
DUBLIN CITY UNIVERSITY

GUINNESS IRELAND GROUP

**SOME NOVEL ANALYTICAL APPROACHES TO THE
ASSESSMENT OF BEER QUALITY**

Thesis submitted for the award of Ph.D. by David Madigan B.Sc.

DECLARATION

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph.D. in Analytical Chemistry, is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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Date: 12 June 1996

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Some Novel Analytical Approaches to the Assessment of Beer Quality.

David Madigan.

ABSTRACT

The shelf-life of a packaged beer is determined by its ability to resist detrimental changes to its flavour, aroma and clarity. The most common symptoms of beer deterioration are the development of colloidal (non-biological) haze, formation of off-flavours, precipitation of sediment, and, in extreme cases, the growth of spoilage organisms. The susceptibility of a beer to spoilage by any of these mechanisms may be predicted by measurement of appropriate indicator compounds. The aim of this thesis was to develop new or improved liquid chromatographic methods for the determination of these compounds, using selective detection systems which allowed direct injection of samples following dilution and/or clarification. Where appropriate, these methods were applied to the development of optimised stabilisation protocols.

The area studied in greatest detail was the application of analytical technology to the investigation of beer colloidal stability. Novel methods for the determination of flavanols in beer were developed, and new procedures for the isolation of flavanol standards were described. These methods were then applied to the detailed study of the role of these compounds in beer haze and flavour instability. The stabilisation process was also studied in detail, and optimised stabilisation protocols were recommended.

Volatile phenolics in beer were also studied. Novel methods for the determination of phenolic-off-flavour (POF) causing compounds were developed, and were applied to the optimisation of brewing regimes as well as to the classification of POF-producing yeasts.

Oxalate may cause hazes and sediments in beers. A method was therefore developed for the determination of soluble and insoluble oxalate in beer and beer sediments, and the factors leading to the formation of such sediments were examined with a view to recommending procedures which could prevent the incidence of oxalate complaints in beer.

Novel analytical methods using electrochemical detection were also developed for the determination of carbohydrates in beers, worts and non-alcoholic beverages, and for the determination of ascorbic acid in beer and malt beverages. These methods were then applied to the assessment of beer quality in general.

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Dedication: This work is dedicated to my parents Alan and Margaret, my brother Andy, and my best friend Triona O'Sullivan.

1. INTRODUCTION: THE ROLE OF ANALYTICAL CHEMISTRY IN THE BREWING INDUSTRY

1.1. A BRIEF INTRODUCTION TO BREWING

Brewing is one of the oldest applications of biotechnology, and it is believed to have been practised as early as ancient Egyptian times. Needless to say, production techniques have changed considerably since then, but the basic principles of brewing have remained the same for centuries. A detailed description of the brewing process would be far beyond the scope of this chapter, and the interested reader is directed to some excellent texts which have been written on the subject [1-5]. What follows is a brief outline which should serve to make clear to the reader unfamiliar with brewing some of the terms used later in this thesis.

The brewing process generally consists of a linear chain of events as depicted in Figure 1.1. This process may be broken down as follows:

Malting

Barley (*Hordeum vulgare*) contains starch as nearly two-thirds of its mass. In order to make this starch extractable into the brewing water, however, the structure of the barley must be modified. The first stage in this process involves steeping the barley to raise its moisture content to about 40%. The hydrated barley is then germinated under controlled conditions. During germination, a number of carbohydrases and proteases develop, and these enzymes render the internal structure of the grain more suitable to subsequent extraction. The germination process is terminated prematurely at the optimum stage of modification, and the malt grains are kiln-dried to reduce the moisture content. The malting process is often carried out at a location remote from the brewery, and the prepared malt is then delivered and stored in silos in the brewery itself.

Mashing

Mashing is the process by which the fermentable sugars are extracted from the

malt. Malt grains are first milled to release the starchy endosperm material from the husks, and are then mixed with warm water to form a thick mash, with a consistency somewhat similar to porridge. The mash is maintained warm at 50-65°C, depending on the type of product required, to allow either the proteolytic or amylolytic enzymes to function. At the end of mashing, water at 78°C is used to sparge the mash, and remove the end product, sweet wort, which is rich in fermentable carbohydrates and amino-acids. A balance must be struck at this stage between, on the one hand, removing as much material as possible from the grains, and on the other hand, over-extracting, resulting in a weak sweet wort. Some breweries perform the entire mashing operation in one vessel, called a mash tun, whereas others employ one vessel for the mashing (mash conversion vessel), and another for the sparging process (lauter tun or kieve).

Boiling

The sweet worts produced during mashing are potentially unstable due to their high sugar content, and are therefore transferred immediately to a boiling vessel called a copper or kettle. The worts are then boiled for 60-120 min, effectively sterilising the contents, and it is at this stage that hop cones, the flowers of the hop plant (*Humulus lupulus*) are typically added to the wort, to provide bitterness in the final beer. Although hops themselves are not bitter, they contain substances (α -acids) which isomerize under the conditions of boiling to produce iso- α -acids, which are responsible for the uniquely bitter taste of beer. Another important process occurs during boiling, and this is the combination of proteinaceous material extracted from the mash, with polyphenols which are extracted from the mash and from the hops, to form 'break' or 'trub'. 'Hot-break' precipitates out during boiling, whereas 'cold-break' results from the cooling of the hopped wort after boiling.

Fermentation

The hopped wort is cooled to between 10 and 30°C and aerated en-route to the fermenter, where it is pitched with controlled amounts of brewing yeast (*Saccharomyces cerevisiae* or *S. uvarum* (formerly *S. carlsbergensis*)). The added oxygen in the wort is quickly consumed as the yeasts enter the logarithmic growth stage, the energy for which is supplied by the fermentable carbohydrates. During the

fermentation process, which typically takes about 2-10 days, the fermentable carbohydrates (glucose, fructose, sucrose, maltose and maltotriose), are converted to ethanol and carbon dioxide. As the nutrients become depleted, the yeast approaches the death-phase of growth, and it is important that the brewer separates the yeast from the fermented 'green beer' before autolysis of cells begins. Usually the fermentation process is monitored by measuring the gravity (specific density) of the wort, which diminishes as the carbohydrates are consumed.

Conditioning

The green beer produced by fermentation is usually transferred to a storage vessel, where it is subjected to a controlled secondary fermentation and storage period which varies according to the product. The purpose of this 'conditioning' is to improve the flavour of the final beer. A small amount of unfermented wort is sometimes added to a much larger volume of the green beer, and the mixture is then stored at a constant temperature (-2 to +18°C). During this process, yeast cells remove certain off-flavours from the beer, while the colloidal stability of the product is improved through the precipitation of protein-polyphenol complexes. Often, brewers add isinglass finings, a pure form of collagen, to accelerate this precipitation.

Stabilization

The conditioned beer is centrifuged or filtered to produce a clear beer. In the case of lager beers, it is customary to prolong the shelf-life of the beer by treatment with polyvinylpolypyrrolidone (PVPP) and/or silica hydrogel (SHG) by removing polyphenols and proteins respectively, which would otherwise eventually precipitate from the beer during storage to form a colloidal 'haze'. Following this treatment, the 'bright beer' is carbonated. At this stage a wide variety of stringent specifications must be met, in order to ensure a product of consistent high quality. Some brewers may further treat their beers through the addition of antioxidants, preservatives or foam stabilizers, though this is becoming less common nowadays through the advent of products with greater consistency of quality.

The finished product is now ready for packaging in cans, bottles or kegs, depending on the target market. The beer is usually pasteurized either en-route to packaging (flash pasteurization) or in package in a tunnel pasteurizer.

Traditional Brewing Processes

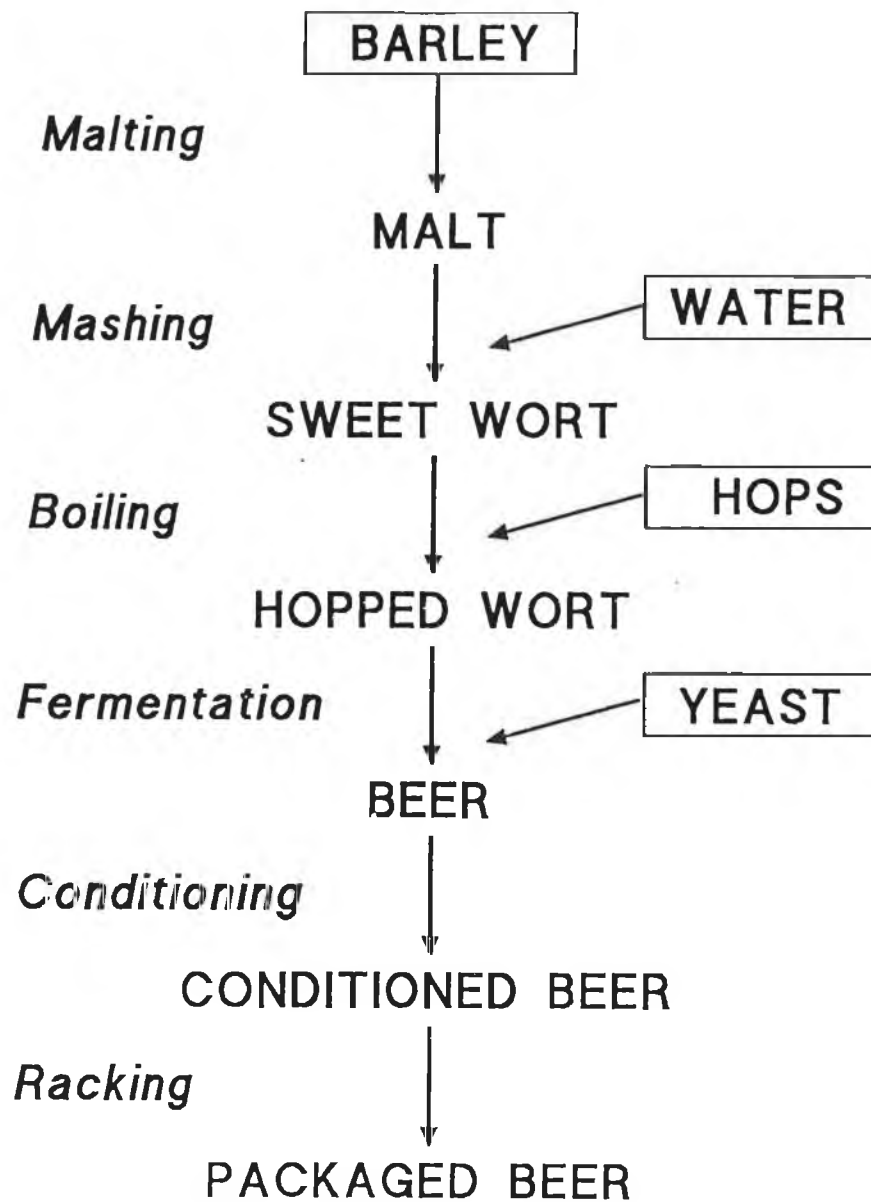


Figure 1.1. Overview of the brewing process.

1.2. REQUIREMENTS FOR ANALYTICAL CHEMISTRY IN THE BREWING INDUSTRY

The ancient Egyptians did not have access to the plethora of instrumental analysis techniques that are available to the modern brewing chemist, but this did not prevent them from brewing beer; indeed the centuries old process has only become heavily reliant on instrumental analysis in the relatively recent past. This situation has evolved in response firstly to the demand from the industry for streamlined, energy efficient and reproducible processes, and secondly, to the demand from the consumer for a consistently high quality product. The quality of a beer is generally regarded to be judged by the consumer on the basis of four criteria, namely:

- (1) foam or head;
- (2) clarity;
- (3) colour; and
- (4) flavour.

Although sensory evaluation (by both the brewer and the consumer) is the definitive test for the quality of a particular batch of beer, analytical chemistry plays a key role in assessing both the initial quality of the product, and the predisposition of the product towards deterioration. The analytical evaluation of beer may be divided into Routine Quality Measurements (RQM's), and Advanced Analytical Evaluations (AAE's). RQM's include colour, pH, bitterness, original gravity (OG), present gravity (PG), percentage alcohol by volume (ABV), dissolved oxygen (DO), carbon dioxide content, haze and other variables which are directly related to process control or to the requirements of the beer specification. RQM's are routinely measured in purpose-built brewery laboratories, or by on-line measurement systems in the plant. AAE's include such measurements as headspace volatile compounds, organic acids, flavanols, polyphenols, volatile phenols, amino acids, lipids, and other such compounds which, though their measurement may not be critical to the day-to-day production of beer, can provide useful indicators of beer quality, or advance warning of either plant or product difficulties. In fact, AAE's typically become most important when troubleshooting plant or product problems. While many breweries have on-site facilities for the

measurement of AAE's, there is a general trend towards contracting out specialized analysis, and maintaining the bare essentials on-site. This trend is in part responsible for the rapid growth in the number of consultancies offering analytical services to the industry over the last 10 years.

1.3. ADVANCED ANALYTICAL EVALUATIONS IN THE ASSESSMENT OF THE QUALITY OF BEER PRODUCTS

The most widely used methods of instrumental analysis for investigating AAE's in beer are as follows:

(i) High-Performance Liquid Chromatography (HPLC)

HPLC is widely used in the brewing industry, and the most common applications include the measurement of hop compounds [6-12], phenolics [13,14], carbohydrates [16,17], permitted additives [18], non-volatile flavour components, organic acids [19] and inorganic ions [20].

(ii) Gas Chromatography (GC)

GC is widely applied to the analysis of flavour-active volatile compounds in beer. The most obvious application is in the measurement of ethanol produced by fermentation, but other important applications include the analysis of fusel alcohols, esters, ketones, aldehydes, pyrazines and sulphur-containing compounds [21].

(iii) UV/VIS spectrophotometry and colorimetry

Although largely replaced by chromatographic methods which offer improved selectivity, there are still a number of important analyses which are carried out by spectrophotometry or colorimetry in the brewing industry. The most obvious example is the international method for beer bitterness measurement [22], which involves measuring the absorbance at 275 nm of an acidified iso-octane extract of beer, and converting this to 'bitterness units' by multiplication. The method is based on the assumption that all absorbing material at this wavelength is hop-derived. Other colorimetric methods include the measurement of total flavanols [23], total polyphenols [24], and staling sensitivity [25].

(iv) Spectroscopy

The two most widely used spectroscopic techniques in the brewing industry are atomic absorption spectroscopy (AAS) and near infra-red spectroscopy (NIRS). AAS is used for the determination of metals in beer. Some metals, such as Ca, Mg, Na and K, are present in high concentrations, and may be critical to beer flavour and the type of brewing procedure used. Trace metals, such as Cu, Fe, Pb, Zn, Cd, Co, Mn, Ni, Cr, Sn, Al, and As, must be routinely measured to satisfy legal requirements, and also as measures of beer quality. Fe and Cu, for example, may catalyse the oxidation of beer components, leading to deleterious effects on beer flavour or colloidal stability.

NIRS is widely used to measure carbohydrate, protein and water in samples of barley, malt and hops. The major advantages of this technique include minimal sample preparation, high versatility, low cost, safety, and portability of the instrumentation.

A detailed discussion of all of the instrumental techniques used in the brewing industry would be beyond the scope of this chapter, but some recent reviews [14, 21] discuss the techniques in greater detail. HPLC was the primary analytical method used in the experiments described in this thesis, and is therefore the only method which will be discussed in detail. Some recent reviews [14,15] describe in detail the main areas of application of HPLC in brewing science. In particular, the latter reference contains a detailed review of the application of HPLC to the analysis of bittering substances in foods, which was carried out as part of the research for this thesis in collaboration with Dr. I. McMurrough. From this review it was apparent that HPLC was the method of choice for the analysis of non-volatile bittering substances, and that this was the case not only in the brewing industry, but also in the food and beverage industry generally. A number of obvious trends emerged during the compilation of the review. The most popular detection system used with HPLC was by far the UV/VIS absorbance detector, no doubt due to its versatility and ease of use. In fact, out of 52 cited HPLC applications (Tables 2-4, reference 15), 50 used UV/VIS detection. In many such cases, however, it was necessary to perform one or more sample preparation procedures, involving typically liquid-liquid extraction (LLE) or solid phase extraction (SPE). While both of these procedures can increase selectivity,

LLE has the disadvantages of being often cumbersome to perform, and requiring the use of large volumes of hazardous solvents, whereas SPE, although often simpler and more reproducible to perform, may compromise sample integrity due to either incomplete extraction of the sample or incomplete recovery of the extract from the column. It may often be possible, however, through judicious choices of separation and detection systems, to use direct-injection ('dilute-and-shoot') HPLC to analyse components of beer and other sample matrices at low concentrations, without the need for further sample pretreatment. By developing such techniques, it is possible firstly to ensure that sample integrity is not compromised by the sample preparation, and secondly to greatly reduce the level of manual input required in the analysis.

1.4. OBJECTIVES

The objective of this thesis was to develop sensitive and selective direct-injection HPLC methods for the analysis of compounds of critical interest in the assessment of beer quality. In order to do this, advantage was taken of the latest technologies in both separation and detection systems for HPLC. The developed methods were then applied to the detailed investigation of brewing processes which improved the observed stability and/or quality of the final product. The detailed objectives are listed below.

1. To develop novel analytical methodology to enable automated direct-injection HPLC analysis of simple flavanols normally present in beer and barley.
2. To develop semi-preparative and preparative methods for the isolation of simple flavanol standards for use both as calibration standards for HPLC, and also in amounts sufficient to enable their addition to stabilized beer at levels typical of the unstabilized product.
3. To investigate the effects of PVPP-stabilization of beer on the contents of simple flavanols having different degrees of hydroxylation and polymerization.

4. To examine critically the role of simple flavanols in the colloidal and flavour stability of beer, in particular differences in behaviour between the flavanols with different degrees of either hydroxylation or polymerization.
5. To develop direct-injection HPLC methods for the determination of volatile phenols and their phenolic acid precursors in beer, and to examine the occurrence and significance of these compounds in beer.
6. To apply automated direct injection HPLC methodology to the investigation of oxalate hazes and sediments in beer.
7. To develop an automated direct injection HPLC method for carbohydrate determination in beer and wort.
8. To develop a repeatable direct-injection HPLC method for the analysis of ascorbic acid in vitamin-fortified beers and malt drinks.

Chapter 2 describes the research performed to fulfil objectives 1-4, while chapters 3-6 refer to objectives 5-8 respectively.

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2. DEVELOPMENT OF NOVEL ANALYTICAL METHODOLOGY FOR FLAVANOL ANALYSIS IN BEER AND THE INVESTIGATION OF THE ROLE OF FLAVANOLS IN BEER STABILITY

2.1. INTRODUCTION

2.1.1. Flavanoid compounds in beer

Beers are known to contain a wide variety of phenolic compounds, most of which originate from the raw materials of brewing, i.e. barley and hops [1]. Of these phenolic compounds, the flavanoids are of particular interest to brewers, as they have long been thought to be precursors of non-biological haze in unstabilized beers [2]. The flavanols have been classified into three groups on the basis of their chromatographic behaviour [3]. The first group, the simple flavanols, of which some examples are shown in Figure 2.1, comprise flavanol monomers (e.g. (+)-catechin and (-)-epicatechin), dimers (e.g. procyanidin B3 and prodelphinidin B3), and trimers (e.g. procyanidin C2). The second group, the polymeric flavanols, are formed by oxidation and polymerization of simple flavanols. The complexed flavanols, the third group, result from the interaction of polyphenols with proteins to form complex structures [4].

For many years it has been recognised that packaged beer undergoes gradual changes during storage. This is signalled by a deterioration in flavour, increases in permanent and chill hazes, and an increase in colour [5,6]. These changes have in common a proven connection with the availability of oxygen in the product package [7]. Indeed, the ageing of beer is caused primarily by a variety of oxidative reactions, through which various susceptible compounds are transformed into products that compromise the standard criteria of quality [5, 8]. Flavanoid polyphenols are one of the more readily oxidized beer constituents [8], and their direct involvement in colloidal (haze) instability is beyond question [9]. Less certain, however, is the role of flavanoid polyphenols in flavour instability [10, 11]. Both negative and positive

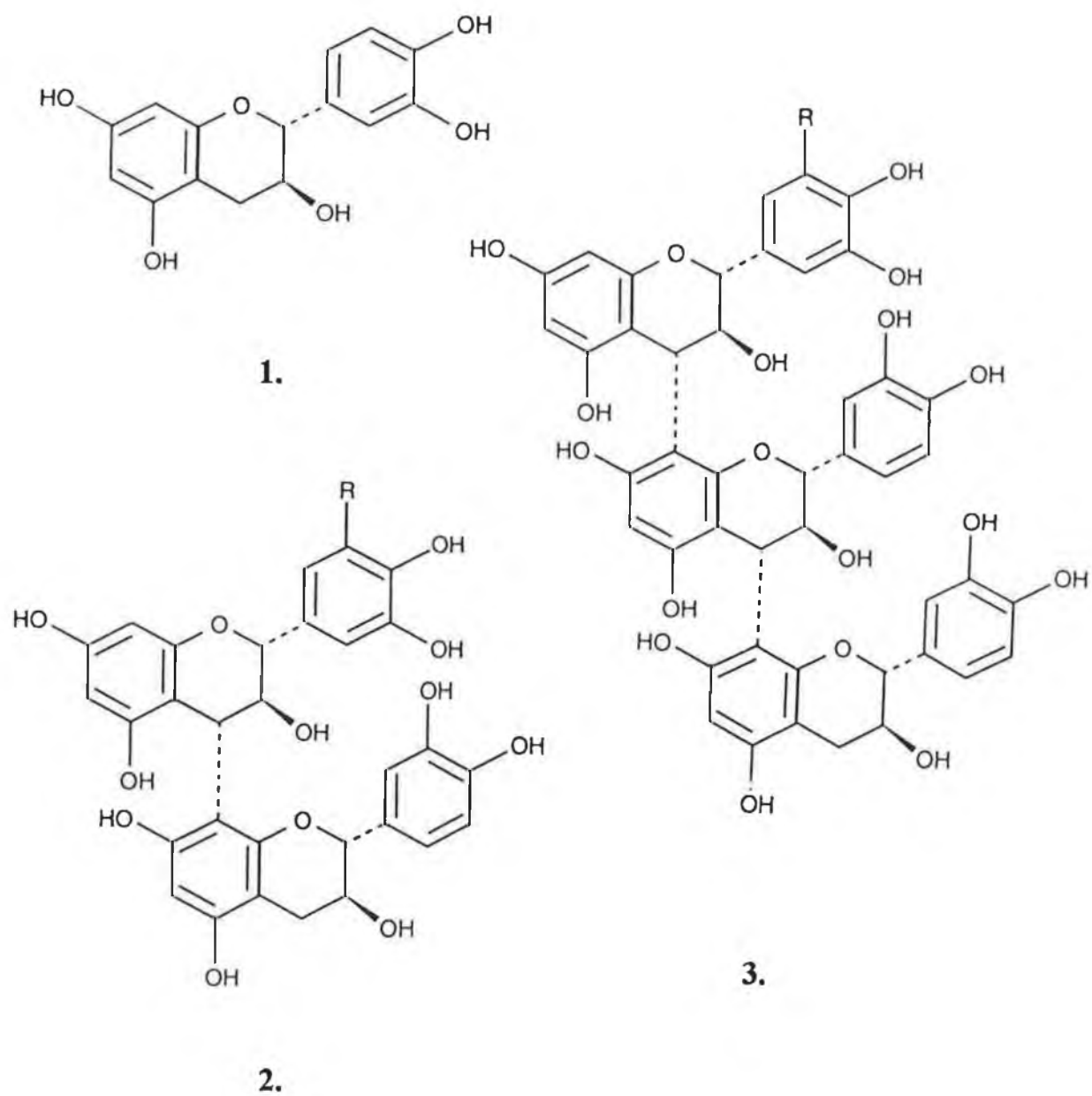


Figure 2.1. Simple flavanols 1 = (+)-catechin; 2 = prodelphinidin B3 (R = OH) or Procyanidin B3 (R = H); 3 = procyanidin C2 (R = H)

flavour properties have been ascribed to polyphenols, though the evidence on which these properties have been assigned has often been far from conclusive [12]. In comparison with the evidence of their culpability in haze instability [9], the facts concerning the role of polyphenols in flavour stability are meagre. Following the finding that oxygen in the bottle headspace was incorporated into an uncharacterized "tannin" fraction from beer [7], beer polyphenols have acquired a reputation for having beneficial antioxidant powers. Although it has been claimed that polyphenols influence beer stability in general [5,8,13], there is no agreement on the most favourable concentration or type of polyphenols.

2.1.2. Stabilization of beer against haze formation

The stabilization of beer against haze formation may be achieved by decreasing the concentration of flavanols. Increasingly, this stabilization is being achieved in breweries by the treatment of beer with polyvinylpolypyrrolidone (PVPP) prior to packaging [14,15]. PVPP was introduced commercially as an adsorbent for beer phenolics as long ago as 1961, and since then has been widely used as an agent for prolonging the stability of beers against haze formation [14-20]. PVPP is typically added to the beer as an aqueous slurry after fermentation and yeast removal; the mixture is then stirred at a temperature of 0-2°C, and the PVPP removed by sheet filtration. PVPP is available either as a single-use product, or in a recoverable grade, which is regenerated by washing with hot NaOH solution (such as is generally used for the sterilization of brewery plant). The mode of action of PVPP is based on the interaction of flavanoids with the exposed pyrrolidone functional units of the polymer (Figure 2.2). On a molecular level, PVPP is made up of a saturated and highly cross-linked carbon backbone from which pyrrolidone rings protrude. The carbonyl oxygen of pyrrolidone is made highly electronegative by interaction with the ring nitrogen, and may therefore provide sites to which flavanoid protons may bind by both hydrogen- and charge-transfer-bonding [20].

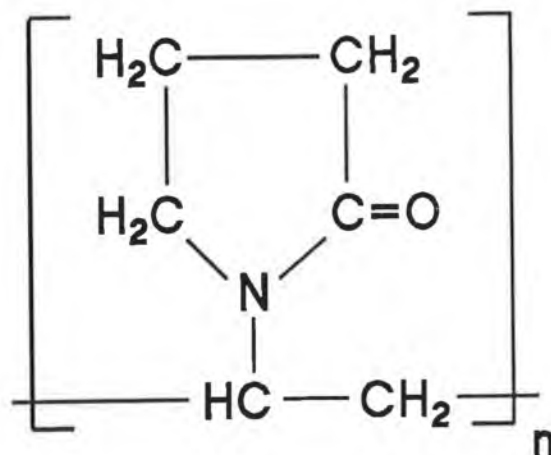


Figure 2.2. The vinylpyrrolidone monomeric unit of polyvinylpyrrolidone (PVPP)

2.1.3. Effects of flavanoids on beer quality

The partial removal of the flavanoids from beer has been reported to decrease the rate of oxygen uptake, accompanied by a retardation in the development of 'oxidized' flavour [21]. Even so, careful control over the use of adsorbents, such as PVPP, to decrease polyphenol contents has been advocated [22]. The removal of polyphenols from wort before boiling was reportedly disastrous for the flavour stability of the resulting beer [23]. Lately, it has been affirmed that a level of polyphenols sufficient to provide flavour stability should be maintained [24]. It has also been claimed that colloidal stabilization by PVPP treatment resulted in a less flavour-stable product than did treatment with silica hydrogel (SHG, an adsorbent of beer proteins) [25]. Excessive treatment of beer with PVPP considerably diminished assay values for the endogenous reducing power in beer, and the extents of these decreases correlated well with the observed decreases in polyphenols [8, 26]. There is, however, no proven relationship between beer stability and reducing power [27]. Another

opinion [28] is that the involvement of polyphenols in beer staling is more of theoretical interest than of practical consequence; this view was supported, in practice, by the finding that PVPP treatment produced no significant differences in flavour stability.

The widely proclaimed interdependence of flavour and haze instability [6,12,13,22,29] is predicated on the generation of radical species during the process of beer oxidation [30-33]. The formation of reactive oxygen species from molecular oxygen [8, 34] can be catalysed by Cu(II) ions, and polyphenols have been supposed to be critically involved in the electron-transfer system in beer [5]. In this respect, it has been claimed that trihydroxy polyphenols such as 3',4',5'-hydroxyflavans (e.g. prodelphinidin B3, Figure 2.1) are required as coupled reducing agents, so that Cu(II) can be recycled through its lower oxidation state (Figure 2.3). Accordingly, the trihydroxyflavans in beer, such as prodelphinidin B3, were identified as putative prooxidants, because of their supposed propensity to drive the staling process. In contrast, dihydroxyflavans such as (+)-catechin and procyanidin B3 (Figure 2.1), were believed to prevent staling by protecting susceptible substrates from oxidation, and to be incapable of acting as prooxidants. The overall effect on flavour stability of the presence of polyphenols in beer would, it was reasoned, depend on the absolute and relative concentrations of the dihydroxy and trihydroxy flavanoid species [34] (Figure 2.3).

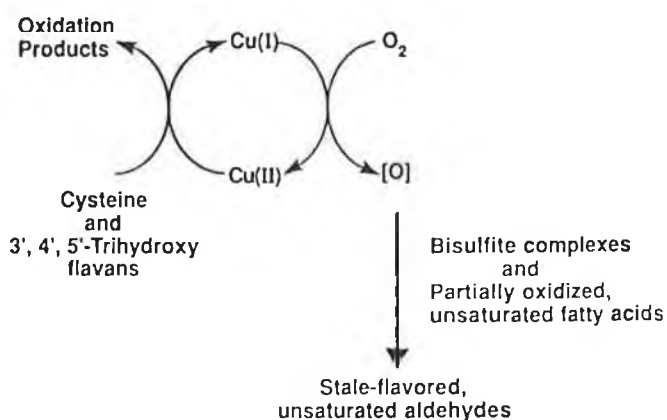


Figure 2.3. The proposed role of copper, oxygen and trihydroxy-flavanoids in beer stability (adapted from ref. 34)

2.1.4. Selectivity of PVPP Adsorbents for Beer Treatment

Haze-stabilization of beer by treatment with PVPP is always accompanied by decreases in total polyphenols, total flavanols, simple phenolic acids, flavonol glycosides, catechins, proanthocyanidins, and complexes of polyphenols and proteins [35], but limited data is available regarding the selectivity of PVPP towards different types of flavanoid compounds. Selectivity in the adsorption was noted, albeit cursorily by Weyh *et al.* [36], and there have been only a few systematic studies on the adsorption of specific phenolic compounds. A strong correlation was drawn [37] between the degree of PVPP-mediated stabilization and the observed decreases in identified hydroxyflavans (proanthocyanidins). Gas chromatographic analysis of treated beers indicated a pronounced selectivity of adsorption of the proanthocyanidins rather than the catechins. The effect of variable PVPP dosage rate and the adsorption of polyphenols was complex; other workers [38] proposed that the removal of polyphenols should be proportional to a geometric or Freundlich progression of increased dosage.

Systematic studies in model systems [39,40] indicated that selective adsorption of phenolics depended on their degree of hydroxylation. Mennett and Nakayama [41] concluded that the selectivity of adsorption of hydroxybenzoic acids by PVPP in very simple model systems was low. Recently, it was found [42] that binding of flavonoids by PVPP, with a few exceptions, increased with the number of hydroxyl groups on the flavonoid nucleus.

2.1.5. Analysis of flavanoid compounds in beer

A large variety of methods for the analysis of phenolic compounds in beer and brewing materials have been published, and some of these have been reviewed recently [43]. Qualitative separations by paper chromatography have revealed a large number of phenolic substances, of which relatively few have been positively identified [44]. Gas chromatography has been used, albeit infrequently, for the quantification of simple flavanols in beer [37]. More extensive use has been made of HPLC with

ultraviolet (UV) absorbance detection for the analysis of flavanols, flavonols and phenolic acids [43, 45]. More recently, the use of HPLC with electrochemical detection (HPLC-ED) for the analysis of phenolic compounds has received much attention [14,15,46-50].

One of the limitations of some previously published HPLC methods for flavanol determination, in particular those employing UV detection, has been the tedious sample preparation required, usually entailing one or more liquid-liquid or solid-phase extraction steps [43,45,49,50]. In addition to being time-consuming, these steps are possible sources of quantitative inaccuracies.

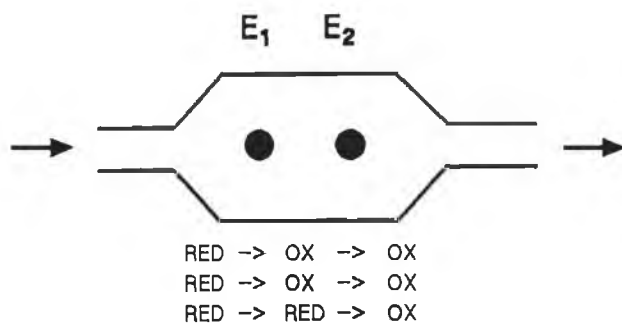
The use of dual-electrode electrochemical detection for HPLC was pioneered in the early 1980's [51-55]; this was followed by rapid-scanning voltammetric detectors [56-59], and more recently by multielectrode array systems [60-65]. The basic principle of using more than one electrode to perform multiple oxidations or reductions (or combinations of both) in a flowing system remains the same. The use of working electrodes in series or parallel at different operating potentials can be used to provide enhanced identification of sample components. It has been shown [50] that the collection efficiency, i.e., the ratio of the downstream to the upstream currents at electrodes operated in series at positive followed by negative potentials [51], can be used as an aid to the structural classification of phenolic compounds. For instance, catechol-substituted flavanols, which exhibit high collection efficiencies, can be distinguished from monohydroxy or trihydroxy-substituted ring systems, which exhibit significantly lower collection efficiencies. This concept is illustrated in Figure 2.4.

Over a decade since the advent of multi-electrode detection systems for HPLC, this technology is still only gradually gaining widespread use in the food and beverage industry (see also chapters 3,4, and 5). Yet, where it is applicable, electrochemical detection offers many advantages over more commonly used detection techniques, such as UV absorbance detection. These advantages were capitalised upon in the development of the novel analytical methodology applied herein.

(1) Coulometric Screening

$$E_2 \gg E_1$$

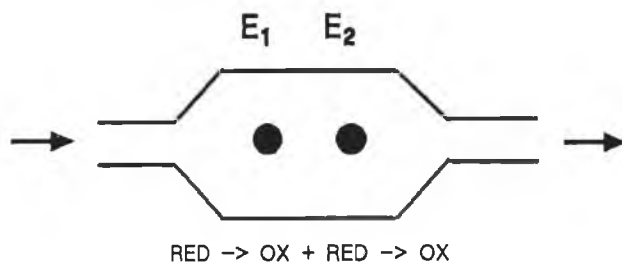
*Electrode 1 screens out impurities;
electrode 2 oxidizes analyte(s)*



(2) Dual Potential Oxidation

$$E_2 > E_1$$

*Electrode 1 partially oxidizes
analyte; electrode 2 causes
further oxidation*



(3) Combined Oxidation / Reduction

$$E_2 \ll E_1$$

*Electrode 1 oxidizes analyte;
electrode 2 reduces oxidized
form of analyte*

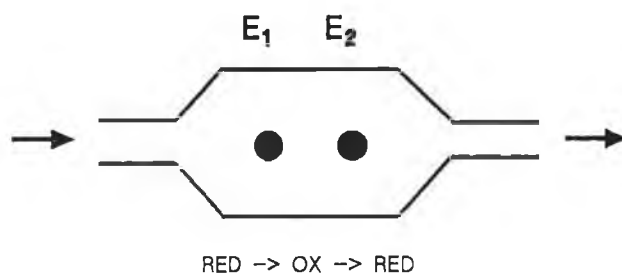


Figure 2.4. Modes of operation of a typical dual-electrode electrochemical detector for HPLC

2.1.6. Commercial Availability of Proanthocyanidins and Catechins

The monomeric flavanols (+)-catechin and (-)-epicatechin (Figure 2.1) are available commercially in a high state of purity [66], and as such may be purchased for use as analytical standards for chromatography. The dimeric flavanols, however, due both to difficulties in their synthesis and also to their instability, are not readily available as pure compounds. A prerequisite to the successful analysis of these compounds, however, is the availability of pure calibration standards. Due to the difficulties associated with the synthesis of oligomeric flavanols, these compounds are typically isolated from plant extracts, and in the case of the flavanols of interest to the brewing industry the extracts are normally obtained from barley. Typically, the isolations have involved lengthy separations of beer or barley extracts on Sephadex LH-20, followed by further purification on LH-20, by semi-preparative reversed phase HPLC [67-70], or, alternatively, by thin-layer chromatography (TLC) [14].

2.1.7. Scope of this Chapter

In this chapter, a novel semi-preparative method for the isolation of small amounts (50-200 μg) of dimeric and trimeric proanthocyanidins from barley is described, as well as a novel preparative procedure for the isolation of large (ca. 100 mg) amounts of the dimeric proanthocyanidins, procyanidin B3 and prodelfinidin B3, also from barley. Using the isolated compounds as standards, a new analytical method was developed using dual-electrode electrochemical detection to analyse proanthocyanidins and catechins in beer and in extracts of barley samples. This method was then used to examine in detail the effects of PVPP-stabilization of beer, and to investigate possible influences of PVPP-stabilization on the flavour stability of beer. By using the new preparative method for the isolation of dimeric proanthocyanidins from barley, it was possible to replace these compounds individually in stabilized beer, to measure and compare the individual effects of the simple flavanols on beer stability. The novel analytical methodology enabled previously tedious and insensitive analyses to be performed with increased confidence,

and therefore allowed new insights to be gained into the role of flavanols in beer stability.

