Optimization and Kinetic Studies of Ultrasound-Assisted Extraction on Polyphenols from Satsuma Mandarin (*Citrus Unshiu* Marc.) Leaves

Ciğeroğlu, Zeynep+●; Kırbaşlar, Ş. İsmail; Şahin, Selin; Köprücü, Gökben***

Istanbul University, Engineering Faculty, Department of Chemical Engineering, 34320 Avcılar, Istanbul, TURKEY

ABSTRACT: *The present article includes the Ultrasound-Assisted Extraction (UAE) of Citrus unsiu Marc. leaves rich in polyphenols. The best possible combinations of solvent, pH of the media, solvent/solid ratio, extraction time, extraction temperature and particle size were obtained for the maximum extraction of Total Phenolic Compounds (TPC) and Total Flavonoid Compounds (TFC) by using One-Factor-at-a-Time (OFAT) approach. The optimum extraction conditions of TPC were as follows: pH 4 in water; solvent/solid ratio of 20:1; extraction time, 54 min; and extraction temperature, 53°C. On the other hand, pH 2 in water, solvent/solid ratio of 42:1, 48 min and 58°C were found to be the optimal conditions for the extraction of TFC. The solvent selection was the most effective parameter for the related system. Additionally, several kinetic models (Film theory, Peleg model, first-order mechanism model and second order mechanism model) were employed to examine the kinetics of UAE.*

KEYWORDS: *Citrus unshiu Marc. leaves; Ultrasound-assisted extraction; Surfactant; One-factor-at-a-time optimization; Total phenolic content; Total flavonoid content.*

INTRODUCTION

Free radicals called Reactive Oxygen Species (ROS) are known to be harmful to DNA and cell membranes [1]. Phytochemicals containing antioxidants, are scavengers of free radicals, thus reducing these harmful effects [2]. Polyphenolic substances, especially flavonoids are beneficial to human health as shown in several studies [3-5]. Flavonoids have been the subject of numerous studies because of their antioxidant, antimicrobial, antitumor, antiviral and anti-inflammatory properties [6]. Studies have shown that flavonoids are effective against the common cold and breast cancer and several studies also discuss the positive effects of flavonoids on protecting the body against 6 different cancer types (colon, prostate, lung, estrogen receptor positive (ER+), melanoma, and Estrogen Receptor Negative (ER−)) [7-9]. It has been shown in the research that flavonoids are also effective against degenerative diseases such as Alzheimer's, and heart disease [10,11]. These cited properties have generated considerable interest in flavonoids amongst researchers.

* *To whom correspondence should be addressed.*

\+ *E-mail: zilby@gmail.com*

● *Other Address: Uşak University, Engineering Faculty, Department of Chemical Engineering, 64200, Uşak, TURKEY*

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Optimization of the extraction process in terms of yield is of great importance due to its economic profitability. There are lots of scientific papers investigating and comparing different traditional methods (Soxhlet, maceration) and novel ones (microwave, high-pressure, ultrasound) to extract phenolic compounds, flavonoids, polysaccharides and oil from various cells [12-14]. Time-consuming and expensive nature of traditional extraction methods led researchers to investigate new alternatives. Moreover, the conventional methods could have drawbacks of the product's quality giving rise to target compounds with unpleasant aromas due to the long extraction time [15]. One of the novel technologies, ultrasound-assisted extraction causes the plant cell to swell up within the solvent and burst spectacularly. This level of efficiency lowers the amount of solvent, thus showing the method to be more economic. The success of this method mostly depends on the cavitation, mechanical, and thermal abilities which can bring about the break of cell walls, particle size reduction, and improved mass transfer across cell membranes [16]. The superior factor giving rise to the improvement of extraction is ultrasonic cavitation, which leads to local hot spot point, free radicals, and locally high pressures [17,18]. In plant glands where the target components are placed within cells, size reduction by ultrasound treatment favors the complete extraction by maximizing the surface area [19].

Citrus unsiu Marc., known as Satsuma Mandarin, is the most common mandarin species grown in Turkey. Moreover, polyphenolic substances, especially flavonoids are plentiful in this species [20]. *Ma et al.* studied phenolic compounds and antioxidant activity of extracts by using ultrasonic treatment of Satsuma Mandarin peels [21]. To our knowledge, there is a lack of literature on the flavonoid and phenolic contents of the satsuma mandarin leaves. The first aim of this study was to identify a set of parameters which will give higher yields in the UAE process. The investigation was done using the best-known and the simplest optimization method, OFAT. The second purpose of this study was to analyze kinetic models through the extraction process.

