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ANALYSIS

APPLICATION OF TLC METHOD WITH VIDEO SCANNING IN ESTIMATION OF DAILY DIETARY INTAKE OF SPECIFIC FLAVONOIDS – PRELIMINARY STUDIES

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Abstract: Flavonoids, substances present in foods of plant origin, play an important role in many metabolic processes. Numerous properties of these substances were described, including their anti-allergic, antitumor and antioxidant properties. Therefore, an increased intake of these nutrients may play a beneficial role in human health. The aim of the presented study was to estimate the daily intake of specific flavonoid compounds using thin layer chromatography (TLC) combined with densitometric qualitative and quantitative analysis. Performed investigations revealed the presence of two flavonoids in the extracts from daily food rations – naringenin and hesperidin. Naringenin content in the daily food ration of women was calculated to be 179-537 mg, whereas in the group of men it ranged around 181-550 mg, depending on the conducted method of extraction and solvent system used. Daily dietary intake of hesperidin was calculated to be 193-534 mg in the group of women and 194-562 mg in the group of men. The highest degree of extraction of these flavonoids was obtained for the mixture of acetone and water (7 : 3, v/v) by means of accelerated solvent extraction (ASE).

Keywords: naringenin, hesperidin, diets, TLC

Nutrition studies which take into account the intake of specific nutrients are designed to show and correct abnormalities in human nutrition. One of the aims of such investigations is to pay particular attention to increasing or reducing intake of specific groups of foods which are sources of selected nutrients. The vast majority of these studies is concerned on the intake of building, energetic or regulatory components, such as macro- and microelements and vitamins. However, in recent years, the new light was shed on the intake of polyphenols, substances present in the plant originated foods (1). Numerous properties of these substances were described, including their anti-allergic, antitumor and antioxidant properties. Therefore, it is believed that the increased intake of substances with antioxidant properties may result in the prevention of diseases in which free radicals play the direct or indirect role. Many studies indicate the positive role of polyphenols in the prevention and even treatment of cardiovascular and eye diseases, as well as in the therapy of AIDS (2-5). One of the main, yet also the most structurally different group of exogenous antioxidants consists of natural polyphenolic compounds, among which the largest one is the group of flavonoids.

Separation of extracts obtained from daily food rations of students using thin layer chromatography (TLC) combined with densitometric qualitative and quantitative analysis was the main purpose of that study. Obtained chromatographic results after appropriate conversion allowed to estimate daily intake of specific flavonoid compounds.

EXPERIMENTAL

Chemicals and reagents

The standards of naringenin and hesperidin were purchased from Sigma Aldrich (St. Louis, MO, USA). Acetone, methanol and dichloromethane (reagent grade) came from Polish Reagents (POCh, Gliwice). Ultrasound bath SONOREX DIGITAL 10P, BANDELIN and Dionex ASE 100 apparatus were used to prepare the extracts. Videoscanner

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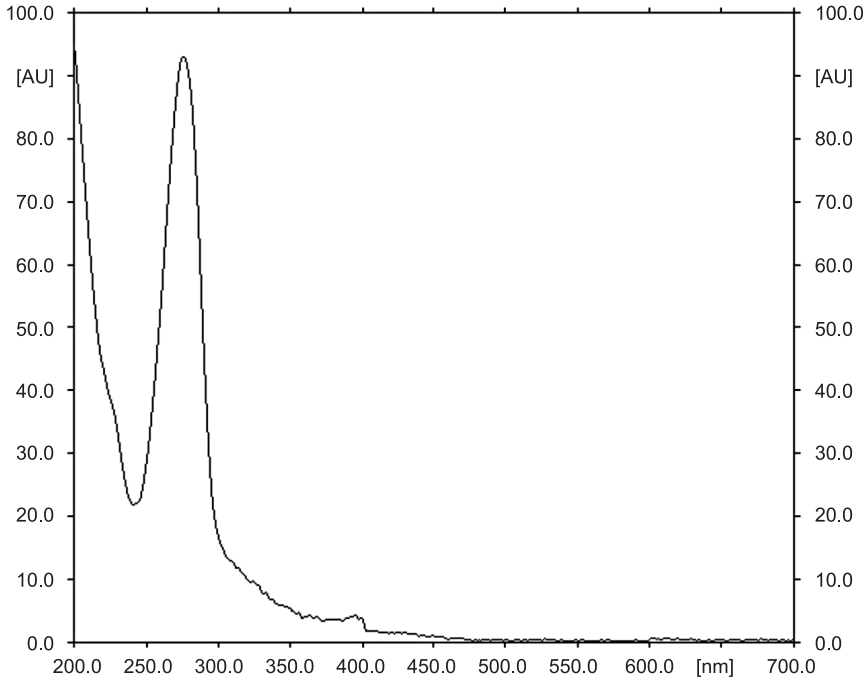

