Quality and Quantity of Prevailing Flavonoid Glycosides of Yellow and Green French Beans (*Phaseolus vulgaris* L.)

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The flavonoid composition of six varieties in either case of yellow and green French beans was investigated. Fresh beans were chopped under liquid nitrogen just after harvest and lyophilized subsequently. The flavonoids were exhaustively extracted, purified by selective adsorption to and stepwise elution from polyamide, and separated by RP-HPLC with UV detection. With one exception the qualitative chromatographic pattern of the 12 varieties was very similar. The main flavonoids were identified as 3-*O*-glucuronides (-3-*O*-glucur) and 3-*O*-rutinosides (-3-*O*-rut) of quercetin (Q) and kaempferol (K) by LC-TSP-MS comparing retention time, UV spectra, and mass spectra with those of pure flavonoids. The areas of HPLC peaks with identical aglycon were summed up and converted into the -3-*O*-rut of Q or K with the respective calibration curves. The total content of Q-3-*O*-glycosides ranged from 19.1 to 183.5 μg/g and that of K-3-*O*-glycosides from 5.6 to 14.8 μg/g of fresh bean, demonstrating a dependence on the variety without general difference between yellow and green representatives.

Keywords: *French bean (Phaseolus vulgaris L.); flavonoids; HPLC; LC-TSP-MS*

INTRODUCTION

Flavonoids are widespread plant secondary metabolites. As components of vegetables and fruits they are regularly contained in human food. Data on the content of flavonoids of a great number of vegetables commonly consumed in the Netherlands show that French bean (*Phaseolus vulgaris* L.) is among the important sources (Hertog et al., 1992). That investigation is based on flavonoid aglycons resulting from acid hydrolysis of the natural glycosides. There is no evidence for glycosidic individuals of the main flavonoids Q and K. But from the necessary conditions of hydrolysis the major glycosides are suggested to be glucuronides. Flavonoid glycosides have been investigated so far in cotyledons, seedlings, or leaves of beans (Endres et al., 1939; Marsh, 1955; Harborne, 1965; Tanguy et al., 1972; Fiussello et al., 1977). Q-3-*O*-glucur, Q-3-*O*-rut, Q-3-*O*-xylosylglucoside, K-3-*O*-glucur, and K-3-*O*-rut have been identified. Since qualitative and quantitative data of flavonoid glycosides in the unripe fruit of French beans including varietal differences are not available, we selected six varieties in either case of yellow and green French beans to study the flavonoid composition and to find out whether the yellow varieties have higher flavonoid contents which might be expected from their coloration.

MATERIALS AND METHODS

Sources and Preparation of Samples. Fresh beans were harvested in late August 1993, 12 weeks after sowing. Six yellow and six green varieties were kindly provided by the Bundessortenamt Hannover, Prüfstelle Rethmar. The beans were cleaned, roughly cut, immediately frozen in liquid nitrogen, chopped in a Waring commercial blender, and lyophilized. With regard to the stability of flavonoids in freeze-dried foods reported by Hertog et al. (1992), the samples were stored at −20 °C for some weeks until analyses were completed.

Extraction and Purification of Flavonoids. A sample of 3.5 g of pulverized beans was dispersed in 50 mL of chloroform with an Ultra-Turrax homogenizer (Janke & Kunkel, Staufen, Germany) for 4 min. After removal of the solvent by suction on a G4 glass filter covered with paper filter, the extraction of chlorophyll and carotenoids was repeated three times by adding 25 mL of chloroform, sonicating for 5 min, and sucking off the chloroform. The combined chloroform extracts were tested for the occurrence of flavonoid glycosides and aglycons; flavonoids could not be detected. A 3.0 g air-dried sample was then exhaustively extracted with 70% aqueous methanol (v/v) as follows: 100 mL of solvent with 4 min mixing by Ultra-Turrax homogenizer, centrifuging for 30 min at 5000*g*, and decanting the extract; 75 mL of solvent with 5 min sonicating and separating the extract as above; and 75 mL of solvent with 15 min rotation at 40 °C and separating the extract as above. To test whether the extraction was completed a further step was added with one bean variety: 75 mL of solvent with 30 min rotation at 60 °C and separating the extract. The filtrates were pooled—or kept separately when investigating the extraction course—and evaporated at 40 °C to remove methanol. The extract was made up to 20 mL with distilled water. To purify the phenolic substances 3 mL of the aqueous extract was added onto a preconditioned (3 mL of methanol; 5 mL of water) polyamide (PA) cartridge (500 mg of PA, Chromabond, Macherey-Nagel, Düren, Germany). After washing with water the flavonoids were eluted stepwise with 9 mL of methanol, and with 9 mL of a mixture of 99.5 parts of methanol and 0.5 part of 25% ammonia (v/v) to eluate the stronger bound flavonoids as, e.g., the glucuronides. The eluates were evaporated to dryness and the residues dissolved with 1.75 mL *N*,*N*-dimethylformamide (DMF)/water (2:1, v/v) prior to filtration through Lida PTFE 0.2 μm filter (Lida, Kenosha, WI). The final solutions were used for HPLC and liquid chromatography—thermospray mass spectrometry (LC—TSP-MS).

