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

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Abstract

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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

Possible presence of exogenous proteins (EP) in wine could essentially result from fining procedures or by the use of technological enzymes

In winemaking the term "fining" indicates the step of adding one (or several) adsorptive/reactive substances (finings) of animal, vegetal or mineral origin in order to reduce the concentration or to remove undesirable compounds. This process not only clarifies and makes wine stable but also improves organoleptic characteristics by reducing astringency and ameliorating colour and flavour of the final product (Yokotsuka *et al.* 1995; Marchal *et al.* 2002b).

If fining agents are used and removed according to a good manufacturing practice, it can be assumed that these substances are not present in the final wine. Good manufacturing practice for fining is essentially defined as the use of the smallest amount of fining agent needed to achieve the desired result when followed by racking and pre-bottling filtration processes. To date, however, there is limited evidence that commercial wines are free from residues of protein fining agents.

In law, wine-fining agents (similarly to enzymes) are generally considered as "processing aids", i.e. substances added to a food during processing but subsequently removed before the food reaches its finished form, or it is converted into components that naturally occur in the food and have no technical or functional effect in the finished food.

According to the Food and Drug Administration's (FDA) regulations [21 CFR 101.100 (a) (3) (ii)], the definition of processing aids is:

(a) Substances that are added to a food during the processing of such food but are removed in some manner from the food before it is packaged in its finished form;

(b) Substances that are added to a food during processing, are converted into constituents normally present in the food, and do not significantly increase the amount of the constituents naturally found in food;

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(c) Substances that are added to a food for their technical or functional effect in the processing but are present in the finished food at insignificant levels and do not have any technical or functional effect in that food.

With slight differences, but with a more specific attention to the consumer health, the UK Food Labelling Regulations (1996) define a processing aid: '… any substance not consumed as a food by itself, intentionally used in the processing of raw materials, foods or their ingredients, to fulfil a certain technological purpose during treatment or processing, and which may result in the unintentional but technically unavoidable presence of residues of the substance or its derivatives in the final product, provided that these residues do not present any health risk and do not have any technological effect on the finished product'.

Canadian regulations classify the wine fining agent as either a food additive or a processing aid. In the latter case, the final concentration of the agent would be, by definition, negligible; thus, no significant risk would exist for protein-allergic wine consumers and its indication on the product label would not be mandatory. However, the Canadian authorities do not indicate the concentration threshold to enable such a distinction (Government of Canada Food and Drug Act 2011).

In any case, processing aids are widely not required to be declared in the ingredient list on the food label because, by definition, they are "incidental additives that are present in a food at insignificant levels" (FDA 2013).

For these reasons, the potential permanence in the final products must be evaluated, and the choice of a particular fining agent must be accurate, taking into account not only the specific mechanism of action and separation in a given fluid, but also the potential consequence of its action and the effects of eventual remnants on the consumer's health.

Legal (and technical) hitches in the use of wine-fining agents derive from the great heterogeneity of their origin and mechanism of action.

As an example, in the process known as "blue fining" (a non-protein fining), potassium ferrocyanide is sometimes used to remove metals from wines. The most important metallic ions involved in casse formation are iron and copper deriving from grapes, soil contaminants, fungicidal residues, or winery equipment. Probably ferrocyanide is the most efficient fining for metal removal, as it precipitates most metal ions but unfortunately it may form hydrogen cyanide. Its use is highly regulated and in many countries is illegal (Boulton *et al.* 1996).

Another (non-protein) organic compound used in beverage fining is polyvinylpolypyrrolidone (PVPP) that is particularly useful in the selective removal of flavans and mono- and dimeric-phenolics, lowering bitterness. PVPP is also efficient in preventing oxidative browning or in removing its brown by-products from white wines after their formation. On the other hand, PVPP removes also quercitin (Laborde *et al.* 2006) and resveratrol (Threlfall *et al.* 1999), representing components that bring health benefit associated with moderate wine consumption (Castellari *et al.* 1998). Furthermore, PVPP is contemporaneously reported as Generally Recognized as Safe (GRAS) approved for many uses by FDA, even if there have been documented cases of allergic reactions to polyvinylpyrrolidone (Ronnau *et al.* 2000; Adachi *et al.* 2003; Yoshida *et al.* 2008).

