Quantitative Analysis of Crocetin Colorants in Gardenias (Gardenia jasminoides Ellis) by LC/DAD/MS

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Abstract: For the quantification of yellow coloring materials in gardenia utilized as natural food colorants and for dyeing fabrics, the fruits of fully matured Gardenia jasminoides were characterized by LC/DAD/MS analysis. The five yellow coloring materials with crocetin chromophore were analyzed by HPLC and characterized by DAD and MS analysis. The molar compositions of each crocetin colorant were measured as follows; trans-crocetin di(β-gentiobiosyl) ester 1 [58 mol %], trans-crocetin mono(β-gentiobiosyl) monoglucosyl ester 2 [13 mol %], trans-crocetin diglucosyl ester 3 [20 mol %], crocetin 4 [7 mol %] and trans-crocetin monoglucosyl ester 5 [2 mol %]. With molar absorptivity of the major colorant I that was first measured as ε 103000 dm$^3$ mol$^{-1}$ cm$^{-1}$ at λ max 440 nm, total crocetin content per mass of dried whole gardenia fruit was quantified as 3.4 μ mole/g of crocetin aglycone.

Keywords: crocin, crocetin, gardenia jasminoides, HPLC/MS analysis, molar absorptivity

Introduction

Recently, a lot of interest has been focused on manufacturing traditional dyeing with natural vegetable colorants [1,2], and industrial activities are beginning to revive [3]. Yellow colorants from gardenias have been widely used for coloring textiles [4] and food [5]. It is also interesting that the major colorant, crocin, has long been used as a food additive, and may act as an antioxidant by quenching singlet oxygen or free radicals [6]. Crocin, digentiobiosyl ester of crocetin, is the main pigment of the fruit of gardenia (Gardenia jasminoides E,) and is one of the freely soluble carotenoids found in nature.

With growing concern over the safety of synthetic dye as well as over synthetic food additives, the importance of natural substitutes suitable for use in food and textile dyeing has gained increasing attention. Thus, the characterization of its physical and chemical properties is worthwhile. Although many researchers have reported on isolation and characterization of the yellow crocetin derivatives [7], a quantitative analysis of the crocetin derivatives found in gardenia has not yet been reported. Our recent complete characterization of crocin from gardenia can provide a clue to a quantitative analysis [8]. The objective of this study was to further quantitatively analyze the yellow colorant crocetin derivatives from gardenia using modern chromatographic and spectroscopic techniques (HPLC-UV/Visible Diode array detector (DAD)-MS).

Experimental

Material

Fully matured gardenias were harvested on December 10, 1999 at Yangsan, Kyungnam Province, dried in the shade at ambient temperature and stored in a refrigerator. HPLC grade solvents, methanol and water were purchased from Merck. Formic acid for HPLC/MS was purchased from Duksan Chemical Co., LTD.

Sample Preparation and Measurement of UV/Visible Spectrum of Whole Gardenia Extract and Measurement of Molar Absorptivity of Crocin

Air-dried whole gardenia samples were dried in an oven
at 70 °C for 3 days. A whole dried fruit was extracted with 300 mL of water three times at room temperature for 24 h with stirring. The combined extracts were filtered through a pad of celite, and the filtrate was diluted with distilled water to 1000 mL. The aliquot of the yellow solution was placed in a 1-cm light path quartz cuvette, and the UV/Vis spectra were obtained on a Shimadzu UV 2100 spectrophotometer in the 200–700 nm range.

The absorption spectrum of pure crocin was recorded in distilled water using a concentration of 5.771 × 10⁻⁶ mol/L, λ max (ε max) = 440 nm (103000) and 466 (93000). The isolation and purification of pure crocin had been reported in the previous paper [8].

HPLC/DAD/MS Analysis of Whole Gardenia Extract

The aliquot of the yellow aqueous solution was diluted 10 times with distilled water for HPLC analysis, before being filtered through a syringe filter (OSMONICS INC., Cameo 13N Nylon, 0.22 Micron, 13 mm) prior to HPLC injection. Analysis of the whole gardenia extract samples was performed using a Hewlett Packard LC/DAD/MS mass spectrometer (HP 1100 model). The chromato graphy was done on an analytical reverse column (HP Eclipse XDB-C18, 4.6 × 150 mm, 3.5 μ) with a gradient. The gradient started at 50% (MeOH/water, 25 mM formic acid), and increased linearly to 80% (MeOH/ water, 25 mM formic acid) over a 30 min period. Three dimensional HPLC chromatograms were scanned by DAD and stored at 200 to 700 nm with 4 nm resolution. Mass spectra were obtained in API-ES positive scan mode at m/z 100 to 1200 every two seconds. Ionization was conducted at a flow rate of 10 L/min with nitrogen drying gas under Nebulizing press 40 psi and in positive 70 eV fragmentator polarity.

