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1 Characterization, Antigenicity, and Detection of Fish

2 Gelatine and Isinglass used as Processing Aids in Wines

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4
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14 15 **ABSTRACT**

16 Fish gelatine (FG) and isinglass (IG) are widely used in the pharmaceutical industry and as
17 ingredients or processing aids in the food production. Both products are the focus of interest
18 since several nations, particularly the member states of the EU and Japan, USA, Australia,
19 and New Zealand, have introduced special labelling regulations for allergenic foodstuffs, such
20 as fish and products thereof. Thus, there is a demand for a reliable and sensitive method for
21 the detection of FG and IG in foodstuffs. In this study, the characterization of various FGs
22 and IGs, polyclonal antibodies raised against fish collagen and the development of a sensitive
23 indirect ELISA for the detection of FG and IG is described. The ELISA method detected \leq
24 $0.11 \mu\text{g ml}^{-1}$ from all FGs and IGs tested. The indirect ELISA was applied to various
25 experimental wines where FG and IG had been used as processing aids and to several

1
2
3 26 commercial wines. No residues of FG and IG were detected in the experimental wines
4
5 27 additionally treated with bentonite, a strong adsorbent for proteins, and successive filtration.
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7
8 28 Additionally, no residues were found in the commercial wines. However, amounts of up to
9
10 29 0.33 $\mu\text{g ml}^{-1}$ were found in some experimental wines not treated with bentonite and
11
12 30 successive filtration. Therefore, wines may contain traces of FG and IG that have been used
13
14
15 31 as processing aids during the wine production. However, the additional treatment with
16
17 32 bentonite in combination with additional filtration was found to show a clear impact onto
18
19
20 33 these residues.
21
22 34
23
24 35 **KEYWORDS:-** Fish gelatine; isinglass; fish collagen; wine; bentonite; ELISA; food allergy
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36 INTRODUCTION

37 Fish gelatine (FG) and isinglass (IG) are widely used in the food industry and are important
38 for the production of beverages, such as wine, beer, juice, cider, and mead (Taylor *et al.* 2004,
39 Baziwane *et al.* 2003). FG is used in dairies, confectioneries, butcheries, for the production of
40 candies, desserts, delicatessen, as dietary supplement and as carrier for dyestuffs, flavors, and
41 vitamins. It is an important alternative for bovine and porcine gelatine due to bovine
42 spongiform encephalitis, and the demand for halal and kosher foodstuffs. Moreover, FG has
43 an importance in the pharmaceutical industry, particularly for the preparation of capsules,
44 pills, plasma substitutes, and as a stabilizer in vaccines.

45
46 Raw materials for the production of FG are mixtures of skins from various bony fishes
47 (*Osteichthyes*) belonging to the subclass *Actinopterygii*. Important genera to note are cod,
48 tilapia, tuna, perch, pollock, haddock, hake, cusk, flatfish, and redfish. IG is derived from the
49 swim bladders of the same and other genera, such as sturgeon, catfish, croaker, and threadfin.
50 Skins and swim bladders are waste materials in the fish industry, which are abundantly
51 available and high in content for the desired type I collagen.

52
53 Both FG and IG products are in the focus of interest as several nations, particularly the
54 member states of the EU, Japan, USA, Australia and New Zealand, have introduced special
55 labelling regulations for allergenic foodstuffs based on a recommendation by the Codex
56 Alimentarius Commission in 1999. These regulations include fish and products thereof. The
57 IgE-binding properties of FG and the fish collagen protein, which is the main compound of
58 FG and IG, were proven *in vitro* by several workgroups in some fish allergic patients with a
59 high prevalence of cross-reactivities among various fish species (Sakaguchi *et al.* 2000a,
60 Sakaguchi *et al.* 2000b, Sakaguchi *et al.* 1999, André *et al.* 2003, Hamada *et al.* 2001,
61 Hamada *et al.* 2003). Evidence for the allergenicity has neither been reported after the

1
2
3 62 consumption of foods nor after the administration of pharmaceuticals containing FG or fish
4
5 63 collagen. No adverse reactions were observed in a double-blind placebo-controlled food
6
7
8 64 challenge (DBPCFC) with FG performed by Andre *et al.* (2003) and only one mild, subjective
9
10 65 symptom occurred in a DBPCFC performed by Hansen *et al.* (2004) on 30 fish allergic
11
12 66 patients after the consumption of a cumulative dosage of 7.6 g of FG. Positive skin prick tests
13
14
15 67 and positive histamine release tests, though, were obtained against FG in some fish allergic
16
17 68 patients (Hansen *et al.* 2004). Published cases of allergic reactions to gelatine-containing
18
19 69 foods are rare and were referred to as mammalian gelatine (Sakaguchi *et al.* 1996) or to
20
21 70 gelatine with uncharacterized origin (Sakaguchi *et al.* 1995, Kelso *et al.* 1993, Wang *et al.*
22
23 71 2005; Wahl *et al.* 1989). That also applies to pharmaceuticals where known cases of allergic
24
25 72 reactions were referred to as mammalian gelatine (Sakaguchi *et al.* 1999, Sakaguchi *et al.*
26
27 73 1996, Nakayama *et al.* 2004, Sakaguchi *et al.* 2001) or to gelatine with uncharacterized origin
28
29 74 (Sakaguchi *et al.* 1995, Nakayama *et al.* 1999, Sakaguchi *et al.* 1997) that were not
30
31 75 administered by the oral route. The latter three studies referred to vaccines from Japan which
32
33 76 are usually produced with mammalian gelatine (Sakaguchi *et al.* 1999). Differentiation
34
35 77 between mammalian gelatine, FG, and fish collagen is very important since *in vitro* cross-
36
37 78 reactivities were not observed (André *et al.* 2003, Hamada *et al.* 2001) or rather weak
38
39 79 (Sakaguchi *et al.* 2000a, Sakaguchi *et al.* 2000b, Sakaguchi *et al.* 1999, Kelso *et al.* 1993).
40
41 80 Based on these facts, an allergic potential of FG and the fish collagen protein was not proven.
42
43 81 However, there is the risk that allergic reactions are not referred to the consumption of FG or
44
45 82 fish collagen containing foods or pharmaceuticals. Many foodstuffs or pharmaceuticals are
46
47 83 prepared by the usage of these substances but they are usually not considered to contain fish
48
49 84 proteins by the fish allergic consumer, e. g. due to improper labelling. Furthermore, the
50
51 85 adherence of fish muscle tissue, including the major fish allergen parvalbumin, must be
52
53 86 considered in FG and IG even though the information does not exist. Apart from this,
54
55 87 vegetarians and vegans wish to abstain from any animal substances.
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3 88 In conclusion, there is a demand for a reliable and sensitive method in detecting FG and IG in
4
5 89 order to assure an adequate labelling of foodstuffs and to detect contamination of foods and
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7
8 90 pharmaceuticals for consumer protection. A common method for the determination of protein
9
10 91 traces is the enzyme-linked immunosorbent assay (ELISA).

