Exploration of Using Cyclodextrins as Novel Additives and Fining Agents in Wine

A thesis presented in fulfilment of the requirements

for the degree of Doctor of Philosophy

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Abstract

Volatile phenols are responsible for some off-odours in wine, for example, the objectionable smoky, ashy notes associated with smoke taint and the sweaty, horsey, barnyard character associated with *Brettanomyces*. Various strategies have been evaluated for use by the wine industry to ameliorate wines with excessive levels of volatile phenols. The current project aimed to explore cyclodextrins (CDs) as a novel approach to amelioration. CDs are a group of glucose-based oligosaccharides, which are characterised by their truncated cone structure, and hydrophilic outer surface and hydrophobic inner cavity. These properties offer CDs the ability to form inclusion complexes with hydrophobic guest molecules in aqueous environments, which has been exploited by various industries, including the food and beverage industries.

Chapter 1 comprises a review of literature concerning smoke taint and CDs. The limited knowledge regarding interaction between CDs and wine constituents is discussed, and then the research aims are stated.

Chapter 2 comprises a study into the formation of inclusion complexes between CDs and volatile phenols in model wine, confirmed by sensory and nuclear magnetic resonance (NMR) analysis techniques. A limitation associated with conventional headspace gas chromatography mass spectrometry (HS GC-MS), namely the interaction of CD with the normalising standard is identified. A new HS GC-MS method is therefore developed to address this issue by isolating the normalising standard from the sample mixture as an additional liquid phase that still shares the same headspace during sample extraction. The inclusion of volatile phenols by CDs is then

characterised using the validated method, demonstrating the reduction in headspace residuals of volatile phenols, following the addition of CD.

Chapter 3 explores the potential of CD polymers to remove volatile phenols from a model wine system. The preparation of hexamethylene diisocyanate (HDI) crosslinked CD polymers is described. Several parameters influencing the removal of volatile phenols by CD polymers are then characterised, for example, the time required to achieve adsorption equilibrium, isotherms (Langmuir and Freundlich models), adsorption capacity and reusability. A batch adsorption test described in this chapter achieved up to 77% removal of volatile phenols.

Chapter 4 adopts the methods and materials developed in previous chapters, and describes the impact of CDs and CD polymers, when used as treatments for smoke taint, on wine parameters. The treatments were applied to smoke affected must at different stages of fermentation. Reductions in the headspace concentrations of volatile phenols was observed following treatment, but certain aroma compounds were also lost, based on HS GC-MS and sensory analysis. The potential for a CD polymer to remove volatile phenol glycoconjugates was also investigated.

Chapter 5 summarises key findings from this thesis and discusses future directions for related research.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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Publications

This thesis comprises a collection of manuscripts prepared for submission to peer-reviewed scientific journals. Authorship statements are included in Chapters which incorporate a manuscript.

In Chapter 2: **Development and Validation of a Novel SPME GC-MS Method for Determining the Retention of Volatile Phenols by Cyclodextrins in Model Wine**; prepared for submission to the Journal of Agricultural and Food Chemistry.

In Chapter 3: **Removal of Volatile Phenols from Wine Using Crosslinked Cyclodextrin Polymers**; prepared for submission to the Journal of Agricultural and Food Chemistry.

In Chapter 4: **Amelioration of Smoke Taint in Wine by Cyclodextrins and Crosslinked Cyclodextrin Polymers**; prepared for submission to the Journal of Agricultural and Food Chemistry.

Symposia

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CHAPTER 1

Literature review and introduction

Literature review and introduction

In 2014, the Australian Research Council's Training Centre for Innovative Wine Production (ARC's TC-IWP) was initiated. The current PhD research project is one of the TC-IWP's first round of projects, which aimed to address wine production issues related to the changing climate [1]. The original goal of the current study was to use cyclodextrins (CDs) as a sink for grape sugars to produce lower alcohol wines. However, after unsuccessful initial trials using two bacterial strains to produce cyclodextrins in grape juice, it was decided that the project should re-direct its focus. The revised focus of this thesis instead aimed to exploit the chemical functionality of CDs to form inclusion complexes by retaining/removing wine constituents associated with taint and/or spoilage. In this way, the research still addresses challenges faced by the wine industry, i.e., smoke taint and microbial spoilage.

Climate change and wine production

Since the early 2000s, there has been a surge in the number of reports concerning the impact of climate change on the wine industry [2-4]. These reports review the key challenges faced by the wine industry today, including the increase in growing season temperature, large variation in annual precipitation, extreme weather events, and hazards related to these conditions, such as bushfires [5]. These conditions influence the wine industry through exerting pressure on the vegetative and/or reproductive

growth of the perennial grapevine [3], resulting in physico-chemical changes to grapes, which further interact with the processing techniques employed in winemaking [4].

A recent study [6] analysed viticultural records from 44 vineyard blocks with data going back as long as 115 years and showed an overall trend of increased growing season temperatures and earlier ripening dates for wine grapes from multiple regions in Australia. Similar trends have also been reported for wine regions in other countries, such as the Rheingau and Baden in Germany [7, 8], California [9], Bordeaux, Alsace and Rhône in France [10-12] and the northeast of Spain [13]. Some studies suggest there could potentially be disassociation between grape sugar ripeness and flavour ripeness [14, 15]. Since grape fermentable sugars are substrates for production of alcohol by the wine making yeasts to produce alcohol, high alcohol levels in wine has become a major concern for winemakers, particularly in warmer wine regions [9].

The increasing grape sugar levels at ripeness and the resulting higher alcohol content of wine have been confirmed by several studies in recent decades [4, 6]. From a viticulture perspective, this can be traced back to synergistic effects of temperature [16, 17], radiation [18], carbon dioxide [19, 20], and water availability [21, 22] on ripening. The high sugar content brings various challenges to winemaking. Firstly, the metabolic response of *Saccharomyces cerevisiae* to the osmotic pressure caused by high sugar levels is highly strain dependent [23, 24]. As fermentation progresses, the high alcohol content may increase the chance of stuck or sluggish fermentations, due to the toxicity

of ethanol to yeast [25, 26]. The high membrane pressure caused by high alcohol content also inhibits the growth of malolactic fermentation bacteria [27, 28]. As a result, the chance of producing unbalanced wine is increased [25, 29-31]. The sensorial impacts of high ethanol in wine mainly include masked/altered aromas [32-34] and an increased perception of astringency, bitterness, hotness and body on the palate [35-37]. Such sensorial characters can be undesirable when out of balance. Other drawbacks associated with higher levels of ethanol in wine relate to the social and health impacts of alcohol consumption [38].

As mentioned above, due to the potential disassociation of sugar ripening and flavour ripening in wine grapes, there are several aroma and flavour related challenges in wine production, induced by the changing climate. For example, with consideration of controlling the sugar levels of grapes, Sauvignon Blanc, Semillon or Cabernet Sauvignon grapes can be harvested earlier but may exhibit unripe aroma/flavour profiles characterised by undesired grassy and herbaceous characters. Methoxypyrazines, such as 2-methoxy-3-isobutylpyrazine (IBMP), 2-methoxy-3-isopropylpyrazine (IPMP) and 2-methoxy-3-sec-butylpyrazine (SBMP), have been identified as contributors of herbaceous aroma, with IBMP being the most abundant methoxypyrazine found in grapes [39, 40]. Several studies have shown an inverse correlation between the concentration of methoxypyrazines and grape sugar levels, after fruit reaches 50% sugar maturation [39, 41, 42]. The sensory thresholds of methoxypyrazines are extremely low, with IBMP being sensorially active at just 2 ng/L

[43]. As such excessive methoxypyrazines in grapes harvested earlier likely need amelioration before being used for winemaking.

The smoke taint problem

It has been well established that exposure to smoke can alter the sensory attributes of food, introducing smoky flavours [44, 45]. The same principle also applies to wine grapes, but grapevine exposure to smoke can lead to off-odours in wine, namely smoke taint, when the exposure is excessive [5]. This is another challenge faced by the wine industry resulting from environmental conditions attributed to the changing climate. Globally, an increasing number of bushfires have occurred in close proximity to prominent wine regions due to warmer and drier climate conditions. In some instances these have caused significant losses in fruit yield, quality and/or profit [46, 47]. As a consequence, smoke taint is increasingly concern for grape and wine producers not only in Australia, but around the world [48].

Early studies investigated the components of wood smoke that contribute to the characteristic aromas and flavours of smoke, and volatile phenols, such as guiacol, 4-methylguaiacol and syringol were found to be important volatile compounds [45, 49]. As a result, trace levels of these phenol compounds could be found in wines aged in toasted oak barrels [50]. A number of studies have been conducted to gain an understanding of the impact of smoke on grapes and wine, including both field and

fermentation trials [51]. Kennison and co-workers conducted one of the earliest studies to demonstrate the presence of smoke taint in wine and reported elevated concentrations of volatile phenols, i.e., guaiacol, 4-methylguaiacol, 4-ethylphenol, and 4-ethylguaiacol, in wines made from Verdelho grapes exposed to smoke post-harvest [5]. In her second study, Kennison and colleagues demonstrated the release of phenols during fermentation of smoke affected Merlot grapes, with guaiacol being detected at levels 10 times above the perception threshold level [52, 53]. Two subsequent field trials demonstrated grapevine exposure to smoke post-verainson had a more profound impact than smoke exposure during the earlier stages of growth, resulting in wines with more intense levels of smoke taint [54, 55]. Parker et al. [53, 56] further studied the perception threshold and sensorial importance of various volatile phenols in smoke affected wines, and suggested guaiacol, 4-methylguaiacol, syringol, 4-methylsyringol, *o*-, *p*- and *m*-cresol to be used as markers of smoke taint.

Volatile phenols were not the whole picture of smoke taint. *In vivo* glycoconjugation of guaiacol in grapes was found by tracing deuterium labelled guaiacol applied to grapevine leaves and fruit bunches [57]. Kennison, in her fermentation study with smoked Merlot fruit [52], suggested that the increase in volatile phenols during fermentation could be a result of acid and enzyme hydrolysis of glycosylated phenols in grape juice [58]. This phenomenon can potentially lead to significant under-estimation of smoke taint in wine, given the hydrolysis of bound volatile phenols is continuous after fermentation. Later, monoglucosides and disaccharide glucosides of

volatile phenols were identified using an HPLC-MS/MS method [59]. Synthesised *β*-D-glucosides of guaiacol and *m*-cresol were reported to have a smoky flavour on the palate in a sensory study [53]. In a bottle ageing study [60], researchers monitored volatile phenol concentrations in smoke affected wine for 3 years after bottling and reported mild accumulation of guaiacol as a result of continuous slow glucoside hydrolysis during that period. In a more recent study [61], small changes in volatile phenol levels were observed in smoke affected wines following 5-6 years of bottle ageing, however, changes in the sensory perception of smoke taint in some wines were attributed to the diminished intensity of fruit aromas and flavours, rather than any significant increase in volatile phenol concentrations.

To date, researchers have established reliable parameters for assessing smoke taint using different analytical techniques. Headspace gas chromatography-mass spectrometry (HS GC-MS) is suitable for quantification of free volatile phenols and has been used in several studies [5, 62-66], whereas liquid chromatography-mass spectrometry methods have been developed to quantify a number of volatile phenol glycoconjugates in other studies [53, 57, 59, 62, 67-69].

The amelioration of smoke taint in wine

Many methods have been trialled to remove smoke taint from wine, but only a few have given promising results, e.g., fining agents and reverse osmosis. Fudge et al. [70] used a technique that combined reverse osmosis and an adsorption resin. This technique preserved larger molecules in wine with a size exclusion membrane used in reverse osmosis, however, it still inevitably had impact on some of the desirable aromas, while the smoke taint can potentially return due to ongoing hydrolysis of the glucosides. In another study, Fudge and team [71] evaluated 13 different commercial fining agents to treat the smoked control sample. Two fining agents, an activated carbon and a synthetic mineral, were found to be effective in the removal of smoke derived volatile phenols, according to GC-MS quantification, and reduction in the intensity of smoke-related sensory attributes, with activated carbon being more effective than the synthetic mineral.

Several other studies have investigated the removal of volatile phenols from wine, particularly 4-ethylphenol and 4-ethylguaiacol, the volatiles associated with *Brettanomyces* spoilage. Reverse osmosis and hydrophobic adsorption resin were used in an early study that aimed to remove 4-ethylphenol and 4-ethylguaiacol from wine [72]. The results were comparable to that of Fudge et al. [70], which showed significant removal of the volatile phenols with some loss of desired aromas. Lisanti et al. [73] successfully removed 4-ethylphenol and 4-ethylguaiacol from red wine using commercial fining agents, such as activated charcoal, zeolite and PVPP. They reported significant decreases in the concentrations of target compounds following treatment with activated charcoal and PVPP, with charcoal being more effective. However, these

treatments negatively influenced the wine aroma profile. Larcher et al. [74] examined the ability of insoluble esterified cellulose to deplete volatile phenols. Esterified celluloses are used to produce transparent films, and were found to be able to reduce volatile phenol concentrations by around 32% (at levels up to 2.0 mg/L) without noticeable depletion of colour or tannins. However, it is not known to what extent this treatment might affect wine aroma profiles. The yeast lees of *Saccharomyces cerevisae* were found to have a sorptive effect on 4-ethylphenol and 4-ethylguaiacol [75]. Polyaniline based polymers were synthesised and found to be capable of removing 4-ethylphenol and 4-ethylguaiacol from model wine, with the presence of 2 g/L polyphenols [76]. Molecularly imprinted polymers, prepared from 4-vinylpyridine using divinylbenzene-80 as a crosslinker, were used to remove 2,4,6-trichloroanisole, the compound that causes cork taint from wine [77]. The study also investigated the removal of 4-ethylphenol and 4-ethylguaiacol and found the molecularly imprinted polymer gave higher adsorption rates for the volatile phenols compared to non-imprinted polymer, achieving 89% retention. However, the loss of desirable aroma compounds was as much as 95%.

It can be summarised that smoke taint treatment is better the earlier it is applied. More options are available for the wine producer during grape processing, whereas when the taint is present in wine, most current treatments especially direct adsorption, are based on hydrophobic interactions and lack of selectivity, causing loss of other wine attributes. An earlier report [78] suggested that a group of carbohydrates, namely cyclodextrins

(CDs), can form inclusion complexes with 4-ethylphenol and 4-ethylguaiacol in red wine, resulting in reduction of volatility of the volatile phenols. This group of carbohydrates raised interest for the current project.

Cyclodextrins

Carbohydrates are a ubiquitous group of compounds, which exist in many forms, including glucose, sucrose, starch and cellulose [79]. They provide substrates and energy for metabolic activities of plants and animals, alike. The fundamental principle of wine making is the conversion of carbohydrates or grape fermentable sugars (glucose and fructose) to ethanol by yeast [80]. The discovery of cyclic oligosaccharides, known as cyclodextrins, excited many researchers [81].

A brief history of cyclodextrin

Cyclodextrins, hereafter referred to as CDs, are cyclic oligosaccharides containing α-1,4 linked glucopyranose subunits [82]. The first recorded isolation of cyclodextrin from degraded starch is thought to have been in the late 19^{th} century [83]. Records showed the isolated crystalline compounds clearly exhibited non-reducing characters and resistance to acid hydrolysis, similar to that of cellulose [84]. These compounds are suspected to have been α- and β-CDs [83]. Later, Austrian scientist Franz Schardinger studied extensively the cellulose-like compounds isolated from potato starch and laid

down much of the fundamental knowledge that exists today on CDs [83, 85, 86]. It was suggested that the compounds were oligomers produced by bacterial activity using starch as a substrate. The microorganism that was principally responsible for producing the oligomers was isolated and named *Bacillus macerans* [82, 86, 87]. For years afterwards, "Schardinger dextrins" was the designated name for the compounds that are today referred to as cyclodextrins.

In the 1930s, Freudenberg's team characterised the α-1,4-glycosidic bonds present in CDs, using acetolysis and hydrolysis methods [88, 89]. They also proposed the cyclic conformation of the α-1,4-linked molecules with a central cavity and developed the earliest enzymatic production and isolation protocols for pure CD fractions. Another type of CD, larger than those previously identified, was discovered and named γ-CD [90]. Subsequent work by Freudenberg and Cramer [91] explained the structure of γ-CD and suggested the possible existence of larger CDs. This was confirmed by French and colleagues [92], with results from their studies confirming the molecular size and structure of the larger δ-, ε-, ζ-, and η-CDs.

According to Szejtli [93], there were some early reviews by French (no online record of the literature) that provided misleading information on the toxicity of CDs. In the review, an unpublished study suggested that diets containing purified β-CD fed to rats led to them rejecting food, while those animals that did eat, died within a week. Szejtli [93] argued that the putative toxicity of CDs could not be judged with the absence of

key parameters, such as the presence of residual organic solvents and prescribed doses. A number of CD toxicity studies that were conducted later showed no evidence of toxicity in rats, dogs, monkeys or human at doses up to 10 g/kg per day [93-95]. Therefore, CDs remained of interest to scientists, with lab-scale methods for the production and analysis of CDs being developed by the 1960s.

Enzymatic production of CDs using bacteria and starch has been achieved on an industrial level [96]. Studies into the applications of CDs and their derivatives fostered CD-related research by various industries. Numerous patents have been filed for industrial production and utilisation of CDs in the food, pharmaceutical, flavour, and cosmetic industries [82, 83, 97, 98]. By 2003, it was estimated that more than 26,000 studies on CDs had been published [99], while the cost of CDs has dropped from US$2,000/kg to just $5/kg between the 1960s and the late 1990s [93].

Basic cyclodextrin chemistry

The most common natural CDs are α-, β-, and γ-CDs, with 6, 7 and 8 glucopyranose subunits, respectively [82]. The cyclic structure of glucopyranose subunits in the molecule is achieved by the formation of α-1,4 glycosidic oxygen bonds (Figure 1). CDs with larger numbers of glucopyranose subunits also exist naturally, but are less common, whereas there are no CDs with less than 6 building blocks, due to steric hinderance [100]. From a 3-dimensional perspective, the cyclic structure of CDs

resembles a truncated-cone (Figure 2), with the primary hydroxyl groups situated at the narrow end of the cone and the secondary hydroxyl groups at the wider end. On the exterior surface of the cone structure, the C-1, C-2 and C-4 hydrocarbon groups are pointed outwards, offering a hydrophilic character and relatively high water solubility to CD's external surface [101]. The interior surface of the molecule is formed by the non-bonding electrons of the glycosidic oxygen bridge and the hydrocarbon groups of the C-3 and C-5 positions, which exhibit much lower polarity [85, 101]. Therefore, in an aqueous environment, the interior of a CD molecule can be considered hydrophobic, while the molecule is readily soluble, due to its hydrophilic exterior. As a result of these amphipathic characters, CDs are known to be able to form inclusion complexes with large number of hydrophobic compounds, encapsulating the guest compounds in their cavity [97]. The success of encapsulation depends on the size, shape and polarity of both the host and guest compounds [102]. Table 1 provides some basic information about the cavity of α-, β- and γ-CDs [103]. It is this ability to form inclusion complexes that provides industrial applications for CDs; for example, CDs can be used for delivering flavours, drugs, removing or retaining unwanted odours and tastes, and preserving colour [83, 97, 98]. Among the various CDs, β-CD has relatively low solubility and particularly high inclusion capability [104]. This may be due to the formation of a hydrogen bond between the C2-OH group of the glucopyranose subunit and the C3-OH group of the adjacent subunit, which contributes to a complete and rigid arrangement [81]. This secondary belt does not exist in other CDs, due to conformational distortion [93].

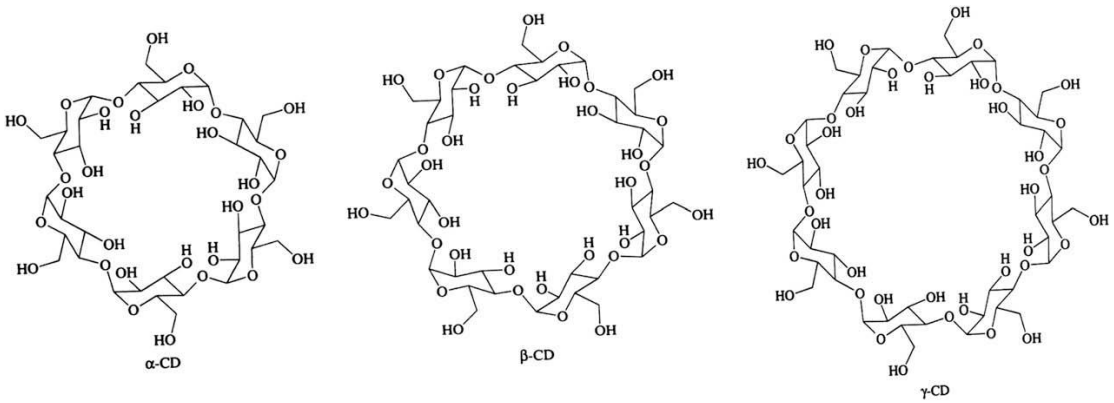

Figure 1. Chemical structures of α-, β-, and γ-CDs [97].

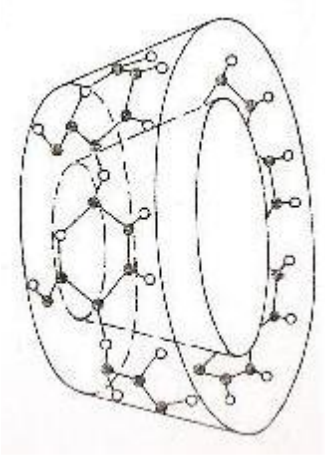

Figure 2. The truncated cone shape of β-CD [105].

Table 1. Structural features of α-, β- and γ-CDs [103].

Features	α-CD	β-CD	γ-CD
Number of glucopyranose subunits	6	7	8
Molecular weight	972	1135	1297
Solubility in water (g/100 mL) at 25℃	14.5	1.85	23.2
$[\alpha]_D$ at 25℃	150 ± 0.5	162.5 ± 0.5	177.4 ± 0.5
Cavity diameter (Å)	4.7-5.3	6.0-6.5	7.5-8.3
Height of torus (Å)	7.9 ± 0.1	7.9 ± 0.1	7.9 ± 0.1
Diameter of outer periphery (Å)	14.6 ± 0.4	15.4 ± 0.4	17.5 ± 0.4
Approximate volume of cavity (Å)	174	262	427

Cyclodextrin glycosyltransferases

CDs are produced naturally by various microorganisms that use α-glucan substrates and enzymes categorised as cyclodextrin glycosyltransferases, or CGTases [106]. CGTase enzymes are capable of catalysing hydrolysis of large α-glucan molecules, such as starch, and assimilating the cleaved glucose chains into cyclic rings. The most common CGTase producing microorganisms are bacteria, especially those of the *Bacillus* genus, such as *B. macerans*, *B. circulans*, *B. megaterium*, *B. coagulans*, *B. sphaericus*, and alkalophilic *Bacillus sp*. [107]. To date, around 50 microorganisms have been identified and characterised as producers of CGTases and CDs, with more than a dozen being found quite recently [108]. For most of these microorganisms, CGTase is an extracellular enzyme, catalysing several reactions during the microbial metabolism of carbohydrates, including cyclisation, coupling, disproportionation and hydrolysation [109]. For cyclisation, CGTase cleaves the α-1,4-glucans at donor sites and acceptor

sites, and then performs an intramolecular trans-glycosylation at the donor site to yield the cyclic CD structure. Coupling can be considered as the reverse reaction of cyclisation, during which the CD rings are opened and transferred to acceptors through intermolecular trans-glycosylation, forming linear α-1,4-glucan chains. The disproportionation reaction is similar to that of the coupling, except the transferred linear oligosaccharides are not formed from an opened CD. Disproportionation is encouraged when the substrate's degree of polymerisation is less than 6, i.e., the minimum number of subunits required to form a CD ring. CGTases are also known to be able to hydrolyse CDs when the acceptors for intermolecular trans-glycosylation are water molecules.

Although the metabolic pathways of CD production remains unclear [110], it is known that CGTases and α-amylase are key enzymes used by microbes during the degradation process of starch. Some studies suggest that CDs are intermediate products in the microbial effort to metabolise sugars in the starch molecules [110, 111]. A group of researchers [112] proposed a hypothesis that CGTases evolved from α-amylase and provided evidence that CGTases work in concert with α-amylase for an efficient saccharification of starch. A theory was proposed to explain the number of glucopyranose subunits in CDs that CGTases could identify 6, 7 or 8 glucopyranose units from the non-reducing end of the linear α-1,4-glucans and cleave the adjacent glycosidic bond of these units to donate the reducing end, which is to be linked to the non-reducing end by cyclisation [113]. However, work by Terada et al. [114] using

CGTases from alkalophilic *Bacillus* sp. A2-5a suggests that the initial cleaving activity may not be specifically targeting α-, β- and γ-CDs. A degree of polymerisation of glucopyranose in the initial products between 6 and 60 was found through anion exchange HPLC analysis, with the larger CDs being more prone to coupling reactions, which drives the equilibrium towards production of smaller α-, β- and γ-CDs.

Production of cyclodextrins

In most cases, CGTases from microorganisms can be differentiated in many ways, including their structure, the proportion of different end products, optimal conditions of reaction, and the efficiency of usage of substrates. Previous studies have used various microorganisms and culture conditions for producing CGTases and CDs. As a result, the information is somewhat inconsistent due to the high volume of publications, nominated microorganisms and variables involved in experimental conditions. So far, a comprehensive review of these studies is lacking. Several key publications are summarised in Table 2.

Table 2. CD producing microorganisms and their optimal growth conditions.

Species	**Strain**	**Product**	**pH**	**Temperature (°C)**	**Carbon source**	**Reference**
B. agaradhaerens	DSM8721, DSM9948, LS-3C, WN-I	*β*-CD	9.0	55 – 60	starch, maltodextrin	[115, 116]
B. circulans	ATCC9995, ATCC21783, No. 38-2, C31, E192, No. 8, DF9R, 251, A11	*α*-, *β*-CD	4.5 – 9.5	30 – 65	amylose, cyclodextrin, glycogen, maltodextrin, starch	[117-125]
B. alkalophilus	B-3103, BA-4229	*β*-CD	8.0 – 9.0	45 – 65	starch	[126]
B. autolyticus	11149	*β*-CD	5.0 – 6.0	60	starch	[127]
B. cereus	NCIMB13123	*α*-CD	5.0 – 7.0	40	starch, glucose, dextrin	[128]
B. clarkii	7384, 7364	*γ*-CD	10.0		starch	[129]
B. firmus	290-3, 7b, NCIMB5119, 37	*α*-, *β*-CD	5.5 – 7.0	50 – 65	starch	[130-134]
B. coagulans	BIO-13m	*α*-CD	6.0	30 – 70	starch	[135]
B. lentus	N/A	*α*-, *β*-CD	6.5 – 8.5	45 – 55	starch	[136]
B. licheniformis	B-4025, BIO-9m	*α*-, *β*-CD	5.0 – 6.5	30 – 65	starch	[126, 137]

Bacillus sp.	BL-31, No. 5, IT25, AL-6, 1011, ATCC39612, G1, INMIA1919, 17-1, B1018, INMIAT4, BE101, TS1-1, KC201, ATCC21595	*α*-, *β*-, *γ*-CD	4.0 – 10.0	30 – 70	starch	[138-145]
B. megaterium	No5	*α*-, *β*-CD	5.0 – 7.0	37 – 60	starch	[146-148]
B. pseudalcaliphilus	20RF, 8SB	*β*-CD	6.0 – 9.0	55 – 60	starch	[149, 150]
B. subtilis	313, NA-1	*γ*-CD	8.0	65	starch	[151]
G. stearothermophilus	TC-60, N2, TC-91, ET1	*β*-, *γ*-CD	5.0 – 6.0	40 – 80	starch, cyclodextrins, amylose	[152-154]
K. pneumonia	M5, M5a1	N/A	6.8	N/A	starch	[155]

Starch is the most commonly used and optimal carbon source for both industrial and lab CD production [156, 157], with differences in CGTase activity observed for different types of starch [158]. Several studies have reported that some monosaccharides and oligosaccharides, including D-glucose, D-xylose and acarbose have an inhibitory effect on the production of CD [159-162]. This may be because these compounds act as good acceptors with reducing features in the coupling and disproportionation reactions, which are more likely to result in intermolecular trans-glycosylation with the non-reducing ends of glucan chains [163]. However, as mentioned above, the preference of culture conditions by microorganisms for CGTase and CD production are very strain specific. In the study by Jamuna and colleagues [128], a glucose based carbon source was preferentially used by *Bacillus cereus* as a substrate for microbial CGTase synthesis. This result contradicts most other studies that report suppression of CGTase and CD synthesis from mono- and disaccharides.

