

Rutin Transformation During Its Analysis Involving Extraction Process for Sample Preparation

Andrzej L. Dawidowicz¹ · Katarzyna Bernacik¹ · Rafal Typek¹

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Abstract Rutin (quercetin-3-*O*-rutinoside) is one of the most popular phenolic compounds found in various plant species. Its miscellaneous biological activity and, especially, the ability to inhibit blood plates aggregation (which makes blood thinner and improves circulation in the blood circulation system), causes permanent interest in its properties and natural occurrence. Liquid-solid extraction is the most popular method for rutin isolation from plants. We found that at least 23 compounds (rutin transformation and degradation products and their methyl derivatives) were formed from rutin during its simulated and real extraction from elderberry flower by methanol and methanol/water mixtures. Twelve of them have not been reported yet. We observed that the amount of each formed compound depends not only on the extraction time, alcohol concentration and extractant pH but also on the components of plant matrix from which rutin was extracted.

Keywords Rutin · Rutoside · Rutin transformation · Rutin degradation · Rutin stability · Extraction

Introduction

Rutin, also called rutoside or sophorin, is one of the most popular phenolic compounds found in various plant species. Its name comes from *Ruta graveolens* L., one of the main natural sources of this compound (Kostova et al. 1999).

Structurally, rutoside (quercetin-3-*O*-rutinoside) is a glycoside between flavonol quercetin and disaccharide rutinose (Wang et al. 2011). It is known as an aggregation inhibitor of blood plates making the blood thinner and improving circulation in the blood circulation system (Navarro-Núñez et al. 2008). Furthermore, it exhibits a few biological activities such as strong antioxidant, antibacterial, anti-inflammatory and antiviral properties, and therefore, it is widely used as an ingredient of numerous medications as well as dietary supplements (Biesaga 2011; Yang et al. 2008; You et al. 2010). The studies have also confirmed that rutin can inhibit some cancerous and pre-cancerous conditions (Mohd Zainol et al. 2009; Wach et al. 2007). The current knowledge about a broad range of beneficial biological properties of rutin (Yang et al. 2008) is the reason for more detailed investigations of its properties and its effective isolation methods from plant material for preparative and analytical purposes (Paniwnyk et al. 2001).

Liquid-solid extraction is the most popular method of plant component isolation. Its efficiency depends on such parameters as the type and pH of the applied extractant, as well as the temperature and time of the extraction process. It is also well known, however, that the isolated plant components may undergo various transformations during this process (Dawidowicz and Typek 2010, 2011, 2012; Wianowska 2014). The transformations constitute an essential problem as they may lead to misleading conclusions about the native composition of plant material and to the decrease of the yields of plant component on the preparative/industrial scale. In most cases, the probability of plant component transformations in the extraction process is greater for substances with a complex structure. This fact is confirmed by the recently published results concerning chlorogenic acid isomerization and transformation during its extraction from plants (Dawidowicz and Typek 2010, 2011, 2012). As the molecular structure of rutin is also complex, it was decided to determine the

✉ Andrzej L. Dawidowicz
dawid@poczta.umcs.lublin.pl

¹ Faculty of Chemistry, Maria Curie Skłodowska University, Pl. Maria Curie Skłodowska 3, 20-031 Lublin, Poland

transformation probability of this compound during the extraction process from plant materials.

Methanol and methanol-water mixtures are most frequently applied as extrahents for the isolation of rutin from plants. Hence, in the present study, we investigate rutin transformation processes during its

- Heating with methanol and methanol/water mixtures under reflux (simulated extraction under reflux) and
- Extraction from elderberry flower with methanol and methanol/water mixtures by heating the plant with the extractant under reflux (classical extraction under reflux)

The impact of these extraction parameters, i.e. temperature, time and pH, on the rutin transformation processes in the above-mentioned systems is examined.

Materials and Methods

Materials

The flowers of *Sambucus nigra* L. (elderberry) used in the experiments came from the eastern part of Poland. Large clusters of these small cream-colored flowers were cut, air-dried and separated from the branches. Before extraction, the plant material was ground and its exactly weighed portions were subjected to the extraction procedure.

Acetonitrile (HPLC), methanol, sodium phosphate and phosphoric acid were purchased from the Polish Chemical Plant POCh (Gliwice, Poland), formic acid—from Sigma-Aldrich (Seelze, Germany) and rutin—from Brenntag (Germany). Water was purified on the Milli-Q system from Millipore (Millipore, Bedford, MA, USA).

Sample Preparation

The investigations of the rutin transformation process were performed on two different systems:

- By heating under reflux its solutions in methanol, methanol/water and methanol/buffer mixtures and
- By rutin extraction from the elderberry flower by heating this plant material under reflux in the same solvents

The methanol/water solutions of rutin contained 50 or 75 % v/v of alcohol, whereas alcohol concentration in its methanol/buffer solutions (pH 4.5, 6.5 or 8.5) was 75 % v/v. The same methanol concentrations in methanol/water and methanol/buffer (pH 4.5, 6.5 or 8.5) solutions were applied to rutin extraction from the plant. Phosphate buffers were used in the experiments. The glass equipment for the experiments consisted of a boiling flask (100 mL) and a small condenser.

The heated rutin solutions contained 10 mg of the compound in 50 mL of a given solvent. To obtain a similar rutin concentration in the elderberry flower suspension in 50 mL of a given solvent, 300 mg of the plant material was used. Individual solutions and plant suspensions were heated for 10 min or 1, 3 or 5 h. Subsequently, each obtained solvent and supernatant isolated by centrifuging the plant suspension were subjected to LC-MS-PDA analysis.

HPLC Measurements

The chromatographic measurements were performed using LC/MS from Finnigan (LCQ Advantage Max) equipped with the ion-trap mass spectrometric system (ThermoElectron Corporation, San Jose, CA) and a diode array detector from Finnigan (Surveyor PDA Plus Detector). The Gemini C18 column (4.6×100 mm, 3 μm) (Phenomenex, USA) was employed for chromatographic separation, which was performed using gradient elution. Mobile phase A was 25 mM formic acid in water; mobile phase B was 25 mM formic acid in acetonitrile. The gradient program started at 5 % B, increasing to 35 % for 60 min, next 35 % B to 95 % B for 12 min, and ended with isocratic elution which followed (95 % B) for 3 min. The total run time was 75 min at the mobile phase flow rate 0.4 mL/min.

In the course of each run, PDA spectra in the range 190–600 nm and MS spectra in the range of 100–2000 *m/z* were collected continuously.

In all rutin solutions and elderberry flower extracts, the SIM function was used to better visualize the chromatographic separation and to remove the signal connected with rutin, plant matrix and buffer components. The time periods and monitored ions were as follows:

0–16.1 min (197 *m/z*), 16.1–17 min (211 *m/z*), 17–17.9 min (179 *m/z*), 17.9–18.5 min (305 *m/z*), 18.5–19.5 min (335 *m/z*), 19.5–20.5 min (193 *m/z*), 20.5–25 min (317 *m/z*), 25–28.5 min (331 *m/z*), 28.5–30.5 min (257 *m/z*), 30.5–32 min (319 *m/z*), 32–34 min (271 *m/z*), 34–41 min (349 *m/z*), 41–44 min (463 *m/z*), 44–45.9 min (623 *m/z*), 45.9–46.8 min (347 *m/z*), 46.8–47.2 min (447 *m/z*), 47.2–53 min (477 *m/z*), 53–59 min (461 *m/z*), 59–64 min (301 *m/z*), 64–66 min (273 *m/z*), 66–69 min (361 *m/z*), 69–71 min (315 *m/z*), 71–74 min (287 *m/z*)

The column effluent was ionized by electrospray (electrospray ionization, ESI). The ESI needle potential was 4.5 kV in the negative ionization mode. To identify rutin derivatives (its hydrolysis, transformation and oxidation products), the function of secondary (MS²) ion fragmentation was applied. The collision energy for each examined compound was chosen individually. For confirmation, HRMS analysis was additionally performed.

