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Flavonoid Quantification in Onion by Spectrophotometric and High Performance Liquid Chromatography Analysis

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Abstract. **Direct spectrophotometric determination of quercetin content in onions (*Allium cepa* L.) was investigated as a possible alternative to high-performance liquid chromatography (HPLC) analysis. Quercetin content in five onion varieties was monitored at 362 nm and quantified using simple spectrophotometric and HPLC methods. HPLC revealed that 3,4'-Qdg and 4'-Qmg comprised up to 93% of total flavonol content detected in the studied varieties. These major quercetin conjugates combined (3,4'-Qdg + 4'-Qmg) and total flavonol conjugates quantified by HPLC correlated closely with spectrophotometer values. Correlation coefficients were 0.96 ($P < 0.0001$) for 3,4'-Qdg + 4'-Qmg and 0.97 ($P < 0.0001$) for total flavonol conjugates in onion. Simple spectrophotometric procedure proved to be a valid, efficient, and cost-effective method for the quantification of total quercetin in onion. Chemical names used: quercetin-3,4'-*O*-diglucoside (3,4'-Qdg); quercetin-4'-*O*-glucoside (4'-Qmg).**

Phytochemicals are biologically active compounds present in edible foods that when ingested, have the potential to prevent or delay the onset of disease (as reviewed by Guhr and Lachance, 1997). Flavonoids are a specific class of phenolic plant phytochemicals represented by over 5000 compounds, subdivided into 13 categories that include anthocyanidins, catechins, flavonols, and flavones (as reviewed by Croft, 1998). The flavonol quercetin has shown much promise as an antioxidant agent, imparting a protective effect in reducing the risk of developing cardiovascular disease (Hertog and Hollman, 1996; Keli et al., 1996; Knekt et al., 1996), and certain types of cancer (Knekt et al., 1997; Leighton et al., 1992). High concentrations of quercetin occur in onion (*Allium cepa* L.) (Hertog et al., 1992; Leighton et al., 1992; Price and Rhodes, 1997), and the amounts vary with bulb color and type (Bilyk et al., 1984; Patil et al., 1995). Three predominant forms of quercetin in onion are quercetin aglycone, quercetin-3,4'-*O*-diglucoside and quercetin-4'-*O*-glucoside (Leighton et al., 1992; Price and Rhodes, 1997) (Fig. 1).

Quantification of flavonoid compounds often employ methods of separation combined with ultraviolet (UV) detection (Harborne, 1984; Jurd, 1962; Mabry et al., 1970; Price and Rhodes, 1997; Seikel, 1962). In onions, concentrations of quercetin aglycone isolated by TLC (thin layer chromatography) have been estimated spectrophotometrically using VO_2^+ (vanadyl) metal ions as a shift reagent (Kaushal et al., 1978). These techniques have largely been replaced by high-performance liquid chromatography (HPLC), which has become one of the most important tools in the separation and identification of flavonoids from raw plant extracts. Indeed HPLC has been used for the quantification of quercetin in onions by several research teams (Bilyk et al., 1984; Crozier et al., 1997; Hertog et al., 1992; Patil et al., 1995; Price and Rhodes, 1997; Rhodes and Price, 1996).

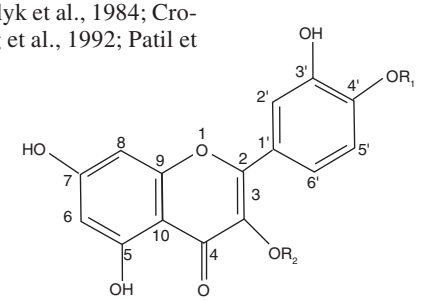
Evaluation of quercetin content in onion is of interest to plant breeders, food technologists and nutritionists alike to assess breeding lines for genetic selection purposes or monitor levels during storage or processing. This interest has underscored the need for rapid, efficient quantification methods. HPLC methods, although more precise, are costly and require a trained operator. Simple spectrophotometric assays allow more rapid analysis (Mayhew et al., 1984; Lin and Wagner, 1994). We address here the precision of spectrophotometer values as a reflection of total quercetin, which represents the majority of flavonol content in onion (Price and Rhodes, 1997). We compared values obtained with simple spectrophotometer readings to the cumulative amounts of individual flavonols identified through HPLC analysis.