There are two industrial processes for producing CDs, known as the solvent process and the non-solvent process. The difference between the two is whether or not organic complexing agents are used to selectively precipitate the target CD, thereby continuously driving the equilibrium towards CD production [164]. The non-solvent process is more suitable for production of the less soluble β-CD, without the use of organic solvents [165].

Figure 3 describes the steps involved in the solvent and non-solvent based approaches to CD production [164]. In the solvent process, the raw material (starch) is usually liquefied and cleaved by jet cooking. This can also be achieved with heat de-activated α-amylase, hydrochloric acid, and mechanical decohesion [106]. CGTase and the organic complexing agent, usually toluene, ethanol, or acetone, are then added to the cooled substrate for enzymatic CD production. Once the reaction stops, the precipitated CD-solvent complex is isolated, washed, and cleaved (via heating or liquid-liquid extraction). The purified CDs are then crystallised and isolated. As for the non-solvent process, β-CD production is carried out without the addition of organic solvent. Instead, the pH of the reaction mixture is typically lower, with amylase added at the end of the process to convert the residual carbohydrates to smaller sugars to assist purification of CD [165].

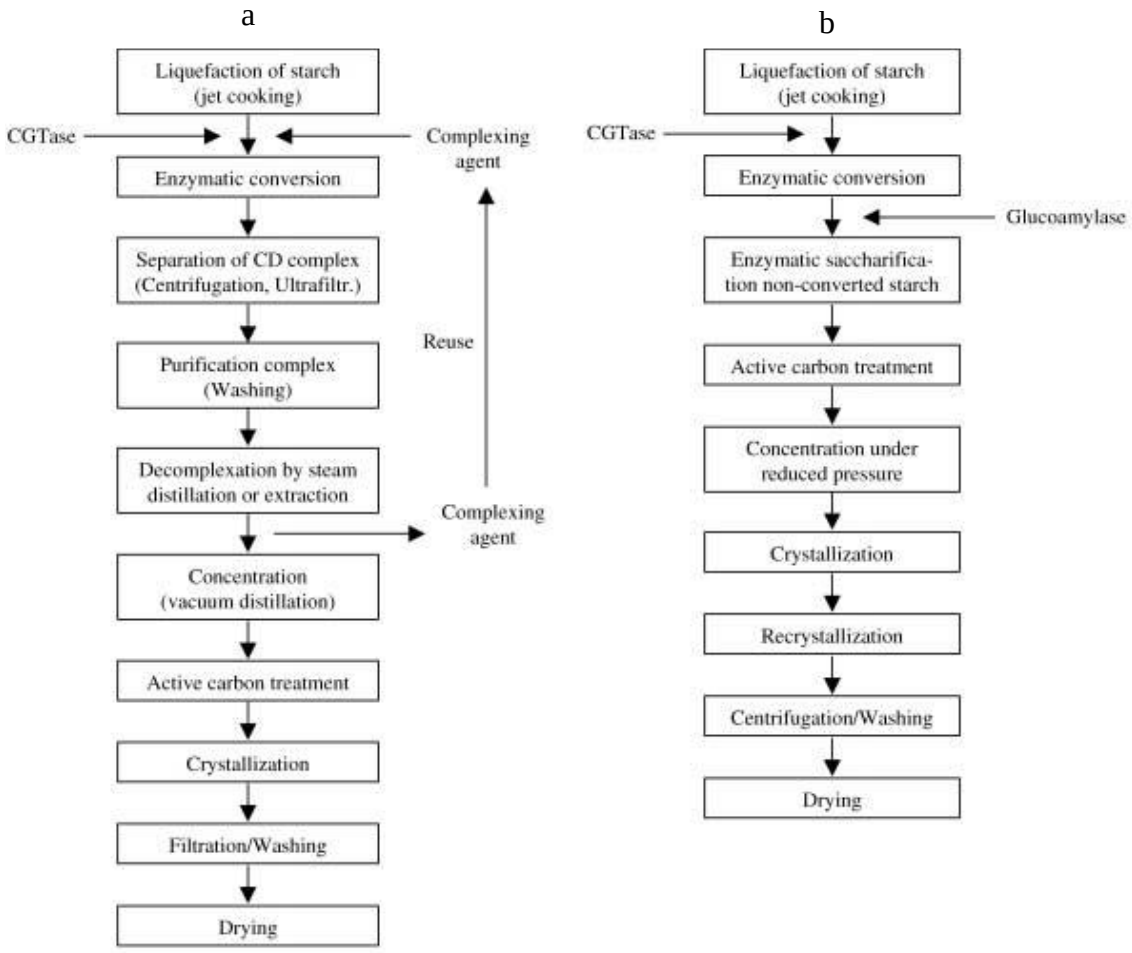

Figure 3. Flowcharts showing (a) solvent and (b) non-solvent processes for CD production [164].

Cyclodextrin inclusion complex

An inclusion reaction is described as a reaction in which a molecule is encapsulated by another molecule or set of molecules [166]. In an aqueous solution of CD, water molecules occupy the hydrophobic and less polar CD cavity [93]. This is energetically unstable due to the polar and non-polar interaction [105]. Some studies [167, 168] found that the polarity of β-CD is similar to that of ethanol. Therefore, hydrophobic molecules of suitable size present in the solution will

preferentially replace the water molecules in the cavity, to yield a more stable heterogeneous hydrophobic encapsulation matrix [169]. Figure 4 depicts the formation of CD inclusion with *p*-xylene [103].

The most common CD complexation consists of one guest molecule and one host molecule [105]. However, guest-to-host ratios of 1:2, 2:1 or of even more complicated arrangements are also found [105]. As a result, CDs can host a large range of chemicals through hydrophobic interaction, van der Waals force, hydrogen bonding, or London dispersion forces [102]. Challa and co-workers [170] explained that the type of CD, size of CD cavity, guest hydrophobicity, the size, pH and ionisation status of the solution, and temperature are all key factors influencing the formation of CD inclusion complexes.

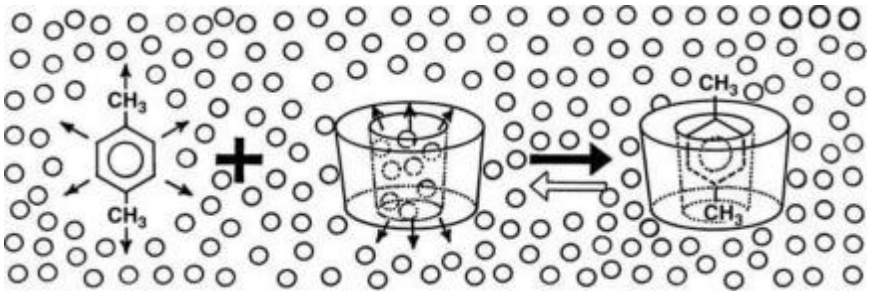

Figure 4. Formation of an inclusion complex between *p*-xylene and cyclodextrin in an aqueous solution [103].

Generally, several changes in the host and guest molecules can be observed with encapsulation [97, 103]:

1. The proportion of dissolved guest molecules significantly increases, while the concentration of dissolved CDs may or may not decrease, depending on the solvent.

2. Spectral properties of the encapsulated molecule, e.g., UV spectral bands, fluorescence and NMR spectra, can be modified.
3. Reactivity of the encapsulated molecule decreases.
4. Volatility of encapsulated molecule decreases.
5. Hydrophobic guests become hydrophilic, changing its mobility in chromatographic analyses.

Use of cyclodextrin in food and beverage industries

The food industry has been using CD as an additive for decades. Generally, the addition of CDs and CD complexations serve the following purposes: (1) preservation of food components from light or air induced degradation [97]; (2) removal or masking of unwanted components, such as off flavours, bitter tastes or cholesterol [171, 172]; (3) stabilisation of volatile fragrances or colouring components [173]; (4) solubilisation and delivery of selected desired nutrients or flavour compounds [174].

One problem that the juice industry has was solved using CDs is browning caused by polyphenol oxidase, which is released after mechanical damage of fruit [102]. It was shown that CDs could protect phenolic compounds from enzymatic browning through inclusion complexation. A series of studies into the anti-browning effect of different CDs in apple, peach, pear and banana juice were conducted by Spanish researchers [173, 175-177]. It was shown that modified β-CD gave the best results as a secondary antioxidant by preserving the primary antioxidant, e.g., ascorbic acid, from oxidation, which prevented enzymatic browning of apple and pear juice. In contrast, α-CD had the highest affinity and best preserving effect in peach juice. In terms of banana juice, CDs

were unable to form complexes with polyphenols, and instead promoted browning. Just 0.2% of *β*-CD was found to stabilise the natural food colouring compounds used in tomato ketchup, with colour persisting despite longer cooking times than for the corresponding control [103]. A colour enhancing effect was also reported by De and Vinho [78] when *β*-CD was added to red wine.

Another common problem encountered by the juice industry is the occurrence of bitter tastes, which cause rejection of products, especially in orange juice [97]. The bitterness detected in orange juice tends not to exist in the fresh product, but instead develops from flavonoid and limonoid components during storage, depending on temperature and pH [171]. Shaw and colleagues [171] reported the effectiveness of *α*- and *β*-CDs in removing bitterness in navel orange and grapefruit juice. It was shown that *β*-CD was more effective in encapsulating flavonoids, whereas both CDs were equally effective in encapsulating limonoids.

Use of cyclodextrin in the aroma and flavour industries

The literature provides several examples of essential oils and volatiles that can form complexes with CDs [85, 97]. There are two ways in which CD inclusion complexes are exploited: (1) using the crystalline guest-CD complex to achieve a gradual release of an aroma compound; or (2) adding CD into a sample to mask or remove an unwanted aroma. It was found that complexations in crystalline form can achieve a much longer release of aroma compounds than in aqueous solutions [178]. On the other hand, odours can be removed through formation of inclusion complexes in aqueous solution. A number of studies have shown CDs can encapsulate a wide variety of compounds within the cavity of their truncated cone structure [85, 93, 97, 174, 179-183].

These findings have lead to important applications of CDs in the food, beverage and flavour industries [82, 97, 98, 170]. Currently, α-CD and β-CD are listed as novel foods in the US, EU and Japan, whereas γ-CD is approved novel food in the EU, but its approval status varies in the US and Japan. In Australia and New Zealand, both α-CD and γ-CD are listed as novel foods by the Australia New Zealand Food Standards Code, whereas β-CD is listed as a food processing aid. A recent report suggests there have been more than 200 foods produced with CDs, as ingredients [85].

It is worth mentioning that only a few studies have investigated CD applications to fermentation, and any subsequent influence on wine aroma profiles. An early study [184] indicates that β-CD addition improved the fermentative rate of *Saccharomyces cerevisiae*. This experiment was repeated later by Okolie [185], yielding similar results. Liang and Wang [186] found β-CD increases the ethanol tolerance of *Saccharomyces cerevisiae* using starch as a substrate. A study in bread baking also showed that the presence of trace amounts of β-CD improved the volume of bread, which is also an indication of increased fermentation rates [187].

Cyclodextrin polymers

The complexation between CDs and various groups of aroma compounds has been widely studied, however, there is a scarcity of available literature on the potential uses of CDs in masking off-odours in wine. A realistic barrier to the application of CDs in wine would be the fact that the winemaking process is legally regulated in many countries, and for now, CDs are not listed as permitted winemaking additives. In recent years, there have been increasing interests to develop

insoluble CD polymers to broaden CD applications in various areas, which may facilitate CD applications in winemaking. CDs can be polymerised with other molecules, known as crosslinkers. These compounds contain at least two functional groups that can react with the hydroxyl groups in CD glucopyranose subunits, linking the molecules into a chain structure [188]. These polymers were found to be useful in removing phenols and dyes from waste water. Several CD crosslinkers have been studied. Crini et al. [189] crosslinked *β*-CD with epichlorohydrin and reported sorption capability of the polymer towards benzene derivatives, such as phenol, *p*-nitrophenol, and benzoic acid. Yamasaki et al. [190] used hexamethylene diisocyanate (HDI) and toluene-2,6-diisocyanate as crosslinkers, and showed adsorption of cresols, phenol and xylenol from waste water by the resulting polymers. Binello et al. [191] produced a range of CD based polymers, using chitosan and cellulose as crosslinkers, and subsequently used them to suppress bitterness from limonoids and flavonoids. Other studies used chitosan and citric acid to form phenol absorbing CD polymers in water pollutant removal experiments [192-195].

Aims of the current study

Microbial synthesis of CGTase and CD under winemaking conditions was conducted at the beginning of the project under the hypothesis that CDs can be produced as a sink for grape fermentable sugars in the production of lower alcohol wine. Commercial CGTase was also used in grape juice to produce CDs. This initial test was a foreseeable challenge and didn't yield any successful results, given only one literature reported production of CGTase and CDs from glucose based substrate [128], whereas considerable literature indicated a suppressing effect from mono-

sugars [159, 160, 162]. This may reflect the acidic nature of grape juice, with most of the known CGTase and CD producing microorganisms preferring neutral or alkaline environment [143].

The aims of this study were therefore adjusted to focusing on the potential for CD inclusion complexes to form with wine constituents, so as to remove off-odours associated hydrophobic volatile phenols, using analytical methods, e.g., gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR). The use of polymerised CDs, as adsorption resins, to remove volatile phenols from wine will also be investigated. Another aim of this thesis is to evaluate the potential impact of these treatments on the fermentation process, wine aroma profile, colour, and sensory attributes, providing key information for potential CD applications in the wine industry.

CHAPTER 2

Development and validation of a novel HS-SPME GC-MS method

Statement of Authorship

Title of Paper	Development and Validation of a Novel SPME GC-MS Method for Determining the Retention of Volatile Phenols by Cyclodextrins in Model Wine
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted w ork w ritten in manuscript style
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Principal Author

Name of Principal Author (Candidate)	Chao Dang
Contribution to the Paper	Designed and conducted experiments; collected, processed, analysed, and interpreted data; drafted and edited manuscript.
Overall percentage (%)	70%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 07-11-18

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Kerry L. Wilkinson
Contribution to the Paper	Designed experiments; analysed and interpreted data; edited manuscript; co-author.
Signature	Date 19/12/18

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Contribution to the Paper	Designed experiments; analysed and interpreted data; edited manuscript; co-author.
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Name of Co-Author	Dennis Taylor		
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Contribution to the Paper			
Signature		Date	

Please cut and paste additional co-author panels here as required.

Development and Validation of a Novel SPME GC-MS Method for Determining the Retention of Volatile Phenols by Cyclodextrins in Model Wine

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ABSTRACT

Volatile phenols exist in wine and can be markers for *Brettanomyces* and smoke taint off-odors. Cyclodextrins (CDs) were found to be able to form inclusion complexes with volatile phenols. Cross peaks on 2D ^{1}H ROESY NMR spectra, together with sensory analysis, showed insertion of volatile phenols into the β-CD cavity, resulting in reduction of guest molecule volatility. However, a conventional headspace solid phase microextraction method using an isotopically labelled normalizing standard failed to quantify the residual volatile phenols by gas chromatography-mass spectrometry due to inclusion of the standard by the CDs. A new method involving an additional liquid phase was developed and validated for quantitation of volatile phenols in the presence of CDs. The retention of eight volatile phenols by α-, β-, and γ-CD was subsequently studied.

Keywords: *Brettanomyces*, cyclodextrins, headspace solid phase microextraction, gas chromatography-mass spectrometry, smoke taint, volatile phenols, wine

INTRODUCTION

Volatile phenols are an important group of wine aroma compounds. Some volatile phenols, for example guaiacol, 4-methylguaiacol, vanillin and eugenol, are routinely identified in wines aged in oak barrels, as a consequence of thermal degradation of lignin during the toasting process of cooperage. These volatile phenols contribute the smoky, vanilla and clove characters often associated with oak maturation[1-2]. However, volatile phenols are also responsible for certain off-odors in wine. *Brettanomyces* and/or *Dekkera* spoilage can result in the accumulation of 4-ethylguaiacol and 4-ethylphenol in wine, which at elevated concentrations can impart undesirable barnyard, sweaty, medicinal and/or horsy notes[3]. Guaiacols, cresols and syringols have been identified as markers of smoke taint, i.e. the objectionable smoky, ashy character observed in wines made from grapes exposed to bushfire smoke for prolonged periods of time[4-6].

The wine industry has long sought strategies for mitigating various wine off-odors, including those attributable to volatile phenols. Most amelioration strategies have involved the addition of sorptive materials such as yeast lees[7-8], activated carbon[9], and polyvinylpolypyrrolidone[10] to remove taint compounds from wine. However, these materials can also bind volatiles responsible for desirable wine aromas and flavors. Reverse osmosis fractionation of wine prior to solid phase adsorption treatment has been used to achieve more selective removal of taint compounds[11-12]; while novel sorbents, including esterified cellulose[13], polyaniline-based compounds[14] and molecularly imprinted polymers[15-16] have also been evaluated for the amelioration of taint due to the presence of volatile phenols in wine.

Cyclodextrins (CDs) are cyclic oligosaccharides comprising α-1,4-linked glucose units, the most common being α-CD, β-CD and γ-CD, which comprise 6, 7 and 8 glucose units, respectively[17] (Supplementary data). The spatial arrangement of sugars gives CDs a characteristic ring shape, whereby the hydrophilic outer surface affords water solubility, while the hydrophobic inner cavity

enables the formation of host-guest inclusion complexes with various molecules, including volatile phenols[17-18]. The encapsulation of volatiles has been exploited by numerous industries, including those involved in the production of foods, beverages and cosmetics, to stabilize, preserve and/or mask aromas, flavors and fragrances[19-22]. However, to date, there are few studies concerning the use of CDs in winemaking. The potential for *β*-CD to reduce the intensity of off-odors associated with *Brettanomyces* spoilage of red wine has been demonstrated[23]. *β*-CD has also been used to extract stilbenes, flavonols and flavan-3-ols from grapes and pomace[24]. A key aim of the current study was therefore to determine whether or not the volatile phenols associated with *Brettanomyces* spoilage and smoke taint can form inclusion complexes with CDs, so as to mitigate their impact on wine sensory properties. In order to achieve this aim, the concentration of volatile phenols should be determined before and after the addition of CD to wine. Headspace solid-phase microextraction (HS-SPME) has been shown to be a fast and effective sampling method for gas chromatography-mass spectrometry (GC-MS) analysis and it has been used extensively for determination of volatile compounds in wine[25-26], including volatile phenols[27]. However, quantitative analysis relies on the addition of an appropriate normalizing standard, for example an isotopically labelled analogue in the case of stable isotope dilution assays[28], and the standards are equally subject to treatments on the sample mixture, such as the addition of CDs. Whereas conventional HS-SPME employs a three-phase extraction system, comprising the sample, its headspace and the SPME fiber, in the current study, an additional liquid phase was introduced to overcome interactions between CDs and normalizing standards. This was achieved by inserting a glass ampoule containing the internal standard solution into the headspace vial, prior to analysis. This study describes the development and validation of a novel four-phase HS-SPME GC-MS method for determining the retention of volatile phenols by CD in model wine.

MATERIALS AND METHODS

Chemicals. Analytical grade volatile phenols (guaiacol, 4-methylguaiacol, 4-ethylguaiacol, 4-ethylphenol, *o*-cresol, *m*-cresol, *p*-cresol and eugenol) and deuterated NMR solvents (d_6-ethanol, D_2O and DCl) were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Deuterium-labelled internal standards (d_3-guaiacol, d_3-4-methylguaiacol and d_4-4-ethylphenol) were sourced from CDN Isotopes (Pointe-Claire, Quebec, Canada). Analytical grade ethanol, tartaric acid and sodium hydroxide were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Food grade (>98% purity) α-, β-, and γ-CDs were supplied by IMCD Group (Adelaide, SA, Australia). Model wine was prepared by dissolving tartaric acid (5 g/L) in aqueous ethanol (12% alcohol by volume) and adjusting the pH to 3.5 by dropwise addition of 1M sodium hydroxide. Stock solutions of standards were prepared volumetrically in absolute ethanol and stored at –20 ºC, with working solutions prepared in model wine and stored at 4 °C.

Nuclear Magnetic Resonance (NMR) Analysis. Complexation of volatile phenols by CDs was investigated by 2-dimensional nuclear magnetic resonance rotating frame Overhauser effect spectroscopy (^{1}H 2D ROESY). Samples were prepared by adding volatile phenols (10^{-3} mol/L) and CDs (10^{-2} mol/L) to deuterated model wine (i.e. 12% d_5-ethanol in D_2O, pD adjusted to 3.5 by dropwise addition of DCl). Spectra were recorded with an Agilent DD2 600 MHz spectrometer filled with a cryoprobe (Agilent Technologies, Santa Clara, CA, USA) operating at 600 MHz with a delay time of 300 ms.

Sensory Analysis. Triangle tests were performed to investigate the sensory impact of volatile phenol retention by β-CD, using previously reported methodology[29]. The panel comprised 38 postgraduate Wine Business students (8 male and 30 female, aged between 21 and 50 years) from the University of Adelaide. Model wines were presented in three-digit coded, covered XL5 wine glasses, using a balanced, randomized presentation order comprising all possible configurations, i.e. ABB, ABA,

AAB, BAA, BAB and BBA, where A denotes model wine spiked with volatile phenols and B denotes model wine spiked with volatile phenols and treated with *β*-CD (10 g/L). Panelists evaluated two brackets of wines: one representing smoke taint, comprising model wines spiked with guaiacol, 4-methylguaiacol and *p*-cresol (1 mg/L each); and one representing *Brettanomyces* spoilage, comprising model wines spiked with 4-ethylphenol and 4-ethylguaiacol (1 mg/L each). Panelists smelled but did not taste wines, then identified the sample in each bracket that was considered to be different.

GC-MS Instrumental Analysis. Analysis of samples was performed with an Agilent GC-MS system (Santa Clara, CA, USA) comprising a 7890A gas chromatograph equipped with a Gerstel MPS autosampler (Mülheim, Germany) coupled to a 5975C mass selective detector. A DB-Wax column (60 m, 0.25 mm i.d., 0.25 µm film thickness, Agilent J&W, Folsom, CA) was used for separation. The carrier gas was helium (BOC Gas, Adelaide, SA, Australia), at a constant flow of 1.5 mL/min. The inlet temperature was set at 240 °C and the oven temperature started at 40 °C for 1 min, increased to 200 °C at 5 °C/min and was held at 200 °C for 5 min, before being increased to 250 °C at 10 °C/min and remaining at 250 °C for 10 min, giving a total run time of 52 min. The transfer line was set at 230 °C and positive ion electron impact spectra at 70 ev were recorded in the range *m/z* 25 to 215 for scan runs. For quantification of volatile phenols, mass spectra were recorded in Selected Ion Monitoring (SIM) mode. The ions monitored in SIM mode were: *m/z* 109, *124* for guaiacol; *m/z* 109, *127* for d_3-guaiacol; *m/z* 123, *138* for 4-methylguaiacol; *m/z* 126, *141* for d_3-4-methylguaiacol; *m/z* 77, 90, *108* for *o*-cresol; *m/z* 122, 137, *152* for 4-ethylguaiacol; *m/z* 77, *107* for *p*-cresol; *m/z* 79, *108* for *m*-cresol; *m/z* 77, *122* for 4-ethylphenol; *m/z* 77, *126* for d_4-4-ethylphenol; and *m/z* 149, *164* for eugenol; with italicized ions used for quantitation. Volatile phenol concentrations are reported as relative peak areas (RPA), i.e. as the ratio of the peak area of the analyte (A_s) relative to the peak area of the isotopic standard (A_i).

Three-Phase HS-SPME GC-MS Analysis of Volatile Phenols with CD Addition. The HS-SPME GC-MS method developed by other studies for determination of volatile phenols in wine[27] was initially employed in the current study to determine changes in volatile phenol levels following CD addition in model wine. Model wine was spiked with guaiacol, 4-methylguaiacol or 4-ethylphenol at 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75 and 2 mg/L, and an aliquot of normalizing internal standard solution (containing 100 mg/L each of d_3-guaiacol, d_3-4-methylguaiacol and d_4-4-ethylphenol) added, prior to SPME GC-MS analysis to develop calibration functions (Supplementary Table S1). High linearity was observed over the working range, with correlation coefficients greater than 0.9995. A preliminary experiment using the above SPME GC-MS method involving the addition of α-CD, β-CD or γ-CD (of 25 g/L) to model wine solutions containing guaiacol, 4-methylguaiacol and 4-ethylphenol (1 mg/L each) suggested no significant binding of volatile phenols by the CDs; i.e. no significant differences were observed between the RPAs of compound-to-standard for volatile phenols with and without CD addition (Table 1). However, the absolute peak areas of analytes (and internal standards) were observed to be considerably smaller in samples with CD addition, e.g., the peak areas of 4-ethylphenol and d_4-4-ethylphenol reduced from approximately 100,000 to 20,000 (abundance). Initially this was thought to reflect either variation in fiber performance or fiber degradation, but subsequent sensory and NMR analyses (described below) confirmed CD binding of volatile phenols. This led to the conclusion that CDs were also binding the isotopically labelled standards and prompted the development of a novel HS-SPME method, involving introduction of the internal standard solution via an additional liquid phase, so as to prevent inclusion of standards by CDs. This was achieved by inserting a 2 mL glass ampoule (Gerresheimer Shuangfang Pharmaceutical Packaging, Zhenjiang, China) containing the internal standard solution into the headspace vial (Sigma Aldrich, Castle Hill, NSW, Australia), as shown in Figure 1. A series of experiments (using a solution of methylene blue) were performed to ensure there was no mixing of samples in the SPME vial and the glass ampoule during sample preparation or the transfer, agitation and extraction (data not shown). The influence of internal standard volume, agitation, incubation

(temperature and duration), and the duration of sample extraction on the repeatability and accuracy of the novel SPME method were also evaluated, as method development and validation.

Four-Phase HS-SPME GC-MS Method Development

Influence of Agitation, Internal Standard Volume, and Pre-analysis Equilibration Time. A 6 mL aliquot of model wine containing 1 mg/L of guaiacol, 4-methylguaiacol and 4-ethylphenol was transferred into headspace vials. The volume of the sample was chosen to be a trade-off to maximize sample volume and keep the ampoule tube from being submerged. The inserted normalizing standard solution contained three isotopic standards (d_3-guaiacol, d_3-4-methylguaiacol and d_4-4-ethylphenol) at 10 mg/L each. In preliminary benchtop experiments, both agitation and the volume of inserted liquid were found to change during the pre-analysis equilibration time (data not shown). Therefore, a multiple factorial design was adopted to optimize extraction conditions (performed in triplicate). Four different volumes of internal standard were used (0.1, 0.5, 1.0 and 2.0 mL). Samples were analyzed over a 24-hour period (at 3-hour intervals, in triplicate) to determine the optimal equilibration time. Agitation, when used, was set at 250 rpm. The autosampler incubation and extraction times were 10 and 15 min, respectively, and the extraction temperature was 35 °C.

Influence of Extraction Time, Extraction Temperature and Internal Standard Concentration. Using the optimal parameters identified above, several additional parameters were evaluated. Extraction temperatures of 35, 50, 65 and 80 °C, extraction times of 15, 30, 45 and 60 min, and concentrations of internal standard solution of 5, 10, 20, 30, 40 and 50 mg/L were evaluated. Samples were prepared in triplicate.