The HRMS analysis was carried out on the HPLC system coupled to a linear trap quadrupole-Orbitrap mass spectrometer (LTQ-Orbitrap Velos from Thermo Fisher Scientific, San Jose, CA) equipped with an ESI source. The ESI was operated in negative polarity modes under the following specific conditions: spray voltage—3.5 kV; sheath gas—40 arb. units; auxiliary gas—10 arb. units; sweep gas—10 arb. units; and capillary temperature—320 °C. Nitrogen (>99.98 %) was employed as sheath, auxiliary and sweep gas. The scan cycle used a full-scan event at the resolution of 60,000.

Due to the lack of standards of rutin derivatives, their amounts were estimated by relating their chromatographic responses to the calibration curve for rutin and quercetin. The calibration curve for rutin was used for estimating the amounts of the following glycoside rutin derivatives:

- 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxochromen-4-one (isoquercitrin)
- 2-(3,4-dihydroxyphenyl)-5-methoxy-7-hydroxy-3-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxochromen-4-one (methyl-isoquercitrin)
- 2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-3-[(2S,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyl-2-tetrahydropyranyl]oxy]-4-chromenone (quercitrin)
- 2-(3,4-Dihydroxyphenyl)-5-methoxy-7-hydroxy-3-[(2S,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyl-2-tetrahydropyranyl]oxy]-4-chromenone (methyl-quercitrin)
- 2-(3,4-dihydroxyphenyl)-5-methoxy-7-hydroxy-3-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-[(2R,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyl-2-yl]oxymethyl]oxan-2-yl]oxochromen-4-one (methyl-rutin)

The calibration curve for quercetin was applied for estimating the amounts of the following aglicon rutin derivatives:

- 2-(3,4-Dihydroxybenzoyl)-2,4,6-trihydroxy-1-benzofuran-3(2H)-one (DTB)
- 2-[(3,4-Dihydroxyphenyl)carbonyl]-4,6-dihydroxy-2-methoxy-1-benzofuran-3(2H)-one (methyl-DTB)
- Oxo(2,4,6-trihydroxyphenyl)acetic acid (OTA)
- Methyl oxo(2,4,6-trihydroxyphenyl)acetate (methyl-OTA)
- 2-[Carboxy(3,4-dihydroxyphenyl)methoxy]-4,6-dihydroxybenzoic acid (CDA)
- 2-[1-(3,4-Dihydroxyphenyl)-2-methoxy-2-oxoethoxy]-4,6-dihydroxybenzoic acid (methyl-CDA)
- 2-[(3,4-Dihydroxybenzoyl)oxy]-4,6-dihydroxybenzoic acid (DDA)
- Methyl 2-[(3,4-dihydroxybenzoyl)oxy]-4,6-dihydroxybenzoate (methyl-DDA)
- 4,6-Dihydroxy-1-benzofuran-2,3-dione (DBD)

- 6-Hydroxy-4-methoxy-1-benzofuran-2,3-dione (methyl-DBD)
- 7-(3,4-Dihydroxyphenyl)bicyclo[4.2.0]octa-1,3,5,7-tetraene-2,4,8-triol (DBOT)
- 7-(3,4-Dihydroxyphenyl)-8-methoxybicyclo[4.2.0]octa-1,3,5,7-tetraene-2,4-diol (methyl-DBOT)
- 3,5-Dihydroxy-2-[methoxy(oxo)acetyl]phenyl-3,4-dihydroxybenzoate (DPD)
- 5-Hydroxy-3-methoxy-2-[methoxy(oxo)acetyl]phenyl 3,4-dihydroxybenzoate (methyl-DPD)
- 2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one (quercetin)
- 2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-3-methoxy-4H-chromen-4-one (methyl-quercetin)
- 2-(3,4-Dihydroxyphenyl)-1-benzofuran-3,4,6-triol (DBT)
- 2-(3,4-Dihydroxyphenyl)-3-methoxy-1-benzofuran-4,6-diol (methyl-DBT)

Statistical Analysis

All the results are presented as the mean of three independent measurements ($n=3$). Differences in the concentration of the formed rutin derivatives were compared using the analysis of variance (ANOVA, $p=0.05$). Differences in the studied group were considered significant for p values lower than 0.05 and F values higher than 4.26. The analysis of variance revealed statistically significant differences only for a few tested groups.

Results and Discussion

Figure 1(A–C) presents the exemplary chromatogram of methanolic (A), methanol/water (B) and buffered methanol/water solution of rutin (C), all heated under reflux for 3 h, whereas Fig. 1(A'–C') shows the exemplary chromatograms of elderberry flower extracts prepared by 3 h of hot extraction process under reflux, applying, respectively, the same solvents as extractants.

The analysis of the chromatogram in Fig. 1(A) shows that the methanolic rutin solution heated under reflux contains, besides the parent substance, 23 additional compounds formed as a result of the rutin transformation and degradation process. All of them were identified and confirmed on the basis of the retention data of their standards, the PDA, MS², HRMS and literature data (Dubber et al. 2005; Wang et al. 2011; Zenkevich et al. 2007; Zvezdanović et al. 2012). The MS² and HRMS data for the identified compounds are collected in Tables 1 and 2, respectively. Chemical structures of all compounds are presented in Fig. 2. Some of them were described in the literature as products of rutin and/or quercetin degradation resulting from high-temperature treatment, UV

