DIETARY SUPPLEMENTS

# Validation Assay for Total Flavonoids, as Rutin Equivalents, from *Trichilia catigua* Adr. Juss (Meliaceae) and *Ptychopetalum olacoides* Bentham (Olacaceae) Commercial Extract

ANDRÉ ROLIM, CAROLINA P.M. MACIEL, TELMA M. KANEKO, VLADI O. CONSIGLIERI, IDALINA M.N. SALGADO-SANTOS, and MARIA VALÉRIA R. VELASCO

University of São Paulo, Department of Pharmacy, Faculty of Pharmaceutical Sciences, 580 Prof. Lineu Prestes Ave, Bl. 13, Conjunto das Químicas, 05508-900, São Paulo, Brazil

**An ultraviolet spectrophotometric method was validated for total flavonoid quantitation, as rutin equivalents, present in the *Trichilia catigua* Adr. Juss (Meliaceae) and *Ptychopetalum olacoides* Bentham (Olacaceae) commercial extract. Parameters as linearity, interval (range), specificity, estimated limit of detection (LOD, μg/mL), estimated limit of quantitation (LOQ, μg/mL), recovery (R, %), precision or relative standard deviation (RSD, %), and accuracy (E, %) were established. The analytical method was validated according to the experimental results: correlation coefficient (r = 0.9997); interval (RSD = 0.15–0.47%; E = 98.98–101.24%); specificity to total flavonoids quantitation, as rutin equivalents, at wavelength 361.0 nm; LOD = 0.09 μg/mL and LOQ = 0.27 μg/mL; R = 99.36–102.14%; adequate intra- and interrun precision (0.30–0.49% and 0.31–0.81%), and intra- and interrun accuracy (100.60–102.38% and 98.58–100.38%).**

A major difficulty concerning addition of natural raw materials in pharmaceutical or cosmetic products is to enable the analytical method to quantify the active substances. Botanical extracts present numerous chemical substances that may act as interferents during determination of active or standard markers, beyond the matrix excipients, when incorporated in pharmaceutical or cosmetic products.

A method validation should be conducted to ensure that an analytical methodology is accurate, specific, and reproducible over the specified range that samples will be analyzed (1). The procedure must guarantee, through experimental studies, that the method meets the requirements of its application, ensuring the reliability of the results, although there are many possible sources of inaccuracies, including inadequate or impure reference materials, poor precision and accuracy, and unsuitable data or techniques (2, 3). Procedure and methodology used must present suitable specificity, linearity, interval (range), precision, accuracy, detection and quantitation limits (4–6).

Precision and accuracy are the most important criteria in determining whether the method is suited to a particular test and whether data generated under routine use of an analytical method are acceptable (7, 8).

*Trichilia catigua* Adr. Juss (Meliaceae) and *Ptychopetalum olacoides* Bentham (Olacaceae) commercial extract is a mixture of 2 South American plants, standardized by addition of rutin. Phytochemical trials of the plants have indicated the presence of alkaloids, tannins, aromatic oils, saponins, terpenes, steroids, fatty resins, behenic acid, lupeol, flavonoids, and flavalignans (9–11).

Rutin (3((6-O-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl)oxy)-2-(3′,4′-dihydroxyphenyl)-5,7-dihydroxy-4H-1-benzopyran-4-one), in Figure 1 (12), a flavonoid type compound possessing broad biological properties, promotes beneficial effects on diseases in which uncontrolled lipid peroxidation is involved (13, 14). The ability to interact with protein phosphorylation, and the antioxidant, iron-chelating, and free radical scavenging activities may account for the wide pharmacological and cosmetic profile of rutin and related flavonoids. Other biochemical and pharmacological activities reported are the effect on the immune system, capillary preservation, inflammatory cell functions, and even anticarcinogenic effects (15–19).