Protein fining is a frequent applied procedure that allows wine clarification and stabilisation, preventing colloidal precipitation (Yokotsuka *et al.* 1995; Marchal *et al.* 2002a), improving wine organoleptic characteristics (Maury *et al.* 2003) and reducing bitterness and astringency (Yokotsuka *et al.* 1995). The possible permanence of "exogenous" components (i.e. not derived from grape, yeast, or other fermenting bacteria) in wine, as the residual fining proteins, can represent a risk for the consumers sensitive to the protein used.

Actually, only few reports illustrate cases of allergy to wine, especially in Mediterranean countries, associated with grape proteins (Borghesan *et al.* 2004; Kalogeromitros *et al.* 2006).

Rolland *et al.* (2006) described a double-blind placebo-controlled (DBPC) wine challenge using fined and non-fined wines. Unfortunately in this study, the number of patients was very low (five patients with allergy to egg and only one patient with allergy to milk) and the few specific reactions to fined wines were not significant compared to reactions to control wines (non-fined wines). These data were supported by the same authors reporting that casein and egg protein concentrations in fined wines were under the limit of detection (1 µg ovalbumin/l and 8 µg casein/l, respectively) of non-commercial enzyme-linked immunosorbent assays (ELISA) (Rolland *et al.* 2008).

Finally, Kirschner *et al.* (2009) investigated the tolerability of casein-, ovalbumin-, and isinglass-fined wines in 14 allergic patients by skin prick tests (SPT), as well as by DBPC food challenge with fined and filtered wines. The fining agents gave a positive reaction in the SPT, but no patient reacted adversely to the oral challenge of the fined and filtered wines.

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Even if the reported literature seems to indicate the inconsistency of an allergological risk, the EU Directive 2007/68/EC establishes that all wines labelled after May 31, 2009 must declare if allergens like egg and milk were used during production. An extension of time until June 30, 2012 was decided by the Standing Committee on the Food Chain and Animal Health of the European Union.

It must be taken into account that the forced inclusion of a statement such as "contains egg proteins" on the wine label can cause doubts in consumers (allergic or not) and, thereby, damage the perception of the product. This concern has increased the winemakers' attention toward this problem.

Nevertheless, other fining agents (e.g. glutens, lupin, and pea proteins, etc.) or technological enzymes are used and could be present in trace amount in wine, generating risks to health, but they are not considered in the EU Directive 2007/68/EC.

Moreover, the use of animal proteins could represent a concern not only for the human health but also for some ethical practices, such as vegetarianism or veganism, or for religious faith (mainly Judaism), since the use of animal proteins is regulated and generally rather avoided.

Proteins used in winemaking and possible drawbacks related to their permanence in final products

Animal gelatin (sometimes in combination with Kieselsol, a silica colloid) is widely used as a fining agent thanks to its ability to clarify red wine, to reduce wine astringency, and for the low cost (Yokotsuka *et al.* 1995). This fining is primarily used to soften red wines by removing the excess of tannins. Gelatin is a mixture of peptides and proteins produced by partial hydrolysis of collagen extracted from connective tissues of animals. The first concern stemmed from the explosion of Bovine Spongiform Encephalopathy (BSE) case, commonly known as "mad cow disease". The consumption of specific animal tissue derivatives is correlated with the new variant of Creutzfeldt-Jakob disease in humans. In particular, the disease may be transmitted to humans by food contaminated with the protein from brain, spinal cord or digestive tract of infected animals (Ramasamy *et al.* 2003).

Even if the risk was minimal, bovine gelatin use has been mentioned as a possible source of wine contamination with prions associated with BSE. Although the real risk of gelatin use to the human health was unknown, actually most gelatin preparations were derived from pig skins, a source free of BSE.

