

1 **Extracting Conditions for Megrim (*Lepidorhombus bosci*) Skin**  
2 **Collagen Affect Functional Properties of the Resultant Gelatin**

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10 Running head: Extracting conditions of megrim skin collagen...

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2 **ABSTRACT**

3 Various procedures for extracting collagen and/or gelatin from megrim skins were  
4 compared on the basis of gelatin functional properties. It was possible to prepare a dry  
5 powder of soluble collagen with good viscoelastic and gelling properties, which can be  
6 converted into gelatin by dissolving at temperatures above 45°C. Physical properties of  
7 gelatins are influenced more by extracting conditions than by imino acid composition. A  
8 high-quality, readily-dissolved gelatin was obtained from megrim skins using a pre-  
9 treatment of the skins with NaCl and dilute NaOH, then swelling with 0.05 M acetic  
10 acid followed by an extraction step in water at 45°C.

11

12 **Key words:** gelatin, collagen, fish skin, extraction, functional properties.

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## 2 INTRODUCTION

3 The volume of gelatin used annually by the food industry worldwide is considerable and  
4 growing. Gelatin is not only used for its functional properties, but also to increase  
5 protein content. It can enhance the elasticity, consistency, and stability of food products,  
6 and it is also used as an outer film to protect against drying, light, and oxygen. The  
7 quality of a food grade gelatin therefore depends to a large extent on its rheological  
8 properties (essentially gel strength and viscosity), but it is also determined by other  
9 characteristics, particularly color, transparency, flavor, and easy dissolution.

10 Soluble gelatin is obtained industrially from collagen in bones or skins;  
11 therefore, the source, age of the animal, and type of collagen, all influence the properties  
12 of the gelatins (Johnston-Banks, 1990). Gelatin and collagen are originally the same  
13 macromolecule, so that the gelation of gelatin could be described as a partial  
14 reformation of the collagen structure (Ledward, 1986). The method of manufacture  
15 greatly affects the physicochemical properties of the gelatin. Collagen must be pre-  
16 treated to convert it into a form suitable for gelatin extraction, which is normally done  
17 with water above 45°C. The degree of conversion of collagen into gelatin is related to  
18 the severity of both the pre-treatment and the extraction processes, which depends on  
19 pH, temperature, and extraction time. A mild acid pre-treatment is usually used for  
20 collagenous material containing a low concentration of intra- and interchain non-  
21 reducible crosslinks, i. e., highly soluble collagens as with fish skins (Ledward, 1986;  
22 Norland, 1990; Montero et al., 1990, 1995). The collagen rod is extracted in acid and  
23 solubilized without altering its original triple-helix configuration. Subsequent thermal  
24 treatment cleaves hydrogen and covalent bonds; this destabilizes the triple helix by  
25 means of a helix-to-coil transition, leading to conversion into gelatin (Djabourov et al.,

1 1993). At 40°C and above, gelatin is soluble and is assumed to exist as a random coil.  
2 However, as reviewed by Ledward (1986), certain regions of the gelatin chains may  
3 retain some helical structure, the actual amount of structure depending on the number of  
4 pyrrolidine residues (proline and hydroxyproline) in the gelatin .

5         Although conventional gelatin from mammals has been widely studied, less  
6 work has been done on fish gelatin and its extraction procedures (Norland, 1990;  
7 Leuenberger, 1991; Grossman and Bergman, 1992; Gudmundsson and Hafsteinsson,  
8 1997). The main difficulties in using fish skin to produce gelatin lies, on the one hand,  
9 in the dark color and strong odor of the skin of most fish species, and, on the other hand,  
10 in that gelatin gels are less stable, as they melt at a lower temperature. Nonetheless,  
11 there is considerable interest in the use of fish skins, for two reasons: utilization of by-  
12 products, and, from a socio-cultural standpoint, as an alternative to mammalian gelatin,  
13 whose consumption is rejected in some cultures. Several marine species have been  
14 examined as a source of raw material for edible gelatin, such as tilapia (Grossman and  
15 Bergman, 1992), cod (Gudmundsson and Hafsteinsson, 1997), lumpfish (Osborne et al.,  
16 1990), or conger eel and arrow squid (Kim and Cho, 1996), but no information is  
17 available for a flat fish such as megrim (*Lepidorhombus boscii*), which frequently is  
18 deskinning and increasingly commercialized as fresh or frozen fillets.

