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Italian cider:
a new potentially typical product to be characterized

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Una sola parola, logora,
ma che brilla come una vecchia moneta: "Grazie!"

Pablo Neruda

CONTENTS

SUMMARY

The objective of this PhD research project has been to evaluate some characteristics (e.g., allergens content, polyphenols profile and antioxidant potential) of the Italian ciders that could affect the human health.

In this way we tried to promote the product contributing to improve the knowledge needed to better the production techniques.

The first aspect described in this PhD study was the recovery of cider proteins (Chapter 2). The rationale was the knowledge that, in other fermented beverages, these molecules are described to be involved in specific organoleptic characteristics such as foam stability, in the interaction with the aroma compounds in the formation of hazes and sediments. In addition, proteins could elicit allergenic reactions in sensitive individuals.

After the setup of the recovery methods, proteins purified from 18 commercial ciders were identified by LC-MS/MS technique. This high sensitive method revealed the presence of Mal d 2 and Mal d 3 proteins in all the cider samples analyzed. In addition, this technique showed that 12 out of 18 ciders are contaminated by hen egg lysozyme that is a well-known allergen, even if its use and presence was not declared on the label as stated by the EU Directive. Since the LC-MS/MS analysis detects only fragments of protein that could not be immunologically active, ELISA and western blot analyses were achieved for the detection of lysozyme. Two samples out of 18 were both positive to the assays applied and this confirm the immunological activity of the protein and the existence of a serious health concern to drink cider for people sensitized to egg (Chapter 3).

The following step was to study the effect of cidermaking procedure on apple allergen. For this reason, the immunological activity of Mal d 2 and Mal d 3 proteins was tested with specific antibodies developed in rabbit. Six and four samples out of the 13 ciders considered were positive to Mal d 2 and Mal d 3, respectively. It is important to highlight that not all the samples showed a positive match to both the apple allergens. This could indicate that the technological process in cidermaking could achieve a selective removal of specific allergens. Moreover, as regarding Mal d 3 the antibody detected an ambiguous signal at the level of the stacking gel of almost all samples (Chapter 4).

Finally, an aspect related to the wholesomeness of cider was investigated. The polyphenols profiles of 18 Italian ciders were correlated with their antioxidant activity, and a multiple

linear regression model was drawn to predict the antioxidant potential of the beverage, knowing the concentration of specific phenols (namely: chlorogenic acid, catechin, procyanidins B1 and B2, quercetins 3-glucoside, 3-xyloside and 3-arabinopyranoside). This suggests that these phenols could be the mainly contributors of the antioxidant activity, but further studies should be performed to exclude that this correlation could be only an epiphenomenon consequence of particular interactions among different molecules. Anyway the total antioxidant activities of Italian ciders results on average lower than those reported in literature for Asturian ones and this could be probably related to the total polyphenols content and their profiles (Chapter 5).

This observation suggests that the Italian cidermaking process could be ameliorated taking in consideration both the allergenic profile of the beverage and the preferences of the Italian consumers.

RIASSUNTO

L'obiettivo di questa tesi di dottorato è stato quello di valutare alcune caratteristiche dei sidri Italiani che potrebbero incidere sulla salute umana, come ad esempio, gli allergeni, il contenuto di polifenoli e il potenziale antiossidante, incrementando in questo modo il valore aggiunto della bevanda.

In un certo senso, si è cercato di promuovere la commercializzazione del prodotto, contribuendo a migliorare le conoscenze necessarie per ottimizzarne le tecniche di produzione.

Il primo aspetto descritto in questo studio è stato la purificazione preparativa delle proteine del sidro (Capitolo 2). La logica di questo approccio è costituita dalla conoscenza che, in altre bevande fermentate, queste molecole sono descritte come coinvolte nel determinare caratteristiche organolettiche specifiche, quali la stabilità della schiuma, l'interazione con i composti aromatici e la formazione di intorbidamenti e di sedimenti. Va inoltre tenuto presente che, le proteine del sidro potrebbero essere coinvolte nello scatenamento di reazioni allergiche in soggetti sensibili.

Dopo la messa a punto dei metodi preparativi, le proteine purificate da 18 sidri commerciali sono state identificate con la tecnica LC-MS/MS. Questo metodo ad alta sensibilità ha rivelato la presenza delle proteine Mal d 2 e Mal d 3 in tutti i campioni analizzati. Inoltre, questa tecnica ha dimostrato come 12 sidri su 18 campioni siano contaminati da lisozima di uovo di gallina. Quest'ultima proteina è un noto allergene, anche se la sua presenza non risultava dichiarata in etichetta, come invece sarebbe previsto dalla Direttiva dell'Unione Europea 2007/68/CE. Poiché l'analisi LC-MS/MS rileva solo frammenti di proteina che potrebbero non essere immunologicamente attivi, sono stati eseguiti un test ELISA e l'analisi con la metodica western blot. Due campioni su 18 sono risultati positivi ai test applicati e questo ha confermato l'attività immunologica della proteina e l'esistenza di un serio rischio per la salute delle persone sensibilizzate alle uova, qualora dovessero bere del sidro (Capitolo 3).

Il passo successivo è stato quello di studiare l'effetto della procedura di produzione del sidro sugli allergeni della mela. Per questo motivo, l'attività immunologica delle proteine Mal d 2 e Mal d 3 è stata testata con anticorpi (IgG) specifici sviluppati in coniglio. Rispettivamente sei e quattro sidri su 13 campioni sono risultati positivi per Mal d 2 e Mal d 3. È importante sottolineare che non tutti i campioni hanno mostrato un risultato positivo per entrambi questi

allergeni di mela. Questo indicherebbe che alcuni passaggi del processo tecnologico di produzione del sidro potrebbero produrre una rimozione selettiva di specifici allergeni. Tuttavia, riguardo alla proteina Mal d 3, il saggio anticorpale in western blot, ha rilevato un segnale ambiguo a livello del gel di impaccamento di quasi tutti i campioni (Capitolo 4). Questo dato dovrà essere oggetto di ulteriori approfondimenti.

Infine, è stato studiato un aspetto che è correlato alla salubrità del sidro. I profili polifenolici di 18 sidri Italiani sono stati correlati con la loro attività antiossidante, ed è stato realizzato un modello di regressione lineare multipla al fine di correlare il potenziale antiossidante della bevanda con la concentrazione di polifenoli specifici (in particolare: acido clorogenico, catechina, procianidine B1 e B2, quercetine 3-glucoside, 3-xiloside e 3-arabinopiranoside). I risultati ottenuti potrebbero suggerire che questi polifenoli siano quelli che principalmente contribuiscono all'attività antiossidante della bevanda, ma ulteriori studi dovranno essere eseguiti per escludere che questa correlazione non possa essere solo un epifenomeno dovuto, per esempio, a particolari interazioni di molecole diverse. In ogni caso, l'attività antiossidante dei sidri Italiani è risultata mediamente inferiore a quella riportata in letteratura per i sidri delle Asturie, e questo potrebbe probabilmente essere dovuto al contenuto di polifenoli totali e al loro profilo (Capitolo 5).

Tutte queste osservazioni suggeriscono che il sidro, attualmente prodotto in Italia, sia un prodotto che potrebbe essere migliorato sotto il punto di vista salutistico pur tenendo in considerazione le peculiarità organolettiche ricercate dal consumatore locale.

CHAPTER 1

GENERAL INTRODUCTION

APPLE

The nutrition awareness of people in most Westernized countries is increasing. In particular, European citizens are conscious that the frequent intake of fruit and vegetables is a part of a healthy balanced diet, and World Health Organization (WHO) statistics report that the fruit and vegetables intake in Spain, Finland, France, Portugal, Greece, and Italy is above the recommended level (400 g per day per person) (1, 2).

Apple (*Malus domestica* L. Borkh) belong to the *Rosaceae* family that also includes apricots, peaches, cherries, pears, raspberries, strawberries, almonds, and plums (*3*) It represent one of the most important flowering plant thanks to its great spread, to the relevance of its fruit in the human diet, and to their wide economic interest. As a consequence of their availability throughout the year, apples are the most important source of secondary plant metabolites (e.g., polyphenols) both in the American and in the European diet (*2*). Among the fleshy fruits, apples are one of the most produced fruit in the European Union, and Italy is the third worldwide exporter after China and Poland. In about 50 years the apple production worldwide has increased by 444%; from 17 million tons in 1961 it reached more than 76 million tons in 2012 (*4*). According to the Food and Agriculture Organization of the United Nations (FAO) statistics, in 2013 Europe produced about 16 million tons of apples, and Italy was the second largest apple producer country in EU with about 2.4 million tons (*5*).

It is estimated that over 20,000 varieties of apples are grown throughout the world, many of which are ancient, uncommon and characterized by a high content of biologically active substances (*6*). However, a limited number of varieties dominate the European market; in fact over 70% of the total harvest is made up of "Golden Delicious", "Jonagold" and "Red Delicious" cultivars (*6*). The reason for this varietal selection is the need to satisfy the demands of different markets, as well as the consumer's preferences for specific organoleptic characteristics such as size, color, and sweetness, or the shelf-life (*6*). However, at the present, many cultivars of apples have been selected for different specific uses such as: raw consumption, cooking and preparation of processed products such as jams, juices and ciders (*7*).

Apples are among the main foods that provide phytochemicals to the human diet (*8*), for this reason its consumption is highly recommended by nutritionists.

Apples contain more than 84% water, minerals as K, Mg, Ca and Na and trace elements such as Zn, Mn, Cu, Fe, B, F, Se and Mo, vitamins mainly C and the B complex. Fibre content as

pectines, celluloses, hemicelluloses and lignins is higher in apples in comparison to other fresh fruits (*9, 10*). Moreover, starch is present in unripe apples, depending on varieties (*11*). Proteins and lipids are in small concentrations.

Sun et al. (*12*) reported that apples have a content of phenolic substances that can be 3-4 times higher compared to other commonly consumed fruits, such as oranges, lemons, peaches and pears. In relation to cultivars and their ripening phase, apples are rich in phenolic compounds, in particular quercetin, catechin, epicatechin, phloridzin and chlorogenic acid, that show strong antioxidant capacity (*2, 13-15*).

The phenols present in apples can be classified into six different groups: the most represented class consists of the proanthocyanidins (oligomeric flavanols, also known as condensed tannins) followed by hydroxycinnamic acids, dihydrochalcones, flavonols, monomeric anthocyanins and flavanols (catechins) (*16*).

The concentration of these compounds may depend on several factors, e.g., the cultivar, the harvest and the storage modality and time, and the procedure of processing of apples (*17*). Studies on 31 different apple cultivars, in fact, have reported a significant difference in the total polyphenol content (*18*). However, it was also observed that the content of such substances and the antioxidant capacity seem to be regulated both by environmental factors, such as the ripening or light exposure, and post-harvest factors, such as storage and processing (*18*). Moreover, the distribution of the phenolic compounds can also vary between different tissues of the fruit (pulp and peel) (*19*).

Many recent studies suggest that the consumption of apples and derivatives may have beneficial effects in preventing diseases, e.g., the fruit intake might be linked to the reduced risk of several forms of cancer, cardiovascular disease, and asthma (*20-24*).

In general, the health benefit of apples is associated to the presence of polyphenols. Scientific data describe that apple consumption may reduce the risk of chronic diseases (*25*).

The procyanidins (catechin and epicatechin), for example, have a strong antioxidant activity and may inhibit the oxidation of low density lipoproteins (LDL) *in vitro*, and may increase the high density lipoprotein (HDL) *in vivo* (*26-28*).

Moreover, apple consumption may have beneficial effects in decreasing the risk of cardiovascular and neurodegenerative diseases (such as Alzheimer's and Parkinson's), counteracting the aging process (*29-31*), may prevent type 2 diabetes, helping weight management, and may protect the gastrointestinal tract from drug injury (*32, 33*). Indeed, in

the collective unconscious, apples have long been associated with a healthful diet. “An apple a day keeps the doctor away” is a public health message that has been delivered by parents and teachers since the 19th century (*34*).

However, despite the beneficial substances contained in apples and derivatives, it is well known the presence of proteins that can cause allergic reactions in sensitive children and adults. Up to 2% of the population of central Europe suffers from allergic reactions to apples (*35*).

The effects on human health will be deeply discussed in the following chapters.

It must be noted that fruits can also be consumed as processed product, e.g., fruit juices, jams, purees, and ciders. In addition, several studies showed that bioactive compounds of the raw fruits could persist in their derivatives and by-product. For example, concentration and antioxidant activity of polyphenols have been widely studied in apple juices and pomace. The latter is a by-product of apple juice and cider production and was proposed as a source of dietary fiber (*13, 17, 36*). Under this point of view, studies were also achieved on the effects of technological processing on fruit allergens (*37, 38*).

Cider is a popular fermented beverage produced worldwide from different apple cultivars. The main operations of cidermaking are similar, but they may vary among countries (*39*).

The trend of the market to ask for low alcoholic beverages has increased the spread of cider consumption worldwide. In the last years, the popularity of cider increased in Italy as well (*40*).

CIDER

In North America the word “cider” indicates a non-alcoholic juice of apple (*41*), however throughout Europe and in the rest of the world, cider refers to a slightly alcoholic beverage obtained from the fermentation of apple juice.

In 2012 the global cider market was 19.9 million hectolitres, and Heineken N.V. Company leads the global cider category with a 20% market share (*42*).

By studying the consumption in 53 countries, the Nielsen Company produced a report that highlights a progressive increase in consumption of alcoholic beverages. In particular, cider is the fermented drink that shows the higher increase in consumption (14%) in 2008 (*40*). From

2011 to 2012 in Italy a Customized Research & Analysis survey on 1550 people aged 18-55 showed a 9% increase of the cider consumers (from 15% in 2011 to 24% in 2012). This investigation, commissioned by Heineken Italia S.p.A., revealed that, in the same period, the interest for the beverage was higher in young adults with an increase of consumers from 5.4% to 27% in the ages ranging from 18 to 24, and from 16.8% to 30.4% in the ages ranging from 25 to 34. The rising consumption of the beverage may be in relation to the low alcohol content and calories, and the healthy properties attributed by the consumers to apples.

At the present, the UK is the first cider producer worldwide (with more than 3.7 million of hectoliters annually), followed by France, Spain, Ireland, Germany and Switzerland. However, other countries such as North America, New Zealand, Canada and Italy are rapidly increasing their cider production (*43*).

The cidermaking process may be different depending on the country traditions. French ciders (from Normady or Brittany) are low in alcohol (ranging from 2 to 4% vol.) and naturally carbonated, and contain high amount of residual sugars; German ciders hail mainly from the Frankfurt and Trier regions, are very dry and acidic, gently carbonated with an average alcohol content of 5%; Spanish ciders are mainly produced in the Asturian and Basque regions, and are characterized by high volatile acidity and high foaming; English ciders are commonly vat-aged and artificially carbonated, with an alcohol content that may range from 5% up to 10% vol. (*43, 44*).

Ciders can be made from almost any type of apple. The surplus of cooking or dessert apples are used for cidermaking in some regions of the UK, little or no distinction is made between cider, juice and dessert apples in Germany. French cidermakers do a selection of apple varieties, named as "true cider cultivars" that are grown for no other purpose, except for cider production (*45, 46*).

In Italy ciders are produced in few regions (mainly the northern ones), especially Trentino-Alto Adige, Friuli-Venezia Giulia, Piemonte e Valle d'Aosta. The reasons way cider has not been so popular in Italy are mainly historical and cultural. The first reason concerns the deep-rooted Italian wine culture, and the second the laws promulgated during the Fascist period. As a consequence of these laws fruit-based fermented beverageswere heavily taxed, and the goal was to help the Italian wine industry to flourish and to eliminate competition (*47-49*).

Unlike the European tradition, Italian ciders are mainly produced with dessert apples, so the final product is usually characterized by low acidity. For this reason, malolactic fermentation

(desirable in Spanish and French ciders) is avoided in the Italian cidermaking, since this reaction converts malic acid into lactic acid, aiming at decreasing the acidity of the final product.

True cider cultivars usually have higher sugar content than dessert apples. Thanks to their structure, pressing is easier, and the juice yield is higher. Their polyphenols concentration is relatively high, contributing to the mouthfeel, flavor and colour of the beverage. In addition, polyphenols have also weak anti-microbial properties (*43, 45, 50*).

Rarely, cider is made up of a single cultivar of apple, mainly because the final product has to achieve a balance between sugar, acid and tannin (*51*). The Long Ashton Research Station classified apples according to the acidity and tannin content (Table 1.1).

Apple group	Tannin (g/100g)	Acidity (Malic acid g/100g)
Bittersharp	> 0.2	> 0.45
Bittersweet	> 0.2	< 0.45
Sharp	< 0.2	> 0.45
Sweet	< 0.2	< 0.45

Table 1.1 Cider apples classified according to the acidity and tannin content (*50*).

So, a blend of apple approximatively incuding 50% of sweet, 35% of acidic, and 5% of astringent apples are usually suggested to obtain a balanced final product with a plesant taste and aroma (*41, 50*). In addition, the importance of having ripe and healthy apples should not be overlooked; green apples will make a poor cider, whereas rotten ones will promote spoilage bacteria growth (*46*). Well-ripened apples are a source of different compounds which contribute to the quality of the final product. Apple skin contains essential oils and aromatic compounds that can modulate the perception of the organoleptic characteristic of the cider (*52*). Apple pulp represents the 95% of the total weight of the fruit, and it is made up of soluble components, mainly water (ranging from 75% to 90%), sugars (mainly glucose, fructose, and saccharose), malic acid and tannins; and a part of unsoluble material, such as cellulose, starch, pectins and nitrougenous material.

CIDERMAKING PROCESS

After the harvest, apples should be stored for a week to 10 days at room temperature to make the fruits easier to grind and to convert starch to sugar. Then, rotten apples are eliminated and healty fruits are washed to remove leaves, debris, and insects to avoid the growth of spoilage bacteria. Afterwards, the whole apple is milled to a pulp, from which the juice can be pressed out. The finer is the pulp grinded, the greater will be the yield of juice (*43, 46*).

In large cider factories, a horizontal piston press is used to gradually squeeze the apple pulp and to recover the juice. This process lasts about half an hour, and contacts with air should be minimized to avoid off-flavors and brown colours in the juice.

Both apples and yeasts contain natural pectolytic enzymes that can break down the pectin during fermentation, but they are little efficient. So to ensure that the final cider is clear, broad-spectrum pectinases can be added to the apple must. Pectin is a heteropolysaccharide of the cell wall that allows plant growth, primary cell wall extension and binding between cells. Alcohol precipitates pectin, so it may lead to hazes in the final cider. Dessert and long-stored apples usually release more pectin than bittersweet fruit, thought giving cloudy ciders.

In the Basque region, the traditional technology implies a maceration step before the milled apples are pressed. Pectolytic enzymes are not used, and spontaneous clarification and fermentation of the must are promoted (*53*). This maceration step may lead to the proliferation of undesiderable micro-organism (e.g., oxidative yeast or lactic and acetic bacteria) that can compete for nutrients with the fermentative yeasts (*53*).

Unlike grape must or beer wort, apple juice is low in yeast nutrients, so 0.2 mg/L of thiamine, 250 mg/L of ammonium phosphate (or sulphate) are usually added to improve the fermentation rate. The addition of sugars (e.g., honey, white sugar, or concentrated apple juice) aims to increase the alcohol content in the final cider.

Like in winemaking, sulphur dioxide (SO_2) is used for its antimicrobial and antioxidant properties (*54*). It inhibits the growth of spoilage bacteria, promoting the fermentation by *Saccharomyces* spp., inhibits the oxidative enzymes, and prevents darkening of oxidizated compounds (*46, 50*). However, it must be noted that some people are sensitive to SO_2, so the Food and Agricultural Organization of the United Nations (FAO) and the World Health Organization (WHO) recommend its total elimination or at least its reduction (*55*).

In France, the traditional cidermaking implies that neither exogenous yeasts nor sulphite are added to the apple juice, since the internal fruit microflora starts the spontaneous fermentation

of must, giving rise to flavour complexity (*44*). The fermentation rate is usually slow and lasts after month, so high levels of residual sugars remain in the cider.

On the contrary, English and Italian cidermaking practice uses SO_2 and pure culture of *Saccharomyces* wine yeasts inocula. So the most troublesome bacteria and yeasts are eliminated, because the yeast cells inoculated multiply quickly. The fermentation lasts in few weeks, as long as there is sugar available for yeast. A complete fermentation can lead to 8-12% alcohol (*51*).

In French and Spanish ciders a second fermentation (malo-lactic fermentation, MLF) can occur sometimes currently to the yeast fermentation. This reaction converts L(-) malic to L(+) lactic acid, aiming at decreasing the cider acidity (*56*), and it consequently produces carbon dioxide. The decrease of acidity also affects the organoleptic characteristic of the beverage, giving rise to a flavour more complex and rounded (*46, 50*).

Once the fermentation is complete, ciders are racked and clarified. The latter step can be performed by natural settling, centrifugation or fining with gelatin. The UK regulations permit the addition of water to correct the alcohol strenght of the final product, together with the addition of sweeteners, malic acid, preservatives, food colorants, and carbon dioxide. Whereas in France, Germany and Spain legislations are more restrictive. Finally, the cider can be bottled. Some cidermaking practices imply a pasteurization step to prevent indesiderable fermentation.

The main steps of the cidermaking process are depicted in Figure 1.1 (*57*).

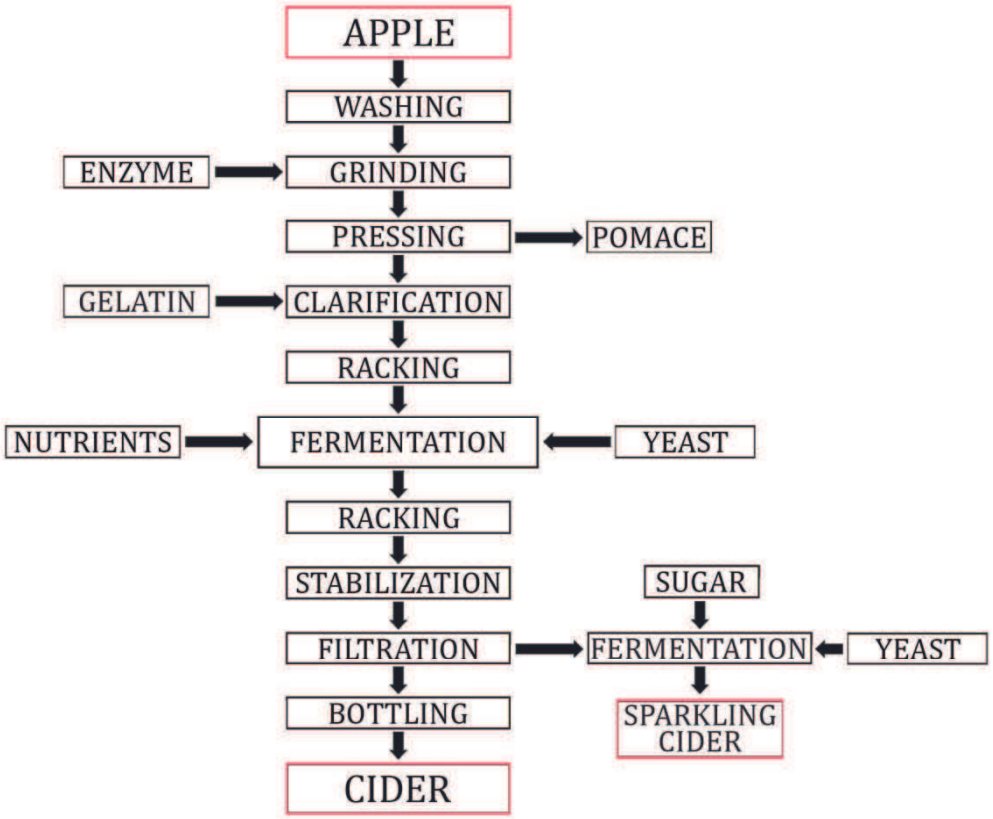

Figure 1.1 Main steps of cider making process .

POLYPHENOLS AND ANTIOXIDANTS OF CIDER

Since the late XVII century the wholesome values of apple cider have been praised, and thanks to "*its specifick Vertues, there is not any Drink more effectual against the* Scurvy. *It is also prevalent against the* Stone*, and by its mundifying qualities, is good against the Diseases of the* Spleen*, and is esteem'd excellent against* Melancholy." (*58*).

As above mentioned, various studies demonstrate a beneficial effect of apples and their derivatives on human health (*59*). It is not clear what are the molecular mechanisms involved in the protection against generative diseases. One hypothesis to explain this effect is the presence of antioxidants and vitamins in fruits.

The main known benefits deriving from apple constituents are summarized in Table 1.2.

		Characteristics
Health Parameters	**Polyphenols**	- Divided into 6 groups: Proanthocyanidins, hydroxycinnamic acids, dihydrocalcones, flavonols, monomeric anthocyanins and flavanols - Antioxidant activity - Prevention of cardiovascular disease, cancer and osteoporosis - Organoleptic characteristics (astringency and bitter taste)
	Vitamins	- Antioxidant activity - Contribution to synthesis and functionality of organs and tissues - Modulate gastrointestinal functions - Immunomodulator - Micronutrient deficiency disorders
	Fibers	- Prevention of heart diseases and cancer - Modulate gastrointestinal functions - Prebiotic activity

Table 1.2 Main apple constituents and their characteristics (*60*).

Polyphenols are the main responsible of the antioxidant activity of apple, and furthermore they contribute to the organoleptic characteristic of the fruit and its derivatives.

Under the quantitative viewpoint, flavan-3-ols, procyanidins, flavonols, dihydrochalcones and hydroxycinnamic acids are the most represented polyphenols in cider form different countries (*61*).

These substances are involved in color, bitterness, and astringency and generally in the mouthfeel of cider. They are precursors of volatile compounds, they are involved in the fermentative process, by controlling spoilage of bacteria (*53*), and they can also inhibit

enzymes used during cidermaking (*62*). Polyphenols profile varies in relation to apple cultivars and their ripening phase, cultural coinditions, climate and soil, and their presence in cider is also influenced by the cidermaking processes, such as maceration, pressing, enzymatic clarification, centrifugation, filtration, and fining (*39*).

The antioxidant activity and the polyphenols profile of English, French, Basque, and Asturian ciders were characterized in many studies (*63-67*). At the present, no information about phenols and antioxidants of Italian ciders are avaiable.

One of the aim of this PhD thesis is to contribute to fill this informational gap.

FOOD ALLERGIES

The relationship between food intake and "adverse reactions" in some sensitive individuals has been known since antiquity. Hippocrates, for example, reported events of adverse reactions to milk in writings of more than 2000 years ago.

Among all the adverse reaction to food, this chapter will deal with food allergies.

The first real approach to the understanding of abnormal immuneresponse to food dates back to 1920s, when Prausnitz and Kustner showed that the substance responsible for the allergic reaction to fish was present in blood serum and could be transferred from a sensitive to a non-sensitive individual (*68*).

The US National Institute of Allergy and Infectious Disease (NIAID) defines *food allergy* as “an adverse health effect arising from a specific immune response that occurs on reproducibly exposure (consumption or other contact) to a given food” (*69, 70*). This immunological reaction only affects sensitive or “sensitized” people to a specific food allergen, which is normally tolerated by the rest of the population.

The European Academy of Allergy and Clinical Immunology (EAACI) defines food allergy as a type of *hypersensitivity* (distinct from food intolerance), that can be divided into IgE-mediated food allergy (such as to egg, milk, and peanuts) and non-IgE-mediated food allergy (such as gluten intolerance) (*71*).

The sensitization to a given allergen is the first step of an IgE-mediated food allergic reaction, and it is characterized by the production of a class of antibodies, called Immunoglobulin isotype E (IgE).

"During the sensitization phase, the IgE antibodies bind by high-affinity to specific receptors (FcεRI) on the surface of mainly tissue mast cells and blood basophils, but also by low affinity to receptors (FcεRII) on macrophages, monocytes, lymphocytes and platelets (*72*). When a new exposure occurs after the sensitization, the specific sites of the antigen (= allergen), named *epitopes*, bind two or more IgE molecules linked to the FcεRI. This determines the cross-linking of the receptors with the consequent massive release of mediators, in particular the vasoactive histamine, by the stimulated cells (*73*). Epitopes could be either an amino acid sequence (*sequential epitope*) or a region on the surface of the antigen generated by sequences held together by the three-dimensional folding of the protein (*conformational epitope*) that might have been partially conserved during the evolution (*74*)" (*75*).

Food hypersensitivity symptoms usually appear within few minutes and last for several hours, after the ingestion of a food to which an individual is sensitized, and they disappear avoiding the food (*76-78*). Symptoms of IgE-mediated food allergy have a range of gravity. Itching, numbness, chest pain, abdominal pain, and nausea are (subjective) symptoms; eczema, hives, swelling (e.g., tongue, lips, throat, or face), trouble breathing, wheezing, nasal congestion, diarrhoea, dizziness and drop in blood pressure are all (objective) signs with increasingly severity until anaphylactic shock (*79*).

The food adverse reactions can be triggered by few milligrams (10-100 mg) of food ingestion, but the threshold that can trigger the allergic reactions varies across the population of patients. So it is not possible to predict neither the level of sensitivity of a consumer to a food allergen, nor the level of severity of the allergic reaction, because it depends on the health of the individual, whether he took medicines or got physical exercises (*78, 80*).

Up to now, the only prevention to hypersensitivity is the strict avoidance of foods to which a patient is sensitized. Therefore, allergic individuals can be at risk from accidental exposure when allergens either cross-contaminate foods, or are incorrectly described or labelled (*79*).

This topic will be discussed in the following chapters.

The prevalence of allergies is increasing in the industrialized countries and presumably in the rest of the world (*81*). EAACI statistics estimated that about 17 million people in Europe suffer from food allergies (*82*), while globally this number is around 220 to 520 million (*83*).

More than 170 foods are known to be involved in allergic reactions, but more than 90% of the cases are elicited by ten allergenic foods: egg, milk, soya, peanut, tree nuts (e.g. almonds,

walnuts, Brazil nuts, hazelnuts, pistachios, chestnuts), wheat, fish, shellfish (both crustacean, e.g. shrimp, prawn, crab, crayfish; and molluscan, e.g. snails, cuttlefish, oysters, clams, octopus), sesame and mustard (*76, 84-87*).

Today, problems related to food allergies are becoming more and more complex.

From one side, "the westernized life style and food globalization (e.g., the increased accessibility to new foods such as kiwi and papaya) turn out to increase the exposition to new potential allergens" (*75*).

On the other side, the food chain underwent the introduction of new ingredients and new production formulas, so it is increasingly difficult to identify the different components of a food product. Strict regulations have been introduced by governments (e.g., label requirement), but these rules are not always effective.

Prevalence studies in Europe and North America demonstrated that 4-8% of young children and 2-4% of adults suffer from severe adverse reaction to food (*79*). "It must be noted that, although hundreds of different foods are represented in the human diet, only a relatively small number is described as capable of inducing allergic reactions in specific ages of life" (*75*).

In children milk, eggs, peanuts, wheat, and soya proteins trigger nearly 90% of the hypersensitivity reactions, whereas in adolescents and adults the 85% of food-allergic reactions are provoked by peanuts, shellfish, and tree nuts ingestion (*70, 88-92*). However, some vegetables (such as tomato, carrot, and potato) and fruits (such as peach, kiwi, cherry, and apple) can elicit allergic reactions (*81*).

Moreover, some food allergies may arise after sensitization to inhalant allergens that share a similarly structure (sequential and/or conformational epitopes) to the food allergens (*87, 89, 90*).

Food allergens are usually proteins (typically with a molecular weight between 10 and 70 kDa), although it has been reported that some non-protein substances (e.g., some food additives and certain drugs) can elicit IgE-mediated allergic reactions, probably by acting as haptens. These small molecules act like allergens only when binded to a carrier protein (*75*).

As above mentioned, each allergen may have multiple epitopes (sequential or conformational) that, interacting with the immunosystem, can trigger hypersensitivity reactions in sensitive individuals. When the food allergens enter the mucosa barrier of the gastrointestinal tract they bind specific sites of IgE antibodies (bound on the surface of specific cells), and elicit the

release of mediators such as histamine, leukotrienes and prostaglandins. These molecules induce the immediate hypersensitivity reaction that causes inflammatory alterations.

It is important to note that if the structure of epitopes undergoes alteration, also its IgE antibody binding will be modified. So, the immunoreactive properties of allergens are affected by their three-dimensional conformational epitopes (*72, 93, 94*). Some allergens, such as some of vegetable origin, are rather labile and normally cause allergic reactions in the oral mucosa. They loose their allergenicity as a result of heat treatment or after digestion by enzymes. Other allergens are more stable, and can reach the mucosa of the gastrointestinal tract in immunologically active form. So the structure of antigenic epitopes can be specifically recognized by IgE antibodies. These types of epitopes (*structural epitopes*) usually consist of a few amino acids arranged in a linear order in the polypeptide chain. They are stable, because their allergenicity depends on the primary structure of the protein, and they are not affected by proteolytic cleavage. For this reason, they seem to be more involved in food allergies (*95*).

