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Use of liquefied cold temperature dimethyl ether for extraction of pigments from fresh vegetable tissues

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Abstract: Dimethyl ether (DME) is known as a useful precursor to other organic compounds and is a promising alternative fuel without issues of toxicity, production, infrastructure, and transportation as is the case with various other fuels. Recently, DME has attracted the attention of scientists and engineers since it behaves as a subcritical solvent or a low-temperature solvent applicable for the extraction of organic molecules from bio-materials. This paper presents the extraction of chlorophylls and carotenoids from green peel and yellow cortex of Japanese squash, spinach leaves and carrot roots using low-temperature liquefied DME. Spectroscopic and fluorescence analyses of the extracted pigments revealed that chlorophylls were successfully extracted by liquefied DME from green materials (squash peel and spinach leaves). HPLC analysis further confirmed that chlorophylls extracted include both chlorophylls *a* and *b*. By using liquefied DME, carotenoids were extracted from all vegetable samples examined. The performance of DME as a novel pigment extracting agent is confirmed in this work and its use as a "green" solvent, as opposed to conventional solvents, for the preparation and extraction of various plant pigments is highly encouraged from an environmental point of view.

1. Introduction

Dimethyl ether (DME), the simplest ether with the formula CH_3OCH_3, is known to be a useful precursor to other organic compounds such as liquefied petroleum gas (LPG) (Zhu *et al*., 2007) and small molecular hydrocarbons (Zhu *et al*., 2008). DME is recognized as a promising alternative fuel for diesel engines, petrol engines, and gas turbines (Gupta *et al*., 2010), enabling clean and high-efficiency combustion with reduced emission of NO_x, SO_x, and particulate matter (Semelsberger *et al*., 2006). Furthermore, DME can be efficiently reformed to H_2 at low temperatures, and most importantly, DME does not have large issues with toxicity, production, infrastructure, or transportation as is the case with various other fuels (Semelsberger *et al*., 2006).

Apart from its use as a fuel or a precursor to other organic chemicals, DME has attracted the attention of scientists and engineers since it behaves as a subcritical solvent. For instance, performance of subcritical DME as an effective media for the extraction of medicinal, flavoring, and pungent agents from some spices (Ginger, black pepper, and chili powder) has been demonstrated (Catchpole *et al*., 2003). In addition to the subcritical approach, DME can be used as a low-temperature solvent or extraction agent, and thus, it is applicable to laboratory procedures for the extraction of organic molecules from bio-materials (Kanda and Makino, 2010; Kanda and Li, 2011). Recently, an industrial use of liquefied DME was reported for rapid removal of water from sub-bituminous coal without any heating process (Kanda and Makino, 2010). This approach encourages us to testify the performance of liquefied DME in de-watering of biomaterials including watery horticultural crops.

One may believe that the usefulness of DME as a solvent is limited by its low boiling point (-23°C). However, this property could be highly beneficial as it may facilitate the removal of the solvent from the extracted samples. In biochemical exercises at ambient temperature, a variety of conventional solvents such as alcohols, acetone, hexane, etc. are used for extraction of organic substances of interest including flavoring agents and pigments from various biological samples. However, extraction of some reactive or highly degradable chemicals requires handling at

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low temperature. Due to the nature of DME in liquefied form at ambient pressure (or relatively low pressure) at low temperature, it may allow us to perform extraction of pigments at low temperature, preferably below 0ºC. In the present study, we examine the application of liquefied DME for the extraction of model plant pigments such as chlorophylls and carotenoids from fresh vegetable tissues by passing the low-temperature solvent through the layer of homogenates of the tissues.

2. Materials and Methods

Plant materials

The pericarp (fruits) of Japanese squash (*Cucurbita moschata*, cv. Ebisu, cultivated in Hokkaido, Japan), leaves of spinach (*Spinacia oleracea* L., cv. Hunter, cultivated in Fukuoka prefecture, Japan), and roots of carrot (*Daucus carota* L., cv. Koyo-2, cultivated in Hokkaido, Japan) were obtained from a local market and used for the extraction of pigments. Squash pericarp was dissected into green peel (1 mm from the surface) and yellow tissue while spinach leaves and carrot roots were used without such separation. Following homogenization of the vegetable tissues in a mixer, the obtained pastes (1-2 g fresh weight, g fw) were layered in the sample holder in the extraction chamber.

