EXTRACTION OF GELATIN FROM MEGRIM (*Lepidorhombus boscii*) SKINS WITH SEVERAL ORGANIC ACIDS

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ABSTRACT

Light-colored dry collagen was obtained which, after dissolving in warm water, turned into soluble gelatin. The type of acid used influenced the gelatin viscoelastic and gelling properties. Acetic and propionic acid extracts produced the gelatins with the highest elastic modulus, viscous modulus, melting temperature, and gel strength, especially when skins were previously treated with dilute NaOH. After such treatment, lactic acid was also shown to be suitable for collagen or gelatin extraction. The lowest degree of turbidity was achieved by using citric acid, whereas propionic acid led to the most turbid gelatin. No improvements of rheological properties were observed when acid concentration for extraction was increased above 0.05 M.

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

INTRODUCTION

The term "gelatin" is applied to a series of food protein products derived by hydrolysis of animal collagen, contained in bones and skins. Although gelatins from beef and pork have been widely studied, less work has been published on extraction procedures and functional properties of gelatins from cold-blooded animals such as fish (Norland 1990; Osborne and others 1990; Leuenberger 1991; Grossman and Bergman 1992; Kim and Cho 1996; Gudmundsson and Hafsteinsson 1997). To convert insoluble native collagen to gelatin, a treatment is required which will break noncovalent bonds so as to disorganize the protein structure, thus producing adequate swelling and cleavage of *intra-* and *inter*molecular bonds leading to subsequent collagen solubilization (Stainsby 1987). Because of the acid lability of cross-linking in fish skin collagen (Montero and others 1990, 1995), reasonably mild treatment with acid should be enough to effect solubilization (Norland 1990). Such treatment leads to a type A gelatin with an isoelectric point between approximately pH 6 and 9, which carries a net positive charge in most food uses (Stainsby 1987). Numerous studies on collagen from different species have focused on acetic acid extractions (Gustavson 1956; Sato and others 1987; Montero and others 1990, 1995). However, for the manufacture of food grade gelatin from fish, citric acid is widely used, because it does not impart objectionable color or odor to the gelatin (Grossman and Bergman 1992; Gudmundsson and Hafsteinsson 1997). The type of acid used, the ionic strength, and the pH that the acid produces, strongly influence swelling properties and solubilization of collagen. Increasing H^+ ions favors the access of water to the collagen fibers, and this water is held

in by electrostatic forces between charged polar groups (electrostatic swelling), or by hydrogen bonding between uncharged polar groups and negative atoms (lyotropic hydration) (Gustavson 1956). Molecules of high molecular weight may arise in gelatin through the persistence of some of the crosslinks between chains, which can vary depending on the nature of the solubilizing process. Johnston-Banks (1990) briefly compared the effects of several organic acids on the rate of viscosity loss of gelatins, but not in terms of functional properties of gelatin from fish skins.

The purpose of this study was to compare the effectiveness of several organic acids for collagen extraction from fish skins by evaluating viscoelastic and gelling properties of the resultant gelatins. Also, the effect of a previous treatment of skins with dilute NaOH, as a preliminary step in each collagen extraction, was tested.

MATERIALS AND METHODS

Fresh (within 18-24 h in ice after capture) megrim (*Lepidorhombus boscii* (Risso)) skins (length: 22 cm $\pm$ 4 cm; width: 12 cm $\pm$ 3 cm) were obtained from a local fish shop in Madrid; they were immediately frozen and stored at –20 ºC until use (within 5 days of frozen storage). All reagents were of analytical grade.

Cleaning of fish skins

Thawed skins, all coming from the same batch, were washed with tap water (1:6 w/v) in a Stephan homogenizer (position II, very vigorous stirring)

(Model UM5; Stephan und Söhne GmbH & Co., Hameln, Germany) at 5 ºC for 10 min, and were rinsed with abundant running tap water. Skins were further cleaned with 0.8 M NaCl (1:6 w/v), again in the Stephan homogenizer at 5 ºC for 10 min, and were rinsed with abundant running tap water. This step was repeated three times. Excess water was removed by draining the cleaned skins and manual squeezing.

