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IDENTIFICATION OF COW MILK ALLERGEN IN THE PRODUCTS OF GRAPE PROCESSING

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ABSTRACT

The aim of research was the identification of cow milk allergen in grapes, must, federweisser and wine by immunochemical method ELISA. Milk allergens (casein) are mostly used together with egg proteins during wine clarification. The results show that quality of calibration curves has significant importance for objective evaluation of quality detection. The degree of the variability calibration samples expressed R^2 was not less than 0.9 in both calibration curves (0.9485 and 0.9659). In grape samples, concentrations of cow's milk casein below the detection limit were determined, that is recommended by the ELISA kit manufacturer. All grape samples showed casein concentration less than the value corresponding to 0 ppm standard (from 0.039 to 0.127 mg/kg). Low concentrations were recorded in three samples of must (from 0.056 to 0.077 mg/kg) as well. In case of the federweisser, the casein concentration ranged from 0.367 to 1.301 mg/kg, which is still less than 1.5 ppm standard (1.373 mg/kg). Most of the wines were found to be in the ELISA detection range. The exceptions were samples no. 3 and 9, whose absorbance was above the highest standard (45 mg/kg). These samples were then reconsidered after the first dilution, the resulting cow milk casein concentration was 67.22 mg/kg and 48.66 mg/L. Higher concentrations of this protein contained white wines (from 21.473 to 67.22 mg/L). In red wines, the milk protein concentrations ranged from 1.634 to 16.715 mg/L.

