

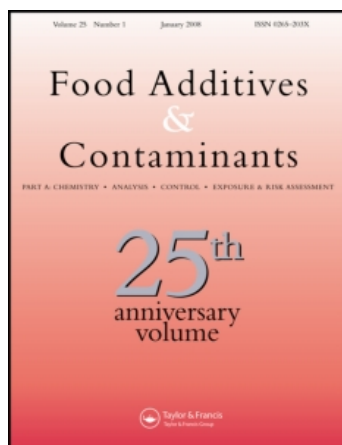
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Characterization, antigenicity and detection of fish gelatine and isinglass used as processing aids in wines

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Fish gelatine (FG) and isinglass (IG) are widely used in the pharmaceutical industry and as ingredients or processing aids in food production. Both products are the focus of interest since several countries, particularly the member states of the EU and Japan, USA, Australia and New Zealand, have introduced special labelling regulations for allergenic foodstuffs, such as fish and products thereof. Thus, there is a demand for a reliable and sensitive method for the detection of FG and IG in foodstuffs. In this study, the characterization of various FGs and IGs, polyclonal antibodies raised against fish collagen and the development of a sensitive indirect ELISA for the detection of FG and IG is described. The ELISA method detected $\leq 0.11 \mu\text{g ml}^{-1}$ from all FGs and IGs tested. The indirect ELISA was applied to various experimental wines where FG and IG had been used as processing aids and to several commercial wines. No residues of FG and IG were detected in the experimental wines additionally treated with bentonite, a strong adsorbent for proteins, and successive filtration. Additionally, no residues were found in the commercial wines. However, amounts of up to $0.33 \mu\text{g ml}^{-1}$ were found in some experimental wines not treated with bentonite and successive filtration. Therefore, wines may contain traces of FG and IG that were used as processing aids during wine production. However, treatment with bentonite in combination with additional filtration had a clear impact on these residues.

Keywords: chromatography; electrophoresis; immunoassays; allergens; residues; animal; fish and fish products; wine

Introduction

Fish gelatine (FG) and isinglass (IG) are widely used in the food industry, particularly for the production of beverages, such as wine, beer, juice, cider and mead (Taylor et al. 2004, Baziwane and He 2003). FG is used in the dairy and confectionery industries for the production of candies, desserts, etc., as a dietary supplement and as a carrier for dyes, flavours and vitamins. It is an important alternative to bovine and porcine gelatine, due to bovine spongiform encephalitis and the demand for halal and kosher foods. Moreover, FG is important in the pharmaceutical industry, particularly for the preparation of capsules, pills, plasma substitutes, and as a stabilizer in vaccines.

Raw materials used in the production of FG are mixtures of skins from various bony fishes (*Osteichthyes*) belonging to the subclass *Actinopterygii*, including the genera of cod, tilapia, tuna, perch, pollock, haddock, hake, cusk, flatfish and redfish. IG is derived from the swim bladders of the same and other genera, such as sturgeon, catfish, croaker and threadfin. Skins and swim bladders are waste materials in the fish industry, which are

abundantly available and high in content of the desired type I collagen.

FG and IG products have provoked interest in several countries, particularly the member states of the EU, Japan, USA, Australia and New Zealand, who have introduced special labelling regulations for allergenic foodstuffs based on a recommendation by the Codex Alimentarius Commission (1999). These regulations include fish and fish products. In vitro, IgE-binding properties of FG and the fish collagen protein, which is the main component of FG and IG, have been shown in some fish allergic patients with a high prevalence of cross-reactivities among various fish species (Sakaguchi and Inouye 2000, Sakaguchi et al. 1999, 2000, André et al. 2003, Hamada et al. 2001, 2003). Evidence of allergenicity has not been found after consumption of foods or after administration of pharmaceuticals containing FG or fish collagen. No adverse reactions were observed in a double-blind placebo-controlled food challenge (DBPCFC) with FG (André et al. 2003) and only one mild, subjective symptom occurred in a DBPCFC performed by Hansen et al. (2004) on 30 fish allergic patients after

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the consumption of a cumulative dosage of 7.6 g of FG. Positive skin prick tests and positive histamine release tests were obtained against FG in some fish allergic patients (Hansen et al. 2004). Published cases of allergic reactions to gelatine-containing foods are rare and refer to mammalian gelatine (Sakaguchi et al. 1996) or to gelatine of uncharacterized origin (Sakaguchi et al. 1995, Kelso et al. 1993, Wang and Sicherer 2005; Wahl and Kleinhans 1989). This also applies to pharmaceuticals, where known cases of allergic reactions refer to mammalian gelatine (Sakaguchi et al. 1996, 1999, Sakaguchi and Inouye 2001, Nakayama et al. 2004) or to gelatine of uncharacterized origin (Sakaguchi et al. 1995, 1997, Nakayama et al. 1999) that were not administered by the oral route. The latter three studies refer to vaccines from Japan, which are usually produced with mammalian gelatine (Sakaguchi et al. 1999). Differentiation between mammalian gelatine, FG and fish collagen is very important since *in vitro* cross-reactivities were not observed (André et al. 2003, Hamada et al. 2001) or rather weak (Sakaguchi et al. 1999, 2000, Sakaguchi and Inouye 2000, Kelso et al. 1993).

