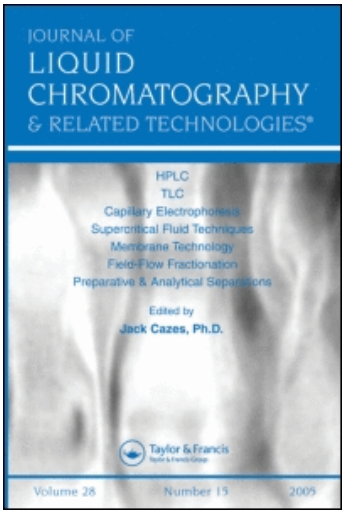


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# PRESSURIZED LIQUID EXTRACTION OF CURCUMINOIDS AND CURCUMINOID DEGRADATION PRODUCTS FROM TURMERIC (*CURCUMA LONGA*) WITH SUBSEQUENT HPLC ASSAYS

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## ABSTRACT

A procedure involving pressurized liquid extraction is used to extract curcuminoids and curcuminoid degradation products from turmeric (*Curcuma longa*) root-powder samples. Subsequent HPLC assay results for total curcuminoids are comparable to that obtained by Soxhlet extraction and slightly, but significantly higher, than that obtained by single and multiple ultrasonically assisted extractions. An efficient HPLC procedure for assaying some of the known curcumin degradation products is also presented.

## INTRODUCTION

Turmeric (*Curcuma longa*) is a spice that also possesses anti-inflammatory, choligogic, hepatoprotective, antitumor, immunostimulating, and antiviral activity.[1] The most important biologically active ingredients in turmeric are



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three curcuminoids that have free-radical scavenging antioxidant properties[2,3] and, like cichoric acid in *Echinacea*,[4] are inhibitors of human immunodeficiency virus type 1 (HIV-1) integrase.[5]

Curcumin or diferulomethane is generally the most predominant curcuminoid with desmethoxycurcumin and bisdesmethoxycurcumin present in lesser amounts. Early work on separation of these three curcuminoids by HPLC yielded poor resolution with $C_{18}$ columns,[6] somewhat better resolution with polymeric PRP-1 columns[7] and $NH_2$ columns.[8] However, peak efficiency appeared to be rather poor with the latter. Recently, reversed-phase methods on $C_{18}$ columns employing an isocratic aqueous/tetrahydrofuran mobile phase buffered at pH 3.0 with citric acid,[9] an aqueous citric acid/acetonitrile gradient,[10] or an aqueous acetic acid/acetonitrile gradient,[11] yielded both good resolution and peak shape. (The latter two were geared toward MS detection).

The extraction of curcumin from turmeric root powder is generally performed under reflux conditions for 1 to 2.5 hr[7,11,12] or, in one case, by pressurized liquid extraction.[12]

Curcumin is light sensitive in solution and solid form[13] and undergoes hydrolytic degradation in solution at high pH. Degradation products include *p*-hydroxybenzoic acid, ferulic aldehyde, *p*-hydroxybenzaldehyde, vanillic acid, vanillin, and ferulic acid, with the latter four being the most prevalent.[13] A weak cation/exchange column used with an organic eluent and a trace of water provided a poorly resolved, inefficient ion-exclusion type separation.[13]

The purpose of the present work was to compare the extractabilities of the curcuminoids by single and multiple ultrasonically-assisted extractions, soxhlet extractions, and pressurized liquid extractions with monitoring by a more recent, efficient HPLC separation. In addition, a more efficient separation of the more important observed curcuminoid degradation products was also sought.

## EXPERIMENTAL

### Reagents and Chemicals

Curcumin was obtained from Chromadex, Laguna Hills, CA and used as received. It was found to be 89.3% pure, as found by HPLC (*vide infra*) at 425 nm. The major impurity was desmethoxycurcumin at 10.3 area % at 425 nm. The curcumin standard was corrected for this impurity and used to determine total curcuminoids from the sum of the areas of the three major peaks in each sample. Vanillic acid, ferulic acid, and 4-hydroxybenzoic acid were obtained from Sigma, while vanillin, 4-hydroxybenzaldehyde, and ferulic aldehyde (4-hydroxy-3-methoxycinnamaldehyde) were obtained from Aldrich. They were used as received. All other reagents were reagent grade, and used as received. Turmeric

root powder, lot number 13300C, was obtained from Stryka Botanicals Inc., Rancho Cordova, CA. Two old turmeric spice samples were obtained in their original tins at an antique auction. They were French's Turmeric, R.T. French Co., Rochester, NY, estimated to be from the 1940s or 1950s and Golden Sun Turmeric, estimated to be from the 1930s. (The latter had a patent date of 1921 embossed on the can).