By contrast, the *conformational epitopes* are constituted by amino acid residues distant from each other in the linear sequence, but close together in the tertiary structure of the protein in its native form. Their structure is generally stabilized by weak bonds; therefore it is more labile and sensitive to the small changes of the native structure of the protein. Heat, enzymes or other treatments that a food undergoes to and that can modify the protein folding, may decrease or inactivate the allergenicity of the molecule. However, new epitopes previously hidden within the three-dimensional structure of the protein could be exposed after the protein unfolding.

Various studies describe cases of allergy to fruit and vegetables, but the most common allergic reactions are triggered by apple, peach and kiwi. Because fruit contains different types of allergens, clinical manifestations may range from mild oral symptoms, to skin reaction, rhinitis, and asthma, up to serious systemic reactions or anaphylactic shock (*96*). It is thought that the fruit allergy is mainly caused by a primary sensitization due to inhaled pollen. However, the gastrointestinal sensitization can also occur directly. This is possible only if the protein is able to resist to proteolysis in the digestive system (*96*).

ALLERGY TO APPLE

Apples, like other fruits belonging to the *Rosaceae* family, can cause the onset of allergic reactions in sensitive individuals (*97, 98*).

Therefore, it would be important to select apple cultivars with low allergens content (*99*), without neglecting the importance of the nutritional properties of the apples.

Up to 2% of the central European population suffer from allergic reactions to apples (*35*), but the geographic distribution of allergies manifestations shows different trends in the Northern hemisphere. In central and northern Europe symptoms are mostly mild, such as Oral Allergy Syndrome (OAS) and are mainly associated with allergy to birch pollen, whereas in Mediterrean European countries, such as Spain and Italy, fruits belonging to the *Rosaceae* family can induce severe allergic reactions (*96*).

Four main allergens have been identified in apple, and different clinical relevance depending on the geographical area is reported. According to the rules of the Committee for Allergen Nomenclature, approved by the World Health Organization and the International Union of Immunological Societies (WHO/IUIS), they are named: Mal d 1, Mal d 2, Mal d 3, and Mal d 4 (*97, 100*). According to the Allergen Nomenclature Sub-Committee of the International Union of Immunological Societies (IUIS), allergen names are given by abbreviating the scientific name of its source, and an Arabic numeral, so Mal d 1 is the first allergen described from apple (*Malus domestica*) (*101*). The main characteristics of apple allergens will be described below and they are summerized in Table 1.3.

MAL d 1

Mal d 1 is the major apple allergen that mainly triggers reaction in the northern and central European apple allergic population. It is a protein of about 17 kDa and belongs to the PR-10 (Pathogenesis-Related Proteins) group (*97*).

Major allergens are defined as “proteins to which more than 50% of patients with an allergy to its source react” (*102*).

Gao et al. (*103*) demonstrated that Mal d 1 is encoded by different gene families, and its expression depends on both biotic factors (e.g., pathogens) and abiotic factors (e.g., environmental stress, wounds) (*104*).

Mal d 1 is present both in the pulp and skin of the fruit (*35*), and its concentration is closely related to the apple cultivar (*105*). In fact, apple varieties are grouped in cultivar with high,

medium or low allergenic potential (*106*). It is not known the exact physiological role of Mal d 1, but it seems to be involved in the binding and transport of the plant steroids (*35, 97, 106*). Mal d 1 is thermolabile, unstable to digestion, and sensitive to oxidation, these characteristics explain why individuals allergic to Mal d 1 tollerate cooked apples (*107*). Garcia et al. (*105*) demonstrated that polyphenol oxidase (PPO) and peroxidase (POD) present in the fruit may reduce the allergenicity of Mal d 1. The mechanism of action is not completely elucidated, but the decrease of immunoreactivity is likely to be due to covalent interactions that cause changes in the tertiary structure of Mal d 1. In this way, the allergenicity is decreased or depleted because some epitopes can not be accessible to the IgE binding sites (*105*). As a result of this characteristic, allergic reactions caused by the ingestion of this protein are usually mild, such as itching of the mouth and swelling of the lips, also known as Oral Allergy Syndrome (OAS).

The use of antioxidants such as sulfite and / or ascorbic acid in food preparations could preserve the allergenicity of Mal d 1. Similarly, diethyldithiocarbamic acid (DIECA) seems to preserve the IgE-binding capacity of Mal d 1 by inhibiting reactions between proteins and phenolic compounds (*105*).

Mal d 1 shows a high sequence homology and structural similarity with the major allergen of birch pollen (Bet v 1) which is a member of the group of PR-10 protein (*104*). The OAS to apple seems to be mainly the result of sensitization to Bet v 1 (*81*) and to pollens of the *Fagaceae* family, that are able to give cross-reaction with Mal d 1. This phenomenon affects 70-88% of apple allergic patients of the central Europe (*107*).

MAL d 2

Mal d 2 is a Thaumatin-like Protein (TLP) of about 31 kDa, which belongs to the pathogenesis-related protein 5 (PR-5) family (*104, 108*).

It has homology with the birch allergen Bet v 2 (*104*). It is present in the ripe fruit, and is equally distributed in the pulp and peel, thus removing the apple peel can not avoid exposure to the allergen (*109*).

Mal d 2 is considered a minor allergen associated to the OAS (*108*). It is very resistant to proteases and heat treatments, thanks to the presence of eight disulphide bridges, which are essential for the protein folding and possibly for its allergenicity. Its resistance to heat

denaturation and gastrointestinal conditions suggests that Mal d 2 might trigger systemic symptoms (*109*).
It is involved in the defence of the plant, due to its antifungal and antibacterial activity and participates in the formation of extracellular polymers of the lipophilic surface (*100*).

MAL d 3

Mal d 3 is a not specific Lipid Transfer Protein (nsLTP) of about 9 kDa. It belongs to the family 14 of pathogenesis-related protein (PR-14), and it is involved in the transport of suberin monomers during synthesis of cutin (*97*).
The protein is highly resistant to thermal denaturation and digestion by pepsin, thanks to its four disulphide bridges, that possibly contribute to the mechanism of sensitization through the gastrointestinal tract (*107*). The protein was found especially in the apple skin and it is not associated with allergy to birch pollen. It mostly occurs in allergic patients in the Mediterranean area and can cause severe allergic reactions (*81, 110*).
It has been shown that the amount of Mal d 3 depends on the orchard, the apple variety, the maturity of the fruit, and the post-harvest treatments. In fact, Mal d 3 levels increase in the ripe fruit, but decrease during the storage of apples (*107, 110*).

MAL d 4

Mal d 4 is a minor allergen of about 12-15 kDa. This protein belongs to the group of profilins, and plays important roles in the plant development, such as cell elongation, flowering and cell development (*97*). Like Mal d 3, symptoms related to Mal d 4 are mainly prevalent in patients of the Mediterranean countries (*97*).
Profilins are considered as a minor allergen, but thanks to the 70-85% of amino acid sequence similarity with the profilins in other species, the possible cross-reactivity in allergic patients should not be neglected. Up to 20% of patients with food allergies and allergy to birch pollen can have IgE mediated response to profilins (*107*).
Mal d 4 is sensitive to heat treatment and enzymatic digestions, and these characteristics possibly cause mild symptoms related to the OAS (*107*).

		Characteristics
Allergens	**Mal d 1**	- Protein of about 17 kDa, present both in the flesh and in the peel of the fruit - Belonging to the PR-10 (Pathogenesis-Related Proteins) group - Sensitive to heat, oxidation and pepsin digestion.
	Mal d 2	- Protein of about 23 kDa, present in ripe fruit - Belonging to the group of Thaumatin-like proteins with antifungal activity - Very resistant to proteases and heat treatments
	Mal d 3	- Protein of about 9 kDa - A not-specific Lipid Transfer Protein
	Mal d 4	- Protein of about 12-15 kDa - Belonging to the group of Profiline

Table 1.3 Known apple allergens and their main characteristics.

ALLEGY TO FERMENTED BEVERAGES

Allergy-like symptoms after ingestion of alcoholic beverage are not usual experiences. The reaction can be very specific (e.g., for a sole type of wine), or can be caused by particular types of alcoholic beverage (like beer) and, in few cases, by a specific brand of beer (*111*).

"Allergy" to beer and wine may be essentially a form of alcohol intolerance that can cause unpleasant reactions immediately after the consumption of the drink. The most common manifestation or symptoms of intolerance to the fermented beverage are skin flushing and nasal congestion (*112, 113*). Conditions like these are sometimes inaccurately referred as "beer or wine allergy".

Intolerance to alcohol is rarely a consequence of genetic conditions in which the body shows a reduced metabolism of alcohol, more frequently it is an effect of drugs (or substances) that inhibit acetaldehyde dehydrogenase. Under this point of view, the only way to prevent adverse reaction is to completely avoid the consumption of alcoholic beverages. People with true intolerance to alcohol may react even to very small amounts of beer or wine (about 10 mL) which may provoke the appearance of severe rashes, sudden difficulty in breathing, painful stomach cramps or even collapse, although this condition (it is necessary to underline) is very rare (*114-116*).

True beer/wine allergy must be due to a specific immune reaction (IgE-mediated) to an ingredient in the beverage, such as a bio/chemical component (deriving from the fermented matrix), or a processing aid. Only occasionally, yeasts have been connected with allergic reactions caused by alcoholic beverages (*117*).

As above described, a true allergic reaction to fermented beverages involves the production of IgE antibodies, which cause the allergic reaction (*79*). It is more important to remember that alcohol *per se* could increase the risk of suffering from a severe allergic reaction. Alcohol indeed increases the gut permeability, which permits the passage of more intact (i.e., undigested) food molecules into the body.

LABELLING OF ALLERGENS IN EU REGULATION

As above described, food allergy is an emerging public health concern, and for this reason a large number of national agencies and intergovernmental organizations provide regulations focused to minimize the onset risk. The Food and Agriculture Organization of the United Nations joined with the World Health Organization (FAO/WHO) established a Commission in 1961 that drafted a collection of internationally recognized guidelines, codes of practice, and recommendations in relation to foods, food safety and production. This collection, named Codex Alimentarius, is an international point of reference for food safety and consumer protection.

Since FAO review estimated that eight allergenic foods are responsible of more than 90% of all food allergies (*118*), the food containing allergens that have to be labelled according to the Codex Alimentarius (*119*) are milk, eggs, fish, crustacean, peanuts, soybeans, tree nuts, and cereals (most specifically wheat) that contain gluten. Indeed, gluten intolerance is deemed as an allergy, even if it is not an IgE-mediated adverse reaction. Furthermore, sulphites at levels higher than 10 mg/kg must to be declared because they could elicit adverse effects on asthmatic people.

However, there are examples of other allergies to specific food, whose prevalence has a particular distribution in the different Countries. This is the case of buckwheat and rice allergy commonly found in Japan, and celery allergy in Switzerland/Austria (*76, 79, 84, 86, 120*).

This implies that the requirements for mandatory food allergen declarations are different across many Countries, where the allergen labelling lists are focused on the local prevalence patterns (*121*). For example, in Japan besides the allergenic foods listed in the Codex Alimentarius, buckwheat must be labelled if present in food. In the EU, scientific evidences

indicate that sesame seeds, mustard, celery and lupine are involved in allergic/adverse reaction occurring in people of some of the Member States. For this reason, the list of mandatory labelling allergens is longer than the Codex standard (*122-125*).

The presence of allergens in a food may derive from two potential sources: the allergen is a component intentionally added as ingredient (that must be necessarily labelled), or it is the result of an unintentional contamination due to shared equipment in different food preparations, use of processing aids containing allergenic products, or lack of expertise of the producer (*80*). A substance represents a "hidden allergen" when it is present but not clearly declared on the label of the product. Thus, the possibility for sensitive people to be inadvertently exposed to the offending food represents a cause of great concern.

However, some contaminations are virtually unavoidable even if "good manufacture practices" are applied. For example, it would be normal to find contamination by wheat grains in oats if the latter is harvested from fields where wheat was grown in precedence. Other substances used as processing aids, even if virtually absent by definition, could persist at trace level in the finished product. This is the case of the "exogenous proteins" used in the production of fermented beverages (*126*), such as pectolytic enzymes, that are used to enhance juice yield and prevent haze formation, proteins from animal origin (e.g., gelatines, egg-white proteins or milk caseins) that are exploited to clarify the beverage, and hen egg white lysozyme (HEWL) is used as a fermentation controller (*127-129*).

In winemaking, contaminations with potentially allergenic proteins are tolerated and/or regulated by national agencies and intergovernmental organization. Indeed, in EU Member States starting from the 2012 vintage wines produced using egg or milk as fining agents must state an allergen warning on the label, if their residues are under the detection and quantification limits of 0.25 mg/L and 0.5 mg/L, respectively, according to the OIV analytical techniques (*130, 131*). It must be noted that this regulation is exclusive for wine and not extended to other fermented beverages. Furthermore, this is an arbitrary limit. In fact, the dose-response assessment and the threshold dose to a given allergen cannot be established *a priori* (*132*), since cases of allergic reactions were elicited by few μg of proteins (*133*).

If it would not be possible avoid food contaminations by allergens, producers may declare on the food precautionary warnings for potential allergen cross-contamination, e.g., 'made in a factory which handles...' or 'may contain…'.

METHODS FOR THE STUDY OF ALLERGENS IN FERMENTED BEVERAGES

One of the aims of this PhD thesis was to determine the allergological risk related to cider consumption by individuals allergic to apple or to other compounds in cider. As a matter of fact, the effects of cidermaking on pontential allergens in the beverage are not described in literature. For this reason, this introductive paragraph necessarly refers to the study of other fermented beverages.

As a consequence of the large number of substances in fermented beverages, purification of allergens is usually difficult. Indeed, the protein extraction from recalcitrant matrices implies the use of denaturing and reducing conditions that could degrade the epitopes structure and thus modify the antigen-antibody binding.

The utopian extraction method should be suitable for all type of matrices. As a matter of fact, a common phosphate-buffered saline (PBS) is not efficient for complex solid samples. The most popular approaches to purify proteins from beverages involve chemical or physical methods (*134, 135*). Previous studies describe different methods to remove interfering compounds (phenols, carbohydrates or salts) or at least reduce their effect, that might hinder the qualitative and quantitative analyses of proteins and allergens (*136-140*). Moreover, it should be remembered that quantitative assays are achievable only if the extraction has been exhaustive.

Finally, dealing with fermented beverages, another problem in studying protein/allergens is represented by their low concentration. This implicates the need to perform a concentration step to increase the sensitivity of detection, and furthermore specific and accurate methods for the study of allergens should be set up. At the present, several methods for the detection of endogenous and exogenous proteins in fermented beverages have been reported in the literature, with detection limits ranging from few micrograms to several milligrams per litre (*126, 129, 141*).

Different analytical methods heve been developed for the quantitative and qualitative detection of allergens in foods. These methods could provide food companies with important data about allergen presence and concentration that are essential information for risk assessment and management. The most frequently used approaches are based on binding of allergen to specific antibodies. These methods include Enzyme-linked Immunosorbent Assay

(ELISA), Western Immuno Blot (WIB), Lateral Flow Assay (LFA). Other approaches for the allergen detection are represented by Polymerase Chain Reaction (PCR) method and Mass Spectrometry (MS) analysis. Immunological and PCR methods are currently commercialized and used by the food industry (*142*).

In the present PhD research thesis ELISA, WIB and MS analyses were used for the characterization of allergenic profile in the cider. Since the PCR-based method detects genes rather than protein, and it is not useful to analyse the allergological profile of a processed food, the approach has been rejected. LFA method is rapid, easy to perform, relatively inexpensive and based on antigen-antibody binding. Nevertheless, its limit of detection is higher than ELISA and WIB, thus it has not been used.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) AND WESTERN IMMUNO BLOT (WIB)

The food industry widely uses ELISA method for the detection of allergens (*143, 144*) for different reasons. It is relatively rapid, so it can be run during the food processing, thus providing a timely assessment of allergen risk, compatible with the Hazard Analysis and Critical Control Point (HACCP) in food manufacturing facilities. Its detection is specific and sensitive, because its detection limits are in the range of few μg/L (*145*), so it might guarantee the safety of the allergic consumers. It can be quantitative, but above all it gives information about the conservation of epitopes, which could be related to an immunological activity of the allergen.

In the common use, WIB technique is coupled with electrophoretic protein separation in denaturing condition (i.e., SDS-PAGE), thereby allowing the study sequential epitopes of allergens. In this way, this technique allows to evaluate the conservation of the primary structure of a given protein as indicated by its molecular weight.

MASS SPECTROMETRY ANALYSIS

Mass Spectrometry (MS) analysis is a method that rarely can be suitable for the food industry, because its equipment is expensive, and it needs of highly trained technicians. However, MS analysis generates large amount of data in a single run, making possible the simultaneous identification of different allergens.

MS approach is not based on an antigen-antibody interaction that could be influenced by food processing (*142*). As a matter of fact, in that case the sensitivity of methods such as ELISA decreases, owing to the protein modifications that make them undectable in the assay (*146*). MS analysis implies an enzymatic protein hydrolysis step, that generates peptides 15-20 residues long, and through the fingerprinting technique it is possible to identify the original protein. It is important to select the appropriate proteolitic enzyme, because food processing may alter (e.g., Maillard reaction) the clevage sites of protein. The most used enzyme is trypsin, that is very stable and active.

MS technique possesses high accuracy sensitivity, and reproducibility (*147*), thus allowing the detection of trace amounts of allergens and making the identification of a protein independent from its structure (*148, 149*). MS approach does not give information about epitopes conservation.

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CHAPTER 2

SET UP OF A PROCEDURE FOR CIDER PROTEINS RECOVERY AND QUANTIFICATION

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ABSTRACT

Cider contains low amount of proteins that, nonetheless, can affect its stability, foam formation and potential allergenicity. At present, scarce information is available on cider proteins, probably due to the lack of methods for their recovery and analysis.

The aim of the present study was to set up a method for recovering and quantifying cider proteins. To this purpose, the proteins from 13 Italian commercial ciders were recovered by dialysis, gel filtration, Trichloracetic acid/acetone (TCA/acetone) and Potassium-Dodecyl Sulfate (KDS) precipitation. The protein content of the samples was then determined by Bicinchoninic acid (BCA), Bradford and o-phthaldialdehyde (OPA) assays. The results were compared to quantitative data obtained by densitometry of electrophoretic gels. The most reliable protocol resulted the KDS method followed by OPA assay. KDS, in addition, allowed also to separate proteins from glycocompounds.

KDS/OPA is the method of choice for cider proteins precipitation and quantification.

Keywords: cider, protein recovery, protein quantification, KDS, o-phthaldialdehyde (OPA) assay

INTRODUCTION

Cider is a slightly alcoholic beverage (generally from 3 to 8% of alcohol volume) resulting from the fermentation of apple must. It is produced in more than 25 countries and it is widespread throughout the world (*1*). The main producers in Europe are the UK, Ireland, France and Northern Spain (*2*).

As other fermented beverages, cider is a complex matrix, whose main constituents are water, ethanol, sugars (mainly fructose), organic acids (essentially malic acid), phenolic and aromatic compounds (*3*). Minor constituents are proteins that might play different important roles in the product characteristics (*4-6*). Indeed, studies on other fermented beverages have shown that proteins are involved in the foam stability, can interact with aroma compounds (*7-9*), and are also responsible for the formation of hazes and sediments (*10-15*). It is well-known that proteins may undergo modifications (such as hydrolysis, denaturation and/or aggregation) during the food processing (*16, 17*). These physicochemical changes could modify not only the protein functional properties, but also their digestibility and potential allergenicity (*18*). For these reasons, it is important to have reliable methods to study cider protein profiles.

Because of the large number of compounds present in fermented beverages, purification and characterization of proteins usually represent a difficult task. Previous works report different methods to remove compounds, like phenols, carbohydrates or salts, that can interfere with the qualitative and quantitative analyses of proteins (*10, 19-22*).

The most common approaches to isolate proteins from beverages involve physical (i.e. dialysis, ultrafiltration and gel filtration) or chemical methods (e.g. precipitation by organic solvents, TCA, ammonium sulphate, sulphosalicylic or phosphotungstic acid) (*23*). Dialysis against water is a well-known non-denaturing method for purifying proteins (*7, 24, 25*). Nevertheless, it is time-consuming, as it takes days to purify proteins from large amounts of sample (*6*).

Ultrafiltration is a good alternative to dialysis, as it is faster, but it is based on membranes that can present scarce selectivity and are highly prone to clog when cloudy samples are used (*7, 24, 26*). In fact, Blanco et al. (*6*) reported that a filtration step is needed before performing ultrafiltration on cider, but this process cause a protein loss if proteins are aggregated. Detergent addition, such as urea or SDS might help protein recovery, but chaotropic agents persist in the samples and could affect further analyses (*6*). Protein purification can also be

performed by chromatography, but the purified protein fractions may need a buffer exchange and/or lyophilisation/concentration steps (*7*).

Chemical methods for proteins recovery are also feasible, but they present some limitations, for example, acetone precipitation requires a large volume of organic solvent, while the use of either TCA/acetone or TCA led to a less satisfying recovery than the acetone alone (*27*). Moreover, the low quality of the recovered proteins is probably due to the strong denaturing effect of TCA, that comes out in an irreversible shrinking and aggregation of proteins that can result in a protein pellet difficult to handle (*19*).

KDS method takes advantage of SDS that dissolves proteins and prevents interactions (e.g. with phenols), that could occur during cider protein concentration (*6*). The addition of potassium ions induces the protein-detergent insolubilization and allows their recovery (*28*). KDS method has been proven useful for protein recovery from fermented beverages as it overcomes the main drawbacks of other protein purification methods (*10, 19, 22, 29*).

As far as protein quantification is concerned, a wide range of techniques are available but frequently they are hampered by the presence of interfering compounds present in fruit juices and musts (*30*). Furthermore, the response of quantification methods (as an example the Bradford assay) is frequently biased in favour of particular classes of amino acids (*22, 31*) that could vary in their abundance in different proteins. This implies the need of appropriate protein standards. For wine protein quantification, as reported by Smith et al. (*20*) the use of invertase was proposed as a possible standard protein in the colorimetric assays, but for cider no standard is actually proposed.

Methods based on protein hydrolysis and amino acid analysis (e.g. Kjeldahl method) are not specific for protein and have limited usefulness for fermented beverages (*12*). Indeed, as reported by Ferreira et al. (*32*) the protein values obtained by nitrogen determination are not representative and are overestimated owing to the fact that fermented beverages contain a considerable amount of non-protein nitrogenous substances. Moreover, apples contain about 0.2% protein measured as crude (Kjeldahl) nitrogen, depending mainly on the age of the orchards and the amount of the fertilizers used (*33*) but just 50-80% of this nitrogen is derived from protein (*34*).

Finally, the o-phthaldialdehyde (OPA) method is described as a rapid, sensitive and convenient spectrophotometric assay. The reagent reacts with the primary amines, and is unaffected by local environment when proteins are denatured by SDS (*35*).

Similarly to quantification, other qualitative assays (e.g. electrophoresis) on proteins from wine, beer and cider are hindered by other compounds (as phenols) present in the beverages (*4, 36, 37*).

The aim of the present study is to set up a method for the recovery and quantification of cider proteins, in order to obtain samples useful for further studies such the physicochemical and immunological modifications that can occur during the cider making.

MATERIALS AND METHODS

Seven Italian ciders were purchased at the local market and six Italian commercial ciders were kindly supplied by L.M. di Maria Lucia Melchiori & C. s.n.c. (Tres, Trento, Italy) and Maley s.r.l. (Brissogne, Aosta, Italy). The thirteen ciders were: 1 still cider (no. 1), 9 sparkling ciders produced with the Charmat method (nos. 2-10) and 3 sparkling ciders produced by the Champenoise method (nos. 11-13).

Titratable acidity (expressed in g/L malic acid equivalents) and pH were measured for all ciders. Sugar content (fructose, sucrose, glucose) was measured using an enzymatic method (Megazyme Sucrose/Fructose/D-Glucose Assay Kit, Megazyme International, Ireland) and expressed as g/L of total sugar. Total polyphenols were quantified following the method described by Folin-Ciocalteau (*38*).

All the chemical reagents, where not specified, were purchased at Sigma-Aldrich (Steinheim, Germany).

Protein recovery procedures

Each cider sample was previously degassed in a ultrasonic bath Branson 5210 (Branson Ultrasonics, Danbury, CT, USA) for 30 min.

Proteins were recovered from each sample by using 4 different approaches, namely dialysis, gel filtration, TCA/acetone and KDS precipitation.

Dialysis. Ciders were dialyzed (3.5 kDa cut-off) (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) against distilled water at 4°C for 24 hours, and lyophilized.

TCA/acetone precipitation. Protein precipitation was carried out as described by Smith et al. (*20*). Two volumes of 10% TCA in cold acetone (-20°C) were added to 1 volume of cider. The mixture was incubated for 45 min at –20°C and centrifuged for 15 min at 21000 *g* at 4°C. The pellets obtained were washed once with cold acetone, centrifuged for 10 min at 21000 *g*

at 4°C and air-dried.

Gel filtration. Cider samples were loaded onto Sephadex G-25 desalting columns (GE Healthcare, Buckinghamshire, UK) with a 5 kDa cut off. According to the manufacturer's instruction, 2.5 ml of sample were loaded on the column. The proteins were eluted by gravity with 3.5 ml of 5% ethanol and lyophilized.

KDS precipitation. Proteins were recovered as described by Vincenzi et al. (*19*). Briefly, 10% (w/v) SDS was added to the ciders to a final concentration of 0.1% (w/v). Samples were then heated in boiling water for 10 min. 2M KCl was added to reach a final concentration of 200 mM and samples were gently mixed for 45 min at 4°C. KDS protein pellets were finally recovered by centrifugation for 15 min at 21000 *g* and 4°C.

Glycocompound recovery

The glycocompound fraction (KDS-A) was recovered by acetone precipitation from the supernatants obtained after KDS precipitation (*10*). Four volumes of cold acetone were added to each sample. After 30 min of incubation on ice, samples were centrifuged at 21000 *g* for 15 min at 4°C. The pellets obtained were air-dried.

SDS-PAGE, gel staining and densitometric analysis

Protein samples, recovered from a volume of 2 ml of each cider were solubilized in 0.2M Tris-HCl pH 8.2 containing 6M urea, 2% SDS, 8% glycerol, and 5% β-mercaptoethanol. SDS-PAGE (14% T) was carried out at 18 mA constant current until the tracking dye bromophenol blue reached the bottom of the gel. Low range molecular weight markers (Bio-Rad, St. Louis, MO, USA) were used. Proteins were revealed by fixing gels in 50% methanol and 10% acetic acid for 2 hours, then gels were incubated overnight with SYPRO Ruby stain (Bio-Rad), de-stained in 10% methanol and 7% acetic acid for 45 min and rinsed in distilled water (*39*). Carbohydrates were stained on electrophoretic gels by the Periodic Acid Schiff (PAS) method (*40*). In the present study, we named glycocompounds all compounds stained by PAS, while we defined glycoproteins all molecules resolved by SDS-PAGE and stained by PAS.

Digitalized images of the SDS-PAGE gels were acquired by a Chemi Doc XRS apparatus (Bio-Rad) and densitometric values were calculated with the Quantity One software (Bio-Rad). Densitometric titration curves were obtained using data from five different gels in

which increasing amounts of BSA (0.25 to 4 μg) were loaded. All experimental conditions, including staining and destaining steps, were the same as those used for cider proteins analysis.

Protein quantification

All colorimetric assays were performed on 96-wells microplates (Sarstedt, Nümbrecht Germany) and absorbances measured by a Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA) at the specific wavelength.

BCA and Bradford Assays. Protein samples were dissolved in water and concentrations were quantified using respectively the BCA™ Protein Assay kit (Pierce, Rockford, IL, USA) and a commercial kit based on the Bradford method (Sigma-Aldrich). Bovine Serum Albumin (BSA), in a range from 1 mg/ml to 31.25 μg/ml, was used as standard, and the absorbances at 562 nm wavelength were measured.

OPA Assay. The OPA solution was prepared immediately before use as described by Dinnella et al. (*41*). The following reagents were mixed and diluted to a final volume of 50 ml with distilled water: 25 ml of 0.1 M sodium tetraborate, 5 ml of 10% SDS, 100 μl of β-mercaptoethanol and 40 mg of OPA (dissolved in 1 ml of methanol). BSA, ranging from 4 mg/ml to 15.62 μg/ml, was used as standard. 200 μl of the reagent were added to 25 μl of each sample, and the absorbance at 340 nm was measured. Protein samples were dissolved in 6M urea.

Statistical Analysis

All measurements are presented as mean values ± SEM (standard error of the mean). Regression analysis was performed using the software SigmaPlot 8.0 (Systat Software, San Jose, CA, USA). Pearson correlation coefficient was calculated using the software GraphPad Prism 6 for Windows, (GraphPad Software, La Jolla, CA, USA). The statistical significance level was fixed at $P < 0.05$.

RESULTS AND DISCUSSION

The great variability of cider-making in Europe determines significant differences in the product characteristics.

For these reasons, we report in Table 2.1 the main chemical characteristics of the 13 considered cider samples.

No.	Ethanol % vol.	pH	Titrable acidity g/L eq malic acid [a]	Residual sugar g/L [a]	Total Polyphenols mg/L eq gallic acid [a]
1	8.0	3.36	5.14 ± 0.12	2.35 ± 0.20	204.5 ± 5.1
2	4.8	3.48	4.74 ± 0.17	2.11 ± 0.19	154.9 ± 9.0
3	7.0	3.38	5.19 ± 0.14	2.32 ± 0.15	260.8 ± 7.3
4	8.0	3.60	3.23 ± 0.17	2.36 ± 0.17	223.3 ± 7.0
5	5.5	3.44	4.57 ± 0.02	2.29 ± 0.31	366.7 ± 8.6
6	8.0	3.64	3.08 ± 0.19	2.06 ± 0.22	285.4 ± 13.7
7	6.0	3.51	4.86 ± 0.10	2.29 ± 0.18	322.7 ± 11.6
8	3.5	3.45	5.31 ± 0.02	2.33 ± 0.22	412.8 ± 6.0
9	5.6	3.52	4.64 ± 0.12	2.47 ± 0.15	469.0 ± 8.2
10	5.0	3.54	5.28 ± 0.07	2.07 ± 0.11	246.2 ± 8.2
11	7.5	3.42	5.46 ± 0.09	2.09 ± 0.21	372.7 ± 11.2
12	8.0	3.48	6.65 ± 0.17	2.23 ± 0.14	178.6 ± 2.0
13	7.0	3.71	3.12 ± 0.14	6.35 ± 0.25	414.0 ± 12.0

Table 2.1 Chemical composition of the selected ciders. Results are the means of three determinations. Data are expressed as mean values ± SEM. No.1: Still cider. No.2-10: Sparkling ciders Charmat method. No.11-13: Sparkling ciders Champenoise method.

Protein recovery and electrophoresis

A first attempt of protein concentration was conducted by centrifuging each cider with a Centricon YM-3 membrane (cut off of 3000 Da). The process was slow and inefficient as the membrane clogged, and for this reason, ultrafiltration has been abandoned.

The SDS-PAGE patterns of the dialyzed cider samples were generally not satisfying, revealing few and faint protein bands with scarce focalization (data not shown).

The electrophoretic profiles of TCA/acetone-precipitated samples showed a more efficient recovery of the proteins in comparison to the dialyzed ones, even though difficulties in the resolubilization of samples were observed as highlighted by the presence of smears and aggregates at the top of the gel (data not shown) (See also Mainente et al. 2014 (*37*)).

The proteins recovered by gel filtration gave well defined SDS-PAGE profiles. The almost total absence of smears and aggregates suggests that proteins recovered were probably not associated with interfering substances. The electrophoretic profile of each cider presented sharp and more intense bands than the previous two methods, with only faintly detectable smears (Figure 2.1S of the Supplementary Materials).

The pellets obtained by KDS precipitation were easily solubilized. The major drawback of the method is the persistence of dodecyl sulfate in the final samples that hampers certain analyses (e.g. the Bradford quantification method). However, the complete removal of the detergent is possible as previously described (*37, 42-44*). As observed for other fermented beverages, the KDS method seems to be the best choice for protein recovery from cider in term of electrophoretic resolution and number of protein bands (Figure 2.1).