19         The objective of this study was to obtain an edible gelatin from fish skin with  
20 good functional properties, comparable to mammalian gelatins. For this purpose various  
21 physicochemical collagen and gelatin extraction methods were examined.

22

## 23 **MATERIALS & METHODS**

24 Fresh (whitin 18-24 hours in ice after capture) megrim (*Lepidorhombus boscii* (Risso))  
25 and hake (*Merluccius merluccius*, L.) skins were obtained from a local fish shop in

1 Madrid, and were immediately stored at  $-20^{\circ}\text{C}$  until use. All reagents used were  
2 analytical grade.

3

#### 4 *Cleaning of fish skins*

5 Thawed skins (0.65 kg) were washed with tap water (1:6 w/v) in a Stephan machine  
6 (position II) (Model UM5; Stephan und Söhne GmbH & Co., Hameln, Germany) at  $5^{\circ}\text{C}$   
7 for 10 min, and were rinsed with abundant running tap water. Skins were further  
8 cleaned with 0.8 N NaCl (1:6 w/v), again in the Stephan (position I) at  $5^{\circ}\text{C}$  for 10 min,  
9 and again were rinsed with abundant running tap water. This step was repeated three  
10 times. Excess water was removed by draining the cleaned skins and manual squeezing.  
11 This process was used for all the extraction procedures described below, except MG1  
12 and HG, where washing with NaCl was omitted.

13

#### 14 *Collagen extraction*

15 Cleaned skins (white megrim side) were stirred with acetic acid 0.5 N (1:6 w/v) in the  
16 Stephan (position I) at room temperature for 20 min and then chopped for 2 min in an  
17 Omni-mixer (model 17106; Omni-International, Waterbury, CT, USA). The resulting  
18 homogenate was filtered in a Büchner funnel with Whatman no. 4 filterpaper  
19 (Whatman, Maidenstone, England) and the filtrate air-dried in a convection oven at 40-  
20  $42^{\circ}\text{C}$  until moisture was less than 15%. This collagen sample was called **EC** (total  
21 extracted collagen). Soluble collagen (**SC**) was obtained in the same manner as EC but  
22 with a centrifugation step at  $10,000 \times g$  in a Beckman J2-MC centrifuge (rotor JA 14)  
23 (Beckman Instruments Inc., Palo Alto, CA, USA) for 45 min, after which the precipitate  
24 was removed. Soluble collagen in 0.15 N citric acid (**SCcit**), instead of 0.5 N acetic  
25 acid, was obtained following the same procedure described for SC. Freeze-dried

1 extracted collagen (**LEC**) and soluble collagen (**LSC**) were obtained by freeze-drying  
2 instead of air-drying. Heated soluble collagen (**SCh**) and heated extracted collagen  
3 (**ECh**) were obtained by a heating step (55°C for 30 min) prior to air-drying.

#### 4 *Gelatin extraction*

6 Gelatin (**MG1**) was basically obtained following the procedure described by  
7 Gudmundsson and Hafsteinsson (1997): washed dark megrim skins were stirred with  
8 cold (2°C) 0.2 N sodium hydroxide (1:6 w/v) for 40 min (repeated three times), and then  
9 with 0.2 N sulphuric acid with the same conditions (repeated three times). Samples were  
10 drained and rinsed with tap water after each step. Skins were then treated with 0.7%  
11 citric acid for 40 min with continuous stirring and after that extracted with distilled  
12 water overnight at 45°C. The mixture with the remains of the skins was then filtered in a  
13 Büchner funnel with Whatman no. 4 filter paper and the clear filtrate was then air-dried  
14 in a convection oven at 40-42°C until moisture was less than 15%. Exactly the same  
15 extraction procedure was used for hake skin gelatin extraction (**HG**).

16 A modification of this extraction method was used to improve physical properties of  
17 gelatin (**MG2**): cleaned skins (with 0.8 N NaCl and rinsed with abundant running tap  
18 water) were treated with 0.2 N NaOH (1:6 w/v) at 5°C for 30 min with constant stirring,  
19 and again were rinsed with abundant running tap water (repeated three times). Skins  
20 were caused to swell with 0.05 N acetic acid (1:10 w/v) at room temperature for 3 hr,  
21 rinsed with tap water and then extracted with distilled water overnight at 45°C.  
22 Subsequent processing was as described for MG1.