Based on these facts, the allergic potential of FG and the fish collagen protein has not been proven. However, there is a risk that allergic reactions are not associated with the consumption of FG- or fish collagen-containing foods or pharmaceuticals. Many foodstuffs or pharmaceuticals are prepared with these substances but they are not usually considered to contain fish proteins by the fish allergic consumer due, for example, to improper labelling. Furthermore, the adherence of fish muscle tissue, including the major fish allergen parvalbumin, must be taken into account even though there is no information on this subject. Apart from this, vegetarians and vegans wish to abstain from any animal substances. There is, therefore, a demand for a reliable and sensitive method for the detection of FG and IG to assure adequate labelling of foodstuffs and to discover contaminations of foods and pharmaceuticals for consumer protection. A common method for the determination of protein traces is the enzyme-linked immunosorbent assay (ELISA).

In this study, the characterization of polyclonal antibodies raised against fish collagen and the development of a sensitive indirect ELISA for the determination of FG and IG is described. This includes the characterization of various FGs and IGs by SDS-PAGE and the investigation of their affinity to polyclonal antibodies by Western blot. The application of the ELISA in wines is demonstrated, where FG and IG are used in the production to reduce the polyphenol and protein content, as described previously (Weber et al. 2007). This treatment contributes to the organoleptic properties of wine and prevents proteinogenic haze. A range of experimental and

commercial wines were examined for the presence of FG and IG residues. Bentonite treatment—an important fining step which reduces the protein content in wines—was also considered in the production of experimental wines.

Materials and methods

Food-grade gelatines and collagens

A total of seven commercially available FG and IG samples were sourced from four different suppliers: Erbslöh GmbH, Germany; Begerow GmbH, Germany; Norland Products Inc., USA; Sigma-Aldrich Co., USA). Three FGs, designated A–C, were derived from fish skin and were provided as yellow powders; two acid-hydrolyzed IG, designated A and B, were provided as acidic gel with 2% IG derived from swim bladder collagen; one hydrolyzed FG, designated A, was provided as a white powder derived from acidic and enzymatic hydrolysis of fish skin collagen; one native IG was derived from swim bladder and was provided as yellow sheets.

Three mammalian gelatines were purchased as yellow powders from local markets or pharmacies: one mammalian gelatine composed of porcine and bovine gelatine, one porcine gelatine and one bovine gelatine. Protein contents were measured by the Kjeldahl method, as published previously (Mattisek et al. 1992).

Polyclonal antibodies against fish collagen

Primary antibodies against fish collagen were produced in New Zealand white rabbits in accordance with institutional guidelines (Eurogentec SA, Belgium). Briefly, 100 µg of acid-hydrolyzed IG A was mixed with 400 µl saline (0.9%) and 500 µl of complete Freund's adjuvant and injected intradermally into three rabbits. Injection was repeated three times with the incomplete Freund's adjuvant at 4-week intervals. Blood samples were taken 10 days after the second, third and fourth injection, and the serum was obtained by centrifugation. The third serum sample (~60 ml) was purified by affinity chromatography using 10 mg hydrolyzed IG A immobilized by CNBr Sepharose. The column was washed with 10 ml elution buffer (100 mM glycine, pH 2.5) and 15 ml phosphate-buffered saline (PBS). Thereafter, the serum was loaded at a flow-rate of 0.5–1 ml min⁻¹ and the column was washed thoroughly with PBS. The antibodies were eluted with elution buffer and neutralized immediately with a solution of 1.5 M Tris(hydroxymethyl)-amino-methane (Tris), pH 8.5. After dialysis against PBS overnight, the final solution consisted of 15.2 mg antibodies in 8 ml of PBS. The antibody purity was greater than 85%, estimated by SDS-PAGE.

Then, 0.1% bovine serum albumin and 0.01% Thimerosal was added for stabilization and the purified serum was stored at -80°C .

Fish samples

Five fish-muscle samples from species commonly utilised in FG and IG production were purchased filleted and frozen from local markets: cod (*Gadus* spp.), pollock (*Pollochiu* *virens*), tuna (*Thunnus* spp.), sturgeon (*Acipenser* spp.) and tilapia (*Oreochromis* spp.).

Wine samples

Sixty commercial white wine samples were purchased from local stores. The selection pattern was restricted to the country of origin (23 German wines, 18 French wines and 19 Italian wines) and to important European grape varieties (Chardonnay, Pinot gris, Pinot blanc, Riesling, Sauvignon blanc and Silvaner). Fining with fish skin gelatine was known for two of these commercial white wine samples whereas fining was not known for the remaining 58 samples.

Experimental wines were prepared from four grape varieties (Riesling Mosel, Riesling Rheingau, Pinot blanc and Pinot gris) by treatment with two different dosages of FG B, hydrolyzed IG A, hydrolyzed IG B and the native IG, respectively. This process is described elsewhere (Weber et al. 2007), in which "isinglass 1" corresponds to hydrolyzed IG A, "isinglass 2" to hydrolyzed IG B, "isinglass 3" to the native IG and "fish gelatine" to FG B. Additionally, wines without bentonite treatment were prepared to evaluate its impact on residual amounts of FG and IG. Thus, wines were bottled directly after separation from the fining precipitate and, therefore, lacked the final cross-flow and membrane filtration.

