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At-line testing of chlorophenol and chloroanisole contaminants in commercial wine production.

INSTITUTE OF BIOSCIENCE AND TECHNOLOGY

PhD THESIS

CRANFIELD UNIVERSITY
INSTITUTE OF BIOSCIENCE AND TECHNOLOGY
CRANFIELD CENTRE FOR ANALYTICAL SCIENCE

PhD THESIS

Academic years 2001 – 2004

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September 2004

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude towards my supervisors Prof. Selly Saini and Dr Steven Setford for their help and support, and for giving me the opportunity to work and study at the Institute of BioScience and Technology, Cranfield University.

My thanks also to personnel involved in the FOODSAFE, EU-collaboration together with colleagues and support staff at Cranfield for their patience and tolerance. I am particularly grateful to the four partners involved in the supporting EU project, Dr. Begoña Alfaro and Dr. Beogoña Perez of AZTI, Bilbao, Spain. Dr. John Wijdenes and Dr. Claudine Vermot-Desroches of Diaclone, Besancon, France. Dr. Helen Berney and Dr. Michelle Sheehan of NMRC, Cork, Eire and all of the support staff who have assisted in this project. Financial support from the EU, Quality of Life and Management of Living Resources programme, FOODSAFE (QLRT-1999-30518) is also gratefully acknowledged.

My thanks also to my wife for her eternal support and encouragement and to the many new colleagues that I have met at Cranfield University, who have helped to revitalise my perennial interest in new areas of science.

Finally my thanks go to Robert Frost the author of the poem “The road not taken”, which has inspired me to consider new directions.

Two roads diverged in a yellow wood,
And sorry I could not travel both
And be one traveller, long I stood,
And looked down one as far as I could
To where it bent in the undergrowth;

Then took the other, as just as fair,
And having perhaps the better claim,
Because it was grassy and wanted wear;
Though as for that the passing there
Had worn them really about the same,

And both that morning equally lay
In leaves, no step had trodden black.
Oh, I kept the first for another day!
Yet knowing how way leads on to way,
I doubted if I should ever come back.

I shall be telling this with a sigh
Somewhere ages and ages hence:
Two roads diverged in a wood, and I-
I took the one less travelled by,
And that has made all the difference. by Robert Frost.

ABSTRACT

The research described in this thesis concerns the development of at-line test procedures for the detection of trace levels of chlorophenols and chloroanisoles in wine and related materials.

Competitive ELISA assays were developed and optimised for pentachlorophenol and pentachloroanisole to enable the detection of chlorophenols and chloroanisoles in the range 0.1 to 100ng/ml in purified sample extracts, using antibodies supplied by the French consortium partner, Diaclone, together with synthesised conjugate materials, based on the enzyme horseradish peroxidase. The cross reactivity of the assay towards chlorophenol and chloroanisole congeners in wine was investigated and the pentachlorophenol assay was selected as the most efficient antibody to detect and quantify both chlorophenol and chloroanisole congeners.

The use of synthetic receptors based on molecularly imprinting technology was also investigated for pentachlorophenol and pentachloroanisole, and a new assay format (Displacement Imprinted Polymer Analysis (DIPRA)) was established to measure chlorophenols in contaminated materials at a concentration range of 0.1 to 50 μg/ml, based on the displacement of a reporter molecule rebound to the synthetic receptor sites. Two alternative reporter molecules were synthesised by linking a pentachlorophenol derivative (2,3,5,6-tetrachloro-4-hydroxy phenoxy acetic acid) to the enzyme horseradish peroxidase or 7-amino-4-methylcoumarin-3-acetyl hydrazide.

Alternative hyphenated sample extraction and purification methods based on solid phase extraction, steam distillation and liquid/liquid partition were evaluated to enable the ELISA and DIPRA test methods to be employed using the limited facilities of a local winery laboratory. The application of the procedures to the analysis of sample types such as wine, corks and packaging materials was undertaken to investigate the suitability of the test methods for inclusion in quality control and incident analysis protocols.

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2,3,4,5-TeCA	2,3,4,5-tetrachloroanisole
2,3,4,5-TeCP	2,3,4,5-tetrachlorophenol
2,3,4,6-TeCA	2,3,4,6-tetrachloroanisole
2,3,4,6-TeCP	2,3,4,6-tetrachlorophenol
2,3,5,6-TeCA	2,3,5,6-tetrachloroanisole
2,3,5,6-TeCP	2,3,5,6-tetrachlorophenol
2,4,5-TCA	2,4,5-trichloroanisole
2,4,5-TCP	2,4,5-trichlorophenol
2,4,6-TCA	2,4,6-trichloroanisole
2,4,6-TCP	2,4,6-trichlorophenol
ABTS	2,2'-azino-di-(3 ethylbenzthiazoline sulfonic acid)
ALP	Alkaline Phosphatase
AMAC	7-amino-4-methylcoumarin-3-acetyl hydrazide
AZTI	AZTI / Food Technology Department. Fundazioa, Fundacion AZTI, Txatxarramendi Ugartea Z/G, 48395, Sukarrieta, Spain.
BRIX	The unit of measurment for soluble solids (sugar) in ripening grapes. A reading of one degree brix equals one percent sugar in the juice.
BSA	N,O-Bis(trimethylsilyl)acetamide
CA	Chloroanisole
CETAB	Cetyl trimethyl ammonium bromide

CHAPS	3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate
C I	Confidence Interval
CP	Chlorophenol
CR	Cross reactivity
CV	Coefficient of Variation
DIPRA	Displacement Imprinted Polymer Analysis
DDBSA	Dodecyl benzenesulfonic acid
DMF	Dimethyl formamide
DMSO	Dimethyl sulphoxide
EC	Electron Capture
EDAC	N-9(3-Dimethylaminopropyl)-N'-ethylcarbodiimide
ELISA	Enzyme Linked Immunosorbent Assay
EPA	Environmental Protection Agency (USA)
EU	European Union
FIA	Flow Injection Analysis
GC	Gas Chromatography
H_2O_2	Hydrogen Peroxide
HACCP	Hazard Analysis Critical Control Point
HCB	Hexachlorobenzene
HPLC	High Performance Liquid Chromatography
HRP	Horseradish Peroxidase
IgG	Immunoglobulin G
IST	International Sorbent Technology

KLH	Keyhole limpet hemocyanin
LOQ	Limit of Quantitation
MES	2-(N-Morpholino)ethane sulphonic acid
MIP	Molecularly Imprinted Polymer
MN	Macherey Nagel
MNNG	1-methyl-3-nitro-1-nitrosoguanidine
MS	Mass Spectrometry
MWCO	Molecular weight cut off
NIRS	Near Infra Red Spectroscopy
NMRC	National Microelectronics Research Centre, Cork, Eire.
OPD	Orthophthalaldehyde
PAH	Polycyclic Aromatic Hydrocarbon
PBS	Phosphate Buffered Saline
PCA	Pentachloroanisole
PCB	Poly chlorinated biphenyl
PCP	Pentachlorophenol
PDVB	Polydivinyl benzene-co-N-vinylpyrolidione
pKa	Acid dissociation constant
ppb	Parts per billion (ng/g)
ppm	Part per million (μg/g)
ppt	Part per trillion (pg/g)
PRG	Pesticide residue grade
RO water	Water purified by reverse osmosis
RSD	Relative Standard Deviation
S/N	Signal to Noise

SAW	Surface Acoustic Wave
SCE	Standard Calomel Electrode
SD	Standard Deviation
SDI	Strategic Diagnostics Inc.
SFE	Super Critical Fluid Extraction
SPE	Solid Phase Extraction
SPR	Surface Plasmon Resonance
TA	Total acidity
TCA	2,4,6-trichloroanisole
TCP	2,4,6-trichlorophenol
TeCA	2,3,4,6-tetrachloroanisole
TeClHPAA	2,3,5,6-tetrachloro-4-hydroxy phenoxy acetic acid
TeCP	2,3,4,6-tetrachlorophenol
TMB	2,2',4,4'-tetramethylbenzidine
Tween20	poly oxy ethylene sorbitan monolaurate
U	Unit of Enzyme Activity
UV/vis	Ultra-violet/ visible
v/v	Volume per Volume
v/w	Volume per Weight
WE	Working electrode
WHO	World Health Organisation

LIST OF SUPPLIERS

Agilent (formerly Hewlett Packard)	Palo Alto, California, USA.
Agmet ESL	Reading, England.
AZTI / Food Technology Department.	Fundazioa, Fundacion AZTI, Txatxarramendi Ugartea Z/G, 48395, Sukarrieta, Spain.
Bio Analytical Systems (BAS)	West Lafayette, IN, USA.
Cadilac Plastics	Swindon, England.
Campo Viejo wine	Ebro Valley, La Rioja, Spain.
(Juan Alcorta Winery)	
Corning	Acton, MA, USA
(Corning Incorporated Life Sciences)	
Dako	Ely, Cambs, England.
DEK	Weymouth, England.
Denley Scientific	Slough,Berks, England.
Diaclone	Besançon, France.
ESA, Inc.	Chelmsford, MA, USA.
Flow Labs	Helsinki, Finland
Fluka Chemie	Part of Sigma-Aldrich Laborchemikalien GmbH.

Griffin & George	No longer trading buisiness purchased by Merck.
IDEX Corp. (Rheodyne)	Rohnert Park, CA, USA
IGEN International Inc.	Gaithersburg, MD, USA.
Machery Nagel	Dũren, Germany.
MCA Services Ltd	Melbourn, Cambs., England.
Merck	St Paul, MN, USA
Millipore	Billerica, MA, USA.
Molecular Probes Inc.	Eugene, OR, USA.
Nichols Institute Diagnostics	San Juan Capistrano, CA, USA
Nunc (Nalge Nunc International)	Rochester, NY, USA.
Perkin Elmer (PE)	Wellesley, MA, USA.
Pharmacia Biotech (Amersham Biosciences)	Amersham, Bucks, England.
Pierce	Rockford, Illinois, USA.
Rhone diagnostics	West of Scotland Science Park, Glasgow, Scotland.
SDI Europe	Hants, England.
Shimadzu	Kyoto, Japan.
Sigma-Aldrich	Poole, Dorset, UK
Varian	Palo Alto, CA, USA.
Waters Corporation	Milford, MA, USA.

1.0 INTRODUCTION

The work presented in this thesis concerns the development of analytical tools for the detection of trace levels of chlorophenols and chloroanisoles contaminants in wine using the limited resources of a small winery laboratory.

1.1 At-line test methods

Modern commercial wineries include a small area in which to conduct analytical tests to enable the winemaker to better understand their product and to confirm that the production process is proceeding normally. These procedures, referred to as at-line test methods, are generally designed to be simple to operate so that they can be performed locally by staff with limited analytical training and provide useful information to enable production problems to be traced to the source and appropriate remedial action to be taken quickly.

This chapter is concerned with, the importance of food contaminants on flavour and in particular their impact on the wine industry. In the following chapters a review is presented of at-line procedures that are currently available to test chlorophenol and chloroanisole contaminants followed by the experimental section which describes work conducted to develop at-line test methods based on selective binding by natural and synthetic receptors.

1.2 Food contamination by chlorophenols and chloroanisoles

The impact of trace levels of chlorophenols and chloroanisoles on the human food chain has been of considerable concern for many years and both the United States and the European Union have included chlorophenols in their priority pollutants list. The nineteen chlorophenols shown in Table 1.1 are routinely monitored in potable water using either HPLC or GC with MS or EC detection.

Table 1.1 – Chlorophenols in potable water.

2-chlorophenol (EU)	2,3,4-trichlorophenol (EU)
3-chlorophenol (EU)	2,3,5-trichlorophenol (EU)
4-chlorophenol (EU)	2,3,6-trichlorophenol (EU)
	2,4,5-trichlorophenol (EU)
2,3-dichlorophenol	2,4,6-trichlorophenol (EU)
2,4-dichlorophenol	3,4,5-trichlorophenol
2,5-dichlorophenol	
2,6-dichlorophenol	2,3,4,5-tetrachlorophenol
3,4-dichlorophenol	2,3,4,6-tetrachlorophenol
3,5-dicholorophenol	2,3,5,6-tetrachlorophenol
	pentachlorophenol

(EU) Included in the EU Priority Pollutants list

The main sources of contaminants stem from the ubiquitous use of bleach for the sterilisation of water and food packaging materials together with the use of pentachlorophenol (PCP) for wood preservation and 2,4,6-trichlorophenol (TCP) in over the counter pharmaceutical products.

The human sensory system is highly sensitive to a number of food contaminants, such as chlorophenols and chloroanisoles, their primary metabolites, which can be detected by a taste panel at very low concentrations (e.g. TCA 0.01ng/ml – Buser *et al.*, 1982). In general, the level of these contaminants in food products is below the level that would

normally require action based on WHO/ EU, Health and Safety regulations (WHO, 1996).

The main issue in the food industry concerns the impact of these contaminants on the flavour and quality of food products. The anisole contaminants may impart a disagreeable odour described as reminiscent of the smell of musty old books; phenol contaminants tend to impart an antiseptic ‘disinfectant’ taste.

In the food and wine industry, taint from the presence of contaminants can have a significant impact on profitability. In some cases, the loss of confidence in specific products due to adverse taints may result in the loss of large production runs or the complete withdrawal of the product. It is therefore essential that low level identification of contaminants is achieved at source and appropriate corrective action taken to minimise production losses.

Chlorophenols have been used for many years as broad spectrum antibiotics and preservatives for detergents, wood, paints, paper and leather. In addition they are used as herbicides, fungicides and insecticides and as intermediates in the production of pharmaceuticals and dyes.

Environmental contamination by chlorophenols arising from pesticide usage and waste incineration is a world-wide problem that is currently being addressed by a United Nations co-operative international programme on persistent organic pollutants (Rotterdam Convention, 2004). Chlorophenols have been studies with other more persistent chemical pollutants such as

dioxins, PCBs, PAHs etc. as they are frequently implicated as metabolites or components of sewage waste and incinerator emissions.

Major concerns have also been raised about the bioaccumulation of chlorophenols in invertebrates, the formation of dioxin related compounds and their transfer to the human food chain (WHO 1987,1989).

The contamination of food by chlorophenols and chloroanisoles has been widely reported (Table 1.2). These compounds have been found in a multitude of food products: wine, canned carbonated beverages, reconstituted fruit juice, poultry, bulk chocolate, modified flour, canned fruit, cocoa powder, packaged biscuits, chilled meat etc.

Chlorophenols are presently widespread in the environment. Even in the most remote natural environments, the presence of chlorophenols in both aquatic and terrestrial food chains has been recorded. These pervasive compounds have been used for a wide range of domestic, agricultural, and industrial purposes for more than 50 years. In addition to industrial production and usage, chlorophenols are produced from naturally occurring phenols, resulting from bleaching of wood pulp in the paper industry and through the chlorination of domestic water supplies and swimming pools. The seriousness of this problem has been addressed by the EC, which issued a directive in 1991 severely restricting the usage of pentachlorophenol and its compounds.

Table 1.2 - Reported cases of food contaminated with chlorophenols and chloroanisoles

Food contaminated	Compounds involved	Origin
Cheese	2,4,6-TCA 2,3,4,6-TeCA 2,3,5,6-TeCA	Wooden pallets initially contaminated with chlorophenols. (Maarse, 1985)
Chilled meat	2,4,6-TCP 2,4,6-TCA	Wooden insulation in cavity walls of chiller cold-store initially contaminated with chlorophenol reaction products or residues. (Whitfield, 1986)
Cocoa powder	2,4-DCA 2,6-DCA 2,4,6-TCP 2,3,4,6-TeCA PCA	Multi-walled paper sacks initially contaminated with chlorophenol residues. (Whitfield, 1986)
Fish	PCP 2,4,6-TCP 2,4-DCP	Screening study. (Araki *et al*., 2001)
Packaged biscuits	2,4,6-TCP 2,3,4,6-TeCP	Adhesive containing chlorophenols as a preservative. (Whitfield, 1986)
Packaged dried fruit	2,4,6-TCA 2,3,4,6-TeCA	Fibreboard packaging material initially contaminated with chlorophenol residues. (Whitfield *et al*. 1991, Aung *et al.,* 1996)
Packaged flour	2,4,6-TCA 2,3,4,6-TeCA	Jute sacks initially contaminated with chlorophenols present either as preservatives or formed by an in-plant chlorination reaction.

		(Whitfield, 1986)
Food contaminated	Compounds involved	Origin
Poultry	2,4,6-TCA	Cage litter containing wood shavings contaminated with wood preservatives. (Land *et al.,* 1975)
Wines and Corks	PCP 2,3,4,6-TeCA 2,4,6-TCA 2,4-DCA	Contaminated wine attributed to cork . (Pollnitz *et al.* 1996, Pena-Nera *et al.,* 2000)

In certain sectors of the food industry, such as wine production, issues concerned with taste and taints are of particular concern. Wine production is a complex process that may require several years to prepare the final product. In view of this, the industry is particularly vulnerable to external contaminants that may influence the taste, aroma and overall experience of wine drinking and special care is necessary to maintain the high quality image of wine production.

1.3 Contaminants in the wine Industry

Wine is a very significant industry in many parts of the world: Europe, US, South America Africa, Australia/ New Zealand, parts of Asia and Russia all have well-established industries. Wine production provides a thriving agricultural system in many arid or upland areas where conventional crop

production would be difficult to achieve. In Europe, viticulture is a major industry; Spain has over one million hectares of land under vine, France and Italy have just less than one million. Portugal, Greece, Germany, Austria and many of the newer European countries also have well established wine industries.

In general terms the key objectives of the winemaker are to develop the flavour components from the grape in a way that imparts a characteristic and desirable range of flavours and fragrances. Some wines develop these characteristics very rapidly, notably the modern style of rich fruity wines designed for high volume consumption e.g. from Australia and America. Many of the more classical European wines develop their delicate flavour characteristics over many years and employ manufacturing and storage procedures that have been handed down from generation to generation. The individual differences of local grape variety, climate, soil type, growing conditions, production and storage methods all contribute to the development of a complex and often unique set of flavour elements that together characterize a particular wine.

Good wines contain a highly complex mixture of flavour components. To ensure continued sales of a good product the winemaker must strive to maintain the product quality. In most traditional wineries, details of the complex and subtle chemical changes behind the development of a particular flavour are probably unknown. However, by keeping precisely to traditional

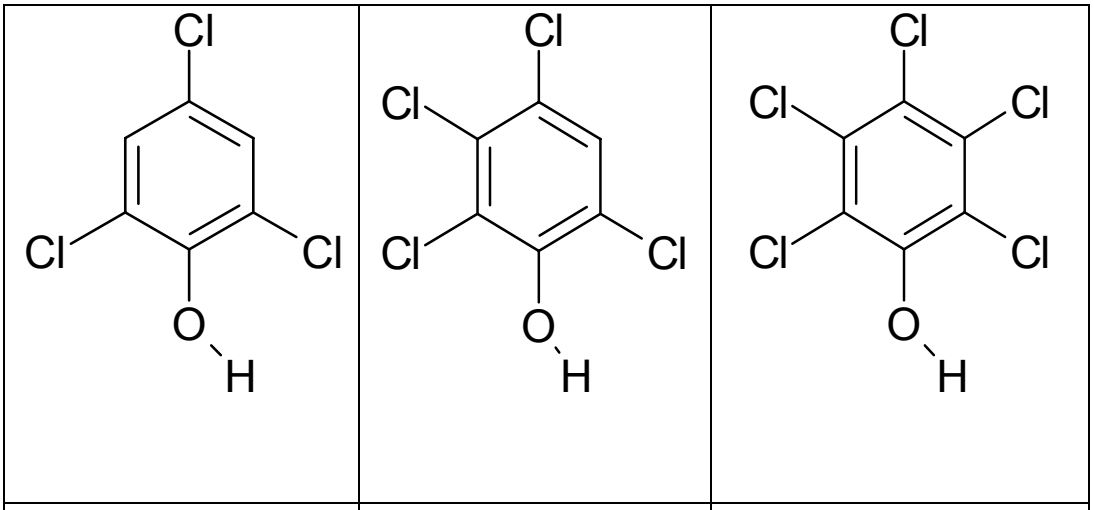
production processes together with ensuring a high level of staff retention, a consistently high quality product can be produced.

Occasionally inferior quality material may be produced due to irregular external inputs such as contamination from e.g. a change of supplier. Unfortunately in the wine industry the consequence of the inclusion of a component that has an adverse effect on flavour is very significant and may not be observed until a considerable period has elapsed or when a particular batch is marketed.

In the wine industry, like many parts of the food and beverage industries, the perception of quality is almost as important as the true quality of the product. The marketed image of wine is easily tarnished and may take many years for public confidence to be regained. In recent years, the UK public perception of Spanish wines appears to be that they are of poorer quality than other European wines. Considerable efforts have therefore been expended to improve the product quality and many wineries have equipped themselves with a small local laboratory. Appropriate production control tests have been introduced to monitor the production process (e.g. sugar content at various stages of fermentation) and to indicate whether any control measures such as the addition of lactic acid are required to optimise product quality.

One of the important quality issues in recent years has been the impact of contaminated corks, arising from the use of chlorine bleaching to remove natural fungal and bacterial contamination or polychlorophenolic biocides in cork-oak forests. The strength of the hypochlorite solution employed may

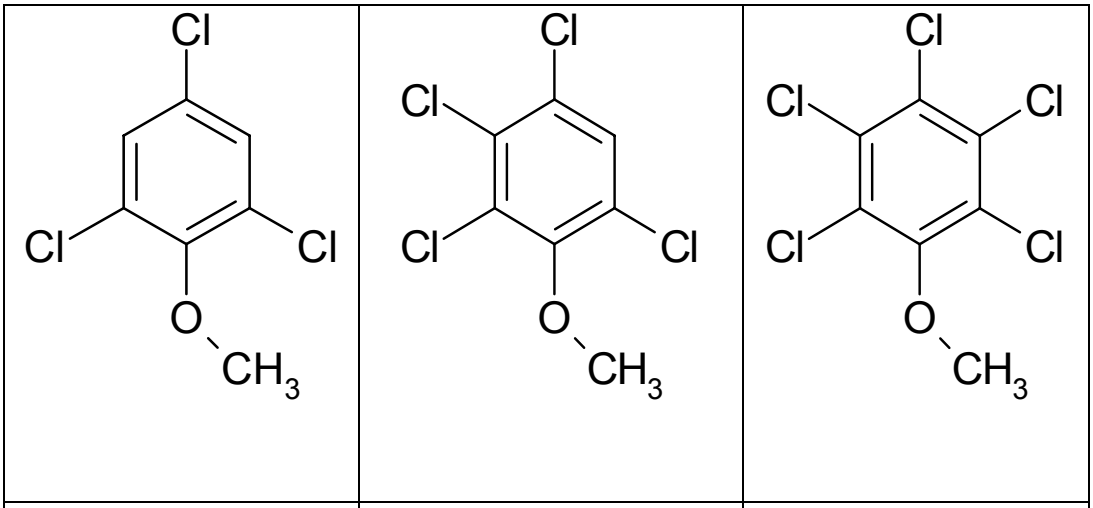
result in the chlorination of natural phenolic materials and may not be sufficient to kill all fungi. Residual natural phenolic materials in the cork are chlorinated to form chlorophenols and further biomethylated by fungi resistant to the concentration of hypochlorite employed to chloroanisoles. These processes may lead to the formation of a set of congener substituted chlorophenols and chloroanisoles (Figures 1.1 & 1.2). The predominant member of the group is 2,4,6-trichloroanisole, capable of imparting a strong musty flavour often referred to as ‘corked’ wine but other aromatic compounds such as 2,3,4,6,-tetrachloroanisole and pentachloroanisole (Figures 1.2b and 1.2c) may also contribute to the cork off-flavour (Buser *et al.*, 1982). In 1993 the European Cork Industry Federation commissioned a study of the cork industry called project Quercus (Quercus report, 1996). The study confirmed suspicions that ‘corked‘ wines were caused by TCA produced by a fungus within the corks themselves and new guidelines were issued for the manufacture and storage of corks.



a) 2,4,6-trichlorophenol	b) 2,3,4,6-tetrachlorophenol	c) Pentachlorophenol

Figure 1.1 – Structures of three common chlorophenol flavour contaminants

In commercial wine production, the risk of contamination by chlorophenols and chloroanisoles extends beyond the cork contamination issue. Hypochlorite is frequently used to wash barrels and chlorophenolic biocides are the most popular method of preservation of softwood products used in wooden pallets and storage racks, cardboard cartons and other packaging materials. The environmental contamination of wine cellars is also considered a principle cause of this problem. The semi-volatile nature of chlorophenols and chloroanisosles provides a significant vapour pressure, which may cause atmospheric contamination leading to the translocation of contaminants within the winery.



a) 2,4,6-trichloroanisole	b) 2,3,4,6-tetrachloroanisole	c) Pentachloroanisole

Figure 1.2 – Three common chloroanisole flavour contaminants.

However, contaminated cork is a very serious source of chlorophenol and chloroanisole contamination, which is currently being addressed world-wide by wine producers.

The industry response to the contaminated cork issue has generally been to produce numerous alternative polymeric cork substitute materials. Many new polymer corks have been evaluated as cork substitutes particularly in products having a relatively short shelf life alternatively screw cap closures are being used increasingly for cheaper wines. Early problems with plasticiser contamination have been virtually eliminated and polymer corks have made a significant impact on this contamination issue in products designed for high volume consumption (Caputo - 1999). However, many traditional wine producers continue to select cork as the closure material of

choice for classical vintage wines to avoid the risk of changing the subtle flavour components which characterise their particular product.

Gaseous exchange via pores in the closure material and slow oxidative processes are essential for the development of the full flavour of vintage wines. In this regard, synthetic corks or coated cork provides a seal that is generally far superior and different from the seal provided by cork. Therefore, the extent of gaseous exchange and consequently the rate of natural oxidation may be significantly altered by changing the material from which the closure is produced. In view of this, many more traditional wineries are cautious about changing to synthetic corks due to the limited information available on the longer-term impact on high added value vintage wines.

1.4 Sensory panel detection of wine contaminants

The human olfactory system is highly sensitive to chlorophenols and chloroanisoles (TCA-0.03 ng/ml – Quercus report 1996). Taste panel experts appear to be able to selectively detect and identify these components at extremely low concentrations within the complex red wine bouquet Table 1.3 (personal communication - Perez-Villarreal, 2001)

Table1.3 - Concentration threshold for chlorophenols/chloroanisoles detectable by taste panel members. in red wine

Compound	Sensitivity (ng/L)
2,4,6,-trichloroanisole	1
2,4,6,-trichlorophenol	10
2,3,4,6-tetrachloroanisole	10
2,3,4,6-tetrachlorophenol	10
Pentachloroanisole	100
Pentachlorophenol	100

Problems of contamination by chlorophenols and chloroanisoles have also been frequently encountered in potable water (WHO – Guidelines for drinking water quality 1998) and also encountered in the production of fruit juices, dried fruit and coffee although these are less frequently reported (Aung *et al*. 1996).

A range of wine samples, from different European wineries, returned due to taint problems were tested by the Spanish consortium partner, AZTI laboratories during 2001/2. Results shown in Appendix 1 indicate that all samples contain trace levels of chlorophenols and chloroanisoles.

The frequency of contamination by each contaminant detected in the AZTI samples is indicated in Table 1.4. Samples contain 2,4,6TCA, 2,3,4,6-TeCA, PCA, 2,4,6TCP and PCP at levels from 5ng/l. One sample contained 2,4,6TCP at greater than 130ng/L.

Table 1.4 – Frequency distribution for contamination AZTI red wines by individual CP/CA congeners

Compound	**Number of contaminated samples**
2,4,6-TCP	19
2,3,4,6-TeCA	16
PCP	16
PCA	13
2,4,6-TCA	3
2,4,5-TCA	0
2,3,5,6-TeCA	0
2,3,4,5-TeCA	0
2,4,5-TCP	0
2,3,5,6-TeCP	0
2,3,4,6,-TeCP	0
2,3,4,5-TeCP	0

These data confirm that the principle contaminants are PCP, TCP, PCA TeCA and TCA. Several unconnected wineries in different European countries have experienced wine contamination problems in recent years and although 2,4,6-TCA continues to be a major contaminant many different sources of chlorophenol/ chloroanisole contamination exist that must be identified and removed by action locally.

Despite recent restrictions on the use of chlorophenol containing products, low-level contamination by chlorophenols and their metabolites continues to have a significant impact on wine production. One of the main problems with contamination by chlorophenols and their metabolites is the numerous potential sources of contamination. Chlorophenols and their metabolites are generally classified as semi-volatile compounds and under standard environmental condition they exhibit a significant vapour pressure. In view of this chlorophenols may be readily transferred from treated or contaminated surfaces. Concentrations measured may involve gross contamination arising from the close proximity of contaminated or treated timber, leather, textiles, paper etc. Examples include the use of pesticides bearing a chlorophenolic moiety used for the vineyard control of weeds, invertebrates and fungi: The use of contaminated process inputs such as egg albumin from poultry reared on contaminated wood-shavings: Trace contamination arising from the use of chlorinated water, disinfectants and cleaning agents in the production of

wine bottles and corks etc. and their subsequent contact with contaminated packaging materials.

Chlorophenol contaminants are also highly susceptible to chemical and microbiological metabolism e.g. by Aspergillus and Penicillium species primarily to the corresponding chloroanisole. The characteristic musty/mouldy aroma described as similar to the aroma of 'musty old books' and often used to describe 'corked' wines is considered to be primarily due to cork contamination by 2,4,6,-trichloroanisole (Buser *et al.* 1982).

All of the principle chlorophenolic contaminants are susceptible to chemical or biological methylation and in general the human sensory system is more sensitive to trace concentrations from the anisolic metabolites.

1.5 The winemaking process

The successful production of high quality wine is a combination between good viticultural and winemaking (oenological) practices. The potential problems associated with wine production are similar for all wine types. Principle differences are generally related to the type of wine and variations in regional production methods.

In view of these differences it is not possible to provide a description of the potential problems encountered in all winemaking process. Where production problems occur, solutions may be mainly concerned with local and regional issues experienced by the winery. However, to outline the steps required in

problem analysis and the use of at-line test methods the production of a high quality red wine has been selected as providing a suitable model on which to base the problem analysis framework.

5.1 Viticulture and Oenology

Many different types of wine are available to the consumer depending on the selection of grape variety and quality and production methods. White wines tend to be made from white grapes, although they can be made from dark skinned grapes. Rosé wines are made in a similar manner to white wines, the juice being tinted from a brief contact with dark skins before fermentation. Dessert wines and fortified wines are produced from very ripe grapes. Red wines are exclusively produced from dark skinned grape varieties.

Viticulture: The spring and summer are the main growth periods and careful control of temperature, irrigation sprinklers are required to maintain the required environmental conditions. A wide range of pesticides is also required to the control of herbicides, insecticides and fungicides. The extent of pesticide usage in the wine industry is very variable as grapes grown in many different locations and conditions that require different treatments

Oenology: In the northern hemisphere the harvest takes place in September and October and from February to April in the southern hemisphere. Grapes

are increasingly harvested by machine but in some areas, the mountainous terrain and the traditions of the region still favour hand picking.

At the time of harvest, the grapes must have also reached the correct maturity when the sugar concentration (Brix) and Total Acidity (TA) levels indicate maturity of wine.

Receiving: Upon receipt at the winery the grapes may be manually sorted and cleaned using large sorting tables.

Destemming and crushing: Destemming includes the removal of stem, leaves and grape stalks, before crushing. Destemmers usually contain a perforated cylinder allowing berries to pass through but prevent the passage of stem, stalks and leaves. Crushing typically immediately follows stemming, since some crushing of the fruit occurs during stemming. The most common crushing processes involve pressing the fruit against a perforated wall or passing the fruit through a set of rollers.

Addition of sulphur-dioxide: Sulphur dioxide is commonly applied to the must using a pump. Sulphur dioxide additions perform a number of tasks: (1) dissolved oxygen in the must is eliminated protecting against further oxidation (2) the 'must' is protected against microbial spoilage eliminating most of the harmful yeasts and bacteria, thus avoiding fermentation problems and (3) the addition of sulphur dioxide also aids the dissolution of phenolic compounds, giving colour and flavour to the wine. Where grape varieties known to exhibit rotting problems are use the flavour is also improved.

Fermentation: In this stage the crushed grapes are introduced in the tanks where maceration and subsequent fermentation takes place. Moreover the addition of oenological products such as yeast, tartaric acid takes place. The alcoholic fermentation is a biological transformation of sugars into ethanol.

Drawing off: In this operation the juice is taken out from the fermentor, once the alcoholic fermentation has been completed. The pomace remaining in the tank is discharged later and driven to the presses.

Pressing: The pomace discharged from the fermentor is driven to the press, where most of the remaining juice is extracted. This juice will be of lower quality. Depending on the press type (horizontal, pneumatic, continuous screw presses) , the produced juice and wine fractions vary in terms of their physicochemical properties.

Malolactic fermentation: A wine can be considered finished when there are no significant quantities of residual sugar or malic acid left. Malic acid imparts an unpleasant taste and microbial instability of wine. In this stage, malic acid is transformed into lactic acid by bacteria of the genera Lactobacillus and Oenococcus, improving the wine taste qualities.

Racking: In this stage the wine is transferred from one tank to another, separating the sediment (lees).

Maturation (Aging in barrels): The maturation step takes place in barrels. During maturation a range of physical and chemical interactions occurs among the barrel, the surrounding atmosphere, and the maturing wine, leading to transformation of flavour and composition of wine. During this period, several components of the wood (most of them phenolics) are extracted and contribute to the wine tannins. The temperature must be constant in this step and the relative humidity around 75-80%.

Clarification/Filtration/Stabilization: The main objective of these operations is to obtain a clear wine, with no clouding or any sign of spoilage and to remove any colloidal particulate material in suspension. The clarification operation consists in adding clarifying agents to the wine such as bentonite, gelatine or egg albumin. These are materials that are able to induce the formation aggregates, which precipitate, removing compounds involved in haze production. Once the coarser particles found in suspension have been removed to avoid rapid plugging of the filter, the wine passes through a porous filter layer (diatomaceous earth) which retains impurities, yeast and bacteria in suspension. After filtering the product is stabilized using the on by chilling the wine to near its freezing point (tartrate stabilization)and then filtering or centrifuging to remove the crystals or protein stabilization with absorption, or neutralization by fining agents e.g. bentonite.

Bottling: Wine is bottled in glass bottles the preferred closure material is cork, however alternative closures based on polymer composites are increasingly being used for wine. The use of metal screw caps has also increased significantly in recent years. . The bottles must pass a decontaminating step and an inspection control to assure the absence of any defects and the stability of the product until its consumption.

Storage (Aging in bottles): After bottling aged wines are taken to the bottle holders, where they are stored horizontally. In this phase temperature can affect the wine volume and eventually loosen the cork seal, leading to leakage, oxidation and possible microbial formation resulting in spoilage of bottled wine.

After maturation of wine, the bottles are cleaned, labelled and encapsulated.

A summary of the production process for high quality red wine is outlined in Figure 1.3.

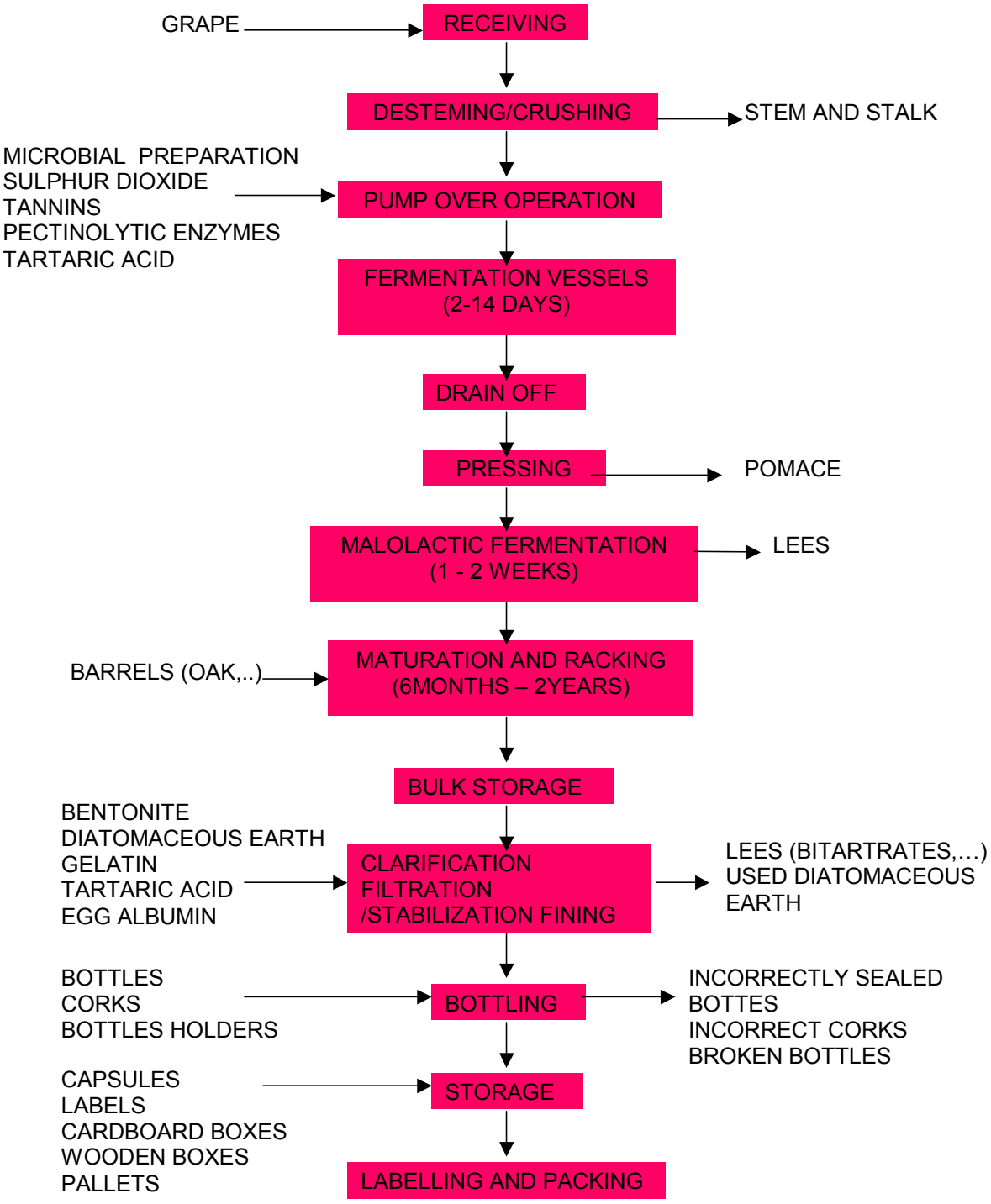


Figure 1.3 – Summary of production processes for a mature red wine.

1.6 Wine quality control

In the wine industry high quality production methods are of paramount importance. Product quality is assessed mainly by tasting according to standard descriptors. This approach may be acceptable for describing wines locally but may present significant problems when the same descriptors are used internationally. For example 'gooseberry' may be used as a classical descriptor of a white wine such as Sauvignon Blanc, its acid flavours match this wine almost exactly, and most British tasters use this term. However, the gooseberry is not a well-known fruit in the US and wine tasters rarely use this descriptor.

Clearly there are many potential problems with this subjective approach and some form of standardization of sensory evaluation procedures is necessary to enable it to be applied internationally. In many laboratories nine principle descriptors are commonly used in sensory evaluation. These are colour, aroma intensity, purity, complexity subtlety, palette strength, length, balance and longevity. However, quality assessment in the wine industry remains a highly subjective judgement that depends on a complex combination of semi-abstract terms.

In many wineries analytical quality control tests are increasingly being used in support of sensory assessment. Traditional methods together with highly sophisticated procedures using GC-MS and ^{13}C NMR to study intact wine bottles (Weekley *et al.,* 2003; Gill *et al*., 2003) and near-infrared

spectroscopy (NIRS) (Dambergs, *et al*., 2003) have recently been applied to the assessment of wine quality.

The characteristics of individual wines are largely governed by the basic characteristics of the fruit and the skills of the winemaker. A master winemaker is able to control and adjust viticultural and oenological procedures to emphasize desirable aromas and flavours in the final product. However, in wine production, quality is a very subjective judgement that is dependent on many factors that are often a compromise between current fashions and established standard products.

Wine is largely purchased for its special flavour and aroma characteristics, such factors making the product particularly vulnerable to influences from external biological or chemical contaminants that may impart adverse flavour characteristics.

Some of the principle external contaminants experienced in wine production are as follows:

Mould growth on grapes. This is quite common and is generally considered to be undesirable as it leads to fruit deterioration and many aroma modifications may occur. Common moulds involved in vineyard spoilage include Penicillium, Aspergillus, Mucor, Rhizous and Botrytis. Mould growth normally involves the secretion of enzymes to partially hydrolyse complex carbohydrates adjacent to fungal hypae. The hydrolysed materials are then absorbed through cell membranes. During this process other side reactions

may also occur e.g. the hydrolysis of monoterpenes with the formation of unpleasant odours described as ‘phenolic’ (Nishimura and Mauda, 1983). Populations of contaminant bacteria such as Gluconobacter and Acetobacter may also affect wine production by distorting the normal levels of volatile fatty acids in wine must.

Chemical contaminants are of particular concern, particularly pesticides/ biocides and their metabolites which represent a widely administered class of synthetic organic compounds that can cause serious health and quality issues to the consumer and also introduce ‘off flavours’ into the final product. Such materials may enter the production process by many diverse routes, contaminating materials during both agricultural production and processing. A wide variety of pesticide sprays are used for the control of mammals, invertebrates, weeds and fungi. When used as directed residual levels of pesticides monitored in the EU monitoring programme were above the MRL in only two percent of grapes tested and did not influence fermentation or wine quality. (EU report SANCO/20/03, 2001). However, systemic pesticides may be found in the must and the wine. Many chlorophenolic pesticides are also used for the preservation of paper and card packaging materials used to store process inputs. Other contact materials such as wine corks may also contain pesticide residues or metabolites that may leech into the wine.

1.7 Phenolic components in commercial wine production

One of the main obstacles to the establishment of a simple at-line test method for chlorophenols and chloroanisoles in wine and related materials are potential interference effects from co-extracted materials. Simple phenols and more complex polyphenolic materials are considered to be the most likely cause of interferences. In wine many different phenolic compounds are present. These range from relatively simple compounds found in grapes to complex tannin-like substances extracted from barrel wood during ageing. Phenols may also be derived from special flavouring or other agents added to the wine. Previous studies, (Singleton and Esau, 1969; Singleton and Nobel, 1976) have indicated over 50 different phenolic compounds in various wines.

The principle classes of compounds are as follows:

Anthocyanins – These compounds are responsible for the pigments in grapes and are normally present in the glucoside or diglucoside form, as shown in Table 1.5.

Other phenolics are grouped into the following classes:

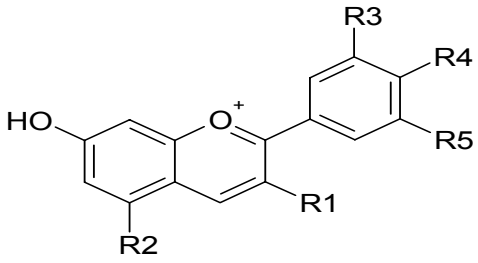
Anthocyanogens

Catechins

Flavenols

Flavenones

Table 1.5 – Naturally occurring anthocyanins in wine



	R_3	R_4	R_5
Cyanidin	OH	OH	H
Peonidin	OCH_3	OH	H
Delphinidin	OH	OH	OH
Petunidin	OCH_3	OH	OH
Malvidin	OCH_3	OH	OCH_3

There are also numerous non flavenoid compounds such as phenol, cresol, ethyl phenol, benzoic acid, and benzaldehyde, cinnamic acid and cinnamaldehyde. Many different coumerins and their precursors such as caffeic and ferulic acids.

All of these compounds have the potential to interfere with the measurement of chlorophenols and chloroanisoles in the proposed at-line test method. The extent of the interference will depend upon the degree of cross reactivity of the test and the concentration present.

In general the presence of phenols can be classified into volatile and non volatile materials (Oughand and Amerin, 1988), the main contributors are listed in Table 1.6. This table also indicates that the highest levels of phenolics are encountered in the non-volatile group therefore a sample purification procedure based on volatility may provide an efficient method of separating many of the potential interfering compounds.

Table 1.6 – Amounts of some non flavenoid compounds in red wine

Compound	**Amount (ng/ml)**
Volatile	
Phenol	<10
p-Cresol	10
4-Ethyl Phenol	350
4-Hydroxy acetophenone	120
4-Ethyl quiacol	80
Vanillin	40
Eugenol	10
Tyrosol	4900
Syringaldehyde	35
Non Volatile	
Salycylic acid	16,000
Vanillic acid	6,000
Getisic acid	4,500
p-Coumaric acid	5,500
Gallic acid	35,000
Ferrulic acid	3,500
Caffeic acid	5,000

1.8 Origin of chlorophenols/ chloroanisole contaminants in the wine industry.

1.8.1 Chlorophenols

Clorophenol contamination may arise due to the accidental or uncontrolled use of pesticides containing the chlorophenoxy moiety e.g the use of 2,4,D to control vinyard weeds. A major potential source of contamination is from constructional timber and paper packaging . Pentachlorophenol has been the principal chlorophenol used for antifungal and biocide use in many products such as non-food packaging, particularly in water-based adhesives. Another common cause of chlorophenol contamination of the wine industry results from in-plant chlorination of phenol. The major source of chlorine in such cases is the treated water supply or chlorine-based sterilising agents. There are a multitude of sources of trace quantities of phenol in food processing plants. Plastic and resin fitting and paints are a common source of this compound but phenol can also be derived from the food itself, from printing inks and packaging materials, and even from boiler water additives. All of these are susceptible to in plant chlorination. Other ingredients in process chemical formulations e.g. Tricolsan™, a chemical used for its antibacterial properties containing chlorophenol moieties and incorporated into many commonly used detergent formulations.

