ELSEVIER

Food Chemistry 75 (2001) 139–144

Food Chemistry

www.elsevier.com/locate/foodchem

Proposed mechanism for the degradation of pelargonidin 3-glucoside by caffeic acid *o*-quinone

F. Kader [a], M. Irmouli [b], J.P. Nicolas [c], M. Metche [a,c,*]

[a] *Laboratoire de Biochimie Appliquée, Ecole Nationale Supérieure d'Agronomie et des Industries Alimentaires, 2 Avenue de la Forêt de Haye BP 172-54505 Vandoeuvre-lès-Nancy Cedex, France*

[b] *Ecole Supérieure du Bois-Atlanpole, 3 rue Christian Pauc BP 10605 44306 Nantes Cedex 03, France*

[c] *Laboratoire de Biochimie Nutritionnelle, Unite INSERM 308, Faculté de Médecine de Nancy, 9 Avenue de la Forêt de Haye BP 184-54500 Vandoeuvre-lés-Nancy Cedex, France*

Received 3 May 2000; received in revised form 30 October 2000; accepted 30 October 2000

Abstract

Caffeic acid *o*-quinone (CQ) was prepared by oxidation of caffeic acid with *o*-chloranil in organic media. The reaction between the purified CQ and pelargonidin 3-glucoside (Pg 3-glc, non-*o*-diphenolic anthocyanin) was monitored by HPLC and spectrophotometric analysis. Quantitative analyses were performed to establish the stoichiometry of the reactions. The results showed that Pg 3-glc reacted with the hydroxycaffeic acid *o*-quinone formed by dismutation of CQ in aqueous media. This reaction led to the formation of condensation products with a stoichiometry close to 1:1. The degradation products were isolated by gel filtration on Sephadex G-25. The UV-vis spectra and chemical analysis of the degradation products suggested that they contained both caffeic acid (or secondary products of oxidation) and anthocyanin moieties. HPLC analysis showed that the purified fractions contained several degradation products.

Keywords: Caffeic acid; *o*-quinone; Anthocyanins; Stoichiometry

1. Introduction

The oxidative browning of fruits is mainly due to polyphenol oxidase (PPO; Vamos-Vigyazo, 1981). The phenolic composition of Highbush blueberry fruits *(Vaccinium corymbosum)* has been studied by Kader, Rovel, Girardin and Metche (1996). Chlorogenic acid (CG) was the major hydroxycinnamic derivative found in blueberries. This compound is a good substrate for blueberry PPO (Kader, Rovel, Girardin & Metche, 1997a). The mechanism of browning in fresh Highbush blueberry has been investigated by Kader, Rovel, Girardin, and Metche (1997b). The results have shown that oxygen, CG, anthocyanins and PPO played a dominant role in the process of browning. In the presence of oxygen, PPO catalysed the oxidation of CG into the corresponding *o*–quinone (chlorogenoquinone, CGQ). This quinone reacts with anthocyanins leading to the formation of brown condensation products.

Model solutions containing purified substrates are useful in understanding the mechanism of anthocyanin degradation (Kader, Haluk, Nicolas & Metche, 1998; Kader, Nicolas & Metche, 1999; Sarni, Fulcrand, Souillol, Souquet & Cheynier, 1995). Cyanidin 3-glucoside (*ortho*-diphenolic anthocyanin, Cy 3-glc) is degraded by a mechanism of coupled oxidation involving the enzymatically generated *o*-quinone with partial regeneration of the *o*-diphenolic cosubstrate (CG; Kader et al., 1998). The ratio of degraded Cy 3-glc to oxidised CG is constant and equal to 2, which means that part of CG is incorporated into the degradation products of Cy 3-glc. Non-*o*–diphenolic anthocyanins, such as pelargonidin 3-glucoside (Pg 3-glc), are degraded by a mechanism involving a reaction between the *o*-quinone and/or secondary products of oxidation formed from the quinone and the anthocyanin pigment (Kader, Nicolas & Metche, 1999). Whatever the structure of the anthocyanin pigment, no degradation occurs in the presence of PPO alone (Kader et al., 1998; Kader, Nicolas & Metche, 1999; Sarni et al., 1995; Sarni-Manchado, Cheynier & Moutounet, 1997). Sarni et al. (1995) demonstrated that malvidin 3-glucoside (Mv 3-glc) reacts with the enzyma-

* Corresponding author. Fax: +33-3-83-15-34-56.
E-mail address: nicolas@u308.nancy.inserm.fr (M. Metche).

