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# Seasonal variation in fat-soluble vitamins, cholesterol and fatty acid profile of lipid classes of *Rapana venosa*

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Nowadays, invasive species *Rapana venosa* is commercially important and widely harvested in the Black Sea. The aim of this study was to evaluate the seasonal changes in lipid bioactive compounds as fat-soluble vitamins (A, E and $D_3$), cholesterol, carotenoids (astaxanthin, beta-carotene), lipid classes and their fatty acid profiles. Among fat soluble vitamins, the amounts of vitamin $D_3$ supplied 100% of recommended daily intake for adults according to Bulgarian food standards. Vitamin E increased in summer period (up to 2825 μg.100$^{-1}$g ww), whereas vitamin A showed opposite trend. Both carotenoid pigments: beta-carotene and astaxanthin insignificantly decreased in summer season. Strong seasonal influence on the lipid classes and their fatty acid (FA) profile was determined. Despite of the seasons, polar lipids (PL) prevailed in total lipids (TL). In both seasons, FA groups showed similar distribution: PUFA> SFA>MUFA, regardless of lipid class. The most abundant FAs in the polyunsaturated FA group were eicosapentaenoic acid (C20:5 n-3, EPA), docosahexaenoic acid (C22:6 n-3, DHA) and docosapentaenoic acid (C22:5 n-3, DPA), which accounted more than 65% of total PUFA. The high content of vitamin $D_3$, long chain omega-3 FAs, different FA ratios, low values of cholesterol and nutrition quality indices (atherogenic and thrombogenic indices) confirm the very good functional properties of rapana lipids. In conclusion, the studied rapana harvested from Bulgarian Black Sea coast presents preferable lipid quality in terms of human health protection. Further investigation is required to determine the biological potential of rapana lipids during the year.

***Keywords**: Rapana venosa, lipid classes, fat-soluble vitamins, carotenoids, seasonal changes*

## INTRODUCTION

Health benefits associated with seafood consumption have been intensively studied over the past three decades. Mollusks are rich sources of polyunsaturated fatty acids (PUFA), phospholipids, sterols, carotenoids, vitamins (vitamin $D_3$ and $B_{12}$) and various micronutrients and essential amino acids. The veined rapa whelk *Rapana venosa* (Valenciennes, 1846) is a large predatory marine gastropod, an invasive species in the Black Sea with nutritional and economic importance in Bulgaria. The main food sources of rapana are *Mytilus galloprovincialis* and *Chamelea gallina*. This species is in high demand on the Asian market, which has lead to increase catch and exportation to Japan, South Korea, China, USA and even Germany. *Rapana venosa* is known to be very resilient and tolerant to temperature, salinity and pollution fluctuations [1]. Chemical composition of mollusks is strongly influenced by season and reproductive cycle [2, 3]. There is scarce literature on the seasonal changes of fat-soluble vitamins, cholesterol and fatty acid profile of *Rapana venosa* from the Black Sea.

## EXPERIMENTAL

### *Sampling*

Live samples of Rapana venosa were purchased from a local enterprise for fish and seafood processing near Varna, Bulgaria in April and August, 2017. Animals were transported to the laboratory in ice boxes. They were brushed, washed and processed immediately. Body length was measured and edible soft tissue was taken for further analyses. Specimens were chosen in same length to avoid misinterpretation of the results due to different life stages.

### *Lipid extraction, separation and purification*

Total lipids were extracted by the method of Bligh and Dyer (1959) [4]. An aliquot of the lipid extract (10 mg/ml in chloroform) was used for separation of lipid classes. Neutral lipids (NL) and polar lipids (PL) were fractionated by column chromatography using a glass column (10 mm dia × 20 cm) packed with slurry of activated silicic acid (70 to 230 mesh; Merck, Darmstadt, Germany) in chloroform. The fraction containing NL was eluted with chloroform, while PL – with methanol. The amounts of total lipids and lipid classes were determined gravimetrically. The purity of each fraction was tested by thin-layer chromatography, using Silica gel F254 plates (thickness = 0.25 mm; Merck, Darmstadt, Germany).