1.8.2 Chloroanisoles

The formation of chloroanisoles normally begins from precursor molecules that contain the hydroxy and chlorine substituted benzene ring. They may be produced by a) synthesis from simple phenols or b) by degradation of more complex molecules.

In both cases the main sources of contaminants mainly arise from the ubiquitous use of bleach for the sterilisation of water and food packaging materials together with the use of chlorinated pesticides for the preservation of wood products.

An example of a synthetic route is the chlorination of phenol or anisole itself, which can be readily chlorinated at the 2 and 4 positions under mild conditions. Biochemical chlorination has also been reported by Jerina *et al* who showed the enzymatic conversion of anisole to Roth chloroanisole by chloroperoxisases.

The principle bio degredative routes originate from the use of halo-organic chemicals such as hexachlorobenzene (HCB), pentachlorophenol, 5-chloro-2-(2,4-dichlorophenoxy) phenol (Triclosan™) and their metabolites.

In addition to these principle routes the formation of chloroanisoles is further complicated by the large number of phenolic and anisolic materials present in plant materials. Many of these be synthetically chlorinated and eventually degrade to simple chloroanisoles.

In wine production the taste and odour of the product is paramount and may critically affect the annual batch production process. The impact of chlorophenol or chloroanisole contamination is therefore highly significant in wine producing regions.

It is essential that appropriate quality control procedures and testing of materials and other inputs is employed to control contaminant levels. This may be achieved by sending samples to a reference laboratory for analysis but the rapid identification of contaminants is best achieved at the production site to enable appropriate corrective action taken rapidly and thereby minimise the spread of contaminants within the production process.

2.0 AT-LINE TESTING PROCEDURES

In this chapter alternative strategies for the development of at-line test methods are considered.

2.1 The design of at-line test procedures

The analytical capabilities of wineries varies widely from traditional 'wet chemistry' methods such as ethanol content, sugar at harvest and titratable acidity to the use of advanced analytical techniques such as gas chromatography-mass spectrometry. In situations were the minimum analytical requirements are installed, problems may arise due to recurring manufacturing difficulties, and the possibility of failed batches due to process contamination. Batch production methods that employ large vessels such as those used in the wine industry are highly susceptible to major contamination of whole production runs and in these circumstances, it is essential that the identification of process contaminants be undertaken rapidly, without the delays that may be associated with the use of an external service laboratory. Where simple test methods are available, at-line analysis is preferred because it enables the source of the contamination to be traced locally and rapid remedial action to be taken. To reduce the risk of future contamination it is also important to set-up local control sampling points for new consignments of materials and to introduce routine contaminant testing procedures at critical production control points. By employing critical path analysis procedures, problem analysis and preventative action can be taken quickly to prevent further production losses.

The design features of equipment used for field measurements are normally similar in basic design to that used in the laboratory. Recently the miniaturisation of measurement devices has produced equipment capable of to providing data of similar quality to the reference laboratory.

An ideal portable biosensor would feature the following characteristics:

1) Equipment should be small, self contained, cheap and robust.
2) A simple user interface.
3) The test specimen should be added by the user and all further reagent handling should be simple or automatic.
4) Results unaffected by the sample matrix by incorporating appropriate sample purification procedures.
5) A measurement time of less than 30 minutes.
6) A good correlation of results with established test methods together with some form of internal calibration check to confirm that the equipment is working satisfactorily.

In production control situations, at-line test methods/devices are normally employed as an indicator of out-of-control process conditions and to trigger confirmatory analysis and corrective actions. In general acceptable data obtained using at-line testing devices may be less accurate than reference laboratory data and appropriate control limits are established which take into account the performance characteristics of the measurement procedure.

2.2 Sample pretreatment

Contaminant analysis in both at-line methods and reference laboratories are governed by similar rules concerned with measurement errors at trace levels. Measurements made at parts per trillion levels (ppt) are subject to significantly higher errors than those made at higher concentrations. The relationship between measurement error and concentration level is described by the Horowitz equation (Horwitz and Albert, 1991).

$$RSD_R = 2^{(1- 0.5 \log C)}$$

This equation indicates that the RSD doubles for every decrease of two orders of magnitude in concentration.

In view of this, the precision of both at-line and laboratory measurements is significantly improved by making measurement at higher concentrations.

The precision and accuracy of at-line and laboratory measurements is also affected by the background signal interference. This may be due to undesirable co-extractives in the matrix of the sample or instrumental noise arising from poor design characteristics. To obtain the highest sensitivity requires an optimisation of the signal and background characteristics of the whole procedure employed to perform the test measurement.

Sample preparation procedures provide good opportunities to concentrate and purify the target analyte. In low level trace analysis, detection systems are prone to interference from relatively high concentrations of co-extracted materials. To reduce the effect of these co-extractives, detection systems may be designed that focus on specific molecular features of the analyte. However, when the concentration of the interference is several orders of magnitude higher than the target analyte, significant interferences are highly likely to occur.

An initial sample preparation/ concentration step that is able to concentrate the target compounds by 100 or if possible 1000 fold is highly desirable to reduce measurement errors.

2.3 Extract purification and concentration.

Pesticide analysis provides an excellent model on which to base this component of the field-testing device. In most trace pesticide analysis, following the extraction and preliminary purification of the active component a second separation may be employed to purify sample extracts prior to determination (Thier H *et al.* 1989). Traditionally this was achieved using a silica or alumina mini column, although more recently specific antibody columns have been developed which provide a very effective secondary purification (e.g. Rhone-diagnostics). In field testing devices, antibodies can also be employed to provide a very specific analyte separation. Alternatively, special polymers have recently been reported which may be employed as

'plastic antibodies' to semi-specifically trap target analytes (Surugiu *et al.*, 2000).

2.4 Influence of analyte concentration.

Conventional laboratory analytical detection methods such as UV/visible spectrophotometric procedures are unable to achieve the required detection limit of less than 1ng/ml in water or wine samples for the six analytes specified above. For the analysis of CP/CA by direct flow injection or HPLC with UV detection a sample concentration procedure is normally required to achieve a detection limit lower than 1μg/ml (Frebortoova and Tatarkovicova, 1994; Kot-Wasik *et al.,* 2004). Greater sensitivity (0.01 – 0.1 μg/ml) can be achieved using HPLC with electrochemical detection or by direct electrochemical methods (Trippel *et al.,* 1985; Cardellicchio *et al.,* 1997). Unfortunately direct electrochemical methods are unsuitable for the analysis of chloroanisoles as they are not directly electroactive.

To achieve the desired target concentration of 0.01 ng/ml in wine for all target analytes a 100 fold concentrator column will be required to enable a final measurement detection range of 1.0 - 100 ng/ml to be used. If possible the performance of the concentrator column should provide a concentration factor better than 100 fold to achieve the desired sensitivity.

2.5 Detection methods

The following final detection methods are considered to provide detection at the sub 1ppb level.

1) Gas phase analysis with column or membrane separation and detection by mass selective (miniature GC-MS) or surface acoustic wave detection.
2) Liquid phase analysis with direct, enzymatic or antibody conjugation procedures together with colorimetric, fluorescence, chemiluminescence, amperometric, potentiometric/ ion selective electrode detection, bulk or surface acoustic wave detection.

Gas phase procedures are suitable for the analysis of chloroanisoles in a wide range of sample matrices but these procedures are unsuitable for the direct analysis of chlorophenols due to their susceptibility to adsorption and polymerisation on hot glass and metal surfaces. Chlorophenols can however be measured after the formation of a stable derivative such as the acetyl derivative (Diserens, 2001). In field test methods gas phase methods tend to be highly complex and prone to leaks. Supplies of suitable gasses and high capital cost are also frequently found to restrict the use of this type of equipment.

Liquid phase systems are generally considered the preferred alternative as it is possible to measure both chloroanisoles and chlorophenols without prior derivatization.

2.5.1 UV/visible detection methods

Colorimetric methods provide very robust, selective procedures suitable for inclusion in field test methods. In their simplest format, quantitation employs the use of a comparator chart or stepped comparison cell. Simple portable colorimeter devices are also frequently employed such as water test kit methods (Nanocolor test 75, Macherey-Nagel, Dũren, Germany).

2.5.2 Fluorescence

Where higher sensitivity is required fluorescence or chemiluminescence can be used. However, test procedures employing these detection methods are prone to numerous non-specific matrix quenching effects and instrumentation required for fluorescence measurement is generally complex and easily damaged in field use. However, the recent development of evanescent wave sensors provides a very simple and robust high sensitivity detection system (Buerck *et al.*,1994). This procedure employs an optical fibre and relies on interference of internal reflectance at the fibre surface. Applying antibody systems employing fluorescent labels to the fibre surface provides a very simple optical dip probe. Laser induced fluorescence has also been particularly successful as micro HPLC and Capillary Electrophoresis detection systems. This approach may also be employed for more general flow analysis systems with clean sample extracts. In experiments with amino acids labelled with 9-cyano-6-diethylyaminoxanthene-3-ylene)ethyl-(6-oxo-6-succinimido-oxyhexyl ammonium chloride or Rhodamine, detection limits of

less than 10^{-10} molar were detected with a signal to noise ratio greater than 19 (argon-ion laser at 10mW - Zetalif™, ESA, Inc., MA, USA)

2.5.3 Chemiluminescence

Chemiluminescent methods appear to provide improved sensitivity compared to fluorescence methods (e.g. alkaline phosphatase, 10^{-9}molar – Lin J *et a*l., 1997) and can be used without the need for complex optical systems. Selected reagents (e.g. acridinium esters) can be attached directly to antibodies or antigens. After washing the ester linkage is cleaved under alkaline conditions to release the unstable compound N-methylacridone which decomposes producing a 'flash' of light. In this method it is essential that reagent additions are made automatically or using flow systems to ensure that the signal captured is reproducible. Several commercial systems are available such as the ELECSYS ™ system from Roche Diagnostics, USA and the Nichols 'Advantage ™' system (Nichols Institute Diagnostics, CA, USA).

In some examples the excited state is formed electrochemically at the surface of an electrode e.g. the ORIGEN Tricorder ™ (IGEN International Inc Maryland, USA.). However, the possibility of significant quenching effects by very simple molecules means that matrix components must be very low to enable this method to be successful in field applications.

2.5.4 Enzyme labels

Enzyme labels are now used more widely than any other type of label such as direct fluorescence. The catalytic properties of enzymes are normally employed to generate uv/vis, fluorescent or luminescent products that can be measured at very low concentrations (10^{-7} - 10^{-10} molar). The main disadvantage of enzyme labels is that they have a susceptibility to numerous types of interferences. The enzyme substrate incubation is sensitive to time, temperature, pH, and the presence of many inhibitory substances. Some enzymes require cofactors, such as Mg^{2+} ions or they may be enhanced by co-extractives in the sample matrix. The two enzymes most commonly employed in immunoassays are horse radish peroxidase (HRP) and alkaline phosphatase (ALP). HRP is normally used with hydrogen peroxide (H_2O_2). It is considered to be the most sensitive enzyme label but also the most susceptible to interferences. The HRP/H_2O_2 system is a very strong oxidant capable of oxidising many wine sample co-extractives and it should be noted that many of the components in wine are reducing agents, such as ascorbic acid, vitamin E etc. The activity of HRP is also affected by chlorophenols a fact that has been previously used in the construction of an chlorophenol biosensor based on HRP enhancement (Degiuli and Blum, 2000). To minimise method errors the test solution must be free of these components prior to adding the HRP enzyme label. Alternative enzymes such as ALP is almost as sensitive as HRP particularly when fluorescent substrates are employed but far less susceptible to interferences. Many other enzymes have been used e.g.acetyl choline esterase, acetate kinase, firefly luciferase,

xanthine oxidase, glucose oxidase but all of these are less sensitive than HRP and ALP.

To take full advantage of the special sensitivity of HRP it may be necessary to eliminate sample matrix effects. To achieve this using the competitive assay format it may be possible to employ an intermediate small molecule hapten conjugate that is not directly bound to HRP. Moieties such as phosphotyrosine (Cummings *et al.,* 1999) and biotin have previously been employed in this manner to reduce HRP matrix effects. A secondary antibody - HRP (e.g. anti phosphotyrosine-HRP) or streptavidin-HRP label is then added after washing the primary hapten conjugate free of sample matrix components.

2.5.5 Surface plasmon resonance

The surface plasmon resonance sensor technique is an extension of the evanescent wave principle. In this approach, monochromatic light is reflected internally from the interface of a high refractive index transparent material coated with a thin layer (50nm) of an appropriate metal e.g. gold. When conducting affinity based binding studies, the metal surface is coated with the binding agent. The angle of reflection is dependent on the refractive index of the surface layer which in turn is dependent on hapten – conjugate competition. This technique is developing rapidly and many ultra-high sensitivity and wide dynamic range devices are currently under development (Nikitin *et al.,* 1999). The technique appears to show great potential for use in at-line sensor methods.

2.5.6 Electrochemical methods

Amperometric devices are very popular in field test methods, as they are inexpensive and disposable electrodes are very simple to fabricate. High sensitivity versions generally employ oxidation-reduction enzyme systems such as horse radish peroxidase (HRP) or glucose oxidase. Sensitivity is equivalent to visible spectrum optical methods using similar enzymatic procedures. Any redox compounds present, such as oxygen, ascorbate, thiols, phenols etc. may interfere with the system; to avoid interference effects, chemical mediators may be used. Alternatively a capture and separate secondary antibody labelling system can be employed to remove enzyme interferences. The electrode systems may require regular replacement to avoid matrix contamination effects (Wang, 2000).

Potentiometric sensors rely on the measurement of changes in potential that arise from the reaction of an analyte with a specific sensor. Variants on this approach include field effect transistors and the use of ion selective electrodes to measure the product of a high sensitivity enzymatic reaction such as the liberation of fluoride ions from 4-fluorophenol by HRP (Diomede *et al.,* 1988).

2.5.7 Acoustic methods

Acoustic methods can be used as biosensors in various forms. Employing quartz crystal microbalance or surface acoustic wave devices are cost effective and experimentally very simple to set-up. The response of these systems is dependent on the effect of mass changes on oscillation frequency. For molecules with masses of less than 1000 daltons they are inherently non-specific. The sensitivity is significantly improved when the analyteis bound to an antibody or synthetic receptor molecule (Luo *et al*., 2001). Several high sensitivity devices have been developed for the analysis of pesticides in the liquid phase and these methods show great promise in the form of crystal arrays for simultaneous multiple assays.

The potential benefits and shortcomings of the various sensor devices are summarised in Table 2.1. The amperometric and potentiometric sensors have been intensively investigated over many decades but with the exception of the glucose biosensor, their development has not provided the range of cheap versatile devices originally conceived.

Mass-detection piezoelectric sensors in various forms and advances in microwave technology appear to provide greater potential for incorporation for ‘new wave’ biosensors. These devices appear to be capable of detecting very low concentrations of contaminants but due to their non-specific mass detection principle, they require a highly selective separation procedure to

enable their use in field-testing devices and may be more suitable to gas phase systems.

Optical detection methods, particularly fluorescence, may be more sensitive than other methods if the dynamic range of the competitive assay procedure is configured appropriately and semi-specific sensors can be produced using fluorescent labels or fluorescent enzyme substrates. Both optical and electrochemical systems are highly suitable for automation and inclusion in portable flow devices.

The primary requirements of the detection method for incorporation into an at-line measurement device for chlorophenols and chloroanisoles in wine and related materials is high sensitivity. However, in view of the numerous co-extractives in red wine, methods that demonstrate the highest sensitivity may be inappropriate for incorporation into the procedure unless they are linked to procedures designed to reduce background contaminant levels.

This study will concentrate largely on the potential of the two optical methods, fluorescence and visible spectroscopy together with two electrochemical procedures employing an amperometric detection procedure.

Table 2.1 – Summary of detection method characteristics

Working range	**Sensor Technology**	**Main advantages**	**Main disadvantages**
Femtomolar	Luminescence	High sensitivity. Simple optics, low production costs	Matrix interference effects at low concentrations.
Picomolar	Fluorescence	High sensitivity. Simple to automate.	Complex optical systems. Less robust. Some interference effects at low concentrations.
Micromolar	Optical - visible	Simple to automate	Complex optical systems, less sensitive than fluorescence or luminescence .
Micromolar	Potentiometric	Simple device configuration. Low cost. Generally interference free.	Limited availability of high sensitivity electrode systems
Micromolar	Amperometric	Proven in clinical chemistry applications. Low cost.	Susceptible to interferences.
Micromolar	Surface plasmon resonance		High production costs. Sensitive to high mass interferences.
Micromolar	Surface acoustic wave	Low production costs.	Systems not proven. Low-mass errors highly significant. Subject to environmental influences (e.g. pH, temp)

2.6 COMMERCIALLY AVAILABLE AT-LINE TEST METHODS AND EQUIPMENT

2.6.1 Immunoassay test kits

Several test kits based on immunoassay technologies are available for the analysis of PCP in environmental samples, largely water and soil. Such methods rely on the selective binding of anti.

.bodies. Antibodies are proteins that bind very tightly to their targets (antigens), they are produced in vertebrates as a defence against infection. Antibodies are produced by a class of white blood cells called B lymphocytes. Each B lymphocyte cell carries a different membrane bound antibody receptor molecule on the surface. When the antigen binds to this receptor , the B cell is stimulated to divide and to secrete large amounts of the same antibody. The repetitive introduction of the antigen at intervals of several weeks into a host animal such as a laboratory mouse stimulates the production of different types of antibodies.

Each antibody molecule is made of two identical immunoglobulins (Ig) normally composed of four polypeptides– two heavy chains and two light chains joined to form a "Y" shaped molecule. They are may be crudely divided into five major classes, IgM, IgG, IgA, IgD and IgE, based on their structure and immune function. IgM are the first antibody produced in response to an immunogen . IgG are monomeric antibodies usually produced later in the immune response than IgM. IgG are the most prevalent antibody and normally shows the strongest affinity towards the specific antigen. IgA

are also produced later and generally have a more specific protective barrier function. IgE and IgD also produced later are normally present only at very low concentrations and have very specific functions.

After repeated injections of the antigen only B lymphocyte cell clones with a high affinity (IgG) are able to bind effectively; these are normally selected for monoclonal antibody production.

The production of monoclonal antibodies was pioneered by Kohler and Milstein in 1975. In their procedure surface bound antibodies from spleen cells with are fused with immortal Myeloma cell produced by invitro cell culture techniques. These cells are grown on suitable medium fro 2-3 weeks and the supernatent is assayed for antibodies with the required specificity. The selected monoclonal antibody is then propagated using cell culture techniques or by injecting into a new host animal. Antibodies are extracted, purified and retained the desired application. A stylised structure of an antibody is show in Figure 2.1.

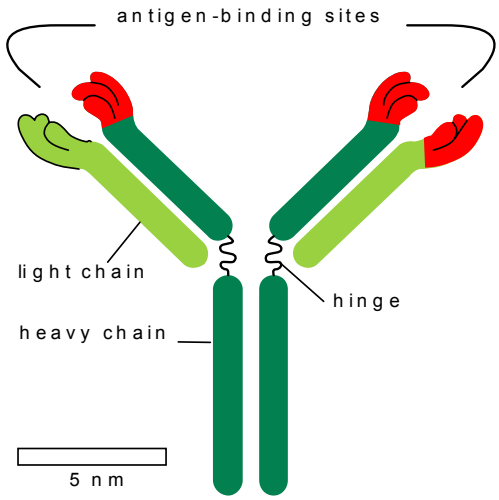


Figure 2.1 – Diagram indicating the principle functional regions of an antibody

Analytical applications normally involve binding either the antibody or antigen to a surface such as a 96 well microplate or an iron cored 'magnetic' bead and performing direct or competitive binding with the surface bound molecule. Many different assay formats have been developed for specific applications. The choice of assay format is dependent on the availability and size of binding sites. The simplest assay format is referred to as direct ELISA. In this case the antigen is attached to the solid phase and reacted directly with an enzyme labelled antiserum. In pesticide analysis using ELISA procedures the most common assay format is the competitive ELISA format (Dankwardt, 2000). This approach is appropriate where the antigen is a small molecule and a single highly specific binding site is available. The principle of the assay relies on the construction of a binding surface with a limited number of available sites. An antigen-mimic is also required that is able to link to the binding site and has an attached reporter label which may be an enzyme, a fluorescent tag or another label that enables a high sensitivity detection method to be used. The antigen from the sample solution and added antigen-mimic then compete for the available binding site. The unbound antigen and antigen mimic guests are removed, in the ELISA assay by washing and the reporter label on the antigen-mimic is activated e.g. by the addition of a high sensitivity enzyme substrate. This procedure is summarised in Figure 2.2.

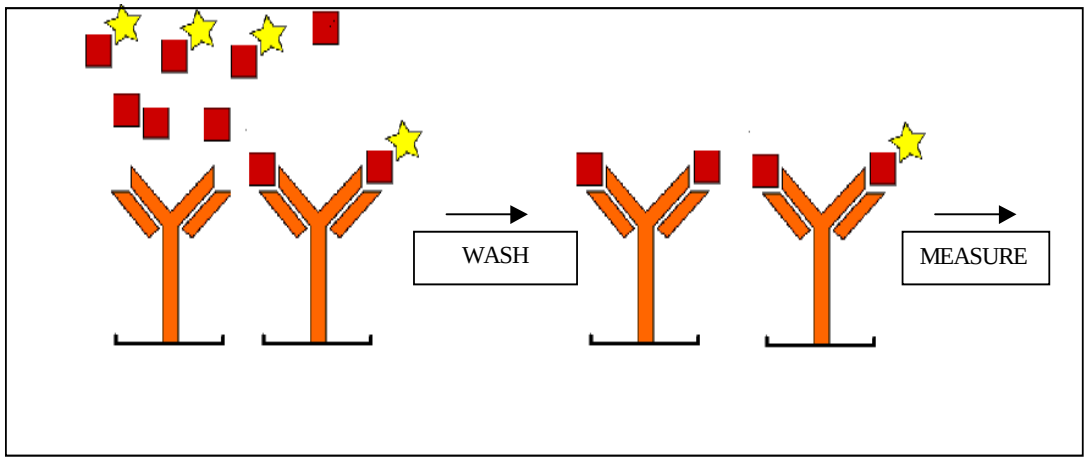


Figure 2.2. – Diagramatic representation of the competitive ELISA assay format.

Potential problems associated with antibody procedures are largely involve cross-reactivity due to antibody response to other molecules. These normally contain structurally related groups, however any molecule that has a strong binding affinity for the antibody receptor site may interfere with competitive binding and produce a false positive response. In view of this ELISA methods of analysis for small molecules are generally only suitable for very pure samples or sample extracts that have been rigorously purified prior to analysis.

The following three systems were reviewed in 1995 by the US, EPA (US, EPA report EPA/540/R-95/514 -1995).

1) Penta RISc™ test system developed by EnSYS, Incorporated.

2) The RaPID™ assay developed by Ohmicron Corporation.

3) The EnviroGard™ pentachlorophenol test kit developed by the Millipore Corp, MA, USA.

The report recommended that result obtained using the EnSys Penta RISc semi-quantitative test kit should be used as screening data only. It was found to be highly temperature sensitive and did not meet all of the specified performance characteristics.

The Ohmicron PCP RaPID assay was slightly better but this was also shown to be temperature sensitive but it could be used for initial site surveys.

The Millipore EnviroGard produced false negative results and poor precision at high concentrations of PCP.

2.6.2 Portable analytical instruments

One portable instrumental device is commercially available for at-line analysis of chloroanisoles in wine. This is called the Znose, illustrated in Figure 2.3 (Staples, 2000). The device is based on a miniaturised headspace GC design with detection using a surface acoustic wave (SAW) detector. The instrument is limited to the analysis of volatile components (chloroanisoles) and its sensitivity is limited to 1ppb. It is unsuitable for the analysis of chlorophenols because these components tend to be adsorbed or chemically modified by sample co-extractives during sample introduction. The unit has been adopted by a few large wineries in the US although and the unit cost is similar to reference laboratory GC instruments.

Figure 2.3 - Znose commercial wine testing device

Other devices have been used for field analysis of wine that are based on a portable GC-MS using a short column or membrane inlet system. In general they have not been widely accepted by the industry due to their high cost.

3.0 REFERENCE GAS CHROMATOGRAPHY PROCEDURE USING ELECTRON CAPTURE DETECTION

The purpose of this chapter is to describe the analytical method used for detecting, and measuring the chlorophenol and chloroanisole congeners most commonly implicated in wine contamination incidents. The intent is not to provide an exhaustive list of analytical methods but to identify and describe a well-established method that may be used as a reference procedure for the analysis of wine at nano-grammes per litre and other sample types such as cork and wood products at parts per billion concentrations.

3.1 Development of a reference GC-ECD procedure.

Pesticide residue analysis using gas chromatography methods have been employed for many years to achieve the level of resolution and sensitivity for wine analysis. Early methods involved methylation of chlorophenols with diazomethane (Cheng and Kilgore, 1966). However, diazomethane is now considered to be a toxic substance therefore alternative, safer derivatizing agents are currently recommended. The most commonly used methods employ acylation using acetic anhydride to form the corresponding acetate ester, alkylation with diazomethane or pentafluorobenzyl bromide for higher sensitivity using electron capture detection (EPA 8041A 2000). Diakylacetals have also been frequently used to form methyl esters and several silylation methods are available using e.g. BSA to form trimethylsilyl ethers (Danielson *et al.,* 2000).

A popular method for suspected chlorophenol/chloroanisole contamination of wine in a reference or service laboratory involves extraction and concentration by liquid/liquid partition followed by gas chromatographic analysis using sophisticated GC-ECD or GC-MS procedures (Cooper *et al.,* 1994). Chloroanisoles may be chromatographed directly but chlorophenols, which are less stable and are more susceptible to decomposition and polymerisation on hot surfaces, require derivatization prior to GC analysis.

The chromatographic method detailed in the following section was employed to measure chlorophenols and chloroanisoles wine samples and extracts of cork, wood and miscellaneous products. The method was also employed to test various sample fractions separated during method development experiments.

3.1.1 Materials and methods

Wine sample

A representative sample of Rioja wine (Campo Viejo, Cranzia 1997) from the winery Campo Viejo in Northern Spain was selected as it was produced from the most commonly used Spanish grape varieties. This wine was produced from Temperanillo, Garnacha and Mazuelo grapes, matured in oak barrels for 12 months.

Each bottle of wine was subsampled into 50ml aliquots in sealed under nitrogen in amber bottles and stored at 4^0C until required for analysis.

Fortified wine sample.

A working standard solution containing a mixture of the three principal chlorophenol and three chloroanisole contaminants in wine (see Chapter 1 Section 1.4) were prepared in ethanol. A 1ml volume of the working standards were diluted to 100ml using the selected wine sample. The following CA/CP reference materials were used:

Pentachlorophenol

Pentachloroanisole

2,3,4,6-tetrachlorophenol

2,3,5,6-tetrachloroanisole

2,4,6-trichlorophenol

2,4,6-trichloroanisole

Reference standard solutions

Six reference standard solution were prepared by dilution of 0, 5, 10, 15, 20, 25 ml of the working standard solution to 100ml in a blank wine sample to give the following calibration ranges:

Chlorophenols 2 – 200 ng/ml

Chloroanisoles 4 – 400 ng/ml

These solutions were extracted and derivatized in an identical manner to the wine samples and extracts and were employed for instrumental calibration.

3.1.2 Determination of CA/CP content by gas chromatography

Cleaning of laboratory glassware

At low concentrations chlorophenols bind strongly to active sites on the glassware employed for extraction purposes and to the internal surfaces of the gas chromatographic inlet system. In extracts or samples containing few co-extractives it is possible to achieve a satisfactory separation by direct injection using specially deactivated glassware, inlet liner and precolumn but where samples contain high levels of co-extractives the retention characteristics of chlorophenols by the inlet liner and column may change after each sample injected. This produces distorted peaks with inconsistent retention characteristics. In view of this the determination of chlorophenols in wine extracts was carried out using glassware that was scrupulously cleaned by soaking overnight in 3% v/v pesticide grade detergent (Decon 90, Aldrich), rinsing and soaking in 7% v/v nitric acid. Glassware was rinsed in RO water, HPLC grade water and HPLC grade methanol before being dried immediately prior to use (according to the ASTM Annual Book of Standards, 1984). Standard solutions and wine extracts for chlorophenol analysis were derivatised with acetic anhydride to form the acetyl derivatives. Chloroanisoles were extracted and chromatographed simultaneously, no derivatization was required.

3.1.3 Derivatisation procedure

A sample of the test solution (1 ml) was transferred into a 25 ml volumetric flask and 4ml 1M phosphoric acid and 2 ml hexane solution containing internal standard (540ng/ml Mirex, Fluka Chemie, GmbH) were added with mixing. The mixture was shaken for 10 min using a wrist action shaker (Griffin and George, UK) and allowed to stand for 30 min HPLC water was then added with care until the phase boundary was within the neck of the flask. The flask was allowed to stand for a further 30 min to ensure complete separation. Where separation difficulties were experienced samples were placed in an ultrasonic cleaning bath for 5 min to accelerate the phase separation process.

A sample of the upper hexane layer (1ml) was transferred to a 2ml gas chromatography vial and 100µl pyridine and 100µl high purity acetic anhydride (Fluka Chemie, GmbH) was added with mixing. The vial was sealed and heated for 20 min at 60 ^{o}C in a waterbath. After cooling, the sample was injected onto the gas chromatograph using the instrumental conditions listed in Table 3.1. A good resolution of the three chlorophenol and chloroanisole congeners was achieved (Figure 3.1).

Table 3.1 – Gas chromatography instrument conditions

Instrument	Hewlett Packard 5890 series II gas chromatograph
Carrier gas	Helium at 1ml/min
Injector	1µl by autosampler, split/splitless injection
Column	30M x 0.25mm(ID)x0.25µm(film) J&W DB-5
Temperature	Initial - 50 ^{o}C for 2mins Ramp - 5 ^{o}C/min to 250 ^{o}C Hold - 40 minutes
Detector	Ni 63 electron capture with N_2 makeup gas
Data handling	HP Chemstation
Internal standard	**Dodecachloropentacyclodecane (Mirex)**

3.1.4 Calibration

Instrument calibration was achieved using six reference standard solutions described in 3.1.1 above. The repeatability of the determination was assessed by measuring the CA and CP content of ten wine samples fortified at 100 ng/ml approximately.

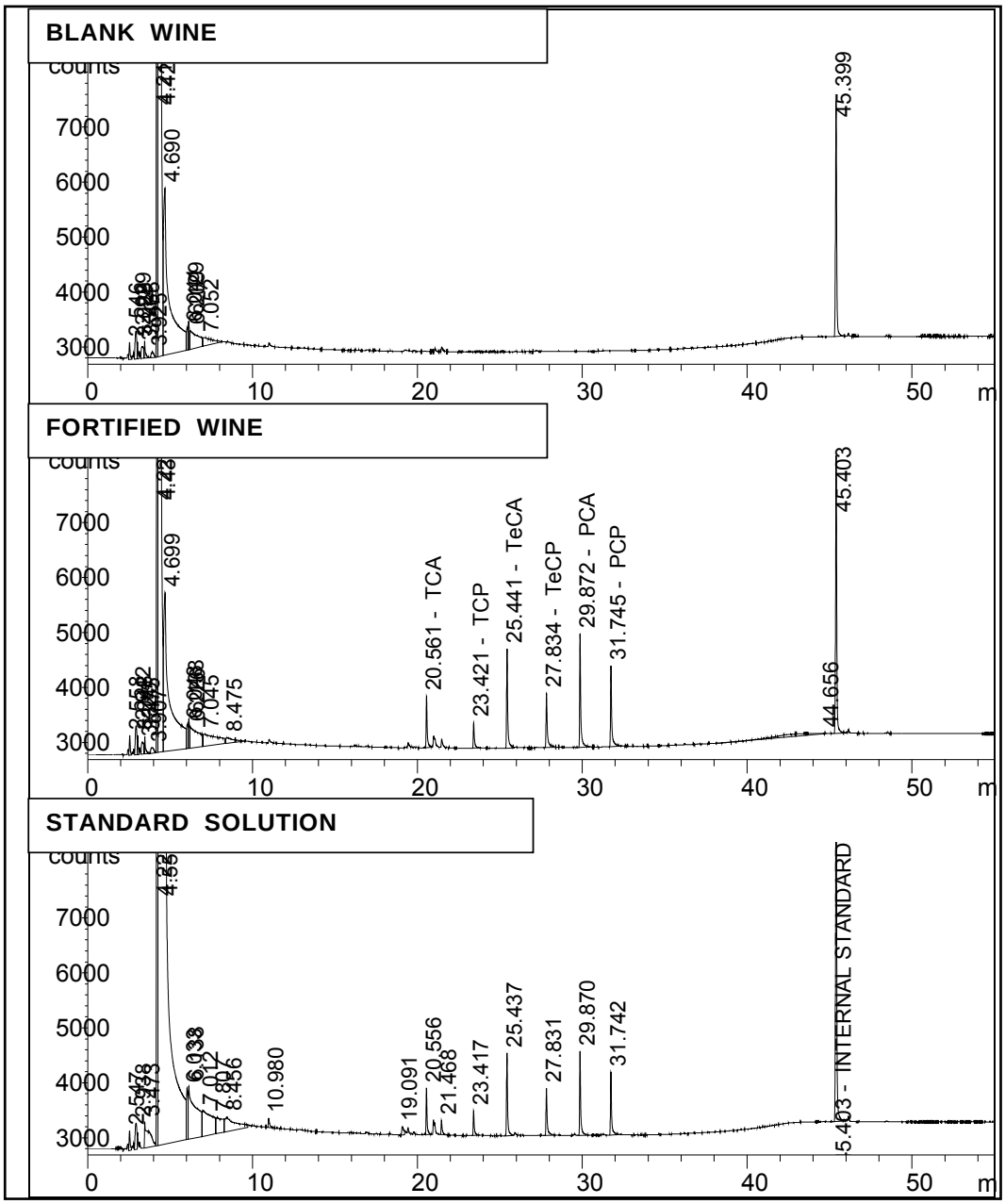


Figure 3.1 – Example chromatogram, showing GC-ECD profile for a six component reference standard solution, together with fortified and blank wine extracts.

3.2 Performance Characteristics of GC-ECD procedure

3.2.1 Linearity

A linear calibration graph was obtained for each calibration reference standard material. In each case the regression coefficient was greater than 0.990.

3.2.2 Repeatability

Confidence intervals (CI) for the fortified wine samples were calculated using the following standard formula

$$CI = t(s/\sqrt{n})$$

where is the Students 't' statistic, s is the standard deviation of the samples et and n the number of samples tested. Results are shown in Table 3.2.

Table 3.2 – Performance characteristics of analytical method using ten fortified wine samples

Compound	TCP	TeCP	PCP	TCA	TeCA	PCA
CI (± %)	4.5	4.1	3.1	4.9	6.5	6.0

The adopted method appears to be robust and straightforward in routine operation. It is also capable of handling a wide range of sample types with few chromatographic interferences.

4.0 PRELIMINARY INVESTIGATIONS

In view of the high sensitivity required and the complexity of the sample matrix, preliminary experiments were conducted to explore key issues in the development of a suitable method.

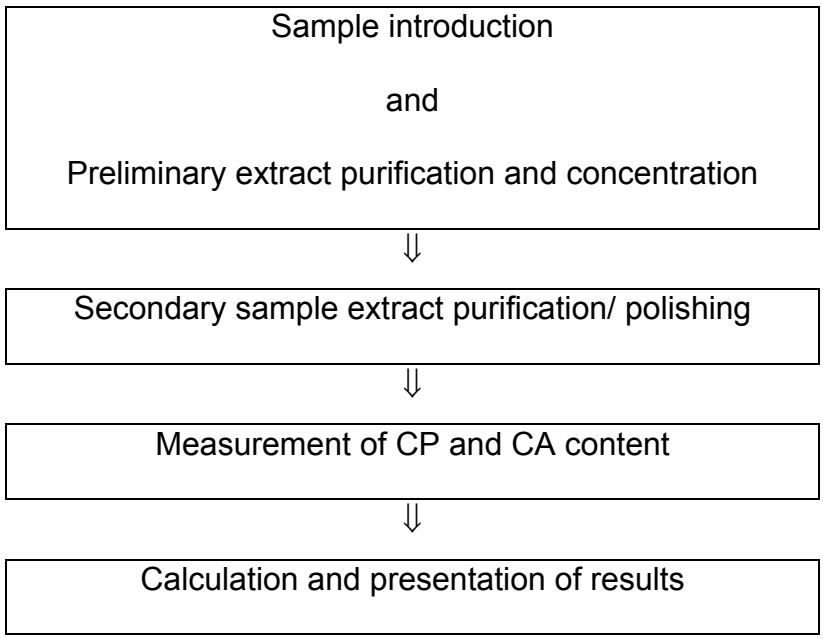
4.1 Preliminary investigations into alternative detection procedures and matrix interference effects.

A limited preliminary investigation was undertaken to investigate the following two key issues and their impact on the design of a proposed pocedure:

(i) Detection methods.

(ii) Matrix interference effects.

The basic construction of an at-line procedure or device was considered to require the following four key components:



4.2 - Alternative detection methods.

The limit of detection of the final measurement stage of the assay is highly dependent on the sample purification procedures employed. The use of colorimetric/ fluorescence or electrochemical measurement systems may provide good opportunities for detection in the range 0.1 – 10 ng/ml.

4.2.1 Colorimetric/ fluorescence procedures

Using direct colorimetric or HPLC methods of analysis with UV detection, the detection limit for phenols is normally in the region of 500 ng/ml (Macherey-Nagel test kits, Dũren, Germany). Using a commercially available competitive chlorophenol antibody test kit (SDI , Hants, UK) with detection based on a horseradish peroxidase enzymatic oxidation of 3,3',5,5'tetramethyl benzidine the detection limit can be improved to 0.1 ng/ml, an amplification factor of 5000 times.

Additional experiments indicate that the standard detection limit of the test kit can be further improved by a factor of 100 by using an enzyme substrate that produces a fluorescent oxidation product. Preliminary experiments using a commercially available substrate QuantaBlu^{TM} (Pierce, Rockford, Illinois) indicate that a practical detection limit of 0.001 ng/ml can be achieved in water samples.

4.2.2 Electrochemical procedures

Direct electrochemical procedures have been used extensively for the amperometric determination of chlorophenols at levels of around 0.1μg/ml. They can also be employed indirectly to measure the concentration of products of the enzyme catalysed reaction of selected redox substrates (mediators) e.g. horse-radish peroxidase and 1,1'dimethyl ferrocene or glucose oxidase and glucose. A competitive ELISA procedure is the preferred option, as it would also enable the measurement of chloroanisoles, which are not electro-active.

Electrochemical methods provide a low cost measurement system, which may have significant advantages in the design of a portable device. The principle advantages of this procedure are their low cost and suitability for incorporation into portable biosensor devices. Disposable electrodes may be produced using screen printing methods (Kröger & Turner, 1997) which involves the controlled deposition of successive layers of specially formulated inks onto a cellulose acetate sheet. The principal disadvantage of this approach is considered to be the greater risk of false positive results caused by electrode poisoning or other matrix effects.

4.3 - Evaluation of the SDI PCP test kit with electrochemical detection using screen-printed carbon electrodes.

The objective of this preliminary experiment was to assess the potential of electrochemical amperometric detection methods as an improved detection method to the standard visible spectroscopic procedure employed by the standard SDI kit method.

4.3.1 Summary of procedure

The four standards provided in the SDI kit (0, 0.1, 1.0 and 10 ng/ml PCP) were pipetted into small test tubes and the enzyme conjugate was added with mixing. The antibody bound to colloidal magnetic particles was then added and after 30 minutes the bound material was separated using the SDI magnetic separator rack and the residue washed three times with the wash solution provided. The residue was retained for the electrochemical measurement of PCP.

Electrochemical measurement was performed using a sample cell fabricated by cementing a small cylindrical, polypropylene sample chamber (13mm dia. x 15mm) to the screen printed electrode.

4.3.2 Electrode fabrication

A DEK 248 printer (DEK, Weymouth, UK) incorporating screens with appropriate stencil designs (60 per screen) was used for precision ink deposition. The base material for electrode fabrication was 0.25 mm

thickness polyester sheeting (Cadillac Plastics, Swindon, UK) onto which was deposited 3 successive ink layers. These were: basal tracks and working/counter electrode - I45R carbon ink (MCA Services Ltd., Melbourn, Cambs. UK); reference electrode – 15% w/w silver chloride in silver paste (MCA); insulation shroud – 242-SB epoxy-based protective coating ink (Agmet ESL, Reading UK). The electrodes were then heat-treated at 125°C for 2 h to cure the insulation shroud and stabilise the working electrode to allow prolonged device usage in aqueous environments. A diagram of the electrode screen-printed assembly is shown in Figure 4.1.

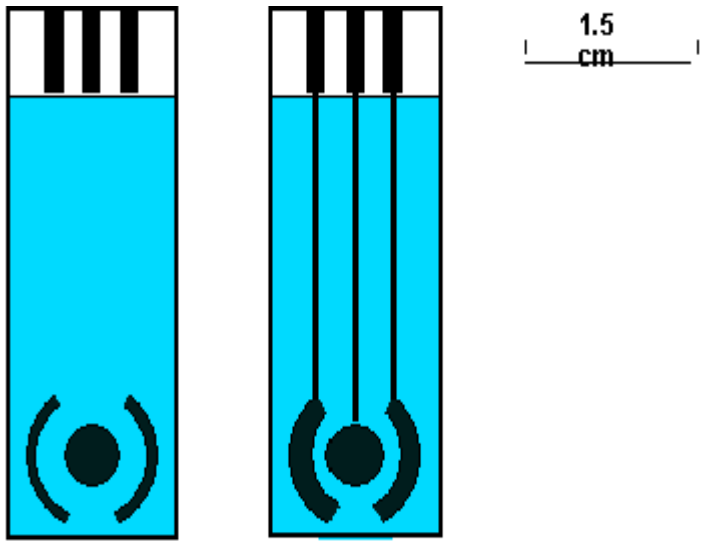


Figure 4.1 - Screen-printed 3-electrode assembly. (a) front view showing the 3 electrodes and electrode connectors, (b) rear view showing underlying basal tracks. The central circular working electrode (WE) and counter (left of WE) and reference (right of WE) electrodes can be seen).

Electrochemical measurements were achieved using a 2cm^2 sample cell fabricated by cementing a small cylindrical, polypropylene sample chamber (13mm dia. x 15mm) to the screen printed electrode (Figure 4.2).

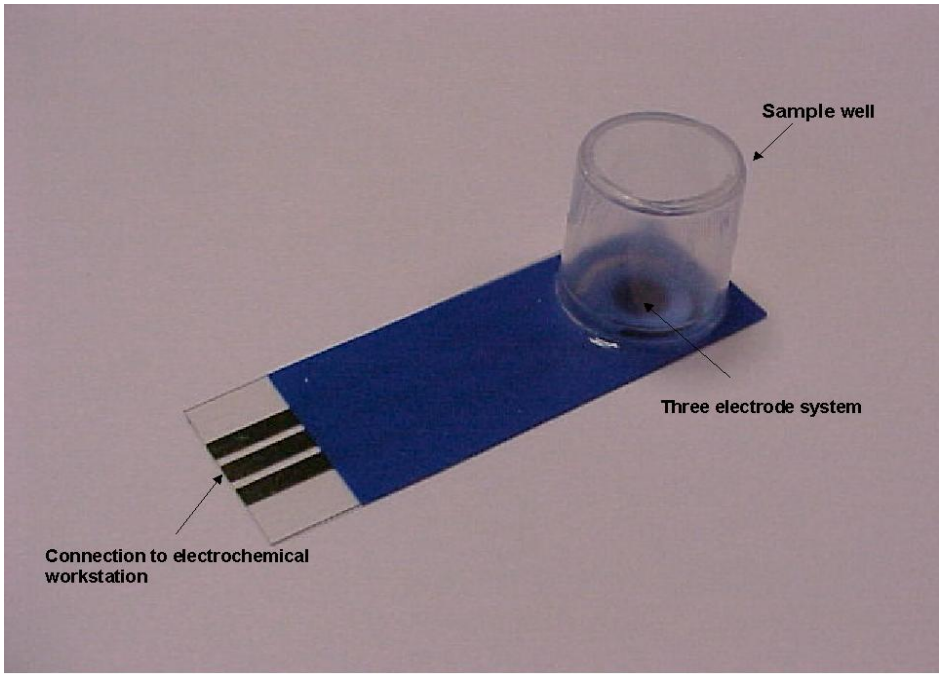


Figure 4.2 – Modified screen printed electrode

4.3.3 Experimental procedure

All electrochemical measurements were carried out with a computer controlled BAS100B/W electrochemical workstation (Bioanalytical Systems Inc., Congleton, UK) using an ambient cell with a three electrode configuration at 22-23^{o}C. The reference electrode was an Ag-AgCl electrode with a carbon auxiliary electrode and the carbon working electrode optimised to maximum sensitivity at –100mV.