Reagents and buffers

All chemicals used were of analytical grade. Urea solution was prepared from 8M urea and 2% cyclohexylaminopropane sulfonic acid in double distilled water. Colloidal Coomassie Brilliant Blue (CBB) staining solution was prepared by dissolving 1g of CBB G250 in 20ml double-distilled water (solution 1). Then, 16ml of solution 1 was added to 16ml *o*-phosphoric acid (85%), 80g ammonium sulphate and 768ml double-distilled water. A 200-ml aliquot of methanol was slowly added immediately before use.

Electrode solutions for Western blot were as follows: cathode solution (pH 7.6) was prepared with 2.6g 6-aminohexanoic acid, 0.05g SDS, 0.05g sodium azide and 100ml ethanol, filled to 500ml with double distilled water. Anode I solution (pH 10.4) consisted of

18.15g Tris, 0.05g sodium azide, and 100ml ethanol, which was filled to 500ml with double-distilled water. Anode II solution (pH 10.4) contained 1.52g of Tris, 0.05g sodium azide and 100ml ethanol, filled to 500ml with double-distilled water.

For immunostaining, the following solutions were prepared. The Tris solution contained 50mM Tris, 150mM NaCl and 0.5% polyethylene-sorbitan monolaurate (Tween 20) in double-distilled water (Szepfalusi et al. 1993). The TMB solution consisted of 12mg 3,3',5,5'-tetramethylbenzidine (TMB) and 40mg diocytlsodiumsulfosuccinate dissolved in 10ml ethanol. Citric buffer (pH 5.0) was prepared with 9.4g of citric acid and 18.2g of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ dissolved in 1l double-distilled water. The substrate solution for immunostaining was carefully mixed from 5ml TMB solution, 15ml citric buffer and 10 μl of H_2O_2 (30%). Re-buffer solution was prepared with 121.2mg Tris, filled to 80ml with double-distilled water, then adjusted to pH 6.0 with 4M hydrochloric acid and finally filled to 100ml with double-distilled water. Ponceau red solution was prepared from 2% Ponceau red and 3% trichloroacetic acid in double-distilled water.

Goat anti-rabbit horseradish peroxidase (HRP) immunoglobulins were purchased from Dako (order no. P0448, Dako GmbH, Germany). Carbonate buffer for ELISA was prepared from 75mM Na_2CO_3 and 175mM NaHCO_3 in double distilled water (pH 9.5). The washing solution, substrate solution and stop solution for ELISA were prepared according to Weber et al. (2007).

Preparation of FG and IG solutions

FGs and hydrolyzed FG A were completely soluble in double-distilled water. The urea solution was added to the insoluble IGs, giving a concentration of approximately $200\mu\text{g ml}^{-1}$. The suspension was stirred at room temperature and repeatedly heated at 80°C until the collagen was dissolved (24–72h). Samples in urea were diluted at least 20-fold with carbonate buffer to allow renaturation for the indirect ELISA.

Preparation of fish extracts

An 2.4-g thawed fish muscle sample was homogenized for 2min with 20ml of urea solution in an Ultra-Turrax at 8000rpm. Then, the homogenate was extracted for 2h via continuous shaking and centrifuged for 15min at 6700g. The clear supernatant was removed and used for SDS-PAGE analysis. Spare extracts were stored at -24°C .

SDS-PAGE

SDS-PAGE was performed on Invitrogen equipment (Invitrogen Corp., Carlsbad, CA, USA). Samples were separated between 200 and 3.5 kDa at 200 V for 45–50 min with 1-mm thick pre-cast NuPAGE Novex Bis-Tris 12-well gels with 12% acrylamide (Invitrogen), NuPAGE MES SDS running buffer as electrolyte (Invitrogen), and calibrated against the Serva Mark12 molecular weight (MW) ladder (Invitrogen). Proteins with MWs between 460 and 31 kDa were separated at 150 V for 70 min on 1-mm thick pre-cast NuPAGE Novex Tris-acetate 10-well gels with 3–8% acrylamide (Invitrogen), NuPAGE Tris-acetate SDS running buffer (Invitrogen), and calibrated against the pre-stained HiMark MW ladder (Invitrogen).

Colloidal CBB staining

Gels were stained with 50 ml of colloidal CBB staining solution for 16 h. Thereafter, the background was decolorized via incubation in double-distilled water with soaked paper tissues.

Semidry Western blot and immunostaining (Immunoblot)

Semidry Western blot was performed on a LKB Bromma 2117 Multiphor II blotting apparatus with Pharmacia Biotech Nova Blot graphite electrodes (Pharmacia Biotech, Piscataway, NJ, USA) and a Powerease 500 power supply (Invitrogen) onto nitrocellulose transfer membranes with a 0.2- μ m pore size (Whatman PROTRAN; Whatman GmbH, Germany).

Initially, four layers of thin cloth were soaked in cathode solution and placed on the cathode. The gel was then laid onto the cloth pile. The nitrocellulose transfer membrane was soaked with anode solution II and transferred onto the gel. Areas where no transfer was desired were covered with plastic slides. Two layers of cloth were soaked with anode buffer II and placed onto the blot membrane. Four layers of cloth were soaked with the anode solution I and then transferred onto the pile. Finally, the graphite anode was placed on the pile and blotting performed for each gel for 80 min at 60 mA. The air-dried membranes were blocked twice with Tris solution for 1 h each and incubated for 1.5 h with polyclonal antibodies against fish collagen (1 : 500 diluted in Tris solution). Washing was done three times with the Tris solution for 5 min each and the membrane incubated for 1.5 h with goat anti-rabbit HRP immunoglobulins (diluted 1 : 2000 in Tris solution). The membrane was washed four times with the Tris solution for 5 min each, incubated for 1 min in re-buffer solution and, finally, incubated with substrate solution for immunostaining until the desired colour intensity was achieved. The reaction was

stopped by washing with double-distilled water. The Serva Mark12 MW ladder was stained by the addition of 50 ml Ponceau red solution for 1.5 h followed by a thorough washing with double-distilled water.