2.2. EXPERIMENTAL

2.2.1. Reagents

Deionized water was prepared using an Elga Maxima system (Elga Ltd., Berks., England). Polyvinylpyrrolidone (Povidone) K90 was obtained from Fluka Chemical Co., Poole, Dorset, U.K. Prodelphinidin B3 and Procyanidin B3 were prepared as described in sections 2.3.2.2 and 2.3.2.3. Polyclar 10 (single-use PVPP), Polyclar Super R (recoverable PVPP) and experimental samples of PVPP were obtained from International Specialty Products, Guildford, Surrey, U.K. All other chemicals were of analytical reagent grade.

2.2.2. Instrumentation, Conditions and Procedures

2.2.2.1. Analytical Determination of Simple Flavanols

The liquid chromatograph used was a Perkin-Elmer Integral 4000 (Perkin-Elmer, Beaconsfield, Buckinghamshire, U.K.). The column eluent was diverted from the diode-array detector fitted to this system, and was passed through an ESA Analytical Coulochem II electrochemical detector equipped with a Model 5011 analytical cell (ESA Analytical, Huntingdon, Cambridgeshire, U.K.). This cell contained a porous graphite high conversion efficiency electrode (electrode 1; also referred to as a coulometric electrode) connected in series to a glassy carbon thin-layer type amperometric electrode (electrode 2). The working electrodes were independently programmable. The internal reference electrode used was a palladium electrode, which exhibits a -300 mV shift with respect to the more commonly used Ag/AgCl reference electrode, at about pH 3.0. This reference electrode was also pH sensitive, and moved about +60 mV per pH unit increase [71].

Chromatographic separations were carried out on a 30 cm x 4.6 mm i.d. reverse-phase Nucleosil 10 μ m C₁₈ stainless-steel column (Macherey Nagel, Düren, Germany). A Waters Guard-Pak pre-column was used with a Nova-Pak C₁₈ disposable

insert (Waters U.K. Ltd., Watford, Hertfordshire, U.K.). The experimental parameters used were as follows: flow rate, 1.0 ml min⁻¹; injection volume, 10 µl; mobile phase A, 2.5% v/v acetic acid; and mobile phase B, 10% v/v acetic acid. The gradient programme was as shown in Table 2.1.

Table 2.1. Gradient programme for the analytical separation of proanthocyanidins and catechins

Time (min)	A (%)*	B (%)
0	100	0
60	0	100
70	0	100
80	100	0
100	100	0

*A = 2.5% v/v CH₃COOH; B = 10% v/v CH₃COOH

The electrochemical detector settings were: Electrode 1: potential, +350 mV; output range, 5 µA; offset, +5%; polarity, positive; and filter, 2 s. Electrode 2: potential, -650 mV; output range, 2 µA; offset, +5%; polarity, negative; and filter, 2 s. Electronic data acquisition and processing were performed using a Waters Maxima 820 chromatography workstation.

The experimental procedures were as follows:

Preparation of standard solutions

Solutions containing 1.0 mg L⁻¹ of protocatechuic acid and 1, 2, 3, and 4.0 mg

L⁻¹ of (+)-catechin, (-)-epicatechin, prodelpinidin B3, and procyanidin B3 were prepared in methanol and stored under a nitrogen atmosphere at -30°C prior to use. A stock solution of 10 mg L⁻¹ procatechuic acid (internal standard) was prepared in methanol and stored at -30°C.

Sample preparation

Beer samples were degassed by at least 20 transfers between beakers, and were filtered through Anotop 10 plus 0.22 µm membrane filters (Whatman Scientific, Maidstone, Kent, U.K.). Procatechuic acid (1.0 ml of a 10 mg L⁻¹ solution) was then added to 9.0 ml of beer to give an internal standard concentration of 1.0 mg L⁻¹.

Barley was extracted by a method developed previously [70,72]. Samples (10 g) were milled for 2 min in a blender and extracted with 3 x 100 ml of cooled 75% v/v acetone for 30 min under a N₂ atmosphere. The extracts were diluted two-fold with water and filtered through 0.22 µm filters. Procatechuic acid stock standard solution (1.0 ml) was then added to 9.0 ml of dilute extract.

Spiked samples were prepared by combining equal volumes of 2.0 mg L⁻¹ standard solutions with samples prepared as described above. Caustic PVPP regenerant solution (1 M NaOH) was neutralized with 1 M HCl, diluted, and internal standard was added.

2.2.2.2. Semi-preparative Isolation of Dimeric and Trimeric Proanthocyanidins from Barley

Instrumentation

The liquid chromatograph used for the semi-preparative procedure consisted of a Waters Model 510 HPLC pump, a Waters Model 710B WISP autosampler, and a Waters Lambda-Max Model 481 LC spectrophotometer. The column used was a 30 cm x 10 mm Superdex 75 HR 10/30 high performance gel filtration column (Pharmacia LKB Biotechnology, Uppsala, Sweden). Analytical determinations were performed as described in section 2.2.2.1. UV spectra were recorded with a Hitachi Model U-2000 double-beam spectrophotometer.

Procedures

Extraction of Barley

Two extraction procedures were developed, and were optimized either for extraction of dimeric proanthocyanidins only, or for both dimers and trimers:

Method 1 (dimers only):

A 50 g sample of Irish Barley (Blenheim variety, 1994 crop) was ground for 2 min in an IKA Analysis Mill A10 (Janke and Kunkel KG, Staufen, Germany). The ground barley was extracted with 150 ml methanol under a CO₂ atmosphere for 1 hr, after which the extract was filtered through sintered glass and reduced to 10 ml by evaporation *in vacuo* at 35°C. The solution was filtered through a 0.22 µm membrane filter and retained for semi-preparative separation.

Method 2 (dimers and trimers):

Barley was ground as described above, but the extraction was performed in 150 ml acetone-water (3:1). The extract was filtered through sintered glass and was salted out by vigorous shaking with excess NaCl (ca. 5 g) for 10 min, followed by standing

for 1-2 hr. The upper acetone phase which formed on standing was evaporated *in vacuo* until only approx. 7 ml of aqueous solution remained. This solution was filtered through a 0.22 μm membrane filter and was retained for semi-preparative separation.

Semi-Preparative Separation

200 μl aliquots of the barley extracts were injected onto the semi-preparative column, which was eluted with methanol at 1.0 ml min⁻¹. The absorbance of the column eluent was monitored at 280 nm, and fractions corresponding to the peaks of interest were collected manually. For extracts prepared using method 1, the column was eluted for 3 hr, and fractions were collected corresponding to procyanidin B3 and prodelfphinidin B3. Extracts prepared by method 2 were eluted for an extended run time of 9 hours to permit collection of four trimer fractions in addition to the dimeric flavanols. Fractions were collected only near the peak apices, to avoid any possible contamination due to overlapping of neighbouring peaks. Collected fractions were evaporated *in vacuo* to near dryness, and were reconstituted in methanol (5 ml for trimer fractions; 15 ml for dimers). This provided concentrated solutions of standards which could be diluted to the normal calibration range of the analytical HPLC system as required. Standards were stored at -40°C under N₂ when not in use.

Analytical Separation

Analytical separations were performed as described in section 2.2.2.1. The described method was found to be suitable for separation of the monomeric, dimeric and trimeric proanthocyanidins without modification.

Measurement of Concentration of Standards

Spectra of the different flavanoids in the concentrated fractions were obtained in the range 190-400 nm, and the concentrations of the flavanols were estimated from

their optical densities measured at 280 nm, using solutions of (+)-catechin at 1-30 mg L⁻¹ as standards. The solutions were then analysed by reaction with *p*-dimethylaminocinnamaldehyde (DAC) using (+)-catechin (1-30 mg L⁻¹) as a calibration standard [73]. Concentrations of the flavanol standards were expressed as (+)-catechin equivalents.

2.2.2.3. Preparative Isolation of Dimeric Flavanols from Barley

Kiln-dried Irish-grown barley (1.7 kg, variety Blenheim) was milled in a Buhler-Miag Mill at a particle size setting of 0.2 mm. The milled barley was extracted for 1.5 hr with 10 L acetone-water (3:1) under N₂. The extracted barley was removed by filtration through a sintered glass filter to give approximately 8 L of extract. This extract was saturated with 200 g NaCl by stirring under an atmosphere of N₂. The upper organic phase that formed on standing was removed and evaporated under vacuum at 30°C to a final volume of about 500 ml aqueous solution. This solution was extracted with 5 portions (500 ml each) of ethyl acetate, and the combined ethyl acetate was evaporated almost to dryness at 30°C. The residue was dissolved in methanol (100 ml) which was then pumped onto a column (82 cm x 2.5 cm i.d.) containing Sephadex LH-20 swollen in methanol. The column was eluted at 5 ml min⁻¹ with methanol supplied by a Waters Model 510 HPLC pump. The absorbance of the eluate was monitored at 280 nm with a dual path monitor (Model UV-2, Pharmacia LKB Biotechnology) and was collected in fractions (15 ml) using a fraction collector (Model FRAC-300, Pharmacia LKB Biotechnology). The chromatogram of this primary separation is shown in Figure 2.5.

Selected fractions from this separation were analysed by HPLC-ED as described in section 2.3.2.1. Figure 2.6 shows HPLC-ED chromatograms of some of these fractions. From the HPLC-ED analysis it was deduced that prodelphinidin B3 and procyanidin B3 were found unresolved in a single peak following the elution of catechin (regions 4,5 in Figure 2.5; fractions 40-70 in Figure 2.6) from the LH-20 column.

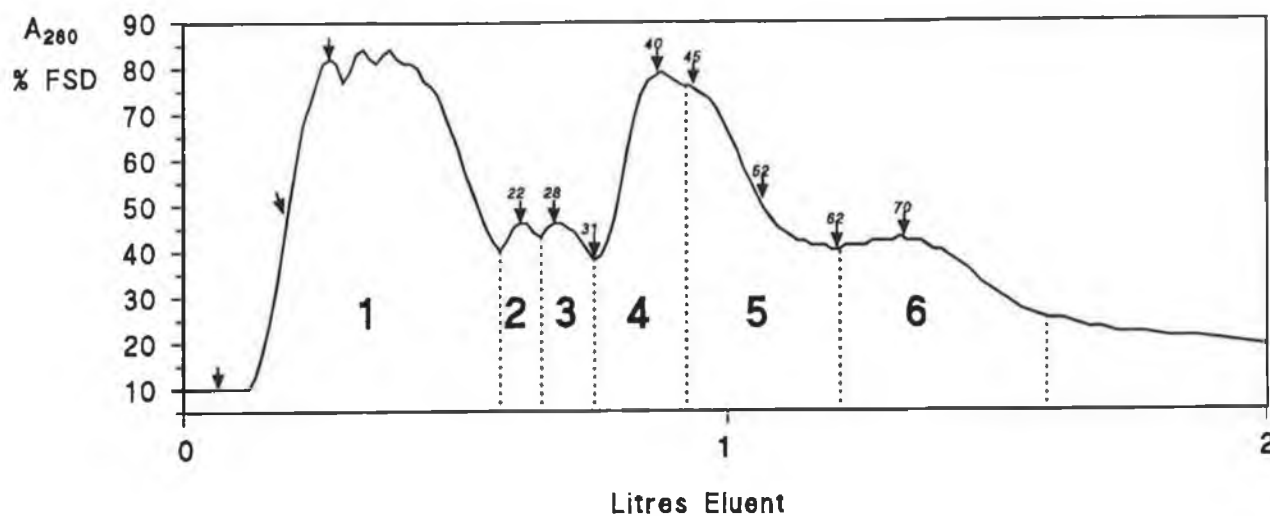


Figure 2.5. Primary separation of barley extract on Sephadex LH-20. A column (82 cm x 2.5 cm i.d.) containing LH-20 swollen in methanol was eluted with methanol at 4.95 ml min⁻¹. The bold numbers refer to the elution of 1, early fraction; 2,3, monomeric flavanols; 4, procyanidin B3; 5, prodelphinidin B3; and 6, trimeric flavanols. The italic numbers and arrows refer to the numbers of the fractions analysed by HPLC-ED and shown in Figure 2.6.

Fractions of eluate that contained the dimers were combined and evaporated *in vacuo* to 2 ml. This concentrate was injected onto two columns in series (effective dimensions 171 cm x 2.5 cm i.d.) of Sephadex LH-20 swollen in ethanol, and the columns were eluted with ethanol at 2.0 ml min⁻¹. The absorbances of diluted samples of selected fractions were measured at 280 nm, and after correction for dilution, were expressed as total absorbance units (TAU₂₈₀) to produce the profile shown in Figure 2.7.

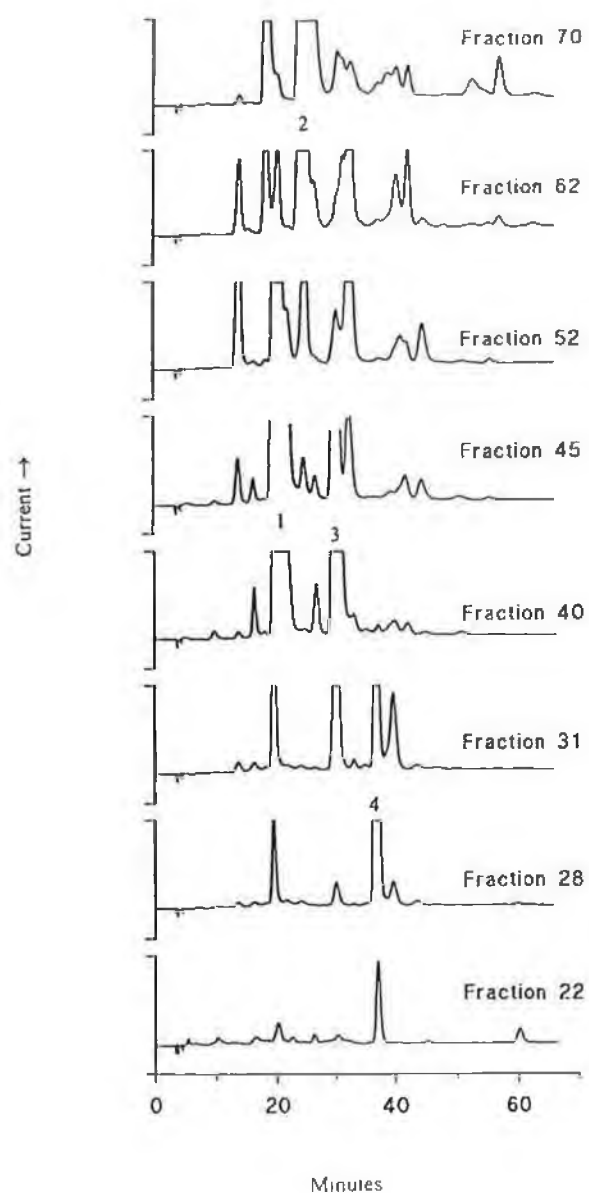


Figure 2.6. HPLC-ED chromatograms of selected fractions from the primary separation on LH-20 of the barley extract. Fraction numbers refer to those given in Figure 2.5. Identified peaks: 1 = prodelphinidin B3; 2 = trimeric flavanols; 3 = procyanidin B3; and 4 = (+)-catechin.

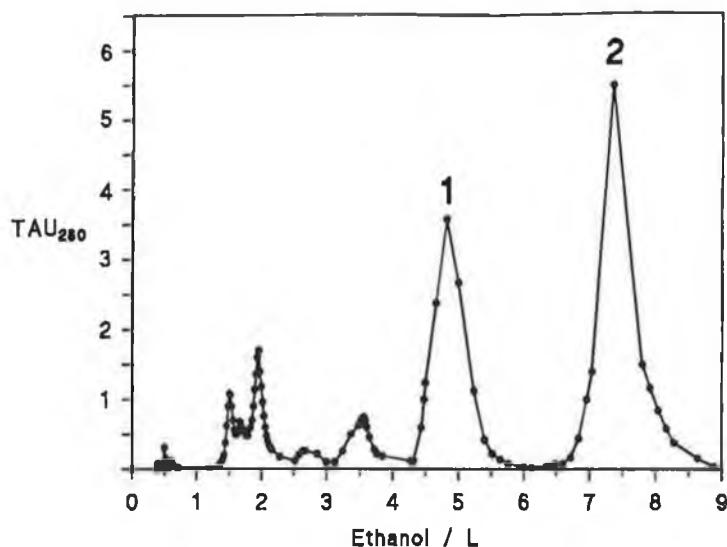


Figure 2.7. Secondary separation of dimeric flavanols on Sephadex LH-20. A column (171 cm x 2.5 cm i.d.) was eluted with ethanol at 2.0 ml min⁻¹. Identified peaks, 1 = procyanidin B3; 2 = prodelphinidin B3.

Clearly, this chromatographic procedure effectively resolved the dimeric flavanols. Combined eluate fractions containing each of the dimers were each evaporated to 100 ml and stored at -40°C under N₂. The identity of the dimers was confirmed by ¹H nuclear magnetic resonance spectroscopy [69, 74] and electrochemical collection efficiency measurement [51]. The purities of the fractions collected during the isolation of dimeric flavanols from barley were assessed using HPLC as described in section 2.3.2.1, but with isocratic elution (3.5% CH₃COOH, 1.0 ml min⁻¹) with a diode array detector (Perkin-Elmer Integral 4000) included upstream of the electrochemical detector. The amounts of purified procyanidin B3 and prodelphinidin B3 recovered were 85 mg and 112 mg respectively. A schematic summary of the preparative separation is shown in Figure 2.8.

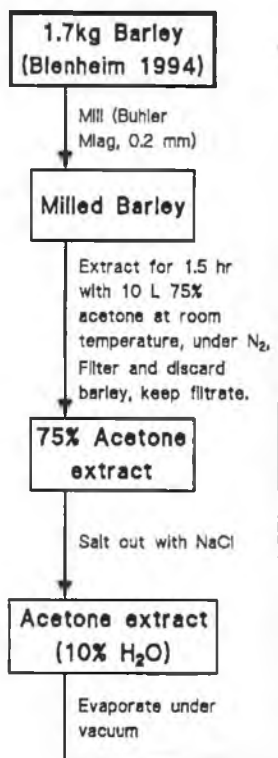
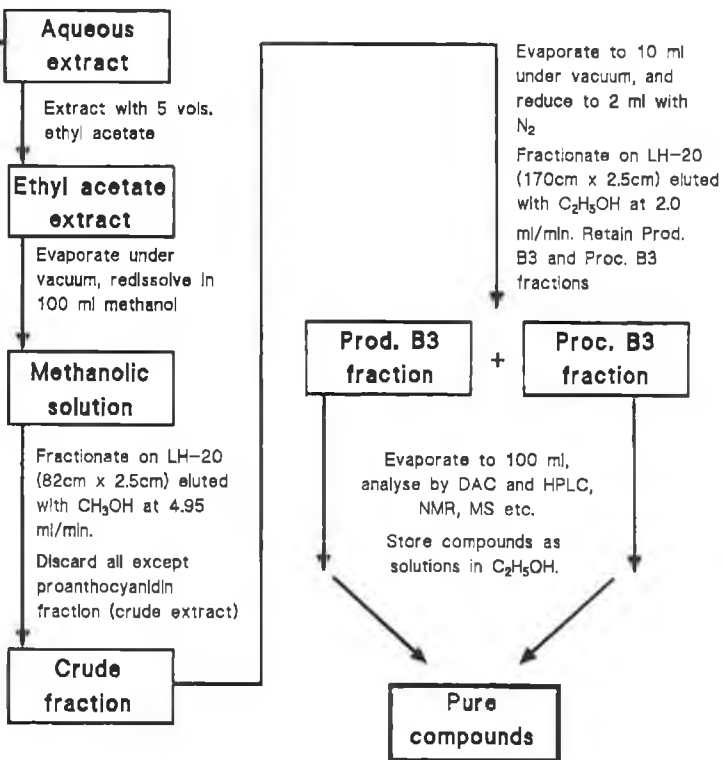


Figure 2.8. Summary of the procedure for preparative isolation of dimeric proanthocyanidins from barley.



2.2.2.4. Adsorption by Polyvinylpyrrolidone of Catechins and Proanthocyanidins from Beer

Instrumentation

Two HPLC systems were used as follows:

(1) For the measurement of hydroxybenzoic acids in model solutions:

The system consisted of a Waters Model 710B WISP autosampler, a Waters Model 510 pump, and a Waters Model 481 U.V. spectrophotometer. The column was a 25 cm x 4 mm i.d. Nucleosil C₁₈ 10 µm (Machery Nagel, Düren, Germany) used with a Waters Guard-Pak column containing a Nova-Pak C₁₈ insert. The mobile phase was CH₃OH/H₂O/H₃PO₄ (350:640:10 by volume) and was pumped at a flow rate of 1.0 ml min⁻¹. The injection volume was 20 µl and the detection wavelength 228 nm. The run time was 20 min, and calibration of the detector was by external standardisation using solutions of hydroxybenzoic acids at concentrations in the range 1-100 mg L⁻¹.

(2) For the measurement of simple flavanols in beer.

The system described in section 2.2.2.1 was used.

Preparation of Prodelphinidin B3 and Procyanidin B3 Dosage Solution

The procedure described previously [70] was modified to allow a quick extraction of prodelphinidin B3 and procyanidin B3: Each of two samples (350 g) of Irish Blenheim variety barley (1993 crop) was ground in a blender for 2 min. The ground samples were both extracted with 1 L of acetone-water (3:1) at 0°C for 1 hr under N₂. The extracts were filtered through sintered glass, and the acetone was removed by rotary evaporation at 30°C. The aqueous extracts were then extracted with three volumes of ethyl acetate. The ethyl acetate was evaporated to dryness, and the solids were redissolved in 10 ml of CH₃OH. This resulted in solutions containing

approximately 2 mg ml⁻¹ each of prodelpinidin B3 and procyanidin B3 and little other UV absorbing material. The methanolic solution was stored under N₂ at -40°C until use.

Sampling of PVPP

For laboratory-scale treatments, representative subsamples of the PVPP powders were taken from bulk (25 kg drums). Because of the dust hazard and the waste associated with coning and quartering such a large amount, representative samples (ca. 100 g) from the top, bottom, centre, and three points near the circumference were taken with a core sampler designed originally for the sampling of cereal grains. These samples were then combined and mixed well, and the combined sample (ca. 600 g) was termed "homogenised" PVPP.

Adsorption of Phenolic Acids from a Model System

A mixture of benzoic and hydroxybenzoic acids was prepared, with each acid present at 0.1 mM in 5% ethanol containing 0.1 M KH₂PO₄ adjusted to pH 4.0 with H₃PO₄. Samples (100-500 ml) of these solutions were treated with homogenised Polyclar 10 at rates of 0, 5, 10, 15, 20, 25, 30, 40, 50, 75, 100, 150, 200, 500 and 1,000 g hL⁻¹. The PVPP was allowed to react for 1 hr at 0°C with stirring. Samples (10 ml) were then centrifuged at 4,500 x g for 15 min, and the supernatants were analysed by HPLC as described above.

Adsorption of Flavanols from Beer

A sample (10 L) of unstabilized lager beer was taken from the storage vessel of a lager brewery and centrifuged at 4,000 x g for 30 min. A subsample (5 L) of this beer was then treated with an excess (approximately 5,000 g hL⁻¹) of Polyclar 10 for 1 hr at 0°C to produce a "flavanol-free" beer. This beer was centrifuged at 3,000 x g for 2 min to remove the PVPP. The flavanol-free beer was then spiked with (+)-catechin (Sigma) and prodelpinidin B3 and procyanidin B3 (from barley) at 10 mg L⁻¹ each. (+)-Catechin was weighed dry and added directly to the beer. Dimeric

proanthocyanidins were added as concentrated methanolic solutions prepared as described above. The beer was agitated well to promote dispersal and dissolution of the flavanols. Samples (100-500 ml) of the spiked beer were then stirred with homogenised Polyclar 10 at rates of 0, 5, 10, 15, 20, 25, 30, 40, 50, 75, 100, 150, 200, 500 and 1,000 g hL⁻¹ for 1 hr at 0°C. Samples (10 ml) of the treated beer were centrifuged at 4,500 x g and 0°C for 30 min to remove spent PVPP. The supernatants were analysed by HPLC-ED as described above. The entire procedure was then repeated with homogenised Polyclar Super R.

2.2.2.5. Determination of the Effect of Flavanoid Polyphenols on Beer Stability

Filtration and Stabilization of Beer

(a) Pilot Scale

Unstabilized lager beer A (original gravity = 13.5°P), brewed from a barley malt and maize mixed-grist by decoction mashing was obtained from a storage vessel of a conventional lager brewery after two days cold storage, and then diluted as required in 1 hL batches to 10°P with deaerated water. Processing was carried out in a pilot plant as described previously [14], but without the addition of silica hydrogel. Polyclar Super R was added either at 0, 10, 30 or 50 g hL⁻¹ to the coarse-filtered beer batches before their fine-filtration. The polished beers were then carbonated (6 g L⁻¹ CO₂), packaged in dark-brown 284-ml bottles and pasteurised (20 PU). Some of the bottles were then stored at 2°C; the rest were force-aged at 37°C.

(b) Bench Scale

Filtration was carried out using a scaled-down version of the pilot scale procedure described above, the main difference being that CO₂ pressure was used to drive the filtration instead of a positive displacement pump. Diluted Beer A (10°P) was primary-filtered on a plate and frame filter containing two 20 cm x 20 cm filter sheets (total effective filter area 0.061 m², type KDS 15; Beco Filtration Systems, Sudbury, Suffolk, England), using a filter aid precoat (0.4 kg m⁻²) of 40% Dicalite 418 (Steetley Ltd., Worksop, Nottinghamshire, England), 40% Dicalite 438 and 20% Clarcel CBL (Irish Ceca Ltd., Naas, Ireland). A control subsample (18 L) of the beer was filtered without PVPP stabilization, and a second subsample (54 L) was filtered with Polyclar Super R added at 100 g hL⁻¹.

All of the unstabilized control beer, and a portion (18 L) of the PVPP-stabilized beer, were separately carbonated (6 g L⁻¹ CO₂) and packaged in dark brown 284-ml glass bottles (mean dissolved O₂ content = 0.14 mg L⁻¹) and pasteurised (20 PU). Contents of iron in selected samples of bottled beers analysed by flame atomic

absorption spectroscopy averaged 0.05 mg L^{-1} (RSD = 22%), while copper contents averaged 0.13 mg L^{-1} (RSD = 17%). Treatment with PVPP had no effect on either of these assay values. The remaining stabilized beer was used for treatment with specific flavanols.

Replacement of Flavanols in Stabilized Beer A

Solutions (3 ml each) of procyanidin B3 and prodelfinidin B3 (prepared as described in sec. 2.2.2.2) and (+)-catechin (Sigma) in ethanol were each prepared separately at concentrations of 568, 1136, 2272 and 4544 mg L^{-1} . This was done so that concentrations of 1, 2, 4, and 8 mg L^{-1} of added flavanol were obtained by the addition of 0.5 ml of the appropriate concentrate to the beer in a 284-ml bottle. Accordingly, bottles which had been flushed with CO_2 were filled manually, one at a time, with cold beer that had been overstabilized (100 g hL^{-1} Polyclar Super R) and then carbonated ($6 \text{ g L}^{-1} \text{ CO}_2$). After each filling, 0.5 ml of the selected flavanol concentrate or ethanol blank was quickly added by syringe injection to the beer at a point 10 cm below the surface; sufficient fob was allowed to overflow from the bottles to prevent the ingress of air before crown sealing. Five bottles were prepared at each flavanol addition rate and the mean dissolved oxygen content was determined on five of the 25 blank samples as being 0.12 mg L^{-1} . The remaining bottles were pasteurised (20 PU) and were then either analysed immediately or force-aged at 60°C for 7 days.

Analysis of Simple Flavanols by HPLC-ED

Beers were analysed for simple flavanols as described in section 2.2.2.1 above.

Analysis of Total Polyphenols

Total polyphenols were measured by reaction with Fe(III) by standard procedures [75,76] using (\pm)-catechin as a calibration standard.

Analysis of Total Flavanols

Total Flavanols were determined by reaction with *p*-dimethylaminocinnamaldehyde (DAC) as described previously by Delcour and Janssens de Varebeke [73], using (\pm)-catechin as a calibration standard.

Haze Measurement

Haze (light-scattering) measurements were made on samples in duplicate using a HZ-103 haze meter (LG-Automatic ApS, Hillerod, Denmark). The instrument was calibrated in EBC haze units with turbidity standards supplied by Advanced Polymer Systems, Inc., Redwood City, CA, USA. Permanent haze was measured on degassed beers at room temperature, and total haze was measured on the same samples following overnight storage at 0°C. Chill haze was calculated as the difference between these two values.

Reducing Capacity

The formation of a red complex by the reduction of ferric dipyriddy (DPFe₃) by different beer samples was followed by the adaptation [77] of the original method of Chapon and Chapon [26]. Using this method, the effect of treatment with PVPP on the reducing capacity of three different beer types (Beers A, B, and C) was examined. Beer A was the same storage lager (original gravity = 10°P) as was used for pilot scale trials, and was prepared from a 20% maize-80% barley malt grist. Beer B was storage lager (original gravity = 12.4°P) prepared from an all barley-malt grist. Beer C was trade stout beer (original gravity = 10°P). Subsamples (500 ml) of each beer were stirred with varying amounts of Polyclar Super R (0-100 g hL⁻¹) for 1 hr at 0°C, under a blanket of CO₂. The stabilized samples were then centrifuged at 4,500 × g for 25 min to remove spent PVPP and the clear supernatants were tested immediately for reducing activity. Assay values, in milliequivalents per litre (meq L⁻¹) were shown to be linearly related ($r = 0.9991$, $n = 7$) to the amounts of reducing substances present in incrementally diluted samples of the control beer, and the repeatability was acceptable (RSD = 2.2%, $n = 6$).

Force-Ageing

Two conditions were used for accelerating the ageing processes in beer samples:

- 1) bottles of beers intended for both haze and flavour stability assessments were held for up to twelve weeks at 37°C and were examined at weekly intervals; and
- 2) samples that contained added flavanols were aged more rapidly at 60°C, for only one week.

Staling Sensitivity (TBA-test)

The procedure of Grigsby and Palamand [78] was modified as follows:

5 ml of beer was extracted vigorously with 25 ml chloroform for 2 min. 20 ml of the chloroform was evaporated to dryness *in vacuo*, and reconstituted in 2 ml 2-thiobarbituric acid (TBA) reagent (0.33% w/v TBA in 1:1 propan-2-ol:water). The mixture was heated for 1 hr at 60°C and the absorbance was measured at 445 nm against a heated reagent control. Staling sensitivities were measured on beers before and after force-ageing.

Taste Panel Evaluations

(a) Staleness Intensity:

Each of a sixteen member panel of trained tasters was asked to score the intensity of staleness in samples, tasted twice, on a continuous scale of 0-5 where 0 was awarded to fresh, totally unoxidised beer and 5 to extremely stale beer. Staleness was defined as a papery, cardboard, oxidized or metallic flavour [25], but beers were not scored individually for these different attributes.

(b) Difference Testing:

Flavour differences between selected beers were established in single tastings

by the panel using 'triangular' comparisons, in which random arrangements of three glasses of the test beers, in which one sample is always different to the other two, are tasted, and the panelist must select the odd-one-out, and state his or her preference. Panelists were requested also to identify flavour differences using recognized terminology [79].

Analysis of *trans*-2-nonenal

Analysis of *trans*-2-nonenal was kindly carried out by ISP Ltd., Guildford, Surrey, using gas chromatography-mass spectrometry, using a modified version of the method of Bonte and Dupire [103].

2.3. RESULTS AND DISCUSSION

2.3.1. HPLC-ED Analysis of Proanthocyanidins and Catechins in Beer and Barley

Development of HPLC Gradient Conditions

In previously reported work [14,15] a gradient of 0-40% v/v methanol in 20 mM phosphoric acid was used over 60 min for the separation of beer flavanols. This gradient was designed originally to permit the elution of UV-absorbing substances in beer well ahead of the analytes of interest. By replacing this gradient with the acetic acid gradient (2.5-10% v/v) reported by Jerumanis [80], a smoother baseline was achieved for electrochemical detection, along with obviously increased resolution of flavanols (Figure 2.9). In particular, the resolution between protocatechuic acid and prodelphinidin B3 was enhanced by changing to the acetic acid gradient.

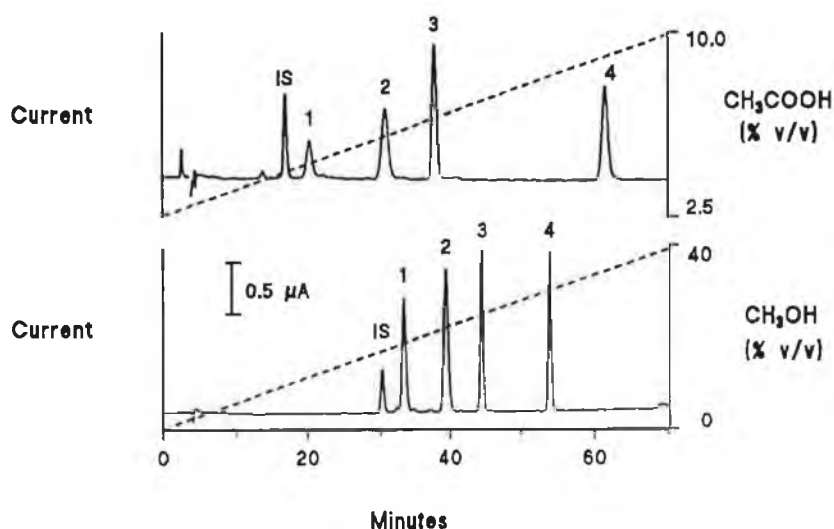


Figure 2.9. Comparison of acetic acid and methanolic gradients for the chromatographic separation of flavanols in beer

Electrochemical Detection Conditions

The capabilities of dual-channel electrochemical detection are most commonly exploited by three different approaches for the determination of oxidizable sample components: coulometric screening, dual-potential oxidation, and combined oxidation-reduction (Figure 2.4).

Coulometric screening: The high conversion efficiency of a coulometric electrode means that almost all of the analyte passing through it becomes oxidized, in contrast to a thin layer amperometric electrode, which typically only oxidizes about 2-10% of the analyte. The coulometric electrode may therefore be used as an effective screening system [81,82]. Coulometric screening in general involves operating the coulometric electrode at a potential lower than the oxidation potentials of the analytes, but sufficiently high to oxidize the most readily oxidized components of the sample matrix that might otherwise interfere in the chromatogram. The downstream amperometric electrode is set to operate at a higher potential, and is capable of detecting the analytes of interest. This device, in effect, utilizes the coulometric electrode as a filter to 'remove' unwanted interferences from the column eluent. In this particular application, the applicability of coulometric screening was limited by the fact that the analytes were oxidized at lower potentials than many of the interferences of the sample matrix.

Dual potential oxidation: Dual-potential oxidation involves operating the two electrodes in series at increasingly more positive potentials, both potentials having been chosen to cause some degree of analyte oxidation. The ratio of detector signals resulting from analyte oxidation at the two chosen potentials can then serve as an indicator of peak purity. This approach was examined using working electrode potentials of +10 and +350 mV for electrodes 1 and 2, respectively. The resulting chromatograms are shown in Figure 2.10.

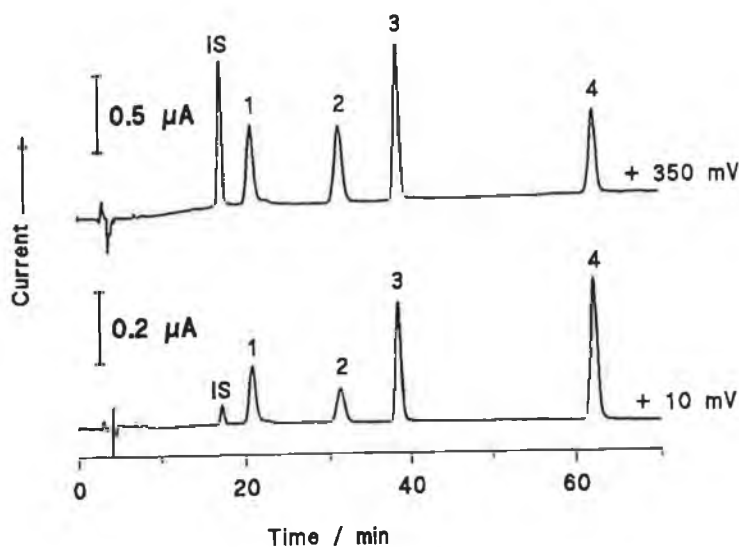


Figure 2.10. Use of dual potential oxidation to detect flavanols in standard solutions. Identified peaks: IS, internal standard; 1, prodelphinidin B3; 2, procyanidin B3; 3, (+)-catechin; and 4, (-)-epicatechin. Potential: (a) + 350 mV; and (b) + 10 mV.