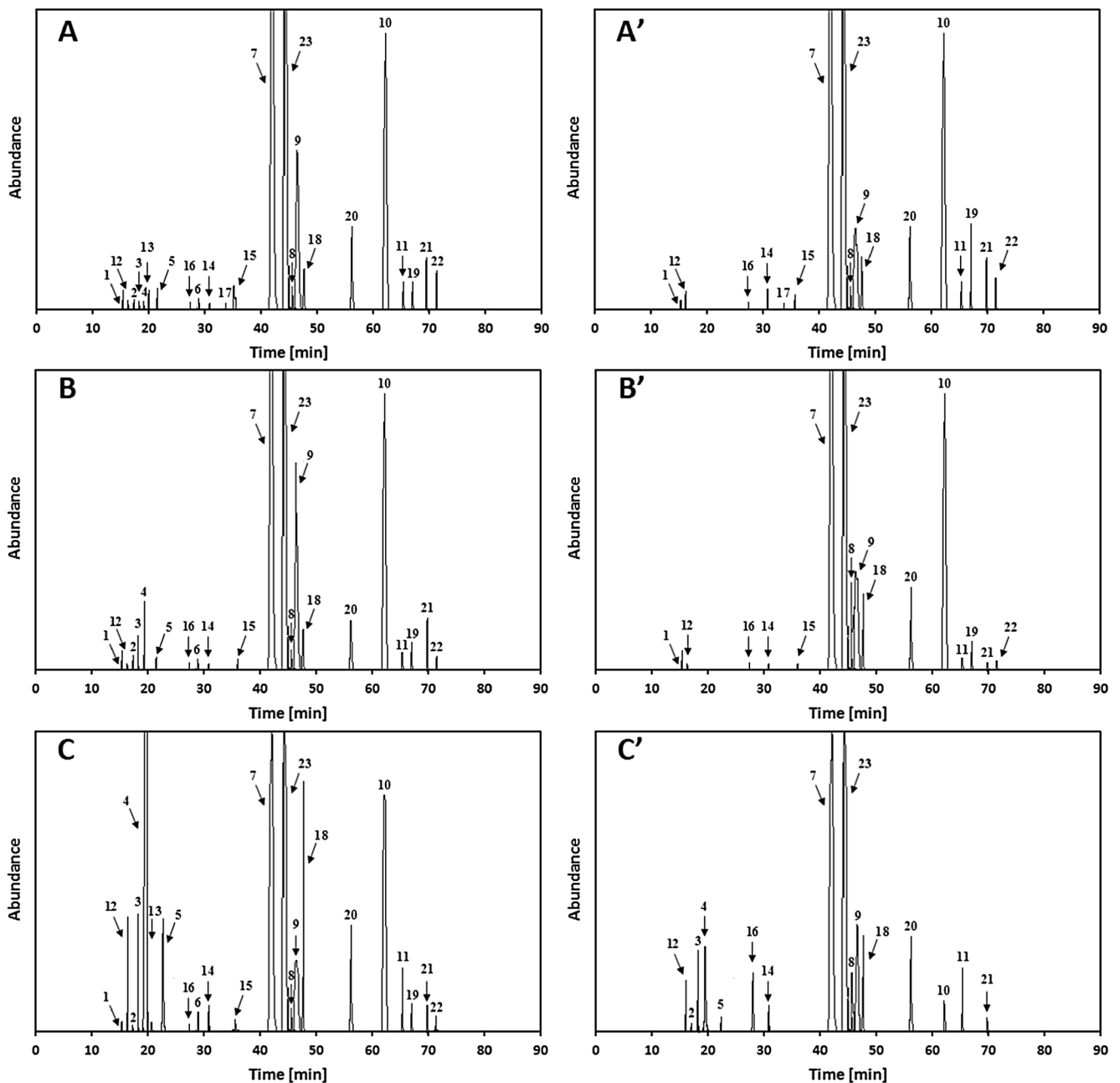


Fig. 1 Chromatograms of methanolic (A), methanol/water (75/25 v/v) (B) and methanol/phosphoric buffer (pH=6.5) (75/25 v/v) (C) solution of rutin and methanolic (A'), methanol/water (75/25 v/v) (B') and

methanol/phosphoric buffer (pH=6.5) (75/25 v/v) (C') extract of elderberry flower, all heated under reflux for 3 h. Peak numbers correspond to compound numbers reported in Fig. 2

irradiation or the influence of aggressive environments: DTB, OTA, CDA, DDA, DBD, DBOT, DPD, quercetin, DBT, isoquercitrin and quercitrin (Dubber et al. 2005; Rohn et al. 2007; Wang et al. 2011; Zenkevich et al. 2007; Zvezdanović et al. 2012). Yet our experiments have shown that these compounds are formed not only in aggressive conditions but also in the conditions typical for the extraction process. The remaining compounds (methyl-DTB, methyl-OTA, methyl-CDA, methyl-DDA, methyl-DBD, methyl-DBOT, methyl-DPD, methyl-quercetin, methyl-DBT, methyl-isoquercitrin,

methyl-quercitrin and methyl-rutin) have not been described in literature yet. As pure methanol was applied as a solvent, the presence of methyl derivatives among the rutin transformation products is not surprising. Their molecular weights are greater by 15 Da in relation to their precursors (DTB, OTA, CDA, DDA, DBD, DBOT, DPD, quercetin, DBT, isoquercitrin, quercitrin and rutin). This mass corresponds to the methyl substituent. The retention times of methyl derivatives are longer than in the case of their precursors because—in the RP system—blocking the polar hydroxyl group by alkyl

Table 1 Negative ion MSⁿ data for rutin transformation products

Peak number	MS ¹	MS ²			Compounds
	Base peak	Base peak	Secondary peak		
	<i>m/z</i>	<i>m/z</i>	<i>m/z</i>	Intensity (%)	
1	197.1	153.1	125.2	24.7	OTA
2	178.9	151.2	123.1	3.7	DBD
3	305.2	261.1	168.1	27.1	DDA
			196.2	12.9	
4	335.1	291.2	247.1	35.7	CDA
5	317.2	179.1	151.2	8.8	DTB
6	257.1	229.1	201.1	2.4	DBOT
			179.2	35.4	
7	463.2	301.2	255.1	23.9	Isoquercitrin
			283.1	2.9	
8	347.1	332.2	153.2	26.8	DPD
			178.1	13.9	
			179.1	31.1	
9	447.2	301.2	255.1	20.8	Quercitrin
			283.2	3.7	
10	301.2	255.1	227.1	27.2	Quercetin
			283.2	42.9	
11	273.2	229.2	–	–	DBT
12	211.1	196.2	153.2	25.7	Methyl-OTA
			125.1	4.9	
13	193.1	178.2	123.1	1.9	Methyl-DBD
			151.1	22.3	
14	319.2	304.1	168.1	8.5	Methyl-DDA
			196.1	6.8	
			261.2	35.7	
15	349.0	334.2	247.1	9.8	Methyl-CDA
			291.1	23.3	
16	331.1	316.2	151.1	1.3	Methyl-DTB
			179.1	13.8	
17	271.2	256.2	201.1	1.1	Methyl-DBOT
			229.2	11.8	
18	477.2	462.1	179.2	1.8	Methyl-isoquercitrin
			273.1	13.3	
			257.2	26.7	
			301.2	38.4	
19	361.1	346.1	153.2	12.2	Methyl-DPD
			178.1	9.9	
			332.1	27.7	
20	461.2	446.1	179.2	1.4	Methyl-quercitrin
			273.2	6.8	
			257.1	7.3	
			301.1	14.3	
21	315.1	300.1	179.2	29.6	Methyl-quercetin
			255.2	18.5	
			283.1	7.2	
22	287.1	272.1	229.2	25.8	Methyl-DBT
23	623.3	608.1	301.1	31.9	Methyl-rutin

substituent increases the hydrophobicity of the molecule, thus leading to a stronger hydrophobic interaction with the stationary phase and the elongation of its retention time. Moreover,

in most cases, the hydrogen substitution in the OH group bound to the aromatic ring is more probable than the analogous substitution in the OH group coupled to aliphatic

