

DEVELOPMENT OF REVERSED-PHASE HPLC METHOD FOR ISOLATION AND QUANTIFICATION OF RUTIN AND QUERCETIN FROM FLOWER BUDS OF SOPHORA JAPONICA L.

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Abstract. In the article, the results of the extraction of rutin and quercetin, which formed the basis of the flower buds of *Sophora Japonica* L. grown in the territory of Syrdarya region, and the quantitative analysis of the extracted polyphenolic compounds by the method of high-performance liquid chromatography (HPLC) were described. A linear relationship with the coefficient of determination (R^2) was observed for rutin and quercetin in the range of 25-500 μg . This relationship was found to be $y=13970x+13878$ and $y=46919x+287504$ at retention times of 2.407 and 6.452 minutes, respectively. The LODs for rutin and quercetin were found to be 12.74 and 13.48 $\mu\text{g/ml}$, respectively. The LOQs were found to be 38.59 and 40.86 $\mu\text{g/ml}$, respectively. Rutin was the most flavonoid analyzed in *Sophora Japonica* L. and when isolated by the extraction method, the purity rate for HPLC was found to be 94.483%. Quercetin, obtained by acid hydrolysis of rutin, was found to have an HPLC purity of 94.238%.

Keywords: *Sophora Japonica* L., HPLC analysis, Rutin, Quercetin, Calibration curve, Validation, LOD and LOQ.

Introduction. Flavonoids are widespread in the plant kingdom and are currently considered an integral part of the food, pharmaceutical, medical and cosmetic industries. In vitro and in vivo studies by many scientists have shown that flavonoids are potent anti-inflammatory compounds that scavenge free radicals, regulate cellular metabolism and treat stress-related diseases [1,2]. Due to their strong antioxidant, anticarcinogenic, antibacterial, antiviral and anti-inflammatory effects [3,4], flavonoids have attracted increasing interest from chemists and pharmacologists. According to the literature, flavonoids have been reported to modulate various cancer cells (e.g., colon, pancreatic, breast, lung, liver and leukocytes) [5-8], and their increase can be prevented by limiting edema. Rutin and quercetin, along with their derivatives, are particularly significant in the food and pharmaceutical industries due to their high biological activity. Consequently, the search for rapid and efficient methods to locate, isolate, and identify natural sources of rutin and quercetin is a pressing issue.

The aim of the work is to isolate, purify and clean rutin from the flower buds of *Sophora Japonica* L. - a medicinal plant of industrial importance, to synthesize quercetin from pure rutin and to study it by high-performance liquid chromatography (HPLC).

Research Methodology

The research involved distillation, extraction, filtration, drying and chromatography methods. The following solutions were used for the research: freshly distilled (refined) alcohol, acetonitrile, methanol, acetic acid, and chemically pure organic solvents.

Chromatographic conditions. HPLC analysis (Shimpack GIST C18 (250 × 4.6 mm; 5 μm, Shimadzu, Japan) of reverse phase column and standard solution, sample extract (Shimpack GIST C18 (150 × 4.6 mm; 5 μm, Shimadzu, Japan)) of reverse phase column and gradient mobile phase consisting of aqueous solution of acetonitrile (A) and acetic acid (B) in a ratio of 60:40, with an injection volume of 40 μl, the analysis was carried out at 254 and 356 nm. The duration of the chromatographic cycle is 10 minutes. Rutin and quercetin solutions (0.5 mg/ml) were prepared in acetonitrile, from which their standard solutions were prepared by serial dilutions.

Preparation of extracts. Flower buds of *Sophora japonica* L. were collected in June-July and dried to constant weight in a room protected from sunlight. 1 g of dried flower buds of the plants was added to a 100 ml flat-bottomed flask, 50 ml (1:50) of distilled water was added, a reflux cooler was connected and the mixture was extracted at 100 °C for 3 hours. The aqueous extract was filtered hot (through 2 layers of gray) and re-extracted 2 more times with 50 ml of water. The filtrates were combined and the extract was left in a refrigerator (+5 °C) for 24 hours. The precipitated technical rutin was filtered and recrystallized twice in ethanol. The resulting dry residue was first dried at room temperature and then dried in a drying cabinet at 80 °C. The obtained rutin was dissolved in acetonitrile, filtered through a 0.45 mm filter and analyzed.

