Journal of the Serbian Chemical Society
JSCS@tmf.bg.ac.rs • www.shd.org.rs/JSCS
J. Serb. Chem. Soc. 87 (9) S307–S316 (2022) *Supplementary material*

SUPPLEMENTARY MATERIAL TO

Melissopalynology analysis, determination of physicochemical parameters, sugars and phenolics in Maltese honey collected in different seasons

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J. Serb. Chem. Soc. 87 (9) (2022) 983–995

Methods

Melissopalynological analysis

Amount of 10 g of sample was diluted in 20 mL of distilled water and centrifuged at 2500 RPM for 10 minutes. After supernatant is discarded the resulting sediment was suspended in 10 mL of distilled water and 1 % of the volume is transferred to a microscopic slide for quantitative pollen analysis. The remaining sample was centrifuged again at 2500 RPM for 10 minutes and the entire amount of resulting sediment was transferred to a microscopic slide for qualitative pollen analysis. Microscopic slide mounting medium, a glycerol gelatin mixture that contains gelatin, glycerin, phenol, distilled water, and basic fuchsine, was prepared in the Laboratory for Palynology, University of Novi Sad. Both slides were mounted with the glycerol-gelatin mixture. Quantitative pollen analysis has been performed by counting all pollen suspended in 1 % of the total sediment extracted from 10 g of honey sample, which enabled the calculation of pollen concentration in 10 g of honey (PG per 10 g).

Sugar analysis

The evaluation of the carbohydrate content of the honey samples was obtained from the calibration curves of pure compounds. The calibration was performed with standard solutions of sugars dissolved in ultrapure water. Each individual standard was dissolved in ultrapure

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water. Stock solutions with concentrations of 1000 mg L^{-1} were prepared and working solutions in the concentration ranges were as follows: for glucose and fructose from 10.0 to 100.0 mg L^{-1}; for sucrose from 1.0 to 10.0 mg L^{-1}; for isomaltose from 0.5 to 5.0 mg L^{-1}, while for all the other standards, the concentration range was from 0.1 to 1.0 mg L^{-1}. Each honey sample was injected with an ICS AS-DV 50 autosampler (Dionex). ICS 3000 DP liquid chromatograph was equipped with a quaternary gradient pump (Dionex, Sunnyvale, CA). The carbohydrates were eluted with the flow rate set to 0.7 mL min^{-1}, using a gradient program constituted from 600 mM sodium hydroxide (eluent A), 500 mM sodium acetate (eluent B) and ultrapure water (eluent C). The gradient program was as previously described in Gašić *et al.*[1] The carbohydrates were separated on a CarboPac1 PA10 anion-exchange column (4×250 mm) at 30 °C.

Analysis of phenolic compounds

A 1000 mg L^{-1} stock solution of a mixture of all phenolic compounds was prepared in methanol. Dilution of the stock solution with methanol yielded the working solutions of concentrations 0.025, 0.050, 0.100, 0.250, 0.500, 0.750, and 1.000 mg L^{-1}. The calibration curves were obtained by plotting the peak areas of the standards against their concentration. The calibration curves revealed good linearity, with r^2 values exceeding 0.99 (peak areas *vs.* concentration). The elution was performed at 40 °C on a Syncronis C18 column. The mobile phase consisted of water + 0.1 % formic acid (A) and acetonitrile (B). A TSQ Quantum Access Max triple-quadrupole mass spectrometer equipped with heated electrospray ionization (HESI) source was used for detection of compounds of interest. Chromatographic conditions and mass spectrometry settings were previously published[21]. Xcalibur software 2.2 (Thermo Fisher, Bremen, Germany) was used for instrument control. The phenolic compounds were identified by direct comparison with commercial standards and expressed as mg kg^{-1}.

Fig. S-1. Regional map of Malta (A); map of sampling sites on Malta (B) with the assigned locations (L1–L14).

