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Simultaneous HPLC determination of fat soluble vitamins, carotenoids and cholesterol in seaweed and mussel tissue

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The aim of the present study was to develop simple method for simultaneous determination of fat soluble vitamins (all-trans retinol, cholecalciferol, ergocalciferol and α-tocopherol), carotenoids (β-carotene and astaxanthin) and cholesterol in seaweeds and mussel tissue. Reversed-phase high performance liquid chromatography system combine with UV and fluorescent detection was the method characterized with rapid, sensitive and accurate detection of all components. Extraction procedure requires small amounts of sample. The sample preparation included saponification and liquid-liquid extraction of the analytes. The method precision (relative standard deviation) was below 10% for all analytes. The method shows good linearity of all investigated components and analysis time – 32 min. The method was applied on real seaweed and mussel tissue samples and the results for the tested fat soluble analyte contents were in a good agreement with the data given by other authors.

Key words: *all-trans retinol, calciferol, α-tocopherol, astaxanthin, cholesterol*

INTRODUCTION

Many studies suggest that marine mollusks and edible seaweeds are valuable healthy food, low in calories and fats, and high in proteins and bioactive compounds [1, 2]. Seaweeds also have been used as permanent source of the raw materials used in pharmaceutical, food industries, medicine and cosmetics, as fodder and fertilizer [3]. Mollusks and different seaweeds are amongst the most important dietary sources of fat soluble vitamins – A, D_3 and E. They are also rich in carotenoids, which act as antioxidants [4, 5]. Overall mollusk production in Bulgaria relies on two sources – commercial fishing (for mussel and sea snail *Rapana*) and marine aquaculture (consists of mussel only) [6].

Lipid content of different marine algae is only 1-5% of dry matter and exhibits an interesting polyunsaturated fatty acid composition (particularly ω-3 and ω-6) [7]. Seaweed and mollusks tissue is a good source of some water- (B_1, B_2, B_{12} and C) and fat soluble (β-carotene with vitamin A activity, ergocalciferol and cholecalciferol and α-tocopherol) vitamins. Vitamins derived from algae are very important due to their antioxidant activity, biochemical functions and other health benefits - prevention of cardiovascular diseases (β-carotene), decreasing blood pressure (vitamin C), reducing the risk of developing cancer (vitamins E and carotenoids) [8].

Information available about the fat soluble vitamins, carotenoids and cholesterol content of Black mussel and different algae from Black Sea is scarce. The aim of the present work was to develop a simple and accurate method for simultaneous determination of seven fat soluble biologically active components: all-trans-retinol (vitamin A), cholecalciferol (vitamin D_3), ergocalciferol (vitamin D_2), α-tocopherol (vitamin E), β-carotene, astaxanthin and cholesterol in matrices from animal and plant origin.

EXPERIMENTAL

Instrumentation and chemicals

The chromatographic analysis was performed on HPLC system (Thermo Scientific Spectra SYSTEM) equipped with UV2000 and FL3000 detectors. All solvents were of HPLC grade specification, obtained by Sigma-AldrichTM, USA. Substances of all-trans-retinol, ergocalciferol, cholecalciferol, α-tocopherol, β-carotene, astaxanthin, cholesterol and L-ascorbic acid were all with analytical standard specifications and were supplied by Supelco (Sigma-AldrichTM, USA).

Sample preparation

Two mussel and four seaweed samples were used for evaluation the all-trans-retinol, ergocalciferol, cholecalciferol, α-tocopherol, β-carotene, astaxanthin and cholesterol contents. Sample preparation procedure was performed following the method of Dobreva *et al*. (2011) with some modifications [9]. Edible mussel and shredded algal tissue was homogenized using

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kitchen homogenizer for 3 min. An aliquot of the homogenized sample (1.000 ± 0.005 g) was weighed into a glass tube with a screw cap and 1% of methanolic L-ascorbic acid and 0.5M methanolic potassium hydroxide were added. Six parallel tests of each mussel and algae samples were prepared and subjected to saponification at 50°C for 30 min. After cooling the analytes were extracted twice with *n*-hexane: dichloromethane = 2:1 (v/v) solution. Combined extracts was evaporated under nitrogen flow and dry residue was dissolved in methanol: dichloromethane solution, filtered (0.45 µm syringe filter) and injected (20µl) into the HPLC system.