Table 2 The HRMS data for rutin transformation products

Compound	Elemental composition [M-H] ⁻	Theoretical mass [M-H] ⁻ (Da)	Experimental mass [M-H] ⁻ (Da)	Δ mDa	Δ ppm
OTA	C ₈ H ₅ O ₆	197.00862	197.00822	-0.40	2.03
DBD	C ₈ H ₃ O ₅	178.99805	178.99884	0.79	4.41
DDA	C ₁₄ H ₉ O ₈	305.02975	305.02898	-0.77	2.52
CDA	C ₁₅ H ₁₁ O ₉	335.04031	335.03893	-1.38	4.12
DTB	C ₁₅ H ₉ O ₈	317.02975	317.02910	-0.65	2.05
DBOT	C ₁₄ H ₉ O ₅	257.04500	257.04468	-0.32	1.24
Isoquercitrin	C ₂₁ H ₁₉ O ₁₂	463.08766	463.08705	-0.61	1.32
DPD	C ₁₆ H ₁₁ O ₉	347.04031	347.03932	-0.99	2.85
Quercitrin	C ₂₁ H ₁₉ O ₁₁	447.09274	447.09499	2.25	5.03
Quercetin	C ₁₅ H ₉ O ₇	301.03483	301.03525	0.42	1.40
DBT	C ₁₄ H ₉ O ₆	273.03992	273.03866	-1.26	4.61
Methyl-OTA	C ₉ H ₇ O ₆	211.02427	211.02347	-0.80	3.79
Methyl-DBD	C ₉ H ₅ O ₅	193.01370	193.01238	-1.32	6.84
Methyl-DDA	C ₁₅ H ₁₁ O ₈	319.04540	319.04496	-0.44	1.38
Methyl-CDA	C ₁₆ H ₁₃ O ₉	349.05596	349.05584	-0.12	0.34
Methyl-DTB	C ₁₆ H ₁₁ O ₈	331.04540	331.04588	0.48	1.45
Methyl-DBOT	C ₁₅ H ₁₁ O ₅	271.06065	271.05956	-1.09	4.02
Methyl-isoquercitrin	C ₂₂ H ₂₁ O ₁₂	477.10331	477.10298	-0.33	0.69
Methyl-DPD	C ₁₇ H ₁₃ O ₉	361.05596	361.05514	-0.82	2.27
Methyl-quercitrin	C ₂₂ H ₂₁ O ₁₁	461.10839	461.10910	0.71	1.54
Methyl-quercetin	C ₁₆ H ₁₁ O ₇	315.05048	315.04901	-1.47	4.67
Methyl-DBT	C ₁₅ H ₁₁ O ₆	287.05557	287.05446	-1.11	3.87
Methyl-rutin	C ₂₈ H ₃₁ O ₁₆	623.16122	623.16211	0.89	1.43

structure (McMurry 2007). It is difficult to define unequivocally which OH group in the aromatic ring was methylated. For this reason, the structures of methyl derivatives in Fig. 2 were marked with stars as tentative. It should be emphasized, however, that these structures are the most thermodynamically probable.

As results from the performed experiments, all rutin transformation and degradation products in methanol exist in two forms: as non-methyl and methyl derivatives. In this system, two possible processes of the rutin transformation and degradation should be considered:

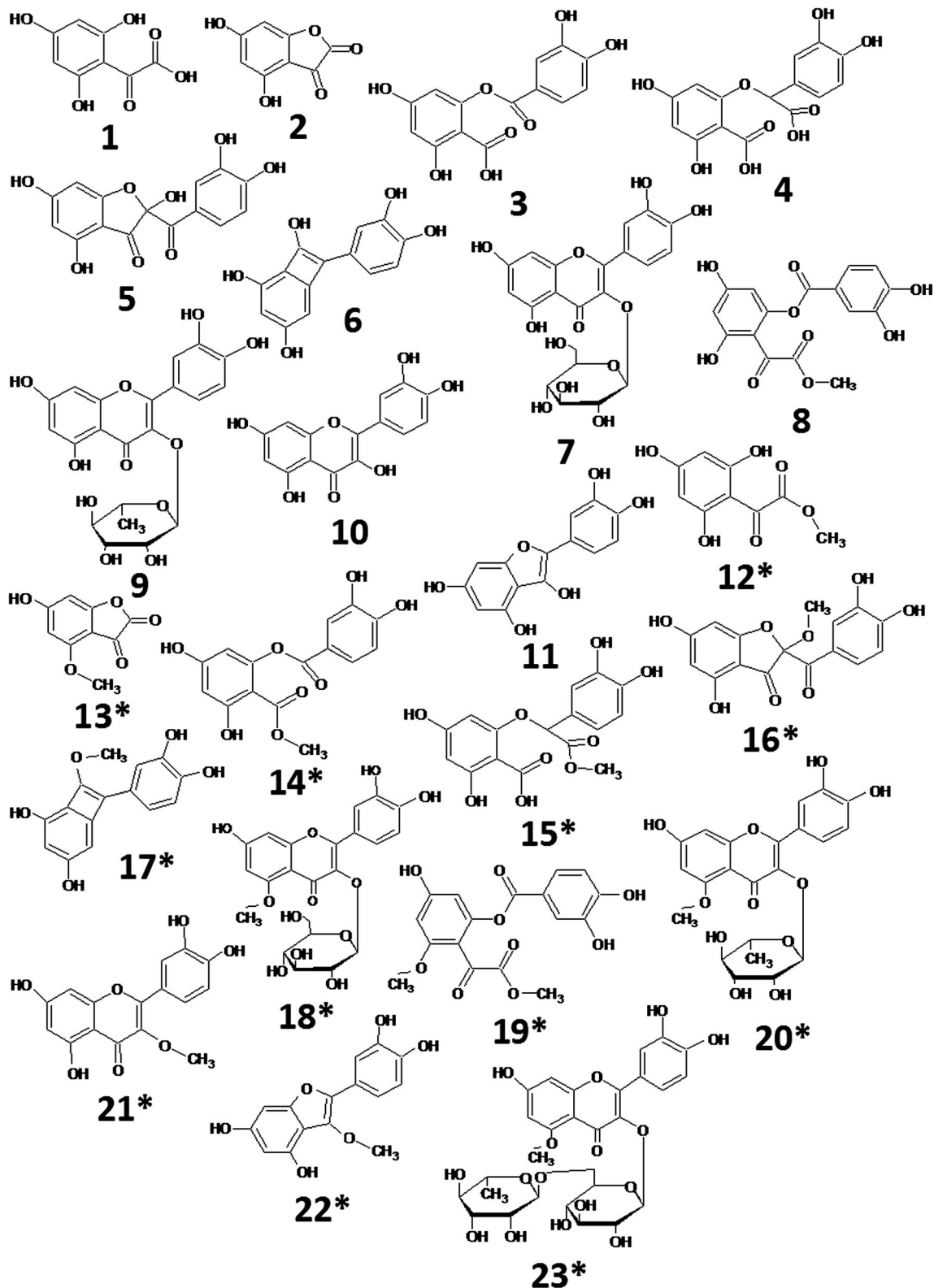
- Methyl derivative formation in the reaction of non-methyl rutin derivatives with methanol which are the final products of rutin transformation and degradation
- Methyl derivative formation as intermediate products in the process of non-methyl derivative formation

Figure 1(A') shows the chromatogram of the methanolic elderberry flower extract prepared by hot extraction process under reflux. Its analysis shows that it contains most of the rutin derivatives which appear in the heated methanolic rutin solution (cf. Fig. 1(A)), and no DTB, CDA, DDA, DBOT, DBD or methyl-DBD in the extract is present. The absence of chromatographic peaks for these rutin derivatives