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*Fatty acid derivatization and analysis*

Lipid fractions were derivatized by acid-catalyzed transesterification with 2% $H_2SO_4$ in anhydrous methanol and n-hexane [5]. Fatty acid compositions of TL, NL and PL were determined by gas chromatography with masspectrometry (GC/MS) of the corresponding fatty acid methyl esters (FAME). Chromatographic separation was performed by Thermo Scientific FOCUS Gas Chromatograph on a TR-5 MS capillary column (30 m, 0.25 mm i.d.). For identification and quantification of FAME peaks authentic standards – SUPELCO FAME Mix C4-C24 and PUFA №3 from Menhaden oil (Sigma-Aldrich, Merck) were used.

*Fat-soluble vitamins and carotenoids analysis*

Analyses of the non-saponifiable lipids – vitamin A, $D_3$, E, cholesterol, astaxanthin and β-carotene were performed by following the method of Dobreva *et al*. [6]. Briefly, soft edible tissue was subjected to direct alkaline hydrolysis with methanolic potassium hydroxide. Consequently, analytes of interest were extracted with a mixture of hexane: dichloromethane and simultaneously analyzed by high performance liquid chromatography.

*Nutrition quality indices (NQI)*

Several indices and ratios were used to evaluate lipid quality of rapana meat: omega-6/omega-3 (n-6/n-3) and polyunsaturated fatty acids/saturated fatty acids (PUFA/SFA) ratios, indices of atherogenicity (AI) and thrombogenicity (TI), and cholesterolemic index (h/H).

*Statistical analysis*

Student's t-test was employed to estimate the significance of defferences. Statistical significance was indicated at $p<0.05$.

## RESULTS AND DISCUSSION

*Total lipids and lipid classes composition*

The results for total lipids (TL), neutral lipids (NL) and polar lipids (PL) are presented in Table 1. *Rapana venosa* was characterized by very low lipid content, which is in agreement with previously published data [7-10].

**Table 1.** Total lipids (TL), neutral lipids (NL) and polar lipids (PL) content in *Rapana venosa* meat

| | Spring | Summer |
|---|---|---|
| Mean water temperature, °C | 9.4 | 25.3 |
| Mean length, cm | 6.27±0.9 | |
| TL, g.100g-1 ww | 0.51±0.02 | 0.48±0.05 |
| NL, % of TL | 31.9±0.8* | 60.0±0.8* |
| PL, % of TL | 68.0±0.4* | 40.0±1.1* |

*Statistical differences, p<0.05

TL content in *Rapana venosa* edible meat is very low and no significant differences between seasons were observed. Sea snails edible meat (also called "foot") is not considered as lipid storage organ, therefore its lipid content is usually low and not affect by seasonal changes [11,12]. On the other hand, large differences were observed in the distribution of lipid classes. PL were higher than NL content in spring, while summer samples showed the opposite trend (NL>PL). The observed variations are probably related to different roles of the lipid classes during the reproductive cycle of rapana.

*Fatty acids composition*

Studies on fatty acid composition of marine species provide important information for the sources of fatty acids, especially very long-chain PUFA, which exhibit physiological and structural benefits for organisms. There are numerous studies devoted to biochemical composition of marine invertebrates, but studies on lipid components of Black Sea mollusks are scarce. Fatty acids composition of total lipids and lipid classes of spring and summer *Rapana venosa* are presented in Table 2.

Fatty acids composition of marine invertebrates is usually affected by the environmental factors such as dietary sources, season, salinity, pollution, sum radiation, etc. [13]. PUFA was the most abundant FA group in both spring and summer samples, followed by SFA and MUFA. Same pattern was observed for total lipids and lipid classes in both seasons PUFA>SFA>MUFA. In previous studies [7-9] authors reported same distribution of FA groups for Black Sea *Rapana venosa* lipids. On the other hand, autumn samples of raw and cooked *Rapana venosa* showed slightly higher amounts of SFA, compared to PUFA in total lipids [14].