A second drawback in the use of gelatin is related to its intrinsic origin that can cause ethical drawbacks independently of the protein persistence in the final products but it is related to their use *tout court*. From the Kosher (Jewish law) point of view, animal derivatives need to be produced from suitably slaughtered animals. Nevertheless, gelatin from fish skin is edible and kosher.

Another "food ethics" problem of gelatin (common with other fining agents of animal origin such as isinglass, chitosan, casein, and egg albumen) is related to the respect of vegetarian and vegan diet.

Isinglass is a very pure gelatin, originally prepared from the air bladders, but successively obtained from other fish tissues. It is very effective in clarifying white wines by removing tannins. A drawback is a voluminous sediment formation that tends to plug filters (Chagas *et al.* 2012). The health concern of isinglass is represented by its allergological potential. Salmon, tuna, and halibut are the most common allergenic fishes, but fish-allergic people are often sensitive to almost all kind of fish (Van Do *et al.* 2005). Sub-trace amounts of fish allergens in wines are still able to elicit IgE-mediated skin responses and *in vitro* basophil activation in sensitised patients. On the other hand, Vassilopoulou *et al.* (2011) described that the magnitude of the responses elicited with wine treated with isinglass was quite low, as could be expected from small allergen concentrations.

Similar concern is related to the use of chitosan (Chagas *et al.* 2012) that could be contaminated by crustacean proteins that could be released during fining.

Recently, fish by-products have been used for fining, because they eliminate religious obstacles surrounding the animal gelatin consumption. But, isinglass fined products could not be considered as vegetarian/vegan friendly.

Egg white proteins (albumin or albumen) are a very effective fining agent, long used for clarifying red wines and still widely used in modern winemaking. Egg white proteins are ideal to soft wine astringency by binding and reducing the tannin content, therefore they are most appropriate for highly tannic wines or oak-aged wines (Cosme *et al.* 2007). The resulting aggregates are insoluble and can be eliminated by racking and/or filtration prior to bottling or further maturation.

Hen's egg white albumin preparations are in the form of either fresh or frozen egg white, or as freeze-dried

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powder. The commercial preparations are often sold as "ovalbumin" but it is a mixture of egg white proteins (EFSA 2011b): the major one is albumin (Ovalbumin) which represents 54% of the egg white protein. Other major proteins are Conalbumin (Ovotransferrin) (12%), Ovomucoid (11%), Ovomucin (3.5%), and Lysozyme (3.4%) (Powrie *et al.* 1985; Walsh *et al.* 1988).

Even though Ovalbumin is probably one of the most frequently studied antigens in immunology, it does not appear to be the most allergenic molecule in humans. In a study of 34 adults with confirmed egg allergy, Conalbumin and Ovomucoid were demonstrated to be the most prevalent allergens with a frequency of reactivity of 53 and 38%, while Ovalbumin and Lysozyme showed a frequency of reactivity of 32 and 15%, respectively (Aabin *et al.* 1996).

From an epidemiologic point of view, egg allergy appears mainly in children (i.e. in a population that does not consume wine), being the second most common food allergy at paediatric age, but this disease is not exclusive of children. It has been reported that the prevalence of allergy to egg proteins (particularly to ovalbumin) is around 0.3% among the adult population (EFSA 2011b).

Furthermore, it must be remembered that egg whites are powerful histamine liberators, also provoking a pseudo-allergic response in some people, a condition considered food intolerance instead of a true IgE-based allergic reaction. In this situation, proteins of egg white directly trigger the release of histamine from mast cells on contact (Cantani 2008).

To our knowledge (and as reported by Uberti *et al.* 2014) no case of an allergic reaction after wine consumption due to the presence of residues of egg white proteins has been reported in the literature. Nevertheless, this does not exclude that egg proteins are absent in wine, as there is a threshold value for triggering an allergic reaction that is difficult to establish. Bindslev-Jensen *et al.* (2002) defined a threshold value for egg of 8.6 mg that would protect 99% of egg-allergic individuals. Moneret-Vautrin and Kanny (2004) reported that 18% of egg-allergic individuals can react to a concentration equal to or lower than 65 mg, while the threshold for egg white able of triggering an allergic reaction in 1% of sensitised people was between 1 and 2 mg. Similarly, Morisset *et al.* (2003) performed a DBPC food challenge with egg-allergic individuals and reported that the lowest adverse effect level for crude egg was 2 mg.