Figure 1. UV spectrum of the standard of naringenin at λ = 200-700 nm

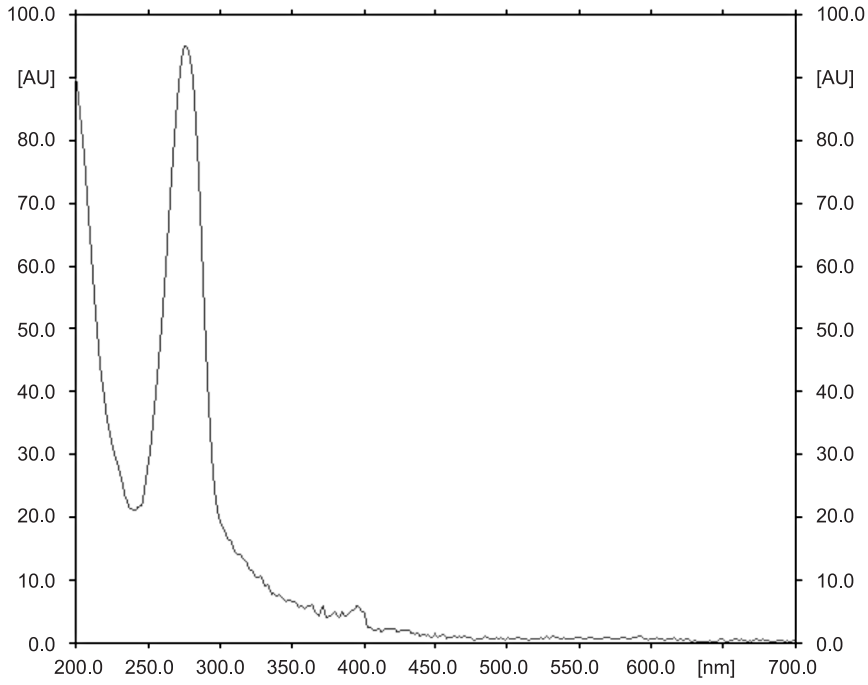

Figure 2. UV spectrum of the standard of hesperidin at λ = 200-700 nm

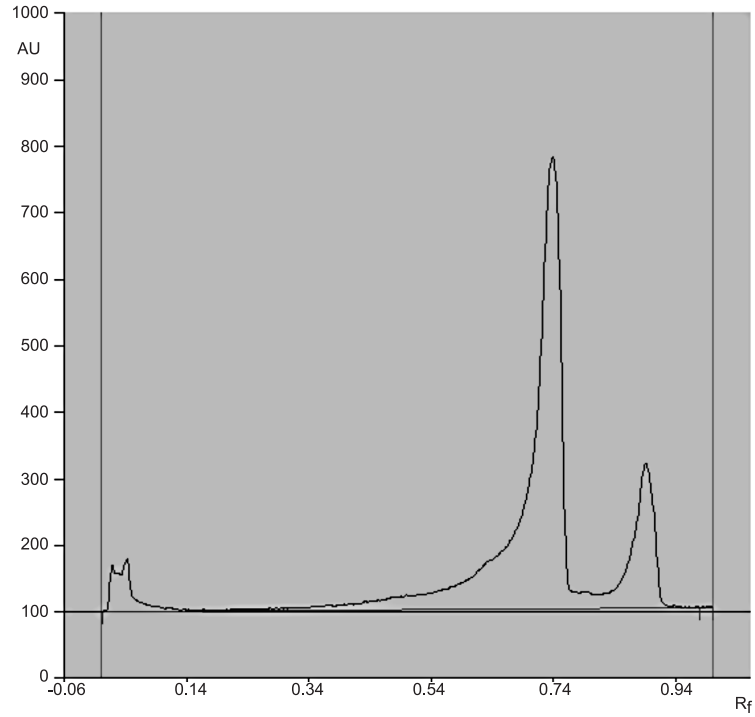

Figure 3. Densitogram of naringenin obtained using videoscanner Camag. R_f value = 0.74