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12 92
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15 93 In this study, the characterization of polyclonal antibodies raised against fish collagen and the
16
17 94 development of a sensitive indirect ELISA for the determination of FG and IG is described.
18
19 95 This includes the characterization of various FGs and IGs by SDS-PAGE and the
20
21 96 investigation of their affinity to the polyclonal antibodies by Western blot. Furthermore, the
22
23 97 application of the ELISA for the investigation of wines is demonstrated. FG and IG are used
24
25 98 in wine production to reduce the polyphenol and protein content as described previously
26
27 99 (Weber *et al.* 2007). The treatment contributes to the organoleptic properties of the wine and
28
29 100 to the prevention of proteinogenic haze. A range of experimental and commercial wines were
30
31 101 investigated for the presence of FG and IG residues. Bentonite treatment – an important fining
32
33 102 step in the winery process, which reduces the protein content in wines – was considered in the
34
35 103 production of experimental wines.
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42 43 105 **MATERIALS & METHODS**

44 45 106 *Food-grade gelatines and collagens*

46
47 107 A total of seven commercially available FG and IG samples were collected from four
48
49 108 different suppliers (Erbslöh GmbH, Germany; Begerow GmbH, Germany; Norland Products
50
51 109 Inc., USA; Sigma Aldrich Co., USA): Three FGs A - C (derived from fish skin and provided
52
53 110 as yellow powders), two acid-hydrolyzed IG A and B (provided as acidic gel with 2 % IG
54
55 111 derived from swim bladder collagen), one hydrolyzed FG A (provided as white powder
56
57 112 derived from acidic and enzymatic hydrolysis from fish skin collagen), and one native IG
58
59 113 (derived from swim bladder and provided as yellow sheets).

1
2
3 114 Three mammalian gelatines were purchased as yellow powders from local markets or
4
5 115 pharmacies: One mammalian gelatine composed of porcine and bovine gelatine, one porcine
6
7 116 gelatine, and one bovine gelatine. Protein contents were measured by the Kjeldahl method as
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9
10 117 published earlier (Mattisek *et al.* 1992).
11

118

119 *Polyclonal antibodies against fish collagen*

120 Primary antibodies against fish collagen were produced in New Zealand white rabbits in
121 accordance with institutional guidelines (Eurogentec SA, Belgium). Briefly, 100 µg of acid-
122 hydrolyzed IG A was mixed with 400 µl saline (0.9%) and 500 µl of complete Freund's
123 adjuvant and were injected intradermally to three rabbits each. Injection was repeated three
124 times with the incomplete Freund's adjuvant in four-week intervals. Blood samples were
125 taken 10 days after the second, third and fourth injection and the serum was obtained by
126 centrifugation. The third serum sample (~ 60 ml) was purified by affinity chromatography
127 using 10 mg hydrolyzed IG A immobilized at CNBr Sepharose. The column was washed with
128 10 ml elution buffer (100 mM glycine, pH 2.5) and 15 ml phosphate-buffered saline (PBS).
129 Afterwards, the serum was loaded with a flow rate of 0.5 – 1 ml/min and the column was
130 washed thoroughly with PBS. Then, antibodies were eluted with elution buffer and
131 neutralized immediately with a solution of 1.5 M Tris(hydroxymethyl)-aminomethane (Tris),
132 pH 8.5. After dialysis against PBS over night, the final solution consisted of 15.2 mg
133 antibodies in 8 ml of PBS. The antibody purity was greater than 85 %, estimated by SDS-
134 PAGE. 0.1% bovine serum albumin and 0.01 % Thimerosal was added for stabilization and
135 the purified serum was stored at -80 °C.

136

137 *Fish samples*

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3 138 Five fish muscle samples from species important to FG and IG production were purchased
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5 139 filleted and frozen from local markets; cod (*Gadus ssp.*), pollock (*Pollochius virens*), tuna
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7
8 140 (*Thunnus ssp.*), sturgeon (*Acipenser ssp.*) and tilapia (*Oreochromis ssp.*).
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10 141

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12 142 *Wine samples*

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15 143 Sixty commercial white wine samples were purchased from local stores. The selection pattern
16
17 144 was restricted to the country of origin (23 German wines, 18 French wines and 19 Italian
18
19 145 wines) and to important European grape varieties (Chardonnay, Pinot gris, Pinot blanc,
20
21 146 Riesling, Sauvignon blanc and Silvaner). Fining with fish skin gelatine was known for two of
22
23 147 these commercial white wine samples whereas fining was not known for the remaining 58
24
25 148 samples.
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31 150 Experimental wines were prepared from four grape varieties (Riesling Mosel, Riesling
32
33 151 Rheingau, Pinot blanc and Pinot gris) by treatment with two different dosages of FG B,
34
35 152 hydrolyzed IG A, hydrolyzed IG B and the native IG, respectively. This process is described
36
37 153 elsewhere (Weber *et al.* 2007) in which “isinglass 1” corresponds to hydrolyzed IG A,
38
39 154 “isinglass 2” to hydrolyzed IG B, “isinglass 3” to the native IG and “fish gelatine” to FG B.
40
41 155 Additionally, wines without bentonite treatment were prepared to evaluate the impact onto
42
43 156 residual amounts of FG and IG. Therefore, wines were bottled directly after separation from
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45 157 the fining precipitate and, thus, lacked of the final cross-flow and membrane filtration.
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53 159 *Reagents and buffers*

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55 160 All chemicals used were of analytical grade.

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57 161 Urea solution was prepared from 8 M urea and 2 % cyclohexylaminopropane sulfonic acid in
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59 162 double distilled water.
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3 163 Colloidal Coomassie Brilliant Blue (CBB) staining solution was prepared by dissolving 1 g of
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5 164 CBB G250 in 20 ml double distilled water (solution 1). Then, 16 ml of solution 1 was added
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7
8 165 to 16 ml *o*-phosphoric acid (85%), 80 g ammonium sulphate, and 768 ml double distilled
9
10 166 water. 200 ml of methanol was slowly added immediately before use.

11
12 167 Electrode solutions for the Western blot were composed as follows: Cathode solution (pH 7.6)
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14
15 168 was prepared with 2.6 g 6-aminohexanoic acid, 0.05 g SDS, 0.05 g sodium azide, and 100 ml
16
17 169 ethanol, filled to 500 ml with double distilled water. Anode I solution (pH 10.4) consisted of
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19
20 170 18.15 g Tris, 0.05 g sodium azide, and 100 ml ethanol, which was filled to 500 ml with
21
22 171 double distilled water. The anode II solution (pH 10.4) contained 1.52 g of Tris, 0.05 g
23
24 172 sodium azide, and 100 ml ethanol, filled to 500 ml with double distilled water.
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29 174 For immunostaining, the following solutions were prepared: The Tris solution contained 50
30
31 175 mM Tris, 150 mM NaCl, and 0.5 % polyethylene-sorbitan monolaurate (Tween 20) in double
32
33 176 distilled water (Szepfalusi *et al.* 1993). The TMB solution consisted of 12 mg 3,3',5,5'-
34
35 177 tetramethylbenzidine (TMB) and 40 mg dioctylsodiumsulfosuccinate dissolved in 10 ml
36
37 178 ethanol. Citric buffer (pH 5.0) was prepared with 9.4 g of citric acid and 18.2 g of Na₂HPO₄ *
38
39 179 2 H₂O dissolved in 1 l double distilled water. The substrate solution for immunostaining was
40
41 180 carefully mixed from 5 ml TMB solution, 15 ml citric buffer and 10 µl of H₂O₂ (30%). Re-
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43 181 buffer solution was prepared with 121.2 mg Tris, filled up to 80 ml with double distilled
44
45 182 water, then adjusted to pH 6.0 with 4 M hydrochloric acid and finally filled up to 100 ml with
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47 183 double distilled water. Ponceau red solution was prepared from 2 % Ponceau red and 3 %
48
49 184 trichloroacetic acid in double distilled water.
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185
186 Goat anti-rabbit horseradish peroxidase (HRP) immunoglobulins were purchased from Dako
187 (order no. P0448, Dako GmbH, Germany).

