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NEW APPROACHES TO REMOVING ALKYL-METHOXYPYRAZINES FROM GRAPE JUICE AND WINE

Gary Pickering[1,2,3], Debra Inglis[1,2,4], Andreea Botezatu[2,3], Ailin Beh[2], Eric Humes[1], and Ian Brindle[5]

[1] Dept of Biological Sciences, [2] Cool Climate Oenology and Viticulture Institute, [3]Environmental Sustainability Research Centre, [4] Centre for Biotechnology, [5]Dept of Chemistry, Brock University, 500 Glenridge Ave, St. Catharines, ON L2S 3A1, Canada; Phone:1-905-688-5550;

gpickering@brocku.ca, dinglis@brocku.ca, abotezatu@brocku.ca, abeh@brocku.ca, eric.humes@brocku.ca, ibrindle@brocku.ca.

corresponding author email: gpickering@brocku.ca. presenting author: ibotezatu@brocku.ca

Abstract

Grape-based products, including wine, are amongst the world's most important value-added horticultural commodities, both economically and culturally. 3-isobutyl- (IBMP) and 3-isopropyl- (IPMP) 2-methoxypyrazine are important grape- and insect-derived flavour compounds in some grape juices and wine, and are responsible for undesirable green characters associated with under-ripe grapes or infestation from Coccinellidae. Here we present data on two novel approaches to removing these compounds from juice and wine. Firstly, we describe a protein-based technology that binds and removes IPMP and IBMP in juice. The lipocalin Mouse Major Urinary Protein 1 (mMUP) was expressed in Pichia pastoris, secreted, and purified using anion exchange chromatography. mMUP, combined with a 10 KD cut-off PES membrane filtration system, resulted in a reduction of IPMP and IBMP in juice of > 98%. However, removal of methoxypyrazines from wine using this technique may be limited by ethanol-induced changes in the mMUP structure. Therefore, a 2nd approach is being developed that takes advantage of the sorptive properties of various polymeric materials. A range of food-grade polyethylene-, polypropylene- and silicon- based polymers were evaluated for their capacity to remove IPMP and IBMP from red wine. Candidate polymers were standardized to a common surface area and added to red wine for 2 hrs. Quantification of IPMP and IBMP using HS-SPME-MD-GC-MS showed reductions of up to 40% for some polymers, and minimal changes to the sensory characteristics of the wine. We conclude there is capacity to significantly mediate the impact of methoxypyrazines on juice and wine quality using these biotechnology tools.

Keywords: *methoxypyrazines, wine taint, ladybug taint, Coccinellidae, remediation.*

INTRODUCTION

Flavour is a critical driver of consumer acceptance of and preference for agri-food products. Alkyl-methoxypyrazines (MPs) are aroma-active constituents of several species of vegetables, nuts (Boubee et al., 2000; Buchbauer et al., 2000; Sala et al., 2002), fruits (Schieberle et al., 2003) and spices (Jagella and Grosch, 1999). MPs have also been identified in several grape cultivars and their wines. In wine, they can have a positive impact on the aroma profile of certain varietals, particularly Sauvignon blanc (Allen and Lacey, 1998), but in general are considered detrimental to quality, contributing undesirable characters such as "green" and "unripe" (Allen et al., 1991). 3-isobutyl- (IBMP), 3-sec-butyl- (SBMP) and 3-isopropyl- (IPMP) 2-methoxypyrazine have all been confirmed in grape and wine, with IBMP the most prevalent. IBMP concentrations in grape decrease with ripening; thus elevated levels in wine are indicative of the use of grapes of sub-optimum maturity or poor viticultural management (Lacey and others, 1991).

In addition to being found as intrinsic compounds in grapes, MPs in wine may also be derived from Coccinellidae (ladybeetles); specifically, *Harmonia axyridis* (Pallas) and *Coccinella septempunctata* (Botezatu et al., 2013). When grapes are harvested, beetles resident in grape clusters may also be inadvertently collected, and following crushing and pressing operations in the winery, impart the resulting juice and wine with an unpleasant aroma and flavour coined 'ladybug taint' (Figure 1).

Figure 1. *Harmonia axyridis* beetles can infest grapes and taint the resulting juice and wine with alkyl-methoxypyrazines (photo courtesy of Kevin Ker and Ryan Brewster).

