

CHAPTER 19

Gluten-Free Spirits and Drinks

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A b s t r a c t

Alcoholic beverages are those containing more than 0,5% (vol/vol) of alcohol. They can be obtained by various processes (fermentation, addition, distillation, extraction, etc.). As there is no established classification of alcoholic beverages, alcoholic concentration is the most commonly used: 1) fermented alcoholic beverages such as beer, cider and wine, and 2) distilled beverages and spirits (higher in alcoholic concentration).

The manufacture of some fermented alcoholic beverages and spirits sometimes involves the use of gluten-containing raw materials (cereals such as barley, wheat or rye). For this reason, in many cases it has been thought that they should not be included in the diet of celiacs. It is also common to add plant material to clarify alcoholic beverages in order to filter or to remove particles in suspension and sometimes malt proteins or hydrolyzed preparations containing wheat gluten are used. As well as this, the addition of flavorings to some spirits is permitted and most of these are obtained from the fermented raw materials. Despite this, nowadays it is possible to find types of fermented alcoholic beverages and spirits that do not contain gluten and are suitable for the celiac population on the market.

As a result, gluten analysis of fermented alcoholic beverages and spirits is often needed to confirm their claim to be the gluten-free. But as gluten is sometimes hydrolyzed, the sandwich R5 ELISA method is not appropriate when foods and beverages are treated with proteolytic enzymes or when they are fermented. In these cases, other techniques such as mass spectrometry (MS) or competitive R5 ELISA have some advantages over the sandwich method.

Keywords

Gluten-free beverages, beer, spirits, gluten analysis.

1. Introduction

Alcoholic beverages are those containing more than 0,5% (vol/vol) of alcohol and can be obtained by various processes (fermentation, addition, distillation, extraction, etc.). There is no definite classification of alcoholic beverages by alcohol content, development process, carbonic content, etc. So the most commonly used classification is the following one: fermented alcoholic beverages such as beer, cider and wine, and distilled beverages and spirits (with high alcoholic concentration).

Fermented alcoholic beverages have been known since the earliest civilizations. Cereals, fruits and juices were left in containers and fermented spontaneously, producing an alcoholic liquid. With the Greek, Roman, Egyptian and Assyrian civilizations methods of making wine and beer were improved¹. Beers are made from grains, cider from apples, and wine and wine derived beverages are made from grapes. Distillation processes for the production of spirits were developed later.

The process of making some fermented alcoholic beverages and spirits sometimes involves the use of gluten-containing raw materials (grains such as barley, wheat or rye). For this reason, in many cases it has been thought that they should not be included in the diet of celiacs. But nowadays it is possible to find on the market types of fermented alcoholic beverages and spirits that do not contain gluten and are suitable for celiacs.

Regulation (EU) No 1169/2011 of the European Parliament and of the Council², on the provision of food information to consumers, lists in Annex II the substances or products causing allergies or intolerances. The substances and products include those cereals containing gluten, namely: wheat, rye, barley, oats, spelt, kamut or their hybridised strains, and products thereof. But it also includes some exceptions: a) wheat based glucose syrups including dextrose; b) wheat based maltodextrins; c) glucose syrups based on barley and d) cereals containing wheat based glucose syrups. Some of these products are used in the manufacture of some gluten-free spirits and drinks.

2. Fermented Alcoholic Beverages

2.1. Beer

Beer is one of the most frequently consumed alcoholic beverages. It is a very popular beverage in most European and American countries and can constitute an important part of diet and leisure time. The average annual consumption in 2011 was established in about 68,2 kg/capita in Europe and 78,3 kg/capita in Northern America³.

Beer is a non-distilled alcoholic beverage produced by the fermentation of malted barley grains or other cereals (wheat, rye and oats) with yeast. Most beer is also flavored with hops, which adds a bitter taste and acts as a natural preservative⁴. Other flavorings such as herbs or fruit may occasionally be included.

In traditional beer-making or brewing, barley (*Hordeum vulgare var. distichum*) is the most important raw material. The barley is germinated in a controlled manner to develop the enzyme system responsible for transforming the starch into sugars. This process is known as malting: enzymes are synthesized and mobilized, and the starch granules are mobilized in the endosperm. Malting is halted by drying and the malted barley is then toasted to deactivate the enzymes, denature proteins and produce characteristic colours and aromas.

