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Resolution of Molecular Species of the Triacylglycerol Containing Petroselinic Acid (*cis*-$C_{18:1\omega12}$) by Silver Ion-HPLC

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Abstract : On the analysis of triacylglycerol (TG) from the kernels of *Acanthopanax sessiliflorus* by reversed phase-HPLC, it was separated into three main fractions of PN 44, 46 and 48, according to partition number (PN). On the contrary, it could be clearly classified into seven fractions of SMM, MMM, SMD, MMD, SDD, MDD and MDT by silver ion-HPLC by the number of double bond in the acyl chains of TG species. But resolution of so-called critical pairs of TG molecular species such as molecular pairs of P_eLL [$C_{18:1\omega12}/(C_{18:2\omega6})_2$] and OLL [$C_{18:1\omega9}/(C_{18:2\omega6})_2$] and OOL [$(C_{18:1\omega9})_2/C_{18:2\omega6}$], and P_eP_eL [$(C_{18:2\omega12})_2/C_{18:1\omega6}$] was not achieved ($P_e$; petroselinic acid, L; linoleic acid, O; oleic acid). On the other hand, TG extracted from *Aralia continentalis* kernels were also fractionated into seven groups of SSM, SMM, MMM, SMD, MMD, SDD and MDD (S; saturated acid, M; monoenoic acid, D; dienoic acid) by silver ion-HPLC, although it's were classified into three groups of PN 44, 46 and 48 by reversed phase-HPLC. The fractions of SMM, MMM, MMD and MDD were divided into two subfractions, respectively; the fractions of SMM, MMM, MMD and MDD were resolved into the subfraction of PP_e/P_e and POO (critical pairs from each other), that of $P_e/P_e/P_e$ and OOO, that of $P_e/P_e/L$ and OOL, and that of $P_e/L/L$ and OLL.

Keywords : *silver ion-HPLC, reversed phase-HPLC, Acanthopanax sessiliflorus, Aralia continentalis, critical pair of triacylglycerol.*

1. Introduction

The composition of triacylglycerol (TG) molecular species in the vegetable oils is very complicated, even though it contains the limited number of fatty acid. Actually TGs with 'n' numbers of fatty acid may have n^3 TG molecular species. Distribution of fatty acid to the position of TG molecule is directly related to the physico-chemical properties of TG. For example, the melting point of cocoa butter is quite different with that of mutton tallow though they have a close similarity in fatty acid composition[1], and the autoxidation rate of TG molecules depends on the positions of unsaturated acyl chain linked to the glycerol backbone[2]. In

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addition, pancreatic lipase does not split acyl moiety attached to β-position of the glycerol backbone of TG molecule[1]. Thus, from the view point of food science, nutrition and lipid chemistry it is very important to corroborate the distribution of fatty acids in TG molecule, particularly in those comprising critical pairs.

Reversed-phase high performance liquid chromatography (RP-HPLC) using column(s) with octadecylsilanyl packing material has been widely used for the structural analysis of triacylglycerol (TG)[3], retinoyl fatty acyl esters[4-6], and steryl fatty acyl esters[7-9]. In the RP-HPLC the elution profile of components is determined by partition number, defined as the effective number of carbon atoms in all the acyl and alkyl moiety less twice the number of double bonds in a molecule, the elution order of components is not easy to interpret when the TG species comprise high level of positional isomer(s) of given unsaturated fatty acyl moiety.

Recently, silver-ion HPLC has been developed and extensively used for analyses of fatty acid methyl esters[10-13], cholesterol esters[14], TG from seed oils[15-18], and fish oils[19,20] with resolution, because this method is simple and rapid, and gives clean fractions without contamination by silver ions and other artifacts often experienced with thin-layer chromatography.

The kernel oils of *Acanthopanax sessiliflorus* and *Aralia continentalis* were found to have a high level of petroselinic acid (*cis*-$C_{18:1\omega12}$, 56% and 29.0% to total fatty acids, respectively)[23]. This fatty acid has the double bond closer to the carboxylic group than oleic acid, so its physico-chemical properties such as higher melting point[1] and more immune to lipase[26] are quite different from those of the latter. In addition, the unusual position of the double bond in this fatty acid provides an opportunity for production of valuable raw materials, lauric acid ($C_{12:0}$) and adipic acid (dicarboxylic acid), both with important applications in the manufacture of soaps and plastics [27-29]. However, little is known of the detailed chemical and physical properties of the TGs containing petroselinic acid as well as of the metabolic mechanism in human. One approach to this problems is to clarify the structure of TGs.

