JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

# Ultra Performance Liquid Chromatography–Tandem Quadrupole Mass Spectrometry Profiling of Anthocyanins and Flavonols in Cowpea (*Vigna unguiculata*) of Varying Genotypes

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**ABSTRACT:** The structure of flavonoids in food plants affects bioactivity and important nutritional attributes, like micronutrient bioavailability. This study investigated flavonol and anthocyanin compositions of cowpea (*Vigna unguiculata*) of varying genotypes. Black, red, green, white, light brown, and golden brown cowpea phenotypes were analyzed for anthocyanins and flavonols using ultra performance liquid chromatography–tandem quadrupole mass spectrometry. Eight anthocyanins and 23 flavonols (15 newly identified in cowpea) were characterized. Mono-, di-, and tri(acyl)glycosides of quercetin were predominant in most phenotypes; myricetin and kaempferol glycosides were present only in specific phenotypes. The red phenotypes had the highest flavonol content (880–1060 μg/g), whereas green and white phenotypes had the lowest (270–350 μg/g). Only black (1676–2094 μg/g) and green (875 μg/g) phenotypes had anthocyanins, predominantly delphinidin and cyanidin 3-*O*-glucosides. Cowpea phenotype influenced the type and amount of flavonoids accumulated in the seed; this may have implications in selecting varieties for nutrition and health applications.

**KEYWORDS:** *cowpea, anthocyanin, flavonol, UPLC, TQD mass spectrometer*

## INTRODUCTION

Cowpea (*Vigna unguiculata*) is a drought- and heat-tolerant legume with low input requirements that is a major source of nutrition in Africa and other parts of the world. Like other legumes, cowpea is a good source of proteins and trace minerals. The attractive nutritional and agronomic properties of cowpea have seen its global production and consumption grow steadily, expanding by 50% between 1999 and 2009.[1] Africa accounted for 70% of the 5.2 MMT global cowpea production and consumption in 2009;[1] thus, the crop is a major source of dietary proteins and micronutrients, iron, and zinc, among nutritionally vulnerable groups. With rising global temperatures and water scarcity, cowpea will likely play a greater role in global food security.

Grain legumes contain significant quantities of flavonoids, particularly flavonols, flavan-3-ols, and anthocyanins;[2,3] these flavonoids have been associated with various health benefits. Flavonoids also affect plant and grain characteristics, including plant secondary color and seed color. Seed coat color is used by consumers in many regions of the world as a major preference criterion for legumes. Given that the type of flavonoids present in plant can determine various bioactive mechanisms, such as estrogenic[4] and anti-inflammatory[5] responses, information on how the flavonoids accumulated by cowpea influence seed coat color is an important first step in exploring the health properties of cowpea.

Also important is the fact that the flavonoid structure can strongly influence their ability to bind to divalent metal ions, particularly iron and zinc. For example, the presence of glycosyl substitution and the position of the substitution have a major impact on the iron-binding ability of quercetin.[6] In common beans, high levels of kaempferol were reported to significantly contribute to reduced iron bioavailability in red- and black-colored beans as compared to white ones.[7] Given that iron and zinc malnutrition are among the biggest global health challenges, particularly among people who rely on plant-based diets, accurate information on cowpea flavonoids is important. Evidence also suggests that flavonoid accumulation in cowpea seed or leaves is correlated with improved plant defense against insects;[8,9] specific compounds involved have not been identified.

Most of the investigations characterizing flavonoids in legumes have focused on common beans (*Phaseolus vulgaris*). Thus, even though a limited number of flavonoids have been identified in cowpea,[10,11] very little is known about how seed coat color or other phenotypic traits influence their accumulation in cowpea. Most studies of flavonoids in cowpea rely on hydrolysis products, partly due to the difficulties involved in resolving complex flavonoid mixtures via high-performance liquid chromatography (HPLC) and establishing their identity without appropriate standards.[12,13] In this study, we take advantage of the speed and resolution power of an ultra performance liquid chromatography–mass spectrometry (UPLC-MS) system to separate and characterize flavonols and anthocyanins in distinct cowpea phenotypes.

## MATERIALS AND METHODS

**Plant Materials.** A total of 105 cowpea lines largely derived from International Institute of Tropical Agriculture (IITA) accessions and maintained by University of California-Riverside, CA, and Texas A&M University, College Station, TX, were originally screened for

Received: December 22, 2011
Revised: March 8, 2012
Accepted: March 19, 2012
Published: March 19, 2012

polyphenols, tannins, and antioxidants, using established protocols.[14] From the preliminary screening, we selected a set of 10 samples grouped into six distinct phenotypes that represented the spectrum of variations observed in the collection: black, red, green, white, light brown (cream), and golden brown. The 10 samples were grown in a uniform environment in the university test plot at College Station, TX, in the months of April–July, 2010; flood irrigation was used as needed to eliminate drought-related stress. Samples were grown in two rows. All samples were harvested at maturity (dry seed stage) and stored at 4 °C until used. The physical properties of the seeds recorded during harvest are shown in Table 1. The seeds were ground using a coffee grinder (Cuisinart, model DCG-20N series) to pass through a 60 mesh sieve and stored at −20 °C until used. The moisture content was determined using standard protocol.[15]

**Table 1. Description of Cowpea Cultivars Used in the Study**

| variety | seed weight[a] (g/100 seeds) | seed coat color and texture |
|---|---|---|
| IT95K-1105-5 | 23.4 ± 0.37 | black, smooth |
| IT98K-1092-1 | 11.6 ± 0.35 | black, smooth |
| IT82D-889 | 11.3 ± 0.17 | red, smooth |
| IT97K-1042-3 | 13.1 ± 0.20 | red, smooth |
| TX2028-1-3-1 | 21.6 ± 0.45 | green, freckled, black-eye |
| IAR-48 | 22.6 ± 0.22 | light brown, rough |
| 09FCV-CC-27M | 14.9 ± 0.40 | light brown, smooth |
| IFE BROWN | 15.8 ± 0.16 | golden brown, rough |
| IT84S-2246 | 17.5 ± 0.08 | golden brown, smooth |
| EARLY ACRE | 11.6 ± 0.16 | white, rough, brown-eye |

[a]Seed weight expressed as the mean ± SD of triplicate weights of 100 seeds. All samples were grown in Texas A&M University Test Plots in College Station, TX, in 2011.

**Chemicals and Reagents.** All reagents were analytical grade. Kaempferol, quercetin, and quercetin-3-*O*-rutinoside (rutin) were purchased from Sigma-Aldrich (St. Louis, MO); myricetin was obtained from ACROS Organics (Morris Plains, NJ). Delphinidin-3-*O*-glucoside, cyanidin chloride, cyanidin-3-*O*-glucoside, malvidin-3-*O*-glucoside, petunidin chloride, peonidin chloride, peonidin-3-*O*-glucoside, and quercetin-3-*O*-glucoside were purchased from Extrasynthese Natural Products (Genay Cedex, France).

**Extraction of Polyphenols for UPLC-MS Analysis.** Approximately 5.0 g of ground cowpeas was separately weighed into capped polypropylene centrifuge tubes and soaked in 15 mL of 70% acetone (or methanol for anthocyanins) acidified with 1% formic acid for 12 h at 4 °C. The two extraction procedures were necessary due to the known reactivity of anthocyanin with acetone to form pyranoanthocyanins.[16,17] Then, the mixtures were shaken intermittently for 4 h in a shaking water bath set at 37 °C. The extracts were centrifuged (10000*g* force for 10 min) using a Heraeus Megafuge 11R Centrifuge (Thermo Fisher Scientific, Asheville, NC) at 4 °C, and the supernatants were transferred into new falcon tubes. The solid residues were washed twice with 10 mL of the extraction solvent and further centrifuged. The supernatants were then combined and concentrated under reduced pressure (Buchmann R110 Rotavapor, Westbury, NY).

