# Flavonoid Concentration Changes in Maturing Broad Bean Pods

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Five flavonoid aglycons and eight flavonol glycosides were isolated from broad bean pods. By chemical, chromatographic, and spectral methods the aglycons were identified as 7,3′,4′-trihydroxyflavone, 7,4′-dihydroxyflavone, geraldone, butein, and kaempferol and the glycosides as kaempferol 3-glucoside, 7-glucoside, 3-(2″-rhamnosyl)galactoside 7-rhamnoside, 3-galactoside 7-rhamnoside, 3-rhamnosyl-(6″-acetyl)galactoside 7-rhamnoside, and 3-(6″-acetyl)galactoside 7-rhamnoside and as quercetin 3-galactoside 7-rhamnoside and 3-(6″-acetyl)galactoside 7-rhamnoside. The concentration changes of these flavonoids in pod tissues with progressive stages of maturity were evaluated by HPLC analysis. A significant increase in the concentration of both flavonoid aglycons and glycosides was observed when the pods matured and became reddish and brown. Some of the flavonoid aglycons detected in broad bean pods had previously been reported as *Rhizobium* nodulation gene inducers.

## INTRODUCTION

Analysis of flavonoids of plants used in food and feed is important because some of these secondary metabolites have nutritional relevance. Antiscorbutic properties (Regnault-Roger, 1988) and mutagenic activity of flavonol aglycons (Brown, 1980) have been reported.

Broad bean (*Vicia faba*) is cultivated for its pods, seeds, and green forage. A recent study showed that *V. faba* leaves are rich in flavonol glycosides and can be used as a source of such flavonoids (Tomás-Lorente et al., 1989). Flavonol aglycons (myricetin, quercetin, and kaempferol) have been detected in the seed coat (Herrmann and Woldecke, 1977; Nozzolillo et al., 1989), but the phenolic constituents of the pods remain unknown.

Thus, the aim of the present work was the isolation and identification of the flavonoid compounds, both free aglycons and glycosides, in broad bean pods during various stages of maturity.

As broad bean matures, the color of the pod changes from green to reddish and finally to dark brown. On the assumption that the flavonoid content of the pods could be associated with these color changes, as recently reported for peanut hull (Daigle et al., 1988), a study of the changes of flavonoid aglycon and glycoside content of maturing broad bean pods was undertaken by means of HPLC.

## MATERIALS AND METHODS

**Plant Material.** Broad bean pods (cv. Aguadulce) were collected from the Cooperativa de Corvera orchards (Murcia) during January 1989 and January 1990. Pods were separated into nine groups on the basis of length, color, seed size, and stage of maturity. Thus, stage I included pods below 6 cm; II, pods 6–8 cm; III, pods 8–10 cm; IV, pods 10–13 cm; V, 13–15 cm; VI, green pods approximately 20 cm with fully developed seeds; VII, fully developed pods with reddish spots; VIII, fully developed pods with intense red color; and IX, fully developed pods with dark brown color. Samples were collected for 2 years to confirm the results obtained.

**Extraction of Flavonoids.** Fresh, mature pods (stage VIII) (2.5 kg) from which seeds had been removed were cut in thin slices and exhaustively extracted (macerated) with methanol for 24 h at room temperature, and the methanol was removed under reduced pressure. From the remaining aqueous solution, the flavonoid aglycons were selectively extracted with ethyl ether, and successively the flavonoid glycosides were extracted with *n*-butanol.