RESULTS AND DISCUSSION

Melissopalynology analysis of Maltese honey samples

Besides *Lotus* and *Eucalyptus, Rubus* pollen was present in ~two-fold lower frequency, followed by *Reseda*-type and *Thymus* pollen (Fig. S-2). In addition to *Lotus*, honey samples also contained additional four types of pollen that belong to Fabaceae (*Ceratonia silique, Trifolium, Acacia* and *Peltophorum*). Considering Rosaceae family, there were four different types, while for Asteraceae and Boraginaceae the appearance of two pollen types was noted for each family (Fig. S-2). The quantitative pollen analysis identified three times higher pollen concentration for spring honey than those in autumn and summer samples. Furthermore, the average value of the estimated pollen concentration for spring honey was higher than for autumn and summer samples pairwise (Fig. S-2). High frequency of *Lotus* pollen type coincided with high pollen concentration, indicating that this small pollen gets notably overrepresented in samples. In the first season of harvesting, the pollen types that was found in lower amounts were *Rubus*, *Tropaeolum majus*, *Ceratonia silique*, *Carduus*, *Pelthophorum* (Table S-II). However, in honey samples, there was also pollen from plants whose flowering period was several months before such as *Lotus* (flowering period from February to June), *Thymus* (May-July), and *Brassica* pollen (March-June). Presence of *Reseda*-type (July-September), as well as *Eucalyptus* (June-September), *Triofilium* (July-October), *Ceratonia silique* (October-November) and *Smilax aspera* pollen (September-October) in the spring season (Table S-II) was not expected, as their flowering period does not correspond to this period of sampling. Besides *Lotus*, *Reseda*-type and *Thymus* pollen, in summer samples there was also appearance of other pollen such as *Eucalyptus*, *Rubus*, *Citrus*, *Brassica*, *Carduus*, *Triofilium*, *Pistacia* (Table S-II), which flowering occurs before or during the period of sampling. Furthermore, the presence of *Tropaeolum majus* (October-April), *Ceratonia siliqua* (October-November), *Acacia* (March-May) and *Smilax aspera* (September-October) in summer samples was in contrast to their flowering period.

PCA performed on determined parameters

PCA performed on data of physicochemical parameters, resulted in a four-component model, which explains 92.38 % of the total data variance. The principal components (PC's) accounted for the following data variances (from PC1 to PC4 – 49.26, 30.08, 7.99 and 5.06 %, respectively). Two principal components explained approximately 80 % of the total data variances and revealed distinctly grouping of the samples according to seasonal variability (Fig. 1). PCA performed on data on phenolic and sugar content, resulted in a four-component model, which explains 69.30 % of the total data variance. First two

PCs explained approximately 50 % of the total data variances (PC1 – 31.68 %, PC2 – 16.98 %) (Fig. 1).

