

Extraction, purification, and quantification of hesperidin from the immature *Citrus grandis/maxima* fruit Nepal cultivar

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Abstract. Manikyam HK, Tripathi P, Patil SB, Lamichhane J, Chaitanya MVNL, Patil AR. 2022. Extraction, purification, and quantification of hesperidin from the immature *Citrus grandis/maxima* fruit Nepal cultivar. *Asian J Nat Prod Biochem* 20: 21-26. Hesperidin, a flavanone group of flavonoids and a 7-O-rutinoside of hesperitin, is abundant in citrus fruits. Pomelo or *Citrus grandis* (L.) Osbeck/ *C. maxima* (Burm.) Merr. is grown and cultivated in Nepal as seasonal edible fruit. Pomelo has flavanone and other chemical constituents. Flavanones like naringin, hesperidin, neohesperidin, and naringenin are present in the fruits of *C. grandis*. Many extraction techniques have been reported in the extraction and purification of hesperidin citrus fruits like sweet lemons, grapefruits, mandarins, etc. However, little information has been provided on hesperidin content in pomelo fruits. In order to estimate the quantity of hesperidin in the pomelo fruits of Nepal, we have first made a study. The immature pomelo fruits of size 2-7 cm were collected from the cultivation field and dried completely. The pulverized fruit powder with moisture content less than 15% was subjected to 5% methanolic acetic extraction and Dimethylformamide (DMF) solvent extraction in Soxhlet apparatus by refluxing at 90°C in 1:15 ratio in three subsequent extractions of each 1 hour. The concentrates were crystallized at freezing temperatures in methanol and further purified by repeating the crystallization process. The white amorphous powder was subjected to RP-HPLC analysis method, chromatographic conditions 285 nm wavelength, C18 column 4.6 mm x 15 cm x 5 µm, flow rate 1.2 mL/minute with injection volume 10 µL run time of 30 minutes, column temperature 40°C. Hesperidin yields were 0.12 g/100 g with acetic acid methanol with a purity of 87% and 0.17 g/100 g in DMF extraction with a purity of 90% with recoveries of 87% in acetic acid methanol extraction, and 90% in DMF extraction when compared with standard 93%. Further extraction does not yield any hesperidin content indicating a total of 0.15-0.17 g/100 g of hesperidin content in the whole immature fruit of *C. grandis/maxima*. Thus we found DMF and 5% methanolic acetic solvents as preferable solvents to extract hesperidin.

Keywords: Acetic acid, *Citrus grandis/maxima*, dimethylformamide, hesperidin, methanol, Nepal cultivar, RP-HPLC UV method

INTRODUCTION

Citrus grandis (L.) Osbeck/ *C. maxima* (Burm.) Merr. commonly called pomelo (red pomelo), is an important member of the Rutaceae family, well known for its high morphological variability, nutritional and commercial values (Alam et al. 2014; Susandarini et al. 2020). It was found in Barbados and commercially grown in Morocco, Israel, Spain, South Africa, Brazil, the USA, and some Asian countries (Jokic et al., 2019). In Nepal, it is called Bhogate and is eaten as seasonal fruit. In Nepal, they make Bhogate sadheko, a common salad eaten by local people. In India commonly called Chakotra. China is the major producer of pomelo, which is more than 60% of the world's cultivation (Man et al., 2015). Many bioactive compounds like flavanones, flavones, flavonols, and anthocyanin are abundantly present in the pomelo fruit. Chemical ingredients like limonoid glycones and aglycones, coumarins, naringin, hesperidin flavanones, etc., are the most abundant compounds in pomelo fruits.

Naringin is the most abundant flavanone present, around 295 to 377 mg/L in grapefruits and pomelo. Many research studies have proved that the extracts of pomelo have a therapeutic effect on cancer, inflammation, diabetes, and neurodegenerative and cardiovascular diseases with powerful antioxidant activity (Damon et al. 1987; Ma et al. 2008). It was studied that active components in the pomelo extract by using high-performance liquid chromatography (HPLC) had naringin (11.90 ± 0.21 mg/g dried extract), hesperidin (12.04 ± 0.12 mg/g dried extract), neohesperidin (25.4 ± 0.12 mg/g dried extract), and naringenin (9.20 ± 0.19 mg/g dried extract) (El-Shafae and El-Domiaty 2001; Luckner et al. 2002; Iglesias-Carres et al. 2019).