Indirect ELISA procedure

For the indirect ELISA assay, standard solutions were prepared in carbonate buffer and wine samples were diluted 1 : 10 with the carbonate buffer. 150 μ l of these solutions were loaded in the wells of a polystyrene microtiter plate (Maxisorb F96 certified; Nunc, Roskilde, Denmark). The plate was incubated for 16 h at 8–9°C and washed three times with 200 μ l well⁻¹ of the washing solution. Free binding sites of the wells were blocked with 250 μ l well⁻¹ washing solution for 2 h at room temperature. The plate was washed three times with 250 μ l washing solution and the ELISA proceeded by addition of 150 μ l well⁻¹ polyclonal antibodies against fish collagen (diluted 1 : 2000 in washing solution). After a 1.5-h incubation at room temperature, the plate was washed three times with 200 μ l well⁻¹ washing solution. Thereafter, 150 μ l well⁻¹ of goat anti-rabbit HRP immunoglobulins (diluted 1 : 2500 in washing solution) were added and incubated for 1.5 h at room temperature. After four washing steps with 250 μ l well⁻¹ washing solution, 150 μ l well⁻¹ substrate solution was added and reacted for exactly 9 min in darkness at 8–9°C. The enzymatic colorimetric reaction was stopped by adding 75 μ l stop solution. The optical density (OD) was measured at 450 nm against a reference wavelength of 630 nm using a microtiter plate reader (MRX, Dynex Technologies, Chantilly, VA, USA).

The attained ODs were plotted against the logarithm of the protein concentration. Quantitative evaluations were performed with AssayZap software (Biosoft, Cambridge, UK) using a four-parametric regression. All ODs were reduced by the mean blank OD. Thus, the limit of decision (LODC; 50% probability that the measured values lie within the spread of the blank values) was defined as zero plus 3-fold the standard deviation of the blank OD and the limit of detection (LOD; 99.8% probability that the measured values are larger than the spread of the blank values) was defined as zero plus 6-fold the standard deviation of the blank OD (Einax et al. 1997, Doerffel 1987).

Intra-assay variation coefficients were calculated from five independent standard curves on the same microtiter plate, whereas inter-assay variation coefficients were calculated from five independent standard curves on five different microtiter plates and on five different days.

The effect of heating and urea treatment during the preparation of the IG solutions to the antigenicity was mathematically considered. Therefore, correction

factors (CF) were calculated from the ODs derived from the respective concentration of FG A, treated and not treated with urea and heat: $CF = OD_{\text{untreated}} / OD_{\text{treated}}$. Values derived from IGs dissolved in urea and heating were corrected by this correction factor: $OD_{\text{corrected}} = OD \times CF$.

The investigation of wine samples was performed four times.

Results

Characterization of various fish and mammalian gelatines and collagens by SDS-PAGE and colloidal Coomassie staining

Figure 1 shows the heterogeneous composition of various gelatines and IGs. FGs showed proteins and numerous fragments between 280 and 22 kDa. Single bands were visible for FG B at approximately 240 kDa, probably corresponding to dimeric collagens (β -collagens), 120 and 117 kDa, probably corresponding to different monomeric collagens (α -collagens), and 60 kDa. Hydrolyzed IGs were found to consist partly of smaller protein fragments. Hydrolyzed IG A showed

distinct ~400 kDa (probably γ - or tropo-collagen), ~240 kDa (probably β -collagen), and ~177 and 120 kDa (probably α -collagen) bands, in addition to various collagen fragments in the range 460–8 kDa. In contrast, hydrolyzed IG B and hydrolyzed FG A revealed collagen fragments of 100–8 kDa, of which hydrolyzed FG A showed only protein bands between 40 and 8 kDa. Native IG displayed three distinct bands at MWs of approximately 400, 240, and 120 kDa. These bands possibly represent γ -, β -, and α -collagens, as previously described.

Porcine and mammalian gelatine had a similar composition to FGs, indicating proteins and numerous fragments between ~250 and 30 kDa. Distinct bands were visible for porcine gelatine at ~240, 120, 117 and 60 kDa. However, bovine gelatine showed considerable differences to fish and other mammalian gelatines. MWs were determined between 50 and 8 kDa for bovine gelatine. Thus, this bovine gelatine was more characteristic of hydrolyzed IG or hydrolyzed FG than FG, indicating that the bovine gelatine had been comprehensively processed.

