ANALYTICAL METHODS FOR THE SELECTION OF POLYPHENOLIC PROPERTIES IN MALTING BARLEY BREEDING

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by

Walter James Pitze

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ABSTRACT

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The phenolic properties of barley, that is, the total polyphenolic content and the polyphenol oxidase and peroxidase enzyme systems have a significant effect on beer flavour and stability. In beer storage, phenolic constituents and "sensitive" protein complex to form haze and an off-flavour which markedly reduces the shelf-life of the beer. Oxidation enzymes present in the barley malt are believed to lessen the amount of these haze precursors in the beer by oxidizing the phenols during malt mashing in the brewery. In the oxidized state, polyphenols more readily condense intramolecularly and react with protein in the mash to form insoluble complexes which are partially removed in mash filtration.

In brewing, the amount of polyphenols in beer is reduced somewhat by various treatments that include adsorption onto nylon 66 or Polyclar AT. In this study, the possibility of regulation of polyphenols in the beer by barley breeding was examined. Methodology for the determination of polyphenolic components and for the assay of oxidase enzymes in barley and malt was studied; and the extent of genetic variability in barley was determined.

A number of methods were investigated for their utility in the estimation of phenolic properties of barley from plant breeding programs. The most practical method for the estimation of total polyphenols in barley appeared to be an automated colorimetric procedure based on the reaction between the

polyphenols in the barley and ammoniacal 4-amino antipyrine and potassium ferricyanide. Aqueous dimethyl formamide was used as an extractant and the test was calibrated with solutions of gallic acid. Data obtained by this method correlated highly with those of other procedures. The most suitable assays for polyphenol oxidase and peroxidase were found to be automated procedures in which the rate of the enzyme-catalyzed oxidation of substrates, catechol and catechol/hydrogen peroxide, were measured colorimetrically at 430 nm. and 510 nm. Optimum activity was at pH 6.6.

The results of tests made on plant breeders' lines and parental material showed that there are significant varietal differences in total polyphenols and enzyme activities. Gas chromatographic analyses suggested that there are qualitative differences in the phenolic composition of barley cultivars. A study of some properties of enzymes indicated that, in malting, their activities decrease during steeping and in the early stages of germination and then increase rapidly as germination proceeds; an application of gibberellic acid (GA) in malting stimulates enzyme syntheses and; there appears to be varietal differences in response to GA. Diethylammonium diethyldithiocarbamate effectively inhibits these enzyme systems.

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REVIEW OF THE LITERATURE

Introduction

The two most important properties of beer, stability and flavour characteristics, depend largely on the behaviour of its phenolic constituents. Freshly brewed beer contains a very complex mixture of simple and moderately complex polyphenolic materials derived directly from barley and also polymers and co-polymers formed during the malting and brewing processes. As the beer ages, it undergoes a series of deteriorative phenomena that includes development of an off-flavour and excessive colour and an accelerated formation of haze. The changes during malting, brewing and beer storage are mainly due to interactions of polyphenols with peptides and proteins in the presence of catalytic amounts of metals and molecular oxygen or through several anaerobic enzyme - catalyzed processes.

The main aim of the brewer is to produce beer with characteristics that appeal to a wide segment of the public. The extension of fresh flavour and improved stability over a longer period of time has obvious marketing and economic implications. This accounts for the intensive work on the polyphenol problem in Canada (22), Britain (8), the international Haze and Foam Group of the European Brewery Convention and at several industrial laboratories in the U.S.A. Although the problems associated with polyphenols have been appreciated for some time, the number of reports in the scientific literature of the brewing industry has recently increased. It is possible that technological changes in malting and brewing, while beneficial in some ways, may have aggravated the polyphenol situation, or that the availability of more sophisticated laboratory techniques has intensified the research. The influence of polyphenols can be

modified by various treatments during malting and brewing, but it is not unlikely that, for reasons in addition to those of a technical nature, minimization of treatment is desirable.

