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Original research paper

Effect of *in vitro* gastrointestinal digestion on the chemical composition and antioxidant properties of *Ginkgo biloba* leaves decoction and commercial capsules

YUBO ZHOU*
YINGXIN YANG
MINYAN MA
LINGYUN XIE
AIJUAN YAN
WEN CAO

College of Pharmacy, Zhejiang Pharmaceutical College
Ningbo, Zhejiang, 315100, China

ABSTRACT

In this study *Ginkgo biloba* leaves (GBL) decoction and commercial capsules were digested using an *in vitro* model. Thirty-six active compounds were identified and quantified by HPLC-ESI-MS analysis based on the MS/MS patterns (precursor ions and product ions) and retention times, in comparison with reference standards. Most compounds in GBL showed a significant decrease during intestinal digestion, with an exception of vanillic acid and biflavonoids. Bioaccessibility values of chemical compositions varied between decoction and capsules samples. Also, significant reductions of total flavonoids and total phenolic content was observed after *in vitro* digestion. Both, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) scavenging capacity decreased after gastric digestion, but increased during intestinal digestion. Nevertheless, different behaviour was observed in reducing antioxidant power (FRAP) assay. Compared to the pH of digestion, the influence of digestive enzymes on the chemical composition and antioxidant activity of GBL was relatively minor. Overall, these results may help provide a valid foundation for further investigations on bioactive compounds and the pharmacodynamics of GBL.

Keywords: Ginkgo biloba L., *in vitro* digestion, HPLC-MS/MS, chemical composition, antioxidant property, bioaccessibility

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Ginkgo biloba L., as an important herbal medicine and a health dietary supplement, has been widely accepted and received increased attention over recent years (1, 2). *Ginkgo biloba* leave (GBL) and its preparations are used to treat tinnitus, hypertension, cardiovascular and cerebrovascular diseases, Alzheimer's disease, *etc*. (3–5). The major bioactive compounds in GBL are reported to be flavonoids, terpene lactones and phenolic acids.

Oral administration is the main route of delivery for traditional medicines; GBL is mostly prepared in the form of decoction, capsule, tablet or syrup and ingested *per os*. The

* Correspondence; e-mail: wszyb9132000@126.com

chemical composition and biological actions of GBL and its preparations could be affected by chemical transformations after human digestion. Only bioactive compounds released from the matrix during gastrointestinal (GI) digestion can be bioaccessible and then available for absorption (bioavailable) to exert health benefits (6). Bioaccessibility has been defined as the fraction of a compound that is released from its matrix in the GI tract and thus becomes available for intestinal absorption (7, 8). Therefore, it is important to investigate the stability, chemical transformations and bioaccessibility of the compounds during digestion in the GI tract to gain a better understanding of their biological effects. In recent years, for making a first screening of the behaviour of the chemical composition in food materials after ingestion, *in vitro* digestion models are carried out as the most common approach, and bioaccessibility of dietary compounds in food are determined (9–11). *In vitro* digestion models simulating the physical and chemical processes that occur during GI digestion are relatively simple, inexpensive and rapid, present fewer ethical restrictions, and conditions can be effectively controlled (10–12). In addition, it is found that *in vitro* digestion models are well correlated with clinical and *in vivo* studies (13).

Here we performed a simulated *in vitro* gastrointestinal digestion (14) of *Ginkgo biloba* leaves decoction (GBLD) and commercial capsules (GBLC). The aim of the current work was to explore the chemical transformations of GBLD and GBLC which occurred during the digestion process in terms of: (*i*) the content of individual compounds and bioaccessibility indexes, (*ii*) total flavonoid and total phenolic content, (*iii*) antioxidant activity. To the best of our knowledge, this is the most complete study reporting the effect of *in vitro* digestion on the chemical composition and antioxidant properties of GBLD and GBLC.

EXPERIMENTAL

Samples

The dried *Ginkgo biloba* leaves and commercial capsules (Shanghai Sine Promod Pharmaceutical Co., Ltd., China) were obtained from a local pharmacy. Each capsule contains a mixture of 40 mg of GBL extract (not less than 9.6 mg flavonol glycosides and 2.4 mg terpene lactones) with excipients; total mass 200 mg.

Reagents

Reference compounds (all ≥ 97 % purity) were all purchased from Chengdu Must Biotechnology Co., Ltd. (China) (Table I). Chemicals including bile salt (a mixture of various taurocholic acid, mainly glycodeoxycholate, taurodeoxycholate and taurocholate), pepsin, pancreatin (a mixture of various enzymes extracted from porcine pancreas, mainly trypsin, pancreatic amylase and pancreatic lipase), Folin-Ciocalteu reagent, potassium persulfate, ferrous sulfate heptahydrate, ferric chloride, ferrous chloride tetrahydrate, aluminium nitrate nonahydrate, hydrochloric acid, sodium acetate, sodium bicarbonate, sodium hydroxide, sodium nitrite, sodium carbonate, ethylenediaminetetraacetic acid and sodium chloride were purchased from Sinopharm Chemical Reagent Co. Ltd (China). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-*S*-triazine (TPTZ), 2,2′-azino-bis(3-ethylbenzthiazoline)-6-sulphonic acid (ABTS), Trolox, L-ascorbic acid and 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p*′-disulfonic acid monosodium salt hydrate were obtained from

Table I. Retention times and optimized mass spectrometry parameters of 36 active compounds under negative MRM mode by HPLC-MS/MS[a]

No.	Reference standard	t_R (min)	M_r	Precusor ion (*m/z*)	Product ion (*m/z*)	Declustering potential (V)	Collision energy (V)
1	Gallic acid	0.83	170.12	169.00	125.00	–40	–20
2	Epigallocatechin	1.61	306.27	304.60	124.60	–100	–26
3	Vanillic acid	2.53	168.14	166.90	107.80	–50	–29
4	Caffeic acid	2.63	180.15	178.80	134.60	–60	–22
5	Clitorin	4.13	740.66	739.10	283.70	–170	–53
6	Ferulic acid	4.25	194.19	192.80	134.00	–45	–20
7	Rutin	4.36	610.51	609.00	299.80	–100	–50
8	Myricitrin	4.38	464.38	463.10	315.50	–100	–40
9	Bilobalide	4.44	326.30	325.10	162.80	–75	–22
10	Ginkgolide C	4.46	440.40	439.00	383.00	–85	–25
11	Galuteolin	4.56	448.38	447.10	284.50	–130	–40
12	Isoquercitrin	4.57	464.38	463.00	299.80	–130	–35
13	Kaempferol-3-*O*-glucorhamnoside	4.58	594.52	593.00	283.30	–130	–45
14	Hyperin	4.61	464.38	462.80	299.90	–120	–40
15	Quercetin-3-(2″-glucosyl)rhamnoside	4.92	610.52	609.10	299.70	–140	–48
16	Kaempferol-3-*O*-β-D-rutinoside	4.98	594.52	593.00	284.40	–130	–40
17	Narcissin	5.07	624.56	623.10	314.30	–140	–48
18	Quercitrin	5.29	448.38	447.00	299.50	–120	–35
19	Apigenin-7-*O*-glucoside	5.39	432.38	431.00	267.80	–120	–50

No.	Reference standard	t_R (min)	M_r	Precusor ion (*m*/*z*)	Product ion (*m*/*z*)	Declustering potential (V)	Collision energy (V)
20	Myricetin	5.93	318.24	317.00	150.60	–95	–32
21	Quercetin-3-*O*-2′′-(6′′′-*p*-coumaroyl) glucosyl-rhamnoside	6.14	756.66	755.10	608.80	–120	–40
22	Ginkgolide A	6.69	408.40	407.10	351.10	–40	–23
23	Kaempferol-3-*O*-2′′-(6′′′-*p*-coumaroyl) glucosyl-rhamnoside	6.69	740.66	740.00	593.00	–120	–40
24	Ginkgolide B	6.70	424.40	423.00	367.00	–80	–24
25	Luteolin	7.27	286.23	284.70	132.80	–90	–40
26	Quercetin	7.39	302.24	300.90	150.90	–140	–30
27	Naringenin	8.38	272.25	271.00	118.80	–80	–30
28	Genistein	8.48	270.24	269.00	132.70	–80	–40
29	Apigenin	8.57	270.24	268.90	116.90	–100	–50
30	Kaempferol	8.88	286.23	285.00	116.80	–100	–56
31	Isorhamnetin	9.02	316.26	314.90	299.60	–90	–28
32	Amentoflavone	10.27	538.46	537.00	374.90	–130	–50
33	Bilobetin	11.55	552.48	551.00	518.70	–160	–40
34	Genkwanin	11.70	284.27	282.80	267.80	–90	–35
35	Ginkgetin	12.80	566.51	564.80	532.60	–200	–42
36	Isoginkgetin	12.82	566.51	565.00	532.80	–160	–40

[a] See Fig. 1.

MRM – multi reaction monitoring

Aladdin (China). Acetone (HPLC grade) was obtained from Tedia (USA). Methanol, alcohol, and all other solvents were of analytical purity grade and purchased from Sinopharm Chemical Reagent Co.

Preparation of GBLD

The *Ginkgo biloba* leaves were rinsed with distilled water and then dried at 60 °C until constant mass. The dried leaves weighing 15.0 g were refluxed twice for 1 hour with the addition of water (150 mL). The two decoctions were merged and concentrated to a volume of 150 mL by rotatory evaporation under reduced pressure.

Gastrointestinal digestion in vitro

The *in vitro* digestion was carried out using the reported method with some changes (14) and composed of two steps (gastric and intestinal digestion). The salivary step was not included because the residence time of decoction or capsule in the mouth is negligible after directly drinking or swallowing. The procedure consisted of two separate digestions (separate gastric and small intestinal digestion) and one continuous digestion (complete gastrointestinal digestion).

