Bulgarian Chemical Communications, Volume 51, Special Issue D (pp. 256– 261) 2019

Effect of thermal stress on the biologically active lipids of *Mytilus galloprovincialis*

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Received November 5, 2018; Revised January 4, 2019

Black mussels (*Mytilus galloprovincialis*) are the most important marine aquaculture species in Bulgaria. They are widely traded and consumed fresh and frozen. To our knowledge, there is limited information in the scientific literature about changes in lipid composition of Black Sea mussel after cooking and prolonged storage. The aim of the present study was to investigate the changes in total lipids, lipid classes, fatty acid composition, fat-soluble vitamins (A, D_3 and E), carotenoids and cholesterol of *Mytilus galloprovincialis* following culinary treatment (steaming) and three month-storage at -20 °C. Lipids were extracted by the method of Bligh & Dyer and subsequently separated by column and thin-layer chromatography. Fat-soluble vitamins and cholesterol were determined by RP-HPLC/UV/FL. Fatty acid methyl esters were analyzed by GC/MS. There were significant differences in the amounts of total lipids and moisture between raw and steamed mussels, but no changes occurred after the period of freezer storage. Mussel lipids were characterized by higher contents of polar lipids (PL) than neutral lipids (NL). The most abundant FAs in the polyunsaturated FA group were eicosapentaenoic acid (C20:5n-3, EPA) and docosahexaenoic acid (C22:6n-6, DHA). The amounts of astaxanthin, α-tocopherol and cholesterol did not change after cooking, but decreased significantly after storage. Vitamin D_3 increased after thermal treatment but decreased twofold in the freezer. Vitamin A and β-carotene were most affected by temperature changes. Despite the changes observed, the present study confirmed that steamed and frozen *Mytilus galloprovincialis* from the Black Sea is a good natural source of high quality nutritional lipids.

Keywords: *Mytilus galloprovincialis*, lipid classes, fat-soluble vitamins, carotenoids, cooking, freezing

INTRODUCTION

In recent years, marine molluscs have attracted much attention as inexpensive food, low in calories but high in nutrients. The health-beneficial effect of seafood consumption depends on the lipid content, lipid classes and fatty acid composition. Other essential components such as fat-soluble vitamins, carotenoids and cholesterol, complemented and enhanced the marine lipids quality. Black mussels (*Mytilus galloprovincialis*) are the most important marine aquaculture species in Bulgaria. They are widely traded and consumed fresh and frozen. One of the preferable cooking methods for healthier diet is steaming. Other widely used method for food long storage is freezing. This method makes able to preserve the nutrition quality of stored food. Moreover, mussel meat is an easy perishable food that strongly depends on the storage conditions during distribution and commercialization. During frozen storage and cooking process, the mussel tissue is subjected to different physicochemical qualitative changes [1]. Among other tissue constituents, lipids are the major labile components which are significantly affected after steaming and freezing processes. The rate of lipid changes depends on the degree of fatty acid saturation, storage time and temperature treatment. To our knowledge there is limited or scattered information in the scientific literature about changes in lipid composition of Black Sea mussel after cooking and prolonged storage. In relation to the foregoing, the aim of the present study is to investigate the changes in total lipids, lipid classes, fatty acid composition, fat-soluble vitamins (A, D_3, D_2 and E), carotenoids and cholesterol of *Mytilus galloprovincialis* following culinary treatment (steaming) and three months-storage at -20 °C.

EXPERIMENTAL

Sample preparation

Mussel samples were purchased alive from a mussel farm located along the northern Black Sea coast in November 2017. One hundred animals were chosen randomly to determine the sample mean. Specimens were divided into three groups: first group (n=35) – raw samples; second group (n=32) – steamed mussels (at 90 °C for 6 min); third group (n=34) – frozen samples (at -20 °C for 3 months). At each group sampling, mussels of similar size class were selected (Table 1). The moisture content was determined by drying in an oven for 3 h at 105 °C until constant weight [2].

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Extraction of total lipids (TL)

Three replicate samples of raw, cooked and frozen meat homogenates (2.000±0.001 g) were extracted following the Bligh and Dyer procedure [3]. Lipid content was determined gravimetrically and the results were expressed as g per 100 g wet weight.