Four-Phase HS-SPME GC-MS Method Validation. The optimized SPME method comprised the following conditions: An ampoule tube containing 0.5 mL of model wine and 10 mg/L of internal standard solution was inserted into 6 mL of sample (i.e., model wine spiked with volatile phenols) in

a 20 mL headspace sampling vial. Equilibrium in the headspace vial was achieved via 15 min incubation at 35 °C, before 15 min extraction without agitation. To validate the method, calibration curves were generated for guaiacol, 4-methylguaiacol, 4-ethylguaiacol, 4-ethylphenol, *o*-cresol, *m*-cresol, *p*-cresol, and eugenol at 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75 and 2.0 mg/L. Guaiacol, 4-methylguaiacol and 4-ethylphenol were quantified against their isotopically labelled equivalents, whereas 4-ethylguaiacol, *o*-cresol, *m*-cresol, *p*-cresol, and eugenol were quantified against d_3-2-methoxy-phenol. The linear range of detection was tested at concentrations of volatile phenols up to 50 mg/L. All samples were analyzed by GC-MS in triplicate.

Retention of Volatile Phenols in Model Wine by Cyclodextrin. A model wine solution comprising 1 mg/L of guaiacol, 4-methylguaiacol, 4-ethylguaiacol, 4-ethylphenol, *o*-cresol, *m*-cresol, *p*-cresol and eugenol was prepared. Aliquots (6 mL) were placed in 20 mL SPME headspace vials, to which α-CD, β-CD or γ-CD were added (to achieve CD concentrations of 25 g/L). Samples were then heated to 35 °C in an incubator (Ratek, Boronia, VIC, Australia) with agitation (200 rpm) for 20 min, after which samples were cooled to ambient temperature and analyzed by GC-MS. Samples were prepared in triplicate. Control samples (i.e. without the addition of any CD) were also prepared in triplicate. The residual volatile phenol levels were determined using the optimized four-phase SPME GC-MS method. Semi-quantification based on standard addition was used to calculate the percentage difference between the RPA of residual volatile phenols following CD addition and control samples.

Data Analysis. Data are presented as mean values of three replicates ± standard error. One-way ANOVA was conducted to determine differences between sample means, with a T-test at $p = 0.05$, using XLSTAT software (version 2015.3, Addinsoft, Paris, France).

RESULTS AND DISCUSSION

Three-Phase HS-SPME GC-MS Method, NMR and Sensory Analysis. The conventional three-phase HS-SPME GCMS method yielded excellent repeatability and linearity for quantification of guaiacol, 4-methylguaiacol and 4-ethylphenol in model wine (Supplementary Table S1). In the CD treatment assay, 25 g/L of α-CD, β-CD and γ-CD were dissolved in the mixture before the internal standard was added. The RPAs were compared between the treatment groups and the controls, but no significant differences in volatile phenol levels were observed for any of the CD treatments (Table 1). These results contradicted previous reports that β-CD significantly reduced the sensory perception of 4-ethylphenol in wine[23]. It was speculated that the CDs may have formed an inclusion complex with both the volatile phenols and the internal standards, equalizing changes in the relative response of both samples and standards following CD addition. In agreement with previously published work[26-28, 30], the absolute peak areas suffered greatly in reproducibility, particularly when different fibers were used, and thus were not suitable for use in quantification.

To establish whether or not binding had occurred between CDs and volatile phenols, NMR analyses were carried out on the mixture of β-CD and several volatile phenols (Figure 2). Cross peaks arising from the Nuclear Overhauser Effect (NOE) were observed between protons in the β-CD cavity and volatile phenol protons, confirming the close spatial arrangement of these protons in an inclusion complex. This was further supported by sensory analysis, with 24 and 20 panelists (of 38) perceiving a difference in the "smoke taint" bracket and "Brett" bracket, respectively, suggesting significant changes in the volatile phenol in the headspace with β-CD treatment. These results confirmed that the three-phase headspace SPME method, involving addition of the internal standard to the sample containing CDs, was not suitable for quantitative analysis.

Development of A Four-Phase HS-SPME GC-MS Method. In the current study, an ampoule comprising an additional liquid phase containing the internal standard was used to prevent CD interference. This ensured the standard could not be directly encapsulated by the CDs, but this

modification significantly affected the kinetics of the existing SPME method. Conventionally, quantitative headspace SPME method is performed when a partition equilibrium of the target compound is achieved between three phases, namely the sample matrix, headspace and the fiber. The extraction of a given compound can then be expressed as:

$$C_0*V_s = C_s*V_s + C_h*V_h + C_f*V_f$$

where C_0 is the original concentration of the compound in the sample, C_s is the residual concentration remaining in the sample, V_s is the volume of the sample (liquid phase), C_h is the concentration of the compound in the headspace, V_h is the volume of the headspace (gas phase), C_f is the concentration of the compound on the SPME fiber coating, and V_f is the volume of the fiber (solid phase).

There are two equilibria in this process, i.e. the equilibrium between the sample and the headspace (K_1) and that between the headspace and the fiber coating (K_2). The equilibrium constants, i.e., Henry's Law Constants, K_1 and K_2, are expressed as:

$$K_1 = C_s/C_h;$$

$$K_2 = C_h/C_f$$

where the equilibration time is longer than ideal, good precision can be achieved, provided extraction conditions such as temperature, fiber penetration and agitation are well controlled[31]. In the current study, the equilibrium is more complex, comprising distribution of volatile compounds in four phases during extraction, due to the presence of an additional liquid phase:

$$C_0*V_s = C_s*V_s + C_i*V_i + C_h*V_h + C_f*V_f$$

where C_i is the concentration of the internal standard in the additional liquid phase, and V_i is the volume of the additional liquid phase.

An additional equilibrium constant, K_3, exists for partitioning between the headspace and the additional liquid phase:

$$K_3 = C_h/C_i$$

In the current study, it was hypothesized that the sample mixture containing the CDs would not meaningfully interfere with the volatile phenols present in the headspace due to the relatively short extraction time, so C_i*V_i was considered to be zero. The method development employed in this study did not focus on modelling the overall process, but rather the practicality of the process in determining the retention of volatile phenols by CDs.

Effect of Agitation. The RPAs obtained for some samples changed significantly with time, before more constant levels were achieved. With the same volume of internal standard, agitated samples yielded significantly higher RPAs than non-agitated samples, particularly for lower equilibration times (Figure 3). To further investigate, absolute peak areas for *m/z* 124 (guaiacol) and *m/z* 127 (d_3-guaiacol) were compared. Despite being unable to quantify the compounds, the absolute peak areas were used to establish a hypothesis, based on samples being analyzed in triplicate, using relatively new fibers, with no sign of degradation. The response of *m/z* 124 was generally higher in agitated samples than in non-agitated samples, provided the same volume of internal standard was used. In contrast, the opposite was observed for the response of *m/z* 127 (data not shown). To provide an explanation, the auto-sampler's agitation process was evaluated, and it was found that agitation had a variable effect on both the sample and the internal standard solution. At 250 rpm the agitator moved the headspace vial in a horizontal circular trajectory, with the inserted ampoule spinning within the vial. This caused the ampoule to sit at an angle in the vial, which impacted the relative abundance of *m/z* 124 in the headspace of agitated samples. According to Dalton's Law, the total pressure in a gas phase equals the sum of pressure of each individual component. In the current case, the headspace pressure in the vial is comprised of the vapor pressure of both the sample and the internal standard solution. The distribution of each volatile component is defined by its Henry's Law constant. In the concept of HS-SPME, Pawliszyn[31] mentioned that Henry's Law constant is only dependent on the system temperature and the liquid phase matrix. Considering the sample vials were left unagitated prior to extraction, it can be concluded that agitation-induced partial pressure differences during

extraction disrupted the headspace pressure distribution. However, this disruption doesn't alter the Henry's Law constant of any of the volatile compounds, or the final equilibrium, and it becomes less effective as the system approaches equilibrium. As a consequence, samples were extracted without agitation in the final HS-SPME method developed in this study.

Effect of Volume of Internal Standard Solution. According to Henry's Law Constant equation: $K_1=C_s/C_h$ can be rearranged to give:

$$C_h=C_0/(K_1+\beta)$$

where β is the phase ratio between the headspace and the liquid phase of the sample.

In the recently updated Henry's Constant Compilation[32], the value of K for guaiacol partitioning between water and air is around 2.2×10^4 at 25 °C (converted from reported H_{cp} mol/m^3Pa). In the current study, the phase ratio (β) between the additional liquid phase and the headspace phase ranged from 6 to 139, which is insignificant when added to K (the volume of the glass material of the ampoule was deemed negligible). It can be inferred that the concentration of internal standard in the headspace at equilibrium would be within similar ranges for the various internal standard volumes used. It was concluded that agitation disrupted the equilibrating process, albeit only small deviations were observed in the RPA of agitated samples when the internal standard volume was 0.1 mL (Figure 3). This indicated that the system approached equilibrium sooner with smaller internal standard volumes. As such, lower volumes of internal standard were used in the new HS-SPME method. Taking into account the possible depletion of the deuterium labels in the internal standard[33], 0.5 mL was chosen as the functional volume for the internal standard.

Effect of Extraction Temperature, Extraction Time and Internal Standard Concentration. Once the most key analytical parameters had been optimized, several other factors, i.e., extraction temperature, duration and internal standard concentration, were evaluated. Increasing RPAs for

volatile phenols were observed when extraction temperature was increased from 35 to 80 °C (Figure 4). As mentioned above, Henry's Law constant (K) is temperature dependent, so K decreased as extraction temperature increased for most volatile phenols[32]. Wieland and colleagues[34] reported a 100-fold decrease in Henry's law constant for guaiacol, when the temperature increased from 35 to 80 °C. The phase ratio (β) for the internal standard and sample was 27 and 2.25, respectively (with 0.5 mL inserted standard). According to $C_h = C_0/(K+\beta)$, with decreasing K, C_h will have greater increases at low β values. Three things need to be taken into consideration when choosing extraction temperature: experimental sensitivity; the stability of CD complexation; and the potential for volatile compounds to re-dissolve in either of the liquid phases (i.e., the sample or the internal standard solution). In the present study, a temperature of 35 °C was therefore chosen as a trade-off.

Since the equilibrium problem was resolved by avoiding agitation and minimizing the volume of internal standard, the extraction time factor mainly addressed analytical sensitivity. The RPA for volatile phenols did not show significant differences between extraction times, ranging from 15 to 60 min (Figure 5). Accordingly, 15 min was chosen as the extraction time for the new HS-SPME method.

In terms of internal standard concentration, according to $C_h = C_0/(K+\beta)$ and $RPA = A_s/A_i$, it is expected that the RPA will follow a rational function with linear increases in concentration of the internal standard. Experimental data supported this notion (Figure 6). Indeed, in quantification studies using an isotopically labelled standard, the concentration is normally irrelevant to the analysis of compounds of interest, except where the standard is used for calibration [35]. The concern with choosing a higher concentration of ISTD is the adsorption capacity of the SPME fiber and competition for absorption between the standard and the compounds of interest. In the current study, 10 mg/L gave a RPA range close to 1.0.

Experimental Conditions for the Validated Four-Phase HS-SPME GC-MS Method. The final validated method involved a 6 mL aliquot of model wine solution spiked with volatile phenols (at 1 mg/L) being placed into a 20 mL headspace vial. A 0.5 mL aliquot of internal standard solution (10 mg/L) was added to a 2 mL glass ampoule, which was then inserted into the SPME vial. The vial was incubated for 10 min at 35 °C before extraction with the SPME fiber for 15 min. No agitation was used during extraction. The new HS-SPME GC MS method gave excellent linearity, repeatability and reproducibility (Table 2). A calibration function was constructed for guaiacol, 4-methylguaiacol and 4-ethylphenol ranging from 0.25 to 2 mg/L, and also gave excellent linearity, with an R2 value ≥ 0.9956.

Retention of Volatile Phenols by *α*-CD, *β*-CD, and *γ*-CD in Model Wine. Different CDs exhibited varying degrees of binding with the volatile phenols studied, i.e., guaiacol, 4-methylguaiacol, 4-ethylphenol, *o*-cresol, *m*-cresol, *p*-cresol, 4-ethylguaiacol, and eugenol (Table 3). In the current study, *β*-CD retained the highest proportion of volatile phenols, with the overall headspace concentration of volatile phenols reduced to 48.3% following 25 g/L addition of *β*-CD. This was expected given *β*-CD is the most reported inclusion complex host in other CD studies, due to its cavity size and hydrophobicity[18]. Guaiacol proved to be the most difficult compound to retain within the CDs, with 25 g/L of *γ*-CD giving the best result reducing guaiacol levels in the headspace to 70.6%. In contrast, 4-ethylphenol was the most susceptible to CD complexation, with *β*-CD reducing the headspace concentration of 4-ethylphenol to just 23.1%. The ranking of volatile phenols by the extent to which they decreased in the headspace following *β*-CD addition was 4-ethylphenol > *p*-cresol > eugenol > *m*-cresol > 4-ethylguaiacol > *o*-cresol > 4-methylguaiacol > guaiacol. Differences in reactivity were attributed to the differences in molecular structures. It has long been established that the hydrophobicity, molecular structure and size of guest molecules are among the most influential factors in the formation of CD inclusion complexes[17, 19]. Factors that influence binding between CDs

and guest molecules have been previously studied[36]. In the current study, it was obvious that the molecular geometry and the polarity of chemical functional groups in the guest molecule played a major role in binding. The more highly retained 4-ethylphenol and *p*-cresol have the most "aligned" structure, with the non-polar alkyl groups attached in the *para* position of the benzene ring (Supplementary Figure S1), whereas the less highly retained phenols, namely guaiacol and 4-methylguaiacol, have more polar methoxy groups at their *ortho* positions, which likely act to sterically hinder the molecule from entering into the *β*-CD cavity.

The newly developed four-phase HS-SPME GCMS method overcame the difficulties associated with analyzing volatile compounds with dissolved treatment. Whilst this method doesn't completely prevent interactions between isotopically labelled standards and dissolved CDs, it mitigates interactions by introducing the standard via a separate liquid phase. Therefore, modification and verification need to be conducted when using this method for the analysis of other volatile compounds. Nevertheless, the improvements offered by this method enabled complexation between CDs and volatile phenols to be studied. CDs formed inclusion complexes with volatile phenols in model wine, resulting in the reduction in volatility of these compounds, and therefore, most importantly, a reduction in the intensity of off-odors.

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SUPPORTING INFORMATION

Cyclodextrin molecular structure; Molecular modelling of the complexation between 4-ethylphenol and β-CD; Calibration curve using conventional HS-SPME GC-MS method.

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Table 1. Residual volatile phenol levels following addition of *α*-, *β*- and γ-CDs to model wine, based on conventional three-phase HS-SPME GC-MS analysis.

	guaiacol	4-methylguaiacol	4-ethylphenol
control	0.99 ± 0.02	1.00 ± 0.01	1.22 ± 0.02
α-CD	0.99 ± 0.02 (100.0%)	1.00 ± 0.02 (100.0%)	1.23 ± 0.02 (100.8%)
β-CD	0.99 ± 0.01 (100.0%)	1.00 ± 0.01 (100.0%)	1.29 ± 0.02 (105.7%)
γ-CD	0.99 ± 0.02 (100.0%)	1.00 ± 0.02 (100.0%)	1.24 ± 0.03 (101.6%)

Values are means of three replicates ± standard error (and percentage of control). Values within columns were not significantly different (one-way ANOVA, $p = 0.05$).

Table 2. Validation of the four-phase HS-SPME GCMS method.

	guaiacol			4-methylguaiacol			4-ethylphenol		
Repeatability	Mean RPA	CV [a] (%)	n [b]	Mean RPA	CV (%)	n	Mean RPA	CV (%)	n
0.25 mg/L	0.35	2.7	3	0.38	2.5	3	0.48	1.7	3
1.0 mg/L	1.12	7.5	3	1.16	8.9	3	1.50	9.3	3
2.0 mg/L	2.31	0.7	3	2.36	0.8	3	2.97	2.4	3
Reproducibility	Mean RPA	CV (%)	n	Mean RPA	CV (%)	n	Mean RPA	CV (%)	n
0.5 mg/L	0.63 (0.65) [c]	3.1 (4.5)	3	0.68 (0.69)	2.2 (5.8)	3	0.86 (0.90)	5.4 (8.9)	3
1.25 mg/L	1.61 (1.53)	0.6 (5.4)	3	1.74 (1.58)	0.8 (5.8)	3	2.27 (1.99)	6.5 (7.0)	3
1.75 mg/L	2.12 (2.10)	0.1 (2.2)	3	2.27 (2.17)	0.2 (3.1)	3	3.01 (2.84)	6.6 (6.1)	3

[a] Coefficient of variation.
[b] Number of replicates.
[c] Repeat analysis performed after 1 month.

Table 3. Residual volatile phenol levels following addition of c *α*-, *β*- and γ-CDs to model wine, using four-phase HS-SPME GC-MS analysis.

		guaiacol	4-methylguaiacol	4-ethylphenol	*o*-cresol	*m*-cresol	*p*-cresol	4-ethylguaiacol	eugenol
control		1.26 a ± 0.05	1.36 a ± 0.06	1.85 a ± 0.10	1.64 a ± 0.11	0.95 a ± 0.05	1.86 a ± 0.06	0.94 a ± 0.01	0.66 a ± 0.03
α-CD	5 g/L	1.14 abc ± 0.02 (90.3%)	1.19 ab ± 0.04 (87.9%)	1.51 ab ± 0.10 (81.6%)	1.39 abc ± 0.06 (85.0%)	0.81 b ± 0.02 (85.1%)	0.92 b ± 0.03 (84.8%)	0.82 b ± 0.01 (87.3%)	0.59 ab ± 0.03 (71.1%)
	25 g/L	1.17 ab ± 0.05 (92.9%)	1.20 ab ± 0.04 (88.2%)	1.42 b ± 0.11 (76.9%)	1.41 ab ± 0.07 (86.2%)	0.75 bc ± 0.03 (79.3%)	0.85 bc ± 0.04 (78.3%)	0.76 b ± 0.02 (80.4%)	0.47 c ± 0.01 (71.1%)
β-CD	5 g/L	1.01 cde ± 0.03 (80.2%)	1.04 bcd ± 0.04 (76.7%)	0.80 c ± 0.03 (43.1%)	1.14 cd ± 0.03 (70.0%)	0.61 d ± 0.01 (63.9%)	0.61 d ± 0.01 (56.5%)	0.68 c ± 0.02 (72.6%)	0.46 c ± 0.01 (70.0%)
	25 g/L	0.98 de ± 0.00 (77.5%)	0.95 cd ± 0.02 (69.8%)	0.43 d ± 0.02 (23.1%)	0.92 d ± 0.01 (56.0%)	0.41 e ± 0.01 (42.6%)	0.34 e ± 0.01 (31.1%)	0.47 e ± 0.01 (50.0%)	0.24 e ± 0.01 (36.9%)
γ-CD	5 g/L	1.07 bcd ± 0.00 (84.8%)	1.10 bc ± 0.00 (81.1%)	1.29 b ± 0.01 (69.8%)	1.24 bc ± 0.01 (75.7%)	0.67 cd ± 0.01 (70.1%)	0.76 c ± 0.01 (70.0%)	0.79 b ± 0.00 (84.2%)	0.56 b ± 0.01 (84.7%)
	25 g/L	0.89 e ± 0.02 (70.6%)	0.87 d ± 0.03 (64.1%)	0.77 cd ± 0.03 (41.5%)	0.93 d ± 0.01 (57.2%)	0.49 e ± 0.01 (51.1%)	0.56 d ± 0.01 (51.5%)	0.57 d ± 0.01 (60.0%)	0.35 d ± 0.01 (53.0%)

Values are means of three replicates ± standard error (and percentage of control). Values followed by different letters are significantly different (one-way ANOVA, $p = 0.05$).

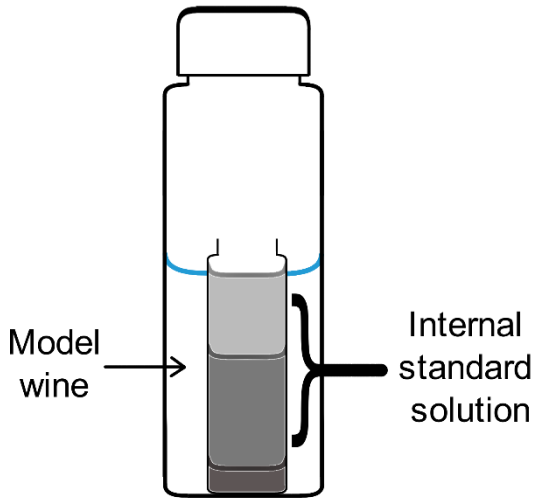


Figure 1. Headspace vial containing model wine samples with different volumes (as indicated by shading) of internal standard introduced via a glass ampoule.

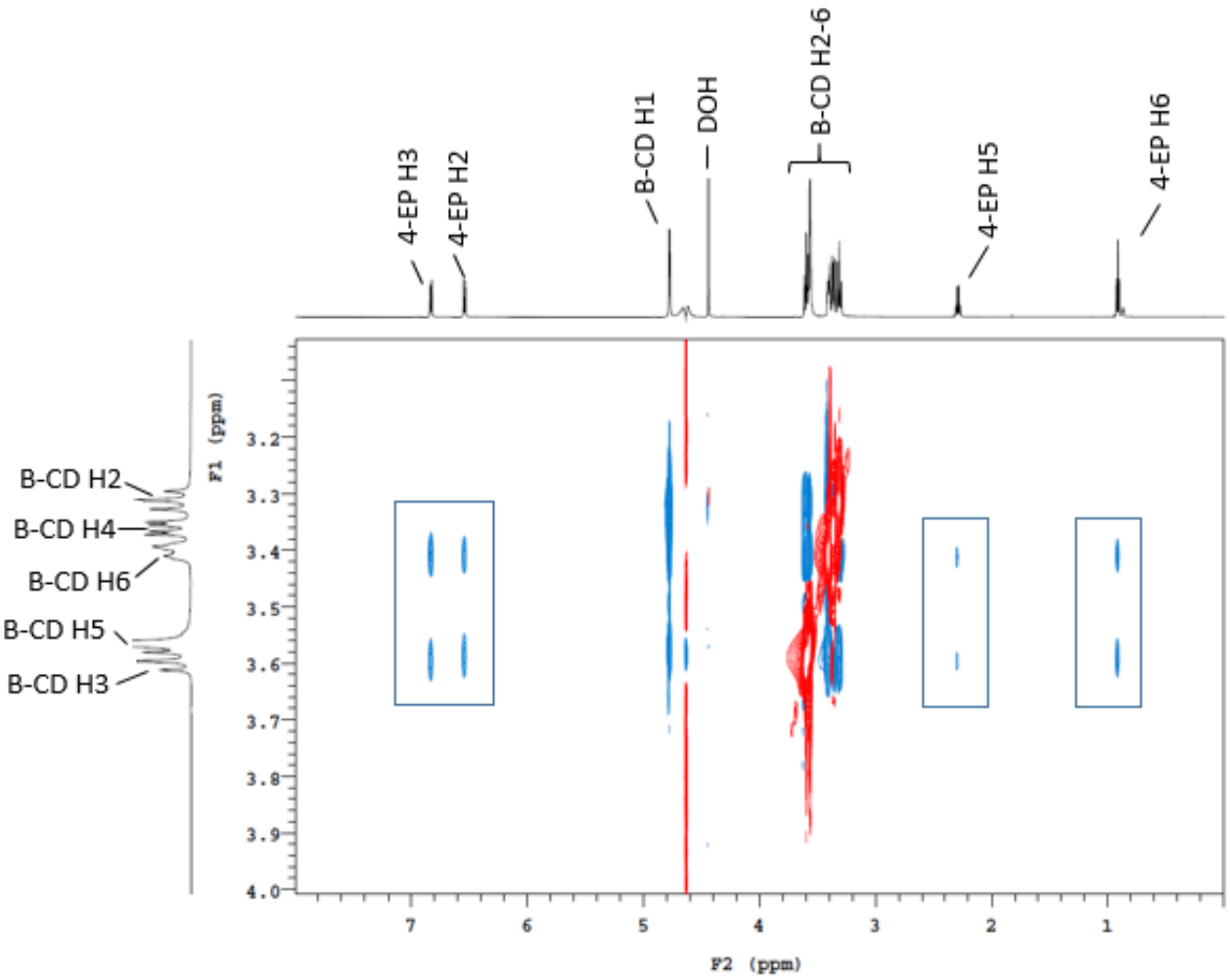


Figure 2. ^{1}H 2D ROESY NMR (600 MHz, pD 3.5 and 25 °C) spectrum of a D_2O and d_5-ethanol model wine containing 10^{-3} mol/L of 4-ethylphenol and 10^{-2} mol/L of *β*-CD. Rectangles indicate the cross-peaks arising from NOE interactions between the annular H3, H5 and H6 protons of the CD and the aromatic and methyl protons of 4-ethylphenol.

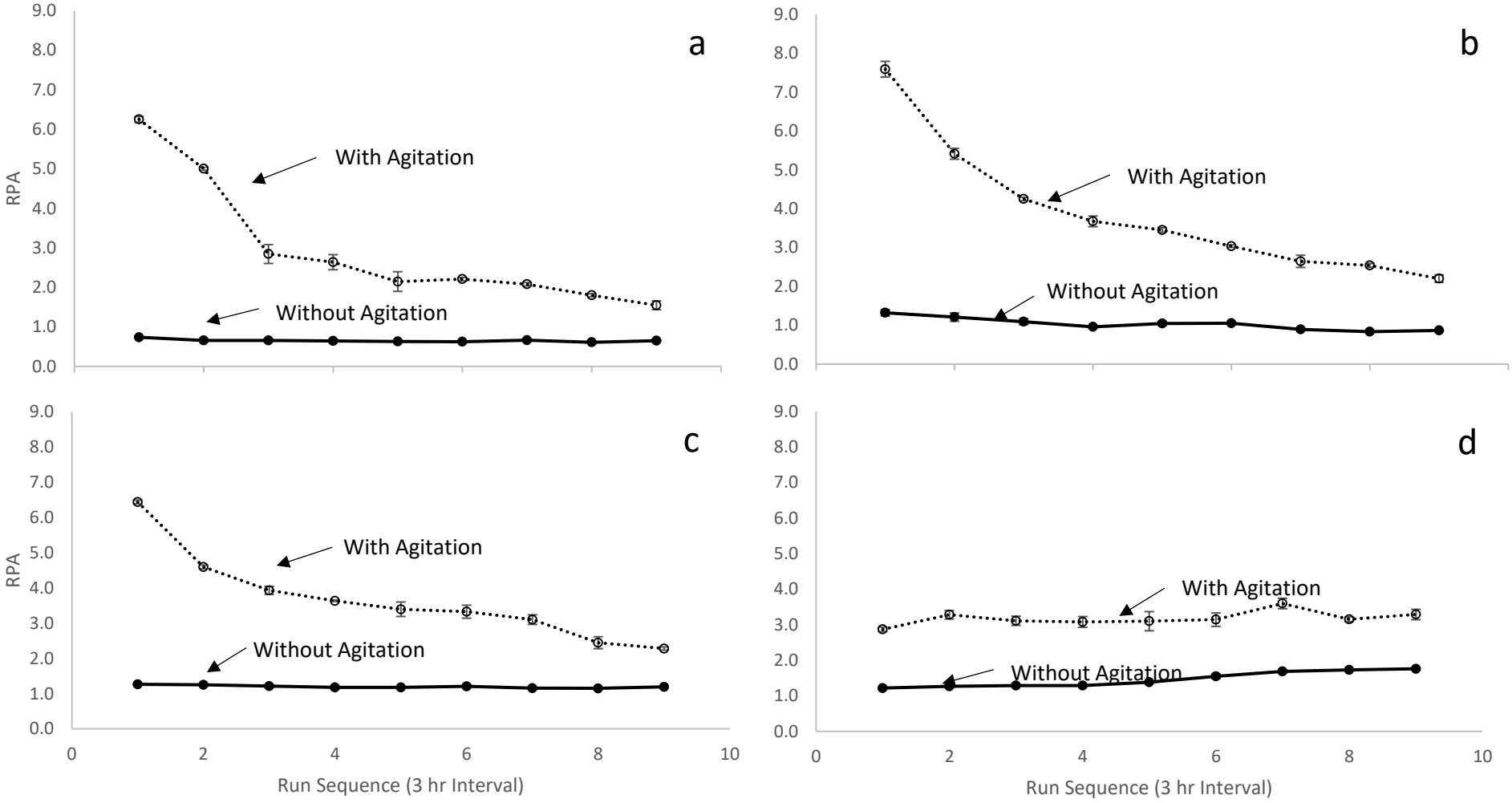


Figure 3. Effect of agitation and internal standard volume on relative peak area (RPA) of guaiacol, according to pre-analysis equilibration time (expressed as run sequence with 3-hour interval after the vial was closed). Internal standard volume: (a) 2 mL; (b) 1 mL; (c) 0.5 mL; (d) 0.1 mL. Values are means of three replicates ± standard error.

