Utilisation of oleuropein as a crosslinking agent in collagenic films

A.P.M. Antunes[1], G. Attenburrow[1], A.D. Covington[1], J. Ding[2]

[1]British School of Leather Technology, The University of Northampton, Boughton Green Road, Northampton, NN2 7AL, United Kingdom.
[2]Department of Chemistry and Materials, Manchester Metropolitan University, John Dalton Building, Chester Street, Manchester, M1 5GD, United Kingdom

Abstract

The phenolic compound oleuropein, isolated from the olive tree (*Olea europaea* L.), was investigated as a crosslinking agent for collagen. Oleuropein undergoes deglucosidation by the enzyme β-glucosidase to produce an aglycone structure of oleuropein which is the active form. Various parameters such as oleuropein concentration, enzyme concentration and incubation period were investigated. A concentration of 0.2% w/v of the oleuropein and 0.5 U/ml of the enzyme was found to be efficient and rapid under these conditions producing a denaturation temperature of 68.3ºC for the collagen-oleuropein aglycone matrix.

Keywords: Collagen, Crosslinking, Iridoid glycoside, Oleuropein, Thermal Stability.

1. Introduction

The utilisation of collagen as a biomaterial in the medical industry has been well documented in review articles[1,2,3]. Matrices such as collagen films are widely utilised as barrier membranes to prevent infection and/or to act as drug delivery systems for the treatment of wounds[1,2]. Collagen, as a biopolymer, has a distinct advantage over synthetic polymers in that the cell is able to interact with the material. Other advantages include biocompatibility with other biopolymers, biodegradability and low antigenicity. Collagen is usually solubilised and reconstituted to produce gels, films and sponges. Reconstituted collagen has been shown to lack mechanical strength and may fragment easily when manipulated and therefore requires crosslinking. The material may be crosslinked by chemical or physical methods depending on the requirements of the reconstituted product. The chemical methods include agents such as aldehydes, carbodiimides, diisocyanates, epoxides and acyl azides; while physical methods include UV or gamma radiation, dehydrothermal and photo-oxidation[1,2].

These treatments are used to create intramolecular and intermolecular crosslinks in the triple helix to impart strength to collagenic material in order to resist mechanical forces and enzymatic degradation[1,2]. Glutaraldehyde is widely used as a crosslinking agent for biomaterials with collagen as a substrate because it is economical, effective and the reaction rate is rapid, however, glutaraldehyde has been found to be cytotoxic and calcification of implants may occur[2,3]. Alternative crosslinking agents conferring similar thermal and mechanical properties would be advantageous.

Oleuropein, a naturally-occurring iridoid glycoside, isolated from the olive tree (*Olea europaea* L.), is characterised as a secoiridoid glycoside. Iridoid glucosides contain a glucose molecule which is cleaved by the enzyme, β-glucosidase, to produce an aglycone[5]. Iridoid glucosides guard the plant against assault such as infections and herbivorous animals. The resultant aglycone molecule is responsible for the denaturation of proteins as well as being a crosslinking agent[4,5].

The deglucosidation and oxidation of oleuropein produces a poly α, β-unsaturated aldehyde similar in structure to polymerised glutaraldehyde structures. This unsaturated moiety is associated with the irreversible covalent crosslinking ability of the oleuropein aglycone (Figure 1)[4]. Aldehydes are well established as crosslinking agents and have a strong affinity for nucleophilic binding sites such as amino and thiol functional groups[6]. Konno, *et. al.*[4], showed that the crosslinking ability of oleuropein outperformed other secoiridoid glycosides such as aucubin and geniposide.

Studies have also shown oleuropein to have a broad spectrum of antimicrobial activity which may also be attributed to the aglycone form[5], whilst antioxidant activity is thought to be specifically due to the *o*-dihydroxy moiety of the molecule. The degree of efficacy of oleuropein to act as a antimicrobial agent[7,8], antioxidant[9], free radical scavenger[10,11], and anti-tumour agent[12] is dependent on the cultivar, ripening, development and processing of the fruit[13].

