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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 fermentation for its ability to control the growth of Gram-positive bacteria and spoilage bacteria, without inhibiting yeast growth, and it allows a reduction in 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 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 were found to be positive both by 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 unintended allergen 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 (Taylor, Nordlee, Niemann, & Lambrecht, 2009). 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 EU (Directive 2007/68/EC) 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 (Tolin et al., 2012a; Tolin, Pasini, Simonato, Mainente, & Arrigoni, 2012b; Penas, Di Lorenzo, Uberti, & Restani, 2015; Rizzi, Mainente, Pasini, & Simonato, 2016), and changes in wine label regulations have been introduced (Regulation EU 579/2012). 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 (OIV, 2010; OIV-Comex, 2012). Nevertheless, to the best of our knowledge, no studies have been conducted to detect hidden allergens in other alcoholic beverages.

In North America cider refers to a non-alcoholic juice of apple (Watson, 2013), 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 wine-making 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 (Kashyap, Vohra, Chopra, & Tewari, 2001; Tapre & Jain, 2014; Penas et al., 2015). 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 potential of 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 (Rizzi et al., 2016). 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. (2016). 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 mm² 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. (2015). Briefly, gel pieces were dehydrated with acetonitrile (ACN) and dried under vacuum. 150 µL of 10 mM dithiothreitol (DTT) in 50 mM NH₄HCO₃ 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 NH₄HCO₃ for 45 min at room temperature and in the dark.

Samples were washed twice with 200 µL of 25 mM NH₄HCO₃ 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 NH₄HCO₃, Promega Corporation, WI, USA) and 25 µL of 25 mM NH₄HCO₃. 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 C₁₈ 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 μ m 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. Chemiluminescence 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 S1-S18). In general, the amount and type of proteins present in each cider were variable, with some contaminating proteins (namely human keratins) being almost invariably the dominant proteins 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 (Xu et al., 2011). The presence of keratins in other types of beverages (e.g. wine) has been rarely documented (Tolin et al., 2012b). 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 (Wigand, Tenzer, Schild, & Decker, 2009; Jaeckels et al., 2015; Meier et al., 2016; Jaeckels et al., 2017). 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*, *Oenococcus*), 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*, *Oenococcus*), which is sometimes desirable to decrease cider acidity (Reuss et al., 2010). 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 (Tapre & Jain, 2014). Among the proteins from *Malus domestica* that are more frequently identified, there are Thaumatin-like proteins (Mal d2) and non-specific Lipid-Transfer Protein (Mal d3). 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 d1) and Profilins (Mal d4) are also known apple allergens, but they have not been detected in the samples. These proteins, described as thermolabile

and prone to proteolysis (Oberhuber et al., 2008), 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 1). 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 (Liburdi, Benucci, & Esti, 2014). It represents therefore an advantageous method to prevent undesirable fermentation allowing a reduction in the use of sulphur dioxide (Liburdi et al., 2014). 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 (Reuss et al., 2010; Quendler & Martinello, 2011; Watson, 2013). 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 (Bartowsky, 2009; Sonni, Bastante, Chinnici, Natali, & Riponi, 2009). Although the use of HEWL as food preservative is permitted in the European Union and in other countries (EU Regulation 2066/2001), HEWL is also described as an allergen (Gal d4) that contains both sequential and conformational epitopes (Mine & Zhang, 2002; Walsh, Hill, Macoun, Cairns, & Howden, 2005; Weber et al., 2009). Aabin et al. (1996) indicate a frequency of reactivity of 15% in adults with confirmed egg allergy, and Frémont et al. (1997) 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), 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 (OIV-Comex, 2012). 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* (Hischenhuber et al., 2006), since cases of allergic reactions were elicited by few μg of proteins (Madsen, 2001). 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 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 $\text{Fc}\epsilon\text{RI}$, to determine the massive release of mediators by the stimulated cells (Sicherer & Sampson, 2009). 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 (Mine & Zhang, 2002; Weber et al., 2009). 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 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 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.

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 (Taylor et al., 2009). As mentioned above, cider-makers and wine-makers 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 cider-makers and Authorities to safeguard the consumers' health and the quality of the product.