Hesperidin bio-flavanone, also known as hesperitin 7-rutinoside or 7-O-glycoside hesperitin (3,5,7 trihydroxyflavanone 7-rhamnoglucoside, C₂₈H₃₄O₁₅). Figure 1, abundantly present in citrus fruits with a molecular weight of 610.57 with a melting point of 250-255°C and boiling point of 576.17°C, respectively. It is not soluble in water and highly soluble in alkaline solutions but

moderately soluble in propylene glycol and Poly ethylene glycol 400 (Kanaze et al. 2004). It is highly soluble in DMSO and DMF. Poorly soluble in solvents like ethanol, isopropanol, and methanol (when purified) (Pandey et al. 2015).

Hesperidin is generally considered safe for topical and systemic administrations, with 2% in topical applications; it has shown no adverse cutaneous reactions in mice for 9 days of administration (Xiong et al. 2019). In addition, the oral formulation Daflon-500 mg containing 10% hesperidin with a daily dosage of 100 mg showed no adverse effects in rats (Razavi and Hosseinzadeh 2019).

Multiple therapeutic effects of hesperidin include anti-inflammatory, lipid-lowering, analgesic, antioxidant, and anticancer activities. Other than mentioned health benefits, hesperidin is well-known to treat venous insufficiency, varicose veins, venous ulcers, and hemorrhoids (Paudyal and Haq, 2008; Man et al., 2019). By stimulating the release of the appetite-regulating hormone cholecystokinin (CCK) in enteroendocrine STC-1 cells, both hesperitin and hesperidin help treat obesity by controlling appetite (Magwaza et al., 2015). In one of the studies on the neuroblastoma cell line, hesperidin has exerted a neuroprotective effect against rotenone by its antioxidant effect and maintaining mitochondrial functioning (Wu et al. 2007).

In recent studies, attempted extraction of hesperidin from peels of *Citrus reticulata* Blanco mandarin using deep eutectic solvents (DESs), a sustainable green extraction technique using choline chloride: acetamide (1:2) and water DESs had shown most efficient extraction of hesperidin 112.14 mg/g (Hendrickson and Kesterson 1954). Extraction of hesperidin by ultrasound assistance has shown that solvent has a profound effect on the yields, along with an increase in temperature and extraction time (Kim and Lim 2020). The highest flavonoid yields were achieved by 58.4% ethanol concentration at 80°C, 40 mL/g solvents to feed concentration for 30 minutes, and hesperidin yields of 66.6%. The extraction conditions for the highest flavonoid yields based on a response surface methodology were 80.3°C, 58.4% (ethanol concentration), 40 mL/g (solvent/feed), and 30 min (Tamilselvam et al., 2013). Ethanol extraction was found to be more effective when compared to methanol (yield 57.3%) and acetone (yield 37.7%) (Tamilselvam et al., 2013). Some studies showed that the yield of hesperidin was higher in 70% methanol than in 80% ethanol at 35°C from mandarin peels (Caengprasath et al. 2013), and *C. sinensis* pulp was higher in 90% methanol than 90% ethanol at 55°C (Kim and Lim 2020).

In the present study for the first time, for the first time, we are extracting hesperidin from immature fruits of the *C. grandis/maxima* (pomelo) cultivar of Nepal and quantifying it.

MATERIALS AND METHODS

Plant material preparation: *C. grandis/maxima* fruits were obtained from the local cultivation field Chisapani area, Kathmandu, Nepal, and immature fruits of size 2-7

cm see Figure 2, were dried till moisture content reached 15%. The 200 g of dried immature fruits of 2-7 cm were pulverized to 180 microns mesh size.

Extraction of hesperidin using acetic acid methanol solvent

Weight separately 100 g of dried immature fruit powder of 2-7 cm size and place it in Soxhlet apparatus with 5% acetic in methanol (1:15 feed/solvent) and continued extraction for 1 hour at 100°C with a circulation of solvent. A total of 3 extractions were carried out at an interval of 1 hour with a 1:15 feed to solvent ratio consequently. The total extract is collected, filtered, and concentrated to 80% before chilling. The 80% reduced liquid concentrate was chilled at 5°C for a few hours and filtered. Finally, the precipitate of hesperidin is filtered and washed with pure methanol till a white-colored amorphous powder appears and is further quantified.