Fig. 2 Molecular structures of rutin transformation products: (1) ▶ oxo(2,4,6-trihydroxyphenyl)acetic acid; (2) 4,6-dihydroxy-1-benzofuran-2,3-dione; (3) 2-[(3,4-dihydroxybenzoyl)oxy]-4,6-dihydroxybenzoic acid; (4) 2-[carboxy(3,4-dihydroxyphenyl)methoxy]-4,6-dihydroxybenzoic acid; (5) 2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxy-1-benzofuran-3(2H)-one; (6) 7-(3,4-dihydroxyphenyl)bicyclo[4.2.0]octa-1,3,5,7-tetraene-2,4,8-triol; (7) 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxychromen-4-one; (8) 3,5-dihydroxy-2-[methoxy(oxo)acetyl]phenyl-3,4-dihydroxybenzoate; (9) 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[[2S,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyl-2-tetrahydropyranyl]oxy]-4-chromenone; (10) 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one; (11) 2-(3,4-dihydroxyphenyl)-1-benzofuran-3,4,6-triol; (12*) methyl oxo(2,4,6-trihydroxyphenyl)acetate; (13*) 6-hydroxy-4-methoxy-1-benzofuran-2,3-dione; (14*) methyl 2-[(3,4-dihydroxybenzoyl)oxy]-4,6-dihydroxybenzoate; (15*) 2-[1-(3,4-dihydroxyphenyl)-2-methoxy-2-oxoethoxy]-4,6-dihydroxybenzoic acid; (16*) 2-[(3,4-dihydroxyphenyl)carbonyl]-4,6-dihydroxy-2-methoxy-1-benzofuran-3(2H)-one; (17*) 7-(3,4-dihydroxyphenyl)-8-methoxybicyclo[4.2.0]octa-1,3,5,7-tetraene-2,4-diol; (18*) 2-(3,4-dihydroxyphenyl)-5-methoxy-7-hydroxy-3-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxychromen-4-one; (19*) 5-hydroxy-3-methoxy-2-[methoxy(oxo)acetyl]phenyl 3,4-dihydroxybenzoate; (20*) 2-(3,4-dihydroxyphenyl)-5-methoxy-7-hydroxy-3-[[2S,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyl-2-tetrahydropyranyl]oxy]-4-chromenone; (21*) 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-methoxy-4H-chromen-4-one; (22*) 2-(3,4-dihydroxyphenyl)-3-methoxy-1-benzofuran-4,6-diol; (23*) 2-(3,4-dihydroxyphenyl)-5-methoxy-7-hydroxy-3-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-[[2R,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyl-2-yl]oxymethyl]oxan-2-yl]oxymethyl]oxan-2-yl]oxychromen-4-one. The stars indicate the tentative structures of methyl derivatives—see explanation in the discussion

can result from their low concentration in the obtained extracts. However, the absence of DTB, CDA, DDA and DBOT at the simultaneous presence of their methyl derivatives (methyl-DTB, methyl-CDA, methyl-DDA and

methyl-DBOT) in the methanolic elderberry flower extract is striking and suggests another explanation. The absence of the mentioned non-methyl rutin degradation products can result either from



- The catalytic influence of the plant matrix components on the rutin transformation process and the formation of methyl derivatives in the reaction of non-methyl rutin degradation products with methanol, or
- The inhibiting influence of the plant matrix components on the rutin transformation process, when the methyl derivatives play the role of intermediate substances and are ultimately transformed to non-methyl rutin degradation products

Figure 1(B) shows that the methanol/water rutin solution heated under reflux contains, beside the parent substance, 21 additional compounds—the rutin transformation and degradation products. The absence of methyl-DBD and methyl-DBOT in the solution is the main difference in the composition of methanol and methanol/water rutin extracts (cf. Fig. 1(A)). The exemplary chromatogram of the methanol/water elderberry flower extract prepared by hot extraction process under reflux is presented in Fig. 1(B'). As can be seen, one of the rutin transformation products, namely methyl-DBOT, is missing at the chromatogram as compared with the analogous one obtained from the methanolic solution (cf. with Fig. 1(A')). The absence of some methyl derivatives of rutin transformation products in the methanol/water extracts of rutin and elderberry flowers can be explained by lower methanol concentration in the extraction systems.

Figure 1(C) shows the chromatogram of buffered methanol/water rutin solution (pH=6.5) heated under reflux. Relating it to those presented in chromatograms A and B, this extract contains one substance less, methyl-DBOT, than the

methanolic extract of rutin, and one substance more, methyl-DBD, than the methanol/water rutin solution.

The exemplary chromatogram of the elderberry flower extract prepared by hot extraction process under reflux using buffered methanol/water mixture (pH=6.5) is presented in Fig. 1(C'). Its analysis shows that the extract contains only 16 rutin derivatives and that certain derivatives are absent in it: OTA, DBOT, methyl-DBD, methyl-CDA, methyl-DBOT, methyl-DPD and methyl-DBT. Taking into account that the pH of elderberry flower suspension in water is equal to about 5.5, the comparison of the results in Fig. 1(B' and C') shows that a more acidic environment favours the formation of methyl derivative rutin degradation products. This behaviour agrees with the literature reports concerning the influence of pH on the methylation process (McMurry 2007).

The influence of heating time on the amount of individual rutin derivatives formed during rutin transformation and degradation in methanol is presented in Figs. 3(A1) (non-methyl derivatives of rutin degradation products) and 3(A2) (methyl derivatives of rutin degradation products). The presented relationships demonstrate two concentration trends resulting from the increase of heating time:

- OTA, CDA, DTB, DBOT, isoquercitrin, DPD, methyl-OTA, methyl-DTB, methyl-DBOT, methyl-quercitrin and methyl-rutin concentrations increase. This trend is obvious as the thermal degradation of compounds increases with heating time.
- Initial concentration increases followed by its decrease for the remaining compounds. The shape of these

