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# Immunoreactivity of Selected Wines Commercially Available in Poland

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**Abstract**

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Taking into consideration the allergic consumers safety, the European Union imposed the duty of labelling allergenic substances used in the wine production on the wine producers. Although the rule entered into force in the course of the last three years, not properly labelled alcoholic beverages are still available on the Polish market. 25% of the beverage samples tested without the casein declaration on the label did not contain any traces of this protein. The rest of them were found to contain casein while three contained such an amount of casein which could be considered risky for hypersensitive individuals. The results were obtained with a commercial ELISA kit. The samples were also tested using slot-blot technique, which was recognised less sensitive than ELISA and therefore also less reliable. Due to the fact that the tested beverages were not properly labelled, hypersensitive people still should not buy them unless they contain clear information: "the product does not contain casein". Simultaneously, manufacturers are required to withdraw them from the market.

**Keywords**: casein; ELISA test; milk allergy; slot-blot analysis; wine

The European Federation of Allergy and Airways Disease Patients' Associations reports that the problem of food allergies affects 4% of adults and 8% of children. The main allergens of animal origin include cow's milk, eggs and fish. Undesired reactions after the consumption of milk or dairy products are observed in 2–3% infants in the general population in the developed countries, but they disappear in 85–90% of individuals once they pass three years of age (Høst 2002). However, if diagnosed in adulthood, these reactions may take a very severe form (Eigenmann 2002), this problem having been reported in approx. 0.5–1% of the adult population.

Allergic reactions to milk proteins are most commonly associated with alpha S1-casein and its derivatives, such as caseinates. Milk proteins are frequent components of non-dairy origin food while hidden allergens cause about one fourth of all allergic reactions (Wróblewska & Kaliszewska 2012).

Potassium caseinate is also used as a fining agent for white or rose wines to remove phenolic and tannin compounds. The EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) of the European Commission was asked to consider the possibility of permanent exemption from labelling of fining agents of milk origin. However, it has been proven that the fining agents used in wine processing are not completely removed by settling or/and filtration (Marchal *et al*. 2002, 2003). Thus, the EFSA Panel concluded that even if the concentration of casein in ready-to-consume beverages is trace or zero, wines should be labelled as it is typically done and their producers are not exempt from this duty (EFSA 2011).

It is generally considered that 90 mg of casein is the lowest dose of casein which may trigger allergic reactions (Lam *et al.* 2008). However, some reports indicate that a dangerous threshold may be significantly lower. Momeret-Vautrin and Kanny (2004) reported that less than 30 mg of milk proteins is the dose inducing symptoms typical of the IgE-dependent allergy in 5% allergic patients and in 1% of them as little as about 1 mg provokes an adverse effect. However, it should

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be kept in mind that the volume of samples in oral-provocation tests is small, usually, less than 100 ml.

Thus, wine needs to be examined for allergen contents, even if no case of the anaphylactic reaction or allergic symptoms has been observed after the consumption of wine. Moreover, because of the need of wine controlling, reliable and fast analytical tests should be developed (Lifrani *et al.* 2009; Weber *et al*. 2009). Currently there are some commercially available tests, based on the immunochemical detection of casein proteins with the ELISA method.

Such a commercially produced test was used in the presented experiments to examine the content of casein proteins in wines commercially available in Poland to analyse their immunoreactivity for the individuals suffering on the intake of casein. The applicability of slot-blot analysis with commercially available anti-casein antibodies was also verified.

## MATERIAL AND METHODS

***Experimental material***. White wines and one brand of cider commercially available in Poland from discounts or wine shops were used as the experimental material.

***Protein content analysis***. The concentration of protein in the analysed samples was determined according to Bradford (1976).

***ELISA test***. Commercially available enzyme immunoassay (EuroClone EAE001096) for the quantitative determination of casein in wine were applied to detect casein content in the samples. The test is based on the sandwich, direct ELISA format, with the second antibody conjugated with horseradish peroxidase. The analysed samples without any dilution and casein standards (4, 2, 0.5, 0.25, and 0 µg/ml) were applied to the microplate coated with the first detection antibody. The specificity of the test was declared only for casein fractions (α-casein 100%, β-casein 50%, κ-casein 50%), there was no cross reactivity with rice and egg. The samples were analysed in triplicate.