Characterization of the polyclonal antibody's specificity by semidry Western blot and immunostaining

Specificity of the polyclonal antibody was initially evaluated by various fish and mammalian gelatines and IG (Figure 2). Proteins were detected in all FGs and IGs. FGs bound antibodies to proteins and their fragments between MWs of approximately 460–40 kDa. Smaller proteins, despite being detected by CBB staining, were not detected by this antibody. Binding to the hydrolyzed IG and FG was, with the exception of hydrolyzed IG A, considerably weaker than FGs. Hydrolyzed IG B revealed a weak antibody binding to MWs between 100 and 38 kDa, whereas hydrolyzed FG A showed a single immunostained protein fragment at 14 kDa. On the contrary, hydrolyzed IG A, which had been used for antibody production, was well recognized by the antibody in the range 460–8 kDa.

Bovine and mammalian gelatine were not recognized by the antibody, whereas porcine gelatine revealed a slight antibody binding in the range 400–120 kDa.

The specificity of the polyclonal antibody to fish collagen was also assessed in various fish muscle extracts (Figure 3). Colloidal CBB showed proteins between >200 and 8 kDa for all fish species, except tuna. Binding of the primary antibody by immunostaining occurred almost exclusively to proteins with MWs ≥ 120 kDa. Detection of these proteins is expected as they represent the major collagen fractions (see Figure 3, lane 6). Only weak staining occurred in proteins of ~55 and ~36 kDa. Major fish proteins with

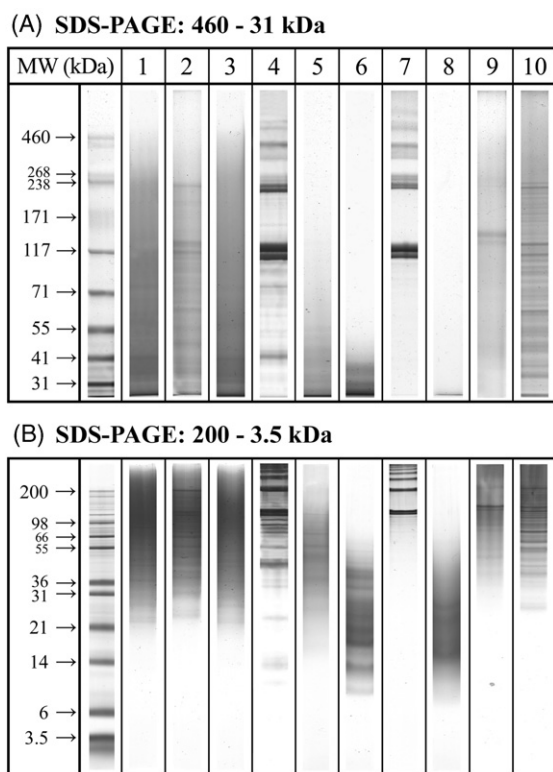
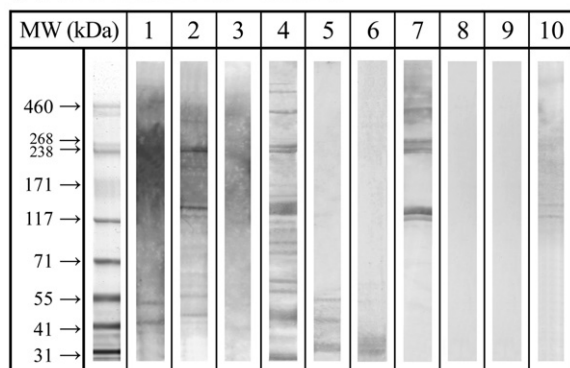


Figure 1. SDS-PAGE with colloidal Coomassie brilliant blue staining of various gelatines and collagen products derived from fish and mammals. (A) MW between 460 and 31 kDa; (B) MW between 200 and 3.5 kDa. M: MW marker; 1: fish gelatine A; 2: fish gelatine B; 3: fish gelatine C; 4: hydrolyzed isinglass A; 5: hydrolyzed isinglass B; 6: hydrolyzed fish gelatine A; 7: native isinglass; 8: bovine gelatine; 9: mammalian gelatine; 10: porcine gelatine.

(A) SDS-PAGE: 460 - 31 kDa



(B) SDS-PAGE: 200 - 3.5 kDa

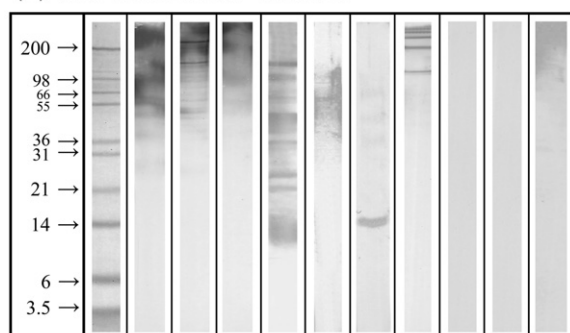


Figure 2. Immunoblot of various gelatines and collagen products derived from fish and mammals. (A) MW between 460 and 31 kDa; (B) MW between 200 and 3.5 kDa. M: MW marker; 1: fish gelatine A; 2: fish gelatine B; 3: fish gelatine C; 4: hydrolyzed isinglass A; 5: hydrolyzed isinglass B; 6: hydrolyzed fish gelatine A; 7: native isinglass; 8: bovine gelatine; 9: mammalian gelatine; 10: porcine gelatine.