In this study, a resolution of the TG species has been carried out for the first time by silver ion-HPLC.

2. Experimental

2.1. Lipids samples and reagents

The seeds of *Panax schinseng*, *A. continentalis* and *A. sessiliflorus*, grown in herb-cultivating fields, were collected in the area of Sunchun, Chun-Nam Province, in November, 1999. The air-dried, smashed seeds were extracted according to the method of Bligh & Dyer[21] in a stream of nitrogen. All solvents and reagents were HPLC-grade and supplied by Merck Ltd. Standard reagents of fatty acid and TG were purchased from Sigma-Aldrich and Nu-Chek Prep, Inc (Elysian, MN, USA)

2.2. Silver ion-HPLC of FAMEs[22]

25 μL of an internal standard of methyl henecosanoate ($C_{21:0}$, 5.8 mg in 25 mL hexane) along with 1 ml of 0.01% butylated hydroxytoluene (BHT) dissolved in hexane, were added to all the fractions collected from a single HPLC run. All the TG molecular species of each fraction were transmethylated with sodium methoxidemethanol solution for 5 min at ambient temperature, and the methyl esters were then recovered with hexane. For quantitative analysis, a Hewlett-Packard Model 5890 Series II gas chromatograph with split/ splitless injection system was equipped with a capillary column (25 m × 0.22 mm, i.d., 0.25 μm film) of fused silica coated with BPX 70 (70% cyanopropylpolysilphenylene siloxane, SEG, Austin, TX, USA). The initial

temperature of 160℃ was held for 3 min and then programmed up to 220℃ at 3℃/min, with a final hold for 10 min. Hydrogen was the carrier gas, and the area percentage of each peak was calculated by electronic integration. Fatty acid composition was obtained by multiplying a correction factor to the percentage of peak area recommended by Christie [22]. The results are given in Table 1. Each figure is the mean of triplicate measurements.

2.3. Reversed phase-HPLC analysis of TGs[16,18,19]

HPLC analysis was carried out with a Hewlett-Packard model 1050 solvent delivery system, a Sedex model 55 light-scattering detector (Alfortville, Cedex, France) and a Hewlett-Packard HP 3395 integrator. A stream-splitter (approx. 8 : 2) was installed between the column and the detector. Reversed phase HPLC analysis was performed using two ChromSpher™ C18 columns in serial (100 × 4.6 mm, i.d., 3 μm, Chrompack International Co., Middelburg, the Netherlands). TG fractionation was carried out using binary solvent gradient system composed of dichloromethane (DCM)/ acetonitrile (ACN) (20 : 80, v/v) (A) and DCM/ ACN (30 : 70, v/v) (B). The mobile phase profile was 85% solvent A/ 15% solvent B over 20 min and then changed linearly to 100% solvent B over 60 min. The elution continued for 20 min more with the final solvent. The column was kept at 18℃, and the flow rate was 0.8 mL/min. Each sample (1-1.2 mg) was dissolved in DCM (1 mL) and its small portion (10 μL) was injected onto the column. Each sample (1-1.2 mg) was dissolved in DCM (1 mL) and its small portion (10 μL) was injected onto the column.

2.4. Silver ion-HPLC analysis of TG[16-19]

The HPLC analysis was performed on the same instrument mentioned as the reversed phase-HPLC. A silver-ion column (Xpertex Nucleosil™SA, 250 × 4.6 mm, i.d., 5 μm, P. J. Cobert Associates, Inc., St. Louis, MO, USA) was prepared as described else-where[12]. The resolution of TG molecules was conducted with ternary gradient system, which consisted of 1, 2-dichloroethane (DCE)-DCM (1 : 1, v/v) (A), acetone (B), and acetone-ACN (9 : 1, v/v) (C). The mobile phase composition was changed linearly from 100% solvent A to 50% solvent A/ 50% solvent B over 5 min, then to 20% solvent A/ 50% solvent B/ 30% solvent C over 80 min, and finally 50% solvent B/ 50% solvent C was eluted for a further 5 min. The column was kept at 18℃ and the flow rate was 0.8 mL/min. The sample (1-1.2 mg) was dissolved in DCE (1 mL) and an aliquot (10 μL) was injected onto the column.