Collagen extraction with different organic acids

Cleaned skins were constantly and slowly stirred for 16-18 h at 20 ºC, with different solutions of 0.05 M, 0.1 M, and 0.5 M of several acids (1:20 w/v): formic, acetic, propionic, lactic, malic, tartaric, and citric acid. The mixture with the remains of the skins was then filtered in a Büchner funnel with Whatman no. 4 (Maidenstone, England) filter paper and the clear filtrate was then air-dried in a convection oven at 40ºC, in the form of very thin layers, until moisture was less than 15%. Extractions with 0.05 M of each acid were also done after a pre-treatment of skins with 0.2 N NaOH (1:6 w/v) at 5 ºC for 30 min with constant stirring, and rinsing with abundant running tap water (this washing cycle was repeated three times).

Preparation of gelatins

The dried collagens, all extracted with the different acids, were dissolved in distilled water at 45 ºC for 30 min, at a concentration of 6.67% (w/v).

Measurement of pH

Determination of the natural pH of the extracted gelatin was performed at 20 ºC, after dissolving the dried collagen in water at 20ºC (6.67% concentration), using a PHM93 pH-meter (Radiometer, Copenhagen, Denmark). When pre-treatment of skins with NaOH was done, the pH of the extracting acid was adjusted to 4.5-5 with 0.2 N NaOH.

Viscoelastic properties

Dynamic viscoelastic studies on the gelatins were performed with a Bohlin CSR-10 rheometer rotary viscometer (Bohlin Instruments Ltd., Gloucestershire, UK) using a cone-plate geometry (cone angle = 4 º; gap = 150 mm). Cooling and heating from 50 to 5 ºC and back to 50 ºC were performed at a scan rate of 0.5 ºC/min, frequency 1 Hz, and oscillating applied stress 3.0 of Pa. Dried collagens were dissolved in distilled water just prior to the start of the test. The melting temperature was taken as the point at which the phase angle (δ) peaks immediately after a sharp increase. Setting time (gel onset time) was determined as the time, in minutes, elapsing between the last temperature of maximum δ and the first temperature of minimum δ (gelling point). The elastic modulus (G') and viscous modulus (G'') (in Pa) were taken at 5 ºC to compare characteristics at a given standard temperature. Results were averages of four runs.

Gel strength

Gel strength was determined on a gelatin gel of 6.67% concentration, formed by dissolving 2.66g of dried collagen in 40 mL of distilled water at 60 ºC.

The dimension of the sample in the container was 6 cm in diameter and 2 cm in height. The solution was cooled in a refrigerator at 7 ºC (maturation temperature) for 16-18 h. Measurements were done at 8-9 ºC with an Instron model 4501 Universal Testing Machine (Instron Co., Canton, Mass., U.S.A.) with a load cell of 5 kN, cross-head speed 1 mm/s, equipped with a 1.27-cm-diameter cylindrical teflon plunger. Maximum force (expressed in grams), when the plunger had penetrated 4 mm into the gelatin gels, are averages of five determinations expressed in grams.

Analysis of turbidity

Turbidity of gelatins was determined by measuring absorbance at 340 nm, using a Spectronic 20D turbidometer (Milton Roy Co., Rochester, NY, U.S.A.), after dissolving the dried collagens for 30 min in distilled water at 60 ºC (at 6.67%).

Statistical analysis

One-way analysis of variance was carried out. The computer program used was SPSS® (SPSS Inc., Chicago, Ill., U.S.A.). The difference of means between pairs was resolved by confidence intervals using a Tukey test. The level of significance was set for $P<0.05$.

RESULTS AND DISCUSSION

Elastic modulus (G'), plotted as a function of melting temperature of soluble gelatin, obtained after dissolving the light-colored dried skin collagens,

extracted with several organic acids at varying concentrations, are shown in Fig. 1. Additional information about other viscoelastic properties is given in Table 1.