Juice and wine affected by ladybug taint are characterised by undesirable peanut and bell-pepper aroma and increased bitterness (Pickering et al., 2004). IPMP – a component of Coccinellidae haemolymph – has been identified as the main compound responsible for the taint (Pickering et al., 2005), while other MPs may contribute to a lesser extent (Botezatu et al., 2013).

Importantly, the human threshold for both IPMP and IBMP in juice and wine is very low - down to 300 pg/L (Pickering et al., 2007) - meaning little is needed to compromise wine quality, and creating challenges for efforts aimed at remediating affected juice and wine. Indeed, traditional winery processes and fining agents have only proven partially effective in reducing MP levels (Pickering et al., 2006; Kotseridis et al., 2008; Kögel et al., 2014), in part due to a lack of specificity for MPs. Interestingly, some preliminary data suggests that polyethylene-based polymers may have capacity for removing MPs from wine through sorption (Blake et al., 2009; Pickering et al., 2010).

The objectives of the current study were to assess the efficacy of 2 technologies at reducing IPMP and IBMP in grape juice and wine. In the first study, an odorant binding protein (mMUP) with high specificity for MPs was trialled for use in grape juice. In the second study, a range of polyethylene and other polymers was evaluated for their potential to remove MPs from wine.

METHODS AND MATERIALS

In Study 1, we evaluated 2 lipocalins with purported high affinity for MPs (Kd values as low as 0.8 µm) - Mouse Major Urinary Protein 1 (mMUP) and Porcine Odorant Binding Protein (plOBP) - for their MP-fining potential in buffer and juice; we report on the results for mMUP here. mMUP was expressed in the methylotrophic yeast *Pichia pastoris*, and the secreted protein was purified using anion exchange chromatography. The protein was then added to MP-spiked Phosphate Citrate Buffers or a Chardonnay juice. In related assays, we also assessed the capacity of a polyethersulfone membrane (10kDa MW cutoff) and a bentonite fining agent to remove the mMUP-MP complexes from the buffer/juice. Full details can be found in Inglis et al. (2010).

In a second study, we investigated the capacity of a range of 14 synthetic and natural polymers to remove MPs from red wine. These included low-, medium-, and high- density polyethylenes, polypropylene, and related copolymers. The 3 best-performing polymers were selected for further study, which involved standardizing their area to approx 68 cm^2 and soaking them in a 200mL aliquot of red wine (Cabernet sauvignon and Merlot blend) spiked with MPs, for 2 hrs.

IPMP and IBMP were quantified in both studies using the sensitive headspace solid-phase microextraction multidimensional gas chromatography-mass spectrometry method of Botezatu et al. (2013). Wine samples were treated with NaOH, diluted to volume and then volatiles extracted from the headspace using a Gerstel autosampler and a DVB/Carboxen/PDMS Stable Flex fiber. Analyses were performed using an Agilent 7890A Gas - Chromatographer coupled with an Agilent MS 5975 Mass Spectrometer with triple detector. The GC was equipped with a Dean Switch that permitted switching the effluent flow between the two columns (Column 1: HP5MS; Column 2: DBWAX). For IPMP, ions 137 and 152 were monitored as quantifying and qualifying ions, respectively. For [$^2H_3$] – IPMP, ions 140 and 127 were monitored as the quantifying and qualifying ion, respectively. The selected mass channels for IBMP were m/z 124 and 81, and for [$^2H_3$] – IBMP they were 127 and 154. Ions 124 and 127 were used as quantifying ions while 81 and 154 were qualifying ions. The MS was operated in total ion chromatogram mode (TIC), with a gain factor of 7.

RESULTS AND DISCUSSION

Study 1

As shown in Figure 2, IPMP and IBMP were reduced from 300ng/L to < 2ng/L and 5 ng/L, respectively, in the juice.

Importantly, the mMUP-methoxypyrazine complexes were successfully counter-fined out of the juice using bentonite; a commonly employed fining agent used in juice and wine production (data not shown). In a related trial, we demonstrated that bovine serum albumin, used as a non-specific protein control, had no impact on reducing MPs (Inglis et al., 2010).