**Extract Purification.** To minimize peak overlap that was apparent when crude extracts were directly injected in UPLC system, the aqueous acetone cowpea extracts were fractionated on a Sep-Pak Solid phase Octadecylsilane (C18) cartridges (Sigma, St. Louis, MO) following methods described by Prior et al.[18] and Monagas et al.[19] but with some modifications. Briefly, the C18 cartridges were preconditioned with 25 mL of methanol:water (1:1) for 1 h and washed with 50 mL of distilled water. Five milliliters of the concentrated extracts was deposited into the cartridges and washed with 5 mL of distilled water to remove the sugars and proteins. Then, catechins and an oligomeric proanthocyanins-rich fraction were eluted with 15 mL of ethyl acetate (fraction I). The flavonol-rich fraction was then eluted with 15 mL of methanol acidified with 0.1% formic acid (fraction II) and concentrated to dryness using a rotoevaporator (Buchmann R110 Rotavapor, Westbury, NY). Samples were redissolved in methanol acidified with 0.1% formic acid. For anthocyanins, crude methanolic extracts were directly used for analysis because there were no interfering signals or peak overlap.

**UPLC–Electrospray Ionization (ESI)/MS Analysis.** A Waters-ACQUITY UPLC/MS system (Waters Corp., Milford, MA) was used. The UPLC was equipped with a binary solvent manager, sample manager, column heater, and photodiode array e$\lambda$ detector and interfaced with a tandem quadrupole (TQD) mass spectrometer equipped with an ESI source. Anthocyanins were monitored at 520 nm, while flavonols were monitored at 360 nm. The column used was a Kinetex C18 column, 150 mm × 2.10 mm, 2.6 $\mu$m (Phenomenex, Torrance, CA). The column temperature for anthocyanins and flavonols was thermostatted at 50 and 40 °C, respectively. Mass spectrometric data of the eluted compounds from the column were acquired in positive mode for anthocyanins and negative mode for flavonols. Data acquisition and processing were performed using Empower 2 software (Waters Corp.). The MS scan was recorded in the range of 100–1000 Da. Nitrogen was used both as a drying gas and as nebulizing gas, while argon was used as the collision gas (AOC, Bryan, TX). The nitrogen gas flow conditions were 800 and 50 L/h for desolvation and at the cone, respectively. The source block temperature and desolvation temperature were set at 150 and 400 °C, respectively. Optimization of ionization conditions was based on the intensity of the mass signals of protonated/deprotonated molecules and aglycones fragments and was performed for each individual peak/compound detected. Mass parameters were optimized as follows: capillary voltage, 3.5/3.0 kV; and cone voltage, 40/30 V for positive/negative ionization, respectively. The MS/MS scan was optimized as follows: cone voltage, 40/30–55 V; and collision energy, 20/15–40 V for anthocyanins/flavonols, respectively. The purified phenolic extracts were dissolved in methanol/water (1:1) acidified with 0.05% formic acid. The solution was filtered by a syringe filter with a 0.2 $\mu$m PTFE membrane, and 1 $\mu$L was injected onto the LC column for UPLC-ESI-MS/MS analyses.

The solvents were 2% formic acid in $H_2O$ (solvent A) and acetonitrile (solvent B) for anthocyanin analysis and 0.05% formic acid in $H_2O$ (solvent A) and acetonitrile (solvent B) for flavonols. The gradient was 5% B from 0 to 2 min, 5–75% B from 2 to 27 min, 75% B isocratic from 27 to 30 min, 75 to 5% B from 30 to 31 min, followed by 5% B isocratic for 5 min to allow for column equilibration before the next injection. The flow rate was 0.4 mL/min.

**Quantification of Anthocyanins and Flavonols.** The method optimized for separation as described for UPLC above was tweaked for quantitative analysis using an Agilent 1200 HPLC system (Agilent Technologies, MD)[20] as described below. A Kinetex C18 column, 150 mm × 4.6 mm (instead of 2.1 mm), 2.6 $\mu$m, was used; the flow rate was adjusted to 1 mL/min. The HPLC procedure was used to allow for higher column loading; 5 $\mu$L of sample was injected. The gradient profile was programmed at 0% B from 0 to 2 min, 0–10% B from 2 to 8 min, 10–20% B from 8 to 15 min, 20–50% B from 15 to 30 min, 50–80% B from 30 to 35 min, 80–100% B from 35 to 40 min, followed by 5 min of isocratic elution at 100% B. A 2 min post-time was allowed for a system equilibration before each sequential injection. Other conditions were similar as described for UPLC system. Concentrations of the identified compounds in cowpea were calculated from the HPLC diode array detection (DAD) signal peak area, by interpolation based on calibration curves obtained using pure standards, and were expressed as micrograms per gram ($\mu$g/g) of dry seed ± standard deviation based on three separate runs. The assumption made for quantification of anthocyanin and flavonol derivatives with no available standards was that their molar absorptivity was similar to those of their aglycones, monoglycosides, or related diglycosides. In such a case, the standard used as the basis for quantification was specified. For flavonol compounds that eluted in both ethyl acetate and methanol fractions (obtained as described under "Extract Purification" above), peak areas from matching DAD signals in both fractions were added together to obtain total amount of the given compound in a sample.

## RESULTS AND DISCUSSION

**Anthocyanin Composition of Cowpeas; Influence of Seed Coat Color.** Using the UPLC system, complete separation of anthocyanins in cowpeas was completed within 7 min (Figure 1); by contrast, typical HPLC analysis of anthocyanins takes 40−60 min.[21] In the UPLC chromatograms acquired at 520 nm, eight distinct anthocyanin peaks were detected only in the black (IT95K-1105-5 and IT98K-1092-1) and green (TX2028-1-3-1) cowpea phenotypes. Identification of the anthocyanins was achieved by comparing elution profile, UV−vis spectra, and molecular and product ions to pure standards and literature information.[22,23] The eight anthocyanins identified in black and green cowpea phenotypes are listed in Table 2. The fact that anthocyanins were not detected in other colored cowpea phenotypes suggests that anthocyanin synthesis in cowpea has a strong genetic component.

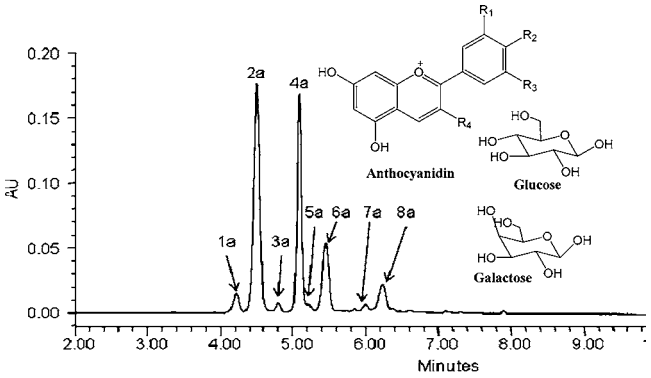


**Figure 1.** Representative UPLC chromatogram and structural backbones of anthocyanins found in the black and green cowpea varieties monitored at 520 nm. See Table 2 for peak identities.

