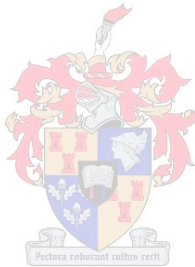


THE OPTIMIZATION OF THE EXTRACTION AND
PURIFICATION OF HORSERADISH PEROXIDASE FROM
HORSERADISH ROOTS

by

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*Thesis presented in partial fulfilment of the requirements for the degree
Master of Science in Biochemistry at the University of Stellenbosch*



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September 2012

DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the owner of the copyright thereof and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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ABSTRACT

This study describes:

- a) the optimization of the current industrial-scale extraction and purification of Horseradish peroxidase from horseradish at BBI Enzymes, focussing on:
 - a. Raw material quality,
 - b. Extraction,
 - c. Ultra-filtration,
 - d. Salt fractionation,
 - e. Diafiltration,
 - f. Ion Exchange Chromatography,
- b) developing an new in-process microtitre plate calorimetric assay,
- c) characterization of main groups of HRP relevant to BBI Enzymes by SDS-PAGE- and HPLC analysis

OPSOMMING

Hierdie studie beskryf:

- a) die optimisering van die huidige industriële-skaal ekstraksie en suiwing van peperwortel-peroksidase vanuit peperwortel by BBI Enzymes, deur te fokus op:
 - a. Rou material kwaliteit,
 - b. Ekstraksie,
 - c. Ultra-filtrasie,
 - d. Sout fraksionering,
 - e. Diafiltrasie,
 - f. Ioon-uitruilchromatografie

- b) Ontwikkeling van 'n nuwe in-proses mikro-titer gebaseerde kalorimetriese toetsmetode

- c) die karakterisering van die hoof groepe peperwortel-peroksidase belangrik vir BBI Enzymes

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ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
A/S	Ammonium sulphate
BU	Billion units*
B/T	Break-through
CIP	Clean-in-place
CM	Carboxy-methyl. Negatively charge group that is linked to CM Sepharose [®] resin beads. This group gives the CM Sepharose [®] its characteristic weak cation exchange property.
DEAE	Diethylaminoethyl
DF	Diafiltration, a tangential flow membrane filtration process.
EC	Enzyme Commission number, a numerical classification of enzymes.
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
HRP C	Horseradish peroxidase isoenzyme C
Hz	Hertz
IAA	Indole-3-acetic acid
k_{cat}	Catalytic constant
kDa	kilo-Dalton
K_m	Michaelis constant
MM	Molecular weight marker

Mr	Molecular weight
mS	milli-Siemens
MTP	Microtitre plate
MU	Million units*
MWCO	Molecular weight cut-off
NaCl	Sodium chloride
PES	Polyethersulfone
PAS	Periodic acid Shift method of staining for Carbohydrates
P_{in}	Membrane inlet pressure
P_{out}	Membrane outlet pressure
PAGE	Poly-acrylamide gel electrophoresis
QC	Quality control. A system of maintaining standards of all factors that are involved with production in order to ensure consistency and quality of product.
RO	Reverse-osmosis
Rz	“Reinheitzahl” value is the unit of measure representing the ratio of absorbance at 403nm (wavelength at which haem groups of proteins absorb light) to the absorbance at 275nm (wavelength at which aromatic groups of proteins absorb light). This is used as a measure of the purity of the HRP specific protein in the HRP process at different steps.
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate poly-acrylamide gel electrophoresis
S/N	Supernatant
t	Tonne (1000kg)

TMB	3,3',5,5'-tetramethylbenzidine
Tris	Tris(hydroxymethyl)aminomethane buffer
TFF	Tangential flow filtration
UF	Ultra-filtration
U.S.A.	United States of America
V_{\max}	Maximal reaction rate

**Guaiacol enzyme units*. Unit definition: The amount of enzyme which catalyzes the conversion of one micromole of hydrogen peroxide per minute at 25°C.

CHAPTER 1: INTRODUCTION

Horseradish peroxidase (HRP) is a haemoprotein classed in the oxidoreductases group of enzymes (EC 1.11.1.7) due to its ability to oxidize a wide range of substrates in the presence of an oxidizing agent. Substrates catalyzed by HRP include ascorbate, ferrocyanide and cytochrome C. This class of enzyme is expressed in both pro- and eukaryotic cells. HRP isoenzyme C (HRP C), the most abundant isoenzyme in horseradish (*Armoracia rusticana*), is one of the best studied enzymes of this class due to its wide range of applications. Studies of isoenzyme C have proposed applications including cancer therapy, immunodiagnostics, biotransformation, enzyme-linked assays and treatment of wastewater.

BBI Enzymes is one of the world's largest manufacturers of natural enzymes derived from plant and animal tissue. The company is located in Cape Town, South Africa and is one of the world's largest HRP manufacturers, selling more than 10 billion HRP enzyme units (Guaiacol assay method) (1), annually. The methods employed in the current extraction and purification process were selected due to the scale of the operation. These methods are, however, time consuming, inconsistent, inefficient and yield low volumes of low purity HRP. Because of the complexity of manufacturing and inadequate in-process control methodology, it was not possible to optimally track the product yield of the previous HRP isolation and purification processes.

The in-process quantitative enzyme analysis is very time-consuming and susceptible to a number of factors that influence the accuracy of the method. These processes delay production as operations cannot continue without results and lead to delays in the delivery of product to our customers.

The aim of this project was to determine whether the currently applied methods for the extraction, purification and in-process quantitative analysis of HRP from horseradish are

optimal. The scope of improvements will include equipment and enzyme assays/detection methods, whilst taking into consideration time, cost, expertise and quality of the final product.

An overview of the general properties of the HRP enzyme is given in Chapter two. The main properties of the enzyme important to its successful extraction and purification are discussed. The optimization of the extraction and purification of HRP is discussed in Chapter three. The proposed focus for this optimization was extraction of the enzyme, concentration and ammonium sulphate fractionation (A/S) of the extract, diafiltration and chromatographic purification of HRP.

In Chapter four, the results of experiments aimed at the development of a new in-process microtitre-based colorimetric assay for HRP is discussed. Parameters that will be reviewed during development will be aimed at desensitizing the substrate, whilst generating a robust, consistent assay. The characterization of the main groups of HRP isoenzymes, important to BBI Enzymes, was also performed in this study and the results are discussed in Chapter five. In Chapter six, the work performed in the thesis is discussed and conclusions are made.

CHAPTER 2: HORSERADISH PEROXIDASE: AN OVERVIEW

HRP is a haem-containing, 44kDa glycoprotein. It is classified in the oxidoreductases group of enzymes (EC 1.11.1.7) due to its ability to oxidize a wide range of substrates in the presence of an oxidizing agent such as hydrogen peroxide. It is one of the best studied enzymes of the oxidoreductases group (2; 3). Interest in HRP is due to its wide range of biomedical applications including cancer therapy, immunodiagnostics, biotransformation, enzyme-linked assays and treatment of wastewater (4; 5; 6). For example, in cancer therapy, cancer cells are transfected with HRP cDNA. Indole-3-acetic acid (IAA), a HRP substrate acting as a prodrug, is oxidized as part of a gene-directed enzyme/prodrug therapy inducing apoptosis in certain cancer cells (7). Enzyme-linked assays, on the other hand, exploit the vast range of substrates oxidized by HRP. An example of this application is where antibody-conjugated HRP create a colorimetric signal by oxidizing selected colourless substrates to coloured measurable metabolites. The colour reaction enables the user to determine the presence of the target molecule captured by the conjugate antibody. The oxidative coupling of phenolic compounds by HRP is a three-step cyclic reaction.

HRP consists of a 308 amino acid polypeptide, with four disulphide bonds supporting the structure of the active enzyme. Different HRP isoenzymes were first reported in 1942 (8). Of the seven main isoenzymes characterized in literature, HRP isoenzyme C (HRP C) is the most abundant, and subsequently the most studied (9). HRP C has also been adopted as the main isoenzyme from horseradish for use in various applications. Catalytically active isoenzyme C contains two metal centres, two calcium atoms and an iron (III) protohaemin IX or haem group (9). Gajhede *et al.* was the first to elucidate the X-ray crystal structure of HRP C (10). Their work is shown in Figure 1. From this figure it is clear that the amino acid backbone of the enzyme structure consists mainly of α -helices, with a number of β -sheet regions. HRP C has a total of 9 glycosylation sites and is glycosylated at 8 of these positions on the protein surface by asparagine linking. The role of *N*-linked glycans in the stabilization of glycoproteins has been studied with contradictory results (11; 12). The role of these groups in HRP has been shown to be related to the kinetic stability of the enzyme (13). These groups

are of importance in a number of applications, as it is used in the preparation of conjugates (14). Conserving these groups during the optimization of the HRP isolation and purification process will be reviewed later in Chapter 5.

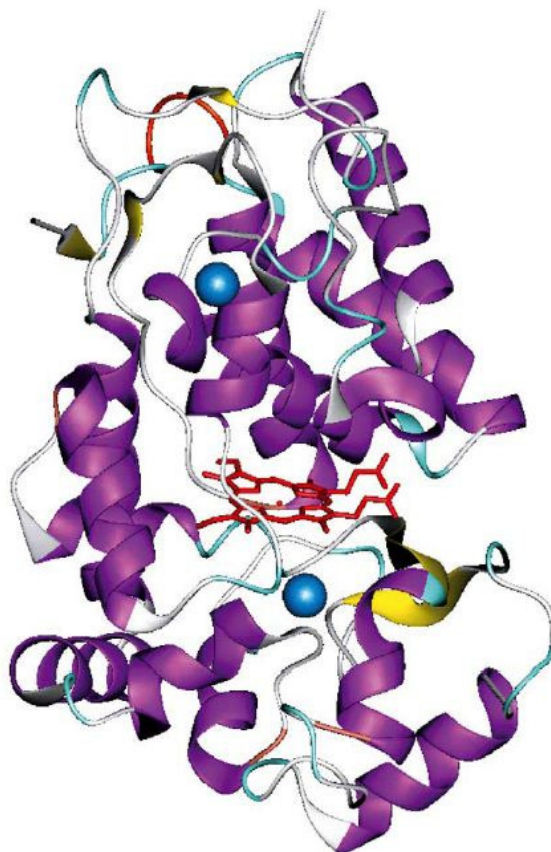
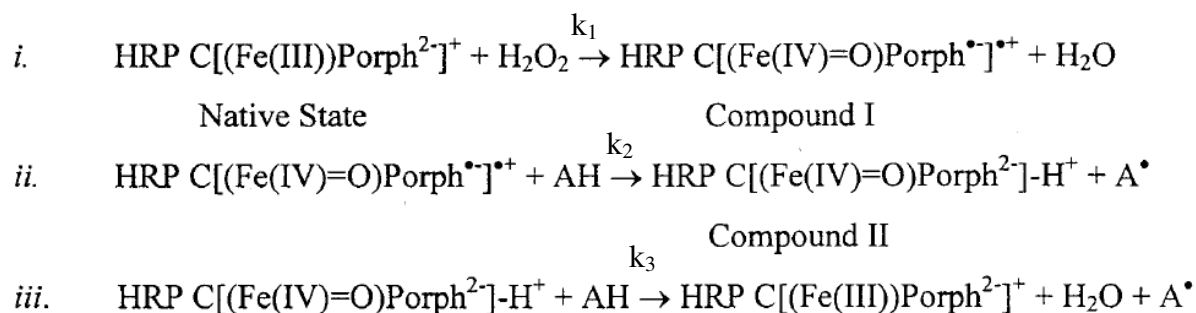


Figure 1. *X-ray crystal structure of HRP C*. The position of the two calcium atoms is shown by the two blue spheres, with the iron (III) protoporphyrin IX coloured in red. The α -helix regions are shown in purple, with the β -sheet regions identified in yellow (15).

2.1. Mechanism of action, kinetic characteristics and functional role

HRP oxidizes a wide range of substrates in the presence of an oxidizing agent such as hydrogen peroxide. The reaction starts by the reduction of HRP by hydrogen peroxide

(oxidizing agent) forming compound I, followed by the oxidation of substrate (represented by “AH” in the peroxidase cycle scheme below) by the enzyme:



The catalytic constant (k_{cat}) and Michaelis constant (K_m) of isoenzyme C for different HRP substrates are shown in Table 1. The data illustrates that 3,3',5,5'-tetramethylbenzidine (TMB) has the highest k_{cat} of the substrates reviewed (16; 17; 18). 2,2'-Azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) also have relatively high k_{cat} value, when compared to that of the oxidation of hydrogen peroxide (H_2O_2) by HRP during the formation of Compound I. The catalytic efficiency of the various substrates were compared by determining the value of k_{cat}/K_m . From the data, it is shown that TMB is the most efficient of the three substrates reviewed. For reaction cycles involving H_2O_2 and Guaiacol (2-methoxyphenol), the rate limiting reaction would be the oxidation of Guaiacol. The higher K_m value for the oxidation of H_2O_2 by HRP than that of the reaction with TMB (8.5 times higher) and ABTS (8.4 times higher) indicates lower affinity by the enzyme for H_2O_2 .

Table 1. Kinetic characteristics for HRP as determined at 25°C (16; 17; 18).

Substrate	k_{cat}	K_m	k_{cat}/K_m
Hydrogen peroxide (H_2O_2) ¹	$3.48 \times 10^3 \text{ s}^{-1}$	$3.7 \times 10^{-3} \text{ M}$	9.4×10^5
3,3',5,5'-tetramethylbenzidine (TMB) ¹	$4 \times 10^3 \text{ s}^{-1}$	$0.434 \times 10^{-3} \text{ M}$	9.2×10^6
2,2'-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) ²	$226 \pm 14 \text{ s}^{-1}$	$(0.44 \pm 0.03) \times 10^{-3} \text{ M}$	5.1×10^5
Guaiacol (2-methoxyphenol) ³	13.8 s^{-1}	$2.8 \times 10^{-3} \text{ M}$	4.9×10^3

¹ [TMB] = 2.5mM, [HRP] = $2.5 \times 10^{-11} \text{ M}$, [H_2O_2] = $1 \times 10^{-3} \text{ M}$.

² Reaction in 0.1M phosphate buffer, pH6.0, [HRP] = $1 \times 10^{-10} \text{ M}$, [H_2O_2] = $7 \times 10^{-4} \text{ M}$.

³ Reaction in 0.02M potassium phosphate, pH7.0, [H_2O_2] = $2 \times 10^{-4} \text{ M}$, [HRP] = 6nM.

The affinity of HRP for ABTS and other substrates is pH dependant. The maximal reaction rate for ABTS at a concentration of 2mM/L is achieved at pH4.2 (19). By increasing the pH of the reaction solution, the enzyme affinity for the substrate is reduced. This property of the enzyme-substrate interaction will be exploited during the desensitizing of the colorimetric assay discussed in Chapter 5(Figure 43).

Gaspar *et al.* reported observations linking a cascade variation of peroxidase isoenzyme levels in relation to growth and developmental processes such as abscission, root formation and flower initiation (20). Increased levels of free radicals and peroxides during stress-induced membrane depolarization initiated lipid peroxidation. The changes in membrane integrity lead to changes in ionic fluxes (potassium and calcium) and the secretion of basic peroxidases into the intracellular matrix, aiding in the reduction of peroxides and free radicals. During the stress-response and other growth and developmental processes, basic peroxidases was found to bind cell membrane structures and play a role in the increase in acidic peroxidase activity. Acidic peroxidase is linked to the lignification of damaged or developing cell walls (3). The mechanism of stress-response in the plant is exploited during the cultivation of high HRP content horseradish and will be discussed during the review of raw material quality (Chapter 3).

2.2. Enzyme stability

HRP is stable at neutral to basic pH between pH 5.0 – 10.0. Thermal stability of the enzyme is affected by a number of factors, including buffer conditions, calcium ion bond strength, disulphide bridges and glycosylation (21). It has been shown that high concentrations of sodium phosphate buffer, pH 7.0, reduce the thermal stability of the enzyme (22). This is of importance as phosphate buffer is used in the current HRP preparation process at BBI. Thermal or pH-inactivation leads to the de-stabilization of α -helix and haem regions of the tertiary structure and is irreversible (23). The use of 3,3',5,5'-tetramethylbenzidine (TMB) and luminol has been shown to increase the stability of HRP in solution (24). This property

was exploited during the development of the microtitre-based HRP assay described in Chapter 5.

2.3. Inhibitors

HRP inhibitors include divalent ions such as dichromate, lead (Pb^{+2}), cobalt (Co^{+2}) cadmium (Cd^{+2}), manganese (Mn^{+2}), copper (Cu^{+2}) and nickel (Ni^{+2}). Other inhibitors include vanadate, sodium azide, cyanide, fluoride, ethylenethiourea, hydroxylamine, sulphide, L-cystine, p-aminobenzoic acid, and carbon monoxide (25; 26). A number of these small molecules bind to the iron atom of the haem group, leading to the formation of six-coordinate peroxidase complexes with reduced or no catalytic activity. This is also the haem group formation observed at alkaline pH where the enzyme has reduced reduction-oxidation potential (27). Cobalt, a non-competitive inhibitor, slowly binds to the enzyme and induces conformational changes at higher concentrations. The most significant of these changes take place in the active site of the enzyme (28).

The commercial grade of ammonium sulphate (A/S) used for the saturation of the HRP solution during fractionation and chromatography, on an industrial scale, contains sulphide ions. Sulphide is a known reversible inhibitor of HRP and the level of inhibition was investigated by Theorell *et al* (26). In Figure 2, the suppression of HRP activity is illustrated with an increase in A/S concentration. The data shown in this figure is from a single experiment performed. A single experiment was performed following observed losses across fractionation steps. This suppression of enzyme activity is suspected to be an irreversible inhibition, as exhaustive dialysis of HRP could not reconstitute the activity. This ineffective reversal of the inhibition may contribute to the lower enzyme activity. Other inhibiting contaminants present in this grade of A/S include Lead, Cobalt and Manganese (29). The solution would be to use a higher purity grade of A/S with a lower concentration of sulphide. Alternatively, the use of sodium phosphate can be investigated. The use of commercial grade A/S for the initial fractionation of the HRP extract at the end of primary processing will,

however, be continued in the near future due to the financial implications of changing to a more expensive higher grade of A/S.

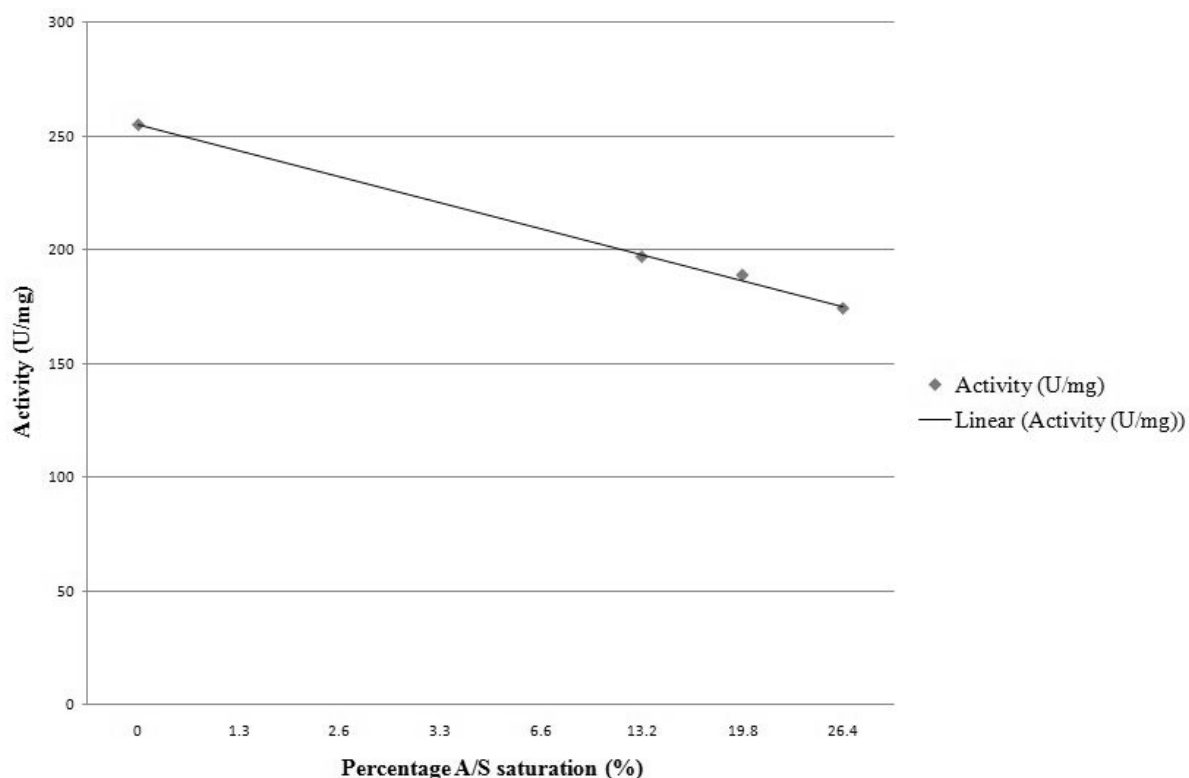


Figure 2. *Effect of percentage A/S saturation on the activity of 161451-grade HRP.* Solutions of reconstituted HRP was saturated with A/S, dialysed, lyophilized and the HRP activity tested (161451-Grade HRP is a BBI specific product grade).

HRP activity, as measured by conversion of dianisidine, is increased in the presence of imidazole, an alkaloid organic compound (30). The peroxidation of dianisidine is also increased significantly by nitrogenous compounds such as ammonia, or A/S (30). This effect on dianisidine conversion has lead to use of different substrates for in-process HRP activity testing due to the presence of high levels of A/S.

The enzyme stability, activators and inhibitors are important factors to consider in the extraction and purification of HRP. Introduction of new grades or types of chemicals, used directly or indirectly (cleaning or sanitizing of equipment) in production, needs to be reviewed for their effects on the enzyme stability and activity. Trace contaminants are one of the main risks and a review of the chemical composition of the product, together with lab-scale production trials were performed to minimize the risk of activity loss and enzyme yield. The reduced thermal stability of HRP in sodium phosphate buffer, for instance, has led to the removal of this buffer from the process. Critical control points during the process include a maximum temperature limit and a defined pH range. These factors ensure enzyme stability during the process. All these factors were considered during the optimization of the HRP production process and will be addressed in subsequent chapters.

CHAPTER 3: OPTIMIZATION OF THE CURRENT INDUSTRIAL-SCALE EXTRACTION AND PURIFICATION OF HRP FROM HORSERADISH AT BBI ENZYMES

3.1. INTRODUCTION

HRP was first extracted and purified by Seravac Laboratories (today BBI Enzymes) in 1958. Since then BBI Enzymes has been a bulk producer of HRP for 53 years.^a Initial markets were mainly enzymes for biochemical/biomedical research, but as the use of enzymes as components in immunodiagnostic test systems were identified, the market for HRP grew. The method employed to extract and purify HRP was an adaptation of the process developed by Keilin *et al.* in 1950 (31). The method was adapted to a previous revision of the current method in 1966, after Shannon *et al.* published their review of the peroxidase isoenzymes present in horseradish (9). Between 2001 and 2010, BBI Enzymes produced and sold an average of 7 billion units (BU) of HRP enzyme annually (1).^b At the high-grade final product yield achieved in 2007 of 8 million units (MU) of enzyme per tonne of raw material input, 7 BU of HRP equates to the processing of 875 tonnes of horseradish roots.^c This yield (8MU/tonne) was, however, not commercially viable due to the rising costs of raw materials and reagents (Table 2).

^a Lifescan - Horseradish Peroxidase from SERAVAC. Cape Town : SERAVAC, 1993.

^b BBI Enzymes. *Sales - Products*. Cape Town, Western Province, South Africa. 22 July 2003.

^c BBI Enzymes. 161457 BBI Peroxidase Product Specification. BBI Enzymes Quality Management System. Cape Town : s.n., 2010, p. 161457BBI.

Table 2. *Raw material and reagent cost increases for HRP production over the past 13 years.*

Raw material or reagent	1998	2011	Percentage increase
	Cost (ZAR/Kg)	Cost (ZAR/Kg)	
Horseradish roots: South African	R6.50	R12.75	196%
Horseradish roots: North American	–	R14.04	216%*
Ammonium sulfate (A/S)	R1.53	R4.537	297%

* Compared to the price for horseradish roots (South African) in 1998.

As enzyme yield and isoenzyme distribution are crucial factors for the viable processing of horseradish, there has always been a large emphasis on the quality of the raw material processed by BBI enzymes.

The extraction and purification of HRP can be achieved using a variety of techniques. The important considerations, when deciding on the use of these techniques, are the cost, robustness and consistency at the scale of operations. Affinity chromatography matrices including Sepharose-bound Concanavalin A and p-acylamidobenzoic acid have been developed and lead to rapid purification of HRP (32; 33). The use of these resins are impractical in the large-scale production of the enzyme due to the low throughput, cost of the matrices, low pH stability and their relatively short cycle life, which can be as little as 20-30 cycles (34). In addition the specificity of p-acylamidobenzoic acid towards isoenzyme C as capturing group during affinity chromatography does not satisfy the aim of BBI Enzymes to purify the full complement of neutral, basic and acidic isoenzymes (35).

The original method for HRP production at BBI Enzymes is divided into primary and secondary processing. Primary processing entails maceration of the horseradish roots and liberation of the enzyme from the plant material by exposing it to a slightly saline, aqueous solution. The extracted plant material is then removed by centrifugation and the supernatant clarified using diatomaceous earth. The clarified extract is then concentrated to approx. 15 –

30% of the original 16000L volume by using Ultra-filtration with a molecular weight cut off (MWCO) membrane of 10 kDa (Figure 3).

Secondary processing at BBI Enzymes begins with salt fractionation. This step reduces the amount of proteins that do not form part of the product in the extract. The ionic strength of the fractionated HRP extract is then reduced by dialysis. The HRP solution is purified through a series of ion exchange and hydrophobic interaction chromatography steps (Figure 4). The final product is a salt-free lyophilized powder. In this chapter the factors affecting raw material quality and the optimization of the HRP extraction and purification process followed at BBI Enzymes will be reviewed.

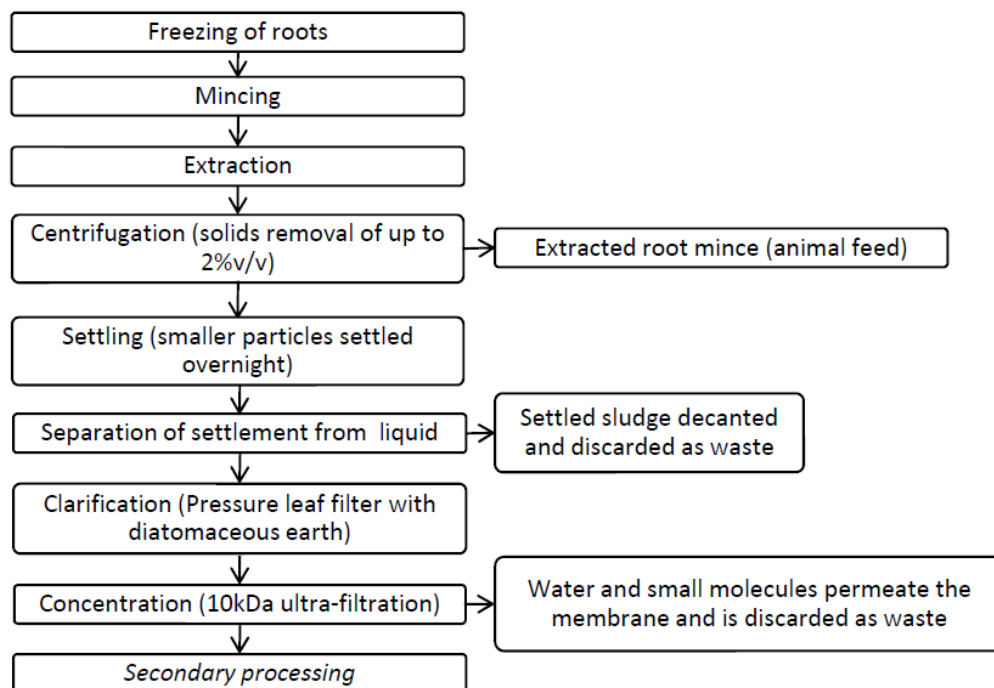


Figure 3. *Current primary processing of HRP at BBI Enzymes.*

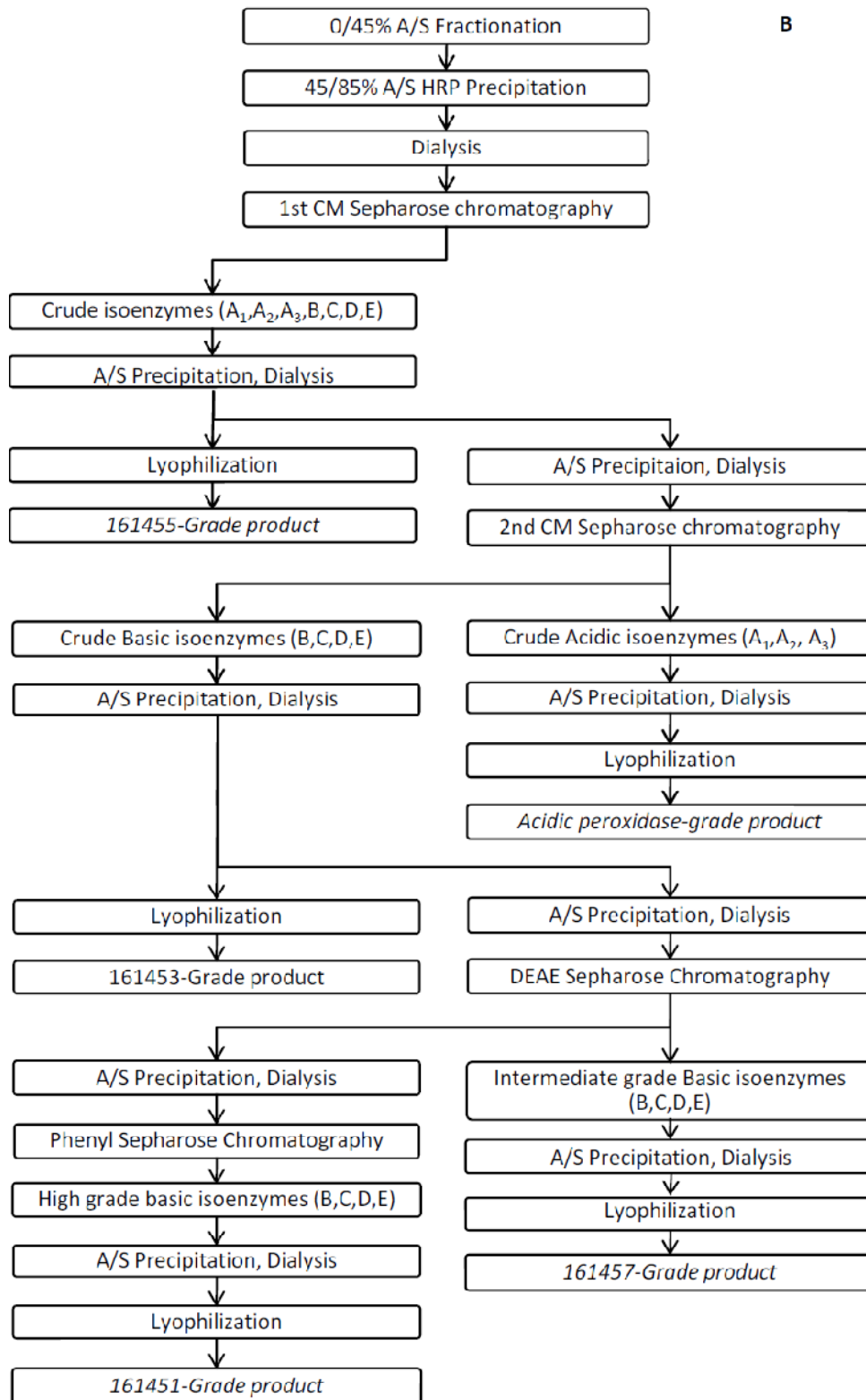


Figure 4. *Current secondary processing (purification) of HRP at BBI Enzymes.* The HRP product grades 161455, 161453, 161457 and 161451 will be discussed later in this document.