EXPERIMENTAL SECTION

Materials

Citrus unshiu Marc. leaves were collected during the harvesting period in October 2012 by Batı Akdeniz Agricultural Research Institute (BATEM) which is located in Antalya, Turkey. The leaves were dried in a vacuum oven at 35ºC for 12 hours. The dried leaves were ground to their particle size through 500-710, 710-1000 and 1000-2000 µm sieves, respectively.

Methanol, ethanol, 2-propanol, sodium carbonate, (+)-catechin, gallic acid, Folin-Ciocalteu reagent, sodium hydroxide and hydrochloric acid were supplied from Sigma Aldrich Chemicals. Sodium nitrite and aluminum chloride were purchased from Merck & Co. A Millipore Milli Q water purification system was used for obtaining deionized (18 mΩ) water.

Ultrasound-assisted extraction

The extraction was carried out in an ultrasonic bath (Protech) with a frequency of 40 kHz, at several temperatures as reported in our previous studies [22,23]. Samples were put into vials, and stored at -20ºC until biochemical measurements. Each experiment was done in triplicate.

Total phenols determination

UV-Vis spectrophotometry (PG Instruments, TG-40) was used in order to measure the concentration of total phenolic content in the extracts. The procedure of Malik and Bradford [24] was applied to determine the total phenolic content, using the Folin-Ciocalteu reagent as an oxidizing agent. The absorbance was measured at 760 nm. The total phenolic content was calculated as the gallic acid equivalent per g of the dried leaf (mg-GAE/g-DL) using a calibration curve.

Total flavonoid determination

Total flavonoid content was determined by using a slightly modified version of the calorimetric method described by *Sakanaka et al.* [25]. The absorbance was read at 510 nm against a blank by UV-Vis spectrophotometry. The amount of total flavonoid content was expressed in mg (+) - catechin equivalent per g of the dried leaf (mg-CE/g-DL).

Optimization of each experimental condition with OFAT

The design factors were the selection of solvents (ethanol, methanol, 2-propanol, 50% 2-propanol, 50% ethanol, 50% methanol, water), pH of the media (2-12), solvent/solid ratio (10:1-50:1), extraction time (10-60 min), extraction temperature (32-62°C) and mean particle size (605-1500 µm). The effects of these independent parameters on TPC and TFC were investigated, respectively.

One-Factor-at-a-Time (OFAT) is a traditional method of experimental design. While the effect of one factor is observed on response/responses, the other factors are kept constant. The effects of solvent type, pH, solvent volume, and extraction time and extraction temperature were examined step by step on the extraction of TPC and TFC from mandarin. To select the optimal values of the factors, curve fitting function in SigmaPlot (ver 12) was used. Experimental results were expressed as means ±Standard Deviations (SD) of three replicate determinations.

Kinetics of ultrasound-assisted extraction

Kinetical models such as first order, second order, Peleg and film theory are commonly applied to extraction process of this kind [26-29]. Film theory contains two-stage mechanism with two parameters. The first stage is the washing stage and second stage is the slow extraction which is defined in Eq. (1) [26].

$$\frac{C_{(t)}}{C_{(e)}} = 1-(1-b).e^{-kt} \tag{1}$$

Where *C(t)*, concentration depends on time *t*; *Ce*, the concentration at equilibrium; *t*, extraction time; *b*, washing coefficient; and k, slow extraction coefficient.

In case of extraction, Peleg model can be employed as in Eq. (2) [27].

$$c(t) = \frac{t}{K_1 + K_2} \tag{2}$$

Where K_1, Peleg rate coefficient, K_2, Peleg's capacity coefficient.

First order mechanism model is based on the steady-state model which was explained by *Spiro and Jago*. First-order mechanism model is linearized as in Eq. (3) [28].

$$\ln\frac{c_e}{c_t} = k_{obs}.t \tag{3}$$

Where k_{obs}, is overall rate constant.