Most of the anthocyanins identified have previously been reported in black cowpea.[22,24] The only new pigment that we detected in all three anthocyanin-containing samples in minor quantities was tentatively identified as petunidin-3-*O*-galactoside. The identity of this compound (peak 5a, $t_R$ = 5.20 min; Figure 1) was based on comparison to the petunidin-3-*O*-glucoside elusion profile (peak 6a, $t_R$ = 5.45 min), MS/MS profile (molecular ion at *m*/*z* 479, and one product ion at *m*/*z* 317 (M − 162 amu), and UV−vis profile (identical to the glucoside [$\lambda_{max}$ = 526 nm]) (Table 2). Flavonoid galactosides elute before glucosides on reversed phase HPLC.[18,25]

**Anthocyanin Content in Black and Green Cowpeas.** The anthocyanin contents of the raw black and green cowpeas are presented in Table 2. As expected, the black cowpea phenotypes had higher anthocyanin contents than the green cowpea. Among the black phenotypes, the larger seeded IT95K-1105-5 cowpea variety had a higher monomeric anthocyanin content (2094 μg/g) than the smaller seeded black IT98K-1092-1 variety (1676 μg/g); this was the opposite of what would be expected given that the pigments in cowpea are localized in the seed coat, and the smaller seeded line has a higher proportion of seed coat. This observation can be attributed to variety. Reports on the anthocyanin content of cowpea are limited. Ha et al.[26] recently reported 25 mg/g anthocyanins in the seed coat of a black cowpea variety; this agrees with our data given that the seed coat accounts for about 70−100 mg/g of cowpea seed weight.

In all three varieties, the most dominant pigment was delphinidin-3-*O*-glucoside (231−681 μg/g), followed by cyanidin-3-*O*-glucoside (209−549 μg/g) and then petunidin-3-*O*-glucoside (119−265 μg/g) (Table 3). Petunidin-3-*O*-galactoside, the newly indentified anthocyanin in cowpea, was the least (16−35 μg/g). The quantitative profile generally agrees with Ha et al.[26] and Chang and Wong,[24] who identified 3-*O*-glucosides of delphinidin and cyanidin as the major anthocyanin peaks in black cowpea seed coat. The similarity among anthocyanin profiles of the different cowpea varieties indicates that the nature of anthocyanins in cowpea might be largely species driven. Delphinidin-3-*O*-glucoside was also reported as the major anthocyanin in beans (*P. vulgaris*).[27]

**Flavonols in Different Cowpea Phenotypes.** The UPLC system enabled us to resolve all major flavonoid peaks in cowpea within 11 min (Figures 2 and 3). In general, fractionation of the extracts into methanol and ethyl acetate fractions improved UPLC resolution of some peaks and minimized coelution but was not useful as a tool to concentrate flavonols in a given fraction. A large number of the flavonols were detected in both the ethyl acetate and the methanol fractions, although in different proportions (Figures 2 and 3). This indicates that the use of solid phase fractionation can significantly distort overall profile of flavonols in a given sample, if all fractions are not subsequently characterized. The chromatographic information from both fractions (Figures 2 and 3) was thus useful for profiling the cowpea flavonols. Crude

**Table 2. Identification and Concentration (μg/g, d.w.) of Monomeric Anthocyanins in Black and Green Cowpea Varieties[a]**

| peak no. | $t_R$ (min) | $\lambda_{max}$ (nm) | MS [M + H]$^+$ | MS/MS [M + H]$^+$ | proposed compd ID | IT95K-1105-5 (black) | IT98K-1092-1 (black) | TX2028-1-3-1 (green) |
|---|---|---|---|---|---|---|---|---|
| 1a | 4.22 | 524 | 465 | 303 | delphinidin-3-*O*-galactoside[b] | 169 ± 5.2 | 143 ± 2.4 | 98.7 ± 0.8 |
| 2a | 4.50 | 524 | 465 | 303 | delphinidin-3-*O*-glucoside | 681 ± 5.3 | 508 ± 6.4 | 231 ± 8.3 |
| 3a | 4.79 | 516 | 449 | 287 | cyanidin-3-*O*-galactoside[c] | 152 ± 4.9 | 142 ± 3.2 | 62.2 ± 3.3 |
| 4a | 5.08 | 515 | 449 | 287 | cyanidin-3-*O*-glucoside | 549 ± 8.5 | 444 ± 6.0 | 209 ± 4.4 |
| 5a | 5.20 | 526 | 479 | 317 | petunidin-3-*O*-galactoside[d] | 35.2 ± 1.1 | 24.1 ± 3.0 | 15.7 ± 2.3 |
| 6a | 5.45 | 526 | 479 | 317 | petunidin-3-*O*-glucoside | 265 ± 3.6 | 202 ± 3.5 | 119 ± 4.4 |
| 7a | 5.99 | 525 | 463 | 301 | peonidin-3-*O*-glucoside | 41.1 ± 2.1 | 38.4 ± 1.1 | 29.3 ± 3.0 |
| 8a | 6.23 | 528 | 493 | 331 | malvidin-3-*O*-glucoside | 202 ± 4.8 | 174 ± 2.2 | 110 ± 1.9 |
| | | | | | total | 2094 ± 36 | 1676 ± 28 | 875 ± 29 |

[a]Peak numbers are referenced to Figure 1. All values are expressed as means ± SDs of triplicates on a dry weight basis (based on UPLC peak areas relative to pure standards). [b]Expressed as delphinidin-3-*O*-glucoside equivalent. [c]Expressed as cyanidin-3-*O*-glucoside equivalent. [d]Expressed as malvidin-3-*O*-glucoside equivalent.


