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Original article

Validated HPTLC Method for the Quantitative Analysis of Rosmarinic Acid in Several *Salvia* Sp.

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Rosmarinic acid (RA), which is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, is one of the most significant phenolic compound in the family Lamiaceae, which is restricted to the subfamily Nepetoideae, including *Salvia* species. Since various biological effects have been attributed to RA content of medicinal plants, has recently gained a great importance. We aimed to develop a rapid, simple and sensitive method for the quantitative determination of RA in extracts. The method was then practiced for comparative analysis of RA contents in seven *Salvia* species; *S. candidissima*, *S. dichroantha*, *S. heldreichiana*, *S. sclarea*, *S. tomentosa*, *S. triloba* and as well as the official sage *S. officinalis*. The methanol extracts obtained from the aerial parts of the plant materials were submitted to chromatographic separation on silica gel 60 F_{254} HPTLC plates with toluene: ethyl acetate:formic acid (5:4:1) as mobile phase and the densitometric detection of RA was carried out at 330 nm by HPTLC. Method was then validated in terms of accuracy, precision, repeatability, reproducibility, linearity, limit of detection/quantification, sensitivity and specificity. The newly developed HPTLC method provides a powerful approach to estimate RA content which is a generally phytomarker in many plant extracts.

Key words: HPTLC, Rosmarinic acid, *Salvia*, Sage, Lamiaceae.

Bazı *Salvia* Türlerinde Rozmarinik Asit'in Kantitatif Analizi İçin Valide Edilmiş YPİTK Yöntemi

Kafeik asit ve 3,4-dihidroksifenillaktik asit esteri olan rozmarinik asit (RA) özellikle Lamiaceae familyasının alt familyası olan Nepetoideae' ye bağlı Salvia türlerinin en önemli fenolik bileşiklerindendir. Bu çalışmamızda son zamanlarda pek çok aktiviteden sorumlu olduğu öne sürülen RA' nın bitki ekstrelerinde miktar tayininin kolayca yapılabilmesi için hızlı, basit ve hassas bir metod geliştirdik. Geliştirdiğimiz metodu Türkiye' nin çeşitli bölgelerinden topladığımız ve satın aldığımız yedi farklı *Salvia* türüne (*S. candidissima, S. dichroantha, S. heldreichiana, S. sclarea, S. tomentosa, S. triloba ve S. officinalis*) uyguladık. Bitkilerin toprak üstü kısımlarının metanol ekstreleri silika jel 60 F_{254} YPİTK plaklarına mobil faz olarak toluen: etil asetat: formik asit (5:4:1) kullanılarak uygulanmış ve 330 nm' de RA miktarı densitometrik olarak tayin edilmiştir. Sonuç olarak geliştirdiğimiz ve valide ettiğimiz yöntemimizin, RA miktar tayini için basit, güvenilir ve de kullanışlı bir yöntem olduğu kanıtlanmıştır.

Anahtar kelimeler: YPİTK, Rozmarinik asit, *Salvia*, Adaçayı, Lamiaceae.

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INTRODUCTION

Even before the chemical structure of rosmarinic acid (RA) (Figure 1) was elucidated, RA and similar compounds have been known as "Labiatengerbstoffe", a type of tannin known from species of the family Lamiaceae. After, two Italian chemists, Scarpati and Oriente (1958) isolated for the first time RA as a pure compound from *Rosmarinus officinalis* was named rosmarinic acid referring the plant it was isolated (1). The structure was elucidated as an ester of caffeic acid and 3,4 - dihydroxyphenyllactic acid.