Milk casein is a well-known phosphoprotein that, in association with sodium or potassium, forms a flocculate that absorbs and precipitates suspended particles. Casein is primarily used as a decolourant in white wines for reducing browning resulting from oxidation. It is also recommended for reducing the tannin content in over-oaked white wines (Weber *et al.* 2007b; Cosme *et al.* 2012).

The four proteins α-lactalbumin, β-lactoglobulin (both whey proteins), α-casein, and β-casein are considered as the major allergens in bovine milk (Shi *et al.* 2014).

Cow's milk allergy (CMA) is the most common food allergy in infants and young children (Exl *et al.* 2001; Skripak *et al.* 2007). Depending on different studies the prevalence of CMA in children ranges from 1% to 7.5% and from 0.1% to 0.5% in adults (Sicherer *et al.* 2010; Hochwallner *et al.* 2014). Most of the children become milk tolerant after 3 years of age (Sicherer *et al.* 2010; Hochwallner *et al.* 2014) but, even if CMA in adults is rare (EFSA 2011a), according to Lam *et al.* (2008) it may be fatal.

No information is available about the water-soluble milk whey proteins as contaminants in casein preparations used for wine fining, whose permanence could represent a risk to milk-sensitive subjects (Weber *et al.* 2009).

Moreover, caseins and egg white proteins, as previously described for gelatin, are not vegan friendly.

Vegetal proteins could be used during fining as alternative to animal ones, especially those derived from legumes and wheat (CE Regulation No. 2165/2005b; FSANZ 2004) showed a very good fining ability (Maury *et al.* 2003; Cosme *et al.* 2012).

Nevertheless, these vegetal proteins could represent a potential risk to the health of allergic subjects like casein and egg albumen proteins.

Allergic reactions provoked by the ingestion of pea are common. The allergenic potential of this crop is enhanced due to cross reactivity particularly with lentil and chickpea. Two allergens have been identified in pea proteins: vicilin and convicilin (Verma *et al.* 2013).

Wheat proteins are involved in both food allergy and coeliac disease, which occur in sensitive individuals after consumption of wheat products (Hischenhuber *et al.* 2006). In particular, it has been demonstrated that several wheat-gluten proteins, such as α/β-, γ-, ω-gliadins, are involved in wheat allergy (Shewry 2009). Gliadins, in particular the α-gliadins, are also responsible for coeliac disease, producing the most severe effects (Howdle *et al.* 1984).

Recently, other vegetal proteins have been proposed as fining agents, such as maize zeins (Simonato *et al.*

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2009, 2013) and patatin P (Gambuti *et al.* 2012). Zeins can be extracted directly from the so-called "corn gluten" produced in high quantities as the main by-product of the starch industry. They own a good fining efficiency (Simonato *et al.* 2009) and do not negatively affect the wine aroma (Simonato *et al.* 2013). Finally, the American FDA designates zeins as GRAS.

Patatin P is a family of glycoproteins recovered from a potato aqueous by-product, and has gained increased interest. Technically, it is a suitable alternative to animal proteins used as fining agent to decrease total phenolics and tannins of wines, and to lower astringency (Gambuti *et al.* 2012).

Potato allergy has been described rarely, generally in relation to the Oral Allergy Syndrome (OAS) (Seppala *et al.* 1999). Recently, patatin was identified as a major cross-reactive protein in the latex-associated potato allergy and appears to be relevant for atopic dermatitis (Schmidt *et al.* 2002).

In conclusion, as a consequence of the low prevalence of potato allergy and the long history of safe use of the potato in the human diet to meet nutritional requirements, no study (to our knowledge) has been performed to investigate the persistence of potato protein in wine after patatin fining.