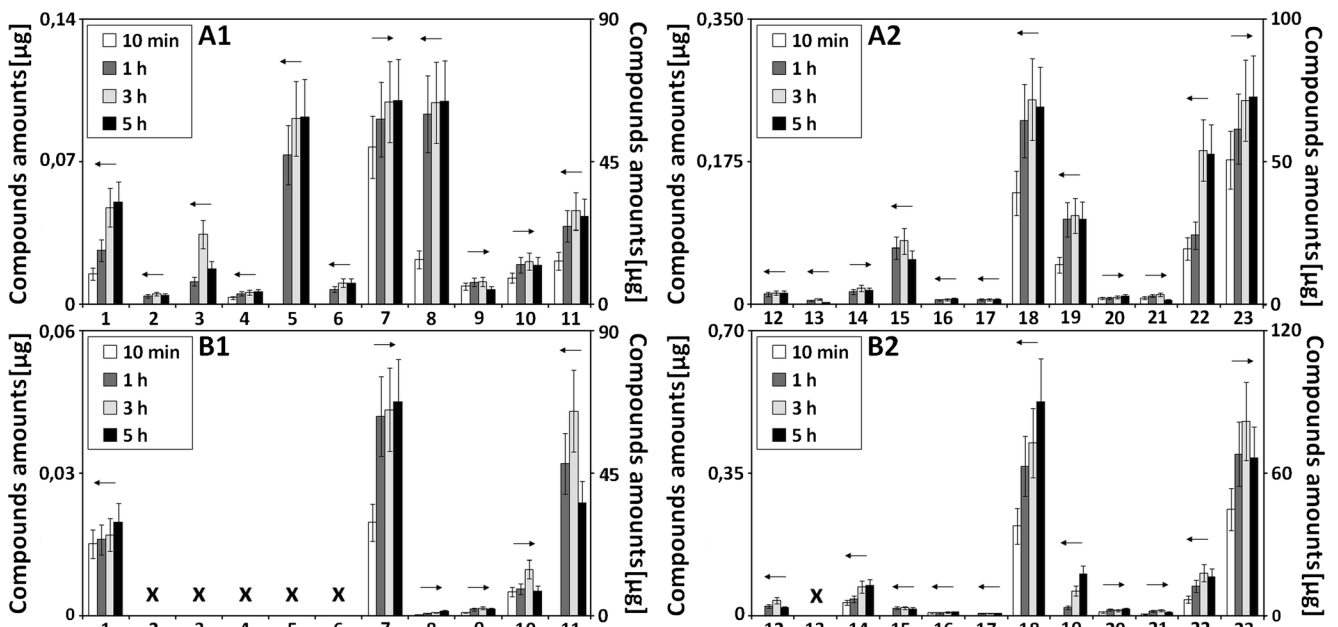


Fig. 3 The influence of heating time on the amounts of non-methyl and methyl rutin degradation products formed during the heating of methanolic rutin solution (A1 and A2, respectively) and during rutin extraction

from elderberry flower using methanol (B1 and B2, respectively). Numbers under bars correspond to compound numbers reported in Fig. 2. Appropriate concentration axis is indicated by arrows

relationships can be explained by the occurrence of two competitive reactions: the formation of transformation products and their degradation.

However, not all the observed trends are statistically unequivocal.

Similar concentration trends are observed examining the influence of heating time on the amount of individual rutin derivatives formed during rutin transformation and degradation in methanolic elderberry flower extract—see Figs. 3(B1) (non-methyl derivatives of rutin) and 3(B2) (methyl derivatives of rutin). As results from the comparison of Figs. 3(A and B), the increase of heating time causes analogous concentration changes for most rutin derivatives in both examined system types (methanolic rutin solution vs. methanolic elderberry flower extract). Only in the case of methyl-OTA, methyl-DDA, methyl-isoquercitrin, methyl-DPD and methyl-rutin are the shapes of the relationships a little bit different. As individual trends are statistically unequivocal, it can be concluded that the matrix components do not influence significantly the shape of the relationships between the concentration of a rutin transformation and degradation products and heating time in the extraction process, but do influence the concentration and number of the forming rutin derivatives. Hence, the catalytic/inhibiting influence of plant matrix components on the rutin transformation process cannot be excluded.

The same conclusions can be drawn analysing the results shown in Figs. 4 and 5, which present the influence of heating

time on the amount of individual rutin derivatives formed during rutin transformation and degradation in

- The methanol/water rutin solution (Fig. 4(A)) and the methanol/water elderberry flower extract (Fig. 4(B))
- Buffered methanol/water rutin solution (Fig. 5(A)) as well as buffered methanol/water elderberry flower extract (Fig. 5(B))

In these experiments, methanol/water (50/50 v/v) mixture (Fig. 4) and methanol/phosphoric buffer (pH=6.5) mixture (75/25 v/v) (Fig. 5) were applied. The influence of heating time on the amount of individual rutin derivatives formed during rutin transformation and degradation in methanol/water (75/25 v/v) mixture and in methanol/phosphoric buffer (pH=4.5 or 8.5) mixture (75/25 v/v) is presented in Figs. 6, 7 and 8.

The comparison of the results presented in Figs. 3, 4 and 6 shows that in most cases the increase of water concentration in the extractant causes the concentration increase of non-methyl and the concentration decrease of methyl rutin degradation products. Probably, it results from increasingly lower methanol concentration in the extraction mixture or from the hydrolysis process of the formed methyl rutin derivatives.

As appears from the comparison of the results shown in Figs. 5, 7 and 8, the decrease of the extractant pH leads in most cases to the formation of a greater number of methyl rutin derivatives. This phenomenon agrees with the literature reporting on the easier formation of methyl derivatives of phenolic compounds in more acidic environments (McMurry 2007).

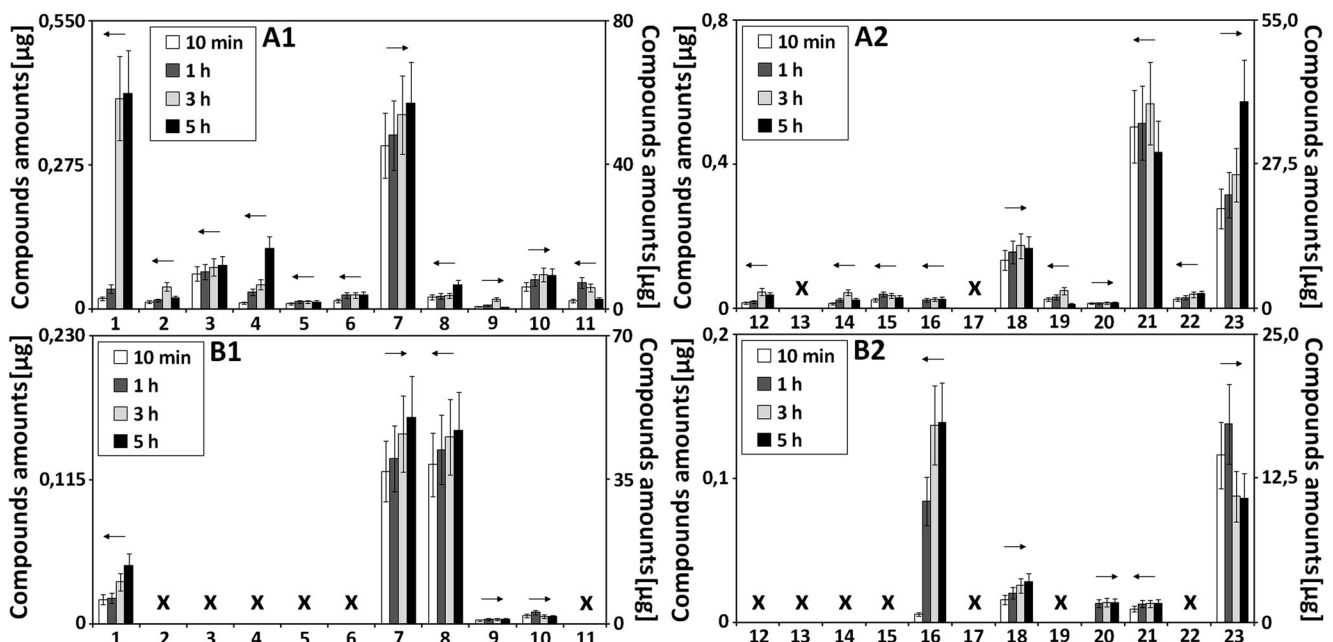


Fig. 4 The influence of heating time on the amounts of non-methyl and methyl rutin degradation products formed during the heating of methanol/water (50/50 v/v) rutin solution (A1 and A2, respectively) and during rutin extraction from elderberry flower using methanol/water (50/50 v/v)

mixture (B1 and B2, respectively). Numbers under bars correspond to compound numbers reported in Fig. 2. Appropriate concentration axis is indicated by arrows