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3 188 Carbonate buffer for ELISA was prepared from 75 mM Na₂CO₃ and 175 mM NaHCO₃ in
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5 189 double distilled water (pH 9.5). The washing solution, substrate solution, and stop solution for
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7 190 ELISA were prepared according to Weber *et al.* (2007).
8
9

10 191
11
12 192 *Preparation of FG and IG solutions*
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14 193 FGs and hydrolyzed FG A were completely soluble in double distilled water. The urea
15
16 194 solution was added to the insoluble IGs, giving a concentration of approximately 200 µg ml⁻¹.
17
18 195 The suspension was stirred at room temperature and repeatedly heated at 80 °C until the
19
20 196 collagen was dissolved (24-72 h). Samples soluted in urea were diluted at least 20-fold with
21
22 197 the carbonate buffer to allow renaturation for the indirect ELISA.
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29 199 *Preparation of fish extracts*
30

31 200 An amount of 2.4 g thawed fish muscle was homogenized for 2 min with 20 ml of the urea
32
33 201 solution using an Ultra-Turrax with 8000 rpm. Then, the homogenate was extracted for 2 h
34
35 202 through continuous shaking and centrifuged for 15 min at 6700 g. The clear supernatant was
36
37 203 removed and used for SDS-PAGE analysis. Spare extracts were stored at – 24 °C.
38
39
40

41 204
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43 205 *SDS-PAGE*
44

45 206 SDS-PAGE was performed on Invitrogen equipment (Invitrogen Corp., USA). Samples were
46
47 207 separated between 200 – 3.5 kDa at 200 V for 45-50 min with 1mm thick pre-cast NuPAGE
48
49 208 Novex Bis-Tris 12-well gels with 12% acrylamide (Invitrogen Corp.), NuPAGE MES SDS
50
51 209 running buffer as electrolyte (Invitrogen Corp.), and were calibrated against the Serva Mark12
52
53 210 molecular weight (MW) ladder (Invitrogen Corp.). Proteins with MWs between 460 - 31 kDa
54
55 211 were separated at 150 V for 70 min on 1mm thick pre-cast NuPAGE Novex Tris-Acetate 10-
56
57 212 well gels with 3-8% acrylamide (Invitrogen Corp.), NuPAGE Tris-Acetate SDS running
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3 213 buffer (Invitrogen Corp.), and were calibrated against the pre-stained HiMark MW ladder
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5 214 (Invitrogen Corp.).
6
7
8 215

9
10 216 *Colloidal CBB staining*
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12 217 Gels were stained with 50 ml of colloidal CBB staining solution for 16 h. After that, the
13
14 218 background was decolorized through incubation in double distilled water with soaked paper
15
16 219 tissues.
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22 221 *Semidry Western blot and immunostaining (Immunoblot)*
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24 222 Semidry Western blot was performed on a LKB Bromma 2117 Multiphor II blotting
25
26 223 apparatus with Pharmacia Biotech Nova Blot graphite electrodes (Pharmacia Biotech, USA)
27
28 224 and a Powerease 500 power supply (Invitrogen Corp.) onto nitrocellulose transfer membranes
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30 225 with a 0.2 μm pore size (Whatman PROTRAN, Whatman GmbH, Germany).
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34 226 Initially, four layers of thin cloth were soaked with cathode solution and put on the cathode.

35
36 227 The gel was then laid onto the cloth pile. Afterwards, the nitrocellulose transfer membrane
37
38 228 was soaked with anode solution II and was transferred onto the gel. Areas where no transfer
39
40 229 was desired were covered up with plastic slides. Two layers of cloth were soaked with the
41
42 230 anode buffer II and were put onto the blot membrane. After that, four layers of cloth were
43
44 231 soaked with the anode solution I and were transferred onto the pile. Finally, the graphite
45
46 232 anode was put onto the pile and the blotting was performed for one gel for 80 min at 60 mA.
47
48

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50 233 Afterwards, the air-dried membrane was blocked two times with Tris solution for 1 h each
51
52 234 and was incubated for 1.5 h with polyclonal antibodies against fish collagen (1:500 diluted in
53
54 235 Tris solution). Then, washing was done three times with the Tris solution for 5 min each and
55
56 236 the membrane was incubated for 1.5 h with goat anti-rabbit HRP immunoglobulins (diluted
57
58 237 1:2000 in Tris solution). The membrane was washed four times with the Tris solution and for
59
60 238 5 min each, was incubated for 1 min in re-buffer solution, and finally was incubated with

1
2
3 239 substrate solution for immunostaining until the desired color intensity was obtained. The
4
5
6 240 reaction was stopped by washing with double distilled water. The Serva Mark12 MW ladder
7
8 241 was stained by the addition of 50 ml Ponceau red solution for 1.5 h followed by a thorough
9
10 242 washing with double distilled water.

11 243

12 244 *Indirect ELISA Procedure*

13
14
15 245 For the indirect ELISA assay, standard solutions were prepared in carbonate buffer and wine
16
17 246 samples were diluted 1:10 with the carbonate buffer. 150 μ l of these solutions were loaded in
18
19 247 the wells of a polystyrene microtiter plate (Maxisorb F96, certified, Nunc, Denmark). The
20
21 248 plate was incubated for 16 h at 8-9 $^{\circ}$ C and washed three times with 200 μ l/well of the
22
23 249 washing solution. Afterwards, free binding sites of the wells were blocked with 250 μ l/well
24
25 250 washing solution for 2 h at room temperature. The plate was washed three times with 250 μ l
26
27 251 washing solution and the ELISA was proceeded by addition of 150 μ l/well polyclonal
28
29 252 antibodies against fish collagen (diluted 1:2000 in washing solution). After 1.5 h of
30
31 253 incubation at room temperature, the plate was washed three times with 200 μ l/well washing
32
33 254 solution. Thereafter, 150 μ l/well of goat anti-rabbit HRP immunoglobulins (diluted 1:2500 in
34
35 255 washing solution) were added and incubated for 1.5 h at room temperature. After four
36
37 256 washing steps with 250 μ l/well washing solution, 150 μ l/well substrate solution was added and
38
39 257 reacted for exactly 9 min in darkness, at 8-9 $^{\circ}$ C. The enzymatic colorimetric reaction was
40
41 258 stopped by adding 75 μ l stop solution. The optical density (OD) was measured at 450 nm
42
43 259 against a reference wavelength of 630 nm using a microtiter plate reader (MRX, Dynex
44
45 260 Technologies, USA).