dx.doi.org/10.1021/jf2052948 | J. Agric. Food Chem. 2012, 60, 3735−3744

**Table 3. Flavonols Identified in Different Cowpea Phenotypes**

| peak no. | $t_R$ (min) | $\lambda_{max}$; band I, band II (nm) | $[M - H]^-$ ($m/z$) | MS/MS fragments ($m/z$) | proposed compd ID |
|---|---|---|---|---|---|
| 1 | 5.80 | 354, 254 | 787 | 301(35), 300(100) | quercetin-3-*O*-triglucoside |
| 2 | 6.08 | 345, 266, 256 | 787 | 625(100), 300(6), 301(33) | quercetin-3-*O*-diglucoside-4′-O-glucoside |
| 3[a] | 6.29 | 357, 256 | 641 | 317(15), 316(100) | myricetin-3-*O*-diglucoside |
| 4 | 6.65 | 354, 256 | 757 | 301(7), 300(100) | quercetin-3-*O*-arabinosyl-diglucoside |
| 5 | 6.84 | 353, 255 | 625 | 301(15), 300(100) | quercetin-3-*O*-digalactoside |
| 6 | 6.92 | 352, 256 | 625 | 463(12), 301(100), 300(64) | quercetin-3,7-diglucoside |
| 7 | 6.97 | 354, 256 | 625 | 301(100) | quercetin-3-*O*-galactosylglucoside |
| 8[a] | 7.02 | 354, 256 | 625 | 301(43), 300(100) | quercetin-3-*O*-diglucoside |
| 9[a] | 7.10 | 357, 257 | 479 | 317(8), 316(100), 287(10) | myricetin-3-*O*-glucoside |
| 10 | 7.51 | 349, 265 | 609 | 285(80), 284(100) | kaempferol-3-*O*-diglucoside |
| 11 | 7.58 | 353, 256 | 595 | 301(5), 300(100) | quercetin-3-*O*-arabinosylglucoside |
| 12 | 7.65 | 355, 256 | 609 | 301(27), 300(100) | quercetin-3-*O*-galactosylrhamnoside |
| 13 | 7.76 | 354, 256 | 609 | 301(35), 300(100) | quercetin-3-*O*-glucosylrhamnoside |
| 14[a] | 7.81 | 371, 255 | 463 | 301(100), 300(15) | quercetin-7-*O*-glucoside |
| 15[a] | 7.93 | 355, 255 | 463 | 301(20), 300(100) | quercetin-3-*O*-galactoside |
| 16[a] | 8.05 | 354, 255 | 463 | 301(21), 300(100) | quercetin-3-*O*-glucoside |
| 17[b] | 8.29 | 379, 255 | 737 | 407(33), 394(100), 287(35), 271(50), 229(33) | unknown |
| 18 | 8.46 | 354, 256 | 549 | 505(13) 301(19), 300(100), 181(20) | quercetin-3-(6″-malonyl)-glucoside |
| 19[a] | 8.84 | 334, 250 | 801 | 625(100), 301(17), 300(10) | quercetin-3-(6″-feruloyl)-diglucoside |
| 20 | 9.16 | 363, 257 | 709 | 625(16), 301(36), 300(100) | quercetin-3-(6″-diacetoyl)-diglucoside |
| 21 | 9.51 | 355, 255 | 563 | 463 (7), 301 (30), 300 (100) | quercetin-3-(6″-succinoyl)-glucoside |
| 22 | 9.96 | 332, 251 | 815 | 607(15), 301(51), 300(100) | quercetin-3-(6″-sinapoyl)-rutinoside |
| 23[a] | 11.05 | 370, 255 | 301 | 179(44), 151(100), 121(24), 107(13) | quercetin |

[a]Previously identified in cowpea.[10,11] Values in parentheses represent relative signal intensity. [b]Identity not established.

extracts, however, produced fewer total peaks due to the problem of coelution (data not shown). Twenty-two flavonols were structurally characterized in the 10 cowpea varieties analyzed (Table 3). Out of the 23 flavonols, only eight had been previously identified in cowpea.[10,11] Identification and peak assignments for flavonols were primarily based on comparison of their retention times ($t_R$), UV−vis, and mass spectrometric data to authentic standards and literature information. Chemical structures of flavonols and their substituents identified in cowpea are presented in Figure 4.

Peak 1 ($t_R$ = 5.80 min, $\lambda_{max}$ = 354, 255 nm) was only present in the white cowpea and had a $[M - H]^-$ at $m/z$ 787. A single major MS/MS product was observed at $m/z$ 301 (M − 486), suggesting loss of three glycosyl units (3 × 162 amu) attached at the same position. The MS/MS product ion, coupled with an UV−vis profile, indicate that the aglycone of this compound is quercetin. Because MS/MS cleavage of glycosyl units occurs at the glycosidic bonds between the C ring and the sugars directly attached to it,[28] this compound is likely a triglycoside of quercetin. The position of attachment of the sugars can be deduced from the UV−vis profile of this compound relative to the quercetin aglycone (Table 3). The large hypsochromic shift in band I absorbance $\lambda_{max}$ (−16 nm) (Table 3) is characteristic of glycosyl substitution at position 3 or 5 of flavonols.[29] However, substitution of the −OH group at position 5 is normally accompanied by a concomitant hypsochromic shift in band II,[29] which was not observed in this case. This confirms that peak 1 is a 3-*O*-glycoside; thus, we identified this compound as quercetin-3-*O*-triglucoside, given that glucose is the most common hexose identified in cowpea and other pulses.

Similar to peak 1, peak 2 ($\lambda_{max}$ = 345, 266, 254 nm) had $[M - H]^-$ at $m/z$ 787 (Table 3). However, the peak 2 fragmentation pattern was different from that of peak 1; the MS/MS ion at $m/z$ 625 (M − 162 amu) corresponded to loss of one glucosyl moiety attached at one position, while the ion at $m/z$ 301 (M − 162 − 324 amu) corresponded to a further loss of two glucosyl moieties attached at a different position on a quercetin aglycone. Additionally, there was a large hypsochromic shift in band I $\lambda_{max}$ relative quercetin (−25 nm) as well as the appearance of an additional peak in band II spectrum at 266 nm. These factors are strongly indicative of glycosylation at positions 3 and 4′ of quercetin.[30] Thus, on the basis of a match of the UV−vis profile to literature[30] and the MS/MS data, we propose this compound to be quercetin-3-*O*-diglucoside-4′-*O*-glucoside, although the diglucoside could be at either position and vice versa. This compound was a minor component of the black, green, and white phenotypes. Glucosides of quercetin substituted at both positions 3 and 4′ are common, particularly in onions,[31] but have not been previously identified in pulses.

Peak 3 ($t_R$ = 6.29 min, $\lambda_{max}$ = 357, 256 nm) had $[M - H]^-$ at $m/z$ 641, which gave a fragment at $m/z$ 317 (M − 324 amu) corresponding to loss of two glycosyl units attached at the same position (Table 3). On the basis of its UV−vis profile, elution time, and MS data, peak 3 was identified as myricetin-3-*O*-diglucoside; this compound was previously reported in a black cowpea variety.[11] In this study, this compound was only detected in the black and red cowpea phenotypes.

Peaks 4 ($t_R$ = 6.65 min) had $[M - H]^-$ at $m/z$ 757. The MS/MS data showed only one fragment at $m/z$ 301 (M − 456 amu), corresponding to loss of two hexose units (2 × 162 amu) and one pentose unit (132 amu) linked together and bonded to a quercetin aglycone at one position. The most common pentose glycosides in nature are arabinose or xylose, with the former usually preferred.[32] Thus, on the basis of UV−vis profile and MS data, peak 4 was proposed to be quercetin-3-*O*-arabinosyl-diglucoside. The position of glycosylation was determined based on the logic provided for peak 1 above. This compound was present in all phenotypes. This is the first