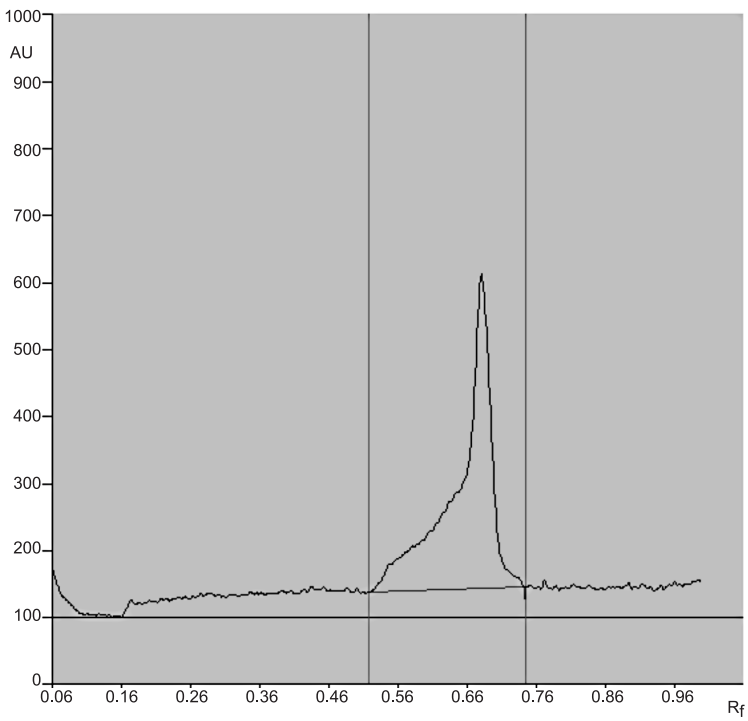

Figure 4. Densitogram of hesperidin obtained using videoscanner Camag. R_f value = 0.70

