The colloidal stability of craft beers obtained with gluten-free adjuncts: an assessment of aspects related to technology, composition and analysis

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ABSTRACT

It is generally known that haze in beer is directly related to the formation of complexes resulting from the interaction of haze active polyphenols and a protein fraction.

This project aims to study issues related to the physical and chemical stability of craft beers. Specifically, the plan is to analyze how some variables in the malting process (i.e., pH of the first steeping water) and formulation (use of gluten-free adjuncts) can contribute to the colloidal stability (shelf-life) of the final beer.

Polypeptides responsible for haze formation originate mainly from barley and are rich in the amino acid proline. Incidentally, these proteins are also responsible for the immune reaction experienced by coeliacs; therefore haze prevention in beer and rendering the beer “gluten-free” could be compatible practices.

The primary aim of the study was to carry out micro malting tests in the laboratory on four gluten-free cereals/pseudocereals (millet, amaranth, buckwheat and quinoa) in order to identify the optimal conditions for obtaining malts suitable for the production of craft beers. The use of an alkaline solution in the first steeping may facilitate the extraction of proteinaceous and phenolic fractions from seeds; for this reason the pH of the first steeping water was modified. Quality of malts was checked analysing diastatic power, beta glucans content and Kolbach index (soluble nitrogen content as a percentage of total nitrogen).

Nine beers with different formulations were produced in the laboratory (2 L, micro-brewing): beer made with 100% barley malt (reference sample), beers

with 40% substitute gluten-free malt using the best malts obtained from the micromalting tests and 60% of barley malt. The use of gluten-free substitutes was intended to reduce the base level of the protein fractions rich in proline.

The evaluation of beer stability was carried out analyzing some indices validated by EBC (European Brewery Convention) (sensitive proteins and cold turbidity respectively), and two unconventional methods (gluten analysis and antioxidant activity, AA) to verify the possible correlation among these analyses and the official methods. Measurements of beer AA, which is mainly due to the polyphenol content, could give useful information on the colloidal stability of the final beer product.

Subsequently, the experimental plan was transferred on a larger scale; three beers were produced at the pilot plant of University of Udine (capacity of 200 L) using only the malt with the highest diastatic power (buckwheat): the first with 100% malted barley (reference sample), the other two with increasing amounts of malted buckwheat (20 and 40%). The colloidal stability of beer samples was tested with the same four analyses used to verify physico-chemical stability of the laboratory produced samples.

The results obtained from the micromalting tests were in line with the expectations: the four gluten-free cereal/pseudocereals were under-modified if compared to traditional malts, but potentially suitable as adjuncts (especially buckwheat).

The unconventional methods used to study the physico-chemical stability of craft beers provided encouraging results: either gluten analysis or the crocin test (AA) data showed trends correlated with those obtained with the validated method (alcohol chill haze). Specifically, the gluten analysis discriminated the beers produced at the pilot plant better than sensitive proteins (EBC method).

Based on the collected results, it can be concluded that the use of gluten-free adjuncts, combined with relevant process conditions (i.e. alkalinized first steeping water in the malting process), could lead to more stable final products with a gluten content less than 100 mg/L and so potentially suitable by coeliacs.

The colloidal stability monitoring of the beers produced at the pilot plant has already been planned, as well as the descriptive sensory analysis of the same samples.

RIASSUNTO

È noto che l'intorbidamento nella birra sia la diretta conseguenza della formazione di complessi risultanti dall'interazione tra frazioni polifenoliche e proteiche cosiddette attive.

Questo progetto ha lo scopo di studiare le problematiche legate alla stabilità chimico fisica delle birre artigianali. Nello specifico, l'obiettivo è stato quello di analizzare come alcune variabili del processo di maltazione (ad esempio il pH dell'acqua utilizzata nella prima bagnatura) o come la formulazione (utilizzo di succedanei privi di glutine), possano contribuire alla stabilità colloidale (*shelf-life*) del prodotto finale.

I polipeptidi responsabili dell'intorbidamento derivano principalmente dall'orzo e sono ricchi nella loro sequenza amminoacidica dell'amminoacido prolina. Queste frazioni proteiche sono inoltre responsabili delle reazioni di intolleranza nelle persone affette da celiachia; da ciò deriva l'idea che abbassare il contenuto di glutine nella birra possa stabilizzare la stessa verso i fenomeni di intorbidamento.

Quindi, il primo obiettivo di questo lavoro è stato individuare le condizioni ottimali di maltazione di quattro cereali/pseudocereali privi di glutine (miglio, amaranto, grano saraceno e quinoa) al fine di ottenere malti idonei per la produzione della birra artigianale, attraverso delle prove di micro maltazione effettuate in laboratorio. L'utilizzo di una soluzione alcalina nella prima bagnatura può facilitare l'estrazione dai semi di frazioni proteiche e polifenoliche; per questo motivo è stato modificato il pH dell'acqua impiegata nella prima bagnatura. La qualità dei malti ottenuti è stata controllata attraverso le analisi del potere diastatico, dei beta glucani e dell'indice di Kolbach (rapporto tra azoto solubile e totale).

Sono state, quindi, prodotte nove birre in laboratorio (micro-birrificazioni da 2 L): un riferimento ottenuto con solo malto d'orzo (100% p/p), e birre prodotte con il 40% p/p di succedaneo senza glutine (ottenuto dalle prove di micro maltazione), e il restante 60% p/p di malto d'orzo. L'impiego di succedanei senza glutine aveva l'obiettivo di ridurre il livello di base delle frazioni proteiche con un alto contenuto dell'amminoacido prolina.

La valutazione della stabilità colloidale delle birre è stata effettuata attraverso due indici dell'EBC (*European Brewery Convention*) (rispettivamente proteine sensibili e torbidità a freddo), e due metodi non convenzionali (analisi del glutine e dell'attività antiossidante, AA), ed è stato valutato il grado di correlazione tra le diverse metodiche. La misura dell'AA dei campioni di birra, principalmente determinata dai composti polifenolici contenuti nei medesimi campioni, potrebbe fornire informazioni utili sulla stabilità colloidale della birra.

Successivamente, il piano sperimentale è stato trasferito su scala maggiore; sono state prodotte tre birre presso l'impianto pilota universitario (micro-birrificazioni da 200 L): la prima con il 100% di malto d'orzo, e le altre due con quantità crescenti di grano saraceno maltato (20 e 40%). La valutazione della stabilità colloidale delle birre è stata realizzata con le medesime analisi utilizzate per i campioni di birra prodotti in laboratorio.

I risultati ottenuti con le prove di micro-maltazione sono in linea con le attese: i quattro cereali/pseudocereali senza glutine sono risultati sotto-modificati rispetto ad un tradizionale malto, ma potenzialmente impiegabili come succedanei per la produzione di birra (in modo particolare il grano saraceno).

Le due metodiche non convenzionali, utilizzate per valutare la stabilità chimico fisica delle birre artigianali, hanno fornito risultati incoraggianti: sia i dati ottenuti con l'analisi del glutine che quelli ottenuti con il test della crocina (AA)

hanno evidenziato andamenti correlabili a quelli ottenuti con il metodo validato (test della torbidità a freddo). Nello specifico, l'analisi del glutine discriminava meglio le birre prodotte presso l'impianto pilota rispetto al test delle proteine sensibili (metodo EBC).

Sulla base dei risultati raccolti, può essere dedotto che l'impiego di succedanei privi di glutine, affiancato ad adeguate condizioni di processo (ad esempio l'alcalinizzazione dell'acqua impiegata nella prima bagnatura del processo di maltazione), potrebbe portare alla produzione di birre più stabili e con un contenuto di glutine inferiore a 100 mg/L e, quindi, idonee per le persone affette da celiachia.

Inoltre, è stato pianificato sia il monitoraggio della stabilità colloidale che l'analisi sensoriale delle birre prodotte presso l'impianto pilota.

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1. OVERVIEW OF THE BREWING PROCESS

1.1 BEER

Along with bread and wine, beer is one of the oldest foods created by man. The first documented beer recipe dates back to 4000 B.C.: it was the Sumerians who created the first fermented alcoholic beverage that could be considered beer.

Beer should for all intents and purposes be considered both a beverage and a food. In fact, it contains many substances that are necessary for our bodies: water, mineral salts, vitamins, aminoacids, maltodextrin, enzymes, antioxidants and fiber, all of which make beer a useful complement to a healthy and balanced diet. In addition to the nutrients it contains, we have to consider that beer has a low alcohol content and that the Mediterranean diet entails usually drinking it in moderation and with meals. According to a vast amount of international scientific literature, moderate beer consumption may have beneficial effects on health.

Italy's brewing sector is an increasingly important part of the Italian agrifood industry, that can, and wishes to, contribute to the country's economic and social revitalization. It produces wealth for the country, and its share of the domestic market is continuously increasing. It has strengthened its ties with the nation's agricultural sector and with local communities, in which it is an increasingly significant player. It has consolidated its image abroad, thanks to a product that is on par with that of other countries with a strong beer tradition. These are the highlights that emerge from AssoBirra's 2012 Annual Report: increasing production by 0.5%, essentially maintaining the value of exports (which in 2011 reached an all-time high of 2 million hL), increasing direct occupation by 4%, generating a total of 4 billion euros in tax revenues for the country.

Facilities (16 industrial plants and approximately 500 microbreweries) in Italy produced 13,482,000 hL of beer, equivalent to +0.5% compared to 2011 (13,410,000 hL), 1,990,000 (14.8% of the total) of which were exported, while the remaining part satisfied 65.1% of domestic demand for beer, which amounted to 17,636,000 hL (-0.5% compared to 2011).

In terms of destinations, compared to 2011 the portion absorbed by the EU market, with the United Kingdom holding the lion's share with over 1 million hL, decreased (from 74% to 66%); among non-European countries, noteworthy performance was seen in the United States (over 217,000 hL, +16% compared to 2011), Australia (over 20,000 hL, +53.8%) and South Africa (almost 205,000 hL, +66%), all of which are English-speaking countries with a strong beer-drinking tradition.

Germany confirmed itself as the main exporter of beer to Italy, with almost 3,200,000 hL (52% of the total), followed by the Netherlands (9.7%), Belgium/Luxembourg (7.4%), Denmark (5.3%), Slovenia (4%) and the United Kingdom (3,4%). Overall, almost all (96%) of the demand for beer in Italy that is not met by domestic production is still covered by imports from other EU countries, accounting for 34.9% of the domestic market.

In 2012 too, as in 2011, Europe's brewing sector experienced slower growth than the Italian one: the production of 29 countries (the 27 countries of the EU as of 2012, plus Switzerland and Norway) amounted to 389,470,000 hL, -0.4% compared to 2011. Italy strengthened its standing among beer producing countries, at ninth place. The leading producer remains Germany, which alone accounts for almost 24% of total production, followed by the UK (10.8%) (Figure 1.1, Table 1.1) (www.assobirra.it).

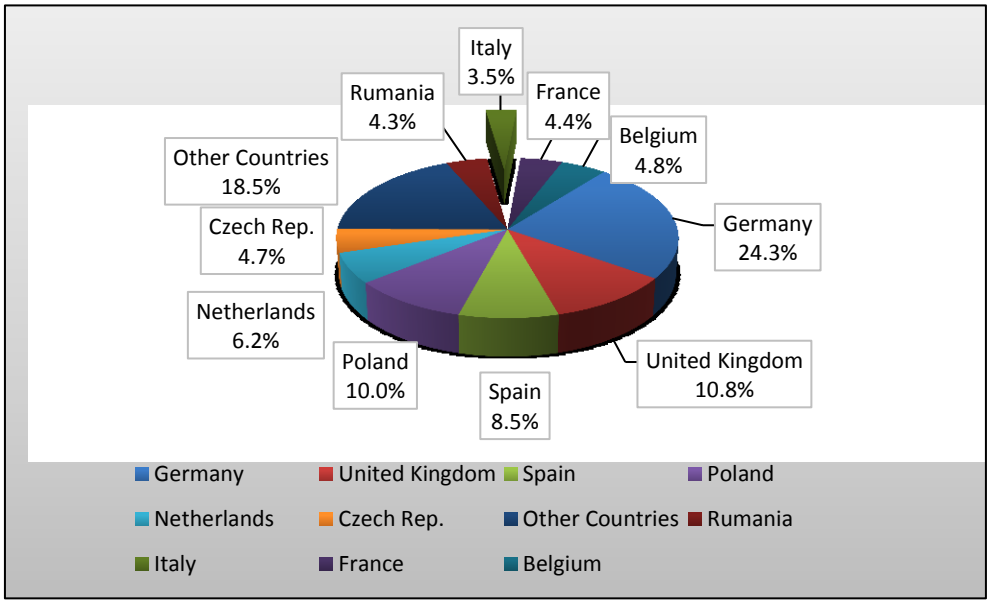


Figure 1.1 Beer production in Europe (as % of total production): most significative countries (Source: AssoBirra & The Brewers of Europe 2012, www.assobirra.it).

Table 1.1 Beer in Europe: PRODUCTION 2007-2012 (000 hL) (Source: www.assobirra.it).

	2007	2008	2009	2010	2011	2012
Germany	100.628	99.910	98.078	95.863	95.545	94.618
United Kingdom	51.341	49.469	45.141	44.997	45.701	42.049
Spain	34.343	33.402	33.825	33.375	33.573	33.000
Poland	36.895	37.108	35.992	36.621	37.854	39.294
Netherlands	27.259	27.181	25.376	24.218	23.644	24.200
Czech Rep.	19.897	19.806	18.187 *	17.020	17.705	18.165
Belgium	18.480	18.044	18.009	18.123	17.705	18.751
France	15.094	14.777	14.731	14.290	15.910	17.132
Italy	**13.462**	**13.343**	**12.776**	**12.814**	**13.410**	**13.482**
Austria	9.044	8.937	8.728	8.670	8.917	8.927
Denmark	7.604	6.474	6.046	6.335	6.335 ***	6.080
Reland	9.270	8.846	8.041	8.249	8.249 ***	8.195
Hungary	7.584	7.102	6.348	6.2956.295	6.249	6.159
Portugal	8.191	8.208	7.833	8.312	8.312 ***	7.986
Finland	4.547	4.470	4.491	4.491	4.491	4.030
Slovakia	3.683	3.558	3.264	3.112	3.124	3.206
Greece	4.340	4.374	4.177	4.178	4.178 ***	4.178 ***
Sweden	4.428 *	4.288 *	4.455	4.354	4.354 ***	4.354 ***
Lithuania	3.225 *	3.074 *	2.794	2.664	3.050	3.050 °
Luxemburg	322 **	312 **	325 **	302 **	302 ***	302 ***
Slovenia	1.546 **	1.553	1.443	1.390	1.640	1.556
Malta	110 **	112 **	104 **	414 **	128	128 °
Cyprus	386	399	355	340	316	316 °
Bulgaria	5.298	5.358	4.825	4.800	4.820	4.820 °
Latvia	1.410	1.307	1.357	1.455	1.455 ***	1.455 ***
Estonia	1.413	1.275	1.234	1.312	1.312 ***	1.312 ***
Swiss	3.532	3.625	3.555	3.539	3.546	3.515
Norway	2.553	2.560	2.516	2.435	2.346	2.310
Rumania	19.554	20.640	17.600	16.920	16.900	16.900 °
Total	**415.438**	**409.512**	**391.606**	**386.888**	**391.071**	**389.470**

*** Eurostat ** Canadian Global beer trend 2009 edition *** Dato 2010_*Datum 2010* ° Dato 2011_*Datum 2011***

In 2012 average consumption per capita in the EU (plus Switzerland and Norway) went down to 71.5 L (-4.2%), with more or less accentuated decreases in all the major beer-consuming countries (Czech Republic 144, Austria 107.8, Germany 105, Ireland 85.6, Luxembourg 85, Belgium 74, United Kingdom 68.5). This led to the result that Italy, although remaining last in terms of consumption, for the fourth consecutive year narrowed - albeit slightly - the gap with the EU

average. In 2012 Italy's consumption accounted for 29.5 L per capita, compared to 29.8% in 2011 (Table 1.2). However, this consumption is still 3 to 5 times less than that of Europe's leading consumers and less than that of countries similar to Italy in terms of history and geography such as Spain, Portugal, Greece and France.

Table 1.2 Beer in Europe: liters per capita consumption 2011-2012 (Source: www.assobirra.it).

	2011	2012
Czech Rep	154.0	144.0
Germany	107.2	105.5
Austria	108.3	107.8
Ireland	90.0 ***	85.6
Luxemburg	85.0 ***	85.0 ***
United Kingdom	71.6	68.5
Belgium	145.0	74.0
Spain	48.2	47.5
Denmark	68.0	90.0
Slovakia	70.2	72.9
Netherlands	71.7	72.3
Poland	95.0	98.0
Slovenia	81.0	77.8
Bulgaria	69.0	69.0 °
Portugal	59.0	49.0
Sweden	53.0 ***	53.0 ***
Malta	45.2 *	45.2 °
Greece	38.0 ***	38.0 ***
Swiss	57.9	57.3
France	30.0	30.0
Rumania	89.0	89.0 °
Norway	59.0	45.9
Italy	29.8 ****	29.5
Total	74.7	71.5

*** Canadian Global beer trend 2009 edition**
**** Non disponibile_*Not available***
***** Dato 2010_*Datum 2010***
° Dato 2011_*Datum 2011*
****** *Datum rectified following the 2011 census by ISTAT***

Across the past two decades, the global beer industry has become globalized in the same sense as other familiar branded products which originate in one country and later are manufactured and consumed throughout the world. The pace of globalization for beer has greatly accelerated over this period with the increased activity of multinational beer enterprises acquiring existing breweries and constructing new facilities in emerging markets, as well as licensing production of their brands outside their home countries.

The Global Beer Market grew by over 2% (per capita alcohol consumption continues to rise), pushed forwards by impressive performances in the key emerging markets; Africa, Asia and Latin America. Whilst more mature markets (West Europe and North America) fell, being restricted by weak economies (Figure 1.2).

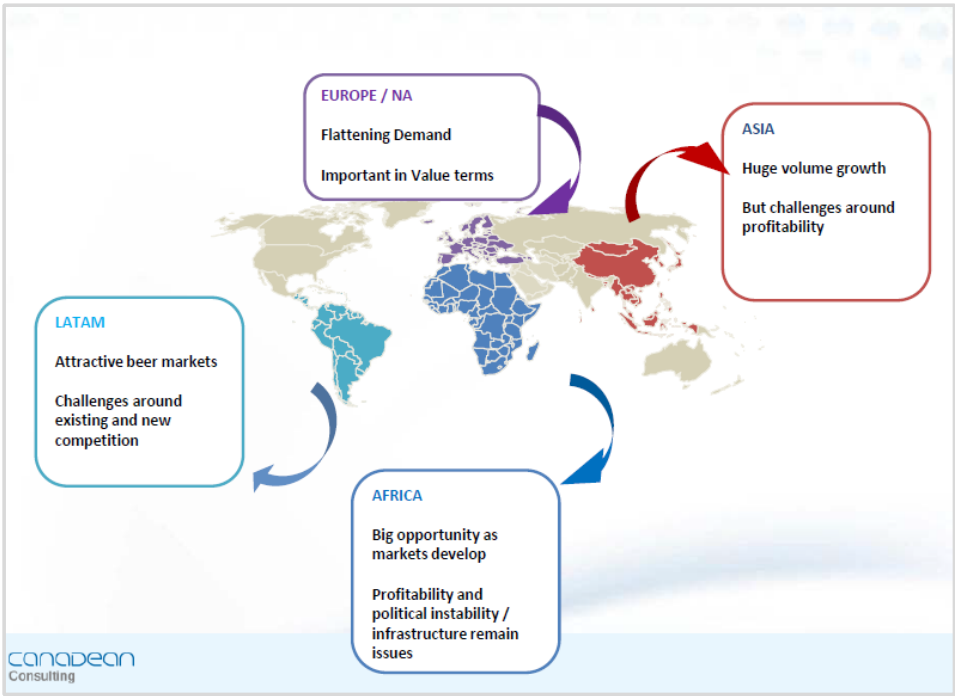


Figure 1.2 Global beer market trends (Source: www.canadean.com).

1.2 RAW MATERIALS

1.2.1 Barley

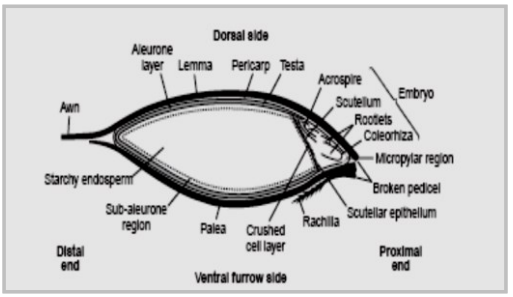


Figure 1.3 A schematic longitudinal section of a barley grain, to one side of the ventral furrow and the sheaf cells (Source: Briggs et al., 2004).

The most simple preparation of European-style beers involves (a) ground up cereal grains (usually barley malt) with warm water. Sometimes the ground malt is mixed with other starchy materials and/or enzymes. (b) The solution obtained is boiled with hops or hop preparations. (c) The boiled solution is clarified and cooled. (d) The cooled liquid is fermented by added yeast.

Barley, almost always in the form of malt, provides the bulk of the extract for most worts, and is an essential source of non-sugar nutrition for yeast comprising amino acids, vitamins and minerals. The barley grain or corn has a complex structure (Figure 1.3), and is a single-seeded fruit (acaryopsis). Barley varieties differ in their suitabilities for malting. Barley plants are annual grasses. Some are planted in the autumn (winter barleys) while others are planted in the spring (spring barleys). Grains are arranged in rows, borne on the head, or ear. The number of rows varies, being two in two-rowed varieties and six in six-rowed forms. Grains vary in size, shape and chemical composition. The barley corn is elongated and tapers at the ends (Figure 1.3). The dorsal, or rounded side is covered by the lemma, while the ventral, grooved or furrow side is covered by the palea. Together these units constitute the husk. Within the testa, at the base of the grain, is the small embryo. This is situated towards the dorsal side of the grain.

The embryonic axis consists of the coleoptile (the maltster's `acrospire') pointing towards the apex of the grain and the root sheath (coleorhiza) which surrounds several (typically five) embryonic roots. This appears at the end of the grain, at the onset of germination, as the `chit'. The axis is the part of the embryo that can grow into a small plant. It is recessed into an expanded part of the embryo called the *scutellum* (Latin, `little shield'). Unlike the *scutellum* in oats, in barley this organ does not grow. Its inner surface, which is faced with a specialized epithelial layer, is pressed against the largest tissue of the grain, the starchy endosperm. With the exception of the embryo all the tissues mentioned so far are dead. The starchy endosperm is a dead tissue of thin-walled cells packed with starch granules embedded in a protein matrix. The outer region of the starchy endosperm, the sub-aleurone layer, is relatively richer in protein (including β-amylase) and small starch granules but poor in large starch granules. Where the starchy endosperm fits against the *scutellum* the cells are devoid of contents and the cell walls are pressed together, comprising the crushed-cell or depleted layer. The starchy endosperm, away from the sheaf cells, is surrounded by the aleurone layer (which botanically is also endosperm tissue). Malting can be understood only by reference to the grain structure and the interactions which occur between the tissues (Briggs *et al*., 2004).

For making malt, barley must be of a suitable malting variety, sufficiently low in protein (11% ÷ 13% as N × 6.25), adequately free of dockage and skinned and broken materials, highly viable (at least 96%) and quite low in moisture (12% ÷ 14%), and the lot should have a high proportion of plump grains. The enemies of barley in storage are microbes, insects and grain respiration and neither dryness nor coolness protects them from all the enemies; the grain must be stored cool and dry. The grain must be moved and cleaned on a regular basis. Prolonged dry storage permits the grain to pass through dormancy and water sensitivity (most

easily construed as residual dormancy) until it is ready for malting (Bamforth, 1999).

1.2.2 Adjuncts (gluten-free cereals/pseudocereals)

Cereals not containing gluten include: rice (*Oryza sativa*), maize (*Zea mais*), sorghum *(Sorghum bicolor*), and millets (e.g. *Panicum miliaceum*, *Setaria italica*, *Pennisetum typhoideum* and *Eleusine coracana*). Other carbohydrate-rich pseudocereals without gluten are buckwheat (*Fagopyrum esculentum*), quinoa (*Chenopodium quinoa*), and amaranth (*Amaranthus*) (Zarnkow *et al*., 2005).

Millet

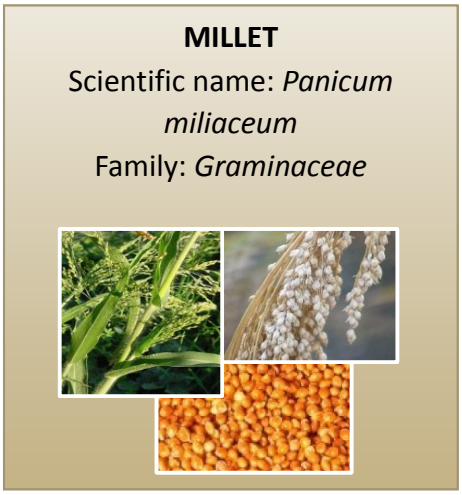


Figure 1.4 (Source: http://faostat.fao.org/).

Millets are not a single species, or even different species within a single genus. They are simply cultivated grasses (cereals) that have small kernels and they are grouped together solely on this basis. The word millet is derived from the French word “mille” meaning thousand, implying that a handful of millet contains thousands of grains. In fact, as can be seen in Table 1.3 there are many different millets, some of which are closely related, like proso millet and little millet, and others which are not, in particular finger millet and teff, which belong to a different tribe to most of the other millets. The study of millet literature is problematical because different common names are used for the same species and even different proper species names are in widespread use. In this account, the English name as given in

the table will be used when discussing each species but the list of vernacular names should help when reading the literature (Arendt and Dal Bello, 2008).

Proso millet (*Panicum miliaceum L.*) (Figure 1.4) is a small C4- metabolism cereal plant that yields flattened kernels in a short time (60-90 days). The inflorescence is a slender panicle up to 45 cm long, which may be open or compact. The caryopses (2 mm long and 2 mm wide) are covered with smooth, hard and shiny glumes (lemma and palea). The kernels are generally white, oval and smooth (Angold, 1979; Hulse *et al*., 1980) with a 1000-kernel weight of approximately 5 g. Proso millet starch granules of the corneous endosperm are angular, whereas the ones located in the floury area are spherical (Lorenz, 1977), their size ranges from 3 ÷ 21 µm (8 ÷ 16.5 µm in the peripheral, 3 ÷ 19 µm in the corneous and 11 ÷ 21 µm in the floury zone) (www.brenda-enzymes.info).

Table 1.3 The different millet species. Information mainly from the USDA Germplasm Resources Information Network (GRIN) (Source: www.ars-grin.gov/cgi-bin/npgs).