***Slot-blot analysis***. Casein standards, which were components of the EuroClone kit, were also used in this experiment. The series of 15 standards (obtained *via* 2-fold dilution) in the range from 60 mg/ml down to 0.004 mg/ml were prepared. 100 µl of each of the prepared standard were applied on the PVDF membrane (Merck Millipore, Warszawa, Poland), while the volume of the alcoholic beverage samples applied was 150 µl. Pork gelatin (1% in Tris-buffered saline, pH 7.4; Sigma, Poznań, Poland) was used as the blocking agent (1-h incubation). The rabbit anticasein polyclonal antibody was the detection antibody (ab 166596; Abcam, Cambridge, UK) diluted at 1 : 2500. The goat polyclonal antibody against the whole IgG molecule, conjugated with alkaline phosphatase, was applied as a secondary one (A3686; Sigma) at the dilution of 1 : 20 000. The incubation with these antibodies took 2 h for each of them. As the substrate for the enzyme BCIP/NBT (5-bromo-4-chloro-3'-indolyphosphate and nitro-blue tetrazolium; Calbiochem) was used. 0.1 ml of the prepared stock solution of BCIP (50 mg/ml) and 1 ml NBT (10 mg/ml) were mixed with distilled water make the volume of to 10 ml. The stock and working solutions were prepared immediately before their application onto the membrane. The prepared solutions of BCIP (50 mg/ml) and NBT (10 mg/ml) were mixed and made up to 10 ml with distilled water. The substrate was prepared immediately before its application onto the membrane. The reaction was stopped with water followed by the drying of the membrane. Optical density was measured to quantify the casein content in the analysed samples (Image Studio Lite program; Li-cor, Bad Homburg, Germany). The experiment was repeated twice.

## RESULTS AND DISCUSSION

The EFSA Panel reported that 26% French wines and 20% German wines are fined with casein, while 15% French wines are fined with casein mixed with PVPP or bentonite. Globally 30% wines are treated with casein (EFSA 2011). The samples of the purchased wine (23 samples) and cider (1 sample) were characterised at first on the basis of information presented on the label (Table 1). Each sample was bought in a Polish shop, but only two of them had been bottled in Poland and only cider had been produced in this country. While rose wine may also be clarified with casein, only white wines were selected, both grape and fruit wines, sweet, semi-sweet, semi-dry, and white. None of the samples was labelled with the declaration of the use of casein during filtration/clarification processes. At the same time, each sample contained the declaration about the sulphite content. According to the currently binding law, the use of any potentially allergenic material applied during food processing should be declared by the producers on

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Table 1. Characteristic of the analysed samples on the basis of producers declarations

| No. | Name of wine | Country of origin | Production year | Alcohol content (%) | Wine sorts | Bottled in |
|---|---|---|---|---|---|---|
| 1 | Slavia, Blanc de Blancs | Bulgaria | 2013 | 11.5 | semi-dry | Bulgaria |
| 2 | Sophia Muskat | Bulgaria | 2013 | 10.5 | semi-sweet | Bulgaria |
| 3 | Carlo Rossi II | USA | 2008 | 9.5 | semi-dry | USA |
| 4 | Chardonay | Australia | 2012 | 11.5 | semi-dry | Australia |
| 5 | O Roncal Godello | Spain | 2013 | 13.5 | dry | Spain |
| 6 | Blossom Hill, California | USA | 2013 | 11.5 | semi-dry | Italy |
| 7 | Rioja Vega | Spain | 2012 | 11 | dry | Spain |
| 8 | Carlo Rossi I | USA | 2008 | 9.5 | semi-dry | USA |
| 9 | California Oak View | USA | 2013 | 11 | semi-dry | Germany |
| 10 | Cape Zebra, Chemin Blanc | RPA | 2012 | 12 | semi-dry | RPA |
| 11 | Sophia, Blanc de Blanc | Bulgaria | 2012 | 12.5 | semi-dry | Bulgaria |
| 12 | Kadarka | Bulgaria | 2014 | 11 | semi-sweet | Poland |
| 13 | Chardonay | France | 2012 | 12.5 | sweet | Germany |
| 14 | Cote | Bulgaria | 2013 | 12.5 | semi-dry | Bulgaria |
| 15 | Viña Tardida | Spain | 2014 | 12 | semi-sweet | Spain |
| 16 | Dobroński cider with cinnamon | Poland | 2013 | 4.5 | sweet | Poland |
| 17 | Labirynth | Bulgaria | 2012 | 12.5 | semi-dry | Bulgaria |
| 18 | Carlo Rossi II | USA | 2010 | 9.5 | semi-dry | USA |
| 19 | Mikado | Ukraine | 2011 | 11 | sweet | Ukraine |
| 20 | Chardoney | France | 2012 | 12.5% | dry | Germany |
| 21 | Vernaccia di San Gimignano | Italy | 2012 | 12.5 | dry | Italy |
| 22 | Liebfraumilch | Germany | 2012 | 8.5 | semi-sweet | Germany |
| 23 | Gadiva Douro Branco | Portugal | 2012 | 13 | dry | Portugal |
| 24 | Tokaji Furmint | Hungary | 2009 | 12 | semi-sweet | Hungary |
| 25 | Baron Romero | Spain | 2013 | 10.5 | semi-dry | Spain |