Chromatographic conditions

All-trans-retinol, ergocalciferol, cholecalciferol, α-tocopherol, astaxanthin, β-carotene and total cholesterol were determined simultaneously using HPLC/UV/FL system equipped with RP analytical column. Chromatographic separation was performed by a Synergi 4µ Hydro-RP 80A pore 250x4.6 mm reversed-phase column, through a gradient at 1.1 mL/min. Solvent A consisted of methanol : water (93:7), solvent B was acetonitrile and solvent C – 2-propanol. The gradient changed as follows: 0-16.0 min, 100 % solvent A; 20.0-30.0 min, 60% solvent B and 40 % solvent C; 30.0-40.0 min, 50 % of solvent B and 50% solvent C. The gradient was then returned to 100 % of solvent A.

Detection of ergocalciferol (λ= 265 nm), cholecalciferol (λ= 265 nm), astaxanthin (λ= 474 nm), β-carotene (λ= 450 nm) and cholesterol (λ=208 nm) was performed by UV detector. Concentrations of all-trans-retinol (at λ_{ex} = 334 nm and λ_{em} = 460 nm) and α-tocopherol (at λ_{ex} = 288 nm and λ_{em} = 332 nm) were measured by fluorescence detection.

Standards and quantification

Stock standard solutions of each analyte (1.0 mg/ml) were prepared as follows: retinol, ergocalciferol, cholecalciferol, α-tocopherol and cholesterol in 100% methanol; astaxanthin and β-carotene in 100% dichloromethane. The solutions were stored at −20°C away from light. Working solutions were prepared prior to analysis by dilution proportionally with methanol/dichloromethane. In all cases, the stock solution was analyzed together with the samples. Analyte concentrations in samples were estimated on the basis of peak areas. All samples were analyzed in triplicate. Contents of each analytes were presented as means ± standard deviation.

RESULTS AND DISCUSSION

Chromatography

Chromatograms of combined standard solution are presented on figure 1. The quantitation was performed by the method of the external calibration, comparing the chromatographic peak areas of the samples with those of the corresponding standards.

Selected chromatographic conditions provided good separation of the analytes, in less than 40 min. Retention times (t_R) were as follows: astaxanthin t_R = 4.5 min, retinol t_R = 4.9 min, ergocalcipherol t_R = 11.1 min, cholecalciferol t_R = 11.7 min, cholesterol t_R = 19.6 min, α-tocopherol t_R = 12.5 min and β-carotene t_R = 40.3 min. Resolution factors were greater than 1, indicating a sufficient separation.

Linearity

Each calibration curve was constructed by measuring five diluted standards from the corresponding standard solutions and by plotting peak area response against concentration of the standards. The least square linear regression analysis was applied to estimate equation of the line.