Generally used English name	Other common vernacular names	Taxonomy
Finger millet	Ragi Wimbi	Tribe Eragrostideae *Eleucine coracana* L. Gaertn.
Teff	Tef Teff grass Abyssinian lovegrass	Tribe Eragrostideae *Eragrostis tef* (Zuccagni) Trotter
Job's tears	Adlay Adlay millet	Tribe Andropogoneae *Coix lacryma-jobi* L.
White fonio	Fonio Acha Fonio millet Hungry rice	Tribe Paniceae *Digitaria exilis* (Kippist) Stapf
Black fonio	Black acha Hungry rice	Tribe Paniceae *Digitaria iburua* Stapf
Japanese millet	Japanese barnyard millet	Tribe Paniceae *Echinochloa esculenta* (A. Braun) H. Scholz
Sawa millet	Shama millet Awnless barnyard grass Corn panic grass Deccan grass Jungle ricegrass Jungle rice	Tribe Paniceae *Echinochloa colona* (L.) Link
Proso millet	Common millet Broom millet Hog millet Panic millet	Tribe Paniceae *Panicum miliaceum* L. subsp. *miliaceum*
Little millet	Blue panic Sama	Tribe Paniceae *Panicum sumatrense* Roth.
Kodo millet	Creeping paspalum Ditch millet Indian paspalum Water couch	Tribe Paniceae *Paspalum scrobiculatum* L.
Foxtail millet	Italian millet Foxtail bristle grass German millet Hungarian millet	Tribe Paniceae *Setaria italica* (L.) P. Beauv. subsp. *italica*
Pearl millet	Bulrush millet Cattail millet Babala Bajra/Bajira	Tribe Paniceae *Pennisetum glaucum* (L.) R. Br.
Guinea millet	False signal grass	Tribe Paniceae *Urochloa deflexa* (Schumach.) H. Scholz

Germplasm Resources Information Network (2007).

Buckwheat

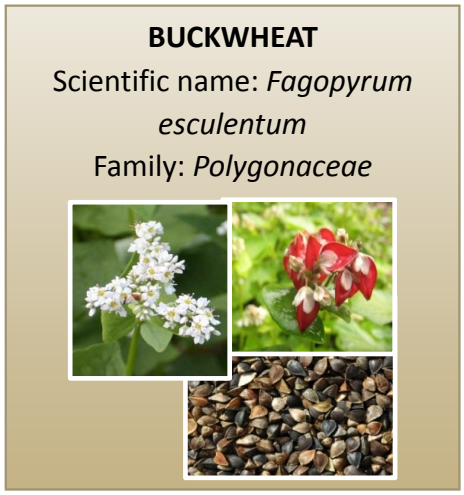


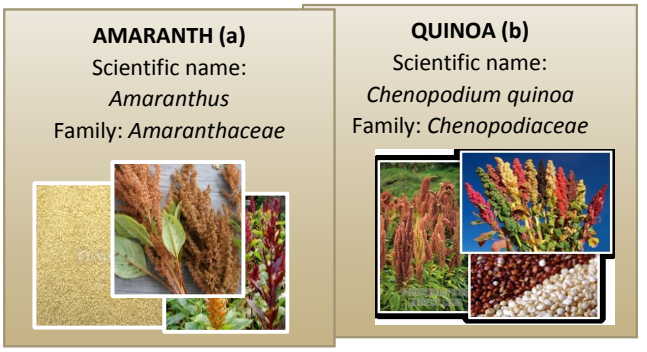
Figure 1.5 (Source: http://faostat.fao.org/).

Buckwheat (*Polygonaceae* family) (Figure 1.5) is a traditional crop in Asia and Central and Eastern Europe. There are three types of buckwheat: *cymosum* (wild), *tartaricum* (tartary) and *esculentum* (common) (Aufhammer, 2000). *Fagopyrum esculentum* is the most economically important species, making up approximately 90% of the world production of buckwheat (Mazza, 1993). Almost all of the buckwheat plant can be utilized for a variety of applications. The buckwheat flower is used as an excellent honey source, the hull is used for the filling of pillows and the grain is used as a basic material for a wide range of products (i.e., pancakes and pasta). Buckwheat can also be used to make malt (Belton and Taylor, 2004; Wijngaard *et al*., 2006; Nic Phiarais *et al*., 2005).

In recent years, buckwheat has regained importance as an alternative crop for organic cultivation and as an ingredient for health food products (Skrabanja *et al*., 2004). Buckwheat achenes have proven to be similar to cereal grains: they consist predominantly of starch, they are edible and they possess a starchy endosperm and a non-starchy aleurone layer (Bonafaccia *et al*., 2003). On the other hand, buckwheat shows botanical differences to cereal grains. Buckwheat is a dicotyledonic plant, whereas barley is monocotyledonic. Therefore, buckwheat does not belong to the grass and cereal family (*Poaceae*). It is classified as a so-called pseudocereal (Wijngaard and Arendt, 2006).

Amaranth and quinoa

Amaranth (*Amaranthaceae* family) (Figure1.6a) **a**nd quinoa (*Chenopodiaceae* family) (Figure 1.6b) were major crops for the Pre-Colombian cultures in Latin-America. After the Spanish conquest, however, consumption and cultivation of these crops was suppressed and thereafter only continued in a small scale. Since it has been shown that both grains show good nutritional properties, the interest in them has risen again. The production of quinoa was 25,329 tonnes in Bolivia, 652 tonnes in Ecuador, and 32,590 tonnes in Peru in the year 2006 (FAOSTAT, 2006). Amaranth and quinoa cultivation remain relatively low, amaranth is not even listed in the FAO statistics on production data, although an appreciable commercial cultivation of amaranth for human nutrition does take place. Besides Latin American countries, it is produced in the USA, China, and Europe. Amaranth and quinoa are dicotyledonous plants and thus not cereals (monocotyledonous), but since they produce starch-rich seeds like cereals they are called pseudocereals. Over 60 species of amaranth are known worldwide. The main grain amaranth species used today are *Amaranthus caudatus* L. (syn. *edulis* Spegazzini), *Amaranthus cruentus* L. (syn. *paniculatus* L.), and *Amaranthus hypochondriacus*. Among quinoa sweet and bitter varieties exist, dependent on the content of saponins (i.e. if the saponin content is below 0.11% the variety is considered to be a sweet variety) (Koziol, 1991). Amaranth seeds are lentil-shaped



Figures 1.6a, 1.6b (Source: http://faostat.fao.org/).

and measure about 1 mm in diameter. The 1000 kernel weight is only 0.5-1.4 g. Quinoa seeds are slightly larger than amaranth seeds, the 1000 kernel weight is approximately 1.9-4.3 g.

1.2.3 Water

Historically, different regions became famous for particular types of beer and in part these beer types were defined by the waters available for brewing (Table 1.4). Thus Pilsen, famous for very pale and delicate lagers has, like Melbourne, very soft water. Burton-on-Trent, with its extremely hard water, rich in calcium sulphate, is famous for its pale ales while Munich is well-known for its dark lagers, and Dublin (which has similar soft water) for its stouts. Breweries may receive water from different sources, which may be changed without warning. Water supplies may vary in their salt contents between day and night, from year to year and between seasons (Rudin, 1976). It is now usual for breweries to adjust the composition of the water they use.

Breweries use large amounts of water, (`liquor' in the UK). The actual amounts of water used ranging from three to (exceptionally) 30 times the volumes of beer produced. As beers usually have water contents of 91-98% (or even 89% in the cases of barley wines), and the amounts lost by evaporation and with by-products are relatively small it follows that large volumes of waste water are produced. Apart from brewing, sparging and dilution liquors, water is used for a range of other purposes. These include cleaning the plant using manual or cleaning-in-place (CIP) systems, cooling, heating (either as hot water or after conversion into steam in a boiler), water to occupy the lines before and after running beer through them, for loading filter aids such as *kieselguhr*, for washing

yeast and for slurrying and conveying away wastes as well as for washing beer containers such as tankers, kegs, casks and returnable bottles (Koch and Allyn, 2011). The acquisition and treatment of liquor and the disposal of the brewery effluents are expensive processes and have long been studied. Most regions have strict regulations, which must be met before water is classified as being potable, and these provide the *minimum* standards for brewing waters (Armitt, 1981; Bak *et al*., 2001; Baxter and Hughes, 2001). These regulations are often reviewed, the upper permitted limits for specified substances are frequently reduced and the numbers of substances mentioned are increased. Tables 1.5a and 1.5b (see pp. 18 ÷ 20) indicate how complex these `*minimum* standards' can be. The requirements may be grouped as `aesthetic' (color, turbidity, odor and taste), microbiological standards (particularly the absence of pathogens), the levels of organic and inorganic materials that are in solution and the presence of radioactive materials.

Ions present in brewing water have a range of effects on the production process and the quality of the product. Calcium ions (Ca^{2+}) serve several important functions in brewing. They stabilize the enzyme α-amylase during mashing and, by interacting with phosphate, phytate, peptides and proteins in the mash and during boil, the pH values of the mash and the wort are usefully reduced. If bicarbonate ions are also present (the water has temporary hardness) these can more than offset the effect of calcium and cause arise in pH. Perhaps the concentration of calcium ions should not greatly exceed 100 mg/L in the mashing liquor as no great advantage is gained from higher doses and there is the risk that too much phosphate may be removed from the wort, and the yeast may then have an inadequate supply. Another recommendation is that calcium should be in the range 20 ÷ 150 mg/L depending on the beer being made. Iron ions (Fe^{2+}, ferrous and Fe^{3+}, ferric) can occur in solution, for example, as ferrous bicarbonate or complexed with organic materials. Ferrous water is undesirable for brewing

purposes, since it can deposit slimes (probably after oxidation, as red-brown hydrated ferric hydroxide), which can block pipes, filters, ion exchange columns, reverse osmosis equipment, etc. The ions, possibly because of their ability to act as oxidation/reduction catalysts, favor haze formation and flavor instability. At concentrations of >1 mg/L iron ions are harmful to yeasts. Perhaps concentrations should be reduced to less than 0.1 mg Fe/L. For all these reasons, and because of the difficulties that they can cause in some water treatments, it is usual to reduce the levels of dissolved iron early in a water treatment process. Copper (Cu^{2+}) presented problems in brewing when vessels and pipework were made of copper but since these have come to be made of stainless steel there have been fewer problems with dissolved copper in breweries. Copper ions are toxic and mutagenic to yeasts, which accumulate them and develop `yeast weakness'. Another source of copper ions was the older, copper-based fungicides applied to hops. Copper ions are oxidation/reduction catalysts and their presence favors flavor instability and haze formation in beer. Brewing liquor should contain <0.1 mg copper/L. Zinc (Zn^{2+}), if present in appreciable amounts in brewing water, usually indicates that this ion has been picked up during transfer or storage. High concentrations in ground waters are unusual. At high levels this substance can be toxic, the upper permitted concentration in potable water is 5 mg/L (Table 1.5a). High concentrations are damaging to yeasts but small amounts are essential. Not infrequently the levels of zinc in worts are insufficient to maintain good fermentations and in these cases the worts may be supplemented with additions of zinc chloride (0.15 ÷ 0.2 mg/L). The recommended range in brewing liquor is 0.15 ÷ 0.5 mg/L (Briggs *et al*., 2004; Denny, 2009).

Table 1.4 Analyses of some waters from famous brewing centres, (expressed as mg/L). The analyses of these, or any waters do not remain constant with time (Source: Moll, 1995; Mailer et al.,1989).

Parameter	Pilsen	Burton-on-Trent		München (Munich)		London	Wien	Melbourne
Tot. dry solids	51	-	1226	536	273	320	984	25
Ca^{2+}	7.1	352	268	109	80	90	163	1.3
Mg^{2+}	3.4	24	62	21	19	4	68	0.8
HCO_3^-	14	320	-	171	-	-	243	-
CO_3^{2-}	-	-	141	-	164	123	-	3.6
SO_4^{2-}	4.8	820	638	7.9	5	58	216	0.9
NO_3^-	tr.	18	31	53	3	3	tr.	0.2
Cl^-	5.0	16	36	36	1	18	39	6.5
Na^+	-	-	30	-	1	24	-	4.5

tr. = Traces.
- = Not given.

Table 1.5a A list of the maximum (minimum) concentrations of substances that may not be exceeded in drinking water in the UK in 2001 (Source: Briggs et al., 2004).

Parameter	Units	Concentration or value
Colour	mg/L (Pt/Co scale)	20
Turbidity	Formazin units	1
Odour	Dilution number	3 at 25°C
Taste	Dilution number	3 at 25°C
Temperature	°C	25
pH (limits)	pH units	6.5-10.0
Conductivity	µS/cm at 20°C	2500
Permanganate value	O_2, mg/L	5
Permanganate value	C, mg/L	no significant increase
Total coliform bacteria	number/100mL	0
Faecal coliform bacteria	number/100mL	0
Faecal Streptococci, Enterococci	number/100mL	0
Clostridium perfringens	number/100mL	0
Sulphate reducing Clostridia	number/20mL	≤1

Parameter	Units	Concentration or value
Colony counts	number/mL at 25 or 37°C	no significant increase
Radioactivity (total indicative dose)	MSv/year	0.1
Tritium	Bq/L	100
Boron	B mg/L	1
Chloride	Cl, mg/L	250
Calcium	Ca, mg/L	250
Total hardness	Ca, mg/L	60 (minimum)
Alkalinity	HCO_3, mg/L	30 (minimum)
Sulphate	SO_4, mg/L	250
Magnesium	Mg, mg/L	50
Sodium	Na, mg/L	200
Potassium	K, mg/L	12
Dry residues (after 180°C)	mg/L (Pt/Co scale)	1500
Nitrate	NO_3, mg/L	50
Nitrite	NO_2, mg/L	0.5
Ammonia, ammonium ions	NH_4, mg/	0.5
Kjeldahl nitrogen	N, mg/L	1.0
Dissolved or emulsified hydrocarbons		
Mineral oils	µg/L	10
Benzene	µg/L	1
Phenols	C_6H_5OH, µg/L	0.5
Surfactants (detergents)	as lauryl sulphate, µg/L	200
Aluminium	Al, µg/L	200
Iron	Fe, µg/L	200
Manganese	Mn, µg/L	50
Copper	Cu, mg/L	2
Zinc	Zn, mg/L	5
Phosphate	P, mg/L	2.2
Fluoride	F, mg/L	1.5
Silver	Ag, µg/L	10
Arsenic	As, µg/L	10
Bromate	BrO_3, µg/L	10
Cadmium	Cd, µg/L	5

Table 1.5b A list of the maximum (minimum) concentrations of substances that may not be exceeded in drinking water in the UK in 2001 (Source: Briggs et al., 2004).

Parameter	Units	Concentration or value
Cyanide	CN, µg/L	50
Chromium	Cr, µg/L	50
Mercury	Hg, µg/L	1
Nickel	Ni, µg/L	20
Lead	Pb, g/l (will be reduced in 2013)	25
Antimony	Sb, µg/L	5
(Elsewhere limits are set on other substances, such as thallium, beryllium, uranium and asbestos)		
Acrylamide	µg/L	0.1
Vinyl chloride	µg/L	0.5
Epichlorohydrin	µg/L	0.1
Aldrin	µg/L	0.03
Dieldrin	µg/L	0.03
Heptachlor	µg/L	0.03
Heptochlorepoxide	µg/L	0.03
Other pesticides	µg/L	0.1
Pesticides, total	µg/L	0.5
Polycyclic aromatic hydrocarbons*	µg/L	0.1
Benzo(a)-3,4-pyrene	µg/L	10
1,2-Dichloroethane	µg/L	3
Tetrachloromethane	µg/L	3
Trichloroethane	µg/L	10
Tetrachloroethane & trichloroethene	µg/L	10
Trihalomethanes, total**	µg/L	100
Substances extractable in chloroform	mg/L, dry residue	1

***Sum of individual concentrations of members of a list of substances benzo[b]fluoranthene, benzo[k]fluor- anthene, benzo-11,12-fluoranthene, benzo[ghi]perylene and indeno-[1,2,3-cd]pyrene.**

****Sum of chloroform, bromoform, dibromochloromethane and dibromodichloromethane.**

1.2.4 Yeast

Kurtzman and Fell (1998) define yeasts as being fungi with vegetative states that reproduce by budding or fission resulting in growth that is predominantly in the form of single cells. Yeasts do not produce sexual states within or upon a specialized fruiting body. This definition is relatively imprecise since many fungi are dimorphic. During certain phases in their life cycles, such fungi adopt a yeast-like unicellular form and at others they take on a filamentous hyphal habit and develop into a mycelium. Brewing yeast strains are ascomycetous types classified within the genus *Saccharomyces*. The precise taxonomy of the fungi in general and the *Saccharomyces* in particular is still subject to debate and continual revision. A current version is given in Table 1.6. At present, the genus *Saccharomyces* is divided into 14 species (Briggs *et al*., 2004; Koch and Allyn, 2011).

Table 1.6 Classification of Saccharomyces cerevisiae. (Source: Briggs et al., 2004).

Taxon	Name	Comments
Kingdom	Fungi	
Phylum	*Ascomycotina*	Teliomorphic forms characterized by formation of ascospores enclosed within ascus
Sub-phylum	*Saccharomycotina* (syn. *Hemiascomycotina*)	
Class	Saccharomycetes (syn. *Hemiascomycetes*)	Single ascus not enclosed in ascocarp developing directly from zygotes
Order	*Saccharomycetales* (syn. *Endomycetales*)	Yeast-like cells, rarely developing hyphae
Family	*Saccharomycetaceae*	
Genus	*Saccharomyces*	Globose, ellipsoidal or cylindroidal cells. Vegetative reproduction by multilateral budding. Pseudohyphae may be formed but hyphae are not septate. The vegetative form is predominantly diploid, or of higher ploidy. Diploid ascopores may be formed that are globose to short ellipsoidal with a smooth wall. There are usually 1-4 ascopores per ascus
Type species	*S. cerevisiae*	

Taxonomists seem to have struggled for a number of years with the names that should be ascribed to brewing strains. Ale yeast has long been referred to as *Saccharomyces cerevisiae* and that practice remains. It is the bottom-fermenting lager yeasts that have received different names as research has developed. Successively, they have been named *S. carlsbergensis*, *S. uvarum* and *S. cerevisiae* lager-type. Now, they are strictly termed *S. pastorianus*. It is understood that *S. pastorianus* evolved from a melding of *S. cerevisiae* with *S. bayanus*, resulting in the larger and more complex genome of lager strains. In brewing practice yeast

grows under very restricted conditions caused primarily by the absence of oxygen (fermentation), relatively low temperature and recycling practices. The conditions used exercise a selective pressure on the population, and yeasts become adapted to certain brewing practices under which they perform satisfactorily. Fermentation results in the inefficient extraction of energy from fermentable sugar and so, relative to the large amount of sugar and other metabolites utilized, the yield of new yeast mass is quite small. This means that a good deal of material is left behind as metabolic waste products and appears in the beer as alcohol and carbon dioxide (CO_2) primarily (along with glycerol and flavor compounds). Contrast this to aerobic metabolism where much yeast mass accumulates and the end-products are essentially carbon dioxide (CO_2) and water (H_2O). The brewer's task is to manipulate wort qualities and the conditions of fermentation in such a way that beer of consistent flavor quality is made efficiently. Thus, controlled yeast growth (rate and amount) is the key to successful beer production. Taking a simple mass-balance approach to fermentation inputs and outputs (Figure 1.7), it is clear that additional yeast growth must subtract from formation of alcohol/carbon dioxide and/or flavor compounds and *vice versa* (Lewis and Bamforth, 2006).

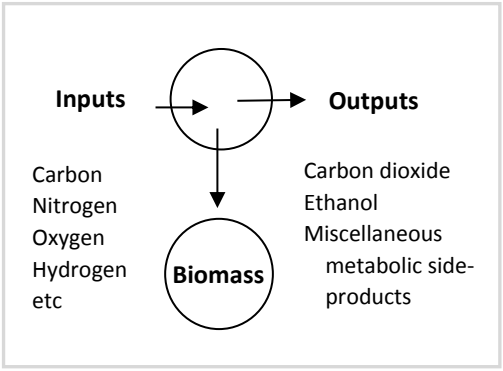


Figure 1.7 Inputs and outputs in fermentation (Source: Lewis and Bamforth, 2006).

1.2.5 Hop

Hop (*Humulus lupulus* L.) is a perennial climbing plant; the aerial part dies off in the autumn but the root stock stays in the soil, sometimes for many years. The plant needs a support up which to grow. In the wild, hops are found in hedgerows but for cultivation they are trained up strings attached to permanent wirework.

In the spring the stem tissue in the upper part of the root stock produces numerous buds from which many shoots develop. The farmer selects the strongest shoots and trains them clockwise up the strings. As the bines climb, young flowerings hoots develop in the leaf axils –the so-called “pin” stage- which then form the young female inflorescence with papillated stigmas the “burr” (Figure 1.8). From this the strobiles or hop cones develop. The cones consist of a central strig with bracts and bracteoles attached. Most of the lupulin glands are formed at the base of the bracteoles but they are readily detached and adhere to the bracts, strig and seed (Figure 1.8).

A few lupulin glands are found on the undersides of hop leaves but not enough to make these useful for brewing. The lupulin glands can contain as much as 57% of α-acids and the sum of the (α + β)-acids is equal to 75 ± 6% of the weight of the gland. The ratio α/β can range from 0 to about 4. The amount of resin/gland is fairly constant; the “high-alpha” varieties contain many more glands than the “low-alpha” varieties.

The hop is dioecious, male and female flowers are produced on different plants. Male flowers have five sepals and five anthers but since the flowers drop off after flowering any brewing value is lost. However, the male flowers produce pollen which can be carried long distances by the wind so any female plant in the

vicinity will be fertilized and produce seeds at the base of the bracteoles. Despite many demonstrations that excellent lager beers can be produced with seeded hops, lager brewers do not like seeds so most varieties are grown “seedless”.

Hops are added in brewing in either or both of two places: in the kettle and/or after fermentation. The objective is the same in each case: to make beer bitter to an exact, consistent and repeatable level (Briggs *et al*., 2004; Denny, 2009; Koch and Allyn, 2011).

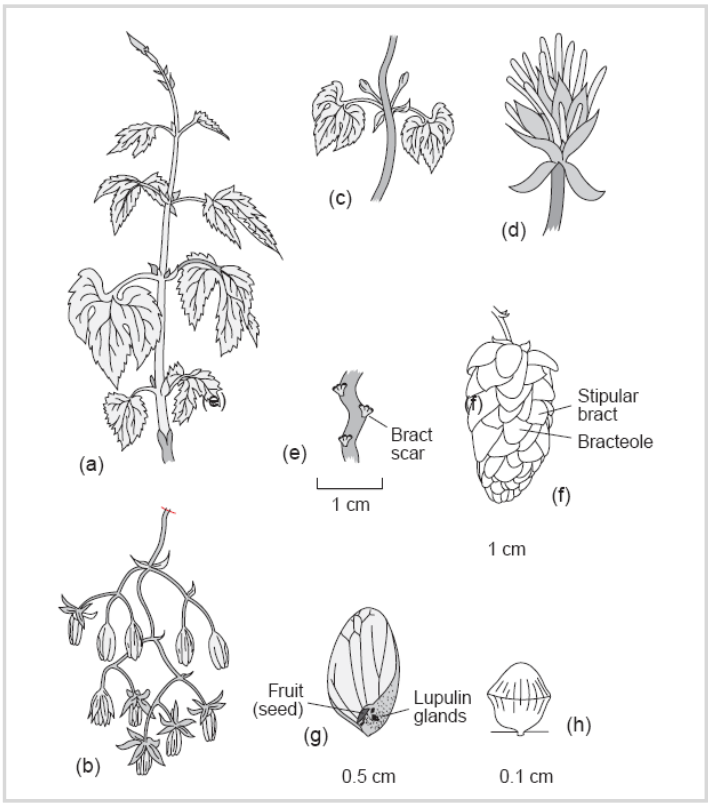


Figure 1.8 Hop (Humulus lupulus L.) (a) young shoot; (b) male flowers; (c) `pin', young flowering shoot developing in the leaf axils; (d) `burr', young female inflorescence with papillated stigmas; (e) part of axis (`strig') of cone; (f) single mature hop cone; (g) bracteole with seed and lupulin gland; and (h) lupulin gland (Source: Briggs et al., 2004).

1.3 MALTING AND BREWING PROCESSES

1.3.1 Malt production

The purpose of malting is to produce enzymes in the grain kernel and to cause defined changes in its chemical constituents (Kunze, 1996a). The malting process involves the cleaning and grading of stocks of barley, steeping the grain in water, germinating the grain and finally drying and curing it on the kiln (Figure 1.9).

Before malting, grain is screened and aspirated to remove large and small impurities and thin corns. To initiate malting it is hydrated. This is achieved by `steeping' (Figure 1.9), immersing the grain in water or "steep liquor". Later, the moisture content may be increased by spraying the grain. The steep-water temperature should be controlled. At elevated temperatures water uptake is faster but microbial growth is accelerated and the grain may be damaged or killed. The best temperature for steeping immature (partly dormant) grain is low (about 12°C). For less dormant grain a value of 16-18°C is often used. As the grain hydrates it swells to 1.3-1.4 times its original volume. Steep water, which checks grain germination and growth if re-used, is periodically drained from the grain and replaced with fresh. The minimum acceptable number of water changes are used since both the supply of fresh water and the disposal of steep effluent are costly (Briggs *et al*., 2004). Respiration (an oxygen consuming process) rises and throughout the steeping process malters provide adequate aeration to prevent stifling of the grain. Over about 48 hours, the moisture content of barley rises from about 12% to a target moisture content in the range 42% to 48% depending on the malter's objective and the characteristics of the barley. Generally, high steep-out moisture is used to make colored malt or to achieve high modification (at the cost of high malting loss) or if the barley is slow to germinate for some reason. Pale

malt is generally made from vigorous barley, and the steep-out moisture is therefore at the low end of the range. Air rests are used between steeps (Lewis and Bamforth, 2006).

After a steep has been drained air (air rest) (Figure 1.9), which should be humid and at the correct temperature, is sucked down through the grain. Such downward ventilation, or `carbon dioxide extraction', assists drainage, provides the grain with oxygen, removes the growth-inhibiting carbon dioxide and removes some of the heat generated by the metabolizing grain.

The onset of germination (Figure 1.9) is indicated by the appearance of the small, white “chit”, the root sheath (coleorhiza) that protrudes from the base of each germinated grain. At this stage the grain is transferred to a germination vessel (or floor in older maltings) or, if it is in a steeping/germination vessel, the equipment will be set into the germination mode. The grain grows, producing a tuft of rootlets (culms) at the base of the grain and, less obviously, the coleoptile or “acrospires” grows along the dorsal side of the grain, beneath the husk. The extent of acrospire growth, expressed as a proportion of the length of the grain, is used as an approximate guide to the advance of the malting process. Variations in acrospire lengths indicate heterogeneity in growth. The living tissues respire and carbon dioxide and water are generated resulting in a loss of dry matter. The energy liberated supports growth and is liberated as heat. Many hydrolytic enzymes, which are needed when malt is mashed, appear or increase in amount. Some of these catalyse the physical modification of the starchy endosperm (Briggs *et al*., 2004). Germination takes about four days, during which time the temperature of the grain bed rises from about 15°C to about 20°C, despite constant application of a stream of cool humid air throughout the period of germination and regular turning of the grain to promote even air flow and prevent

entanglement of rootlets. As the grains grows during germination, it breaks down its own storage substance (the endosperm materials) to provide energy and matter for embryo growth; this causes heating up of the grain bed and malting loss (i.e., the loss of dry substance as carbon dioxide and water are formed during ATP generation) (Lewis and Bamforth, 2006). When the acrospires have grown to about 3/4 to 7/8 the length of the grain and the level of soluble nitrogenous substances cease to increase with increasing germination time, and the fine-coarse extract difference has almost stopped decreasing although friability is still increasing and the viscosity of grain extracts may still be declining. Enzyme levels may or may not be increasing, depending on the malting conditions. Usually germination is terminated at this stage by kilning. Longer germination periods waste malthouse capacity and result in extra malting losses (Briggs *et al*., 2004).