Materials and Methods

Plant material. Five long-day, storage-type onion varieties consisting of one red skinned variety purchased from a local grocery store (Albertsons, Lubbock, Texas, presumed to be of one variety) and four yellow skinned varieties: Rio Rita, RNX 10968, Predator (Rio Colorado Seed Co., Bakersfield, Calif.) and Tamara (Bejo Zaden Seed Co., Warmenhuizen, Netherlands) were used in this study. Ten bulbs were selected randomly from each of the five varieties and stored at 4 °C until sampled.

Sample preparation. Over the course of 10 d, one bulb from each of the five varieties was selected randomly each day for quercetin extraction. Outer, inedible portions from bulbs were removed. Onions were quartered; one quarter section was weighed then frozen in liquid nitrogen. Frozen tissue was ground to a fine powder using a coffee grinder (Braun, Boston).

Extraction. Quercetin was extracted from 20-g ground onion powder in 80 mL of 80% EtOH (Patil et al., 1995) by filtering twice through number 8 (Fisher Scientific, Houston) and grade 42 (Whatman; Clifton, N.J.) filter paper. Filtrate was collected in 2-mL Eppendorf tubes and placed into storage at –20 °C until analysis.



Compound	R_1	R_2
isoquercitrin (quercetin-3-O-glucoside)	H	glucose
quertetin aglycone	H	H
quercetin-3, 4'-O-diglucoside	glucose	glucose
quercetin-4'-O-glucoside	glucose	H

Fig. 1. Quercetin compounds measured. Redrawn in part from Price and Rhodes (1997).

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Spectrophotometer analysis. Ethanolic extracts were thawed, vortexed, and diluted 10:1 with 80% EtOH to a total of 5 mL. Absorbance (AU) readings were made in duplicate at 362 nm using a Spectronic Genesys 5 (Waltham, Mass.) spectrophotometer.

HPLC analysis. HPLC analyses were conducted using a Dionex GP 40 gradient pump, AD 20 UV detector fixed at a wavelength of 362 nm, combined with an AS 3500 auto sampler (Dionex Corp, Sunnyvale, Calif.). Quercetin conjugates were separated by injecting a 50-µL sample onto a Waters µBondapak C18 (Milford, Mass.) (3.9 × 300 mm diameter) column coupled to a Waters µBondapak C18 guard column. Two separate injections were made for each sample extract. HPLC grade solvents were used in a gradient run with 98% H_2O : 2% THF : 0.1% TFA (solution A), and 100% acetonitrile (solution B) at a flow rate of 1 mL/min (Price and Rhodes, 1997) over a 40 min modified gradient as follows to optimize peak separation: isocratic 17% B for 2 min; gradient to 90% B for 20 min; gradient to 95% B for 1 min: isocratic 95% B for 2 min; gradient to 17% B for 2 min; and isocratic 17% B for 15 min to reequilibrate the column.

Quercetin quantification. Identical standard curves of isoquercitrin (quercetin-3-*O*-glucoside; Extrasynthese; Genay, France) were made for quercetin quantification by spectrophotometer and HPLC methods. Maximum absorbancy for isoquercitrin was determined using a scanning spectrophotometer (Beckman model DU530 UV-vis, Fullerton, CA) in 1-cm quartz cuvettes. Absorbancy values (spectrophotometer) and peak areas (HPLC) for each level of the standard curve were recorded between every 25 samples for calibration purposes. Quercetin dihydrate (3,3',4',5,7-pentahydroxyflavone), and kaempferol (3,5,7,4'-tetrahydroxy flavone) (Sigma-Aldrich; St. Louis) were also used as standards for identification of other flavonol compounds during HPLC analyses.