An investigation of the ability of the aglycone form of oleuropein to crosslink collagen was undertaken. Biochemical and chemical properties of the crosslinked material were measured.

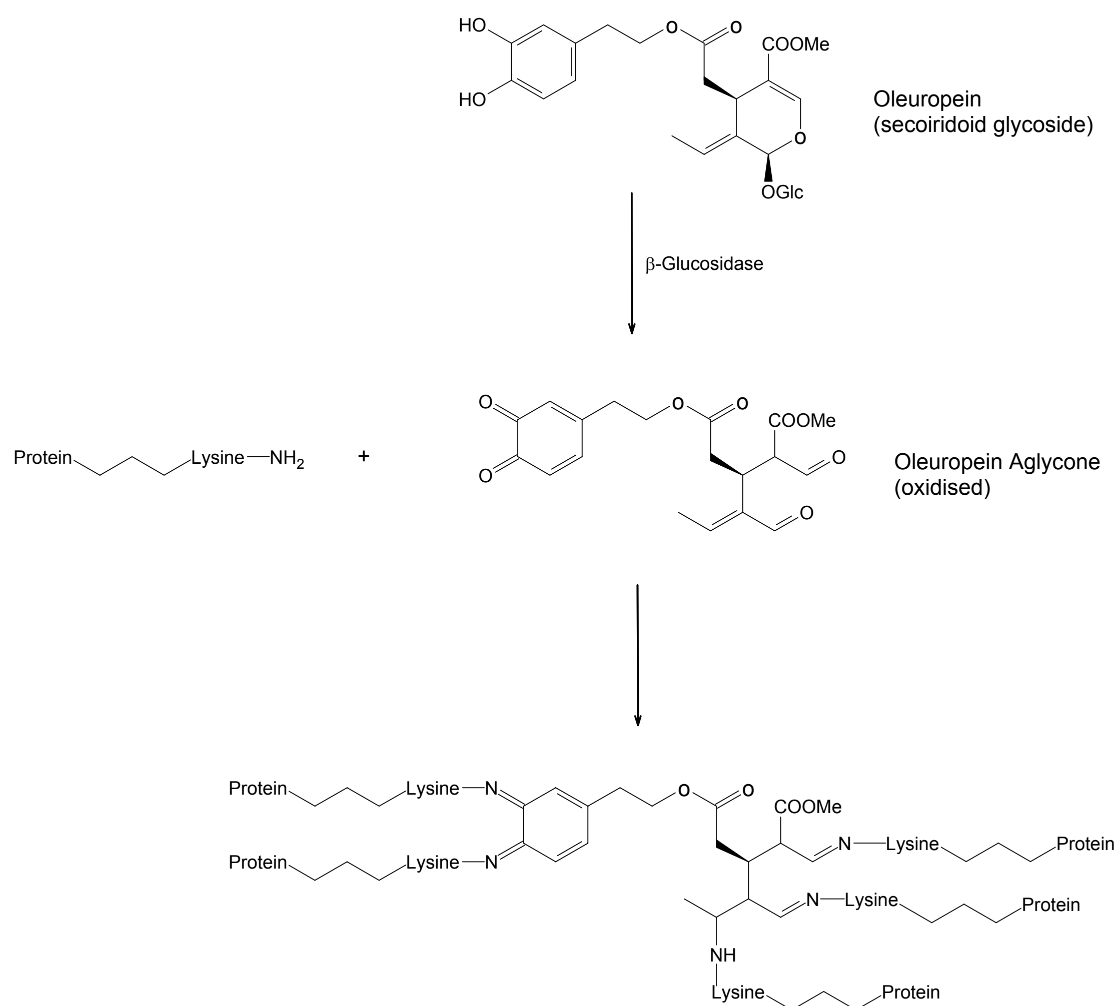

Figure 1: Schematic representation of the reaction mechanism thought to be involved in the activation of oleuropein to the aglycone form which reacts Lysine residues on proteins[1,14].