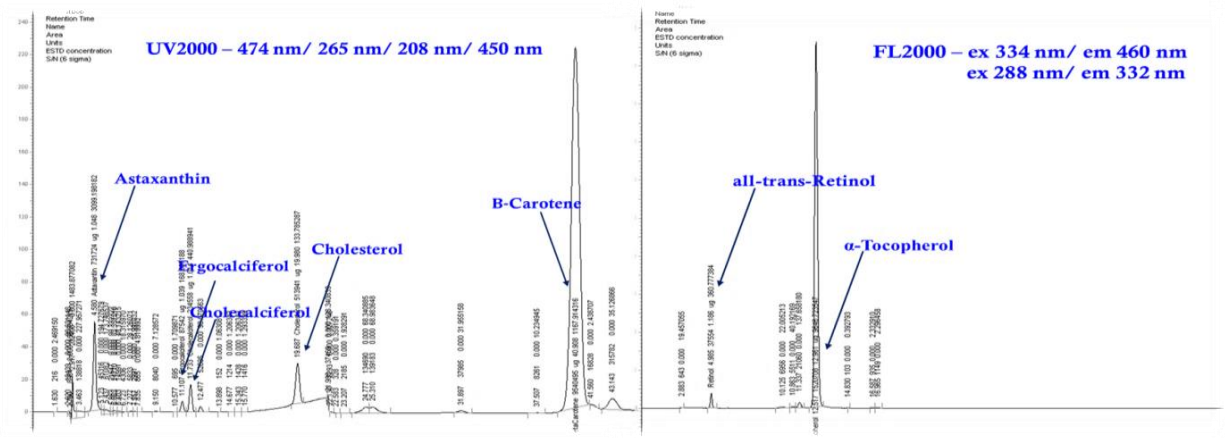

Fig. 1. HPLC chromatograms of standard solution

The received equations were: y = 2.74 ^{e005} µg for retinol; y = 1.01 ^{e005} µg for ergocalciferol; y = 3.67 ^{e006} µg for cholecalciferol, y = 3.29 ^{e005} µg for cholesterol, y = 4.27 ^{e006} µg for β-carotene, y = 1.20 ^{e006} µg for astaxanthin, and y = 7.73 ^{e006} µg for α-tocopherol. Correlation coefficients ranged from 0.9804 to 0.9983 (n = 3).

Precision

Six replicate determinations of each algae and mussel tissue sample were performed under optimum conditions to determine repeatability. The relative standard deviations (RSDs) were ≤10%. Recovery rates were determined by comparing the observed concentration with a spiked concentration. After hydrolysis, consequent extraction and chromatographic separation, analytical recovery exceeded 70% for astaxanthin and all-trans retinol, 80 % for β-carotene and cholecalciferol, 90% for ergocalciferol and α-tocopherol, and 102% for cholesterol.

Verification

Five seaweed and two mussel samples were analyzed to assess practical applicability of the method (figure 2, 3). Chromatograms from UV and FL detectors of the green algae (*Ulva rigida)* sample are presented on figure 2. Peaks of cholecalciferol and all-trans-retinol are missing.

Figure 3 shows the UV and FL chromatograms of a wild mussel extract. In contrast to figure 2, peaks for all analytes are present. This is due to sample composition – edible tissue included meat and stomach content (algae).

There are many studies about optimum saponification conditions for the extraction of lipid-soluble biologically active components from different matrices [10, 11, 12, 13]. The large scale extraction methods are expensive in terms of apparatuses required, cost of solvents, and time.

Therefore, we decided to develop a few steps simple and rapid method for simultaneous determination of fat soluble vitamins (all-trans retinol, cholecalciferol, ergocalciferol and α-tocopherol), carotenoids (β-carotene and astaxanthin) and cholesterol in seaweeds and mussel tissue.