RA is commonly found in species of the Boraginaceae and the subfamily Nepetoideae of Lamiaceae. A multitude of biological activities have been described for RA, mainly astringent, antioxidant, antiinflammatory, antimutagenic, antibacterial and antiviral (2). Particularly due to its potent antioxidant activity, RA acts as a bioactive compound to alleviate many disorders. Among these, RA supplementation was found to prevent cardiac abnormalities and hypertension in experimental animals, alleviate drug-induced nephrotoxicity and hepatotoxicity (3-5). Recent investigations have also revealed that RA may have beneficial effect in diabetic patients to improve libido via increasing serum testosterone level and exerts an early renal protective role to diabetic nephropathy (6,7). Moreover, RA was nominated as a good candidate for a new therapeutic approach in bone metastasis from breast carcinoma (8). In further studies, RA was isolated as one of the neuroprotective constituents against Aβ42 insult, a proteolytic derivative of the large trans membrane amyloid precursor protein, which plays a crucial role in Alzheimer's disease (9). For those reasons it is an important constituent of many medicines, e. g. Neurex™ (Smart), Persen™ (Lek Pharmaceuticals d.d.), nutritional additives, e.g. PAX+™ (Arcopharm), Life Extension™ (Herb soul), or preservatives e.g. Aquarox™ (Vitira). Usually rosmarinic acid is introduced in these preparations as ground dried leaves (aerial parts) of some natural plants, known to have human beneficial and health promoting effects, or powders obtained by evaporation of liquid extracts from these plants (10).

The genus *Salvia* (sage) is represented by around 90 species in the flora of Turkey half of which are endemic (11). Some *Salvia* species, particularly *S. triloba*, are traditionally consumed as herbal tea in Turkey. They are indicated as spasmolytic, carminative, diuretic, antiseptic, wound healing agents, as well as against colds (12). Research into historical literature has also revealed that sage was considered as a "cure all" medicine and it was believed that sage extends life to the point of immortality. Undoubtedly, RA is the most abundant caffeic acid dimer in *Salvia* species and has been reported to be the major phenolic constituent responsible for the several biological effects (*i.e.*: antioxidant activity) of *Salvia* species (13).

Due to widespread occurrence in Lamiaceae plants and broad biological effects, RA has been used as a marker component for the standardization of herbal crude drugs from this family including sage, lemon balm and rosemary or phytotherapeutics. Several methods have been developed for the quantitative analysis of RA in herbal extracts, such as solid state differential pulse voltametry, UHPLC, ESI/MS^n, HPLC (14-18). However, there are only few reported HPTLC methods for qualitative and quantitative determination of RA in Lamiaceae plant samples. In the successive publications of Fecka and colleagues, they investigated the polyphenol contents along with RA in several Lamiaceae spices, *i.e.*, thyme, marjoram, lemon balm and mint samples and compared the results from HPTLC-densitometric analysis with those from HPLC-UV (19,20). In another study, HPTLC technique was used to analyze the polyphenolic content including RA of *Satureja hortensis*, but the method was not validated (21). By considering the demand of this herb and the rosmarinic acid, there is a widespread need for simple and rapid analytical method for the manufacturer of plant-based medicines. There are various analytical techniques for the quality control of medicinal plants, such as NMR, LC - MS, HPLC, TLC, and HPTLC. Among these "High Performance

Thin Layer Chromatography" offers several advantages such as; comparatively shorter analysis time, comparison of many samples on a single plate, ease of optimization of fingerprint for certain compounds, ease of sample preparation, extreme flexibility of detection and so many. Hence HPTLC has become increasingly practiced at industrial level for routine analysis of many herbal medicines. The objective of the present work was to develop a HPTLC method for the estimation of RA composition present in herbal drugs and test the validation of method through analysis of RA content in various *Salvia* species.

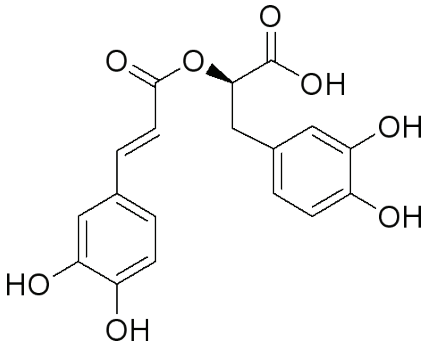

Figure 1. Chemical structure of Rosmarinic acid (RA)

EXPERIMENTAL

Plant material

Each *Salvia* species were collected from different regions of Turkey. *Salvia candidissima* Vahl. subsp. *occidentalis* Hedge (Akaydın 13349) and *Salvia heldreichiana* Boiss. ex Bentham (Akaydın 13366) were collected from Konya in July 2010, while *Salvia dichroantha* Stapf. (Akaydın 10539), *Salvia sclarea* L. (Akaydın 11086) and *Salvia tomentosa* Miller (Akaydın 10934) were gathered from Eskişehir, in July 2008, Turkey. The plant materials were identified by one of us (Dr. Galip Akaydın) and the voucher specimens are kept at the Herbarium of the Faculty of Education, Hacettepe University, Ankara, Turkey. *Salvia triloba* L. and *Salvia officinalis* L. were purchased from a herb dealer and the local market, successively. The above-ground parts of each *Salvia* species were dried at shadow and fresh air and stored at 25 ºC in air tight containers till further use.

