

1 **Introduction**

2 Collagen, from which gelatin is derived, is the major structural protein found in skin
3 and bones of animals. Collagen molecules, composed of three α -chains
4 intertwined in the so-called collagen triple helix, adopt a 3D structure which is an
5 ideal geometry for interchain hydrogen bonding (Johnston-Banks, 1990; te
6 Nijenhuis, 1997). In fact, the hydroxyproline content, through its –OH group, has
7 been reported to be largely responsible for the triple helix stabilisation (Burjandze,
8 1979; Ledward, 1986). There are also very short terminal regions, called
9 telopeptides, which do not form triple helical structure, in which intra- and inter-
10 molecular covalent crosslinks, mainly involving lysine and hydroxylysine residues,
11 are located.

12
13 Because of the acid lability of cross-linking in fish skin collagen, mild acid treatment
14 is normally enough to produce adequate swelling and cleavage of noncovalent
15 intra- and inter-molecular bonds (Stainsby, 1987; Montero, Borderías, Turnay &
16 Lizarbe, 1990; Norland, 1990). Subsequent thermal treatment above 40°C cleaves
17 hydrogen and a number of covalent bonds; this destabilises the triple helix by
18 means of a helix-to-coil transition, leading to conversion into soluble gelatin
19 (Djabourov, Lechaire & Gaill, 1993). High molecular weight polymers may arise in
20 the resulting gelatin through the possible persistence of cross-links depending on
21 the nature and degree of solubilisation.

22
23 High pressure above 150 MPa has been shown to induce protein denaturation by
24 disturbing the balance of the non-covalent interactions that stabilise the native
25 conformation of many proteins (Masson, 1992). However, collagen has been
26 reported to be scarcely affected by high pressure, given that hydrogen bonds are
27 chiefly pressure-insensitive (Gekko & Koga, 1983; Heremans, 1995). At relatively
28 low pressure they could appear to be even somewhat strengthened, due to an
29 associated small reduction in volume (Cheftel & Culioli, 1997). No significant
30 effects by high pressure treatment around 300-400 MPa were found on
31 intramuscular collagen solubility from beef (Suzuki, Watanabe, Ikeuchi, Saito &

1 Takahasi, 1993) or pacific blue whiting (Fernández-Martín, Pérez-Mateos &
2 Montero, 1998). However, changes in bovine collagen solubility have been shown
3 to varied with applied pressure; 200 MPa increased solubility, whereas 400 MPa
4 reduced it (Kwiatkowska, Jankowska & Korzeniowski, 2001).

5
6 Regarding high pressure utilisation in gelatin, there are a few works focused on
7 aspects related to induced gelation (Walkenstrom & Hermansson, 1997; Montero,
8 Fernández-Díaz & Gómez-Guillén, 2002). However, no published articles are
9 available in relation to gelatin extraction assisted by high pressure.

10
11 Gómez-Guillén and Montero (2001) reported a procedure for extracting gelatin with
12 high gelling capacity from fish skins; the procedure was essentially based on a mild
13 acid pre-treatment for collagen swelling followed by extraction in water at moderate
14 temperature (45°C). The entire process takes around 24 hours. The point of
15 applying high pressure in the fish gelatin extraction process is mainly to cut down
16 the treatment times and/or to improve the quality of the gelatin produced. In
17 conventional gelatin manufacturing procedure, high pressure can be applied at two
18 stages: either during pre-treatment in acid at 10°C to facilitate destabilization of
19 acid labile crosslinks, or during extraction in water at 45°C to accelerate collagen
20 hydrolysis.

21
22 The aim of this work was to analyse the yield, molecular weight distribution and
23 viscoelastic properties of gelatin extracted from fish skins using high pressure (250
24 or 400 MPa) either during mild acid pre-treatment at 10°C or during extraction in
25 water at 45°C.

26 27 **Material and methods**

28 29 *Gelatin extraction*

30 Skins from Dover sole (*Solea vulgaris*) were collected and stored frozen at –20°C
31 until use. Cleaning of thawed fish skins and gelatin extraction procedure was

1 carried out as previously described (Gómez-Guillén & Montero, 2000). Briefly, the
2 method consist in a mild acid (50 mM acetic acid) swelling step for 3h and
3 subsequent overnight (16-18h) gelatin extraction in distilled water at moderate
4 temperature (45°C). Extracted gelatin was air-dried until moisture was less than
5 15%. This procedure was considered as conventional treatment and the resulting
6 gelatin was called “control gelatin”, in order to compare with the gelatins obtained
7 by means of high pressure.