Results and Discussion

The five yellow coloring materials with visible chromophore from the fruits of fully matured Gardenia jasminoides were detected by HPLC analysis. The HPLC chromatogram-UV/Visible spectra monitored with DAD is presented in Figure 1. Three components were notable, yet the other two were negligible in quantity. The five colorants had the same absorption spectral properties which exhibited characteristic absorption bands at 466 nm and 440 nm with a shoulder around 418 nm. This suggests that the structure of chromophore is trans-crocin, which has been completely characterized by UV/Visible and NMR techniques [8]. In recent work, Ichi and coworkers. reported that seven yellow pigments from the fully matured gardenia were characterized as crocin ester derivatives, yet the two cis-crocin derivatives, cis-crocin monogentiobiosyl ester and cis-crocin monoglucosyl ester, were insignificant [5]. In our samples, cis-crocin derivatives could be negligibly present, as they were not detected.

Each component in the extract was further characterized with an LC/Mass spectrometer. The major colorant at retention time (RT) 6.8 in HPLC chromatogram was identical with the authentic crocin isolated by a preparative HPLC technique, and fully characterized as trans-crocin di(β-gentiobiosyl) ester 1 (crocin) from the previous study by NMR and UV/Visible spectroscopy [8]. It was further confirmed as crocin with molecular formula C₄₄H₅₀O₁₄ (976.379 amu) by LC/MS (Figure 2-A). Here a peak at m/z 999.6 (100%) as a base peak with expected isotopic clusters at m/z 1000.8 (46%) and m/z 1001.6 (15%) is clearly shown. Often in LC/MS analysis with API-ES ionization, the molecular ions appeared as Na⁺ complexes, resulting in a peak at 23 amu higher than expected [9]. The expected molecular ion [M⁺] appeared at m/z 976.4 with only a 3% relative abundance.

The second component at RT 10.1 in HPLC was identified as crocin monogentiobiosyl monoglucosyl ester 2 with molecular formula C₄₆H₅₂O₁₆ (814.326 amu). The molecular ion [M + Na⁺] appeared as a base peak at m/z 837.6 with [M + Na + 1] at m/z 838.4 (43%) and [M + Na + 2] at m/z 839.6 (7%) shown in Figure 2-B. The third component at RT 22.6 is the second major colorant, crocin diglucosyl ester 3 with molecular formula C₅₀H₅₄O₁₄ (652.273 amu). The molecular ion cluster consists of m/z 675.6 (100%) [M + Na⁺], m/z...
Figure 2. The API-ES mass spectra of (A) trans-crocin di(β-gentiobiosyl) ester 1 (crocin), (B) crocin monogentiobiosyl monoglucosyl ester 2, (C) crocin diglucosyl ester 3, (D) crocetin 4, and (E) crocetin monoglucosyl ester 5.

676.4 (42%) and m/z 677.6 (10%), as shown in Figure 2-C. The fourth component at RT 23.0 was analyzed as crocetin itself 4 from the base peak at m/z 353.2, shown in Figure 2-D. This base peak appeared at 2 amu higher than expected at m/z 351.2 for [M + Na]^+, which could not be interpreted reasonably. However, the peaks at m/z 361.2 (12%) and 369.2 (9%) could be assigned as [M + CH₃OH + H]^+ and [M + Na + H₂O]^+, respectively, as these kinds of solvated species are often observed [9]. The least minor component at RT 25.1 was unambiguously identified as crocetin monoglucosyl ester 5 with molecular formula C₃₀H₄₀O₁₄ (490.220 amu), as shown in Figure 2-E.