The wort is prepared by mixing the starch source (normally malted barley) with hot water, is known as "mashing". The malt is ground and made into a paste with brewing water and partially degraded and solubilized with malt enzymes. Wort is boiled with hops or hop products to hydrolyze and dissolve proteins and to dissolve hop ingredients. Boiling also destroys any enzymes remaining from the mashing stage. Next, alcoholic fermentation is produced with the addition of *Saccharomyces cerevisiae* (for ale) or *Saccharomyces uvarum* (for lager). During fermentation, the wort becomes beer in a process which takes from a week to some months, depending on the yeast and beer type. Fermentation is complete when the desired alcohol content has been reached. Nowadays, the majority of beers receive a relatively short

conditioning period after fermentation and before filtration. This conditioning is performed to drop proteins out of solution to prevent cloudiness in the bottled or otherwise packaged product^{4,5}.

Beer can be classified according to its dry malt extract (DME), which is the total solid organic ingredient that the wort contains before fermentation. It is expressed in g/100g wort (%). Traditional beers have DME \geq 11%; Special beers: DME \geq 13%; Extra Special Beers: DME \geq 15%; and Alcohol free beers have a variable DME between 2-4 %.

Depending on the type of fermentation beer can be classified into top fermented beers and bottom fermented beers. Top fermented beers are fermented at temperatures up to 20°C and yeasts rise to the surface during fermentation creating a very thick, rich yeast head. Ale, Stout, Porter are the most known. British beers as well as the wheat beers, German Altbier and Kölsch are also top fermented beers.

Bottom fermented beers or lager are made at low temperatures ranging from 7 to 15°C. At these temperatures, lager yeasts grow less rapidly than ale yeasts, and they tend to settle out to the bottom of the fermentation container. They can be stored for a long time (months) and they are commonly identified according to the place where they are from: Munich, Vienna, Pilsner, etc. Some beers are made with spontaneous fermentation with natural or wild yeast strains. Lambic, Gueuze and Faro are some of the latter.

Beer contains an average of 0.2-0.6 g/100 mL of proteins or peptides that mainly come from barley. This quantity is bigger than that found in other alcoholic beverages, such as wine (0.1-0.2 g/100mL). Proteins remain relatively unmodified, but they may suffer proteolysis and other chemical modification events. Prolamin concentration in beer depends on the malt type, the mashing technology, type of fermentation, maturation and stabilization process. Because of the use of malted barley or wheat, the possible presence of toxic proteins in beer has been discussed for a long time, and therefore, beer is excluded from the diet of celiacs.

Although most celiacs could drink one beer with low levels of gluten, each person displays a different level at which his or her autoimmune response will be activated. In addition, the amounts of dietary gluten that each one can ingest without damaging the mucosa of the small intestine is generally unknown and should be kept below 50 mg/day, as suggested by Catassi et al.⁶.

In this sense, many adult celiacs are unhappy that this beverage is not permitted in their diet or that they can drink only one glass a week. For this reason, over the past years several beers have been launched in the market advertised as "gluten-free" or "gluten-removed". The availability of safe gluten-free beers would improve celiac patient well-being and perception of a normal social life.

2.1.1. Strategies to Brew Gluten-Free Beers

The most obvious strategy to brew gluten-free beer is the use of raw material without gluten. Gluten-free beers are made from cereals without gluten, such as millet, rice, sorghum, corn and teff. The use of oat in gluten-free beers is controversial because not all people with gluten intolerance can include this cereal in their diet without adverse effects.

The use of pseudocereals like buckwheat, quinoa or amaranth to brew beers for the celiac market is well known^{7,8,9,10}. These are taxonomically different from *Poaceae* (grass family) and are considered gluten free^{11,12}. Another option is the use of other vegetable products as raw materials (potatoes and sweet potatoes, chestnut chips, or chips made from almonds or hazelnuts, and other fermentable sugar sources and syrups)^{12,13}.

Nevertheless, rice (*Oryza sativa*) is probably the most commonly used gluten free grain, industrially and for research objectives. However, there are few data concerning malting and brewing with 100% rice.

Some works^{14,15} have shown that the rice malts obtained have much lower extract contents and, on the whole, a lower enzymatic activity than barley malt.

Compared to barley malt, rice malts had a lower soluble protein percentage and low soluble/total protein ratio, which implies that they were poorly modified during extraction process.

Using these gluten free alternatives, the brewing process is similar to that of malted barley but obviously parameters such as germination and fermentation conditions, pH of mashing, yeast strain used, temperatures and storage conditions have to be adjusted depending on the raw material used^{7,8,9,10}.