Keywords: allergen, casein, allergen in wine, production of wine, ELISA

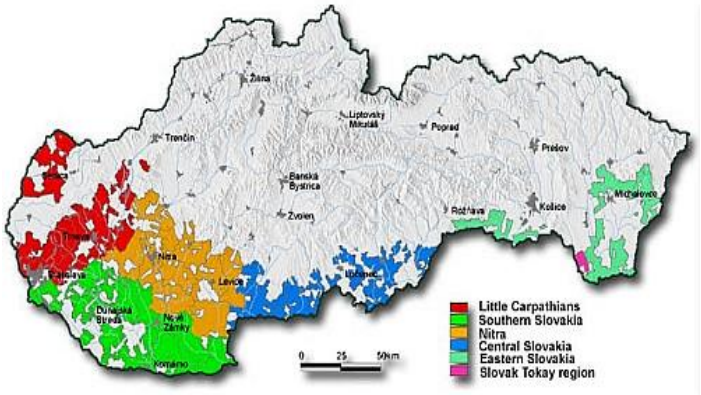
INTRODUCTION

Grape is the main fruit crop in several countries. Although many grape-based food products can be found in the market, studies have shown that around 75% of the world grape production is destined for the wine industry. Grape pomace is an abundant by-product from the wine industry, which consists of the remaining skin, seeds and stalks and represents around 25% of total grape weight used in the winemaking process (**Beres *et al.*, 2017**). Wine consumption, if it is drunk sensibly and in moderation, is not harmful to the human body and forms an appropriate part of the diet and is beneficial for health. It is proved by the content of the total polyphenols, especially specific substances such as trans-resveratrol, quercetin and anthocyanins in red wines. In addition, Slovak wines have significant anti-radical capabilities, which allow them to compete with high-quality foreign wines **(Gažarová *et al.*, 2008, 2010, 2016).**
Wine is a beverage resulting from the fermentation of grape must with appropriate processing and additives. The diversity and quality of wine result from the grape variety, soil composition, location, climate and the enological processes used (**Peňas *et al.*, 2015**).
The use of several products is permitted during winemaking. Some of them are additives and are still present in bottled wine; others are normally removed after treatment and do not leave any residue in the final beverage (**Castillo-Sánchez *et al.*, 2006; Castillo-Vergara *et al.*, 2015**). Some additives and processing aids used in vinification are proteins, and some of them are provided by foods included amongst the most important allergens (such as milk proteins, egg white proteins, *etc.*) (**Peňas *et al.*, 2015**). In principle, proteins can affect wine stability and clarity, a variety of procedures have been developed for their removal from wines (**Ferreira *et al.*, 2002**). Proteins are present in wines at low levels, most of them having a remarkable technological and economical relevance. Milk and egg proteins are also typically utilized by the winery industry as fining agents to promote wine clarity and to improve wine color, flavor and physical stability (**Yokosuka and Singleton, 1995**). The formation of protein-polyphenols complexes and tannin-protein aggregates has been often described (**Siebert, 1999**). These complexes can be further removed by decantation or filtration steps (**Castillo-Sánchez *et al.*, 2006**).
Among milk proteins, caseins are universally known as suitable agents for binding phenolic compounds and reducing off-flavour ingredients that may affect wine taste and colour. Although it is assumed that fining agents are nearly quantitatively removed during the manufacturing process, to date there is no evidence that the consumer ready product is truly free of residues (**Monaci *et al.*, 2017**).
Commercially available bottled wines made using standardized processes, fining, maturation, and filtration, do not therefore represent any risk of anaphylactic reactions in sensitized people (**Lifrani *et al.*, 2017; Cho *et al.*, 2015; Munblit and Verhasselt, 2016**). **Rolland *et al.* (2006)** investigated whether wines fined with allergenic proteins (such as milk proteins, isinglass and egg proteins) can provoke significant clinical allergic reactions in sensitive patients. Although the consumption of milk protein-fined wine did not induce anaphylaxis, some mild reactions were observed. In view of this, it is of paramount importance to have at disposal sensitive analytical methods able to detect traces of milk and egg allergens in food (**de Angelis *et al.*, 2017**).
Several analytical methods exist for the quantitative and qualitative detection of residues of priority allergenic foods. These include methods such as enzyme-linked immunosorbent assays (ELISAs), lateral flow assays, and polymerase chain reaction (PCR) methods, which are currently available commercially for detecting residues from allergenic sources. Methods such as mass spectrometry (MS) and surface plasmon resonance (SPR) biosensors have only recently been applied to the detection and quantification of allergenic residues in wine. In this context criteria for the methods of quantification of potentially allergenic residues of fining agent proteins in wine were examined (**Žiarovská *et al.*, 2018; Baumert, 2013; Rona *et al.*, 2007; Poms *et al.*, 2004 etc**.). Several ELISA procedures have been developed to detect allergenic residues in wines. However, the complexity of the wine matrix can inhibit the immunoenzymatic reaction (**Koestel *et al.*, 2016**). In paper of **Monaci *et al.* (2017)** a method using a capillary LC separation combined with ESI-Q-TOF mass spectrometry for the

unequivocal identification of peptides from caseins is described. The method has been applied to white wine fined with caseinate where some peptides, arising from α and β caseins, present as residues in wine extracts could be detected and identified. The method appears to be very useful for screening purposes as well as a confirmative method to corroborate positive results obtained by ELISA.
The exemption for the wine labeling regarding casein and ovalbumin, according the **European Directive 2003/89/EC**, has been revoked following the negative Scientific Opinion of European Food Safety Authority. EFSA concludes that wines fined with casein / caseinate / milk products / egg derivates may trigger adverse reactions in susceptible individuals. Thereby, allergen labeling of wines become compulsory from June 2012. In the wine manufacturing process, casein and egg albumin are frequently used as fining agent proteins for the fining of white, red wines and rosé. **The European Regulation 1266/2010 (EC)** establishes that all wines, placed on the European market or labeled after 30 June 2012, shall comply with the labeling rules. **Commission** implementing **Regulation (EU) No 579/2012** establishes the requirement to indicate any potentially allergenic ingredient on the labelling of any beverages containing more than 1.2 % by volume of alcohol, and especially egg-based or milk-based products used in making wines.