**Isolation of Flavonoids.** The ether extract was chromatographed on Whatman No. 1 paper with a 30% aqueous solution of acetic acid. Five major fractions were separated and eluted from the powdered paper packed into a column with methanol. Compounds were then purified by passing through a column of

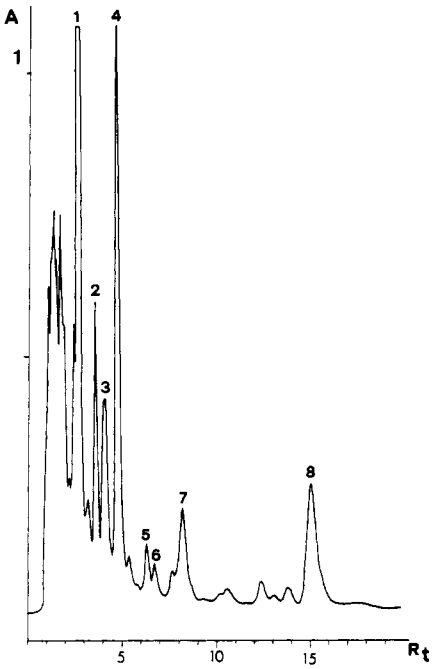


**Figure 1.** HPLC analysis of broad bean pod free flavonoid aglycons and kaempferol 3-glucoside and 7-glucoside. (A) Absorbance 340 nm. ($R_t$) Retention times. (1) THF; (2) DHF; (3) geraldone; (4) butein; (5) Km 3-glucoside; (6) Km 7-glucoside; (7) kaempferol (Km); (8) unidentified quercetin methyl ether.

**Table I. Free Flavonoid Aglycons in *V. faba* Pods: Variation with Maturity**[a]

| | stage of maturity | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| flavonoids | I | II | III | IV | V | VI | VII | VIII | IX |
| THF | 32 | 20 | 7 | 1 | 2 | 1 | 30 | 330 | 160 |
| DHF | 11 | 10 | 13 | 6 | 4 | 6 | 12 | 60 | 40 |
| geraldone | 5 | 2 | 2 | 2 | 2 | 8 | 17 | 50 | 90 |
| butein | 9 | 4 | 2 | 2 | 2 | 3 | 20 | 100 | 50 |
| Km | 90 | 1 | | | | | 8 | 20 | 45 |

[a] Values are micrograms of flavonoid/100 g of fresh plant material. THF, 7,3′,4′-trihydroxyflavone; DHF, 7,4′-dihydroxyflavone; geraldone, 7,4′-dihydroxy-3′-methoxyflavone; butein, 2′,3,4,4′-tetrahydroxychalcone; Km, 3,5,7,4′-tetrahydroxyflavone. Stages I–IX represent pods with different size, color, and maturity.

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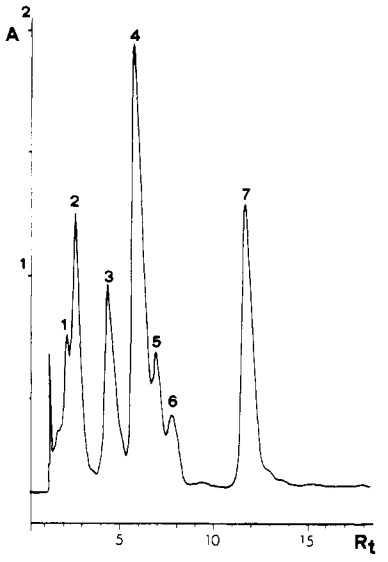


**Figure 2.** HPLC analysis of broad bean pod flavonoid diglycosides and triglycosides. (A) Absorbance 340 nm. ($R_t$) Retention times. (1) Qu triglyc; (2) Km 3RG-7R; (3) Qu 3G-7R; (4) Km 3G-7R; (5) Qu 3AcG-7R; (6) Km 3AcRG-7R; (7) Km 3AcG-7R.

Sephadex LH-20 with methanol. The purity of each compound was tested by reversed-phase HPLC using a photodiode array detector. The *n*-butanol extract, containing flavonol glycosides, was fractionated by paper chromatography with *n*-BuOH–HOAc–$H_2O$ (4:1:5, upper phase), and the separated flavonoid compounds were visualized under UV light (360 nm). Nine compounds were isolated by a combination of chromatography on Whatman No. 3 paper with 2% aqueous acetic acid and low-pressure column chromatography [Lobar C-8 with MeOH–$H_2O$ (7:13), solvent flow rate 3 mL/min]. The isolated compounds were purified on a Sephadex LH-20 column with methanol as eluant. Flavonoid glycosides were identified by spectroscopic methods (UV, $^1H$ NMR) as described previously for broad bean leaf flavonoids (Tomás-Lorente et al., 1989).