Amperometric oxidation potentials in excess of +350 mV resulted in a strong drift in baseline current as the percentage of organic modifier in the mobile phase increased. This effect was observed as a positive baseline drift with the acetic acid gradient, and a negative drift when a methanolic gradient was used. This problem was also observed when attempting to operate other amperometric electrode systems at high potentials in conjunction with gradient HPLC.

Combined oxidation-reduction: Detection by combined oxidation-reduction involves choosing a primary potential at the coulometric electrode that is sufficient to oxidize the analytes, and a secondary amperometric potential sufficiently negative to reduce the analytes to their original states, provided that the reaction is partly or wholly reversible. The ratio of peak currents may then be used as an indicator of peak purity when comparisons of sample and standard chromatograms are made. This information may possibly be used further to elucidate structural information from the chromatographic separation of unknown compounds, on the basis of their

electrochemical behaviour. Because of this important advantage, this was the approach adopted in this study to develop a method for the analysis of beer and barley extract samples.

Choice of Detector Potentials for Combined Oxidation-Reduction Detection

To optimize the coulometric oxidation potential for detection of the analytes of interest, hydrodynamic voltammetry was carried out on a solution containing 1.0 mg L^{-1} of protocatechuic acid and 4.0 mg L^{-1} of each of the proanthocyanidins and catechins. Although more time consuming to perform than cyclic voltammetry, hydrodynamic experiments, in which chromatograms are obtained at a range of selected potentials, take account of factors such as cell geometry and mobile phase effects, and are, therefore, of direct practical use. Moreover, the chromatovoltammogram simultaneously yields results for several chromatographically pure compounds.

In this study, the chromatovoltammogram constructed was restricted to potentials between -250 and $+650 \text{ mV}$. Although the manufacturers recommended a maximum potential of $+900 \text{ mV}$ for the coulometric detector, it was observed that at potentials above about $+700 \text{ mV}$ the chromatogram was noisy and the background current was high (see also chapter 6, section 6.3.3, Figure 6.4).

Whereas the hydrodynamic experiments (Figure 2.11) indicated that a potential of $+650 \text{ mV}$ might be appropriate, the analysis of beer at this potential resulted in a chromatogram containing a large amount of unresolved interfering material. Figure 2.12 shows the effect of variations in the oxidation potential on the chromatogram for a sample of unstabilized beer. From these experiments, an oxidation potential of $+350 \text{ mV}$ was selected. Although at the cost of some sensitivity, this resulted in a detection system that provided enhanced selectivity for the proanthocyanidins and catechins of interest. Of particular interest in this experiment was the observation of two peaks that eluted between 26 and 30 min and showed a dramatic increase in size at potentials in excess of $+450 \text{ mV}$. At $+350 \text{ mV}$ the interference from these peaks did not hinder identification of the analytes, and so this was the analytical potential chosen for all work reported herein.

The negative potential for amperometric reduction was chosen by examining

potentials in the range from -100 to -750 mV. It was found that a potential of -650 mV provided maximum analyte reduction without significant baseline drift. More negative potentials, however, caused the baseline to drift in a similar way to that observed at high positive potentials.

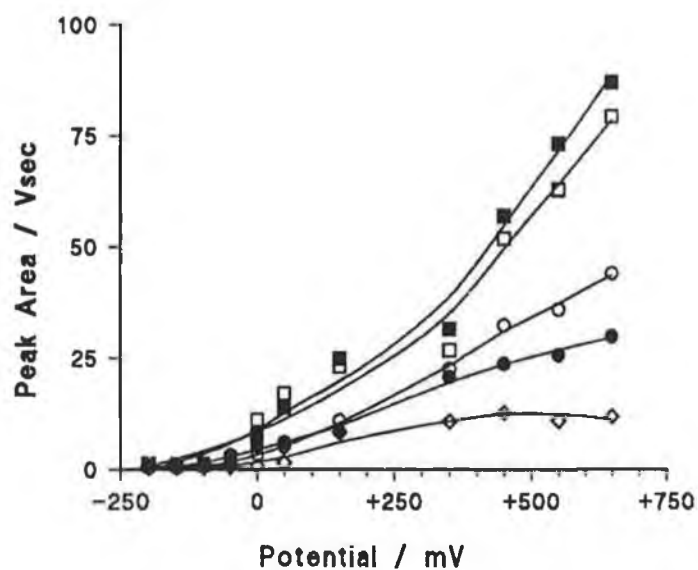


Figure 2.11. Hydrodynamic voltammograms obtained at a coulometric electrode. (■) = (+)-catechin; (□) = (-)-epicatechin; (○) = procyanidin B3; (●) = prodelphinidin B3; and (◇) = protocatechuic acid.

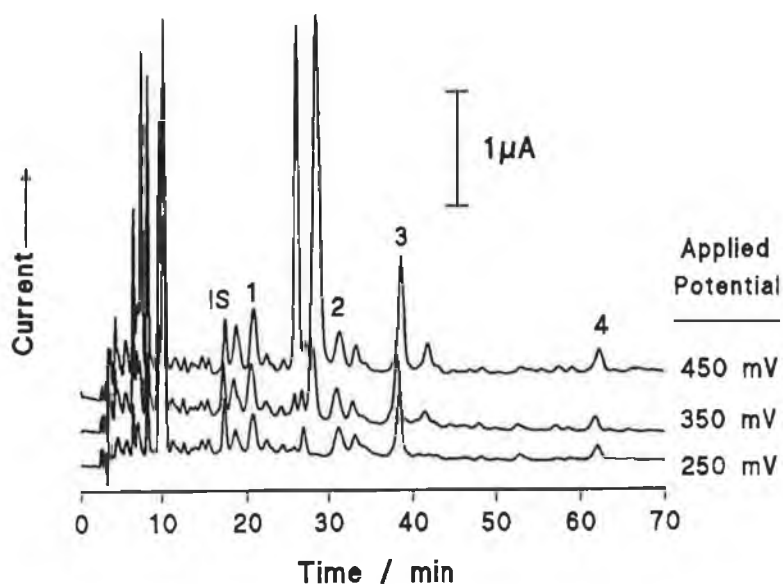


Figure 2.12. Effects of potential on the observed beer chromatograms. Identified peaks as in Figure 2.11.

Calibration of the Detection System

Standard solutions were analysed under the optimized conditions. Peak area increased linearly with increasing concentration in the range $0.1\text{--}4.0\text{ mg L}^{-1}$ for both oxidation and reduction currents for the flavanols examined. This range was sufficient for the analysis of proanthocyanidins and catechins at the concentrations normally encountered in beers. The detection limits (i.e., the point at which the signal-to-noise ratio was equal to 2) of the flavanols were determined to be the following: prodelphinidin B3, 0.2 mg L^{-1} ; procyanidin B3, 0.1 mg L^{-1} ; (+)-catechin, 0.1 mg L^{-1} ; and (-)-epicatechin, 0.1 mg L^{-1} .

Linear regression plots of corrected peak area (using protocathechuic acid as an internal standard) *versus* flavanol concentration were plotted for the oxidation and reduction peaks; the correlation coefficients obtained are shown in Table 2.2.

Table 2.2. Linearity of the Analytical Method

Flavanol	Correlation coefficient (<i>r</i>)	
	Oxidation peak	Reduction peak
Prodelphinidin B3	0.9978	0.9974
Procyanidin B3	0.9982	0.9992
(+)-Catechin	0.9978	0.9993
(-)-Epicatechin	0.9980	0.9995

The ratio of oxidation to reduction corrected peak areas was calculated for the analysis of eight standard solutions of varying concentration between 1.0 and 4.0 mg L⁻¹; the results are shown in Table 2.3. The higher relative standard deviation observed for prodelphinidin B3 was due to the low collection efficiency exhibited by the trihydroxy-substituted ring; this resulted in small chromatographic peaks at the amperometric electrode.

The pronounced difference in electrochemical behaviour between the trihydroxy- and dihydroxy-substituted dimers, and between monomers and dimers, can be used to predict possible structural classifications of unknown flavanols present in beer samples.

The oxidation-reduction peak area ratios determined in this work were used as aids to confirmation of peak identity in the subsequent analysis of beer and barley samples.

Table 2.3. Oxidation to Reduction Peak Area Ratios

Flavanol	Oxidation: reduction peak area ratio ⁺	s_r * (%)
Prodelphinidin B3	5.7	5.0
Procyanidin B3	1.8	2.0
(+)-Catechin	1.1	2.1
(-)-Epicatechin	1.0	2.6

⁺ Corrected for the internal standard area

* s_r = Relative standard deviation (n = 8)

Repeatability of the Analysis

The analysis of standard solutions containing 4.0 mg L⁻¹ of the flavanols of interest and 1.0 mg L⁻¹ of protocatechuic acid gave the relative standard deviations in detector response (for five injections) listed in Table 2.4.

Table 2.4. Repeatability of the Analysis

Compound	s_r^* (%)	
	Oxidation Peak	Reduction Peak
Protocatechuic acid	1.9	0.6
Prodelphinidin B3	1.8	2.3
Procyanidin B3	1.6	1.6
(+)-Catechin	1.1	1.2
(-)-Epicatechin	1.9	1.1

* s_r = Relative standard deviation (n = 5)

Analysis of Beers and Extracts of Barley

Chromatograms of beer and barley samples prepared as described earlier and analysed under the optimized conditions are shown in Figures 2.13 and 2.14. Lager beer samples taken from the storage vessel (before stabilization) and from filled kegs (after stabilization) showed marked differences in flavanol content. The results of this comparison are given in Table 2.5. The effect of treating the beer with PVPP was evinced as a near total removal of the dimers present, and a significant removal of the monomer content. The behaviour of PVPP in this respect is studied in detail in sections 2.4.4 and 2.4.5.

Three barley varieties grown in Ireland, Alexis; Blenheim; and Grit, from the 1992 crop, were analysed for flavanol content. These results are shown in Table 2.5. A remarkable varietal similarity in content of prodelphinidin B3 was observed, but the content of procyanidin B3 showed considerable variability. The contents of flavanol

monomers in all three varieties were low, (-)-epicatechin being undetectable under the described conditions.

The use of collection efficiency measurements throughout these experiments allowed verification of peak integrity. Further confirmation of peak identity was obtained by the analysis of spiked sample solutions. In all spiked samples, the quantitative increases in peak areas were entirely in accordance with expectations.

Table 2.5. Results of the Beer and Barley Analyses

Sample	Concentration / mg L ⁻¹			
	Prodelphinidin B3	Procyanidin B3	(+)-Catechin	(-)-Epicatechin
Unstabilized lager	3.3	3.1	4.2	1.1
Stabilized lager	0.5	0.3	2.7	0.6
Alexis barley ⁺	229	97	13	ND*
Blenheim Barley	234	88	10	ND
Grit Barley	229	142	14	ND

⁺ Concentrations expressed as mg kg⁻¹

* ND = Not detected.

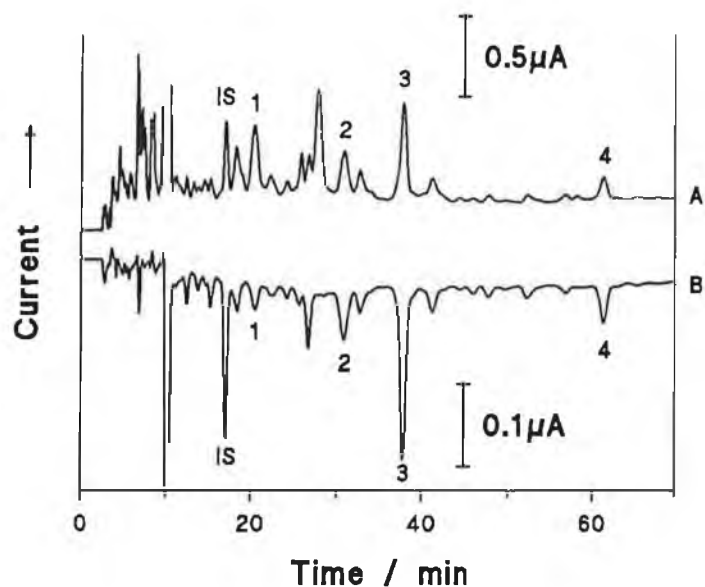


Figure 2.13. Analysis of unstabilized beer. Identified peaks: IS, Internal standard; 1, prodelphinidin B3; 2, procyanidin B3; 3, (+)-catechin; and 4, (-)-epicatechin. Potential: A, +350 mV; and B, -650 mV.

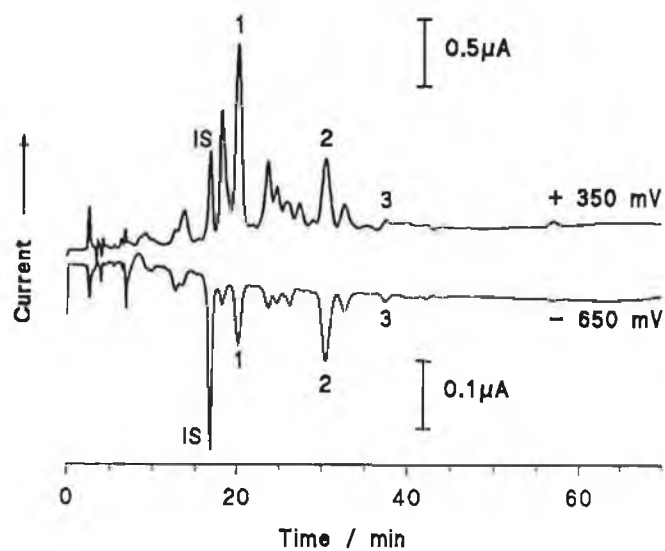


Figure 2.14. Analysis of an acetone-water (3:1) extract of barley by HPLC-ED. Peaks and potentials as in Figure 2.13 (above).

2.3.2. Semi-Preparative Isolation of Dimeric and Trimeric Proanthocyanidins from Barley.

Extraction Conditions

Method 1: This procedure was developed as a convenient method for the extraction of dimeric proanthocyanidins from barley, with the aim of minimizing chromatographic pretreatment protocols. Different solvents were tested firstly for their effectiveness in extracting barley flavanoids. 50 g portions of barley were extracted with 150 ml of either methanol, acetone or acetone-water (3:1) with stirring for 1 hr under a CO₂ atmosphere. The solutions were filtered through sintered glass, and the barley samples were washed with a further 50 ml of extraction solvent. Figure 2.15. shows a chromatogram of reverse phase HPLC-ED separations of samples from these three barley extracts. Measurements for total flavanols in the extracts using *p*-dimethylaminocinnamaldehyde reagent [73] indicated that the relative efficiency of extraction by methanol, acetone and acetone-water (3:1) was respectively 6.5:1:15. Clearly, 100% acetone was a very ineffective extractant of flavanols. Methanol, however, extracted large amounts of the dimeric proanthocyanidins, but showed poor extractability for the trimers, and was therefore the extraction solvent of choice for Method 1. Acetone-water (3:1) has previously been used as an extraction solvent for the quantitative analysis of barley flavanoids [70], but for preparative work this solvent was less convenient than methanol, which could be easily reduced to dryness by evaporation under vacuum, allowing large-scale extractions to be performed with relative ease of recovery of the proanthocyanidins.

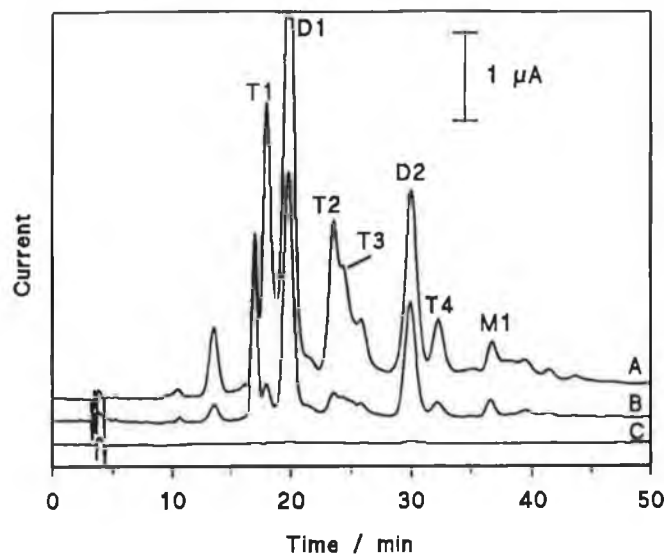


Figure 2.15. Reversed phase HPLC-ED analysis of extracts of barley: A= acetone-water (3:1); B = methanol; and C = acetone. Identified peaks: M1, (+)-catechin; D1, prodelphinidin B3; D2, procyanidin B3; T1, trimer1; T2, trimer 2; T3, trimer 3; and T4, trimer 4.

Method 2: It was evident that acetone-water (3:1) extracted large amounts of both the dimers and the trimers, so this solvent was chosen for extraction Method 2. To isolate the proanthocyanidins from this extract, it was found convenient to salt out the extract solution, thereby removing water from the solvent mixture. HPLC-ED analysis showed that, following salting out with excess NaCl, most of the proanthocyanidins remained in the upper acetone phase. Following evaporation of the acetone phase, the bulk of the remaining proanthocyanidins were recovered in the remaining water. The small amount of residue precipitated during evaporation contained only traces of detectable flavanols, and was therefore discarded.

Semi-Preparative Separations

Separation Conditions

The high affinity of Sephadex LH-20, a hydroxypropylated dextran gel, for flavanoid compounds has made it the current method of choice for the isolation of large amounts of proanthocyanidins [68-70]. However, LH-20 does not have the correct physical characteristics to facilitate high performance resolution, so further purifications by either gel filtration or HPLC are often necessary in order to provide isolates of sufficient quality for use as chromatographic standards. Superdex 75 HR, on the other hand, is a dextran polymer which has a mean bead diameter of 13 μm and it can withstand backpressures of up to 1.8 MPa (260 psi). Although commercially available columns of this material are designed and marketed specifically for the separation of proteins, the separation characteristics were examined with a view to improving on the separations of flavanoids obtainable with Sephadex LH-20. When small volumes (200 μl) of extracts from barley were injected onto the Sephadex column and eluted with methanol, the separation of dimers and trimers was superior to any previously reported for LH-20 [68-70]. A typical separation of dimers and trimers from a barley extract is shown in Figure 2.16.

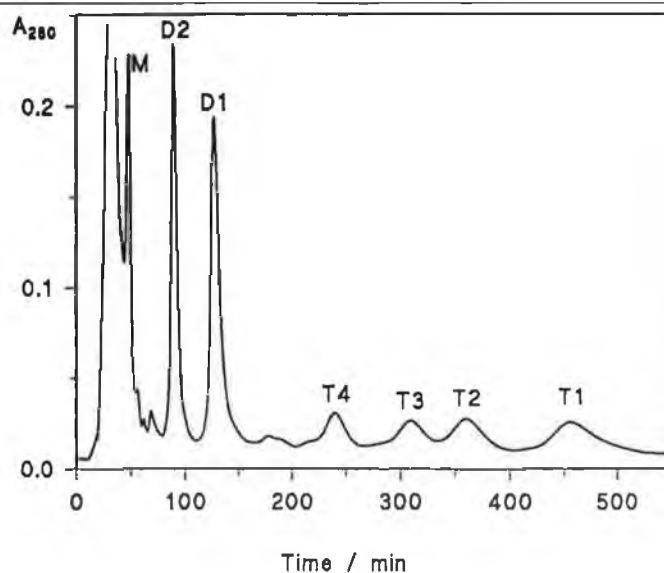


Figure 2.16. Elution of dimeric and trimeric proanthocyanidins from Superdex 75. Identified peaks as in Figure 2.15 (above).

The areas of the peaks in Figure 2.16 were in the ratio 3:4:1:1.1:1.3:1.5 for D2:D1:T4:T3:T2:T1, which indicated the relative proportions of the compounds present in the extract. This system was adopted as a routine method for the isolation of proanthocyanidins for use as chromatographic standards in the analysis of beers and extracts of barley by HPLC-ED.

Identity of Collected Fractions

Concentrated fractions from the semi-preparative separation were analysed by HPLC-ED as described in section 2.3.2.1. Figure 2.17 shows a chromatogram of a) the barley extract used for the semi-preparative isolation, and b) an overlay of the chromatograms of the individual isolated fractions.

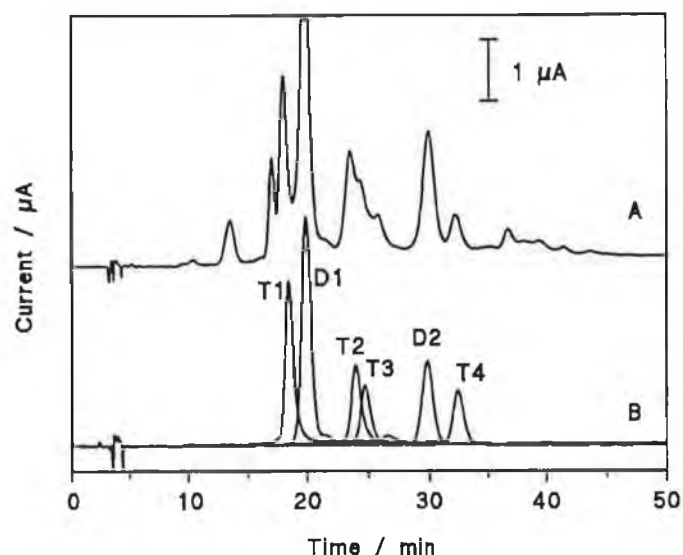


Figure 2.17. Reversed-phase HPLC-ED analysis of: A, an acetone-water (3:1) extract of barley; and B, an overlay of individual chromatograms of the fractions isolated by semi-preparative chromatography. Identified peaks as in Figure 2.15 (p. 54).

For consistency the dimer and trimer fractions were numbered D1-D2 and T1-T4 respectively, in the order of their elution from the reversed-phase HPLC column,

in compliance with the nomenclature described previously by Outtrup [83] and used by McMurrough *et al.* [70]. It was evident that all of the compounds were isolated in a high state of purity, and their identities were deduced as follows.

1. UV spectra in the range 190-400 nm exhibited maxima at 280 ± 2 nm, identical with (+)-catechin and authentic dimeric proanthocyanidins, and typical of polyhydric phenols in which no carbonyl conjugation is present.
2. All of the isolated peaks gave strong positive reactions with *p*-dimethylaminocinnamaldehyde, indicating that they were flavanoid in nature [72,73].
3. The relative retention times on an octadecylsilane reverse phase column of the compounds isolated were in agreement with those observed by Jerumanis [67], Outtrup [83], Mulkay *et al.* [84,85], and by other workers in this laboratory [68,70], and were therefore designated as follows:

D1 = Prodelphinidin B3;

D2 = Procyanidin B3;

T1 = Prodelphinidin trimer with structure (gallo catechin-gallo catechin-catechin);

T2 = Prodelphinidin trimer with structure (gallo catechin-catechin-catechin);

T3 = Prodelphinidin trimer with structure (catechin-gallo catechin-catechin);

T4 = Procyanidin C2 (catechin-catechin-catechin).

4. Collection efficiency [51] is defined as the ratio of the current at the downstream electrode to that at the upstream electrode in a dual-electrode electrochemical detection system, when the downstream electrode is set at a potential sufficient to reverse the oxidative effect of the upstream electrode. For example, a reversible reaction, such as the oxidation of hydroquinone, will give a higher collection efficiency than an irreversible reaction, such as the

oxidation of gallic acid [51]. The collection efficiencies observed for prodelphinidin B3 and procyanidin B3 were in agreement with those observed in section 2.4.1 above. The collection efficiencies of the trimers increased in order of increasing catechin to gallocatechin ratios, as observed for the dimeric proanthocyanidins, and as would be expected from the predicted higher stability of catechin structural units in the oxidized form.

5. Hydrolysis of concentrated methanolic solutions (1 ml) of the isolated prodelphinidin B3 and procyanidin B3 fractions in 5:1 butanol: HCl (2 ml) for 1 hr under reflux yielded compounds with absorbance maxima at either 556 or 546 nm, in accordance with the expected production of delphinidin and cyanidin respectively [86,87].

It is significant that the orders of elution of the flavanoid dimers and trimers on normal phase Superdex 75 (Figure 2.16) was not the exact reverse of that displayed on the reversed-phase column (Figures 2.15, 2.17, 2.18). Retention of solutes on the dextran gel through hydrogen bonding increased primarily with increasing molecular size, but with a secondary influence of the number of gallocatechin versus catechin units per molecule (c.f. D2 versus D1, D2 versus T4, D1 versus T1). Thereupon, different possible sequences of one gallocatechin residue and two catechin residues provided yet another selective influence on elution order (c.f. T3 versus T2). In contrast, the retention to C₁₈ silica of phenolic substances is thought to increase generally with decreasing polarity, and consequently with decreasing aromatic hydroxylation. From Figures 2.15-2.18 it is seen that the separate dependencies of elution order on molecular size, degree of hydroxylation and intermolecular sequence were not clearly distinguishable in their relative strengths. For instance, the retention of (+)-catechin oligomers increased in the order, dimer (D2), trimer (T4), monomer (M1) (Figure 2.15, 2.18.).

Quantification of the Collected Fractions

To quantify the concentrations of each fraction in catechin equivalents, UV spectra of the collected fractions were acquired in the range 190-400 nm, and the

absorbances of the solutions at 280 nm were related to the absorbances of standard solutions of (+)-catechin in methanol (Table 2.6). 1 ml aliquots of the fractions were also analysed with DAC reagent [72,73], and compared also with standard solutions of (+)-catechin between 0 and 30 mg L⁻¹. Results obtained with DAC solution (Table 2.6) differed significantly from those based on UV absorbance, in accordance with expectations [2,88]. It has been shown previously that the molar colour yields obtained with DAC reagent decrease almost in proportion with the degree of polymerization of flavanoid oligomers. Accordingly the average ratios for the concentrations measured in catechin equivalents by UV absorbance and DAC reactivity were respectively 2.0 for the dimers and 2.6 for the trimers (Table 2.5). It was decided, however, to rely on the results of the direct UV absorbance analysis to quantify the fractions. It should be noted that the relative proportions of the flavanols that were isolated do not necessarily reflect the proportions present in the original barley extract (Figure 2.16), because fractions were collected only near the apices of the chromatographic peaks during the semi-preparative separation.

Use of the Quantified Fractions to Calibrate Reversed-phase HPLC-ED Analysis

The quantified dimer and trimer fractions were used to calibrate their determination in unknown samples, by area integration of peaks separated by reversed-phase HPLC-ED. As a test of this improved method, a sample of lager beer, brewed with an all barley malt, was stabilized on a bench scale (1 L) by treatment with 50 g hL⁻¹ of PVPP at 0°C for 1 hr. Samples of the lager before and after stabilization were centrifuged and then analysed by HPLC-ED as described in section 2.3.2.1 and shown in Figure 2.18. The results of this analysis are given in Table 2.7. This ability to measure trimers in beer provides an important addition to the capability of the analytical method described in section 2.3.2.1, and discussed in section 2.4.1. In the beer used as an example here, the trimeric proanthocyanidins accounted for 14% of the total flavanols measurable by HPLC-ED, but it has been claimed that their contribution to haze formation may be more significant than is their relative quantitative presence [89]. Furthermore, PVPP stabilization caused a 38% decrease in the concentration of these compounds, indicating that the measurement of trimers may be another useful marker for the assessment of beer stabilization protocols. It is

evident from Figures 2.15 and 2.17 that the barley extracts made with acetone-water (3:1) contained electroactive compounds other than the flavanols that were separated as distinct peaks on Superdex 75. These unidentified compounds may also be oligomeric proanthocyanidins, as judged by their HPLC-ED behaviour. Their importance in the assessment of beer stabilization is in doubt, however, since there is no evidence that they were removed from beer by PVPP stabilization (Figure 2.18)

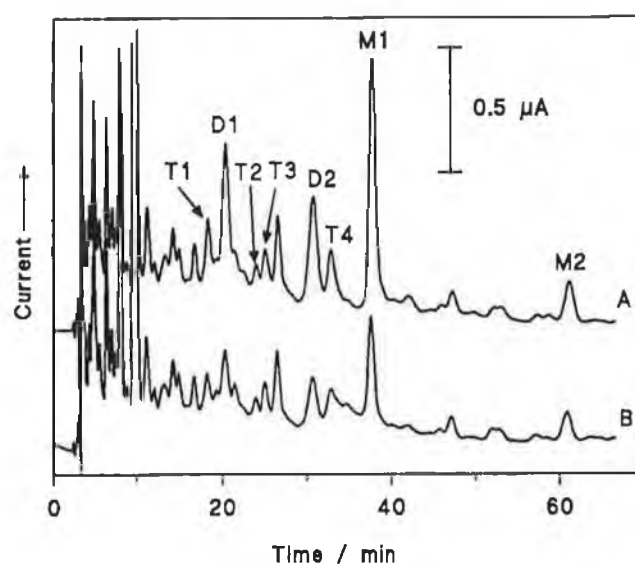


Figure 2.18. Reversed-phase HPLC-ED of a sample of lager beer, A) before, and B) after stabilization with 50 g hL⁻¹ of PVPP. Identified peaks: As in Figure 2.15 above; M2 = (-)-epicatechin.

Table 2.6. Quantification of Concentrated Fractions from Semi-preparative separation by UV Absorbance Spectrophotometry and Colorimetry with *p*-Dimethylaminocinnamadehyde (DAC)

Collected Peak concentrate	Concentration in (+)-catechin equivalents (mg L ⁻¹)		
	UV (280 nm)	DAC	UV / DAC
Dimer 1	14.9	8.3	1.8
Dimer 2	10.3	4.7	2.2
Trimer 1	18.1	9.2	2.0
Trimer 2	13.1	4.4	3.0
Trimer 3	11.8	4.7	2.5
Trimer 4	12.9	4.4	2.9

Table 2.7. Effect of Stabilization of Beer with 50 g hL⁻¹ PVPP on Contents of Proanthocyanidins and Catechins

Flavanol	Flavanol concentration (mg L ⁻¹)		
	Unstabilized lager	Stabilized lager	Percentage decrease
(+)-Catechin	4.3	1.7	60
(-)-Epicatechin	1.3	0.9	31
Total monomers	5.6	2.6	54
D1 (Prodelphinidin B3)	2.5	1.1	56
D2 (Procyanidin B3)	1.7	0.7	59
Total dimers	4.2	1.8	57
T1 (Prodelphinidin trimer)	0.7	0.4	43
T2 (Prodelphinidin trimer)	0.1	0.1	0
T3 (Prodelphinidin trimer)	0.3	0.2	33
T4 (Procyanidin trimer)	0.5	0.3	40
Total trimers	1.6	1.0	38
Total flavanols measurable by HPLC	11.5	5.5	52

2.3.3. Preparative Isolation of Dimeric Flavanols from Barley using Chromatography on Sephadex LH-20

The preparative isolation procedure described in section 2.3.2.3 was used to produce sufficient quantities of proanthocyanidins in pure form to enable their addition to bottled beer at levels of 1-8 mg L⁻¹. The procedure was based on that described previously [70], but was modified so that large amounts of the dimers could be isolated in pure form without the need for further purification by semi-preparative HPLC, as was the case prior to the development of this new procedure.

Extraction of Barley

The extraction solvent acetone-water (3:1) was chosen on the basis of the experiments described in section 2.4.2, and it was decided to use salting-out as an alternative to freeze-drying for the removal of the bulk of the aqueous phase from the barley extract. The extract was deliberately maintained partially aqueous so as to allow an ethyl-acetate extraction to be performed following removal of the acetone, thereby increasing the purity of the final extract. The reconstitution of the ethyl-acetate soluble material in methanol provided one additional purification step prior to the chromatographic separation (Figure 2.5).

Primary Purification by Gel Filtration

The initial separation of this extract on LH-20 was primarily designed to separate the simple flavanols from lipids, phenolic acids, proteins and other high molecular weight material. Therefore, methanol, which is a strong elution solvent for simple flavanols, was used at a flow rate sufficient to achieve a fast primary separation. By analysing every 2-3 fractions collected using HPLC-ED, it was possible to identify the peaks corresponding to the elution of the simple flavanols. Figure 2.6 shows the HPLC-ED chromatograms corresponding to the most important fractions. Fraction 22 contained (-)-epicatechin as well as (+)-catechin, and fraction 28 was mainly (+)-catechin, while fractions 40-62 contained most of the dimeric flavanols. Fraction 70 was rich in trimeric flavanols. This elution order was similar

to that observed on Superdex 75, although the resolution was not nearly as good.

Secondary Purification by Gel Filtration

The crude dimer fraction isolated from the primary separation was reduced in volume and subjected to a secondary LH-20 purification. This time two columns were combined in series to achieve more than double the number of theoretical plates available for the primary separation. Furthermore, a weaker eluent (ethanol) was used to further enhance the system resolution. This resulted in a very efficient separation, as shown by the chromatogram in Figure 2.7. The excellent resolution of the dimeric proanthocyanidins precluded the need for further HPLC purification of the isolated compounds.

Determination of Purity of Isolated Proanthocyanidins

The isolated dimeric proanthocyanidins were subjected to a number of tests of their purity. Fractions from the leading and trailing edges as well as the apices of the peaks in the secondary purification were analysed by HPLC-ED, and were shown to contain compounds which co-eluted with prodelphinidin B3 and procyanidin B3, and which exhibited similar collection efficiencies to those observed previously for these compounds (section 2.4.1). Figure 2.19 shows typical HPLC-ED chromatograms of these peaks.

The isolated fractions were also analysed by isocratic HPLC with UV absorbance detection, in order to detect any non-electroactive interferents which may have been present. Figure 2.20 shows the HPLC-UV chromatograms of the isolated compounds.

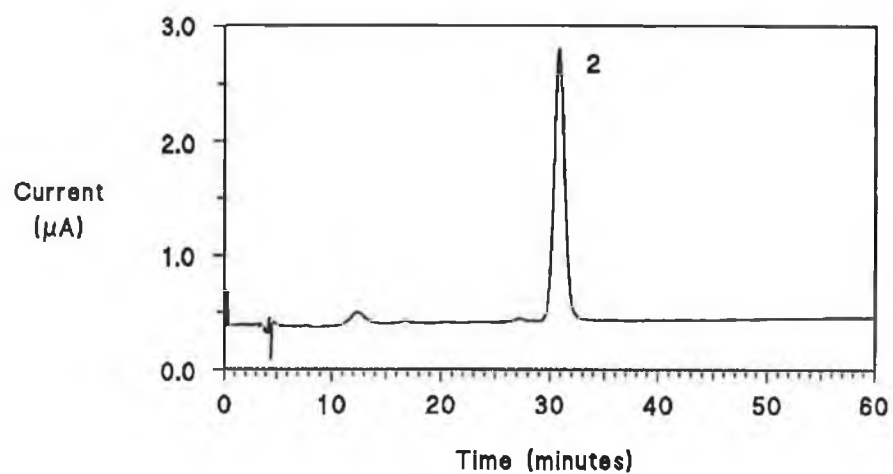
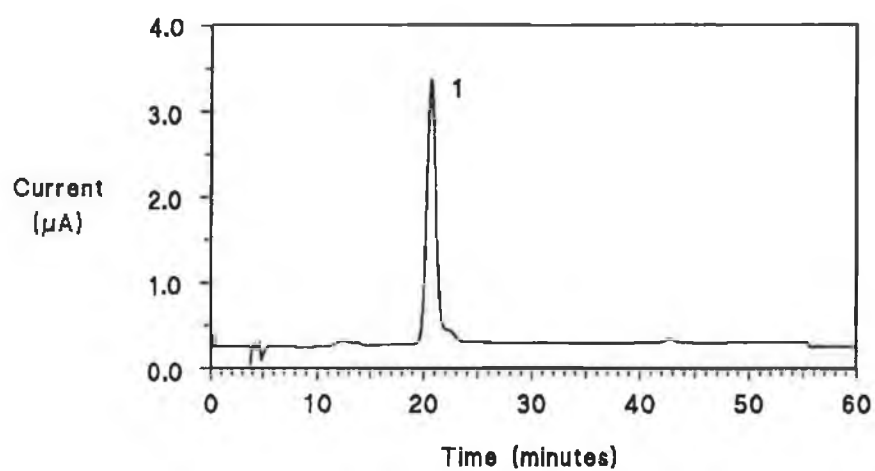


Figure 2.19. HPLC-ED chromatograms of the isolated proanthocyanidins. Identified peaks: 1 = prodelphinidin B3; 2 = procyanidin B3.