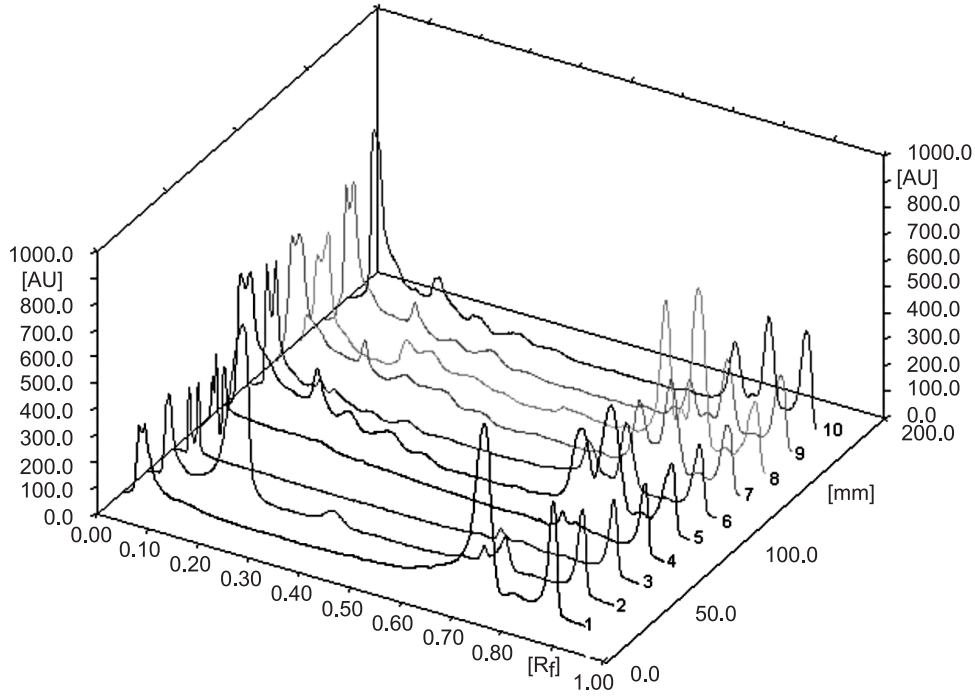

Figure 5. Densitogram of the TLC plate no. 1

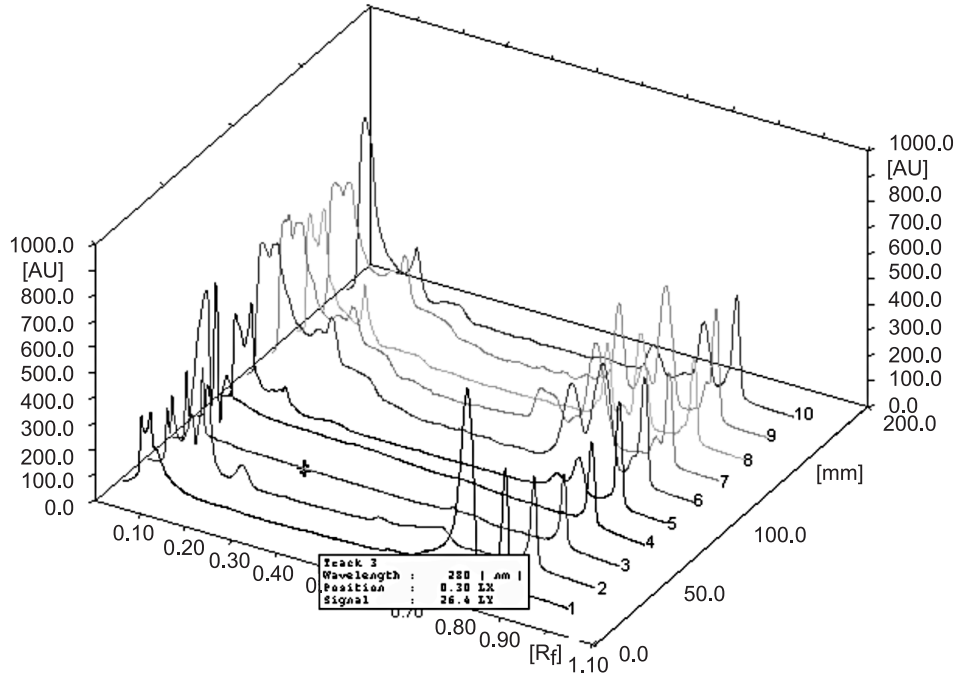

Figure 6. Densitogram of the TLC plate no. 2

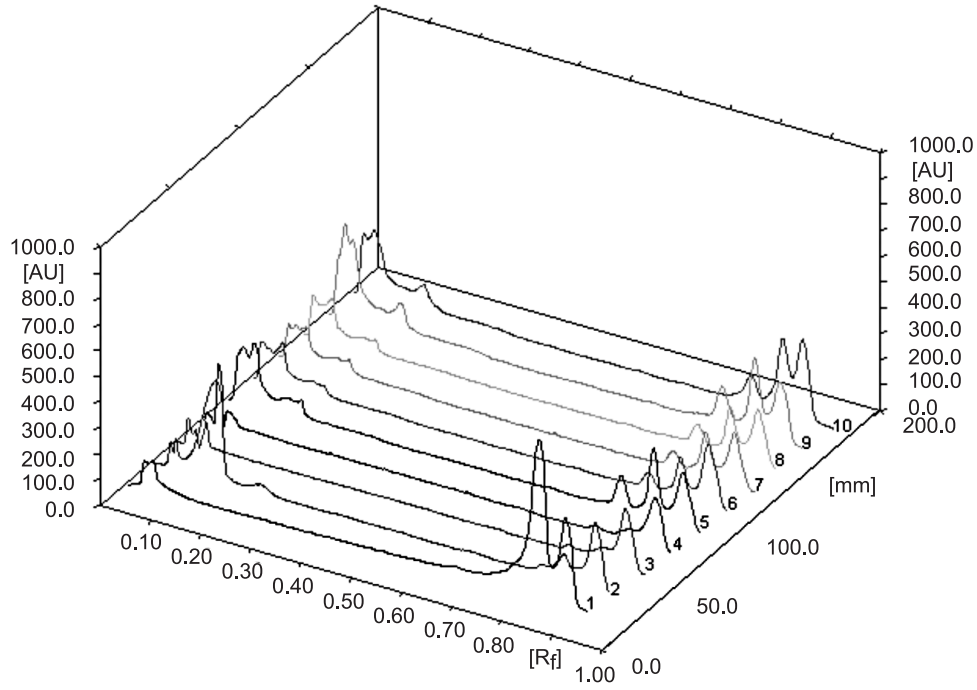

Figure 7. Densitogram of the TLC plate no. 3

CAMAG TLC SCANNER 3 with winCATS software version 1.4.4.6337 was applied in the qualitative and quantitative analysis of the extracts.