Irrespective of the concentration of acid used, there was a strong and positive correlation (R^2 = 0.90-0.99) between G' and the melting temperature of gelatins. With 0.5 M concentration a suitable dried collagen was only achieved with formic, acetic and propionic acids; with all the other acids, the end product was very sticky. This could have been due to strong aggregation during drying as a consequence of excessive ionic strength. With malic and tartaric acids, gelatins showed viscoelastic behavior only when prepared at low concentration (0.05 M), although exhibiting a considerably high phase angle, which denoted very poor gel development. A similar result was obtained in the case of the extraction with lactic acid at 0.1 M. These findings could be explained by assuming that a non-excessive, suitable ionic strength is needed for extraction. Of all the organic acids used, irrespective of the concentration, the highest viscoelastic properties were registered with propionic, acetic, and formic. These are the acids with the smallest molecular size, also having a low ionization constant (*K*), and low ionic strength. Acetic acid, followed by propionic acid and formic acid, was the acid that gave visually the highest swelling capacity, revealed by a considerable increase in thickness and in degree of transparency. Swelling is important because it can favor protein unfolding by disruption of noncovalent bonding and predispose the collagen to subsequent extraction and solubilization (Stainsby 1987). It is known that the acetic acid molecule can cleave hydrogen bonds and become associated with the carboxyl group of the peptide bond (Gustavson 1956). According to Asghar and Henricksson (1982), the lyotropic effect of carboxylic acids on collagen seems to dominate the

swelling capacity, rather than a specific ion effect, since it is the nonionized acid which acts as the swelling agent by competing with the peptide group involved in *inter*molecular linking of the protein chain, mainly due to the hydrogen bonding power of the acid.

Another contributing factors to the different behaviors of the various extracting acids is the pH attained after extraction: a low pH can favor a maximum extraction rate, but is detrimental to the physical properties as it produces more degradation and proliferation of lower-molecular-weight peptides (Johnston-Banks 1990). Fig. 2 shows the modulus of viscosity (G'') and the phase angle as a function of extraction pH, for extractions at 0.05 M acid concentration, which was the unique concentration that allowed a comparison among all. There was a strong and positive correlation (R^2 = 0.91) between G'' and pH values. Propionic acid-extracted gelatin exhibited the highest values of G'', coinciding with a higher pH than the rest. The clear tendency for G'' to decrease at lower pH values would confirm the degrading effect of low-pH extraction, through decreasing viscosity, likely related to an increased number of lower-molecular-weight peptides (Johnston-Banks 1990). The phase angle (δ) was considerably higher with tartaric and malic acids, and slightly higher with citric acid than with the other acids, coinciding again with lower pH values. This confirmed that a weaker gel developed in these samples, with a higher prevalence of the viscous component. The effect of pH also has to be taken into account during the renaturation process: at low pH levels, the excess anions from the added acid screen the positive charges of the protein (Asghar and Henricksson 1982); this effect may inhibit the ability of the chains to come into

suitable juxtaposition for the formation of junction sites during renaturation (Ledward 1986).

Table 2 shows the viscoelastic properties of gelatins from collagen extracted with the various acids at 0.05 M concentration, including a pre-treatment of skins with NaOH. The pH of the extracting acid solution was adjusted to 4.5-5 in all cases where the natural pH of extraction was lower, in order to minimize possible pH effects. The lactic, malic, tartaric, and citric acid samples had to be adjusted. The slight alkaline treatment produced an increase in G', G'', and in the melting point, in all cases and particularly with the more ionized and larger carboxyl chain acids. The increase produced in the case of lactic acid is particularly interesting, given that it is virtually odorless and is widely used in the food industry. The phase angle (δ) was very low in all cases, but especially with acetic and lactic acids, which indicates that a suitable gel was formed, by predominance of the elastic component. The lime pre-treatment of skins may act by reducing protein molecular size through slight hydrolysis of the polar regions (Johnston-Banks 1990). On the one hand, this facilitates lyotropic hydration or swelling of collagen protein by organic acids, especially in the case of the larger-molecular-size acids, improving collagen solubilization. And on the other hand, during collagen renaturation a reduced molecular size would favor *inter*molecular association and would hence facilitate better stabilization of the collagen helix, thus enhancing rheological properties (Ledward 1986), although, as shown in Table 1, it produced a slight delay in setting time.

