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**The removal of volatile phenols from  
*Brettanomyces*-contaminated wine using  
Molecularly Imprinted Polymers**

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**List of abbreviations**

4-EG	4-ethylguaiacol
4-EP	4-ethylphenol
ABCHC	1.1'-azobis cyclohexanecarbonitrile
AF	alcoholic fermentation
APY	2-acetylpyrroline (APY)
ATHP	2-acetyltetrahydropyridine
CA	cellulose acetate
CAB	cellulose acetate butyrate
CAP	cellulose acetate propionate
CFU	Colony forming unit
CIE	International Commission on Illumination
CP	cellulose propionate
DAD	diode array detector
DAO	diamine oxidase
DDS	drug delivery system
EDMA	ethylene dimethacrylate
EGDMA	ethylene glycol dimethacrylate
ETHP	2-ethyltetrahydropyridine
FLD	fluorescence detector
FTIR	Fourier-transform infra-red spectroscopy
GC	gas chromatography
HPLC	high performance liquid chromatography
ISEC	inverse size exclusion chromatography
LAN	local area network
LOD	limit of detection
LOQ	limit of qualification
MAE	microwave-assisted extraction

MIM	molecularly imprinted membrane
MIP	molecularly imprinted polymer
MLF	malolactic fermentation
MPS	multi-purpose sampler
MS	mass spectrometry
MW	molecular weight (mass)
NADH	nicotinamide adenine dinucleotide, reduced form
NAD <sup>+</sup>	nicotinamide adenine dinucleotide, oxidized form
NIP	non-imprinted polymer
NMR	solid-state nuclear magnetic resonance
OIV	International Organisation of Vine and Wine
OSF	optimal spatial fit
PAD	phenolic (cinnamic) acid decarboxylase
PADI	phenylacrylic acid decarboxylase gene
PANI-EB	polyaniline-emeraldine base
PANI-ES	polyaniline-emeraldine salt
PC	personal computer
PDMS-OTT	polydimethylsiloxane-open tubular traps
PET	polyethylene terephthalate
PFP	pentafluorophenyl
POF <sup>+</sup>	phenolic off-flavour producing gene
PVPP	polyvinylpolypyrrolidone
QCM	quartz crystal microbalance
R-COOH	carboxyl group
R-OH	hydroxyl group
RPM	rotations per minute
SBSE	stir-bar sorptive extraction
SEM	scanning electron microscopy
SPE	solid phase extraction

SPME	solid phase micro-extraction
TCA	tricarboxylic acid cycle
TDU	thermal desorption unit
UV	ultraviolet
UV-VIS	ultraviolet-visible spectroscopy
VBNC	viable but not culturable
VPR	vinyl phenol reductase

## Summary

*Brettanomyces* infections represent one of the major red wine quality spoilers affecting the industry worldwide and will most likely continue to be so in the years to come.

When attempting to correct or “cure” a *Brettanomyces* tainted wine, the most problematic metabolites to be removed are volatile phenols, particularly 4-ethylphenol (4-EP).

Molecular imprinting represents a branch of knowledge with implications still to be further understood through research and a developing technology which is yet to reach full maturity. Molecularly imprinted polymers are created by mixing and polymerizing together functional, cross-linking monomers, template molecules and an initiator in a solvent. The complex formed between the template molecule and functional monomers is stabilized in the resulting highly cross-linked polymer. After the template molecule has been extracted, the resulting imprinted polymer possesses a permanent memory for the imprinted species formed, enabling the resultant polymer to selectively rebind the imprinted molecule from a mixture of other compounds.

Molecular imprinting represents one of the technologies which promise to bridge the gap between the precision and selectivity of chemical analytical methods for investigating liquid matrices, and that of industrial filtration and separation technologies currently in use.

In this work, the removal of 4-ethylphenol, as the most representative marker of *Brettanomyces* infection in red wine, with the aid of molecularly imprinted polymers (MIPs) was studied.

The molecularly imprinted polymers could demonstrate their removal effectiveness and selectivity for 4-ethylphenol (4-EP) in model solutions, with a contact time and dose dependent imprinting effect.

The 4-EP removal efficiency was scalable when changing from lab-scale to small-scale winemaking conditions. The polymer could be successfully eluted of bound 4-EP and regenerated with alkaline and acid solutions.

The polymers facilitated an almost 10-fold reduction of 4-EP from a contaminated red wine, using repeated filtration and regeneration cycles, but the imprinting effect, evident in lab-

scale conditions, was no longer observable as both imprinted and non-imprinted polymers performed very similarly at a small experimental scale.

In terms of removal selectivity, the tested polymers did not remove any free anthocyanins, whereas PVPP and activated carbon treatments caused stronger removal effects.

Embedding the active polymeric material into filter sheets caused a fourfold reduction in 4-EP removal efficacy, compared to the same polymer in a powder format.

The polymer-embedded filter sheets, although brand new when used, were in fact a source of 4-EP contamination for treated wines, explained as 4-EP had been used as a template for molecular imprinting during the manufacturing process.

The polymer-embedded filter sheets were half as effective as charcoal at removing 4-EP from a contaminated wine, whilst being at a five times higher dose. While the contact time was much longer for the charcoal treatment, it nevertheless displayed comparable reductions in measured esters, alcohols, and volatile aroma compounds.

Sensory analysis revealed the polymer-treated samples to be the least preferred out of all tested samples, including the regular contaminated wines. The polymer-embedded filter sheets conferred to the wines an off-flavour described as “Bretty” and chemical, and which was not successfully removed from the experimental filter sheets using water and ethanol rinses or using steam sanitization.

## 1 Introduction

The so-called “Brett character” is a relatively common and well-known defect in red wines. It is associated with a class of chemical compounds called volatile phenols, which confer off-odours and determine losses of positive sensorial qualities in the affected wines. This unwanted character has been found to originate from the presence of yeasts of the *Brettanomyces* genus, which have been linked to many forms of wine spoilage: volatile acidity (Loureiro & Malfeito-Ferreira, 2006), biogenic amines (Caruso et al., 2002), cloudiness (Van der Walt & Van Kerken, 1958), film formation (Joseph & Bisson, 2004), anthocyanin degradation (Suárez et al., 2007), formation of unpleasant flavours and suppression of the sensory perception of desirable fruity or floral aromas (Ribéreau-Gayon et al., 2006).

The problem of *Brettanomyces* spoilage is a significant and complex one for the wine industry. It also sometimes represents a source of contentious arguments in wine enthusiast circles, where some are more sympathetic and forgiving with the occurrence of this defect than others. Indeed, small amounts of volatile phenols may play a positive role in enhancing sensorial complexity in young red wines (Fugelsang et al., 2006). Some authors indicate that volatile phenol concentrations lower than 400 µg/L may contribute to wine complexity, imparting sensory descriptors like “spice”, “leather”, “smoke” or “game” (Loureiro and Malfeito-Ferreira, 2003). Other authors recognize that even at low concentrations of 600-700 µg/L of volatile phenols the aroma is undoubtedly negatively altered, masking typical wine characteristics (Ribéreau-Gayon et al., 2006).

*Brettanomyces* yeasts have been identified in all wine-producing areas of the world (Fugelsang et al., 2006) and have established themselves as a major worldwide oenological concern (Oelofse et al., 2009).

Currently there are many methods proposed which aim to address the issue by focusing on detection, growth prevention, spoilage management through removal of yeast cells and removal of the off flavours.

Much progress has been achieved in the areas of detection, prevention and removal of *Brettanomyces* yeast cells from affected wines through filtration or special fining agents.

However, the aspect pertaining to removing off-flavours from affected wine is still missing a powerful, reliable and gentle method that can be successfully used to selectively remove the unwanted volatile phenols while preserving the wine's positive organoleptic qualities to as high a degree as possible.

This present work is focused on exploring the potential for the technological innovation of molecularly imprinted polymers to successfully provide an elegant and effective curative solution for the "Brett" problem in wine.

## 2 Literature review

### 2.1 Wine quality and wine defects

It can be regarded as self-evident, even axiomatic, that there is a direct relationship between wine quality and the lack of any organoleptic defects in the concerned wine.

Indeed, the absence of defects, or “technical correctness”, in a wine is considered a crucial determinant of its quality, or at least a necessary precursor to, if not part of quality itself (Schuster, 1992).

In the wine industry, the concept of a wine defect is quite variable and can benefit from some clarification and disambiguation. It is usually an alteration of the wine that can be directly perceived through the visual, olfactory, or gustatory senses. It is important to note that, although most of known wine defects can be identified through direct sensory evaluation, there are several known instances where undesired substances may compromise wine quality or even product safety without there being obvious visual, olfactory or gustatory cues to inform the specialist about their presence. A well-known example is that of biogenic amines (Gafner, 2003). In these cases, specific analytical chemical methods are necessary as part of responsible wine quality control measures.

An expedient means to classify wine defects is to group them into faults or taints, according to whether the wine defect is of internal or external origin.

A wine fault represents an internal contamination that leads to spoilage, usually derived from inappropriate winemaking practices or storage conditions. Some examples of faults are volatile acidity, *Brettanomyces* infection, mousiness, reduction, oxidation etc.

A wine taint represents an external contamination of the wine with a foreign odour or flavour, rendering the wine unappealing. Examples are cork taint, smoke taint, Ladybug taint etc.

In practice, however, the words fault and taint are quite interchangeable, when casually used.

In some cases, the distinction between internal or external contamination is not clear-cut.

An example of this kind of ambiguity can be seen in the case of the so-called Geranium defect in wines. This occurs when sorbic acid is added to an off-dry wine to prevent refermentation.



If lactic acid bacteria, specifically *Oenococcus*, are present, they will esterify sorbic acid into sorbyl alcohol, which will subsequently rearrange itself to a more stable chemical form as 2-ethoxyhexa-3,5-diene, which has a characteristic and potent odour of crushed geranium leaves, which many people find particularly unappealing (Fugelsang & Edwards, 2007).

Lactic acid bacteria generally belong to the must and wine microflora unless they are specifically removed or inactivated through various methods. Therefore, one could call the Geranium odour a fault. However, the precursor, sorbic acid, is not usually part of must or wine composition. It must be added. From this perspective, the geranium odour should be called a taint.

Therefore, in the interest of simplicity, the terms defect, fault, or taint have generally been used interchangeably throughout the relevant literature.

Attitudes to the occurrence of wine defects are variable, depending on the relationship or professional function of those who encounter these situations. For the wine maker, detection of a defect in one of his wine lots can be a call for immediate remedial action, before the taint intensifies, sometimes irreversibly. For the wine merchant, a faulty wine represents a difficult or unsafe product to sell, and spells possible financial losses or other complications.

For the wine drinking citizen, a defect represents a justified cause for rejecting the wine and requesting another, as is the courteous case in restaurants. In a broader sense, and certainly if rejecting or returning the wine is not possible, a defect represents, from the customer's perspective, a breach of trust and a failure to have the most basic expectation of "technical correctness" for his wine purchase fulfilled.

In short, wine defects weaken the reputation of any affected wine producer and that of the wine industry in general.

However, what exactly constitutes a wine fault, or defect to put it broadly, is very often an open subject, as Ronald S. Jackson highlights (2008, Wine Science 3rd edition):

*"There is no precise definition of what makes a wine faulty. Human perception is too variable. In addition, it is the vinous equivalent of incorrect grammar, and therefore open to interpretation."*

Several wine faults are caused by higher-than-usual levels of specific chemical compounds in the wine matrix. Quite often, some of the salient sensory characteristics of special types of wine (such as Port, Sherry, Madeira or Jura wines etc.) are based on high levels of compounds which would otherwise have been considered as excessive or faulty, had they been found in regular table wines. The sensory perception of faults and their identification as such is also conditioned by culture, tradition and by the customer's expectations in general.

Another caveat worth paying attention to is that a series of unprecedented worldwide changes are impacting the wine industry, so that an accurate understanding of off flavours in general is further complicated.

Firstly, one could rightfully point out the globalization of wine (Schirmer, 2011): the planting of international wine varieties and expansion of brands across the globe, the emergence of flying winemakers and rapid diffusion of modern winemaking techniques.

Secondly, an increasingly clear impact of climate change can be seen in manifold ways (IPCC, 2007; Millán, 2014), from sporadic effects like the occurrence of forest fires or unexpected extreme weather events to long term changes in climate which translate into fundamental changes in wine composition such as increases in sugar content, alcohol and pH, all of which subtly affect the microbiology and chemical balance of wines in myriad ways.

And thirdly, another highly significant aspect to consider is the breakdown of biogeographical barriers (Mooney & Cleland, 2001) which is a combined result of climate change and globalization, and its associated worldwide increase in mobility of people, goods, and resources. The usually unintended introduction of animal, plant or insect species to new maladapted habitats can transform them into invasive pests (Pimentel et al., 2000), that upset local trophic chains, sometimes with powerful negative effects for viticulture, as shown by the recent example of the fruit fly *Drosophila suzukii* damaging fruit and grape crops around the world in recent years (Calabria et al., 2010; Chabert, et al., 2012).

## 2.2 Worldwide occurrence of wine defects

According to the International Organisation for Vine and Wine (OIV) 2019 statistical report on world viticulture, the average world wine production for the past 17 years has been around 270 million hectolitres per year.

In 2018, approximately 292 million hectolitres of wine were produced. The most important wine producing countries were Italy (54.8 mhL), France (48.6 mhL), Spain (44.4 mhL), USA (23.9 mhL), Argentina (14.5 mhL) and Chile (12.9 mhL). It is worth mentioning that in 2018 the three biggest wine producing countries, Italy, France and Spain, accounted for precisely half of the world's wine production.

When it comes to wine defects, no such reliable statistics can easily be accessed, for political and economic reasons which are easy to infer. It is therefore difficult to estimate the true magnitude of the impact, economic or otherwise, that the occurrences of various wine defects have on the worldwide wine industry.

However, a useful starting point would be to begin looking at the percentage of defective or problematic wines identified in wine tasting competitions. This approach, by its nature, offers two advantages.

First, wine competitions tend to employ highly diverse and international groups of judges and gather many wines from different wine-producing countries. This helps to correct and, in a sense, normalize individual variability and any idiosyncrasies of the judges. More significantly, it helps with keeping the data strictly about defects in wines, in a general or even global sense, without going into specifics about which country or wine region encounters more defects than others, therefore avoiding or minimizing the risk of getting involved in contentious arguments, where national brand images and mostly negative publicity can be at stake.

Secondly, the occurrence of wine defects in wine competitions can point to possible internal quality control issues at an individual wine producer or distributor level which may be worthy of addressing. Any wine that is introduced into competition has been previously selected by a responsible person somewhere. Therefore, while there are many possible wine defects that can occur during the winemaking process and are readily corrected by the responsible

specialist before the wine is ready to be sold, the percentage of defects found in wine competitions points to the proportion of detectable wine quality issues that, for any number of reasons, have gone either unaddressed or undetected by the wine producer or distributor. According to a journalistic report from 2012 of Tim Atkin, Master of Wine, over the course of a 7 year-long logging of data of the International Wine Challenge (IWC) in London (where, for a point of reference, about 14.000 wines were evaluated in the 2015 edition) there seem to be an average of 10.4% of wines with a defect. The most common defects were cork taint (2.8%), sulphides (2.7%), oxidation (2.6%) and Brett (1.4%).

In an informal survey which I have conducted in 2016 in the Berliner Wein Trophy (wine competition held bi-annually under the patronage of the OIV), with the aid of 12 other jury members, we found that 155 wines out of the lot of 1661 which we had collectively examined had manifested a defect. This amounts to a percentage of 9.3% of wines with a defect. The most important ones were cork taint (2.8%), oxidation (2.5%), sulphuric/reductive (1.3%) and Brett (1.4%).

It is significant to highlight that the percentage of wines with cork taint, oxidation and Brett are virtually identical in the two independent surveys.

The entry of wines into such competitions usually costs money. Therefore, wineries or distributors select wines to send and they also pay an admission fee, indicating that these wines have already passed at least one form of quality check before arriving at the jury tasting.

When considering the rough estimate that 1.4% of wines in competitions were regarded as Brett-tainted, one should remember that this defect is found almost exclusively in red wines, for reasons that will be made evident in the following sections of the literature review. The instances of *Brettanomyces* defects occurring in white or rosé wines are rare, although not impossible. If one were to evaluate the incidence of Brett taint in red wines strictly, to the exclusion of white and rosé wines, then the percentage would certainly appear higher. It is also quite likely that the rate of occurrence of different defects in wines during the winemaking and storage processes is at least the same if not higher than that of defects encountered in wine competitions, for obvious reasons pertaining to internal quality control

measures inside individual wine producing facilities. In other words, the estimated 1.4% rate of incidence of Brett in red wines should be considered as highly conservative.

## 2.3 The wine defect caused by *Brettanomyces bruxellensis* in wine

### 2.3.1 Historical background

The so-called “Brett” character of a wine is generally considered a defect and is attributed to certain metabolites produced by spoilage yeast of the genus *Brettanomyces* (or its spore-forming teleomorph *Dekkera*). This affects the chemical composition of wine especially during its maturation and ageing process. At low concentrations, the aroma compounds produced by this yeast may arguably be regarded as contributing to the complexity of a wine’s aroma. However, at higher levels these metabolites impart overwhelmingly intense specific aromas that lead to an unpleasant sensory experience and an unfavourable assessment of a wine (Chatonnet & Pons, 1990).

*Brettanomyces* yeast was first described by Niels Hjelte Claussen who, in 1904 at the Carlsberg Brewery in Denmark, has initially isolated it from British spontaneously fermented beer, where it was deemed responsible for the secondary fermentation and the specific flavour of English stock ales (Claussen, 1904). Because of this origin, he named it *Brettanomyces*, formed from the Greek words “Brettano” (British) and “Myces” (fungus) (Licker et al., 1998). This initial instance of *Brettanomyces* isolation resulted in it becoming the first patented microorganism in history (UK patent GB190328184). After several years in which more and more isolates were obtained from lambic beers, *Brettanomyces* was proposed as a genus (Kufferath & van Laer, 1921).

Within the *Brettanomyces* genus, the species names that were given reflected their original isolation from beers: *B. lambicus* from Belgian lambic beer, *carlsbergensis* from the Carlsberg Brewery, *claussenii* from English beer both isolated by Niels Claussen, and *bruxellensis* from the region of Brussels (Smith & Divol, 2016).

After identification in beers, *Brettanomyces* started to be noticed in wine as well: in German grape must in 1933 (Krumbholz & Tauschanoff, 1933) but initially referred to as *Mycotorula intermedia*, and then in French wine in the 1950s (Barret et al., 1955, Peynaud & Domercq, 1956).

Additionally, following investigation, it was revealed that *Brettanomyces* yeasts were present in wineries, not just on the equipment utilized there, but in the air, on cellar walls, drains, pumps, transfer lines and other particularly difficult to sanitize pieces of equipment (Peynaud, 1959; Van der Walt, 1984; Neva et al., 1998; Fugelsang, 1998; Connel et al., 2002).

Being continually detected and identified in wine during the 1960s (Webb et al., 1967) and 1970s (Dubois & Brulè, 1970), interest in and awareness of *Brettanomyces* spoilage has since continued to develop during the past 40 years (Buglass, 2011).

Their negative impact on the organoleptic properties of wine as well as the formation of acetic acid in aerobic conditions established *Brettanomyces* as a spoilage yeast (Peynaud & Domercq, 1959). In South Africa, *Brettanomyces intermedius* and *Brettanomyces schanderlii* were linked to haze formation in wines (Van der Walt & van Kerken, 1958, 1959, 1961; Van Zyl, 1962).

Beginning with the 1940s *Brettanomyces* was reported in Italy, Australia, and New Zealand (Sponholz, 2003).

*Brettanomyces* yeasts were also isolated following detection in the cider industry, from conveyor channels and waters used for washing apples (Davenport, 1976) and from cider fermentation and maturation processes (Beech, 1993; Michel et al., 1988; Morrissey et al., 2004).

*Brettanomyces* yeasts were detected even in the fermentation mash used in the Tequila industry (Lachance, 1995).

Even though this yeast has become famous for its negative effects on wine quality, the presence of *Brettanomyces* can be desirable, however, in particular situations concerning foods and beverages and is starting to become a valuable contributor in certain novel industrial fermentation applications (Steensels et al, 2015). For example, it is an important

contributor to the flavour profile of Belgian lambic and gueuze beers (Dequin et al., 2003; Dufour et al., 2003). Indeed, this species of yeast is known for other positive contributions to the aroma of various other foods and beverages, such as the fermented and sweetened Kombucha tea (Mayser et al., 1995., Teoh et al., 2004), feta cheese (Fadda et al., 2001) and sour dough bread (Meroth et al., 2003).

Additionally, *Brettanomyces* yeast have found application in the bioethanol industry, as they are tolerant to low pH levels, have a nutrient efficient metabolism and can produce high concentrations of ethanol (Passoth et al., 2007).

In wine, however, the fermentation activity of *Brettanomyces* yeast produces metabolites with odour profiles described as “animal”, “medicinal”, “Elastoplast”, “sweaty leather”, “barnyard”, “spicy” or “clove-like”, which are considered detrimental to the organoleptic properties of the final product, and, therefore, has become a major concern to winemaking in recent years (Oelofse et al., 2008). Consequently, the control of *Brettanomyces* yeast development has become one of the most important microbiological challenges in modern winemaking (Boulton et al, 1996; Fugelsang & Edwards, 2007).

Currently, based on molecular analysis of the genera, there are five species belonging to the genus *Brettanomyces*: *B. custersianus*, *B. naardenensis*, *B. nanus*, *B. anomalus* (and *Dekkera anomala*) and *B. bruxellensis* (and *Dekkera bruxellensis*) (Kurtzmann et al., 2011; Steensels et al., 2015). The latter, *B. bruxellensis* (Figure 1), is the one most commonly affecting wine (Wedral et al., 2010), primarily because of its metabolism’s by-products.

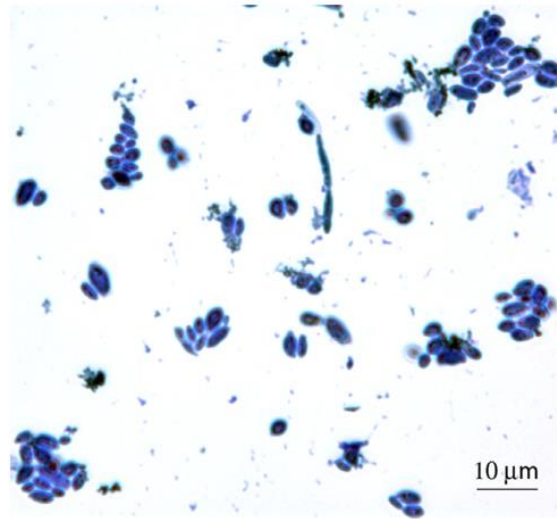


Figure 1. Photomicrograph of *B. bruxellensis* (Wedral et al., 2010)

### 2.3.2 Spoilage of wine by *Brettanomyces/Dekkera*

The *Brettanomyces* yeasts can break down several sugar types (Galafassi et al., 2011). In the process of glycolysis, a glucose molecule is broken down into two pyruvate molecules, which are then used by yeasts for either respiration or fermentation (Schifferdecker et al., 2014). Even though respiration is energetically favourable to fermentation, the latter usually being performed only when oxygen availability decreases, several yeast species, such as *S. cerevisiae* and *B. bruxellensis* can switch the metabolic pathway for pyruvate from respiration to fermentation, even in aerobic conditions, displaying a “Crabtree-positive” phenotype (Pronk et al., 1996; Rozpedowska et al., 2011). This is referring to the Crabtree effect (reverse Pasteur Effect), a phenomenon in which yeast produce ethanol in aerobic conditions rather than producing biomass via the tricarboxylic acid cycle (TCA) (de Deken, 1966). Normally, oxygen availability determines the expression of genes, but this is also regulated by substrate availability (Hutkins, 2006). If the glucose concentration is high enough, the transcription of catabolic genes (including the ones coding for TCA enzymes) will be repressed by glucose, and the metabolism will be fermentative (Hutkins, 2006).

The way this plays out in *B. bruxellensis* is that as oxygen availability becomes limited, the fermentation of glucose to ethanol is blocked. In this anaerobic environment, the options for this yeast to convert nicotinamide adenine dinucleotide, reduced form (NADH) to



nicotinamide adenine dinucleotide, oxidized form (NAD<sup>+</sup>) are limited (Steensels et al., 2015). With a deficit of NAD<sup>+</sup> comes a blockage in glycolysis (Wijsman et al., 1984). While *S. cerevisiae* can bypass this by producing glycerol, *Brettanomyces* does not have this option, lacking the appropriate enzyme expression (Tiukova et al., 2013). These findings explain two things which are of practical value to the winemaker: Brett is disadvantaged in nutrient-rich environments and is advantaged in nutrient-deficient environments, as glycerol production is energy consuming (Steensels et al., 2015). During winemaking, as the must is gradually transformed into wine and the environment is gradually depleted of nutrients, a considerable lag phase (Figure 2) occurs for *B. bruxellensis*, as the environment becomes anaerobic (Steensels et al., 2015).

The metabolic pathway that supports the production of volatile phenols is linked to the presence of the vinylphenol reductase (VPR) enzyme (Heresztyn 1986). Curtin et al. (2013) highlighted that oxygen deficiency in the environment will enhance the VPR activity in the yeast. This enzyme uses NADH as a cofactor in reducing hydroxystyrenes to their ethyl derivatives, seeming to play a role in maintaining the redox balance of the cell (Steensels et al., 2015).

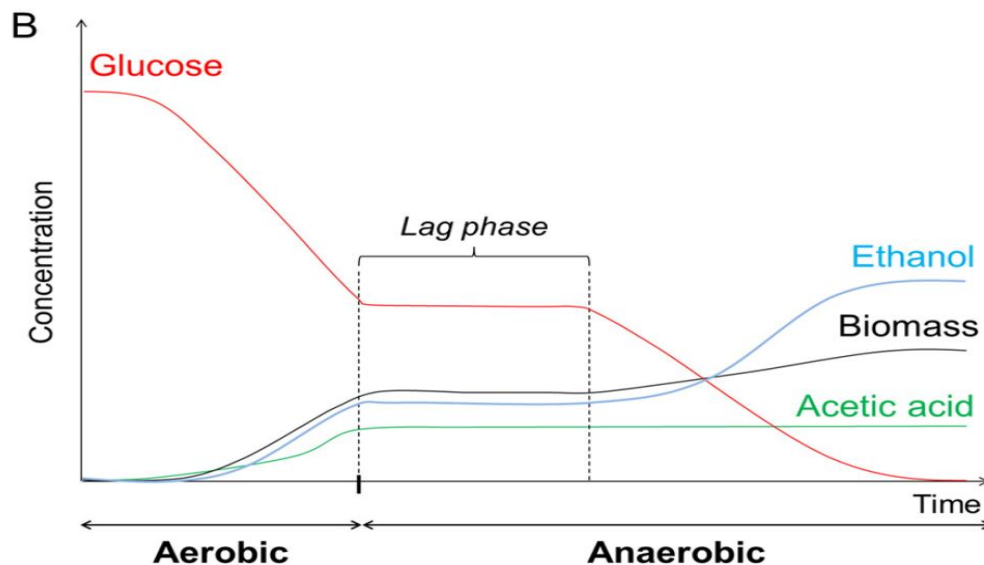


Figure 2. Effect of a shift from aerobic to anaerobic culture conditions on the growth kinetics of *B. bruxellensis* (Steensels et al., 2015)

### 2.3.2.1 Production of volatile phenols

The most problematic metabolites produced by this yeast species belong to the category of volatile phenols (Table 1), a large family of substances, possessing a strong odour, influencing the aroma of numerous fermented food and beverage products (cheese, fermented milk, beer, wine, cider, tequila etc.) (Boidron et al., 1988; Chatonnet et al., 1992; Oelofse et al., 2008). The usual concentration found in wines ranges from ~10 to several hundred  $\mu\text{g/L}$ , with serious infections leading to concentrations of a few thousand  $\mu\text{g/L}$ .

**Table 1. The main volatile phenols responsible for the *Brettanomyces* aroma in red wine (adapted from Curtin et al., 2005)**

Compound	Molecular weight (g/mol)	Taint aroma	Odour threshold ( $\mu\text{g/L}$ )	Concentration in red wine ( $\mu\text{g/L}$ )
4-Vinylphenol	120.15	Phenol, medicinal	20***	8-43
4-Vinylguaiacol	150.17	Clove-like	10*	0.2-15
4-Ethylphenol	122.16	Phenol, Band-Aid®, medicinal, barnyard	30-60**	118-3696
4-Ethylguaiacol	152.19	Spice, clove	33*/110**	1-432
4-Ethylcatechol	138.16	Horsey	30-60**	27-427
*model wine, ** red wine, *** water.				

Figure 3 highlights the chain of reactions that leads to the formation of the three most important volatile phenols responsible for the Brett defect: 4-ethylphenol, 4-ethylguaiacol, 4-ethylcatechol.

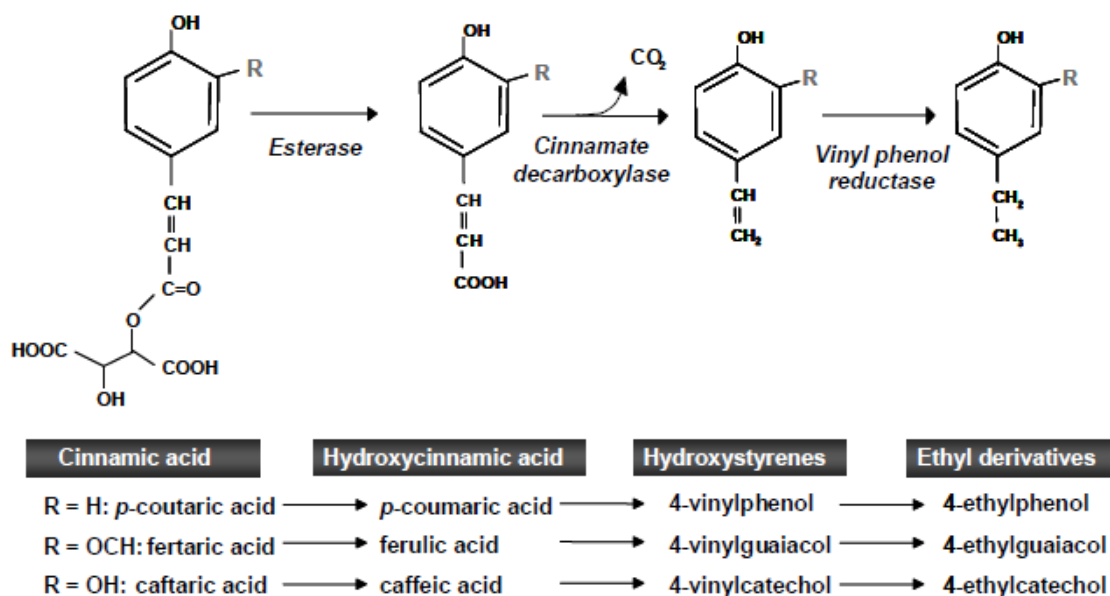
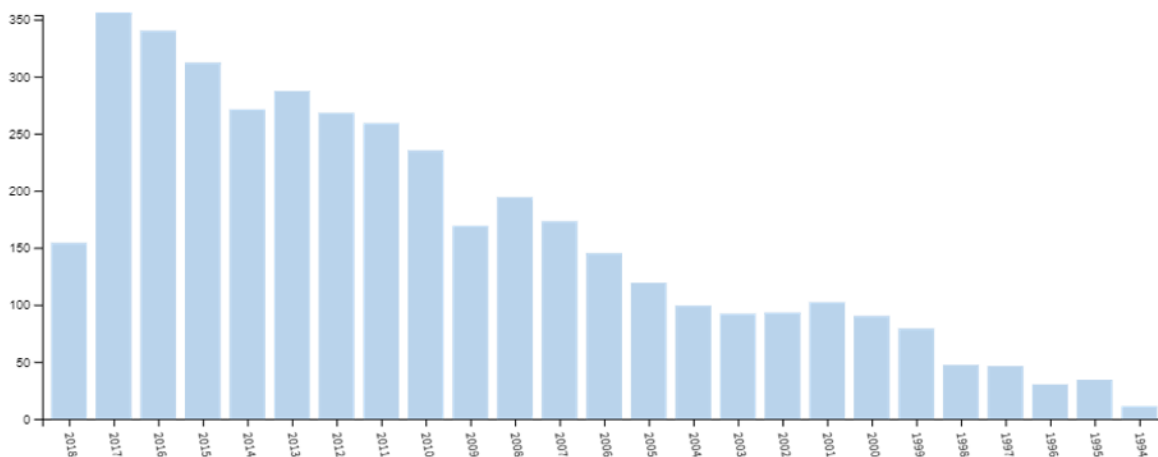


Figure 3. Formation of volatile phenols via the decarboxylation of hydroxycinnamic acids (Oelofse et al., 2008)

The availability of phenolic acid precursors (coumaric, ferulic and caffeic acid) is an important starting point for subsequent defect development.

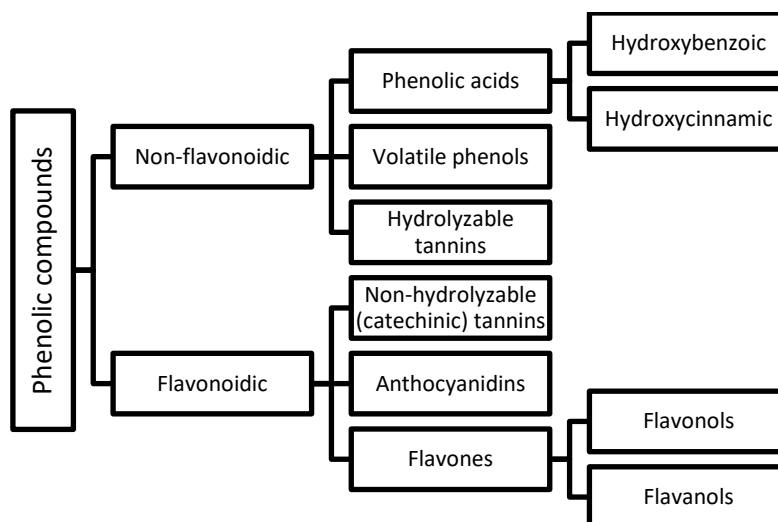
Phenolic acids are a specific subcategory of the broad spectrum of phenolic compounds which are found in wine. Phenolic compounds are, from an oenological perspective, most important for wine quality, being responsible for virtually all differences between red and white wines, particularly their colour and flavour characteristics (Ribéreau-Gayon et al., 2006b). Additionally, phenolic compounds have bactericide, antioxidant, and vitamin properties, and seem to play some role in protection from cardiovascular disease (Ribéreau-Gayon et al., 2006b), resveratrol being a well-known example (Cotea et al., 2014). The manifold properties of phenolic compounds in wine have led to them becoming an increasingly popular research topic for the scientific community (Figure 4).



**Figure 4.** Number of scientific publications containing the keywords “phenolic compounds” and “wine” published per year, as of 3.07.2018 (Data from [www.webofknowledge.com](http://www.webofknowledge.com))

Chemically, phenols are substances derived from aromatic hydrocarbons (arenes) to which one or more hydroxyl (-OH) group can be attached. Due to the mobility of the hydrogen atom from the -OH groups, phenolic compounds are generally highly reactive substances, and slightly acidic (Cotea et al., 2014).

The broad category of phenolic compounds can be structured and compartmentalized according to the presence in the molecule of other functional groups apart from the -OH one. A schematic classification of phenolic compounds can be found in Figure 5.



**Figure 5.** Classification of phenolic compounds (adapted from Cotea et al., 2014)

Phenolic acids contain a carboxyl (-COOH) group attached either directly or indirectly to the benzene nucleus. Hydroxybenzoic acids have the -COOH group attached directly to the benzene ring, while hydroxycinnamic acids have the same functional group indirectly attached to the benzene ring.

The usual concentrations of phenolic acids are around 100-200 mg/L in red wine, and 10-20 mg/L in white wines (Ribéreau-Gayon et al., 2006b).

The content of phenolic acids can vary depending on grape variety. For example, the French oenologist Vincent Renouf partitions red wine varieties in three categories, depending on their phenolic acid content: those rich in phenolic acids (such as Tannat, Grenache, Negrette), those with moderate phenolic acid content (such as Merlot, Malbec, Syrah, Gamay), and those with low phenolic acid content (such as Pinot Noir) (Renouf, 2005).

As seen from the results of Curtin et al. (2005) and Oelofse et al. (2008), the main precursors for unwanted volatile phenols due to *Brettanomyces bruxellensis* infections are represented by the three main hydroxycinnamic acids found in wine: p-coumaric, ferulic and caffeic acids.

As an example of the usual values for these hydroxycinnamic acids in red and white wines from China, one can look at the work of Tang et al. (2015):

**Table 2. Values of hydroxycinnamic acids in red and white Chinese wines; all values are in mg/L (adapted from Tang et al., 2015)**

<b>Compound</b>	<b>Carménère (n=20)</b>	<b>Cabernet Sauvignon (n=29)</b>	<b>Merlot (n=14)</b>	<b>Chardonnay (n=19)</b>	<b>Italian Riesling (n=24)</b>
p-coumaric acid	10.56 ± 3.4	10.54 ± 5.4	8.25 ± 2.6	3.24 ± 0.7	2.98 ± 0.8
Ferulic acid	1.71 ± 1.1	2.56 ± 1.7	1.98 ± 1.2	nd	nd
Caffeic acid	15.36 ± 6.1	15.55 ± 5.9	12.92 ± 3.1	6.78 ± 3.1	6.31 ± 2.3

The ratio of p-coumaric to ferulic acid has been observed to be identical to the ratio between the corresponding volatile phenols for which they are precursors, and this ratio varies between 3.5:1 and 16:1 (Chatonnet et al., 1992; Pollnitz et al., 2000; Romano et al., 2008)

The amount and ratio of these cinnamic acids available in a wine is influenced by the grape cultivar (Lampíř, 2013), and one can infer that, as is the case for phenolic compounds in general, geographical region, climatic conditions and winemaking methods may also play some influencing role.

A further point in the refinement of understanding may come from the work of Salameh et al. (2008), where the adsorption of 10-50% of *p*-coumaric acid on *Brettanomyces* sp. cell walls was demonstrated, all the while no vinylphenol or ethylphenol were being produced by this yeast in the medium. This is an example of the polyphenolic adsorption phenomena on yeast cell walls, also documented by other authors (Suárez et al., 2007; Medina et al., 2005; Morata et al., 2003; Morata et al., 2005; O'Neill et al., 1996). In fact, it seems that *Brettanomyces bruxellensis* yeasts can only potentially transform 50-60% of the total content of *p*-coumaric acid into 4-ethylphenol (Chatonnet et al., 1992).

Be that as it may, it is essential to highlight that practically all red wines contain potentially enough hydroxycinnamic acids to produce several milligrams of volatile phenols per litre of wine (Renouf, 2015).

These acids are not always freely available in the grape must. Unlike hydroxybenzoic acids, hydroxycinnamic acids can combine with tartaric acid and form esters (*p*-coumaroyl tartaric or coutaric, caffeoyl tartaric or caftaric and feruoyl tartaric or fertaric acids) (Cotea et al., 2014). As these acids are usually found in grape skins, they are extracted during maceration (Renouf, 2015).

Although these esters of hydroxycinnamic acids with tartaric acid will naturally hydrolyse in a spontaneous and progressive manner, this process can be catalysed by an increase of cinnamoyl esterase enzymes, either due to *Botrytis cinerea* infections in the grapes or due to using insufficiently purified enzymatic preparations (such as pectolytic enzymes) during the winemaking process (Renouf, 2015). This has the effect of increasing the content of free hydroxycinnamic acids available for subsequent *Brettanomyces* development at an early stage of winemaking, which is a crucial detail for practical winemaking purposes.

The next enzymatic reaction, the decarboxylation step, has been linked to POF<sup>+</sup> (phenolic off-flavour producing) and PADI (phenyl acrylic acid decarboxylase) genes of *S. cerevisiae*

(Clausen et al., 1994) and can also be facilitated by several other bacteria (such as lactic acid bacteria), fungi and yeast species present during the winemaking process, as they also possess the required enzymatic activity (Heresztyn, 1986a; Chatonnet et al., 1992; Cavin et al., 1993; Degraasi et al., 1995; Edlin et al., 1995; Cavin et al., 1997; Edlin et al., 1998; Shinohara et al., 2000; Van Beek & Priest, 2000; Barata et al., 2006; Couto et al., 2006).

The final step is carried out by the vinyl phenol reductase (VPR) enzyme of the *Brettanomyces* yeast, resulting in the typical “Brett” aroma. This reduction step is not commonly facilitated by microorganisms (Chatonnet et al., 1995). Although low amounts of ethylphenols can be produced by *Lactobacillus* spp. (Chatonnet et al., 1995; Couto et al., 2006), or *Pichia guilliermondii* (Dias et al., 2003), there is currently no other species of microorganism able to approach the magnitude of 4-ethylphenol production of *Brettanomyces* yeasts (Barata et al., 2006).

The most significant volatile phenols responsible for this taint are 4-ethylphenol and 4-ethylguaiacol. The former is the main component responsible for the “Brett” aroma. Its precursor is p-coumaric acid, a phenolic acid, which is transported into the cell, decarboxylated to its vinyl derivate by a phenolic (cinnamic) acid decarboxylase (PAD), and then enzymatically reduced to the ethyl derivate, 4-ethylphenol, by a vinyl phenol reductase (VPR) enzyme (Wedral et al., 2010). In a corresponding manner to p-coumaric acid, ferulic acid is similarly converted first into 4-vinylguaiacol and then into 4-ethylguaiacol, with its characteristic sweet clove-like flavour (Bartowsky & Pretorius, 2009).

In white wines, most of volatile phenols are represented by vinylphenols, and in red wines by ethylphenols. (Boidron et al., 1988). It has been observed that hydroxycinnamic acids can be decarboxylated enzymatically to vinylphenol in enough quantities to actively participate in the aroma of certain white wines (Boidron et al., 1988). One significant difference between red and white wines in this case is that certain grape phenols, especially procyanidins, can inhibit the activity of the cinnamate decarboxylase enzyme of *S. cerevisiae* (Chatonnet et al., 1989). This can help explain why vinylphenol levels can be higher in white wines than in red wines, even as the quantity of available precursors is much higher in the latter. Red wines can potentially reach high levels of ethylphenols, while white wines may potentially reach

high levels of vinylphenols, but not ethylphenols; rosé wines in general are situated somewhere in between white and red ones in this regard (Chatonnet et al., 1992).

In vineyard studies, there have been no significant differences observed in the microflora present on the surface of white grapes as opposed to red grapes, *Brettanomyces* yeasts being detected in both cases (Renouf, 2015).

Despite this, *Brettanomyces* yeasts have rarely been isolated from white wines (Licker et al., 1998). Firstly, as aromatic ripeness usually is accomplished before phenolic ripeness, grapes for white or rosé wines are generally harvested earlier than those for red wine. This earlier harvesting date ensures a reduced microbial charge on the grapes, as the population of *Brettanomyces* and of yeasts in general increases with the advancement of the ripening process, while the bacterial population remains stable (Renouf, 2015).

Their loss of viability in white wine and consequent lack of ethylphenols in said wines has been ascribed to generally lower pH levels (Renouf, 2015), and the subsequently increased efficiency of sulphur dioxide at lower pH levels (Loureiro & Malfeito-Ferreira, 2006), as well as to the absence of the corresponding precursors (Chatonnet et al., 1992). Additionally, white, and rosé musts are clarified before fermentation, which lowers the yeast biomass and removes suspended solids which usually act as potential nutrient sources for unwanted microorganisms (Renouf, 2015). In white and rosé winemaking, the fermentation times are generally shorter, and the use of selected yeasts is widespread. Additionally, the phenolic acids (p-coumaric and ferulic acid) which are precursors for volatile phenols, are involved in the browning of white grape must (Cheynier et al., 1995), and are generally removed by the winemaker by a fining during the must clarification stage or by controlled oxidation of the must, thus further removing their availability for future microbiological problems (Renouf, 2015).

Red wines, on the contrary, are highly susceptible to infections, due to their lower acidity, higher polyphenol content (and therefore of p-coumaric and ferulic acids) and ageing in barrels (Wedral et al., 2010). Although between different red varieties of *V. vinifera* quantitative differences in precursor polyphenols can be observed, it is not possible to infer that this can lead to large differences in the amounts of volatile phenols produced in cases of



*Brettanomyces* infections. Pinot Noir, for example, has a low p-coumaric acid content (Wedral et al., 2010). However, this does not necessarily imply a low probability for the development of the taint. A study performed in Burgundy on Pinot Noir revealed that at least 50% of wines undergoing maturation contained *Brettanomyces* yeasts, and 25% of already bottled wine do as well (Gerbaux et al., 2000). In addition, other studies show no difference in ethylphenol concentrations produced in wines from Cabernet Franc, Cabernet Sauvignon, Merlot, Pinot Noir or Syrah varieties (Rayne & Eggers, 2008).

There are several wine matrix effects noted regarding the volatility of 4-ethylphenol and 4-ethylguaiacol. For example, Petrozziello et al. (2014) report statistically significant decreases of the volatility of 4-EP and 4-EG with increases in alcohol content and in polyphenol content. An explanation for the influence of the polyphenol content is given by the results of Jung et al. (2000) referring to Pi stacking of aromatic rings being involved in the chemical interactions between polyphenols and certain flavouring compounds with aromatic cycles. These types of interactions are stabilized by the presence of hydroxyl groups and the formation of hydrogen bonds and can explain the decrease in 4-EP volatility in the presence of high doses of polyphenols. The magnitude of these interactions may depend on different molecular weights and structures of the involved polyphenols (Aronson & Ebeler, 2004). Production of volatile phenols, whilst being the main defect that *Brettanomyces* yeast are known for, does not represent their only negative impact on wine.

### **2.3.2.2 Production of volatile acidity and volatile fatty acids**

The metabolism of *Brettanomyces* can produce acetic acid (Licker et al., 1998; Loureiro & Malfeito-Ferreira, 2006), which is a major component of volatile acidity, together with acetaldehyde and ethyl acetate (Guillamón & Mas, 2011). It is considered that when the acetic acid concentration is below 0.72 g/L that it is neither easy to detect on the palate, nor does it particularly impart any positive effect (Ribéreau-Gayon et al., 2006a), with values above this level beginning to convey the typical olfactory impression of sourness and the gustatory perception of sourness and bitterness.

The European Union adopts, for still wines, the maximum values for an acceptable volatile acidity as 18 meq/L (1.07 g/L of acetic acid) for white and rosé wines, and 20 meq/l (1.2 g/l of acetic acid) for red wines.

Additionally, *Brettanomyces* yeasts can also produce, by the degradation of L-leucine, L-isoleucine and L-valine, several volatile fatty acids, such as isovaleric acid (3-methylbutanoic acid), 2-methylbutyric and isobutyric acid (Oelofse et al., 2008) with attributed “rancid”, “sweaty” or “cheesy” aromas (Coulter et al., 2004) (Figure 6).

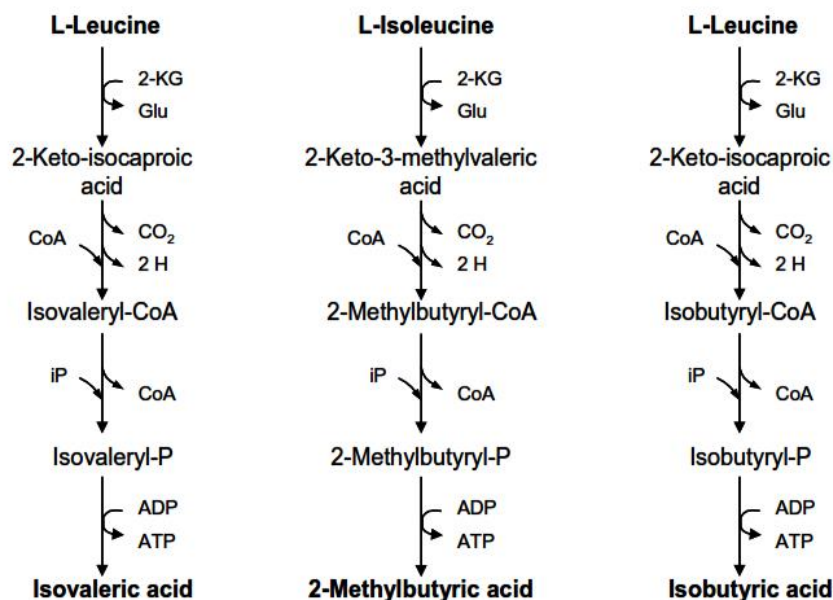


Figure 6. The formation of isovaleric, 2-methylbutyric and isobutyric acids (Harwood & Canale-Parola, 1981, cited in Oelofse, 2008)

The relationship between volatile phenols and these volatile fatty acids is one of perceptual interaction. According to some authors, although an increase in the content of volatile fatty acid does not correlate with high levels of ethylphenols, they are assumed to have a sensorially synergistic effect, in strengthening each other’s impact (Coulter et al., 2004; Oelofse et al., 2008).

Conversely, according to Romano et al. (2009), the role of isobutyric and isovaleric acids has been shown to produce an olfactory masking effect onto volatile phenols by increasing the detection threshold for 4-ethylphenol. This also comes in to help explain various empirically

observed inconsistencies, as described by Renouf (2015) between the sensory impression of the Brett taint and its volatile phenol content.

### 2.3.2.3 Mousiness

The so-called “mousy” off flavour in wine was first identified and described in the 1980s (Heresztyn, 1986b). It can be caused by *Lactobacillus* and *Brettanomyces* species. The occurrence of mousiness does not always entail a Brett infection, but Brett infections could contribute to the defect called mousiness.

It seems to be caused by 2-acetyltetrahydropyridine (ATHP), 2-ethyltetrahydropyridine (ETHP) and 2-acetylpyrroline (APY) (Grbin et al., 1995), of which *Brettanomyces* can produce the first two (Oelofse et al., 2008). ATHP is detected in this case in wine with concentrations of 4.8-106 µg/L (Grbin et al., 1995). Its sensory threshold value is reported to be 1.6 µg/L (Teranashi et al., 1975). ETHP on the other hand, can be found in wine, but in much lower concentrations than its sensory threshold value (Grbin et al., 1995), which is 150 µg/L (Craig & Heresztyn, 1984).

The precursors for the formation of ATHP and ETHP are the amino acid L-Lysine (Heresztyn, 1986b) and ethanol (Snowdon et al., 2006), with oxygen presence acting as a stimulating agent (Grbin, 1998). It has been proven that certain strains of *Brettanomyces* can produce the mousy off-flavour even in the absence of L-Lysine (Snowdon et al., 2006), as these yeasts can synthesize L-Lysine endogenously (Bode et al., 1990).

Snowdon et al. (2006) propose a chemical pathway to produce ATHP and ETHP from *Brettanomyces* yeast, as can be seen in Figure 7.

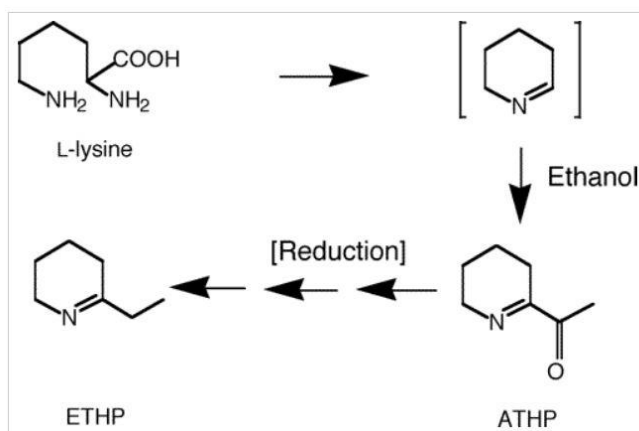


Figure 7. Proposed pathway for the formation of the mousy heterocycles ATHP and ETHP by *Dekkera/Brettanomyces* in wine (Snowdon et al., 2006)

Although the aroma descriptors of ATHP have been given as “cracker biscuit” and “popcorn”, this compound makes itself sensorially noticeable at the usual pH levels in wine through a bitter, metallic aftertaste (Oelofse et al., 2008).

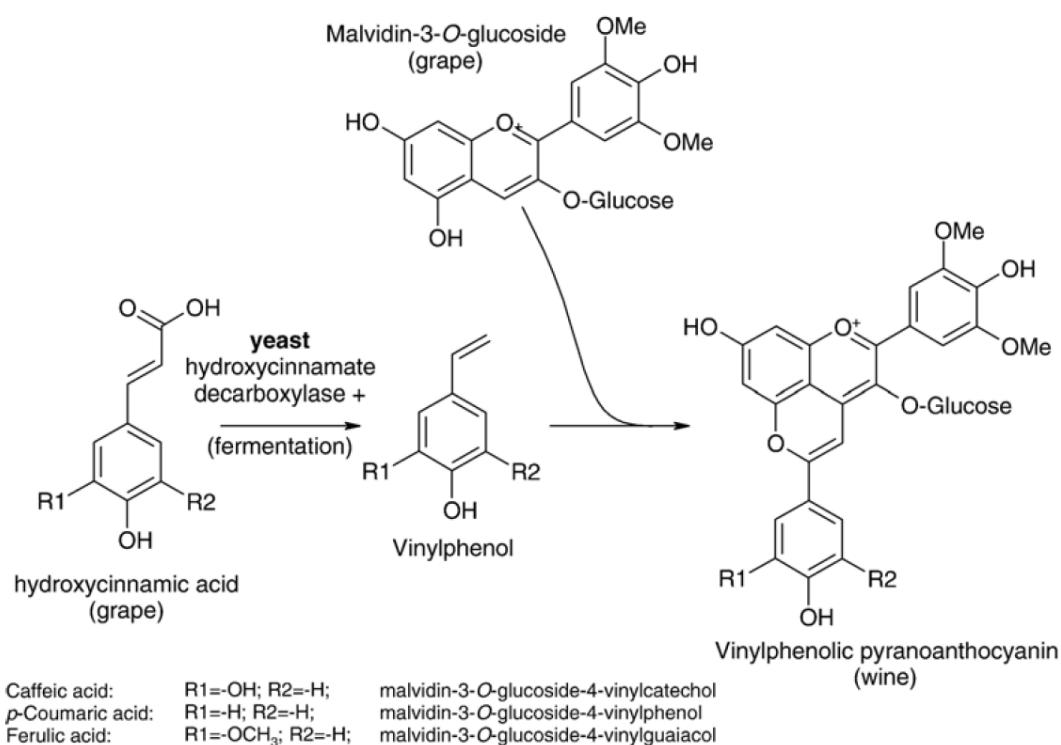
#### 2.3.2.4 Decolouration

It has been reported that contamination with *Brettanomyces* results in wines having undesirable colour (Oelofse et al., 2008). It has been demonstrated that some *Brettanomyces* yeast have the enzyme  $\beta$ -glucosidase, and therefore display glycosidic activity (Fugelsang et al., 1993).

Although this can be interpreted positively, in the direction of enhancing wine aroma and complexity (Oelofse et al., 2008), this enzymatic activity affects the mono-glucosylated anthocyanins, the red pigments in young wine, and may be the reason why the wines infected with this yeast have an undesirable colour (Suárez et al., 2007), because after the hydrolysis of glucose, the corresponding anthocyanin may be converted to a colourless pseudo base, with negative implications for colour (Mansfield et al., 2002).

Because of the condensation of vinylphenols with grape anthocyanins, such as malvidin-3-O-glucoside, vinylphenolic pyranoanthocyanins are formed (Oelofse et al., 2008). These are one of the three kinds of pyranoanthocyanin pigments (vitisins, pyrano-anthocyanin-

flavanols, vinylphenolic adducts) to which are attributed increases in colour intensity and stability during ageing by Morata et al., 2007. According to these authors, fermentation carried out by hydroxycinnamate decarboxylase activity containing *S. cerevisiae* yeast strains favours the formation of vinylphenolic pyranoanthocyanins, as can be seen in Figure 8.



**Figure 8.** Formation of vinylphenolic pyranoanthocyanins in musts to which hydroxycinnamic acids were added, during fermentation with yeasts showing hydroxycinnamate decarboxylase activity (Morata et al., 2007)

### 2.3.2.5 Biogenic amines

Through the metabolic activity of microorganisms, biogenic amines can be formed via decarboxylation of amino acids (Oelofse et al., 2008). The production of biogenic amines in wines by yeasts has been studied by Caruso et al. (2002). They observed that, in a comparison with other yeasts, *B. bruxellensis* tend to produce the highest concentration of biogenic amines (15 mg/L on average). These were: ethanolamine, methylamine, tryptamine, putresceine, cadaverine, histamine, agmatine and 2-phenylamine. It seems that 2-

phenylamine is the biogenic amine produced in the most significant quantity (Caruso et al., 2002; Granchi et al., 2005).

Although in these studies most biogenic amines produced (except agmatine and 2-phenylalanine) were found in amounts close to their detection limits, they are nevertheless of great interest as they have been linked with physiological feelings of bodily discomfort, such as headaches, nausea, diarrhoea and red skin coloration in some people who display amine intolerance (Gafner, 2003).

Additionally, while the human body has the capacity to convert biogenic amines into harmless products through the activity of the diamine oxidase (DAO) enzyme, it has been shown that ethanol entering the body from alcohol consumption drastically inhibits the activity of this enzyme (Gafner, 2003).

### **2.3.3 Sources of *Brettanomyces* infection in wine**

There are multiple sources for *Brettanomyces* spp. occurrence in wine. A vineyard provides ample sources, such as the soil, roots, bark, leaves or grapes. However, the poor detection of this yeast species on grapes could be due to their low cell numbers in a diverse microbial yeast system (Oelofse et al., 2008).

The most frequently cited location of *Brettanomyces* is in the oak wood (Fugelsang & Edwards, 2007), as these yeasts can utilize cellobiose (Blondin et al., 1982), which results from the toasting of barrels. As this carbohydrate is found in a higher concentration in new barrels, they are more preferred sites for the growth of *Brettanomyces* yeast than older barrels.

Additionally, they may grow in any difficult to clean area: in processing equipment, transfer lines or valves where organic deposits may accumulate throughout the season, or in drains or isolated pockets of juice and wine (Fugelsang & Edwards, 2007).

They increase in numbers when other favourable conditions suit their slow growth manner, such as after alcoholic fermentation, when traces of residual sugar, low levels of free sulphur dioxide and release of nutrients from yeast autolysis improve their proliferation effectiveness (Oelofse et al., 2008). Even if a wine is considered dry, it may still support growth of

*Brettanomyces* as even concentrations of residual sugar as low as 0.275 g/L glucose, fructose, galactose and trehalose are enough to support the growth of this spoilage yeast and damage the aroma of the wine (Chatonnet et al., 1995). Even so, not all strains of *B. bruxellensis* can produce the taint compounds (Wedral et al., 2010; Conterno et al., 2006). The differences in ethyl phenol production between different *Brettanomyces* strains have been better correlated to the availability of nitrogen sources than to the amount of residual sugars present (Conterno et al., 2007).

Malolactic fermentation and ageing in previously used oak barrels are also critical stages for contamination during the production of wine (Oelofse et al., 2008). Also, during storage, long periods of barrel ageing, low SO<sub>2</sub> levels and tendency to either perform a lighter filtration of the wine or not filter it at all are also linked to increases in *Brettanomyces* populations in the wine (Oelofse et al., 2008).

*Brettanomyces* yeasts grow very slowly in comparison to other yeasts found in wines. They typically reach maximum population density in a wine about 5 to 7 months after vinification (Fugelsang & Zoecklein, 2003).

Several winemaking trends in recent years have been correlated to the increase in incidence of *Brettanomyces* spoilage (Yap et al., 2007):

- Wines with high pH and/or residual sugar.
- Decreases in filtration use and SO<sub>2</sub> doses.
- Insufficient cellar hygiene and sanitation of barrels.
- The spread of *Brettanomyces* between wineries or regions due to use of second-hand contaminated barrels.
- Transfer of contaminated wine between wineries.

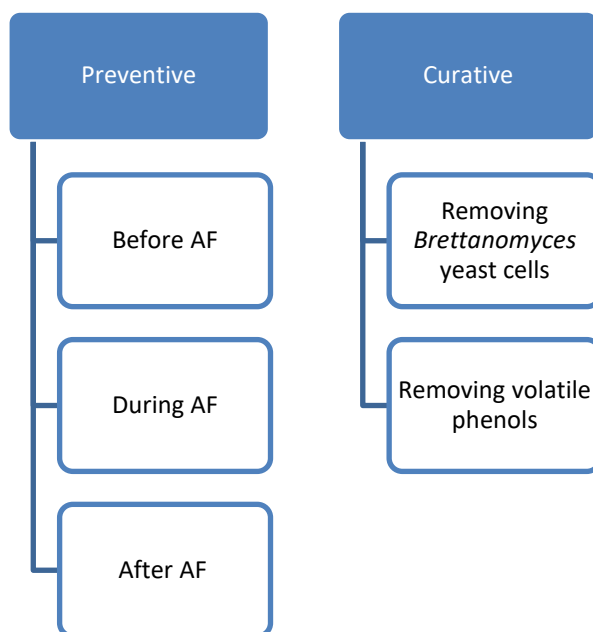
Another tendency observed in Australian wine making establishments is the preference towards smaller and more frequent SO<sub>2</sub> additions, as opposed to fewer, larger ones. This can lead to unintentional selection and build-up of more resistant yeast species (Coulter et al., 2004).

### 2.3.4 Curative and preventive methods for management of *Brettanomyces* taint in wines

In the efforts to control or remedy the problems caused by this yeast strain and its taint in wine, several approaches have been considered. There are things an oenologist can do to prevent *Brettanomyces* taint in his wines, and things he can do to remedy this problem once it has presented itself. So, one can talk about preventive and curative approaches (Figure 9). The essence of all such preventive approaches is to inhibit the development of *Brettanomyces* yeast. This encompasses all measures taken from the start of viticulture activities, throughout all oenological decision making, culminating with wine bottling that have an effect of inhibiting the development or proliferation of *Brettanomyces* yeast. Such an integrated, holistic approach is very tightly connected to general oenological knowledge and covers a broad spectrum of possible activities. One can divide the spectrum of preventive methods according to the respective stage of the winemaking process. One can talk about options available before alcoholic fermentation (AF) starts, options available during fermentation and options available for the post-fermentative period, associated with wine maturation and ageing. Further discussion on the broad range of preventive measures possible is beyond the scope of this review.

The second type of approach is curative in nature. Here, the winemaker has encountered a *Brettanomyces* contamination which he needs to contain and resolve. A curative approach must consider that two distinct actions are to be considered: the removal of *Brettanomyces* yeast cells and the removal of their metabolites (volatile phenols) which create the sensory perception typical of Brett.





**Figure 9.** The basic dimensions of preventive and curative approaches towards Brett infections

Wine treatments intended to reduce *Brettanomyces* populations and/or their qualitative impact during the ageing process can be regarded as preventive approaches, in that the concentration of yeast cells is to be kept low so that there is no chance for significant quantities of volatile phenols and of other disagreeable metabolites to be produced.

Wine treatments intended to reduce the concentration of volatile phenols and other metabolites produced by *Brettanomyces* yeast can be regarded as curative approaches, since the taint can, in this case, already be detected by smelling and tasting, therefore the wine is considered sub-optimal. Nevertheless, if a Brett-type defect has been identified in a wine, the need to remove *Brettanomyces* yeast cells is paramount to a successful strategy, which is why it should be considered a curative approach as well.

Treatments intended for cleaning barrels, wooden vats, and other approaches to cellar sanitation can be viewed as both curative (for contaminated vessels) and preventive processes (by allowing continued safe usage of vessels).

Yeast selection can play a significant role, as different strains of *S. cerevisiae*, for example, differ in their ability to produce vinylphenols (Nelson, 2008). Selecting, therefore, a low vinylphenol producing strain can lead to less problematic quantities of ethylphenols.

However, a particular benefit of vinylphenols lies in their role in forming pyranoanthocyanins, which can help increase the stability and intensity of the wine colour (Morata et al., 2007).

While some sources cite concentrations of 30 mg/L free SO<sub>2</sub> as adequate protection (Chatonnet et al., 1992), others observe growth of *Brettanomyces* even at higher values (Froudière & Larue, 1988). However, irrespective of the free form of SO<sub>2</sub>, the actual effectiveness of its molecular form may be a clearer indicator (Boulton et al., 1996; Ribéreau-Gayon et al., 2006a). Molecular SO<sub>2</sub> is mainly dependent on pH but also on other variations in wine composition (ethanol, temperature, anthocyanin content and nutrient content) (Smith, 1996). A value of 0.5 mg/L of molecular SO<sub>2</sub> has been proposed as a standard reference for protection of wine against *Brettanomyces* yeast proliferation (Renouf, 2015).

Some success is registered with fining agents, such as liquid gelatine, which achieves a reduction in *Brettanomyces* populations in an experiment from  $1.2 \times 10^4$  colony forming units (CFU)/mL to 270 and 170 CFU/mL after dosages of 0.3 and 0.6 mg/L respectively (Suárez et al., 2007). However, such fining is sometimes rejected by winemakers since it allegedly also reduces wine aroma and colour.

The use of chitosan and lysozyme mixes were reported as successful by Renouf (2015) in reducing *Brettanomyces* yeast cell content during red wine ageing. Two mixtures, one of chitosan (25%, lysozyme (50) and glucanase (25%) and the other only of chitosan (33%) and lysozyme (67%), when applied in doses of 30 g/hl, were successful in reducing *Brettanomyces* populations when compared to two SO<sub>2</sub> treated wines and one control. Especially the mixture of chitosan and lysozyme reduced the *Brettanomyces* concentration from  $1 \cdot 10^3$  to 1 CFU/ml in two days, where it was kept stable for the next 20 days.

Filtration has been used effectively to remove *Brettanomyces* cells with membranes with pore sizes smaller than 0.45 µm (Calderón et al., 2004). However, it has been seen that *Brettanomyces* cells can reduce their size when entering a VBNC (viable but not culturable) state and can pass through the pores of such filters (Millet & Lonvaud-Funel, 2000). Therefore, some wines appear to be subject to *Brettanomyces/Dekkera* spoilage, after they have been declared sterile by agar plate enumerations (Oelofse et al., 2008).

Another approach to solving the issue of the Brett taint is the selective removal of the unpleasant smelling volatile phenols.

The use of reverse osmosis and adsorption using a hydrophobic resin and a tangential-flow membrane filtration have been described in the literature (Ugarte et al., 2005) with a 77% reduction in the total ethylphenols, however this also determined a reduction in aromatic compounds (for example in ethyl and methyl vanillate and other esters).

Polyvinylpyrrolidone (PVPP) and charcoal have been used to lower ethylphenol levels (Suárez et al., 2007) with prescribed amounts ranging from 60-480 mg/L for PVPP and 15-240 mg/L for charcoal, depending on the intensity of the taint.