Separation of lipid classes

Total lipids were separated into different classes by column chromatography. The applied procedure for lipid separation was described in details by Merdzhanova *et al.* (2018) [4].

Preparation of fatty acids methyl esters (FAME) and GS-MS analysis

The dry residues of each fraction were methylated using 2% H_2SO_4 in methanol and n-hexane [5]. FAMEs were separated by gas chromatograph Thermo Scientific FOCUS with TR-5 MS capillary column (30 m, 0.25 mm i.d.) and MS detector (Polaris Q). For peaks identification two parameters were used: mass spectra of FAME mixed standard (SUPELCO 37 F.A.M.E. Mix C4 - C24) and internal data base (Thermo Sciences Mass Library, USA). Results for total lipid fraction were calculated using conversion factor for molluscs and for the individual lipid classes – as a percentage of each FA with respect to the total FAs. [6].

Saponification, extraction of vitamins, pigments, cholesterol and HPLC analysis

Alkaline saponification was used to analyse β-carotene, astaxanthin, cholesterol, all-*trans*-retinol, alpha-tocopherol, ergocalciferol, and cholecalciferol content in mussel tissue. All-*trans*-retinol, ergocalciferol, cholecalciferol, alpha-tocopherol, β-carotene, astaxanthin and cholesterol were determined simultaneously using high performance liquid chromatography (HPLC). Sample preparation procedure and HPLC analysis were performed following the method of Dobreva *et al.* [7].

RESULTS AND DISCUSSION

Total lipids, lipid classes and moisture content

The results for biometric characteristic, total lipids (TL), neutral lipids (NL), phospholipids (PL) and moisture content are presented in Table 1. Black Sea mussels were characterized by low lipid contents, regardless of the thermal treatment.

Table 1. Biometric characteristic, total lipids (TL), neutral lipids (NL), phospholipids (PL) and moisture content in raw, steamed and 3 month-stored Black Sea mussel edible tissues

	Raw	Steamed	3 Month-stored
Length (cm)		4.55±0.15	
TL, g 100 g^{-1} ww	1.20±0.03	2.92±0.10[a]	1.87±0.05[b]
NL, % of TL	41.20±0.4	43.20±0.4	32.9±0.2 [b]
PL, % of TL	58.80±0.5	56.80±0.1	67.1±0.8 [b]
Moisture, %	83.30±0.8	74.80±0.5[a]	75.38±0.4[b]

[a] $p<0.001$(Raw *vs* Steamed); [b] $p<0.001$(Raw *vs* Stored); [c-] $p<0.001$(Steamed *vs* Stored)

Significant differences were observed in the moisture content of raw, cooked and frozen samples. The water content was reduced by approximately 11-12% after cooking and storage processes. According to Fokina *et al.* (2018) cellular dehydration occurring in freezing, provokes reduction of cell volume in this way securing tolerance to ice formations of bivalve molluscs' tissues [8]. Moreover, higher levels of PL in mussel tissues probably perform a protective role against freezing. In our research, the amounts of TLs and PL increased significantly after heat treatment (140%) and freezing (56%), while the NL levels decreased up to 20% (after storage). Observed results for increase of PL levels during low-temperature storage confirm the suggestion of Fokina *et al*. Other possible explanation of noted PL increase after 3 months of storage is elevation of extractability of PL due to the protein denaturation occurring during prolonged frozen storage [9]. Zhou *et al.* (2014) reported a similar increase of PL levels after steaming and high-pressure treated New Zealand Greenshell mussel (*Perna canaliculus*) [10]. The same author supposed that the activity of the main enzyme which catalyzes ester bonds hydrolysis (lipase) in lipids during mussel storage may be protected or enhanced by temperature treatments. However, the lipid class distributions (PL>NL) in all analysed mussel samples remained unchanged.

Fatty acids composition of total lipids and lipid classes

The FA alignment of analyzed mussel samples was dominated by PUFA, followed by SFA and MUFA in all lipid fractions, regardless of the temperature treatment. FA composition of TL, NL and PL fractions of raw, steamed and 3 month-stored molluscs are presented in Table 2.