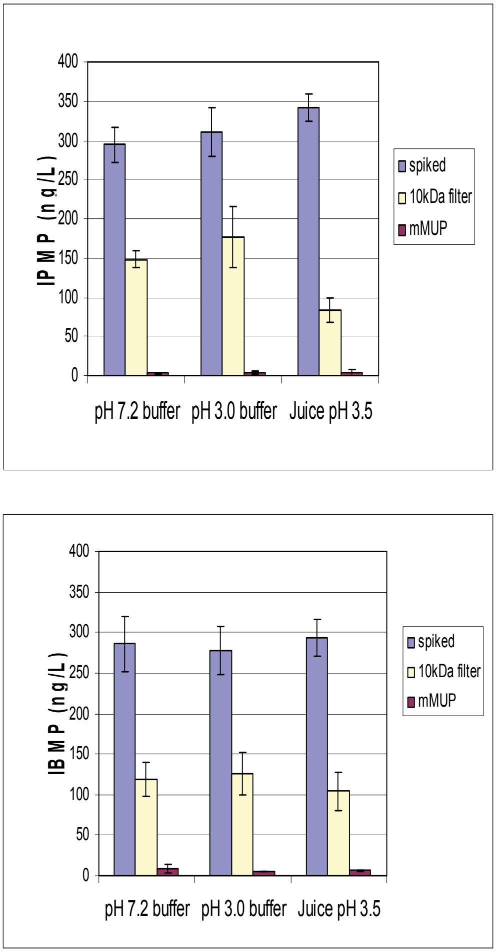

Figure 2. Reduction of 3-isopropyl- (top) and 3-isobutyl- (bottom) 2-methoxypyrazine in Phosphate Citrate Buffer and Chardonnay juice using mMUP and the 10 MWCO PES filtration system. Data represent the mean values of 9 replicates for each matrix. Figure adapted from Inglis et al., (2010).

Study 2

As shown in Figure 3, all 3 polymers significantly reduced both IPMP and IBMP concentrations in the wine. On-going research in our labs is focused on optimising Polymers B and C for use on a commercial scale.

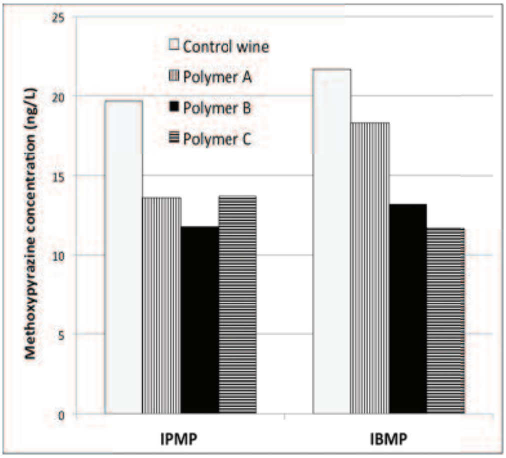

Figure 3. Reduction of 3-isopropyl- (IPMP) and 3-isobutyl- (IBMP) 2-methoxypyrazine in red wine after treatment with selected polymers.

Other considerations

Reduction of both IPMP and IBMP in juice to below their limit of quantitation with the mMUP system represents an important result, given the resistance of these compounds to more conventional additives and treatments (Pickering et al., 2006). We speculate this system will be similarly effective with other fruit and vegetable juices containing elevated MPs, given its efficacy across a wide pH range. The significant reduction in MPs due to the membrane alone (Figure 2) is unexpected, however likely to have limited commercial applicability; the 10 kDa pore size would be too small for industrial juice and wine processing. Further research from our labs is directed at immobilizing mMUP on solid supports for the extraction of MPs from wine and juice where membrane pore size can be varied. The immobilization of proteins on solid supports often results in increased protein stability, which would be advantageous in developing a system that may be regenerated for use multiple times. Preliminary results indicate reduced efficacy of the mMUP system in wine - possibly due to ethanol-induced changes in the protein's structure - and is also under active study in our labs.

While the reduction in IPMP and IBMP in wine using the various polymers is relatively modest, it may be sufficient to decrease their concentration to below the human detection threshold, particularly if combined with treatment of juice with mMUP prior to fermentation. Additionally, the sensory quality of polymer-treated MP-enhanced wines shows minimal differences compared to non-treated control wines (data not shown). On-going work in both the mMUP and polymer studies is assessing the specificity of these treatments for MPs by assessing their impact on other key volatile compounds in juice and wine.

CONCLUSIONS

Alkyl-methoxypyrazines are high impact, taint compounds found in some juices and wines, and are generally resistant to traditional remediation approaches. We developed and evaluated 2 treatments aimed at removing 3-isobutyl- and 3-isopropyl- 2-methoxypyrazine from grape juice and wine. The lipocalin mMUP was very effective, and selected polymers were moderately effective, at reducing these compounds in juice and wine, respectively. Current research is focused on optimising these tools for use on a commercial scale.

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