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Biotransformation of flavonols and taxifolin in hepatocyte *in vitro* systems as determined by liquid chromatography with various stationary phases and electrospray ionization-quadrupole time-of-flight mass spectrometry

Jan Vacek[a,*], Barbora Papoušková[b], Pavel Kosina[a], Jiří Vrba[a], Vladimír Křen[c], Jitka Ulrichová[a]

[a] Department of Medical Chemistry and Biochemistry, Faculty of Medicine and Dentistry, Palacký University, Hněvotínská 3, CZ 775 15 Olomouc, Czech Republic
[b] Regional Centre of Advanced Technologies and Materials, Department of Analytical Chemistry, Faculty of Science, Palacký University, 17. listopadu 12, CZ 771 46 Olomouc, Czech Republic
[c] Institute of Microbiology, Center of Biocatalysis and Biotransformation, Academy of Sciences of the Czech Republic, Vídeňská 1083, CZ 142 20 Prague, Czech Republic

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ABSTRACT

Liquid chromatography (LC) on various stationary phases was used for the metabolite profile analysis of quercetin, rutin, isoquercitrin and taxifolin. The metabolites were obtained using an *in vitro* model system of human and rat hepatocytes in the form of cell suspensions and the primary cultures. For separations of the parent compounds and their metabolites, stationary phases based on C_{18}, C_8, cyanopropyl (CNP) or phenyl (PHE) modifications of silica were tested. CNP and PHE stationary phases operating in reversed-phase mode have been shown to be efficient for separation of parent flavonoids and their polar metabolites. Individual metabolites were identified on the basis of an elemental composition determination using electrospray ionization-quadrupole time-of-flight mass spectrometry (ESI-QqTOF MS) on-line connected with an LC system. Detailed analytical parameters such as retention times, selectivity, resolution of chromatographic peaks, MS fragmentation and UV–vis absorption maxima were determined for individual metabolites, namely for phase II biotransformation products. The predominant metabolites were methylated flavonols and flavonol glucuronides. The highest biotransformation rate was found with taxifolin, which was mainly converted to sulfates. The HPLC/ESI-QqTOF MS analyses revealed that quercetin and taxifolin were metabolized more extensively than the studied glycosides, rutin and isoquercitrin.

1. Introduction

Flavonoids are a large group of natural polyphenols found in food of plant origin. The recommended daily intake of these polyphenols is *ca.* 1 g per person [1,2]. Flavonoids and their metabolites are thought to have a number of chemoprotective effects. [3]. The antioxidant, anti-inflammatory, antitumor and antibacterial activities of various flavonoids have been shown in many *in vitro* studies [3–5]. Apart from their chemoprotective effects, some data indicate that flavonoids can be involved in pro-oxidant effects after complexing with transition metals [6]. The detailed conditions under which these compounds and their biotransformation products behave as antioxidants or pro-oxidants are not known. This is important for understanding the impact of polyphenols on human health.

The metabolism of flavonoids mainly occurs in enterocytes and hepatocytes, and extensive biotransformation processes take place in the colon as a result of microbial fermentation [2]. The bioavailability of most flavonoids is relatively low, for example the plasma levels of quercetin usually do not exceed 1 μM [7]. Since the flavonoid skeleton has hydroxyl groups attached, flavonoids may readily undergo conjugation reactions (phase II biotransformation [8]) followed by relatively rapid urinary excretion [9]. Despite the considerable attention devoted to the study of flavonoids, relatively little is known about their biotransformation and the biological activities of their metabolites.

Chromatographic methods are an important tool in the analysis of flavonoids in biological matrices [10]. The techniques used for the extraction and analysis of flavonoids in plant material [10,11] and in mammalian tissues and clinical samples [2] have been recently reviewed. The separation procedures are mostly based on reversed-phase systems, both classical one-dimensional and in an orthogonal/2D arrangement [12] in combination with ESI-MS. Due to the high water-solubility of flavonoids (frequently conjugated with carbohydrate moieties [13]) we have developed effective LC methods based on more polar stationary phases than classical C_{18} [11,14]. Cyanopropyl- (CNP) and phenyl- (PHE) modified sorbents were successfully used in classical HPLC as well as U-HPLC (ultra-high pressure LC) systems operating at high pressure, typically over 6000 psi [11,14].

* Corresponding author. Tel.: +420 585 632 303.
E-mail address: jan.vacek@upol.cz (J. Vacek).

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This study describes the application of LC with more polar sorbents in combination with ESI-MS in the analysis of flavonols (quercetin, rutin and isoquercitrin), taxifolin and their metabolites. A robust analytical LC/MS metabolic study focused on flavonols and flavanonols has not yet been published to the best of our knowledge. LC separation of the above-mentioned compounds can present problems, since the retention times of biotransformation phase II metabolites and of other conjugated species (*e.g.* glycosides) are often identical [2]. The application of more polar CNP- and PHE-based stationary phases can thus resolve problems with co-elutions of the polar analytes. Moreover, the biotransformation of flavonol glycosides and the flavanonol taxifolin has not yet been investigated in the model of human hepatocytes [15–17], which is a universally accepted *in vitro* model system for cytotoxicity and metabolic studies.