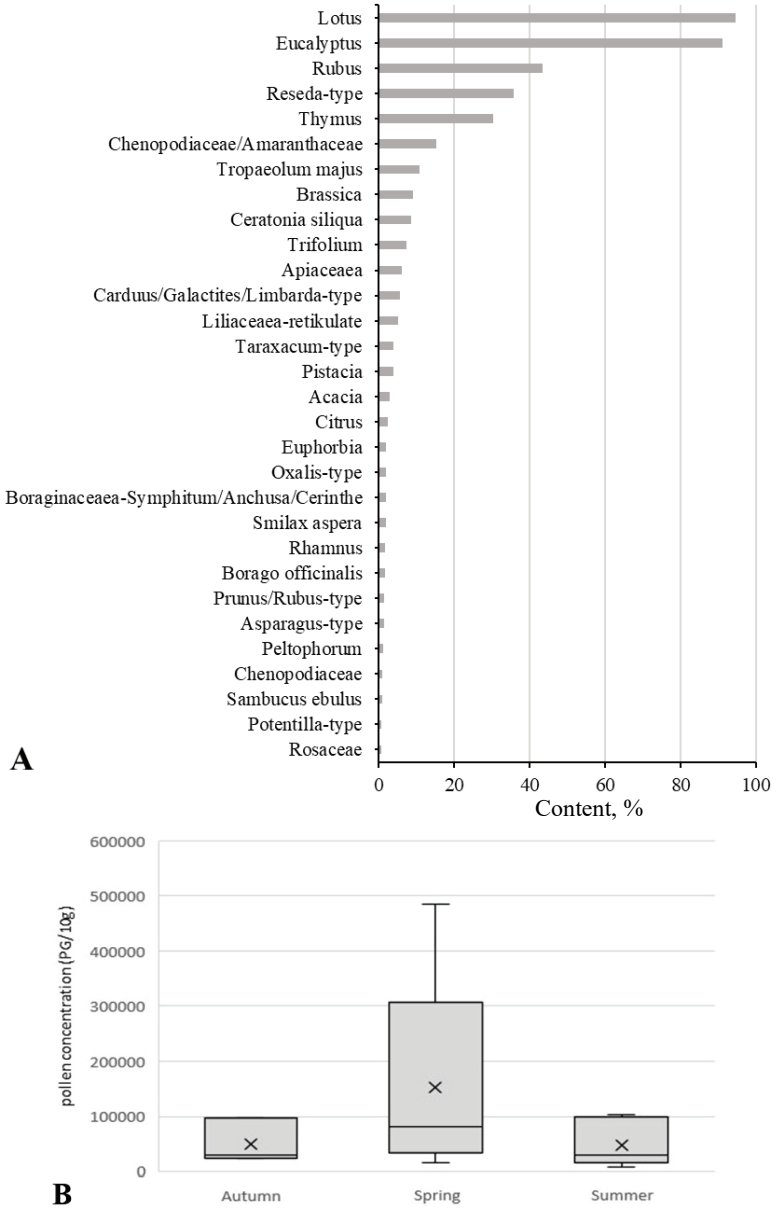

Fig. S-2. Distinctive melissopalynological features of analysed honey samples from Malta: (A) Maximum frequency of 30 identified pollen types, (B) pollen concentrations in samples from different seasons (x denotes the average and horizontal line as the median value).

TABLE S-I. Melissopalynological analysis of 14 honey samples from Malta, harvested in three seasons (autumn, 2014; spring, 2015; summer, 2015); percentage of pollen and melissopalynological assessment of honey samples.

Period of sampling	Honey sample	Pollen conc. PG per 10 g	Predominant pollen (>45 %)	Secondary pollen (16-45 %)	Important minor pollen (3-15 %)	Minor pollen (<3 %)
Autumn, 2014	AU1	23,700	*Eucalyptus* (91 %)		*Brassica* (4 %), *Ceratonia siliqua* (4 %)	*Lotus* (1 %)
	AU 2	30,700	-	*Rubus* (44 %), *Eucalyptus* (17 %)	*Lotus* (13 %), *Reseda* (10 %), *Brassica* (9 %), *Thymus* (3 %)	Apiaceaea (2 %), *Peltophorum* (1 %)
	AU 3	97,000	*Eucalyptus* (68 %)	-	*Reseda* (12 %), *Lotus* (8 %), *Rubus* (4 %)	*Ceratonia siliqua* (2 %), *Carduus/Galactites/Limbarda*-type (2 %), *Brassica* (1 %), *Liliaceaea-retikulate* (1 %), *Tropaeolum majus* (1 %)
Spring, 2015	SP1	128,800	*Lotus* (95 %)	-	-	*Acacia* (1 %), *Carduus/Galactites/Limbarda*-type (1 %), *Peltophorum* (1 %), *Pistacia* (1 %), *Trifolium* (1 %)
	SP2	80,800	-	*Lotus* (43 %)	*Eucalyptus* (15 %), *Tropaeolum majus* (11 %), *Ceratonia siliqua* (9 %), *Taraxacum*-type (4 %), *Acacia* (3 %), *Trifolium* (3 %)	*Liliaceaea-retikulate* (2 %), *Reseda*-type (2 %), *Borago officinalis* (1 %), *Brassica* (1 %), *Citrus* (1 %), *Euphorbia* (1 %), *Oxalis*-type (1 %), *Prunus/Rubus*-type (1 %), *Smilax aspera* (1 %)
	SP3	484,600	*Lotus* (87 %)	-	*Carduus/Galactites/Limbarda*-type (6 %)	*Citrus* (2 %), *Smilax aspera* (2 %), *Prunus/Rubus*-type (1 %), *Trifolium* (1 %)