**HPLC Analysis of Flavonoid Aglycons and Glycosides in Pods of Different Maturity.** Three samples (15 g) of seedless pod tissues of each maturity stage (I–IX) were extracted with methanol overnight at room temperature. The extracts were filtered and the methanol was removed under reduced pressure. The remaining aqueous solution was diluted with water to reach 100 mL and extracted twice with ethyl ether (25 mL) and *n*-butanol (25 mL) successively. The ether extracts, containing flavonoid aglycons, were concentrated under reduced pressure and redissolved in 1 mL of methanol. These methanol solutions and the isolated flavonoid aglycons were analyzed by HPLC on a reversed-phase column [LiChrospher 100 RP-18 (5 μm)] using as mobile phase methanol–5% aqueous formic acid (1:1) isocratically with a flow rate of 1 mL/min and detected with a photodiode array detector. Quantitative analyses were performed after calculation of the extinction coefficient of the isolated substances or authentic markers (Apin Chemicals and Roth) for the detection wavelength (340 nm). The *n*-butanol extracts containing the flavonoid glycosides were dried in vacuo and redissolved in 1 mL of methanol. The methanol solutions and the isolated glycosides were analyzed by HPLC under the same conditions as the aglycons except that the mobile phase was methanol–5% aqueous formic acid solution (3:7) and detected at 340 nm. Quantitative analyses were performed after the calculation of the extinction coefficient for the major flavonol glycoside [kaempferol 3-(2″-rhamnosyl)galactoside 7-rhamnoside]. The quantitative results for flavonoid glycosides were related to this substance.

**Identification of Flavonoids.** Flavonoids were identified by their UV spectra in methanol, after the addition of the classical shift reagents (Mabry et al., 1970), and by chromatographic comparisons with authentic markers and chemical derivatizations. Thus, THF, DHF, and kaempferol showed UV properties as reported previously for these substances (Mabry et al., 1970) and were identified by chromatographic comparisons and co-

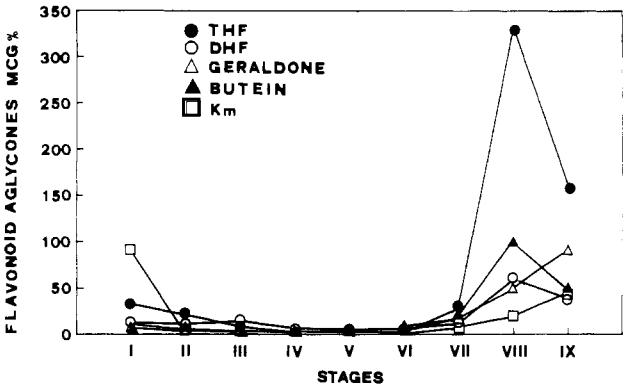


**Figure 3.** Free flavonoid aglycons in *V. faba* pods. Variation with maturity. Flavonoid content in micrograms per 100 g of fresh plant material. Abbreviations are as for Table I.

chromatography on TLC (cellulose 30% acetic acid) and HPLC with authentic markers. Geraldone was identified by UV spectra and was confirmed by diazomethane permethylation, yielding a derivative which coincided with that obtained from THF. Butein was tentatively identified by its typical UV spectra in methanol after addition of shift reagents (Mabry et al., 1970), and its chemical nature was confirmed by its chromatographic behavior (TLC and HPLC).

**7,3′,4′-Trihydroxyflavone** showed blue fluorescence under UV light (360 nm) which changed to yellow when fumed with ammonia. UV data $\lambda_{max}$ (nm) in methanol: 340, 320, 233 sh; +NaOMe 400, 256; +$AlCl_3$ 455 sh, 374, 302; +$AlCl_3$ + HCl 403, 345, 308; +NaOAc 377, 255; +NaOAc + $H_3BO_3$ 364, 303, 255 sh.