MATERIAL AND METHODS

The aim of our research was milk allergen (casein) determination in the process of wine production. For this reason the detection of casein was performed in 35 samples of grapes, must, federweisser (SW – stormy wine) and wine which originated from the wine producers from different wine-growing regions of



Picture 1 Wine-growing regions of Slovakia

Slovakia. Grape samples of different varieties (9 samples), must (3 samples) and federweisser (6 samples) originated from Nitra wine region and Central Slovakian wine region- district Krupina (autumn 2017). Red and white wines (17 samples) used for analysis originated from Nitra wine region and Eastern Slovakian wine region, vintage 2014 – 2017.
All samples were stored in frozen conditions until the analysis by ELISA kits. Casein ELISA Kit is intended for the quantitative determination of casein in raw as well as heat treated foodstuffs. With use of ELISA Kits were determined all samples in triplicate. Time required for the sample preparation and extraction for 10 samples was about 1 hour. Time required for ELISA determination (96 wells micro-titration plate) was 2 hours 50 min. Limit of detection (LOD): 0.24 ppm (mg/kg), limit of quantification (LOQ): 1.30 ppm (mg/kg), calibration scale range: 1.5 – 45 ppm (mg/kg).

Principle of analysis

The determination of casein is based on its immunochemical reaction with a specific antibody. Casein present in analysed sample and casein, having been marked with biotin prior to the analysis, react in the first step with a specific antibody coated on walls of wells, as arrayed in a microtitration plate. As a net result, casein is bound to the wells´ walls, while both casein of the sample and that marked with biotin, complete for access to binding spots of the antibody against casein; these spots are limited in their count. Following the step of wells washing, added to the wells is the horse-radish peroxidase conjugated with streptavidin, to undergo an incubation phase. After expiry of the necessary incubation period comes washing out the wells and then the addition of a chromogenic substrate (tetramethylbenzidine) will enable to detect the remaining coated (immobilised) peroxidase. The intensity of colouration thus developed is inversely proportional to the concentration of casein in calibrators, check samples and analysed samples.

Sample preparation

Grape samples were grinded in grinding mortar to obtain powder material. Liquid samples (must, federweisser and wine) were processed directly. To 1.00 g or 1.00 ml of sample contained in a clean closeable flasks was added 10 mlL of extraction buffer solution. The extraction process was running under continuous shaking for 5 min. After completing the extraction, the flask content was centrifuged and the supernatant liquid was sampled. Conditions of centrifuging: R.C.F = 1.800 x g; time 20 min.

Determination procedure

- STEP 1 (Pipetting) – into every well 150 µL of the calibrator or the sample + 50 µL biotinilated casein,
- STEP 2 (Incubation) – to cover frame with lid, incubate for 90 min. at 18 – 25 °C, no shaking,
- STEP 3 (Washing) – suck off and 4 times rinse with the diluted washing solution,
- STEP 4 (Pipetting) – into every well 200 µL of the diluted solution of the conjugate,
- STEP 5 (Incubation) – to cover frame with lid, incubate for 60 min. at 18 – 25 °C, no shaking,
- STEP 6 (Washing) – suck off and 4 times rinse with the diluted washing solution,
- STEP 7 (Pipetting and incubation) – into every well 200 µL of TMP substrate. Incubate for 20 min. at 18 – 25 °C in dark,
- STEP 8 (Measurement) – to stop reaction by adding 50 µL of the STOP solution, to measure colour change at 450 nm.

RESULTS AND DISCUSSION

Proteinaceous products are widely used as fining agents during winemaking to remove unwanted insoluble particles and undissolved microscopic particles (colloidal material) from the must or wine to improve stability. Some of them (egg white, caseinates, and fish gelatine) have allergenic potential and the presence of their residues in the final product could represent a risk for allergic individuals (**Peñas *et al.*, 2015**). Slovakia is home for almost 400 active winemakers producing varietal and quality wines with protected geographical indication and wines with designation of origin from the 19 634 hectares of vineyards in 390 municipalities in 6 main wine regions (Picture 1). The Slovak winemakers may be wine-growers themselves, or supply from the growers in neighbouring regions or both.
Prior to the analysis of 35 samples, quality control of ELISA tests was done. C.V. of results (n = 10) for inter and intra assay was 5.6% and 4.85%. In accordance with the producer´s declared quantitation range, it is possible correctly quantify the contamination between 0 – 45 ppm (mg/kg) of cow casein presence in the examined samples. The starting point for obtaining of relevant data was to create 2 calibration curves from the values given in the table 1.

Table 1 The values for the creation of calibration curve for the detection of cow milk casein in samples by ELISA tests.