3.2. RAW MATERIAL QUALITY

When reviewing new horseradish raw material, the main parameters reviewed is the workability of the material over the primary production processes, HRP enzyme content and isoenzyme distribution, specifically isoenzyme C content. The rigidity of the root is of concern and reflects the level of lignification (36). This is of importance as more lignified roots show better workability and may be correlated to high levels of HRP. The colour may also be an indication of the degree of lignification of the root. The process of lignification is started mainly by the involvement of acidic peroxidases, whilst it is suspected that the initiation, due to hormonal stimulation, is controlled by basic HRP isoenzymes (37; 38). Lignification in roots, grown for a single season, may also be related to a higher HRP content, as reports in the literature suggests that HRP play an important role in the lignification and root formation (39). Evidence suggests that roots left in the soil to mature for an additional growing season yields lower HRP isoenzyme C (basic isoenzyme) content (2). This may be due to the down-regulation of HRP involved with the lignification of the plant during the rapid growth phase and stress response stimulated by the decrease in soil temperature during autumn and winter months (20). Down-regulation will take place once the plant has been protected to a level where it is resistant to the stressors (2). By standardizing the harvesting procedure, variability in the isoenzyme complement is reduced. This is of importance as certain customers require minimum levels of isoenzyme C. Failure to achieve this minimum level will lead to additional costs incurred whilst specifically purifying this isoenzyme, reducing the final product yield.

Historically, horseradish roots have predominantly been sourced from South African farmers, exclusively producing the material for BBI Enzymes. Trials have also been done on northern hemisphere sources of horseradish roots, including North American (U.S.A.), German, Hungarian, Polish, Israeli, British and Chinese sources.^d Sources other than U.S.A. were not favoured due to low workability, price and supplier capacity.

^d BBI Enzymes. *HRP Assay Log Books*. Cape Town : QC Department, 1958 - 2006.

In 2007, increased demand for HRP from BBI Enzymes, the seasonal availability of local raw material, storage costs and storage capacity lead to the identification and sourcing of horseradish roots from the U.S.A. In addition to identifying a second supplier of raw material, having a northern hemisphere supplier ensures a constant supply of fresh roots throughout the year. Initial lots of roots showed poor yields of HRP (Figure 5) and low workability. During the mincing and extraction of U.S.A. roots, received in 2007, it was observed that a higher concentration of finer particles (fines) was released into the aqueous extract. These higher levels of fines put increased pressure on the production equipment, as the fines needed to be removed prior to Ultra-filtration. The removal process was challenging, as the fines stay suspended, even after overnight settling of the extract. As a result, increased amounts of filtration aid (diatomaceous earth and potable water) and operating hours were required to clarify the material, increasing the product unit cost.

An investigation was launched into the poor yields from the U.S.A. roots, when compared to yields achieved from raw material obtained locally. Initially, a morphological comparison was performed (Figure 6). The morphological comparison highlighted a difference in the colour of the roots, as well as their rigidity. The causes for the lower workability and yields observed for this consignment of U.S.A. roots were proposed to be the variety cultivated, low lignification of the roots and the absence of stress prior to harvesting.

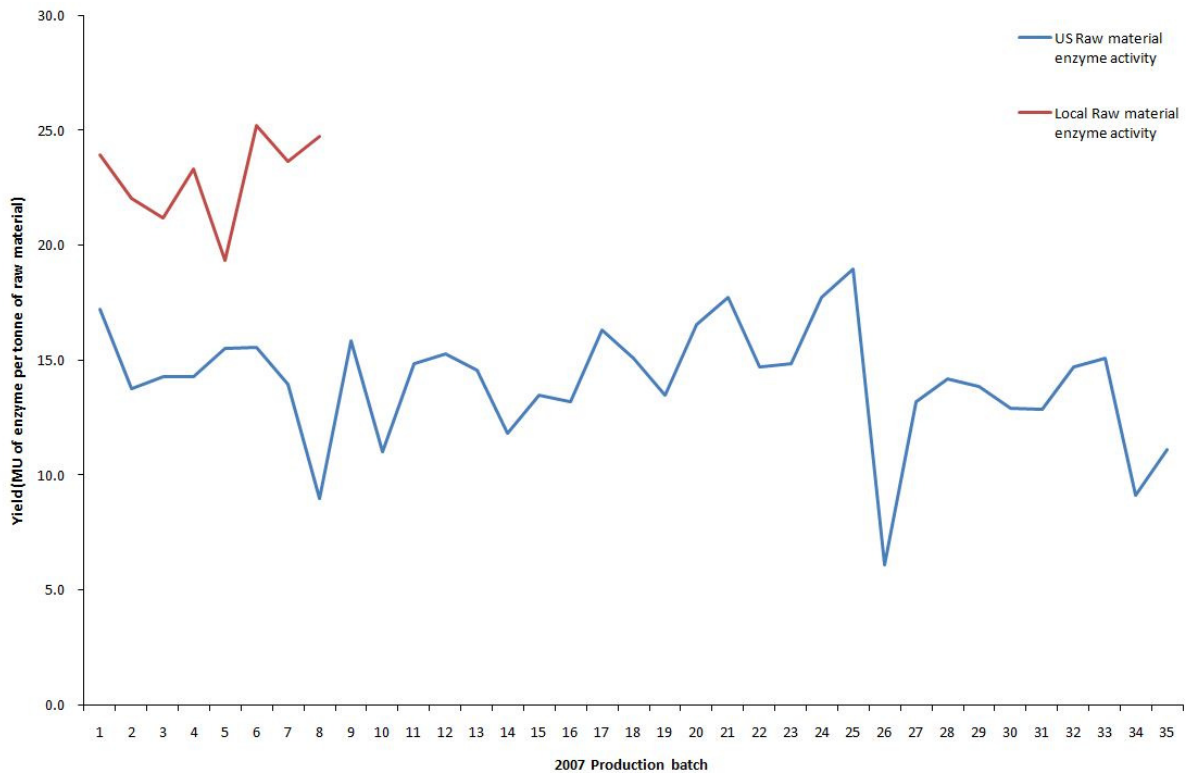


Figure 5. Comparative line graph indicating the HRP enzyme yield recovered at the end of primary processing per batch processed in 2007. The two lines represent local and U.S.A. sourced raw material. The process yield for locally sourced raw material follows the trend as illustrated by the 8 batches shown.

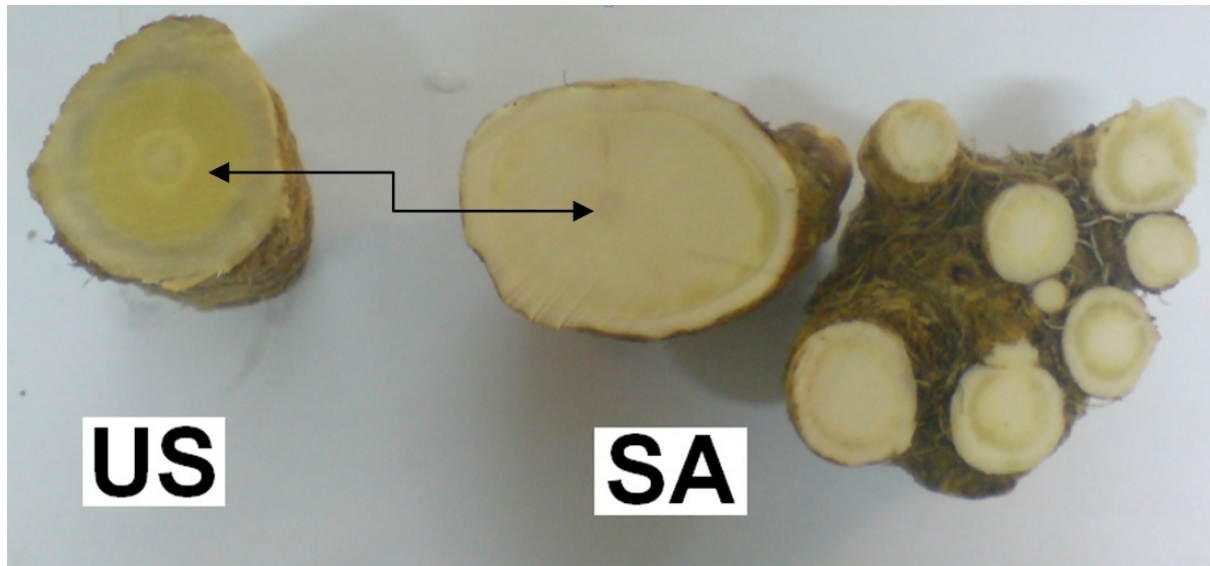


Figure 6. Cross-section of horseradish roots of local and U.S.A. source. The arrow indicates where the colour of the South African roots was significantly different to roots received from the U.S.A.

3.2.1. *Cultivation of horseradish*

Horseradish is cultivated commercially from root cuttings, typically about 15cm long. During the induction of development of the planted horseradish cutting, the total level of peroxidase activity increases (2). At the point where growth is initiated, the total level of peroxidase activity reaches a maximum. At this point, the level of basic peroxidases decreases, with the levels of the acidic peroxidase continuing to rise (2). A basal level of basic and acidic peroxidases remain present throughout the growing season of the horseradish, as there is a continuous need to expand the lignin support structure of the plant.

The increase in peroxidase activity and content in plants under stress have been well documented (40). Bastin demonstrated that an increase in peroxidase activity, in response to injury, takes place in most tissues (41). Gaspar *et al.* documented the stress response pathway in HRP (20) (Figure 7). It was observed that there is a cascade variation of isoperoxidases, starting with the immediate migration of basic peroxidases from the cytoplasm to the site of the injury, followed by the upregulation and increased concentration of basic peroxidases (20).

^e BBI Enzymes. Horseradish roots Raw material specification . *BBI Enzymes Quality Management System*. Cape Town : s.n., 2010, pp. BP6.4.2. - RM45.

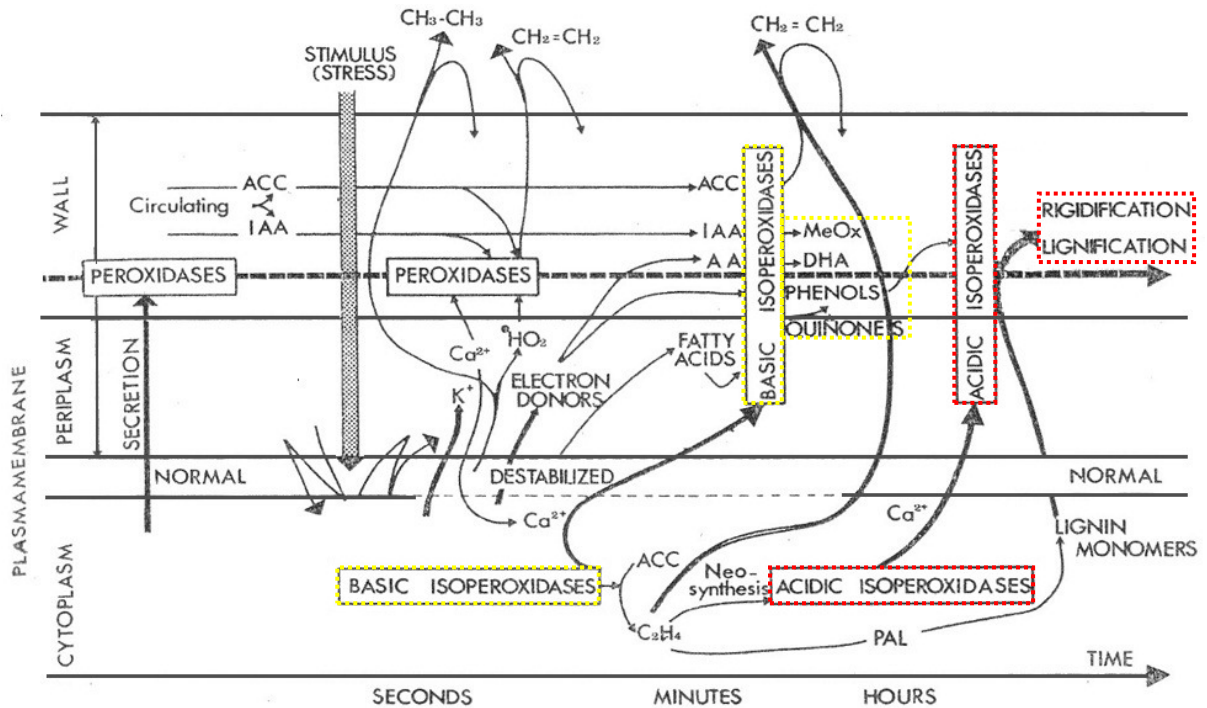


Figure 7. Illustration of the suggested general pathway in response to different physical and chemical stimuli in some cell compartments. It shows the two-step control of cathodic (yellow) and anodic (red) peroxidases and their interdependent roles in lignification, as mediated by ethylene. The vertical arrow indicates the stimuli. The arrow along the bottom of figure indicates the time elapsed (20).

This evidence support the theory for the production of high HRP content roots by introducing a stressor (defoliation, lower soil temperature) prior to harvesting and cultivation in areas where the plant is exposed to seasonal soil temperatures that drop below 10°C.

The cultivation practices of South African farmers also involve stress induction. The aim is to cultivate raw material with a high HRP, and specifically isoenzyme C, content. The protocol for stress induction and harvesting of horseradish cultivated specifically for high HRP concentrations is as follows:

1. *Harvesting commences once the first frost in autumn appears.* Soil (Environmental) temperature falls to below 50°F (10°C).
2. *Defoliation.* Remove all leaves from the plant (bush cutter), leaving the roots in the ground.

3. *Two weeks after defoliation*, harvest the roots (removal from soil).
4. *Freeze slowly* to at least 5° F (-15°C). This stops all cell activities and eliminates the down-regulation of HRP at the end of the stress response.^e

This procedure has ensured that the raw material that BBI receives is of consistent quality, enabling BBI Enzymes to predict the product output. The protocol, together with the requirement for varieties of horseradish root highly lignified root material in order to increase workability of the material, was presented to the U.S. growers.^f Compliance to the stressing/harvesting procedure is ensured by supplier quality agreements and annual audits of the facilities and documents.

The U.S. growers cultivate 3500 to 4000 acres of horseradish annually.^f This is predominantly for the consumer market and varieties are selected to contain the lowest levels of lignin (visual estimation by panel of growers, consumers) possible, as more rigid, barky roots are not wanted. Southern Illinois University (Illinois), in collaboration with the Illinois Horseradish growers, cultivated up to 3000 seedlings in greenhouses annually.^f Twenty-eight of these seedlings were selected by the Illinois Horseradish growers and propagated in test plots and four of these varieties were subsequently commercially cultivated.^f This has enabled the U.S. growers to supply BBI Enzymes with material from different varieties for production-scale samples in order to identify the variety that best fit BBI Enzymes' specification.

^f BBI Enzymes. Report on visit to U.S. Horseradish growers. Cape Town : s.n., 2008.

A total of four U.S.A. horseradish varieties were shipped to BBI Enzymes from the 2008/2009 harvesting season. The material was processed and two varieties were identified as the most suitable, 630D and K15[§]. Variety 630D yielded significantly higher levels of isoenzyme C than the minimum 40% content required, with a number of final product lots exceeding 55%. Variety K15 had significantly higher total enzyme content. Higher levels of total HRP enzyme were also shown in a number of the other varieties, but their workability was low and resulted in lower recoveries due to additional processing. As a result, growers were instructed to subsequently only cultivate 630D and K15 varieties horseradish for BBI Enzymes. This allowed for the review of the extraction procedures with reduced risk of variability in raw material.

[§] BBI Enzymes. *HRP Assay Log Books*. Cape Town : QC Department, 1958 - 2006.

3.3. EXTRACTION PROCEDURES

Horseradish peroxidase is extracted from horseradish by maceration of the roots, followed by suspension of the solids in a saline medium. The challenge to commercial extraction and purification practices lies in the liberation of the maximum amount of HRP, whilst limiting the amount of non-specific species extracted. Non-specific molecules can potentially reduce the performance of downstream filtration and purification techniques. The proposal was to determine the optimum level of maceration and feasible (achievable with processing equipment constraints) ratio between the macerated horseradish and the extraction medium. The downstream workability of the extract was also reviewed for each of the improvements investigated, ensuring that the changes made would not reduce the performance of the process downstream.

On a daily basis, 7 days a week, a minimum of 3.5 tonnes (single batch input) of horseradish roots are minced through a 10mm hole plate and subjected to extraction in 12500L of extraction medium consisting of: 12750L potable municipal water, 222kg of sodium chloride and 25L formaldehyde (bacteriostatic preservative).

The extraction of horseradish is performed at a root: liquid ratio of 3.6 to 1. Sodium chloride is added to increase the solubility of extracted proteins (42). Formaldehyde reduces the bioburden of the extract, limiting the risk of fermentation of the extracts during production. Extraction is performed for a minimum of 12 hours overnight. The raw material input, extraction time and methods for enzyme liberation was reviewed.

The temperature of the extract is not controlled and the addition of formaldehyde is of particular importance due to the seasonal variances in ambient temperature. The seasonal variance in ambient temperature for Cape Town (site location) is illustrated in Figure 8. The high temperature observed during the first and last months of the year lead to the extract

temperature rising to favourable conditions for microbial growth. The addition of formaldehyde has shown to have bactericidal and bacteriostatic effects on microbial growth (43)

It was determined that a 4 hour extraction period is sufficient for maximal extraction of HRP enzyme (Figure 9). No major increases in HRP activity in the extract was observed after 4 hours. This illustrated that the current extraction process could be shortened to 4 hours, in order to increase the daily raw material input.

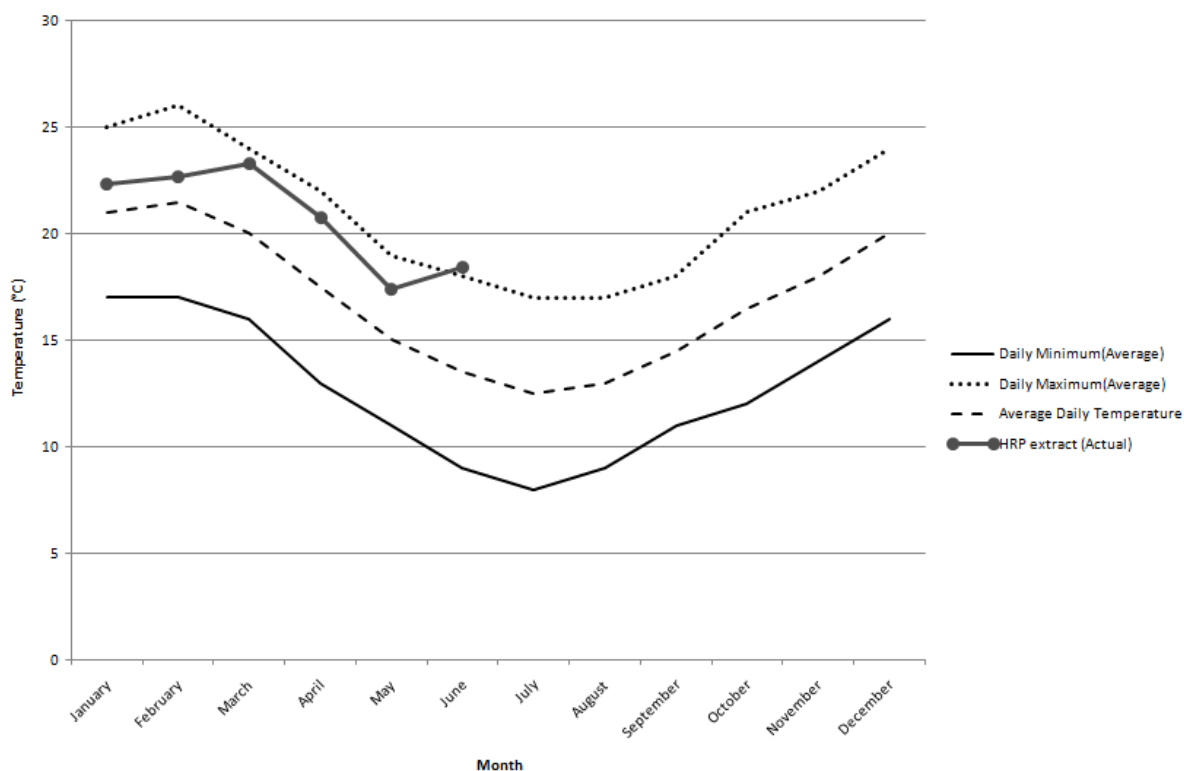


Figure 8. Average ambient and HRP extract temperature variances in Cape Town. The actual HRP extract, daily average, average minimum and maximum temperatures are shown (44).

A higher ratio between minced raw material and extraction medium (more liquid) was investigated. The hypothesis was that an increased diffusion gradient of HRP between the

raw material and the extraction medium may increase the efficiency of the extraction. Results obtained from extraction trials illustrated that a higher HRP content were extracted when using a higher ratio. The current average extracted HRP activity obtained by employing a 1: 3.6 ratio is 23.44 MU/tonne (t). An average of 42.2% more HRP activity (33.3 MU/t) was extracted using a 1: 5 ratio (Figure 10). A 1: 5 root to liquid ratio is the highest ratio achievable with the current equipment due to tank volume constraints. The proposal therefore would be to extract 2.5 t batches at a 1: 5 ratio rather than the current 3.5 tonnes at 1: 3.6 ratio. At the improved yield of 33.3MU/t, the total amount of HRP enzyme extracted per week when extracting 2.5tonne batches (83.3MU of HRP), would exceed that achieved when extracting 3.5tonnes at the current root to liquid ratio (82.0MU of HRP).

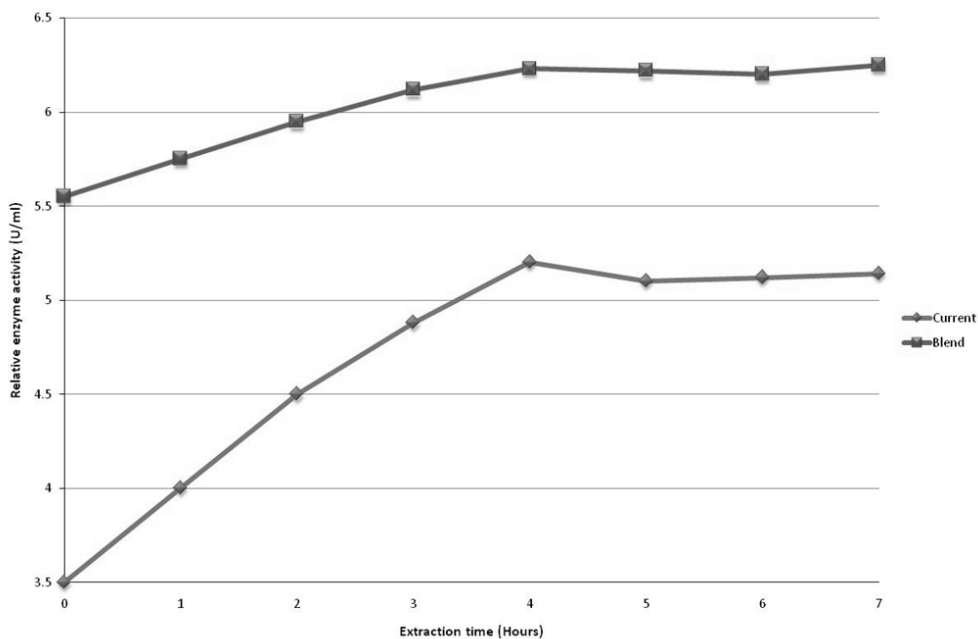


Figure 9. Increase in enzyme activity of the extract in the current process and when introducing blending of the roots. There is an increase in the initial free HRP in the extract after blending.

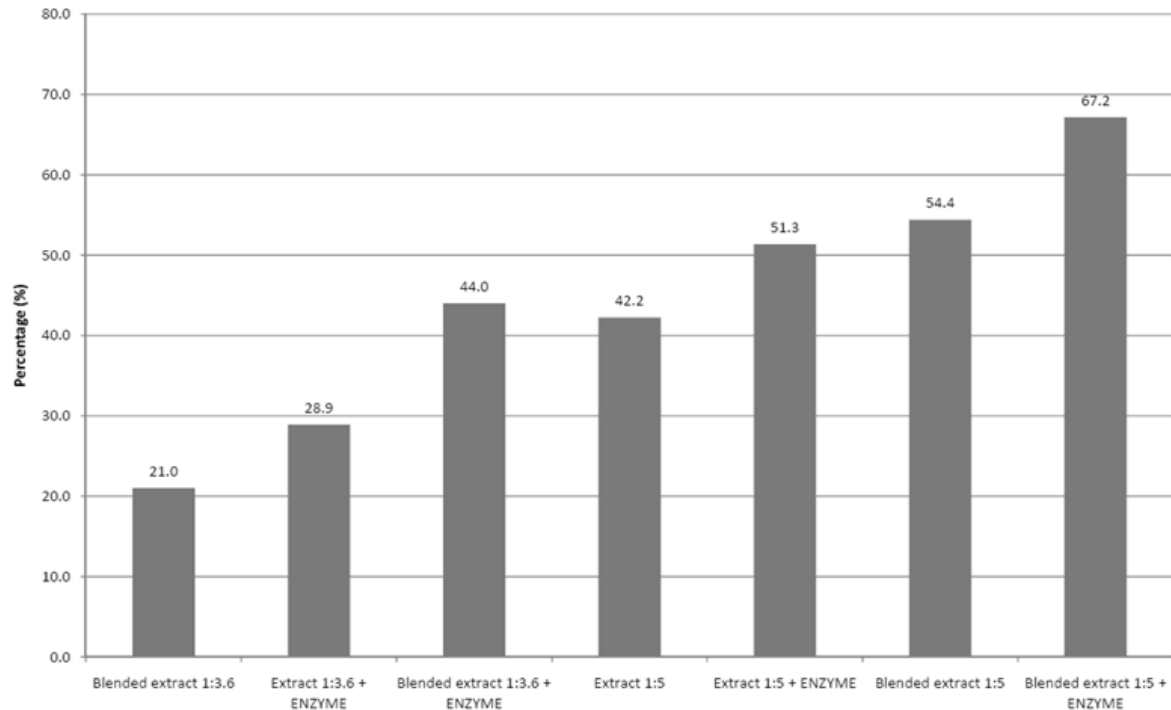


Figure 10. *Percentage improvement in extracted HRP after 4 hours.* The current extraction performance (control) was compared to extraction after additional maceration (“Blended”), a higher liquid to root ratio (“1:5” ratio), in the presence of cell-wall macerating enzymes (“ENZYME”) and combinations of these possible improvements. Results shown are averages from multiple repeats of the experiments

The degree of agitation of the extraction medium affects the amount of HRP released by the roots. By simply introducing more vigorous stirring, there is a 6.6% increase in activity. After the effect of additional vigorous stirring was observed, attention was aimed at finding more efficient methods of liberation of the enzyme. Blending (homogenization of the raw material to a fine pulp), as well as enzymatic maceration was investigated. It was found that blending of the raw material instantly liberated about 89% of the extractable HRP (Figure 9). The increase in total extracted HRP enzyme was 21% higher than what is achieved in the current process. Blending of roots at a ratio of 1: 5 resulted in a 54.4% increase in extracted HRP.

Pectinases are a group of enzymes that degrade pectin, a polysaccharide that is found in the cell walls of plants. The hypothesis was that the addition of a cell wall macerating enzymes to the extraction medium would increase the liberation of HRP from the cell wall, as Kawaoka

et al. illustrated that the levels of HRP in the soluble fraction horseradish decreased during wounding (45). It was also shown that the HRP content of the cell wall fraction increased significantly, supporting the proposal for pectinase digestion as part of extraction (45). It was found that the addition of pectinase increased the amount of HRP liberated by 28.9% and 51.3% for a 1:3.6 and 1:5 ratio extractions, respectively (Figure 10). However, the digestion of the pectin cell support structure would ultimately lead to higher levels of fines, reducing the workability of the extract. Additional liberation of fines requires a larger filtration surface than what is available during clarification.

Introducing a foreign enzyme would also be seen as a major change to the current process, and extensive validations would be required by customers before they will accept this major change to the process. The cost of the addition of enzymes would also be high, as the levels of pectinase enzyme, shown to increase liberation of the enzyme significantly, are 240mg per litre of extraction medium (46). In addition the introduced enzyme will also have to be removed later in the process and relative amounts of enzyme, still present after removal, would need to be tested. It was also found that some pectinases show cross reactivity with the Guaiacol assay used for in-process control of HRP (47).

After evaluating the different methods used to increase the efficiency of the extraction process, a combination of the improvements was trialled together. In light of the risk involved with the inclusion of a foreign enzyme in the extract during the increase in extraction efficiency, only changing maceration and the liquid to root ratio, were evaluated. A combination of additional maceration (blending), a higher liquid to root ratio (1: 5) and the addition of pectinase enzymes resulted in a 67.2% increase in extracted HRP. Extraction of blended material at a higher liquid to root ratio increased the extracted enzyme level by 54.4%. A higher liquid to root ratio and increased maceration of the roots was implemented.

3.4. CONCENTRATION OF HORSERADISH EXTRACT

One mechanism of concentration is tangential flow filtration (TFF). This process can be performed in combination with A/S fractionation and chromatography to enhance either product yield or purity. In tangential flow or cross-flow filtration, the feed is passed across the filter membrane (tangentially) at positive pressure relative to the filtrate side (Figure 11). Compounds in the feed with molecular masses lower than the membrane pore size pass through the membrane as permeate or filtrate; all other molecules/compounds should be retained on the feed side of the membrane as retentate. The tangential motion of the bulk of the fluid across the membrane causes trapped particles or foulants on the filter surface to be removed. This means that a cross-flow filter should operate continuously at relatively high solids loads without blinding or fouling.

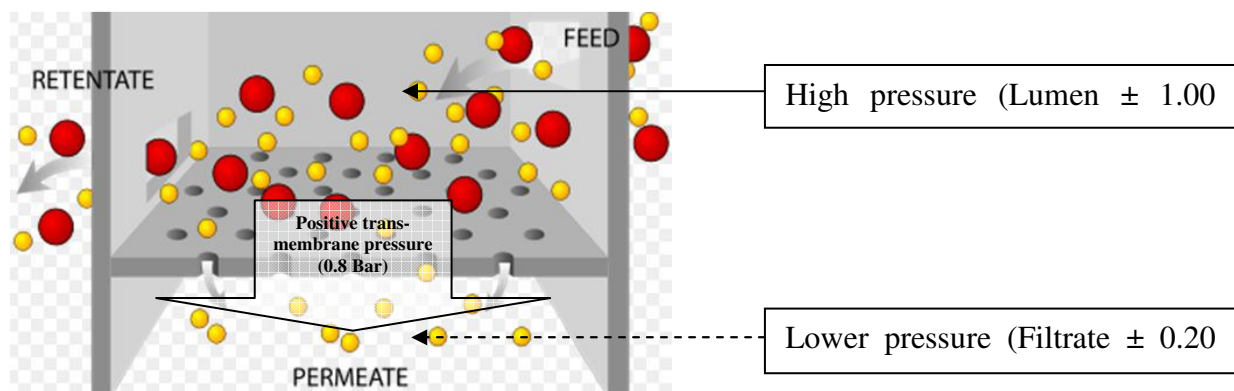


Figure 11. *Diagram illustrating the principle of TFF. Feed is passed across the filter membrane (tangentially) at positive pressure (e.g. 1.00 Bar) relative to the filtrate side (e.g. 0.20 Bar). This creates a positive trans-membrane pressure, favouring filtrate flux (48).*

In order to process the extract through the secondary stages of the process, the volume needs to be decreased by concentrating the clarified horseradish extract at an economically acceptable rate of flux. Previously, $\pm 16000\text{L}$ of clarified extract was concentrated to 15 – 30% of its original volume. The expected concentration to 10% of the original volume was not achieved due to inefficiencies of the ultra-filtration (UF) system employed. An investigation was conducted into the identification of a suitable replacement system, as well

as the optimum conditions for the effective, sustainable concentration of the clarified HRP extract. The increase in efficiency of the system also needed to double the capacity of the concentration step to allow for HRP processing at a higher throughput. The required capacity for the reduction in extract volume of the new UF system would need to be 1600L/hour, in order to reduce 32000L (two extracts) of extraction medium to 3200L in 18 hours.