This study aimed to (i) compare the retention times and other basic chromatographic parameters of quercetin, rutin, isoquercitrin and taxifolin metabolites using C_{18}, C_8, CNP- and PHE stationary phases; (ii) use these stationary phases in the separation of flavonols, taxifolin and their metabolites in a cell samples; (iii) use ESI/QqTOF MS to identify the whole spectrum of metabolites of selected flavonols and taxifolin in cell suspensions and primary cultures of human and rat hepatocytes.

2. Experimental

2.1. Chemicals

Quercetin was purchased from Sigma–Aldrich (St. Louis, MO, USA), rutin (97% purity) was obtained from Merck (Sao Luis, Brazil) and taxifolin (96% purity), with no admixture of quercetin (HPLC), was obtained from Amagro (Prague, Czech Republic). Isoquercitrin (98% purity) was prepared by an enzymatic procedure [18]. Buffer components and other chemicals were purchased from Sigma–Aldrich. All solutions were prepared using reverse-osmosis deionized water (Ultrapur, Watrex, Prague, Czech Republic). Nitrogen, argon and helium (99.999% for all) were obtained from Linde Gas (Prague, Czech Republic).

2.2. Hepatocytes

2.2.1. Preparation and incubation of human hepatocyte suspensions

Liver samples ($n = 3$) were obtained from multi-organ donors. The tissue acquisition protocol conformed to the requirements of the Ethics Commission of the Faculty Hospital in Olomouc. Three independent isolations of human hepatocytes (three multi-organ donors; two men, 31 y and 61 y, one woman, 66 y) were carried out. Hepatocytes were isolated according to Pichard et al. [19]. The obtained cells were resuspended in serum-free medium, based on a 1:1 mixture of Williams' E and Ham F12 culture media with additives, fully defined in [19]. Human hepatocytes (4×10^6 cells mL^{-1}) were incubated with flavonols and taxifolin (to a final conc. of 50 μM) in a rotary incubator for 1 and 2 h at 37 °C. The cell viability at the end of the incubations was checked using the trypan blue test [20]. After incubation, the hepatocytes were centrifuged ($100 \times g$) for 3 min at room temperature, and cells and culture medium were separated.

2.2.2. Preparation and incubation of primary cultures of human hepatocytes

Human hepatocytes ($n = 3$) isolated as described above (Section 2.2.1) were resuspended in culture medium (Williams'E and Ham F12, 1:1) with 5% bovine serum. Cells were seeded on collagen-coated 6-well plates (area 9.4 cm^2) at a density of 1.5×10^5 cells/cm^2. The cell cultivation and treatment were carried out under sterile conditions, using a humidified incubator at 37 °C and an atmosphere containing 5% CO_2. After 12 h, the culture medium was replaced with a serum-free medium. After 24 h of stabilization, the culture medium was replaced with fresh serum-free medium and the flavonols and taxifolin (50 μM to the final concentration) were added to the cells. After 24 h of incubation, the cells were harvested into the culture medium, centrifuged ($100 \times g$) for 3 min at room temperature, and the cells and culture medium were then separated.

2.2.3. Preparation and incubation of primary cultures of rat hepatocytes

The study was approved by the Ethics Committee, Ministry of Education, Czech Republic and conducted in compliance with the Experimental Animal Protection Act No. 167/1993 L.C. Male Wistar rats were purchased from BioTest Ltd., Konarovice, Czech Republic. The rats were acclimatized for at least one week before the experiment. Hepatocytes were isolated by two-step collagenase perfusion according to Moldéus et al. [21]. The liver tissue was perfused with a calcium-free buffer solution for 5 min at 37 °C. The tissue was then perfused for 5 min with the buffer solution containing calcium and collagenase (Collagenase NB from Serva Electrophoresis, Heidelberg, Germany) to digest the tissue. The hepatocytes were collected in a centrifugation solution containing 160 mM NaCl, 3.09 mM KCl, 0.69 mM Na_2HPO_4, 32.7 mM HEPES and 6.75 mM $CaCl_2$, pH 7.4. Cells were filtered through gauze and washed three times with centrifugation ($50 \times g$), and finally resuspended in William's medium E with additives, fully described in [22]. They were plated on collagen-coated 6-well plates at a cell density of 1×10^5 cells/cm^2 in Williams' medium E supplemented with the above-mentioned supplements and enriched with 10% fetal calf serum. After 4 h of stabilization, the culture medium was replaced with fresh serum-free medium and the flavonoids (50 μM to the final concentration) were added to the cells. After 24 h of incubation, the cells were scraped into the culture medium, centrifuged ($100 \times g$) for 3 min at room temperature, the cells and culture medium were then separated. Primary cultures were maintained for stabilization and incubation in a humidified incubator with CO_2 (5%, vol.) at 37 °C under sterile conditions.