Commonly, malt is produced from the barley or wheat and some authors had showed important differences in celiac immunotoxicity of barley varieties¹⁶ or wheat varieties which are naturally reduced in celiac disease related gluten epitopes¹⁷.

Thus, a second strategy for brewing low gluten beers is to select cereals with fewer immunogenic epitopes for the process. Therefore, when gluten free beer is produced from traditional raw materials by elimination of toxic proteins and peptides, the right choice of malt facilitates this process.

Furthermore, sometimes it is necessary the use of industrial enzyme preparations and gluten free adjuncts because gluten free malt is not as suitable as barley malt for brewing¹¹. For example, the addition of enzymes like beta-amylase and amyloglucosidase increases the amount of fermentable sugars in the sorghum malt worts¹⁸.

Despite of this, most brewers have created different types of beers from gluten free raw material: Ale, Pale Ale, Pilsner, Lager, lemon-flavored beer, etc. However, the colour, flavour and the taste may be quite different from traditional beers made from barley or wheat.

Another method of brewing gluten-free beer is to make gluten-removed beer. Brewers use barley to produce the malt, which gives the traditional flavor of beer. Then they add microbial peptidases or grain endopeptidases^{11,19,20}. So it does not actually remove the gluten from the beer. Instead the gluten is broken into small fragments, which are supposed to be too small to be toxic to individuals with celiac disease. But it is difficult to

quantify the amount of gluten in a product, such as beer, when the protein has been hydrolyzed^{20,21,22,23,24}.

As a result, in some states of the USA manufacturers of gluten-removed beers who label the beer with the claim “gluten levels are below 20 mg/kg”, must add that the “product is fermented from grains containing gluten and is processed to remove it”²⁰.

Proteases from germinated gluten cereals are produced during fermentation and cleave celiac toxic peptides into non-toxic fragments. It is also possible to add a prolyl endopeptidase (e.g. a proline-specific endo-protease) that breaks down gluten molecules and other proteins at the carboxyl end of the amino acid proline. Prolyl endopeptidases from microbial origin has been used in brewing industries to prevent haze and when they are added during fermentation or at the end of the process, produces gluten free beer or with low quantities of gluten^{11,19,25}.

Sometimes, the use of both types of enzymes (from grain and microbial prolyl endopeptidase) has been used to obtain a beer with lower concentration of gluten (< 20 mg/kg)^{25,26}.

Another approach to reach gluten free beer is based on precipitation of hordeins. During years haze formation has been considered a defect in beer brewing. The proteins that produce haze are derived from the prolin-rich barley hordeins. To prevent this defect, substances like tannins, unflavored gelatins and silica hydrogels have been used for the beer stabilization. These substances form complexes with the proline present in barley hordein than can be removed by precipitation and/or filtration^{11,27,28}. The process eliminates the haze and simultaneously reduces the gluten content. This fourth strategy does not ensure the complete removal of gluten but sometimes the level reached is acceptable for celiac.

Finally, manufacture of all these gluten-free beers has to be carried out in an entirely gluten-free environment.

2.2. Wine

Wine is a product exclusively obtained from the alcoholic fermentation of grapes. Fresh grapes, crushed grapes or grape juice may be used as raw material for further full or partial fermentation^{1,4}.

White and red grapes are used, but white grapes produce only white wine. Red grapes can make white, rosé or red wine. This depends on the cultivar and the vinification process. There are cultivars of red grapes that yield white juice and, if solids are separated before fermentation begins, white wine is obtained. Red grapes which have white or red juice, yield either rosé or red wine if the fermentation is done with the whole fruit because red pigments or anthocyanins are presented in the skins. A limited period of maceration brings about rosé wines⁵.

None of the raw materials required for the production of wine contains gluten so it is considered as a gluten-free fermented beverage. But sometimes, it is necessary to clarify the wine in order to remove particles in suspension because they can affect the appearance and the flavor of the wine. This procedure has to ensure long-term clarity and prevent sediment during storage. The fining process relies on adding substances that induce flocculation and settling in cloudy or in non-stabilized wines. Filtration can remove particles such as grape fragments and dead yeast but fining can remove soluble substances (phenolic compounds such as tannins, and coloring matter in red wine).