During kilning (Figure 1.9) of malt enzyme destruction does occur and the enzymatic quality of dry malt is a shadow of the green malt from which it is made, both in terms of the amount and kinds of enzymes present; only these surviving enzymes are carried forward into mashing in the brewery. Although traces of many enzymes might survive kilning, brewers evaluate malt on the presence of only the starch-digesting amylases: they measure DP or diastatic power. From the point of view of wort quality, it is best to assume that the primary action of enzymes, other than amylases, is confined to the malting process and that their action in mashing is minimal. Many pale malts are cured at about 80°C, but some will be “finished” at higher temperatures, up to 105°C. Under these conditions colour formation is minimized. In the manufacture of some coloured malts the temperature is increased while the grain is still comparatively wet to promote the formation of free sugars and aminoacids and the interaction of these and other substances form the coloured melanoidins, flavoursome and aromatic substances (Maillard reaction). In these malts enzyme levels are comparatively low and, in

extreme cases, enzyme destruction is complete. After kilning malts are dressed (de-culmed or de-rooted and cleaned). The cooled malt is agitated to break up the brittle rootlets and these, and dust, are separated by sieving and aspiration with air currents. (Lewis and Bamforth, 2006; Briggs *et al*., 2004).

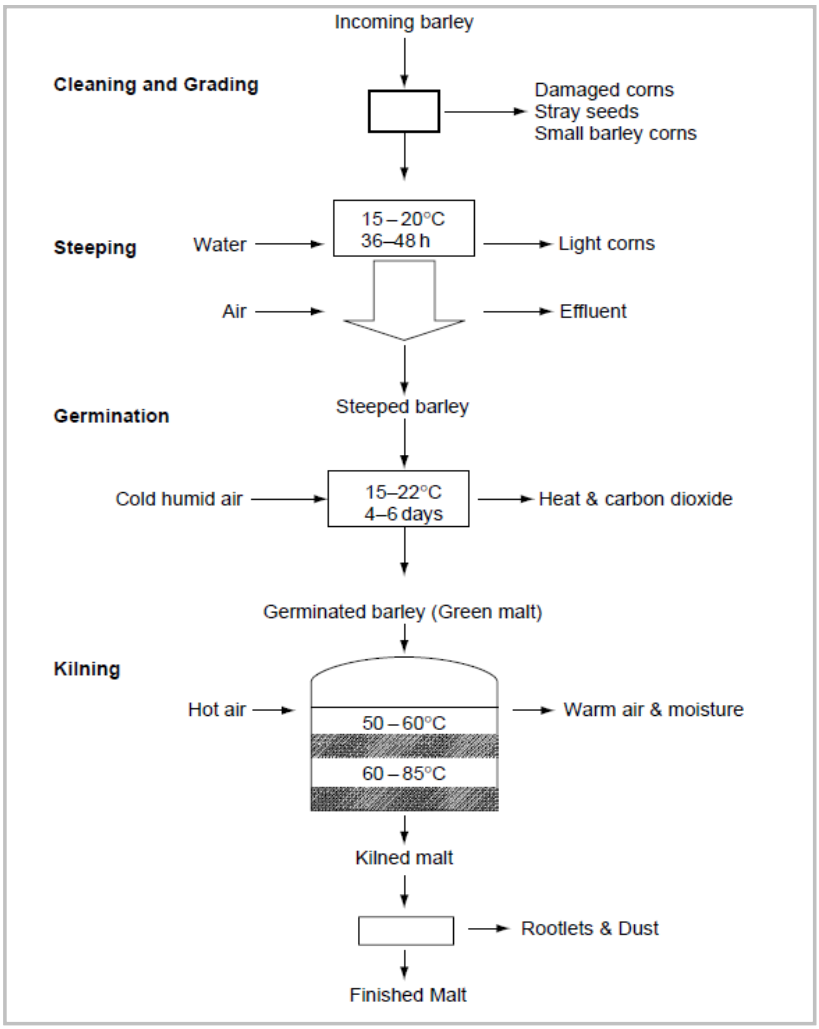


Figure 1.9 Flow diagram of the malting process (Source: Arendt and Dal Bello, 2008).

1.3.2 Beer production

The two most important processes in beer production are the degradation of starch to sugar during mashing followed by the fermentation of these sugars to form alcohol and carbon dioxide (Kunze, 1996b). Brewing in its simplest form involves seven steps (Figure 1.10):

- The malt, sometimes premixed with particular adjuncts, is broken up to a controlled extent by milling to create the `grist'. The type of mill used and the extent to which the malt (and adjunct) is broken down is chosen to suit the types of mashing and wort-separation systems being used.
- At mashing-in the grist is intimately mixed with brewing liquor, both flowing at controlled rates, into a mashing vessel at an exactly controlled temperature. Malt enzymes (especially α- and β-amylase), which were produced during malting, are encouraged to solubilize the degraded endosperm of the ground malt at their optimum temperatures to give as much soluble extract as possible; a mash should be held at a chosen temperature (or at successive different temperatures), for pre-determined times, to allow enzymes to `convert' (degrade) the starch and dextrins to soluble sugars, to cause the partial breakdown of proteins, to degrade nucleic acids and other substances.
- In the lauter tun, the soluble extract in the wort is separated from the insoluble spent solids (grain husk) (lautering phase). Furthermore water is sprayed from the top of the tank onto the mash to increase extract (sparging).
- The wort is then boiled in the wort kettle with hops. This halts enzyme action, sterilizes the wort, coagulates some proteins and polyphenols

fractions (*hot trub*), imparts distinctive flavors and aromas to the wort from the hops. Evaporation of the wort, reduces the volume by 7 ± 10%, and so it is concentrated. Unwanted flavour-rich and aromatic volatile substances are removed. During the boil flavour changes and a darkening of the colour occurs (Maillard reactions). The hop-boil consumes about half of the energy use in brewing.

- At the end of the boil the wort contains flocs of *trub* (the hot break or *hot trub*) and suspended fragments of hops. The hop fragments (if present) and the *trub* are usually separated in a `whirlpool tank'. The clear `hopped wort' is cooled and so it can be inoculated (`pitched') with yeast. The wort is aerated or even oxygenated, to provide oxygen for the yeast in the initial stages of fermentation.
- Fermentation may be carried out in many different types of vessel (Boulton and Quain, 2001). Fermenters may be open or completely closed or they may allow part of the yeast to be exposed to the air for part of the fermentation period. The variety of fermenters remains because yeasts working in different vessels produce beers with different flavours. Yeast strains vary in their properties and the flavours they impart. Traditionally, ale beers are fermented with `top yeasts' which rise to the top of the beer in the head of foam. These are pitched at about 16°C and fermentation is carried out at 15 ÷ 20°C for 2 ± 3 days. Traditional lager beers are fermented with `bottom yeasts', which settle to the base of the fermenter. These are pitched at lower temperatures (e.g., 7 ÷ 10°C) and fermentations are also carried out at lower temperatures (e.g., 10 ÷ 15°C), consequently they take longer than ale fermentations. The carbohydrates present are converted into alcohol and carbon dioxide. Other yeast metabolites contribute to flavour and aroma.

- When the main, or `primary' fermentation is nearly complete the yeast density is reduced to a pre-determined value. The `green' or immature beer (it is not green in colour, but has an unacceptable, immature flavour) is held for a period of maturation or secondary fermentation called lagering process: lagern is German and means stored or deposited. During this process the flavour of the mature beer is refined. Now, after legering process, most beers are chilled and filtered or centrifuged to remove residual yeast. These completely bright beers are carbonated (their carbon dioxide content is adjusted), than are transferred into bottles, cans, kegs, or bulk tanks. Before packaging the beer may be sterile filtered, a process that avoids flavour damage but it follows that all subsequent beer movements must be made under rigidly aseptic conditions. More often the beer is subjected to a carefully regulated heat treatment (pasteurization process) (Briggs *et al*., 2004; Arendt and Dal Bello, 2008). Refermentation of fermenting beers in bottles is a frequently used process in small craft breweries. Unfiltered finished beer is mixed with fermentable extract and subsequently bottled (Van Landschoot *et al*., 2004).

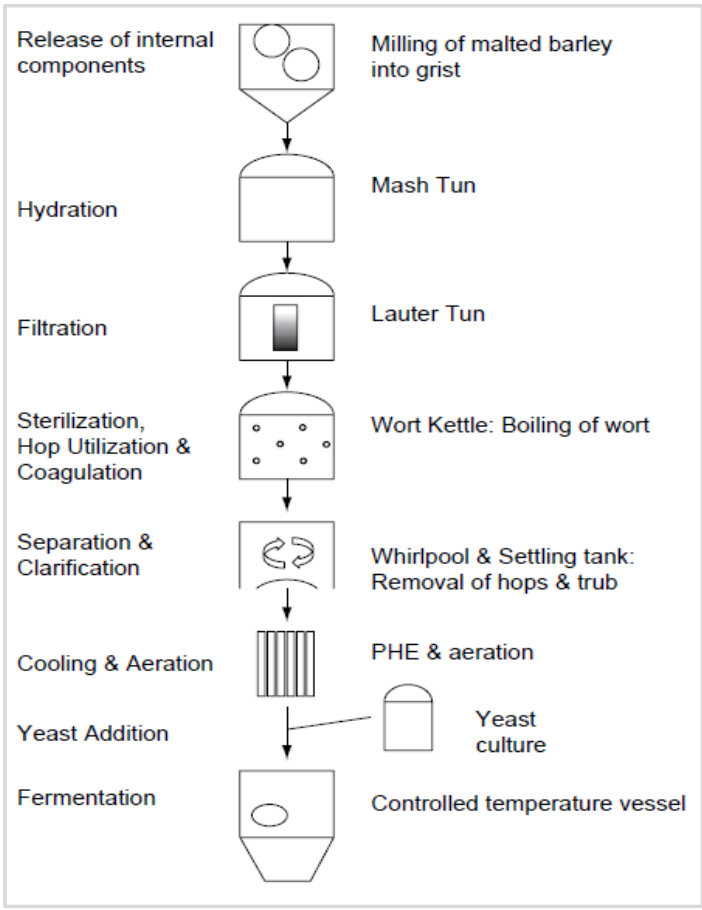


Figure 1.10 Flow diagram of the brewing process (Source: Arendt E. K., Dal Bello, 2008).

1.4 COLLOIDAL STABILITY OF BEER

1.4.1 Introduction

The quality of the beer foam and its clarity should match the consumer's expectations for that style of beer, because these are the first characteristics by which a consumer judges the quality of his or her beer. It follows that beer foam and storage haze stability are characteristics of critical importance to brewers (Goldberg and Bamforth, 2010). To establish and maintain brand appeal, brewers desire foam with optimum stability, quantity, lacing, whiteness, "creaminess", and strength. Brewers generally desire that minimal haze is formed during the anticipated storage life of the product. Formation of haze is considered to be a sign of aging or contamination (Evans and Sheehan, 2002; Yang *et al*., 2006). There are, of course, exceptions that provide characteristics of beer styles, such as "bottle/cask-conditioned" beers, in which the yeast added to the product to enable carbonation is present to give a hazy impression, and wheat beers, in which a fine haze is produced by protein-polyphenol interactions (Delvaux *et al*., 2000; Delvaux *et al.*, 2001; Evans *et al*., 2003).

1.4.2 Treatments to stabilize beer against colloidal haze formation

The primary source of haze-forming materials in brewing is malt. This is the source of specific haze-potentiating proteins and polyphenols. Hops also contribute polyphenols. Brewers therefore select low-protein barleys that are easily modified for malting, so that the survival of protein into beer is minimized at the outset. It is also possible these days to select barley that has a low content of polyphenol (anthocyanogen-free or ant-free barley) that is highly effective in yielding haze-stable beer. A related strategy for control of such hazes is to use thoroughly well-modified malt, and thus, malters' strategies for good modification are a part of the defence against haze; i.e., the use of an alkaline solution in the first steeping may facilitate the extraction of proteinaceous and phenolic fractions from seeds (Briggs, 1998; Briggs *et al*., 2004).

Brewhouse processes are vital opportunities for the deposition of protein and polyphenol; milling, of course, exposes the husk and endosperm to extraction by brewing water in mashing. Brewers assume excessive milling promotes undesirable extraction of husk polyphenols, but experience with hammer-milled malt suggests that this concern is misplaced. In the early, low temperature stages of a temperature-programmed mash, protein and polyphenol dissolve from the grain. However, as the mash rises toward conversion temperature, protein and polyphenol react and proteins substantially (about 80%) precipitate in the mash and so exit the process in the spent grains (which comprises about 30% crude protein, dry weight). Not only the *amount*, but also the *kinds* of proteins present in wort are affected by this precipitation.

What is less arguable is the fact that oxygen ingress in the brewhouse does impact the colloidal stability of beer. It was Dennis Briggs who first made additions

of an “active” form of oxygen, hydrogen peroxide, into mashes to oxidize polyphenols and cause their agglomeration with proteins and removal at the wort-separation stage. As a result, lower levels of haze precursors emerged into the finished wort, and the resultant beers had increased resistance to haze development. Oxygen entering into a mashing system reacts with the so-called gel proteins. The sulfhydryl side chains in these proteins (provided by cysteine residues) react with the oxygen and, as a result, cross-link (Figure 1.11). The resultant protein agglomerates serve to slow down wort separation as they form a clay-like mass in the grain beds. Hydrogen peroxide is produced and this forms a substrate for peroxidase, which catalyzes the oxidation of polyphenols to form red oxidation products (these increase the colour of the wort). The oxidized products also cross-link with hordein-derived polypeptides in the wort to form insoluble complexes that can be filtered out. As a result, there is less of these polypeptides and polyphenol left to go forward to the finished beer (Lewis and Bamforth, 2006).

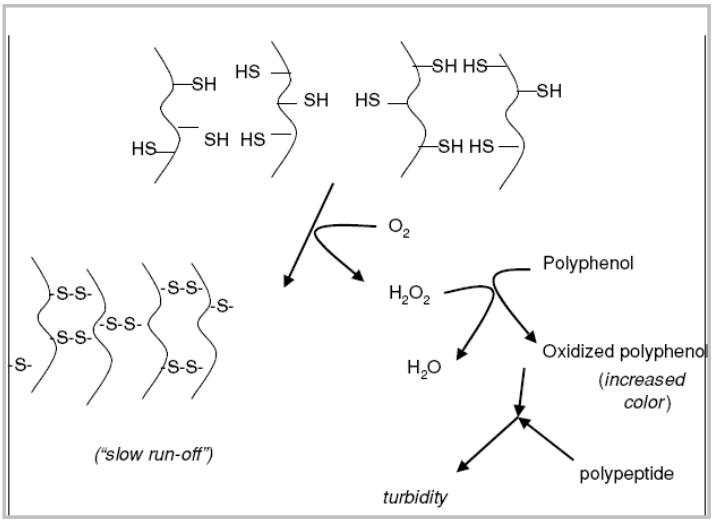


Figure 1.11 Oxidative reactions in mashing (Source: Lewis and Bamforth, 2006).

During wort boiling, more protein-polyphenol complex is precipitated as "hot break" (or *hot trub*). The amount precipitated is a function of a vigorous boil (a "full rolling" boil being essential) and the length of the boil. The *hot trub* and any insoluble material from hop is taken out of the wort by centrifugation or by a whirlpool tank (Figure 1.12).

Nevertheless, the prolonged time and low temperature of fermentation and, especially, finishing processes undoubtedly favour further precipitation of protein-polyphenol complexes (Briggs *et al*., 2004).

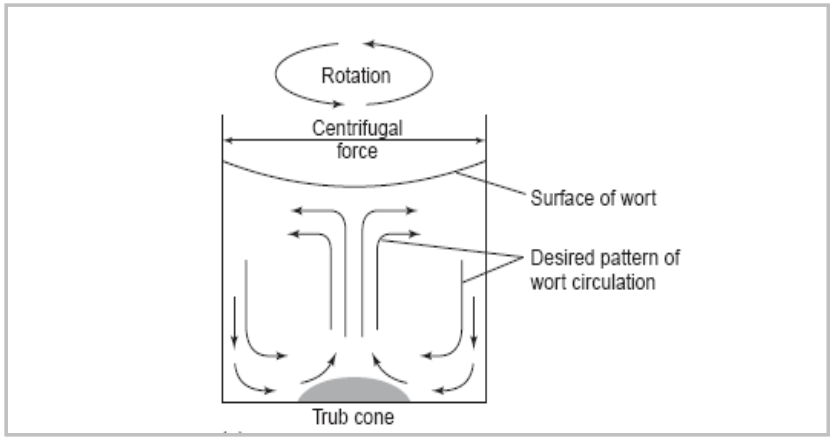


Figure 1.12 Currents in whirlpool tanks. The ideal flow pattern in a whirlpool (Source: Briggs et al., 2004).

Brewers routinely employ a range of methods to stabilize final beer against colloidal haze formation for the intended shelf life of a product. These include various combinations of cold storage, fining, adsorbents, proteolytic enzymes and filtration. In some cases the procedures have undesirable side effects, most often impairment of beer foam performance. Proteins have not to be eliminated

completely because they are associated with important characteristics of beer; it is not clearly established if the haze forming and foam-forming proteins are different. It is important to use coadjuvants that are able to remove constituents of haze from the final product without reducing foam stability, flavour and taste (Hough *et al*., 1982; Evans *et al*., 2003; Kosin *et al*., 2010).

Bamforth (1999) reported three different strategies: protein removal, polyphenol removal or remove a proportion of each. Many different substances can be used to improve the stability of beer and to remove polyphenols. Polyvinylpolypyrrolidone (PVPP) is commonly used; Siebert and Lynn (1998) have showed that the structure of PVPP strongly resembles the structure of polyproline (Figure 4.1: subsection 4.1, p. 116) and that it binds polyphenols in the same way proteins rich in proline bind with polyphenols. PVPP removes both haze active polyphenols (about 50%) and non-haze active polyphenols from beer (Siebert and Lynn, 1997). Simple flavanoids, proanthocyanidins and tannoids are sorbed (McMurrough *et al*., 1997). PVPP treatment is reported to decrease the reducing activity of beer (O'Reilly, 1994) and some authors did not find any significant effect of PVPP treatment on flavour stability (McMurrough *et al*., 1997); while others came to opposite conclusions (Mussche, 1994; Mikyška *et al*., 2002; Dienstbier *et al*., 2011).

Papain was one of the first stabilizers used in brewing. It is a proteolytic enzyme; it hydrolyzes peptides but it reduces the foam quality (Bamforth, 1999).

Also tannic acid and bentonite, used as a specific precipitant of haze-active proteins, can damage foam in beer. It is most common to use silica gels that bind to proline residue in the protein with minimal negative effects on the protein fraction involved in beer foam-active quality (Siebert and Lynn, 1998).

Lopez and Edens (2005) have proposed alternatives to the traditional stabilization compounds. A proline-specific protease in wort that can hydrolyze proteins rich in proline has been used, yielding a peptide fraction that is unable to form a haze without negative effect on foam stability.

Evans *et al.* (2003) have proposed a different approach; since haze activity is dependent on the distribution of proline in the hordein, they have studied immunological methods that can predict the potential of malt samples to produce beer with superior foam and haze stability.

1.5 ANTIOXIDANT ACTIVITY

1.5.1 Free radical mechanism

Most unsaturated organic compounds react with oxygen when exposed to air, heat or light. This oxidation has undesirable effects on flavour and odours, nutritional properties and safety of lipid containing foods. The use of various antioxidants is an important method for the control of oxidation in foods and biological systems, where free radical reactions are now implicated in the development of many degenerative diseases. To understand better how antioxidants operate, it is necessary to understand the main aspects of the mechanism of lipid oxidation. The oxidation of unsaturated lipids is generally a free radical chain reaction that includes three processes: initiation, propagation and termination.

To break the free radical chain effectively the structure of an active antioxidant is designed to produce a radical in which the unpaired electron is

delocalized round the aromatic structure and is stabilized by high resonance energy.

1.5.2 Oxidative processes in beer

Maintaining beer quality through the various stages of maturation, distribution and shelf storage remains an extensive challenge. While several attributes are used to establish overall beer quality, two aspects in particular have received considerable attention: colloidal and flavour stability. About the issue of flavour stability remains a challenge, especially for pale lager beers that are more sensitive to flavour deterioration during aging. Most aged-beer flavours have been attributed to oxidative mechanisms.

Under normal conditions, molecular oxygen in its triplet ground state cannot directly react with molecules that possess paired electrons with anti-parallel spins, molecules such as polyphenols that exist in their singlet state. This would violate Pauli's exclusion principle, and thus the reaction could only take place if spin inversion were to occur, a process that would require a large and unlikely energy input. The activation energy required for oxygen to react with a lipid is also relatively large, between 35 and 65 kcal/mol (Labuza, 1971).

Reactions involving oxygen are thus thought to proceed in one-electron steps via the formation of free radicals (Danilewicz, 2003), a process that can be catalyzed by transition metals (Kaneda *et al*., 1989). In the presence of a metal catalyst such as Fe^{2+} or Cu^{+}, oxygen can capture an electron to form superoxide anion (O_2^{-}). Upon protonation, superoxide forms the perhydroxyl radical (OOH·). Generally most of the superoxide (pKa 4.8) originating in beer (pH ~4.5) exists in

this protonated and more reactive state (Lewis and Young, 2002; Vanderhaegen *et al*., 2006).

Superoxide may also undergo reduction to form peroxide anion (O_2^{2-}). Peroxide ion can in turn become protonated to form hydrogen peroxide (H_2O_2) (Irwin *et al*., 1991). Furthermore, iron can catalyze the generation of hydroxyl (OH·) and peroxyl radicals (OOH·) from hydrogen peroxide (H_2O_2) via the Haber-Weiss and Fenton reactions (Figure 1.13).

Bamforth *et al*. (1993) provide a comprehensive review of oxygen and oxygen radical chemistry pertaining to malting and brewing.

The involvement of other metals in radical generation has not been as thoroughly investigated, but d-block elements such as manganese are capable of catalyzing reactions that produce reactive oxygen species (ROS) and may act synergistically along with iron and copper to catalyze oxidative staling reactions (Mochaba *et al*., 1996; Kaneda *et al*., 1999; Aron and Shellhammer, 2010).

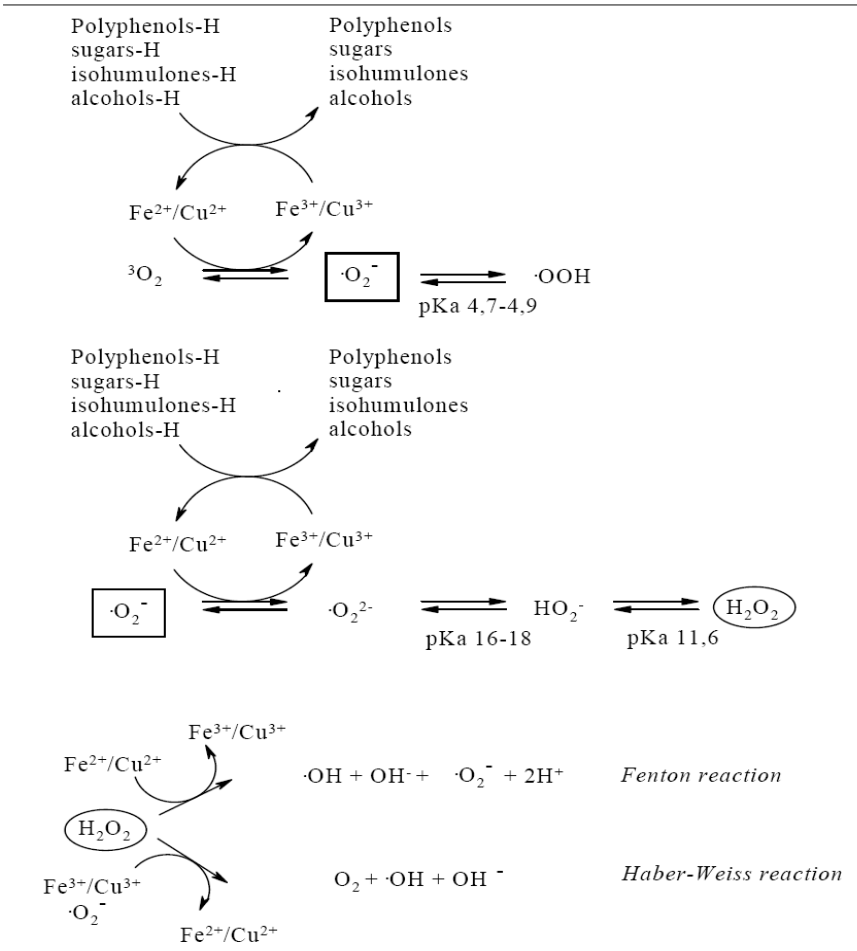


Figure 1.13 Reactions of active oxygen in beer (Source: Kaneda et al., 1999).

During mashing some lipid seems to disappear because it is oxidized, by oxygen dissolved in the mash, to more polar substances, some of which reach the beer and, during storage, give rise to unsaturated aldehydes (such as trans-2 nonenal and trans-2, cis-6-nonadienal) which give the beer an unpleasant, cardboard like flavour. The chain of reactions is complicated (Figure 1.14). Lipids

are hydrolysed by lipases (lipid hydrolases) and esterases to free fatty acids, a major proportion of which is linoleic and linolenic acids, which are unsaturated. Some of these acids may have been oxidized while still combined in the original lipid.

Malt acrospires are rich in lipases and lipid degrading enzymes. Lipases are active to some extent during mashing. The unsaturated acids are partly oxidized by oxygen in the presence of lipoxidase enzymes (LOX). LOX, is a very heat-sensitive enzyme produced in the barley embryo during germination. LOX is substantially destroyed during kilning. It will survive mashing at lower temperatures, but is rapidly destroyed at 65°C. It has been argued that if this enzyme has any relevance in mashing, then it can only be at the point of initial striking of malt with brewing water, at which point alone there seems to be sufficient substrate and enzyme for the enzyme to act.

However, linoleic acid is susceptible to oxidation even in the absence of enzymes. The reaction is autocatalytic and needs only a small amount of initial "trigger" to start the cascade of radical reactions. Radical scavengers, which halt this cascade by trapping radicals without forming fresh radicals, may include polyphenols and melanoidins (Briggs *et al*., 2004; Bamforth, 2008). Flavour and haze stability are key attributes of beer and the importance of polyphenols has been discussed for many years. Contradictory influences on those two attributes results from their nature. Some of them can act as antioxidants and improve flavour stability, others deteriorate haze stability.

Dependence of reducing activity and beer flavour stability based on the malt polyphenol content and composition has been reported by some authors (Kaneda *et al*., 1995) and the significant effect of hop polyphenols on reducing activity and stale flavour carbonyl formation in beer was demonstrated by other

authors (Lermusieau *et al*., 1999; Noël *et al*., 1999). It is well known that oxidation during packaging causes deterioration of beer quality, haze and flavour stability. Generally accepted opinion is that the oxygen in the headspace is incorporated into compounds in the beer, especially polyphenols, carbonyl compounds and isohumulones during storage.

However, the oxidized polyphenol might itself act as a donor, or oxidant molecule, under some circumstances, especially the presence of metal ions (again copper and iron). Polyphenols with hydroxyl groups at the 3' and 4' positions on the flavan ring (i.e., catechin) are antioxidants because they scavenge oxygen radicals. Those with an additional 5' hydroxyl group (i.e., delphinidin) promote staling because they can reduce transition metal ions to their more potent lower valence forms (Lewis and Bamforth, 2006).

Indeed, the Strecker degradation, between α-dicarbonyls and amino compounds, provides an opportunity to form aldehydes during wort boiling that might influence beer flavour. Reactions such as this might also explain the suggestion that melanoidins (products of the Maillard reaction that also can involve the Strecker degradation) are involved in formation of aldehydes, though brewers observe that dark beers are intrinsically more stable to flavour change by oxidation than pale beers (Bamforth, 2008; Cortés *et al*., 2010).