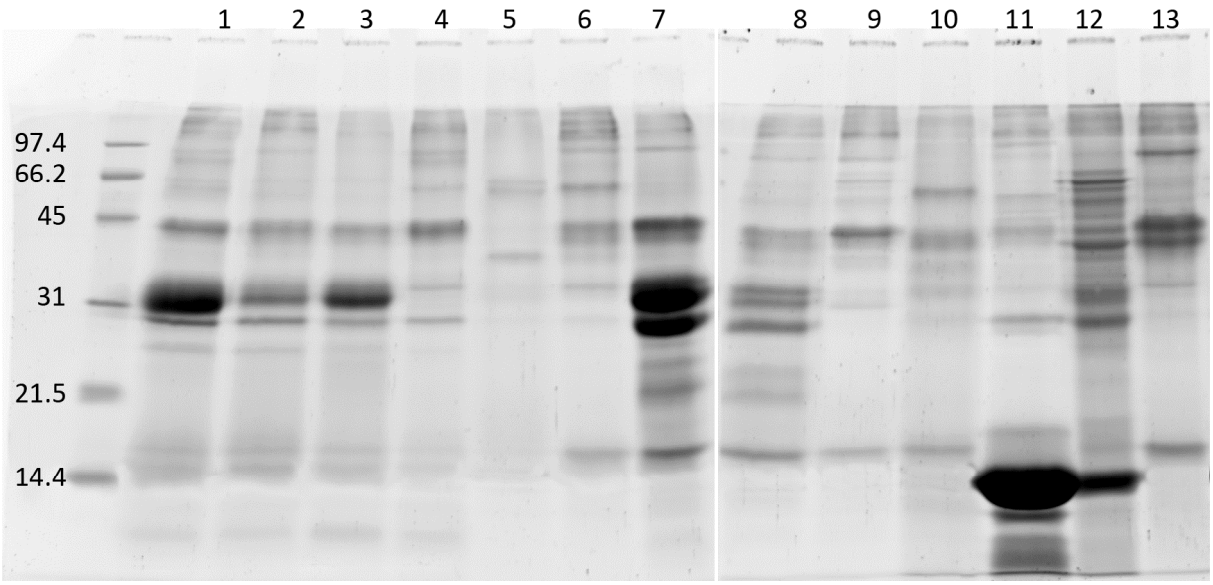

Figure 2.1 SDS-PAGE of cider proteins recovered by KDS, Sypro Ruby stained. Each lane was loaded with protein recovered from 2 ml of the sample. Lane 1: Still cider, Lanes 2-10: Sparkling ciders Charmat method, Lanes 11-13: Sparkling ciders Champenoise method. M.W.s are indicated on the left.

The quality of the electrophoretic profiles of KDS samples is comparable to that of recovered by gel filtration, but the intensity of the stained bands appears stronger thus indicating that KDS performs better than gel filtration. Protein bands observed were in the range of M.W.s of

those described by Blanco et al. (*6*), with the exception of the band at 14.4 kDa.

All samples (except no. 5, 11 and 12) were characterized by faint bands at about 14 kDa. Samples no. 1, 2, 3, 4, 7, 8 and 12 was mainly characterized by proteins with M.W.s between 30 and 45 kDa. Samples no. 5, 6, 9, 10 and 13 showed weak but clear bands at about 65 kDa. In addition, samples no. 11 and 12 showed a marked band below 14 kDa, and cider no. 11 presented further low M.W. proteins.

Generally, ciders produced with the Charmat method (Figure 2.1, lanes 2-10) showed a similar electrophoretic pattern even if the beverages were produced by different cellars. Moreover, these patterns resemble the one of the still cider (Figure 2.1, lane 1). On the contrary, the protein profiles of ciders produced by the Champenoise method (Figure 2.1, lanes 11-13) seem not to present bands in common, except for the ones below 14 kDa, previously described only in ciders no. 11 and 12.

KDS method is described to allow a differential recovery of glycocompounds from fermented beverages (*10, 29*) upon treatment of KDS supernatants with acetone (KDS-A). As described for other fermented beverages, KDS precipitated low amounts of glycoproteins from all cider samples.

All KDS samples, stained with PAS, showed signals at the top of the resolving gel, indicating the presence of slow migrating glycoproteins and a band at about 45 kDa. The SDS-PAGE pattern of the KDS-A samples showed high amounts of glycocompounds embedded in the stacking and at the top of the resolving gel, in particular in ciders method Champenoise. The SDS-PAGE of KDS-A samples stained with Sypro Ruby did not show any signals (data not shown). The presence of high amounts of glycocompounds could explain the scarce correlation among the values obtained with the protein quantification assays in the samples recovered by dialysis, gel filtration and TCA/acetone precipitation (see below). In fact, all protein samples recovered with the above mentioned methods showed to contain these glycocompounds (data not shown), that are acknowledged to be overestimated by BCA and underestimated by Bradford methods (*45*).

Quantification Assays

All the protein samples recovered using the different approaches described above (Dialysis, TCA/Acetone, Gel filtration and KDS) were quantified by Bradford, BCA and OPA assays. Bradford method is well-known to be incompatible with detergents, for this reason the data on

KDS proteins were omitted. The results reported in Table 2.2 showed a high degree of variation even within the samples

No.	DIALISYS			TCA/ACETONE			GEL FILTRATION			KDS		
	BCA	Bradford	OPA	BCA	Bradford	OPA	BCA	Bradford	OPA	BCA	OPA
1	680.43±68.69	17.48±6.49	63.78±2.49	37.65±2.48	5.61±2.00	6.70±0.95	507.61±54.63	4.77±0.92	64.59±1.15	410.64±7.62	59.15±4.29
2	689.68±60.78	13.16±4.98	43.58±3.10	31.64±1.25	5.25±2.33	2.76±0.59	284.72±20.92	8.36±0.79	80.95±5.18	311.5±16.22	27.83±6.87
3	976.10±75.32	15.54±8.25	34.75±1.47	24.50±2.56	1.91±1.00	4.42±0.52	128.68±6.91	10.29±1.17	63.65±1.89	249.18±9.46	36.75±4.26
4	864.68±97.84	38.94±20.39	41.48±2.18	23.07±3.01	6.26±1.82	1.40±0.41	191.76±14.06	17.29±2.83	234.07±2.86	267.85±2.48	30.62±5.13
5	827.48±27.66	29.12±9.35	38.03±3.05	18.85±1.32	5.29±1.60	2.25±0.51	187.16±1.47	1.49±0.40	57.95±3.64	215.±6.09	19.01±3.13
6	520.23±60.48	27.48±5.06	24.33±8.72	37.63±4.92	2.78±1.04	4.51±0.59	243.31±10.57	8.42±2.26	77.55±5.56	275.54±3.66	30.35±3.82
7	532.44±46.53	18.91±3.72	48.65±6.95	36.44±5.61	6.89±0.99	5.97±1.85	142.47±8.11	9.47±4.78	61.79±16.67	466.23±2.15	77.18±3.94
8	339.29±32.15	11.64±1.90	21.97±8.30	42.90±3.78	7.46±1.05	6.84±1.57	321.47±3.09	10.10±5.04	75.98±3.37	328.75±14.71	50.61±7.58
9	708.55±52.02	10.08±2.46	32.14±7.61	21.41±4.28	1.85±0.26	5.61±2.85	219.78±10.18	15.14±7.05	23.09±4.45	252.87±11.66	23±5.40
10	700.58±43.29	27.08±3.67	61.09±7.46	18.88±8.58	3.09±1.30	3.69±1.62	189.95±18.03	34.05±4.62	46.26±11.19	316.19±13.78	39.06±3.62
11	2871.24±77.46	23.22±4.84	39.99±2.29	26.17±2.87	7.36±1.73	19.36±1.86	204.69±7.39	33.87±2.66	120.10±7.81	341.62±4.24	76.05±3.5
12	604.85±80.51	15.79±2.89	42.52±8.25	28.07±7.29	10.47±3.12	20.52±5.64	425.25±17.99	4.042±2.76	54.43±5.56	444.82±9.37	56.07±4.73
13	808.25±53.24	12.95±3.39	61.51±12.76	24.61±7.53	10.26±2.25	3.72±1.82	615.16±17.38	1.49±0.59	63.45±8.37	377.55±10.29	62.58±4.81

Table 2.2 Protein quantification obtained with three different assays for each recovery method. Results are the means of six determinations. Data are expressed as µg/ml ± SEM. No.1: Still cider. No.2-10: Sparkling ciders Charmat method. No.11-13: Sparkling ciders Champenoise method.

obtained using the same recovery method. In particular, BCA assay gave values higher than those obtained by Bradford quantification. A high variability in protein contents were observed also when different recovery methods were used on the same cider sample. Both recovery and quantification methods determine the uncertainty of protein determination in ciders. To select the best combination of protein recovery and quantification methods we first resort to correlation analysis using the data measured for all the assayed ciders. The results are shown in Table 2.3.

Recovery Methods	Quantification Methods	Pearson r	P
DIALISYS	Bradford vs BCA	0.177	0.563
	Bradford vs OPA	0.025	0.935
	OPA vs BCA	0.026	0.932
TCA ACETONE	Bradford vs BCA	0.189	0.536
	Bradford vs OPA	0.486	0.092
	OPA vs BCA	0.100	0.746
GEL FILTRATION	Bradford vs BCA	-0.472	0.103
	Bradford vs OPA	0.238	0.433
	OPA vs BCA	-0.173	0.571
KDS	OPA vs BCA	0.835	**0.0004**

Table 2.3 Pairwise correlation of protein quantification data for each recovery method. Pearson correlation coefficient r was computed by pairwise comparison of data obtained using the indicated quantification methods on samples drawn from the 13 selected ciders. Significant correlation ($P < 0.05$) is indicated in bold.

Within the same recovery method (dialysis, TCA/acetone and gel filtration), no significant correlation between the results of the quantification assays was observed. This suggests that dialysis, TCA/Acetone precipitation and gel filtration recovery methods lead to protein samples that are contaminated by substances that interfere with the quantification methods. The quantitative data obtained by BCA and OPA assays on KDS proteins showed instead a high positive correlation ($r = 0.835$, $P=0.0004$; see Table 3). However, in KDS protein samples BCA assay returned values generally higher than those obtained by OPA (see Table 2.2).

It must be noted that an important factor, when choosing a specific protein quantification method, is the relative response of each assay, in relation to the relative abundance of amino

acid and glycosilation of proteins (*22*). As a consequence of the large assay-dependent variation, a wide range of protein content (from less than 1 mg/l to greater than of 1 g/l) has been reported for the same fermented beverage (*30*). This leads to the obvious question of which quantification assay is the most reliable. Moreover, the absence of a specific standard protein could provide an erroneous quantification. Bovine serum albumin is frequently used as reference in protein quantification of other fermented beverages (*22, 46*). Thus, we performed densitometric quantification of SDS-PAGEs of proteins recovered with the different methods, by using BSA as standard.

Even if densitometry is based on the evaluation of protein staining that might be affected by protein composition (*47*), the analysis of an electrophoretic profile represents an objective control of the quality of the recovered proteins in terms of solubility and aggregative state. In addition, the presence of a strong detergent and a reducing agent in SDS-PAGE allows the solubilization of almost all the proteins that can be recovered from a recalcitrant matrix like cider. A pairwise correlation of the quantitative data calculated by densitometry versus those obtained by spectrophotometric assays is shown in Table 2.4.

Recovery Methods	Quantification Methods	Pearson r	P
DIALYSIS	DENSITOMETRY vs BCA	-0.0443	0.934
	DENSITOMETRY vs Bradford	-0.0291	0.956
	DENSITOMETRY vs OPA	0.7662	0.076
TCA ACETONE	DENSITOMETRY vs BCA	0.0237	0.965
	DENSITOMETRY vs Bradford	0.6691	0.146
	DENSITOMETRY vs OPA	0.5796	0.228
GEL FILTRATION	DENSITOMETRY vs BCA	0.3716	0.468
	DENSITOMETRY vs Bradford	0.5936	0.214
	DENSITOMETRY vs OPA	-0.0088	0.987
KDS	DENSITOMETRY vs BCA	0.8934	**< 0.0001**
	DENSITOMETRY vs OPA	0.8981	**< 0.0001**

Table 2.4 Pairwise correlation between protein quantification values obtained by densitometry vs colorimetric methods for each recovery method. Pearson correlation coefficient r was computed by pairwise comparison of data obtained using the indicated quantification methods on samples drawn from the 13 selected ciders. Significant correlation ($P < 0.05$) is indicated in bold.

A high and significant correlation with densitometry measurements was observed for data obtained by OPA ($r = 0.8934$ and $P<0.0001$) and BCA ($r = 0.8981$ and $P<0.0001$) quantification of proteins recovered by KDS method.

Figure 2.2 shows how both BCA and OPA quantification methods on KDS protein samples perform with respect to densitometry. If we take densitometry as the reference standard of protein quantification, then it is clear that OPA performs much better than BCA that, on its turn, appears to overestimate the amount of proteins in KDS samples.

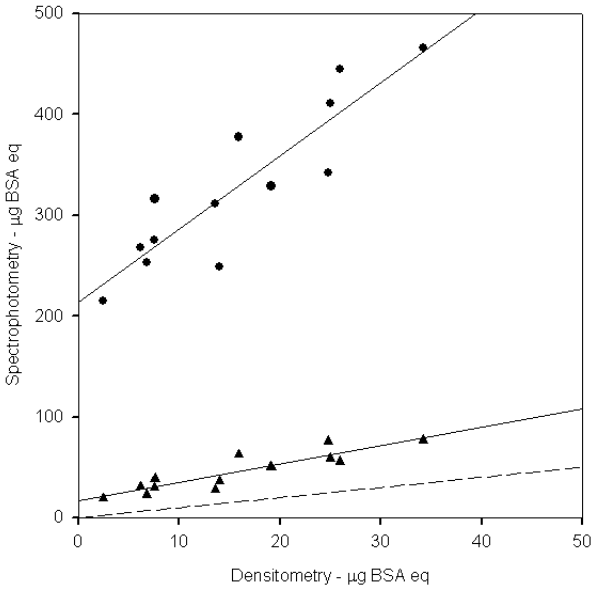

Figure 2.2 Experimental data by OPA (▲) and BCA (●) assays fitted with the densitometric values with a first-order polynomial line (solid lines), the dashed line represented the theoretical condition in which densitometric data perfectly correlate with the spectrophotometric values.

In any case, this result further confirms that the KDS method is an effective approach for protein recovery from cider as also described for other fermented beverages (*10, 19, 20, 29*).

CONCLUSIONS

Protein concentration in cider is very low, but these molecules can nonetheless affect product characteristics, such as foam formation and stability. In addition, it is well-known that apples contains proteins that can cause allergic reaction in sensitized individuals, a condition that affect 2% of the Northern and central European population (*48*). Finally, as already observed with wines (*49*), proteins from other sources can be used as processing aids during cider preparation and become exogenous hidden allergens. The development of suitable methods for protein recovery and analysis is therefore important to either improve knowledge on cider proteins and investigate on the presence of potential hidden allergens in ciders, and thus provide information to consumers at risk.

In the present paper different recovery and quantification methods were compared, and our findings point to KDS precipitation followed by OPA quantification as the most reliable method to study cider proteins.

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SUPPLEMENTARY MATERIALS

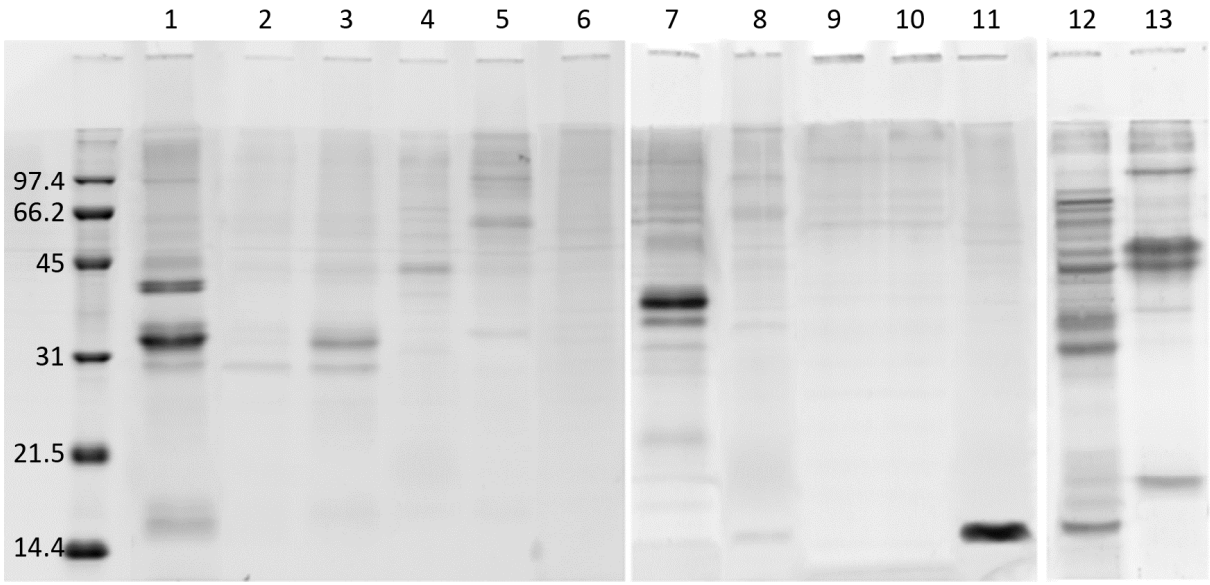

Figure 2.1S SDS-PAGE of cider proteins recovered by gel filtration, Sypro Ruby stained. Each lane was loaded with protein recovered from 2 ml of the sample. Lane 1: Still cider, Lanes 2-10: Sparkling ciders Charmat method, Lanes 11-13: Sparkling ciders Champenoise method. M.W. are indicated on the left.

CHAPTER 3

HEN EGG WHITE LYSOZYME IS A HIDDEN ALLERGEN IN ITALIAN COMMERCIAL CIDERS

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ABSTRACT

Hen egg white lysozyme (HEWL) is an enzyme used in alcoholic fermentations for its ability to control the growth of Gram-positive bacteria and spoilage bacteria, without inhibiting yeast growth, and it allows reducing the use of sulphur dioxide. Nevertheless, considering the potential allergenicity of this protein, the presence of HEWL should be declared on the label of the final product. In this work, we analysed 18 commercial Italian ciders by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), and found traces of HEWL in 12 samples without label declaration. We used western blot and Enzyme-linked Immunosorbent Assay (ELISA) to verify the immunological activity of HEWL, and to quantify its content in the ciders. Two out of 18 samples resulted positive both to Immunoblot and ELISA. Our results indicate the requirement of a more stringent control of the commercial ciders and the need of label declaration for ciders treated with such compounds.

Keywords: cider, LC-MS/MS, ELISA, western blot, lysozyme, hidden allergens, EU Regulation

INTRODUCTION

Hidden allergens in food represent a cause of great concern for sensitive people as they can be inadvertently exposed to the offending food and undergo to hypersensitivity reactions. A substance represents a "hidden allergen" when it is unrecognised or not declared on the product label. The unintentional intake of such substances can be a consequence of unaware allergens contamination by sharing equipment in different food preparations, by the use of processing aids containing allergenic products, or due to the lack of information on labels (*1*). For example, proteins from milk and egg represent potential risks for sensitive subjects, and are therefore included in the list of allergenic substances drafted by the European Union (Directive 2007/68/EC)(*2*) when used as food additives. These proteins are also used as processing aids during winemaking, and they could be considered as hidden allergens if residues remain in the wine.

Recently, many studies on the presence of hidden allergens in wine have been carried out (*3-6*), and changes in wine label regulations have been introduced (Regulation EU 579/2012) (*7*). So, starting from the 2012 vintage wines produced using egg or milk derivatives as fining agents must state an allergen warning on the label. As an exception to the new regulation, such processing aids could be undeclared if their residues are under the detection and quantification limits of 0.25 mg/L and 0.5 mg/L, respectively, according to the OIV analytical techniques (*8, 9*). Nevertheless, to the best of our knowledge, no studies have been conducted to detect hidden allergens in other alcoholic beverages.

In North America cider indicates a non-alcoholic juice of apple (*10*), however throughout Europe and in the rest of the world, cider refers to a slightly alcoholic beverage obtained from the fermentation of apple juice. When cider is produced on industrial scale, different additives and processing aids are used, similarly to what occurs in the winemaking process. Pectolytic enzymes are used to enhance juice yield and prevent haze formation, fining agents such as proteins from animal origin (e.g., gelatines, egg-white proteins or milk caseins) are exploited to clarify the beverage, and hen egg white lysozyme (HEWL) is used as a fermentation controller (*3, 11, 12*). These substances could persist in the final product. For this reason, investigations on the presence of hidden allergens in ciders are important to avoid risks of allergic reactions to sensitized consumers, and to evaluate the need for improvements in labelling regulations.

The present study aimed at assessing the presence of hidden allergens in commercial Italian ciders by exploiting the potentials of liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and related proteomic techniques. This approach possesses high sensitivity, accuracy, and reproducibility, and permits the detection of a large number of proteins simultaneously, even if they are present in trace amounts. However, protein identification by LC-MS/MS is independent of the protein structure, and the real immunological activity (which might be important for allergen analysis) of the target protein is then not considered (*4*). For this reason in the present study, ciders were also analysed both by Enzyme-linked Immunosorbent Assay (ELISA) and immunoblotting.

MATERIALS AND METHODS

Eighteen Italian ciders of 11 different brands were purchased at a local supermarket. The samples were: 1 still cider (no. 1), 14 sparkling ciders produced with the Charmat method (nos. 2-15), and 3 sparkling ciders produced by the Champenoise method (nos. 16-18).
All chemical reagents, if not specified, were purchased from Sigma-Aldrich (Steinheim, Germany) and where of MS grade.

Protein recovery

Proteins of each cider were recovered as described by Mainente et al. (*13*). Briefly, to remove carbon dioxide samples were degassed in an ultrasonic bath Branson 5210 (Branson Ultrasonics, Danbury, CT, USA) for 30 min. SDS was added to a final concentration of 0.1% (w/v). After boiling samples at 100 °C for 10 min, KCl was added to reach a final concentration of 200 mM. Samples were gently mixed for 45 min at 4 °C, and proteins were recovered by centrifugation for 15 min at 21000 *g* and at 4 °C.

Analytical and preparative SDS-PAGE

Protein samples obtained from a volume of 5 mL of each cider were solubilized in 50 µL of 0.2M Tris-HCl pH 8.2 containing 6M urea, 2% SDS, 8% glycerol, 5% β-mercaptoethanol, and bromophenol blue as tracking dye. SDS-PAGE at 14% polyacrylamide was carried out at 18 mA constant.

In the analytical method SDS-PAGE was performed until the tracking dye reached the bottom of the gel. Gels were stained with Sypro Ruby (Bio-Rad Laboratories, St. Louis, MO, USA) according to the manufacturer's instructions, or used for the western blot assay.
In the preparative protocol proteins were allowed to enter the gel for approximately 1 cm, and a single gel band of about 10 mm^2 was excised from each lane. Samples were stored at -20 °C for the LC-MS/MS analysis.

In-gel digestion and LC-MS/MS analysis

Proteins were digested in gel as described by Gerotto et al. (*14*). Briefly, gel pieces were dehydrated with acetonitrile (ACN) and dried under vacuum. 150 µL of 10 mM dithiothreitol (DTT) in 50 mM NH_4HCO_3 were added to each sample and proteins were reduced for 1 h at 56 °C. The solution was discarded and alkylation was performed by adding 150 µL of 55 mM iodoacetamide in 50 mM NH_4HCO_3 for 45 min at room temperature and in the dark.
Samples were washed twice with 200 µL of 25 mM NH_4HCO_3 and then with 200 µL of ACN and dried under vacuum. In-gel digestion was performed overnight by adding to each sample 25 µL of sequencing grade modified trypsin (12.5 ng/µL in 50 mM NH_4HCO_3, Promega Corporation, WI, USA) and 25 µL of 25 mM NH_4HCO_3. Peptides were extracted from the gel by 3 passages of 200 µL of 50% (v/v) ACN/0.1% formic acid. Samples were dried under vacuum and stored at -20 °C until LC-MS/MS analyses were performed.
Each sample was suspended in 15 µL of 3% ACN/0.1% formic acid and 5 µL were injected into a pico-frit column (New Objective) packed in house with C_{18} material (Aeris Peptide 3.6 mmXBC18; Phenomenex, CA, USA) with a nano-HPLC Ultimate 3000 (Dionex – Thermo Fisher Scientific, MA, USA) coupled to a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific). Peptides were separated with a linear gradient of ACN/0.1% formic acid from 3% to 40% in 20 min at a flow rate of 250 nL/min. The instrument operated in a data dependent mode acquisition, with a full scan from 300 to 1700 Da at high resolution (60000) in the Orbitrap, followed by MS/MS scans on the 10 most intense ions acquired in the linear ion trap. To avoid carry over, a blank injection with the same instrumental and chromatographic conditions was performed after the analysis of each cider sample.

Protein identification

Raw data files were analysed with the software Proteome Discoverer 1.4 (Thermo Fisher Scientific) interfaced to a Mascot server (version 2.2.4, Matrix Science) against a Swissprot database (version February 2016) with no taxonomy restriction (550552 entries). Peptide and fragment tolerance windows were set to 10 ppm and 0.6 Da respectively, and 1 missed cleavage was allowed. Carbamidomethyl cysteine was set as fixed modification and methionine oxidation as variable modification. The algorithm Percolator was used to assess the false discovery rate (FDR) using a randomized database and results were filtered to consider only proteins identified with at least 2 unique peptides with high confidence ($q < 0.01$). Proteins were grouped into families, according to the principle of maximum parsimony. Blank samples were analysed in the same way and no significant carry over was observed.

Western blot and ELISA

Proteins separated by SDS–PAGE were electroblotted in a tank apparatus (Elettrofor S.A.S., Rovigo, Italy) using 0.2 µm pore-size nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK) at constant voltage (20 V for 16 h). The membrane was blocked with Phosphate Saline Buffer (PBS) containing 3% skimmed milk and 0.05% Tween 20 (Buffer 1), and immunodetection was carried out for 2 h with a polyclonal IgG anti-lysozyme (hen egg white) developed in rabbit (Rockland Immunochemicals Inc., PA, USA) diluted 1:5000 in Buffer 1. As secondary antibody an anti-rabbit IgG HRP-conjugated was used at 1:5000 dilution in Buffer 1. Chemioluminescence was revealed by luminol (Millipore Corporation, MA, USA), and the signals were acquired by a ChemiDoc XRS apparatus (Bio-Rad).

ELISA was performed using the RIDASCREEN®FAST Lysozym kit (R-biopharm, Darmstadt, Germany) according to the manufacturer's instructions. Absorbance was measured by a microplate reader (Bio-Tek Instruments, Winooski, VT, USA) at 450 nm.

RESULTS AND DISCUSSION

Proteins recovered from a panel of 18 commercial Italian ciders were analysed by LC-MS/MS as specified above. All information regarding proteins and peptides identification is reported as supplementary material (Tables 3.S1-S18 of the Supporting Materials). In general, amount and type of proteins present in each cider are variable, with some contaminating proteins (namely human keratins) being almost invariably the dominant species in the samples. Whether these contaminants are really present in the ciders or they derive from sample preparation and manipulation procedures it cannot be established on the base of the present results. However, the fact that in some ciders the level of contaminant proteins appears to be much higher than in other samples suggests that (at least partially) the source of contamination could be located during the phases of apple harvest and cider production.

It must be highlighted that contamination by human keratins is extremely common in proteomics experiments, overall when gel-based procedures are adopted (*15*). The presence of keratins in other types of beverages (e.g. wine) has been rarely documented (*5*). However in most of the cases keratins tend to be considered as contaminants that are introduced in the sample during the processes of sample preparation and manipulation and are therefore usually discarded from the final list of identified proteins (*16, 17, 17-19*). To assess whether these proteins were originally present in the samples would require different and more complex types of investigation. Beside contaminant proteins of human origin, several other proteins could be identified belonging to apple (*Malus domestica*), yeast (*Saccharomyces cerevisiae*), bacteria (*Lactobacillus*, *Oenoccocus*), and mould (*Aspergillus*). Obviously the presence of proteins from apple had to be expected, and the existence of proteins from the other microorganisms is not surprising, as they are involved in alcoholic fermentation (*Saccharomyces*) and in malolactic fermentation (*Lactobacillus*, *Oenoccocus*), which is sometimes desirable to decrease cider acidity (*20*). The presence of proteins derived from *Aspergillus* can be justified considering that pectolytic enzymes (extracted by this fungus) are added during the cider production process to enhance apple juice yield and prevent haze (*11*). Among the proteins from *Malus domestica* that are more frequently identified, there are Thaumatin-like proteins (Mal d 2) and non-specific Lipid-Transfer Protein (Mal d 3). These are well-known apple allergens, involved in the systemic allergic reactions, being resistant to gastric digestion and thermal treatments. Pathogenesis-Related (PR) 10 proteins (Mal d 1) and Profilins (Mal d 4) are also known apple allergens, but they have not been detected in the

samples. These proteins, described as thermolabile and prone to proteolysis (*21*), are probably degraded during the cidermaking process.

It is of particular interest to note that, quite unexpectedly, 12 out of the 18 ciders analysed in the present study show the presence of peptides belonging to HEWL (Table 3.1).

Cider	LC-MS/MS	ELISA	western blot
1	+	-	-
2	+	-	-
3	-	-	-
4	+	-	-
5	+	-	-
6	+	-	-
7	+	-	-
8	+	-	-
9	-	-	-
10	+	-	-
11	-	-	-
12	-	-	-
13	+	-	-
14	+	-	-
15	-	-	-
16	+	+	+
17	+	+	+
18	-	-	-

Table 3.1 Ciders positive to HEWL detection with LC-MS/MS, ELISA and western blot techniques.

HEWL is a natural enzyme used in the food industry for its lytic activity on Gram-positive bacteria and it does not inhibit yeast growth (*22*). It represents therefore an advantageous method to prevent undesirable fermentation allowing to reduce the use of sulphur dioxide (*22*). It must be noted that Italian ciders are mainly produced with dessert apples, so that the final product is usually characterized by low acidity. For this reason malolactic fermentation (desirable in Spanish and French ciders) is avoided in the Italian cidermaking, since this reaction converts malic acid into lactic acid, aiming at decreasing the acidity of the final product (*10, 20, 23*). The main technological interest in exploiting the antibacterial properties of lysozyme is the possibility to reduce the traditional use of sulphur dioxide, which can cause health concerns in consumers (*24, 25*). Although the use of HEWL as food preservative is permitted in the European Union and in other countries (EU Regulation 2066/2001), (*26*) HEWL is also described as an allergen (Gal d4) that contains both sequential and conformational epitopes (*27-29*). Aabin et al. (*30*) indicate a frequency of reactivity of 15% in adults with confirmed egg allergy, and Frémont et al. (*31*) describe the presence of anti-

lysozyme IgE in 35% of the egg-allergic patients. For this reason, even if HEWL is admitted in food products, its presence as hen egg derivatives must be declared (EU Regulation 1169/2011) (*32*), because it could represent a risk for the allergic consumers. Exception is represented by wine, which undergoes a different regulation (Regulation EU 579/2012) as mentioned above.

In the present study the identification of HEWL (like all other proteins) was based on the detection of at least two unique peptides identified with high confidence, indicating a good level of reliability. Moreover, to avoid any possible cross-contamination effect, each sample had been prepared using new dedicated vials for ultra-centrifugation. Furthermore, to avoid possible carry over effect on the chromatographic column, after the analysis of each cider a blank injection was performed, and a complete LC-MS/MS analysis was carried out under the same instrumental and chromatographic conditions. In none of the blanks we could identify HEWL (data not shown), thus clearly indicating that, when present in the cider, this enzyme cannot be attributable to a cross contamination.

The identification of HEWL in the large majority of ciders tested (12 out of 18) prompted us to apply a quantitative method to determine the amount of enzyme present in the different samples. We therefore used an ELISA kit (RIDASCREEN®FAST Lysozym) that is routinely exploited for the detection of lysozyme in wine. The declared LOD and LOQ for this method are 0.02 mg/L and 0.05 mg/L, respectively. According to the OIV Resolution, HEWL concentration in wine higher than 0.25 mg/L must be declared (*9*). However, it must be noted that this is an arbitrary limit. In fact, the dose-response assessment and the threshold dose to a given allergen cannot be established *a priori* (*33*), since cases of allergic reactions were elicited by few μg of proteins (*34*). Nevertheless, the average alcoholic degree of wine should *per se* represent a reasonable limit for the assumption of the possible allergens. Cider instead is characterized by a lower alcoholic degree with respect to wine, so it might be expected that a consumer could drink larger quantities of cider than wine. This would result in a higher HEWL intake, thus the admitted HEWL concentration limit should be proportionally reduced. It is important to note here that cider, differently from wine, does not represent an exception to the EU Directive 2007/68/EC, therefore the presence in it of egg derivatives should have been labelled even if HEWL is present at concentrations lower than 0.25 mg/L.