Investigated material

The study was performed in 2012 and involved 162 randomly chosen students, 102 women and 60 men, from Medical University in Lublin. All students were volunteers; their lifestyles were characterized by moderate physical activity. The investigations were carried out using 24-hour dietary recall technique. On the basis of the information concerning qualitative and quantitative parameters of diets provided by students and using Dietetyk 2006 software, average diets for both, women and men, were reconstructed. All the products used to prepare food rations were from the retail market of the Lublin region. The daily rations included the plate portions of main meals identical with the ones consumed by a particular individual and all other foodstuffs and beverages consumed daily. Diets duplicates were prepared according to generally accepted culinary techniques. Each average diet duplicate was homogenized and subsequently extracted.

Preparation of extracts

Maceration

Three 75 g portions were weighted from each average daily diet into conical flasks of 300 mL volume and covered with a portion of 75 mL of methanol, methanol-water mixture (1 : 1, v/v) and acetone-water mixture (7 : 3, v/v). The mixture was extracted with shaking for 48 h, the solution was filtered and the residue poured with another portion of the fresh extractant. This action was repeated 3 times. The combined filtrates were evaporated under reduced pressure at a temperature not exceeding 40^{o}C using a rotary evaporator. The dry residue was redissolved in 100 mL of methanol-water (1 : 1, v / v).

Ultrasound-assisted maceration

Three 10 g portions were weighted from each average daily diet into conical flasks of 300 mL volume and covered with a portion of 20 mL of methanol, methanol-water mixture (1 : 1, v/v) and acetone-water mixture (7 : 3, v/v). The mixtures were placed in an ultrasonic bath in 40^{o}C and then extracted for 45 min. Then, the extracts were evaporated with a rotary evaporator. The dry residue was

Table 1. The characteristic of all paths on the TLC plate No. 1.

Path no.	Applied extract	Aplication volume [µL]
1	Diosmetin	20
2	Naringenin	25
3	Diosmin	40
4	Hesperidin	40
5	Women, maceration, acetone/water	15
6	Women, ASE, acetone/water	27
7	Men, maceration, methanol/water	15
8	Women, maceration, methanol/water	20
9	Men, ultrasound maceration, methanol/water	25
10	Women, ultrasound maceration, methanol	20

Table 2. The characteristic of all paths on the TLC plate No. 2.

Path no.	Applied extract	Aplication volume [µL]
1	Diosmetin	25
2	Naringenin	25
3	Diosmin	50
4	Hesperidin	30
5	Men, ASE, acetone/water	25
6	Women, maceration, methanol	25
7	Men, maceration, acetone/water	25
8	Women, ultrasound maceration, methanol/water	25
9	Men, maceration, methanol	25
10	Men, ultrasound maceration, acetone/water	25

Table 3. The characteristic of all paths on the TLC plate No. 3.

Path no.	Applied extract	Aplication volume [µL]
1	Diosmetin	25
2	Naringenin	25
3	Diosmin	50
4	Hesperidin	30
5	Women, ASE, methanol/water	25
6	Men, ASE, methanol/water	25
7	Men, ASE, methanol	25
8	Women, ASE, methanol	25
9	Women, ultrasound maceration, acetone/water	25
10	Men, ultrasound maceration, methanol	25

Table 4. Naringenin and hesperidin content in daily food ration of men extracted by maceration with solvent system considered.

SOLVENT SYSTEM	Sample no.	NARINGENIN [mg]	HESPERIDIN [mg]
METHANOL	1	210	228
	2	220	230
	3	215	230
	AVERAGE	215	229
METHANOL/WATER (1 : 1, v/v)	1	201	220
	2	219	228
	3	211	230
	AVERAGE	210	226
ACETONE/WATER (7 : 3, v/v)	1	180	193
	2	181	204
	3	191	197
	AVERAGE	184	198

Table 5. Naringenin and hesperidin content in daily food ration of women extracted by maceration with solvent system considered.