Chemicals

Reagents for separation and analysis of pigments such as HPLC grade acetone and methanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and ammonium acetate (Sigma-Aldrich Japan, Tokyo, Japan) were obtained through local vendors.

Apparatus for cryo-liquefaction of DME and extraction of pigments

The DME gas cylinder was obtained from a local gas vendor (Air-Gases Kitakyushu Inc.) and supply of gaseous DME was made through 0.5 MPa gauge. The newly-designed apparatus consisted of (1) DME gas cylinder connected to a flow meter, (2) cooling unit, (3) extraction chamber, and (4) liquid trapping port (Fig. 1A). Each of the units was connected with aluminum capillaries. Within the extraction chamber, there was a disk-shaped sample holder for vegetable paste layering. Since the extraction chamber of acryl resin was transparent, the amount of cryo-liquefied DME could be monitored and manually controlled (Fig. 1B, C).

Treatment of plant materials with liquefied DME

For extraction of model pigments from the homogenized vegetable tissues, the apparatus shown in Figure 1 was used. DME gas, supplied directly from the gas cylinder at 0.4 MPa, was rapidly cryo-liquefied in the cooling unit. By handling (opening and closing) of a stopcock between the cooling unit and extraction chamber, collection and release of liquid DME precipitated in the cooling chamber was manually controlled. After loading *ca*. 15 ml liquid DME into the extraction chamber, the vegetable sample was allowed to have contact with liquefied DME only for a few seconds. The mixture of passed DME and accompanying liquid was collected in a test tube by opening the stopcock between the extraction chamber and the collection unit (Fig. 1D, E). In turn, dry matter was left in the extraction chamber. Following passive DME removal under ambient pressure, mixtures of vegetable sap (water) and vegetable-derived oily materials containing pigments were recovered.

Spectroscopic analysis

Pigments recovered after extraction with DME were diluted with acetone and used for spectroscopic scanning and fluorescence analyses using a spectrophotometer (U-3310, Hitachi High-technologies, Tokyo, Japan) and a fluorescence spectrophotometer (F-4500, Hitachi High-technologies, Tokyo, Japan), respectively. For fluorometric analysis of pigments, a three-dimensional (3D) contour plot was performed as described elsewhere (Kawano *et al*., 1999). The fluorescence contour profile was obtained with an excitation range between 250 nm and 700 nm (slit size, 5 nm; sampling interval, 5 nm) and an emission range between 300 nm and 750 nm (slit size, 5 nm; sampling interval, 5 nm), at scanning speed of 30000 nm/min. Recovery of chlorophyll *a* in the extracts was spectroscopically quantified according to the formula proposed by Wellburn (1994) as follows: [chlorophyll *a*] (μg/ml) = 12.25 x $A_{663.2\ nm}$ - 2.79 x $A_{646.8\ nm}$. Similarly, concentration of total carotenoids in the extracts was spectroscopically quantified using the following formula proposed by the National Agriculture and Food Research Organization of Japan: [total carotenoids] (mg/L) = 4.143 x $A_{475\ nm}$ - 0.561.

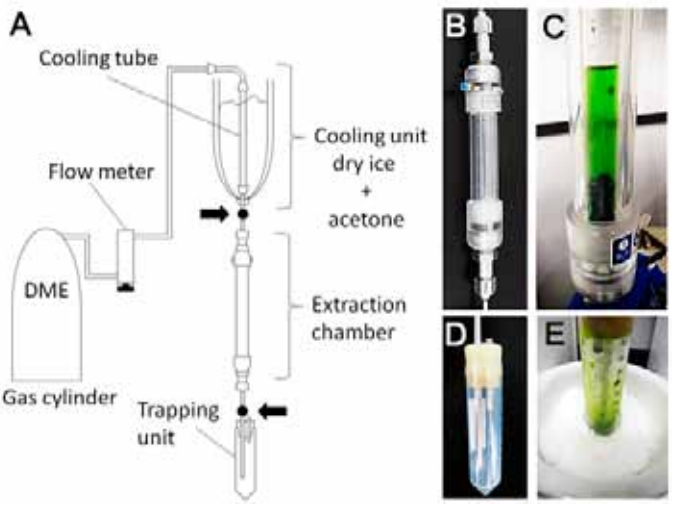

Fig. 1 - Apparatus for pigment extraction with liquefied DME. (A) Composition of the system. Arrows indicate the positions of two stopcocks designed for fine controls of gaseous and liquefied DME flows. (B, C) Extraction chamber. (D, E) Trapping port with a plastic tube.