Extraction of hesperidin with dimethylformamide

Weight separately 100 g of dried immature fruit powder of 2-7 cm size, place it in the maceration apparatus in DMF (1:15 feed/solvent), and continue stirring for 1 hour at 100°C with a solvent circulation. A total of 3 extractions were carried out at an interval of each 1 hour with a 1:15 feed to solvent ratio consequently. First, the extract is collected, filtered, and concentrated to 80% before chilling. Next, the 80% reduced liquid concentrate was chilled at 5°C for a few hours and filtered. Finally, the precipitate of hesperidin is filtered and washed with pure methanol till a white-colored amorphous powder appears and is further quantified.

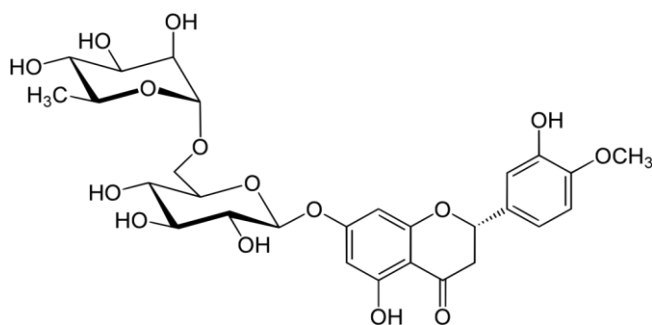


Figure 1. Structure of hesperidin



Figure 2. Immature fruit (2-7 cm) of *Citrus grandis/maxima*

Methods

Loss on drying

Accurately weigh 1.0~2.0 g of sample, and spread it on a dry flat weighing bottle previously weighed. Dry the bottle at $135 \pm 2^\circ\text{C}$ for 4 hours. Calculate loss on drying by the following formula, which should be less than 5.0%.

$$\text{Loss on drying}\% = (M1 - M2/M1 - M) \times 100\%$$

Where:

M : Tare weight of the weighing bottle (g)

M1 : Weight of the weighing bottle and sample before drying (g)

M2 : Weight of the weighing bottle and sample after drying (g)

Assay (RP-HPLC)

Chromatographic system

Chromatography system: HPLC with UV detector; Column: 4.6 mm x 15 cm x 5 μm packing C18; Wavelength: UV 284 nm; Flow rate: 1.2 mL/min; Column temperature: 40°C ; Injection volume: 10 μL ; Run time: 30 minutes

Mobile phase: Methanol: water: Glacial acetic Acid: acetonitrile = 28:66:6:2 (V/V), diluent for sample preparation: dimethyl sulphoxide

Preparation of solution

Test solution: Accurately weigh 25 mg of sample to a 50 mL volumetric flask, dissolve and dilute to exactly 50 mL with dimethyl sulphoxide, and mix well.

Reference solution: Accurately weigh 25 mg of a hesperidin reference standard to a 50 mL volumetric flask, dissolve and dilute to exactly 50 mL with dimethyl sulphoxide, and mix well.

Test procedure

Inject reference solution, test solution, and record the chromatograms. Calculate the assay of hesperidin according to a standard external method based on an anhydrous basis. The result of the assay should be not less than 90%.

$$\text{Assay (\%w/w)} = (A_T/A_R \times W_S/W_R) \times P \times 100\%/1\text{-LOD}$$

Where:

A_T : Main peak area in the chromatograms obtained with test solution;

A_R : Main peak area in the chromatograms obtained with reference solution;

W_S : Weight of sample, mg;

W_R : Weight of reference standard, mg;

P : Assay of reference standard, %;

LOD : Loss on drying of sample, %.

Method validation

Parameters like linearity, precision, accuracy, and limits of detection (LOD) and quantification (LOQ) were developed to determine the hesperidin content by the HPLC method. Calibration graphs for linearity were

determined using standard solution preparation of hesperidin at different concentrations of 20, 60, and 100 $\mu\text{g/mL}$. The dilutions were injected in series, and the peak area was calculated for each concentration plotted against the peak area. By adding standard at three concentrations of 20, 60, and 100 $\mu\text{g/mL}$, percentage recovery was calculated for each concentration to determine accuracy. Hesperidin was injected three times on the same day and on 3 different days to determine intra-day and inter-day variations to determine system precision. Both cases considered related standard deviation (RSD), a precision measure. By determining the LOD and LOQ, sensitivity was evaluated. The amount of analyte which gives a peak with a signal-to-noise ratio of 3 was defined as LOD, and the lowest amount of analyte with a signal-to-noise ratio of 10 was considered LOQ. The International Conference on Harmonization (ICH) was considered to validate the method.