## Apparatus

The pressurized-liquid extraction apparatus described previously[14] was used, with the exception that the final nitrogen purge was omitted. The HPLC used was a Waters Model 2690 Separations Module with a Model 996 Photodiode Array Detector, or a Model 2295 Module with a Model 2487 Dual $\lambda$ Absorbance Detector. The photodiode array detector was used for initial studies, while the more sensitive absorbance detector was used for the final degradation product study. Both systems were operated with the Millenium32 Chromatography Manager version 3.20 software. The column for the curcuminoid assay was a $150 \times 4.6$-mm, 5 μm Phenomenex Luna $C_{18}$. The eluent was based on that used by He and coworkers[11] and consisted of a 0.25% aqueous acetic acid/acetonitrile gradient, and was as follows: 40% to 60% acetonitrile in 10 min, held at that composition for 10 min, changed to the initial conditions in the next 2 min, and held there for 2 min. The flow rate was 1.0 mL/min with a column temperature of 48°C, an injection volume of 5 μL, and a monitoring wavelength of 425 nm. For curcuminoid degradation products, the HPLC system used for the analysis of phenolic acids in *Echinacea* was used.[15] It consisted of a $250 \times 4.6$-mm, 5 μm Phenomenex Prodigy $C_{18}$ column with a 0.3% aqueous phosphoric acid/acetonitrile gradient as follows: 10% to 22% acetonitrile in 13 min changed to 40% acetonitrile in the following minute, held at 40% for 0.5 min, changed to initial conditions (10% acetonitrile) in 0.5 min, and held there for 5 min. The flow rate was 1.5 mL/min with a column temperature of 35°C, an injection volume of 10 μL, and a monitoring wavelength of 300 nm. These conditions are similar to that used for the method developed for *Echinacea* phenolic acids by the Institute of Nutraceutical Advancement and presented on the internet.[16] All turmeric and curcumin solutions were stored in actinic glassware or otherwise protected from light, unless otherwise noted.

## Initial Investigation of Sample Preparation

Initially, the two-part procedure of Anderson and Burney[17] for validating the efficiency of simple extractions of analytes from herb samples was utilized.

It consisted, basically of a sample size study followed by a repeated extraction study. In the first part, the maximum sample weight that could be extracted ultrasonically with 20.0 mL of methanol without a decrease in assay results was determined. In the second part, single extraction results using the optimum weight were compared with multiple extraction results to estimate % extracted (or recovered) in the single extraction. The multiple extractions were commenced by transferring an accurately weighed quantity of sample to a 50-mL centrifuge tube. About 20 mL of methanol was added to the tube and the tube placed in a sonicator for about 30 min. The tube was centrifuged and as much of the supernatant as possible was quantitatively transferred with methanol washings to a 100-mL volumetric flask. The residue was broken up with a spatula and an additional 20 mL of methanol was added, washing off the spatula. The tube was then sonicated again for 30 min. This procedure was repeated until the turmeric in the tube had been extracted four times, with all of the extractions collected in the 100 mL volumetric flask, which was then diluted to volume with methanol. The entire single and multiple extraction results were then compared with 5, between-day, 6-hour Soxhlet extraction results obtained with methanol on 1-g samples.



## Pressurized Liquid Extraction

The pressurized-liquid extraction procedure was identical to that described for goldenseal,[14] except that methanol was used as the solvent and the nitrogen purge was omitted. It was replaced by a pressurized methanol purge of a few mL/min at the nominal conditions of 1500 psi and 100°C to fill the actinic 50-mL volumetric flask nearly to the mark. The flask was diluted to the mark with methanol when cool. A study was performed examining extractability of curcuminoids vs. number of 5-min cycles at 1500 psi and 100°C.

Results obtained by the various extraction procedures were compared, using one-way analysis of variance (ANOVA) as calculated with the ORIGIN 6.0 graphic and statistical software package (Microcal, Northampton, MA). Significance of differences between mean values was assessed at the 95% confidence level.

## Assay of Curcuminoid Degradation Products

Samples were taken from the pressurized-liquid extraction solutions and stored in amber vials at −20°C until used (usually one day). Some turmeric sample solutions were stored in transparent, colorless glass vials in bright sunlight for 1 to 4 days.