Various methods have been described to quantify total flavonoids, as rutin equivalents, in plant extracts and pharmaceutical or cosmetic products: direct and derivative spectrophotometry (20, 21), liquid chromatography (22–24), capillary electrophoresis (25), fluorimetry, chemiluminescence (26), and mass spectrometry (27, 28). Direct UV spectrophotometry and its analytical validation present application advantages over some routine methods: simplicity, rapidity, adequate sensitivity, operational convenience, and relatively low cost of equipment and reagents. Analytical method validation involving UV spectrophotometric quantitation for total flavonoids, as rutin equivalents, is poorly described in the scientific literature.

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Corresponding author's e-mail: andrerb@usp.br

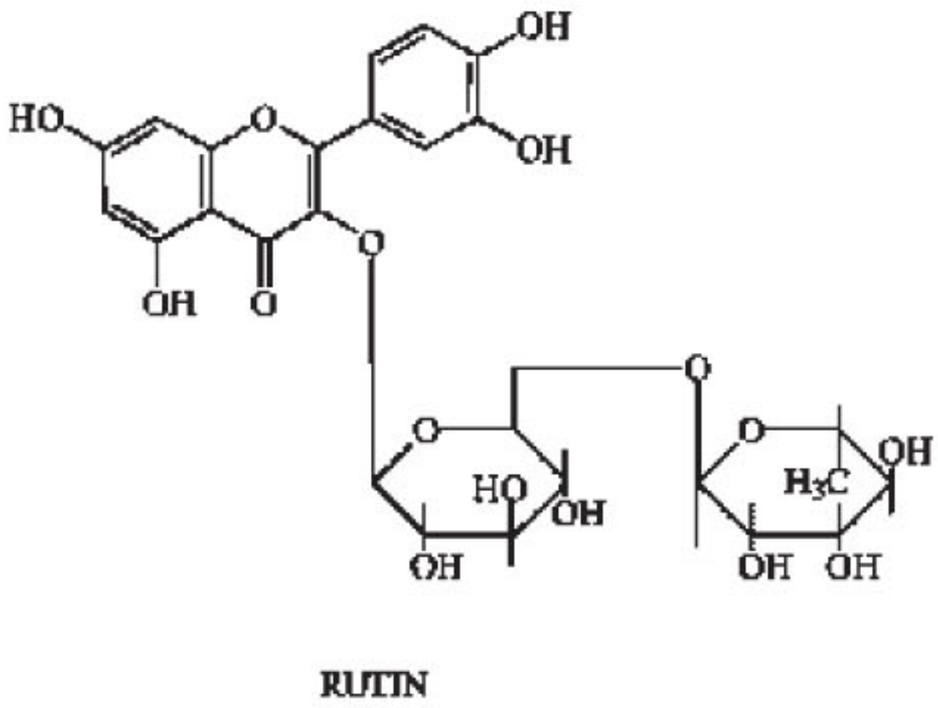


**Figure 1. Structure of flavonoid rutin (ref. 12).**

This research is intended to validate a simplified UV spectrophotometric method for quantitation of total flavonoids, as rutin equivalents, present in the *T. catigua* Adr. Juss (Meliace) and *P. olacoides* Bentham (Olacaceae) commercial extract, ChemyUnion® Ltd. (Sorocaba, Brazil), standardized in rutin content. It focuses on the parameters established in the literature and official compendiums for linearity, interval (range), specificity, estimated quantitation limit, recovery, precision, and accuracy (1, 4–6, 29–31).

## Experimental

### *Calibration Curve and Linearity*

Standard rutin (PVP, Parnaíba, Brazil), NF XI secondary standard without any further purification (96.1%, NF XI, lot 02134, expiration date 07/2005), was dissolved in 95.0% ethanol and 0.02M acetic acid (99 + 1; LabSynth, São Paulo, Brazil) to concentrations from 5.0 to 15.0 µg/mL. The absorbance measurements were obtained at 361.0 nm using Spectrophotometer Beckman DU–640 with a 1 cm quartz cuvette. Means of 6 replicates of each concentration value were used to construct the calibration curve. The linearity was analyzed and confirmed by statistical analysis (4, 32).

### *Interval (Range)*

The interval of concentrations used to construct the calibration curve was evaluated by determining the precision or relative standard deviation (RSD, %) and accuracy (E, %) for diluted solutions of the standard rutin (1, 32).