46
47 261
48
49 262 The attained ODs were plotted against the logarithm of the protein concentration. Quantitative
50
51 263 evaluations were performed by AssayZap Software (Biosoft, United Kingdom) using 4-
52
53 264 parametric regression. All ODs were reduced by the mean blank OD. Thus, the limit of

1
2
3 265 decision (LODC; 50% probability that the measured values lie within the spread of the blank
4
5 266 values) was defined as zero plus 3-fold the standard deviation of the blank OD and the limit
6
7
8 267 of detection (LOD; 99.8% probability that the measured values are larger than the spread of
9
10 268 the blank values) was defined as zero plus 6-fold the standard deviation of the blank OD
11
12 269 (Einax *et al.* 1997, Doerffel 1987).
13
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16
17 271 Intraassay variation coefficients were calculated from five independent standard curves on the
18
19 272 same microtiter plate whereas interassay variation coefficients were calculated from five
20
21 273 independent standard curves on five different microtiter plates and on five different days.
22
23

24 274 The effect of heating and urea treatment during the preparation of the IG solutions to the
25
26 275 antigenicity was mathematically considered. Therefore, correction factors (CF) were
27
28 276 calculated from the ODs derived from the respective concentration of FG A treated and not
29
30 277 treated with urea and heat: $CF = OD_{\text{untreated}}/OD_{\text{treated}}$. Then, values derived from IGs soluted
31
32 278 with urea and heating were corrected by this correction factor: $OD_{\text{corrected}} = OD * CF$.
33
34

35
36 279 The investigation of wine samples was performed four-fold.
37
38
39 280

40 41 281 **RESULTS**

42
43 282 *Characterization of various fish and mammalian gelatines and collagens by SDS-PAGE and*
44
45 283 *colloidal Coomassie staining.*
46
47

48 284 Figure 1 shows the heterogeneous composition of various gelatines and IGs. FGs showed
49
50 285 proteins and many fragments between approximately 280 - 22 kDa. Single bands were visible
51
52 286 for FG B at approximately 240 kDa, probably corresponding to dimeric collagens (β -
53
54 287 collagens), 120 and 117 kDa, probably corresponding to different monomeric collagens (α -
55
56 288 collagens), and 60 kDa. Hydrolyzed IGs were found to consist partly of smaller protein
57
58 289 fragments. Hydrolyzed IG A showed distinct ~ 400 kDa (probably γ - or tropo-collagen), ~240
59
60 290 kDa (probably β -collagen), and ~177 and 120 kDa (probably α -collagen) bands beside various

1
2
3 291 collagen fragments in the range of approximately 460 – 8 kDa. In contrast, hydrolyzed IG B
4
5 292 and hydrolyzed FG A revealed collagen fragments of approximately 100 – 8 kDa. Thereof,
6
7 293 hydrolyzed FG A showed only protein bands between 40 – 8 kDa. Native IG indicated three
8
9
10 294 distinct bands at MWs of approximately 400, 240, and 120 kDa. These bands very likely
11
12 295 represent γ -, β -, and α -collagens as previously described.
13
14

15 296
16
17 297 Porcine and the mammalian gelatine had a similar composition to FGs, indicating proteins
18
19 298 and many fragments between ~ 250 – 30 kDa. Distinct bands were visible for porcine gelatine
20
21 299 at ~240, 120, 117 and 60 kDa. However, bovine gelatine showed considerable differences to
22
23 300 fish and other mammalian gelatines. MWs were determined between 50 – 8 kDa for bovine
24
25 301 gelatine. Thus, this bovine gelatine has a character rather similar to hydrolyzed IG or
26
27 302 hydrolyzed FG than to FG. This indicated that the bovine gelatine had been comprehensively
28
29 303 processed.
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34 304
35
36 305 *Characterization of the polyclonal antibody's specificity by semidry Western blot and*
37
38 306 *immunostaining.*
39

40
41 307 Specificity of the polyclonal antibody was initially evaluated by various fish and mammalian
42
43 308 gelatines and IG (Figure 2). Proteins were detected in all FGs and IGs. FGs bound antibodies
44
45 309 to proteins and their fragments between MWs of approximately 460 – 40 kDa. Smaller
46
47 310 proteins, despite being detected by CBB staining, were not detected by this antibody. Binding
48
49 311 to the hydrolyzed IG and FG was, with the exception of hydrolyzed IG A, considerably
50
51 312 weaker compared to FGs. Hydrolyzed IG B revealed a weak antibody binding to MWs
52
53 313 between 100 - 38 kDa whereas hydrolyzed FG A showed a single immunostained protein
54
55 314 fragment at 14 kDa. On the contrary, hydrolyzed IG A, which had been used for the antibody
56
57 315 production, was well recognized by the antibody in the range of 460 - 8 kDa.
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59
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2
3 316 Bovine and mammalian gelatine were not recognized by the antibody whereas porcine
4
5 317 gelatine revealed a slight antibody binding in the range between 400 - 120 kDa.
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8 318

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10 319 Furthermore, the specificity of the polyclonal antibody to fish collagen was assessed by the
11
12 320 investigation of various fish muscle extracts (Figure 3). Colloidal CBB showed proteins
13
14 321 between > 200 - 8 kDa for all fish species except for tuna. Binding of the primary antibody by
15
16 322 immuno-staining occurred almost exclusively to proteins with MWs \geq 120 kDa. Detection of
17
18 323 these proteins can be expected as they represent the major collagen fractions (see Figure 3,
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20 324 lane 6). Only weak staining occurred in proteins of ~ 55 and ~ 36 kDa. Major fish proteins
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22 325 with MWs lower than collagen, e. g. definite bands between 50 – 36 kDa, were barely
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24 326 detected.
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30 328 *Indirect ELISA evaluation*

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32 329 The indirect ELISA standard curves were derived by serial dilution of gelatine and IG
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34 330 samples in the range of 0.001 – 10 $\mu\text{g ml}^{-1}$ protein. Figure 4 shows a significant difference in
35
36 331 the slopes of the various products. The relative reactivity of FGs and IGs related to FG A had
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38 332 values between 70 – 13 % within the steepest range of 0.3 – 3 $\mu\text{g ml}^{-1}$. Calculation of
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40 333 recovery rates was not possible for hydrolyzed IG A because of a greater sensitivity. LODCs
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42 334 were calculated in the range of 0.001 – 0.11 $\mu\text{g ml}^{-1}$ and LOD were calculated in the range of
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44 335 0.002 – 0.18 $\mu\text{g ml}^{-1}$ for all FGs and IGs (Table 1).
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52 337 Gelatines from mammals displayed higher LODCs and LODs of 0.14 – 0.8 $\mu\text{g ml}^{-1}$ and 0.27 –
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54 338 2.05 $\mu\text{g ml}^{-1}$, respectively. Bovine gelatine was not detectable by indirect ELISA. Relative
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56 339 reactivities related to FG A were between 30 – 1% and thus, were lower compared to FG and
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58 340 IG. Intra-assay and inter-assay variation coefficients were calculated for FG A. Intra-assay
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60 341 variation coefficients were less than 10 % (1.1 – 6.3 %) and inter-assay variation coefficients