Concentrations of quercetin conjugates and other flavonols identified by HPLC in sample extracts were calculated by linear regression onto the standard curve of isoquercitrin. Likewise, concentrations of total flavonols derived by spectrophotometer measurement were calculated by regressing absorbancy readings from sample extracts onto the standard curve of isoquercitrin. The relationship of peak area vs. concentration for HPLC analyses and AU vs. concentration for spectrophotometer analyses were used to express results from both methods on a relative basis in mg·kg^{-1} fresh weight (fwt).

Data analysis. Analysis of variance (n = 10 bulbs/variety) was performed in SAS (SAS Inst., Cary, N.C.) using general linear model (Proc GLM) and correlation procedure (Proc Corr Cov). Means were separated by Fisher's protected least significant difference (LSD) test ($P < 0.05$).

Three correlation analyses were performed on values obtained by the spectrophotometer compared to values obtained by HPLC and included: 1) spectrophotometer values compared to main quercetin glucoside (3,4'-Qdg + 4'-Qmg) combined (HPLC); 2) spectrophotometer values compared to total quercetin (3,4'-Qdg + 3-Qmg + 4'-Qmg + Q combined; HPLC); and 3) spectrophotometer values compared to TF represented by 3,4'-Qdg + 3-Qmg + 4'-Qmg + 3-Img+ Q + K (HPLC). In order to perform correlation analyses, spectrophotometer and HPLC values (expressed in mg·kg^{-1} fwt) were grouped separately and all varieties were pooled (n = 50).

Results and Discussion

Isoquercitrin was used for quantification purposes as an external standard because it is similar chemically to the major quercetin glucosides found in onions (Hirota et al., 1998) (Fig. 1). The standard curve of isoquercitrin was established to resolve the ranges of quercetin concentration in onions as reported by Bilyk et al. (1984) and Patil et al. (1995). Serial dilutions ranged from 0.00 to 87.50 µg·mL^{-1} in concentration.

The maximum absorbancy spectra of flavonoids occur in two primary regions when measured by scanning spectrophotometer: 240–285 nm (band II) and 300–550 nm (band I) (Markham, 1982). Depending upon the external standard used for quantification of quercetin in onion, 280 nm (Bilyk et al., 1984), 360 nm (Hirota et al., 1998), and 374 nm (Patil et al., 1995) have been reported. To establish the maximum absorbancy of the external standard for this study, scanning spectrophotometer measurements were taken between 190–500 nm which showed that maximum absorbancy for isoquercitrin in 80% EtOH occurred at 258 nm in band II and 362 nm in band I (Fig. 2), agreeing with literature values (Jurd, 1962).

Quercetin content in both yellow and red onions was examined, however anthocyanidin

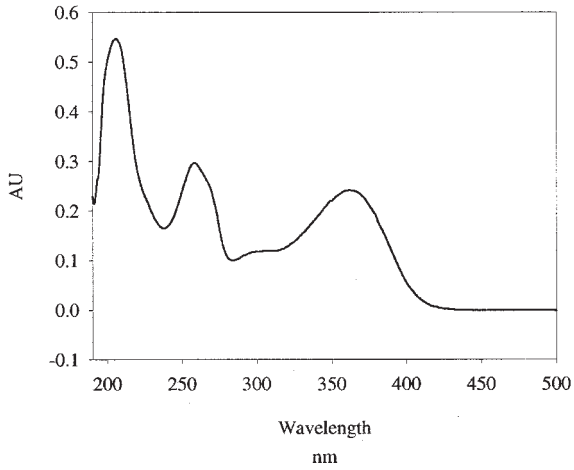

Fig. 2. Absorbance spectra (190–500 nm) of isoquercitrin (quercetin-3-*O*-glucoside) (Extrasynthase, Genay, France) standard in 80% EtOH. Absorbance maximum of isoquercitrin occurred at 258 nm in band II and 362 nm in band I.

Table 1. Quercetin concentration in onion (*A. cepa*) entries quantified with the spectrophotometer and HPLC (362 nm).