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2 *Gel strength*

3 Gel strength was determined on a 6.67% gel (w/v), formed by dissolving the dry  
4 powder in distilled water at 60°C, and cooling the solution in a refrigerator at 7°C  
5 (maturation temperature) for 16-18 hr. Gel strength at 8-9°C was determined on an  
6 Instron model 4501 Universal Testing Machine (Instron Co., Canton, MA, USA) with a  
7 load cell of 5 kN, cross-head speed 1mm/s, equipped with a 1.27-cm-diameter flat-faced  
8 cylindrical Teflon<sup>®</sup> plunger. Maximum force (in g), taken when the plunger had  
9 penetrated 4 mm into the gelatin gels, are averages of five determinations.

10

11 *Viscoelastic properties*

12 Dynamic viscoelastic studies were performed on a Bohlin CSR-10 rheometer rotary  
13 viscometer (Bohlin Instruments Ltd., Gloucestershire, UK) using a cone-plate geometry  
14 (cone angle 4°, gap=150 mm). Cooling and heating from 50 to 5°C and back to 50°C  
15 were performed at a scan rate of 0.5°C/min, frequency 1 Hz, and oscillating applied  
16 stress 3.0 Pa. Dry powder was dissolved at 45°C (at 6.67% concentration) just before  
17 the start of the test. The melting temperature was taken as the point at which the phase  
18 angle peaks immediately after a sharp increase. Setting time (gel onset time) was  
19 determined as the time in minutes elapsing between last temperature of maximum phase  
20 angle and first temperature of minimum phase angle (gelling point)). In order to allow  
21 suitable gelling in all samples studied, reference  $G'$  and  $G''$  (Pa) values were taken at  
22 5°C to compare characteristics at a standard temperature. Results were averages of four  
23 measurements. The error in the reproducibility of the parameters considered in different  
24 determinations of a single sample was 6% or less.

25

### 1 *Apparent viscosity*

2 Apparent viscosity (at 6.67% concentration) was performed in the cone-plate cell of the  
3 Bohlin rheometer at 60, 40, and 25°C at a constant shear stress of 3.0 Pa. Results are  
4 averages of five determinations and were expressed as cPa.

5

### 6 *Amino acid composition*

7 For the analysis of amino acids, the dry powders were reconstituted with distilled water  
8 at 1mg/mL. Approximately 50µg of sample was treated by acid hydrolysis (HCl 5.6N)  
9 at 108°C for 18h, and injected into a Beckman 6300 analyzer (Beckman Instruments  
10 Inc., Palo Alto, CA, USA). Imino acid content was expressed as the number of residues  
11 of Pro + Hyp per 1000 residues. Hydrophobic amino acid content was expressed as the  
12 sum of residues of Ala + Val + Leu + Iso + Pro + Phe + Met per 1000 residues.

13

## 14 **RESULTS & DISCUSSION**

15 Megrim is a flat fish with a light-colored skin on one side. This can be used for  
16 extraction of collagen, so that after the drying process a whitish powder is obtained.  
17 This collagen dry powder is converted into gelatin by dissolving in water at  
18 temperatures above 42°C. A comparison was made of different procedures based on the  
19 extraction of fish gelatin or its precursor, collagen. Fig. 1 shows the dynamic  
20 viscoelastic properties of the different materials: modulus of elasticity ( $G'$ ), modulus of  
21 viscosity ( $G''$ ) and setting time are plotted as a function of melting temperature.

22       Regarding the collagen extraction procedures, SCcit exhibited the lowest values  
23 for melting temperature,  $G'$ ,  $G''$ , and setting time. This was mainly attributed to the  
24 poor extraction capacity of citric acid at 0.15 N concentration (dilute citric acid is  
25 commonly used for pretreatment in gelatin manufacturing) as compared to 0.5 N acetic



1 acid, which was used in all other collagen extractions. As reported by Norland (1990), it  
2 is difficult to dissolve collagen in dilute acid since the molecules are covalently cross-  
3 linked into fibrils that may swell, but do not dissolve completely. This means that yield  
4 is low and very-high-molecular-weight components remain, which are known to reduce  
5 the setting time (Johnston-Banks, 1990). It is this highly covalent cross-linked fraction  
6 that is removed by centrifuging the total extracted collagen (EC) to obtain the soluble  
7 collagen or procollagen fraction (SC). The latter is transparent and after drying is more  
8 readily dissolved than EC.