0308-8146/01/$ - see front matter
PII: S0308-8146(00)00301-0

tically generated caftaric acid *o*-quinone to form adducts with a stoichiometry of 1:1. The UV-vis spectrum of the new pigment detected by HPLC suggests that it contains both caftaric acid and anthocyanin moieties.

The use of model systems has shown the role played by the *o*-quinones in the process of anthocyanin degradation. However, kinetic studies do not determine the exact mechanisms of anthocyanin degradation.

The use of a solution of pure quinone has been investigated by few authors (Kader, Irmouli, Zitouni, Nicolas & Metche, 1999; Sarni-Manchado et al., 1997). This new approach has permitted simplification of the model reactions. Sarni-Manchado et al. (1997) have studied the reaction of Mv 3-glc and caftaric acid *o*-quinone. Liquid chromatography with ion spray mass spectrometry indicates that the reaction products are adducts of Mv 3-glc and caftaric acid. The results show that different adducts can be formed, depending on the anthocyanin involved.

More recently, Kader, Irmouli et al. (1999) have studied the reaction of Cy 3-glc with caffeic acid *o*-quinone (CQ). The results confirm that Cy 3-glc is degraded by a coupled oxidation mechanism with integration of CQ into the slightly coloured products of degradation. The addition of purified PPO to the medium results in the oxidation of the caffeic acid, formed from the reaction of coupled oxidation (Cy 3-glc/CQ), into the corresponding *o*-quinone (CQ) that subsequently polymerises. This result suggests that the brown condensation products are formed from the polymerisation of the CQ. The degradation products of Cy 3-glc have been isolated by gel filtration on Sephadex G-25. UV-vis spectra and chemical analyses (acidic hydrolysis) of the degradation products suggest that they contain both anthocyanin and caffeic acid moieties.

The purpose of this work was to study the reaction of Pg 3-glc with CQ. The reaction was monitored by HPLC in order to determine the stoichiometry and the mechanism of the reaction. The reaction products were separated by gel filtration and characterised by their UV-visible spectra.

2. Materials and methods

2.1. Reagents

Pg 3-glc (Callistephin) was of HPLC grade from Extrasynthèse (Genay, France). Sephadex G-25 (fine, particule size: 20–80 μm) was from Pharmacia (Uppsala, Sweden). Ortho-chloranil was obtained from Aldrich (Strasbourg, France). Caffeic acid, ascorbic acid, trifluoroacetic acid (99.5% of purity), orthophosphoric acid (mm. 85% of purity) and benzene sulphinic acid were obtained from Sigma Chemicals (St. Quentin Fallavier, France). Methanol (HPLC grade), acetic acid (99.5% of purity), chloroform (99.5% of purity), ethyl acetate (99.5% of purity), propan-2-ol (analytical grade), and all other chemicals were of reagent grade from Merck (Darmstadt, Germany). Pg 3-glc (2 mM) was dissolved in McIlvaine buffer, pH 3.5. This buffer was prepared from 0.1 M citric acid adjusted to the correct pH by adding 0.2 M dibasic potassium phosphate.

2.2. Preparation and purification of the CQ

Caffeic acid and *o*-chloranil solutions were prepared in anhydrous methanol. CQ was prepared from caffeic acid by oxidation with *o*-chloranil at 25°C. The reaction mixture contained 0.2 ml of 50 mM caffeic acid, 0.2 ml of 100 mM *o*-chloranil and 1.6 ml of chloroform. The CQ was purified by HPLC (semipreparative scale) as described by Kader, Irmouli et al. (1999). The purified CQ was collected and used immediately.