Simulation of digestion for GBLD in vitro. – In the gastric digestion step, the decoction (15 mL) was acidified to pH 2.0 with HCl (6 mol L^{-1}) and mixed with 1 mL of a solution of pepsin (16 %, *m/V*, pepsin in 0.1 mol L^{-1} HCl with proper electrolytes), then incubated for 2 h. In the small intestinal digestion step, the decoction (15 mL) was adjusted to pH 5.3 with a saturated solution of $NaHCO_3$ and mixed with 5 mL of pancreatin-bile solution (0.4 % pancreatin and 2.5 % bile salt, *m/V*, in 0.1 mol L^{-1} $NaHCO_3$), then the sample was re-adjusted with a saturated solution of $NaHCO_3$ to pH 7.0 and digested for 4 h.

In the complete digestion, both the gastric and intestinal digestion were the same as described above and combined; after the gastric digestion phase, the sample was treated for intestinal digestion. In order to test the influence of digestive enzymes on *in vitro* digestion, GBLD underwent the same gastrointestinal digestion procedure without adding enzymes. In the gastric digestion step, the acidified decoction (pH 2.0) was mixed with 1 mL of a solution of 0.1 mol L^{-1} HCl with proper electrolytes, in the small intestinal digestion step, the decoction (adjusted to pH 5.3) was mixed with 5 mL of a bile salt solution (2.5 %, *m/V*, in 0.1 mol L^{-1} $NaHCO_3$), then the sample was re-adjusted to pH 7.0 and digested for 4 h. Water was used as a control. Samples were incubated in a water bath shaker at 37 °C and shaken (100 rpm) during all digestion steps. The final samples were diluted with water and centrifuged (4000 rpm, 30 min), then the supernatant was filtered through an Amicon filter (10 kDa, Millipore, USA).

Simulation of digestion for GBLC in vitro. – The contents of twenty *Ginkgo biloba* leaf commercial capsules (0.2 g per capsule) were quantitatively transferred to a 100-mL flask and homogenized. Then the aliquot (0.1 g) was mixed with 15 mL of water, and digestion steps were carried out as detailed above.

HPLC-ESI-MS/MS

A reversed-phase C18 column (50 × 2.1 mm, 2.6 μm, Phenomenex, USA) was used in an HPLC system consisting of an LC-20A liquid chromatograph (Shimadzu, Japan). It was

equipped with SIL-20A/20AC autosampler and two LC-20ADXR gradient pumps, connected to a triple quadrupole-linear ion trap composite mass spectrometer (QTRAP 4500, AB SCIEX, USA) equipped with an electrospray ionization (ESI) source.

The samples were eluted with aq. formic acid (0.1 %, *V/V*, solvent A) and acetonitrile (solvent B) as follows: 0–10 min B linearly increased from 10 to 40 %, 10–15 min B linearly increased from 40 to 90 %, 15–16 min B maintained at 90 %, 16–17 min B linearly decreased from 90 to 10 %, 17–20 min B maintained at 10 %. The flow rate was 0.2 mL min^{-1} and the injection volume was 1 μL.

The ESI source was operated in a negative polarity mode since it produced more efficient ionization of the compounds relative to the positive polarity mode. The optimal MS parameters were set as follows: ion spray voltage 4500 V, source temperature 500 °C, curtain gas 68.9 kPa, collision gas 62.1 kPa, ion source gas 1 and ion source gas 2: 344.7 kPa, drying gas N_2, scan range *m/z* 100–1000. Detection of the analytes was performed in multiple reaction monitoring (MRM) mode and the conditions are indicated in Table I which was optimized by infusing individual standard solutions into the ion source by using a Harvard Apparatus syringe pump (flow rate of 10 μL min^{-1}, Harvard Apparatus, USA). Each sample was monitored with the precursor ion/product ion transitions and MRM peak area was used for quantification. Individual compounds were quantified based on external standard calibration curves of reference standards and the content expressed as μg per 1 g (dm) GBL sample studied. LC-ESI-MS/MS control and data analysis were performed by Analyst software (version 1.6.3, Applied Biosystems, Foster City, CA, USA).

Total flavonoid content (TFC)

TFC was measured by the procedure of Zhishen *et al*. (15) with some modifications. Sample solution (0.3 mL) and 5 % $NaNO_2$ (0.3 mL) were mixed in a test tube and 10 % $Al(NO_3)_3$ solution (0.3 mL) was added 6 min later. After another 6 min, 4 % NaOH (4 mL) was added, mixed and kept for 1 min. Then the solution was diluted with 50 % aq. ethanol to 10 mL and incubated at room temperature for 15 min. The absorbance was recorded at 510 nm. TFC was calculated from a standard curve of rutin and expressed as mg rutin equivalents (RE) per 1 g of GBL or GBLC samples.

Total phenolic content (TPC)

Sample solution (0.1 mL) was mixed with Folin-Ciocalteu reagent (2.5 mL, diluted 1:10 with water) in a test tube and 10 % Na_2CO_3 solution (2.5 mL) was added 4 min later (16). Then, the solution was diluted with distilled water to 10 mL and incubated at room temperature for 2 h. The absorbance was measured at 765 nm immediately. TPC of the sample was expressed as mg gallic acid equivalents (GAE) per 1 g of GBL or GBLC samples.

Antioxidant activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay. – The DPPH radical scavenging activity was evaluated using the method described by Gordon *et al*. (17) with some modifications. The sample solution was mixed with DPPH (in methanol) and methanol. Then the absorbance was read at 517 nm after 30 min of incubation in the dark at room temperature. Results were expressed as μmol of Trolox equivalents (TE) per 1 g of sample.

2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay. – The ABTS radical scavenging activity was determined by a previous method (18) with some modifications. $ABTS^+$ was generated by mixing ABTS solution and $K_2S_2O_8$ solution. The solution was incubated in the dark for 16 h and then diluted with methanol to the absorbance of 0.7 ± 0.02 (734 nm). The sample solution was diluted with methanol and mixed with fresh $ABTS^+$. Absorbance was measured after 30 min and the results were expressed as μmol TE g^{-1} of the sample.

Ferric reducing antioxidant power (FRAP). – FRAP of the samples was determined by the known method (19) with minor changes as follows: the sample solution was diluted with methanol and mixed with FRAP reagent. After 30 min of incubation in the dark at room temperature, the absorbance was recorded at 593 nm. The ferric reducing antioxidant power was expressed as μmol TE g^{-1} of the sample.

All the results are expressed on a dry mass sample basis.

Bioaccessibility of individual compounds

In vitro models have been used to simulate the bioaccessibility of active compounds in plant-like polyphenols extensively (20). In our work, GBLC and GBLD were submitted to an *in vitro* digestion process in order to evaluate the stability and bioaccessibility of active compounds under gastrointestinal conditions. Bioaccessibility of the sample components was calculated by the following formula:

$$\text{Bioaccessibility (\%)} = \frac{A_{\text{digest}}}{A_{\text{sample}}} \times 100$$

where A_{digest} denotes the amount of a compound in the gastrointestinal digested fraction and A_{sample} is the amount of the compound in GBLD or GBLC before digestion.

Statistical analysis

All the data measured in triplicate were presented as mean ± standard deviation. Statistical analysis of the significant differences was carried out using Statistical Package for the Social Sciences (SPSS) software (version 26 for Windows, USA). The Least Significant Difference (LSD) and Student-Newman-Keuls (S-N-K) tests were used to determine any significant difference and Pearson correlation coefficient was used to measure the correlation matrix. Statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

Model validation of HPLC-MS/MS analysis

To assess the validity of the HPLC-MS/MS method, validation tests were run using reference standards according to the guidelines (21, 22). The stock solution containing 36 reference substances was prepared in HPLC-grade methanol and then appropriately diluted to yield a series of concentrations for constructing calibration curves at seven levels. All 36 reference substances showed good linearity with correlation coefficients (R) higher than 0.999 in the corresponding concentration ranges (Table II). *LODs* ranged from 0.6 to 3.3 ng mL^{-1} and *LOQs* were found in the range from 2.1 to 12.1 ng mL^{-1}, indicating good