9         However, as regards the viscoelastic properties, no great differences were found  
10 between the two lots. The reason for this may be that such covalent cross-links are  
11 normally located at the end of the chain (telopeptide zone), whereas it is the imino acid-  
12 rich regions, distant from the terminal zones, which form the stable junctions that  
13 determine the melting point and the  $G'$  (Ledward, 1992). Moderate heating at 55°C,  
14 which was carried out to convert the total extracted collagen and the soluble collagen  
15 into gelatin prior to drying (ECh and SCh, respectively), clearly reduced  $G'$  and  $G''$  in  
16 both cases, but there was not the same definite effect on melting temperature. Such  
17 heating favors the collagen molecule helix-to-coil transition and helps to obtain a more  
18 soluble and transparent product, as reported by Norland (1990). Freeze-drying of the  
19 soluble collagen (LSC) and the total extracted collagen (LEC) produced an increase in  
20 the viscoelastic properties as compared with the corresponding samples air-dried at  
21 45°C. A positive effect from freeze-drying has also been reported elsewhere  
22 (Gudmundsson and Haffsteinsson, 1997). The air-drying process at such relatively high  
23 temperature could have produced partial breakdown of the collagen or, on occasions,  
24 partial conversion into gelatin, which is detrimental to physical properties.

1 With a direct gelatin extraction, both megrim and hake gelatins (MG1 and HG,  
2 respectively), pre-treated with diluted citric acid, were colorless, transparent, and  
3 odorless, but their melting temperature and G' values were very low compared to  
4 collagen extractions in 0.5 N acetic acid. The melting temperature and setting time for  
5 HG were slightly higher than for MG1. In megrim gelatin extraction MG2, a mixed  
6 procedure was used. This consisted of an alkaline pre-treatment with diluted NaOH (to  
7 eliminate the strong fishy odor and raise the pH), pre-extraction of collagen with 0.05 N  
8 acetic acid (to efficiently denature the collagen triple helix) and gelatin extraction in  
9 heated water at 45°C (to favor controlled hydrolysis of the opened helices and release of  
10  $\alpha$ - and  $\beta$ -chains). This method allows the use of white or dark pigmented skins with  
11 around 10% yield (42% on a dry basis), and eliminates the strong odor from the fish  
12 skins and the acetic acid, giving a high degree of transparency, and easy dissolution.

13 MG2 had a higher melting temperature and G' value than any of the collagen  
14 samples. The lime pre-treatment may produce a reduced molecular size through slight  
15 hydrolysis of the polar regions. Stabilization of the poly-L-proline helix is more likely  
16 to occur primarily by intermolecular rather than intramolecular associations, which thus  
17 increases rheological properties (Ledward, 1986). The setting time was reduced, and  
18 was similar to MG1. This is attributed to the relative similarity of the two procedures,  
19 given that the main extraction was done by heating in water overnight, favoring the  
20 helix-to-coil transition typical of normal gelatin extraction (Djabourov et al., 1993).

21 The other cases mainly involved collagen, although by dissolving the powder at  
22 45°C some of it was successfully converted to gelatin. The acid pre-treatment is the  
23 main point of difference between the two extraction processes: MG1 was treated with  
24 citric acid for 40 min, and MG2 with acetic acid for a longer time (3 hr). Both, type of  
25 acid used and time of swelling, appeared to be decisive for the physical properties.

1

2           As shown in Fig. 2, the viscosity of LSC at 25°C was much greater than the rest  
3 of the samples. This is because LSC was freeze-dried, pure SC in which the temperature  
4 had at no point exceeded 7-8°C, and which largely retained its original triple helix. The  
5 viscosity of LEC at 25°C was considerably lower than LSC, probably due to the  
6 presence of the highly cross-linked insoluble collagen fraction. It is precisely because of  
7 the lack of this fraction in the SC sample that benefits more than EC by freeze-drying as  
8 compared to air-drying.

9           However, when SC was heated at 55°C prior to drying (SCh), viscosity was  
10 greatly reduced, whereas this did not occur in the case of total extracted collagen. This  
11 indicates that, for the production of gelatin, it is better to retain the insoluble fraction of  
12 collagen. The viscosity of LSC decreased sharply at 40°C and 60°C. At such high  
13 temperatures the SC becomes denatured, losing its triple helix to become gelatin. At  
14 60°C, LEC had the highest viscosity of all, a fact attributed to a higher proportion of  
15 cross-linked collagen that is not susceptible to thermal hydrolysis. Gelatins MG1, HG,  
16 and MG2 were the least viscous at all experimental temperatures, the rest consisting  
17 mainly of collagen. These gelatins were subjected to an alkaline pre-treatment, and the  
18 combination of this with the heating for extraction almost certainly reduced the number  
19 of inter-chain covalent linkages, leading to reduced viscosity (Johnston-Banks, 1990).