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3 342 were less than 25 % (3.6 – 21.5 %) for concentrations between 0.1 – 10 $\mu\text{g ml}^{-1}$. Matrix
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5 343 effects were assessed by spiking experiments with all four experimental wines by spiking
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7 344 with FG A and hydrolyzed IG A, respectively (Table 2). Considering the 10-fold dilution,
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10 345 recovery rates for FG A were between 73 – 125 % within the steepest range of 3 – 20 $\mu\text{g ml}^{-1}$.
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12 346 Thus, the limit of quantification (LOQ) was found to be 3 $\mu\text{g ml}^{-1}$, which resulted in a
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14 347 practical working range of 3 – 20 $\mu\text{g ml}^{-1}$ for the white wines. The LODCs were calculated
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16 348 between 0.5 – 0.6 $\mu\text{g ml}^{-1}$ and the LODs between 0.7 – 0.9 $\mu\text{g ml}^{-1}$ for all spiked wines.
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18 349 Consequently, LODC and LOD of FG A were only insignificantly affected by the wine
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20 350 matrix.
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27 352 Hydrolyzed IG A was detected in 10-fold diluted experimental wines with recovery rates
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29 353 between 69 – 113 % in the range of 0.03 – 1 $\mu\text{g ml}^{-1}$. The LOQ was found to be 0.03 $\mu\text{g ml}^{-1}$,
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31 354 establishing a practical working range of 0.03 – 1 $\mu\text{g ml}^{-1}$. The LODCs were calculated
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33 355 between 0.01 – 0.02 $\mu\text{g ml}^{-1}$ and the LOD result was 0.02 $\mu\text{g ml}^{-1}$ for all investigated wines.
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35 356 Thus, the LODC and LOD of hydrolyzed IG A were also not significantly affected by the
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37 357 wine matrix.
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43 359 *Determination of fish gelatine and isinglass in wines by indirect ELISA*
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45 360 Of the 60 commercial white wine samples none were found to give signals significantly
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47 361 different from the blank OD. This included two wines with the known treatment of fish skin
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49 362 gelatine. All signals were significantly lower than the LODC. Based on the LODC values that
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51 363 were achieved for the various FG and IG (Table 1), the amounts of FG or IG were considered
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53 364 lower than 1.1 $\mu\text{g ml}^{-1}$ in these wines.
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59 366 Investigation of the experimental wines fined with FG and various IGs revealed negative
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367 results for all wines fined with FG B, hydrolyzed IG B and native IG. This result was

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3 368 independent of fining agent dosage and bentonite usage. Positive results were obtained for
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5 369 wines fined with hydrolyzed IG A. Quantification was performed against a standard curve
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8 370 prepared with hydrolyzed IG A. Detected residues were up to the level of $0.33 \mu\text{g ml}^{-1}$ in
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10 371 wines not treated with bentonite (Table 3). Thereof, amounts in wines treated with usual
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12 372 dosages of hydrolyzed IG A were up to the level of $0.09 \mu\text{g ml}^{-1}$ while treatment with an
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15 373 excessive dosage led to residues between $0.04 - 0.33 \mu\text{g ml}^{-1}$. Negative results were obtained
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17 374 for all wines treated with hydrolyzed IG A and bentonite. Thus, amounts of hydrolyzed IG A
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19 375 were lower than $0.01 \mu\text{g ml}^{-1}$ in these wines.
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23 24 377 **DISCUSSION**

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26 378 FGs and IGs are produced from various fish species and by various methods. Methods for the
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28 379 FG production include heating, acidic extraction, and further acidic and/or enzymatic
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30 380 hydrolysis depending on the required properties (Baziwane *et al.* 2003). IG is produced from
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32 381 swim bladders by washing and cleaning, conditioning in hot water, and the removal of muscle
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34 382 layers and blood vessels (Koochekian *et al.* 2006, EFSA 2007). Thus, the final products vary
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36 383 in their species composition and alterations in their collagen structure. This may interfere with
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38 384 the detection of various FGs and IGs with only a single method. In this regard, Figure 1
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40 385 illustrates the highly heterogeneous composition of various FGs and IGs. Depending on the
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42 386 degree of hydrolysis, their constitutions range from native products with the characteristic α -,
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44 387 β -, and γ -collagens with MWs larger than 117 kDa to highly hydrolyzed products with MWs
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46 388 lower than 40 kDa. However, all products bound to the primary antibody as displayed by
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48 389 Western blot in Figure 2. As previously assumed, a strong impact of processing could be
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50 390 demonstrated to the primary antibody's binding. Highly hydrolyzed products such as
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52 391 hydrolyzed IG B and hydrolyzed FG A were less detected by the primary antibody raised
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54 392 against fish collagen. Other products with their various native and fragmented proteins
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56 393 showed good affinity to the antibody. Consequently, the detection of FG and IG by indirect

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3 394 ELISA should be possible, but the extent of cross-reactivity between the various products
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5 395 must be clarified. This is a very critical step because, sometimes, it is not clear which product
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8 396 was used in the manufacturing process. Thus, a method predominantly independent from the
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10 397 used product is needed. In the case of the ELISA, a high cross-reactivity among the various
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12 398 FGs and IG products would lead to such a method.

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17 400 The indirect ELISA revealed different affinities of the primary antibody to various FGs and
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19 401 IGs. Hydrolyzed IG A showed the highest affinity to the antibody. This can be simply
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21 402 explained as the product had been used for the antibody production. If hydrolyzed IG A is
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23 403 ignored, the three FGs showed the highest antigenicity. Hydrolyzed IG B and hydrolyzed FG
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25 404 A, which had been further hydrolyzed by acidic and/or enzymatic digestion, revealed the
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27 405 lowest antigenicity. Interestingly, native IG indicated an antigenicity between FG and
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29 406 hydrolyzed IG. The complex tertiary and quaternary structure of native IG could be
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31 407 considered as a possible factor for the masking of epitopes since the product had not been
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33 408 processed. These structures are destroyed during the production of FG, resulting in a higher
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35 409 antigenicity. In conclusion, the antibody binding occurs seemingly in a processing dependent
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37 410 manner with the lowest affinity belonging to highly processed hydrolyzed IGs and the highest
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39 411 affinity to moderately processed FGs. However, differences in the affinity of FGs and IGs to
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41 412 the microtiter plate must also be considered for some products demonstrated by competitive
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43 413 ELISA (results not shown). Furthermore, sequential differences of collagens from various fish
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45 414 species must be kept in mind since the sequential information is currently inaccessible,
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47 415 therefore, differences or conformities in the amino acid sequences are unknown.