SOLVENT SYSTEM	Sample no.	NARINGENIN [mg]	HESPERIDIN [mg]
METHANOL	1	175	199
	2	178	190
	3	184	190
	AVERAGE	179	193
METHANOL/WATER (1 : 1, v/v)	1	297	315
	2	309	323
	3	304	338
	AVERAGE	303	325
ACETONE/WATER (7 : 3, v/v)	1	243	263
	2	296	281
	3	257	273
	AVERAGE	265	272

Table 6. Naringenin and hesperidin content in daily food ration of men extracted by ultrasound-assisted maceration with solvent system considered.

SOLVENT SYSTEM	Sample no.	NARINGENIN [mg]	HESPERIDIN [mg]
METHANOL	1	240	263
	2	241	264
	3	248	259
	AVERAGE	243	262
METHANOL/WATER (1 : 1, v/v)	1	282	283
	2	270	293
	3	264	309
	AVERAGE	272	295
ACETONE/WATER (7 : 3, v/v)	1	362	390
	2	373	393
	3	364	399
	AVERAGE	366	394

Table 7. Naringenin and hesperidin content in daily food ration of women extracted by ultrasound-assisted maceration with solvent system considered.

SOLVENT SYSTEM	Sample no.	NARINGENIN [mg]	HESPERIDIN [mg]
METHANOL	1	530	582
	2	542	584
	3	538	568
	AVERAGE	537	578
METHANOL/WATER (1 : 1, v/v)	1	438	472
	2	442	477
	3	454	484
	AVERAGE	445	478
ACETONE/WATER (7 : 3, v/v)	1	229	256
	2	250	265
	3	251	274
	AVERAGE	243	265

Table 8. Naringenin and hesperidin content in daily food ration of men extracted by accelerated solvent extraction (ASE) with solvent system considered.

SOLVENT SYSTEM	Sample no.	NARINGENIN [mg]	HESPERIDIN [mg]
METHANOL	1	177	196
	2	182	192
	3	184	194
	AVERAGE	181	194
METHANOL/WATER (1 : 1, v/v)	1	343	370
	2	342	379
	3	355	376
	AVERAGE	347	375
ACETONE/WATER (7 : 3, v/v)	1	572	511
	2	522	580
	3	557	595
	AVERAGE	550	562

dissolved in 50 mL of methanol-water (1 : 1, v/v). The obtained extracts were subjected to further analysis. The parameters of extraction did not cause degradation of the flavonoid compounds.

Accelerated solvent extraction (ASE)

To extract the investigated diets by ASE method, part of the homogenized daily food ration was evaporated to dryness under reduced pressure using a rotary evaporator. From the obtained dry residue of average diets three 1.0 g samples were collected and extracted using Dionex ASE 100 apparatus, using three extraction systems. Three solvents systems were used: methanol, methanol-water (1 : 1, v/v) and a mixture of acetone-water (7 : 3, v/v). The extractions were carried out at 75^{o}C and a pressure of 90 bar. The elution volume was set at 60% of the cell's volume, static time was set at 5 min and flush time at 100 s. Cellulose filters used were Dionex Corporation 30 mm.

Separation of extracts using TLC method

One-way thin layer chromatography was used to separate and determine the quantity of specific flavonoid compounds in the obtained extracts. Extracts obtained from diets together with selected standards of flavonoids were applied on the silica gel coated aluminum plates (NP, 60 F254, Merck,

Table 9. Naringenin and hesperidin content in daily food ration of women extracted by accelerated solvent extraction (ASE) with solvent system considered.