the labels, both fining agents and preservatives. This rule has been obligatory in the European Union since 15.12.2014 (European Commission 2011). However, even if it may be concluded that the producers accurately declared the use of sulphites, none of them indicated the use of other allergenic processing agents (milk or egg protein).

According to the recommendation of EFSA, wine also has to be labelled as a product potentially containing casein, even if its amount in the final product is small or not detectable.

At first, the concentration of total protein was controlled in the analysed samples. The presence of protein in wines may indicate the residual presence of dead yeast or lactic acid bacteria cells, microscopic fragments of skins, grape seeds, and protein fining agents (gelatin, milk, or egg proteins) (D'Amato *et al.* 2010]. These elements are usually removed during the fining process, because they form a suspension and destabilise wines. There were no proteins detected in any wine below the level of detection of 50 mg/l using the Bradford assay. None of the analysed samples contained any sediment. This additionally confirmed that the wines had been treated with a fining agent.

However, the absence of protein in such an amount does not guarantee health safety of the products for allergic patients. Thus, immunochemical analyses were performed.

At first, the commercial ELISA test was performed. The calculated results of the casein content in the samples are presented in Table 2. Only six of the examined samples showed no trace content of casein (the obtained results below the limit of detection), which indicates that they could be fined using another fining agent and the lack of declaration on the label could have been reasonable.

It was proven that even if small concentrations of fining agents are used in wine processing, some

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Table 2. The content of casein in wines determined with ELISA method

| No. | Name of wine | Casein content (mg/l) |
|---|---|---|
| 1 | Slavia, Blanc de Blancs | nd |
| 2 | Sophia Muskat | 1.395±0.001 |
| 3 | Carlo Rossi II | nd |
| 4 | Chardonay | nd |
| 5 | O Roncal Godello | nd |
| 6 | Blossom Hill, California | below the LOQ |
| 7 | Rioja Vega | below the LOQ |
| 8 | Carlo Rossi I | nd |
| 9 | California Oak View | 0.493 ± 0.002 |
| 10 | Cape Zebra, Chemin Blanc | below the LOQ |
| 11 | Sophia, Blanc de Blanc | 0.476 ± 0.001 |
| 12 | Kadarka | below the LOQ |
| 13 | Chardonay | below the LOQ |
| 14 | Cote | 0.485 ± 0.001 |
| 15 | Vińa Tardida | 0.927 ± 0.002 |
| 16 | Dobroński cider with cinnamon | 1.211 ± 0.002 |
| 17 | Labirynth | below the LOQ |
| 18 | Carlo Rossi II | 0.869 ± 0.002 |
| 19 | Mikado | 0.718 ± 0.000 |
| 20 | Chardoney | 0.526 ± 0.000 |
| 21 | Vernaccia di San Gimignano | below the LOQ |
| 22 | Liebfraumilch | 0.760 ± 0.000 |
| 23 | Gadiva Douro Branco | 0.501 ± 0.001 |
| 24 | Tokaji Furmint | below the LOQ |
| 25 | Baron Romero | nd |

nd – the content under the limit of detection; under the LOQ – the content under the limit of quantification; result are presented as an average value and SD

amounts of animal fining agents remain after filtration and/or settling processes (Marchal *et al.* 2002, 2003). Thus, the other analysed samples showed the presence of casein trace amounts, including eight of them below the limit of quantification. All of these samples should be labelled as a 'product which may contain trace amounts of casein' or 'produced with the casein use'. Moreover, two wine samples and the cider sample showed casein content close to 1 mg. Thus, it may be expected that for some extremely hypersensitive individuals, the consumption of those products may be not completely safe. It was confirmed that wines fined with casein give some positive results in skin prick tests (Kirschner *et al.* 2009) and some patients may react also after the wine consumption (Sbornik *et al.* 2007).