2.3. Sample processing for the HPLC/ESI-QqTOF MS analysis

The hepatocytes were washed three times in 0.5 mL of phosphate buffered saline (PBS), resuspended in 0.4 mL of methanol (5% acetic acid, vol.) and sonicated (cycle 0.5, amplitude 50, 10-times) using a UP 200S ultrasonic homogenizer (Hielscher Ultrasonics GmbH, Teltow, Germany). The samples were then centrifuged ($14{,}000 \times g$) for 2 min at room temperature and supernatants were analyzed by HPLC/ESI-QqTOF MS. The culture medium was diluted (1:1, v/v) in methanol (5% acetic acid, vol.), centrifuged ($14{,}000 \times g$) for 2 min at room temperature and the supernatants were analyzed.

2.4. HPLC/ESI-QqTOF MS

Chromatography was performed on an ACQUITY UPLC system (Waters, Milford, MA, USA) equipped with a binary solvent manager, sample manager, column manager and PDA detector. The autosampler was conditioned at 4 °C. The separation was performed in Agilent Zorbax Eclipse XDB columns (150 mm × 2.1 mm i.d., 5 μm; Agilent Technologies, CA, USA) and the retention behaviour of the analytes was tested on four stationary phases: C_{18}, C_8, CNP and PHE. Binary gradient elution was performed at a flow rate of 0.4 mL/min, column temperature was maintained at 35 °C. Mobile phase A consisted of 1% acetic acid (*aq*) and methanol 90:10 (v/v). Mobile phase B was pure methanol. The gradient profile was

as follows: 0–14 min 10–50% B; 14–16 min 50–100% B; 16–18 min 100–10% B; 18–20 min 10% B. The injection volume was 5 μL.

A Waters QqTOF Premier Mass Spectrometer (Waters Corp., Manchester, UK) was connected to the UPLC system *via* an electrospray ionization (ESI) interface. The ESI source operated in negative ionization mode with the capillary voltage at 2.1 kV and the sampling cone at 40 V. The source temperature and desolvation temperature were set at 120 °C and 300 °C, respectively. The cone and desolvation gas flow rates were 0 L h^{-1} and 500 L h^{-1}, respectively. Data were acquired from 50 to 1000 Da with a 0.5 s scan time and processed using MassLynx™ V4.1 software (Waters, Milford, MA, USA). The mass spectrometer was calibrated across the mass range of 50–1000 Da using a solution of sodium formate. Data were centroided and mass corrected during acquisition using an external reference, Leucine-enkephalin (200 μg/L in water:acetonitrile:formic acid mixture (100:100:0.2) at a flow rate of 5 μL/min).

Data were acquired with two independent scan functions (MS and MS^E experiments), which enabled the simultaneous acquisition of both low collision energy and high collision energy mass spectra during a single experiment. The collision energy was set at 5 V for Function 1 and at 20–35 V for Function 2. Acquisition data enabled the collection of intact precursor ions as well as fragment ion information in an unbiased manner. Post-acquisition processing of the data was done using the program MetaboLynx V4.1 (Waters Corp., Milford, MA, USA).

3. Results and discussion

3.1. Chromatography of flavonols and taxifolin on various stationary phases

Our investigation was focused on the chromatography of flavonols (quercetin and glycosides – rutin and isoquercitrin), the flavanonol taxifolin (Fig. 1) and their metabolites on columns with identical geometrical parameters but different chemical modifications of the silica stationary phase (C_{18}, C_8, CNP and PHE). The compounds were separated by gradient elution in the system methanol/1% acetic acid (*aq*) and methanol 90:10 (v/v). This mobile phase with a low acetic acid concentration was previously shown to be suitable for polyphenol HPLC/MS analysis [14,23]. Under the above conditions, the compounds can be separated in only a single run on PHE and also partially separated on CNP column, which correlates with the more polar character of these stationary phases. Co-elution of glycosides was observed with a C_{18}/C_8 stationary phase (Table 1). ESI operating in the negative mode and QqTOF MS instrument on-line connected to the LC system was used to identify the parent compounds and subsequently their metabolites. For details on the MS analyses, see Section 2.4.

3.2. Identification of metabolites by HPLC/ESI-QqTOF MS

The metabolites of the above-mentioned flavonoids were produced using metabolically active liver cells (human and rat hepatocytes) prepared by a two-step collagenase perfusion of human and rat liver tissue ($n = 3$ for both), see Section 2.2. The metabolites were identified by calculating their elemental composition based on accurate mass measurement by ESI-QqTOF MS. Verification was based on a comparison of isotopic profiles (measured *vs.* generated) and also on a detailed analysis of MS^E spectra. Retention times, MS^E and UV–vis parameters of the metabolites obtained (using various stationary phases) in the experiments with human and rat hepatocytes are given in Table 1. Multiple chromatographic peaks reflecting a given metabolic process (methylation, glucuronidation, *etc.*) are associated with the presence of different positional isomers. Chromatograms of flavonol and taxifolin metabolites in a PHE column are shown in Fig. 2 and full MS spectra and MS^E spectra of the predominant metabolites are presented in Fig. 3.