20

21           Gelatin MG2 registered the highest gel strength (Fig. 3), even though it had the  
22 lowest viscosity at 60°C. This is not surprising given that when gel strength is measured  
23 at a temperature below 10°C, the many short-chain peptides present in such low-  
24 viscosity gelatins tend to strengthen the gel (Johnston-Banks, 1990).

1 Values were significantly higher in freeze-dried collagen than in the  
2 corresponding air-dried collagen samples. The effect of heating at 55°C, to convert  
3 collagen into gelatin prior to drying, was only apparent in the SC samples, where there  
4 was a clear fall in SCh with respect to SC. This confirms that the insoluble fraction of  
5 collagen should not be removed to produce gelatin.

6 The MG1 extraction procedure applied to hake skin (HG) produced a gelatin  
7 with lower gel strength, even though it had the highest melting temperature. There may  
8 be inter-species differences in amino acid composition which would influence the gel  
9 properties. The stability of collagen and gelatin has been reported to be proportional to  
10 the total content in pyrrolidine imino acids, given that it is the Pro+Hyp rich zones of  
11 the molecules that are most likely to be involved in the formation of junction zones  
12 stabilized by hydrogen bonding (Ledward, 1986; Johnston-Banks, 1990). Fig. 4 shows  
13 the proportion of imino acids (Pro + Hyp) and hydrophobic amino acids in samples  
14 produced by the most representative extraction procedures. Imino acid content (Fig. 4a)  
15 did not differ in the various megrim extractions and was slightly higher than in the hake  
16 gelatin (HG). The hake gelatin contained more hydrophobic amino acids than the  
17 megrim gelatin (MG1) when extracted in the same conditions (Fig. 4b). At the same  
18 time, gel strength and viscosity at 25°C in HG were lower than in MG1, and melting  
19 temperature and setting time were slightly higher.

20 Of the megrim samples, LEC (cold-extracted) had a higher proportion of  
21 hydrophobic amino acids than MG1, ECh, or SCh. Imino acid and hydrophobic amino  
22 acid composition, therefore, influenced the physical properties of gelatins from different  
23 species; however, within the same species, the extraction conditions influenced these  
24 properties through changes in hydrophobic amino acid composition rather than imino  
25 acid.

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## CONCLUSIONS

A high-quality, readily-dissolved gelatin was obtained from megrim skins using a pre-treatment of the skins with NaCl and diluted NaOH, then swelling with acetic acid followed by an extraction step in water at 45°C. It is possible to prepare a dry powder of soluble collagen with good viscoelastic and gelling properties, which can be converted into gelatin by dissolving at temperatures above 45°C. Moreover, elimination of the highly cross-linked insoluble fraction of collagen heightens the benefit of freeze-drying as opposed to air-drying. However, to manufacture the best gelatin, the insoluble collagen fraction should not be removed.

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12 composition.

13

## 1 LEGEND TO FIGURES

2  
3 Fig. 1.- Elastic modulus (a) and viscous modulus (b), measured at 5°C, and setting time  
4 (c) plotted as functions of melting temperature of both gelatin and collagen samples.

5 SCcit: soluble collagen extracted in 0.15 N citric acid; SC: soluble collagen extracted in 0.5 N acetic acid;

6 EC: total extracted collagen in 0.5 N acetic acid; SCh: soluble collagen extracted in 0.5 N acetic acid and

7 heated at 55°C; ECh: total extracted collagen in 0.5 N acetic acid and heated at 55°C; LSC: lyophilized

8 soluble collagen extracted in 0.5 N acetic acid; LEC: lyophilized total extracted collagen in 0.5 N acetic

9 acid; MG1: gelatin pretreated with citric acid 0.7% for 40 min; HG: gelatin from hake skin pretreated

10 with 0.7% citric acid for 40 min; MG2: gelatin pretreated with 0.05 N acetic acid for 3 hr.

11  
12 Fig. 2.- Apparent viscosity measured at 25, 40, and 60°C of gelatin and collagen  
13 samples.

14 Sample identification as described in Fig. 1. Bars represent standard error.

15  
16 Fig. 3.- Gel strength of gels made of gelatin and collagen samples, matured at 7°C for  
17 16-18 hr.