Finally, the use of grape seed proteins as wine fining agents is becoming of great interest, since their application would avoid the introduction of exogenous proteins (Vincenzi *et al.* 2013). Indeed, the extraction of small amounts of grape seed components normally occurs during winemaking, and the presence of traces of grape seed proteins has been historically reported in red wine (Yokotsuka *et al.* 1995). From this point of view, they are considered "normal" or "endogenous" components of the beverage without allergological or food ethic problems.

Other proteins used in winemaking are enzymes. The FDA, the Alcohol and Tobacco Tax and Trade Bureau (TTB), and the Organisation Internationale de la Vigne et du Vin (OIV) approved enzyme preparations in winemaking, e.g. to promote clarification and filtration (pectinases, xylanases, glucanases, proteases) or/and to release varietal aromas (glycosidase). These preparations are sold as enzyme blends, having more than one function (Guérin *et al.* 2009), but no specification on their exact composition is given in datasheet.

It is well-known that pectinase-rich enzyme preparations are obtained from *Aspergillus* sp., and are commonly used in red winemaking (Ducasse *et al.* 2010).

A few cases of oral allergy to fungal enzymes (even if they are referred to bread making) are described in literature (Baur *et al.* 1994). Wine certainly constitutes a protein-denaturing environment but the real effect on technological enzyme epitopes and the possible persistence of them in finished products deserve attention.

In addition to the enzyme preparation above mentioned, lysozyme has been proposed as an alternative to sulphur dioxide to control the proliferation of lactic acid bacteria (LAB) in red and white wine or as a means of delaying malolactic fermentation (MLF) in winemaking (Gerbaux *et al.* 1997). Regulation EC No. 2066/2001 (see the section Exogenous proteins in wine and legislation) allows up to 500 mg/l of lysozyme to be added to wine or must.

Lysozyme or muramidase (Proctor *et al.* 1988) is an enzyme ubiquitous in nature and is contained in almost all secretions, body fluids and tissues of the animal organism. Hen's egg white is an important source of muramidase and the principal commercial source of lysozyme. Hen's egg lysozyme is an important food allergen and these preparations are frequently characterised by a contamination with other egg proteins (for the risks see the section Egg white proteins).

Methods for the study of exogenous proteins in wines

Despite the recommendations of good winemaking practices, a lack of standardisation of their use has been noted (EFSA 2011b) and for this reason the possible risk of persistence of proteins in the final product is difficult to evaluate.

These problems affected winemakers of countries such as Australia, New Zealand, and United States, where a specific regulation was introduced (Weber *et al.* 2007b).

For these reasons, the availability of accurate and sensitive detection methods for allergens in wine is crucial. Nevertheless, to the best of our knowledge, there are few and conflicting data on the detection of residual proteins in final products.

Actually, several methods for the detection of residual exogenous proteins in wine have been reported in the literature, with detection limits ranging from a few micrograms to several milligrams per litre.

The most frequently applied strategies are essentially based on immunodetection or mass spectrometry assay. All the proposed methods are calibrated in order to obtain performances that satisfy the criteria issued by OIV, although problems related to outliers and low recoveries have been occasionally encountered.

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It is difficult to list the proposed methods in relation to the origin of the specific protein residue in wines, because the authors frequently described multiple applications of a single protocol. For this reason we separate the immunological approaches from the mass spectrometry ones following a chronological description of the literature results.

Immunological methods for exogenous protein detection in wine. Weber *et al.* (2007a), investigating four different German white wines by applying ELISA, detected lysozyme residues in all wines but egg albumin in only one wine fined with a massive dosage (20 g/hl) of dried egg white. Rolland *et al.* (2008) developed a specific and sensitive ELISA method and tested 153 commercial Australian wines. Their finding showed a lack of residual egg or milk proteins derived from the processing aids in final bottled wines. Lifrani *et al.* (2009) detected the fining agent remainder in fined wines by using sandwich ELISA methods. They analysed 400 commercially available wines, 37 of which were organic, and the tests for the detection of fining agents (albumen, caseinate or isinglass) were positive in 11% wines. Some organic winemakers choose not to filter their wines after fining, which could explain the high level of detection of fining agents.