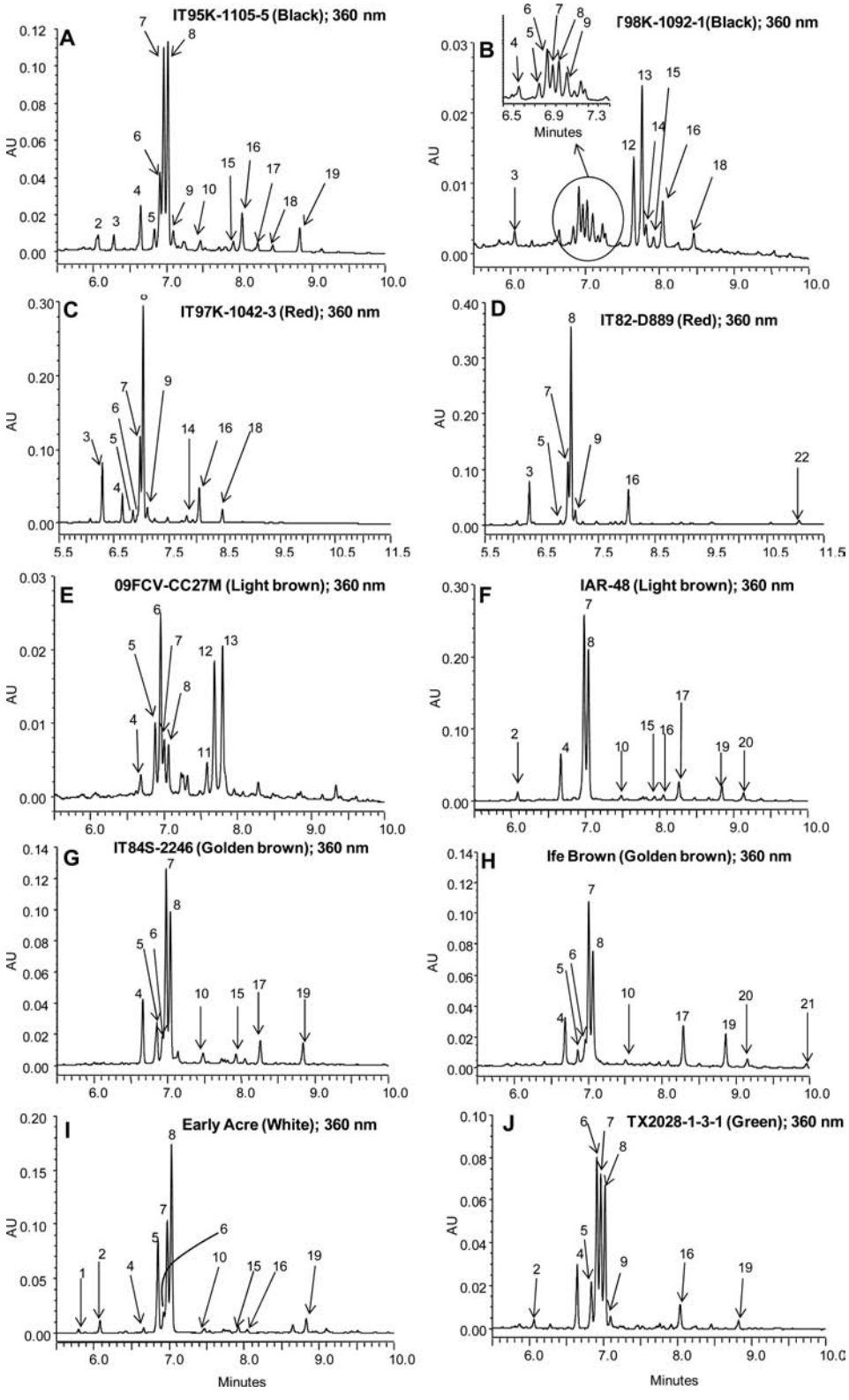


**Figure 2.** Reverse-phase UPLC chromatogram of methanol fractions of phenolic extracts from cowpea monitored at 360 nm. Peak identities are listed in Table 3.

time that pentose-substituted flavonols have been found in cowpea.

Peaks 5, 7, and 8 had similar MS parent ions ($[M - H]^-$ at $m/z$ 625) and produced one major MS/MS fragment at $m/z$ 301 corresponding to the loss of 324 amu (a dihexose unit) from a quercetin backbone. On the basis of the similarity of MS data and UV−vis spectra, peaks 5 ($t_R$ = 6.84 min), 7 ($t_R$ = 6.97 min), and 8 ($t_R$ = 7.02 min) were likely diglycoside isomers of quercetin substituted at the same position (C-3, given the hypsochromic shift in band I). Going by the retention times, it is logical to assume that the major difference among these peaks is the sugar moiety attached. The two common hexosides


dx.doi.org/10.1021/jf2052948 | J. Agric. Food Chem. 2012, 60, 3735−3744

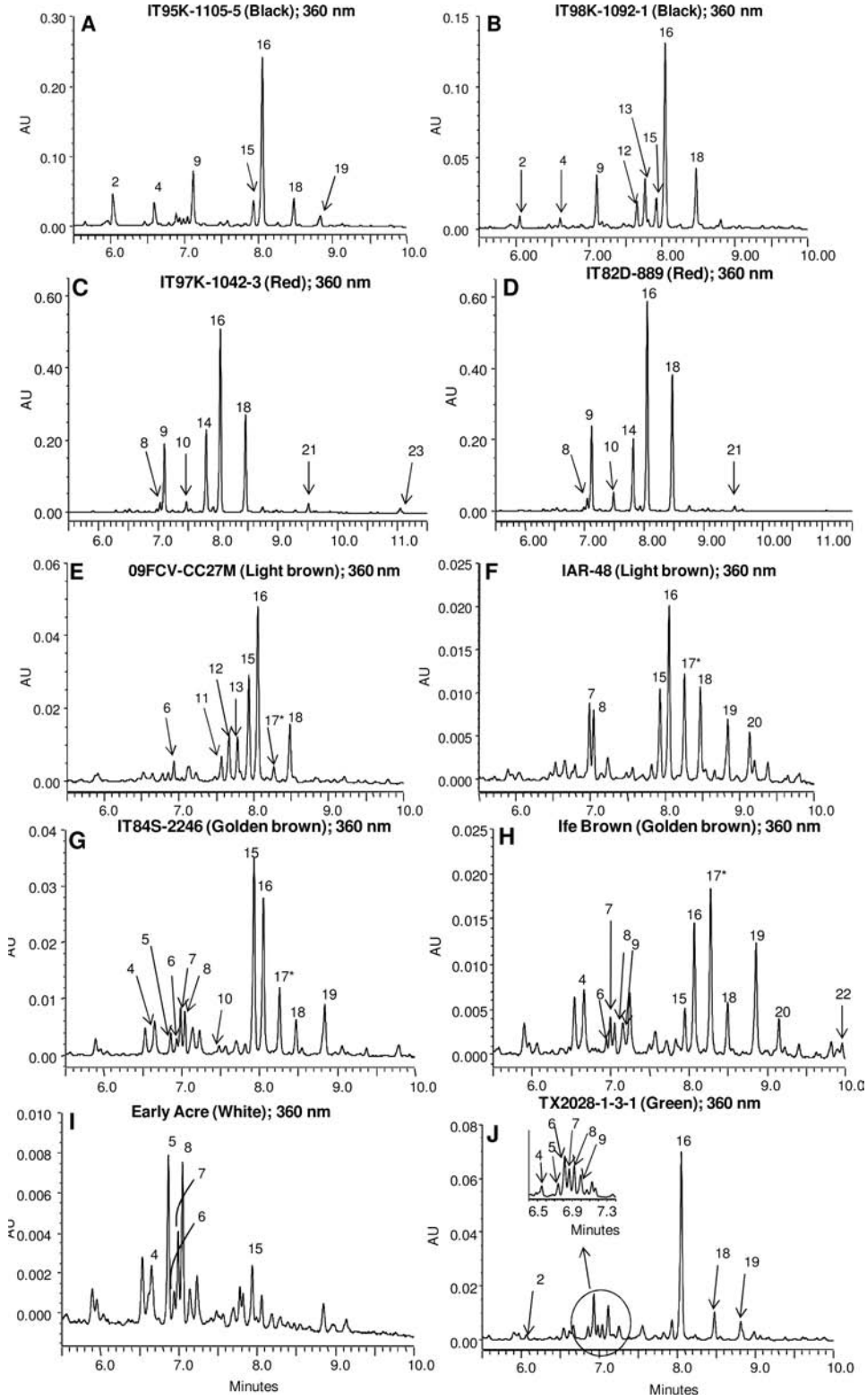


**Figure 3.** Reverse-phase UPLC chromatogram of ethyl acetate fractions of phenolic extracts from cowpea monitored at 360 nm. The peak marked (*) was unidentified. Peak identities are listed in Table 3.

of flavonols in pulses (including cowpea) are glucose and galactose.[10,11] These two sugars produce similar UV−vis profiles with flavonols; however, galactose typically elutes before glucose in reversed phase chromatography.[18,25] On the basis of the above reasoning, we assigned peak peak 5 as quercetin-3-*O*-digalactoside, peak 7 as quercetin-3-*O*-galactosylglucoside, and peak 8 as quercetin-3-*O*-diglucoside. Quercetin-3-*O*-diglucoside was previously identified in cowpeas.[10,11] However, digalactoside and galactosylglucoside of quercetin have not been reported in cowpea.