3.4.1. Membrane fouling

As discussed in the review of the raw material, it is important to process roots that are well lignified. By doing so, less fines are generated that needs to be removed during clarification. Any level of fines not removed during clarification, directly affects the performance of the UF system due to membrane fouling (Figure 12). Fouling is a process where the membrane surface and pores are coated and blocked by the deposit of particles present in the solution filtered. The starch content of the roots, when harvested at the incorrect time of the season, also has a major effect on the rate and onset of fouling. Harvesting in the autumn, when there is overnight freezing, can alleviate this problem as the lower ambient temperature promotes the conversion of sugars to starch (49). The main factors that influence the onset of membrane fouling and membrane performance is:

- Nature of the solution (feed) filtered.
- Temperature.
- pH.
- Hydrodynamic environment of the feed.
- Transmembrane pressure (TMP).
- Feed rate.
- Filtrate flux.
- Membrane properties.
- Hydrophobicity or hydrophilicity of the membrane surface molecules.
- Surface charge.
- Pore size and distribution.

The onset and rate of membrane fouling is measured by monitoring the TMP and filtrate flux. As the membranes foul, the membrane permeability reduces and a reduced flux can be observed. As a result of the reduced pressure dissipation across the membrane due to the reduced flux, the TMP also increases.

Bourgeois *et al* described three types of membrane fouling as gel/cake formation, pore plugging and pore narrowing (50). Gel formation is the term describing a coating of the whole membrane (concentration polarization) with a layer of particles larger than the membrane pores, with pore plugging and narrowing describing the effect of smaller particles on the membrane pores (Figure 12). The most important factors to be considered in fouling reduction are: flow conditions, extract pre-treatment, rinse water quality, membrane properties and operational factors (51).

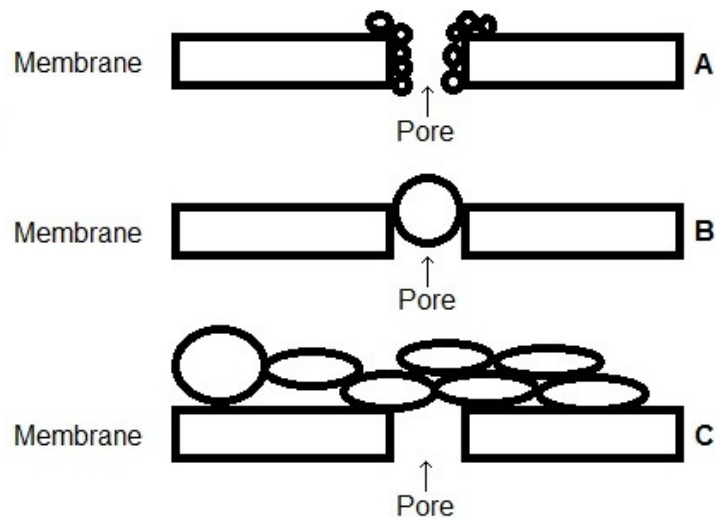


Figure 12. *Mechanisms of membrane fouling*. Three are illustrated: “A” pore narrowing; “B” pore plugging; and “C” gel formation (50).

By increasing the cross-flow velocity over the membrane surface and reducing the TMP, the thickness of the polarization layer is reduced (51; 52). This is achieved due to the reduced applied pressure allowing for the adsorption of the foulant and shear force that reduces the

secondary membrane by collision with unbound particles in the extract. It is, however, important to always consider the amount of energy (electricity input) and change in equipment required for the change in hydrodynamics.

The horseradish extract is pre-treated by filtration. Diatomaceous earth filter beds are prepared in a Pressure Leaf Filter and Cloth filter Press. The first bed is prepared using a coarser powder (Celite[®] 545), retaining particles up to ± 4 micron (nominal) in size (53). Final clarification through a Celite[®] Hyflo Super-cel filter bed reduces the nominal particle size in the extract to ± 1 micron (53).

The pH of the extract is currently monitored to be within the pH range of 5 – 6. Herrero *et al* concluded that filtration at a pH far from the isoelectric point should reduce the rate of onset of fouling (54). Following trials performed on the optimization of extract pre-treatment, it was found that the pH of the extract during pre-treatment is important. Maintaining a pH closer to the upper limit of the primary processing pH range showed increased performance (percentage reduction in volume per hour) during ultra-filtration (Figure 13). This may be due to the increased aggregation of impurities at the higher pH, leading to removal of a higher concentration of non-specific molecules during clarification. The lower concentration of impurities would ultimately reduce the rate and extent of fouling. As a result, the pH range over the primary stages of the process was amended to be 5.5 - 6.0.

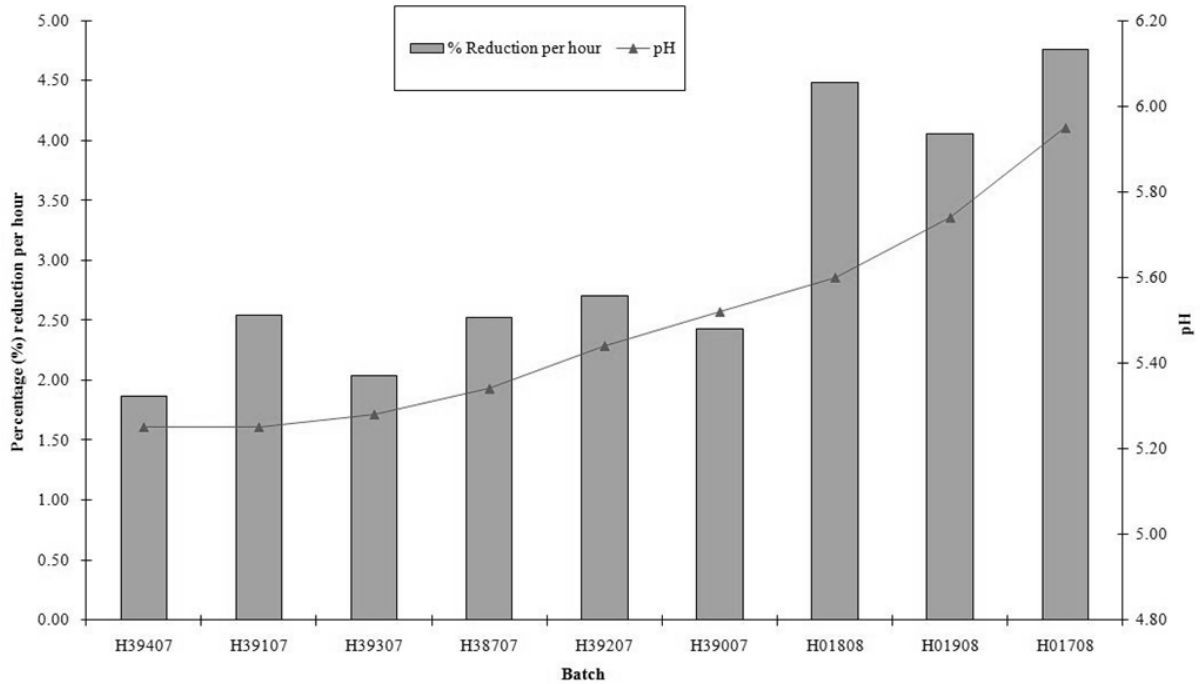


Figure 13. Concentration performance: Percentage reduction (per hour) in extract volume at a certain pH during clarification and UF. The pH shown was maintained during clarification and UF.

3.4.2. UF System development

Trials were conducted on a small-scale (0.1m^2 membrane surface area, 30cm fibre length) using membranes from three manufacturers of tangential flow filtration (TFF) systems, General Electric (GE), Pall Corporation and Sartorius. GE and Pall validated hollow fibre membranes, whilst Sartorius focussed on membrane cassettes. These trials would allow for the identification of a membrane capable of concentrating clarified extract, whilst retaining HRP activity. Initially, a nominal water permeability (NWP) run was performed to establish a benchmark for determining the efficiency of the cleaning procedure, as well as monitoring of the performance of the membranes after use (Figure 14). This experiment was performed once prior to use of the membrane.

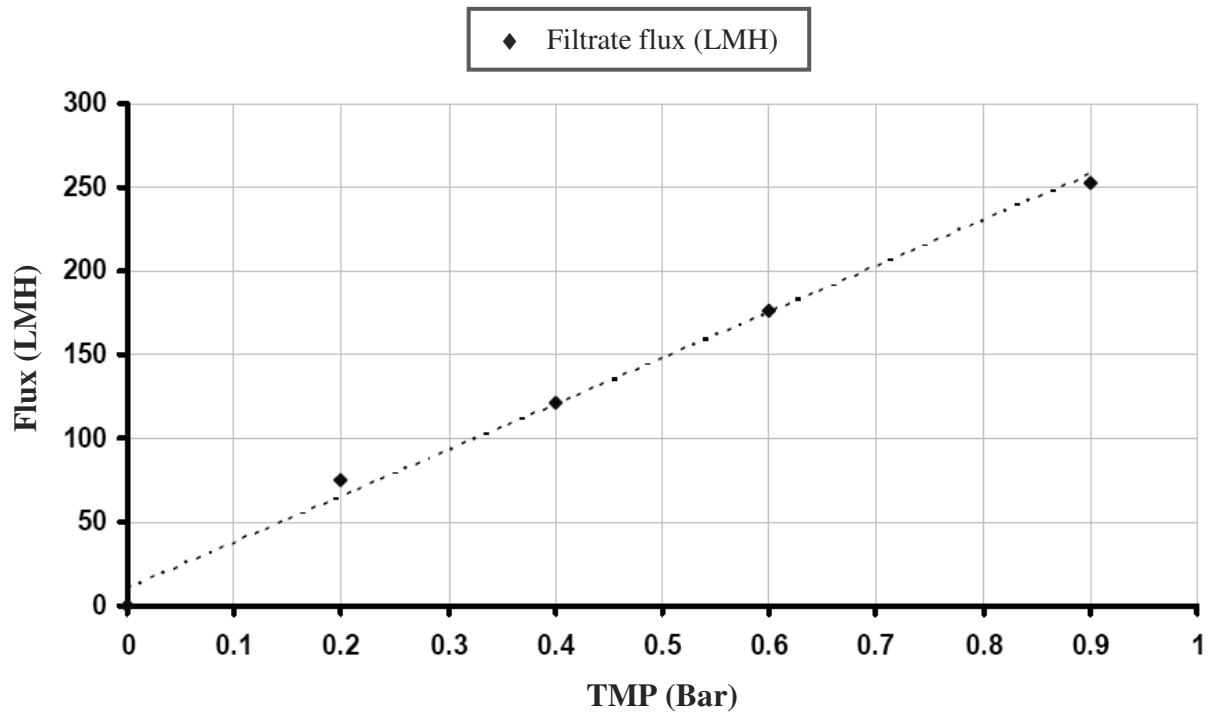


Figure 14. *Initial NWP data of Pall Microza SLP 3053 polyethersulfone (PES) membranes with clean water.* The linear correlation between TMP and filtrate flux indicate a clean membrane.

All three vendors validated MWCO membrane modules of 10kDa. The membranes were manufactured from polyethersulfone (PES), a hydrophilic, thermoplastic polymer. PES was chosen for its resistance to pressure, low protein retention and wide range of pH stability (pH 1 – 14) (55). The low protein retention property of this type of membrane is ideal for the processing of proteinaceous biological material. During ultra filtration with membranes from all three manufacturers HRP activity was retained. A 10 times concentration (and higher) of the clarified extract was observed without the cartridges/cassettes showing severe fouling during the initial run. However, the membranes could only recover 85% of their original NWP after the second product run. Additional cleaning could not recover the lost performance. This may be attributed to the irreversible blocking of intra-membrane pores (internal fouling) with small particles.

An optimization plot shows the relationship between permeate flux and TMP and illustrates the TMP at which the relationship is not linear (Figure 15). The point at which the relationship between TMP and filtrate flux is not linear indicates the maximum efficiency

(filtrate flux) at the TMP investigated. A cross-flow of 1m/s and 1.5m/s was selected and a filtrate flux of 66L/m²/hour (LMH) was achieved at 1.5m/s (Figure 15).

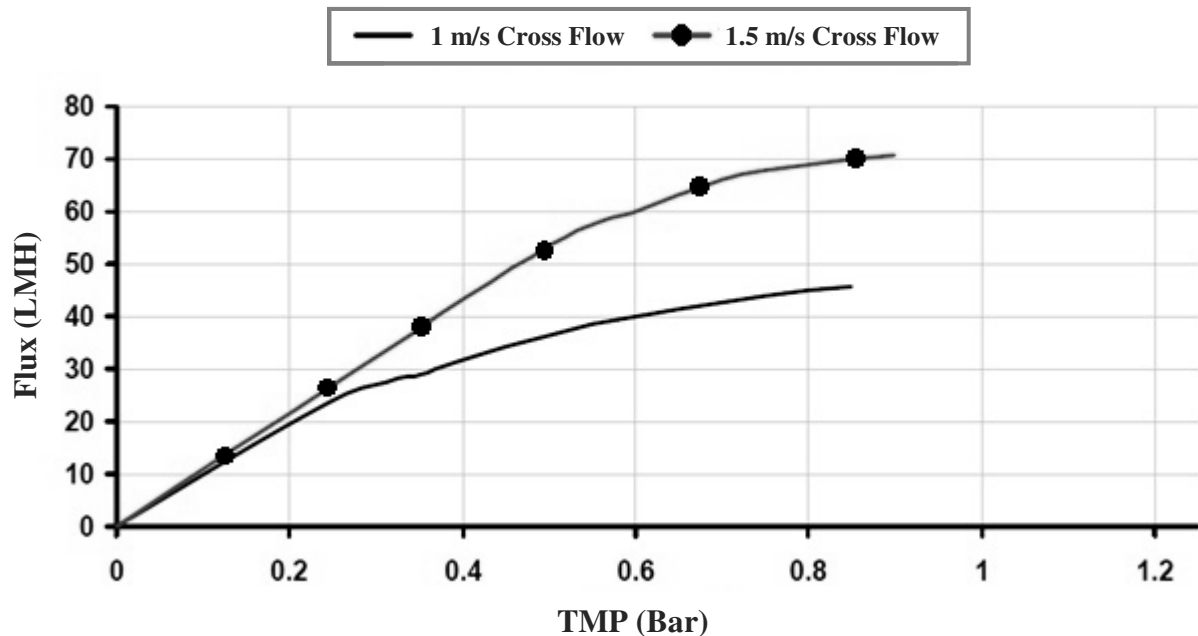


Figure 15. Optimization plot for Pall Microza SLP-3053 PES membranes with clarified HRP extract as feed. The Filtrate flux at various TMP setpoints is illustrated for a 1m/s and 1.5m/s crossflow.

The appropriate cross-flow was determined after taking into consideration the following disadvantages and advantages of high feed flow in the application:

- Higher cross-flow will lead to higher shear (more passes of product through pump).
- Increased energy input (larger pump, more electricity).
- Larger piping, leading to larger hold-up volume and indirectly product losses (unrecoverable holdup).

On the basis of the trials performed, it was shown that an average filtrate flux of 40LMH could be achieved with a new membrane (Figure 16). In order to achieve 1800L/hour flux, a minimum membrane surface area of 45 m² would be required. A surface area of 72m² was

proposed. This figure was extrapolated from the initial concentration plot, taking into account the 10-15% loss in performance due to irreversible fouling of smaller intra-membrane pores observed in the trial membrane modules after continuous use. Due to the increased path length of the full-scale membrane modules (1129cm in length compared to the 30cm trial module), a reduced average filtrate flux is expected and additional 25% membrane surface area was included. A 15% buffer capacity was also incorporated under recommendation of the supplier.

A higher crossflow was not investigated, as the required energy input to achieve higher flow rates would not be sustainable. The largest rotary lobe pump commercially available would also not be able to achieve such high feed rates for a system with the required amount of membrane surface area.

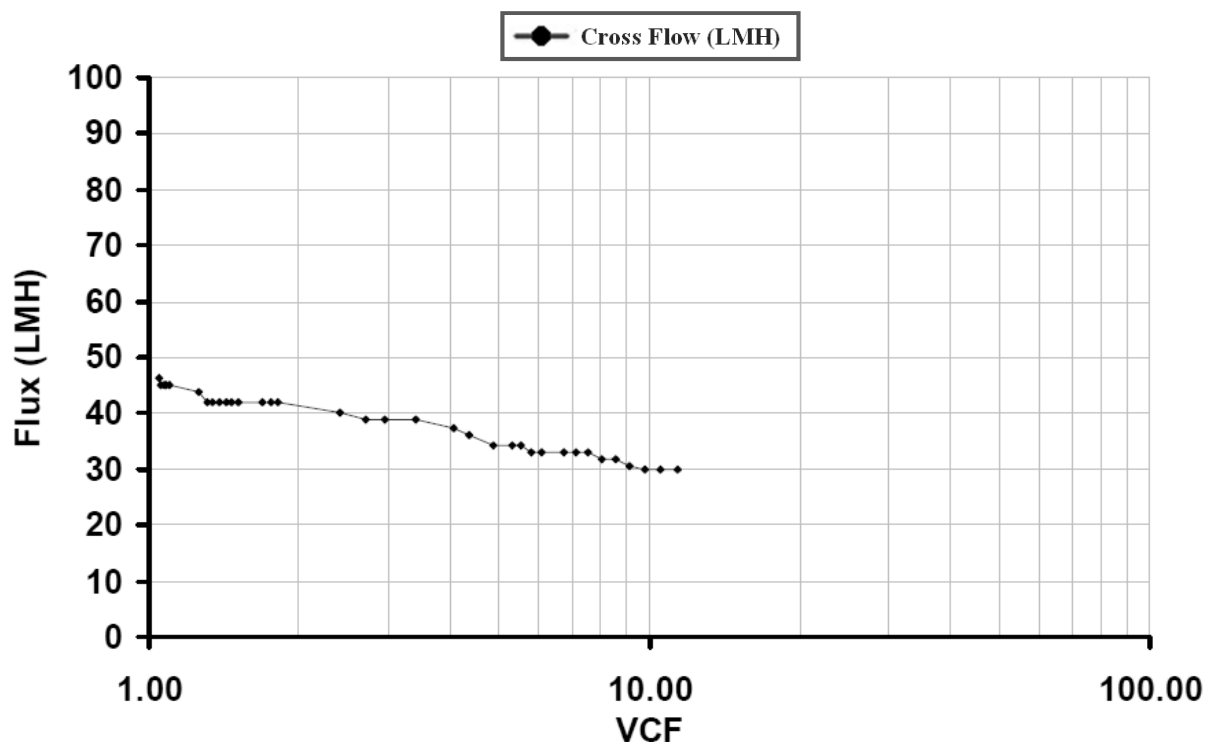


Figure 16. Concentration plot illustrating the filtrate flux as a function of the log volumetric concentration factor (VCF) with clarified HRP extract. A gradual decrease in flux is shown as the VCF increases.

Pall Life Sciences was identified as the preferred supplier for the large-scale unit. The main reason for the decision was lead-time on delivery of the large-scale UF system and cost (Figure 17).

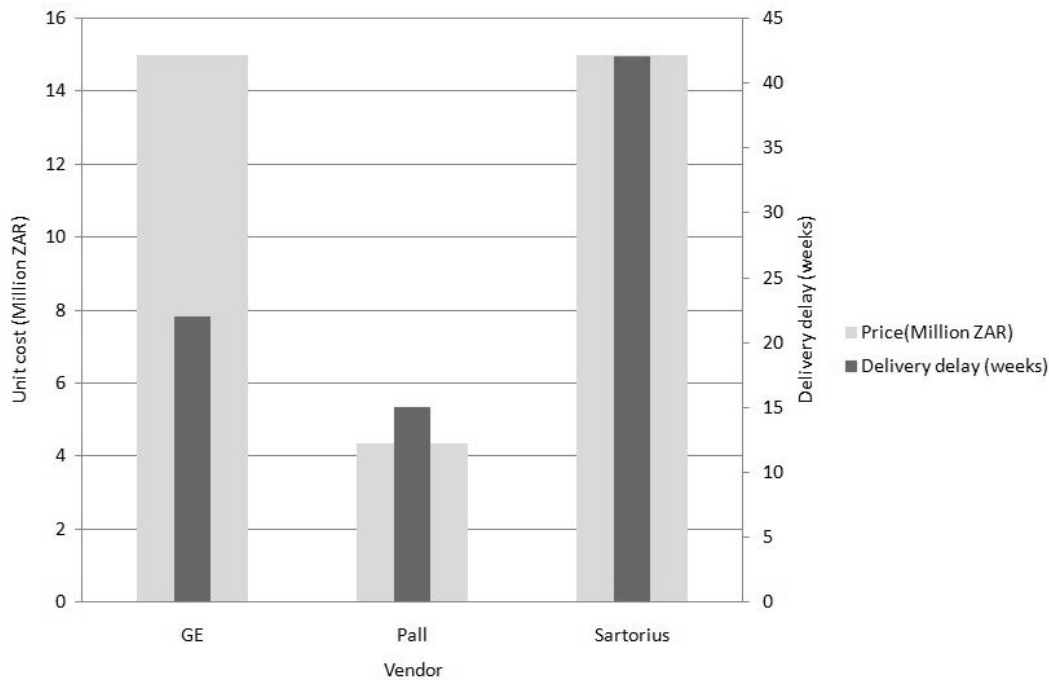


Figure 17. *UF system vendor comparison.* The cost and time to delivery of a system that meets BBI Enzymes' requirements are illustrated.

Pall Microza membranes have properties identical to a PES membrane, with the additional benefit of being a double-skin tube. This unique membrane construction increases the MWCO specificity and increases the strength of the fibre. The double skin allows for reverse filtration, specifically reverse-flushing in this application, to break down foulant build up.

A fully automated Pall Microflow 20/16 AUTO UF system was acquired. This system was fitted with 16 Pall Microza SLP3053 Membranes (72m² membrane surface area), with the option of increasing the membrane surface area to 90m². The system was fully automated and required only the delivery of the extract to the inlet, as well as batch information input. Due

to the lower shear rate observed for a rotary lobe pump, when compared to a centrifugal pump, this type of pump was fitted in all applications where product was pumped. Parameters that could be adjusted and controlled were:

- Filtrate flux (L/min).
- System pressure (Inlet pressure (P_{in}), Outlet pressure (P_{out}) and filtrate pressure).
- Concentration factor.
- Cross-flow velocity by means of controlling the pump speed (as a percentage of the maximum rate).
- Backflush intensity and frequency.
- Clean in place temperature.
- TMP.

During the performance qualification of the Microflow system, the process conditions were optimized. Different trial runs were performed. During the first runs, it was observed that the performance of the system was reduced due to excessive fouling. It is the view that this was due to concentration polarization of the proteins in the extract (54). The “closed system” approach of the Microflow system was one of the main impediments to the optimal performance of the system. During concentration, the concentrated retentate is not fed back to the extract holding tank, but to a small UF system holding tank (3500L). As the extract volume in the UF system is reduced, the system tops up the 3500L holding tank with balance material in the larger 30000L holding tank. This approach entails that the concentration in the system (3500L holding tank plus circulation loop), is significantly higher than that of the total extract in the 30000L holding tank and system combined, from the start of production when the first filtrate is generated. For example, if the total volume of clarified extract in the UF system (3500L tank plus circulation loop) is 3650L, and after approx. 4 hours 3650L of filtrate has been produced, the effective concentration factor (CF) in the UF system is ± 2 . However, the actual CF of the total volume of extract is only 1.14 (if the total extract volume to be concentrated is 30000L).

A number of system and process parameter settings were tested in order to optimize the UF process (Figure 18). The system and process parameters settings changed between trial runs were crossflow, TMP and whether the system was run as an open or closed unit or whether a pit stop was performed (Table 3). The trial runs performed were:

1. *A Cycle mode “Pit Stop”* run with the total batch volume divided into parts. A pit stop involves stopping the concentration run, recovering the concentrated product to a holding tank and performing a hot water rinse of the system. After completion of the rinse, the concentration run is resumed.
2. *Open system.* The CF of the liquid inside of the 72m² membrane surface area UF system is spread over the total volume of the clarified extract batch by allowing for bleeding of concentrate from the UF system back to the 30000L extract holding tank.
3. *Open system with a higher cross-flow.* The system is open as in Trial 2, but at a higher cross-flow rate.
4. *Open, higher membrane surface area system and crossflow.* The system is open as in Trial 2 (above), but 4 additional membrane modules were fitted to the system, increasing the total membrane surface area to 90m². The system is also run at a higher cross-flow rate.
5. *Open higher membrane surface area system, with adjusted TMP and high crossflow.* The system is open as in Trial 2 (above), but 4 additional membrane modules was fitted to the system, increasing the total membrane surface area to 90m². The feed rate and TMP are both higher.

Table 3. *System and process parameter settings used for each trial run.*

Trial	Open or closed system	Pit stop performed	Feed rate (% Pump speed)	Crossflow (m/s)	TMP
1	Closed	Yes	75%	1.2	0.9
2	Open	No	75%	1.2	0.8
3	Open	No	93%	1.5	0.8
4	Open	No	93%	1.5	0.8
5	Open	No	93%	1.5	1

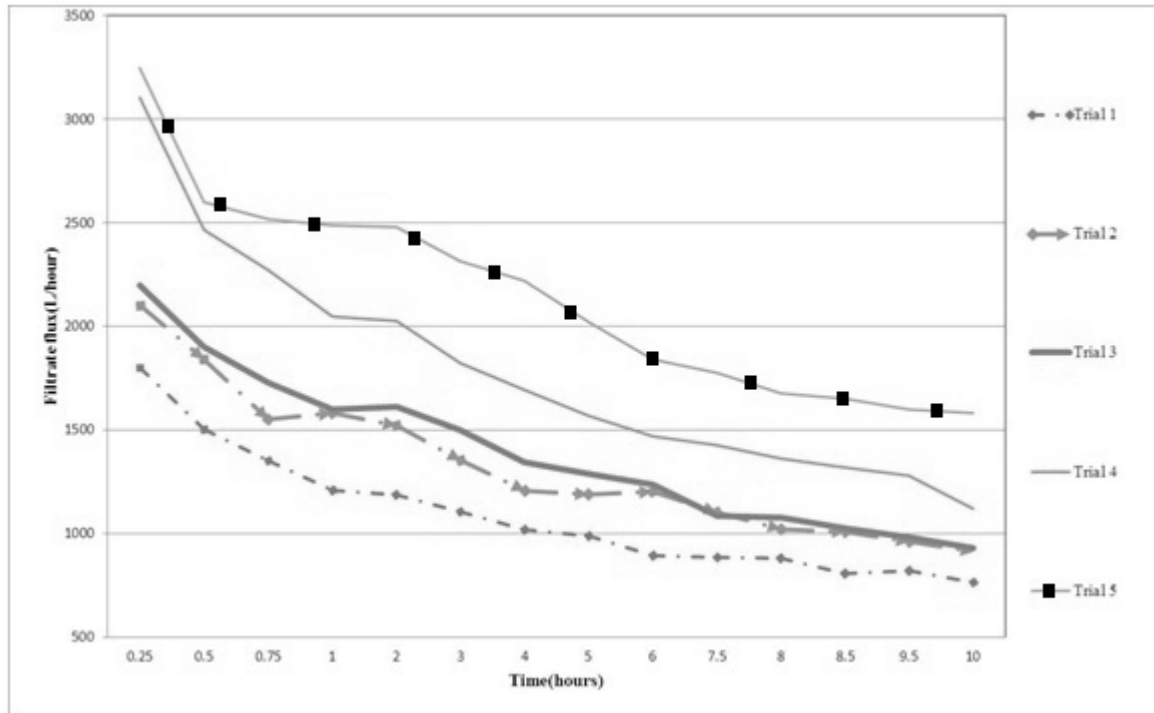


Figure 18. *Filtrate flux (L/hour) comparison all trial runs.* The differences in conditions for the trials are discussed earlier in this document.

It was found that a combination of higher membrane surface area, high crossflow, TMP of 1 Bar and the implementation of an open system was the most efficient setting for the concentration of clarified extract (Trial 5). The Microflow parameters were therefore set to the following:

- Filtrate flux (L/min) – No control
- System pressure
- Inlet pressure (P_{in}) = 1.1 Bar
- Outlet pressure (P_{out}) = 0.9 Bar
- Filtrate pressure = 0 Bar
- Concentration factor = 10
- Cross-flow velocity = 1.5m/s

- Backflush intensity and frequency during production = Every 20 minutes, for 15 seconds
- Production maximum temperature limit = 30°C
- Clean in place (CIP)
- Chemical composition = 0.5M Sodium hydroxide (NaOH), 500ppm Sodium hypochloride (NaOCl)
- Temperature of CIP solution = 45 – 55°C
- TMP = 1 Bar

During the first month of operation of the optimized UF system, input cost of the production line was reduced by ZAR1 133 064. The main contributor to this saving was the reduced volumes of concentrate subjected to downstream A/S fractionation.

3.5. AMMONIUM SULPHATE FRACTIONATION

The separation or fractionation of proteins in an aqueous solution with the aid of A/S is one of the most common initial separation techniques employed during the purification of proteins. The sulphate ion of A/S is kosmotropic, making this a kosmotropic salt. Kosmotropic molecules aid in stabilizing water-water interactions, leading to the increased intermolecular interaction of proteins (56). The increased interaction between the proteins leads to aggregation and subsequent precipitate formation. The solubility of the different proteins in an aqueous solution is exploited during this process. Ammonium sulphate is preferred due to its stabilizing effect to proteins, availability, price and its solubility (57).

During the primary processing of HRP extract, a two-step A/S fractionation is performed. The percentage saturation is calculated using temperature dependent tables prepared according to work published in Data for Biochemical Research (58). The first, saturation to

45% is aimed at removal of unwanted molecules. The material is clarified through diatomaceous earth to retain the precipitated material. HRP in the filtrate precipitates at 85% A/S saturation. During the optimization of the concentration step previously described, the final CF of the extract was increased from 3 to 10. To determine the percentage A/S saturation of HRP extract that would lead to maximal recovery of HRP, whilst removing the maximum amount of unwanted protein, both the CF 3 and CF 10 HRP extracts were subjected to A/S saturations ranging from 30% - 45%. To determine the percentage A/S saturation of HRP extract material that would precipitate 95% of the total HRP activity, both CF 3 and CF 10 extracts were subjected to A/S saturations ranging from 70% - 85%. Trials performed on the CF 10 extract illustrated that saturation percentages of 45% and 75% was sufficient for recovering the maximum HRP during A/S precipitation (Table 4).

Table 4. *Experimental and Production data and interpretation of A/S fractionation of concentrated (CF of 10) extract.* Activity shown was the total activity measured in one millilitre of extract. A correction of the results for the fractionated samples was incorporated to compensate for the dilution effected by the addition of ammonium sulphate.

Sample stage	Experiment 1		Experiment 2		Experiment 3		Average	Production	
	Activity (U/mL)	Percentage recovery (%)	Activity (U/mL)	Percentage recovery (%)	Activity (U/mL)	Percentage recovery (%)	Percentage recovery (%)	Activity (U/mL)	Percentage recovery (%)
Concentrate	11.68	100	21.68	100	23.89	100	100	66	100
35% Satn. supernatant	11.52	110	20.63	106	20.94	98	105	65.5	110.65
45% Satn. supernatant	10.23	102	17.92	100	18.28	101	101		
35/70% Satn. supernatant	0.063	99	0.12	99	0.412	98	99		
35/75% Satn. supernatant	0.022	100	0.028	100	0.222	99	99	1.5	97.06

These results illustrate that the fractionation of more concentrated HRP extract at the current A/S saturation of 45% ensures the HRP recovery over the stage, while keeping the amount of non-specific protein carried to the chromatography stages at the same level as in the current process. The precipitation of HRP from a more concentrated solution was also achieved at a lower 75% A/S saturation. This reduces the A/S consumption of the process and leads to an average monthly saving of ZAR10 500. The saturation of the clarified 45% saturated concentrate to 75% and not 70% saturation was implemented to reduce the risk of product loss. During large-scale production, the 75% A/S filtrate is only sampled for reference and pumped to a waste tank.