Weber *et al.* (2009) investigated a panel of various white wines fined with different caseinate dosages and 61 commercial wines with unknown fining by using an indirect ELISA method. They detected α- and β-caseins residues in white wine samples, even if processes such as bentonite addition or membrane filtration contributed to a significant decrease of casein residues in wines. According to this work, allergic reactions due to the consumption of casein treated wines cannot fully be excluded. Restani *et al.* (2012) analysed 16 experimental and 63 commercial wines fined with caseinates, using a specifically developed ELISA as well as an immunoblotting technique in which membranes were incubated with specific anti-caseinate antibody, and no detectable allergenic residues were found in any sample.

More recently Uberti *et al.* (2014) analysed, both by ELISAs and immunoblotting methods, 78 commercial (essentially red) wines. This considerable study provides robust results and must be described in detail. The authors used European, Australian, and New Zealand wines with a complete description of their oenological practice. The wines were then analysed by: (1) sandwich ELISA kit (Euroclone SpA, Pero, Milano), specifically developed, in agreement with the OIV Resolution 427/2010, modified in 2012 (OIV-COMEX 2012), for the quantification of egg white proteins in wine (Restani *et al.* 2014); (2) immunoblotting with a detection limit for egg white proteins corresponding to 0.122 mg/l in the wine sample; (3) ELISA test specifically developed to detect traces of egg white proteins in wine, and validated by a collaborative inter-laboratory study involving 11 laboratories (Restani *et al.* 2014).

In this study, no egg proteins were detected in the 78 commercial wines analysed (detection limit of 0.0564 mg/l).

These results apparently suppress any doubt on the allergenic risk related to egg protein fining. ELISA surely represents a test with a good sensitivity, fast and easy in execution for the detection of specific allergens in foodstuffs. Nevertheless, drawbacks of these techniques could be due to the presence of interfering compounds in a specific matrix. As a matter of fact, a high content of polyphenols in the matrix (as in the case of red wines) could generate interactions both with proteins and antibodies (Weber *et al.* 2007b). Moreover, in these immunological tests, also the adsorption to solid matrices of the allergens could alter epitopes (Kaul *et al.* 2007) compromising the reaction sensitivity in relation to the antibodies used.

From this point of view, there still remains a reasonable suspicion that residues of proteins used for fining processes can remain in wine after filtration, in an amount sufficient to elicit an allergic reaction in sensitised consumers (Lacorn *et al.* 2011). This is the rationale of additional recently proposed studies.

Lacorn *et al.* (2014) utilised the sandwich ELISA kits for the quantification of egg (RIDASCREEN®FAST Ei/Egg R6402) and caseins (RIDASCREEN®FAST Casein R4612) in wine provided by R-Biopharm AG. In this collaborative test participated 18 laboratories with consolidated expertise in immunological tests.

In this study, the determination of casein in white wine and egg white protein in red wine fits the performance criteria set by the OIV resolutions. However, the authors recognised that if a few laboratories struggled when using this assay, most of the participants showed a variation of results.

Experimental evidences suggest that filtration of wines should remove egg and milk proteins almost completely and result in residual concentrations below the LODs of analytical tests and far below allergy-eliciting concentrations. However, this paper confirms a previous report (Lacorn *et al.* 2011) indicating that insufficient, incomplete, or erroneous

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filtration results in measurable allergen concentrations, which might harm predisposed individuals.

A further recent study investigates the efficiency of oenological procedures on lysozyme depletion in wine by a specifically developed indirect ELISA method (Carstens *et al.* 2014). For the assessment of the effect of winemaking procedures, two wines (a white one and a red one) were produced following the standard oenological procedures. In the tested condition, all the oenological procedures (filtrations, centrifugation, flash pasteurisation, fining with silica and bentonite) are able to reduce the lysozyme amount that remains very high in the final products. Bentonite is the most effective in lysozyme removal, but it fails with the lysozyme concentration over 1 g/l. Hence, the authors concluded that, depending on the production technique employed, lysozyme might potentially be present in the final product representing a risk to sensitised individuals. Regarding future developments, the authors concluded, in agreement with Liburdi *et al.* (2012), that lysozyme must be used in immobilised form.