Table II. Method validation of HPLC–MS/MS analysis

Analyte	Regression line equation[a]	Linearity range (μg mL^{-1})	R^2[b]	*LOD/LOQ* (ng mL^{-1})[c]	Precision (RSD, %)[d]		Recovery (%)			
					Interday	Intraday	GBLD		GBLC	
							Mean	RSD (%)	Mean	RSD (%)
Gallic acid	$y = 1256500x - 121105$	0.0480–1.20	0.9999	2.3/7.5	1.9	3.4	90.5	7.3	93.7	3.5
Epigallocatechin	$y = 72679x - 16730$	0.0500–2.50	0.9998	1.1/4.9	1.8	2.3	95.2	6.3	106.2	4.2
Vanillic acid	$y = 91506x - 10110$	0.0510–2.55	0.9995	0.9/3.5	1.3	1.9	106.4	5.3	96.7	5.5
Caffeic acid	$y = 1882024x - 32197$	0.0540–1.35	0.9998	2.1/5.9	1.6	3.3	91.2	5.9	95.6	5.3
Clitorin	$y = 312795x - 54230$	0.1130–2.26	0.9998	1.2/5.0	3.1	4.3	93.7	2.0	106.4	3.8
Ferulic acid	$y = 538721x - 4906$	0.0500–2.50	0.9994	2.9/10.0	0.9	2.6	87.5	3.7	89.3	4.6
Rutin	$y = 987591x - 152369$	0.0500–1.25	0.9996	2.5/9.2	2.7	3.8	108.7	6.3	102.3	6.3
Myricitrin	$y = 206481x - 10812$	0.0112–0.45	0.9997	0.7/3.1	2.2	4.5	93.9	3.9	91.5	4.7
Bilobalide	$y = 493058x + 37064$	0.0450–2.25	0.9992	2.7/10.7	1.0	1.9	87.1	2.5	88.5	2.7
Ginkgolide C	$y = 552442x - 55331$	0.0600–1.50	0.9998	2.5/10.1	2.6	4.3	108.7	3.9	93.6	4.3
Galuteolin	$y = 263576x - 75247$	0.0450–2.25	0.9996	1.8/6.8	2.4	3.8	107.1	5.6	110.5	5.9
Isoquercitrin	$y = 663026x + 8451$	0.0525–2.62	0.9995	1.2/3.9	1.7	3.5	92.9	6.4	108.2	4.8
Kaempferol-3-*O*-glucorhamnoside	$y = 30913x - 4170$	0.0450–2.25	0.9998	0.7/2.1	0.7	2.8	89.4	2.6	91.5	3.8
Hyperin	$y = 1015711x - 110155$	0.0428–2.14	0.9994	0.7/3.0	0.8	2.3	90.9	5.3	89.8	5.9
Quercetin-3-(2″-glucosyl)rhamnoside	$y = 610735x - 63120$	0.0404–2.02	0.9995	1.1/4.3	1.9	4.0	105.5	4.3	103.4	3.5
Kaempferol-3-*O*-β-D-rutinoside	$y = 107067x - 28774$	0.0582–2.91	0.9998	0.9/3.9	1.3	4.0	105.0	2.5	96.4	6.7
Narcissin	$y = 218261x - 33547$	0.0564–2.82	0.9995	1.4/5.2	2.6	2.8	95.6	3.8	93.9	4.5
Quercitrin	$y = 174546x - 9353$	0.0490–2.45	0.9999	1.8/8.7	1.8	3.3	98.7	3.7	98.2	3.9
Apigenin-7-*O*-glucoside	$y = 1000363x + 242904$	0.0616–3.08	0.9995	0.9/3.4	1.0	2.2	88.7	4.7	91.5	3.6
Myricetin	$y = 311900x - 137882$	0.0550–2.75	0.9991	1.5/5.4	0.6	3.3	101.6	7.0	88.9	4.5

Analyte	Regression line equation[a]	Linearity range (μg mL^{-1})	R^2[b]	*LOD*/*LOQ* (ng mL^{-1})[c]	Precision (RSD, %)[d]		Recovery (%)			
					Interday	Intraday	GBLD		GBLC	
							Mean	RSD (%)	Mean	RSD (%)
Quercetin-3-*O*-2″-(6‴-*p*-coumaroyl) glucosyl-rhamnoside	$y = 128611x - 11441$	0.0567–2.83	0.9992	0.8/4.0	2.0	3.1	102.7	7.7	105.3	3.9
Ginkgolide A	$y = 7884x+1049$	0.0550–2.75	0.9996	0.6/2.2	1.7	4.6	93.7	7.0	97.9	2.4
Kaempferol-3-*O*-2″-(6‴-*p*-coumaroyl) glucosyl-rhamnoside	$y = 21745x–3717$	0.0525–2.62	0.9998	2.2/8.3	1.4	3.1	91.8	5.3	96.6	5.3
Ginkgolide B	$y = 1277892x - 15119$	0.0500–2.50	0.9999	0.7/2.3	2.4	4.0	100.7	5.6	93.5	3.7
Luteolin	$y = 951832x - 145682$	0.0450–2.25	0.9997	1.2/4.5	1.7	2.6	108.5	3.1	90.8	3.9
Quercetin	$y = 34148x - 21195$	0.0540–2.70	0.9992	3.3/12.1	3.3	5.3	87.1	2.5	85.6	7.1
Naringenin	$y = 866519x + 25281$	0.0525–2.62	0.9999	1.8/6.8	1.7	3.4	87.8	4.9	89.5	4.9
Genistein	$y = 98059x + 18721$	0.0450–2.25	0.9993	2.3/8.4	1.3	3.8	91.5	3.8	110.4	5.5
Apigenin	$y = 1327725x - 67133$	0.0128–1.27	0.9999	0.9/4.1	0.5	2.9	93.6	4.2	103.7	2.4
Kaempferol	$y = 589467x - 13160$	0.0480–2.40	0.9992	2.1/8.0	3.6	4.4	102.0	5.7	82.8	5.5
Isorhamnetin	$y = 33690300x - 534168$	0.0430–2.15	0.9998	0.6/2.3	1.3	4.2	89.2	5.4	87.7	7.8
Amentoflavone	$y = 809978x - 175868$	0.0490–2.45	0.9993	1.1/3.4	1.0	2.4	87.3	6.5	82.3	3.5
Bilobetin	$y = 569111x - 16405$	0.0135–1.35	0.9992	1.9/6.2	2.2	3.8	98.2	4.0	110.7	5.3
Genkwanin	$y = 59994200x + 286872$	0.0130–1.30	0.9999	1.5/5.5	0.8	2.7	104.2	3.7	84.16	5.7
Ginkgetin	$y = 138681x - 18656$	0.0148–1.48	0.9990	1.0/4.5	2.6	4.6	87.5	4.8	103.1	6.3
Isoginkgetin	$y = 1175859x - 2304$	0.0150–1.50	0.9995	2.5/8.9	3.1	3.8	92.0	6.4	115.5	4.6

[a] x – concentration (μg mL^{-1}), y – counts (peak area);[b] R – correlation coefficient; [c] *LOD* (limit of detection): signal to noise (*S*/*N*) = 3; *LOQ* (limit of quantification): signal to noise (*S*/*N*) = 10.

[d] Intra-day: obtained by analysing the same standard mixture solution six times on the same day; inter-day: obtained by analysing the standard mixture solution once a day on three consecutive days;

[e] Recovery was evaluated by using the standard addition method and the assay for the GBL samples.

sensitivity. Intra-day and inter-day precision RSDs ranged from 0.5 to 3.6 % and 1.9 to 5.3 %, resp. The sample solution stability was assessed under different conditions (15 °C for 2 h, 4 °C for 4 h, and −20 °C for 2 weeks) and the result showed the analytes were stable at all tested conditions with RSD values < 7.6 %. The results of recovery were satisfactory (87.1–108.7 % for GBLD and 82.3–115.5 % for GBLC), as shown in Table II. The method specificity was tested by measuring retention time (t_R) stability (3 times over a 3-days period) and setting a pair of precursor/product ions; t_R deviation was ≤ 1.8 %. The matrix effect was evaluated by dividing the slopes of calibration curves obtained from GBL samples spiked at different concentrations by the slope of the calibration curve (the results gained for standards). The matrix effects were between 87.8 and 112.4 %, which indicate an acceptable matrix effect (ion suppression/enhancement). The established HPLC-MS/MS method described was applicable for simultaneous determination of the analyzed 36 constitutions.

Analysis and bioaccessibility of samples components

Based on the MS/MS patterns (precursor ions and product ions) and retention times in comparison with the data of reference standards 36 compounds were identified and quantified in the samples and the results are summarized in Tables III–V. Values in brackets represent each compound recovery, the percentage of the compound that remains stable after *in vitro* digestion process. The chromatograms of GBLD and GBLC at different

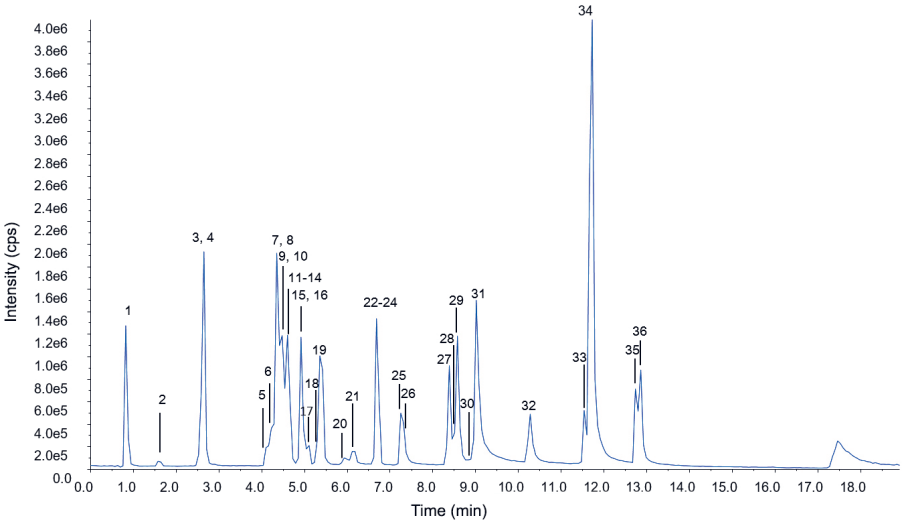

Fig. 1. The HPLC-QQQ-MS/MS chromatogram of a standard mixture: 1 – gallic acid, 2 – epigallocatechin, 3 – vanillic acid, 4 – caffeic acid, 5 – clitorin, 6 – ferulic acid, 7 – rutin, 8 – myricitrin, 9 – bilobalide, 10 – ginkgolide C, 11 – galuteolin, 12 – isoquercitrin, 13 – kaempferol–3-*O*-glucorhamnoside, 14 – hyperin, 15 – quercetin-3-2″-glucosyl-rhamnoside, 16 – kaempferol-3-*O*-β-D-rutinoside, 17 – narcissin, 18 – quercitrin, 19 – apigenin-7-*O*-glucoside, 20 – myricetin, 21 – quercetin-3-*O*-2″-6‴-*p*-coumaroyl-glucosyl-rhamnoside, 22 – ginkgolide A, 23 – kaempferol-3-*O*-2″-6‴-*p*-coumaroyl-glucosyl-rhamnoside, 24 – ginkgolide B, 25 – luteolin, 26 – quercetin, 27 – naringenin, 28 – genistein, 29 – apigenin, 30 – kaempferol, 31 – isorhamnetin, 32 – amentoflavone, 33 – bilobetin, 34 – genkwanin, 35 – ginkgetin, 36 – isoginkgetin.