Fining is carried out with protein-based products which are often a mixture of denatured or partially denatured proteins that trap undesirable substances, resulting in a precipitate. Proteins used for fining have been typically of animal origin. In red wine the most commonly used are egg albumin, serum albumin, casein, isinglass or gelatin from fish. These protein-phenol complexes are then removed by decanting, centrifugation or filtration. Nowadays winemakers are seeking a substitute for these animal proteins due to the restrictions imposed because of bovine spongiform encephalopathy (BSE) in animals and its possible transmission to humans. In this regard, the

legislation in several countries of the European Union has been adopted to avoid the use of blood powder and serum albumin^{29,30}.

Plant proteins have proved to show potential in this context. Malt proteins, sorghum prolamins and legume proteins have been used to clarify wines. Wheat gluten, especially hydrolyzed preparations, allows a very efficient clarification of the wine, with a selective precipitation of condensed tannins from red wine.

Since proteins derived from plants are considered good wine fining agents, it seems to be important to quantify their residual amount in the fined wine, as some plant proteins could cause severe immunological responses or chronic intolerance. The possibility that gluten proteins remain in the wine after treatment cannot be excluded, representing a potential hazard for persons who have celiac disease^{29,30}.

Labeling legislation in European Union, Canada, USA and Australia requires that potentially allergenic compounds must be stated on the labels but there are few data about whether any plant proteins derived from fining agents are present in the finished wine. Despite this fact, the published results provide evidence that the gluten concentration in the treated wines is by far below even the most restrictive legal threshold for gluten-free drinks.

2.3. Cider

Cider is a fermented alcoholic beverage made from apple juice¹. Cider alcohol strength varies from 1.2% to 8.5% (vol/vol).

This is the general process for making cider. Apples are crushed and the pulp obtained is then transferred to the cider press where it is pressed until all the 'must' or juice is squeezed from the apple pulp. Then the fermentation takes place in casks or barrels. The proportion of alcohol content depends directly of the total sugar content in its must, because during the fermentation process the sugar is transformed into carbonic anhydride and alcohol. Then the cider is transferred to another cask to maintain the quality and after some months the process is completed and it is ready for bottling.

As in the case of wine, all raw materials used in the manufacture of cider are gluten free. Therefore, if the whole process is carried out in an entirely gluten-free environment, cider is in general considered to be a gluten-free beverage.

However, low quantities of malted barley are added to some special ciders which are then filtered to guarantee gluten removal.

3. Spirits

The *Official Journal of the European Union*³¹ gives the following definition: spirit drinks are alcoholic drinks intended for human consumption. By definition, spirit drinks possess particular organoleptic qualities and have a minimum alcoholic strength of 15% vol. But some of them reach 40% vol.

Spirit drinks are produced by two ways. The first one is directly, a) by distillation, with or without added flavorings, of naturally fermented products; b) by maceration of plant materials in ethyl alcohol of agricultural origin and/or distillates of agricultural origin, and/or spirit drinks; c) by the addition of flavorings, sugars or other sweetening products and/or other agricultural products and/or foodstuffs to ethyl alcohol of agricultural origin and/or to distillates of agricultural origin and/or to spirit drinks. The second way is by the mixture of a spirit drink with one or more of: a) other spirit drinks; b) ethyl alcohol of agricultural origin or distillates of agricultural origin; c) other alcoholic beverages; and/or d) drinks.

The spirit drinks are classified by category (rum, whisky vodka, etc.).

3.1. Rum

Rum is a spirit drink produced exclusively by alcoholic fermentation and distillation, either from molasses or syrup produced in the manufacture of cane sugar or from sugar-cane juice itself³¹. Addition of alcohol or flavorings is forbidden and rum may only contain added caramel for coloring. The minimum alcoholic strength by volume of rum is 37.5 %.

Rum is considered a gluten-free beverage because in the manufacturing process cereals containing gluten are not used.

However, pre-made drink mixes with rum, such as those intended for piña colada, mojito, daiquiri, etc. may contain gluten ingredients as flavorings.

3.2. Whisky or Whiskey

Whisky or whiskey is a spirit drink produced exclusively by distillation of a mash made from malted cereals with or without whole grains of other cereals, which has been saccharified by the diastase of the malt contained therein, with or without other natural enzymes and fermented by the action of yeast^{31,32}. The distillation is carried out one or more times so that the distillate has an aroma and taste derived from the raw materials used. At the end, the final distillate is matured for at least three years in wooden casks. This distillate, to which only water and plain caramel (for coloring) may be added, retains its color, aroma and taste derived from the production process. The minimum alcoholic strength by volume of whisky is 40 %.