Finally, the paper of Deckwart *et al.* (2014) focused on the development of a sensitive ELISA for the casein detection in wine must be cited. In this paper, an indirect ELISA for the investigation of wine is described. The performance of the system is a LOD of 0.2 mg/l for red wine while for white wine it depends on the calibration standard: 0.1 mg/l for the fining agent casein and 0.01 mg/l for casein from a chemical trader. It is also shown that the use of different technological procedures during winemaking leads to no detectable amounts of casein in various wine samples.

In this paper the above-mentioned drawbacks of ELISA methods are described. In red wine the interfering compounds in the matrix are mainly polyphenols as well described by Weber *et al.* (2007b) and Monaci *et al.* (2010).

MS-based methods for the detection of exogenous protein in wine. The high sensitivity, accuracy, and reproducibility of the MS techniques (Picariello *et al.* 2011) allow the detection of trace amounts of proteins and make the identification independent of the protein structure (Kaul *et al.* 2007; Kirsch *et al.* 2009).

A further big benefit of MS analysis compared to ELISA is the possibility to detect more than one protein simultaneously. However, a drawback of MS methods is that it is a non-immunological method and the antigenicity of the target protein is not considered, which might be important for allergen analysis.

Monaci *et al.* (2010) proposed a method based on capillary liquid chromatography combined with electrospray ionisation-tandem mass spectrometry that allowed the detection of some peptides arising from α- and β-caseins present as residues in fined white wines. Nevertheless, protein analysis is more difficult in red wines, because of the above-mentioned presence of a large quantity of interfering compounds, such as polyphenols, in particular tannins, and polysaccharides (Moreno-Arribas *et al.* 2002). Therefore, it is necessary to develop methods that, in addition to concentrate the proteins, allow the removal of the interfering compounds from the concentrated protein preparation.

Some authors reported a method based on a preliminary enrichment step, performed by combinatorial peptide ligand libraries, coupled to MS for identifying traces of casein in red and white wines (Cereda *et al.* 2010; D'Amato *et al.* 2010). The proposed method included a partial removal of phenolic substances by overnight incubation with PVPP, absorption and subsequent desorption of the captured proteins from the beads and then an SDS-PAGE step followed by in-gel trypsin digestion and finally LC-MS analysis.

A method based on the recovery and identification of proteins by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) in a gel-free approach has been recently described for the detection of residual gluten and egg albumen proteins in red wines fined in laboratory (Simonato *et al.* 2011; Tolin *et al.* 2012a,b). This method is very simple and rapid, being based on the first step of recovery of proteins by precipitation using the KDS method (Vincenzi *et al.* 2005) and, after dodecyl sulphate removal, on the second step of protein identification by LC-MS/MS. Moreover, this technique allows overcoming the problems of phenolic compound and carbohydrate removal and has the advantage of being a gel-free approach, allowing the simplification of the analytical procedure and avoiding the loss of components not detectable on electrophoretic gels. The proposed method was also applied for the detection of possible residual milk and egg white fining proteins in 25 commercial wines (12 red, 12 white, and 1 rosé) and found proteins of animal origin in 8 samples (Tolin *et al.* 2012b). The quantification of the allergenic residues found in egg or milk treated wines was not the aim of the paper, but it represents uniquely a system to assess the presence and not the quantity of potential allergens in wines. From this point of view, it must be remembered that the quantity of allergen that can elicit the allergic reaction cannot be established a priori (Hischenhuber *et al.* 2006)

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and therefore the target should be the development of analytical methods able to exclude the presence of any residual amount of allergenic substances.

A further approach was proposed by Losito *et al.* (2013). These authors developed a method for the detection and quantification of caseinate traces resulting from fining processes in white wines by using the combination of size exclusion-solid phase extraction and ultrafiltration, followed by tryptic digestion and analysis of the protein digest by liquid chromatography-electrospray ionisation-3D ion trap-mass spectrometry (LC-ESI-3D IT-MS).