Standards	Concentration of cow milk casein in the standard [mg/kg]	Absorbance at 450 nm	
		Analysis of grape, must and federweisser	Analysis of wine
1	0	1.197	1.375
2	1.5	0.683	0.763
3	4.5	0.44	0.492
4	15	0.28	0.322
5	45	0.252	0.275
	Control		
Negative control	0.046	1.990	1.890
Positive control	62.376	0.200	0.220

The results show that accuracy of detection is directly affected by the quality of calibration curve that has significant importance for objective evaluation of the quality detection. As it is presented in the figures 1 and 2, logarithmically modified data needed for creation of calibration curves had linear dependence and detection reliability was described by regression equations. The degree of the variability calibration as expressed R^2 was not less than 0.9 in both calibration curves (0.9485 and 0.9659, respectively).

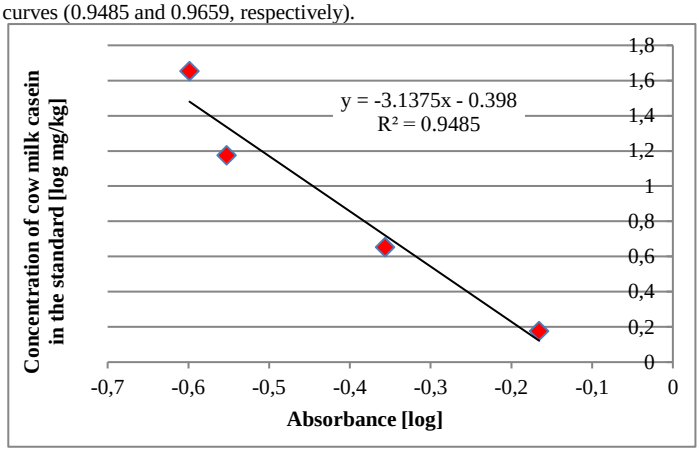
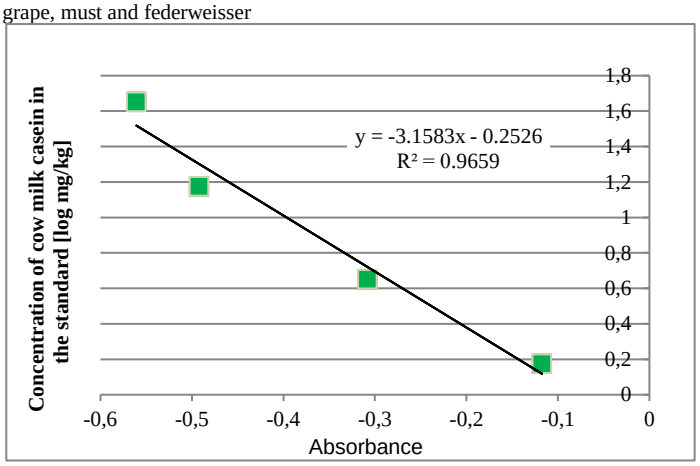


Figure 1 Calibration curve for the detection of cow milk casein in the sample of grape, must and federweisser



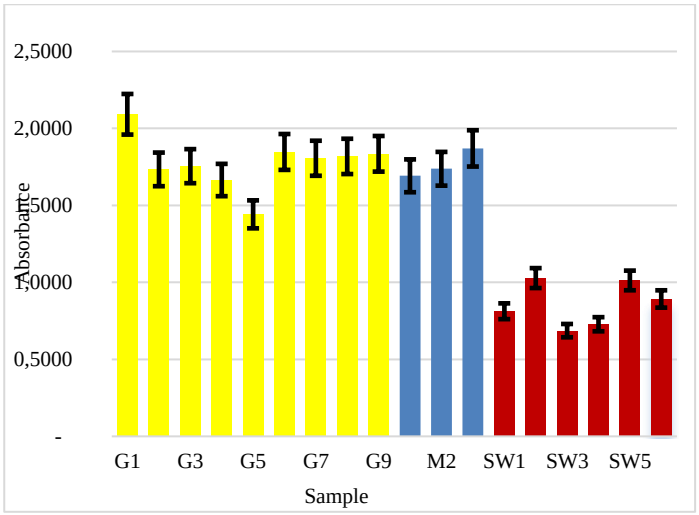


Figure 3 Absorbance of milk casein in the sample of grape, must and federweisser (SW)