The predominant biotransformation processes for quercetin in human hepatocytes are methylation and glucuronidation (Table 2). The MS^E spectrum of the parent ion of methylquercetin (*m/z* 315.0513, 2.5 ppm) features as the major ion at *m/z* 300, which corresponds to the radical cleavage of the methyl group (Fig. 3A). The spectrum also contains ions at *m/z* 107 and *m/z* 151, which corresponds to the cleavage of the methylated derivative of quercetin – isorhamnetin [24,25]. These data confirm the previously published findings [26] that the *O*-methylation of quercetin takes place primarily on ring B. Chromatographic records of quercetin metabolites are shown in Fig. 2A.

As with quercetin, the main biotransformation products of rutin and isoquercitrin are their methyl derivatives (Table 2), which was confirmed by precise mass measurements and investigation of fragmentation spectra. The methyl derivative of rutin (*m/z* 623.1625, 2.1 ppm) in a MS^E spectrum undergoes a neutral loss of a sugar residue ($\Delta m/z$ 308) followed by radical splitting of the methyl (Fig. 3B). In contrast to quercetin and rutin, methylisoquercitrin exhibited two positional isomers (Fig. 2C), which were separated in all columns in the study with the exception of CNP (compare in Table 1). A nonstandard radical splitting of the sugar moiety in MS^E spectra, which accompanied the typical neutral loss $\Delta m/z$ 162, was shown to also take place in the isoquercitrin standard (the most abundant fragment in the MS^E experiment was *m/z* 300 [27]) and is thus typical for the given setting of mass spectrometer in collision experiments (see Section 2.4). Exact mass measurements confirmed the presence of methylation products. Peak 1 (Fig. 2C) gave the value *m/z* 477.1029 (−0.8 ppm), the major product (peak 1′) had *m/z* 477.1043 (2.1 ppm).

The major taxifolin metabolite is its sulfated conjugate (Fig. 2D). The MS spectrum features, in addition to the quasimolecular ion (*m/z* 383.0072, −0.3 ppm), also an adduct with sodium (*m/z* 404.9903, 2.7 ppm) (Fig. 3D). The MS^E spectrum also contains two high-intensity ions, *m/z* 285 and *m/z* 125. The *m/z* 285 ion corresponds to a neutral loss of sulfate and water giving rise to a fully aromatic ion, whereas the *m/z* 125 ion is most likely a fragment of ring A carrying three hydroxy groups. This observation indicates that the biotransformation of taxifolin is not based on modifications of ring A.

3.3. Chromatographic behaviour of metabolites of flavonols and taxifolin

The MS identification is substantiated by retention data and an interpretation of the retention behaviour of the studied metabolites. The retention times of methylated derivatives are higher than the retention times of the parent flavonoids or their glucuronides and sulfates (see '1' in Fig. 2). The effect of methylation on prolonging retention time has recently been confirmed in a comparative study dealing with hydroxy derivatives and methoxy derivatives of benzoic and cinnamic acid [14]. On the other hand, the retention times of highly water-soluble glucuronides and sulfates and their methylated derivatives (mixed conjugates) are lower than those of the parent compounds (see '2–4' in Fig. 2).

For a better comparison of the chromatographic behaviour of the metabolites in the four studied columns, a calculation of relative retention (selectivity, α) and resolution (R) was done (Tables 3 and 4). The selectivity was calculated as a ratio of the reduced (adjusted) retention times of metabolites and reduced retention times of the parent flavonoids relative to whichever compound eluted first, so the resulting number was higher or equal to one. Only the peak parameters of the parent flavonoid and its

Table 1
Retention times for different HPLC stationary phases and other qualitative ESI-QqTOF MS and UV–vis parameters of quercetin, rutin, isoquercitrin, taxifolin and their metabolites in human and rat hepatocytes. The presence of more than one peak for one metabolite (metabolic process) corresponds to the positional isomerism. Molecular peaks are marked in bold. Average values are presented ($n = 3$), n.a., not analyzed.