Peak 6 ($[M - H]^-$ at $m/z$ 625) had a similar parent ion as peaks 5, 7, and 8, but its fragmentation pattern was different


dx.doi.org/10.1021/jf2052948 | J. Agric. Food Chem. 2012, 60, 3735−3744

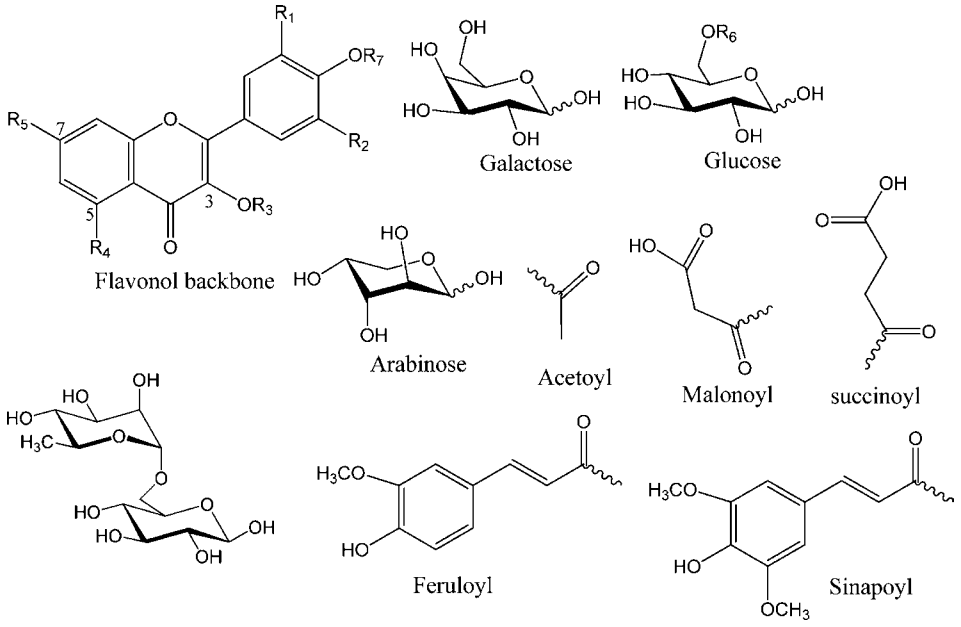


**Figure 4.** Chemical structures of flavonol derivatives identified in cowpea. $R_1 = R_2 = H$; $R_3 = R_4 = R_5 = OH$, kaempferol; $R_1 = R_3 = R_4 = R_5 = OH$, $R_2 = H$, quercetin; and $R_1 = R_2 = R_3 = R_4 = R_5 = OH$, myricetin. Glycosides: $R_3$; $R_5$, $R_7$ = galactose, glucose, arabinose, and rutinose. Acyl groups: $R_6$ = succinoyl, malonoyl, acetoyl, feruloyl, and sinapoyl residues.

(Table 3). Two sequential losses of 162 amu indicated that the hexoses were linked at different positions. On the basis of the similarity of its UV−vis profile to the other peaks (5, 7, and 8), one sugar was substituted at C-3. The fact that the other sugar substitution did not affect the UV−vis profile is indicative of glycosylation at position 7;[29,30] thus, this compound was most likely a quercetin-3,7-diglucoside. In general, the four diglucosides of quercetin were present in most varieties of cowpea as among the major flavonol peaks.

Peak 9 ($t_R$ = 7.10 min, $\lambda_{max}$ = 357 nm) produced a $[M - H]^-$ at $m/z$ 479 and an MS/MS spectrum with the predominant ion at $m/z$ 317 (M − 162 amu, loss of a glucosyl unit) (Table 3). On the basis of this MS spectrum and literature data, we established peak 9 as myricetin-3-*O*-glucoside. This compound was identified in all lines, except the light brown phenotypes; it was previously identified in cowpea.[10,11] Peak 10 ($t_R$ = 7.51 min, $\lambda_{max}$ = 349 nm) had MS profiles $[M - H]^-$ at $m/z$ 609 (Table 3). Upon MS/MS fragmentation, it produced a major signal at $m/z$ 285 (loss of 324 amu, corresponding to the loss two glycosyl units attached at the same position). The daughter ion, along with UV−vis profile, indicates that this compound is a diglycoside of kaempferol.[33] Coupled with literature data,[34] we identified this peak as kaempferol-3-*O*-diglucoside. This compound was a minor component of most cowpea lines studied.

Peak 11 ($\lambda_{max}$ = 353 nm), which was only present in the light brown 09FCV-CC27 M variety (Figure 2), had $[M - H]^-$ at $m/z$ 595 (Table 3). The major signal in MS/MS was $m/z$ 301 (M − 294), corresponding to loss of pentose (132 amu) + hexose (162 amu) attached at the same position on the aglycone. Because arabinose is the most common pentose,[32] peak 11 was designated as quercetin-3-*O*-arabinosylglucoside. Interestingly, the other compound with a pentose, quercetin-3-*O*-arabinosyldiglucoside (peak 4), was present in all phenotypes.

Peaks 12 and 13 had similar parent ion as peak 10 ($[M - H]^-$ at $m/z$ 609) . However, these peaks fragmented differently and also had different UV−vis profiles (Table 4). They both showed a loss of 308 amu ($m/z$ 301), corresponding to loss of a hexose (162 amu) + rhamnose (146 amu) attached at the same position. The daughter ion corresponded to quercetin. The two compounds were thus structurally related, with the only likely difference being the type of the hexose unit. On the basis of their relative retention times, peak 12 was proposed to be quercetin-3-*O*-galactosylrhamnoside, while peak 13 was positively identified as quercetin-3-*O*-glucosylrhamnoside (rutin) by comparison to authentic standard. These two compounds were major flavonol components of one light brown (09FCV-CC27M) and one black (IT98K-1092-1) variety (particularly in the methanol fraction) but were absent in all other lines (Figure 2). This suggests a strong genetic component regulating their synthesis in cowpea. Not surprisingly, they have not previously been identified in cowpea.

Peaks 14 ($t_R$ = 7.81 min), 15 ($t_R$ = 7.93 min), and 16 ($t_R$ = 8.04 min) showed $[M - H]^-$ at $m/z$ 463. They all had one major fragment ion at $m/z$ 301 (−162 amu) denoting loss of a hexose unit. All compounds were thus monoglycosides of quercetin. On the basis of UV−vis spectra, particularly the similarity in $\lambda_{max}$ of bands I and II to quercetin aglycone (Table 3), coupled with literature evidence,[11] peak 14 was identified as quercetin-7-*O*-glucoside. Peaks 15 and 16 were both glycosylated at position 3, based on the hypsochromic shift in their UV−vis spectra. Thus, going by their retention times and literature evidence, these compounds were positively identified as quercetin-3-*O*-galactoside and quercetin-3-*O*-glucoside, respectively. All three compounds have been identified in cowpea.[10,11] The 3-*O*-monoglycosides were present in all cowpea phenotypes, whereas the 7-*O*-glucoside was only present in the red phenotypes (Table 4 and Figure 2).