The H_2O_2/TMB substrate solution (Sigma T8665) was placed in the cell with mixing and the colloidal suspension of magnetic particle retained conjugate was then transferred to the measurement cell.

A record of the current passing through the working electrode as a function of time was recorded for a further 400 seconds.

Each of the four standards provided in the SDI kit was measured in an identical manner.

4.3.4 Results

Experimental data is shown graphically in Figures 4.3, 4.4 and Table 4.1.

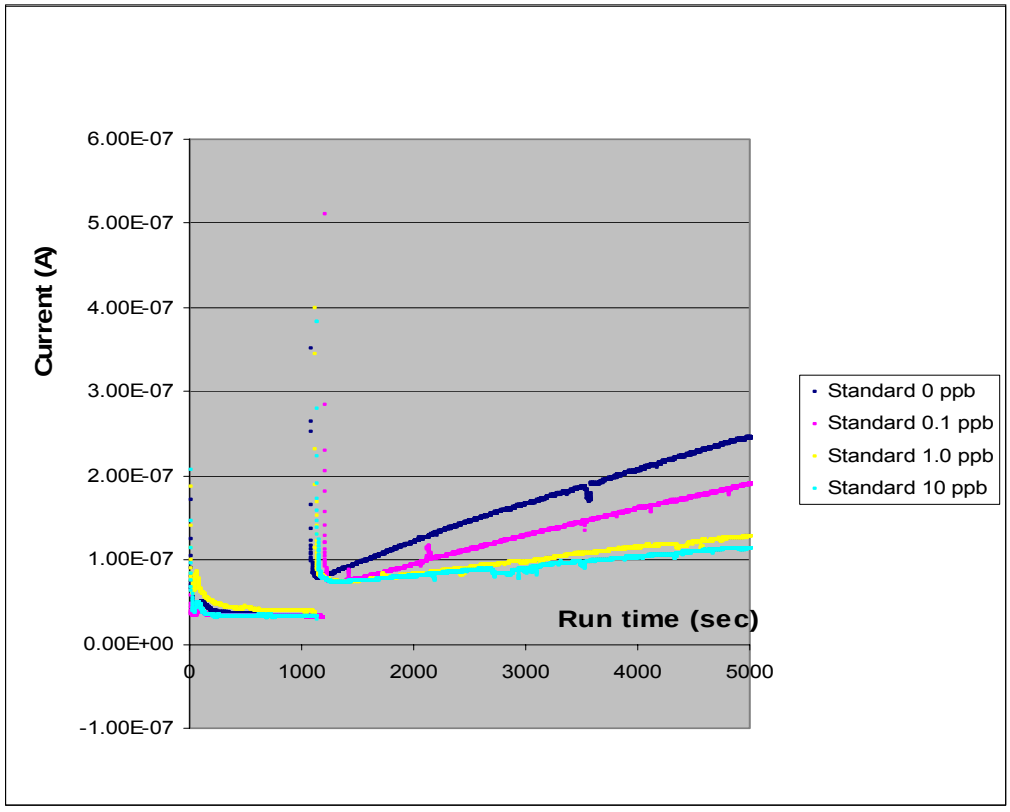


Figure 4.3 – Plot of current vs time for PCP standard solutions

The initial enzymic rate calculated by determining the slope of the standards graphs is shown in Table 4.1. A calibration graph was constructed based on the ratio of the initial rate (S/S_0) where S and S_0 are the reaction rates for the specified standard and blank.

Table 4.1 – Initial rates of enzyme reaction for the SDI test kit with electrochemical detection.

Standards conc. (ng/ml)	Log conc.	Slope(S) pamps/sec	%S/So
0	-	40.9	100
0.1	-1	31.2	76.3
1.0	0	15.4	37.5
10	1	11.8	28.7

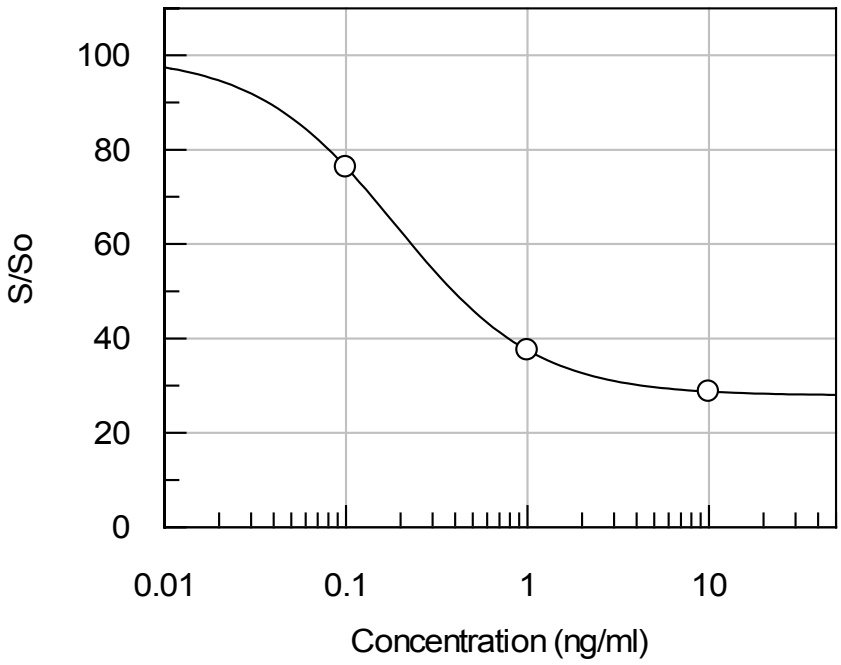


Fig.4.4 – Calibration curve for PCP in diluent buffer using SDI anti-PCP immunoassay test kit with electrochemical detection.

Results demonstrate that amperometric detection procedures are able to provide an alternative detection method to the colorimetric assay procedure provided in the SDI test kit of equivalent sensitivity. This approach may therefore provide a more suitable alternative detection method for incorporation into a portable test kit device.

4.4 Preliminary investigation into matrix interference effects.

Laboratory methods of analysis for chloroanisoles and chlorophenols normally involve complex sample concentration and purification procedures together with the use of GC or HPLC methods. At –line test methods are normally in kit form with all of the required sample preparation and measurement equipment/ devices contained within a portable case. At-line devices for various phenols have been available in the water industry for many years and involve colorimetric measurement following a chemical reaction (e.g. the formation of an azo dye). However these methods are limited to measuring only mg/l concentrations in pure water samples compared to a requirement for µg/l concentrations in wine taint analysis. One ELISA type method from the company SDI Europe has recently become available for chlorophenol analysis with a detection limit of 0.1 ng/ml.

Preliminary experiments were conducted using this test kit to investigate the requirement for additional purification procedures and to estimate the extent of non-specific interferences on the measurement of pentachlorophenol. The SDI kit uses a simple competitive assay for PCP using a horseradish peroxidase-PCP enzyme conjugate and a colorimetric final measurement.

4.4.1 Effect of ethanol content of wine on assay calibration.

A set of six PCP standards (range 0.1 – 10 ng/ml) were prepared and each standard was spiked with an equal amount of the following samples:

1. Control (HPLC grade water)
2. 6 % v/v ethanol in water
3. 12 % v/v ethanol in water

Using the standard SDI procedure PCP concentrations were measured in each sample and results assessed according to the deviation of the standard graph using pure water (Figure 4.5)

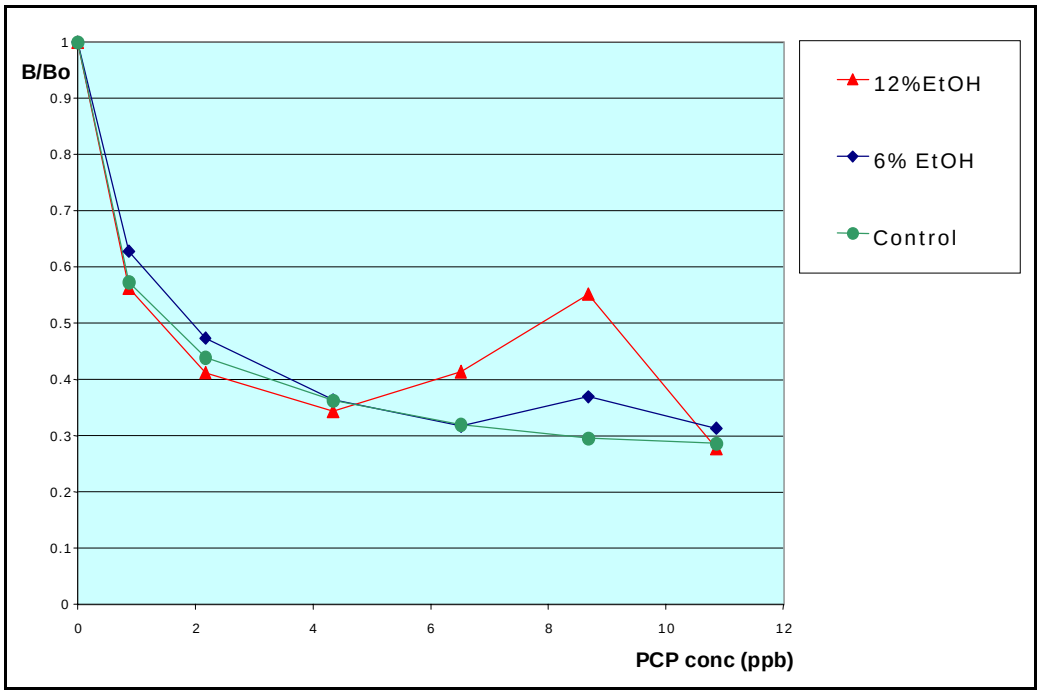


Figure 4.5 – Effect of Ethanol on PCP response using the SDI test kit.

Results indicated that ethanol content has a significant impact on the calibration graph for PCP causing results to be higher than the control sample particularly at higher concentrations (6 –10 ppb). This was considered to be due to disruption of the antibody – antigen binding interaction by conformational changes or effects on the PCP-HRP vs PCP competitive equilibrium (Setford, 2000). In this assay procedure the presence of ethanol in the sample matrix is likely to produce false positive results.

4.4.2 Effect of wine matrix

The following selection of wines, purchased in the UK from established supermarkets were also tested using the SDI kit:

1. Full bodied red wine from the Rioja region (northern Spain).
2. Full bodied red wine from the Valdepenas region (southern Spain).
3. Sweet white wine from Valencia (eastern Spain).
4. Dry white wine from Valencia (eastern Spain).
5. Sweet sherry from Jerez.
6. Sparkling Cava wine.

Table 4.2 – Results of SDI test kit assay on selected Spanish wines

Rioja red	Valdepenas red	Valencia sweet white	Valencia dry white	Jarez sherry	Cava sparking wine
3.1ng/ml	4.0ng/ml	<0.1ng/ml	0.5ng/ml	2.5ng/ml	<0.1ng/ml

Significant concentrations of PCP were measured in four of the selected wines (Table 4.2).

These results obtained were considered to be much higher than typical results. In view of this, these data are considered to significantly higher than the true values and hence represent false positive values. This may be due to non-specific antibody binding or irregular enzymic reactions. Unfortunately,

when these experiments were conducted the reference method was not available to measure the true concentrations of PCP.

Further work may be required to determine the effect of the wine sample matrix. However this preliminary investigation indicates that it is highly likely that samples will require preliminary purification to minimise the risk of false positive results.

4.5 Alternative purification procedures

Several alternative preliminary purification procedures are available to separate the target analytes them from other matrix components. Based on the need to establish a simple at-line procedure using very limited laboratory facilities The following are considered to offer good potential as the preliminary stage of the procedure:

4.5.1 Steam distillation (Azeotropic co-distillation)

Steam distillation is a classical separation method used for the analysis of phenols in many different sample matrices (US, EPA method 9065). The apparatus required is shown diagrammatically in Figure 4.6, in practice Quickfit™ glassware was used to construct the steam distillation unit.

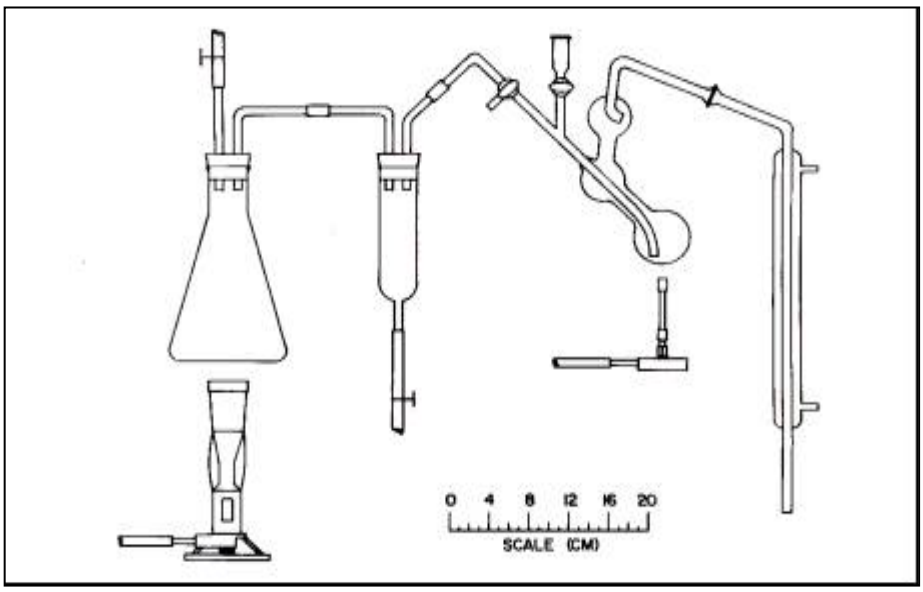


Figure 4.6 – Diagramatic representation of steam distillation apparatus.

Preliminary experiments indicate the procedure is a very effective cleanup for wine samples. A recovery rate of greater than 80% PCP at the 1ppm level was obtained.

4.5.2 Solid Phase Extraction (SPE)

SPE involves the separation of contaminants according to their binding characteristics. This is dependent on the surface chemistry of the adsorbent and molecular features of the target compound. The use of adsorbents that are surface coated with C18 organosilanes or anion exchange resins are frequently employed procedures for the concentration and purification of water samples. The separation of the phenolic group of compounds may be improved by using the specific adsorption properties of the common -OH or -

OCH_3 group on the primary carbon atom of CP and CA. The oxygen moiety in phenols is normally acidic and this feature is further enhanced by the ring chlorine atoms (p*K*a PCP = 4.9). This imparts a high dipole moment to the molecule that enhances the adsorption characteristics on polar surfaces. New commercial polyamide resins have recently become available that appear to exhibit greater selectivity towards CP and CA. Preliminary experiments with the Oasis^{TM} SPE cartridges (Waters Corp, Milford, USA) indicate recoveries better than 90% for PCP at the 1ppm level.

4.5.3 Liquid/Liquid extraction

This procedure involves the separation of the target analyte between two immiscible solvents Solvent partitioning of CP and CA between the largely aqueous sample matrix and an organic solvent such as hexane or hexane:ether mixtures may also be enhanced by pH control. It has been incorporated into standard methods for analysis of wine and other alcoholic beverages for many years. Excellent recoveries are obtained at ppt levels; however the use of this procedure may be difficult to incorporate into a portable unit.

4.5.4 Membrane separation

Dialysis membranes are able to provide a good separation of small and large molecules by molecular filtration. However, the procedure is diffusion

controlled and therefore may be too slow for a portable device unless the potential gradient and enhance the kinetics of the system can be improved.

4.6 Preliminary investigation of matrix effects on HRP enzyme conjugate reaction.

Antibody technology provides a range of biochemical procedures that can be employed to selectively trap analytes on immobilised antibody binding sites. Residual non-bound analyte and sample matrix can then be removed using washing procedures.

The principle disadvantage of antibody/enzymatic detection procedures is that the accuracy is highly dependent on the influence of other compounds in the sample matrix and their ability to occupy the analyte binding site (cross reactivity) or participate in non specific binding effects e.g. binding to active surface materials . This feature may produce a significant number of false positive results.

Selected red and white wine samples were purified using steam distillation, SPE and liquid liquid partition. The interference of the extract solutions and ethanol on the HRP catalysed reaction was investigated.

Two samples of wine Campo Viejo Rioja (red) and Chablis (white) were purified and concentrated according to the procedures set out in Chapter 7. The extracts were applied to a solution of the standard HRP-PCP conjugate

and the HRP activity was developed by adding TMB/peroxide substrate. After incubation for 30minutes the reaction was stopped using 2M sulphuric acid and the intensity of the yellow colour produced was measured using a 2mm cell at 450nm.

Results shown in Table 4.3 indicate the presence of a significant matrix effect on the reporter enzyme HRP confirming that an essential component of the procedure for wine samples must include a rigorous sample clean-up procedure.

Table 4.3- Results of matrix interference experiment using PCP-HRP conjugate

Sample description	Mean Relative Response (%) @450nm (n=3)
PCP (100 μg/ml)	98
White wine + 100 μg/ml PCP	54
White wine + 100 μg/ml PCP purified by LL partition	50
White wine + 100 μg/ml PCP purified by SPE + LL partition	80
White wine + 100 μg/ml PCP purified by steam distillation + LL partition	94
Red wine+ 100 μg/ml PCP	37
Red wine + 100 μg/ml PCP purified by LL partition	54
Red wine + 100 μg/ml PCP purified by SPE + LL partition	81
Red wine + 100 μg/ml PCP purified by steam distillation + LL partition	93
12% Ethanol + 100 μg/ml PCP	96

These data indicate significant interference of the wine matrix components on the activity on the HRP reporter enzyme. Steam distillation combined with liquid- liquid partition appears to produce an extract that interferres least with the HRP activity, confirming the need for a rigorous sample purification procedure. For additional work on sample purification procedures please refer to Chapter 7. Interestingly ethanol at a 12%(v/v) does not appear to have a significant adverse effect on the HRP activity.

To enable the use of a simple SPE sample purification procedure as part of the at-line test method further work is required to develop a detection method in which the ELISA reporter enzyme is not in direct contact with the wine sample matrix. This may be achieved by using an ELISA conjugate in which the hapten is linked to a secondary antibody or binding protein e.g. streptavidin. In the latter case the initial competitive PCP ELISA incubation takes place between the antibodies PCP and sample matrix and a PCP-streptavidin conjugate. The bound material is rigorously washed removing any of the original wine sample matrix. A biotin linked reporter enzyme is employed to form a quantitative link to the streptavidin moieties and may be detected using a suitable enzyme substrate. For further work on this assay format please refer to Chapter 5.

5.0 DEVELOPMENT OF ELISA TEST PROCEDURE

Enzyme-Linked Immunosorbent Assays (ELISA) are the most widely used solid-phase immunoassays with unparalleled applicability. In this chapter the development of ELISA assays for chlorophenols and chloroanisoles is reported together with the synthesis of the assay components and optimisation of the procedures.

5.1 Assay components

Competitive or reagent limited ELISA methods are very effective for small molecules with a single binding site. To develop appropriate methodology three principle reagent components are required.

1) Monoclonal antibodies against PCP and PCA – These were supplied by the French consortium partner, Diaclone.
2) Antigen – Pentachlorophenol (PCP) or pentachloroanisole (PCA) were selected as the reference standard materials.
3) Antigen-conjugate – These were prepared by linking the antigen to the enzyme horseradish peroxidase as a reporter moiety.

5.1.1 Monoclonal antibodies

Animals normally make many thousands of different antibodies as a defence mechanism against viruses, bacteria or other toxins and for each toxin there is normally a different antibody binding site. The molecular size of toxins and their corresponding antibody binding sites varies widely but in general they are produced for relatively large molecules such as proteins, viruses and bacteria with a molecular size of several kilo Daltons as depicted in Figure 5.1.

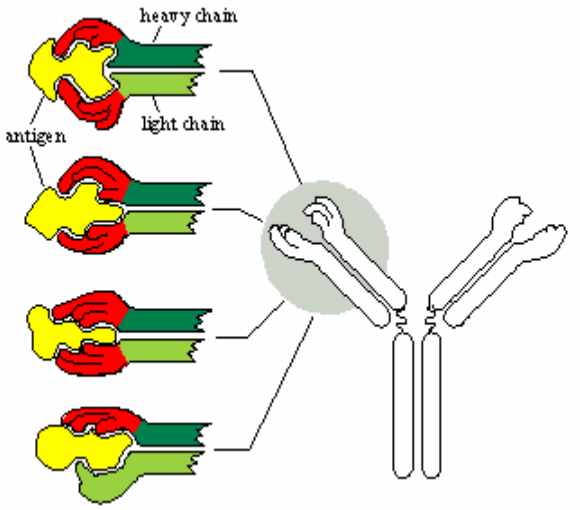


Figure 5.1 – Antibody binding sites indicating large molecule specificity.

For small molecules such as PCP and PCA difficulties may arise with the formation of suitable receptor sites. In preliminary experiments conducted at Diaclone to produce PCP and PCA antibodies with no chemical modification,

no antigen selectivity was observed. To generate antibodies with high binding characteristics it was necessary to link PCP and PCA to a suitable carrier protein. Unfortunately, the use of a large carrier protein molecule stimulates the production of antibodies against antigen plus the protein carrier which may reduce the specificity of the antibodies produced.

5.1.2 Preparation of protein carrier conjugate (Diaclone)

The carrier proteins Keyhole Limpet Hematocyanin (KLH) and Bovine Serum Albumin (BSA) were linked to PCP and PCA respectively using carbodiimide chemistry and fractionated by cholumn chromatography using a PD-10 column (PharmaciaBiotech., Uppsala, Sweden).

5.1.3 Immunisation of mice (Diaclone)

Mice were immunised by intraperitoneal injection with 100 µl of the immunogen PCA-BSA or PCP-KLH (100 µg/ml) with an equal volume of complete Freund's adjuvant for the first injection. At least, two additional injections were performed every two weeks with 100 µl of the immunogen (100 µg/ml) in an equal volume of adjuvant. Four days prior the spleen

collection, the mice were boosted with 100 µl of the immunogen preparation (100 µg/ml) in the tail vein.

5.1.4 Separation of monoclonal antibodies (Diaclone)

Splenocytes from the immunised mice were mixed with murine myeloma cells (X63-AG8) at a ratio of 5:1 and fused by the procedure described by Köehler and Milstein (1975) with modifications. The fused cells were cultured in hypoxanthine aminopterin thymidine medium. Following the immunogen used, the culture medium of each hybridoma was screened for anti-PCP or PCA antibody by ELISA. Hybridoma cell populations secreting anti PCP or PCA antibody were cloned at least three times by the limiting dilution method. Isotyping of antibody was performed with a Mouse mAb Isotyping Kit (Diaclone, France). The cloned hybridoma cell line was injected into Freund-primed mice. The anti PCP or PCA mAbs were purified from the ascite supernatants by affinity chromatography using a protein A-agarose gel column (Pharmacia Biotech., Uppsala, Sweden).

5.2 Development of high sensitivity ELISA test procedure for chlorophenols and chloroanisoles.

To enable the analysis of extracts from wine samples and other materials such as corks and packaging materials to be tested, a high sensitivity method with an operational range of 0.1 - 100ng/ml was considered to be required.

This was developed by optimising and improving the performance characteristics of the micro-plate method and developing alternative reporter conjugate and detection procedures.

High sensitivity pesticide residue ELISA test procedures are normally configured in the competitive assay format. In this approach competitive binding takes place between the antigen, antigen-conjugate and the antibody with either the antibodies or antigen conjugate bound to the surface (see Chapter 2, Section 2.6.1 for further information). Many different assay formats have been developed for specific applications and the final choice of assay format is dependent on the availability and size of binding sites. The principle of the competitive assay relies on the dynamic competition between an antigen-conjugate and the antigen for a restricted number of antibody binding sites The antigen-conjugate has an attached reporter label that enables a high sensitivity detection method to be used. In this assay format an inverse sigmiodal concentration dependent response is observed i.e. high signal at low concentrations as shown in Figure 5.2.

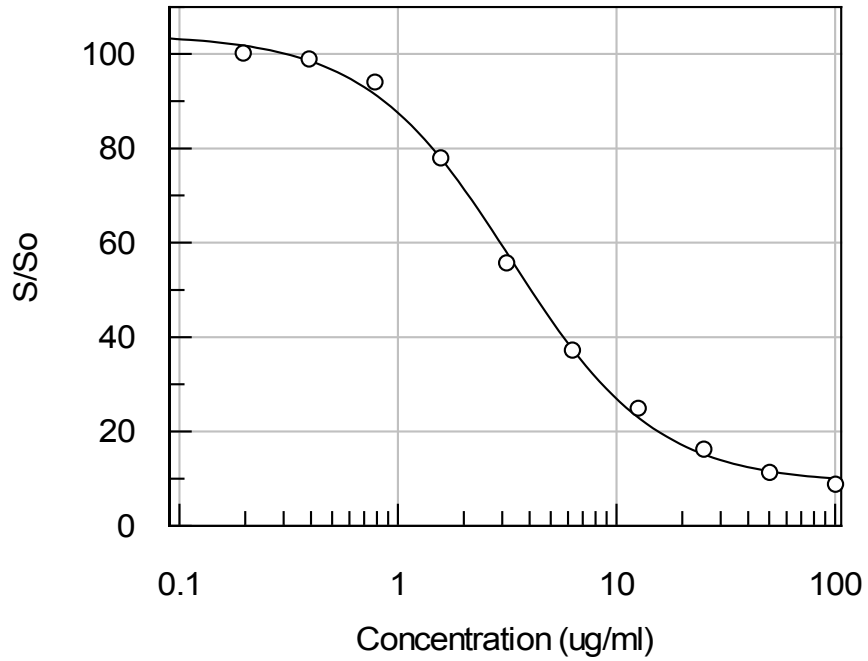
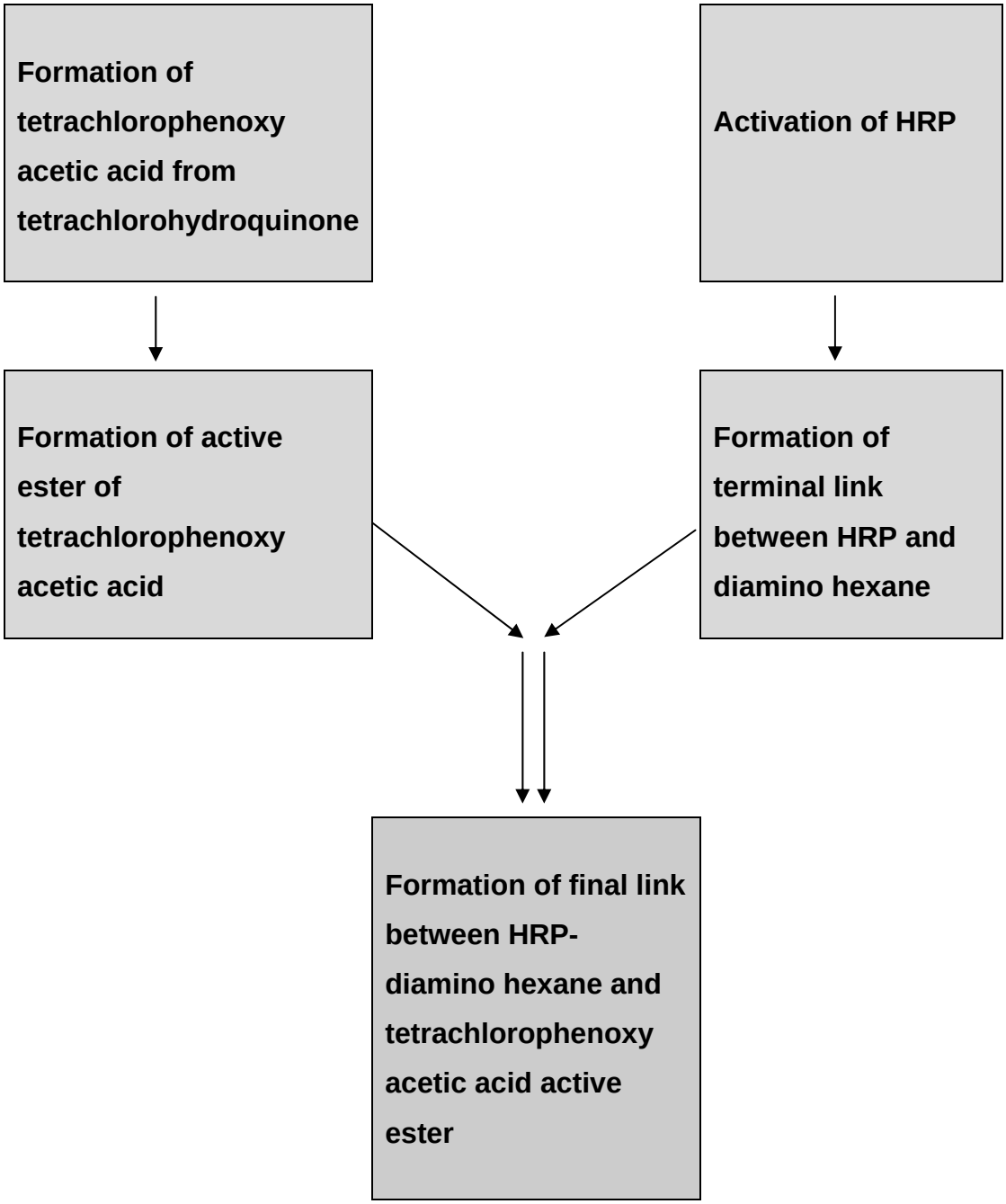


Figure 5.2 – Typical calibration graph for high level ELISA analysis S/So are the sample and background signals expressed on a percentage basis.

5.1.2 Synthesis of reporter conjugates

Reporter conjugates for both the PCP and PCA based ELISA assay were synthesised based on horseradish peroxidase conjugated to a 2,3,5,6 tetrchloro-4-hydroxyphenoxy acetic acid moiety. The performance characteristics of the PCP conjugate were demonstrated to be similar to those used in the commercial SDI PCP test kit.

The antigen conjugate was prepared using a five-stage synthesis from 2,3,5,6-tetrachlorohydroquinone using the following reaction sequence:



5.1.3 Preparation of antigen-conjugate

The preparation of the intermediate 2,3,4,5,tetrachloro-4-hydroxyphenoxy acetic acid was performed according to the reaction shown in Figure 5.3 (Li *et al.,* 2001) 2,3,5,6-Tetrachlorohydroquinone (4.96g) 20.0mmol) was dissolved in 30ml of a 35%w/v aqueous sodium hydroxide solution, followed by the addition of 15ml water in a standard 250ml three necked Quickfit flask. The resulting solution was heated and stirred for 20 minutes in an oil bath at 80^{o}C under a low stream of nitrogen.

A solution of 24.1g bromoacetic acid (173.4mmol) in 20 ml water was added drop wise over a period of approximately 1hr. Heating was then continued for a further four hours.

After cooling the buff /brown solid was separated by filtration under vacuum using a Whatmann GFC paper and recrystallized using approximately 100ml 95%v/v ethanol/water. Yield of recrystallized buff powder 4.3g (70.3%)

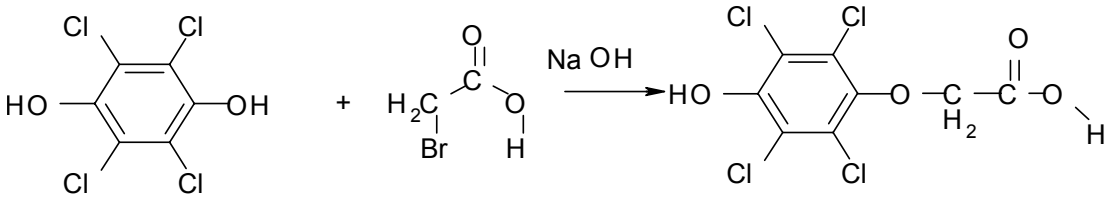


Figure 5.3 – Synthesis of 2,3,4,5,tetrachloro-4-hydroxyphenoxy acetic acid

Elemental and mass spectral analysis of the recrystallized material ,conducted externally, indicates good agreement with theoretical values. NMR indicates three proton environments on the molecule but these data are unclear due to poor solubility, some ethanol contamination was also suspected (a summary of the data is shown in Table 5.1).

Table 5.1 - Analysis of 2,3,5,6 Tetrachloro-4-hydroxy phenoxyacetic acid product

Elemental analysis	Theory %	Found %
C	31.3	30.2, 30.0
H	1.3	2.29, 2.23
N	0	0.01, 0.00
Cl	46	45.9, 45.5
MS	Assignment	
304,306,308, 310	306 – 4Cl isomer pattern	
245,246,247,248*, 249,250,251,252	Corresponds to cleavage of phenoxy acid group.	
NMR	Integ. ratio	
Triplet 1.0ppm		EtOH
Quadruplet 3.45ppm		EtOH
Doublet 3.8ppm	2	-CH2-
Singlet 4.2ppm	1	Bz-OH
Singlet 4.3ppm	1	-COOH

HPLC analysis using a 15 cm Eclipse XDB-C18 3.0x 15mm column (Zorbax, Varian)) and a solvent mixture containing 50%v/v methanol:water and 1%v/v phosphoric acid and UV detection at 254nm indicated one major peak (85.3 peak area%) and three impurity peaks at 1.2, 8.2 and 3.5 %. The retention time of the largest impurity peak (8.2%) corresponded to the retention time of the starting material tetrachlorohydroquinone. Additional purification of this material was not undertaken at this stage as the principle impurity did not participate in subsequent reactions and was removed during the purification of the final HRP conjugate material.

5.1.4 Activation of horseradish peroxidase with Sodium Periodate

The enzyme horseradish peroxidase was activated by oxidising pendant glucose moieties to produce active aldehyde groups using periodate. This enables the formation of a covalent bond with one of the diamino hexane amine groups according to the procedure of Dzantiév *et al. (*1996)

A sample of horseradish peroxidase 0.20g (Sigma P8375, 250-300 units/mg) was dissolved in 10ml water in a foil-covered vial. A solution of 0,188g sodium periodate in 10ml water was prepared and stored in a foil covered vial to exclude light. 1ml of the periodate solution was added to the horseradish peroxidase (HRP) solution and the mixture was stirred using a magnetic stirrer for 20 minutes in the dark. A colour change from amber/ brown to khaki/green was observed. Four 10cm × 1cm dia. columns of Sepadex G-25 were prepared using a column buffer containing 0.01M sodium dihydrogen orthophosphate and 0.15M sodium chloride to pH 7.2 and

2ml of the oxidized HRP was applied to each column at a drip rate of approximately one drip/second. The dark green band was eluted directly from the column and 2ml of dark green solution was collected from each column. Identical extract fractions from four columns were combined, mixed and re-aliquoted into 0.5ml fractions for freezer storage.

Samples of the stored material were freeze-dried to confirm the HRP concentration using a freeze drier. Approximately 20mg of material was recovered from four vials.

5.1.5 Addition of C6 spacer to HRP using 1,6-Diaminohexane

To avoid potential problems that may influence hapten access to binding sites, a C6 spacer molecule (diamino hexane) was employed to separate the hapten from the HRP protein groups (Hermanson *et al.,* 1992) .

The previously prepared frozen oxidized HRP extract 1.5ml (approx. 15mg) was mixed with 3mg 1,6-diaminohexane (Sigma H2381) in a foil-covered vial with mixing using a small magnetic stirrer. Unreacted residual oxidized HRP moieties were then reduced by adding 200μl of a solution of 4mg/ml sodium borohydride, added in 20μl aliquots. Gas bubbles were observed during the borohydride addition. The mixture was stirred for 20minutes at room temperature.

Purification of the derivatized HRP was achieved using a 10cm Sephadex G-25 column eluted with 0.01M 2-[N-Morpholino]ethanesuphonic acid (MES)

buffer at pH 6.1. The initial 5ml of eluent was discarded and ten 2ml fractions were collected.

Each fraction was tested for HRP activity by incubating with 1ml 3,3',5,5'tetramethylbenzidine/peroxide solution (Sigma T8665) for 10minutes and stopping the reaction with 1ml 2Msulphuric acid. Solutions were read using a uv/vis bench spectrometer at 450nm

Fractions two and three were identified as containing the active HRP as shown in Figure 5.4.

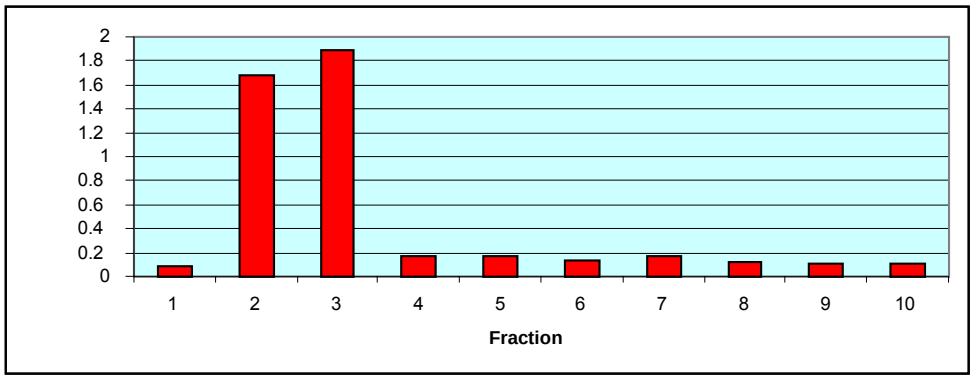


Figure 5.4 – Optical density of HRP-conjugate fractions

5.1.6 Formation of active ester of 2,3,5,6 tetrachloro-4-hydroxyphenoxy acetic acid (TeClHPAA)

The active ester method of conjugating hapten to an enzyme was employed to facilitate the efficient linking of TeClHPAA to the modified HRP because it is considered to produce fewer co-products than traditional direct carbodiimide type procedures (Herrmanson *et al.,* 1992). In this method the free carboxylic acid group was esterified to form a reactive ester using 1.7mg N-hydroxysuccinimide, 2.8mg N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide (EDAC) hydrochloride with 3.1mg TeClHPAA in dimethylformamide as depicted in Figure 5.5.

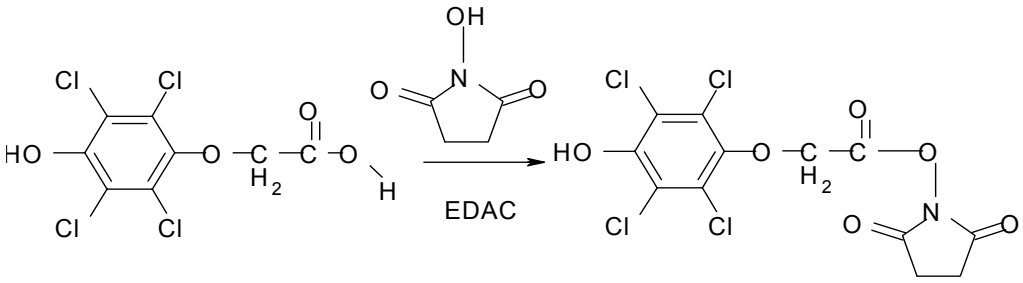


Figure 5.5 – Formation of TeClHPAA active ester

The procedure was performed in a close foil covered vial with mixing for 2hrs using a small magnetic stirrer. The mixture was initially observed to be cloudy but becomes clear and turns purple within 10 minutes.

The active ester was purified by two liquid/liquid extractions using 1ml ethyl acetate, the purple component was not retained.

5.1.7 Methylation of active ester for the PCA assay.

The preparation of the HRP conjugate employed in the PCA assay required the methylation of the phenolic –OH group. This was achieved using diazomethane synthesized using the recommended Aldrich procedure and apparatus (Aldrich Z419761).

The hydroxysuccinimide ester of 2,3,5,6 tetrachloro-4-hydroxyphenoxy acetic acid from stage 5.1.4 was methylated using a solution of diazomethane in diethyl ether.

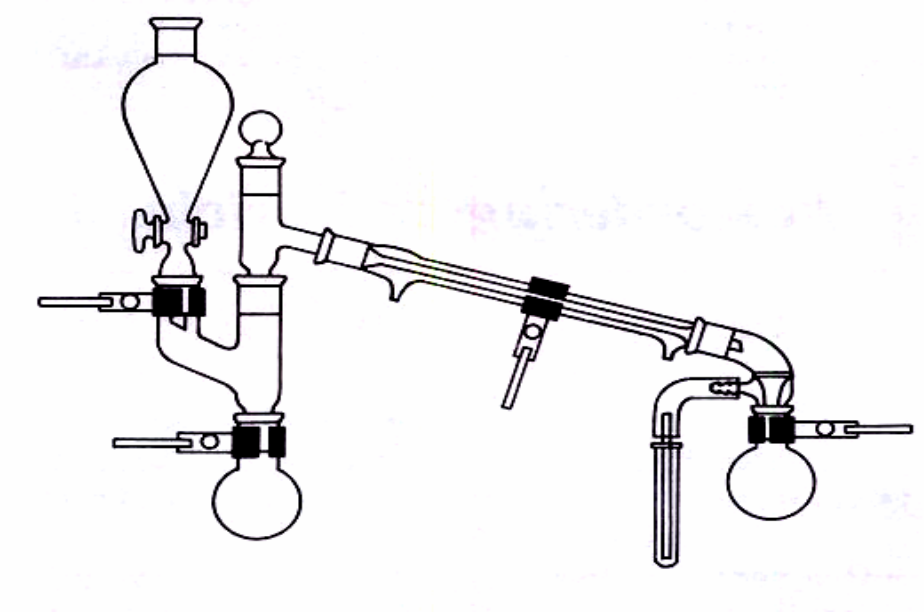


Figure 5.6. – Apparatus used for diazomethane production.

In view of safety considerations, diazomethane gas was produced in ethereal solution using a special apparatus (Aldrich Diazald kit) as shown in Figure 5.6.

The apparatus was assembled as shown in Figure 5.6 and 10ml ethanol was added to the reaction flask together with a solution of 5g potassium hydroxide in 8ml water. The receiver vessel was cooled in a sodium chloride ice bath. The separating funnel was charged with a solution of 3.4g 1-methyl-3-nitro-1-nitrosoguaanidine (MNNG) in dry ether. The reaction vessel was warmed to 65^{o}c using an oil bath and the MNNG was added drop-wise over a period of 20 minutes. Ether, 10ml was then added and the distillation continued until the distillate was colourless.

The diazomethane solution (1ml) was added to the active ester solution in ethyl acetate from stage 5 with mixing for 30 minutes and stored overnight at.4^{o}C.

The methylated mixture was evaporated almost to dryness using a stream of nitrogen.

5.1.8 Formation of HRP-conjugate by linking the TeClHPAA active ester to HRP with hexamino- spacer.

The concentrated mixture from stage 5.1.4 or 5.1.5 was reconstituted using 1ml dimethylformamide and added to the combined fractions 2 and 3 from stage 5.1.3 containing HRP with a six carbon linking amide as shown in Figure 5.7. The mixture was stirred at room temperature for 2hrs and purified by dialysis using a 10,000 MWCO Slide-A-Lyser™ (Pierce) dialysis cassette overnight with 0.01M MES buffer pH 6.1.

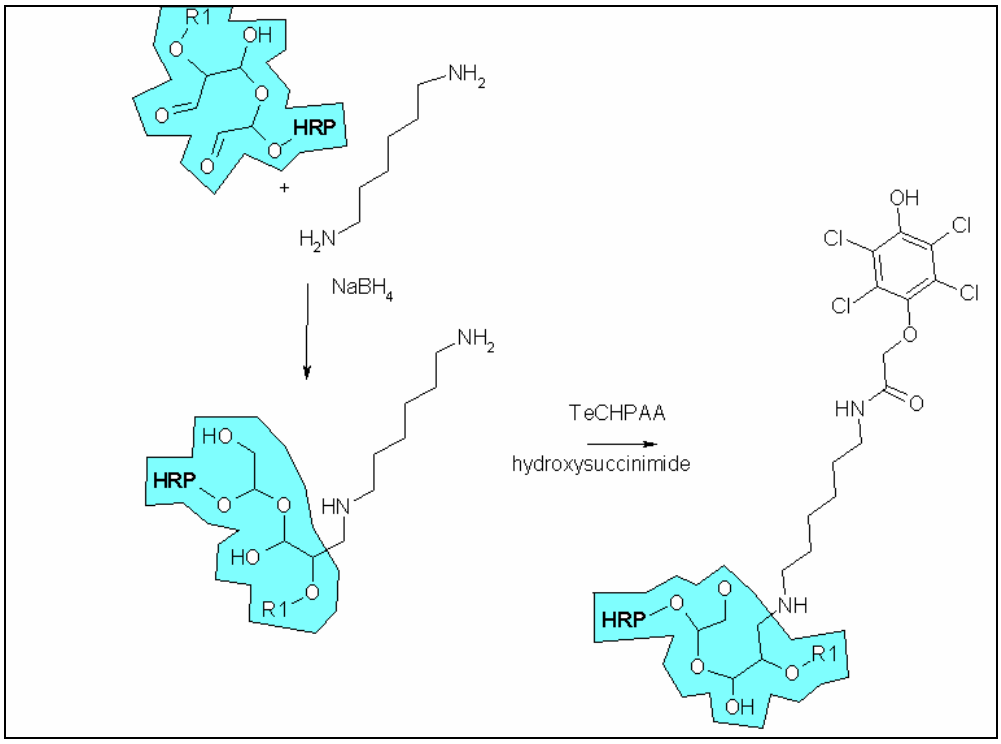


Figure 5.7 – TeClHPAA coupling to HRP

5.1.9 Storage of antigen-conjugate

The dialysed fraction (approximately 3.5 ml) was mixed with 1mg/ml BSA, 0.5 mg/ml horse heart cytochrome C and 3ml glycerol approximately to enhance the storage stability of the conjugate.