Sulphite is capable of forming addition complexes with carbonyl containing compounds, the resultant “adducts” display no perceptible flavour at the concentrations likely to be found in beer (Barker *et al*., 1983). It has been suggested that carbonyls produced upstream bind to the sulphite produced by yeast, thereby carrying through into the finished beer, to be progressively released as sulphur dioxide (SO_2) is consumed in other (as yet unknown) reactions (Ilett and Simpson, 1995). It has been suggested that the greater significance of sulphite for

protecting against staling is through its role as an antioxidant (Kaneda *et al*., 1994). In this regard, Dufour *et al*. (1999) indicate that SO_2-carbonyl binding actually occurs through the C=C of the unsaturated aldehyde, rather than at the carbonyl group and, as such, is non-reversible (Bamforth, 2008).

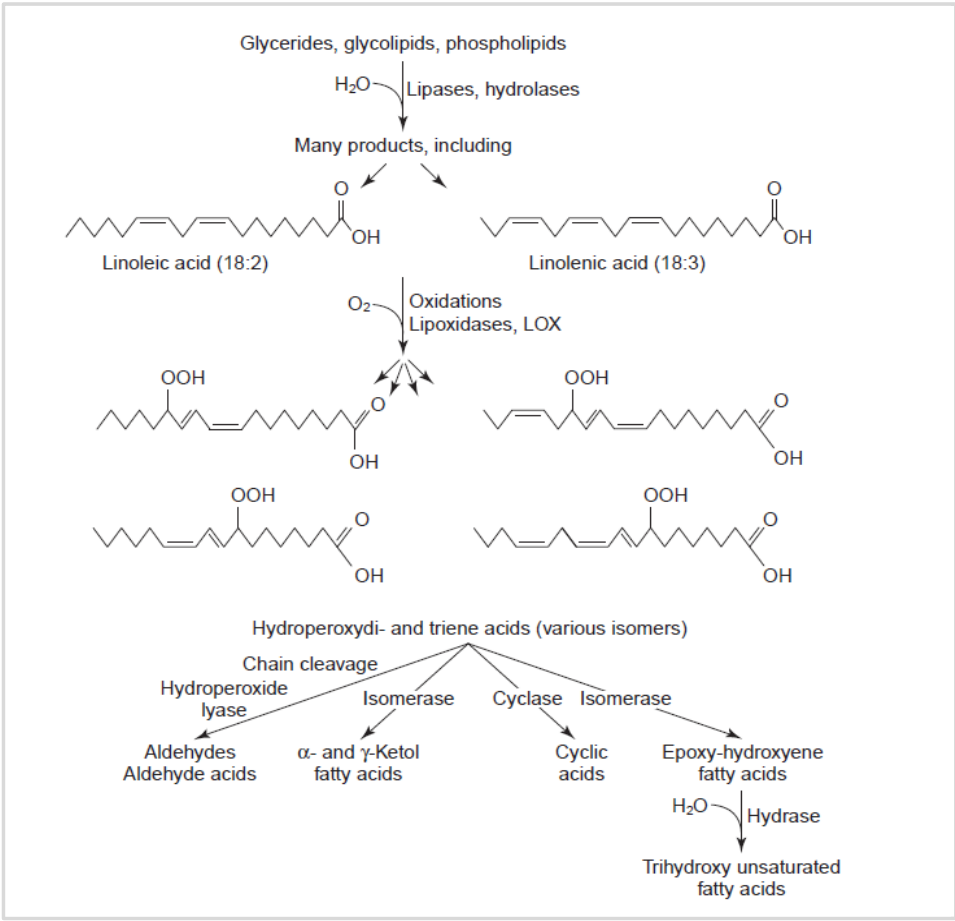


Figure 1.14 Possible stages in the oxidative breakdown of the major unsaturated fatty acids during mashing. The number of possible products is very large indeed. It is thought that the unsaturated trihydroxy-fatty acids are the precursors of staling flavour compounds in beers (Source: Briggs et al., 2004).

1.6 GLUTEN-FREE BEER

1.6.1 Introduction

The proteins of haze material primarily arise in the hordein or prolamin (storage) fraction of barley. These alcohol-soluble proteins have a high content of proline, the residue of which seems to be essential for haze formation. Incidentally, these proteins are also responsible for the immune reaction experienced by coeliacs; haze prevention in beer and rendering the beer "gluten-free" are therefore compatible practices. More directly, brewers can dilute, by up to 50%, the malt used in mashing with adjunct materials such as preparations of rice or corn (maize) that are naturally low in protein and polyphenol. Such beers are intrinsically more haze stable than all-malt products (Lewis and Bamforth, 2006).

1.6.2 Celiac disease

Celiac disease is an immune-mediated enteropathy triggered by the ingestion of gluten in genetically susceptible individuals. Celiac disease is one of the most common lifelong disorders on a worldwide basis. The condition can manifest with a previously unsuspected range of clinical presentations, including the typical malabsorption syndrome (chronic diarrhea, weight loss, abdominal distention) and a spectrum of symptoms potentially affecting any organ or body system. Since celiac disease is often atypical or even silent on clinical grounds, many cases remain undiagnosed, leading to the risk of long-term complications, such as osteoporosis, infertility or cancer (Fasano and Catassi, 2001). There is a growing interest in the social dimension of celiac disease, since the burden of illness related to this condition is doubtless higher than previously thought

(American Gastroenterological Association, 2001). Although celiac disease can present at any age, including the elderly, typical cases often manifest in early childhood.

Celiac disease prevalence has been estimated to be 1 in about 100 people worldwide (Hamer, 2005; Sollid and Khosla, 2005). The only effective treatment is a strict adherence to a diet that avoids ingestion of cereals (wheat, spelt, triticale, rye, and barley) that contain gluten and their products throughout the patient's lifetime (Ellis *et al*., 1990).

1.6.3 Regulations

Ninety-eight per cent of all governments worldwide are member of the Codex Alimentarius Commission. One of the tasks of the Commission is to adopt Codex Standards, which give guidance to governments for food legislation and are mandatory for the food industry when participating in global trade. Almost all governments around the world are incorporating the Codex Standards into national legislation.

The Association of European Coeliac Societies (AOECS), the umbrella organization of national European celiac societies, was given Observer status in the Codex and contributed to the development of the working paper. This paper contains the proposal that gluten-containing cereals and their products should always be declared. Also that other foods or ingredients, which may cause intolerance or allergy, should be added to the list. Because it covers intolerances as well as allergies, the list is called the "list of hypersensitivity."

Switzerland was the first country in Europe to adopt the Codex list of hypersensitivity plus celery and fruits for national legislation by May 1, 2002. In the European Union the labeling of gluten-containing starches was incorporated into law first, the rest of the labeling improvements followed later. Bearing in mind that the AOECS has been informing the European Commission and the members of the European Parliament about the inadequate labeling of gluten-containing ingredients in foodstuffs since 1989, it is clear that changes in legislation take some time. In 1995 the European Parliament voted for the declaration of gluten-containing starches and in March 2000 Directive 2000/13/EC was published (European Directive, 2000).

In November 2003 the European Parliament and the Council adopted Directive 2003/89/EC, which amended Directive 2000/13/EC (European Directive, 2003). Annex IIIa of this Directive is mostly in accordance with the Codex list of hypersensitivity. "*Cereals containing gluten (…) and products thereof*" remained the first group in the list.

Cereals containing gluten and products thereof always have to be declared without any exception if the ingredient is part of a compound ingredient or has been added for technological reasons or processing purposes. Directive 2003/89 specified this issue in Article 1 (c) (iv): "*substances which are not additives but are used in the same way and with the same purpose as processing aids and are still present in the finished product, even if in altered form*."

In Article 2 Member States were requested:

to bring into force, by 25 November 2004 the laws, regulations and administrative provisions necessary to permit, as from 25 November 2004, the sale of products that comply with this Directive and prohibit, as from 25 November 2005, the sale

of products that do not comply with this Directive but which have been placed on the market or labelled prior to this date may, however, be sold while stocks last.

In Article 1 paragraph 10 the following instructions are given:

(…) any substance used in production of a foodstuff and still present in the finished product, even if in altered form, and originating from ingredients listed in Annex IIIa shall be considered as an ingredient and shall be indicated on the level with a clear reference to the name of the ingredient from which it originates.

However, as a consequence, exemption of “allergen labeling” is needed to avoid confusion: if an ingredient or product has been rendered from gluten-containing cereals and the gluten content has been removed, it is misleading to list “wheat” in the ingredients of a prepackaged food. For example, wheat contains gluten but ethanol, the alcohol derived from wheat, does not. Article 1 paragraph 11 informs that the list in Annex IIIa shall be systematically re-examined and, where necessary, updated. Updating could also include the deletion from Annex IIIa, if it has been scientifically established that some substances do not cause adverse reaction (Arendt and Dal Bello, 2008).

The European Commission, using recent internationally recognised scientific evidence, has introduced compositional and labelling standards (Commission Regulation (EC) n. 41/2009) that set levels of gluten for foods claiming to be either 'gluten-free' or 'very low gluten', which came into force in January 2012.

These levels are:

- 'gluten-free': at 20 mg/Kg of gluten or less
- 'very low gluten': at 100 mg/Kg of gluten or less - however, only foods with cereal ingredients that have been specially processed to remove the gluten may make a 'very low gluten' claim

These regulations apply to all foods, pre-packed or sold loose, such as in health food stores or in catering establishments.

The new labelling standards are an important public health measure to help protect the long term health of coeliacs. These labelling standards will enable coeliacs to make informed choices about the foods that are safe for them to eat.

Where caterers are unable to justify 'gluten-free' or 'very low gluten' claims because of the risk of cross-contamination, if steps have been taken to control this contamination, caterers will be able to indicate which foods do not have gluten-containing ingredients, allowing coeliacs to make choices based on their individual levels of sensitivity (www.food.gov.uk).

1.6.4 Gluten-free beer consumption

The market for gluten-free cereal products is expected to rise significantly as consumer demand increases in reaction to increased levels of diagnosis of celiac disease and also as specific consumers make the conscious choice to remove gluten from their diets. The difficulty associated with the development of the gluten-free market has been attributed to the strict processing requirements of the sector and also the perceived size of the market.

What are the market requirements? When consuming a gluten-free diet is a necessity for consumers, they are looking for gluten-free cereal products with the same appearance and texture as conventional products. The increasing number of people with celiac disease being diagnosed each year and their desire for more better-tasting and better-textured products offers great market opportunities for food manufacturers.

Across food markets, according to Milton (2003), the key food areas for future new product development (NPD) include: convenience foods, foods with perceived health benefits, low fat and organic products, range extensions, extending brands, product improvements, new categories, and premium quality foods (www.naturalproductsinsider.com).

This market opportunity also provides a means of product differentiation from mass-produced goods from industrial foods and beverages. This product differentiation can be seen in Anheuser-Busch's sorghum beer Redbridge that was developed as a hand-crafted specialty beer made without wheat or barley (Nutra Ingredients USA, 2007). This niche market product is clearly targeted at those consumers who wish to exclude gluten from their diets. A niche market like this can be attractive to firms as it typically attracts fewer competitors (Kotler, 2000).

The search for new gluten-free brewing materials is still in its infancy. Limited studies are opening a new area of brewing and once process conditions are adjusted to accommodate gluten-free raw materials, the production of satisfactory gluten-free beers and products will be more realistic and should lead to a greater variety of products for people with celiac disease. Initial research on sorghum was not to find gluten-free alternatives but was in response to the 1988 ban on importation of barley malt into Nigeria. While acceptable to a large proportion of beer drinkers in Africa, the taste and flavour of sorghum beer may

not be acceptable to countries outside this region. Further extensive research work is necessary to develop products that meet the tastes and consumer habits of the industrialized countries. A search of the internet reveals that there are a number of micro-breweries producing gluten-free beer (Arendt and Dal Bello, 2008).

However, a detailed analysis of the ingredient list of some of those so-called gluten-free beers shows that a percentage of malt was included in the recipes and this contamination could not be suitable for some patients with celiac disease.

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2. DEVELOPMENT OF APPROPRIATE MALTING CONDITIONS OF FOUR GLUTEN-FREE CEREALS/PSEUDOCEREALS

Part of this chapter is in combination with the work presented at:

18th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Conegliano (2013) Passaghe, P. The colloidal stability of craft beers: an assessment of aspects related to technology, composition and analysis.

2.1 INTRODUCTION

It was once believed that beer could not be produced without barley; however it is well documented that opaque beers made from cereals like sorghum (Goode *et al*., 2003; Nso *et al*., 2003), millet (Eneje *et al*., 2001) and maize (Shephard *et al*., 2005) have the potential to be alternative substrates for conventional beer brewing.

Malting of Millet

In southern Africa, pearl millet is traditionally processed by malting and fermentation. Malted pearl millet is used to make weaning foods for infants with reduced viscosity. An optimal malting procedure for pearl millet, which involves steeping at 25°C, with a cycle of 2 hours wet and 2 hours air rest for a total of 8 hours, germination at 25-30°C for 72-96 hours and finally a kilning regime at 50°C for 24 hours has been suggested. These conditions resulted in high diastatic power (α- and β-amylase activities), a good quantity of free amino nitrogen and a moderate malting loss (Pelembe *et al*., 2002).

Malting of Amaranth

Kanensi *et al* (2011) soaked amaranth seeds for 5 hours at 30°C and germinated them for up to 24 hours. These malting conditions varied significantly from those reported by Alvarez-Jubete *et al* (2010), who recommended a steeping-air rest time of 24 hours at 15°C, and a germination temperature of 10°C for 98 hours. However more research is still required to evaluate the use of amaranth as a malting and brewing.

Malting of Buckwheat

Investigation of the impact of steeping time and temperature on the quality of buckwheat malt has revealed that the optimal moisture content at the end of steeping is 35-40%, and the recommended steeping time is 7 to 13 hours at a temperature of 10°C (Wijngaard *et al*., 2005a, 2005c). At these moisture levels the malting loss falls within an acceptable range, and malt quality is optimized. Optimal enzymatic activity in buckwheat malt can be obtained when buckwheat is germinated for 96 hours at 15°C (Wijngaard *et al*., 2005b, 2006). At this time, the grains are sufficiently modified and nutrients have not been exhausted yet. Moreover, the quantity of rutin, a polyphenol with functional properties, increases significantly during malting (Arendt and Dal Bello, 2008).

Several optimal conditions have been recently proposed for buckwheat malting. It was found that both α-amylase and β-amylase activities were low in malted buckwheat in comparison to malted barley. The maximum activity level of α-amylase was obtained in buckwheat without hull, which germinated at 16.5°C. In addition, maximum apparent fermentability (56%) was reached when buckwheat germinated at 20.2°C.

Based on the results of different studies, it can be concluded that prolonged kilning at 40°C (more than 24 hours) causes greater inactivation of endo-β-glucanase and α-amylase activity in comparison to β-amylase and protease activities. However the latter is still affected by the kilning regime. The results collected so far strongly suggest that buckwheat, when optimally malted, shows potential as a health-preserving, gluten-free alternative to sorghum malt for brewing purposes (Nic Phiarais *et al*., 2005).

Malting of Quinoa

When malted for 36 hours, the α-amylase activity of quinoa increased 4-fold (Atwell *et al*., 1988). However, the starch granules of the perisperm do not appear to be extensively degraded by amylase during germination (Varriano-Marston and De Francischi, 1984). Some authors have optimized the malting conditions of quinoa as follows: steeping time of 24 hours, air rest for 3 hours, and a germination temperature of 10°C for 82 hours (Alvarez-Jubete *et al*., 2010).

The aim of this study was to carry out malting tests in the laboratory on four gluten-free cereals/pseudocereals (proso millet, amaranth, buckwheat and quinoa) in order to identify the optimal conditions for obtaining malts suitable for the production of craft beers.

2.2 MATERIALS

2.2.1 Reagents and samples

Hydrogen peroxide (37% v/v), soluble starch, acetic acid, sodium acetate, sodium hydroxide (1 M), thymolphthalein, iodine, potassium iodide, sulphuric acid (98% v/v), sodium thiosulphate, disodium tetraborate, potassium sulphate, copper sulphate pentahydrate, boric acid, bromocresol green screened indicator and hydrochloric acid were purchased from Sigma-Aldrich (Milan, Italy). Dibasic sodium phosphate, absolute ethanol (99.9% v/v) purchased from Carlo Erba (Milan, Italy), and reagents purchased from β-Glucan assay kit (Megazyme International, Ireland Ltd.). Solvents and other chemicals were of analytical grade. Ultrapure water was obtained by Milli-Q® Advantage A10® system (Millipore, Billerica, MA, USA).

The cereal/pseudocereal samples (proso millet, amaranth, buckwheat and quinoa) were purchased from Dr. Schär S.p.a. (Trieste, Italy).

2.3 EXPERIMENTAL PROTOCOL

2.3.1 Cereal/pseudocereals analysis

The germination capacity and germinative energy can give useful information about vitality and dormancy (respectively) of the seeds and suggest the appropriate micro malting conditions (times-temperatures) for each sample (gluten-free adjuncts).

Germination capacity (European Brewery Convention-EBC method 3.5.2)

The determination of the percentage of living corns in a sample (cereal/pseudocereal) was carried out using a hydrogen peroxide assisted growth test. The samples (100 seeds of cereal/pseudocereal) were steeped in a hydrogen peroxide solution (7.5 g/L) at 19.5 ± 1.5°C. The seeds showing either root or acrospire growth after 48 h of incubation were counted, those that had not developed both root or acrospires growth were placed into a fresh hydrogen peroxide solution (7.5 g/L) at 19.5 ± 1.5°C for another 48 h.

The number of total seeds which had developed both root or acrospires growth was recorded and expressed as %.

The germination capacity (GC) test is a typical case of binomial distribution. If reasonable confidence in the values from the germination test is required, it is necessary to interpret the results using a standard statistical procedure based on the number of grains in the test. For example, if it is desired to reject all material with less than 93% viability, the acceptance level for 100 seeds used in the test has to be 98% (based on a 95% probability level) (Table 2.1). However, the same degree of protection can be obtained using an acceptance level of 96% provided that the number of seeds is increased to 350 and an acceptance level of 95% if the number of seeds is 500 (EBC, method 3.5.2).

Table 2.1 Lower limit of confidence (probability level 95%) depending on the number of seeds in the test (n) (Source: Analytica-EBC, 1998-2007).

GC	Germination Capacity, % Lower Limit of Confidence		
	n		
	100	350	500
94	87	91	92
96	90	93	94
98	93	96	96

Germinative energy (EBC method 3.6.2)

Germinative energy is the measurement of the percentage of seeds which can be expected to germinate fully if the sample is malted normally at the time of the test. A sheet of cotton wool was cut out to the size of the germination paper (Whatman No. 4 circular filter) and it was spread on a germination plate (Petri dish). The cotton wool layer was moistened with 3 mL of ultrapure water. Subsequently, 100 seeds were spread on the whole surface of the paper. The seeds were covered with the second sheet of germination paper (Whatman No. 4 circular filter). The plate thus prepared was moistened with the rest of the ultrapure water (1 mL) and it was put into a thermostat oven (with temperature 20 ± 1°C and relative humidity 95 ± 5% at any point). After 48 h the germinated seeds were counted and removed. A seed was considered germinated if rootlets or the acrospire, visible to the naked eye were developed. The plate with the non-

germinated seeds was put back immediately into the oven. Finally after 72 h, the number of the total germinated seeds was recorded and expressed as %.

2.3.2 Malt analysis

Malt quality (obtained from the micro malting tests) was checked analyzing moisture content, diastatic power, Kolbach index and β-Glucan content. The malt analyses were carried out according to Analytica EBC.

Moisture content of malt (EBC method4.2)

The determination of the moisture content of all malt samples was carried out recording their loss in mass upon drying under specified conditions. The malt sample (5 g) was milled and immediately placed in a clean, dry moisture dish, previously tared to 0.001 g. Then, the dish with the ground sample was weighed to 0.001 g (W1). The dish without the cover was placed in the preheated oven (105-106°C) for 3 h ± 5 min. Subsequently, the dish with the lid was cooled at room temperature in a desiccator. Then, it was reweighed to 0.001 g (W2). The moisture content of the malt samples was calculated according to the formula:

$$\text{Moisture } \%\ (\text{m/m}) = \frac{W1 - W2}{W1} \text{ x } 100$$

where:

W1 = mass in g of sample before drying

W2 = mass in g of sample after drying

The results (moisture percentage) were reported to one decimal place. To produce a stable and storable product, the malt traditionally is dried to < 4.5% moisture.

Diastatic power (EBC method 4.12)

Diastatic power is the determination of combined activity of alpha- and beta-amylase of malt under standardized reaction conditions (WK units). The enzymes were extracted from malt samples: the beaker with 20.0 g of milled malt sample and 480 mL of cool water were placed in a mashing bath (attemperated to 40°C) and maintained at this temperature for 1 h ± 2 min stirring its content continuously. Subsequently, 50 mL of filtered extract were collected (called malt extract). Thus, 100 mL of starch solution (20 g/L in water) and 5 mL of acetate buffer (pH 4.3) were pipetted into a 200 mL volumetric flask. The flask was placed into a water bath attemperated to 20°C and after 20 min 5 mL of the malt extract were added. After 30 min, the amount of reducing sugars formed by amylolytic action was estimated iodometrically (enzymes inactivated after the addition of 4 mL of sodium hydroxide 1 M), and the alkalinity of the solution was checked by adding a drop of thymolphthalein. Thus, 50 mL of the digest, 25 mL of iodine solution (12.7 g of iodine and 20 g of potassium iodide in 1 L of water) and 3 mL of sodium hydroxide were transferred into a 150 mL volumetric flask. Subsequently, after 15 min, 4.5 mL of sulphuric acid (0.5 M) were added and the unreacted iodine with thiosulphate solution (0.1 M) was titrated. Contextually, a blank test was prepared: 100 mL of starch solution (20 g/L in water) and 2.35 mL of sodium

hydroxide were pipetted into a 200 mL volumetric flask. Subsequently, 5 mL of malt extract were added and the volume was made up to 200 mL with water. The amount of maltose produced in the sample test under the hydrolysis conditions was calculated according to the formula:

$$DP\ (WK) = F \cdot (V_B - V_T)$$

where:

DP = diastatic power of sample, in Windisch-Kolbach units

V_B = titration value of unreacted iodine (mL) in blank test

V_T = titration value of unreacted iodine (mL) in sample test

F = correction factor to obtain the result per 100 g of malt used for the extraction.

The results were expressed as WK units to the nearest whole number (i.e. 200 WK represent malts with a good enzymatic activity).

Kolbach index (EBC method 4.9.1)

Kolbach index is the determination of soluble wort nitrogen content (prepared during the course of malt analysis) as a percentage of the total malt nitrogen content by a Kjeldahl procedure (Kjeldahl Gerhardt, Germany). The nitrogenous compounds in the malt/wort samples (1.0 g finely ground or 1.0 mL of wort) were digested with hot sulphuric acid 98% v/v (20 mL) in the presence of 10

g of a powdered catalyst mixture (potassium sulphate 70 parts m/m and copper sulphate pentahydrate 30 parts m/m) to give ammonium sulphate. The digest was made alkaline with 70 mL of sodium hydroxide solution (450 g of sodium hydroxide pellets in 1 L of water) and the released ammonia was distilled into an excess of boric acid solution (20 g/L in water). Thus, the ammonia was titrated with a standard acid solution (hydrochloric acid, 0.1 M). The soluble nitrogen content as a percentage of the total nitrogen content (Kolbach index) was calculated with the following equation:

$$N_K\,(\%) = \frac{N_S \cdot 100}{N}$$

where:

N_K = soluble nitrogen content as a percentage of total nitrogen in % (m/m)

N_S = soluble nitrogen content on dry malt in % (m/m) (EBC method 4.5.1)

N = total nitrogen content on dry malt in % (m/m).

The results were expressed as % (m/m) to the nearest whole number. A Kolbach index between 35 and 41% represents a malt with a good modification.

β-Glucan content (EBC method 4.16.3)

β-Glucan content is the determination of soluble high molecular weight mixed linkage (1,3)(1,4) β-D-glucan fraction in malt by spectrophotometric analysis (Varian Cary 1E UV-Visible).

To 1.0 g of milled malt sample 5.0 mL of aqueous ethanol (50% v/v) were added into a glass test tube (12 mL capacity). The mixture was incubated in a boiling water bath for 5 min. Thus, further 5.0 mL of 50% (v/v) aqueous ethanol were added into the test tube. The content of the test tube was centrifuged for 10 min at 1000 rpm. Subsequently, the pellet was re-suspended in 10.0 mL of 50% (v/v) aqueous ethanol and centrifuged again. The pellet (supernatant was discarded) was suspended in 5.0 mL of sodium phosphate buffer (20 mM, pH 6.5). Afterward, 0.2 mL of lichenase were added (enzyme purchased from Megazyme) and the test tube was incubated in a boiling water bath (attemperated to 40°C) for 1 h. The volume of the test tube was adjusted to 30.0 mL by addition of ultrapure water. The content of the tube was thoroughly mixed on a vortex mixer and an aliquot was filtered through a Whatman No. 41 circular filter. The filtrate (0.1 mL) was carefully and accurately (by Gilson Pipetman®) transferred to the bottom of three test tubes. An aliquot (0.1 mL) of sodium acetate buffer (50 mM, pH 4.0) was added to one of the test tubes (the blank), while to the other two (the samples) were added 0.1 mL of β-glucosidase (enzyme in 50 mM acetate buffer, pH 4.0, from Megazyme). The three test tubes were incubated at 40°C for 15 min. Then, 3.0 mL of the Gopod Reagent (p-hydroxybenzoic acid and sodium azide buffer purchased from Megazyme) were added to each tube. The three tubes were subsequently incubated at 40°C for 20 min. Finally, the absorbance of the content of the three test tubes was measured spectrophotometrically at 510 nm. The determination of β-glucan content was carried out using the formula:

$$\beta - \text{glucan (\% w/w)} = \Delta A \text{ x } F \text{ x } 300 \text{ x } \frac{1}{1000} \text{ x } \frac{100}{w} \text{ x } \frac{162}{180}$$

where:

ΔA = Absorbance after β-glucosidase treatment (reaction-sample) minus reaction-blank absorbance

F = A factor for the conversion of absorbance values to μg of glucose

$$= \frac{100 \text{ μg of D} - \text{glucose, standard solution purchased from Megazyme}}{\text{absorbance of 100 μg of D} - \text{glucose}}$$

300 = Volume correction (i.e. 0.1 mL taken from 30.0 mL)

$\frac{1}{1000}$ = Conversion from μg to mg

$\frac{100}{W}$ = Factor to express $\beta -$ glucan content as a percentage of dry flour weight

W = The calculated dry weight of the sample analysed (mg)

$\frac{162}{180}$ = A Factor to convert from $\text{D} -$ glucose, as determined, to anhydro $-\text{D} -$ glucose, as occurs in $\beta -$ glucan

The results were expressed as % (w/w) to the nearest whole number. When malts contain substantial levels of β-glucans (> 4.5% w/w), the modification

is incomplete and the polysaccharide itself may cause problems in the brewing process.

2.3.3 Statistical analysis

All analyses were performed in triplicate (n = 3). The statistical analysis was conducted using Student's test with α (0.05/number of Student's test replicas) corrected according to the Bonferroni test to assess any differences between group means (Excel 2003; Microsoft, Redmond, USA).

2.4 RESULTS AND DISCUSSION

Three micro-malting cycles for every sample (350 g of cereal and pseudocereals) were planned: for the first cycle literature parameters were considered (time and temperature) (Pelembe *et al*., 2002; Wijngaard *et al*., 2005b, c; Zarnkow *et al*., 2007; Alvarez-Jubete *et al*., 2010; De Meo *et al*., 2011), with changes to the steeping time based on the data obtained with germination capacity and germinative energy tests (Table 2.2).