Period of sampling	Honey sample	Pollen conc. PG per 10 g	Predominant pollen (>45 %)	Secondary pollen (16-45 %)	Important minor pollen (3-15 %)	Minor pollen (<3 %)
Spring, 2015	SP4	15,400	*Lotus* (89 %)	-	*Rubus* (4 %)	Apiaceaea (2 %), *Carduus/Galactites/Limbarda*-type (2 %), *Peltophorum* (1 %)
	SP5	53,200	-	*Lotus* (35 %), *Reseda* (36 %)	*Trifolium* (7 %), *Eucalyptus* (5 %), *Pistacia* (4 %)	*Euphorbia* (2 %), *Acacia* (1 %), Apiaceaea (1 %), *Brassica* (1 %), Boraginaceaea-*Symphitum/Anchusa/Cerinth*e (1 %), *Carduus/Galactites/Limbarda*-type (1 %), *Citrus* (1 %), *Sambucus ebulus* (1 %), *Oxalis*-type (1 %), *Potentila*-type (1 %), *Smilax aspera* (1 %)
Summer, 2015	SU1	24,300	*Lotus* (56 %)	*Reseda* (21 %)	*Liliaceaea-retikulate* (5 %), *Carduus/Galactites/Limbarda*-type (4 %), *Trifolium* (3 %)	*Borago officenalis* (2 %), *Rhamnus* (2 %), *Rubus* (2 %), *Tymus* (2 %), *Citrus* (1 %)
	SU2	97,000	*Lotus* (49 %)	*Reseda* (28 %)	Apiaceaea (6 %), *Liliaceaea-retikulate* (4 %), *Trifolium* (4 %), *Carduus/Galactites/Limbarda*-type (3 %)	*Brassica* (2 %), *Eucalyptus* (2 %), *Acacia* (1 %), Apiaceaea (1 %), *Ceratonia siliqua* (1 %), *Prunus/Rubus*-type (1 %), *Smilax aspera* (1 %), *Tropaeolum majus* (1 %), *Tymus* (1 %)

Period of sampling	Honey sample	Pollen conc. PG per 10 g	Predominant pollen (>45 %)	Secondary pollen (16-45 %)	Important minor pollen (3-15 %)	Minor pollen (<3 %)
Summer, 2015	SU3	7,900	-	*Thymus* (30 %), *Reseda* (17 %)	Chenopodiaceae/ Amaranthaceae (15 %), Apiaceaea (6 %), *Lotus* (5 %), *Brassica* (4 %), *Carduus/Galactites/Limbarda*-type (4 %), *Rubus* (4 %)	*Trifolium* (2 %), *Tropaeolum majus* (2 %), *Acacia* (1 %), *Asparagus*-type (1 %), Chenopodiaceae (1 %), *Eucalyptus* (1 %), *Prunus/Rubus*-type (1 %), *Smilax aspera* (1 %)
	SU4	18,900	*Lotus* (74 %)	-	*Reseda* (10 %), *Brassica* (3 %), *Tymus* (3 %)	*Euphorbia* (2 %) *Rhamnus* (2 %), Boraginaceaea-*Symphitum/Anchusa/Cerinthe* (1 %), *Oxalis*-type (1 %), *Trifolium* (1 %)
	SU5	34,000	*Lotus* (72 %)	*Thymus* (17 %)	*Reseda*-type (4 %)	Apiaceaea (1 %), *Brassica* (1 %), *Pistacia* (1 %), *Liliaceaea-retikulate* (1 %), *Trifolium* (1 %)
	SU6	103,900	*Lotus* (60 %)	-	*Thymus* (15 %), *Eucalyptus* (4 %), *Acacia* (3 %), *Brassica* (3 %)	Boraginaceaea-*Symphitum/Anchusa/Cerinthe* (2 %), *Oxalis*-type (2 %), *Rubus* (2 %), Apiaceaea (1 %), *Borago officinalis* (1 %), *Carduus/Galactites/Limbarda*-type (1 %), *Ceratonia siliqua* (1 %), *Potentila*-type (1 %), *Reseda*-type (1 %), Rosaceae (1 %),