Both wine and model solution experiments were reported (Chassagne et al., 2005) in which active dried yeast of *S. cerevisiae* was able to remove 33% and 26% of total 4-ethylphenol and 4-ethylguaiacol concentrations. The affinity of the yeast lees to the volatile phenol adsorption was sensitive to the degree of yeast autolysis and physical-chemical parameters, such as ethanol, temperature, and pH. However, the amount of yeast lees necessary for this reduction was 32 g/L, and the equilibrium time required for sorption was 3 hours.

Focusing on the treatment approach of reducing the undesired yeast content in the wine barrel, several other substances have been shown to have an effect against *Brettanomyces* development, possibly due to their general antimicrobial effects. These approaches include chitosan application (Hill, 2009; Taillandier et al., 2014), sorbic acid application (Stopforth et al., 2005), natamycin (Gerbaux et al., 2000; Delves-Broughton et al., 2005), diethyl dicarbonate and dimethyl dicarbonate (Golden et al., 2005).

In 2014, Marican et al. published results regarding polyaniline-based materials for removal of 4-ethylguaiacol in red wines. While amounts of 10 mg polyaniline-emeraldine salt (PANI-ES) and polyaniline-emeraldine base (PANI-EB) managed to remove 4-EG by amounts up to 50% from a contaminated wine, they also had a strong impact on the total phenolic content, reducing it by 0.54 g/L and 1.06 g/L respectively. The next year, in 2015, Carrasco-Sánchez et al. expanded on this topic to also remove 4-ethylphenol from red wines, managing to remove with a 10 mg/mL treatment of PANI-EB 67.8% of 4-EP, 50% of 4-EG and 41.38% of total phenols.

Another approach has been the removal of 4-Ethylphenol and 4-Ethylguaiacol in wine by using esterified cellulose (Larcher et al., 2012). In this example, different cellulose polymers were tested, including cellulose acetate (CA), cellulose acetate propionate (CAP), cellulose acetate butyrate (CAB), cellulose propionate (CP). Out of these, doses of 4 g/L CAP and CP were the most effective, with average percentages of 38% and 37% reduction for 4-EP and 4-EG, respectively. The increasing of the dose up to a maximum of 20 g/L had an effect, attaining reductions of about 70%, however the increase in reduction of 4-EP and 4-EG as influenced by the increase in dosage slows down after 4-6 g/L. While contact times of up to 60 minutes were explored, a plateau in reduction was reached during the first 15 minutes. After treatments, the reductions in ethylphenol content were statistically significant from the control. Also, statistically significant reductions in the colour intensity and total anthocyanins were noted, however were considered technologically irrelevant, with average reductions of 4.6% and 6.6% respectively. In a sensory analysis panel, the treated wines were perceived as both different and preferable to the control samples. Ease of regeneration and re-use of CAP and CP fibres recommends them as an interesting curative solution to removing the typical *Brettanomyces* taint odour, to a certain degree.

With regards to barrel cleaning and maintenance, the risk of biofilm formation by *Brettanomyces/Dekkera* is significant, with Joseph and Bisson (2004) reporting that 50% of the 35 isolated strains having this ability. This is a cause for concern as biofilms can be up to 1000 times more resistant to chemical cleaning agents and sanitizers (Lewis, 2001). The recommendation for SO<sub>2</sub> sanitation is at least 7 g of gas per barrel (Chatonnet et al., 1992). While ozone treatment has been reported both as ineffective (Cantacuzene et al., 2003) and as 99% effective in reducing *Brettanomyces* populations (Coggan, 2003), it has been used effectively in the sanitation of stainless-steel tanks (Oelofse et al., 2008).

Other approaches explored involve barrel shaving and re-firing, with a reduction of 4-EP and 4-EG in wines stored in shaved and re-fired barrels, as compared to untreated barrels (Pollnitz et al., 2000). Also, sonication seems to achieve a 97% population reduction with an ultrasound power of 50 watts for 90 to 120 seconds in laboratory tests (Yap et al., 2007).

Unfortunately, although many treatment strategies are being developed, there are currently no reliable techniques that allow the detection of *Brettanomyces/Dekkera* in wood (Oelofse et al., 2008).

The removal of volatile phenols from wine using molecularly imprinted polymers has been attempted previously and there are several published scientific articles on this topic.

Specifically, Garde-Cerdán et al. (2008) investigated 4-vinylpyridine based molecularly imprinted polymers that use pentachlorophenol as a template for removing 2,4,6-Trichloroanisole, 4-Ethylphenol and 4-Ethylguaiacol from aged red wines. Teixeira et al. (2015) also investigated 4-vinylpyridine based molecularly imprinted polymers that used both 4-Ethylphenol and 4-Ethylguaiacol as templates for removing 4-Ethylphenol and 4-Ethylguaiacol from tainted wines and from model solutions.

Most recently, Filipe-Ribeiro et al. (2020) investigated ethylene glycol methyl ether acrylate based molecularly imprinted polymers for achieving the same goal of reducing 4-Ethylphenol and 4-Ethylguaiacol levels in red wines.

Although detailed comparisons between the results found in these most relevant reference literature items and the results found in this work will be given and discussed in the results section, as part of the appropriate experiments, there are a few general aspects that can be highlighted right away.

Firstly, in all cases, high removal rates for volatile phenols (4-EP and 4-EG) are reported: up to 63% (Filipe-Ribeiro et al. 2020), 89-92% (Garde-Cerdán et al., 2008) and 55% (Teixera et al. 2015).

Secondly, doses of polymer required for such effects were in the 2-10 g/L range, contact times were of several hours and treated wine volumes were usually 25-100mL. Exact levels of 4-ethylphenol and 4-ethylguaiacol taint in tested wines were not disclosed in all studies. In some there were only removal percentages disclosed. In others, concentrations between 2.5 and 10 mg/L were given. These conditions are reflective of a laboratory scale experiment. Ultimately, the choice of these parameters is highly significant since it is possible to generate high removal rates using concentrations of taint substance and polymer dose that are later difficult to reproduce when scaling up from laboratory to a winery environment.

Thirdly, and most crucially, these studies rely on recipes for molecularly imprinted polymers that use either 4-vinylpyridine or ethylene glycol methyl ether acrylate as their functional monomers. The importance of selecting a suitable monomer for creating MIPs is critical to determining how that polymer behaves in its intended matrix, as will be discussed in the next section of literature review. Both 4-vinylpyridine or ethylene glycol methyl ether acrylate are toxic, flammable and corrosive compounds (Sigma-Aldrich SDS-V3204, Sigma Aldrich SDS-408913). Their use in food-contact applications is therefore not possible. This means that while the comparative results of these studies are of scientific interest, any upscaling from laboratory to practical winemaking conditions using these substances is rendered impossible by the toxic nature of the monomers. By way of contrast, the monomers and the MIP recipes used in this study are non-toxic and food-contact compliant.

## 2.4 Molecularly imprinted polymers

### 2.4.1 Introduction to the technology of molecular imprinting

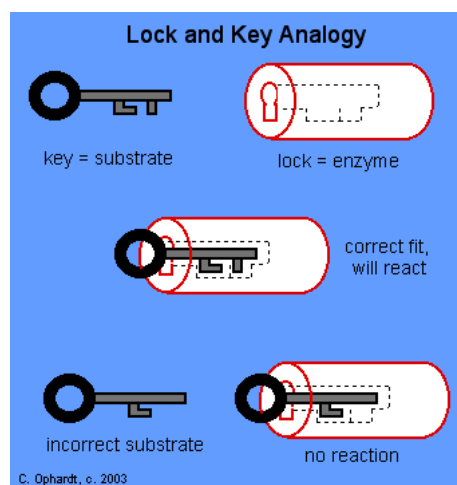
The technology of molecular imprinting is a continually developing technique for preparation of polymers that display specific affinities (molecular recognition) for a given chemical compound or one of its analogues (Dickey, 1949; Owens et al., 1999). The synthesis of a molecularly imprinted polymer (MIP) involves the mixing of the template molecule with functional monomers, cross-linking monomers, and an initiator in a solvent, which is usually aprotic and non-polar. The polymerization is initiated by UV light irradiation or by heat. The complex formed between the template molecule and functional monomers is stabilized in the resulting highly cross-linked polymer. After this, the template molecule is extracted and the resulting imprinted polymer possesses a permanent memory for the imprinted species formed, enabling the resultant polymer to selectively rebind the imprinted molecule from a mixture of other compounds, related or not. The cavities formed are complementary in shape and in chemical functionality to that of the template. The high degree of cross-linking enables the cavities to maintain their shape and functional groups in an optimal configuration to be bound once again with the template, allowing for molecular recognition (Shea & Sasaki, 1988; Rimmer 1998).

MIPs have demonstrated chemical and thermal stability, being suitable for use in harsh or aggressive media (Vlatakis et al., 2003). Other advantages include low cost, ease of operation and storage, ability to regenerate the polymer for repeated use and mechanical robustness (Yan & Row, 2006). For these reasons, MIPs have become increasingly interesting for research in the fields of chemistry and biology, as affinity materials for sensors (Kriz et al., 1997; Haupt & Mosbach, 2000), artificial antibodies (Lavignac et al., 2004), adsorbents for solid phase extraction (Molinelli et al., 2002) and stationary phases for chromatography (Hwang & Lee, 2001).

### 2.4.2 A brief history of milestones in MIP research and development

Molecular imprinting has been a topic for scientific research that can be traced back to 1931 with what is considered to be the first work done in this field by M.V. Polyakov who demonstrated that silica gel prepared in the presence of a solvent additive showed preferred binding to the same solvent. His group used  $(\text{NH}_4)_2\text{CO}_3$  as a gelating agent to assist the polymerization of sodium silicate in water. After two weeks, additives are added, such as benzene, toluene, or xylene. After 20-30 days in which the silica can dry, the additive was removed by washing extensively with hot water. Adsorption studies revealed a higher capacity for uptake of the additive by the silica than for structurally related ligands, making apparent that, in the cases of benzene and toluene, a memory for the additive had been manifested by the polymer. This observed selectivity was explained as the result of structural changes in the silica reflecting the nature of the additive (Polyakov et al., 1933; Polyakov et al., 1937).

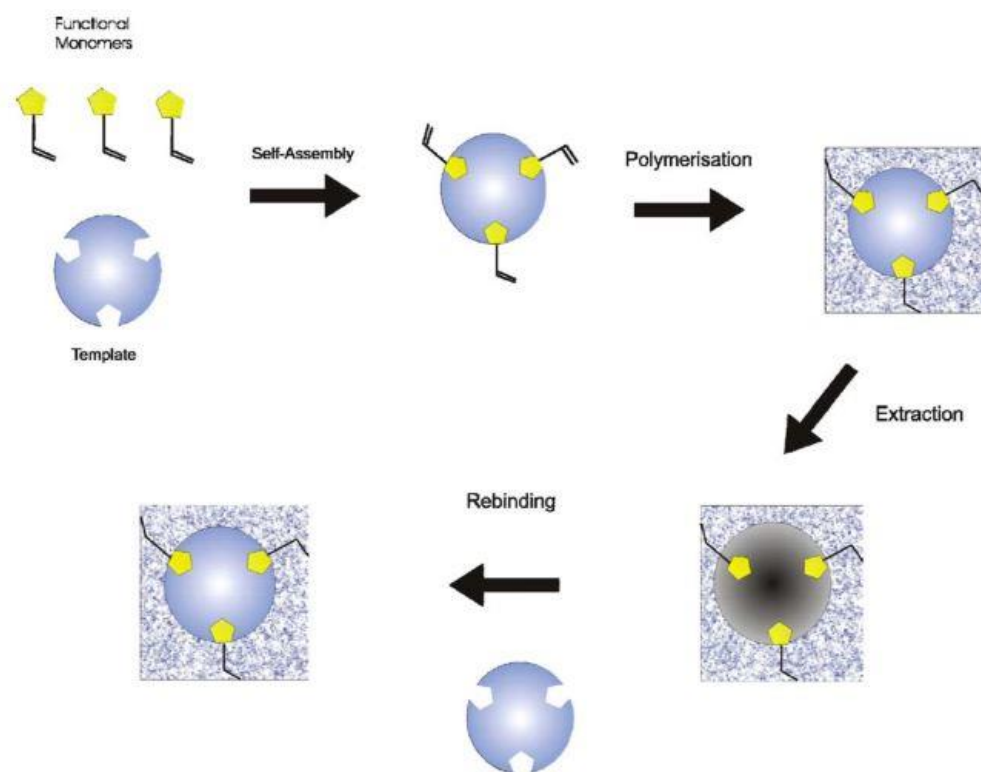
The biomimetic property reflected in the molecular recognition has been likened to the substrate-selectivity mechanisms analogous to that of antibodies or enzymes. A fitting exemplification was a theory originating in biochemistry, the lock-and-key mechanism postulated by Emil Fischer in 1894. In his analogy, the lock is the enzyme and the key the substrate. Only the correctly sized key (substrate) fits into the keyhole (active site) of the lock (enzyme) (Figure 10).



**Figure 10. Lock and key analogy for enzyme-substrate complex**  
(<http://chemistry.elmhurst.edu/vchembook/571lockkey.html>)



However not all experimental evidence could be explained adequately with this model, and it was consequently refuted by X-ray diffraction studies. This gave rise to the induced fit theory and showed that enzymes have a more flexible structure than initially indicated by the lock-and-key mechanism (O'Mahony et al., 2004). Nevertheless, the idea of a matrix designed to recognise a specific substrate has remained as the cornerstone of molecular imprinting theory and remained a primary goal of research into this field since the 1970s (O'Mahony et al., 2004).



**Figure 11. Schematic representation of non-covalent imprinting (O'Mahony et al., 2004)**

While the example of the mechanism of enzyme reactivity was helpful to comprehend the basic principle of operation of a MIP (Figure 11), it was the study of antibody formation that heralded further insights into the understanding of the creation of selectively reactive sites of the MIP.

At the time of Polyakov's findings, the origin of the selectivity of antibodies of the immune system was a debated topic. One proposed theory was the so-called instructional theory of

antibody formation (Breinl & Haurowitz, 1930; Mudd 1932). They suggested that the effect of an antigen in determining the structure of an antibody molecule might involve the ordering of the amino-acid residues in the polypeptide chains in a way different from that in the normal globulin.

The renowned chemist Linus Pauling further refined this view and formulated his own theory of the structure and process of formation of antibodies, published in 1940. He assumed that all antibody molecules contain the same polypeptide chains as normal globulin and differ from normal globulin only in the configuration of the chain, the way it is coiled in the molecule. In other words, he proposed that antibody formation takes place in the presence of an antigen, captured by the cell, which served as a template for antibody formation. The primary structure of any antibody would have to be identical, and the template-induced conformational effect should give rise to the remarkable selectivity that antibodies manifest. Dickey (1949), who was working in Pauling's team, would later create the precursor to modern MIPs by imprinting silica gels with alkyl orange dyes. The resulting silica gel's pronounced selectivity for the dye which had been present during polymerization was explained as follows:

*“[T]he adsorbent in the process of formation has accessible to it a very great number of structures which differ only slightly in stability, and ... in the presence of a foreign molecule those structures that are stabilized through attraction for the foreign molecule are preferentially assumed. The adsorbent is thus pictured as automatically forming pockets that fit closely enough to the foreign molecule to hold it by van der Waals' forces, hydrogen bonds, interionic attractions, and other types of intermolecular interaction.”*

This footprint or imprint mechanism was refuted by Morrison et al. (1959) who also conducted research in the same experimental area. They concluded that the observed retention was not due to molecules occupying specific cavities but was due to a self-association mechanism in which template molecules, trapped after the synthesis step, act as nucleation sites, and are the underlying basis for the observed selectivity.

Following the logic of the nucleation theory, superior retention should be observed when a larger amount of template remains in the polymer work. This was refuted by Beckett and Youssef (1963), as they observed an inverse relation between the amount of trapped template and amount of analyte retained. Moreover, a nucleation mechanism would cause an enantiomer, as trapped template, to form a racemic compound with its opposite enantiomer and yield a more stable complex with the opposite enantiomer and thus selectively retain it as well. However, they observed that the reverse is true: matrixes imprinted with enantiomers show selectivity only for their respective enantiomer.

While the footprint mechanism of recognition was most supported by the data, the exact mechanism was not entirely elucidated, and was a topic of controversy throughout the late 1950s and the 1960s.

More recent work by Baggiani et al. (2001) demonstrated that covalently bound template molecules can indeed act as nucleation points for the formation of template molecule clusters. The binding sites in a MIP are created by these clusters rather than individual molecules. However, in this case it is not clear whether this effect requires template molecules to remain trapped in the matrix to create nucleation points, or if it can occur as a cooperative effect between template molecules upon rebinding (O'Mahony, 2004). Other findings (Andersson et al., 1999) in a chromatographic study on a MIP for nicotine indicate that template-template complexes are formed, and that binding sites are created for these complexes specifically. Overall, the contribution of self-association mechanisms to the creation of an imprinting effect is not precisely quantified and further work in this area is required (O'Mahony, 2004). After two decades of intense research, the decline in molecular imprinting in silica corresponds to the introduction of molecular imprinting in organic polymers (Wulff & Sarhan, 1972; Takagishi & Klotz, 1972). Another important milestone is the introduction of non-covalent imprinting by Andersson & Mosbach (1990). They identify the limitations of covalent binding, as very few reversible covalent bonds are suitable for use and point to the possibility of using hydrogen bonding and carboxylic acids. This evolution allowed for molecular imprinting to become a practical analytical tool that is easily adaptable (O'Mahony, 2004).

### 2.4.3 Potential applications of MIP technology

The reason for the interest in MIPs is their affinity and selectivity, like that of natural receptors, stability superior to natural biomolecules and ease of preparation and adaptation to different practical applications (Piletsky et al., 1999; Andersson et al., 1995; Vlatakis et al., 1993).

The main areas for research are material science and separation technology, with the main commercially applicable fields being separation, assays, sensors and finally catalysis (Piletsky et al., 2001).

In 2001, Piletsky et al. projected that the US market for separation techniques was US\$ 19 billion, of which MIP materials could claim 1-3%, especially in the chromatography column sector, valued at the time at US\$500 million per year. The most interesting prospect remained contract manufacturing for MIP materials, valued at US\$100m-US\$300m, for use in solid phase extraction (SPE), as sorbents or as enantioselective materials by the pharmaceutical industry, as well as manufacturing tailor-made sensing devices for health care, testing of product quality, or authenticity assessment of products such as perfumes and wines.

#### 2.4.3.1 Use of MIPs in drug delivery systems

To maximize efficacy and safety of medicines, drug delivery systems (DDS) must be able to regulate the rate of release and targeting the drug to a specific site. This is supported by the need to deliver the desired therapeutic dose, at the most appropriate place in the body, to expand the duration of pharmacological action and reduce adverse effects (Puoci et al., 2010). In this situation, molecular imprinting technology can offer systems with the ability to recognize specific bioactive molecule, hinting at the future potential of imprinted DDS systems.

However, a compromise has to be reached between rigidity and flexibility, as the imprinted cavity should be stable enough to maintain its conformation in the absence of the template, but also flexible enough to allow for a fast equilibrium between release and re-uptake of the template in the cavity.

Also, MIPs for drug delivery should be stable enough to resist enzymatic, chemical, or mechanical stress, as they are meant to be in contact with biological fluids of complex composition and varied pH. Also, as they would come in contact with sensitive tissues, safety and toxicological concerns have to be addressed, and the MIP in such a case should not be toxic, nor should its components, residual monomers, impurities or possible products of degradation (Aydin et al., 2002). The challenge in designing MIPs for DDS systems is combining intelligent drug delivery with molecular recognition. The predictable intelligent drug release of therapeutic agents would be a response to specific stimuli, such as the presence of another specific molecule, small changes in temperature, pH, solvent composition, ionic strength, electric field or incident light (Gil & Hudson, 2004; Peppas & Leobandung, 2004).

#### **2.4.3.2 Use of MIPs as chemo or biosensors**

Sensors are devices that respond to physical or chemical stimuli by producing a response signal, which is usually electric in nature. Unlike more common stimuli such as temperature, weight or light, when a sensor's target is a molecule, ion, or atom, the response signal to an effect can be specific or non-specific, either informative or misleading (Puoci et al., 2010). It is common for an analyte of interest to be accompanied by several different species, present at different concentrations, further complicating the analytical issue.

Expedient methods of chemical/biological detection are highly researched and sought after throughout the world, especially in relation to medical diagnosis, environmental monitoring, food safety and public security, as such bio/chemo sensors can potentially be less time-consuming, less expensive and simpler to construct and operate than the established laboratory analysis methods.

The key to improving the performance of chemical sensors is improving their selectivity, so that a chemical species can be detected without the need for separation stages. This has been accomplished with the development of biosensors (Updike & Hicks, 1967), which consist of two components: the receptor and the transducer. The receptor is a chemosensory material that can selectively bind the target analyte, while the transducer is transforming the binding

events into a readable signal output related to the analyte concentration in the sample (Eggins, 2002).

Typical sensing elements used are biological macromolecules (antibodies, enzymes, receptors, ion channel proteins, nucleic acids, peptide nucleic chains) or biological systems (microorganisms, isolate whole cells, ex vivo tissue) (Puoci et al., 2010). Nanomaterials have recently found applications as the foundation for chemo sensors, due to their unique electrical, optical, catalytic, or magnetic properties (Chen et al., 2004). Their significant advantages of large adsorptive surfaces (Xie et al., 2008) and specific molecular affinity (Puoci et al., 2010) are countered by the complex procedures and high costs required for their synthesis, and by their instability (Whitcombe et al., 2000; Haupt & Mosbach, 2000).

This wider context has left molecular imprinting as one of the most efficient ways to design artificial recognition systems with the template polymerization technique (Spivak, 2005; Zhang et al., 2006).

Since their initial development about 25 years ago (Andersson et al., 1990) MIP based electrochemical sensors have made remarkable progress in recent years, by using conductometric/potentiometric measurements and MIP nanomaterials, significantly extending their range of detected targets and improving their sensitivity, selectivity and simplicity (Zhou et al., 2003).

Also, optical sensors based on fluorescent on/off mechanisms have been shown to be useful for analysis of small molecules in difficult environments, due to their high signal output and feasible measurements (Holthoff & Bright, 2007).

However, mass sensitive devices have proven to be the best fit for MIP based chemo sensing, achieving potentially universal applicability, good limits of detection, low cost and ease of miniaturization and automation (Haupt et al., 1999). The measurement of mass is the most general method suitable for the detection of an analyte, as mass can be regarded as a universal property of matter. As such, piezoelectric devices such as quartz crystal microbalances (QCM) may provide highly sensitive measurements to the mass of analyte binding at the surface of piezoelectric materials. Changes in the mass of the piezoelectric material result in a change in the resonant frequency, which can be precisely measured. For example, in a 10

MHz resonating system, a 1 ng change in mass results in a 1 Hz change in the resonant frequency. When a molecular imprinted polymer is used as the surface of a QCM it can be used to measure template specific binding with a high degree of sensitivity. Such an application of MIP nanomaterials in piezoelectric sensors can be used in a range from small molecules to biomacromolecules and even to large analytes such as microorganisms and cells (Puoci et al., 2010).

#### **2.4.3.3 MIPs as artificial antibodies and receptors**

One of the most ambitious and anticipated goals for bioorganic chemistry has been the design and production of biomimetic receptor systems, which can bind target molecules with similar affinities and specificities as their natural counterparts (Puoci et al., 2010). Due to their binding characteristics, chemical and physical stability and low cost, molecularly imprinted polymers have been proposed as alternative artificial antibodies (Ye & Haupt, 2004). However, it is worth remembering that molecularly imprinted polymers are large, rigid, and insoluble amorphous bulk materials, while antibodies are molecular in nature, small, flexible and soluble (Spivak, 2005).

The most common MIPs are organic polymers synthesized from vinyl or acrylic monomers by radical polymerization, using non-covalent interactions. However, these monomers are unable to form a stable complex as their association constant with the template is too low. Therefore, during non-covalent imprinting, functional monomers are used in excess to shift the equilibrium towards complex formation, resulting in randomly distributed functional groups in the polymer matrix. Non-specific binding arises because of this process, and is a considerable drawback, compared to natural antibodies. More sophisticated monomers are being designed to form stable interactions with the template molecule or substructure, with the purpose of being used in a stoichiometric ratio (Puoci et al., 2010).

#### 2.4.3.4 Molecularly imprinted membranes

A MIM (molecularly imprinted membrane) is a membrane either composed of a MIP or containing a MIP. A general problem of the “conventional” MIP technology is the simultaneous and random creation of the imprinted sites along with the formation of the polymer matrix including its pore structure. Consequently, random distribution and uneven accessibility of receptor sites in the volume of a MIP material are characteristic (Ulbricht M., 2004). Three main strategies can be envisioned for the preparation of MIM:

1. Sequential approach—preparation of membranes from previously synthesized “conventional” MIPs, i.e. particles,
2. Simultaneous formation of MIP structure and membrane morphology,
3. Sequential approach—preparation of MIPs on or in support membranes with suited morphology.

The template binding to MIP sites in a MIM can be coupled with a selective transport through the MIM thus enabling a membrane separation. The transport pathways in a polymer membrane can be either the free volume between polymer chains, the solvent fraction of a swollen polymer gel or connected pores in a solid polymer.

There are two major mechanisms for selective transport (Ulbricht M., 2004): facilitated permeation and retarded permeation.

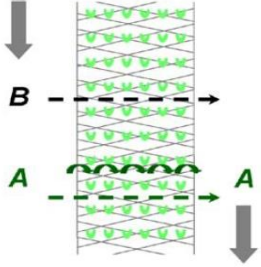
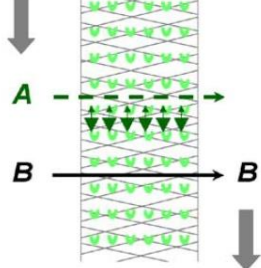
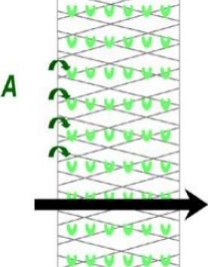
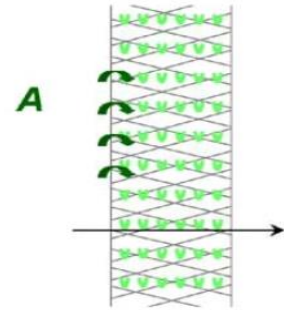
Facilitated permeation is driven by preferential sorption of the template due to affinity binding and allows a slower transport of the solutes. In this case, influenced by the membrane structure as well as MIP site concentration and distribution, transport may occur via carrier-mediated transport, in real membranes coupled with diffusion. Coupling with non-selective diffusion means that separation selectivity can only be achieved for small diameters of transmembrane pores.

Retarded permeation is due to affinity binding and is a faster transport of other solutes, until a saturation of MIP site with template is reached. In this, the saturation behaviour means that separation efficiency will be determined by the MIP binding capacity. The MIM can be considered as a membrane absorber.



The template binding can also change the barrier properties of the MIM, due to an altered membrane swelling (Table 3).

**Table 3. Four types of separation mechanisms for MIM, due to the binding selectivity obtained by imprinting for a substance (Ulbricht M, 2004 – adapted)**

Schematic	Description of separation mechanism
	<p>Transport of A driven by concentration gradient is facilitated by binding/desorption to neighbored MIP sites; non-specific transport of B by diffusion is hindered by the micropore structure of the membrane (fixed carrier membrane).</p>
	<p>Transport of A is prevented by binding or binding/desorption to MIP sites on the surface of trans-membrane pores; B has no specific interactions with the membrane surface, will be transported by diffusion or convection (membrane absorber)</p>
	<p>The MIM permeability is increased, due to an increase in membrane swelling because of A binding to MIP sites.</p>
	<p>The MIM permeability is decreased due to a decrease of membrane swelling because of A binding to MIP sites.</p>

Active development is devoted to the synthesis of MIPs as nanoparticles and microgels. With small particles of well-defined morphology in a colloidal dispersion, the specific binding capacity of MIPs can be increased significantly (Ulbricht M., 2004).

Microgels can mimic the three-dimensional structure of biomacromolecules, as they have a molecular weight in the same range as that of proteins. However, the handling of such small entities requires mechanisms which are suited for colloids or biomacromolecules, for which membrane separation can be a good fit (Ulbricht M., 2004).

The unique feature of MIM is the interplay of selective binding and transmembrane transport of molecules, making them potentially superior to state-of-the-art synthetic separation membranes already applied in various industries (Ulbricht M., 2004). The main problem in MIM preparation is the optimization of MIP recognition and membrane transport properties at the same time.

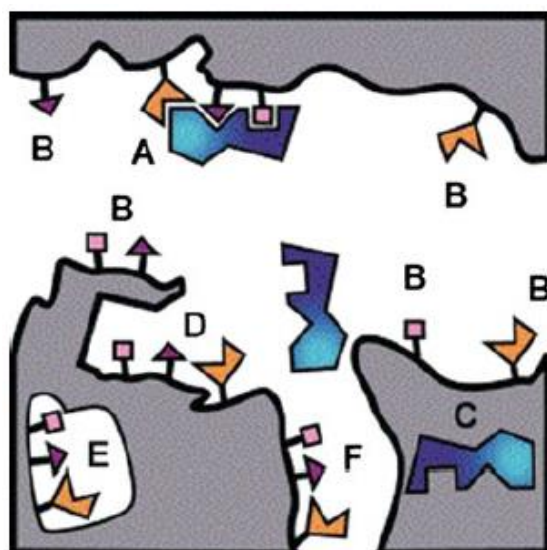
#### **2.4.4 Approaches to molecular imprinting**

The defining feature of MIP formation is represented by the technique of molecular imprinting, which allows for the formation of specific substrate recognition sites in the polymers. Molecular recognition is attributed to a combination of characteristics: the complementary size, shape and binding sites created by the template molecules (Puoci et al., 2010). However, the specific interaction between the template and the functional group is essential in determining the final binding properties of a MIP. The choice of functional monomers is the first major decision to be made to achieve molecular imprinting performance (Curcio et al., 2009).

Usually, three imprinting approaches are used for MIP synthesis: non-covalent, covalent or semi covalent (Caro et al., 2002).

The non-covalent approach is most widely used as it is experimentally simple. The template is mixed with an appropriate functional monomer, or monomers, in a suitable solvent (Joshi et al., 1998). After synthesis, the template is removed from the resultant polymer by solvent washing. The non-covalent interactions are the basis for the step of rebinding the template to the MIP. Also called self-assembly protocol, the non-covalent approach is based on

interactions such as hydrogen bonds, van der Waals forces or hydrophobic effects. The association between monomer and template is governed by equilibrium with the functional monomer being added in excess relative to the template to form the complex, resulting in a heterogeneous binding site distribution (Figure 12) (Tse Sum Bui & Haupt, 2010). The non-covalent approach has a series of limitations related to its conditions of molecular recognition; for example, the interaction between monomer and template is stabilized under hydrophobic environments and can be easily disturbed by polar environments (Yan & Row, 2006). It is suggested that the number of functional groups in the polymer binding site is not determined directly by the pre-polymer complex, but rather is determined during polymerization (Yan & Row, 2006).



**Figure 12. The heterogeneity of the binding sites: high affinity site in the macropore (A) and micropore (F), lower affinity sites (B) in macropore, trapped template (C), embedded site (E), highest affinity site (D) with shape selectivity from the polymer (Tse Sum Bui & Haupt, 2010)**

The covalent approach involves the formation of covalent bonds between template and functional monomer before polymerization, requiring a chemical synthesis prior to the synthesis of the MIP. The cleaving of covalent bonds is necessary to remove the template from the polymer matrix, and for this purpose specific reagents are to be used in solution (Ikegami et al., 2004). Binding of the analyte occurs through covalent interactions. In this method the monomer-template complex is stoichiometric, resulting in a homogenous

population of binding sites with the polymer. However, the necessity to re-establish covalent bonds upon binding the target to the MIP causes the binding kinetics to be slow (Wulf & Sarhan, 1972; Shea & Thompson, 1978). Overall, this method has several disadvantages, as the choices regarding the possible reversible covalent interactions and the number of potential templates is very limited, and often an acid hydrolysis procedure is needed to cleave the covalent bonds between template and functional monomer (Yan & Row, 2006).

The semi-covalent approach represents a hybrid between the non-covalent and covalent methods. While covalent bonds are established between template and functional monomer, the subsequent rebinding of the analyte to the MIP is accomplished through non-covalent interactions (Whitcombe et al., 1995).

Depending on which interactions the imprinting process is based on, different characteristics will be expressed by the MIP. Generally, the average affinity of the binding site formed with non-covalent bonds is weaker than with covalent ones (Puoci et al., 2010), as the electrostatic, hydrogen bonding,  $\pi$ - $\pi$  and hydrophobic interactions between the template and functional monomers are used exclusively in forming the molecular assemblies (Hwang & Lee, 2002). When covalent bonds are established between template and functional monomer prior to polymerization, their stability ensures that the binding sites are better defined and more homogeneous than in the non-covalent approach.

However, as stated above, since the work of Andersson & Mosbach (1990) non-covalent imprinting has been widely used, as it is more advantageous than the covalent approach from the point of view of synthesis. The extensive use of non-covalent molecular imprinting can be explained by three arguments. Firstly, the non-covalent approach is easy to conduct, and avoids the extra steps needed to form a pre-polymerization complex as is the case in the covalent approach. Secondly, the removal of the template is quite easy, accomplishable by continuous extraction. Thirdly, this method allows for a greater variety of functionalities to be introduced into the binding sites of the MIPs (Yan & Row, 2006).

Even in non-covalently prepared polymers, the binding of the template to the polymer can be too strong for the template to be washed from the polymer (Martin et al., 2003). In such a case, small amounts of template bleeding from the polymer can occur. To overcome this, the

synthesis of MIPs with an analogue of the target molecule as a template has been proposed (Dirion et al., 2002). This would ensure that even if template bleeding occurred, it would not interfere in the quantification of the target analyte.

Several advanced methods of template removal from an MIP have been proposed, including thermal annihilation, microwave-assisted extraction (MAE) and desorption of the template with supercritical fluids (Ellwanger et al., 2001).

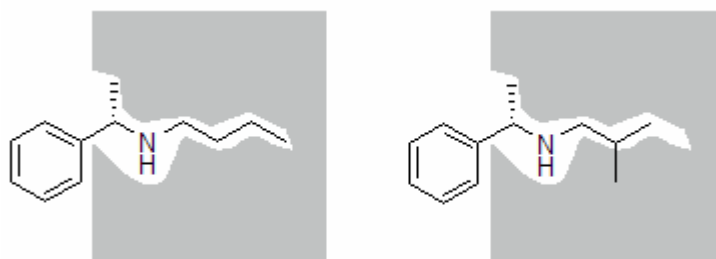
#### **2.4.5 Considerations regarding molecular recognition in MIPs**

The defining trait of MIPs, molecular recognition, is governed by two mechanisms (Simon, 2005): pre-organization of functional groups (which is a factor determining selectivity when it comes to distance between functional monomers in the binding site; the complementary positioning of the functional groups in the binding site is another key aspect of pre-organization) and shape specificity of the binding site.

Conformational flexibility in functional monomers can adversely affect selectivity. Shape selectivity is important in molecular recognition in MIPs, and there is a relationship between the MIP selectivity and the size of hydrocarbon side chain on templates (Simon, 2005).

The selectivity for an analyte decreases as the number of carbons in the side chain of the analyte increases beyond the number in the template used. When the analyte is smaller than the imprinted compound, the selectivity for that analyte is reduced on that polymer. When a molecular probe is analysed on a polymer that was imprinted with a compound that had a larger hydrocarbon side chain, the selectivity is reduced (“non-optimal spatial fit”) (Simon, 2005). When the highest selectivity for a polymer is observed, it is called “optimal spatial fit” (OSF).

A steric exclusion effect can happen for analytes larger than the imprinted analyte. If a given analyte has too many carbon atoms to fit into the space allotted by the template molecule, then the steric effect arises from its size. If the topological arrangement of atoms in an analyte is too big to fit into the allotted space, then its steric exclusion is a result of the shape of the molecule (Figure 13).



**Figure 13. Illustration of steric exclusion resulting from topological arrangement of atoms (Simon, 2005)**

An important aspect in the evaluation of a MIP is the assessment of its specific binding capacity and selectivity towards the analyte (Sellergren, 1998; Andersson et al., 1995). To assess the incidence of random non-specific interaction with the polymer, the MIP is usually compared with a non-imprinted control polymer (NIP), which is prepared in the same manner as the MIP but in the absence of the template, or with a control polymer, prepared with a non-related template (Kempe & Kempe, 2010). A MIP with a different control template would make for a better tool to estimate non-specific interactions as the presence of a template modifies the morphology of the polymer (porosity, surface area), which results in binding differences compared to the control, not due to the presence of the imprinted sites (Kempe & Kempe, 2010). However, experiments with non-imprinted polymers could provide useful information regarding the choice of functional monomer usage, as the interaction between monomer and template can be calculated (Karim et al., 2005). The monomers giving the highest binding scores could represent better candidates for ulterior MIP preparation.

The presence of salt ions seems to influence the binding capabilities of MIPs (Kempe & Kempe, 2010). While the pH and ionic strength of the rebinding solvent mixture have been recognized as important parameters affecting binding capacity (Andersson, 1996), it seems that the type of salt also can have an influence. While working with polymers imprinted for penicillin G and propranolol, Kempe & Kempe (2010) have augmented the binding by the addition of salts (NaCl, KCl and CsCl). However, while the binding was significantly increased, it appeared to be of non-specific nature.

Temperature is an important factor both for polymerization and during recognition for MIP efficiency (Karim et al., 2005).

#### 2.4.6 Methods of polymerization

There are several methods of polymer synthesis, of which free radical polymerization is usually the preferred choice for MIP preparation (Cormack & Elorza, 2004):

**Free radical polymerization** is characterized by three stages: initiation, propagation, and termination. Typically, the rate of propagation is usually much faster than the rate of initiation. As soon as a new polymer chain starts to grow, it will propagate to high molecular weight in a short time. Therefore, even when low amounts of monomer have been consumed, high molecular weight polymers are already present in the system. Also, the initiator is usually active throughout the whole duration of the polymerization, meaning that at any given time, the unreacted monomer and initiator, the growing polymer chains and the finished high molecular weight polymer chains are all present in the system.

**Free radical copolymerization** refers to the simultaneous polymerization of two or more vinyl monomers within the same reaction vessel to give copolymers. It allows products to be prepared with chemically distinct properties to the polymers obtained from polymerizing each monomer independently. An important point of consideration is the relative reactivity values of the monomers involved, which have been tabulated and can be found in the form of reactivity ratios for monomer pairs. These values are used to predict the likely outcome of copolymerization. Reactivity ratios values usually vary in the range 0-1. Low reactivity ratio implies low reactivity and high value implies high reactivity. If two monomers with moderate values of  $\sim 0.5$  each are combined, the copolymer will have a similar composition to the monomer, but not necessarily identical. If both monomers have low values of  $\sim 0$ , then copolymerization will be slow and tend to form specifically alternating copolymer. If one reactivity ratio value is high while the other is low the tendency is to consume preferentially one polymer near the beginning of the copolymerization and the second monomer near the end, giving rise to a mix of two homopolymers, rather than a copolymer.

**Cross-linked polymerization** can be advantageous by allowing non-linear polymer architectures to be prepared. By using monomers with two or more vinyl groups that can polymerize, commercially interesting structures such as branched macromolecules, microgels or macroscopic networks can be obtained (Figure 14).

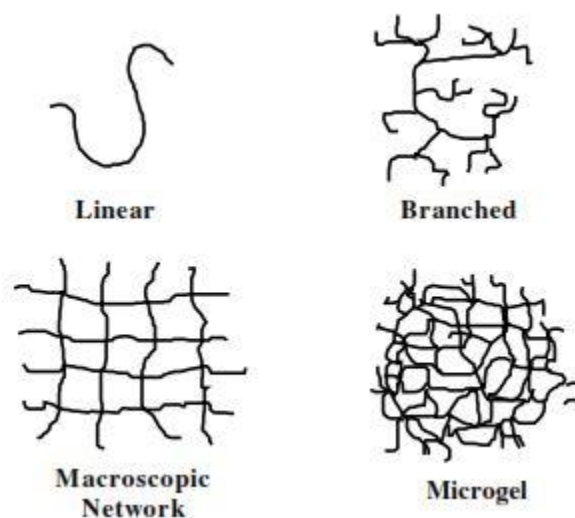


Figure 14. Schematic representation showing polymers with different topologies: linear, branched, macroscopic network and microgel (Cormack & Elorza, 2004)

**Gel-type polymers, macroporous polymers and microgel powders** are different kinds of speciality materials that can be obtained by adjusting two experimental parameters: the cross-link ratio (the percentage of cross-linker with respect to the total number of moles of monomer) and the volume of the solvent used.

Gel-type polymers occur at either low or high cross-linker ratios in the presence of low volumes of solvent, compatible with the polymer network. In such a case phase separation occurs separate from polymerization, resulting in a lightly solvated gel-type polymer. However, the structure collapses upon drying, having low specific surface area in the dry state. Their poor mechanical properties have prevented them from finding applications in molecular imprinting so far.

Macroporous polymers are obtained at higher cross-link ratios and in the presence of high volumes of solvent and are characterized by permanently porous structures in dry state, and



higher specific surface area than gel-type resins. Being more mechanically robust, they are used in the creation of molecularly imprinting polymers.

Microgels are obtained when the volume of solvent used is increased beyond the values used for macroporous polymer preparation. Under a more diluted condition the primary polymer particles are too spread out to form gel-type polymers or macroporous polymer. They rather remain in a non-aggregated state and are recovered as microgel powders. Their importance in molecular imprinting is increasing as the process of synthesis of micrometre-sized spherical polymers attains good yields (Wang et al., 2007).

#### **2.4.7 The synthesis of molecularly imprinted polymers and the variables involved**

Several experimental variables are involved in the process of designing and synthesising a MIP, making it a complicated process. These variables are the template molecule, the selection of suitable functional monomers, cross-linkers, solvents, initiators as well as the polymerization procedures themselves.

Before these factors are detailed, several criteria for the synthesized polymer are considered essential (Wulff, 1995):

- stiffness of the polymer structure: to retain cavity shape and spatial relationships of functional groups;
- high flexibility of the polymer structure, essential for rebinding kinetics and rapid equilibration with the substrate, although it contravenes the previous criteria of stiffness;
- good accessibility of as many cavities as possible, achievable by forming polymer morphologies;
- mechanical stability,
- thermal stability, as higher temperatures warrant favourable equilibration kinetics.

In general, it has been concluded that lower temperatures are more conducive to greater selectivity in the MIPs. Higher temperatures render the initiation reaction extremely fast and hard to control. Higher temperatures also diminish the complex stability, reduce reproducibility of the stationary phases, and produces high column pressure drops (Yan &

Row, 2006). The postulated argument for preferring lower temperatures for molecular imprinting is Le Chatelier's principle, which predicts that lower temperatures drive the pre-polymer complex towards complex formation, increasing the number and quality of the binding sites formed (Yan & Row, 2006).

#### **2.4.7.1 Template**

The template in the molecular imprinting process is of central importance, directing the organization of the functional groups of the monomers. Ideally templates should be chemically inert under the polymerization conditions. If the template participates in radical reactions or is unstable under the polymerization conditions, alternative strategies need to be found. (Cormack & Elorza, 2004).

When considering using a template, several issues need to be understood and addressed: the existence of polymerizable groups in the template, the possibility that any functionality of the template could inhibit free radical polymerisation, and the stability of the template at moderately elevated temperatures or upon UV radiation exposure (Cormack & Elorza, 2004; Yan & Row, 2006).

A risk factor associated with the use of MIPs is the leaching of the template from the polymer, especially since residual template can be trapped in highly cross-linked parts of the polymer network (Figure 12). This is then released in small portions upon solvent changes and volume changes of the materials, constituting problems or quantification errors in the case of SPE trace analysis protocols (Tse Sum Bui & Haupt, 2010). This issue recommends the use of an analogue of the template for solving the problem of template leaching or bleeding.




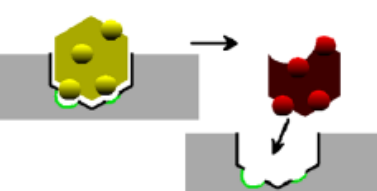
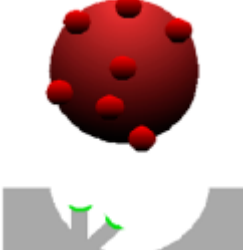
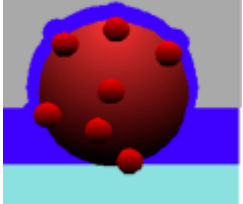
Besides the significant issue of template bleeding, there are a number of other reasons for selecting a target analogue as template: the target may be toxic, or very expensive, the direct use of the target molecule as template could cause potential interference problems, reactivity problems. In general, when selecting an analogue molecule as template, it should be readily available in large quantities at low price, exhibit solubility under imprinting conditions and should lead to formation of sites manifesting good cross-reactivity with the target analyte (Sällergren & Hall, 2013).

When targets are complex, have poor solubility or are unavailable in enough quantities for imprinting, then alternative strategies need to be used, as traditional imprinting would not find success. In the case of macromolecular biomolecules (proteins, nucleic acids, polysaccharides) the polymer needs to be synthesized in aqueous medium to solubilize and stabilize the target. However, the strong hydration forces and polar environment of water prevent such stable interactions between target and functional monomers (Sellergren & Hall, 2013). This is solved by using as template certain fragments complementary to substructures of the target, as was first developed for peptides (Rachkov & Minoura, 2001) and simple vitamins (Quaglia et al., 2001).

In some cases, MIPs for complex natural products may be made using simpler templates that exhibit either part or all the functional groups placed at geometrically complementary positions to the target (Nemoto et al., 2007).

Therefore, several approaches to molecular imprinting have been developed, regarding the nature of the template (Table 4) (Schirhagl, 2013).

Table 4. Different strategies for molecular imprinting (adapted from Schirhagl, 2013)

Type of imprinting	Imprinting image	Imprinting description
Bulk Imprinting		<p>A template molecule is added to the pre-polymer. Selective cavities are distributed all over the bulk. When the template is large, it may not diffuse through the cross-linked polymer.</p>
Surface Imprinting		<p>Surface imprinting with stamps is used for large templates, that would not properly diffuse through the cross-linked polymer in bulk imprinting</p>
Substructure Imprinting		<p>Small characteristic substructures are used instead of the whole molecule. This is like natural antibodies which target epitopes instead of whole molecules. This is of use if the analyte has a surface where the arrangement of functional groups is changing, such as membrane proteins of cells.</p>
Substructural Analogues		<p>Favourable if the target itself is rare, toxic, or not stable under imprinting conditions. Also, if the template molecule is hard to remove from the polymer or inconvenient to use.</p>
Antibody Replica		<p>Natural antibodies for the desired target molecule are used as the starting material. These are imprinted onto a polymer, which then imprints another polymer, resulting in receptors that mimic the structure of the initial antibody material.</p>
Sacrifice Layer		<p>This allows the use of a polymer that would otherwise react with the template. The template is covered with a monolayer of molecules, forming the binding site, and which are covalently bound to the polymer.</p>

### 2.4.7.2 Functional monomers

Functional monomers are selected based on their possible complementary interactions with the template and substrates (Figure 15) and are usually added in excess to the amount of template, and their functionality should be matched in a complementary fashion (such as H-bond donor with H-bond acceptor) in order to maximise complex formation and the imprinting effect (Cormack & Elorza, 2004). As stated above, when using multiple functional monomers for copolymerisation, the reactivity ratios should be considered.

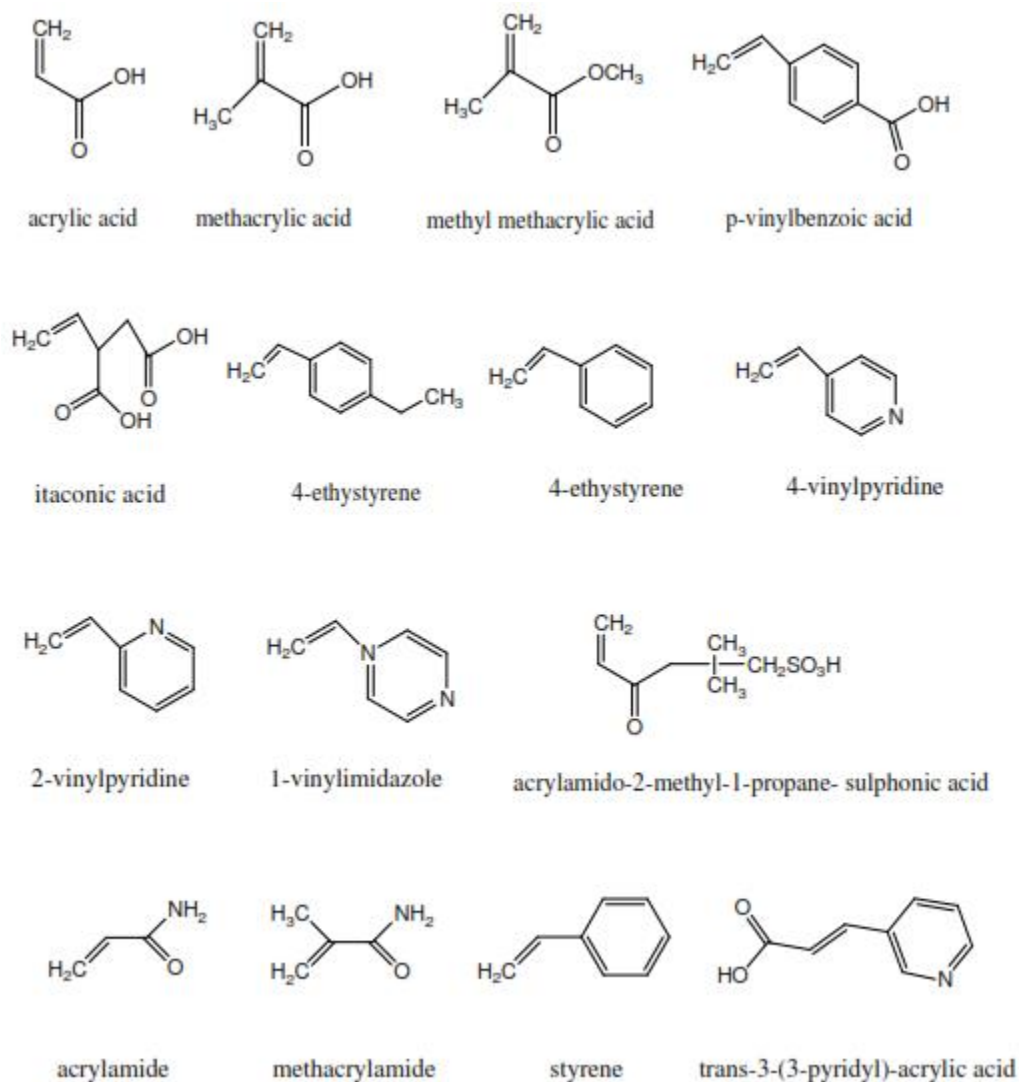


Figure 15. Common functional monomers used in non-covalent molecular imprinting procedures (Yan & Row, 2006)

An important step in polymer synthesis is the prearrangement phase when a complex is formed between the template and one or more types of functional monomer. It is postulated that the quantity and quality of the molecularly imprinted polymer recognition sites is a direct function of the mechanisms and extent that the monomer-template interactions present in the pre-polymerisation complex (Karim et al., 2005). In other words, it is assumed that the more stable and stronger this complex is, the more selective the MIP will be.

There is a broad range of functional monomers available that makes it possible to design MIPs for any type of stable chemical compound. The difficulty consists of performing detailed thermodynamic calculations on multi-component systems, a screening of monomer combinations which is a lengthy and costly process (Karim et al., 2005). For example, the task of checking even simple two-component combinations out of 100 monomers, it is necessary to synthesize and test around 5000 polymers, without including the possibility that these monomers could be used in monomer mixtures in different ratios. Also, the pressure applied at the time of polymerisation, the reaction time and the size of the holding container are also parameters that have an impact on the affinity and interactions of the binding sites to the template (Figure 16) (Karim et al., 2005). In the case of non-covalent polymers, the pre-polymerization solution is a mixture of functional monomers and template either non-complexed, partially, or totally complexed. This tends to generate highly heterogeneous binding sites and decreased binding (Karim et al., 2005).

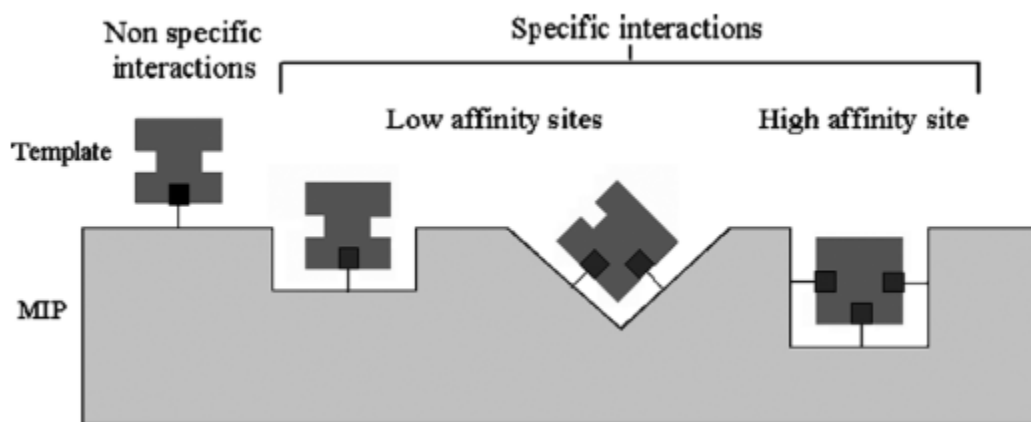


Figure 16. Representation of binding sites in heterogeneous imprinted polymers (Karim et al., 2005)

The main paradigm of molecular imprinting has been described by Karim et al. (2005) as:

*“[T]he strength and type of interactions, existing between monomers and template in monomer mixture will determine the recognition properties of the synthesized polymer.”*

This paradigm assumes that complexes formed in monomer mixture will be sustained throughout the polymerization stage, and their structure will remain preserved in the synthesized polymer.

However, the choice of functional monomer does not rely only on the interaction strength between monomer and template. The price of functional monomers is an important factor, as well as their solubility or polarity, stability under polymerization conditions and lack of competing photoreactions or degradations.

#### **2.4.7.3 Cross-linkers**

The type and amount of cross-linker used (Figure 17) has a great influence on the ulterior selectivity of the synthesized polymer (Yan & Row, 2006). The cross-linkers are important to the imprinting process and have three major functions. They control the morphology of the polymer matrix, stabilize the imprinting site, and impart mechanical stability to the polymer matrix (Cormack & Elorza, 2004). High cross-link ratios are generally preferred, with those higher than 80% being usually the norm. This can be explained as, according to Wulff (1995), polymer selectivity has been found to be a function of the type and amount of cross-linking agent. Below concentrations of 10% cross-linker, no specificity is observed, and the shape of the cavities cannot be stabilized. At values above 70% cross-linker the selectivity of the polymers obtained increases dramatically, and even values of 95% cross-linker still demonstrate further increases in selectivity. Some cross-linkers, such as divinylbenzene increase the stiffness of the polymer network severely, decreasing the accessibility of the cavities, while others, such as tetramethylene dimethylacrylate are too flexible as cross-linkers, and are unable to sufficiently stabilize the cavities (Wulff, 1995).

Consequently, a compromise is to be reached as an inflexible arrangement of the polymer chain gives high selectivity, while a higher degree of flexibility allows for better access to the imprinted cavities and a faster binding process.

Since crosslinking agents were observed to make significant contributions to template complexation (Shovari et al., 2014), the interaction between functional monomer and crosslinker needs consideration when designing molecularly imprinted polymers.

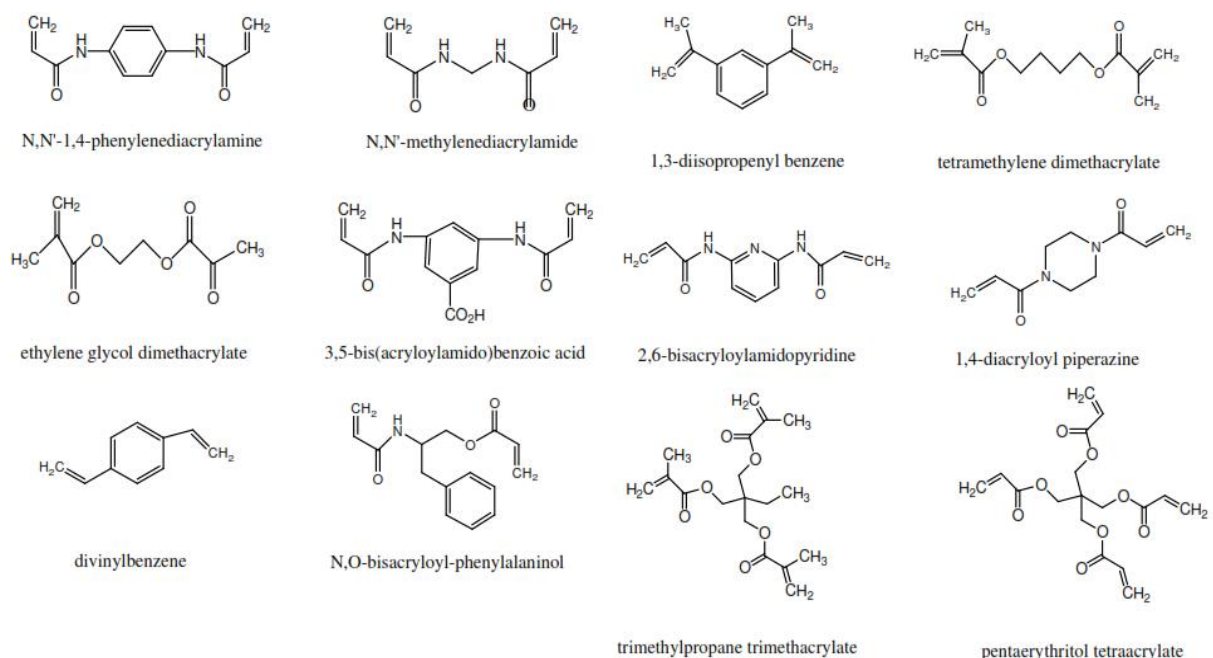


Figure 17. Cross-linkers commonly used in non-covalent molecular imprinting (Yan & Row, 2006)

#### 2.4.7.4 Porogenic solvents

The solvent is used to bring the template, functional monomers, cross-linker, and initiator into one phase. It plays an important role as it is responsible for creating the pores in macroporous polymers, hence the naming as porogen. Its nature and quantity used determines the strength of the non-covalent interaction and influences the morphology and total pore volume (Yan & Row, 2006). In choosing an adequate solvent, first the template molecule, initiator, monomer, and cross-linker must be soluble in it. Then, the porogenic solvent should be able to produce large pores, to allow good flow-through properties of the resulting



polymer. Finally, the solvent should have low polarity, to diminish interferences during complex formation between the imprinted molecule and the monomer, which is of significance in achieving high MIP selectivity (Yan & Row, 2006). Generally, thermodynamically good solvents are preferred as they lead to the formation of polymers with well-developed pore structures and high specific surface areas (Cormack & Elorza, 2004).

Beyond its role as a solvent and as a pore forming agent, the chosen solvent in non-covalent molecular imprinting must also simultaneously maximize the likelihood of template-functional monomer complex formation (Yan & Row, 2006).

#### **2.4.7.5 Initiators**

The initiator substance chosen depends on the system's conditions. In free radical polymerization, several chemical initiators can be used (Figure 18). They are generally used at low levels, 1% of the total number of moles of polymerizable double bonds (Yan & Row, 2006). If the template is photochemically or thermally unstable, then initiators which respond to different triggers are preferred. If hydrogen bonding is the driver of polymerization, then lower temperatures are preferred, in which case photochemically active initiators are used, as they are operating efficiently at low temperatures (Cormack & Elorza, 2004).

The presence of oxygen slows free radical polymerisations, and it is advisable to remove all dissolved oxygen by means of ultrasonication or by sparging the monomer solution with inert gas in order to maximize the rates of monomer propagation and ensure good reproducibility between batches (Yan & Row, 2006).

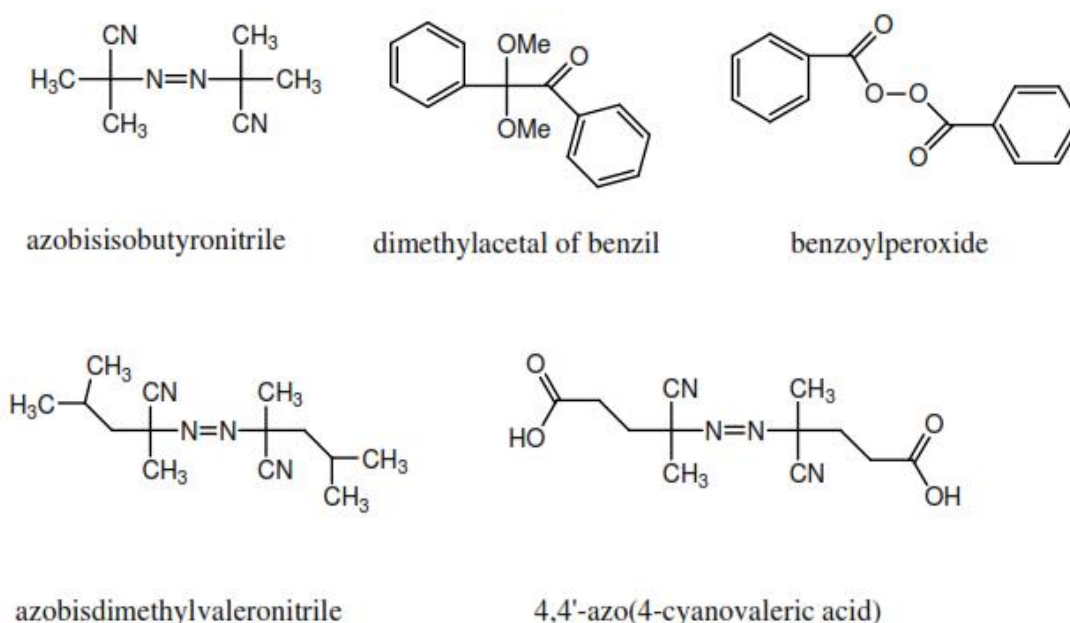


Figure 18. Chemical structure of common initiators used in non-covalent molecular imprinting (Yan & Row, 2006)

#### 2.4.8 MIP preparation methods

Molecularly imprinted polymers can be prepared in various physical forms to suit the desired application. The conventional method for MIP preparation is solution polymerization to form a bulk polymer, usually as a brittle rod, which is then mechanically grinded to small particles of the desired size range, usually circa  $30\ \mu\text{m}$  (Rimmer, 1998; Yan & Row, 2006). This has been a popular method but riddled by many drawbacks and problems. Firstly, the grinding of the rods of MIP polymer is not economical on a large scale and difficult to control. This results in high variabilities from batch to batch, as only 50% of the material can be recovered to be used. Secondly, the highly cross-linked nature of the network renders the removal of the template difficult. Thirdly, the inherent brittleness of the polymer prepared in such a way limits its opportunities for being used in a variety of applications (Rimmer, 1998).

Another significant problem with bulk thermal- (or photo-) polymerization is that during the cumbersome grinding and sieving process, most of the lost polymer is a very fine sub-micrometric powder, which adheres to bigger particles and increases the back pressure in SPE columns to excessive levels during the extraction procedures (Baggiani et al., 2007).

All these drawbacks have given rise to several alternative methods. These will be summarized below (Table 5), highlighting the main advantages and drawbacks of each preparation method, but not the accurate description of the processes themselves, since each method of MIP preparation has itself several possible approaches and techniques. For a detailed overview of each method, see Yan & Row (2006) pp 166-171. Overall, the most promising strategies are those that manage to separate the physical property features of a MIP from its selective binding requirements (Rimmer, 1998).

**Table 5 Summary of advantages and disadvantages for different MIP preparation methods (Yan & Row, 2006)**

<b>MIP format</b>	<b>Benefits</b>	<b>Limitations</b>
Bulk polymerization	Simple polymerization Does not require special skills or any sophisticated instrumentation	Tedious procedure of grinding, sieving, and column packaging, with high losses of useful polymer (50-75%) Irregular particle in size and shape Low performance Cannot be up scaled
Suspension polymerization	Spherical particles Highly reproducible results Ease of up-scaling	Special surfactant polymers required Need for phase partitioning and special liquid perfluorocarbons in the continuous phase
Multi-step swelling polymerization	Monodisperse beads of controlled diameter Useful for HPLC analysis	Complicated procedures and reaction conditions Need for aqueous emulsions
Precipitation polymerization	Imprinted microspheres Uniform size and high yields	Large amount of template High dilution factor
Surface polymerization	Monodisperse product Thin imprinted layers	Complicated system Time consuming
In-situ polymerization	One step preparation Cost efficient, good porosity	Extensive optimization required for each new template system

### **2.4.9 Methods of characterization of polymer structure**

Due to the hard to control and insoluble nature of macroscopic network polymers, their precise characterization has been regarded as notoriously difficult (Cormack & Elorza, 2004) and encompasses chemical, morphological, and molecular recognition behaviour characterization.

#### **2.4.9.1 Chemical characterization**

A few convenient analytical methods have been successfully used with solid samples (Cormack & Elorza, 2004):

- elemental micro-analysis is used to measure the percentage by mass of carbon, hydrogen, nitrogen etc. in the samples, and these are used to calculate the monomer used in the polymer. However, it is not sensitive enough to detect trace quantities of template remaining in the MIP;
- Fourier-transform infra-red spectroscopy (FTIR) is used to extract quantitative information on the composition of the polymer, and can give well resolved diagnostic signals, as well as probing non-covalent interactions;
- solid-state nuclear magnetic resonance (NMR) is useful for working with insoluble products and can give information regarding the chemical composition of the sample.

#### **2.4.9.2 Morphological characterization**

The imprinted polymers' morphology can be inspected, with useful insights regarding the specific pore volume and size, as well as pore size distribution and specific surface areas of the materials, by using the following methods (Cormack & Elorza, 2004):

- solvent uptake experiments are used to estimate specific pore volume, by measuring the amount of solvent uptake by a polymer;
- nitrogen sorption porosimetry is a useful analytical technique for detailed micro (smaller than 2 nm) and meso (between 2 nm and 50 nm) pore analysis. It uses a fixed mass of dry polymer which is exposed to a gas such as nitrogen at a series of

determined pressures. Sorption isotherms are constructed by measuring the amount of gas sorbed as a function of pressure. Useful information such as specific surface area, specific pore volume, average pore diameter and pore size distribution can be deduced;

- mercury intrusion porosimetry involves forcing mercury into a fixed mass of dry polymer under pressure. Like nitrogen sorption porosimetry, it is more sensitive at probing macro pores (larger than 50 nm);
- inverse size exclusion chromatography (ISEC) enables the probing of polymer structure in a wet state, making it a good complementary technique to nitrogen sorption or mercury intrusion porosimetry;
- microscopy of different kinds can be used to assess imprinted polymers. Light microscopy can be used to probe the structural integrity of the beads, while scanning electron microscopy (SEM) can be used to image macropores.