8
9 For the pressure-assisted extractions, fish skins were frozen, thawed and cleaned
10 as mentioned above. Pretreatment in 50 mM acetic acid at 10°C was performed
11 during 10 min at 250 MPa, followed by extraction in water at 45°C during 16-18°C
12 at atmospheric pressure (S₂₅₀). Samples extracted in water at 45°C by high
13 pressure were all preceded of a pre-treatment (at atmospheric pressure) in 50 mM
14 acetic acid at 10°C during 3 hours. In substitution of a conventional overnight
15 extraction during 16-18 h, high pressure was applied at 250 MPa for 10 min (E₂₅₀₋
16 10') or 20 min (E_{250-20'}), and at 400 MPa for 10 min (E_{400-10'}) or by two pulses of 5
17 min each (E_{400-2x5'}).

18 19 *Electrophoretic analysis (SDS-PAGE)*

20 Dry gelatin was dissolved in distilled water at 60°C and then 3-fold-concentrated
21 loading buffer containing β-mercaptoethanol was added until reach a final
22 concentration of 2 mg/ml of gelatine, as described previously by Gómez-Guillén *et*
23 *al.* (2002). Protein samples were heat-denatured 5 min at 90°C and analysed by
24 PAGE-SDS according to Laemmli (1970) using 3% stacking gels and 5% resolving
25 gels in a Mini Protean II unit (Bio-Rad Laboratories, Hercules, CA) at 25 mA/gel.
26 The loading volume was 15 µl in all lines. Protein bands were stained with
27 Coomassie brilliant Blue R250. Type I and type III collagens from fetal calf skin
28 (Sigma Aldrich) were used as markers of α-chain, β- and γ-component mobilities.

29 *Viscoelastic properties*

30 Dynamic studies were performed on a Bohlin CRS-10 controlled stress rheometer
31 rotary viscometer (Bohlin Instrumments Ltd., Gloucestershire, U.K.) using a cone-

1 plate geometry (cone angle 4°, gap=0.15mm), according to Gómez-Guillén,
2 Turnay, Fernández-Díaz, Olmo, Lizarbe & Montero (2002). Solutions were
3 prepared by mechanical stirring for 15–20 min of pre-weighed (6.67% w/v) dry
4 gelatin in water at 40°C. Also the samples were cooled in a refrigerator at 7°C
5 (maturation temperature) for 16–18 h overnight to analyse the effect of maturation
6 time on gelatin.

7 Temperature ramps were performed at a scan rate of 1°C/min, frequency 1Hz, and
8 oscillating applied stress of 3.0 Pa. The phase angle (°) was represented as a
9 function of temperature. Values of elastic modulus (G') and viscosity modulus (G'')
10 were taken at 6°C.

11 The ramps were implemented from 40°C to 6°C and back to 40°C for newly
12 dissolved gelatin, to study gelatin gelation and subsequent melting. Gelatin
13 matured overnight (16 –18 h) at 6-7°C was also subjected to temperature ramps
14 from 6°C to 40 and then from 40 to 7°C, to evaluate the effects of maturation time
15 on the gelatin gel.

16 Measurements were done at least in triplicate and results were expressed as mean
17 values and standard deviation.

19 **Results and discussion**

20 The utility of the high pressure treatment was tested at two stages: during acid
21 swelling to facilitate the conversion of the insoluble collagen into a form suitable for
22 subsequent extraction; and during the actual gelatin extraction step in water at
23 45°C. As a control for comparison of the gelatins from different extraction
24 procedures, gelatin was extracted conventionally following the procedure described
25 by Gómez-Guillén and Montero (2000), with a mild acid pre-treatment followed by
26 overnight extraction at 45°C.

28 *Yield of extraction*

29 Pressure-assisted acid swelling was performed at 250 MPa/10 minutes at 10°C
30 (S_{250}), followed by an overnight extraction in water. These conditions were assayed
31 with 50 mM acetic acid as in the control treatment, and also with 10 mM acetic

1 acid. Preliminary results showed that no significant amount of gelatin could be
2 extracted at the low acetic acid concentration, even with 20 min pressurising. All
3 the preliminary collagen swelling (pre-treatment) assays in the extracting step were
4 therefore performed with 50 mM acetic acid.