Period of sampling	Honey sample	Pollen conc. PG per 10 g	Predominant pollen (>45 %)	Secondary pollen (16-45 %)	Important minor pollen (3-15 %)	Minor pollen (<3 %)
						Trifolium (1 %), *Tropaeolum majus* (1 %)

TABLE S-II Physicochemical parameters in 14 analyzed honey samples from Malta collected in three seasons.

Parameter	Autumn, 2014, (N=3)			Spring 2015, (N=5)					Summer 2015, (N=6)					
	AU1	AU2	AU3	SP1	SP2	SP3	SP4	SP5	SU1	SU2	SU3	SU4	SU5	SU6
Moisture, %	18.08	19.51	19.70	16.67	15.92	15.59	15.58	17.63	17.62	17.96	17.55	18.80	18.24	19.76
Brix, %	80.24	78.85	78.66	81.62	82.35	82.72	82.67	80.68	80.69	80.36	80.77	79.53	80.08	78.61
σ / mS cm^{-1}	1.21	0.87	1.52	0.49	0.54	0.76	0.66	0.98	0.51	0.71	0.46	0.52	0.48	0.52
pH	4.00	3.71	3.92	3.58	3.78	3.88	3.87	3.93	3.59	3.78	3.83	3.67	3.78	3.79
Acidity, mmol kg^{-1}	26.00	34.00	29.50	26.50	26.60	25.57	22.97	26.20	57.40	44.00	38.03	54.77	42.83	36.30
HMF, mg kg^{-1}	18.80	12.80	16.00	11.96	2.33	2.22	0.89	7.33	6.25	15.48	15.61	11.82	12.42	9.14
Proline, mg kg^{-1}	558.69	602.93	611.60	652.66	377.70	431.69	372.97	562.08	899.52	726.80	434.45	928.53	667.10	815.29
DA, Schade units[1]	9.58	7.67	8.54	8.09	5.79	4.58	6.82	4.04	10.34	6.33	11.05	10.71	11.74	12.62
	Content, g kg^{-1}													
Glucose	390.89	316.05	342.85	346.95	362.41	335.07	305.51	373.48	381.02	343.49	342.96	359.62	327.80	350.80
Fructose	392.74	320.27	343.23	345.23	365.19	345.06	309.20	384.96	369.44	338.92	377.10	385.56	366.94	388.98
Arabinose	0.49	0.34	0.27	0.39	0.39	0.36	0.30	0.46	0.30	0.39	0.44	0.36	0.47	0.43
Succrose	2.78	4.75	7.04	7.04	8.23	7.17	5.19	9.09	5.91	6.43	1.79	8.78	4.99	8.65
Maltose	4.71	2.89	3.62	7.50	7.58	7.91	6.45	7.87	5.08	5.52	4.72	5.23	5.26	5.54
Isomaltose	1.03	2.88	4.26	1.53	7.59	2.45	2.10	6.22	3.78	5.20	1.00	1.87	1.30	2.35
Trehalose	0.66	0.07	0.28	0.35	0.19	0.23	0.41	0.09	0.21	0.72	0.46	0.17	0.32	0.82
Turanose	3.18	2.96	2.16	3.62	4.10	4.40	4.37	5.75	2.54	2.86	3.29	2.70	3.11	3.59
Melibiose	1.50	0.98	0.98	0.51	0.72	0.46	0.48	1.06	0.81	0.91	1.03	0.92	0.94	0.96
Gentiobiose	1.24	1.15	0.69	1.05	1.21	1.44	1.17	1.54	0.72	0.90	1.20	0.97	1.20	1.03
Melezitose	2.47	2.01	1.39	2.64	2.97	3.84	3.19	3.75	1.45	2.37	2.62	1.87	2.40	2.40
Raffinose	2.45	1.64	2.04	2.39	2.47	2.85	2.42	2.98	2.38	2.58	1.59	1.98	1.93	2.47
Maltotriose	0.13	0.43	0.17	0.10	0.10	0.06	0.05	0.19	0.32	0.47	0.37	0.41	0.33	0.29
Isomaltotriose	0.41	0.98	0.90	0.68	0.68	0.63	0.60	0.71	0.69	0.77	0.09	0.84	0.08	0.05
Panose	0.58	0.66	0.60	0.85	0.89	0.33	0.18	1.27	1.11	2.63	0.80	0.84	0.58	0.67
Monosaccharides[2]	784.12	636.66	686.36	692.57	728.00	680.49	615.01	758.90	750.76	682.81	720.50	745.54	695.21	740.21
Disaccharides[2]	15.10	15.67	19.02	21.59	29.62	24.06	20.17	31.63	19.04	22.54	13.47	20.63	17.11	22.94
Trisaccharides[2]	6.03	5.72	5.10	6.66	7.11	7.70	6.43	8.89	5.95	8.82	5.47	5.95	5.32	5.89
Sugars[2]	805.25	658.05	710.48	720.82	764.73	712.25	641.61	799.42	775.74	714.17	739.45	772.12	717.63	769.04