Numerous producers and sellers offer their own softwares for imunoanalytical data processing and these are also the part of fotometric analysers (four-parametric logistic model and spatial comparison method). **Czerwenka *et al.* (2010), Zeleňáková *et al.* (2010, 2011, 2016a), Zarranz and Izco (2007), Asensio *et al.* (2008)** have studied the calibration relationships in frame of chromatographic and ELISA detection of cow milk in the wide spectrum of food. In research of **Zeleňáková *et al.* (2016a)** the R^2 values ranged from 0.9981 up to 0.9956 for the linear regression and R^2 were 1 in two experiments for the polynomial regression models within interspecies milk adulteration.

Some of the wine samples were succesfully quantified due to their decimal dilution prior to the analysis (table 3). The presence of cow milk casein in samples was calculated multiplying by diluting factor. The producer of ELISA kit does not recommend samples to be quantified over/under the detection limit. In grape samples, we detected concentrations of cow's milk casein below the detection limit that is required by the ELISA kit manufacturer. All grape samples had the casein concentration less than the value corresponding to 0 ppm standard (0.039 – 0.127 mg/kg). Equally low concentrations were recorded in three samples of must (0.056 – 0.077 mg/L). It seems that, under regular consumption of grapes and musts, human health should not be affected in view of the possible allergic reaction to cow's milk protein. In case of federweisser, the casein concentration ranged from 0.367 – 1.301 mg/L, that is still less than the 1.5 ppm standard (1.373 mg/L).

Table 2 Concentration of cow milk casein in the samples of grafe (G), must (M) and federweisser (SW)

Sample	Grape variety	Wine-growing region of Slovakia	Average of absorbance (n=3)	Log (abs)	Equation	Concentration of cow milk casein (mg/kg and mg/L)
G1	Pálava	Nitra	2.092	0.321	-1.404	0.039 (*)
G2	Dornfelder	Nitra	1.734	0.239	-1.148	0.071 (*)
G3	Rizling vlašský	Nitra	1.755	0.244	-1.164	0.068 (*)
G4	Cabernet Savignon	Nitra	1.665	0.221	-1.093	0.081 (*)
G5	Müller Thurgau	Nitra	1.442	0.159	-0.897	0.127 (*)
G6	Frankovka modrá	Nitra	1.847	0.267	-1.234	0.058 (*)
G7	Othello	Central Slovakia	1.806	0.257	-1.204	0.063 (*)
G8	Concordia	Central Slovakia	1.818	0.260	-1.213	0.061 (*)
G9	Iršai	Nitra	1.835	0.264	-1.225	0.060 (*)
M1	Pálava	Nitra	1.692	0.228	-1.115	0.077 (*)
M2	Dornfelder	Nitra	1.738	0.240	-1.151	0.071 (*)
M3	Othello + Concordia	Central Slovakia	1.870	0.272	-1.251	0.056 (*)
SW1	Othello	Central Slovakia	0.813	-0.090	-0.115	0.767
SW2	Modrá concordia	Central Slovakia	1.028	0.012	-0.436	0.367
SW3	Müller Thurgau	Nitra	0.687	-0.163	0.114	1.301
SW4	Andre	Nitra	0.728	-0.138	0.034	1.081
SW5	Svätovavrinecké	Nitra	1.013	0.005	-0.415	0.384
SW6	Savignon Blanc	Nitra	0.892	-0.049	-0.243	0.572

* the Producer of ELISA kit does not recommend that samples to be quantified under the detection limit

Increased concentrations of milk casein in federweisser can be found due to the first fermentation or federweisser clarification. At grape processing and pressing, sludge particles are getting to the must containing tannins, polyphenols, chemical residues, wild yeasts and other substances that negatively affect the fermentation process and the overall quality of the wine produced. For this reason, these substances and microorganisms are removed from the federweisser by decanting. This process is often associated with the process of clarification using milk proteins. Milk and egg proteins are commonly used as fining agents for wine production. They remove undesirable substances such as phenolic compounds to prevent coagulation of colloidal particles, reduce bitterness and astringency, resulting in pure wines with no foreign odours (**Tolin *et al.*, 2012**). In table 3 are shown the cow's milk casein concentrations [mg/L] that have been quantified in wine samples.