The conjugate was aliquoted (500μl) into small polypropylene vials and stored at -20^{o}C.

5.2 Preliminary experiments base on the PCP assay to optimise micro-plate performance

The following experiments were conducted to investigate and optimise the assay performance

5.2.1 Alternative coated microplates,

Three alternative coated microplates were investigated, MaxiSorp™, PolySorp™ (Nunc) and Costar™ high binding microplate (Corning). The Nunc Maxisorp™ microplate produced the highest response at the 1-100μg/ml level as shown in Figure 5.8.

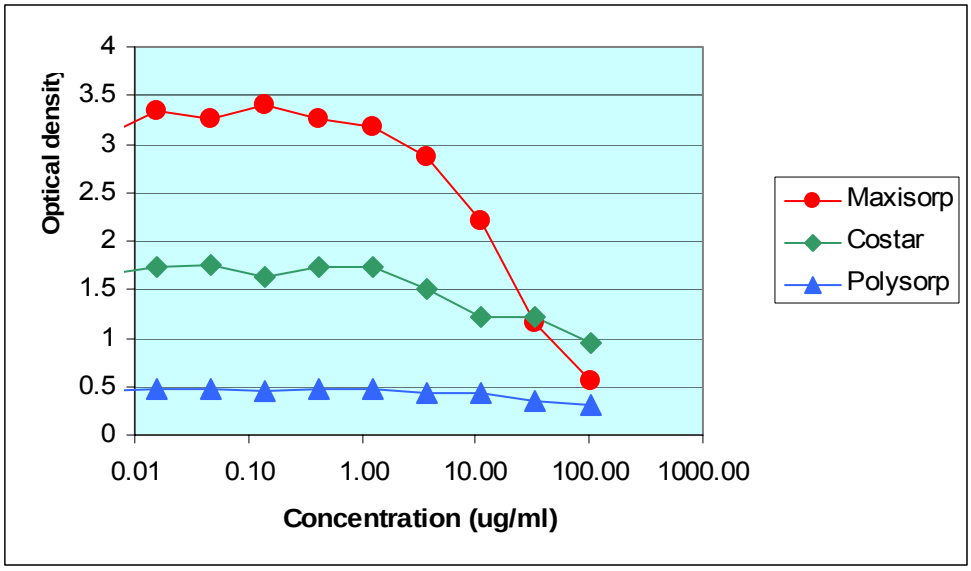


Figure 5.8 – Performance of three alternative microplates showing increased level of antibody binding using Maxisorp plates.

5.2.1 Alternative coating buffers

Alternative antibody coating buffers were evaluated. Antibody coating buffers containing carbonate/bicarbonate at pH9 approximately, showed improved performance when applied to the standard Nunc MaxiSorp microplate.

5.2.2 Working range

To enable the analysis of wine extracts to be attempted at 1-100ng/ml opportunities to reduce the working range of the assay by limiting the level of bound antibodies were investigated. Preliminary experiments at low antibody levels indicated that absorbance readings obtained using TMB as the HRP substrate were very low (<0.1) and very variable. Two alternative fluorescent HRP substrates, QuantaBlu™ (Pierce) and Amplex Red ™ (Molecular Probes) were investigated. Both gave good quantitative results at 1-100ng/ml. QuantaBlu™ was selected as the preferred substrate because it showed slightly higher sensitivity and better storage stability. The performance characteristics of QuantaBlu were evaluated using the Nunc Maxisorp plate at the low range (1-50 μg/ml) with a range of antibody concentrations (0.1-3.2 μg/ml). Calibration graphs indicated a variable response as indicated in Figure 5.9. The best response was obtained at an antibody coating level of 0.8 – 3.2 μg/ml.

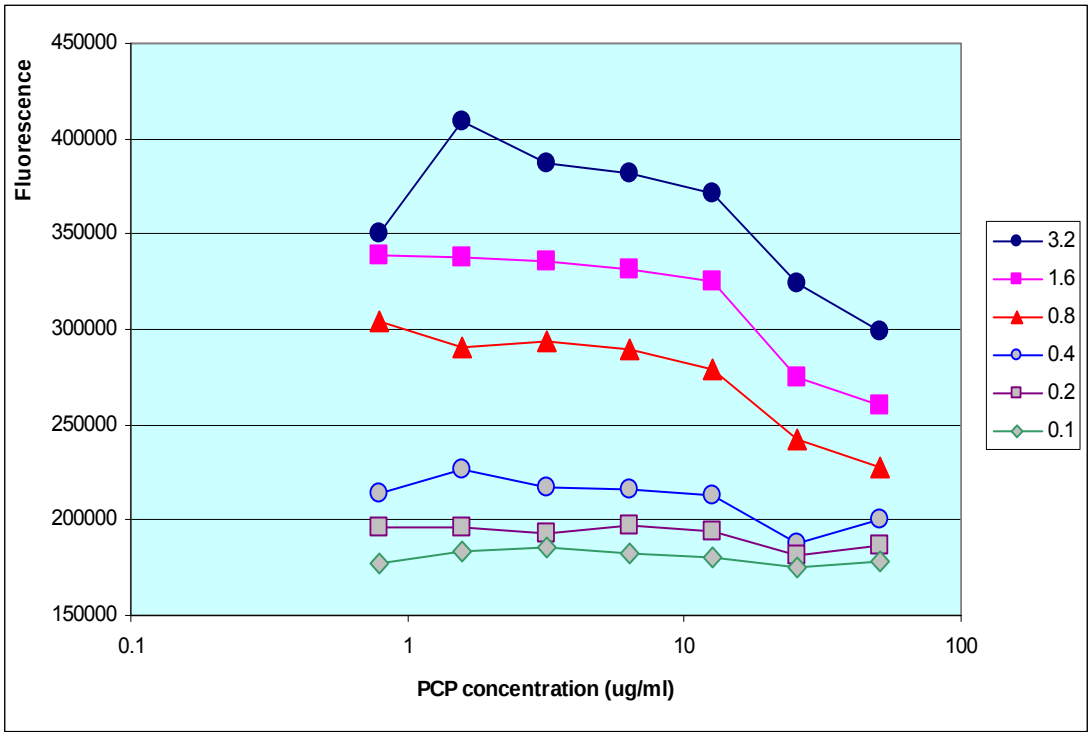


Figure 5.9 – Response profile of Maxisorp™ plates coated with PCP antibodies at 0.1 – 3.2 ug/ml and fluorescence detection.

5.2.3 Experiments to improve the assay reproducibility

Improvements to the overall reproducibility were based on observations that controlling the orientation of the antibody ligand had a significant impact on plate performance (Subramanian and Verlander, 1996; Gosling, 2000; Brogan *et al.,* 2001). Poorly controlled binding by simple physical adsoption was considered to result in inefficient plate coating in which only a small proportion of antibodies applied were being oriented in a desirable manner as depicted in Figure 5.10. Uncontrolled binding was considered to result in higher levels of disordered antibodies and a very poor concentration

dependent relationship. Attachment of the antibodies using covalent or non-covalent immobilization methods was investigated to orient the antibodies more favourably.

Common methods used to achieve controlled binding are shown in Table 5.2. In each case, the aim is to sequester the ligand in a manner that preserves the full binding activity. The selection of the most appropriate method will depend on the skills of the analyst but in general, the use of non-covalent attachment is considered least likely to affect the three-dimensional structure of the antibody and hence have minimal impact on the active binding sites.

In view of the need to exercise control of attachment at very low concentrations, methods 8 and 9 of table 13 were considered the most suitable for evaluation, as they are likely to impose least conformational changes on the antibody structure.

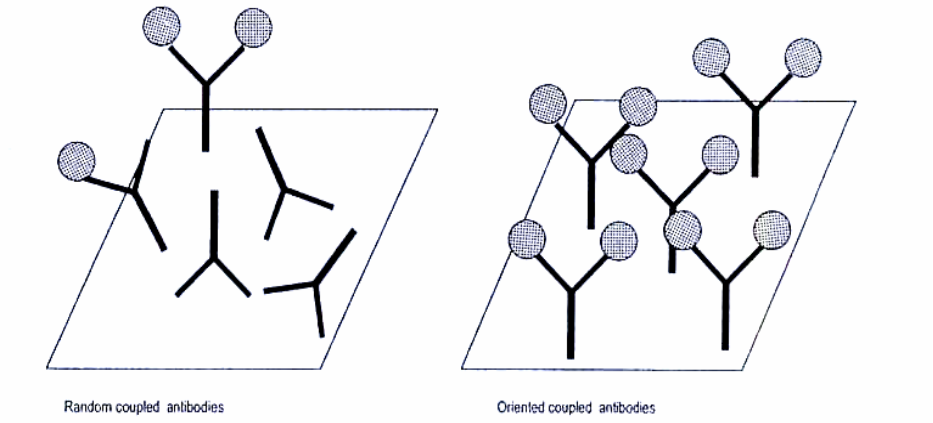


Figure 5.10 – Diagrammatic representation of random and oriented antibody binding.

Table 5.2 - Common methods used to attach antibodies to surfaces (Gosling, 2000).

	Ligand linking group	Solid-phase reactive group
1	Amino	Aldehydes, acyl azides, anhydrides, aryl halides,carbodiimides, imidoesters, isothiocyanates
2	Thiol	Alkyl halides, haloacetyls, maleimides
3	Carboxyl	Carbodiimides, diazoalkanes, hydrazides
4	Hydroxyl	Alkyl halogens, chloroformate, epoxides isocyanates.
5	Aldehyde	Hydrazide, amino(Schiff base), Mannich reaction
6	Oxidisable carbohydrate	Hydrazide, Schiff's base.
7	Free sulphydryl	Maleimide, alkyl halide, haloacetals
8	Fc-region	Antibody agains FC, Protein A, Protein G
9	attached Biotin	Streptavidin

5.2.3 Optimization of anti-mouse microplate

An alternative microplate, pre-coated with anti-mouse antibodies, (Pierce, Rockford, USA) was evaluated using QuantaBlu™ as the HRP substrate. This procedure produced consistently better reproducibility and an improved concentration dependent relationship.

Alternative concentrations of antibody coating of the anti-mouse microplate was investigated at various HRP-reporter conjugate levels. The binding characteristics of this microplate were found to be independent of antibody concentration provided that a minimum concentration of approximately 1μg/ml of antibody was present in the coating solution. This suggested that at concentrations above 1μg/ml of antibody the anti-mouse coating controlled the binding capacity.

The performance characteristics of the optimised procedure gave a very good, concentration dependent relationship, over the measurement range of 1- 100ng/ml PCP and a standard deviation of less than 5% at all levels (n=6) as shown in Figure 5.11.

Results were fitted to the four-parameter logistic model, (Nix and Wild, 2000) using the GraFit™ computer program (Leatherbarrow, 1998). This fits data to the following equation (Eq. 1) based on IC_{50} enzyme inhibition data:

$$y = \frac{\text{Range}}{1+\{x/IC_{50}\}^{s}} + \text{Background} \qquad \text{(Eq 1)}$$

In this equation, Range is the fitted uninhibited value minus the Background, and s is a slope factor (The equation assumes that y decreases with increasing x. as shown in Figure 18. In this usage the term IC_{50} refers to the position of inflection between the conjugate excess plateau at low concentrations and conjugate limiting region at high concentrations. A lower IC_{50} indicates that less analyte is required for a change to be detected, indicating a higher sensitivity procedure. Therefore IC_{50} values can be used to assess the relative sensitivities of the procedures (Noguera *et al.*, 2002; Galve *et al.,* 2002).

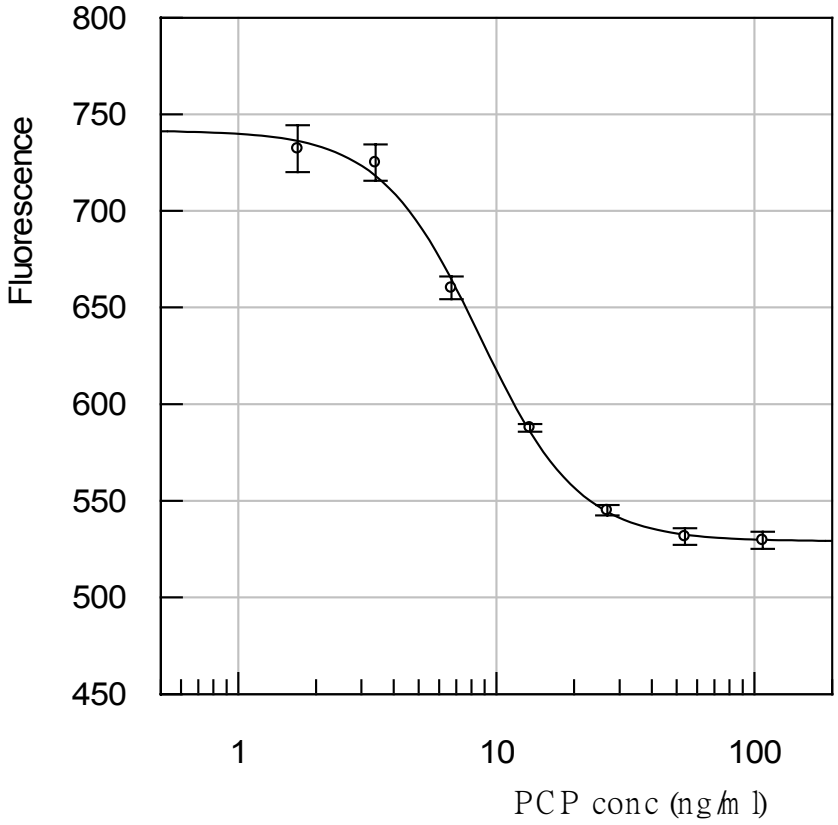


Figure 5.11 – Standard calibration graph for PCP procedure (n=5)

5.3 PCP immunoassay procedure using PCP-HRP conjugate.

The following standard operating procedure was adopted for subsequent ELISA assays.

5.3.1 Materials

5.3.1.1 Reagents

Water (distilled or deionized), solvents and chemicals must be free of chlorophenol/anisole contaminants, therefore pre-tested or 'pesticide grade' chemicals must be used throughout. It is essential that at least three sample blanks are included in each batch to confirm that chemicals are free of contaminants.

Phosphate buffered saline solution (PBS) - Dissolve one tablet PBS (Sigma P-4417) in 200ml of water to obtain 0.01M phosphate buffer pH7 in saline solution.

Monoclonal antibodies anti-PCP (Diaclone BL36 -1μg/ml approximately, in storage buffer). Dilute 10μl of the supplied antibody solution (1mg/ml Diaclone BL36) with 10ml PBS, using aseptic techniques, mix.

PCP-HRP conjugate – Add 1ml stock solution (containing 0.01mg/ml approx. PCP-HRP conjugate in PBS) to 10ml PBS buffer using aseptic techniques, mix.

PCP stock standard solution (approximately 1280 μg/ml) - Weigh accurately approximately 128mg PCP solid and dilute in 100ml pesticide grade methanol.

PCP standard solution (approximately 12.8μg/ml) - Dilute 1ml 1000μg/ml PCP stock standard solution to 100ml in PBS.

PCP working standard solution (approximately 128ng/ml) - Dilute 1ml, 12.8μg/ml PCP standard solution to 100ml in PBS

PCP standard solutions - Prepare seven standard reference solutions in PBS by a serial dilution of the 128ng/ml working standard solution at the following approximate concentrations: 64, 32, 16, 8, 4, and 2 ng/ml. Prepare an identical blank solution vial containing PBS.

Wash buffer – 0.1%(w/v) Tween 20 in PBS buffer.

Blocking buffer – 1% (v/v) mouse serum (Dako - X0910) in PBS.

HRP substrate - QuantaBlu™ Fluorogenic Peroxidase substrate, stable peroxide and stop solution (Pierce - 15169)

5.3.2 Apparatus

ELISA micro-plates (Pierce Reacti-Bind™ goat anti-mouse coated 96 well plates).

Automatic plate washer (e.g. Denley Well-wash 4).

ELISA fluorescent plate reader (e.g. Perkin Elmer LS50B with plate reader accessory).

Small polypropylene test tubes, approximately 1ml volume.

Tube vortex mixer.

Micro-plate mixer (e.g. Flow Laboratories Titretek DSG).

5.3.3 Preparation of microtitre plates

Wash micro-plates three times using 300μl wash buffer.

Remove any residual buffer by inverting the plate and blotting against clean paper towelling.

Pipette 100μl of the diluted antibody preparation into each well and incubate overnight at 4^{o}C.

Wash micro-plate three times using 300 μl wash buffer, blot.

Block plate by adding 200μl mouse serum blocking buffer per well. Incubate for 2hrs at room temperature using the microplate mixer.

Wash micro-plate three times using 300 μl wash buffer, blot.

5.3.4 Assay procedure

Prepare standards and sample extracts for analysis and check pH. This should be approximately pH7, adjust pH if required using 0.1M NaH_2PO_4 or 0.1M Na_2CO_3.

Pipette 200μl of each standard and sample solution into a small tube.

Add 200μl diluted HRP-conjugate solution and mix using the vortex mixer.

Prepare a blank for each sample by adding 200μl PBS buffer and sample to a separate tube.

Pipette 100μl of each standard, blank or sample solutions into each well. All measurements must be performed in triplicate.

Cover with an adhesive strip or plastic cover and incubate for 1hr using the micro-plate mixer

Wash micro-plate three times using 300 μl wash buffer, blot.

Add 1ml of the QuantaBlu™ stable peroxide to the QuantaBlu™ substrate solution and mix using the vortex mixer.

Pipette 100μl of the mixture into each well, cover and incubate for 1hr using the micro-plate mixer.

Add 100μl QuantaBlu™ stop solution and mix for 1min using the micro-plate mixer.

Measure the emitted light from each well using the Perkin Elmer LS50B fluorescent plate reader or similar using instrumental conditions shown in Table 5.3.

Table 5.3 - Perkin Elmer LS50B instrument settings

Excitation wavelength	325nm
Excitation slit width	8.0
Emission wavelength	420nm
Emission slit width	8.0
Emission cut-off filter	390nm

5.3.5 Results

Results were inspected and triplicate data values averaged.

A calibration graph was plotted using semi-log graph paper or a suitable computer based data analysis program such as Grafit. The concentration of samples was determined from the graph allowing for any dilution factors etc.

Sample blanks were checked to identify any sample extracts showing natural fluorescence. Samples with positive blanks may contain residues of fluorescent contaminants such as polycyclic aromatic hydrocarbons.

5.4 – Development of Pentachloroanisole (PCA) assay procedure

The basis of this assay is very similar to the procedure employed in the high sensitivity fluorescence assay for pentachlorophenol (PCP) in sample extracts, reported in section 5.3 with PCA relevant components.

In this case the sample to be tested and the PCA-HRP conjugate are initially mixed and applied to the wells of a microtitre plate coated with anti-PCA antibodies. The sample PCA and PCA-HRP conjugate are incubated for 30 minutes and compete for the available antibody sites.

In this way, an inverse concentration dependent relationship was established in the range 0.1 to 100 ng/ml PCA as shown in Figure 5.12.

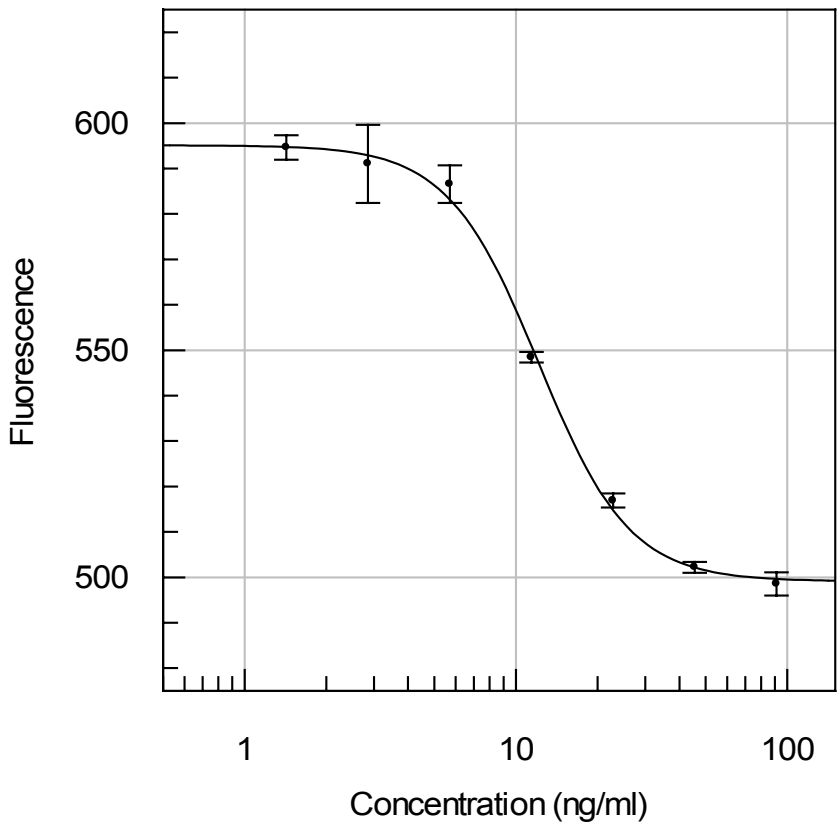


Figure 5.12 – Standard calibration graph for pentachloroanisole (n=5)

5.5 - CROSS REACTIVITY INVESTIGATIONS

The specificity of the immunoassay was evaluated for both the PCP and PCA assays by testing seven structurally related phenols over the concentrations ranges shown in Table 5.4.

Table 5.4 – Compounds tested and concentration ranges for crossreactivity studies

Compound	Range (ng/ml)
Pentachlorophenol	1.7 – 107.6
2,3,4,6-tetrachlorophenol	1.6 – 101.6
2,4,6,-trichlorophenol	1.6 – 101.1
24Dichlorophenol	1.5 – 98.6
Phenol	1.6 – 99.8
Pentachloroanisole	1.7 – 90.2
2,3,4,6-tetrachloroanisole	1.4 – 90.2
2,4,6,-trichloroanisole	1.6 – 105.0
24Dichloroanisole	1.8 – 115.8

Each plate was prepared using antibodies against PCP and PCA.

A serial 2X dilution of each standard was prepared and applied to the plate in the competitive assays format as described previously.

5.5.1 Results

Results are shown graphically in Figures 5.13 and 5.14. for a range of chlorophenols. Anti PCA results are shown in Figures 5.15 and 5.16. IC_{50} values and %cross-reactivity are listed in Table 5.5.

Results for antimouse-anti-PCP coated plates

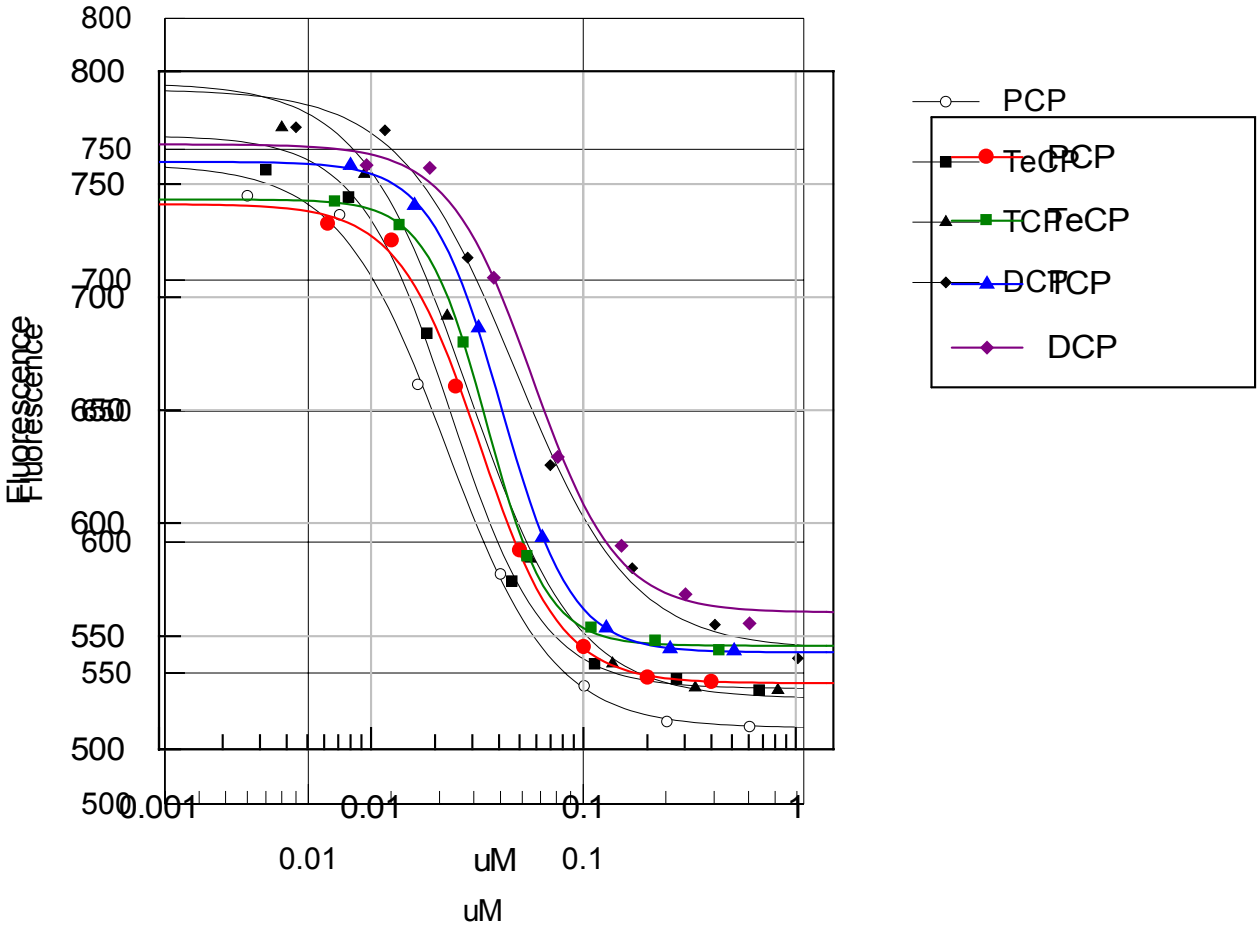


Figure 5.13 – Typical concentration relationship for PCP and cross reactivity of chlorophenols (n=3)

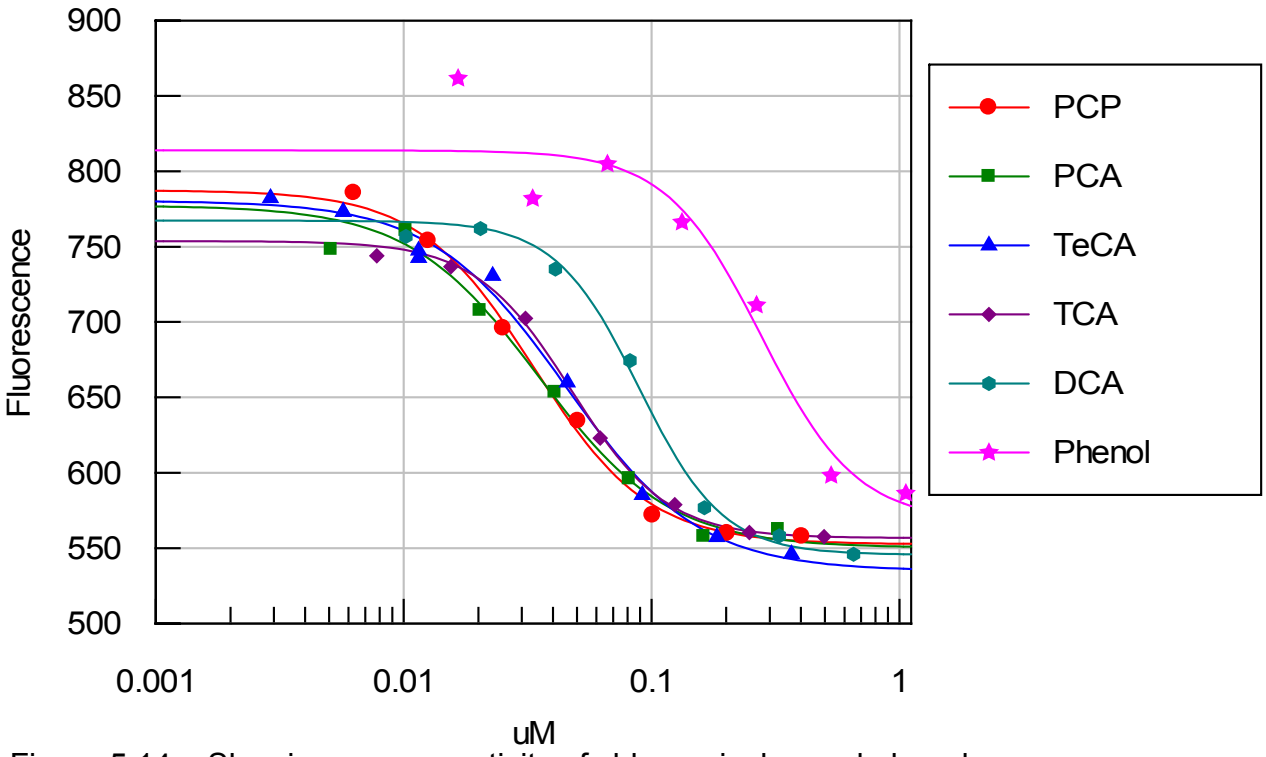


Figure 5.14 – Showing cross-reactivity of chloroanisoles and phenol.

Table 5.5- Interference with PCP-HRP conjugate binding caused by structurally related chemicals, expressed by their IC_{50} and the percentage of cross-reactivity * using anti-PCP coated plates

Compound	IC50		% CR
		Replicates	
PCP	0.033	5	100
TeCP	0.035	3	106.1
TCP	0.041	3	80.5
DCP	0.058	3	56.9
PCA	0.035	2	94.2
TeCA	0.045	2	73.5
TCA	0.047	2	69.8
DCA	0.089	2	37.1
Phenol	0.277	2	11.9

* Cross-reactivity is expressed as % of the IC_{50} of PCP/IC_{50} of the phenolic compound

5.5.2 Results for antimouse-anti-PCA coated plates

Results for anti-PCA coated plates are shown in Figures 5.14 and 5.15 and IC_{50}/ crossreactivity values in Table 5.6.

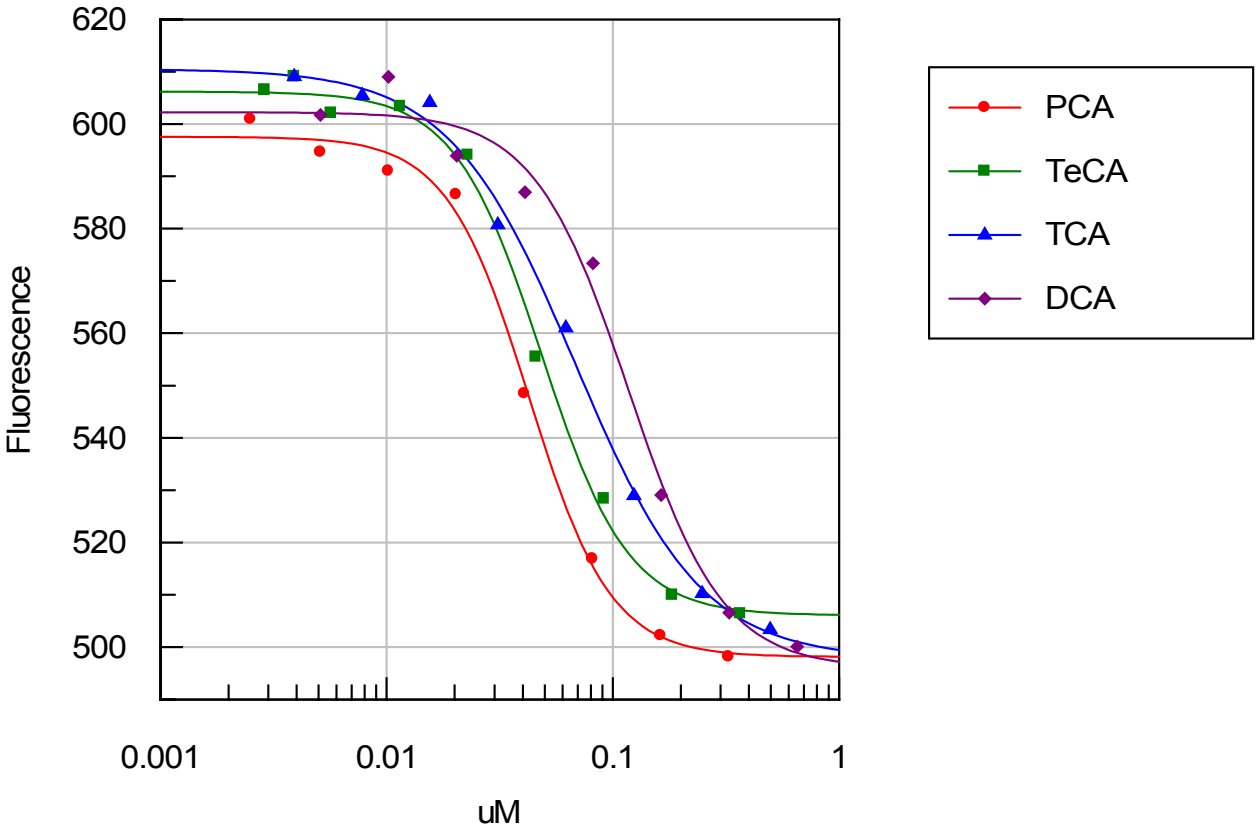


Figure 5.15 – Cross-reactivity using antimouse-anti-PCA coated plates (n=3)

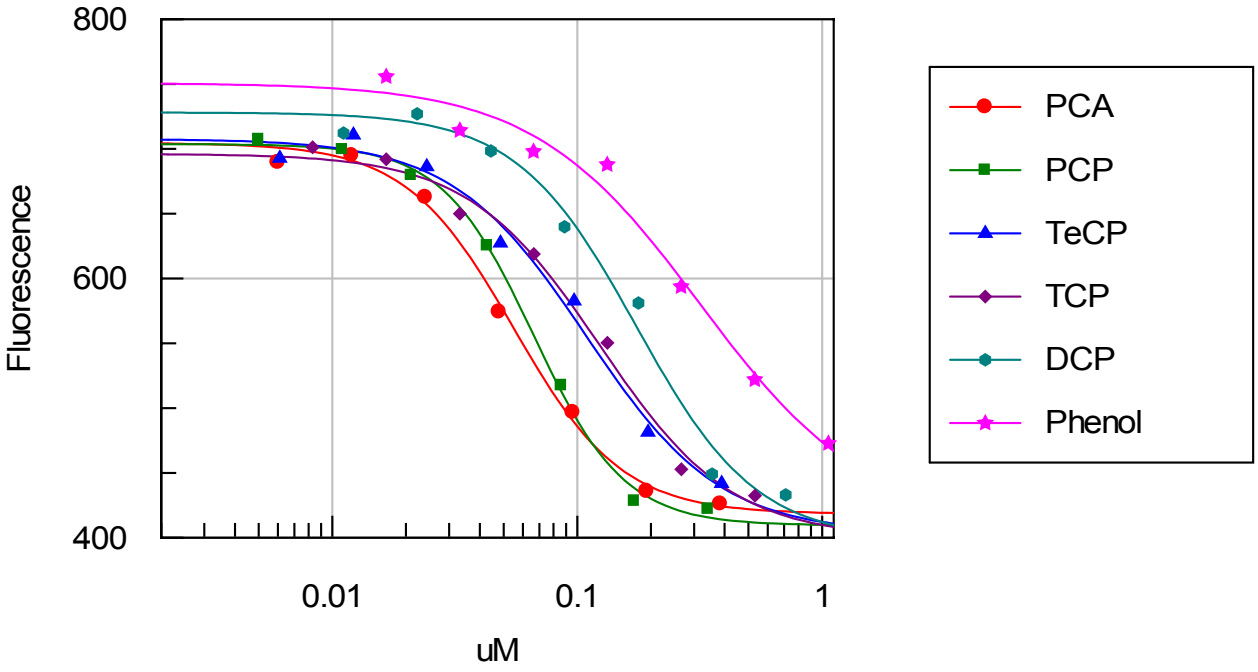


Figure 5.16 – Cross-reactivity studies using antimouse-anti-PCA plates (n=2)

Table 5.6 - Interference with PCA-HRP binding caused by structurally related chemicals, expressed by their IC_{50} and the percentage of cross-reactivity * using anti-PCA coated plates.

Compound	IC50		% CR
		Replicates	
PCA	0.049	5	100
TeCA	0.049	3	100
TCA	0.068	3	72.1
DCA	0.118	3	41.5
PCP	0.064	2	76.4
TeCP	0.109	2	45.0
TCP	0.124	2	39.5
DCP	0.174	2	28.2
Phenol	0.318	2	15.4

* Cross-reactivity (CR) is expressed as % of the IC_{50} of PCP/IC_{50} of the phenolic compound.

5.5.3 Discussion

The analysis of data using the computer program IC_{50} model indicates that the PCP assay is generally more sensitive than the PCA assay, as indicated by consistently lower IC_{50} values.

Significant cross reactivity is exhibited by both antibodies, however procedure employing the anti-PCP antibodies gave generally higher level of cross-reactivity except for phenol which showed lower cross reactivity when used with the anti-PCP anbtibodies. These data indicate that using either antibody the level of cross reactivity appeared to correspond to the degree of chlorination of the aromatic ring, PCP>TeCP>TCP>DCP>Phenol.

Owing to its higher sensitivity, the PCP procedure the Diaclone BL36 antibodies were selected as the preferred antibodies for future work to develop a at-line test kit procedure. The higher levels of cross-reactivity were considered to enable a single test method to be used for the detection of both chlorophenol and chloroanisole congeners with good discrimination against other co-extracted phenolic materials.

5.6 Development of PCP ELISA magnetic bead assay format

To facilitate the use of the ELISA procedure in a small winery laboratory a simple test tube assay was developed using antibodies bound to magnetic beads.

Magnetic beads from three suppliers were evaluated by coating them with Diaclone, anti-PCP antibodies according to the manufacturers recommendations and evaluated using a standard competitive procedure with fluorescence detection.:

1) Dynabeads® Tosylactivated - Dynal M-450

2) Magnabind ™ Goat anti-Mouse IgG beads – Pierce 21354

3) Dynabeads® Sheep anti-mouse IgG - Dynal M-280

Preliminary results indicated that only the anti-mouse IgG Dynabeads® showed selective binding towards PCP. In view of this the procedures used for the preparation of unsuccessful magnetic bead materials 1) and 2) have not been reported.

5.6.1 Preparation of anti-PCP coated Dynabeads® and optimisation of antibody concentration

5.6.2 Materials and methods

5.6.2.1 Chemical and reagents

Dynabeads M-280 sheep anti-mouse IgG were supplied as 2ml of a suspension containing 6-7x10^8 beads/ml in PBS at pH7.4 with 0.1% BSA and 0.2% NaN_3 preservative. Approximately 2.8μm diameter.

Buffer solution 0.1%w/vBSA (Sigma B4287) in PBS buffer (Sigma 4417).

Magnetic separations rack – SDI Europe

5.7.3 – Bead preparation procedure

The magnetic bead suspension (100μl) was transferred aseptically into a 10 ml vial and washed three times using 0.1% BSA in PBS solution. The magnetic beads were separated using a small boron/neodymium magnet.

The supplied 1mg/ml anti-PCP antibody solution was diluted to 10, 1, and 0.2μg/ml using the PBS/BSA buffer. The diluted antibodies (10μl) in 10ml buffer were added to the washed magnetic beads and mixed overnight at room temperature.

The coated beads were separated from the mixture, washed and blocked using 0.1% (v/v) mouse serum with mixing for two hours at room temperature.

5.6.4 Assay procedure

The sample solution (500μl) and HRP-PCP conjugate (500μl) were mixed in a 5ml glass test tube and incubated for 30mins with the magnetic bead suspensions (500μl) at room temperature. The mixture was separated and washed three times using the SDI magnetic separations rack. The fluorescent substrate QuabtaBlu (1ml) was added with mixing. After 30 minutes the reaction was stopped using 1ml QuantaBlu stop solution.

The fluorescence of solutions was measured using a Perkin Elmer LS-50 Luminometer using the settings listed in Table 5.7.

Table 5.7 - Perkin Elmer LS50B instrument settings for magnetic bead assay

Excitation wavelength	325nm
Excitation slit width	10.0
Emission wavelength	420nm
Emission slit width	10.0
Emission cut-off filter	390nm

5.6.5 Results and Discussion

Results are shown graphically in Figure 5.17

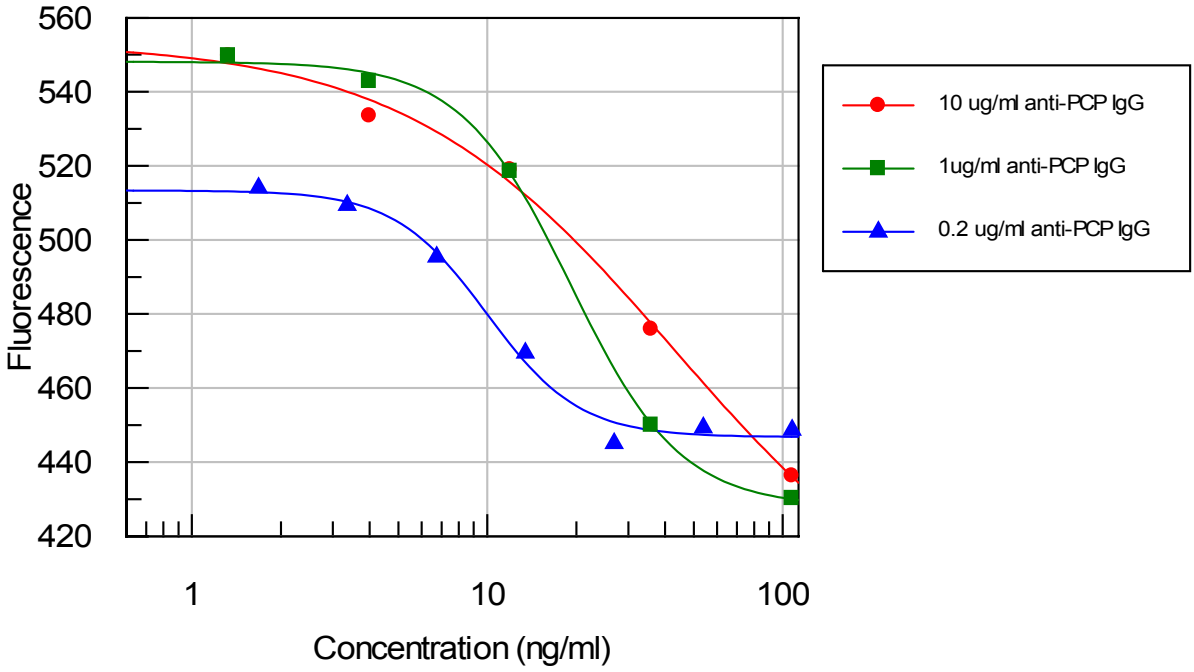


Figure 5.17 – Comparison of assay performance of Pierce antimouse coated microplates at three antibody concentrations (n=2)

Table 5.8 – A comparison of IC_{50} values for magnetic beads coated at various antibody concentrations.

IgG concentration (μg/ml)	IC50 (μg/ml)	Measurement range (μg/ml)
0.2	9.97	2 - 66.6
1.0	19.10	2 - 120.2
10.0	43.17	2 - 166.9

Results shown in Table 5.8 indicate that an antibody concentration of 0.2 μg/ml gave the lowest IC_{50} value and would therefore provide the lowest detection levels. Using this magnetic bead antibody configuration the

measurement range appears to be significantly reduced compared to the other two antibody concentrations studied In view of this an antibody concentration of 1.0 μg/ml, (equivalent to 10ng IgG per 6-7x10^7 beads) was considered to provide the best compromise between sensitivity and limit of detection.

5.7 Development of magnetic particle assay using alternative PCP-biotin and PCP-streptavidin intermediates.

As previously discussed, the use of an inert competitive conjugate is considered to provide significant benefits when testing real-world samples. The principle potential benefits to be gained from using a PCP analogue conjugated to streptavidin or biotin are as follows:

Avoiding unpredictable cross reactions between HRP or HRP conjugates and wine extract coextractives - This would probably reduce the reactivity of the enzyme producing false positive results.

Signal amplification – The binding of a biotinylated reporter enzyme is known to involve several biotin groups for each streptavidin molecule. Thus considerable signal amplification may be gained using this procedure.

The use of streptavidin conjugated material with real-world samples may also have one potential weaknesses. Wine samples may contain some natural biotin that could bind to the PCP-streptavidin conjugate and thereby make it unavailable for competitive binding equilibria. In view of this sample extracts

must be pre-treated with a solution containing streptavidin to block any natural sources of biotin before adding the PCP-streptavidin conjugate.

5.7.1 Synthesis of PCP-biotin conjugate

Materials and methods

Chemicals and reagents

Biotin LC hydrazide - Pierce (cat. - 21340)

Aldehyde-agarose – Sigma (cat. - A9951)

Procedure

This conjugate was prepared from 2,3,5,6,tetrachloro-4-hydroxy phenoxy acetic acid

via the hydroxysuccinimide active ester as previously described in this chapter and purified by extraction into ethyl acetate. The solution in ethyl acetate was reduced to dryness using a stream of nitrogen and reconstituted in 50 μl of dimethylformamide. The ester was linked to a commercially available biotin LC hydrazide (LC indicates 12 atom spacer) as shown in Figure 5.18.