By applying this approach to the 18 Italian commercial ciders we found that in cider n. 16 and cider n. 17 (both obtained by the Champenoise method by different producers) the

concentration of HEWL was 2.0 and 0.2 mg/L respectively, while for all other samples the amount of lysozyme was below the limit of detection (0.02 mg/L). Thus, our results indicate that HEWL can escape detection if the official method adopted for wine is applied to ciders. We must point out that for the LC-MS/MS analysis we recovered the proteins starting from 5 mL of cider while, according to the manufacturer's instruction, the ELISA test (which does not require a protein concentration step) was performed on samples diluted 1:20 and 5 µL was the volume of cider effectively used for the ELISA. Considering that probably part of the proteins are lost during our recovery procedure and that only 1/3 of the processed sample is injected into the column for the LC-MS/MS analysis, we can speculate that our approach allows to analyse about 300 times higher amount of proteins with respect to the standard ELISA. This might explain why our approach led to the identification of HEWL in many more samples than the ELISA. However, sandwich ELISA allows quantifying proteins with at least two epitopes conserved, which can be related to an immunological activity. As a matter of fact, in the allergic reaction it is necessary that allergens bind two or more IgE molecules linked to the FcεRI, to determine the massive release of mediators by the stimulated cells (*35*). On the contrary, LC-MS/MS analysis can detect small fragments of proteins that might have no immunological activity.

The western blot technique was performed to study the immunological activity of sequential epitopes of HEWL, that are described to be important for allergenicity of hen egg white proteins (*27, 29*). At the same time this technique allows to evaluate the conservation of the primary structure of the protein as indicated by its molecular weight.

Figure 3.1 shows the results of the western blot analysis for the immunodetection of HEWL in the cider samples. Since it was immediately clear that the presence of HEWL was confirmed in ciders 16 and 17, the membrane was purposely overexposed (at the cost of loss in linearity) in an attempt to reveal the presence of traces of HEWL in the other samples. This explains the lack of correspondence between the concentration of HEWL measured by ELISA and the relative intensity of the bands visible in Figure 3.1.

It must be noted that protein samples loaded in each lane correspond to 5 mL of cider, i.e., about 3 and 1000 times higher than those analysed by LC-MS/MS and ELISA, respectively. In lane C+ it can be observed the signal of the positive control, while in lanes 16 and 17, the ones of ciders positive to ELISA. Presence of HEWL has not been detected in any of the other samples. The apparent molecular weight of the bands is 14.4 kDa, corresponding to the

conserved primary structure of HEWL. This means that almost all the sequential epitopes of HEWL are maintained in the ciders, suggesting a possible risk for the allergic consumers.

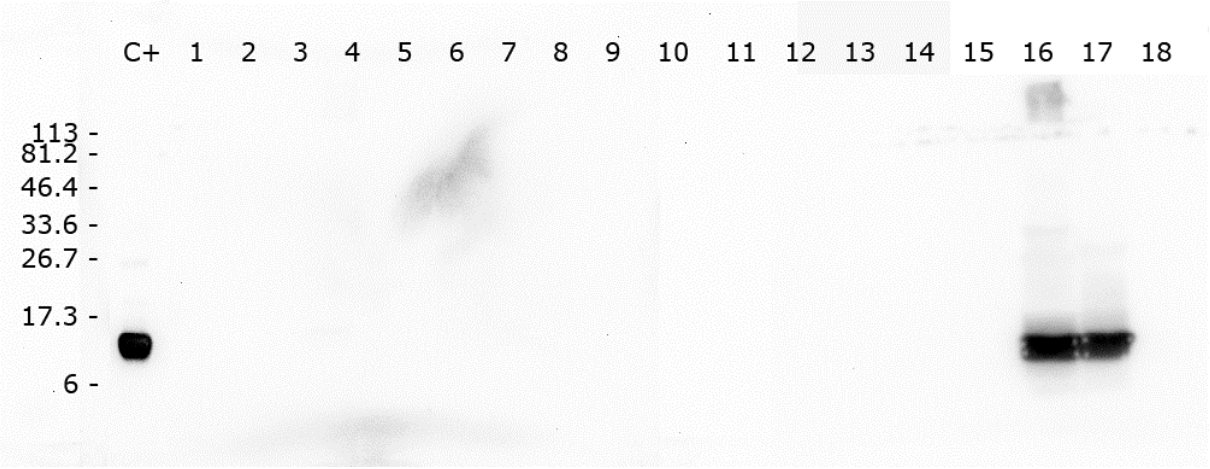

Figure 3.1 Western blot analysis of cider proteins. For SDS-PAGE 14% each lane was loaded with protein recovered from 5 mL of sample. Immunoblotting was developed with anti-lysozyme (hen egg white) polyclonal antibody. C+: positive control. Lane 1: still cider, lanes 2-15: sparkling ciders Charmat method, lanes 16-18: sparkling ciders Champenoise method.

Among the analytical methods exploited in this study, LC-MS/MS appears to be the most sensitive technique for the detection of trace amount of HEWL, even if it does not give information about the immunological activity of allergens, and it is not a quantitative approach in the way it was used in the present study. For this reason, we believe that the application of quantitative MS-based methods such as Single Reaction Monitoring (SRM) on triple quadrupole type of instruments is the best choice to detect and quantify hidden allergens in food. Moreover, the possibility to use high mass accuracy/high resolution (HM/HR) instruments with a data-independent acquisition strategy (such as Parallel Reaction Monitoring, or SWATH acquisition) might represent the best trade-off between rigorous quantitative data and the possibility to identify even unsuspected hidden allergens.

Going back to the presence of HEWL in the samples, it would be important to identify if this enzyme is deliberately introduced in ciders during the making process, or if it can rather be considered as a contamination of which the producers might not be aware of. For the two samples in which the amount of HEWL was measured to be above the LOD of the ELISA (ciders 16 and 17), we can speculate that the enzyme was intentionally added by the producers to limit or avoid the use of sulphur dioxide. The omission by the producers to indicate the presence of HEWL in the label could be due to a lack of information regarding the substances listed as allergens by the European Commission or other Agencies. In the other cases, when

LC-MS/MS analysis identified HEWL below the LOD of ELISA, it is conceivable that it was unintentionally added to the final product, probably as a consequence of contamination by sharing equipment in different beverage preparations, or by the use of processing aids containing allergenic products (*1*). As mentioned above, cidermakers and winemakers often share the same processing aids, for this reason contamination of HEWL might be unavoidable. This might result in the need of including the cider in the EU Regulation 579/2012, which excluded wine from mandatory labelling if HEWL concentration is lower than 0.25 mg/L. But it must be noted that cider is a low alcohol beverage, so the consumer could assume higher amount of HEWL than drinking wine. This implicates the need of specific regulations or alternatively much sensitive detection methods for hidden allergens in cider.

In conclusion our work shows the widespread presence (often at trace level) of HEWL in Italian ciders, suggesting the need for official methods for allergens detection. An implementation of the current regulations would be desirable to allow cidermakers and Authorities to safeguard the consumers' health and the quality of the product.

Acknowledgement

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SUPPLEMENTARY MATERIALS

Table 3.S1-S18: list of proteins identified by LC-MS/MS in commercial Italian ciders. Each sheet refers to a different commercial cider (samples 1-18). For each protein the score, the number of peptides and the sequence coverage are reported, together with the list of all peptides detected, the confidence of their identification, the amino acid sequence, and the Mascot score.

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
Q9FSG7	Thaumatin-like protein 1a OS=Malus domestica GN=TL1 PE=1 SV=1 - [TP1A_MALDO]	968,46	48,78	1	3	7	42	246	25,7	5,15
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	645,74	25,47	1	12	13	16	644	66,0	8,12
P83336	Thaumatin-like protein 1b (Fragment) OS=Malus domestica PE=2 SV=1 - [TP1B_MALDO]	639,85	41,98	1	2	6	30	212	22,1	4,54
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	536,59	27,74	1	15	15	17	584	58,8	5,21
P00698	Lysozyme C OS=Gallus gallus GN=LYZ PE=1 SV=1 - [LYSC_CHICK]	524,20	60,54	1	7	7	13	147	16,2	9,07
P00830	ATP synthase subunit beta, mitochondrial OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=ATP2 PE=1 SV=2 - [ATPB_YEAST]	517,26	19,77	1	7	7	14	511	54,8	5,71
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	419,53	22,85	1	12	13	17	639	65,4	8,00
Q6CQ83	Heat shock protein SSB OS=Kluyveromyces lactis (strain ATCC 8585 / CBS 2359 / DSM 70799 / NBRC 1267 / NRRL Y-1140 / WM37) GN=SSB PE=3 SV=3 - [HSP75_KLULA]	377,95	11,26	1	3	5	8	613	66,2	5,43
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	356,81	14,61	1	6	6	8	623	62,0	5,24
A2R3I1	Probable pectin lyase A OS=Aspergillus niger (strain CBS 513.88 / FGSC A1513) GN=pelA PE=3 SV=1 - [PELA_ASPNC]	336,56	14,51	2	4	4	8	379	39,8	4,34
P87222	Heat shock protein SSB1 OS=Candida albicans (strain WO-1) GN=SSB1 PE=3 SV=2 - [HSP75_CANAW]	316,35	10,60	1	2	4	7	613	66,4	5,38
P49375	ATP synthase subunit alpha, mitochondrial OS=Kluyveromyces lactis (strain ATCC 8585 / CBS 2359 / DSM 70799 / NBRC 1267 / NRRL Y-1140 / WM37) GN=ATP1 PE=1 SV=1 - [ATPA_KLULA]	270,10	11,31	1	5	5	7	548	59,1	9,36
P10591	Heat shock protein SSA1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=SSA1 PE=1 SV=4 - [HSP71_YEAST]	252,74	8,88	5	5	5	8	642	69,6	5,11
P00761	Trypsin OS=Sus scrofa PE=1 SV=1 - [TRYP_PIG]	210,13	16,45	1	3	3	6	231	24,4	7,18
P0CY35	Elongation factor 1-alpha 1 OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=TEF1 PE=3 SV=1 - [EF1A1_CANAL]	203,29	14,63	2	5	5	9	458	50,0	9,03
Q874B9	Elongation factor 2 OS=Komagataella pastoris GN=EFT1 PE=3 SV=1 - [EF2_PICPA]	182,91	6,89	1	4	4	4	842	93,4	6,71
Q07421	Plasma membrane ATPase OS=Ajellomyces capsulatus GN=PMA1 PE=3 SV=1 - [PMA1_AJECA]	153,15	1,75	1	2	2	2	916	98,8	5,43
P27080	ADP,ATP carrier protein OS=Chlamydomonas reinhardtii GN=ABT PE=2 SV=1 - [ADT_CHLRE]	145,58	7,79	1	2	2	3	308	33,5	9,74
P15703	Glucan 1,3-beta-glucosidase OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=BGL2 PE=1 SV=1 - [BGL2_YEAST]	142,65	16,93	1	4	4	4	313	34,1	4,51
Q756H2	Enolase OS=Ashbya gossypii (strain ATCC 10895 / CBS 109.51 / FGSC 9923 / NRRL Y-1056) GN=ENO PE=3 SV=1 - [ENO_ASHGO]	132,29	7,55	1	2	2	3	437	46,6	5,55
Q08193	1,3-beta-glucanosyltransferase GAS5 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS5 PE=1 SV=1 - [GAS5_YEAST]	119,68	6,40	1	2	2	2	484	51,8	4,64
O74258	Actin OS=Ogataea parapolymorpha (strain DL-1 / ATCC 26012 / NRRL Y-7560) GN=ACT PE=3 SV=2 - [ACT_OGAPD]	109,69	21,01	6	6	6	7	376	41,7	5,57
Q92211	Glyceraldehyde-3-phosphate dehydrogenase OS=Candida albicans (strain WO-1) GN=TDH1 PE=1 SV=2 - [G3P_CANAW]	107,38	11,64	1	2	3	4	335	35,8	7,12
O13639	Adenosylhomocysteinase OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=pi047 PE=3 SV=1 - [SAHH_SCHPO]	104,24	5,54	1	2	2	2	433	47,4	5,94

Table 3.S1

Q12230	Sphingolipid long chain base-responsive protein LSP1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=LSP1 PE=1 SV=1 - [LSP1_YEAST]	103,02	7,92	1	2	2	2	341	38,0	4,70
P00890	Citrate synthase, mitochondrial OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=CIT1 PE=1 SV=2 - [CISY1_YEAST]	101,44	6,68	1	2	2	2	479	53,3	8,29
P0CS90	Heat shock protein SSC1, mitochondrial OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=SSC1 PE=1 SV=1 - [HSP77_YEAST]	100,57	4,59	3	2	2	2	654	70,6	5,59
P18819	NADP-specific glutamate dehydrogenase OS=Emericella nidulans (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) GN=gdhA PE=3 SV=2 - [DHE4_EMENI]	97,71	6,75	1	2	2	2	459	49,6	6,47
Q9M5X7	Non-specific lipid-transfer protein OS=Malus domestica GN=MALD3 PE=1 SV=1 - [NLTP_MALDO]	93,33	24,35	1	2	2	2	115	11,4	8,92
A6ZL22	Cell wall protein ECM33 OS=Saccharomyces cerevisiae (strain YJM789) GN=ECM33 PE=3 SV=2 - [ECM33_YEAS7]	82,45	5,83	4	2	2	2	429	43,7	4,91
O59841	Glyceraldehyde-3-phosphate dehydrogenase OS=Ogataea parapolymorpha (strain DL-1 / ATCC 26012 / NRRL Y-7560) GN=GPD PE=3 SV=2 - [G3P_OGAPD]	80,80	10,45	1	2	3	3	335	35,9	7,17
O75000	60S ribosomal protein L12 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=rpl1201 PE=1 SV=1 - [RL12_SCHPO]	71,21	14,55	1	2	2	2	165	17,7	9,31
P0CX55	40S ribosomal protein S18-A OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=RPS18A PE=1 SV=1 - [RS18A_YEAST]	69,41	13,01	1	2	2	2	146	17,0	10,27
Q6BXM5	60S ribosomal protein L3 OS=Debaryomyces hansenii (strain ATCC 36239 / CBS 767 / JCM 1990 / NBRC 0083 / IGC 2968) GN=RPL3 PE=3 SV=1 - [RL3_DEBHA]	64,31	4,88	1	2	2	5	389	43,7	10,32
P0CX47	40S ribosomal protein S11-A OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=RPS11A PE=1 SV=1 - [RS11A_YEAST]	60,27	12,82	1	2	2	2	156	17,7	10,78
P46598	Heat shock protein 90 homolog OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=HSP90 PE=1 SV=1 - [HSP90_CANAL]	52,62	3,39	1	2	2	2	707	80,8	4,88
P0CG84	Polyubiquitin (Fragment) OS=Nicotiana sylvestris GN=UBI4 PE=2 SV=1 - [UBI4P_NICSY]	50,64	38,46	53	2	2	3	377	42,2	7,58
P38288	Protein TOS1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=TOS1 PE=1 SV=1 - [TOS1_YEAST]	40,19	8,79	1	2	2	2	455	48,0	4,67
P0CX45	60S ribosomal protein L2-A OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=RPL2A PE=1 SV=1 - [RL2A_YEAST]	33,63	12,99	3	2	2	2	254	27,4	11,11

...continua Table 3.S1

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	1606,68	45,34	1	21	24	45	644	66,0	8,12
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	1457,02	52,27	1	20	25	42	639	65,4	8,00
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	1350,05	54,90	1	18	18	36	623	62,0	5,24
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	1014,13	37,67	1	17	18	29	584	58,8	5,21
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	592,13	36,44	1	7	13	18	472	51,5	5,16
P08779	Keratin, type I cytoskeletal 16 OS=Homo sapiens GN=KRT16 PE=1 SV=4 - [K1C16_HUMAN]	494,60	29,60	1	5	11	14	473	51,2	5,05
P13647	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN]	427,29	24,75	1	10	13	14	590	62,3	7,74
P83336	Thaumatin-like protein 1b (Fragment) OS=Malus domestica PE=2 SV=1 - [TP1B_MALDO]	409,83	22,17	1	1	4	12	212	22,1	4,54
Q9FSG7	Thaumatin-like protein 1a OS=Malus domestica GN=TL1 PE=1 SV=1 - [TP1A_MALDO]	385,34	36,99	1	2	5	12	246	25,7	5,15
P15703	Glucan 1,3-beta-glucosidase OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=BGL2 PE=1 SV=1 - [BGL2_YEAST]	283,35	21,09	1	6	6	8	313	34,1	4,51
P02538	Keratin, type II cytoskeletal 6A OS=Homo sapiens GN=KRT6A PE=1 SV=3 - [K2C6A_HUMAN]	256,05	13,30	1	3	7	8	564	60,0	8,00
P00698	Lysozyme C OS=Gallus gallus GN=LYZ PE=1 SV=1 - [LYSC_CHICK]	221,87	33,33	1	4	4	5	147	16,2	9,07
P00761	Trypsin OS=Sus scrofa PE=1 SV=1 - [TRYP_PIG]	215,78	16,45	1	3	3	7	231	24,4	7,18
Q08193	1,3-beta-glucanosyltransferase GAS5 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS5 PE=1 SV=1 - [GAS5_YEAST]	207,72	6,40	1	2	2	3	484	51,8	4,64
A2R3I1	Probable pectin lyase A OS=Aspergillus niger (strain CBS 513.88 / FGSC A1513) GN=pelA PE=3 SV=1 - [PELA_ASPNC]	201,80	14,51	2	4	4	6	379	39,8	4,34
P22146	1,3-beta-glucanosyltransferase GAS1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS1 PE=1 SV=2 - [GAS1_YEAST]	198,40	4,47	1	3	3	4	559	59,5	4,67
Q9M5X7	Non-specific lipid-transfer protein OS=Malus domestica GN=MALD3 PE=1 SV=1 - [NLTP_MALDO]	121,74	36,52	1	3	3	3	115	11,4	8,92
P53301	Probable glycosidase CRH1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=CRH1 PE=1 SV=1 - [CRH1_YEAST]	111,12	10,45	1	5	5	5	507	52,7	4,65
Q86YZ3	Hornerin OS=Homo sapiens GN=HRNR PE=1 SV=2 - [HORN_HUMAN]	99,16	4,49	1	2	2	5	2850	282,2	10,04
P81605	Dermcidin OS=Homo sapiens GN=DCD PE=1 SV=2 - [DCD_HUMAN]	92,42	20,00	1	2	2	2	110	11,3	6,54
P38288	Protein TOS1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=TOS1 PE=1 SV=1 - [TOS1_YEAST]	87,72	5,93	1	2	2	2	455	48,0	4,67
B5VL27	Cell wall mannoprotein CIS3 OS=Saccharomyces cerevisiae (strain AWRI1631) GN=CIS3 PE=3 SV=1 - [CIS3_YEAS6]	65,93	8,00	2	2	2	2	225	23,0	4,68

Table 3.S2

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	1456,28	42,70	1	19	22	40	644	66,0	8,12
P83336	Thaumatin-like protein 1b (Fragment) OS=Malus domestica PE=2 SV=1 - [TP1B_MALDO]	878,74	41,98	1	2	6	33	212	22,1	4,54
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	875,18	32,91	1	11	11	18	623	62,0	5,24
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	808,82	38,03	1	16	21	27	639	65,4	8,00
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	762,08	30,48	1	15	16	21	584	58,8	5,21
Q9FSG7	Thaumatin-like protein 1a OS=Malus domestica GN=TL1 PE=1 SV=1 - [TP1A_MALDO]	727,24	34,96	1	2	6	27	246	25,7	5,15
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	405,19	26,06	1	4	10	13	472	51,5	5,16
P08779	Keratin, type I cytoskeletal 16 OS=Homo sapiens GN=KRT16 PE=1 SV=4 - [K1C16_HUMAN]	331,22	23,47	1	3	9	11	473	51,2	5,05
P13647	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN]	296,47	17,97	1	8	10	11	590	62,3	7,74
P04259	Keratin, type II cytoskeletal 6B OS=Homo sapiens GN=KRT6B PE=1 SV=5 - [K2C6B_HUMAN]	226,34	11,17	1	2	6	7	564	60,0	8,00
O43790	Keratin, type II cuticular Hb6 OS=Homo sapiens GN=KRT86 PE=1 SV=1 - [KRT86_HUMAN]	192,54	9,47	1	3	3	3	486	53,5	5,66
Q9M5X7	Non-specific lipid-transfer protein OS=Malus domestica GN=MALD3 PE=1 SV=1 - [NLTP_MALDO]	154,43	36,52	1	3	3	4	115	11,4	8,92
P00761	Trypsin OS=Sus scrofa PE=1 SV=1 - [TRYP_PIG]	137,07	16,45	1	3	3	8	231	24,4	7,18
P15703	Glucan 1,3-beta-glucosidase OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=BGL2 PE=1 SV=1 - [BGL2_YEAST]	82,04	7,99	1	2	2	2	313	34,1	4,51
P81605	Dermcidin OS=Homo sapiens GN=DCD PE=1 SV=2 - [DCD_HUMAN]	80,40	20,00	1	2	2	2	110	11,3	6,54
O76011	Keratin, type I cuticular Ha4 OS=Homo sapiens GN=KRT34 PE=2 SV=2 - [KRT34_HUMAN]	66,78	4,59	1	2	2	2	436	49,4	5,06
Q05091	Polygalacturonase inhibitor OS=Pyrus communis GN=PGIP PE=1 SV=1 - [PGIP_PYRCO]	40,50	6,67	1	2	2	2	330	36,5	6,67
Q86YZ3	Hornerin OS=Homo sapiens GN=HRNR PE=1 SV=2 - [HORN_HUMAN]	0,00	5,54	1	2	2	2	2850	282,2	10,04

Table 3.S3

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	1212,88	37,58	1	15	16	31	644	66,0	8,12
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	891,67	32,19	1	15	17	29	584	58,8	5,21
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	834,21	31,14	1	12	12	23	623	62,0	5,24
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	731,20	32,71	1	14	16	23	639	65,4	8,00
P08779	Keratin, type I cytoskeletal 16 OS=Homo sapiens GN=KRT16 PE=1 SV=4 - [K1C16_HUMAN]	462,19	34,88	1	6	13	17	473	51,2	5,05
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	343,58	20,76	1	2	9	14	472	51,5	5,16
P83336	Thaumatin-like protein 1b (Fragment) OS=Malus domestica PE=2 SV=1 - [TP1B_MALDO]	288,08	22,17	1	4	4	11	212	22,1	4,54
A2R3I1	Probable pectin lyase A OS=Aspergillus niger (strain CBS 513.88 / FGSC A1513) GN=pelA PE=3 SV=1 - [PELA_ASPNC]	278,86	20,84	1	5	5	8	379	39,8	4,34
Q08193	1,3-beta-glucanosyltransferase GAS5 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS5 PE=1 SV=1 - [GAS5_YEAST]	277,47	11,57	1	4	4	7	484	51,8	4,64
P00761	Trypsin OS=Sus scrofa PE=1 SV=1 - [TRYP_PIG]	258,95	16,45	1	3	3	12	231	24,4	7,18
P02538	Keratin, type II cytoskeletal 6A OS=Homo sapiens GN=KRT6A PE=1 SV=3 - [K2C6A_HUMAN]	249,29	17,91	1	6	8	9	564	60,0	8,00
P22146	1,3-beta-glucanosyltransferase GAS1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS1 PE=1 SV=2 - [GAS1_YEAST]	222,21	4,83	1	4	4	6	559	59,5	4,67
A6ZL22	Cell wall protein ECM33 OS=Saccharomyces cerevisiae (strain YJM789) GN=ECM33 PE=3 SV=2 - [ECM33_YEAS7]	196,38	9,56	4	4	4	6	429	43,7	4,91
P13647	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN]	183,24	10,85	1	5	6	7	590	62,3	7,74
A2QK82	Probable pectinesterase A OS=Aspergillus niger (strain CBS 513.88 / FGSC A1513) GN=pmeA PE=3 SV=1 - [PMEA_ASPNC]	171,92	9,79	1	2	2	3	327	34,6	4,39
P81605	Dermcidin OS=Homo sapiens GN=DCD PE=1 SV=2 - [DCD_HUMAN]	109,40	20,00	1	2	2	3	110	11,3	6,54
P53301	Probable glycosidase CRH1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=CRH1 PE=1 SV=1 - [CRH1_YEAST]	108,22	6,31	1	3	3	3	507	52,7	4,65
A2QK83	Probable endo-xylogalacturonan hydrolase A OS=Aspergillus niger (strain CBS 513.88 / FGSC A1513) GN=xghA PE=3 SV=1 - [XGHA_ASPNC]	101,44	5,67	2	2	2	2	406	42,1	4,51
P00698	Lysozyme C OS=Gallus gallus GN=LYZ PE=1 SV=1 - [LYSC_CHICK]	101,30	19,05	2	2	2	2	147	16,2	9,07
Q9M5X7	Non-specific lipid-transfer protein OS=Malus domestica GN=MALD3 PE=1 SV=1 - [NLTP_MALDO]	81,06	24,35	1	2	2	2	115	11,4	8,92
Q03674	Lysophospholipase 2 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=PLB2 PE=1 SV=1 - [PLB2_YEAST]	42,07	2,83	1	2	2	2	706	75,4	4,70

Table 3.S4

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	1325,42	40,84	1	19	21	37	644	66,0	8,12
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	945,76	41,00	1	15	20	32	639	65,4	8,00
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	929,39	35,45	1	17	18	29	584	58,8	5,21
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	773,18	30,34	1	12	12	23	623	62,0	5,24
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	510,91	28,39	1	4	11	16	472	51,5	5,16
P08779	Keratin, type I cytoskeletal 16 OS=Homo sapiens GN=KRT16 PE=1 SV=4 - [K1C16_HUMAN]	449,51	34,46	1	6	13	16	473	51,2	5,05
Q08193	1,3-beta-glucanosyltransferase GAS5 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS5 PE=1 SV=1 - [GAS5_YEAST]	297,59	11,57	1	4	4	7	484	51,8	4,64
P48668	Keratin, type II cytoskeletal 6C OS=Homo sapiens GN=KRT6C PE=1 SV=3 - [K2C6C_HUMAN]	266,13	14,89	1	4	8	10	564	60,0	8,00
P13647	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN]	259,93	20,34	1	8	11	11	590	62,3	7,74
P00698	Lysozyme C OS=Gallus gallus GN=LYZ PE=1 SV=1 - [LYSC_CHICK]	229,50	25,17	1	3	3	6	147	16,2	9,07
P00761	Trypsin OS=Sus scrofa PE=1 SV=1 - [TRYP_PIG]	179,48	16,45	1	3	3	8	231	24,4	7,18
Q9M5X7	Non-specific lipid-transfer protein OS=Malus domestica GN=MALD3 PE=1 SV=1 - [NLTP_MALDO]	149,96	46,09	1	4	4	5	115	11,4	8,92
P83336	Thaumatin-like protein 1b (Fragment) OS=Malus domestica PE=2 SV=1 - [TP1B_MALDO]	144,30	22,17	1	4	4	5	212	22,1	4,54
P22146	1,3-beta-glucanosyltransferase GAS1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS1 PE=1 SV=2 - [GAS1_YEAST]	71,88	4,47	1	2	2	2	559	59,5	4,67
P15703	Glucan 1,3-beta-glucosidase OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=BGL2 PE=1 SV=1 - [BGL2_YEAST]	59,74	9,27	1	2	2	2	313	34,1	4,51

Table 3.S5

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	1484,56	46,74	1	22	25	46	644	66,0	8,12
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	894,54	35,47	1	13	13	27	623	62,0	5,24
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	812,76	26,03	1	14	15	29	584	58,8	5,21
P00698	Lysozyme C OS=Gallus gallus GN=LYZ PE=1 SV=1 - [LYSC_CHICK]	787,21	60,54	1	7	7	22	147	16,2	9,07
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	737,84	34,90	1	15	19	27	639	65,4	8,00
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	358,01	22,46	1	7	8	12	472	51,5	5,16
P00761	Trypsin OS=Sus scrofa PE=1 SV=1 - [TRYP_PIG]	269,97	16,45	1	3	3	8	231	24,4	7,18
P13647	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN]	197,74	13,56	1	6	8	8	590	62,3	7,74
P02025	Hemoglobin subunit beta OS=Hylobates lar GN=HBB PE=1 SV=1 - [HBB_HYLLA]	89,50	14,38	13	2	2	4	146	15,9	7,28
P83336	Thaumatin-like protein 1b (Fragment) OS=Malus domestica PE=2 SV=1 - [TP1B_MALDO]	57,81	11,32	1	2	2	3	212	22,1	4,54

Table 3.S6

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	1887,56	50,31	1	23	28	62	644	66,0	8,12
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	1507,60	54,62	1	21	27	48	639	65,4	8,00
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	1368,66	59,07	1	20	20	42	623	62,0	5,24
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	1013,66	32,19	1	17	19	31	584	58,8	5,21
P08779	Keratin, type I cytoskeletal 16 OS=Homo sapiens GN=KRT16 PE=1 SV=4 - [K1C16_HUMAN]	877,98	50,32	1	11	20	32	473	51,2	5,05
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	764,05	39,41	1	6	15	23	472	51,5	5,16
P02538	Keratin, type II cytoskeletal 6A OS=Homo sapiens GN=KRT6A PE=1 SV=3 - [K2C6A_HUMAN]	619,49	34,04	1	3	19	25	564	60,0	8,00
P04259	Keratin, type II cytoskeletal 6B OS=Homo sapiens GN=KRT6B PE=1 SV=5 - [K2C6B_HUMAN]	584,31	32,45	1	2	18	25	564	60,0	8,00
P13647	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN]	570,15	31,36	1	12	18	21	590	62,3	7,74
P00761	Trypsin OS=Sus scrofa PE=1 SV=1 - [TRYP_PIG]	409,86	25,11	1	4	4	22	231	24,4	7,18
P00698	Lysozyme C OS=Gallus gallus GN=LYZ PE=1 SV=1 - [LYSC_CHICK]	250,09	48,98	1	5	5	8	147	16,2	9,07
P83336	Thaumatin-like protein 1b (Fragment) OS=Malus domestica PE=2 SV=1 - [TP1B_MALDO]	207,35	22,17	1	4	4	7	212	22,1	4,54
P81605	Dermcidin OS=Homo sapiens GN=DCD PE=1 SV=2 - [DCD_HUMAN]	123,43	20,00	1	2	2	3	110	11,3	6,54
P15924	Desmoplakin OS=Homo sapiens GN=DSP PE=1 SV=3 - [DESP_HUMAN]	89,72	1,08	1	3	3	3	2871	331,6	6,81
P14923	Junction plakoglobin OS=Homo sapiens GN=JUP PE=1 SV=3 - [PLAK_HUMAN]	80,41	2,95	5	2	2	2	745	81,7	6,14
P0CT31	Elongation factor 1-alpha OS=Dictyostelium discoideum GN=eef1a1 PE=1 SV=1 - [EF1A1_DICDI]	73,37	4,19	1	2	2	2	453	49,6	8,98
Q86YZ3	Hornerin OS=Homo sapiens GN=HRNR PE=1 SV=2 - [HORN_HUMAN]	72,95	6,77	1	4	4	6	2850	282,2	10,04
Q5T749	Keratinocyte proline-rich protein OS=Homo sapiens GN=KPRP PE=1 SV=1 - [KPRP_HUMAN]	31,66	3,97	1	2	2	2	579	64,1	8,27