Table 3 Concentration of cow milk casein in the samples of wine (W)

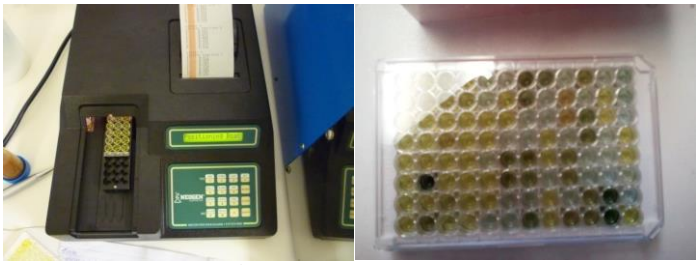
Sample	Grape variety	Wine-growing region of Slovakia	Average of absorbance (n=3)	Log (abs)	Equation	Concentration of cow milk casein (mg/L)
W1	Muškát moravský (2016)	Eastern Slovakia	0.278	-0.554	1496	31.692
W2	Rulandské šedé (2016)	Eastern Slovakia	0.315	-0.502	1.332	21.473
W3a	Rizling vlašský (2016)	Eastern Slovakia	0.455	-0.342	0.828	67.22 (** undiluted sample)
W4	Dievčie hrozno (2016)	Eastern Slovakia	0.263	-0,580	1.579	37.962
W5	Frankovka modrá (2016)	Eastern Slovakia	0.618	-0.209	0.408	2.556
W6	Cabernet savignon (2016)	Eastern Slovakia	0.507	-0.295	0.679	4.776
W7	Rulandské modré (2016)	Eastern Slovakia	0.712	-0.148	0.213	1.634
W8	Dornfelder (2016)	Eastern Slovakia	0.389	-0.410	1.042	11.027
W9a	Chardonay (2015)	Nitra	0.504	-0.298	0.687	48.66 (** undiluted sample)
W10	Fragolino Bianco (2016)	Nitra	0.275	-0.561	1.518	32.973
W11	Ríbezľové víno (2017)	Nitra	0.654	-0.184	0.330	2.137
W12	Frankovka Modrá (2014)	Nitra	0.341	-0.467	1.223	16.715
W13	Frankovka modrá (2015)	Nitra	0.479	-0.320	0.757	5.715
W14	Frankovka modrá (2016)	Nitra	0.643	-0.192	0.353	2.225
W15	Svätovavrinecké (2016)	Nitra	0.327	-0.485	1.281	19.081
W16	Pálava	Nitra	0.354	-0.451	1.172	14.852
W17	Dornfelder	Nitra	0.357	-0.447	1.160	14.461

** the Producer of ELISA kit does not recommend that samples to be quantified over the detection limit

As it is shown in Table 3, most of wine samples were determined to be in the ELISA detection range. The exceptions were just samples no. 3 and 9, whose absorbance was above the highest standard (45 mg/kg). These samples were then reconsidered after the first dilution, the resulting cow milk casein concentration was 67.22 mg/kg and 48.66 mg/L. Higher concentrations of this protein contained white wines (from 21.473 to 67.22 mg/L). In red wines, the milk protein concentrations ranged from 1.634 to 16.715 mg/L.

Since July 1st 2012, according to the **European Regulation 1266/2010 (EC)** all wines placed on the European market shall comply with the labeling rules. The **2003/89/EC directive** requests allergen labeling for wine if egg and milk protein were used during the winemaking process and are present at levels 0.25 mg/L (0.25 ppm) or higher.

Milk proteins (casein, potassium caseinate) are used in the process to remove phenols and tannins from white wine, and egg proteins are used to remove tannin compounds from red wine. The proteins are added to the wine and the precipitates are removed (**Rolland *et al.*, 2006**). The mechanism of action consists in their interaction with polyphenols to form complexes which can be further removed by decantation or filtration (**Castillo-Sánchez, 2006**). These proteins are included in the list of allergenic substances and must appear on the label when they are added to the food as ingredients. Conversely, when used as additives for wine production, these products were temporarily excluded from the obligation to label them because of the lack of scientific evidence of their actual presence as residual proteins in wines (**Tolin *et. al.*, 2012**). Laboratory analyzes are much more complicated in red than in white wines because it is difficult to obtain and analyze proteins because of the presence of a large number of polyphenols and carbohydrates **(Moreno-Arribas *et. al*., 2002**).