Monaci *et al.* (2013) developed a technique based on the combination of ultrafiltration, tryptic digestion, and LC/ESI-MS analysis with a high-resolution mass spectrometer that enabled the development of an analytical method able to detect and quantify simultaneously traces of caseinate and egg white powders potentially remaining in white wines upon fining.

Finally, Mattarozzi *et al.* (2014) recently proposed chromatography-tandem mass spectrometry methods for the simultaneous detection of casein and ovalbumin in wine. In this case, an easy protein cut-off concentration protocol combined with size-exclusion-based purification (6 kDa-SE column) was developed. In comparison with a conventional PVPP treatment, SE is able to provide improved protein recovery and extract purity. The work-flow proposed combined with LC-MS/MS analysis results sensitive enough to identify and quantify allergens in red wine protein extracts at very low levels (about µg protein/ml wine), making this method useful to assist in the protection of the health of allergic consumers.

Exogenous proteins in wine and legislation

Hidden allergens represent a cause of great concerns for sensitive people as they can be inadvertently exposed to the triggering food. Hidden allergens can induce a wide variety of hypersensitivity reactions. Currently it is not possible to determine the exact prevalence of these reactions but they are clearly a rising problem.

A hidden allergen is a substance that is unrecognised or not declared on the product label. The unintentional intake of such ingredient can be a consequence of allergen contamination by using shared equipment in different foodstuff preparations, by adding of allergic processing (Taylor *et al.* 2009). For such reasons, the European Parliament drafted Directive 2003/89/EC, last amended by Directive 2007/68/EC indicating a list of food ingredients known as food allergens (including milk, egg derivatives, and gluten proteins) that necessarily must be declared in the appropriate labels. Nevertheless, these compounds were temporarily excluded from the labelling requirements when used as processing aids in wine fining, because scientific data were missing proving their involvement in allergic reactions in such utilisation (Regulation UE 2010/1266; Directive 2003/89/EC; Directive 2005a/26/EC; Directive 2007/68/EC).

The use of advisory labels (such as "May Contain") on packaged foods was voluntary, and there are no guidelines for their use. Since July 2012 European winemakers have been obliged, like those of Australia and New Zealand (Weber *et al.* 2007b), to indicate the use of egg albumin and milk caseins on the wine labels whenever they are used as fining agents. The threshold adopted by the European Union legislation is 0.25 mg/l (Regulation (EU) 579/2012).

The new European legislative frame represents an important tool for assuring both the winemaking that could give statements in a clear and consistent manner, and the consumers that are informed on the real composition of the products.

This legal achievement regarding wine labelling represents a very important result not only with regard to the knowledge of the actual composition of the product, but also especially for subjects affected by allergic diseases to certain proteins used as fining agents, who could be exposed to unknown allergenic risks (FSANZ 2004; CE Regulation No. 2165/2005b).

However, it is interesting to note that this Regulation does not take into account the vegetal proteins admitted as fining agents as some of them are well-known food allergens, such as pea and gluten proteins (Shewry 2009; Verma *et al.* 2013).

In the field of vegetal protein application to food, it sometimes happens that the industry asks a "safety opinion" to the authority. An example could be given by protein isolated from potato that specifically are not currently listed in the Code of Federal Regulations as an approved food additive in the US. However, in 2002 the United States FDA issued a letter of no objection in response to a Notice of Generally Recognized as Safe self-determination for coagulated potato protein in hydrolysed and unhydrolysed form ("potato protein preparations") for addition to a variety of food products as a water binder, foaming aid, or emulsifier at use-levels in the range of 0.1–3.0% resulting in dietary exposures of 1.9 g/day (GRN 000086) (Post 2002).

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Nevertheless, in the present state of things no label indication is required for these kinds of proteins, but it is hoped that also the vegetal protein indication on a wine label will be taken into account and it will be mandatory in the future.

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