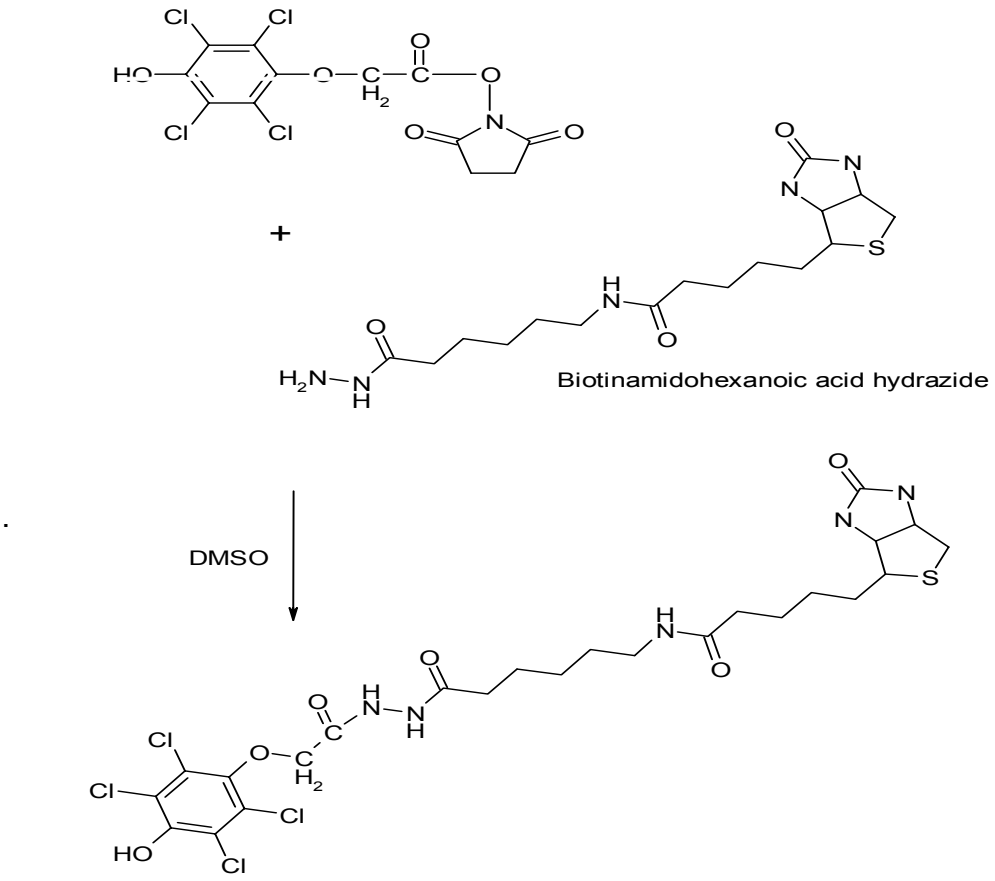


Figure 5.18 – Reaction scheme for the synthesis of PCP-biotin conjugate

The mixture was purified by passing the mixture through a mini column containing aldehyde-agarose to remove any unreacted biotin hydrazide and eluting with MES buffer.

5.8 Synthesis of PCP-Streptavidin conjugate

In this alternative approach the conjugate configuration is reversed and a hapten-streptavidin conjugate and biotin-reporter are employed .

5.8.1 Materials and Methods

5.8.1.1 Chemicals and reagents

Streptavidin hydrazide – Pierce (21120)

5.8.2 Procedure

A solution of 2,3,5,6,tetrachloro-4-hydroxyphenoxy acetic acid was prepared as previously described in section 5.1.2.

The solution in ethyl acetate was reduced to and reconstituted in 50 μl of dimethylformamide. Streptavidin hydrazide solid (2mg) was dissolved in 2ml water and added with mixing for 2hr and then overnight at 4^{o}C. The observed cloudy precipitate was removed using a 0.2μm syringe filter and the mixture purified by stirred dialysis at room temperature using a 10,000 MWCO Slide-A-Lyser^{TM} (Pierce) dialysis cassette in 0.01M MES buffer (pH 6.1).

5.9 Evaluation of PCP-biotin and PCP-streptavidin conjugates.

5.9.1 Preliminary evaluation conjugates using the SDI test kit.

The PCP-biotin and PCP-steptavidin conjugates were evaluated by replacement of the PCP-HRP conjugate in the SDI PCP test kit procedure.

The initial competitive incubation was performed using a 1.0 μg/ml PCP standard solution (500μl) and the previously prepared PCP-biotin or streptavidin conjugate (diluted 1000-1 in MES buffer) (500μl). Following the standard incubation and washing procedures, a solution of a commercially available streptavidin/ biotin-HRP reporter conjugate was added. The level of bound HRP was then detected using a TMB substrate.

A strong blue colour was observed with both conjugates indicating significant competitive binding.

5.9.2 Evaluation of PCP-biotin conjugate using Diaclone, BL-36 anti PCP IgG antibodies using the magnetic particle procedure

A simple test tube format was used similar to that used in the SDI PCP test kit.

A series of standard solutions (500μl) were incubated with the PCP-biotin conjugate (dil. 1000-1) and anti-PCP coated magnetic particles (500μl) for 30 minutes. The mixture was separated using the magnetic separations rack and washed three times with 0.1%w\v Tween20 in PBS. A solution of the commercial HRP-streptavidin conjugate was added (500μl) and incubated for 10 minutes. The mixture was then washed three times and the enzyme

substrate QuantaBlu was added incubated for 30 minutes and the reaction stopped prior to reading the fluorescence using the standard settings. Results are shown graphically in Figure 5.19.

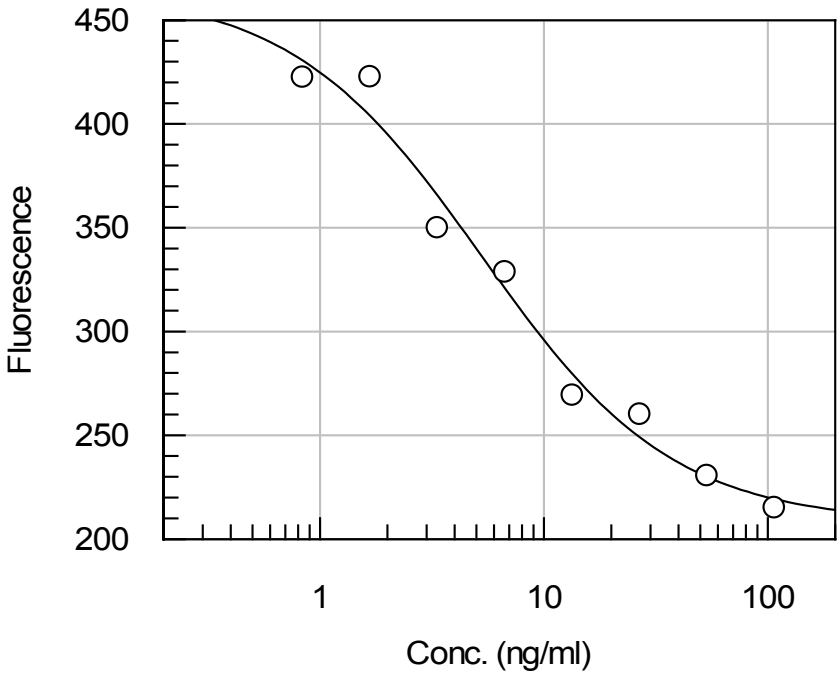


Figure 5.19 - Calibration relationship for PCP magnetic particle assay using PCP-biotin conjugate

5.9.3 Evaluation of PCP-streptavidin conjugate using Diaclone, BL-36 anti PCP IgG antibodies

A similar magnetic particle assay procedure for PCP was employed to evaluate the prepared PCP-streptavidin conjugate. In this case, the bound PCP-streptavidin was visualized using a commercially available HRP-biotin conjugate. Initially the bound and HRP conjugated residues were detected by fluorescence using QuantaBlu fluorescent substrate in a similar manner to the previous procedure. In this case, the levels detected were above the detection range of the instrument and a 10-fold dilution was required to enable the micro titre plate to be read using the previously established instrumental conditions.

In view of this additional sensitivity, a second set of standard solutions was prepared and read using a bench spectrophotometer set at 450nm.

Results are shown graphically in Figure 5.20.

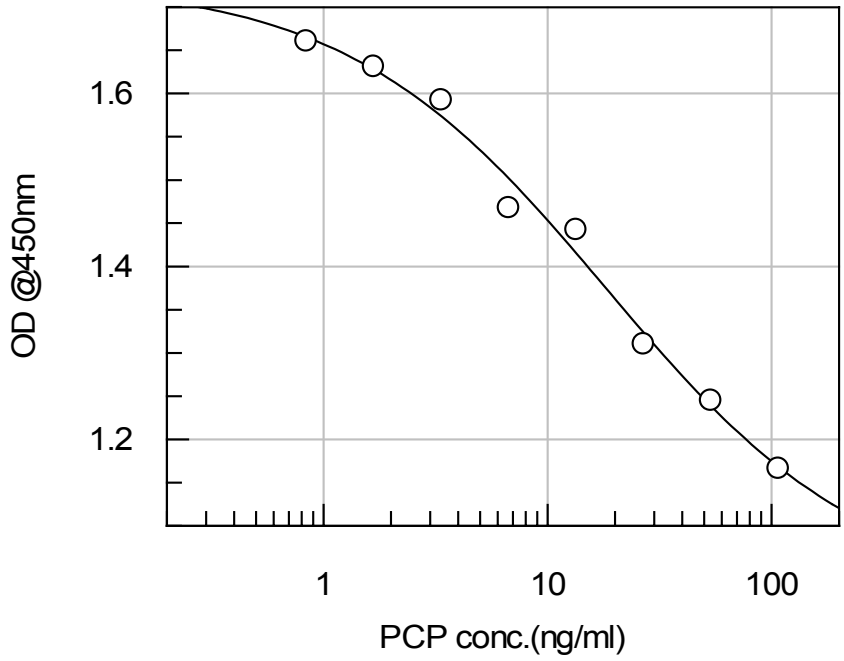


Figure 5.20 PCP magnetic separations assay using PCP-streptavidin conjugate and uv/vis detection

5.10 Discussion

The analysis of data for the standard assay described in Section 5.5 using the computer program IC_{50} model indicates that the anti-PCP antibodies are is generally more sensitive than the anti-PCA antibodies, as indicated by consistently lower IC_{50} values for speciific analytes.

Significant cross reactivity is exhibited by both antibodies, however procedure employing the anti-PCP antibodies gave generally higher level of cross-reactivity except for phenol which showed lower cross reactivity when used with the anti-PCP anbtibodies. These data indicate that using either antibody the level of cross reactivity appeared to correspond to the degree of

chlorination of the aromatic ring, PCP>TeCP>TCP>DCP>Phenol as shown in Figure 5.21.

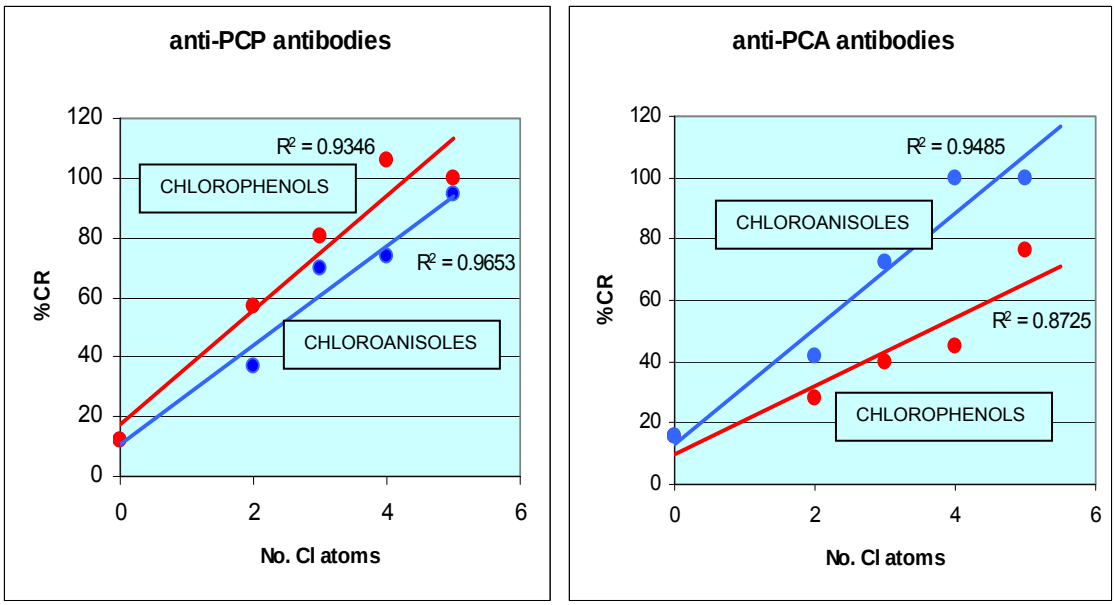


Figure 5.21 - Correlation between cross reactivity of anti-PCP and ant-PCA antibodies and chlorine atom substitution.

Owing to its higher sensitivity, the PCP procedure the Diaclone BL36 antibodies were selected as the preferred antibodies for future work to develop a at-line test kit procedure. The higher levels of cross-reactivity observed using the anti-PCP antibodies were considered to enable the detection of both chlorophenol and chloroanisole congeners with good discrimination against other co-extracted phenolic materials.

Biotin/streptavidin conjugates

These experiments demonstrated that both biotin and streptavidin conjugates of the PCP analogue could be prepared using simple chemical procedures and that in both cases a good concentration dependent relationship were established using the Diaclone BL36 antibodies in the magnetic particle assay format.

Using the PCP-streptavidin conjugate a significant enhancement of sensitivity was observed which enabled the use of a simple UV/vis. detection method. The additional sensitivity afforded by the use of a hapten-streptavidin conjugate is considered to provide opportunities for the use of alternative detection methods of equivalent sensitivity such as electrochemical detection methods that may be more suitable for incorporation into a portable detection and measurement device.

6.0 DISPLACEMENT IMPRINTED POLYMER RECEPTOR ANALYSIS (DIPRA).

During the past few years interest in the development of selective binding assays using molecularly imprinted polymers (MIPs) has increased exponentially. The use of MIPs is now a well established method of providing efficient and selective synthetic binding sites that may be used for a wide range of applications (Piletsky *et al*., 1994; Haupt, 2001; Chianella *et al*., 2002 ; Bartsch and Maeda, 1997; Komiyama *et al.,* 2003). Selective binding provides numerous opportunities for the development of rapid test procedures and many methods have been reported based on selective binding analogous to natural antibodies (Wulff, 1995).

Recent developments in the preparation of synthetic or artificial antibodies based on molecular imprinting technology offers low cost, selective binding sites that are relatively simple to prepare, have improved storage stability and may also be used in the presence of organic solvents. Previously the main uses of MIPs have been as a highly selective solid phase extraction procedure or as a direct replacement for antibodies in ELISA format assays. In this study the equilibrium binding characteristics of MIPs have been utilized to provide a displacement assay format that has significant advantages when compared to antibody based ELISA assays.

6.1 Molecularly imprinted polymers as artificial antibodies

Standard competitive binding methods used for pesticide and trace contaminant analysis normally employ antibodies that are selected for their high binding affinity for a specific antigen in a competitive assay format. The resultant procedures produce a characteristic inverted sigmoidal calibration graph as shown in Figure 6.1.

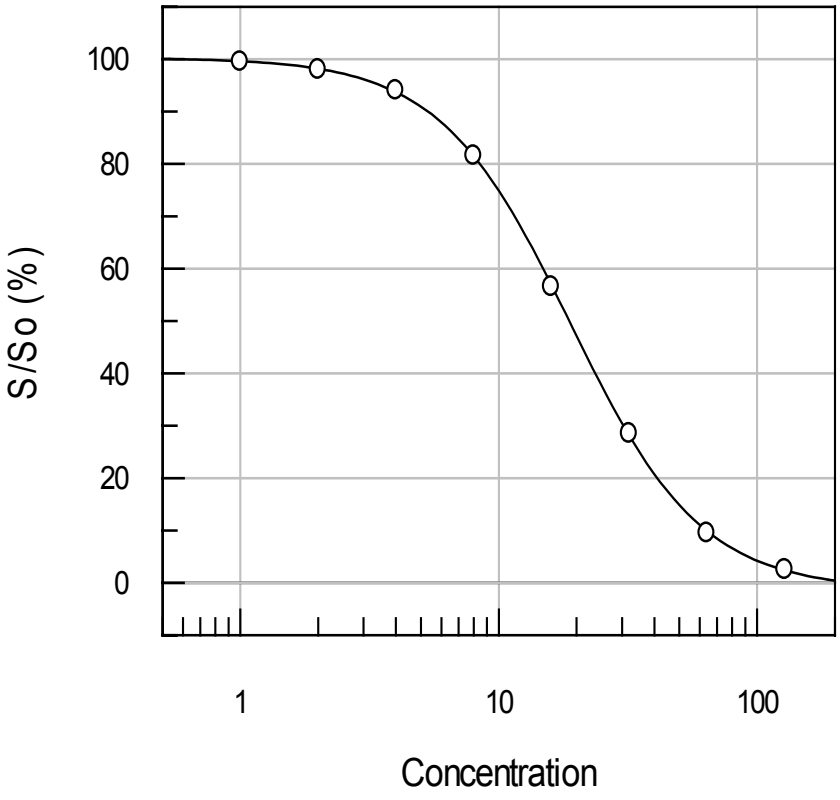


Figure 6.1 – Format of standard calibration graph for PCP, ELISA procedure.

In this example of a competitive ELISA format a high signal intensity is observed at low concentrations and the sensitivity of the method to a small concentration change is reduced in this region of the calibration graph resulting in relatively large errors at low concentrations.

In this chapter the development of a competitive binding assay using synthetic receptors is described. Initially, a competitive ELISA format assay was developed using synthetic receptors and the HRP-PCP conjugate and reagents used for the ELISA procedure described in Chapter 5 and a similar inverted sigmoidal concentration dependent relationship was observed. In order to obtain a signal response that was more directly proportional to concentration the potential use of a displacement format assay was investigated. In this displacement mode of operation the MIP cavities were refilled using a reporter molecule and the measurement signal was produced by the displacement of the reporter molecule by the analyte as depicted in Figure 6.2. In this case the signal produced is directly related to analyte concentration i.e. a low signal response is observed at low concentrations.

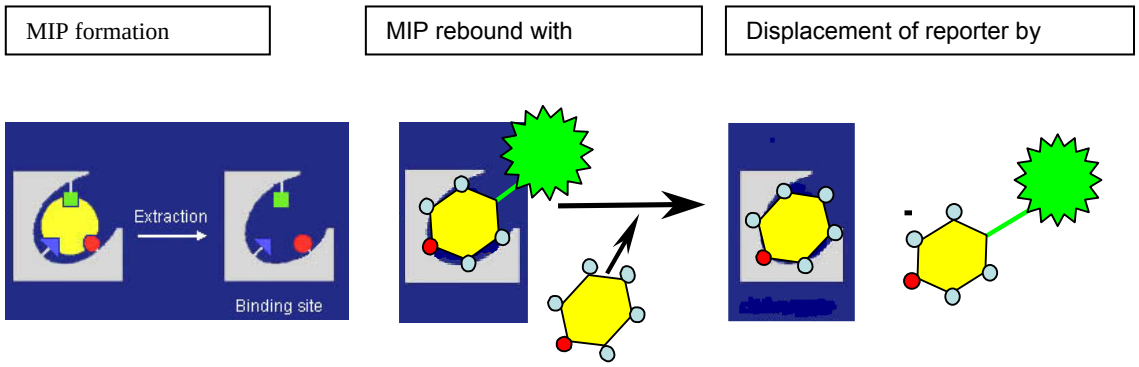


Figure 6.2 - Representation of Displacement Imprinted Polymer Receptor Analysis (DIPRA) procedure .

6.2 Synthesis and purification of Molecularly Imprinted Polymers

In this study molecularly imprinted polymer receptors were produced using a six stage procedure as follows:

6.2.1 The molecular features of the test molecule were modeled computationally using Silicon Graphics workstation with SYBYL 6.9^{TM} software, adopted by Cranfield University for the rational design of MIPs to characterize the potential intra-molecular binding forces (Chianella *et al*., 2002). These may be hydrostatic, π-π, hydrogen bonding, Van der Waals or other non-covalent binding forces. The computer program is designed to search a database of over 100 candidate monomers and select those that show the best binding characteristics to the template molecule as shown in Table 6.1. One or more polymer monomers are then selected for their ability to interact with the target molecule.

Table 6.1 – Binding energies between 2,3,5,6-tetrachlorophenol and candidate functional monomers.

Monomer	**Energy (kjoules mol^{-1})**
Urocanic acid	-76.94
2 - vinyl pyridine	-34.64
4 - vinyl pyridine	-30.04
Vinyl benzene	-26.65
meta Divinyl benzene	-26.11

6.2.2 The template molecule was dissolved in a suitable solvent together with the functional monomer. The mixture is equilibrated under nitrogen to facilitate the establishment of solution interactions with the print molecule. The number and quality of the interactions are controlled by the specific molecular features of the print molecule and the functional monomers.

6.2.3 A large molar excess of cross-linking monomer is added together with the free radical initiator. Since free-radical polymerization is inhibited by the presence of oxygen, the solution is normally purged with nitrogen at this stage. 6.2.4 The polymerization is then initiated by raising the temperature to 60 - 70°C depending on the initiator decomposition temperature, thus starting the homolytic cleavage of the initiator to form free radicals. The radical formation then initiates the polymerization of the functional monomers and the cross-linker, which leads to the formation of a rigid polymer.

6.2.5 Before the molecularly imprinted polymer may be used the original print molecule must be extracted from the polymer matrix. Soxhlet solvent extraction procedures are normally used to remove the template molecule. These procedures are often combined with washing the polymer with

alternating large quantities of methanol containing acids (e.g. acetic acid) and bases (e.g. triethylamine) as appropriate.

6.2.6 The prepared molecularly imprinted polymer is subjected to a work-up scheme tailored to the selected application. This may involve further purification and particle fragmentation, by grinding and sieving.

The polymer mass is then rebound with a guest molecule selected for its ability to fit into the binding cavity but bind less strongly than the target analyte. The residence time and performance characteristics of the guest molecule are also influenced by the selected solvent. The rebound polymer is then washed extensively to remove any surplus guest molecules that may be retained by surface adsorption not associated with the synthetic receptor cavities.

The selection of solvents and the washing procedures employed are critical components in the establishment of displacement equilibria with cavity bound guest molecules. If the guest molecule is strongly bound and the selected solvent is unable to elute of the non-cavity material, the analyte will also be strongly bound to the polymer restricting displacement equilibria. For the Displacement Imprinted Polymer Receptor analysis (DIPRA) assay to be successful the washing procedure must remove most of the non-cavity material and a slight background level due to the elution of non-cavity material should be observed. The analyte is then able to selectively displace the guest molecule

6.3 - PREPARATION OF MOLECULARLY IMPRINTED POLYMERS FOR PENTACHLOROPHENOL AND PENTACHLOROANISOLE

Imprinted polymers were prepared for pentachlorophenol and pentachloroanisole using the following procedure:

6.3.1 - Chemicals

Pesticide residue grade (PRG) water (distilled or deionized), solvents and chemicals must be free of chlorophenol/chloroanisole contaminants, therefore pre-tested or 'pesticide grade' chemicals must be used throughout. It is essential that at least two sample blanks are included with each analytical batch to confirm that chemicals are free of contaminants.

Methanol – Merck (AR grade)

Urocanic acid – Aldrich 85,979-6

Trimethylolpropane trimethacrylate (TRIM) – Aldrich 24,684-0

4,4'Azobis(4-cyanovaleric acid) – Aldrich 11,816-8

Pentachlorophenol

Pentachloroanisole

6.3.2 Reagents

4:1(v/v) Methanol:water - Degas by sparging with helium or nitrogen gas for 30 minutes.

6.3.3 Apparatus

250ml three neck Quickfit flask

Nitrogen inlet

Condenser

Oil bath

6.3.4 Procedure

The apparatus was assembled as shown in Figure 6.3.

Figure 6.3 – Apparatus used for the preparation of molecularly imprinted polymers.

Urocanic acid (0.414g - 3mM) and pentachlorophenol (0.27g - 1mM) or pentachloroanisole (0.28g) was weighed into a three-necked flask and dissolved in 60ml 4:1(v/v) methanol:water. Urocanic acid is sparingly soluble in non-aqueous solvents therefore warming and aid of an ultrasonic cleaning bath may be required to dissolve the mixture. The solution was mixed for 10 minutes under nitrogen to allow the formation of non covalent bonds between

the monomer and template molecules. TRIM (5.08g -15mM) was added and the weighing vessel rinsed using 10ml of 4:1 methanol:water. The mixture was stirred fro for 5minutes and 0.037g cyanovaleric acid were added with mixing. The flask was heated to 70^{o}c and mixed for a period of 4hrs. A white flocculent precipitate was observed after 10 minutes at 70^{o}C. The product was filtered under vacuum, washed using methanol and oven dried overnight at 60^{o}C. Yield approximately 3g

6.3.5 Template removal

Polymers were washed four times by transferring the material into a beaker; mixing and filtering under vacuum using methanol. They were then Soxhlet extracted with three acetone solvent changes. After each solvent change the extractor was refluxed for four hours.

6.3.6 Particle fragmentation

The product was ground in methanol suspension using a large pestle and mortar. The fine material was removed by filtration using a 20 micron test sieve collecting the material passing through the sieve. The retained material was washed back into to the mortar and reground gently for 10 minutes. The process of grinding and sieving was continued until sufficient material had been produced.

Molecularly imprinted polymers prepared in this manner are highly deliquescent and should be stored in a sealed desiccated container.

6.4 SYNTHESIS AND PURIFICATION OF PCP CONJUGATES

Synthetic receptors based on the templates pentachlorophenol (PCP) and pentachloroanisole (PCA) were prepared using the suspension polymerisation procedure described previously in section 6.3. The polymer cavities were rebound using alternative guest molecules to enable an evaluation of a DIPRA procedure using alternative detection methods. The following guest molecules were employed as reporter labels:

Enzymatic - Pentachlorophenol-horseradish peroxidase (PCP-HRP) conjugate using a PCP polymer binding site.

Fluorescent - Pentachlorophenol-aminomethylcoumarinacetate (PCP-AMCA) conjugate using a PCP receptor and aminomethylcoumarinacetate (PCA-AMCA) conjugate with a PCA imprinted polymer.

Electrochemical - 2,3,5,6-Tetrachlorohydroquinone with a PCP binding site for the displacement analysis of PCA.

6.4.1 Enzymatic labels

Enzyme labels are used in a variety of assay formats. A wide range of enzymes and enzyme substrates are also available, which enable them to be detected to extremely low concentrations. The most commonly used enzyme labels are horseradish peroxidase, glucose oxidase, alkaline phosphatase and acetycholinesterase. These enzymes are considered to offer good sensitivity and repeatability. The performance characteristic of each enzyme

may be tailored to a specific detection method with the chosen method being a compromise between sensitivity and robustness. Substrates that react with the enzyme label to produce a coloured product are generally more robust and require very simple apparatus but are generally less sensitive, whereas fluorescent methods exhibit higher sensitivity but are more susceptible to chemical and spectroscopic interferences. Chemiluminescent methods exhibit similar sensitivity to fluorescent methods but require simpler (cheaper) instrumentation, although they are more prone to chemical interferences.

The main disadvantage of using enzymatic labels is the need for numerous incubation stages, each of which needs to be optimised and regulated, and each may also have local interference issues that need to be controlled.

In this study a PCP analogue conjugated to horseradish peroxidase with fluorescence detection was selected to obtain good levels of sensitivity and robustness. The PCP-HRP conjugate was prepared using a six-stage synthesis. The HRP enzyme was oxidised and linked to a six carbon atom hexamino spacer molecule that was combined with 2,3,5,6,tetrachloro-4-hydroxy phenoxyacetic acid using a hydroxy-succinimide ester intermediate. Details of the synthesis are described in Chapter 5.

A variety of substrates may be used for quantifying peroxidase-based assays ,such as 3,3',5,5'-tetramethylbenzidine (TMB), o-phenylendiamine dihydrochloride (OPD), and 2,2'-azinobis [3-ethyl-benzothiazoline-6-sulfonic acid] diammonium salt (ABTS), QuantaBlu^{TM} (Pierce). Pierces fluorogenic peroxidase substrate QuantaBlu , a formulation based on 3-(p-Hydroxyphenyl)propionic acid, has been shown to be more sensitive than

traditionally used chromogenic substrates (Savage *et al.,* 1998). The minimum detection concentration of a QuantaBlu-based peroxidase assay is considered to be between 4.5 and 35 times lower than equivalent TMB, OPD, ABTS-based assays. QuantaBlu peroxidase substrate product also exhibits a large Stokes' shift (the difference in wavelength between excitation and emission) of 95nm (excitation 325nm – emission 420nm) which reduces spectral interference effects.

6.4.2 Fluorescent labels

Certain fluorophores can be detected at very low concentrations without the need for enzymatic amplification. The effectiveness of fluorescence detection is also highly dependent on the presence of quenching compounds and spectroscopic interferences from other materials such as detergents that may emit stray light at the selected operating wavelengths. A reporter molecule with a high Stokes shift is highly desirable to minimise spectroscopic interferences.

In this study 7-amino-4-methylcoumarin-3-acetyl hydrazide was linked to 2,3,5,6,tetrachloro-4-hydroxy phenoxy acetic acid via the hydroxysuccinimide derivative (Figure 6.4).

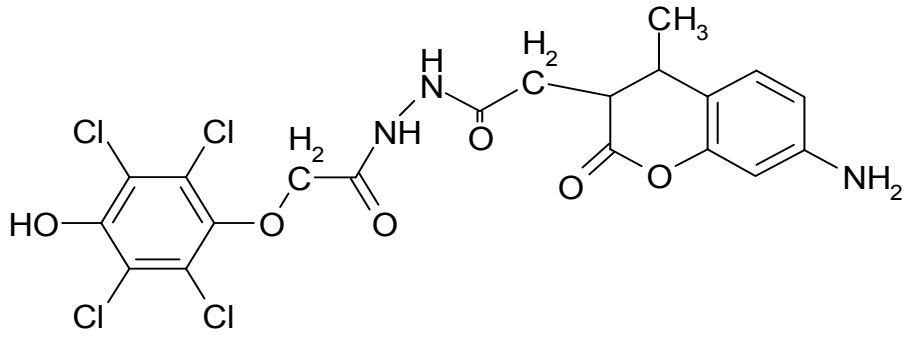


Figure 6.4 - Molecular structure of PCP- AMCA derivative - (2,3,5,6-Tetrachloro-4-hydroxy-phenoxy)-acetic acid N'-[2-7-amino-4-methyl-2-oxo-chroman-3-yl)-acetyl]-hydrazide

The PCP-AMCA conjugate also exhibits a large Stokes' shift (94nm) and a pale blue fluorescence (excitation 350nm – emission 444nm).

The main advantage of this conjugate is the very high fluorescence efficiency which enables the label to be used directly with no additional amplification procedures.

The excitation wavelength of 350nm is also very convenient as it enables the doping of polymers to be monitored using a simple UV viewing lamp set at the long wavelength setting (365nm).

6.4.3 Preparation of PCP-AMAC fluorescent conjugate

The hydroxysuccinimide active ester intermediate of (2,3,5,6-tetrachoro-4-hydroxy phenoxy) acetic acid (TeClHPAA) was formed by mixing 1.7mg N-hydroxysuccinimide with 3.1mg TECLHPAA and 2.8mg 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDAC) in 200μl dry dimethylformamide (DMF). The mixture was stirred for two hours in the

dark. The active ester was extracted into ethyl acetate evaporated to dryness under nitrogen and redissolved in 100μl DMF. 7-amino-4-methylcoumarin-3-acetyl hydrazide (Pierce) 2.5mg was dissolved in 900μl DMF was added and mixed for two hours. The solution was stored at 4^{o}C and the PCP-AMAC conjugate was used for rebinding studies without further purification.

A PCA-AMCA conjugate was prepared in a similar manner using the methylated 2,3,5,6-tetrachloro-4-hydroxy phenoxyacetic acid hydroxy succinimide ester.

6.4.2.2 Characterization of PCP-AMCA conjugate

The PCP-AMCA conjugate was analysed by Warwick Analytical Services Ltd using a Waters LCT electrospray L(ES) mass spectrometer (Waters, MA, USA) in positive ion mode. The samples were dissolved in acetonitrile/water (3:1v/v) and directly infused into the source. Results indicate three principle components . Detailed mass spectra were supplied on the main component, which exhibited a molecular ion at 558 Daltons and typical four chlorine isotope pattern corresponding to the sodium adduct of the target molecule (exact mass 535 Daltons).

HPLC analysis of the product solution was performed using a 3mm x 15 cm Zorbax Eclipse XDB-C18 column (Agilent, USA) with a gradient solvent change from of 5%v/v acetonitrile:1% H_3PO_4 to 50 % v/v acetonitrile: 1% H_3PO_4 over a period of 20 minutes. Detection was by uv at 254nm to obtain the best general response.

Results show three principle peaks at 5.5, 8.1 and 19.2 minutes as shown in Figure 6.5. The identity of the peaks was not confirmed by mass spectrometry but the retention time of the first peak corresponded to DMF and the peak at 19.2 was found to exhibit fluorescent characteristics. A crude estimate of the product purity based on area percent at 254nm is approximately 80% (excluding the DMF component).

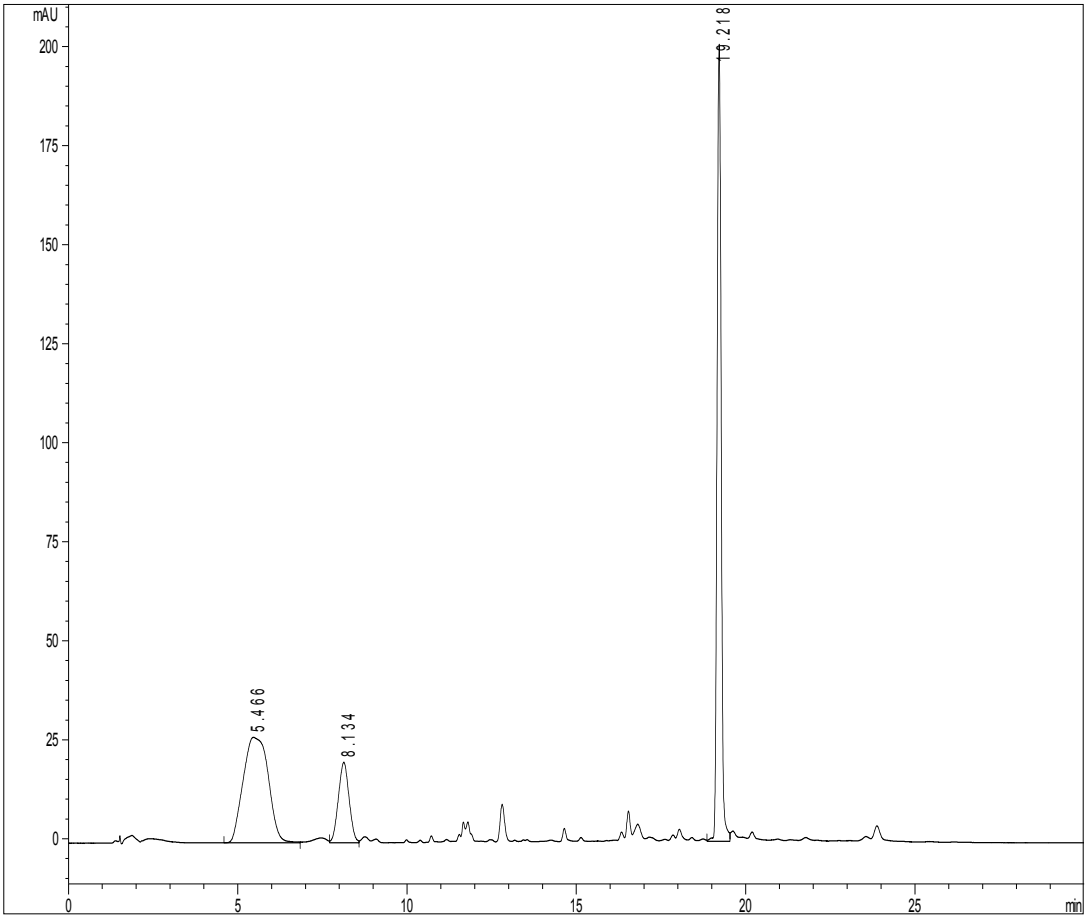


Figure 6.5 – HPLC chromatogram of PCPAMCA conjugate solution

6.4.4 Electrochemical labels

Alternative detection methods based on electrochemical measurements are highly desirable because using this detection method a simple portable and highly sensitive detection device can be configured. In the DIPRA procedure an electroactive guest molecule can be selected to enable electrochemical detection of analyte molecules that are not naturally electroactive (e.g. pentachloroanisole).

The molecule 2,3,4,5,tetrachlorohydroquinone (TeClHQ) is stoichiometrically very similar to pentachloroanisole but unlike pentachloroanisole is readily oxidised at the electrode surface to 2,3,4,5,tetrachloroquinol (chloranil). Tetrachlorohydroquinone may therefore be used as a suitable guest molecule for the analysis of chlorophenols and chloroanisoles as reported in section 6.7.

6.5 PRELIMINARY ASSESSMENT OF MIPS USING AN ELISA FORMAT COMPETITIVE BINDING ASSAY.

A competitive ELISA assay was established using a type R1 Millipore MultiScreen microplate assembly (Millopore, Mass., USA).

This commercially available MultiScreen system incorporates the use of a microwell plate fitted with a hydrophilic PTFE membrane. A special vacuum manifold or bench centrifuge fitted with a microplate attachment is used to facilitate the removal of liquid residues. The use of a standard microplate washing apparatus is unsuitable in these applications as the polymer is not bound to the well but present as a free suspension which would be removed during standard washing operations.

A MIP suspension (50μl) containing approximately 10mg/ml polymer in water was added to each well and a mixture containing 100μl of the PCP-HRP conjugate together with 100μl of a PCP standard solution were added. The plate was incubated for 30 minutes at room temperature with slow vortex mixing.

The supernatent was removed by vacuum filtration and the residue washed three times using 200μl of a 0.1 % Tween 20/ PBS solution with filtering between washes.

The bound PCP-HRP conjugate was detected by adding 100μl

Quantablu flurorescent substrate (Pierce, Rockford, Mass.), incubating for 30 minutes and stopping the reaction using the QuantaBlu stop solution.

The microplate was then filtered and the filtrate was retained for fluorescence measurements using a simple flow injection system consisting

of an Agilent 1050 HPLC pump (Agilent, USA), Rheodyne injector (IDEX Corp,USA) and Shimadzu RF-551 ftuorescence detector (Shimadzu Corp., Japan) using 10% v/v methanol:water mobile phase and a 20μl injection loop. Results are shown in Figure 6.6.

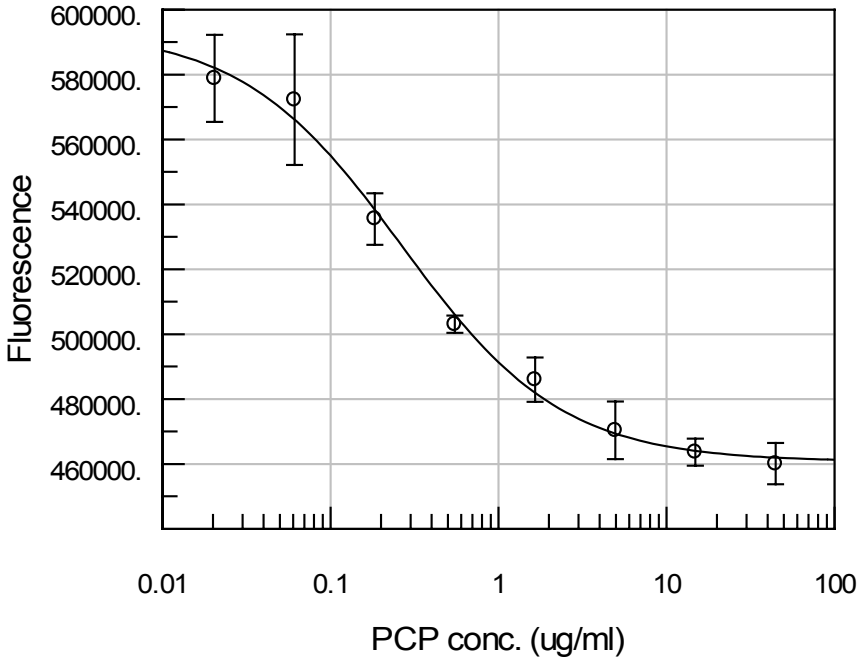


Figure 6.6 – Calibration graph for PCP MIP ELISA format assay (n=4 error bars at 2 x standard deviation).

Results indicate a similar calibration format to the standard ELISA calibration curve with a similar inverse concentration dependence that could be fitted to an IC_{50} model and higher variance at low concentrations. In view of this, the potential of an alternative displacement procedure was investigated.

6.6 MIP DISPLACEMENT ANALYSIS

To facilitate the use of the MIP in a displacement mode the following procedure was employed to rebind the synthetic receptor cavities with a reporter molecule that may be displaced by the analyte.

6.6.1 Chemicals

Isopropyl alcohol - Merck

Conjugate stock solutions: -

PCP-HRP conjugate – 0.01mg/ml approximately.

PCP-AMCA conjugate -1mg/ml approximately

Tetrachlorohydroquinone - 1mg/ml approximately prepared in degassed water

QuantaBlu flurogenic peroxidase substrate – Containing Substrate Solution, Stable Peroxide Solution and Stop Solution (Pierce 15169)

6.6.2 Reagents

Phosphate buffered saline solution (PBS) - Dissolve one tablet PBS (Sigma P-4417) in 200ml of water to obtain 0.01M phosphate buffer pH7 in saline solution.

Tween20/PBS buffer – 0.1%(w/v) Tween 20 in PBS buffer.

Polymer doping reagents

HRP-PCP conjugate – For PCP analysis dilute the PCP- HRP conjugate stock solution 500μl to 5.0ml in PBS.

AMCA-PCP for PCP analysis - dilute 50μl to 5.0ml in PBS.

Tetrachlorohydroquinone for PCP analysis - dilute 50μl to 5.0ml in degassed PBS.

AMCA conjugate - PCA analysis dilute the conjugate 500μl to 5.0ml in 10%(v/v) isopropanol:hexane.

QuantaBlu peroxidase working solution – Mix 9ml Substrate Solution with 1ml 'Stable Peroxide Solution'. The working solution is stable for 24 hours at room temperature.

6.6.3 Apparatus

Centrifugal filter tubes for polymer preparation – Vectaspin 20, Polysulphone 0.2μm Whatmann). Filter tubes fitted with an inert PTFE filter may also be used but alternative materials such as Nylon and PVDF may cause surface binding problems, particularly when using the PCP-HRP conjugate.

Centrifugal filter tubes for analysis - Vecta Spin Micro Polysulphone 0.2μm (Whatman).

Alternatively for large batches the Multiscreen^{TM} 96-well filtration plate (Millipore), R1 hydrophilic PTFE membrane was used.

Rotamixer – e.g. Blood tube rotator SB1 (Jencons) or similar

Centrifuge – e.g. Beckman Microfuge.

6.6.4 Polymer rebinding procedure

A suspension of the polymer (1mg/ml) was prepared in propan-2-ol. The suspension 20 ml was transferred to two Vectacspin 20 tubes and centrifuge at approximately 3000rpm for 2 minutes. Wash the polymer residue with 2x 20ml Tween 20 /PBS buffer.

The polymer was resuspended in 5ml of the selected conjugate and mixed for 1hr. For PCP analysis use PCP-HRP, PCP-AMCA conjugates or 100μg/ml Tetrachlorohydroquinone in PBS. For PCA analysis use PCA-AMCA conjugate in 10%(v/v) propan-2-ol:hexane. The doped polymer was then centrifuged and wash five times using an appropriate solvent and resuspended in 5ml of the wash solvent and transfer to a standard test tube. The polymer suspension (100μl) was transferred to a Vecta Spin Micro tube and 400μl of sample extract was added. Care is required to ensure that identical solvents are used for polymer suspension and sample extracts. The suspension was mixed for 1hr using the Rotomixer device and centrifuged for 2 minutes at 3000 rpm. The polymer residue was retained for the measurement of displaced conjugate.

6.6.6 Measurement of the displaced conjugate

QuantaBlu peroxidase working solution (100μl) was transfered to the filtrate in the centrifuge tube and incubate for 30 minutes at room temperature.

QuantaBlu Stop Solution (100μl) was added and the solutions were mixed.

The fluorescence emission was measured using a fluorimeter or 96-well fluorescent plate reader with an excitation wavelength of 325nm and emission wavelength 420nm.

6.6.7 PCP-AMC conjugate

The emitted fluorescence was read directly using a fluorimeter or 96-well fluorescent plate reader using an excitation wavelength of 350nm and emission wavelength 444nm.

6.6.8 Tetrachlorohydroquinone

A solution of the base electrolyte (PBS) (1.0ml) was added to the electrochemical cell (described in section 4.2) and a stable base current was established. A solution of the sample extract (500μl) was added and the resultant current change was measured after mixing for approximately 30 seconds.