High-performance liquid chromatography (HPLC)

Aliquots of the extracts containing pigments were analyzed by the reverse-phase HPLC system according to Maeda *et al*. (1998). HPLC (Waters 2690 Separations Module, Waters, USA) equipped with Inertsil ODS-80A column (GL Science, Tokyo, Japan) and two types of detectors, namely, a detector with photodiode arrays (water Alliance PDA system, Waters, USA) and a fluorescence detector (Waters 474 Scanning Fluorescence Detector, Waters, USA), for the separation and detection of pigments.

Analysis of chlorophylls was carried out using the solvent system which consisted of A (1:4 mixture of 1 M ammonium acetate and 80% methanol) and B (1:4 mixture of acetone and methanol). The program started with a linear gradient from A to B (15 min), continued with an isocratic run with B (5 min), and returned to A (2 min), and continued isocratically for 8 min at a flow rate of 1 ml/min. Peaks corresponding to pigments were detected with a fluorescence detector (excitation at 405 nm, emission at 660 nm).

Analysis of carotenoids was carried out using the solvent system consisting of 9:1 mixture of methanol and ethanol at flow rate of 1 ml/min. Peaks corresponding to pigments, chiefly carotenoids, were detected by monitoring absorbance at 455 nm using a detector with PDA.

3. Results and Discussion

Preparation of samples and pigment extraction

Fresh tissues of Japanese squash (green peel and yellow cortex), spinach (green leaves) and carrot (roots) were homogenized and the resultant pastes were prepared for extraction with liquid DME (Fig. 2A-K). After passing liquid DME through the plant sample layer (homogenates) packed in the column of the extraction chamber, a dry powder resembling acetone powder was left in the apparatus (Fig 2L-O). In turn, liquid samples mostly the mixtures consisted of extracted water (due to de-watering action of DME), oil (due to solvent action of DME), and the carrier liquid DME, were collected in the tubes placed beneath the extraction apparatus. As DME can be readily evaporated out under conditions of ambient pressure and temperature, solvent removal from the collected liquid was passively allowed by leaving the tubes with liquid samples at ambient temperature for at least 30 min. As a consequence, dense colored aqueous samples remained in the tubes (Fig. 2P-S) which were a mixture of oil and water. This indicated that both water and oils were extracted from the plant samples by liquid DME. Note that pigments were separately extracted from the green peel (Fig. 2A, D, H, L, P) and yellow cortex (pericarp; Fig. 2A, E, I, M, Q) of Japanese squash. The above extraction processes were repeated three times.

The total yield of oil/water mixture extracted with DME flow largely depends on the water content in the vegetable samples; the highest yield was found with