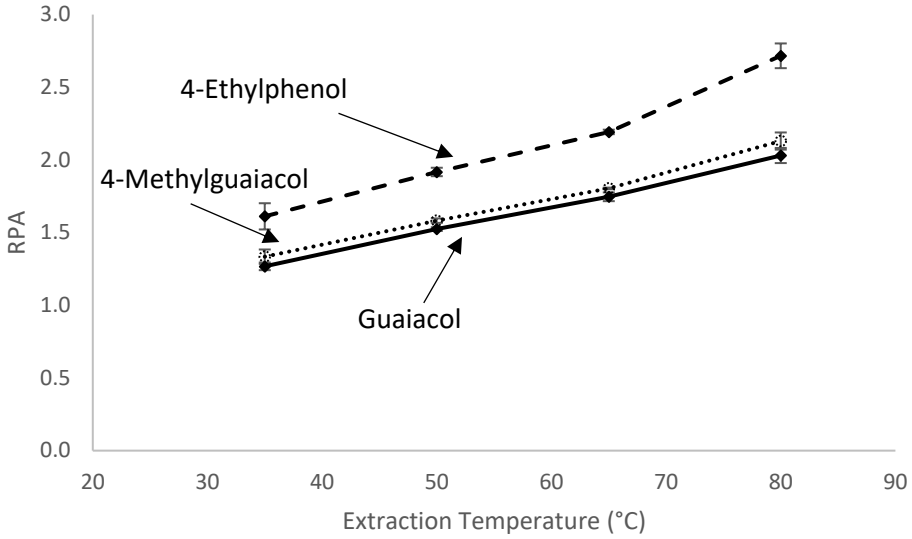


Figure 4. Effect of extraction temperature on relative peak area (RPA) of guaiacol, 4-methylguaiacol and 4-ethylphenol. Values are means of three replicates ± standard error.

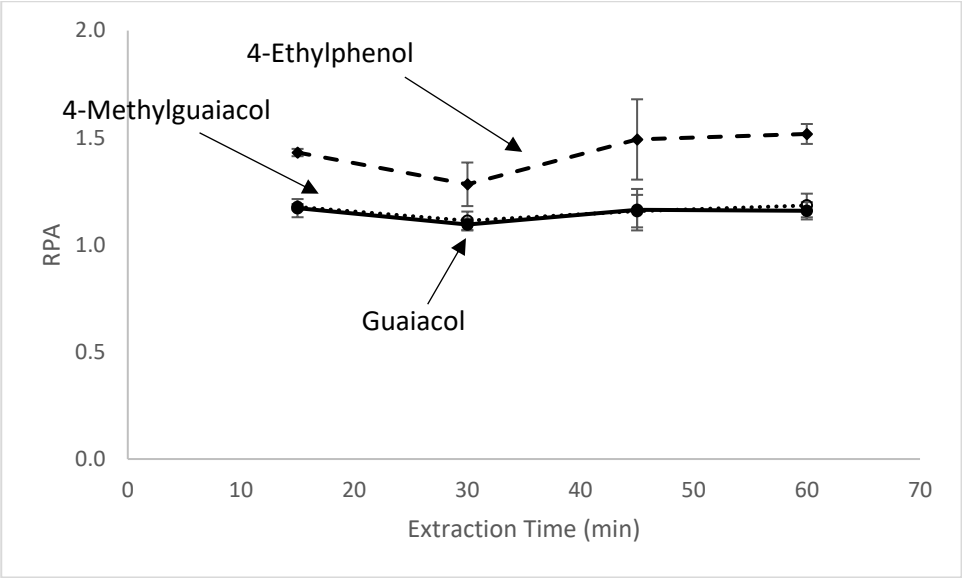


Figure 5. Effect of extraction time on relative peak area (RPA) of guaiacol, 4-methylguaiacol and 4-ethylphenol. Values are means of three replicates ± standard error.

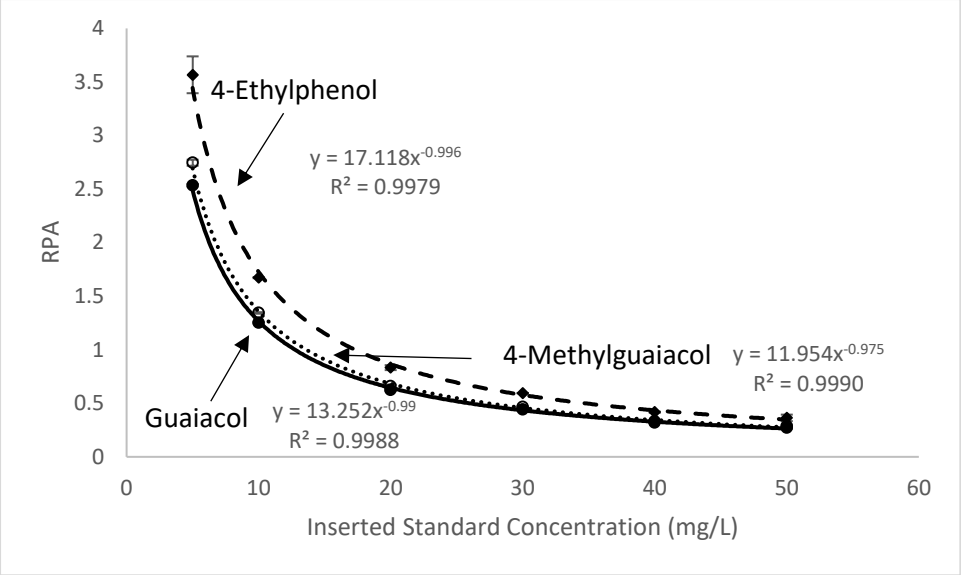
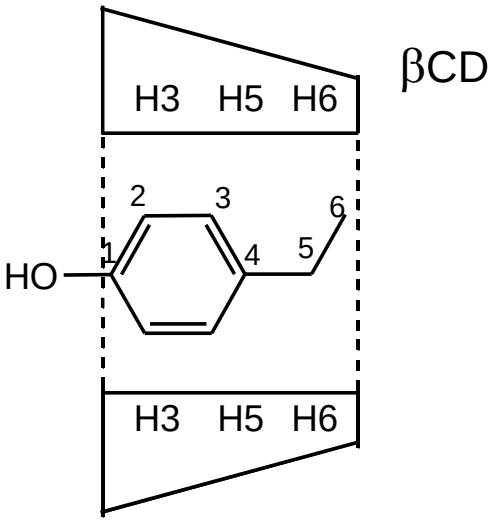


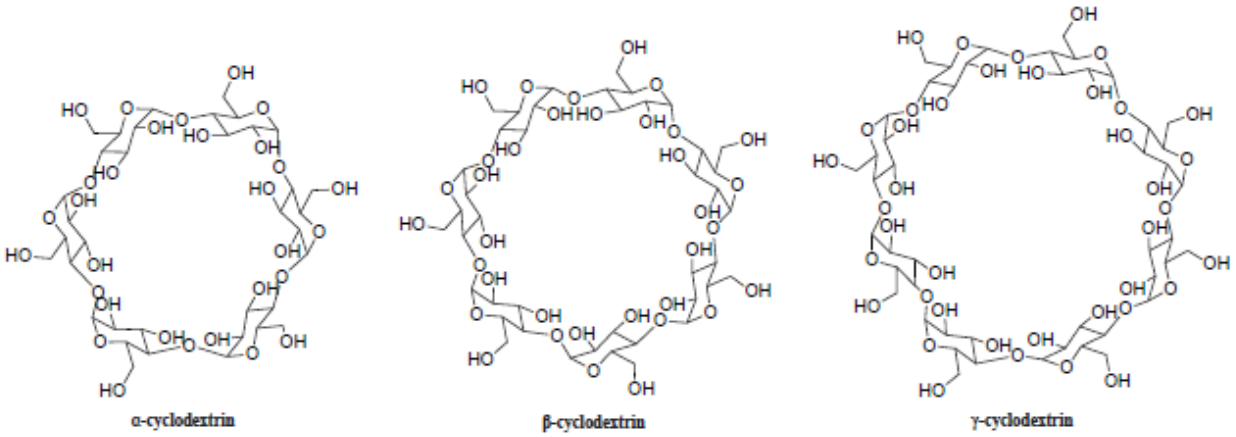
Figure 6. Effect of internal standard concentration on relative peak area (RPA) of guaiacol, 4-methylguaiacol and 4-ethylphenol. Values are means of three replicates ± standard error.

Supplementary Table S1. Calibration curve using conventional HS-SPME GC-MS method.

	guaiacol	4-methylguaiacol	4-ethylphenol
Slope	0.980	1.014	1.328
Intercept	0.003	0.007	0.006
R^2	0.9997	0.9996	0.9996



Supplementary Figure S1. Encapsulation of 4-ethylphenol by *β*-CD.



Supplementary Figure S2. Structures of α-CD, β-CD and γ-CD.

CHAPTER 3

Removal of volatile phenols from wine using crosslinked cyclodextrin polymers

Statement of Authorship

Title of Paper	Removal of Volatile Phenols Using Crosslinked Cyclodextrin Polymers
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Principal Author

Name of Principal Author (Candidate)	Chao Dang
Contribution to the Paper	Designed and conducted experiments; collected, processed, analysed, and interpreted data; drafted and edited manuscript.
Overall percentage (%)	70%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 25-11-18

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Removal of Volatile Phenols from Wine Using Crosslinked Cyclodextrin Polymers

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ABSTRACT

Volatile phenols have been implicated as contributors to off-odors associated with taints from bushfire smoke and microbial spoilage. Various methods for the amelioration of off-odors have been evaluated, but to date, cyclodextrin (CD) polymers have not been trialed. In the current study, two CD polymers were prepared from *β*- and *γ*-CD using hexamethylene diisocyanate (HDI) as a crosslinking agent. Adsorption tests were conducted with four volatile phenols (guaiacol, 4-methylguaiacol, 4-ethylguaiacol and 4-ethylphenol) at concentrations up to 1 mg/L. The removal of volatile phenols by CD polymers achieved equilibrium almost instantly, with the isotherm tests suggesting an adsorption capacity of 20.7 µg of volatile phenol per gram of polymer. Langmuir and Freundlich models were subsequently used to fit the data. In a batch adsorption test, the CD polymers achieved 45 to 77% removal of volatile phenols (at a 1 mg/L starting concentration). Reusability of the polymers was also evaluated and found to be excellent. A comparison between volatile phenol adsorption of CD polymers and the reduction in volatility achieved following CD addition suggested the CD polymers offer several advantages for use in the wine industry.

Keywords: *Brettanomyces*, cyclodextrin, cyclodextrin polymer, headspace solid phase microextraction, gas chromatography-mass spectrometry, smoke taint, wine

INTRODUCTION

Aroma plays an important role in determining wine quality, so optimizing the aroma profile of wine remains to be the winemaker's pursuit. Despite the evolution of viticultural and enological techniques that facilitate this effort, challenges still exist for the wine industry, including off-odors resulting from elevated concentrations of volatile phenols. In recent years, the impact of climate change has aggravated the risk of off-odors occurring in wine[1]. One of the known sources of off-odor volatile phenols is *Brettanomyces*/*Dekker* spp., a spoilage yeast capable of producing 4-ethylphenol and 4-ethylguaiacol, which typically impart animal, horse stable, sweat, and medicinal characters at excessive concentrations[2-3]. As a consequence of the warmer ripening conditions associated with climate change, grapes and therefore wines, tend to have less acidity (i.e., higher pHs) and higher sugar concentrations (i.e., conditions which favor the growth of spoilage yeast)[4]. The warmer, drier weather conditions also increase the potential for bushfires to occur near wine regions, which can lead to another volatile phenol related off-odors, commonly known as smoke taint[5-6]. Elevated concentrations of guaiacol, 4-methylguaiacol, 4-ethylguaiacol and 4-ethylphenol in smoke affected wines have been found, and their smoke-related sensory characters can negatively impact wine quality[7].

Conventional techniques have been used to prevent volatile phenol related off-odors, and include: (1) the addition of sulfur dioxide to control the growth of *Brettanomyces*; and (2) reducing the duration of skin contact or degree of pressing so as to minimize volatile phenol extraction from smoke affected fruit[8-9]. In recent years, several studies have evaluated the potential for volatile phenols to be removed from wine using charcoal[10], polyvinylpolypyrrolidone[11], yeast lees[12], yeast cell wall[13], cellulose[14] additions, reverse osmosis membrane filtration[7] or treatment with molecularly imprinted polymers[15]. However, these removal techniques inevitably impact the intensity of desired aromas as well. In a recent study, cyclodextrins (CDs), a group of compounds that have long been used by other industries,

were reported to be able to form inclusion complexes with volatile phenols in wine, reducing the volatility from these off-odor volatiles[10].

The most common naturally occurring CDs are α-CD, β-CD and γ-CD, which are cyclic oligosaccharides consisting of 6, 7 and 8 α-1,4-linked glucopyranose subunits, respectively, derived from starch[16]. The arrangement of the C-H groups of subunits within the ring-shaped molecules result in a lipophilic cavity (H-3 and H-5) and a hydrophilic surface (H-1, H-2 and H-4), a structure that enables CDs to form inclusion complexes with the non-polar moieties of guest molecules[17]. This binding process exists as a dynamic equilibrium in aqueous environments, and is mainly driven by non-covalent van de Waals forces, resulting in a more stable status with lower energy when enthalpy-rich water molecules are replaced by non-polar guests[18]. The size, hydrophobicity, and conformation of the guest molecule will influence encapsulation in aqueous conditions[19]. Formation of the CD-guest complex leads to a series of changes in both the physical and chemical properties of the guest molecule, including increased solubility of insoluble compounds, protection from degradation, reduced volatility, and therefore reduced aroma and flavor impact, as well as shifts in spectral peaks and chromatographic separation[16]. As a result of these functions, CDs have been exploited as additives by the food, beverage and flavor industries[18, 20-22]. Currently, α-CD and β-CD are listed as novel foods in the US, EU and Japan. γ-CD is an approved novel food in the EU, but its approval status varies in the US and Japan. In Australia and New Zealand, both α-CD and γ-CD are listed as novel foods by Food Standards, whereas β-CD is classified as a food processing aid. A recent report suggests there are more than 200 food products containing CDs as ingredients[23].

The complexation that occurs between CDs and various aroma volatiles has been widely studied, however, there is limited literature concerning the use of CDs in wine. A key barrier to the uptake of CDs in wine production is the legal regulation of permitted winemaking additives, which currently does not include CDs. There is, nevertheless, increasing interest in developing insoluble CD polymers

to broaden the applications of CDs. CDs can be polymerized with various molecules, known as crosslinkers, with these compounds containing at least two functional groups that can react with the hydroxyl groups on the glucopyranose subunits of CD, linking the molecules in a chain structure[24]. These polymers were found to be useful to remove phenols and dyes from waste water[25-28]. Several CD crosslinkers have been studied. Crini and colleagues[25] crosslinked *β*-CD with epichlorohydrin and studied the sorption capability of the resulting polymer with benzene derivatives, such as phenol, *p*-nitrophenol, and benzoic acid. Yamasaki[26] used hexamethylene diisocyanate (HDI) and toluene-2,6-diisocyanate as CD crosslinkers, and showed adsorption of cresols, phenol and xylenol from waste water by the polymers. Other studies have used chitosan and citric acid to form phenol absorbing CD polymers for removing pollutants from water[27-30].

The present study aimed to evaluate the potential for CD polymers to remove volatile phenols from tainted wine. Two insoluble CD polymers were prepared from *β*- and *γ*-CD using HDI as crosslinker. Adsorption tests were conducted to evaluate the preference and capability of the polymers to adsorb volatile phenols associated with smoke taint and *Brettanomyces* spoilage. In order to compare volatile phenols removed following addition of CDs and CD polymers, a newly developed four-phase headspace solid phase microextraction (HS-SPME) method for gas chromatography-mass spectrometry (GC-MS) was employed to quantify changes in volatile phenol concentrations without interference between CD additives and internal standards.

MATERIALS AND METHODS

Chemicals. Analytical grade volatile phenols (guaiacol, 4-methylguaiacol, 4-ethylguaiacol, and 4-ethylphenol), hexamethylene diisocyanate (HDI), N,N-dimethylformamide (DMF), dibutyltin dilaurate (BTL) and deuterated NMR solvents (*d*$_6$-ethanol, D_2O and DCl) were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Analytical grade chloroform was purchased from Chem-supply (Adelaide, SA, Australia) for polymer precipitation. The deuterium-labelled

normalizing standard, d_3-4-methylguaiacol, was purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada). Food grade (>98% purity) β-, and γ-CD were sourced from IMCD Group (Adelaide, SA, Australia). Volatile phenol and deuterated standard stock solutions were made in pure ethanol (Thermo Fisher Scientific, Waltham, MA, USA) and stored at –20 ºC. Working solutions were prepared in model wine containing 5 g/L of tartaric acid (Thermo Fisher Scientific) and 12% v/v ethanol, and were stored at –4 ºC. A commercial red wine sample (2017 Yalumba Cabernet Sauvignon) was used for the adsorption study.

Preparation of HDI Cross-linked CD Polymers (CD-HDI)

CD-HDI polymers were prepared according to the method developed by Yamasaki and co-workers[26]. In the previously published method, polymerization was achieved with a range of molar ratios of HDI to CD due to the number of hydroxyl groups available for crosslinking within the CD structure. In the current study, the HDI:CD ratio was 4:1. To yield 5 g of polymer, 1.8 mM of β- or γ-CD was dissolved in 15 mL of DMF under a nitrogen atmosphere, with stirring. Two drops of dibutyltin dilaurate (BTL) initiator were added to the mixture before 7.2 mM of HDI in DMF (5 mL) was added. The crude product was heated in an oil bath at 70 °C under nitrogen. After 24 hours, the resulting gel like mixture was transferred into 50 mL of chloroform and stirred for 12 hours to facilitate precipitation. The suspension was filtered and washed repeatedly with around 10 L of de-ionized water. The polymer was then dried at 60 °C for 6 hours before being ground in a multi-directional planetary QXQM-1 ball mill (Tencan, Changsha, China). The ground polymer was finally passed through a 150 µm sieve to be ready to use.

Adsorption Experiments with CD polymers. Kinetic adsorption tests were carried out (in triplicate) by adding 1% w/w polymer to model wine (10 mL) spiked with 1 mg/L of each of the various volatile phenols (guaiacol, 4-methylguaiacol, 4-ethylguaiacol and 4-ethylphenol). The sample was agitated at 120 rpm at 25 °C for 2 hours, during which time aliquots (1 mL) were periodically withdrawn (i.e.,

at 5, 20, 40, 80 and 120 min after the start of mixing), centrifuged (2,000 g for 5 min) and analyzed by HS-SPME GC-MS.

The initial test showed that CD-HDI polymers reached adsorption equilibrium within 2 hours of incubation, hence all analyses thereafter were conducted using this incubation time. To study the equilibrium adsorption capacity of the adsorbent in model wine, 2% w/v of polymer was added to 10 mL of model wine containing various concentrations of one of the selected volatile phenols (guaiacol, 4-methylguaiacol, 4-ethylguaiacol and 4-ethylphenol). The concentration of volatile phenols ranged from 0.05 to 1.0 mg/L. Controls were prepared without the addition of polymer. The equilibrium adsorption capacity of the polymer is expressed as q_e (mg/g):

$$q_e = (C_0 - C_e)*V/W$$

where C_0 is the starting concentration of volatile phenols, C_e is the remaining concentration of volatile phenols in wine samples at equilibrium, V is the volume of the wine sample, and W is the weight of the polymer. The concentration of volatile phenols in the wine was determined by measuring the headspace residual of the compounds.

To evaluate volatile phenol adsorption in a real wine environment, a batch experiment was conducted (in triplicate) to compare the adsorption efficiency (expressed as a percentage of the RPA for the controls) using various amounts of CD-HDI polymers added to spiked red wine samples. The amount of CD-HDI polymer was 1%, 2% and 5% w/v. The reusability of the polymers was also tested. Pre-exposed polymers were collected from the wine samples and soaked in methanol at room temperature for 24 hours with 120 rpm agitation, then filtered and dried for reuse. The regenerated polymers were subjected to five rounds of batch adsorption to test their regeneration efficiency.

Binding Experiments with CDs. The two functional CDs, *β*- and *γ*-CD (in their monomer forms), were also included in the current study to enable a comparison against the performance of the CD polymers. CDs (20 g/L) were added to red wine samples spiked with 1 mg/L volatile phenol. Samples were then incubated at 35 °C with 120 rpm agitation for 20 min, prior to GC-MS analysis. Controls without any CD addition were also prepared and analyzed (in triplicate).

Four-Phase HS-SPME GC-MS Analysis. A previously developed four-phase HS-SPME GC-MS method was used with modification (see Chapter 2). An ampoule containing 0.2 mL of model wine solution was spiked with the isotopically labelled standard (d_3-4-methylguaiacol, at 2 mg/L) was inserted into a 20 mL headspace sampling vial (Sigma Aldrich, Castle Hill, NSW, Australia), which initially contained 0.2 mL of red wine sample. The vial was incubated for 5 min at 35 °C. The fiber extraction of headspace aroma compounds occurred over 15 min without agitation. Samples were then analyzed using an Agilent 6890 GC-MS system coupled to a 5973 mass selective detector (Santa Clara, CA, USA), and filled with Gerstel MPS autosampler (Mülheim, Germany). The column used for GC separation was a 60 m DB-Wax column with 0.25 mm internal diameter and 0.25 µm film thickness (Agilent J&W, Folsom, CA). Helium (BOC Gas, Adelaide, SA, Australia) was used as the carrier gas at a constant flow of 1.5 mL/min. The inlet temperature was set at 240 °C. The oven temperature started at 40 °C for 5 min, then increased to 250 °C at 3 °C/min and was held at 250 °C for 5 min, to give a total run time of 80 min. Selected Ion Monitoring (SIM) mode was used to record the mass spectra of target ions. The ions monitored in SIM mode were: *m/z* 109, *124* for guaiacol; *m/z* 123, *138* for 4-methylguaiacol; *m/z* 126, *141* for d_3-4-methylguaiacol; *m/z* 122, 137, *152* for 4-ethylguaiacol; *m/z* 77, *107* for *p*-cresol; and *m/z* 77, *122* for 4-ethylphenol, with italicized ions used for quantitation. Volatile phenol concentrations are reported as relative peak areas (RPA), i.e. as the ratio of the peak area of the analyte relative to the peak area of the normalizing standard (d_3-4-methylguaiacol).

Nuclear Magnetic Resonance (NMR) Analysis. To compare the encapsulation of tested volatile phenols by CDs, 2-dimensional nuclear magnetic resonance rotating frame Overhauser effect spectroscopy (^{1}H 2D ROESY) was used, as described in Chapter 2, with an Agilent DD2 600 MHz spectrometer connected to a cryoprobe (Agilent Technologies, Santa Clara, CA, USA), operating at 600 MHz with a delay time of 300 ms.

Data Analysis. Data are presented as mean values of three replicates ± standard error. One-way ANOVA was conducted to determine differences between sample means, with a T-test at $p = 0.05$, using XLSTAT software (version 2015.3, Addinsoft, Paris, France).

RESULTS AND DISCUSSION

Equilibrium Time. The polymerization process (Figure 1) yielded a slightly yellow colored polymer with *β*-CD, and a white colored polymer with *γ*-CD. After ball mill grounding, the powdered polymers were weighed and added to the wine sample. The time required for CD polymers to achieve adsorption equilibrium is dependent on the sample mixture, the chemical properties of the target molecule and temperature[30-31]. In the current study, 1% w/w of polymer was added to spiked model wine samples at 25 °C, and an incubation time of less than 5 min was required for the adsorbents to achieve equilibrium. No significant differences in the RPAs for the four volatile phenols were observed between aliquots collected from the reaction mixture at 5, 20, 40, 80, and 120 min intervals after polymer treatments (Figure 2). The rapid equilibria observed were consistent with the findings of Yamasaki and colleagues[26], who used a *β*-CD-HDI polymer to remove phenols from waste water. Alsbaiee and team[28], who used a different type of *β*-CD polymer with tetrafluoroterephthalonitrile as crosslinker, also reported rapid equilibrium, i.e., within 10 min. Other studies have reported different equilibrium times when using different CD polymers on various adsorbates. For example, Romo[29] and Crini[31] used epichlorohydrin crosslinked *β*-CD polymer to adsorb dyes and phenols, with 2 hours

of incubation required to achieve equilibrium. The differences in time to equilibrium may be attributed to the polymer type, the initial concentration of adsorbate, and/or the sample matrix.

Adsorption Capacity. The four-phase HS-SPME GC-MS method showed excellent repeatability in developing calibration curves for the volatile phenols across studied concentrations, ranging from 0.5 to 1.0 mg/L in model wine. To evaluate the adsorption capacity of CD polymers, 2% w/v of *β*-CD-HDI or *γ*-CD-HDI polymers were added to model wine samples containing sequential concentrations of volatile phenols, as used for development of calibration curves. The RPA of treated samples represents the concentration of free volatile phenols, with the corresponding concentrations (C_e) being calculated from the calibration curves. The difference between C_0 and C_e was considered to be due to volatile phenol adsorption by the polymer, and was used to calculate the adsorption capacity q_e. Plots of q_e against the concentration of remaining volatile phenols, once the adsorption equilibrium was achieved, are shown in Figure 3.

Overall, the adsorption capacity of the *β*-CD-HDI and *γ*-CD-HDI polymers was similar, with the *γ*-CD-HDI polymer showing greater affinity towards guaiacol and 4-methylguaiacol, and the *β*-CD-HDI polymer showing higher affinity for 4-ethylguaiacol and 4-ethylphenol. This difference in binding preference by *β*-CD and *γ*-CD is consistent with results from other experiments in the current study (outlined below). Among the four volatile phenols tested, it was obvious that 4-ethylphenol was by far the most readily removed compound. The adsorption capacity of both CD polymers for all four volatile phenols at equilibrium was around 20 µg/g, and the adsorption capacity increased with increasing volatile phenol concentration. The magnitude of this adsorption rate is quite low compared with other studies[27, 29-30, 32]. This was attributed to the range of concentrations used in the current study, which were very low by comparison. Other studies have demonstrated that CD polymers, including *β*-CD-HDI, could achieve much higher adsorption rates at higher concentrations of

adsorbate. For example, the citric acid crosslinked *β*-CD polymer, used by Zhao and colleagues[27], exhibited a 13.8 mg/g adsorption capacity for phenol, with an equilibrium concentration around 400 mg/L, and a capacity of 3.8 mg/g at an equilibrium concentration of 85.4 mg/L. The epichlorohydrin and chitosan crosslinked *β*-CD polymers, used by Li and co-workers[30], showed similar adsorption capacities for phenol, *p*-nitrophenol and *p*-chlorophenol at equilibrium concentrations above 200 mg/L. Romo[29] tested the *β*-CD-HDI polymer at higher concentrations of phenol, and reported adsorption capacity of around 15 (mol_{phenol}/mol_{CD}) at an equilibrium concentration of 0.94 g/L. In the current study, the starting and equilibrium concentrations of volatile phenols were no more than 1 mg/L, which is more reflective of concentrations observed in wine than concentrations reported in studies targeting pollutant removal from water. There is indeed limited literature reporting adsorption rates at these lower concentrations, so adsorption isotherm models were used in the current study to predict the maximum adsorption capacity of the studied polymers. Several isotherm models have been developed to describe the adsorption of gas/liquid molecules onto an adsorbent surface. In the current study, Langmuir and Freundlich isotherm models were used to fit the data (Table 1).