Picture 4 Measurement of absorbance by spectrophotometer at 450 nm and visualization of ELISA test after addition Stop solution

Milk and egg are renowned allergens often used as fining agents to promote clarification of wines, therefore any residual amount in the end-products could represent a menace for allergic individuals (**de Angelis *et al*., 2017**). The aim of the study of **Lifrani *et al.* (2009)** was to design sandwich ELISA tests specific to each fining agent in order to detect their residue antigenicity, both during wine processing and in commercially available bottled wines. Sensitized mice and sandwich ELISA methods were established to test a vast panel of wines. The results showed that they were positive to our highly sensitive sandwich-ELISA tests. ELISA is the most widely used form of immunoassay in milk analysis and has advantages of high sensitivity, low cost and fast application. It is easy to use, reliable, rapid and readily automated (**Zeleňáková *et al. 2008, 2011, 2016b*; Costa *et al*., 2008**). To implement ELISA assay for the detection of ovalbumin in red wines using commercially available antibodies tested Koestel et al. (2016). The specificity of the acquired antibodies and the absence of cross reactivity were assessed by immunoblotting and ELISA. ELISA assay with LOD of 14.2 µg/L and a LOQ of 56.4 µg/L of ovalbumin in aqueous solution was obtained (**Koestel *et al.*, 2016**).

The O.I.V. (International Organization of Vine and Wine) through the Oiv-comex 502-2012 resolution: revision of the limit of detection and limit of quantification related to potentially allergenic residues of fining agent proteins in wine establishes the following requirements for ELISA test systems: LOD = 0.25 ppm and LOQ = 0.5 ppm. ELISA kit used in our analysis had these parameters: Limit of detection (LOD): 0.24 ppm (mg/kg), limit of quantification (LOQ): 1.30 ppm (mg/kg), calibration scale range: 1.5 – 45 ppm (mg/kg).

The devised UF based method coupled with peptide on-line pre-enrichment enabled to reach the lowest LODs down at 0.036 mg/mL and 0.05 mg/mL for egg and milk allergens respectively, proving to be the most sensitive strategy for monitoring allergens contamination in wine (**de Angelis *et al.*, 2017**). Concerning wine samples, the widespread method used for the detection of caseins is based on antibody recognition. Several enzyme-linked-immunosorbent-assay (ELISA) formats have been recently developed for detection of casein residues in wine samples, with the lowest limit of detection 8 ng/ml. Quantitative ELISA method for determination of caseins in white and rose wines ranged from 0.01 to 10 mg/L, was reported by **Weber *et al*. (2007).** Sensitive and specific enzyme-linked immunosorbent assays (ELISA) were developed and established for the proteins casein, ovalbumin, and peanut. Lower limit of detection of these proteins was 8 ng/mL. Samples of 153 commercially available bottled Australian wines were tested by these assays and except for two red wines known to contain added whole eggs, residuals of these food allergens were not detected in any wine. These findings are consistent with a lack of residual potentially allergenic egg-, milk-, or nut-derived processing aids in final bottled wines produced in Australia according to good manufacturing practice at a concentration that could cause an adverse reaction in egg, milk, or peanut/tree-nut allergic adult consumers (**Rolland *et al.*, 2008**).

CONCLUSION

Method based on immunoenzymatic reaction for the detection and identification of casein in products of grape processing was described. This is important step towards the development of more sensitive method for the detection/identification of markers of potentially allergenic milk proteins used as

wine fining agents. The findings obtained in the present investigation appear to be important also from the consumer health point of view. Higher concentrations of this protein contained white wines (from 21.473 to 67.22 mg/L). In red wines, the milk protein concentrations ranged from 1.634 to 16.715 mg/L. Since caseins may trigger allergic reactions in sensitive consumers, it important to check for their presence also in these products.

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