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53 417 The ELISA results showed that cross-reactivities between various FGs and IGs are rather
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55 418 unacceptable for a reliable quantification if the used product is unknown. Cross-reactivities,
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57 419 as demonstrated by the relative reactivity related to FG A, were between 13-70 % if

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3 420 hydrolyzed IG A is ignored (Table 1). However, all FGs and IG products were detected with a
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5 421 sensitivity of less than $0.11 \mu\text{g ml}^{-1}$. The impact of urea treatment and heating as applied for
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7 422 the solubilization of some IGs was considered to have only a minor effect on these findings.
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9 423 OD values in the steepest range between $0.3 - 3 \mu\text{g ml}^{-1}$ were found to be between 104 % (0.3
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11 424 $\mu\text{g ml}^{-1}$) and 63 % ($3 \mu\text{g ml}^{-1}$) of the respective untreated material as demonstrated with FG A
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13 425 (results not shown). Furthermore, the effects were mathematically considered as explained in
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15 426 the introduction.
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22 428 Cross-reactivity between fish and mammalian gelatine was demonstrated as weak by SDS-
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24 429 PAGE (Figure 2) and indirect ELISA (Table 1). Thus, bovine gelatine was not detected by the
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26 430 primary antibody. This is most likely due to high sequential differences and also due to
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28 431 different hydrolysis levels. The SDS-PAGE (Figure 1) indicated a stronger hydrolysis of the
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30 432 used bovine gelatine compared to the fish, porcine, and mammalian gelatines. However, the
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32 433 observation of low cross-reactivity between fish and mammalian gelatine is in accordance
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34 434 with the published findings described in the introduction. This allows the fish and bovine
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36 435 gelatine traces to differentiate, but not the fish and porcine gelatine traces by indirect ELISA.
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40 436 In conclusion, the indirect ELISA can be used for the sensitive qualitative detection of minute
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42 437 amounts of $\leq 0.11 \mu\text{g ml}^{-1}$ rather than as a quantitative method for the detection of FGs and
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44 438 IGs. The possibility of differentiation between fish and mammalian products is limited.
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50 440 The extent of the use of FG and IG within wine production is barely known and largely
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52 441 depends on grape variety, vintage, region, and the winemaker's practice. It is estimated that
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54 442 about 16% of French and 7% of German wines have been fined with products composed of
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56 443 fish collagen (personal communication: Deutscher Weinbauverband, Germany, and Union des
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58 444 Fabricants, des Laboratoires et des Industries Oenologiques, France, 2006). The appliance of
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60 445 this indirect ELISA to white wines indicated only minor effects of the wine's matrix to the

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3 446 assay's sensitivity and accuracy, demonstrated for one FG and one IG (Table 2). However,
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5 447 the investigation of red wines was impossible because of the strong impact of the red wine's
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8 448 matrix. This led to an extremely high background noise causing false positive results.
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10 449 Polyphenols are the main suspects as they strongly interact with proteins as previously stated
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12 450 (Weber *et al.* 2007). Separation of polyphenols by the use of solid-phase extraction, dialysis,
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15 451 protein precipitation, or by masking the polyphenols by adding other proteins, like skim milk
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17 452 powder or egg white powder, led to unacceptable results (results not shown). Thus, the
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20 453 analysis of wines was focused on white wines.
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24 455 Residues of FG or IG were not detected in any of the 60 commercial white wine samples
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27 456 including the two samples known to be fined with FG. Experimental wines were found to
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29 457 contain traces of hydrolyzed IG A, less than $0.33 \mu\text{g ml}^{-1}$, when these wines were not treated
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31 458 with bentonite and lacked of the successive cross-flow and membrane filtration (Table 3).
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34 459 Bentonite is an important substance for the stabilization of white wines as previously
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36 460 mentioned. Thus, it should be applied to most white wines. No traces of FG or IG were
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39 461 detected in any of the experimental wines treated with bentonite and successive cross-flow
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41 462 and membrane filtration, indicating amounts of $< 0.02 \mu\text{g ml}^{-1}$.
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45 464 Bentonite treatment of red wines is a critical step because it can reduce the red colour of the
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48 465 wine. Furthermore, red wines are more stable against protein clouding because of their natural
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50 466 protein precipitating properties, which is due to their high polyphenol content. Thus, bentonite
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53 467 is frequently avoided in red wine production. However, residues of foreign proteins, such as
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55 468 FG and IG, can be expected to be considerably lower in those untreated red wines compared
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58 469 to the untreated white wines as earlier demonstrated (Weber *et al.* 2009). This is most likely
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60 470 due to the high content of polyphenols.

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3 471 Overall, no residues of FG and IG could be detected in wines if good manufacturing practices
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5 472 were used combined with thorough filtration. The lack of bentonite usage and additional
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8 473 filtration can result in minute amounts of IG in white wines. However, there is no evidence
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10 474 that FG or IG can present a risk to human health. Combining this lack of evidence with the
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12 475 results of detectable residues as from little to none, fining of wines with FG and IG seemed to
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15 476 be of no risk for the consumers allergic to fish. This conclusion is in accordance with Taylor
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17 477 *et al.* (2004). Labelling of FG and IG on wines for the purpose of protection of fish allergic
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19 478 persons has been exempted within the EU by the initiation of the EU-Directive 2007/68/EC in
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22 479 November 2007.

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27 481 In conclusion, an indirect ELISA method for the sensitive detection of FG and IG has been
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29 482 developed. This method is suitable to detect $\leq 0.11 \mu\text{g ml}^{-1}$ independent of the analyte's
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31 483 composition and processing stage. Involving a 10-fold dilution, this method can be directly
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33 484 applied to the investigation of white wines. No residues of FG and IG were found in
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35 485 commercially available wines and in experimental wines treated with bentonite, a strong
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37 486 adsorbent for proteins, and successive cross-flow and membrane filtration. Only $\leq 0.33 \mu\text{g ml}^{-1}$
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40 487 ¹ were found in some white wines not treated with bentonite or with successive cross-flow
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42
43 488 and membrane filtration.

