

CHANGES IN THE PROFILE OF VOLATILE COMPOUND IN SEED STORAGE OF *RUBUS FRAXINIFOLIUS*

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Abstract

Storage conditions are one of the factors that affect seed quality. However, storage cannot improve seed quality, but it can maintain seeds in good condition, including their volatile compounds. Therefore, the study aimed to determine the changes of volatile compounds during seed storage on *Rubus fraxinifolius* seeds. The seeds were obtained from Cibodas Botanical Garden and divided into three groups i.e., seed without storage, seed stored at room temperature for four months, and seed stored in the refrigerator for four months. The seed extract using ethanol and methanol solvents was analyzed by Gas Chromatography-Mass Spectrometry (GC-MS). The results showed that the total volatile compounds detected in the ethanol and methanol extracts of *R. fraxinifolius* seed were 113 and 80, respectively. Seven types of main volatile compounds are found in all seed treatments and solvents included γ -Sitosterol, γ -Tocopherol, 9-Octadecenamide, (Z)-, 9,12-Octadecadienoic acid (Z,Z)-, 9,19-Cyclolanostan-3-ol, 24-methylene-, (3.beta.)-, dl- α -Tocopherol, and Squalene. Compounds were belong to sterols, vitamin E, fatty acids, and triterpenoids. In addition, seed storage treatment increased the percentage of specific volatile compounds, such as γ -Sitosterol, γ -Tocopherol, 2,4-Decadienal, (E,E)-, 2,4-Decadienal, (E,Z)-, 9-Octadecenamide, (Z)-, 9,12-Octadecadienoic acid (Z,Z)-, 9,19-Cyclolanostan-3-ol, 24-methylene-, (3.beta.)-, dl- α -Tocopherol, Eicosane, n-Hexadecanoic acid, and Squalene.

Keywords: GC-MS, Metabolite, Raspberry seed, *Rubus*, Seed

Introduction

Metabolites are small organic compounds that can be divided into primary and secondary metabolites based on their functions and biosynthesis pathways. Primary metabolites such as amino acids, sugars, and fatty acids are essential for plant growth and

development, whereas secondary metabolites are nonessential, but they play crucial roles in plant defense, ecological interactions, and medicinal properties (Yang *et al.*, 2018; Ali *et al.*, 2023). *Rubus fraxinifolius* belongs to crop wild relative of

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raspberry that has highly potential as food and medicine (Surya *et al.*, 2018; Dewi *et al.*, 2019). The seed of *Rubus* is rich in primary and secondary metabolites that confer several benefits to the plant and have potential applications in the pharmaceutical, nutraceutical, and food industries (Monforte *et al.*, 2018; Fraga-Corral *et al.*, 2021).

Generally, the seed quality is not improved by storage. It means the germination and vigor index of the seed began to decrease as seed storage. The seed storage of *Rubus* contains a variety of primary metabolites such as carbohydrates, proteins and lipids that provide energy and nutrition for the developing embryo (Jan *et al.*, 2021). Carbohydrates are the main source of energy for plants, and seeds store carbohydrates in the form of starch, sucrose and fructose. These carbohydrates are hydrolyzed to glucose during germination, which the embryo uses to produce ATP and other important molecules (Aguirre *et al.*, 2018; Kaur *et al.*, 2021). Protein is also stored in the seed reservoir and serves as a source of amino acids for the developing embryo (Fujiwara *et al.*, 2002; Famiani and Walker, 2009). Rubisco is the most abundant protein in *Rubus* seeds and plays an important role in carbon fixation during photosynthesis (Roush, 2001; Famiani and Walker, 2009). Lipids are another important component of seed storage, providing seedlings with a focused source of energy during germination (Weselake, 2020). *Rubus* seeds contain both saturated and unsaturated fatty acids, stored primarily in the form of triglycerides (Oomah *et al.*, 2000; Ash *et al.*, 2011). In addition to primary metabolites, *Rubus*'s seed storage might also contain secondary metabolites such as phenolics, flavonoids, and terpenoids, which have diverse biological activities such as antimicrobial, antioxidant, anticancer, and anti-inflammatory properties (Desmiaty *et al.*, 2018, 2021; Dewi *et al.*, 2019).