### Standard Solutions

Curcumin standard solutions were prepared by serial dilution with methanol to yield concentrations in the range 30 to 185 μg/mL. They were found to be stable for at least 20 weeks when stored in well-sealed amber vials at −20°C. Standard solutions of 4-hydroxybenzoic acid, vanillic acid, 4-hydroxybenzaledehyde, vanillin, ferulic acid, and ferulic aldehyde were similarly prepared in the range 0.8 to 10 μg/mL. Typical linearity data for curcumin and degradation products determined over the above ranges by plotting peak area at 425 nm for curcumin and 254 nm, 300 nm, or 335 nm for the degradation products, are presented in Table 1. Assay values were generally determined from application of the standard curve. Trace levels of impurities, i.e., at concentrations less than that found in the respective standard curve, were determined by single-point ratio calculations based on the lowest concentration standard.

Encapsulated turmeric samples were stored in sealed bottles in a chamber held at 40°C and 70% relative humidity, and assayed at 3 months for total curcuminoids and degradation products. Samples from the same batch were similarly stored at 25°C and 60% relative humidity and assayed at 6, 9, and 12 months. Extracted methanolic sample solutions were stored in sunlight for 1–4 days and similarly assayed.

## RESULTS AND DISCUSSION

### Determination of Curcuminoids

A sample chromatogram of the root-powder sample is shown in Fig. 1. Final results are summarized in Table 2 and described below.

Duplicate assays of four sample weights ranging from 0.125 to 1.0 g yielded no trend in data and a low RSD, as shown in Table 2. Multiple extractions yielded results comparable to that for the single extractions, but with a higher RSD, probably resulting from the additional sample handling. Both sets of results were about 3.5% below that obtained by 5 between-day Soxhlet extractions. Although small, this difference was statistically significant by one-way ANOVA at the 95% confidence level. Pressurized liquid extraction yielded comparable results for 3, 4, and 5 cycles. Seven between-day extractions (four cycles) yielded results comparable to that for Soxhlet extractions, as shown in Table 2. A 4-cycle re-extraction of one of the pressurized liquid-extraction residues indicated less than 1% remained unextracted.

A sample solution stored 1 day in bright sunlight in a transparent, colorless vial lost about 80% of its curcumins. However, encapsulated powder samples stored in closed opaque containers at 40°C and 75% humidity for 3 months and

***Table 1.*** Linearity of Curcuminoids and Degradation Products

| Parameter | Total Curcuminoids | 4-Hydroxybenzoic Acid | Vanillic Acid | 4-Hydroxybenzaldehyde | Vanillin | Ferulic Acid | Ferulic Aldehyde |
|---|---|---|---|---|---|---|---|
| Wavelength, nm | 425 | 254 | 300 | 300 | 300 | 300 | 335 |
| Correlation coefficient | 0.999994 | 0.9999998 | 0.99998 | 0.999985 | 0.99992 | 0.99998 | 0.990 |
| Slope, area units mL/μg | 36,562 | 41,392 | 10,226 | 29,030 | 22,800 | 28,408 | 43,264 |
| Intercept, area units | 199,933 | 674 | 197 | 130 | 3,238 | −2,459 | 30,804 |
| Standard error of the estimate ($S_{y/x}$), area units | 13,979 | 111 | 189 | 937 | 1,663 | 907 | 17,821 |
| % Intercept[a] | 0.6 | 0.5 | 0.8 | 0.1 | 2.7 | −1.9 | 22.4 |
| % Variation[b] | 0.4 | 0.1 | 0.7 | 0.7 | 1.4 | 0.7 | 12.9 |

[a]($y$ intercept/$\bar{y}$) × 100, where $\bar{y}$ is the average $y$.[18]
[b]($S_{y/x}/\bar{y}$) × 100.

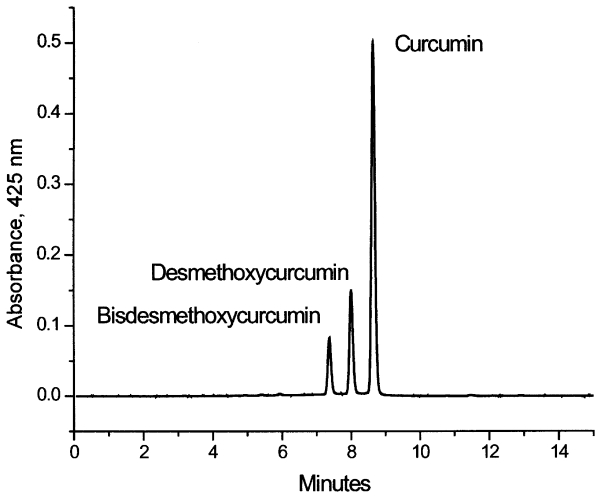


***Figure 1.*** Typical chromatogram of a turmeric root-powder sample. Four hundred and twenty five nanometers detection wavelength.