Second order mechanism model states that there are two simultaneous processes through extraction. The yield rises very rapidly with time at first and later reduces gradually with time during extraction process [29,30]. The integrated form for second order mechanism model is depicted in Eq. (4).

$$c(t) = \frac{c_s^2 kt}{1 + c_s kt} \tag{4}$$

Where *k* is the second-order extraction rate constant, *Cs* is the extraction capacity (concentration of extract yields at saturation).

The linear form of Eq. (4) becomes as Eq. (5);

$$\frac{t}{c_t} = \left(\frac{1}{kc_e^2}\right) + \left(\frac{t}{c_e}\right) \tag{5}$$

SigmaPlot version 12 was used to determinate the correlation coefficients and parameters for different kinetic methods.

RESULTS AND DISCUSSIONS

Effect of solvent type on TPC and TFC

The extraction yields (TPC and TFC) obtained by UAE with water, methanol (MeOH), ethanol (EtOH), 2-propanol, 50%MeOH, 50% EtOH, 50% 2-propanol at the operating condition of 30 min, 32ºC and solvent/solid ratio of 20:1 (v/w) is illustrated by Fig. 1.

Water was found in the most effective solvent. Expectedly, properties of the solvent such as polarity, dielectric constant, dipole moment and viscosity effects the diffusion rate thus effecting extraction efficiency [31,32]. In this study, properties of the water such as polarity (highest polarity index (9) amongst the solvents used) and dielectric constant (80) explain why water is the most effective on the TPC and TFP content. This might be explained by the fact that similar substances dissolve in similar solvents by nature [33-35]. Many of the researchers worked with water mixtures of EtOH and MeOH with different ratios instead of pure solvents found out higher phenolic content [33,36,37]. Our results are consistent with that of *Goli et al.* [38], who studied the effect of solvent type on the TPC extracted from pistachio hull. Similarly, *Khochar and Magnusdottir* [39] found that water is the most effective solvent comparing to 80% and 70% ethanol in the extraction of catechin and caffeine from the tea plant.

Effect of pH media on TPC and TFC

0.1 N of Hydrochloric acid and 0.1 N of sodium hydroxide were used to adjust pH levels. After water

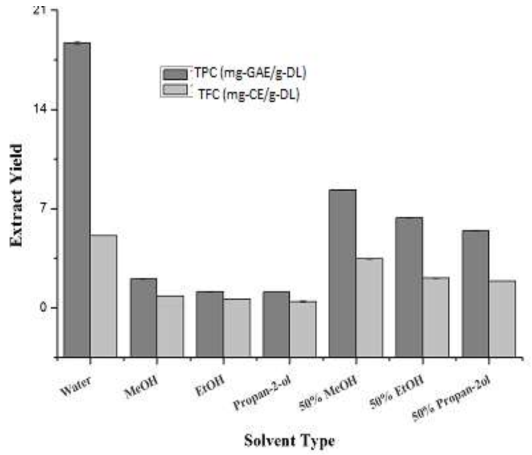

Fig. 1: Effect of solvent type on TPC and TFC in Citrus unsiu* Marc. *leaf extracts obtained by UAE (30 min, 32ºC, pH 6, 20:1 (v/w)and 1000-2000 µm particle size). Reported values are the mean ± SD (n=3).

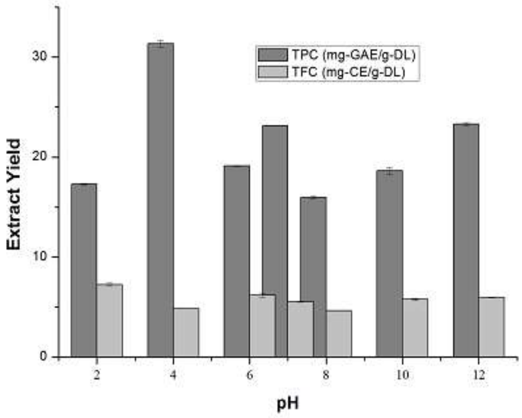

Fig. 2: Effect of pH on TPC and TFC in Citrus unsiu* Marc. *leaf extracts obtained by UAE with water (30 min, 32ºC, 20:1 (v/w) and 1000-2000 µm).Reported values are the mean ± SD (n=3).

was chosen as the best solvent, the pH levels of water were increased by factors of 2 from 2 to 12. Fig. 2 exhibits the TPC and TFC of the extracts obtained by UAE with water at 30 min, 32ºC and solvent/solid ratio of 40:1 (v/w). The sharp increase in TPC was seen at pH 4, whereas the highest yield of TFC was found at pH 2. The highest amounts of both TPC and TFC were achieved in acidic media. This might be explicated by the fact that an acidic pH supports the cleavage of phenolics bonded to proteins and carbohydrate polymers [40].