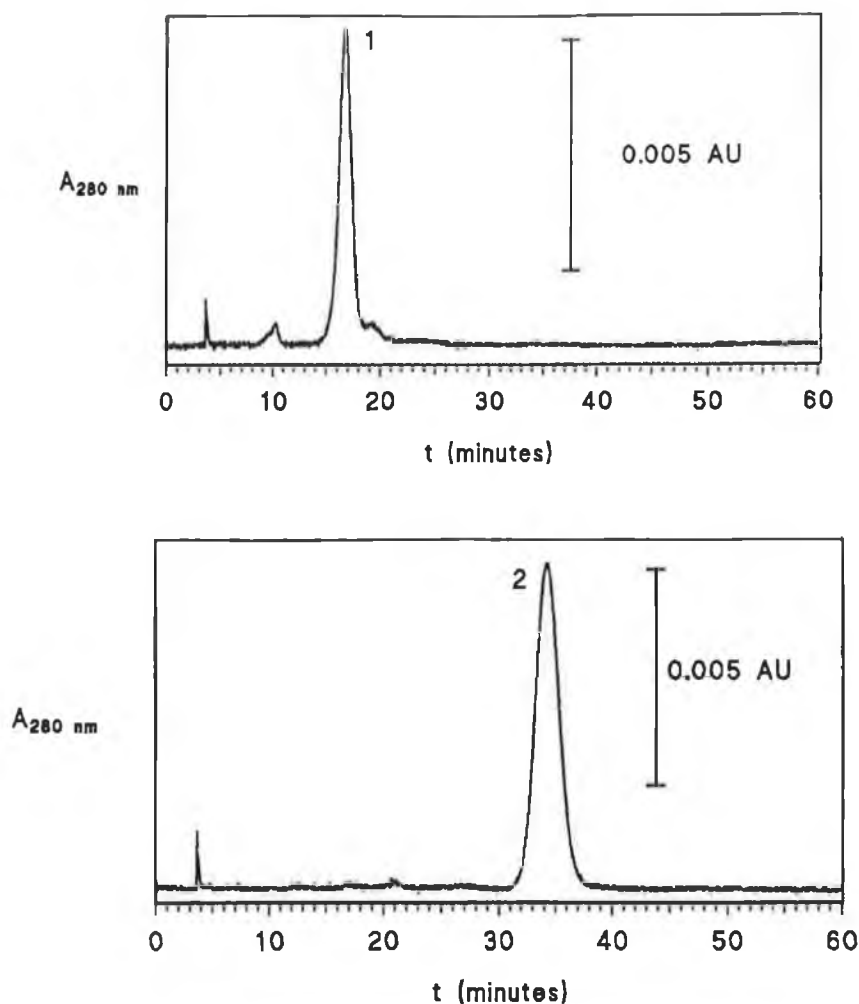


Figure 2.20. HPLC-UV chromatograms of the isolated proanthocyanidins. Identified peaks: 1 = prodelphinidin B3; 2 = procyanidin B3

The purities of the isolated prodelphinidin B3 and procyanidin B3 as determined by HPLC-ED were 94.7% and 93.4% respectively, and by HPLC-UV the respective purities were 94.6% and 100%. HPLC with diode-array detection was used to assess the peak purities of the compounds, and both dimers were found to be isolated in high states of purity, with neither dimer being contaminated by the other. As well as analysis by diode-array HPLC, UV absorbance spectra were acquired with methanolic solutions of the proanthocyanidins. These spectra are shown in Figures 2.21 and 2.22. The absorbance maxima at $280\text{ nm} \pm 2\text{ nm}$ were typical of polyhydric flavanol compounds.

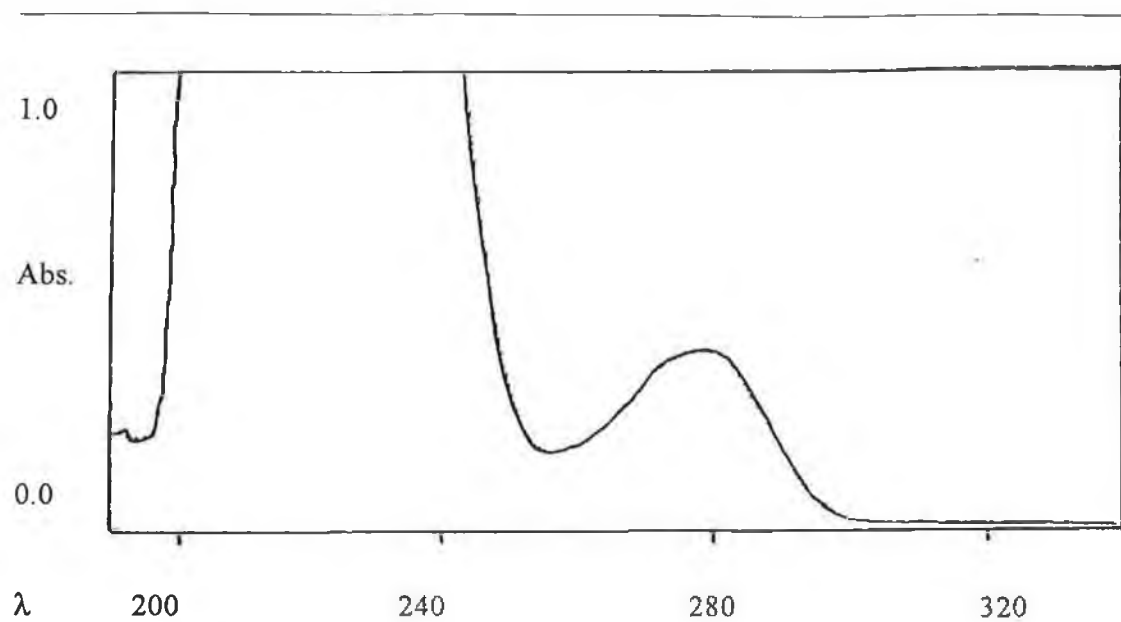


Figure 2.21. Ultraviolet absorbance spectrum of purified prodelphinidin B3 in methanol

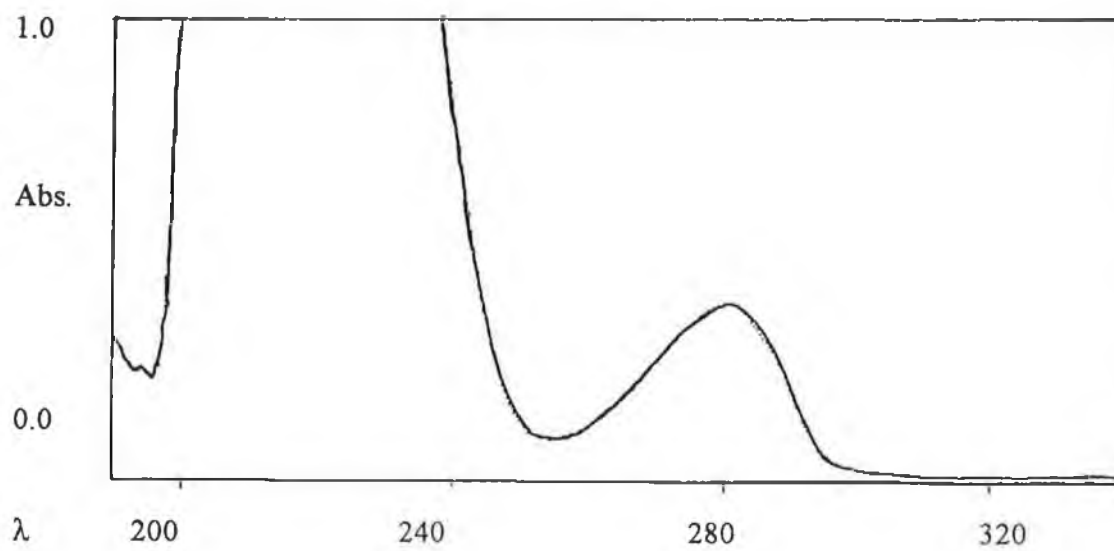


Figure 2.22. Ultraviolet absorbance spectrum of purified procyanidin B3 in methanol

Ethanol solutions of the proanthocyanidins were dried under argon, and the samples were reconstituted in deuterated acetone and analysed by ^1H nuclear magnetic resonance (nmr) spectroscopy at 400 MHz. The nmr spectra are shown in Figures 2.23 and 2.24.

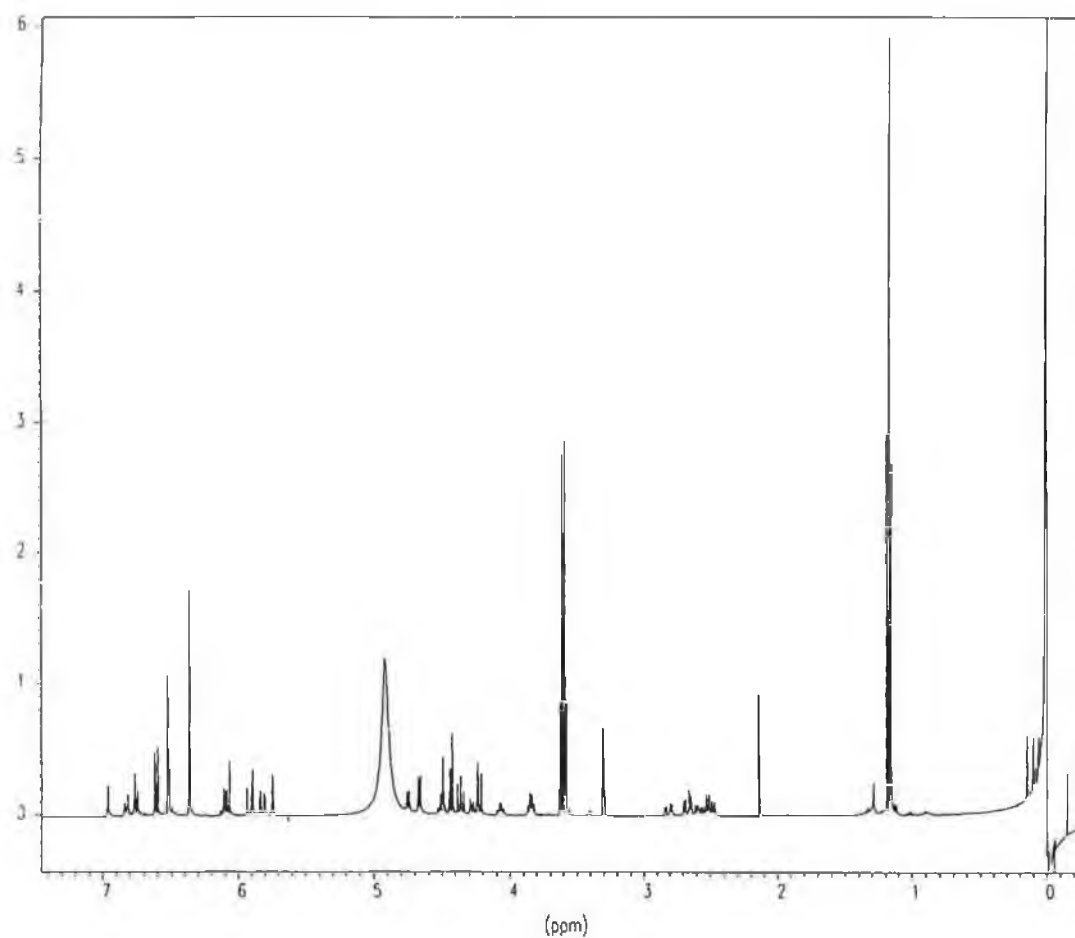


Figure 2.23. ^1H nmr spectrum of purified prodelphinidin B3. Peaks at $\delta = 1.2$ and $\delta = 3.6$ are due to ethanol; peaks at $\delta = 3.3$ are due to methanol, and peak at $\delta = 4.9$ is due to water.

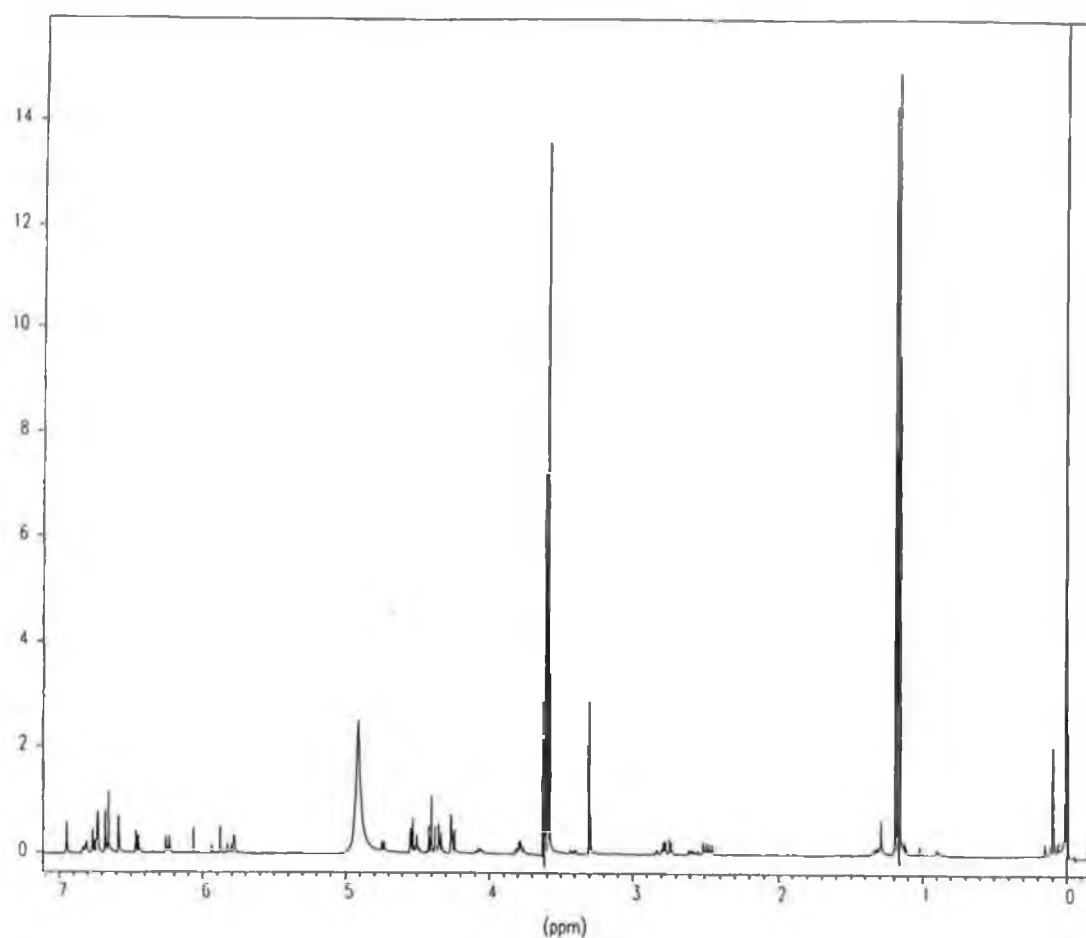


Figure 2.24. ¹H nmr spectrum of purified procyanidin B3. Peaks at $\delta = 1.2$ and $\delta = 3.6$ are due to ethanol; peaks at $\delta = 3.3$ are due to methanol, and peak at $\delta = 4.9$ is due to water.

The ^1H nmr spectra of the proanthocyanidins were interpreted and compared with previously acquired spectra of similar compounds [73,74], and it was concluded on the basis of these spectra, combined with the other evidence obtained, that pure prodelphinidin B3 and procyanidin B3 had been obtained. Acidic hydrolysis of the isolated proanthocyanidins in butanol: hydrochloric acid (5:1) for 1 hr under reflux conditions gave products with absorbance maxima at 556 nm for prodelphinidin B3 and at 546 nm for procyanidin B3, in accordance with the expected production of delphinidin and cyanidin [86,87; see also section 2.4.2]. The amounts of the dimers isolated were calculated by (a) reaction of the compounds with *p*-dimethylaminocinnamaldehyde (DAC) reagent using (+)-catechin as a calibration standard, and (b) by comparison of the absorbance of the isolated compounds at 280 nm with that of solutions of (+)-catechin. To obtain results in weight per unit volume using the DAC assay, the results in (+)-catechin mg L⁻¹ equivalents were multiplied by 2.04 for prodelphinidin B3 and 1.99 for procyanidin B3 to correct for the differences in molecular weight of the dimers relative to (+)-catechin. This assumed that the molar response of the dimeric flavanols was similar to that of (+)-catechin in the DAC assay. This was found previously [73] to be the case, at least for the dimeric flavanols. On the basis of this calibration, the described procedure produced 112 mg prodelphinidin B3 and 85 mg procyanidin B3.

2.3.4. The Adsorption of Catechins and Proanthocyanidins from Beer by Polyvinylpyrrolidone

Selective Adsorption by PVPP of Hydroxybenzoic Acids from a Model System

The isotherms obtained for the removal of hydroxybenzoic acids from a model mixture by Polyclar 10 are shown in Figure 2.25. This adsorbent demonstrated greatly increased affinity for compounds with higher degrees of hydroxylation. Benzoic acid was only slightly adsorbed, even at very high PVPP dosage levels, but the substitution of hydroxyl groups into the aromatic nucleus caused progressive increases in the extent of the adsorption. The affinity of the sorbent for the different acids was dependent on the equilibrium concentrations for each species, so that the complete removal of even the most strongly adsorbed acid was not possible. A similar exponential relationship for the adsorption of polyphenols from beer by PVPP has been reported by Narziss [90].

Adsorption of Simple Flavanols from Beer by Commercial PVPP

To investigate the selective adsorption of specific beer polyphenols, it was necessary to diminish the background of phenolic interferents in the test beer matrix. This was achieved by treatment of the beer with a large excess of Polyclar 10 (5,000 g hL⁻¹). The flavanol-free beer produced in this way gave the chromatogram shown in Figure 2.26 (A), and subsequent addition of pure (+)-catechin and the barley proanthocyanidin mixture gave the chromatogram shown in Figure 2.26 (B).

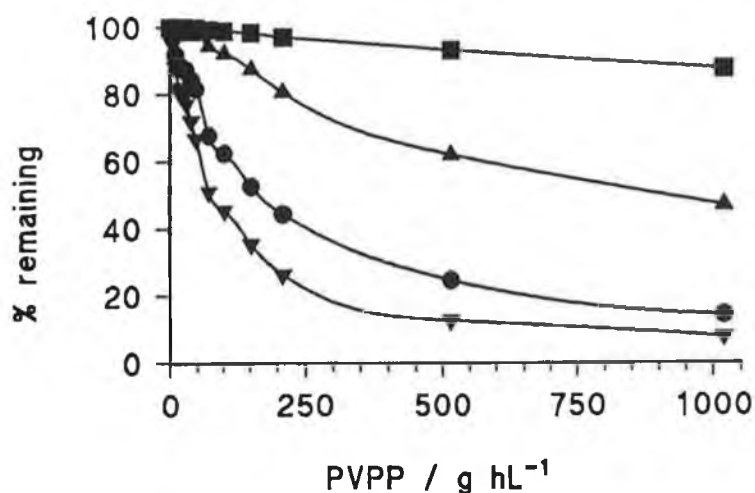


Figure 2.25. Adsorption of phenolic acids from a model system by Polyclar 10: ■ = benzoic acid; ▲ = *p*-hydroxybenzoic acid; ● = 3,4-dihydroxybenzoic acid; ▼ = 3,4,5-trihydroxybenzoic acid. The phenolic acids (0.1 mM) were prepared in 5% ethanol containing 0.1 M KH₂PO₄, pH 4.0. Homogenized Polyclar 10 was added and allowed to react for 1 hr at 0°C with continuous stirring. Centrifuged samples were analysed for phenolic acids by HPLC.

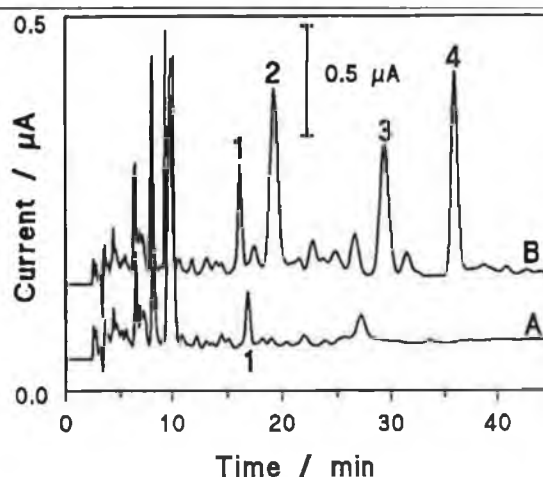


Figure 2.26. HPLC chromatograms of (A) flavanol-free beer after treatment with excess PVPP and addition of 1.0 mg L⁻¹ protocatechuic acid (internal standard, peak 1) and (B) the same beer following addition of 2 mg L⁻¹ protocatechuic acid (internal standard, peak 1) and 10 mg L⁻¹ each of prodelfphinidin B3 (peak 2), procyanidin B3 (peak 3), and (+)-catechin (peak 4). Chromatographic conditions were as described in section 2.3.2.1.

The high sensitivity of the detection system towards phenolic compounds [68; section 2.3.1] confirmed that only small amounts of unidentified substances were added adventitiously to the beer in the barley proanthocyanidin mixture.

Treatment of the spiked beer with various levels of Polyclar 10 and Polyclar Super R produced the isotherms shown in Figures 2.27 and 2.28.

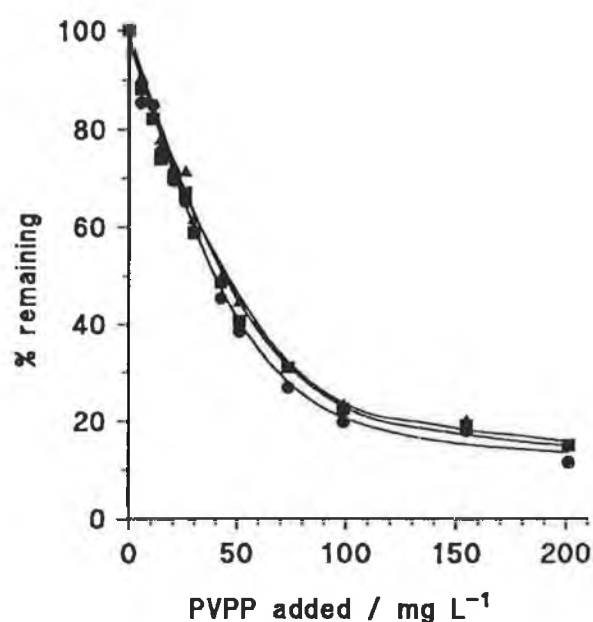


Figure 2.27. Adsorption of flavanols from flavanol free beer, spiked with flavanols (10 mg L^{-1}), by Polyclar Super R: ● = prodelphinidin B3; ▲ = procyanidin B3; ■ = (+)-catechin.

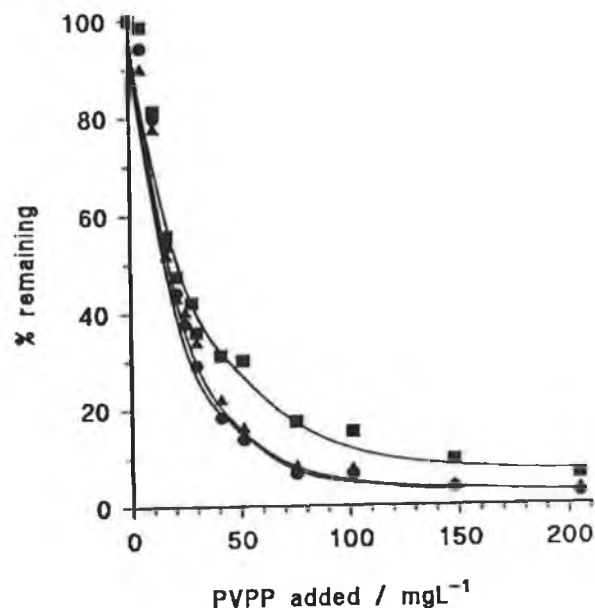


Figure 2.28. Adsorption of flavanols from flavanol free beer, spiked with flavanols (10 mg L^{-1}), by Polyclar 10: ● = prodelfphinidin B3; ▲ = procyanidin B3; ■ = (+)-catechin.

From these figures it can be seen that Polyclar 10 had a much higher adsorption capacity for flavanols than did Polyclar Super R. This was explicable by the greater particle size of Polyclar Super R, and consequently, lower specific surface area for adsorption [20]. The results indicated that neither adsorbent showed significant differentiation between 3',4'- and 3',4',5'-hydroxy-substituted flavanols, though the isotherms for Polyclar 10 were different from those obtained with Polyclar Super R. To quantify the differences displayed in adsorption behaviour, the data were plotted in the form of Freundlich isotherms. The general parabolic Freundlich isotherm can be used as an empirical model of the adsorption of gases on solids or the adsorption of solutes at low concentrations by a solid sorbent. The Freundlich equation [91,92] is as follows:

$$x/m = kc^{1/n} \quad 2.1$$

where x = mass of solute adsorbed (mg), m = mass of adsorbent (g), c = equilibrium concentration of solute after adsorption (mg L^{-1}), k = adsorption value, the amount of solute adsorbed at unit concentration by unit mass of adsorbent (a constant dependent on temperature, surface area of the adsorbent, and the relative attraction of the solutes in a mixture for the solid surface), and $1/n$ = adsorption exponent (a temperature-dependent constant, characteristic of the particular system being studied and always less than unity when the adsorbent surface is partially covered).

Equation 2.1 may be written as a linear transformation as follows:

$$\log x/m = \log k + (1/n)(\log c) \quad 2.2$$

From this equation it can be seen that the amount of solute adsorbed in dilute solution is determined by both a concentration-independent term ($\log k$) and a concentration-dependent term ($1/n \log c$).

Figure 2.29 shows, as one example, the Freundlich plot of $\log x/m$ versus $\log c$ for the adsorption of (+)-catechin on Polyclar 10, and from this were obtained values of 0.77 and 0.89 for $\log k$ and $1/n$, respectively. The values for the constants k and $1/n$ were calculated also for the adsorption of each of the hydroxybenzoic acids (Figure 2.25) and simple flavanols on Polyclar 10 (Figure 2.28) and Polyclar Super R (Figure 2.27) from the corresponding plots. Table 2.8 compares values of $1/n$ and k for the different hydroxybenzoic acids on Polyclar 10 and for flavanols on both adsorbents. The test for the applicability of the isotherm is the linearity of the double-log plot. It must be stated that few data points were obtained for benzoic acid and *p*-hydroxybenzoic acid, due to the low amounts of these compounds adsorbed, so the artificially high correlation coefficients reported for these compounds do not necessarily imply better fits to the Freundlich isotherms.

The data reported here are for solutes in competition for active sites on the sorbent, so solute displacement effects would be expected to have some influence on the isotherms obtained, even though the solutes were present individually at very low concentrations [91]. This model was designed to mimic the anticipated solute behaviour during production-scale beer stabilization.

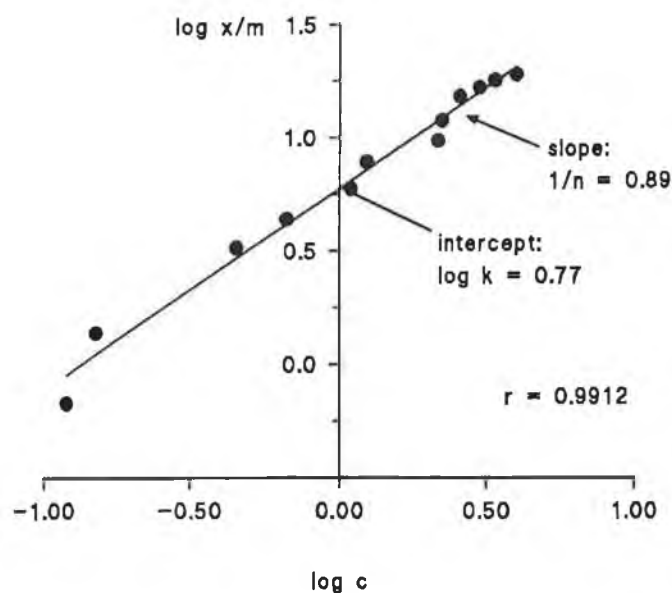


Figure 2.29. Freundlich isotherm for the adsorption of (+)-catechin from beer by Polyclar 10.

There was a very obvious increase in the adsorption value, k , obtained for Polyclar 10 with increasing hydroxylation of the benzoic acids (Table 2.8). The adsorption value increased 10-fold, 34-fold, and 70-fold, respectively, with the introduction of one, two, and three hydroxyl groups per molecule. The adsorption value for (+)-catechin (Figure 2.29) was considerably greater, however, than would have been predicted solely from its four aromatic hydroxyl groups per molecule, which suggested that structural dispositions were of importance also. The values of k obtained for the flavanoid dimers were significantly greater than that for (+)-catechin (Table 2.8). As judged by the correlation data obtained for the dimers, however, their adsorption values were determined with much less accuracy than that for (+)-catechin.

Table 2.8. Determination by Linear Regression of Freundlich Constants ($k, 1/n$) for the Adsorption of Phenolic Acids and Beer Flavanols by Commercial PVPP.

Compound	PVPP grade	k (mg g ⁻¹)	$1/n$	r^a
Benzoic acid	Polyclar 10	0.02	0.78	0.9999 ($N^b = 4$)
p-Hydroxybenzoic acid	Polyclar 10	0.20	0.77	0.9834 ($N = 4$)
Protocatechuic acid	Polyclar 10	0.67	0.91	0.9822 ($N = 9$)
Gallic acid	Polyclar 10	1.40	0.91	0.9894 ($N = 10$)
Prodelfinidin B3	Polyclar 10	9.20	0.85	0.9251 ($N = 12$)
Procyanidin B3	Polyclar 10	10.0	0.81	0.9154 ($N = 12$)
(+)-Catechin	Polyclar 10	6.00	0.89	0.9912 ($N = 12$)
Prodelfinidin B3	Polyclar Super R	4.10	0.65	0.9842 ($N = 11$)
Procyanidin B3	Polyclar Super R	3.50	0.60	0.9610 ($N = 15$)
(+)-Catechin	Polyclar Super R	3.30	0.69	0.9830 ($N = 11$)

^a r = correlation coefficient. ^b N = number of data points

Lower values for k for flavanol adsorption were observed with Polyclar Super R relative to Polyclar 10, and this is at least partially explained by the lower surface area per unit mass of the former sorbent. Freundlich has stated (91) that for two sorbents with identical surface characteristics, differing only in specific surface area, the adsorption isotherms should be affine, i.e. one curve should be the product of a linear transformation of the other. This would result in parallel isotherms, and hence the constant $1/n$ would be similar for both sorbents. A comparison of values for $1/n$ for Polyclar 10 ($1/n = 0.81-0.88$) and Polyclar Super R ($1/n = 0.60-0.69$) suggests a quantitative difference in adsorption behaviour for flavanols in addition to that dictated

by differences in effective surface area. The two adsorbents also showed some differences in selectivity toward the flavanols. Values for k indicated a slight preferential adsorption of the dimers over (+)-catechin by Polyclar 10 but a much less obvious preference for dimers by Polyclar Super R. No consistent preference for the more highly hydroxylated dimer, prodelphinidin B3, over procyanidin B3 was obvious. Unlike the hydroxybenzoic acids, the complex molecular structures of the flavanols might sterically limit the number of hydroxyl groups that effectively participate in the adsorption process. Polyclar Super R is a regenerable sorbent and is manufactured to a high physical and mechanical stability specification, whereas Polyclar 10 is designed as a single-use disposable product. The observed differences in adsorptive behaviour may, therefore, be due to differences in the extent of polymer cross-linking achieved during manufacturing of the sorbents.

2.3.5. Studies on the Role of Flavanoid Polyphenols in Beer Stability

Reducing Capacities of Different Beer Types

The reducing capacities of Beer A (low original gravity lager), Beer B (high original gravity lager), and Beer C (trade stout beer) are given in Table 2.9.

Table 2.9. Effect of PVPP Treatment on Reducing Capacities of Different Beers

Sample	Reducing Capacity (meq L ⁻¹)*		
	0 g hL ⁻¹ PVPP	50 g hL ⁻¹ PVPP	100 g hL ⁻¹ PVPP
Lager A	1.04	0.86	0.76
Lager B	1.55	1.11	0.96
Stout C	2.01	1.89	1.82

* Reduction of Iron(III) dipyridyl

In accordance with theoretical expectations [8,26,77] the reducing capacity of the untreated stout beer (C) was the highest and the reducing capacity of the low original gravity lager (A) was the lowest. Treatment with excess PVPP diminished the reducing capacity of the stout by only 9%, whereas the corresponding decreases for lagers A and B were 27% and 38% respectively.

The Effect of PVPP Treatment on Reducing Capacity, Total Polyphenols and Total Flavanols in Beers A, B, and C.

Increasing dosages of PVPP progressively decreased the reducing capacities of the beers, as exemplified in Table 2.10 for Beer A. Moreover, the reducing capacities of the treated samples were linearly correlated with their contents of total polyphenols and total flavanols ($r = 0.9837 \rightarrow 0.9930$). Extrapolation of the regression line for total flavanols versus reducing capacities to zero flavanol content indicated that flavanols accounted for variable amounts of the total reducing capacity. For instance, for Beer B (Figure 2.30) the proportion of the total reducing capacity accounted for by total flavanols was 60%. The corresponding proportion was 50% for Beer A and was as little as 23% for Stout Beer C, which contained the lowest amount of total flavanols (13 mg L^{-1}).

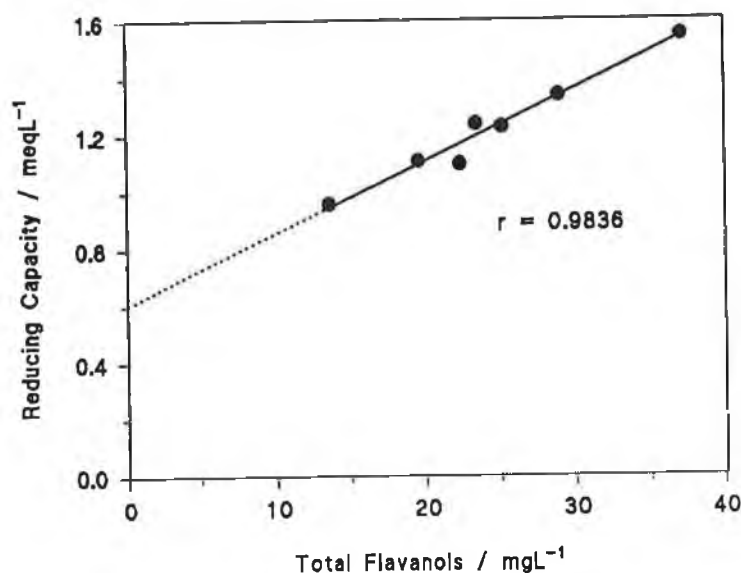


Figure 2.30. Dependence of reducing capacity of lager Beer B on flavanol concentration after samples had been dosed with different levels of PVPP ($0\text{-}100 \text{ g hL}^{-1}$).

Restoration of Reducing Capacity in Lager Beer B

When treated with an excess of PVPP (100 g hL^{-1}) the reducing capacity of Lager B was decreased by 38% (Table 2.9). The depleted reducing capacity was increased linearly when various additions of (+)-catechin, prodelphinidin B3 and procyanidin B3 were made separately to different samples of the treated beer (Figure 2.31).

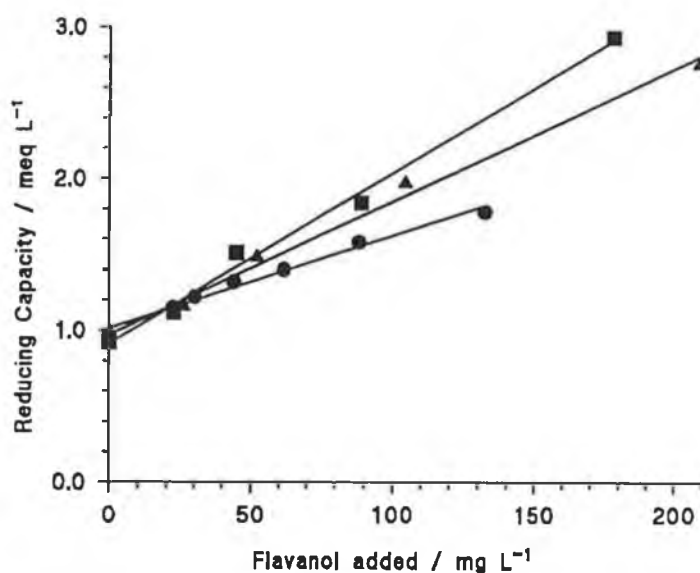


Figure 2.31. Restoration of reducing capacity of over-stabilized (100 g hL^{-1} PVPP) lager Beer B by addition of simple flavanols. ■ = procyanidin B3; ▲ = prodelphinidin B3; ● = (+)-catechin.

From slopes of the regression lines ($r = 0.9918 \rightarrow 0.9968$) for each flavanol added the specific reducing capacities (meq g^{-1}) were calculated (Table 2.11). Corresponding data were obtained also for additions of ascorbic acid, an established antioxidant (see Chapter 5). The reducing power of ascorbic acid at a concentration of 30 mg L^{-1} was measured as 0.3 meq L^{-1} , which is similar to that reported previously

by Chapon [8].

The specific reducing capacities of the three flavanols were similar to that of ascorbic acid and were ranked: procyanidin B3 > ascorbic acid > prodelphinidin B3 > (+)-catechin. The decrease in reducing capacity of Beer B caused by treatment with an excess of PVPP was calculated to be equivalent to the removal of about 65 mg L⁻¹ of ascorbic acid or 90 mg L⁻¹ of (+)-catechin. The measured decrease in total polyphenols was about 300 mg L⁻¹ in (+)-catechin equivalents.

Table 2.10. Effect of PVPP Treatment on Reducing Capacity, Total Polyphenols and Total Flavanols of Lager Beer A.