### *Specificity*

*T. catigua* Adr. Juss and *P. olacoides* Bentham commercial extract (1.02% rutin content; ChemyUnion Ltd.) was dissolved in 95.0% ethanol and 0.02M acetic acid (99 + 1) to a concentration of 10.0 µg/mL, as rutin equivalents. The absorbance spectra for the range 200.0–400.0 nm were obtained and overlapped with the standard rutin (10.0 µg/mL) spectra absorbance to evaluate the presence of possible interferents and the specificity of the method (32, 33).

### *Estimated Limits of Detection (LOD) and Quantitation (LOQ)*

The LOD and LOQ were estimated by the slope and mean SD of concentrations used to construct the calibration curve, according to Equations 1 and 2 (34):

$$\mathrm{LOD} = \frac{3.3\,\sigma}{S} \qquad (1)$$

where LOD is the estimated detection limit (µg/mL); σ is the mean SD; S is the slope of the calibration curve.

$$\mathrm{LOQ} = \frac{10\,\sigma}{S} \qquad (2)$$

where LOQ is the estimated quantitation limit (µg/mL); σ is the mean SD; S is the slope of the calibration curve.

### *Recovery*

Recovery was evaluated by adding standard rutin to *T. catigua* Adr. Juss and *P. olacoides* Bentham commercial extract solutions to obtain total flavonoids, as rutin equivalents, final concentrations 8.0, 10.0, and 12.0 µg/mL (3, 34). The assay was conducted with 9 replicates. Recovery (R, %) was calculated according to Equation 3.

$$\mathrm{Recovery} = \mathrm{R}\,(\%) = \frac{\mathrm{Cse} - \mathrm{Ce}}{\mathrm{Cs}} \times 100 \qquad (3)$$

where R (%) is recovery; Cse is the concentration of total flavonoids, as rutin equivalents, in samples containing *T. catigua* Adr. Juss and *P. olacoides* Bentham commercial extract and standard rutin (µg/mL); Ce is the concentration of total flavonoids, as rutin equivalents, in samples containing *T. catigua* Adr. Juss and *P. olacoides* Bentham commercial extract (µg/mL); Cs is the concentration of standard rutin (µg/mL).

**Table 1. Statistical data to set calibration curve for standard rutin and interval (range) evaluation**

| Standard rutin, µg/mL | Parameters[a] | | | |
|---|---|---|---|---|
| | A ($n$ = 6) | SD | RSD, % | E, % |
| 5.0 | 0.1612 | 0.0007 | 0.41 | 101.24 |
| 8.0 | 0.2449 | 0.0011 | 0.47 | 98.98 |
| 10.0 | 0.3078 | 0.0006 | 0.20 | 100.66 |
| 12.0 | 0.3616 | 0.0010 | 0.27 | 99.18 |
| 15.0 | 0.4546 | 0.0007 | 0.15 | 100.50 |

[a] A = Mean absorbance of 6 replicates; SD = standard deviation of the absorbances; RSD = relative standard deviation (precision); E = accuracy.

**Table 2. Recovery, precision and accuracy assays[a]**

| | | Intrarun | | | | Interrun | | | |
|---|---|---|---|---|---|---|---|---|---|
| TC[b], μg/mL | Recovery, % | C, μg/mL | SD | RSD, % | E, % | C, μg/mL | SD | RSD, % | E, % |
| 8.0 | 102.14 | 8.19 | 0.04 | 0.49 | 102.38 | 8.03 | 0.06 | 0.81 | 100.38 |
| 10.0 | 99.36 | 10.06 | 0.03 | 0.30 | 100.60 | 9.87 | 0.04 | 0.41 | 98.70 |
| 12.0 | 99.98 | 12.08 | 0.04 | 0.32 | 100.67 | 11.83 | 0.04 | 0.31 | 98.58 |

[a] Intrarun assays were evaluated in 1 day and interrun assays were evaluated in 3 consecutive days.

