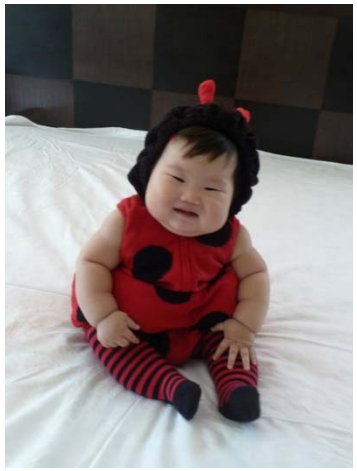
- Natural Sciences and Engineering Research Council of Canada (NSERC) Strategic Grants Program



THANK YOU!!!

QUESTIONS ??????????????