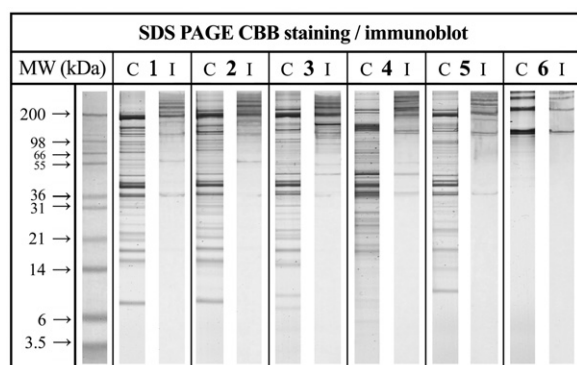


Figure 3. SDS-PAGE and immunoblot of extracts derived from various fish species. C: colloidal CBB staining; I: immunostaining; 1: cod; 2: pollock; 3: tilapia; 4: tuna; 5: sturgeon; 6: native isinglass.

MWs lower than collagen, i.e. definitive bands between 50 and 36 kDa, were barely detected.

Indirect ELISA evaluation

The indirect ELISA standard curves were derived by serial dilution of gelatine and IG samples in the range of

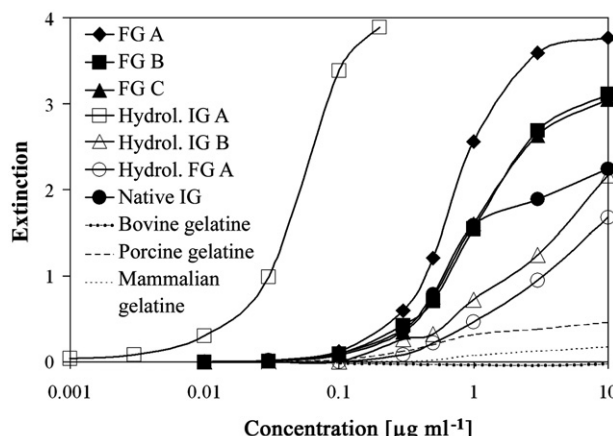


Figure 4. Indirect ELISA curves of various gelatines and collagen products derived from fish and mammals.

Table 1. LODC, LOD and relative reactivities of various gelatines and IGs determined by indirect ELISA.

Product	LODC [$\mu\text{g ml}^{-1}$]	LOD [$\mu\text{g ml}^{-1}$]	Relative reactivity related to FG A [%]
FG A	0.04	0.08	—
FG B	0.04	0.06	45.8–70.1
FG C	0.03	0.04	36.2–68.4
Hydrolyzed IG A	0.001	0.002	—
Hydrolyzed IG B	0.11	0.18	16.7–53.3
Hydrolyzed FG A	0.11	0.13	13.4–30.5
Native IG	0.02	0.04	23.7–70.0
Porcine gelatine	0.14	0.27	6.4–30.3
Mammalian gelatine	0.80	2.05	0.6–5.2
Bovine gelatine	n. d.	n. d.	—

0.001–10 $\mu\text{g ml}^{-1}$ protein. Figure 4 shows a significant difference in the slopes of the various products. The relative reactivity of FGs and IGs related to FG A had values between 70 and 13% within the steepest range of 0.3–3 $\mu\text{g ml}^{-1}$. Calculation of recovery rates was not possible for hydrolyzed IG A due to a greater sensitivity. LODCs were calculated in the range of 0.001–0.11 $\mu\text{g ml}^{-1}$ and LOD were calculated in the range of 0.002–0.18 $\mu\text{g ml}^{-1}$ for all FGs and IGs (Table 1).

Gelatines from mammals displayed higher LODCs and LODs of 0.14–0.8 and 0.27–2.05 $\mu\text{g ml}^{-1}$, respectively. Bovine gelatine was not detectable by indirect ELISA. Relative reactivities related to FG A were between 30 and 1% and, thus, were lower than FG or IG. Intra- and inter-assay variation coefficients were calculated for FG A. Intra-assay variation coefficients were less than 10% (1.1–6.3%) and inter-assay variation coefficients were less than 25% (3.6–21.5%) for concentrations between 0.1 and 10 $\mu\text{g ml}^{-1}$. Matrix effects were assessed by spiking all four experimental wines with FG A and hydrolyzed IG A (Table 2). Considering the 10-fold dilution, recovery rates for FG A were between 73 and 125% within the steepest range

Table 2. LODC, LOD and recovery rates of FG A and hydrolyzed IG A in various wines determined by indirect ELISA.

Grape variety	LODC [$\mu\text{g ml}^{-1}$]		LOD [$\mu\text{g ml}^{-1}$]		Recovery [%]	
	FG A	Hydrolyzed IG A	FG A	Hydrolyzed IG A	FG A (3–20 $\mu\text{g ml}^{-1}$)	Hydrolyzed IG A (0.03–1 $\mu\text{g ml}^{-1}$)
Riesling Mosel	0.6	0.02	0.9	0.02	80–121	83–116
Riesling Rheingau	0.6	0.01	0.9	0.02	78–83	69–93
Pinot blanc	0.5	0.01	0.7	0.02	87–118	70–107
Pinot gris	0.6	0.01	0.7	0.02	73–125	82–113

of 3–20 $\mu\text{g ml}^{-1}$. Thus, the limit of quantification (LOQ) was found to be 3 $\mu\text{g ml}^{-1}$, which resulted in a practical working range of 3–20 $\mu\text{g ml}^{-1}$ for the white wines. The LODCs were calculated between 0.5 and 0.6 $\mu\text{g ml}^{-1}$ and the LODs between 0.7 and 0.9 $\mu\text{g ml}^{-1}$ for all spiked wines. Consequently, LODC and LOD of FG A were not significantly affected by the wine matrix.