	Spectrophotometer	HPLC						
	TF[z]	3,4'-Qdg[x]	4'-Qmg[w]	3,4'-Qdg + 4'-Qmg		Total quercetin[u]		TF[z]
Entry	mg·kg^{-1} fwt	mg·kg^{-1} fwt	mg·kg^{-1} fwt	mg·kg^{-1} fwt	% TF[v]	mg·kg^{-1} fwt	% TF[t]	mg·kg^{-1} fwt
Tamara	296.8 c ± 29.0	113.7 ± 13.1	137.7 ± 18.0	251.3 ± 30.6	88%	253.6 c ± 30.9	89%	285.5 c ± 33.5
Predator	318.0 c ± 18.9	125.1 ± 9.8	149.4 ± 15.1	274.6 ± 24.2	90%	278.2 c ± 24.9	91%	306.3 c ± 26.5
Rio Rita	437.9 b ± 48.0	134.9 ± 9.3	241.6 ± 26.0	376.5 ± 35.1	84%	399.0 b ± 44.9	88%	453.9 b ± 48.3
RNX 10968	525.1 ab ± 31.3	211.1 ± 16.0	267.5 ± 17.0	478.6 ± 30.5	93%	481.0 ab ± 30.7	93%	516.4 ab ± 33.6
Red	574.9 a ± 42.4	202.2 ± 12.8	300.1 ± 23.5	502.3 ± 36.0	86%	513.3 a ± 37.3	88%	580.9 a ± 42.4

[z] Total flavonol(TF) content obtained by combining peak areas fitting flavonol profile observed between 5 and 25 min (Price and Rhodes, 1997) (see Fig. 3); Means within a column with the same letter are not significantly different; Fisher's protected LSD ($P < 0.05$).
[y] mg/kg fresh weight (fwt) ± SEM.
[x] 3,4'-quercetin-O-diglucoside.
[w] 4'-quercetin-O-glucoside.
[v] 3,4'-Qdg + 4'-Qmg/TF (HPLC) × 100%.
[u] total of 3,4'-Qdg + 3-Qmg + 4'-Qmg + Q.
[t] 3,4'-Qdg + 3-Qmg + 4'-Qmg + Q/TF (HPLC) × 100%.
n = 10 bulbs/variety.

and anthocyanin composition responsible for red color (Fenwick and Hanley, 1990; Rhodes and Price, 1996) was not measured. To avoid spectral interferance from anthocyanidins and anthocyanins that absorb between 270–280 nm in band II (Jurd, 1962; Markham, 1982), band I maximum absorbancy of isoquercitrin at 362 nm was monitored.

Quantification. Total flavonol (TF) content as recorded from simple spectrophotometer readings are reported in Table 1. Significant differences in concentration were observed among the five varieties ranging in TF from 296.8 mg·kg^{-1} fwt to 574.9 mg·kg^{-1} fwt.

Chromatograms obtained during HPLC revealed six peaks in most samples, two major peaks and four minor peaks. This profile is similar to those reported in other studies quantifying quercetin in onion (Leighton et al., 1992; Patil, 1994; Price and Rhodes, 1997; Rhodes and Price, 1996). A typical chromatogram showing components eluted between 5.0–25.0 min appears in Fig. 3. This region represents total flavonol content in onions according to Price and Rhodes (1997). Three minor flavonol components were identified as quercetin-3-*O*-glucoside (3-Qmg or isoquercitrin) (retention time [Rt] 9.55 min), quercetin aglycone (Q) (Rt 12.93 min), and kaempferol (K) (Rt 14.38). A fourth minor flavonol, isorhamnetin-3-*O*-glucoside (3-Img), was identified based on a retention time by Price and Rhodes (1997). Combined, these four compounds accounted for 7% to 17% of total flavonol content in all varieties.

Two large peaks occurring at Rts of 7.87 and 10.48 min, identified as quercetin-3,4'-*O*-diglucoside (3,4'-Qdg) and quercetin-4'-*O*-glucoside (4'-Qmg), respectively, by chromatographic comparison (Price and Rhodes, 1997) and relative position of these peaks compared to authentic standards. Combined, 3,4'-Qdg and 4'-Qmg accounted for 84% to 93% of total flavonol compounds, depending on the variety (Fig. 3 and Table 1). Our findings agree with those of Price and Rhodes (1997) who report these two main quercetin conjugates together accounted for over 80% of total flavonol content in two varieties of onions. In our study, quercetin-4'-*O*-glucoside represented the main quercetin glucoside in all five varieties, agreeing with Leighton et al. (1992) and Tsushida and Suzuki (1995), contrary to Price and Rhodes (1997) who report 3,4'-Qdg to be the main quercetin conjugate in onion.