2.3. Model system of the degradation of Pg 3-glc by CQ

The reaction mixture contained 0.1 ml of 1 mM Pg 3-glc, 0.25 ml of 0.235 mM CQ and 0.65 ml of McIlvaine buffer, pH 3.5. To determine the CQ concentration, the reaction mixture contained 0.25 ml of CQ, 0.05 ml of ascorbic acid or sodium benzenesulphinate (20 mM in McIlvaine buffer pH 3.5), and 0.7 ml of McIlvaine buffer, pH 3.5. After 60 min of reaction, 0.1 ml of the reaction mixture was analysed by HPLC (Merck-Hitachi L-6200 Intelligent pump equipped with a diode array detector Merck-Hitachi L-3000 connected to a Chromojet integrator). The constituents of the medium were separated on a Lichrosorb 100 RP-18 reversed phase column (250×4 mm i.d., 5 μm packing; Merck, Darmstadt, Germany) using a mobile phase consisting of water-acetic acid (97.5:2.5, v/v), (solvent A) and acetonitrile-solvent A (80:20, v/v) (solvent B). The gradient profile was 0–5 min, 5% B; 5–20 min, 5–40% B; 20–35 min, 40–80% B; 35–45 min, 80–100% B. Elution was performed at a flow rate of 1.0 ml/min, and 0.1 ml of the reaction mixture was injected using a Basic$^+$ Marathon automatic injector (Spark Holland). Caffeic acid and Pg 3-glc were detected at 280 nm.

The CQ was determined in equivalents of caffeic acid by reduction of the quinone by ascorbic acid (Kader, Irmouli et al., 1999). Caffeic acid and Pg 3-glc concentrations were determined by HPLC, as described above using a calibration curve ranging from 0.01 to 0.1 mM.

Pg 3-glc degradation was also monitored across a spectrum between 250 and 600 nm recorded every 2-mm using a Shimadzu UV 260 spectrophotometer.

For each assay, two analyses were conducted in duplicate. Each data point is therefore the mean of four measurements.

2.4. Purification of the reaction products

The reaction mixture contained 0.6 ml of McIlvaine buffer, pH 3.5, 0.4 ml of 1 mM Pg 3-glc and 2 ml of 0.2 mM CQ. The reaction was started by adding the CQ solution. After a reaction time of 1 h, the solvent was removed under reduced pressure using a rotary evaporator at 25°C. The resulting solutions were applied onto a Sephadex G-25 column (15×3 cm, i.d.) previously equilibrated with distilled water, at a flow rate of 15 ml/h. The reaction products were eluted with distilled water, and the absorbances at 280 and 325 nm were recorded on each 3-ml fraction.

2.5. Characterisation of the reaction products

The UV-vis spectra were recorded from 220 to 600 nm using a Shimadzu UV-260 spectrophotometer. The reaction products (0.5 mg) occurring in the fractions P1 were hydrolysed by heating at 85°C in 3 ml of 2 M trifluoroacetic acid in methanol under nitrogen for 45 min. The hydrolysed solutions were cooled and 2 ml of water was added. Methanol and trifluoroacetic acid were removed under vacuum at 35°C. The residual aqueous solution was extracted four times with ethyl acetate (1:1, v/v). The aqueous phase was concentrated in a rotary evaporator under vacuum at 35°C. The resulting solution (0.2 ml) was chromatographed to identify glucose on a silica gel plate (Merck, ref. 5553) with the propan-2-ol/ethyl acetate/water (50:40:10, v/v) system. The glucose was located on TLC plates by spraying with 2% (w/v) naphthoresorcinol solution in acetone and 9% orthophosphoric acid (5:1, v/v) followed by heating in an oven at 105°C for 10 min.

2.6. HPLC of the purified products

The fraction P1 was analysed by HPLC using the same apparatus and column as described for the degradation of Pg 3-glc by CQ. The elution conditions were as follows: solvent A, 1% acetic acid in distilled water (v/v); solvent B, methanol; flow rate, 1 mil/min; linear gradients from 0 to 100% B in 50 min; 0.2 ml of the reaction mixture was injected using a Basic$^+$ Marathon automatic injector (Spark Holland). The reaction products were detected at 280 nm.