Peak 18 ($t_R$ = 8.46 min, $\lambda_{max}$ = 355 nm) had $[M - H]^-$ at $m/z$ 549 and showed MS/MS fragment at $m/z$ 301 (Table 3), corresponding to loss of 248 amu. This indicated a loss of hexose (162 amu) + malonic acid (86 amu). Acyl groups such as acetyl or malonyl usually occur as 6″-*O*-acetylglucoside or 6″-

Table 4. Concentration ($\mu$g/g, d.w.) of Identified Flavonols in Different Cowpea Phenotypes[a]

| phenotype | black | | red | | light brown | | golden brown | | green | white |
|---|---|---|---|---|---|---|---|---|---|---|
| variety | IT95K-1105-5 | IT98K-1092-1 | IT82D-889 | IT97K-1042-3 | IAR-48 | 09FCV-CC27M | Ife Brown | IT84S-2246 | TX2028-1-3-1 | Early Acre |
| quercetin-3-*O*-triglc[c] | ND[b] | ND | ND | ND | ND | ND | ND | ND | ND | 14.9 ± 2.2 |
| quercetin-3-*O*-diglc-4′-O-glc[c] | 34.1 ± 1.1 | 30.5 ± 3.1 | 20.6 ± 7.9 | 34.0 ± 3.1 | 27.5 ± 1.7 | ND | trace | 14.9 ± 2.2 | 35.2 ± 2.8 | 17.4 ± 1.6 |
| quercetin-3-*O*-arabinosyl-diglc[c] | 44.2 ± 0.7 | 23.9 ± 0.2 | 51.1 ± 8.2 | 93.0 ± 3.9 | 75.4 ± 4.9 | 23.7 ± 2.9 | 40.9 ± 8.24 | 91.9 ± 4.7 | 55.4 ± 4.5 | 11.4 ± 0.9 |
| quercetin-3-*O*-digal[c] | 17.8 ± 0.3 | 12.6 ± 0.3 | 12.5 ± 1.2 | trace | ND | 43.0 ± 8.7 | 32.4 ± 6.3 | 16.1 ± 1.3 | 22.9 ± 1.6 | 48.5 ± 6.3 |
| quercetin-3,7-diglc[c] | 12.3 ± 0.6 | 8.5 ± 1.3 | ND | ND | ND | 50.9 ± 2.8 | trace | 21.6 ± 2.9 | 84.8 ± 7.8 | 31.4 ± 9.2 |
| quercetin-3-*O*-galactosyl-glc[c] | 55.4 ± 1.4 | 12.5 ± 2.8 | 82.0 ± 7.0 | 70.9 ± 2.3 | 314 ± 3.2 | 14.7 ± 2.4 | 177 ± 4.5 | 188 ± 4.1 | 34.6 ± 3.1 | 33.7 ± 1.1 |
| quercetin-3-*O*-diglc[c] | 59.0 ± 2.0 | 16.1 ± 1.0 | 313 ± 6.2 | 334 ± 5.8 | 212 ± 5.0 | 16.1 ± 2.8 | 148 ± 2.1 | 157 ± 5.7 | 42.1 ± 1.4 | 77.2 ± 2.8 |
| quercetin-3-*O*-arabinosyl-glc[c] | ND | ND | ND | ND | ND | 24.0 ± 3.1 | ND | ND | ND | ND |
| quercetin-3-*O*-galactosyl-rha[d] | ND | 53.8 ± 3.4 | ND | ND | ND | 96.0 ± 4.7 | ND | ND | ND | ND |
| quercetin-3-*O*-rut | ND | 67.1 ± 3.6 | ND | ND | ND | 88.0 ± 3.4 | ND | ND | ND | ND |
| quercetin-7-*O*-glc[c] | ND | ND | 40.3 ± 0.8 | 52.2 ± 1.3 | ND | ND | ND | ND | ND | ND |
| quercetin-3-*O*-gal[c] | 23.0 ± 0.7 | 21.2 ± 2.8 | 12.6 ± 1.8 | 13.1 ± 0.9 | 37.7 ± 1.9 | 41.2 ± 4.1 | 34.0 ± 1.4 | 17.1 ± 2.1 | trace | 5.0 ± 0.7 |
| quercetin-3-*O*-glc | 76.9 ± 3.0 | 63.3 ± 7.8 | 114 ± 3.1 | 100 ± 2.6 | 46.8 ± 1.3 | 52.1 ± 3.4 | 23.8 ± 1.3 | 30.6 ± 2.9 | 48.7 ± 3.3 | trace |
| quercetin-3-(6″-malonoyl)-glc[c] | 20.8 ± 0.2 | 15.4 ± 0.7 | 56.5 ± 3.1 | 120 ± 4.1 | 25.8 ± 0.2 | 13.6 ± 0.7 | trace | trace | trace | ND |
| quercetin-3-(6″-feruloyl)-diglc[c] | 10.8 ± 0.2 | trace | ND | ND | 22.5 ± 1.6 | trace | 30.4 ± 4.7 | 18.4 ± 2.5 | 12.5 ± 1.1 | 14.5 ± 1.6 |
| quercetin-3-(6″-diacetoyl)-diglc | ND | ND | ND | ND | 20.0 ± 0.3 | ND | 20.2 ± 2.8 | trace | ND | ND |
| quercetin-3-(6″-sinapoyl)-rut[d] | ND | ND | ND | ND | ND | ND | 10.1 ± 1.9 | ND | ND | ND |
| myricetin-3-*O*-diglc[e] | 23.8 ± 1.4 | 19.3 ± 0.9 | 73.3 ± 2.1 | 75.4 ± 2.4 | ND | ND | ND | ND | trace | ND |
| myricetin-3-*O*-glc[e] | 23.2 ± 1.0 | 26.1 ± 1.8 | 85.4 ± 6.2 | 128 ± 5.6 | ND | ND | trace | ND | 15.7 ± 4.9 | trace |
| kaempferol-3-*O*-diglc[f] | 13.6 ± 2.4 | trace | 18.7 ± 1.0 | 38.7 ± 1.4 | 13.8 ± 0.9 | ND | 12.2 ± 1.9 | 13.0 ± 2.5 | ND | 15.8 ± 2.3 |
| total flavonols | 415 ± 14 | 370 ± 36 | 880 ± 35 | 1060 ± 30 | 796 ± 24 | 461 ± 33 | 528 ± 32 | 569 ± 24 | 352 ± 27 | 270 ± 29 |

[a]Data are based on HPLC quantification of major peaks only and are expressed as means ± standard deviations ($n$ = 3). [b]ND, not detected; trace, below the limit of determination. [c]As quercetin-3-glucoside equivalents. [d]As rutin equivalents. [e]As myricetin equivalents. [f]As kaempferol equivalents. Peaks that were not structurally identified are not included. Glc, glucoside; diglc, diglucoside; triglc, triglucoside; gal, galactoside; digal, digalactoside; rha, rhamnoside; and rut, rutinoside.