Chemicals and materials

The standard RA was purchased from Sigma-Aldrich (Steinheim, Germany). Further analytical-grade ethyl acetate, formic acid and methanol were obtained from Sigma-Aldrich (Steinheim, Germany), toluene was obtained from Riedel-de Haën (Germany).

HPTLC instrumentation

The sample solutions were spotted in the form of bands of width 8 mm with Camag microlitre syringe (Hamilton, Switzerland) on precoated silica gel aluminum HPTLC plates 60 F_{254} (20 cm × 10 cm; Merck, Germany) using a Camag Linomat V automatic sample applicator (Camag, Switzerland). A constant application rate was applied and the spaces between tracks were 12.3 mm. Densitometric scanning was performed with Camag TLC Scanner III (Camag, Switzerland) in the reflectance-absorbance mode at 330 nm and operated with *WinCATS* software. The slit dimension was kept at 8.00 × 0.40 mm, macro, and the scanning speed was set at 20 mm/s. Concentrations of the compounds chromatographed were determined from the intensity of diffusely reflected light. Evaluation was carried out by comparing peak areas with linear regression. The mobile phase consisted of toluene (A) : ethyl acetate (B) : formic acid (C) (a : b : c, 5 : 4 : 1) where a:b:c are the proportions (by volume) of the components A, B and C, respectively (v/v%) (22). 20 mL of mobile phase was used per chromatography and each time it was prepared freshly. Linear ascending development was carried out in 20 cm × 10 cm twin through glass chamber (Camag, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 45 min. Before and after the development, plates were

dried at the oven. The length of chromatogram run was 9 cm.

Preparation of standard solution

A standard solution of RA (50 µg/mL) was prepared by dissolving 5 mg of accurately weighed RA in methanol and adjusting the volume of solution to 100 mL with methanol. Then the solution was filtered through a 0.45 µm microfilter. Later the standard solution of RA (50 µg/mL) was diluted to 200, 250, 300, 400 and 500 ng.

Preparation of sample solutions

10 g of each aerial parts of *Salvia* species were powdered. The powdered plant materials were separately left to maceration over a night in methanol (125 mL) and then extracted in rotary evaporator at 45 °C for 3 hours. The following day the same process was repeated for each material. Each of the pooled extracts was concentrated by using rotary evaporator under reduced pressure. The *Salvia* extracts were obtained with the yields as follows 12.1% for *S. heldreichiana*, 12.5% for *S. tomentosa*, 13.2% for *S. candidissima*, 14% for *S. dichroantha*, 15.4% for *S. officinalis*, 16.3% for *S. sclarea*, and 18% for *S. triloba*. 15 mg of each extract was accurately weighed and dissolved in 10 mL of methanol and then filtered through a 0.45 µm microfilter.

Quantification of Rosmarinic acid in seven different Salvia extracts

3 µL of *S. heldreichiana*, 3.5 µL of *S. tomentosa*, 5 µL of *S. dichroantha* and *S. candidissima*, 6 µL of *S. sclarea* and *S. triloba* and 14 µL of *S. officinalis* methanol extracts with the concentrations of 1500 µg/mL were applied in triplicate on aluminum silica gel 60 F_{254} HPTLC plates. The plates were developed and scanned as mentioned above. The amounts of RA in seven different *Salvia* extracts were calculated by using a calibration curve of the standard RA (50 µg/mL).

METHOD VALIDATION

The method was validated for specificity, linearity, accuracy and repeatability of measurement as well as repeatability of sample application as per ICH guidelines (23-25).