#### **2.4.10 Molecularly imprinted polymers and electrospun nano- and microstructures**

One of the recent tendencies in the field of molecular imprinting is the synthesis of MIPs either in the form of nanomaterials or in a combination with other materials in nanocomposites, to obtain novel structures with augmented properties (Bompart et al., 2012). Nanofibers are a type of innovative material, characterized as long, three-dimensional ultrafine fibres, with thicknesses between 100 nm and 1  $\mu\text{m}$ , and lengths of up to kilometres (Chronakis & Ye, 2013). As such, they present a series of unique and highly attractive properties, one of the most significant being an extraordinarily large surface area and small pore size. For example, nanofibers with a diameter of 100 nm have a ratio of geometrical surface area to mass of approximately 100  $\text{m}^2/\text{g}$  (Frenot & Chronakis, 2003).

Additionally, they are highly customizable and can attain very high porosities, tuneable pore size and surface functionalities, adjustable layer thickness, allow easy inclusion of nano- and microparticles, have a high permeability and low density (Chronakis & Ye, 2013). Also, electrospun nanofibers may either dissipate or retain electrostatic charges, depending on the electrical properties of the polymer (Frenot & Chronakis, 2003).

Electrospinning is the most well-known and cost-effective technique for the fabrication of nano- and microfiber materials (Frenot & Chronakis, 2003; Li & Xia, 2004). This technique has been successfully used to create nanofibers with MIP nanoparticles included (Chronakis et al., 2006; Piperno et al., 2011).

The process of electrospinning has been known for a long time, since the first patent was issued to Formhals in 1934 (US Patent, 1-975-504). However, the technology has received renewed interest at a much later date, in 1996, when the possibility to use a wide range of polymer solutions in the electrospinning process was first demonstrated (Reneker & Chun, 1996). Currently electrospinning is applicable for a large variety of polymers such as polyolefin, polyamides, polyester, aramide, acrylic acid, as well as biopolymers like proteins, DNA, polypeptides or others like electric conducting and photonic polymers (Frenot & Chronakis, 2003).

#### **2.4.10.1 Electrospinning process**

The electrospinning process is a non-mechanical electrostatic technique. It involves the use of a high voltage electrostatic field to charge the surface of a droplet of polymer solution, inducing the ejection of a liquid jet through a spinneret (Chronakis & Ye, 2013). Figure 19 illustrates such a setup, which comprises a high voltage power supply, and a syringe needle connected to it, and a counter-electrode collector. The inserts in the Figure show a drawing of the electrified Taylor cone, the subsequent bending instability, which is a transversal vibration of the electrospinning jet enhanced by electrostatic repulsion and suppressed by surface tension, and a SEM image of a nonwoven mat of polyethylene terephthalate (PET) nanofibers deposited on the collector (Chronakis & Ye, 2013).

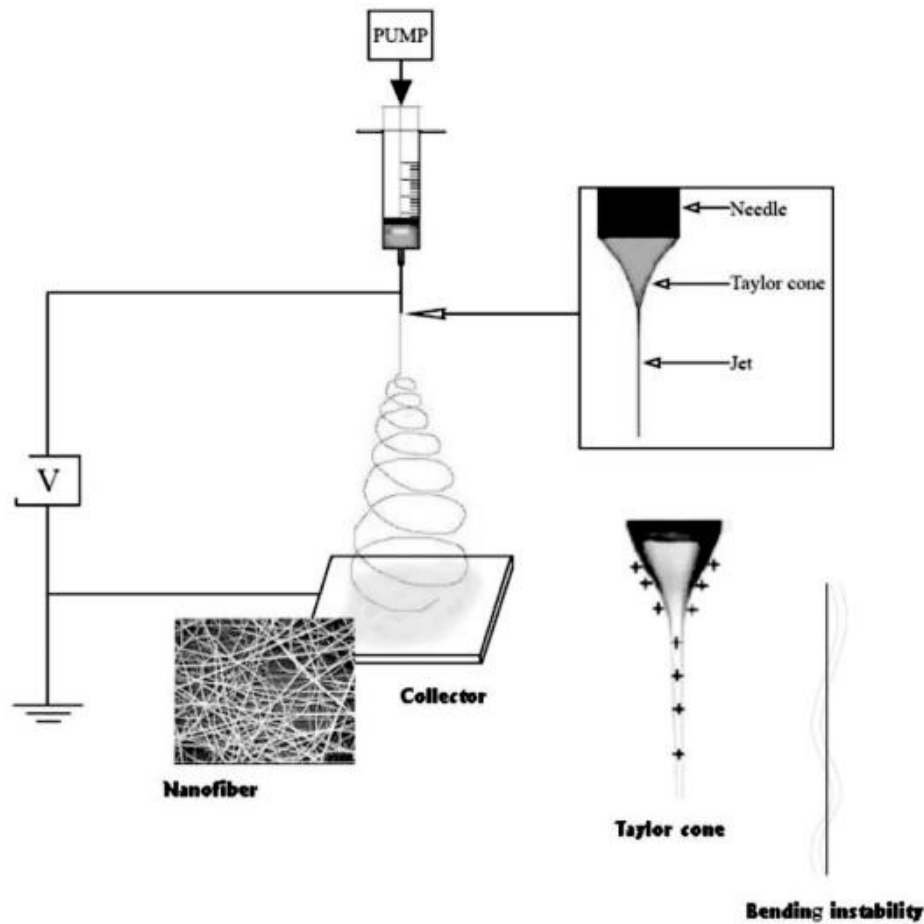


Figure 19. A conventional electrospinning setup (Chronakis & Ye, 2013)

The term “Taylor cone” is given after Sir Geoffrey Ingram Taylor, who first described this phenomenon in 1964, and refers to the cone of polymer liquid droplet solution observed at the tip of the needle. Such an isolated charged liquid droplet becomes unstable, and fission takes place when the charge becomes sufficiently large compared to the stabilizing effect of the surface tension (Rayleigh, 1884). Therefore, sessile, and pendant droplets of polymer solutions acquire stable shapes when they are electrically charged, by applying an electrical potential difference between the droplet and a flat plane (Yarin & Reneker, 2001). When a critical potential is reached, and any further increase will destroy the equilibrium, the liquid body acquires a conical shape, called Taylor cone.

When the repulsive electrostatic force of the applied electric field overcomes the surface tension of the droplet, then a charged jet of polymer solution is ejected from the tip of the Taylor cone. During this process, it stretches immensely and, at the same time, solidifies either as the solvent evaporates or the solution cools. The resulting product is the electrically charged fibre which can be directed or accelerated by electrical forces and then collected as sheets or other physical shapes (Chronakis & Ye, 2013).

Such electrospun nanofibers, in light of their many unique characteristics, as described above, have found numerous potential applications in multifunctional membranes, biomedical structural elements, protection in speciality fabrics, filter media for submicron particles in the filtration industry, composite reinforcement, structures for nano-electronic machines (Frenot & Chronakis, 2003), recovery of metal ions, drug release control, catalyst and enzyme carriers, sensors and energy storage (Fang et al., 2008).

To supply electrospun nanofibers in enough quantities necessary for commercial applications, improvements to the conventional needle-based electrospinning setup were warranted. Specifically, ways to increase throughput without affecting the quality and structure of the fibre were investigated.

One self-evident way to increase throughput is to increase the number of needle jet spinnerets. This has been experimented with several times (Varesano et al., 2009; Yang et al., 2010), however it has led to poor fibre production rates and diminished quality, as the proximity of the jets leads to a strong repulsion force among each other (Niu et al., 2011). This process has been stabilized with the addition of an extra-cylindrical electrode to cover the multi-jet spinneret (Kim et al., 2006) and, while this increased the production rate, coarser fibres were observed.

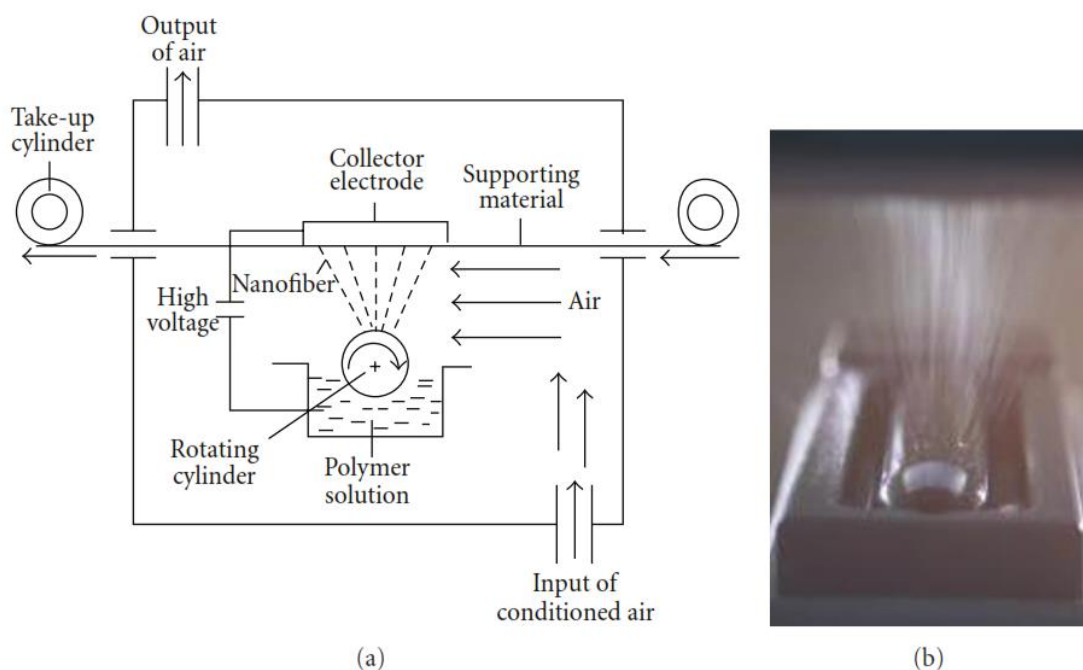
As the ejected solution jets carry a large electrostatic charge, such interferences in multi-jet electrospinning setups cannot be eliminated. This, together with the fact that each individual needle nozzle requires regular cleaning to avoid blockage, indicate that needle-based electrospinning will not be successfully deployed for mass production of nanofibers (Niu et al., 2011).



### 2.4.10.2 Needleless electrospinning

As seen above, despite the numerous potential applications, electrospun nanofibers, produced either with the conventional needle configuration (Figure 19) or derivative variants, have serious disadvantages in their commercial viability (Niu et al., 2011). Consequently, several other technologies have emerged for large scale nanofiber production, one of the most successful of these being upward needleless electrospinning.

An example of a commercial solution for needleless electrospinning is the Nanospider™, developed by Emlarco Co. It is based on the setup first developed by Jirsak et al. (2005) and involves using a rotary roller as the nanofiber generator. When the roller is partially immersed into a polymer solution and slowly rotates, the polymer solution is loaded onto the upper roller surface. After a high voltage is applied to the system, many solution jets are generated from the roller surface upward (Figure 20).



**Figure 20. Schematic (a) of a needleless electrospinning setup with a rolling spinner and (b) a picture of the roller during the process (Jirsak et al., 2010)**

In needleless electrospinning the solution bath is normally open to air, therefore, the evaporation of the solvent from the solution can increase its viscosity and decrease its uniformity. Therefore, it is important that the solution in the bath is precisely calculated.

Also, because many solution jets are formed in a small space, high concentrations of organic solvent can build up in the electrospinning zone, making the task of air circulation and ventilation recommended in order to efficiently recycle the organic solvent (Niu et al., 2011).

### 3 Materials and methods

This study was carried out with the intention of accurately determining the ability of molecularly imprinted polymers to successfully aid in the curative treatment of red wines affected by unwanted proliferation of *Brettanomyces* yeast.

More specifically their ability to remove 4-ethylphenol, which is the main unwanted aroma marker associated with *Brettanomyces*, from red wines, was evaluated, by liquid chromatography and by gas chromatography methods.

Additionally, their degree of selectivity was also evaluated, determining how much of other positive aroma or phenolic compounds were being removed by the treatment.

The efficiency of the regeneration of these polymers was also evaluated with successive trials.

As this work has been carried out over several years and in several locations and has been very much an exploratory work into a novel topic, the specific wines used for testing and analysis were frequently different from one test to the next. Therefore, the wine types used are not enumerated here, but are always specified in the results section of the work. There, each successive trial is presented and explained in a logical sequence as it would fit into the structure of experimentation with these new materials.

#### 3.1 Molecularly imprinted polymers

The molecularly imprinted polymers tested in this work have been created by Ligar Polymers, a company based in New Zealand. Two different iterations, which have been codified in this work as MIP1 (with NIP1 as its non-imprinted equivalent) and MIP2 (with NIP2 as its non-imprinted equivalent) were tested. MIP1 (and NIP1) is a non-covalent bulk polymerized cross linked poly- ethylenedimethacrylate (EDMA) co-bilirubin polymer. MIP2 (and NIP2) is a non-covalent binding functionalized ethylene glycol dimethacrylate (EGDMA) polymer.

The imprinting molecule (Template) is incorporated during the polymerization or crosslinking process and then later removed. The polymerization is an alkene polymerization. The mixture of monomers is composed of crosslinked bilirubin and ethylenedimethacrylate (EDMA) but could also include other monoalkenes such as methacrylic acid, vinylpyridines, hydroxyethylmethacrylate or acrylamide. The type of interaction that lays the foundation for molecular recognition in this case is represented by non-covalent interactions, which consist of electrostatic interactions, hydrogen bond formation or hydrophobic interactions. The mole ratio of comonomer to crosslinker is in the ratio 0:1 to 1:15, preferably 0:1 to 1:10. The preferred mole ratio of bilirubin to the crosslinker is 1:20 to 1:1, preferably 1:20 to 1:4. The polymer mass is ground down to small size particles in order to reduce non-specific binding. The particle size would preferably be in the range 38 to 150 microns (50-80 microns), with more than 80% of the material consisting of particles of this size. The solvent was 40% methanol water. The initiator was 1,1'-azobis cyclohexanecarbonitrile (ABCHC). The temperature of polymerization was 70 °C.

Any additional information regarding the production of these materials might represent a trade secret and is therefore not available for disclosure.

Table 6 highlights the main physical and chemical properties as well as the recommended operation conditions for these polymers.

**Table 6. Data sheet for molecularly imprinted polymers tested in this work**

<b>Physical and chemical properties</b>	
Mean Particle Size*	0.02-0.03 mm
Poured Dry Bulk Density Approx.	0.30 g/mL
Settled Wet Bulk Density Approx.	0.38 g/mL
Water Retention (atmospheric pressure)	2.60 mL/g
Surface Area	97.44 m <sup>2</sup> /g
Stability Temperature Range	up to 250 °C
<b>Recommended operating conditions</b>	
Operating pH Range	2-8
Contact Time	<1 min
Polymer Loading	2.5 w/v %
Eluent (Sodium Hydroxide)	0.1 M
Regenerant (citric acid)	0.1 M
Rinse Water Requirement	NA

### 3.2 Methods of analysis

Several wine analyses were performed throughout the course of this work.

Several of the wine analyses, especially those pertaining to conventional oenological parameters, were done in accordance with the methods described in the *OIV Compendium of International Methods of Analysis of Wines and Musts* Vol. 1 and Vol. 2, from 2014. These are summarized in Table 7, with an internal reference to the *OIV Compendium*.

**Table 7. General wine analyses performed and reference methodology**

<b>Analysis</b>	<b>Reference</b>
Density at 20 °C and specific gravity at 20 °C measured by electronic densimeter using an oscillating cell	OIV-MA-AS2-01A
Measurement of the alcoholic strength by refractometry	OIV-MA-AS312-01B
Total dry matter, sugar free extract, rest extract	OIV-MA-AS2-03B
pH	OIV-MA-AS313-15
Total acidity	OIV-MA-AS313-01
Sulphur dioxide (free and total)	OIV-MA-AS323-04B

Apart from these foundational methods, several other analytical methods were employed, which will be explained in detail in the sections below.

#### 3.2.1 Determination of chromatic characteristics according to CIELab

This spectrophotometric method is based on the CIE-L\*a\*b\* colour space, as it was defined by the International Commission on Illumination (CIE) in 1976. Its purpose is to define the process of measuring and calculating the chromatic characteristics of wines, by using three attributes or qualities of visual sensation: tonality, luminosity and chromatism (OIV *Compendium of International Methods of Analysis of Wines and Musts* Vol. 1, 2018, Method OIV-MA-AS2-11).

The colorimetric coordinates determined by this method will define the chromatic characteristics of a wine, and they are: clarity (L\*), red/green colour component (a\*),

blue/yellow colour component ( $b^*$ ), chroma ( $C^*$ ), tone ( $H^*$ ) and chromacity [ $(a^*, b^*)$  or ( $C^*, H^*$ )] (OIV *Compendium of International Methods of Analysis of Wines and Musts* Vol. 1, 2018, Method OIV-MA-AS2-11).

Additionally, the overall colorimetric difference between two wines ( $\Delta E^*$ ) can be calculated using the following formula:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

The spectrophotometric analyses performed in this work were done on a photoLab® 7600 UV-VIS (ultraviolet-visible spectroscopy) spectrophotometer, using disposable plastic cuvettes with a volume capacity of 3 mL. The blank value was always taken using deionized water. The interval of light absorption is between 380 and 780 nm, and the data with  $L^*a^*b^*$  values is extracted separately for each wine, and then further processed using the computer program Microsoft Excel.

### 3.2.2 Folin-Ciocalteu Index

This method is applied to obtain information about the total quantity of phenolic compounds in a wine, and the information is expressed by the Folin-Ciocalteu Index (OIV *Compendium of International Methods of Analysis of Wines and Musts* Vol. 1, 2018, Method OIV-MA-AS2-10), and appears written in mg/L of catechin.

The principle of this method is that all the phenolic compounds in a wine will be oxidized by the Folin-Ciocalteu reagent. This reagent is formed from a mixture of phosphotungstic acid ( $H_3PW_{12}O_{40}$ ) and phosphomolybdic acid ( $H_3PMo_{12}O_{40}$ ). After the phenols are oxidized, this mixture is reduced to one of blue oxides of tungsten ( $W_8O_{23}$ ) and molybdenum ( $Mo_8O_{23}$ ), which has a blue coloration. The maximum absorption of this blue coloration is in the 750nm spectrophotometric region and is proportional to the total quantity of phenolic compounds originally present (OIV *Compendium of International Methods of Analysis of Wines and Musts* Vol. 1, 2018, Method OIV-MA-AS2-10).

Because fructose (in higher levels), ascorbic acid, iron (II) ions and sulphur dioxide may disturb the reaction and skew the results, hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) is used to oxidize these substances.

Additionally, the reaction of phenol oxidation, by which all hetero-poly-acids are reduced to blue oxides, should be complete. In this sense, an alkaline medium is necessary, for the phenols to go through phenolation and then to oxidize to quinones. Therefore, sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) is added to ensure alkalinity.

The reagents needed for this analysis are: Folin-Ciocalteu reagent, Sodium Carbonate solution (200 g/L  $\text{Na}_2\text{CO}_3$ ), Hydrogen Peroxide solution (30%  $\text{H}_2\text{O}_2$ ) and catechin, and are purchased from Merck, Darmstadt.

The measurement is made using a Dr Lange LP2W Digital Photometer at 720 nm wavelength.

First, by addition of 0.2 mL  $\text{H}_2\text{O}_2$  (wait time: 30 minutes) to the wine sample,  $\text{SO}_2$  or ascorbic acid are neutralized and eliminated from playing a disturbing role in the reaction. Afterwards, 1 mL of prepared sample is added to a 100 mL volumetric flask, to which 75 mL of distilled water have previously been added. Next, 5 mL of the Folin-Ciocalteu reagent are added to the flask, which is then shortly mixed and left to sit for exactly 3 minutes. Afterwards 10 mL of sodium carbonate are added, and the flask volume is filled to 100 mL with distilled water. After precisely one hour of waiting time, a 3 mL volume of the prepared sample in a plastic cuvette is measured at 720 nm absorption length. This value is then converted mathematically into mg/L of catechin, by merit of a standard linear calibration curve ( $r^2 = 0.9991$  between 0 and 1 g/L catechin).

To create a standard calibration curve of catechin, a 50 mg/100 mL catechin in 98% alcohol stock solution is created. Afterwards, in 10 mL volumetric flasks, the following volumes are pipetted: 1, 2, 4, 6 and 8 mL; then the flasks are filled with 98% alcohol to the 10 mL volume mark. These dilutions will correspond to 50, 100, 200, 300 and 400 mg/L of catechin.



### 3.2.3 Sensory analysis

The sensory evaluation of the wines was conducted in the sensory laboratories of the University of Geisenheim (Hochschule Geisenheim, Germany), in the department of Oenology, and the procedure, equipment and working space were organized in accordance with the standard DIN EN ISO 5492.

The sensory analysis data was processed using the program FIZZ™ Networks, Biosystemes France, version 2.51a86 and the statistical analysis performed was done using the FIZZ™ Calculations, Biosystemes, France, version 2.60.00.1512.

### 3.2.4 4-ethylphenol determination

#### 3.2.4.1 SBSE-GCMS method

The method developed and available in Hochschule Geisenheim for the quantitative determination of 4-ethylphenol (and 4-ethylguaiacol) is based on stir-bar sorptive extraction (SBSE) and subsequent thermal desorption by gas chromatography (GC) - mass spectrometry (MS). The gas chromatograph is a HP 6890 Series GC system with a Rxi 5ms column. This is a nonpolar column containing 5% polar groups. The measures of the column are 60 m (length) x 0.32 mm (inner diameter) x 0.25  $\mu\text{m}$  (coating thickness in the capillary) and the detector is a HP 5973 Mass Selective Detector (mass spectrometer). The detection limits for 4-EP and 4-EG are 2  $\mu\text{g/l}$  and 1  $\mu\text{g/l}$ .

The SBSE method is specifically applied to overcome the limitations of other techniques, particularly the recovery of medium-to-high volatile compounds sampled in liquid phase using polydimethylsiloxane-open tubular traps (PDMS-OTT). Additionally, this method can improve on the limited recovery that is achieved with solid phase micro-extraction (SPME) for ultra-trace analysis.

The method of SBSE is applied for sampling from liquid phases and relies on the sorption of the fraction containing the analytes of interest on a thick PDMS film, coated onto a glass-coated magnetic stir bar (Twister®, Gerstel GmbH, Mülheim, Germany).

A volume of 10 mL of each sample is pipetted into a glass vial in which 3.5 g of NaCl has been added. The NaCl enables slightly polar substances to enter the Twister®. Normally, this stir bar exclusively absorbs nonpolar analytes. The internal standards for 4-ethylphenol and 4-ethylguaiacol are then added (50 µL of 4-ethylphenol-d<sub>10</sub> with a concentration of 500.1 µg/L and of 2-ethylphenol with a concentration of 546.4 µg/L) for the reference samples.

The sampling is achieved by the direct introduction of the SBSE device in the aqueous sample. After a specific contact time of 60 minutes and stirring at 1000 rotations per minute (RPM) at 22 °C, which allow the solvent-less extraction to take place, the non-polar Twister is removed, rinsed with deionized water and placed in a thermal desorption unit (TDU tube), which is then sent for subsequent GC-MS analysis. There, the analytes are recovered from the Twister® by thermal desorption. The gaseous sample is then cryo-focused with liquid nitrogen at a temperature of -150 °C. Afterwards, the sample is rapidly heated up, which leads to a vaporization, enabling the sample to better access the GC column, providing higher definition and sharper chromatogram peaks. The compounds in the sample are split on the GC column and the split compounds are detected by electron impact ionization in the MS unit.

The mass spectrometry identification takes place under vacuum and three major units are involved in the analytical process: an ion source (the electron impact ionization), a mass analyser (quadrupole) and a detector (electron multiplier horn). The MS measures only single positively charged ions and the ratio between mass and charge ( $m/z$ ). Therefore, ions are generated by collisions of the sample components with electrons. The lens system focuses the ions and transports them towards the quadrupole, where its four elements possess the same polarization. Both direct and alternative current is run through the four quadrupole elements. For each ion passing the quadrupole, a unique combination of direct and alternating voltages is determined. After passing through the quadrupole and entering the electron multiplier horn, the positively charged ions are discharged and push out the electrons with which the electron multiplier is lined with. The released energy is transformed into a signal, showing up as a peak in the chromatogram. The concentrations are calculated from the chromatogram by integrating the areas of the ethylphenols and the internal standards and

observing their relationship, resulting in a relative peak area. The following equation is used to calculate the relative peak area:

$$\text{relative peak area} = \frac{A (EP)}{A (IStd)}$$

Where A(EP) represents the area of the chromatogram peak corresponding to the measured ethylphenol, and A (Istd) corresponds to the chromatogram peak corresponding to the measured internal standard.

The specific parameters of the GC-MS setup used in Hochschule Geisenheim are described in Table 8.

**Table 8. Characteristics of the SBSE GC-MS method of analysis done in Hochschule Geisenheim**

<b>Front Inlet (CIS3)</b>		
Mode	Split	
Initial temperature	0 °C (Off)	
Pressure	37.6 kPa (On)	
Split ratio	20:1	
Split flow 21.9 mL	21.9 mL/min	
Total flow	25.8 mL/min	
Gas saver	On	
Saver flow	30.0 mL/min	
Saver time	5.00 min	
Gas type	Helium	
<b>Oven</b>		
Initial temperature	50 °C (On)	
Maximum temperature	325 °C	
Initial time	1.00 min	
Equilibration time	0.50 min	
<b>Ramps:</b>		
# Rate	Final temperature	Final time
1 20.00	120 °C	0.00 min
2 3.00	145 °C	0.00 min
3 15.00	300 °C	6.83 min
4 0.0 (off)		
Post temperature	50 °C	
Post time	0 min	
Run time	30 min	
<b>Column</b>		
Capillary column	(not installed)	
Model number	Restek 13427 RXI-5ms	
Max temperature	350 °C	
Nominal length	60.0 m	
Nominal diameter	320.00 µm	
Nominal film thickness	0.25 µm	
Mode	Constant flow	
Initial flow	1.1 mL/min	
Nominal initial pressure	37.6 kPa	
Average velocity	27 cm/sec	
Inlet	Front inlet	
Outlet	Mass selective detector (MSD)	
Outlet pressure	Vacuum	

### 3.2.4.2 HPLC-DAD-FLD method

Despite the precision and accuracy of the previously described gas chromatography method, it was not feasible to employ it when analysing large numbers of samples, for several practical reasons, especially the time consuming and labour-intensive manual process of sample preparation. For this purpose, a different method was sought, one that could handle the analysis of several hundreds of samples effectively.

Therefore, an analytical method based on high pressure liquid chromatography (HPLC) – diode array detector (DAD) – Fluorescence detector (FLD) was adapted from Caboni et al. (2007) and applied.

The 4-Ethylphenol standard was prepared using a dual-range semi-micro Shimadzu™ AUW-D Series analytical balance in a 25 mL volumetric flask (class A) at 20 °C. One gram of 4-Ethylphenol solid substance (99%, Sigma-Aldrich™) is dissolved in 6.5 mL Ethanol (99%, Chemical Company, Iași). The rest of the volume up to 25 mL is filled in with deionised water (18.2 MΩ cm at 25 °C).

Afterwards, using a Gerstel MPS (Multi-Purpose Sampler) for filtration, dilution and vortexing (mVortex Module) of standards, eight dilution points are obtained: 1, 2, 20, 50, 100, 250, 500 and 1000 ppm. All solutions are filtered through 0.45 μm nylon 2mm syringe filters. The LC (liquid chromatography) analysis is performed using a pentafluorophenyl (PFP) column. For the 4-ethylphenol analyses, samples were processed on a Shimadzu™ HPLC setup (Figure 21), consisting of: Shimadzu™ Prominence series quaternary pump (solvent delivery unit) (LC-20AD) with five-channel degasser (DGU-20A5), Shimadzu™ Prominence series auto sampler (SIL-20AC) (injection volume: 1 μL, sample temperature 20 °C), Shimadzu™ Prominence series column oven (CTO-20AC), Shimadzu™ Prominence series diode array detector (200-440nm) (SPD-M20A), fluorescence detector (Shimadzu FLD RF-10Ax1), and in order to achieve a double spectral certification for analytes, Shimadzu™ Prominence series chromatographic system controller (CBM-20A) with personal computer (PC) connectivity via local area network (LAN).



Figure 21. The HPLC-DAD-FLD setup used for the 4-ethylphenol analyses, Oenology Laboratory Iași, Romania

The mobile phases for gradient elution are:

- A. Acetonitrile (AcCN 1%) adjusted with 0.5% phosphoric acid to pH 1.9
- B. Acetonitrile (AcCN 50%) adjusted with 0.5% phosphoric acid to Ph 2.22

The gradient was optimized using a filtered watery solution of phosphoric acid as an eluent acidification of 1% AcCN (A channel) and 50% AcCN (B channel). All eluents were purified by a glass vacuum filtration device using 47mm nylon filters.

The column has installed a SecurityGuard ULTRA Cartridge UHPLC 5×4.6 mm mounted on a SecurityGuard ULTRA Cartridge holder. The column is a Kinetex® 2.6  $\mu$  PFP 100A 150×4.6 mm column, manufactured by Phenomenex.

The flow is 1.5 mL/min. and the oven temperature is 40 °C. The injection of 1  $\mu$ L is done at 100% A and is maintained for 2 minutes. From minute 2 until minute 15 a linear gradient of

B eluent is made and maintained for 5 minutes, afterwards, the initial condition is established over the course of 3 minutes and for 2 minutes the system is re-equilibrated.

The DAD has a 40 Hz acquisition rate from 220 to 500 nm, with a reference correction at 400 nm with a reference bandwidth of 20nm. The FLD is set to excite at 270 nm for an emission of 300 nm with a 2.5 Hz acquisition rate.

The working range of the calibration is 10-6000 mg/L for DAD (Figure 23) and up to 100 mg/L for FLD (Figure 22).

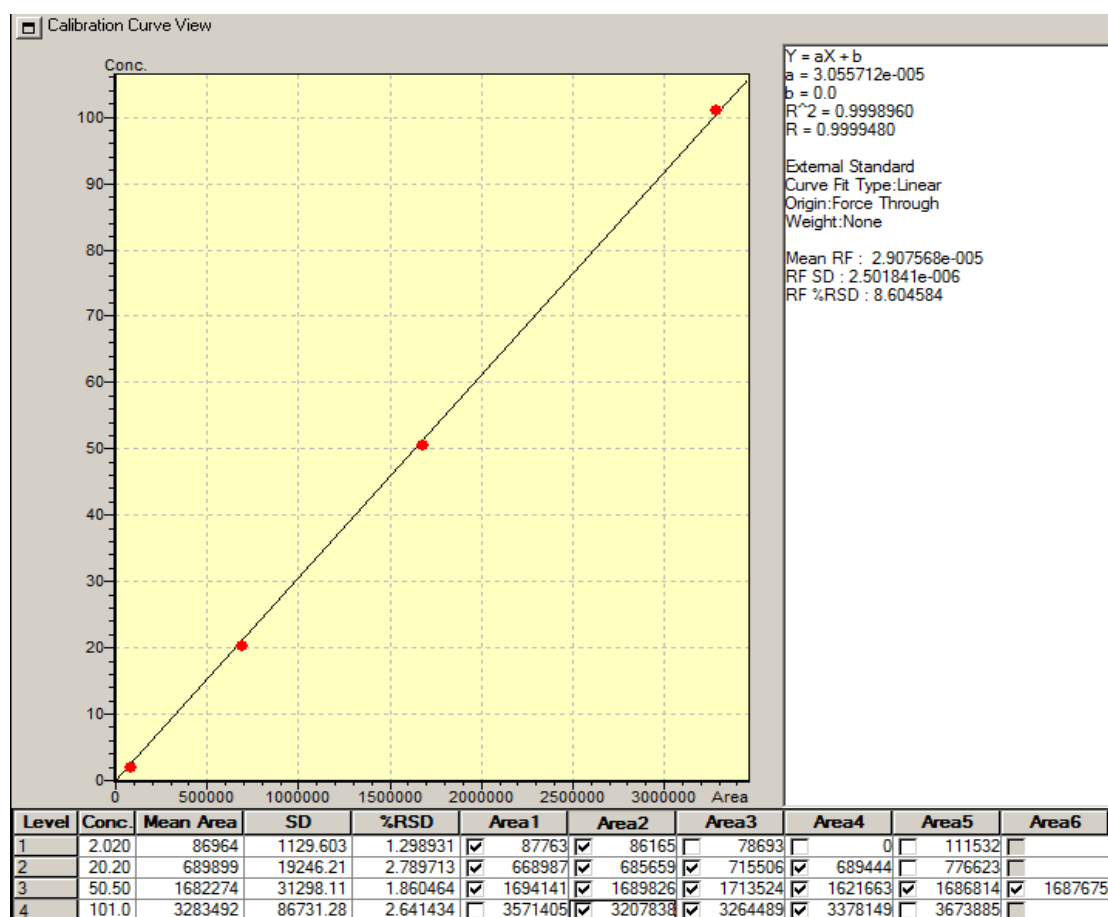


Figure 22. Calibration curve FLD

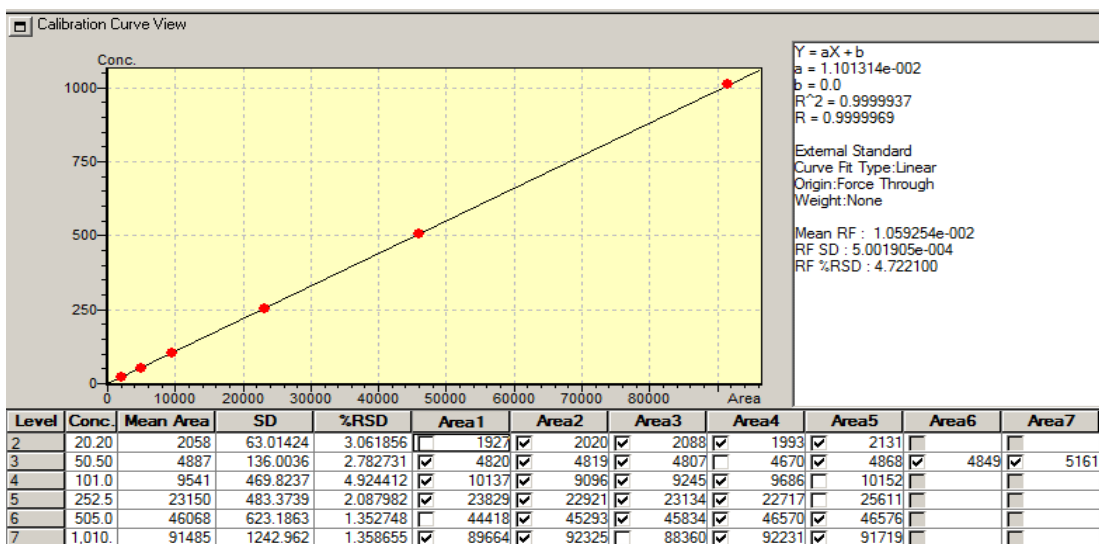
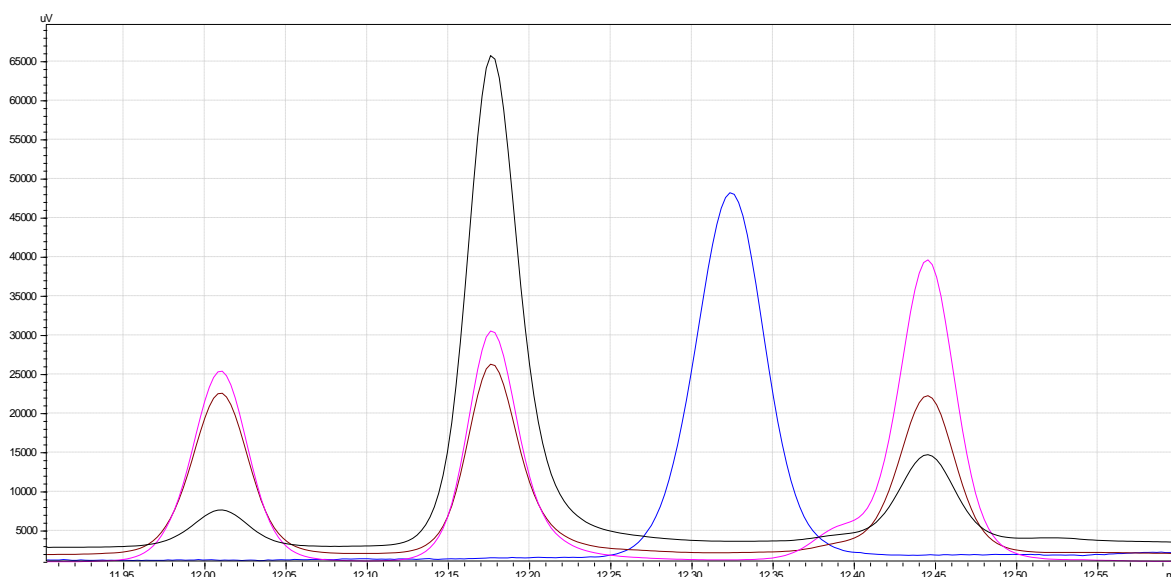


Figure 23. Calibration curve DAD

The limit of detection (LOD) for DAD is 1.65 ppm and for FLD is 0.25 ppm. The limit of quantification (LOQ) for DAD is 5.02 ppm and for FLD is 0.76 ppm. The fluorescence optimisation for analysis of 4-ethylphenol is:  $\lambda_{\text{excitation}}$  at 273.63 nm for maximum at elution point for 4-ethylphenol and  $\lambda_{\text{emission}}$  at 299 nm for maximum at elution point for 4-ethylphenol.

The decision was made to opt for two inline detectors (DAD and FLD) to make sure that the elution time for 4-ethylphenol does not interfere with the elution of other phenolic compounds, which would render identification and quantitative assessment problematic. 4-ethylphenol has a maximum absorbance at 278 nm (Figure 24), which is close to that of other phenolic compounds and proteins. By using the fluorescence detector (FLD  $\lambda_{\text{excitation}}$  273.63 nm,  $\lambda_{\text{emission}}$  299 nm) one can achieve a double confirmation of 4-ethylphenol presence and quantitation. Additionally, the lower limit of detection is improved.





**Figure 24.** A chromatogram highlighting the elution time and peak for 4-ethylphenol using this method. Blue line: 278 nm, peak is 4-ethylphenol, eluting between 12:25 and 12:40 (mm:ss)

### 3.2.5 NMR fingerprinting

Treated wines were analysed using nuclear magnetic resonance ( $^1\text{H}$  NMR) to quantify several compounds including alcohols and aldehydes (Godelmann et al., 2016, 2013). First, a 90% sample dilution was prepared using a  $\text{K}_2\text{PO}_4$  buffer (1M, pH = 2.4) in  $\text{D}_2\text{O}$ , with 3-(trimethylsilyl)-propanoic acid sodium salt as internal standard to reference the chemical shift to 0 ppm. The pH of the mixture was adjusted to  $3.10 \pm 0.02$  using 1M NaOH and HCl solutions. A total of 600  $\mu\text{L}$  were transferred to a 5 mm NMR tube and measured immediately. Measurements were made with a 400 MHz Avance III NMR spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) equipped with a 5 mm  $^1\text{H}/\text{D}$ -TXI probe head with  $z$ -gradient, automated tuning and matching accessory, and BTO-2000 for temperature control. Measurements were made under water and ethanol suppression with the correction of eventual interferences. Signal-to-noise (S/N) ratio was 10:1 and recycling time was 6 s. Temperature was set to  $300 \pm 0.2$  K without rotation. The pulse angle was maintained for the calibration and measurement of wine samples and the sweep width (SW) was 18 ppm. Compound quantification was performed by Bruker BioSpin GmbH according to Godelmann et al. (2016).

### 3.3 Experimental methodology

In this section, the methodology and further clarifications pertaining to it will be presented for each experiment undertaken.

#### 3.3.1 First MIP1 trial – methodology

Red wine (*V. vinifera*, mixture of varieties) from Hochschule Geisenheim was used. The wine did not evidently display a *Brettanomyces* taint, although its 4-ethylphenol value was approximately 200 µg/L. Samples of wine in a volume of 100 mL were treated with 37.5 mg MIP each. They were either previously spiked with a solution of 4-ethylphenol (FG, 99%, Sigma-Aldrich) or not spiked (control wine). The first generation 4-ethylphenol molecularly imprinted polymer (MIP1) provided by Ligar Polymers was used.

The amount of MIP added corresponded to the ratio of 250 mg polymer for 1 mg/L 4-ethylphenol, which was the initial recommendation of the polymer producer. As the spiked wines had about 1.5 mg/L 4-ethylphenol, and the volume for each sample was 100 ml, the 37.5 mg MIP value was calculated. Three contact times were explored: 5 minutes, 2 hours, 24 hours. Therefore, eight sample conditions were prepared in total (Table 9).

Table 9. First MIP1 Trial sample conditions

Sample number	Wine type	MIP treatment	Contact time [hh:mm]
1	Control wine	No	-
2	Control wine	37.5 mg MIP / 100mL	00:05
3	Control wine	37.5 mg MIP / 100mL	02:00
4	Control wine	37.5 mg MIP / 100mL	24:00
5	Spiked wine	No	-
6	Spiked wine	37.5 mg MIP / 100mL	00:05
7	Spiked wine	37.5 mg MIP / 100mL	02:00
8	Spiked wine	37.5 mg MIP / 100mL	24:00

The MIP was continuously stirred in the wine with a magnetic stirrer throughout the entire duration required in each respective case. Once the time was expired in each case, the MIP was separated from the wine with filter paper, and 50 mL vials were filled with the wine samples and sent for stir bar sorptive extraction gas chromatography mass spectroscopy (SBSE GC-MS) analysis, using a Restek RTX-5 GC column. Each sample corresponding to a specific experimental condition was prepared in duplicate, and each sample was itself analysed in duplicate.

### 3.3.2 Second MIP1 trial – methodology

For this experiment, the MIP dosage used was 500 mg/100 mL wine, and the contact times were 1 minute, 15 minutes, one hour and two hours (Table 10). The control wine and the spiked wine were both subjected to the same polymer treatment in parallel only for the 1-minute contact time experimental condition. In addition to 4-ethylphenol, the 4-ethylguaiacol values were also observed.

**Table 10. Second MIP1 Trial sample conditions**

<b>Sample number</b>	<b>Sample code</b>	<b>Wine type</b>	<b>MIP treatment</b>	<b>Contact time [hh:mm]</b>
1	K	Control wine	No	-
2	K1m	Control wine	500 mg MIP / 100mL	00:01
3	S	Spiked wine	No	-
4	S1m	Spiked wine	500 mg MIP / 100mL	00:01
5	S15m	Spiked wine	500 mg MIP / 100mL	00:15
6	S1h	Spiked wine	500 mg MIP / 100mL	01:00
7	S2h	Spiked wine	500 mg MIP / 100mL	02:00

### 3.3.3 MIP1 filtration experiment – methodology

The filter sheets used were based on the Seitz™ K200 grade (Pall GmbH, Bad Kreuznach, Germany). The filter sheets were round and 6cm in diameter and contained 2.2 grams of embedded polymer each. Two types of filter sheets were used, embedded with MIP1 and with NIP1, the non-imprinted analogue polymer. For comparison, the MIP1 and NIP1 powder was also used to treat an equivalent volume of wine, the dosage being identical: 2.2 g polymer per Litre of wine.

For the wine treated with polymer-embedded filter-sheets, the treatment setup was devised around a pressurized tank with a 5 L maximal capacity (Figure 25). The appropriate volume of wine was loaded inside, and the tank was closed. A pressurized air outlet equipped with manometer was connected to the tank. This would provide the necessary pressure to move the wine through the filter sheet. The polymer-embedded filter sheet was placed inside a Seitz™ circular filter holder, connected to the pressurized tank. The volume of wine was always 1000 mL in all experiments, spiked at 1 mg/L 4-ethylphenol. All the wine would pass through the filter and be collected in a 1000 mL volumetric flask, placed on a digital lab scale. The lab scale was connected to a computer, which could take a reading of mass each second, allowing for an accurate representation of average filtration rate and duration.

For the wine treated with polymer powder, the treatment setup was devised around a glass collection flask with a 3 L maximal capacity, connected to a vacuum pump (Figure 26). On top of the flask was a metallic funnel with a sintered metallic mesh support on which a 0.65 µm cellulose nitrate membrane (Sartorius AG) was installed. A suspension of polymer in distilled water was prepared first and filtered through the membrane to create a layer of polymer on top. This polymer layer would be then used to treat the spiked red wine. The wine was poured into the funnel and the vacuum pump provided the necessary negative pressure to pull the wine through the polymer layer and the membrane into the glass collection flask. Using a pump bypass, it was possible to modulate the negative pressure so that the filtration times would be equal in both filtration setups.



Figure 25. Treatment setup for polymer-embedded filter sheets



Figure 26. Treatment setup for polymer powder pre-coated on a membrane

The experimental conditions of this experiment are outlined in Table 11.

**Table 11. MIP1 filtration trial sample conditions**

<b>Sample name</b>	<b>Wine type</b>	<b>Treatment</b>
C	Control wine	None
S	Spiked wine	None
K200	Spiked wine	K200 filtration
F MIP1	Spiked wine	Filtration with MIP1 embedded filter sheet
F NIP1	Spiked wine	Filtration with NIP1 embedded filter sheet
P MIP1	Spiked wine	Filtration with MIP1 powder coated on membrane
P NIP1	Spiked wine	Filtration with NIP1 powder coated on membrane

### **3.3.4 MIP1 regeneration experiment – methodology**

The methodology, like the previous experiment, consists of working with polymer powder pre-coated onto cellulose nitrate 0.65  $\mu\text{m}$  membranes (Sartorius AG) and with a vacuum pump. This time, the volume of liquid was 100 mL, and all treatment contact times were below 1 minute: between 35 and 55 seconds. For this reason, marginal differences may be accounted for due to this variation in contact time, but the results are still useful in showcasing a general trend. The polymer dose was kept identical to the previous experiment: 2.15 g/L polymer (215 mg polymer / 100 mL wine). The protocol followed for polymer regeneration was given by the polymer manufacturer, and was a sequential 5-step process:

1. Water rinse
2. 0.1M NaOH solution rinse
3. Water rinse
4. 0.1M citric acid solution rinse
5. Water rinse

In this experiment, each of the five regeneration fractions had a volume of 20 mL, meaning that the total sum of regeneration solution was 100 mL, equal to the volume of wine treated

either before or after regeneration. The experimental conditions of the samples are presented in Table 12.

**Table 12. Sample names and descriptions for the MIP1 regeneration experiment**

<b>Sample name</b>	<b>Description</b>
C	Control wine
S	Spiked wine (1 mg/L 4-EP)
MIP S	Spiked wine treated with MIP
NIP S	Spiked wine treated with NIP
MIP S/R/C	Control wine treated with a regenerated MIP that had previously treated a spiked wine
NIP S/R/C	Control wine treated with a regenerated NIP that had previously treated a spiked wine
MIP S/R/S	Spiked wine treated with a regenerated MIP that had previously treated a spiked wine
NIP S/R/S	Spiked wine treated with a regenerated NIP that had previously treated a spiked wine

Samples C, S, MIP S and NIP S are simple to understand. They are, approximately, a repetition of the previous experiment, done at one level of magnitude lower (100 mL wine samples instead of 1000 mL wine samples).

Samples MIP S/R/C and NIP S/R/C explore the situation when the polymer is regenerated after treating a tainted wine. Were this polymer to be put in contact with a clean wine, with no 4-EP, it is possible that the wine would be contaminated with 4-EP leaching out of the polymer. If some of the 4-EP bound by the polymer does not get removed by the regeneration protocol, there is a possibility that it will desorb it back into a clean wine.

Samples MIP S/R/S and NIP S/R/S explore the situation when the polymer is regenerated after treating a tainted wine, and then re-used to continue treating wine of the same 4-EP

concentration as before. Its 4-EP removal capability after being regenerated is to be compared to that of it before the regeneration.

### **3.3.5 Polymer binding experiment – methodology**

#### **3.3.5.1 Concentration constant, variable polymer mass experiment – methodology**

In this experiment, a 4-EP solution in distilled water (25 mg/L, 15% vol. ethanol) is used, with a constant concentration, as the mass of polymer varied. Six grams of each (MIP and NIP) polymer was suspended in a 15% vol. ethanol solution, at a 100 mg/mL concentration. The suspensions were prepared 24 hours in advance of testing.

Sample volumes were 10 mL, and a solution of 15% vol. ethanol was added to account for the differences in volume due to changing the polymer suspension quantities, and to keep all volumes identical and with the same concentration in ethanol. Each sample was prepared in triplicate for both the MIP and the NIP (Table 13).

The contact time for the testing was 5 minutes, considered as a reasonable amount of contact between polymer and target, without being long enough to cause too much of a selectivity decrease. The samples were placed on an orbital shaker at 100 RPM for 5 minutes. Afterwards, the samples were passed through a 0.45  $\mu\text{m}$  cellulose acetate syringe filter (Minisart®, Sartorius AG) to separate the solution from the polymer. The solution was retained for HPLC analysis (described in Section 3.2.4.).

For the 200 mg polymer samples, the 200 mg was weighed directly into the sample vial and suspended in 1 mL of 15% vol. ethanol solution overnight, due to difficulties in accurately pipetting a suspension of more than 100 mg/mL. The remaining 9 mL of test solution (25 mg/L 4-EP solution in distilled water, 15% vol. ethanol) was added afterwards, and the sample was mixed at 100 RPM for 5 minutes.



**Table 13. List of sample preparation conditions; trial with concentration constant, variable polymer mass**

<b>Sample</b>	<b>Polymer (mg)</b>	<b>100 mg/mL polymer suspension (μL)</b>	<b>15% EtOH (μL)</b>	<b>25 mg/L TS (mL)</b>
1	200	n/a	n/a	9
2	100	1000	0	9
3	80	800	200	9
4	60	600	400	9
5	40	400	600	9
6	20	200	800	9
7	10	100	900	9
8	5	50	950	9
9	2.5	25	975	9
10	0.5	5	995	9
11	0	0	1000	9

### 3.3.5.2 Polymer mass constant, concentration variable experiment – methodology

For this experiment, a polymer suspension of 40 mg/mL was used. This concentration was kept constant, while the concentration in 4-EP of the test solution changed. The 40 mg/mL polymer suspensions were made by diluting 16 mL of 100 mg/mL polymer suspensions with 24 mL of 15% vol. ethanol solution. The sample volumes, contact time and stirring method were the same as in the previous experiment: 10 mL, 5 minutes at 100 RPM. Afterwards, the sample filtration and HPLC analysis were performed identically to the case of the previous experiment. Each of the samples was prepared in triplicate for both the MIP and the NIP (Table 14).

**Table 14. List of sample preparation conditions; trial with polymer mass constant, concentration variable**

<b>Sample</b>	<b>Test solution (mg/L 4-ethylphenol)</b>	<b>Test solution (mL)</b>	<b>40 mg/mL polymer suspension (mL)</b>
1	200	9.0	1.0
2	100	9.0	1.0
3	80	9.0	1.0
4	60	9.0	1.0
5	40	9.0	1.0
6	20	9.0	1.0
7	10	9.0	1.0
8	5	9.0	1.0
9	2.5	9.0	1.0
10	0.5	9.0	1.0
11	0	9.0	1.0

### 3.3.6 Characterization of polymer binding behaviour – methodology

#### 3.3.6.1 Contact time effect – methodology

Two types of molecularly imprinted polymers were used, along with their respective non-imprinted analogues MIP1, NIP1 and MIP2, NIP2. The polymers were tested both in dry and in wet state.

For the dry polymer experiments, 50 mg of polymer were weighted into 1.5 mL plastic centrifuge tubes, using an analytical balance (Shimadzu™ AUW220D) (Figure 27).



**Figure 27. Sample preparation with analytical balance (Shimadzu™ AUW220D)**

For the wet polymer experiments, a solution of 100 mg/mL polymer was prepared in 15% vol. ethanol and let sit for 24 hours before use.

One mL of 25 ppm 4-EP solution prepared in 15% vol. ethanol was pipetted in the centrifuge tubes and mixed with the polymer (either dry or in wet suspension) by manual agitation if contact time was below 5 minutes, or by depositing on an Orbital shaker at 150 RPM, for contact times between 10 minutes and one hour.

For the samples that were deposited on an orbital shaker, they were then centrifuged at 13.300 RPM for 5 minutes in a Thermo Fisher Scientific MicroCL 17R microcentrifuge. Afterwards they were returned and treated identically as the other samples.

When the mixing time was finished, each sample in the centrifugal tube had its liquid extracted with a 1.5 mL pipette and deposited into a 3 mL single use syringe equipped with a 0.45  $\mu\text{m}$  nylon filter tip, through which they passed into a 2 mL HPLC grade vial, secured with 9mm polypropylene bonded screw cap with septa.

The explored contact times were: 7 seconds, 15s, 30s, 1 minute, 2 min., 3 min., 4 min., 5 min., 10min., 15 min., 30 min., 60 min. Every sample condition was prepared in triplicate.

The polymer doses used in this experiment (50 mg/mL, equivalent to 50 g/L), as well as the 4-EP concentration (25 mg/L) are too high to be possibly representative of actual winemaking conditions. Nevertheless, they are useful in highlighting certain trends in the data.

#### **3.3.6.2 4-ethylphenol concentration effect – methodology**

In this case a solution of 100 mg/ml of polymer was prepared with 15% vol. ethanol solution and let to sit for 24 hours before use. 100  $\mu$ L of this solution was pipetted into each centrifugal tube. Afterwards, to each tube were added 900  $\mu$ L of 4-EP solutions made in 15% vol. ethanol of varying 4-EP concentrations, in the following range (mg/mL): 0.1, 0.25, 0.5, 1, 2, 3, 4, 5, 7.5, 10. These testing conditions correspond to a 10 g/L polymer treatment.

After addition, each tube was agitated using a vortex mixer, and then filtered following the same method as in Experiment 1. The overall contact time between polymer and 4-EP solution was around 7 seconds, which represented the shortest possible time necessary for mixing and filtration of each sample into an HPLC vial.

Two testing conditions were explored: MIP2 addition and NIP2 addition. Another testing condition was also used, pertaining to the control samples, by adding 15% vol. ethanol solution addition instead of any polymer solution. The percentage bindings of the polymers are calculating by referencing to the 4-EP values from the samples which have received no polymer addition (the control).

#### **3.3.6.3 Polymer dose effect – methodology**

Varying doses of polymer solution (100 mg/mL) were added to the centrifugal tubes, to obtain the following polymer concentrations in a 1 mL volume (mg/mL, equivalent to g/L): 0.5, 1, 2.5, 5, 7.5, 10, 15, 20. The total volume of polymer solution added was increased, therefore, from 5, 10, 25, 50, 75, 100, 150, until 200  $\mu$ L. To this volume, 15% vol. ethanol solution was added until the total volume reached 200  $\mu$ L, after which 800  $\mu$ L of 4-EP solution (either 1  $\mu$ g / 800  $\mu$ L or 5  $\mu$ g / 800  $\mu$ L) was added to the centrifugal tube. Afterwards, each tube was agitated and filtered following the same methodology as in the previous

experiments. The contact time was that of a pass-through (~7 seconds). Each sample was prepared in triplicate.

#### **3.3.6.4 Polymer elution behaviour – methodology**

For this experiment, 5 mL of MIP2 and NIP2 polymer suspensions (100 mg/mL) were added to 10 mL syringes, equipped with nylon mesh filters and 0.45 µm nylon filter tips. After the liquid solution of the polymer suspension passed through and after drying the polymers loaded in the syringes overnight, 10 mL of 25 ppm 4-EP solution in 15% vol. ethanol was loaded in the syringes and passed through the polymer bed. Every 1 mL was separately collected in 2 mL HPLC vials and stored for analysis. After this, 5 mL of water was passed through, each mL being separately collected, in the same way as above. Subsequently 5 mL of NaOH 2M solution was passed through, each mL being collected separately. Subsequently, 5 mL water was passed through, each mL being collected separately. Subsequently, 5 mL citric acid 2M was passed through, each mL being collected separately. Finally, 10 mL of water was passed through, each mL being collected separately.

For the samples collected from the 5 mL NaOH solution and the 5 mL of water inserted after it, they were diluted 1:1 with 11% formic acid solution, to prevent damaging the HPLC column.

After a first pass of 10 mL 25 mg/L 4-EP test solution through 500 mg polymers (equivalent of a 50 g/L dose), the MIP bound 75.4% of the total 4-EP, while the NIP bound 99.5% of the total 4-EP. Although the high dose should theoretically allow for both polymers to completely bind the 4-EP, it is possible due to the syringe filter setup that filter channelling occurred through the MIP powder, leading to an inferior binding performance. Nevertheless, one can still look at the ability of the elution liquids to clean the considerable 4-EP which was bound to the polymers.

### 3.3.6.5 Particle size effect – methodology

A screening test was performed. 500 mg of a NIP were weighted into a 100 mL beaker to which 50 mL of drum acetone was added. Afterwards the mixture was stirred using a magnetic stirrer at 400 RPM for 5 minutes. After the stirring stopped, 1 mL samples were taken from the liquid suspension immediately afterwards, after 5, 15 and 30 minutes, and after 1, 2, 3, 4, 5 and 6 hours. Each millilitre was placed in a scintillation vial and placed in an alcove to evaporate the acetone. The vials were inspected afterwards to check whether there was any polymer powder present in the suspension.

Based on the results of the screening test, three sedimentation times were considered: 5 minutes, 30 minutes, and 2 hours.

One gram of polymer (for the 5 and 30-minute contact times) and two grams of polymer (for the 2 hours contact time) were mixed with 100 mL of acetone for 5 minutes, under constant stirring at 400 RPM. After the respective times (5 minutes, 30 minutes, 2 hours) had passed, the acetone containing suspended polymer was pipetted into a new beaker and placed in an alcove to evaporate the liquid. This kind of fraction was labelled “Fine”. At the bottom of the initial beaker, the larger, heavier polymer particles formed a sediment and were collected separately. These fractions were labelled “Coarse”.

After the separation of the polymer into two fractions with this method, they were tested using the same methodology as in the experiment on section 4.6.1, at a pass-through contact time of 7 seconds, with a 50 mg polymer dose and a 1 mL 15% EtOH test solution with a 25 mg/L 4-EP concentration.

### 3.3.6.6 Lab scale wine trial – methodology

This experimental setup is comparable to the one in section 3.3.6.1 in terms of exploring different contact times. Specifically, three contact times were explored: 7 seconds (the shortest pass-through time possible under these experimental conditions), 15 seconds and 30 seconds. The wine was spiked at two 4-EP levels (1 mg/L and 5 mg/L) analogous to the spiking done in the experiment from section 4.6.3. The polymer dose was 10 mg/mL (equivalent to 10 g/L), analogous to the dose used in the experimental methodologies from

sections 3.3.6.2 and 3.3.6.3. Therefore, one can compare results obtained in this experiment with the equivalent data points pertaining to identical testing conditions from the experiments in sections 4.6.2 and 4.6.3 and draw conclusions.

### **3.3.6.7 Test solution filtration – methodology**

For this experiment two cycles of 10 L test solution (15% EtOH, 1 mg/L 4-EP concentration) passed through a plate frame filter loaded with two 20 cm<sup>2</sup> filter sheets with a retention rating of 0.5 µm (equivalent to a Seitz™ EK filter grade, sterilizing grade filtration), onto which 25 g of MIP2 polymer was pre-loaded. The loading of the polymer was accomplished by suspending the 25 g of polymer in deionized water and recirculating the liquid through the filtration system until clear, indicating that all the polymer particles were loaded onto the two filter sheets.

After each volume of 10 L of test solution passed through the filter, the regeneration solutions were passed through in the following sequence: 200 mL deionized water, 800 mL 2M NaOH solution, 400 mL deionized water, 2M 400 mL citric acid and, finally, 200 mL deionized water.

### **3.3.6.8 Wine filtration with MIP2 – methodology**

A *Vitis vinifera cv.* Cabernet Sauvignon red wine was used. The vintage was 2016 and several Bag-in-Box containers of this kind were purchased from a local supermarket. The total volume of wine used for the filtration trial was 10 L (spiked at 5 mg/L 4-EP) and this was put through six cycles of filtration with filter sheets onto which MIPs were pre-coated. After each filtration, the wine was collected in a vessel and the polymer was regenerated. The amount of polymer used was 25 g, which was pre-loaded onto the two filters, using the same method described in the previous section, by preparing a polymer suspension in deionized water and recirculating the suspension through the filter until the liquid became clear and all the polymer powder had been deposited onto the filter sheets.



### 3.3.7 Wine filtration with MIP2 in HS-Geisenheim – methodology

The setup for this experimental filtration is seen in Figure 28. A stainless-steel pressure container with a maximum capacity of 6 litres is connected to an air inlet, seen on the left side of the image. Compressed air is used for pushing the wine through the filter casing, seen to the right of the image. Therefore, the air pressure replaces, functionally, the pump. The wine is collected in a glass container placed on an electronic scale connected to a laptop which takes weight measurements every second. This allows one to deduce with precision the total time for a filtration, as well as the flow rate.



Figure 28. Filtration setup at Hochschule Geisenheim

In the previous wine filtration, 10 litres of wine were treated, however the pump needed to be primed with liquid to safely start. Therefore, between 1 and 2 litres of wine would enter the system to eliminate air inside the pump before the filtration could begin. While this liquid was recovered after the filtration was finished, it was still not possible to recover the entire volume of 10 L from the system. There were unaccounted losses of about 0.5-0.6 L of wine per each treatment and regeneration cycle. At the same time, the water used to flush the regeneration solution out of the system was also not completely evacuated from the system, which resulted in possible dilutions of wine with water.



The experimental setup at Hochschule Geisenheim is better suited for overcoming this issue by using the air pressure to flush all liquid from the system before moving on to the next step (treatment or regeneration).

However, it must be noted that, while the vessel and pipes do not contain liquid, there is indeed liquid in the filter sheet which can only be displaced by flushing with another liquid. Therefore, simply because of this way of working, there are losses and possible dilutions which are unavoidable. Specifically, to this point, a 22.2 g round filter sheet (13 cm diameter) absorbs about 50 mL of liquid, which cannot be removed except for flushing the filter with another liquid. During the actual filtration, it is likely that more than 50 mL are taken up by the filter, possibly close to 100 mL. When wine passes through a wetted filter, it mixes up with the water present inside. Therefore, there are losses of wine and possible dilutions due to the liquid being absorbed by the filter sheet. Also, about 10-25 mL are left in the pressure tank and cannot be filtered but can be recovered.

The filtration done in section 4.6.8 was performed with 10 L of red wine, spiked at 5 mg/L 4-ethylphenol, which is an extremely high dose, among the highest observed in the scientific literature for wine contaminated with *Brettanomyces bruxellensis*.

25 g of MIP2 were used to treat 10 L of wine. The polymer was first introduced into deionized water which circulated through the two 20x20cm filter sheets with a retention rating of about 0.5 microns, until all the polymer was coated on the 0.08 m<sup>2</sup> filtration surface. Assuming that the distribution of polymer was uniform, this corresponds to a polymer layer thickness of 0.822 mm.

The filter sheet that would fit the experimental setup in Geisenheim would be a round sheet with a diameter of 13 cm for the active filtration surface. This corresponds to a filtration surface of 0.01327 m<sup>2</sup>. Therefore, the appropriate amount of polymer to be pre-coated on this surface is 4.1468 g, which would allow for the same theoretical polymer layer thickness.

The volume of 5 mg/L 4-EP spiked wine that could be treated by this amount of polymer is 1.658 L, to maintain all the parameters proportional to the previous experiment.

The pressure was adjusted with the expectation that it can help modulate the flow rate, and therefore the contact time between polymer layer and spiked red wine. A series of pre-trials

with MIP1 were done to find the optimal pressure. A pressure of 1.5 Bar was selected as it allowed for a contact time of about 2 seconds, which was the closest to the calculated contact time of 2.2 seconds obtained during the wine filtration trials in New Zealand.

The volume of the regeneration sequence was:

1. Distilled water, 500 mL
2. NaOH 300 mL 1M
3. Distilled water 500 mL
4. Citric acid 150 mL 1M
5. Distilled water, 500 mL

This corresponds to the volumes used in the previous filtration experiments at Ligar. The amount of NaOH and citric acid used correspond to the necessary amounts needed to successfully elute a polymer to which 30% of the 4-EP in the spiked wine would be bound. The 30% value is derived from the bench trials performed in New Zealand, which show that for the given parameters, a 30% reduction is the maximum that can be expected per cycle. The summary of the highlighted differences between the two filtrations (in New Zealand and in Germany) can be seen in Table 15.

**Table 15. Comparison of experimental parameters between two filtration setups in different locations**

<b>Parameter</b>	<b>Ligar, NZ</b>	<b>HS Geisenheim, DE</b>
<b>4-EP concentration</b>	5 µg/L	5 µg/L
<b>Volume of wine</b>	10 L	1.658 L
<b>Filtration surface</b>	800 cm <sup>2</sup>	132.7 cm <sup>2</sup>
<b>Polymer amount</b>	25 g	4.15 g
<b>g of polymer / L wine</b>	2.5 g/L	2.5 g/L
<b>Polymer layer thickness</b>	0.8223 mm	0.8223 mm
<b>Contact time (seconds)</b>	3.99 s	3.55 s average (from 1.9 to 5 s)
<b>Filter plate orientation</b>	Vertical	Horizontal
<b>Fluid is moved by</b>	Rotary pump	Air pressure
<b>Filter retention rating</b>	0.5 µm	0.5 µm
<b>Polymer tested</b>	MIP2	MIP2, NIP2, no polymer
Regeneration process	5 step process containing 1M alkaline and 1M acid solutions	Identical process with adapted volumes to maintain ratios

### 3.3.8 Polymer selectivity analysis – methodology

The experimental conditions can be seen in Table 16. Several treatments with different materials were applied, all used at the same dose of 2.5 g/L: MIP1, NIP1, MIP2, NIP2, PVPP and activated carbon. A blank sample was also prepared, where no material was used. The wine used in this experiment was a 2016 red wine c.v. Fetească Neagră, from the Iași region of Romania. The wine had been stored in a 225 L oak barrel for 12 months, which means that, as a wine matrix, this was quite an accurate representation of the typical wine which is at risk of a Brett infection. The wine was spiked at two 4-EP doses, one high (5000 µg/L) and one low (1000 µg/L). The interest was to see whether having more 4-EP available for the polymer to bind could lead to differences in selectivity from the same polymer, presumably as the higher dose would lead to more of the molecularly imprinted binding sites in the polymer being used, therefore reducing the possibility for non-selective binding. All samples

had a volume of 0.5 L and were filtered all in the same way using a vacuum pump like the setup shown in Figure 26. The membrane filters used were Pall Supor<sup>®</sup> PES Membrane Disk Filters, with a pore size of 0.45  $\mu\text{m}$ .