1 g of the obtained rutin was dissolved in 100 ml of ethanol and hydrolyzed with HCl at a temperature of 100 °C, and pure quercetin was obtained by recrystallization of technical quercetin in 96% ethanol 2 times. The obtained quercetin was dissolved in acetonitrile and analyzed by filtration through a 0.45 mm filter.

Determination of rutin and quercetin extracted from buds using HPLC-PDA. HPLC system (LC-40D) with pump, column oven (CTO-40C), and SPD-M40 (Photo Diode Array) Detector was employed. HPLC analysis of rutin and quercetin isolated and purified from *Sophora Japonica* flower bud extract was used with C18 column Shim-pack (Shim-pack GIST C18, 250 mm×4.6 mm, 5 μm) with mobile phase gradient program, consisting of 0,5% acetic acid and acetonitrile as depicted in Table 1, at a flow rate of 1.0 mL/min. PDA detector at 356 nm was used for rutin and quercetin determination. The injection volume used was 10 μL for both standards and sample solutions. Rutin and quercetin were identified in *Sophora Japonica* buds by comparing their retention times to those of the standards. That is concentration of rutin and quercetin in the extracts were calculated using the peak area of the calibration curves obtained from standard solutions.

Method Validation. *Linearity and Range.* The linearity of the analytical method optimized for quercetin quantification was determined using nine quercetin standard solutions (0.14 and 245 μg/mL). Analysis was performed over different days in triplicate, and a calibration line comprising all analyses was constructed. One of the strategies to evaluate linearity is the RSD of the slope [11,12]. Accordingly, RSD was calculated using Equation (1):

$$RSD\% = \frac{SM}{M} \times 100 \quad (1)$$

where “SM” represents the slope standard deviation and “M” represents the slope.

Limit of Detection and Limit of Quantification. As recommended, LOD and LOQ were determined based on the calibration curve (specifically, using the calibration curve slope) and standard deviation of the response obtained through the analysis of 10 blank samples analyzed in triplicate. These parameters were determined employing Equations (2) and (3), respectively [11]:

$$LOD = 3.3 \times \sigma / S \quad (2)$$

$$LOQ = 10 \times \sigma / S \quad (3)$$

where “σ” represents the standard deviation of the response, and “S” represents the calibration curve slope.

Precision. Intra- and intraday precisions were calculated by analyzing quercetin standard solutions in five distinct concentrations (0.35, 0.57, 5, 125, and 185 µg/mL) in triplicate and subsequently expressed under RSD [12] (Equation (4)):

$$RSD\% = \frac{S}{\bar{X}} \times 100 \quad (4)$$

where “S” represents the standard deviation of a series of measurements, and “X” represents the mean value from the independent variable.

Accuracy. To determine the accuracy of the method, six quercetin standard solutions with known concentrations (0.35, 0.5, 7.5, 150 and 196 µg/mL) were injected in triplicate. The area values obtained for each standard were used to back-calculate their corresponding concentrations using the calibration equation. These calculated concentrations were then compared to the known (actual) concentrations of the standard solutions using Equation (5) (n = 5) to assess the accuracy of the method:

$$\text{Accuracy} = \frac{\text{Experimental value}}{\text{Real value}} \times 100 \quad (5)$$

Analysis and results

To select optimal conditions for the HPLC analysis of Rutin and Quercetin. When the solution of rutin and quercetin standards in acetonitrile was analyzed by HPLC, rutin had maximum absorption at wavelengths of 254 and 356 nm and quercetin at 254 and 370 nm (Fig. 1).