2. Materials and methods

2.1. Chemicals

Type I collagen was isolated and purified from bovine Achilles tendon (BAT). All chemicals used were of analytical grade. Sodium hydrogen carbonate ($NaHCO_3$), acetic acid (HAc) and sodium chloride (NaCl) were obtained from Scientific Laboratory Supplies Limited, UK. All other chemicals were obtained from Sigma-Aldrich, USA, unless otherwise specified. Oleuropein (crude) was kindly donated by Dr.-Ing. Günther Laufenberg, University of Bonn, Bonn, Germany.

2.2. Collagen isolation and purification

The primary step was to establish a reliable source of collagen, in the form of BAT. The material is comprised of predominantly Type I collagen, and thus the need for extensive purification is minimal. Mature BAT was obtained from an abattoir and washed in cold phosphate buffered saline solution (PBS, pH 7.4, 4°C) to remove extraneous material and then macerated. The isolation and purification of pepsin-soluble, Type I collagen was followed according to the method cited in Miller and Gay[16]. Collagen was solubilised in acetic acid (0.5 M) containing pepsin (100:1 w/w). The collagen solution was subjected to salt precipitation (0.9M) and redissolved in acetic acid (0.5M, 1:25 w/v). The salt precipitation and acetic acid solubilisation was repeated twice, then the final collagen solution was dialysed overnight with water to remove salt and acetic acid. Once the purification was complete, 100 ml batches of collagen solution (~6 mg/ml) were placed in flasks and frozen until required.

2.3. Collagen matrix preparation

The oleuropein concentration, the enzyme (β-glucosidase) concentration, and incubation period required for optimal crosslinking was investigated.

Oleuropein was dissolved in PBS (range of 0-0.5% w/v) and unsolubilised oleuropein was removed by filtration. β-Glucosidase enzyme (0 - 4 U/ml), was added to the oleuropein solution to produce a final volume of 5 ml. The resultant oleuropein-enzyme solution was placed in a thermostatically controlled orbital shaker at 25°C with an agitation speed of 150 rpm for 0 - 4 hours.

After incubation, the solution was cooled on ice (to terminate the reaction) and added to the isolated collagen solution (5 ml, 6 mg/ml) to produce a final volume of 10 ml. Both solutions were mixed slowly and thoroughly. The collagen-oleuropein solution was poured into a petri-dish (50 mm x 5 mm) and placed in an incubator at 37°C for one hour in order for collagen fibrillogenesis to occur. The collagen-oleuropein gel was then allowed to air-dry at a constant temperature of 22°C. Biochemical and thermal analyses of the material was undertaken.

2.4. Degree of Crosslinking

The thermal stability of the matrix was measured using differential scanning calorimetry (DSC822e, Mettler-Toledo, UK). Studies have shown that the thermal stability of the material is related to the degree of crosslinking[6,15]. The air-dried samples were placed in PBS at physiological pH and allowed to hydrate for two hours. Excess water was removed by blotting. A heating rate of 5°C/min was applied to the sample over a temperature range of 15°C to 100°C, and the denaturation temperature of the sample was recorded as T_D.

2.5. Swelling

The ability of the matrix to swell is dependent on the degree of crosslinking and studies have shown that the greater the degree of crosslinking the lower the ability of the material to swell[15]. Samples of known weight were placed in PBS, pH 7.4 at room temperature, and allowed to hydrate for two hours.

The percentage swelling was calculated according to the following formula:

$$\textbf{\textit{Percentage Swelling}} = \frac{W_f - W_i}{W_i} \times 100 \qquad \textbf{[1]}$$

$\mathbf{W}_i$: Initial weight of matrix before swelling.
$\mathbf{W}_f$: Final weight of matrix after swelling.