3. Results and Discussion

The TG from each of the kernel oils was analyzed by RP-HPLC equipped with C_{18} column(s) using a linear gradient elution system. The TG class from the kernel oils of *A. sessiliflorus* was rich in petroselinic acid (56 mol%) and was resolved simplely into three fractions, PN 44, PN 46 and PN 48 fraction (PN; partition number, defined as the effective number of carbon atoms in all acyl residues minus twice the number of double bonds in a molecule) [24] as presented in Fig. 1. The PN 44 fraction contained mainly the species of P_eLL [$C_{18:1\omega12}/(C_{18:2\omega6})_2$], OLL [$C_{18:1\omega9}/(C_{18:2\omega6})_2$], PLL [$C_{16:0}/(C_{18:2\omega6})_2$] and $P_eP_eL_n$ [$(C_{18:2\omega6})_2/C_{18:3\omega3}$], and the PN 46 fraction was the most abundant and it was composed of the species of P_eP_eL [$(C_{18:1\omega12})_2/C_{18:2\omega6}$], PP_eL [$C_{16:0}/C_{18:1\omega12}/C_{18:2\omega6}$], P_eOL [$C_{18:1\omega12}/C_{18:1\omega9}/C_{18:2\omega6}$] and OOL [$(C_{18:1\omega9})_2/C_{18:2\omega6}$]. The last PN 48 fraction comprised $P_eP_eP_e$ [$C_{18:1\omega12}/C_{18:1\omega12}/C_{18:1\omega12}$], P_eP_eO [$C_{18:1\omega12}/C_{18:1\omega12}/C_{18:1\omega9}$]. The abbreviations P, P_o, P_e, O, A_s, S_t, L, L_n and

Table 1. Fatty Acid Composition (Mol% of Total Fatty Acids) of Triacylglycerol Fractions Obtained by Silver Ion-HPLC from the Kernel Oils of *A. sessiliflorus* and *A. continentalis*

(1)

Fatty acid	Total (mol%)		Fraction											
			1 SSM		2 SMM		3 SMM		4 MMM		5 MMM		6 SMD	
	A	B	A	B	A*	B	A*	B	A*	B	A	B	A	B
				PPP_eP PO	PP_eO**	PP_eP_e		POO	$P_eP_eP_e$, P_eP_eO	P_eP_eO		P_eOO	POL, S_tOL	POL, PP_eL
$C_{16:0}$	3.4	14.			21.			34.3					25.5	30.1
$C_{16:1\omega7}$	0.1	2		48.6	8	30.0		0.1		0.2		0.3	0.1	0.2
$C_{18:0}$	0.7	0.7						4.3					6.6	2.6
$C_{18:1\omega12}$	55.	1.2		0.1	5.7	0.3		4.3	62.4	65.6		33.5	4.8	14.5
$C_{18:1\omega9}$	7	28.			32.			55.7	37.6	32.3		65.0	40.3	24.2
$C_{18:1\omega7}$	6.1	6		8.2	8	5.1		1.1		1.8		1.1	0.3	0.4
$C_{18:2\omega6}$	1.1	29.			37.								22.3	27.8
$C_{18:3\omega3}$	30.	8		14.4	3	56.2								
$C_{20:1\omega9}$	4	1.0			2.4			0.2	0.1	0.2		0.1	0.1	0.2
	2.3	23.		28.7		7.0								
	0.2	9												
		tr				1.4								
		0.5												

A; *A. sessiliflorus*, B; *A. continentalis*,
*; critical pairs were not resolved,
**; main TG molecular species,

Abbreviation; S (saturated), M (monoene), D (diene), T (triene),
P (palmitic acid), P_e (petroselinic acid), O (oleic acid), A_s (asclepic acid),
S_t (stearic acid), L (linoleic acid), L_n (linolenic acid).