PVPP (g hL ⁻¹)	Reducing capacity (meq L ⁻¹)	Total Polyphenols (mg L ⁻¹)	Total Flavanols (mg L ⁻¹)
0	1.04	56	19.5
10	0.97	51	17.0
20	0.92	37	14.7
30	0.89	36	13.6
40	0.87	33	12.6
50	0.86	30	12.0
75	0.79	24	10.6
100	0.76	16	9.2

Pilot Scale Stabilization of Lager Beer A

General Specifications:

Analysis of selected samples revealed that trial beers were bottled according to production specifications for original gravity (10°P), alcohol content (4.3% v/v), bitterness (19-20 IBU), colour (7-8 EBC), pH (4.2), head retention (105-110 sec. by Rudin), carbonation (5.75-6.16 g L⁻¹), air (<0.3% by vol.) and total haze (<0.65 EBC).

Total Polyphenols, Total Flavanols and Simple Flavanols:

The effect of variable dosage (0-50 g hL⁻¹) of PVPP is shown in Table 2.12. At maximum PVPP dosage the proportional decreases obtained for total polyphenols, total

flavanols, prodelphinidin B3, procyanidin B3, (+)-catechin and (-)-epicatechin were 41%, 74%, 83%, 71%, 81%, and 76% respectively. These results indicated that PVPP very effectively adsorbed flavanoid compounds.

Table 2.11. Specific Reducing Capacities of Simple Flavanols and Ascorbic Acid

Reducing substance	Specific Reducing Capacity (meq g ⁻¹)*
(+)-catechin	6.1
prodelphinidin B3	8.8
ascorbic acid	9.4
procyanidin B3	11.2

*Added to Beer B.

Table 2.12. The Effect of Pilot-scale PVPP Treatment on Polyphenol Contents of Beer*

PVPP Dosage (g hL ⁻¹)	Contents (mg L ⁻¹)					
	Total Polyphenols	Total Flavanols	Prod. B3	Proc. B3	(+)-Cat.	(-)-Epi.
0	97	13.3	1.6	1.4	2.2	0.6
10	86	9.6	1.2	1.1	1.5	0.4
30	79	7.3	0.8	0.6	0.8	0.3
50	57	3.5	0.3	0.4	0.4	0.2

* Lager Beer A, original gravity = 10°P

Notes: Prod. B3 = prodelphinidin B3; Proc. B3 = procyanidin B3; (+)-Cat. = (+)-catechin; (-)-Epi. = (-)-epicatechin.

Colloidal Stability at 37°C:

Measurements of total hazes taken at weekly intervals during warm storage are given in Figure 2.32. Permanent Haze values did not increase significantly from their starting values (0.6-0.7 EBC); so any increases were due to chill haze. The forced shelf-lives resulting from the different treatments were calculated as the times required for total hazes to increase to 2 EBC units: control = 0.4 weeks; 10 g hL⁻¹ PVPP = 1 week; 30 g hL⁻¹ PVPP = 4 weeks; 50 g hL⁻¹ PVPP = 8.3 weeks.

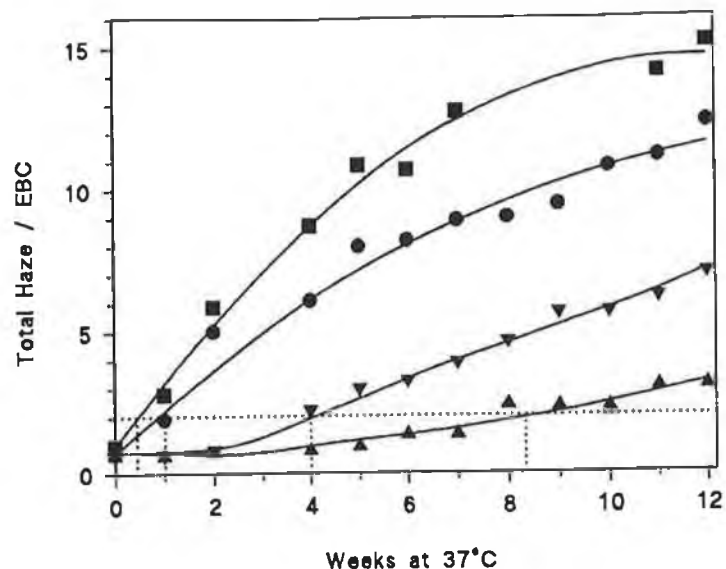


Figure 2.32. Haze development in PVPP-treated beers during storage at 37°C. ■ = control beer; ● = 10 g hL⁻¹ PVPP; ▼ = 30 g hL⁻¹ PVPP; and ▲ = 50 g hL⁻¹ PVPP. The vertical broken lines indicate the storage times necessary to produce a haze of 2.0 EBC.

Colloidal Stability at 60°C:

Values obtained for total hazes in beers stored for one week at 60°C closely matched those obtained after 9 weeks storage at 37°C ($r = 0.9866$, $n = 7$). The relationship was:

$$\text{Haze at } 37^{\circ}\text{C} = 0.977 (\text{Haze at } 60^{\circ}\text{C}) + 0.469.$$

Staleness Intensity:

Mean values for sensory evaluations of staleness intensity performed at weekly intervals on control beer stored at 37°C for up to three weeks indicated a progressive, though unsensational, increase in oxidized flavour character, from slight (mean = 0.9,

RSD = 5%) to noticeable (mean = 2.1, RSD = 5%). The corresponding staleness intensity for control beer stored at 2°C for three weeks was 1.7 (RSD = 6%). Much greater variability was found for the sensory evaluations of the trial beers after three weeks warm storage. For beers treated with PVPP at 10 g hL⁻¹, 30 g hL⁻¹, and 50 g hL⁻¹ respectively the staleness intensities were 2.1 (RSD = 19%), 1.6 (RSD = 29%) and 1.8 (RSD = 35%). These results indicated that, with respect to oxidized flavours, there were no significant differences between the control beer and the experimental beers. However, when a triplet comparison was made between the three-week warm-stored control and warm-stored beer that had been treated with 50 g hL⁻¹ PVPP, a very significant difference (11 correct out of 16, $p \leq 0.01$) was found, with a significant preference (7 out of 11 correct identifications, $p \leq 0.05$) for the trial beer. Preferences for the trial beer were generally attributed to its less harsh / astringent character, which was unsurprising, as the polyphenols are generally recognised as providing bitterness and astringent flavour notes.

Staling Sensitivity:

The staling sensitivities of beers that had been treated respectively with 10, 30 and 50 g hL⁻¹ PVPP were on average 2.0, 1.7, and 1.8, after storage for 12 weeks at 37°C. These values were only slightly lower than that of the untreated control beer after storage, i.e. 2.4. The repeatability of the method was acceptable (RSD < 4%) and the correlation (r) between these values and staleness intensities was 0.8352.

Losses of Simple Flavanols During Force-Ageing:

The untreated control beer A contained readily measurable quantities of prodelphinidin B3, procyanidin B3, (+)-catechin and (-)-epicatechin at bottling (Table 2.12). Considerable losses of these components occurred during storage of the beer samples at 37°C (Figure 2.33). The rates of loss were greatest during the first 4-5 weeks of storage, but continued at decreased rates throughout prolonged ageing. The relative rates of disappearance of the two dimers, prodelphinidin B3 and procyanidin B3, were indistinguishable from one another but were perceptibly greater than the monomers. After 12 weeks storage only about 25% of the dimers originally present

in the bottled beer remained, whereas the residual monomers amounted to at least 40%.

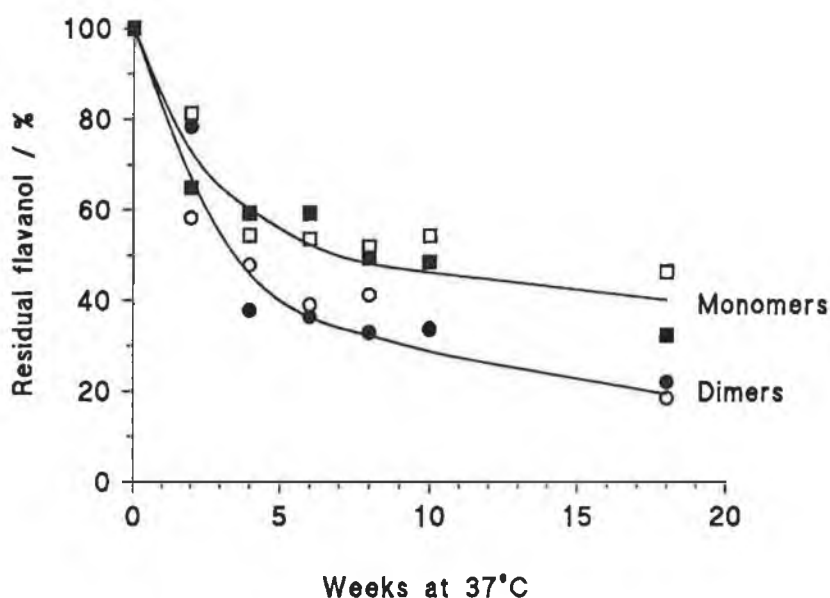


Figure 2.33. Losses of simple flavanols from lager beer A during storage at 37°C. □ = (+)-catechin; ■ = (-)-epicatechin; ○ = prodelphinidin B3; and ● = procyanidin B3.

Destabilization of Lager Beer A

Several tests of stability were performed on a subsample of the lager: firstly after it had been treated with an excess of PVPP (100 g hL⁻¹) on a bench scale and, secondly, following its destabilization by different additions of different simple flavanols. Stabilities were challenged by storage at 60°C for up to seven days.

Polyphenols Adsorbed by PVPP:

Treatment with PVPP at 100 g hL⁻¹ removed 48% of the total polyphenols, 78% of the total flavanols, 90% of the prodelphinidin B3, 96% of the procyanidin B3, 79% of the (+)-catechin and 88% of the (-)-epicatechin present in the beer.

Colloidal Stability at 60°C:

Measurements made at daily intervals for seven days showed that chill haze in samples of unstabilized beer increased linearly with time to 9.86 EBC ($r = 0.9943$), while haze in beer that had been stabilized with PVPP (100 g hL^{-1}) increased only to 0.28 EBC ($r = 0.9146$). Various additions (1, 2, 4, and 8 mg L^{-1}) of prodelphinidin B3, procyanidin B3 and (+)-catechin to the over-stabilized beer caused no increases in chill haze in samples immediately after pasteurization, but chill haze development was obvious during heat punishment. In Figure 2.34 it is shown that increasing amounts of simple flavanols caused increasing amounts of chill haze development; the increases in chill haze for equal amounts of (+)-catechin and procyanidin B3 were similar, and prodelphinidin B3 was the most effective inducer of haze development.

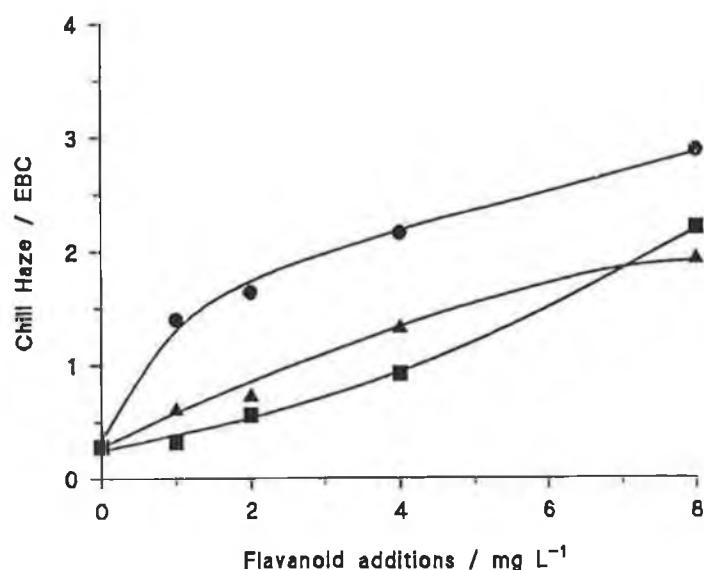


Figure 2.34. Effect of the addition of simple flavanols to over-stabilized lager Beer A on the development of chill haze during storage (1 week) at 60°C. ● = prodelphinidin B3; ▲ = procyanidin B3; and ■ = (+)-catechin.

Conversely, the effect of excessive PVPP treatment in causing decreases in simple flavanols (i.e. (+)-catechin = 2.6 mg L^{-1} , prodelfphinidin B3 = 1.2 mg L^{-1} , procyanidin B3 = 1.3 mg L^{-1}) was calculated from Fig. 2.33 as accounting for only about 2.4 EBC units of the chill haze decrease of 9.5 EBC units obtained on stabilization. This implied that PVPP treatment removed substances from beer other than the named simple flavanols that contributed even more extensively to haze instability.

Changes in Flavanoid Polyphenols During Ageing at 60°C:

Significant losses of added flavanols occurred in spiked beers, after both pasteurization and storage. The correlations ($r = 0.9968 \rightarrow 0.9987$) between the contents of simple flavanols in samples taken before and after pasteurization are given for (+)-catechin and procyanidin B3 as examples in Figure 2.35.

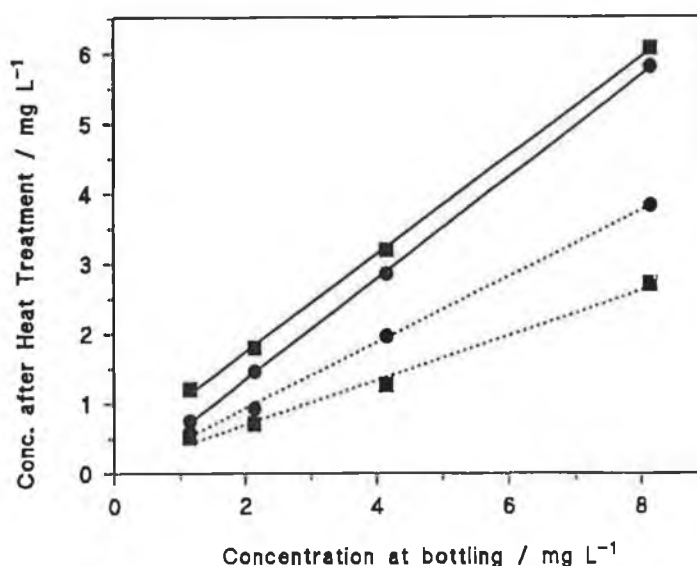


Figure 2.35. Post-bottling changes in concentrations of (+)-catechin and procyanidin B3 from over-stabilized beers dosed with simple flavanols, following heat treatment by pasteurization and warm storage. Solid lines indicate concentrations after pasteurization (20 PU). Broken lines indicate concentrations after pasteurization and subsequent storage at 60°C for 7 days. ■ = procyanidin B3; ● = (+)-catechin

The average losses of added flavanols after pasteurization were respectively 28%, 30% and 34% for (+)-catechin, procyanidin B3 and prodelphinidin B3. Losses continued throughout storage so that after seven days the corresponding decreases were 53%, 68% and 75%. There were, however, no accompanying changes measured colorimetrically in values for either total polyphenols or total flavanols. This was largely due to the insensitivity of the colorimetric method to very small changes ($1\text{--}4\text{ mg L}^{-1}$ in simple flavanol contents). This is an important demonstration of the advantage of HPLC-ED over other less specific and less sensitive methods for measuring the effects both of PVPP-stabilization and also of force-ageing on the contents of beer simple flavanols.

Staling Sensitivities of Unstabilized, Stabilized and Destabilized beers:

As judged by the reaction with thiobarbituric acid [78], there were no discernible differences between unstabilized control beer, and samples of stabilized beer to which up to 4 mg L^{-1} of the different simple flavanols had been added (Table 2.13). The average absorbance value obtained for the samples before storage was 0.75 (RSD = 1.7%) and this increased to 2.57 (RSD = 3%) after force-ageing.

***trans*-2-Nonenal Analysis**

The analysis for T2N reportedly gave poor repeatability and precision, and the results were not reliable enough to interpret with any certainty. The method did not show any evidence of there being a detectable relationship between the concentration of flavanoid polyphenols in beer, and the T2N present after ageing. Therefore, further improvement of the analytical method would be required before any definite conclusions may be made in this area.

Table 2.13. Staling Sensitivity Values Measured Before and After Forced Ageing of PVPP-Stabilized Beer Containing Various Additions of Simple Flavanols.

Sample	Addition (4 mg L ⁻¹)	Staling Sensitivity*	
		Before Storage	After Storage**
Unstabilized beer	None	0.77	2.59
Stabilized beer***	None	0.74	2.69
Stabilized beer	procyanidin B3	0.75	2.58
Stabilized beer	prodelphinidin B3	0.75	2.54
Stabilized beer	(+)-catechin	0.76	2.47

* Absorbance at 455 nm in thiobarbituric acid test [78]

** At 60°C for 7 days

***Treated with 100 g hL⁻¹ PVPP.

Overall Observations on the Role of Flavanols in Beer Stability

One impediment to the universal use of PVPP as a haze stabilizer has been an apprehension that partial removal of polyphenols would, *ipso facto*, diminish the endogenous reducing capacity of the subject beer [8,26], thereby rendering it more susceptible to oxidative flavour damage. The experimental rationale adopted here to investigate these possibilities involved two tactics: firstly, the effects of removing polyphenols from beer by moderate PVPP treatments were measured; secondly the effects of replacing specific polyphenols in beer that had been treated with an excess of PVPP were measured.

The most obvious and important effects of the removal and replacement of polyphenols were on the colloidal stability of beer. Pilot scale treatment of Beer A

with different dosages of PVPP up to recommended levels removed polyphenols to various extents (Table 2.12) with striking effects on haze stability (Figure 2.32). The total haze that developed in the untreated control beer during forcing exceeded 2 EBC units after only one-half week. This rate of haze development in the control was almost five times greater than that measured in a similar beer studied three years earlier [14]. The main difference between the beers was that Beer A in this study was cold-stored for only three days before filtration, whereas the subject of the earlier study was cold-stored for 21 days. Increasing dosage of Beer A with PVPP up to 50 g hL⁻¹ progressively extended the 'shelf-life' to more than eight weeks at 37°C. Further comparisons with the earlier study [14] suggested that curtailments in cold storage can be compensated for by increasing PVPP dosage, with important implications for cost reductions, particularly in the light of the high price of electrical energy and therefore high costs associated with controlled-temperature storage.

Several authors have measured the haze destabilizing potential of individual polyphenols using either model systems or different beers that had been stabilized to different extents [24,35,89,93-98]. These results are in line with the general consensus that simple polyphenols have little or no chill haze forming capabilities until they have been oxidatively polymerized. This occurred readily in bottled beer during heat punishment (Figure 2.33) even in the presence of low oxygen levels. Polyphenols were categorized by Gramshaw [35] as being 'simple' when they are of a molecular size sufficiently low as to be chromatographically mobile as discrete entities. Simple flavanoid polyphenols are readily oxidized, of course, as evinced by their sensitive chromatographic detection at low electrochemical potentials (section 2.3.1). The electrochemical behaviour of prodelphinidin B3, a trihydroxy species, indicates that its oxidation product is highly unstable, and likely therefore to take part rapidly in further complexation. In comparison, both procyanidin B3 and (+)-catechin form more stable oxidation products, so they might be expected to display slower kinetics of haze formation. The practical observations reported here (Figure 2.34) support such suppositions. Doubtless, a small proportion of the haze was also produced from traces [68] of flavanoid trimers [85,89,94,97,98] that were also present in the subject beer (flavanoid trimers accounted for 14% of the HPLC-resolvable flavanols in an earlier studied beer, see section 2.3.2).

Even though the simple flavanols were potent initiators of chill haze formation

in stabilized beer, these results also suggest that about 75% of the haze formed in control beer A was formed from complexed polyphenols [35,99]. It is suspected that the contents of such chill haze progenitors would have been higher in control Beer A than would be present in a beer subjected to longer pre-filtration cold storage. For many years, it has been known that PVPP removes such protein-polyphenol complexes from beer as well as the simple polyphenols [35,99]. From other sources [45], it was estimated that less than 20% of the haze formed during beer storage was caused by the polymerization of 'free' catechins and proanthocyanidins; 'bound' or complexed flavanoid polyphenols formed early during brewing accounted for the difference. It is not known, however, to what extent the soluble protein-polyphenol complexes can, independently of the oxidation of simple flavanols, self-associate during storage and thereby exceed the limits of their solubility.

The removal of oxidizable polyphenols by PVPP extended the haze shelf-life by as much as twenty times but simultaneously diminished the endogenous reducing capacity by up to approximately 20% (Table 2.10). These results showed that the reducing capacity of different beers, as estimated by reaction with iron(III) dipyriddy, were diminished by treatment with recommended dosages of PVPP, but more than 60-90% remained even after treatment with excess PVPP (Table 2.10). Clearly, in the different beers examined, a variable proportion (20-60%) of the total reducing capacity was accounted for by polyphenols (Table 2.11, Figure 2.30), the remainder being attributable probably to melanoidins or other reductones [29,78]. Treatment of Beer B with excess PVPP (100 g hL^{-1}) decreased the assay value for total polyphenols by about 300 mg L^{-1} of (+)-catechin colorimetric equivalents but diminished the reducing capacity only by about the equivalent of 90 mg L^{-1} of (+)-catechin (Table 2.12). This result signifies that the bulk of those substances assayed as polyphenols in Beer B possessed a specific reducing capacity much below that of (+)-catechin. The simple flavanols were significant reducing agents: the specific reducing capacities (Figure 2.31, Table 2.12) of the proanthocyanidins bracketted that of ascorbic acid while the corresponding value for (+)-catechin was one-third lower. Even so, the calculated reducing power for the simple flavanols (catechins and proanthocyanidins) removed by excess PVPP was sufficient to account for only about one quarter of the total decrease. This result implies that the substances responsible for the bulk of the 'non-melanoidin' reducing capacity of Beer B were polyphenol species, possibly complex

flavanols. These substances were quantitatively more significant than the simple flavanols though their specific reducing capacities were lower. It must be borne in mind, however, that it has yet to be established whether any connection exists between the propensities of certain beer components to reduce the iron(III) ion, as in the test for reducing capacity, and the resistance of beer to oxidative flavour deterioration. However, a very significant correlation has been claimed recently between the reducing activity of a beer for the 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical [100] and its flavour stability [101]. A significant positive correlation between the DPPH-reducing activity and contents of total flavanoid polyphenols was also shown.

Storage of trial beers at 37°C was accompanied by several changes other than the development of haze (Figure 2.32). For instance, the chemically determined staling sensitivity of all samples increased dramatically over 12 weeks, though the differences between the different beers were slight. Similarly, 'typical' sensory staleness increased rapidly but did not vary convincingly between samples dosed at different rates with PVPP. In contrast, there was no doubt that beer treated with PVPP at the recommended rate was significantly less harsh/astringent than the untreated control following heat punishment. This flavour deterioration is probably tracked by the geometric rates of loss of simple flavanols during warm storage (Figure 2.33). The initially rapid decreases during the first five weeks of storage probably signify the consumption and incorporation of headspace oxygen into polymerized flavanoid tannins [7,8,21]. It is believed that the oxidative polymerization of simple polyphenols to tannins is responsible for the development of astringency [6,12,21,22]. The observation that the dimeric flavanols disappeared more rapidly than the monomers, both occurring without the formation of chromatographically detectable products, is in agreement with the findings of others [32].

The attempts to test separately the impacts on staling of prodelphinidin B3 and procyanidin B3, proposed respectively as prooxidant and antioxidant [34], were complicated by the relatively small amounts of these materials available. Added to this were anticipated difficulties in objectively evaluating their contributions to stale flavour development by sensory scoring [5,6,12,22,29,100,102]. There is no doubt that beer staling is associated with the development of a variety of volatile, long-chain, unsaturated carbonyl compounds [5,10,12,30,34,78,102], especially *trans*-2-nonenal (T2N). These considerations caused us to limit our studies to (a) the non-specific

assay of staling sensitivity [19] and (b) the more specific measurement of *trans*-2-nonenal [103] as a staleness indicator. The latter analysis, however, which was kindly carried out by International Specialty Products Ltd., was prone to poor repeatability and so results were interpreted with caution. Neither of these methods distinguished untreated control beer from over-stabilized beer after force-ageing. These methods were, therefore, insensitive to the effects of there being gross differences in polyphenol contents and reducing capacities in these two beers. For this reason, it was not possible to discern any differences between the other samples (Table 2.13) that contained various additions of simple flavanols to stabilized beer.

The test with 2-thiobarbituric acid was published [78] as a method for assessing the effects of different agents that might promote or retard the sensitivity of beer to oxidation. It seems contradictory, therefore, that the addition (up to 30 mg L⁻¹) of several antioxidants, including a polyphenol, had no significant effect on the development of TBA-reactive material. It seems possible that the test, as it was applied here, marked mainly the generation of furfurals during thermal degradation of beer carbohydrates. For this reason, the results of the TBA test must be interpreted cautiously.

Although the amounts of T2N that were produced during force ageing were small and the precision of analysis was poor, differences should have been detected between T2N contents in PVPP-treated and untreated beer if polyphenols played a decisive role in T2N production, and this was not the case. Moreover, samples contained sufficient amounts of copper, headspace air and selected flavanol additions to test whether prodelphinidin B3 and procyanidin B3 performed convincingly in their proposed roles as prooxidant and antioxidant [34].

It must be restated, however, that whatever effects polyphenols might have on the flavour stability of the final product, it was predicted that this influence might be exerted most forcefully early in the brewing sequence, e.g., in the brewhouse. Accordingly, the partial removal of polyphenols from the final beer for colloidal stabilization might have much less serious consequences on flavour stability than had their removal from the unboiled wort [23].

2.4. CONCLUSIONS

The described analytical method for simple flavanol analysis lends itself well to application in brewery laboratories. The minimal sample preparation involved due to the direct-injection capability enables large numbers of samples to be processed in batch form. The sensitivity of the method is adequate for the determination of the levels of proanthocyanidins and catechins normally encountered in production beers.

Furthermore, the use of dual-electrode electrochemical detection allows increased confidence in sample component identification. Although a similar effect may be obtained using dual-wavelength monitoring or photodiode array detection, these techniques require a sample enrichment step in order to achieve the required sensitivity. Also, diode-array measurements cannot be used to predict accurately the degree of hydroxylation of flavanols within a particular class [50].

The versatility of this method is exemplified by its application to raw materials of brewing and to beers, and by its wide applicability in the studies of flavanols described in this chapter.

The semi-preparative method for the isolation of dimeric and trimeric proanthocyanidins using a commercially available high performance column which had been previously unexplored in this context provided an important addition to the analytical method described above. The ability to isolate dimeric and trimeric proanthocyanidins in order to calibrate their measurement in beer enables significant ease of application of the HPLC-ED analysis of beer flavanols, and is of particular interest for monitoring the removal of flavanol dimers and trimers from beer by stabilization with PVPP.

LH-20 has long been recognized as being ideally suited to the separation of flavanols, but the preparative method described in this chapter allowed a simplified separation of large amounts of the dimeric proanthocyanidins, without any requirement for further purification. This method was useful for the isolation of these compounds for addition to beer, but may also be of use to other researchers wishing to isolate large amounts of these compounds for further experiments of their own.

The studies on the adsorption of proanthocyanidins and catechins from beer by PVPP provided some interesting conclusions. The adsorption of simple flavanols

from beer was characterized by classical Freundlich isotherms over a much wider range of PVPP concentrations ($0-1,000 \text{ g hL}^{-1}$) than is typically used by the brewing industry (i.e. $0-50 \text{ g hL}^{-1}$). Polyclar 10 displayed almost twice the adsorptive capacity (k) for simple flavanols as did Polyclar Super R. Moreover, Polyclar 10 exhibited a slight preferential adsorption of dimeric proanthocyanidins in the presence of (+)-catechin, unlike Polyclar Super R. Neither of the adsorbents, however, showed any significant adsorption specificity for 3',4',5'-trihydroxy flavanols as compared with 3',4'-dihydroxy flavanols. Presumably, the high affinity of PVPP for the flavanoid moiety masked any possible discrimination between the extent of aromatic B-ring hydroxylation. As a consequence, treatment of beer with commercial PVPP will effectively decrease the contents of all of these haze precursors and is unlikely to materially alter the concentration ratio of the putative prooxidants to the antioxidants. Further investigation will be required to determine separately the effects of PVPP treatment on flavour stability.

These findings explain, enhance and expand upon the results obtained by Vancraenenbroeck *et al.* [37], which were too few to interpret by classical adsorption rationale. The results explain how the empirically determined relationship [18] between haze stabilization and variable PVPP dosage rate (which is always nonlinear) is due to the effects on specific haze precursors. Such operational certainties support a logical approach to procedural optimization based on judicious correction of product composition. Although this study was undertaken with the requirements of the brewing industry in mind and was confined to the examination of lager beer, the emergent principles should be more generally applicable to the removal of polyphenols from aqueous plant extracts in general.

The studies on the role of beer flavanols in determining the colloidal and flavour stability of the product indicated that treatment of lager beer with recommended dosages of PVPP effectively extended colloidal stabilities without compromising the flavour stability in any detectable manner. Although the partial removal of flavanoid polyphenols by adsorption onto PVPP did decrease the assay values for endogenous reducing capacity, the resulting beers did not appear to have been rendered more susceptible to oxidative flavour damage. While these results add further testimony to the complicity of polyphenols in colloidal instability [9], their assumed participation as major determinants of flavour instability is challenged.

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3. PHENOLIC OFF FLAVOURS (POF) IN BEER: A NEW ANALYTICAL METHOD AND ITS APPLICATION TO THE INVESTIGATION OF POF CONDITIONS IN BREWING.

3.1. INTRODUCTION

Having studied the role of flavanoid polyphenols in beer stability, it was felt appropriate to investigate the significance of some lower molecular weight phenolic compounds in relation to beer flavour and flavour instability.

Of the myriad of compounds responsible for the flavour of beer, phenols are not the most flavour active, especially when compared with some carbonyl or sulphur-containing compounds [1]. However, certain phenols, in particular 4-vinyl guaiacol (4-VG, Figure 3.1) and 4-vinyl phenol (4-VP) contribute part of the essential character to some beer types such as Weizenbier, Rauchbier and Altbier [2-4]. The flavour of 4-VG is described as clove-like or spicy [2,5,6] and has a threshold in beer of 0.2-0.4 mg L⁻¹. In some beverages 4-VG is appreciated whereas 4-VP is considered an off-flavour [3,7]. In most beers, however, both phenols are considered undesirable [5,6,8-11] and 4-VG is known to impart a rotten flavour to poorly stored orange juice [12]. The main influences on the formation of phenols in beer are the proportion of wheat malt in the grist, the mashing conditions, the wort boiling, the yeast strain, the fermentation procedure, and the presence of contaminants [13,14].

The primary sources of 4-VG and 4-VP in beer are known to be respectively, ferulic acid (*trans*-4-hydroxy-3-methoxycinnamic acid, Figure 3.1) and *p*-coumaric acid (*trans*-4-hydroxycinnamic acid). These phenolic acids are present at all stages during beer production, and are not very flavour-active [4,8]; the flavour threshold for ferulic acid was reported as 660 mg L⁻¹. By a variety of agencies, however, the hydroxycinnamic acids can be decarboxylated to form the corresponding phenols [14].

Usually, phenolic acids are bound covalently to macromolecular cell wall components [15,16]. Phenolic acids such as ferulic acid and *p*-coumaric acid are esterified to cell-wall polysaccharides [7,17], but are released on treatment with cold aqueous alkali [15,17]. Such hydroxycinnamic acids are thought to form ester links with arabinoxylans and they may also form ether links with proteins [18]. Hydroxybenzoic acids, such as vanillic and syringic acids, have long been recognized as major constituents of lignin [19]. Phenolic acids have been recovered from acetone-water extracts of unmalted barley in the form of glycosides or other conjugates, from which they were liberated for assay by either acidic or alkaline hydrolysis. Using such methods, the sum of the six most common phenolic acids in grains of brewing barley was estimated as 80-110 mg kg⁻¹ [20]. During barley germination, ferulic acid was released from the aleurone layers mainly as ester-linked feruloyl arabinoxylans, following the action of several endohydrolases [21]. In contrast, non-conjugated or 'free' species were relatively scarce, amounting to only 15 mg kg⁻¹ in unmalted barley [8], but contents doubled during malting and were then increased on mashing to the equivalent of about 200 mg per kg of dry grain. This release of phenolic acids from bound forms was confirmed during the production of worts for Bavarian Weizenbier, and was shown to depend on both pH and temperature [13]. Little is known about the esterases responsible for releasing ferulic acid from the limit arabinoxylans of barley but hydroxycinnamic acids were freed from their corresponding tartaric acid esters by a cinnamoyl esterase during wine-making [22].

Most of the information gained on the possible changes in the contents of phenols during the brewing cycle has been obtained in the context of wheat-beer production [4]. Conditions leading to the highest content of ferulic acid in the wort gave the highest contents of 4-VG and 4-VP in the young beer [13]. Accordingly, during mashing the liberation of ferulic acid was greatest at 43-45°C and at a pH of 5.8 [13]. In another study, some of the ferulic acid in the wort was decarboxylated during boiling [7]. Thermal decarboxylation of ferulic acid, with the formation of 4-VG, was also shown to occur during the roasting of barley [23].

The formation of 4-VG from ferulic acid by yeast activity is dependent on both temperature, being most rapid at 20°C, and on the strain of yeast [4]. Only ferulic acid that has been released from the pentosans is accessible to yeast for conversion to 4-VG [13]. The enzyme, ferulate decarboxylase (or cinnamate decarboxylase), which

transforms ferulic acid to 4-VG, is widely distributed amongst microorganisms, including species of *Saccharomyces* [2,3,24,25]. The constitutive cinnamate decarboxylase isolated from a strain of *Saccharomyces cerevisiae* was, however, incapable of decarboxylating hydroxybenzoic acids to phenols [2]. The capacity for decarboxylating hydroxycinnamic acids, known as Pof⁺ (for phenolic off-flavour), is due to a single dominant nuclear gene denoted as *POF1* [5,11,24,26]. Bottom-fermenting yeasts used in lager brewing usually lack this characteristic [27], so they are Pof⁻ phenotypes [24]. In contrast, many wild yeasts and top-fermenting yeasts are strikingly positive [4,6,9,11]. All the *Saccharomyces diastaticus* strains that were examined in one study were Pof⁺ [13,27].

Following fermentation, further changes in the contents of phenolics might be evident. It has been suggested that 4-VG formation may occur during prolonged lagering and after pasteurisation [28]. Moreover, the labile double bond of 4-VG is readily susceptible to oxidation, leading to the possible formation of other oxygenated products [29]. For instance, 4-VG and 4-VP decreased markedly during storage of Riesling wine as they were partly transformed into the corresponding ethoxyethyl phenols, with an accompanying diminution of the unpleasant off-flavour [22].

In several brands of Bavarian wheatbeer (Weizenbier) the contents of 4-VG varied from 0.2 - 4.3 mg L⁻¹ while 4-VP varied from 0.02 - 2.7 mg L⁻¹ [14]. The corresponding variations in the phenolic acids were 5.6 - 12.2 mg L⁻¹ and 0.2 - 1.5 mg L⁻¹ respectively for *trans*-ferulic acid and *p*-coumaric acid, while in Mexican beers the range 0.04 - 0.17 mg L⁻¹ was reported [11]. Earlier, it had been shown [8] that the contents of phenolic acids, including ferulic and *p*-coumaric acids, in Irish beers were significantly lower than in several others of different origin.

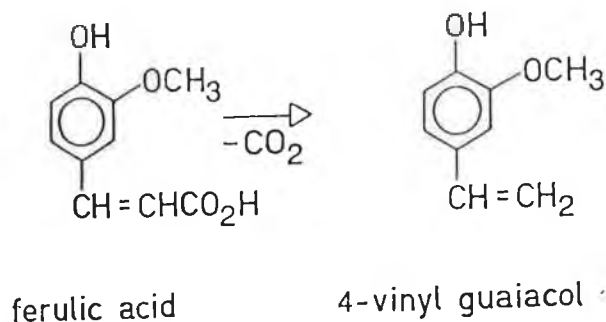


Figure 3.1. Decarboxylation of ferulic acid to 4-vinyl guaiacol.

In view of the variable perception of 4-VG in different beers, it is of both interest and importance for the brewer to be able to measure this compound and its phenolic acid precursor accurately at low levels. A limited number of analytical procedures for the determination of 4-VG have been reported [9,11,12,27,30-32]. Early work in this area mainly used gas chromatographic procedures [9,11,12,31], but these procedures involved lengthy sample preparation and/or derivatization steps, and there was some doubt as to the stability of the analytes on-column. More recently, there has been some interest in the use of high performance liquid chromatography (HPLC) for this analysis [12,30,32]. Wiesner and Franke [32] reported the determination of 4-VG and 4-VP in beer with ultraviolet absorbance detection and achieved a limit of detection of 50 ppb for 4-VG. Lee and Nagy [12] reported the analysis of 4-VG in stored orange juice using HPLC with fluorimetric detection. This method offered improved sensitivity with a limit of detection of 10 ppb for 4-vinyl guaiacol, but required a solid-phase extraction clean-up stage before the chromatographic analysis. Siegrist *et al.* [30] reported the determination of 4-VG, 4-ethyl guaiacol (4-EG), 4-VP, and 4-ethyl phenol (4-EP) in wine with limits of detection of 20-40 ppb. This method also involved a preliminary solid-phase

extraction step.