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6 As Table 1 shows, the yield (expressed as grams of dry gelatin per 100 g of clean
7 skin) was slightly higher when pressurisation was used for collagen pre-
8 solubilization (S_{250}), than in conventional gelatin extraction (control). To the
9 contrary, the yield was considerably lower in all cases when pressurisation was
10 applied in the extraction step. The general impression was that increasing the
11 pressure or prolonging pressurisation tended to reduce the yield. As regards the
12 pressure level, more gelatin was extracted with 250 MPa than with 400 MPa.
13 Prolonging of treatment with 250 MPa from 10 min to 20 min also caused a
14 reduction in the extraction yield. In the case of pressurising at 400 MPa, when
15 samples were pressurised in two consecutive 5-min cycles, the amount of gelatin
16 extracted was only 2% greater than when samples were pressurised continuously
17 for 10 min in the same conditions.

18 19 *Molecular weight distribution*

20 The electrophoretic (SDS-PAGE) profiles of the various gelatin preparations are
21 shown in Fig. 1. Samples extracted with high pressure at 400 MPa ($E_{400-10'}$ and
22 $E_{400-2x5'}$) were characterised by a typical gelatin/collagen type I profile, with a
23 notable amount of α -chains (α_1/α_2 ratio around 2), a considerable presence of β -
24 components (α -chain dimers), and particularly of higher molecular weight polymers
25 including γ -components (α -chain trimers) (Gómez-Guillén et al., 2002). There were
26 noticeably more of these high molecular weight peptides than with the conventional
27 extraction procedure; the reason may be either that high pressure favours more
28 polymer extraction, or else that there is some aggregation during hydrolysis. Both
29 in the swelling step (S_{250}) and in the water extraction step ($E_{250-10'}$), pressurisation
30 at 250 MPa for 10 min led to gelatin preparations in which peptides of $MW \leq 100$
31 kDa predominated and dimers and trimers were practically absent. The

1 electrophoretic profiles and the higher yield of these pressure-assisted gelatin
2 preparations indicate a higher degree of collagen solubilization or hydrolysis. When
3 pressurisation at 250 MPa was prolonged to 20 min (E_{250-20}) during extraction in
4 water, the profile contained appreciably more high molecular weight polymers,
5 which suggests a degree of pressure-induced aggregation.

6 7 *Viscoelastic properties of newly dissolved gelatins*

8 Dry gelatins were dissolved at 40°C. Figure 2 shows changes in the phase angle
9 (δ) of the newly dissolved gelatin preparations (concentration 6.67% w/v) upon
10 cooling to 6°C and subsequent heating. As the cooling ramp shows, the transition
11 curve from solution to gel was rapid and similar in all samples, the only variation
12 being in the gelling temperatures. All samples presented low phase angles at 6°C
13 ($\delta < 5^\circ$), indicating good gelling gelatins. The same was true during subsequent
14 heating; the studied gelatins differed in melting temperature but all presented a
15 sharp melting transition. The onset of gelling and melting occurred at slightly higher
16 temperatures than in the control in gelatins extracted under 400 MPa pressure, but
17 at considerably lower temperatures than the control in gelatins extracted under 250
18 MPa pressure for 10 min (S_{250} and E_{250}). However, transition temperatures were
19 higher when the pressurisation time was prolonged (E_{250-20}). As reported by
20 Johnston-Banks (1990), the increase in the gelling/melting temperatures is directly
21 related to the increase in the average molecular weight of the studied gelatins.

22
23 Table 2 shows gelling and melting temperatures and elastic modulus (G') and
24 viscous modulus (G'') values at 6°C and 40°C. The samples pressurised at 250
25 MPa for 10 min, both during swelling (S_{250}) and during extraction in water (E_{250-}
26 $10'$), presented significantly lower G' and G'' values as measured at 6°C than the
27 other gelatins. In samples pressurised at 400 MPa in two 5-min cycles, viscoelastic
28 properties were again lower than with continuous pressurisation for 10 min. On the
29 other hand, values of G' in 40°C samples were low upon subsequent heating,
30 indicating that the gels were all perfectly thermo-reversible and melted completely.