The results of the present study revealed that polar lipids and neutral lipids of rapana contained predominantly C16:0 (13.56%-20.10%), C18:0 (7.00%-12.84%), C20:5n3 (24.15%-33.84%), C22:6n3 (10.79%-16.31%) and C22:5n3 (1.20%-15.06%), while C8:0, C10:0, C12:0, C15:0, C17:0, C20:0, C24:0, C14:1, C22:1, C24:1, C18:4n3, C18:3n6, C18:3n3, C20:3n3 and C20:2n6 were minor components of mollusks lipids. Palmitic acid (C16:0) was the most abundant FA in SFA group, palmitoleic acid (C16:1n7) in MUFA, and eicosapentaenoic acid (C20:5 n3, EPA) in PUFA group in all lipid fractions for both seasons.

**Table 2.** FA (as relative %) composition of TL, NL and PL in spring and summer *Rapana venosa*

| FA, % of TFA | TL | | NL | | PL | |
|---|---|---|---|---|---|---|
| | **Spring** | **Summer** | **Spring** | **Summer** | **Spring** | **Summer** |
| 8:0 | 0.06±0.01 | 0.03±0.01 | 0.01±0.0 | 0.06±0.01 | 0.02±0.0 | 0.09±0.01 |
| 10:0 | 0.11±0.01 | 0.02±0.0 | 0.02±0.0 | 0.12±0.01 | 0.02±0.0 | 0.06±0.0 |
| 12:0 | 0.21±0.01 | 0.04±0.0 | 0.03±0.0 | 0.12±0.01 | 0.09±0.01 | 0.06±0.0 |
| 14:0 | 2.54±0.23 | 2.01±0.21 | 3.84±0.3 | 0.85±0.09 | 1.58±0.14 | 2.10±0.23 |
| 15:0 | nd | 0.03±0.0 | 0.07±0.01 | 0.16±0.01 | 0.04±0.0 | 0.99±0.1 |
| 16:0 | 15.6±0.9 | 13.56±0.9* | 20.10±1.7 | 16.89±1.1* | 18.91±1.3 | 15.70±1.0* |
| 17:0 | nd | 0.15±0.01 | nd | 0.20±0.01 | nd | 1.03±0.1 |
| 18:0 | 7.6±0.7 | 9.89±0.9 | 7.00±0.7 | 11.39±0.9 | 8.42±0.9 | 12.8±0.9 |
| 20:0 | 0.68±0.7 | 0.40±0.03 | 0.05±0.0 | 0.48±0.04 | 0.14±0.01 | 0.37±0.03 |
| 22:0 | 1.65±0.16 | 1.03±0.1 | 0.50±0.05 | 0.38±0.03 | 1.56±0.14 | 1.61±0.15 |
| 24:0 | 0.72±0.07 | 0.74±0.07 | 0.36±0.04 | 0.79±0.08 | 0.28±0.02 | 0.65±0.07 |
| **SFA** | **29.26** | **27.90*** | **32.00** | **31.33** | **31.07** | **35.50*** |
| 14:1 | 0.42±0.04 | 0.14±0.01 | 0.48±0.04 | 0.26±0.02 | 0.20±0.01 | 0.06±0.0 |
| 16:1 | 6.67±0.6 | 4.51±0.43* | 6.35±0.6 | 1.05±0.1* | 4.58±0.43 | 2.22±0.21* |
| 18:1 | 3.08±0.3 | 3.95±0.4 | 3.17±0.3 | 1.00±0.1* | 2.41±0.23 | 1.27±0.11* |
| 20:1 | 0.41±0.04 | 1.18±0.11 | 0.61±0.05 | 0.26±0.02 | 1.24±0.11 | 0.60±0.06 |
| 22:1 | 0.38±0.04 | 0.15±0.01 | 0.42±0.04 | 0.21±0.02 | 0.53±0.04 | 0.11±0.01 |
| 24:1 | 0.60±0.05 | 0.20±0.01 | 0.24±0.02 | 0.40±0.04 | 0.23±0.02 | 0.39±0.03 |
| **MUFA** | **11.57** | **10.35** | **11.26** | **3.11*** | **9.19** | **4.73*** |
| 18:4n3 | 0.68±0.06 | 0.39±0.03 | 0.25±0.02 | 0.74±0.07 | 0.80±0.07 | 0.31±0.03 |