Table 3.S7

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	1895,82	47,98	1	21	25	59	644	66,0	8,12
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	1424,71	54,77	1	20	27	58	639	65,4	8,00
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	1180,91	27,74	1	17	18	38	584	58,8	5,21
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	1022,42	44,30	1	17	17	35	623	62,0	5,24
P08779	Keratin, type I cytoskeletal 16 OS=Homo sapiens GN=KRT16 PE=1 SV=4 - [K1C16_HUMAN]	1004,50	43,13	1	10	18	33	473	51,2	5,05
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	904,11	40,04	1	4	15	29	472	51,5	5,16
P02538	Keratin, type II cytoskeletal 6A OS=Homo sapiens GN=KRT6A PE=1 SV=3 - [K2C6A_HUMAN]	772,62	39,72	1	4	23	36	564	60,0	8,00
P04259	Keratin, type II cytoskeletal 6B OS=Homo sapiens GN=KRT6B PE=1 SV=5 - [K2C6B_HUMAN]	764,76	37,94	1	3	22	36	564	60,0	8,00
P13647	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN]	456,44	26,95	1	9	15	24	590	62,3	7,74
Q04695	Keratin, type I cytoskeletal 17 OS=Homo sapiens GN=KRT17 PE=1 SV=2 - [K1C17_HUMAN]	415,44	31,02	1	6	12	19	432	48,1	5,02
P83336	Thaumatin-like protein 1b (Fragment) OS=Malus domestica PE=2 SV=1 - [TP1B_MALDO]	379,29	22,17	1	1	4	14	212	22,1	4,54
Q9FSG7	Thaumatin-like protein 1a OS=Malus domestica GN=TL1 PE=1 SV=1 - [TP1A_MALDO]	376,03	31,71	1	2	5	14	246	25,7	5,15
P00698	Lysozyme C OS=Gallus gallus GN=LYZ PE=1 SV=1 - [LYSC_CHICK]	246,77	25,17	1	3	3	5	147	16,2	9,07
P00761	Trypsin OS=Sus scrofa PE=1 SV=1 - [TRYP_PIG]	240,56	25,11	1	4	4	16	231	24,4	7,18
P81605	Dermcidin OS=Homo sapiens GN=DCD PE=1 SV=2 - [DCD_HUMAN]	113,65	20,00	1	2	2	3	110	11,3	6,54
Q9M5X7	Non-specific lipid-transfer protein OS=Malus domestica GN=MALD3 PE=1 SV=1 - [NLTP_MALDO]	111,91	24,35	1	2	2	3	115	11,4	8,92
P15703	Glucan 1,3-beta-glucosidase OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=BGL2 PE=1 SV=1 - [BGL2_YEAST]	86,80	10,22	1	3	3	4	313	34,1	4,51

Table 3.S8

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	1300,91	41,77	1	18	20	40	644	66,0	8,12
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	717,70	25,86	1	14	15	27	584	58,8	5,21
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	695,26	37,09	1	16	19	31	639	65,4	8,00
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	690,18	27,13	1	10	10	20	623	62,0	5,24
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	263,05	21,40	1	6	7	9	472	51,5	5,16
P00761	Trypsin OS=Sus scrofa PE=1 SV=1 - [TRYP_PIG]	225,35	16,45	1	3	3	13	231	24,4	7,18
P13647	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN]	217,03	12,37	1	6	7	8	590	62,3	7,74
P83336	Thaumatin-like protein 1b (Fragment) OS=Malus domestica PE=2 SV=1 - [TP1B_MALDO]	169,77	22,17	1	4	4	9	212	22,1	4,54
Q9M5X7	Non-specific lipid-transfer protein OS=Malus domestica GN=MALD3 PE=1 SV=1 - [NLTP_MALDO]	132,23	24,35	1	2	2	4	115	11,4	8,92
Q08193	1,3-beta-glucanosyltransferase GAS5 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS5 PE=1 SV=1 - [GAS5_YEAST]	73,37	8,88	1	3	3	3	484	51,8	4,64
P22146	1,3-beta-glucanosyltransferase GAS1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS1 PE=1 SV=2 - [GAS1_YEAST]	39,90	4,47	1	2	2	2	559	59,5	4,67
P15703	Glucan 1,3-beta-glucosidase OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=BGL2 PE=1 SV=1 - [BGL2_YEAST]	38,43	7,35	1	2	2	2	313	34,1	4,51

Table 3.S9

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	1495,02	45,81	1	19	22	42	644	66,0	8,12
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	861,86	36,76	1	14	14	25	623	62,0	5,24
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	781,58	42,41	1	17	21	32	639	65,4	8,00
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	764,39	26,03	1	14	15	27	584	58,8	5,21
Q9FSG7	Thaumatin-like protein 1a OS=Malus domestica GN=TL1 PE=1 SV=1 - [TP1A_MALDO]	477,71	27,64	1	5	5	19	246	25,7	5,15
Q08193	1,3-beta-glucanosyltransferase GAS5 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS5 PE=1 SV=1 - [GAS5_YEAST]	361,32	11,57	1	4	4	9	484	51,8	4,64
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	294,01	19,28	1	6	7	12	472	51,5	5,16
P00761	Trypsin OS=Sus scrofa PE=1 SV=1 - [TRYP_PIG]	226,39	16,45	1	3	3	10	231	24,4	7,18
A6ZL22	Cell wall protein ECM33 OS=Saccharomyces cerevisiae (strain YJM789) GN=ECM33 PE=3 SV=2 - [ECM33_YEAS7]	221,91	6,06	4	3	3	7	429	43,7	4,91
P02538	Keratin, type II cytoskeletal 6A OS=Homo sapiens GN=KRT6A PE=1 SV=3 - [K2C6A_HUMAN]	208,45	9,40	2	2	5	6	564	60,0	8,00
P00698	Lysozyme C OS=Gallus gallus GN=LYZ PE=1 SV=1 - [LYSC_CHICK]	202,48	25,17	1	3	3	4	147	16,2	9,07
P15703	Glucan 1,3-beta-glucosidase OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=BGL2 PE=1 SV=1 - [BGL2_YEAST]	199,45	15,97	1	5	5	9	313	34,1	4,51
P22146	1,3-beta-glucanosyltransferase GAS1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS1 PE=1 SV=2 - [GAS1_YEAST]	189,78	5,90	1	4	4	6	559	59,5	4,67
P13647	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN]	162,44	10,51	1	4	6	6	590	62,3	7,74
P53301	Probable glycosidase CRH1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=CRH1 PE=1 SV=1 - [CRH1_YEAST]	94,70	11,64	1	6	6	6	507	52,7	4,65
P38288	Protein TOS1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=TOS1 PE=1 SV=1 - [TOS1_YEAST]	83,69	8,13	1	3	3	3	455	48,0	4,67
B5VL27	Cell wall mannoprotein CIS3 OS=Saccharomyces cerevisiae (strain AWRI1631) GN=CIS3 PE=3 SV=1 - [CIS3_YEAS6]	78,67	8,00	2	2	2	4	225	23,0	4,68

Table 3.S10

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
P02534	Keratin, type I microfibrillar 48 kDa, component 8C-1 OS=Ovis aries PE=1 SV=2 - [K1M1_SHEEP]	1477,70	63,83	1	9	28	57	412	46,6	4,81
Q148H4	Keratin, type II cuticular Hb1 OS=Bos taurus GN=KRT81 PE=2 SV=1 - [KRT81_BOVIN]	1452,34	42,20	1	5	26	66	500	54,6	5,68
A4FUZ0	Keratin, type II cuticular Hb3 OS=Bos taurus GN=KRT83 PE=2 SV=1 - [KRT83_BOVIN]	1348,86	48,88	1	1	28	63	493	54,0	5,48
P15241	Keratin, type II microfibrillar, component 7C OS=Ovis aries PE=1 SV=1 - [K2M2_SHEEP]	1324,24	45,42	1	2	26	59	491	53,6	5,57
P25691	Keratin, type II microfibrillar, component 5 OS=Ovis aries PE=1 SV=1 - [K2M3_SHEEP]	1024,47	41,43	1	11	23	53	502	55,2	6,46
P25690	Keratin, type I microfibrillar, 47.6 kDa OS=Ovis aries PE=3 SV=2 - [K1M2_SHEEP]	962,13	49,75	1	7	21	43	404	46,0	4,93
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	877,22	43,94	1	19	21	28	644	66,0	8,12
A5A6M5	Keratin, type I cuticular Ha1 OS=Pan troglodytes GN=KRT31 PE=2 SV=1 - [K1H1_PANTR]	731,33	26,20	2	2	13	29	416	47,2	4,88
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	687,31	29,11	1	17	17	21	584	58,8	5,21
Q9FSG7	Thaumatin-like protein 1a OS=Malus domestica GN=TL1 PE=1 SV=1 - [TP1A_MALDO]	584,69	48,78	1	3	7	21	246	25,7	5,15
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	518,84	23,92	1	10	10	15	623	62,0	5,24
P83336	Thaumatin-like protein 1b (Fragment) OS=Malus domestica PE=2 SV=1 - [TP1B_MALDO]	515,77	41,98	1	2	6	16	212	22,1	4,54
Q0P5J7	Keratin, type I cuticular Ha5 OS=Bos taurus GN=KRT35 PE=2 SV=2 - [KRT35_BOVIN]	418,57	27,09	1	6	11	21	454	49,9	5,26
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	398,86	24,57	1	11	13	14	639	65,4	8,00
P00761	Trypsin OS=Sus scrofa PE=1 SV=1 - [TRYP_PIG]	269,36	16,45	1	3	3	14	231	24,4	7,18
Q6R648	Keratin-associated protein 11-1 OS=Capra hircus GN=KRTAP11-1 PE=2 SV=1 - [KR111_CAPHI]	259,20	22,01	1	2	2	6	159	16,8	7,84
Q08193	1,3-beta-glucanosyltransferase GAS5 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS5 PE=1 SV=1 - [GAS5_YEAST]	258,85	11,57	1	4	4	5	484	51,8	4,64
P02442	Keratin, high-sulfur matrix protein, IIIA3 OS=Capra hircus PE=1 SV=1 - [KRA3_CAPHI]	219,73	43,94	1	4	4	10	132	14,2	8,12
P02446	Keratin-associated protein 3-1 OS=Ovis aries GN=KRTAP3-1 PE=1 SV=2 - [KRA31_SHEEP]	174,26	15,31	2	2	2	5	98	10,4	6,96
Q02958	Keratin-associated protein 6-1 OS=Ovis aries GN=KRTAP6-1 PE=1 SV=2 - [KRA61_SHEEP]	156,22	32,53	1	3	3	4	83	8,4	8,22
P15703	Glucan 1,3-beta-glucosidase OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=BGL2 PE=1 SV=1 - [BGL2_YEAST]	132,87	15,65	1	5	5	5	313	34,1	4,51
O13923	Coronin-like protein crn1 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=crn1 PE=1 SV=1 - [CORO_SCHPO]	115,37	1,50	1	2	2	5	601	67,0	6,18
P27996	Histone H4 OS=Solaster stimpsoni PE=3 SV=2 - [H4_SOLST]	94,87	29,13	15	3	3	4	103	11,4	11,19
P02444	Keratin, high sulfur matrix protein, IIIB3 OS=Ovis aries PE=1 SV=1 - [KRA33_SHEEP]	93,68	21,43	2	2	2	2	98	10,5	6,95
A6ZL22	Cell wall protein ECM33 OS=Saccharomyces cerevisiae (strain YJM789) GN=ECM33 PE=3 SV=2 - [ECM33_YEAS7]	91,11	5,83	4	2	2	2	429	43,7	4,91
P53301	Probable glycosidase CRH1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=CRH1 PE=1 SV=1 - [CRH1_YEAST]	85,41	5,92	1	3	3	3	507	52,7	4,65
P22146	1,3-beta-glucanosyltransferase GAS1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS1 PE=1 SV=2 - [GAS1_YEAST]	77,43	2,68	1	2	2	2	559	59,5	4,67

Table 3.S11

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	1585,80	42,55	1	19	21	41	644	66,0	8,12
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	808,34	30,66	1	11	11	20	623	62,0	5,24
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	710,35	30,36	1	13	16	25	639	65,4	8,00
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	696,75	24,49	1	11	15	30	584	58,8	5,21
P83336	Thaumatin-like protein 1b (Fragment) OS=Malus domestica PE=2 SV=1 - [TP1B_MALDO]	664,92	33,49	1	2	6	24	212	22,1	4,54
Q9FSG7	Thaumatin-like protein 1a OS=Malus domestica GN=TL1 PE=1 SV=1 - [TP1A_MALDO]	616,33	41,46	1	3	7	25	246	25,7	5,15
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	406,74	20,34	1	4	8	12	472	51,5	5,16
P13647	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN]	237,94	15,76	1	7	8	8	590	62,3	7,74
Q08193	1,3-beta-glucanosyltransferase GAS5 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS5 PE=1 SV=1 - [GAS5_YEAST]	233,36	11,57	1	4	4	5	484	51,8	4,64
P13646	Keratin, type I cytoskeletal 13 OS=Homo sapiens GN=KRT13 PE=1 SV=4 - [K1C13_HUMAN]	230,13	15,94	1	2	7	8	458	49,6	4,96
P15703	Glucan 1,3-beta-glucosidase OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=BGL2 PE=1 SV=1 - [BGL2_YEAST]	223,43	15,97	1	5	5	6	313	34,1	4,51
P02538	Keratin, type II cytoskeletal 6A OS=Homo sapiens GN=KRT6A PE=1 SV=3 - [K2C6A_HUMAN]	210,37	15,78	1	5	7	8	564	60,0	8,00
P22146	1,3-beta-glucanosyltransferase GAS1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS1 PE=1 SV=2 - [GAS1_YEAST]	198,68	5,90	1	4	4	6	559	59,5	4,67
P02534	Keratin, type I microfibrillar 48 kDa, component 8C-1 OS=Ovis aries PE=1 SV=2 - [K1M1_SHEEP]	177,10	12,38	1	3	4	5	412	46,6	4,81
P00761	Trypsin OS=Sus scrofa PE=1 SV=1 - [TRYP_PIG]	174,37	16,45	1	3	3	9	231	24,4	7,18
P15241	Keratin, type II microfibrillar, component 7C OS=Ovis aries PE=1 SV=1 - [K2M2_SHEEP]	107,38	11,20	2	4	4	4	491	53,6	5,57
P53301	Probable glycosidase CRH1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=CRH1 PE=1 SV=1 - [CRH1_YEAST]	89,07	6,51	1	3	3	3	507	52,7	4,65
B5VL27	Cell wall mannoprotein CIS3 OS=Saccharomyces cerevisiae (strain AWRI1631) GN=CIS3 PE=3 SV=1 - [CIS3_YEAS6]	61,68	8,00	2	2	2	2	225	23,0	4,68

Table 3.S12

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	1286,49	39,29	1	15	17	31	644	66,0	8,12
P00698	Lysozyme C OS=Gallus gallus GN=LYZ PE=1 SV=1 - [LYSC_CHICK]	715,02	60,54	1	6	6	19	147	16,2	9,07
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	712,79	23,12	1	12	13	22	584	58,8	5,21
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	677,51	32,08	1	13	17	23	639	65,4	8,00
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	647,73	25,52	1	9	9	17	623	62,0	5,24
P08779	Keratin, type I cytoskeletal 16 OS=Homo sapiens GN=KRT16 PE=1 SV=4 - [K1C16_HUMAN]	296,56	22,20	1	3	8	10	473	51,2	5,05
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	276,93	18,01	1	2	7	9	472	51,5	5,16
P00761	Trypsin OS=Sus scrofa PE=1 SV=1 - [TRYP_PIG]	246,97	16,45	1	3	3	6	231	24,4	7,18
P02538	Keratin, type II cytoskeletal 6A OS=Homo sapiens GN=KRT6A PE=1 SV=3 - [K2C6A_HUMAN]	202,56	11,88	1	4	6	7	564	60,0	8,00
P83336	Thaumatin-like protein 1b (Fragment) OS=Malus domestica PE=2 SV=1 - [TP1B_MALDO]	166,01	16,04	1	3	3	6	212	22,1	4,54
P13647	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN]	152,76	11,53	1	5	6	6	590	62,3	7,74
Q9M5X7	Non-specific lipid-transfer protein OS=Malus domestica GN=MALD3 PE=1 SV=1 - [NLTP_MALDO]	80,67	24,35	1	2	2	2	115	11,4	8,92

Table 3.S13

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	1162,69	42,08	1	19	21	37	644	66,0	8,12
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	804,28	23,12	1	13	14	28	584	58,8	5,21
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	668,64	28,64	1	14	17	25	639	65,4	8,00
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	614,37	26,65	1	9	9	17	623	62,0	5,24
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	323,75	27,97	1	4	10	15	472	51,5	5,16
P08779	Keratin, type I cytoskeletal 16 OS=Homo sapiens GN=KRT16 PE=1 SV=4 - [K1C16_HUMAN]	265,51	20,30	1	2	8	13	473	51,2	5,05
P83336	Thaumatin-like protein 1b (Fragment) OS=Malus domestica PE=2 SV=1 - [TP1B_MALDO]	227,58	22,17	1	4	4	8	212	22,1	4,54
P00761	Trypsin OS=Sus scrofa PE=1 SV=1 - [TRYP_PIG]	203,69	16,45	1	3	3	12	231	24,4	7,18
P02538	Keratin, type II cytoskeletal 6A OS=Homo sapiens GN=KRT6A PE=1 SV=3 - [K2C6A_HUMAN]	160,16	7,98	2	2	4	6	564	60,0	8,00
P13647	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN]	158,67	12,71	1	6	7	9	590	62,3	7,74
P00698	Lysozyme C OS=Gallus gallus GN=LYZ PE=1 SV=1 - [LYSC_CHICK]	101,15	19,05	2	2	2	3	147	16,2	9,07
P22146	1,3-beta-glucanosyltransferase GAS1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS1 PE=1 SV=2 - [GAS1_YEAST]	44,81	4,47	1	2	2	2	559	59,5	4,67

Table 3.S14

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	1284,48	39,60	1	18	19	34	644	66,0	8,12
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	1035,49	39,00	1	13	13	26	623	62,0	5,24
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	864,12	34,12	1	13	17	34	639	65,4	8,00
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	853,18	33,90	1	14	17	29	584	58,8	5,21
P69327	Glucoamylase OS=Aspergillus awamori GN=GLAA PE=1 SV=1 - [AMYG_ASPAW]	494,48	13,75	1	5	5	9	640	68,3	4,45
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	451,12	27,97	1	6	11	15	472	51,5	5,16
O43790	Keratin, type II cuticular Hb6 OS=Homo sapiens GN=KRT86 PE=1 SV=1 - [KRT86_HUMAN]	400,71	30,25	1	4	10	13	486	53,5	5,66
Q14525	Keratin, type I cuticular Ha3-II OS=Homo sapiens GN=KRT33B PE=2 SV=3 - [KT33B_HUMAN]	309,22	17,57	1	2	7	8	404	46,2	4,84
Q08193	1,3-beta-glucanosyltransferase GAS5 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS5 PE=1 SV=1 - [GAS5_YEAST]	289,20	11,57	1	4	4	7	484	51,8	4,64
P02538	Keratin, type II cytoskeletal 6A OS=Homo sapiens GN=KRT6A PE=1 SV=3 - [K2C6A_HUMAN]	287,81	17,38	1	5	9	12	564	60,0	8,00
P13647	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN]	248,19	16,27	1	6	9	12	590	62,3	7,74
P78385	Keratin, type II cuticular Hb3 OS=Homo sapiens GN=KRT83 PE=1 SV=2 - [KRT83_HUMAN]	241,80	17,85	1	1	7	10	493	54,2	5,64
P00761	Trypsin OS=Sus scrofa PE=1 SV=1 - [TRYP_PIG]	203,26	16,45	1	3	3	7	231	24,4	7,18
A6ZL22	Cell wall protein ECM33 OS=Saccharomyces cerevisiae (strain YJM789) GN=ECM33 PE=3 SV=2 - [ECM33_YEAS7]	192,80	6,06	4	3	3	7	429	43,7	4,91
P78386	Keratin, type II cuticular Hb5 OS=Homo sapiens GN=KRT85 PE=1 SV=1 - [KRT85_HUMAN]	185,92	14,00	1	2	6	9	507	55,8	6,55
O76011	Keratin, type I cuticular Ha4 OS=Homo sapiens GN=KRT34 PE=2 SV=2 - [KRT34_HUMAN]	182,90	15,83	1	2	7	7	436	49,4	5,06
Q04E64	60 kDa chaperonin OS=Oenococcus oeni (strain ATCC BAA-331 / PSU-1) GN=groL PE=3 SV=1 - [CH60_OENOB]	160,77	11,65	1	5	5	5	541	57,3	4,96
P15703	Glucan 1,3-beta-glucosidase OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=BGL2 PE=1 SV=1 - [BGL2_YEAST]	160,42	16,93	1	4	4	4	313	34,1	4,51
P13646	Keratin, type I cytoskeletal 13 OS=Homo sapiens GN=KRT13 PE=1 SV=4 - [K1C13_HUMAN]	150,72	14,85	1	2	6	6	458	49,6	4,96
P22146	1,3-beta-glucanosyltransferase GAS1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS1 PE=1 SV=2 - [GAS1_YEAST]	119,89	4,47	1	3	3	4	559	59,5	4,67
P38288	Protein TOS1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=TOS1 PE=1 SV=1 - [TOS1_YEAST]	95,37	11,65	1	3	3	3	455	48,0	4,67
Q9M5X7	Non-specific lipid-transfer protein OS=Malus domestica GN=MALD3 PE=1 SV=1 - [NLTP_MALDO]	65,78	24,35	1	2	2	2	115	11,4	8,92
P83336	Thaumatin-like protein 1b (Fragment) OS=Malus domestica PE=2 SV=1 - [TP1B_MALDO]	37,75	11,32	1	2	2	2	212	22,1	4,54

Table 3.S15

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
P00698	Lysozyme C OS=Gallus gallus GN=LYZ PE=1 SV=1 - [LYSC_CHICK]	6567,51	72,11	1	6	11	244	147	16,2	9,07
P49663	Lysozyme C OS=Phasianus versicolor GN=LYZ PE=1 SV=1 - [LYSC_PHAVE]	3293,38	40,77	1	2	6	101	130	14,3	8,82
Q7LZQ2	Lysozyme C OS=Aix sponsa GN=LYZ PE=1 SV=1 - [LYSC_AIXSP]	1408,80	49,61	1	2	6	47	129	14,5	8,97
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	1100,14	38,98	1	18	20	26	644	66,0	8,12
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	733,40	34,27	1	14	18	21	639	65,4	8,00
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	653,26	31,14	1	12	12	17	623	62,0	5,24
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	511,68	23,12	1	11	12	13	584	58,8	5,21
P00761	Trypsin OS=Sus scrofa PE=1 SV=1 - [TRYP_PIG]	269,27	25,11	1	4	4	15	231	24,4	7,18
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	180,92	11,44	1	4	5	5	472	51,5	5,16
A6ZL22	Cell wall protein ECM33 OS=Saccharomyces cerevisiae (strain YJM789) GN=ECM33 PE=3 SV=2 - [ECM33_YEAS7]	153,44	6,06	4	3	3	4	429	43,7	4,91
P53301	Probable glycosidase CRH1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=CRH1 PE=1 SV=1 - [CRH1_YEAST]	148,73	9,07	1	5	5	7	507	52,7	4,65
P13647	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN]	146,78	8,47	1	3	5	5	590	62,3	7,74
P22146	1,3-beta-glucanosyltransferase GAS1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS1 PE=1 SV=2 - [GAS1_YEAST]	127,87	4,47	1	2	2	3	559	59,5	4,67
P28319	Cell wall protein CWP1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=CWP1 PE=1 SV=2 - [CWP1_YEAST]	106,78	12,13	1	2	2	3	239	24,3	4,67
P15703	Glucan 1,3-beta-glucosidase OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=BGL2 PE=1 SV=1 - [BGL2_YEAST]	59,33	6,71	1	2	2	2	313	34,1	4,51
P83336	Thaumatin-like protein 1b (Fragment) OS=Malus domestica PE=2 SV=1 - [TP1B_MALDO]	52,17	11,32	1	2	2	2	212	22,1	4,54
P38288	Protein TOS1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=TOS1 PE=1 SV=1 - [TOS1_YEAST]	49,33	8,79	1	2	2	2	455	48,0	4,67
A2R3I1	Probable pectin lyase A OS=Aspergillus niger (strain CBS 513.88 / FGSC A1513) GN=pelA PE=3 SV=1 - [PELA_ASPNC]	35,74	5,80	2	2	2	2	379	39,8	4,34
Q86YZ3	Hornerin OS=Homo sapiens GN=HRNR PE=1 SV=2 - [HORN_HUMAN]	33,61	7,23	1	3	3	5	2850	282,2	10,04

Table 3.S16

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
P00698	Lysozyme C OS=Gallus gallus GN=LYZ PE=1 SV=1 - [LYSC_CHICK]	1764,40	59,86	1	5	7	63	147	16,2	9,07
P49663	Lysozyme C OS=Phasianus versicolor GN=LYZ PE=1 SV=1 - [LYSC_PHAVE]	1079,75	33,85	1	2	4	34	130	14,3	8,82
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	772,28	35,40	1	15	16	21	644	66,0	8,12
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	675,32	21,51	1	9	9	15	623	62,0	5,24
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	565,23	27,74	1	12	13	16	584	58,8	5,21
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	562,38	28,01	1	13	14	17	639	65,4	8,00
Q9FSG7	Thaumatin-like protein 1a OS=Malus domestica GN=TL1 PE=1 SV=1 - [TP1A_MALDO]	499,31	41,46	1	6	6	17	246	25,7	5,15
Q8NK89	Alpha-L-arabinofuranosidase B OS=Aspergillus kawachii (strain NBRC 4308) GN=abfB PE=1 SV=1 - [ABFB_ASPKW]	408,58	23,05	2	6	6	10	499	52,6	4,46
P62694	Exoglucanase 1 OS=Hypocrea jecorina GN=cbh1 PE=1 SV=1 - [GUX1_HYPJE]	253,86	13,84	1	5	5	7	513	54,0	4,81
A2R3I1	Probable pectin lyase A OS=Aspergillus niger (strain CBS 513.88 / FGSC A1513) GN=pelA PE=3 SV=1 - [PELA_ASPNC]	252,37	11,35	2	3	3	7	379	39,8	4,34
P00761	Trypsin OS=Sus scrofa PE=1 SV=1 - [TRYP_PIG]	223,58	16,45	1	3	3	9	231	24,4	7,18
P00343	L-lactate dehydrogenase OS=Lactobacillus casei GN=ldh PE=1 SV=3 - [LDH_LACCA]	180,40	12,58	1	3	3	4	326	35,5	5,45
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	163,53	9,11	2	3	4	6	472	51,5	5,16
P17872	Pectinesterase OS=Aspergillus tubingensis GN=pme1 PE=1 SV=1 - [PME_ASPTU]	146,16	14,80	1	4	4	6	331	35,7	4,36
P22832	Glucoamylase OS=Aspergillus shirousami GN=glaA PE=3 SV=1 - [AMYG_ASPSH]	117,29	7,04	2	2	2	2	639	68,1	4,49
Q03F25	Elongation factor Tu OS=Pediococcus pentosaceus (strain ATCC 25745 / 183-1w) GN=tuf PE=3 SV=1 - [EFTU_PEDPA]	102,64	17,97	1	3	6	7	395	43,3	4,87
Q88YH3	Enolase 1 OS=Lactobacillus plantarum (strain ATCC BAA-793 / NCIMB 8826 / WCFS1) GN=eno1 PE=3 SV=1 - [ENO1_LACPL]	97,03	7,24	1	2	2	2	442	48,0	4,72
Q12679	Endoglucanase A OS=Aspergillus kawachii (strain NBRC 4308) GN=cekA PE=2 SV=2 - [GUNA_ASPKW]	73,97	8,37	1	2	2	2	239	25,8	4,84
Q03QN5	Elongation factor Tu OS=Lactobacillus brevis (strain ATCC 367 / JCM 1170) GN=tuf PE=3 SV=1 - [EFTU_LACBA]	60,64	14,39	1	2	5	5	396	43,6	4,84
B5VL27	Cell wall mannoprotein CIS3 OS=Saccharomyces cerevisiae (strain AWRI1631) GN=CIS3 PE=3 SV=1 - [CIS3_YEAS6]	54,19	8,00	2	2	2	2	225	23,0	4,68
P53301	Probable glycosidase CRH1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=CRH1 PE=1 SV=1 - [CRH1_YEAST]	50,70	4,54	1	2	2	2	507	52,7	4,65
P15703	Glucan 1,3-beta-glucosidase OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=BGL2 PE=1 SV=1 - [BGL2_YEAST]	43,80	6,71	1	2	2	2	313	34,1	4,51

Table 3.S17

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	1142,77	41,15	1	18	20	31	644	66,0	8,12
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	759,77	23,92	1	8	8	16	623	62,0	5,24
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	753,03	37,56	1	16	19	29	639	65,4	8,00
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	637,08	25,86	1	14	16	24	584	58,8	5,21
Q04E64	60 kDa chaperonin OS=Oenococcus oeni (strain ATCC BAA-331 / PSU-1) GN=groL PE=3 SV=1 - [CH60_OENOB]	544,51	26,43	1	13	13	17	541	57,3	4,96
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	353,32	20,34	1	6	8	11	472	51,5	5,16
Q08193	1,3-beta-glucanosyltransferase GAS5 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS5 PE=1 SV=1 - [GAS5_YEAST]	287,46	11,57	1	4	4	8	484	51,8	4,64
P00761	Trypsin OS=Sus scrofa PE=1 SV=1 - [TRYP_PIG]	240,77	16,45	1	3	3	10	231	24,4	7,18
Q48796	Malolactic enzyme OS=Oenococcus oeni GN=mleA PE=3 SV=1 - [MLES_OENOE]	224,72	7,39	1	3	3	4	541	59,1	4,97
P22146	1,3-beta-glucanosyltransferase GAS1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS1 PE=1 SV=2 - [GAS1_YEAST]	195,71	4,47	1	3	3	4	559	59,5	4,67
Q04DH2	Enolase OS=Oenococcus oeni (strain ATCC BAA-331 / PSU-1) GN=eno PE=3 SV=1 - [ENO_OENOB]	193,72	12,72	1	4	4	4	448	48,4	4,82
P15703	Glucan 1,3-beta-glucosidase OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=BGL2 PE=1 SV=1 - [BGL2_YEAST]	182,26	12,14	1	4	4	5	313	34,1	4,51
A6ZL22	Cell wall protein ECM33 OS=Saccharomyces cerevisiae (strain YJM789) GN=ECM33 PE=3 SV=2 - [ECM33_YEAS7]	131,81	6,06	4	3	3	3	429	43,7	4,91
Q04FQ4	Elongation factor Tu OS=Oenococcus oeni (strain ATCC BAA-331 / PSU-1) GN=tuf PE=3 SV=1 - [EFTU_OENOB]	113,44	11,11	1	3	3	3	396	43,6	5,16
P13647	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN]	108,45	7,80	2	4	5	5	590	62,3	7,74
Q04EE1	Chaperone protein DnaK OS=Oenococcus oeni (strain ATCC BAA-331 / PSU-1) GN=dnaK PE=3 SV=1 - [DNAK_OENOB]	96,46	6,00	1	3	3	3	617	66,2	5,06
Q04G42	Phosphoglycerate kinase OS=Oenococcus oeni (strain ATCC BAA-331 / PSU-1) GN=pgk PE=3 SV=1 - [PGK_OENOB]	91,60	9,16	1	4	4	5	404	43,0	5,69
Q9M5X7	Non-specific lipid-transfer protein OS=Malus domestica GN=MALD3 PE=1 SV=1 - [NLTP_MALDO]	90,75	24,35	1	2	2	3	115	11,4	8,92
B5VL27	Cell wall mannoprotein CIS3 OS=Saccharomyces cerevisiae (strain AWRI1631) GN=CIS3 PE=3 SV=1 - [CIS3_YEAS6]	78,37	8,00	2	2	2	3	225	23,0	4,68
Q04G44	Glucose-6-phosphate isomerase OS=Oenococcus oeni (strain ATCC BAA-331 / PSU-1) GN=pgi PE=3 SV=1 - [G6PI_OENOB]	69,80	10,73	1	4	4	4	438	48,6	5,14
P38288	Protein TOS1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=TOS1 PE=1 SV=1 - [TOS1_YEAST]	68,89	5,93	1	2	2	2	455	48,0	4,67

Table 3.S18

CHAPTER 4

PERSISTENCE OF APPLE ALLERGENS (Mal d 2 and Mal d 3) IN THE ITALIAN CIDERS

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Work in progress

INTRODUCTION

Apple (*Malus domestica* L. Borkh) represent one of the most important flowering plant thanks to its great spread, to the relevance of its fruit in the human diet, and to its wide economic interest.

Apples are among the main foods that provide phytochemicals to the human diet (*1*), for this reason its consumption is highly recommended by nutritionists.

Many recent studies suggest that the consumption of apples and derivatives may have beneficial effects in preventing diseases, e.g., the fruit intake might be linked to the reduced risk of several forms of cancer, cardiovascular disease, and asthma (*2-6*).

However, despite the beneficial substances contained in apples and derivatives, it is well known the presence of proteins that can cause allergic reactions in sensitive individuals. Up to 2% of the population of central Europe suffers from allergic reactions to apples (*7*).

Apples, like other fruits belonging to the *Rosaceae* family, can cause the onset of allergic reactions in sensitive individuals (*8, 9*).