2.6. Free amine group content

The free ε-amino concentration of lysine and hydroxylysine residues of the non-crosslinked and crosslinked samples were measured using 2,4,6-trinitrobenzenesulfonic acid (TNBS)[17]. Samples with a weight of between 15-16 mg for were used. $NaHCO_3$ (4%, 1ml) was added to the blank and the test samples. To the blank, HCl (6N, 3 ml) was added before the TNBS to prevent the reaction of TNBS with the primary amine groups. Freshly prepared TNBS (0.5%, 1 ml) was added to the blank and the test sample. The samples were incubated at 40°C for a period of 4 hours. Hydrochloric acid (6N, 3 ml) was then added to the test samples to terminate the reaction. The samples were heated at 100°C overnight to hydrolyse the sample. Once

cooled, 5 ml deionised water was added to all the samples. The hydrolysate was extracted using ethyl ether and an aliquot (5 ml) was placed over a water bath to remove the solvent. Once cooled, further dilution of samples with deionised water was carried out. The absorbance was measured at 346 nm using a CECIL CE 2031 spectrophotometer. The free amine content was calculated according to equation 2, where 1.46×10^4 L/mole.cm, is the molar absorption constant for TNP-Lys, ***b*** is the cell path length and ***x*** is the weight of the collagen sample in g. The results were expressed as moles of free ε-amino groups/g collagen or percentage of free amine groups with the control at 100%.

$$\frac{\text{Moles } \varepsilon\text{-amino groups}}{\text{g collagen}} = \frac{2\,(\text{Absorbance})(\text{total volume})}{(1.46 \times 10^4\ \text{L/mole.cm})(\boldsymbol{b})(\boldsymbol{x})} \qquad [2]$$

2.7. Enzymatic Digestion

Samples of known weight (15-16 mg) were placed in TRIS-HCl, pH 7.4 for one hour prior to the addition of 10 mM $CaCl_2$ and collagenase (0.5 U/ml). Samples were incubated for a period of 5 hours at 37°C in a thermostatically controlled orbital shaker with an agitation speed of 150 rpm. The reaction was terminated by cooling the solution on ice. The samples were then filtered to remove insoluble material. Hydrochloric acid (6N, 1:1) was added to the filtrate and placed in an oven at 100°C for 16 hours. The hydroxyproline content of the hydrolysed samples was measured according to Jamall *et al.*,[18].

3. Results and Discussion

3.1. Optimisation of collagen crosslinking using oleuropein aglycone (OA)

Studies were undertaken to optimise the concentration of oleuropein (0 - 0.5% w/v) required for crosslinking collagen, with a constant β-glucosidase concentration (0.5 U/ml) and incubation period (2 hours). Figure 2 shows a concentration-dependent increase in the denaturation temperature of the collagen-oleuropein aglycone (C-OA)

matrix which begins to plateau at an oleuropein concentration of approximately 0.2% w/v. An inverse relationship was found for the number of moles of free ε-amino groups/g collagen and as the concentration of oleuropein was increased, the moles of free ε-amino groups decreased (Figure 2). This confirms evidence that OA binds to the free ε-amino lysine and hydroxylysine groups on proteins[4].

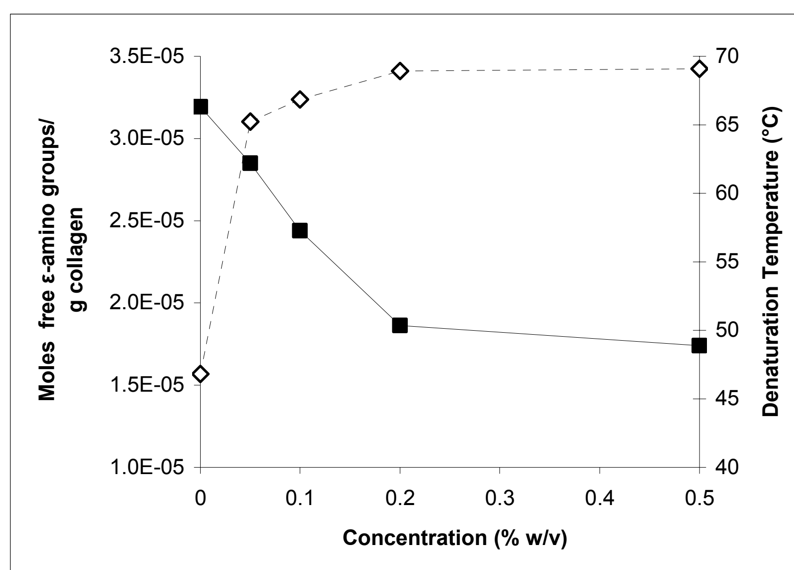

Figure 2: The effect of oleuropein concentration on the moles of free ε-amino groups/g collagen (■) and denaturation temperature (◊) of collagen films. Conditions: β-glucosidase: 0.5 U/ml, pH 7.4, temperature: 25ºC, agitation speed: 150 rpm, and incubation period: 2 hours.