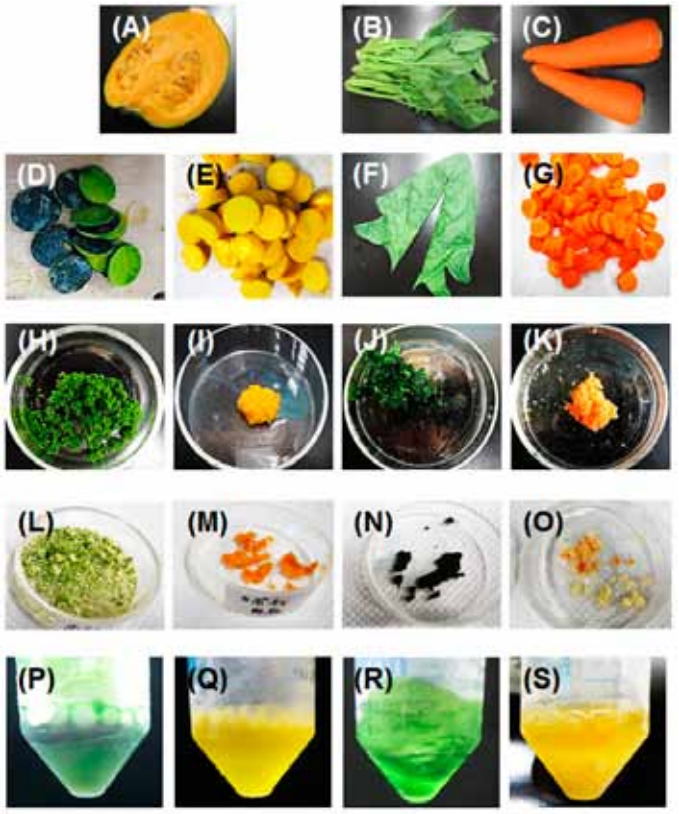

Fig. 2 - Fresh tissues, homogenized paste, dry powder, and pigment mixture of vegetables. Peel and cortex of Japanese squash (A, D, E, H, I, L, M, P, Q), leaves of spinach (B, F, J, N, R) and carrot roots (C, G, K, O, S) were used for extraction of pigments using DME. Whole vegetables (A-C), fresh tissues prior to homogenization (D-E), paste of homogenates (H-K), dry powder remaining after extraction with DME (L-O), and pigment mixtures obtained after evaporation of DME (P-S) are shown.

carrot roots, the most watery sample among those tested (Fig. 3). Since a drastic change in color after DME-based extraction was observed in squash peel (Fig. 2H, L) and carrot root tissues (Fig. 2K, O), we assumed that extraction of pigments was successfully carried out. In

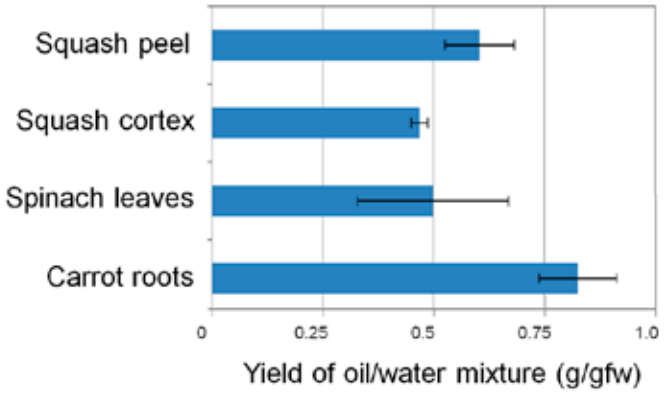

Fig. 3 - Yield of oil/water mixture extracted from vegetables using DME as solvent. Mixture of oil and water containing pigments from vegetables are compared.

contrast, pigment extraction from sticky pastes of yellow squash pericarp and spinach leaves required further modifications (Fig. 2I, J, M, N).

Quantitative and qualitative analysis of extracted pigments

Spectroscopic analyses of the pigments extracted from vegetable samples were performed (Fig. 4 left) and the resultant data was used for quantification (Fig. 5). Carotenoids were observed in all samples. As expected, the presence of chlorophylls was detected only in the samples derived from green tissues. The chlorophyll content in squash peel was found to be higher than that in spinach leaves (Fig. 5 top), possibly due to the loss of chlorophyll during extraction from the leafy sample as previously suggested (Fig. 2N). The total carotenoid contents in vegetable extracts were also compared and revealed that the green peel of squash and carrot roots had the highest values (Fig. 5 bottom).

The presence of chlorophylls (chiefly chlorophyll *a*) in the extracts from squash peel and spinach leaves was further confirmed by fluorescence spectroscopy (Fig. 4 right) and HPLC (Fig. 6). While it was difficult to dissect the spectroscopic and fluorescent signals for chlorophyll *a* from those of concomitantly present chlorophyll *b*, HPLC chromatograms showed clearly separated peaks of both chlorophylls *a* and *b* in the extracts from squash peel and spinach leaves (Fig. 6). With the aid of HPLC, the presence of both α- and β-carotenes were also confirmed in carrot roots while the other three samples showed the presence of β-carotene only (Fig. 7).