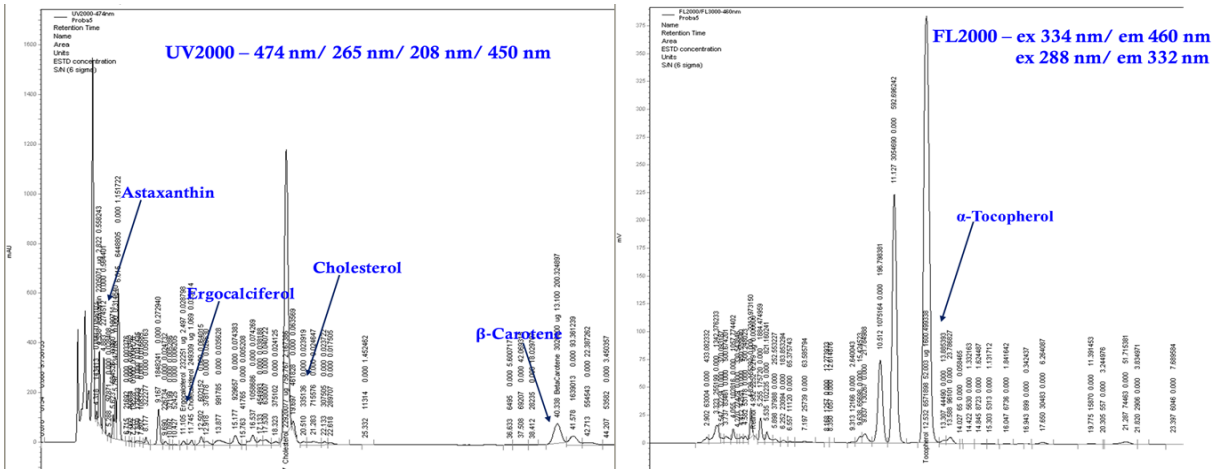

Fig. 2. HPLC chromatograms of *Ulva rigida* sample

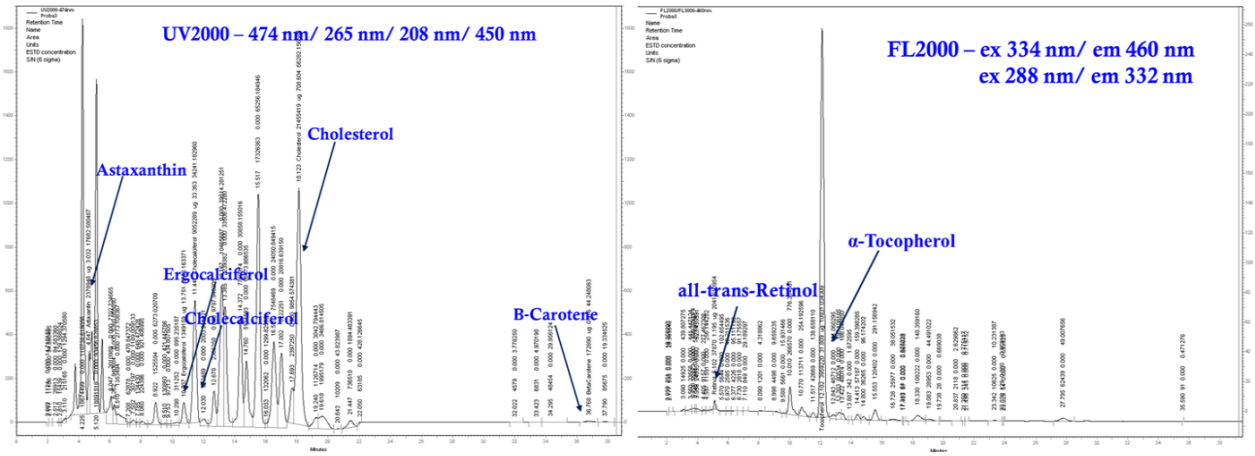

Fig. 3. HPLC chromatograms of wild mussel sample

The method was similar to those described by Dobreva *et al*., 2011 [9]. Generally smaller amounts of sample (1.0 g) and optimal hydrolysis conditions (0.5 M KOH/CH_3OH) were used. Preservation of the analytes from oxidation was assured by adding 1% methanolic ascorbic acid.

To achieve better resolution of the fat soluble components, several compositions of the mobile phases were tested: 100% methanol; 40/60 acetonitrile/methanol (v/v); 97/3 methanol/water (v/v) and 75:20:5 = ACN:MeOH:iPrOH (v/v/v). Best resolution between vitamins D_2 and D_3, and between β-carotene and α-carotene (which are very similar structurally) was achieved when using the mobile phase ACN:MeOH:iPrOH in gradient. The standard curves in mentioned concentration ranges (in accordance with the published data) were linear with coefficients of variation greater than 0.9907 with the exception of β-carotene. The precision of the method was evaluated – it comprised of repeatability and recovery rates. Six replicate determinations of each sample were performed under the described conditions to determine repeatability. RSDs of the repeatability and the estimated recovery indicated that the method can be used for quantitative determination of the above mentioned lipid soluble analytes in seaweed and mussel tissue matrices. The amounts of the seven analyzed compounds are presented in table 1 as miligram per 100 grams wet weight ($mg.100g^{-1}ww$). The results are expressed as average and standard deviation (mean ± SD). They are corrected against the calculated yield of each analyte.

Fat soluble analytes' quantity in edible tissue of wild and aquaculture mussel are closed. Present results are comparable to data published by other authors [1, 2, 4, 5, 6, 8]. Astaxanthin, vitamin D_2 and cholesterol contents in the wild sample were found slightly higher than in farmed one. On the other hand aquaculture species exhibited higher amounts of vitamin A, vitamin E and β-carotene, but results were similar too. These results are in good agreement with those presented by other scientific groups. In comparison to our data the MacDonald (2010) reported lower amounts – 0.039 $mg.100g^{-1}ww$ for vitamin A, 0.740 $mg.100g^{-1}ww$ for vitamin E and 29 $mg.100g^{-1}ww$ cholesterol in raw edible tissue of green shell mussel [14]. Other results confirming data in table 1 are presented by Danish Food Composition Databank (2009) and Öhrvik *et al*. (2012) – 0.08 $mg.100g^{-1}ww$ and 0.067 $mg.100g^{-1}ww$ for vitamin A, and 3.5 $mg.100g^{-1}ww$ and 3.07 $mg.100g^{-1}ww$ for vitamin E, respectively [15, 16]. Pospelova and Nehoroshev analyzed β-carotene content of wild Black mussel harvested in the Black Sea near Sevastopol, Ukraine [17,18] and found about 0.5 $mg.100g^{-1}ww$, which is in the same range as our results.