Viscoelastic properties of overnight cold matured gels

The transition curves of the matured gelatin gels upon melting and subsequent cold renaturation (Fig. 3) again revealed differences among the samples, predominantly in the thermal transition temperatures rather than in the slope. Both melting and gelling temperatures of S₂₅₀ and E_{250-10'} gels were again notably lower than in the others. Also, phase angle values at 6°C were higher in these samples, indicating poorer overall gel forming capacity as a result of cold maturation.

In sample pressurised at 400 MPa for 10 min (E_{400-10'}), the cooling phase angle transition indicated the onset of collagen renaturation at around 20°C, which was considerably higher than with the conventional treatment.

As shown in Table 3, values of G' measured after overnight maturation at refrigeration temperature (around 6°C) in E_{250-20'} and E_{400-10'} gelatin gels were significantly higher than in the control gelatin. Again, all gels were completely melted at 40°C. Upon subsequent cold renaturation, the E_{400-10'} sample attained the highest values of G' and "re-gelling" temperature, which was in fact higher than in the corresponding newly dissolved gelatin. There is clearly a relationship between the effect of pressure and the molecular weight of gelatin fractions. According to Stainsby (1987), the greater presence of α -chain dimers and trimers in these gelatins should make them more susceptible to renaturation to the full native collagen form. In addition, the presence of a significant amount of higher molecular weight components acts also in favour of their slightly higher melting/gelling temperatures (Johnston-Banks, 1990). On the other hand, Sims et al. (1997) indicated that the generation of short triple helices by many interactions of free chains, rather than rapid completion of a longer triple helix, will form a more stable gel. In this sense, the way of triple helix formation upon cooling in E_{400-10'} and E_{400-2x5'} samples having similar molecular weight profile, may explain the difference in their renaturation ability.

High pressure-assisted extraction at 400 MPa for 10 min produced considerably less amount of gelatin than conventional extraction over 16-18h. However, the

1 quality of the gelatin extracted in that short time was high, comparable to that of
2 conventionally- extracted gelatin as reported by Montero and Gómez-Guillén
3 (2000). The molecular weight distribution of the gelatin extracted by high pressure
4 was characterised by a high presence of α -chain, together with β - and γ -
5 components (dimers and trimers of α -chain, respectively), as well as a certain
6 amount of greater polymers. All of them are key factors for high viscoelastic
7 properties, gel strength and melting/gelling temperatures. After maturing overnight
8 at 6-7°C, the modulus of elasticity (G') of the gel from pressure-processed gelatin
9 was greater than that of a conventional gelatin, and the melting temperature was
10 slightly higher. This gelatin also presented greater renaturation ability upon
11 subsequent cooling, but only with continuous extraction ($E_{400-10'}$); when extraction
12 was carried out at 400 MPa in two 5-min cycles ($E_{400-2 \times 5'}$) there was no
13 improvement in renaturation ability even although the molecular weight profile was
14 similar to that of gelatin $E_{400-10'}$, from which we deduce that it is not only the
15 amount but also the configuration of these fragments that matters.

16
17 Pressurising at 250 MPa for 20 minutes produced a gelatin with less amount of
18 dimers and trimers than with 400 MPa, however, viscoelastic properties were
19 similar, and the value of G' after overnight maturation was even higher. With
20 pressurising at 250 MPa, time was a key factor in the quality of the extracted
21 gelatin: with 10 min pressurisation in these conditions, the peptides extracted were
22 largely of relatively low molecular weight and there were insufficient α -chains for
23 proper annealing of collagen molecules upon cooling. Although the yield was
24 higher, this gelatin exhibited considerably poorer gel-forming ability than any of the
25 others, including the control.

26
27 In light of these results, we conclude that the use of high pressure to extract gelatin
28 from fish skins is a useful alternative to the conventional procedure, essentially in
29 that it drastically reduces the duration of the longest part of the process, so that a
30 high-quality gelatin can be produced in a matter of minutes. Successive extractions
31 at atmospheric pressure and increasing temperatures would improve the yield from

1 original raw material. Further studies would be needed to optimise
2 pressure/time/temperature settings so as to increase the yield in the first extraction
3 and in successive extractions from the same original material.