Calibration studies

The application concentrations of the standard solution of RA (50 µg/mL) were 200, 250, 300, 400 and 500 ng $spot^{-1}$ (band length: 8 mm, distance between tracks: 12.3 mm) on aluminum silica gel 60 F_{254} HPTLC plates using a Camag Linomat IV automatic sample applicator. The plates were dried in the oven before and after the development in a solvent system in each time freshly prepared of toluene: ethyl acetate: formic acid (5:4:1) in a Camag glass twin through chamber (20 cm × 10 cm) up to distance of 1 mm. The plates were scanned at 330 nm by using Camag TLC Scanner 3 and the *WinCATS* software. The integration limits were adjusted as 15 mm to 60 mm. Calibration curve of standard RA was obtained by plotting peak areas versus concentration of RA applied. Regression analysis of calibration data revealed a good linear relationship ($r^2 \geq 0.998$) for the peak area data, in the concentration range 200 - 500 ng/spot. A peak area versus concentration was subjected to least square linear regression analysis and the slope, intercept and correlation coefficient for the calibration were determined. Limit of detection (LOD) and limit of quantification (LOQ) were determined from the calibration curve, using the following equations: $3\sigma/S$ and $10\sigma/S$, where σ is the standard deviation of *Y-intercept* and *S* is the slope of the calibration curve.

Precision studies

Instrumental precision was checked by repeated screening of the same spot of the standard RA (250 ng) for seven times and was expressed as coefficient of variance (CV%). Variability of the method was studied by analyzing aliquots of standard solution of RA (250, 300, 400 ng/spot) on the same day (intra-day precision) and on different days (inter-day

precision) and the results were expressed as CV%.

Repeatability

Repeatability of the method was affirmed by analyzing 250 ng/spot of the standard RA solution after application on HPTLC plate for five times and was expressed as CV%.

Accuracy

One of the *Salvia* extracts was spiked with known amount of the standard solution. 1 mL of *S. heldreichiana* methanol extract (1500 µg/mL) was spiked with 25, 50 and 100 µg/mL of RA solution. In order to calculate percentage recovery at three levels, each time 4 µL of spiked solutions and diluted sample solution were used and the calculated and the experimentally determined amounts were compared.

Specificity

The identities of the RA bands in the corresponding samples were demonstrated by comparing the R_f values and the UV spectra with those of standard solutions. The purities of RA in the samples were confirmed by overlaying the UV spectra of the standard RA and the samples.

RESULTS AND DISCUSSION

Linearity and chromatographic conditions

We report a HPTLC method for rapid quantification of RA, a bioactive polyphenolic acid which is widely distributed in Lamiaceae. HPTLC procedure was optimized with the ratios of toluene: ethyl acetate: formic acid (5:4:1) gave a good resolution as well as well-defined peak at R_f value of 0.31 (Figures 2, 3). Other chromatographic conditions like chamber saturation time, run length, sample application rate and volume, sample application positions, distance between tracks, detection wavelengths were optimized to give reproducible R_f values, better resolution and symmetrical peak shape for the marker. The HPTLC chromatograms were taken at 330 nm and all the spectra were compared (Figure 4). The identity of the band of RA in the sample extracts were confirmed by overlaying its UV absorption spectra with that of the standard RA. Purity of the RA bands in the sample extracts were confirmed by comparing the absorption spectra at start, middle and end position of the band spectrum (Figure 5). RA showed good correlation coefficient when peak area of the resolved spot was plotted against concentration in the range of 250-500 ng/spot. The equation of the regression line was $Y = 451.6 + 11.46 \times X$ ($r^2 = 0.99898$) (Table 1).

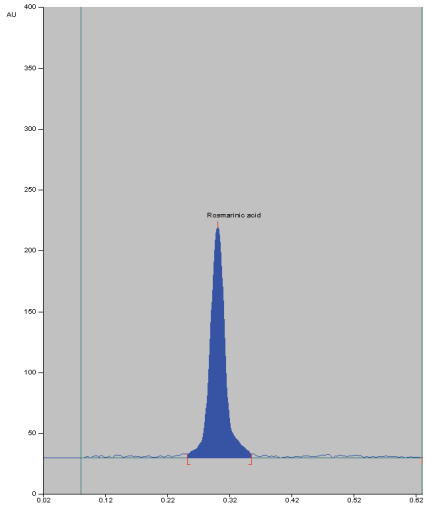

Figure 2. HPTLC chromatogram of the standard RA (R_f = 0.31)