The results for the seaweed samples varied from species to species (table 1). The green and red algae present lower amount of vitamin D_2 and astaxanthin, compared to brown seaweed species. On the other hand, both brown seaweeds arecharacterized by high vitamin E concentrations. Algae samples, especially red species, contain very high levels of β-carotene and cholesterol. β-carotene amounts exceeded those found in fresh carrot (0.088 mg/g), which is known as excellent source of this nutrient [19]. Astaxanthin was also detected in high contents (1.39 $mg.100g^{-1}$ ww) in *Cystoseira barbata* – brown algal sample. This seaweed sample is also rich in α-tocopherol – 12.36 $mg.100g^{-1}$ ww. The high concentration of this form of vitamin E is in accordance with other published results [20], where brown algae Cystoseira spp. are characterized by higher amount compared to other samples. Comparing the results in table 1 with literature data, is obvious their good accordance.

Table 1. Analyte's contents in raw mussel and seaweed tissue ($mg.100g^{-1}$ ww)

Analyte	Mussel		Seaweed				
	Aquaculture	Wild	Ulva rigida (green)	Chaetomorpha linum (green)	Gelidium crinale (red)	Cystoseira barbata (brown)	Cystoseira crinita (brown)
Vitamin A	0.309±0.018	0.167±0.017	-	-	-	-	-
Vitamin D_2	0.035±0.002	0.071±0.009	0.031±0.01	0.025±0.007	0.006±0.002	0.12±0.03	0.16±0.05
Vitamin D_3	0.024±0.002	0.031±0.001	-	-	-	-	-
Vitamin E	4.70±0.16	2.69±0.19	1.223±0.03	1.14±0.7	1.53±0.09	3.13±0.15	12.36±0.22
Astaxanthin	0.211±0.036	0.62±0.03	0.110±0.01	0.015±0.001	0.20±0.01	0.30±0.03	1.39±0.12
β-carotene	0.232±0.017	0.066±0.009	1.702±0.04	0.017±0.003	3.38±0.09	5.57±0.20	1.88±1.31
Cholesterol	60.050±0.918	70.81±0.811	3.911±0.25	6.08±0.30	52.6±1.07	0.89±0.06	1.52±0.13

CONCLUSIONS

The method presented in this study has analytical characteristics that allow its use for quantitative simultaneous analyses of retinol, α-tocopherol, cholecalciferol, ergocalciferol, cholesterol, astaxanthin and β-carotene in mussel and seaweed matrices. The sample preparation, including extraction in a single reaction tube, characterizes the method with minimum manipulation. The method shows RSD below 10% for all analytes, good linearity for all components and short analysis time – less than 32 min.

The high concentrations of most investigated biologically active compounds (including antioxidants astaxanthin and β-carotene) indicate the possible application of Black Sea Black mussel and especially seaweeds as supplements in food and pharmaceutical industries.

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СЪВМЕСТНО ВЕТХ ОПРЕДЕЛЯНЕ НА МАСТНОРАЗТВОРИМИ ВИТАМИНИ, КАРОТЕНОИДИ И ХОЛЕСТЕРОЛ В ТЪКАН НА ВОДОРАСЛИ И МИДИ

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(Резюме)

Целта на настоящото изследване бе да се разработи хроматографски метод за едновременно количествено определяне на мастноразтворими витамини (ретинол, холекалциферол, ергокалциферол и α-токоферол), каротеноиди (β-каротен и астаксантин) и холестерол в тъкан на водорасли и миди. Високоефективната течно-хроматографска система с UV и FL детектори е метод, характеризиращ се с бързина, чувствителнност и точност при откриването на всички компоненти. Екстракционната процедура се провежда с минимални количества на пробите. Пробоподготовката включва осапунване и течно-течна екстракция на аналитите. Точността на метода (относително стандартно отклонение) е под 10% за всички изследвани вещества. Методът се характеризира с добра линейност при всички изследвани компоненти и време за анализ - до 32 мин. Методът бе приложен при проби от тъкани на водорасли и миди, като получените резултати за изследваните мастноразтворими аналити са близки до данни, публикувани от други автори.

Ключови думи: *ретинол, калциферол, α-токоферол, астаксантин, холестерол*