4

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1 **LEGEND TO FIGURES**

2
3 Figure 1.- Electrophoretic analysis of gelatin preparations (SDS-PAGE) in the
4 presence of 2- β -mercaptoethanol. (a) control, (b) S₂₅₀, (c) E_{250-10'}, (d)
5 E_{400-10'}, (e) E_{400-2x5'} and (f) E_{250-20'}.
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7 Figure 2.- Changes in phase angle δ , monitored upon cooling from 40°C to 6°C and
8 subsequent heating from 6°C to 40°C, of newly dissolved gelatins at
9 6.67% (w/v).
10 after overnight maturation at 7°C, phase angle was monitored from 7°C
11 to 40°C and subsequent renatured from 40°C to 7°C (ii).
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13 Figure 3.- Changes in phase angle δ , monitored upon heating from 6°C to 40°C
14 and subsequent cooling from 40°C to 6°C, of gelatin gels (6.67% w/v,
15 16-8 hours maturation at 6-7°C).
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Table 1.- Yield of extraction (expressed
as g dry gelatin per 100 g of cleaned skins

SAMPLES	YIELD OF EXTRACTION (%)
Control	21.3
S ₂₅₀	22.8
E ₂₅₀ -10'	10.2
E ₂₅₀ -20'	4.7
E ₄₀₀ -10'	3.4
E ₄₀₀ -2x5'	5.7

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Table 2.- Viscoelastic properties of newly dissolved gelatins

SAMPLES	Cooling ramp			Heating ramp		
	G' _{6°C}	G'' _{6°C}	g.t	G' _{40°C}	G'' _{40°C}	m.t
	(Pa)	(Pa)	(°C)	(Pa)	(Pa)	(Pa)
Control	2520 ± 63	54.6 ± 1.4	13	0.64 ± 0.02	0.41 ± 0.01	22
S ₂₅₀	868 ± 22	35.6 ± 0.9	9	0.67 ± 0.02	0.31 ± 0.01	18
E ₂₅₀ -10'	731 ± 18	45.9 ± 1.1	8	0.66 ± 0.01	0.35 ± 0.02	17
E ₂₅₀ -20'	2720 ± 68	69.4 ± 1.7	12	0.35 ± 0.01	0.42 ± 0.03	21
E ₄₀₀ -10'	2700 ± 70	63.6 ± 1.6	13	0.54 ± 0.03	0.42 ± 0.02	23
E ₄₀₀ -2x5'	1630 ± 41	35.7 ± 0.9	13	0.47 ± 0.02	0.45 ± 0.02	23

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8 g.t: gelation temperature
9 m.t: melting temperature

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Table 3.- Viscoelastic properties of overnight matured gelatins

SAMPLES	Heating ramp				m.t (°C)	Cooling ramp		
	G' _{6°C} (Pa)	G'' _{6°C} (Pa)	G' _{40°C} (Pa)	G'' _{40°C} (Pa)		G' _{6°C} (Pa)	G'' _{6°C} (Pa)	g.t (Pa)
Control	5240 ± 131	361 ± 9.0	0.72 ± 0.02	0.50 ± 0.02	24	2870 ± 72	61.6 ± 1.5	14
S ₂₅₀	566 ± 14	104 ± 2.6	0.66 ± 0.02	0.30 ± 0.01	20	312 ± 7.8	24.9 ± 0.6	8
E ₂₅₀ -10'	882 ± 22	169 ± 4.2	0.52 ± 0.01	0.33 ± 0.01	20	602 ± 15.5	45.6 ± 1.1	9
E ₂₅₀ -20'	8030 ± 201	413 ± 10.3	0.48 ± 0.02	0.28 ± 0.02	23	3460 ± 86	94.3 ± 2.3	13
E ₄₀₀ -10'	6160 ± 154	318 ± 7.9	0.53 ± 0.03	0.67 ± 0.03	24	4720 ± 118	122.6 ± 3.1	17
E ₄₀₀ -2x5'	5370 ± 134	303 ± 7.6	0.44 ± 0.02	0.60 ± 0.02	24	2680 ± 67	54.9 ± 1.4	15