6.6.9 - Results

PCP-horseradish peroxidase conjugate for pentachlorophenol analysis

A typical calibration graph for the PCP-HRP conjugate and QuantaBlu substrate mixture is shown in Figure 6.7. This guest molecule shows a very good concentration dependent relationship from 0.1 to 5 μg/ml. At higher PCP concentrations (>2ug/ml approximately) the large PCP-HRP molecule appears to be displaced less readily. This would be expected if some of the reporter molecule were incompletely bound to the receptor sites and therefore more easily displaced by the analyte.

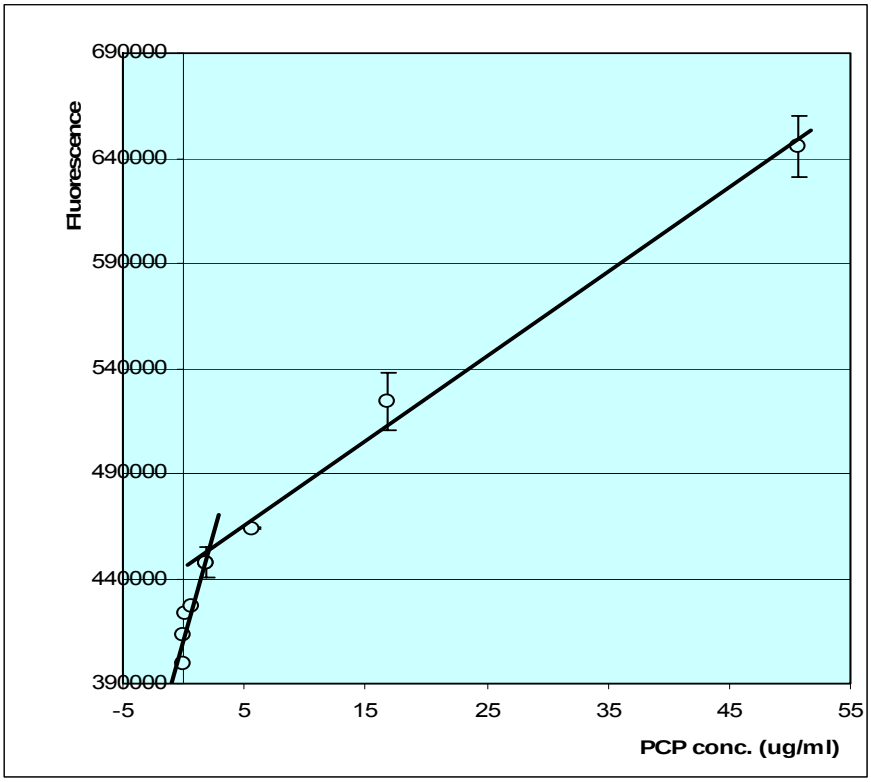


Figure 6.7 – PCP DIPRA calibration graph using PCP-HRP conjugate (n=3, error bars 2x standard deviations). Measurement by FIA using a Shimadzu RF-551 fluorimetric detector and 10%v/v methanol/water mobile phase.

PCP-AMCA conjugate for pentachlorophenol analysis

Using the PCP-AMCA conjugate, a uniform calibration graph was produced over

the range 0.1 to 50 μg/ml (Figure 6.8). In this case the calibration graph is approximately linear to 20 μg/ml.

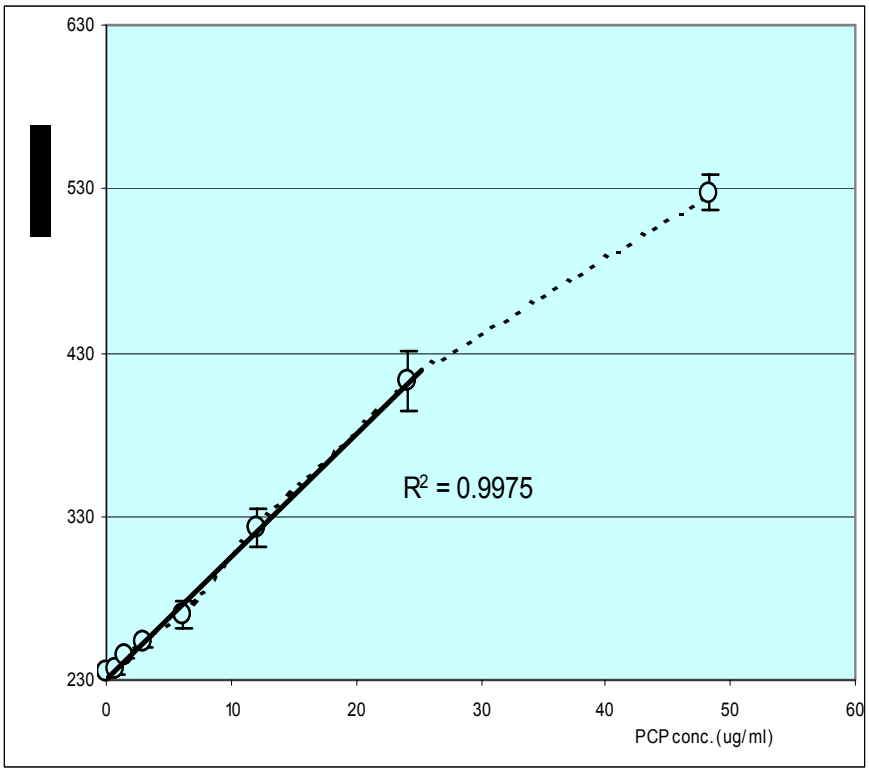


Figure 6.8 – PCP DIPRA calibration graph using PCP-AMCA conjugate (n=3, error bars at 2x standard deviations). Fluorescence measured using a Perkin Elmer LS50B Luminometer.

6.7 DIPRA procedure using PCA-AMCA conjugate for pentachloroanisole analysis in non-aqueous solvents.

Chloroanisoles are only sparingly soluble in water therefore the use of a non-polar organic solvent is preferred for the efficient extraction of these contaminants. A range of non-aqueous solvents were investigated for the preparation of a PCA-AMCA doped polymer and to enable the direct measurement of PCA in sample extracts using a DIPRA approach.

In preliminary experiments a range of solvents of increasing polarity were used to rebind the PCA-AMCA conjugate to the polymer, remove surface bound material and set-up the desirable elution equilibria with cavity bound material.

A solvent mixture containing approximately 10% v/v propan-2-ol in hexane was considered to provide suitable performance characteristics based on their ability to extract chloroanisoles efficiently but only partially remove weakly bound PCA-ANCA conjugate from the polymer surface.

Preliminary studies shown in Table 6.2 indicate a good concentration dependent relationship. The use of non-aqueous solvent is particularly attractive with fluorescence detection because fluorescence quenching effects may be significantly reduced in organic solvents.

Table 6.2 – Preliminary calibration for non-aqueous DIPRA procedure.

PCA concentration (μg/ml)	Fluorescence reading	Reading - blank
0	166.9	0
17.4	242.6	75.7
34.7	266.4	99.5

This very limited experiment is reported here to indicate the potential of the DIPRA procedure for use in non-aqueous solvents. Additional work is necessary to fully characterize the performance of this procedure.

6.8 DIPRA procedure using tetrachlorohydroquinone for pentachloroanisole analysis and electrochemical detection.

In this application the resin was synthesized using PCP as the template and rebound using tetrachlorohydroquinone as the guest molecule. The doped resin was then used for the analysis of a non-electrochemically active PCA molecule.

The analysis was attempted at approximately 35μg/ml in aqueous solution but it was found that PCA was insufficiently soluble in PBS electrolyte and this range of concentrations. In view of this 10%(v/v) methanol:PBS buffer solution was used to wash the doped resin and as the DIPRA solvent.

Screen printed carbon and silver/silver chloride reference electrodes described in section 4.2 were immersed in 1.0 ml of a standard methanol:buffer solution poised at –1.10V. The sample solution (500μl) was added and mixed for 30 seconds and the resulting current change was recorded. Preliminary data shown in Table 6.3 indicates that the electro-active species tetrachlorohydroquinone is displaced from the polymer cavities resulting in a concentration dependent relationship.

Table 6.3 – Amperometric response for PCA reference solutions using a tetrachlorohydroquinone guest molecule.

PCA concentration (μg/ml)	Averaged background signal x 10^{-6} (amps)	Averaged sample signal x 10^{-6} (amps)
0	3.22	3.26
17.4	3.29	3.72
34.7	3.36	4.37

The use of degassed solvents to avoid potential problems with atmospheric oxidation is considered to restrict the application of this method in real-world samples. Further work is required to confirm these findings and investigate the use of alternative guest molecules suitable for electrochemical detection such as 2,3,5,6-tetrachloroquinol (the oxidised form of 2,3,5,6-

tetrachlorohydroquinone). Alternative pulsed electrochemical measurement procedures may also enhance the selectivity of this procedure.

6.9 Cross-reactivity studies

Cross reactivity was evaluated by applying solutions of different pesticides commonly encountered in water samples to the doped polymer and assessing the fluorescence of the filtrate using the standard PCP calibration graph. Three chlorophenol congeners were also tested in a similar manner. Results shown in Figure 6.9 indicated that the polymer binding site shows good selectivity towards pesticides with similar molecular features. The blank subtracted response to PAHs, phenol, detergents and soil contaminants such as humic acid is very low. Other pesticide molecules such as dieldrin which are significantly larger than the template showed low displacement efficiency (<20%) as did as small molecules such as glyphosate. However, pesticides with a chlorinated aromatic ring or chlorinated triazine moiety were detected using the procedure and halo-phenolic pesticides such as Bromoxynil were detected with similar efficiency to PCP.

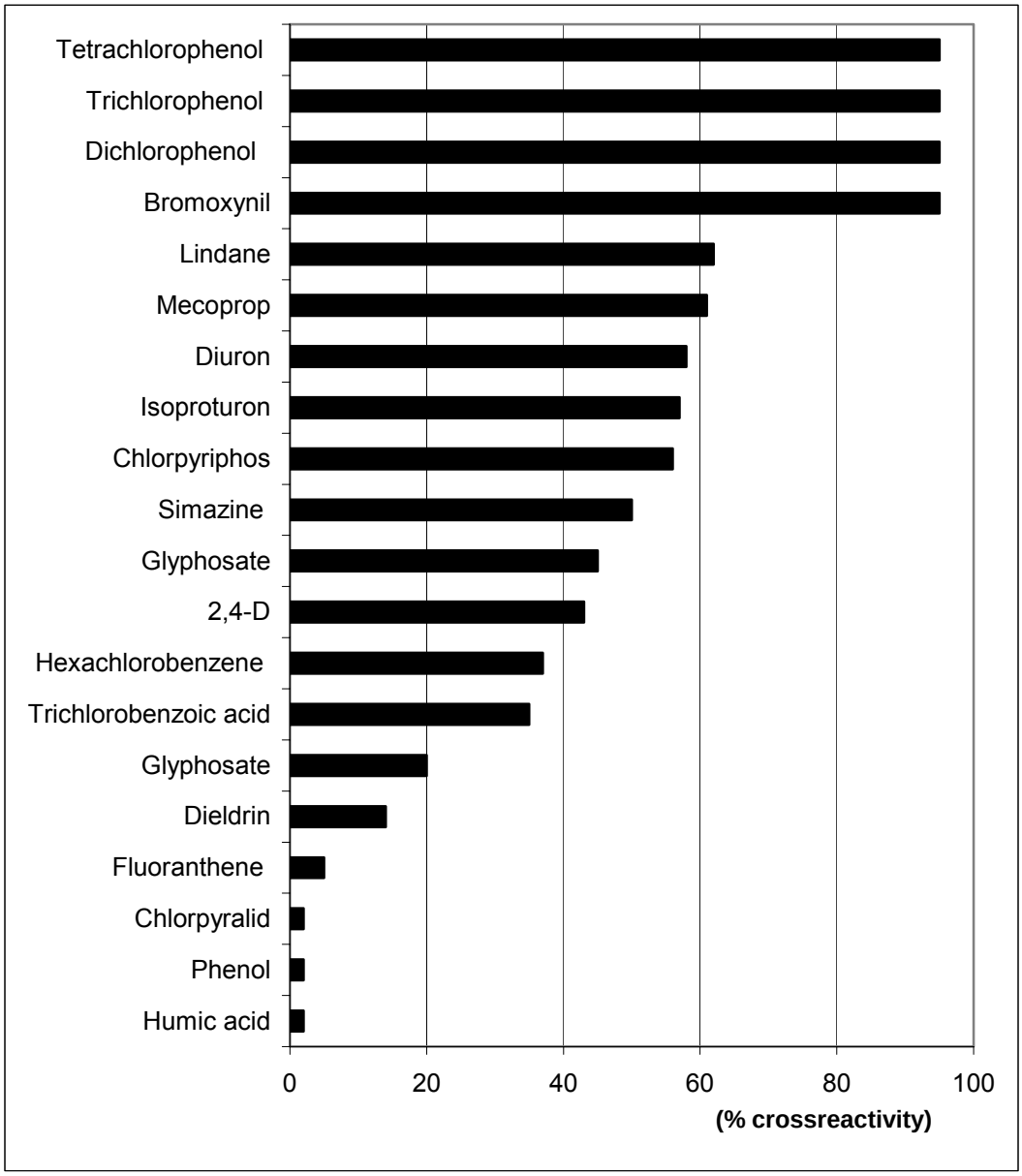


Figure 6.9 – Cross reactivity of PCP MIP with various pesticides

6.10 Discussion

In this chapter it has been demonstrated that the preparation of imprinted polymer receptors is straightforward and may be employed as a replacement for natural antibodies in a competitive binding assay format. In this mode of operation the assay shows a high level of low concentration errors and in this respect performs in a similar manner to natural antibodies. The alternative displacement (DIPRA) procedure features significantly lower errors at low concentrations and provides a signal response that is directly concentration dependent . Two alternative reporter conjugates were employed to demonstrate the flexibility of the system, a synthesized fluorescent conjugate based on a PCP or PCA analogue and the structurally related but more electrochemically active tetrachlorohydroquinone. In each case a concentration dependent relationship was established. This indicates that the DIPRA procedure provides the basis for the development of a screening procedure for many sample types using alternative reporter guest molecules which enables the procedure to be tailored to the requirements of the selected detection system. This is particularly suitable where the target analyte has few molecular features on which to base a system of measurement. The DIPRA procedure is particularly suitable for use with MIPs because any strongly retained template molecules from the imprinting process do not interfere with the analysis. Doped synthetic receptors are inexpensive to prepare and may be stored for many months without loss of activity. They are therefore highly suitable for incorporation into at-line test

methods or simple sensor devices using optical or electrochemical detection methods.

7.0 SAMPLE PURIFICATION AND CONCENTRATION PROCEDURES

Preliminary analysis presented in Chapter 4 indicted the potential analytical problems due to structurally related polyphenolic and other materials commonly found in wine, cork and other wood/paper products. This is considered to be a major concern with the use of receptor methods due to the high risk that these matrix components may be able to interfere with the receptor or the reporter moiety and thereby produce a false positive result.

7.1 Alternative sample purification procedures.

In commercial winery laboratories the procedure most commonly used for the extraction and concentration of chlorophenol / chloroanisole contaminants in wine prior to analysis involves liquid-liquid extraction. The extraction efficiency of the procedure for chlorophenols is enhanced significantly by pH control due to the simple acid base equilibrium shown in Figure 7.1. Pentachlorophenol has a pKa of 4.7 and above pH 6.7 chlorophenol congeners are present as the dissociated phenate ion form. If the pH is lowered to below 2.7 chlorophenols are undissociated (Crosby *et al.,* 1981).

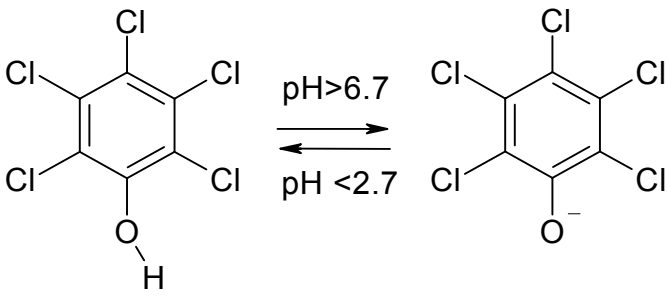


Figure 7.1 – Acid base equilibria for pentachlorophenol

In aqueous solution, the pentachlorophenol molecule is acidic and has the highest pKa value of the group (pKa = 4.7). However, to a greater or lesser extent all of the 19 chlorophenol congeners are susceptible to pH changes in a similar manner. These pH dependent equilibria have a significant influence on the solubility of the chlorophenol molecule in water or organic solvents. Therefore changing the pH of a wine samples can be used to selectively extract and concentrate chlorophenols and to remove interferents from the wine matrix.

Organic solvents selected for liquid-liquid extraction of wine are normally light hydrocarbons, pentane, hexane or petroleum spirit. Using an acidified wine sample (<pH2) an extraction efficiency of greater than 95% can be achieved by this method (Cooper *et al.* - 1994).

Potential problems with this method are that non-polar materials such as terpenes and fatty acid esters are also extracted into the organic phase. These materials may give rise to false positive results using a simple field-test procedure, whereas in laboratory analyses extracts of wine samples are normally tested using GC-MS with selective ion monitoring to reduce any detector interferences. GC methods that employ electron capture detection alone may also be sufficiently selective for use with this extraction method.

Alternative sample purification systems suitable for incorporation into a simple field test method have been outlined in Chapter 4. In this chapter, two alternative procedures 1) solid phase extraction and 2) steam distillation which were considered suitable for use in a small winery laboratory were selected for further evaluation.

7.2 Solid phase extraction

Solid-phase extraction (SPE) uses adsorption and solution equilibria between a solid phase and a liquid phase to selectively recover groups of compounds having similar adsorption properties from a solution. It is usually used to clean up a sample before using a chromatographic or other analytical method to quantify the amount of analyte(s) in the sample. Traditionally the materials employed as column packing sorbents are similar to HPLC column materials based on modified silica or alumina. More recently many different polymers with different surface adsorption properties have been developed for specific applications.

The standard procedure involves loading a solution onto the SPE phase, washing away undesired components, and then washing off the desired analytes with another solvent into a collection tube as shown in Figure 7.2.

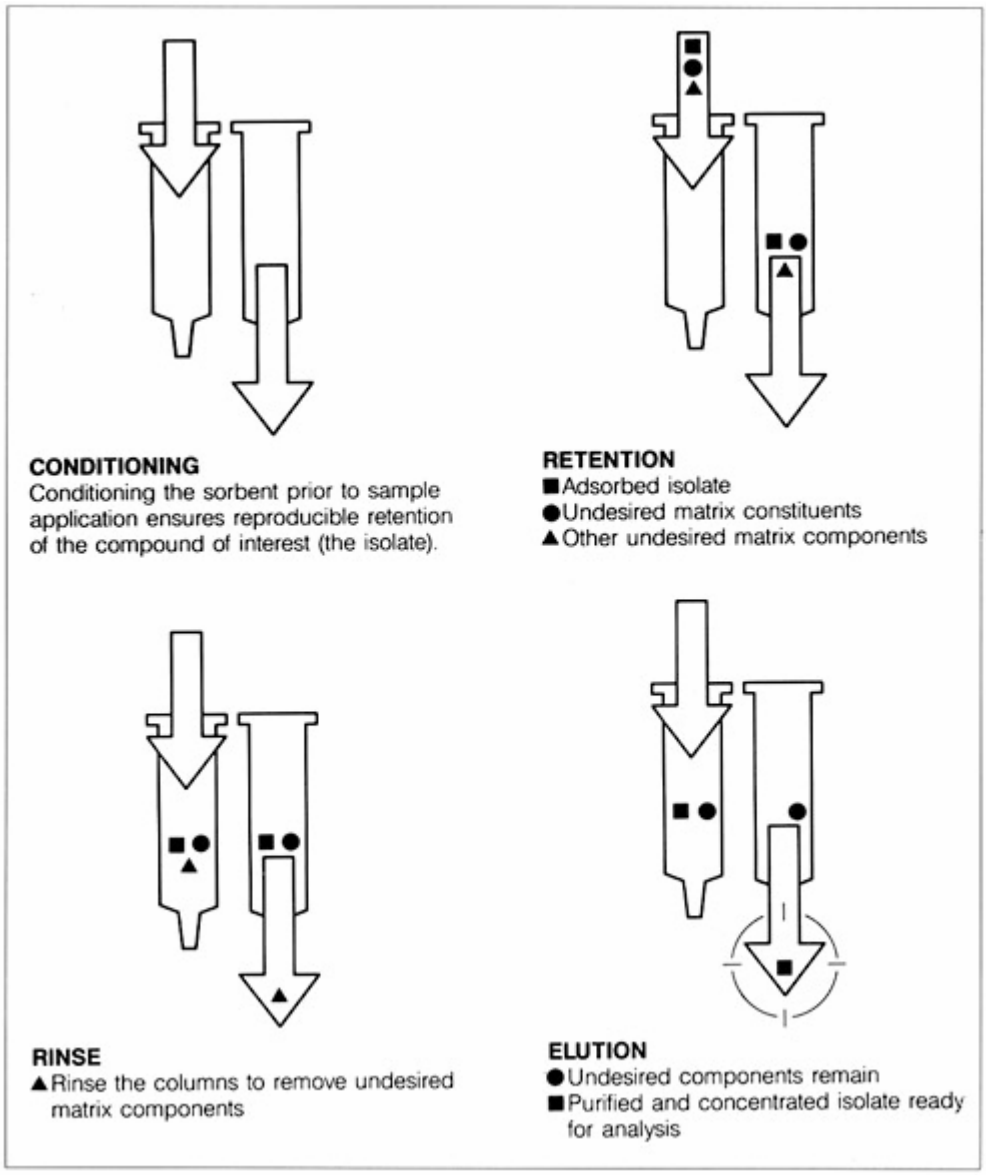


Figure 7.2 – Diagram indicating the key stages the SPE cleanup procedure

SPE procedures have been used extensively for the purification and concentration of sample extracts for a wide variety of analytical applications. Several commercially available materials have been adopted as standard reference procedures in the food, drug and environmental industries. SPE is particularly attractive for use in a field test kit format as it is simple to use and does not require sophisticated laboratory facilities. Many applications have

been reported that use SPE in field test methods, particularly in environmental studies. Several polymer materials have been specifically developed for the extraction of chlorophenols from water and other environmental samples such the Bond Elut PPL ™ (Varian, Palo Alto, California, USA) recommended in the EPA reference method 528 and the Oasis™ sample extraction products (Waters Corp, Milford, USA). These have not previously been employed for the analysis of chlorophenols in wine although the technique has been used for the characterization of other phenolic components in wine (Martinez-Ortega *et al.,* 2004; Matejicek *et al.,* 2003*;* Lopez *et al.,* 2002).

7.2.1 Investigation into alternative polymer materials

Five commercially available polymer materials from different producers that were recommended for the analysis of chlorophenols were selected for evaluation (listed in Table 7.1).

Table 7.1 – Materials selected for SPE purification experiments.

Material	**Commercial name**	**Supplier**
Octadecyl silane bonded silica (end capped)	Bondelut ™ C18	Varian Associates, Sunnyvale, CA, USA.
Polyamide 6	DPA6S™	Supelco UK, Poole, Dorset, UK.
Poly divinylbenzene/ vinylpyrolidone	Oasis™ HLB	Waters Corporation, Milford, MA, USA.
Hydroxylated polystyrene/divinylbenzene	Env+™	International Sorbent Technology Ltd., Hengoed, Mid Glamorgdan, UK.
Polyacrylate	Amberlite™ XAD-7HP	**Rohm and Haas** Philadelphia, PA, USA.

The performance of each material was characterised using two procedures.

1) Sorption breakthrough – In these experiments aliquots of artificially contaminated wine were applied to the resin to assess the breakthrough volume.
2) Elution recovery - Each of the materials used in stage 1) above were sequentially eluted with an eluotropic series of solvents shown in Table 7.2.

Eluted materials were tested using the GC-ECD procedure described in Chapter 3

Materials and methods

Wine sample

A sample of Rioja wine (Campo Viejo Cranzia 1997) from the winery Campo Viejo in Northern Spain was selected. This wine was produced from Temperanillo, Garnacha and Mazuelo grapes, matured in oak barrels for 12 months and was considered to be a good representation of the wines from the Rioja region of Spain .

Preparation of spiked wine sample.

A working standard solution containing three chloroanisoles and three chlorophenols was prepared in ethanol (Merck, pesticide grade). A 2ml volume of the working standard were diluted to 100ml using the selected

wine sample. The CA/CP concentrations show in Table 19 were employed in the experiment

Table 7.2 – Concentration of contaminants used to fortify wine samples

Reference material	**Concentration added to wine (ng/ml)**
pentachlorophenol	91.0
2,3,4,6-tetrachlorophenol	101.5
2,4,6-trichlorophenol	69.5
pentachloroanisole	80.0
2,3,5,6-tetrachloroanisole	76.0
2,4,6-trichloroanisole	72.5

Sorption breakthrough experiments

Nine 10ml aliquots of artificially contaminated wine were applied to 500 mg of each material contained within a 6ml syringe cartridge. The flow rate of each column was adjusted to approximately approx. 6 ml /min (1 drip per second). The eluant from each application was collected and tested for chlorophenol and chloroanisole content.

Elution recovery experiments

Each of the materials used were sequentially eluted with an eluotropic series of solvents. 10ml of the solvent mixtures shown in Table 7.3 were employed.

Table 7.3 – Solvent mixtures employed for elution of SPE cartridges

20 %(v/v) ethanol in water
40 %(v/v) ethanol in water
60 %(v/v) ethanol in water
1 %(v/v) glacial acetic acid in acetone

Ethanol was selected in this experiment due to its relatively low toxicity and hence suitability for at-line use in food production environments. The final eluting solvent (1%(v/v) glacial acetic acid in acetone) was recommended by several SPE suppliers for the analysis of substituted phenols. The flow rate through the mini-columns was adjusted by applying a low vacuum (5mm Hg) to draw the solvent through the columns. The flow rate of each column was adjusted individually to approximately 6ml/min using a PTFE tap fitted between the column and the vacuum manifold.

Each fraction was retained for analysis by gas chromatography with electron capture detection (GC-ECD). Chlorophenols were measured as their acetyl derivatives, chloroanisoles were underivatised. The analytical procedure employed to measure the chlorophenol and chloroanisole content of sample extracts is described in Chapter 3.

The optical density of each eluant was also recorded at 520nm using a 2mm cell as a measure of the red anthocyanin pigments in the wine fractions to indicate the extent to which the wine matrix components were selectively removed by each resin.

Results

Sorption breakthrough

Breakthrough of the six added contaminants is expressed as a percentage of the mass of each contaminant applied to the column in each fraction collected. is The elution of red (anthocyanin) pigments was employed as an indicator of the ability of each column material to retain complex polyphenolic moieties, which may present interference problems in the final at-line test procedure. Visible spectroscopy absorbance measurements may be used to estimation of various pigmented phenolic components in wine (Zoecklein *et al* 1995). Figure 7.3 shows the uv/visible spectrum of a standard Rioja wine measured using a Perkin Elmer (MA,USA) spectrometer fitted with 2mm path length cuvette. The optical density at 520 nm of each fraction eluted from SPE columns is shown in Table 7.4.

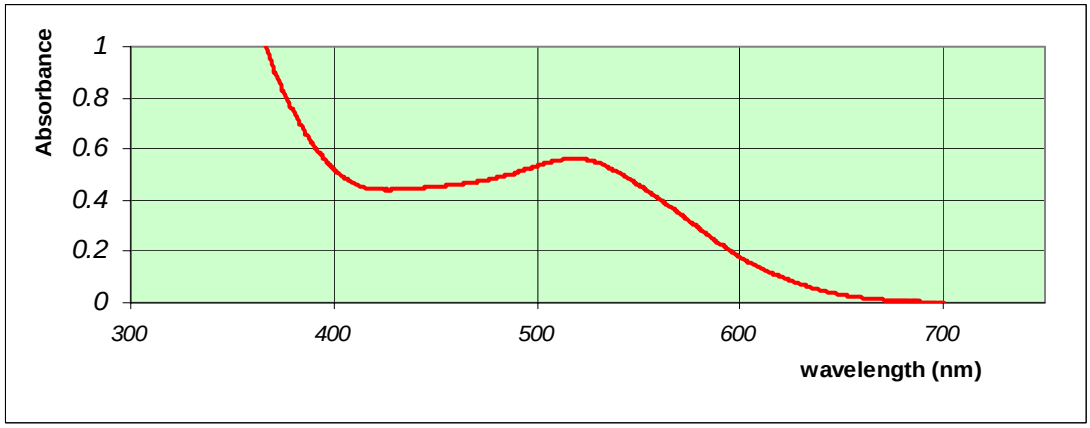
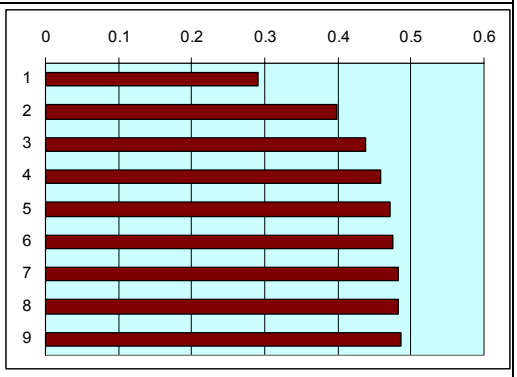
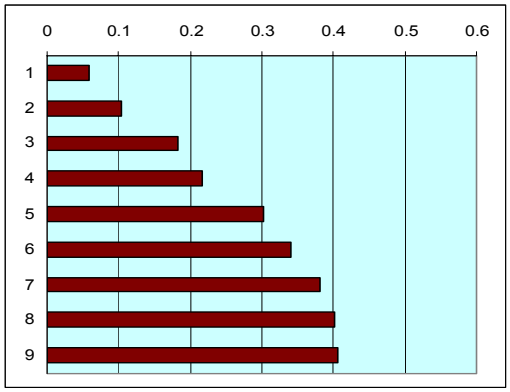
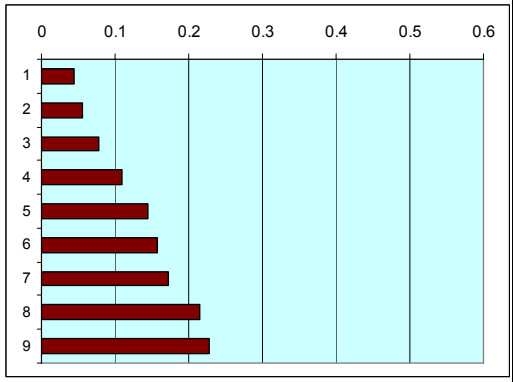
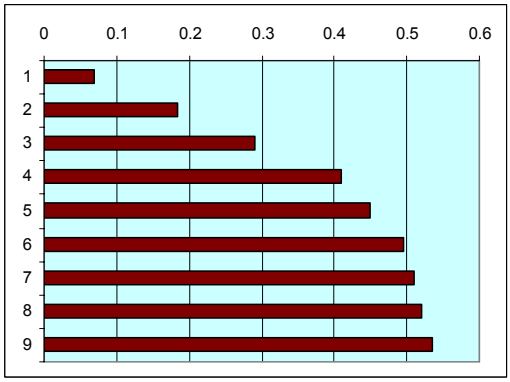
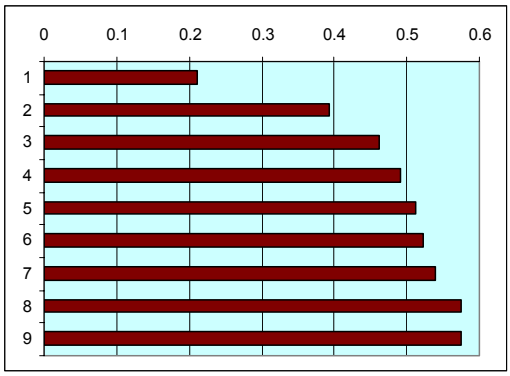


Figure 7.3 – UV/visible scan of Rioja wine sample

Table 7.4 - Breakthrough levels expressed as a percentage of the mass of material applied to the column and graphical representation of the extract absorbance.

Material	Vol. (ml)	Breakthrough (%)	UV/vis absorbance at 520nm
Octadecyl silane bonded silica (end capped)	10	<1	
	20	<1	
	30	<1	
	40	<1	
	50	<1	
	60	<1	
	70	<1	
	80	<1	
	90	<1	

Material	Vol. (ml)	Breakthrough (%)	Visible abs. at 520nm
Polyamide 6	10	<1	
	20	<1	
	30	<1	
	40	<1	
	50	4 (TCP)	
	60	9 (TCP)	
	70	12 (TCP)	
	80	14 (TCP)	
	90	15 (TCP)	
Polydivinylbenzene/vinylpyrolidone	10	<1	
	20	<1	
	30	<1	
	40	<1	
	50	<1	
	60	<1	
	70	<1	
	80	<1	
	90	<1	
Hydroxylated polystyrene	10	<1	
	20	<1	
	30	<1	
	40	<1	
	50	<1	
	60	<1	
	70	<1	
	80	<1	
	90	<1	

Material	Vol. (ml)	Breakthrough (%)	Visible abs. at 520nm
Polyacrylate	10	<1	
	20	<1	
	30	<1	
	40	<1	
	50	<1	
	60	<1	
	70	<1	
	80	<1	
	90	<1	

Elution characteristics

The elution characteristics of the selected resins are shown in Figures 7.4 – 7.8. Extraction efficiencies were based on the mass of each component recovered expressed as a percentage of the mass of the component applied to the column.

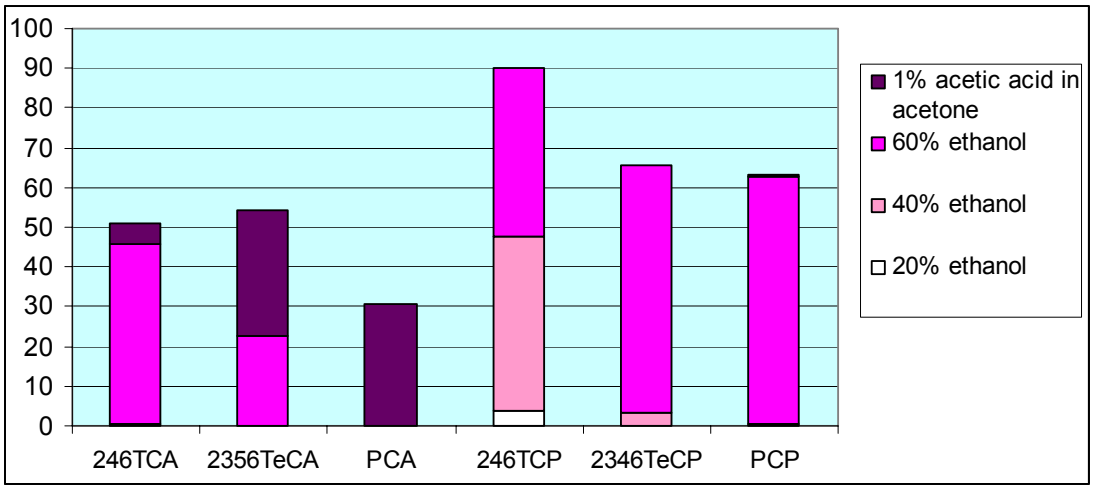


Figure 7.4 – Elution pattern for Bondelut C18.

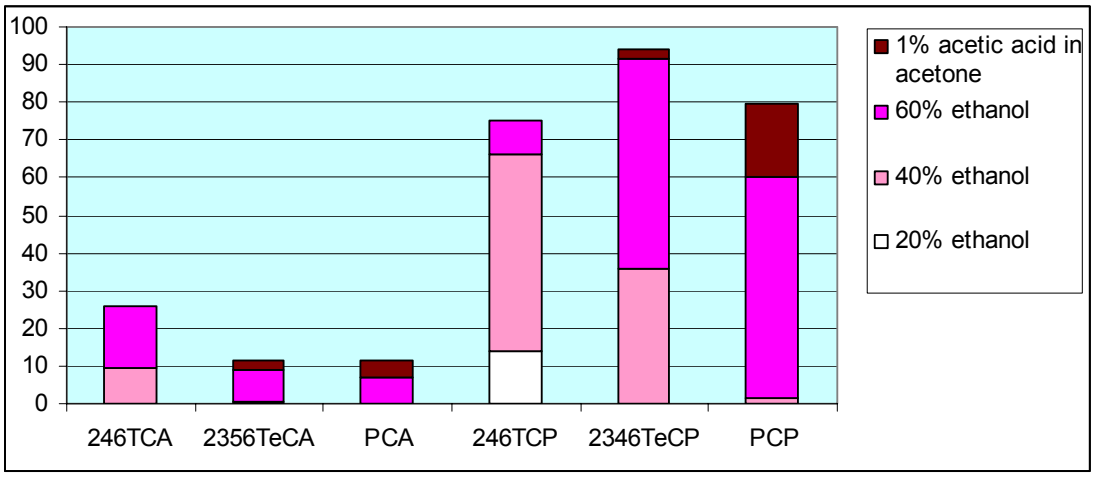


Figure 7.5 – Elution pattern for Supelco DPA 6S, polyamide 6 resin.

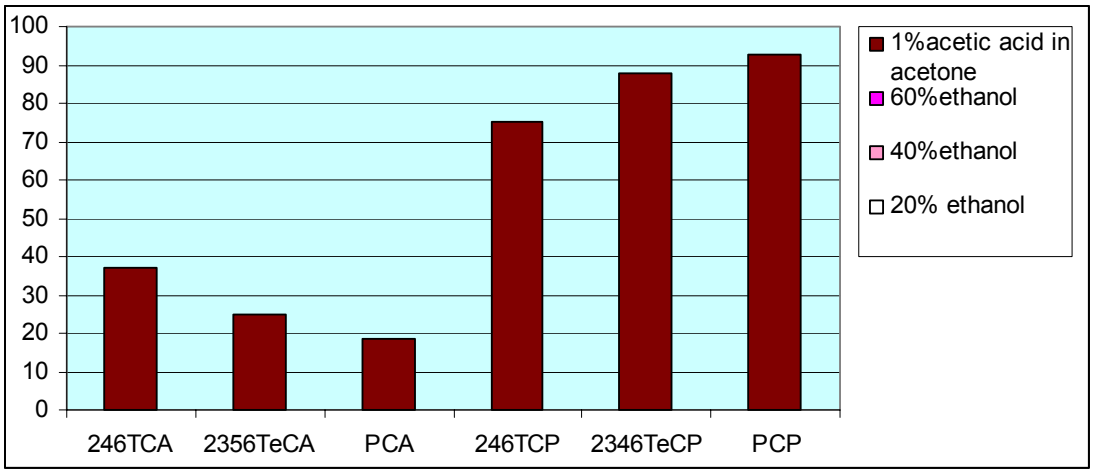


Figure 7.6 – Elution of Oasis HLB, polydivinylbenzene/vinylpyrolidone resin

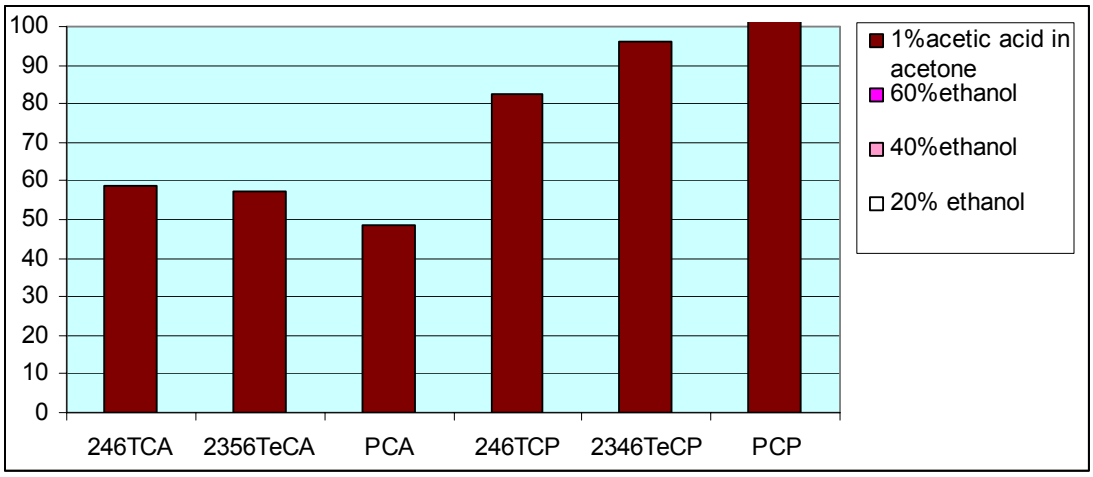


Figure 7.7 – Elution of IST, Env+ hydroxylated polystyrene/divinylbenzene

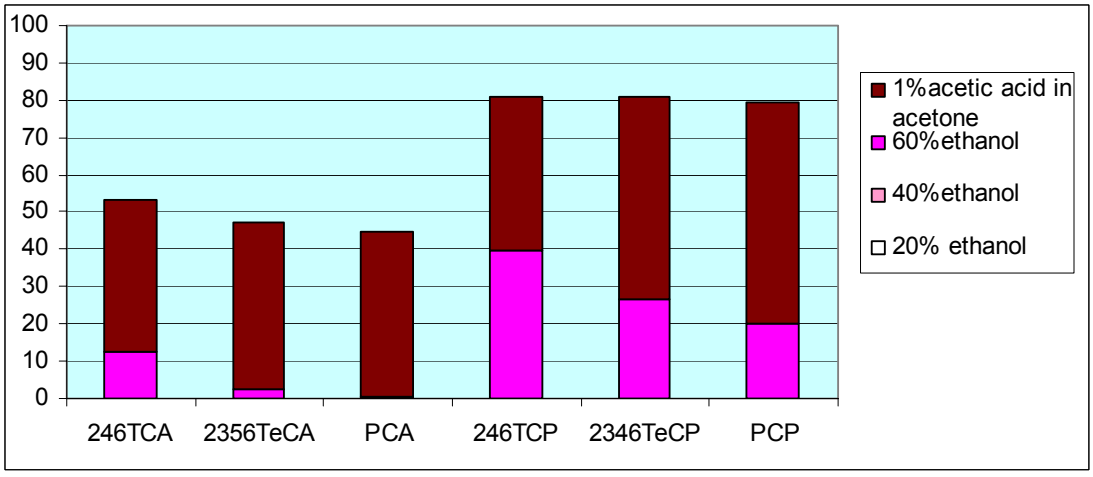


Figure 7.8 – Elution of Amberlite XAD-7HP, Polyacrylate resin

Discussion

Sorption breakthrough

All of the materials selected demonstrated good retention of the selected chlorophenols and chloroanisoles. Breakthrough was observed during the elution of 90ml wine the polyamide resin. However, the extent of breakthrough was less than 10 %of the total amount loaded onto the column.

Elution patterns

Octadecyl silane bonded silica (C18)

This silica based material marketed by Varian instruments as Bondelut ^{TM}C18 has been used most extensively for the extraction and purification of a wide range of organic contaminants in the water supply and food industries . The principle separation method employed by this material is liquid/liquid partition into the surface bound C18 layer. The elution performance of the material indicates that the polar poyphenolic components associated with the red colour of wine are largely unretained by this material. This suggests that C18 materials may facilitate the selective separation of CP/CAs from other contaminants.

The distribution pattern of eluted fractions shown in Figure 7.3, indicates that the majority of the CP/CAs are eluted using a solvent of equivalent or greater eluting power than 60% (v/v) ethanol.

The sorbent material appears to function moderately well for the three phenolic contaminants but recoveries for the anisoles particularly 2,3,5,6-TeCA and PCA were less that 60%.

Polyamide 6

This polymeric material is recommended for the extraction of substituted phenols by several major HPLC suppliers e.g. Supelco and Macherey-Nagel. Polyamides are also recommended for use in the gas chromatography technique solid phase microextraction (SPME). The surface sorption is largely due to hydrogen bonding to pendant amine groups which makes it particularly suitable for the extraction of polar phenolic materials.

Breakthrough experiments for polyamide 6 show good retention of all materials except 246TCP, which appears in the eluent from fractions 5-9.

Figure 7.4 shows the distribution pattern of eluted fractions for Polyamide 6 (Supelco DPA6S).

This indicates that CP can be measured effectively with this material although it appears to be unsuitable for chloroanisoles due to the low recoveries observed and the high levels of coloured materials that are co-eluted with these fractions.

Polydivinylbenzene/vinylpyrolidone

This relatively new material called Oasis HLB™ from the HPLC supplier, Waters Chromatography,Mass., USA is a macroporous polymer [polydivinyl benzene-co-N-vinylpyrolidione (PDVB)] material that is reprted to contain both hydrophobic and lipophylic retention characteristics. It is recommended for the analysis of chlorophenols in water and environmental samples. PDVB materials are also regularly used in brewing and viticulture as fining agents to remove undesirable proteins and polyphenolic materials.

The sorption of spiked red wine onto this material shows that the polyphenolic materials in wine are retained to a high degree from each fraction of the 90ml sample used. This indicated a high efficiency of the material to retain phenols and polyphenols but this could also cause interference problems with some samples. The distribution pattern shown in Figure 7.5 indicates that the chlorophenols and chloroanisoles investigated were all strongly retained and none of the added components were eluted with ethanol:water solvents. CAs and CPs were both eluted at fraction four using acidified acetone and the absorbance of this fraction at 520 nm was low (0.2), indicating low levels of polyphenolic coextractives. CPs are eluted with good efficiency using this solvent although CA recovery levels are all below 60 %.