Quantification of hesperidin in concentrated methanolic acetic acid extract

The total methanolic acetic acid extracts thus collected after 3 subsequent extractions were combined and concentrated. The concentrated extract was then subjected to RP-HPLC chromatography as mentioned in the assay method for standard hesperidin, and the area of peak in correlation to the retention time of standard was analyzed. Finally, the amount of hesperidin was calculated from the calibration plot obtained by a regression equation.

RESULTS AND DISCUSSION

Although hesperidin is a bioflavonoid in all citrus fruits, it is also known as Hesperitin 7-rutinoside or 7-O-glycoside hesperitin (3, 5, 7 trihydroxyfavanone 7-rhamnoglucoside). It is a highly economically viable phytochemical in demand for the Pharmaceutical, Nutraceutical, and cosmeceutical industries. It is a precursor molecule to synthesize Diosmin and a molecule used to treat neurological problems. Extraction of hesperidin using economically viable methods is needed in the present scenario looking at the abundant availability of raw material resources and market demand. Most immature citrus fruits varying in size from 2 mm- 3 mm contain 10-12% of hesperidin. Pomelo (*C. grandis/maxima*) is abundantly grown in Nepal as edible fruit. Pomelo fruit contains vitamin C, beta carotene, anthocyanin, hesperidin, and Naringin ingredients. Even though hesperidin content is found to be less in pomelo fruits, we attempted to quantify hesperidin in immature dried fruits for the first time in the Nepal cultivar of pomelo.

Many works have suggested using ethanol and methanol as solvents to extract hesperidin. In our present work, we have used two solvent methods, such as a 5% acetic acid in methanol and dimethylformamide, to extract hesperidin. The pH and temperature directly affect the recovery of the extraction of hesperidin. In experiments conducted by Hendrickson and Kesterson Florida horticulture society in 1955, they found hesperidin had

poor solubility in high-pH alcoholic solvents and high solubility in low-pH alcoholic solvents. Also, the optimal temperature for extraction should be from 90°C to 100°C (Alam et al., 2014). Research shows hesperidin solubility in DMF and DMSO (30 mg/mL) and soluble in ethanol and methanol (~1 mg/mL). Two separate experiments were carried out to extract hesperidin using 5% acetic acid in methanol and DMF in 1:15 feed to the solvent ratio for 3 hours consecutively at 100°C while stirring, and later the liquid extract was filtered and concentrated up to 80%. The concentrate was chilled at 5 °C for a few hours. Thus, the precipitate was filtered and purified by washing it with pure methanol. The obtained hesperidin was quantified using RP-HPLC UV analysis method, chromatographic conditions 285 nm wavelength, C18 column 4.6 mm x 15cm x 5 µm, flow rate 1.2 mL/minute with injection volume 10 µL run time of 30 minutes, column temperature 40°C. Hesperidin yields were 0.12 g/100g with acetic acid methanol with a purity of 87%, and 0.17 g/100 g in DMF extraction with a purity of 90% with recoveries of 87% in acetic acid methanol extraction, and 90% in DMF extraction when compared with standard 93% see Figure 3 and 4. Further extraction does not yield any hesperidin content indicating a total of 0.15-0.17 g/100 g of hesperidin content in the whole immature fruit of *C. grandis/maxima*. Thus we suggest using acetic acid methanol, methanol, or DMF as good solvents to extract high yields of hesperidin with good purity.

Method validation

Linearity, LOD, and LOQ

It was confirmed that the linearity of the method for concentrations ranging from 20 to 100 µg/mL of hesperidin. Good linear regressions observed with calibration curves $y = 0.3456x + 0.1573$ $R^2 = 0.9973$ (Figure 5 and Table 1). The minimum amount of analyte plant extract LOD used for detection and LOQ was 6.66 µg/mL and 20.19 µg/mL, with a retention time of 7.45 minutes.