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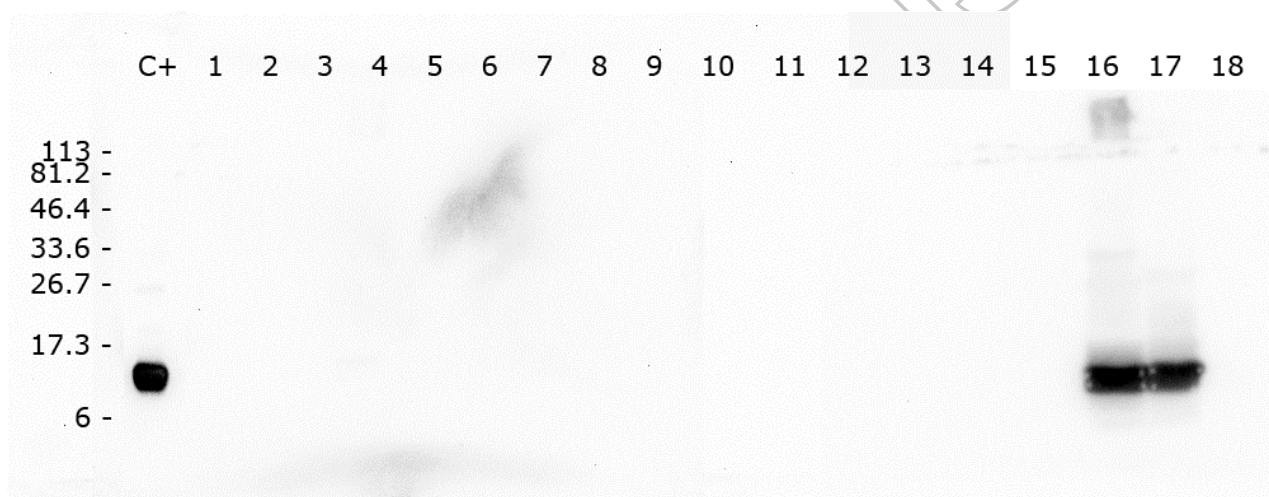
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Figure 1 Western blot analysis of cider proteins. For SDS-PAGE 14% each lane was loaded with protein recovered from 5 mL of sample. Immunoblotting was developed with anti-lysozyme (hen egg white) polyclonal antibody. C+: positive control. Lane 1: still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



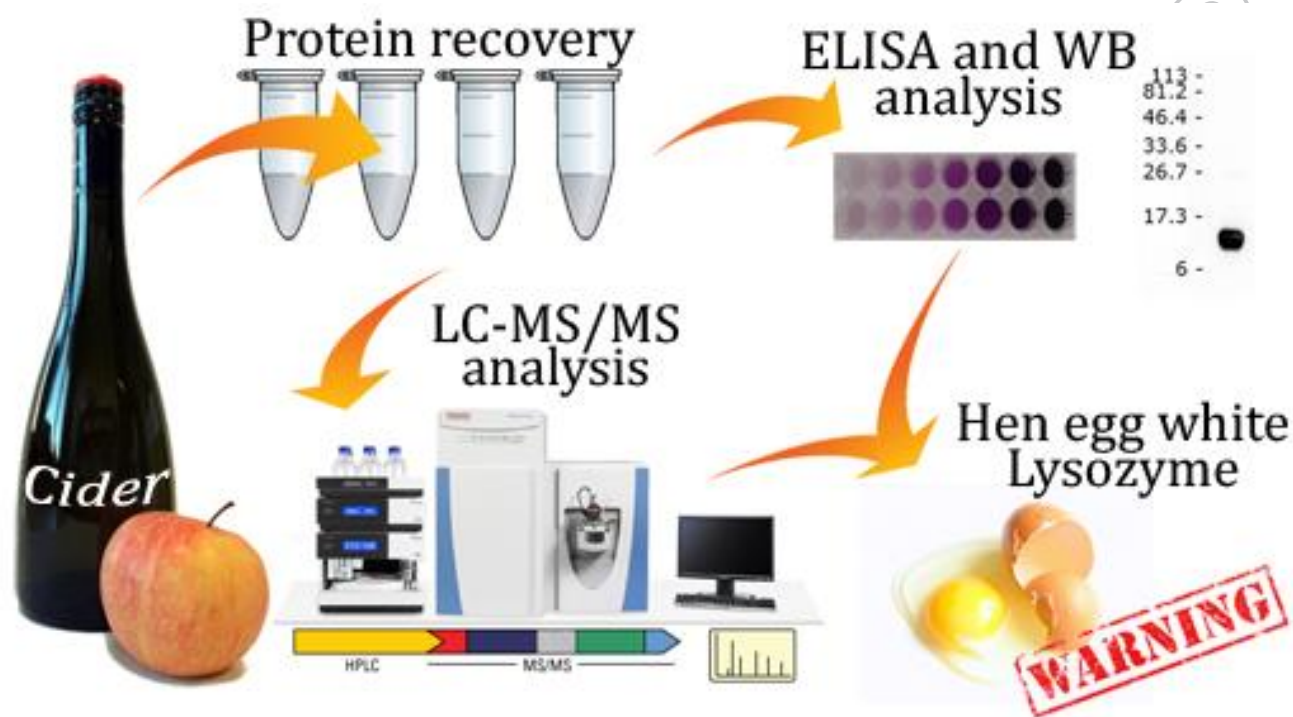


Table 1 Ciders positive to HEWL detection with LC-MS/MS, ELISA and western blot techniques.

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	-	-	-