18 Sample identification as described in Fig. 1. Bars represent standard error.

19  
20 Fig. 4.- Imino acid (a) and hydrophobic amino acid (b) composition of several gelatin  
21 and collagen samples

22 SCh: soluble collagen extracted in 0.5 N acetic acid and heated at 55°C; ECh: total extracted collagen in

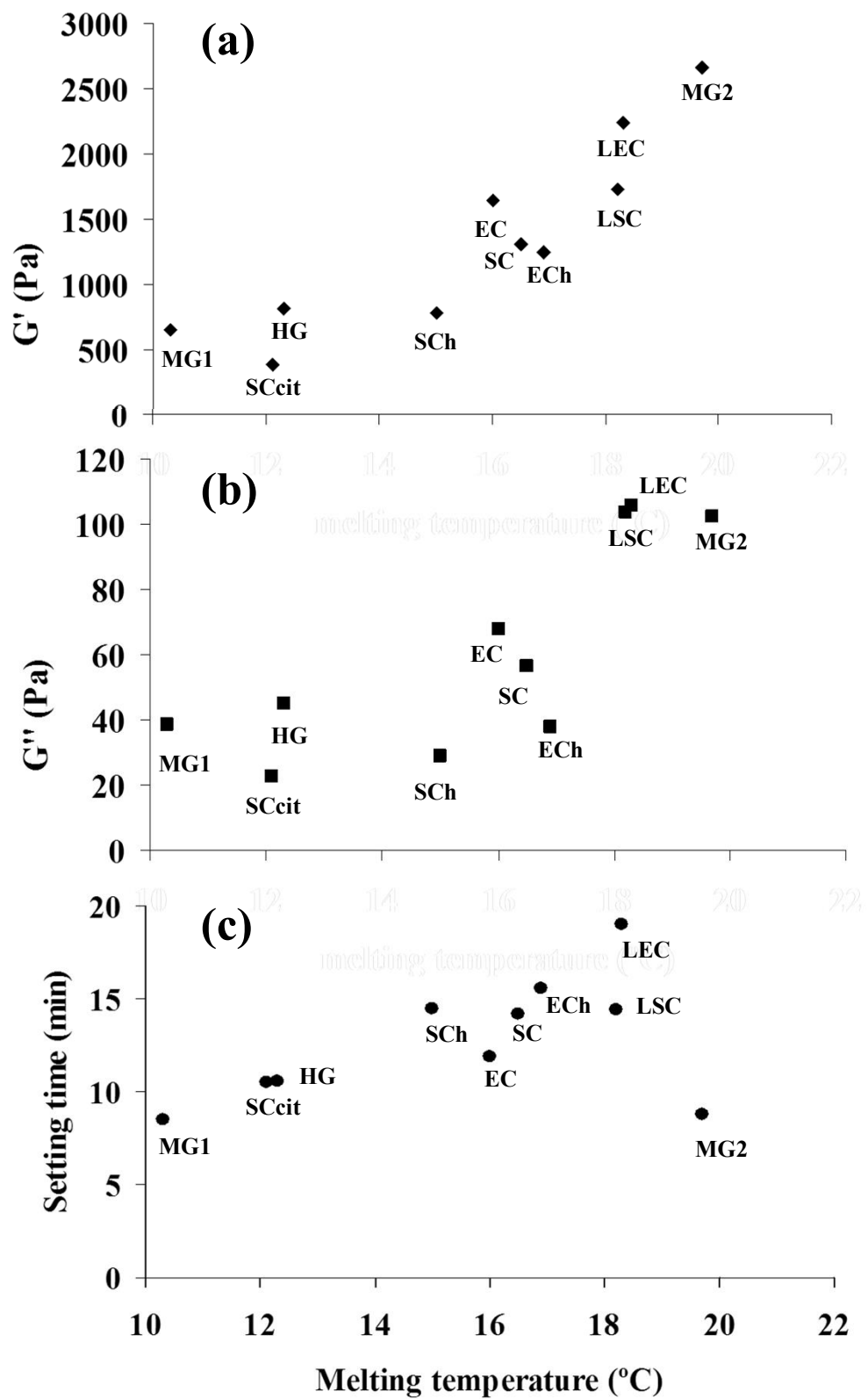
23 0.5 N acetic acid and heated at 55°C; LEC: lyophilized total extracted collagen in 0.5 N acetic acid; MG1:

24 gelatin pretreated with citric acid 0.7% for 40 min; HG: gelatin from hake skin pretreated with 0.7% citric

25 acid for 40 min.