Total quercetin concentrations by HPLC analyses, a summation 3,4'-Qdg, 3-Qmg, 4'-Qmg and Q peaks, ranged from 253.6 to 513.3 mg·kg^{-1} fwt and represented >88% of total flavonol content (Table 1). While our concentrations of total quercetin in onions were considerably lower than those reported by Price and Rhodes (1997) of 1369–1778 mg·kg^{-1} fwt for total quercetin, our values are close to those of Hertog et al. (1992) with a mean of 347 mg·kg^{-1} and the range of 54.34–286.40 mg·kg^{-1} fwt reported by Patil et al. (1995). The discrepancies in concentrations are likely the result of varietal differences in onions grown in the United Kingdom, but may indicate differences in sample techniques.

Spectrophotometer compared to HPLC. When comparing simple spectrophotometric and HPLC quantification methods to one another, identical trends among varieties after the performance of means separation were revealed between total flavonol concentrations in both methods, and between total flavonol concentrations (spectrophotometer) and total quercetin concentration (HPLC) (Table

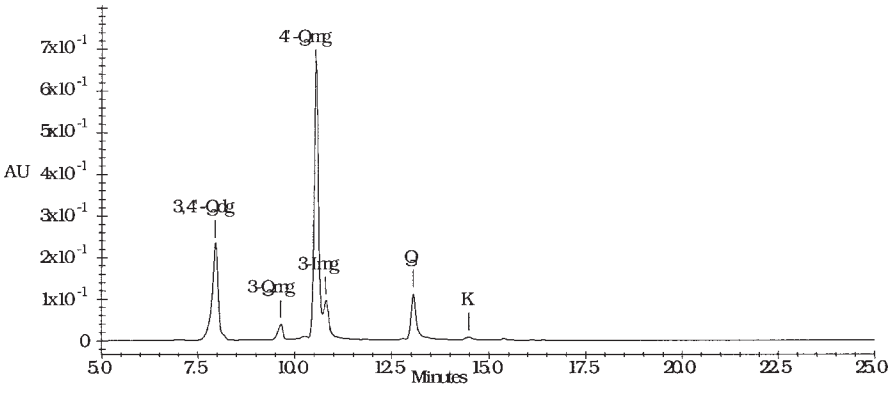

Fig. 3. HPLC chromatogram of an ethanolic extract of 'Rio Rita' onion at 362 nm eluted from 5.0 to 25.0 min. **From left to right:** quercetin-3,4'-*O*-diglucoside (3,4-Qdg); quercetin-3-*O*-glucoside (3-Qmg or isoquercitrin); quercetin-4'-*O*-glucoside (4'-Qmg); isorhamnetin-3-*O*-glucoside (3-Img), quercetin aglycone (Q); and kaempferol (K).