In addition to the optimization of the percentage saturation, the variance in temperature of the extract, due to daily and seasonal changes in ambient temperature, on A/S precipitation efficiency of HRP extracts was also considered. There is an 8°C ($\pm 1^\circ\text{C}$) difference between the daily minimum and maximum temperatures and an 18°C difference between the maximum temperature of the hottest month and temperature minimum of the coldest month (44). Extract temperatures, however, rarely exceed a maximum of 25°C and minimum of 15°C, due to the insulation and height of the HRP facility roof. The current method for A/S fractionation of the HRP extract fractionates all extracts using the concentrations (grams/L) of A/S calculated for fractionation at 25°C (59). When fractionating a batch of extract at 15°C, with the A/S concentration calculated for fractionation at 25°C to the same percentage A/S saturation, the error is 0.5% and 4g/L less A/S is needed for the required saturation. This higher percentage saturation will result in a loss of enzyme due to precipitation together with the discarded fraction and an increase in the cost of processing a 1500L batch of extract by R27.00. This figure is insignificant compared to the total cost of processing a batch of HRP extract to final product. However, taking into consideration the average annual processing of 269 batches of HRP during 2010-2011, this would lead to an additional cost of R7209 or 1.59 tonnes of A/S.

The use of ammonium sulphate for the initial fractional purification of HRP leads to a challenge ahead of downstream chromatography: reducing the ionic strength of the extract. Diafiltration was reviewed to replace the traditional membrane tube dialysis that relies on diffusion.

3.6. DIAFILTRATION

Diafiltration (DF) is a tangential flow filtration (TFF) process that can be performed in combination with A/S fractionation and chromatography to enhance either product yield or purity. During DF, buffer is introduced into the retentate tank while filtrate is removed (Figure 19). In processes where the product required is in the retentate, diafiltration can be used to

remove unwanted low molecular weight contaminants and affect a buffer exchange. When the product is a membrane permeable molecule its passage through the membrane and retention of larger, non-specific contaminants lead to purification.

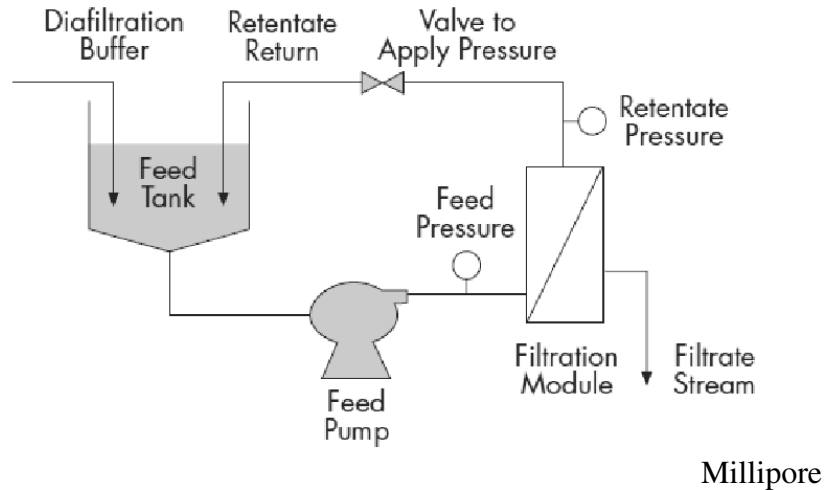


Figure 19. Diagram illustrating the different components of a Diafiltration/TFF system (60).

3.6.1. Application of DF in HRP purification

Currently, a three-step process consisting of 85% saturated A/S precipitation, filtration of the precipitate and passive membrane tube dialysis (diffusion process) is followed between all chromatography steps (Figure 20). This process accounts for approximately 15 days of production during the manufacture of the highest grade of HRP (161451-grade) produced by BBI Enzymes.

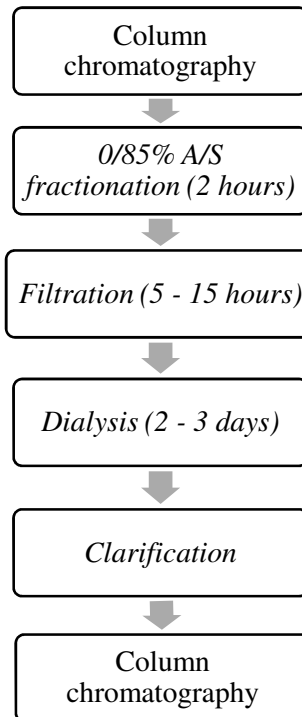


Figure 20. Diagram illustrating the current procedure followed to process material between chromatography stages.

With the optimization of the DF process in the secondary purification stages of HRP extraction and purification, special care was taken to ensure the conservation of the product with a molecular mass of $\approx 40\text{kDa}$. In this application, DF would perform three functions: purification, change of buffer and concentration of the HRP preparations:

Purification. The HRP solution would be purified by removing low molecular weight molecules. Molecules smaller than the MWCO of the membrane pass through the 10kDa MWCO Pall Microza SLP-3053 membrane, thereby reducing the amount of unwanted, low molecular weight protein and other contaminants in the fraction.

Change of buffer. The buffer used to elute the enzyme from the CM chromatography column has a higher conductivity and pH than the buffer used to equilibrate and load the HRP preparations onto the column. The different chromatography media employed also utilizes different types of buffer. The elution conditions of the material

are changed by DF to the conditions required for loading before commencing the next chromatography stage.

Concentration. Large volume chromatography fractions can be concentrated significantly. By doing so at the start of DF, relatively small volumes of diluent liquid are required to dilute the solution during DF. After achieving the required solution conditions, the preparations can be concentrated again. Loading of the column with the minimum volume results in recovery of smaller fractions during chromatography where the target molecules do not interact with the resin.

By implementing DF, the time required to produce 161451-grade material would be shortened by a minimum of 7 full work days (Figure 21).

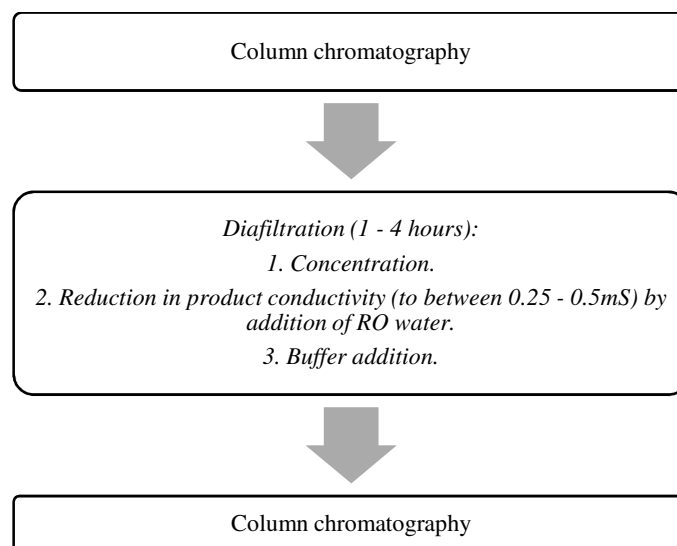


Figure 21. Diagram illustrating the proposed procedure followed to process material between chromatography stages.

3.6.2. *DF trials performed in HRP secondary processing*

Process parameters were optimized during production runs with potable water, as well as during the initial batch DF. Important parameters that needed to be optimized were:

- Temperature.
- Filtrate flux (L/min).
- System pressure (Inlet pressure (P_{in}), Outlet pressure (P_{out}) and filtrate pressure).
- Conductivity (measured in milli-Siemens (mS)).
- Initial concentrate volume.
- Buffer volume.
- Pump speed (measured in Hertz (Hz)).

Twenty-three runs were performed and data collected. The total HRP activity present prior to and post DF was determined, as well as the HRP activity in the filtrate. The trials were performed on batches of material at various stages in the secondary processing (Table 5). In Table 6, the filtrate flux and relative purity, as measured by the Rz-value^h, is illustrated. This data demonstrates that the filtrate flux increased with the purity of the product.

^h “Reinheitzahl” value is the unit of measure representing the ratio of absorbance at 403nm (wavelength at which haem groups of proteins absorb light) to the absorbance at 275nm (wavelength at which aromatic groups of proteins absorb light). This is used as a measure of the purity of the HRP specific protein in the HRP process at different steps.

Table 5. *List of in-process batches diafiltered between Chromatography steps.* The DF of fractions with an asterisk suffix was not taken to completion. Reasons why are given in a later section.

Batch	Fractions subjected to DF
Breakthrough (B/T)	Combined post CM B/T fractions, material washed from the chromatography columns
175/17609	CM1 fraction (pre-CM2)
177/17809	CM1 fraction (pre-CM2); CM2 fraction (pre-CM3); CM3 fraction (pre-DE); DE fraction (Pre-0/45%)*
179/18009	CM2 fraction (pre-CM3); CM3 fraction (pre-CM4); CM4 fraction (pre-DE)*; DE fraction (Pre-0/45%)
181/18209	CM2 fraction (pre-CM3); DE fraction (Pre-0/45%); Phenyl fraction (Pre-F/D)*
183/18409	CM3 fraction (pre-DE)
187/18809	CM2 fraction (pre-CM3); CM3 fraction (pre-DE)*
192/19309	CM2 fraction (pre-CM3); CM3 fraction (pre-DE)*

* DF of these fractions was not taken to completion.

3.6.3. Data and Results

The parameters reviewed and data generated during the DF optimization runs on HRP extract are shown in Table 6. The performance as measured by monitoring filtrate flux for different purities of HRP extract is shown in Table 7.

Table 6. *DF trial run parameters and data generated.*

Run	Fraction	Inlet pressure (P_{in} , kPa)	Outlet pressure (P_{out} , kPa)	Transmembrane pressure (TMP, Bar)	Conductivity (mS)	Initial temperature ($^{\circ}C$)	Final temperature ($^{\circ}C$)	Filtrate volume (L)	Volume buffer/diluent added (L)	Pump speed (Hz)
1	B/T	102	18	0.6	0.87	10.4	22.1	916	500	48
2	CM1 fraction (pre-CM2)	109	27	0.6	1.77	8.6	21.5	785	525	49
3	CM2 fraction (pre-CM3)	100	13	0.6	1.4	12.9	21.7	285	305	48
4	CM1 Fraction	82	08	0.4 5	\uparrow 20	21.3	25	390	400	42
5	Post CM3	92	61	0.8	1.59	20	23	250	200	29
6	Post CM4	70	45	0.6	1.68	21	26.1	225	145	20
7	DEAE fraction	90	60	0.8	4	21	26.1	210	150	20
8	DEAE fraction	100	51	0.8	0.68	21.2	20.1	210	100	25
9	CM2 fraction	90	68	0.8	1.64	20.9	22.1	210	135	40
10	Post CM2	90	69	0.8	1.90	21.8	25.8	280	120	27
11	DEAE fraction	100	52	0.8	0.23	Stopped, high haem and HRP concentrations in filtrate				
12	Post CM2	89	61	0.8	0.80	21.9	24.5	245	475	25
13	DEAE fraction	70	39	0.6	0.31	21.1	22	50	50	25
14	Phenyl fract.	60	30	0.5	\uparrow 20	Stopped, high haem and HRP concentrations in filtrate				
15	Post CM2	76	14	0.5	1.79	16.1	18	350	170	27
16	Post CM2	60	30	0.5	1.43	15.9	21	230	75	20
17	CM2 fraction	60	35	0.5	0.47	21.7	22.3	155	140	20
18, 19	CM3 fraction	90	58	0.8	-	Stopped, high haem and HRP concentrations in filtrate				
20	CM2 fraction	70	48	0.6	1.61	14.5	21.3	285	170	27
21	CM2 fraction	70	48	0.6	1.43	15.1	22	250	145	27
22	CM1 fraction	90	46	0.7	2.78	18.3	24.5	1025	840	27
23	CM1 fraction	89	48	0.7	2.3	19.5	24.4	630	390	27

Table 7. Average Filtrate flux (L/hour) and Rz-values of HRP material of different grades or purity.

Material grade (Ascending)	Average Rz-value of grade material	Filtrate flux (L/min) at 27Hz pump speed
455 (Post CM1)	0.28 – 0.6	3.5
453 (Post CM2)	0.8 – 2.1	5.3
457 (Post DEAE)	2.0 – 3.3	6.25
451 (Post Phenyl)	3.0 and higher	6.7

In order to demonstrate that this process could be used to successfully exchange the CM chromatography 50mM sodium acetate (pH4.9) elution buffer (e.g. from the CM1 column) with the 5mM sodium acetate (pH4.5) “start” buffer (e.g. for application to CM2) a CM column binding demonstration was performed. HRP product was eluted from a lab scale CM column, diafiltered and applied to a second lab scale CM column. In Figure 22 neutral and basic HRP isoenzymes are shown to bind in a red band.

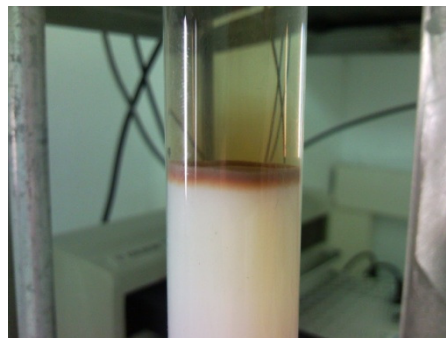


Figure 22. Lab-scale CM Sepharose[®] column loaded with material (H175/17609) diafiltered after CM1 Chromatography. The neutral and basic HRP isoenzymes are shown to bind in a red band at the top of the column.

After confirming that the buffer exchange could be performed successfully with DF, production-scale trials were performed. Figure 24 to 27 are photographs to show the colour of the diafiltrate and include a visible range wavelength scan that will show absorption of haem

at 403nm. The presence of a haem peak will indicate the presence of HRP. However, the presence of active HRP, and not haem derived from denatured HRP, needed to be confirmed by performing a kinetic assay (1). This experiment was carried out and the presence of a peak at 403nm correlated with the presence of active HRP. From the scans a progressive leaking of enzyme (Mr. 44,000kDa) through the 10kDa membrane is illustrated. Wavelength scans (230nm – 550nm) were performed on pink/red coloured filtrate 5 minutes into every run to rapidly identify high levels of haem (HRP sub-unit absorbing at 403nm). As seen in the scans in Figure 26 and 27, a peak is observed at 403nm, indicating the presence of haem corresponding with the presence of HRP (9). The yellow appearing filtrate produced during the DF of lower purity material, was characterized using SDS-PAGE analysis. It was found that a small amount of HRP passed through the membrane, together with smaller proteins or protein fragments (approx. 6kDa) and a high concentration (as a function of the intensity of the band) of a 30 – 40kDa protein (Figure 23).

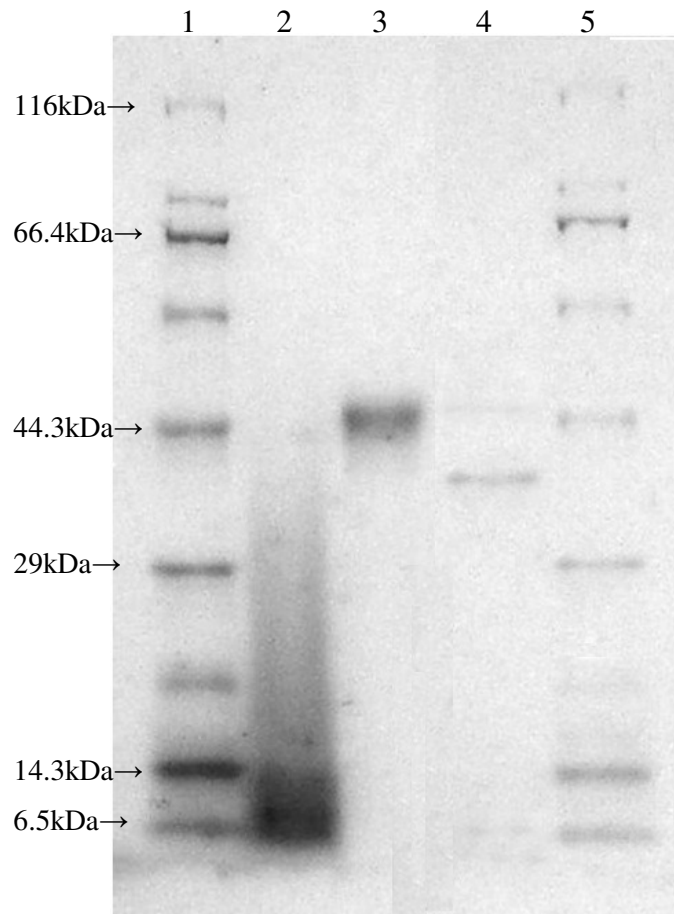


Figure 23. *SDS-PAGE analysis of filtrate from a 10kDa MWCO Pall Microza SLP-3053 produced during DF of 161455-grade HRP material. Lane 1: Molecular weight marker (MM); Lane 2: Alkalized casein (protein control); Lane 3: 161451-Grade HRP Product; Lane 4: DF filtrate; Lane 5: Molecular weight marker (MM). All samples were prepared by dilution of a 10mg/L sample solution with an equal volume of sample buffer. A final amount of 25 μ g of product was loaded per well for each of the samples. The gel was stained with a protein stain as discussed in the Experimental methods at the end of this document.*

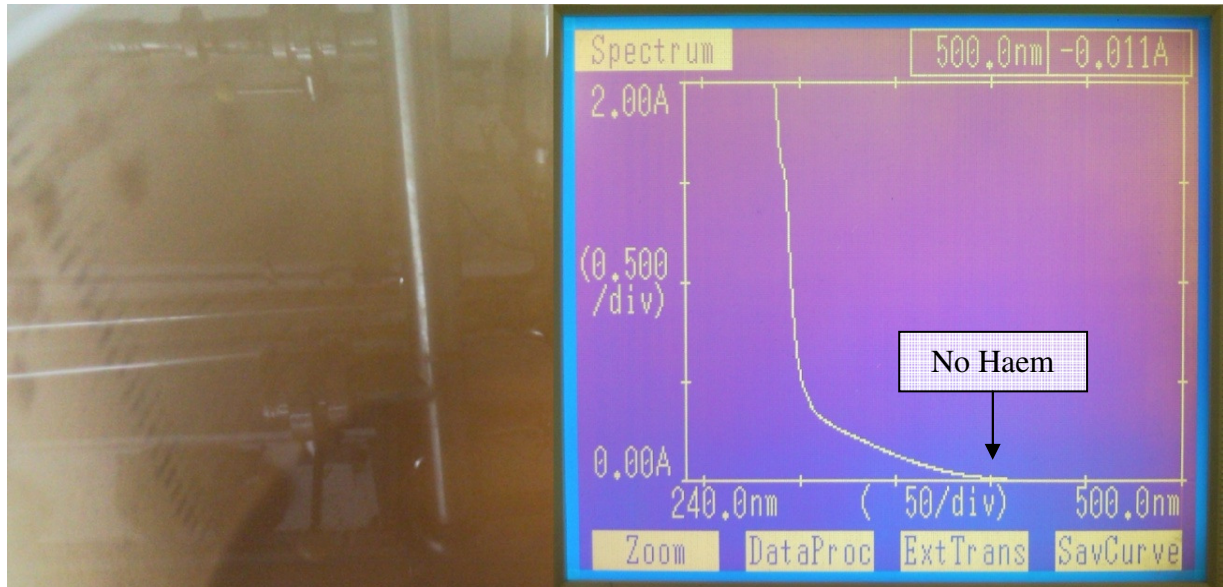


Figure 24. Picture and wavelength scan (230 – 550nm) of typical filtrate generated during the DF of fractions between CM chromatography steps one and two. The Rz-value of a fraction of this purity will be approx. 0.28 – 0.6. The presence of yellow-appearing contaminating molecules is clear in the image of the filtrate.

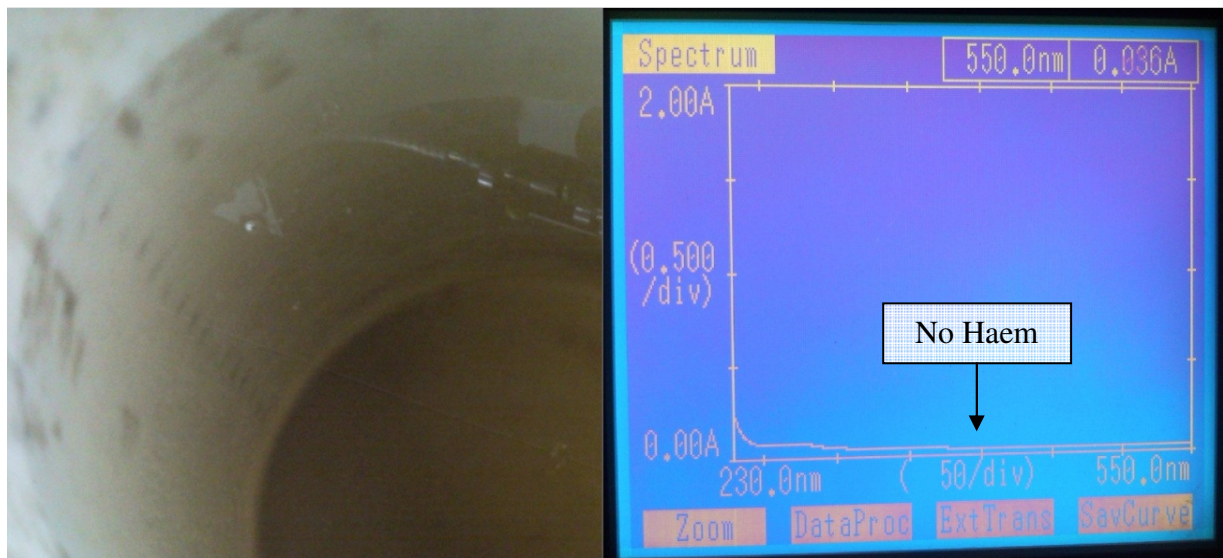


Figure 25. Picture and wavelength scan (230 – 550nm) of typical filtrate generated during the DF of fractions between CM chromatography steps two and three. The Rz-value of a fraction of this purity will be approx. 0.8 – 1.5.

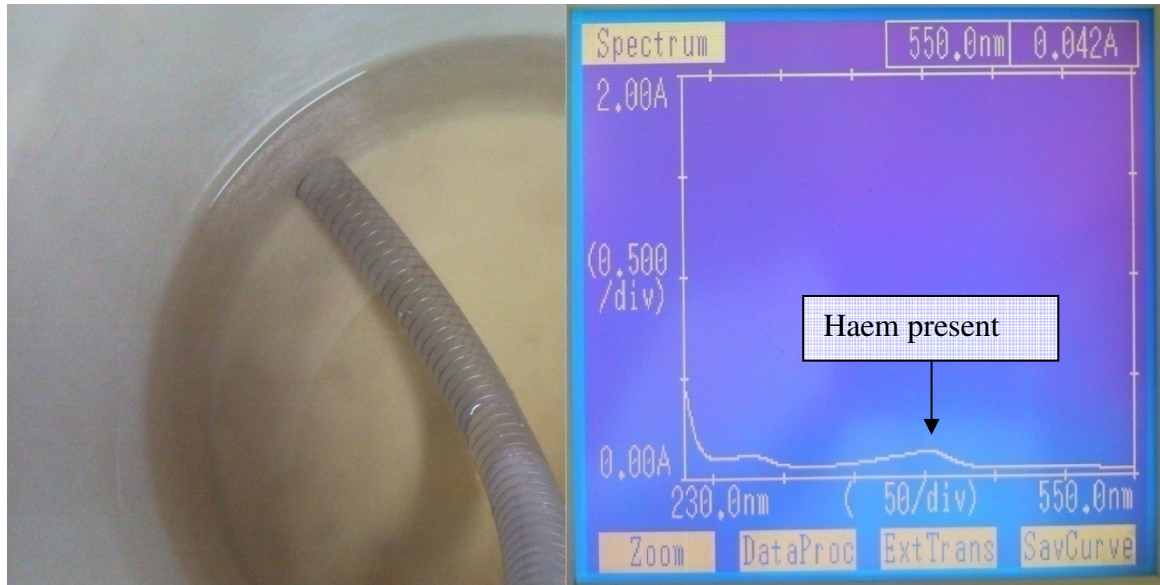


Figure 26. Picture and wavelength scan (230 – 550nm) of typical filtrate generated during the DF of fractions between the final CM chromatography and DEAE chromatography steps. The Rz-value of a fraction of this purity will be approx. 0.8 – 2.1.

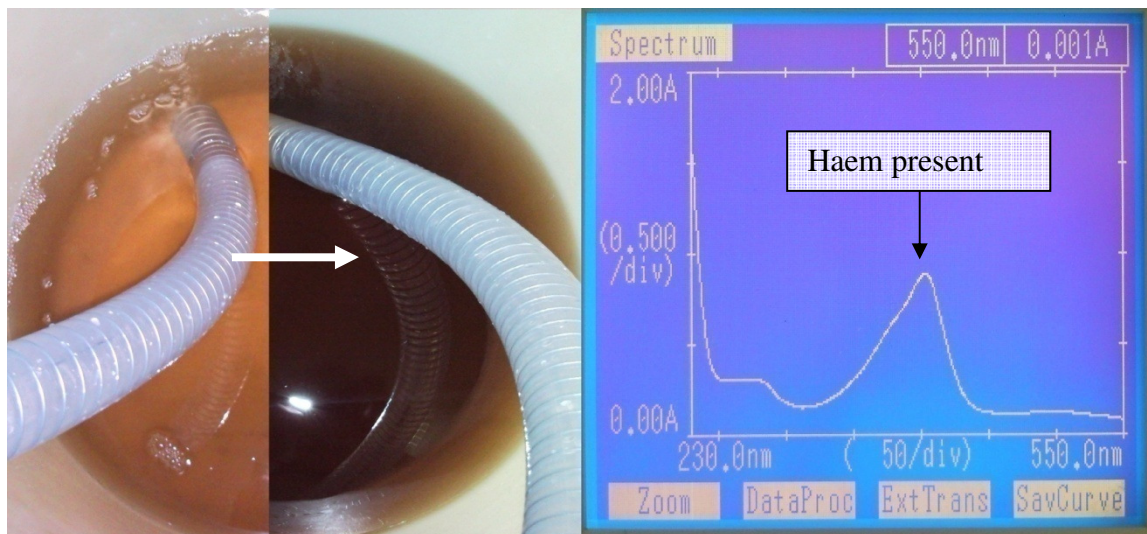


Figure 27. Picture and wavelength scan (230 – 550nm) of filtrate generated during the DF of fractions between the final chromatography steps, DEAE and Phenyl Sepharose® chromatography. The Rz-value of a fraction of this purity will be approximately 3.0 and higher.

Initial trials were performed on material of low purity to optimize the DF. The *filtrate flux* observed for runs performed on batches of lower purity HRP (161455-grade, i.e. CM1 fractions), compared to runs performed on high grade material i.e. post Phenyl Sepharose[®] and DEAE Sepharose[®] chromatography fractions, was significantly lower. This was attributed to the presence of more membrane foulants, decreasing the membrane permeability and was confirmed during the CIP performed after the completion of the runs. During the CIP, yellow to dark-brown coloured retentate and filtrate was observed immediately after the cleaning solution was flushed through the system after DF of 161455-grade material. As the purity of the material subjected to DF increased, the visually observed colour change of the CIP solution (clear to yellow or dark-brown) became insignificant when compared to the lowest grade of material. This indicated a lower concentration of foulants.

During the trials, two of the batches were observed to have a minimum conductivity in excess of the allowed maximum of 0.5 mS. Exceeding this conductivity reduces the efficiency of CM chromatography. It was observed that the conductivity of the material could not be reduced to within the range of 0.25mS to 0.5mS, even when additional DF was performed. The native conductivity of the solution is suspected to originate from a property of the compliment of molecules present in the extract.

The *temperature increase* observed in initial trials was reduced by the addition of cold buffer to the product during DF. With the optimization of the DF process, the duration was also reduced to approx. 1.5 hours from the initial 4 hour runs. Consequently, the amount of time the product was exposed to heat transfer from the pump was significantly reduced.

Trial runs performed on the batches indicated with a suffix (Table 5) were aborted due to the identification of HRP in the filtrate. Diafiltered HRP production batch runs where high concentrations of haem were observed in the filtrate were DEAE and Phenyl chromatography fractions. This lead to an initial hypothesis that there might be some inactivation or conformational changes occurring in the HRP due to the buffers used, as this was not

observed for runs performed on CM fractions. To confirm whether the problem was associated with the integrity of a specific membrane, or the series of nominally rated 10kDa MWCO membranes, a trial run was performed on a new 0.1m² surface area 10kDa MWCO Pall Microza SLP-1053 membrane. High purity material (a phenyl sepharose[®] chromatography fraction, 161451-grade material) was subjected to DF by use of the SLP-1053 membrane under similar conditions to that of the large-scale DF system. After two minutes of concentration, pink filtrate was formed. A wavelength scan was performed, which confirmed the presence of haem. In addition it was found that 7.7% (0.048MU) of the initial amount of HRP (0.619MU at 5mg/ml concentration) subjected to DF had passed through the membrane after 5 minutes. Following the investigation, it was concluded that the purity of the material diafiltered was the determining factor, as all the batches with significant HRP passing through the membrane had higher Rz-values than material that did not show measurable HRP permeability. With the results obtained during the trials, the proposal is to limit the use of the 10kDa DF system to material that does not exceed an Rz-value of 1.5.

The reason for the passing of 44kDa proteins through the 10kDa MWCO membrane is the way in which MWCO of the membrane is determined. Polyethylene glycol (PEG) of known molecular weight is concentrated by the membrane and the percentage passing determined. However, PEG is a linear molecule, whilst HRP is globular. This difference leads to the ability of HRP to pass through the membrane during production runs where low fouling is observed. Fouling tightens the MWCO of the membrane by pore narrowing, leading to the initial qualification of 10kDa Pall Microza membranes for use in concentration of HRP.

With the completion of the *DF* trials necessary for implementation, the major *benefits* to the process at BBI Enzymes became evident. The improvements include:

- *Additional purification.* Low molecular weight molecules pass through the 10kDa molecular weight cut-off (MWCO) Pall Microza SLP-3053 modules. Material of 161455-grade subjected to DF, showed an average increase in Rz-value of 0.25, indicating a significant decrease of contaminating proteins during the DF process.

- *Reduction in Ammonium sulphate consumption.* The fractionation step currently set in motion after the chromatography fraction was collected, is now obsolete for material of 161455 and 161453-grade, where DF showed consistent results*.
- *Reduction in process time.* Because the processes of 0/85% Ammonium sulphate fractionation, Precipitation (filtration) and Dialysis is not performed the duration of the process is also shortened by a minimum of 7 days.
- *Reduction in risk of product loss.* By replacing three steps with one, the consequent reduction in the handling of the product reduces the risk of product loss. Performing of a wavelength scan (from 450nm down to 250nm) will also be part of the method, as a means to rapidly identify product loss to the filtrate side of the membrane.
- *Concentration.* Allows control over the volumes of batches at all stages post DF irrespective of initial batch size.
- *Change of buffer conditions.* Allows rapid and controllable buffer exchange in between chromatography stages.

* Results that indicate no or little loss of product, inclusive of all improvements listed in the discussion.

With the completion of the trials, the desired parameters for the production process were identified and confirmed (Table 8). These parameters and conditions will be compiled in the DF work instruction (BP5.3.2 – WI24) at BBI-enzymes.