As shown in Table 1, an increase in the concentration of oleuropein-aglycone (OA) decreased the water absorptivity (swelling) of the C-OA sample. It is understood that the decrease in swelling is due to a greater association of intra- and intermolecular links between the collagen fibres[15,19].

Table 1: Swelling of collagen films after equilibration in buffer (PBS) as a function of oleuropein concentration (n=3).

Oleuropein (% w/v)	*Swelling (%)*
0	356 ± 14
0.05	151± 20
0.10	112 ± 14
0.20	115 ± 18
0.50	93 ± 15

The results obtained for the T_D, moles of free ε-amino groups/g collagen, and swelling, correlate with one another and T_D results using DSC were utilised for all further optimisation studies. Further optimisation of β-glucosidase concentration and incubation period was also undertaken utilising an oleuropein concentration of 0.2% w/v.

In the optimisation of β-glucosidase concentration the denaturation temperature stabilised at approximately 66.3±1.5°C (ΔT_D = 19.5ºC) from a β-glucosidase concentration of 0.5 U/ml and was used for further experimental work (Figure 3). This confirmed the need to convert the inactive oleuropein to the active aglycone using β-glucosidase.

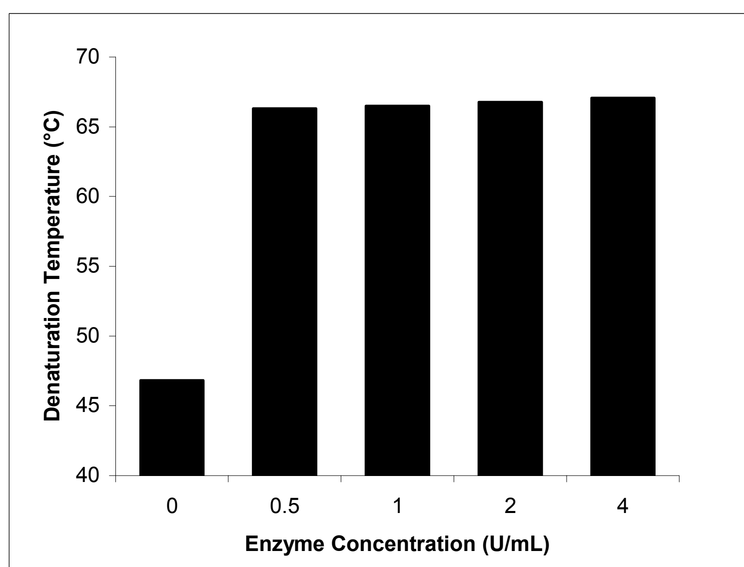

Figure 3: The effect of the concentration of β-glucosidase on the denaturation temperature of the collagen films. Conditions: oleuropein concentration: 0.2% w/v, pH 7.4, room temperature: 25°C, agitation speed: 150 rpm, and incubation period: 2 hours.

Studies undertaken to determine the incubation period required for β-glucosidase to activate oleuropein prior to the reaction with collagen showed that the enzyme catalysed reaction was too rapid to determine under the experimental conditions utilised (Figure 4). At time = 0, the oleuropein-β-glucosidase solution was added to the collagen solution and mixed thoroughly before treatment at 37ºC for 1 hour to encourage the fibrillogenesis of collagen (Section 2.3). A denaturation temperature of 68.3±0.5°C was obtained under these conditions, which is an increase of 21.5°C as

compared with the collagen control. Konno *et al.*[4], found that β-glucosidase is highly specific for oleuropein and was found to remove the glucose molecule rapidly from oleuropein to form the oleuropein-aglycone. The β-glucosidase activation of the oleuropein may still occur within the time taken to produce the collagen gel and an optimal incubation period of 5 minutes was chosen as a precaution.