	t_R C_{18}	t_R C_8	t_R CNP	t_R PHE	MS (>10%)	MS^E (>10%)	UV–vis (nm)
Tested compounds							
Quercetin	12.0	10.4	11.1	11.9	**301**	107, 121, 149, 151, 179	255, 371
Rutin	8.2	6.4	5.9	8.1	**609**, 745	300, 301, 609	256, 356
Isoquercitrin	8.2	6.4	6.2	8.3	**463**	255, 271, 300, 301	256, 354
Taxifolin	5.7	4.8	4.5	6.6	125, 285, **303**, 325	83, 123, 125, 150, 151, 174, 175	289
Quercetin metabolites (man)							
Methylation	15.0[a]	13.3[a]	12.8[a], 12.6	15.0[a]	**315**	107, 151, 271, 283, 300, 315	255, 370
Sulfate conjugation	12.1	10.9	9.5	10.9	301, **381**	151, 300	n.a.
Glucuronide conjugation	11.0[a], 8.2	9.7[a], 6.5	8.4[a], 6.1	10.8[a], 7.9	**477**, 499	301, 499	251, 368
Rutin metabolites (man)							
Methylation	10.5[a]	8.5[a]	6.9[a], 7.1	10.3[a]	**623**	300, 315, **623**	255, 355
Methylation + glucuronide conjugation	7.6[a], 6.8	5.7[a], 5.3	4.1[a]	7.6[a], 7.2	**799**, 821	113, 315, 473, 491, 513, 533, 623, **799**, 821	267, 349
Glucuronide conjugation	7.0, 5.9	5.3, 4.6	4.0, 4.3	6.2, 6.9	**785**, 807	173, 301, 459, 499, 609, **785**, 807	267, 340
Isoquercitrin metabolites (man)							
Methylation	10.6[a], 10.3	8.7[a], 8.5	7.2[a]	10.7[a], 10.3	**477**	271, 299, 314, **477**	255, 353
Methylation + glucuronide conjugation	7.6[a], 6.5, 4.8	5.8[a], 5.1, 3.2	4.3[a], 2.9	7.9[a], 7.0, 5.2	**653**, 675	113, 315, 477, 491, 513, 675	266, 347
Glucuronide conjugation	5.8, 7.1	4.6, 5.5	4.4, 4.2	6.3, 7.2	**639**, 661	301, 463, 499, 661	266
Taxifolin metabolites (man)							
Sulfate conjugation	5.9[a]	5.3[a]	3.5[a]	6.2[a]	**383**, 404	125, 151, 152, 153, 175, 217, 285, **383**, 404	290
Methylation	8.1[a]	7.2[a]	5.8[a]	9.8[a]	289, **317**	**152**	288
Reduction (dehydroxylation)	7.9	7.0	5.9	9.1	259, **287**	n.a.	294
Quercetin metabolites (rat)							
2 × Glucuronide conjugation	7.0	5.5	4.7, 5.4	6.9, 8.0	**653**, 675	301, 499	265, 347
Methylation	12.5	11.0	10.7	12.0	315, 395, 571, 593	**300**	n.a.
Methylation + glucuronide conjugation	10.3, 11.4	8.4, 10.0	6.9, 8.2	10, 11.7	315, **491**	300	258, 351
Rutin metabolites (rat)							
Methylation + glucuronide conjugation	7.4, 6.6, 4.9	5.5, 5.1, 3.3	4.0, 3.3, 2.9	7.4, 6.8, 5.2	**799**, 821	113, 315, 473, 491, 513, 533, 623, **799**, 821	267, 349
Methylation	10.3	8.2	6.6, 6.8	10.1	**623**	300, 315, **623**	255, 352
Glucuronide conjugation	6.8, 5.8, 4.0, 3.1	5.1, 4.4, 3.1, 1.9	4.1, 3.8, 2.3	6.7, 6.0, 4.5, 3.4	**785**, 807	301, 459, 477, 499, 519, 609, **785**, 807	267, 340
Isoquercitrin metabolites (rat)							
Methylation + glucuronide conjugation	7.3, 6.6, 6.3, 6.8, 4.6, 4.2	5.6, 5.3, 4.9, 3.0,2.8	4.2	7.7, 6.9, 7.4, 5.0, 6.1, 4.6	**653**, 675	113, 300, 315, 477, 491, 513, **653**, 675	266, 348
Methylation	10.4, 10.1	8.5, 8.2	6.9	10.5, 10.1	**477**	271, 299, 314, **477**	255, 354
Glucuronide conjugation	4.8, 5.6, 6.9, 4.4, 2.9	3.8, 4.4, 5.3, 3.2, 1.8	n.a.	5.0, 6.2, 7.0, 3.2	222, 283, **639**, 661	301, 463, **639**, 661	266, 347
Taxifolin metabolites (rat)							
Methylation	4.6, 4.0, 5.9, 2.6	4.0, 3.2, 5.3, 2.2	4.1, 2.7, 2.1	5.9, 5.1, 4.0, 7.0, 9.5	289, **317**, 397	125, 152, 192, 289, **317**, 397	251, 283, 333
Methylation + glucuronide conjugation	4.0, 4.8, 2.6, 5.5	3.3, 4.2, 2.2, 4.6	2.7, 3.2, 2.1, 3.5	5.1, 6.5, 4.0, 7.0, 5.6	243, 317, **493**, 515	125, 152, 165, 289, 299, 317, 515	284
Sulfate conjugation	6.0	5.4	4.2	6.3	**383**, 397	152	266, 358