The quinoa seeds show a more pronounced dormancy (lowest germinative energy) compared to the other gluten-free seeds (Table 2.2), and therefore were subjected to a longer steeping phase (Table 2.3a). Furthermore, the amaranth and quinoa samples provided the lowest values of GC (lowest viability) (Table 2.2). The malting conditions (steeping, air rest, germination, kilning times and temperatures) of the first cycle are reported in the Table 2.3a.

Table 2.2 Germination capacity (EBC method 3.5.2) and Germinative energy (EBC method 3.6.2) of four gluten-free adjuncts.

Gluten-free samples	Germination capacity (%)	Germinative energy (%)
Millet	96	94
Amaranth	95	93
Buckwheat	97	94
Quinoa	93	85

The use of an alkaline solution in the first steeping may facilitate the extraction of proteinaceous and phenolic molecules (fractions involved in haze

formations) from seeds (Briggs, 1998), and for this reason the pH of the first steeping water was modified: each cycle was repeated twice changing the first steeping water pH (in one case at pH 6.8 and in the second at pH 8.0) (Tables 2.3a, b and c).

Table 2.3a Duration (hours and days) and temperature (°C) of malting tests (first cycle) performed on 4 adjuncts.

Malting phases	I cycle			
	Millet[1]	Amaranth[2]	Buckwheat[3]	Quinoa[2]
1st steeping*	2 h (25°C)	5 h (13°C)	7 h (16°C)	26 h (13°C)
1st air rest	2 h (25°C)	2 h (13°C)	1 h (16°C)	5 h (13°C)
2nd steeping	2 h (25°C)	15 h (13°C)	16 h (16°C)	24 h (13°C)
2nd air rest	2 h (25°C)	2 h (13°C)	/	/
germination	5 days (25°C)	4 days (13°C)	5 days (16°C)	4 days (13°C)
kilning	24 h (50°C)	24 h (70°C)	24 h (50°C)	24 h (70°C)

*** = Each cycle was repeated twice at different pH of steeping water (6.8 and 8.0).**
([1]Pelembe *et al.*, 2002)
([2]Alvarez-Jubete *et al.*, 2010)
([3]Wijngaard *et al.*, 2005b,c)

In the second cycle the 1st, 2nd steeping and germination times were increased and the 1st air rest time was reduced (Table 2.3b). In the third cycle the 1st, 2nd steeping and germination times were reduced and the 1st air rest time was increased (Table 2.3c).

Table 2.3b Duration (hours and days) and temperature (°C) of malting tests (second cycle) performed on 4 adjuncts.

Malting phases	II cycle			
	Millet	**Amaranth**	**Buckwheat**	**Quinoa**
1st steeping*	6 h (25°C)	9 h (13°C)	11 h (16°C)	30 h (13°C)
1st air rest	1 h (25°C)	1 h (13°C)	1 h (16°C)	4 h (13°C)
2nd steeping	6 h (25°C)	19 h (13°C)	20 h (16°C)	28 h (13°C)
2nd air rest	2 h (25°C)	2 h (13°C)	/	/
germination	6 days (25°C)	5 days (13°C)	6 days (16°C)	5 days (13°C)
kilning	24 h (50°C)	24 h (70°C)	24 h (50°C)	24 h (70°C)

*** = Each cycle was repeated twice at different pH of steeping water (6.8 and 8.0).**

Table 2.3c Duration (hours and days) and temperature (°C) of malting tests (third cycle) performed on 4 adjuncts.

Malting phases	III cycle			
	Millet	**Amaranth**	**Buckwheat**	**Quinoa**
1st steeping*	1 h (25°C)	1 h (13°C)	3 h (16°C)	22 h (13°C)
1st air rest	3 h (25°C)	3 h (13°C)	2 h (16°C)	6 h (13°C)
2nd steeping	1 h (25°C)	11 h (13°C)	12 h (16°C)	20 h (13°C)
2nd air rest	2 h (25°C)	2 h (13°C)	/	/
germination	4 days (25°C)	3 days (13°C)	4 days (16°C)	3 days (13°C)
kilning	24 h (50°C)	24 h (70°C)	24 h (50°C)	24 h (70°C)

*** = Each cycle was repeated twice at different pH of steeping water (6.8 and 8.0).**

The malting trials (three cycles) were carried out in triplicate (n = 3).

The analyses carried out on the obtained malts allowed to underline the following considerations about the results collected (Figures 2.1a, b, c; 2.2 a, b, c; 2.3a, b, c and 2.4a, b, c):

The variation of malting parameters (steeping, air rest, germination times, and pH of first steeping water) does not affect the final malt quality. The pairwise comparisons among quality indices (diastatic power, Kolbach index) of all gluten-free malts obtained with the three cycles show no significant differences (Student's test) (α=0.002, corrected according to the Bonferroni test) (Tables 2.4a, b).

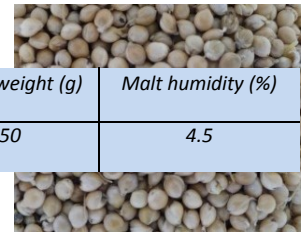
The buckwheat malt presents the highest diastatic power, which is the most important index to define the suitability of the malt for brewing, especially its "yield of extract" during the mashing phase: 148 ± 13 WK (I cycle-first steeping water pH 6.8) (Figure 2.3a), where 200 WK represents malts with a good enzymatic activity (activity of α- and β-amylase). During buckwheat malting β-amylase is solubilised, which is similar to what is observed during barley malting. The difference between buckwheat and barley malting is that in buckwheat additional β-amylase is produced (Wijngaard *et al.*, 2005c). The temperature during kilning was kept constant at 50°C for 24 h in all three cycles; in fact, several authors have demonstrated that prolonged kilning at 40°C (for 48 h) causes greater inactivation of α-amylase activity in comparison to β-amylase. However, the latter is still affected by the kilning regime. For this reason, the kilning regime has been set at 50°C for shorter times (24 h) than those cited in the literature (Nic Phiarais *et al.*, 2005), in order to have an adequate decrease in malt humidity (4.3% in all buckwheat malts) and to ensure survival of the amylolytic enzymes.

The Kolbach index values obtained from all gluten-free malts is in the range of 15 ÷ 36%, considering that Kolbach index values between 35 and 41%

represent a malt with a good modification (Figures 2.1b; 2.2b; 2.3b; 2.4b); the buckwheat malt presents the highest Kolbach index (36 ± 5 %, III cycle-first steeping water pH 6.8) (Figure 2.3b).

The β-glucan content is below the critical value of 4.5% in all the gluten-free malts (Figures 2.1c; 2.2c; 2.3c; 2.4c); this result suggests that there would be no significant problems during brewing process, especially in the lautering phase (i.e. obstructions caused by the wort viscosity).

Proso millet (*Panicum miliaceum*)



Sample weight (g)	Malt humidity (%)
350	4.5

Figure 2.1 a

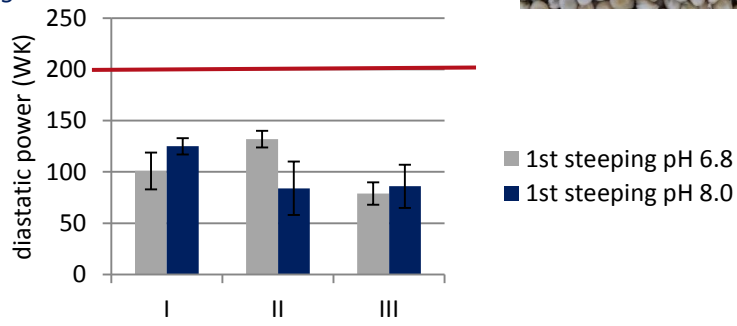


Figure 2.1 b

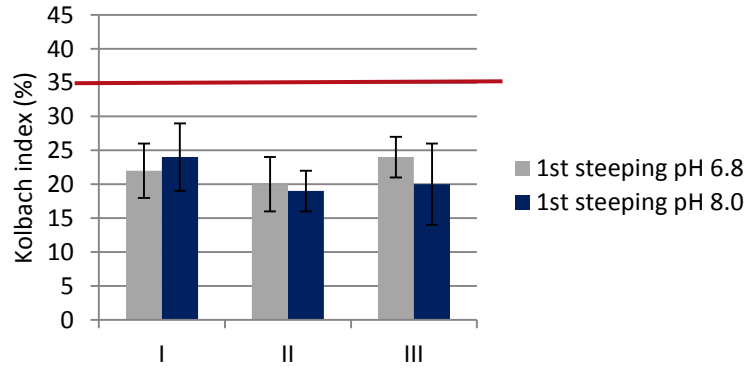
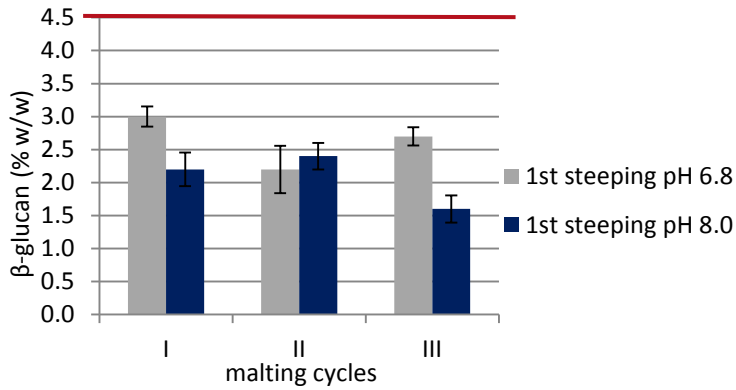
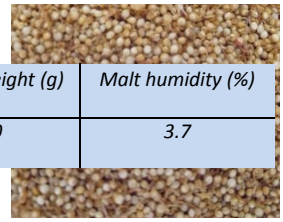


Figure 2.1 c



Figures 2.1: a- diastatic power (WK), b- Kolbach index (%), c- β-glucan (%w/w) of malted millet obtained with the three cycles (I, II and III). All analyses were replicated (n = 3) and are reported as mean values. The error bars represent the standard deviation (SD).

Amaranth (*Amaranthus caudatus*)



Sample weight (g)	Malt humidity (%)
350	3.7

Figure 2.2 a

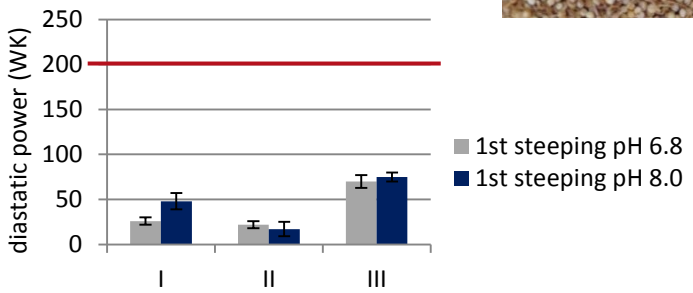


Figure 2.2 b

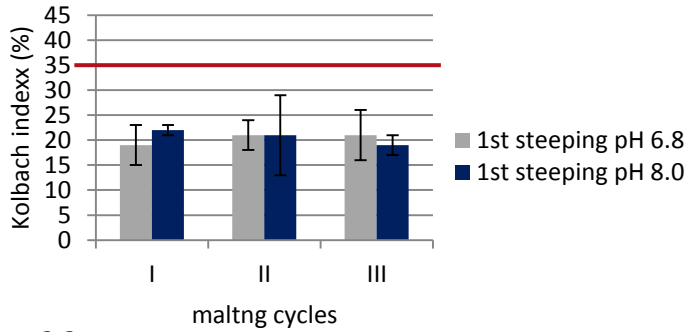
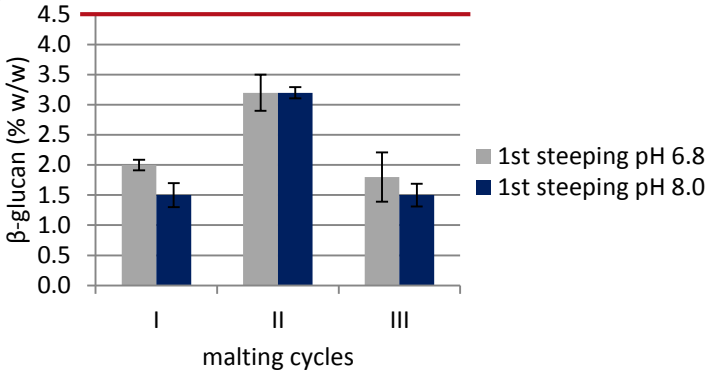
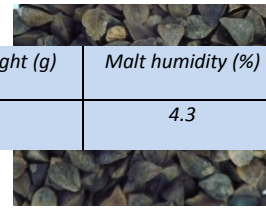


Figure 2.2 c



Figures 2.2: a- diastatic power (WK), b- Kolbach index (%), c- β-glucan (%w/w) of malted amaranth obtained with the three cycles (I, II and III). All analyses were replicated (n = 3) and are reported as mean values. The error bars represent the standard deviation (SD).

Buckwheat (*Fagopyrum esculentum*)



Sample weight (g)	*Malt humidity (%)*
350	*4.3*

Figure 2.3 a

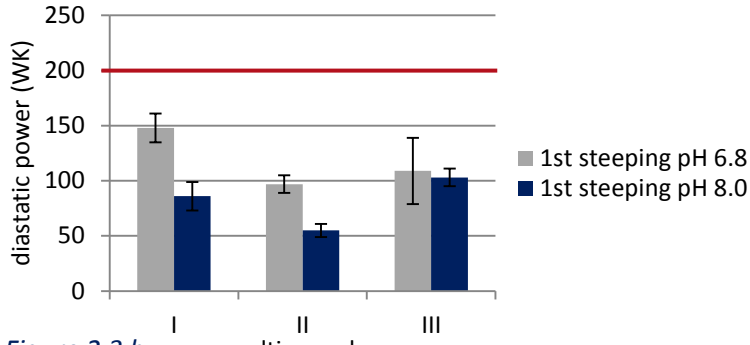


Figure 2.3 b

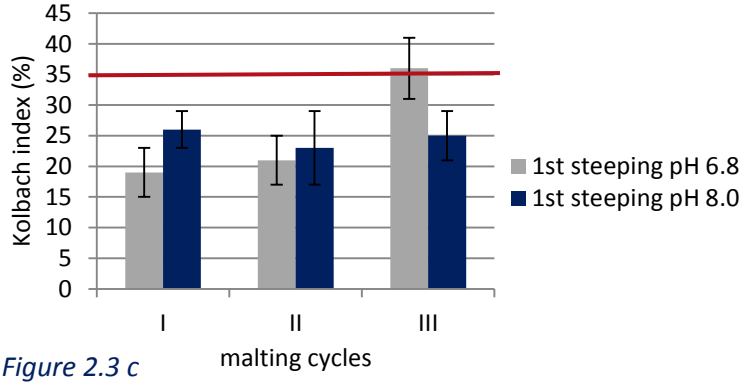
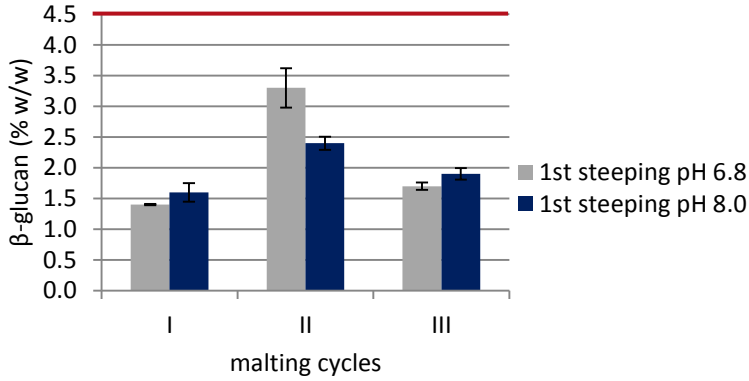
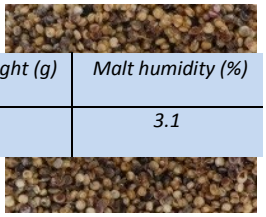


Figure 2.3 c



Figures 2.3: a- diastatic power (WK), b- Kolbach index (%), c- β-glucan (%w/w) of malted buckwheat obtained with the three cycles (I, II and III). All analyses were replicated (n = 3) and are reported as mean values. The error bars represent the standard deviation (SD).

Quinoa (*Chenopodium quinoa*)



Sample weight (g)	Malt humidity (%)
350	3.1

Figure 2.4 a

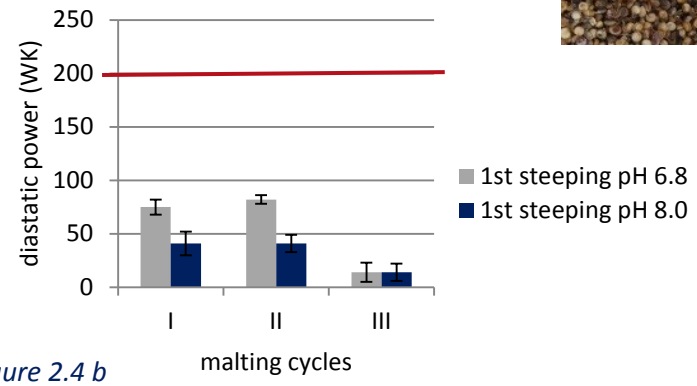


Figure 2.4 b

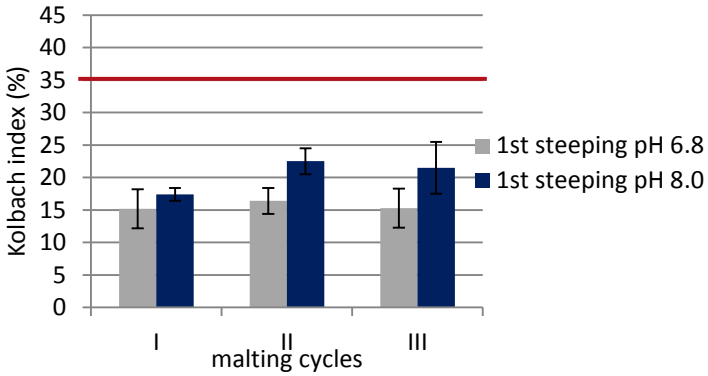
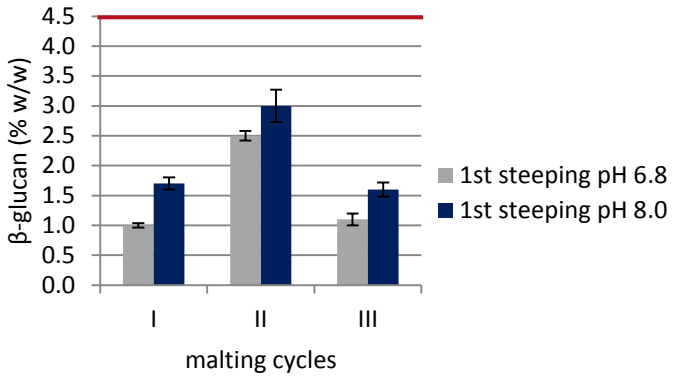


Figure 2.4 c



Figures 2.4: a- diastatic power (WK), b- Kolbach index (%), c- β-glucan (%w/w) of malted quinoa obtained with the three cycles (I, II and III). All analyses were replicated (n = 3) and are reported as mean values. The error bars represent the standard deviation (SD).

Table 2.4a Pairwise comparisons among the diastatic power (DP) (mean values) of malts obtained with the three cycles (I, II and III). All the differences were not statistically significant (Student's test with α corrected according to the Bonferroni test, $p < 0.002$).

Comparisons among DP values of malts obtained with different cycles (without alkaline treatment)	(p-value)	Comparisons among DP values of malts obtained with same cycle (with and without alkaline treatment)	(p-value)
Millet I-II	0.493	Millet I-I	0.743
Millet II-III	0.033	Millet II-II	0.162
Millet I-III	0.128	Millet III-III	1.000
Amaranth I-II	0.467	Amaranth I-I	0.173
Amaranth II-III	0.034	Amaranth II-II	0.686
Amaranth I-III	0.031	Amaranth III-III	0.172
Buckwheat I-II	0.060	Buckwheat I-I	0.041
Buckwheat II-III	0.301	Buckwheat II-II	0.060
Buckwheat I-III	0.765	Buckwheat III-III	0.356
Quinoa I-II	0.274	Quinoa I-I	0.158
Quinoa II-III	0.044	Quinoa II-II	0.045
Quinoa I-III	0.031	Quinoa III-III	0.918

Table 2.4b Pairwise comparisons among the Kolbach index (KI) (mean values) of malts obtained with the three cycles (I, II and III). All the differences were not statistically significant (Student's test with α corrected according to the Bonferroni test, p < 0.002).

Comparisons among KI values of malts obtained with different cycles (without alkaline treatment)	(p-value)	Comparisons among KI values of malts obtained with same cycle (with and without alkaline treatment)	(p-value)
Millet I-II	0.629	Millet I-I	0.217
Millet II-III	0.123	Millet II-II	1.000
Millet I-III	0.186	Millet III-III	0.712
Amaranth I-II	0.504	Amaranth I-I	0.325
Amaranth II-III	0.334	Amaranth II-II	0.639
Amaranth I-III	0.210	Amaranth III-III	0.264
Buckwheat I-II	0.200	Buckwheat I-I	0.096
Buckwheat II-III	0.081	Buckwheat II-II	0.430
Buckwheat I-III	0.038	Buckwheat III-III	0.121
Quinoa I-II	0.883	Quinoa I-I	0.775
Quinoa II-III	0.887	Quinoa II-II	0.237
Quinoa I-III	0.996	Quinoa III-III	0.758

2.5 CONCLUSIONS

The investigated experimental factors, malting conditions and pH of first steeping water do not show a significant effect on malt quality; the pairwise comparisons among quality indices (diastatic power and Kolbach index) of malts obtained with the three cycles show no significant differences. In this respect, the type of cereal is much more important than the malting conditions for the final malt quality.

The results obtained from the micromalting test are in line with the expectations: the four gluten-free cereals/pseudocereals are under-modified if compared to barley malt, but potentially suitable as adjuncts.

Furthermore, the results collected suggest that the raw material with the most prevalent potential for brewing appears to be buckwheat.

For each of the four gluten-free adjuncts a couple of malts was chosen (with and without the alkaline treatment), obtained from the cycle that had given the best indices (diastatic power and Kolbach index) for brewing on a laboratory scale.

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3. BREWING OF GLUTEN-FREE MALTS AT LAB SCALE AND AT THE EXPERIMENTAL PILOT PLANT

Part of this chapter is in combination with the work presented at:

18th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Conegliano (2013) Passaghe, P. The colloidal stability of craft beers: an assessment of aspects related to technology, composition and analysis.

3.1 INTRODUCTION

Several studies have focused on the production of beer from gluten-free cereals such as rice, maize, millet, and pseudocereals such as buckwheat, quinoa, and amaranth (Bauer *et al*., 2005; Nic Phiarais *et al*., 2005, 2006; Wijngaard and Arendt, 2006; Wijngaard *et al*., 2006) because of the absence of gluten and the presence of compounds that are claimed to have positive effects on health (Zarnkow *et al*., 2005; Kreisz *et al*., 2005).

Brewing of Millet

Several studies have suggested that millet could be used in brewing European type lager beer (Nout and Davies, 1982; Agu, 1995). Pearl millet is used in Mozambique for brewing traditional beer called *uphutsu* (Pelembe *et al*., 2002). The protein contents in most millets are comparable to those of wheat, maize, and rice, but finger millet is nutritionally superior because of its high levels of methionine, making it the best material for malting and brewing (Shewry, 2002). Moir (1989) attributes beer quality to colour, clarity, foam appearance, and flavour and comparative studies of barley, sorghum, and millet showed that beer brewed

from millet malt met these qualities (Agu, 1995). The fact that a suitable mashing program has been developed for extracting sorghum malt, whose starch, like that of millet, gelatinizes at a high temperature, suggests that millet malt can be extracted in a similar way (Palmer, 1989). Eneje *et al*. (2001) evaluated whether similar mashing methods developed for extracting sorghum malt would be suitable for extracting millet malt. It can be concluded that it is possible to produce a lager beer from millet although extensive work is needed to improve the flavour and colour of the beer.

Brewing of Amaranth

Only limited data on amaranth brewing is available in the literature. Fenzl *et al*. (1997) examined whether products pre-gelatinized through extrusion cooking are suitable as a partial substitute for barley malt in the production of lager beers. It was found that a 20% substitution is technically feasible without problems. Compared with the pure barley malt beer, the beer produced with amaranth was judged as better on smell, taste, bitterness quality, and full body taste, and was judged as worse on two of the evaluated characteristics (bitterness intensity and freshness of flavour). Considering the literature available and without further studies into its brewing potential, amaranth could be promoted as a low-alcohol innovative functional beverage (Zarnkow *et al*., 2005).

Brewing of Buckwheat

The first step in the production of buckwheat beer that needs to be optimized is mashing wort derived from malted buckwheat. It showed low fermentability values and high viscosity levels in comparison to wort derived from barley malt (Nic Phiarais *et al*., 2005; Wijngaard *et al*., 2005b). These worts were

obtained by congress mashing, which did not appear to be optimal for buckwheat malt. The optimization of mashing procedures was performed combining rheological tests with traditional mashing experiments (Goode *et al*., 2005a, 2005b; Wijngaard and Arendt, 2006). Improved lautering performance of the mash was observed when unhulled buckwheat was used instead of hulled buckwheat. Maccagnan *et al*. (2004) used buckwheat mainly as an unmalted adjunct in micro brewing for the production of gluten-free beer. The results of this study revealed that buckwheat has suitable beer-making properties with regard to both appearance and taste. Still more extensive work is required to optimize fermentation performance and beer characteristics (i.e. flavour, aroma, and foam development).

Brewing of Quinoa

To date, little research has been carried out on quinoa as a brewing ingredient, and mainly studies on the properties of quinoa starch are available. Quinoa starch, being high in amylopectin, gelatinizes at a low temperature, comparable with the temperate cereals wheat and barley, and rather lower than the tropical cereals such as maize and sorghum. Gelatinization temperature ranges of 57 ÷ 64°C and 60 ÷ 71°C have been reported. This suggests that an adjusted mashing procedure would not be required to extract quinoa malt. Quinoa starch exhibits a much higher viscosity than wheat and amaranth (Atwell *et al*., 1983; Qian and Kuhn, 1999). With regard to the use of quinoa as a brewing ingredient, Kreisz *et al*. (2005) performed malt analysis on optimally malted quinoa and found a slightly higher extract than for barley malt. A subsequent study by Zarnkow *et al*. (2005) showed that beer made from quinoa malt contained a similar alcohol level to barley beer and therefore has the potential to be used as a brewing ingredient.

Research on malt and beer based on gluten-free raw materials has focused mainly on sorghum, and as previously stated, the objective of this work has been to focus on the use of gluten-free cereal such as millet, as well as pseudocereals such as amaranth, buckwheat and quinoa, as alternatives to sorghum. The use of gluten-free substitutes for brewing was intended to reduce the base level of the sensitive protein fractions rich in proline in final beer, which take part in the turbidity process responsible for quality decay of the product.

3.2 MATERIALS

3.2.1 Reagents and samples

Iso-octane (2,2,4-trimethyl pentane) and hydrochloric acid were purchased from Sigma-Aldrich (Milan, Italy). Ethanol absolute (99.9% v/v) purchased from Carlo Erba (Milan, Italy). Solvents and other chemicals were of analytical grade. Ultrapure water was obtained by Milli-Q® Advantage A10® system (Millipore, Billerica, MA, USA).

The barley malt (Pale Ale type) was purchased from Weyermann Specialty Malting Company (Bamberg, Germany). The dry ale yeasts (Safale S-04) and hops (Hallertau perle, in pellets) were purchased from PAB (Udine, Italy).