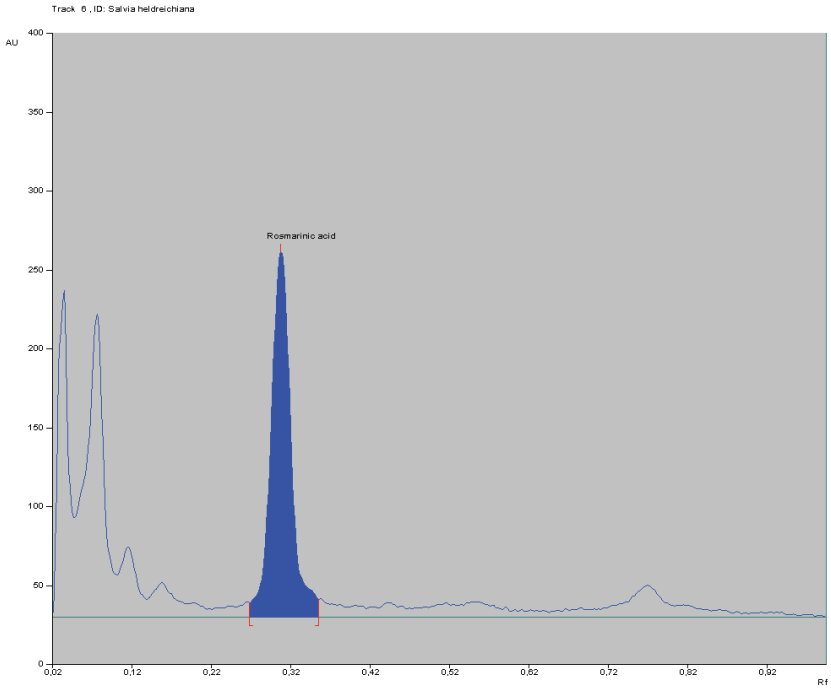

Figure 3. HPTLC chromatogram of one of the *Salvia* methanol extracts at 330 nm (*S.heldreichiana*, $R_f = 0.31$)

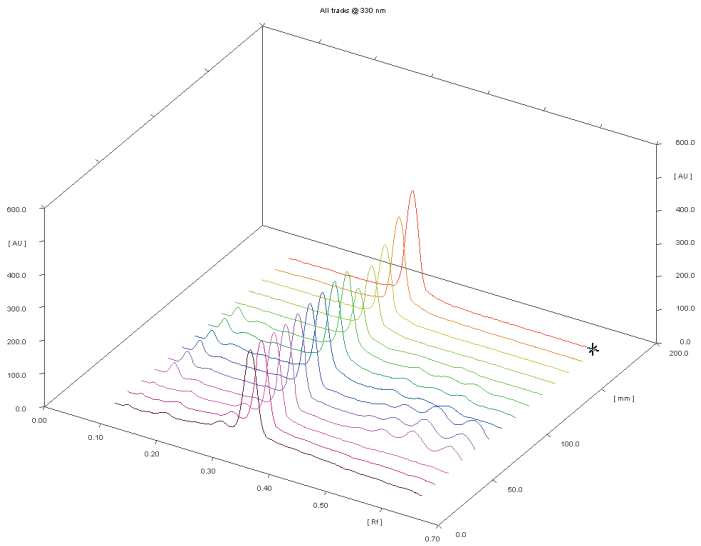

Figure 4. Three-dimensional overlay of HPTLC densitograms of the calibration spots of the standard RA and the *Salvia* extracts (*S. tomentosa*, *S. triloba* and *S. sclarea*)