3. Results and discussion

3.1. Degradation of Pg 3-glc by CQ in McIlvaine buffer (pH 3.5)

The reaction between Pg 3-glc and CQ was monitored across a spectrum between 250 and 600 nm and recorded every 2-min (Fig. 1). The decrease in absorbance at 495 nm indicates the loss of colour, meaning that the anthocyanin pigment degrades. We also observed a decrease in absorbance at 422 nm. At this wavelength, both Pg 3-glc and CQ absorb, indicating that the CQ and the pigment disappear from the reaction mixture. The increase in absorption at 330 nm may be due to a new product being formed during the reaction process. These spectra provide only qualitative information. The reaction between CQ and Pg 3-glc was also analysed by HPLC after 1 h of reaction (Fig. 2). The HPLC profile shows the presence of three peaks. Peaks 1 and 2 were identified, respectively, as caffeic acid and Pg 3-glc, on the basis of their retention time and UV-vis spectra. Comparison with a chromatogram profile, obtained from a CQ-free control, confirms that reactions between Pg 3-glc and CQ take place. Because the anthocyanin does not have an *o*-diphenolic structure, it would not be expected to generate caffeic acid in the reaction mixture (Kader, Nicolas & Metche, 1999; Sarni et al., 1995).

The formation of caffeic acid in model systems containing Pg 3-glc and CQ could be due to the hydroxylation of CQ by water (Reaction 1), leading to the formation of hydroxycaffeic acid (i.e. 2′, 4′, 5′ hydroxy cinnamic acid). This compound immediately reacts with another molecule of CQ to regenerate caffeic acid and to give the hydroxycaffeic acid *o*-quinone HCQ (Reaction 2). Such a pathway has already been proposed by Richard-Forget, Rouet-Mayer, Goupy, Philippon and Nicolas (1992) for the enzymatic degradation of 4-methylcatechol in acidic media.

$$\mathrm{CQ} + \mathrm{H_2O} \rightarrow \text{hydroxycaffeic acid} \quad (1)$$

$$\text{Hydroxycaffeic acid} + \mathrm{CQ} \rightarrow \text{caffeic acid} + \mathrm{HCQ} \quad (2)$$

$$2\ \mathrm{CQ} + \mathrm{H_2O} \rightarrow \text{caffeic acid} + \mathrm{HCQ} \quad (3)$$

Quantitative analyses were carried out to establish the stoichiometry of the reaction between CQ and Pg 3-glc (Table 1). The amount of Pg 3-glc that reacted with the CQ corresponds to the initial amount of Pg 3-glc minus the amount of Pg 3-glc (0.1–0.077 μmol) remaining. Consequently, 0.023 μmol of Pg 3-glc disappeared from the reaction mixture. At the same time, 0.026 μmol of caffeic acid was formed. If caffeic acid is generated according to the mechanism described above (Reaction 3), 0.052 μmol of CQ is required to generate 0.026 μmol of HCQ and 0.026 μmol of caffeic acid. It may be that the HCQ reacts with the Pg 3-glc with a stoichiometry close to 1:1. The degradation of Pg 3-glc by PPO, in the presence of chlorogenic acid, has been studied by Kader, Nicolas and Metche (1999). We have shown that the degradation of Pg 3-glc is subject to a delay, which may correspond to the formation of secondary products