**Table 16. Experimental conditions for polymer selectivity analysis**

<b>Sample number</b>	<b>4-EP concentration [<math>\mu\text{g/L}</math>]</b>	<b>Treatment type</b>	<b>Treatment dosage [g/L]</b>	<b>Average filtration time [hh:mm:ss]</b>
1	1000	MIP1	2.5	00:03:45
2	5000	MIP1	2.5	00:03:45
3	1000	NIP1	2.5	00:03:50
4	5000	NIP1	2.5	00:03:50
5	1000	MIP2	2.5	00:04:20
6	5000	MIP2	2.5	00:04:20
7	1000	NIP2	2.5	00:05:20
8	5000	NIP2	2.5	00:05:20
9	1000	Blank	2.5	00:15:30
10	5000	Blank	2.5	00:15:30
11	1000	PVPP	2.5	06:00:00
12	5000	PVPP	2.5	06:00:00
13	1000	Carbon	2.5	00:23:15
14	5000	Carbon	2.5	00:23:15

### 3.3.9 Particle size distribution – methodology

An analysis was performed in September 2017 at Pall Filtersystems GmbH in Bad Kreuznach, Germany, to examine the particle size distribution of the four types of polymers (MIP1, NIP1, MIP2 and NIP2). The analysis was performed using a Masterizer 2000 laser diffraction particle size analyser (Malvern Panalitical) for particle size analysis. All results represent averages from three independent measurements done for each polymer type.

### 3.3.10 Polymer total capacity analysis – methodology

For this experiment, MIP1, NIP1, MIP2 and NIP2 were all tested. A quantity of 250 mg of each type of polymer was pre-coated onto a separate Seitz™ EK filter sheet, of 6 cm in diameter. This kind of filter sheet was used to filter a total volume of 1 L red wine, spiked at 1 mg/L 4-ethylphenol. The volume of 1 L was filtered in ten distinct 100 mL fractions, and a sample was taken from each fraction for subsequent analysis of volatile phenols. All filtration experimental conditions were prepared in triplicate. All analyses were performed in triplicate. Shown values represent averages of these results.

### 3.3.11 MIP2 filter sheet experiments

These filter sheets are 20x20 cm in size and either contain an amount of imprinted polymer (MIP2) corresponding to approximately 30% of their mass (further called MIP2 sheets), or contain no polymer, and thus represent blank filter sheets (further called BKP sheets)

The main physical parameters of these filter sheets are highlighted in Table 17.

Table 17. Main characteristics of experimental polymer-embedded filter sheets

Type of filter sheet	Surface size	Weight	Measured g/m <sup>2</sup>	Polymer content
MIP2 sheet	20x20 cm	46.45 ± 0.4 g	1161.3 ± 9	13.935 g
BKP sheet	20x20 cm	54.1 ± 1 g	1352 ± 27	0 g

To begin testing these filter sheets, a simple comparison test was made to evaluate their propensity to absorb colouring matter and phenolic compounds from red wine. The blank filter sheet (called BKP) was compared to a Seitz™ K900 filter sheet (in that their g/m<sup>2</sup> values were similar).

Each sheet used was 6cm in diameter, and through it, a volume of 1 L red wine was filtered. Afterwards, the sheets were rinsed with the regeneration protocol recommended by the filter sheet producer (Ligar), and then re-used, for six filtration and regeneration cycles.

### **3.3.11.1 4-ethylphenol binding and elution trials using MIP-embedded filter sheets – methodology**

The MIP2 filter sheets have been compared with a standard Seitz™ K900 filter sheet which was pre-coated with 1 g MIP1 (first generation polymer). Therefore, the amount of polymer is, in both cases, identical. The difference is that MIP1 was pre-coated onto a standard filter sheet, whereas the MIP2 was already embedded into an experimental filter sheet by the polymer producer.

The comparison between the filter sheet with embedded MIP2, the filter sheet with pre-coated MIP1 and the blank control filter sheet, has been made over the course of six filtration and polymer regeneration cycles. The regeneration procedure has been slightly adapted according to the indications of the polymer manufacturer, consisting of a 5-step process, involving passing through the filter of the following liquids, in the following order:

1. Distilled water (200 mL)
2. NaOH 0.1M solution (200 mL)
3. Distilled water (200 mL)
4. Citric acid 2M solution (200 mL)
5. Distilled water (200 mL)

### **3.3.11.2 Assessment of different elution and regeneration protocols – methodology**

The general outline of the previous experiment was used, with some adjustments. A polymer dose of 1 g was used (corresponding to the 1 g of MIP2 being embedded in a 6cm in diameter filter sheet). Through it, volumes of 100 mL of red wine, spiked at 2.29 mg/L 4-ethylphenol were filtered. Samples were collected for HPLC analysis of 4-EP.

Five filtration and regeneration cycles were used. It is important to highlight that in this case of filtration cycles, a new spiked wine was used each time, unlike the previous experiment where the wine was collected and re-filtered. This experiment therefore highlights how the performance of the polymer maintains itself throughout several regeneration cycles. Additionally, there were four different variants of the regeneration cycle tested:

1. Standard regeneration procedure (Regen Var. 1):

- a. Distilled water, 100 mL
  - b. Sodium Hydroxide 0.1M, 100 mL
  - c. Distilled water, 100 mL
  - d. Citric acid, 2M, 100 mL
  - e. Distilled water, 100 mL
2. Regeneration procedure with double volumes for steps b and c (Regen Var. 2):
- a. Distilled water, 100 mL
  - b. Sodium Hydroxide 0.1M, 200 mL
  - c. Distilled water, 200 mL
  - d. Citric acid, 2M, 100 mL
  - e. Distilled water, 100 mL
3. Regeneration procedure in which the regeneration solutions of steps b and c would be used, collected separately, and recirculated one more time (Regen Var. 3):
- a. Distilled water, 100 mL
  - b. Sodium Hydroxide 0.1M, 100 mL (collected separately)
  - c. Distilled water, 100 mL (collected separately)
  - d. Sodium Hydroxide 0.1M, 100 mL (same as step b, reused)
  - e. Distilled water, 100 mL (same as step c, reused)
  - f. Citric acid, 2M, 100 mL
  - g. Distilled water, 100 mL
4. Regeneration procedure in which there are two additional steps, containing identical new solutions corresponding to steps b and c (Regen Var. 4):
- a. Distilled water, 100 mL
  - b. Sodium Hydroxide 0.1M, 100 mL
  - c. Distilled water, 100 mL
  - d. Sodium Hydroxide 0.1M, 100 mL
  - e. Distilled water, 100 mL
  - f. Citric acid, 2M, 100 mL
  - g. Distilled water, 100 mL

### 3.3.12 Final filtration of wine with polymer-embedded filter plates – methodology

All previous experiments were carried out with normal red wine which had been spiked with 4-ethylphenol. For this experiment, a naturally *Brettanomyces*-contaminated red wine was found and used. This wine was of the variety Spätburgunder, vintage 2014, from the Rheingau viticultural region of Germany. Its 4-ethylphenol content was 1200 µg/L and its 4-ethylguaiacol content was 300 µg/L. To compare a *Brettanomyces*-tainted wine with a non-tainted wine spiked to identical levels of ethylphenols, a Merlot wine, vintage 2017, also from the Rheingau viticultural region of Germany was used, both as standard (un-tainted) and as spiked to identical levels of 4-ethylphenol and 4-ethylguaiacol as the Spätburgunder. Four different types of filtrations were explored for the contaminated Spätburgunder wine: standard filtration using a Seitz™ EK filter sheet, filtration with MIP2 embedded filter sheets, filtration with NIP2 embedded filter sheets and, finally, charcoal fining (Fenol-Free, Enartis) and subsequent sterilizing grade filtration using Seitz™ EK filter sheets. In addition to these, the normal wine (non-filtered) was also tested in its actual state at the time.

For the Merlot wine, three conditions were compared: no filtration, sterilizing grade filtration (EK filter sheets) and filtration using MIP2 embedded filter sheets. The purpose of this set of conditions was to explore the degree to which a molecularly imprinted polymer embedded filter sheet imparts any unwanted characteristics to a wine which is otherwise considered correct and free of any defects.

For the spiked merlot wine, five conditions were explored: the spiked wine before any filtration, sterilizing grade filtration, filtration with MIP2 embedded filter sheets, charcoal fining with sterilizing grade filtration and finally, sterilizing grade filtration followed by treatment through adsorption using a column filled with MIP2 beads. This column with polymer beads was presented as an alternative format to the filter sheet format. According to the polymer manufacturer's specifications, the column containing 84.6 g of polymer beads was intended to work with a volume of only 2.25 litres of tainted wine.

All experimental conditions and wines tested are listed and described in Table 18.



**Table 18. List of wines treated with polymer-embedded filter sheets, experimental conditions as well as wine codes for subsequent figures**

Sample	Wine code	Variety	Year	Experimental condition
1	B	Spätburgunder	2014	<i>Brettanomyces</i> contaminated wine
2	BE	Spätburgunder	2014	<i>Brettanomyces</i> contaminated wine, sterilizing grade filtration
3	BM	Spätburgunder	2014	<i>Brettanomyces</i> contaminated wine, MIP2 filtration
4	BN	Spätburgunder	2014	<i>Brettanomyces</i> contaminated wine, NIP2 filtration
5	BC	Spätburgunder	2014	<i>Brettanomyces</i> contaminated wine, charcoal fining + sterilizing grade filtration
6	K	Merlot	2017	Control wine (0 µg/L volatile phenols)
7	KE	Merlot	2017	Control wine, sterilizing grade filtration
8	KM	Merlot	2017	Control wine, MIP2 filtration
9	S	Merlot	2017	Spiked wine (volatile phenols 1200 µg/L 4-EP and 300 µg/L 4-EG)
10	SE	Merlot	2017	Spiked wine, sterilizing grade filtration
11	SM	Merlot	2017	Spiked wine, MIP2 filtration
12	SC	Merlot	2017	Spiked wine, charcoal fining + sterilizing grade filtration
13	SMC OL	Merlot	2017	Spiked wine, sterilizing grade filtration, absorption treatment with MIP2 beads column

For all filtrations, a 500 L pressurized stainless steel tank was used to hold the wine to be filtered. The wine in the tank was covered with a nitrogen gas blanket to prevent any oxidation, as the different filtrations and treatments occurred in successive days. The wine was filtered using three 20x20 cm filter sheets mounted on a KHS Innopro Pilot filter plate

device. As the wine tank was pressurized, the nitrogen over-pressure ensured that the filtration could be performed without the need of any pumping device. The stainless-steel kegs into which a volume of 50 L of filtrate would be collected were placed on a pallet jack with an in-built scale, which allowed a continuous measurement of mass, permitting fine-tuning of pressure from the wine tank to the filter, in order to ensure all filtrations were performed with the same constant filtration speed, as recommended by the Seitz™ EK filter sheet instruction manual.

A total number of three filter sheets were used in each experimental condition, and the volume of wine filtered was 50 L in every case. This means that the ratio between polymer quantity and wine volume was that of 0.9 g polymer / litre wine. Each filter sheet contained 15 g of polymer (MIP2 and NIP2 respectively), and three filter sheets were used per each filtration event of 50 L of wine. An image of this filtration setup can be seen in Figure 29.

In the case of the wines treated with charcoal before filtration, a volume of 50 L of wine was directly transferred from the pressurized tank to a stainless-steel keg, to which 10 g of activated carbon was added (dosage of 20 g/hL) and kept in contact with the wine for 72 hours, which represent the recommended activated carbon dosage and contact time for wines contaminated with *Brettanomyces* taint, according to the activated carbon producer. The wine was afterwards filtered using the same setup as described above, into a new stainless-steel keg. This slightly modified filtration setup can be seen in Figure 30.



**Figure 29.** Wine filtration setup including pressurized stainless-steel tank, nitrogen gas tank, filtration device with plates, stainless steel keg for collecting filtrate and scale





**Figure 30.** Filtration setup for wine treated with activated carbon beforehand. The wine to be filtered is stored in a stainless-steel keg, and the overpressure from a nitrogen gas tank is used to enable the filtration through Seitz™ EK filter sheets into a new, clean stainless-steel keg

Finally, the treatment setup using the adsorptive column containing MIP2 beads was quite different from the previous filtrations (Figure 31). According to the specifications indicated by the polymer manufacturer, a column containing 84 g of MIP2 beads was meant to be used to treat a volume of 2.25 L of contaminated wine (equivalent of 3 bottles). This small-scale setup was achieved using a 3 L glass balloon flask which contained the wine to be filtered. A peristaltic pump was used to push the wine through the column, in an upwards directed flow, at a flow rate of 20 mL per minute, which was according to the manufacturer's specifications. The wine was collected in three wine bottles, which were, after the end of the treatment, mixed and homogenized together, before being sent to subsequent analysis. The flowrate was controlled using the peristaltic pump speed setting and fine-tuned by checking the real-time flow speed using a lab scale.

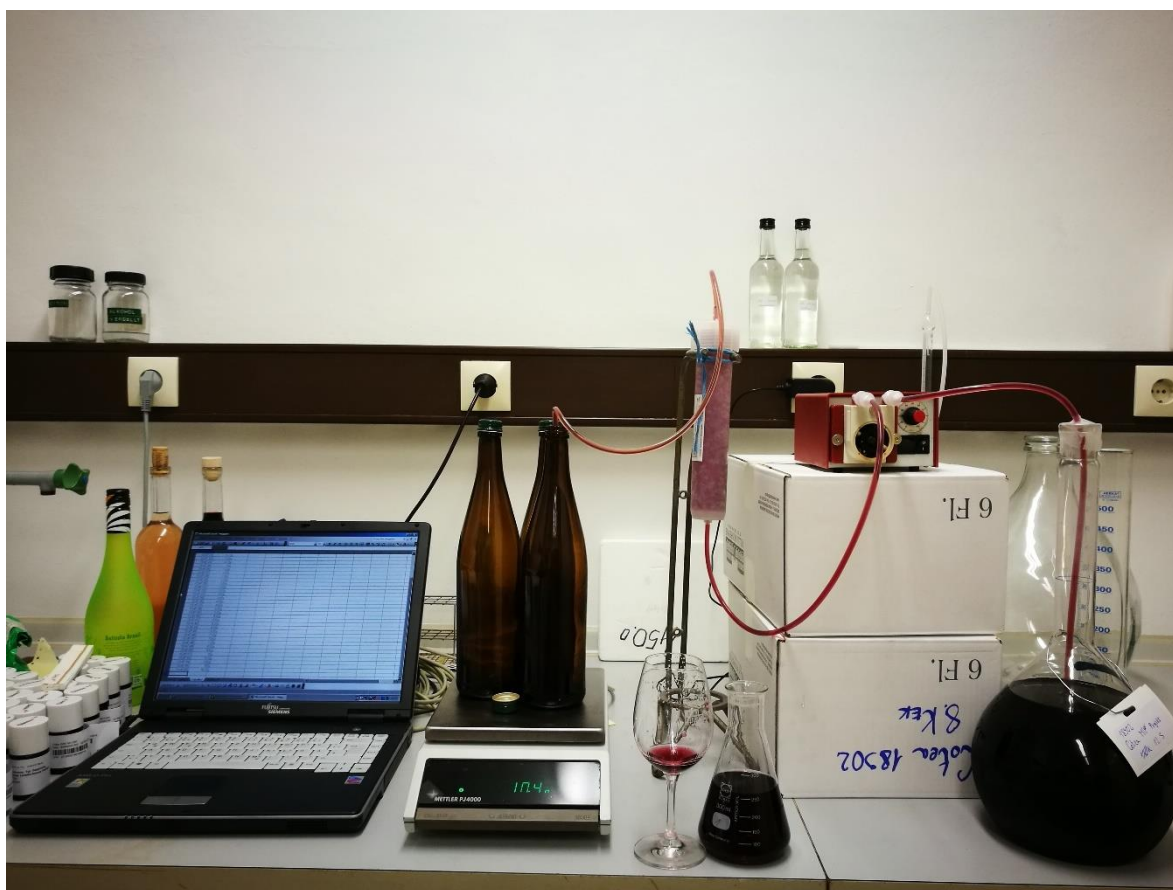


Figure 31. Treatment setup for using the adsorptive column containing MIP2 beads

Once all wine treatments were complete, the resulting wines were bottled using screwcap closures and deposited in cardboard boxes in a cool-temperature storage room, awaiting analysis.

#### **3.3.12.1 Wine physical-chemical analysis – methodology**

This methodology has been described in section 3.2.

#### **3.3.12.2 Sensory analysis – methodology**

A tasting panel was formed, consisting of 18 judges which regularly participate in wine sensory analysis tasting panels and trainings and are familiar with most wine defects.

The judges were asked to write down the most prominent olfactory and gustatory impressions that they formed based on tasting each wine.

The number of times each sensory descriptor mentioned by a taster was quantified, and the dominant olfactory and gustatory characteristics were considered those which were mentioned by at least 25% of the tasters in at least one of the four tasted variants.

#### **3.3.12.3 Filter sheet pre-cleaning procedures – methodology**

Standard Seitz™ EK filter sheets were used as a comparison reference for the MIP embedded filter sheets. Three 20x20 cm filter sheets from each kind were first rinsed with 25 L of water, and then used to filter a volume of 10 L of water. Afterwards, they were steamed for 20 minutes, after which they were once again rinsed with 25 L of water and then used to filter and collect separately another volume of 10 L water.

Out of the 10 L volumes of water filtered and separately collected (both before and after steam treatment), nine bottles from each treatment condition were filled and used for a series of triangle tests and a ranking test with the aid of a sensory analysis panel totalling 15 participants. The experimental conditions are highlighted in Table 19.

**Table 19. Experimental conditions for the filter sheet cleaning experiment**

<b>Sample code</b>	<b>Treatment type</b>
EB	Water filtered with Seitz™ EK filter sheets (rinsed but not steam cleaned)
EN	Water filtered with Seitz™ EK filter sheets (rinsed and steam cleaned)
MB	Water filtered with MIP filter sheets (rinsed but not steam cleaned)
MN	Water filtered with MIP filter sheets (rinsed and steam cleaned)
RW	Clean, municipal water, not filtered with any of the two filter sheets

Before collecting filtered water and bottling it for sensory analysis, they needed to be rinsed beforehand, as it is common for brand new filter sheets to give off a light paper taste when first in use. In the case of Seitz™ EK filter sheets, the recommended water rinsing volume is 50 L/m<sup>2</sup>. This means that for three 20x20 cm filter sheets ( $3 \times 0.04 \text{ m}^2 = 0.12 \text{ m}^2$ ), a volume of 6 L would have been theoretically necessary. Still, in the interest of redundancy and safety, the filter sheets were rinsed with 25 L, to represent a scenario where the MIP containing filter sheets are rinsed much more thoroughly than what is commonly deemed necessary for normal filter sheets.

The volume of 10 L of filtered water which was used for sensory analysis was collected separately from the rinsing water. Therefore, these water samples are taken from an output which was coming from the filter sheet at a time when theoretically no additional smell or taste should be taken up by the water. Ideally, no differences between the samples are to be expected. Any significant difference noticed may serve as a reasonable basis for recommending the application of more careful cleaning protocols and stricter quality control measures on the part of the polymer and polymer-embedded filter sheet manufacturer.

## 4 Results and discussions

### 4.1 First MIP1 Trial

The methodology and further clarifications pertaining to this experiment have been outlined in section 3.3.1.

This initial trial was conducted in October 2015. The aim of the trial was to create a starting point for assessing the performance of MIPs for treatment of various wine taints. It was conceived to give a first impression about how effective a MIP can be under a set of given conditions, and what kind of contact time is a better fit, as well as serving as an opening statement for communication with the producer of molecularly imprinted polymers, whose feedback and input was of help with the improvement and superior design of future trials. The results obtained are highlighted in Table 20.

**Table 20. Results from the first MIP1 trial: the 4-ethylphenol content of the control and spiked wine after treatment with MIPs at various contact times**

<b>Wine type</b>	<b>4-ethylphenol level <math>\mu\text{g/L} \pm \text{SD}</math></b>	<b>4-ethylphenol removed (%)</b>
Control wine	201.8 $\pm$ 1.1	-
Control wine +MIP treatment (5 min. contact time)	189.6 $\pm$ 2.2	6.03
Control wine +MIP treatment (2h contact time)	183.4 $\pm$ 2.4	9.1
Control wine +MIP treatment (24h contact time)	180.8 $\pm$ 1.9	10.39
Spiked wine	1529.7 $\pm$ 14.1	-
Spiked wine +MIP treatment (5 min. contact time)	1485.9 $\pm$ 17.2	2.86
Spiked wine +MIP treatment (2h contact time)	1445.0 $\pm$ 14.9	5.54
Spiked wine +MIP treatment (24h contact time)	1390.1 $\pm$ 9.3	9.13

What is immediately evident is the low amount of removal of 4-ethylphenol from the wine matrix by the MIP. In the case of the spiked wine, where the initial concentration of 4-



ethylphenol was close to 1500  $\mu\text{g/L}$ , the MIP managed to remove 43.8, 84.8 and 139.6  $\mu\text{g/L}$  for 5 minutes, 2 hours and 24 hours contact times, respectively. Transformed into percentages, these correspond to removal rates of 2.86%, 5.54% and 9.13%.

It is significant to highlight that when looking at the performance of the MIP when treating the control wine, which also contained a small amount of 4-ethylphenol, the removal rates are like those for the spiked wine. In fact, when looking at the longest contact time (24 hours), at the same dose of 250 mg MIP / L wine, the removal rate of 4-ethylphenol from the control wine was 10.39%. That of the spiked wine was 9.13%, which means they are similar values. This seems to indicate that the polymer can remove a certain percentage of the initial concentration of 4-ethylphenol, quite independent of whether that initial concentration appears to be high or low.

#### 4.2 Second MIP1 Trial

The methodology and further clarifications pertaining to this experiment have been outlined in section 3.3.2.

To improve on the results of the previous trial, the MIP dosage was increased, the contact times explored were shorter and the stirring of the samples was reduced to 100 RPM.

The results obtained from this experiment are highlighted in Table 21.

The first thing to be ascertained is that the removal percentages of 4-ethylphenol are certainly higher than in the previous experiment. This can be attributed to the greatly increased polymer dosage. If the initial recommendation from the polymer manufacturer was 250 mg MIP per 1 mg 4-ethylphenol / litre, then the revised recommendation was 500 mg MIP per 100 mL of wine spiked at 1.5 mg/L. Therefore, if the MIP dose in the first experiment was 375 mg/L, here it is 5 g/L, an approximately 13-fold increase.

This increase of the MIP dosage did find itself reflected in the removal percentage of 4-ethylphenol, almost proportionally. Looking at the 2-hour contact time samples from this experiment and the previous one, with 375 mg/L MIP dosage, the removal was 5.54%, whereas with the 5 g/L dosage the removal was 52.2%, so an almost 10-fold increase.

**Table 21. Results from the second MIP1 trial: the 4-ethylphenol content of the control and spiked wine after treatment with MIP1 at various contact times**

<b>Sample</b>	<b>4-ethylphenol µg/L ± SD</b>	<b>4-ethylphenol removed (%)</b>	<b>4-ethylguaiacol µg/L ± SD</b>	<b>4-ethylguaiacol removed (%)</b>
K	351.5 ± 1.6	-	62 ± 0.4	-
K1m	203.5 ± 2.1	42.1	34.1 ± 1.2	45
S	1671 ± 17.3	-	67.8 ± 1.2	-
S1m	1112.6 ± 18.4	33.4	38.6 ± 0.9	43.1
S15m	884.8 ± 2.2	47	28.5 ± 1.5	58
S1h	799.6 ± 4.2	52.2	21.8 ± 0.7	67.8
S2h	758.9 ± 0.3	54.6	19.7 ± 0.7	71

What one notices as well is that most of the 4-ethylphenol binding happens in the first minute of contact. The longer the contact time, the less effective the binding becomes per unit of time. Therefore, looking at the binding of 4-ethylphenol from the spiked wine sample over two hours of contact time, one can notice that out of the removal percentage of 54.6%, 33.4% was achieved by the first minute, 13.6% was removed over the next 14 minutes, 5.1% was removed over the next 45 minutes, and the last 2.4% was removed in the final hour of contact time.

Until now it has been made evident that the polymer necessitates large doses (in the order of grams per litre) to be effective, but that it does not need a long contact time. Almost two thirds of the potential 4-EP binding that would happen over the course of two hours is achieved within the first minute of contact time.

Additionally, when looking at the removal rate of 4-Ethylguaiacol, one notices a very similar result to that of 4-Ethylphenol in terms of percentages removed, even if the concentration of 4-EG in wine was much smaller than that of 4-EP. This result is important in terms of evaluating selectivity of the polymer. Although 4-EG has an additional Methoxy functional group (O-CH<sub>3</sub>) and 25% more molar mass than 4-EP, they both seemed to be removed almost in identical proportion by the polymer, which speaks against its selectivity, even though 4-EG would also be a desirable compound to remove from a Brett-contaminated wine.

These results can be contrasted to those published by Teixeira et al. (2015), where a red wine with a 4-EP level of 1659  $\mu\text{g/L}$  and a 4-EG level of 150  $\mu\text{g/L}$  was treated. Those 4-EP and 4-EG levels are quite comparable to the levels in the spiked wine of this experiment. They used a polymer dose of 2 g/L, and a four-hour contact time. There the 4-EP MIP removed 55% of 4-EP and 54% of 4-EG, the 4-EG-MIP removed 55% of 4-EP and 40% of 4-EG and the NIP removed 46% of 4-EP and 43% of 4-EG.

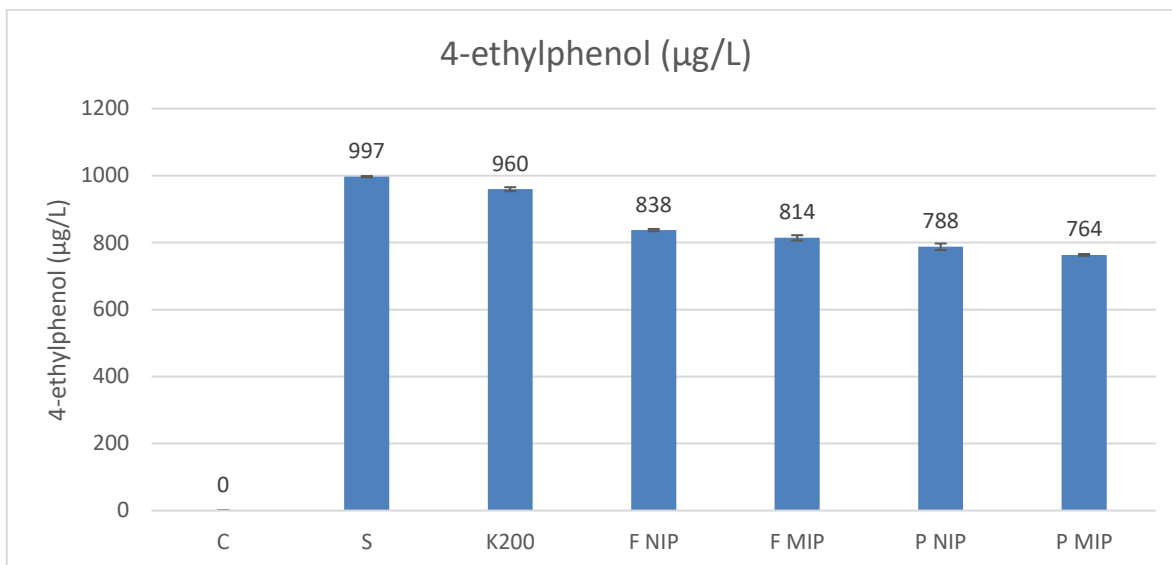
In this experiment, somewhat comparable removals of 54% 4-EP and 71% of 4-EG were reached after 2 hours of contact time, but with a larger polymer dose of 5 g/L. Since the increase in 4-EP removal follows closely a logarithmic model ( $y = 4.4652\ln(x) + 31.581$ ), one can infer that in this experiment, a 58.3% 4-EP removal would have been achieved if the contact time was extended to 4 hours, similar to this literature reference.

It is difficult to extrapolate from the 0.375 and 5 g/L polymer dosage results (which were 6 and 54% 4-EP removal respectively), what would be the result in this experiment with a 2 g/L polymer dosage, to accurately compare to the result in the cited literature. A rough estimate would be a potential removal rating between 23% and 34% 4-EP removal for the same contact time of 4 hours. This is a lower result than that obtained in the literature using 4-vinylpyridine polymers.

### **4.3 MIP1 Filtration experiment**

The methodology and further clarifications pertaining to this experiment have been outlined in section 3.3.3.

Following the results from the previous two experiments, polymer-embedded filter sheets were produced and tested. The 4-ethylphenol measurements are illustrated in Figure 32.



**Figure 32. The removal of 4-ethylphenol by imprinted and non-imprinted polymers, in powder form and embedded in filter sheets (C – control wine; S – spiked wine; F – polymers as embedded in filter sheets; P – polymers as powder)**

When looking at the 4-ethylphenol measurements, the first thing to highlight is that this is the first experiment in which non-imprinted polymers (NIPs) are used. These are chemically identical to the molecularly imprinted polymers (MIPs) but lack the imprinting step in their production process. Ideally, these polymers should show no binding to 4-ethylphenol. The larger the difference between MIPs and NIPs are, the larger the imprinting effect should be. These results show that with the NIP embedded filter sheets, the 4-Ethylphenol level decreased from  $997 \pm 1.87 \mu\text{g/L}$  to  $838 \pm 3.28 \mu\text{g/L}$ , which represents a 16% removal. The MIP embedded filter sheets decreased the 4-EP level to  $814 \pm 7.94 \mu\text{g/L}$ , which represents a 18.3% removal. Therefore, only 2.3% of the 4-EP removal could reasonably be attributed to the imprinting of the polymer.

The K200 filtration lowered the 4-EP level to  $960 \pm 5.9 \mu\text{g/L}$ , which is a 3.8% removal.

When looking at the use of polymers in powder form the results seem superior, in that the NIP powder reduced the 4-EP level to  $788 \pm 9.7 \mu\text{g/L}$  (a 21% removal) and the MIP powder reduced the 4-EP level to  $764 \pm 2.7 \mu\text{g/L}$  (a 23.4% removal). Therefore, in the case of using polymer powder pre-coated onto a cellulose nitrate membrane, the imprinting effect is 2.4%, almost identical to that from the filtration experiments. The superior 4-EP removal when

using polymer powder could be attributed to the fact that the flow rates were lower when working with the polymer powder than when working with the polymer-embedded filter sheets. The flow rate was 24.5 L/h for the standard K200 filtration, 30 L/h for the MIP and NIP embedded filter sheets and 10 L/h for the pre-coated polymer powder treatments. As this difference in flow rate corresponds to a different treatment time (3:30 minutes on average for polymer-embedded filters, 6 minutes on average for powder-coated membranes), it can be assumed, based also on the tendencies observed from the previous two experiments that the longer contact time was associated with more 4-EP binding.

The novel piece of information extracted from this experiment was that the imprinting effect only accounted for a small percentage of 4-EP binding, regardless of contact time. Much of the 4-EP binding seems to be due to the polymeric material's inherent structure.

Looking at the standard oenological parameters (Table 22), one can notice that any of the four polymer treatments hardly had any influence on the general wine composition and parameters, especially in comparison to the K200 filtration.

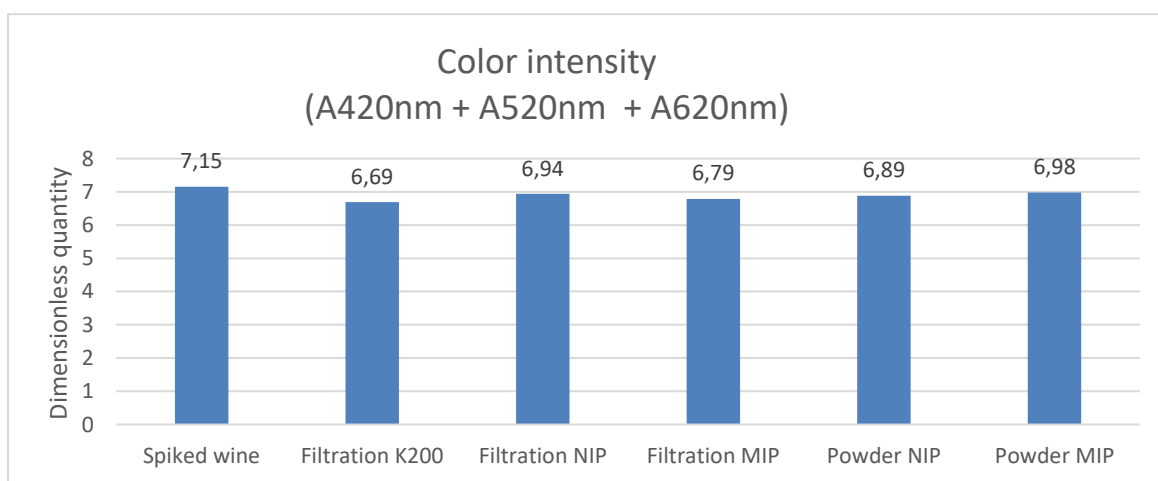
**Table 22. General oenological parameters of wines treated with polymers in either filter sheet embedded or powder formats**

<b>Wine analysis</b>	<b>Spiked wine</b>	<b>K200</b>	<b>Filtration NIP</b>	<b>Filtration MIP</b>	<b>Powder NIP</b>	<b>Powder MIP</b>
Total alcohol (% vol)	12.2	12.05	12.05	12.1	12	12.05
Total extract (g/L)	27.9	27.25	27.45	27.35	27.5	27.6
Sugar free extract (g/L)	23.3	21.95	22.35	21.75	23.05	22.8
Rest extract (g/L)	11.2	10.3	10.1	9.95	11.4	11.2
Sugar before inversion (g/L)	5.6	6.3	6.1	6.6	5.5	5.8
Sugar after inversion (g/L)	5.6	6.3	6.1	6.6	5.5	5.8
Fermentable sugar (g/L)	4.6	5.3	5.1	5.6	4.45	4.8
pH	3.5	3.45	3.4	3.4	3.4	3.4
Total acidity (g/L)	5.1	4.85	4.85	4.85	4.75	4.75
Volatile acidity (g/L)	0.43	0.51	0.43	0.49	0.44	0.44
Free sulphurous acid (mg/L)	26	21	20	21	19	19
Total sulphurous acid (mg/L)	42	41	38	36	35	34
Relative density (20/20)	0.9951	0.9951	0.9952	0.9951	0.9952	0.9952
Refraction index	42	41.4	41.45	41.45	41.5	41.6
Total phenols (mg/L)	2317 ±2	2151.75 ±8.25	2160.5 ±3	2151.75 ±20.25	2170.75 ±1.15	2185.25 ±10.25

The parameter that saw the most prominent changes was the total phenolic index, which was diminished by 7% after the K200 filtration. After the treatment with the MIP and NIP

embedded filter sheets, the total phenolic index decreased by 7.1% and 6.7% respectively. After the treatment with the MIP and NIP powders, the total phenols decreased by 5.7% and 6.3%, respectively. The fact that the reduction in total phenols was quite similar in all filtered samples, regardless of whether polymer (MIP or NIP) was present seems to indicate that the polymers in themselves do not play a central role in the absorption of undifferentiated phenolic compounds, but rather the filter media does, which is common established knowledge. This is further supported by the fact that the total phenol reduction tended to be smaller when working with the polymer in powder form whilst at the same polymer doses the treatment times were longer, amounting to an almost doubled contact time between wine and polymer.

Additionally, looking at the colour characteristics, colour intensity (Figure 33) and colour hue (Figure 33), no meaningful differences can be observed. Colour hue remains virtually identical. Colour intensity is lowest after the K200 filtration, while the treatment with the same filter sheet containing MIP and NIP seems to have a less pronounced effect. The treatment with the polymer powders decreased the colour intensity to an even lower degree than the filter-embedded polymers. Colour intensity and hue are dimensionless quantities, relevant only for comparison between each other.



**Figure 33.** Colour intensity of the samples treated with polymers in either filter-sheet embedded or powder formats

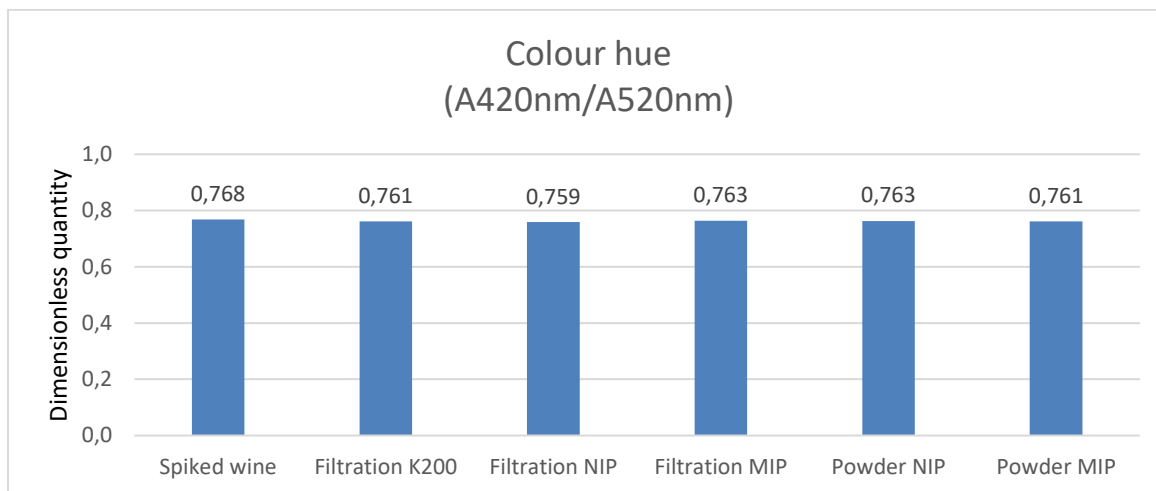


Figure 34. Colour hue of the samples treated with polymers in either filter-sheet embedded or powder formats

#### 4.4 MIP1 regeneration experiment

The methodology and further clarifications pertaining to this experiment have been outlined in section 3.3.4.

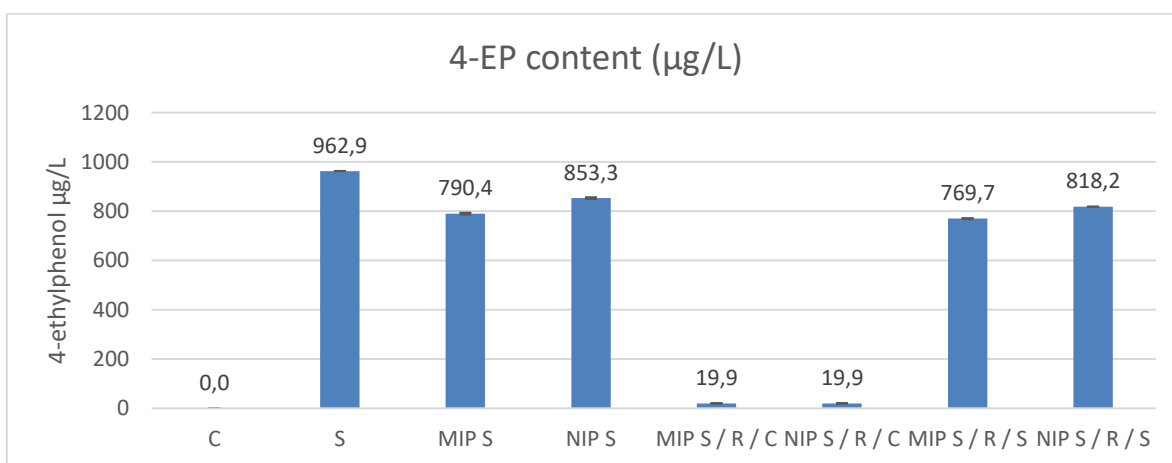
After the previous experiments, which explored only the ability of the polymers to bind 4-Ethylphenol under various circumstances, this experiment aims to take a first look at the polymer regeneration, and the ability of the polymer to release the bound 4-EP under different situations.

The results can be seen in Figure 35. When working with short contact times (sub 1 minute) the difference between the MIP and NIP treatments appears more evident, with the MIP powder reducing the 4-EP content from  $963 \pm 0.7 \mu\text{g/L}$  to  $790 \pm 3.8 \mu\text{g/L}$  (an 18% reduction) and the NIP powder reducing the 4-EP content to  $853 \pm 3.5 \mu\text{g/L}$  (an 11% reduction). In the case of such short, below 1 minute contact times, the 7% difference in removal capacity between MIP and NIP highlights the imprinting effect.

Looking at the MIP S/R/C and NIP S/R/C samples, one notices that both contain almost  $20 \mu\text{g/L}$  4-EP. To reiterate, these samples illustrate the 4-EP concentration of a clean wine which passes through the MIP and NIP powders after they have been used to treat a spiked wine and then regenerated according to the polymer producer's specifications. It is evident that there is a small amount of 4-ethylphenol leaching out of the material back into the wine.



Looking at the MIP S/R/S and NIP S/R/S samples, these illustrate what happens when, instead of a clean wine, the same kind of spiked wine is treated with the polymers after they have been used to treat a spiked wine and then regenerated. In this case, the MIP reduced the 4-EP concentration to  $770 \pm 0.4$  (a 20% reduction) and the NIP reduced it to  $818 \pm 2.5$  (a 15%) reduction. Following the regeneration step, the effectiveness of the polymers certainly maintained itself and, one could say, even slightly increased in this experiment. The difference between imprinted and non-imprinted diminished to account for only 5% of the 4-EP removal.



**Figure 35. The effectiveness of MIP and NIP treatment before and after polymer regeneration**

The next part of this experiment looked at the ability of the regeneration solution to elute the 4-ethylphenol bound by the polymer after treating spiked wine (Figure 36). Apart from the regeneration solution (which is described by the sequential 5-step process above), distilled water and control wine were also used as alternative fluids to provide a point of comparison. The idea of this comparison was to see how effective water and control wine were at removing 4-ethylphenol from the bound polymer in comparison to the designated regeneration solution, once the polymer had been used beforehand to treat spiked wine (equivalent to samples MIP S and NIP S from Figure 35). As the volume of spiked treated wine, of regeneration solution and of water and control wine used in lieu of regeneration

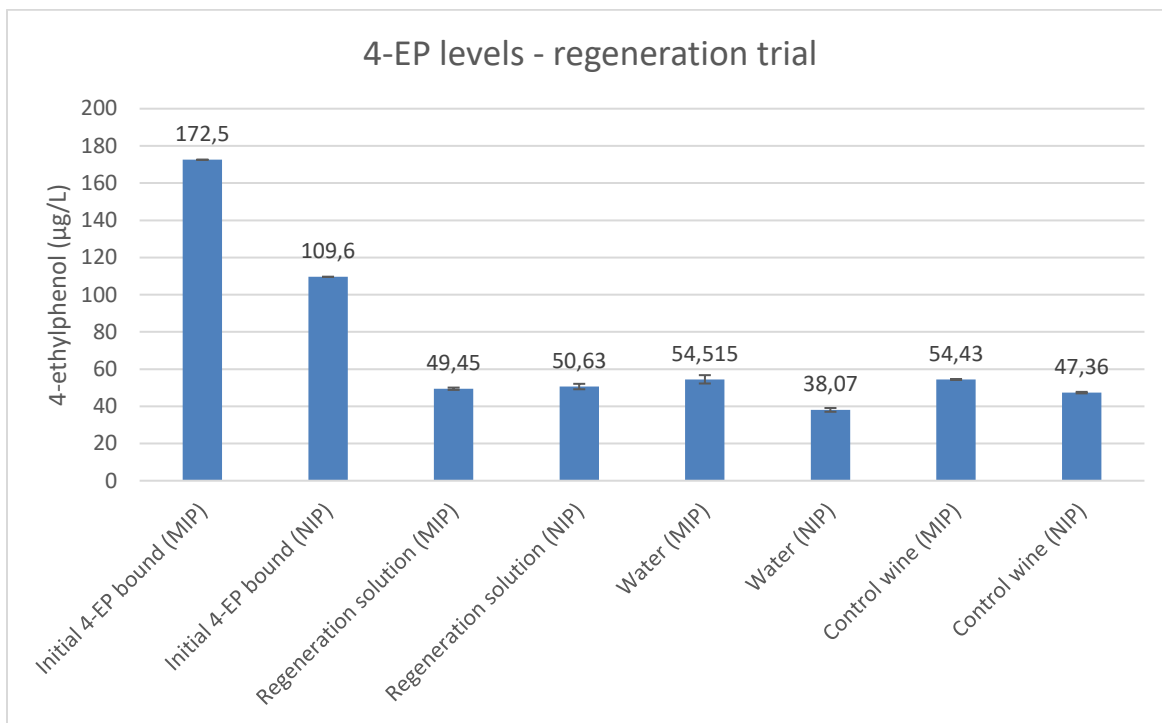
solution was always the same, a direct comparison expressed in  $\mu\text{g/L}$  4-ethylphenol remains valid and accurate throughout the experiment.

The first two samples, Initial 4-EP bound (MIP) and Initial 4-EP bound (NIP), indicate the total amount of 4-EP that can be regenerated from the polymer, as they represent the difference between the 4-EP concentration of the spiked wine before contact with the polymers and after contact with the polymers.

The first striking observation from this experiment is that the regeneration solution, the water, and the control wine all eluted approximately identical amounts of 4-ethylphenol. There is no clear difference between using either of these to elute 4-ethylphenol from the polymers.

Therefore, if one looks at the results in the case of the imprinted polymer, out of the total potential concentration of  $172.5 \mu\text{g/L}$  (which would correspond to 100% removal), the regeneration solution eluted almost  $50 \mu\text{g/L}$  4-EP (29% removal), distilled water eluted  $54.5 \mu\text{g/L}$  (32% removal) and control wine eluted  $54.4 \mu\text{g/L}$  (32% removal). In this case, all fluids eluted less than a third of the total available 4-EP, and the regeneration solution was marginally worse than the other two fluids.

In the case of the non-imprinted analogue, out of the total potential concentration of  $109.6 \mu\text{g/L}$  (corresponding to a 100% removal), the regeneration solution eluted  $50.6 \mu\text{g/L}$  (46% removal), water eluted  $38 \mu\text{g/L}$  (35% removal) and control wine eluted  $47 \mu\text{g/L}$  (43% removal). Although the percentages appear higher in the case of the NIP, it is important to remember that the total 4-EP concentration in the NIP is lower than that in the MIP, while the absolute values of 4-EP eluted are very similar across all samples.



**Figure 36.** The uptake of 4-EP of different fluids from a polymer (MIP and NIP variants) that has already been used in treating tainted wine

#### 4.5 Polymer binding experiment

In the previous experiments, several approaches were explored: polymer powder addition, polymer immobilized in a filter sheet and polymer pre-coated onto a membrane.

Initial doses of polymer were too low to obtain a strong effect, so they had to be dramatically increased to 2-5 g/L. Additionally, while longer contact times led to more 4-EP removed, the differences between the MIP and the NIP were best highlighted when the contact times were kept short, less than 1 minute.

Also, general physical-chemical characteristics (pH, alcohol, total and volatile acidity, colour intensity, nuance etc.) of wine were largely unaffected by the polymer treatment.

Following these results, the decision was made together with the polymer producer to go further with two binding experiments to determine the maximum amount of 4-ethylphenol target in a test solution that can be taken up per mg of polymer for a given contact time. These

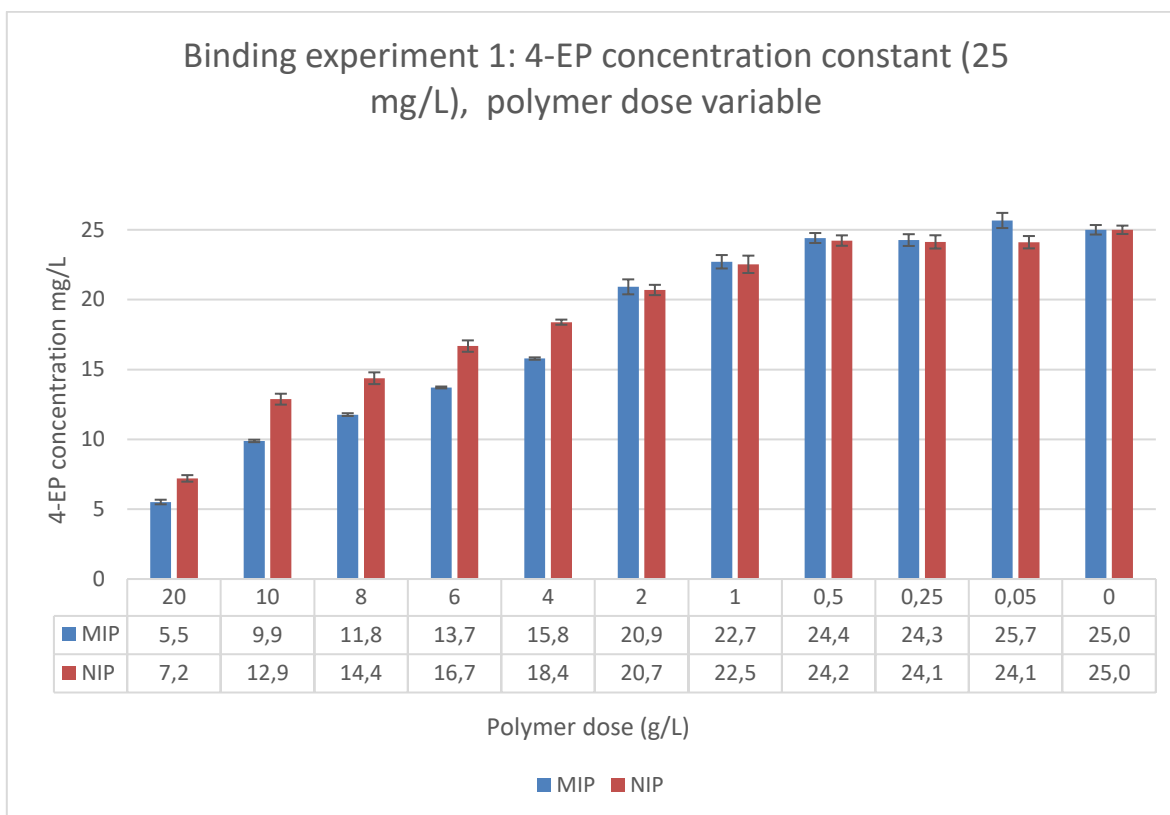
experiments would highlight the difference between imprinted and non-imprinted polymer and explore a wider range of combinations between different polymer and 4-EP concentrations.

The first binding experiment kept the 4-EP concentration constant and the polymer mass variable, and the second binding experiment kept the polymer concentration constant and the 4-EP concentration variable.

#### **4.5.1 Concentration constant, variable polymer mass experiment**

The methodology and further clarifications pertaining to this experiment have been outlined in section 3.3.5.1.

Figure 37 highlights the results of this experiment. The tendencies observed are quite clear: the larger the polymer dose, the stronger the effect. If one compares with the 0 g/L polymer dose samples, then doses of up to 1 g/L polymer (either MIP or NIP) produce small effects, approaching 10% reduction. Beginning from doses of 4 g/L and higher, the difference between the MIP and the NIP becomes noticeable, accounting for a maximum of 12% more 4-EP binding from the MIP at the 10 g/L polymer dose.



**Figure 37. Binding experiment 1: 4-EP concentration constant (25 mg/L), polymer dose variable**

By subtracting the amount of 4-EP left in the sample from the amount of 4-EP in the 0 g/L polymer sample and dividing that by grams of polymer used, one can infer how many mg 4-EP are removed per gram of polymer (Table 23). From this it can be observed that for the MIP, the efficiency per gram of material generally stays between 1 and 2.3 mg 4-EP removed per gram of polymer per litre, whereas for the NIP it is marginally lower. A removal efficiency of 1-2.3 mg 4-EP per g MIP per litre of wine would be quite useful in practical winemaking conditions, as levels of 4-ethylphenol due to *Brettanomyces* infections are to be found generally between 0.4 and 6 mg/L. However, the previous experiments seem to show that at more realistic concentrations of 4-EP, such efficiencies were not seen. These values could be the consequence of working with high doses of both 4-EP and of polymers in model solutions.

In the case of the negative value obtained from the MIP at a 0.25 g/L dose and the high positive value obtained by the NIP at the same dose, this is due to low absolute effect levels

and the fact that the 4-EP levels were identical to, if not mathematically slightly higher than those of the control (0 g/L polymer), leading to skewed results. The lower the polymer doses the greater the margin for error and the less robust the results. Based on the small effects noticeable, the accuracy regarding the amount of 4-EP removed per gram of material is not as reliable for doses of less than 1 g/L polymer than in the case of higher doses.

The next experiment, pertaining to a constant polymer dose and variable 4-EP concentration, will further explore this subject.

**Table 23. The amount of 4-EP (mg/L) removed per gram of polymer per litre**

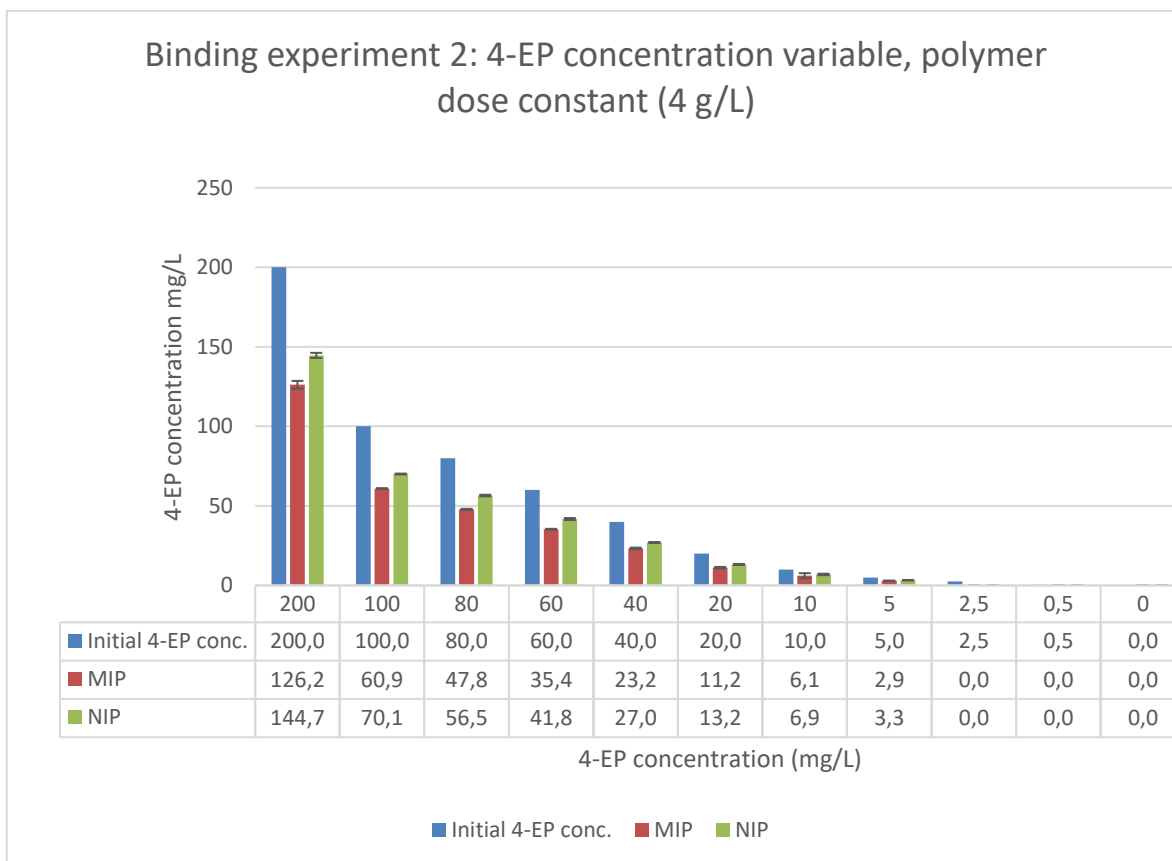
<b>mg 4-EP removed / g polymer / L wine</b>		
<b>polymer dose (g/L)</b>	<b>MIP</b>	<b>NIP</b>
20	1.0	0.9
10	1.5	1.2
8	1.7	1.3
6	1.9	1.4
4	2.3	1.7
2	2.0	2.2
1	2.3	2.5
0.5	1.2	1.5
0.25	2.9	3.5
0.05	-13.3	17.8

#### **4.5.2 Polymer mass constant, concentration variable experiment**

The methodology and further clarifications pertaining to this experiment have been outlined in section 3.3.5.2.

The results of this experiment are illustrated in Figure 38. To begin, it is important to consider that the polymer dosage was identical in all testing conditions (4 g/L). This clearly highlights how the polymer's ability to remove 4-EP is highly dependent on the initial 4-EP concentration in the solution. It manages to absorb 4-EP until it reaches an equilibrium state

with the liquid. If we compare the performance of the MIP with the 200 mg/L 4-EP solution to that of the 5 mg/L 4-EP solution, we notice how 4 g/L MIP managed to remove 74 mg/L 4-EP in the former, but only 2.1 mg/L in the latter.



**Figure 38. Binding experiment 2: 4-EP concentration variable, polymer dose constant (4 g/L)**

If one were to examine this data in terms of removal percentages (Table 24), one observes removal efficiencies of about 40% for the MIP, and about 30% for the NIP, with the imprinting effect accounting for the approximately 10% difference between the two. This means that the MIP generally removes 25% more 4-EP than the NIP, throughout this experimental setup.

When looking at the 2.5 and 0.5 mg/L 4-EP concentrations, here the doses of 4 g/L MIP and NIP seem to have equally succeeded in completely removing the 4-EP from the model solution. This interpretation is not quite valid as these values were below the limit of

detection for both HPLC detectors used in this method (FLD and DAD). If one looks again at the results from the second MIP1 experiment in Table 10, which were analysed by SBSE-GCMS (which is much more sensitive than the HPLC method at low 4-EP concentrations but has a very narrow range of detection), here the removal efficiency of the imprinted polymer was around 33.4% at a 5 g/L dose for a 1.5 mg/L 4-EP concentration in wine with 1 minute contact time, and reached 47% when the contact time was extended to 15 minutes. This data point from another experiment supports the assumption that the 100% removals seen in this experiment are due to the inability of the method to accurately measure such low concentrations well enough. Additionally, it supports the observation that given the experimental conditions of this binding trial, 40% binding for the MIP seems to be the norm, regardless of 4-EP concentration.

**Table 24. 4-EP removal efficiency (expressed as a % of initial concentration) for solutions of 4-EP of varying concentrations**

4-EP concentration (mg/L)	4-EP removal (%)		
	MIP	NIP	Imprinting effect
200	36.9	27.7	9.3
100	39.1	29.9	9.2
80	40.2	29.4	10.8
60	41.1	30.4	10.6
40	41.9	32.5	9.4
20	44.0	34.1	9.9
10	38.7	30.7	8.0
5	41.3	34.2	7.1
2.5	100.0	100.0	0.0
0.5	100.0	100.0	0.0
0	0.0	0.0	0.0

Looking again at the per gram efficiency of a 4 g/L polymer dose of both MIP and NIP in removing 4-EP, one notices very clear linear relationships between the 4-EP removal



efficiency per gram and the 4-EP concentration of the solution (Figure 39). Here, too, the imprinting effect can be seen, allowing the MIP to generally remove 25% more than the NIP.

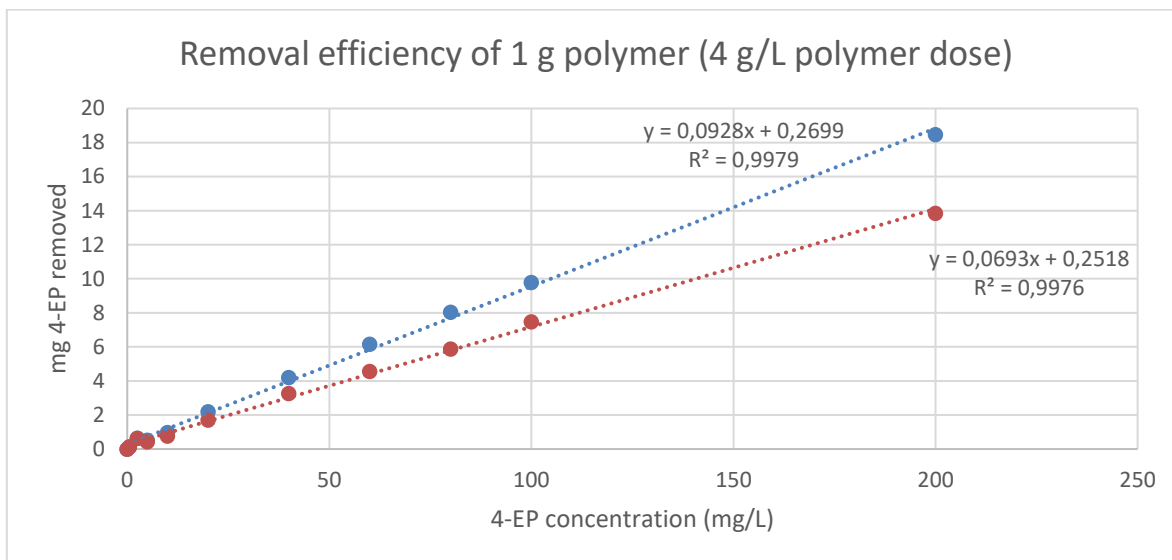


Figure 39. The removal efficiency of 1 g imprinted (blue) and non-imprinted (red) polymer (at 4 g/L dosage)

#### 4.6 Characterization of polymer binding behaviour

The results from the previous experiment highlighted that the removal effect of the polymers is proportional to their dosage, that at any given dosage and contact time they remove a certain percentage of the initial 4-EP concentration, regardless of how high or low the concentration is, and that the imprinting effect is noticeable particularly at higher polymer doses and at higher 4-EP concentrations.

With the aim to further understand the binding behaviour of the polymer, the decision was made to conduct a series of experiments at the laboratory of the polymer producer (Ligar Polymers), in Hamilton, New Zealand, in February-March 2017.

In the meantime, a second generation of molecularly imprinted polymer had been developed by the manufacturer, incorporating feedback from the results of the previous experiments into the design of a new variant (named henceforth MIP2, and its non-imprinted analogue NIP2). This second generation was tested together with the first generation of polymer.

#### 4.6.1 Contact time effect

The methodology and further clarifications pertaining to this experiment have been outlined in section 3.3.6.1.

The aim of this experiment has been to establish the optimal contact time between liquid and polymer for the uptake of 4-EP, and to establish how does the contact time influence the difference between selective binding (attributable to the molecularly imprinted cavities in the polymer structure) and non-selective binding.

The results from the experiments working with dry polymer can be seen in Figure 40.

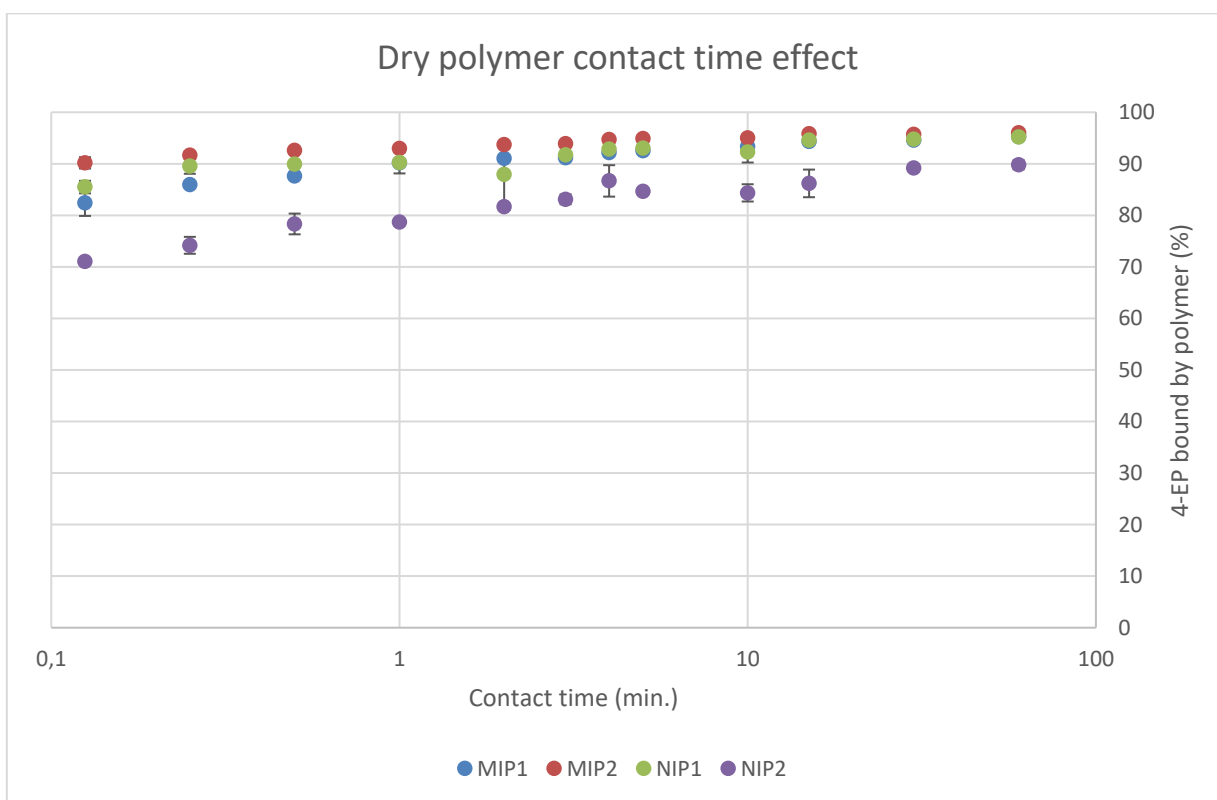


Figure 40. Dry polymer experiment, contact time effect

When comparing the two imprinted polymers (MIP1 in blue, MIP2 in red), one can see that the MIP2 consistently removes more 4-EP than MIP1 in contact times lower than 1 minute. When comparing the imprinted polymers to their non-imprinted analogues, NIP1 and MIP1 seem to be performing almost identically, in several examples being virtually superimposed on one another, So here the imprinting effect is effectively absent. In contrast, the difference

between MIP2 and NIP2 is considerable and quite consistent throughout the whole range of contact times.