Moreover, an information related to metabolite of *R. fraxinifolius* is still limited. Most of *R. fraxinifolius* studies was focused on the leaves and fruit metabolites (Bakar *et al.*, 2016; Surya *et al.*, 2018); Desmiaty and Elya, 2021; Dewi *et al.*, 2019; Shamsudin *et al.*, 2019; Desmiaty *et al.*, 2018, 2021. There is no information related to the metabolites of *R. fraxinifolius* seed after storage. Therefore, we need to explore the metabolite of *Rubus* seed storage. So, the aim of this study was to determine the changes of volatile compounds during seed storage on *R. fraxinifolius* seeds.

Materials and Methods

Sample of *R. fraxinifolius* seed were obtained from mature fruit in the Cibodas Botanical Garden harvested on September 2022. Seeds were extracted by squashing fruit under running water using a strainer as a filter. Seeds were mashed against the strainer as needed to completely remove the fruit pulp and cleaned seeds were spread on paper towels to air dried for seven days until the seed moisture content was 9%. Moreover, it was divided into three groups i.e. seed without stored, seed stored for four months at room temperature ($27\pm 3^{\circ}\text{C}$) and seed stored in the refrigerator ($4\pm 2^{\circ}\text{C}$).

R. fraxinifolius seed are mashed up into powder. Five milligrams of the powder are put into an erlenmeyer, then soaked using 25 mL of solvent. Two types of solvents were ethanol and methanol, where methanol has more polar organic properties than ethanol. Each sample was covered by aluminum foil and macerated for 24 hours. After that, the pulp is separated from the filtrate by Whatman No. 1 filter paper. The filtrate was placed on a porcelain cup and concentrated by air dried for six days and the weight extract was constant. The extract of each solvent was analyzed for the secondary metabolite profile using Gas Chromatography-Mass Spectrometry (GC-MS). Moreover, GC-MS analysis was performed using GC-MS merk Agilent 7890B (GC) and 5977A (MSD). Column type 19091S-433: 93.92873 DB-5MS UI 5% Phenyl Methyl Silox, Dimention: 0°C - 325°C (325°C): 30 m x 250 μm x 0.25 μm . Initial Temperature: 40°C , Hold Time 1 min. Post Run 300°C . Volume injection: 1 μL . The mass spectra of the compounds found in the extract was matched with the National Institute of Standards and Technology (NIST20) library. The diagram of total volatile compound in each treatment was constructed with the Jvenn tool and presented as a Venn diagram.

Results and Discussion

Various kinds of volatile compounds are found in *R. fraxinifolius* seeds extract. Seed extracted with ethanol provides a more abundant volatile compound than methanol (Figure 1).

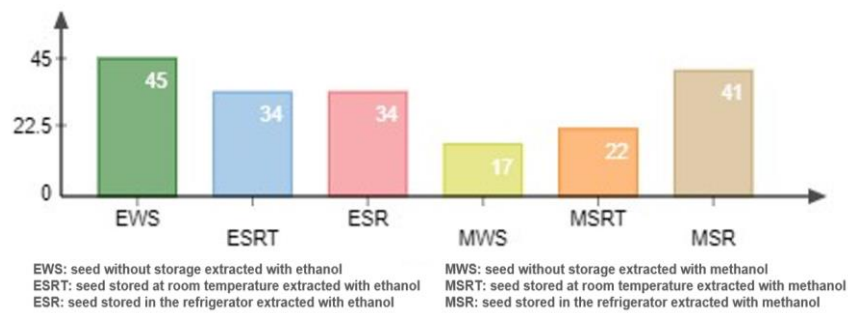


Figure 1. Number of volatile compounds based on treatments

Ngo *et al.* (2017) stated that the solvent affected the antioxidant capacity of the roots of *Salacia chinensis* L. It suggested that methanol was a better solvent for extracting than ethanol. As shown in Table 1, the main volatile compound in the ethanol extract of seed without storage was γ -Tocopherol. Once the seed is stored, its volatile compound profile changes. The most abundant compounds found in seeds stored at room temperature was 9,12-Octadecadienoic acid (Z,Z)-, and in the refrigerator was γ -Tocopherol (Table 1). In methanol extract, γ -Sitosterol was the main volatile compound in seeds without storage and stored in the refrigerator (Table 2). However, the highest volatile compound in seeds stored at room temperature was 9,12-Octadecadienoic acid (Z,Z)-. Based on observations, volatile compounds tend to increase during seed storage, especially when stored in the refrigerator. These results indicate that refrigerator can keep the seeds in condition. In a previous study, *Lycopersicum esculentum* seeds stored in the refrigerator had a higher viability than those stored at room temperature (Kolo and Tefa, 2016).