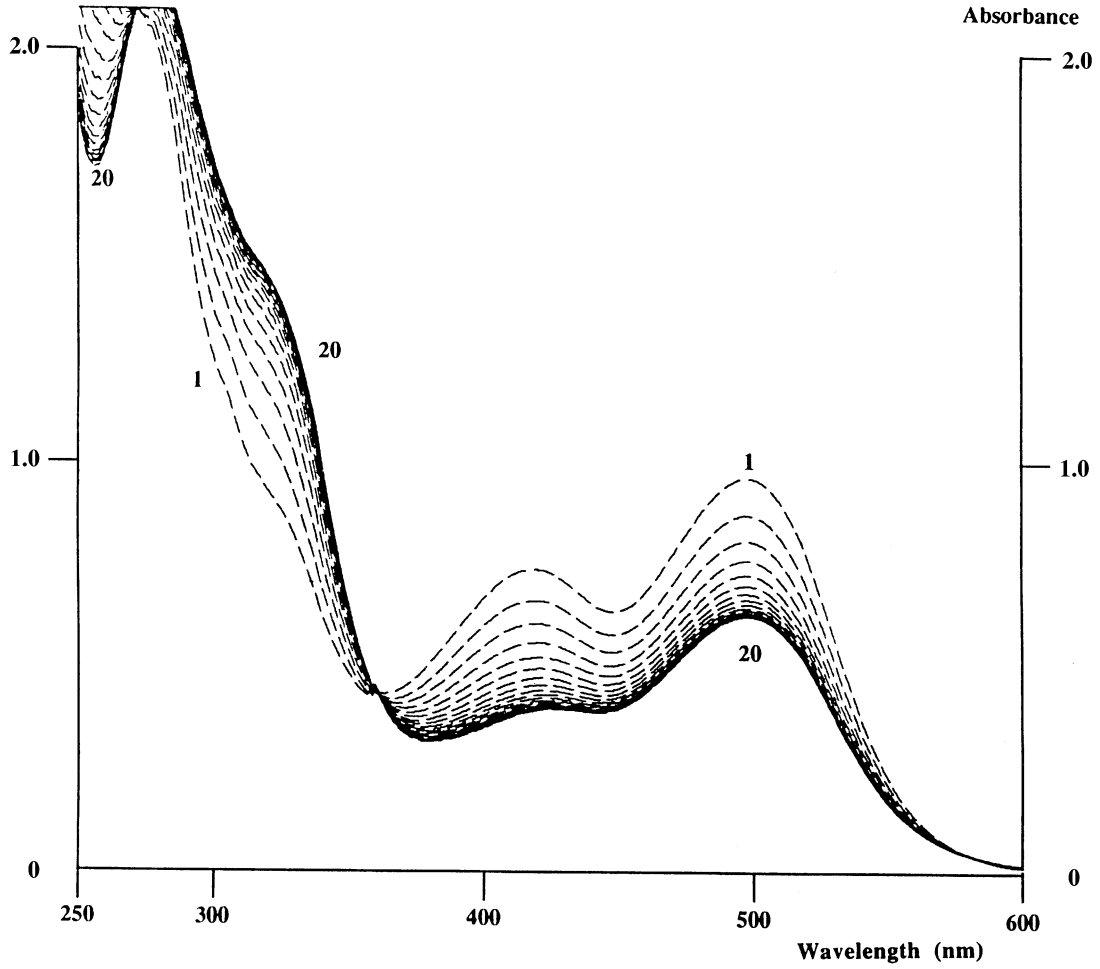

Fig. 1. Absorbance spectra (600–350 nm) of the model system containing Pg 3-glc (0.1 mM) + CQ (0.06 mM) in McIlvaine buffer, pH 3.5. All scans were at time intervals of 2 min.

of oxidation (formed from the chlorogeno-quinone). These results clearly explain the observed delay, and we can assume that the HCQ may react with the Pg 3-glc leading to combinations with a stoichiometry close to 1:1.

3.2. Partial purification of the reaction products

The reaction products obtained after 60 min of reaction between the Pg 3-glc and CQ were also chromatographed on a Sephadex G-25 column, The elution profile (Fig. 3) shows three peaks; two small ones (P1 and P2) which absorb at 280 and 330 nm, followed by a large peak (P3) which HPLC analysis showed to contain both caffeic acid and the remaining Pg 3-glc.

The fractions corresponding to peaks P1 and P2 were pooled individually and concentrated under vacuum in a rotary evaporator. The UV-vis spectra were recorded between 220 and 600 nm (Fig. 4). The spectra of P1 (Fig. 4A) and P2 (Fig. 4B) showed maxima at 276 and 280 nm and a shoulder at 335–320 nm, respectively. The shoulder at 315–320 nm might suggest the incorporation of the caffeic acid moiety (or degradation products formed from the CQ) into the degradation products of the Pg 3-glc. The degradation products occurring in peak P1 (the most abundant) were hydrolysed by heating at 85°C in 2 M trifluoroacetic acid-methanol (v/v) for 45 min. The fractions obtained after acidic hydrolysis were