What is also evident is that MIP2 achieves 90% of binding within the first 7 seconds of contact time. After 60 minutes of contact, the binding increases only by an extra 5%: This further supports the idea that the relevant 4-EP binding effect is happening within the first few seconds of contact. Additionally, longer contact times are not particularly desirable, as they allow the non-imprinted analogue to bind more 4-EP, and slowly catch up to the MIP. Considering that the NIP binds non-selectively, any increase in contact time detracts from polymer selectivity, allowing for non-specific binding of potentially positive flavour or aroma compounds, when used in a wine matrix.

Looking at Figure 41, pertaining to the same experiment carried out with pre-wetted polymers, the general tendencies are the same as when working with the dry polymer. MIP2 has the best 4-EP binding immediately. Extended contact time does not improve this significantly, but only allows the other polymers to catch up. In wet condition, NIP1 was binding more than MIP1 at small contact times (7 and 15 seconds), but afterwards they become equal. The NIP2 is consistently binding less than MIP2, although the difference gets smaller with increased contact time. The main conclusions one can draw from this experimental series is that the second-generation polymer (MIP2) exhibited a better performance than the first generation, with consistent 4-EP binding of more than 90% within the first 7 seconds, and with the lower non-specific binding attributable to its non-imprinted analogue (NIP2). No meaningful differences were noticeable between working with the polymers in the dry state or the wet state. The decision was made, therefore, to continue the experiments with MIP2 and NIP2 and only at the shortest contact times of 7 seconds, which allow the polymer to perform at its best (high 4-EP binding, lowest non-specific binding).

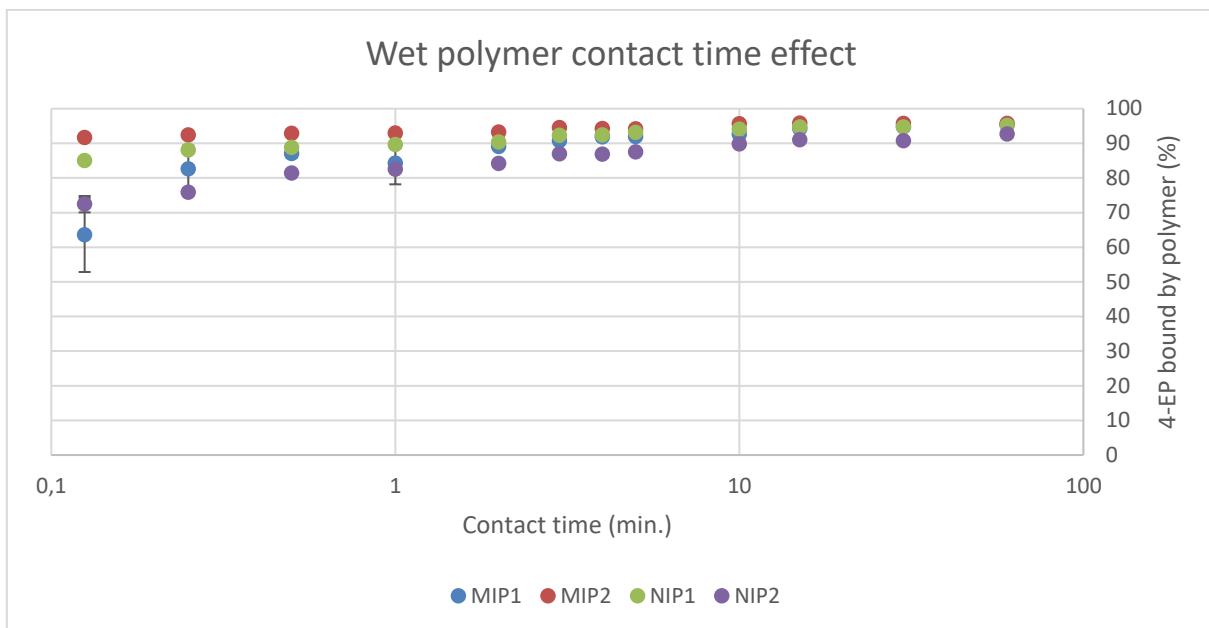


Figure 41. Wet polymer experiment, contact time effect

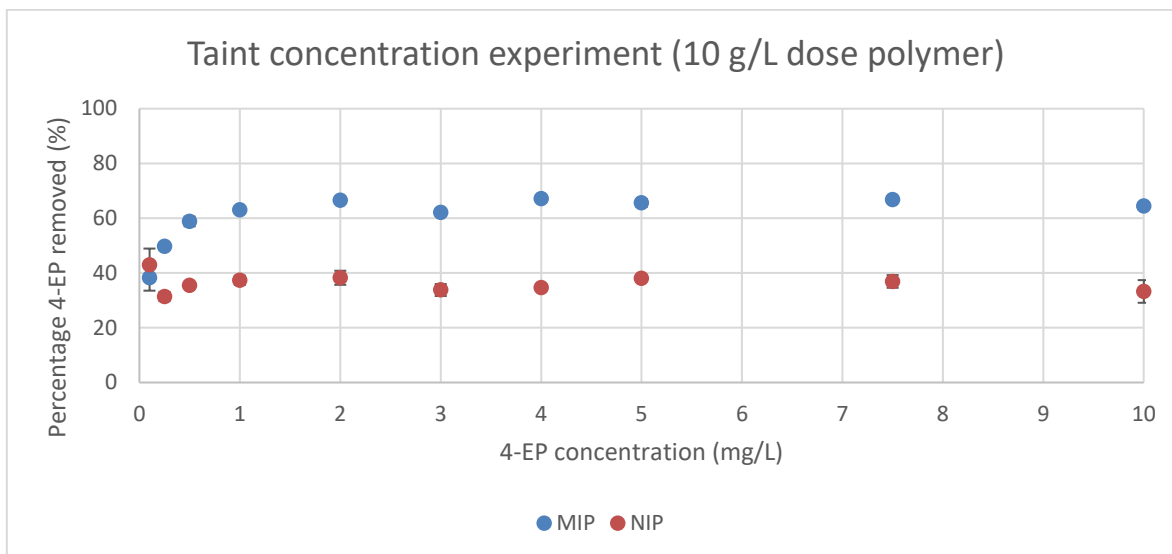
#### 4.6.2 4-ethylphenol concentration effect

The methodology and further clarifications pertaining to this experiment have been outlined in section 3.3.6.2.

Following the results of the previous experiment, the shortest time possible (7 seconds) and the wet polymer state were selected as optimal, as shorter contact times allowed the biggest difference between MIPs and NIPs in terms of 4-EP uptake and working in wet polymer condition allowed for a more practical and accurate way of handling and dosing of the polymer.

Also, because MIP2 was more successful than MIP1 in removing 4-EP at shorter contact times, and because NIP2 removed less 4-EP than NIP1 at these contact times, MIP2 and its corresponding analogue NIP2 were selected to be used in further experiments.

The aim of this experiment is to establish how does a given amount of polymer remove 4-EP depending on the initial concentration of 4-EP.



**Figure 42.** Taint concentration experiment using a polymer dose equivalent to 10 g/L

Looking at the results from Figure 42, it can be clearly observed that the removal of 4-EP tends to be a fixed percentage of its initial concentration, irrespective of the ratio between polymer concentration and 4-EP concentration. This suggests that the binding might be limited by diffusion. This could be improved by increasing the contact time, but this would reduce specificity and diminish the difference between MIP and NIP performance.

Except for the situations with low doses of 4-EP (0.1 and 0.25 mg/L), the MIP consistently removed over 60% of the phenol, whereas the NIP consistently removed less than 40% of the phenol. This experiment shows a clear example of the imprinting effect, accounting for the approximately 20% difference in 4-EP binding between the two polymer variants.

These results lend themselves well to a comparison of results obtained by Teixeira et al. (2015) in their rebinding study using 4-vinylpyridine based MIPs for removing volatile phenols and relying on the SPE method. This method involves a simple passthrough of wine through the polymer and is quite comparable to the method applied here. In both cases, the contact times are kept as short as possible.

Specifically, they highlight reductions of 29-59% of both 4-EP and 4-EG when working with 2g/L MIP dosages and 2,5 mg/L volatile phenol concentrations in test solution. They also highlight 50-60% reductions of both 4-EP and 4-EG when working with 8g/L MIP dosages

and 10 mg/L volatile phenol concentrations in test solution. It is not specified whether the 4-EP MIP or 4-EG MIP is used. No NIP was used in this testing context.

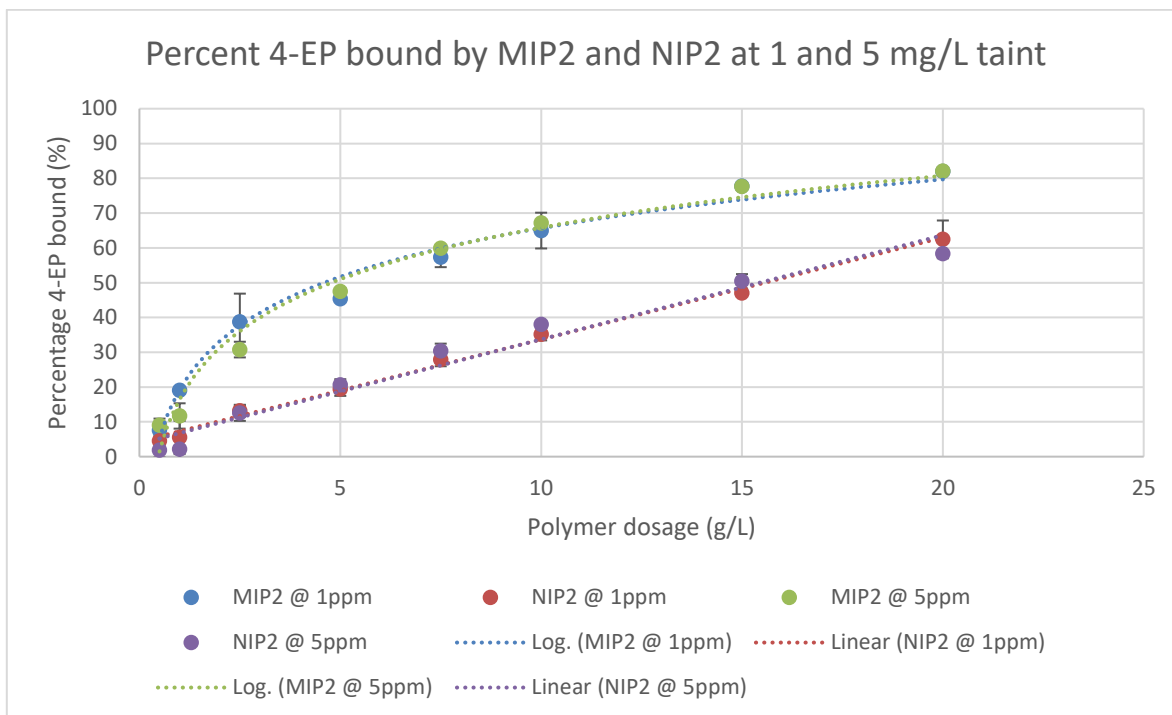
In this experiment, a removal rate of above 60% was obtained in all cases where the 4-EP concentration was above 1 mg/L. The polymer dose in this experiment was higher than that used in this literature reference. Perhaps the most striking difference is the smaller variability in results seen with this polymer, when compared to the wider ranges for removal percentages reported by Teixeira et al. (2015).

#### **4.6.3 Polymer dose effect**

The methodology and further clarifications pertaining to this experiment has been outlined in section 3.3.6.3.

Following the results of the previous experiment, which highlighted that regardless of the 4-EP concentration, the same amount of MIP2 removed between 60% and 65% of the 4-EP whereas the NIP2 removed between 30% and 35% of the 4-EP, two 4-EP concentrations were explored further in greater detail: 1 mg/L 4-EP (corresponding to a medium-intensity taint) and 5 mg/L (corresponding to a very strong taint).

The aims of this experiment were to determine what doses of polymer are most effective in achieving a 4-EP reduction, and when can the polymer dosage become redundantly high, and how low of a dose can one use and still obtain a noticeable effect.



**Figure 43. Polymer concentration experiment at two 4-EP dosages, corresponding to an average and a high taint**

The results from this experiment (Figure 43) corroborate the ones from the previous one, in that, for any given polymer concentration the amount of 4-EP removed is shown to be a fixed percentage of the initial 4-EP concentration. Adding more imprinted polymer leads to more 4-EP removed, but in going beyond 7 mg/mL one encounters diminishing returns, as the percentage removal of the NIP increases more than that of the MIP beyond this point. These results further highlight that diffusion is limiting the binding of 4-EP.

Another important finding from this experiment is that the increase in 4-EP percentage binding associated by increasing the dose of polymer is linear in the case of the non-imprinted polymers but is logarithmic in the case of the imprinted polymers (Table 25). This highlights the effect of the molecular imprinting step, leading to a superior and preferential binding of 4-EP by the MIP, and that the rate of 4-EP binding is accelerated by increasing the MIP dosage, up to a point.

**Table 25. Best fit regression models for polymer binding characteristics**

Experimental condition	Regression model	R <sup>2</sup>	Formula
MIP2 @ 1 ppm	Logarithmic	0.985	$y = 20.198\ln(x) + 19.164$
MIP2 @ 5 ppm	Logarithmic	0.9767	$y = 21.477\ln(x) + 16.375$
NIP2 @ 1 ppm	Linear	0.9953	$y = 2.9309x + 4.3582$
NIP2 @ 5 ppm	Linear	0.9669	$y = 2.9947x + 3.7577$

If one were to plot the two models for the MIP and the NIP at the 1 mg/L 4-EP concentration (Figure 44), one can notice that at a certain point the performance of the MIP and of the NIP will become identical. The model suggests that at doses higher than 28 g/L, the imprinting effect will be completely nullified. Additionally, one can see that the imprinting effect rapidly increases when one increases the polymer dosage and peaks at being responsible for about 33%, when the polymer dose is 7 g/L (Figure 45). The imprinting effect decreases for polymer doses larger than 7 g/L.

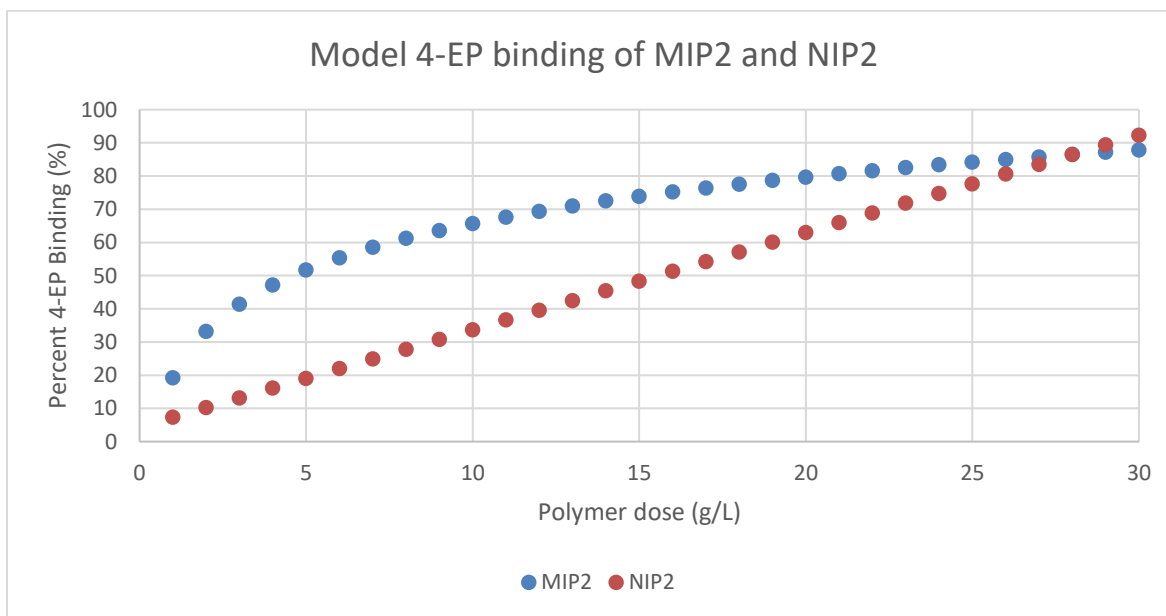
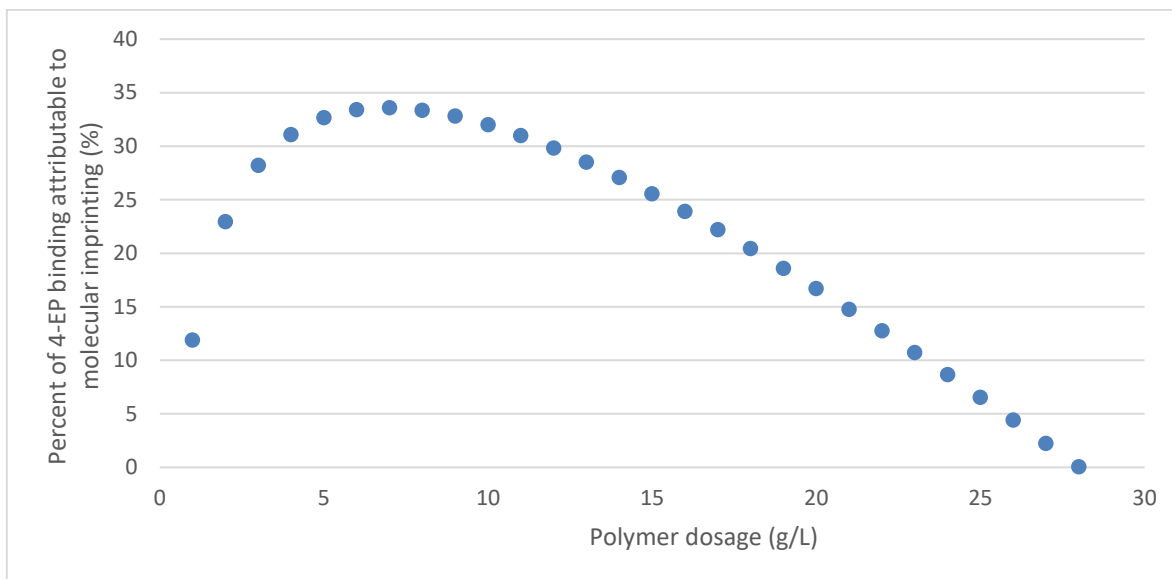


Figure 44. Model 4-EP binding of MIP2 and NIP2





**Figure 45. Percentage of 4-EP binding attributable to the effect of molecular imprinting**

Looking at the data obtained so far, one can initially conclude that for removing 4-ethylphenol from a model solution using a MIP which maintains its maximum possible specificity, that contact times should be kept as short as possible (7 seconds) and that polymer doses should not exceed 7 g/L.

Because these results highlight that, given a particular concentration of MIP/NIP in this experiment, one can expect a certain percentage removal that is rather independent of the starting 4-EP concentration. As can be seen above in figure 43, the removal percentages are the same, whether one has a 1mg/L taint or a 5 mg/L taint. This means it is possible to extrapolate based on the regression models in table 25 to compare with the results obtained by Teixeira et al (2015).

Specifically, at 2 g/L polymer dose of MIP2, it would be responsible for 33% 4-EP removal and at 10 g/L polymer dose of MIP2, it would be responsible for 66% 4-EP removal.

Teixeira et al (2015) report 29-59% removal at 2 g/L 4-vinylpyridine polymer dosage and 51-60% removal at 8 g/L 4-vinylpyridine polymer dosage. In this regard, one can say that these two types of polymers have a similar performance.

#### 4.6.4 Polymer elution behaviour

The methodology and further clarifications pertaining to this experiment have been outlined in section 3.3.6.4.

Next, the focus was shifted to the behaviour of the polymers when regenerated. The aim of this study was to establish the optimal regeneration procedure for the polymer.

For the MIP treatment, 75.4% of the total 4-EP content of the test solution (250  $\mu\text{g}$ ) was successfully bound by the polymer, out of which 40% could be successfully eluted with the polymer regeneration step, while the remaining 35% could not be eluted and remained in the polymer.

In the case of the NIP treatment, it managed to bind 99.4% of the total 4-EP content, out of which only 23% could be eluted by the regeneration step, while the remaining 76% was still bound in the polymer. These initial results highlight the fact that when working with this syringe filtration setup, the risk of channelling can lead to skewed results, leading to the necessity of both a more accurate testing method and increased repetitions, to get a clearer sense of the margin for error.

The exact behaviour of the two polymers is best contrasted when looking at what happens throughout all the binding and elution stages, and this finer resolution view is seen in Figure 46. On the Y axis, one can see the amount of 4-EP (expressed in  $\mu\text{g}$ ) that elutes downstream of the polymer bed (either MIP, represented by the blue line, or NIP represented by the orange line). On the X axis, one has a visual representation of the progression of the experiment: the first stage, highlighted in grey, is the binding stage when 10 mL of a test solution (25 mg/L 4-EP in 15% vol. ethanol) passes through the polymer bed, either MIP or NIP. Therefore, the precise amount of 4-EP present in each of these 10 mL of test solution is 25  $\mu\text{g}$ . The amount of 4-EP present downstream of the contact between test solution and polymer bed is shown, therefore, on the graph. When the value is at or close to zero, this means that for that particular millilitre all the 4-EP present in the test solution was bound by the polymer. When the value starts to increase (such as in the case of the MIP) this means that the polymer did not completely bind all the 4-EP in the test solution, and it begins to elute downstream.

The next five stages represent the stages of the polymer regeneration, where first distilled water is used to rinse (5 mL, first section highlighted in blue), then 10 mL of NaOH 2M is used to elute the 4-EP (10 mL, section highlighted in green), then a second distilled water rinse (10 mL, highlighted in blue), then a citric acid 2M rinse, to lower the pH level and bring the polymer into an optimal state for future 4-EP binding (5 mL, highlighted in yellow), and, finally, the third and last water rinse (5 mL, highlighted in blue). Any non-zero amount of 4-EP present in any of these samples on the graph signifies the elution of the 4-EP from the polymer bed into the various liquids that are involved in the regeneration process.

The dots on the lines represent each individual mL of test solution and afterwards regeneration fluids passing through the polymer bed.

The first thing to notice, when looking at Figure 44, is that the 4-EP was almost completely bound by the NIP but only about 75% bound by the MIP. The first 2 mL of test solution are eluting without any 4-EP from the MIP, but from the 3<sup>rd</sup> mL onward, more and more 4-EP is gradually eluting downstream through the polymer. The last mL of test solution came out with a little over 15  $\mu\text{g}$  4-EP out of its initial 25  $\mu\text{g}$ , indicating that polymer binding was still happening.

Moving further, looking at the regeneration steps, one sees a slight elution of 4-EP into the first pass of cleaning water from the MIP but none from the NIP. Moving on at the NaOH solution (2M) one sees an immediate large release of 4-EP from the MIP into the liquid, and then a gradual falloff throughout the whole 10 mL of alkaline rinse. The NIP, on the other hand, did not exhibit this behaviour. It tended to release a lower amount of 4-EP, with an increasing tendency towards the end of the 10 mL volume of alkaline solution. The overall release of 4-EP from the NIP was much lower than that of the MIP.

Moving on to the second water rinse, there is a slight elution of 4-EP in the first half of the 10 mL water volume, and levels at zero from this point on until the end of the whole regeneration process. The MIP releases no more 4-EP either during the citric acid rinse, or during the final water rinse. The NIP, on the other hand released some 4-EP during the second water rinse, with a small spike at its end. Afterward it did not release any more 4-EP during the citric acid rinse or the final water rinse.

Overall, one can say from these results that the elution from the NIP seems to be less effective and more irregular than that of the MIP.

Although time and labour-intensive, this kind of experiment can be refined and expanded upon as it potentially can provide a detailed view into the effectiveness of polymer binding and regeneration.

By way of contrast, the polymer elution used by Garde-Cerdán et al. (2008) relied on methanol and acetic acid solutions at 99:1% and 90:10%, v/v concentrations to accomplish a 98.9% and 53.4% reduction of 4-EP respectively. One can note how the increase in acetic acid concentration improved the elution of 4-EP dramatically. Unfortunately, both methanol and acetic acid are unreasonable eluting agents to be used in any practical application in the wine industry, which is why this work focused on the sequential rinse with citric acid and sodium hydroxide, both common, effective and inexpensive cleaning substances used in the industry.

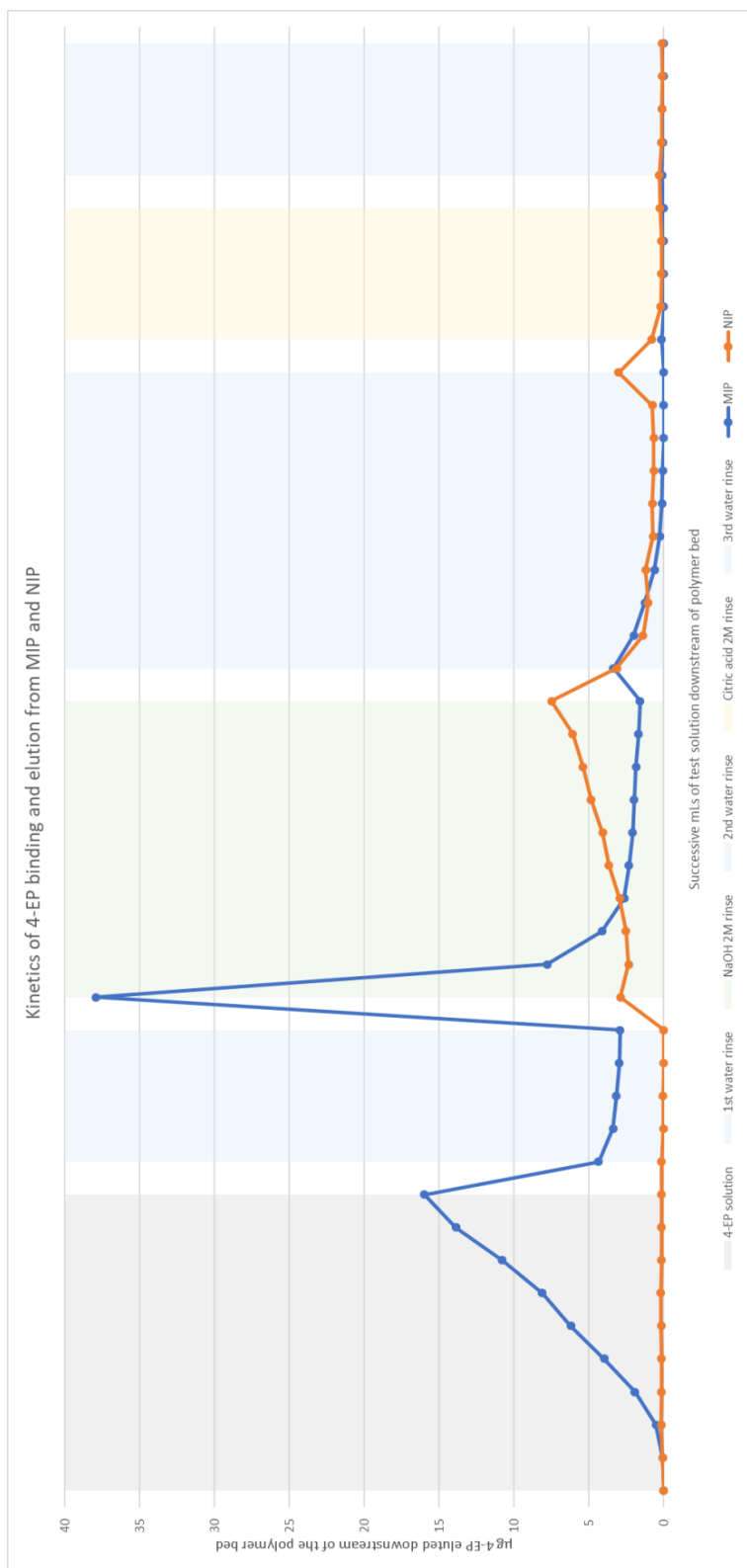


Figure 46. Kinetics of the 4-EP binding from MIP and NIP across the entire binding and regeneration process

#### 4.6.5 Particle size effect

The methodology and further clarifications pertaining to this experiment have been outlined in section 3.3.6.5.

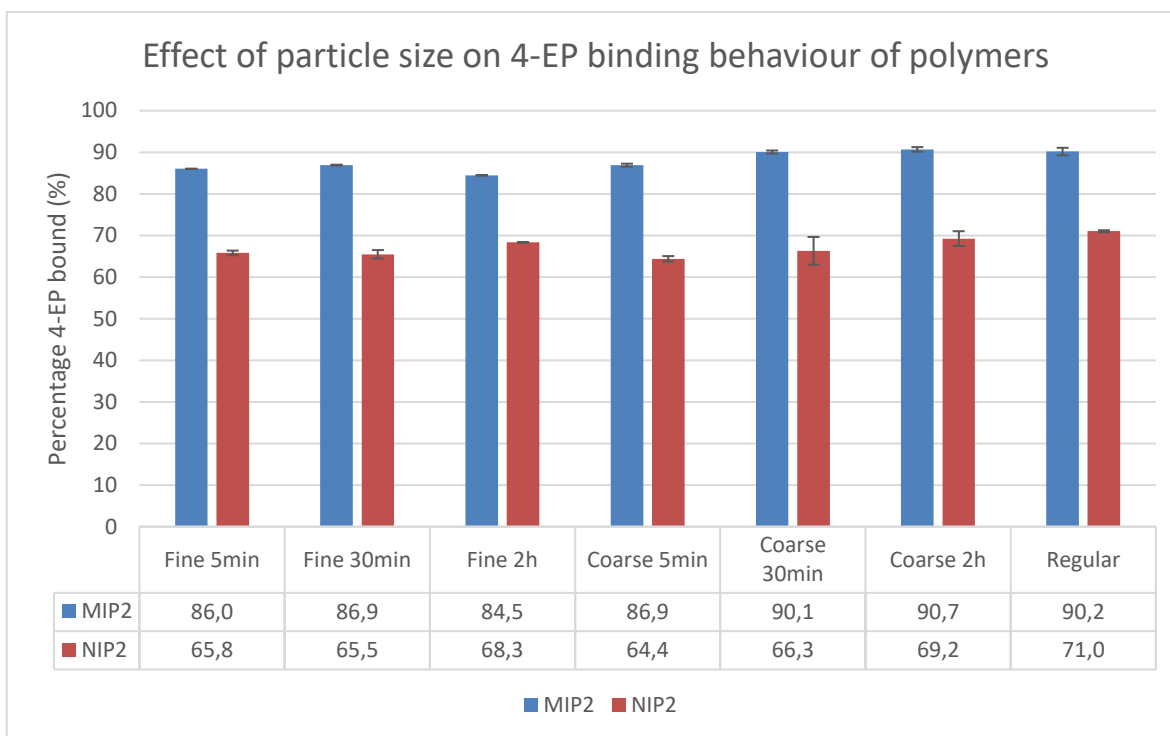
A factor possibly influencing polymer binding capacity can be the particle size of the ground polymer powder. One simple preliminary way to test this possibility was to design a simple experiment, based on separation of particle sizes based on their different speed of sedimentation.

The results of this experiment are highlighted in Figure 47. Overall, one notices little difference in 4-EP binding between the different “Fine” fractions of polymer, regardless of whether it was imprinted or not. The “Coarse” fractions seem to achieve a higher binding than the “Fine” fractions, around 90% in the case of the MIP, and identical to the binding achieved by the regular MIP.

The binding behaviour of the NIP was consistently lower than that of the MIP and is representative of the results seen before in section 4.6.1.

Overall, as the finer fractions of MIP seem to show a very slight decrease in 4-EP binding, the general impression was that this experiment did not reveal any clear advantages in terms of 4-EP binding stemming from separation the polymer according to particle size differences achievable through sedimentation speed.

Additional, more precise work would be beneficial in this direction, to provide a more accurate perspective. Ideally, one would be able to create specific well-defined fractions of polymer according to the real particle size distribution and compare them in terms of 4-EP binding.

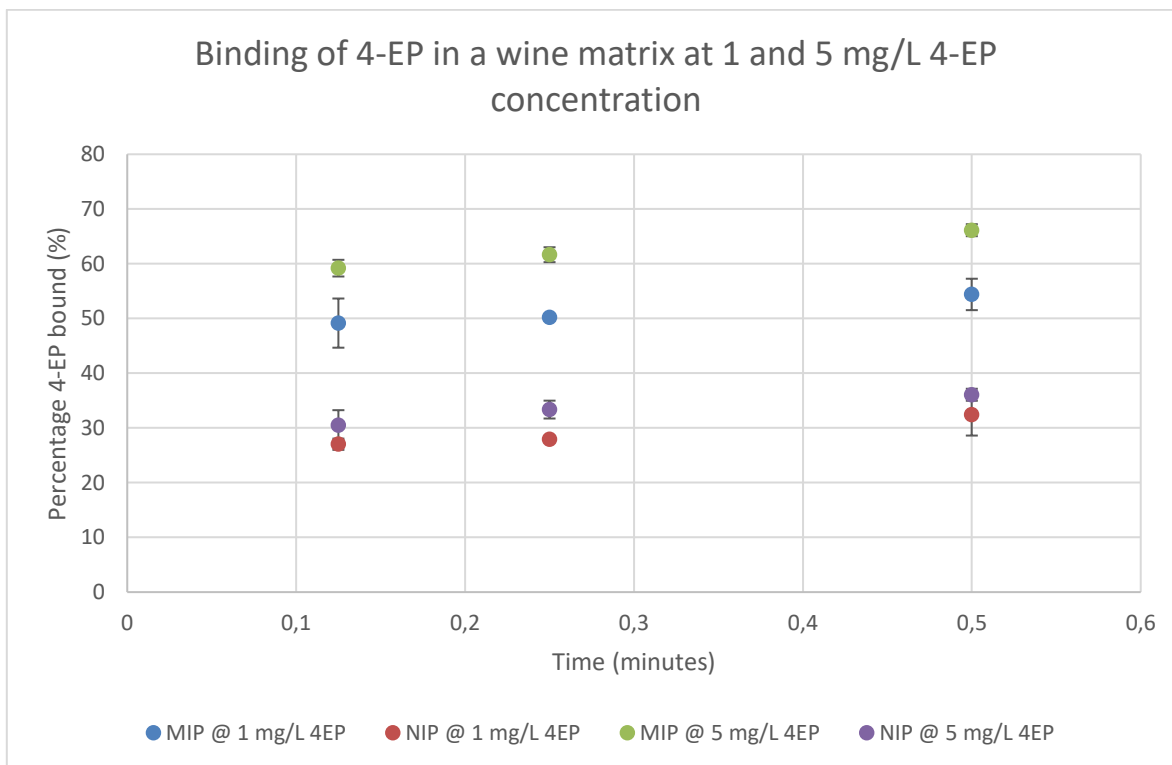


**Figure 47. Effect of particle size on 4-EP binding behaviour of polymers**

#### 4.6.6 Lab scale wine trial

The methodology and further clarifications pertaining to this experiment have been outlined in section 3.3.6.6.

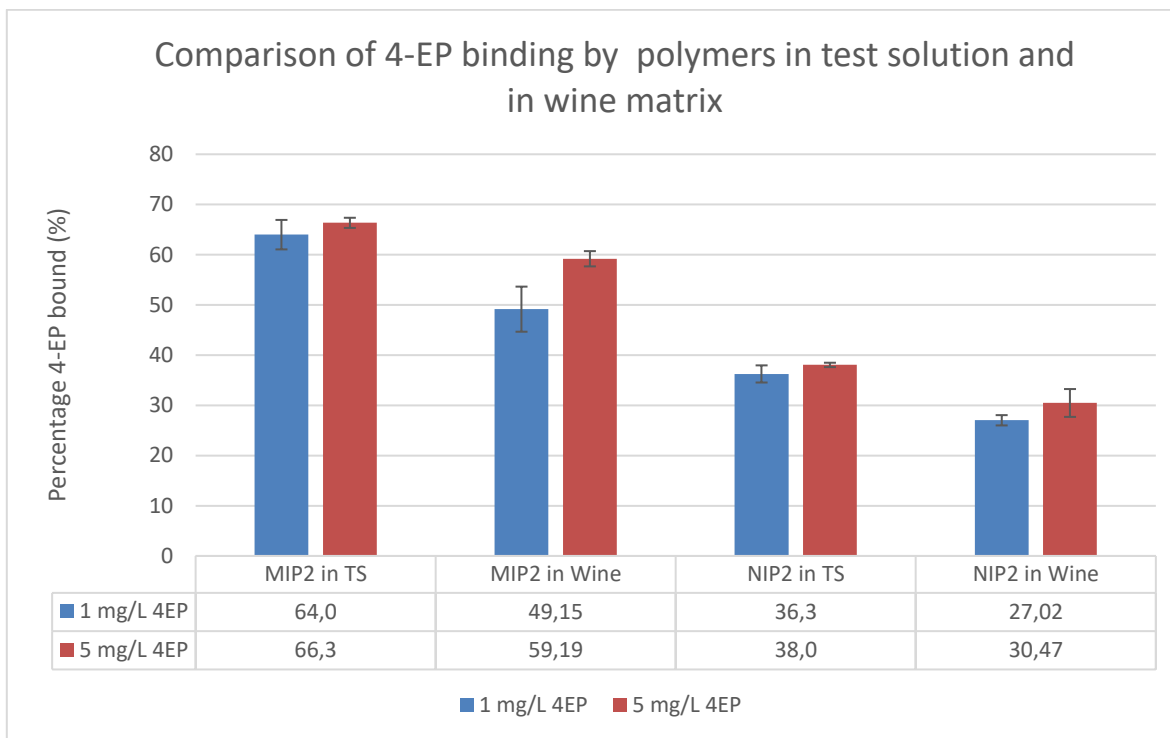
After collecting all the previous information from working with model solution, the decision was made to switch to working in a real wine matrix and observe what happens to the specific and non-specific binding of the polymers in this context.



**Figure 48.** The binding of 4-EP in a red wine matrix at 1 and 5 mg/L 4-EP concentration

Looking at the results displayed in Figure 48, one sees the results from this experiment in the wine matrix only. The trend for increased 4-EP binding with increased contact time is discernible, identical to the case of the experiments in section 4.6.1, which was expected. What is also clearly discernible is the slightly inferior 4-EP binding occurring at the 1 mg/L 4-EP concentration, throughout all three explored contact times. If one looks back at Figures 42 and 43, one notices that at the 10 mg/L polymer dose, at concentrations of either 1 or 5 mg/L 4-EP in 15% EtOH test solution that the binding is around the 63-65% mark. This is indeed the case in the wine matrix for the 5 mg/L 4-EP taint concentration, but the 1 mg/L 4-EP concentration, the binding is 10% lower. To illustrate this difference, in Figure 49 one can see the test results for both MIP and NIP, at 1 and 5 mg/L 4-EP spiking and 7 seconds contact time in test solution (average values from the results in sections 4.6.2 and 4.6.3) and wine matrix.





**Figure 49. Comparison of 4-EP binding by polymers in test solution and in wine matrix**

For both the MIP and the NIP, the switch from test solution to wine matrix is associated with a decrease in polymer binding. The most pronounced difference can be seen in the case of the MIP at the 1 mg/L 4-EP taint concentration.

The effects of the molecular imprinting and of the wine matrix are illustrated in Table 26. Looking at the imprinting effect (which refers to the difference in percentage bound between the MIP and the NIP), one sees that it stays at around 27-28% in both wine and test solution matrixes when using high 4-EP concentrations. When using a lower-to moderate 4-EP concentration, the imprinting effect stays the same in test solution, but it diminishes in wine, accounting for about 22% more binding. This slight drop in effectiveness of the MIP related to its non-imprinted homologue has been observed in the experiment described in section 4.5.2. The imprinting effect there tended to decrease when the 4-EP concentration decreased from 20 mg/L to 10 and lower. This is unfortunate, as levels of 4-EP in tainted wines are generally much lower, between 0.5 and up to 6 mg/L. These results indicate that MIPs are more effective when removing higher concentrations of 4-EP than in the case of lower ones.

In practice, if one were to treat a wine, these results indicate that it is more desirable to treat a highly tainted wine first, and then blend it with another less tainted or non-tainted wine, than to blend first and treat the mixture afterwards.

The effect of switching to a wine matrix led to a lowering of the 4-EP binding performance of both polymers, observable at both 4-EP concentrations. The effect was more pronounced when working with lower 4-EP concentrations, and most pronounced when working with the MIP, in the case of which the wine matrix accounted for an almost 15% drop in polymer binding performance at 1 mg/L 4-EP taint levels, and a 7.2% drop in polymer binding performance at 5 mg/L 4-EP taint levels.

These results highlight that lowering the 4-EP concentration (to more realistic levels) and working in a wine matrix both make it more challenging for the MIP to remove 4-EP as effectively as it was able to do in test solutions and at high polymer doses and taint concentrations. This switch also lowers its advantage over the NIP to a lower but still noticeable degree.

**Table 26. The effects of molecular imprinting and of the wine matrix on the 4-EP binding characteristics of the polymers**

<b>Percentage of 4-EP Binding by polymers</b>				
<b>Taint concentration</b>	<b>Imprinting effect</b>		<b>Wine matrix effect</b>	
	<b>TS</b>	<b>Wine</b>	<b>MIP</b>	<b>NIP</b>
1 mg/L 4-EP	+27.7%	+22.1%	-14.8%	-9.2%
5 mg/L 4-EP	+28.3%	+28.7%	-7.2%	-7.6%

Garde-Cerdán et al. (2008) have compared the performance of 4-vinylpyridine MIPs to the non-imprinted analogues in wine matrix as well. With a 4 g/L polymer dose and a pass-through contact time equivalent, they have observed an improvement in MIP binding from 55% using the NIP to 92.3% using the MIP that used pentachlorophenol as a template.

The current work shows that at comparatively larger polymer dosages (10 g/L) using MIP2 and NIP2, the 4-ethylphenol binding was around 50-60% for the imprinted polymer and 27-30% for the non-imprinted polymer. Similar to the results from the cited literature reference,

there is a clear difference in binding between the imprinted and non-imprinted variants. The polymers studied in this work show poorer binding than the 4-vinylpyridine ones explored by Garde-Cerdán et al. (2008). The advantage of the polymers used in this work is that they are created using non-toxic monomers.

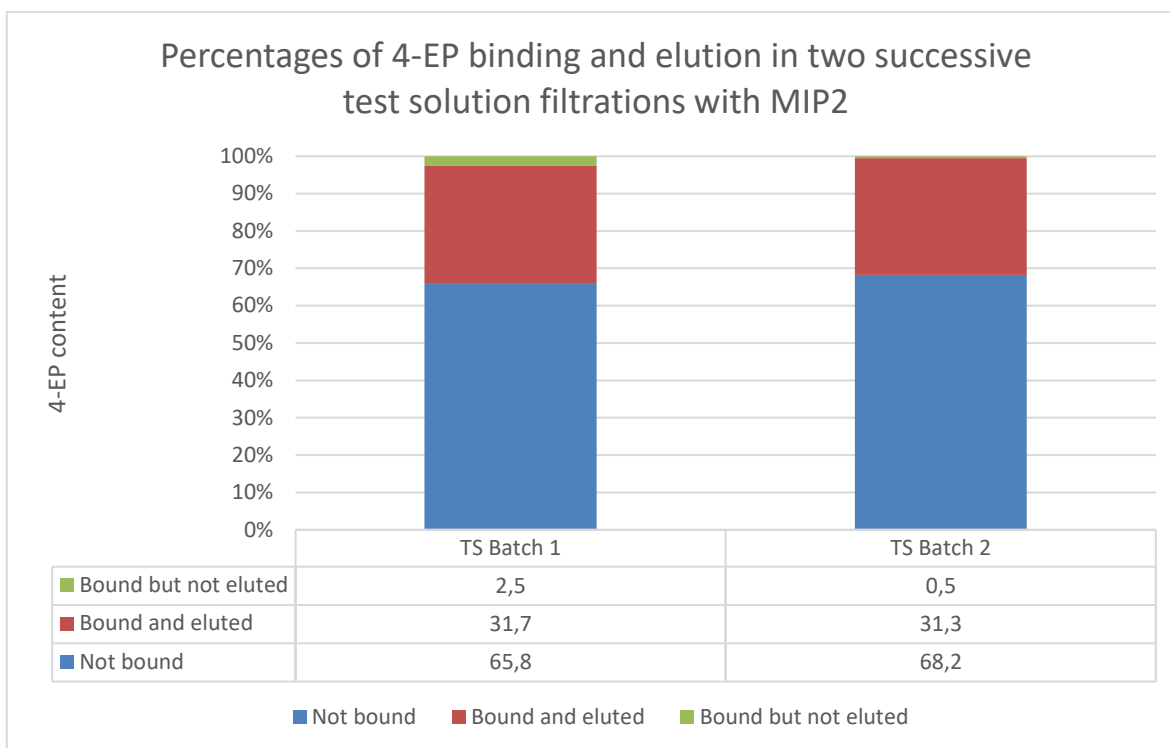
Additionally, while the 4-EP binding in case of either high (5 mg/L) or low (1 mg/L) taint concentrations was the same in this experiment, the cited literature reference discussed here does not disclose the true 4-ethylphenol concentration of the wine sample in the article. They indicate the removal as a percentage of the total.

#### **4.6.7 Test solution filtration**

The methodology and further clarifications pertaining to this experiment have been outlined in section 3.3.6.7.

Having observed the way polymer binding occurs when working with small (1 mL) volumes of liquid in centrifugal tubes, the interest naturally shifted to investigating what happens with the specific and non-specific binding when scaling up to working with 10 L batches of test solution filtered through a plate and frame filter. As the polymer can be regenerated and re-used, two successive treatment and regeneration cycles were performed to get an initial assessment of the continued functionality of the polymer.

The result of this experiment can be seen in Figure 50. TS Batch 1 represents the first batch of 10 L test solution filtered by the (yet) unused polymer. TS Batch 2 represents the second batch of test solution, which was filtered after the polymer has been regenerated, following the treatment of batch 1. Therefore, one can look at differences between batches 1 and 2 for signs of loss of polymer effectiveness.



**Figure 50.** The percentage binding and elution of 4-EP in two successive test solution (1 mg/L 4-EP, 15% EtOH) filtrations with MIP2

The differences between the two batches are, however, minimal. The first batch had its 4-EP concentration reduced by 34% and the second batch by almost 32%. A general slight loss in polymer binding can be noticed, but it would take multiple repetitions of such an experiment to get a clear sense of the margin for error.

The dose of polymer used was 25 g and the volume of test solution was 10 L, with a 1 mg/L 4-EP concentration. This experiment shows, therefore, a 4-EP reduction of more than 30% at a pass-through contact time, with a dose of 2.5 g/L MIP2.

The direct point of comparison from lab-scale trials can be seen in section 4.6.3. There, the MIP2 at a 2.5 g/L dose, removed  $38.8 \pm 8.1\%$  of the 4-EP from a 1 mg/L concentration solution. The removal of 4-EP in the MIP assisted filtration is slightly lower than the average removal in the bench trials in the lab, but certainly within one standard deviation of the mean. So, while this tendency points to a lowering of performance, the effect does not appear to be large, at a first observation.

What is remarkable is that the regeneration succeeded in eluting almost all the 4-EP bound by the MIP in both test batches. For batch 1 (34% 4-EP removal), 93% of the bound 4-EP was successfully eluted by the regeneration step, while for batch 2, (32% 4-EP removal) 98% of the 4-EP was successfully eluted by the regeneration step. This points to a much more successful result in polymer clean-up when compared to previous such experiments (Section 4.6.4) where the levels of eluted and non-eluted 4-EP from the MIP were almost equal.

A way to explore this contrast in performance is to observe differences between these experiments in terms of their testing conditions.

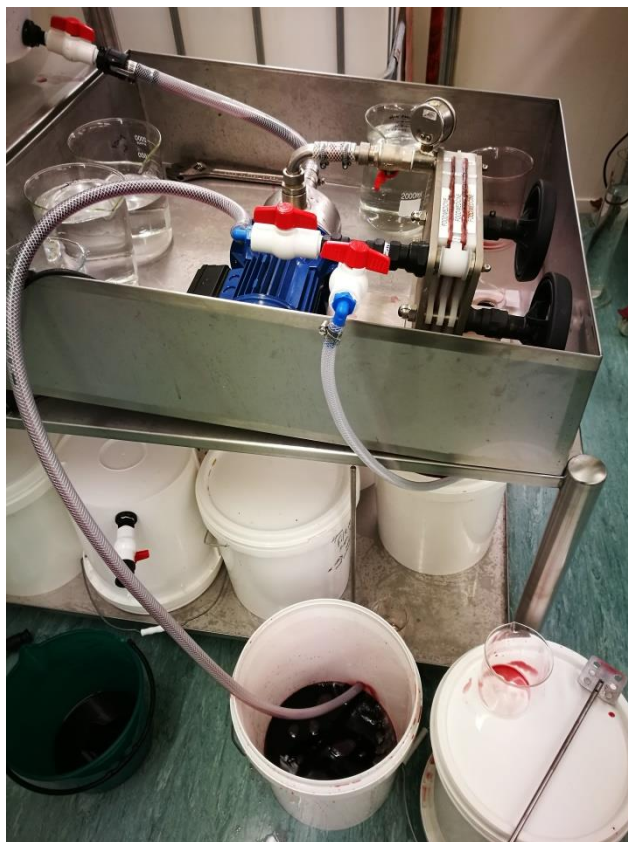
In the experiments in section 4.6.4, working with the Syringe setup, a volume of 10 mL of NaOH 2M solution was used to elute 500 mg polymer, with 52.6% of the bound 4-EP being successfully eluted. This represents a dosage of 20 mL NaOH 2M solution per gram of polymer.

In these filtration experiments, as 800 mL of NaOH 2M solution were used to elute 4-EP from 25 g polymer, the dosage was 32 mL NaOH 2M solution per gram of polymer. This certainly supports the higher elution rates of 93% and 98% 4-EP elution from the two batches of test solution filtration. Out of these results, a simple process of optimization of the regeneration solution can be attempted by the polymer manufacturer to find the minimal quantity of NaOH that would ensure a consistent elution of 100% of the bound 4-EP on the polymer.

#### **4.6.8 Wine filtration with MIP2**

The methodology and further clarifications pertaining to this experiment have been outlined in section 3.3.6.8.

The next step was to perform a filtration using the same methodology as in section 4.6.7 but replace the test solution with a red wine. A close-up view of the plate filter and associated pump can be seen in Figure 51.



**Figure 51. Plate filter and pump setup used at Ligar Polymers, NZ**

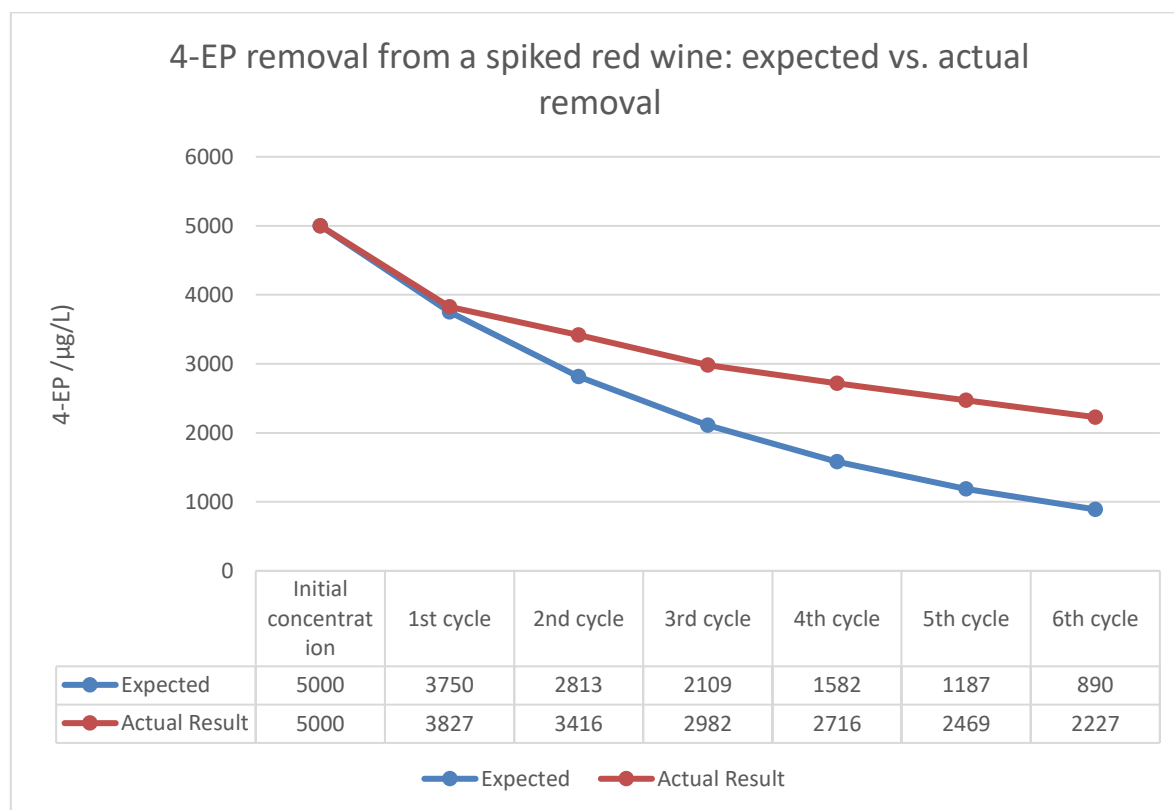
Therefore, the purpose of this experiment was to observe how well the MIP filtration treatment removes 4-EP from a red wine spiked at 5 mg/L 4-EP, which would represent a very severe taint.

Looking back at the previous experiments, the results from section 4.6.3 describe that the expected removal rate is around 30% when using a 2.5 g/L polymer dosage in a 5 mg/L 4-EP test solution. Results from section 4.6.6 indicated that when switching from test solution to wine, under similar polymer and taint concentrations that the drop in removal efficiency would be around 7%. Finally, the results seen in section 4.6.7 indicate that when scaling up from 1 mL volumes to 10 L volumes that the drop of removal efficiency was about 6%. Looking at these values, it was conjectured that one should expect to see a 25% or lower removal of 4-EP per cycle of treatment.

Figure 52 illustrates the result of this filtration trial. What is initially noticeable is that for the first filtration cycle the actual result was like the expected result, a 23% reduction was indeed achieved. The following filtration cycles did not maintain the same removal efficiency. They succeeded in removing 11% for the 2<sup>nd</sup> cycle, 13% for the 3<sup>rd</sup>, 9% for the 4<sup>th</sup> and the 5<sup>th</sup> and finally 10% for the 6<sup>th</sup> cycle.

This loss of effectiveness can be partly attributed to channelling issues with the polymer layer on the filter sheets and their vertical orientation, losses of wine and dilutions due to a volume of liquid immobilized in the wetted filter sheets after each rinsing. The distressing of the filter sheets due to the use of sodium hydroxide and citric acid, both at 2M concentrations could also have compounded the problem.

The main conclusion to be drawn from this experiment is that the polymer, under specific conditions, does indeed work, and can reduce the 4-EP content of a wine quite remarkably. A repetition of this experiment with a more precise filtration setup was scheduled to follow this one.



**Figure 52. Removal of 4-EP by filter sheets pre-coated with MIPs: a comparison between expected reduction, 25% based on previous data, and actual performance**

Just like in the previous experiment, after each volume of 10 L of test solution passed through the filter, the regeneration solutions were passed through the filter sheets with MIP powder on them in the following sequence: 200 mL deionized water, 800 mL 2M NaOH solution, 400 mL deionized water, 2M 400 mL citric acid and, finally, 200 mL deionized water.

Another explanation for the loss of polymer effectiveness can be hinted at by examining the percentage of 4-EP that was successfully eluted by the regeneration process (Table 27).

The elution never managed to remove more than 64% of the 4-EP bound to the polymer. Seeing how the elution across all cycles managed to remove roughly only 50% of the 4-EP bound, one can easily infer that there must be a relationship with the fact that the 4-EP removal was, for all filtration cycles except the first, around half or less than half of that which was expected.

**Table 27. The percentage of 4-EP that was eluted from the polymer powder after each regeneration cycle**

<b>4-EP eluted from polymer</b>	
1st cycle	63%
2nd cycle	64%
3rd cycle	48%
4th cycle	55%
5th cycle	46%
6th cycle	57%

Additionally, one can look at Table 28 for some basic chemical parameters of the wine before the MIP filtration treatments (Control and Spiked), as well as the wine after each successive filtration event.

Some trends are quite clear. The alcohol content is decreasing after each filtration cycle. The relative density is increasing accordingly. This points to a dilution of the wine due to the significant volume of liquid still inside the plates after the regeneration step has been



completed. The fact that the total acidity slightly increases could signify that the citric acid used to elevate the pH of the MIP powder after the elution was performed could still be found inside the filter sheets and therefore lead to this increase.

The total polyphenolic content is decreasing with each filtration step, partly due to dilution, and partly because the filter sheets, which normally absorb some colour from the red wine when first used, are being thoroughly cleaned with alkaline and acidic solutions, and therefore continue to take up colour from the wine each time it is passed through.

**Table 28. Basic chemical parameters of the wine, after each filtration cycle (from T1 to T6)**

	<b>Control</b>	<b>Spiked</b>	<b>T1</b>	<b>T2</b>	<b>T3</b>	<b>T4</b>	<b>T5</b>	<b>T6</b>
<b>Alcohol content (% Vol.)</b>	13.7	13.6	13	12.8	12.7	12.5	12.2	12
<b>pH</b>	3.5	3.5	3.7	3.5	3.4	3.4	3.4	3.4
<b>Total acidity (g/L) as tartaric acid</b>	5	5.12	5.12	5.37	5.75	5.87	6	6.25
<b>Relative density @ 20 °C</b>	0.9946	0.9948	0.9949	0.9953	0.9957	0.9959	0.9961	0.9963
<b>Refraction index</b>	46.7	46.7	44.5	45.5	44.8	44.5	43.9	43.4
<b>Total polyphenolic index (mg/L Catechin)</b>	2623	2672	2467	2363	2290	2154	2059	2024

Ultimately, these trials highlight some promising results from the MIP powder for 4-EP removal from wine and point to the need to perform a repetition of this experiment using a filtration setup more carefully corrected for dilution and possible alterations of wine from the cleaning steps.

#### 4.7 Wine filtration with MIP2 in HS Geisenheim

The methodology and further clarifications pertaining to this experiment have been outlined in section 3.3.7.

The aim of this experiment is to perform a repetition of the previous wine filtration trial (Section 4.6.8) using a similar filtration setup that would allow for better control of possible losses or dilutions.

The MIP2 and NIP2 polymers were tested for this experiment. This second generation of MIP was selected to be used in further experiments based on previous results highlighted in section 4.7.1.

The first observation to mention is that MIP2 is more easily mixed in water than MIP1. This can be first seen by the aspect of the pressure vessel after 4.15 g of polymer are added to 3 litres of deionized water and then filtered through. Note the difference between Figures 53 and 54. The MIP2 mixed in better with water than MIP1 and was therefore more easily pre-coated on the filter surface.

While keeping all other parameters identical (same wine, filter sheet, volume, polymer quantity, pressure etc.) except for the type of polymer (MIP1 and MIP2), the filtrations with MIP2 were slower than those with MIP1.

When filtering with an air pressure of 1.5 bars, filtration times of around 4 minutes ( $\pm$  30 seconds) were observed with MIP1 filtration, whereas the MIP2 filtrations took between 5 minutes and 54 seconds to 7 minutes and 45 seconds.



**Figure 53.** The aspect of the pressurized container after 4.15 g of MIP1 suspended in 3 L water are pushed through the filter sheet



**Figure 54.** The aspect of the pressurized container after 4.15 g MIP2 suspended in 3 L water are pushed through the filter sheet

When looking at the aspect of the filter after the treatments were finished, it was clear to see that the MIP1 had a compact, dry appearance (Figure 55), whereas MIP2 seemed to have formed a paste, and looked like wet clay (Figure 56), which could explain the longer times necessary to filter the wine through it.



**Figure 55. Aspect of MIP1 after finishing the treatment and regeneration cycles**



**Figure 56. Aspect of MIP2 after finishing the treatment and regeneration cycles**

The data in Table 29 can highlight the slight changes in contact time with the polymer, treatment time, flow rate as well as the volume of wine (input and output) after each treatment. There are significant losses due to the nature of the setup. 50 mL of wine are taken out after each treatment to perform the 4-EP analysis. Additionally, a small volume of wine

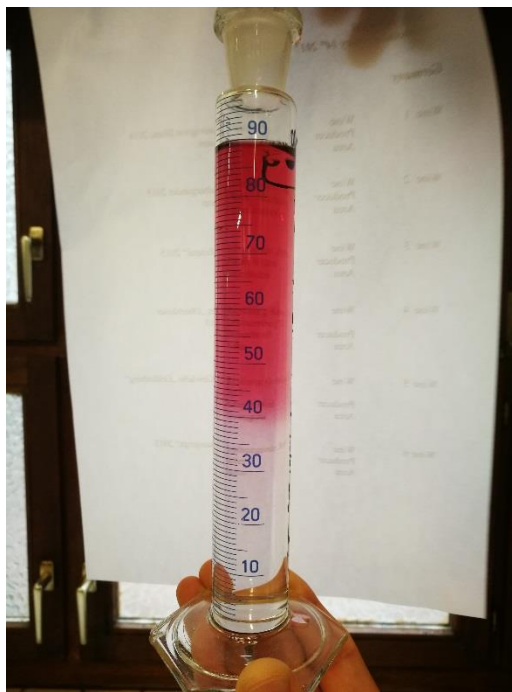
is added back to the filtered wine, corresponding to the wine on the bottom of the pressurized vessel which was sitting too low to reach the tip of the stainless-steel pipe that would carry it through the filter.

**Table 29. General filtration parameters of the MIP2 treatment cycles**

<b>Treatment cycle</b>	<b>Treatment time (mm:ss)</b>	<b>Contact time w. MIP (s)</b>	<b>Flow L/h</b>	<b>Input volume (mL)</b>	<b>Output volume (mL)</b>
T1	05:54	2.43	16.1	1750	1584.5
T2	07:14	3.33	11.8	1550	1422.1
T3	07:19	3.76	10.42	1401.1	1269.7
T4	07:43	4.54	8.65	1244.2	1112.2
T5	07:17	4.93	7.96	1066.3	965.7
T6	06:40	5.31	7.39	936.2	820.5

The volume of the regeneration solutions used was also recorded. Additionally, a sample was taken from the regeneration solutions and subjected to pH analysis and an analysis of the Total Phenolic Index (expressed in mg/L catechin). As there was unavoidable mixing in of wine and water throughout each treatment and regeneration cycle, there would clearly be a small volume of wine present in the regeneration solution. The analysis of the total phenols of each regeneration solution could cumulatively indicate how much wine was being lost at each step.

Additionally, the first 90 mL of liquid coming out of the filter when a wine filtration was started were collected separately and discarded, since they contained a mixture of wine and water, as seen in Figure 57. It is therefore likely that the wine could also be slightly diluted with some water, and other liquid regeneration solutions that were still present in the filter sheet, despite the attempts to thoroughly clean it, in the 5-step process.



**Figure 57. The first 90 mL of liquid at the beginning of each wine filtration highlighting the water present in the filter and the mixing of wine and water**

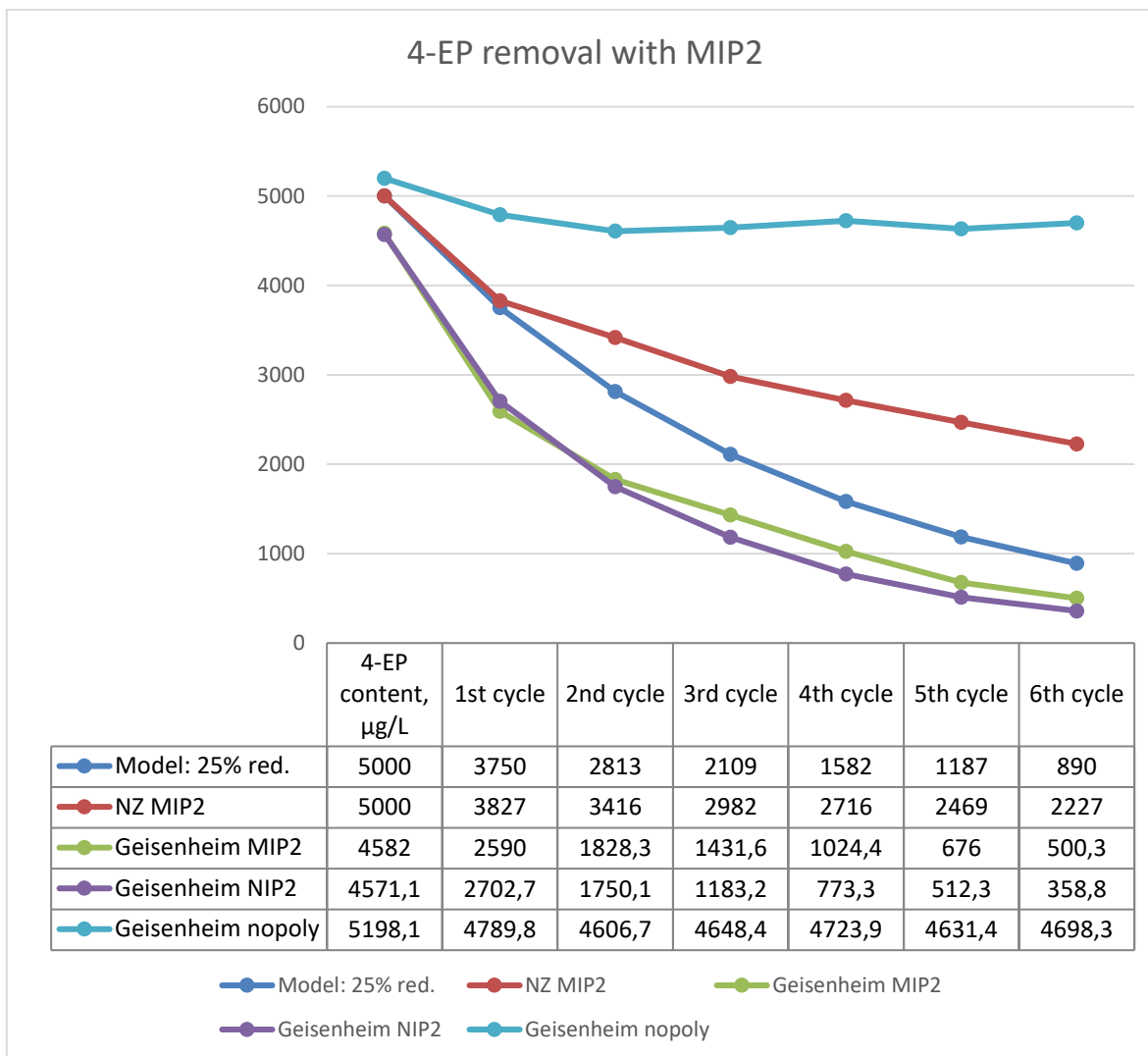
The polymer on the filter sheet was recovered by washing with water. This water containing the polymer was placed into a wide beaker and left in a drying oven to evaporate the water. Afterwards the dry polymer was collected and weighted. Out of the initial 4.15 g of MIP2, 3.55 g were recovered. The difference of 0.6 g was represented by the polymer that was not recovered, some was either adhering too strongly to the filter sheet and could not be flushed with water, and some was represented by too fine particles in the drying beaker to be successfully collected and weighted. As a point of comparison, for MIP1, out of the initial 4.15 g, 4.02 g were recovered, meaning that only 0.13 g were lost. This seems to indicate that MIP2 particles manage to adhere stronger to the filter sheet and cannot be easily removed by washing it with water. Even light scratches on the wet filter sheet do not remove any polymer but damage the filter sheet surface instead. Even after drying of the filter sheet, it was not possible to remove any polymer particles from its surface.