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3 570 TABLES
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7
8 572 Table 1: LODC, LOD, and relative reactivities of various gelatines and IGs determined by

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10 573 indirect ELISA.
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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.	-

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

Grape variety	LODC [$\mu\text{g ml}^{-1}$]		LOD [$\mu\text{g ml}^{-1}$]		Recovery [%]	
	FG A	Hydrol. IG A	FG A	Hydrol. IG A	FG A (3-20 $\mu\text{g ml}^{-1}$) 1)	Hydrol. 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

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3 578 Table 3: Fining agent contents in white wines fined with hydrolyzed IG A and not treated
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5 579 with bentonite. Results were acquired by indirect ELISA with a standard curve prepared of
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8 580 hydrolyzed IG A.
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Wine	Fining agent content for a common fining agent dosage of 50 ml/hl [$\mu\text{g ml}^{-1}$]	Fining agent content for an excessive fining agent dosage of 250 ml/hl [$\mu\text{g ml}^{-1}$]
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

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30 581 LODC = value was significantly equal to the limit of decision (t-Test); LOD = value was
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32 582 significantly equal to the limit of detection (t-Test); variation values represent the standard
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3 584 FIGURE CAPTION
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8 586 Figure 1 SDS-PAGE with colloidal Coomassie Brilliant Blue staining of various gelatines and
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10 587 collagen products derived from fish and mammals.
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15 589 *Figure 1 subtitle*

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17 590 A: MW between 460 – 31 kDa; B: MW between 200 – 3.5 kDa; M: MW marker; 1: Fish
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19 591 gelatine A; 2: Fish gelatine B, 3: Fish gelatine C; 4: Hydrolyzed isinglass A; 5: Hydrolyzed
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21 592 isinglass B; 6: Hydrolyzed fish gelatine A; 7: Native isinglass; 8: Bovine gelatine; 9:
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23 593 Mammalian gelatine; 10: Porcine gelatine.
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29 595 Figure 2 Immunoblot of various gelatines and collagen products derived from fish and
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31 596 mammals
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36 598 *Figure 2 subtitle*

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38 599 A: MW between 460 – 31 kDa; B: MW between 200 – 3.5 kDa; M: MW marker; 1: Fish
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40 600 gelatine A; 2: Fish gelatine B, 3: Fish gelatine C; 4: Hydrolyzed isinglass A; 5: Hydrolyzed
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42 601 isinglass B; 6: Hydrolyzed fish gelatine A; 7: Native isinglass; 8: Bovine gelatine; 9:
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44 602 Mammalian gelatine; 10: Porcine gelatine.
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50 604 Figure 3 SDS-PAGE and immunoblot of extracts derived from various fish species
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55 606 *Figure 3 subtitle*

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57 607 C: Colloidal CBB staining; I: Immunostaining; 1: Cod; 2: Pollock; 3: Tilapia; 4: Tuna; 5:
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59 608 Sturgeon; 6: Native isinglass.
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3 610 Figure 4 Indirect ELISA curves of various gelatines and collagen products derived from fish
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5 611 and mammals
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1 Table 1: LODC, LOD, and relative reactivities of various gelatines and IGs determined by
 2 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.	-

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

Grape variety	LODC [$\mu\text{g ml}^{-1}$]		LOD [$\mu\text{g ml}^{-1}$]		Recovery [%]	
	FG A	Hydrol. IG A	FG A	Hydrol. IG A	FG A (3-20 $\mu\text{g ml}^{-1}$))	Hydrol. 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

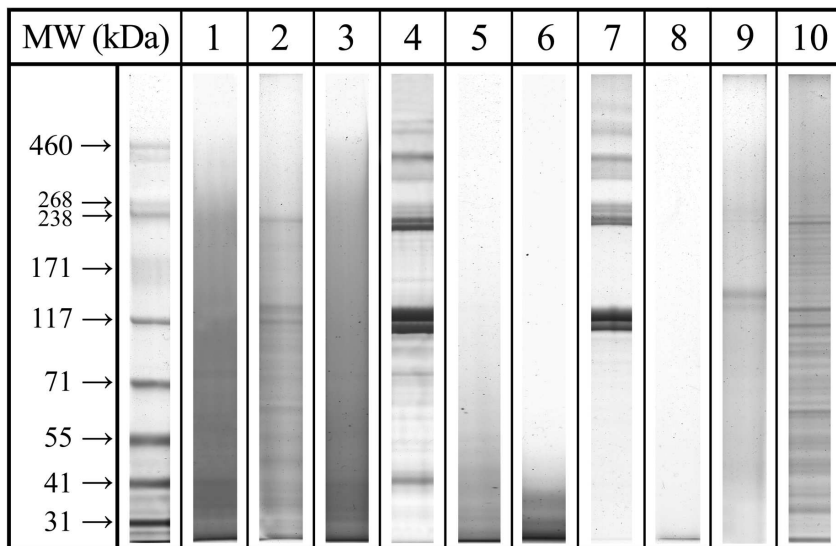
1 Table 3: Fining agent contents in white wines fined with hydrolyzed IG A and not treated
 2 with bentonite. Results were acquired by indirect ELISA with a standard curve prepared of
 3 hydrolyzed IG A.

Wine	Fining agent content for a common fining agent dosage of 50 ml/hl [$\mu\text{g ml}^{-1}$]	Fining agent content for an excessive fining agent dosage of 250 ml/hl [$\mu\text{g ml}^{-1}$]
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

4 LODC = value was significantly equal to the limit of decision (t-Test); LOD = value was
 5 significantly equal to the limit of detection (t-Test); variation values represent the standard
 6 deviation.

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A SDS-PAGE: 460 - 31 kDa



B SDS-PAGE: 200 - 3.5 kDa

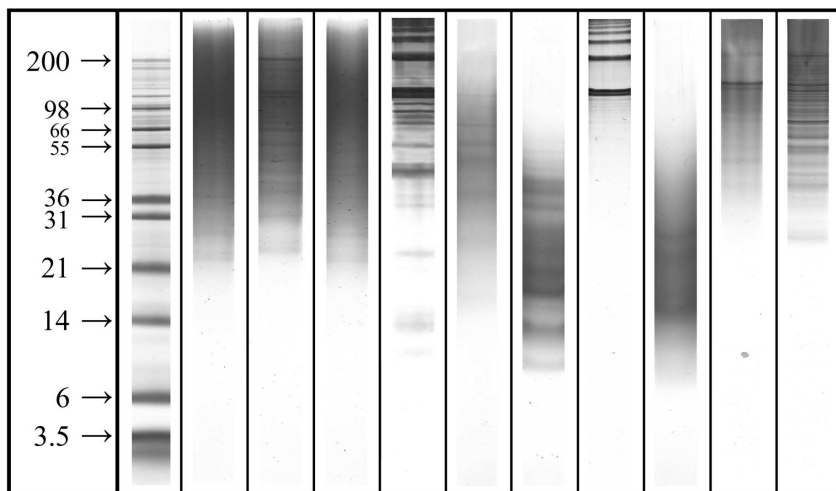


Figure 1 SDS-PAGE with colloidal Coomassie Brilliant Blue staining of various gelatines and collagen products derived from fish and mammals.

Figure 1 subtitle

A: MW between 460 – 31 kDa; B: MW between 200 – 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.