The Langmuir isotherm assumes single-layer adsorption of an adsorbate onto a homogenous surface with an identical cavity[30]. It is especially useful in describing adsorption at lower pressures (concentration of adsorbate), i.e., when the adsorption capacity curve (Figure 3) appears to be close to linear[33]. It can be expressed as:

$$q_e = 1/q_{max} K_{L +} C_e/q_{max}$$

where q_e is the adsorption capacity per unit weight of polymer, q_{max} is the maximum adsorption capacity of the system, C_e is the residual concentration of volatile phenols at equilibrium, and K_L is the Langmuir isotherm constant. The equation can be re-arranged to give:

$$C_e/q_e = 1/q_m * C_e + 1/K_L q_m$$

It is obvious that if C_e/q_e is plotted against C_e, values of K_L and q_m can be calculated from the slope and intercept of the regression. In the current study, a Langmuir model fitted the data well with good linearity. The calculated values of K_L and q_m are shown in Table 1. The modelled maximum adsorption capacity (q_m) for *β*-CD-HDI and *γ*-CD-HDI binding guaiacol, 4-methylguaiacol, 4-ethylguaiacol and 4-ethylphenol is between 17.7 mg/g (for *β*-CD-HDI adsorbing 4-methylguaiacol) and 22.7 mg/g (for *β*-CD-HDI adsorbing 4-ethylphenol), which falls within the ranges reported previously[27, 30-31].

The Freundlich isotherm can be regarded as a special case of the Langmuir isotherm at intermediate adsorbate concentrations, where the adsorption capacity curve starts to plateau[34], and can be expressed as:

$$\log q_e = \log K_F + 1/n \log C_e$$

where K_F is the maximum adsorption capacity of the system, and 1/n is a natural value normally smaller than 1, which describes the extent of curving of the plotted $\log q_e$ against $\log C_e$, which relates to the "adsorption intensity" or mobility of adsorbate at the adsorbent surface. The Freundlich isotherm fitted the data well in the current study. The K_F and 1/n for the Freundlich isotherm are also shown in Table 1. The K_F value is similar to the q_m predicted by the Langmuir isotherm, with the value ranging from 22.4 mg/g (for *β*-CD-HDI adsorbing 4-ethylguaiacol) and 33.1 mg/g (for *β*-CD-HDI adsorbing 4-ethylphenol). The 1/n value suggests homogeneity of the adsorption process across the experimental concentrations when it is close to 1, whereas a value close to 0 suggests heterogeneity. In the present study, the 1/n value ranged from 0.347 (for *γ*-CD-HDI adsorbing 4-ethylphenol) to 0.762 (for *γ*-CD-HDI adsorbing 4-methylguaiacol).

Batch Adsorption and CD Addition in Red Wine. Three concentrations of the CD polymers were used in the batch adsorption test, with the percentage removed of volatile phenols shown in Table 2. Not surprisingly, with increasing amounts of polymer addition, the amount of volatile phenol being adsorbed also increased. Following addition of β-CD-HDI and γ-CD-HDI polymers (5% w/v), the residual concentrations of guaiacol, 4-methylguaiacol, 4-ethylguaiacol and 4-ethylphenol were 55% and 54%, 54% and 51%, 51% and 46%, and 43% and 37%, respectively. When comparing these adsorption results with CD addition, it is worth mentioning that the concentrations of CD added were close to the limit of solubility, according to the supplier, which is a limiting factor for CD functionality. Nevertheless, the reduction in volatile phenol levels following addition of CDs was positively correlated with CD concentration, with 77 and 66% removal of guaiacol, 75 and 63% removal of 4-methylguaiacol, 58 and 57% removal of 4-ethylguaiacol, and 32 and 48% removal of 4-ethylphenol following addition of β-CD and γ-CD (at 20 g/L), respectively. 4-Ethylphenol was consistently the most readily remove volatile phenol. The ranking of volatile phenols by efficiency of removal (irrespective of polymer type) was: 4-ethylphenol > 4-ethylguaiacol > 4-methylguaiacol > guaiacol. It has been established that hydrophobicity and size are among the most influential factors in determining the extent of encapsulation of a guest molecule by CD[19, 23]. The log P value is the logarithm of a compound's partition coefficient, and it is often used to describe the hydrophobicity of a compound, with higher log P values correlating with higher hydrophobicity. As expected, 4-ethylphenol has the highest hydrophobicity of the four volatile phenols studied, followed by 4-ethylguaiacol with guaiacol being the lowest. When comparing the guaiacol based volatile phenols, it is clear 4-methylguaiacol and 4-ethylguaiacol have greater tendency to be encapsulated within the CD cavity due to the higher hydrophobicity granted by the alkyl group. This was apparent by 2D NMR analysis (Figure 4). The cross peaks indicate the close spatial correlation between protons. The graph was cropped to specifically show interactions between the aromatic and alkyl protons of the volatile phenols with the CD cavity. It is obvious that the methyl and ethyl groups facilitate insertion of 4-methylguaiacol and 4-ethylguaiacol into the CD cavity.

Reusability Test. The CD polymers were recovered and washed with methanol after treatment, before being added to new wine samples to test their reusability. No significant differences were found between the RPA for residual volatile phenols across four series of adsorption tests. As mentioned above, the mechanism of CD encapsulation is mainly based on non-covalent bond hydrophobic interactions. The process can therefore be reversed by placing the CD polymers in a more hydrophobic environment.

The two types of CD polymers tested in the current study, i.e., β-CD-HDI and γ-CD-HDI, were found to be capable of removing volatile phenols from both model wine and red wine samples, at volatile phenol concentrations that reflect tainted wines. Considering the practical use of CDs for off-odor removal in wine, the use of polymers shows advantages over CDs; not only does the solubility of CDs limit its functionality, the addition of CD may also have greater negative impact on overall wine quality, which needs to be investigated further. In comparison, polymerized CDs can be used as fining agents (pending classification as permitted winemaking additives), with polymerization appearing to enhance the binding capability of the CD cavity. Opportunities remain to further validate the use of CD polymers as an innovative tool for flavor management by the wine industry.

FUNDING

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Table 1. Langmuir and Freundlich adsorption isotherms for the adsorption of volatile phenols by *β*-CD-HDI and *γ*-CD-HDI in model wine at 25 °C.

	Langmuir adsorption isotherm			Freundlich adsorption isotherm		
	q_m (mg/g)	K_L (L/mg)	r	K_F	1/n	r
guaiacol						
β-CD-HDI	22.3	3.0	0.989	21.7	0.67	0.992
γ -CD-HDI	19.6	5.8	0.992	22.3	0.56	0.991
4-methylguaiacol						
β-CD-HDI	20.1	4.9	0.990	21.5	0.56	0.987
γ -CD-HDI	21.2	5.8	0.988	23.5	0.54	0.995
4-ethylguaiacol						
β-CD-HDI	20.0	10.4	0.995	23.9	0.44	0.968
γ -CD-HDI	20.5	7.7	0.997	24.2	0.50	0.959
4-ethylphenol						
β-CD-HDI	22.1	8.1	0.986	34.0	0.56	0.957
γ -CD-HDI	20.6	23.1	0.976	25.9	0.35	0.985

Table 2. Relative peak area (RPA) of batch adsorption test and percentage of headspace residual volatile phenol of the control.

		guaiacol		4-methylguaiacol		4-ethylguaiacol		4-ethylphenol	
Control		2.34 a ± 0.03		2.18 a ± 0.04		1.89 a ± 0.03		0.97 a ± 0.03	
	1% w/v	2.07 b ± 0.03	89%	1.96 a ± 0.01	90%	1.63 b ± 0.01	86%	0.81 b ± 0.01	84%
β-CD-HDI	2% w/v	1.69 c ± 0.05	72%	1.57 b ± 0.03	72%	1.25 c ± 0.02	66%	0.55 c ± 0.01	57%
	5% w/v	1.29 d ± 0.05	55%	1.17 c ± 0.05	55%	0.82 d ± 0.02	43%	0.23 d ± 0.01	23%
	1% w/v	1.97 b ± 0.06	84%	1.90 a ± 0.05	87%	1.63 b ± 0.05	86%	0.83 ab ± 0.04	86%
γ-CD-HDI	2% w/v	1.61 c ± 0.10	69%	1.50 b ± 0.10	69%	1.31 c ± 0.00	69%	0.64 c ± 0.00	66%
	5% w/v	1.27 d ± 0.04	54%	1.11 c ± 0.10	51%	0.88 d ± 0.08	46%	0.36 d ± 0.06	37%

Values are means of three replicates ± standard error (and percentage of control). Values followed by different letters are significantly different (one-way ANOVA, $p = 0.05$).

Table 3. Relative peak area (RPA) of CD addition and percentage of headspace residual volatile phenol of the control.

		guaiacol		*4-methylguaiacol*		*4-ethylguaiacol*		*4-ethylphenol*	
Control		2.43 a ± 0.06		2.30 a ± 0.09		1.99 a ± 0.08		1.04 a ± 0.05	
β-CD	5 g/L	2.06 bc ± 0.02	85%	1.88 b ± 0.07	82%	1.62 b ± 0.07	81%	0.79 b ± 0.01	76%
	10 g/L	1.96 bc ± 0.02	81%	1.80 b ± 0.07	78%	1.38 bc ± 0.07	69%	0.49 c ± 0.01	47%
	20 g/L	1.87 d ± 0.01	77%	1.73 bc ± 0.02	75%	1.15 c ± 0.03	58%	0.33 c ± 0.00	32%
γ -CD	5 g/L	2.19 b ± 0.06	90%	1.97 ab ± 0.06	85%	1.64 b ± 0.05	82%	0.89 ab ± 0.03	86%
	10 g/L	1.83 cd ± 0.08	75%	1.68 bc ± 0.10	73%	1.41 bc ± 0.09	71%	0.76 b ± 0.07	73%
	20 g/L	1.61 d ± 0.05	66%	1.45 c ± 0.10	63%	1.13 c ± 0.04	57%	0.49 c ± 0.01	48%

Values are means of three replicates ± standard error (and percentage of control). Values followed by different letters are significantly different (one-way ANOVA, $p = 0.05$).

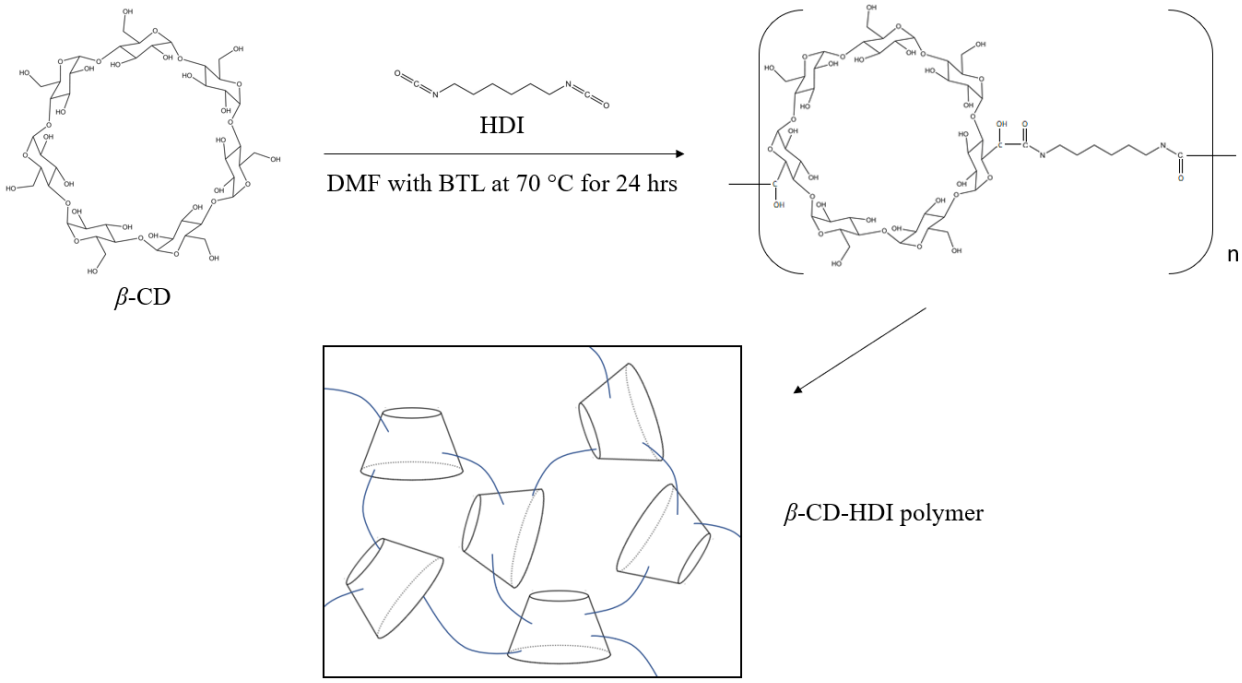

Figure 1. Preparation of *β*-CD-HDI polymer and its hypothetical structure.

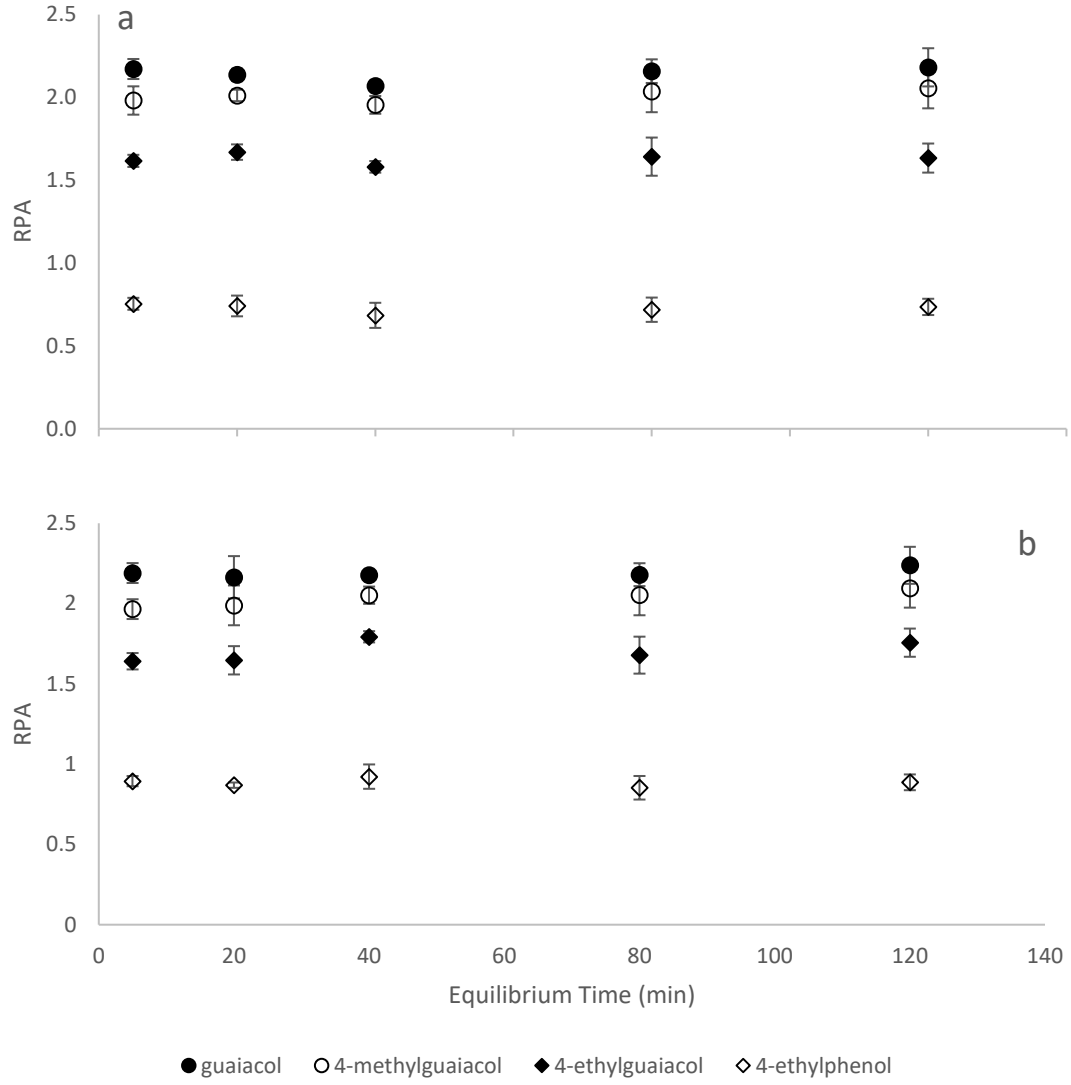

Figure 2. Equilibrium time for binding of volatile phenols by CD-HDI polymers expressed as relative response area (RPA): (a) *β*-CD-HDI and (b) *γ*-CD-HDI. Values were not significantly different (one-way ANOVA, $p = 0.05$). Values are means of three replicates ± standard error.

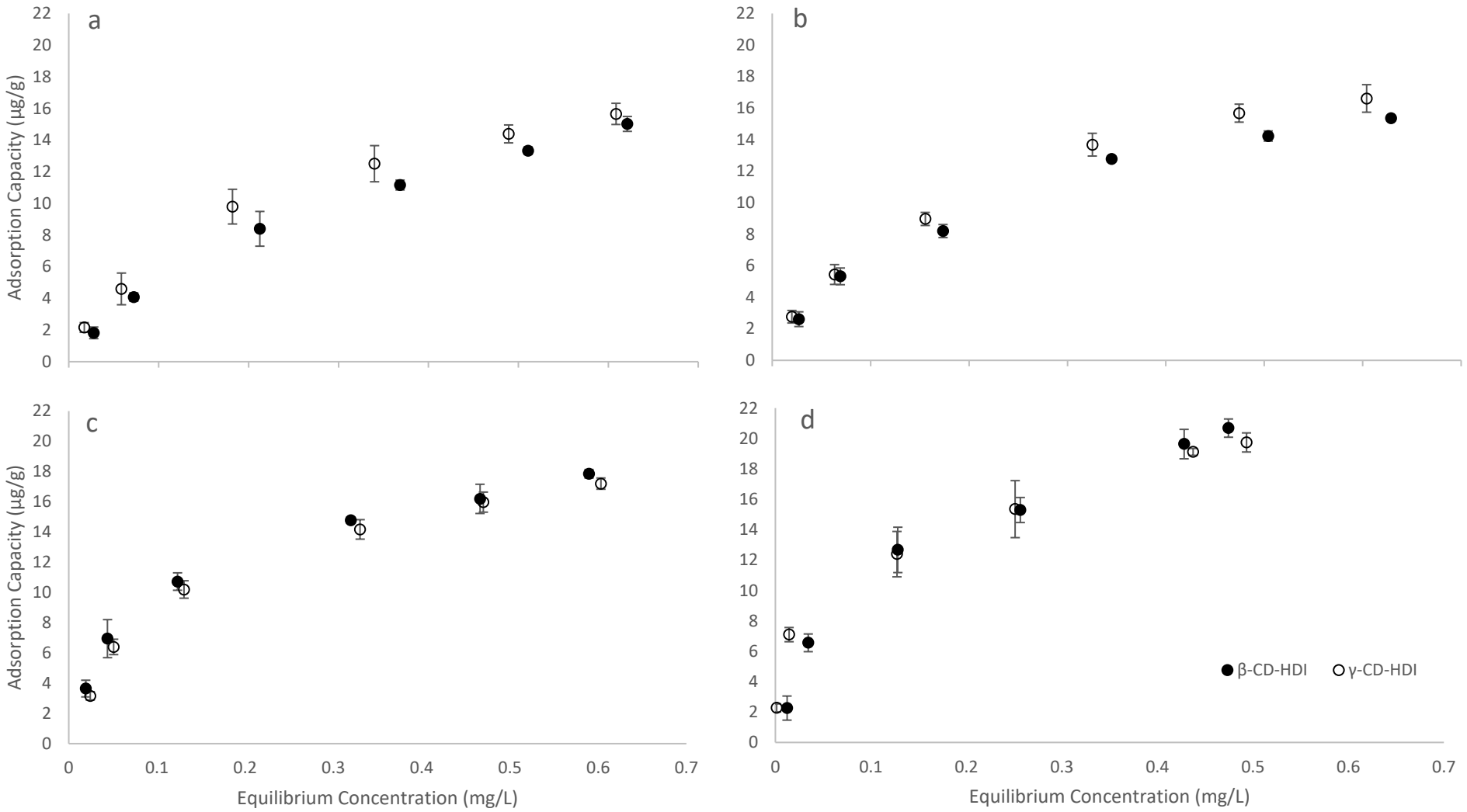

Figure 3. Adsorption capacity of (a) guaiacol, (b) 4-methylguaiacol, (c) 4-ethylguaiacol and (d) 4-ethylphenol by *β*-CD-HDI and *γ*-CD-HDI polymers in model wine at pH 3.5 and 25 °C. Values are means of three replicates ± standard error.

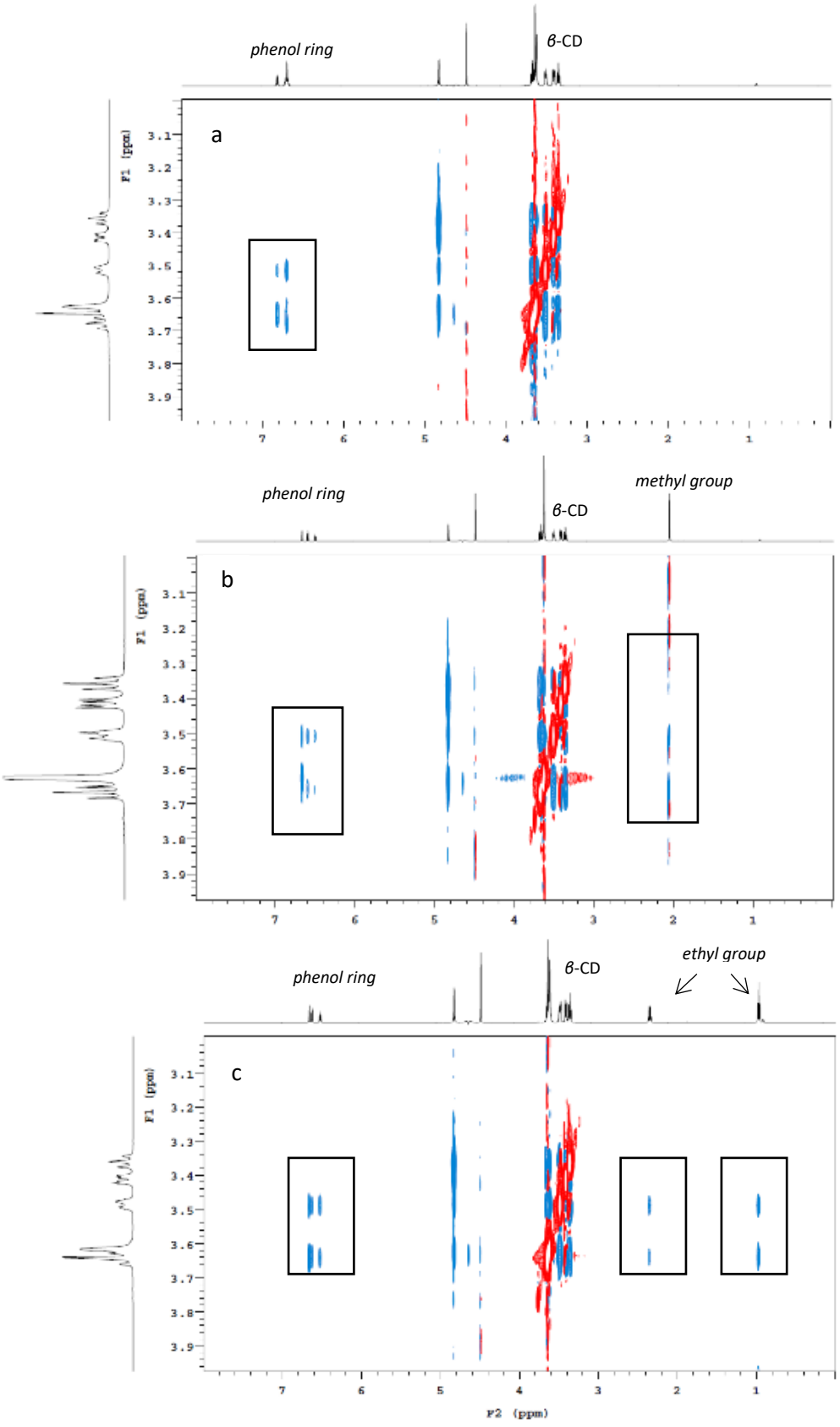

Figure 4. ^{1}H 2D ROESY NMR (600 MHz, pD 3.5 and 25 °C) spectrum of a deuterated model wine solution of *β*-CD with (a) guaiacol, (b) 4-methylguaiacol and (c) 4-ethylguaiacol. Rectangles indicate the cross-peaks arising from NOE interactions between protons of volatile phenols and the *β*-CD cavity.

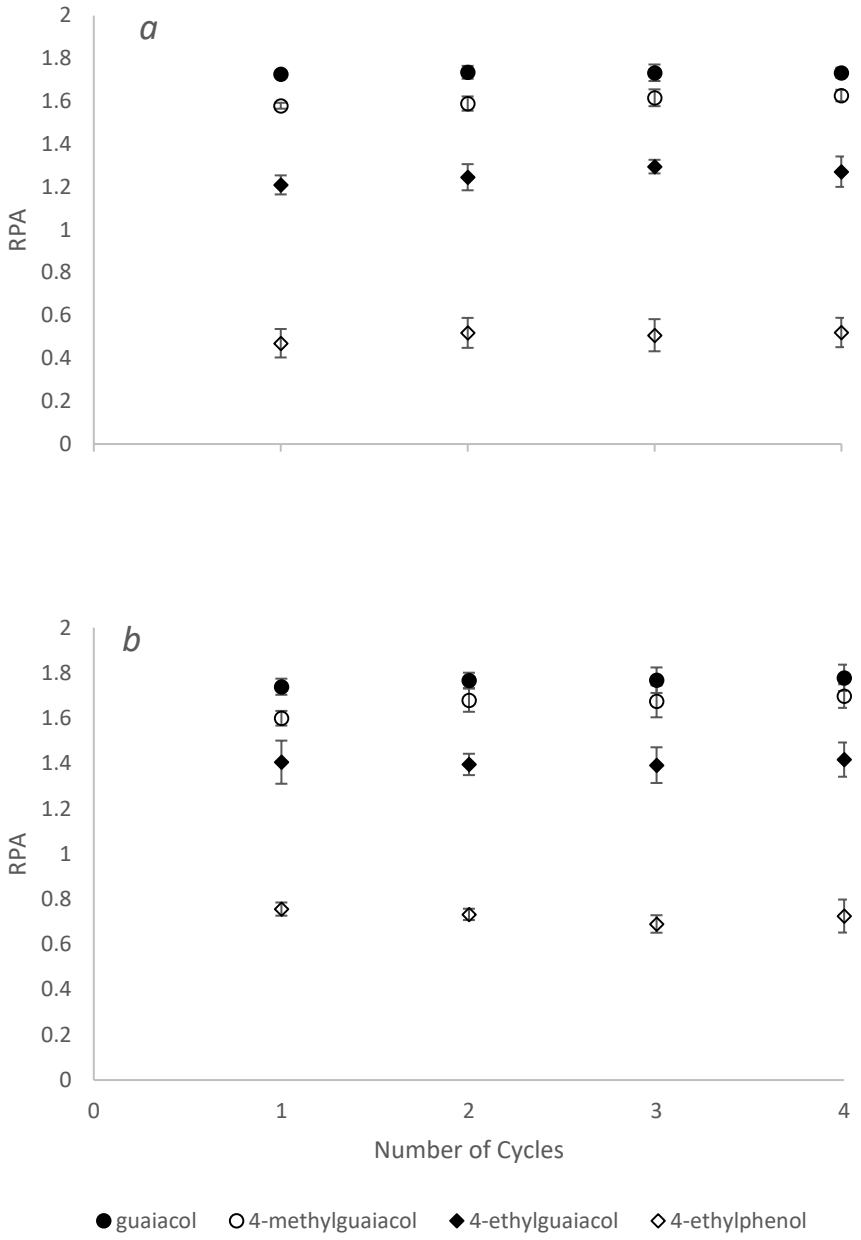

Figure 5. Reusability of CD-HDI polymers expressed as relative response area (RPA): (a) β-CD-HDI and (b) γ-CD-HDI. Values are means of three replicates ± standard error. Values are not significantly different (one-way ANOVA, $p = 0.05$).