Oasis HLB appears to offer good potential for the purification of red wine samples. Further work is required to investigate and improve the elution efficiency of CA from this material by employing a solvent of greater eluting

strength (e.g. acidified ethyl acetate) and to investigate its performance with other sample types.

Hydroxylated polystyrene/divinylbenzene

Several HPLC equipment suppliers such as International Sorbent Technology, Macherey-Nagel market examples of this polymer for use in SPE cleanup methods for substituted phenols.

The sorption characteristics indicate lower retention of red, polyphenolic materials than the Oasis HLB column. In this case all of the added chlorophenols and chloroanisoles appear to be efficiently retained and none of the contaminants were eluted in fractions 1-3 as shown in Figure 7.6 the lowest recovery is 48% for PCA. The absorbance of this solution at 520 nm is also very low (0.16) indicating that the fraction should be relatively free of other phenolic contaminants that could interfere with the final determination. Further work to optimise the final elution solvent mixture is necessary to improve the PCA recovery.

Polyacylate

Polyacrylate polymers have frequently been employed to remove phenolic materials in the water industry and for the analysis of substituted phenols in atmospheric samples. Amberlite XAD-7 HP resin **was obtained from**

Supelco in a 20-60 mesh size. This was reduced to 50 to 100 microns by wet grinding and sieving.

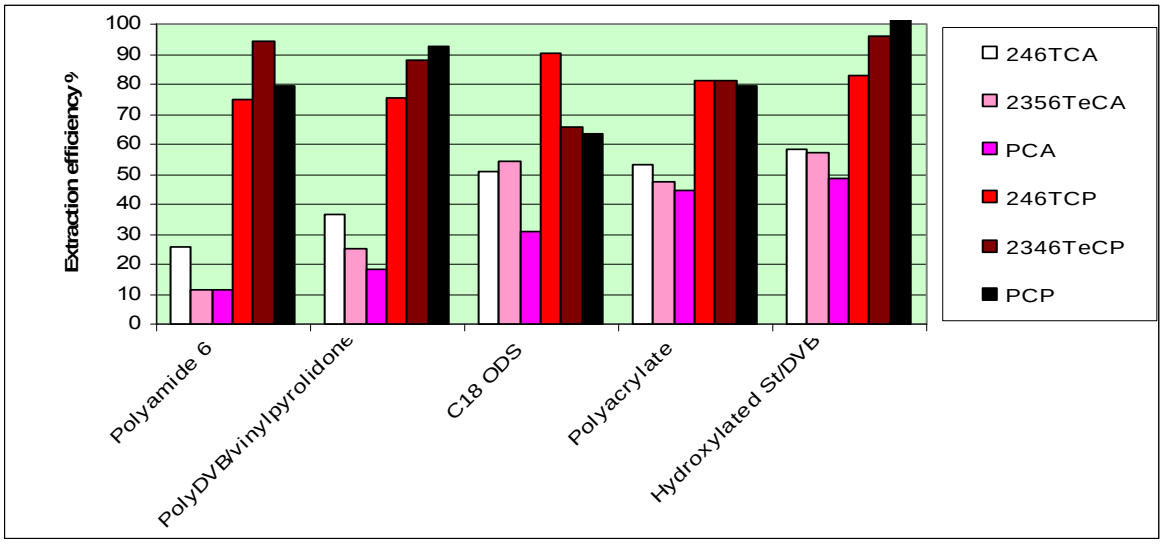
The sorption behaviour of this polymer indicates low retention of co-extractives and the elution characteristics were generally better than Bondelut C18ODS and the polyamide 6 resin. All of the added chloroanisoles were eluted with a recovery better than 40% as shown in Figure 7.7. However, elution fraction 3 was very dark red in colour indicating that there may be high levels of co-extracted contaminants in this fraction. Further work to compare alternative commercially available acrylate/methacrylate polymers may indicate suitable alternative materials.

Sorbent comparison

The five materials tested all demonstrated good retention of the test chlorophenol and chloroanisole mixture. Some breakthrough was observed with two of the polymers investigated (poly(4-vinylpyridine) and polyamide 6) but this occurred only after the application of 40ml of contaminated wine to the columns.

All of the materials tested showed good overall recoveries for the added phenols, although anisole recoveries were generally less than 60%.

Three of the polymers tested polyacrylate (Amberlite XAD-7HP), polyDVB/vinylpyrolidone (Waters Oasis HLB) and hydroxylated polystyrene/DVB (IST Env+) appeared to be significantly better than the **remainder in their ability to separate a clean phenol/anisole fraction as**



shown in Figure 7.9. It was found that the IST Env+ and the Waters Oasis HLB materials could be eluted with 60% v/v ethanol:water with no elution of the test contaminants.

Figure 7.9 – Elution efficiency of the six resins selected in Experiment 7.2

Only one material (hydroxylated polystyrene/divinylbenzene - IST Env+) gave satisfactory recovery performance for the selected anisoles. Similar performance characteristics were observed with the Waters Oasis HLB column but in this case anisole contaminants were retained more strongly by the column resulting in lower recoveries.

7.2.2 Investigation into alternative elution solvents

Summary of experimental procedure

The commercially available polymer materials described in 7.1.1 which exhibited the best extraction efficiency (hydroxylated polystyrene/divinylbenzene) was selected for further optimisation.

In the previous experiments SPE columns were eluted with an eluotropic series of solvents based on an ethanol organic modifier. In this experiment only one material was selected and a range of elution solvents were employed to further optimize the performance of the material. The solvents selected were those typically used in HPLC separation methods.

Materials and methods

Wine sample

A sample of Rioja wine (Campo Viejo Cranzia 1997) from the winery Campo Viejo in Northern Spain was selected as a representative wine sample.

Preparation of spiked wine sample.

A working standard solution containing three selected chloroanisoles and three chlorophenols was prepared in ethanol. A 2ml volume of the working standard were diluted to 100 ml using the selected wine sample.

Concentrations of CA/CPs (Table 7.5) that may be experienced in contaminated wine samples were employed to fortify wine samples.

Table 7.5 – Concentration of CA/CPs used to fortify wines in experiment to investigate alternative elution solvents.

Reference material	**Concentration in wine (ng/ml)**
pentachlorophenol	3.6
2,3,4,6-tetrachlorophenol	6.5
2,4,6-trichlorophenol	5.5
pentachloroanisole	4.7
2,3,5,6-tetrachloroanisole	4.5
2,4,6-trichloroanisole	5.4

Sorbtion breakthrough experiments

Nine 10ml aliquots of artificially contaminated wine were applied to 200mg of each sorbent contained within a 3ml syringe cartridge. The eluent from each application was collected for chloroanisole and chlorophenol analysis by gas chromatography as described in section 3.4 .

Elution recovery experiments

The performance of each material was characterised using mixtures of the following solvents ethanol, methanol, acetonitrile and acetone at 20, 40, 60 and 80 %(v/v) followed by a final strongly eluting solvent composed of 5%(v/v) isopropanol in acetone with 1% v/v acetic acid:

Each fraction was retained for analysis by GC-ECD as described in Chapter 3. The UV absorption at 520nm of each eluant was also recorded as a measure of the red colour of the wine fractions.

Results

The percentage of each component eluting in each of the four eluted fractions (f1-f4) for each solvent system is shown in Table 7.6

Table 7.6 – Results for experiment to investigate the use of alternative elution solvents.

Ethanol							
	246TCA	2356TeCA	PCA	246TCP	2346TeCP	PCP	UVabs 520nm
20% ethanol	<1	<1	<1	0	<1	<1	0.12
40%ethanol	<1	<1	<1	2	1	0<1	0.30
60%ethanol	<1	<1	<1	8	4	3	0.14
80%ethanol	16	8	<1	57	56	39	0.08
1%acetic acid in acetone+5% propan-2-ol	58	72	73	7	28	55	0.05
Total	75	81	73	74	89	97	
Methanol							
	246TCA	2356TeCA	PCA	246TCP	2346TeCP	PCP	UVabs 520nm
20% methanol	<1	<1	<1	<1	<1	<1	0.08
40%methanol	<1	<1	<1	<1	<1	<1	0.17
60%methanol	<1	<1	<1	<1	<1	<1	0.17
80%methanol	<1	<1	<1	35	3	<1	0.11
1%acetic acid in acetone+5% propan-2-ol	95	91	88	73	103	106	0.07
Total	95	91	88	107	105	106	

Acetonitrile							
	246TCA	2356TeCA	PCA	246TCP	2346TeCP	PCP	UVabs 520nm
20% ACN	<1	<1	<1	<1	<1	<1	0.47
40% ACN	<1	<1	<1	<1	<1	<1	0.23
60% ACN	15	4	<1	92	71	28	0.06
80% ACN	59	57	34	6	32	54	0.05
1%acetic acid in acetone+5% propan-2ol	8	20	41	2	4	16	0.05
Total	81	81	76	100	107	97	
Acetone							
	246TCA	2356TeCA	PCA	246TCP	2346TeCP	PCP	UVabs 520nm
20% Acetone	<1	<1	<1	<1	<1	<1	0.21
40% Acetone	<1	<1	<1	<1	<!	<1	0.22
60% Acetone	25	<1	<1	105	95	67	0.10
80% Acetone	38	59	54	<1	11	20	0.06
1%acetic acid in acetone+5% propan-2-ol	<1	<1	<1	<1	<1	<1	0.05
Total	83	105	59	106	54	87	

Discussion

A comparison of the performance characteristics of the solvent systems is shown in Figure 7.10.

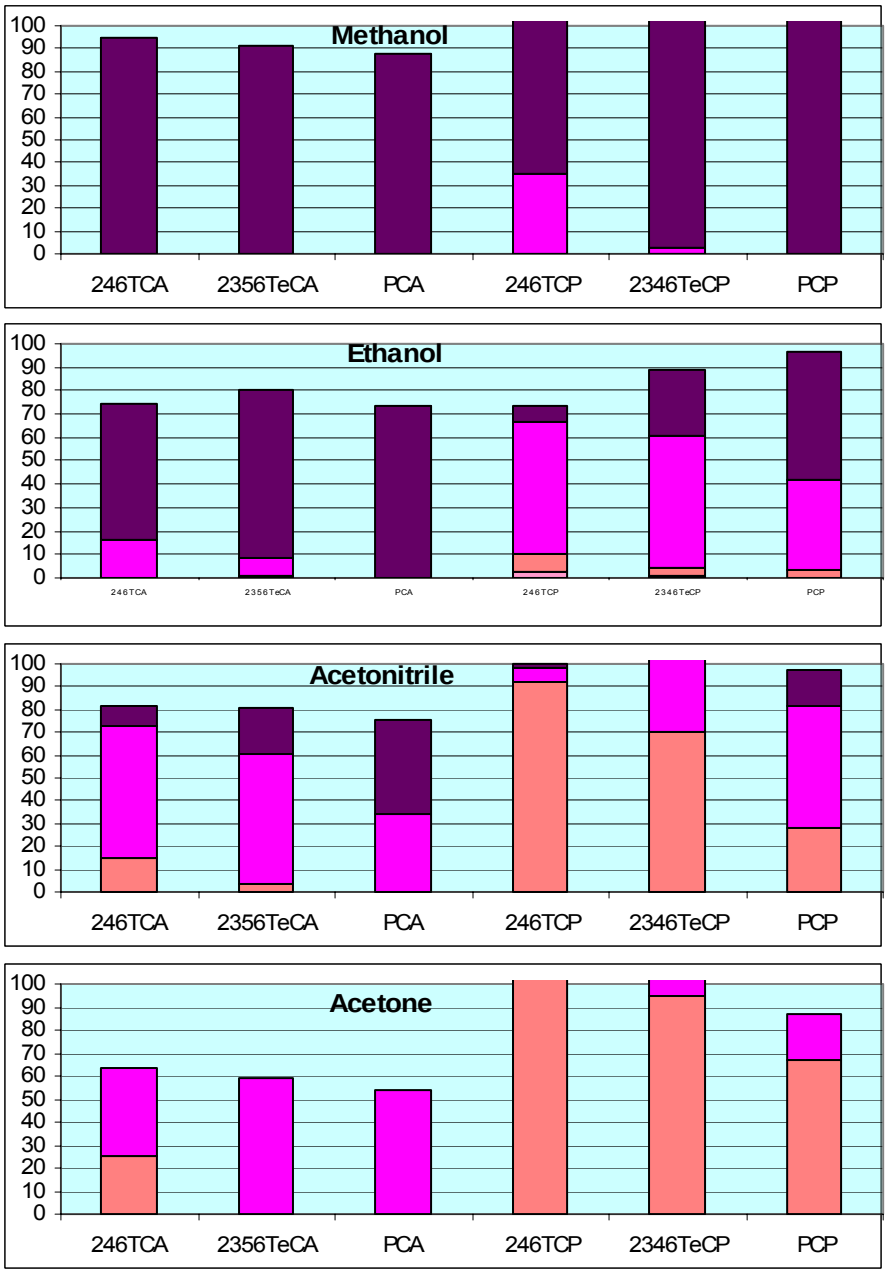


Fig. 7.10 – Comparison of elution patterns for alternative solvent systems tested.

Results indicate that a solvent system based on methanol is more selective and more efficient than the three alternative solvent systems based on ethanol, acetonitrile and acetone. Interestingly the methanol system appears to retain all of the tested analytes until concentrations greater than 80% (v/v) are employed. In view of this a solvent system based on a methanol eluotropic series should enable samples of wood, barrel packaging materials, cork etc. to be extracted using 40% (v/v) methanol and then concentrated by applying them directly to the concentrator column, washing with 60%(v/v) methanol to remove co-extractives and then eluting with the final solvent. Preliminary experiments indicate that this approach may provide a common route for the analysis of a wide range of materials.

7.2.3 Investigation into alternative polystyrene/divinyl benzene resins

In section 7.2.1 five different types of commercially available solid phase extraction materials were assessed for their ability to selectively concentrate chlorophenols. One material, a copolymer of polystyrene/divinyl benzene exhibited superior characteristics for the retention of chlorophenols and chloroanisoles. However, the extraction efficiency for the anisole group is considered to require further characterization before the concentrator column could be considered sufficiently robust for inclusion in the field analytical procedure. Styrene divinyl benzene polymer products are reported by the manufacturer to be most suitable in applications where the analytes are very water soluble. This is confirmed in the previous experiments which indicate

that the more water soluble chlorophenols are more efficiently retained that the chloroanisoles. However the retention characteristic of polymers produced using different production methods is considered highly likely to influence the sorption mechanism. In view of this a new experiment was designed too explore the performance characteristics of six alternative commercially available polystyrene/ divinylbenzene resins using the previously optimised solvent system.

Materials and methods

Eight commercially available polystyrene/divinyl benzene (PS/DVB) polymers were investigated as shown in Table 7.7.

Table 7.7 – Styrene/Divinylbenzene polymer materials evaluated

Commercial name	**Form**	**Supplier**
Amberchrom™ CG161	Macroreticular,50-100µm	Supelco UK, Poole, Dorset,UK
PLRP-S 100A°™	Macroporous PS/DVB, 50-70µm	Polymer Laboratories Church Stretton, Salop, UK
Chromabond HR-P™	PS/DVB, 100 Å, 100µm	Macherey-Nagel Ltd. Middleton Cheney, Oxfordshire, UK.
Envi-Chrom P™	PS/DVB 20-300 Å ,80-160µm	Supelco UK, Poole, Dorset,UK
Chromabond 'Easy'™	Polar PS/DVB, 100 Å , 100µm	Macherey-Nagel Ltd. Middleton Cheney, Oxfordshire, UK.
Lichrolut EN™	PS/DVB,40-120, 100µm	Merck Eurolab, Poole, Dorset, UK.
Isolut 101™	PS/DVB, 60µm	International Sorbent Technology Ltd., Hengoed, Mid Glamorgan, UK.
Isolut Env+™	Hydroxylated PS/DVB, 90µm	International Sorbent Technology Ltd., Hengoed, Mid Glamorgdan, UK.

Preparation of spiked wine sample.

CA/CP solutions were prepared at a concentrations shown in Table 7.8 in the selected wine sample to approximately represent naturally contaminated wines.

Table 7.8 – Concentration of CA/CPs used to fortify wines in Section 7.2.3.

Reference material	**Concentration in wine ng/ml)**
pentachlorophenol	3.6
2,3,4,6-tetrachlorophenol	6.5
2,4,6-trichlorophenol	5.5
pentachloroanisole	4.7
2,3,5,6-tetrachloroanisole	4.5
2,4,6-trichloroanisole	5.4

Experimental procedures

Sorption breakthrough experiments.

Polymer materials shown in Table 24 (200 mg of each) were slurry packed into 3ml tubes supplied by IST, Hengoed, UK and fitted with a 10µm 4mm frit above and below the packed polymer bed. 90 ml of a fortified red wine sample (approx. 10µg/l) was passed through the column at approximately 10ml/minute. Each wine sample was spiked with six contaminants listed in

Table 7.8. Fractions (10ml) of the eluant were collected and each fraction was tested for the six added contaminants using the previously reported standard gas chromatography procedure described in section 3.4.

Extraction efficiency experiments.

Columns similar to those used in the sorption breakthrough experiments were prepared and the fortified wine (50ml) was applied to each column. Columns were eluted with an elutropic series of solvents consisting of 10ml methanol: water mixtures at 20, 40, 60, 80%(v/v) and a final acetone fraction containing 1%(v/v) glacial acetic acid and 5% (v/v) propan-2-ol. Each fraction was collected and tested for the six added contaminants using the standard gas chromatography method described in section 3.4.

Levels of impurities in each fraction were also estimated by measuring the residual red wine colour using a spectrophotometer at 520 nm.

Results

Sorption breakthrough experiments.

Each column material showed no breakthrough of the six added contaminants in the nine eluted fractions.

Elution extraction efficiency experiments

Results for extraction efficiency are shown in Table 7.9.

Table 7.9 – Elution profile of materials from styrene divinyl benzene columns.

Supelco, Amberchrom CG161							
	246TCA	246TCP	2356TeCA	2346TeCP	PCA	PCP	Abs.52
20%MeOH	<1	<1	<1	<1	<1	<1	0.07
40%MeOH	<1	<1	<1	<1	<1	<1	0.39
60%MeOH	<1	<1	<1	<1	<1	<1	0.88
80%MeOH	<1	65	<1	11	<1	<1	0.24
1%HOAc+5%propan-2-ol in acetone	65	44	61	89	58	83	0.07
Total	65	109	61	101	58	83	
Polymer Labs, PLRP-S							
	246TCA	246TCP	2356TeC	2346TeC	PCA	PCP	Abs.52
20%MeOH	<1	<1	<1	<1	<1	<1	0.08
40%MeOH	<1	<1	<1	<1	<1	<1	0.43
60%MeOH	<1		<1	<1	<1	<1	0.55
80%MeOH	<1	89	<1	53	<1	43	0.10
1%HOAc+5% prpopan-2-ol in acetone	72	14	69	47	62	54	0.07
Total	72	102	69	100	62	97	

Table 7.9 continued

Macherey-Nagel, Chromabond HR-P							
	246TCA	246TCP	2356TeC	2346TeC	PCA	PCP	Abs.52
20%MeOH	<1	<1	<1	<1	<1	<1	0.04
40%MeOH	<1	<1	<	<1	<1	<1	0.05
60%MeOH	<1	<1	<1	<1	<1	<1	0.05
80%MeOH	<1	50	<1	8	<1	<1	0.05
1%HOAc+5% prpan-2-ol in acetone	72	54	74	88	60	91	1.05
Total	72	104	74	97	60	91	
Supelco, Envi-Chrom P							
	246TCA	246TCP	2356TeC	2346TeC	PCA	PCP	Abs.52
20%MeOH	<1	<1	<1	<1	<1	<1	0.06
40%MeOH	<1	<1	<1	<1	<1	<1	0.30
60%MeOH	<1	<1	<1	<!	<1	<1	0.63
80%MeOH	<1	29	<1	<1	<1	<1	0.25
1%HOAc+5% propan-2-ol in acetone	77	77	76	100	69	92	0.08
Total	77	106	76	100	69	92	
Macherey-Nagel, Chromabond 'Easy'							
	246TCA	246TCP	2356TeCA	2346TeCP	PCA	PCP	Abs.52
20%MeOH	<1	<1	<1	<1	<1	<1	0.05
40%MeOH	<1	<1	<1	<1	<1	<1	0.05
60%MeOH	<1	<1	<1	<1	<1	<1	0.07
80%MeOH	<1	58	<1	4	<1	<1	0.07
1%HOAc+5%propan-2-ol in acetone	83	44	82	92	72	85	0.05
Total	83	103	82	96	72	85	

Table 7.9 continued

Merk, Lichrolut EN							
	246TCA	246TCP	2356TeCA	2346TeCP	PCA	PCP	Abs.52
20%MeOH	<1	<1	<1	<1	<1	<1	0.047
40%MeOH	<1	<1	<1	<1	<1	<1	0.048
60%MeOH	<1	<1	<1	<1	<1	<1	0.051
80%MeOH	<1	58	<1	4	<1	<1	0.058
1%HOAc+5% propan-2-ol in acetone	83	44	82	92	72	85	0.054
Total	83	103	82	96	72	85	
International Sorbent Technology, Isolut 101							
	246TCA	246TCP	2356TeCA	2346TeCP	PCA	PCP	Abs.52
20%MeOH	<1	<1	<1	<1	<1	<1	0.09
40%MeOH	<1	<1	<1	<1	<1	<1	0.45
60%MeOH	<1	<1	<1	<1	<1	<1	0.50
80%MeOH	<1	105	8	61	<1	40	0.10
1%HOAc+5% propan-2-ol in acetone	84	<!	83	46	79	67	0.07
Total	84	105	91	107	79	107	
International Sorbent Technology, Isolut Env+							
	246TCA	246TCP	2356TeCA	2346TeCP	PCA	PCP	Abs.52
20%MeOH	<1	<1	<1	<1	<1	<1	0.08
40%MeOH	<1	<1	<1	<1	<1	<1	0.17
60%MeOH	<1	<1	<1	<1	<1	<1	0.17
80%MeOH	<1	<1	<1	35	3	<1	0.11
1%HOAc+5% propan-2-ol in acetone	95	91	88	73	103	106	0.07
Total	95	91	88	107	105	106	

Discussion

In this experiment eight commercially available styrene/divinyl-benzene polymers were compared. All of the material showed very similar elution patterns, in each case fortified analytes were completely retained with no detectable breakthrough and eluted in fractions 4 and 5. Figure 7.11 summarises the elution efficiency of each material.

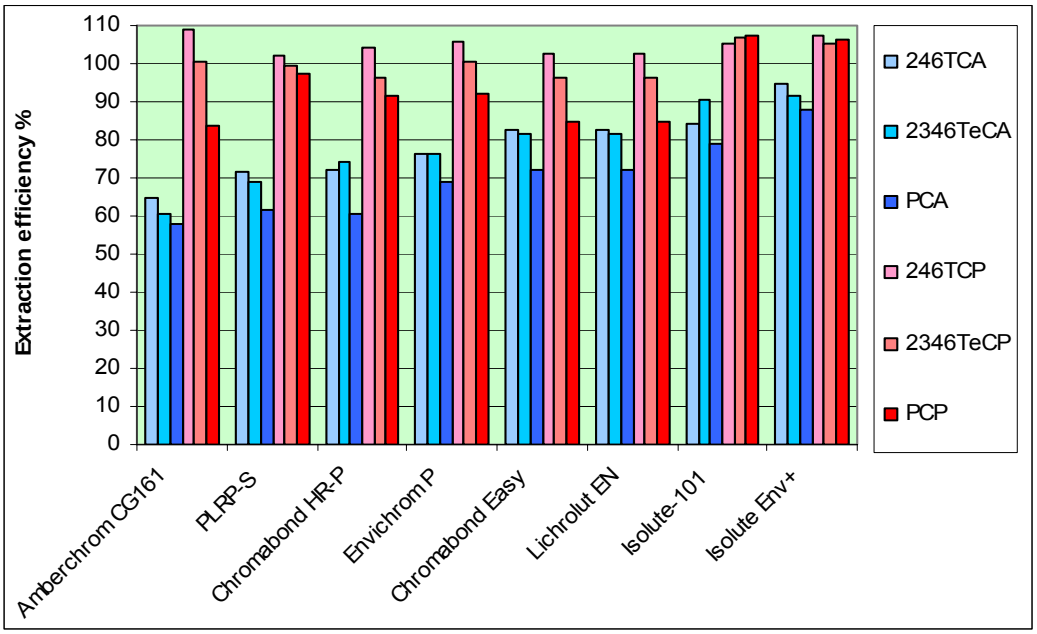


Figure 7.11 – Performance characterisation of eight PS/DVB polymers compared in Experiment 7.2.3.

The ISE, Isolute Env + material appears to provide the most efficient recovery of analytes from fortified wine samples at the 5ppb level. However, four of the materials tested, Chromabond Easy, Lichrolut EN, IST 101 and ISE, Isolute Env + provided acceptable recoveries (>70% for all analytes).

In each case the added contaminants were eluted in fractions four (80 %methanol:water) and five (acetone mixture). Absorbance measurements for these fractions (shown in Figure 7.12) indicated that extracts from Chromabond Easy and Merck, Lichrolut EN contained less of the red wine pigments indicating that these materials may provide a more selective purification system but with lower extraction efficiency. Unfortunately, the manufacturers literature for Merck, Lichrolut EN indicated that a loss of efficiency may be experienced with this material if columns are inadvertently dried. In view of this information this material was considered less suitable for inclusion in a robust at-line test kit procedure.

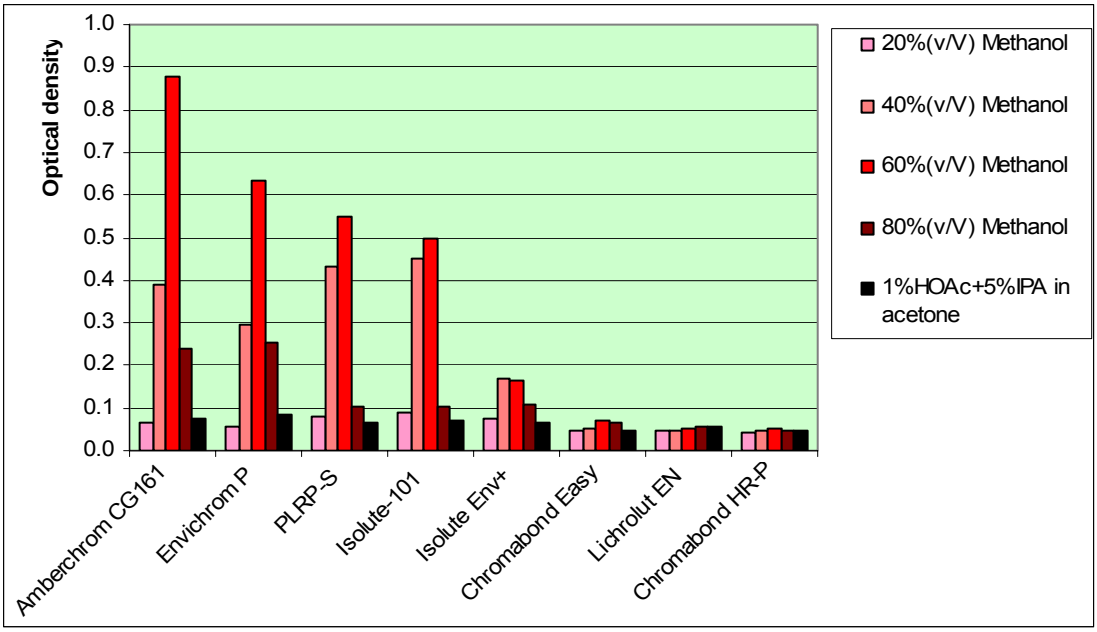


Figure 7.12 – Optical density of fractions eluted from SPE column indicating levels of red pigmentation eluted in each solvent mixture.

Two of the six resins tested demonstrated superior performance characteristics.

1) Isolut Env+ - The material showed the highest extraction efficiencies for the six specified contaminants.

2) Macherey Nagel Chromabond ‘Easy’ - This resin exhibited slightly lower extraction efficiencies than the Isolut Env+ resin but extracts were less pigmented indicating improved specificity with this resin.

7.3 STEAM DISTILLATION

The second candidate purification method to be considered is steam distillation. This procedure is an important commercial process that is use in the purification of a wide variety of semi volatile materials. To be suitable for steam distillation a candidate compound must be stable and relatively insoluble in water. It must also have a vapour pressure in boiling water in excess of 1kPa. Steam distillation is most commonly used for the isolation of essential oils from natural products. These are a complex group of compounds including semi-volatle terpenes, phenolics aldehydes, esters and ketones.

In the food industry many volatile components may also be separated by steam distillation and the determination of phenol index by steam distillation and titration has been used for many years (US, EPA method 9065). In order to separate phenols their solubility in water must be significantly reduced by acidifying the mixture to a pH of less than 2.7 using mineral acids such as 2M sulphuric acid.

The steam volatility of PCP is reported to be 0.167 g/100g water. [Crosby et al 1981] this is the least volatile of all chlorophenol or chloroanisole congeners. All congeners should therefore be quantitatively released from an acidified sample of wine.

To confirm the suitability of steam distillation an experiment was established to measure the efficiency of the distillation procedure for PCP and PCA from a fortified red wine sample.

Method validation experiments

Apparatus

The apparatus was set-up as shown in Figure 7.13

Figure 7.13 - Steam distillation apparatus. Featuring steam generating flask (A) sample reservoir (B) condenser and sample trap (C)

Distillation and fraction collection

In separate experiments an uncontaminated standard red wine (Campo Viejo, Rioja) was fortified to a final concentration of 218.8ng/ml PCP and a separate sample to 45.6 ng/ml PCA.

To each sample (50ml) sulphuric acid 2M (4ml) was added and the sample was steam distilled. Fractions of the distillate (10ml) collected using 0.1M sodium carbonate (2ml) as the trapping solution . Fractions were then tested for PCP or PCA content using the standard GC procedure described in Chapter 3, section 3.4. Results for PCP recovery as a percentage of added analyte are shown graphically in Figure 7.14.

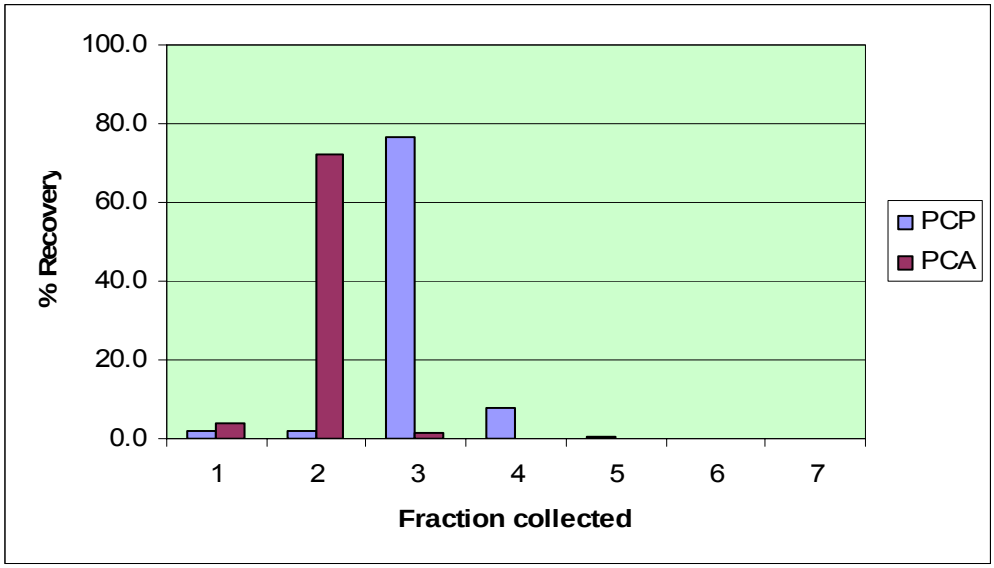


Figure 7.14 – Recovery of PCP in steam distillation fractions.

Results indicate that the collection of 5 x 10ml fractions will enable the chlorophenol and chloroanisole content of samples and extracts to be efficiently separated.

Low level recovery experiment

The standard wine was fortified using the six principal analytes at concentration shown in Table 7.10 Five separate samples (50ml) were steam distilled as previously described.

Table 7.10 – Wine fortification for Section 7.3 experiments

Compound	**Concentration in fortified wine (ng/ml)**
pentachlorophenol	8.8
2,3,4,6-tetrachlorophenol	8.1
2,4,6-trichlorophenol	11.3
pentachloroanisole	18.2
2,3,5,6-tetrachloroanisole	22.6
2,4,6-trichloroanisole	16.8

Results for the low-level recovery experiment are shown in Table 7.11.

These data confirm that recoveries in excess of 70% and an average overall coefficient of variation of 9.1% was obtained for the three principle

chlorophenol and chloroanisole congeners, at a concentration range of 8 to 23 ng/ml.

Table 7.11 – Purification by steam distillation, recovery experiment (n=5)

Compound	Mean (ng/ml)	C.V. (%)	Recovery (%)
pentachlorophenol	6.3	11.7	72.1
2,3,4,6-tetrachlorophenol	6.3	9.0	77.0
2,4,6-trichlorophenol	8.6	13.3	76.1
pentachloroanisole	13.3	9.8	73.0
2,3,5,6-tetrachloroanisole	17.8	7.5	79.0
2,4,6-trichloroanisole	15.3	3.3	91.0

Results indicated that steam distillation may provide a suitable sample purification method that could be used in a simple winery laboratory. However the procedure is valuable only as a purification method. The final distillate volume (50ml) does not amplify the sensitivity of the measurement method by concentrating the sample extract. The distillate also contains all of the ethanol content of the wine sample, which has previously been shown to interfere with the ELISA test method. An additional concentration step is therefore necessary to produce an extract that is suitable for the ELISA test method.

Two alternative approaches were considered;

a) Steam distillation + SPE concentrator column.

b) Steam distillation + liquid/liquid partition using pentane.

7.3.2 Steam distillation + SPE

To investigate the effect of the ethanol content of the wine sample on the efficiency of the SPE procedure a PCP fortified wine sample was prepared containing four levels of ethanol and applied to the standard SPE concentrator column. The standard wine sample was fortified to a concentration of 74.8 ng/ml PCP and ethanol was added at a concentration of 30, 40 ,50 and 60 % by volume. Samples (50ml) were then steam distilled using the procedure described previously in section 7.3; 50 ml of distillate was collected. The PCP content of the sample distillate was concentrated by applying it to an SPE column (100mg Env+) in the manner previously described for wine samples. The column was washed using 50%(v/v) methanol dried and finally eluted using 2ml 1%(v/v) acetic acid in acetone.

The percentage recovery of the added PCP shown in Figure 7.15 indicates that an ethanol content greater than 30%(v/v) lead to significant column losses therefore to maintain the efficiency of the SPE concentrator column the volume of distillate collected must be greater than 50 percent of the original sample volume. In this way a wine sample that contains e.g. 12v/v) ethanol would produce an extract containing a maximum of 24% (v/v) ethanol.

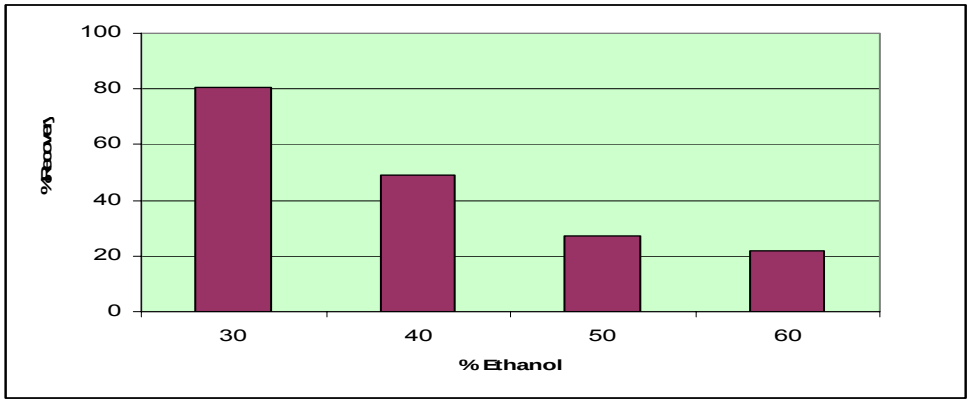


Figure 7.15 – Variation of % recovered PCP with ethanol content of sample distillation extracts

Two wine samples (50ml) were purified by steam distillation and solid phase extraction . Results fro the original wine and recoveries fortified by the addition of 109.4ng/L PCP are shown in Table 7.12.

Table 7.12 - Wine tested by the steam distillation-SPE cleanup (n=3).

Wine	GC (ng/L)	Recovery (109.4ng/L)	Recovery (%)
Rioja - Campo Viejo, Spain.	<20	79.5	72.7
Rioja - Marques de Grignon, Spain.	<20	77.2	70.6

Using this procedure both of the original samples tested were below the detection limit of the method and in both cases spiked samples produced recoveries in excess of 70%.

7.2.3 – Steam distillation + liquid/liquid partition

The potential of steam distillation followed by liquid/liquid partition was investigated as an alternative procedure to concentrate sample extracts for analysis.

Methods and materials

A fortified wine sample was prepared by dilution of a stock standard solution containing PCP,TeCP and TCP (10ml) and PCA,TeCA and TCA (1ml) to 500ml with a naturally contaminated wine sample (Gallo – Ruby Cabernet 2000).

Procedure

Three samples of a naturally contaminated wine and three artificially fortified samples (50ml) were steam distilled using the standard procedure. The distillate (50ml) was collected in a 100ml volumetric flask containing 10 ml 0.1M sodium carbonate solution.

The distillates were acidified to pH2 using 2M sulphuric acid and pentane (2ml) was added. The mixture was shaken using a wrist action shaker for 10 minutes to extract the organic content and allowed to stand for 30minutes for phase separation. The upper pentane layer (1ml) was pipetted into a GC sample vial and 0.5ml of internal standard solution was added. A series of standards was prepared in a similar manner and both standards and sample solutions were acetylated as described in section 3.4; pyridine (100μl) and acetic anhydride (100μl) and warming at 60^{o}C for ten minutes using a water bath.

7.3 Results

Results shown in Table 7.13 indicate that recoveries greater than 70% were obtained for the three chlorophenols tested.

Table 7.13 – Recovery data distillation-liquid/liquid partition experiment (n=3)

Compound	Sample Mean (ng/ml)	C.V. (%)	Wine + Spike Mean (ng/ml)	C.V. (%)	Recovery (%)
pentachlorophenol	0.134	30.4	0.240	8.9	72.4
2,3,4,6-tetrachlorophenol	0.217	3.7	0.450	2.6	86.8
2,4,6-trichlorophenol	0.146	5.7	0.301	0.6	70.3
pentachloroanisole	0.107	6.3	0.125	8.7	92.0
2,3,5,6-tetrachloroanisole	0.100	4.8	0.110	4.6	59.2
2,4,6-trichloroanisole	0.074	5.9	0.086	3.3	57.3

Recoveries for the three chloroanisoles was less efficient, particularly for TCA (57%). In view of the lower fortification level of chloroanisoles and coefficient of variation (less than 9%) results are considered to be suitable for the estimation of chloroanisoles and chlorophenols in wine.

Recovery and repeatability results for fortified samples tested using the steam distillation-SPE and steam distillation-liquid/liquid partition show similar results and indicate that both procedures may be suitable for incorporation into an at-line test procedure for use in a local winery laboratory.

7.4 Discussion

Results for the purification of wine samples indicated that the two alternative purification methods evaluated were both able to purify wine extracts efficiently for GC analysis.

Steam distillation is also considered to be a robust, a well tried method that could be used within the limited resources of a simple winery laboratory.

SPE purification methods were selected for evaluation as they are well established for the analysis of water using field test methods and for liquid food matrixes however no work has been reported for the analysis of wine using this cleanup method. This study indicates that the extraction of chlorophenols and chloroanisoles from wine can be efficiently achieved using a styrene/divinyl benzene copolymer sorbent material. The study also indicates that significant differences in performance characteristics between

styrene/divinyl benzene sorbents from different manufacturers may be observed. Results suggest that differences in polymer preparation methods can affect the efficiency of extraction chlorophenols. Efficiencies for chlorophenols were generally in the region of 90 - 110% although some resins exhibited lower efficiency for the more polar congeners such as PCP. A greater variation was observed in the analysis of chloroanisole congeners ranging from 58% PCA for the Amberchrom CG161 resin (Supelco) to 103% for the Env+ resin (IST). In addition to efficient trapping of the analyte an important characteristic of the selected resin was its selectivity for chlorophenols and chloroanisoles and exclusion of other matrix phenols. This was measured very crudely by recording absobance at 520nm. Figure 7.9 shows that four of the resins tested were considerably better at rejecting pigmented wine materials .

Two materials Isolut Env+ (IST) and Chromabond ‘Easy’ (Macherey Nagel) were recommended for further work using this procedure.

Following the initial evaluation of the two candidate procedures preliminary experiments were conducted to evaluate the suitability of the purified extracts for use in the ELISA test method. The experiments, reported in Chapter 8.0 indicated that sample extracts prepared using a single stage cleanup method were still contaminated by materials that produced a false positive ELISA response. In view of this two alternative dual stage cleanup methods were considered. These were steam distillation-SPE and steam distillation-LL partition. The efficiency of both hyphenated techniques was considered

adequate by GC-ECD. However, the extended time taken to perform the steam distillation-SPE method was considered to limit the practical value of this procedure.

8.0 AT- LINE SAMPLE TESTING PROTOCOLS

In this chapter the analysis of a range of sample types that are likely to be encountered in a winery contamination incident. is considered using the ELISA and DIPRA measurement procedures reported in Chapters 5 and 6.

To enable a small winery laboratory to locate the source of contamination simply and at low cost three sample types have been investigated. These sample groups were selected because they have previously been reported to represent high-risk potential sources of contamination in the wine industry (Buser *et al.*, 1982; Maarse, 1985; Pollnitz *et al.* 1996; Pena-Nera *et al.,* 2000).

1) Wine and raw(grape) materials – The analysis of wine and grape juice is essential to confirm that contamination is attributable to chlorophenols or chloroanisoles.
2) Corks – Cork contamination by 2,4,6-trichloroanisole has been widely reported and was attributed to hypochlorite bleaching (Quercus report 1996). The use of alternative sterilization chemicals has resulted in a reduction of contamination from this source but contaminated cork remains a principle source of contamination .
3) Packaging/storage materials – This is considered to be a principle source of contamination and arises from the use of chlorophenols as preservatives in wood, cardboard and paper products. Chlorophenols are strongly bound to structural carbohydrates and readily converted to

chloroanisoles, which are known to be very volatile and easily translocated (Whitfield *et* al 1991).

These sample types are considered to represent the principal potential contamination sources that may be encountered**.**

8.1 Wine

The measurement of chlorophenols and chloroanisoles in wine was investigated using GC-ECD and the ELISA test methods as described in Chapter 5.0, Section 5.3.

8.1.1 Preliminary experiments

Following the initial development of the competitive ELISA test procedure several experiments were conducted to measure CP/CA in the original unpurified wine. Results indicated that both contaminated and uncontaminated red and white wines gave an unacceptable number of false positive results due to the presence of interfering matrix components.

A number of wine samples were also tested using extracts prepared using liquid/liquid partition, SPE and steam distillation sample preparation procedures as described in Chapter 7.0. These analyses were also largely unsuccessful.

Using the SPE procedure without further modification produced a reasonable correlation between GC and ELISA, approximately 30% false positive results were observed. Further improvements were considered to be necessary to reduce the number of false positive results by introducing an additional purification stage employing either steam distillation or liquid/liquid partition.

8.1.2 Experiments to investigatet the use of SPE+liquid/liquid partition for the purification of wine samples.

Materials and methods

Wine samples (100ml) were purified and concentrated using the SPE procedure described in Chapter 7, Section 7.2. The final sample extracts were eluted using 2ml of 1% acetic acid in acetone into a 25ml volumetric flask. The eluant was acidified using 2ml of 2M sulphuric acid and shaken using a wrist action shaker for ten minutes with 2ml pentane to extracted chlorophenols and chloroanisoles. The mixture was diluted until the pentane layer was within the narrow neck of the volumetric flask and the pentane layer was removed to a conical base glass tube. The extract was evaporated to almost dryness under a stream of nitrogen and redissolved in 100μl 0.1M Na_2CO_3. The pH of the solution was adjusted to approximately pH 7 by the addition of 100μl of NaH_2PO_4 .The chlorophenol and chloroanisole content was measured using GC-ECD (Section 3.1) and the modified ELISA microplate procedures using the streptavidin-PCP conjugate (Section 5.11).

Spiked sample were also prepared by adding a diluted PCP solution (500μl for white and 1ml for red wines of 10.94 ng/ml PCP solution) to the original wine sample (100ml). Spiked samples were extracted by SPE and testing using the ELISA procedure.

Results obtained using the GC and ELISA procedures are shown in Table 8.1 and 8.2. A calibration relationship was established for each technique. For GC a linear regression model was employed by the ChemStation Plus™ software (Agilent Technologies) to calculate results. In the ELISA procedure a four parameter logistic IC50 model was employed using an Excel™ spreadsheet. Values obtained by GC were determined as the six individual contaminants but reported as PCP on a mole ratio basis.