25°C and 40% humidity for 6, 9, and 12 months showed no loss of curcumins. The old turmeric spice samples, estimated to have been in their original containers for 40–70 years, yielded 41.9 mg/g total curcuminoids for French's and 15.9 mg/g for Golden Sun.

## Determination of Curcumin Degradation Products

A chromatogram of 5–10 μg/mL 4-hydroxybenzoic acid, vanillic acid, 4-hydroxybenzaldehyde, vanillin, ferulic acid, and ferulic aldehyde standards

***Table 2.*** Summary of Total Curcuminoid Assay Results

| Procedure | *n* | Total Curcuminoids, mg/g |
|---|---|---|
| Single ultrasonic extraction, 0.125–1.0 g sample size | 8 | 17.95 ± 0.05 (0.3%) |
| Multiple ultrasonic extractions | 6 | 17.8 ± 0.4 (2.3%) |
| Soxhlet extraction | 5 | 18.6 ± 0.5 (2.6%) |
| Pressurized solvent extraction four cycles | 7 | 18.7 ± 0.3 (1.5%) |

obtained at 254 nm, a wavelength not optimum for all of the analytes, is shown in Fig. 2. Wavelengths for actual assays were chosen as follows: 254 nm for 4-hydroxybenzoic acid, 300 nm for vanillic acid, 4-hydroxybenzaldehyde, vanillin, and ferulic acid, and 335 nm for ferulic aldehyde. Degradation product assay results for a turmeric root powder, as a function of sample solution storage conditions, and for old turmeric spice samples, are shown in Table 3. Thus, photolytic degradation does cause an increase in the degradation product content. Photodiode-array on-the-fly spectra of all the observed degradation products occurring at sufficient levels to produce useable spectra, were consistent with that obtained from authentic standards. Since 4-hydroxybenzoic acid and ferulic aldehyde were found to form at lower levels than the other four degradation products, require different wavelengths for optimum sensitivity, and ferulic aldehyde suffers from interference by the gradient artifact, method development centered on the other four degradation products. A sample chromatogram of the turmeric root powder, obtained at 300 nm, is shown in Fig. 3. (Slight differences in retention time from that in Fig. 2 result from use of different HPLC equipment). Examination of low-concentration solutions of the four main degradation products with the more sensitive variable wavelength absorbance detector set to 300 nm, indicated that concentrations of about 10 ng/mL yielded peak heights corresponding to about 10 times the average



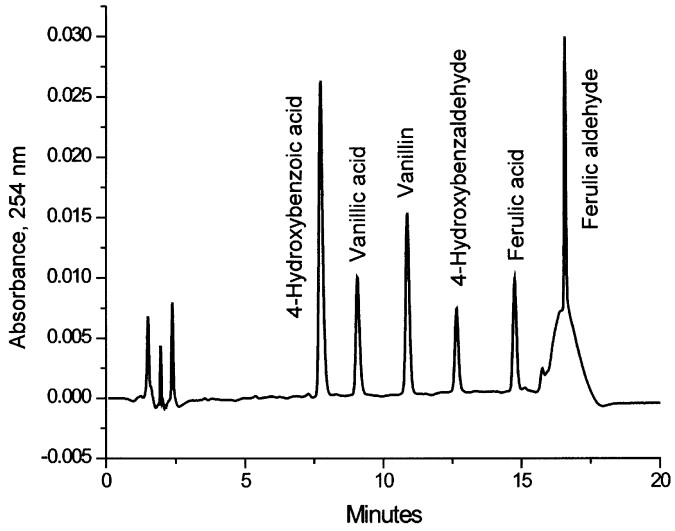


***Figure 2.*** Chromatogram of 5–10 μg/mL curcumin degradation product standards. Hump starting at about 16 min is a gradient artifact. Two hundred and fifty four nanometers detection wavelength.