**7,4′-Dihydroxyflavone** showed blue fluorescence under UV light. UV $\lambda_{max}$ (nm) in methanol: 320, 290 sh, 255 sh, 234 sh; +NaOMe 384, 331, 250; +$AlCl_3$ 329, 308; $AlCl_3$ + HCl 332, 306; NaOAc 357, 307, 264; +NaOAc + $H_3BO_3$ 320, 254 sh.

**7,4′-Dihydroxy-3′-methoxyflavone** also showed blue fluorescence under UV light. UV $\lambda_{max}$ (nm) in methanol: 331, 311, 287 sh, 256 sh; +NaOMe 388, 332, 252; +$AlCl_3$ 331, 311, 293 sh; +$AlCl_3$ + HCl 390, 310; +NaOAc 360 sh, 341, 256; +NaOAc + $H_3BO_3$ 336.

**2′,3,4,4′-Tetrahydroxychalcone** showed purple color under UV light, changing to red when fumed with ammonia. UV $\lambda_{max}$ (nm) in methanol: 379, 288, 265; +NaOMe 448, 340, 284; +$AlCl_3$ 486, 288; +$AlCl_3$ + HCl 426, 312, 273; +NaOAc 414, 278; +NaOAc + $H_3BO_3$ 414, 282.

**3,5,7,4′-Tetrahydroxyflavone** showed a yellow fluorescence under UV light. UV $\lambda_{max}$ (nm) in methanol: 366, 320 sh, 296 sh, 266, 254 sh; +NaOMe 420 (dec), 315, 278; +$AlCl_3$ 420, 350, 302 sh, 269, 258 sh; +$AlCl_3$ + HCl 418, 345, 302 sh, 269, 255 sh; +NaOAc 386, 302, 273; +NaOAc + $H_3BO_3$ 372, 320 sh, 296 sh, 266.

## RESULTS AND DISCUSSION

**Free Flavonoid Aglycons.** From the mature pods (stage VIII) of *V. faba* (cv. Aguadulce) the following flavonoid aglycons were isolated and identified: 7,3′,4′-trihydroxyflavone (THF); 7,4′-dihydroxyflavone (DHF); 7,4′-dihydroxy-3′-methoxyflavone (geraldone); 3,5,7,4′-tetrahydroxyflavone (kaempferol); and 2′,3,4,4′-tetrahydroxychalcone (butein). The quantitative and qualitative variation of these flavonoid aglycons with the maturity of broad bean pods was evaluated by means of HPLC analysis (Figure 1), and results are quoted in Table I. Significant differences have been observed in the flavonoid aglycon content with the progress of pod maturation and senescence (Figure 3). For THF, which is the main flavonoid aglycon in broad bean pods, the content reaches a concentration of 32 μg/100 g of fresh plant material in the early ripening stage (I), decreases with maturation until a minimum of 1 μg/100 g of fresh weight is obtained in stage VI (green, fully developed pods), and increases considerably to 330 μg/100 g of fresh weight in stage VIII