Table 8. *Parameters for the DF production process.*

Parameter	Setting
<i>Pump setting</i>	27Hz
<i>Inlet pressure</i>	90kPa
<i>Outlet pressure</i>	47 – 55kPa
<i>Filtrate pressure</i>	17 – 22kPa
<i>TMP</i>	± 50kPa
<i>Maximum product temperature</i>	25°C

The TMP was set lower than that of the production UF conditions due to the absence of a back-flush system. During batch process runs, where the filtrate flux decreased, performance was recovered by stopping the filtrate flow for a short period, while maintaining the cross-flow velocity. In Figure 28, the flow-dynamics in the DF system under these conditions are shown. It is observed that pressure difference between the membrane lumen inlet and outlet, as well as the effective pressure on the filtrate side lead to a net directional force favouring the movement of foulant away from the membrane surface towards the outlet of the membrane. The lower overall differential pressure between the inside and outside of the membrane also reduces the force favouring fouling.

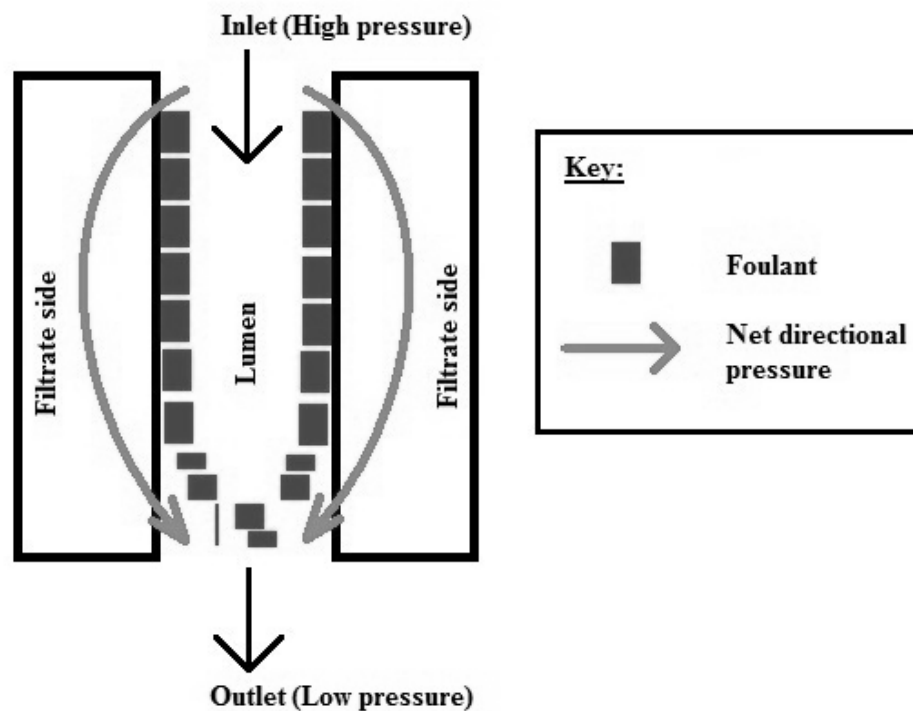


Figure 28. Diagram illustrating the self-cleaning of an TFF membrane during conditions of no filtrate flux. The net directional pressure is indicated by the gray arrow.

3.7. CHROMATOGRAPHY

The chromatography practices employed at BBI Enzymes in the separation and purification of HRP include ion-exchange and hydrophobic interaction chromatography (HIC). Ion exchange chromatography consists of carboxymethyl (CM) - and diethylaminoethyl (DEAE)-Sephacrose[®] resin processing. HIC is performed by use of Phenyl Sepharose[®] resin (Figure 4). The characteristic red colour of HRP due to its haem-group allows for the collection of fractions containing the enzyme by visual determination.

Cation exchange (CM) chromatography is first performed on the HRP-containing material precipitated during the A/S fractionations post primary concentration. The aim is to separate basic and neutral HRP isoenzymes from acidic isoenzyme and other proteins with low isoelectric points. By removing these proteins, the following HIC chromatography step requires less of the more expensive chromatography media. The HRP material is buffered with a 5mM sodium acetate buffer to a pH of 4.5 and loaded onto the column.

Two CM column runs are performed per batch of product. During the first CM run, most of the cathodic (basic) and neutral isoenzymes bind to the resin, with the anionic (acidic) HRP isoenzymes being collected in the flow-through (9). The first CM column is run as a clarification step, removing a grey contaminant that strongly associates with and competes for binding sites on the CM resin beads with HRP. The bound HRP fraction is eluted by applying 50mM sodium acetate buffer, pH4.9 to the resin. This buffer does not elute the grey contaminant. The flow-through and eluate are combined and prepared for the second CM chromatography step. The grey contaminant is removed during regeneration of the resin by use of 1M NaCl.

The second chromatography step is performed under the same conditions binding all cathodic (basic) and neutral isoenzymes to the resin, separating it from the anionic (acidic) HRP

isoenzymes (9). The fraction that does not bind to the column (flow-through) contains the acidic HRP isoenzymes. A portion of yellow-appearing molecules are removed from the HRP solution prior to the start of collection of the acidic isoenzymes. The acidic peroxidase is precipitated with an 85% A/S saturation and is now a commercial product. The eluted fraction (neutral and basic isoenzymes) is processed to 161455- or 161453-grade product, or further purified by anion exchange (DEAE) chromatography (See Figure 4).

The DEAE Sepharose[®] column is run at a condition (high pH) that favours only partial binding of the neutral and basic isoenzymes and removes proteins that co-eluted with these isoenzymes on CM Chromatography, as well as trace amounts of the acidic isoenzymes that may be present due to inefficient CM chromatography. The resin is equilibrated and washed with 5mM Glycine buffer, pH 10.2. Acidic isoenzymes bind to the column, while the neutral and basic isoenzymes are isolated by fractionation of the flow-through. During the column wash, more of the yellow-appearing molecules are removed from the neutral and basic isoenzyme solution as it is also washed from the resin before these isoenzymes. The flow-through fraction (neutral and basic isoenzymes) is processed to 161457-grade product, or subjected to a final chromatography step (Figure 4).

The final chromatography step is a hydrophobic interaction (HIC) process. The Phenyl Sepharose[®] resin is equilibrated with 5mM sodium acetate buffer saturated to 45% with A/S, pH5.0. During this step, the neutral and basic HRP isoenzymes bind to the resin. Purification is achieved by washing retained impurities (yellow-appearing molecules) from the resin. The HRP fraction is eluted and processed to 161451-grade product, BBI Enzymes South Africa's highest purity of HRP (Figure 4).

Currently all the chromatography processes (CM, DEAE and Phenyl) are performed with resin packed in open top Perspex columns and yield highly variable results. During the addition of buffer or product, the resin bed is disturbed and cavitates, causing an uneven loading of the product. The columns are also run by gravitational flow and differences in the

volume of buffer loaded to the top of the column will cause differences in the flow rate. The columns are packed on a filter paper disc and the bottom of the column is drained through 10 holes at different positions. This film of paper and the drain holes are ineffective in allowing for the uniform flow from the bottom of the column. During regeneration, the resin is removed from the column in order to remove the filter paper disc which becomes blocked by the combination of CIP solution and the eluted impurities. This handling of the resin causes significant losses of the chromatography media. All of these factors reduce the consistency, output and quality of the chromatography performed.

Control of resin bed surface, height and compression, flow rate and distribution by implementation of semi-automated columns was investigated. Due to the scale of production at BBI Enzymes, three high volume (120L capacity) Amicon[®] Moduline[®] P630 x 500 columns were reviewed and optimized for use during CM Chromatography. The columns were equipped with adjustable flow distribution cells and 30 micron polyethylene sinters optimized for the even flow of liquid onto and from the resin and a range of bed heights (61). The differences between the open top Perspex columns, used in the past, and the Amicon[®] Moduline[®] P630 x 500 are illustrated in Figure 29. The difference in effectiveness of the outlet flow distribution is evident between the two pieces of equipment.

In order to allow for semi-automated operation of these columns, a positive displacement pump, with adjustable flow rate control (variable frequency drive), was fitted to the column to allow for product and buffers to be pumped onto the column automatically and at a constant feeding rate. An in-line pressure gauge was fitted after the pump to measure the feeding pressure of the column. The feed pressure of the column must be maintained at a constant value and is controlled by the feeding rate. This ensures that the batch-to-batch performance of the resin bed is not compromised due to cavitation or compacting. The maximum head pressure on the resin bed, as proposed by the manufacturer, was limited by the installation of an in-line pressure sensor and trip switch, shutting down feed to the column in the event of a sudden pressure increase. To protect the column and pump from potential

damage (delays due to re-packing) caused when air is allowed into the system, it was fitted with a run dry protection sensor that shuts off the pump when air passes through the feed line.

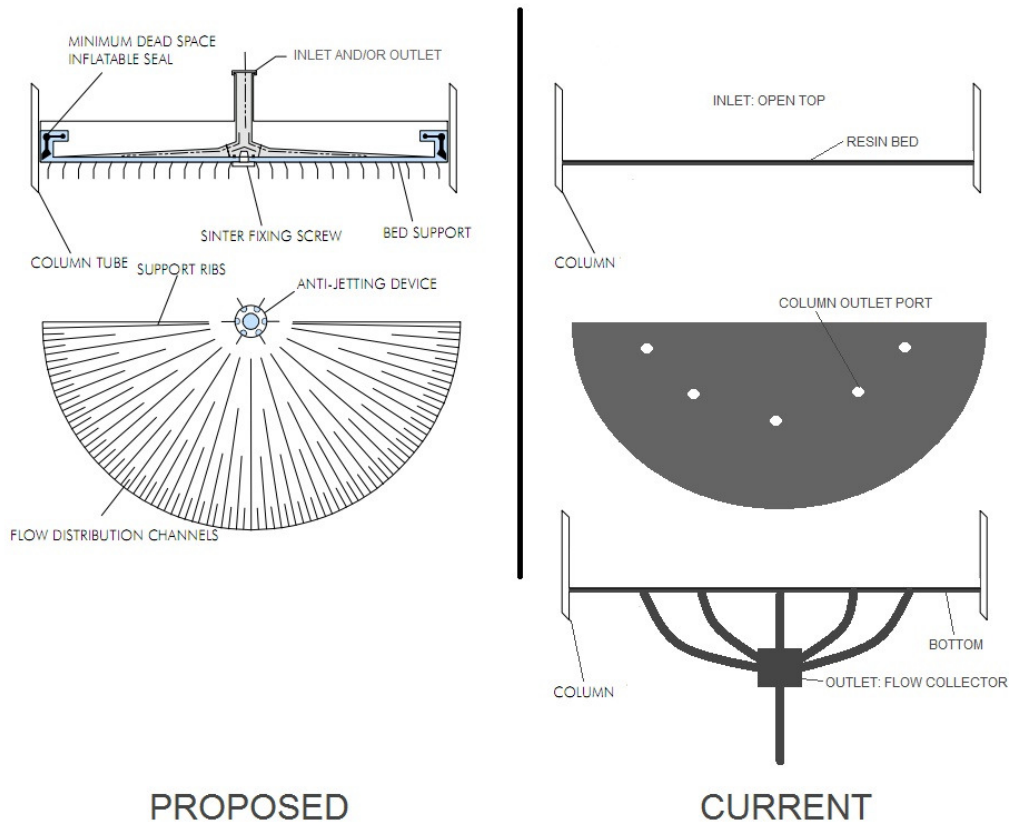


Figure 29. Diagrams of the inlet and outlets designs of the current (open top Perspex) and proposed (Amicon® Moduline®) chromatography columns (61).

With the current open top column system, resins were regularly subjected to contamination and/or blockage with foreign particles, insoluble protein in the extract or large inorganic material. To prevent this in the new system, in-line filters (20 and 5 micron nominal retention rating). The filters were fitted in series after the pump, in order to also eliminate fragments of the pump components to enter the column in the event of a failure (Figure 30).

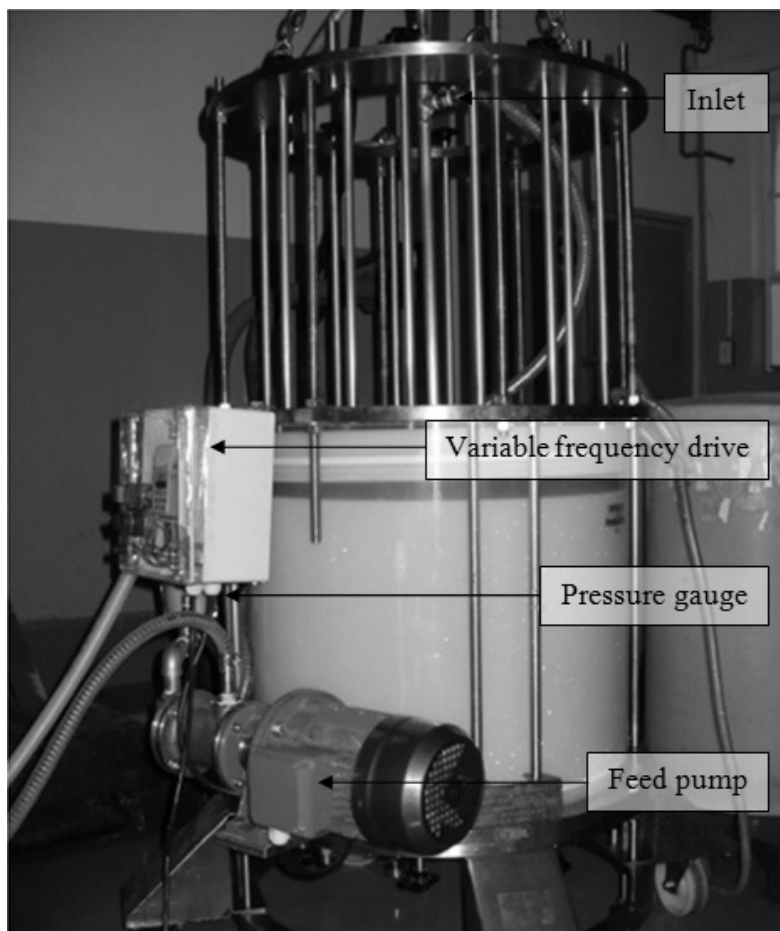


Figure 30. *BBI Enzymes Amicon® Moduline® chromatography column*. The feed pump, feed pressure gauge and variable frequency drive is shown. The in-line pre-filters are not visible. T column is packed with 120L of CM Sepharose® Fast Flow resin.

All three P630 x 500 columns were filled with 120L of regenerated CM Sepharose® Fast Flow (CM, weak cation exchanger) resin. HRP material produced during A/S precipitation was subjected to cation exchange chromatography on the CM resin, equilibrated with 5mM sodium acetate buffer, pH4.5. During the loading, washing and elution of the column, clear, horizontal bands were observed (Figure 31).

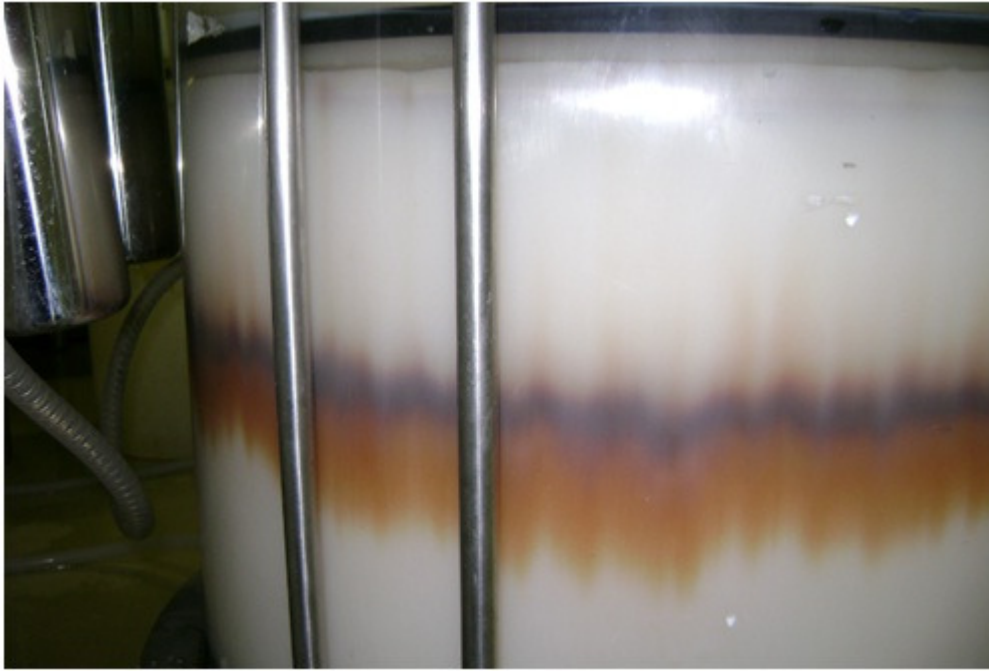


Figure 31. *Elution of the BBI Enzymes Amicon® Moduline® chromatography CM Sepharose® Fast Flow column. Clear horizontal bands are visible, indicating a good, even progression of product through the column. All visible bands are combined as product.*

The implementation of semi-automated columns improved the efficiency of the chromatography step. As discussed, the Amicon® Moduline® columns were equipped with flow distribution cells and sinters on the inlet and outlet side of the resin. This eliminates the cavitation and bed disturbance observed in the previous system. The 30 micron sinter discs on the in- and outlet further improves flow distribution, but also eliminates the requirement for the removal of the resin from the column. The Amicon® columns allow for quick buffer exchange of the resin by lowering the adjustable top flow cell onto the resin bed. The fitment of a feed pump reduces the variability of the linear velocity through the column, which in turn increases the consistency of the process from run to run. A production run is started on the chromatography system, with the required amount of equilibration or wash buffer prepared in the buffer container. The system will then proceed to complete the equilibration or washing step of the resin unsupervised, stopping once the buffer is depleted. This is managed by the run dry detection system. Overhead costs are thus reduced, and the allowed daily production can exceed normal working hours, performing regeneration and equilibration or washing steps of the column after hours. The in-process performance of the

columns during trial processing of production batches indicated an average increase of the Rz-value of the HRP extract over the conventional CM chromatography step of Rz 0.6 – 2.00^h. Similar columns were subsequently introduced for the DEAE and Phenyl Sepharose[®] chromatography steps.

By implementing DF, as discussed earlier in this document, the processing of fractions that were further purified was shortened. It was also discovered that levels of yellow-appearing contaminating molecules in the product was reduced during DF, as the molecule passed through the membrane to the discarded filtrate (Figure 24). The improved chromatography and additional purification achieved by implementation of DF eliminated the requirement for a second cation exchange chromatography step when producing intermediate or higher grade product. This second CM run was previously performed due to the inefficiencies of the system, and the high concentration of non-specific protein that bound and saturated a major part of the resin binding capacity under the same conditions as the neutral and basic isoenzymes. The levels of contaminating proteins were reduced during DF by passing through the membrane and concentration polarization (fouling). Concentration polarization led to the reversible precipitation of non-specific proteins on the DF membrane surface, which was subsequently removed during CIP. The chromatography media and process conditions were not changed, as such changes would affect the different, established, product grades BBI Enzymes produces.

^h BBI Enzymes. *HRP Assay Logbook*. Cape Town : QC Department, 2009.

3.8. COMPARATIVE ANALYSIS OF PRODUCT OBTAINED FROM CURRENT AND OPTIMIZED EXTRACTION AND PURIFICATION PROCESSES

Comparative analyses between current product and that produced from the optimized process were performed in order to ensure the quality of the product produced would be higher, yet within the specification of the customer. The *carbohydrate content* of material generated by following the proposed new process was compared to two batches of product from the current method. Carbohydrates present in HRP preparations are normally glycans associated to the enzyme at glycosylation sites (62). The main risk to product quality is the presence of glycans not bound to glycosylation sites. Four PAGE analyses were performed on the material. Two of the gels were stained for protein (Coomassie, Figure 32 and 34), and two were stained for carbohydrate following a PAS (Periodic Acid Schiff) protocol (Figure 33 and 35). Two of the gels (Figure 34 and 35) were deliberately overloaded in order to demonstrate the purity of the preparation.

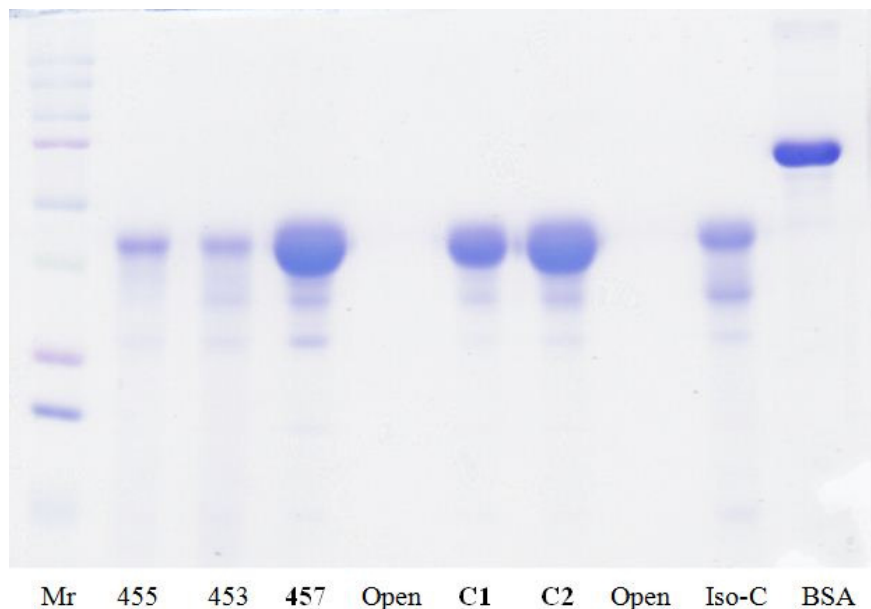


Figure 32. PAGE analyses: Protein visualization by Coomassie staining. Material generated following the proposed HRP process. Molecular marker (Mr) was run in lane 1. Lane 455 represents 161455-grade material. Lane 453 represents 161453-grade material. Lane 457 represents 161457-grade

material. Lanes C1 and C2 were loaded with two 161457-grade control batches (C1 and C2). Lane Iso-C was loaded with a sample of purified Isoenzyme C (purified by gradient Ion Exchange Chromatography). Lane BSA was loaded with a BSA standard. All samples were prepared by dilution of a 10mg/L sample solution with an equal volume of sample buffer. A final amount of 25µg of product was loaded per well for each of the samples. The gel was stained with a protein stain as discussed in the Experimental methods at the end of this document. Coomassie staining was then performed on the gel to visualize protein bands. (63).

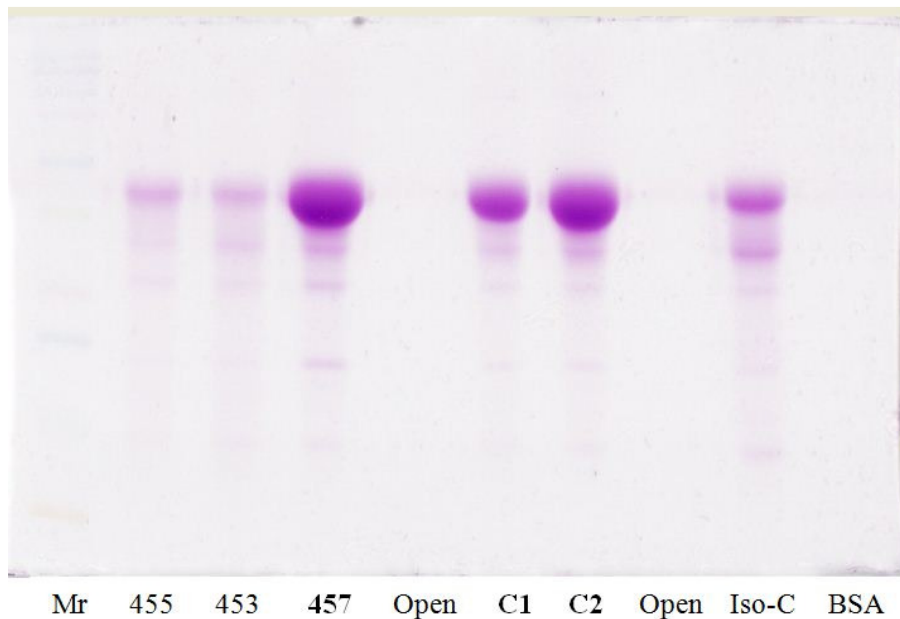


Figure 33. *PAGE preparation: Carbohydrate visualization by PAS staining.* Material generated following the proposed HRP process. Molecular marker (Mr) was run in lane 1. Lane 455 represents 161455-grade material. Lane 453 represents 161453-grade material. Lane 457 represents 161457-grade material. Lanes C1 and C2 were loaded with two 161457-grade control batches (C1 and C2). Lane Iso-C was loaded with a sample of purified Isoenzyme C (purified by gradient Ion Exchange Chromatography). Lane BSA was loaded with a BSA standard. All samples were prepared by dilution of a 10mg/L sample solution with an equal volume of sample buffer. A final amount of 25µg of product was loaded per well for each of the samples. The gel was stained with a protein stain as discussed in the Experimental methods at the end of this document. PAS staining was then performed on the gel to visualize carbohydrates. Molecular marker (Mr) was run in lane 1 (63).

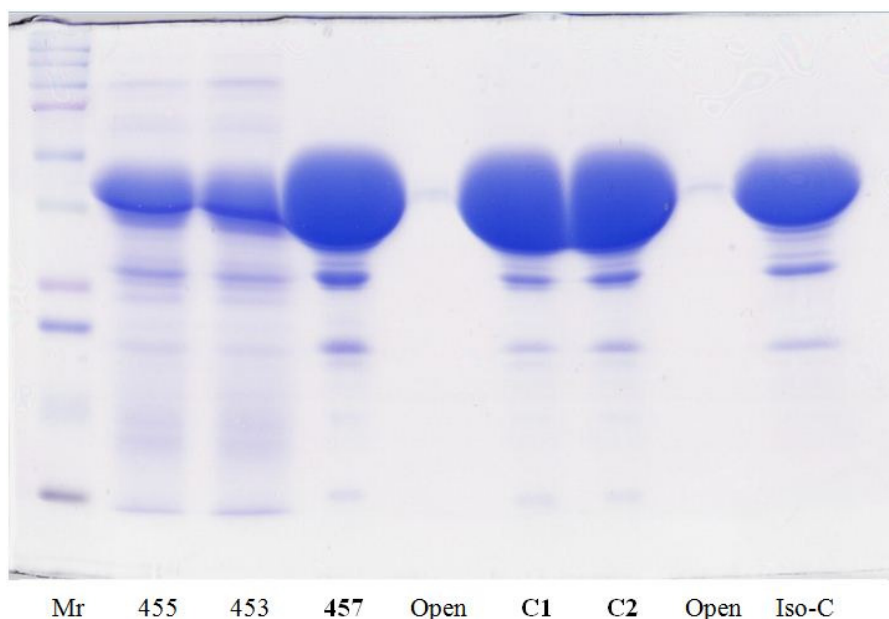


Figure 34. *PAGE preparation: Coomassie staining.* Material generated following the proposed HRP process. Molecular marker (Mr) was run in lane 1. Lane 455 represents 161455-grade material. Lane 453 represents 161453-grade material. Lane 457 represents 161457-grade material. Lanes C1 and C2 were loaded with two 161457-grade control batches (C1 and C2). Lane Iso-C was loaded with a sample of purified Isoenzyme C (purified by gradient Ion Exchange Chromatography). The gel was deliberately overloaded in order to demonstrate the purity of the preparation. All samples were prepared by dilution of a 10mg/L sample solution with an equal volume of sample buffer. A final amount of 50 μ g of product was loaded per well for each of the samples. The gel was stained with a protein stain as discussed in the Experimental methods at the end of this document. Coomassie staining was then performed on the gel to visualize protein bands. Molecular marker (Mr) was run in lane 1 (63).

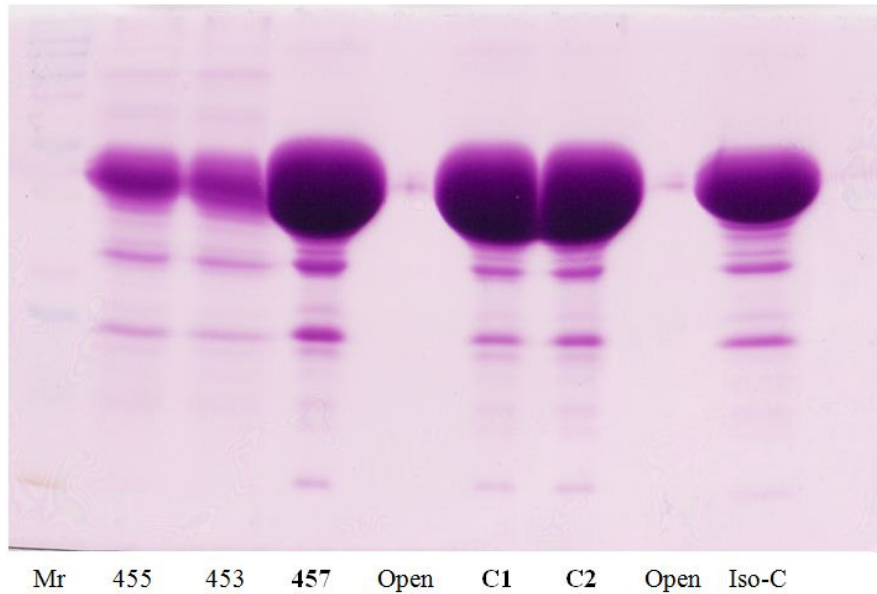


Figure 35. *PAGE preparation: PAS staining.* Material generated following the proposed HRP process. Molecular marker (Mr) was run in lane 1. Lane 455 represents 161455-grade material. Lane 453 represents 161453-grade material. Lane 457 represents 161457-grade material. Lanes C1 and C2 were loaded with two 161457-grade control batches (C1 and C2). Lane Iso-C was loaded with a sample of purified Isoenzyme C (purified by gradient Ion Exchange Chromatography). The gel was deliberately overloaded in order to demonstrate the purity of the preparation. All samples were prepared by dilution of a 10mg/L sample solution with an equal volume of sample buffer. A final amount of 50 μ g of product was loaded per well for each of the samples. The gel was stained with a protein stain as discussed in the Experimental methods at the end of this document. PAS staining was then performed on the gel to visualize carbohydrates. Molecular mass marker (Mr) was run in lane 1 (63).

From these results it is evident that the preparations obtained were of high purity. All the proteins in the enzyme preparations are heavily and seemingly equally glycosylated. It is important to note that the only carbohydrate present in the gel is bound to the protein. As both the origin and leading front of the gel are shown in the figures it is clear that there was no free carbohydrate in the samples applied to the gel. The non glycosylated protein, Bovine Serum Albumin (BSA), loaded in last lane of the first two gels was shown to only stain with Coomassie, not the PAS stain. This confirmed the staining to be specific to glycoproteins and is further confirmed by the absence of PAS staining of the non-glycosylated molecular

weight markers. The current and proposed process yielded product that is indistinguishable by PAGE analysis and selective staining.

3.9. DISCUSSION

The optimization of the extraction and purification of HRP have resulted in an increased quality, efficiency, throughput and control over the manufacture of all grades of HRP enzyme produced by BBI Enzymes. The standardization of raw material pre-harvest treatment has shown to improve the HRP content and workability of the roots supplied to BBI Enzymes. Extraction at a higher liquid to root ratio, with improved maceration has shown to liberate significantly higher concentrations of HRP. Optimization and up-scaling of the UF system, including the pre-treatment of the extract allows for more control over the concentration step, higher throughput and a reduction in A/S usage due to its increased concentrating ability. Pre-treatment was found to be essential and efficacy of the step crucial to the performance of the UF system. This included more control over the extract pH and use of different grades of diatomaceous earth in order to reduce the extract particle size. These improvements lead to the investigation and optimization of the A/S fractionation step.

The changes in the A/S saturation conditions increased enzyme recovery and reduced A/S consumption as a consequence of the higher concentration factor achieved. DF reduced production lead times significantly by replacing additional A/S precipitation and passive dialysis, and added to the purification of the product, due to the semi-permeable nature of the membranes. The semi-automated chromatography columns implemented increased control, consistency and robustness of the chromatography practiced. Overhead cost per enzyme unit was also reduced due to the option of running the machine unattended. Literature linking reduced thermal stability of HRP with sodium phosphate buffer has lead to the adoption of use of acetate buffer as a replacement.