[b] TC = Theoretical concentrations of standard rutin; C = mean of the calculated concentrations ($n$ = 9); SD = standard deviation; RSD = relative standard deviation (precision); E = accuracy.

### *Precision and Accuracy*

Precision and accuracy were achieved by using 3 replicates of each concentration of the *T. catigua* Adr. Juss and *P. olacoides* Bentham commercial extract solutions (8.0, 10.0, and 12.0 μg/mL, as rutin equivalents). Assays were performed in 1 day (intrarun) and in 3 consecutive days (interrun).

Precision was evaluated by assessing the closeness of results obtained in a series of measurements of a multiple sampling of the same sample; accuracy represented the degree of match between the individual results found and a theoretical value accepted as reference (31, 32).

Precision and accuracy were calculated according to Equations 4 and 5, respectively:

$$\text{Precision} = \text{RSD}(\%) = \frac{\text{SD} \times 100}{\text{C}} \qquad (4)$$

where RSD (%) is precision; SD is standard deviation; C is mean of calculated concentrations.

$$\text{Accuracy} = \text{E}(\%) = \frac{\text{C} \times 100}{\text{TC}} \qquad (5)$$

where E (%) is accuracy; C is mean of calculated concentrations; TC is theoretical concentration.

## Results and Discussion

The UV spectrophotometric method offered linearity for concentrations 5.0, 8.0, 10.0, 12.0, and 15 μg/mL of standard rutin in 95.0% ethanol and 0.02M acetic acid (99 + 1), resulting in a straight line and coefficient correlation r = 0.9997. The acceptable minimal criteria of correlation coefficient must be $r \geq 0.99$ (2, 3, 32, 35).

The interval (range; Table 1), presented suitable data for the precision (RSD, %) and accuracy (E, %) analyzed for concentrations 5.0–15.0 μg/mL of standard rutin, indicating that the concentration range used was adequate to conduct the analytical validation process. Data were statistically analyzed by least-squares fit method (3, 32), and the equation obtained by the linear regression for the calibration curve was

$$y = 0.0293x + 0.0129 \qquad (6)$$

where $Y$ is absorbance and $x$ is concentration of rutin (μg/mL).

An analytical method is specific when it can provide truthful measurements of the compound in the presence of other components, impurities, degradation products, and components of the matrix (31, 32).

The standard rutin absorbance spectra presented 2 peaks of maxima absorbance, 258.0 and 361.0 nm. Overlapping the standard rutin (10 μg/mL) and *T. catigua* Adr. Juss and *P. olacoides* Bentham commercial extract (10 μg/mL, as rutin equivalents) absorbance spectrum (Figure 2), the method provided specificity for the quantitative assay for total flavonoids, as rutin equivalents, at the wavelength 361.0 nm. Absorbance spectra have resulted in overlapped and identical maxima absorbance peaks at this wavelength for equal concentrations, being adequate to quantify the total flavonoids, as rutin equivalents, in the presence of other chemical components of the commercial extract.

Recovery assay provided adequate range values of 99.36–102.14%. Precision and accuracy (intra- and interrun) are shown in Table 2. The intrarun assays for precision and accuracy resulted in values of 0.30–0.49% and 100.60–102.38%, respectively; and interrun assays were 0.31–0.81% and 98.58–100.38% for precision and accuracy, respectively. Precision must not exceed 5% of the RSD,

**Table 3. Estimated limits of detection and quantitation**

| TC[a], μg/mL | SD | MSD | Slope | LOD, μg/mL | LOQ, μg/mL |
|---|---|---|---|---|---|
| 5.0 | 0.0007 | | | | |
| 8.0 | 0.0011 | | | | |
| 10.0 | 0.0006 | 0.0008 | 0.0293 | 0.09 | 0.27 |
| 12.0 | 0.0010 | | | | |
| 15.0 | 0.0007 | | | | |

[a] TC = theoretical concentrations of standard rutin (μg/mL); SD = standard deviation ($n$ = 6); MSD = mean standard deviation; LOD = estimated limit of detection (μg/mL); LOQ = estimated limit of quantitation (μg/mL).