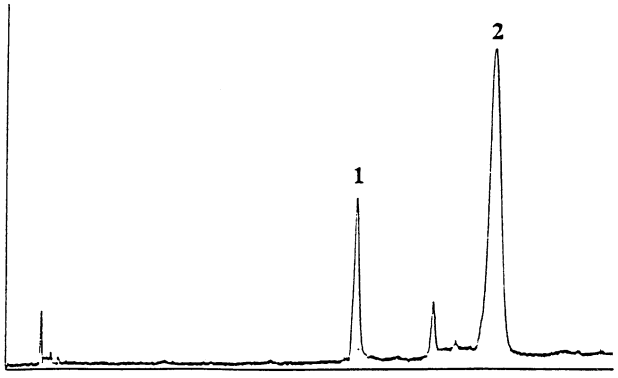

Fig. 2. HPLC elution profile at 280 nm of a solution of Pg 3-glc (0.1 mM) incubated for 60 min with the purified CQ (0.06 mM) in McIlvaine buffer, pH 3.5. Peak 1, caffeic acid; Peak 2, remaining Pg 3-glc.

Table 1
Degradation of Pg 3-glc by CQ in McIlvaine Buffer (pH 3.5): determination of the stoichiometry of the reaction[a]

Initial amount of Pg 3-glc (μmol)	Initial amount of CQ (μmol)	Amount of acid formed (μmol)	Amount of remaining Pg 3-glc (μmol)
0.1±0.05	0.045±0.005	0.026±0.003	0.077±0.004

[a] The different compounds were quantified by HPLC using calibration curves. For each assay two analyses were conducted on duplicate experimentations. Each data point is the mean four measurements. CQ, caffeic acid *o*-quinone.

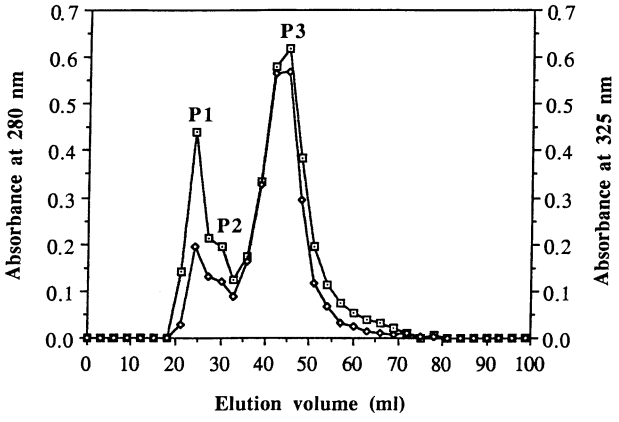

Fig. 3. Elution profile, on Sephadex G-25, of a solution of Pg 3-glc (0.133 mM) incubated for 60 mm with the purified CQ (0.133 mM) at pH 3.5. Absorbance at 280 nm (□); absorbance at 335 nm (◇).

analysed by TLC (see Section 2). After spraying the developed chromatogram, we identified glucose which is linked at the C-3-position of the pelargonidin. The overall results suggest that the products of anthocyanin degradation contain both caffeic acid and anthocyanin moieties. This is consistent with previous papers on the oxidative degradation of Cy 3-glc and Mv 3-glc in the presence of caffeoyltartaric acid and grape PPO (Sarni et al., 1995), which reported that the degradation products of Cy 3-glc and Mv 3-glc contained both caffeoyltartaric acid and anthocyanin moieties. Moreover, these degradation products were gradually replaced by colourless products as a result of further oxidative degradation. More recently, Sarni-Manchado et al. (1997) have shown that the reaction between Mv 3-glc and caftaric acid *o*-quinone led to the formation of adducts. This indicated that the hemiacetal form of the pigment is more reactive than the flavylium form.

The HPLC analysis of the fraction P1 (Fig. 5) shows that several products of degradation can be formed from the reaction between anthocyanins and CQ, which supports the idea of the complex degradation process mentioned by Kader, Nicolas and Metche (1999).

Recently, we studied the mechanism of anthocyanin degradation in model solutions containing CG, blueberry PPO and purified Pg 3-glc (Kader, Nicolas & Metche, 1999). Kinetic studies have shown that Pg 3-glc reacts with CGQ and/or secondary products of oxidation (formed from the CGQ) to yield anthocyanin-CG