Apparatus. HPLC analyses were carried out on Merck-Hitachi equipment consisting of an autosampler AS-2000 A, a gradient pump L-6200 A, a diode array detector L-4500 A, and an interface D-6000 (all units from Merck-Hitachi, Darmstadt, Germany) with diode array detector-system-manager

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software D-6500. LC−TSP-MS analyses were carried out using two solvent delivery modules 116 (Beckman Instruments, San Ramon, CA) with pump 1 coupled to a low pressure gradient former M 250 B (Gynkotek, Germering, Germany) and pump 2 to add a buffering solution after the column but before the UV detector (Merck-Hitachi, Darmstadt, Germany). Data were evaluated with a CR-6A integrator (Shimadzu Europe, Duisburg, Germany) and the MS data system. The MS unit comprised a thermospray interface TSP1 with control unit and a mass spectrometer SSQ 710 (Finnigan MAT, Bremen, Germany) with the DEC station 5000/33 and Finnigan ICIS II software as data system.

Standards. Flavonoid standards were purchased from Extrasynthèse. The stock solution was prepared by dissolving Q-3-*O*-rut trihydrate and K-3-*O*-rut each in 5.0 mL of DMF/water (2:1, v/v) and mixing of equal volumes. The stock solution was diluted to five different concentrations ranging from 10 to 160 μg/mL of either standard. With three repetitions of 50 μL injections both compounds had linear calibration curves (peak area vs concentration) through the origin with R^2 values greater than 0.9995. The other two flavonoid standards used for identification only (Q-3-*O*-glucur and K-3-*O*-glucur) were kindly provided by R. Galensa from the Institute of Food Chemistry of the Technical University, Braunschweig, Germany.

LC−TSP-MS. Chromatographic separations were carried out on a Ultrasphere ODS (Beckman Instruments, San Ramon, CA) column (4.6 × 250 mm, 5 μm). Flavonoid standards and the methanol and methanol/ammonia PA eluates of the flavonoid extract of one bean variety were eluted with the solvent system: A = acetonitrile, B = 2% acetic acid in water; 5% A in B (v/v) linearly to 30% A in B (v/v) in 40 min and holding for 5 min. The flow rate was 1 mL/min. Analytes were monitored at 354 nm. Before mass spectrometry 0.1 M ammonia acetate in 10% methanol was added at a flow rate of 0.8 mL for buffer ionization to enable positive ion detection (Vestal, 1990). To identify flavonoid peaks of the bean which have retention time identical to a standard in HPLC and show congruent UV spectra, the ion mass spectra were compared.

HPLC. Chromatographic separations were performed on a LiChrospher 100 RP-18 endcapped (Merck, Darmstadt, Germany) column (4 × 250 mm, 5 μm) protected by a guard column (4 × 4 mm) with the same stationary phase. Elution was carried out at 25 °C at a flow rate of 0.8 mL/min with the solvents A = acetonitrile and B = 2% acetic acid in water as follows: 10% A in B (v/v) linearly to 30% A in B (v/v) in 35 min followed by A linearly to 45% in B in 2 min and holding for 5 min and back to 10% A in B in 2 min and holding for 21 min to equilibrate for the next injection. The effluent was scanned between 240 and 390 nm to compare spectra of peaks with those of references in the library. Analytes were monitored at 354 nm. Quantification was based on peak area after having classified the peaks according to their UV spectra. Peaks of Q glycosides were integrated at 354 nm, those of K glycosides at 346 nm. The total peak areas were converted into equivalents of Q-3-*O*-rut or K-3-*O*-rut respectively using the standard calibration curves.