While brewing technology and industrial research projects will make the main contribution to the solution of beer quality problems, the characteristics of new cultivars of barley produced by plant breeders can be expected to have a significant effect. Most of the work reported by brewing research institutes relates to wort and beer, with some reference to barley and malt. Reports on varietal differences in barley and malt are very rare and concern only two or three cultivars. As far as is known, no attention is being given to the possible role of plant breeding. The purpose of the present work is, therefore, to examine methodology for the determination of polyphenolic components and for the assay of oxidase enzymes in barley and malt; to determine the extent of variability in these properties in barley; and to develop techniques for screening early generation breeding material. (It is noteworthy that, although polyphenols are a component of the extractable matter in hops, Harris (34) found that the contribution of barley malt to haze formation was more significant than that of hops).

The Nature of the Problem

The role of polyphenols in the colloidal stability of beer has been recognized for a long time, but the detailed mechanism of haze formation is still largely unknown. Over the past twenty-five years however there have been many reports (for example 34, 82, 31, 90, 36 and 91). Generally it is claimed that haze formation involves the gradual polymerization of polyphenols and their subsequent reaction or oxidative condensation with protein to form

insoluble complexes, and that acid and metal catalysts may be involved. The proteins in beer may vary in their sensitivity toward polyphenols (64) and protein sulphydryl groups may have some influence (17, 30). Hydrogen bonding may be involved (90) and evidence has been reviewed (9) that suggests a much firmer covalent linkage involving cystine may exist. The high level of cystine found in haze (25) would support this claim. Not all polyphenolic materials in malt contribute to haze formation, and evidence has been presented (79) which suggests that products in an intermediate state of oxidation are the active precipitants of beer protein. Molecular size and ability to polymerize influence the ability of phenolics to react with protein (22,31,90).

In contrast to their role in stability, the impact of polyphenols on flavour properties of beer was emphasized only recently (20). Phenolic acids and substituted phenols are a source of various undesirable flavours in beer (23). On the other hand, complete removal of polyphenols by adsorption on polyamide resins, results in a beer with poor organoleptic properties (16). The astringency of polyphenols (tannins) and its influence on taste is well known. Reduced polyphenols impart a "fresh" taste to beer while oxidized polyphenols produce an "aged" flavour (11).

The adverse effect of polyphenols on beer properties is associated with oxidation (83). Oxidation by molecular oxygen occurs during malt mashing and wort boiling and is catalyzed by phenol oxidases and peroxidases present in barley and malt. The role of polyphenol oxidases and peroxidases in malting and brewing with respect to haze formation, colour, flavour and collodial stability is therefore very important. The tanning power of malt polyphenols increases markedly on mashing as a result of oxidation, enabling them to react

with wort proteins to form insoluble complexes (15). It is generally accepted that the polymerization of polyphenols in mashing, enhanced by oxidase activity, is beneficial in that the polymerized polyphenols are complexed with protein and left behing in the lauter tun with the grains. However, some undesirable oxidized polyphenols which unfavourably affect the taste are also formed.

The activities of both these enzyme systems are reported to increase during malting germination (60), and they are able to survive in mashing at 70°C for one hour (85, 86). However, increased kilning temperatures led to a distinct increase in tannin and anthocyanogen contents of both worts and beers (51). This was attributed to a greater inactivation of phenol oxidases and polyphenol oxidases which prevented a further degradation of tannins during mashing. More extensive modification during malting, and presumably more synthesis of oxidases, has been related to better beer stability (14, 75). Brewing trials with green-malt containing substantial amounts of oxidases produced beer with good collodial stability (59). However, while promoting oxidation during mashing enhances stability, it may produce a harsh-flavoured beer (83). Well-modified and green-malts can be expected to have high levels of proteolytic as well as oxidative activities and there may be some modification of the protein moiety under these conditions.

Malt modification and its effect upon tannoid levels and stability of beer have been discussed by Gramshaw (32). Malts contain insoluble protein which complexes with polyphenols during mashing. These complexes are removed in filtration and remain in the lauter tun with the spent grains. Beers made from high nitrogen and undermodified malts contain more high molecular weight protein - that is, sensitive protein - than beers from well-modified malts. Beers high in sensitive protein are prone to haze formation, as only a small degree of poly-

merization of polyphenols will cause insoluble complexes (haze) to form. Beers with little sensitive protein require considerably more polymerization of polyphenols, even when the content of polyphenols is relatively high, before insoluble complexes appear. Although well-modified malts give beers of better collodial stability, it has been noted that such malts yield worts of higher rather than lower tannin contents (14, 74). This evidence suggests that in addition to the concentration of beer phenolics, their chemical composition may influence beer stability. A similar relationship between malt modification and beer phenolics have been noted by other workers (40, 52 and 61).