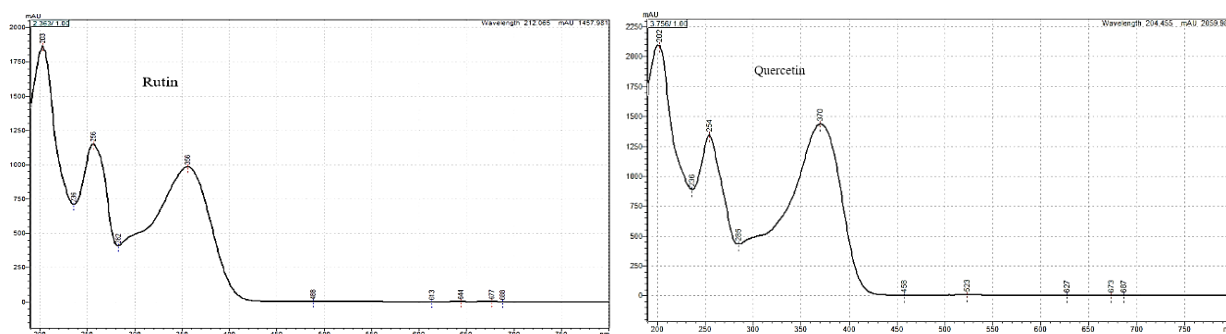


Fig. 1. Absorption spectra of standard rutin and quercetin in 0.5%acetic acid-acetonitril (60:40, v/v)

The optimal wavelength of 356 nm was selected for the simultaneous chromatographic determination of the amount of quercetin and rutin in the extract of flower buds of “Sophora Japonica L.” (Table 1).

Table 1

Conditions of chromatography

Parameter	Description
Column	250×4,6 mm, 5 mkm Shimpack GIST C ₁₈ column HPLC, Shimadzu (Japan)
Mobile phase	0,5% acetic acid : acetonitrile (60:40)
Elution mode	Gradient
Detector	PDA
Wavelength	356 nm
Time to perform analysis	10 min
Flow rate	1 ml/min

Injection volume	10 μ l
Temperature	25 $^{\circ}$ C

Chromatograms of standard rutin and quercetin obtained on the basis of the chromatographic parameters shown in Table 1 are presented in Fig. 2.

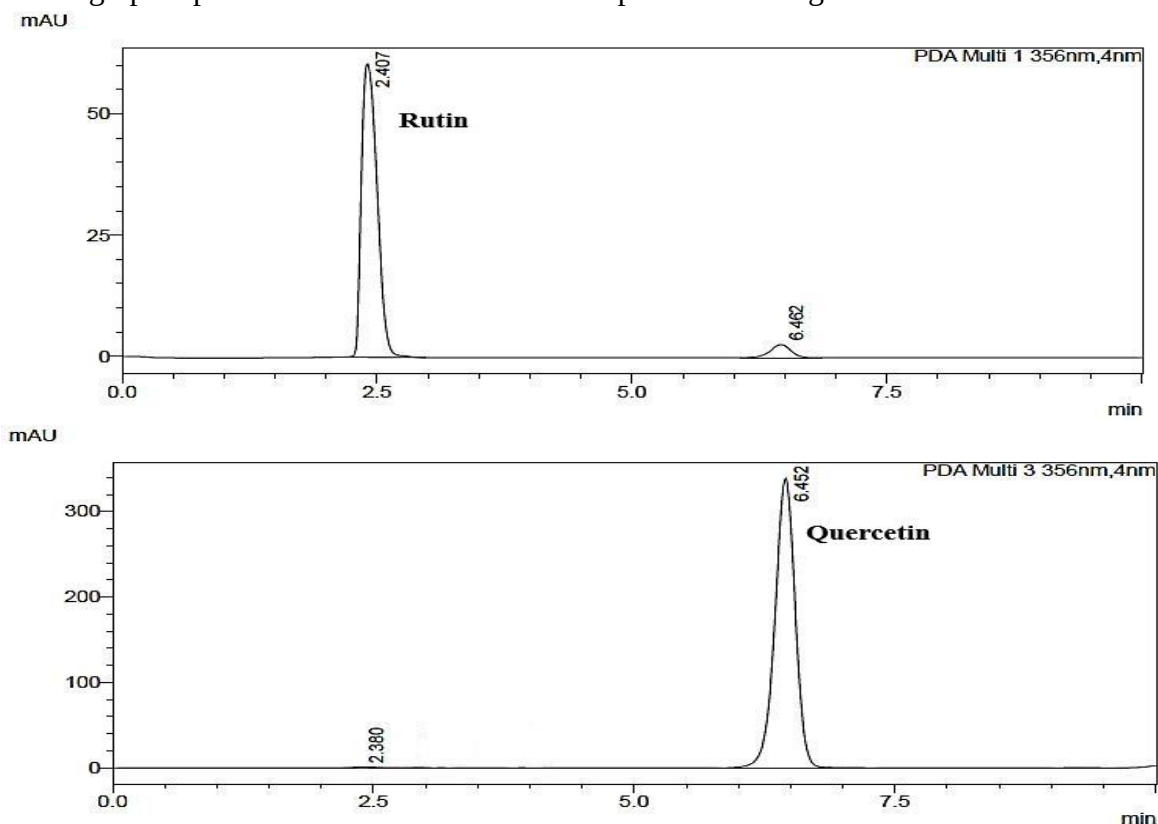


Fig. 2: HPLC Chromatogram of Standard Rutin and Quercetin at 356 nm.