RESULTS AND DISCUSSION

Most of the extractable flavonoids were collected with the first three steps of extraction (Figure 1). This was the basis to analyze and compare the bean varieties. With the exception of the methanol PA eluate of the green variety "Ingo", the qualitative chromatographic pattern was very similar for the six yellow and six green varieties within the methanol PA eluates and the methanol/ammonia eluates. Figure 2 presents both the chromatograms of the green variety "Tuf" as typical representatives and the exceptional one of Ingo. The main peaks in the Tuf chromatograms were identified since they had the same retention time, UV spectrum and MS spectrum as the reference compounds. Q-3-*O*-glucur was the dominant flavonoid of all the bean

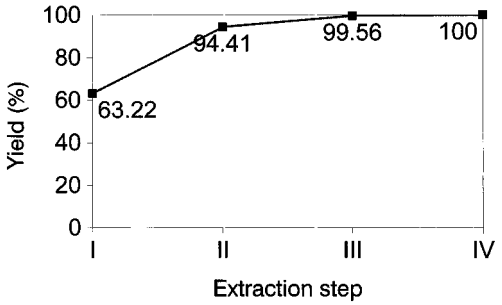

Figure 1. Cumulated amount (%) of flavonoids in the course of extraction of the green bean variety Tuf (steps I−IV).

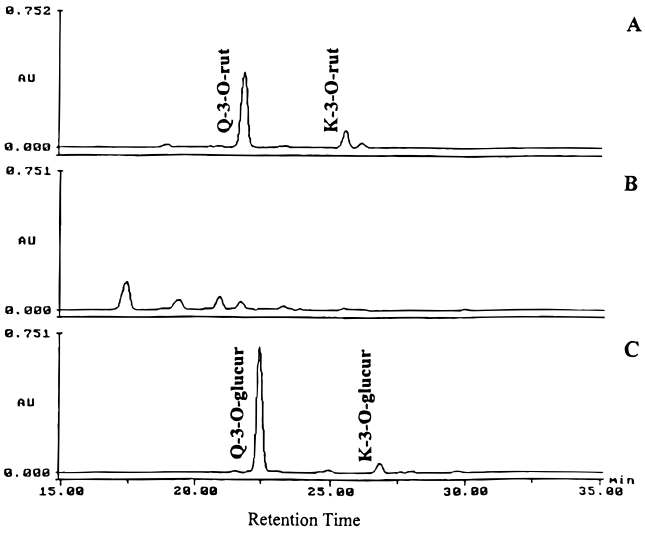

Figure 2. Separation of bean flavonoids by RP-HPLC with acetonitrile/2% acetic acid gradient (λ = 354 nm): methanol PA eluate of Tuf (A) and Ingo (B), methanol/ammonia PA eluate of Tuf (C).

varieties investigated. The additional peaks of the Ingo methanol PA eluate were not identified. The UV spectrum of the last two peaks in front of Q-3-*O*-rut could be related to that of Q derivatives. From the small difference between the retention times of Q-3-*O*-rut (21.87 min) and Q-3-*O*-glucur (22.43 min), it became obvious that the stepwise elution of flavonoids from PA allowed a useful fractionation as precondition for further separation. Summing up the areas of all peaks relatable to Q and K derivatives, respectively, and quantifying the values as the corresponding -3-*O*-rut showed the preponderance of Q over K derivatives (Table 1). The reproducibility of the working-up procedure from the lyophilized bean to the Q-3-*O*-glucur peak area of the chromatogram was proved with three replicates. The standard deviation was found to be ±3.5%. There was a strong influence of the bean variety on the flavonoid content but no general difference between yellow and green representatives. The total content of Q-3-*O*-glycosides was found to be between 19.1 μg/g for the yellow bean "Vollenda" and 183.5 μg/g of fresh bean for the variety Tuf. K-3-*O*-glycosides ranged from 5.6 μg/g for Vollenda to 14.8 μg/g of fresh bean for the yellow variety "Goldetta". Hertog et al. (1992) found 39 μg of Q and <12 μg of K per gram of fresh bean after hydrolysis of all the glycosides from French bean. This would correspond to 79 μg of Q-3-*O*-rut and <25 μg of K-3-*O*-rut per gram of fresh bean. Apart from the difference due to the variety and the growing conditions, this might be explained by the fact that hydrolysis to aglycons comprises also the minor flavonoid glycosides.