SOLVENT SYSTEM	Sample no.	NARINGENIN [mg]	HESPERIDIN [mg]
METHANOL	1	276	298
	2	281	313
	3	287	304
	AVERAGE	281	305
METHANOL/WATER (1 : 1, v/v)	1	429	478
	2	435	475
	3	447	465
	AVERAGE	437	473
ACETONE/WATER (7 : 3, v/v)	1	490	528
	2	491	540
	3	498	533
	AVERAGE	493	534

Darmstadt) using an autosampler (Camag TLC Automatic Sampler 3). Application distance from the X-axis was set at 18 mm and from the Y-axis – at 15 mm, while the distance between tracks was set at 18 mm. The study was conducted at a wavelength of 280 nm. Densitometer slit dimensions were set at 6.00 × 0.20 mm. After application of the samples, the plates were pre-conditioned with solvent: methanol-methylene chloride 15 : 85 (v/v) and then developed in a vertical glass chamber. The mobile phase was a mixture of methanol-methylene chloride 15 : 85 (v/v). After the plates were developed and the mobile phase evaporated to dryness, the obtained chromatograms were observed under UV light at a wavelength $\lambda = 365$ nm and $\lambda = 254$ nm. The plates were then scanned using a videoscanner Camag TLC Scanner 3. The basis for the identification of flavonoids was the comparison of Rf-values and UV spectra of individual spots with those of corresponding flavonoids' standards.

The following standards were used in the study: diosmetin, diosmin, naringenin, hesperidin. The applied denstitometric analysis confirmed the presence of two flavonoid compounds in the daily food ration samples – naringenin and hesperidin. The UV spectra of naringenin and hesperidin are presented in Figures 1 and 2. On Figures 3 and 4 the densitograms of standards are presented.

The scans of the obtained TLC plates with the applied standards and prepared extracts are presented in Figure 5. The detailed characteristic of all paths on the described TLC plates are presented in Tables 1, 2 and 3.

RESULTS

Taking into account the peak area of standards and tested extracts, as well as their application volume, the content of specific flavonoid compounds in obtained extracts was determined and then calculated to dietary intake with daily food rations. The obtained results showing the applied extraction methods and chosen solvent systems are presented in Tables 4-9.

DISCUSSION AND CONCLUSION

Separation of the received extracts by thin layer chromatography (TLC) and densitometic analysis of obtained chromatograms resulted in the identification of two flavonoids – naringenin and hesperidin in the extracts coming from reconstituted diets. The presence of diosmetin and diosmin in the extracts from daily food rations was not confirmed. It should be emphasized that this does not mean that the above-mentioned flavonoids were not present in the diets. Perhaps the methods of extraction, separation or analysis of the results were not sensitive enough or require refinement.

Daily dietary intake of naringenin determined and calculated with the described method of extraction and solvent system ranged around 179-537 mg in the group of women and 181-550 mg in the group of men whereas the intake of hesperidin was 193-534 mg and 194-562 mg in the group of women and men, respectively. Such a large discrepancy of the

results was probably associated with the use of different techniques of extraction and elution systems. However, it appears that the dietary intake of naringenin and hesperidin, determined especially in the extracts obtained by ASE is slightly overestimated. The results from multiple maceration resembled the other studies performed on similar material [1, 6]. The outcomes of ASE extraction need further optimization and confirmation of this high content of flavonoids in the extracts. Furthermore, it is worth consideration to introduce additional purification steps for the crude extract before its application on TLC plate. It could allow to avoid the process of peak overlapping and eventual increase in the peak areas.

The most effective method of recovery of the substances from the diets was accelerated solvent extraction (ASE). However, it is worth noting that the very great importance of this method was dependent on the applied solvent system. Definitely, the most effective mixture of solvents was 70% aqueous solution of acetone. However, the usage of pure methanol allowed to obtain recovery comparable with those obtained by simple multiple maceration. In the case of the latter method of extraction the results are inconclusive - the highest recovery of the flavonoid compounds from the men's diet was obtained using pure methanol, whereas from the women's diet with a mixture of methanol and water (1 : 1, v/v).

The ultrasound-assisted maceration was proved to be a very effective technique as well. However, also in this case, selection of the best solvent system was not easy, and the results were inconclusive. The highest degree of extraction of both naringenin and hesperidin in the diet of men was obtained using a mixture of acetone and water (7 : 3, v/v), while in the diet of women using pure methanol.

In general, presented studies shed new light on the application of TLC method coupled with densitometric scanning to the analytical determination of dietary intake of specific flavonoids. According to the obtained results the developed method may be an alternative to HPLC chromatography, which enables the qualitative and quantitative determination of flavonoids content in daily food rations. It was confirmed that both the extraction technique and the solvent system used had a significant influence on the evaluation of flavonoid intake with daily food rations. However, it should be emphasized that performed investigations have a character of preliminary studies and have to be elaborated in the future.

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