Liang and Xu [41] studied the impact of pH on the extract yield from tea in the range of 1.1-11 in their study. They gained the highest yield at pH 1.1. There was a sharp drop in the yield from pH 1.1 to pH 2 and fluctuated from 2 to 11. *Besides, Chethan and Maleshi* [42] analyzed the polyphenolic content in precipitate and supernatant from finger millet between the pH 1 to 10. They obtained best extract efficiency at pH 1. However, there was no trend showing a proportional relation from acidity to alkalinity. Besides, when alkalinity was used, the ratio of precipitate increased. While in acidity media, polyphenols dissolved. These results are in agreement with those of the present study.

Effect of solvent/solid ratio on TPC and TFC

Fig. 3 shows the TPC and TFC of the extracts obtained by UAE with water at 30 min and 32ºC. In this stage, the solvent volume was increased by factors of 5 from 5 to 25 mL.

The following pH levels of water were chosen: pH 4 for TPC and pH 2 for TFC. As seen in Fig. 3., the fast-rising of TFC was observed up to solvent/solid ratio of 50:1 (v/w). When more than solvent/solid of 30:1 (v/w) was used, the smaller increase in TPC and TFC were obtained. Higher quantities of solvent might help improve the mass transfer rate, so the extract yield can increase up to a certain point. *Prasad et al.* [43] and *Bucić-Kojić et al.* [44] observed the effect of solid/solvent ratio on extract yield from different plant matrixes. They found that solvent volume favored the extraction of polyphenolic contents. Their reports were consistent with our results. In this study, optimal solvent volumes were selected solid/solvent ratio of 20:1 (v/w) and solvent/solvent ratio 42:1 (v/w) for TPC and TFC, respectively.

Effect of extraction time on TPC and TFC

Extraction time was increased by factors of 10 mins from 10 to 60 mins with water at 32ºC as seen in Fig. 4. The pH and solvent volume were as follows: pH 4 and solvent/solid ratio of 20:1 (v/w) for TPC; and pH 2 and solvent/solid ratio 42:1 (v/w) for TFC.

A rapid rise was seen up to 30 min since the diffusion rate increased. After this point, the slope of the line was gradually decreased. The concentration comes to equilibrium at saturation level, thus diffusion rate kept constant. The TPC yields of *Wang et al.* [37] and *Thoo et al.* [45] are good harmony with this study. Moreover, they noticed that the extract yield decreased after

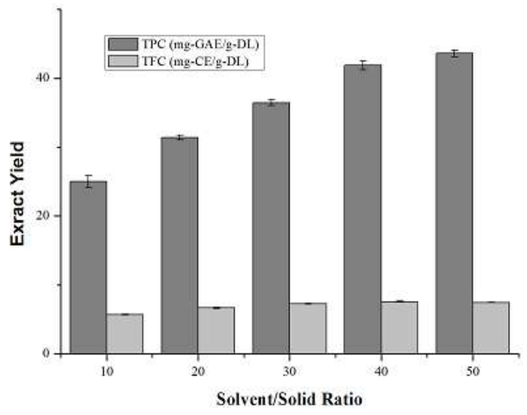

Fig. 3: ***Effect of solvent volume on TPC (30 min, 32ºC, pH 4 and 1000-2000 µm) and TFC (30 min, 32ºC, pH 2 and 1000-2000 µm) in Citrus unsiu*** **Marc.** ***leaf extracts obtained by UAE with water. Reported values are the mean ± SD (n=3).***