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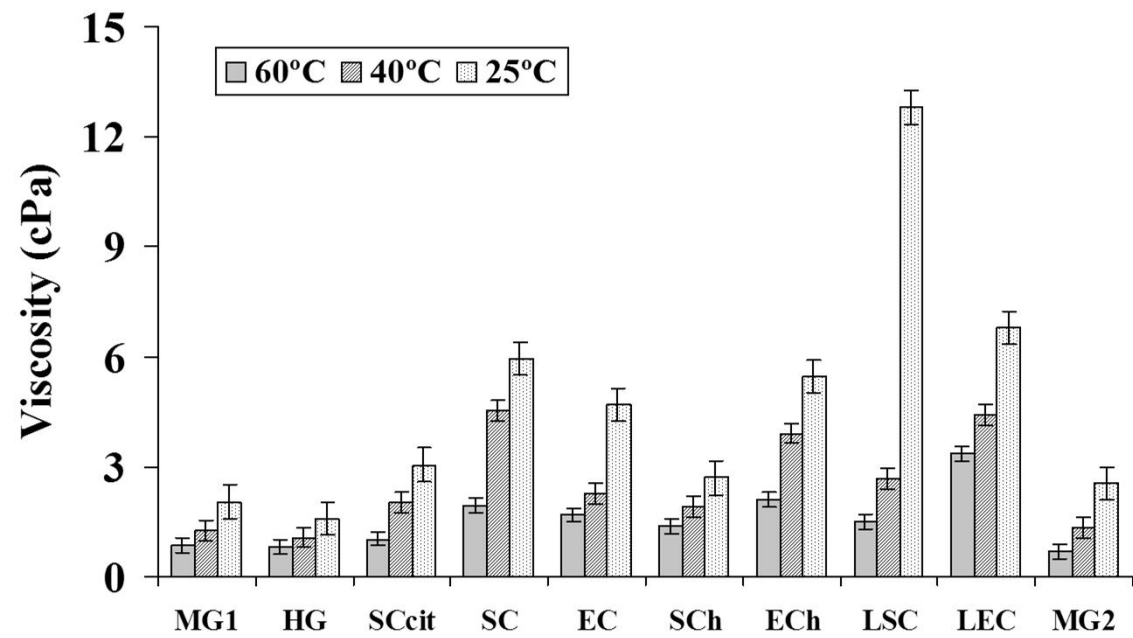