The temperature during storage affects the quality of the seeds and also affects the volatile compounds in them. Our results showed there are several numbers of specific volatile compounds that are present in each treatment (Figure 2), as shown in Table 3. Based on the data, the content of γ -Sitosterol in the seeds stored in the refrigerator was higher than at room temperature (Table 3). According to Tripathi *et al.*, (2013), γ -Sitosterol was the first phytosterol identified in *Girardinia heterophylla* leaves. *Acacia nilotica* leaves extract, *Gossypium* seeds, and *Andrographis producta* root

and stem also contain those compounds (Tripathi *et al.*, 2013; Krishnaveni *et al.*, 2014; Dalawai and Murthy, 2021). Furthermore, this compound shows antidiabetic and anticancer activity (Sundarraj *et al.*, 2012; Tripathi *et al.*, 2013; Dalawai and Murthy, 2021).

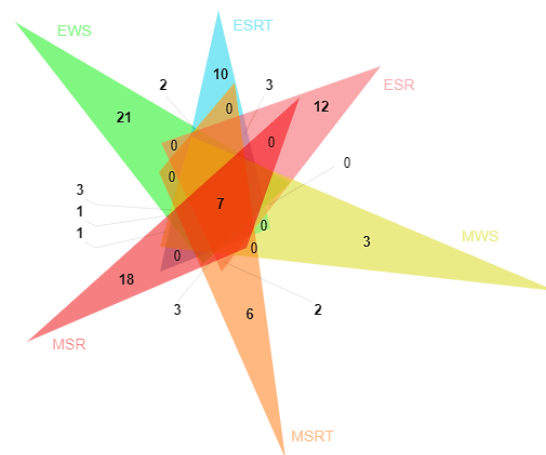


Figure 2. Venn diagram of the common volatile compound based on all treatments (EWS: seed without storage extracted with ethanol; ESRT: seed storage at room temperature extracted with ethanol; ESR: seed stored in the refrigerator extracted with ethanol; MWS: seed without storage extracted with methanol; MSRT: seed storage at room temperature extracted with methanol; MSR: seed stored in the refrigerator extracted with methanol)