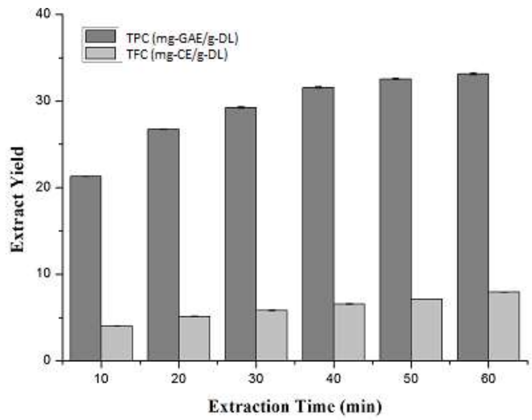

Fig. 4: ***Effect of extraction time on TPC (32ºC, pH 4, solid/water ratio of 20:1 and 1000-2000 µm particle size), TFC (32ºC, pH 2, solid/water ratio of 42:1 and 1000-2000 µm particle size) in Citrus unsiu*** **Marc.** ***leaf extracts obtained by UAE with water. Reported values are the mean ± SD (n=3).***

a certain time. Long extraction time might cause the degradation. 54 and 48 min were determined as the optimal extraction time for TPC and TFC, respectively.

Effect of extraction temperature on TPC and TFC

Fig. 5 shows the effect of temperature on the yields of TPC and TFC of Citrus unsiu Marc. leaf extracts obtained by water through UAE. The extraction temperature was increased by factors of 10ºC from 32 to 62ºC.

The selected extraction times for TPC and TFC were 54 mins and 48 mins. The remaining parameters were kept fixed as stated in the previous stage. First, TPC increased rapidly between 32 ˚C and 42˚C. The slope of line gradually increased between 42 ˚C and 52˚C. As for TFC, there was not an increment apparently. This might be explained by the fact that the density of cavitation reduced with increasing temperature [46,47]. According to the Einstein equation, temperature decreases solvent viscosity, leading to high diffusion rate [48]. In addition to Einstein equation, Teorell formula reveals that diffusion coefficient is proportional to temperature [49,50]. These phenomena were also reported by many researchers. For example, Wang et al. [51] extracted phenolic compounds from the peel of pomegranate. They observed that temperature resulted in the increase in the extract yield. They concluded that this situation was relevant to Einstein mobility which stated that temperature raised mass transfer. Furthermore, *Spigno et al.* [50] also examined total polyphenols from grapes. They showed the same conclusion as that of *Wang et al.* [51]. Consequently, the optimal temperatures were found as 53 °C and 58 °C for TPC and TFC, respectively.

Effect of particle size on TPC and TFC

In the final step, three different particle sizes (500-710, 710-1000, 1000-2000 µm) of the plant material were used to investigate the effect of solid particle size in the UAE of Citrus unsiu Marc._leaves (Fig. 6). While the remaining parameters were kept the same as in the previous stage, the following extraction temperatures were chosen: 53ºC for TPC and 58ºC for TFC.

Particle size affected the TPC in the extracts much more comparing to TFC as seen in Fig. 6. Both of the yields increased by decreasing the particle size, as a result of the higher surface area leading to rising in the mass transfer rate. Our results are in agreement with those of *Wang et al.* [50], *Capelo et al.* [52] *Zhao et al.* [53]. On the other hand,

the particle size has not a huge impact on the yield, since UAE method already favors the rupture of the plant cell walls [23]. This phenomenon here has two steps. The first step, dissolution of soluble components on surfaces of the plant matrix take places, which is known also as 'washing'. The other step, mass transfer of the solute from the plant matrix into the solvent by osmotic pressure and diffusion, which is known as 'slow extraction'.

Table 1: Values for kinetic parameters (k, b, K_1, K_2, k_1, k_2) and correlation coefficients (R^2) and adjustment correlation coefficients (Adj R^2), and the Root Mean Squared Deviation (RMSD) of each model for TPC and TFC through UAE.

	Kinetic Model	Parameters of Models	Value of Coefficients	R^2	Adj R^2	RMSD
TPC (mg-GAE/g-DL)	Film Theory	k	0.0740	0.9800	0.9734	0.7708
		b	0.1454			
	Peleg's	K_1	0.2077	0.9962	0.9953	0.2528
		K_2	0.0267			
	First Order Mechanism Model	k_1	0.0783	0.9760	0.9760	1.4016
	Second Order Mechanism Model	k_2	6818.2785	0.9934	0.9934	1.8806
TFC (mg-CE/g-DL)	Film Theory	k	0.0385	0.9858	0.9811	0.2811
		b	0.2284			
	Peleg's	K_1	1.7294	0.9462	0.9327	4.1354
		K_2	0.1047			
	First Order Mechanism Model	k_1	0.0456	0.9452	0.9452	0.5192
	Second Order Mechanism Model	k_2	50.1990	0.9882	0.9882	0.5197