**Table II. Flavonoid Monoglycosides, Diglycosides, and Triglycosides in *V. faba* Pods: Variation with Maturity[a]**

| flavonoids | stage of maturity | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | I | II | III | IV | V | VI | VII | VIII | IX |
| monoglucosides* | | | | | | | | | |
| Km 3-Glc | 3.0 | 1.0 | | | | | 3.0 | 6.0 | 15.0 |
| Km 7-Glc | 20.0 | 20.0 | 20.0 | 10.0 | 3.0 | 3.0 | 4.0 | 6.0 | 6.0 |
| diglycosides | | | | | | | | | |
| Km 3G-7R | 1.51 | 1.95 | 1.60 | 0.93 | 0.08 | 0.37 | 0.64 | 1.40 | 4.00 |
| Km 3AcG-7R | 8.33 | 11.07 | 6.24 | 6.50 | 1.55 | 1.30 | 2.00 | 2.00 | 10.00 |
| Qu 3G-7R | 2.20 | 2.93 | 1.50 | 1.56 | 1.53 | 0.91 | 3.17 | 6.10 | 22.20 |
| Qu 3AcG-7R | 0.72 | 2.15 | 2.07 | 1.26 | 1.21 | 1.10 | 2.80 | 1.80 | 10.80 |
| triglycosides | | | | | | | | | |
| Km 3RG-7R | 3.32 | 1.72 | 1.30 | 0.75 | 0.74 | 0.33 | 0.58 | 0.90 | 1.80 |
| Km 3RAcG-7R | 9.80 | 7.07 | 3.12 | 2.30 | 1.90 | 0.83 | 1.50 | 2.40 | 5.80 |
| Qu trigly | 1.80 | 0.66 | 0.79 | 0.36 | 0.38 | 0.18 | 1.08 | 0.80 | 0.63 |
| total glycosides | 27.7 | 27.5 | 16.6 | 13.7 | 7.4 | 5.0 | 11.7 | 15.4 | 55.2 |

[a] Values are milligrams (* micrograms in the case of monoglycosides) of flavonoid/100 g of fresh plant material. Km 3G-7R, kaempferol 3-galactoside 7-rhamnoside; Km 3AcG-7R, kaempferol 3-(6″-acetyl)galactoside 7-rhamnoside; Qu 3G-7R, quercetin 3-galactoside 7-rhamnoside; Qu 3AcG-7R, quercetin 3-(6″-acetyl)galactoside 7-rhamnoside; Km 3RG-7R, kaempferol 3-(2″-rhamnosyl)galactoside 7-rhamnoside; Km 3RAcG-7R, kaempferol 3-(2″-rhamnosyl-6″-acetyl)galactoside 7-rhamnoside; Qu trigly, quercetin triglycoside. Stages I–IX represent pods with different size, color, and maturity.

(mature pods with intense red color). The other aglycons follow a similar pattern, with slight differences in the case of geraldone and kaempferol. When the pods become dark brown (stage IX), there is generally a decrease in the concentration of flavonoid aglycons, but the amount of these substances is still important.

The minimum value for flavonoid concentration (both aglycons and glycosides) coincides with the group that is usually consumed fresh (stage VI), while the mature pods (stages VIII and IX), which are used as cooked food, contain the highest amounts of flavonoids.

Flavonoid aglycons without oxygenation at the 5-position have been reported from leaves of Leguminosae (Harborne, 1967), but their occurrence in pods has not been reported to date. These substances are of biological and agricultural interest, since it has recently been shown that some flavonoids act as chemical signals produced by legume plants (exudated by seeds and roots) which induce nodulation genes in susceptible *Rhizobium* species (Firmin et al., 1986; Peters et al., 1986; Redmon et al., 1986). Moreover, the induction of nodulation genes of *Rhizobium trifolii* by DHF and geraldone detected in the root exudates of clover (*Trifolium repens*) (Redmon et al., 1986) and the induction of those of *R. leguminosarum* by a commercial sample of THF (Firmin et al., 1986) have been reported. In addition, we have detected THF and DHF in the seed exudates of *V. faba* (unpublished results). These substances increase markedly with senescence in the pod shells.

**Flavonoid Glycosides.** The flavonoid glycosides present in broad bean pods were identified as kaempferol 3-glucoside and 7-glucoside, 3-galactoside 7-rhamnoside, and 3-(2″-rhamnosyl)galactoside 7-rhamnoside as well as the 6″-acetylated derivatives of the two latter compounds and quercetin (3,5,7,3′,4′-pentahydroxyflavone) 3-galactoside 7-rhamnoside, quercetin 3-(6″-acetyl)galactoside 7-rhamnoside, and an unidentified quercetin triglycoside. These compounds were reported, with the exception of the monoglycosides and the quercetin triglycoside, from the leaves of this species (Tomás-Lorente et al., 1989). Their structures were established by chemical, chromatographic, and spectroscopic methods ($^1$H NMR). The quantitative and qualitative progression of these substances with maturity was studied by HPLC analyses (Figures 1 and 2), and the results obtained are shown in Table II.