Table III. Content of individual phenolic and ginkgolide compounds before and after gastric, intestinal and complete in vitro digestion

Analyte (μg g^{-1}) [a,b]	Sample	Control	Digestion[c]					
			Without enzymes			With enzymes		
			Gastric	Intestinal	Complete	Gastric	Intestinal	Complete
Phenolics								
Gallic acid	GBLD	12.39 ± 0.50[d]	14.06 ± 1.23 (114)[d]	ND	ND	13.01 ± 1.39 (105)[d]	ND	ND
	GBLC	20.77 ± 0.81[d]	15.61 ± 1.47 (75)[f]	ND	ND	17.49 ± 1.52 (84)[e]	ND	ND
Epigallo-catechin	GBLD	9.90 ± 0.41[d]	0.94 ± 0.13 (10)[f]	ND	ND	1.28 ± 0.16 (13)[e]	ND	ND
	GBLC	98.32 ± 4.81[d]	37.11 ± 3.50 (38)[f]	ND	ND	70.02 ± 8.01 (71)[e]	ND	ND
Vanillic acid	GBLD	27.60 ± 1.05[e]	29.75 ± 1.67 (108)[e]	35.00 ± 2.56 (126)[d]	34.31 ± 2.41 (124)[d]	28.87 ±2.43 (105)[e]	33.95 ± 1.45 (123)[d]	32.22 ± 2.58 (116)[d,e]
	GBLC	57.46 ± 3.20[f]	61.81 ± 5.41 (108)[f]	82.39 ± 6.01 (143)[d]	74.58 ± 5.97(130)[d,e]	61.89 ± 4.58 (108)[f]	80.37 ± 4.64 (140)[d]	70.26 ± 6.54 (122)[e]
Caffeic acid	GBLD	5.44 ± 0.27[d]	5.64 ± 0.62 (104)[d]	ND	ND	5.40 ± 0.43 (99)[d]	ND	ND
	GBLC	36.60 ± 1.46[d]	33.95 ± 2.66 (93)[d,e]	ND	ND	31.68 ± 3.24 (87)[e]	ND	ND
Ferulic acid	GBLD	5.45 ± 0.23[d]	5.92 ± 0.56 (109)[d]	1.26 ± 0.13 (23)[g]	2.12 ± 0.23 (39)[f]	5.94 ± 0.48 (109)[d]	3.37 ± 0.35 (62)[e]	1.88 ± 0.20 (34)[f]
	GBLC	14.83 ± 0.74[e]	19.08 ± 1.72 (129)[d]	6.21 ± 0.57 (42)[f]	4.69 ± 0.55 (31)[f,g]	18.36 ± 1.54 (123)[d]	3.58 ± 0.31 (24)[g,h]	2.87 ± 0.32 (19)[h]
Ginkgolides								
Bilobalide	GBLD	56.58 ± 2.36[e]	68.70 ± 4.19(121)[d]	ND	ND	67.38 ± 3.93 (119)[d]	ND	ND
	GBLC	2539.58 ± 98.50[e]	2893.68 ± 231.49 (114)[d]	ND	ND	2890.39 ± 202.89 (114)[d]	ND	ND
Ginkgolide A	GBLD	146.78 ± 7.49[e]	153.75 ± 8.30 (105)[e]	33.42 ± 2.01 (23)[f,g]	44.31 ± 3.42 (30)[f,g]	186.33 ± 19.50 (127)[d]	31.10 ± 2.14 (21)[g]	48.47 ± 4.28 (33)[f]
	GBLC	2808.33 ± 95.48[d]	2555.17 ± 125.20 (91)[e]	114.75 ± 8.03 (4.1)[f]	203.64 ± 12.01(7.3)[f]	2755.26 ± 195.62 (98)[d]	133.36 ± 8.11 (4.7)[d]	193.32 ± 13.79 (6.9)[d]

Analyte ($\mu g\ g^{-1}$)[a,b]	Sample	Control	Digestion[c]					
			Without enzymes			With enzymes		
			Gastric	Intestinal	Complete	Gastric	Intestinal	Complete
Ginkgolide B	GBLD	73.45 ± 3.97[d]	74.33 ± 4.05 (101)[d]	21.17 ± 1.57 (29)[e]	24.03 ± 1.98 (33)[e]	74.45 ± 3.87 (101)[d]	19.88 ± 1.88 (27)[e]	24.37 ± 2.01 (33)[e]
	GBLC	948.17 ± 39.82[d]	958.63 ± 48.29 (101)[d]	182.34 ± 12.40 (19)[e]	221.56 ± 12.85 (23)[e]	975.73 ± 42.01 (103)[d]	171.99 ± 11.40 (18)[c]	183.34 ± 13.48 (19)[e]
Ginkgolide C	GBLD	92.52 ± 3.61[e]	104.73 ± 4.23 (113)[d]	8.37 ± 0.72 (9)[f]	9.96 ± 0.67(11)[f]	100.85 ± 5.85 (109)[d]	7.38 ± 0.71 (8)[f]	11.10 ± 0.97 (12)[f]
	GBLC	1397.92 ± 55.92[e]	1564.44 ± 68.84 (112)[d]	32.91 ± 2.47 (2.4)[f]	45.00 ±3.52 (3.2)[f]	1535.29 ± 112.08 (110)[d]	32.16 ± 2.37 (2.3)[f]	32.69 ± 2.52 (2.3)[f]

[a] Dry mass basis; [b] Mean ± SD (n = 3); [c] Value in parenthesis indicate bioaccessibility (%) of an individual compound.
Values in the same row with different letters (d-h) indicate statistically significant differences ($p < 0.05$); ND – not detected

Table IV. Content of individual flavonol aglycones and biflavonoids before and after the gastric, intestinal and complete in vitro digestion

Analyte ($\mu g\ g^{-1}$)[a,b]	Sample	Control	Digestion[c]					
			Without enzymes			With enzymes		
			Gastric	Intestinal	Complete	Gastric	Intestinal	Complete
Myricetin	GBLD	1.92 ± 0.12[d]	1.33 ± 0.09 (69)[e]	0.77 ± 0.06 (40)[f]	0.85 ± 0.07 (44)[f]	1.34 ± 0.11 (70)[e]	0.80 ± 0.06 (42)[f]	0.81 ± 0.07 (42)[f]
	GBLC	31.50 ± 1.67[d]	25.80 ± 1.87 (82)[e]	11.15 ± 1.08 (35)[g]	11.03 ± 0.97 (35)[g]	21.68 ± 1.55 (69)[f]	11.21 ± 0.83 (36)[g]	11.38 ± 1.01 (36)[g]
Luteolin	GBLD	3.16 ± 0.17[d]	2.15 ± 0.14 (68)[e]	0.38 ± 0.03 (12)[g]	0.61 ± 0.05 (19)[f]	2.14 ± 0.13 (68)[e]	0.39 ± 0.03 (12)[g]	0.72 ±0.07 (23)[f]
	GBLC	9.87 ± 0.93[d]	8.72 ± 0.72 (89)[d,e]	5.73 ± 0.55 (58)[g,h]	4.68 ± 0.50 (47)[h]	7.86 ± 0.74 (80)[e,f]	8.66 ± 1.21 (88)[d,e]	6.51 ± 0.66 (66)[f,g]
Quercetin	GBLD	9.97 ± 0.58[d]	5.95 ± 0.41 (60)[e]	ND	ND	5.17 ± 0.37 (52)[f]	ND	ND
	GBLC	38.77 ± 2.48[e]	41.02 ± 2.77 (106)[d,e]	ND	ND	43.49 ± 3.35 (112)[d]	ND	ND
Naringenin	GBLD	6.33 ± 0.31[d]	6.33 ± 0.37 (100)[d]	0.94 ± 0.10 (15)[f]	1.46 ± 0.13 (23)[e]	6.78 ± 0.58 (107)[d]	1.56 ± 0.15 (25)[e]	1.62 ± 0.14 (26)[e]
	GBLC	1.26 ±0.12[e]	1.62 ± 0.15 (128)[d]	0.40 ± 0.05 (32)[g]	0.38 ± 0.04 (31)[g]	1.47 ± 0.12 (117)[d]	0.98 ± 0.23 (78)[f]	0.58 ± 0.07 (46)[g]