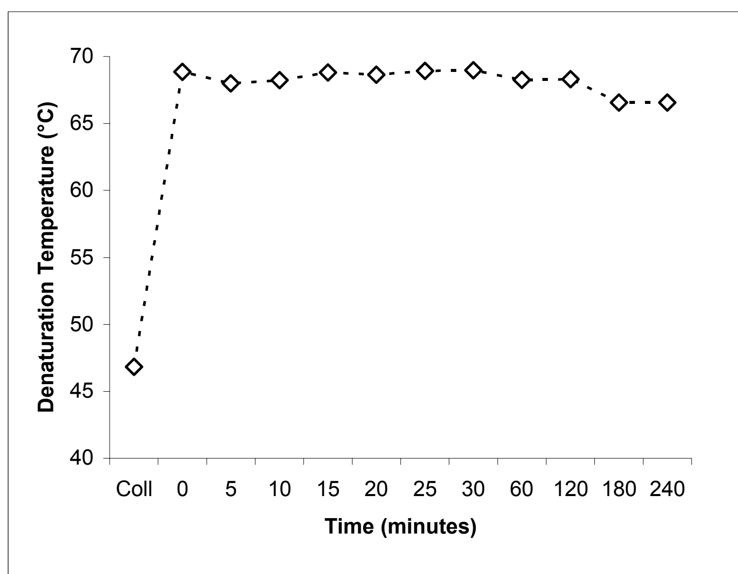

Figure 4: Denaturation temperature of C-OA films as a function of time. Conditions: oleuropein: 0.2% w/v, pH 7.4, β-glucosidase: 0.5 U/ml, temperature: 25°C, and agitation speed: 150 rpm.

In addition an inverse relationship was established (R^2 = 0.967) between the denaturation temperature and moles of free ε-amino groups/g collagen for the incubation period investigated.

3.2. Comparison of oleuropein aglycone with glutaraldehyde as a crosslinking agent

Enyzmatic digestion of equimolar OA and glutaraldehyde crosslinked collagen matrices showed that digested collagen was only detected in the control sample (Table 2) and that the crosslinked matrices are more stable than the control. The denaturation temperatures show that glutaraldehyde crosslinked material is slightly more thermally stable than the C-OA films (Table 2). However, when measuring the free ε-amino group content (Table 2) the results suggest that OA is capable of crosslinking more amino groups than glutaraldehyde (mole for mole at the optimum concentration of

oleuropein, 0.2% w/v). This is likely to be due to the presence of five bonding sites proposed for OA (Figure 1) versus two for glutaraldehyde.

Table 2: Biochemical and chemical properties of collagen-based films using OA and glutaraldehyde as crosslinking agents, at an equimolar concentration of 4.5×10^{-5} moles.

Sample	*Solubilised Collagen[a] (mg/ml)*	*Denaturation Temperature (ºC)*	*Crosslinked Amine Groups (%)*
Control	1.7×10^{-2}	50.2	0
Collagen-OA	UD	67.4	47.9
Collagen-glutaraldehyde	UD	68.3	22.6

a: based on hydroxyproline analysis
UD: undetectable

It is well known that glutaraldehyde binds to the primary amine group of lysine and hydroxylysine amino acids[1,6,12]. The reaction mechanism of glutaraldehyde has still not been fully elucidated, although investigation with sheep dermal collagen have shown that the reaction with lysine and hydroxylysine residues to form α,β-unsaturated Schiff bases is rapid and reversible[6]. Further stabilisation is likely to occur by the reaction of the Schiff base with another glutaraldehyde molecule to form polymeric crosslinking structures[1,6,12]. The resultant α,β-unsaturated products are thought to be involved in the crosslinking reaction between the primary amine groups and glutaraldehyde. Amine groups bind relatively easily to the β-carbon of an α,β-unsaturated aldehyde *via* Michael addition and the adduct formed is resistant to acid hydrolysis[4]. Konno *et al.*[4], suggested that the aglycone form of oleuropein is chemically similar to condensed glutaraldehyde structures, i.e., as an α,β-unsaturated aldehyde, however, the oleuropein aglycone was shown to be a much stronger lysine-binding agent[4].