[1]Diastase activity [2]Total

TABLE S-III Correlation between physicochemical parameters through the seasons (based on their mean values, $p<0.05$).

	Brix	Moisture	HMF	Proline	Acidity	Diastase activity	pH	Electrical conductivity
Brix	1							
Moisture content	-1.00	1						
HMF	-0.99	0.99	1					
Proline	-0.63	0.63	0.54	1				
Acidity	-0.44	0.44	0.34	0.97	1			
Diastase activity	-0.77	0.77	0.70	0.98	0.91	1		
pH	-0.27	0.27	0.37	-0.58	-0.74	-0.40	1	
Electrical conductivity	-0.54	0.54	0.63	-0.31	-0.51	-0.11	0.96	1

TABLE S-IV. Content of quantified phenolic compounds in honey samples from Malta harvested in three seasons (lod – limit of detection)

No	Phenolic compound	Autum 2014, (N=3)			Spring 2015, (N=5)					Summer 2015 (N=6)					
		AU1	AU2	AU3	SP1	SP2	SP3	SP4	SP5	SU1	SU2	SU3	SU4	SU5	SU6
		Content, mg kg^{-1}													
1	Protocatechuic acid	0.02	<lod	0.05	<lod	<lod	<lod	<lod	<lod	<lod	<lod	<lod	<lod	<lod	<lod
2	Aesculin	0.06	<lod	0.06	<lod	<lod	<lod	<lod	0.13	0.05	<lod	<lod	0.06	<lod	<lod
3	Chlorogenic acid	0.11	0.12	0.14	<lod	0.11	<lod	<lod	0.14	0.12	0.12	0.11	0.11	0.11	0.12
4	*p*-Hydroxybenzoic acid	0.47	0.80	0.81	0.25	0.42	0.22	0.24	0.39	0.71	0.97	0.61	0.81	0.58	0.73
5	Catechin	<lod	<lod	<lod	<lod	<lod	<lod	0.14	<lod	<lod	<lod	<lod	<lod	<lod	<lod
6	*p*-Hydroxyphenylacetic acid	<lod	0.31	<lod	<lod	<lod	<lod	<lod	<lod	0.33	<lod	0.69	0.62	0.67	0.67
7	Caffeic acid	0.17	0.37	0.21	0.17	0.23	0.20	0.19	0.19	0.16	0.34	0.22	0.31	0.22	0.21
8	Vanillic acid	0.29	0.48	0.43	0.08	0.15	0.11	0.11	0.15	0.40	0.44	0.90	1.02	0.83	0.87
9	Syringic acid	0.05	0.11	0.42	0.03	0.03	0.06	0.05	0.09	0.05	0.19	0.10	0.22	0.13	0.35
10	Rutin	0.13	0.13	0.21	0.26	<lod	0.22	0.24	0.23	0.29	<lod	0.11	0.14	0.15	0.14
11	*p*-Coumaric acid	1.97	3.44	3.06	4.59	5.07	6.04	5.69	3.48	2.58	4.19	3.68	2.53	2.62	2.44