| 18:3n6 | 0.16±0.01 | 0.08±0.01 | 0.15±0.01 | nd | 0.10±0.01 | 0.08±0.01 |
| 18:3n3 | 0.14±0.01 | 0.14±0.01 | 0.56±0.05 | 0.20±0.01 | 0.12±0.01 | 0.22±0.02 |
| 18:2n6 | 1.37±0.1 | 0.80±0.07* | 1.27±0.1 | 0.74±0.07* | 2.00±0.2 | 0.90±0.07* |
| 20:5n3 | 24.5±2.3 | 28.7±2.6* | 24.1±2.3 | 33.8±3.1* | 25.7±2.3 | 32.7±3.2* |
| 20:4n6 | 4.17±0.4 | 2.37±0.2* | 3.60±0.3 | 2.20±0.2* | 4.17±0.4 | 3.31±0.3* |
| 20:3n6 | 1.99±0.2 | 1.54±0.2 | 0.34±0.3 | 0.78±0.07 | 2.01±0.2 | 2.36±0.2 |
| 20:3n3 | 0.80±0.07 | 0.40±0.04 | 0.07±0.0 | 0.70±0.07 | 0.22±0.01 | 0.63±0.07 |
| 20:2n6 | 0.44±0.04 | 0.36±0.04 | 0.24±0.01 | 0.36±0.02 | 1.00±0.1 | 0.30±0.02 |
| 22:2n6 | 2.03±0.2 | 1.15±0.1 | 0.21±0.01 | 2.39±0.2 | 1.69±0.2 | 0.55±0.04 |
| 22:6n3 | 16.31±1.5 | 10.79±0.9* | 14.05±1.3 | 13.15±1.2 | 11.63±1.2 | 12.04±1.2 |
| 22:5n3 | 6.60±0.6 | 15.06±1.5* | 8.10±0.7 | 8.67±0.8 | 8.71±0.8 | 1.20±0.1* |
| 22:4n3 | nd | 0.40±0.04 | 3.55±0.3 | 1.64±0.2 | 1.55±0.1 | 0.63±0.06 |
| **PUFA** | **59.17** | **61.91** | **56.54** | **65.40*** | **59.74** | **59.79** |

Statistical differences, $p<0.05$ (Spring vs Summer)

The amount of palmitic acid decreased significantly from spring to summer. Koral and Kiran [9] reported same tendency for C16:0 in *Rapana venosa* from several sites of the Black Sea. SFA increased significantly in PL due to the increase of the amount of C18:0 in summer. Total SFA content decreased in total lipid and neutral fractions during the summer season, while MUFA content decreased significantly in both NL and PL. One possible reason could be that the sea snails metabolize NL and TL in colder seasons and stores them in hotter seasons, especially for reproductive purposes. In the Black Sea the observed reproductive period of *Rapana venosa* is from July to September [15]. The decrease in MUFA was mainly due to the content of C16:1n7 and C18:1n9. The latter fatty acid promotes membrane fluidity in colder seasons, thus there is inverse correlation between MUFA content and environmental temperatures. Summer samples showed higher content of PUFA in TL and NL fractions and no change in the PL class. Ekin *et al.* [13] reported similar changes for fatty acid profiles of TL, NL and PL classes of the freshwater snail *Melanopsis praemorsa*. However, in this study sea snail *Rapana venosa* lipids presented significantly higher PUFA content, compared to the freshwater snail.