In this chapter, sensitive methods for the determination of 4-VG and its precursor, ferulic acid, in beer and wort using fluorimetric detection (for the analysis of 4-VG alone) and amperometric detection (for the simultaneous analysis of ferulic acid and 4-VG), are presented. Quantitative analysis was restricted to these two compounds, as they are the two examples of a phenolic acid and its decarboxylation product which are known generally to be present in the greatest quantities in beer [8]; also, these compounds are both available commercially as high purity calibration standards which permits accurate and repeatable analysis. The methods described are more sensitive than previously reported procedures, and they require minimal sample preparation. Furthermore, they are totally automated, allowing the unattended analysis of large batches of samples overnight.

These methods were applied to the monitoring of 4-VG production and ferulic acid utilization during fermentation, as well as to the quantification of these compounds in production beers and beers suspected of possessing phenolic off-flavours. Further studies of the methods revealed their broader applicability to the analysis of a wide range of phenolic compounds in beer, and also to the quantitative assignment of Pof phenotypes to test yeasts. Resulting from the development of these methods, detailed investigations into the control of ferulic acid and 4-VG throughout the brewing process were made possible. The results of these studies allowed the optimization of brewing raw materials and procedures for the production of specified levels of 4-VG in the final products.

3.2. EXPERIMENTAL

3.2.1. Reagents and materials

Guaiacol, 4-vinyl guaiacol, 4-ethyl guaiacol and 4-methyl guaiacol were supplied by Oxford Chemicals Ltd. (Brackley, Northants., U.K.). 4-vinyl phenol, which was not commercially available, was prepared in sufficient quantity to serve as a chromatographic marker by the enzymatic decarboxylation of *p*-coumaric acid using a culture of *S. diastaticus*, which is a highly Pof⁺ yeast, grown in defined medium. Ferulic acid, protocatechuic acid and cinnamic acid were supplied by Sigma Chemical Co. (Poole, Dorset, U.K.). 2,4-dimethoxycinnamic acid, 2,5-dimethoxycinnamic acid, 3,4-dimethoxycinnamic acid, 3,5-dimethoxycinnamic acid, gallic acid, *p*-hydroxybenzoic acid, syringic acid, vanillin, phenol, *o*-coumaric acid, salicylic acid and 3,5-dimethoxybenzoic acid were supplied by BDH Chemical Co. (Poole, Dorset, U.K.). Tyrosol and *p*-coumaric acid were supplied by Koch-Light Ltd. (Buckinghamshire, U.K.). All solvents were HPLC grade and all other chemicals were analytical reagent grade. Deionized water was prepared using an Elga Maxima purification system (Elga Ltd., High Wycombe, Buckinghamshire, U.K.).

3.2.2. Instrumentation and Conditions

Fluorescence spectra and wavelength optimization prescans were performed on a Perkin Elmer model LS-50B luminescence spectrometer (Perkin Elmer Ltd., Beaconsfield, England). HPLC was performed on a system consisting of a Waters Model 510 HPLC pump, a Waters Model 710B WISP automatic sample injector, a Waters Model 490 programmeable multiwavelength U.V. detector, a Waters Model 460 electrochemical detector, a Shimadzu Model RF-535 fluorescence HPLC monitor, a Shimadzu CR-3A integrator, and a Waters Maxima 820 data handling system. The Maxima data system was used to acquire data from the UV spectral and fluorescence detectors, and the output of the electrochemical detector was monitored using the integrator. Chromatographic separation was achieved using a 25-cm x 4-mm i.d. Nucleosil C₁₈ 10-μm column (Machery-Nagel, Düren, Germany) and a Waters Guard-

Pak guard column containing a disposable insert packed with Nova-Pak C_{18} silica. Depending on the application, three different instrument configurations were employed for different purposes:

1. For Method 1, i.e. the analysis of 4-VG alone, the mobile phase (mobile phase 1) consisted of $H_2O/CH_3OH/H_3PO_4$ (54:45:1 by vol.) pumped at a flow rate of 1 ml min^{-1} at room temperature. The sample injection volume was $25\text{ }\mu\text{l}$ and the run time was 25 min. Chromatograms (Figure 3.2) were obtained by fluorescence monitoring using an excitation wavelength of 260 nm and an emission wavelength of 340 nm. The fluorescence monitor was operated at maximum sensitivity and minimum range, with the response time set to 'fast'.

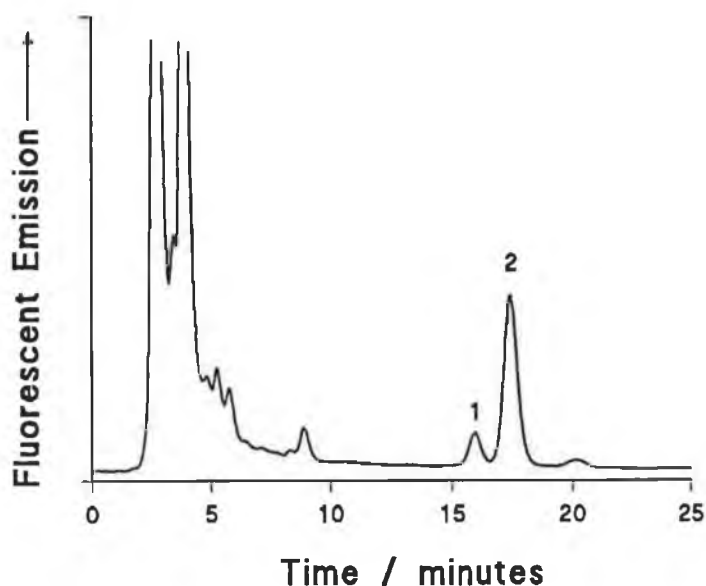


Figure 3.2. Chromatogram of a tainted beer containing 4-VG (0.6 mg L^{-1}). A filtered lager beer ($25\text{ }\mu\text{l}$) was injected onto a 25-cm C_{18} column and eluted with mobile phase 1. Fluorescent emission was monitored at 340 nm, using an excitation wavelength of 260 nm. Identified peaks: 1 = 4-VP, 2 = 4-VG.

2. For Method 2, i.e. the simultaneous analysis of 4-VG and ferulic acid in beer and wort, the mobile phase consisted of $H_2O/CH_3OH/H_3PO_4$ (64:35:1 by vol.)

pumped at a flow rate of 1.0 ml min^{-1} at room temperature. The injection volume was $25 \text{ }\mu\text{l}$, and the run time was 45 min. Measurement of both analytes (Figure 3.3) was performed by amperometric detection using the glassy carbon working electrode supplied with the Waters 460 detector, operated at a potential of $+0.90 \text{ V vs. Ag/AgCl}$. The output range of the detector was 20 nA .

3. The third instrument configuration was used for investigating other phenolic compounds in beer, and was as described in Method 2 above, except that the fluorescence and electrochemical detectors were preceded by the Waters 460 absorbance detector, which was operated at 280 nm and 1.0 AUFS . The run time was extended to 60 min when using this configuration.

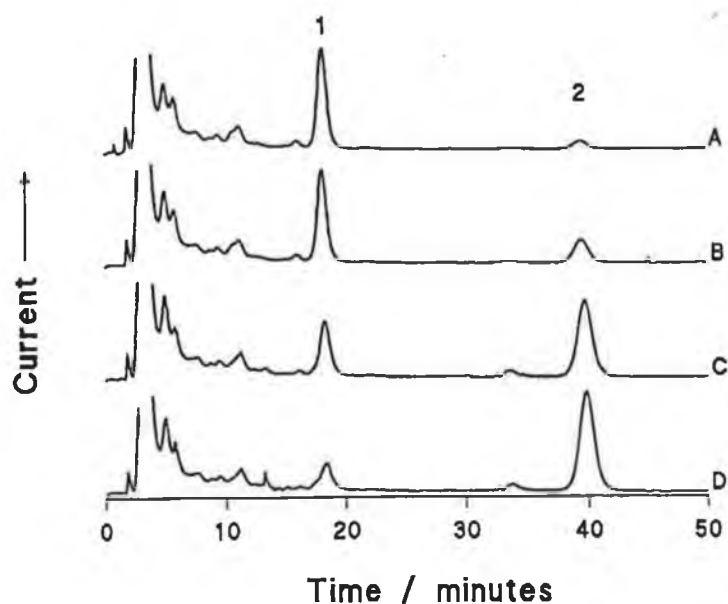


Figure 3.3. HPLC separation of ferulic acid (peak 1) and 4-VG (peak 2) in samples of fermenting lager wort initially containing added ferulic acid (5 mg L^{-1}). Wort was sampled at days 1, 5, 6, and 7 (traces A, B, C, and D, respectively) during fermentation with *S. diastaticus*. Samples were analysed by HPLC on a 25-cm C_{18} column eluted with mobile phase B at 1 ml min^{-1} . Electrochemical detection was achieved using a glassy carbon working electrode at a potential of $+0.9 \text{ V vs Ag/AgCl}$.

3.2.3. Sample preparation

Lagers and other pale beers were degassed by beaker transfer, followed by sonication for 10 min. Samples were then filtered through 0.22 μm alumina membrane filters (Anotop 10 Plus, Whatman U.K. Ltd., Maidstone, Kent, England) into autosampler vials. The first 1 ml of filtrate was discarded. The vials were maintained at 4°C until analysis. Aliquots (5 ml) of stouts, worts, and other cloudy samples were diluted to 10 ml with methanol, mixed well, brought back to 10 ml if necessary, and then centrifuged at 4,500 \times g for 15 min on a benchtop centrifuge at 2°C. The supernatants were retained and chilled until analysis.

3.2.4. Calibration

The 4-vinyl guaiacol standard was stored at -40°C when not in use. Standard solutions of 4-VG were prepared in the range 0.01 - 4.0 mg L⁻¹ in methanol, and these solutions were also stored at -40°C. Standard solutions of *trans*-ferulic acid were prepared in the range 0.1 - 4.0 mg L⁻¹. These solutions were prepared in subdued light, and were immediately transferred to blackened sample vials that were chilled until use. This was to prevent isomerization of the *trans*-ferulic acid to a *cis/trans*-mixture, which occurred rapidly in sunlight [33]. Standard solutions were analysed under the described chromatographic conditions.

3.3. RESULTS AND DISCUSSION

3.3.1. Choice of Detection System

For the analysis of 4-VG alone, Method 1 (Figure 3.2) with fluorescence monitoring was very convenient. This method exhibited high speed, sensitivity, and reliability and was the preferred routine method. This method was not suitable for the simultaneous analysis of 4-VG and ferulic acid, because the ferulic acid was insufficiently separated from fast-eluting interferences. Moreover, under the optimal fluorescence conditions for 4-VG, ferulic acid exhibited no detectable fluorescence. Fluorescence wavelength optimization prescans on methanolic solutions of ferulic acid showed the optimum excitation and emission wavelengths to be respectively 369 and 415 nm (compared with 259 and 341 nm for 4-VG). Optimum sensitivity for both analytes could have been obtained, therefore, only with programmed variations in fluorescence conditions, an option which was not available with the instrumentation used here. As an alternative, amperometric detection with a working electrode potential of +0.9 V vs. Ag/AgCl gave sufficiently sensitive simultaneous detection of both analytes, although the high sensitivity of the fluorescence detector for 4-VG was not matched.

Although not necessary for routine determinations, it was sometimes of interest to perform dual-detector monitoring. The advantage of such a system was the increased confidence in the identification of the 4-VG peak. Peak identity was confirmed by comparing the fluorescence and electrochemical detector peak area ratios obtained for 4-VG in samples and standard solutions.

3.3.2. Development of Chromatographic Conditions

The isocratic chromatographic conditions were developed either to achieve sufficient resolution of ferulic acid from the numerous earlier-eluting beer components, or for the elution of 4-VG as a sharp peak in the shortest possible analysis time. Not only were isocratic conditions more acceptable for rapid throughput of samples, as

compared with gradient elution, but isocratic elution is more compatible with electrochemical detection systems, allowing more stable baselines and rapid equilibration, particularly at high operating potentials such as those used in this study.

After the analysis of each sample queue (40-50 samples), the column was washed with water (50 ml) followed by methanol (100 ml). Although only minimal sample clean-up was performed, there was no detectable deterioration in column performance after the analysis of over 1,000 samples.

3.3.3. Method Performance

Method 1. Fluorescence detection (Figure 3.2) was linear in the range 0.01-5.0 mg L⁻¹ for 4-VG. Five injections of a standard solution containing 1.8 mg L⁻¹ of 4-VG gave a relative standard deviation (RSD) of 0.9% in peak area. The limit of detection for 4-VG (defined here as a signal to noise ratio of 2) was 0.002 mg L⁻¹, which is five times as sensitive as that reported previously [12].

Method 2. Amperometric detection (Figure 3.3) was linear in the range 0.05 - 1.0 mg L⁻¹ for 4-VG and 0.1 - 4.0 mg L⁻¹ for *trans*-ferulic acid. Five injections of a 0.5 mg L⁻¹ solution of 4-VG gave an RSD in peak area of 0.3%, and five injections of a standard solution containing 1.4 mg L⁻¹ *trans*-ferulic acid gave a peak area RSD of 1.1%. The limit of detection for *trans*-ferulic acid by amperometric detection was 0.02 mg L⁻¹, and for 4-VG the limit of detection was 0.01 mg L⁻¹.

The repeatability of the analysis of various beers using both methods was always better than RSD = 2%; the performance of the fluorescence detector being in general slightly better, probably as a result of gradual deterioration of the glassy carbon electrode through irreversible adsorption of sample components. The working electrode was dismantled and cleaned regularly with mild detergent and deionized water. Occasionally, freshly prepared chromic acid was used to refresh the electrode surface. The system was routinely recalibrated after cleaning.

3.3.4. Application of the Method to the Analysis of Other Phenolic Compounds in Beer

Quantitative analytical evaluations of beers in this study were restricted to the measurement of ferulic acid and 4-VG. However, it was of interest to examine the separation of a number of other available phenolic compounds known or suspected to be present in beers either naturally or as a result of experimental brewing procedures. Table 3.1 gives the elution order of the compounds studied using Method 1. The use of Method 2 did not change the elution order, but increased retention times approximately twofold, allowing the identification and analysis of all the phenolic acids that are known to occur in beer [8].

3.3.5. Investigation of Tainted Beers

Several hundred samples of stout, ale, and lager beer were screened for 4-VG by Method 1. Nearly all samples that had been described by tasters as having spicy or clovelike flavour notes contained elevated contents of 4-VG (0.2 - 1.4 mg L⁻¹). A number of different flavour thresholds have been reported for 4-VG [1,2], and the threshold is clearly dependent on the type of matrix in which the substrate is present. Evidence from studies carried out in Guinness Ireland Group suggests that the flavour threshold for 4-VG is about 0.2 mg L⁻¹ in lightly flavoured beers, whereas up to 0.3 mg L⁻¹ may not be detected in some stouts. A small peak that eluted shortly before 4-VG (Figures 3.2 and 3.3) was tentatively identified as 4-vinyl phenol (4-VP) on the basis of relative retention time [32], and also because it coeluted with the peak produced when a solution of *p*-coumaric acid was decarboxylated using a Pof⁺ yeast. This compound is known to occur in Weissbiers in lesser concentrations than the 4-VG present [14].

Table 3.1. HPLC Retention Times (t_R) of Various Phenolic Compounds Eluted with Mobile Phase 1.

Compound	t_R (min)
Gallic acid	3.1
Protocatechuic acid	3.6
Tyrosol	4.0
<i>p</i> -Hydroxybenzoic acid	4.7
Vanillic acid	4.8
Syringic acid	5.0
Vanillin	5.6
Phenol	6.0
Guaiacol	6.6
<i>p</i> -Coumaric acid	6.9
3,4-Dimethoxybenzoic acid	7.1
Ferulic acid	7.2
<i>m</i> -Coumaric acid	7.9
<i>o</i> -Coumaric acid	9.4
4-Methyl guaiacol	10.2
Salicylic acid	11.6
3,4-Dimethoxy cinnamic acid	12.7
4-Vinyl phenol	13.5
3,5-Dimethoxy benzoic acid	14.7
4-Vinyl guaiacol	14.8
Cinnamic acid	16.2
4-Ethyl guaiacol	17.8
2,5-Dimethoxy cinnamic acid	25.5
2,4-Dimethoxy cinnamic acid	28.6
3,5-Dimethoxycinnamic acid	29.0

3.3.6. Analysis of Packaged Production Beers

Table 3.2 shows typical levels of ferulic acid and 4-VG in beers available in Ireland, as well as a range of products sold in the U.S.A. Although a wide variation in ferulic acid concentrations ($0.5 - 7 \text{ mg L}^{-1}$) was observed, only the Weissbier samples contained levels of 4-VG above the flavour threshold. Surprisingly, the Weissbiers contained relatively low levels of ferulic acid, which suggests that the yeasts used in the production of these beers were selected for their efficient decarboxylating activity. Conversely, the very low levels of 4-VG in the other products indicated efficient exclusion of Pof^+ yeasts from the production process, as would be expected with well functioning modern brewery plant and brewing conditions.

3.3.7. Stability of 4-VG in Packaged Beer

Based on the results of a number of studies on the effect of warm storage on the 4-VG content of beer, it appeared that 4-VG underwent a temperature-dependent decay in packaged beer during storage, displaying a half-life of about 60 days at 18°C . This result was been confirmed for a number of beers containing a range of initial 4-VG concentrations. Figure 3.4 shows typical decay curves obtained for samples of a high gravity (18°P) stout containing 0.6 mg L^{-1} added 4-VG, which were subsequently stored at both 18°C and 37°C .

Table 3.2. Ferulic Acid and 4-Vinyl Guaiacol Concentrations in a Range of Beers.

Beer type	Ferulic Acid (mg L ⁻¹)	4-VG (mg L ⁻¹)
Irish Ale	0.5	0.04
Irish Lager	1.1	0.07
Irish Stout	0.9	0.04
Irish Weissbier	2.5	0.68
American Ale 1	6.6	0.01
American Ale 2	2.8	0.02
American Weissbier	0.7	0.57
American Lager 1	5.4	0.00
American Lager 2	4.8	0.05
American Lager 3	4.1	0.09
American Lager 4	3.7	0.00
American Lager 5	3.5	0.03
American Lager 6	3.4	0.03
American Lager 7	3.2	0.05
American Lager 8	2.9	0.03
American Lager 9	2.4	0.02
American Lager 10	2.0	0.04
American Lager 11	1.9	0.02
American Lager 12	1.6	0.02
American Lager 13	1.6	0.02
American Lager 14	1.5	0.00
American Lager 15	1.0	0.01

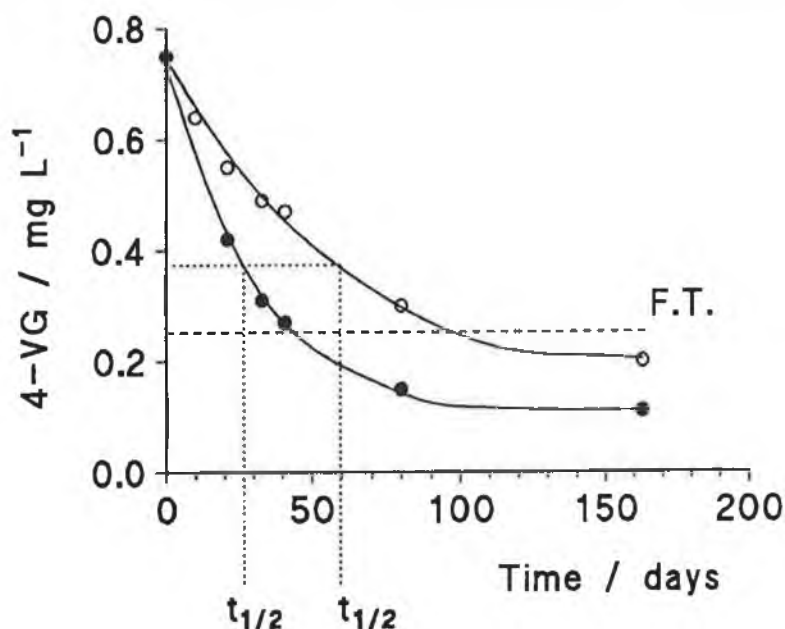


Figure 3.4. Losses of 4-VG from a high gravity stout containing 0.6 mg L^{-1} added 4-VG. ○ = storage at 18°C , ● = storage at 37°C . F.T. = Flavour Threshold, $t_{1/2}$ = Half-life of 4-VG.

Similar experiments were also performed with 0.3 mg L^{-1} of 4-VG added to the same stout and with an unspiked control. From Figure 3.4 it is evident that even with a highly elevated starting level of $>0.7 \text{ mg L}^{-1}$ of 4-VG, which was sufficient to impart a strong phenolic note that was totally unacceptable in stout, after 100 days storage at 18°C the contents had decreased below the flavour threshold to become 0.25 mg L^{-1} in this particular beer. Storage at 37°C caused an equivalent decrease in <50 days. Similar findings were obtained when lager beers that initially contained sub-threshold levels ($<0.2 \text{ mg L}^{-1}$) of 4-VG were stored at different temperatures ($4 - 37^\circ\text{C}$). This degradation of 4-VG, possibly to form 4-(1-ethoxyethyl) guaiacol [22], may be used to advantage when low levels of this phenol cause a slight flavour defect in young beers. Such a perceived flavour problem may, as has been found in several instances in Guinness Ireland Group, be self-correcting during subsequent storage.

A number of commercial Weissbiers which contained levels of 4-VG in the range $0.8 - 1.5 \text{ mg L}^{-1}$ were also examined. For these beers a similar temperature

dependent decay of 4-VG during storage was seen, which amounted to about 30% after 2 months, even at storage temperatures as low as 6°C. Clearly, inappropriate storage of such beers will result in losses of desirable flavours.

3.3.8. Measurement of the Decarboxylation of Ferulic Acid During Fermentation of Lager Wort

Samples (10 ml) of sterile hopped lager wort containing added ferulic acid (5 mg L⁻¹) were inoculated with *Saccharomyces diastaticus*, a Pof⁺ yeast strain, and were incubated in McCartney bottles under microaerophilic conditions at 15 - 16°C. Subsamples were taken daily and analysed for ferulic acid and 4-VG by Method 2 over a period of eight days. The results are shown in Table 3.3. The initial presence of 4-VG was probably due to thermal fragmentation of ferulic acid during wort production or sterilization. Over the period, a decrease of almost 70% of the initial ferulic acid concentration was observed. Although a net decrease of 3.8 mg L⁻¹ was observed, the corresponding increase in 4-VG was only 3.1 mg L⁻¹, instead of the stoichiometrically predicted 3.9 mg L⁻¹. This apparent discrepancy is explained first by the volatility of 4-VG, some of which was lost in the headspace of the fermentation, and secondly by the instability of 4-VG which was demonstrated in package and is likely to be even greater during fermentative conditions.

3.3.9. Specificity of Phenolic Decarboxylation by a Pof⁺ Yeast

It is well recognized that Pof⁺ strains of *Saccharomyces* are capable of non-oxidatively decarboxylating *p*-coumaric and ferulic acids, but not the corresponding benzoic acids [2]. This property is due to the presence in the cytoplasm of a constitutive cinnamate decarboxylase which is also stereospecific for *trans*-isomers. It has been reported [24] that the *POF1* decarboxylation system is specific for certain substituted cinnamic acids, yet paradoxically, does not require substrates to have a particular substitution pattern in the benzene ring. A number of phenolic substrates were used to test the decarboxylating activity of whole cells [24] with a view to

further examining the nature of this specificity.

S. bayanus var. *saccardo*, the most actively Pof⁺ yeast in the Guinness Ireland Group collection was used to attempt decarboxylation of the substrates shown in Table 3.3. The substrates and their reaction products were analysed using method 3 (section 3.2.2). The retention times of the substrates are given in Table 3.1, and the susceptibility of these compounds to degradation during incubation are given in Table 3.3. After fermentation with *S. bayanus*, ferulic acid and *p*-coumaric acid were greatly depleted as expected [2], and their products were detected by all three detection systems. The decarboxylation product of ferulic acid (i.e. 4-VG) had a strong spicy/clovelike aroma, while the product from *p*-coumaric acid (i.e. 4-VP) had a medicinal/phenolic aroma. Cinnamic acid was completely removed by fermentation, and although the culture had a strong solvent-like smell of styrene, this product was not detectable under the described HPLC conditions. Both 2,4-dimethoxycinnamic acid and 3,4-dimethoxycinnamic acid were significantly depleted by fermentation, yielding products that were readily detectable by both their fluorescence and UV absorbance, and also by their smoky aromas. In contrast, the concentrations of neither 2,5-dimethoxycinnamic acid nor 3,5-dimethoxycinnamic acid were decreased during incubation.

Contrary to the findings of some authors [2], but in agreement with others [24], it was found with one Pof⁺ yeast, that neither hydroxy nor methoxy substitution of the aromatic ring are requirements for decarboxylation, since cinnamic acid itself was readily transformed. From this it seems that the *POF1* gene encodes for a versatile cinnamate decarboxylase rather than a hydroxycinnamate decarboxylase. Certain substitution patterns, i.e. 2,5- and 3,5-, appeared to be totally resistant to decarboxylation by the cells, though it has yet to be ascertained whether this was due to the failure of these compounds to cross the cell membrane or to their resistance to the decarboxylase. One possibility is that the enzyme requires two adjacent unsubstituted aromatic carbon positions adjacent to the carboxylic acid-substituted position, and hence 2,5- and 3,5- substitution patterns resulted in spatial inhibition of enzyme function.

Table 3.3. Susceptibility of Phenolic and Substituted Cinnamic Acids to Degradation During Incubation with *S. bayanus*

Acid	Depletion of substrate (%)	Product(s) detectable by*:		
		UV	ED	FL
Ferulic acid	74	YES	YES	YES
Cinnamic acid	100	NO	NO	NO
<i>p</i> -Coumaric acid	80	YES	YES	YES
2,4-DMCA ⁺	75	YES	NO	YES
2,5-DMCA	0	NO	NO	NO
3,4-DMCA	98	YES	NO	YES
3,5-DMCA	0	NO	NO	NO

* UV = Ultraviolet spectral detection; ED = Electrochemical detection; FL = Fluorescence detection.

⁺ DMCA = Dimethoxycinnamic acid.

3.3.10 Control of Ferulic Acid and 4-Vinyl Guaiacol in Brewing

The most important applications of the methods described were in the investigation of the brewing conditions most likely to produce beers that were free from phenolic off-flavours. The high sensitivity of the methods, combined with the fact that simultaneous analysis of precursor and product was possible, gave an ease of application that was hitherto unavailable. A number of practical brewing experiments were carried out in the GBW Research Centre and the conclusions, based on the analytical results obtained, were as follows.

- a) The extraction of free ferulic acid from both stout and lager malts by water was temperature dependent, having an optimum of approx. 45°C. Extractability was very poor above 60°C, suggesting an enzyme-catalysed reaction which was inhibited at high temperature. Extraction of free ferulic acid from steamed flaked barley was poor, and was unaffected by temperature, suggesting that the steaming process denatured any enzymes present initially in the barley.
- b) Mashing programmes which started at 65°C produced significantly less ferulic acid in the wort than programmes which commenced with 45°C stages, due to inactivation of the decarboxylase enzymes at the higher temperature. However, high-temperature mashing alone did not necessarily suppress ferulic acid extraction sufficiently to preclude the formation of phenolic off-flavours in the final beer if a Pof⁺ yeast was used in the fermentation.
- c) Significant quantities of 4-VG were produced during wort boiling, the amounts produced being related to the amounts of ferulic acid extracted during mashing. Worts containing >6 mg L⁻¹ ferulic acid at the start of boiling produced 4-VG concentrations above the flavour threshold in the green beer.
- d) The most significant factor in the production of phenolic off-flavours in beer was the yeast chosen. Pof⁺ yeasts produced strong off-flavours from even very low ferulic acid concentrations. Most worts contained sufficient free ferulic acid to allow the production of phenolic off-flavours if a sufficiently Pof⁺ yeast was used in the fermentation. Conversely, if a Pof⁻ yeast was used, there was very little probability of the development of off flavours, even in the presence of high ferulic acid concentrations.

3.3.11 Detection of the Pof Phenotype in Test Yeasts

The original method for detection of Pof⁺ yeasts described by Thurston [10] involved the addition of ferulic acid or *p*-coumaric acid to sterile wort, followed by

inoculation of the wort with the test yeast. Following fermentation in a closed container for 24 hr, a strong spicy or clovelike smell indicated the Pof⁺ phenotype in the test yeast. A large number of similar tests were carried out in the GBW research centre on lager wort supplemented with 100 mg L⁻¹ ferulic acid. Analysis of the incubated solutions for 4-VG gave a confirmatory result, and provided some quantitative measure of the degree of expression of the Pof phenotype. Following the testing of a wide variety of yeasts, it became evident that the results generally fell into three categories: in the first category, designated Pof⁻, 4-VG levels following incubation were always less than 0.05 mg L⁻¹; yeasts from the second category, designated Pof^{+/-}, produced 0.06-2.5 mg L⁻¹ 4-VG, indicating some slight decarboxylase activity whereas yeasts from the third category, designated Pof⁺, produced levels above 2.5 mg L⁻¹ of 4-VG, with typical examples lying in the 10-20 mg L⁻¹ range. Table 3.4 shows the results obtained with some typical test yeasts. Of 59 isolated strains tested, 28 were Pof⁻ and this group included all of the Guinness Brewing yeasts. 14 contaminant strains tested were found to be Pof⁺, and could therefore cause significant phenolic off-flavour problems if they were allowed to contaminate a brewery fermentation. Of the remaining 17 samples which were designated Pof^{+/-}, six were used in wheat beer production, four were ale brewing yeasts, two were wine yeasts, one was a distillery yeast and the remainder were isolates of wild yeast contaminants.

The ability to quantify the expression of the Pof phenotype quite obviously provides advantages in the selection of yeasts for the production of different products. In particular, Pof^{+/-} yeasts can be chosen on the basis of this and similar tests for the production of particular beers in which a phenolic flavour-note is desirable such as Weissbiers or other speciality beers.

Table 3.4. Assignment of the Pof Phenotype to Members of the Genus
Saccharomyces

Code*	Classification	4-VG in screening test (mg L ⁻¹)	Designated Phenotype
C1	<i>S. bayanus</i>	25.8	Pof ⁺
C2	<i>S. diastaticus</i>	20.8	Pof ⁺
C3	<i>S. diastaticus</i>	11.0	Pof ⁺
C4	<i>S. cerevisiae</i>	9.8	Pof ⁺
TFW1	<i>S. cerevisiae</i>	2.33	Pof ^{+/-}
TFW2	<i>S. cerevisiae</i>	1.40	Pof ^{+/-}
TFW3	<i>S. cerevisiae</i>	0.80	Pof ^{+/-}
TFW4	<i>S. cerevisiae</i>	0.13	Pof ^{+/-}
D1	<i>S. diastaticus</i>	1.14	Pof ^{+/-}
TF1	<i>S. cerevisiae</i>	1.39	Pof ^{+/-}
TF2	<i>S. cerevisiae</i>	1.14	Pof ^{+/-}
TF3	<i>S. cerevisiae</i>	0.01	Pof ⁻
TF4	<i>S. cerevisiae</i>	0.01	Pof ⁻
BF1	<i>S. uvarum</i>	0.02	Pof ⁻
BF2	<i>S. uvarum</i>	0.01	Pof ⁺

*C = Wild yeast contaminant

TF = Top fermenting ale yeast

BF = Bottom fermenting lager yeast

TFW = Top fermenting yeast used in Weissbier production

D = Distillery yeast

3.4. CONCLUSIONS

Method 1, with fluorescence detection, was a suitable method for the routine analysis of production beers for 4-VG. Besides requiring minimal sample preparation, this method is totally automated, and is therefore capable of processing over 50 samples in 24 hr.

Method 2, using electrochemical detection, was designed for simultaneous analysis of 4-VG and its precursor, ferulic acid. This method can be applied to the investigation of the decarboxylation of ferulic acid to 4-VG and can indicate the potential production of 4-VG in worts and beers. Another use is in the screening of yeast cultures for the phenolic off-flavour (Pof) character. To the best of my knowledge, this is the first instance in which electrochemical detection has been applied to the simultaneous analysis of ferulic acid and 4-VG in beer.

The application of these direct-injection methods has led to significant advancement of current knowledge in the area of POF conditions in brewing. The simplicity of sample preparation afforded by the use of selective detection systems allowed large numbers of samples to be processed in a short time, with no associated loss of sample integrity due to the sample preparation procedures. This permitted detailed investigations to be carried out into the sources of POF conditions, and the control of these conditions in practical brewing.

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4. APPLICATION OF ION CHROMATOGRAPHY TO THE ANALYSIS OF OXALATE IN BEER AND BEER SEDIMENTS.

4.1. INTRODUCTION

The next area in which research was carried out was that of oxalate sediments and hazes in beer. Most beers are known to contain oxalate, either as an ionic species in solution, or as its insoluble calcium salt. A number of studies have been undertaken on oxalate levels in beer [1-3], and typically reported levels were in the range 6-27 mg L⁻¹ [1]. The primary source of oxalate in beer is barley malt, which may contain up to 600 mg kg⁻¹ oxalate. Secondary sources include hops (up to 4,000 mg kg⁻¹) and brewer's yeast (500-800 mg kg⁻¹) [4]. Toxicologically, the oxalate content of beers is insignificant, and the sensory impact is not noticeable at typical levels. The presence of oxalate in beer has, however, been implicated in beer gushing [5], and the presence of calcium and oxalate ions in beer may cause the formation of an insoluble white calcium oxalate precipitate or haze [5]. Alternatively, a stringy precipitate containing polyphenol, protein, β -glucan, dextrins and calcium oxalate can result, especially when the beer is inadvertently frozen and rethawed before consumption [6].

A method commonly used by brewers to decrease the oxalate content in beer is to add gypsum (calcium sulphate dihydrate) during brewing [7,8], either to the brewing water at the mashing stage, or during wort boiling. The dissolved calcium ions combine with anionic oxalate, producing a virtually insoluble calcium oxalate precipitate, which may be removed by settling, centrifugation or filtration before product packaging.

The measurement of oxalate levels is therefore important in determining the effect of gypsum addition on the formation of oxalate sediment during brewing and in packaged beers. To monitor the effect of gypsum addition, a method is required to determine oxalate levels in solution in beers and worts. The analysis of sediments, on the other hand, requires a method for analysis of insoluble constituents after

isolation of the precipitate. The simplest method of examination of isolated sediments is microscopy, for which there are many examples in the literature [4,6,9]. There are several methods for the determination of oxalate in beer solution involving enzymatic [1,2,6], colorimetric [10], or titrimetric [4] techniques. Recently, an ion chromatographic method was published for the determination of oxalate in the presence of other organic acids and inorganic anions [11]. This method is, however, lengthy to perform and requires the use of gradient ion chromatography.

In this chapter, a rapid isocratic ion chromatographic method for the determination of oxalate in the presence of inorganic anions (chloride, nitrate, phosphate and sulphate) based on that reported previously by Jancar *et al.* [12], which is equally applicable to the direct measurement of oxalate in beer and in oxalate-containing extracts of beer sediments, is described. The application of this method to the investigation of a number of beers and sediments, as well as the study of the fate of oxalate in the brewing process, is also detailed.

4.2 EXPERIMENTAL

4.2.1. Reagents and Materials

All reagents were of analytical reagent grade. Deionized water, prepared using an Elga Maxima purification unit (Elga Ltd., High Wycombe, Bucks, England), was used for all experiments. Alumina membrane filters (Anotop 10 plus 0.22 μm) were obtained from Whatman Scientific Ltd., Maidstone, Kent, England.

4.2.2. Instrumentation and Conditions

Separations were performed on a Dionex 2000i ion chromatograph (Dionex U.K. Ltd., Camberley, England), equipped with an HPIC-AG4 guard column, HPIC-AS4 separator column, and an anion micro-membrane suppressor. The eluent used was 0.0021 *M* NaHCO_3 /0.0017 *M* Na_2CO_3 , and the regenerant solution was 0.0125 *M* H_2SO_4 . The sample loop size was 50 μl , and the eluent flow rate was 1.8 ml min^{-1} . The conductivity detector was operated at a sensitivity of 10 μS full-scale deflection, with a 1-V analog output and a temperature compensation setting of 1.7. Samples were injected automatically using a Dionex Automated Sampling Module (ASM).

Data acquisition and processing were performed with a Waters Maxima 820 data-handling system (Waters Chromatography Ltd., Hertfordshire, England). This system was interfaced with the Dionex ASM using a Dionex Automation Interface Box (part no. 000143).

Calcium was determined by atomic absorption spectroscopy using an Instrumentation Laboratory (Wilmington, MA, USA) model IL 257 spectrophotometer with an air-acetylene flame and a calcium hollow cathode lamp operated at a current of 5 mA.

4.2.3. Procedures

Beer and wort samples were agitated to resuspend any sediment and were degassed by sonication. Aliquots (25 ml) were centrifuged in duplicate at 10,000 \times

g for 30 min in a Sorvall RC-5B centrifuge at ambient temperature. The supernatants were retained for analysis of soluble oxalate and calcium. The centrifuge tubes were inverted and allowed to stand for about 10 min to allow complete drainage of supernatant. The precipitates were not washed, to avoid losses of calcium oxalate. To each tube was added 2.5 ml of 0.1 M HCl, and the tubes were placed in an ultrasonic bath for 15 min to promote extraction of the calcium oxalate. The tubes were then recentrifuged at 10,000 x g for 10 min, and the supernatants were retained for analysis of calcium and oxalate extracted from the sediment.