For all these reasons, it was highly desirable to repeat this experiment, upholding the exact same methodology except for not using any polymer to pre-coat the filtration surface. The

comparison between the 4-EP content of the wine fractions, the TPI values and pH values found in the regeneration solutions, and the comparison between the general wine analysis parameters of the control wine, the wine treated with MIP2 for 6 cycles and the wine treated with the same method excluding the polymer for 6 cycles, could paint a more accurate picture of exactly how much 4-ethylphenol is being removed due to the presence of the polymer and how much due to various dilutions and losses inherent to the filtration setup.

The results of this experiment pertaining to the level of 4-ethylphenol after the filtration events can be observed in Figure 58. It is evident that the performance of the polymer (both MIP2 and NIP2 variants) is better in the filtration setup in HS Geisenheim than in the setup in New Zealand. In the first filtration cycle, both MIP2 and NIP2 reduced the 4-EP content from values around 4500  $\mu\text{g/L}$  to 2500  $\mu\text{g/L}$ . By the 6<sup>th</sup> filtration cycle, the MIP2 has reduced the 4-EP level to a low value of 500  $\mu\text{g/L}$  (89% reduction) and NIP2 reduced the level to 358  $\mu\text{g/L}$  (92% reduction).





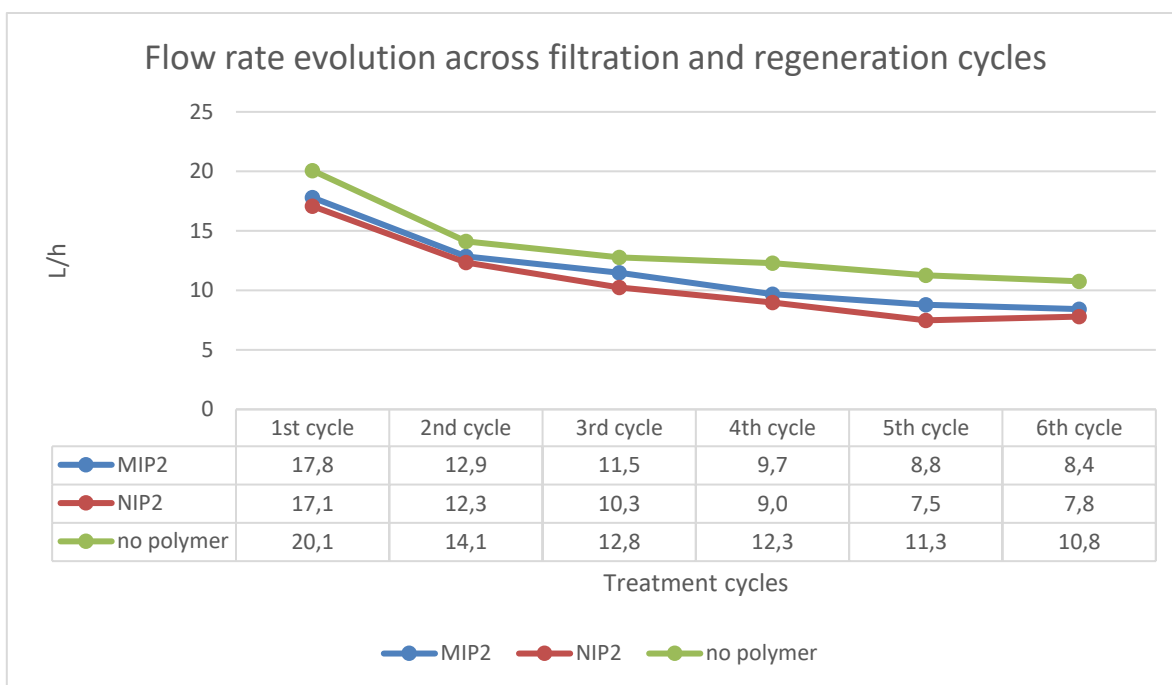
**Figure 58. Reduction of 4-EP from filtrations in HS Geisenheim with MIP2 and NIP2, in comparison to the filtration using no polymer, as well as the result of the filtration from the previous experiment from section 4.6.8**

The filtration run which did not contain any type of polymer (“Geisenheim nopoly”) is having its level of 4-EP constant, not decreasing through the successive filtration and regeneration cycles. This highlights that the removal of 4-EP is strictly a function of the polymers and not due to any other characteristic of the filtration process.

The difference between MIP2 and NIP2 is not obvious, with NIP2, if anything, displaying even a slightly superior capacity to remove 4-EP.



With each successive filtration and regeneration, the flow rate has been observed to diminish (Figure 59), possibly as a function of the interaction between the cleaning chemicals and filter sheets, as this decrease in flow rate was observed also in the filtration runs which did not contain any polymer.



**Figure 59. Evolution of flow rate across six filtration and regeneration cycles, on three different types of filtration runs**

The effect of this reduction in flow rate meant that the contact time between wine and the bed of polymer was increased (Figure 60). As has been seen before, in experiments from section 4.6.1, the longer contact times favour the non-imprinted polymer, allowing it to catch up in effectiveness to its imprinted analogue. This could point to the slightly superior results seen from the NIP2 in this experiment.

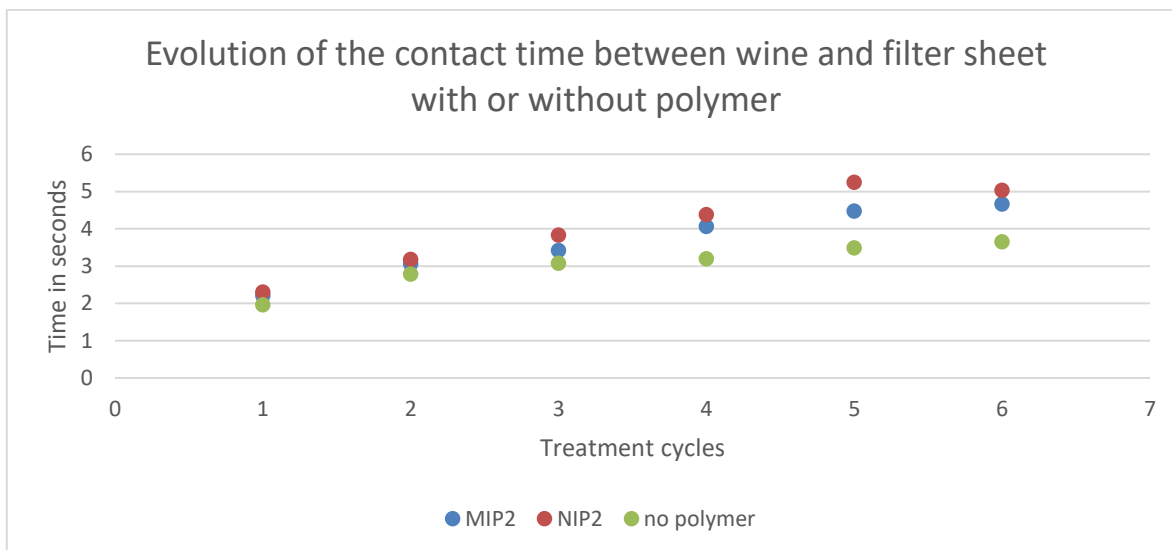


Figure 60. Evolution of contact time between wine and filter sheet with/without polymer

Regarding the efficiency of polymer regeneration and re-usability, the following Figures describe the regeneration process with the MIP2 (Figure 61), NIP2 (Figure 62) and the filter sheet without any polymer (Figure 63).

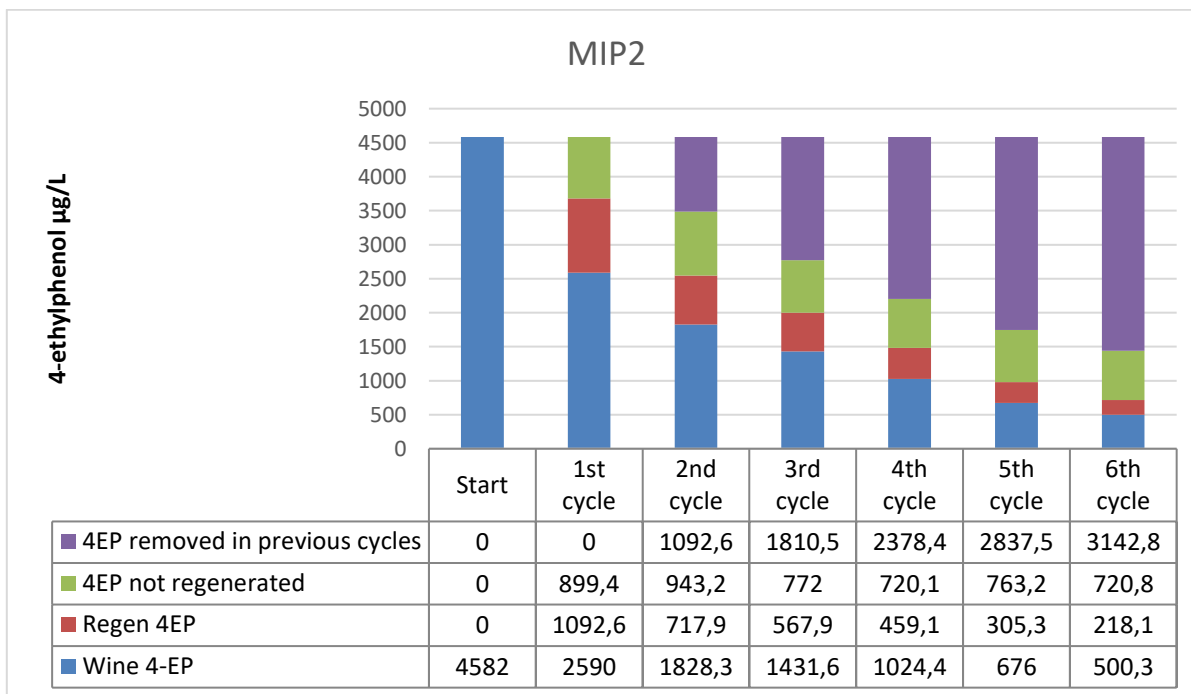


Figure 61. 4-EP levels throughout the 6 filtration and regeneration cycles for MIP2

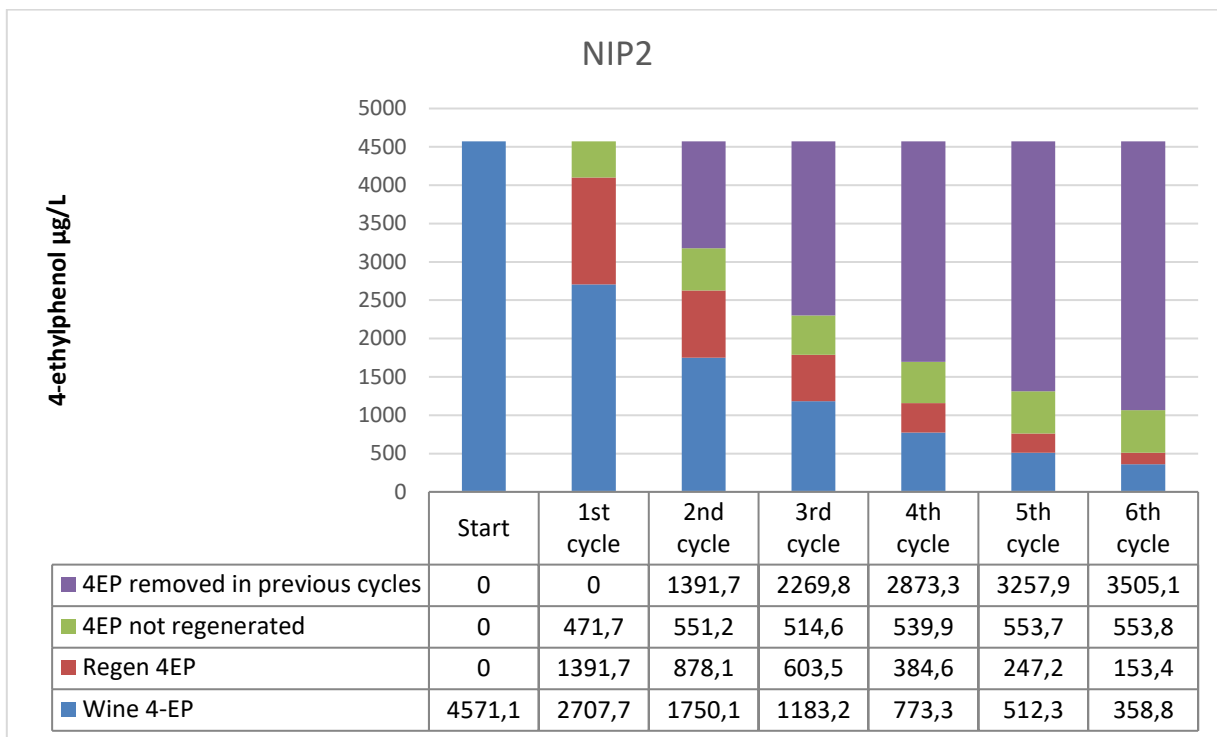


Figure 62. 4-EP levels throughout the 6 filtration and regeneration cycles for NIP2

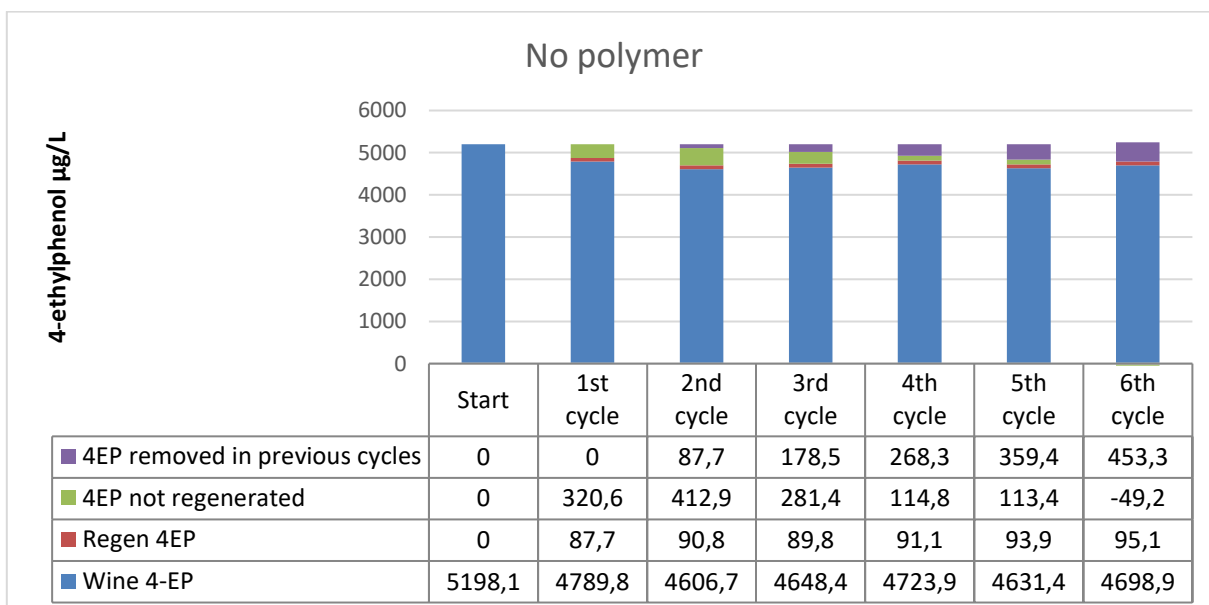


Figure 63. 4-EP levels throughout the 6 filtration and regeneration cycles for standard filter sheet with no polymer

It is clear from Figure 63 that when no polymer is used, the 4-EP content of the wine stays for the most part the same. Figures 61 and 62, displaying the behaviour of the MIP2 and NIP2 when regenerated, highlight how beginning with the 1<sup>st</sup> regeneration cycle there is an amount of 4-EP bound to the polymer that is not getting eluted. This quantity is observed throughout all filtration and regeneration cycles to be quite constant.

**Table 30. Oenological parameters of the wines at the end of the 6th filtration cycle**

<b>Oenological parameters</b>	<b>Control</b>	<b>MIP2 T6</b>	<b>NIP2 T6</b>	<b>no polymer T6</b>
<b>Total alcohol (% vol)</b>	12	11.5	11.5	12.1
<b>Total extract (g/L)</b>	25.9	24.1	24.1	27.2
<b>Sugar content (g/L)</b>	5	3.5	3.6	5.2
<b>Ferm. Sugar (g/L)</b>	4	2.5	2.6	4.2
<b>pH</b>	3.6	3.5	3.5	3.4
<b>Total acidity (g/L)</b>	4.3	4.5	4.5	5.1
<b>Free SO<sub>2</sub> (mg/L)</b>	40	31	30	23
<b>Total SO<sub>2</sub> (mg/L)</b>	72	56	53	45
<b>Relative density</b>	0.9945	0.9943	0.9943	0.9949
<b>Refraction index</b>	41	39.5	39.5	41.6
<b>Total phenols (mg/L)</b>	2357	1339	1223	1333

Looking at Table 30, one can see the standard measured oenological parameters of the control wine, and the wine filtered through 6 cycles with the MIP2, NIP2 and the filter sheet without any polymer, respectively. In contrast to the relative density measurements seen in Table 28, here the relative density remains virtually unchanged after six filtration and regeneration cycles, indicating that no wine dilution took place. Additionally, by looking at the total phenols, their level decreased by almost 50% in all three filtration events, regardless of whether they were with MIP2, NIP2 or without any polymer. This indicates that virtually all removal of phenolic compounds is not due to the polymers but due to the filtration setup. More specifically, the use of alkaline and acidic cleaning between each two filtration cycles

means that an amount of phenolics and colour compounds are washed out of the filter sheet with each regeneration event, leading to these cumulative losses.

Indeed, looking at the colour measurements (Table 31), observed through spectrophotometric analysis, the colour intensity of the filtered samples decreased by almost half. Computing the  $\Delta E$ , all three filtered wines were assessed as displaying perceptible differences compared to the control. However, the filtered samples were not perceptibly different among each other, with the  $\Delta E$  for the MIP2 and NIP2 samples being less than 0.5, when the reference was taken as the wine filtered without polymer.

**Table 31. Colour measurements of the filtered wines, compared to the control**

<b>Colour measurements</b>	<b>Control</b>	<b>MIP2</b>	<b>NIP2</b>	<b>No polymer</b>
<b>Colour intensity</b>	7.395	3.579	3.204	3.502
<b>Colour nuance</b>	0.676	0.684	0.689	0.680
<b><math>\Delta E</math></b>	0	2.427	2.672	2.460
<b><math>\Delta E</math></b>	2.460	0.0487	0.217	0

#### **4.8 Polymer selectivity analysis**

The methodology and further clarifications pertaining to this experiment have been outlined in section 3.3.8.

This experiment turns the attention towards the topic of polymer selectivity. The average filtration times were different, due to the nature of the materials. MIP1 and NIP1 had the shortest filtration time, with MIP2 and NIP2 having slightly longer filtration times. The blank sample had a longer filtration time than the polymer filtrations, possibly because the solids in the wine formed an even layer over the filter membrane, thereby reducing flow, whereas the polymers acted as a pre-coat, breaking up the formation of a single layer of solids. Activated carbon had a lower filtration rate, and PVPP, having slightly swollen from contact with the liquid, led to extremely long filtration times.

First, looking at the 4-EP removal of the different materials, Figure 64 shows the high 4-EP dose results and Figure 65 shows the low 4-EP dose results.

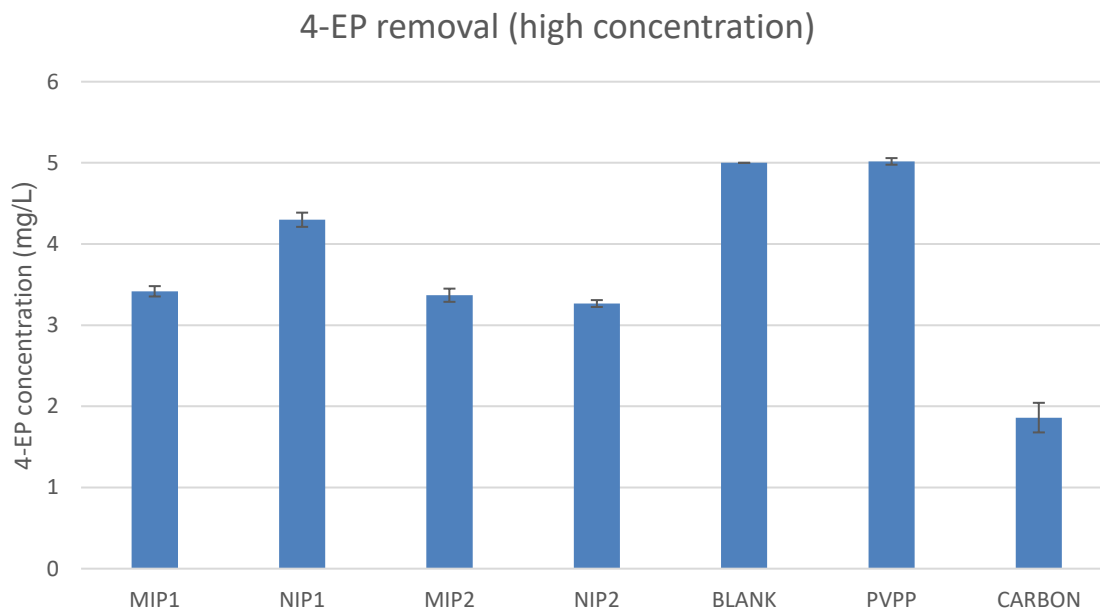


Figure 64. The 4-EP removal in the high 4-EP concentration case (5 mg/L)

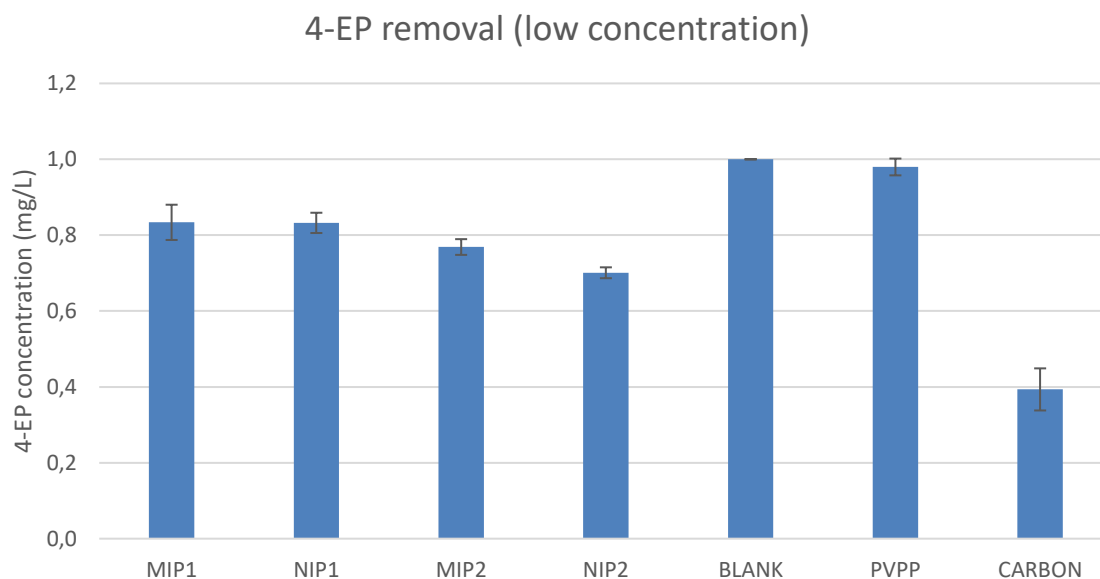
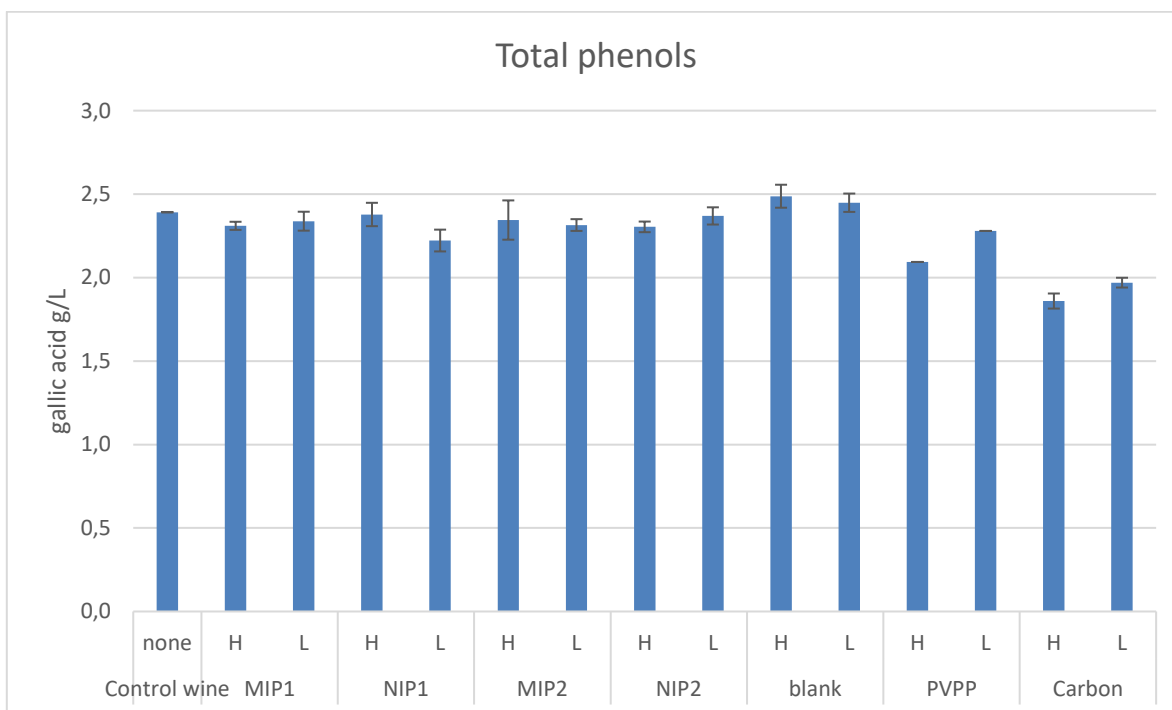


Figure 65. The 4-EP removal in the high 4-EP concentration case (1 mg/L)

The same trend as in the previous experiments reveals itself. The second generation of polymer is more effective than the first, with small differences between the MIP and the NIP in both cases. PVPP had virtually no effect on 4-EP concentration. Activated carbon removed the most 4-EP in both cases.

The effect of the treatments on the total polyphenol content can be seen in Figure 66. Overall, the differences between the polymer treatments and the control are small and non-significant, whereas the effects of PVPP and especially activated carbon were more powerful. This points to the polymers (regardless of generation or whether they are imprinted or not) being more selective than activated carbon, as the latter reduces several hundred milligrams of phenolic compounds more than the formers.



**Figure 66.** Total polyphenol content of wine following the treatments. H - high 4-EP dose (5000 µg/L), L - low 4-EP dose (1000 µg/L)

Teixeira et al. (2015) report reductions of total phenolic compounds of 24.5% from their MIP treatment and 18.2% from their NIP treatment. In this experiment, only the activated carbon treatment resulted in similar reductions in total phenols, 22%, and the PVPP treatment

resulted in a 12.5% reduction as well. In contrast, the strongest reduction from any of the polymers (NIP1) in this experiment was of about 7% reduction. Of course, one must be aware that the testing conditions are somewhat different. The cited literature reference here used 2 g/L polymer doses and 4h contact times, whereas this experiment had a polymer dosage closer to 2.5 g/L and used a pass-through short contact time.

Nevertheless, it must be pointed out that the 4-vinylpyridine MIPs in the work of Teixeira et al. (2015) led to 55% 4-EP removal from the 4-EP MIP and 46% 4-EP removal from the NIP. In this experiment, the differences between imprinted and non-imprinted were not discernible, for either polymer recipe (MIP1 or MIP2). Specifically, in this experiment 4-EP reductions of 20-32% and 24-34% for MIP1 and MIP2 respectively were observed. And 4-EP reductions of 16-17% and 30-36% for NIP1 and NIP2 respectively were observed.

Next, looking at the five most important free anthocyanins in wine, cyanidin, delphinidin, peonidin, petunidin, malvidin, the differences between the MIPs and NIPs and activated carbon can be very clearly highlighted.

For cyanidin (Figure 67), the MIPs and NIPs had no strong effect while activated carbon essentially eliminated it from the wine. In the case of delphinidin (Figure 68), the activated carbon did reduce its content, without having as dramatic an impact as in the case of cyaniding. Here, the polymers did not reduce this anthocyanin at all. If anything, it seems to be higher. As the filtration times of the polymers are lower than that of the control, this higher amount of delphinidin can hint at diminished wine oxidation in the MIP and NIP treated samples when compared to the blank filtration. For peonidin (Figure 69), petunidin (Figure 70) and malvidin (Figure 71) the same trend can be seen as in the case of delphinidin, with the MIP and NIP treated samples having a slightly higher level of these anthocyanins than the control, possibly due to less oxidation, whereas the activated carbon treated samples have these anthocyanin levels almost halved.



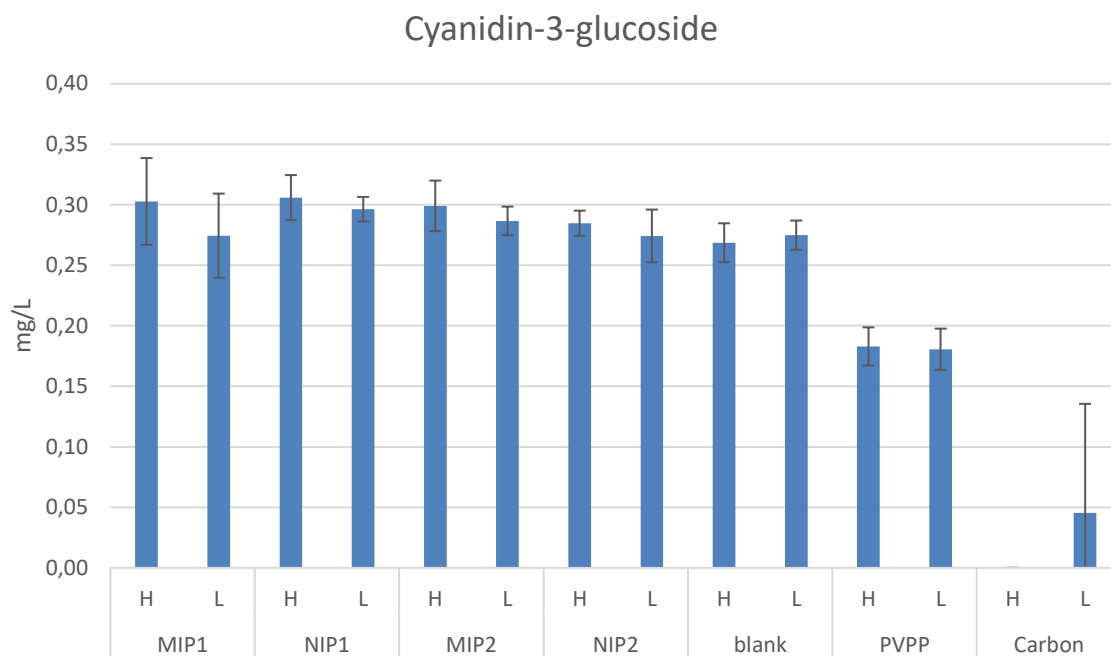


Figure 67. Effect of treatments on Cyanidin-3-glucoside. H - high 4-EP dose (5000  $\mu\text{g/L}$ ), L - low 4-EP dose (1000  $\mu\text{g/L}$ )

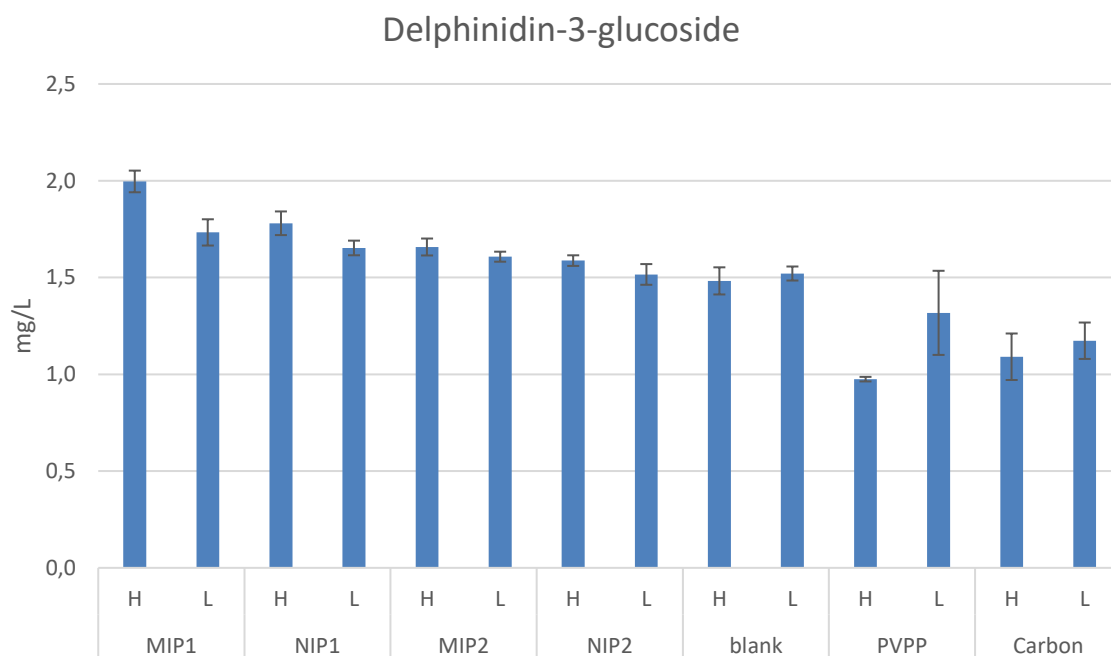
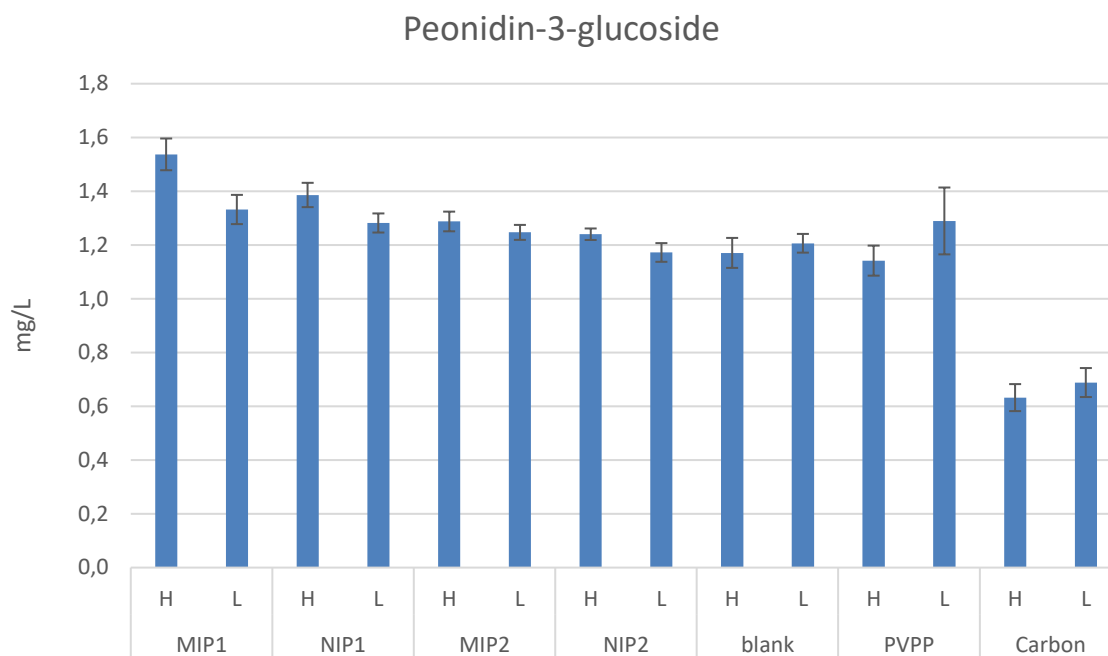
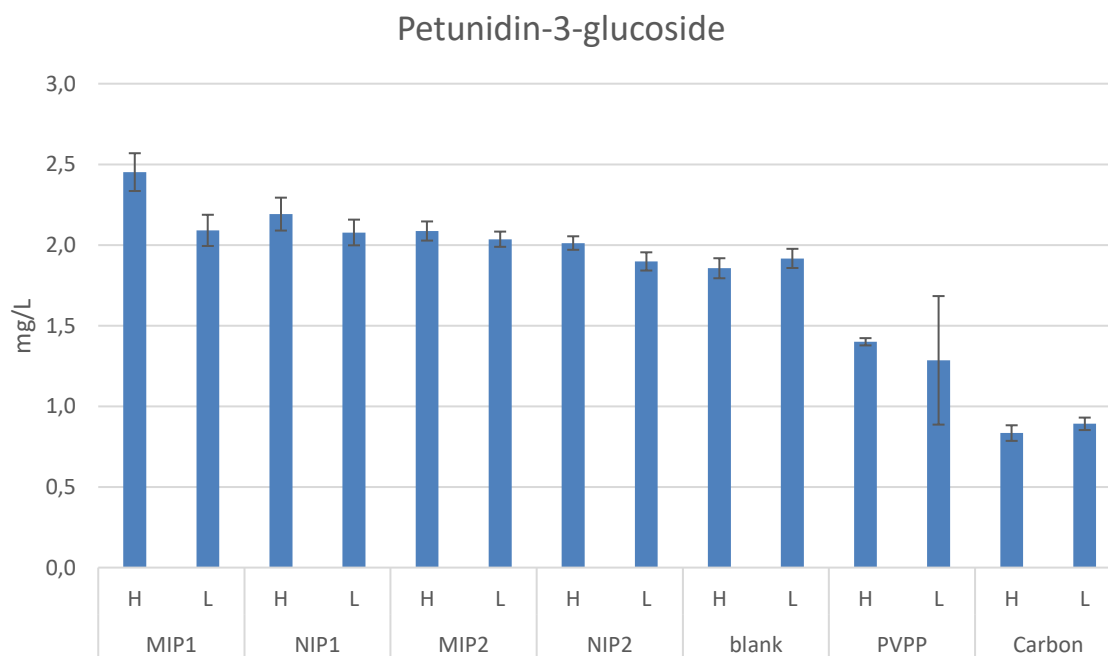


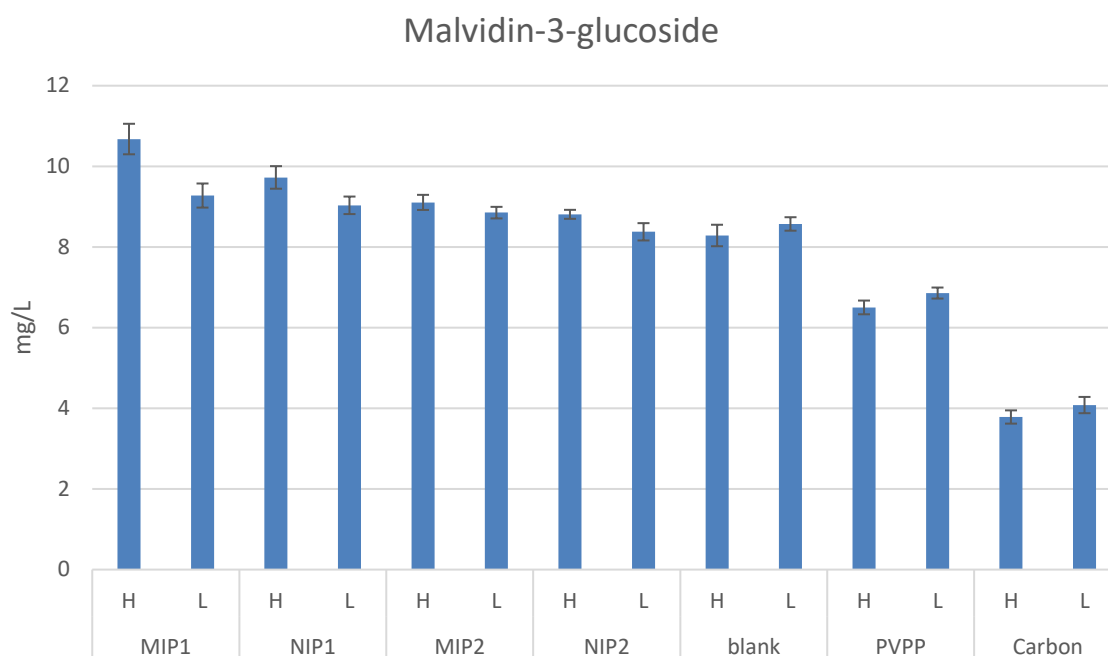
Figure 68. Effect of treatments on Delphinidin-3-glucoside. H - high 4-EP dose (5000  $\mu\text{g/L}$ ), L - low 4-EP dose (1000  $\mu\text{g/L}$ )



**Figure 69.** Effect of treatments on Peonidin-3-glucoside. H - high 4-EP dose (5000  $\mu\text{g/L}$ ), L - low 4-EP dose (1000  $\mu\text{g/L}$ )

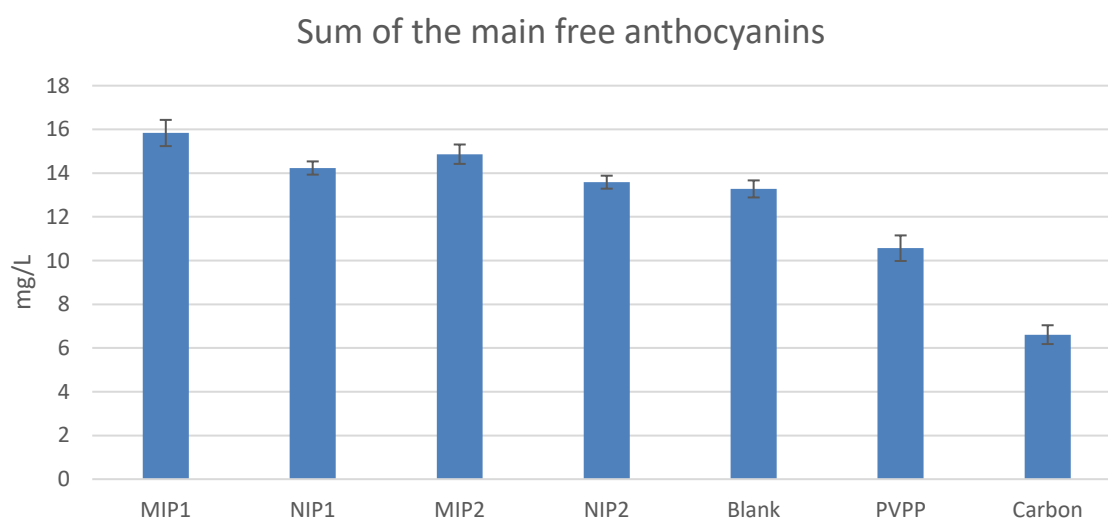


**Figure 70.** Effect of treatments on Petunidin-3-glucoside. H - high 4-EP dose (5000  $\mu\text{g/L}$ ), L - low 4-EP dose (1000  $\mu\text{g/L}$ )



**Figure 71. Effect of treatments on Malvidin-3-glucoside. H - high 4-EP dose (5000  $\mu\text{g/L}$ ), L - low 4-EP dose (1000  $\mu\text{g/L}$ )**

Overall, if one were to sum up all the main free anthocyanins present in the wine (averaged from both high and low taint concentrations) as they are affected by the various treatments, the difference between the polymers and activated carbon is clear (Figure 72).



**Figure 72. Sum of main free anthocyanins in wine after the different treatments**

All polymers have essentially no discernible effect on the anthocyanin content of the wine. If anything, as they improved the filterability of the wine in this experimental setup, they indirectly improved the anthocyanin content by minimizing wine oxidation. PVPP reduced the anthocyanin content by approximately 20%, while activated carbon reduced it by 50%. As the filtration times were much longer in the case of PVPP than in the case of carbon, these differences cannot be attributed to just wine oxidation. Carbon removes a large quantity of phenolic compounds and anthocyanins, and that the polymers have minimal, if any, effect. In the work of Teixeira et al. (2015), the effect of the 4-EP MIP based on 4-vinylpyridine was of 24% removal. In accordance to their observations, malvidin-3-O-glucoside also represented a majority portion of the main free anthocyanins in the wine selected for this experiment (64%). In contrast to the findings in the cited literature reference above, none of the tested polymers exhibited any negative impact on the free anthocyanins in this experiment.

#### **4.9 Particle size distribution**

The methodology and further clarifications pertaining to this experiment has been outlined in section 3.3.9.

The particle size distributions can be seen in Figure 73. Essentially all polymers have most of their individual particles range from 5 to 100  $\mu\text{m}$  in diameter, with the largest proportion of particles being in the 20-30  $\mu\text{m}$  range. The particle distribution of the NIP2 is having a broader peak than all the others. It is not clear what the origin of this difference could be, but a possible reason could be a change in the polymer monolith grinding process. There is no characteristic observed in the performance of this type of polymer that could be related to this difference in particle size distribution. The results observed in section 4.6.5, which also pertained to the effect of particle size, also did not show any clear difference in this regard. With the data currently generated within the scope of these experiment it is not known whether a tighter or a wider particle size distribution of the polymer could lead to changes in their effectiveness or in the size of the standard deviation from repeat experiments. Further

effects due to polymer particle sizes and their distribution were not investigated further within the scope of this work.

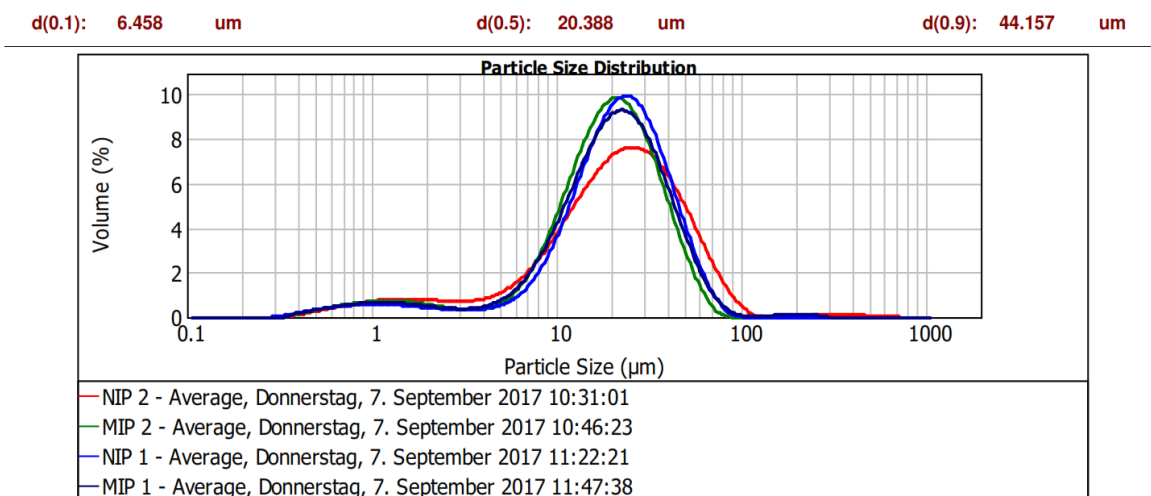
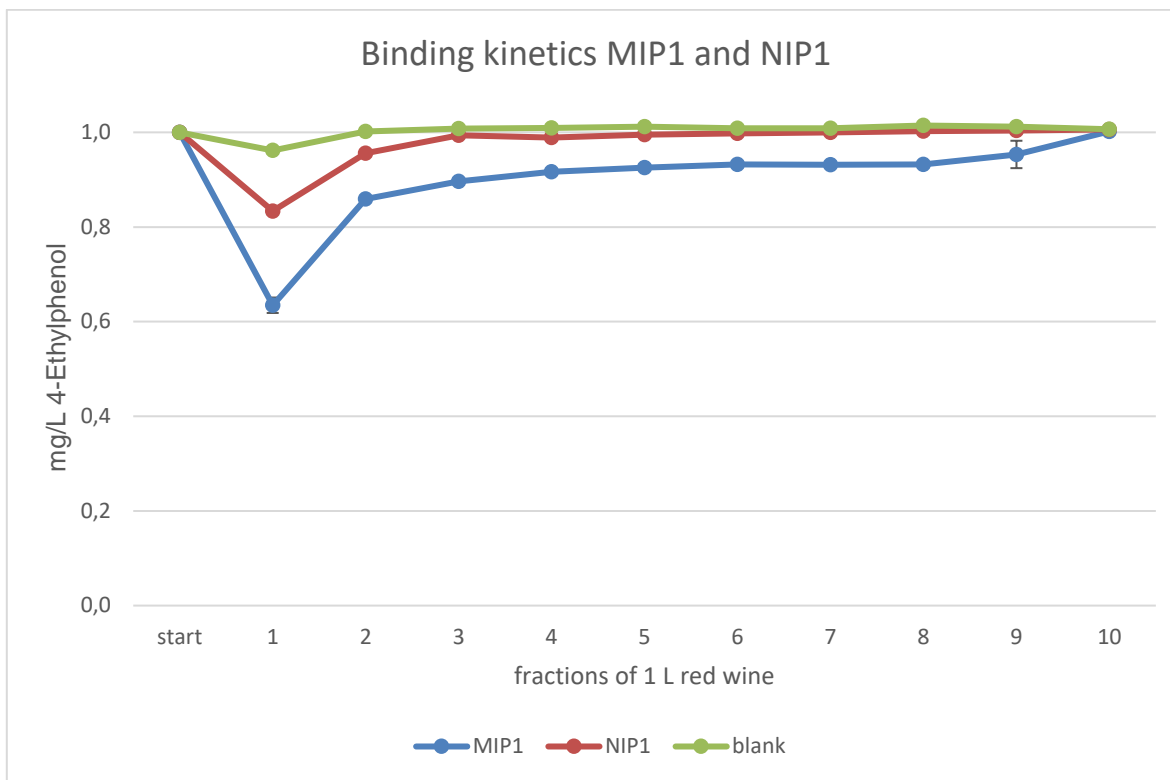


Figure 73. Particle size distribution of tested molecularly imprinted polymers and their analogues

#### 4.10 Polymer total capacity analysis

The methodology and further clarifications pertaining to this experiment has been outlined in section 3.3.10.

The aim of this experiment is to explore the total capacity of the polymers to bind 4-ethylphenol from a fluid stream. More specifically, they aim to identify after the filtration of what quantity of liquid does the 4-EP binding capacity of the polymers become zero, and how does their binding capacity look like up until that point.



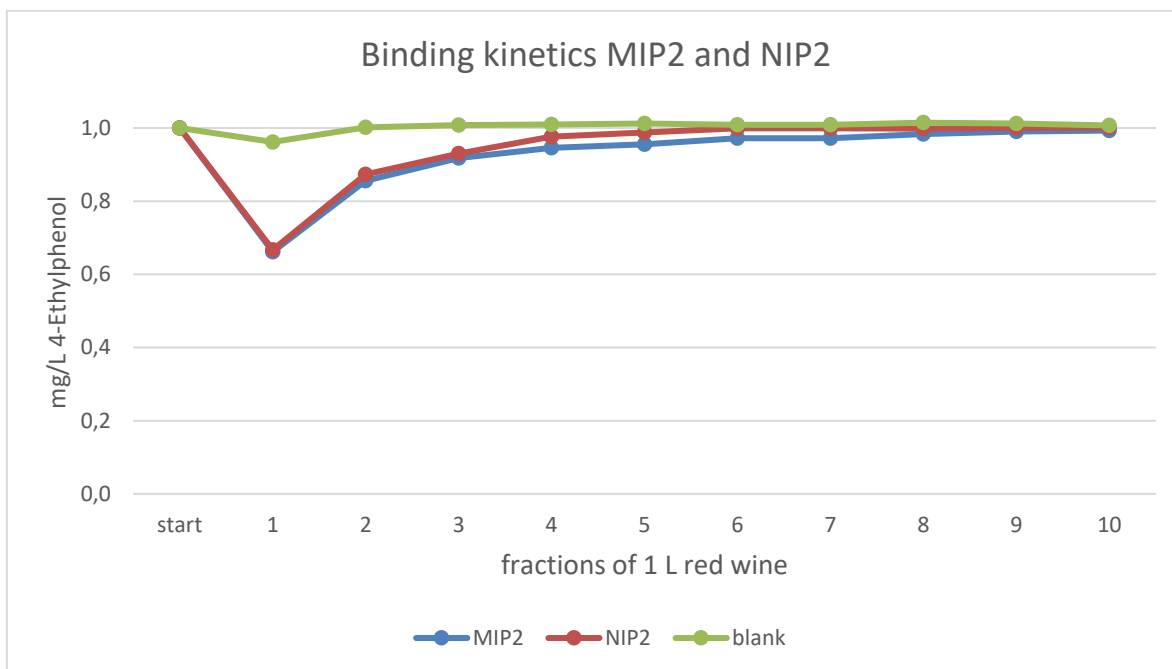
**Figure 74. Binding kinetics for MIP1 and NIP1**

Figure 74 highlights the total capacity of the first generation of polymer (MIP1 and NIP1). The results obtained with the blank filter sheet are also displayed, to contrast with the effect of the polymers. The binding was seen to be strongest in the initial fraction of wine, with the level of 4-EP dropping from 1 mg/L to about 0.6 mg/L.

What is also noticeable is that after the strong binding of 4-EP in the first fraction, the NIP1 essentially stops any other binding activity, whereas MIP1 continues to bind 4-EP, albeit clearly less than in the initial fraction, until it appears to have exhausted its binding capacity, at the 10<sup>th</sup> and final wine fraction. This clear difference between the binding performed by the MIP1 and the NIP1 highlights the effectiveness of the molecular imprinting step in the polymer preparation process.

For the MIP1, the total reduction of 4-EP was 10.16%, with one third of this amount being removed in the first fraction, and two thirds being removed in the subsequent fractions.

For the NIP1, the total reduction of 4-EP was 2.22%, with three quarters of it being removed in the first fraction, and the last quarter being removed in the second fraction, and essentially nothing else being removed in the subsequent fractions.



**Figure 75. Binding kinetics for MIP1 and NIP1**

Figure 75 highlights the results obtained with the second generation of polymers (MIP2 and NIP2) in the same experimental conditions. The initial peak in 4-EP binding for the first wine fraction is also noticeable here. However, in this case, the imprinted and the non-imprinted polymer seem to have performed rather similarly.

For the MIP2, the total reduction of 4-EP was 7.53%, with a little under half of that happening in the first wine fraction.

For the NIP2, the total reduction of 4-EP was 5.68%, with almost 60% of the 4-EP removed in the first fraction.

In the case of this second generation of polymers, it seems that the 4-EP binding efficacy is lower, and that the imprinting effect is diminished, when compared to the first generation of polymers. These results are summarized in Table 32.

Table 32. Total capacity of polymers to bind 4-EP

Polymer type	% removal (250 mg polymer per 1 L red wine with 1 mg/L 4-EP)	Total 4-EP removal capacity per gram polymer under these conditions
MIP1	10.16%	406.44 $\mu\text{g/L}$ 4-EP
NIP1	2.22%	88.83 $\mu\text{g/L}$ 4-EP
MIP2	7.53%	301.28 $\mu\text{g/L}$ 4-EP
NIP2	5.68%	227.20 $\mu\text{g/L}$ 4-EP

#### 4.11 MIP2 filter sheet experiments

The methodology and further clarifications pertaining to this experiment have been outlined in section 3.3.11.

Following the results generated from the previous work, several experimental filter sheets containing molecularly imprinted polymers, as well as blank (non-polymer containing) filter sheets were offered by the polymer producer for testing purposes.

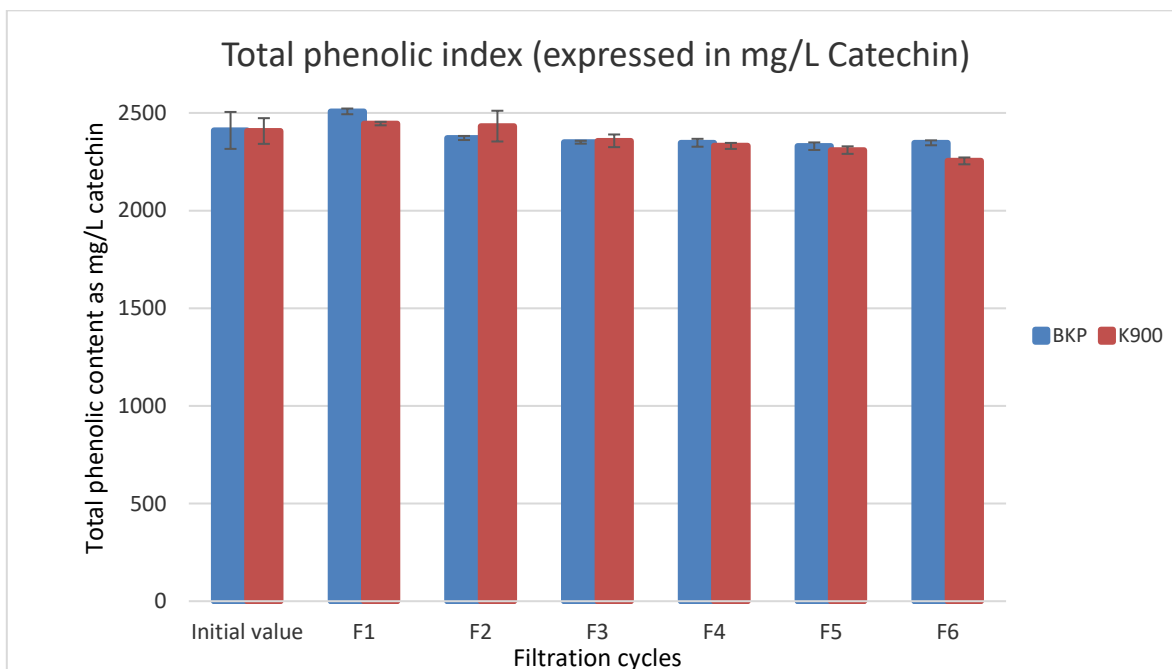


Figure 76. Total polyphenolic index of red wine after six successive filtrations with two kinds of filter sheets



As can be seen in Figure 76, the general trend for both filter types is to gradually decrease the total phenolic content of the wine, however the differences between the two filter types are marginal, and the overall total phenolic content reduction is modest enough to lack practical significance.

Looking at a comparison of colour measurements (Table 33), one can see how with each filtration cycle, the colorimetric difference becomes more and more accentuated. Overall, the BKP filter sheet seems to generate a weaker colour change than the Seitz™ K900 filter sheet.

**Table 33. Comparison of colour differences occurring from successive filtrations (F – filtration)**

	Sheet	Initial	F 1	F 2	F 3	F 4	F 5	F 6
$\Delta E$	<b>BKP</b>	0	0.4	0.693	1.105	1.469	1.648	2.192
$\pm SD$	<b>sheet</b>	$\pm 0.037$	$\pm 0.058$	$\pm 0.126$	$\pm 0.006$	$\pm 0.010$	$\pm 0.091$	$\pm 0.077$
$\Delta E$	<b>Seitz™</b>	0	0.519	0.960	1.146	1.915	2.521	3.172
$\pm SD$	<b>K900</b>	$\pm 0.106$	$\pm 0.086$	$\pm 0.075$	$\pm 0.020$	$\pm 0.063$	$\pm 0.119$	$\pm 0.103$

The significances of the  $\Delta E$  values are highlighted in Table 34 and point to the visually observable colorimetric differences between a sample and a reference.

**Table 34. Significance of  $\Delta E$  values**

$\Delta E$	Colour difference
0.0 ... 0.5	Virtually none
0.5 ... 1.0	Very slight
1.0 ... 2.0	Usually unremarkable
2.0 ... 4.0	Noticeable
4.0 ... 5.0	Large
> 5.0	Different colour

Following the descriptions in Table 34, one could state that it is only after the sixth filtration using the BKP filter sheets that the colour of the wine becomes noticeably different, whereas this noticeable colour difference can be discerned after the fifth filtration using the Seitz™ K900 filter sheet.

#### **4.11.1 4-ethylphenol binding and elution trials using MIP-embedded filter sheets**

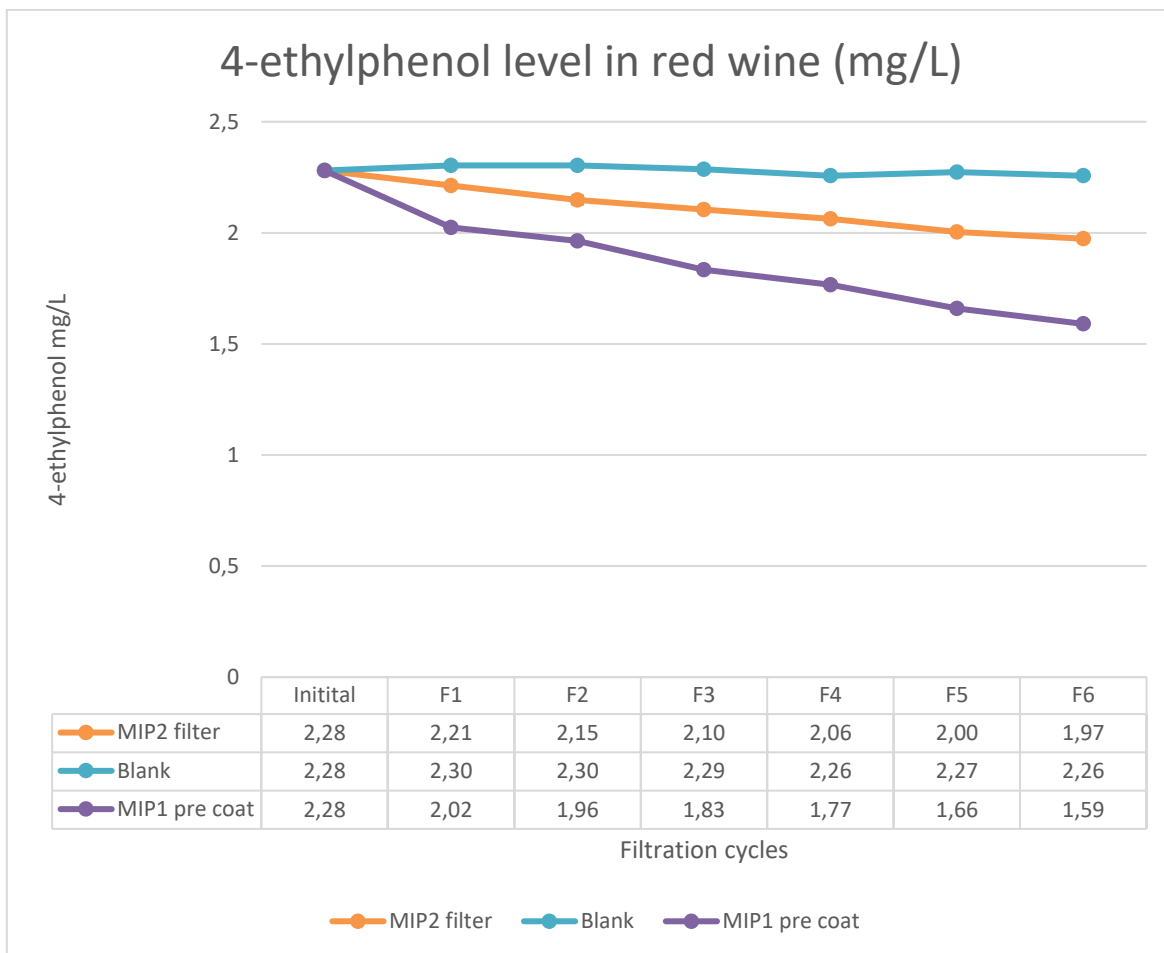
The methodology and further clarifications pertaining to this experiment has been outlined in section 3.3.11.1.

One of the reasons for proceeding with exploring the viability of MIP-embedded filter sheets is that the application of polymer powder has led to extremely heterogeneous results in terms of 4-EP binding (between 30% and 80% for identical experimental conditions) that the polymer producer has been reporting in internal testing conditions.

The first objective of this experiment is to assess if working with MIP2 embedded filter sheets helps with reducing this variability, therefore obtaining more homogeneous results.

The second objective is to assess whether an improved filtration setup will help make the polymer regeneration step more effective and less variable. In previous experiments a removal of 40-60% of bound 4-EP has been possible.

The third objective is to evaluate how the 4-EP content is reduced throughout six successive treatment and regeneration cycles. There are several data points from previous experiments that can be used as comparison points.



**Figure 77.** The level of 4-ethylphenol present in the wine after each successive filtration cycle, after using either the MIP2 embedded filter, a blank filter or a MIP1 powder precoated filter

As can be seen from Figure 77, the starting concentration of the red wine was 2.28 mg/L 4-ethylphenol. Throughout the six cycles, it is evident that the blank filter had no effect on the 4-EP concentration. However, the two MIP containing filters clearly reduced the 4-EP concentration. Whereas the MIP1 precoated filter ultimately achieved a 30% reduction in 4-ethylphenol over the six cycles, the reduction using the MIP2 embedded filter sheets was much more modest, at 13.6%. So, the newer version of the polymer, in the context of its embedding into a filter sheet underperformed when compared to the older version of the polymer which had simply been pre-coated onto a standard filter sheet.

In terms of color characteristics, both color intensity and color nuance were largely unaffected by the treatments.

The total phenolic content was slightly lowered by all treatments, albeit less than in previous experiments. That the regeneration step with alkaline rinse had its concentration reduced from 2M to 0.1M, as per polymer producer's recommendations, could have played a role in preventing the color from eluting out of the filter sheets once used, which further hindered their successive absorption of more and more phenolic compounds with each successive filtration cycle, as was seen in previous experiments (sections 4.6.8 and 4.7).