Table 1. Fatty Acid Composition (Mol% of Total Fatty Acids) of Triacylglycerol Fractions Obtained by Silver Ion-HPLC from the Kernel Oils of *A. sessiliflorus* and *A. continentalis*

(2)

Fatty acid	Total (mol%)		Fraction											
			7 MMD		8 MMD		9 SDD		10 MDD		11 MDD		12 MDT	
	A	B	A*	B	A*	B	A	B	A*	B	A*	B	A	B
			P_eP_eL	P_eP_eL		OOL	PLL, S_tLL	PLL	P_eLL	P_eLL, OLL		OLL, P_eLL, A_sLL,	P_eLL_n, OLL_n	
$C_{16:0}$	3.4	14.					18.2	30.4						
$C_{16:1\omega7}$	0.1	2	2.8						3.6				1.5	
$C_{18:0}$	0.7	0.7					16.6	7.5						
$C_{18:1\omega12}$	55.	1.2	47.	57.		9.2			33.5	32.5.		4.4	5.0	
$C_{18:1\omega9}$	7	28.	5	3		50.			4.6	10.0		25.6	19.5	
$C_{18:1\omega7}$	6.1	6	21.	7.4		3			5.3	1.1		1.3	15.8	
$C_{18:2\omega6}$	1.1	29.	8	0.7		2.0	65.2	62.1	53.0	56.4		68.6	30.2	
$C_{18:3\omega3}$	30.	8	2.3	34.		38.							28.0	
$C_{20:1\omega9}$	4	1.0	25.	4		4								
	2.3	23.	5											
	0.2	9		0.2		0.1								
		tr	0.1											
		0.5												

A; *A. sessiliflorus*, B; *A. continentalis*,
*; critical pairs were not resolved,
**; main TG molecular species.

Abbreviation; S (saturated), M (monoene), D (diene), T (triene),
P (palmitic acid), P_e (petroselinic acid), O (oleic acid), A_s (asclepic acid),
S_t (stearic acid), L (linoleic acid), L_n (linolenic acid).

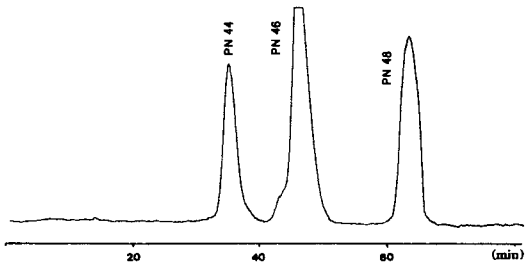

Fig. 1. Resolution of the triacylglycerol from the kernel oils of *A. sessiliflorus* by reversed phase- PLC.
- Operation conditions are described in Experimental,
- PN; partition number.

G_o stand for palmitic ($C_{16:0}$), palmitoleic ($C_{16:1\omega 7}$), petroselinic ($C_{18:1\omega 12}$), oleic ($C_{18:1\omega 9}$), asclepic ($C_{18:1\omega 7}$), stearic ($C_{18:0}$), linoleic acid ($C_{18:2\omega 6}$), α-linolenic acid ($C_{18:3\omega 3}$) and gondoic acid ($C_{20:1\omega 9}$), respectively. On the other hand, with silver ion-HPLC the TG fraction was separated clearly into seven main groups according to the number of double bonds in the following order; SMM> MMM> SMD> MMD> SDD> MDD> MDT (S; saturated, M; monoenoic, D; dienoic and T; trienoic acid) (Fig. 2). The fraction SMM is a tiny one and is mainly composed of the molecular species of PP_eO with small quantities of S_tP_eO and POA_s, while the fraction MMM is the second largest one consisting of the species of $P_eP_eP_e$ and P_eP_eO, and the fraction SMD is a small one containing the species of POL and S_tOL. The MMD fraction is the most abundant and mainly consists of the species of P_eP_eL and P_eOL with a small amount of P_oP_eL as expected. The fraction SDD having the species of PLL and S_tLL is a small one, but the MDD fraction is the third largest and has the species of P_eLL and P_oLL [$C_{16:1\omega 7}/C_{18:2\omega 6}/C_{18:2\omega 6}$] with small quantities of A_sLL [$C_{18:1\omega 7}/C_{18:2\omega 6}/C_{18:2\omega 6}$], and the last fraction of MDT was the smallest and was primarily composed of P_eLL_n [$C_{18:1\omega 12}/C_{18:2\omega 6}/C_{18:3\omega 3}$], A_sLL_n[$C_{18:1\omega 7}/C_{18:2\omega 6}/C_{18:3\omega 3}$], P_oLL_n [$C_{18:1\omega 12}/C_{18:2\omega 6}/C_{18:3\omega 3}$], and G_oLL_n [$C_{20:1\omega 9}/C_{18:2\omega 6}/C_{18:3\omega 3}$] (Table 1).