Table 2. Fatty acid composition of total lipids (TL), neutral lipids (NL), phospholipids (PL) in raw, cooked and 3 month-stored Black Sea mussel edible tissues

	Raw			Steamed			3 months-stored		
Fatty Acid	TL	NL	PL	TL	NL	PL	TL	NL	PL
C 8:0	0.03 ±0.01	0.02±0.01	0.02±0.01	0.03±0.01	0.07±0.01	0.12±0.01	0.02 ±0.01	Nd	0.02±0.01
C 10:0	0.02±0.01	0.03±0.01	0.07±0.05	0.17±0.02	0.18±0.02	Nd	Nd	0.01±0.01	0.05±0.01
C 12:0	0.61±0.02	0.20 ± 0.05	1.82 ± 0.10	0.37±0.03	0.11±0.01	0.06±0.01 [a]	Nd	Nd	0.01 ± 0.01[b]
C 14:0	1.07±0.08	1.10 ± 0.03	1.16 ± 0.04	1.03±0.05	2.26±0.10 [a]	1.54±0.30	0.94±0.07	1.39 ± 0.07	0.92 ± 0.04[a]
C 16:0	16.28±1.05	17.40 ± 1.10	19.40± 1.25	15.53±0.50	21.71±2.05 [a]	19.85±1.5	18.25±0.56	25.28±1.10	20.61 ± 1.25[a]
C 18:0	4.20±0.35	3.85 ± 0.30	7.95 ± 0.56	3.17±0.30 [a]	3.62±0.15	7.89±0.50	6.90±0.16	10.13±0.30	7.14 ± 0.56[a]
C 20:0	0.04±0.01	0.01 ± 0.01	Nd	0.06±0.01	0.02±0.01	Nd	0.04±0.01	0.12 ± 0.01	0.05±0.01
C 21:0	Nd	Nd	Nd	0.05±0.01	Nd	Nd	Nd	Nd	Nd
C 22:0	0.20±0.01	0.06 ± 0.01	0.10 ± 0.02	0.06±0.01	1.10±0.20	Nd	0.42±0.01	2.29 ± 0.01	1.49 ± 0.02
C 23:0	Nd	Nd	Nd	0.04±0.01	0.07±0.03	Nd	0.03±0.02	0.27±0.02	0.01±0.01
C 24:0	0.24±0.02	0.15± 0.02	0.38 ± 0.05	0.64±0.08	1.51±0.10	1.63±0.15	0.60±0.24	0.76± 0.02	0.53± 0.01
SFA	22.69±0.90	22.60±0.65	29.97±0.65	21.47±0.85	30.65±3.10 [a]	31.59±2.25 [a]	28.77±0.43	41.51±1.65	32.60±1.68
C 14:1 n 5	0.10±0.01	0.08 ± 0.01	0.17± 0.03	0.10±0.01	0.08±0.01	0.24±0.02	0.09±0.04	0.02 ± 0.01	0.05± 0.03
C 16:1 n 7	3.81±0.58	3.40±0.15	1.98 ± 0.12	4.60±0.20	5.34±0.15 [a]	1.65±0.04 [a]	2.62±0.09 [c]	6.97±0.15 [b]	1.86 ± 0.12 [b]
C 18:1 n 9	2.62±0.47	2.19 ± 0.10	1.33 ± 0.05	3.65±0.15	2.72±0.10	0.97±0.01	2.19±0.47 [c]	3.67 ± 0.10 [b, c]	0.90 ± 0.05[b]
C 20:1 n 9	0.54±0.02	0.77 ± 0.03	0.67 ± 0.08	0.98±0.08	2.19±0.05	0.62±0.02	0.54±0.02	0.65 ± 0.03	0.08 ± 0.08[b]