Analyte (μg g^{-1})[a,b]	Sample	Control	Digestion[c]					
			Without enzymes			With enzymes		
			Gastric	Intestinal	Complete	Gastric	Intestinal	Complete
Genistein	GBLD	ND	ND	ND	ND	ND	ND	ND
	GBLC	ND	ND	ND	ND	ND	ND	ND
Apigenin	GBLD	3.15 ± 0.27^{d}	2.17 ± 0.18 (69)^{e}	1.34 ± 0.15 (43)^{f,g}	1.52 ± 0.18(48)^{f}	2.20 ± 0.21 (70)^{e}	0.90 ± 0.12 (29)^{h}	1.04 ± 0.16 (33)^{g,h}
	GBLC	2.67 ± 0.27^{d,e}	3.07 ± 0.42 (115)^{d}	3.16 ± 0.37 (118)^{d}	2.70 ± 0.35 (101)^{d,e}	2.24 ± 0.30 (84)^{e}	2.40 ± 0.31 (90)^{e}	2.44 ± 0.40 (91)^{e}
Kaempferol	GBLD	6.25 ± 0.36^{d}	4.66 ± 0.67 (75)^{e}	ND	ND	4.54 ± 0.30 (73)^{e}	ND	ND
	GBLC	13.52 ± 1.49^{d,e}	14.37 ± 1.91 (106)^{d}	ND	ND	12.01 ± 1.44 (89)^{e}	ND	ND
Isorhamnetin	GBLD	1.71 ± 0.09^{d}	1.10 ± 0.08 (65)^{e}	ND	ND	1.13 ± 0.11 (66)^{e}	ND	ND
	GBLC	5.99 ± 0.34^{d}	6.16 ± 0.62 (103)^{d}	ND	ND	5.60 ± 0.60 (94)^{d}	ND	ND
Genkwanin	GBLD	0.43 ± 0.05^{d}	0.15 ± 0.02 (34)^{g}	0.24 ± 0.02 (55)^{e}	0.27 ± 0.03 (64)^{e}	0.16 ± 0.02(37)^{f,g}	0.19 ± 0.02 (45)^{f}	0.19 ± 0.03 (45)^{f}
	GBLC	ND	ND	ND	ND	ND	ND	ND
Amento-flavone	GBLD	0.51 ± 0.05^{d,e}	0.39 ± 0.03 (78)^{f}	0.50 ± 0.06 (100)^{d,e}	0.52 ± 0.05 (103)^{d}	0.40 ± 0.04 (79)^{f}	0.45 ± 0.05 (90)^{e,f}	0.53 ± 0.06 (106)^{d}
	GBLC	5.73 ± 0.36^{e}	5.55 ± 0.61 (97)^{e}	6.01 ± 0.72 (105)^{d,e}	5.79 ± 0.60 (101)^{e}	5.58 ± 0.49 (97)^{e}	7.04 ± 0.82 (123)^{d}	6.55 ± 0.59 (114)^{d,e}
Bilobetin	GBLD	0.72 ± 0.08^{d}	0.087 ± 0.011 (12)^{h}	0.50 ± 0.06 (70)^{f}	0.39 ± 0.05 (54)^{g}	0.094 ± 0.015 (13)^{h}	0.49 ± 0.04 (69)^{f}	0.60 ± 0.08 (83)^{e}
	GBLC	1.19 ± 0.13^{d,e}	0.93 ± 0.12 (78)^{e,f}	1.41 ± 0.23 (118)^{d}	1.23 ± 0.19 (103)^{d,e}	0.84 ± 0.10 (70)^{f}	1.30 ± 0.25 (109)^{d}	1.36 ± 0.22 (114)^{d}
Ginkgetin	GBLD	0.79 ± 0.15^{d}	0.27 ± 0.03 (35)^{g,h}	0.32 ± 0.02 (40)^{f,g,h}	0.35 ± 0.04 (45)^{f,g}	0.24 ± 0.02 (30)^{h}	0.40 ± 0.06 (51)^{f}	0.51 ± 0.05 (65)^{e}
	GBLC	4.17 ± 0.40^{d}	3.82 ± 0.51 (92)^{d}	3.73 ± 0.33 (89)^{d}	3.57 ± 0.31 (86)^{d}	3.52 ± 0.43(84)^{d}	4.44 ± 0.52 (106)^{d}	3.77 ± 0.29 (90)^{d}
Isoginkgetin	GBLD	0.78 ± 0.12^{d}	0.07 ± 0.02 (8.3)^{h,i}	0.12 ± 0.02 (16)^{g,h}	0.17 ± 0.03 (21)^{g}	0.03 ± 0.01 (4.0)^{i}	0.30 ± 0.01 (39)^{f}	0.49 ± 0.05(63)^{e}
	GBLC	1.27 ± 0.23^{d}	0.52 ± 0.05 (41)^{f}	0.65 ± 0.04 (51)^{f}	0.57 ± 0.07 (45)^{f}	0.34 ± 0.04 (27)^{f}	0.88 ± 0.13 (69)^{e}	0.93 ± 0.11 (74)^{e}

[a] Dry mass basis; [b] Mean ± SD (n = 3); [c] Values in parentheses indicate bioaccessibility (%) of an individual compound.
Values in the same row with different letters (d-i) indicate statistically significant differences ($p < 0.05$); ND – not detected

Table V. Contents of individual flavonol glycosides before and after the gastric, intestinal and complete in vitro digestion

Analyte ($\mu g\ g^{-1}$)[a,b]	Sample	Control	Digestion[c]					
			Without enzymes			With enzymes		
			Gastric	Intestinal	Complete	Gastric	Intestinal	Complete
Clitorin	GBLD	176.94 ± 9.56[d]	180.33 ± 10.27 (102)[d]	48.63 ± 2.85 (27)[g]	71.98 ± 5.33 (41)[f]	168.17 ± 8.74 (95)[d]	101.32 ± 6.59 (57)[e]	100.42 ± 8.23 (57)[e]
	GBLC	2474.75 ±111.36[d,e]	2657.97 ± 108.98 (107)[d]	1885.73 ± 116.92 (76)[g]	1448.96 ± 137.89 (59)[h]	2440.69 ± 118.59 (98)[d,e,f]	2240.55 ± 152.36 (91)[f]	2283.77 ± 157.58 (92)[e,f]
Rutin	GBLD	90.13 ± 1.25[e]	100.93 ± 6.56 (112)[d]	1.64 ± 0.15 (1.8)[h]	13.66 ± 1.41 (15)[g]	101.55 ± 8.21 (113)[d]	6.34 ± 0.60 (7.0)[h]	22.00 ± 2.37(24)[f]
	GBLC	1763.67 ± 29.56[d]	1696.34 ± 60.27 (96)[d]	473.55 ± 32.61 (27)[f]	179.16 ± 15.63 (10)[g]	1750.75 ± 85.28 (99)[d]	974.63 ± 85.36 (55)[e]	1049.14 ± 77.29 (59)[e]
Myricitrin	GBLD	1.09 ± 0.03[e]	1.40 ± 0.11 (129)[d]	ND	ND	1.44 ± 0.15 (132)[d]	ND	ND
	GBLC	50.56 ± 4.50[e]	65.37 ± 6.55 (129)[d]	ND	ND	61.69 ± 6.37 (122)[d]	ND	ND
Galuteolin	GBLD	2.58 ± 0.13[d]	2.48 ± 0.15 (96)[d]	0.60 ± 0.06 (23)[f]	0.98 ± 0.10 (38)[e]	2.47 ± 0.11 (96)[d]	0.65 ± 0.06 (25)[f]	1.04 ± 0.11 (41)[e]
	GBLC	58.15 ± 2.52[e]	64.67 ± 2.58 (111)[d]	25.40 ± 2.75 (44)[g]	18.07 ± 1.36 (31)[h]	63.71 ± 3.71 (110)[d]	34.50 ± 3.67 (59)[f]	35.86 ± 2.99 (62)[f]
Isoquercitrin	GBLD	24.44 ± 0.67[e]	26.58 ± 0.88 (109)[d]	0.16 ± 0.02 (0.6)[h]	1.72 ± 0.10 (7.0)[f,g]	26.00 ± 1.71 (106)[d]	0.75 ± 0.06 (3.1)[g,h]	2.88 ± 0.25 (12)[f]
	GBLC	687.92 ±17.52[e]	728.44 ± 23.89 (106)[d]	111.98 ± 10.82 (16)[g]	36.69 ± 2.77 (5.3)[h]	708.96 ± 29.32 (103)[d]	290.80 ± 13.25 (42)[f]	310.74 ± 16.96 (45)[f]
Kaempferol-3-*O*-gluco-rhamnoside	GBLD	4.48 ± 0.25[d]	4.83 ± 0.31 (108)[d]	0.74 ± 0.06 (16)[g]	1.22 ± 0.10 (27)[f]	4.63 ± 0.35 (103)[d]	1.75 ± 0.15 (39)[e]	1.72 ± 0.16 (38)[e]
	GBLC	42.03 ± 2.31[d]	43.58 ± 3.22 (103)[d]	26.60 ± 2.05 (63)[f]	15.20 ± 1.51 (36)[g]	44.49 ± 2.59 (106)[d]	37.39 ± 2.91 (89)[e]	34.80 ± 3.01 (83)[e]
Hyperin	GBLD	19.32 ± 0.59[e]	20.05 ± 1.17 (104)[d,e]	0.32 ± 0.03 (1.7)[h]	1.64 ± 0.16 (8.5)[g]	20.85 ± 0.96 (108)[d]	0.82 ± 0.07 (4.2)[g,h]	2.71 ± 0.21 (14)[f]
	GBLC	510.50 ± 20.93[e]	586.51 ± 53.88 (115)[d]	93.26 ± 8.96 (18)[g]	33.75 ± 3.17 (6.6)[g]	547.80 ± 36.12 (107)[d,e]	233.68 ± 16.25 (46)[f]	249.80 ± 12.37 (49)[f]
Quercetin-3-(2′′-glucosyl)-rhamnoside	GBLD	64.02 ± 4.02[d]	58.83 ± 2.11 (92)[e]	12.74 ± 0.84 (20)[g]	26.98 ± 1.92 (42)[f]	59.50 ± 2.87 (93)[e]	23.42 ± 1.90 (37)[f]	27.53 ± 1.82 (43)[f]
	GBLC	1872.25 ± 68.63[d]	1952.11 ± 50.75 (104)[d]	1428.64 ± 72.86 (76)[f]	1132.56 ± 39.64 (60)[g]	1956.46 ± 54.78 (104)[d]	1652.49 ± 109.06 (88)[e]	1514.36 ± 106.03 (81)[f]