Several methods for increasing beer stability are used in the brewing process. The use of well modified malts, combined with mashing conditions that favour proteolysis is said to ensure that the content of sensitive proteins (that is, those that readily combine with polymerized polyphenols) is low. Chilling of beer before final filtration and longer cold storage aid stability. Low content in the beer of catalytic metals, iron and copper, and, low air content in bottles are important factors because of their influence on polyphenol polymerization. Adsorption of polyphenols on polyamides such as nylon 66 (34) and Polyclar AT (46) effectively reduce haze, and proteolytic enzymes added to the beer have been beneficial. The addition of formaldehyde during malt mashing (42) and in barley steeping (88) enhance the stability of the finished beer, as does the addition of hydrogen peroxide during mashing

or to filtered wort (59). All these techniques indicate that polyphenols and their oxidative polymerization are major factors in beer stability.

Review of Analytical Methods

A. Estimation of Total Polyphenols

1. Colorimetric methods: Measurement of the polyphenolic complex in barley and malt is difficult. This is because (a) the complex is heterogenous both with respect to the type of phenolic nuclei and the degree of their polymerization, and (b) no reagent is known that measures even one type of phenolic nucleus under these conditions. Nevertheless, a number of techniques have been used to provide at least an estimate of polyphenol content.

Harris and Ricketts (35) reported a method for the estimation of anthocyanogens in beer which was based on the well-known reaction of these compounds with acid to produce a red colour. The beer components were adsorbed on a polyamide resin, nylon 66; after heating with butanol-hydrochloric acid, the absorbance of the resulting red solution was read at 550 nm. Dadic (17, 18, 21) using an adaptation of this method, made simultaneous determination of anthocyanogens and catechins. He pointed out that the red product of these two groups has two absorption maxima, 550 nm. and 450nm., with anthocyanogens having a higher maximum at 550 nm.and catechins a maximum at 450 nm. This enabled Dadic to use a mathematical binary analysis technique to calculate the contribution of anthocyanogens and catechins to a total "tanninogen" value.

Most phenolic compounds are readily attacked by various oxidizing agents such as phosphomolybdate or permanganate. Many react with diazotized aromatic amines forming coloured compounds; and some form coloured complexes with certain metals. Based on these properties, other colorimetric methods for the estimation of phenolics have been developed.

The chromogenic reaction between iron salts and tannins has been used by DeClerck (24) and Jerumanis (39) to estimate polyphenols in brewing materials. The polyphenols were extracted by aqueous dimethyl formamide and the extract treated with alkaline (ammoniacal) ferric ammonium citrate. The absorbance of the product was read at 525 nm. The present tentative official method for polyphenol determination of the European Brewery Convention is an adaptation of Jerumanis' method. MacFarlane (43) and Ng and Mocek (53) used 4-amino phenazone (4-amino antipyrine) as a chromogenic reagent. The colour developed by the reaction of sample, the reagent and ammoniacal ferricyanide was read at 510 nm.

Singleton and Rossi (67) investigated colorimetric methods for total phenolic determinations based on phospho-tungstic and phospho-molybdic acid reagents. They concluded that the Folin-Ciocalteu reagent yielded better reproducibility and had less interference from non-phenols than the Folin-Denis reagent, on which the official method for measurement of tannins in wines and spirits is based (4). Singleton proposed the use of gallic acid as a reference standard and suggested that, although his work had been applied to wine, it is equally useful in the analysis of beer (66). Other reagents of the Folin type are also available. Snell (70) proposed the use of arseno-tungstic acid.

The reaction between diazotized aromatic amines and phenols was used by Woof and Pierce (89) as the basis of a semi-automatic method which proved to be useful in the study of the extraction of polyphenols from malt grist during brewery mashing. The amine used was p-aminobenzoic acid and the automated phase employed an auto-analyzer. The use of other amines (10, 41) and of