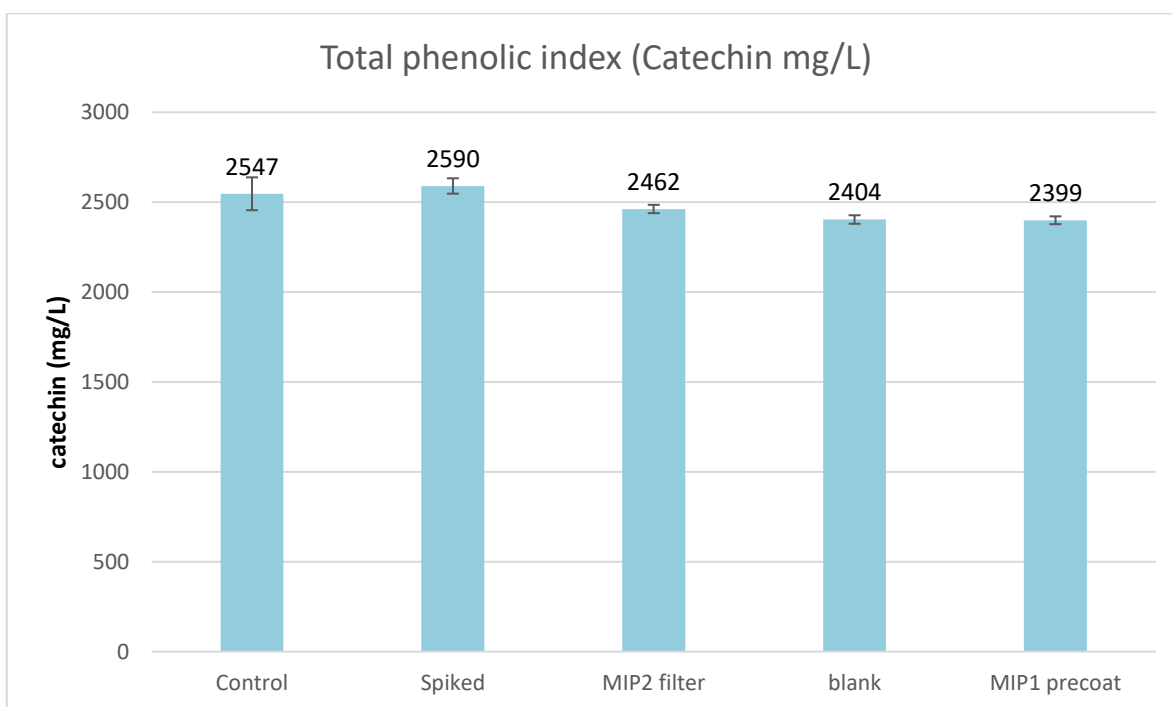


Figure 78. Total phenolic content of the treated samples

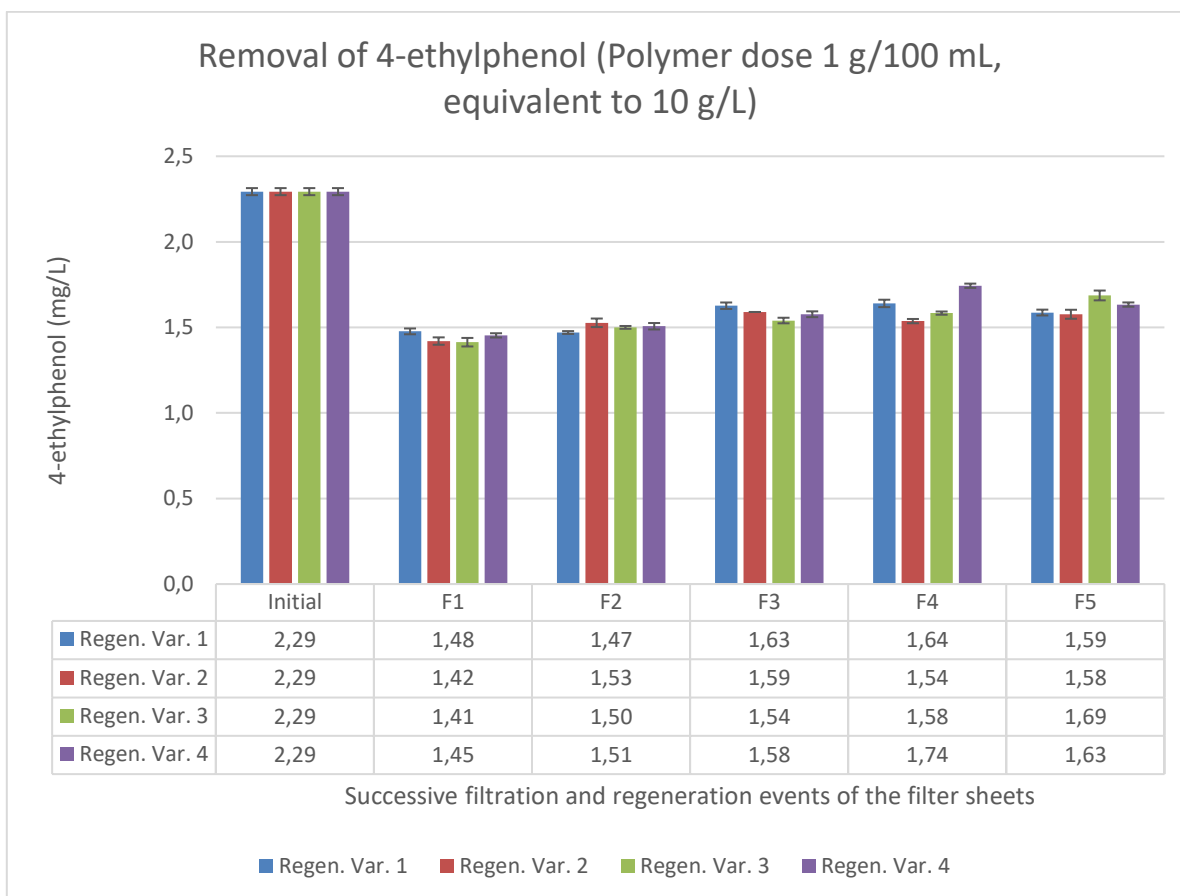
Overall, as can be seen in Figure 78, the strongest reduction of total phenolic content was observed in the MIP1 precoat, a reduction of about 7.4%, compared to that of the spiked sample, with the MIP2 filter removing slightly less than that.

#### 4.11.2 Assessment of different elution and regeneration protocols

The methodology and further clarifications pertaining to this experiment have been outlined in section 3.3.11.2.

The topic of regeneration efficiency was brought into question, seeing how data from the polymer producer showed that the concentration of the NaOH in the second rinse could be lowered from 2M to 0.1M and still maintain its function of removing the bound 4-ethylphenol from the polymer.

The results of this experiment can be seen in Figure 79. First thing to notice is, as this experiment has a higher ratio between polymer quantity and wine volume, the 4-EP removal looks much stronger than in previous experiments, at a little over 36%, from the first filtration cycle. However, no clear differences or trend could be observed from comparing the different regeneration variants between themselves. It seems that the standard regeneration cycle is enough for adequately returning the polymer to an optimal state for the next treatment cycle. All other variations or improvements on the standard protocol did not yield any meaningful improvement. What can also be discerned from this figure is that, although only five cleaning cycles were tested, there is a noticeable linear tendency for the polymer to bind less and less 4-EP than it did in previous cycles. This data points to a limit in the number of times the polymer can be regenerated and re-used. Once that limit is reached, either the polymer's functionality is exhausted or a different, more drastic kind of regeneration must be attempted.



**Figure 79. Results of 4-EP removal from MIP2-embedded filters which have been cleaned with four different regeneration protocol variants**

Differences in wine color between the initial and treated wines were marginal, as can be seen from Table 35.

**Table 35. Differences in color characteristics, total phenolic index, acidity, and pH for a red wine before and after MIP2 filtration**

	<b>Before MIP2 filtration</b>	<b>After MIP2 filtration</b>
<b>Color intensity</b>	5.62	5.43
<b>Color nuance</b>	0.9	0.85
<b>Total phenols</b>	2037 mg/L catechin	1906 mg/L catechin
<b>Total acidity</b>	5.1 g/L tartaric acid	5 g/L tartaric acid
<b>pH</b>	3.7	3.8

## 4.12 Final filtration of wine with polymer-embedded filter plates

Ultimately, the purpose of receiving the MIP2 embedded filter plates was to use them to perform a small-scale filtration of red wine, not in laboratory, but in normal winery conditions. The subsequently resultant wines would be bottled and stored. Later they would be subjected to a sensory analysis with a trained tasting panel, as well as to chemical analysis at the university of Geisenheim.

### 4.12.1 Wine physical-chemical analysis

The methodology and further clarifications pertaining to this experiment have been outlined in sections 3.3.12. and 3.2.

A series of chemical analyses were performed on the resulting wines. Some of the main relevant oenological parameters analysed can be seen in Table 36.

No relevant changes in alcohol content or pH can be seen. Total acidity remained quite constant (accounting for instrumental measurement variations), except for the last sample, treated with the polymer bead column, in which case total acidity dropped by 0.4 g/L from the spiked wine sample.

Looking at the changes in total phenols, as was expected, even the standard filtration had the effect of absorbing some, amounting to a 10% reduction in the case of the naturally Brett contaminated Spätburgunder, and a 3-4% reduction in the case of the Merlot wine (either spiked or clean).

The treatment with a MIP-embedded filter sheet caused a slightly stronger reduction in total phenols compared to the standard Seitz™ EK filter sheet, which, considering the size of standard deviations for this type of analysis, amounts to a small effect: 13% total phenol reduction in the case of the Spätburgunder, and a 4% removal in the case of the Merlot wine. The NIP containing filter sheet removed the same amount of total phenols as the MIP containing filter sheet.

A slightly stronger reduction in total phenols comes, as would be expected, from the samples treated with activated carbon, with a 15% reduction in the case of the Spätburgunder, and a

5.6% reduction in the case of the Merlot wine. However, the differences are not as high as one might expect, considering the dose of 20 g/ hL (200 mg/L and 72h contact time). Another conclusion easily drawn from these observations is that the phenolic content of the Merlot wine was more stable than that of the Spätburgunder wine, and was, therefore, less susceptible to be removed by contact with filter sheet materials. This is in accord with literature observations that a *Brettanomyces* infection (as is the case of the Spätburgunder wine) will tend to decrease the stability of a red wine's colouring matter, of which phenolic compounds represent a substantial component (Mansfield et al., 2002).

The strongest reduction of total phenols was observed in the sample treated with a column filled with MIP2 beads. This was expected as the dose of polymer was much higher, and the contact time was longer, facilitating a greater absorption of phenolic compounds. With a total phenolic content of 1239 mg/L, the sample treated with polymer beads had more than a third of its total phenols removed. This result clearly points to non-specific binding of phenols to the polymer beads, facilitated by the long contact time and high dosage of material.

Finally, the total dry extract values remain quite constant, with minimal changes between different treatments and the starting points. The only exception is the sample treated with the polymer bead column, for which this treatment led to a loss of more than one gram per litre of total dry extract.



Table 36. Main oenological parameters of the treated wines

Wine code	Alcohol %	pH	Total acidity g/L tartaric acid	Total phenolic index mg/L Catechin	Total dry extract g/L
B	12.3	3.2	5.8	1652.0 ± 4.9	23.8
BE	12.3	3.4	5.7	1486.0 ± 23.3	23.5
BM	12.3	3.3	5.6	1440.0 ± 19.6	23.5
BN	12.3	3.3	5.6	1447.7 ± 9.7	23.4
BC	12.2	3.3	5.5	1407.0 ± 10.2	23.3
K	13.2	3.6	5.3	1995.0 ± 5.6	27.1
KE	13.1	3.6	5.3	1917.7 ± 20.9	26.6
KM	13.1	3.6	5.3	1893.0 ± 21.3	26.7
S	13.1	3.6	5.2	1950.7 ± 26.6	26.7
SE	13.0	3.6	5.1	1896.3 ± 9.7	26.5
SM	13.0	3.6	5.1	1881.3 ± 43.6	26.5
SC	13.0	3.6	5.1	1846.7 ± 36.2	26.4
SMCOL	12.8	3.6	4.8	1239.3 ± 16.8	25.3

In addition to the main oenological parameters, a breakdown of the main organic acids present in these wines is shown in Table 37, as analysed using a WineScan™ SO<sub>2</sub>, which relies on FTIR spectral measurements (Foss Analytics, Denmark).

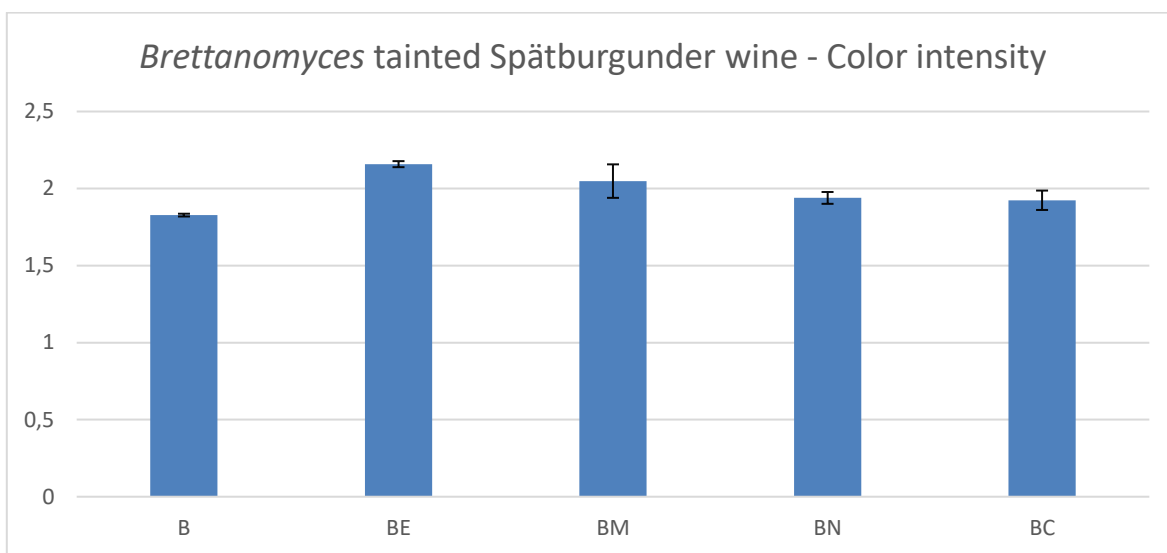
Looking at these organic acids, the first observation is that the *Brettanomyces*-contaminated wine had a higher volatile acidity than the untainted wine, which is in accord with the literature on *Brettanomyces* defects, where increases in acetic acid are often associated with an infection. Broadly speaking, none of the major organic acids present in the wine was affected by any of the treatments, as the variations seen in the Table are quite minor, and common in FTIR spectral analyses.

Another parameter measured with FTIR was the total glycerine content, which was unaffected by any of the treatments in any wine (results not shown).

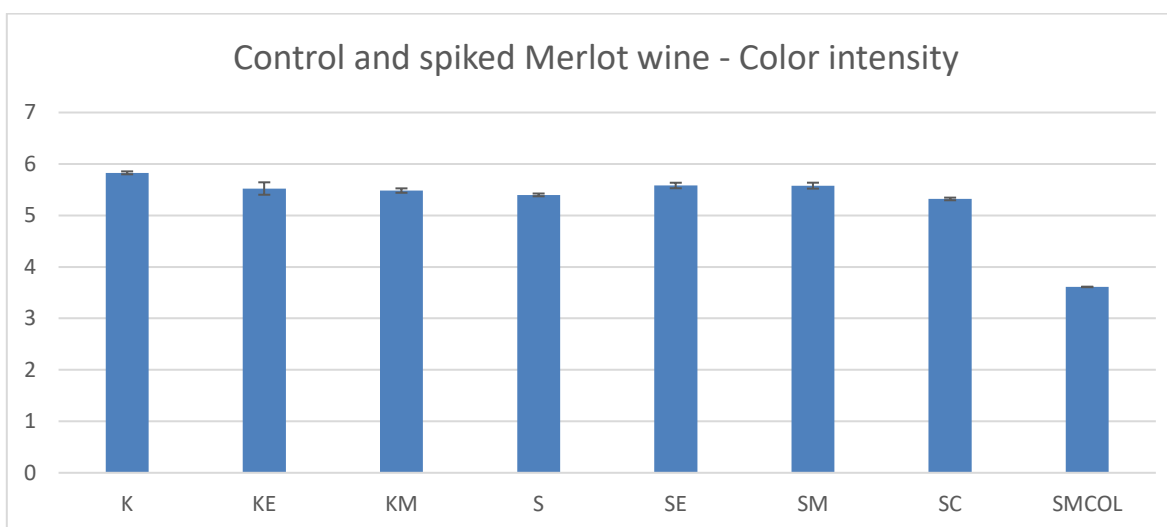
Table 37. Organic acid content of wines under the different treatment conditions

Wine code	Total acids g/L	Tartaric acid g/L	Malic acid g/L	Lactic acid g/L	Acetic acid g/L	Other organic acids g/L
B	5.6	1.9	0	2.4	0.7	0.6
BE	5.3	2.0	0	2.3	0.8	0.2
BM	5.3	1.9	0	2.3	0.8	0.3
BN	5.3	1.9	0	2.3	0.8	0.3
BC	5.2	1.9	0	2.3	0.8	0.2
K	4.9	2.3	0.1	1.6	0.5	0.4
KE	4.9	2.3	0.1	1.6	0.4	0.5
KM	5.0	2.2	0.1	1.8	0.5	0.4
S	5.0	2.1	0.1	1.8	0.5	0.5
SE	4.9	2.1	0.1	1.8	0.4	0.5
SM	5	2.1	0.2	1.8	0.5	0.4
SC	4.9	2.1	0.1	1.8	0.5	0.4
SMCOL	4.8	1.9	0.2	1.7	0.5	0.5

Regarding wine colour changes as induced by the treatments, no meaningful change was observed in colour nuance (results not shown). When looking at the *Brettanomyces*-tainted Spätburgunder wine treatments (Figure 80), one can observe that all treatments led to a slight increase in colour intensity. This may be because the wine had been left unfiltered, and essentially all treatments included a filtration step, which may have helped to reduce any slight turbidity or haze present, thereby conferring a higher colour intensity to the wines. Looking at the Merlot wines (Figure 81), the colour intensities of the treated wines do not deviate strongly from the control or the spiked untreated variants. The one outlier is the MIP2 column treated sample, where the colour intensity was reduced by almost one third. This reduction of one third of the colour intensity is congruent with the reduction of one third of the total phenolic content if this treated sample, as seen in Table 36.



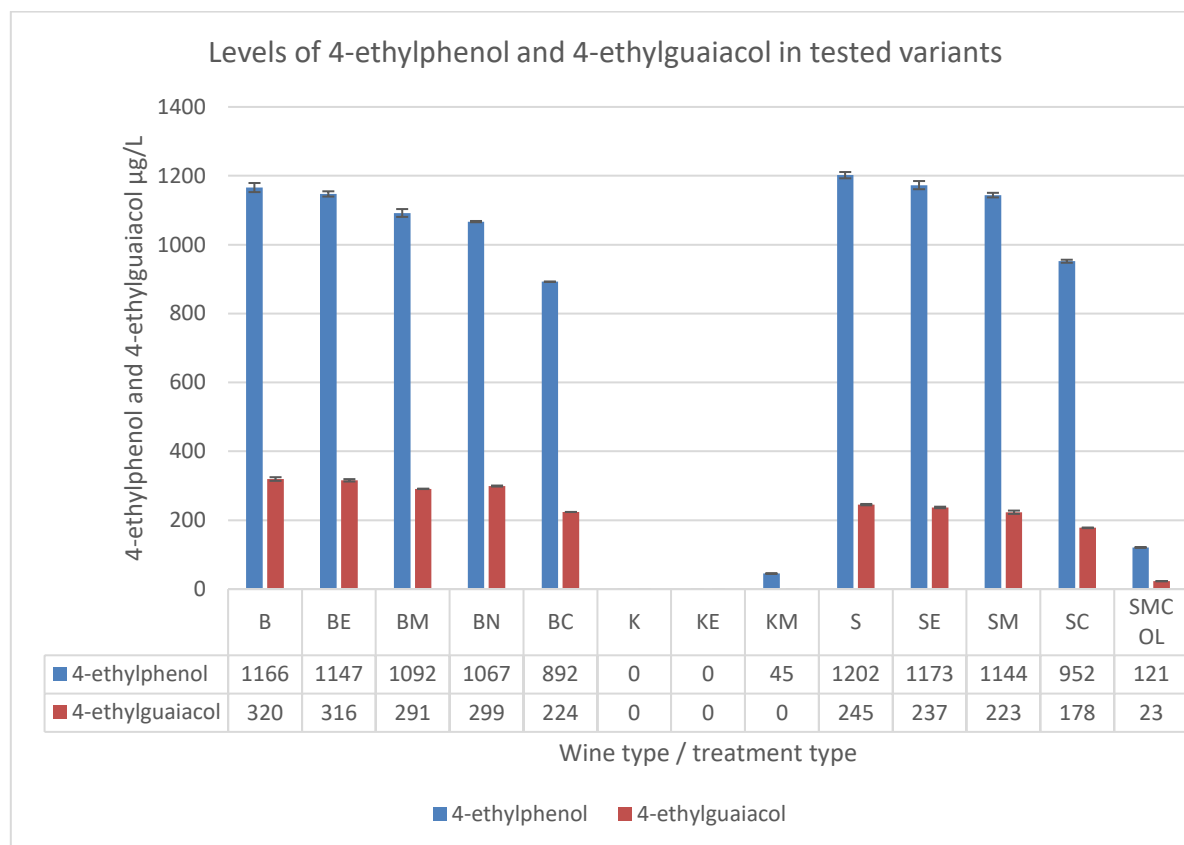
**Figure 80.** Color intensity of *Brettanomyces* tainted Spätburgunder wines (dimensionless quantity)



**Figure 81.** Color intensity of control and spiked Merlot wines (dimensionless quantity)

By way of contrast, in the work of Teixeira et al. (2015), the MIP treatment showed a loss of colour compared to the treated sample and the NIP treatment exhibiting the lowest colorant intensity. Such results were not encountered in this work. Overall the MIP recipes studied here did not affect the colour of the wine, with the exception of the column treatment, for the reasons discussed above.

Next, in Figure 82 are highlighted the changes in 4-ethylphenol and 4-ethylguaiacol of the different treatments.



**Figure 82. Levels of 4-ethylphenol and 4-ethylguaiacol after filtration with polymer-embedded filter sheets**

To begin, comparing the samples filtered with standard Seitz™ EK filter sheets and the non-filtered samples reveals minimal to no volatile phenol reduction, as was expected. In the case of the Spätburgunder, the filter sheets containing imprinted polymer (MIP2 embedded) achieved a 6.4% 4-EP reduction and a 10% 4-EG reduction, and those containing non-imprinted polymer (NIP2 embedded) achieved an 8.5% 4-EP reduction and a 6.6% 4-EG reduction. In the case of the Merlot spiked wine sample, the MIP2-embedded filtration achieved a 4.9% 4-EP reduction and a 9% 4-EG reduction.

These levels of 4-EP removal by the imprinted polymer are slightly better than those observed with the same kind of embedded filter sheets in a lab scale experiment (section

4.11.1) in which, under similar conditions, a reduction of only 3.1% was observed when using the MIP2 embedded filter sheets.

On the other hand, the results obtained with just MIP2 powder pre-coated onto standard filter sheets (section 4.10) were clearly superior, with 1 g of MIP2 having the capacity to remove about 300  $\mu\text{g}$  4-EP per litre of wine with an average initial 4-EP content of 1 mg/L. In this case, the removal was about 70  $\mu\text{g}$  4-EP per litre of wine, for the same amount of polymer. This indicates an almost fourfold decrease in polymer effectiveness once the MIP becomes embedded in filter sheets, compared to its normal powder form.

Next, the results from using activated carbon treatment and sterilizing grade filtration will be discussed. In the case of the Spätburgunder, treatment with activated carbon resulted in a 23.5% reduction in 4-ethylphenol and a 30% reduction in 4-ethylguaiacol. In the case of the Merlot, the reduction in 4-ethylphenol was 21%, and the reduction in 4-ethylguaiacol was 27.4%. It is evident that the activated charcoal treatment was much more effective than the polymer (either imprinted or non-imprinted) treatment at removing volatile phenols.

The strongest 4-EP and 4-EG removal comes from the column with MIP2 beads, with a 90% reduction of 4-EP and a 90% reduction of 4-EG. These impressive results were made possible by the extremely high ratio between polymer and wine since the column contained 84 g polymer and would be allowed to treat 2.25 L of wine. This corresponds to a dose of almost 37 g polymer per litre of wine, which is, of course, extremely high and outside the potential scope of any reasonable practical application.

The last result that is worthy of highlighting from this Figure is, perhaps, the most interesting and revealing of all. When a clean wine with zero volatile phenols is filtered with the MIP2 embedded filter sheets, its level of 4-EP rises to 45  $\mu\text{g}/\text{L}$ . This indicates that the MIP2 embedded filter sheets represent a source of contamination with 4-EP, the very substance the polymers are designed to remove from a wine matrix. It is known that the template used for molecular imprinting is often the very same analyte later intended to be removed by the polymer. Ideally, during the solvent washing phase of the polymer, all remnants of template are removed, but this is not always a guarantee. This result points to a weakness in the preparation method of this molecularly imprinted polymer, which results in contaminating a

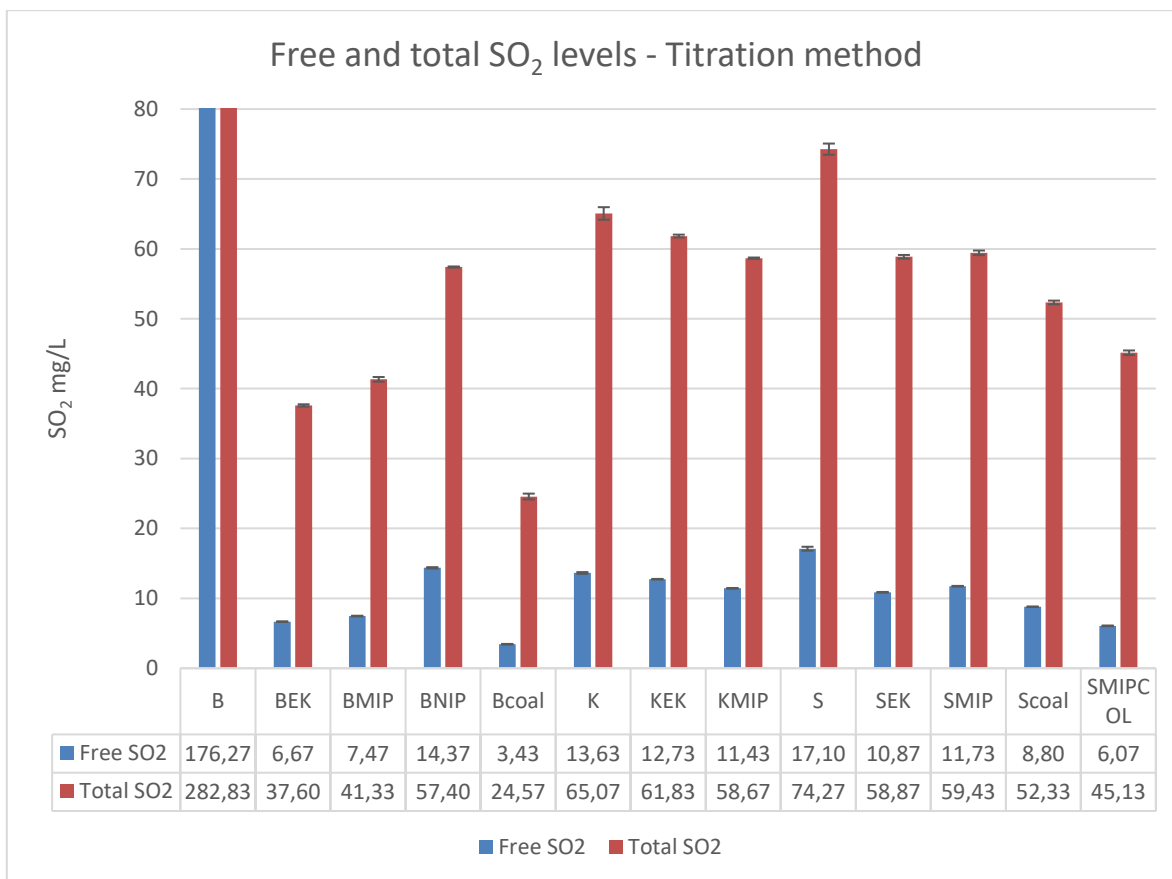
clean wine. If one considers that 50 litres of wine were filtered, and that their concentration at the end was 45 µg/L, then a total of 2.25 mg 4-ethylphenol leached off the three MIP2-embedded filter sheets. This corresponds to 750 mg 4-ethylphenol per filter sheet. It is possible that this already present 4-ethylphenol in the filter sheets could have played an influencing role in the reduced performance of the polymer embedded in these sheets. Whether the fourfold reduction in efficiency when moving from powder to embedded filter sheets is all due to the change in medium or (at least partly) due to 4-EP contamination in this batch of polymer remains an open question to be addressed by the polymer producer.

Next, one can see in Figure 83 the overall free and total sulphur dioxide values of the wines in all treatment conditions.

It is interesting to note that the initial *Brettanomyces*-contaminated wine displayed an exceedingly high level of SO<sub>2</sub>, possibly due to a decision to combat the Brett infection in this way. Considering that the pH of this infected Spätburgunder wine was 3.2 and its alcohol content was 12.3%, then just a dose of 15 mg/L of free SO<sub>2</sub> would suffice in guaranteeing at least 0.5 mg/L of molecular SO<sub>2</sub> in the wine, which would be enough to combat the growth of *Brettanomyces* in the medium. The fact that this wine displays such a high initial value of free and total SO<sub>2</sub> indicates that this addition was probably performed after the wine had already been spoiled. Nevertheless, following a single sterilizing grade filtration with Seitz™ EK filter sheets, these initial high values of total and free SO<sub>2</sub> were reduced to much lower levels. These high values (176 mg/L free SO<sub>2</sub> and 282 mg/L total SO<sub>2</sub>) seem not to be artefacts or measurement errors, as they have been corroborated by a separate FTIR analysis (results not shown). In the case of the *Brettanomyces*-tainted Spätburgunder, the charcoal treatment led to the lowest overall values of free and total SO<sub>2</sub>, whereas the MIP2 treatment was closest to the standard sterilizing grade filtration. The NIP2 treatment resulted in the highest free and total SO<sub>2</sub> values in this wine.

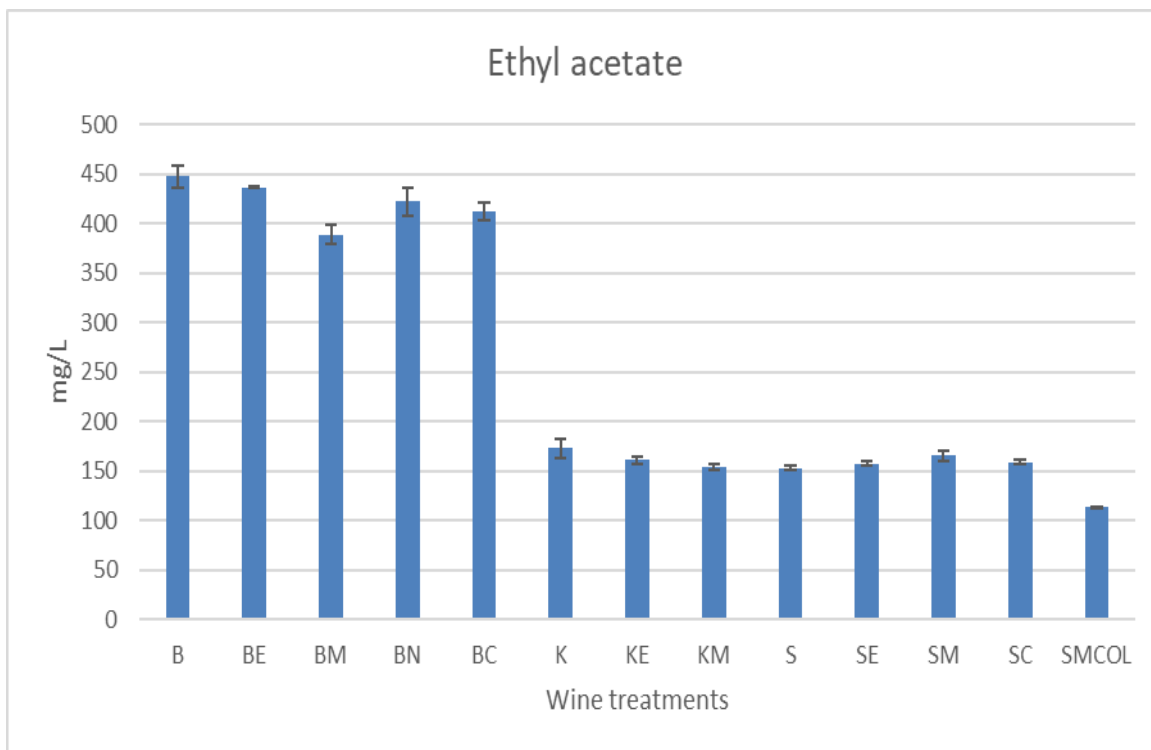
Looking at the Merlot wine, either spiked or non-spiked, one can see small differences between the initial wines (marked either K for control or S for spiked Merlot wine) and the other treatments. The MIP2 and NIP2 treatments were like the sterilizing grade filtration, having minimal effect on free and total sulphur levels. The charcoal treatment led to slightly

lower free and total sulphur levels. The MIP2 beads column treatment determined the lowest levels of free and total SO<sub>2</sub>, but, considering the extremely high dose of the polymer, these losses should better be attributed to the time that the wine spent being slowly processed through the column at a rate of 20 mL/minute which facilitated oxygen uptake, rather than to the higher dose of polymer.



**Figure 83. Free and total levels of sulfur dioxide of treated wines**

Next, one can look at results displaying various esters and aroma compounds present in these wines and the degree to which these treatments have affected their concentration. These esters were quantified using gas-chromatography and mass spectrometry in the department of Microbiology at Hochschule Geisenheim, in Germany.



**Figure 84. Ethyl acetate levels in treated samples**

Ethyl acetate, one of the most common wine esters, was present in higher concentrations in the Spätburgunder wine, where the MIP2 treatment led to the strongest reduction, more than the charcoal treatment (Figure 84). For the Merlot wine, all samples seem unaffected, except for the MIP2 column treatment, where a reduction from approximately 150 to 100 mg/L was observed.

Barring differences in baseline levels of compounds attributable to the differences in grape variety and age of wine (Spätburgunder from vintage 2014 and Merlot from vintage 2017), for compounds such as isobutanol (Figure 85), isoamyl alcohol (Figure 86), 2-methyl-1-butanol (Figure 87) one can observe, that the MIP2 filtration, the MIP2 column treatment and the charcoal treatment showed slightly stronger effects in lowering their concentrations than other treatments, although the differences are, overall, quite marginal. Caprylic acid displays similar effects, albeit more pronounced (Figure 91).



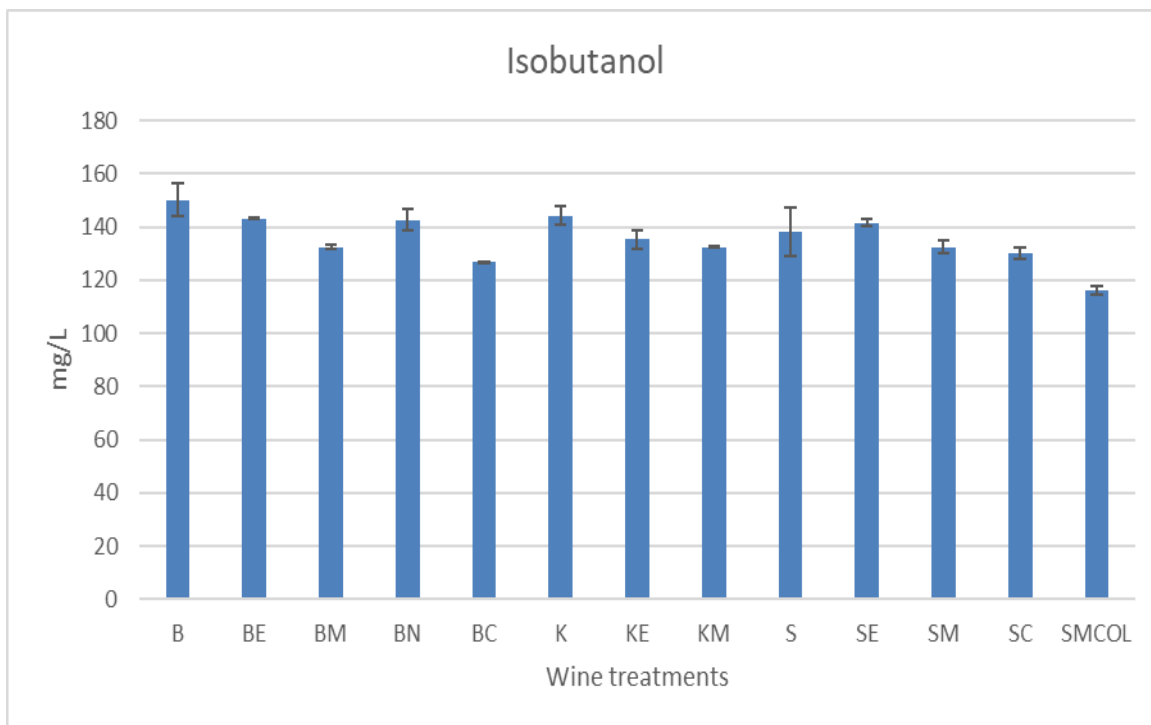


Figure 85. Isobutanol levels in treated samples

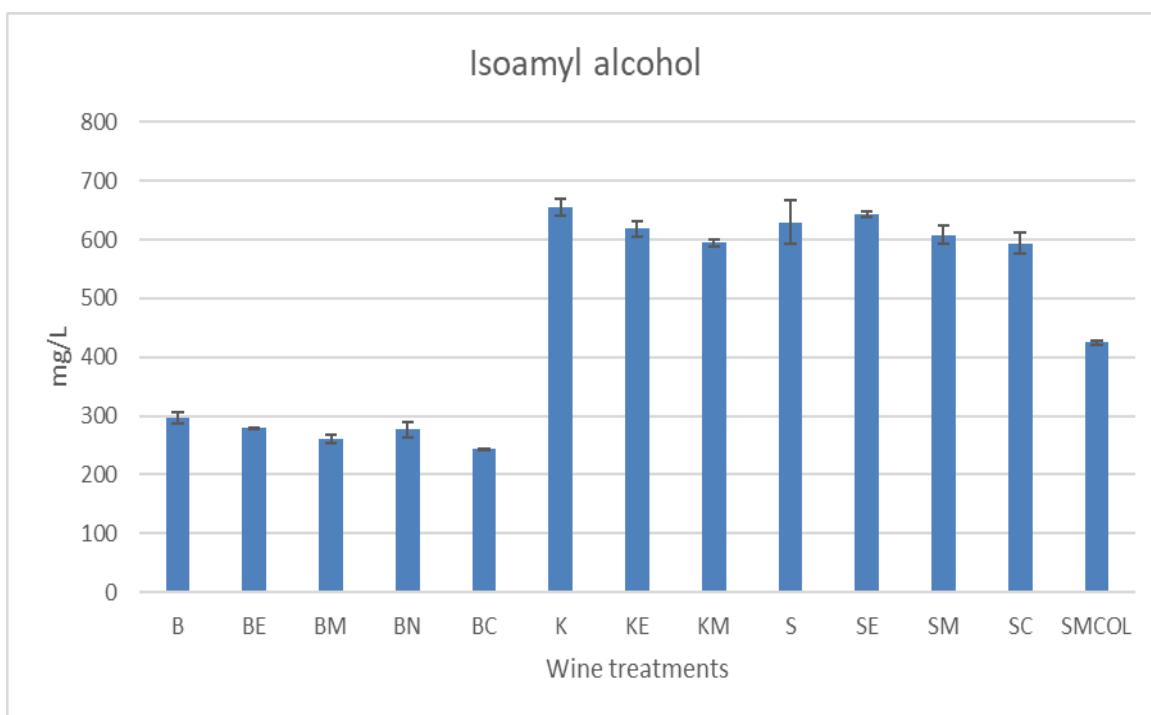


Figure 86. Isoamyl alcohol levels in treated wines

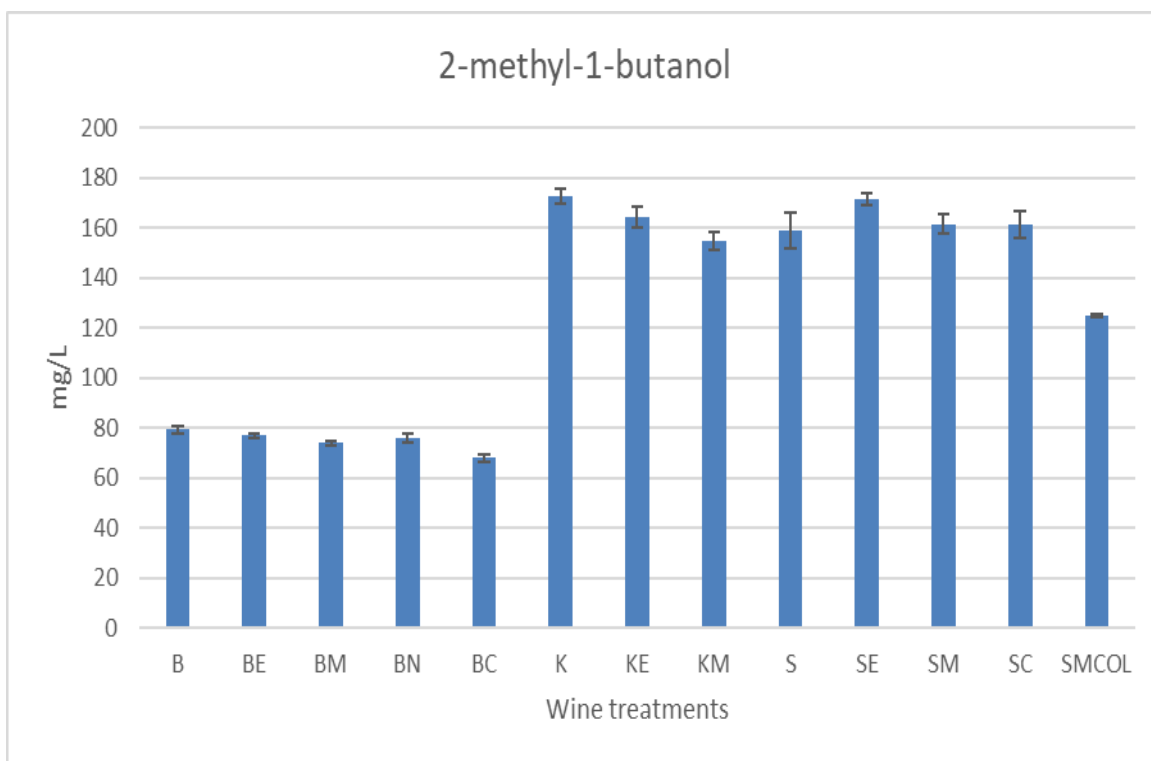


Figure 87. 2-methyl-1-butanol levels in treated wines

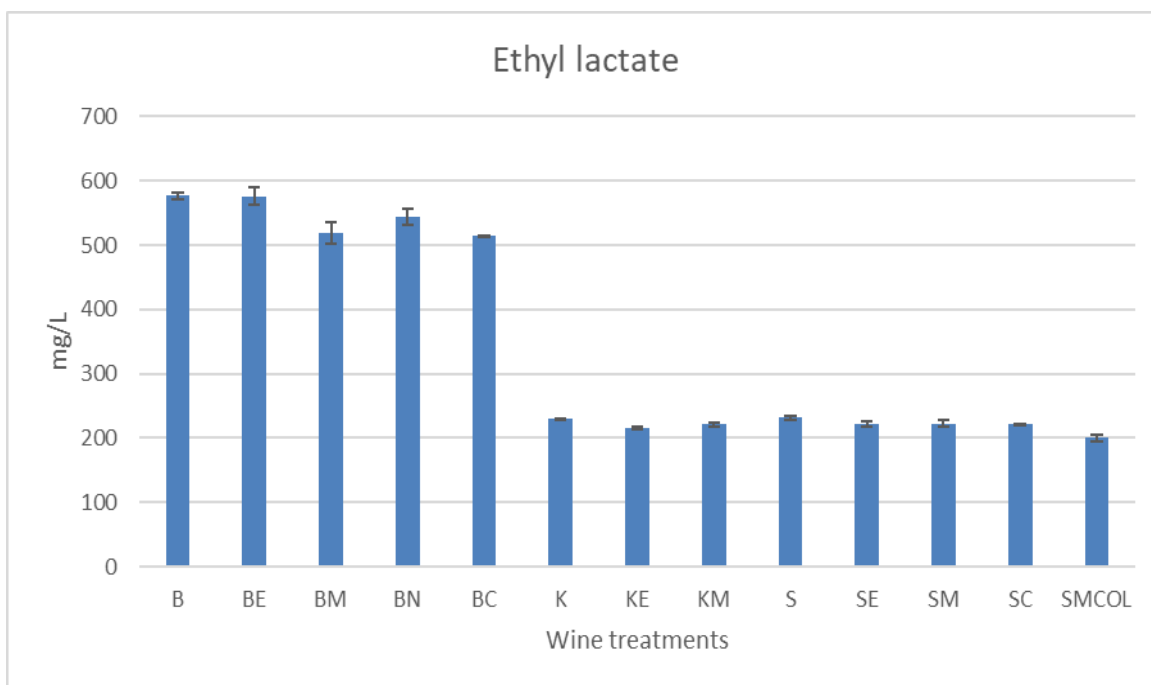
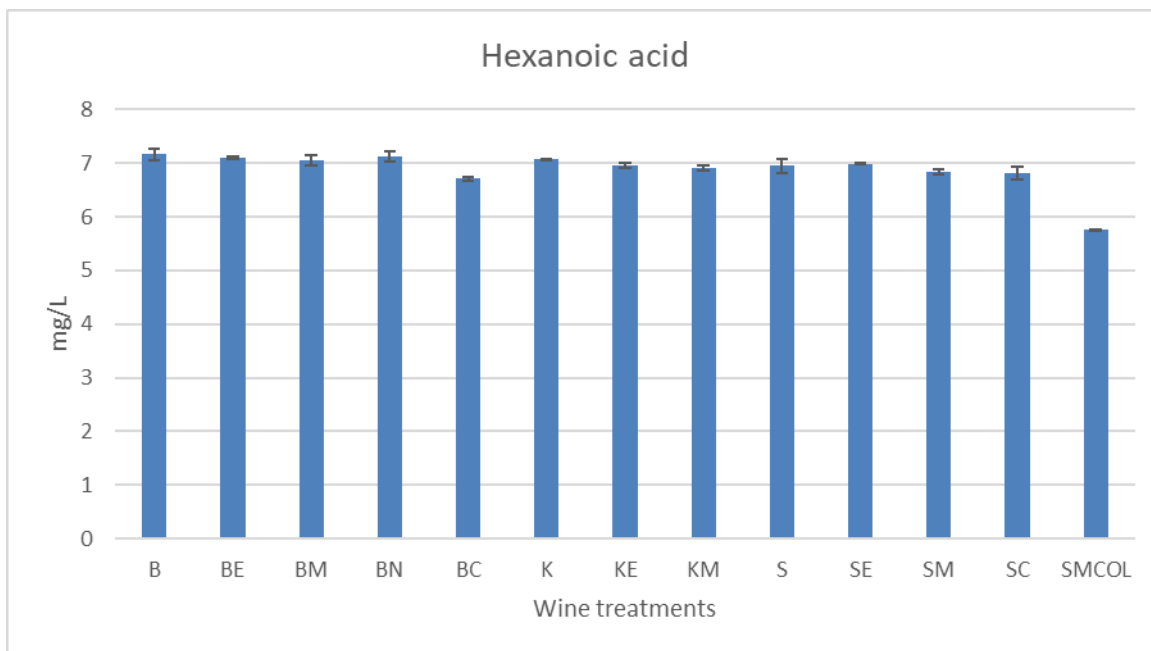


Figure 88. Ethyl lactate levels in treated wines



**Figure 89. Hexanoic acid levels in treated wines**

For compounds such as ethyl lactate (Figure 88) and hexanoic acid (Figure 89), the treatments did not seem to have any meaningful effect, the concentrations of these compounds remaining quite stable, except for slightly stronger reductions due to the MIP2 column treatment. This is also the case for ethyl propionate (Figure 93), isovaleric acid (Figure 95), hexanol (Figure 96), 2-hydroxy-4-methyl-valerianic acid ester (Figure 99) and diethyl succinate (Figure 101).

In the case of compounds such as 2-phenyl ethanol (Figure 90), one can observe that the MIP2 column treatment had the most dramatic effects, removing almost half of that compound, while all other treatments left this compound unaffected.

For caprylic acid (Figure 91), the treatments display similar effects as in the cases of isobutanol, isoamyl alcohol and 2-methyl-1-butanol, albeit more pronounced. Indeed, here the NIP2 shows a clear reduction effect, and the charcoal treatment removed clearly more than the MIP2 treatment. The MIP2 column treatment still displays the strongest reduction. Phenylethylacetate was also a similar case (Figure 92), where almost all treatments, except for the standard sterilizing grade filtration using Seitz™ EK filter sheets, resulted in quite

strong reductions in this compound. The strongest were due to the MIP2 column treatment, followed by the charcoal treatment, MIP2 filtration, and finally NIP2 filtration.

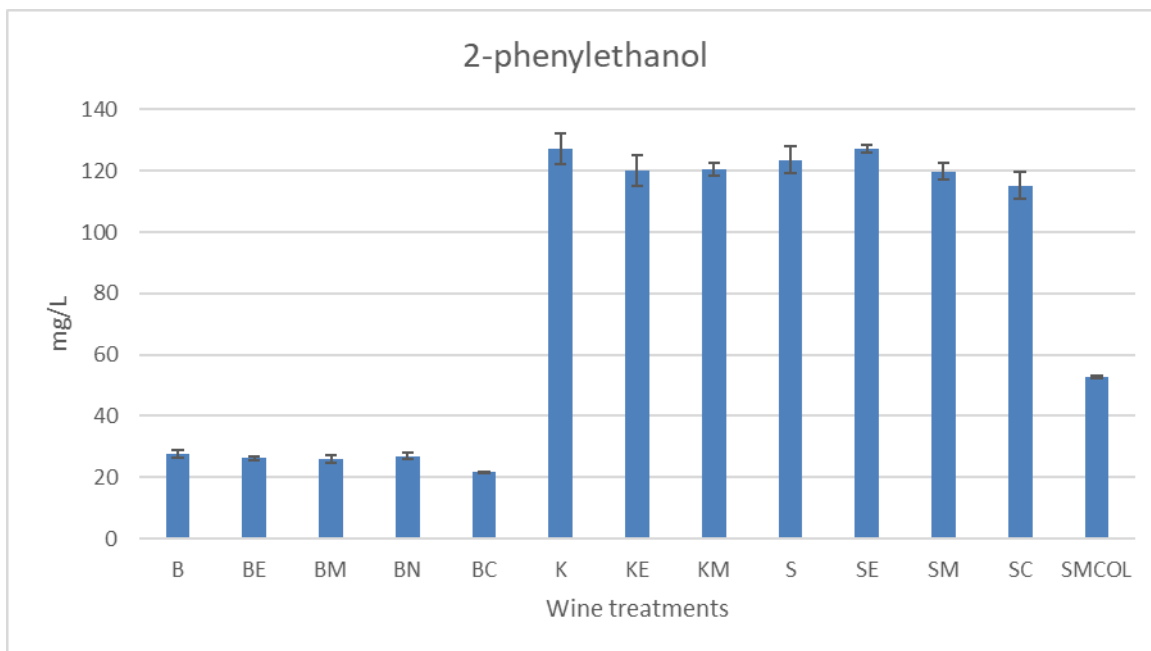


Figure 90. 2-phenylethanol levels in treated wines

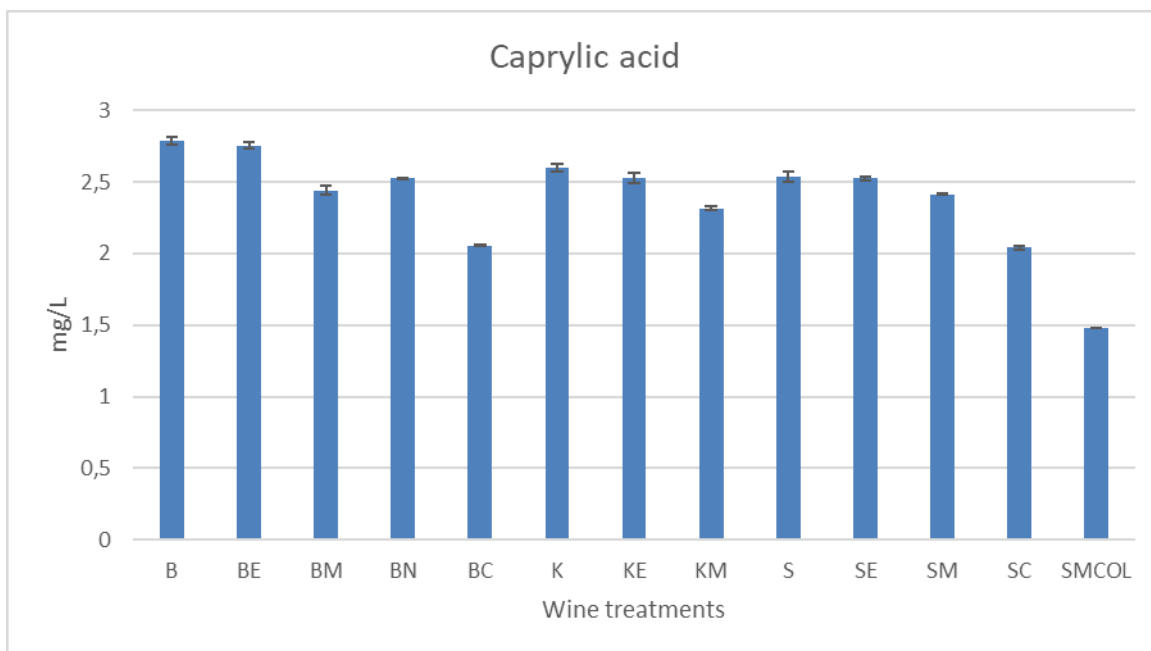


Figure 91. Caprylic acid levels in treated wines

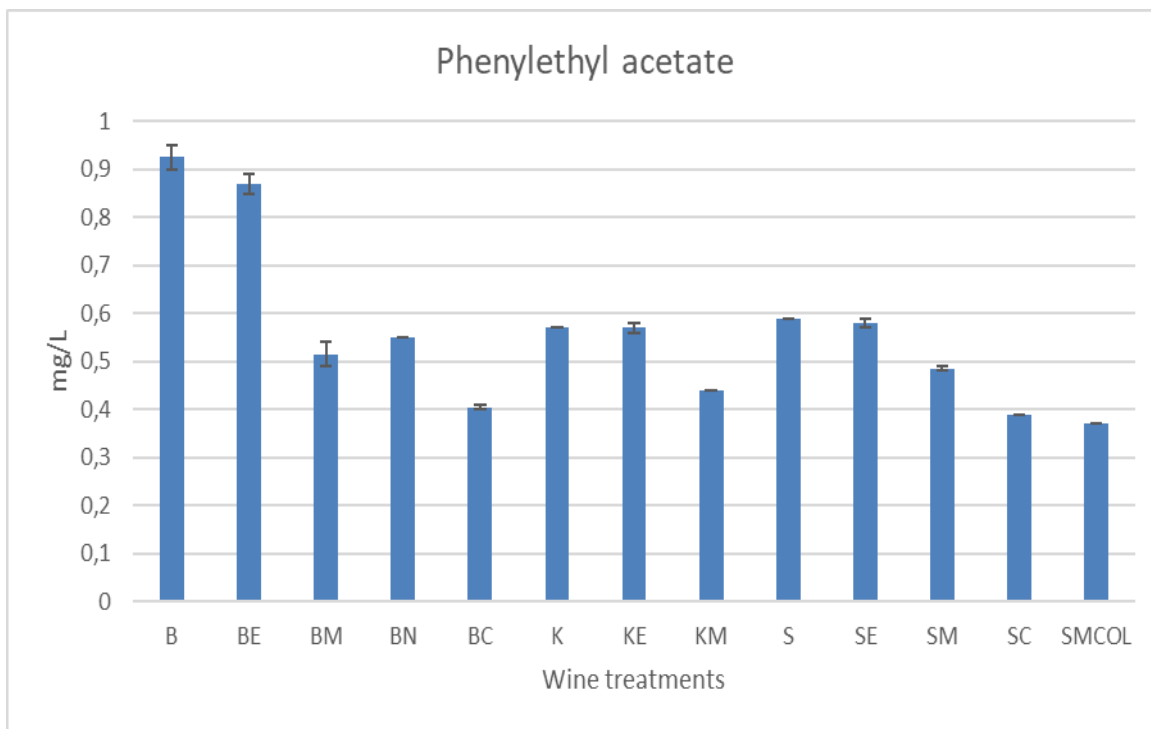


Figure 92. Phenylethyl acetate levels in treated wines

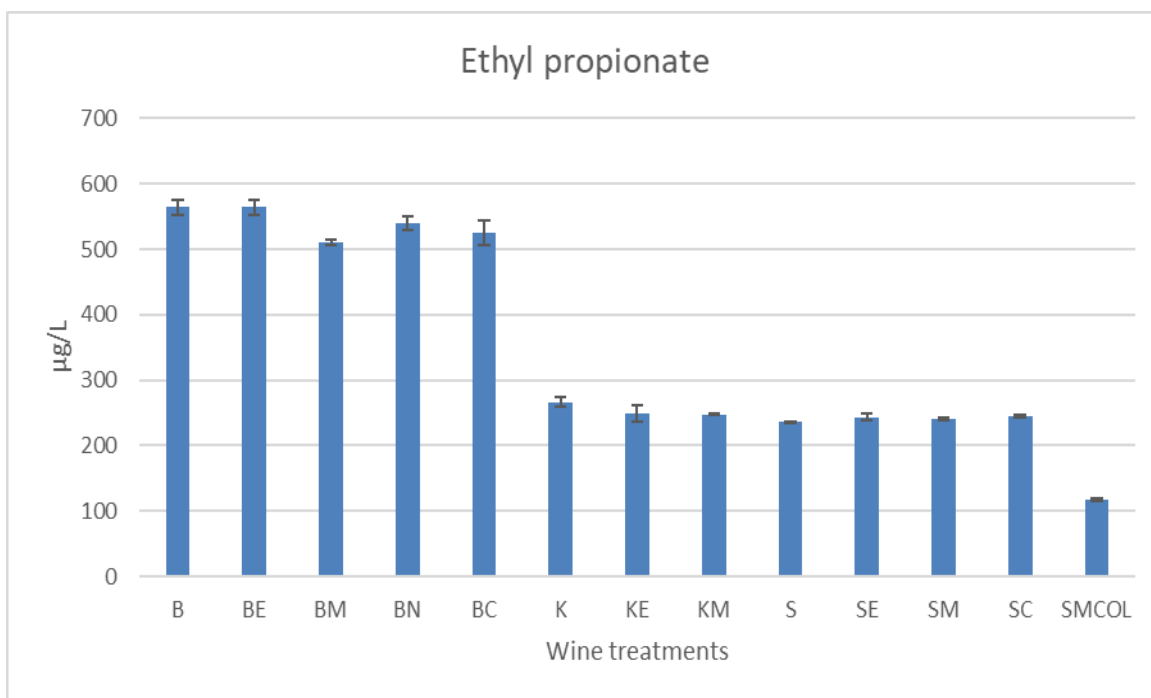


Figure 93. Ethyl propionate levels in treated wines

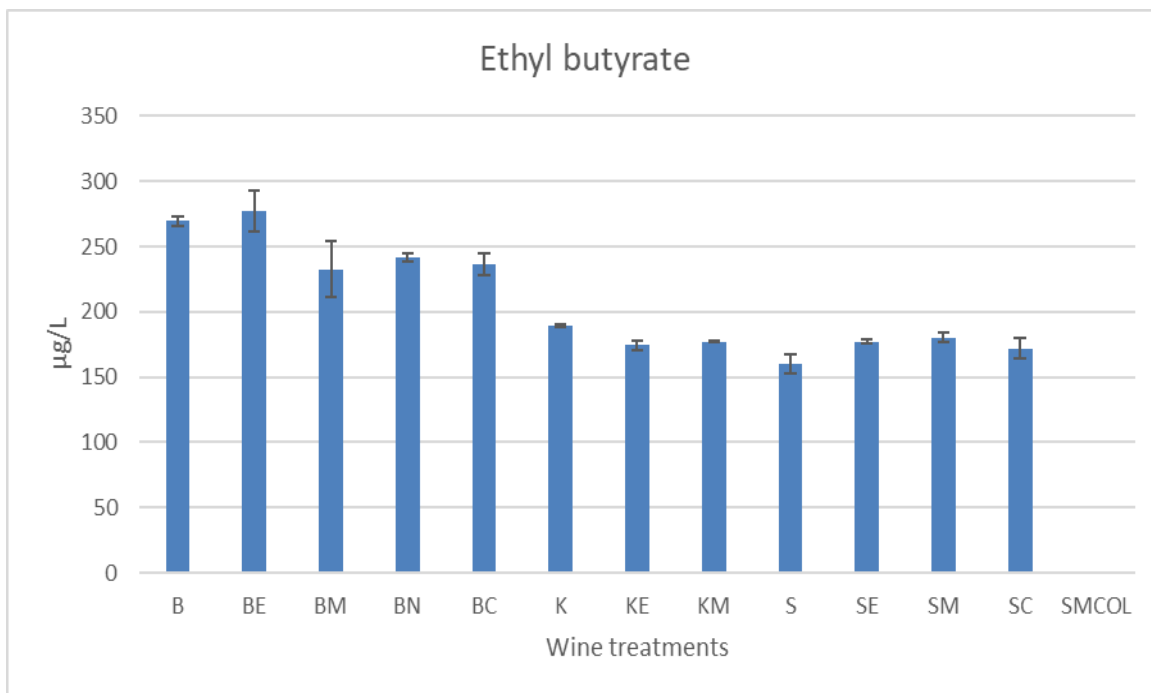


Figure 94. Ethyl butyrate levels in treated wines

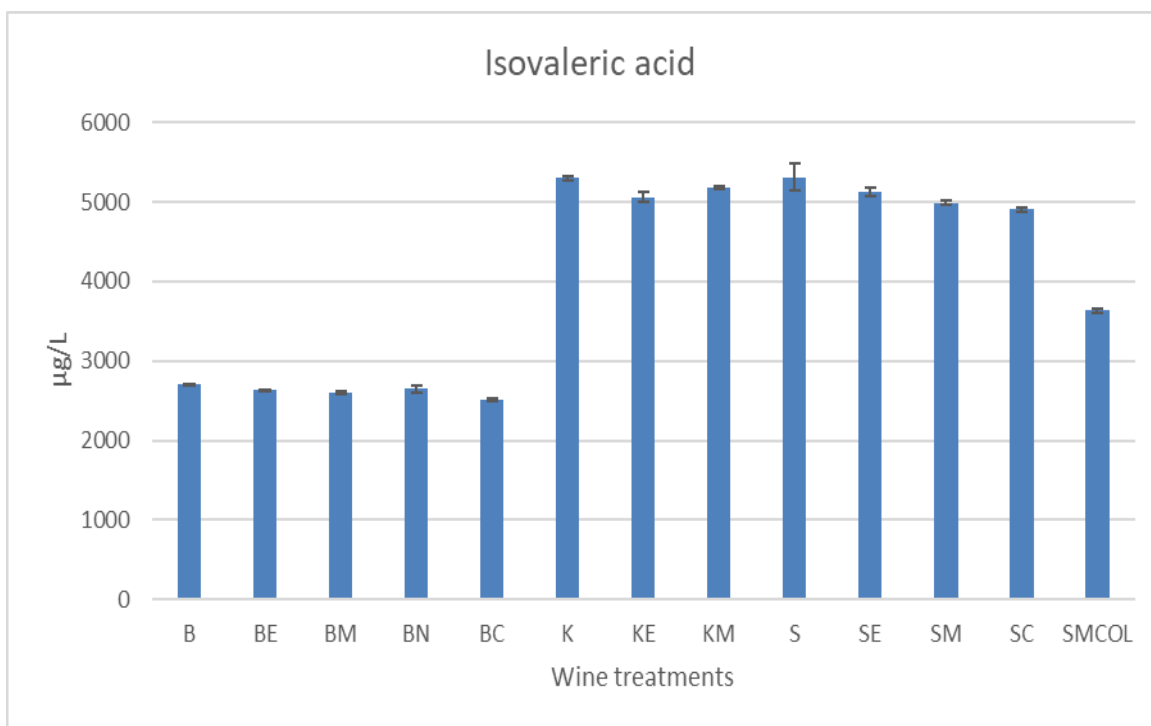
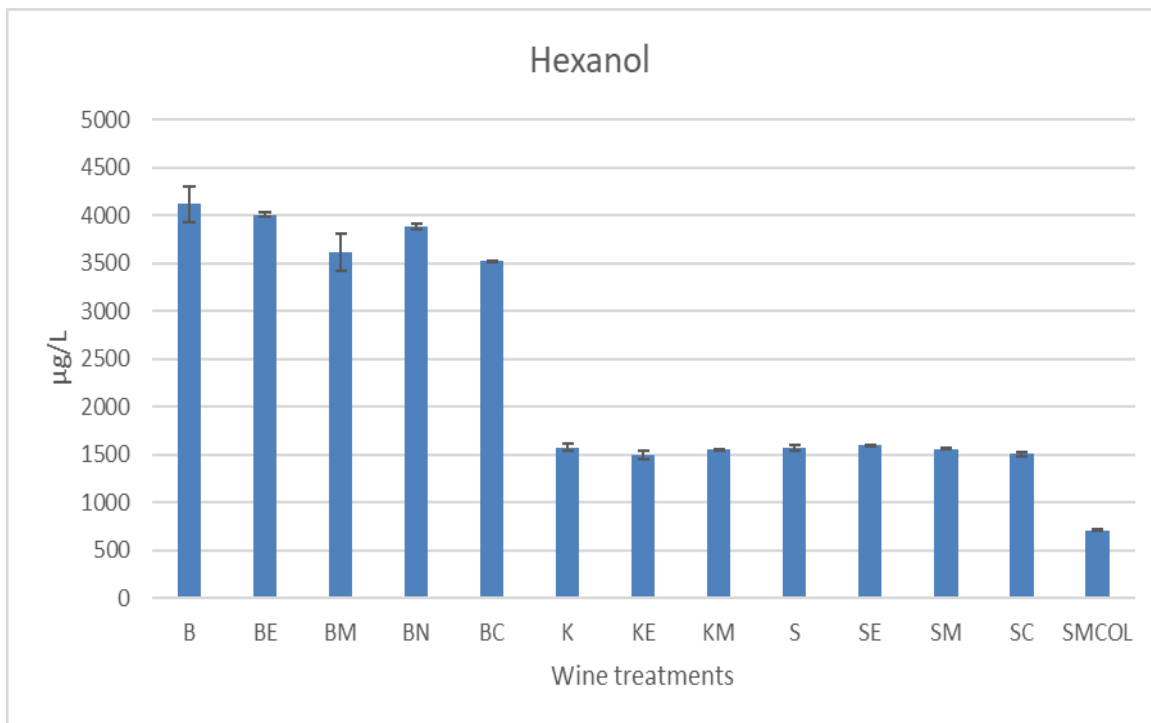


Figure 95. Isovaleric acid levels in treated wines

Ethyl butyrate saw only slight reductions from the MIP2 filtration, in the case of the Spätburgunder wine. The MIP2 column treatment eliminated this compound from the wine matrix (Figure 94). This is also the case for isoamyl acetate (Figure 97) and 2-methylbutyl acetate (Figure 98), both esters, responsible for fruity aromas in wines and were removed in totality by the column treatment.