3.3 EXPERIMENTAL PROTOCOL

The malts were analysed in accordance with standard analyses (EBC methods) (subsection 2.3.2, pp. 69 ÷ 75). However, standard analyses results are not always a reliable indicator of how well the malts will perform in a specific brewery. For this reason the aim of this section was to find the best conditions for brewing with these gluten-free adjuncts.

Malts with the best indices (beta-glucans, diastatic power and Kolbach index) obtained from the micro malting tests were microbrewed (2 L) producing nine beers (four beers replicated twice plus the reference), in order to define a specific mashing program. Consequently, the mashing program was optimized at the pilot plant of the University of Udine (Figure 3.1).

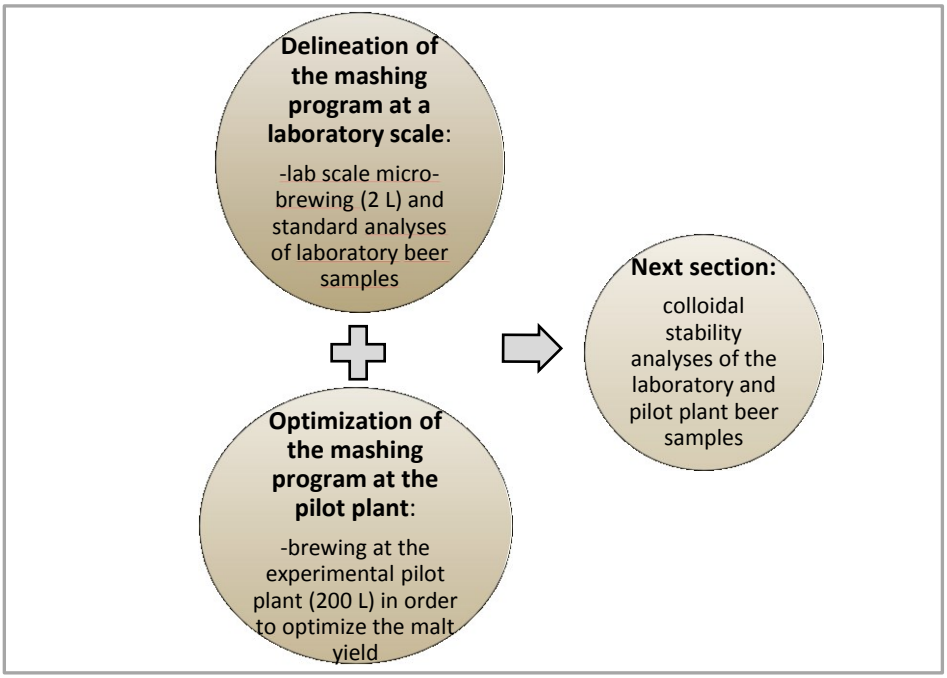

Figure 3.1 Experimental protocol.

3.3.1 Brewing conditions

The process conditions were kept constant for all the brews (Table 3.1); the original gravity was standardized (°P) for all the laboratory beer samples by acting on the volume of water used during the sparging phase (subsection 1.3.2, p. 30).

For each beer the same ingredients were used (Table 3.1): gluten-free adjuncts (40% w/w), barley malt (60% w/w), hop (α-acid 8.9%) and dry ale yeast (fermentation temperatures range: 15 ÷ 24°C). The latter two were employed in order to have standardized beer samples in terms of alcohol content and bitterness. Only barley malt (Pale Ale type) was used for the reference beer sample (Ba1).

Barley malt and gluten-free malts were milled at settings 1.2 mm and 0.2 mm respectively, and mashed-in with deionised water; a grist: water ratio of 1:3 was used. The following specific temperature-time profile was used: 30 min at 50°C, increase to 63°C (1°C/min), 45 min at 63°C.

After separation of the wort and the spent grains in a small scale lauter tun, the wort was hopped to obtain a bitterness of 20 IBU (International Bitterness Units – mg of bitter substances per litre of wort) boiled for 60 min in a glycerol bath (106°C) and it rested for 1 hour.

The hop was used in pellet (Hallertau perle) form because of its ease of use and availability. The hop amount was calculated using the following predictive formula:

$$\text{Hop quantity (g)} = \frac{\text{IBU}}{\text{a x 3}} \text{ x V}$$

where:

IBU = International Bitterness Units-mg of bitter substances per litre of beer

a = alpha acids content (%)

3 = constant that represents the hop yield (30%)

V = volume of beer (L)

The formula is dependent on the variety of hop pellet, the alpha acid percentage and the time of addition to the 60 min boil (Table 3.1). Two 800 mL worts were decanted (hot *trub* separation) and combined in a 2L fermentation vessel.

The wort was further cooled to 20°C, oxygenated, pitched with Safale S-04 (50-80 g/hL); during the cooling period, one package (11 g) Safale S-04 dry ale yeast was prepared according to package directions.

Approximately 100 mL sterilized (boiled) water at 20-25°C was placed into a sanitized 150 mL beaker. Yeast (0.5 g/L) was gently sprinkled on top of the water.

The yeast was allowed to hydrate for 15 min, and further pitched into the cooled wort.

The primary fermentation process took place at 20 °C. When the specific gravity measurement remained constant for two consecutive days, a cold maturation was carried out for 20 days at 1 °C.

All the laboratory samples were bottle conditioned: re-fermented at 23°C for 1 month. The determinations of alcohol content, pH, IBU were carried out one month after bottling.

Considering the alcohol content, the refermentation in bottle conditioned samples caused an increase in its value (i.e. from 4.6 % to about 5 % v/v). This increase is accounted for the fermentable sugar added and for this reason, the pH of beer samples was measured only one month after bottling.

Table 3.1 Flow diagram for laboratory beer brewing procedure.

Raw materials
Barley malt (Pale Ale type, 360 g), Gluten-free malt (240 g)
Hallertau Perle (α-acids, 8.9%)
SafAle S-04 (dry ale yeast)
Brewing process
Milling
Pale Ale and gluten-free malts (mill setting at 1.2 mm and 0.2 mm respectively)
Mashing
grist: water ratio of 1:3 (600 g/1.8 L)
temperature-time profile: 30 min at 50°C, increase to 63°C (1°C/min), 45 min at 63°C
Wort separation-Sparging
sparge water at 70°C (volume in the range of 200 ÷ 400 mL)
Wort boiling
temperature-time profile: 60 min at 106°C
add hops at following intervals (boil time remaining):
60 min, 3 g (Hallertau Perle in pellets), 20 IBU predicted
Wort cooling
to 20°C (fermentation temperature)
Primary fermentation
dry ale yeast (Safale S-04, 0.5 g/L)

temperature-time : 4÷8 days at 20°C
Cold maturation
temperature-time: 20 days at 1°C
Condition (refermentation)
temperature-time: 1 month at 23°C

3.3.2 Standard analysis

The beers produced in the laboratory, as indicated in subsection 3.3.1, were subjected to the following standard analyses: pH, alcohol, Original Gravity (O.G.), Extract Density (E.D.) and bitterness. Standard beer analyses (pH and bitterness) were carried out according to Analytica EBC.

pH of beer (EBC methods 1.5 and 9.35)

The pH of laboratory beer samples was evaluated at 20 °C according to the EBC method 9.35 using a pH meter (*Crison* micropH 2001). The results are reported to two decimal places (Tables 3.2 and 3.4).

Alcohol, E.D and O.G. of beer

The determination of alcohol content was carried out using the Alcolyzer Beer Analyzing System. The beer samples were degassed and thermostated (20°C):

50 mL were filtered through a Whatman No. 4 circular filter. The filtered beers were collected into a becker (100 mL) and immediately analyzed.

The system consists of the Alcolyzer Plus beer measuring module and the Anton Paar density meter. At the heart of this system is the selective alcohol measurement: a narrow, highly alcohol-specific range of the NIR spectrum is evaluated using a specially developed spectrometer and suitable algorithms. In this particular spectral range, the influence of other beer ingredients is so small that extremely accurate alcohol results are obtained. While the Alcolyzer Plus determines the alcohol content, an Anton Paar oscillating U-tube density meter determines the density of the beer samples. It calculates extract density (E.D. in g/L) from the primary measuring values, density and alcohol, using a specific algorithm.

From this, original gravity (O.G.) that is expressed in °P (Plato), which measures the concentration in weight/weight terms as g of solids per 100 g of wort, is determined by the Anton Paar instrument. The extract is expressed both as E.D and °P. The measured/calculated data are displayed and sent to a printer.

Studies comparing the Alcolyzer Plus beer analyzing system with the distillation method (EBC method 9.2.1) have shown no significant deviation of the mean values and a reproducibility standard deviation of 0.025% v/v (Zanker and Benes, 2004).

The alcohol content (% v/v) and O.G. (°P) values are reported to two decimal places (Tables 3.2 and 3.4). The E.D. (g/L) values are reported to three decimal places (Tables 3.3. and 3.5).

Bitterness of beer (EBC method 9.8)

The bitter substances (mainly iso-α-acids) were extracted with iso-octane (20 mL) from acidified (0.5 mL of 6 M hydrochloric acid) beer (10 mL of degassed beer sample). After centrifugation (3 min at 3000 rpm, Beckman; model TJ-6 centrifuge, Ireland), the absorbance of the iso-octane layer is measured at 275 nm (Varian Cary 1E UV-Visibile spectrophotometer) against a reference of pure iso-octane. The bitterness values were calculated according to the formula:

$$\text{Bitterness (IBU)} = 50 \text{ x } A_{275}$$

where:

A_{275} = the absorbance at 275 nm measured against a reference of pure iso-octane.

The results are reported as IBU values to the nearest whole number (Tables 3.2 and 3.4).

The malt yields were calculated with the following formula:

$$\text{Yield (\%)} = \frac{\text{O. G x E. D. x V}}{\text{W}} \text{x } 100$$

where:

O.G. = Original Gravity of the wort in °P (Plato)

E.D. = Extract Density of the wort (g/L)

V= Volume of beer produced (L or hL)

W = weight of the malt employed

The results are expressed as % (w/w) to the nearest whole number (Table 3.3 and 3.5). The yield for a typical barley malt (Pale Ale type) is in the range of 60 ÷ 70%, and the beer sample obtained with only barley malt was used as the reference for the other beer samples.

3.3.3 Standard analyses results for laboratory beer samples

According to the literature, the best results in terms of yield and volume of beer produced, with the four gluten-free malts (i.e. buckwheat malts without alkaline treatment, 40% and 2.1 L respectively) (Table 3.3), were obtained when a mashing-in temperature was used in the range of 45°C to 50°C (Goode *et al.*, 2005a, 2005b; Wijngaard and Arendt, 2006).

These mashing-in temperatures were used to design an optimal mashing procedure (Table 3.1). The obtained results confirm the expectations; the malts with a higher modification degree (diastatic power and kolbach index, subsection 2.4, pp. 76 ÷ 86) produced higher extract yields (Table 3.3). Quinoa and Amaranth malts (with and without the alkaline treatment), which provided the lowest

diastatic power values, gave the lowest yields (28-30% and 30-32% respectively) and consequently the lowest volumes of the final beers (1.5-1.6 L and 1.6-1.7 L respectively) (Table 3.3). Instead, the buckwheat malt (without alkaline treatment) gave both a higher diastatic power and the best yield (40%), compared to the beers obtained with the other gluten-free malts (Table 3.3).

The O.G. values obtained from all the laboratory beer samples are in the range of 10.72 ÷ 11.52 °P, obviously the reference Ba1 provided the highest value (Table 3.2). The alcohol content (% v/v) obtained for all the beer samples is in the range of 4.59 ÷ 4.96% v/v (Table 3.2).

Regarding grist size, optimum results were obtained when the grist was milled as small as possible. For such a small grist size, a small scale lauter tun was used in order to optimize the lautering phase (separation of the wort from the insoluble spent solids). The malts were not difficult to handle, i.e. they did not cause process problems (i.e. during lautering phase); in fact the β-glucan content was below the critical value of 4.5% in all the gluten-free malts (subsection 2.4, p. 80).

The bitterness units obtained for all the beer samples are in the range of 18 ÷ 23 IBU (Table 3.2). Furthermore, the pH of the laboratory beer samples is in the range of 3.81 ÷ 4.91 (Table 3.2); some authors (Kunz and Methner, 2009; Kunz *et al*., 2010) have demonstrated that in the range of 5.50 to 3.25 a decrease in the pH leads to a stronger chill haze formation.

Table 3.2 Standard chemical analyses of laboratory beer samples.

Laboratory beer samples	Code	pH (20°C)	O.G. (°P)	Alcohol (% v/v)	Bitterness (IBU)
Reference sample	Ba1	4.72	11.52	4.96	21
Beer obtained with millet*	M1	4.51	10.98	4.69	23
Beer obtained with millet**	M2	4.67	11.02	4.72	20
Beer obtained with amaranth*	A1	4.91	10.93	4.68	18
Beer obtained with amaranth**	A2	4.90	10.82	4.63	19
Beer obtained with buckwheat*	Bu1	4.63	11.04	4.72	20
Beer obtained with buckwheat**	Bu2	4.45	11.24	4.81	21
Beer obtained with quinoa*	Q1	3.81	10.72	4.59	22
Beer obtained with quinoa**	Q2	4.11	10.77	4.61	21

***=first steeping water pH (6.8)**
****=first steeping water pH (8.0)**

Table 3.3 Gluten-free malts yield in laboratory brewing.

	Sample Code								
	Ba1	M1	M2	A1	A2	Bu1	Bu2	Q1	Q2
E.D. (g/L)	1.046	1.044	1.044	1.043	1.044	1.044	1.045	1.043	1.043
Volume of beer (L)	2.5	1.9	1.8	1.6	1.7	2.1	2.0	1.5	1.6
Malt yield (%)	50	36	35	30	32	40	39	28	30

3.3.4 Brewing at the experimental pilot plant

After a micro-mashing program was delineated at the laboratory scale, the experimental plan was transferred on a larger scale (capacity of 200 L).

The buckwheat seeds gave the best malts in terms both the diastatic power (subsection 2.4, p. 79) and yield (Table 3.3: subsection 3.3.3, p. 105). According to these results buckwheat malt was used for brewing at the pilot plant.

Three beers with different formulations were produced: the first with 100% malted barley (reference sample Ba2), the other two with increasing amounts of malted buckwheat (20% and 40% w/w).

The same ingredients (hop and dry ale yeast) and conditions used in the laboratory brewing procedure (Table 3.1) were adopted: adjuncts (20% and 40% w/w), barley malt (60% w/w), hop (α-acid 8.9%) and dry ale yeast (fermentation temperatures range: 15-24 °C).

Also in this case the beers were standardized in terms of °P, alcohol and bitterness (Table 3.4). The aim was to test on a larger scale the technological applicability of the laboratory brewing conditions, in terms of yield (Table 3.5) and potential filtration problems. All the pilot plant samples were bottle conditioned.

The laboratory mashing was not completely optimised in terms of extract recoveries (yield); the reference and the beers obtained with buckwheat malt provided yields in a range of 39 ÷ 50% (Table 3.3). The mashing program was improved (yields in a range of 54 ÷ 60%) (Table 3.5) with the mashing system (lauter tun) and the whirlpool tank (wort separation technique) of the pilot plant. As expected, with an increased buckwheat amount in the recipe (from 20% to 40%

w/w) a moderate decrease in the total yield was observed (from 56% to 54%) (Table 3.5).

Table 3.4 Standard chemical analyses of pilot plant beer samples.

Laboratory beer samples	Code	pH (20°C)	O.G. (°P)	Alcohol (% v/v)	Bitterness (IBU)
Reference sample obtained with 100% barley malt	Ba2	4.38	11.72	4.86	23
Beer obtained with 20% of buckwheat malt*	Bu20%	4.35	11.20	4.72	23
Beer obtained with 40% of buckwheat malt*	Bu40%	4.41	11.32	4.69	21

***=first steeping water pH (6.8)**

Table 3.5 Gluten-free malts yield in pilot plant brewing.

	Sample Code		
	Ba2	Bu20%	Bu40%
E.D. (g/L)	1.047	1.045	1.045
Volume of beer (L)	172	166	160
Malt yield (%)	60	56	54

3.4 CONCLUSIONS

Malts with the best indices (beta-glucans, diastatic power and Kolbach index) obtained from the micro malting tests were microbrewed (2 L) producing nine beers with quite similar Plato degree values (O.G. in the range of 10.72 ÷ 11.52°P) and bitterness (in the range of 18 ÷ 23 IBU) (Table 3.2). The seeds should be milled as finely as possible, and a grist-water ratio of 1:3 provided the best results (in terms of O.G., D.E., alcohol and volume) for all the beers produced (Tables 3.2 and 3.3).

The same process conditions were adopted on a larger scale (35 Kg of malt for a pilot plant capacity of 200 L) for brewing with increasing amounts of buckwheat (0%, 20% and 40% w/w). The aim was to improve the laboratory mashing program.

The buckwheat malt provided an acceptable extract yield; the extract yield obtained (56% for beer obtained with 20% w/w of buckwheat malt) compared to the reference sample Ba2 (60%) (Table 3.5) can be considered quite good. The buckwheat gave yields higher than those obtained with the micro-brewing process (Tables 3.3 and 3.5).

Furthermore, the buckwheat seeds had operated satisfactorily under laboratory conditions and throughout the brewing process at the pilot plant; the malt, was not difficult to handle and therefore it did not cause process problems (i.e. during lautering phase).

According to the obtained results, it is possible to state that the gluten-free adjuncts considered can potentially be used for brewing. A mashing program was successfully optimized for buckwheat malt. However, future studies should be

performed to improve the brewing conditions, especially for amaranth and quinoa, which showed lower extract yields compared to the other gluten-free adjuncts (Table 3.3).

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4. COLLOIDAL STABILITY OF LAB AND PILOT PLANT BEER SAMPLES

Part of this chapter is in combination with the works presented at:

-34th EBC Congress, Luxembourg (2013) Buiatti, S., Bertoli, S., and Passaghe, P. Evaluation of chemical physical stability of craft beers through unconventional methods.

-18th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Conegliano (2013) Passaghe, P. The colloidal stability of craft beers: an assessment of aspects related to technology, composition and analysis.

4.1 INTRODUCTION

Hazes can arise from numerous causes. Although the reaction between polyphenols and proteins is undoubtedly the most common cause of haze in modern brewing and brewers' primary action for control, compounds like starch, metal ions, β-glucans, pentosans, hop products, oxalate, foam stabilizers, filter aid (and so on) can also cause the so-called non-biological haze (Lewis and Bamforth, 2006).

These compounds (proteins and polyphenols) form weak, temperature sensitive hydrogen bonds that produce haze at low temperatures (0-4°C) and are broken as the beer's temperature increases (chill haze, with particle size between 0.1 and 1.0 µm). The other form of haze is permanent (with particle between 1 and 10 µm), and is characterized by strong covalent bonds in which constituent atoms share the available electrons to achieve a more stable energy state (Bamforth, 1999).

There is a fundamental compromise between beer foam and clarity; proteins (though different ones) drive both phenomena, and in general, factors that improve foam could aggravate the haze.

Most beers contain approximately 300 ÷ 1,000 mg/L total-N equivalent (0.11 ÷ 0.63% protein) (Briggs *et al*., 2004). Polypeptides responsible for haze formation (known as sensitive or haze-active proteins) originate mainly from barley, ranging in size from 10 kD to 30 kD, and are rich in the amino acid proline (Figure 4.1). They are heavily glycosylated with glucose and account for only 3-7% of total beer proteins (Siebert and Lynn, 1997; Leiper *et al*., 2003; Leiper *et al*., 2005). A quantity as low as 2 mg/l of protein can produce a haze of 1 EBC (European Brewery Convention), equivalent to 69 FTU (formazin turbidity units) (Fontana and Buiatti, 2009).

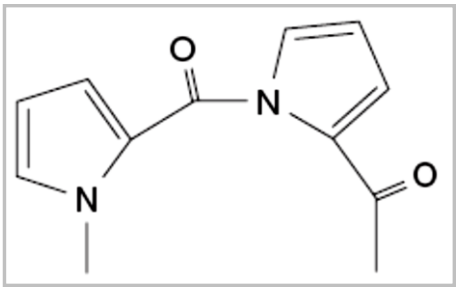

Figure 4.1 A fraction of polyproline (Source: Asano, 1982).

The pyrrolidine rings of proline forming proteins have unfolded molecular structures that facilitate the entry of polyphenols into them. Furthermore, the pyrrolidine ring of proline cannot form intramolecular and intermolecular hydrogen bonds with oxygen atoms of peptide bonds and, consequently, these

free oxygen atoms readily form hydrogen bonds with hydroxyl groups of polyphenols. Moreover, proline participates in hydrophobic bonding between the haze-forming proteins and polyphenols (Asano, 1982).

Polyphenols in beer originate from barley and hops. Their structure is based on phenol (monohydroxylated benzene) and the term “polyphenol” covers all molecules with two or more phenol rings (Bamforth, 1999).

Beer contains approximately 100 ÷ 300 mg/L polyphenol (McMurrough and O’Rourke, 1997) and these can be divided into derivatives of hydrobenzoic and hydroxycinnamic acids, as well as flavanols and their derivatives (Hough *et al*., 1982). The latter group accounts for 10% of total beer polyphenols and contain the species related to colloidal instability. Flavanoids (oligomers of flavanols) all have the same basic structure of two aromatic rings linked by a three carbon unit and they are often hydroxylated to varying degrees, and these groups are sometimes glycosylated or methylated (Doner *et al*., 1993).

Flavanols found in beer are catechins, epicatechins, gallocatechins and epigallocatechins (Siebert and Lynn, 1998) (Figure 4.2). These can exist as monomers, but are more commonly joined to form flavanoids as dimers, trimers or larger polymers. Polyphenols are lost throughout the brewing process, particularly during mashing, boiling, wort cooling and cold conditioning. Flavanoids found in beer consist of monomers, dimers and a few trimers at a level of approximately 15 mg/L (McMurrough and O’Rourke, 1997). Two dimers have been particularly associated with haze formation: procyanidin B3 (catechin-catechin) and prodelphinidin B3 (gallocatechin-catechin). These are known as proanthocyanidins and come from malt and hops, accounting for only 3.3% of total beer polyphenols. Monomers on their own do not appear to be involved in haze formation.

The number and position of hydroxyl (OH) groups on the flavanoid's aromatic rings influence protein binding. Thus rings with only one OH group are almost inactive, whereas those with two OH groups are more active, especially when they are adjacent (vicinal), and the activity further increases with three OH groups. Thus prodelphinidin B3 is more haze active than procyanidin B3, as gallocatechin has three vicinal OH groups while catechin has two (Leiper *et al*., 2005).

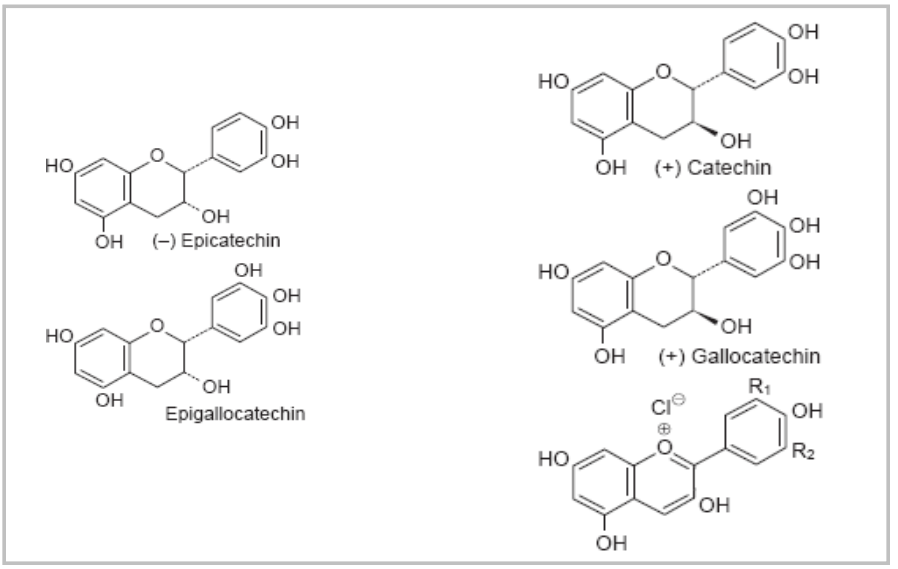

Figure 4.2 Flavanols found in beer: catechin, epicatechin, gallocatechin and epigallocatechin (Source: Briggs et al., 2004).

4.1.2 Mechanism for haze formation

4.1.2.1 Chapon model

The exact mechanism by which flavanoids bind to polypeptides and cause haze is uncertain, however it has long been recognized that the most frequent cause of haze in packaged beer is protein-polyphenol interaction (Siebert and Lynn, 1997).

Fresh beer contains acidic proteins and numerous polyphenols. These can come together by loose hydrogen bonding, but the associations formed are too small to be seen by the naked eye. These polyphenols, called flavanoids, can further polymerize and oxidize to produce condensed polyphenols, which have been called tannoids (Figure 4.3) (Chapon, 1993).

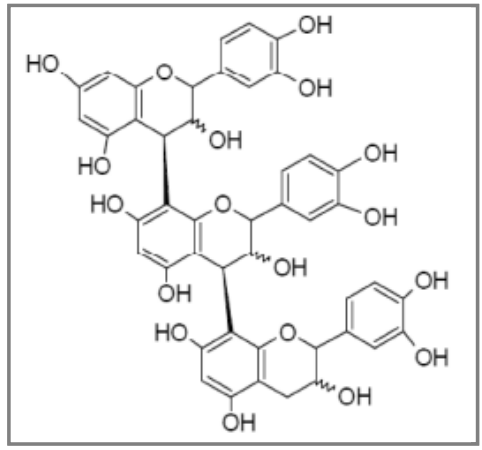

Figure 4.3 Proanthocyanidin (condensed tannin) structures (Source: Aron and Shellhammer, 2010).

These tannoids (originated from the oxidative processes) can `bridge' by hydrogen bonding across a number of proteins to form a reversible chill haze.

This haze forms at around 0°C, but redissolves when the beer is warmed to 15°C. After further storage of the beer, strong bonds can form between the tannoids and proteins and permanent haze is formed (Figure 4.4).

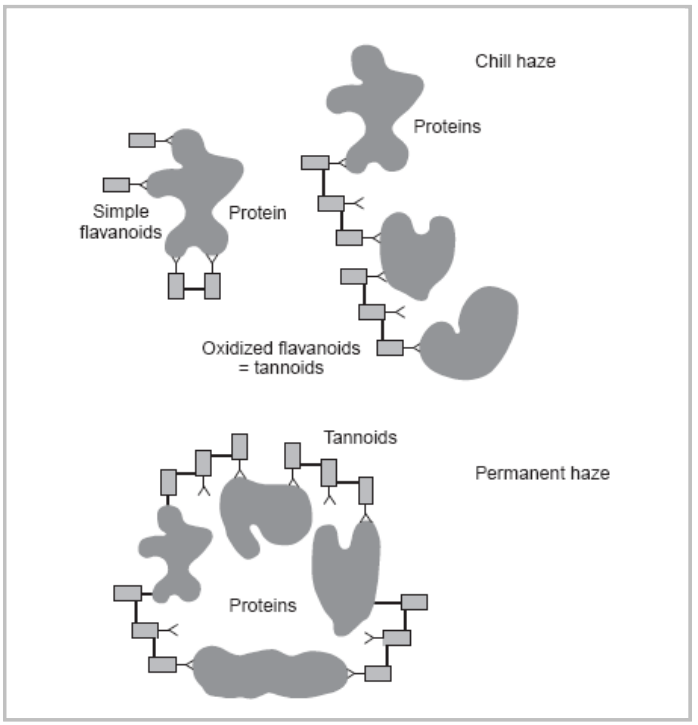

Figure 4.4 Models of chill and permanent hazes development in beer (Source: Gopal et al., 2005).