CHAPTER 4

Amelioration of smoke taint in wine by cyclodextrins and crosslinked cyclodextrin polymers

Statement of Authorship

Title of Paper	Amelioration of Smoke Taint in Wine by Cyclodextrins and Crosslinked Cyclodextrin Polymers
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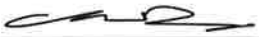
Principal Author

Name of Principal Author (Candidate)	Chao Dang		
Contribution to the Paper	Designed and conducted experiments; collected, processed, analysed, and interpreted data; drafted and edited manuscript.		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	01-12-18

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Amelioration of Smoke Taint in Wine by Cyclodextrins and Crosslinked Cyclodextrin Polymers

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ABSTRACT

Smoke taint is an off-odor in wine caused by grapevine exposure to smoke from bushfires or prescribed burns. Elevated concentrations of volatile phenols have been found to contribute to the taint, which can lead to rejection of fruit or wine due to loss of quality. The current study investigated the potential for β- and γ-cyclodextrins (CDs) and crosslinked CD polymers to ameliorate smoke taint following addition to grape must or wine. Chemical and sensory analysis was performed on smoke affected Merlot wines and showed that volatile phenols were effectively retained or removed by CDs and CD polymers, with the headspace concentration of volatile phenols decreasing to between 32 and 67% of their initial levels depending on the treatment. These results were better than that achieved following addition of charcoal or PVPP at recommended doses. There were some losses of desirable aroma compounds, which was reflected by significant differences in the overall aroma intensity of wines, based on sensory analysis. Pre- and post-fermentation treatments were also applied to smoke-affected Sauvignon Blanc fermentation, with pre-fermentation treatment showing better preservation of desirable aroma compounds, whilst still reducing the level of volatile phenols. Volatile phenol glycoconjugates were also removed from finished wine by β-CD polymer addition at 5% w/v dose, albeit only a small proportion of the overall glycoconjugate pool.

Keywords: cyclodextrin, cyclodextrin polymer, GC-MS, LC-MS, smoke taint, wine, wine aroma

INTRODUCTION

Around the world, an increasing number of bushfires have been reported in proximity to wine regions in recent years, as a consequence of the changing climate[1-2]. The potential occurrence of fire events has resulted in smoke taint becoming an ongoing concern for the wine industry, given the substantial impact on grape and wine quality, which can result in significant financial losses for producers[3]. Numerous studies have been conducted, predominantly in Australia, to gain an understanding of the impact of smoke taint through field trials on grapevines and fermentation trials using smoked grapes[4-7]. It has previously been established that elevated concentrations of volatile phenols, including guaiacol, 4-methylguaiacol, cresols and syringol are predominantly responsible for smoke taint in wine, and contribute to the unpleasant smoky and ashy aromas[6, 8]. Some of these volatile phenols are also associated with *Brettanomyces* related off-odors in wine[9]. Recent studies indicate that volatile phenols accumulate in smoke affected grapes in glycoconjugate forms[10]. Hydrolysis of these glycoconjugates during fermentation and/or storage can release additional volatile phenols[7, 11-12]. As such both free and bound forms of volatile phenols need to be considered to avoid under-estimation of the severity of smoke taint.

Researchers have developed novel analytical methods for detecting smoke taint[13]. Headspace gas chromatography-mass spectrometry (HS GC-MS) is predominantly used for measuring volatile phenols[4, 13-14], whereas liquid chromatography-mass spectrometry (LC-MS) has been used to detect and quantify a number of volatile phenol glycoconjugates[11, 15].

Various techniques have been evaluated as strategies for mitigating smoke taint in grapes and wine. Early advice encourage industry to minimize extraction of smoke taint related compounds by avoiding the inclusion of leaf material[16], whole-bunch pressing fruit[3], and

reducing the degree of pressing[5, 7]. Different yeast strains were found to release different amounts of volatile phenols[5]. Other studies investigated the removal of volatile phenols from wine using various methods, including reverse osmosis in combination with adsorption resin[17], the addition of fining agents[3, 18], yeast autolysis[19], polyaniline based polymer[20], molecularly imprinted polymers[21], or esterified cellulose[22].

This study aims to evaluate the use of cyclodextrins (CDs) for the amelioration of smoke taint in wine. Inspired by studies in other fields which have exploited the ability of CDs and CD polymers to encapsulate or remove hydrophobic compounds[23-27], the current study investigated the encapsulation of volatile phenols by CDs and crosslinked CD polymers. It has previously been established that CDs and CD polymers can retain or remove volatile phenols from red wine[24], lowering their headspace concentration and sensory impact. However, the overall influence on wine aroma profiles has yet to be investigated.

CDs are able to encapsulate a wide variety of compounds within the cavity of their truncated cone structure[28]. In aqueous based solutions, this process occurs by replacing the enthalpy rich water molecule with a hydrophobic guest molecule through the formation of non-covalent bonds between the guest and the inner surface of the CD cavity[29]. Hydrophobicity, size and conformation of the guest molecule have been shown to influence the selectivity of CDs, and a range of studies have been conducted, demonstrating encapsulation of aroma and flavor compounds by CDs[25, 30-36]. It is reasonable to assume, based on the literature, that CDs and CD polymers can therefore influence wine aroma by encapsulating volatile phenols so as to ameliorate smoke taint.

To date, the use of CDs during the fermentation process has not been well studied, and the potential influence on yeast remains largely unknown. Early studies suggested the addition of *β*-CD improved the fermentative performance of *Saccharomyces cerevisiae*[37-40], but did not consider ameliorate properties.

In the current study, the potential for *β*-CD, *γ*-CD and their polymers to ameliorate smoke taint was evaluated both during fermentation and in finished wine, with consideration of the overall influence of treatment on the wine aroma profile.

MATERIALS AND METHODS

Chemicals. Food grade (>98% purity) CDs were supplied by IMCD Group (Adelaide, SA, Australia). Analytical grade reagents used for producing CD polymer were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia) and Chem-supply (Adelaide, SA, Australia). All analytical grade (purity > 97%) reference standards of aroma compounds for GC-MS analysis were also purchased from Sigma-Aldrich. Deuterium-labelled HS-SPME GC-MS normalizing standards were acquired from CDN Isotopes (Pointe-Claire, Quebec, Canada). All analytical grade reagents used for the synthesis of the guaiacol *β*-glucoside were purchased from Sigma-Aldrich. Labelled glycoconjugate internal standard (*d*3-syringol-*β*-D-gentiobioside) for LCMS analysis was synthesized at the Australian Wine Research Institute (Adelaide, SA, Australia). NMR solvents (d_6-ethanol, D_2O and DCl) were purchased from Sigma-Aldrich. Volatile phenol and deuterated standard stock solutions were prepared in pure ethanol (Thermo Fisher Scientific, Waltham, MA, USA) and stored at –20 ºC. Working solutions were prepared in model wine (containing 5 g/L of tartaric acid and 12% v/v ethanol) and stored at –4 ºC.

Preparation of CD Polymers. CD polymers were synthesized using hexamethylene diisocyanate (HDI) as the crosslinker according to the methodology previously reported[26], but with modification as outlined Chapter 3 of this thesis.

Smoke Tainted Wine Samples. A smoke-affected Merlot wine was available from a previous trial[41]. Merlot grapes were sourced from vineyards in the Adelaide Hills that were subjected to one hour of smoke treatment, a week after veraison. The wine was made following small batch wine making protocols, without secondary fermentation[42]. Finished wine was packaged in 375 mL bottles under screw cap, stored at 15 °C in darkness. Bench top sensory trials confirmed the Merlot wine had high levels of smoke taint at the time of current study.

A total of 10 treatments were applied to the smoke-affected Merlot wine: β-CD and γ-CD addition at two concentrations of 5 or 20 g/L; β-CD-HDI and γ-CD-HDI polymer addition at rates of 1 or 5% w/v; activated charcoal addition at 2 g/L; and PVPP addition at 0.5 g/L. Samples treated with CDs were heated at 35 °C for 15 min to ensure the CDs completely dissolved, while those treated with polymer and fining agents were shaken at room temperature (21 °C) for 15 min before centrifugation (2,000 g for 5 min) to facilitate separation. All samples were prepared in triplicate.

Wine Making Trial. Sauvignon Blanc grapes were sourced from a vineyard in the Orange region (NSW, Australia), which experienced a bushfire that burned between the 10th and 14th of February 2018. The fruit was thought to have been exposed to smoke during this time and was harvested at maturity on 20th of March. Grapes (around 300 kg) were whole bunch pressed, and the juice was placed in a stainless-steel tank to settle at 3 °C for four days. About 50 mg/L

potassium metabisulfite (PMS) was added. The juice was then portioned into 10 L plastic containers, frozen and transported from Orange to Adelaide overnight by refrigerated freight.

The wine making procedure was based on that described by Holt and colleagues[42] with some modifications. Briefly, the juice was thawed and racked off sediment. Juice analysis confirmed total soluble solids of 13.6 °Baumé and a pH of 3.15. A 10 L juice sample was divided into 17 experimental treatments, including a control, established in triplicate (Figure 1). Each comprised 150 mL of juice ferment in a 250 mL Schott bottle equipped with a fermentation airlock. Pre-fermentation treatments were applied to juice with β-CD and γ-CD added at 5 or 20 g/L, and β-CD-HDI and γ-CD-HDI polymers at 1 or 5% w/v dose rates. Treatment preparation was the same as for Merlot wines. Prior to fermentation, 270 mg/L of diammonium phosphate (DAP) and 55 mg/L of PMS were added. Then, PDM yeast (Maurivin, QLD, Australia) was rehydrated with warm water and added to the samples to give a 300 mg/L dose for inoculation. Fermentation temperatures were maintained at 25 °C. Sugar levels were monitored with a hydrometer daily until the end of fermentation. After 10 days, all fermentations were considered to be finished, with sugar levels below 2 g/L (Clinitest®, Bayer, Pymble, NSW, Australia). The post-fermentation treatments were then applied following the same method as outlined above for Merlot wine. Eventually, all wines were decanted into 50 and 100 mL glass bottles with nitrogen sparged on top of the liquid phase. Samples were stored at 4 °C in darkness before analysis.

Four-Phase HS-SPME GC-MS Analysis. Conventional three-phase HS-SPME GC-MS was not able to distinguish the influence of CD addition to the sample due to the binding of the deuterated normalizing standard (as described in Chapter 2). Hence the four-phase HS-SPME GC-MS method, which uses an additional liquid phase to isolate the normalizing standard from

the CD treatment, was used. Selected Ion Monitoring (SIM) mode was set up to record the mass spectra of target ions for both the normalizing standards and 28 aroma compounds, including 8 volatile phenols. The selection of quantifying and qualifying ions was based on the report by Wang and colleagues[43]. Identification of aroma compounds was achieved through comparison of retention time and mass spectra of authentic standards with reference to the Wiley Library database. All GC-MS data were expressed as relative peak area (RPA) of the quantifying ion of the target compound compared to *d*3-guaiacol as the normalizing internal standard. The modification of the extraction method was intended to minimize absorption of the headspace normalizing standards by the sample, so as to overcome errors associated with fiber aging and inter-fiber variation. Aroma compounds were quantified using the modified HS-SPME GC-MS method with external calibration curves of corresponding standards. The percentage of remaining headspace concentration of aroma compounds was calculated based on RPA.

HPLC-MS Analysis of Phenol Glycoconjugates. The concentration of volatile phenol glycosides present in the Sauvignon Blanc wines were determined using an HPLC-MS method previously described by Hayasaka and co-wokers[15] with modification. Briefly, juice samples with treatments and the control were centrifuged at 2000 g for 5 min to give a 2 mL aliquot of the supernatant to be loaded onto an SPE cartridge (Extract Clean C18-HF, Grace Davison Discovery Sciences, Australia). After washing with 10 mL of water, the adsorbed contents of the cartridge were eluted with 1 mL of methanol. The mixture was dried under nitrogen at 40 °C, and the extract was reconstituted with 0.3 mL of water. The resulting solution was filtered (0.45 μm) ahead of analysis. Finished wine samples were also centrifuged to give 1 mL of supernatant that was transferred to sample vials for analysis. Each juice or wine sample contained *d*3-syringol-β-D-gentiobioside (*d*3-syringol-GG) as an internal standard (at 2 mg/L).

The HPLC-MS instrument settings were as described by Hayasaka and colleagues[15]. Quantification was achieved using an external calibration curve of the syringol-GG standard, hence concentrations of all glycosides are expressed as syringol-GG equivalent.

Synthesis of guaiacol-*β*-D-glucopyranoside. Guaiacol-*β*-D-glucopyranoside was prepared for nuclear magnetic resonance (NMR) analysis as a *β*-CD complex. The synthesis method was based on previous works[11, 44] with modifications. Briefly, 2,3,4,5-tetra-*O*-pivaloyl-*α*-D-glucopyranosyl bromide was mixed with guaiacol at a 1:1 molar ratio in dichloromethane containing silver triflate and 2,6-lutidine at a 1.5 molar ratio to guaiacol. The mixture was stirred in darkness for 16 hours at room temperature before adding saturated sodium bicarbonate solution to halt the reaction. The resulting product was extracted in 2 x 15 mL addition of dichloromethane and washed with 2 x 15 mL brine. The crude product was purified using a silica column and eluted by hexane containing 20% ethyl acetate. The resulting white crystal was re-dissolved in methanol, followed by addition of sodium metal dissolved in methanol solution (36 mg of sodium dissolved in 5 mL of methanol). Acidified Amberlite IRC-50 (H) resin was added to the mixture to protonate the resulting glucopyranoside. Guaiacol-*β*-D-glucopyranoside was then filtered and re-crystallized in ethanol.

Nuclear Magnetic Resonance (NMR) Analysis. The potential encapsulation of guaiacol-*β*-D-glucopyranoside by *β*-CD was investigated by 2-dimensional NMR rotating frame Overhauser effect spectroscopy (^{1}H 2D ROESY) using an Agilent DD2 600 MHz spectrometer (Agilent Technologies, Santa Clara, CA, USA) connected to a cryoprobe, operating at 600 MHz with a delay time of 300 ms. The sample solution was a deuterated model wine (12% d_5-ethanol in D_2O, pD adjusted to 3.5 with DCl) containing 10^{-3} mol/L of guaiacol-*β*-D-glucopyranoside and 10^{-2} mol/L of *β*-CD.

Sensory Evaluation of Merlot Wines. A paired comparison test was conducted to evaluate the intensity of smoky aromas and overall aroma intensity of the treated Merlot samples. A total of 26 assessors, comprising research students (13 male and 13 female, aged between 22 and 36 years) from the wine science department of the University of Adelaide, participated in the test. Two groups of samples were prepared with each group comprising 4 brackets of wine, featuring addition of β- and γ-CD at two concentrations of 5 or 20 g/L, respectively. Each bracket comprised two wines, i.e. a control and a treated wine, presented in black tasting glasses, designated with random 3-digit codes. The order of presentation for each assessor was also randomized. Assessors were presented with one bracket at a time and only smelled samples. For the first group, panelists were asked to identify the wine that exhibited more intense "smoky" aroma, whereas for the second group, panelists identified the wine with higher "overall aroma intensity". Sensory data were recorded on answer sheets.

Basic Analysis of Merlot Wines. The influence of CD and CD polymer treatments on the pH and color of Merlot wines was evaluated in triplicate. A Cintra 2020 (GBC Scientific Equipment, Braeside, VIC, Australia) spectrophotometer was used to record spectra data at A_{420}, A_{520} and A_{620} (where A_λ represents the absorbance at wavelength λ). Wine color intensity was calculated as a sum of the absorbance. Wine pH was measured using a Mettler Toledo pH meter (Melbourne, VIC, Australia).

Data analysis. Chemical data are presented as mean values of three replicates ± standard error. One-way ANOVA was conducted to determine differences between sample means, with a T-test at $p = 0.05$, using XLSTAT software (version 2015.3, Addinsoft, Paris, France). The least

number of correct responses for significant difference in the sensory evaluation was determined using a *p* value of 0.05.

RESULTS AND DISCUSSION

Optimization of the Four-Phase HS-SPME GC-MS. The conventional HS-SPME method includes three phases during extraction, i.e. the sample liquid, headspace, and fiber coating[45], whereas the new HS-SPME method comprised an additional liquid phase that contained the normalizing standard, thereby complicating the equilibrium of the overall system. The purpose of using a normalizing standard and RPA in HS-SPME GC-MS analysis was verified by multiple studies to overcome the fiber age and inter-fiber variation problem[46-48]. As shown in Chapter 2, conventional HS-SPME GC-MS analysis results in inclusion of the normalizing standards by the CD additive, which affected both the signals for the compounds of interest and the standard, exhibiting no apparent differences in relative peak areas (RPAs) between the treated and control samples. This problem was solved by introducing another liquid phase containing the normalizing standard.

When using the HS-SPME method for quantification purposes, the response was correlated to the headspace concentration of target compounds[49], which reached equilibrium prior to analysis. With the new four-phase method, a certain volatile compound is distributed in three phases at equilibrium prior to the extraction, which can be described as:

$$C_0*V_s = C_s*V_s + C_i*V_i + C_h*V_h$$

with two partition constants involved:

$$K_s = C_s/C_h$$

$$K_i = C_i/C_h$$

where C_0 is the starting concentration of volatile compound in the sample, V_s is the sample volume, C_s is the equilibrium concentration in the sample phase, C_i is the equilibrium concentration in the inserted phase, V_i is the volume of inserted liquid phase, C_h is the equilibrium concentration in the headspace, V_h is the volume of headspace, K_s is the partition constant of the compound between sample phase and headspace, and K_i is the partition constant of the compound between inserted phase and headspace.

Adopting the K equations, the equilibrium equation can be rearranged as:

$$C_0*V_s = C_h*K_s*V_s + C_h*Ki*V_i + C_h*V_h$$

and C_h can be expressed as:

$$C_h = C_0*V_s/(V_s*K_s + K_i*V_i + V_h)$$

The original four-phase HS-SPME method developed in Chapter 2 adopted volumes of 6 and 0.5 mL for the sample and the normalizing standard, respectively. This method provided excellent repeatability and reproducibility. However, this method assumed a perfect scenario, where the absorption of the normalizing standard by CDs added to the sample was small and therefore neglected. The success of *d*3-guaiacol as a normalizing standard as reported in Chapter 2 proved it to be a suitable candidate, but when there was no availability of such a standard, other compounds need to be considered. The absorption of the normalizing standard should be addressed when using various compounds, especially those that are highly reactive with CDs.

When CD was added to the sample, the partition constant (K_s) of a susceptible volatile compound between sample phase and headspace increases. According to the C_h equation, if the changes of C_h with increasing K_s are to be mitigated, reducing sample volume to close to zero

can be effective. A series of trials (data not shown) were conducted with various isotopically labelled normalizing standards (including *d*3-hexyl acetate, *d*5-ethyl nonanoate, *d*13-hexanol, *d*3-4-methylguaiacol, *d*3-guaiacol and *d*4-4-ethylphenol) using different sample volumes, so as to test absorption of normalizing standard by the sample mixture. Results showed that 0.2 mL sample volume gave least absorption of the standards and provided excellent repeatability and reproducibility. In the current study, *d*3-guaiacol was used as the normalizing standard for all quantification analyses.

Retention/Removal of Volatile Phenols. The residual levels of volatile phenols following the addition of various amelioration are shown in Table 1. The smoke-affected Merlot wine initially contained 143.4 µg/L of total volatile phenols, with guaiacol, *m*-cresol and 4-methylguaiacol being the most abundant, at 73.7, 21.4 and 18.7 µg/L, respective, i.e., concentrations that exceeded these compounds' perception thresholds[4, 8]. The treatments were found to be able to remove volatile phenols from the wine sample, with most CD and CD polymer treatments giving significantly different residual volatile phenol levels, except for guaiacol and 4-methylguaiacol removal at several lower dosage rates. The headspace residual of total volatile phenols decreased to 52 and 47% following *β*-CD addition at 5 and 20 g/L, respectively, and to 66 and 42% following *γ*-CD addition at 5 and 20 g/L. For polymer addition, volatile phenol levels decreased to 67 and 50% by *β*-CD-HDI polymer dosage at 1 and 5% w/v, and to 66 and 32% after *γ*-CD-HDI polymer was added. The two commercial fining agents, charcoal and PVPP, however, achieved no significant removal of volatile phenols at their recommended doses, with the exception of *m*-cresol following charcoal treatment.

The preference of CDs for individual volatile phenols was consistent with the results described in previous chapters, i.e., *β*-CD and its polymer were more effective at removing cresols, 4-

ethylguaiacol and 4-ethylphenol, whereas γ-CD and its polymer showed greater encapsulation with guaiacol and 4-methylguaiacol. Overall, β-CD had greater inclusion capability due to its more rigid structure, which leads to higher hydrophobicity[28]. However, the structure and size of the guest molecule also plays a role in the inclusion reaction, i.e. the methoxy group present on guaiacol and 4-methylguaiacol hinders these molecules from entering into the β-CD cavity, but not the γ-CD cavity.

Impact of Treatment on Other Aroma Compounds. The impact of CD and CD polymer treatments on several other aroma compounds was considered (Table 2, Table 3, Supplementary Table S2). It appeared that esters were less prone to retention by β-CD than γ-CD, whereas higher alcohols and fatty acids were readily encapsulated. At higher doses, the residual concentrations of ethyl hexanoate, ethyl octanoate and isoamyl acetate were above 90% following β-CD addition at 20 g/L, whereas isoamyl acetate was not significantly different from the control. In contrast, γ-CD addition significantly reduced levels of ethyl hexanoate and isoamyl acetate (to 75% and 84%, respectively). Most higher alcohols and fatty acids were retained to a greater extent by β-CD, yielding residual concentrations at 45-67%, compared to 70-100% residual concentrations by γ-CD treatment. Exceptions were isobutyl alcohol and butanoic acid, which were not significantly different after addition of either CD, even at high doses. In terms of CD polymers, at higher doses, β-CD-HDI generally had more impact on aroma compounds. It appeared that polymerization changed the behavior of β-CD, enabling more readily formed inclusion complexes with esters, but less inclusion with fatty acids. At their recommended doses, charcoal and PVPP had little impact on any of the aroma compounds, including the volatile phenols. Only concentrations of hexanol (88%), butanoic acid (68%), and octanoic acid (98%) were significantly different after charcoal addition, and octanoic acid (76%) after PVPP addition.

The literature suggests that hydrophobicity, size and structure of the guest molecule are key factors for the formation of CD inclusion complexes[23, 30, 50]. The percentage of headspace aroma compounds remaining after the addition of 20 g/L CD and the calculated Log P value (acquired from the PubChem Project: https://pubchem.ncbi.nlm.nih.gov/) were plotted to give Figure 2. Although only 15.6% and 27.7% of the difference could be explained by the Log P value when all compounds were plotted, higher correlations were found within individual chemical classes. For example, the R^2 values for regression of the plots with β-CD were 0.809, 0.859, 0.774 and 0.987 for volatile phenols, esters, alcohols and acids, respectively. The partial explanation of CD complexation by hydrophobicity has been discussed in previous studies already[30, 51].

Impact of CD Treatments on Wine pH, Color and Aroma. No significant differences in pH were found between wines following the different treatments (Table 4). Color intensity, however, was significantly influenced, except for addition of γ-CD. PVPP reduced the color intensity by as much as 53%, whereas other treatments only gave up to 20% color variation. The color pigments in wine are mainly composed of monomeric and polymerized flavonoids[52]. Complexation between β-CD and flavonoids was reported decades ago, including the ability of β-CD to bind with anthocyanins[53] and other flavonoids[54], so the results obtained in the current study were not unexpected.

No significant difference in smoky aroma intensity were observed between control wines and wines treated with CDs at 5 g/L. However, significant differences in the overall aroma intensity were observed. When CD additions were made at 20 g/L, significant differences in both the intensity of smoke aroma and overall aroma were perceived. Theses results demonstrate the

need for higher dose rates, but also highlight the impact of treatment on desirable wine volatile compounds.

Pre-Fermentation *vs* Post-Fermentation Treatments. The smoked Sauvignon Blanc fruit had much lower levels of volatile phenols than expected, with volatile phenol concentrations all below 10 µg/L and no obvious taint character perceived during benchtop sensory trials. The concentrations of volatile compounds by chemical classes are presented in Table 5 (with data for individual compounds reported in Supplementary Table S2). It is interesting to note from the percentage residual values that pre-fermentation treatments with the CD polymers yielded similar results, compared to the post-fermentation treatments. This was unexpected considering the potential release of volatile phenols from glycoconjugates during fermentation[5, 7], albeit this may reflect low levels of smoke taint in these samples.

Overall, pre-fermentation treatments had less impact on the overall aroma profile compared with the post-fermentation treatments. Ethyl butyrate, ethyl hexanoate, ethyl octanoate, isoamyl acetate, isobutyl alcohol, butanoic acid, hexanoic acid and octanoic acid showed no significant difference between the pre-fermentation treatments. Ethyl butyrate, isobutyl alcohol, butanoic acid and hexanoic acid also showed no significant differences between post-fermentation treatments, whereas all other compounds were significantly different in post-fermentation treatments. Diethyl succinate was the only compound that gave significantly different values for pre-fermentation treatment vs post-fermentation treatments. There was no significant increase in aroma volatile concentrations following treatment of juice, which was consistent with a previous study that used molecularly imprinted polymers to remove methoxypyrazines prior to fermentation[55]. This might reflect the high sugar levels present in juice. The observed differences were all negative, judging from the percentage of residual. The

pre-fermentation treatments had the most negative impact on diethyl succinate, followed by hexanol and isoamyl alcohol, whereas post-fermentation treatments influenced the concentration of a wider range of compounds with similar results obtained as for the Merlot experiments.

Concentrations of volatile phenol glycoconjugates were monitored by LC-MS, with six disaccharides being quantified, including syringol-gentiobioside (SyGG), cresol-arabinosyl-glycoside (CrRG), guaiacol-arabinosyl-glycoside (GuRG), 4-methylguaiacol-arabinosyl-glycoside (MGuRG), 4-methylsyringol-gentiobioside (MSyGG) and phenol-arabinosyl-glycoside (PhRG). Glycoconjugate levels in Sauvignon Blanc juice and wine after CD polymer treatments are shown in Table 6. The juice initially contained 118 µg/L of total volatile phenol glycoconjugates, which decreased to 93.1 µg/L after fermentation, presumably due to enzyme and acid hydrolysis[7]. *β*-CD-HDI and *γ*-CD-HDI polymers were applied to the juice at 1 and 5% w/v levels. Only SyGG was found to be significantly different after treatment, with around 10% removed by *β*-CD-HDI at low and high doses. The same treatments were also applied to the Sauvignon Blanc wine and yielded different results. The concentrations of four disaccharides were found to be significantly different after addition of 5% *β*-CD-HDI polymer; SyGG, CrRG, GuRG and MGuRG concentrations were reduced by 22, 28, 32 and 22%, respectively. The total glycoside removal by 5% *β*-CD-HDI polymer treatment was 24% of the control.

Guaiacol-*β*-glucoside was synthesized, and its binding with *β*-CD in model wine was analyzed by ^{1}H 2D ROESY NMR (Figure 3). Analysis of the NMR spectra was complicated by the overlap of *α*-glucose signals from the CD and *β*-glucose signals from the glucoside. However, from comparisons of NMR spectra with and without *β*-CD addition, it was clear that cross

peaks arose due to complexation between the phenol ring of the glucoside and the *β*-CD, which explains the partial removal of the glycoconjugates by *β*-CD-HDI polymer.