Comparative analyses between current product and that produced from the optimized process showed that the preparations obtained were of high purity. The current and proposed process yielded product that is indistinguishable by PAGE analysis and selective staining. This ensured that the product produced would comply with the existing specifications. These results confirm the effectiveness of the optimized method for the production of HRP. The implementation of the improvements discussed in this document, forms part of the HRP purification process to the different BBI Enzymes product grades from the clarified root as shown in Figure 36. The characterization of the main groups of HRP relevant to BBI Enzymes as product was also performed consisted of further PAGE and high performance liquid chromatography (HPLC) as discussed in Chapter 4.

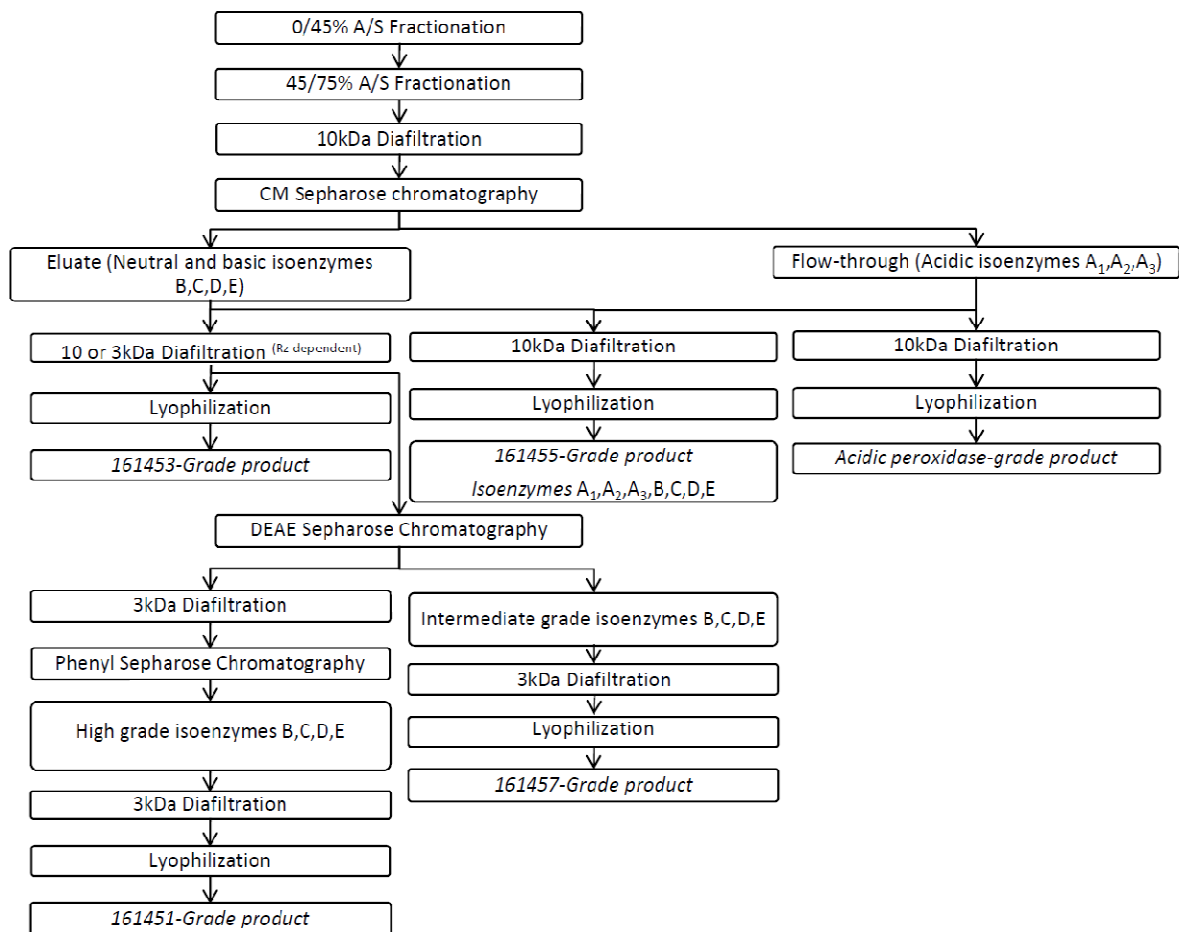


Figure 36. New secondary process flow for HRP purification. The stages where the 5 grades of HRP product (161455-,161453-,161457-,161451- and Acidic peroxidase grades) produced by BBI Enzymes are yielded is shown.

CHAPTER 4: CHARACTERIZATION OF MAIN GROUPS OF HRP RELEVANT TO BBI ENZYMES BY SDS-PAGE AND HPLC ANALYSIS

4.1. INTRODUCTION

HRP is extracted and purified at BBI Enzymes employing an adaptation of the process developed by Keilin *et al.* in 1950 (31). The method was adapted to a previous revision of the current method in 1966, after Shannon *et al.* published their review of the peroxidase isoenzymes present in horseradish (9). They described the contributions to total HRP activity of the various isoenzymes in a horseradish extract (9). These values are shown in Table 9. It is important to note that the isoenzyme content and ratios are ultimately dependent on raw material quality, as discussed during the review of the raw material.

Table 9. *The seven main HRP isoenzymes and their percentage contribution to the total HRP activity of a horseradish extract (9).*

Isoenzyme group	Isoenzyme	% Total HRP activity*	Comment
Acidic	A1	13	Removed at CM
Acidic	A2	2	Removed at CM
Acidic	A3	5	Removed at CM
Neutral	B	20	Eluted from CM
Neutral	C	36	Eluted from CM
Basic	D	4	Eluted from CM
Basic	E	6	Eluted from CM

*Activity of homogenate = 100%

Isoenzymes B and C are the main contributors to the HRP activity compliment of the extract. As discussed in the review of Chromatography, neutral and basic isoenzymes are purified together. In Figure 36, the new secondary process flow for HRP purification at BBI Enzymes

is shown and the separation of groups of isoenzymes is indicated. The different BBI Enzymes product grades and their minimum purity requirements are shown in Table 10.

BBI Enzymes customers for higher grades of HRP (161457- and 161451-grade) have a minimum isoenzyme C requirement. The reason for this is the separation of the different isoenzymes in this grade of product by the customers themselves. Isoenzyme C is separated by customers and purified for use in enzyme conjugation (64). BBI Enzymes do not sell pure isoenzyme C, primarily for the reason that there is no significant market that would absorb the levels of isoenzyme B that would be produced in the process (20% of HRP activity present in the extract).

Table 10. *BBI Enzymes HRP product specification minimum purity requirements.*

Product grade	Minimum specific activity*	Minimum Rz-value
161455	60U/mg	1.00
161453	100U/mg	2.00
161457	200U/mg	2.50
161451	250U/mg	3.00

* Specific activity expressed as Guaiacol HRP Activity units per milligram of lyophilized product (1).

The different grades of HRP produced by BBI Enzymes are used for different applications. The higher purity grades (161457 and 161451) uses include coupling to immunoglobulins for use in immunodiagnosics, chemiluminescent assays and recently in cancer therapy (65). It is also used in combination with one of the other enzymes in BBI Enzymes' product catalogue, glucose oxidase, to monitor glucose levels in diabetes test kits. Lower grades (161455 and 161453) are generally sold to customers that further purify the enzymes. The neutral and basic HRP isoenzymes are selectively purified to yield two main fractions, high purity isoenzyme C and the balance of neutral and basic isoenzymes (64). The balance of HRP is used as indicator enzyme in systems where one of the by-products of the reaction identified is hydrogen peroxide. It is also used for the preparation of apo-peroxidase. This denatured form of HRP, produced by removal of the haem group, is used for the blocking of peroxidase antibodies in immunohistochemistry applications (66).

During the review of the chromatography performed at BBI Enzymes, the aim of each of the purification stages and the step-wise purification and separation of the different grades of HRP was reviewed. The characterization of these grades of HRP will be discussed in this chapter.

4.2. SDS-PAGE ANALYSIS

Gel electrophoresis was performed in order to characterize the different grades of HRP product produced at BBI Enzymes. SDS-PAGE analyses of 161457- and 161451-grades yielded a single band corresponding to a molecular mass of 40,000 kDa (Figure 37). Because of the heterogeneous glycosylation of HRP it is not possible to obtain a well-defined sharp band as is the case for non-glycosylated proteins. When the gel was overloaded, a low concentration of lower molecular mass proteins can be observed as shown in Figure 34. Analysis of samples of 161455- and 161453-grade both show that, although there are several other proteins present in these preparations, HRP is still the major protein component (Figure 37).

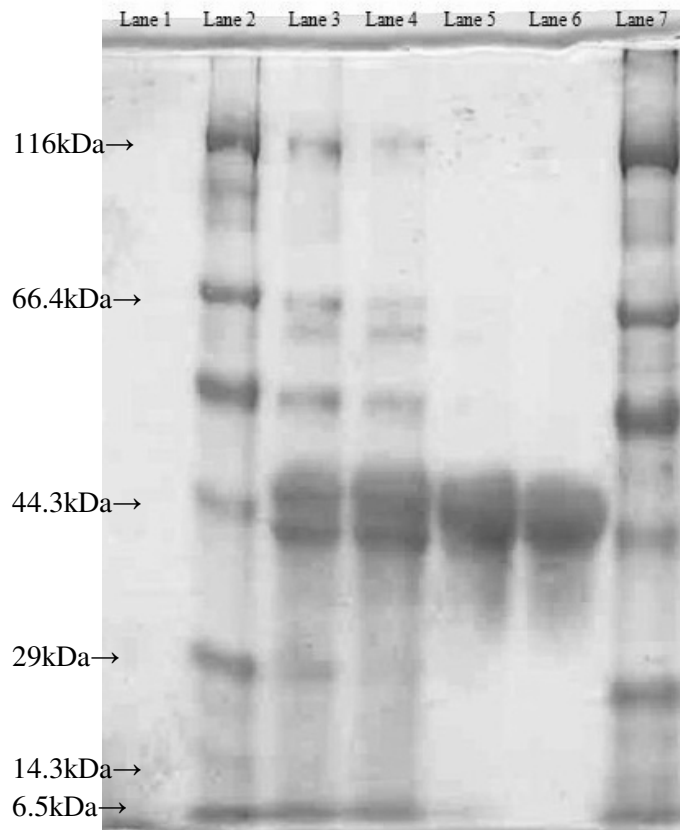


Figure 37. *SDS-PAGE analysis of the different grades of HRP product produced by BBI Enzymes.* Lane 1: Empty; Lane 2: Molecular mass marker (MM); Lane 3: 161455-Grade Product; Lane 4: 161453-Grade Product; Lane 5: 161457-Grade Product; Lane 6: 161451-Grade Product; Lane 7: Molecular mass marker (Mr) was run in Lanes 2 and 7. All samples were prepared by dilution of a 10mg/L sample solution with an equal volume of sample buffer. A final amount of 25 μ g of product was loaded per well for each of the samples. The gel was stained with a protein stain as discussed in the Experimental methods at the end of this document.

4.3. HPLC ANALYSIS

Cation exchange (CM) HPLC was performed on the BBI Enzymes HRP product range. Acidic peroxidase was not characterized using this method, as the run conditions of the system and resin used do not support separation of the components in this product. Figure 38

to 41 represent the CM-HPLC chromatograms of samples of 161455-, 161453-, 161457- and 161451-grade products, respectively. The identification of the different peaks was performed by determining the retention of isoenzyme standards acquired from Roche Applied Science. From these chromatograms the reduction in contaminating proteins can be clearly observed as the product increases in purity. In Figure 38, a CM-HPLC chromatogram of the lowest grade of HRP sold by BBI Enzymes is shown. The large acidic HRP contribution is eluted from the CM column within the first minutes of the chromatography run. Contaminating proteins are eluted at increased ionic strength buffer after the neutral and basic isoenzymes and is represented by the large, broad peak towards the end of the chromatogram.

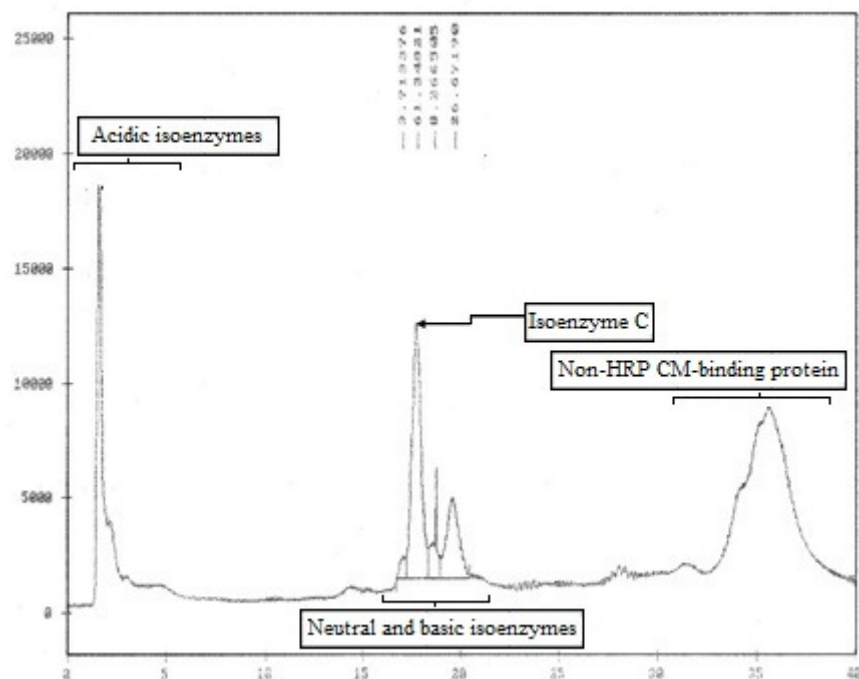


Figure 38. *Elution profile of 161455-grade HRP from a CM-HPLC column.* The complement of peaks representing the neutral and basic, non-HRP resin binding protein and acidic isoenzymes are indicated. The peak representing isoenzyme C is also identified. Absorbance was detected at 280nm for the column eluate. A Waters Protein PAK CM, 8-HR, 8m column was used. A linear gradient consisting of eluent A (0.01 M sodium acetate, pH 4.4) and eluent B (0.01 M sodium acetate, 0.2 M NaCl, pH 4.4) at a flow rate of 0.8 ml/min was used during separations.

The chromatogram of 161453-grade material (Figure 39) illustrates the successful removal of the majority of the contaminating proteins. Due to the significant purification performed during preparative CM-chromatography, acidic peroxidase is regularly added back to the eluate to reduce the purity, ensuring compliance with customer specifications.

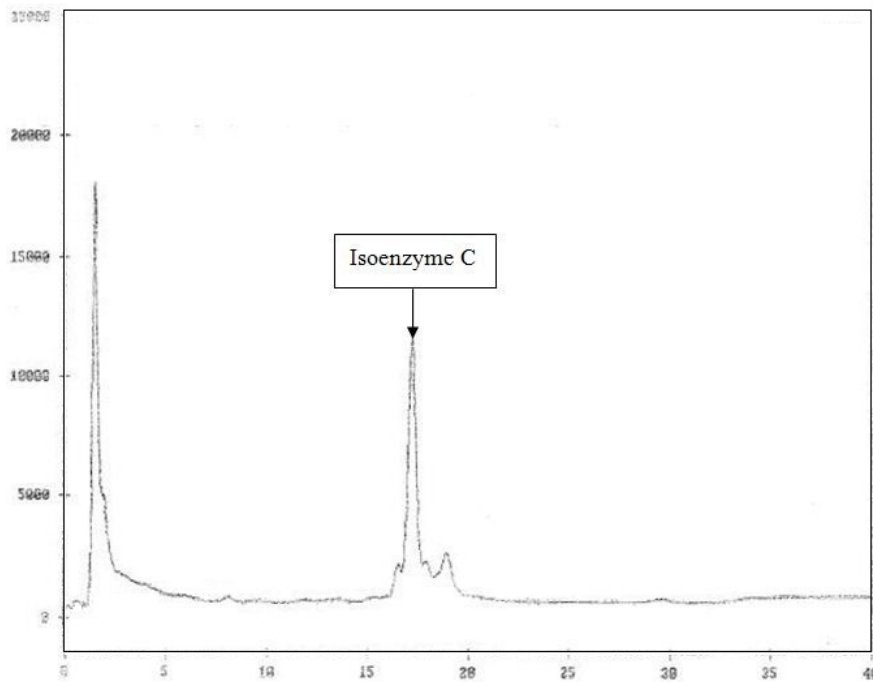
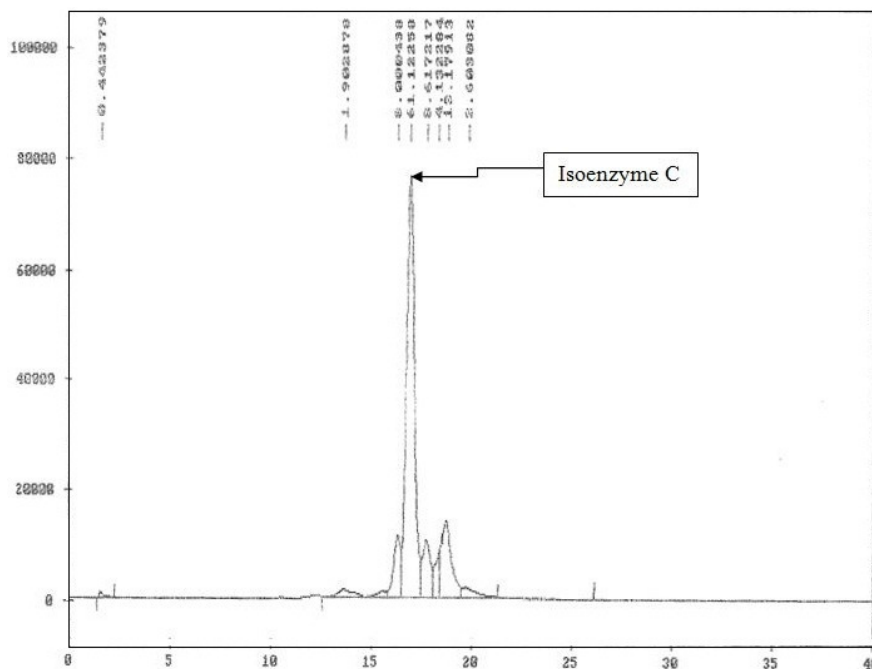


Figure 39. *Elution profile of 161453-grade HRP from a CM-HPLC column.* The peak representing isoenzyme C is indicated. Absorbance was detected at 280nm for the column eluate. A Waters Protein PAK CM, 8-HR, 8m column was used. A linear gradient consisting of eluent A (0.01 M sodium acetate, pH 4.4) and eluent B (0.01 M sodium acetate, 0.2 M NaCl, pH 4.4) at a flow rate of 0.8 ml/min was used during separations.

The production of 161457-grade product includes purification through a second ion-exchange chromatography step (Figure 40). This step removes proteins that were separated with the neutral and basic isoenzymes on CM Chromatography, as well as trace amounts of the acidic isoenzymes that may be present due to inefficient cation exchange chromatography.



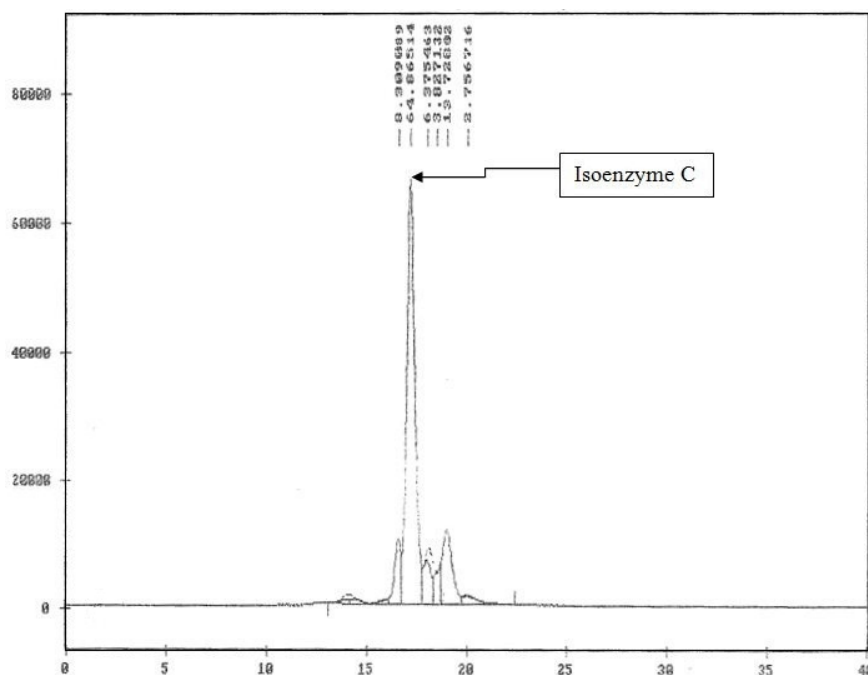


Figure 41. *Elution profile of 161451-grade HRP from a CM-HPLC column.* The peak representing isoenzyme C (64.86% of the total protein) is indicated. Absorbance was detected at 280nm for the column eluate. A Waters Protein PAK CM, 8-HR, 8mm column was used. A linear gradient consisting of eluent A (0.01 M sodium acetate, pH 4.4) and eluent B (0.01 M sodium acetate, 0.2 M NaCl, pH 4.4) at a flow rate of 0.8 ml/min was used during separations.

4.4. DISCUSSION

The characterization of the different product grades by SDS-PAGE and CM-HPLC demonstrated that material with a higher specific activity (Activity) and an increased percentage of the neutral isoenzyme C was obtained after each successive purification step. The increase in HRP isoenzyme C content is of major importance to the customers of BBI Enzymes, as the market trend still leans towards the use of mainly isoenzyme C in most applications. As illustrated in the SDS-PAGE gel analyses, discussed earlier in this chapter, and the comparative analysis discussed in the first chapter, the proteins in the enzyme preparations are heavily and seemingly equally glycosylated. The only carbohydrate present in the gel is bound to the protein. The review of the main groups of HRP isoenzymes relevant to BBI Enzymes highlighted the importance of producing a product range that satisfies

market requirements and allows for the marketability of the full HRP isoenzyme compliment. All the improvements discussed in this document rely on the accurate, real-time availability of enzyme activity results. The development of a new microtitre plate-based colorimetric HRP assay will be discussed in Chapter 5.

CHAPTER 5: DEVELOPING A NEW IN-PROCESS MICROTITRE PLATE COLORIMETRIC ASSAY

5.1.INTRODUCTION

The number of HRP in-process samples submitted to the Quality control (QC) laboratory fluctuates on a daily basis between 5 and 22. The main reason for this variance is that there are several batches of HRP extract at different stages of the process at any given time. The option of running production 20.5 hours a day will lead to even higher amounts of samples submitted for testing. The current in-process QC method (kinetic Guaiacol assay) is time-consuming and susceptible to both temperature and pipetting errors due to high dilution factors. The results generated are not confirmed by duplication. As a consequence, the daily assay capacity of a QC analyst is a maximum of 20 samples. The day is divided into 1.5 hours of set-up time, 12 minutes per run, 3 minutes for interpretation of each run and 30 minutes at the end of the day to update the Assay Logbook. An analyst also has to work exclusively on testing HRP samples, and overtime is incurred on a weekly basis in order to limit the accumulation of untested samples. This leads to the delay of results required by the production staff in real-time. These factors stressed the need to develop a rapid, quantitative microtitre plate(MTP) based calorimetric assay. The assay must be capable of processing multiple samples simultaneously and deliver accurate, real-time results to production to overcome the constraints of the current method.

Most assays measuring HRP seek the maximum sensitivity. These assays aim to detect the enzyme at picograms quantities per millilitre sample. As a consequence of the scale of HRP operations at BBI Enzymes, large amounts of HRP can be found in ubiquitous deposits. Even with routine cleaning, the levels present on all surfaces in the facility will react with sensitive substrates. This created a problem unique to BBI Enzymes. The objective was therefore to develop a sub-optimal substrate test system that would be quantitative but relatively insensitive in order to limit background interference by the ubiquitous HRP.

5.2. IDENTIFICATION OF SUITABLE SUBSTRATE FOR COLORIMETRIC ASSAY

Several substrates, specific to HRP, are commercially available. The substrates can be grouped into different categories, chromogenic, chemiluminescent or chemifluorescent (67). The key factors that need to be taken into consideration when choosing detection signal type is shown in Table 11.

Table 11. *Factors to consider when choosing a detection signal type (67).*

Colorimetric Substrates	Chemifluorescent Substrates	Chemiluminescent substrates
Medium/low sensitivity	High sensitivity	High sensitivity
Generally less expensive	Generally more expensive	Generally more expensive
Wide range of substrates available	Few substrates available	Few substrates available
Slow signal detection	Rapid signal generation	Rapid signal generation
Enzyme catalyzed rapidly	Enzyme activity retained	Enzyme catalyzed rapidly
Small linear range/poor low-end linearity	Large linear range/enhanced low-end linearity	Large linear range/enhanced low-end linearity
Flexible (stopped, non-stopped and kinetic assays)	Flexible (stopped, non-stopped and kinetic assays)	Non-flexible
Spectrophotometer detection	Fluorometer detection	Luminometer detection

Chromogenic substrates were investigated, due to its lower sensitivity, cost, slow signal development and flexibility. Chromogenic reactions are detected using standard

spectrophotometers. The challenge with the use of a chromogenic substrate signal type is identifying the linear range of the standard curve and establishing standard dilutions of the in-process samples that would be measurable in this range. The use of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) or ABTS was investigated, due to its relatively low sensitivity when compared to other commercially available chromogenic substrates (68). The oxidised ABTS substrate yields a blue-green colour that can be optimally measured in a range of 405 nm to 415 nm (68).

5.3. DETERMINATION OF ASSAY CONDITIONS

The low sensitivity substrate was further desensitized by sub-optimal assay conditions. Tris(hydroxymethyl)aminomethane (Tris) and phosphate buffer was investigated as it is effective as a buffer between pH 7.1 – 9.0, a range which is sub-optimal to HRP activity (69). Other factors that reduce the sensitivity were also investigated, including the addition of detergents. Sodium perborate is used as electron source. This donor was identified due to its stability and as a consequence long shelf life as part of a substrate buffer.

5.3.1. Detergents

The addition of Tween 20 and octyl- β -D-glucopyranoside to the assay buffer was investigated for their ability to stabilize or reduce the enzyme activity by partial protein denaturation and interference with substrate-enzyme interaction (70). In Figure 42, the effect of the two detergents on HRP enzyme activity is illustrated at different concentrations.

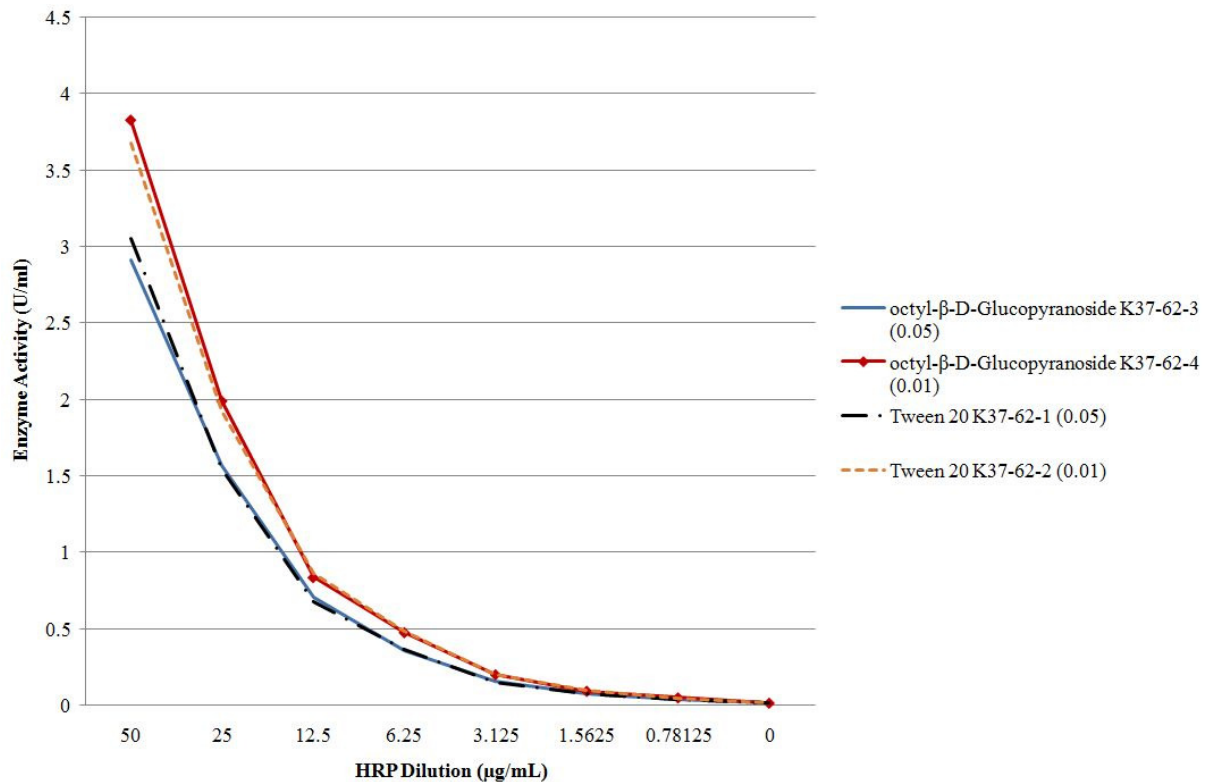


Figure 42. *Effect of detergents on HRP enzyme activity.* Enzyme activity was determined at different HRP concentrations in the presence of Tween 20 or octyl-β-D-glucopyranoside.

Both Tween 20 and octyl-β-D-glucopyranoside show inhibition of enzyme activity on a comparable scale.

5.3.2. pH

An increase in the pH of the assay system away from the optimum pH of HRP was investigated to slow down the reaction. The optimum pH for HRP enzyme activity is in the range of pH 6.0 to 6.5 (71). In Figure 43, the effect of different pH conditions on the enzyme reaction is shown. The pH was maintained by use of a suitable Tris buffer.

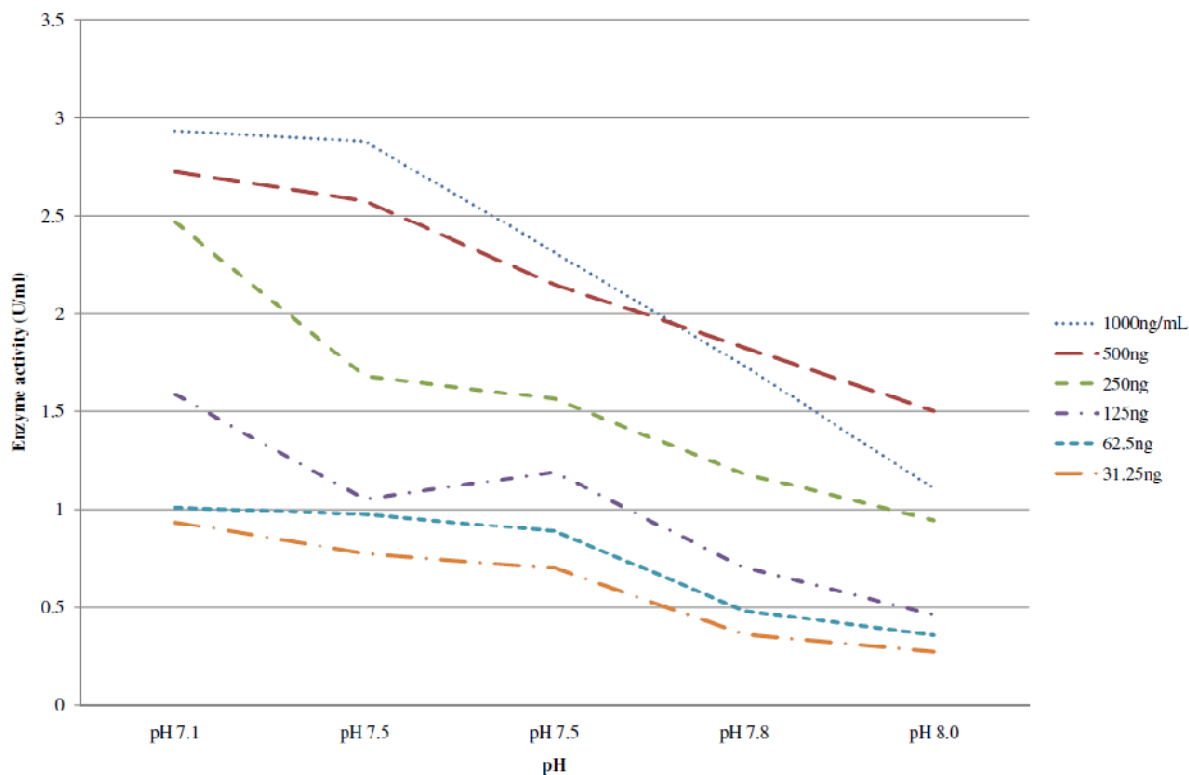


Figure 43. *Effect of buffer pH on HRP enzyme activity.* Enzyme activity was determined at four pH values for different concentrations of HRP.