Centrifuged beer and wort samples were diluted fivefold with deionized water and were filtered through 0.22- μ m membrane filters before analysis. Hydrochloric acid extracts of beer and wort sediments were filtered through 0.22- μ m filters and injected directly.

Calcium analysis of beers and beer extracts was carried out by flame atomic absorption spectroscopy. Samples were diluted to a calcium concentration range of 1-2 mgL⁻¹, and La₂O₃ (1,000 mg L⁻¹) was added to prevent phosphate masking of calcium ions.

4.3. RESULTS AND DISCUSSION

4.3.1. Method Performance

The ion chromatographic assay was linear in the range 0 - 40 mg L⁻¹ oxalate in standard solutions, and linearity was also observed in beers spiked with oxalate at these levels. Five replicate injections of a 10 mg L⁻¹ standard solution of oxalate gave a relative standard deviation (RSD) of 1.9% in peak area. Five replicate injections of a diluted beer sample gave an RSD of 2.4%.

Insoluble oxalate was measured in four different beer samples, and each sample was prepared and analysed in quadruplicate. The RSD of the analyses ranged from 2.7 to 9.0%, with a mean value of 5.9%. Insoluble oxalate levels in these samples ranged from 0.13 to 2.86 mg L⁻¹ with a mean of 0.82 mg L⁻¹.

Figure 4.1 shows a typical chromatogram of direct injection of 50 µl of a diluted beer sample, and Figure 4.2 shows a chromatogram of direct injection of 50 µl of a hydrochloric acid extract of a beer precipitate. The large peak at the beginning of this latter chromatogram is caused by chloride ions, which are present in excess, but which do not affect resolution of the oxalate peak, although a slight difference in the retention time of oxalate was evident between the two chromatograms (Figures 4.1 and 4.2).

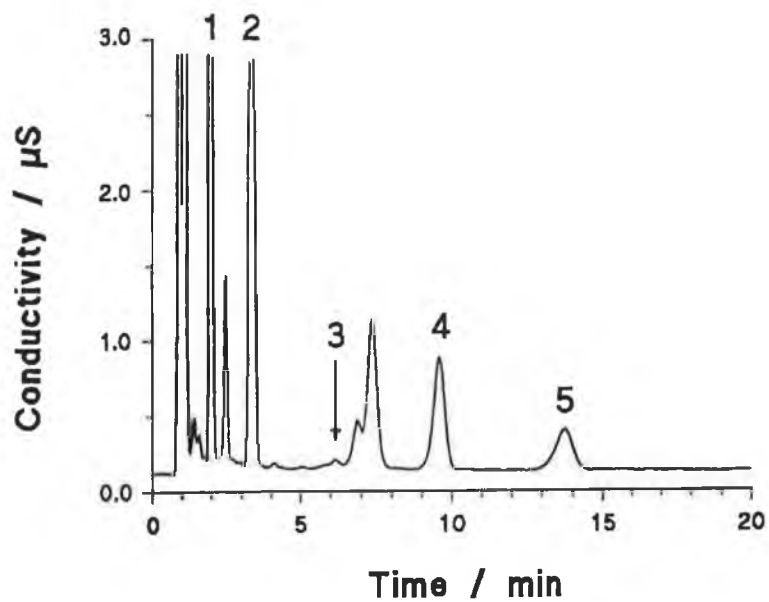


Figure 4.1. Ion chromatographic analysis of anionic species in beer. Identified peaks: 1 = chloride, 2 = phosphate, 3 = nitrate, 4 = sulphate, 5 = oxalate.

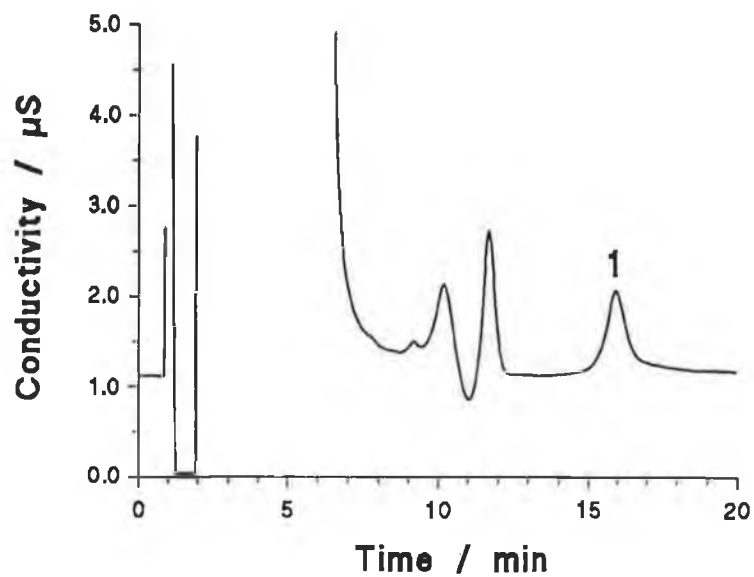


Figure 4.2. Ion chromatographic analysis of oxalate in beer sediment. Identified peak: 1 = oxalate.

4.3.2. Identification of Calcium Oxalate in Beer Sediments

Settled Sediments: The particulate elements which were quickest to settle from samples of commercial beers were usually identifiable as mainly calcium oxalate. Such particles settled rapidly from shaken samples and could be recovered as a thin white film on the base of the container after 2 to 4 hours of undisturbed standing. Samples collected in this way were usually free from major contamination with other microscopic particles. A wide range of both the morphology and size of particles recognisable as "oxalate sediment" [9] were found in different samples. Apart from the typical octahedral crystals, both needle-shaped and barrel-shaped crystals were common, as well as were rosette and amorphous forms [5]. Sediments recovered from beer by settling were usually almost totally soluble in dilute HCl. Analysis of the acidic extracts revealed calcium and oxalate to be present in almost equimolar quantities.

Total Sediments: Microscopy of total sediments recovered from samples by centrifugation revealed heterogeneous mixtures of oxalate crystals (recognisable as octahedral, barrel and rosette-shaped [5,9]), protein particles and nonviable microorganisms. Treatment with dilute acid dissolved only the calcium oxalate, leaving the microorganisms and protein particles unaffected. When these acid extracts of total sediments were analysed, a slight excess of calcium to oxalate molar equivalency was usually observed, due probably to the presence of other calcium salts in the beer. In several consumer complaint samples that contained sediments readily visible to the naked eye, the insoluble calcium oxalate accounted for almost 25% of the total oxalate content of the beer, indicating that significant precipitation had occurred in package. In several such cases, the morphology of the sediments was not that of typical octahedral crystals of calcium oxalate. Instead, the sediments were composed of barrel-shaped or granular precipitates agglomerated with protein and carbohydrate to form long (1-2 mm) threads or needles. It is believed that these sediments may be formed by slow accretion at the bases of bottles and cans, and that, in extreme cases, these sediments could be misidentified as insect parts by the consumer.

A random selection of 57 samples of sweet worts, hopped worts and beers from a range of production brews were then analysed for insoluble calcium and oxalate levels with a view to determining whether the two ions were present in the correct theoretical molar proportions of calcium oxalate. Figure 4.3 shows the plot of insoluble oxalate vs. insoluble calcium contents.

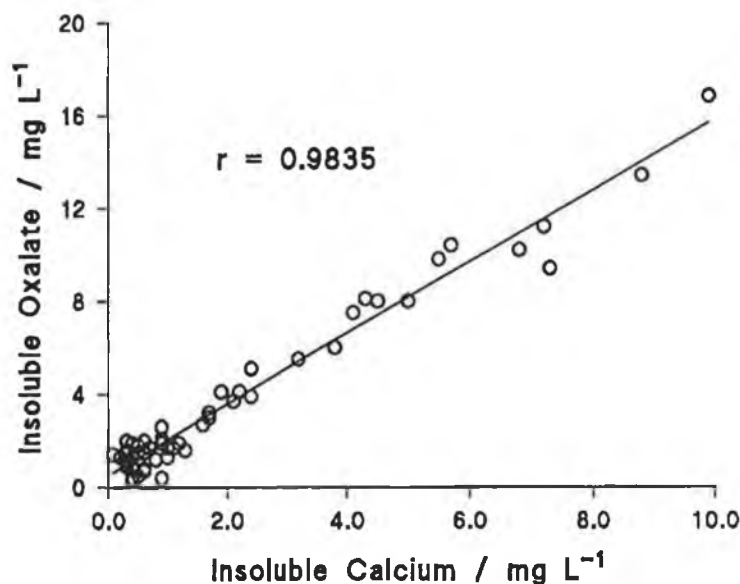


Figure 4.3. Relationship between insoluble calcium and insoluble oxalate contents of a range of stout beers and worts.

There was a strong correlation between levels of insoluble oxalate and calcium ($r = 0.9835$), but the slope of the line (i.e. 1.53) was lower than the theoretical mass ratio of calcium oxalate (i.e. 2.20), indicating that other calcium salts were probably present in the sediment, or that there was calcium present in bound forms which, although detectable by atomic absorption, was not available for reaction with oxalate ions.

4.3.3. Fate of Oxalate During the Brewing Process

As one example of the application of the analytical method, changes in the concentrations of oxalates were followed during an experimental high-gravity (15°P) stout brew. Samples were drawn at various stages and analysed for soluble and insoluble ions. The results are shown in Table 4.1.

Table 4.1. Fate of Oxalate During the Brewing Process (Gypsum-free Brew)

Sampling Stage	Oxalate, mg L ⁻¹			Soluble calcium (mg L ⁻¹)
	Sol.*	Insol.	Total	
Kettle at filling	47.1	2.6	49.7	26
Kettle after hopping	48.6	5.9	54.5	24
Kettle at strike-off	51.6	6.7	58.3	28
Fermented Wort	55.2	3.7	58.9	23
Centrifuged Beer	51.4	3.7	55.1	26
Diluted to trade gravity	29.3	3.0	32.2	27
Polished Bright Beer	25.5	0.6	26.1	26

*Sol. = Soluble; Insol. = Insoluble

This brew was performed without gypsum addition and therefore the mean calcium level of 26 mg L⁻¹ was low enough to permit high soluble oxalate levels during all stages of brewing. The boiling time was 90 min, and the wort was hopped at the start of boiling. The oxalate content of 49.7 mg L⁻¹ at filling increased to 58.3 mg L⁻¹ at the end of boiling, with the proportion of insoluble oxalate rising from 5 to 11%. The increase in total oxalate may be attributed mainly to the 10% volume reduction effected during boiling, but also due to some extraction from the hops. The

content of insoluble oxalate in the cooled worts was highest in samples taken at strike-off, in keeping with the highest concentration of total oxalate. These results indicated that most of the oxalate present in the wort originated from the malt, and that the contributions from the hops were minor. Analysis of the fermented and clarified beers showed that fermentation had little effect on the total oxalate content of the beer, although the ratio of soluble to insoluble oxalate increased. This may indicate the formation of small amounts of oxalate by growing yeast cells [4], coupled with a removal of some insoluble oxalate attached to yeast cells. Centrifugation decreased the total amount of oxalate by 3.8 mg L^{-1} , almost exactly equal to the amount of insoluble oxalate in the unclarified beer. The presence of insoluble oxalate in a beer sample taken after centrifugation suggested that the equilibrium of soluble to insoluble oxalate was restored quickly before analysis took place.

Dilution of the beer to trade gravity resulted in a predictable decrease in oxalate concentration, and subsequent polishing centrifugation achieved a very significant removal of insoluble oxalate, decreasing its concentration from 3.0 to 0.6 mg L^{-1} . No significant change in the calcium concentration occurred during dilution because the dilution water contained a calcium level similar to that present in the undiluted beer. Clearly, the final concentrations of oxalate and calcium in the beer were determined by the equilibrium conditions established during wort boiling. The final concentration of soluble oxalate in the beer of 25.5 mg L^{-1} was sufficient to predetermine the propensity of this beer to precipitation of calcium oxalate crystals during long-term storage [5]. From the work carried out here, and in the general experience of staff at Guinness Ireland Group, the elimination of oxalate sediment formation on storage requires the reduction of oxalate levels at packaging to about 15 mg L^{-1} .

4.3.4. Relationship Between Calcium and Oxalate Ions in Solution

To examine the relationship between calcium and oxalate ions in solution in polished stout beer, a range of 41 gypsum-treated and non gypsum-treated high-gravity brews (15°P) were analysed. Using the pooled results, a plot of oxalate vs. calcium

ions in solution was prepared (see Figure 4.4). From this plot, there was a clear negative correlation between calcium and oxalate ions in beer. Furthermore, minimum oxalate concentrations (15-20 mg L⁻¹) were achieved with a calcium concentration of about 90 mg L⁻¹, whereas higher calcium concentrations appeared not to decrease the oxalate content below about 15 mg L⁻¹. Dilution of beers containing 80-100 mg L⁻¹ calcium to trade gravity resulted in oxalate contents between 10 and 15 mg L⁻¹, which appeared sufficient to forestall subsequent precipitation of calcium oxalate crystals from the packaged beer. Burger *et al.* [5] found beers with oxalate at less than 14 mg L⁻¹ to be stable with respect to sediment formation and gushing potential.

The observed calcium-oxalate relationship supports previously published observations [5,7,8]. Briggs *et al.* [7] recommended the addition of gypsum to the brewing water to a calcium concentration of 50 mg L⁻¹, and a second similar addition to the copper, to achieve a final calcium concentration in the beer of 60-80 mg L⁻¹. Harrison *et al.* [8] recommended a level of 80-100 mg L⁻¹ calcium in wort, leading to a final calcium content of 60-80 mg L⁻¹ in the beer. The analysis of worts from gypsum-treated brews showed that the effect of calcium addition was to produce large amounts of insoluble oxalate (up to 40% of the total oxalate present) in the cooled hopped wort. This precipitate was removed from the fermented beer with the yeast by centrifugation.

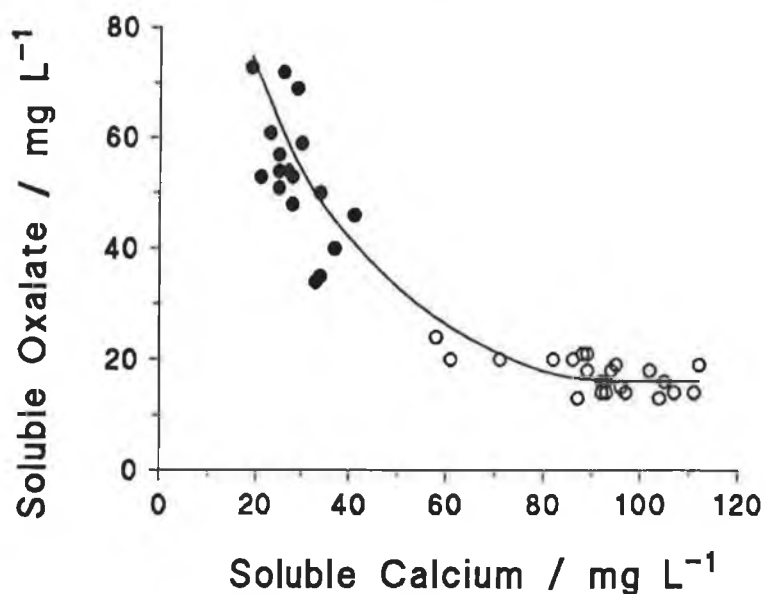


Figure 4.4. Relationship between soluble oxalate and soluble calcium in a range of high-gravity (15°P) stout beers after centrifugation (○ = gypsum brews, ● = non-gypsum brews.)

4.3.5. Relationship between Oxalate and Sulphate Ions in Solution

Sulphate was measured in twenty-five of the beers referred to in section 4.4.4, and the relationship shown in Figure 4.5. was observed. Although there is strictly no theoretical relationship between the amounts of oxalate and sulphate in solution, in the case of these particular beers there was a clear distinction between those which had been treated with gypsum, and therefore had elevated soluble sulphate levels, and those which were untreated, and therefore contained normal sulphate levels, and high concentrations of soluble oxalate. Although sulphate concentration may vary considerably from batch to batch in brewing water, the very obvious clustering of samples about high or low sulphate concentrations may be used as a rapid test to identify beers which have received gypsum treatment. The advantage of such a test is that it can be carried out simultaneously with oxalate analysis, thereby eliminating the necessity of atomic absorption analysis to identify such beers.

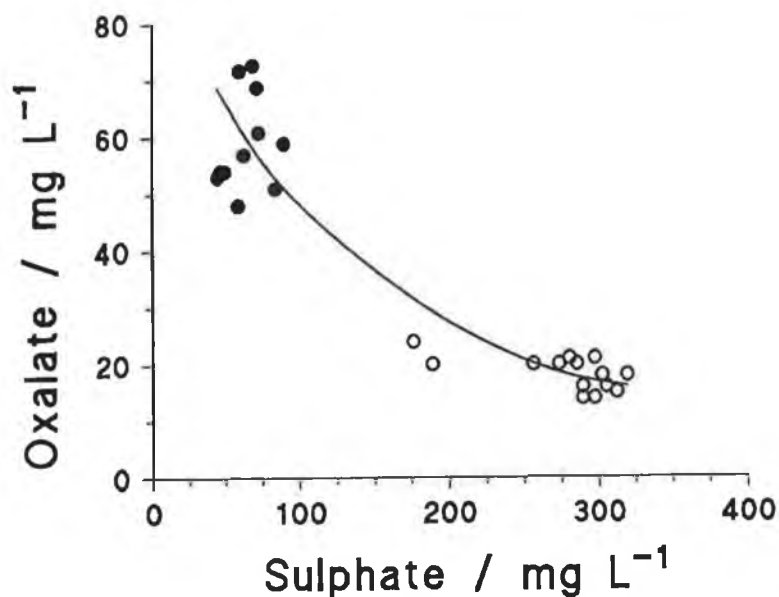


Figure 4.5. Relationship between oxalate and sulphate ions in solution in beers with (○) and without (●) gypsum treatment.

4.4. CONCLUSIONS

The procedures described in this chapter may easily be adopted as routine quality assurance methods in the brewery and used to monitor typical oxalate levels in production beers. The analysis of beer sediments is valuable in the assessment of consumer complaints involving beer hazes and precipitates. The use of the techniques described here is both quicker and more accurate than is the use of titrimetric and/or enzymatic methods. The fact that the method is fully automated makes its use as a quality assurance method attractive; under the conditions described here, over 75 samples may be processed in a 24-hr period.

The use of selective separation and detection systems, coupled with the high physical stability of the polymeric ion-exchange column used, enabled beers and acidic extracts of beer sediments to be analysed by a direct-injection technique, thereby decreasing manual input while also maintaining sample integrity. Development of fast methods such as that described, coupled with simplified sample preparation due to the

selectivity of the system, has enabled detailed studies to be carried out, which would have been impossible without this improved methodology.

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5. ANALYSIS OF CARBOHYDRATES IN BEERS, WORTS AND NON-ALCOHOLIC BEVERAGES USING ION CHROMATOGRAPHY WITH PULSED ELECTROCHEMICAL DETECTION.

5.1. INTRODUCTION

The course of wort fermentation is usually monitored by changes in specific gravity [1]. Concomitant changes in total carbohydrates can be estimated colorimetrically if required [2-4], but most brewers do not routinely monitor changes in individual carbohydrates. The measurement of fermentable carbohydrates (glucose, fructose, maltose and maltotriose) may, however, be valuable for certain purposes. For instance, the metabolic characteristics of novel yeast strains, as well as the fermentability of worts produced either from novel raw materials (e.g., maize and sorghum) or by new brewing procedures (e.g., high-gravity brewing), can be ascertained. The use of brewing adjuncts, alternative carbohydrate sources, and high-gravity brewing is currently increasing, and it is important, therefore, to make available reliable and versatile methods for the measurement of individual carbohydrates.

High performance liquid chromatography (HPLC) is the method of choice at present for carbohydrate analysis in complex matrices [3-11]. It allows selective and sensitive determinations, high accuracy and precision, and the option of automation. Most carbohydrates are polar compounds that elute too quickly from reversed-phase columns for usable resolution. For this reason, the most common methods used for carbohydrate separation use either ion-exchange resins [4,7] or aminopropyl silica columns operated in the normal phase mode, eluted with mobile phases containing high levels of acetonitrile [4,6,8,10-12]. Of 177 HPLC methods for carbohydrate cited in a recent review [11], 87 used refractometric detection either alone or in combination with other detection systems.

High-performance ion chromatography is already an established technique in

the brewing industry, for example, in the analysis of anions [13], anions and organic acids [14], and oxalate (Chapter 3). For the measurement of fermentable carbohydrates in wort, the standard methods of the European Brewery Convention (EBC) [3] and the American Society of Brewing Chemists (ASBC) [2] use cation-exchange chromatography, as does the ASBC method for beer carbohydrate measurement [2]. A recent publication [7] evaluated 10 cation exchange columns for the analysis of carbohydrates in brewing, using water as an eluent, and refractometric detection. In several applications, such as the analysis of worts and syrups, high sensitivity is not a requirement of the HPLC method, so simple non-selective detection systems such as differential refractometers or evaporative light-scattering detectors are suitable.

In this chapter, a method using gradient ion chromatography on a polymeric non-porous pellicular anion-exchange resin (Dionex Carbopac PA1) with pulsed amperometric detection is presented. This method is based on anion-exchange chromatography, made possible because carbohydrates, being very weakly acidic with pK_a values of approximately 12-14, are separable by anion exchange chromatography at high pH. The detection system, which employs electrochemistry on a gold working electrode, is based on the principle that carbohydrates may undergo electrochemical oxidation on noble metal surfaces. A pulsed amperometric system has the advantage that continuous electrochemical cleaning of the electrode is effected, thereby minimizing the need for maintenance and physical cleaning. This technique has already been applied to the analysis of grapefruit juice [15] and orange juice [9]. This chapter describes the optimisation of the technique for application in the brewing industry. The method developed is, moreover, suited to a diverse range of other carbohydrate-containing substances, e.g., milk and soft drinks. This method is more versatile than normal phase HPLC, offering greater sensitivity, selectivity, stability and repeatability. Application of the method to a number of analyses of interest to brewers, and, more broadly to the beverage industry in general, is described.

5.2. EXPERIMENTAL

5.2.1. Reagents

All reagents were of analytical reagent grade. Glucose, fructose, and sodium hydroxide (12.5 *M* solution) were obtained from BDH Chemicals Ltd., Dorset, U.K. Sucrose, melezitose, maltose, maltotriose, lactose, and the other carbohydrates used in this study were obtained from Sigma Chemical Co., Dorset, U.K., and ammonium sulphate and sodium acetate were obtained from E. Merck, Darmstadt, Germany. Deionized water was prepared using an Elga Maxima purification unit (Elga Ltd, Bucks, England).

5.2.2. Instrumentation and Conditions

Analysis was carried out on a Dionex series 4500i ion chromatograph (Dionex U.K. Ltd., Camberley, England), equipped with a programmable gradient pump, a pulsed electrochemical detector, an eluent degassing module, and an automated sampling module. Separations were performed on a Dionex Carbopac PA1 analytical column (25 cm x 4 mm i.d.) with a Dionex PA1-Guard guard column. The eluent was a gradient of increasing sodium acetate in sodium hydroxide (Table 5.1) supplied at a flow rate of 1.0 ml min⁻¹. Although this was a binary gradient, ternary mixing was employed to reduce the frequency of NaOH preparation by using a strong solution of NaOH, thereby reducing the amount of air entering the system. Introduction of air could cause the formation of sodium carbonate, which acts as a powerful eluting agent on the column used. For this reason, the eluents were stored under helium at 5 p.s.i. even when not in use. The sample loop size was 50 µl. The electrochemical detector was operated in the integrated amperometry mode, using a Ag/AgCl reference electrode and a gold working electrode. The potential waveform was as follows: 0-500 msec, *E* = +0.05 V; 500-600 msec, *E* = +0.6 V; 600-650 msec, *E* = -0.6 V. The current was integrated over 300-500 msec. The detector sensitivity was 10 µC full-scale deflection.

Data acquisition and processing were performed with Waters Maxima 820 software (Waters Chromatography Division, Millipore Ltd., Hertfordshire, England). The Dionex system was interfaced with the Maxima software using the Dionex automation interface box (part 000143).

Table 5.1. Gradient Conditions*

Time (min)	%A	%B	%C
0	84	0	16
4	84	0	16
15	62	22	16
20	84	0	16
30	84	0	16

* Solvent A: Deionized H₂O, solvent B: 1.0 M CH₃COONa, solvent C: 1.0 M NaOH.

5.2.3. Procedures

All eluents were stored under 5 psi helium pressure, and were degassed by sparging with helium regularly during use. Sodium hydroxide (1 M) was prepared by thoroughly degassing 920 ml of water by sparging with helium for 1 hr, followed by the addition of 80 ml 12.5 M NaOH below the water surface. The solution was mixed by sparging with helium for 1 hr.

A stock solution of 600 mg L⁻¹ melezitose (internal standard) was prepared in water. Standard solutions containing glucose, fructose, sucrose, maltose and maltotriose were prepared in the range 0.1 - 40 mg L⁻¹; each solution contained 30 mg

L^{-1} melezitose internal standard. Other carbohydrates were included as required (e.g. lactose for dairy milk analysis) at similar concentration ranges. Fermenting worts were inactivated, degassed, and samples ($100\ \mu\text{l}$) were added to internal standard solution ($5\ \text{ml}$) and diluted to $100\ \text{ml}$ with water, resulting in a solution containing $30\ \text{mg}\ \text{L}^{-1}$ melezitose for HPLC analysis. Beers and other beverages were degassed, filtered if necessary, and diluted as required, with the addition of internal standard to $30\ \text{mg}\ \text{L}^{-1}$. Brewing syrups were warmed to 50°C and subsamples were weighed into a $50\ \text{ml}$ volumetric flask and made up with water. This solution was diluted, and internal standard added. Milk samples were treated with an equal volume of saturated ammonium sulphate solution, shaken, and centrifuged to remove denatured protein. The deproteinised solution was diluted and internal standard added. Samples for analysis were placed in Dionex 'polyvials' that contained $10\text{-}\mu\text{m}$ filters in the caps, thereby ensuring that an additional sample filtration was performed. Two vials of water were injected at the start of every set of samples to clear the sample loop and rinse the column and fittings. A linear regression plot of peak area divided by melezitose peak area versus carbohydrate concentration for the standard solutions was constructed, and sample concentrations were calculated from this plot.

After processing every 200 samples, the columns were disconnected from the detector, their order reversed, and a rinse cycle of $50\ \text{ml}$ of $1\ \text{M}\ \text{HCl}$, $100\ \text{ml}$ of water, $50\ \text{ml}$ of $1\ \text{M}\ \text{NaOH}$, and $100\ \text{ml}$ of water, followed by normal elution solvent, was applied. The electrochemical cell was dismantled monthly and thoroughly cleaned, including polishing of the gold electrode if pitting of the surface was evident.

5.3. RESULTS AND DISCUSSION

5.3.1. Development of Gradient Conditions

The gradient described was optimized for the analysis of worts and beers. Two options exist for controlling gradient elution of carbohydrates from the Carbopac PA1 column. Firstly, the sodium hydroxide concentration can be gradually increased, thereby increasing the eluent pH and causing carbohydrates to elute more quickly from the column. Alternatively, the sodium hydroxide concentration may be kept constant, while sodium acetate is added to the eluent to speed carbohydrate elution by competitive binding of acetate ions to the column. Baseline stability was maximised by keeping the NaOH concentration (and therefore the pH) constant. An optimum concentration of 160 mM NaOH was chosen as this was low enough to permit resolution of glucose and fructose, but sufficiently high to prevent the formation of sodium carbonate on the column. The gradient of increasing sodium acetate (from 0 to 220 mM) was suitable for the analysis of fermentable carbohydrates and could be extended to include higher malto-oligosaccharides in the elution profile by continuing the increase in sodium acetate concentration to 300 mM. It was also possible to reduce the run time for the analysis of glucose syrups, milk or soft drinks by shortening the gradient program.

5.3.2. Development of Electrochemical Detection Conditions

The detector was operated in the pulsed amperometric mode, in which a potential waveform was applied to the working electrode. The instrument was supplied with a pre-programmed waveform for carbohydrate analysis [16], which was modified for this application. The recommended electrode cleaning and reducing potentials of +0.6 and -0.6 V, respectively, were maintained while the effect of varying the analytical potential was examined using a standard solution (20 mg L⁻¹) of glucose, fructose, sucrose, melezitose, maltose and maltotriose at nine different analytical potentials (Figure 5.1).

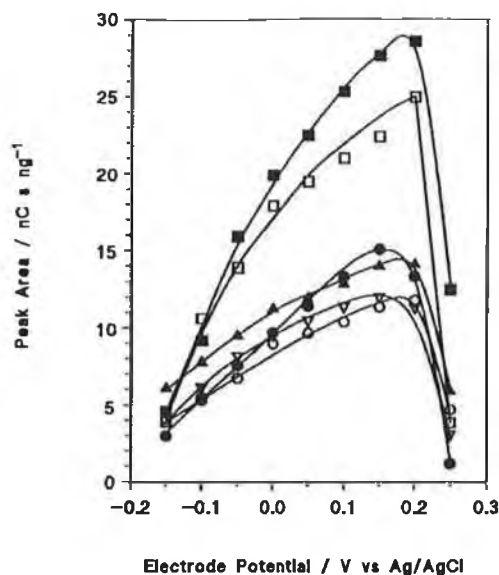


Figure 5.1. The effect of working electrode potential on the recorded peak area for the analysis of a standard solution containing 20 mg L^{-1} each of glucose (■), fructose (□), sucrose (●), melezitose (○), maltose (▲), and maltotriose (▼) by gradient ion chromatography with integrated amperometric detection at a gold working electrode.

From this experiment, an optimum analytical potential of $+0.05 \text{ V}$ was chosen, which provided ample sensitivity and a very low noise level. The effect of varying the integration period was also examined, and this confirmed that the preset integration period of $300 - 500 \text{ ms}$ provided the best compromise between high sensitivity and low baseline noise, as measured by the signal-to-background ratio during normal operation.

5.3.3. Method Performance

The peak response was linear in the range $0.1 - 50 \text{ mg L}^{-1}$ for glucose, fructose, lactose, sucrose, melezitose, maltose and maltotriose. At higher concentrations, the ratio of peak area to concentration began to fall slightly, possibly due to diffusion

limitation of the oxidation reaction, or due to saturation of the electrode surface. Peak area relative standard deviations of between 0.2 and 0.4 % (mean 0.3 %) were obtained for six replicate injections of a solution containing 10 mg L⁻¹ each of glucose, fructose, sucrose, melezitose, maltose and maltotriose. Retention time relative standard deviations for the same injections were between 0.1 and 0.3 % (mean 0.2 %). The column performance was excellent in terms of reproducibility and high plate numbers, provided that the rinsing protocol was adhered to. The detection system was also extremely stable. When not in use, the column was stored in 160 mM NaOH to prevent bacterial growth.

5.3.4. Analysis of Samples

Worts. The method was used to measure carbohydrates in experimental samples of stout, ale, and lager worts, and also in worts prepared from non-standard cereals such as sorghum, wheat and maize. Figure 5.2 (A) shows a chromatogram of a typical hopped lager wort before fermentation. Generally, there was little variation in the relative contents of fermentable carbohydrates in the worts examined, although the mashing conditions and the degree of enzymic treatment used to produce the worts were quite varied. A typical lager wort at 10°P contained (g L⁻¹): glucose, 9.8; fructose, 2.0; sucrose, 0.4; maltose, 49.5; and maltotriose, 11.4.

Amyloglucosidase Assay. The method was used to assay the activity of samples of commercial amyloglucosidase (AMG) enzyme preparations. A standard maltose solution was dosed with a measured amount of commercial AMG, and the hydrolysis of maltose to glucose was measured in samples taken at 10-min intervals over 30 min during incubation at 50°C. The enzyme activity was then calculated from the reaction rate. The degree of inactivation of commercial AMG at high temperature was also examined using a similar method. Most commercial AMG samples showed some inactivation at 60°C and were rapidly deactivated above 80°C. Figure 5.2 shows a chromatogram of a lager wort before (A) and after (B) treatment with an optimized dosage of commercial AMG for 1 hr at 50°C. Such enzyme-treated worts were used in the production of beers with low remaining carbohydrate (e.g., diet beers).

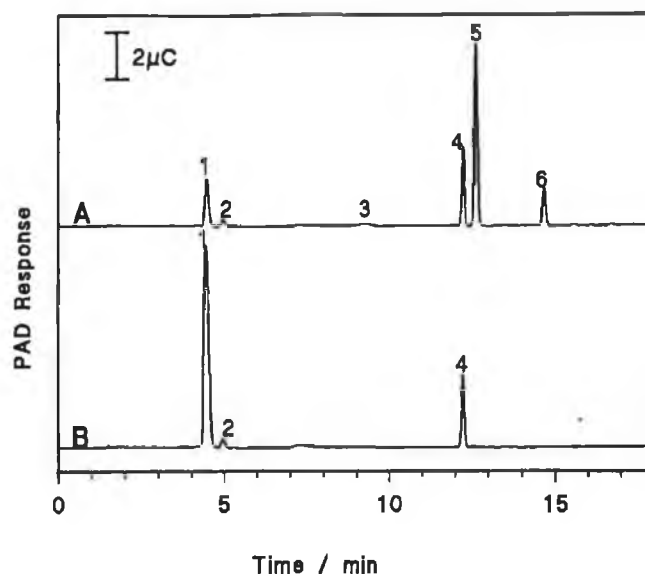


Figure 5.2. Chromatograms of a lager wort sample before (A) and after (B) treatment with an excess of commercial amyloglucosidase enzyme. Samples were diluted 1,000-fold with deionized water, melezitose (internal standard) was added to a concentration of 30 mg L⁻¹, and the samples were analysed by gradient anion-exchange HPLC with integrated amperometric detection. Identified peaks: 1 = glucose, 2 = fructose, 3 = sucrose, 4 = melezitose, 5 = maltose, and 6 = maltotriose.

Beers. The method was used to measure residual carbohydrates in fermented beers and also unfermented carbohydrates in low-alcohol beers, non-alcoholic beers, and sweet beers, e.g., milk stouts. Table 5.2 compares the carbohydrate content of a range of stout products.

Table 5.2. Comparison of the Carbohydrate Content (g L⁻¹) of Commercial Milk Stout (A-D), Sweet Stout (E-H), and Bitter Stout (I-L) Products*

Sample	Gluc.	Fruc.	Suc.	Lac.	Malt.	M3ose.	Total
A	0.4	0.1	0.2	1.4	1.0	1.0	4.1
B	2.3	1.9	1.8	1.6	0.3	ND	7.9
C	2.8	2.4	ND	14.2	0.2	0.3	19.9
D	3.0	0.2	ND	12.1	8.6	3.3	27.2
E	0.2	ND	ND	ND	1.7	3.3	5.2
F	0.9	0.4	0.2	ND	3.0	13.4	17.9
G	7.6	7.9	0.1	ND	10.2	2.5	28.3
H	4.9	4.3	18.7	ND	2.1	0.9	30.9
I	<0.1	<0.1	ND	ND	<0.1	0.1	0.1
J	<0.1	<0.1	ND	ND	0.3	0.2	0.5
K	<0.1	<0.1	ND	ND	0.7	0.3	1.0
L	<0.1	<0.1	ND	ND	<0.1	1.4	1.4

*ND = Not Detected; Gluc. = Glucose, Fruc. = Fructose, Suc. = Sucrose, Lac. = Lactose, Malt. = Maltose, M3ose. = Maltotriose.

Samples A-D were milk stouts containing added lactose. Samples E-H were sweet stout products. Sample F was a high gravity (23°P) stout with an elevated maltotriose level. Sample H was clearly sweetened by the addition of sucrose. In general, fully fermented beers (samples I-L) contained only traces of mono- and disaccharides, whereas maltose and maltotriose varied from 0.0 to 1.1 and 0.0 to 1.4 g L⁻¹, respectively.

The method was also of particular interest in comparing low-alcohol beers (0.05 - 0.5% alcohol by volume), as the carbohydrate profile was usually indicative of the method of manufacture. The products of arrested fermentation contained high

levels of fermentable carbohydrates (Table 5.3, samples B-H). Post-fermentation dealcoholisation, however (sample A, Table 5.3), produced a beer containing only traces of maltose and maltotriose as is usually present in fermented beers. Product A, however, contained unusually elevated glucose and fructose levels, which suggested the addition of extra sugars as sweeteners: the glucose and fructose present may have resulted from natural inversion of added sucrose.

Table 5.3. Comparison of the Fermentable Carbohydrate Content (g L^{-1}) of European Low-alcohol Beers*

Sample	Gluc.	Fruc.	Malt.	M3ose	Total	% alcohol [†]
Group 1 (0.1 % alcohol or less)						
A	4.4	5.4	Trace	Trace	9.8	0.05
B	2.8	0.8	30.0	12.1	45.7	0.1
C	4.4	1.6	30.3	9.8	46.1	0.1
Group 2 (0.5 % alcohol or less)						
D	1.8	0.9	10.6	2.8	16.1	0.5
E	1.8	0.7	12.1	2.8	17.4	0.3
F	3.1	1.2	17.3	5.0	26.6	0.5
G	1.0	0.8	22.5	8.3	32.6	0.5
H	2.3	0.7	30.9	8.5	42.4	0.5

*Sucrose was not present at $>0.2 \text{ g L}^{-1}$ in any of the samples analyzed.