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10 g.t.: gelation temperature
11 m.t.: melting temperature
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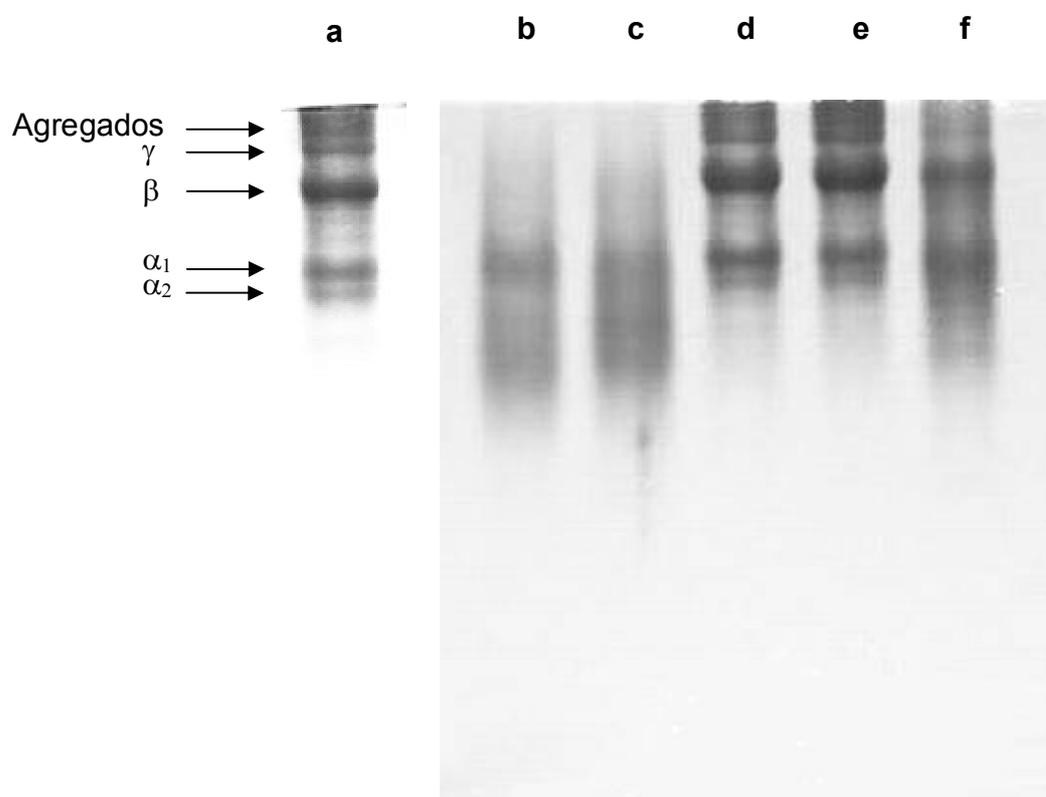