Four main allergens have been identified in apple, and different clinical relevance depending on the geographical area is reported. According to the rules of the Committee for Allergen Nomenclature, approved by the World Health Organization and the International Union of Immunological Societies (WHO/IUIS) (*10*), they are named: Mal d 1, Mal d 2, Mal d 3, and Mal d 4 (*8, 11*).

Cider is a popular fermented beverage produced worldwide from different apple cultivars. The main operations of cidermaking are similar, but they may vary among countries and among the producers (*12*). Since the late XVII century the wholesome values of apple cider have been praised, and thanks to "*its specifick Vertues, there is not any Drink more effectual against the* Scurvy. *It is also prevalent against the* Stone*, and by its mundifying qualities, is good against the Diseases of the* Spleen*, and is esteem'd excellent against* Melancholy." (*13*).

As above mentioned, various studies demonstrate a beneficial effect of apples and their derivatives (e.g., cider) on human health (*14*).

Since it is well-known that the technological processes could modify the allergenicity of a food (*15, 16*), it would be worth to study if the cidermaking reduces or eliminates the apple allergens, mantaining the wholesome characteristics of the fruits.

Among the apple allergens, Mal d 2 and Mal d 3 are proteins resistant to the heat treatment and proteases, so they could persist in the apple derivatives and could pass throught the gastrointestinal tract in their native form, thus triggering a systemic allergic reaction.
In the previous chapter, the persistence of apple allergens (Mal d 2 and Mal d 3) has been demonstrated by LC/MS-MS analysis. As a matter of fact, this techniques appears to be the most sensitive technique for the detection of trace amount of proteins, even if it does not give information about the immunological activity of allergens.
For these reasons, studies to evaluate the persistence of immunological activity of these allergens in Italian ciders are required.

MATERIAL AND METHODS

Thirteen Italian ciders were purchased at a local supermarkets, and were probed with antibodies specific for Mal d 2 and Mal d 3 developed in rabbit. The samples were: 1 still cider, 9 sparkling ciders produced with the Charmat method, and 3 sparkling ciders produced by the Champenoise method.
For each cider sample, protein recovery, SDS-PAGE separation, western blot analysis and LC-MS/MS analysis were achieved following the procedures described in "Material and Methods" section of chapter 3: "*Hen egg white lysozyme is a hidden allergen in Italian commercial ciders"*.
Allergens detection was performed overnight with a polyclonal IgG anti-Mal d 2 (PRIMM, Milano, Italy) diluted 1:6000, or with a polyclonal IgG anti-Mal d 3 (kindly supplied by Dr. Gianni Zoccatelli, University of Verona) diluted 1:500, both developed in rabbit. As secondary antibody an anti-rabbit IgG HRP-conjugated (1:5000 dilution, developed in mouse) was used. Peroxidase activity was detected by chemioluminescence with Immobilon Western Chemiluminescent HRP Substrate (Millipore, Milano, Italy) and image acquisition by a Chemidoc XRS apparatus (Bio-Rad).

RESULTS AND DISCUSSION

Figure 4.1 shows the electrophoretic patterns of the commercial ciders considered in this step of the PhD project reaserch. It must be noted that the protein loaded in each lane were recovered from equal volumes of the beverage (5 mL). The samples were: 1 still cider (lane 1), 9 sparkling ciders produced with the Charmat method (lanes 2-10), and 3 sparkling ciders produced by the Champenoise method (lanes 11-13).

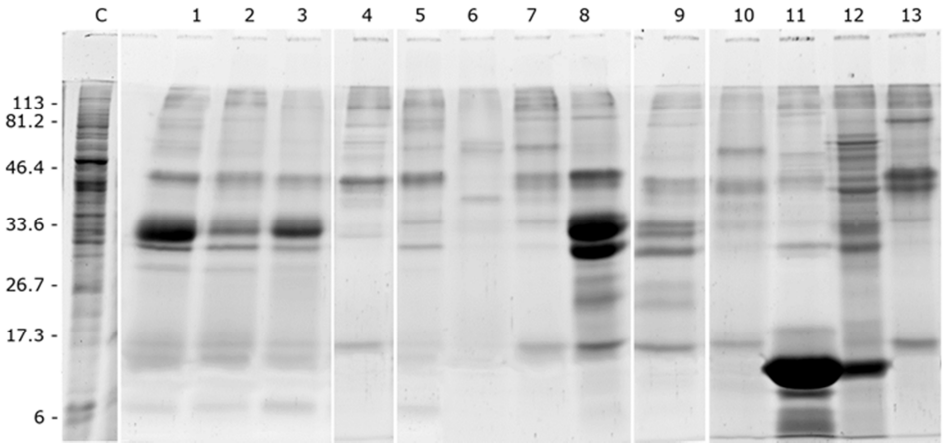

Figure 4.1 SDS-PAGE of cider proteins recovered by KDS, Sypro Ruby stained. Each lane was loaded with protein recovered from 5 ml of the sample. Lane C: total (pulp and skin) apple protein, Lane 1: Still cider, Lanes 2-10: Sparkling ciders Charmat method, Lanes 11-13: Sparkling ciders Champenoise method. M.W. are indicated on the left.

In lane C, a total (pulp and skin) apple extract was loaded. It is evident that protein profile of apple is different from the ones of ciders. This difference could be explained taking into account the potential differences in the raw material used (i.e., apple varieties, ripening state…) and/or the effect of the different cidermaking processes applied (e.g., enzymatic treatment, clarification and fining steps…). Data dealing with the apple varieties and characteristics (e.g., ripening state, acidity, sugar and polyphenols content…), blend, and technological procedures are being collected. With regard to the effect of cidermaking steps on cider protein profiles, it must be noted that, even if samples in lanes 1-3, 5 and 7 are ciders belonging to the same brand (brand A), their protein profiles show some differences. In particular, the band pair migrating at about 31 kDa (M.W. corresponding to Mal d 2, see below) decreases progressively its intensity according to the order samples 1 > 3 > 2 > 5 > 7 . Whereas the band at about 9 kDa (M.W. corresponding to Mal d 3, see below) seems to be present in all samples of brand A ciders, except from sample 7. Similar considerations could

be made for samples 8 and 9 that are produced by the same brand (brand D). In this case, their protein profiles are similar except from the intensity of the bands, that strongly decreases in sample 9.

Ciders 6 and 11 belong to the brand C, and show a very different protein profile. As a matter of fact, a marked band at about 14 kDa (corresponding to the M.W. of hen egg white lysozyme (HEWL), as demonstrated in chapter 3) is the most represented protein in sample 11, and it is also evident in sample 12 (produced by brand F). As a matter of fact, HEWL is used as a processing aids in winemaking to control fermentation and to avoid spoilage bacteria. Its use is allowed and regulated by EU directives, being an allergen. However, the use of HEWL must be declared on label, as stated by EU Regulation 1169/2011. It must be noted that ciders 11 and 12 are produced with the Champenoise method, so the HEWL presence could be related to the complexity of process that could expose the cider to the risk of spoilage. In addition, its use can be avoided as in the case of sample 13, that is a Champenoise cider, in which neither the SDS-PAGE nor the LC/MS-MS analyses detected HEWL (see Table 4.1 and Tables 4.S11-S13 of Supporting Materials).

		IMMUNOBLOT ANALYSIS			**LC-MS/MS ANALYSIS**		
Brand	**Ciders**	**Mal d 2**	**Mal d 3**	**HEWL**	**Mal d 2**	**Mal d 3**	**HEWL**
A	1	+	+	n.d.	+	+	+
A	2	+	n.d.	n.d.	+	+	+
A	3	+	+	n.d.	+	+	n.d.
B	4	n.d.	n.d.	n.d.	+	+	+
A	5	n.d.	+	n.d.	+	+	+
C	6	n.d.	n.d.	n.d.	+	n.d.	+
A	7	n.d.	n.d.	n.d.	+	n.d.	+
D	8	+	n.d.	n.d.	+	n.d.	n.d.
D	9	+	n.d.	n.d.	+	n.d.	n.d.
E	10	n.d.	n.d.	n.d.	+	+	n.d.
C	11	n.d.	n.d.	+	+	n.d.	+
F	12	+	n.d.	+	+	n.d.	+
G	13	n.d.	+	n.d.	n.d.	+	n.d.

Table 4.1 Ciders positive to Mal d 2, Mal d 3 and/or HEWL detection (see chapter 3) with western blot and LC-MS/MS techniques.

Figures 4.2A and 4.2B show the immunodetection of Mal d 2 and Mal d 3 proteins, respectively. Lane C was loaded with a total apple extract, and the M.W.s of the signals detected correspond to those reported in the literature for Mal d 2 (~ 31 kDa) and Mal d 3 (9 kDa).

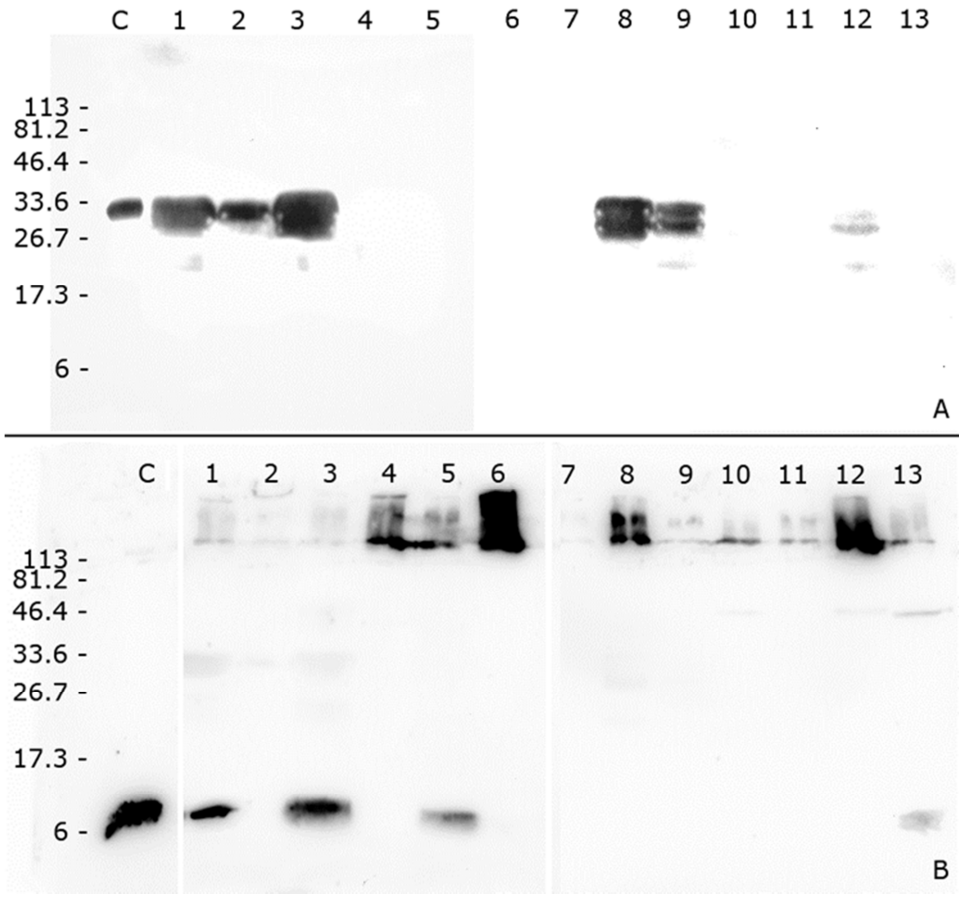

Figure 4.2 Western blot of cider proteins recovered by KDS, resolved by SDS-PAGE (14%T) and detected with antibodies specific for Mal d 2 (A) and for Mal d 3 (B). Each lane was loaded with protein recovered from 5 ml of the sample. Lane C: total (pulp and skin) apple protein, Lane 1: Still cider, Lanes 2-10: Sparkling ciders Charmat method, Lanes 11-13: Sparkling ciders Champenoise method. M.W.s are indicated on the left.

In Figure 4.2A, six samples out of thirteen ciders were positive for immunodetection of Mal d 2 protein. As reported by Marzban et al. (*17, 18*), oxidizing/reducing and thermal treatments may alter the electrophoretic mobility of Mal d 2, generating a band pair in all samples except from sample 2. Moreover, the analysis was achieved in reducing condition because it increases the immunoreactivity of the protein as described by Marzban et al. (*17*). The amount of protein stained with Sypro Ruby (Figure 4.1) seems to directly correlate with the intensity of the immunodetected signals of the western blot (Figure 4.2A).

The LC-MS/MS analysis of ciders protein were performed and validated as reported in "Result and Discussion" of chapter 3. Data obtained are summerized in Table 4.1, and it is important to underline that there is a full correspondence between the immunodetection of Mal d 2 (i.e., lanes 1-3, 8, 9, and 12 of Figure 4.2A) and the LC-MS/MS data. However, thanks to the higher sensitivity of MS, all ciders (except from sample 13) are positive to the presence of Mal d 2.

In Figure 4.2B the signals of Mal d 3 immunodetection are evident. Four ciders (lanes 1, 3, 5 and 13) out of thirteen show a signal with a M.W. corresponding to about 9 kDa. Perhaps it is no accident that ciders 1, 3, and 5 belong to the same brand (brand A). But, in this case, the intesity of the immunodetection seems not to be correlated to the signal revealed with Sypro Ruby on SDS-PAGE (Figure 4.1). As a matter of fact, samples 1-3, and 5 show a faint band at about 9 kDa that could correspond to Mal d 3. However, in cider 2 no signal was immunodetected (Figure 4.2B), even if the Sypro Ruby stain reveals a protein banding at about 9 kDa. This suggests that the protein could be present but not immunologically active, and this inactivation could be a consequence of the epitopes modification due to technological process (e.g., Maillard reaction) (*19*). Alternatively, the protein detected by Sypro Ruby could be a protein that co-migrates with Mal d 3, but not immunologically correlated to the apple allergen. Other signals of immunodetection are visible in the stacking gel for numerous samples, in particular these are stronger in ciders 6, 8 and 12 (Figure 4.2B). It could be hypotesized that technological processes might generate polyphenol-protein aggregates. However, it must be noted that no smears corresponding to proteins are detected by Sypro Ruby stain in the stacking gel (Figure 4.1). It is interesting to note that LC-MS/MS analysis did not reveal Mal d 3 protein in cider 6, 8 and 12 (Table 4.1). To explain this apparent paradox, it must be noted that the LC-MS/MS analysis implies a preparative SDS-PAGE step, in which proteins are allowed to enter the gel for approximately 1 cm, then a fragment of the resolving gel of about 10 mm^2 is excised from each lane (as described in the "Material and Methods" section of chapter 3). So, these aggregates in the stacking gel are then excluded from the MS analysis. Further studies should be performed to better explain the origin of these signals.

As regarding the LC-MS/MS data, all information about proteins and peptides identification is reported in Tables 4.S1-S13 of Supporting Materials. In general, the type of proteins detected in each cider are variable, with some contaminating proteins (namely human

keratins) being almost invariably the dominant species in the samples. Whether these contaminants are really present in the ciders or they derive from sample preparation and manipulation procedures it cannot be established on the base of the present results. However, the fact that in some ciders the level of contaminant proteins appears to be much higher than in other samples suggests that (at least partially) the source of contamination could be located during the phases of apple harvest and cider production.

The first hypotesis is strenghtened by observing that samples 8 and 9, both produced by brand D, are contaminated by keratins from ovines (*Ovis aries*), suggesting that a sheep farm could be present near the cidermaking cellar.

Beside contaminant proteins of human origin, several other proteins could be identified belonging to apple (*Malus domestica*), yeast (*Saccharomyces cerevisiae*), bacteria (*Lactobacillus, Oenoccocus*), and mould (*Aspergillus*). Obviously the presence of apple proteins Mal d 2 and Mal d 3 had to be expected, because they are described to be resistant to technological processes and proteases whereas Mal d 1 and Mal d 4 are absent due to their lability. The presence of proteins from microorganisms is not surprising, as they are involved in alcoholic fermentation (*Saccharomyces*) and in malolactic fermentation (*Lactobacillus, Oenoccocus*), which is sometimes desirable to decrease cider acidity (*20*). The presence of proteins derived from *Aspergillus* in 6 samples (i.e., samples 1, 2, 4, 10, 11 and 12) can be explained by considering that pectolytic enzymes (derived by this fungus) are added during the cider production process to enhance apple juice yield and prevent haze (*21*).

In conclusion, some cidermaking technologies seem to eliminate the allergens of apple, even if hidden allergens could be introduced. However, data reported in this study could be better understood when the information about apple characteristics and technological procedures of cidermaking of each sample will be collected. In this case, it would be possible to produce a wholesome and ipoallergenic beverage, that could be drunk also by sensitive to apple individuals. Nevertheless, experiments with apple allergic patients' sera should be performed.

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SUPPLEMENTARY MATERIALS

Table 4.S1-S13: list of proteins identified by LC-MS/MS in commercial Italian ciders. Each sheet refers to a different commercial cider (samples 1-13). For each protein the score, the number of peptides and the sequence coverage are reported, together with the list of all peptides detected, the confidence of their identification, the amino acid sequence, and the Mascot score.

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
Q9FSG7	Thaumatin-like protein 1a OS=Malus domestica GN=TL1 PE=1 SV=1 - [TP1A_MALDO]	968,46	48,78	1	3	7	42	246	25,7	5,15
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	645,74	25,47	1	12	13	16	644	66,0	8,12
P83336	Thaumatin-like protein 1b (Fragment) OS=Malus domestica PE=2 SV=1 - [TP1B_MALDO]	639,85	41,98	1	2	6	30	212	22,1	4,54
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	536,59	27,74	1	15	15	17	584	58,8	5,21
P00698	Lysozyme C OS=Gallus gallus GN=LYZ PE=1 SV=1 - [LYSC_CHICK]	524,20	60,54	1	7	7	13	147	16,2	9,07
P00830	ATP synthase subunit beta, mitochondrial OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=ATP2 PE=1 SV=2 - [ATPB_YEAST]	517,26	19,77	1	7	7	14	511	54,8	5,71
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	419,53	22,85	1	12	13	17	639	65,4	8,00
Q6CQ83	Heat shock protein SSB OS=Kluyveromyces lactis (strain ATCC 8585 / CBS 2359 / DSM 70799 / NBRC 1267 / NRRL Y-1140 / WM37) GN=SSB PE=3 SV=3 - [HSP75_KLULA]	377,95	11,26	1	3	5	8	613	66,2	5,43
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	356,81	14,61	1	6	6	8	623	62,0	5,24
A2R3I1	Probable pectin lyase A OS=Aspergillus niger (strain CBS 513.88 / FGSC A1513) GN=pelA PE=3 SV=1 - [PELA_ASPNC]	336,56	14,51	2	4	4	8	379	39,8	4,34
P87222	Heat shock protein SSB1 OS=Candida albicans (strain WO-1) GN=SSB1 PE=3 SV=2 - [HSP75_CANAW]	316,35	10,60	1	2	4	7	613	66,4	5,38
P49375	ATP synthase subunit alpha, mitochondrial OS=Kluyveromyces lactis (strain ATCC 8585 / CBS 2359 / DSM 70799 / NBRC 1267 / NRRL Y-1140 / WM37) GN=ATP1 PE=1 SV=1 - [ATPA_KLULA]	270,10	11,31	1	5	5	7	548	59,1	9,36
P10591	Heat shock protein SSA1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=SSA1 PE=1 SV=4 - [HSP71_YEAST]	252,74	8,88	5	5	5	8	642	69,6	5,11
P00761	Trypsin OS=Sus scrofa PE=1 SV=1 - [TRYP_PIG]	210,13	16,45	1	3	3	6	231	24,4	7,18
P0CY35	Elongation factor 1-alpha 1 OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=TEF1 PE=3 SV=1 - [EF1A1_CANAL]	203,29	14,63	2	5	5	9	458	50,0	9,03
Q874B9	Elongation factor 2 OS=Komagataella pastoris GN=EFT1 PE=3 SV=1 - [EF2_PICPA]	182,91	6,89	1	4	4	4	842	93,4	6,71
Q07421	Plasma membrane ATPase OS=Ajellomyces capsulatus GN=PMA1 PE=3 SV=1 - [PMA1_AJECA]	153,15	1,75	1	2	2	2	916	98,8	5,43
P27080	ADP,ATP carrier protein OS=Chlamydomonas reinhardtii GN=ABT PE=2 SV=1 - [ADT_CHLRE]	145,58	7,79	1	2	2	3	308	33,5	9,74
P15703	Glucan 1,3-beta-glucosidase OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=BGL2 PE=1 SV=1 - [BGL2_YEAST]	142,65	16,93	1	4	4	4	313	34,1	4,51
Q756H2	Enolase OS=Ashbya gossypii (strain ATCC 10895 / CBS 109.51 / FGSC 9923 / NRRL Y-1056) GN=ENO PE=3 SV=1 - [ENO_ASHGO]	132,29	7,55	1	2	2	3	437	46,6	5,55
Q08193	1,3-beta-glucanosyltransferase GAS5 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS5 PE=1 SV=1 - [GAS5_YEAST]	119,68	6,40	1	2	2	2	484	51,8	4,64
O74258	Actin OS=Ogataea parapolymorpha (strain DL-1 / ATCC 26012 / NRRL Y-7560) GN=ACT PE=3 SV=2 - [ACT_OGAPD]	109,69	21,01	6	6	6	7	376	41,7	5,57
Q92211	Glyceraldehyde-3-phosphate dehydrogenase OS=Candida albicans (strain WO-1) GN=TDH1 PE=1 SV=2 - [G3P_CANAW]	107,38	11,64	1	2	3	4	335	35,8	7,12
O13639	Adenosylhomocysteinase OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=pi047 PE=3 SV=1 - [SAHH_SCHPO]	104,24	5,54	1	2	2	2	433	47,4	5,94

Table 4.S1

Q12230	Sphingolipid long chain base-responsive protein LSP1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=LSP1 PE=1 SV=1 - [LSP1_YEAST]	103,02	7,92	1	2	2	2	341	38,0	4,70
P00890	Citrate synthase, mitochondrial OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=CIT1 PE=1 SV=2 - [CISY1_YEAST]	101,44	6,68	1	2	2	2	479	53,3	8,29
P0CS90	Heat shock protein SSC1, mitochondrial OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=SSC1 PE=1 SV=1 - [HSP77_YEAST]	100,57	4,59	3	2	2	2	654	70,6	5,59
P18819	NADP-specific glutamate dehydrogenase OS=Emericella nidulans (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) GN=gdhA PE=3 SV=2 - [DHE4_EMENI]	97,71	6,75	1	2	2	2	459	49,6	6,47
Q9M5X7	Non-specific lipid-transfer protein OS=Malus domestica GN=MALD3 PE=1 SV=1 - [NLTP_MALDO]	93,33	24,35	1	2	2	2	115	11,4	8,92
A6ZL22	Cell wall protein ECM33 OS=Saccharomyces cerevisiae (strain YJM789) GN=ECM33 PE=3 SV=2 - [ECM33_YEAS7]	82,45	5,83	4	2	2	2	429	43,7	4,91
O59841	Glyceraldehyde-3-phosphate dehydrogenase OS=Ogataea parapolymorpha (strain DL-1 / ATCC 26012 / NRRL Y-7560) GN=GPD PE=3 SV=2 - [G3P_OGAPD]	80,80	10,45	1	2	3	3	335	35,9	7,17
O75000	60S ribosomal protein L12 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=rpl1201 PE=1 SV=1 - [RL12_SCHPO]	71,21	14,55	1	2	2	2	165	17,7	9,31
P0CX55	40S ribosomal protein S18-A OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=RPS18A PE=1 SV=1 - [RS18A_YEAST]	69,41	13,01	1	2	2	2	146	17,0	10,27
Q6BXM5	60S ribosomal protein L3 OS=Debaryomyces hansenii (strain ATCC 36239 / CBS 767 / JCM 1990 / NBRC 0083 / IGC 2968) GN=RPL3 PE=3 SV=1 - [RL3_DEBHA]	64,31	4,88	1	2	2	5	389	43,7	10,32
P0CX47	40S ribosomal protein S11-A OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=RPS11A PE=1 SV=1 - [RS11A_YEAST]	60,27	12,82	1	2	2	2	156	17,7	10,78
P46598	Heat shock protein 90 homolog OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=HSP90 PE=1 SV=1 - [HSP90_CANAL]	52,62	3,39	1	2	2	2	707	80,8	4,88
P0CG84	Polyubiquitin (Fragment) OS=Nicotiana sylvestris GN=UBI4 PE=2 SV=1 - [UBI4P_NICSY]	50,64	38,46	53	2	2	3	377	42,2	7,58
P38288	Protein TOS1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=TOS1 PE=1 SV=1 - [TOS1_YEAST]	40,19	8,79	1	2	2	2	455	48,0	4,67
P0CX45	60S ribosomal protein L2-A OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=RPL2A PE=1 SV=1 - [RL2A_YEAST]	33,63	12,99	3	2	2	2	254	27,4	11,11

...continua Table 4.S1

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	1606,68	45,34	1	21	24	45	644	66,0	8,12
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	1457,02	52,27	1	20	25	42	639	65,4	8,00
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	1350,05	54,90	1	18	18	36	623	62,0	5,24
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	1014,13	37,67	1	17	18	29	584	58,8	5,21
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	592,13	36,44	1	7	13	18	472	51,5	5,16
P08779	Keratin, type I cytoskeletal 16 OS=Homo sapiens GN=KRT16 PE=1 SV=4 - [K1C16_HUMAN]	494,60	29,60	1	5	11	14	473	51,2	5,05
P13647	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN]	427,29	24,75	1	10	13	14	590	62,3	7,74
P83336	Thaumatin-like protein 1b (Fragment) OS=Malus domestica PE=2 SV=1 - [TP1B_MALDO]	409,83	22,17	1	1	4	12	212	22,1	4,54
Q9FSG7	Thaumatin-like protein 1a OS=Malus domestica GN=TL1 PE=1 SV=1 - [TP1A_MALDO]	385,34	36,99	1	2	5	12	246	25,7	5,15
P15703	Glucan 1,3-beta-glucosidase OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=BGL2 PE=1 SV=1 - [BGL2_YEAST]	283,35	21,09	1	6	6	8	313	34,1	4,51
P02538	Keratin, type II cytoskeletal 6A OS=Homo sapiens GN=KRT6A PE=1 SV=3 - [K2C6A_HUMAN]	256,05	13,30	1	3	7	8	564	60,0	8,00
P00698	Lysozyme C OS=Gallus gallus GN=LYZ PE=1 SV=1 - [LYSC_CHICK]	221,87	33,33	1	4	4	5	147	16,2	9,07
P00761	Trypsin OS=Sus scrofa PE=1 SV=1 - [TRYP_PIG]	215,78	16,45	1	3	3	7	231	24,4	7,18
Q08193	1,3-beta-glucanosyltransferase GAS5 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS5 PE=1 SV=1 - [GAS5_YEAST]	207,72	6,40	1	2	2	3	484	51,8	4,64
A2R3I1	Probable pectin lyase A OS=Aspergillus niger (strain CBS 513.88 / FGSC A1513) GN=pelA PE=3 SV=1 - [PELA_ASPNC]	201,80	14,51	2	4	4	6	379	39,8	4,34
P22146	1,3-beta-glucanosyltransferase GAS1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS1 PE=1 SV=2 - [GAS1_YEAST]	198,40	4,47	1	3	3	4	559	59,5	4,67
Q9M5X7	Non-specific lipid-transfer protein OS=Malus domestica GN=MALD3 PE=1 SV=1 - [NLTP_MALDO]	121,74	36,52	1	3	3	3	115	11,4	8,92
P53301	Probable glycosidase CRH1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=CRH1 PE=1 SV=1 - [CRH1_YEAST]	111,12	10,45	1	5	5	5	507	52,7	4,65
Q86YZ3	Hornerin OS=Homo sapiens GN=HRNR PE=1 SV=2 - [HORN_HUMAN]	99,16	4,49	1	2	2	5	2850	282,2	10,04
P81605	Dermcidin OS=Homo sapiens GN=DCD PE=1 SV=2 - [DCD_HUMAN]	92,42	20,00	1	2	2	2	110	11,3	6,54
P38288	Protein TOS1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=TOS1 PE=1 SV=1 - [TOS1_YEAST]	87,72	5,93	1	2	2	2	455	48,0	4,67
B5VL27	Cell wall mannoprotein CIS3 OS=Saccharomyces cerevisiae (strain AWRI1631) GN=CIS3 PE=3 SV=1 - [CIS3_YEAS6]	65,93	8,00	2	2	2	2	225	23,0	4,68

Table 4.S2

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	1456,28	42,70	1	19	22	40	644	66,0	8,12
P83336	Thaumatin-like protein 1b (Fragment) OS=Malus domestica PE=2 SV=1 - [TP1B_MALDO]	878,74	41,98	1	2	6	33	212	22,1	4,54
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	875,18	32,91	1	11	11	18	623	62,0	5,24
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	808,82	38,03	1	16	21	27	639	65,4	8,00
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	762,08	30,48	1	15	16	21	584	58,8	5,21
Q9FSG7	Thaumatin-like protein 1a OS=Malus domestica GN=TL1 PE=1 SV=1 - [TP1A_MALDO]	727,24	34,96	1	2	6	27	246	25,7	5,15
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	405,19	26,06	1	4	10	13	472	51,5	5,16
P08779	Keratin, type I cytoskeletal 16 OS=Homo sapiens GN=KRT16 PE=1 SV=4 - [K1C16_HUMAN]	331,22	23,47	1	3	9	11	473	51,2	5,05
P13647	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN]	296,47	17,97	1	8	10	11	590	62,3	7,74
P04259	Keratin, type II cytoskeletal 6B OS=Homo sapiens GN=KRT6B PE=1 SV=5 - [K2C6B_HUMAN]	226,34	11,17	1	2	6	7	564	60,0	8,00
O43790	Keratin, type II cuticular Hb6 OS=Homo sapiens GN=KRT86 PE=1 SV=1 - [KRT86_HUMAN]	192,54	9,47	1	3	3	3	486	53,5	5,66
Q9M5X7	Non-specific lipid-transfer protein OS=Malus domestica GN=MALD3 PE=1 SV=1 - [NLTP_MALDO]	154,43	36,52	1	3	3	4	115	11,4	8,92
P00761	Trypsin OS=Sus scrofa PE=1 SV=1 - [TRYP_PIG]	137,07	16,45	1	3	3	8	231	24,4	7,18
P15703	Glucan 1,3-beta-glucosidase OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=BGL2 PE=1 SV=1 - [BGL2_YEAST]	82,04	7,99	1	2	2	2	313	34,1	4,51
P81605	Dermcidin OS=Homo sapiens GN=DCD PE=1 SV=2 - [DCD_HUMAN]	80,40	20,00	1	2	2	2	110	11,3	6,54
O76011	Keratin, type I cuticular Ha4 OS=Homo sapiens GN=KRT34 PE=2 SV=2 - [KRT34_HUMAN]	66,78	4,59	1	2	2	2	436	49,4	5,06
Q05091	Polygalacturonase inhibitor OS=Pyrus communis GN=PGIP PE=1 SV=1 - [PGIP_PYRCO]	40,50	6,67	1	2	2	2	330	36,5	6,67
Q86YZ3	Hornerin OS=Homo sapiens GN=HRNR PE=1 SV=2 - [HORN_HUMAN]	0,00	5,54	1	2	2	2	2850	282,2	10,04