Validation of rutin and quercetin. To determine the accuracy of the method, the field values of five different concentrations of rutin and quercetin (25, 50, 100, 250, 500 μ g/ml) were re-measured three times. The obtained results are presented in Table 2.

Table 2

Identification of Rutin and Quercetin by HPLC analysis

N ^o	Compounds	Retention time (min)	Conc. (μ g/ml)	Peak area (mean $\pm$ SD) (n=3)	Height	%RSD
1	Rutin	2.407	25	361205 $\pm$ 2818	29806	0.78
			50	656616 $\pm$ 4071	53445	0.62
			100	1563910 $\pm$ 12980	129111	0.83
			250	3393376 $\pm$ 25450	304806	0.75
			500	7030528 $\pm$ 41480	830449	0.59
2	Quercetin	6.452	25	1151897 $\pm$ 9330	135854	0.81
			50	2480551 $\pm$ 18356	288384	0.74
			100	6030406 $\pm$ 57289	721655	0.95
			250	11771997 $\pm$ 78872	1434702	0.67
			500	23689919 $\pm$ 151615	2927185	0.64

Based on the results of Table 2, calibration curves and calibration equations for rutin and quercetin were constructed (Fig. 3).

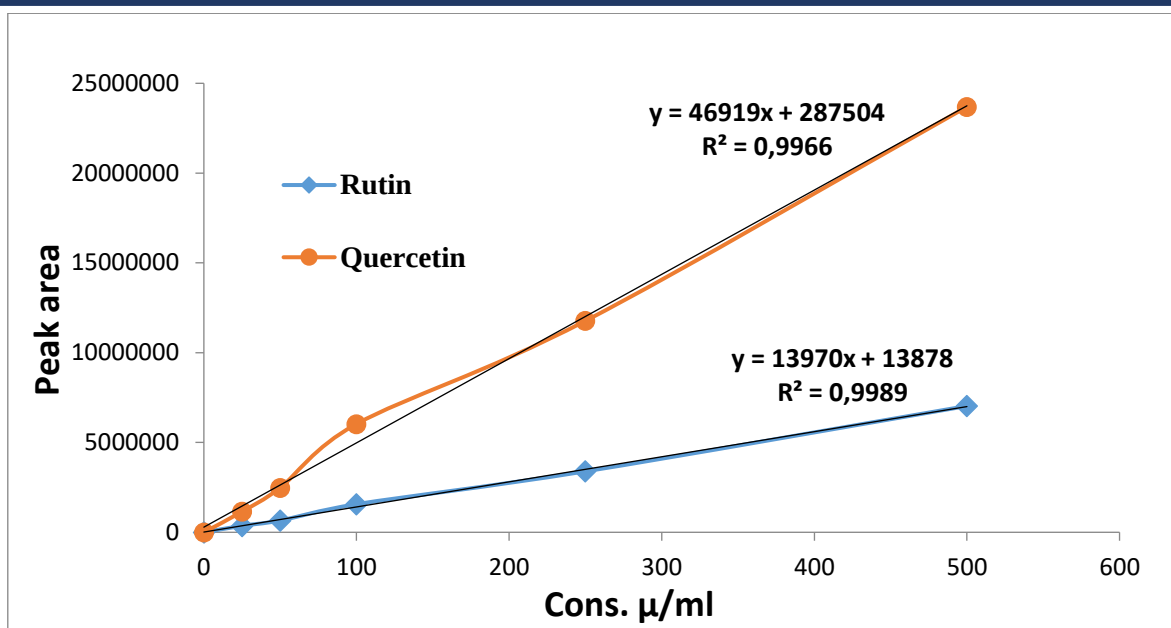


Fig. 3: Calibration curve of Rutin and Quercetin

Based on the calibration results, the detection limit (LOD) and quantification limit (LOQ) of Rutin and quercetin were found and the results are shown in Table 3.