[†]Refers to labelling and not actual analysis.

Note: Gluc. = glucose; Fruc. = fructose; Malt. = maltose; M3ose = maltotriose.

Soft/Non-Alcoholic Beverages. A wide range of soft drinks were analyzed using the described method. Analysis of milk (Figure 5.3 (A)) showed lactose to be the major carbohydrate present. Low-sugar blackcurrant cordial (Figure 5.3 (B)) contained predominantly glucose and fructose, while orange and cola drinks contained mainly glucose, fructose and sucrose. The analysis of malt drinks (Table 5.4) showed, in general, unusually high levels of sucrose relative to the other fermentable sugars present in normal wort. Some samples (D and H, Table 5.4) showed significantly higher proportions of glucose, fructose and sucrose than maltose and maltotriose, suggesting that the supplementation of malt extract with variable amounts of added sugar had occurred.

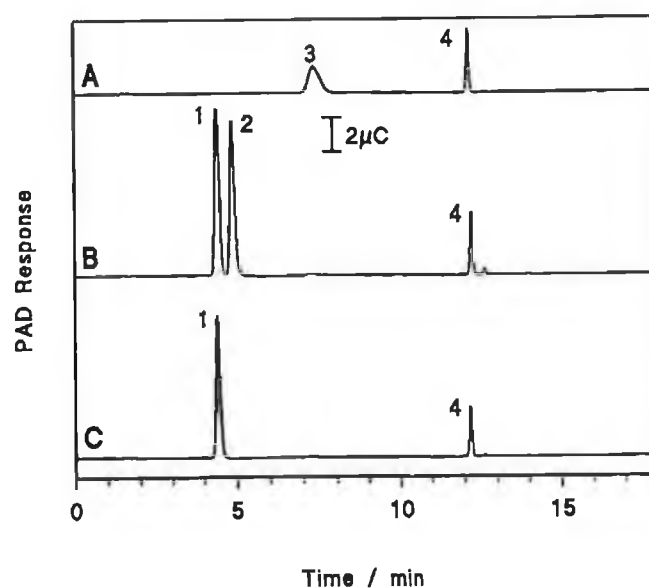


Figure 5.3. Chromatograms of dairy milk (A), low sugar blackcurrant cordial (B), and high-glucose brewing syrup (C). Samples were diluted with deionized water, melezitose (internal standard) was added to a concentration of 30 mg L^{-1} , and the samples were analysed by gradient anion-exchange HPLC with integrated amperometric detection. Identified peaks: 1 = glucose, 2 = fructose, 3 = sucrose, 4 = melezitose.

Table 5.4. Comparison of the Fermentable Carbohydrate Content (g L⁻¹) of Commercially Available Malt Drinks*

Sample	Gluc.	Fruc.	Suc.	Malt.	M3ose.	Total
A	8.7	3.2	18.3	30.5	17.4	78.1
B	13.6	3.4	35.2	27.4	18.6	98.2
C	20.7	1.9	38.8	24.5	12.3	98.2
D	70.1	16.4	10.5	6.5	0.4	103.9
E	16.2	6.4	48.2	22.5	10.8	104.1
F	10.0	2.8	47.1	28.4	16.1	104.4
G	12.1	6.0	42.4	28.3	18.6	107.4
H	34.4	23.4	31.0	16.5	6.9	112.2

*Gluc. = glucose, Fruc. = fructose, Suc. = sucrose, Malt. = Maltose, M3ose = Maltotriose.

Syrups, Malt Extracts and Caramels. A wide variety of glucose syrups for brewing were analysed for fermentable carbohydrates. In general, samples contained maltose, glucose and maltotriose, with no detectable sucrose or fructose. Figure 5.3 (C) shows a chromatogram of a glucose syrup with no other detectable carbohydrates. Malt extracts contained predominantly maltose, maltotriose and glucose. Brewing caramel samples contained glucose, fructose and maltose.

Oligosaccharide Analyses. Due to the high selectivity of the Carbowax column, it was possible to resolve a very wide range of sugars by extended gradient elution. Figure 5.4 shows the resolution of a 19-component mixture prepared from carbohydrate standards. This figure clearly demonstrates the resolution of a series of maltodextrins up to malto-octanose in a run time of 20 min. Normal wort (Fig. 5.2

(A)) contained only traces of small oligosaccharides greater than maltotriose. Most of the unfermentable carbohydrates in wort consisted of high molecular weight limit dextrans or β -glucans that were not detectable as resolved peaks by the described system.

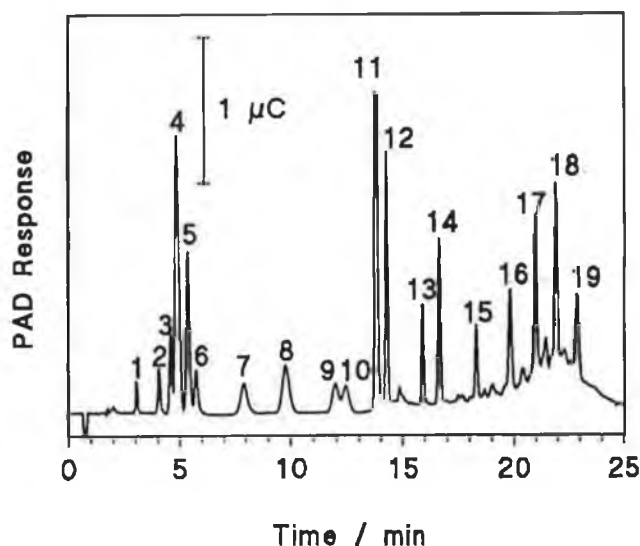


Figure 5.4. Chromatogram of a mixture of carbohydrates resolvable by gradient anion-exchange HPLC with integrated amperometric detection. Identified peaks: 1 = fucose, 2 = arabinose, 3 = mannose, 4 = glucose, 5 = fructose, 6 = ribose, 7 = lactose, 8 = sucrose, 9 = cellobiose, 10 = palatinose, 11 = melezitose, 12 = maltose, 13 = panose, 14 = maltotriose, 15 = maltotetraose, 16 = maltopentaose, 17 = maltohexaose, 18 = maltoheptaose, and 19 = maltooctanose.

5.3.5. Comparison of the Method with Normal-Phase HPLC

The ion-exchange method described above was developed as a replacement for a previously used HPLC method that used a silica column impregnated with 0.1%

tetraethylene pentamine (TEP) that was continuously regenerated by elution with 72% CH₃CN containing 0.02% TEP [11]. This normal-phase system was robust and had a very long lifetime for carbohydrate analysis. Detection of the eluted carbohydrates was achieved by either refractive index or evaporative light-scattering detection, neither of which were without disadvantages. This method in turn had been adopted as a more stable alternative to the use of commercially prepared aminopropyl silica columns, which exhibited unacceptably short working lifetimes when continuously eluted with buffered acetonitrile-containing mobile phases. Having compared gradient ion chromatography and isocratic normal-phase HPLC, it was obvious that the former offered the greater separation efficiency (12,100 theoretical plates per column for maltose compared with 600 using normal-phase separation). Resolution of carbohydrates was also improved, the resolution of maltotriose from maltose (defined as the difference in retention time divided by half the sum of the peak widths) was 68 for the ion chromatographic method compared with 3 for the normal phase separation. Typical precision values for peak response using the normal-phase procedure were in the 2-5% RSD range compared to 0.4% for the ion chromatographic procedure. The amperometric detector was also very sensitive, with nanogram amounts of carbohydrate detectable compared with microgram amounts for evaporative light-scattering or refractometric detection. Furthermore, the long-term stability and short equilibration times associated with the ion chromatographic column made this a preferable procedure.

One limitation of the ion chromatographic method, however, is the incompatibility of the column with organic solvents. This limits the extent to which organic extractions can form part of a sample preparation protocol and dictates that only compounds soluble in aqueous media may be separated by this technique. However, I have not yet encountered a brewing-related application that was precluded by this limitation.

The use of ion chromatography is moderately more expensive in terms of capital expenditure and replacement parts than is normal phase HPLC. However, the cost of consumable reagents for ion chromatography is low, and the elimination of the health and safety risks associated with the use of acetonitrile, particularly in evaporative light scattering detectors which vaporize the solvent, is an added factor which merits important consideration.

5.4. CONCLUSIONS

The ion chromatographic method described uses selective separation and detection techniques to enable direct injection of extremely complex sample matrices such as wort and beer, thereby providing a rapid analytical method which does not compromise sample integrity. The method described has been adopted as a standard method for carbohydrate analysis in the GBW Research Centre for an extended period, and has been found to be versatile and reliable, offering distinct advantages over more commonly used normal phase methods. In particular, automation capability and minimal sample preparation make the method attractive for routine analysis in the brewery, as well as in other industries.

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6. AN IMPROVED METHOD FOR ASCORBIC ACID ANALYSIS AND ITS APPLICATION TO THE ANALYSIS OF BEERS AND VITAMIN-FORTIFIED DRINKS.

6.1. INTRODUCTION

The analysis of ascorbic acid was felt to be an important area in which to carry out research, due to the current level of interest in antioxidants, both from a health and nutritional point of view, and also in relation to the stability of foodstuffs against oxidative damage.

Ascorbic acid (AA) is one of the most powerful antioxidants known, and, unlike many other antioxidants, it has the capability to reduce molecular oxygen [1]. AA is widely used as an antioxidant in the food and beverage industry, and some brewers add AA at 30-50 mg L⁻¹ to beer in order to diminish residual oxygen after packaging. This is thought to delay the development of stale flavours in beer, which are believed to result from oxidative chemical reactions [2]. The sensitivity of AA to oxygen, however, makes the analysis of the reduced form very difficult, particularly if the sample must undergo extraction techniques that permit oxidation. Therefore it is desirable to measure AA by rapid, sensitive methods which minimize contact of the sample with atmospheric oxygen. The standard AOAC method for AA determination [3] uses a titration based on the reduction of 2,6-dichlorophenol indophenol. This method, however, is subject to numerous interferences in samples such as beers or fruit juices, which may contain substantial amounts of reducing substances such as flavanols, melanoidins or reductones. Added to this, the endpoint of the titration is poorly defined, and this increases the possibilities for errors in this method. The use of HPLC with ultra violet absorbance detection is applicable to AA determination [4-6], but is insufficiently sensitive for the determination of very low levels of AA. Sample preconcentration steps may be necessary, therefore, leading to the unwanted risk of absorption of oxygen by the sample. A suitable alternative to these methods is the use of electrochemical detection with HPLC. AA is very easily oxidized at a

glassy carbon working electrode at low operating potentials, thereby providing a sensitive detection system. The ease of oxidation of AA means that potentially interfering analytes with higher oxidation potentials are not detected, so selectivity is high.

While HPLC with electrochemical detection for AA determination has been widely documented [7-11], many reported methods suffer from either a lack of reproducibility or from losses of sensitivity due to fouling of the electrode with sample components. This has necessitated bracketing of samples and standards, which, as well as being troublesome to perform, greatly reduces the number of samples which may be analysed in a given time. In this chapter a method for the determination of AA in beer which provides excellent repeatability and long-term electrode stability, and which may also be applied to other beverages, is presented. Furthermore, the detection system used in this study possessed additional advantages over the more commonly used single-cell amperometric devices: the high conversion efficiency (coulometric) electrode is claimed to be capable of oxidizing all of the sample which passes through it, due to the large electrode surface area. This should therefore provide added sensitivity and repeatability when compared to amperometric detectors, which, due to diffusion limitation, typically only oxidize 2-10% of the analyte. In addition, the response of the downstream amperometric electrode, which is operated at a higher potential, can be used as a valid indicator of peak purity and/or electrode poisoning; the ratio of current responses at the two electrodes is dependent on the shape of the hydrodynamic wave of the analyte, and also on the operating efficiency of the electrodes (see also Chapter 2).

AA measurement has many important applications in the food and beverage industries, and the development of accurate, repeatable methods such as that described here is much in demand, particularly in view of the favourable health factors being attributed to food antioxidants in recent times.

6.2. EXPERIMENTAL

6.2.1. Reagents, Instruments and Conditions

All reagents were of analytical grade. Deionized water was prepared using an Elga Prima/Maxima purification System (Elga Ltd., High Wycombe, Buckinghamshire, UK). HPLC was performed on a Perkin-Elmer Integral 4000 HPLC system equipped with a photodiode array detector (Perkin Elmer Ltd., Beaconsfield, Buckinghamshire, U.K.). An ESA Analytical Coulochem II electrochemical detector (ESA Analytical, Huntingdon, Cambridgeshire, U.K.) fitted with a Model 5011 high sensitivity analytical cell was placed downstream of the photodiode array detector. This cell contains a porous graphite high conversion efficiency electrode (also referred to as a coulometric electrode) placed upstream of a glassy carbon amperometric working electrode. The detector was operated under the following conditions for routine analysis: Channel 1 (high efficiency electrode): potential, +40 mV; output range, 10 μ A; offset, + 5%; and filter, 1 s. Channel 2 (Amperometric electrode): potential, +350 mV; output range, 10 μ A; offset, +5%; and filter, 1 s. The column used was a Waters Radial Compression Module (RCM) containing a cartridge (10 cm x 8 mm i.d.) packed with 5 μ m Resolve octadecyl stationary phase (Waters UK Ltd, Watford, Hertfordshire, U.K.). This stationary phase was recommended for use at low pH in preference to other octadecyl phases available from Waters. The mobile phase was 0.05 M potassium dihydrogen orthophosphate, 500 mg L⁻¹ disodium EDTA, adjusted to pH 3.00 with conc. orthophosphoric acid and pumped at a flow rate of 1.0 ml min⁻¹. The injection volume was 10 μ l.

6.2.2. Calibration

Standard solutions of AA were prepared at concentrations in the range 0.5 - 20 mg L⁻¹ in the mobile phase described above. These solutions were stored cold under N₂ when not in use, and were prepared fresh daily. Before calibration of the HPLC system, two 100 μ l injections of a solution containing 1 g L⁻¹ ascorbic acid were

made, followed by two 100 μ l injections of mobile phase. This removed residual oxidizing species from the system and improved repeatability.

6.2.3. Sample Preparation

Samples (50 ml) were degassed by helium sparging, allowing minimal contact with atmospheric oxygen, and were mixed with an equal volume of mobile phase. A subsample (10 μ l) of the resulting solution was injected immediately.

6.3. RESULTS AND DISCUSSION

6.3.1. Sample Preparation

Significant losses of AA occurred during sample preparation if oxygen was not totally excluded. Even when very gentle agitation was used to release dissolved CO_2 , losses of 10 - 20% of the measurable AA were not uncommon. When helium degassing was used, however, repeatable results and high recoveries were obtained. This severely limits the number of samples which may be processed on a given day, but is an absolute requirement for accuracy. The inclusion of EDTA is also vital: Figure 6.1 shows the effect of the inclusion of EDTA on the repeatability of injection of both standard solutions and beer samples. Solutions containing EDTA did not exhibit the rapid deterioration rate of unstabilized solutions. The rate of loss of AA was faster in beer than in standard solutions.

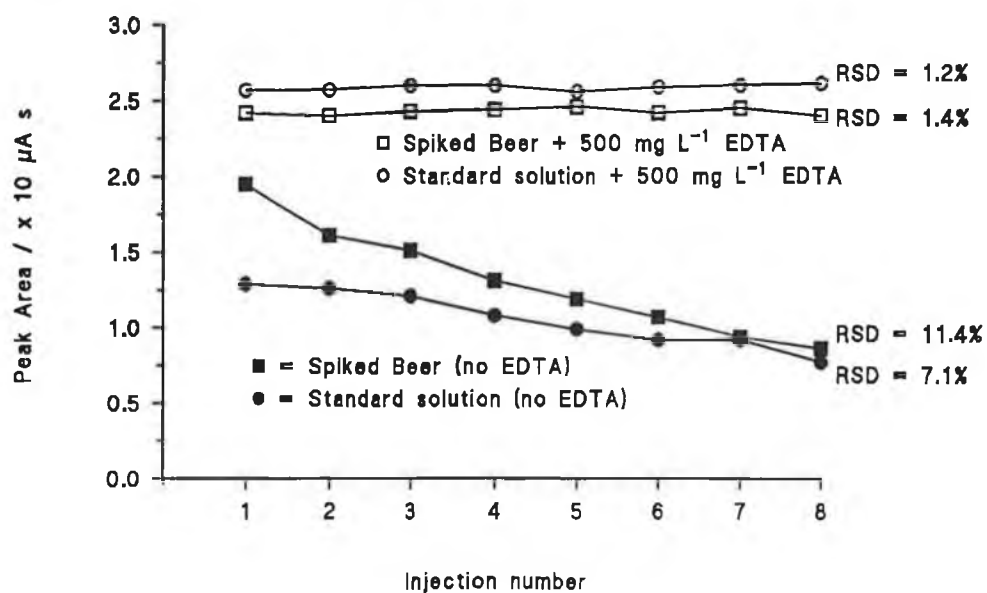


Figure 6.1. Effect of inclusion of EDTA on the repeatability of injection of samples and standard solutions containing ascorbic acid.

6.3.2. Separation Conditions

The method of Rose and Bode [12] was modified. The buffer strength was reduced from 0.2 M to 0.05 M, in order to improve column lifetimes, and disodium EDTA was added to the mobile phase at 500 mg L⁻¹ to inhibit metal-ion-catalysed autoxidation of ascorbic acid. By operating at pH 3.00 it was possible to separate ascorbic acid, which has pK_a values of 4.0 and 11.3 [1], by ion suppression HPLC, and thereby dispense with the requirement for corrosive ion-pairing reagents. The column used was very stable at this pH. Although a Waters Nova-Pak Octadecyl 4 µm stationary phase gave almost equally good resolution of ascorbic acid, the Resolve packing material was chosen because of its long-term durability at low pH.

6.3.3. Optimization of detection conditions

Hydrodynamic voltammograms are illustrated in Figures 6.2 and 6.3. In Figure 6.2, the potential of the upstream high conversion efficiency electrode was varied from -300 to +400 mV, while maintaining the downstream amperometric electrode at a constant potential of +350 mV, whereas in Figure 6.3 the high efficiency electrode was maintained at a constant potential of +40 mV while the downstream amperometric electrode potential was varied. The effect of potential on background current was also investigated (Figure 6.4), and these experiments indicated that at potentials above +350 mV the background current rose sharply, probably due to the oxidation of mobile phase components. At the chosen combination of analytical potentials (+40 mV and +350 mV at the high efficiency and amperometric electrodes respectively) the ratio of peak areas from the two electrodes remained constant within the linear range of the method. Provided that the electrode response is reproducible, any gross departure from this ratio in samples would be indicative either of the presence of interfering compounds or of electrode fouling. This is of particular importance when analysing beer, which contains a wide variety of reducing substances [13].

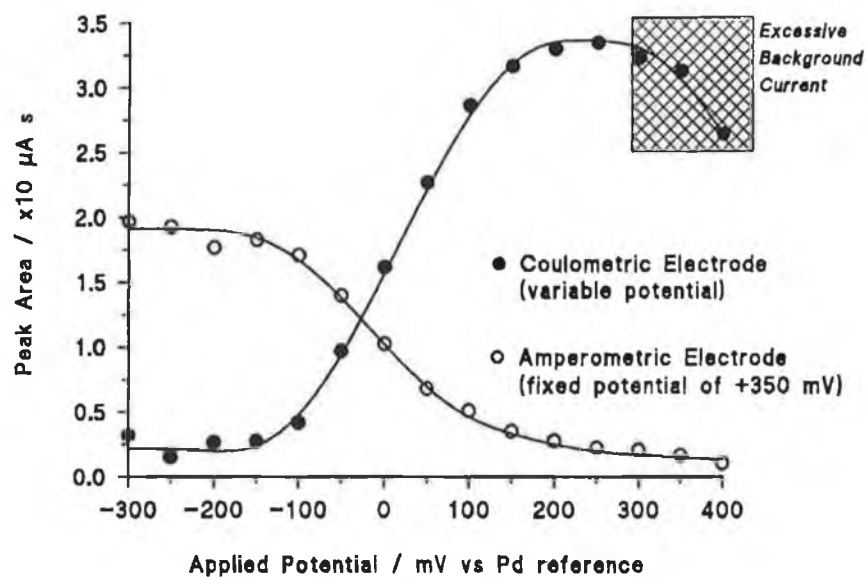


Figure 6.2. Hydrodynamic voltammogram of ascorbic acid at the high conversion efficiency electrode. The potential at the amperometric electrode was maintained at +350 mV.

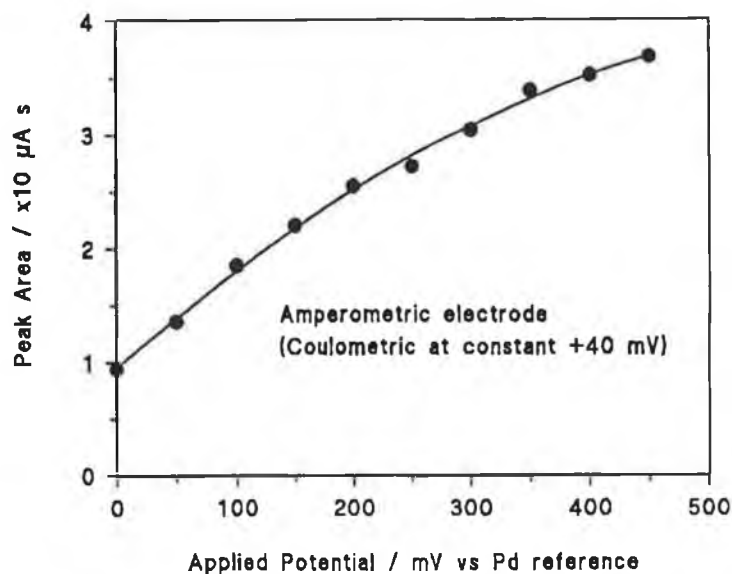


Figure 6.3. Hydrodynamic voltammogram of ascorbic acid at the amperometric electrode. The potential at the high conversion efficiency electrode was maintained at +40 mV.

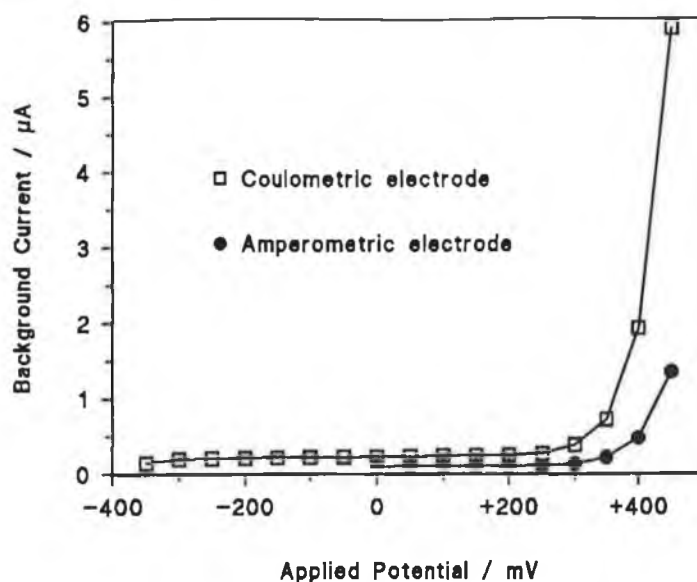


Figure 6.4. Effect of potential on the background current observed at each of the electrodes in the electrochemical detector, under the standard chromatographic conditions.

6.3.4. Method Performance

The method was linear in the range $0.5\text{--}20\text{ mg L}^{-1}$ ascorbic acid, which was suitable for the applications described here. It should be noted that at higher concentrations of AA the high efficiency electrode began to exhibit a non-linear response, whereas the amperometric electrode response remained linear. This is important both from the point of view of calibration and also if peak area ratioing is used as a measure of peak purity. The reason for this non-linearity of response at high concentrations of AA may have been due to saturation of the electrode surface with oxidised AA, resulting in a temporary diminution of electrode response. The limit of detection was 0.2 mg L^{-1} ascorbic acid in beer for the method as described, although higher sensitivity could be obtained by varying the electrode potentials and injection volume. Figure 6.5 shows fifteen consecutive injections of a beer sample containing 10 mg L^{-1} AA, diluted with an equal volume of mobile phase; twenty consecutive injections of this sample gave percentage relative standard deviations in peak area of 1.3% and 1.4% at the coulometric and amperometric detectors respectively, and in

peak height of 1.2% and 0.9% respectively. This demonstrated not only the repeatability of the analysis, but also the resistance of the electrodes to fouling by sample components. The method was also used for the analysis of non-alcoholic vitamin drinks and fruit juices.

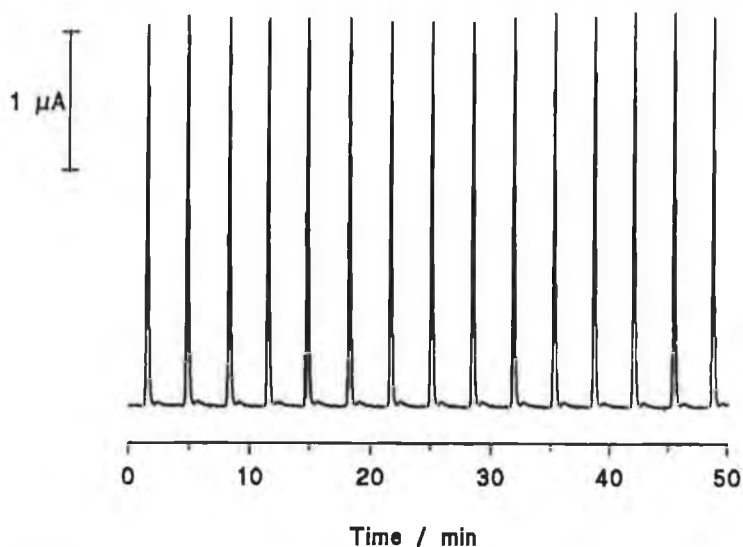


Figure 6.5. Chromatogram of fifteen consecutive injections of a beer sample containing 10 mg L⁻¹ AA, diluted with an equal volume of mobile phase, and analysed by HPLC with dual electrode electrochemical detection. The output shown is that of the high conversion efficiency electrode.

6.3.5. Stability of AA in Fortified Malt Drinks

A malt drink containing 60 mg L⁻¹ AA at bottling was analysed immediately after bottling, after pasteurisation, and thereafter after storage at 4°C and at 30°C. Figure 6.6 shows the results of these analyses. It was obvious from the results obtained that significant AA was lost during mixing, carbonation and bottling of the product. Only a slight amount of AA was lost during pasteurization, but thereafter significant losses occurred during storage, with the decay rate being accelerated at higher temperature. Studies such as this one are useful in predicting the shelf-life for valid labelling of vitamin drinks for public consumption, but have previously been

hampered by imprecise results and sample deterioration before analysis. On the basis of this particular study it was ascertained that in order to guarantee a three month shelf-life during which time a malt drink will contain a minimum amount of 30 mg L⁻¹ AA, it is necessary to add about 60 mg L⁻¹ AA before pasteurization. In tropical climates, such as the target market for this particular product, it may be necessary to increase the addition rate. The very obvious instability of AA following opening of samples and agitation of the contents (Figure 6.3) combined with its instability in package (Figure 6.6) demonstrates the difficulty in relating added amounts of AA during beverage production to the levels subsequently detected in samples which have been poorly stored, handled, or prepared.

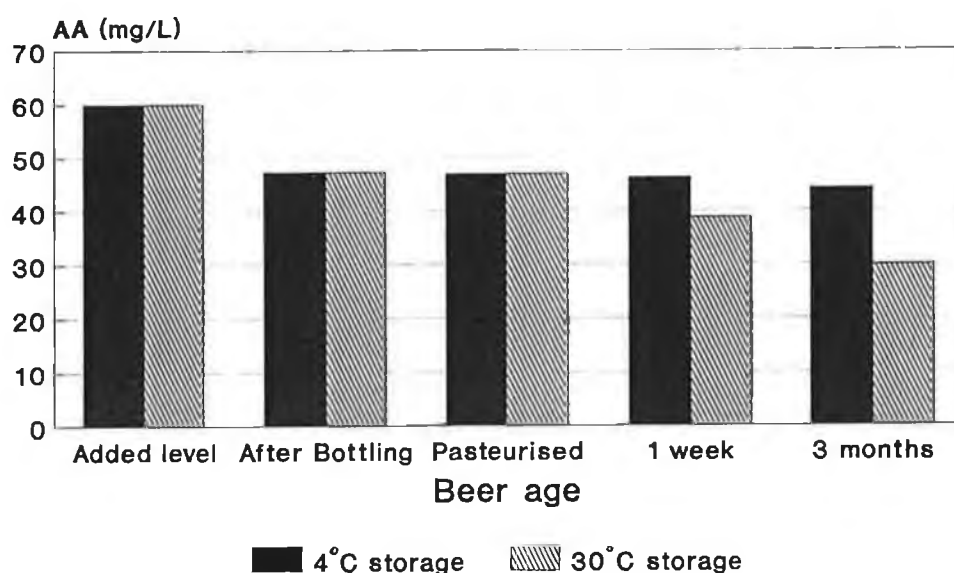


Figure 6.6. Stability of AA in a vitamin-fortified malt drink during storage at 4°C and 30°C

6.4. CONCLUSIONS

The described method was shown to be suitable for the analysis of AA in beer and non-alcoholic beverages, and should be adaptable to the analysis of solid foodstuffs. This method offers distinct advantages over previously reported methods for analysis of AA in beer in terms of accuracy, freedom from interferences, and repeatability. Furthermore, use of the electrochemical detector allows the determination of low residual AA levels in beers that have deteriorated during prolonged storage.

This method provided a final demonstration of the virtues of 'dilute-&-shoot' HPLC methodology. In this instance the analyte was highly unstable, and could easily be depleted through inadvertent reaction with oxygen during sample preparation. A selective and sensitive detection system, however, overcame these difficulties by allowing the analysis to take place with minimal interference with the sample.

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7. OVERALL CONCLUSIONS AND PERSPECTIVES

The research described in this thesis was undertaken with a number of objectives in mind, which are listed in section 1.4. In this chapter I will demonstrate briefly how these individual objectives were met, and suggest the areas in which there is scope for further research which I feel could significantly further current knowledge in these fields.

1. *To develop novel analytical methodology to enable automated direct-injection HPLC analysis of simple flavanols normally present in beer and barley.*

The method developed for the analysis of flavanols provided selectivity and sensitivity which was previously unmatched in this area, and the direct-injection capability allowed the adoption of the method as a routine assay. This in turn permitted detailed studies of the behaviour of flavanols during beer treatment and ageing to be carried out. The use of electrochemical detection for HPLC is still viewed as something of a complicated research tool, and instances of the use of this method in brewery laboratories are relatively rare. However, many enquiries relating to this method were received, and this indicates a possible shift in popular opinion in this regard. Further research in this area will no doubt involve the extension of the assay to include flavonoids and flavonol glycosides such as those present in hops, and, as more of the minor flavanols in beer are identified, the method may be improved to analyse these compounds also, thereby broadening the analytical spectrum of beer polyphenols that are measurable routinely.

2. *To develop semi-preparative and preparative methods for the isolation of simple flavanol standards for use both as calibration standards for HPLC, and also in amounts sufficient to enable their addition to beer at typical levels.*

The semi-preparative method for the isolation of dimeric and trimeric proanthocyanidins from barley represents a significant improvement to the methods currently available for the isolation of these compounds. The convenience of the procedure means that it is now possible for interested researchers to isolate the pure compounds for use as chromatographic standards without the need for particularly specialised equipment or a high degree of manual input. The preparative method described was an improvement upon the currently available separations on LH-20, and the main advantage of this procedure was that it allowed easy preparation of large amounts of dimeric proanthocyanidins without the need for many repetitive purifications by either TLC or HPLC. The availability of such large amounts of these compounds in pure form allowed a number of detailed studies of the behaviour of the compounds to be performed. It is quite possible that using either of these methods, other researchers in this area will now be encouraged to prepare their own proanthocyanidins and investigate further the elusive role of these compounds in beer stability.

3. *To investigate the effects of PVPP-stabilization of beer on the contents of simple flavanols having different degrees of hydroxylation and polymerisation.*

This study provided much important information relating to the performance characteristics of commercial beer stabilization sorbents, and their effects on the contents of particular haze precursors in beer. The conclusions are of direct practical use to the brewer and will no doubt be of assistance in the selection of sorbents for production use, and also in selecting the correct dosage rate. Furthermore, the findings relating to the relative affinities of the sorbents for different flavanol classes provided evidence that the use of PVPP does not significantly alter the ratio of putative prooxidant to antioxidant flavanols in beer, and therefore should not pose any real threat to the stability of beers prepared using PVPP stabilization.

4. *To examine critically the role of simple flavanols in the colloidal and flavour stability of beer, in particular differences between flavanols with differing degrees of either hydroxylation or polymerization.*

While the exact mechanism of action of flavanols in the formation of haze in beer remains uncertain, many conclusions can be drawn, and assumptions reaffirmed on the basis of the research reported here. The role of flavanols in beer flavour instability remains a little more elusive, however, but the studies reported here did indicate that the changes in flavanol content effected by PVPP stabilization of beer are unlikely to detrimentally alter the flavour of a beer to any noticeable extent. This information is of real use to brewers considering possible methods of increasing the shelf-life of their products. The studies on the effect of PVPP stabilization on beer colloidal shelf-life are of direct practical use in designing stabilization protocols to meet defined shelf-life specifications.

Further research in this area will probably focus on the effects of flavanols on beer flavour stability in particular, and much work is being done in Japan by Hirotaka Kaneda and co-workers in this field at present. There is also much interest at present in the development of biosensors based on polyphenol oxidase for the determination of beer flavanols, and the development of such a sensor may result in increased interest in the levels of flavanols in beer due to the increased ease of measurement of these compounds.

5. *To develop direct-injection HPLC methods for the determination of volatile beer phenolics and their phenolic acid precursors in beer, and to examine the occurrence and significance of these compounds in beer.*

The methods developed for the analysis of phenolic acids and their degradation products (in particular ferulic acid and 4-vinyl guaiacol) represented significant improvements on those previously reported. The direct-injection capability of these methods meant that large numbers of samples could be analysed and as a result of this the methods received widespread application in the Guinness breweries worldwide. The resulting discoveries relating to the incidence of phenolic off-flavours and the

control of this phenomenon enabled recommendations to be made for brewing practice which allowed tighter control of phenolic compounds in production beers. Further research in this area may allow cloning of Pof^{+/+} yeasts suitable for the production of speciality beers containing desirable levels of phenolic compounds.

6. *To apply automated direct injection HPLC methodology to the investigation of oxalate hazes and sediments in beer.*

This simple adaptation of what was already a routine method for the analysis of inorganic anions in beer to include the analysis of oxalate in soluble and insoluble forms allowed the incidence of oxalate in beer to be examined in detail. As a result of this, the levels of oxalate likely to cause problems during beer storage were identified, and recommendations for prevention of this phenomenon were made. The method, due to its ease of application, allows quick identification of sediments which may occur during the brewing process, and which require fast identification so that the problem may be rectified if necessary.

7. *To develop an automated direct-injection HPLC method for carbohydrate determination in beer and wort.*

The method of analysis described was a great improvement on methods in use in breweries generally at the time, in terms of both sensitivity and selectivity. The method is also widely applicable, and therefore may be used in many food and beverage applications. The direct injection capability was once again welcome, as it allowed fast sample preparation prior to unattended overnight operation of the analytical instrumentation; a feature which is particularly desirable in today's climate of financial restrictions on spending by industry on scientific support in general.

8. *To develop a repeatable direct-injection HPLC method for the analysis of ascorbic acid in vitamin-fortified beers and malt drinks.*

The analysis of ascorbic acid, and of antioxidants in general, is of widespread interest at present, in particular due to the widely professed role of antioxidants in cancer prevention. The method described here offered advantages over other methods in terms of ease of application and also in improved repeatability. The development of the method allowed studies to be performed on the stability of additions of ascorbic acid to malt drinks as well as to beers in general, and the method was found to give improved performance compared with titrimetric methods which were previously used for this purpose.

APPENDIX A. LIST OF PUBLISHED PAPERS.

1. Analysis of proanthocyanidinss and catechins in beer and barley by HPLC with dual electrode electrochemical detection. D. Madigan, I. McMurrough and M.R. Smyth, *Analyst*, 1994, **119**, 863.
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6. Improved method for the determination of ascorbic acid in beer by using high-performance liquid chromatography with electrochemical detection. D. Madigan, I. McMurrough and M.R. Smyth, *Anal. Comm.*, 1996, **33**, 9.
7. Control of ferulic acid and 4-vinyl guaiacol in brewing. I. McMurrough, D. Madigan, D. Donnelly, J. Hurley, A. Doyle, G. Hennigan, N. McNulty and M R. Smyth, *J. Inst. Brew.*, 1996, **102(5)**, In Press.

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APPENDIX B. LIST OF CONFERENCE PROCEEDINGS

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