C 22:1 n 9	0.35±0.01	0.21 ± 0.01	0.15 ± 0.01	0.63±0.05	0.23±0.02	0.16±0.03	0.35±0.01	0.07 ± 0.01	0.17 ± 0.01
C 24:1 n 9	0.21±0.01	0.43 ± 0.01	0.38 ± 0.01	0.41±0.03	1.72±0.10	0.25±0.01	0.21±0.01	0.59 ± 0.01	0.45 ± 0.01[b]
MUFA	7.60±0.85	7.05±0.18	4.66[a]±0.20	10.36±0.60	12.29±0.40	3.89±0.05	6.88±0.15	12.0±0.36	3.57±0.20
C 18:4 n 3	0.11±0.01	0.36±0.02	0.14± 0.01	0.09±0.02	Nd	0.29±0.02	0.26±0.09	Nd	0.16± 0.01
C 18:3 n 6	Nd	Nd	Nd	0.13±0.03	0.57±0.03	Nd	0.09	Nd	Nd
C 18:2 n 6	2.06±0.15	2.21 ± 0.15	1.63 ± 0.05	1.22±0.06 [a]	1.68±0.10 [a]	2.10±0.10 [a]	0.42±0.04 [b]	1.00±0.07 [b]	0.56 ± 0.02[b, c]
C 18:3 n 3	1.10±0.08	1.59 ± 0.40	1.00 ± 0.05	0.58±0.04	0.70±0.04	0.21±0.02 [a]	0.92±0.08	1.57 ±0.40	1.06 ± 0.05[c]
C 20:3 n 3	0.62±0.01	1.05±0.18	0.90±0.02	1.00±0.10	0.40±0.03	0.12±0.01	1.66±0.01	0.17±0.18	0.11±0.01
C 20:3 n 6	1.44±0.01	0.38±0.02	1.62±0.04	2.61±0.14	1.59±0.10	0.77±0.03	5.60±0.01	1.13±0.02	0.69±0.04
C 20:2 n 6	0.94±0.05	0.61±0.02	0.58±0.02	0.90±0.07	0.56±0.05	0.53±0.03	0.94±0.05	1.22±0.02	0.43±0.02
C 20:4 n 6	6.24±0.25	7.64±0.25	4.38±0.20	5.06±0.40	7.90±0.65	8.50±0.15	7.41±0.25	2.27±0.25	0.91±0.04
C 20:5 n 3	31.70±2.28	27.59±1.30	13.21±0.50	35.04±2.80 [a]	16.84±1.20 [a]	19.44±1.28 [a]	16.49±0.76 [b, c]	4.92±1.30 [b, c]	22.19±0.50 [b, c]
C 22:6 n 3	26.85±2.56	31.04 ± 1.45	42.78±2.5	21.70±1.75 [a]	25.03±1.65 [a]	32.96±2.8 [a]	23.24±1.56	34.24±1.45 [b, c]	37.28±2.56 [b, c]
C 22:2 n 9	0.82±0.01	0.63 ± 0.03	0.90± 0.07	1.14±0.83	3.48±0.60	2.00±0.30 [a]	0.62±0.04	0.25±0.03	0.39± 0.02
PUFA	69.71±3.55	70.35±3.40	65.37±2.50	68.16±3.40	57.07±3.50[a]	64.53±3.30	64.35±0.55 [b]	46.79±2.40 [b, c]	63.83±1.94
Σ n 3	60.38	61.63	58.03	57.78	42.97	53.03	42.66	40.9	60.80
Σ n 6	9.33	8.72	7.34	10.38	14.1	11.5	21.69	5.89	3.03
N 6/ n 3	0.15	0.14	0.13	0.18	0.33	0.22	0.51	0.15	0.10
PUFA/SFA	3.07	3.11	2.18	3.17	1.86	2.04	2.24	1.13	1.93
(EPA+DHA)/C16:0	3.62	3.37	2.88	3.65	1.97	2.64	2.18	1.55	2.88