Although some experts as the American Dietetic Association and Dieticians of Canada considered whisky as a gluten-free beverage³³, the fact that distillation removes the harmful gluten proteins should be demonstrated. In this sense, the whisky industry has undertaken a program of study that has showed the absence of gluten or other allergenic materials in distillates that use wheat and barley as raw materials³⁴.

But some celiacs associations advise against consuming whisky if the consumer is particularly sensitive. They claim that it is possible for distillation not to remove 100% of the gluten, or that a small amount of gluten is added back in as part of processing after distillation. In some cases, whisky manufacturers add caramel coloring (which may contain gluten) or even a small amount of the undistilled grain mash after the distilling process.

3.3. Vodka

Vodka is a spirit drink produced from ethyl alcohol of agricultural origin obtained following fermentation with yeast of either potatoes and/or cereals; or other agricultural raw materials³¹. Then, it is distilled and/or rectified to reduce selectively the organoleptic characteristics of the raw materials used and the products formed during fermentation. This process may be followed by redistillation and/or treatment with appropriate processing aids to give it special sensory qualities. The only flavorings that might be added are natural flavoring compounds present in distillate obtained from the fermented raw materials. In addition, the product may be given special organoleptic characteristics, other than a predominant flavor. The minimum alcoholic strength of vodka is 37.5 % vol.

There are plenty of vodkas made from non-gluten sources, such as potato vodka, corn vodka or grape vodka and they are usually gluten-free. Most of them are labelled as being gluten-free.

3.4. Gin

Gin is a juniper-flavored spirit drink produced by flavouring organoleptically suitable ethyl alcohol of agricultural origin with juniper berries (*Juniperus communis* L.). The minimum alcoholic strength by volume of *gin* shall be 37.5 %³¹. Natural and natural-identical flavouring substances from material of vegetable or animal origin can be added for the production of gin.

Due to the fact that gin is a distilled spirit, some experts consider it as a gluten-free beverage. But it is a controversial subject because other experts do not recommend this alcoholic beverage for celiac since the agricultural alcohol used is made from cereals which may include wheat, barley or rye and flavorings are used.

3.5. Grain, Wine, Fruit, Honey, Cider, Perry, Grape Marc Spirits

There is a wide range of alcoholic beverages produced exclusively by alcoholic fermentation and/or distillation of the raw materials (grains, wine, fruit, honey, cider, perry, etc). The sales denomination is different depending on the type of raw materials used in the manufacture³¹. For example, the sale denomination of fruit spirit shall be ‘spirit’ preceded by the name of the fruit, berry or vegetable, such as: cherry spirit or *kirsch*, mirabelle, peach, apple, pear, apricot, fig, citrus or grape spirit or other fruit spirits. In the case of marc spirits, the sales denomination consists of the name of the fruit followed by “marc spirit”. If marcs of several different fruits are used, the sales denomination shall be “fruit marc spirit”. The Table 1 shows some characteristics of this kind of beverages.

Table 1. Characteristics of spirits made from different raw materials.

Product	Raw material	Process	Alcoholic strength
Grain Spirit	Fermented mash of whole grain cereals	Distillation	≥ 35.0%
Wine Spirit	Wine	Distillation	≥ 37.5%
Grape marc Spirit	Grape marc	Fermentation and Distillation	≥ 37.5%
Fruit marc Spirit	Fruit marc (except grape)	Fermentation and Distillation	≥ 37.5%
Fruit Spirit	Fleshy fruit or must of such fruit, berries or vegetables	Fermentation and Distillation	≥ 37.5%
Cider or Perry Spirit	Cider or Perry	Distillation	≥ 37.5%
Honey Spirit	Honey mash	Fermentation and Distillation	≥ 35.0%

In this kind of spirits the key is the raw material used in the manufacture. Most of them are clearly gluten-free if they are handled in a gluten-free environment, but other, such as grain spirits, may be made from gluten-containing cereal grains and are not required to indicate the type of cereal is used in the manufacture. Despite this, as in the case of other foods, it is necessary to indicate the allergens at the label.

4. Problems in the Quantification of Gluten in Alcoholic Fermented Beverages and Spirits

The assessment of gluten content in beers and other beverages should take into account two important aspects. Firstly, as malt beer is usually produced from barley and wheat cereals, in addition to gliadins, the techniques used have to be able to accurately detect and quantify barley prolamins. The use of a single wheat gliadin standard could be unsuitable for the accurate determination of gluten from cereals which consist of a complex mix of proteins that have different responses to the antibodies used^{22,23,35}. It seems that accurate determination of hordein requires that the hordein standard used to calibrate the assay be similar in composition to the hordeins present in the beverages^{22,35}. Depending on the standard used, the quantification may over- or under-estimate by several orders of magnitude.