In summary, it is clear CDs and CD polymers can retain/remove volatile phenols from juice or wine, either before or after fermentation. It is unclear to what extent CD treatments influenced yeast activity, but the reduced impact on wine aroma observed with pre-fermentation treatments was expected, given most of the wine aroma compounds measured, i.e., higher alcohols, fatty acids and esters, are derived from yeast activity during fermentation[56]. CD polymers appeared to be more aggressive in removing volatiles than CDs, regardless of whether activity was towards volatile phenols or other desirable aroma compounds. The *β*-CD-HDI removed a small amount of volatile phenol glycoconjugates from wine but was not effective in juice. In general, there are advantages with the use of polymers, including the potential to increase dose rates without having CD residues. CD polymers could also be used in combination with other techniques, such as reverse osmosis. On a practical level, the amelioration of smoke taint remains trade-off between retaining/removing smoke-derived volatile phenols and preserving desirable aroma volatiles. Benchtop trials should therefore be conducted to optimize treatment doses as would normally be performed as part of fining trials.

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Table 1. Headspace residual concentrations (µg/L) of volatile phenols in smoke-affected Merlot wines.

	Control	**Dissolved CD treatment**				**CD polymer fining treatment**			**Other fining agents**		
	(i.e., no treatment)	*β*-CD 5 g/L	*β*-CD 20 g/L	*γ*-CD 5 g/L	*γ*-CD 20 g/L	*β*-CD-HDI 1% w/v	*β*-CD-HDI 5% w/v	*γ*-CD-HDI 1% w/v	*γ*-CD-HDI 5% w/v	charcoal 2 g/L	PVPP 0.5 g/L
volatile phenols											
guaiacol	73.7 a ± 4.3	57.9 bcd ± 1.3 79%	53.8 cd ± 1.4 73%	60.2 abcd ± 0.9 82%	46.6 de ± 3.1 63%	61.5 abc ± 2.1 84%	53.3 cd ± 2.8 72%	54.7 cd ± 1.0 74%	34.3 e ± 4.1 47%	71.5 ab ± 0.7 97%	74.1 a ± 4.9 101%
4-methylguaiacol	18.7 ab ± 2.2	8.2 def ± 0.9 44%	6.8 def ± 0.9 36%	10.5 bcde ± 0.3 56%	5.3 ef ± 0.9 28%	17.2 abc ± 1.3 92%	10.2 cdef ± 0.9 54%	13.7 abcd ± 0.2 73%	2.0 f ± 1.4 11%	19.0 a ± 1.4 102%	20.3 a ± 4.0 108%
4-ethylguaiacol	4.1 a ± 0.0	2.3 bc ± 0.2 56%	1.0 de ± 0.2 25%	2.9 b ± 0.3 70%	1.8 cd ± 0.1 44%	2.6 bc ± 0.4 64%	0.6 e ± 0.1 15%	2.6 bc ± 0.4 64%	1.8 cd ± 0.3 45%	5.0 a ± 0.2 121%	4.6 a ± 0.1 111%
o-cresol	4.3 a ± 0.7	tr	tr	0.3 bcd ± 0.3 7%	tr	tr	tr	0.8 bc ± 0. 1 19%	tr	2.6 ab ± 0.8 61%	4.7 a ± 0.6 110%
m-cresol	21.4 ab ± 0.9	6.5 c ± 0.8 30%	5.9 c ± 0.8 28%	12.0 c ± 0.7 56%	7.0 c ± 0.3 33%	9.5 c ± 1.4 44%	8.3 c ± 0.8 39%	11.0 c ± 0.1 51%	8.1 c ± 1.1 38%	16.9 b ± 0.8 79%	22.8 a ± 1.1 107%
p-cresol	10.7 a ± 1.3	tr	tr	3.9 bc ± 0.5 36%	tr	3.4 bcd ± 1.0 32%	tr	5.1 b ± 0.2 48%	tr	10.1 a ± 1.5 95%	10.1 a ± 1.1 94%
4-ethylphenol	10.5 a ± 0.7	tr	tr	4.4 c ± 0.3 41%	tr	1.7 bc ± 0.6 16%	tr	6.0 c ± 0.2 57%	0.3 e ± 0.4 3%	9.4 ab ± 0.3 89%	11.4 a ± 1.0 109%
total	143.4 ± 10.3	75.0 ± 4.5 52%	67.5 ± 5.7 47%	94.0 ±3.2 66%	60.7 ± 6.3 42%	96.0 ± 7.1 67%	72.4 ± 5.4 50%	94.0 ± 2.3 66%	46.5 ± 7.7 32%	134.6 ± 5.7 94%	147.9 ± 12.9 103%

Values are means of three replicates ± standard error (n = 3) and residuals as a percentage of the control.
Values followed by different letters (within a row) indicate statistical significance (one-way ANOVA, $p = 0.05$).
tr = trace levels (i.e., signal detected but below limit of quantification)

Table 2. Headspace residual concentrations (µg/L) of selected aroma volatiles in smoke-affected Merlot wines.

	Control	**Dissolved CD treatment**				**CD polymer fining treatment**				**Other fining agents**	
	(i.e., no treatment)	*β*-CD 5 g/L	*β*-CD 20 g/L	*γ*-CD 5 g/L	*γ*-CD 20 g/L	*β*-CD-HDI 1% w/v	*β*-CD-HDI 5% w/v	*γ*-CD-HDI 1% w/v	*γ*-CD-HDI 5% w/v	charcoal 2 g/L	PVPP 0.5 g/L
ethyl hexanoate	355.8 ab ± 9.4	331.3 abcd ± 12.3 93%	323.1 bcd ± 12.3 91%	303.3 def ± 4.4 85%	266.9 fg ± 10.9 75%	277.5 efg ± 6.4 78%	226.1 h ± 0.8 64%	309.5 cde ± 5.1 87%	264.1 g ± 10.9 74%	345.9 abc ± 2.9 97%	365.1 a ± 3.8 103%
ethyl octanoate	80.6 a ± 0.3	80.3 a ± 0.3 100%	78.4 bc ± 0.2 97%	78.8 b ± 0.4 98%	75.5 d ± 0.4 94%	78.8 b ± 0.3 98%	72.5 e ± 0.0 90%	77.1 c ± 0.3 96%	73.1 e ± 0.2 91%	80.6 a ± 0.3 100%	80.7 a ± 0.2 100%
isoamyl acetate	296.8 ab ± 9.3	305.4 ab ± 10.4 103%	269.6 ab ± 13.6 91%	297.5 ab ± 13.4 100%	241.9 bc ± 8.7 81%	263.7 ab ± 4.2 89%	196.2 c ± 11.1 66%	264.4 ab ± 3.9 89%	243.3 bc ± 19.3 82%	314.9 a ± 1.2 106%	307.4 a ± 13.0 104%
isobutyl alcohol [a]	100.9 a ± 2.4	100.0 a ± 2.4 99%	100.1 a ± 5.7 99%	99.4 a ± 1.4 98%	94.1 a ± 2.0 93%	91.1 a ± 4.8 90%	86.2 a ± 1.7 85%	99.9 a ± 3.0 99%	99.1 a ± 3.5 98%	99.8 a ± 1.6 99%	100.1 a ± 2.2 99%
isoamyl alcohol [a]	457.3 a ± 9.7	419.3 abc ± 8.0 92%	308.2 e ± 10.3 67%	410.2 abc ± 1.1 90%	319.8 de ± 1.4 70%	409.8 abc ± 18.5 90%	316.1 bc ± 17.1 69%	371.0 cd ± 9.2 81%	389.6 de ± 9.7 85%	410.5 abc ± 5.4 90%	444.3 ab ± 20.4 97%
hexanol [a]	4.0 a ± 0.0	3.4 bcd ± 0.1 86%	2.4 e ± 0.1 59%	3.6 abc ± 0.1 91%	3.0 d ± 0.0 75%	3.2 e ± 0.1 79%	2.1 cd ± 0.1 53%	3.5 bc ± 0.1 88%	3.3 bcd ± 0.1 83%	3.5 bc ± 0.1 88%	3.7 ab ± 0.0 93%
butanoic acid [a]	3.7 a ± 0.2	3.7 a ± 0.3 101%	3.6 ab ± 0.1 97%	3.8 a ± 0.1 103%	3.8 a ± 0.1 105%	3.0 ab ± 0.1 82%	2.8 ab ± 0.2 78%	3.6 ab ± 0.3 98%	3.8 a ± 0.3 103%	2.5 b ± 0.3 68%	3.0 ab ± 0.2 82%
hexanoic acid [a]	2.1 ab ± 0.1	2.0 ab ± 0.1 99%	1.3 b ± 0.0 63%	2.0 ab ± 0.2 97%	2.1 ab ± 0.1 100%	2.0 ab ± 0.3 99%	1.8 ab ± 0.4 86%	2.0 ab ± 0.1 96%	2.0 ab ± 0.2 96%	2.0 ab ± 0.0 98%	2.2 a ± 0.2 105%
octanoic acid [a]	3.4 ab ± 0.1	2.6 c ± 0.1 75%	1.5 d ± 0.0 45%	2.9 bc ± 0.1 85%	2.4 c ± 0.0 71%	3.3 b ± 0.2 96%	3.4 ab ± 0.2 99%	3.3 b ± 0.1 97%	4.0 c ± 0.2 117%	3.3 b ± 0.1 98%	2.6 c ± 0.1 76%

Values are means of three replicates ± standard error (n = 3) and residuals as a percentage of the control.
Values followed by different letters (within a row) indicate statistical significance (one-way ANOVA, $p = 0.05$).
tr = trace levels (i.e., signal detected but below limit of quantification).
[a] Concentration expressed as mg/L.

Table 3. Headspace residual concentrations (µg/L) of different chemical classes in smoke-affected Merlot wines.

	Control	**Dissolved CD treatment**				**CD polymer fining treatment**				**Other fining agents**	
	(i.e., no treatment)	*β*-CD 5 g/L	*β*-CD 20 g/L	*γ*-CD 5 g/L	*γ*-CD 20 g/L	*β*-CD-HDI 1% w/v	*β*-CD-HDI 5% w/v	*γ*-CD-HDI 1% w/v	*γ*-CD-HDI 5% w/v	charcoal 2 g/L	PVPP 0.5 g/L
volatile phenols	143.4 ± 10.3	75.0 ± 4.5 52%	67.5 ± 5.7 47%	94.0 ± 3.2 66%	60.7 ± 6.3 42%	96.0 ± 7.1 67%	72.4 ± 5.4 50%	94.0 ± 2.3 66%	46.5 ± 7.7 32%	134.6 ± 5.7 94%	147.9 ± 12.9 103%
esters [a]	7.8 ± 0.3	7.6 ± 0.3 97%	7.3 ± 0.4 93%	7.1 ± 0.2 91%	6.2 ± 0.2 80%	7.4 ± 0.3 94%	5.0 ± 0.2 64%	6.4 ± 0.1 82%	8.5 ± 0.1 70%	7.8 ± 0.1 100%	8.0 ± 0.2 102%
alcohols [a]	596.6 ± 13.2	552.4 ± 11.3 93%	435.3 ± 16.9 73%	542.5 ± 3.0 91%	442.2 ± 3.8 74%	532.9 ± 24.7 89%	247.0 ± 19.7 72%	503.6 ± 12.8 84%	520.0 ± 13.6 87%	545.4 ± 8.1 91%	578.8 ± 24.1 97%
acids [a]	9.1 ± 0.4	8.3 ± 0.4 90%	6.4 ± 0.2 70%	8.7 ± 0.3 95%	8.3 ± 0.3 91%	8.3 ± 0.6 91%	8.0 ± 0.7 88%	8.9 ± 0.5 97%	9.7 ± 0.7 107%	7.9 ± 0.4 86%	7.8 ± 0.4 85%

Values are means of three replicates ± standard error (n = 3) and residuals as a percentage of the control.
Values followed by different letters (within a row) indicate statistical significance (one-way ANOVA, $p = 0.05$).
tr = trace levels (i.e., signal detected but below limit of quantification).
[a] Concentration expressed as mg/L.

Table 4. Color Intensity (based on sum of absorbance at A_{420}, A_{520} and A_{620}) of smoke-affected Merlot wines.

Control	B 5 g/L	B 20 g/L	G 5 g/L	G 20 g/L	B Pol 1%	B Pol 5%	G Pol 1%	G Pol 5%	PVPP	Charcoal
2.73 a ± 0.00	2.48 b ± 0.01	2.41 bc ± 0.02	2.41 bc ± 0.00	2.65 a ± 0.05	2.29 cde ± 0.02	2.37 bcd ± 0.07	2.38 bcd ± 0.00	2.18 de ± 0.00	1.29 f ± 0.00	2.26 de ± 0.00
	91%	88%	88%	97%	84%	87%	87%	80%	47%	83%

Values are means of three replicates ± standard error (n = 3) and residuals as a percentage of the control.
Values followed by different letters (within a row) indicate statistical significance (one-way ANOVA, $p = 0.05$).

Table 5. Headspace residual (µg/L) of different classes of compounds present in smoke affected Sauvignon Blanc samples.

	Control		**Dissolved CD treatment**				**CD polymer fining treatment**			
	(i.e., no treatment)		*β*-CD 5 g/L	*β*-CD 20 g/L	*γ*-CD 5 g/L	*γ*-CD 20 g/L	*β*-CD-HDI 1% w/v	*β*-CD-HDI 5% w/v	*γ*-CD-HDI 1% w/v	*γ*-CD-HDI 5% w/v
volatile phenols	23.8 ± 1.0	pre-	11.4 ± 1.0	3.6 ± 0.1	15.8 ± 0.6	6.5 ± 1.0	14.3 ± 1.0	2.7 ± 0.2	14.6 ± 0.8	2.6 ± 0.1
			48%	15%	67%	28%	60%	11%	62%	11%
		post-	11.3 ± 1.3	2.9 ± 0.2	16.1 ± 1.2	6.5 ± 0.6	14.4 ± 1.0	4.8 ± 0.2	15.9 ± 1.3	1.7 ± 0.1
			48%	12%	68%	27%	61%	20%	67%	7%
Esters [a]	5.7 ± 0.2	pre-	5.2 ± 0.3	4.9 ± 0.3	5.4 ± 0.3	4.5 ± 0.2	5.5 ± 0.3	5.2 ± 0.4	4.7 ± 0.3	4.8 ± 0.2
			91%	85%	94%	78%	96%	68%	83%	83%
		post-	5.3 ± 0.3	5.1 ± 0.3	5.0 ± 0.3	4.1 ± 0.2	4.9 ± 0.3	3.2 ± 0.2	4.6 ± 0.2	3.8 ± 0.2
			93%	89%	88%	72%	85%	56%	80%	66%
Alcohols [a]	219.0 ± 8.6	pre-	186.3 ± 7.4	189.0 ± 9.1	195.1 ± 8.3	187.8 ± 9.1	165.3 ± 7.6	148.3 ± 7.4	154.9 ± 5.9	145.9 ± 8.3
			85%	86%	89%	86%	75%	68%	71%	67%
		post-	211.2 ± 13.7	158.0 ± 6.6	221.4 ± 8.6	165.4 ± 10.4	197.0 ± 8.6	14.7 ± 7.4	194.0 ± 10.1	130.5 ± 8.8
			96%	72%	101%	76%	90%	52%	89%	60%
Acids [a]	6.4 ± 0.4	pre-	6.2 ± 0.3	6.0 ± 0.3	6.2 ± 0.3	5.9 ± 0.3	6.1 ± 0.4	6.1 ± 0.4	6.3 ± 0.4	6.2 ± 0.4
			98%	94%	98%	93%	96%	96%	99%	97%
		post-	5.5 ± 0.5	3.9 ± 0.2	5.8 ± 0.3	5.2 ± 0.3	6.2 ± 0.4	5.7 ± 0.4	6.1 ± 0.4	6.3 ± 0.5
			86%	61%	91%	82%	97%	89%	96%	98%

Values are means of three replicates ± standard error (n = 3) and residuals as a percentage of the control.
Values followed by different letters (within a row) indicate statistical significance (one-way ANOVA, $p = 0.05$).
pre-: pre-fermentation; post-: post-fermentation.
[a] Concentration expressed as mg/L.

Table 6. Residual of phenol glycosides in smoke-affected Sauvignon Blanc samples.

		Juice					**Wine**			
	Control	*β*-CD-HDI 1% w/v	*β*-CD-HDI 5% w/v	*γ*-CD-HDI 1% w/v	*γ*-CD-HDI 5% w/v	*Control*	*β*-CD-HDI 1% w/v	*β*-CD-HDI 5% w/v	*γ*-CD-HDI 1% w/v	*γ*-CD-HDI 5% w/v
glucosides										
SyGG	58.0 a ± 1.1	52.3 c ± 0.9 90%	52.5 bc ± 1.4 91%	53.4 abc ± 0.0 92%	56.9 ab ± 0.7 98%	36.5 a ± 0.7	33.1 ab ± 2.0 91%	28.6 b ± 1.4 78%	34.7 ab ± 0.0 95%	30.7 ab ± 0.7 84%
CrRG	10.3 a ± 0.0	9.8 a ± 0.5 95%	9.1 a ± 0.4 89%	10.6 a ± 0.4 103%	10.4 a ± 0.1 102%	12.2 a ± 0.6	11.6 a ± 0.2 72%	8.8 b ± 0.3 72%	11.5 a ± 0.8 94%	10.5 ab ± 0.7 86%
GuRG	13.1 a ± 0.3	12.2 a ± 0.4 93%	11.9 a ± 0.3 91%	12.3 a ± 0.3 94%	12.5 a ± 0.3 96%	14.4 a ± 0.5	13.8 a ± 0.5 95%	9.8 b ± 0.6 68%	13.3 a ± 0.6 92%	11.9 ab ± 1.1 82%
MGuRG	11.8 a ± 0.1	11.2 a ± 0.6 95%	10.4 a ± 0.4 88%	11.6 a ± 0.1 98%	11.2 a ± 0.3 95%	12.1 a ± 0.6	11.9 ab ± 0.2 98%	9.4 c ± 0.6 78%	12.1 a ± 0.3 100%	10.0 bc ± 0.2 83%
MSyGG	19.4 a ± 0.6	18.7 a ± 1.0 96%	18.1 a ± 0.6 100%	19.4 a ± 0.9 100%	18.7 a ± 0.8 96%	9.4 a ± 0.8	9.3 a ± 0.6 99%	7.4 a ± 1.0 78%	9.1 a ± 0.7 97%	8.6 a ± 0.5 92%
PhRG	5.9 a ± 0.2	5.7 a ± 0.3 97%	5.9 a ± 0.3 100%	5.7 a ± 0.2 97%	6.7 a ± 0.2 113%	8.5 a ± 0.3	7.9 a ± 0.5 92%	7.1 a ± 0.5 84%	8.9 a ± 0.2 104%	7.5 a ± 0.4 88%
total	118.4 ± 2.3	109.9 ± 3.7 93%	107.9 ± 3.4 91%	112.9 ± 2.4 95%	116.4 ± 2.3 98%	93.1 ± 3.4	87.5 ± 4.1 94%	71.1 ± 3.4 76%	89.5 ± 3.4 96%	79.3 ± 2.4 85%

Values are means of three replicates ± standard error (n = 3) and residuals as a percentage of the control.
Values followed by different letters (within a row) indicate statistical significance (one-way ANOVA, $p = 0.05$).
SyGG: syringo-gentiobioside; CrRG: cresol-arabinosyl-glycoside; GuRG: guaiacol-arabinosyl-glycoside; MGuRG: 4-methylguaiacol-arabinosyl-glycoside; MSyGG: 4-methylsyringol-gentiobioside; PhRG: phenol-arabinosyl-glycoside.

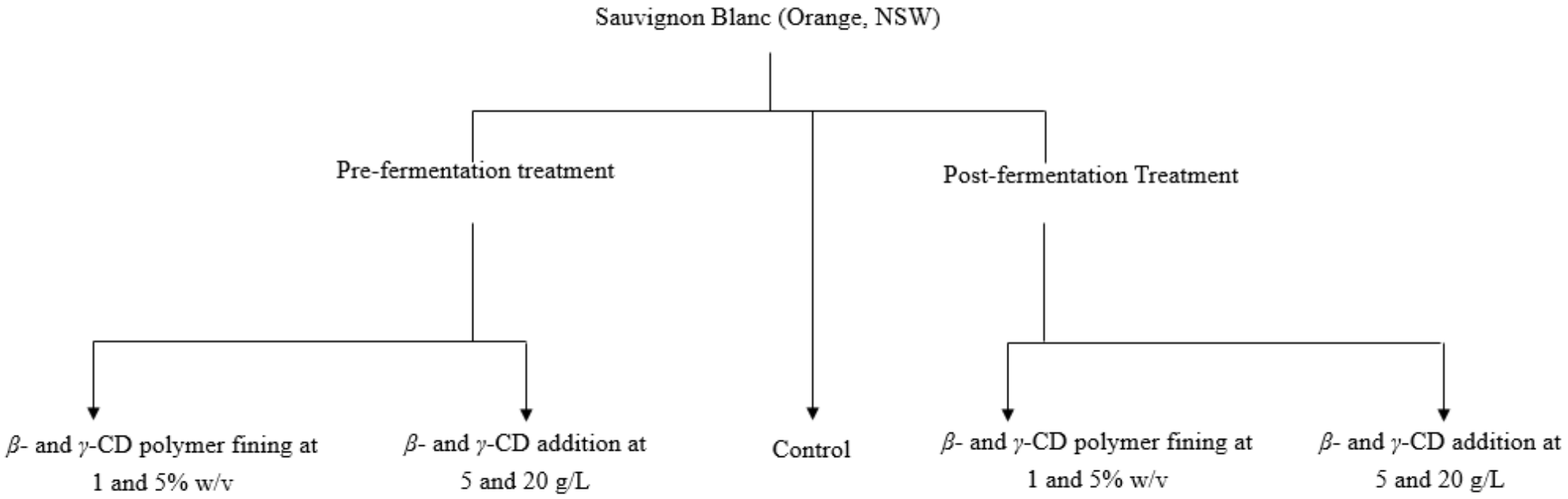

Figure 1. Outline of pre- and post-fermentation treatments with CDs and CD polymers on smoke-affected Sauvignon Blanc samples.

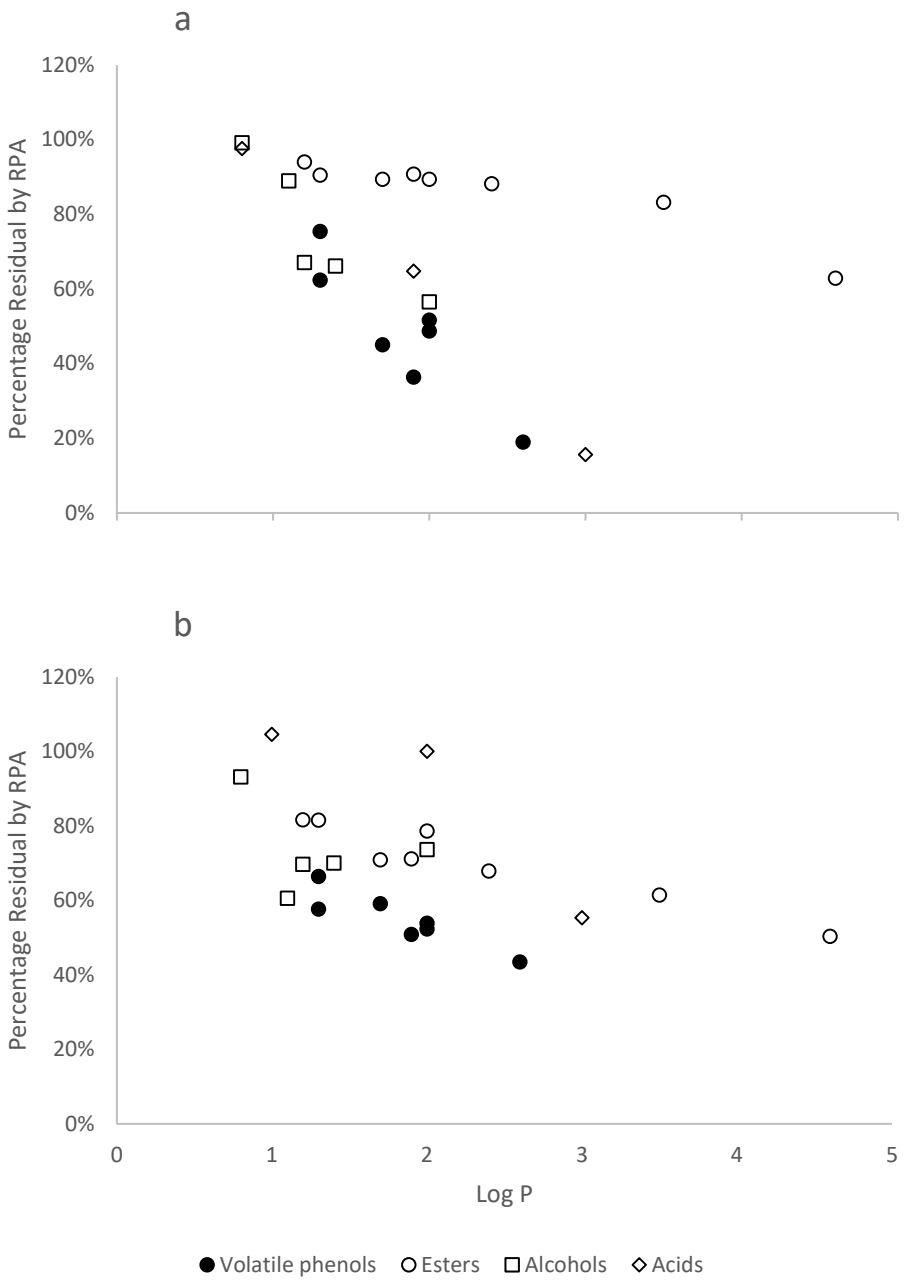

Figure 2. Scatter plot of percentage residual by RPA of various compounds in the smoke-affected Merlot wines with β-CD and γ-CD addition at 20 g/L against the Log P value (acquired from the PubChem Project: https://pubchem.ncbi.nlm.nih.gov/). (a) β-CD-HDI; (b) γ-CD-HDI.

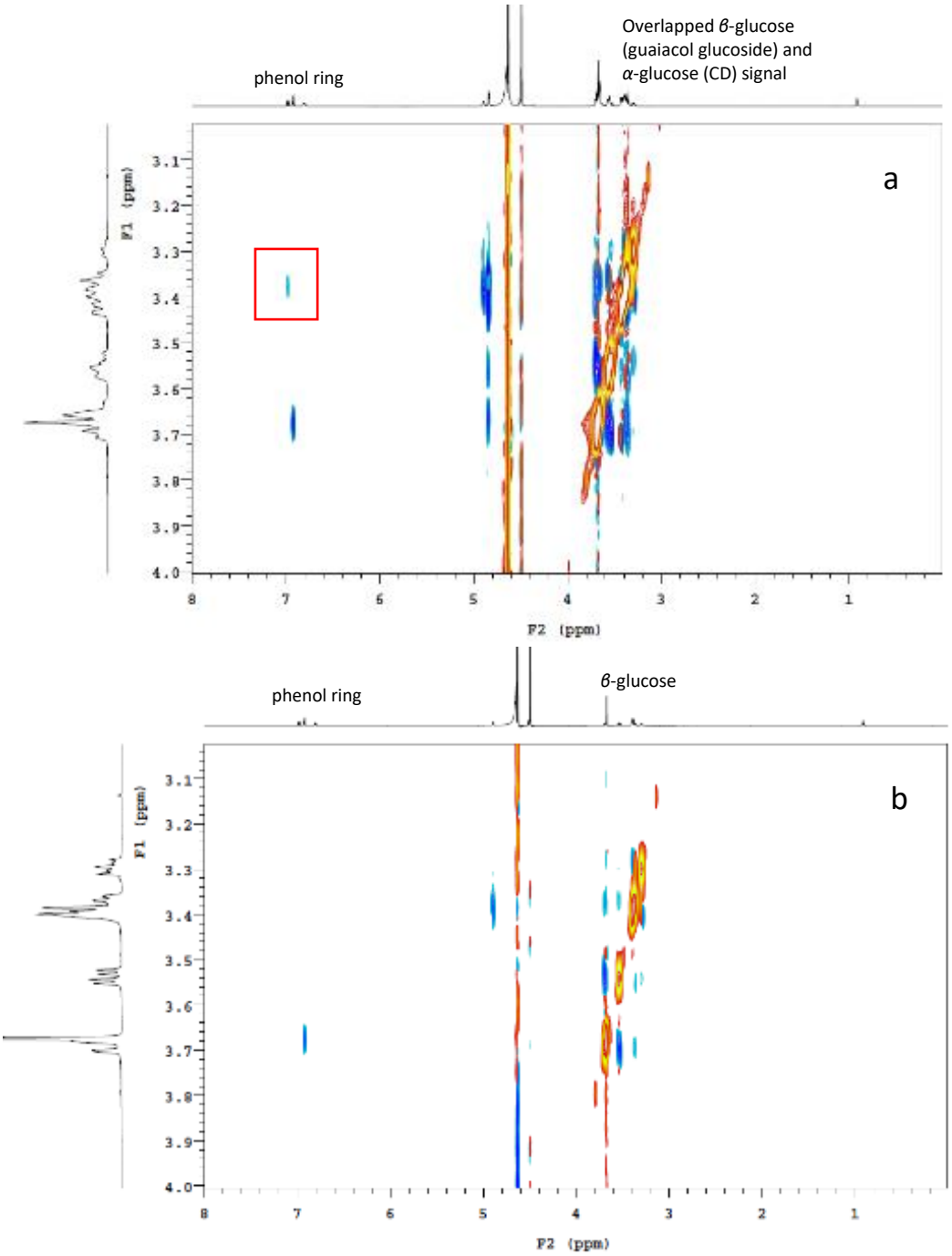

Figure 3. ^{1}H 2D ROESY NMR (600 MHz, pD 3.5 and 25 °C) spectrum of a deuterated model wine solution of guaiacol-β-glucoside (a) with β-CD and (b) without β-CD. Rectangles contain the cross-peaks arising from the NOE interactions between the protons of the phenol glucoside and the β-CD cavity

Supplementary Table S1. Quantification (µg/L) of aroma compounds in smoke-affected Merlot wines.