*O*-malonylglucoside.[35] Thus, peak 18 was tentatively identified as quercetin-3-(6″-malonoyl)-glucoside. Flavonoids containing 6″-malonoyl-glucoside groups have been identified in eggplants and red onions.[36] However, this is the first time that this quercetin derivative is identified in cowpea; it was present in all cowpea phenotypes.

Peak 19 ($\lambda_{max}$ = 334, 250 nm) had precursor ion $[M - H]^-$ at $m/z$ 801. The MS/MS spectrum showed product ions at $m/z$ 625 and at 301 (Table 4), an indication of cleavage of two groups: feruloyl moiety (−176 amu) and a diglucoside (− 324 amu). Fragmentation of acylglycosides can occur at both the glycosidic linkage and the acyl linkage.[21] Thus, even though there are two fragments, the ferulic acid and sugars are likely linked to the quercetin backbone at the same position. Additionally, the UV−vis profile of this compound, particularly the large hypsochromic shift in band I (−36 nm) and smaller shift in band II (−5 nm) relative to quercetin, matched that of a similar compound identified in *Brassica*.[37] Thus, we can designate this peak as quercetin-3-(6″-feruloyl)-diglucoside. This compound was present in all cowpea phenotypes except the red ones (Figure 2); it was previously reported in a black cowpea variety.[11]

Peak 20 showed $[M - H]^-$ at $m/z$ 709; fragmentation of this compound produced ions at $m/z$ 625 (M − 84 amu) and 301 (M − 84 − 324 amu). This indicates the presence of a diacetyl group (2 × 42 amu) and diglucosyl moiety (2 × 162 amu), in which the two acetyl groups are attached onto the two sugar moieties, with a quercetin backbone. Coupled with UV−vis profile and elution time, we propose this peak as quercetin-3-(6″-diacetyol)-diglucoside. The compound was only present in golden brown phenotypes and one light brown variety (IAR-48) (Figure 2) and has not been previously identified in cowpea. Peak 21 had $[M - H]^-$ at $m/z$ 563; its MS/MS fragmentation gave one minor ion at $m/z$ 463 (−100 amu), suggesting loss of a succinyl moiety and major ion at $m/z$ 301 (M − 100 − 162), indicating additional loss of a hexose from a quercetin backbone. On the basis of its elution profile, UV−vis, and MS data, we propose this compound to be quercetin-3-succinoyl glucoside; it was only present in the red phenotypes (Figure 2).

Peak 22 ($\lambda_{max}$ = 332, 251 nm) had $[M - H]^-$ at $m/z$ 815; the UV−vis profile was suggestive of a cinnamic acid−acyl derivative of quercetin as previously explained for peak 19. The fragment at $m/z$ 301 (M − 514 amu) was attributed to the


dx.doi.org/10.1021/jf2052948 | J. Agric. Food Chem. 2012, 60, 3735−3744

loss of rutinose (6-*O*-α-L-rhamnosyl-D-glucose; 308 amu) acylated to sinapoyl moiety (206 amu). Thus, peak 22, which was only found in Ife Brown (golden brown) variety (Figure 2), is tentatively designated as quercetin-3-(6″-sinapoyl)-rutinoside.

Finally, peak 23 ($t_R$ = 11.05 min) had $[M - H]^-$ at $m/z$ 301 and a fragmentation pattern corresponding to the quasi molecular ion of quercetin in the negative ionization mode (Table 4). Its identity as free quercetin was confirmed by matching its chromatographic and MS/MS fragmentation profiles with authentic standard. The free form of quercetin was only present as a minor component in IT82D-889 (red) and white cowpea varieties.

On the basis of our observations, it is obvious that flavonols occur in cowpea primarily as glycosides and acylglycosides. Overall, 23 flavonol compounds were identified in the cowpea varieties, most of which are newly identified in cowpea (Tables 3). The majority (19 compounds) were quercetin derivatives, with diglycosides of galactose and glucose as the most common followed by their monoglycosides. Myricetin derivatives were only found in black, red, and green phenotypes. Only one kaempferol derivative (kaempferol-3-*O*-diglucoside) was detected, and it was present as a minor component of most phenotypes, except green. Even though there are major flavonol compounds common to all cowpea phenotypes, important differences exist in the accumulation of a significant number of flavonols based on phenotype and, to some extent, variety. This information provides a good foundation upon which future studies linking nutritional properties of cowpea with specific phenotypes/varieties can be built.

**Flavonol Content of Different Cowpea Phenotypes.** Seed coat color had a major influence on flavonol composition and content of cowpea. The average flavonol content was highest in the red phenotype (mean = 970 μg/g), whereas the white variety had the lowest (270 μg/g) (Table 4). Most of the other phenotypes had limited variability in their flavonol content with the exception of the light brown phenotypes. Among the light brown lines, the IAR-48 had much higher flavonol content (796 μg/g) than the 09FCV-CC27 M line (461 μg/g). It is important to note that in preliminary screening assays, the IAR-48 had approximately one-third the condensed tannin content (measured as catechin equivalents) of the 09FCV-CC27 M line (data not shown). This suggests that accumulation of flavan-3-ols (tannins) may be sacrificed for flavonol synthesis in light brown cowpea. We are currently investigating flavan-3-ol accumulation in various cowpea phenotypes.

Diglycosides of quercetin were generally the most abundant flavonols in most cowpea varieties (Table 4), whereas monoglycosides were also important components of the red and black phenotypes. Previous studies reported high levels of quercetin (413 μg/g) and myricetin (51.3 μg/g) aglycones in black cowpea accessions after acid hydrolysis.[2] Curiously, quercetin-3-*O*-glucosylrhamnoside (rutin) and quercetin-3-*O*-galactosylrhamnoside accounted for 33 and 40% of flavonols in one black (IT98K-1092-1; 121 μg/g) and one light brown (09FCV-CC27M; 184 μg/g) variety, respectively, but were absent in all other samples. Another major difference was that myricetin glucosides were only detected in the black, red, and green cowpea phenotypes, with much higher levels in the red (159−203 μg/g) than the black and green phenotypes (16−47 μg/g) (Table 4). The myricetin contents reported for some cool season food legumes are as follows: green pea, 36.2 μg/g; yellow pea, 36.7 μg/g; chick pea, 32.1 μg/g; and lentils, 33.3.[38]

The genetic basis for these differences in flavonol composition of cowpea should be investigated. Particularly interesting would be how the variations in flavonol composition of the cowpea phenotypes affect their biological properties, including micronutrient bioavailability and various markers of disease prevention.

Seed coat color has a major impact on the accumulation of specific flavonoid compounds and their levels in cowpea. This is important because seed coat color is a primary basis upon which the selection of legumes for consumption is based in various societies. Knowledge on how the cowpea seed coat color affects flavonoid accumulation is of great relevance to strategies aimed at improving not only bioactive properties of cowpea but also micronutrient bioavailability. In this study, we demonstrate that red cowpea phenotypes accumulate the most flavonols, whereas only black and green phenotypes accumulate anthocyanins. The influence of seed coat color flavan-3-ol composition of cowpea is currently under investigation. Information on the heritability of seed coat color may shed some light on the genetic control of biosynthesis of favonols and anthocyanins in cowpea.

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### Funding

This project was partially sponsored by USAID Dry Grain Pulses CRSP (Grant 61-258).

### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

We thank Drs. Jeff Ehlers and Philip Roberts of the University of California−Riverside and B. B. Singh of Texas A&M University for providing the cowpea samples.

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