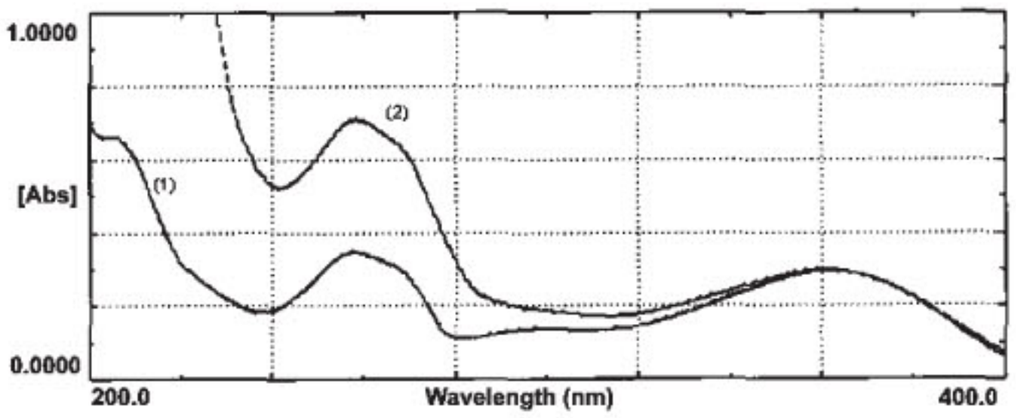


**Figure 2. Specificity of the method. Overlapped (1), standard rutin (10.0 μg/mL); and (2) *T. catigua* Adr. Juss and *P. olacoides* Bentham commercial extract (10.0 μg/mL equivalent in rutin); absorbance spectrum at wavelength range 200.0–400.0 nm. The method provided identical maxima absorbance peaks at 361.0 nm. Abs = absorbance, 211 × 84 mm (96 × 96 DPI).**

% (32). As a result, the UV spectrophotometric method generated closeness of response between the absorbance measurements. By data obtained experimentally for intra- and interrun accuracy, the method demonstrated a high degree of agreement of results (35).

Estimated LOD and LOQ values were 0.09 and 0.27 μg/mL, respectively. Table 3 lists the theoretical concentrations of standard rutin, mean of absorbances ($n = 6$), SD, mean SD of the concentrations, and slope of calibration curve used to calculate LOD and LOQ values.

According to the estimated LOD and LOQ, the method presented sensitivity to identify the flavonoid rutin, differentiating it from the equipment noise, using concentrations >0.09 μg/mL, and to quantify total flavonoids, as rutin equivalents, with adequate precision and accuracy up to the concentration calculated (0.27 μg/mL) for LOQ (32).

## Conclusions

Precision and accuracy proved to be adequate for this study, as shown by evaluations of calibration curve and interval (range). The UV spectrophotometry method and the solvent responded in agreement with criteria for acceptance of linearity and concentration range, providing r = 0.9997.

The method presented specificity for the quantitation assay of total flavonoids, as rutin equivalents, present in the *T. catigua* Adr. Juss (Meliaceae) and *P. olacoides* Bentham (Olacaceae) commercial extract (1.02% rutin content) and absence or minimal response of interferents at the wavelength 361.0 nm (Figure 2). Sensitivity was evaluated by the estimated quantitation limit (0.27 μg/mL). Thus, diluted concentrations of flavonoid rutin may be quantified with acceptable precision and accuracy equal to the quantitation limit.

The analysis of the extract diluted solutions resulted in precise and accurate data: intra- (0.30–0.49% and 100.60–102.38%) and interrun (0.31–0.81% and 98.58–100.38%) assays, according to the experimental values. Recovery was assayed to confirm accuracy, and values obtained ranged from 99.36 to 102.14%.

In agreement with the literature and official compendiums, besides the experimental analysis, the method proved to be validated for the quantitation assay for total flavonoids, as rutin equivalents, from *T. catigua* Adr. Juss (Meliaceae) and *P. olacoides* Bentham (Olacaceae) commercial extract.

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