The effects of the different acids with alkaline pre-treatment of skins were also compared in terms of degree of turbidity of the resultant gelatins (Fig. 3). Gelatin prepared with citric acid showed the lowest absorbance at 340 nm, whereas the one prepared with propionic acid showed the highest and was noticeably turbid.

The gel strength of gelatins obtained from the different extracts, with or without alkaline pre-treatment, after maturation overnight at 7 ºC is shown in Table 3. As already indicated, no improvement was observed when acid concentration was increased, especially with lactic acid, where gelatin attained quite a high gel strength at 0.05 M. Without considering alkaline pretreatment, extractions made with formic acid led to the highest gel strength. This would appear to be connected with the smaller molecular size, which may constitute a lesser obstacle in the correct annealing of protein chains during gel network formation. Alkaline pretreatment of skins before acid extraction produced the highest gel strength in the case of acetic and propionic acid extracts; however, no improvement was observed with formic, citric, lactic or tartaric acids. This indicates that the presumed effect of dilute NaOH in reducing protein molecular size of collagen chains favors gel network formation and stabilization only when acid extraction conditions are optimum for lyotropic swelling, ionic strength, and pH, which is the case with acetic and propionic acid extracts. In extracts with other acids, a number of negative factors converge, such as lower pH, higher ionic strength, and poor swelling during extraction, together with the higher molecular size of the acid, which may inhibit suitable juxtaposition of protein chains during gelation.

Conclusions

It is possible to obtain a light-colored dry collagen extract from megrim skins by solubilizing collagen with constant slow stirring overnight and removing the residual, not solubilized, dark skin. The dried collagen turns into a soluble gelatin after dissolving in warm water. Swelling capacity of collagen, pH of extraction, and ionic strength, which varies depending on the type of acid used, are important for the functional effectiveness of the extraction. Acetic acid and propionic acid produced the highest swelling capacity and pH of extraction, leading to the highest viscoelastic and gelling properties, especially when skins were pretreated with diluted NaOH, and pH was adjusted to 4.5-5. Good results were also obtained by using lactic or formic acid for collagen solubilization. An extremely low pH led to a higher prevalence of the viscous component which were detrimental to gel network development.

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Acknowledgment

This research was financed by the European Comunity under project FAIR CT97.3055, and by the Spanish Comisión Interministerial de Ciencia y Tecnología under project ALI 98 1215 CE.

LEGEND TO FIGURES

Fig. 1.- Elastic modulus (G') measured at 5 ºC, plotted as a function of melting temperature, of gelatins prepared from collagen extracted with different acids at concentrations 0.05M, 0.1M, and 0.5M.

FOR = formic; ACE = acetic; PRO = propionic; LAC = lactic; MAL = malic; TAR = tartaric; CIT = citric.

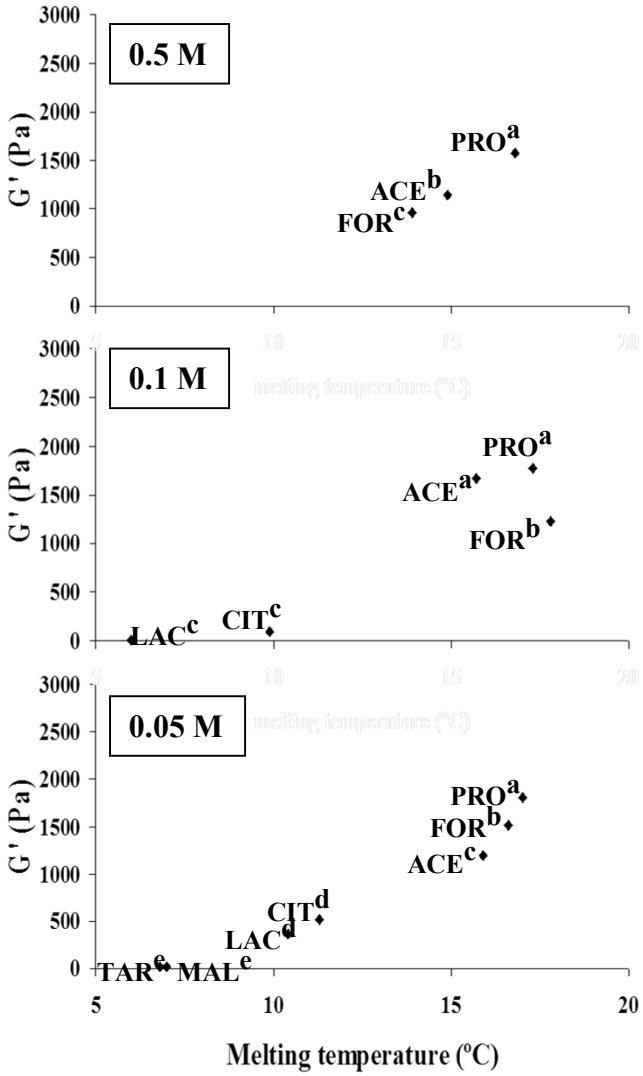
Different letters (a,b,c,...) indicate significant ($P<0.05$) differences in G' among type of acid.