Table 4.S3

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	1212,88	37,58	1	15	16	31	644	66,0	8,12
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	891,67	32,19	1	15	17	29	584	58,8	5,21
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	834,21	31,14	1	12	12	23	623	62,0	5,24
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	731,20	32,71	1	14	16	23	639	65,4	8,00
P08779	Keratin, type I cytoskeletal 16 OS=Homo sapiens GN=KRT16 PE=1 SV=4 - [K1C16_HUMAN]	462,19	34,88	1	6	13	17	473	51,2	5,05
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	343,58	20,76	1	2	9	14	472	51,5	5,16
P83336	Thaumatin-like protein 1b (Fragment) OS=Malus domestica PE=2 SV=1 - [TP1B_MALDO]	288,08	22,17	1	4	4	11	212	22,1	4,54
A2R3I1	Probable pectin lyase A OS=Aspergillus niger (strain CBS 513.88 / FGSC A1513) GN=pelA PE=3 SV=1 - [PELA_ASPNC]	278,86	20,84	1	5	5	8	379	39,8	4,34
Q08193	1,3-beta-glucanosyltransferase GAS5 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS5 PE=1 SV=1 - [GAS5_YEAST]	277,47	11,57	1	4	4	7	484	51,8	4,64
P00761	Trypsin OS=Sus scrofa PE=1 SV=1 - [TRYP_PIG]	258,95	16,45	1	3	3	12	231	24,4	7,18
P02538	Keratin, type II cytoskeletal 6A OS=Homo sapiens GN=KRT6A PE=1 SV=3 - [K2C6A_HUMAN]	249,29	17,91	1	6	8	9	564	60,0	8,00
P22146	1,3-beta-glucanosyltransferase GAS1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS1 PE=1 SV=2 - [GAS1_YEAST]	222,21	4,83	1	4	4	6	559	59,5	4,67
A6ZL22	Cell wall protein ECM33 OS=Saccharomyces cerevisiae (strain YJM789) GN=ECM33 PE=3 SV=2 - [ECM33_YEAS7]	196,38	9,56	4	4	4	6	429	43,7	4,91
P13647	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN]	183,24	10,85	1	5	6	7	590	62,3	7,74
A2QK82	Probable pectinesterase A OS=Aspergillus niger (strain CBS 513.88 / FGSC A1513) GN=pmeA PE=3 SV=1 - [PMEA_ASPNC]	171,92	9,79	1	2	2	3	327	34,6	4,39
P81605	Dermcidin OS=Homo sapiens GN=DCD PE=1 SV=2 - [DCD_HUMAN]	109,40	20,00	1	2	2	3	110	11,3	6,54
P53301	Probable glycosidase CRH1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=CRH1 PE=1 SV=1 - [CRH1_YEAST]	108,22	6,31	1	3	3	3	507	52,7	4,65
A2QK83	Probable endo-xylogalacturonan hydrolase A OS=Aspergillus niger (strain CBS 513.88 / FGSC A1513) GN=xghA PE=3 SV=1 - [XGHA_ASPNC]	101,44	5,67	2	2	2	2	406	42,1	4,51
P00698	Lysozyme C OS=Gallus gallus GN=LYZ PE=1 SV=1 - [LYSC_CHICK]	101,30	19,05	2	2	2	2	147	16,2	9,07
Q9M5X7	Non-specific lipid-transfer protein OS=Malus domestica GN=MALD3 PE=1 SV=1 - [NLTP_MALDO]	81,06	24,35	1	2	2	2	115	11,4	8,92
Q03674	Lysophospholipase 2 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=PLB2 PE=1 SV=1 - [PLB2_YEAST]	42,07	2,83	1	2	2	2	706	75,4	4,70

Table 4.S4

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	1325,42	40,84	1	19	21	37	644	66,0	8,12
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	945,76	41,00	1	15	20	32	639	65,4	8,00
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	929,39	35,45	1	17	18	29	584	58,8	5,21
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	773,18	30,34	1	12	12	23	623	62,0	5,24
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	510,91	28,39	1	4	11	16	472	51,5	5,16
P08779	Keratin, type I cytoskeletal 16 OS=Homo sapiens GN=KRT16 PE=1 SV=4 - [K1C16_HUMAN]	449,51	34,46	1	6	13	16	473	51,2	5,05
Q08193	1,3-beta-glucanosyltransferase GAS5 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS5 PE=1 SV=1 - [GAS5_YEAST]	297,59	11,57	1	4	4	7	484	51,8	4,64
P48668	Keratin, type II cytoskeletal 6C OS=Homo sapiens GN=KRT6C PE=1 SV=3 - [K2C6C_HUMAN]	266,13	14,89	1	4	8	10	564	60,0	8,00
P13647	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN]	259,93	20,34	1	8	11	11	590	62,3	7,74
P00698	Lysozyme C OS=Gallus gallus GN=LYZ PE=1 SV=1 - [LYSC_CHICK]	229,50	25,17	1	3	3	6	147	16,2	9,07
P00761	Trypsin OS=Sus scrofa PE=1 SV=1 - [TRYP_PIG]	179,48	16,45	1	3	3	8	231	24,4	7,18
Q9M5X7	Non-specific lipid-transfer protein OS=Malus domestica GN=MALD3 PE=1 SV=1 - [NLTP_MALDO]	149,96	46,09	1	4	4	5	115	11,4	8,92
P83336	Thaumatin-like protein 1b (Fragment) OS=Malus domestica PE=2 SV=1 - [TP1B_MALDO]	144,30	22,17	1	4	4	5	212	22,1	4,54
P22146	1,3-beta-glucanosyltransferase GAS1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS1 PE=1 SV=2 - [GAS1_YEAST]	71,88	4,47	1	2	2	2	559	59,5	4,67
P15703	Glucan 1,3-beta-glucosidase OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=BGL2 PE=1 SV=1 - [BGL2_YEAST]	59,74	9,27	1	2	2	2	313	34,1	4,51

Table 4.S5

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	1887,56	50,31	1	23	28	62	644	66,0	8,12
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	1507,60	54,62	1	21	27	48	639	65,4	8,00
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	1368,66	59,07	1	20	20	42	623	62,0	5,24
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	1013,66	32,19	1	17	19	31	584	58,8	5,21
P08779	Keratin, type I cytoskeletal 16 OS=Homo sapiens GN=KRT16 PE=1 SV=4 - [K1C16_HUMAN]	877,98	50,32	1	11	20	32	473	51,2	5,05
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	764,05	39,41	1	6	15	23	472	51,5	5,16
P02538	Keratin, type II cytoskeletal 6A OS=Homo sapiens GN=KRT6A PE=1 SV=3 - [K2C6A_HUMAN]	619,49	34,04	1	3	19	25	564	60,0	8,00
P04259	Keratin, type II cytoskeletal 6B OS=Homo sapiens GN=KRT6B PE=1 SV=5 - [K2C6B_HUMAN]	584,31	32,45	1	2	18	25	564	60,0	8,00
P13647	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN]	570,15	31,36	1	12	18	21	590	62,3	7,74
P00761	Trypsin OS=Sus scrofa PE=1 SV=1 - [TRYP_PIG]	409,86	25,11	1	4	4	22	231	24,4	7,18
P00698	Lysozyme C OS=Gallus gallus GN=LYZ PE=1 SV=1 - [LYSC_CHICK]	250,09	48,98	1	5	5	8	147	16,2	9,07
P83336	Thaumatin-like protein 1b (Fragment) OS=Malus domestica PE=2 SV=1 - [TP1B_MALDO]	207,35	22,17	1	4	4	7	212	22,1	4,54
P81605	Dermcidin OS=Homo sapiens GN=DCD PE=1 SV=2 - [DCD_HUMAN]	123,43	20,00	1	2	2	3	110	11,3	6,54
P15924	Desmoplakin OS=Homo sapiens GN=DSP PE=1 SV=3 - [DESP_HUMAN]	89,72	1,08	1	3	3	3	2871	331,6	6,81
P14923	Junction plakoglobin OS=Homo sapiens GN=JUP PE=1 SV=3 - [PLAK_HUMAN]	80,41	2,95	5	2	2	2	745	81,7	6,14
P0CT31	Elongation factor 1-alpha OS=Dictyostelium discoideum GN=eef1a1 PE=1 SV=1 - [EF1A1_DICDI]	73,37	4,19	1	2	2	2	453	49,6	8,98
Q86YZ3	Hornerin OS=Homo sapiens GN=HRNR PE=1 SV=2 - [HORN_HUMAN]	72,95	6,77	1	4	4	6	2850	282,2	10,04
Q5T749	Keratinocyte proline-rich protein OS=Homo sapiens GN=KPRP PE=1 SV=1 - [KPRP_HUMAN]	31,66	3,97	1	2	2	2	579	64,1	8,27

Table 4.S6

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	1495,02	45,81	1	19	22	42	644	66,0	8,12
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	861,86	36,76	1	14	14	25	623	62,0	5,24
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	781,58	42,41	1	17	21	32	639	65,4	8,00
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	764,39	26,03	1	14	15	27	584	58,8	5,21
Q9FSG7	Thaumatin-like protein 1a OS=Malus domestica GN=TL1 PE=1 SV=1 - [TP1A_MALDO]	477,71	27,64	1	5	5	19	246	25,7	5,15
Q08193	1,3-beta-glucanosyltransferase GAS5 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS5 PE=1 SV=1 - [GAS5_YEAST]	361,32	11,57	1	4	4	9	484	51,8	4,64
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	294,01	19,28	1	6	7	12	472	51,5	5,16
P00761	Trypsin OS=Sus scrofa PE=1 SV=1 - [TRYP_PIG]	226,39	16,45	1	3	3	10	231	24,4	7,18
A6ZL22	Cell wall protein ECM33 OS=Saccharomyces cerevisiae (strain YJM789) GN=ECM33 PE=3 SV=2 - [ECM33_YEAS7]	221,91	6,06	4	3	3	7	429	43,7	4,91
P02538	Keratin, type II cytoskeletal 6A OS=Homo sapiens GN=KRT6A PE=1 SV=3 - [K2C6A_HUMAN]	208,45	9,40	2	2	5	6	564	60,0	8,00
P00698	Lysozyme C OS=Gallus gallus GN=LYZ PE=1 SV=1 - [LYSC_CHICK]	202,48	25,17	1	3	3	4	147	16,2	9,07
P15703	Glucan 1,3-beta-glucosidase OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=BGL2 PE=1 SV=1 - [BGL2_YEAST]	199,45	15,97	1	5	5	9	313	34,1	4,51
P22146	1,3-beta-glucanosyltransferase GAS1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS1 PE=1 SV=2 - [GAS1_YEAST]	189,78	5,90	1	4	4	6	559	59,5	4,67
P13647	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN]	162,44	10,51	1	4	6	6	590	62,3	7,74
P53301	Probable glycosidase CRH1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=CRH1 PE=1 SV=1 - [CRH1_YEAST]	94,70	11,64	1	6	6	6	507	52,7	4,65
P38288	Protein TOS1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=TOS1 PE=1 SV=1 - [TOS1_YEAST]	83,69	8,13	1	3	3	3	455	48,0	4,67
B5VL27	Cell wall mannoprotein CIS3 OS=Saccharomyces cerevisiae (strain AWRI1631) GN=CIS3 PE=3 SV=1 - [CIS3_YEAS6]	78,67	8,00	2	2	2	4	225	23,0	4,68

Table 4.S7

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
P02534	Keratin, type I microfibrillar 48 kDa, component 8C-1 OS=Ovis aries PE=1 SV=2 - [K1M1_SHEEP]	1477,70	63,83	1	9	28	57	412	46,6	4,81
Q148H4	Keratin, type II cuticular Hb1 OS=Bos taurus GN=KRT81 PE=2 SV=1 - [KRT81_BOVIN]	1452,34	42,20	1	5	26	66	500	54,6	5,68
A4FUZ0	Keratin, type II cuticular Hb3 OS=Bos taurus GN=KRT83 PE=2 SV=1 - [KRT83_BOVIN]	1348,86	48,88	1	1	28	63	493	54,0	5,48
P15241	Keratin, type II microfibrillar, component 7C OS=Ovis aries PE=1 SV=1 - [K2M2_SHEEP]	1324,24	45,42	1	2	26	59	491	53,6	5,57
P25691	Keratin, type II microfibrillar, component 5 OS=Ovis aries PE=1 SV=1 - [K2M3_SHEEP]	1024,47	41,43	1	11	23	53	502	55,2	6,46
P25690	Keratin, type I microfibrillar, 47.6 kDa OS=Ovis aries PE=3 SV=2 - [K1M2_SHEEP]	962,13	49,75	1	7	21	43	404	46,0	4,93
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	877,22	43,94	1	19	21	28	644	66,0	8,12
A5A6M5	Keratin, type I cuticular Ha1 OS=Pan troglodytes GN=KRT31 PE=2 SV=1 - [K1H1_PANTR]	731,33	26,20	2	2	13	29	416	47,2	4,88
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	687,31	29,11	1	17	17	21	584	58,8	5,21
Q9FSG7	Thaumatin-like protein 1a OS=Malus domestica GN=TL1 PE=1 SV=1 - [TP1A_MALDO]	584,69	48,78	1	3	7	21	246	25,7	5,15
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	518,84	23,92	1	10	10	15	623	62,0	5,24
P83336	Thaumatin-like protein 1b (Fragment) OS=Malus domestica PE=2 SV=1 - [TP1B_MALDO]	515,77	41,98	1	2	6	16	212	22,1	4,54
Q0P5J7	Keratin, type I cuticular Ha5 OS=Bos taurus GN=KRT35 PE=2 SV=2 - [KRT35_BOVIN]	418,57	27,09	1	6	11	21	454	49,9	5,26
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	398,86	24,57	1	11	13	14	639	65,4	8,00
P00761	Trypsin OS=Sus scrofa PE=1 SV=1 - [TRYP_PIG]	269,36	16,45	1	3	3	14	231	24,4	7,18
Q6R648	Keratin-associated protein 11-1 OS=Capra hircus GN=KRTAP11-1 PE=2 SV=1 - [KR111_CAPHI]	259,20	22,01	1	2	2	6	159	16,8	7,84
Q08193	1,3-beta-glucanosyltransferase GAS5 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS5 PE=1 SV=1 - [GAS5_YEAST]	258,85	11,57	1	4	4	5	484	51,8	4,64
P02442	Keratin, high-sulfur matrix protein, IIIA3 OS=Capra hircus PE=1 SV=1 - [KRA3_CAPHI]	219,73	43,94	1	4	4	10	132	14,2	8,12
P02446	Keratin-associated protein 3-1 OS=Ovis aries GN=KRTAP3-1 PE=1 SV=2 - [KRA31_SHEEP]	174,26	15,31	2	2	2	5	98	10,4	6,96
Q02958	Keratin-associated protein 6-1 OS=Ovis aries GN=KRTAP6-1 PE=1 SV=2 - [KRA61_SHEEP]	156,22	32,53	1	3	3	4	83	8,4	8,22
P15703	Glucan 1,3-beta-glucosidase OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=BGL2 PE=1 SV=1 - [BGL2_YEAST]	132,87	15,65	1	5	5	5	313	34,1	4,51
O13923	Coronin-like protein crn1 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=crn1 PE=1 SV=1 - [CORO_SCHPO]	115,37	1,50	1	2	2	5	601	67,0	6,18
P27996	Histone H4 OS=Solaster stimpsoni PE=3 SV=2 - [H4_SOLST]	94,87	29,13	15	3	3	4	103	11,4	11,19
P02444	Keratin, high sulfur matrix protein, IIIB3 OS=Ovis aries PE=1 SV=1 - [KRA33_SHEEP]	93,68	21,43	2	2	2	2	98	10,5	6,95
A6ZL22	Cell wall protein ECM33 OS=Saccharomyces cerevisiae (strain YJM789) GN=ECM33 PE=3 SV=2 - [ECM33_YEAS7]	91,11	5,83	4	2	2	2	429	43,7	4,91
P53301	Probable glycosidase CRH1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=CRH1 PE=1 SV=1 - [CRH1_YEAST]	85,41	5,92	1	3	3	3	507	52,7	4,65
P22146	1,3-beta-glucanosyltransferase GAS1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS1 PE=1 SV=2 - [GAS1_YEAST]	77,43	2,68	1	2	2	2	559	59,5	4,67

Table 4.S8

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	1585,80	42,55	1	19	21	41	644	66,0	8,12
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	808,34	30,66	1	11	11	20	623	62,0	5,24
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	710,35	30,36	1	13	16	25	639	65,4	8,00
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	696,75	24,49	1	11	15	30	584	58,8	5,21
P83336	Thaumatin-like protein 1b (Fragment) OS=Malus domestica PE=2 SV=1 - [TP1B_MALDO]	664,92	33,49	1	2	6	24	212	22,1	4,54
Q9FSG7	Thaumatin-like protein 1a OS=Malus domestica GN=TL1 PE=1 SV=1 - [TP1A_MALDO]	616,33	41,46	1	3	7	25	246	25,7	5,15
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	406,74	20,34	1	4	8	12	472	51,5	5,16
P13647	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN]	237,94	15,76	1	7	8	8	590	62,3	7,74
Q08193	1,3-beta-glucanosyltransferase GAS5 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS5 PE=1 SV=1 - [GAS5_YEAST]	233,36	11,57	1	4	4	5	484	51,8	4,64
P13646	Keratin, type I cytoskeletal 13 OS=Homo sapiens GN=KRT13 PE=1 SV=4 - [K1C13_HUMAN]	230,13	15,94	1	2	7	8	458	49,6	4,96
P15703	Glucan 1,3-beta-glucosidase OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=BGL2 PE=1 SV=1 - [BGL2_YEAST]	223,43	15,97	1	5	5	6	313	34,1	4,51
P02538	Keratin, type II cytoskeletal 6A OS=Homo sapiens GN=KRT6A PE=1 SV=3 - [K2C6A_HUMAN]	210,37	15,78	1	5	7	8	564	60,0	8,00
P22146	1,3-beta-glucanosyltransferase GAS1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS1 PE=1 SV=2 - [GAS1_YEAST]	198,68	5,90	1	4	4	6	559	59,5	4,67
P02534	Keratin, type I microfibrillar 48 kDa, component 8C-1 OS=Ovis aries PE=1 SV=2 - [K1M1_SHEEP]	177,10	12,38	1	3	4	5	412	46,6	4,81
P00761	Trypsin OS=Sus scrofa PE=1 SV=1 - [TRYP_PIG]	174,37	16,45	1	3	3	9	231	24,4	7,18
P15241	Keratin, type II microfibrillar, component 7C OS=Ovis aries PE=1 SV=1 - [K2M2_SHEEP]	107,38	11,20	2	4	4	4	491	53,6	5,57
P53301	Probable glycosidase CRH1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=CRH1 PE=1 SV=1 - [CRH1_YEAST]	89,07	6,51	1	3	3	3	507	52,7	4,65
B5VL27	Cell wall mannoprotein CIS3 OS=Saccharomyces cerevisiae (strain AWRI1631) GN=CIS3 PE=3 SV=1 - [CIS3_YEAS6]	61,68	8,00	2	2	2	2	225	23,0	4,68

Table 4.S9

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	1284,48	39,60	1	18	19	34	644	66,0	8,12
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	1035,49	39,00	1	13	13	26	623	62,0	5,24
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	864,12	34,12	1	13	17	34	639	65,4	8,00
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	853,18	33,90	1	14	17	29	584	58,8	5,21
P69327	Glucoamylase OS=Aspergillus awamori GN=GLAA PE=1 SV=1 - [AMYG_ASPAW]	494,48	13,75	1	5	5	9	640	68,3	4,45
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	451,12	27,97	1	6	11	15	472	51,5	5,16
O43790	Keratin, type II cuticular Hb6 OS=Homo sapiens GN=KRT86 PE=1 SV=1 - [KRT86_HUMAN]	400,71	30,25	1	4	10	13	486	53,5	5,66
Q14525	Keratin, type I cuticular Ha3-II OS=Homo sapiens GN=KRT33B PE=2 SV=3 - [KT33B_HUMAN]	309,22	17,57	1	2	7	8	404	46,2	4,84
Q08193	1,3-beta-glucanosyltransferase GAS5 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS5 PE=1 SV=1 - [GAS5_YEAST]	289,20	11,57	1	4	4	7	484	51,8	4,64
P02538	Keratin, type II cytoskeletal 6A OS=Homo sapiens GN=KRT6A PE=1 SV=3 - [K2C6A_HUMAN]	287,81	17,38	1	5	9	12	564	60,0	8,00
P13647	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN]	248,19	16,27	1	6	9	12	590	62,3	7,74
P78385	Keratin, type II cuticular Hb3 OS=Homo sapiens GN=KRT83 PE=1 SV=2 - [KRT83_HUMAN]	241,80	17,85	1	1	7	10	493	54,2	5,64
P00761	Trypsin OS=Sus scrofa PE=1 SV=1 - [TRYP_PIG]	203,26	16,45	1	3	3	7	231	24,4	7,18
A6ZL22	Cell wall protein ECM33 OS=Saccharomyces cerevisiae (strain YJM789) GN=ECM33 PE=3 SV=2 - [ECM33_YEAS7]	192,80	6,06	4	3	3	7	429	43,7	4,91
P78386	Keratin, type II cuticular Hb5 OS=Homo sapiens GN=KRT85 PE=1 SV=1 - [KRT85_HUMAN]	185,92	14,00	1	2	6	9	507	55,8	6,55
O76011	Keratin, type I cuticular Ha4 OS=Homo sapiens GN=KRT34 PE=2 SV=2 - [KRT34_HUMAN]	182,90	15,83	1	2	7	7	436	49,4	5,06
Q04E64	60 kDa chaperonin OS=Oenococcus oeni (strain ATCC BAA-331 / PSU-1) GN=groL PE=3 SV=1 - [CH60_OENOB]	160,77	11,65	1	5	5	5	541	57,3	4,96
P15703	Glucan 1,3-beta-glucosidase OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=BGL2 PE=1 SV=1 - [BGL2_YEAST]	160,42	16,93	1	4	4	4	313	34,1	4,51
P13646	Keratin, type I cytoskeletal 13 OS=Homo sapiens GN=KRT13 PE=1 SV=4 - [K1C13_HUMAN]	150,72	14,85	1	2	6	6	458	49,6	4,96
P22146	1,3-beta-glucanosyltransferase GAS1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS1 PE=1 SV=2 - [GAS1_YEAST]	119,89	4,47	1	3	3	4	559	59,5	4,67
P38288	Protein TOS1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=TOS1 PE=1 SV=1 - [TOS1_YEAST]	95,37	11,65	1	3	3	3	455	48,0	4,67
Q9M5X7	Non-specific lipid-transfer protein OS=Malus domestica GN=MALD3 PE=1 SV=1 - [NLTP_MALDO]	65,78	24,35	1	2	2	2	115	11,4	8,92
P83336	Thaumatin-like protein 1b (Fragment) OS=Malus domestica PE=2 SV=1 - [TP1B_MALDO]	37,75	11,32	1	2	2	2	212	22,1	4,54

Table 4.S10

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
P00698	Lysozyme C OS=Gallus gallus GN=LYZ PE=1 SV=1 - [LYSC_CHICK]	6567,51	72,11	1	6	11	244	147	16,2	9,07
P49663	Lysozyme C OS=Phasianus versicolor GN=LYZ PE=1 SV=1 - [LYSC_PHAVE]	3293,38	40,77	1	2	6	101	130	14,3	8,82
Q7LZQ2	Lysozyme C OS=Aix sponsa GN=LYZ PE=1 SV=1 - [LYSC_AIXSP]	1408,80	49,61	1	2	6	47	129	14,5	8,97
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	1100,14	38,98	1	18	20	26	644	66,0	8,12
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	733,40	34,27	1	14	18	21	639	65,4	8,00
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	653,26	31,14	1	12	12	17	623	62,0	5,24
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	511,68	23,12	1	11	12	13	584	58,8	5,21
P00761	Trypsin OS=Sus scrofa PE=1 SV=1 - [TRYP_PIG]	269,27	25,11	1	4	4	15	231	24,4	7,18
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	180,92	11,44	1	4	5	5	472	51,5	5,16
A6ZL22	Cell wall protein ECM33 OS=Saccharomyces cerevisiae (strain YJM789) GN=ECM33 PE=3 SV=2 - [ECM33_YEAS7]	153,44	6,06	4	3	3	4	429	43,7	4,91
P53301	Probable glycosidase CRH1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=CRH1 PE=1 SV=1 - [CRH1_YEAST]	148,73	9,07	1	5	5	7	507	52,7	4,65
P13647	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN]	146,78	8,47	1	3	5	5	590	62,3	7,74
P22146	1,3-beta-glucanosyltransferase GAS1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS1 PE=1 SV=2 - [GAS1_YEAST]	127,87	4,47	1	2	2	3	559	59,5	4,67
P28319	Cell wall protein CWP1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=CWP1 PE=1 SV=2 - [CWP1_YEAST]	106,78	12,13	1	2	2	3	239	24,3	4,67
P15703	Glucan 1,3-beta-glucosidase OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=BGL2 PE=1 SV=1 - [BGL2_YEAST]	59,33	6,71	1	2	2	2	313	34,1	4,51
P83336	Thaumatin-like protein 1b (Fragment) OS=Malus domestica PE=2 SV=1 - [TP1B_MALDO]	52,17	11,32	1	2	2	2	212	22,1	4,54
P38288	Protein TOS1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=TOS1 PE=1 SV=1 - [TOS1_YEAST]	49,33	8,79	1	2	2	2	455	48,0	4,67
A2R3I1	Probable pectin lyase A OS=Aspergillus niger (strain CBS 513.88 / FGSC A1513) GN=pelA PE=3 SV=1 - [PELA_ASPNC]	35,74	5,80	2	2	2	2	379	39,8	4,34
Q86YZ3	Hornerin OS=Homo sapiens GN=HRNR PE=1 SV=2 - [HORN_HUMAN]	33,61	7,23	1	3	3	5	2850	282,2	10,04

Table 4.S11

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
P00698	Lysozyme C OS=Gallus gallus GN=LYZ PE=1 SV=1 - [LYSC_CHICK]	1764,40	59,86	1	5	7	63	147	16,2	9,07
P49663	Lysozyme C OS=Phasianus versicolor GN=LYZ PE=1 SV=1 - [LYSC_PHAVE]	1079,75	33,85	1	2	4	34	130	14,3	8,82
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	772,28	35,40	1	15	16	21	644	66,0	8,12
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	675,32	21,51	1	9	9	15	623	62,0	5,24
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	565,23	27,74	1	12	13	16	584	58,8	5,21
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	562,38	28,01	1	13	14	17	639	65,4	8,00
Q9FSG7	Thaumatin-like protein 1a OS=Malus domestica GN=TL1 PE=1 SV=1 - [TP1A_MALDO]	499,31	41,46	1	6	6	17	246	25,7	5,15
Q8NK89	Alpha-L-arabinofuranosidase B OS=Aspergillus kawachii (strain NBRC 4308) GN=abfB PE=1 SV=1 - [ABFB_ASPKW]	408,58	23,05	2	6	6	10	499	52,6	4,46
P62694	Exoglucanase 1 OS=Hypocrea jecorina GN=cbh1 PE=1 SV=1 - [GUX1_HYPJE]	253,86	13,84	1	5	5	7	513	54,0	4,81
A2R3I1	Probable pectin lyase A OS=Aspergillus niger (strain CBS 513.88 / FGSC A1513) GN=pelA PE=3 SV=1 - [PELA_ASPNC]	252,37	11,35	2	3	3	7	379	39,8	4,34
P00761	Trypsin OS=Sus scrofa PE=1 SV=1 - [TRYP_PIG]	223,58	16,45	1	3	3	9	231	24,4	7,18
P00343	L-lactate dehydrogenase OS=Lactobacillus casei GN=ldh PE=1 SV=3 - [LDH_LACCA]	180,40	12,58	1	3	3	4	326	35,5	5,45
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	163,53	9,11	2	3	4	6	472	51,5	5,16
P17872	Pectinesterase OS=Aspergillus tubingensis GN=pme1 PE=1 SV=1 - [PME_ASPTU]	146,16	14,80	1	4	4	6	331	35,7	4,36
P22832	Glucoamylase OS=Aspergillus shirousami GN=glaA PE=3 SV=1 - [AMYG_ASPSH]	117,29	7,04	2	2	2	2	639	68,1	4,49
Q03F25	Elongation factor Tu OS=Pediococcus pentosaceus (strain ATCC 25745 / 183-1w) GN=tuf PE=3 SV=1 - [EFTU_PEDPA]	102,64	17,97	1	3	6	7	395	43,3	4,87
Q88YH3	Enolase 1 OS=Lactobacillus plantarum (strain ATCC BAA-793 / NCIMB 8826 / WCFS1) GN=eno1 PE=3 SV=1 - [ENO1_LACPL]	97,03	7,24	1	2	2	2	442	48,0	4,72
Q12679	Endoglucanase A OS=Aspergillus kawachii (strain NBRC 4308) GN=cekA PE=2 SV=2 - [GUNA_ASPKW]	73,97	8,37	1	2	2	2	239	25,8	4,84
Q03QN5	Elongation factor Tu OS=Lactobacillus brevis (strain ATCC 367 / JCM 1170) GN=tuf PE=3 SV=1 - [EFTU_LACBA]	60,64	14,39	1	2	5	5	396	43,6	4,84
B5VL27	Cell wall mannoprotein CIS3 OS=Saccharomyces cerevisiae (strain AWRI1631) GN=CIS3 PE=3 SV=1 - [CIS3_YEAS6]	54,19	8,00	2	2	2	2	225	23,0	4,68
P53301	Probable glycosidase CRH1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=CRH1 PE=1 SV=1 - [CRH1_YEAST]	50,70	4,54	1	2	2	2	507	52,7	4,65
P15703	Glucan 1,3-beta-glucosidase OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=BGL2 PE=1 SV=1 - [BGL2_YEAST]	43,80	6,71	1	2	2	2	313	34,1	4,51

Table 4.S12

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	1142,77	41,15	1	18	20	31	644	66,0	8,12
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	759,77	23,92	1	8	8	16	623	62,0	5,24
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	753,03	37,56	1	16	19	29	639	65,4	8,00
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	637,08	25,86	1	14	16	24	584	58,8	5,21
Q04E64	60 kDa chaperonin OS=Oenococcus oeni (strain ATCC BAA-331 / PSU-1) GN=groL PE=3 SV=1 - [CH60_OENOB]	544,51	26,43	1	13	13	17	541	57,3	4,96
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	353,32	20,34	1	6	8	11	472	51,5	5,16
Q08193	1,3-beta-glucanosyltransferase GAS5 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS5 PE=1 SV=1 - [GAS5_YEAST]	287,46	11,57	1	4	4	8	484	51,8	4,64
P00761	Trypsin OS=Sus scrofa PE=1 SV=1 - [TRYP_PIG]	240,77	16,45	1	3	3	10	231	24,4	7,18
Q48796	Malolactic enzyme OS=Oenococcus oeni GN=mleA PE=3 SV=1 - [MLES_OENOE]	224,72	7,39	1	3	3	4	541	59,1	4,97
P22146	1,3-beta-glucanosyltransferase GAS1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GAS1 PE=1 SV=2 - [GAS1_YEAST]	195,71	4,47	1	3	3	4	559	59,5	4,67
Q04DH2	Enolase OS=Oenococcus oeni (strain ATCC BAA-331 / PSU-1) GN=eno PE=3 SV=1 - [ENO_OENOB]	193,72	12,72	1	4	4	4	448	48,4	4,82
P15703	Glucan 1,3-beta-glucosidase OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=BGL2 PE=1 SV=1 - [BGL2_YEAST]	182,26	12,14	1	4	4	5	313	34,1	4,51
A6ZL22	Cell wall protein ECM33 OS=Saccharomyces cerevisiae (strain YJM789) GN=ECM33 PE=3 SV=2 - [ECM33_YEAS7]	131,81	6,06	4	3	3	3	429	43,7	4,91
Q04FQ4	Elongation factor Tu OS=Oenococcus oeni (strain ATCC BAA-331 / PSU-1) GN=tuf PE=3 SV=1 - [EFTU_OENOB]	113,44	11,11	1	3	3	3	396	43,6	5,16
P13647	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN]	108,45	7,80	2	4	5	5	590	62,3	7,74
Q04EE1	Chaperone protein DnaK OS=Oenococcus oeni (strain ATCC BAA-331 / PSU-1) GN=dnaK PE=3 SV=1 - [DNAK_OENOB]	96,46	6,00	1	3	3	3	617	66,2	5,06
Q04G42	Phosphoglycerate kinase OS=Oenococcus oeni (strain ATCC BAA-331 / PSU-1) GN=pgk PE=3 SV=1 - [PGK_OENOB]	91,60	9,16	1	4	4	5	404	43,0	5,69
Q9M5X7	Non-specific lipid-transfer protein OS=Malus domestica GN=MALD3 PE=1 SV=1 - [NLTP_MALDO]	90,75	24,35	1	2	2	3	115	11,4	8,92
B5VL27	Cell wall mannoprotein CIS3 OS=Saccharomyces cerevisiae (strain AWRI1631) GN=CIS3 PE=3 SV=1 - [CIS3_YEAS6]	78,37	8,00	2	2	2	3	225	23,0	4,68
Q04G44	Glucose-6-phosphate isomerase OS=Oenococcus oeni (strain ATCC BAA-331 / PSU-1) GN=pgi PE=3 SV=1 - [G6PI_OENOB]	69,80	10,73	1	4	4	4	438	48,6	5,14
P38288	Protein TOS1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=TOS1 PE=1 SV=1 - [TOS1_YEAST]	68,89	5,93	1	2	2	2	455	48,0	4,67

Table 4.S13

CHAPTER 5

POLYPHENOLIC AND ANTIOXIDANT COMPOSITION OF ITALIAN CIDERS

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Work in progress

INTRODUCTION

Cider is a popular fermented beverage produced worldwide, that can be made from almost any type of apples. The surplus of cooking or dessert apples are used for cidermaking in some regions of the UK, little or no distinction is made between cider, juice and dessert apples in Germany. French cidermakers, however, do a selection of apple varieties, named as "true cider cultivars" that are grown for no other purpose, except for cider production (*1*). The main operations of cidermaking are similar, but they may vary among countries (*2*).