The nature of haze formation in this manner likely involves hydrogen bonding and hydrophobic stacking of proline and polyphenol rings associated with

π-bonding (Figure 4.5). Formation of protein-polyhenol haze depends on the beer pH, alcohol content, ionic strength, as well as phenolic composition (Siebert and Lynn, 2006; Siebert and Lynn 2007; Siebert and Lynn 2008; Aron and Shellhammer, 2010).

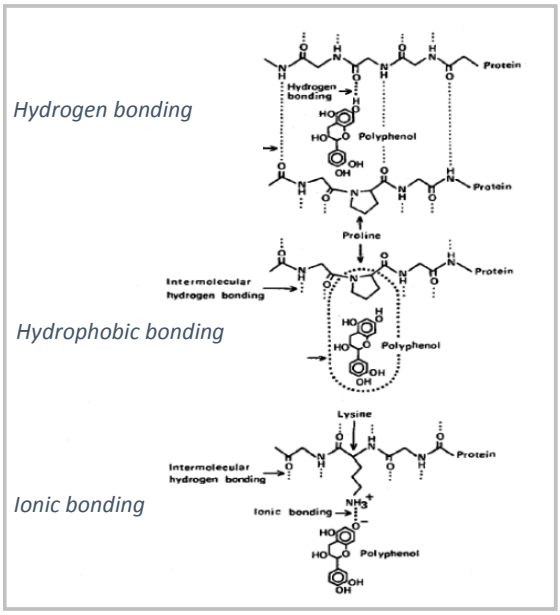

Figure 4.5 Mode of protein combination with polyphenols (Source: Asano et al, 1982).

This model suggests that effective stabilization should be achieved by removing from the beer the constituents of the haze, i.e., the `tannin sensitive' proteins and/or the polyphenols (Briggs *et al*., 2004).

4.1.2.2 Siebert model

An alternative model of haze-formation has been proposed (Siebert *et al*., 1996). This suggests there is a fixed number of binding sites on haze-forming proteins (proline residues) and that haze-forming polyphenols have two binding sites, through which they can join two adjacent protein molecules (Figure 4.6). If there is an excess of proteins with respect to polyphenols, then the polyphenol is involved in binding just two protein molecules together, and these dimers do not constitute insoluble complexes. If the amount of polyphenol greatly exceeds that of protein, then there is a shortage of protein binding sites, and again, haze complexes will not be formed. Hazes are therefore formed when there are equivalent amounts of protein and polyphenol in the beer.

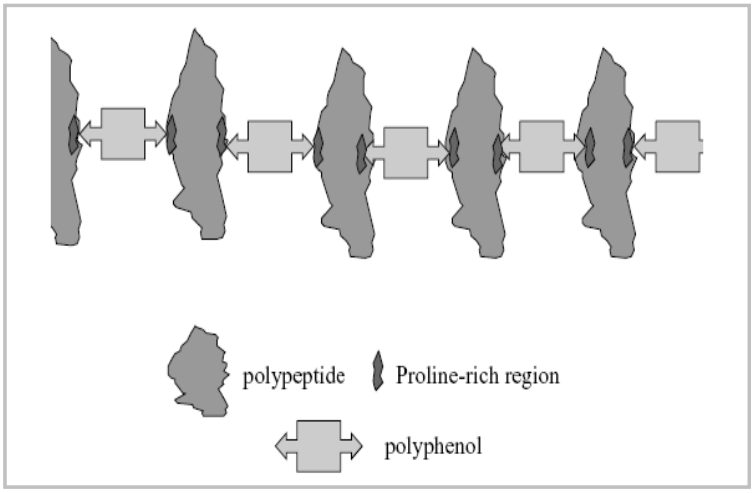

Figure 4.6 The Siebert model for haze formation (Source: Lewis and Bamforth, 2006).

This model suggests an alternative strategy for the prevention of haze, i.e., substantially increasing the amount of either protein or polyphenol. This is not a favoured approach and most brewers will seek to reduce levels of either proteins or polyphenols, or most likely both (Briggs *et al*., 2004; Yang and Siebert, 2001; Leiper *et al*., 2005).

4.1.3 Prediction of haze stability of beer

A diversity of methods have been proposed and used to estimate the physical shelf life of beer. They can be divided into methods that (a) measure specific haze components (b) "force" the beer, thereby accelerating the development of haze (and other elements of colloidal instability notably precipitates and/or bits).

Clearly the first method type has serious inadequacies if only one or relatively few are performed. For example, one method may not reveal a worrisome level of haze-forming protein in beer, but that says nothing about its content of polysaccharides, oxalate and so on. For this reason, some brewers have based their predictive techniques on a combination of a pair of such methods, i.e. protein and polyphenol measurements, but even that may be inadequate.

The second type of method is more reasonable, as (depending on its precise nature) it should assess the tendency of all colloidally-sensitive materials to "drop" out of solution. These methods can be divided into those that challenge the beer by extremes of heat or by hot-cold cycling, and those that involve adding an agent (notably alcohol) that, associated to extreme chilling, will lead to any material that has a tendency to leave solution so to do.

In terms of the former method type we can include:

- for protein: the saturated ammonium sulphate precipitation limit (SASPL) test and the tannic acid precipitation test (EBC method 9.40) (Berg *et al*., 2007; Buckee, 1994; Schneider *et al*., 1997; Analytica-EBC, 1998-2007)
- for polyphenol: the colorimetric determination of total polyphenols (EBC method 9.11), titration with polyvinylpyrollidone (PVP), high performance liquid chromatography (Siebert and Lynn, 2006) and the spectrophotometric determination of flavonoids (EBC method 9.12)

Amongst the forcing tests (O'Neill, 1996) there are:

- The EBC method 9.30 in which beer is held at 60°C for 48 h then cooled to 0°C for 24 hours and the haze is measured
- The Harp method in which the beer is stored for 4 weeks at 37°C followed by 8 hours at 0°C and the haze is measured
- Various cycling methods, such as the one that holds beer for 24 hours at 37°C then for 24 hours at 0°C, this theoretically representing the equivalent of one month of storage at ambient temperatures

Perhaps of higher value are tests in which colloidally sensitive materials are forced out of solution. The most famous of these is the alcohol chill haze or the Chapon test (EBC method 9.41), in which a sample of beer is chilled to –5°C without freezing (added alcohol prevents freezing) and left for 40 min before the chill haze is measured (Bamforth, 2011).

This work aimed to study issues related to the physical and chemical stability of craft beers. Specifically, the plan was to analyze how some variables in the malting process (pH of the first steeping water) and formulation (use of gluten-free adjuncts) can contribute to the colloidal stability (shelf-life) of the final beer. The beers produced in the laboratory and in the pilot plant, as indicated in section 3, were analyzed as described in the following subsection 4.3.

4.2 MATERIALS

4.2.1 Reagents

Tannic acid, sodium carboxymethyl cellulose (CMC), ethylenediaminetetraacetic acid (EDTA), green ammonium iron citrate, hydrochloric acid, methanol, p-dimethyl aminocinnamaldehyde, fish-gelatin liquid, 2,2'-Azo-bis(2-amidinopropane) dihydrochloride (ABAP), Trolox C, ethyl ether, saffron-crocin were purchased from Sigma-Aldrich (Milan, Italy). Dibasic sodium phosphate, absolute ethanol (99.9% v/v) purchased from Carlo Erba (Milan, Italy), and reagents purchased from RIDASCREEN® Gliadin competitive kit (R-Biopharm AG, Darmstadt, Germany.). Solvents and other chemicals were of analytical grade. Ultrapure water was obtained by Milli-Q® Advantage A10® system (Millipore, Billerica, MA, USA).

4.3 COLLOIDAL STABILITY ANALYSIS

The evaluation of beer colloidal stability (laboratory and pilot plant samples) was carried out analyzing two indices validated by EBC (sensitive proteins

and alcohol chill haze respectively), and two unconventional methods (gluten analysis and antioxidant activity, AA) with the aim to verify the possible correlation among unconventional and official methods.

The colloidal stability monitoring (alcohol chill haze) of the beers produced at the laboratory was carried out throughout their storage time (one, three and six months after bottling). Furthermore, the polyphenols and flavanoids content of the pilot plant beer samples was estimated using the two EBC methods (9.11 and 9.12 respectively) in order to verify the possible correlation among polyphenols/flavanoids content and antioxidant activity of beers.

4.3.1 Official methods

4.3.1.1 Sensitive proteins (EBC method 9.40)

A measured volume (5 mL) of tannic acid solution (200 mg/L) was added to an aliquot (200 mL) of degassed beer (by sonication) into a 250 mL beaker. The solution (beer with tannic acid) was stirred at ambient temperature for 40 min. The amount of precipitated proteins (sensitive proteins combined with tannic acid) was measured as an increase in the haze of the beer by a nephelometric measurement (TB1 VELP Scientifica). The values (sensitive proteins) were calculated according to the formula:

$$\text{Sensitive proteins} = \text{FT} - \text{IT}$$

where:

FT= final turbidity of the beer sample after addition of tannic acid solution

IT = initial turbidity of the beer sample (without tannic acid)

The results are reported as EBC units to the nearest whole number.

4.3.1.2 Alcohol chill haze (Chapon test, EBC method 9.41)

A measured volume (6 mL) of ethanol (99% v/v) was added to an aliquot (200 mL) portion of degassed beer (by sonication) into a bottle (330 mL). The bottle was kept in a chill bath (set at -5°C) for 40 min. The amount of alcohol chill haze was measured as an increase in the haze of the beer sample. The values (alcohol chill haze) were calculated according to the formula:

$$\text{Sensitive proteins} = \text{FT} - \text{IT}$$

where:

FT= final turbidity of the beer sample after addition of ethanol (99% v/v) and consequent chilling

IT = initial turbidity of the beer sample (without ethanol and consequent chilling)

The results are reported as EBC units to the nearest whole number.

4.3.1.3 Total polyphenols in beer (EBC method 9.11)

A measured volume (8 mL) of carboxymethyl cellulose/ethylenediaminetetraacetic acid solution (CMC/EDTA) (10 g/L of sodium CMC containing 2 g/L of EDTA) was added to an aliquot (10 mL) of degassed beer (by sonication) into a 25 mL volumetric flask. Then, 0.5 mL of ferric reagent (3.5 g of green ammonium iron citrate in 100 mL of water) was added into the same volumetric flask. The solution was stirred at ambient temperature and after the addition of 0.5 mL of ammonia, its volume was made up to 25 mL with deionized water. Finally, after 10 min, the red colored solution, as a consequence of the reaction between the polyphenols and the ferric ions in alkaline solution, was spectrophotometrically measured (Varian Cary 1E UV-Visible set at 600 nm) against a blank solution (same sample preparation without the ferric reagent addition). The content of polyphenols was obtained using the formula:

$$P = A \times 820 \times F$$

where:

P = polyphenol content (mg/L)

A = absorbance at 600 nm

F = dilution factor (i.e., 2 if a 50 mL volumetric flask was used).

The results are reported as mg/L to the nearest whole number.

4.3.1.4 Flavanoids in beer (EBC method 9.12)

A measured volume (1.0 mL) of degassed beer sample (diluted 1:10) was mixed with 5.0 mL of an acidic solution of the chromogen p-dimethyl aminocinnamaldehyde (500 mg of chromogen were dissolved in a previously cooled mixture composed of 125 mL of hydrochloric acid and 350 mL of methanol). The resultant pigments were determined by measurement of the absorbance value (at 640 nm, Varian Cary 1E UV-Visible) of the mixture against a blank (prepared and treated in the same way, replacing the beer sample with 1.0 mL of water). The concentration of flavanoids was determined directly by means of the following regression equation:

$$\text{Flavanoids} = 335 \text{ x } (A_{640s} - A_{640b})$$

where:

A_{640s} = absorbance of the sample at 640 nm

A_{640b}= absorbance of the blank at 640 nm

335 = correction factor

Since the method was calibrated with (+)-catechin, all the results are reported as (+)-catechin equivalents, mg/L (to one decimal place). Under acidic conditions the chromogen p-dimethyl aminocinnamaldehyde reacts with flavanoids such as (+)-catechin to form colored pigments. This method permits a

quantitative determination of the catechin and proanthocyanidin beer haze precursors.

4.3.2 Unconventional methods

4.3.2.1 Determination of gluten

The gluten content of beer samples was determined using the RIDASCREEN® Gliadin competitive kit (R-Biopharm AG, Darmstadt, Germany). The competitive assay is a test kit for the evaluation of hydrolyzed products, which can contain small peptide fragments. Competitive analysis does not require multiple or repetitive epitopes and can much better determine degraded gluten down to small peptides in products such as beer and wort. The standard for the quantification of hydrolyzed gliadins is the most strongly recognized pentamer, glutamine-glutamine-proline-phenylalanine-proline (QQPFP). The specificity of the R5 antibody is high enough to measure hydrolyzed prolamins down to small sequences of five to 10 amino acids in beer and wort. Following the advice of the kit manufacturer, 1 mL of sample (laboratory and pilot plant beer samples) was mixed with 9 mL of extraction solution (ethanol 60% v/v). The mixture was stirred for 30 min, and then centrifuged at 2500 rpm for 10 min (Beckman; model TJ-6 centrifuge, Ireland).

The kit relies on a microtiter plate where wells are coated with gliadin as an antigen. Gliadin standards calibrated to the QQPFP peptide or sample extracts, together with the peroxidase-labeled antigliadin R5 antibody (conjugate), were added at the same time and incubated for 30 min. The conjugate was bound to the gliadin on the plate and to the prolamin peptides in the solution. Antigen–antibody complexes were formed. During the washing step, the bound enzyme conjugate in

the solution was discarded, and the plate-bound conjugate was left. The substrate, chromogen, was added for 10 min. A bound enzyme conjugate converts the chromogen into a blue product. The addition of a stop solution leads to a color change from blue to yellow. The measurement was made photometrically at 450 nm using a plate reader (Sunrise^{TM} Tecan group Ltd., Switzerland). The absorption is inversely proportional to the prolamin fragment concentration in the sample. The gluten content was determined by using a specific formula provided by the kit. The assay results are expressed in mg/kg (ppm) gliadin.

4.3.2.2 Antioxidant activity of beer (the competitive crocin bleaching test)

The description of the method requires a brief definition of the peroxidative process and the antioxidant mechanism to which the analysis was addressed, highlighting the kinetics of reactions involved in the antioxidant effect. Carbon-centered radicals, generated by thermal decomposition of the diazo-compound (Reaction 1), add molecular oxygen-yielding peroxyl radicals (ROO·) in a diffusion-controlled reaction (Reaction 2). These radicals bleach the carotenoid, crocin, thus allowing the measurement of the reaction rate by following the specific absorbance decrease at 443 nm. In the presence of an antioxidant, competing with crocin for the reaction with radicals, the bleaching rate (Reaction 3) slows down, providing that:

(i) the antioxidant is able to react with peroxyl radicals (Reaction 4)

(ii) the rate of the interaction between the radical of the antioxidant and crocin (Reaction 5) is slower than the rate of Reaction 3.

$$R\text{-}N{=}N\text{-}R \xrightarrow{\text{Heat}} 2\,R\cdot + N_2 \quad [1]$$

$$R\cdot + 0_2 \text{ -----}>ROO\cdot \quad [2]$$

$$ROO\cdot + \text{crocin} \text{ -----}> ROOH + \text{crocin}\cdot \text{ (bleached)} \quad [3]$$

$$ROO\cdot + \text{antiOx} \text{ --->} ROOH + \text{antiOx}\cdot \quad [4]$$

$$\text{antiOx}\cdot + \text{crocin} \text{ -->} \text{antiOx} + \text{Crocin}\cdot \text{ (bleached)} \quad [5]$$

The crocin bleaching by a peroxyl radical ($-\Delta A_0$), corresponding to $V_0 = K_c$ x [C], decreases in the presence of an antioxidant that competes for the peroxyl radical, and according to competition kinetics (Bors *et al.*, 1984), the new bleaching rate (V) corresponds to:

$$V = V0 \text{ x } \frac{Kc[C]}{Kc[C] + Ka[A]} \quad [6]$$

where :

$V_o = K_1$ x [ROO·] x [C];

$V_a = K_2$ x [ROO·] x [A];

$K_c = K_1$ x [ROO·];

$K_a = K_2$ x [ROO·];

V_o = rate of the reaction of crocin with ROO·;

V_a = rate of the reaction of the antioxidant under study with ROO·;

K_1 = rate constant for the reaction between ROO· and crocin;

K_2 = rate constant for the reaction between ROO· and antioxidant;

[C] = concentration of crocin;

[A] = concentration of antioxidant

By transforming, the bleaching rate of crocin ($-\Delta A_o$) decreases in the presence of an antioxidant to a new value ($-\Delta A_a$) fitting the straight line equation:

$$\frac{-\Delta A0}{-\Delta Aa} = \frac{V0}{V} = \frac{Kc[C] + Ka[A]}{Kc[C]} = 1 + \frac{Ka}{Kc} \text{ x } \frac{[A]}{[C]} \qquad [7]$$

The slope K_a/K_c, calculated from the linear regression of the plot of [A]/[C] vs. V_o/V, indicates the relative capacity of different molecules to interact with ROO·. When molecules, although reacting with peroxyl radicals, are transformed into radicals that are able to react with crocin, and thus, by analogy, to propagate peroxidation, this kinetic approach produces ratios K_a/K_c lower than the actual ratio between the absolute rate constants. Thus, this test averages the antioxidant capacity with a possible prooxidant effect of the sample. The kinetic test (Bors *et al*., 1984) was modified by introducing diazo-compounds, 2,2'-Azo-bis (2-amidinopropane) dihydrochloride (ABAP), to produce peroxyl radicals (Tubaro *et al*., 1996). This test is a simple procedure for analyzing the antioxidant capacity of complex matrices (beer samples), which is expressed relative to Trolox C (soluble analog of the α-tocopherol) on a weight basis. Crocin (a natural pigment with strong visible absorption) was isolated from saffron by methanol extraction after

repeated extractions with ethyl ether to eliminate possible interfering substances. The concentration of crocin in methanol was calculated from the absorbtion coefficient (Ɛ=1.33 x 10^5 M^{-1} cm^{-1} at 443 nm). The hydrophilic reaction mixture in the cuvette contained 12 µM crocin (from a 1.2 mM methanolic stock solution), variable amounts of the sample/Trolox (10, 20, 50, 100 µL of Trolox or 30, 50, 100, 200 µL of beer samples), containing the antioxidant to be analyzed and the volume was made up to 2 mL with 10% ethanol in water. The reaction was started by the addition of 5 mM ABAP (from a fresh 0.5 M solution in water) to the complete reaction mixture, which was pre-equilibrated at 40°C. Reactions were carried out at 40°C and the bleaching rate of crocin, linear 1-1.5 min after the addition of the diazo-compound, was recorded for 10 min by a spectrophotometer with a temperature controlled motorized cell holder (Varian Cary 1E UV-Visibile). Blanks without crocin were run to rule out spectral interferences between the molecule under analysis and crocin. The antioxidant activity of beer sample was calculated from the ratio between the slope (K_a/K_c) obtained with the sample and with the Trolox C. The results are expressed as Trolox equivalents.

4.3.3 Statistical analysis

All analysis were performed in triplicate (n = 3). The statistical analysis was conducted using Student's test with α (0.05/number of Student's test replicas) corrected according to the Bonferroni test to assess any differences between group means (Excel 2003; Microsoft, Redmond, USA). Pearson correlation coefficient (r) and p-value were used to show correlations and their significance using the program CoStat 6.204 (1998-2003, CoHort Software, Monterey Ca, Usa). All correlations were considered statistically significant with $p < 0.05$.

4.4 RESULTS AND DISCUSSION

4.4.1 Colloidal stability of laboratory beer samples

The beers produced in the laboratory, as indicated in section 3 and analyzed as per subsection 4.3 (Figures 4.7, 4.8, 4.9 and Table 4.1) show the pH influence (8.0) of the first steeping water on antioxidant activity. The antioxidant activity values obtained for all the beers produced with malts that had been subjected to the alkaline treatment were lower (Figure 4.9) and statistically different from those obtained with the reference Ba1 (Table 4.1). All the laboratory beer samples, on the other hand, provided lower sensitive proteins and gluten values (Figures 4.7 and 4.8 respectively), but not statistically different from the reference beer (Table 4.1).

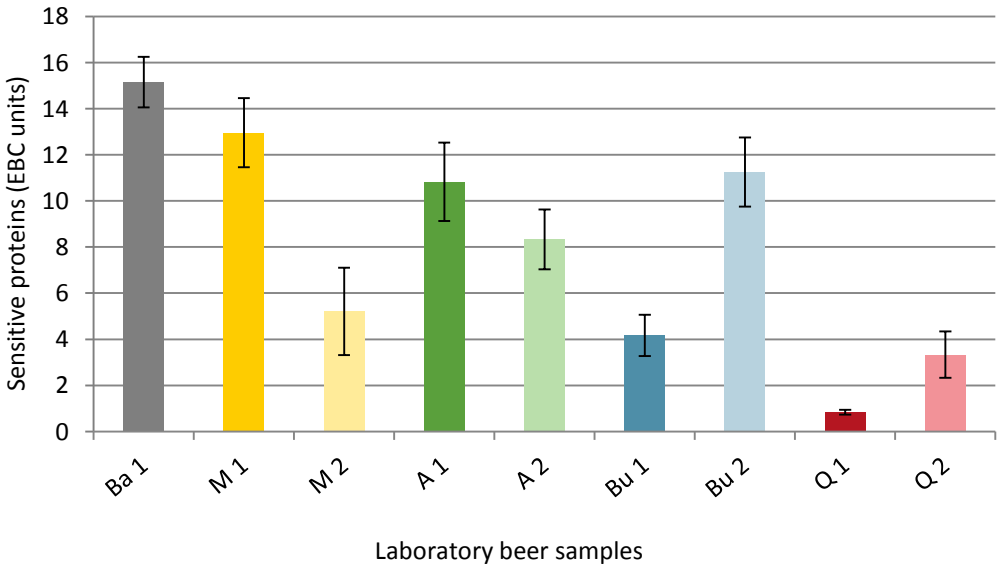

Figure 4.7 Sensitive proteins (EBC units) of the laboratory beer samples. The results are reported as mean values (n = 3). The error bars represent the standard deviation (SD).

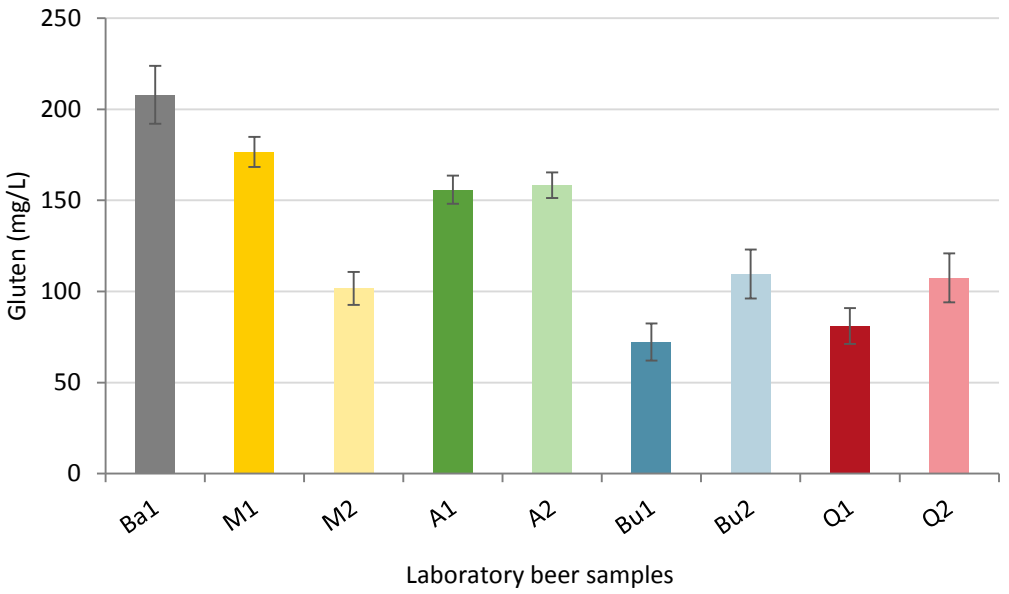

Figure 4.8 Gluten content (mg/L) of the laboratory beer samples. The results are reported as mean values (n = 3). The error bars represent the standard deviation (SD).

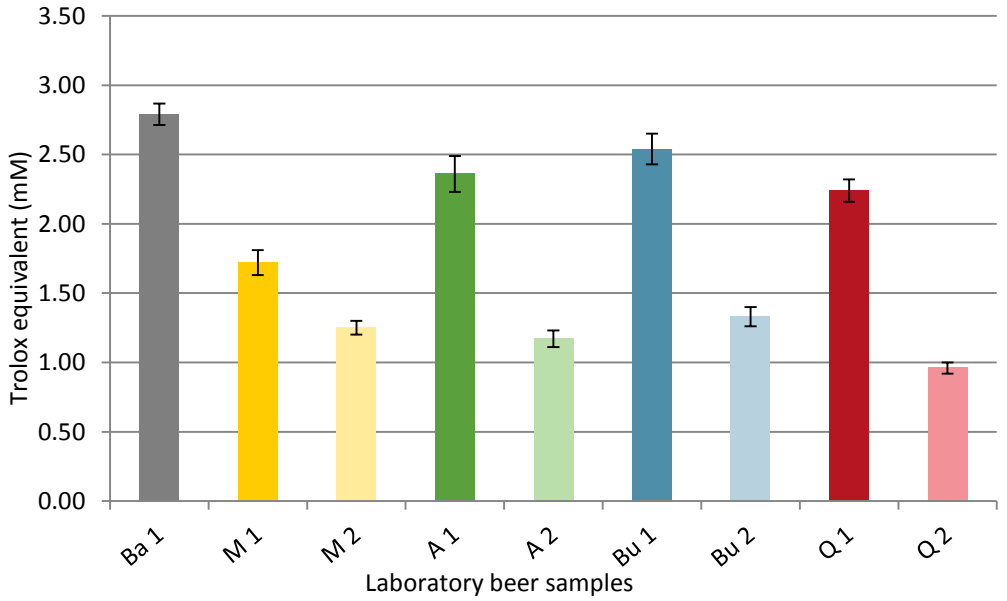

Figure 4.9 Antioxidant activity (Trolox equivalent) of the laboratory beer samples. The results are reported as mean values (n = 3). The error bars represent the standard deviation (SD).

Table 4.1 Comparisons between the colloidal stability analysis values (sensitive proteins, gluten and antioxidant activity) obtained for all beers and the reference Ba1 (Student's test, $p < 0.006$).