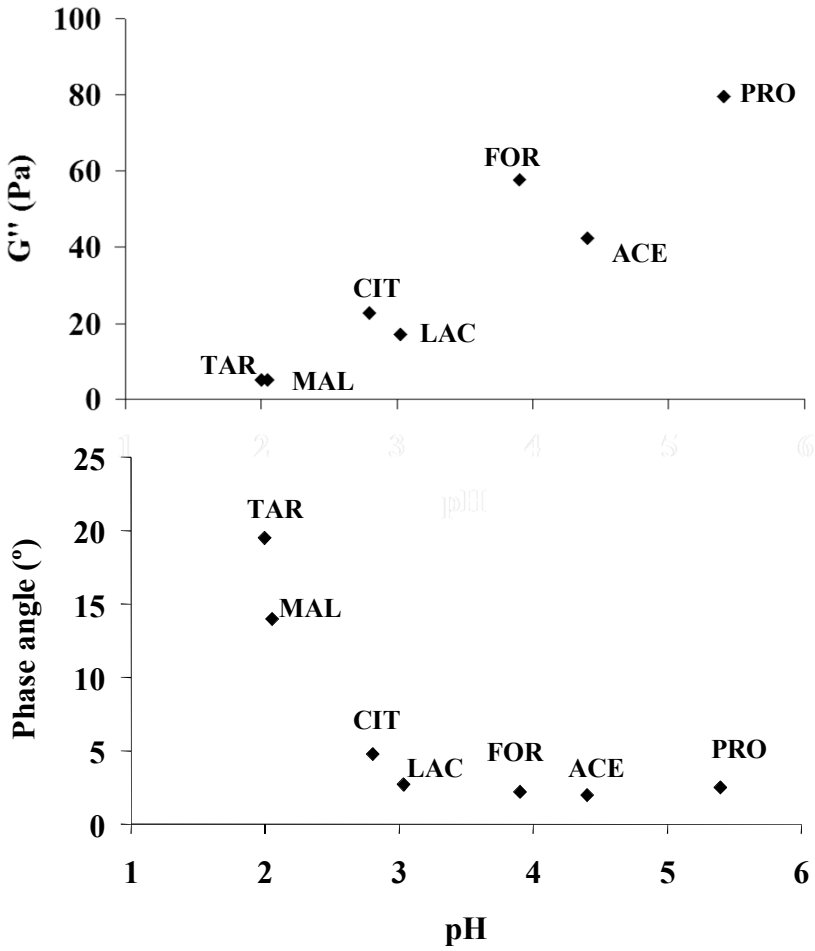
Fig. 2.- Viscous modulus (G'') and phase angle (δ), measured at 5 ºC, plotted as a function of the pH of extraction, of gelatins prepared from collagen extracted with different acids at 0.05 M concentration.

FOR = formic; ACE = acetic; PRO = propionic; LAC = lactic; MAL = malic; TAR = tartaric; CIT = citric.