Although it has been suggested that the oleuropein aglycone crosslinking mechanism is similar to glutaraldehyde[4], further investigation is required to determine the exact reaction mechanism with proteins.

4. Conclusion

The study has shown that β-glucosidase-activated oleuropein has a possibility as a crosslinking agent for collagen. Further investigation is required to elucidate the reaction mechanism between collagen and oleuropein. A potential exists to use waste products such as leaves, wood and processed fruit from the olive industry to isolate iridoid glycosides.

5. References

1) C.H. Lee, A. Singla, Y. Lee (2001) *Int. J. Pharmaceut*., **221**, 1-22.
2) W. Friess (1998) *European J. Pharmaceut. Biopharmaceut.*, **45**: 113-136.
3) Ramshaw, J.A.M., Glattauer, V. and Werkmeister, J.A. (2000) *Biomaterials and Bioengineering Handbook*, D.L. Wise (Ed.), Marcel Dekker, Inc., New York, 717-738
4) Konno, K., Hirayama, C., Yasui, H. and Nakamura, M. (1999) *Procedure of the National Academy of Sciences, USA*, **96**, 9159-9164.
5) Marak, H.B., Biere, A. and van Damme, J.M.M. (2002) *Chemoecology*, **12**, 185-192.
6) Olde Damink, L.H.H., Dijkstra, P.J., Van Luyn, M.J.A., Van Wachem, P.B., Nieuwenhuis, P. and Feijen, J. (1995) *Journal of Materials Science: Materials in Medicine*, **6**, 460-472.
7) Fleming, H.P., Walter, Jr., W.M. and Etchells, J.L. (1973) *Applied Microbiology*, **26(5),** 777-782.
8) Walter, Jr., W.M., Fleming, H.P. and Etchells, J.L. (1973) *Applied Microbiology*, **26(5),** 773-776.
9) Benavente-Gárcia, O., Castillo, J., Lorente, J., Ortuño, A. and Del Rio, J.A. (2000) *Food Chemistry*, **68**, 457-462.
10) Pérez-Bonilla, M., Salido, S., van Beek, T.A., Linares-Palomino, P.J., Altarejos, J., Nogueras, M. and Sánchez, A. (2006) *Journal of Chromatography A* (Article in Press).
11) Visioli, F., Bellomo, G. and Galli, C. (1998) *Biochemical and Biophysical Research Communications*, **247**, 60-64.
12) Hamdi, H.K. and Castellon, R. (2005) *Biochemical and Biophysical Research Communications*, **334**, 769-778.
13) Vinha, A.F., Ferreres, F., Silva, B.M., Valentão, Gonçalves, A., Pereira, J.A., Beatriz Oliveira, M., Seabra, R.M., Andrade, P.B. (2005) *Food Chemistry*, **89**, 561-568.
14) Briante, R., La Cara, F., Febbraio, F., Barone, R., Piccialli, G., Carolla, R., Mainolfi, P., De Napoli, L., Patumi, M., Fontanazza, G. and Nucci, R. (2000) *Journal of Biotechnology,* **77**, 275-286.
15) Friess, W. and Lee, G. (1996) *Biomaterials*, **17**, 2289-2294.
16) Miller, E.J. and Gay, S. *Collagen: An Overview, In: Methods in Enzymology: Structural and Contractile Proteins, Part A.* (1982) Eds., Cunningham, L.W. and Frederiksen, D.W., Academic Press Inc., New York, USA.
17) Ofner, C.M. and Bubnis, W.A. (1996) *Pharmaceutical Research*, **13(12)**, 1821-1827.

18) Jamall, I.S., Finelli, V.N. and Que Hee, S.S. (1981) *Analytical Biochemistry*, **112**, 70-75.
19) Fraga, A.N. and Williams, R.J.J. (1985) *Polymer*, **26**, 113-118.