Table 8.1 – Comparison of data by GC-ECD and ELISA streptavidin procedure for white wines. Results are expressed as ng/L PCP in the original wine samples. Recovery samples were fortified to +54.7 ng/L PCP

Wine sample description	GC (ng/L)	ELISA (ng/L)	ELISA Recovery (ng/L)
Chablis, Jeanne Galette- France	57	28	73
Riesling, Blue Nunn – Germany	<20	<20	49
Chardonay, Gallo – USA	40	27	74
Pino Grigiot, Lamberti - Italy	43	27	55
Riesling – Diaclone 232810 – Germany	29	<20	48

In view of the very low concentrations detected results indicate some agreement between the two methods. Based on averaged data the ELISA results exhibit an approximately 30% low bias compared to the reference method.

Fortified samples were detected in all cases but averaged recoveries for the five samples were approximately 65% of the theoretical value.

Table 8.2 – Comparison of data by GC-ECD and ELISA streptavidin procedure for red wines. Results are expressed as ng/L PCP in the original wine samples. Recovery samples were fortified to +109.4ng/L PCP

Wine sample description	GC (ng/L)	ELISA (ng/L)	ELISA Recovery (ng/L)
Cabernet Sauvignon – Gallo, USA	>200	>200	>200
Coronas – Torres, Spain	73	>200	>200
Beaujolais, France	58	179	>200
Diaclone 13-2810, France	134	114	>200
Diaclone 19-2810, France	120	>200	>200
AZTI 2b, Spain	126	105	>200
AZTI 15b, Spain	117	160	>200
AZTI 18b, Spain	55	50	>200

Results indicated that contaminant levels in red wines were generally overestimated by the ELISA procedure. In all cases, recovery samples showed a clear increase in signal However, absorbance values were above

the standard range of the assay. In view of this, results are generally considered to be over estimated.

These data indicate that this procedure is capable of indicating the presence of contaminants in white and red wines. However, some false positive results are predicted indicating the need for confirmatory analysis for positive samples. Further work should be undertaken using a lower concentration factor to produce an extract containing lower levels of co-extracted materials, which may reduce cross interference effects and thereby produce more reliable results.

8.1.3 - Steam distillation-SPE

Wine samples (50ml) were purified by steam distillation and solid phase extraction was employed to remove co-distilled ethanol and further purify/ concentrate sample extracts, described in Chapter 7, Section 7.3.

Results for GC and ELISA methods together with recovery results fortified by the addition of 109.4ng/L PCP are shown in Table 8.3.

Table 8.3 - Wine tested by the steam distillation-SPE cleanup procedure.

Wine	GC (ng/L)	ELISA PCP (ng/L)	ELISA (ng/L)
Rioja - Campo Viejo, Spain	<20	<40	>200
Rioja - Marques de Grignon, Spain	<20	<40	>200

Using this procedure both samples tested were below the detection limit of the method and in both cases spiked samples produced a clear positive response. In operation was very time consuming and required a full day to test a small set of samples. In view of this, no additional work was conducted

to validate the method because the time taken was considered excessive when compared to other procedures investigated.

8.1.4 - Steam distillation-liquid/liquid partition

Wine samples (50ml) were purified by steam distillation and the acidified extract was then back extracted into pentane as described in Chapter 7 Section 7.2. The pentane layer was transferred to a conical glass test tube, evaporated to dryness under a stream of nitrogen and redissolved in 50μl 0.1M Na_2CO_3. The pH of the solution was adjusted to approximately pH 7 by the addition of 50μl of NaH_2PO_4. Duplicate samples were also fortified to 109.4ng.L and extracted using the steam distillation+liquid/liquid partition procedure, Table 8.4.

The chlorophenol/chloroanisole content was measured using the standard GC and ELISA microplate procedure with fluorescence detection as described in Chapter 5, Section 5.3. Results are shownin Table 8.4

Table 8.4 - Comparison of GC and ELISA results for the steam distillation-liquid/liquid extraction sample preparation procedure applied to red wines and samples fortified to + 109.4ng/L.

Wine	GC (ng/L)	ELISA PCP (ng/L)	ELISA Recovery (ng/L)
Bordeaux – Calvet, France	123	85	192
Bordeaux - Foret Hilar, France	145	192	274
Chianti – Melini, Italy	149	87	238
Rioja – Faustino, Spain	144	118	204

GC chromatograms indicated that the extract produced with this procedure was generally free from additional electron capturing components indicating a high level of sample extract purity. Although the operation of the steam distillation method required operator expertise, in routine use the procedure was considered to be less time consuming than the SPE procedure.

8.2 CORKS

Wine stoppers contaminated by chloroanisole are considered to be one of the principle sources of spoilage of wine and many wineries have discontinued using cork owing to poor quality control of cork closures. TCA is known to be the primary cause of the musty taint that causes 'corked' wines. The origin of chloroanisole contaminants is considered to be an enzyme catalysed biomethylation of chlorophenol contaminants. The enzymes have been identified in several fungi and bacteria found on cork or within the environment of the cork manufacturing facilities. Chlorophenols are less significant as taint contaminants because they are not detected to the same degree as chloroanisoles but their presence in cork does present a significant risk that chloroanisole metabolites may contaminate the wine on long term storage. Compared to chloroanisoles, chlorophenols contribute far less to adverse flavour characteristics of cork. The highly polar nature of the chlorophenol molecule probably causes them to bind strongly to polar cork structural carbohydrates therefore they are largely retained within the cork. However chloroanisoles, which may be formed from the biomethylation of chlorophenols, are less polar and may migrate more readily into the wine. The conversion of chlorophenols to chloroanisoles is a very slow process that takes place as the wine matures therefore it is essential that quality control testing of new

corks for chlorophenols is undertaken by wineries and/or cork producers.

8.2.1 - Cork testing methods

Previously reported methods for the analysis of cork appear to produce very variable results and no standard analytical procedure is recommended by regulatory authorities. Popular methods involve extraction of the milled or whole cork using a variety of organic solvents such as pentane, ethanol or white wine followed by determination of contaminant levels by gas chromatography or odour assessment (Cork Quality Council 2004). The absence of a standardized analytical procedure causes some difficulties in the interpretation of results but experience gained in individual laboratories appears to allow limited recommendations to be made based on data obtained from the in-house test method. In view of the wide differences in polarity of the solvents used it is highly likely that chlorophenols will be extracted to different degrees by different solvents and results obtained will be very variable. Reported extraction methods appear to resist the use of exhaustive methods such as Soxhlet extraction. These methods are likely to produce the highest results and represent the total extractable level of contaminants. However, it may be inappropriate to base judgements on total extractable levels of contaminants as the wine will only be in contact with the outer surface of the cork and penetrate to a limited extent to sub–surface layers via lenticels. To obtain an indication of the level of chlorophenols that may become available over time an accelerated solvent extraction procedure

using whole corks is considered to provide the best indication of potential contamination problems.

In addition to the organic solvents previously reported experiments were also conducted to investigate solvent free extraction procedures using detergents that may be suitable for use in small winery laboratories where solvent fumes would present an unacceptable hazard.

Practical details

Three solvents and four detergents were investigated from each of the following principle detergent classes:

a) Anionic .e.g. dodecyl benzenesulfonic Acid (DDBSA)

b) Cationic e.g. cetyl trimethyl ammonium bromide (CETAB)

c) Non ionic e.g. poly oxy ethylene sorbitan monolaurate (Teen20)

d) Zwitterionic e.g. 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS)

The efficiency of extraction of six principal PCA/PCP wine contaminants were compared using hot and cold extraction procedures. The effect of adding alkali to the detergent mixtures was also investigated.

Three consignments of 100 corks were purchased from a local home brewing supplier. A representative milled sample from each consignment was extracted exhaustively using the Soxhlet method with acetone. The sample

with the highest levels of contaminants was then selected to facilitate optimisation of the extraction procedure.

Samples of eight whole contaminated corks were extracted using the following solvents and detergent solutions:-

Acetone

Ethanol

Hexane

Anionic surfactant

50:50 0.1% w/v DDBSA: Water

50:50 0.1% w/v DDBSA: 0.1MNa_2CO_3

50:50 0.1% w/v DDBSA: 0.1MKOH

Cationic surfactant

50:50 0.1% w/v CETAB: Water

50:50 0.1% w/v CETAB: 0.1MNa_2CO_3

50:50 0.1% w/v CETAB: 0.1MKOH

Nonionic surfactant

50:50 0.1% w/v Tween 20: Water

50:50 0.1% w/v Tween 20: 0.1MNa_2CO_3

50:50 0.1% w/v Tween 20: 0.1MKOH

Zwitterionic surfactant

50:50 0.1% w/v CHAPS: Water

50:50 0.1% w/v CHAPS: 0.1MNa_2CO_3

50:50 0.1% w/v CHAPS: 0.1MKOH

In all three consignments of cork the principal contaminant was pentachlorophenol. Results for hot acetone extraction of whole cork were approximately 50% lower than those obtained using Soxhlet extraction of milled corks, as shown in Figure 8.1.

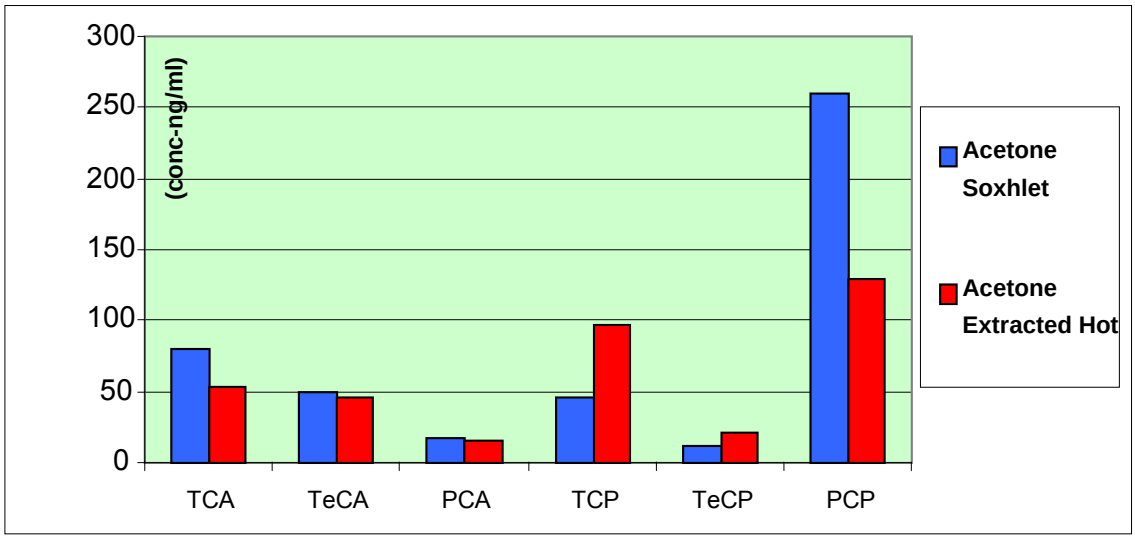


Figure 8.1 – Comparison of extraction methods for milled cork

Extraction of whole cork produced lower results than milled corks probably due to the reduction in exposed surface area and as expected cold extraction produced significantly lower extract concentrations when compared to hot extraction as shown in figure 8.2.

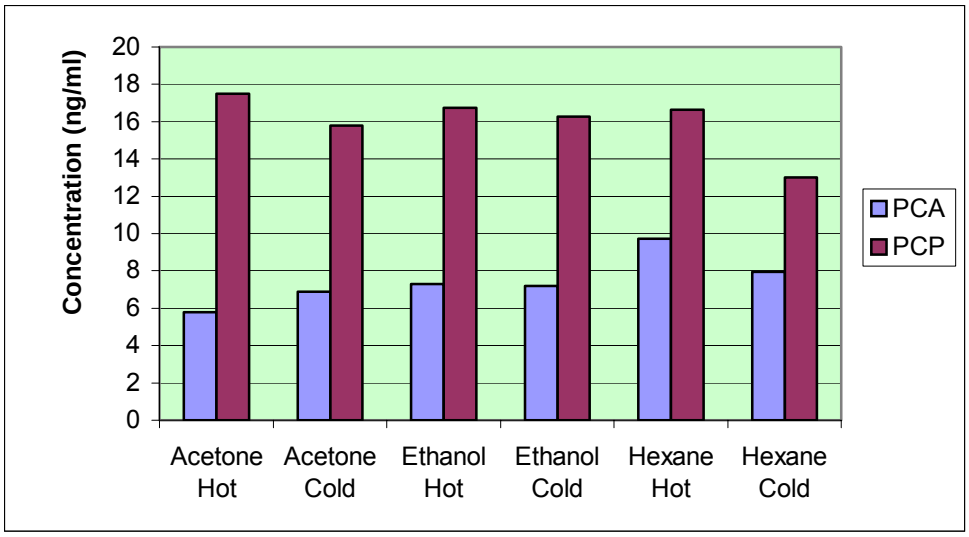


Figure 8.2 – Comparison of hot vs. cold extraction for whole corks

Extracting whole corks with different solvents also influenced the extraction profile as shown in Figure 8.3.

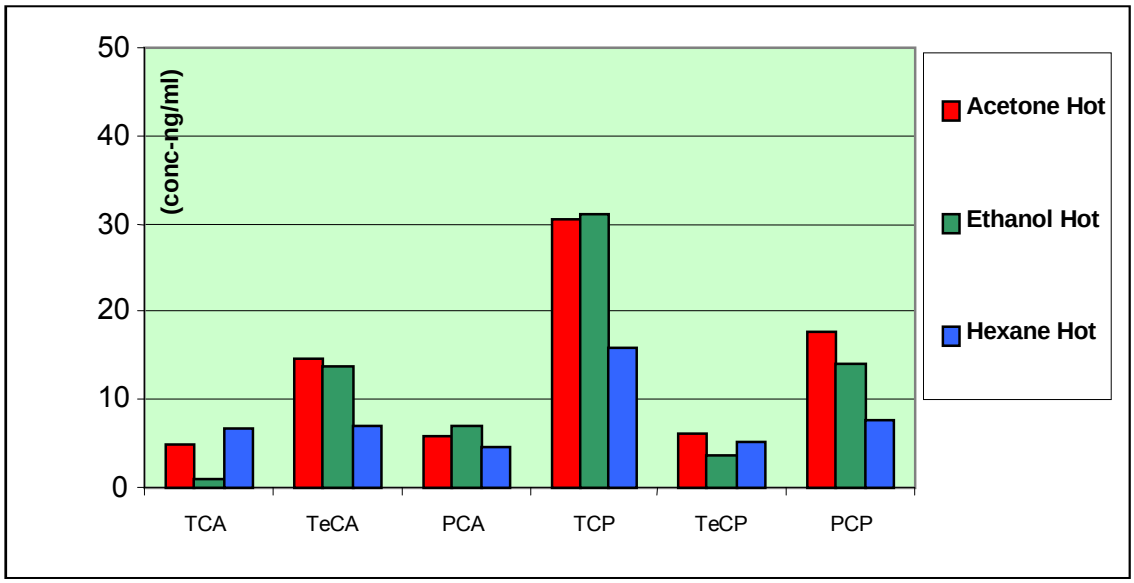


Figure 8.3 - Comparison of extraction efficiency of different solvents using whole corks.

A comparison of detergent extraction efficiency indicated little significant difference between the different detergent classes. However, significant

practical differences were observed due to foaming. With both DDBSA and CETAB excessive foaming was observed which cause practical problems. In general the low foaming non-ionic surfactant Tween 20 performed slightly better. The addition of alkali was found to significantly increase extraction efficiency by approximately 14% but a comparison of carbonate to hydroxide did not show a consistent trend, as indicated in Table 8.5.

Table 8.5 – Comparison of TeCP and PCP levels in corks extracted with various detergents.

		TeCP (ppb)	PCP (ppb			TeCP (ppb)	PCP (ppb)
A	CETAB-hydroxide	12.07	6.25	G	CETAB-carbonate	12.69	5.17
B	CETAB-carbonate	12.69	5.17	H	CETAB-water	11.66	4.49
	A-B	-0.62	1.08		G-H	1.03	0.68
					% change	8.4	14.1
C	DDBSA-hydroxide	15.01	10.79	I	DDBSA-carbonate	14.15	9.64
D	DDBSA-carbonate	14.15	9.64	J	DDBSA-water	12.44	8.60
	C-D	0.87	1.15		I-J	1.70	1.04
					% change	12.8	11.4
E	Tween20-hydroxide	12.88	14.67	K	Tween20-carbonate	13.11	14.92
F	Tween20-carbonate	13.11	14.92	L	Tween20-water	11.4	13.03
	E-F	-0.22	-0.25		K-L	1.8	1.90
					% change	14.4	13.6

In a few cases some of the chlorophenolic contaminants appeared to be methylated using potassium hydroxide detergent mixtures (e.g.CETAB). Tween 20/carbonate was selected as the best overall performer for inclusion in a solvent free extraction procedure.

A comparison of results corks extracted using detergents and solvents indicates that the chlorophenol and chloroanisole content of alkaline/detergent extracts are generally similar to acetone/ethanol extracts but these solvents are more exhaustive extractants than a non-polar solvent such as hexane, as shown in Figure 8.4.

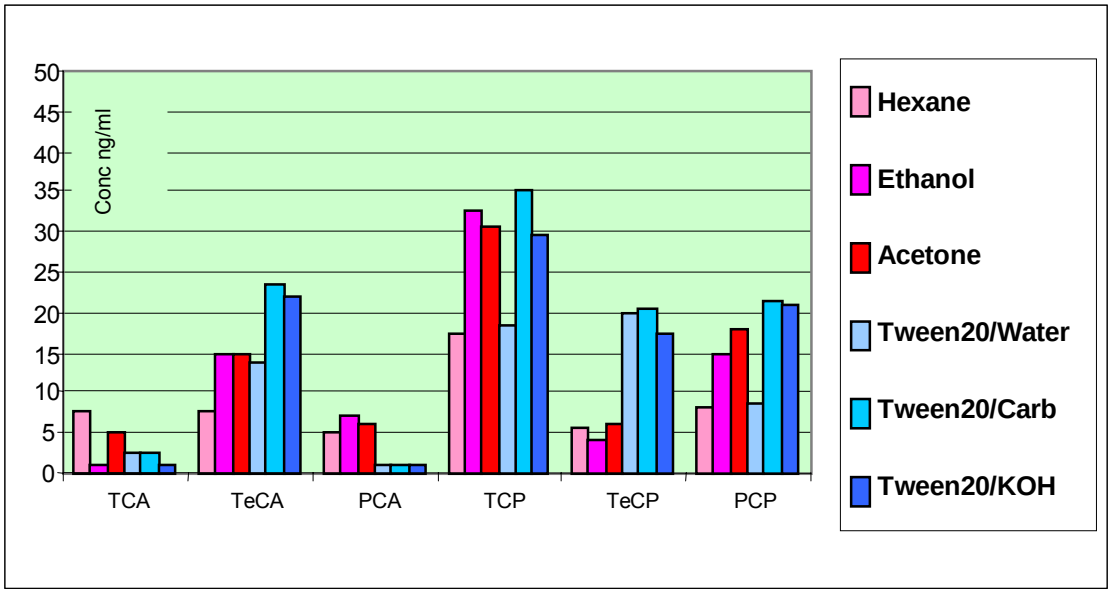


Figure 8.4 – Comparison of solvent and detergent extraction for whole corks

Discussion

These data indicate that the extraction and estimation of contaminants in wine corks is highly method dependent. One approach to the estimation of the risk of contamination may be to base an assessment on data obtained using an exhaustive extraction method such as acetone Soxhlet extraction of the milled cork. However, this procedure is highly likely to lead to an overestimated and distorted assessment of the contamination potential of a consignment of corks as external surfaces of cork normally receive additional treatments during cork production. By measuring milled cork most of the contaminated cork tested would not represent material likely to be in contact with the wine. A better estimate of the potential risk of contamination is available by an accelerated extraction of whole corks using either a hot solvent or alkaline detergent extraction procedure.

8.2.2 – ELISA procedures for the analysis of detergent extracts of cork samples.

Samples of cork were extracted using Tween 20/sodium carbonate alkaline detergent and purified using steam distillation and pentane solvent extraction. The pH-adjusted extracts were then tested for chlorophenols using the ELISA PCP procedure. Table 8.5 lists results for GC, ELISA and samples fortified at 20 ng/ml.

Table 8.5 – Results comparing GC and ELISA results for corks extracted using Tween 20/sodium carbonate Recovery sample extracts were fortified at +20ng/ml PCP

Wine type	GC (ng/ml)	ELISA (ng/ml)	ELISA Recovery (ng/ml)
Campo Viejo (B) – Rioja, Spain	PCP - 34 TeCP - <10	<20	60
Ruby Cabernet - Gallo (B), USA	PCP - 25 TeCP - <10	<20	45
Chablis, France	PCP – 15 TeCP - <10	<20	40

N.B. No chloroanisoles were detected in these samples

Result indicate that the cork samples tested were not significantly contaminated and that the ELISA procedure was able to correctly indicate the quality status. In each case the fortified sample extract was detected but result appear to be slightly elevated when compared to the expected values.

8.3 PACKAGING MATERIALS

Packaging materials were sampled by fine chopping or surface planning (wood). A subsample (5g) was extracted in a sealed bottle by shaking

vigorously with 0.1%Tween 20 in PBS using a wrist action shaker. A portion of the extract (5ml) was filtered using a syringe filter and tested using the GC, Diaclone high level ELISA method (see Appendix 2 and Table 8.6) and the DIPRA procedure described in Chapter 6 Section 6.5 (Table 8.7).

Table 8.6 – Results for packaging materials using the ELISA procedure. Recovery samples were fortified at 250μg/g (n=2)

Sample	GC (μg/g)	ELISA (μg/g)	ELISA Recovery (μg/g)
Cardboard	2.5	<2	>250
Wooden crate	38.8	32.3	>250
Wooden pallet	1.3	5.6	>250
Paper filter	0.8	<2	>250
Pine wood	1.1	2.2	>250

Table 8.7 – Results for extract concentration of packaging materials using the DIPRA procedure.

Sample	GC (μg/g)	DIPRA (μg/g)	DIPRA Samples spiked at approx.250μg/g (μg/g)
Cardboard	2.5	<10	>250
Wooden crate	38.8	37.5	>250
Wooden pallet	1.3	<10	>250
Paper filter	0.8	<10	>250
Pine wood	1.1	<10	>250

Wine bottles were extracted with 100ml 0.1%Tween20 in PBS by shaking vigorously for 30 minutes using a wrist action shaker. All of the bottles tested showed no surface contamination and good recoveries (>80%) at approximately 10μg/ml.

8.4 Discussion

The ELISA and DIPRA methods of analysis have been successfully applied to the three sample types selected, wine, corks and packaging materials.

Wine analysis by the ELISA procedure required very rigorous sample purification procedures using either SPE plus liquid/liquid partition or steam distillation plus liquid/liquid partition to obtain an extract that was sufficiently pure to enable the at-line measurement procedure to be used. The steam distillation-l/l procedure produced an extract that gave fewer interference problems suggesting that the main interfering components were involatile.

Interference problems were also experienced during the analysis of cork samples, and again the steam distillation procedure was successfully employed to separate the target analyte from interfering co-extracted material.

The analysis of packaging materials were also tested using the ELISA and DIPRA procedures. The relatively high concentration of chlorophenol based preservatives in these samples enabled them to be tested by diluting extracts in a suitable buffer, without the use of an additional purification step prior to the measurement of PCP/PCA content.

9.0 GENERAL DISCUSSION

In this study, the primary objective was to develop test methods to enable chlorophenol and chloroanisole contaminants to be measured in a local winery laboratory and rapidly traced back to the source. These objectives have largely been achieved by the development of at-line test kit procedures employing natural and synthetic antibodies and the methods have been successfully applied to the analysis of wine, cork and packaging materials.

9.1 Immunoassays using natural antibodies

Since the 1980's immunoassay procedures have played increasing role in the analysis of foods (Hammock and Mumma 1980, Beier and Stanker 1996). A significant potential role for these assays is developing for the rapid screening of large numbers of samples prior to the use of more sophisticated chromatographic analysis. In the wine industry immunoassay methods are also becoming increasingly important e.g. as a preliminary assay for pre-screening for mycotoxins (Immunoscreen OCHRA, Tecna, Trieste, Italy) and for Botrytis cinerea in grapes and must (Dewey and Meyer, 2004). Only a very limited number of methods for low-level contaminants in wine by immunoassays have been reported e.g. histamine (Diagnostic Systems Laboratories Inc. Texas, USA). The majority of low level assays reported are concerned with field-testing kits relatively high levels of fungicide residues in wine (Bushway and Thome, 1998; Giraudi *et al.* 1999) or the analysis of clean water samples for pesticides (Strategic Diagnostics Inc, Newark, USA) and dioxins (EPA method 4025).

Immunoassays are particularly suited for the analysis of polar water-soluble pesticides and can be significantly quicker than conventional chromatographic methods, particularly when used in a multiwell plate format in automated systems (Newsome, 1985). In addition, immunoassays can be simpler to use than conventional chromatographic methods, require minimal instrumentation and less qualified staff. Despite these advantages, the use of immunoassays for monitoring pesticide residues is less popular than

envisaged mainly due to major problems with interferences but also practical factors may limit their acceptance. One common practical constraint to the use of immunoassays for pesticide analysis in food may be due in part to the unfamiliarity of some analytical chemists with biologically based technology and the aseptic techniques required to prevent degradation of susceptible antibodies and associated materials.

Immunoassays can be frequently less sensitive than chromatographic methods and may exhibit lower levels of reproducibility. Immunoassays are compound/group specific; therefore, they are not suitable for multi-residue analysis. The cross reactivity with other chemicals present in the food is generally considered to be a major limiting factor in the applicability of immunoassay procedures. Problems with cross reactivity must be controlled e.g. by running matrix matched blanks or removing the interfering background substances (Hammock and Mumma, 1980).

Previous work to use immunoassay methods for at-line analysis of wine has been very limited. However, two papers from a joint study on the use of immunoassay procedures for the analysis of wine chlorophenol contaminants were reported in 2003. Lausterer *et al.* reported the production of anti TCA antibodies and Sanvicens *et al.* reported a detection limit of 44ng/L in wine but indicated major matrix interferences with both red and white wine samples.

Immunoassays provide the only practical alternative to chromatographic techniques that require very sophisticated analytical equipment that may be

outside the capabilities of a simple winery test laboratory. However, the levels of natural phenolic materials in wine present major interference problems and to enable wine contaminants to be measured extensive sample purification procedures are required.

9.2 Immunoassay procedures

In this study, test-kit procedures were established for PCP and PCA using multiwell plates and magnetic beads as described in Chapter 5, Section 5.6. The competitive immunoassay format was employed using magnetic beads pre-coated with anti-mouse antibodies. The use of a surface antibody coating was found to enhance the repeatability of the assay due to the establishment of ordered functional antibody layers as described in Chapter 5, Section 5.2.

The cross reactivity of related compounds was determined by constructing a standard dose response curve and determining the relative ratio of the IC_{50} value for the antigen and of the cross reactant, assuming that the displacement curves are parallel to the standard curve over the working range. Data presented in Chapter 5, Tables 5.5 and 5.6 summarise the cross reactivity of anti-PCP and anti-PCA coated microplates to the corresponding chloroanisoles and chlorophenols respectively. These data indicate decreasing cross reactivity with the degree of chlorination of the aromatic ring as shown in Chapter 5 Figure 5.2. A comparison of the performance characteristics of the anti-PCP and anti-PCA antibodies shows that the anti-PCP antibodies are more sensitive to the detection of both PCP and PCA than the anti PCA antibodies (IC_{50} 0.033μm for PCP and 0.35μm

for PCA using anti-PCP antibodies compared to 0.064μm for PCP and 0.049 for PCA using the anti-PCA antibodies). Alternative enzymatic reporter conjugates described in Chapter 5 Section 5.7 were synthesised based on streptavidin- biotin binding to enable interferences from residual co-extracted matrix materials to be removed prior to adding the reporter enzyme. This approach also amplified the sensitivity of the detection method by approximately two orders of magnitude enabling simple colorimetric detection procedures to be used to measure contaminants in sample extracts at microgram per litre concentrations. The use of the streptavidin amplification procedure also enabled alternative electrochemical detection procedures, briefly described in Chapter 4, Section 4.2, to be considered. Using these techniques chlorophenol and chloroanisole contaminants could be measured in the range 0.1 to 100ng/ml in water and purified sample extracts.

9.3 Displacement imprinted receptor analysis (DIPRA) procedures

One of the principal difficulties experienced using natural antibodies in at-line test kit methods is the limited storage life and special handing procedures required to ensure that the kit is viable. In general, the lifetime of commercially available antibody-based assay kits is approximately three to six months (Strategic Diagnostics Inc, Newark, USA). To overcome this difficulty polymers bearing synthetic receptor binding sites were developed based on materials produced by molecularly imprinted polymer technology. In the DIPRA approach a suitable polymer was formed around the analyte

molecule held in place by non-covalent bonding and thus acts as a template for the arrangement of polymer binding sites. The analyte molecule is subsequently removed by solvent extraction to leave behind a molecular cast of the active binding sites. This synthetic-receptor binding site was then rebound using a suitable reporter molecule and the polymer complex was employed in a displacement format test procedure to facilitate the establishment of a simple concentration dependent assay. In the conventional competitive ELISA format the signal generated is normally an inverse sigmoidal relationship, in the DIPRA procedure the measured signal is directly proportional to the analyte concentration. Synthetic antibodies do not suffer from poor storage stability although depending on the type of polymer used the conformation of the polymer receptor may change over a period of many years. The polymers produced in this study were stored and used over a period of two years without loss of activity.

The studies involving synthetic receptors described in Chapter 6 indicate that they may be used either in the standard ELISA assay format or in a displacement mode using enzymatic or high sensitivity fluorescent reporter conjugates synthesised in this study. This approach has additional advantages of better low-level repeatability. In the competitive ELISA format the standard error of both natural and synthetic receptors is significantly higher at low concentrations. When synthetic receptors are used in a displacement mode the standard error is not biased towards low analyte concentrations. The main advantage of using synthetic antibodies is improved robustness and storage stability. In this study we have also

demonstrated that good sensitivity can be achieved using synthetic receptor assays, however, the limit of detection for chlorophenols and chloroanisoles is several orders of magnitude higher than the limit of detection that may be achieved using ELISA procedures. Results indicate a limit of detection of 0.1μg/ml in solution could be achieved by the DIPRA procedure and 0.001μg/ml by ELISA. The DIPRA procedure is therefore recommended for high level testing of e.g. constructional timber and packaging materials and high level contamination of corks and the ELISA procedure is more suitable for wine and the low level analysis of cork contaminants. Again, one of the major problems encountered with synthetic receptors is 'cross reactivity'.

In Chapter 6, Section 6.8 twenty candidate compounds including structurally related pesticides and other materials that may form part of the sample matrix were assessed. The cross reactivity of chlorophenol congeners was highly significant and materials with a chlorinated aromatic ring showed significant cross reactivity but other materials such as humic acids showed less than 5% cross reactivity.

9.4 Application of test procedures to winery samples

9.4.1 Wine

The susceptibility of procedures based on selective binding to cross-reactivity is generally considered as one of their weakest characteristics. The use of monoclonal antibodies may provide a higher level of specificity than polyclonal or synthetic receptors but for highly complex sample matrices that

may contain co-extracted materials of similar molecular geometry, a formal separation procedure is often required to achieve a robust and reliable test results. In general, fewer interference effects are experienced where there are fewer materials of similar molecular geometry in sample extracts. In Chapter 7, experiments are described to investigate alternative methods to purify wine and cork samples.

Compounds that are most likely to become involved in competitive binding are those that have similar molecular features to the target molecules and may be present at concentrations, which are orders of magnitude higher. In both white and red wines, the sample matrix is extremely complex with many complex phenolic materials, some of which are described in Chapter 1, Section 1.7. To achieve the required purification and concentration of wine samples several traditional sample purification methods such as SPE, liquid/liquid partition, steam distillation were assessed. When used alone, all of these procedures failed to produce a suitable purified wine extract that could be successfully applied to a competitive binding measurement assay. To overcome this problem techniques were combined to produce 'hyphenated' cleanup procedures that were still straightforward to operate in a technically unsophisticated winery laboratory.

The following two procedures were successful:

1) SPE - liquid/liquid partition
2) Steam distillation - liquid/liquid partition.

In the method development for the first of these procedures, a number of commercially available resins were investigated to optimise the efficiency of the procedure and two suitable resins were identified that could both concentrate and purify a range of white and red wines. This method is potentially suitable for use in a small winery laboratory because of its efficiency, simplicity and safety considerations.

Extracts prepared using the second, more traditional steam distillation procedure were of a higher purity than the SPE procedure as indicated by GC chromatograms but the preparation of sample extracts by this method would require a higher level of training in laboratory staff.

Both methods were safe to operate in a well-ventilated laboratory, not necessarily equipped with fume extraction facilities. For contaminated and uncontaminated wine samples the steam distillation method was less susceptible to significant variations in the wine matrix and was therefore considered to be more robust. However, the SPE procedure could be set-up to extract overnight and therefore required less staff input. The final choice of method for a specific location will depend on the facilities and laboratory staff available in the winery laboratory.

9.4.2 Contaminated corks

In the wine industry contaminated corks have made a major contribution to wine contamination problems and should therefore be regarded as a special case. The introduction of appropriate sampling and testing procedures and the adoption of international standard cork testing protocols has the potential

to improve the quality of this product and the profitability of this declining industry. Experiments described in Chapter 8, Section 8.2 indicate that the extraction of contaminants from cork is highly method dependent. Cork is composed of a wide range of structural carbohydrates that bind very strongly to chlorophenols and also to a lesser extent to chloroanisoles by surface hydrogen bonding. To extract chloroanisoles from cork the solvent must be reasonably non-polar, however chlorophenols require a more polar solvent to desorb surface bound material. It was found that both contaminants could be extracted with similar efficiency to previously employed organic solvent by using an alkaline detergent mixture. The use of detergent based reagents in a local winery laboratory was also considered preferential for safety considerations. The experiments to investigate alternative extraction solvents described in Chapter 8, Section 8.2 clearly indicate that corks may contain high levels of chlorophenols in addition to the chloroanisoles, which are attributable to the 'corked' descriptor of inferior quality wines. The method of cork sampling is also important as the outer surface of corks may receive a special barrier or purification treatment to prevent wine contamination. Sampling by chopping or milling exposes the inner surface of the cork, which may not come into contact with the wine. The main sampling issue therefore concerns ensuring that 'available' contaminants are measured not the total level of contaminants in the cork. To facilitate the production of data that is valuable to both producer and user it is essential that cork testing procedures are internationally agreed and standardized. Currently the only ISO standard that applies to cork refers to the sampling and measurement of the size of

corks (ISO 4707,1981). Quality control methods based on the sampling procedures recommended in ISO 4707 together with alkaline detergent extraction of whole corks and analysis using the test kit procedures described in Chapter 5 and 6, would provide a more accurate method for the detection of contaminated corks that could be used by both suppliers or wineries to identify contaminated batches of corks.

9.4.3 Contamination from the winery environment

The identification and prevention, of contamination from external sources is considered as a major purpose for the at-line test procedures. Winery management issues such as the recycling of contaminated barrels, the use of preserved constructional timber or packaging materials; the use of inferior quality filter materials and residues on materials contaminated by the excessive use of pesticides and cleaning agents. All of these aspects may contribute to contamination of the final wine and have an impact on the marketability of the final product.

The identification of the source of contamination must therefore be conducted using a structured problem analysis approach together with a simple at-line test method of analysis to extract and measure contaminants locally. The source of environmental contamination will normally contain a level of contamination that is significantly higher than the levels detected in the product, for example surface levels of chlorophenols in treated timber that

could be used for the construction of timber racking etc. is approximately 40μg/cm^2 (Daniels and Swan 1979) and in cardboard used for packaging materials and food containers approximately 1μg/g (Shang-zhi 1983).

For these materials, a very simple extraction method involving shaking the chopped material or shavings in contact with a suitable solvent was investigated and described in Chapter 8 Section 8.3. Results indicated that these procedures were able to identify wood and paper products treated with biocides containing chlorophenols.

10.0 GENERAL CONCLUSIONS AND FUTURE WORK

The procedures developed in this study demonstrate that both natural and synthetic receptors provide unique advantages for the development of at-line test methods in the wine industry. Natural antibodies available for the analysis of chlorophenols and chloroanisoles currently provide the basis for the most sensitive assay procedures.

To enable natural or synthetic receptor based at-line procedures or devices to be widely accepted in the wine industry requirements for both a) a high sensitivity assay, and b) long term storage stability, must be satisfied. At-line test methods that are suitable for industrial problem analysis need to be available at the point of use, to support problem analysis as problems arise. And in this respect MIP technology provides significant advantages over natural antibodies. The measurement of chlorophenols in SPE concentrated water extracts and detergent extracts of various packaging materials contaminated at parts per billion concentrations have been demonstrated using MIP technology. However, for lower level contamination of e.g. corks a simple pre-concentration step is required before applying the sample to the MIP device. Further work is necessary to investigate opportunities for improving cork analysis by combining the detergent extraction - pentane liquid/liquid partition concentration step with the non-aqueous DIPRA assay as described in Chapter 8 Section 8.2 and Chapter 6, Section 6.6 respectively.

Using these synthetic receptors, or the natural antibody procedures described in Chapter 5, a simple and robust local assay for chloroanisoles in wine corks may be established. This would be suitable for use by both cork producers and wineries to check consignments of cork and hence reduce the risk of contamination.

Further work on the development of MIP technology using e.g. more highly ordered polymers produced by surface grafting or as 'dendrimers' (Zimmerman *et al.,* 200*2* and Haupt *2002)* may provide improvements to the kinetics of binding equilibria that could lead to higher sensitivity assays and the application of MIP technology to the at-line analysis of wine. However, the sensitivity and selectivity currently achievable using natural antibodies developed in this study together with appropriate sample purification procedures are sufficient to enable them to be used for local at-line testing. Further work to improve the storage stability of natural antibodies will also enhance their suitability for use in at-line test procedures.

Both natural and synthetic receptors are able to play a significant role in the development of at-line test methods and both are expected to play an important role in the development of low cost at-line sensors to ensure that future food products are contaminant free.

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12.0 APPENDICES

12.1 Appendix 1 - Results of AZTI analysis of wines 2001

	2,4,6-TCA (ng/l)	2,4,5-TCA (ng/l)	2,3,4,6-TeCA (ng/l)	2,3,5,6-TeCA (ng/l)	2,3,4,5-TeCA (ng/l)	PCA (ng/l)	2,4,6-TCP (ng/l)	2,4,5-TCP (ng/l)	2,3,5,6-TeCP (ng/l)	2,3,4,6-TeCP (ng/l)	2,3,4,5-TeCP (ng/l)	PCP (ng/l)
SAMPLE-01	ND	ND	13.0	ND	ND	5.0	26.7	ND	ND	ND	ND	ND
SAMPLE-02	14.0	ND	13.0	ND	ND	256.0	34.3	ND	ND	ND	ND	16.7
SAMPLE-03	ND	ND	5.17	ND	ND	23.5	ND	ND	ND	ND	ND	ND
SAMPLE-04	18.6	ND	27.5	ND	ND	271.0	37.2	ND	ND	ND	ND	37.1
SAMPLE-05	ND	ND	ND	ND	ND	2.49	16.8	ND	ND	ND	ND	16.8
SAMPLE-06	ND	ND	11.58	ND	ND	237.7	43.6	ND	ND	ND	ND	43.7
SAMPLE-07	ND	ND	12.95	ND	ND	70.13	43.5	ND	ND	ND	ND	21.2
SAMPLE-08	ND	ND	ND	ND	ND	ND	28.6	ND	ND	ND	ND	17.4
SAMPLE-09	ND	ND	ND	ND	ND	13.8	33.0	ND	ND	ND	ND	77.8
SAMPLE-10	ND	ND	ND	ND	ND	7.38	55.7	ND	ND	ND	ND	41.9
SAMPLE-11	11.6	ND	ND	ND	ND	8.75	41.4	ND	ND	ND	ND	18.2
SAMPLE-12	ND	ND	12.9	ND	ND	7.43	ND	ND	ND	ND	ND	54.1

SAMPLE-13	ND	ND	14.2	ND	ND	7.26	ND	ND	ND	ND	ND	ND
SAMPLE-14	ND	ND	ND	ND	ND	6.0	42.9	ND	ND	ND	ND	ND
SAMPLE-15	ND	ND	12.98	ND	ND	ND	ND	ND	ND	ND	ND	ND
SAMPLE-16	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	17.1
SAMPLE-17	ND	ND	11.02	ND	ND	ND	ND	ND	ND	ND	ND	ND
SAMPLE-18	ND	ND	ND	ND	ND	ND	20.5	ND	ND	ND	ND	ND
SAMPLE-19	ND	ND	8.67	ND	ND	ND	30.9	ND	ND	ND	ND	80.4
SAMPLE-20	ND	ND	9.13	ND	ND	ND	30.5	ND	ND	ND	ND	20.6
SAMPLE-21	ND	ND	9.94	ND	ND	ND	57.3	ND	ND	ND	ND	ND
SAMPLE-22	ND	ND	9.99	ND	ND	ND	131.8	ND	ND	ND	ND	76.5
SAMPLE-23	ND	ND	ND	ND	ND	ND	39.6	ND	ND	ND	ND	28.2
SAMPLE-24	ND	ND	9.49	ND	ND	ND	16.2	ND	ND	ND	ND	ND
SAMPLE-25	ND	ND	5.62	ND	ND	ND	20.3	ND	ND	ND	ND	48.6
Detection limit (ng/l)	<5.00	<9.00	<5.00	<5.00	<5.00	<5.00	<15.0	<20.0	<20.0	<18.0	<30.0	<15.0

12.2 Appendix 2 - ELISA research protocol. Diaclone

The Elisa competitive immunoassay is based on the binding competition between the antigen in the sample and the labeled antigen for the antibody-coated wells. A monoclonal antibody is used as catcher antibody. After addition of chromogenic substrate and then a stop solution, the resulting color is reported in optical density (OD) units. The intensity of the OD is inversely proportional to the concentration of the antigen in the sample. The concentration is also calculated on the basis of a standard curve. In this model, the reagents required are purified murine antibodies, HRP conjugated PCA or PCP antigens.

MATERIALS

1. REAGENTS REQUIRED.

- Monoclonal antibodies anti PCA (B-G40), anti PCP (B-L36), (Diaclone)
- Antigens HRP PCA BSA, HRP PCP KLH (Diaclone)

2. MATERIALS REQUIRED.

- ELISA microtiter plates (F8 Maxisorp Nunc # 468667, VWR)
- Automatic plate washer (Ultrawash Plus, Dynex Technologies))
- ELISA plate reader with data software (MRX revelation, Dynex Technologies)

3. SOLUTIONS REQUIRED.

- Wash buffer : 0.01% Tween 20 in distillated water
- Blocking buffer: 5%BSA in PBS pH 7.4
- Diluent buffer: PBS
- TMB (#TMBus-1000, Moss Inc)
- Stop solution (H2S04), (#109072-5000, Merck Eurolab)

ELISA PROTOCOL

Plate preparation

1 Transfer 100 µL/well of the capture antibody diluted in PBS at 10 µg/ml to an ELISA plate. Seal plate and incubate overnight at 4-8°C .

2 With an auto washer, aspirate each well and wash with wash buffer (300 µL/well), repeating the process for 4 washes.

3 After the last wash, remove any remaining wash buffer by inverting the plate and blotting it against clean paper toweling.

4 Blocks plates by adding 250 µL of blocking buffer to each well. Incubate at room temperature for 2 hours at room temperature.

5 Repeat the aspiration/wash as in step 2 for 2 washes.

6 When sealed with desiccant, the dried plates can be stored at 4°-8° C for at least two months.

Assay procedure

7 Add 100 µL of samples or control per well. For a qualitative analysis, the standard concentration is 10 g/ml. Use PCP as positive Ag control and BSA as negative control. Use also as negative control for example a sample of the same nature described negative. For a quantitative analysis, use standard diluted to the appropriate concentration, (concentrations recommended in progress). Dilutions of standards should be carried out in polypropylene tubes.

8 Cover with an adhesive strip and incubate 1 hour at room temperature.

9 Repeat the aspiration/wash as in step 2 for 4 washes.

10 Add 100 µL of the HRP conjugated antigen diluted in appropriate diluent to each well.

11 Cover with an adhesive strip and incubate 30 min at room temperature. Dilution of HRP conjugated antigen should be carried out in polypropylene tubes. Repeat the aspiration/wash as in step 2 for 4 washes.

12 Add 100 µL substrate solution to each well. Incubate for 5-10 minutes at room temperature. Avoid placing the plate in direct light.

13 Add 100 µL stop solution to each well.

14 Determine the optical density (O.D.) of each well within 30 minutes to 450 nm.

Analysis of results

For a qualitative analysis, the data obtained with the unknown samples are compared with the negative control.

For a quantitative analysis, the values of the unknown samples are assigned in relation to the standard curve.

12.3 Appendix 3 - Publications

Nicholls, C. R. (2003) Artificial antibodies for the analysis of chlorophenol contaminants in drinking water. Cranfield Conference, Silsoe, Bedfordshire.

Nicholls, C., Karim, K., Piletsky, S., Piletska, S., Setford, S. and Saini, S. (2003). Displacement Imprinted Polymer Receptor Analysis (DIPRA) for Chlorophenol Contaminants in Drinking Water. *Synthetic Receptors 2003*, Lisbon, Portugal.

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Nicholls, C., Setford, S,. Alfaro Redondo, B., Pérez-Villarreal, B., Shehan, M., Berney, M., Wijdenes, J. and Vermot-Desroches, C. (2004). Comparison of different clean-up procedures for the analysis of chlorophenols and chloroanisoles in wine. (in preparation)

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