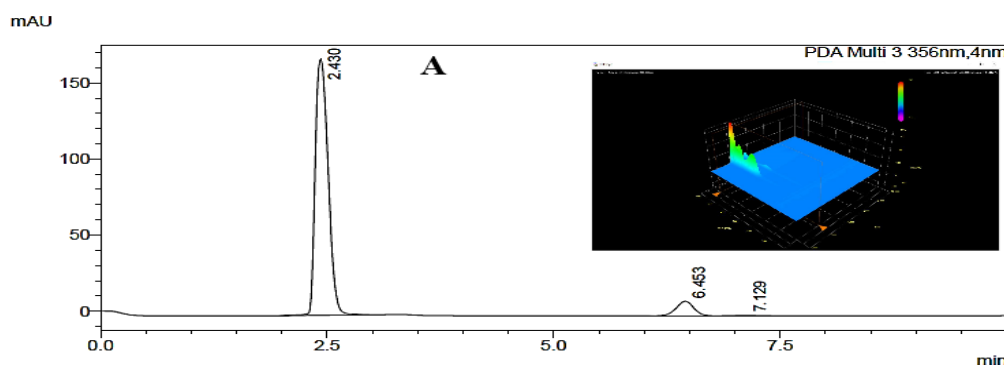
Table 3

Result of validation parameter

Parameter	Rutin	Quercetin
Wavelength (nm)	356	356
Linearity range (µg/ml)	25-500 µg/ml	25-500 µg/ml
Retention time (min)	2.407	6.452
Linear equation	$y = 13970x + 13878$	$y = 46919x + 287504$
Correlation (R^2)	0,9989	0,9966
Slope (b)	13970	46919
Intercept (a)	13878	287504
LOD	12.74	13.48
LOQ	38.59	40.86

HPLC analysis of rutin and quercetin

HPLC analysis of the rutin extract obtained from the flower buds of *Sophora Japonica* revealed a content of 94.483% rutin and 5.517% quercetin. When the quercetin obtained by acid hydrolysis of the isolated rutin was analyzed by HPLC, its composition was found to be 94.238% quercetin and 5.513% rutin. The HPLC chromatograms and their 3D views are presented in Figure 4.



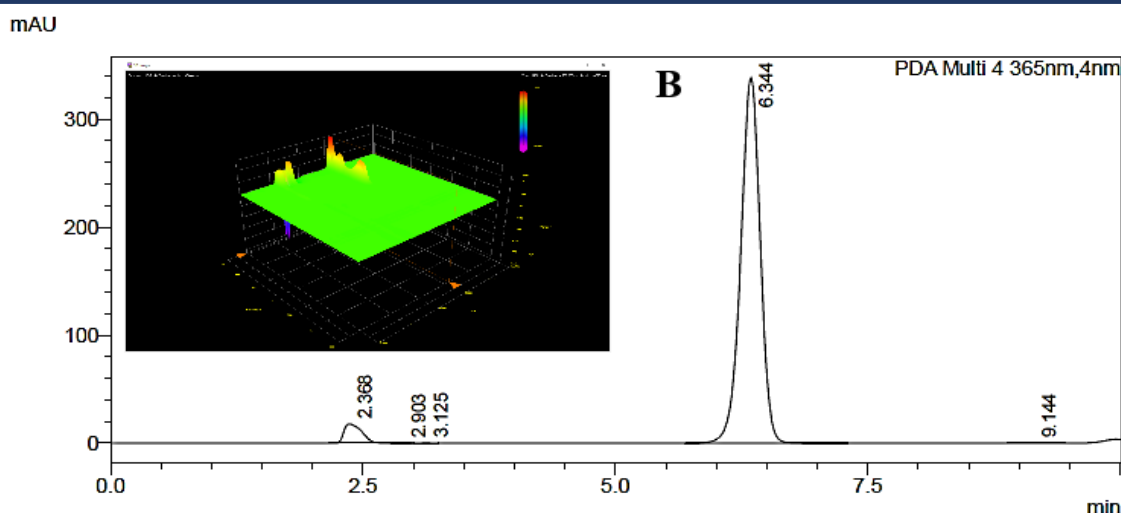


Fig. 4: Chromatograms of Rutin (A) extracted from *Sophora Japonica* flower buds and Quercetin (B) obtained from its hydrolysis and their 3D view