Hydrolyzed IG A was detected in 10-fold diluted experimental wines with recovery rates between 69 and 113% in the range of 0.03–1 $\mu\text{g ml}^{-1}$. The LOQ was found to be 0.03 $\mu\text{g ml}^{-1}$, establishing a practical working range of 0.03–1 $\mu\text{g ml}^{-1}$. The LODCs were calculated between 0.01 and 0.02 $\mu\text{g ml}^{-1}$ and the LOD was 0.02 $\mu\text{g ml}^{-1}$ for all investigated wines. Thus, the LODC and LOD of hydrolyzed IG A were not significantly affected by the wine matrix.

Determination of fish gelatine and isinglass in wines by indirect ELISA

Of the 60 commercial white wine samples, none were found to give signals significantly different from the blank OD. This included two wines with the known treatment of fish skin gelatine. All signals were significantly lower than the LODC. Based on the LODC values that were achieved for the various FGs and IGs (Table 1), the levels of both were considered lower than 1.1 $\mu\text{g ml}^{-1}$ in these wines.

Investigation of the fined experimental wines revealed negative results for all wines fined with FG B, hydrolyzed IG B and native IG. This result was independent of fining agent dosage and bentonite usage. Positive results were obtained for wines fined with hydrolyzed IG A. Quantification was performed against a standard curve prepared with hydrolyzed IG A. Detected residues were up to the level of 0.33 $\mu\text{g ml}^{-1}$ in wines not treated with bentonite (Table 3). Thereof, levels in wines treated with the normal dosages of hydrolyzed IG A were up to 0.09 $\mu\text{g ml}^{-1}$, while treatment with an excessive dosage led to residues between 0.04 and 0.33 $\mu\text{g ml}^{-1}$. Negative results were obtained for all wines treated with hydrolyzed IG A and bentonite, with levels of hydrolyzed IG A lower than 0.01 $\mu\text{g ml}^{-1}$ in these wines.

Table 3. Fining agent contents of white wines fined with hydrolyzed IG A and not treated with bentonite. Results were acquired by indirect ELISA with a standard curve prepared of hydrolyzed IG A.

Wine	Fining agent content for a normal dosage of 50 ml hl ⁻¹ [$\mu\text{g ml}^{-1}$]	Fining agent content for an excessive dosage of 250 ml hl ⁻¹ [$\mu\text{g ml}^{-1}$]
Riesling Mosel	0.09 ± 0.00	0.33 ± 0.01
Riesling Rheingau	LODC	0.04 ± 0.01
Pinot blanc	0.08 ± 0.00	0.32 ± 0.01
Pinot gris	LOD	0.23 ± 0.03

Notes: LODC = value was equal to the limit of decision (*t*-test); LOD = value was equal to the limit of detection (*t*-test). Variations represent the standard deviation.

Discussion

FGs and IGs are produced from various fish species and by various methods. Methods for the FG production include heating, acidic extraction and further acidic and/or enzymatic hydrolysis depending on the required properties (Baziwane and He 2003). IG is produced from swim bladders by washing and cleaning, conditioning in hot water, and the removal of muscle layers and blood vessels (Koochekian et al. 2006, EFSA 2007). Thus, the final products vary in species composition and alterations in their collagen structure. This may interfere with the detection of various FGs and IGs using a single method. In this regard, Figure 1 illustrates the highly heterogeneous composition of various FGs and IGs. Depending on the degree of hydrolysis, their compositions range from native products with characteristic α -, β -, and γ -collagens and MWs larger than 117 kDa to highly hydrolyzed products with MWs lower than 40 kDa. However, all products bound to the primary antibody as displayed by Western blot in Figure 2. As previously assumed, a strong impact of processing could be demonstrated to primary antibody binding. Highly hydrolyzed products, such as hydrolyzed IG B and hydrolyzed FG A, were detected to a lesser degree by

the primary antibody raised against fish collagen. Other products with various native and fragmented proteins showed good affinity to the antibody. Consequently, the detection of FG and IG by indirect ELISA is possible, but the extent of cross-reactivity between the various products needs clarification. This is a critical step because, occasionally, it is unclear which product was used in the manufacturing process. Thus, a method predominantly independent from the used product is required. In the case of the ELISA, a high cross-reactivity among the various FGs and IG products would lead to such a method.

The indirect ELISA revealed different affinities of the primary antibody to various FGs and IGs. Hydrolyzed IG A showed the highest affinity to the antibody, simply because the product had been used for antibody production. If hydrolyzed IG A is ignored, the three FGs showed the highest antigenicity. Hydrolyzed IG B and hydrolyzed FG A, which had been further hydrolyzed by acidic and/or enzymatic digestion, revealed the lowest antigenicity. Interestingly, native IG displayed an antigenicity between FG and hydrolyzed IG. The complex tertiary and quaternary structure of native IG could be considered as a possible factor for the masking of epitopes since the product had not been processed. These structures are destroyed during the production of FG, resulting in a higher antigenicity. In conclusion, antibody binding appears to occur in a process-dependent manner with the lowest affinity belonging to highly processed hydrolyzed IGs and the highest affinity to moderately processed FGs. However, differences in the affinity of FGs and IGs to the microtiter plate must also be considered for some products demonstrated by competitive ELISA (results not shown). Furthermore, sequential differences in collagens from various fish species must be considered since sequential information is currently unavailable and, therefore, differences or agreements in the amino acid sequences are unknown.