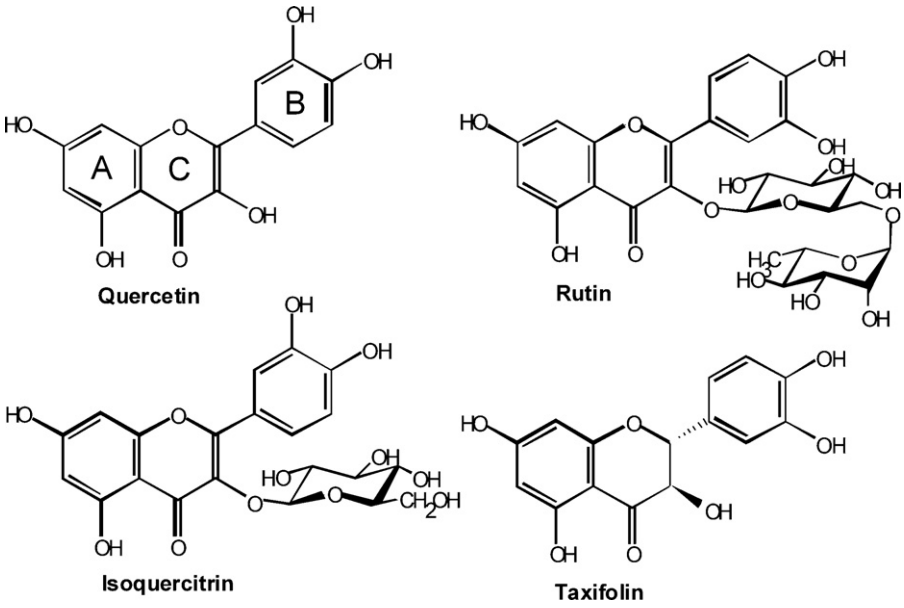

Fig. 1. Chemical structures of studied flavonols (quercetin, rutin and isoquercitrin) and flavanonol taxifolin.

predominant metabolite were used for the calculations. It can be seen from our results that the highest selectivity was reached with the CNP column with the exception of the methylated metabolites, whereas the best results were achieved with the C_8 column (Table 3). However, the resolution of the chromatographic peaks also needs to be taken into consideration (Table 4).

As can be seen, the C_{18} and C_8 columns enabled very high resolution of the methylated metabolites, which are also the most abundant metabolites in three of the four polyphenols studied. On the other hand, co-elution of taxifolin and its main sulfate metabolite was observed in both of these columns. The CNP column provided good resolution of all the main metabolites, though the peak shape was slightly worse than with C_{18} and C_8; a poor

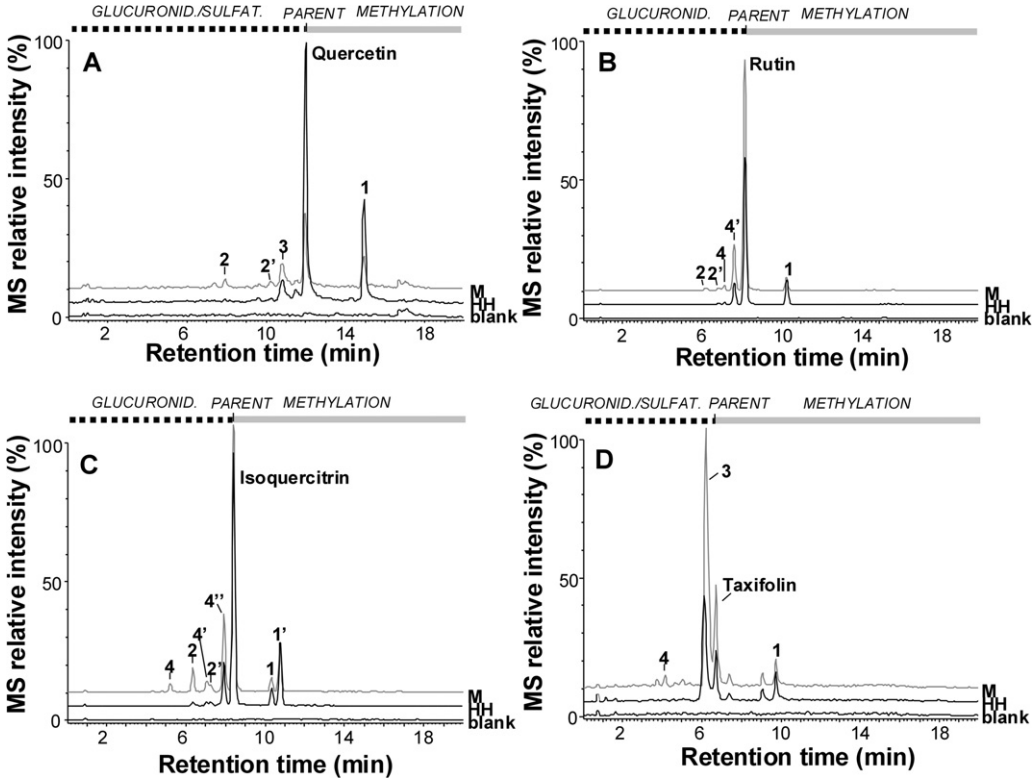

Fig. 2. HPLC/ESI-QqTOF MS chromatograms of quercetin (A), rutin (B), isoquercitrin (C) and taxifolin (D) metabolites using PHE stationary phase. Metabolites: 1, methylated derivatives; 2, glucuronides; 3, sulfates; 4, methylated glucuronides. The chromatograms display a sum of extracted *m/z* ratios for metabolites and parent compounds (50 μM) in culture medium (M) and human hepatocytes (HH) after 2 h incubation with parent compounds (50 μM). The following *m/z* values were monitored using selected-ion monitoring (SIM) mode: 301.03, 380.99, 315.05, 477.07 for A, 799.19, 623.16, 785.17, 609.14 for B, 477.1, 653.14, 639.12, 463.09 for C, and 383.01, 317.07, 287.06, 303.05 for D. Blank, non-treated HH cells. For other details see Section 2.