The decrease in enzyme activity, associated with an increase in pH, may be due to a lower affinity for the substrate by the enzyme (72). This observation will aid in the desensitizing of the assay.

5.3.3. *Substrate concentration and reaction time*

At sub-optimal assay conditions for the HRP enzymatic reaction, it is important to ensure sufficient time is allowed for signal development. In assays run manually the reaction time is determined by the addition of a stop solution. A commercially available stop solution, containing 1% sodium dodecyl sulphate (SDS) in 25 mM HCl, was investigated. The use of an automated plate reader allowed for the accurate, consistent duration of incubation without the use of a stop solution. This was due to the system immediately performing the absorbance

reading of the pre-defined plate content at the end of the incubation period. The concentration of HRP substrate in the reaction mixture also determines the speed and sensitivity of the assay. If the substrate concentration is too low the reaction rate will also be extremely low and the minimum signal required for accurate measurement will not be obtained. As a consequence, it is important to review the development of the reaction over time, and at different substrate concentrations. In Figure 44 the effect of chromogen (ABTS) concentration, at an HRP concentration of $10\mu\text{g/mL}$, on signal development is illustrated. It was determined that an incubation period of 5 – 15 minutes, at room temperature was optimal, as the kinetics is linear during this period. At lower concentrations of HRP ($5\mu\text{g/mL}$), the kinetics was found to be linear between 10 – 20 minutes. The new, MTP-based assay conditions and components are noted in Table 12.

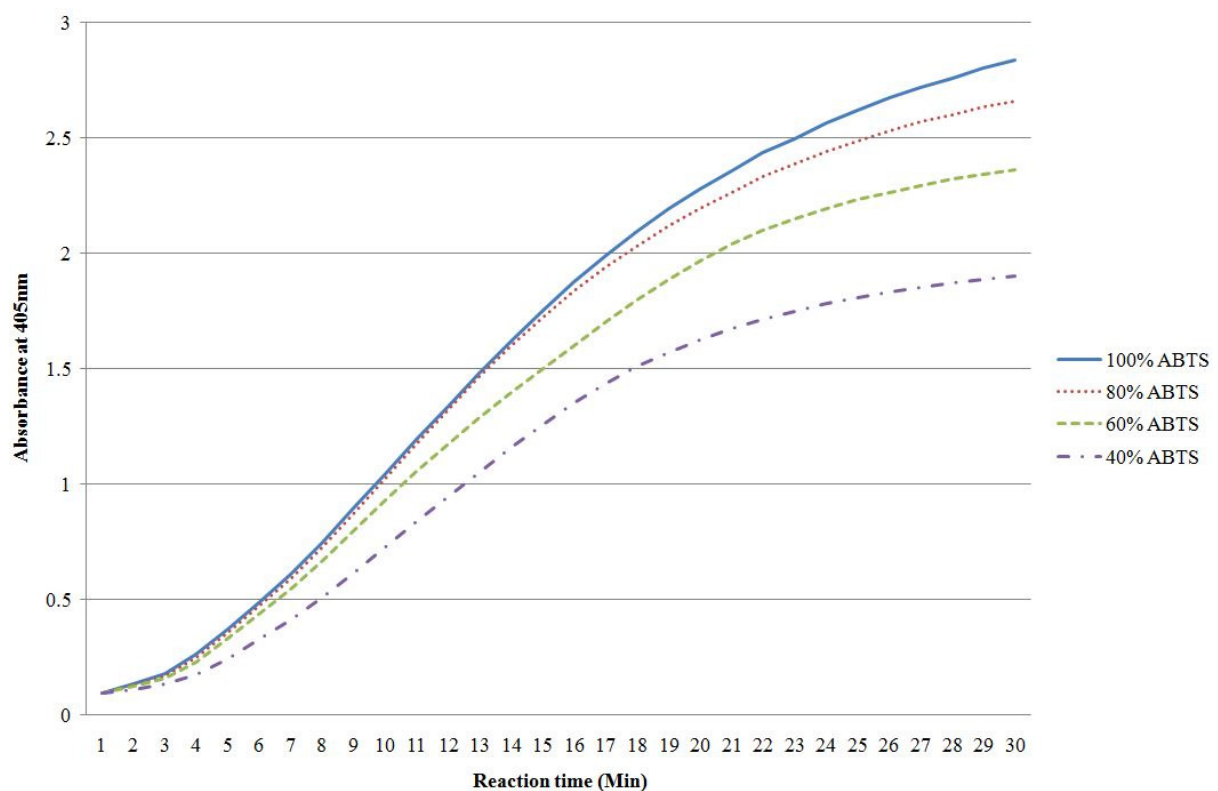


Figure 44. Signal development over time for different concentrations of ABTS at $10\mu\text{g/mL}$ HRP concentration (pH7.5). Colour development of chromogen concentrations of 100% (6×10^{-4} Mol/L), 80% (4.8×10^{-4} Mol/L), 60% (3.6×10^{-4} Mol /L) and 40% (2.4×10^{-4} Mol /L) were monitored at an absorbance of 405 nm for 30minutes at 25°C .

Table 12. *New MTP-based HRP assay conditions and components.*

Description	Composition	Quantity or volume per assay
<i>Sample preparation buffer</i>	100mM Phosphate buffer, pH7.5	Dilution dependent
<i>HRP Standard solution</i>	Range 0,125µg - 5µg per mL	10µL of each prepared dilution
<i>Duration</i>	10 Minutes	
<i>Substrate</i>	In 100 µL: 50µL undiluted ABTS, 50µL 0.01% Tween 20 in 1% Tris buffer (pH7.5)	100µL per well
<i>Stop solution</i> (1% SDS in 25 mM HCl) not required for use with automated plate reader timing incubation.		

Three independent experiments were performed to review the reproducibility of the standard curve. The standard deviation for data points above an enzyme concentration of 1µg/ml were higher than for data points lower than this enzyme concentration. It was determined that the linear part of the standard curve and most accurate results are obtained when read from the curve at an absorbance of between 0.3 – 0.8 (Figure 45). The graph represents the standard curve from one of three independent experiments.

In the event of two of the dilutions prepared for a sample yielding an absorbance within this range, the value closest to 0.6 would be most accurate. An internal control (enzyme prepare of known purity and activity) was also established and included in each test run. The result is required to be within a 5% variance range from the known enzyme content for the test run to be accepted thus ensuring the consistency of the method and assayist.

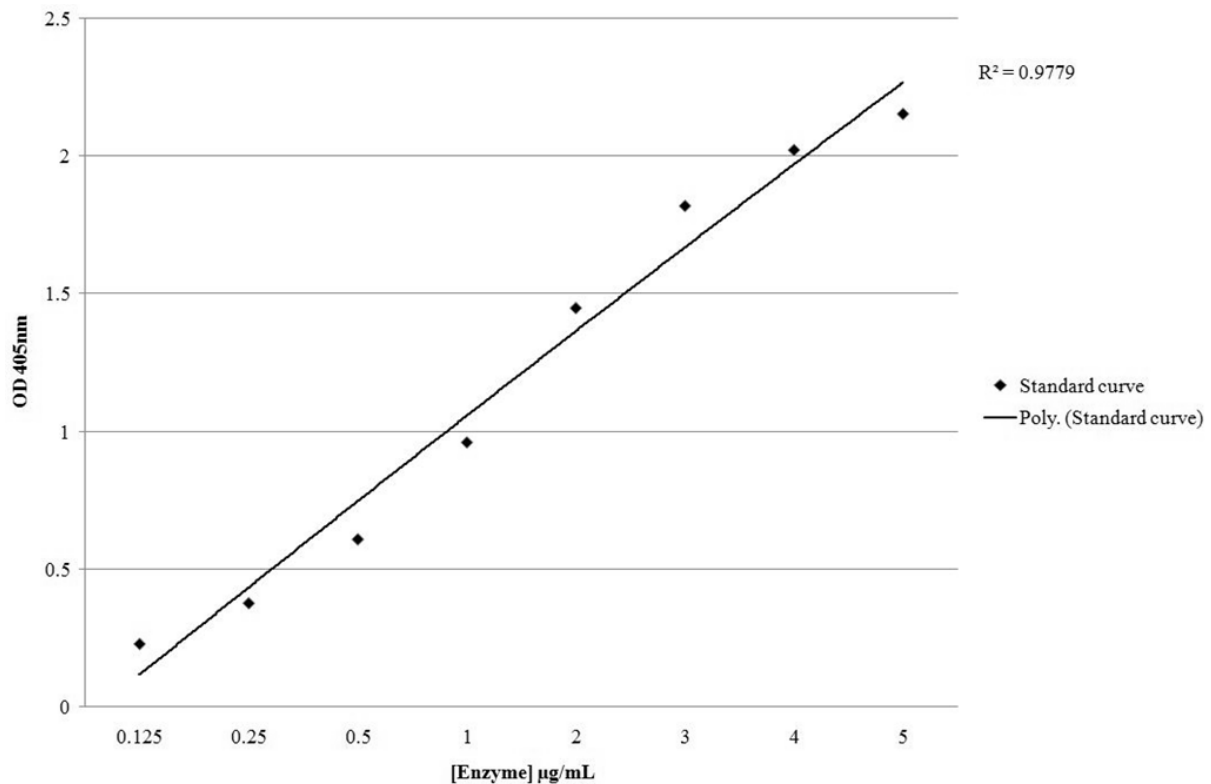


Figure 45. *Standard curve of MTP based HRP assay.* The most linear part of the curve is between 0.3 – 0.8. The R-squared value for the curve is 0.9525.

5.4. DISCUSSION

The current method for determining HRP activity is time-consuming (12 minutes per sample run for an experienced assayist) and susceptible to temperature variations. The results generated for in-process control are not confirmed by duplication. Following the development of a rapid, quantitative microtitre plate based colorimetric assay, it is now possible to accurately characterize the processes of product purification. This MTP-based assay is capable of processing multiple samples simultaneously and delivers accurate, real-time results to production. The system is capable of generating duplicate results of 10 different samples at 3 different dilutions in one run, while the use of a spectrophotometer, which calculates and prints out the concentrations of enzyme activity in the samples, eliminating assayist calculation errors. These enhancements increase the turn-over of results and

eliminates delays incurred due to the repeat analyses of samples that fall outside the accurate range of the system. By running the standard curve and control samples on the same plate during each run, the effect of temperature differences between runs are eliminated.

This newly developed microtitre-based HRP assay is simple, rapid and robust and has been illustrated to be accurate in producing real-time in-process control results. The in-process control points generate samples of varied HRP concentration and as a result different dilutions are required to produce absorbance readings that coincide with the accurate range of the standard curve. As part of the assay development, standard dilutions for every control point sample was determined and documented as part of the assay protocol, in order to reduce the occurrence of out of range resultsⁱ.

ⁱ BBI Enzymes. Peroxidase Microtitre Assay - AM147. *Assay Manual*. Cape Town : s.n., 2009.

CHAPTER 6: DISCUSSION AND CONCLUSION

HRP was first extracted and purified by BBI Enzymes in 1958, and since then the company has been a bulk producer of HRP for 53 years^j. The method employed to extract and purify HRP was an adaptation of the process developed by Keilin *et al.* in 1950 (31). In 1966, after a review of the peroxidase isoenzymes present in horseradish was published by Shannon *et al.*, the method was adapted to the current method developed to extract and purify HRP (9). BBI Enzymes produces an average of 7 billion units (BU) of HRP enzyme annually. At this scale of operations, the emphasis on yield is critical. A review of the HRP enzyme was performed. This allowed for optimization of the process whilst taking into consideration inhibitors, physical properties and stability of the protein. The optimization of the extraction and purification of HRP was investigated in an attempt to increase yield, quality, efficiency, throughput and control over the manufacture of all grades of HRP enzyme produced by BBI Enzymes. This study focussed on the horseradish raw material, techniques used in the extraction and purification of the product and the development of a real-time, robust enzyme assay. The standardization of raw material pre-harvest treatment was shown to improve the HRP content and workability of the roots supplied to BBI Enzymes. Varieties of horseradish were identified that specifically contained higher enzyme, and in particular higher isoenzyme C levels. The scientific basis of pre-harvest treatment was also reviewed and was illustrated to be an essential part of horseradish cultivation for BBI Enzymes. Extraction at a higher liquid to root ratio, with improved maceration was shown to liberate higher concentrations of HRP. Additional mechanical and enzymatic maceration was reviewed but not proposed as part of the optimized process due to challenges concerning the removal of additives and reduced workability of the extract.

^j SERAVAC. Lifescan - Horseradish Peroxidase from SERAVAC. Cape Town : SERAVAC, 1993.

Optimization and up-scaling of the UF system, including the pre-treatment of the extract before UF, was reviewed and it was found that optimising these factors were essential to the optimum performance of the UF system. These factors included more control over the extract pH and removal of particles larger than one micrometer by use of Celite[®] Hyflo diatomaceous earth during clarification, which increased throughput and reduced A/S usage due to increased concentration ability. These improvements lead to the review of the A/S fractionation step. The changes in the saturation conditions increased enzyme recovery and reduced A/S consumption as a consequence of the higher concentration factor achieved during UF concentration. DF was reviewed as an alternative to the time-consuming and labour intensive process followed when including passive dialysis in the production process. The use of DF reduced production lead times significantly by replacing additional A/S precipitation and passive dialysis, and added to the purification of the product. The use of semi-automated chromatography columns was reviewed and implementation increased control, consistency and robustness of the chromatography practiced. Overhead cost per enzyme unit was also reduced due to the option of running the chromatography system unattended.

A review of the composition and quality of current product and that produced from the optimized process was performed in order to ensure the quality of the product produced would be higher, yet within the specification of the customer. This was achieved by performing SDS-PAGE analyses with selective staining. It was shown that the preparations are comparable at the lower grades and equal or purer for the higher grades. This achieved the goal of producing material to fit the customer specifications and purity ranges.

The different product grades produced by BBI Enzymes following the optimized manufacturing process were characterized by use of PAGE- and HPLC-analysis. The review demonstrated that the material obtained after each successive purification step was of higher purity (SDS-PAGE), had a higher specific activity (enzyme activity) and had an increased percentage of the neutral isoenzyme C (CM-HPLC). The increase in isoenzyme C yield is of major importance, as the market trend still leans towards the use of mainly isoenzyme C in

most applications. As illustrated in the SDS-PAGE gel analyses discussed in Chapter 5 and the comparative analyses discussed in Chapter 1, the proteins in the enzyme preparations are heavily and seemingly equally glycosylated. The review of the main groups of HRP isoenzymes relevant to BBI Enzymes highlighted the importance of producing a product range that satisfies market requirements and allows for the marketability of the full HRP isoenzyme compliment.

The development of an MTP-based colorimetric HRP assay was discussed. The new test system is capable of processing multiple samples simultaneously and delivers accurate, real-time results to the production section. Duplicate results of 10 different samples at 3 different dilutions are performed in one analysis. This increases the turn-over of results and eliminates delays incurred due to the repeat of samples that fall outside of the accurate quantification range of the assay system. An internal control was also established and included in each test run. The result is required to be within a 5% variance range from the known enzyme content for the test run to be accepted. This ensures control over the consistency of the method and assayist.

The work discussed in this thesis has lowered input cost for the commercial-scale production of HRP, whilst increasing throughput and product yield by the implementation of new processes, equipment and control.

EXPERIMENTAL METHODS

All large-scale trials were performed on site at BBI Enzymes (Epping 2, Cape Town), utilizing 314-grade stainless steel piping and tanks. For temporary hosing, 2.5" Kanaflex PVC tubing (Kanaflex Corporation, Vernon Hills, IL) with 314-grade stainless steel couplings were used. Paddle stirrers used for the extraction, clarification and concentration stages of HRP production are designed as follows:

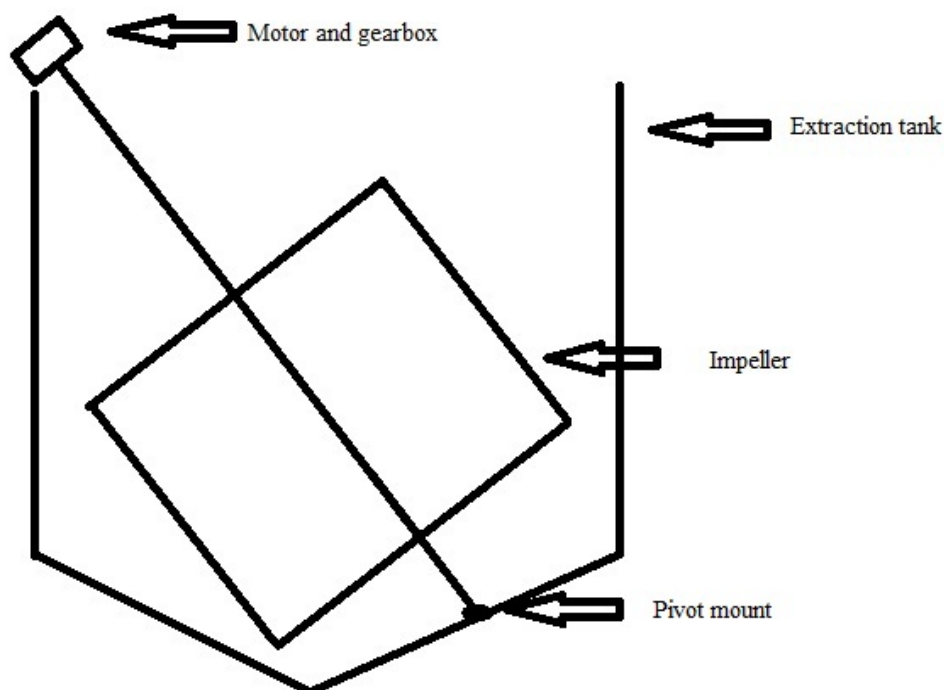


Figure 46. *Illustration of the design of the production scale extraction stirrers and tank at BBI Enzymes.*

Extraction, clarification and concentration holding tank stirrers agitate at a rate of 20RPM. Secondary stirrers agitate at a rate of 40RPM and are three-blade mixing propellers modelled on the marine propeller.

All HRP enzyme activity determinations were performed using the Guaiacol substrate kinetic assay developed by Hans Ulrich Bergmeyer (1). Guaiacol substrate and other reagents were acquired from Merck Chemicals. The assay method is attached as APPENDIX 1 at the end of this document.

Review of U.S. raw material sources

The first U.S. horseradish was received from J.R. Kelly Company (Collinsville, IL) in May 2007. However, as extraction and purification of HRP from this source began, a significant difference in HRP content and workability of the batches over the steps of our process became apparent. The *main findings* were: low concentrations of HRP present; finer mince / root particles were generated during maceration; low lignification – thin, tender cell walls; more, fine particles in Spun Extract (supernatant from centrifuged extract) during processing that is not removed by the Alfa-Laval decanter centrifuge under current conditions; difficulty when clarifying according to standard procedure; inefficient concentration through Hollow fibre membranes due to sub-optimal clarified extract; excessive concentration polarization and/or fouling of membranes.

The main reason for this was suspected to be the root variety received. The varieties received are selected and cultivated specifically for key properties required for the food market including low lignification, whiteness, yield (tonnage per acre), and disease resistance. The selection of these properties lead to contradictory characteristics to what have been established as key properties of horseradish associated with high HRP content, i.e. high lignification and susceptibility to stress (disease, drought, temperature, etc.).

Currently, S.A. farmers are cultivating a single variety of horseradish. These roots have been used as stock for the next growing year, each year, for over 45 years. Consequently, this line of horseradish have been subjected to annual stress and the result is a continually evolving (to adapt to stress) horseradish strain.

A number of different cultivars of horseradish were received from the U.S. farmers and reviewed for performance against the S.A. sourced raw material. The parameters reviewed are described under Raw material quality (Chapter 3).

Raw material quality

Raw material quality was reviewed against three main parameters:

1. *Good workability.* Low fine generation and good performance across clarification stages: clarified extract turbidity reduced to lower than 15 Formazin Turbidity Units (method included at the end of this document as APPENDIX 2).
2. High HRP enzyme content. HRP enzyme content as determined by use of the Guaiacol assay method (refer to APPENDIX 1)
3. *High isoenzyme C content.* A 45% isoenzyme C content is the target concentration, with the minimum concentration of 40% (method included at the end of this document as an appendix).
4. *High HRP content.* Total enzyme activity for the HRP isoenzyme compliment is required to be a minimum of 12MU/tonne of raw material, with an average of 14MU/tonne.

Extraction procedures

The *amount of HRP liberation* that can be achieved was determined by blending different samples of horseradish from S.A. and U.S.A origin at a root to liquid ratio of 1: 3.6 and higher ratios. For each experiment performed on the extraction optimization:

1. Aliquots were cut into 1-3cm³ cubes (from different regions – top, middle, end, outer layers and inner layers – of the root), or aliquots were removed from the production line post mincing to determine current maceration.
2. The cubes were homogenized with a Waring blender for 3 minutes in the presence of potable water or saline (NaCl) solution, in the case of experiments investigating blending.
3. The final volume of water or buffer used during blending or added to mince samples from the production line was dependent on the conditions reviewed.
4. Stirring of the extract was performed using a magnetic desktop stirrer with 3cm magnetic follower. All trials were performed at ambient temperature.
5. The duration of the extraction was dependent on the conditions reviewed.

The effect of the *degree of agitation on extraction* was investigated by stirring extract samples at the current rate of 20RPM and 100RPM. Control experiments were conducted on mince aliquots collected from the production line.

Improved extraction by *use of cell wall macerating enzymes* was reviewed by addition of three different enzyme powders from Shin Nihon Chemical (Showa-cho, Anjyo, Japan):

1. Sumizyme AP2. Pectinase, cellulase and hemicellulase activity.
2. Sumizyme C. Cellulase and hemicellulase activity.
3. Sumizyme MC. Pectinase, amylase and protease activity.

Minced horseradish samples were collected from the production line. It was suspended in 19.92U/mL Sumizyme enzyme solutions of potable water at a ratio of 1: 3.6. The solution was agitated with a desktop magnetic stirrer and 3cm follower. Incubation duration was 1 hour, after which solids separation was achieved by centrifugation at 4200RPM for 7 minutes with a Beckman J-6B. The supernatant enzyme activity was determined as set out in APPENDIX 1. Trials were also performed at a 1: 5 root to liquid ratio at the same enzyme concentration.

Trials on *additional mechanical maceration* were performed by extending the duration of blending with the desktop waring blender by 3 minutes. Mince samples from the production line (minced using Weiler® Grinder with double knife set and 10mm hole plate) was also used as start material. Mince was blended at a 1: 3.6 or 1: 5 ratio in the presence of potable water, for 3 minutes.

Concentration (Ultra-filtration)

The ultra-filtration trials were performed on desktop scale using a Watson Marlow Loadsure peristaltic pump. The inlet, outlet and filtrate pressures were visualized with in-line WIKA manual pressure gauges. The feed-flow was determined by manual measurement with a 100mL Pyrex glass measuring cylinder and TIMEX stopwatch.

The membranes reviewed were:

1. Sartorius Hydrosart® 0.1m² 10kDa (3051443901E-SW) tangential flow filtration stabilized cellulose based membrane cassette.
2. Pall Microza® SLP-1053 0.1m² 10kDa Hollow fibre PES membrane module.
3. GE Healthcare Start AXM 10kDa PES Cross-flow cartridges (UFP-10-C-2U).

Large-scale production trials were performed on the installed Pall Microflow[®] 16/20A UF system and based on the method attached at the end of this document, APPENDIX 4.

Chemicals used for the CIP of the membranes were acquired from Crest Chemicals (Midrand, South Africa): Sodium hypochloride (Sodium Hypo) and sodium hydroxide (Caustic Soda Solution - MBC). All water used is potable. Air pressure feed to the Microflow system was supplied at a pressure of 5.5 Bar, regulated to 4.5 Bar for operation.

Ammonium sulphate fractionation

HRP extract fractionation trials were performed with DSM[®] Fibre Intermediates (Sittard, The Netherlands) ammonium sulphate (161-CPH Ammonium sulphate Technical grade). Calculations for saturation concentrations investigated were determined according to work published in Data for Biochemical Research (58). Fractionations were performed according to the temperature of the concentrate. The effect of ammonium sulphate on HRP and its activity was determined by performing Guaiacol substrate kinetic assay as described in APPENDIX 1. Desktop magnetic stirrers with 3cm magnetic followers were used for the agitation of the concentrate during fractionation. For step-wise fractionation experiments, the fractionate was clarified by use of a Bucher funnel laden with Whatman[®] (1575-grade, Schleicher & Schuell, Germany) filter paper and washed Celite[®] filter aid (Hyflo[®]-grade, World Minerals[®]). The filtrate activity was compared to that of the un-fractionated concentrate.

DF

The DF of HRP was performed with Pall Microza[®] SLP-1053 (lab-scale, 0.12m² membrane surface area) and SLP-3053 (production scale, 4.5m² membrane surface area) hollow-fibres

at conditions as set out in Chapter 3 (3.6). The inlet, outlet and filtrate pressures were visualized with in-line WIKA manual pressure gauges. The feed-flow was determined by manual measurement with a 100mL Pyrex glass measuring cylinder (lab-scale) and a calibrated 20L polybin (production scale) and a TIMEX stopwatch. Chemicals used for the CIP of the membranes were acquired from Crest Chemicals (Midrand, South Africa): Sodium hypochloride (Sodium Hypo) and sodium hydroxide (Caustic Soda Solution - MBC). All water used was potable.

Chromatography

The Ion-exchange and hydrophobic interaction chromatography (HIC) media were all acquired from GE Healthcare (Uppsala, Sweden). Ion exchange chromatography were performed with Carboxymethyl (CM)- Fast flow and Diethylaminoethyl-Sepharose[®] Fast Flow resin. HIC was performed by use of Phenyl Sepharose[®] Fast flow Low Sub resin. Lab-scale trials were performed in 10mL Pharmacia[®] chromatography columns under gravity flow. Buffer salts were concentrated food grade acetic acid and sodium hydroxide (Caustic Soda Solution – MBC) for the preparation of buffers and pH adjustment were acquired from Crest Chemicals. Glycine EP/USP 80 MESH and phosphate buffer crystals were acquired from Warren Chem Specialities (Cape Town). All buffers were prepared using reverse-osmosis (RO) water prepared with an in-house RO system. Ammonium sulphate (161-CPH Ammonium sulphate Technical grade) was acquired from DSM[®] Fibre Intermediates (Sittard, The Netherlands).

Microtitre plate colorimetric assay development

The microtitre plate reader used was a Thermo Multiskan EX with SkanIt Software. PS-Microplate 96-Well flat bottom plates used for the assay was acquired from Greiner Bio-One GmbH. Reagents used were: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonate)

diammonium salt (ABTS) acquired from Roche Applied Science (Code 10102946001); Tween (Code P 5927), Octyl- β -D-Glucopyranoside (Code O8001), SDS $\geq 98.5\%$ (Code L4390), HCl 37% reagent grade (Code 435570) and Tris buffer (Tris-Hydroxymethyl-Aminomethane, (HOCH₂)₃CNH₂) crystals (Code T7443-250G, commercial name Trizma Pre-set Crystals) were acquired from from Sigma-Aldrich®.

SDS-PAGE analysis

SDS-PAGE analysis was performed according to the manufacturer instruction (PIERCE Precise™ Protein Gels – Tris-HEPES-SDS Precast Polyacrylamide Mini Gels booklet). The 4-20% Precise Protein Gels (10 Well) poly-acrylamide gels (25204) was acquired from Thermo Scientific; Laemmli Sample Buffer from BioRad (161-0737); and High Range Rainbow Molecular weight Marker acquired from GE Healthcare (RPN756V). 2-Mercaptoethanol was acquired from Merck Chemicals (Art. 805740). All samples were prepared by dilution of a 10mg/L sample solution with an equal volume of Laemmli Sample Buffer. The diluted samples were heated for 5 min at 100°C and allowed to cool down to room temperature before being loaded onto the gel. A final dissolved amount of 25µg of product was loaded per well for each of the samples.

Electrophoresis was carried out using a Pharmacia Fine Chemicals ECPS 3000/150 at 20mA for 3.5 hours. The BioRad Minicell electrophoresis housing was filled with BupH Tris-HEPES SDS Running Buffer (28398) acquired from Thermo Scientific. After electrophoresis, gels were removed and stained with Simply Blue™ SafeStain for 1 hour. The staining solution was discarded and the gel destained in distilled water overnight.

HPLC analysis

The HPLC procedure, chemicals, equipment and software used is described in APPENDIX 3.

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APPENDICES**APPENDIX 1**

PROCESS: Assay Methods	ISSUE DATE: 19.08.2009	DOC NO: BP6.2.2-WI79
TITLE: Peroxidase	REVISION: 0	PAGE: 1 of 2
AUTHORISED: Senior Technical Officer		APPROVED: QA Manager

CUSTOMER: ROCHE DIAGNOSTICS GmbH**1. REAGENTS****1.1 0,1 M KPO₄ Buffer pH 7,0**

Dissolve 0,53 g KH₂PO₄ (MM 136,09) and 1,06 g K₂HPO₄ (MM 174,18) in distilled H₂O, check pH 7,0 and dilute to 100 ml. Store diluent on ice and buffer at 25°C.

1.2 Guaiacol Solution

Dissolve 0,22 ml guaiacol in 100 ml distilled H₂O.

1.3 H₂O₂ Solution

Add 0,08 ml H₂O₂ (30%) to distilled H₂O (± 100 ml) to yield A₂₄₀ = 0,320 ± 0,010. (light path : 1cm; measure against distilled H₂O).

2. SAMPLE PREPARATION/ENZYME**Test Solution**

Prepare sample at 1 mg HRP/ml buffer (1.1). Dilute with buffer (immediately before assay) to yield 0,15 - 0,30 u/ml.

3. PROCEDURE

λ = 436nm; cell path length = 1 cm; cuvette volume = 3,15 ml; Temp. = 25°C.

Into glass cuvettes pipette:

	BLANK	TEST
Phosphate buffer (1.1)	3,05 ml	3,00 ml
Guaiacol (1.2)	0,050 ml	0,050 ml
Test solution	-----	0,050 ml
Mix and allow to equilibrate. Start reaction by adding:		
H ₂ O ₂ (2.3)	<u>0,050 ml</u>	<u>0,050 ml</u>
	3,15 ml	3,15 ml
Mix and monitor $\Delta A/\text{min}$ (linear portion). 0,02 - 0,025 will be linear portion of curve.		