All of the samples were also analysed with the slot-blot technique using commercially available antibodies. The slot blot method is a fast immunochemical technique, which could be proposed as a reliable technique to detect allergens in wines. The results of this analysis indicates the presence of trace amounts of casein in 8 analysed samples (Figure 1). Casein quantification was possible in the range of 0.117–15 mg/l of casein and the concentration 0.117 was the LOQ, while LOD was 0.029 mg/l. Casein standards containing more than 60 mg/ml, could not be detected, because they caused the membrane overload.

Not all of the samples identified in the ELISA test as containing casein gave a positive result in the slot-blot analysis. At the same time, some detected as casein-free in ELISA test gave a positive result in slot-blot analysis. The differences between the recorded results could be explained by the different specificities of the antibody used. Moreover, if the result of slot-blot analysis was positive, the determined casein content was higher than that obtained with the ELISA test, which could be explained by strong

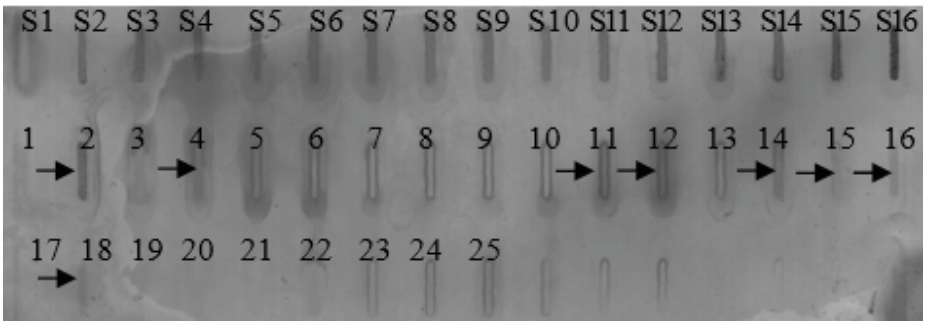


Figure 1. Result of slot-blot analysis for the studied wines

S1–S16 – casein standards in concentrations between S1 – 0 μg/ml and S16 – 60 mg/ml; 1–25 – tested wines sample ordered in the same way as it was presented in the tables

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Table 3. The content of casein in samples of wines determined with slot-blot method after the optical density analysis

| No. of samples | Casein concentration (mg/l) |
|---|---|
| 2 | 6.66 ± 0.03 |
| 4 | 1.44 ± 0.01 |
| 11 | 2.62 ± 0.01 |
| 12 | 1.81 ± 0.00 |
| 14 | 1.22 ± 0.02 |
| 15 | 1.33 ± 0.02 |
| 16 | 2.00 ± 0.00 |
| 18 | 1.33 ± 0.03 |

Samples were numbered in accordance with Table 1; results are presented as an average value and SD

hydrophobic interactions between PVDF membrane and casein proteins. The content of casein in sample No. 2 was even extremely high and unreliable (Table 2). In this situation, it should be considered that the results of ELISA tests were more reliable, because this is the quantitative method usuallz used, while the application of 'blot-type' analysis (western-blot, southern-blot, dot-blot etc.) to protein concentrations determination is still debatable. Additionally, the used ELISA format (sandwich) is considered as highly specific and very sensitive, due to the fact that the immunogen is recognised by two different antibodies – one attached to the microplate and the other applied during the analysis (Immer & Lacorn 2014).

No relationship between the detection of casein and the available metadata (wine sorts, origin, and year of production) has not been observed, either in the results of ELISA test or slot blot analysis. Thus, it was not possible to indicate any group of wines, which could be considered safe for the consumer.

## CONCLUSION

Only 25% wines found on the market were properly labelled, because they did not contain any traces of casein. The other analysed samples should be withdrawn from sale, because according to the EU regulations, each food product ought to be labelled to indicate information on the possible presence of allergenic substances or the use of such substances in their production. Moreover, wines were not excluded from these regulations on the basis of the EFSA expert opinion. Even if no adverse allergic reactions were observed after the consumption of wines, the population must be properly informed. Two of the tested beverages (one wine and cider) contained casein at a level which could be dangerous for hypersensitive individuals.

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