Secondly, the assay ought to accurately quantify partially hydrolyzed prolamins (gliadins and/ or hordeins), though there is no suitable hydrolyzed hordein standard for beer¹¹. Measuring the quantity of hydrolyzed prolamins in these types of products is one of the main problems in gluten analysis because prolamins have been broken into smaller fragments^{20,21,36}. This is what happens, for example, during the brewing process.

ELISA is considered the method type I by Codex Alimentarius for the analysis of gluten-free foods. It is also recommended by the Working Group on Prolamin Analysis and Toxicity (WGPAT) and the Food and Drug Administration (FDA)³⁷.

Sandwich R5 ELISA Méndez method is used to analyse intact prolamins. R5 antibody is capable of recognizing several small repetitive coeliac toxic epitopes and, as the epitope QQPF (glutamine-glutamine-proline-phenylalanine-proline) is present in wheat gliadin, barley hordein and rye secalin, R5 could recognize all fractions of all three grains. This method is based on the requirement that at least two specific epitopes are recognized by the antibody. However, it is not appropriate when foods and beverages are treated with proteolytic enzymes or when they are fermented because there may not be two of this sequence. This is due to prolamins being partially hydrolyzed into fragments containing two or more epitopes and small fragments having only one epitope. Consequently, small hydrolyzed products with a single epitope cannot be reliably determined by using sandwich R5 ELISA^{22,36,38}.

As competitive R5 ELISA requires only one antibody binding epitope, it is more suitable for the detection of hydrolyzed gluten than sandwich R5 ELISA. Both methods have been validated in multi-lab international trials³⁸.

The second FAO accepted sandwich ELISA kit is based on the Skerritt antibody^{39,40}. This antibody was one of the first monoclonal antibodies raised against wheat gliadin³⁹. It recognizes w-gliadins, a subfraction that differs both in their presence and levels within the cereals. In this sense, the Skerritt antibody only has a weak response to the hordeins found in barley and thus may underestimate the gluten content^{23,26,41}. Tanner et al.²³ suggested that this antibody does not seem to be appropriate for the gluten analysis of beers and the use of ELISA sandwich based on it should be discarded to avoid false negatives and dissenting results.

Second generation competitive ELISA methods have been developed using antibodies raised against the dominant immuno-reactive peptides involved in the biological response of celiac disease, e.g. G12 and A1 monoclonal antibodies have been raised against the toxic 33-mer of α -gliadin⁴². These antibodies are able to recognize peptides (besides the 33-mer peptide) from wheat, barley, rye, and varieties of oats which showed immunogenicity in T-cells from celiac patients^{43,44}.

Other competitive ELISA kit has been tested through an interlaboratory study in accordance with AOAC guidelines⁴⁵. The Gluten Tec kit uses a monoclonal antibody that detects a well characterized T cell stimulatory epitope of toxic prolamins. Both intact and small protein fragments, resulting from the hydrolysis of intact proteins, could be detected and, indeed, beer was selected as a food matrix for validation trials.

Competitive ELISA showed some advantages of repeatability and accuracy when analysing alcoholic beverages obtained by hydrolysis processes⁴⁶. However, there are still some unresolved questions concerning competitive ELISA and the analysis of these beverages is summarized in the following paragraphs.

One concern is to establish how prolamins fragmentation into smaller peptides occurs because the relation between prolamins and the fragments can vary from sample to sample. As competitive ELISA relates the total gluten amount in food with possible toxic peptides, a reliable conversion factor into gliadin cannot be given³⁶.

As mentioned, during malting and fermentation endogenous proteolytic enzymes break down the barley/wheat prolamins into short peptide fragments, and even into amino acids. Nevertheless, the heterogeneous mixture of peptides obtained are quite water soluble so they remain in the final product. Many of these peptide fragments contain high proline and glutamine levels. This may suggest that potentially immunogenic epitopes for celiac population may still remain in this beer^{16,43}.

A limitation of the competitive ELISA technique is that the fragment size recognized by the antibodies is not always established as a whole. It would be possible that the antibodies in ELISA competitive assay would also recognize smaller fragments that do not trigger the disease. Therefore, the gluten content is overestimated and products that could be suitable for celiac population would not be labelled as gluten-free.