In Italy ciders are produced in few regions (mainly the northern ones), especially Trentino-Alto Adige, Friuli-Venezia Giulia, Piemonte e Valle d'Aosta. The reasons way cider has not been so popular in Italy are mainly historical and cultural. The first reason concerns the deep-rooted Italian wine culture, and the second the laws promulgated during the Fascist period. As a consequence of these laws fruit-based fermented beverages were heavily taxed, and the goal was to help the Italian wine industry to flourish and to eliminate competition (*3-5*). However, during the last years the consumption of cider has increased in Italy.

Unlike the European tradition, Italian ciders are mainly produced with dessert apples (*1*) following the winemaking protocol. So the final product is similar to a sparkling white wine, usually characterized by low acidity.

The organoleptic properties of the beverage are influenced by several factors, besides the apple variety and their characteristics, the fermentation conditions and yeast strains, the production process and fining treatments (*6*).

Polyphenols are important compounds involved in the organoleptic characteristics; their content and composition are related to colors, astringency, and bitterness and in general to sensory profile of cider. They are precursors of volatile compounds, they are involved in the fermentative process, by controlling spoilage of bacteria (*7*), and they can also inhibit enzymes used during cidermaking (*8*). Various studies indicate that diets rich in polyphenols contribute to reduce the risk of degenerative diseases (*9, 10*), because these compounds act as chelators of metal ions, and terminators of free radicals. It must be noted that polyphenols antioxidant activity depends on their structure (*11*). Numerous studies support a significant relationship between the antioxidant activity and the family of polyphenols, in particular phenolic acids and flavonoids (*11*).

Apples represent an important source of phytochemicals, such as flavonols (mainly quercetin glycosides), flavanols (monomeric and oligomeric), dihydrochalcones, anthocyanidins and phenolic acids (*12*).

Processes applied during cidermaking (i.e., apples grinding and pressing, enzyme addition, fermentation, filtration, and fining) contribute to the extraction and content of polyphenols in the final cider.

The aim of the present study was to evaluate the polyphenolic profile of 18 Italian commercial ciders in relation to their antioxidant activity.

MATERIALS AND METHODS

Eighteen Italian ciders commercially available from 11 different brands were considered in this study. The samples were: 1 still cider (no. 1), 14 sparkling ciders produced with the Charmat method (nos. 2-15), and 3 sparkling ciders produced by the Champenoise method (nos. 16-18). All the chemical reagents, where not specified, were purchased at Sigma-Aldrich (Steinheim, Germany).

Values of pH and titratable acidity (expressed in g/L malic acid equivalents) were measured for all ciders.

Determination of total polyphenolic contents by the Folin-Ciocalteau method

Each cider samples were loaded on a C18 solid-phase cartridge (Waters Corporation, MA, USA) according to the method described by the manufacturer. Total polyphenols were determined by spectrophotometer according to the Folin-Ciocalteu assay (*13*). Total phenols were expressed as mg/L of gallic acid equivalents. Each determination was repeated three times and results are expressed as mean ± SD.

DPPH (2,2'-diphenyl-1-picrylhydrazyl) assay

The antioxidant capacity of each cider was measured according to the protocol described by Vakarelova et al. (*14*) with some modifications. Cider samples were degassed with N_2 to remove free sulfur dioxide. The DPPH solution in methanol (25 mg/L) was prepared daily, and a standard curve was achieved with Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) with concentrations in the range of 1.87-30 μM in methanol. Fifty μL of

cider or standard were mixed to 1.95 mL of DPPH solution, and the decrease in absorbance was measured at 517 nm after 15 minutes.

Identification and quantification of polyphenols by HPLC

Phenolic compounds were separated using an HPLC system (Waters) equipped with a Kinetex C18 column (4.6x150 mm length, 5 µm particle size) from Phenomenex (CA, USA). The eluent solvent were water (solvent A) and methanol (solvent B) both containing 0.1% trifluoracetic acid (TFA), and the flow rate was 1 mL/min. The injection volume of each samples was 10 µl. The gradient conditions are indicated in Table 5.1.

Minutes	% Solvent B
0-2	5%
2-7	12%
7-38	55%
38-39	100%
39-40	100%
40-41	5%
41-45	5%

Table 5.1 Gradient conditions for polyphenols separation by HPLC.

Identification of compounds was realized by comparing the retention times of peaks with those of standards. Quantification was carried out by the external standard method at 350 nm for chlorogenic acid and the quercetin family compounds, and 280 nm for the other phenolic compounds (i.e., gallic acid, tyrosol, epicatechin, catechin, procyanidin B1 and B2, and phloridzin). The integration area measured for each peak was reported in the calibration curve of the corresponding standard. Where no standard was available, standards of the same family were used for quantification, e.g., quercetin-3-xyloside, quercetin-3-arabinofuranoside, and quercetin-3-arabinopiranoside were quantified as quercetin-3-glucoside equivalents.

Statistical analysis

All the statistical analyses (Pearson and Spearman correlations, ANOVA test, multiple regression models) were performed with the software Mathematica (Wolfram Research Inc., 100 Trade Center Drive, Champaign, IL, USA).

RESULTS AND DISCUSSION

Table 5.2 reports the physico-chemical characteristics of each cider, and it shows the heterogeneity of the beverages, e.g., the titrable acidity ranges from 3.02 - 6.77 mg/L malic acid equivalents. This variability could be a consequence of the apple varieties and the technological processes that are not standardized. For example, in Italy dessert apples could be used in cidermaking, giving a final product with a lower acidity in comparison to the cider produced with "true cider" apple cultivar.

No.	Ethanol % vol.	pH	Titrable acidity g/L eq malic acid	Antioxidant Activity mM TROLOX eq	Total Polyphenols mg/L eq gallic acid
1	8.0	3.36	5.23 ± 0.11	0.352	204.5 ± 5.1
2	4.8	3.48	4.63 ± 0.15	0.207	154.9 ± 9.0
3	7.0	3.38	5.29 ± 0.09	0.279	260.8 ± 7.3
4	5.6	3.52	4.72 ± 0.19	0.619	469.0 ± 8.2
5	8.0	3.60	3.35 ± 0.07	0.352	223.3 ± 7.0
6	4.5	3.64	3.02 ± 0.09	1.349	828.8 ± 13.6
7	5.5	3.44	4.59 ± 0.05	0.151	366.7 ± 8.6
8	3.5	3.38	5.86 ± 0.17	0.525	519.2 ± 7.5
9	8.0	3.50	4.86 ± 0.11	0.414	250.8 ± 0.7
10	8.0	3.64	3.22 ± 0.09	0.354	285.4 ± 13.7
11	6.0	3.51	4.92 ± 0.12	0.391	322.7 ± 11.6
12	3.5	3.45	5.29 ± 0.12	0.512	412.8 ± 6.0
13	3.0	3.65	3.32 ± 0.08	0.648	306.9 ± 8.5
14	5.6	3.60	3.69 ± 0.12	0.635	453.4 ± 7.2
15	5.0	3.54	5.22 ± 0.11	0.182	246.2 ± 8.2
16	7.5	3.42	5.52 ± 0.05	0.467	372.7 ± 11.2
17	8.0	3.48	6.77 ± 0.09	0.225	178.6 ± 2.0
18	7.0	3.71	3.02 ± 0.11	0.369	414.0 ± 12.0

Table 5.2 Chemical composition of the selected ciders.

Antioxidant activity of cider

Italian ciders are produced following the white winemaking protocol (*15*), and they may differ from the ciders of other Countries in some production steps. For example, the juice extraction in the Asturian traditional cidermaking is achieved by a pressing procedure that takes 2-3 days, whereas in Italy the automatic process takes a few hours. In addition, sulfur dioxide (SO_2) is added to avoid malolactic fermentation (desirable in French ciders) and spoilage

bacteria growth, and the ascorbic acid is used to prevent undesirable oxidation during the apple grinding step (*15*). This implies a different polyphenols extraction and oxidation, and under this point of view, the final product differs from ciders produced abroad.

The antioxidant activity (AA) of 18 Italian commercial ciders was measured according to the protocol of Vakarelova et al. (*14*) as described above. The possible effect of SO_2 on DPPH assay was excluded by treating ciders by sonication and insufflation of N_2, whereas the absence of residual ascorbic acid was verified by RP-HPLC analysis (data not shown).

Data about the AA range from 0.151 to 1.349 mM TROLOX eq (see Table 5.2), and these results showed a significant correlation with the Total Polyhenols Content (TPC, expressed as mg/L gallic acid equivalents) (see Table 5.3). The Pearson correlation between AA and TPC shows a coefficient of 0.861 (p-value = $4.96e^{-7}$), which is in accordance to that reported by Picinelli-Lobo et al. (11) for Asturian ciders.

The AA should be evaluated by several methods based on different reaction mechanisms (e.g., Ferric Reducing Antioxidant Power versus Oxygen Radical Absorption Capacity) (*16*), because it is well-known that it has to be taken into account the different way of action of antioxidants (*17*). In this preliminary study, the AA of ciders has been evaluated using the DPPH assay only. The TPC was determined by Folin-Ciocalteau assay, and this method is based on the same oxidation/reduction mechanism (i.e., electron transfer) exploited by DPPH assay. The TPC, however, cannot be assumed to be proportional to AA. In fact, it is known that AA is mainly determined by specific families of polyphenols (and not all polyphenols), in particular phenolic acids, flavonols, and flavanols. Thus, it may be important to directly measure the concentration of specific polyphenols and investigate whether it is somehow correlated to AA.

Identification of polyphenols in cider

The preliminary RP-HPLC analysis carried out for all cider samples identified 15 polyphenols, belonging to the group of phenolic acids (chlorogenic and gallic acid), flavanols (catechin, epicatechin, procyanidins B1 and B2), flavonols (quercetin-3-glucoside, quercetin-3-rhamnoside, quercetin-3-xyloside, quercetin-3-galactoside, quercetin-3-arabinofuranoside, and quercetin-3-arabinopiranoside), and dihydrochalcone (phoridzin). The concentration of phenolic compounds is reported in Table 5.3.

Identified phenolics of ciders (mg/L)			Correlation coefficients		
	Mean value ± std dev.	Min, Max	Spearman R	Pearson r	p-value
Gallic acid	0.17 ± 0.25	n.d., 0.81	-0.104	-	0.686
Chlorogenic acid	28.52 ± 34.32	0.90, 116.54	**0.809**	-	$1.30e^{-5}$
Catechin	6.03 ± 3.80	2.00, 15.19	-	**0.866**	$3.28e^{-7}$
Epicatechin	15.91 ± 16.05	n.d., 42.11	**0.846**	-	$1.50e^{-6}$
Procyanidin B1	7.53 ± 5.58	1.18, 22.26	-	**0.796**	$1.44e^{-4}$
Procyanidin B2	5.85 ± 2.19	2.56, 10.15	-	-0.149	0.560
Quercetin-3-glucoside	0.39 ± 0.48	n.d., 1.66	0.222	-	0.381
Quercetin-3-rhamnoside	1.60 ± 0.71	n.d., 2.87	-	0.132	0.607
Quercetin-3-xyloside	0.32 ± 0.23	n.d., 0.69	0.352	-	0.155
Quercetin-3-arabinofuranoside	0.11 ± 0.16	n.d., 0.47	0.067	-	0.795
Quercetin-3-arabinopiranoside	0.29 ± 0.25	n.d., 0.87	0.082	-	0.749
Quercetin-3-galactoside	1.37 ± 0.74	n.d., 2.45	-	**0.708**	0.001
Phloridzin	6.54 ± 4.55	1.03, 14.98	**0.721**	-	$4.32e^{-4}$
Tyrosol	0.68 ± 0.66	0.08, 2.45	-	-0.097	0.707
TPC (mg/L GAE)	348.38 ± 99.98	154.90, 469.00	-	**0.861**	$4.96e^{-7}$
AA (mM Trolox eq)	0.34 ± 0.14	0.15, 0.47			

Table 5.3 Identified phenolic composition of ciders and correlation coefficients.

Their concentration was individually correlated to the AA of ciders by the Spearman's rank statistic. In addition, the Pearson's correlation coefficient was calculated for those polyphenols whose concentration was normally distributed among the 18 ciders. In Table 5.3 the coefficients show that there is no significant correlation for gallic acid, procyanidin B2, the phenols belonging to the flavonols group (except from the quercetin-3-galactoside), and tyrosol. The correlation between other identified polyphenols (i.e., chlorogenic acid, catechin, epicatechin, procyanidin B1, quercetin-3-galactoside, and phloridzin), and AA is statistically significant (see Table 5.2).

The correlation between each polyphenol and the AA of cider is in general limited (i.e., < 0.9) and this could be explained by taking into account the different interaction of some polyphenols in a blend (*11*). For this reason, we tried to identify a pool of molecules (subset) whose concentrations could predict the AA of the Italian ciders. We took into consideration all the 32752 combinations of 2, 3, ..., 15 molecules and we applied a multiple regression model with the corresponding number of predictors, and in the attempt to explain the AA. For each model the ANOVA test was performed to calculate the goodness of the fit.

If we plot the p-values of the ANOVA test versus all the considered subsets, we can see a diagram where some p-values are lower than $2x10^{-7}$ (Figure 5.1).

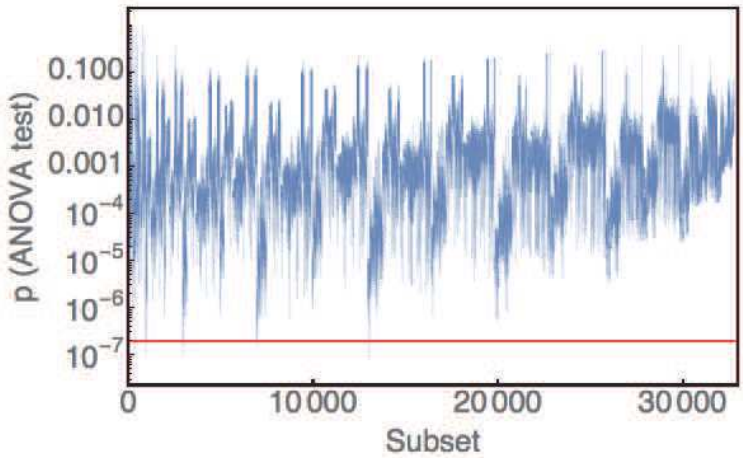

Figure 5.1 Goodness of the fit (p-value of the ANOVA test) vs subsets (different combinations of 2, 3, ..., 15 phenols)

Arbitrarily, we set this cut off value to select the subsets of phenols that better predicted the AA. Seven subsets are below the fixed limit and correspond to the combination of phenols reported in Table 5.4.

Subset	p-value	Molecules							
1	$8.104x10^{-8}$	Chlorogenic acid	Catechin	Procyanidin B1	Procyanidin B2	Quercetin-3-glucoside		Quercetin-3-xyloside	Quercetin-3-arabinopyranoside
2	$9.629x10^{-8}$	Chlorogenic acid	Catechin	Procyanidin B1	Procyanidin B2			Quercetin-3-xyloside	
3	$1.078x10^{-7}$	Chlorogenic acid	Catechin	Procyanidin B1	Procyanidin B2				
4	$1.444x10^{-7}$	Chlorogenic acid	Catechin	Procyanidin B1	Procyanidin B2	Quercetin-3-glucoside		Quercetin-3-xyloside	
5	$1.653x10^{-7}$		Catechin	Procyanidin B1	Procyanidin B2		Quercetin-3-rhamnoside	Quercetin-3-xyloside	
6	$1.681x10^{-7}$	Chlorogenic acid	Catechin	Procyanidin B1	Procyanidin B2				
7	$1.951x10^{-7}$		Catechin	Procyanidin B1	Procyanidin B2				Quercetin-3-arabinopyranoside

Table 5.4 Polyphenols that belong to the subsets that predicted AA with a p-value < $2x10^{-7}$ for the null hypothesis.

Subset no. 1 is the combination of molecules that best describes the correlation between polyphenols concentration and AA of ciders with an adjusted R^2 = 0.968. Parameters estimates for this model are reported in Table 5.5.

All the coefficients are statistically significant since the p-values are lower than 0.05. In addition, the Residual Sum of Squares (RSS) was used to calculate the variance explained by

each variable and the data are also reported in Table 5.5. The variables comparably contributed to explain the variance, even if polyphenols that belongs to the flavonols group (i.e., quercetin-3-glucoside, quercetin-3-xyloside, and quercetin-3-arabinopyranoside) show the smallest contribution to the model (i.e., < 13.99%).

		Estimate	Std Error	t-Statistic	P-Value	Explained variance (%)
	1	0.20662	0.0453844	4.55266	0.000105397	
Procyanidin B1	x1	-0.0295312	0.00627501	-4.70616	0.000833745	16.4772
Catechin	x2	0.0636513	0.0121036	5.25886	0.000368663	20.5747
Procyanidin B2	x3	-0.025748	0.005466	-4.71058	0.000828177	16.5082
Chlorogenic acid	x4	0.00390874	0.000800429	4.8833	0.000638808	17.741
Quercetin-3-glucoside	x5	-0.188918	0.0530635	-3.56023	0.00517926	9.42991
Quercetin-3-xyloside	x6	0.544716	0.125574	4.33781	0.00147141	13.9989
Quercetin-3-arabinopyranoside	x7	-0.305559	0.114805	-2.66154	0.0238363	5.27006

Table 5.5 Estimates of the coefficients in the linear regression model with 7 polyphenols.

It should be noted that chlorogenic acid, catechin, and procyanidins B1 and B2 are present in all the subsets, with the only exception of subsets nos. 5 and 7 where chlorogenic acid is absent. So, according to the "parsimony approach" the combination of these molecules represents the minimum number of phenols that provides the best prediction of the AA of cider.

		Estimate	Std Error	t-Statistic	P-Value	Explained variance (%)
	1	0.249917	0.0536053	4.66217	0.000444614	
Procyanidin B1	x1	-0.0344993	0.00840169	-4.10624	0.00123859	19.1212
Catechin	x2	0.0816244	0.0138396	5.89789	0.0000525427	39.4476
Procyanidin B2	x3	-0.034136	0.00783296	-4.35799	0.000775466	21.5377
Chlorogenic acid	x4	0.00258647	0.000617539	4.18835	0.00106238	19.8936

Table 5.6 Estimates of the coefficients in the linear regression model with 4 polyphenols.

In detail, the coefficients of the multiple regression model for this subset showed an adjusted $R^2 = 0.918$ and p (ANOVA) = 1.078×10^{-7}. Parameters estimates for this model are reported in Table 5.6. The linear regression model with 4 molecules shows that the variables comparably contributed to explain the variance (Table 5.6).

CONCLUSION

The analyses performed on 18 Italian ciders showed that their AA is lower than those observed for the Asturian ones. This could be explained by the lower TPC, that for the Italian cider is on average of 348 ± 100 mg/L GAE, whereas Picinelli-Lobo et al. (*11*) reported a value of about 733 ± 148 mg/L GAE for the Asturian beverages.

In the Italian ciders the average concentration of each identified polyphenols differs from that observed by Picinelli-Lobo. Picinelli-Lobo determined a higher number of polyphenols in their Spanish ciders. It might be interesting to extend our study on Italian ciders to the same polyphenols for comparison purposes.

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CHAPTER 6

GENERAL DISCUSSION and CONCLUSION

The cider currently produced in Italy is a “revival beverage”. The originality of this product lies in the fact that it is obtained trying to meet the taste of an Italian consumer, who is used to the organoleptic characteristics of wine. As a consequence of this market request, the Italian ciders are similar to a sparkling wine (e.g., “Prosecco”) but with a lower alcohol content. But such beverage is not the “cider” as it is intended worldwide.

The good results achieved so far by the producers in meeting the demands of Italian consumers determined an increase in the national beverage market. Even thought it must be noted that, with few exceptions, the Italian ciders are generally made by small industrial companies that rarely invest in the Research and Development and in the deep characterization of their products. The cider is produced with machineries, technologies and procedures completely borrowed from the wine industry.

This means that in Italy the production processes of cidermaking are not yet fully optimized and standardized. On the other hand, not all the wholesome potential of cider is investigated, in spite of what occurs in other Countries, where the potential advantages to the human health related to the beverage consumption is studied, advertised and commercially exploited.

Furthermore, it is definitely less alcoholic than wine whose "healthy" potential, if assumed in moderate amount, was used as a real trigger for the marketing. On this issue, we need only recall the effect of the so-called “French paradox” which led in a significant increase in sales of red wines.

Under this point of view, it is appropriate to remember that in the collective imagination the consumption of apples (and their derivatives) is associated with the well-being and here, it becomes almost useless to repeat the aphorism: "An apple a day keeps the doctor away." At the end, it seems that the cider market has to break through a door already almost opened.

Apples, in relation to varieties and their ripening phase, are rich in phenolic compounds that show high antioxidant capacity. These substances influence colour, bitterness, and astringency and generally the mouthfeel of the beverage. Polyphenols can also influence the fermentative process and control lactic acid bacteria involved in the malolactic fermentation.

Many recent studies suggest a beneficial effect of apple polyphenols on the human health, demonstrating that their intake might be linked to the reduced risk of several forms of cancer, cardiovascular disease, and asthma.

The preliminary results of the evaluation of the Italian ciders showed a good antioxidant activity. This characteristic is related not only to the polyphenol concentration, but also to

specific molecules and their interactions. In this PhD thesis a multiple linear regression model demonstrated that the concentration of chlorogenic acid, catechin, procyanidins B1 and B2, quercetins 3-glucoside, 3-xyloside and 3-arabinopyranoside correlate with the antioxidant activity of ciders. This suggests that these phenols could be the mainly contributors of the antioxidant activity, but further studies should be achieved to exclude that this is an epiphenomenon.

In addition, the polyphenols content of Italian ciders is a lower in respect to Asturian ones. This could be a consequence of the apples used (e.g., cultivar, ripening degree, terroir…) and/or the cidermaking technologies applied. This indicates the necessity to analyse samples collected during all the production chains (from the apple to the finished product). We are confident that, thanks to the relations established with some collaborative cider cellars, this should be possible starting from the next apple harvest. All the information collected during the PhD course will be useful in programming the protocol of sampling.

In the PhD study the allergenic profiles of cider was also investigated. As expected the apple allergens described as more resistant to hydrolysis and thermal treatments (namely, Mal d 2 and Mal d 3) were found in numerous samples. But in some cider the allergens were absent, since no fragments were detected by LC-MS/MS technique.

Taking in advantage of the relationship established with cider cellars, it will be possible to study deeply the effects of different raw material and cidermaking technologies on the allergenic composition of cider. We suppose to identify a process that could eliminate apple allergens, without affecting the polyphenol content and their antioxidant activity.

The fallouts of this study could be extended in different field of the food science and technology. Indeed if it were possible to obtain a hypoallergenic cider, this product could provide a general model to manipulate fruit derivatives (i.e., juice, purée, cakes…) with low allergen content.

Another allergenic concern highlighted by this PhD study has been the detection of hen egg lysozyme in Italian ciders. This protein is a well-known allergen and according to the EU Directive its presence must be labelled. In two analysed samples the protein was immunologically active, whereas in 12 out of 18 samples at least lysozyme fragments were revealed by LC-MS/MS analysis.

Thanks to the established contacts with cider cellars, it emerged that the presence of lysozyme was in some cases an unaware contamination, probably due to a wrong use of the processing aids, whereas in other it was a consequence of ignoring the law.

We can hypothesize that in addition to a deeper characterization of the cider, there is the need of better trained operators in the cider industry. As in the winemaking field, where Research and Development are promoted for viticulture and oenology, also in the cidermaking field it would be important to improve studies in apple and derivatives production technology.

CHAPTER 7

OTHER PUBLICATIONS

Food Chemistry

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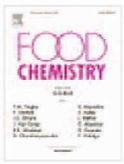

Production of stable food-grade microencapsulated astaxanthin by vibrating nozzle technology

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http://dx.doi.org/10.1016/j.foodchem.2016.10.085

Highlights

- 2% astaxanthin extracted from algae by high pressure homogenization in soybean oil.
- Pectin showed higher (10-fold) oil retention than alginate during drying.
- Kinetics of degradation of encapsulated astaxanthin depends on storage conditions.
- Refrigeration preserved 90% encapsulated astaxanthin during 52 weeks storage.

Abstract

Astaxanthin is a carotenoid known for its strong antioxidant and health-promoting characteristics, but it is also highly degradable and thus unsuited for several applications. We developed a sustainable method for the extraction and the production of stable astaxanthin microencapsulates. Nearly 2% astaxanthin was extracted by high-pressure homogenization of dried *Haematococcus pluvialis* cells in soybean oil. Astaxanthin-enriched oil was encapsulated in alginate and low-methoxyl pectin by Ca^{2+}-mediated vibrating-nozzle extrusion technology. The 3% pectin microbeads resulted the best compromise between sphericity and oil retention upon drying. We monitored the stability of these astaxanthin beads under four different conditions of light, temperature and oxygen exposition. After 52 weeks, the microbeads showed a total-astaxanthin retention of 94.1 ± 4.1% (+4°C/-light/+O_2), 83.1 ± 3.2% (RT/-light/-O_2), 38.3 ± 2.2% (RT/-light /+O2), and 57.0 ± 0.4% (RT/+light/+O_2), with different degradation kinetics. Refrigeration, therefore, resulted the optimal storage condition to preserve astaxanthin stability.

Pomegranate: Chemistry, Processing and Health Benefits
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Chapter 10

ALLERGY TO POMEGRANATE

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ABSTRACT

Food allergy is an immunological disease whose prevalence is increasing worldwide. Pomegranate could determine allergic reactions, even if a limited number of cases is reported in literature. In the present chapter, after an introductive part describing the pathogenesis and the clinical aspects of allergy and more specifically food allergy, we present a review of the scientific literature dealing with allergy to pomegranate, examining more in depth the involvement of non specific Lipid Transfer Proteins (nsLTP). Indeed, these proteins represent the only pomegranate allergens characterized at the molecular level.

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Hidden Exogenous Proteins in Wine: Problems, Methods of Detection and Related Legislation – a Review

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Abstract

Rizzi C., Mainente F., Pasini G., Simonato B. (2016): **Hidden exogenous proteins in wine: problems, methods of detection and related legislation – a review**. Czech J. Food Sci., 34: 93–104.

Fining agents are commonly used in the winemaking process to clarify and stabilise wines. They have different origins (animal, vegetal or mineral) and are added to wines in order to remove specifically undesirable compounds that are discarded. Fining agents should not be present in the final product but their possible persistence, as well as other exogenous residual proteins such as the enzymes utilised in winemaking, cannot be excluded for sure. The principal concern about the presence of exogenous residual proteins is the health of allergic subjects. Nevertheless, the respect of religious creed or other practice of living of the consumer must be considered as well. In the present review we itemise the proteins used in winemaking and possible drawbacks of their permanence in the final products and the related risks, depict the status of the art of the studies performed about the detection of exogenous proteins, and describe the wine labelling laws adopted in different countries to avoid the drawbacks associated with these hidden substances.

Keywords: fining proteins; technological enzymes; residual protein detection; allergy; food ethics; wine labelling laws

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Post-harvest proteomics of grapes infected by *Penicillium* during withering to produce Amarone wine

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ABSTRACT

The study of withered grape infection by *Penicillium*, a potentially toxigenic fungus, is relevant to preserve grape quality during the post-harvest dehydration process. This report describes the first proteomic analysis of Amarone wine grapes, infected by two strains of *Penicillium expansum* (Pe1) and *Penicillium crustosum* (Pc4). Protein identification by MS analysis allowed a better understanding of physiological mechanisms underlying the pathogen attack. The Pe1 strain had a major impact on *Vitis vinifera* protein expression inducing pathogenesis-related proteins and other protein species involved in energy metabolism. A greater expression of new *Penicillium* proteins involved in energy metabolism and some protein species related to redox homeostasis has been observed on grapes infected by Pc4 strain. Moreover, the new induced proteins in infected grapes could represent potential markers in withered grapes, thus creating the chance to develop case-sensitive prevention strategies to inhibit fungal growth.

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Short communication

Red wine proteins: Two dimensional (2-D) electrophoresis and mass spectrometry analysis

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Mass spectrometry

ABSTRACT

The aim of the present study was to optimize protein extraction from red wine (cv. Cabernet) in order to obtain a separation by two-dimensional electrophoresis (2-DE) compatible with mass spectrometry identification.

Proteins were denatured by sodium dodecyl-sulphate (SDS) and precipitated as potassium salts. The potassium-DS (KDS) protein complexes obtained were treated with different solutions in order to remove the detergent. Proteins were solubilized with different buffers and separated by different electrophoretic approaches [native, urea, acid urea PAGEs and isoelectric focusing (IEF)] as the first-dimension (1-DE). The best 2D separation was achieved by using 10% saccharose in the DS removal step, and 6-cyclohexylhexyl β-D-maltoside detergent in the solubilisation buffer combined with the IEF approach. Several well focalized protein spots were obtained and analyzed through mass-spectrometry.

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Recovery and Quantification of Apple and Cider Proteins for the Evaluation of Potential Allergens

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Cider is a complex food matrix rich in interfering substances and no single procedures is effective for all proteins extraction. The first activity of this PhD thesis was then aimed at setting up a method for recovery and quantify proteins from apples and ciders. This would be useful for studying the potential allergens, that can be retained after apple processing and for identifying technological processes that could affect the potential allergenicity of ciders.

Recupero e quantificazione di proteine da mela e sidro per la valutazione dei potenziali allergeni

Il sidro è una matrice alimentare complessa, ricca di sostanze interferenti e non esiste un'unica procedura efficace per l'estrazione di tutte le proteine. La prima attività di questa tesi di dottorato è stata quindi rivolta alla messa a punto di un metodo per il recupero e la quantificazione delle proteine di mela e sidro, con l'intento di studiare i potenziali allergeni, che possono rimanere attivi anche dopo la trasformazione delle mele, e per identificare i processi tecnologici che potrebbero influenzare il potenziale allergenico del sidro.

Key words: Apple proteins; Cider proteins; Allergens; Protein recovery; Protein quantification; SDS-page.

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Health and allergenic properties of apples and ciders

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This PhD thesis research project is aimed at assessing both the healthy properties and the allergenic potential of different apple cultivars and the thus-obtained ciders. The vitamin and phenolic compounds contents as well as the antioxidant potential will be measured in both apples and ciders. In addition, the potential allergenicity of different apple varieties will be evaluated, identifying the allergenic proteins (Mal d1, Mal d2, Mal d3 and Mal d4) described for apple. Moreover, the fate of the apple allergens will be studied in relation to the cider-making steps (fermentation, clarification, tannins addition, microfiltration).

Salute e proprietà allergeniche di mele e sidri

Con il presente progetto di tesi di dottorato si intende valutare sia le proprietà nutrizionali che il potenziale allergenico di diverse varietà di mela e dei sidri da queste ottenuti. Verranno misurati il contenuto di polifenoli, di vitamine e il potere antiossidante sia nel frutto che nella bevanda. Inoltre, si intende caratterizzare quali- e quantitativamente le proteine allergizzanti (Mal d1, Mal d2, Mal d3 e Mal d4) descritti per la mela. Infine, sarà valutata l'evoluzione degli allergeni della mela nei diversi momenti della produzione del sidro (fermentazione, chiarifica, addizione di tannini, microfiltrazione).

ABBREVIATIONS

AA	Antioxidant activity
ACN	Acetonitrile
ANOVA	Analysis of variance
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
DIECA	Diethyldithiocarbamic acid
DPPH	2,2'-diphenyl-1-picrylhydrazyl
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
GAE	Gallic acid equivalent
HCl	Hydrochloridric acid
HDL	High density lipoproteins
HEWL	Hen egg white lysozyme
HPLC	High Pressure Liquid Chromatography
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IUIS	International Union of Immunological Societies
KCl	Potassium chloride
kDa	kiloDalton
KDS	Potassium-Dodecyl Sulfate
LC-MS/MS	Liquid Chromatography-tandem Mass Spectrometry
LDL	Low density lipoproteins
mM	milliMolar
MS	Mass Spectrometry
MW	Molecular weight
NH_4HCO_3	Ammonium carbonate
nsLTP	not specific Lipid Transfer Protein
OAS	Oral Allergy Syndrome
OPA	o-phthaldialdehyde
PR-	Pathogenesis-related protein
RP-HPLC	Reverse-Phase High Pressure Liquid Chromatography
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SO_2	Sulfur dioxide
PAGE	Polyacrylamide gel electrophoresis
PAS	Periodic acid Schiff
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
TLP	Thaumatin-like protein
TPC	Total polyhenols content
Tris	Tris(hydroxymethyl)aminomethane
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
WHO	World Health Organization
WIB	Western immunoblot