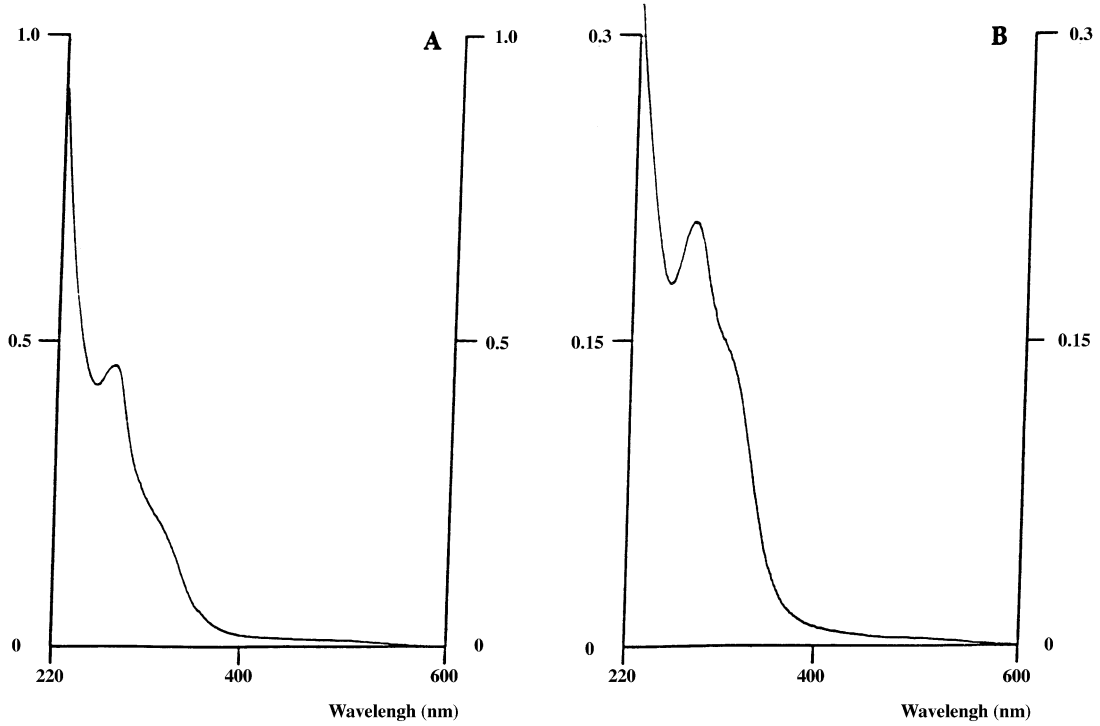

Fig. 4. UV-visible spectra of the concentrated fractions corresponding to (A), peak P1; and (B), peak P2 obtained from the fractionation on Sephadex G-25 of the degraded Pg 3-glc. Spectra were in distilled water.

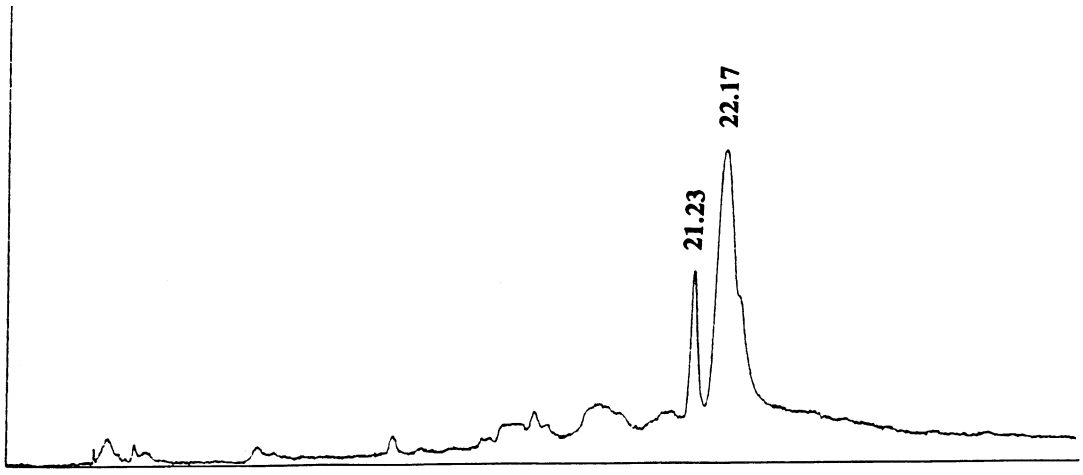

Fig. 5. HPLC elution profile, at 280 nm, of the degradation products eluted in peak P1.

condensation products. The use of simpler model systems containing purified anthocyanins and CQ has confirmed most of the results described above, indicating that CQ reacts with water to form the hydroxycaffeic acid (Reaction 1). Once formed, the hydroxycaffeic acid can react by a mechanism of coupled oxidation with CQ leading to the formation of HCQ and caffeic acid (Reaction 2). Then, HCQ reacts with the Pg 3-glc leading to combinations with the following stoichiometry [1 Pg 3-glc/1 HCQ].