[a] – p<0.001(Raw *vs* Steamed); [b] - p<0.001(Raw *vs* Stored); [c-] p<0.001(Steamed *vs* Stored)

Saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) underwent statistically significant changes during the observed thermal treatments. The levels of SFA groups in NL and PL fractions increased after steaming (up to 35% for NL class) and freezing (up to 80% in NL class), which resulted in elevated content of palmitic acid (C16:0). Among the FA groups, palmitic acid was the most abundant FA in SFA group, palmitoleic acid (C16:1 n7) in MUFA, and eicosapentaenoic acid (C20:5 n3, EPA) and docosahexaenoic acid (C22:6 n3, DHA) in PUFA group. Three SFAs (C16:0>C18:0>C14:0), accounted for 95-98% of the total SFAs in raw samples and decreased up to 87% of total saturates in PL fractions after 3 months of freezing. The major MUFAs (C16:1n7>C18:1n9) showed significant decrease especially in NL fractions after cooking process ($p<0.01$), while storage conditions preserved the levels of these FAs.

Biji *et al*. (2015) also reported higher C16:1N7 compared to C18:1n9 values in New Zealand green mussel tissues and insignificant changes in these FAs after thermal processing [11]. According to Bastias *et al*. (2017) some of the principal changes that occur during thermal processed tissues are due to oxidation [12]. The thermal labile unsaturated C_{20} and C_{22} PUFAs are considered highly unstable and susceptible to oxidation during cooking and freezing storage. Observed results confirm these findings, because the PUFA levels show significant decrease in NL and PL classes after steaming (4%) and especially after 3 months of storage. The major differences were found in NL polyunsaturated FAs (33.5%, $P<0.01$) due to significantly decrease of long chain C_{20} as EPA (80%, $p<0.01$). Opposite trends were observed for C_{22} n-3 PUFAs, where DHA increased in NL (10%). There were a limited information for FA changes in lipid classes of mussel tissues after cooking and especially after storage. Biji *et al*., (2015) reported significant changes in PUFA contents of TLs for green mussel tissue after thermal processing.

According to Joseph (1982), marine molluscs are characterized by high levels of essential omega-3 (n3) PUFAs, mainly EPA and DHA, which accounted half of the total FAs [13]. Our results for Black Sea mussels confirm this statement and showed that both EPA+DHA constitute approximately from 40% up to 59% of total FAs in different lipid classes. Sum of these n3 PUFAs in TL and NL fractions decreased after steaming (30%) and especially after 3 months of storage (34%, $p <0.01$), whereas PL levels increased after storage (5%, $p<0.05$), compared to raw samples. Given the relative importance of essential n3 PUFAs as key dietary markers, these findings suggest that both methods can retain these biologically active FAs throughout steaming and storage at -20 °C processes. The favourable lipid quality of Black mussel tissues was well illustrated by the high values of n3 long chain PUFAs. Nevertheless, thermal processed n3 PUFAs showed 40.9% to 61.63% of total FAs in both lipid classes, whereas omega 6 (n6) PUFAs displayed significantly lower levels (3.03%-21.69%). Moreover, observed increase of n3 PUFA values in PL fractions after storage compared to NL classes, confirm that 3 months of storage can preserve the high bioavailability of these FAs in mussel tissues.

The analyzed samples were assessed from a nutrition quality perspective by two traditionally used FA ratios– n6/ n3 and PUFA/SFA. Depending of the applied treatment, these ratios significantly varied between thermal processed samples (Table 2). According to Simopolous, (2013) the beneficial n6/n3 ratio for the human health is below one [14]. Presented results ranged from 0.1 to 0.5 and confirmed the high quality of Black Sea mussel lipids regardless of treatment. PUFA/SFA ratio described the FA balance in mussel lipids well. In this study PUFA/SFA ratios ranged from 1.13 to 3.17 and were several times higher than recommended from Department of Health (1994) values (should be higher than 0.45) [15]. Based on reported results we may conclude that steaming and 3 months of storage can preserve well-balanced and beneficial lipid class levels, and human-health improved FA profile.

The polyene index (EPA+DHA/16:0) is determined as a valuable indicator of tissue degradations, respective lipid oxidations, during cooking or storage processes [16]. In this study its value ranged from 2.88 (PL) to 3.62 (TL) for raw samples and showed significant reductions in NL fraction after cooking (42%, $p<0.01$) and freezing (56%), whereas in PL fractions it remained unchanged. Due to these findings we can suppose that 3 months of storage at – 20°C affects mostly lipid stability of mussel tissue, especially at NL fraction, compared to PL fraction. One possible reason for the observed changes is that oxidation mechanisms are active during frozen storage and PL fractions are more stable in these conditions. No comparable information is found in scientific literature for this index for steamed and stored Black Sea mussels from the Bulgarian Black Sea coast.