Precision and accuracy

The precision and accuracy tests were performed by injecting the hesperidin sample three times within three consecutive days. Inter-day and Intra-day precision by RSD of the hesperidin peak areas ranged from 0.5-2.1, which is 2.5% under the limit as per ICH guidelines. By calculating the recovery %, accuracy was determined, and for hesperidin, it ranged between 89.99-102%, and the method was proven accurate (Table 2).

Table 1. Calibration data for the proposed HPLC method

Concentration (µg/mL)	Area (10 ⁵ mAU)
20	7.1
40	13.89
60	21.08
80	28.25
100	33.47
120	42.3

Table 2. Recovery and standard deviation data by HPLC

Compound	Amount added (µg/mL)	Amount recovered (µg/mL) ^a	Recovery (%) ^a	RSD (%)	
				Intra day	Inter day
Hesperidin	40	37.09 ± 0.26	92.75 ± 0.26	1.73	1.879
	80	81 ± 0.26	101.25 ± 0.26	2.12	0.156
	100	98.9 ± 1.69	98.9 ± 1.69	0.65	0.48

Note: ^a Mean ± SD (n=3) mean the sample was analyzed three times; ^b Samples were analyzed three times a day; ^c Sample was analyzed once a day over three consecutive days

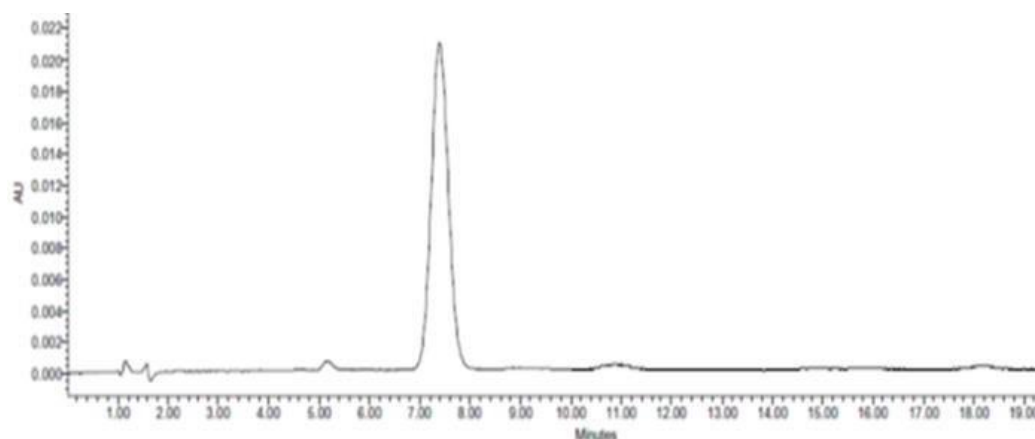


Figure 3. Chromatogram of hesperidin standard 93%

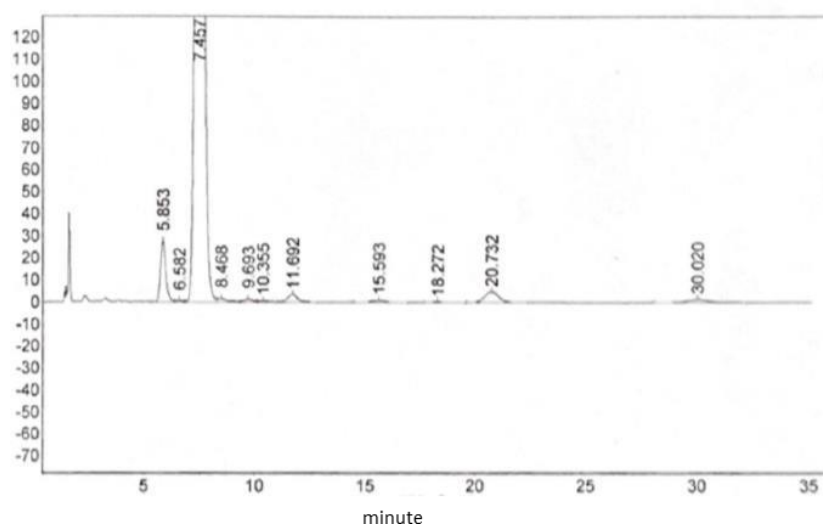


Figure 4. Chromatogram of hesperidin (90% pure) extracted from the dried whole immature fruit of *Citrus grandis/maxima*