Laboratory beer samples	Code	Sensitive proteins (EBC units)	Gluten (mg/L)	Antioxidant activity (mM Trolox)
Reference (100% barley malt)	Ba1	15.2 ± 1.1	207.9 ± 15.9	2.79 ± 0.08
Beer obtained with millet*	M1	13.0 ± 1.5	176.5 ± 8.3	1.72 ± 0.09
Beer obtained with millet**	M2	5.2 ± 1.9	101.7 ± 9.1	1.25 ± 0.05***
Beer obtained with amaranth*	A1	10.8 ± 1.7	155.8 ± 7.7	2.36 ± 0.13
Beer obtained with amaranth**	A2	8.3 ± 1.3	158.3 ± 7.0	1.17 ± 0.06***
Beer obtained with buckwheat*	Bu1	4.2 ± 0.9	72.2 ± 10.2	2.54 ± 0.11
Beer obtained with buckwheat**	Bu2	11.3 ± 1.5	109.5 ± 13.4	1.33 ± 0.07***
Beer obtained with quinoa*	Q1	0.8 ± 0.1	81.0 ± 9.9	2.24 ± 0.08
Beer obtained with quinoa**	Q2	3.3 ± 1.0	107.5 ± 13.4	0.96 ± 0.04***

Results represent mean values ± standard deviation (SD) (n = 3)
*** = first steeping water pH (6.8)**
**** = first steeping water pH (8.0)**
***** = significantly different from the reference sample Ba1**

Finally, the pH, one month after bottling, shows a stronger effect on colloidal stability compared to the use of cereals without gluten which is rich in proline, the aminoacid involved in haze formation (Table 4.2). All beers produced with malts that had been subjected to the alkaline treatment gave alcohol chill haze values lower and statistically different from Ba1, with the only exception of sample A2 (Table 4.2). Among the beers obtained with the malts without the

alkaline treatment, three beers (A1, Bu1 and Q1) did not provide alcohol chill haze values statistically different from Ba1 (Table 4.2).

The influence of the formulation on the beer colloidal stability increases with storage time; three months after bottling, all samples gave lower chill haze values and statistically different from Ba1 (Table 4.2 and Figure 4.10).

Six months after bottling, only Q1 and Q2 (beers obtained with the quinoa malts with and without the alkaline treatment) gave chill haze values not statistically different from Ba1; beers Q1 and Q2 had the lowest pH value (3.81 and 4.11 respectively) (Table 3.2: subsection 3.3.3, p. 105) and showed the highest increase in turbidity six months after bottling (Figure 4.10).

Some authors (Kunz and Methner, 2009; Kunz *et al*., 2010) have demonstrated that in the range of 5.50 to 3.25 the decrease in the pH leads to a stronger chill haze formation: many results clearly show that oxidative processes under the contribution of specific Fenton/Haber-Weiss reaction products like Fe^{3+}, play an important role in the chill haze formation, and their action is influenced by the beer pH.

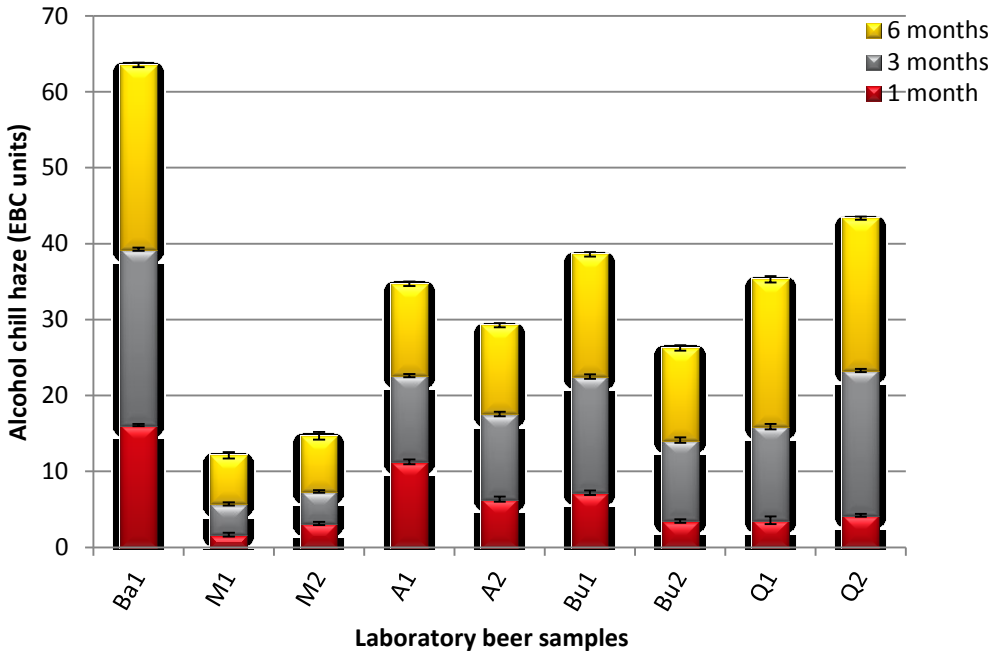

Figure 4.10 Chill haze formation throughout beer shelf life. The error bars represent standard deviation (SD) of three independent measurements.

The Chapon test (alcohol chill haze) is especially valuable because any material that displays a tendency to fall out of solution is likely to be detected in this test, and provides the lower standard deviations if compared to the other methods.

Table 4.2 Comparisons between the chill haze values obtained for all laboratory beer samples and the reference sample Ba1 (Student's test, p < 0.002).

MULTIPLE COMPARISONS				
Laboratory beer samples	**Code**	**Chill haze after 1 month**	**Chill haze after 3 months**	**Chill haze after 6 months**
Reference (100% barley malt)	Ba1	16.1 ± 0.1	23.2 ± 0.2	24.3 ± 0.3
Beer obtained with millet*	M1	1.7 ± 0.2***	4.0 ± 0.2***	6.4 ± 0.4***
Beer obtained with millet**	M2	3.1 ± 0.2***	4.2 ± 0.2***	7.4 ± 0.5***
Beer obtained with amaranth*	A1	11.3 ± 0.3	11.4 ± 0.2***	12.1 ± 0.3***
Beer obtained with amaranth**	A2	6.4 ± 0.3	11.2 ± 0.3***	11.7 ± 0.3***
Beer obtained with buckwheat*	Bu1	7.2 ± 0.3	15.3 ± 0.3***	16.1 ± 0.3***
Beer obtained with buckwheat**	Bu2	3.5 ± 0.2***	10.7 ± 0.4***	12.2 ± 0.4***
Beer obtained with quinoa*	Q1	3.6 ± 0.5	12.3 ± 0.4***	19.4 ± 0.4
Beer obtained with quinoa**	Q2	4.2 ± 0.2***	19.1 ± 0.2***	20.1 ± 0.2

Results represent mean values ± standard deviation (SD) (n = 3)
*** = first steeping water pH (6.8)**
**** = first steeping water pH (8.0)**
***** = significantly different from the reference sample Ba1**

Moreover, in this study, significant correlations were found between conventional (sensitive proteins and alcohol chill haze) and unconventional methods (gluten and antioxidant activity), especially between sensitive proteins test and gluten analysis (p = 0.0001) (Table 4.3); this is probably due to the specificity of the immune-enzymatic kit for the determination of small fragments rich in proline, which incidentally seem to be essential for chill haze formation.

These results suggest that immune-enzymatic and antioxidant analyses, although less informative than the electrophoretic and chromatographic determinations, can be used as a quick screening method for the evaluation of colloidal stability of beer.

Table 4.3 Correlations between official (sensitive proteins, alcohol chill haze) and unconventional methods (gluten analysis and antioxidant activity) used to evaluate the colloidal stability of the laboratory beer samples. CoStat 6.204 (1998-2003, CoHort Software, Monterey Ca, Usa). All correlations were considered statistically significant with $p < 0.05$.

Sample	Gluten content vs. Sensitive proteins (p-value)	Gluten content vs. Alcohol chill haze (p-value)	Antioxidant activity vs. Alcohol chill haze (p-value)
All laboratory beer samples (nine beers)	0.0001*	0.0195*	0.0015*

*** = statistically significant**

4.4.2 Colloidal stability of pilot plant beer samples

The laboratory beers obtained with the buckwheat malt were most stable in terms of colloidal stability compared to the reference beer (Ba1) (Figure 4.10, Table 4.2: subsection 4.4.1, pp. 139 and 140 respectively). According to these results, buckwheat malt was used for brewing at the pilot plant. The pilot plant beers with increasing amounts of malted buckwheat did not give sensitive protein values statistically different from the reference (Ba2) (Figure 4.11 and Table 4.4). However, in this case the samples obtained with 40% w/w of buckwheat (Bu40%) gave lower values of gluten (87.1 ± 0.9 mg/L) (Figure 4.12) and were statistically

different from Ba2 (164.3 ± 1.7 mg/L), unlike the laboratory samples (Tables 4.1 and 4.4). The antioxidant activity of sample Bu40% is lower (0.03 ± 0.02 mM of Trolox equivalent) (Figure 4.13) and statistically different from Ba2 (1.91 ± 0.02 mM of Trolox equivalent), like for the laboratory beer samples obtained with the malt that had been subjected to the alkaline treatment (Bu2) (Tables 4.1 and 4.4).

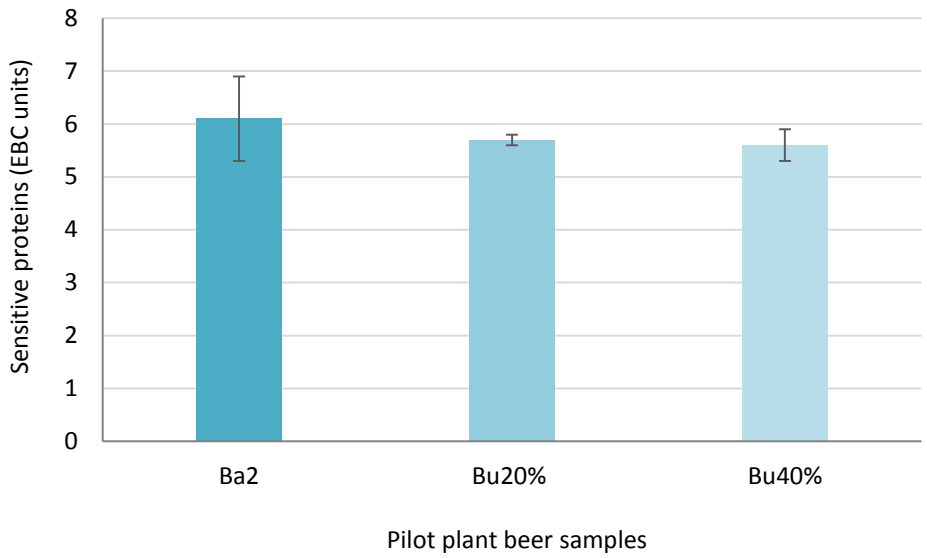

Figure 4.11 Sensitive proteins (EBC units) of the pilot plant beer samples. The results are reported as mean values (n = 3). The error bars represent the standard deviation (SD).

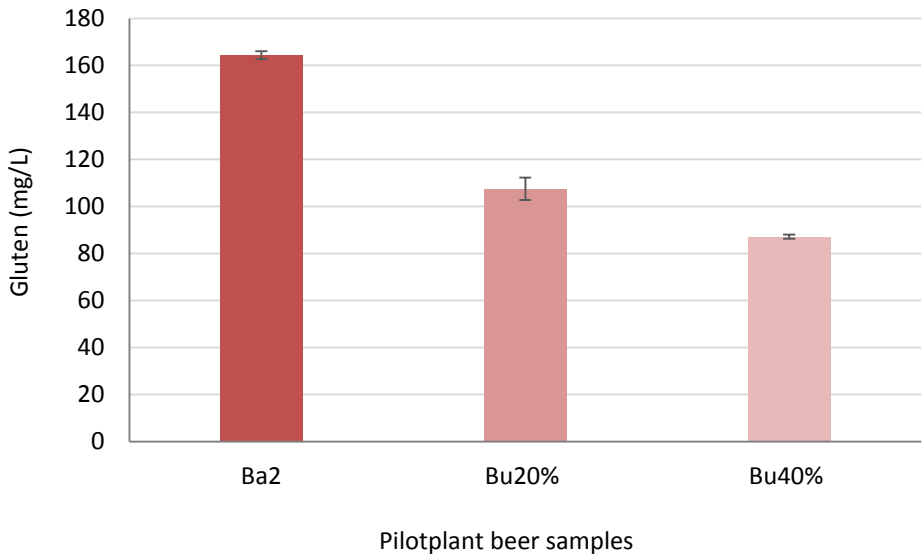

Figure 4.12 Gluten content (mg/L) of the pilot plant beer samples. The results are reported as mean values (n = 3). The error bars represent the standard deviation (SD).

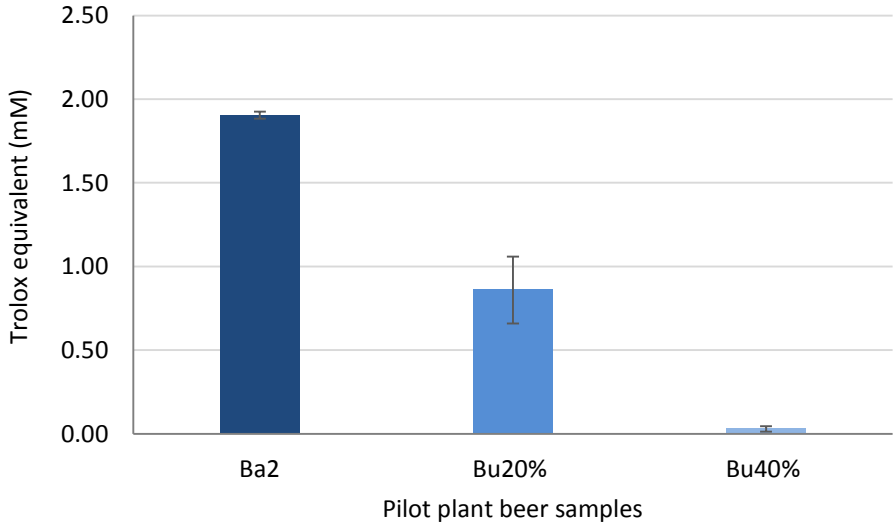

Figure 4.13 Antioxidant activity (Trolox equivalent) of the pilot plant beer samples. The results are reported as mean values (n = 3). The error bars represent the standard deviation (SD).

Table 4.4 Comparisons between the colloidal stability analysis values (sensitive proteins, gluten and antioxidant activity) obtained for all beers and the reference sample Ba2 (Student's test, p < 0.01).

Pilot plant beer samples	Code	Sensitive proteins (EBC units)	Gluten (mg/L)	Antioxidant activity (mM Trolox)
Reference (100% barley malt)	Ba2	6.1 ± 0.8	164.3 ± 1.7	1.91 ± 0.02
20% w/w Buckwheat malt*	Bu20%	5.7 ± 0.1	107.5 ± 4.7	0.86 ± 0.20
40 % w/w Buckwheat malt*	Bu40%	5.6 ± 0.3	87.1 ± 0.9**	0.03 ± 0.02**

Results represent mean values ±standard deviation (SD) (n = 3)
*** = first steeping water pH (6.8)**
**** = significantly different from the reference sample Ba2**

The chill haze values one month after bottling confirm what was observed with the laboratory samples obtained with the malt without the alkaline treatment (Bu1) after 3 and 6 months (Tables 4.2 and 4.5): only Bu40% beers gave lower values and statistically different from Ba2 (Figure 4.14 and Table 4.5).

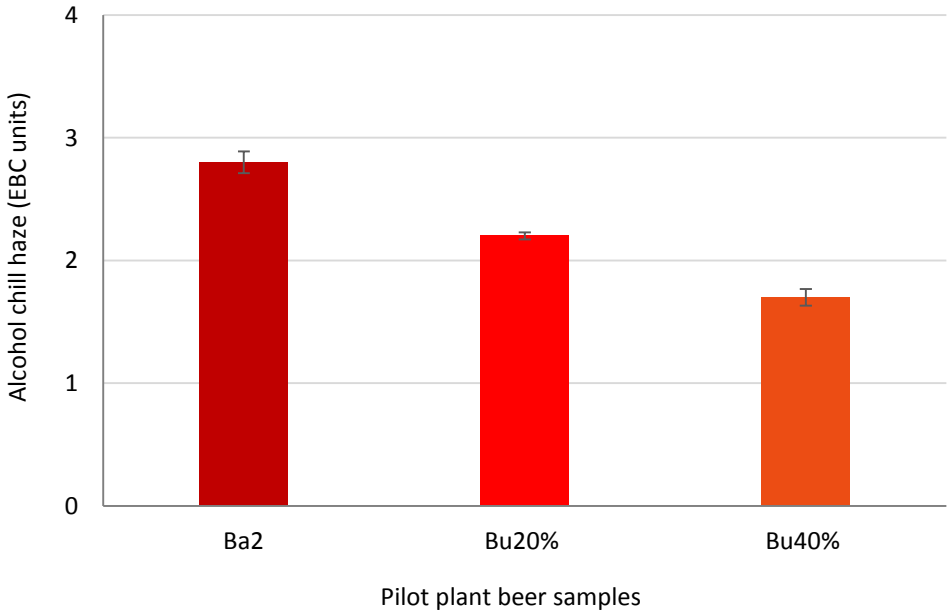

Figure 4.14 Alcohol chill haze values (EBC units) of the pilot plant beer samples. The results are reported as mean values (n = 3). The error bars represent the standard deviation (SD).

Table 4.5 Comparisons between the chill haze values obtained for Bu20% and Bu40% beer samples and the reference sample Ba2 (Student's test, p < 0.01).

Pilot plant beer samples	Code	Alcohol chill haze (EBC units)
Reference (100% barley malt)	Ba2	2.77 ± 0.09
20% w/w Buckwheat malt*	Bu20%	2.16 ± 0.03
40 % w/w Buckwheat malt*	Bu40%	1.71 ± 0.07**

Results represent mean values ±standard deviation (SD) (n = 3)
*** = first steeping water pH (6.8)**
**** = significantly different from the reference sample Ba2**

No significant correlation between sensitive proteins and gluten values was observed (p = 0.3158) (Table 4.6). The hot *trub* and any insoluble material (such as protein and polyphenolic fractions) is taken out of the wort by a whirlpool tank (absent in the laboratory brewing process) (Van Landschoot, 2011), and this may have negatively influenced the correlation among the two methods due to the low difference between the sensitive proteins level in the reference Ba2 and the other two beers (Bu20% and Bu40%). However, statistically significant correlation was obtained between the gluten analysis and alcohol chill haze method (p = 0.0004) (Table 4.6); this result seems to confirm that gluten analysis can discriminate the beers obtained with and without the buckwheat malt in terms of colloidal stability better than the EBC method (sensitive proteins). Significant correlation was found between alcohol chill haze and antioxidant activity values (p = 0.0008) (Table 4.6), like for the values obtained with the laboratory beer samples (Table 4.3).

Table 4.6 Correlations between official (sensitive proteins, alcohol chill haze) and unconventional methods (gluten analysis and antioxidant activity) used to evaluate the colloidal stability of the pilot plant beer samples. CoStat 6.204 (1998-2003, CoHort Software, Monterey Ca, Usa). All correlations were considered statistically significant with p < 0.05.

Sample	Gluten content vs. Sensitive proteins (p-value)	Gluten content vs. Alcohol chill haze (p-value)	Antioxidant activity vs. Alcohol chill haze (p-value)
All pilot plant beer samples (three beers)	0.3158	0.0004*	0.0008*

*** = statistically significant**

Furthermore, for the pilot plant beer samples the total polyphenols and flavonoids contents (two other important specific haze components) were determined and compared to the antioxidant activity values. The flavonoids content of the pilot plant beer samples was the highest in Ba2 (64.7 ± 10.0 mg/L of catechin equivalents) and decreased with the increasing amount of buckwheat malt in beers (58.2 ± 0.2 and 50.7 ± 9.2 mg/L respectively), while the opposite trend was observed for the total polyphenols (Figures 4.15 and 4.16).

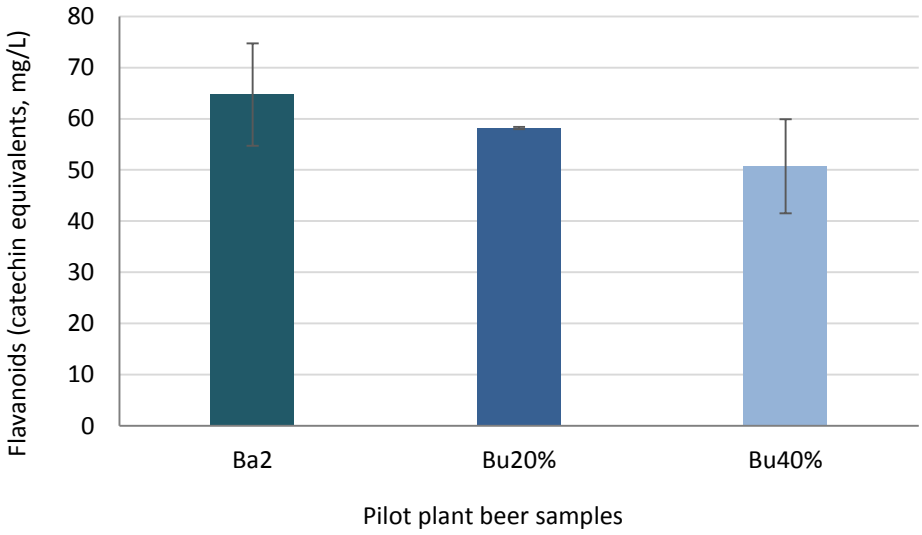

Figure 4.15 Flavanoids (catechin equivalents, mg/L) in the pilot plant beer samples. The results are reported as mean values (n = 3). The error bars represent the standard deviation (SD).

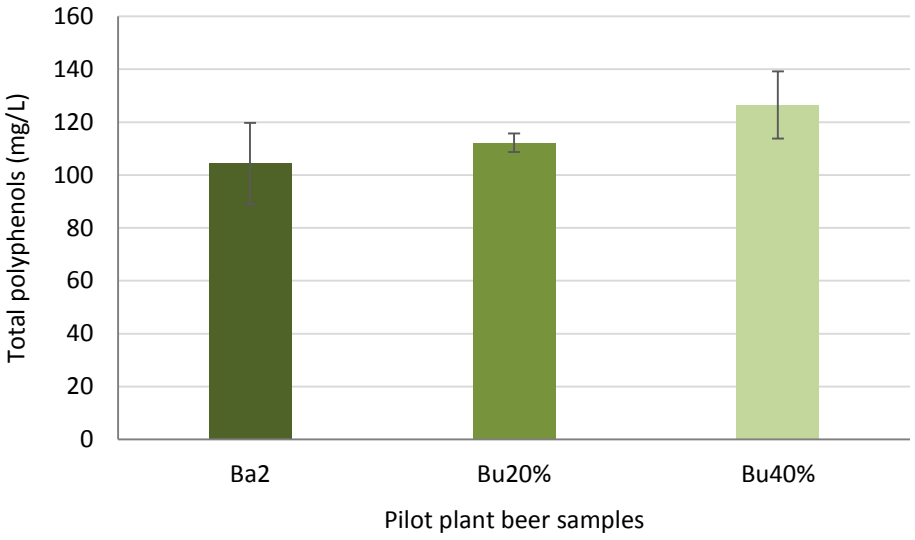

Figure 4.16 Total polyphenols (mg/L) in the pilot plant beer samples. The results are reported as mean values (n = 3). The error bars represent the standard deviation (SD).

The flavanoids and alcohol chill haze values show a similar trend, although the correlation is not statistically significant (p = 0.0719) (Table 4.7). This is unlike the polyphenols (Table 4.7 and Figures 4.14, 4.15, 4.16), probably because they are less specific in explaining the haze formation mechanism.

The total polyphenols and flavonoids values are not correlated with the antioxidant activity values, as shown in Table 4.7. The reference beer sample Ba2 gave a higher antioxidant activity (1.91 ± 0.02 mM of Trolox equivalent) (Figure 4.13 and Table 4.4) and at the same time a lower content of polyphenols (104 ± 15 mg/L) compared to the other two beers (Figure 4.16).

Table 4.7 Correlations between -total polyphenols, flavanoids- and -alcohol chill haze, antioxidant activity- values obtained with the pilot plant beer samples. CoStat 6.204 (1998-2003, CoHort Software, Monterey Ca, Usa). All correlations were considered statistically significant with p < 0.05.

Sample	Total polyphenols vs. Antioxidant activity (p-value)	Flavanoids vs. Antioxidant activity (p-value)	Total polyphenols vs. Alcohol chill haze (p-value)	Flavanoids vs. Alcohol chill haze (p-value)
All pilot plant beer samples (three beers)	0.2716	0.1122	0.2112	0.0719

*** = statistically significant**

This could be explained by having different chemical species involved in antioxidant activities (reducing sugars, lipid transfer proteins, vitamins, Maillard reaction products etc.). The behaviour of these molecules can vary in different ambient conditions (i.e. pH and alcohol) (Wu *et al*., 2012). Schiwek *et al*., have demonstrated that at low pH, the formation of the open chain aldehyde structure of glucose is inhibited and glucose loses the reduction properties against Fe^{3+}. Therefore, both the quantity of reducing sugars and beer pH are interesting in defining the shelf-life (colloidal stability) of bottle-conditioned craft beers and could give information to better understand the obtained results.

Velioglu *et al*. (1998) also observed a non-significant correlation between the antioxidant and total phenolic contents of several products.

Arts *et al*. (2002) and Şensoy *et al*. (2006) suggest that protein and polyphenol interaction may mask part of the antioxidant activity.

Moreover, some polyphenols, i.e. those with an additional 5' hydroxyl group (i.e., delphinidin), could promote staling through their ability to reduce transition metal ions to their more potent lower valence forms (Lewis and Bamforth, 2006).

Oomah and Mazza (1996) found that the polyphenols content in buckwheat seeds was strongly correlated with rutin (rhamnoglucoside of quercetin), but weakly associated with antioxidative activities. Some authors suggest that quercetin readily reduces both Fe^{3+} to Fe^{2+} and Cu^{2+} to Cu^{1+} (pro-oxidant activity), metals responsible for promoting oxidation via Fenton and Haber-Weiss reactions (Figure 1.13, p. 42) (Aron and Shellhammer, 2010), and this could explain the opposite trend between the polyphenols content and antioxidant activity of the pilot plant beer samples.

Moreover, the pilot plant beers with a lower antioxidant activity are not necessarily less stable than those with a higher antioxidant activity (Di Pietro and Bamforth, 2011). Throughout beer shelf life there is a change in its redox properties (all beers were bottle conditioned), during re-fermentation the remnant yeasts both consume oxygen and produce sulphur dioxide (see pp. 44 and 45), with a natural increase in the product stability toward oxidative processes (Jurková *et al*., 2012).

4.5 CONCLUSIONS

Concerning the colloidal stability of all the beer samples, especially for the antioxidant activity, the influence of the first steeping water pH seems to be stronger than the formulation incidence (use of gluten-free adjuncts in the recipe). However, the influence of the formulation is more related with storage time (laboratory beer samples).

The unconventional methods used to study the physico-chemical stability of craft beers (brewing at the lab scale and at the pilot plant) provided encouraging results: both the gluten analysis and the antioxidant activity data showed trends correlated with those obtained with the validated method (alcohol chill haze). Specifically, gluten analysis seems to better discriminate the beers produced at the pilot plant than sensitive proteins (EBC method).

In general, the samples (both laboratory and pilot plant beers) that have provided lower values of gluten and antioxidant activity, have also provided a greater stability towards the cold turbidity (alcohol chill haze) compared to the references.

Furthermore, for the pilot plant beer samples the total polyphenols and flavonoids contents (two other important specific haze components) were determined and compared to the antioxidant activity values. The total polyphenols and flavonoids values are not correlated with the antioxidant activity values; i.e. the reference beer sample Ba2 gave a higher antioxidant and at the same time a lower content of polyphenols compared to the other two beers.

These results highlight the pitfalls of simply assuming that the superior performance of one product in a single assay means that it is inherently preferable as a source of antioxidants-polyphenols.

Following the results collection, quantitative analysis of protein and polyphenol fractions (electrophoretic and chromatographic determinations, respectively) present in the beer samples obtained from these adjuncts will be performed. Other possible variables involved in colloidal stability, such as trace metals in beers, will be analyzed by ICP-MS.

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