FIG. 1

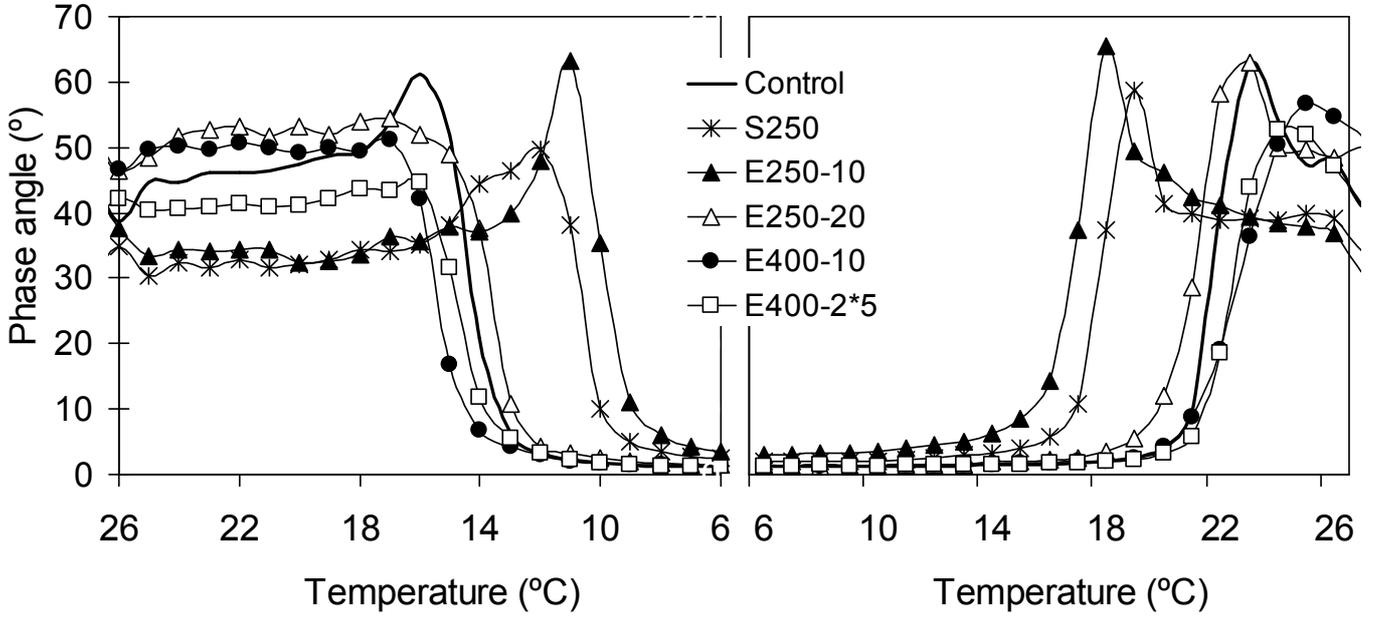


FIG. 2

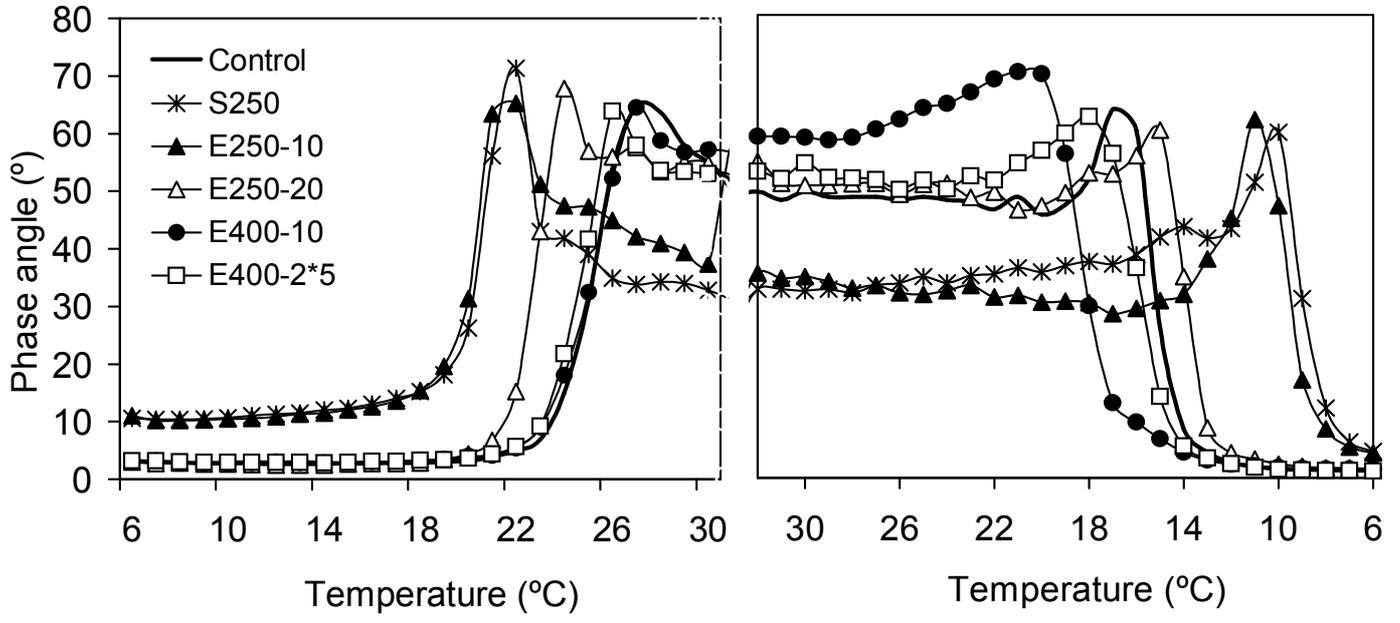


FIG. 3