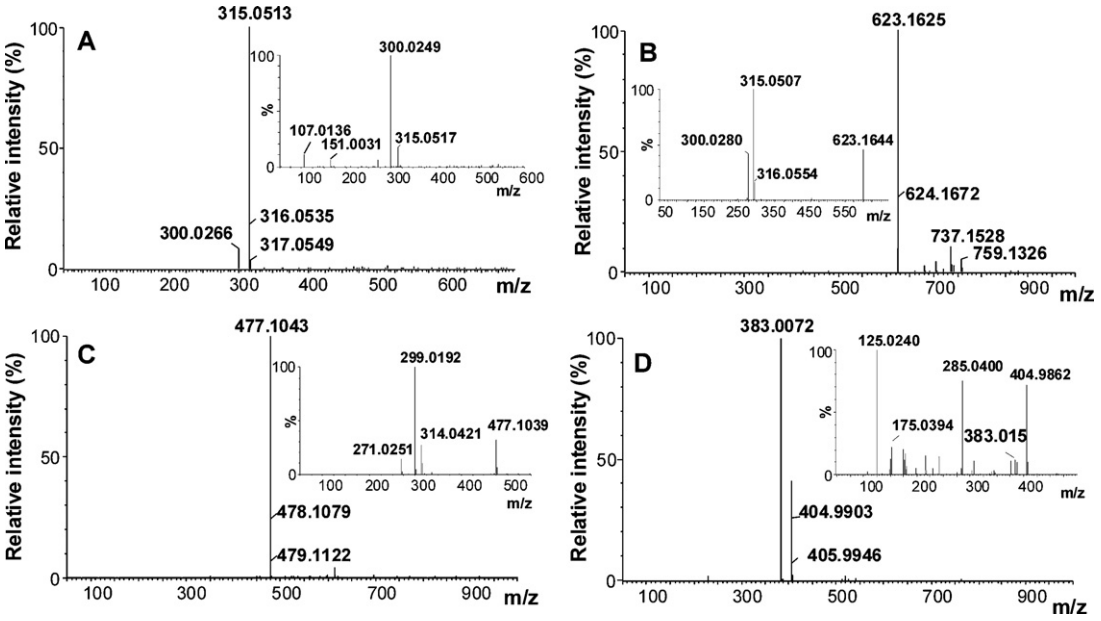

Fig. 3. ESI/QqTOF MS spectra of predominant metabolites of quercetin (A), rutin (B), isoquercitrin (C), and taxifolin (D) in cell suspensions of human hepatocytes after 2 h incubation with parent compounds (50 μM). MS^E spectra of the metabolites with the background subtraction are shown in insets. The MS spectra were subtracted from chromatographic peaks shown in Fig. 2. For other details see Section 2.

retention of minor polar glucuronides was also observed. Therefore, PHE columns can be considered to be a reasonable compromise between the C_{18} and CNP type of stationary phase. It offers a baseline separation of all the main metabolites, whilst having retention for polar metabolites and symmetric peak shapes much like a C_{18} phase.

3.4. Biotransformation patterns of tested flavonoids

The metabolite profile of flavonoids found in the human hepatocytes was similar, in terms of quantitative differences, to the profile obtained in the culture medium (Table 2). The level of metabolites in the hepatocytes and culture medium changed during incubation due to biotransformation kinetics and the excretion of metabolites, respectively. Metabolites identified in the suspensions of hepatocytes were also found in the primary cultures of both human and rat hepatocytes. The differences between biotransformation patterns in rats and humans are in agreement with published interspecies variability data [28].

Biotransformation of the flavonoids may be affected by several factors such as the transport abilities (diffusion and membrane transport parameters), reactivity (stability and interactions with other cell components), solubility (hydrophilicity/lipophilicity) of the parent compounds and their affinity (substrate specificity) to biotransformation enzymes. The HPLC/ESI-QqTOF MS analyses revealed that quercetin and taxifolin were metabolized more extensively than the glycosides, rutin and isoquercitrin. This finding could be explained by differences in the physicochemical parameters of the parent substances, especially their water solubility. Compared to quercetin and taxifolin, the presence of a hydrophilic sugar moiety in rutin and isoquercitrin might decrease the transport of the compounds into the cell and impede their access to the

Table 2
Semi-quantitative percentage representation of quercetin, rutin, isoquercitrin and taxifolin metabolites by HPLC/ESI-QqTOF MS (PHE stationary phase was used). The metabolites were determined in suspensions of human hepatocytes and in culture medium after (1 h and/or 2 h) incubation with parent compounds (50 μM). Σ (parent compound + predominant metabolites) = 100%. Average values are presented (n = 3).