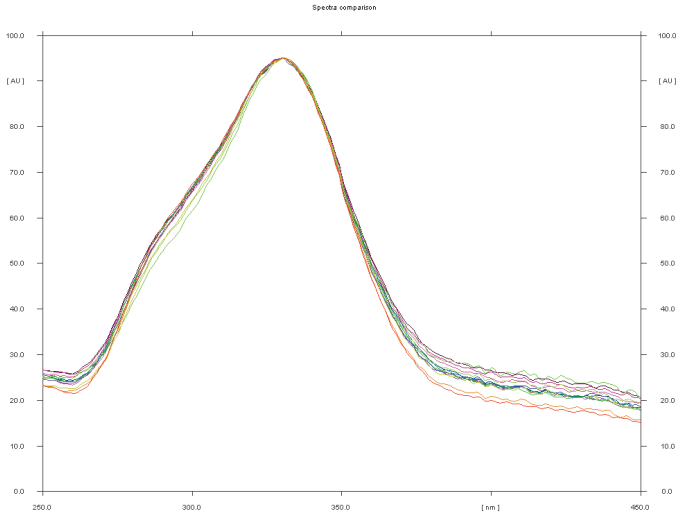

Figure 5. Spectra comparison of the standard RA and the *Salvia* extracts (*S. dichroantha, S. heldreichiana* and *S. candidissima*)

Limit of detection and Limit of quantification

The limit of detection was found to be 76.78 ng while the limit of quantification was found to be 232.67 ng (Table 1).

Precision

The proposed method was found to be precise as indicated by percent RSD not more than 0.5 (Table 2).

Repeatability

Repeatability of the method was given as percent RSD and found as 0.42% (Table 1).

Accuracy

The percentage recovery at three levels of RA was found to be 101.54%, 100.64%, and 100.25% with an average recovery 100.81% (Table 3).

Specificity

The R_f value of RA was calculated as 0.31. The identity of RA was detected also in those of seven *Salvia* samples at R_f of 0.31. Also the identity of RA in *Salvia* species was verified by overlapping the UV spectra of the standard solution and the sample solutions. An absorption maximum for RA was also verified.

Determination of rosmarinic acid content in seven Salvia MeOH extracts

The amount of RA in dry MeOH extracts was found to be in the ranges of 1.2 to 7.3% (Table 4).

Table 1. Summary of validation parameters

No	Parameter	Results
1	Precision (CV%, n = 7)	0.26
2	Repeatability (CV%, n = 5)	0.42
3	Accuracy (average % recovery)	100.81
4	Limit of detection (ng/spot)	76.78
5	Limit of quantification (ng/spot)	232.67
6	Linearity (correlation coefficient)	≥ 0.998
7	Dynamic range (ng/spot)	200 - 500

Table 2. Intra- and Inter-day precision study

Concentration (ng/spot)	Intra-day precision* (CV%)	Inter-day precision* (CV%)
250	0.16	0.29
300	0.33	0.57
400	0.13	0.53

*Mean (n=3)

Table 3. Recovery of rosmarinic acid

Amount of RA present in the sample (µg)	Amount of RA added (µg)	Amount of RA found (µg)	Recovery (%)	Average recovery (%)
110	25	137.08 ± 0.7	101.54 ± 0.54	100.81
110	50	161.03 ± 1.01	100.64 ± 0.63	
110	100	210.53 ± 2.12	100.25 ± 1.01	

*Mean ± SD (n=3)

Table 4. Rosmarinic acid content in different *Salvia* species

No	Sample (1.5 mg/mL)	RA content (w/w %)
1	*Salvia candidissima*	5.0
2	*Salvia dichroantha*	4.2
3	*Salvia heldreichiana*	7.3
4	*Salvia sclarea*	4.1
5	*Salvia tomentosa*	7.2
6	*Salvia triloba*	3.8
7	*Salvia officinalis*	1.2

*Mean (n=12)

CONCLUSION

Considering the broad range of biological activities determined through pharmacological investigations and widespread occurrence of RA in herbal drugs, a HPTLC method was developed for the quantification of free RA in herbal extracts. Further, RA contents of seven *Salvia* species were estimated using the proposed method and the method was found to be simple, precise, specific and sensitive. Based on our results, the extract of *S. officinalis* (1.2%) from the local market bears the least RA content, while *S. heldreichiana* (7.3%) and *S. tomentosa* (7.2%) gathered from nature possess the highest (Table 4). *S. triloba* (3.8%), *S. sclarea* (4.1%), *S. dichroantha* (4.2%), and *S. candidissima* (5.0%) preceded *S. officinalis*, respectively, in increasing order. The above mentioned HPTLC technique was successfully used for estimation of RA in *Salvia* methanol extracts. The developed method is the first validated HPTLC method for the quantification of RA content in *Salvia* species to the best of our knowledge.

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