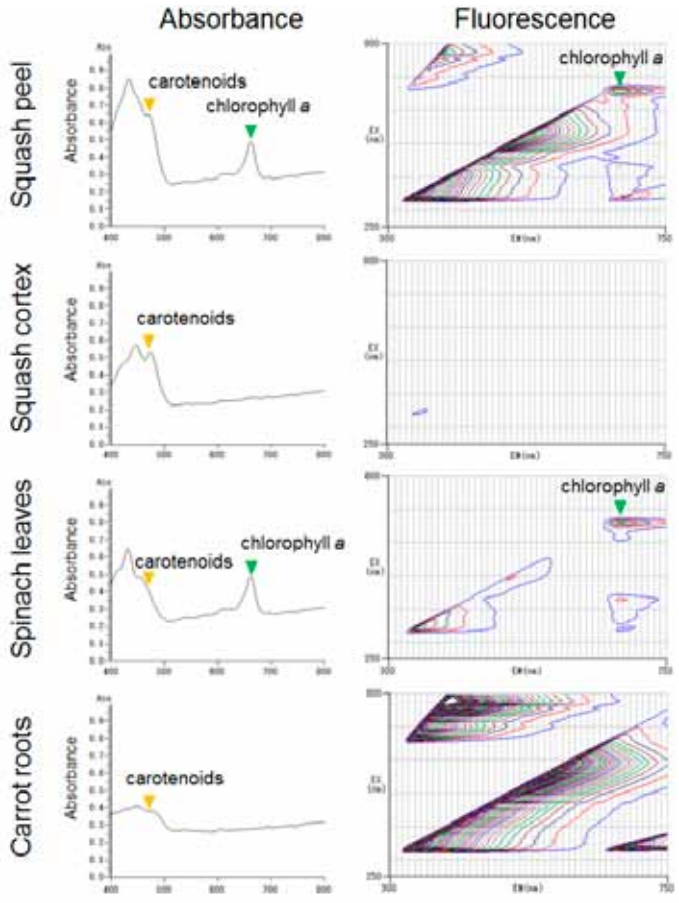

Fig. 4 - Spectroscopic and fluorescent profiles of DME-extracted pigments from vegetable samples. Comparison of absorption spectra (left) and fluorescence spectral contour (right) recorded in extracts from squash peel (top), yellow squash cortex (second line from the top), spinach leaves (third line from the top), and carrot roots (bottom). Prior to assays, extracts (oil/water mixtures) obtained after evaporation of DME were dissolved in acetone.

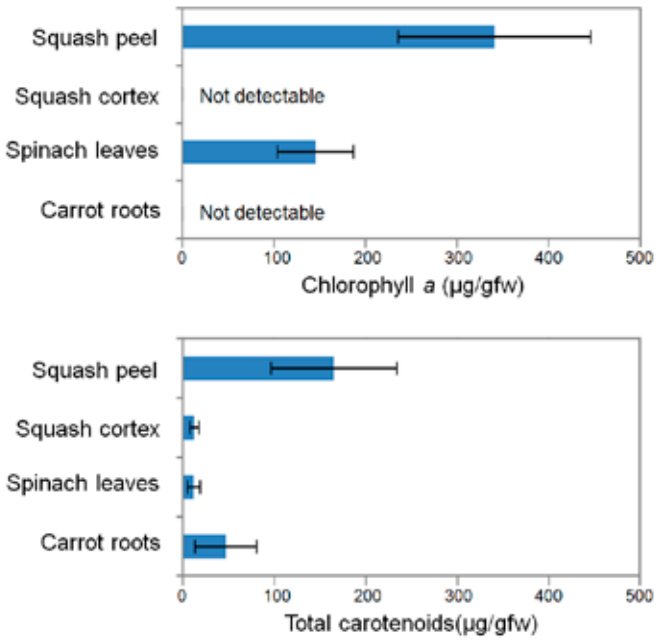

Fig. 5 - Quantification of pigments extracted from vegetable samples. Chlorophyll contents (top) and total carotenoid contents (bottom) in the extracts from green squash peel, yellow squash cortex, spinach leaves, and carrot roots are compared. Bars, S. D. (n = 3).