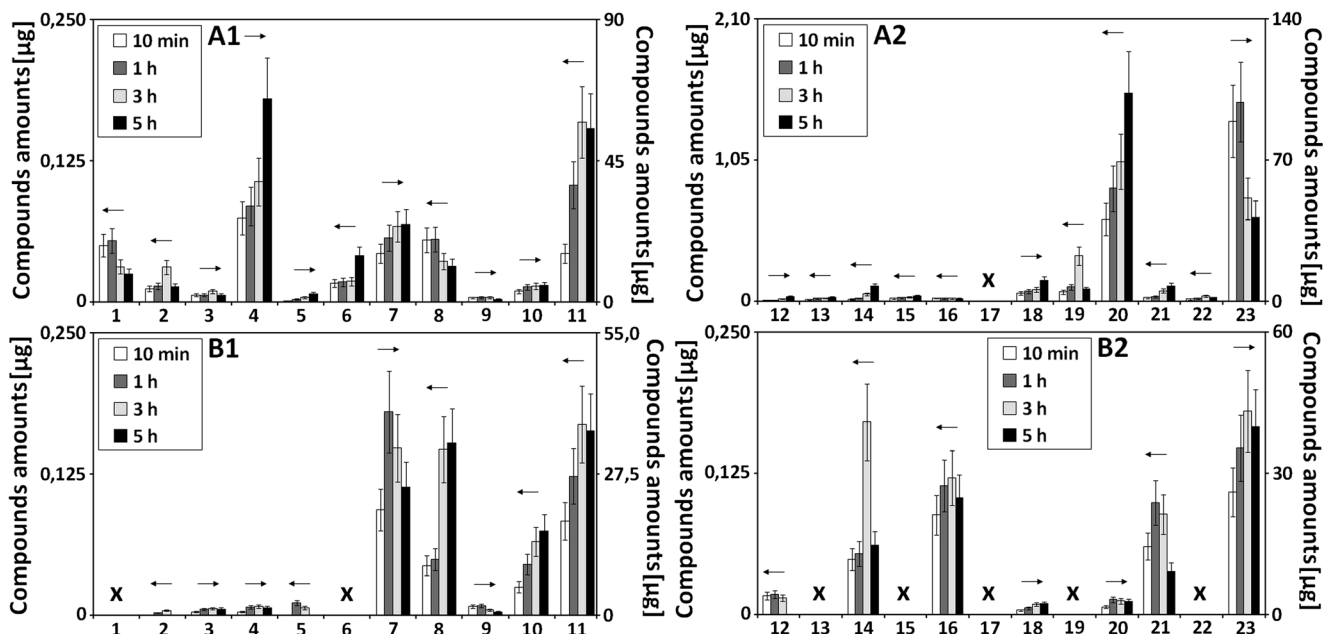


Fig. 5 The influence of heating time on the amounts of non-methyl and methyl rutin degradation products formed during the heating of methanol/phosphoric buffer (pH=6.5) (75/25 v/v) rutin solution (*A1* and *A2*, respectively) and during rutin extraction from elderberry flower using methanol/

phosphoric buffer (pH=6.5) (75/25 v/v) mixture (*B1* and *B2*, respectively). Numbers under bars correspond to compound numbers reported in Fig. 2. Appropriate concentration axis is indicated by arrows

Conclusions

Sample preparation is a crucial step in the chemical analysis of plant material. Liquid extraction is currently applied most frequently for this purpose. Pharmacopoeia formulae of plant

material analysis mainly report the application of liquid extraction methods such as Soxhlet extraction, percolation, maceration and extraction under reflux. For rutin isolation, methanol, methanol/water and buffered methanol/water mixtures are the most frequently employed extractants. The presented results

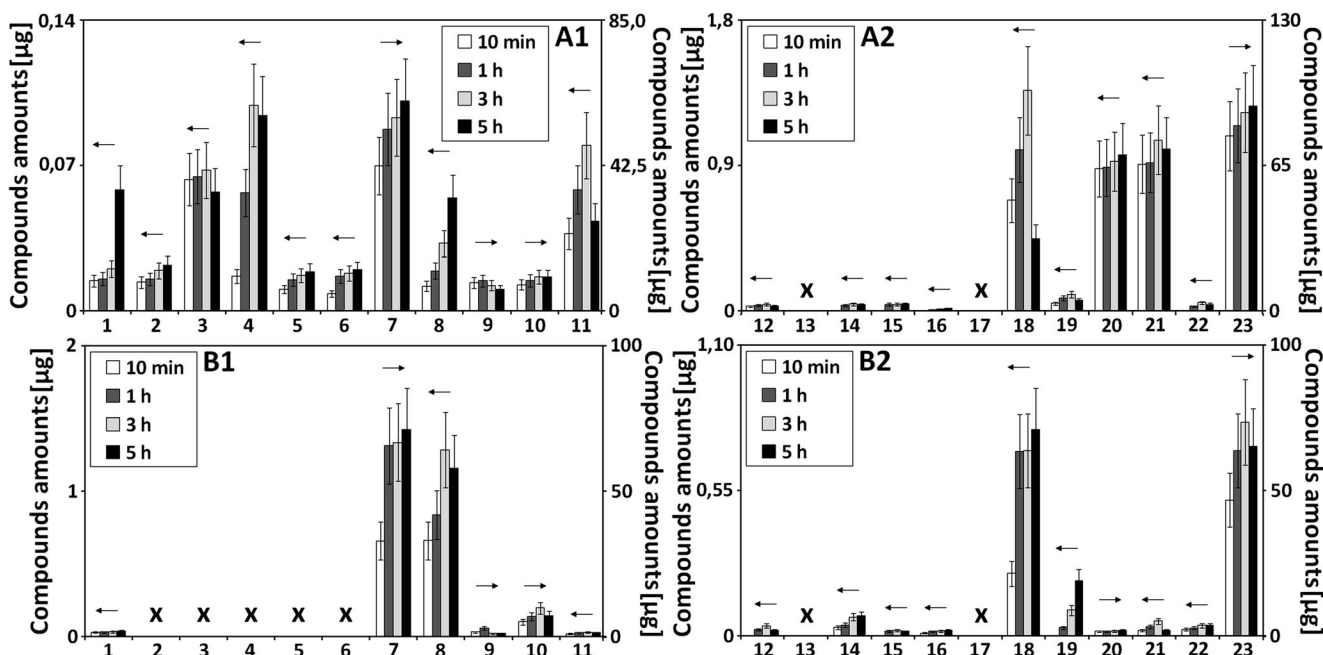


Fig. 6 The influence of heating time on the amounts of non-methyl and methyl rutin degradation products formed during the heating of methanol/water (75/25 v/v) rutin solution (*A1* and *A2*, respectively) and during rutin extraction from elderberry flower using methanol/water (75/25 v/v)

mixture (*B1* and *B2*, respectively). Numbers under bars correspond to compound numbers reported in Fig. 2. Appropriate concentration axis is indicated by arrows