160x222mm (300 x 300 DPI)

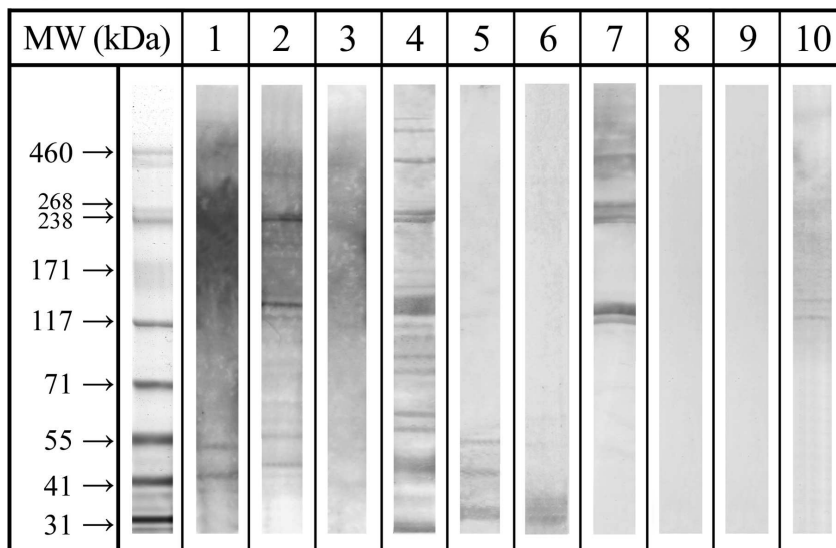
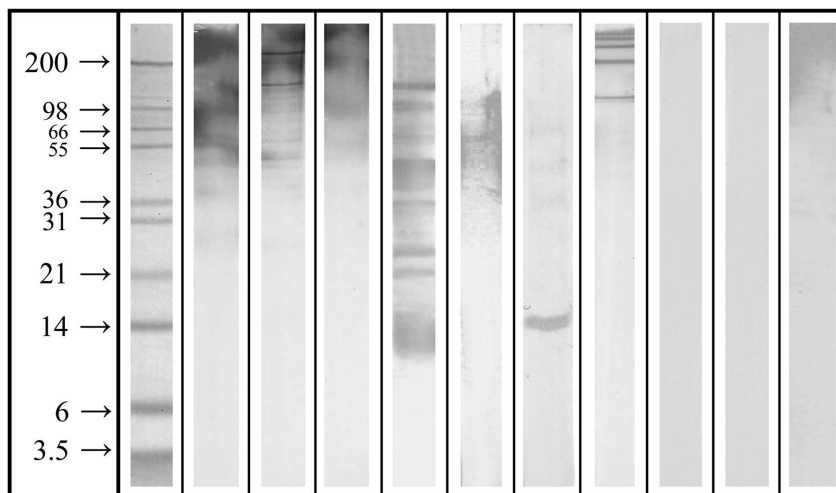
A SDS-PAGE: 460 - 31 kDa**B SDS-PAGE: 200 - 3.5 kDa**

Figure 2 Immunoblot of various gelatines and collagen products derived from fish and mammals

Figure 2 subtitle

A: MW between 460 – 31 kDa; B: MW between 200 – 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.

157x219mm (300 x 300 DPI)

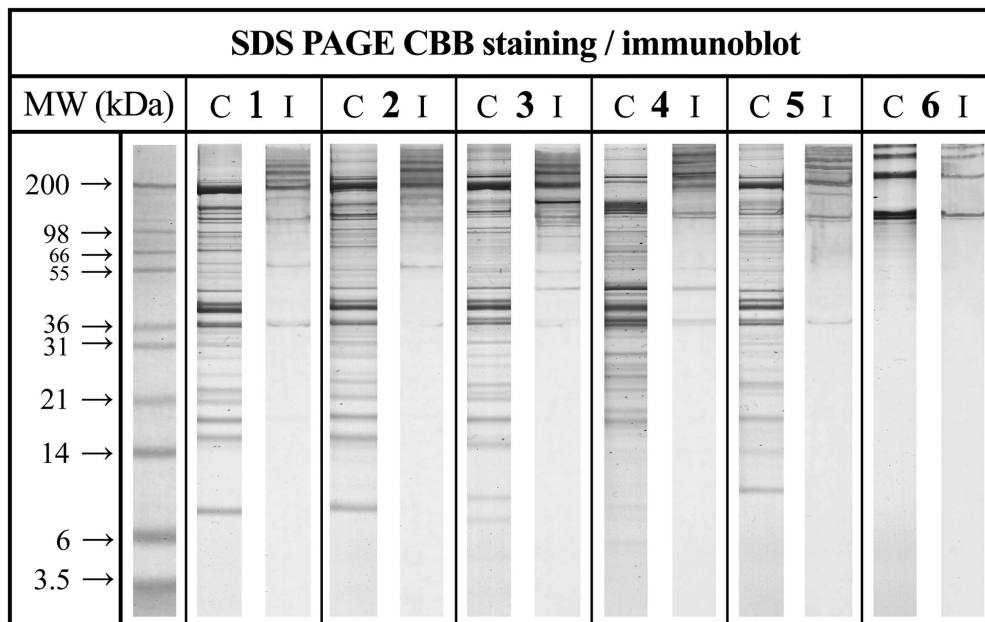


Figure 3 SDS-PAGE and immunoblot of extracts derived from various fish species

Figure 3 subtitle

C: Colloidal CBB staining; I: Immunostaining; 1: Cod; 2: Pollock; 3: Tilapia; 4: Tuna; 5: Sturgeon; 6: Native isinglass.

119x76mm (500 x 500 DPI)

View Only

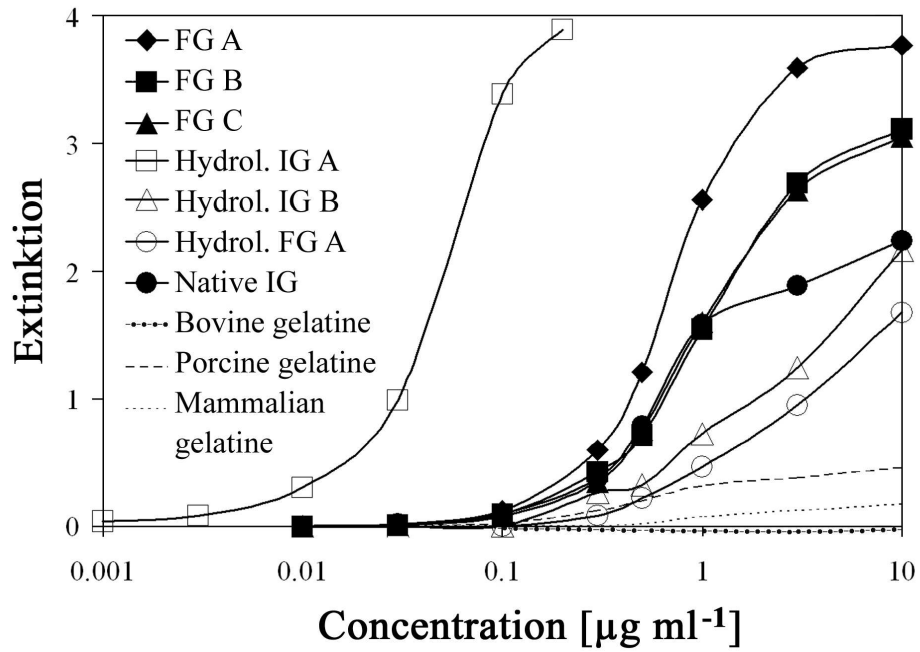


Figure 4 Indirect ELISA curves of various gelatines and collagen products derived from fish and mammals
170x119mm (300 x 300 DPI)