Fig. 3.- Turbidity (measured as absorbance at 340 nm) of gelatins prepared from collagen extracted with different acids at 0.05 M concentration, with alkaline pre-treatment.

FOR = formic; ACE = acetic; PRO = propionic; LAC = lactic; MAL = malic; TAR = tartaric; CIT = citric.


0.5 M
0.1 M
0.05 M
G ' (Pa)
3000
2500
2000
1500
1000
500
0
PRO a
ACE b
FOR c
ACE a
FOR b
LAC c
CIT c
FOR b
ACE c
CIT d
LAC d
LAC e
TAR e
MAL e
5
10
15
20
Melting temperature (°C)


G'' (Pa)
100
80
60
40
20
0
PRO
FOR
ACE
CIT
LAC
TAR
MAL
Phase angle (°)
25
20
15
10
5
0
TAR
MAL
CIT
LAC
FOR
ACE
PRO
1
2
3
4
5
6
pH

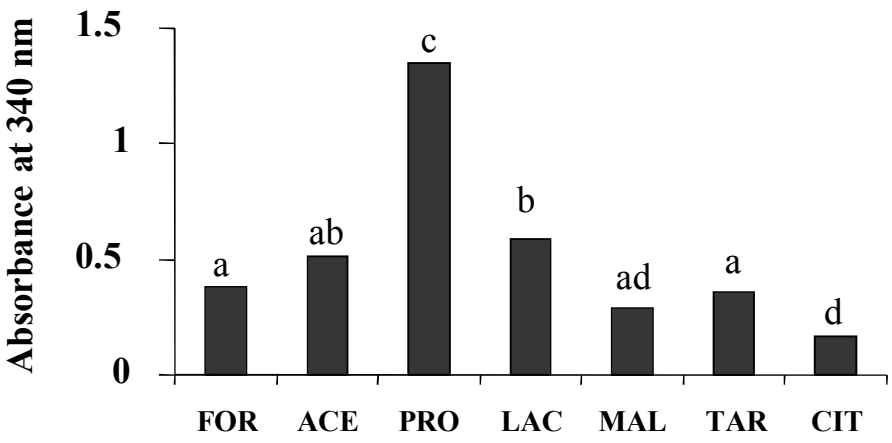

Absorbance at 340 nm
1.5
1
0.5
0
a
ab
c
b
ad
a
d
FOR
ACE
PRO
LAC
MAL
TAR
CIT

Table 2.- Viscoelastic properties of gelatins prepared from collagen extracted with different acids at 0.05M concentration with alkaline pretreatment.

	G' at 5ºC (Pa)	G'' at 5ºC (Pa)	Phase angle at 5ºC (º)	Melting temperature (ºC)	Gelling temperature (ºC)	Setting time (min)
FOR	1414.8^{a}	62.3^{a}	2.5^{a}	18.5^{a}	11.0^{a}	15.0^{a}
ACE	2568.3^{b}	80.5^{b}	1.8^{b}	20.0^{b}	12.5^{a}	15.0^{a}
PRO	2072.4^{c}	87.3^{b}	2.4^{a}	19.1^{ab}	12.1^{a}	14.0^{a}
LAC	1862.7^{c}	57.1^{a}	1.8^{b}	18.0^{ac}	12.2^{a}	11.6^{b}
MAL	834.3^{d}	32.2^{c}	2.2^{ab}	17.4^{ac}	11.2^{a}	12.4^{b}
TAR	698.8^{de}	28.1^{cd}	2.3^{ab}	16.5^{c}	11.5^{a}	10.0^{c}
CIT	405.8^{e}	19.3^{d}	2.7^{a}	15.7^{c}	9.4^{b}	12.6^{b}

FOR=formic; ACE=acetic; PRO=propionic; LAC=lactic; MAL=malic; TAR=tartaric; CIT=citric.
Different letters (a,b,c,d,e) in the same column indicate significant ($P<0.05$) differences.

Table 3.- Gel strength (g) of gelatins prepared from collagen extracted with different acid concentrations (0.05 M, 0.1 M, 0.5 M, and at 0.05M with alkaline pre-treatment).