The ELISA results showed that cross-reactivities between various FGs and IGs are unacceptable for reliable quantification if the used product is unknown. Cross-reactivities, as demonstrated by the relative reactivity related to FG A, were between 13 and 70% if hydrolyzed IG A is ignored (Table 1). However, all FGs and IG products were detected with a sensitivity $<0.11 \mu\text{g ml}^{-1}$. The impact of urea treatment and heating, as applied for the solubilization of some IGs, was considered to have only a minor effect on these findings. OD values in the steepest range between 0.3 and $13 \mu\text{g ml}^{-1}$ were found to be between 104% ($0.3 \mu\text{g ml}^{-1}$) and 63% ($3 \mu\text{g ml}^{-1}$) of the respective untreated material, as demonstrated with FG A (results not shown). Furthermore, the effects were mathematically considered, as explained in the Introduction.

Cross-reactivity between fish and mammalian gelatine was demonstrated as weak by SDS-PAGE (Figure 2) and indirect ELISA (Table 1). Thus, bovine gelatine was not detected by the primary antibody. This is most likely due to high sequential differences and also to different hydrolysis levels. The SDS-PAGE (Figure 1) indicated a stronger hydrolysis of the used bovine gelatine compared to fish, porcine or mammalian gelatines. However, the observation of low cross-reactivity between fish and mammalian gelatine is in accordance with the published findings described in the Introduction. This allows differentiation of the fish and bovine gelatine traces, but not the fish or porcine traces, by indirect ELISA. In conclusion, the indirect ELISA can be used for the sensitive qualitative detection of minute amounts of $\leq 0.11 \mu\text{g ml}^{-1}$ FGs and IGs rather than as a quantitative method. The possibility of differentiating between fish and mammalian products is limited.

The usage of FG and IG within the wine industry is barely known and largely depends on grape variety, vintage, region and the winemaker's practice. It is estimated that ~16% of French and 7% of German wines have been fined with products composed of fish collagen (personal communication: Deutscher Weinbauverband, Germany, and Union des Fabricants, des Laboratoires et des Industries Oenologiques, France, 2006). Application of this indirect ELISA to white wines indicated only minor effects of the wine matrix on assay sensitivity and accuracy, demonstrated for one FG and one IG (Table 2). However, the investigation of red wines was impossible due to the strong impact of the red wines matrix, which gave an extremely high background noise, resulting in false positive results. Polyphenols are the main suspects, as they strongly interact with proteins (Weber et al. 2007). Separation of polyphenols via solid-phase extraction, dialysis, protein precipitation or by masking the polyphenols by adding other proteins, such as skim milk powder or egg white powder, led to unacceptable results (data not shown). Thus, the analysis of wines was focused on white wines only.

Residues of FG or IG were not detected in any of the 60 commercial white wine samples, including the two samples known to be fined with FG. Experimental wines were found to contain traces of hydrolyzed IG A, $<0.33 \mu\text{g ml}^{-1}$, when these wines were not treated with bentonite and in the absence of successive cross-flow and membrane filtration (Table 3). Bentonite is an important substance for the stabilization of white wines, as previously mentioned. Thus, it should be applied to most white wines. No traces of FG or IG were detected in any of the experimental wines treated with bentonite and successive cross-flow and membrane filtration, indicating levels of $<0.02 \mu\text{g ml}^{-1}$.

Bentonite treatment of red wines is a critical step because it can reduce the red colour of the wine.

Furthermore, red wines are more stable against protein clouding owing to their natural protein-precipitating properties, which is due to their high polyphenol content. Thus, bentonite is frequently avoided in red wine production. However, residues of foreign proteins, such as FG and IG, should be considerably lower in untreated red wines compared to untreated white wines (Weber et al. 2009), due, most likely, to the high content of polyphenols.

Overall, no residues of FG and IG should be detected in wines if good manufacturing practices are used in combination with thorough filtration. The absence of bentonite usage and additional filtration can result in minute amounts of IG in white wines. However, there is no evidence that FG or IG presents a risk to human health. Combining this lack of evidence with the results of detectable residues as from little to none, fining of wines with FG and IG appears to be of no risk for consumers allergic to fish. This conclusion is in accordance with Taylor et al. (2004). Labelling of FG and IG on wines for the purpose of protection of fish-allergic consumers has been exempted within the EU since the initiation of EU Directive 2007/68/EC in November 2007.

In conclusion, an indirect ELISA method for the sensitive detection of FG and IG has been developed. This method is suitable to detect levels of $\leq 0.11 \mu\text{g ml}^{-1}$, independent of analyte composition or processing stage. Involving a 10-fold dilution, this method can be directly applied to the investigation of white wines. No residues of FG and IG were found in commercially available wines and in experimental wines treated with bentonite, a strong adsorbent for proteins, and successive cross-flow and membrane filtration. Levels of $\leq 0.33 \mu\text{g ml}^{-1}$ were found in some white wines not treated with bentonite or with successive cross-flow and membrane filtration.

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