By contrast, another aspect to consider is that the enzyme (proline endoprotease) used to produce gluten-removed beer may also destroy the recognized epitope sequence at the amino acid proline^{19,25}. Thus, competitive

ELISA does not detect gluten peptides. Since this enzyme breaks peptides at the prolines and the extent of this breakdown is high, toxic peptides will be broken down too and the gluten remaining in beer will be low. Nevertheless, underestimation of toxic prolamins by antibodies that not discriminate the immunoactivity of the peptide might endanger celiac safety.

In order to avoid these problems, antibodies should be specific and correlated with the potential immunotoxicity of the beer^{16,43,44}.

As well as this, some alcoholic beverages, such as wine or beer contain phenolic compounds. As a result it is necessary to use a protein (e.g. fish gelatin or skimmed milk powder) in the extraction procedure to prevent the phenolic-rich matrix interfering with the ELISA assay. In R5 competitive ELISA kits it is well established that beers are extracted with ethanol containing 10% fish gelatin.

Indeed, when proteins are denatured due to fermentation or by using proteolytic enzymes, a simple ethanol solution is not capable of extracting all the prolamins. Consequently reducing and disaggregating agents such as 2-mercaptoethanol are added to ethanol in the extraction processes by ELISA sandwich³⁸. However, these types of reagents are not compatible with the competitive assay because mercaptoethanol interferes with the specific binding of the antibody, obtaining false results. Some authors⁴⁶ have assayed a cocktail extraction, called UPEX, containing the reducing agent Tris (2-carboxyethyl)-phosphine (TCEP) and the surfactant N-lauroylsarcosine. Other study focusing on hordeins²³ suggest that an alcohol extraction with urea/ dithiothreitol (DTT) successfully extracts the majority of hordeins from barley flour and malt.

MALDI-TOF mass spectrometry was the first non-immunological technique employed to identify prolamins in flours and real complex food samples⁴⁷. Nevertheless, this system did not detect prolamins levels below 20-25 mg/kg and so is not appropriate in food samples with low prolamins levels.

Recently, mass spectrometry (MS) methods for the direct and absolute identification and quantification of food allergens and gluten have been developed^{23,35,41}. Thanks to its high sensitivity, LC-MS allows the detection of

allergenic proteins in trace amounts. In this sense, some authors confirmed that the ELISA sandwich results did not correlate with the relative content of individual hordein peptides as determined by MS, with all barley based beers containing hordein^{22,23,41}. Tanner et al.²³ found that 20% of ELISA results for beers were false negatives compared to results obtained by relative mass spectrometry. They suggested that mass spectrometry could be more reliable than ELISA, as ELISA enumerates only the concentration of particular amino-acid epitopes which may vary between different hordeins and may not be related to the absolute hordein concentration^{35,41}.

Although LC-MS/MS could offer analytical specificity which is superior to that of immunoassays or conventional high performance/pressure liquid chromatography (HPLC), the high initial cost of the equipment is not easily affordable and its throughput is lower than of immunoassays⁴⁸.

Other techniques such as those based on DNA detection have been also developed. Polymerase Chain Reaction (PCR) is useful to confirm other methods but they give only partial information in routine analysis of beers. Mujico et al^{49,50} observed a certain degree of positive correlation between protein and DNA in some hydrolysed food samples. Nevertheless, in food matrixes submitted to an intensive hydrolysis process, as syrups and malt extracts, the DNA was practically undetectable due to massive DNA degradation, and amplification by Q-PCR was not possible. Our Laboratory of gluten analysis UPV/EHU have also tested several techniques to analyze gluten content in alcoholic drinks using sandwich and competitive ELISA, compared with PRC technique.

We reported that despite the detection of gluten traces (5-40 mg/kg) by ELISA methods in some beers, it was not possible to find any WBR-DNA amounts, with an optimized design of a quantitative Real Time WBR-PCR protocol⁵¹. It must be pointed out that DNA extraction in these samples was difficult, and an improvement of this step may be enough to enhance detection of gluten-DNA. When DNA qualitative detection was used, by means of the SureFood® Allergen Gluten Real Time PCR kit, it was possible to detect DNA in some gluten containing beers.

In order to establish the amount of gluten detected in alcoholic beverages it is necessary to point out that gluten detection in spirits or distilled beverages is fairly unusual. Many of the beers consumed contained very low gluten levels, but frequently more than one serving of beer is consumed, which results in a toxic prolamin storage^{19,52,53}.