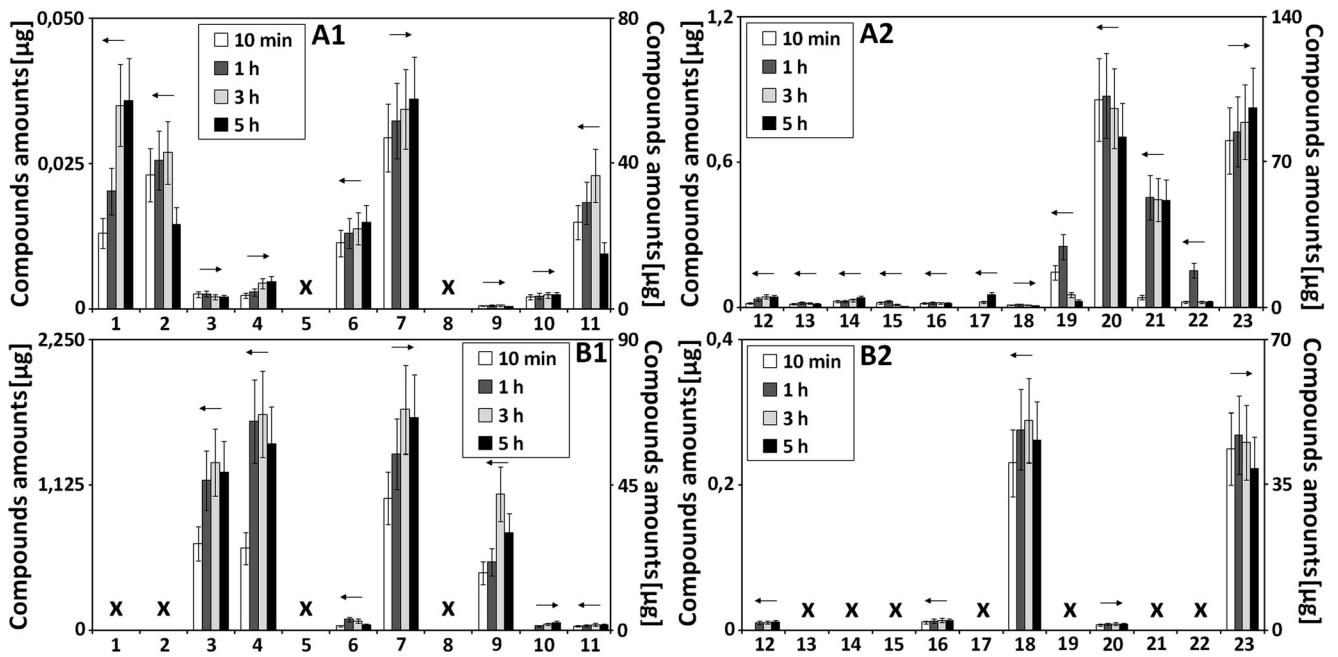


Fig. 7 The influence of heating time on the amounts of non-methyl and methyl rutin degradation products formed during the heating of methanol/phosphoric buffer (pH=4.5) (75/25 v/v) rutin solution (*A1* and *A2*, respectively) and during rutin extraction from elderberry flower using methanol/

phosphoric buffer (pH=4.5) (75/25 v/v) mixture (*B1* and *B2*, respectively). Numbers under bars correspond to compound numbers reported in Fig. 2. Appropriate concentration axis is indicated by arrows

show that at least 23 compounds (rutin transformation and degradation products and their reaction products with alcohol) can be formed from rutin during its simulated and real extraction from plants by methanol and methanol/water mixtures. Eleven of them are known from the literature as rutin and/or quercetin

degradation products forming during their high-temperature treatment, during UV irradiation or in an aggressive environment (Dubber et al. 2005; Rohn et al. 2007; Wang et al. 2011; Zenkevich et al. 2007; Zvezdanović et al. 2012). Twelve compounds have not yet been reported anywhere. The amount of

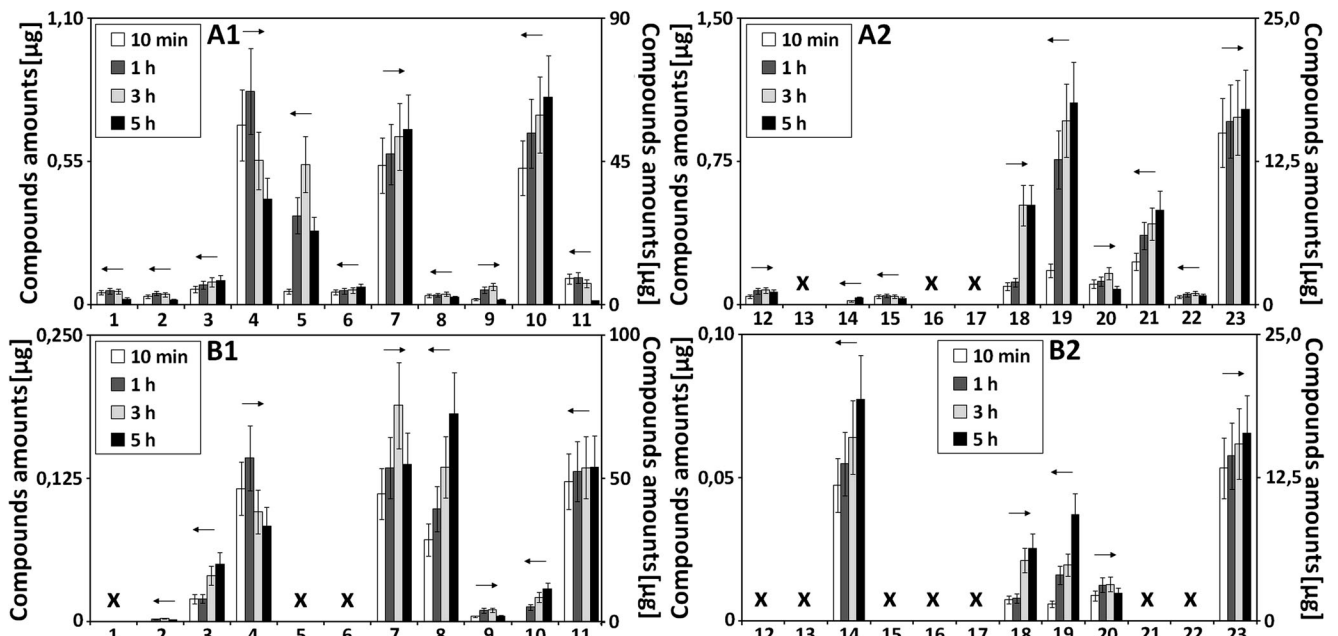


Fig. 8 The influence of heating time on the amounts of non-methyl and methyl rutin degradation products formed during the heating of methanol/phosphoric buffer (pH=8.5) (75/25 v/v) rutin solution (*A1* and *A2*, respectively) and during rutin extraction from elderberry flower using methanol/

phosphoric buffer (pH=8.5) (75/25 v/v) mixture (*B1* and *B2*, respectively). Numbers under bars correspond to compound numbers reported in Fig. 2. Appropriate concentration axis is indicated by arrows

each formed component depends not only on heating time, alcohol concentration and extractant pH but also on matrix components of the plant from which rutin is extracted.

The identified transformation and degradation products of rutin can be mistakenly treated as components naturally present in the examined plant, or can lead to erroneous quantitative estimations of plant composition when some or all components formed from rutin during its extraction by alcohol or alcohol/water solution naturally exist in the examined plant and the transformation process of rutin only increases their amount. In this context, our results are especially useful for researchers investigating plant metabolism and looking for new plant components.

Compliance with Ethics Requirements

Conflict of Interest Andrzej L. Dawidowicz declares that he has no conflict of interest. Katarzyna Bernacik declares that she has no conflict of interest. Rafal Typek declares that he has no conflict of interest. This article does not contain any studies with human or animal subjects.

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