The identification of each component well agrees with the hydrophilicity of the compound under the performed chromatographic condition: the more hydrophilic carbohydrate moieties on crocetin aglycone, the faster elution under the reverse phase chromatography condition. Each compound name, chemical structure, molecular formulae and exact mass are summarized in Chart 1.

The UV/Visible spectrum of isolated pure crocin in water exhibits characteristic absorption bands at 466 nm (ε 93000 dm³ mol⁻¹ cm⁻¹) and 440 nm (ε 103000) with a shoulder around 418 nm, as shown in Figure 3-A. These characteristics are comparable with those taken from methanol solution; where absorption bands were at 458 nm (ε 96600) and 433 nm (ε 103500) with a shoulder around 410 nm [8]. The maximum absorptions slightly shifted to longer wavelengths, but molar absorptivity at wavelength maxima λₘₐₓ 440 nm was almost identical both in water and in methanol.

Each UV/Visible spectrum of the five crocetin derivatives in HPLC chromatogram was taken from a DAD spectrum, and these had the same pattern of absorption characteristics.

A representative UV/Visible spectrum of crocetin diglucosyl ester 3 is shown in Figure 3-B. This shows two distinctive absorption maxima at 440 nm (rel abs 0.521) and 460 nm (rel abs 0.507), and a shoulder around 410 nm, which were identical with those taken from isolated pure crocin aqueous solution.

If the molar absorptivities of crocetin derivatives were assumed to be alike, each component of crocetin derivatives in a gardenia fruit could be quantified from the DAD-HPLC spectrum and the UV absorbance of a whole gardenia. This assumption is reasonable because all crocetin derivatives have the same aglycone chromophore, and sugar substituents transparent in the visible range do not affect UV/visible absorption characteristics. The composition ratios of each component in a whole gardenia could be calculated from maximum absorbances.
Figure 4. UV/Visible spectrum of whole gardenia aqueous extract.

at \( \lambda_{\text{max}} \) 440 nm in DAD-HPLC spectrum, as summarized in Table 1.

A representative UV/Visible spectrum of the whole gardenia aqueous extract is shown in Figure 4. It shows the characteristic absorption maxima due to crocetin chromophore at 440 (0.352) and 466 (0.260) nm, and strong absorption around 320 nm, which is probably due to hydrophilic carbohydrates that could be identified from HPLC/DAD spectrum in Figure 1.

With molecular absorptivity \( 103000 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1} \) at \( \lambda_{\text{max}} \) 440 nm, molar quantity of crocetin aglycone present in unit mass of a whole dried gardenia was quantified as 3.4 mole/g of crocetin aglycone. In Table 2, the absorbance on five samples of gardenias, that is, the absorbances of the extract in 1 L of water, weight and contents of crocetin aglycone are presented. Content of crocetin aglycone per unit mass of dried gardenia was reproducible within experimental error range. From the contents of crocetin aglycone and relative composition ratio of crocetin derivatives present in gardenia fruit, the absolute quantity of the each crocetin derivatives was quantified and then summarized in Table 3.

Weight percentage of each crocetin component was comparable with those reported by Ichi and coworkers, yet the minor components were not detected in the sample [5a]. However, from the molar absorptivity of pure crocin that was first measured, the contents of each crocetin colorant in the gardenia were easily quantified by LC/DAD/MS analysis, and the whole molar content of crocetin colorant was estimated simply through measuring the absorbance of whole gardenia aqueous extract.

### Conclusion

The five yellow coloring materials with crocetin chromophore from the fruits of fully matured *Gardenia jasminoides* were characterized by LC/DAD/MS analysis.
Three components were notable, yet remaining two were negligible in quantity. The molar compositions of each crocetin colorant were measured as follows; trans-crocetin di(β-gentiobiosyl) ester 1 [58 mol%], trans-crocetin mono(β-gentiobiosyl) monoglucosyl ester 2 [13 mol%], trans-crocetin diglucosyl ester 3 [20 mol%], crocetin 4 [7 mol%] and trans-crocetin monoglucosyl ester 5 [2 mol%]. With the molar absorptivity of the major colorant 1 first measured as $\varepsilon = 103000$ dm$^3$ mol$^{-1}$ cm$^{-1}$ at $\lambda_{\text{max}} = 440$ nm, the total crocetin content per mass of whole dried gardenia was quantified as 3.4 µmol/g of crocetin aglycone.

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**References and Notes**