4. Conclusions

In the present study, the extraction of chlorophylls and carotenoids from green peel and yellow cortex of Japanese squash, spinach leaves and carrot roots using low-temperature liquefied DME has been demonstrated. Spectroscopic and fluorescence analysis of the extracted pigments revealed that chlorophylls were successfully extracted by DME from green materials (squash peel and spinach leaves). HPLC analysis further confirmed that the extracted chlorophylls included both chlorophyll *a* and *b*. Carotenoids were shown to be extracted by DME from all vegetable samples examined as confirmed by spectroscopic and HPLC analyses. The performance of DME as a novel pigment extracting agent is confirmed in this work

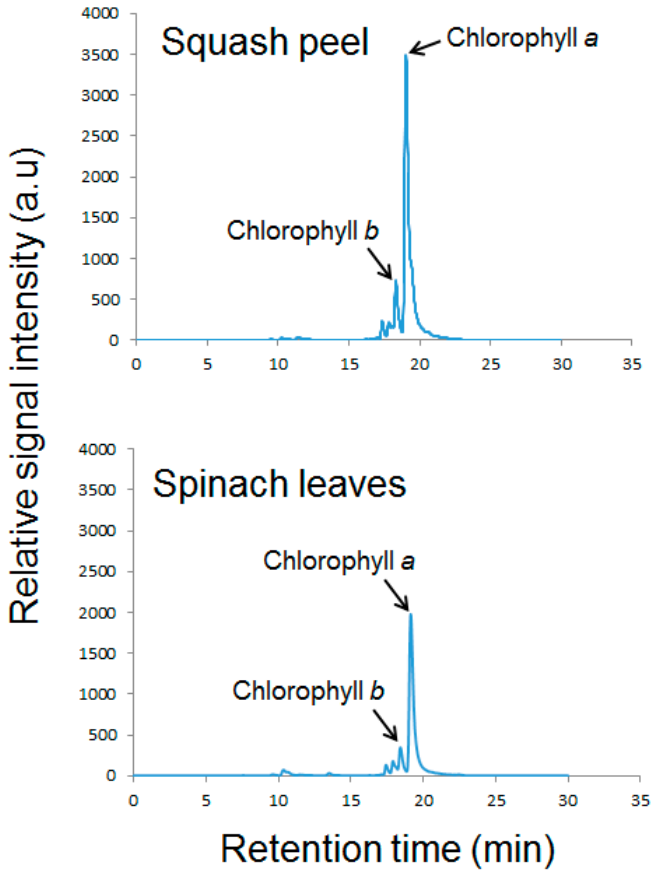

Fig. 6 - Detection of chlorophylls with HPLC. HPLC chromatograms showing the peaks of chlorophylls *a* and *b* extracted from squash peel and spinach leaves using DME. Pigments eluted from Inertsil ODS-80A column were detected by intrinsic fluorescence intensity (excitation, 405 nm; emission, 660 nm).

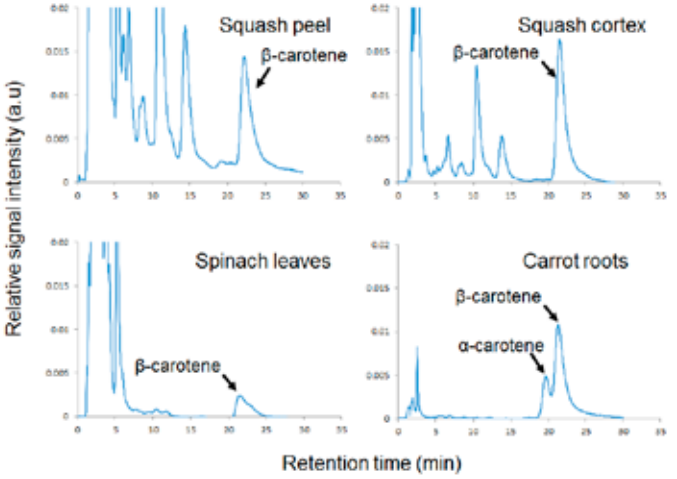

Fig. 7 - Detection of α- and β-carotenes with HPLC. HPLC chromatograms show the peaks of carotenoids extracted with DME from squash peel, squash pericarp, spinach leaves and carrot roots. Pigments eluted from Inertsil ODS-80A column were detected by monitoring the changes in absorption at 455 nm.

and its use as a "green" solvent, as opposed to conventional solvents, for the preparation and extraction of various plant pigments is highly encouraged from an environmental point of view.

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