Table 1. Volatile compounds of *R. fraxinifolius* seeds extracted with ethanol

Volatile compounds	Peak Area (%)		
	WS	SRT	SR
.beta.-Alanine, N-methyl-, ethyl ester	0.23		
γ-Sitosterol	2.72	12.42	16.85
γ-Tocopherol	29.30	13.73	31.81
1-(2-Aminoethylamino)-2-propanol			0.36
1-Heneicosanol	1.13		
1-Heptadecanamine	0.16		0.32
1,2-Ethanediamine, N-(2-aminoethyl)-	0.18		
1,3-Propanediamine, N-methyl-	0.20		
10(E),12(Z)-Conjugated linoleic acid			1.21
11-Octadecenoic acid, methyl ester	0.43		
2-(4,5-Dihydro-3-methyl-5-oxo-1-phenyl-4-pyrazolyl)-5-nitrobenzoic acid	0.74		
2-Butenal, 3-methyl-			0.92
2-Heptenal, (E)-	0.33		
2-Heptenal, (Z)-		0.35	0.82
2-Methyltetracosane		0.14	
2-Pentadecanone, 6,10,14-trimethyl			0.29
2-Piperidin-3-ylethylamine			0.27
2-Propenamide	0.17		
2,4-Decadienal, (E,E)-	0.41	0.77	1.23
2,4-Decadienal, (E,Z)-	0.20	0.56	0.87
2,4-Heptadienal, (E,E)-		0.21	0.38
3-Eicosene, (E)-		0.27	
3,6,6-Trimethyl-cyclohex-2-enol	0.65		
5-Eicosene, (E)-			0.37
5-Octadecene, (E)-	1.12		
7-Hexadecene, (Z)-		0.43	
8-Azabicyclo[4.3.1]decan-10-one, -methyl-			0.18
9-(2',2'-Dimethylpropanoilhydrazono)-3,6-dichloro-2,7-bis- [2-(dierhylamino)-ethoxy]flourene			0.30
9-Octadecenamide, (Z)-	3.00	3.09	2.20
9-Octadecenoic acid, methyl ester, (E)-		0.34	
9,12-Octadecadienoic acid (Z,Z)-	15.02	48.21	18.95
9,12-Octadecadienoic acid (Z,Z)-, 2,3-dihydroxypropyl ester	2.32		
9,12-Octadecadienoic acid (Z,Z)-, methyl ester	0.76		
9,12-Octadecadienoic acid, ethyl ester			2.60
9,19-Cyclolanost-24-en-3-ol, (3.beta.)-		0.84	0.85
9,19-Cyclolanostan-3-ol, 24-methylene-, (3.beta.)-	1.73	4.56	6.59
9(E),11(E)-Conjugated linoleic acid	16.15		
Acetamide, 2-cyano-	0.47		
Benzophenone	0.17		
Bicyclo[10.8.0]eicosane, cis-	0.79		
Bis(2-ethylhexyl) phthalate	0.57	0.26	
Butylated Hydroxytoluene	0.17		
Cyclohexadecane			0.64
Cyclopentane, (1-methylbutyl)-			0.50
Cyclopentane, 1,2,3,4,5-pentamethyl-		0.38	
dl-α-Tocopherol	2.51	1.34	3.43
Docosane, 1-iodo-		0.58	
Docosyl octyl ether	0.93		
Eicosane	0.60	1.20	1.68
Eicosane, 1-iodo-	0.80	0.19	
Ethanol, 2-bromo-	0.77		
Glycyl-D-asparagine	0.62		
Hentriacontane	0.88		
Heptadecane			0.44
Hexacosane		0.13	
Hexadecanamide	1.91	1.01	
Hexadecane	0.33	0.22	0.22
Hexadecanoic acid, methyl ester	0.45	0.34	0.35
Linoelaidic acid	5.66	1.21	
MDMA methylene homolog		0.19	
Methyl 10-trans,12-cis-octadecadienoate		0.25	
Methyl stearate		0.36	0.21
n-Hexadecanoic acid	2.83	4.73	2.91
Naphthalene			0.24
Octadecane	0.19	0.18	0.23
Palmitic Acid, TMS derivative		0.29	
Pentadecane		0.17	
Phenylephrine	0.36		
Phytol		0.37	0.60
Pregn-5-en-3-ol, 20-amino-, (3.beta.,20S)-	0.28		
Squalene	0.71	0.68	0.79
Tetracosane			0.35
Tetracosane, 11-decyl-	0.54		
Triacotane, 1-iodo-	0.30		
Vanillin	0.17		

Note. WS: without stored; SRT: stored at room temperature; SR: stored in the refrigerator