	Control	**Dissolved CD treatment**				**CD Polymer fining treatment**				**Other fining agents**	
	(i.e., no treatment)	*β*-CD 5 g/L	*β*-CD 20 g/L	*γ*-CD 5 g/L	*γ*-CD 20 g/L	*β*-CD-HDI 1% w/v	*β*-CD-HDI 5% w/v	*γ*-CD-HDI 1% w/v	*γ*-CD-HDI 5% w/v	charcoal 2 g/L	PVPP 0.5 g/L
esters											
ethyl butyrate	448.1 a ± 14.7	426.7 ab ± 7.5 95%	409.5 ab ± 6.6 91%	404.0 ab ± 4.9 90%	373.3 bc ± 5.4 83%	403.7 ab ± 4.9 90%	367.7 bc ± 6.4 82%	404.1 ab ± 5.8 90%	391.3 ab ± 25.7 87%	324.0 c ± 5.5 72%	392.0 ab ± 26.8 87%
ethyl 2-methylbutyrate	363.8 a ± 14.8	379.5 a ± 21.7 104%	326.3 ab ± 6.1 89%	325.5 ab ± 16.1 89%	246.0 c ± 16.0 68%	330.0 ab ± 10.4 91%	238.6 c ± 5.9 66%	271.6 bc ± 7.8 75%	168.7 d ± 9.2 46%	331.8 ab ± 2.7 91%	384.2 a ± 14.4 106%
ethyl 3-methylbutyrate	279.1 a ± 10.1	256.8 ab ± 5.4 92%	246.1 abc ± 4.9 88%	241.7 abcd ± 7.5 87%	188.5 de ± 22.3 68%	245.8 abc ± 4.6 88%	159.3 e ± 7.9 57%	194.2 cde ± 9.8 70%	153.2 e ± 9.0 55%	224.1 bcd ± 16.4 80%	274.2 ab ± 3.7 98%
ethyl hexanoate	355.8 ab ± 9.4	331.3 abcd ± 12.3 93%	323.1 bcd ± 12.3 91%	303.3 def ± 4.4 85%	266.9 fg ± 10.9 75%	277.5 efg ± 6.4 78%	226.1 h ± 0.8 64%	309.5 cde ± 5.1 87%	264.1 g ± 10.9 74%	345.9 abc ± 2.9 97%	365.1 a ± 3.8 103%
ethyl octanoate	80.6 a ± 0.3	80.3 a ± 0.3 100%	78.4 bc ± 0.2 97%	78.8 b ± 0.4 98%	75.5 d ± 0.4 94%	78.8 b ± 0.3 98%	72.5 e ± 0.0 90%	77.1 c ± 0.3 96%	73.1 e ± 0.2 91%	80.6 a ± 0.3 100%	80.7 a ± 0.2 100%
ethyl decanoate	42.4 a ± 0.2	40.7 b ± 7.0 96%	39.4 cd ± 6.2 93%	40.1 bc ± 6.5 95%	38.4 ef ± 4.4 91%	41.0 b ± 6.5 97%	38.8 de ± 4.7 92%	40.2 bc ± 5.6 95%	37.5 f ± 4.0 88%	38.9 de ± 6.5 92%	42.4 a ± 6.9 100%
isoamyl acetate	296.8 ab ± 9.3	305.4 ab ± 10.4 103%	269.6 ab ± 13.6 91%	297.5 ab ± 13.4 100%	241.9 bc ± 8.7 81%	263.7 ab ± 4.2 89%	196.2 c ± 11.1 66%	264.4 ab ± 3.9 89%	243.3 bc ± 19.3 82%	314.9 a ± 1.2 106%	307.4 a ± 13.0 104%
diethyl succinate [a]	6.0 ab ± 0.3	5.8 abc ± 0.2 97%	5.6 abc ± 0.4 94%	5.4 abc ± 0.1 91%	4.8 cde ± 0.1 81%	5.7 abc ± 0.2 96%	3.7 e ± 0.2 63%	4.9 bcd ± 0.1 81%	4.2 de ± 0.3 70%	6.2 a ± 0.1 103%	6.1 a ± 0.1 103%
total	7.8 ± 0.3	7.6 ± 0.3 97%	7.3 ± 0.4 93%	7.1 ± 0.2 91%	6.2 ± 0.2 80%	7.4 ± 0.3 94%	5.0 ± 0.2 64%	6.4 ± 0.1 82%	8.5 ± 0.1 70%	7.8 ± 0.1 100%	8.0 ± 0.2 102%
alcohols											
isobutyl alcohol [a]	100.9 a ± 2.4	100.0 a ± 2.4 99%	100.1 a ± 5.7 99%	99.4 a ± 1.4 98%	94.1 a ± 2.0 93%	91.1 a ± 4.8 90%	86.2 a ± 1.7 85%	99.9 a ± 3.0 99%	99.1 a ± 3.5 98%	99.8 a ± 1.6 99%	100.1 a ± 2.2 99%
isoamyl alcohol [a]	457.3 a ± 9.7	419.3 abc ± 8.0 92%	308.2 e ± 10.3 67%	410.2 abc ± 1.1 90%	319.8 de ± 1.4 70%	409.8 abc ± 18.5 90%	316.1 bc ± 17.1 69%	371.0 cd ± 9.2 81%	389.6 de ± 9.7 85%	410.5 abc ± 5.4 90%	444.3 ab ± 20.4 97%

hexanol [a]	4.0 a ± 0.0	3.4 bcd ± 0.1 86%	2.4 e ± 0.1 59%	3.6 abc ± 0.1 91%	3.0 d ± 0.0 75%	3.2 e ± 0.1 79%	2.1 cd ± 0.1 53%	3.5 bc ± 0.1 88%	3.3 bcd ± 0.1 83%	3.5 bc ± 0.1 88%	3.7 ab ± 0.0 93%
benzyl alcohol [a]	1.2 ab ± 0.0	1.1 abc ± 0.0 94%	1.1 abc ± 0.0 92%	1.1 abc ± 0.0 91%	0.9 e ± 0.0 72%	1.1 bcd ± 0.0 87%	1.0 cde ± 0.0 84%	1.0 cde ± 0.1 85%	0.9 de ± 0.0 77%	1.1 abc ± 0.0 95%	1.2 a ± 0.0 103%
phenylethyl alcohol [a]	33.2 a ± 1.1	28.5 bc ± 0.8 86%	23.4 de ± 0.8 71%	28.2 bc ± 0.3 85%	24.5 cde ± 0.4 74%	27.8 bc ± 1.2 84%	21.6 e ± 0.7 65%	28.2 bc ± 0.5 85%	27.1 bcd ± 0.2 82%	30.5 ab ± 0.9 92%	29.4 ab ± 1.4 89%
total [a]	596.6 ± 13.2	552.4 ± 11.3 93%	435.3 ± 16.9 73%	542.5 ± 3.0 91%	442.2 ± 3.8 74%	532.9 ± 24.7 89%	247.0 ± 19.7 72%	503.6 ± 12.8 84%	520.0 ± 13.6 87%	545.4 ± 8.1 91%	578.8 ± 24.1 97%
acids											
butanoic acid [a]	3.7 a ± 0.2	3.7 a ± 0.3 101%	3.6 ab ± 0.1 97%	3.8 a ± 0.1 103%	3.8 a ± 0.1 105%	3.0 ab ± 0.1 82%	2.8 ab ± 0.2 78%	3.6 ab ± 0.3 98%	3.8 a ± 0.3 103%	2.5 b ± 0.3 68%	3.0 ab ± 0.2 82%
hexanoic acid [a]	2.1 ab ± 0.1	2.0 ab ± 0.1 99%	1.3 b ± 0.0 63%	2.0 ab ± 0.2 97%	2.1 ab ± 0.1 100%	2.0 ab ± 0.3 99%	1.8 ab ± 0.4 86%	2.0 ab ± 0.1 96%	2.0 ab ± 0.2 96%	2.0 ab ± 0.0 98%	2.2 a ± 0.2 105%
octanoic acid [a]	3.4 ab ± 0.1	2.6 c ± 0.1 75%	1.5 d ± 0.0 45%	2.9 bc ± 0.1 85%	2.4 c ± 0.0 71%	3.3 b ± 0.2 96%	3.4 ab ± 0.2 99%	3.3 b ± 0.1 97%	4.0 c ± 0.2 117%	3.3 b ± 0.1 98%	2.6 c ± 0.1 76%
total [a]	9.1 ± 0.4	8.3 ± 0.4 90%	6.4 ± 0.2 70%	8.7 ± 0.3 95%	8.3 ± 0.3 91%	8.3 ± 0.6 91%	8.0 ± 0.7 88%	8.9 ± 0.5 97%	9.7 ± 0.7 107%	7.9 ± 0.4 86%	7.8 ± 0.4 85%

Values are means of three replicates ± standard error (n = 3) and residuals as a percentage of the control.
Values followed by different letters (within a row) indicate statistical significance (one-way ANOVA, $p = 0.05$).
tr = trace levels (i.e., signal detected but below limit of quantification).
[a] Concentration expressed as mg/L.

Supplementary Table S2. Quantification (µg/L) of aroma compounds in smoke-affected Sauvignon Blanc samples.

	Control		**Dissolved CD**				**CD Polymer fining**			
	(i.e., no treatment)		*β*-CD 5 g/L	*β*-CD 20 g/L	*γ*-CD 5 g/L	*γ*-CD 20 g/L	*β*-CD-HDI 1% w/v	*β*-CD-HDI 5% w/v	*γ*-CD-HDI 1% w/v	*γ*-CD-HDI 5% w/v
volatile phenols										
guaiacol	4.0 ab ± 0.0	pre-	3.8 a ± 0.3 96%	3.6 ab ± 0.1 90%	3.1 ab ± 0.1 78%	2.8 ab ± 0.4 71%	2.8 ab ± 0.2 71%	2.7 bc ± 0.2 69%	3.0 ab ± 0.1 76%	2.6 bc ± 0.1 66%
		post-	3.4 ab ± 0.2 86%	2.9 ab ± 0.2 73%	3.3 ab ± 0.2 84%	2.7 bc ± 0.2 67%	3.5 ab ± 0.2 89%	3.2 ab ± 0.2 79%	2.6 bc ± 0.2 67%	1.7 c ± 0.1 43%
4-methylguaiacol	2.1 a ± 0.1	pre-	1.4 c ± 0.1 69%	tr	1.5 bc ± 0.1 74%	tr	tr	tr	tr	tr
		post-	1.4 c ± 0.0 69%	tr	1.5 bc ± 0.1 70%	tr	1.9 ab ± 0.1 89%	1.6 bc ± 0.1 76%	1.5 bc ± 0.1 72%	tr
4-ethylguaiacol	3.2 ab ± 0.2	pre-	1.8 ab ± 0.2 57%	tr	1.7 ab ± 0.2 54%	tr	tr	tr	tr	tr
		post-	1.8 a ± 0.2 56%	tr	1.8 b ± 0.1 55%	tr	1.8 b ± 0.1 56%	tr	1.8 b ± 0.1 56%	tr
4-ethylphenol	9.4 a ± 0.4	pre-	4.3 c ± 0.4 45%	tr	5.6 bc ± 0.2 59%	3.7 c ± 0.7 39%	9.5 a ± 0.7 101%	tr	9.3 a ± 0.6 98%	tr
		post-	4.7 bc ± 0.8 50%	tr	6.0 bc ± 0.7 64%	3.8 c ± 0.4 41%	5.2 bc ± 0.6 55%	tr	7.1 ab ± 0.3 75%	tr
p-cresol	5.0 a ± 0.4	pre-	tr	tr	3.8 ab ± 0.1 77%	tr	2.0 c ± 0.1 40%	tr	2.4 bc ± 0.2 47%	tr
		post-	tr	tr	3.5 bc ± 0.2 70%	tr	2.0 c ± 0.1 40%	tr	2.8 bc ± 0.6 56%	tr
esters										
ethyl butyrate	647.9 a ± 31.1	pre-	660.1 a ± 41.4 102%	591.4 a ± 20.9 91%	629.7 a ± 22.9 97%	627.0 a ± 21.5 97%	666.1 a ± 28.4 103%	686.2 a ± 35.0 106%	650.5 a ± 28.8 100%	694.0 a ± 9.3 107%

		post-	590.8 a ± 23.1 91%	555.2 a ± 36.8 86%	549.0 a ± 23.4 85%	545.9 a ± 25.9 84%	621.4 a ± 35.0 96%	542.5 a ± 24.3 84%	612.6 a ± 36.9 95%	561.8 a ± 27.5 87%
ethyl hexanoate	972.7 abc ± 44.6	pre-	976.2 abc ± 60.9 100%	935.1 abcd ± 58.0 96%	1088.9 a ± 78.8 112%	897.9 abcd ± 22.4 92%	990.5 abc ± 96.2 102%	1022.4 ab ± 84.6 105%	944.4 abcd ± 64.0 97%	936.1 abcd ± 80.0 96%
		post-	865.4 abcde ± 74.1 89%	839.1 abcde ± 48.4 86%	819.4 abcde ± 54.1 84%	687.3 cde ± 31.7 71%	735.2 bcde ± 50.5 76%	578.2 e ± 20.7 59%	833.1 abcde ± 40.3 86%	634.8 de ± 48.4 65%
ethyl octanoate [a]	1.5 a ± 0.1	pre-	1.4 ab ± 0.0 97%	1.4 ab ± 0.0 94%	1.5 a ± 0.0 102%	1.3 ab ± 0.0 91%	1.5 a ± 0.1 102%	1.3 ab ± 0.1 90%	1.5 a ± 0.1 103%	1.3 abc ± 0.0 89%
		post-	1.3 abc ± 0.1 87%	1.3 abc ± 0.1 87%	1.2 abc ± 0.1 83%	0.9 cd ± 0.1 62%	1.2 abc ± 0.1 82%	0.5 d ± 0.0 37%	1.0 bc ± 0.1 72%	0.6 d ± 0.0 44%
ethyl decanoate	43.2 abcd ± 0.5	pre-	42.5 bcdef ± 0.4 98%	42.3 bcdefg ± 0.4 98%	42.7 bcde ± 1.0 99%	42.5 bcdef ± 0.3 98%	44.7 ab ± 0.8 103%	45.7 a ± 1.0 106%	43.9 abcd ± 0.3 101%	44.7 abc ± 0.7 103%
		post-	41.4 defg ± 0.7 96%	39.6 fgh ± 0.5 92%	41.1 defg ± 0.4 95%	39.3 gh ± 0.3 91%	41.6 cdefg ± 0.7 96%	39.9 efgh ± 0.3 92%	41.2 defg ± 0.5 95%	37.6 h ± 0.2 87%
isoamyl acetate [a]	1.4 abcd ± 0.0	pre-	1.4 abc ± 0.1 104%	1.4 abcd ± 0.1 103%	1.4 ab ± 0.1 105%	1.4 abcd ± 0.1 100%	1.4 abcd ± 0.0 101%	1.4 abcd ± 0.1 99%	1.3 abcd ± 0.1 97%	1.6 a ± 0.1 119%
		post-	1.3 abcd ± 0.1 95%	1.2 bcd ± 0.1 88%	1.3 abcd ± 0.1 93%	1.0 de ± 0.1 75%	1.1 bcde ± 0.1 81%	0.8 e ± 0.0 57%	1.1 bcde ± 0.0 83%	1.0 cde ± 0.0 76%
diethyl succinate	1229.3 a ± 27.0	pre-	707.0 de ± 63.4 58%	492.0 ef ± 72.4 40%	672.9 de ± 68.9 55%	221.8 g ± 39.2 18%	931.9 bcd ± 48.8 76%	742.5 de ± 76.4 60%	259.9 fg ± 33.3 21%	148.3 g ± 23.2 12%
		post-	1272.9 a ± 48.8 104%	1159.5 abc ± 16.5 94%	1133.1 abc ± 50.9 92%	905.7 bcd ± 32.1 74%	1172.9 ab ± 32.0 95%	720.1 de ± 79.6 59%	895.8 cd ± 47.6 73%	839.2 d ± 48.7 68%
alcohols										
isobutyl alcohol [a]	25.7 a ± 1.2	pre-	24.8 a ± 1.1 96%	24.6 a ± 1.2 96%	24.8 a ± 1.0 96%	24.1 a ± 1.2 94%	25.0 a ± 1.1 97%	23.5 a ± 1.0 91%	23.5 a ± 0.8 92%	25.1 a ± 1.4 98%
		post-	24.9 a ± 1.0 97%	25.2 a ± 1.2 98%	24.9 a ± 0.8 97%	24.4 a ± 1.2 95%	25.6 a ± 1.3 99%	21.1 a ± 1.0 82%	24.2 a ± 1.4 94%	24.8 a ± 1.2 96%
isoamyl alcohol [a]	180.4 a ± 7.2	pre-	149.6 abcd ± 5.8 83%	152.9 abc ± 7.8 85%	158.4 ab ± 6.9 88%	152.2 abc ± 7.4 84%	127.7 bcde ± 5.7 71%	112.4 def ± 5.7 62%	118.0 cdef ± 4.5 65%	107.1 ef ± 6.1 59%

		post-	174.7 a ± 12.2 97%	123.0 bcde ± 5.0 68%	185.1 a ± 7.4 103%	130.3 bcde ± 8.7 72%	160.0 ab ± 6.8 89%	84.7 f ± 6.1 47%	158.5 ab ± 8.4 88%	95.6 ef ± 7.3 53%
hexanol [a]	2.0 a ± 0.1	pre-	1.8 abc ± 0.1 90%	1.6 abcd ± 0.1 83%	1.9 ab ± 0.1 96%	1.8 abc ± 0.1 91%	1.5 bcd ± 0.1 79%	1.3 de ± 0.1 64%	1.9 abc ± 0.1 95%	1.7 abc ± 0.1 84%
		post-	1.6 abcd ± 0.1 83%	1.5 cd ± 0.1 76%	1.8 abc ± 0.1 92%	1.8 abc ± 0.0 90%	1.6 bcd ± 0.1 80%	1.1 e ± 0.1 54%	1.7 abc ± 0.1 84%	1.6 ef ± 0.1 81%
phenylethyl alcohol [a]	11.0 abc ± 0.2	pre-	10.1 abcd ± 0.3 92%	9.8 bcde ± 0.1 89%	10.1 abcde ± 0.3 92%	9.7 bcde ± 0.4 88%	11.1 abc ± 0.6 101%	11.2 abc ± 0.6 102%	11.5 ab ± 0.5 104%	12.1 a ± 0.8 110%
		post-	10.1 abcde ± 0.3 92%	8.3 de ± 0.4 76%	9.7 bcde ± 0.3 88%	9.0 cde ± 0.5 82%	9.8 bcde ± 0.4 89%	7.9 e ± 0.3 72%	9.5 bcde ± 0.2 87%	8.5 de ± 0.2 78%
acids										
butanoic acid	904.6 a ± 135.4	pre-	866.6 a ± 94.4 96%	900.8 a ± 108.2 100%	878.2 a ± 44.0 97%	894.2 a ± 61.0 99%	840.8 a ± 91.4 93%	815.2 a ± 104.8 90%	934.9 a ± 91.2 103%	942.7 a ± 65.0 104%
		post-	824.7 a ± 88.7 91%	941.0 a ± 103.4 104%	883.3 a ± 58.6 98%	866.7 a ± 26.3 96%	692.0 a ± 68.9 77%	654.1 a ± 87.8 72%	873.2 a ± 98.7 97%	859.3 a ± 51.2 95%
hexanoic acid [a]	2.4 a ± 0.1	pre-	2.4 a ± 0.1 102%	2.1 a ± 0.2 86%	2.3 a ± 0.2 98%	2.2 a ± 0.2 91%	2.2 a ± 0.1 94%	2.2 a ± 0.2 93%	2.3 a ± 0.2 98%	2.3 a ± 0.3 95%
		post-	2.3 a ± 0.3 97%	1.4 a ± 0.1 60%	2.2 a ± 0.1 94%	2.1 a ± 0.2 90%	2.3 a ± 0.3 97%	2.0 a ± 0.2 85%	2.3 a ± 0.2 97%	2.3 a ± 0.2 98%
octanoic acid [a]	3.1 a ± 0.1	pre-	2.9 a ± 0.1 95%	3.1 a ± 0.1 99%	3.0 a ± 0.1 98%	2.9 ab ± 0.6 93%	3.0 a ± 0.1 98%	3.1 a ± 0.9 100%	3.0 a ± 0.1 98%	3.0 a ± 0.1 97%
		post-	2.3 bc ± 0.1 75%	1.6 d ± 0.1 50%	2.7 abc ± 0.1 87%	2.2 c ± 0.1 72%	3.2 a ± 0.1 103%	3.0 a ± 0.1 97%	2.9 a ± 0.1 95%	3.1 a ± 0.2 99%

Values are means of three replicates ± standard error (n = 3) and residuals as a percentage of the control.
Values followed by different letters (within a row) indicate statistical significance (one-way ANOVA, $p = 0.05$).
tr = trace levels (i.e., signal detected but below limit of quantification).
pre-: pre-fermentation; post-: post-fermentation.
[a] Concentration expressed as mg/L.

CHAPTER 5

Conclusions and future directions

Conclusions and future directions

Conclusions

Volatile phenol-induced wine faults will continue to challenge the wine industry in the foreseeable future, as the warmer, dryer climatic conditions experienced by wine regions favour the continued occurrence of bushfires and/or growth of spoilage microbes. Accordingly, amelioration techniques need to be developed and/or improved to enable grape and wine producers to manage these issues. The current thesis provides the wine industry an alternative amelioration strategy by evaluating volatile phenol inclusion by cyclodextrins (CDs), the potential removal of volatile phenols and their glycoconjugates by CD polymers, and the impact of CD treatment on other wine quality parameters.

The new extraction method developed for headspace gas chromatography mass spectrometry (HS GC-MS) analysis described in Chapter 2 is an innovative method that resolves an issue encountered by analytical chemistry in which HS GC-MS is used to analyse changes in headspace components with dissolved treatments, which can concurrently react with the normalising standard. The introduction of an additional liquid phase containing the normalising standard changes the dynamics of the headspace extraction equilibrium, but mitigates the interruption of the normalising standard's response by the treatment. This method can also be used in food and beverage aroma component studies which similarly use additives as treatments. Modifications to this method were made in Chapter 4.

The formation of inclusion complexes between CDs and volatile phenols was studied and resulted in the reduction of headspace concentrations of volatile phenols. Among the CDs tested, β-CD

showed greater overall binding capability. 4-Ethylphenol was the volatile phenol most susceptible to CD treatment, with head space residuals as low as 23.1% achieved when β-CD was added at 25 g/L. On the other hand, guaiacol was the least susceptible volatile phenol compound. This was due to conformational differences between the compounds.

The hexamethylene diisocyanate (HDI) crosslinked CD polymers prepared were insoluble and could therefore be used as fining agents, whereas CDs are readily soluble in aqueous solutions so cannot be easily removed after addition. The β- and γ-CD polymers were also able to remove volatile phenols, with up to 45% and 77% removal of total volatile phenols from model wine during a batch adsorption trial.

Like most other fining treatments applied to wine, the retention/removal of off-odour volatile phenols by CDs and CD polymers was achieved to the detriment of some of the wine attributes. Some aroma compounds, particularly those with higher hydrophobicity and more streamlined structures, were negatively impacted by the CD treatments. This was also reflected by sensory analysis, which showed significant differences in the overall aroma intensity, in addition to removing the phenols, and therefore diminishing their sensory impacts. However, when CD polymers were used as fining agents during pre-fermentation treatments, there was considerably less impact on wine aroma compounds. Glycoconjugates forms of volatile phenols can be particularly difficult to ameliorate but were found to be slightly removed by the β-CD polymer. However, the colour of the wine was also affected by the treatments, albeit the impact was far less than that observed following the addition of charcoal or PVPP, at recommended doses. Overall, these findings demonstrate that CD based treatments, despite their ability to effectively

retain/remove volatile phenols from wine, have functional limitations. Another limitation with CDs or CD polymers is that these materials are not yet legally permitted as winemaking additives or processing aids.

Future directions

Cyclodextrins (CDs) have been widely used in food, beverage, flavour, pharmaceutical and cosmetic industries, whereas the literature on CD applications in wine is lacking. The current thesis, with the development of an innovative headspace extraction method, is one of the first attempts to study the interactions between CDs and wine constituents, particularly volatile phenols, using analytical methods. This thesis provides new knowledge into the potential applications of CDs in winemaking, but there are numerous avenues for further research to be pursued, for example:

1. Impact of CD additions on other parameters of wine. Since CDs are listed as foods in EU, US, AUS and many other countries, it would be worth exploring the impact of CD addition on other wine sensorial characters, specifically taste and mouth-feel attributes, the *in vivo* release of aroma compounds from CD inclusion complexes on the palate, and the overall perception of wine quality. Colour preservation by CDs could also be explored, which may lead to the prevention of browning in white wines.

2. Modification of CDs and CD polymers. Chemical groups can be synthetically incorporated into CD molecules, which would change the reactivity of CDs. These modified CDs may offer better selectivity towards binding of volatile phenols, while preserving the desirable aroma compounds. Molecular imprinting could also be used to improve CD polymer selectivity. Based

on the principles of CD inclusion complex, other hydrophobic wine molecules might also be targeted using molecularly imprinted CD polymers. The crosslinkers used to produce CD polymers can be food grade materials, for example, citric acid. The use of food grade reagents will increase the practicality of CD polymer application in wine.

3. Improvement of treatment technique. Reverse osmosis is a fractionation process that separates wine constituents based on sizes and molecular weight. Since many off-odour compounds, such as the volatile phenols, are relatively small in size, compared to other wine components (e.g., tannins, polymerised pigments and proteins, etc.), combining reverse osmosis fractionation and CD polymer treatment may yield better results in levels of preserving desirable aroma compounds in wine, whilst still removing taint compounds.

4. Producing CDs in wine using enzyme or genetically modified microbes. The original aim of this thesis, i.e., to convert grape fermentable sugars into CDs, was not achieved in the trials completed using two bacterial strains. However, it may still be possible to screen a microbe that can produce CDs from sugars in grape must. In this way, CD chemistry could be used to achieve wines with less alcohol.

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