	Acid concentration			
	NaOH/0.05 M	0.05 M	0.1 M	0.5 M
FOR	211.0 $^{a/x}$	276.1 $^{a/y}$	233.6 $^{a/x}$	188.2 $^{a/z}$
ACE	386.7 $^{b/x}$	144.0 $^{b/y}$	145.0 $^{bc/y}$	121.0 $^{b/y}$
PRO	372.5 $^{b/x}$	168.4 $^{b/y}$	185.7 $^{b/y}$	144.2 $^{b/y}$
LAC	227.2 $^{a/x}$	261.7 $^{a/x}$	110.0 $^{c/y}$	-
MAL	139.5 $^{c/x}$	86.7 $^{c/y}$	34.3 $^{d/z}$	-
TAR	120.9 $^{c/x}$	120.0 $^{b/x}$	16.1 $^{e/y}$	-
CIT	67.3 $^{d/x}$	106.0 $^{bc/y}$	54.0 $^{d/z}$	-

FOR=formic; ACE=acetic; PRO=propionic; LAC=lactic; MAL=malic; TAR=tartaric; CIT=citric.

Different letters (a, b, c,..) in the same column indicate significant ($P<0.05$) differences among type of acid. Different letters (x, y, z) indicate significant ($P<0.05$) differences among acid concentration (including alkaline treatment).

Table 1.- Modulus of viscosity (G"), phase angle (δ), gelling temperature, and setting time of gelatins prepared from collagen extracted with different acids at concentrations of 0.05 M, 0.1 M, and 0.5 M.

	G" at 5ºC (Pa)			Phase angle at 5ºC (º)			Gelling temperature (ºC)			Setting time (min)		
	0.05 M	0.1 M	0.5 M	0.05 M	0.1 M	0.5 M	0.05 M	0.1 M	0.5 M	0.05 M	0.1 M	0.5 M
FOR	$57.6^{a/x}$	$3.6^{a/y}$	$28.0^{a/z}$	$2.2^{a/x}$	$1.7^{a/x}$	$1.7^{a/x}$	$11.3^{a/x}$	$10.1^{a/xy}$	$9.2^{a/y}$	$11.2^{a/x}$	$15.6^{a/y}$	$9.8^{a/x}$
ACE	$42.2^{a/xy}$	$50.2^{b/x}$	$28.8^{a/y}$	$2.0^{a/x}$	$1.7^{a/xy}$	$1.4^{a/y}$	$11.5^{a/x}$	$11.5^{ab/x}$	$10.1^{a/x}$	$8.7^{b/x}$	$8.4^{b/x}$	$9.8^{a/x}$
PRO	$79.4^{b/x}$	$36.6^{b/y}$	$46.3^{b/y}$	$2.5^{ab/x}$	$1.2^{a/y}$	$1.7^{a/y}$	$12.2^{a/x}$	$12.3^{b/x}$	$10.5^{a/y}$	$10^{ab/x}$	$10^{b/x}$	$12.6^{b/y}$
LAC	$17.1^{c/x}$	$3.5^{a/y}$	-	$2.7^{b/x}$	$33.1^{b/y}$	-	6.4^{b}	<5	-	8.8^{b}	*	-
MAL	5.0^{d}	-	-	14.0^{c}	-	-	<5	-	-	*	-	-
TAR	5.2^{d}	-	-	19.5^{d}	-	-	<5	-	-	*	-	-
CIT	$22.7^{c/x}$	$7.8^{a/y}$	-	$2.5^{ab/x}$	$4.5^{c/y}$	-	8.0^{b}	<5	-	10.6^{a}	*	-

FOR=formic; ACE=acetic; PRO=propionic; LAC=lactic; MAL=malic; TAR=tartaric; CIT=citric.

*These samples were not completely gelled at 5ºC.

Different letters (a,b,c,d) in the same column indicate significant ($P<0.05$) differences among type of acid; different letters (x,y,z) in the same row indicate significant ($P<0.05$) differences among concentration of acid within one acid type.