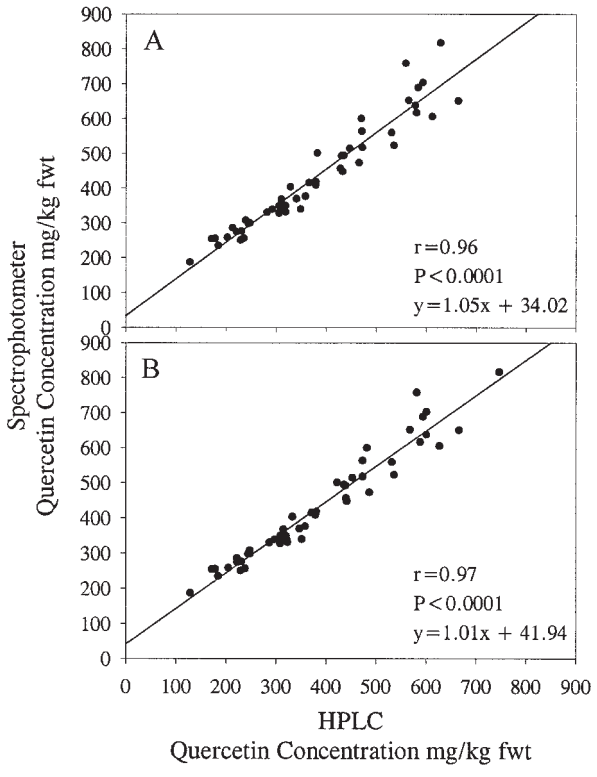

Fig. 4. Correlation between cumulative quercetin conjugates of *A. cepa* ethanolic extracts separated by HPLC and total spectrophotometer values: (**A**) 3,4'-Qdg + 4'-Qmg (r = 0.96); (**B**) total quercetin (3,4'-Qdg + 3-Qmg + 4'-Qmg + Q; r = 0.97). n=50 bulbs.

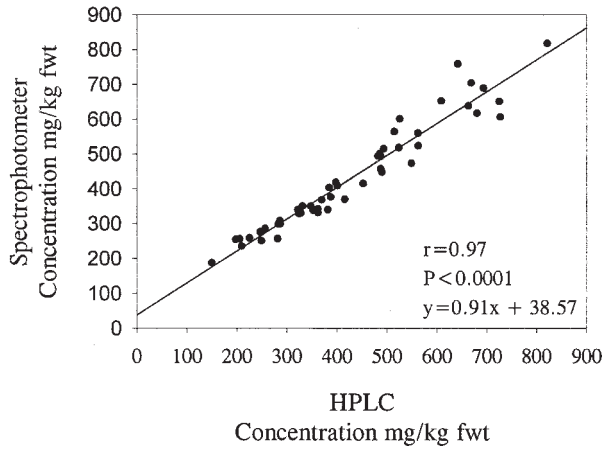

Fig. 5. Correlation between cumulative flavonol compounds of *A. cepa* ethanolic extracts separated by HPLC and total spectrophotometer values: 3,4'-Qdg, 3-Qmg, 4'-Qmg, 3-Img, Q, and K (r = 0.97). n=50

1). Differences between simple spectrophotometric measurement and HPLC analyses in total flavonol content among the five onion varieties ranged from 6–16 mg·kg^{-1} fwt—no more than 4% difference between means of both methods. A slightly higher 8% to 15% difference ranging from 38.9–61.6 mg·kg^{-1} fwt was observed between means of simple spectrophotometer measurements and total quercetin (HPLC).

A high linear relationship (r = 0.96; P < 0.0001) was observed after correlation analyses between spectrophotometer values and combined HPLC values of 3,4'-Qdg + 4'-Qmg (Fig. 4A), indicating a strong positive association between these values. Additionally, high correlation coefficients (r = 0.97; P < 0.0001) were observed when spectrophotometer values were compared to HPLC total quercetin (3,4'-Qdg + 3-Qmg + 4'-Qmg + Q) (Fig. 4B), and HPLC total flavonols (3,4'-Qdg + 4'-Qmg + 3-Img + Q + K) (Fig. 5). From these results it can be concluded that values obtained from the spectrophotometer, in these conditions, do in fact adequately represent total quercetin content in onion extracts, which dominate the flavonol profile.

Conclusion

A high linear relationship was observed when comparing total quercetin content of onions determined by simple spectrophotometric analysis to HPLC analysis. Total quercetin in onions differed significantly among varieties, yet after means separation had been performed, identical trends were shown between simple spectrophotometric and HPLC methods of analyses. The spectrophotometer has also been shown to reliably estimate combined concentrations of 3,4'-Qdg and 4'-Qmg, the two main flavonol conjugates in onions. While the spectrophotometer does not replace the precision achieved with HPLC for the separation and quantification of individual quercetin conjugates, the spectrophotometer represents an inexpensive and efficient method for analysis of high volumes of onions for total quercetin concentration. Considering the health related positive effects of quercetin, the spectrophotometer therefore may be a useful tool for onion breeders interested in selecting for higher amounts of total quercetin and food technologists interested in performing rapid postharvest evaluation of this compound.

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