Fig. 2

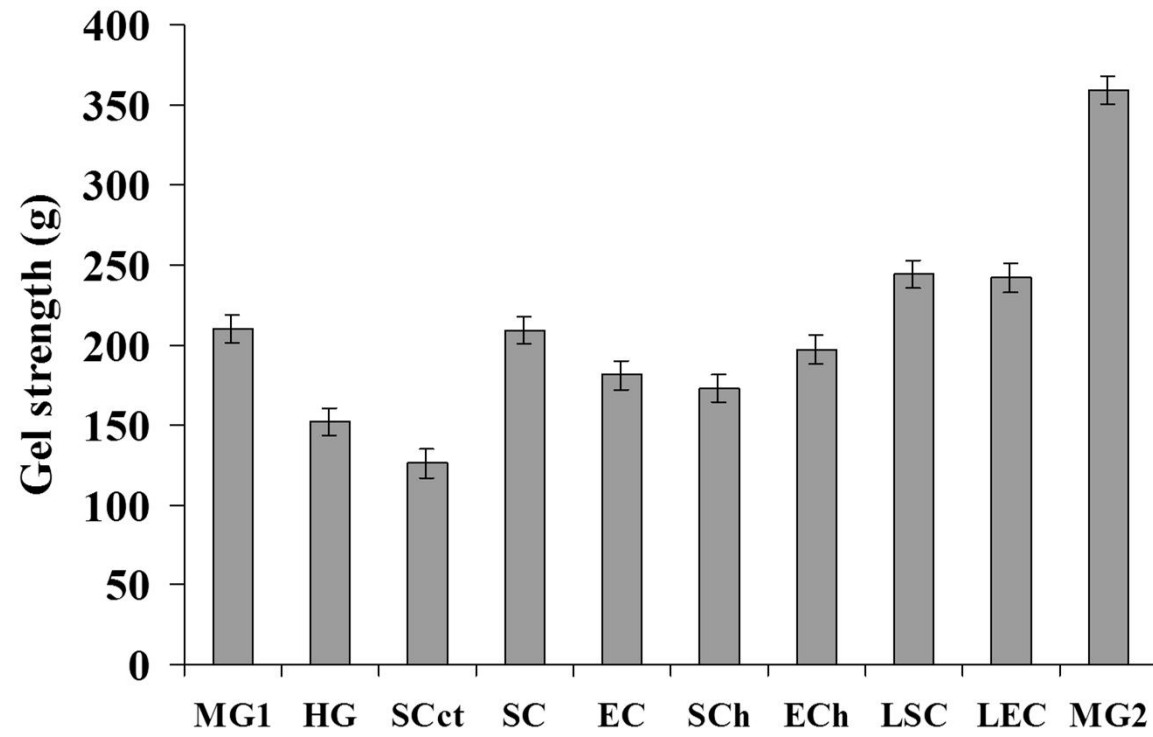


Fig. 3

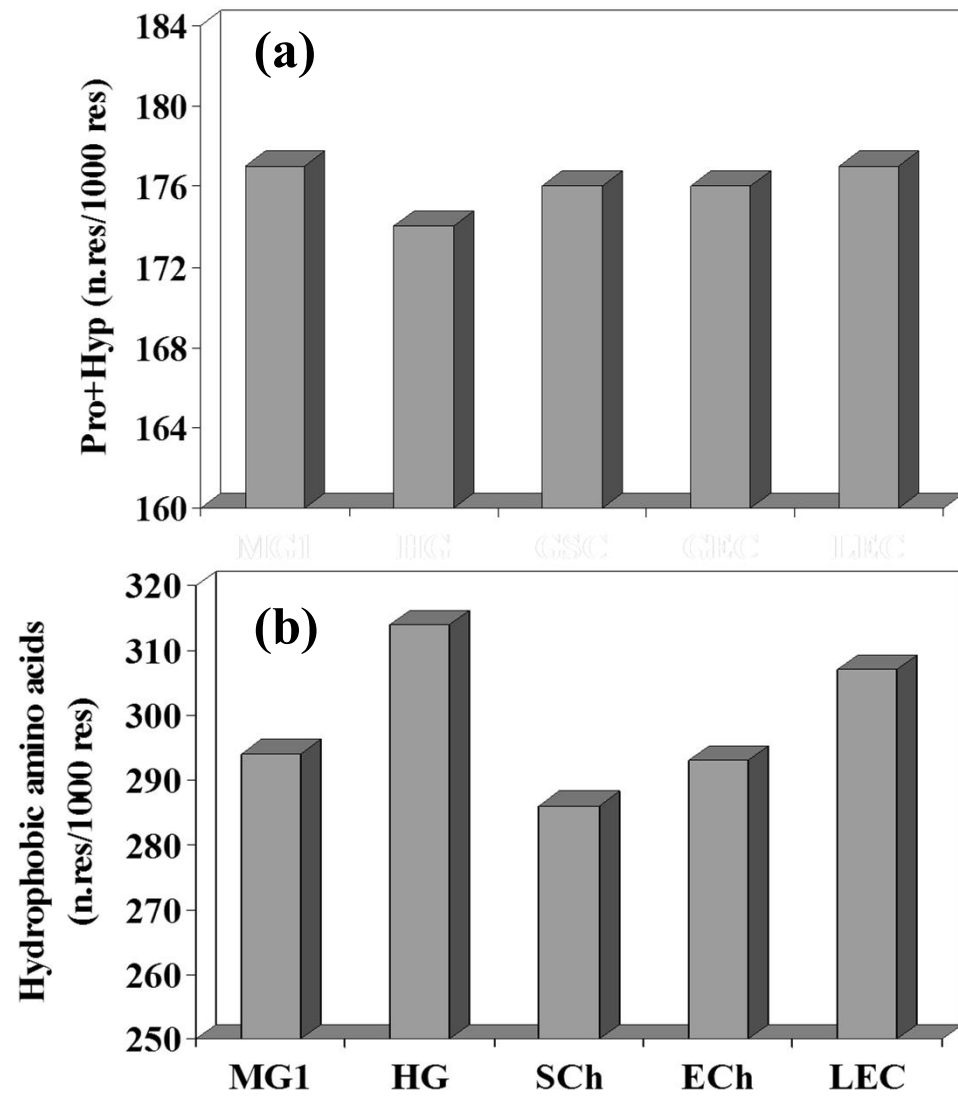


Fig. 4