As mentioned before, when gluten content of beers is measured, several authors described that when using ELISA sandwich some of the negative or very low gluten results could not be considered gluten free when analyzed with competitive ELISA or other techniques^{23,41,43}.

5. Gluten-Content in Beers and Spirits

The studies reviewed showed a high diversity of gluten content in the different beers. This is due, at least in part, to the changes in the brewing processes. There are many differences in filtration processes, enzymatic processes, and/or the use of different varieties of malt barley that modify final content of gluten in beers^{12,19,25,28,52} (Table 2). Moreover, beers often contain significant quantities of gluten free adjuncts, which help to ‘dilute’ the initial raw material gluten content. Also the use of silica gel for removal of proteins may reduce the level of gluten in stabilized beer²⁸.

In addition, nutritional composition of beers showed that final product contains about 0.2-0.6% protein. Dostálek et al.²⁸ found that prolamin content decreased from 100 % in malt to less than 0.2% in beer during the mashing process, fermentation and stabilization process. Which is to say that anti-gliadin antibodies concentration was reduced by at least three orders of magnitude in beer compared with raw malt (on average, malt contained 18780 mg/kg of gluten, wort had 48 mg/kg and beer, 6.0 mg/kg of gluten).

Several studies agree with the fact that beers sold as “gluten free” contained gliadin levels below the detection limit of 6 mg/kg gluten^{11,40,52,54}. Most of the beers analyzed contain relatively low amounts of gluten because the quantity detected is usually between 10 and 50 mg / kg of gluten^{28,26,52}.

Table 2. Factors influencing gluten levels during the brewing process.

Brewing Parameters	Gluten Levels
Grain varieties for malt	Vary
Use of wheat	Highly Increase
Malt process	Vary
Dry Malt extract (DME)	Increase
Original gravity or beer density	Increase
Addition or use of stabilizers (e.g. prolyl or proline-specific endoprotease; Silica gel)	Decrease
Use of specific process equipment (centrifuge, filters)	Decrease

When comparing various types of beer, alcohol free beers usually have a very low content of protein and gluten content is below the detection limit or under the definition of “gluten free” (20 mg/kg)^{28,52}. Comino et al.⁴³ found that 59% of the one hundred analyzed beers contained more than 100 mg/kg of gluten, but other studies showed that many of the lager and ale samples were below 20 mg/kg^{11,26,28,52,53}.

Taking into account the relationship between cereal composition and gluten content, most of the studies reveal that type of cereal is a major element. Beers made of barley tend to produce low gluten levels⁵² whereas wheat or malted wheat beers contain very large quantities of gluten (more than 100 mg/kg and even higher than 500 mg/kg) and, thus wheat beers cannot be included in the diet of celiacs^{23,53,54}.

As suggested before, other factors related to the brewing process affect final content of gluten. Some studies^{25,53} described that many of the barley beer with highest gluten levels (> 100 mg/kg) were not clarified by filtration,

so the level of gluten detected may also correlate with turbidity, which could explain the higher prolamin concentration in some of them. For example, very high gluten content (> 800 mg/kg) is found in the Hefeweizen beer. This German style of wheat beer is a top fermented, unfiltered beer with a noticeable yeast sediment and a cloudy appearance^{23,53,54}.

From the point of view of labelling, beers producers should closely monitor the manufacturing process to ensure consistently low gluten levels before they label beers made from barley, wheat or rye as “gluten free”. With respect to spirits, celiac patients should be aware of the risks of the consumption of these types of highly alcoholic drinks and they should always check the label information and any suspect added ingredients^{55,56}.

6. Conclusion

Beer is the most popular fermented alcoholic beverage and the most likely to contain small amounts of gluten.

The accurate quantification of gluten in beers and other beverages is a challenging problem due to the hydrolysis of gluten and potential immunotoxicity modifications that occur as a result of the processing steps.

Although it is necessary to consider the origin and type of grain used, it is usual that special wheat-beers contain very significant amounts of gluten while other beers brewed with barley rarely exceed 50 mg/kg of gluten.

The remaining fermented beverages, such as wine or cider, rarely contain gluten but it is important to confirm that no gluten-containing additional ingredients are added after brewing.

The manufacturing of distilled drinks or spirits implies that the end product does not usually contain gluten. Nevertheless, it is necessary to check that additional gluten-containing ingredients are not then added.

Analytical techniques used to gluten detection in these alcoholic drinks are not as useful as for other matrices. The competitive ELISA technique has some advantages over other methods but it still needs to be improved.

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