***Table 3.*** Curcumin Degradation Products Assay Results as % of Curcumin[a]

| Sample Solution | *n* | 4-Hydroxybenzoic Acid | Vanillic Acid | 4-Hydroxybenzaldehyde | Vanillin | Ferulic Acid | Ferulic Aldehyde |
|---|---|---|---|---|---|---|---|
| Root powder protected from light | 5 | 0.031 ± 0.002 (7.2%) | 0.076 ± 0.008 (10.9%) | 0.113 ± 0.002 (1.7%) | 0.254 ± 0.007 (2.8%) | 0.28 ± 0.01 (4.1%) | 0.003 ± 0.003 (96.3%) |
| Root powder stored in Sun one day[b] | 1 | 0.1 | 0.3 | 1.1 | 2.4 | 1.5 | 0.1 |
| Root powder stored in Sun four days[c] | 1 | 0.2 | 0.7 | 2.0 | 4.3 | 0.9 | 1.1 |
| French's spice | 1 | — | 0.3 | 0.3 | 0.6 | 0.5 | — |
| Golden Sun spice | 1 | — | 1.3 | 1.2 | 2.1 | 0.9 | — |

[a]Based on total curcuminoid assay value prior to light exposure.
[b]80% curcumin degradation.
[c]Complete curcumin degradation.

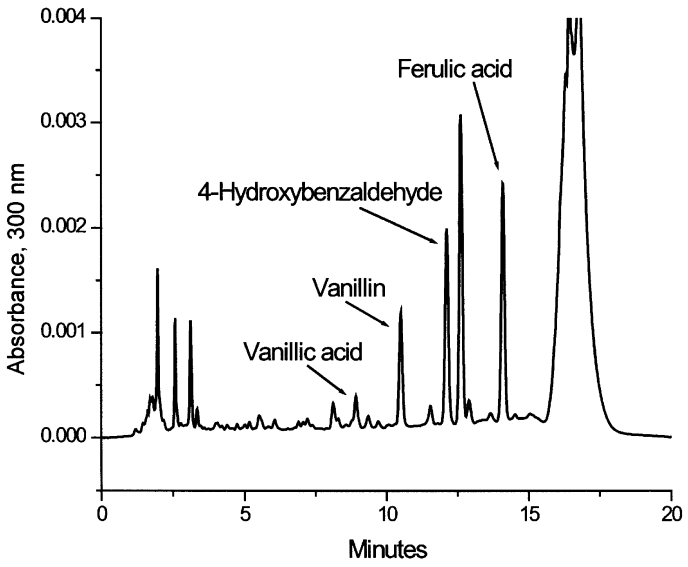


***Figure 3.*** Expanded-scale chromatogram of an actual root-powder sample. Three hundred nanometers detection wavelength.



baseline noise. This estimated quantitation limit corresponds to about 0.005% of total curcumins.

When a standard solution of the six degradation products was chromatographed under curcumin assay conditions, all of the degradation products eluted at low $k'$ values, well before the curcumin peaks.

The 3-, 6-, and 9-month stability samples showed no increase in degradation product levels.

## CONCLUSIONS

Pressurized liquid extraction appears to be a quick alternative procedure to reflux or Soxhlet extraction for ensuring the highest degree of extractability of curcumin and related compounds from turmeric root powder. It also avoids manipulation of cumbersome glassware and heating and cooling equipment. For multiple samples, a commercial multicell automated pressurized liquid extractor might be especially useful.

Although simpler, single or multiple ultrasonically-assisted extractions appear to leave a small, but significant amount of curcumins unextracted. The

results observed here indicate that pressurized liquid extraction might, in some cases, be a better test than multiple extractions for confirming the completeness of extraction for a single ultrasonically-assisted extraction.

The subsequent HPLC assay for total curcuminoids yields good resolution of the three analytes from one another. The fact that the three curcuminoids are well resolved from observed degradation products, which elute at much lower capacity factors, confers stability-indicating properties to the assay.

The degradation product assay is a useful adjunct to the curcuminoid assay for turmeric stability-monitoring purposes and employs the same HPLC parameters as that for the phenolic-acids assay of *Echinacea*.[15,16] Complete photolytic degradation of curcuminoids in solution substantially increases the level of observed degradation products. The curcuminoids appear to be reasonably stable in moderately protected solid powder samples. Very old spice samples displayed a degradation-product level approaching that of completely degraded samples. The substantial amount of curcuminoids left indicates the degradation mechanism may be different in the dry form.



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