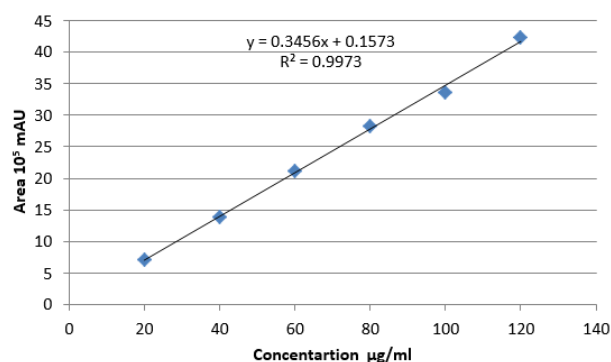


Figure 5. Linearity of hesperidin standard

Discussion

Hesperidin flavanone glycoside is usually found in citrus fruits, and its aglycone is called hesperitin. Plants produce it as a defense mechanism. Different extraction techniques were used earlier to isolate hesperidin with significant yields from different varieties of citrus fruits. It has been found that classical solvent extraction of hesperidin is preferred. The highest flavonoid yield was achieved by 58.4% ethanol concentration at 80°C, 40 mL/g solvents to feed concentration for 30 minutes, and hesperidin yields of 66.6%. The extraction conditions for the highest flavonoid yields based on a response surface methodology were 80.3°C, 58.4% (ethanol concentration), 40 mL/g (solvent/feed), and 30 min (Tamilselvam et al., 2013). Ethanol extraction was found to be more effective when compared to methanol (yield 57.3%) and acetone (yield 37.7%) (Tamilselvam et al., 2013). Some studies showed that the yield of hesperidin was higher in 70% methanol than in 80% ethanol at 35°C from mandarin peels (Caengprasath et al. 2013), and *C. sinensis* pulp was higher in 90% methanol than 90% ethanol at 55°C (Kim and Lim 2020). In our present work, we first time tried to extract

hesperidin and quantify it using DMF and 5% methanolic acetic acid as solvents from the whole dried immature fruit of *C. grandis/maxima* of the Nepal cultivar variety. Two separate experiments were carried out to extract hesperidin using 5% acetic acid in methanol and DMF in 1:15 feed to the solvent ratio for 3 hours consecutively at 100°C while stirring, and later the liquid extract was filtered and concentrated up to 80%. The concentrate was chilled at 5°C for a few hours. Thus, the precipitate was filtered and then purified by washing it with pure methanol. The obtained hesperidin was quantified using RP-HPLC UV analysis method, chromatographic conditions 285 nm wavelength, C18 column 4.6 mm x 15 cm x 5 µm, flow rate 1.2 mL/minute with injection volume 10 µL run time of 30 minutes, column temperature 40°C. Hesperidin yields were 0.09 g/100 g with acetic acid methanol with a purity of 87% and 0.17 g/100 g in DMF extraction with a purity of 90% with recoveries of 87% in acetic acid methanol extraction, and 90% in DMF extraction, when compared with standard 93%, see Figures 3 and 4. It was confirmed that the linearity of the method for concentrations ranging from 20 to 100 µg/mL of hesperidin. Good linear regressions observed with calibration curves $y = 0.3456x + 0.1573$, $R^2 = 0.9973$ (Figure 5 and Table 1). The minimum amount of analyte plant extract LOD used for detection and LOQ was 6.66 µg/mL and 20.19 µg/mL, with a retention time of 7.45 minutes. The precision and accuracy tests were performed by injecting the hesperidin sample three times within the same and three consecutive days. Inter-day and Intra-day precision by RSD of the hesperidin peak areas ranged from 0.5-2.1, which is 2.5% under the limit as per ICH guidelines. By calculating the recovery %, accuracy was determined, and for hesperidin, it ranged between 89.99-102%, and the method was proven accurate (Table 2).

Further extraction does not yield any hesperidin content indicating a total of 0.15-0.17 g/100 g of hesperidin content in the whole immature fruit of *C. grandis/maxima*. Thus, we suggest using acetic acid methanol, methanol, or DMF

as good solvents to extract high yields of hesperidin with good purity.

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