4. **CALCULATION**

$$\Delta A/\text{min} = \Delta A/\text{min} (\text{test}) - \Delta A/\text{min} (\text{blank})$$

$$\text{ACTIVITY (u/ml)} = \frac{\Delta A/\text{min} \times 3,15 \times 4 \times \text{dilution}}{25,5 \times 0,05 \times 1}$$

$$\text{ACTIVITY (u/mg)} = \frac{\text{U/ml}}{\text{mg IHRP/ml original solution}}$$

COMMENTS

1. $\Delta A/\text{min}$ 0,015 - 0,030.
2. Measurement should be at maximal rate of exchange of substrate into end product.
3. Use glass cuvettes and glass pipettes.

APPENDIX 2

PROCESS: HRP	ISSUE DATE: 19.08.2009	DOC NO: BP5.3.2-WI21
TITLE: HANNA Turbidity Meter	REVISION: 0	PAGE: 1 of 3
AUTHORISED: Production Manager		APPROVED: QA & Compliance Manager

1. Purpose

To standardise safe and effective operating procedure for the HANNA HI 93703 Portable Turbidity Meter.

2. Scope

HANNA HI 93703 Portable Turbidity Meter in the HRP Secondary Extraction Facility.

3. Responsibility

3.1 Supervisor

To ensure correct and safe operation of the HANNA HI 93703 Portable Turbidity Meter.

3.2 Operators

To operate the HANNA HI 93703 Portable Turbidity Meter as per procedure (5).

4. Safety Precautions

4.1 Personal protective clothing as set out in BP5.2 shall be worn.

5. Procedure

5.1 Frequency

With every run

5.2 Equipment and material

- HANNA HI 93703 Portable Turbidity Meter
- RO water
- HI 93703-0 at 0 Formazin Turbidity Units (FTU)
- HI 93703-10 at 10 FTU
- HI 93703-50 Cleaning solution
- Cuvet
- Blue cuvet cleaning cloths

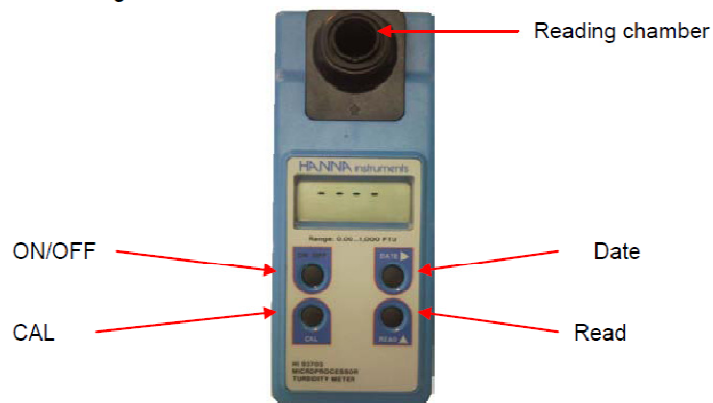


Figure 1. HANNA HI 93703 Portable Turbidity Meter

5.3 **Method (Calibration procedure)**

- 5.3.1 Turn on the meter and wait for the meter to display “---”.
- 5.3.2 Press the CAL key once - the “CAL” message will blink on the display for about 6 seconds, then the calibration mode stops.
- 5.3.3 While the “CAL” message is still blinking, press CAL again. The instrument is now in the calibration mode and a “CL” will appear on the lower part of the display. The date of calibration can be edited now by simply pressing the DATE/⇒ key. To scroll to the correct number press the READ/↑ key. The default blinking parameter is the month, on the left hand of the display (MM.DD).
- 5.3.4 To confirm the displayed data values and to go to the next step, press the CAL key once. A blinking “ZERO” message will appear.
- 5.3.5 Take the **HI93703-0** bottle containing the ZERO FTU³ Standard (or the turbidity-free dilution water) and fill the measurement cuvet.
- NOTE:** In order to minimize any error introduced by the cuvet, it is recommended to use (during calibration) the same cuvet you are going to use to perform the measurement.
- 5.3.6 Insert the cuvet with the **HI 93703-0 @ ZERO FTU** standard solution into the measurement cell and press the CAL key. A blinking SIP message indicates that the instrument is performing the measurement. After approximately 50 Seconds the instrument will ask for the **HI93703-10** standard solution @ 10FTU by displaying “10.0”.
- 5.3.7 Repeat the same procedure with **HI93703-10 @ 10FTU** standard solution.
- 5.3.8 Insert the cuvet with the **HI93703-10** standard at 10 FTU into the measurement cell and press the CAL key again.
- 5.3.9 After about 50 seconds the LCD will display “---”. NOW the machine is calibrated and ready for use.
- NOTE:** All glassware that is in contact with the standards should be properly cleaned and maintained.
- NOTE:** It is recommended to perform calibration **once a month**. Check the last date of calibration by pressing and holding the DATE key down for a few seconds. Record the calibration date, operator and results in logbook BP5.3.2-WI21.

5.4 **Method (Measurement procedure)**

- 5.4.1 Turn the meter on by pressing the ON/OFF key.
- 5.4.2 The meter will carry out a self-test displaying a full set of figures. After the test, the LCD will change to the measurement mode.
- 5.4.3 Then the display shows “---”, the meter is ready for measure.
- 5.4.4 Fill a clean cuvet up to the mark on the cuvet with a thoroughly agitated sample.
- 5.4.5 Allow sufficient time (one minute) for bubbles to escape before securing the cap.
NOTE: Do not over tighten the cap.
- 5.4.6 Wipe the cuvet thoroughly with the blue cloths provided or lint free tissue before inserting into the measurement cell, taking care to remove all fingerprints, dirt and liquid. The cuvet must be completely free of fingerprints or other dirt. Especially in the part where the light travels through. (Approximately 2cm from the bottom of the cuvet.)
- 5.4.7 Place the cuvet into the cell and check that the notch in the cap is positioned securely.
- 5.4.8 The mark on the cuvet cap should point towards the LCD screen.
- 5.4.9 Press the READ key and the LCD will display a blinking “SIP” (Sample In Progress). The turbidity value will appear after approximately 25 seconds.

5.4.10 **Interpretation of data during turbidity determination of HRP Clarified extract:**

- a) A reading of *equal or less than 13 FTUs* is desired for optimal performance of processes downstream of Clarification (Ultra-filtration and Ammonium sulfate fractionation).
- b) If a reading of more than 30FTUs is observed, the extract *should not be allowed* to be subjected to *Ultra-filtration*. Extract should then be re-worked until desirable turbidity is reached of *below 13FTUs*.
- c) Turbidity readings of between 13 – 30FTUs are acceptable, but should be noted and approved by your Supervisor.

5.4.11 **NOTE:** After every 5 measurements, or when a very turbid sample was tested, rinse the cuvet thoroughly using HI 93703-50 cleaning solution. After flushing the cleaning solution, rinse the cuvet with distilled or RO water.

5.4.12 **Cleaning of cuvet:**

- a) Empty the sample material from the cuvet.
- b) Rinse the cuvet three times with RO water in order to remove any sample material.
- c) Decant HI 93703-50 Cleaning solution into the cuvet to a height of 1 cm (in the cuvet). Replace the cap and shake vigorously. Fill the rest of the cuvet with RO water. This is the “storage state”.
- d) Before introducing the next sample material, decant the storage solution to waste and rinse the cuvet with three volumes of RO water.

APPENDIX 3

PROCESS: Assay Methods	ISSUE DATE: 29.04.2010	DOC NO: BP6.2.2-WI107
TITLE: HPLC Assay for Isoenzyme C content	REVISION: 1	PAGE: 1 of 7
AUTHORISED: Laboratory Manager		APPROVED: Quality & Compliance Manager

1. **Purpose**

Procedure to assay for the Isoenzyme C Content in Peroxidase using high performance liquid chromatography (HPLC).

2. **Scope**

Applies to Peroxidase product requiring Isoenzyme C Content assay.

3. **Responsibility**

All competent and HPLC-trained assayists.

4. **Procedure**4.1 **Test Principle**

Different isoenzymes of Peroxidase and contaminating proteins are separated using a weak cation exchanger at pH 4,4 and detected in the UV range at 280 nm. In addition, detection can be performed at 403 nm which is selective for Peroxidase.

4.2 **Instruments, materials**

Chromatograph: Agilent series or comparable
 Pre column: None
 Separation column: Protein PAK CM, 8-HR, 8 µm (Waters WAT-039785)
 Detector: Agilent series 1100 MWD or comparable

4.3 **Reagents**

Acetic acid: Merck 63 (or comparable) 100% analytical grade
 Sodium chloride: Merck 6404 (or comparable)
 Sodium hydroxide solution: Merck 9956 Titrisol (or comparable) approx. 1M
 Peroxidase isoenzyme C: Roche ID.: 815462 (EIA or quality I)
 Peroxidase isoenzyme B: Supplied by the Roche QC group, responsible for release

4.4 Preparation of the solutions

Eluent A (acetate buffer 0,01 mol/l, pH 4,4):

- Add 570 µl acetic acid to approx. 900ml repurified water,
- Adjust the pH value to 4,4 with sodium hydroxide solution.
- Adjust to 1000ml with repurified water.

Eluent B (acetate buffer 0,01mol/l, NaCl 0,2mol/l, pH 4,4):

- Dissolve 570 µl acetic acid and 11,70g sodium chloride in approx. 900ml repurified water.
- Adjust the pH value to 4,4 with sodium hydroxide solution.
- Adjust to 1000ml with repurified water.

4.5 Preparation of samples and standards

Reference solution:

- Prepare a solution of isoenzyme C, according to the preparation of the sample solution.

Identification solution:

- Mix sample solution and reference solution 1 + 1 each.

4.6 Settings of instrument:

Eluent flow rate (ml/min):	0,8	maximum pressure (bar):	80
Part of eluent B (%):	3		
Running time (min):	45		
Injection volume (µl):	10		
Detection:	UV/VIS	wavelength 1 (nm):	280
		Wavelength 2 (nm):	405

Program:

Time (min):	Event
1	%B 3
22	%B 33
25	%B 100
30	%B 100
35	%B 3

4.7 High-performance liquid chromatography (HPLC) – Operating procedures

HPLC operation:

1. Prepare and/or filter buffers. Sonicate all solvents and mobile phase before use for 5 minutes and allow liquid temperature to condition to room temperature.
2. Make sure waste bottle is empty
3. Switch on, in sequence:
Pump A→Pump B→Controller→Detector→AD convertor→Computer, printer automatically switches on/off.
4. Initial conditions:
 - Pump A = Methanol (20%)
 - Pump B = *Milli-Q* water (80%)
5. Start first gradient (Gradient 1) in order to equilibrate system with 100% water. Run for 15 minutes.
6. Remove Methanol bottle (Pump A), dip filter and pump line into “Wash H₂O” to remove methanol. Place Pump A line into Buffer A bottle.
7. Purge Pump A by sucking two Priming syringe volumes (2x10ml) directly from the pump at the “solvent draw-off valve”.
8. Switch Controller to “Isocratic”, divert flow to “Reference valve”, put a container under the outlet and run 100% Pump A at 7ml/min for 1 minute at full speed to clean the line’s void volume.
9. Start Gradient 2 in order to equilibrate system with 100% Buffer A (Pump A).
10. Remove *Milli-Q* Water bottle (Pump B). Place Pump B line into Buffer B bottle.
11. Purge Pump B by sucking two Priming syringe volumes (2x10ml) directly from the pump at the “solvent draw-off valve”.
12. Switch Controller to “Isocratic”, divert flow to “Reference valve”, put a container under the outlet and run 100% Pump B at 7ml/min for 10 seconds at full speed to clean the line’s void volume.
13. Start Gradient 3 in order to equilibrate system, currently equilibrated with 100% Buffer A, with 97% Buffer A (Pump A) and 3% Buffer B (Pump B). Run for 30 minutes. This is the initial conditions for an “Isoenzyme C (HRP) percentage determination” run.
14. Software start up (computer):
 - From Main(start-up screen), go to:
 - i. Acquire→File→Load Acquisition Method→HRP_C
 - Then: Process→Download Acquisition Method→Change the “Raw data file name” to the batch no. of the sample that is going to be run, i.e. 0059→Accept→Accept

Preparation and Injection of a sample:

14. Dissolve 10mg (±2mg) of freeze-dried HRP in 1ml of Buffer A, achieving a final concentration of 10mg/ml. Vortex for 3 seconds.
15. Initialize Gradient 4, “Isoenzyme C (HRP) percentage determination” run, on Controller (Select gradient)
16. Draw a 10µL aliquot of the sample with a Hamilton syringe and load into the system by flipping the “Sample valve” to “load”, removing the plug and injecting the sample.
17. Flip the “Sample valve” to “Inject”. The AD convertor should automatically start (Green Run 1 switch ON), if not press Run 1 button.
18. Allow run to finish (40 minutes) and system to equilibrate for an additional 20 minutes (Total 60 minutes on Controller screen).
19. In *Acquire*, load:
 - Go to→Process
 - In *Process*, load: File→Load Chromatogram→Select one

- Process→Integrate Chromatogram→Edit→Baseline: Do editing and accept. Separate peaks by use of the perpendicular drop method.
 - i. Important: Establish the baseline of the chromatogram at the same level as the zero reading line (picture below).

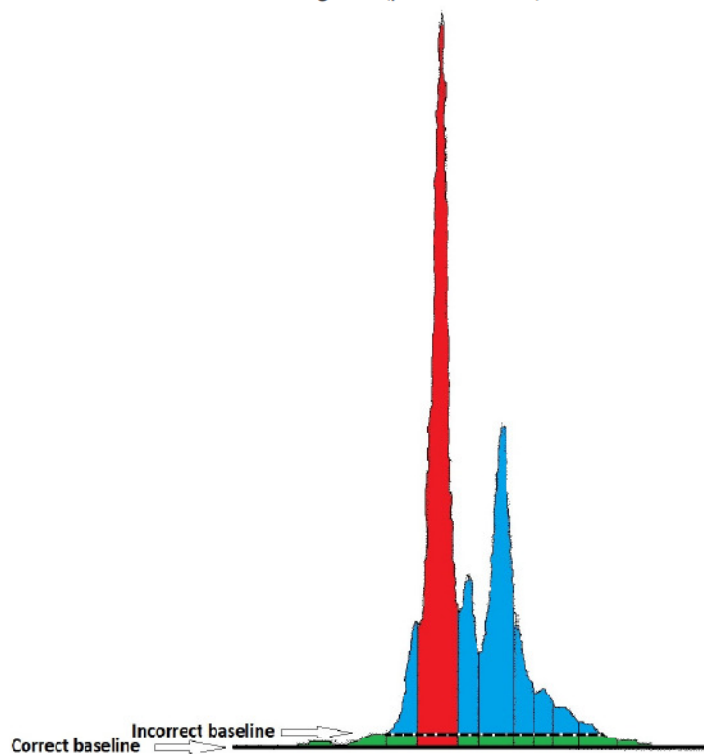


Figure 4. Diagram illustrating correct and incorrect baseline integration.

- Once all editing has been concluded, before printing, press Shift F5 to return graph to original size

To delete chromatograms (integrated):

1. In Process window: File→File Utility→Delete results→AllTo transfer raw data files from software to folder for backup storage:
2. Go to→Main→Applications run
3. In Total Commander, in right hand "window", Create new folder describing files to be saved inside, i.e. date
4. Then, in left hand "window"(c:\Apex\Examples*.*) , select all rd(raw data) files→Move.

To shut down Apex software, go to Main, Exit.

20. After the last run has ended and 60 minutes have elapsed, start Gradient 5 in order to equilibrate system with 100% Buffer A (Pump A). This is the *first step* in the *shut down sequence*.
21. Remove Buffer B bottle (Pump B), dip filter and pump line into "Wash H₂O" to remove buffer. Place Pump B line into *Milli-Q* Water bottle.
22. Purge Pump B by sucking two Priming syringe volumes (2x10ml) directly from the pump at the "solvent draw-off valve".

23. Switch Controller to "Isocratic", divert flow to "Reference valve", put a container under the outlet and run 100% Pump B at 7ml/min for 10 seconds at full speed to clean the line's void volume.
 - Also, clean pump by flushing it manually with distilled water
24. Start Gradient 6 in order to equilibrate system, currently on 100% Buffer A (Pump A), with 100% water (Pump B).
25. Remove Buffer A bottle (Pump A), dip filter and pump line into "Wash H₂O" to remove buffer. Place Pump A line into Methanol bottle.
26. Purge Pump A by sucking two Priming syringe volumes (2x10ml) directly from the pump at the "solvent draw-off valve".
27. Switch Controller to "Isocratic", divert flow to "Reference valve", put a container under the outlet and run 100% Pump A at 7ml/min for 1 minute at full speed to clean the line's void volume.
 - Also, clean pump by flushing it manually with distilled water
28. Start Gradient 7 in order to equilibrate system, currently on 100% water (Pump B), with the "sleeping mix", consisting of 80% water (Pump B) and 20% Methanol (Pump A).
29. Switch off the HPLC system components in the reverse order:
 Computer, printer automatically switches on/off → AD Convertor → Detector → Controller → Pump B → Pump A

4.8 Evaluation

Laboratory data system:

Integration time (min): 40
 Method: Area-% method

Result of analysis: Isoenzyme C content (Area%)

Procedure for integration:

If the chromatographic analysis results in complex chromatograms, which exhibit groups of unresolved peaks, integration will be performed by dropping a line perpendicular to the baseline (see example of chromatogram).

See typical chromatography attached:

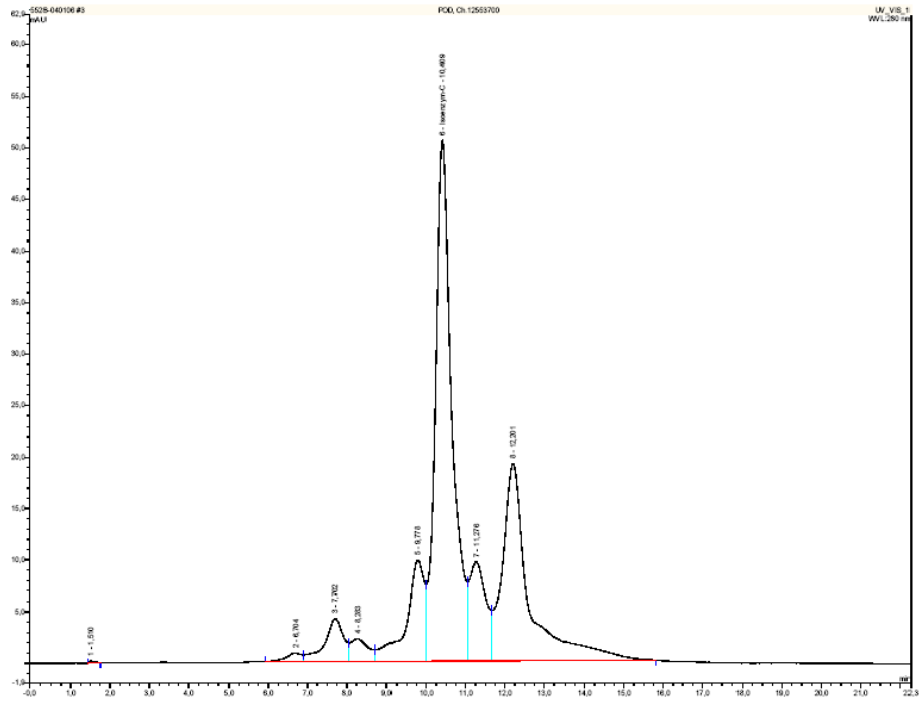


Figure 1. Batch: 0056 Isoenzyme C 280 nm 46 %, 403 nm - 49%

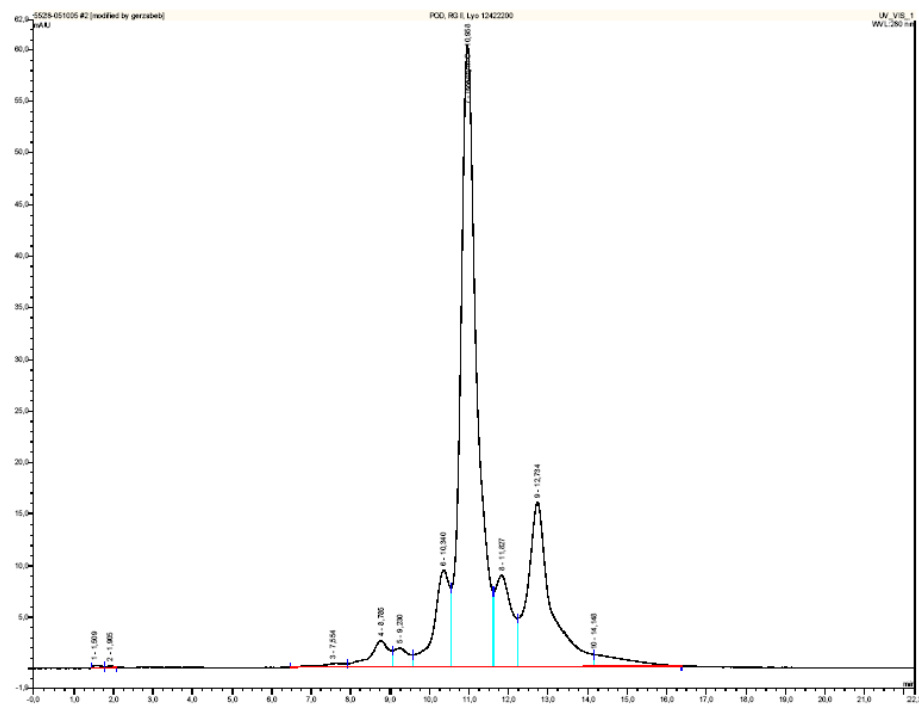


Figure 2. Batch: 161457 Isoenzyme C 280 nm 56 %, 403 nm - 59%.

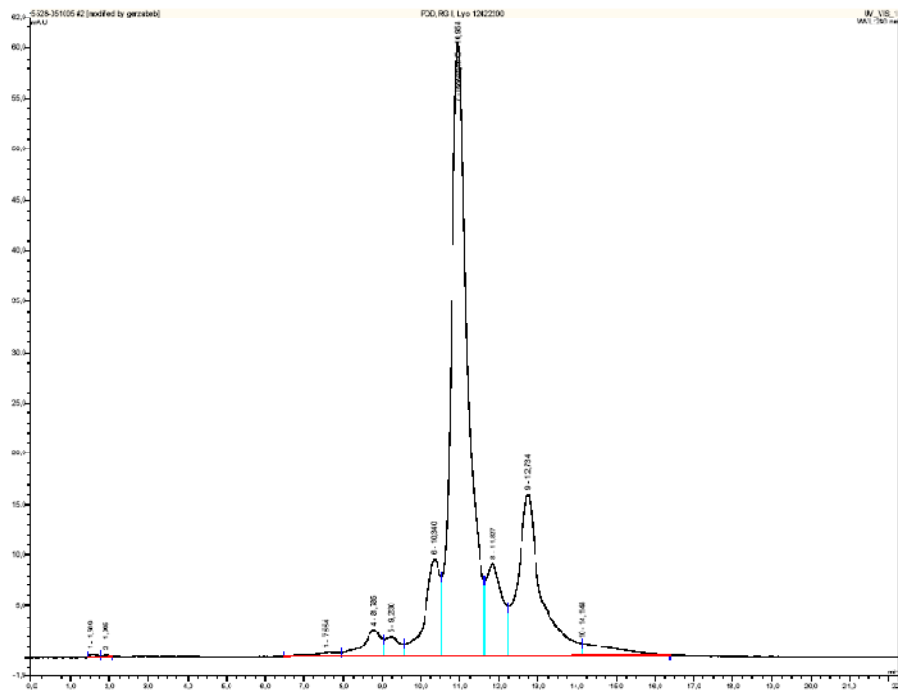


Figure 3. Batch: 0086 Isoenzyme C 280 nm 37 %, 403 nm - 39 %

Reference:

Roche: 5528_POD_E, TV_5528_TEXT_E, 6.0, CURRENT, 13.10.2004

APPENDIX 4

PROCESS: HRP	ISSUE DATE: 19.08.2009	DOC NO: BP5.3.2-WI 23
TITLE: Microflow 16/20A Ultra-Filtration system	REVISION: 0	PAGE: 1 of 9
AUTHORISED: Production Manager		APPROVED: QA & Compliance Manager

1. Purpose

To standardise the safe and effective operating procedure of the Microflow 16/20A Ultra-filtration (UF) system.

2. Scope

Microflow 16/20A UF system in the HRP Secondary Extraction Facility.

3. Responsibility3.1 Supervisor

To ensure correct and safe and accurate operation of Microflow 16/20A UF system.

3.2 Operator

To operate Microflow 16/20A UF system as per procedure (5).

4. Safety Precautions

4.1 Personal protective clothing as set out in BP5.2 shall be worn.

4.2 If any excessive vibrations or noises are noticed, the machine should be switched off immediately and Process Development/Maintenance contacted.

5. Procedure5.1 Frequency

With every run.

5.2 Equipment and material

Microflow 16/20A UF system
Canno flex pipes
Clamps

5.3. Method

5.3.1 Start-up procedure:

- a) Switch on the MAIN SWITCH CP (marked A in Figure 2) on the bottom left of the control panel housing the touch screen interface (screen) shown in Figure 2.
- b) Wait for the system start-up to finish. This will be done when the alarm signal is activated (flashing RESET button appears on screen, marked B in Figure 2, and light on top of control panel flashes whilst sounding the alarm).
- c) Stop the alarm by pressing the RESET button displayed in the top right hand corner on the touch screen interface (marked B in Figure 2).
- d) The display will now show the MAIN MENU. The options displayed are shown in Figure 3. They include:
 1. PRODUCTION
 2. LINE OUT FLUSH
 3. PRODUCT RECOVERY
 4. RINSE

5. DRAIN
6. MANUAL
7. CLEANING
8. SETTINGS
9. BACKFLUSH
10. INTEGRITY TEST
11. CYCLES
12. FILTR FLOW CONTROLLER
13. PUMP P1 CONTROLLER
14. PUMP P2 CONTROLLER
15. PTR PUMP CONTROLLER
16. FEEDLINE IN FLUSH



Figure 1. Front view of the Microflow 16/20A UF system



Figure 2. System control panel. The MAIN SWITCH CP is marked "A", TOUCH SCREEN INTERFACE as "B" and the EMERGENCY STOP as "C".

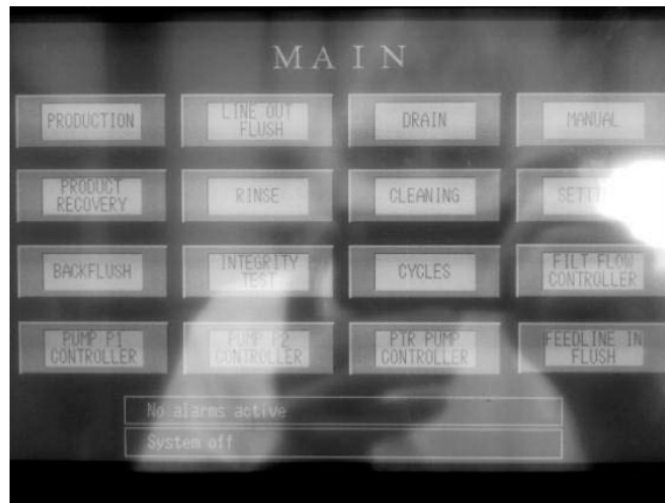


Figure 3. Main menu display on the touch screen interface

The options that you, as operator are allowed to access is:

1. PRODUCTION
2. LINE OUT FLUSH
3. DRAIN
4. PRODUCT RECOVERY
5. RINSE
6. CLEANING
7. BACKFLUSH
8. INTEGRITY TEST
9. CYCLES
10. FEEDLINE IN FLUSH

Limited access is allowed to SETTINGS, as you are allowed to look at the PROJECT HISTORY, under the PROJECT INFO tab. Here you can find a list of the last 200 steps that was performed by the system, including alarms.

e) Three main programs will be used during the daily operation of the system:

1. PRODUCTION

This is the program for the actual concentration. Product to be treated is transferred from customers' main tank in a topped-off mode to the concentration tank T-1 and the filtrate is transferred via the filtrate/back-flush tank T-2 to drain.

The required batch-size is set at the beginning of the filtration process (as per BP5.3.2-WI23-SF01) and when the total volume has been reached, the production program will stop automatically. Concentrate can then be recovered using programs Recovery, Line out flush and Solo back-flush (as discussed later).

In the final part of the Production program, the feed of product to the tank T-1 is stopped and the remaining product in the tank will be concentrated until the lowest required hold-up volume or the highest possible required percentage of solids have been reached.

This process is controlled by pressure, temperature or level and has an overall cut-out time which restricts the duration of the concentration process (timer 99). System and product are

protected against undesirable situations such as:

High temperature, high pressure, over-concentration, dry-run, overflow, power failure, instrument air failure.

Start the Production/Concentration procedure by completing the logbook (BP5.3.2-WI23-SF01, shown below), following the instructions and making sure that all requirements are met and information logged.

2. CLEANING

In this program mode the system performs a clean-in-place (CIP) with a chemical solution after heavy fouling or when the production has to change from one product to another.

The choice can be made either to use hot or cold water and also this program is a combination of various program steps with some special drain steps, rinses and back flushes in between, in order to remove any trapped rest chemicals. The duration of the chemical cleaning can be set as required.

3. CYCLES

This is a unique and very practical program in which all individual programs can be attached to each other and a chain of programs will be active in a logical order.

This means that the unit can be programmed for a total process where next to the production, concentration-recovery- and cleaning steps take place which makes the unit a standalone fully automatic production filter system which does not need to be constantly attended by an operator during the total filtration process due to a repeat function which can be activated and the set programs will be repeated for 1, 2 or 3 times or even in a continue mode.

- f) Start Production by following the instructions and completing the checklist on BP5.3.2-WI23-SF01 shown below.

Template of BP5.3.2-WI23-SF01:

Production (Concentration) Log-sheet	Date				
Was Clarified extract holding tank cleaned before use?					
Was a 100µm filter bag used to retain any Filter aid and/or particles during the filling of the holding tank?					
Pre-filters (large and small) cleaned as per BP5.2.1-WI51?					
Product inlet connected to system valve V1A and all valves opened between the holding tank and V1A?					
Is the system drained? If no, proceed with Drain by selecting DRAIN tab on MAIN menu and then pressing START (top left).					
Start the Production step (in Main menu):					
1. Press START (Top left of screen).					
2. Confirm all questions asked by system through manual inspection. Make sure Product recovery line is connected to a recovery tank and all manual valves (Feedback line valve located in Canno flex line bleeding concentrate back to Tank 3; outlet valve on tank 3 where UF system feeding line is connected; large pre-filters) are open.					
3. CLEAR TOTALIZERS (top left of screen), check and key in batch volume (volume of extract in holding tank 3 connected to system, Max. Input = 30000L). Press ENTER.					
4. Concentration factor (CF) input as 10. Press ENTER, then if all above has been answered YES and correct information entered, press PROCEED.					
5. Confirmation of inputs page appears, PROCEED/YES. Production starts. Complete logbook:					
Time: Start					
End					

<i>Volume (litres):</i>	Batch volume			
	Concentrate			
	Filtrate			
<i>Flow controller:</i>	TMP (Yes/No)			
	Flow (Yes/No)			
<i>Filtrate flux(L/hour, shown in Production screen):</i>	T=0			
	T= End			
<i>Comments:</i>				

IMPORTANT: As soon as Production has been started, Open the valve on the small bleed-back line (Figure 4) that runs back to the 30000L holding tank by turning the valve handle to align in parallel to the Canno flex pipe line. This will allow concentrate from the circulation loop to flow back to the holding tank. If at any stage you are unsure of your selection/options, contact your supervisor of Process Development.

General AI ARMS:

1. PIA 5-1 Inlet pressure too high. This indicates that the pre-filters have blocked and needs to be cleaned. The alarm needs to be reset, the pre-filter cleaned and the Production run re started in the same way as it was when the alarm started.

Air pressure. This indicates a drop in air pressure to below the acceptable level for the system. The air pressure level can be confirmed by reading the pressure on the gauge located below the Control panel. If the pressure is lower than 5 Bar, Maintenance and your Supervisor should be informed, in order to address the problem. As soon as the problem has been eradicated, re-start the system by following instructions on BP5.3.2-WI23-SF01 shown above.