Analyte ($\mu g\ g^{-1}$)[a,b]	Sample	Control	Digestion[c]					
			Without enzymes			With enzymes		
			Gastric	Intestinal	Complete	Gastric	Intestinal	Complete
Kaempferol-3-*O*-β-D-rutinoside	GBLD	239.28 ± 10.77^{d}	251.00 ± 11.55 (105)^{d}	59.40 ± 3.68 (25)^{f}	96.26 ± 7.80 (40)^{e}	242.67 ± 11.65 (101)^{e}	108.28 ± 5.85 (45)^{e}	110.63 ± 4.98 (46)^{e}
	GBLC	3616.67 ±130.20^{d}	3513.04 ± 116.93 (97)^{d}	2617.27 ± 136.10 (72)^{f}	2257.43 ± 160.28 (62)^{g}	3533.53 ± 88.34 (98)^{d}	2955.22 ± 195.04 (82)^{e}	2865.42 ± 231.87 (79)^{e,f}
Narcissin	GBLD	158.47 ± 7.92^{d}	168.33 ± 9.43 (106)^{d}	39.32 ± 2.20 (25)^{g}	63.14 ± 3.91 (40)^{f}	160.88 ± 8.56 (102)^{c}	75.42 ± 3.17 (48)^{d}	77.68 ± 4.89(49)^{e}
	GBLC	2169.17 ± 99.78^{e}	2453.86 ± 132.51 (113)^{d}	1621.76 ± 85.95 (75)^{g}	1426.91 ± 97.03 (66)^{g}	2238.24 ± 194.72 (103)^{c,d}	1897.76 ± 106.27 (87)^{e}	1854.84 ± 127.98 (86)^{f}
Quercitrin	GBLD	24.28 ± 1.02^{e}	26.00 ± 0.68 (107)^{d}	1.70 ± 0.09(7.0)^{h}	5.02 ± 0.32 (21)^{g}	26.37 ± 1.16 (109)^{c}	4.19 ± 0.23 (17)^{f}	6.93 ± 0.54 (29)^{f}
	GBLC	442.92 ± 23.47^{e}	468.15 ± 22.47 (106)^{d,e}	196.91 ± 10.44 (44)^{g}	140.73 ± 5.49 (32)^{h}	482.48 ± 27.98 (109)^{c}	279.35 ± 19.00 (63)^{e}	280.75 ± 19.93 (63)^{f}
Apigenin-7-*O*-glucoside	GBLD	19.15 ± 0.52^{d}	18.48 ± 1.09 (97)^{d,e}	16.07 ± 0.92 (84)^{f}	16.93 ± 1.30 (88)^{e,f}	17.60 ± 1.16 (92)^{c,d,e}	11.42 ± 0.61 (60)^{f}	12.62 ± 0.49 (66)^{g}
	GBLC	331.42 ± 14.25^{d,e}	347.54 ± 21.90 (105)^{d}	315.37 ± 13.56 (95)^{e}	317.64 ± 13.66 (96)^{d,e}	333.58 ± 19.68 (101)^{c,d}	273.63 ± 16.14 (83)^{e}	260.84 ± 18.78 (79)^{f}
Quercetin-3-*O*-2″-(6‴-*p*-coumaroyl)-glucosyl-rhamnoside	GBLD	154.49 ± 8.50^{d}	161.28 ± 5.97 (104)^{d}	40.37 ± 1.78 (26)^{g}	78.65 ± 3.70 (51)^{e}	161.87 ± 9.39 (105)^{c}	41.42 ± 2.73 (27)^{f}	64.83 ± 3.50 (42)^{f}
	GBLC	6041.67 ± 202.08^{d}	6130.89 ± 306.54 (101)^{d}	3892.22 ±233.53 (64)^{f}	3131.81 ± 172.25 (52)^{g}	6408.91 ± 365.31 (106)^{c}	4577.11 ± 224.28 (76)^{d}	4453.13 ± 298.36 (74)^{e}
Kaempferol-3-*O*-2″-(6‴-*p*-coumaroyl) glucosyl-rhamnoside	GBLD	189.78 ± 6.26^{d}	187.17 ± 10.11 (99)^{d}	136.97 ± 8.90 (72)^{f}	154.36 ± 8.80 (81)^{e}	181.67 ± 7.99 (96)^{c}	102.08 ± 6.12(54)^{f}	101.93 ± 5.61 (54)^{g}
	GBLC	6187.50 ± 266.49^{d}	5885.16 ± 235.50 (95)^{d,e}	5743.51 ± 299.69 (93)^{d,e}	5500.50 ± 322.54 (89)^{e,f}	6136.14 ± 315.44 (99)^{c}	5507.46 ± 429.58 (89)^{d,e}	5138.61 ± 377.89 (83)^{f}

[a] Dry mass basis; [b] Mean ± SD (n = 3); [c] Values in parentheses indicate bioaccessibility (%) of an individual compound.
Values in the same row with different letters (d–h) indicate statistically significant differences ($p < 0.05$); ND – not detected

digestion stages compared to the control (the undigested samples) are depicted in Figs. 2 and 3: chromatographic profiles display that MRM peak area of some compounds in GBL samples showed no obvious changes after gastric digestion, whereas the content of most constituents decreased during the intestinal stage and complete gastrointestinal digestion.

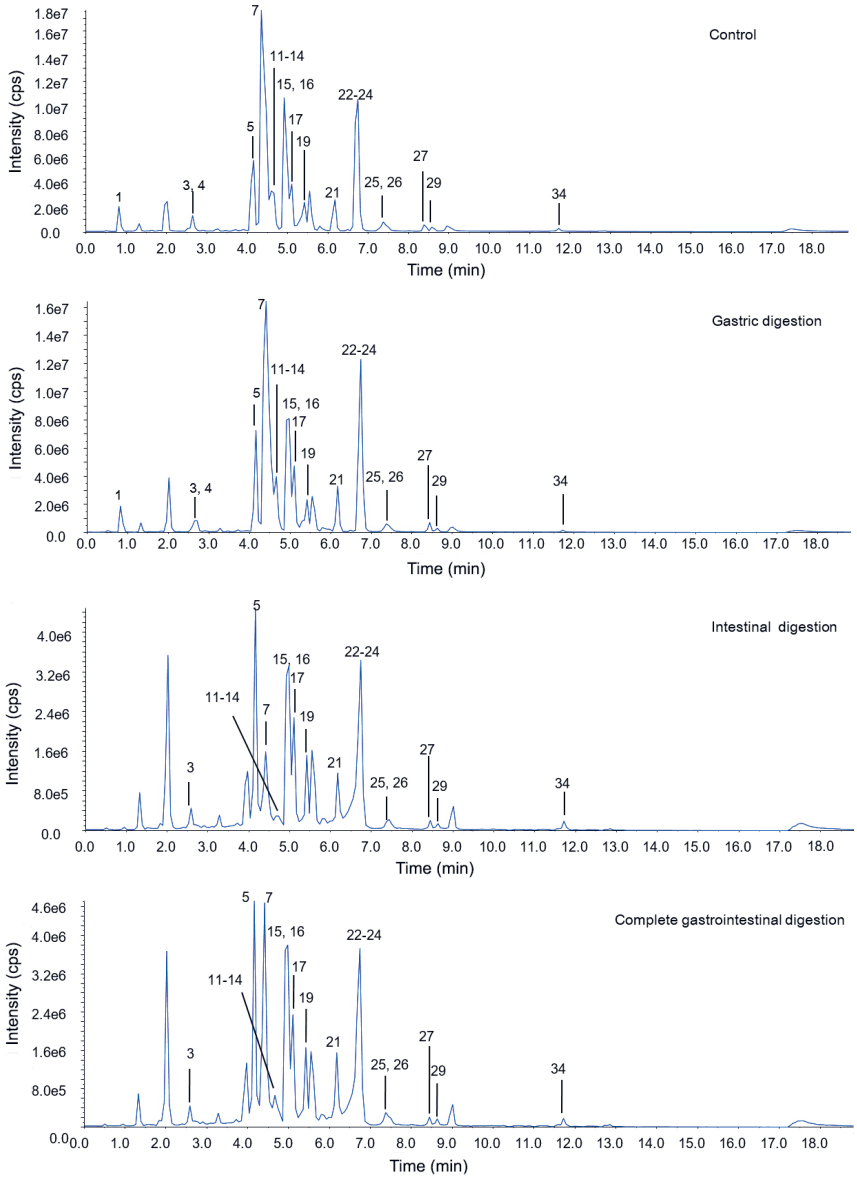

Fig. 2. HPLC profiles of *Ginkgo biloba* leaves decoction (GBLD) samples after *in vitro* digestion process with enzymes (for the keys to the peaks see Fig. 1).