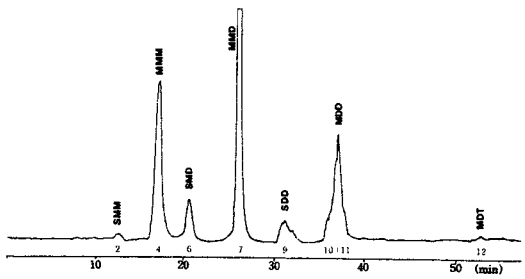

Fig. 2. Resolution of the triacylglycerol from the kernel oils of *A. sessiliflorus* by silver ion-HPLC.
- Operation conditions are described in Experimental,
- Abbreviation; S(saturated), M(monoene), D(diene), T(triene).

It is observed that the molecular species of the MMM and MMD fraction were eluted earlier than those of the SMD and SDD despite of having the same double bond number of three and four, because the interaction of two double bonds in a dienoic acid (D) residue with silver ion was stronger than that of two double bonds in two monoenoic acid (M) residues, presumably because of the localization of π-electrons in diene double bonds.

Fatty acid composition of intact TG from the kernel oils of *A. continentalis* was composed of a nearly equal amount of oleic acid (30.2%), petroselinic acid (29.0%) and linoleic acid (24.1%), along with palmitic acid (13.1%) [23]. This intact TG was also fractionated into three fractions (PN 44, 46 and 48 fraction) according to PN by RP-HPLC. TG molecular species separated in each fraction were as follows; OLL, P_eLL and PLL in the PN 44, POL, OOL and P_eOL in the PN 46, and POO, PP_eP_e and P_eOO in the PN 48 fraction (no chromatogram and data shown). Meanwhile, the TG molecules were well resolved into eleven fractions, which could be categorized in seven groups by silver ion-HPLC: SSM, SMM, MMM, SMD,

MMD, SDD and MDD, as represented in Fig. 3. The fatty acid composition in each of the fractions was identified by GC and the results are listed in Table 1. The first fraction (Fraction 1) contains the molecular species of PPO, PPP_e, PS_tO and PS_tP_e. The second group was resolved into two fractions (Fraction 2 and 3). Fraction 2 and 3 mainly consisted of PP_eP_e and POO, respectively. Fraction 4 and 5 in the third MMM group each had the molecular species of P_eP_eO and P_eOO. Fraction 6 was not divided into subfractions and was composed of the molecular species of POL and S_tOL. Fraction 7 and 8 were the largest groups and were not separated clearly from each other and were contaminated with their neighboring peaks during fractionation because the precise end-point for collecting fraction is not always

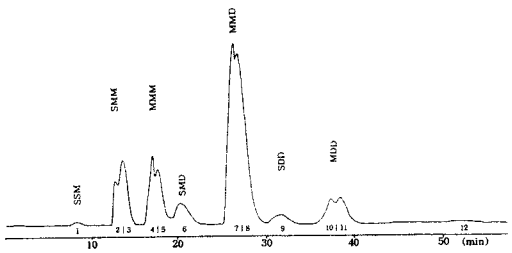

Fig. 3. Resolution of the triacylglyerol from the kernel oils of *A. continentalis* by silver ion-HPLC.
- Operation conditions are described in Experimental,
- Abbreviation; S(saturated), M(monoene), D(diene).

easy to judge. But the former fraction seems to mainly consist of the species of P_eP_eL and P_eOL with a small amount of P_oP_eL and the latter fraction seems to contain the species of OOL. Fraction 9 in the SDD group was a small and broad peak and comprised the species of P_oLL and S_tLL. Though Fraction 11 and 12 in the MDD group were also resolved from each other incompletely as experienced in the analysis of the group of SMM, MMM and MMD, it was easily found that the species of P_eLL existed in Fraction 11 and that of OLL in Fraction 12 by comparison of their fatty acid composition with each other.