Diglycosides (Figure 5) and triglycosides (Figure 6) are

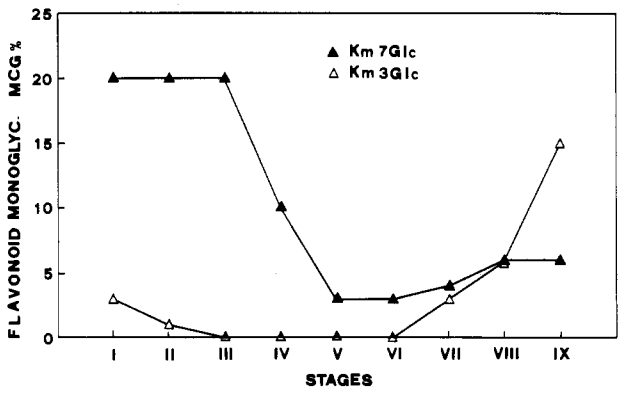


**Figure 4.** Flavonoid monoglycosides in *V. faba* pods. Variations with maturity. Flavonoid content in micrograms per 100 g of fresh plant material. Abbreviations are as for Table II.

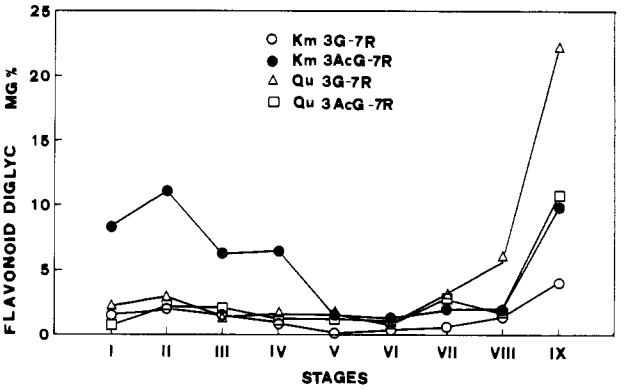


**Figure 5.** Flavonoid diglycosides in *V. faba* pods. Variation with maturity. Flavonoid content in milligrams per 100 g of fresh plant material. Abbreviations are as for Table II.

the major flavonoid compounds in the pod tissues (these are present in concentrations of milligrams of flavonoid/100 g of fresh plant material), while flavonoid aglycons and monoglycosides (Figure 4) are present in smaller amounts (in the microgram range). These glycosides are located in epidermal tissues both in the leaf and in the pod. The concentration of quercetin and kaempferol diglycosides increases slightly until a maximum is reached at stage II (pods 6–8 cm long) and then decreases until stage VI (fully developed green pods). Then they increase again to reach a maximum when the pods become dark brown (stage IX). This final increase paralleling senes-

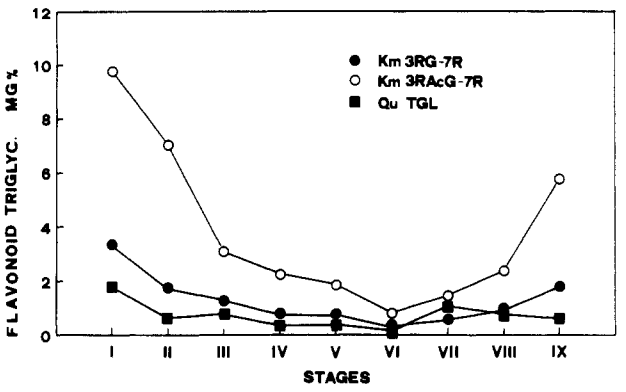


**Figure 6.** Flavonoid triglycosides in *V. faba* pods. Variation with maturity. Flavonoid content in milligrams per 100 g of fresh plant material. Abbreviations are as for Table II.

cence development is relatively more pronounced for the quercetin than for the kaempferol derivatives. Flavonol triglycosides, however, follow a different progression pattern. They accumulate at a maximum concentration in the smallest pods (stage I), decrease to reach a minimum in the green fully developed pods (stage VI), and then increase slightly until senescence (stage IX).

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