Table 3. Fat-soluble vitamins, cholesterol and carotenoid contents in raw, steamed and 3 month-stored Black Sea mussel edible tissues

	Raw	Steamed	3 Months of storage
Vitamin A, μg 100 g^{-1} w.w.	113.4±10.2	32.8±4.5 [a]	31.88±3.8 [b]
Vitamin D_2, μg 100 g^{-1} w.w.	7.93±0.6	7.87±0.5	6.10±0.55
Vitamin D_3, μg 100 g^{-1} w.w.	17.12±0.8	25.20±0.65 [a]	12.74±0.5 [b]
Vitamin E, μg 100 g^{-1} w.w.	5120.89±45.0	5064.3±55.0	1463.46±23.8 [b, c]
Cholesterol, mg 100 g^{-1} w.w.	37.64±0.60	40.68±0.50	20.20±0.15 [b, c]
β-Carotene, μg 100 g^{-1} w.w.	206.39±25.4	80.47±10.3[a]	25.45±12.5 [b, c]
Astaxanthin, μg 100 g^{-1} w.w.	78.28±8.5	80.07±10.0	60.42±5.5 [b, c]

[a] – $p<0.001$(Raw *vs* Steamed); [b] - $p<0.001$(Raw *vs* Stored); [c-] $p<0.001$(Steamed *vs* Stored)

Fat-soluble vitamins, carotenoids and cholesterol

The results for the fat-soluble vitamins, cholesterol and carotenoids contents are presented in Table 3. Vitamin A, D_3, astaxanthin and β-carotene are expressed as micrograms per 100 grams wet weight (μg 100 g^{-1} w.w.), vitamin E and cholesterol is expressed as milligrams per 100 grams wet weight (mg 100 g^{-1} w.w.)

All analysed components significantly decreased after 3 months of storage, as minor changes were observed for astaxanthin (22%), maximal – for vitamin E (70%). Most significant decrease was observed for vitamin A and β-carotene between raw and cooked samples. Vitamin D_3 and cholesterol content was higher in cooked samples, vitamin D_3 was twofold higher than the recommended daily intake [17]. Thus, analysed Black Sea mussels can be classified as an excellent source of vitamin D_3, which increases its beneficial value. Merdzhanova *et al*. (2018) reported lower levels for all fat-soluble vitamins, especially for vitamin D_3 (only 3.1 μg/100 g w.w.) and higher values for cholesterol and carotenoids for aquaculture mussels from the Northern part of Bulgarian Black Sea [18]. Similar increase of cholesterol and vitamin D_3 amounts and decrease of β-carotene and vitamin A were found in raw and cooked *Rapana venosa* TLs from the Bulgarian part of Black Sea [4]. There was no comparable information for changes of analysed fat-soluble components of black mussels from the Bulgarian part of Black Sea after freezing storage. Presented results confirmed that thermally processed Black Sea mussels preserve nutritionally beneficial quality with respect to bioactive lipids with antioxidant properties as β-carotene, astaxanthin, vitamin E compared to raw samples, although some nutrient loss was detected.

CONCLUSIONS

The health-beneficial value of mussels depends strongly on the TL content, lipid classes, FA composition, fat-soluble vitamins, carotenoids and cholesterol contents. The traditionally used methods of processing and storage of black mussels caused different changes in their quality as food. Due to this fact, the evaluation of the lipid composition in steamed and 3 month-stored mussels helps to disclose the levels of resistance of analysed edible tissues to thermal stress processes. Observed changes of biologically active components give the opportunity to assess the food quality preservation during these commonly used methods. Both steaming and freezing methods affected mostly the FA profile of the NL fraction, especially after freezing, whereas FA content of PL fractions remained significantly stable. The similar trends for decrease of fat soluble vitamins, carotenoids and cholesterol contents after 3 months of storage showed that freezing affects more aggressively mussel tissues lipids components compared to steaming process. However, freezing as a commonly used method for mussel edible tissue preservation caused a minimal decrease in nutrition value and naturally occurring components, such as unsaturated n3 PUFAs, vitamin D_3 and antioxidants (vitamin E, β-carotene, and astaxanthin) were significantly retained. It would be important to study the effects of different cooking methods and storage time on biologically active lipids of black mussel tissue over a longer period of time. Regardless of the thermal processes, the analysed mussel samples from Bulgarian coast are well balanced and rich of biologically active lipids to meet the needs of consumers.

Acknowledgement: *The study is a part of a project DM09/2 from15 Dec 2016 "Seasonal variations in lipid profile and thermal stress effect on the lipid composition of Black sea Mytilus galloprovincialis and Rapana venosa".*

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