Table 1. Flavonoid Content (Micrograms per Gram of Fresh Weight) in Bean Varieties Grown in Different Years

	Q derivatives[c]		K derivatives[d]	
variety	1993	1995	1993	1995
Goldetta[a]	81.0	46.2	14.8	4.2
Goldfinger[a]	54.4	n.d.[e]	7.6	n.d.
Berggold[a]	37.9	n.d.	13.5	n.d.
Golddukat[a]	29.6	138.4	8.5	9.3.
Gabriella[a]	27.5	n.d.	6.9	n.d.
Vollenda[a]	19.1	116.0	5.6	8.6
Tuf[b]	183.5	112.5	14.4	8.2
Ingo[b]	64.9	58.9	7.7	6.7
Rovita[b]	42.9	n.d.	8.7	n.d.
Lars[b]	26.1	n.d.	13.7	n.d.
Dublette[b]	29.4	n.d.	10.4	n.d.
Olga[b]	27.4	65.6	6.1	8.3

[a] Yellow varieties. [b] Green varieties. [c] Q derivatives are given as Q-3-*O*-rut. [d] K derivatives are given as K-3-*O*-rut. [e] Not determined.

Flavonoid analysis was repeated with six selected bean varieties grown at the same location as in 1993 and harvested in early August 1995 11 weeks after sowing (Table 1). We obtained chromatograms similar to those of 1993. The exceptional composition of the green variety Ingo was confirmed. As in 1993 the content of Q derivatives was higher than that of K derivatives. The absolute amounts changed, e.g., with Vollenda and Tuf to 116.0 and 112.5 μg of Q-3-*O*-glycosides/g of fresh bean and to 8.6 and 8.2 μg of K-3-*O*-glycosides/g of fresh bean, respectively. A nonuniform change among the bean varieties became obvious, which might be caused by diverse reaction of the genotypes to changing weather conditions. Local weather data document remarkable differences in mean temperature (July and August 4−5 °C higher in 1995 than in 1993) and in precipitation (1995: July, 40.4 mm; first decade of August, 1.7 mm. 1993: July, 113.7 mm; August, 51.4 mm). The differing growth conditions caused variable dry matter content ranging from 9.6 to 13.0% in 1993 and from 15.6 to 27.9% in 1995 with the selected varieties. The comparability of the flavonoid content could be impaired by the fact that it is difficult to estimate the stage of fruit ripeness exactly. While there are obviously several factors determining the flavonoid amount, the chromatographic pattern of flavonoid individuals is dependent on the variety only. Since the flavonoid content did not differ generally between yellow and green beans, we further looked at how the yellow pigmentation is substantiated. We compared semiquantitatively the carotenoid content of the yellow variety Goldetta with that of the green variety Tuf. After three times extraction with chloroform the combined extracts were concentrated to dryness under vacuum. Portions were separated by thin-layer chromatography on silica plates. The carotenoids were eluted with chloroform/methanol (1:1, v/v) and the visible absorption spectra of the combined carotenoids were taken. The spectra showed the typical absorption with maxima at 424/427, 449/455, and 477/482 nm, which are similar to those reported by Lopez-Hernandez et al. (1993). From the absorption at 455 nm, an approximately sixfold higher content of carotenoids was estimated for the green variety Tuf. The pigmentation of yellow beans is therefore not due to the higher content of either flavonoids or carotenoids compared with green beans but is probably caused by the more or less complete degradation of chlorophyll (Vicentini et al., 1995). The results of this investigation were used to make available the identified flavonoid compounds which were tested for their antioxidative and antigenotoxic potencies (Raab et al., 1995).

ABBREVIATIONS USED

DMF, *N*,*N*-dimethylformamide; PA, polyamide; LC−TSP-MS, liquid chromatography followed by thermospray mass spectrometry; Q, quercetin; K, kaempferol; -3-*O*-glucur, -3-*O*-glucuronide; -3-*O*-rut, -3-*O*-rutinoside.

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