In GBLD and GBLC before *in vitro* digestion, flavonol glycosides and ginkgolides were shown to be the most abundant compounds (Tables III, IV and V). The five principal flavonol glycosides identified were kaempferol-3-*O*-β-D-rutinoside, kaempferol-3-*O*-2′′-(6′′′-*p*-coumaroyl)glucosyl-rhamnoside, clitorin, narcissin and quercetin-3-*O*-2′′-(6′′′-*p*-coumaroyl)-

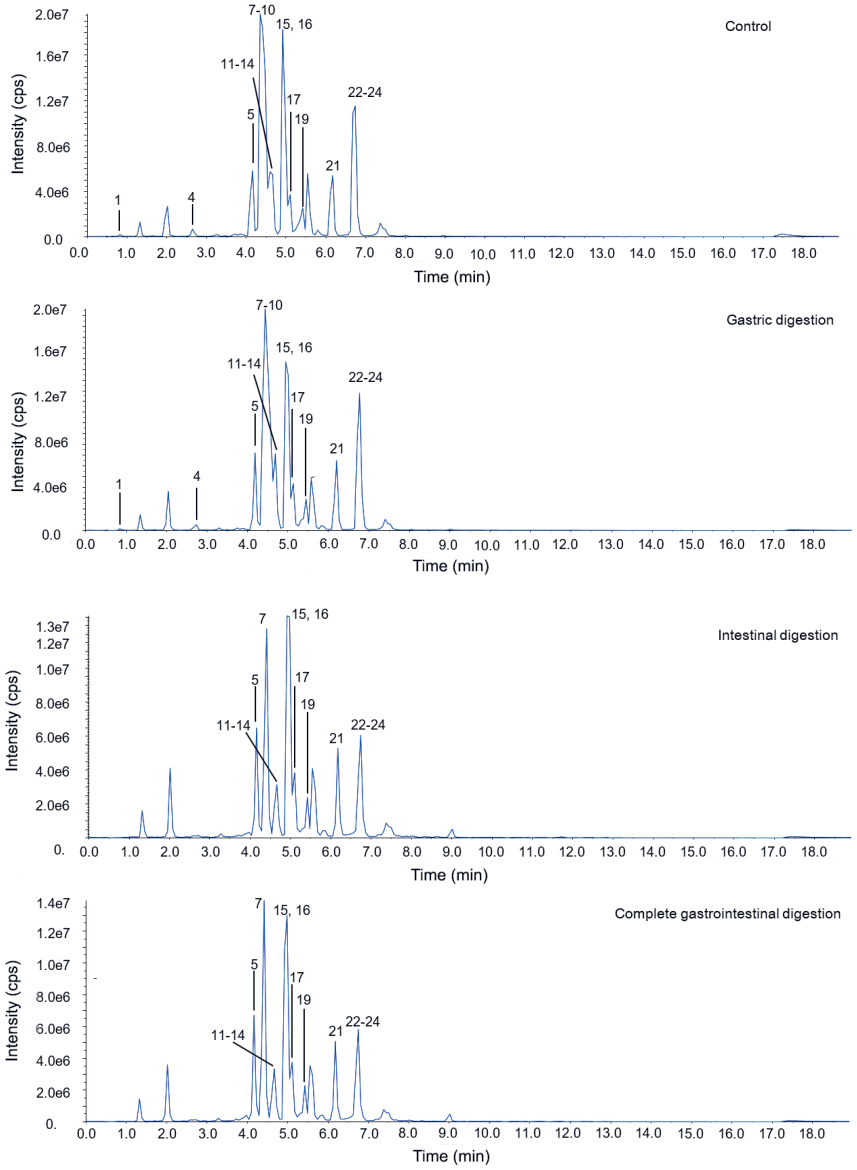

Fig. 3. HPLC profiles of *Ginkgo biloba* leaves commercial capsules (GBLC) samples after *in vitro* digestion process with enzymes (for the keys to the peaks see Fig. 1).

glucosyl-rhamnoside. Bilobalide, ginkgolide A, B and C were the four main ginkgolides in undigested GBLD and GBLC. The contents of flavonol aglycones and biflavones were low in the original GBL sample, and some compounds such as genistein reported to be present (23) could not be detected. After *in vitro* GI digestion, flavonol glycosides and ginkgolides were still the most abundant compounds in digested GBLD and GBLC. The five main flavonol glycosides identified were the same as in the undigested samples, nevertheless, ginkgolides showed a significant decrease and bilobalide was even found undetectable after *in vitro* digestion.

Phenolics. – As shown in Table III, most phenolics, except epigallocatechin, in GBLD and GBLC were relatively stable after the gastric digestion stage, and ferulic acid in GBLC increased by more than 23 %. The increase may be due to the improved release of phenolics bound to the matrix (such as polysaccharides, proteins) at low pH (24). After intestinal and complete gastrointestinal digestion, the content of ferulic acid in GBLD and GBLC decreased significantly ($p < 0.05$). In completely digested GBLD and GBLC, gallic acid, epigallocatechin and caffeic acid were hardly detectable, whereas the content of vanillic acid was found to increase. The higher content of vanillic acid following intestinal and complete gastrointestinal digestion in this study may be attributed to the alkaline environment of the intestine where insoluble vanillic acid conjugates coupled to the plant matrix depolymerized and were subsequently released. Results of intestinal and complete gastrointestinal digestion indicated that the majority of the phenolics were degraded or transformed during intestinal digestion which had been reported for some herbal teas (25) and low bioaccessibility of most phenolic compounds present in GBLD and GBLC could be observed in this study.

Ginkgolides. – Ginkgolides are important active components of *Ginkgo biloba* which possess high medicinal value (5) of improving the memory and learning ability, protecting the cerebral hippocampal neurons against epilepsy, alleviating neuronal injury, *etc*. The main ginkgolides in GBLD and GBLC were quantified and shown in Table III. During the gastric phase, ginkgolides remained stable and the amount of bilobalide and ginkgolide C showed higher values compared to the control ($p < 0.05$) possibly because of improved release from the matrix at low pH. However, ginkgolides showed a significant decrease with low bioaccessibility values (less than 33 %) and bilobalide even disappeared at intestinal and complete digestion stages, indicating that terpene lactones could be hydrolyzed in the alkaline environment of the intestine.

Flavonol aglycones and biflavonoids. – Flavonol aglycones and biflavonoids were detected but their concentrations were low in GBLD and GBLC (Table IV). The stability of most flavonols was compromised after *in vitro* digestion and a significant decrease was observed during intestinal and complete digestion for all flavonol aglycones ($p < 0.05$), with the exception of apigenin. Apigenin was detected in GBLC and showed little variation (recovery was more than 84 %) in concentration with respect to the undigested sample. Biflavonoids in GBL, including amentoflavone, bilobetin, ginkgetin, and isoginkgetin (26), are important active compounds that show neuroprotective, antiviral, antitubercular, anti-inflammatory, inhibition of cytochrome P450 enzymes, *etc*., properties (3). Until now only a little bit of attention has been paid to biflavonoids in GBL. As compared with the control, the amount of amentoflavone changed minimally during *in vitro* digestion, whereas bilobetin, ginkgetin, and isoginkgetin in GBLD varied widely, especially at the gastric digestion stage. The

percentage recovery of all biflavonoids during the gastric phase was lower compared to the value for intestinal and complete digestion stages, in contrast to the results obtained for most flavonol aglycones.

Flavonol glycosides. – In regard to flavonol glycosides (Table V), all of them were quite stable after the gastric digestion (recovery more than 90 %) and the concentration of myricitrin increased significantly ($p < 0.05$), namely, by more than 22 %. The reason for the increase may be similar to phenolics. Nevertheless, after intestinal and complete digestion, the loss of flavonol glycosides was apparent and myricitrin was not detected. Reduction in the amount of flavonol glycoside during *in vitro* digestion may be accompanied by an increase in the number of corresponding aglycones. However, this was not observed. It seems that the aglycones have been greatly degraded after *in vitro* digestion process.

Influence of digestive enzymes. – The difference between the two groups (digested with enzymes and digested without enzymes) varied from compound to compound. There was no significant difference in ginkgolides between the groups, implying that the content of studied ginkgolides in GBL during *in vitro* digestion may be influenced mainly by the pH of the digestion medium. Still, in regard to flavonoid glycosides, a significant difference could be observed between the two groups at the intestinal and complete digestion stages. In the present study, the digested sample with enzymes showed significantly higher content of most flavonoid glycosides, except for apigenin-7-*O*-glucoside and kaempferol-3-*O*-2''-(6'''-*p*-coumaroyl)glucosyl-rhamnoside (they showed lower content in the digested sample with enzymes) compared with the digested sample without enzymes.

Effect of in vitro *digestion on TFC and TPC*

TFC and TPC of *Ginkgo biloba* samples prior and after *in vitro* gastrointestinal digestions are shown in Fig. 4. TFC in GBLD and GBLC decreased during *in vitro* digestion. A significant decrease was observed for TFC after gastric digestion with a recovery of less than 73 %. However, after intestinal and complete digestion, the TFC values significantly showed recovery ratios higher than 83.5 %. Thus, the loss of TFC in GBL mainly happened with gastric digestion, while this finding was different from the result of the change of individual flavonoids measured by HPLC. Compared with the control, TPC in GBLD and GBLC after *in vitro* digestion were significantly lower; apparent reductions of more than 13 % happened during intestinal and complete digestion.

Compared with TFC and TPC in the samples digested with enzymes, no significant difference was observed for those in group digested without enzymes, except for TFC in GBLC, indicating that the digestive enzymes might have a slight influence on the TFC and TPC values in GBLD.

Content changes during different digestion phases were greater for individual flavonoids or phenolics than for TFC and TPC. Colourimetric assays using aluminium nitrate or Folin-Ciocalteu reagent are not highly selective and may not describe the changes of flavonoids or phenolics in the samples exactly. These determinations may be affected by other bioactive compounds (*e.g.*, sugars, carotenoids, amino acids, vitamins and proteins) (27, 28). Therefore, HPLC-related methods, such as LC-MS might be methods of choice (29). Thus, we determined the contents of 36 compounds in GBLD and GBLC prior to and after *in vitro* digestions using HPLC-MS/MS.

Effects of in vitro *digestion on antioxidant activity*

In this study, the antioxidant activity of GBLD and GBLC submitted to *in vitro* digestion was investigated using DPPH, ABTS and FRAP assays as shown in Fig. 5. GBLC showed higher antioxidant activity than GBLD possibly due to the higher concentration of TFC and TPC (Fig. 4), which play a major role in the antioxidant property of many plants (30).

DPPH assay. – As regards to DPPH assay, gastric digestion decreased the antioxidant values in both GBLC and GBLD samples significantly ($p < 0.05$) compared with the control. After intestinal and complete digestion, the increases were observed, and the DPPH values in GBLC were even significantly higher than that of the undigested sample ($p < 0.05$) whereas Tables II, III and IV show significantly reduced content of flavonoid and phenolic compounds when detected chromatographically after intestinal and complete digestion. The result suggested that these flavonoid and phenolic compounds may have been degraded or converted in an alkaline environment to some metabolites which exhibit higher antioxidant activity in radical scavenging capacity against DPPH (31). In addition, we did not detect all the compounds in GBL prior to and after *in vitro* digestions.