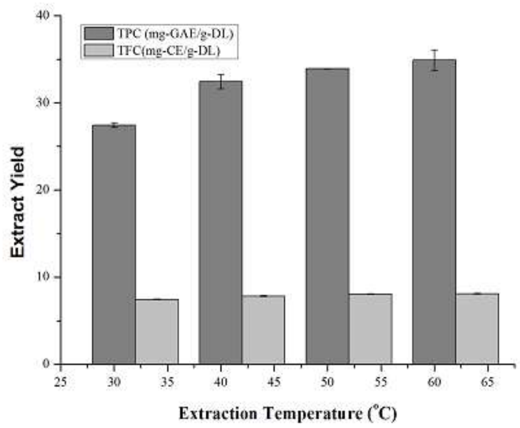

Fig. 5: Effect of extraction temperature on TPC (30 min, pH 4, solid/solvent ratio of 20:1 and 1000-2000 µm) and TFC (30 min, pH 2, solid/solvent ratio of 42:1 and 1000-2000 µm) in Citrus unsiu* Marc. *leaf extracts obtained by UAE with water. Reported values are the mean ± SD (n=3).

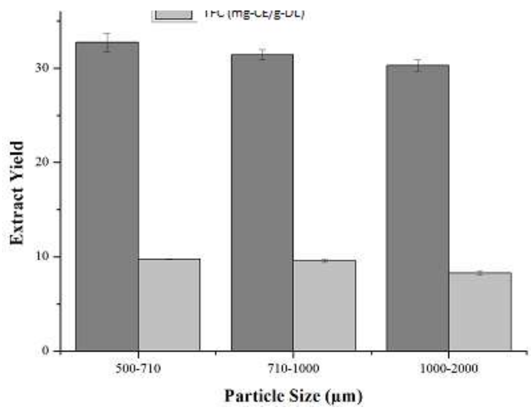

Fig. 6: Effect of particle size on TPC (54 min, pH 4, solid/water ratio of 20:1,53 ^{0}C) and TFC (48 min, pH 2, solid/water ratio of 42:1, 58 ^{0}C) in Citrus unsiu* Marc. *leaf extracts obtained by UAE with water. Reported values are the mean ± SD (n=3).

Kinetics of the UAE process

Table 1 indicates the kinetic parameters of each model (Film Theory, Peleg's, first-order mechanism model and second order mechanism model), the values of correlation coefficient (R^2) and adjustment correlation coefficient (Adj R^2), and the Root Mean Squared Deviation (RMSD) of the kinetic models. These values were calculated by applying the equations of kinetic models mentioned to experimental results depicted in Fig. 4 by means of SigmaPlot.

Correlation coefficient and root mean squared deviation were used to evaluate the relationship between the experimental and calculated values (Table 1). According to the high correlation coefficient (R^2=0.9760–0.9962 for the TPC, and R^2=0.9452–0.9882 for the TFC) and the low root mean squared deviation (RMSD= 0.2528–1.8806 for the TPC, and RMSD=0.2811–4.1354 for the TFC) in all data, the models applied in this study show to be convenient kinetic models for the extracts of Citrus *unsiu* Marc. leaves obtained by UAE.

CONCLUSIONS

Citrus *unsiu* Marc. leaves have proved to be a potential source of high added value compounds. In order to achieve the maximum TPC from the leaves by means of UAE, water (pH 4), solvent/solid ratio of 20:1 (v/w), 54 min and 53°C should be employed as optimal operating conditions. As for TFC, pH 2 in water, solvent/solid ratio of 42:1 (v/w), 48 min and 58°C were found to be the optimal conditions. Selection of the solvent was the most significant parameter affecting the system. Particle size showed a slight effect on the yields comparing to other variables. On the other hand, significant differences observed, when using acidic media at the optimal extraction conditions. The last but not the least, the applied kinetic models are proved to be appropriate to model the UAE extraction kinetics of polyphenols and flavonoids from Citrus *unsiu* Marc. leaves, according to the high correlation coefficient and the low root mean squared deviation in this system.

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