Table 2. Volatile compounds of *R. fraxinifolius* seeds extracted with methanol

Volatile compounds	Peak Area (%)		
	WS	SRT	SR
γ -Sitosterol	57.06	7.48	42.35
γ -Tocopherol	4.81	34.00	15.95
(2-Piperidin-3-ylethyl)amine		0.45	0.22
(Phenylthio) acetamide, N-(2-phenylethyl)-N-methyl-			0.59
(Phenylthio)acetic acid, 1-adamantylmethyl ester	1.05		
1-Docosene			0.16
1-Heptadecanamine			0.16
1-Methyl-4-(1-methylethenyl)-3-[1-methyl-1-(4-methyl-hex-5-enyl)-5,9-dimethyldec-4-enyl] cyclohexene			0.80
1-Tetradecanamine			0.11
1,10-Decanediol		0.40	0.14
1,2-Ethanediamine, N-methyl-			0.10
1H-Imidazole, 4,5-dihydro-2-methyl-			0.44
2-(4,5-Dihydro-3-methyl-5-oxo-1-phenyl-4-pyrazolyl)-5-nitrobenzoic acid	0.72		0.48
2-Butenal, 3-methyl-		1.19	
2-Fluoropyridine			0.19
2,4-Decadienal, (E,Z)-			0.09
2,4-Di-tert-butylphenol		0.39	0.09
3-Methoxyamphetamine	0.82		
3,6,6-Trimethyl-cyclohex-2-enol	0.60		
4-Aminobutyramide, N-methyl-N-[4-(1-pyrrolidinyl)-2-butyryl]-N',N'-bis(trifluoroacetyl)-			0.10
4-Oxatricyclo[20.8.0.0(7,16)]triacenta-1(20),7(16)-diene			1.57
8-Hydroxyguanosine			0.10
9-Octadecanamide, (Z)-	0.30	5.13	1.22
9,12-Octadecadienoic acid (Z,Z)-	1.21	32.09	2.05
9,19-Cyclolanost-24-en-3-ol, (3.beta.)-	4.07		3.31
9,19-Cyclolanostan-3-ol, 24-methylene, (3.beta.)-	21.29	2.81	15.44
Acetamide, 2-cyano-	0.85		
Asparagine, N-dl-alanyl-, l-		0.72	
Atomoxetine			0.20
Benzaldehyde, 2-nitro-, diaminomethylidenhydrazone			0.84
Benzenepropanoic acid, .alpha.-(1-aminoethyl)-, [R-(R*,R*)]-			0.35
Bis(2-ethylhexyl) phthalate		0.68	0.36
dl- α -Tocopherol	0.97	4.77	2.46
Ethanol, 2-bromo-	1.26		
Gorgostane, (5.alpha.)-			0.75
Guanidine, N,N-dimethyl-			0.05
Hentriacontane			0.12
Heptadecane		0.33	
Heptadecanoic acid, 16-methyl-, methyl ester		0.25	
Heptasiloxane, hexadecamethyl-			0.12
Hexadecane			0.09
Hexadecanoic acid, methyl ester		0.40	0.18
Isophthalic acid, 2-ethylbutyl isobutyl ester		0.53	
Isopropyl linoleate		1.78	
Linoelaidic acid			4.51
MDMA methylene homolog	1.80		0.41
Metaraminol			0.06
n-Hexadecanoic acid		3.08	0.93
Naphtho[2,3-b]furan-2-one, 3-[[2-(2-methoxyphenyl)ethylamino]methyl]-8a-methyl-5-methylenedecahydro-			0.38
Octadecanoic acid, dodecyl ester		1.53	
Octatetracontane, 1-iodo-		0.43	
Phthalic acid, isohexyl 3-methylbutyl ester	0.21		
Phytol		0.47	0.19
Squalene	0.22	1.10	0.56
Stigmasta-5,24(28)-dien-3-ol, (3.beta.,24Z)-	2.76		1.73

Note. WS: without stored; SRT: stored at room temperature; SR: stored in the refrigerator

After analysis, the percentage of γ -Tocopherol in fresh seeds did not change significantly after being stored at room temperature and in the refrigerator (Table 3). As an antioxidant, γ -Tocopherol can scavenge lipid peroxy radicals. These compounds are rich in plant seeds, such as peanut, almond, and sunflower seeds. In addition, it is present in vegetable oils such as corn, soybean, and peanut (Jiang *et al.*, 2022). dl- α -Tocopherol is another compound from the same group as γ -Tocopherol. After storage, the percentage of that compound tended to decrease (Table 3). dl- α -Tocopherol and γ -Tocopherol belong to Vitamin E and play a role in skin protection (Huang *et al.*, 2009). In plants, dl- α -Tocopherol was found in the leaves of *Psuederathenum tunicatum* (Ameachi and Chijioke, 2018). Fatty acid groups such as 9-Octadecenamide, (Z)- and 9,12-Octadecadienoic acid (Z,Z)- were identified in all treatments (Table 3). Those compounds increased when stored at room temperature in both solvents. As reported by Saleh (2023), 9-Octadecenamide, (Z)- is a primary fatty acid discovered in *Hypericum perforatum*.

Apart from γ -Sitosterol, the volatile compound with a higher percentage when stored in the refrigerator was 9,19-Cyclolanostan-3-ol, 24-methylene-, (3.beta.)- (Table 3). This compound was a triterpenoid with antimicrobial and antibacterial activity (Ameachi and Chijioke, 2018; Likittrakulwong *et al.*, 2023).