Marine invertebrates are characterized by higher content of essential very long chain n-3 PUFA, namely EPA, docosahexaenoic acid (C22:6 n3, DHA) and docosapentaenoic acid (C22:5n3, DPA). Together, these three FA constitute almost half of the total FAs in spring and more than half of TFA in summer season. There was an increase in TL and NL PUFAs in the summer period, while PL PUFA did not change. Generally, PL fractions of marine mollusks show less seasonal variation compared to the NL fractions, because they play major role in maintaining cellular integrity [16]. The most abundant PUFAs in the present study were EPA, DHA, DPA and arachidonic acid (C20:4n6, AA). The sum of EPA and DHA represented 69% of PUFA in TL in spring and 63.8% – in summer. The amount of EPA increased significantly in all lipid fractions in summer period. On the other hand, DHA content showed the opposite trend in TL and NL, and only a slight increase was observed in the PL fraction. The amount of DPA in the present study increased significantly in the TL and decreased in the PL fractions from spring to summer. Koral and Kiran [9] reported different variations for EPA and DHA in the TL of *Rapana venosa* from several locations along the eastern Black Sea coast. In their study, authors found that AA was the most abundant in the PUFA group. Same pattern was observed for total lipids of the black mussel *Mytilus galloprovincialis* from the northern part of the Bulgarian Black Sea coast [17]. The black mussel comprises the main food source for rapana [1]. Marine invertebrates are good alternative sources of very long chain omega-3 fatty acids, especially EPA and DHA. Together with DPA, they are important membrane lipids with well recognized health benefits. The fact that significant amounts of these fatty acids are bound to PL increases their bioavailability and can therefore be important for achieving the corresponding healthy effects.

*Fat-soluble vitamins and carotenoids and cholesterol*

The results for the fat soluble vitamins, carotenoids and cholesterol, expressed as mean and standard deviation (mean ± SD) are presented in table 3. The results are expressed as microgram per 100 grams wet weight (μg.100$^{-1}$ g ww) for vitamin A, $D_3$, astaxanthin and β-carotene and milligram per 100 grams wet weight (mg.100$^{-1}$ g ww) for cholesterol and vitamin E.

**Table 3.** Fat-soluble vitamins, carotenoids and cholesterol contents in spring and summer *Rapana venosa*

| | Spring | Summer |
|---|---|---|
| Astaxanthin | 20.5±1.7 | 19.7±1.5 |
| β-carotene | 216.9±19 | 180.2±15* |
| Vitamin A | 49.35±3.8 | 37.03±2.5* |
| Vitamin $D_3$ | 15.40±1.5 | 17.60±1.5 |
| Vitamin E, mg.100g$^{-1}$ ww | 2.71±0.5 | 2.82±0.6 |
| Cholesterol, mg.100g$^{-1}$ ww | 23.1±1.8 | 25.4±1.9 |

*Statistical differences, $p<0.05$

Fat-soluble vitamins, especially vitamin $D_3$ are present in high concentration in marine organisms. Their concentrations show seasonal variations related to spawning cycle, environmental conditions and food availability [18]. In this study, β-carotene and vitamin A content decreased significantly from spring to summer. There was no change in astaxanthin amount related to the season. Vitamin E and cholesterol content increased in summer, but the observed change was not significant. Vitamin E and carotenoids (beta-carotene and astaxanthin) are fat-soluble components with high antioxidant activity. One of the main roles of vitamin E is the protection of n-3 PUFA against oxidation [19]. In this study, there was a correlation between vitamin E content and PUFA content in rapana lipids. Higher vitamin E and higher PUFA were found in summer. In our previous study *Rapana venosa* showed significantly higher amounts of the fat-soluble vitamins $D_3$ and E, carotenoids and cholesterol and lower for vitamin A [14]. There is inverse correlation between the levels of β-carotene and temperature. Lower water temperature is related to higher β-carotene content [14,20]. Same variation is observed in the present study.