Table 4

HPLC analysis results of rutin and quercetin

Parameter	A		B	
Compound	Rutin	Quercetin	Rutin	Quercetin
Retention time (min)	2.430	6.453	2.368	6.344
Peak area	645783	37705	232430	3973341
Height	60465	2700	19214	293989
Peak area (%)	94.483	5.517	5.513	94.238

Conclusion

In this presented research, rutin was extracted from the flower buds of *Sophora Japonica* L. using aqueous extraction and purified through recrystallization in ethanol. Quercetin was obtained by acidic hydrolysis of the isolated rutin. A quantitative HPLC method was developed to determine the content of rutin and quercetin by comparing them with standards.

REFERENCES

- Perez-Vizcaino, F., Fraga, C.G. (2018). Research trends in flavonoids and health. *Arch Biochem Biophys*. 646:107-112. <https://doi.org/10.1016/j.abb.2018.03.022>
- Gorlach, S., Fichna, J., Lewandowska, U. (2015). Polyphenols as mitochondria-targeted anticancer drugs. *Cancer Lett*. 366(2):141-149. <https://doi.org/10.1016/j.canlet.2015.07.004>
- Vrhovsek, U., Rigo, A., Tonon, D., Mattivi, F. (2004). Quantitation of polyphenols in different apple varieties. *J Agric Food Chem*. 52(21):6532-6538. <https://doi.org/10.1021/jf049317z>
- Borbulevych, O.Y., Jankun, J., Selman, Sh., Skrzypczak-Jankun, E. (2004). Lipxygenase interactions with natural flavonoid, quercetin, reveal a complex with protocatechuic acid in its X-ray structure at 2.1 Å resolution. *Proteins*. 54(1):13-19. <https://doi.org/10.1002/prot.10579>
- Rodriguez-Garcia, C., Sanchez-Quesada, C., Gaforio, J.J. (2019). Dietary Flavonoids as Cancer Chemopreventive Agents: An Updated Review of Human Studies. *Antioxidants*. 8(5):137. <https://doi.org/10.3390/antiox8050137>

6. Abotaleb, M., Samuel, S.M., Varghese, E., Varghese, S., Kubatka, P., Liskova, A., Busselberg, D. (2019). Flavonoids in Cancer and Apoptosis. *Cancers*. 11(1):28. <https://doi.org/10.3390/cancers11010028>
7. Chirumbolo, S., Bjorklund, G., Lysiuk, R., Vella, A., Lenchyk, L., Upyr, T. (2018). Targeting Cancer with Phytochemicals via Their Fine Tuning of the Cell Survival Signaling Pathways. *International Journal of Molecular Sciences*. 19(11): 3568. <https://doi.org/10.3390/ijms19113568>
8. Kopustinskiene, D.M., Jakstas, V., Savickas, A., Bernatoniene, J. (2020). Flavonoids as Anticancer Agents. *Nutrients*. 12(2): 457. <https://doi.org/10.3390/nu12020457>
9. De Andrade, L., dos Santos, L.F., Pires, D.S., Machado, E.P., Martines, M., Macedo, M., Cardoso, T., Severino, P., Souto, E.B., Kassab, N.M. (2024). A Newly Validated HPLC-DAD Method for the Determination of Ricinoleic Acid (RA) in PLGA Nanocapsules. *Pharmaceuticals*. 17(9): 1220. <https://doi.org/10.3390/ph17091220>
10. Deshmukh, M.D., Patil, M.P. (2024). The Quality by design Approach for Analytical Method Development and Validation of the RP-HPLC Method for Estimation of Quercetin in Pure, Marketed, and Cream Formulation. *Biotech Res Asia*. 21(1). <http://dx.doi.org/10.13005/bbra/3224>
11. Guideline, I.H.T. (2022). Validation of analytical procedures Q2 (R1). *ICH: Geneva, Switzerland*.
12. Carvalho, D., Jesus, A., Pinho, C., Oliveira, R.F., Moreira, F., Oliveira, A.I. (2023). Validation of an HPLC-DAD Method for Quercetin Quantification in Nanoparticles. *Pharmaceuticals*. 16(12): 1736. <https://doi.org/10.3390/ph16121736>