**Figure 96. Hexanol levels in treated wines**

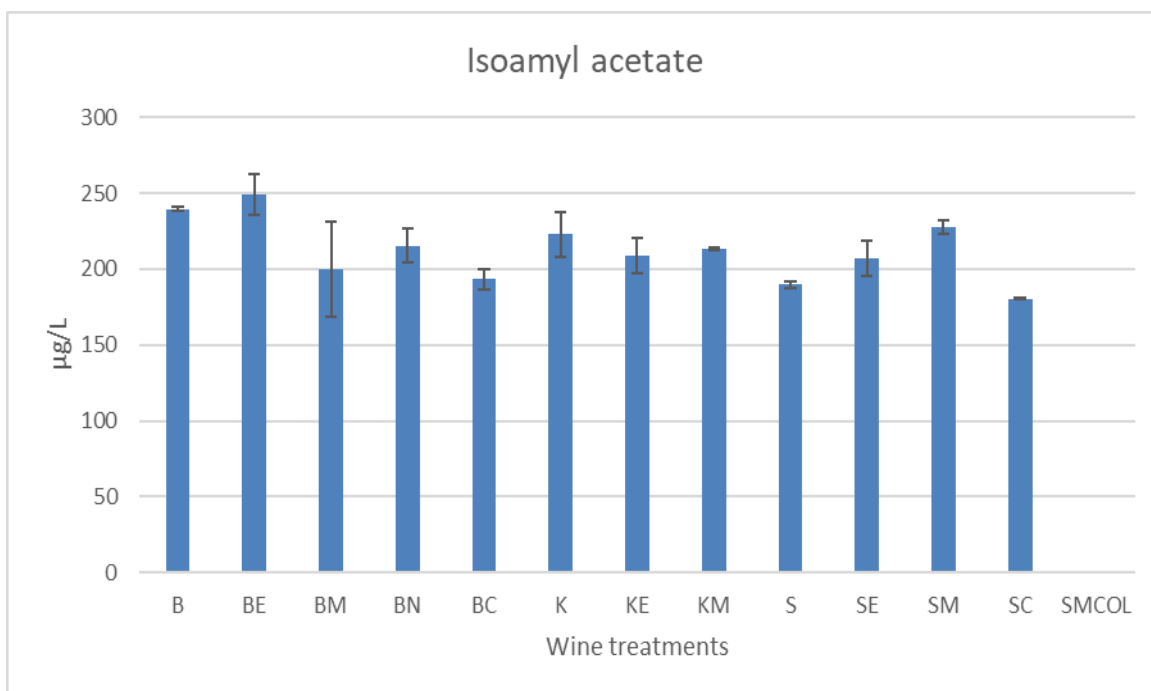


Figure 97. Isoamyl acetate levels in treated wines

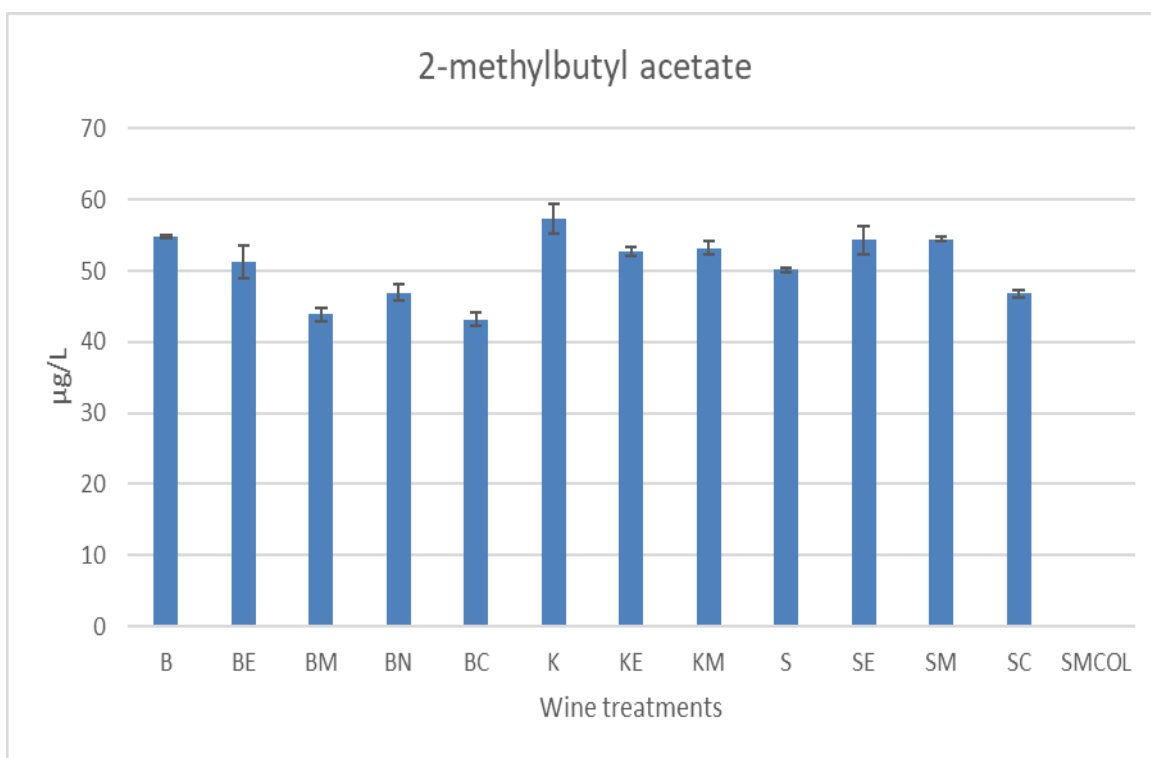


Figure 98. 2-methylbutyl acetate levels in treated wines



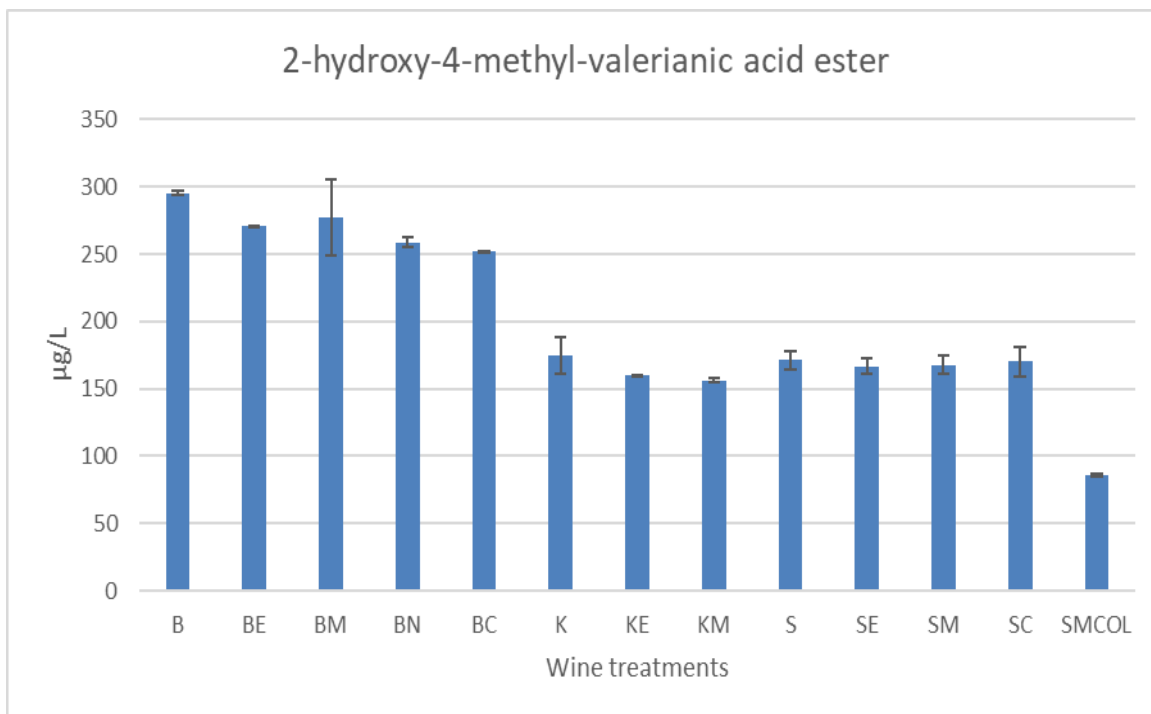


Figure 99. 2-hydroxy-4-methyl-valerianic acid ester levels in treated wines

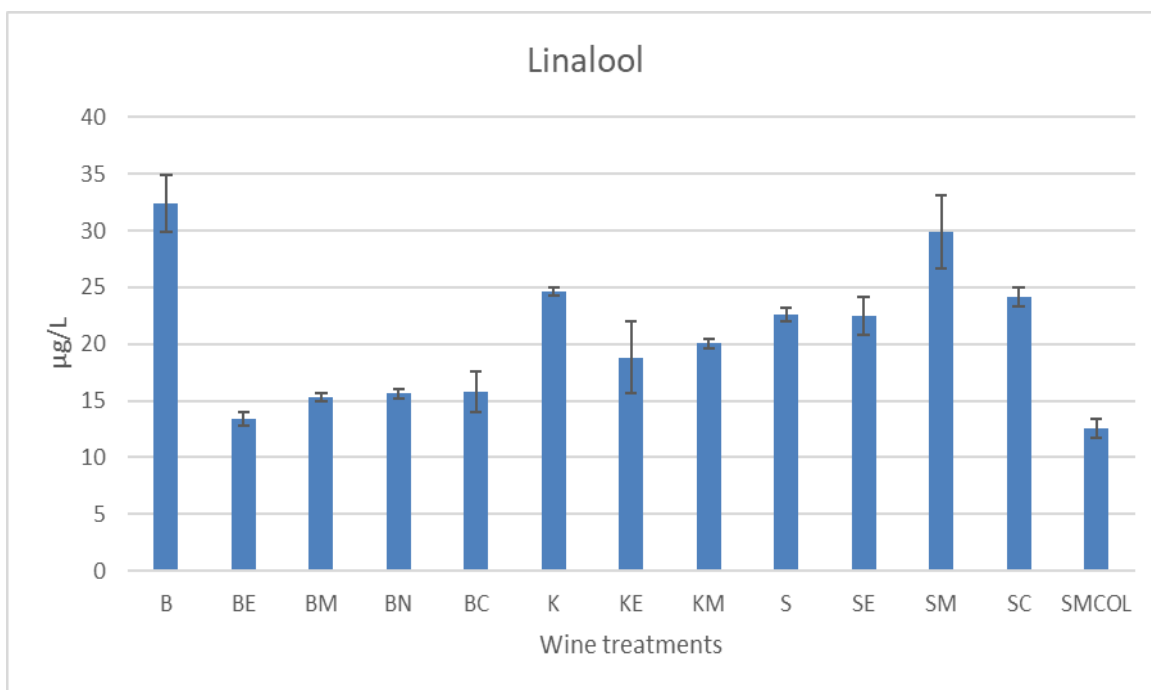
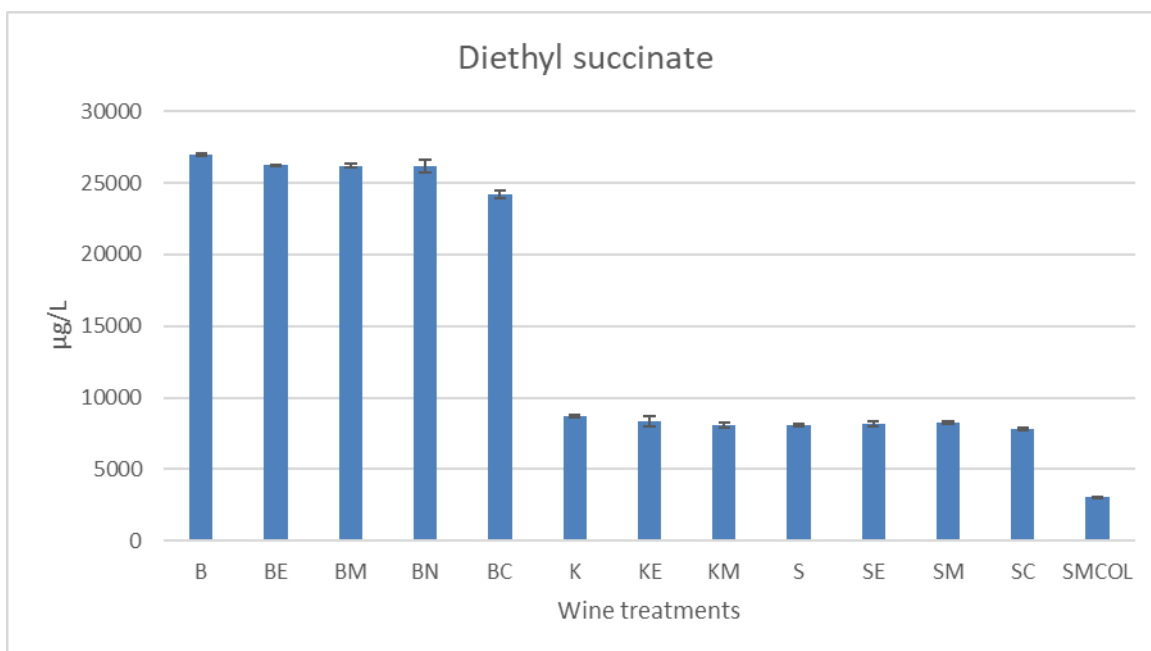


Figure 100. Linalool levels in treated wines



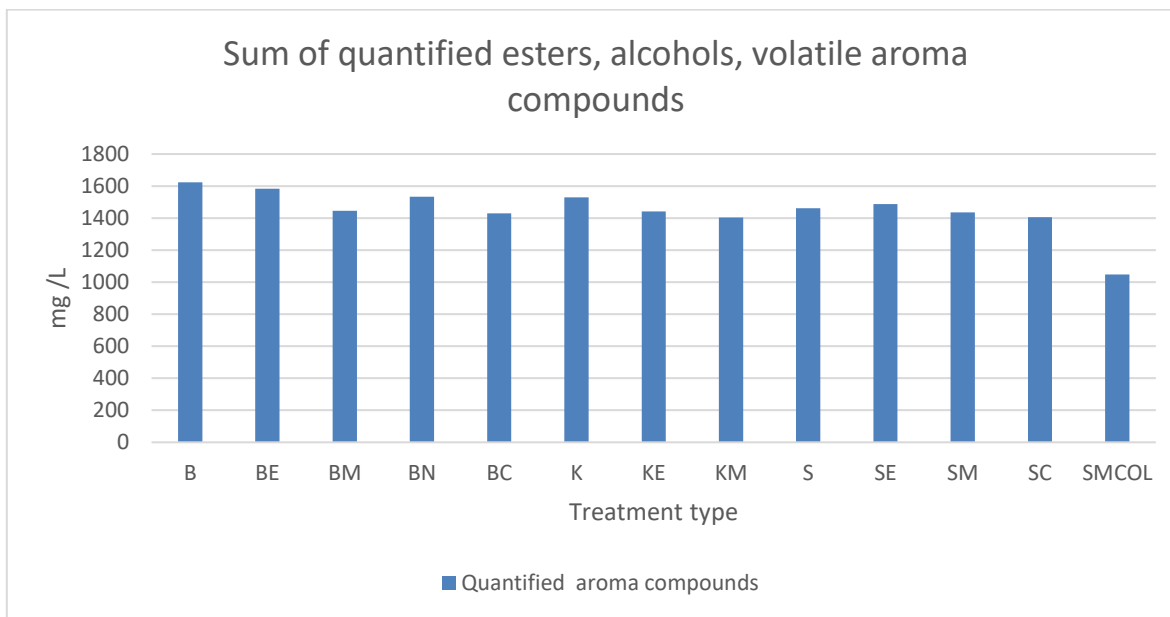
**Figure 101. Diethyl succinate levels in treated wines**

Finally, the concentrations of linalool, a terpene alcohol with floral, spicy flavors, which is commonly found in wine, varied quite strongly with the different treatments (Figure 100). In the case of the Spätburgunder wine, one can see that it was clearly reduced by all treatments, including the standard sterilizing grade filtration. In the case of the Merlot wine, the MIP2 treatment appeared to cause slight increases in this compound, although the larger standard deviations would point towards a lack of precision in the analysis. As the concentration of linalool is among the lowest of the detected compounds, it is reasonable to assume its results to be less reliable, in the absence of more specific analytical methods, targeted at low concentration terpenes in wines, but which would rather be out of scope for this work.

Finally, to take one general look at the overall concentration of quantified aroma compounds in these treatments, one can refer to Figure 102.

In the case of the Spätburgunder wine, the MIP and the charcoal treatments caused a loss of 11 and 12% of all quantified aroma compounds, whereas the losses due to the NIP treatment were lower, at 5.6%. In the case of the treatments on the spiked Merlot wine, the losses were less pronounced, with 1.7% losses due to the MIP treatment and 3.8% losses due to the

charcoal treatment. The MIP column treatment caused the most severe loss of aroma compounds, 28%, as was expected due to its high polymer dose and long contact time.



**Figure 102.** Sum of quantified esters, alcohols, and other volatile aroma compounds in the treated wines

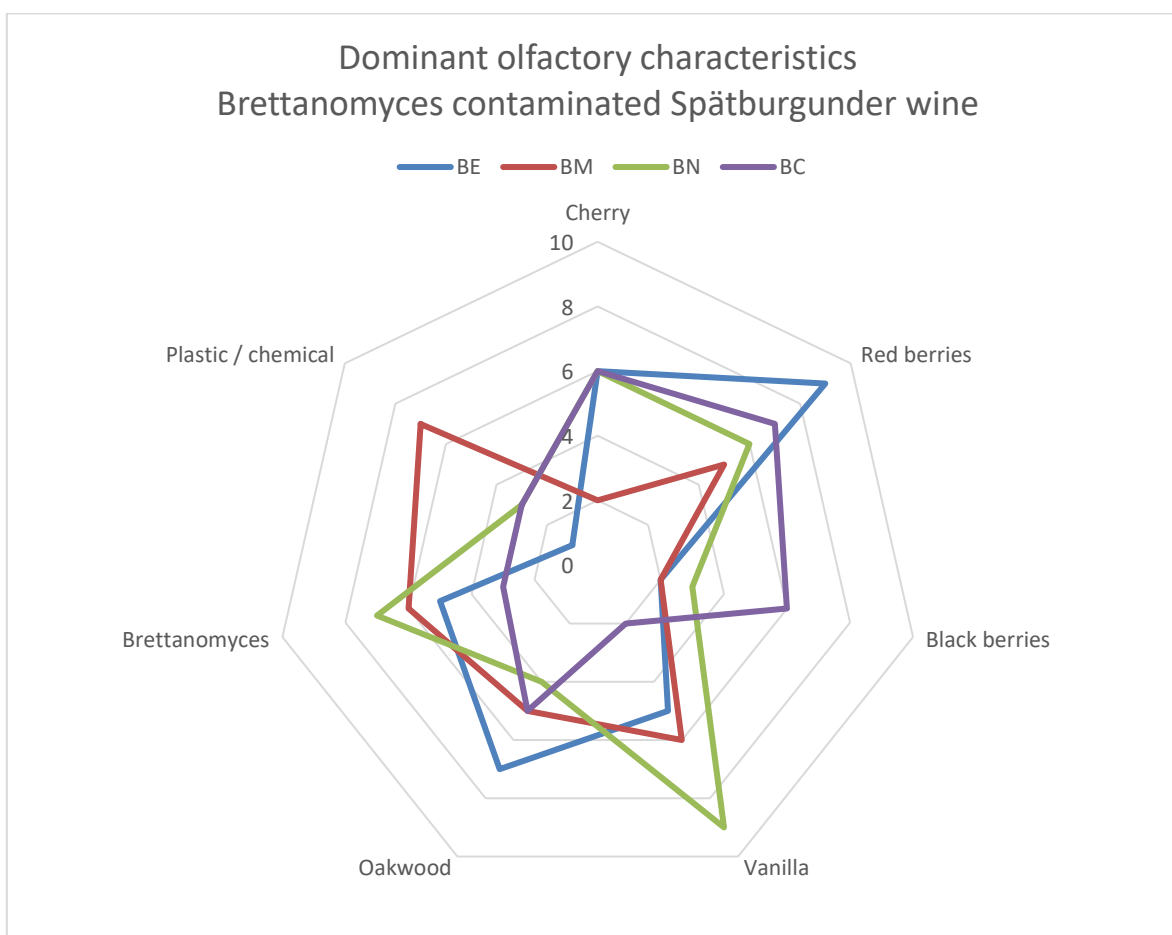
By way of comparison, total losses of volatile aroma compounds of 36% and 32% were observed by Teixeira et al. (2015) for their 4-EP-MIP and NIP respectively. They are larger than what is observed in this experiment, comparable only to the reduction seen with the column of MIP beads, where the polymer dosage relative to the volume of wine treated was extremely high.

#### 4.12.2 Sensory analysis

The methodology and further clarifications pertaining to this experiment have been outlined in sections 3.3.12 and 3.3.12.2.

The wines have been evaluated by a trained tasting panel, at Hochschule Geisenheim, department of Oenology. The naturally *Brettanomyces*-contaminated wines were first evaluated. The dominant olfactory characteristics for the Spätburgunder wines were cherry, red berries, black berries, vanilla, oak wood, *Brettanomyces* and plastic/chemical. The

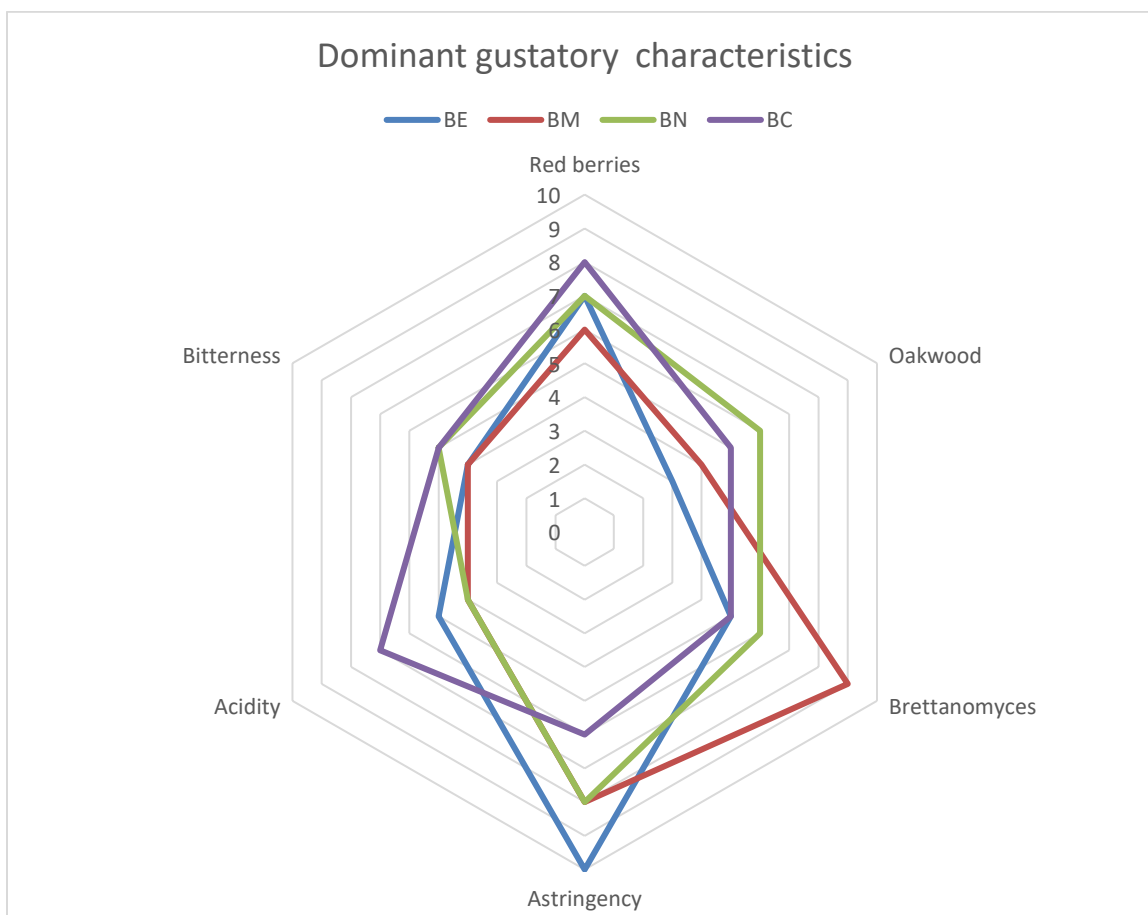
dominant gustatory characteristics were red berries, oak wood, *Brettanomyces*, acidity, astringency, and bitterness. The four Spätburgunder variants were tasted in succession: the wine filtered with Seitz™ EK filter sheets (BE), the wine filtered with MIP2 embedded filter sheets (BM), the wine filtered with NIP2 embedded filter sheets (BN) and the wine treated with activated carbon and then filtered with Seitz™ EK filter sheets (BC).



**Figure 103. Dominant olfactory characteristics of the *Brettanomyces* contaminated Spätburgunder wines. The numbers from 0 to 10 represent the number of tasters out of the total 18 which had indicated a given olfactory perception as dominant in a wine. BE – standard filtration, BM – MIP filtration, BN – NIP filtration, BC – charcoal fining and standard filtration**

Looking at Figure 103, one can get an overall impression of how the different treatments led to different dominant olfactory perceptions in the wines. The sample coded as BE represents the wine filtered with standard Seitz™ EK filter sheets, which had virtually no effect on the concentration of 4-ethylphenol and 4-ethylguaiacol. The dominant olfactory flavors here are

red berries, cherry, oak wood and *Brettanomyces*. However, when one looks at the sample coded as BM, which represents the wine filtered with the filter sheets embedded with the molecularly imprinted polymer, there is a dramatic shift in the olfactory profile of this wine. Tasters started to detect a plastic, chemical-like smell and a few more tasters detected *Brettanomyces* as being a dominant smell. In other words, the MIP2 filtration made the wine appear more tainted with *Brettanomyces*, although analytically, there was a slight reduction in the 4-ethylphenol content, compared to the standard filtration. Another significant feature is the fact that all other positive olfactory impressions are considerably diminished in the case of this treatment. The MIP2 filtration treatment made the wine appear as more Brett-tainted and overall, less fruity, as well as conferred it a strong chemical or plastic-like odor. The NIP2 treated wine showed a similar profile, where even more tasters considered *Brettanomyces*-type aromas to be dominant, fruitiness appearing diminished, and a plastic or chemical-type smell becoming apparent. The only treatment where the *Brettanomyces*-type aromas seemed to slightly diminish was in the charcoal fining treatment, which also saw slight reductions in the oak wood and vanilla odor impressions, and an increase in the black fruit odor impression.



**Figure 104. Dominant gustatory flavor characteristics of the *Brettanomyces* contaminated Spätburgunder wines. The numbers from 0 to 10 represent the number of tasters out of the total 18 which had indicated a given gustatory flavor perception as dominant in a wine; BE – standard filtration, BM – MIP filtration, BN – NIP filtration, BC – charcoal fining and standard filtration**

Figure 104 highlights that the overall gustatory flavor characteristics of all wines remained similar, except for the MIP treatment, where the *Brettanomyces* impression was clearly stronger than in all the other cases.

In terms of dominant taste sensations, the MIP, NIP, and charcoal treatments seemed to reduce the overall astringency impression of the wine, with the charcoal fining displaying the strongest effect. The charcoal fining also had the effect of making the acidity sensation seem more pronounced.

A ranking test was also performed, in which the tasting panel was asked to rank the wines according to their preference, from most preferred to least preferred. According to this, a sum

of their ranks was generated, as can be seen in Table 38. The Friedman test, looking at differences between these rankings, did not achieve enough statistical significance. Nevertheless, one can still get a sense that, although the standard filtration, charcoal filtration and NIP filtration have quite similar sums of ranks, the MIP filtration is the most different from the rest. The larger the sum of ranks of a given sample is, the more it was judged as being less preferred by the tasting panel participants. Therefore, the tendency of the tasting panel was to evaluate the MIP filtered sample as the least preferred sample.

**Table 38. Ranking test of the treatments done on the *Brettanomyces*-contaminated Spätburgunder wine. BE- standard filtration; BM - MIP filtration, BN - NIP filtration, BC - charcoal fining and standard filtration**

Product	Sum of ranks
BE	41
BM	56
BN	43
BC	40

Following the descriptive analysis and ranking test of the wines, a series of triangle tests were performed, comparing different treatment modes against each other to observe whether a significant enough difference can be ascertained. The results of these triangle tests for the Spätburgunder wines can be seen in Table 39.

The MIP-treated and the normal filtered wines were found by this tasting panel to be significantly different from each other, although no clear preference for one or the other wine could be ascertained. The MIP treatment was found to be significantly different from both the charcoal and the NIP treatment. The tasters in general tended to rank the MIP treatment as lower than either the NIP or the charcoal treatment. The last significant difference was between the NIP and the charcoal treatment, with the charcoal treatment being the preferred variant.

The NIP and charcoal treatments were both not found by this tasting panel to be sufficiently different from the standard treatment to render them as significant.

**Table 39. Triangle tests comparing different treatments for the Spätburgunder wine. \* is an indicator of statistical significance; BE – standard filtration, BM – MIP filtration, BN – NIP filtration, BC – charcoal fining and standard filtration**

Spätburgunder wines	Answers	Answers	Signif.	Preference				Signif.
	Taken	Right	(Risk)					(Risk)
BE vs BM	18	10	0.0433*	BE	6	BM	4	0.7539
BE vs BN	18	7	0.3915					
BE vs BC	18	7	0.3915					
BM vs BC	18	12	0.0039**	BM	3	BC	9	0.146
BM vs BN	18	14	0.0001***	BM	5	BN	9	0.424
BN vs BC	18	10	0.0433*	BN	1	BC	9	0.0215*

The overall results with the treatments in the case of the naturally tainted Spätburgunder wines point to the fact that both the MIP and the NIP treatments made the wines worse than they were before, increasing the impression of Brett contamination and conferring a plastic or chemical smell and taste. The charcoal treatment was the only one which improved on the Brett defect, albeit slightly, and was not different enough from the control to be considered statistically significant.

The next set of results pertains to the Merlot wine, which was not *Brettanomyces*-contaminated, but was tested as both control and spiked wine. The idea behind testing a clean red wine was to observe any effects that a MIP filter sheet treatment might have on it. As these materials should, in theory, only bind 4-ethylphenol and leave all other wine compounds intact, the best possible result here would indicate no perceptible difference, either in descriptive analysis or in triangle testing. The idea behind taking a clean wine and spiking it to the same levels of 4-ethylphenol and 4-ethylguaiacol as another naturally tainted wine was to explore the stylistic differences that appear between a real and an induced taint. Another motivation was to examine whether the different origin of ethylphenols in these wines plays any role in their tendency to get removed by the treatments.

In the case of treatments performed on the Merlot wine (vintage 2017, both spiked and non-spiked variants), the dominant olfactory characteristics were cherry, red fruits, vanilla, oakwood, *Brettanomyces*, spicy, smoky and musty-mouldy/chemical. In terms of taste perceptions, the dominant gustatory characteristics were red fruits, black fruits, cherry, oak

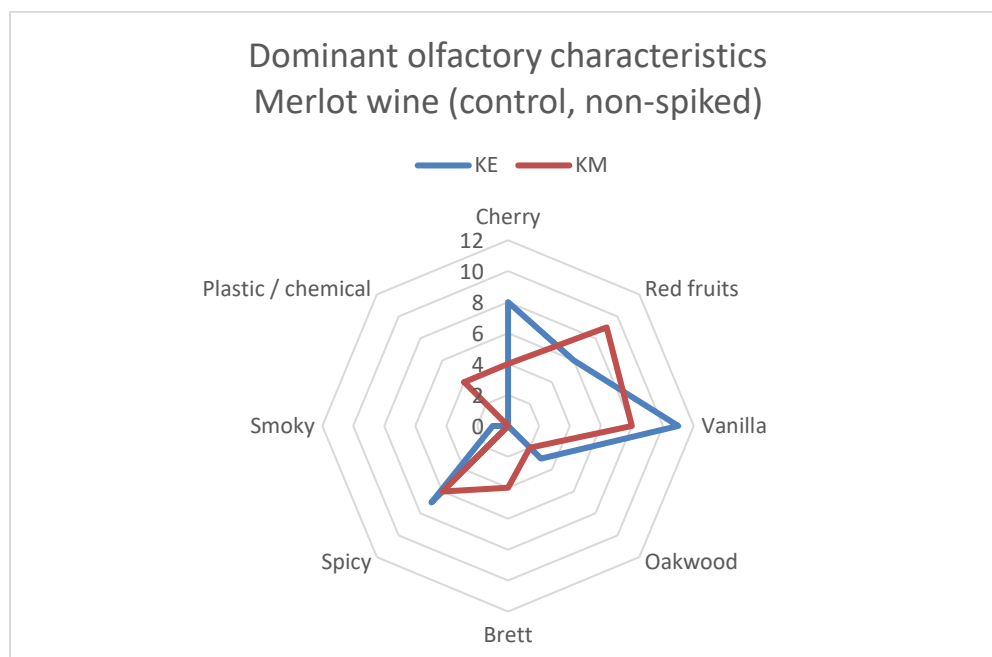


wood, *Brettanomyces*, spicy, plastic/chemical, bitter and astringent. For this series of tastings, the panel was formed of 16 trained members.

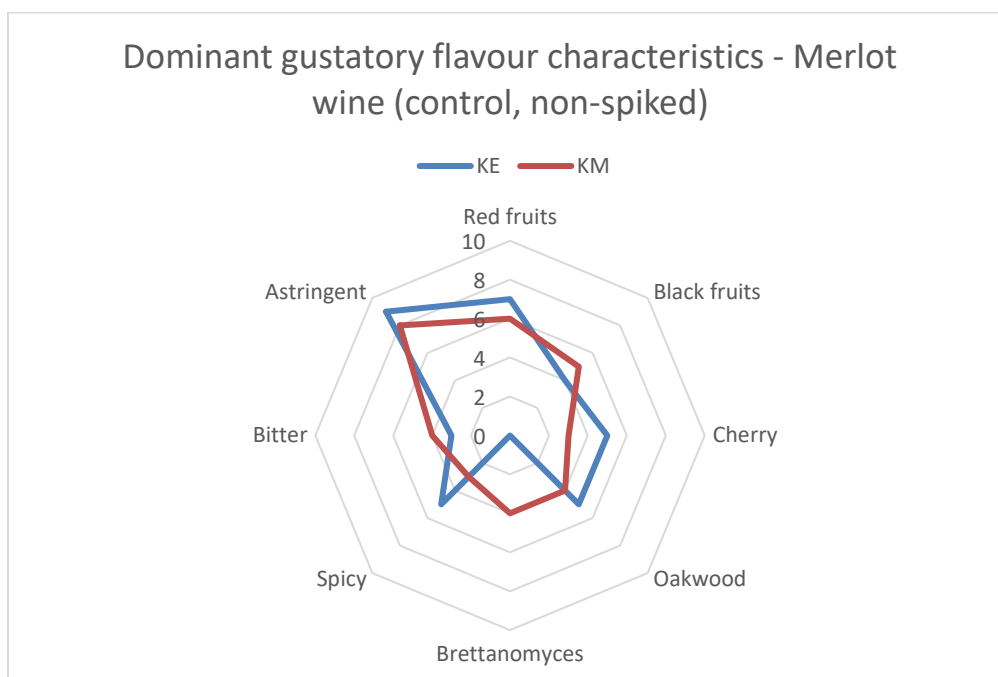
Figure 105 highlights to overall olfactory characteristics of the Merlot non-spiked wine, after either a standard filtration with Seitz™ EK filter sheets or with MIP filter sheets.

One can observe that the wine which had undergone a standard filtration displayed dominant cherry, vanilla and spicy flavours, with no perception of *Brettanomyces* or of any kind of plastic or chemical off-odour. The MIP filtered sample, however, has been found to display both plastic/chemical odours and *Brettanomyces*-type odours. This further corroborates the findings from the 4-ethylphenol measurements, where a slight contamination of 4-ethylphenol was found in the control wine after the MIP treatment. The same kind of plastic, chemical odour was detected in these samples too. This off-odour is originating from the MIP filter sheets. Slight reductions in the perceived dominance of cherry and vanilla aromas were also noted in the MIP treated wine.

The dominant gustatory characteristics can be observed in Figure 106. Although slight variations in fruity flavours, astringency and bitterness can be ascertained, the clearest difference is, once again, in the perception of *Brettanomyces*-related taste sensations, of which the standard filtered wine displays none, whereas several tasters began to evaluate the MIP-filtered wine as affected by this kind of off-flavour.

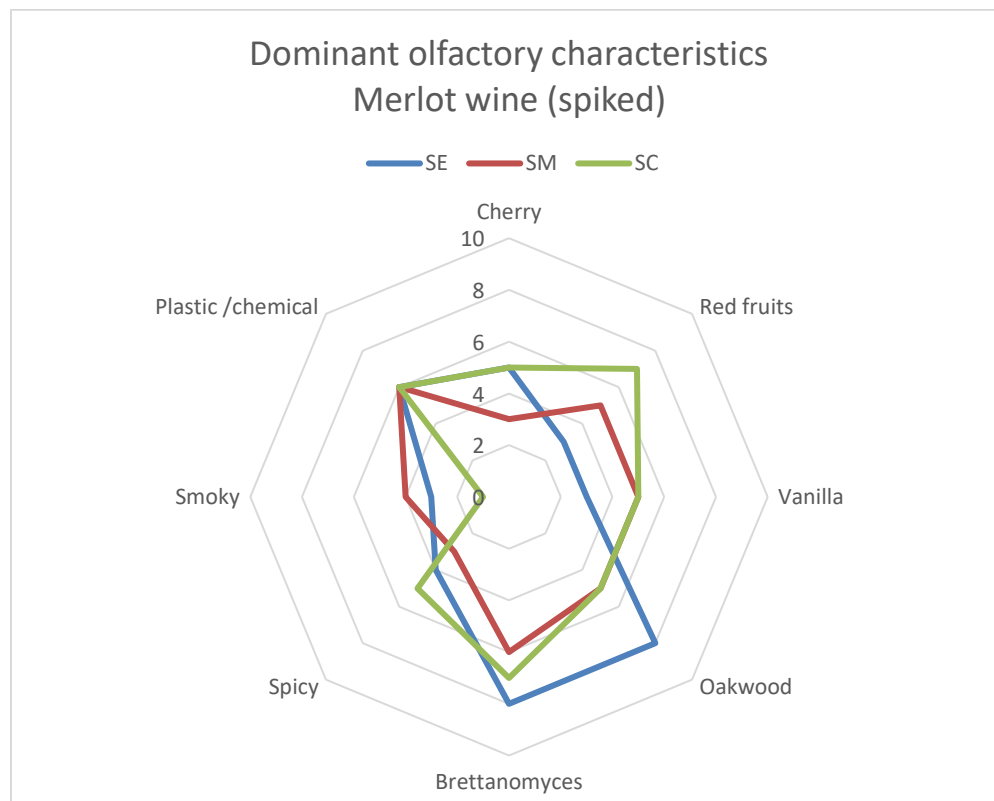


**Figure 105. Dominant olfactory characteristics for the Merlot control wine. KE - standard filtration; KM - filtration with MIP-filter sheets**



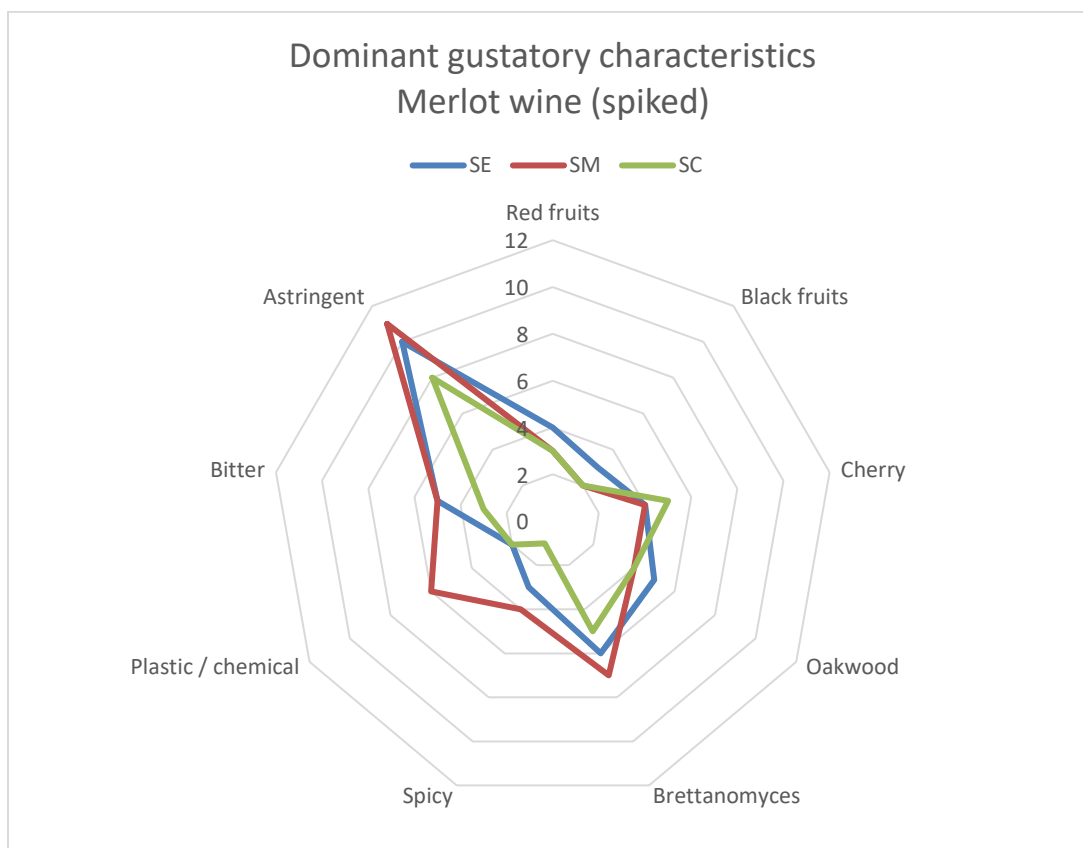
**Figure 106. Dominant gustatory characteristics for the Merlot control wine. KE - standard filtration; KM - filtration with MIP-filter sheets**

Next, looking at the results for the treatments on the spiked wine (Figure 107), all samples were perceived similar in terms of the chemical off-odor. Here, however, there is another compounding factor, coming from the spiking with 4-ethylphenol and 4-ethylguaiacol. These wines were spiked to identical values as in the case of the naturally tainted Spätburgunder wine (meaning 1200 µg/L 4-ethylphenol and 300 µg/L 4-ethylguaiacol). Their smell intensity was easily observed to be much more powerful. This was expected, as a natural *Brettanomyces* contamination results not just in the formation of these two volatile phenols, but of other chemical compounds as well, which have their own influence on the overall aroma profile of the wines. This chemical-like taste was also found to be a characteristic of the standard filtered spiked wine. As this was not the case for the standard filtered control wine, a confounding of the aroma of added volatile phenols and the chemical off-odor coming from the MIP filter sheets was most probably occurring. As the MIP filter sheets caused a slight 4-ethylphenol contamination in the control wines, this fact is not surprising, as it is highly probable that 4-ethylphenol is the source of this off-odor in both cases. Both the MIP and charcoal treatments seemed to diminish the impression of oak wood type flavors, and both seemed to increase the perception of vanilla, cherry, and red fruit type flavors. The fact that both the MIP and the charcoal treatment seemed to show the *Brettanomyces* type aromas as less dominant than in the case of the standard filtered wine could be explained by the fact that, in this wine set, the *Brettanomyces* aromas and the chemical / plastic like aromas reported are quite similar to each other and some tasters did not feel the need to write both descriptors in their evaluation, as they both pointed to the overall same aromatic impression.



**Figure 107. Dominant olfactory characteristics for the Merlot spiked wine. SE - standard filtration; SM - filtration with MIP-filter sheets; SC – charcoal fining and standard filtration**

In terms of the overall dominant gustatory characteristics of the treatments done on the Merlot spiked wines (Figure 108), the profiles of all wines are quite similar, the biggest exception being the perception of the plastic / chemical off-flavor, which was most present in the MIP treatment, and least noticeable in the charcoal treatment. The charcoal treatment also had a marginal effect to reduce the *Brettanomyces*-type flavors. These flavors were most dominant, once again, in the MIP treated sample.



**Figure 108. Dominant gustatory characteristics for the Merlot spiked wine. SE –standard filtration; SM – filtration with MIP-filter sheets; SC – charcoal fining and standard filtration**

The three treated spiked wines were assessed, together with the filtered control wine, in a ranking test (Table 40). A Friedman test was used resulting in statistically significant differences between the sum of ranks. Examining the ranking test results, at a 95% confidence interval, the non-spiked wine was the most preferred, whereas the MIP treated spiked wine was the least preferred. The standard filtered and charcoal treated spiked wine treatments were in the middle, with no clear distinguishable preference between the two among tasters. Even at a more stringent 99% confidence interval, it is still discernible that the spiked wine treated with MIP filter sheets was found to be the least preferred.

**Table 40. Ranking test of spiked and non-spiked Merlot wine treatments and Friedman test results. \* is an indicator of statistical significance. SM – spiked wine, MIP treatment, SC – spiked wine, charcoal treatment, SE – spiked wine, standard filtration, KE, control wine, standard filtration**

Level	Sum of Ranks	Difference test at 1%	Difference test at 5%	Friedman test	
		Groups	Groups	Sum of squares of sums of ranks	7086
SM	59	A	A	F	25.725
SC	40	B	B	corrected F	25.725
SE	39	B	B	Significance F (Risk)	<0.0001***
KE	22	B	C	Significance corrected F (Risk)	<0.0001***

Finally, one can look at the results of the triangle tests for this set of wines (Table 41). The tasting panel was overall less able to successfully identify the different samples than it was in the case of the Spätburgunder wines. Having wines spiked with volatile phenols leads to an unnaturally strong perception of these substances in the wine matrix, which is a probable cause for olfactory fatigue and, if their olfactory impression is strong and lingering, to carryover from one sample to the other. Nevertheless, the only statistically significant result was that the clean Merlot wine was perceived as different from the MIP treated spiked Merlot wine. Although in terms of preference, it did not cross the threshold of statistical significance, the fact that out of 10 tasters who answered right, 8 preferred the clean control sample corroborates the overall impression from the tasting panel, that the MIP treatment did not improve the *Brettanomyces* problem of any of the treated wines, and generally made them less preferable to the tasters.

**Table 41. Triangle tests comparing different treatments for the Spätburgunder wine. \* is an indicator of statistical significance; SE – spiked wine, standard filtration; SM – spiked wine, filtration with MIP-filter sheets; SC –spiked wine, charcoal fining and standard filtration, KE – control wine, standard filtration**

Merlot wines	Answers Taken	Answers Right	Signif. (Risk)	Preference				Signif. (Risk)
				KE	8	SM	2	
SE vs SM	15	6	0.3816					
SE vs SC	15	3	0.9206					
SM vs SC	15	7	0.203					
SE vs KE	15	6	0.3816					
KE vs SM	15	10	0.0085**	KE	8	SM	2	0.1094
KE vs SC	15	6	0.3816					

Overall, these results are less impressive than what is reported by Teixeira et al. (2015) where a beneficial effect was observed in treated samples, with the 4-EP MIP treated sample being the preferred one by the tasting panel (n=6), due to the lowest intensity of volatile phenol off-flavours.

#### **4.12.3 Filter sheet pre-cleaning procedures**

The methodology and further clarifications pertaining to this experiment have been outlined in sections 3.3.12 and 3.3.12.3.

After observing the sensory analysis results for the MIP treated wines, the problem of plastic or chemical-like off-odors came to the forefront. Although these filter sheets have been rinsed with copious amounts of water, this was not enough to render them odorless and tasteless.

A final experiment was organized to check whether steaming the filter sheets would be enough to remove the undesirable off-odor.

The results of the triangle tests can be seen in Table 42.

According to this sensory analysis panel, the water filtered with the Seitz™ EK filter sheets was indistinguishable from the clean municipal tap water (RW), regardless of whether the filter sheets were steamed beforehand (EN) or not (EB). This is an obvious success for this kind of product and was also the expected result. Steaming these filter sheets may be necessary for establishing a sterile filtrate downstream, but it is not necessary for ensuring that the filter sheets do not impart any odour or taste to the filtered product. A simple water rinse is all that is necessary.

Looking at the MIP filter sheets which were rinsed but not steamed (MB), they were found to produce a water filtrate which was highly significantly different from clean tap water, and from the water filtrate of the Seitz™ EK filter sheets. It was also universally disliked, not garnering a single vote of preference from the entire panel. This result points to the fact that these filter sheets, in their current manufacturing condition, are unsuitable for practical use without any additional cleaning step beforehand, the responsibility for which should belong to the polymer and polymer embedded filter sheet producer.

Looking at the MIP filter sheets which were rinsed with water and steamed before use (MN), they were found to be statistically significantly different from the steamed Seitz™ EK filter sheets and from the clean municipal water sample. In both cases, the Seitz™ filter sheets, and the clean tap water samples were almost universally preferred. The rinsed and steamed MIP filter sheet output was, in one triangle test, found to be almost significantly different from that of the non-steamed Seitz™ EK filter sheets, requiring one more right answer to be qualified as such.

When comparing the effect of the steaming step on the filtrate quality of the MIP filter sheets, the result was again highly significant, with a universal preference for the steamed filter sheets.

**Table 42. Triangle tests comparing the effectiveness of different cleaning procedures in rendering water filtrate from Seitz™ EK and the experimental MIP filter sheets as indistinguishable from clean municipal tap water. \* is an indicator of statistical significance; MB – MIP filter sheets, rinse and no steaming, MN – MIP filter sheets, rinse, and steaming, EN – Seitz™ EK filter sheet, rinse and steaming, EB – Seitz™ EK filter sheet, rinse and no steaming, RW – clean tap water**

Water	Answers	Answers	Signif.	Preference				Signif.
$\Delta$ -test	Taken	Right	(Risk)					(Risk)
EB vs EN	15	5	0.5959					
MB vs MN	15	11	0.0018**	MB	0	MN	11	0.0010***
EB vs MB	15	12	0.0003***	EB	11	MN	1	0.0063**
EN vs MN	15	10	0.0085**	EN	8	MN	2	0.1094
EB vs MN	15	8	0.0882					
EN vs MB	15	12	0.0003***	EN	12	MB	0	0.0005***
RW vs EB	15	5	0.5959					
EN vs RW	15	4	0.7908					
RW vs MB	15	14	<0.0001***	RW	14	MB	0	0.0001***
RW vs MN	15	9	0.0308*	RW	8	MN	1	0.0391*

Looking at Table 43, one can see a ranking of the five samples in terms of perceived water purity. There is a clear ranking preference discernible, and it must be stated that, beyond any doubt, the water filtrate from the rinsed but not steamed MIP filter sheets was the least preferred by the tasting panel. The water filtrate from the rinsed and steamed filter sheets was the second least preferred. Beyond that, the Seitz™ EK filtered water samples and the clean tap water sample were quite similarly ranked in the preferences of this tasting panel.



**Table 43. Ranking test of filter sheet cleaning treatments and Friedman test results. \* is an indicator of statistical significance. MB – MIP filter sheets, rinse and no steaming, MN – MIP filter sheets, rinse, and steaming, EN – Seitz™ EK filter sheet, rinse and steaming, EB – Seitz™ EK filter sheet, rinse and no steaming, RW – clean tap water**

Level	Sum of Ranks	Difference test at 5%	Difference test at 1%	Friedman test	
		Groups	Groups	Sum of squares of sums of ranks	
MB	72	A	A		11253
MN	50	B	AB	F	30.08
EN	38	BC	B	corrected F	30.08
RW	35	BC	B	Significance F (Risk)	<0.0001***
EB	30	C	B	Significance corrected F (Risk)	<0.0001***

This filter sheet cleaning experiment indicates that, while steaming the filter sheets thoroughly before use leads to a clear improvement in the filtrate quality when compared to that of using the MIP filter sheets without steaming, it is still highly significantly inferior to the filtrate of a standard Seitz™ EK filter sheet or to the quality of normal tap water at the testing location.

Therefore, the additional steaming step does not represent a complete and viable solution to improve the quality of these experimental materials. The producer must take a different approach to improve their viability and quality.

## 5 Conclusions

### 1. *Brettanomyces* infections will most likely continue to be one of the major red wine quality spoilers in the years to come.

The problematic wine taint caused by *Brettanomyces* infections remains one of the most complex and significant challenges for the wine industry. As this problem concerns red wines, particularly those which spend time maturing in barrels, as the global climatic warming tendency causes grape and wine pH values to increase and as the wine industry, as a culture adhering to the spirit of its time, is both moving and being pushed towards reducing SO<sub>2</sub> addition values, it is reasonable to expect that “Brett” will continue to be a top wine quality concern for the next generations of winemakers, worldwide.

### 2. When attempting to correct or “cure” a *Brettanomyces* tainted wine, the most problematic and challenging metabolites to be removed are volatile phenols.

One can exhaustively group all approaches to solving the “Brett” problem in two main categories: preventive and curative interventions. What one does to prevent “Brett” from occurring overlaps with general sound winemaking practices that a skilled oenologist concerned with quality and cleanliness will naturally adhere to. Curative interventions must address removing the *Brettanomyces* yeasts from the wine, e.g. by filtration, and removing or at least finding a way to diminish the sensory impact of those metabolites of this spoilage yeast which confer to wine a “Bretty” character. The most problematic of these metabolites are the volatile phenols, of which 4-ethylphenol is most iconic with its animal, barnyard-like, chemical odor.

### 3. Molecular imprinting represents a branch of knowledge with implications still to be further understood and a developing technology which is yet to reach full maturity.

The principle of molecular imprinting was first observed by Polyakov in the 1930s and was subsequently harnessed to develop a science and a technology of developing molecularly imprinted polymers. This technology is still in its infancy in terms of range and breadth of

possible applications, of which separation science (high pressure liquid chromatography, thin layer chromatography and solid phase extraction in particular) is currently best established. Other major studied application areas are drug delivery systems, chemo- and biosensors as well as artificial antibodies. Indeed, molecular imprinting has even been postulated by Nobel Prize-winning chemist Paul Lauterbur as an essential mechanism which, as it links with physical chemistry processes and influenced by geochemical systems, gives rise to populations of entities resembling proto-cells, proto-enzymes and proto-ribosomes, in what he called the spontaneous development of biology from chemistry (work published posthumously in 2008).

**4. Molecular imprinting represents one of the technologies which promise to bridge the gap between the precision and selectivity of chemical analytical methods for investigating liquid matrices, and that of industrial filtration and separation technologies currently in use.**

For practical purposes in the wine industry, molecular imprinting is a technology which holds promise for uncovering the “Holy Grail” of truly selective filtration and separation. As chemical analytical methods of investigating wine composition have improved tremendously over the past decades, the ability to literally pin-point wine defects to specific molecules in the wine matrix has opened the door to research into testing various ways of achieving specificity, selectivity and effectiveness with new and special absorbers, of which molecularly imprinted polymers represent an example of.

In this work, the removal of 4-ethylphenol, as the most representative marker of *Brettanomyces* infection in red wine, with the aid of molecularly imprinted polymers (MIPs) was studied.

**5. Molecularly imprinted polymers demonstrate their removal effectiveness and selectivity for 4-ethylphenol in model solutions, with a contact time and dose dependent imprinting effect.**

When testing MIPs in model solutions, their ability to remove 4-ethylphenol is impressive, as is the effect of molecular imprinting, with MIPs achieving 90% reduction, and NIPs (the non-imprinted polymer analogue) achieving 70% reduction in laboratory conditions. One consideration of importance is that this imprinting effect is inversely proportional to the contact time, meaning that the longer the polymer stays in contact with a liquid, the more will the non-imprinted analogue start to catch up in effectiveness. As this is non-specific binding, this effect is to be minimized, thereby recommending the application of MIPs as a short-contact time wine treatment, in some ways like a filtration where wine passes quickly through the medium.

The imprinting effect was also seen to be dose dependent. While the binding of 4-EP increased linearly with increasing the dose of NIP, the increase in the MIPs effectiveness followed a logarithmic (rather than linear) regression model, achieving a peak of binding capacity at a dose of 7 g/L where it had two times the binding capacity of its non-imprinted analogue (MIP removing 58% of 4-EP and NIP removing 24% of 4-EP). After this threshold, it decreased to the point where at doses of 28 g/L and beyond (which are of no practical use, being this high), the imprinting effect is no longer observable.

**6. The 4-EP removal efficiency was scalable when changing from lab-scale to small-scale winemaking conditions. The polymer could be successfully eluted of bound 4-EP and regenerated with alkaline and acid solutions.**

Progressing from model solution to wine matrix and from lab-scale to small winemaking-scale has revealed, as was expected, some losses in the observed binding effectiveness, quantified at about 7% and 6% respectively. The polymer, benefitting from good chemical stability, can be regenerated using alkaline and acid solutions, together with water rinses, with results of up to 98% 4-ethylphenol elution from the polymer when working with model solutions at lab scale.

- 7. The polymers facilitated an almost 10-fold reduction of 4-EP from a contaminated red wine, using repeated filtration and regeneration cycles, but the imprinting effect, evident in lab-scale conditions, was no longer observable as both imprinted and non-imprinted polymers performed very similarly at this experimental scale.**

The strong removal capacity observed in lab bench trials, coupled with the acceptable performance losses from switching to wine matrix and from up-scaling the process, as well as with the knowledge and confidence in the ability to regenerate and re-use the polymer for multiple cycles, has allowed for further experiments, looking at whether a highly tainted wine with a 5000  $\mu\text{g/L}$  4-ethylphenol content could theoretically have this taint level be reduced to below its detection threshold as generally estimated in wine as 400-700  $\mu\text{g/L}$ , according to various authors (Loureiro and Malfeito-Ferreira, 2003; Ribéreau-Gayon et al., 2006).

By pre-coating the polymer onto a standard winemaking-grade filter sheet and cycling the wine through this installation for several cycles of filtration, alternating with polymer elution and regeneration steps, it was observed that the level of 4-ethylphenol was successfully reduced from 4500  $\mu\text{g/L}$  to 500  $\mu\text{g/L}$  over six cycles, representing an 89% reduction starting from a value that is among the highest recorded in the scientific literature for *Brettanomyces*-contaminated wines, and ending with a value that is approaching its theoretical detection threshold in wine.

Both the NIP and the MIP treatments exhibited the same reduction of 4-ethylphenol, which means that, when scaling up, the imprinting effect was no longer noticeable. Throughout subsequent experiments in this work, no other meaningful positive effect caused by the molecular imprinting was discernible. This effect was only established in small-scale experimental conditions but could not be maintained when scaling up, within the experimental conditions and constraints of this work.

An almost 50% loss of total phenolic compounds was also observed because of this six-cycle treatment, however this loss was not due to the polymers, but due to the filter sheet used as a support. This filter sheet was practical as it was used to ensure an even distribution of polymer over its surface, minimizing channeling and ensuring a measurably short contact time between the wine and the material of approximately 3.5 seconds. However, as it was rinsed

with strong alkaline and acidic washes, the filter sheet unloaded the color and phenolic compounds absorbed from the wine after each completed filtration cycle and reabsorbed new color and phenolic compounds with each new cycle. This behavior is normal and expected for filter sheets (which are not designed to be cleaned in this way) and was observed to be identical in the case of the treatment which did not contain any polymer but which only contained a filter sheet. This control treatment exhibited no 4-ethylphenol reduction whatsoever.

**8. In terms of removal selectivity, the tested polymers did not remove any free anthocyanins, whereas PVPP and activated carbon treatments caused stronger removals.**

Experiments looking at polymer selectivity, focused on the non-specific removal of main free anthocyanins in red wine revealed no discernible difference of practical interest between the polymers (either imprinted or non-imprinted) and control wines, whereas PVPP treatments caused a 20% reduction, and activated carbon caused a 50% reduction at identical dosage levels.

**9. Embedding the active polymeric material into filter sheets caused a fourfold reduction in 4-EP removal efficacy, compared to the polymer powder format.**

The last experiments in this work looked at whether embedding the molecularly imprinted polymers into a filter sheet-type product that is easy to use in any winery setting would preserve the effectiveness of the polymer and allow for a convenient and successful treatment of *Brettanomyces*-infected wines. Experimental filter sheets with molecularly imprinted polymer embedded inside, produced by the polymer manufacturing company, were used. When switching from using polymer powder pre-coated onto a standard filter sheet to this polymer embedded-type filter sheet, a fourfold reduction in the polymer effectiveness in removing 4-ethylphenol was observed. In powder format, a removal rate of 300  $\mu\text{g}$  4-EP per g MIP was observed whereas in the embedded MIP format, a removal rate of 70  $\mu\text{g}$  4-EP per g MIP was observed. This negative result could be related to various manufacturing

characteristics that pertain to filter sheet production and represent important feedback given to the polymer manufacturer, who is looking into ways of embedding the material in formats which ensures ease-of-use but also preserves the functionality of the active sites of these imprinted polymers.

**10. The polymer-embedded filter sheets, although new when used, were in fact a source of 4-EP contamination for treated wines, explained by using 4-EP as a template for molecular imprinting during the manufacturing process.**

Another important observation was that the experimental filter sheets represented a source of contamination with 4-ethylphenol for clean wines that were filtered through them. The clean, untainted, wines with 0 µg/L initial 4-ethylphenol levels exhibited concentrations of 45 µg/L after they were filtered with the polymer-embedded filter sheets. This amounts to 750 µg of 4-ethylphenol leaching out of each 20×20 cm polymer-embedded filter sheet. This observation indicates that at some step in the polymer preparation process, the template (which was indeed 4-ethylphenol, judging from these analytical results) was not completely eluted out of the material, and caused a contamination in the filtered product.

**11. The polymer-embedded filter sheets were half as effective as charcoal at removing 4-EP from a contaminated wine, whilst being at a five times higher dose. While the contact time was much longer for the charcoal treatment, it nevertheless displayed similar reductions in measured esters, alcohols, and volatile aroma compounds.**

The loss of polymer effectiveness, when used in this format, also made the MIP-filtration less effective than charcoal fining in removing 4-ethylphenol from a naturally *Brettanomyces* contaminated wine, with charcoal treatment removing 23.5% of 4-ethylphenol and causing a 12% reduction in total measured esters, alcohols and volatile aroma compounds, whereas the MIP treatment removed just 6.4% of 4-ethylphenol and causing a 11% reduction in total measured esters, alcohols and volatile aroma compounds. This unfavorable comparison with activated carbon is further strengthened by the fact that, while the polymer dose was 1 g/L, the charcoal dose was only 0.2 g/L, five times lower. So, in these experiments, activated

carbon removed more 4-ethylphenol than the polymer treatment, and removed a similar amount of the measured esters, alcohols and other volatile aroma compounds, which would establish it as more effective than the polymers, within the context of these experiments.

**12. Sensory analysis revealed the polymer-treated samples to be the least preferred out of all tested samples, including the regular contaminated wines. The polymer-embedded filter sheets displayed and conferred to the wines an off-flavour described as “Bretty” and chemical, and which was not successfully removed using water and ethanol rinses, or steam sanitization.**

In sensory analysis trials performed with a trained tasting panel at Hochschule Geisenheim, all the wines treated with the polymer-embedded filter sheets exhibited unpleasant, chemical and plastic-like odors and flavors which caused the wines to be overall considered actually more intense in “Brett” characteristics and consistently less preferred than the control samples, including the naturally Brett-contaminated wines, and the charcoal-treated samples. This off-odor and off-flavor was originating from the polymer-embedded filter sheets and it could not be successfully eliminated from them by abundant water rinsing, ethanol rinsing or steam cleaning at high temperatures for 20 minutes. This is another important point of feedback for the polymer manufacturer, to improve on this initial design and develop superior iterations of MIP-based products that better preserve their qualities as observed in model solutions and in small scale laboratory conditions.

This work has contributed some new points of reference for future studies into the selective removal of volatile phenols from *Brettanomyces*-contaminated wines. Overall, the materials investigated in this work show promise when investigated in lab-scale conditions with model solutions, and can be effective in treating wine, if the treatment conditions are appropriate and involve multiple regeneration steps. An initial approach to embed these materials into filter sheets has not been successful and, consequently, future studies would benefit from taking a different approach to attaining ease-of-use.



The technology of molecular imprinting probably needs to mature and improve some more before it can attain practical applicability in the wine industry. Although there are many possible lines of improvement to speak of (removal efficiency, selectivity, regeneration ability etc.), the one which this author considers essential is the preservation of the imprinting effect in a wine matrix. As was seen in this work, the imprinting effect was only noticeable in model solutions, and at a lab scale.

Although 4-ethylphenol is the iconic marker of “Brett”, the metabolism of this yeast determines many other changes to the wine matrix, such as increases in volatile acidity, small-chain fatty acid and tetrahydropyridines, all of which play a generally negative role in the olfactory and gustatory perception of these wines. Even a perfect solution to removing 4-ethylphenol with impeccable selectivity would, unfortunately, not represent a complete solution to the “Brett” problem, but it would nevertheless be a significant step forward.

The main insight which this work has made clear to me, regarding *Brettanomyces* taint, is that everything matters, starting from the vineyard management, and ending with decisions made at bottling, and one must understand well how to prevent as well as how to cure. The limits of this understanding can, and should, motivate and direct further research into these topics.

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