*Nutrition quality indices (NQI)*

There are two fatty acids ratios that are common indicators for the relative nutritional values of dietary lipids. In this study the PUFA/SFA ratio was found to be higher than the recommended 0.4 and n-6/n-3 – lower than 4.0 in both seasons [21]. The PUFA/SFA ratio increased, while n-6/n-3 ratio decreased significantly in summer, corresponding to higher PUFA and higher n-3 content (Table 4).

The ratio n-6/n-3 decreased two fold in the summer – from 0.21 to 0.11. Popova *et al.* [8] reported higher n-6/n-3 ratios (0.42-0.56) and lower PUFA/SFA (1.16-1.63) for summer and autumn *Rapana venosa* harvested in the Black Sea. Koral and Kiran [9] also found higher n-6/n-3 ratios and lower PUFA/SFA in the TL of *Rapana venosa* from several locations along the eastern Black Sea coast. The differences are mainly due to the higher content of some SFA (C18:0) and n-6 PUFA (C20:4n6) reported by the authors.

**Table 4.** Fatty acid contents, FA ratios, nutrition quality indices in spring and summer Rapana venosa

| | Spring | Summer |
|---|---|---|
| n-6/n-3 | 0.21 | 0.11* |
| PUFA/SFA | 2.02 | 2.22 |
| AI* | 0.37 | 0.30 |
| TI* | 0.16 | 0.14 |
| h/H* | 2.37 | 2.22 |

*AI= [C12:0+ (4×C14:0) +C16:0]/ (n6PUFA+ n3PUFA+ MUFA);

*TI = (C14:0+C16:0+C18:0)/[(0.5MUFA) + (0.5n6PUFA) + (3n3PUFA) + (n3PUFA/ n6PUFA)] *h/H= (C18:1n9+C18:2n6 +C18:3n3 +C20:4n6+ C20:5n3+ C22:6n3)/ (C14:0 +C16:0)

*Statistical differences, $p<0.05$

The functional properties of *Rapana venosa* total body lipids were also assessed by the following indices: index of atherogenicity (AI), thrombogenicity (TI), and cholesterolemic index (h/H). Their values decreased slightly in the summer, but there were no significant differences between the values of nutrition quality indices among the two seasons. The results showed very low AI (< 1) and TI (<< 1) values and higher h/H (> 1) levels in the mollusks lipids in both seasons. Anti-atherogenic and anti-thrombogenic indexes of both spring and summer samples were found lower than those presented by Popova *et al* [8]. These results confirm the good anti-atherogenic, anti-thrombogenic and hypocholesterolaemic properties (AI, TI < 1 and h/H > 1) of *Rapana venosa* lipids, which could classify the gastropod meat as beneficial for human consumptions.

## CONCLUSIONS

The present study analyzed seasonal changes in biologically active lipid composition in the muscle tissue of the edible sea snail *Rapana venosa* from the Black Sea. Regardless of the observed seasonal fluctuations, *R. venosa* showed very low lipid content, comprising mainly of polar lipids, which increases the bioavailability of the fatty acids. There were significant differences between the lipid classes distribution in the two seasons. The main PUFAs in the present study were EPA and DHA, together representing more than half of total PUFA in *Rapana venosa* TL in both seasons. Although characterized by very low lipid content, Black Sea *Rapana venosa* remains significant source of PUFA, especially those bonded to polar lipids, vitamin $D_3$ and carotenoids. These results of the present study also confirm the good anti-atherogenic, anti-thrombogenic and hypocholesterolaemic properties of *Rapana venosa* meat. In conclusion, the studied rapana harvested from Bulgarian Black Sea coast presents preferable lipid quality in terms of human health protection. Further investigation is required to determine the biological potential of rapana lipids during the year and changes occurring in the antioxidants during various cooking processes and prolonged storage.

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