Parent compound/metabolite	1 h, hepatocytes	1 h, culture medium	2 h, hepatocytes	2 h, culture medium
Quercetin	60.9	50.2	53.2	45.7
Methylation	14.7	10.2	19.1	17.2
Sulfate conjugation	5.6	4.6	7.5	8.6
Glucuronide conjugation	16.4	27.5	16.4	24.7
Methylation + glucuronide conjugation	2.3	6.8	3.7	12.7
Rutin	82.3	91.6	76.2	85.4
Methylation	8.8	1.6	10.6	2.5
Methylation + glucuronide conjugation	7.0	4.5	10.3	9.5
Reduction (dehydroxylation)	1.4	1.5	1.9	1.4
Glucuronide conjugation	0.3	0.8	0.9	1.0
Isoquercitrin	75.8	83.0	69.5	69.8
Methylation	11.8	4.7	14.8	8.8
Methylation + glucuronide conjugation	8.6	7.4	12.0	15.6
Reduction (dehydroxylation)	1.6	1.7	1.5	1.7
Glucuronide conjugation	2.4	3.1	2.2	4.8
Taxifolin	23.1	34.0	19.5	18.8
Sulfate conjugation	50.4	46.5	53.2	59.6
Methylation	18.8	12.5	20.7	14.1
Reduction (dehydroxylation)	6.2	4.3	5.0	3.0
Methylation + glucuronide conjugation	1.5	2.7	1.6	4.4

Table 3
Selectivity (α) of quercetin, rutin, isoquercitrin and taxifolin metabolites on suspensions of human hepatocytes by HPLC/ESI-QqTOF MS on the different stationary phases; n.p., not presented (with respect to the co-elution of positional isomers of the metabolites). The metabolites presented here are described in detail in Table 1.

Metabolites	α C_{18}	α C_8	α CNP	α PHE
Quercetin				
Methylation	1.26	1.31	1.15	1.28
Methylation	n.p.	n.p.	1.17	n.p.
Sulphate conjugation	1.01	1.06	1.19	1.10
Glucuronide conjugation	1.51	1.68	1.97	1.57
Glucuronide conjugation	1.10	1.07	1.36	1.11
Rutin				
Methylation	1.31	1.38	1.20	1.31
Methylation	n.p.	n.p.	1.25	n.p.
Methylation + glucuronide conjugation	1.24	1.24	1.59	1.14
Methylation + glucuronide conjugation	1.09	1.14	n.p.	1.07
Glucuronide conjugation	1.45	1.47	1.62	1.36
Glucuronide conjugation	1.20	1.24	1.48	1.20
Isoquercitrin				
Methylation	1.28	1.37	1.20	1.27
Methylation	1.32	1.41	n.p.	1.32
Methylation + glucuronide conjugation	1.86	2.38	2.69	1.72
Methylation + glucuronide conjugation	1.30	1.32	1.56	1.22
Methylation + glucuronide conjugation	1.09	1.13	n.p.	1.06
Glucuronide conjugation	1.48	1.49	1.61	1.37
Glucuronide conjugation	1.18	1.20	1.52	1.18
Taxifolin				
Sulphate conjugation	1.04	1.13	1.37	1.09
Methylation	1.50	1.61	1.38	1.55
Reduction (dehydroxylation)	1.46	1.55	1.42	1.42

Table 4
Resolution (R) of quercetin, rutin, isoquercitrin and taxifolin major metabolites in suspensions of human hepatocytes by HPLC/ESI-QqTOF MS on the different stationary phases. The metabolites presented here are marked by "a" in Table 1.

Main metabolites	R C_{18}	R C_8	R CNP	R PHE
Quercetin – methylated quercetin	9.2	9.2	3.9	8.3
Rutin – methylated rutin	7.3	7.4	2.8	6.2
Isoquercitrin – methylated isoquercetin	8.0	8.0	2.8	7.6
Taxifolin – taxifolin sulfate	<0.5	0.7	2.7	1.7
Quercetin – quercetin glucuronide	4.1	3.0	6.8	3.1
Rutin – methylated rutin + glucuronide	2.8	3.7	5.6	1.8
Isoquercitrin – methylated isoquercitrin + glucuronide	2.8	3.4	5.8	1.7
Taxifolin – methylated taxifolin	7.0	10.0	4.0	10.4

active sites of the respective biotransformation enzymes. For data on the solubility of the studied flavonoids in an aqueous medium, see Refs. [29,30].

The highest biotransformation rate was found with taxifolin, which was mainly converted to sulfates. In contrast, the studied flavonols were predominantly metabolized by methylation and glucuronidation. The biotransformational differences between flavonols and taxifolin could also be extended to other flavonols and flavanonols, but this will need to be confirmed experimentally. The metabolic profiles of rutin, isoquercitrin and taxifolin obtained with the human hepatocytes have not yet been published, the spectrum of the main metabolites of quercetin analyzed in this study is in agreement with previously published results [25].

4. Conclusion

The metabolic profiles of quercetin, rutin, isoquercitrin and taxifolin in human hepatocytes were characterized. The selection of suitable stationary phases and determination of suitable conditions for MS detection are key factors in conducting successful metabolic analyses. Like other polyphenols, flavonoids containing numerous OH-groups yield a rich spectrum of metabolites and their positional isomers, demanding the use of robust HPLC methods. CNP and PHE stationary phases operating in reversed-phase mode have been shown to be efficient for separation of polar metabolites. Combining them with C_{18}/C_8 columns could in the future be used for 2-D separation systems. This description of the biotransformation patterns and retention behaviour of flavonol and taxifolin metabolites and the methodology presented here is a fundamental step towards effective future metabolic studies of flavonols and flavanonols.

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