ABTS assay. – The ABTS results suggested that the antioxidant activity decreased markedly after the gastric phase, especially in GBLD (from 77.56 to 43.84 μmol Trolox g^{-1}). On the contrary, ABTS values increased significantly after intestinal and complete digestion in GBLC ($p < 0.05$), being in agreement with DPPH assay findings.

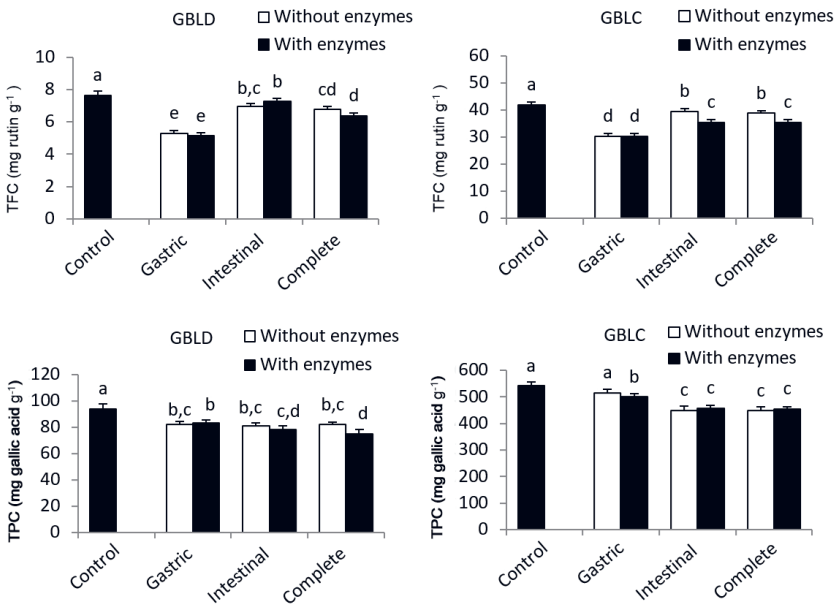

Fig. 4. TFC and TPC of GBL samples after *in vitro* digestion process with or without enzymes. Values are expressed in mg per 1 g of GBL sample (dry mass basis), mean ± SD, $n = 3$. Different letters of each value denote significant difference ($p < 0.05$).

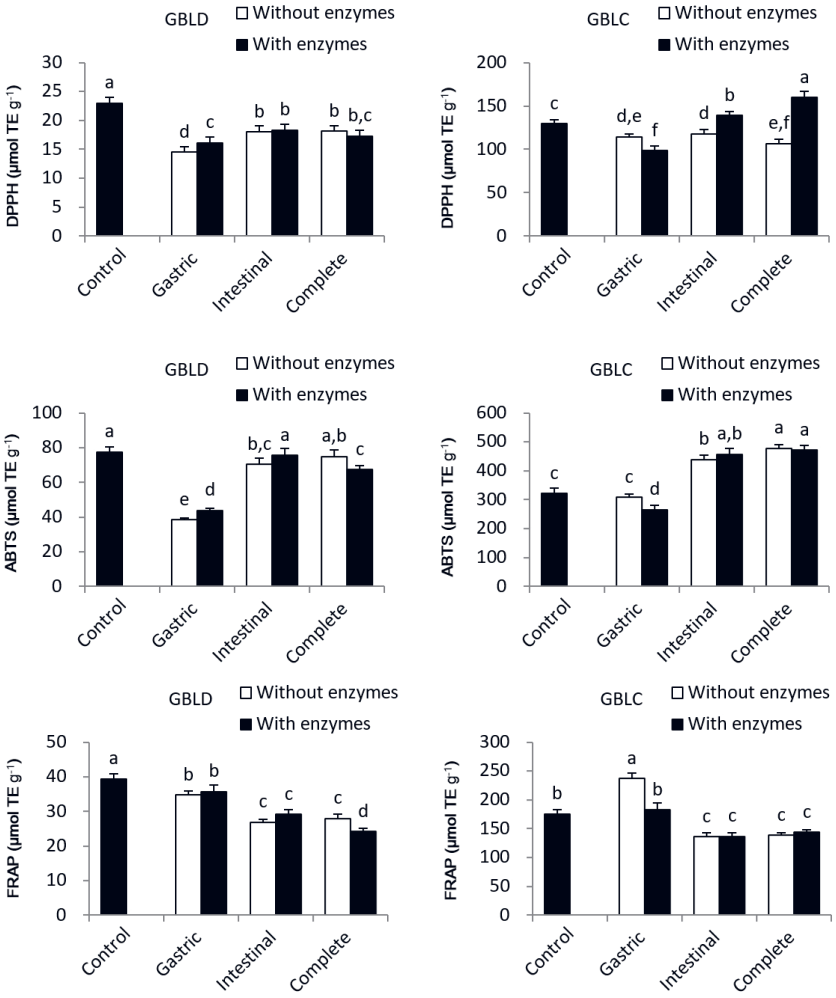

Fig. 5. Antioxidant activities (DPPH, ABTS, FRAP) of GBL samples after *in vitro* digestion process with or without enzymes. Values are expressed in μmol TE per 1 g of sample (dry mass basis); mean ± SD, $n = 3$. Different letters of each value denote significant differences ($p < 0.05$).

FRAP assay. – The antioxidant activity evaluated by FRAP assay decreased significantly after the gastric digestion phase for GBLD and continued to decrease after intestinal and complete digestion, but increased slightly for GBLC after gastric digestion. However, FRAP decreased significantly ($p < 0.05$) after intestinal and complete digestion in GBLC.

The results obtained in the FRAP assay differed from that of ABTS and DPPH assays. ABTS and DPPH values during the intestinal and complete digestion phase were higher than those measured for the gastric phase, whereas different behaviour was observed when FRAP was used; different mechanisms of the assays may result in these discrepancies.

No significant difference was found between GBL samples digested with enzymes and without enzymes, except for the DPPH assay in GBLC (Fig. 5). These results implicate that the antioxidant activity of GBL during *in vitro* digestion may be influenced mainly by the acidity of the digestion medium.

In vitro digestion studies showed that flavonoid and phenolic contents and antioxidant activity of food or plant samples were affected by different ways after digestion (32, 33). Some chlorogenic acid derivatives were stable during digestive conditions, although anthocyanins degraded throughout digestion. The majority of tea's antioxidant activity is rapidly decreased while TPC remained relatively stable (34). However, Pellegrini *et al*. (35) reported antioxidant capacity being increased during *in vitro* gastrointestinal digestion while TPC was highest after gastric digestion and showed a slight decrease after the intestinal phase. These reports showed that the antioxidant activity of compounds in plants was maintained, increased or reduced, depending not only on their stability during *in vitro* digestion, but also on the nature of the derivatives formed after *in vitro* digestion (36, 37). The *in vitro* digestion steps could result in biochemical transformations in the plant matrix (not only in raw material but also in the extract) and lead to changes in antioxidant activity by the way of degradation, formation of new compounds and new pro-oxidants (38, 39).

Correlation analysis

The correlation between TFC, TPC and antioxidant capacities was also calculated. In GBLD, TFC showed significant correlations with DPPH ($R = 0.854$, $p < 0.05$) and ABTS ($R = 0.954$, $p < 0.01$) values before and after the simulated digestion what indicated that TFC might be the key substances in DPPH and ABTS radical scavenging. Still, TPC showed comparable correlations with FRAP values ($R = 0.850$, $p < 0.05$). In GBLC, only TPC was significantly correlated to FRAP ($R = 0.763$, $p < 0.05$) during the simulated digestion. Thus, phenolic compounds contribute significantly to the FRAP values throughout *in vitro* digestion.

CONCLUSIONS

A simulated *in vitro* gastrointestinal digestion was carried out to measure changes in the chemical composition and antioxidant activity of GBLD and GBLC, and to evaluate the bioaccessibilities of individual compounds after *in vitro* digestion. The contents of individual compounds, TFC, TPC and antioxidant activity of *Ginkgo biloba* leaves changed differently during various *in vitro* digestions. During the gastric digestion step, the higher recovery of most compounds could possibly be associated with their greater stability under acidic conditions. On the other hand, the instability of the compounds in neutral to alkaline pH values in the intestine digestion step may result in their lower bioaccessibility. Individual compounds changed more at the intestinal and complete digestion stage, with the exception of biflavonoids. Additionally, the bioaccessibility of individual compounds following exposure to *in vitro* gastrointestinal conditions varied among GBLD and GBLC. The bioaccessibility of flavonol aglycones, glycosides and biflavones in GBLC showed to be higher than in GBLD, whereas ginkgolides in GBLC showed to be lower than in GBLD. This fact is probably due to the complex composition of GBLD and multiple interactions occurring between bioactive compounds. Despite the reduction of TFC and TPC observed after *in vitro* digestion, antioxidant activity obtained from DPPH and ABTS assays showed

an increasing trend during intestinal and complete digestion, suggesting the formation or a strong release of bioactive compounds with high radical scavenging capacity. These changes may be mainly caused by the acidity of the digestion medium, whereas digestive enzymes seemed to have a primary influence on the changes of flavonoid glycosides in GBLD and GBLC, and TFC and DPPH values in GBLC. The results in this research are believed to be a valid foundation for further investigations of bioactive compounds in GBL and may help understand the changes of chemical composition and antioxidant activity of GBL during *in vitro* digestion. The scientific information obtained in this study could provide an important basis for elucidating the pharmacodynamics of GBL.

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Conflict of interest. – The authors declare no conflict of interests.

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