Previous studies discovered 9,19-Cyclolanostan-3-ol, 24-methylene-, (3.beta.)- in *Zingiber niveum* roots, in *Pseuderathenum tunicatum* leaves, in *Musa paradisiaca* stem, and peel extracts (Theanphong *et al.*, 2016; Ameachi and Chijioke, 2018; Sari *et al.*, 2020; Likittrakulwong *et al.*, 2023). Another triterpenoid found in *R. fraxinifolius* seeds was Squalene. Seed storage at room temperature and in the refrigerator for four months enhanced the percentage of Squalene than fresh seeds (Table 3). When

compared to the other six compounds, this compound was the lowest. However, Squalene has potential in the pharmacological, cosmetic, and nutritional fields (Lozano-Grande *et al.*, 2018). The most abundant source of Squalene comes from shark liver, while most plants come from olive (Rosales-Garcia *et al.*, 2017). This discovery may increase the availability of Squalene for industrial applications.

As previously stated, *R. fraxinifolius* seeds extract contained seven dominant compounds, and their percentage increased after being stored at room temperature and in the refrigerator. The presence of γ -Sitosterol and 9,19-Cyclolanostan-3-ol, 24-methylene-, (3.beta.)- was higher when extracted with ethanol (Table 1). Ethanol extraction also enhanced the percentage of 2,4-Decadienal, (E,E)-, 2,4-Decadienal, (E,Z)-, Eicosane, and n-Hexadecanoic acid. On the other hand, γ -Tocopherol, 9-Octadecenamide, (Z)-, 9,12-Octadecadienoic acid (Z,Z)-, dl- α -Tocopherol, and Squalene were more suitable extracted with methanol (Table 2).

There hasn't been much research on the changes in volatile compounds in seeds following storage. As we observed, γ -Sitosterol, 2,4-Decadienal, (E,E)-, 2,4-Decadienal, (E,Z)-, 9,19-Cyclolanostan-3-ol, 24-methylene-, (3.beta.)-, and Eicosane were higher when stored in the refrigerator. However, after being held at room temperature, n-Hexadecanoic acid, γ -Tocopherol, 9-Octadecenamide, (Z)-, 9,12-Octadecadienoic acid (Z,Z)-, dl- α -Tocopherol, and Squalene were found to be higher. According to Yang *et al.* (2018), temperature is one of the environmental factors that influence secondary metabolites in plants. In a study by Morales *et al.*, (2014), long storage at low temperatures resulted in variations in volatile compounds, specifically an increase in terpenoid concentrations in two cultivars of *Rubus idaeus*.

Table 3. Volatile compounds that found in all treatments

Compound	Peak area (%)					
	E ¹ WS	M ² WS	E ¹ SRT	M ² SRT	E ¹ SR	M ² SR
γ -Sitosterol	2.72	57.06	12.42	7.48	42.35	16.85
γ -Tocopherol	29.30	4.81	13.73	34.00	15.95	31.81
9-Octadecenamide, (Z)-	3.00	0.30	3.09	5.13	1.22	2.20
9,12-Octadecadienoic acid (Z,Z)-	15.02	1.21	48.21	32.09	2.05	18.95
9,19-Cyclolanostan-3-ol, 24-methylene-, (3.beta.)-	1.73	21.29	4.56	2.81	15.44	6.59
dl- α -Tocopherol	2.51	4.81	1.34	4.77	2.46	3.43
Squalene	0.71	0.22	0.46	1.10	0.56	0.79

Note. ¹: ethanol seed extract; ²: methanol seed extract; WS: without stored; SRT: stored at room temperature; SR: stored in the refrigerator

Conclusions

The volatile compounds identified in *R. fraxinifolius* seeds extract after storage varied depending on the solvent. There were 113 volatile compounds found in ethanol extract. However, methanol only extracted 80 volatile compounds from *R. fraxinifolius* seeds. The specific compounds found in each treatment were γ -Sitosterol, γ -Tocopherol, 9-Octadecenamide, (Z)-, 9,12-Octadecadienoic acid (Z,Z)-, 9,19-Cyclolanostan-3-ol, 24-methylene-, (3 β)-, dl- α -Tocopherol, and Squalene. These compounds are classified as sterols, vitamin E, fatty acids, and triterpenoids. In addition, storage treatment of *R. fraxinifolius* seeds enhanced the percentage of some volatile compounds.

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