In this experiment, we observed that silver ion-HPLC was more excellent in resolving the molecular species of TG containing unusual fatty acids than RPHPLC, and the molecular species of TG esterified with petroselinic acid(s) (*cis*-$C_{18:1\omega12}$) were eluted earlier than those with oleic acids (*cis*-$C_{18:1\omega9}$) because both the TG species were positional isomers with different location of a double bond despite of the same partition number (PN) and the same double bond number. For comparison, when a mixture of tripetroselinin ($P_eP_eP_e$) and triolein (OOO) available commercially, was co-run on silver ion-HPLC, the species of tripetroselinin was eluted earlier than that of triolein as represented in Fig. 4. It is supported by Nokolova-Damyanova et al [25]; she described that when methyl esters of the positional isomers of octadecamonoenoic acid, *cis*-11-$C_{18:1}$, *cis*-6-$C_{18:1}$ and *cis*-9-$C_{18:1}$, were cochromatographed in silver ion-HPLC using a mixture of DCE-DCM-ACN (50 : 50 : 0.01, v/v/v), the elution order was as follows; *cis*-11-$C_{18:1}$> *cis*-6-$C_{18:1}$> *cis*-9-$C_{18:1}$ because

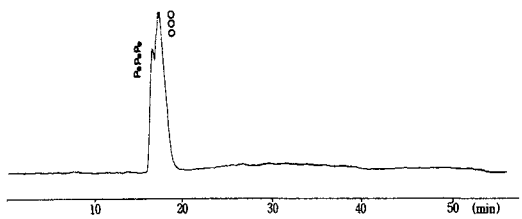

Fig. 4. Resolution of the triacylglycerol standards by silver ion-HPLC.
- Operation conditions are described in Experimental,
- PePePe; petroselinin, OOO; triolein.

strength of interaction between π-electron of the double bond and silver ion became

weaker when a double bond site was located remote from the middle part of acyl chain length of a molecule. Furthermore, Joh [18] reported that the TG molecular species esterified with a conjugated trienoic acid (punicic acid, $C_{18:3c9,11t,13c}$) was eluted earlier than those with a methylene-interrupted conjugated trienoic acid (*cis*-$C_{18:3\omega3}$) despite of having the same number of double bonds, because the conjugated double bond showed much weaker strength with silver ion than methylene-interrupted double bond on an account of delocalization of its π-electrons.

We confirmed again an excellence of silver ion-HPLC using both HPLC in the mode of reversed-phase and silver-ion in this experiment, though critical pairs of the molecular species of TG with a high level of fatty acid positional isomers were not resolved. Now we are continuing to separate the molecular species of TG without any contamination of neighboring peaks and to further a resolution of critical pairs of the TG species using silver ion-HPLC, by modifying operational conditions and improving column efficiency.

4. Conclusions

With the kernel oils of *A. sessiliflorus* and *A. continentalis*, we have carried out the experiment to compare the efficiency of resolution of the TG molecular species including petroselinic acid (*cis*-$C_{18:1\omega12}$) and its positional isomer oleic acid (*cis*-$C_{18:1\omega9}$) with that of other TG molecular species with reversed phase-HPLC and silver ion-HPLC. The TG molecules from the kernels of *A. sessiliflorus* were separated just only into three main fractions according to so-called partition number(PN) by reversed phase-HPLC, while clearly classified into seven fractions by the number of double bond in the acyl chains by silver ion-HPLC. Similarly, the sample extracted from *A. continentalis* kernels was also fractionated into seven groups by silver ion-HPLC, although they were classified into three groups by reversed phase-HPLC, and resolution of so-called critical pairs of TG molecular species such as molecular pairs of P_eLL [$C_{18:1\omega12}/(C_{18:2\omega6})_2$] and OLL [$C_{18:1\omega9}/(C_{18:2\omega6})_2$], and OOL [$(C_{18:1\omega9})_2/C_{18:2\omega6}$] and P_eP_eL [$(C_{18:2\omega12})_2/C_{18:1\omega6}$] was achieved.

In this study, silver ion-HPLC proved to be superior to reversed phase-HPLC and an excellent tool to resolve TG containing unusual fatty acids such as petroselinic acid into the TG molecular species and their critical pairs.

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