12	Luteolin 7-*O*-glucoside	2.08	0.69	1.24	<lod	<lod	<lod	<lod	0.15	<lod	<lod	<lod	<lod	<lod	<lod
13	Quercetin 3-*O*-galactoside	0.05	0.03	0.04	<lod	0.02	0.02	0.02	0.04	0.03	0.03	0.02	0.03	0.02	0.03
14	Ellagic acid	0.58	0.14	0.47	0.07	0.06	<lod	0.02	0.08	0.04	0.12	0.04	0.05	0.04	0.03
15	Apigenin 7-*O*-apioglucoside	<lod	<lod	<lod	<lod	<lod	<lod	<lod	<lod	0.02	<lod	0.02	0.02	0.02	0.02
16	Ferulic acid	0.32	0.29	0.32	0.05	<lod	0.02	0.02	0.13	0.05	0.19	0.04	0.07	0.05	0.07
17	Naringin	0.02	0.01	0.01	0.01	0.01	<lod	<lod	0.05	0.03	0.02	<lod	<lod	<lod	0.01
18	Kaempferol 3-*O*-glucoside	0.01	<lod	<lod	<lod	<lod	<lod	0.01	<lod	<lod	0.01	<lod	<lod	<lod	<lod
19	Aesculetin	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.09	0.09	0.09	<lod	0.08	0.09	0.09
20	Luteolin	0.13	0.26	0.21	0.24	0.30	0.41	0.42	0.24	0.26	0.60	0.24	0.41	0.22	0.24
21	Quercetin	0.32	0.29	0.60	0.14	0.27	0.12	0.12	0.24	0.32	0.46	0.27	0.47	0.26	0.57
22	Cinnamic acid	0.02	0.06	0.14	0.10	0.35	0.08	0.06	0.32	0.08	0.43	0.04	0.08	0.05	0.07
23	Baicalein	0.02	0.17	0.04	<lod	0.07	0.06	0.06	0.12	0.08	0.24	0.19	0.22	0.19	0.15

24	Apigenin	<lod	0.04	0.01	0.03	0.01	<lod	<lod	0.01	<lod	<lod	0.02	0.02	<lod	0.01
25	Naringenin	0.02	0.04	0.03	0.03	0.06	0.02	0.02	0.12	0.03	0.08	0.04	0.05	0.03	0.04
26	Genistein	<lod	0.44	<lod	0.36	0.14	<lod	<lod	0.17	<lod	0.47	0.20	0.27	0.28	0.10
27	Kaempferol	0.17	0.27	0.31	0.19	0.31	0.15	0.17	0.23	0.4	0.43	0.35	0.40	0.21	0.31
28	Amentoflavone	<lod	0.01	<lod	0.01	0.01	0.01	0.01	<lod	0.03	0.13	0.01	0.02	0.01	0.01
29	Chrysin	0.02	0.57	0.05	0.52	0.33	0.06	0.04	0.16	0.02	0.77	0.34	0.35	0.44	0.09
30	Pinocembrin	<lod	0.33	0.02	0.35	0.20	0.06	0.03	0.10	<lod	0.44	0.20	0.24	0.29	0.04
31	Galangin	<lod	0.41	0.07	0.32	0.30	0.07	0.06	0.16	0.05	0.57	0.24	0.28	0.39	0.09

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