Sarni-Manchado et al. (1997) consider that the products of degradation result from nucleophilic attack of the anthocyanin (in the hemiacetal form) onto the caftaric acid *o*–quinone. In this study, the oxidised solutions of Mv 3-glc were analysed after 90 min of reaction. However, on following the reaction as a function of time, caffeic acid is detected in the solutions by HPLC analysis. It is established that non-*o*-diphenolic anthocyanins, such as Pg 3-glc and Mv 3-glc, are not degraded by a mechanism of coupled oxidation. HPLC analyses of Pg 3-glc-free controls containing McIlvaine buffer and purified CQ showed that no caffeic acid appeared in the reaction mixture, confirming that caffeic acid is formed during the process of Pg 3-glc degradation.

However, the exact mechanism remains to be determined by characterising the structure of the degradation products. For this reason, the next step in this work will involve both isolating and determining the structure of the resolved condensation products.

References

Kader, F., Haluk, J. P., Nicolas, J. P., & Metche, M. (1998). Degradation of cyanidin 3-glucoside by blueberry polyphenoloxidase: Kinetic studies and mechanisms. *Journal of Agricultural and Food Chemistry, 46*, 3060–3065.

Kader, F., Irmouli, M., Zitouni, N., Nicolas, J. P., & Metche, M. (1999). Degradation of cyanidin 3-glucoside by caffeic acid *o*-quinone. Determination of the stoichiometry and characterization of the degradation products. *Journal of Agricultural and Food Chemistry, 47*, 4625–4630.

Kader, F., Nicolas, J. P., & Metche, M. (1999). Degradation of pelargonidin 3-glucoside in the presence of chlorogenic acid and blueberry polyphenol oxidase. *Journal of the Science of Food and Agriculture, 79*, 517–522.

Kader, F., Rovel, B., Girardin, M., & Metche, M. (1996). Fractionation and identification of the phenolic compounds of Highbush blueberries (*Vaccinium corymbosum*, L.). *Food Chemistry, 55*, 35–40.

Kader, F., Rovel, B., Girardin, M., & Metche, M. (1997a). Mechanism of browning in fresh Highbush blueberry fruit (*Vaccinium corymbosum*, L.). Partial purification and characterization of blueberry polyphenol oxidase. *Journal of the Science of Food and Agriculture, 73*, 513–516.

Kader, F., Rovel, B., Girardin, M., & Metche, M. (1997b). Mechanism of browning in fresh Highbush blueberry fruit (*Vaccinium corymbosum*, L.). Role of Blueberry polyphenol oxidase, chlorogenic acid and anthocyanins. *Journal of the Science of Food and Agriculture, 74*, 31–34.

Richard-Forget, F. C., Rouet-Mayer, M. A., Goupy, P. M., Philippon, J., & Nicolas, J. J. (1992). Oxidation of chlorogenic acid, catechins, and 4-methylcatechol in model solutions by apple polyphenol oxidase. *Journal of Agricultural and Food Chemistry, 40*, 2114–2122.

Sarni, P., Fulcrand, H., Souillol, V., Souquet, J. M., & Cheynier, V. (1995). Mechanisms of anthocyanin degradation in grape must-like model solutions. *Journal of the Science of Food and Agriculture, 69*, 385–391.

Sarni-Manchado, P., Cheynier, V., & Moutounet, M. (1997). Reactions of polyphenoloxidase generated caftaric acid *o*-quinone with malvidin 3-*O*-glucoside. *Phytochemistry, 45*, 1365–1369.

Vamos-Vigyazo, L. (1981). Polyphenol oxidase and peroxidase in fruits and vegetables. *CRC Critical Reviews in Food Science and Nutrition, 15*, 49–127.