Journal of Chromatography A, 1217 (2010) 4100–4108

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Identification of new phase II metabolites of xanthohumol in rat *in vivo* biotransformation of hop extracts using high-performance liquid chromatography electrospray ionization tandem mass spectrometry

Robert Jirásko[a], Michal Holčapek[a,*], Eva Vrublová[b], Jitka Ulrichová[b], Vilím Šimánek[b]

[a] University of Pardubice, Faculty of Chemical Technology, Department of Analytical Chemistry, Studentská 573, 532 10 Pardubice, Czech Republic
[b] Palacký University, Faculty of Medicine and Dentistry, Department of Medical Chemistry and Biochemistry, Hněvotínská 3, 775 15 Olomouc, Czech Republic

ARTICLE INFO

Article history:
Available online 25 February 2010

Keywords:
Xanthohumol
Hop
Metabolites
Sulfate
Phenolic compounds
HPLC/MS/MS

ABSTRACT

Polyphenolic compounds occurring in hop extracts and their phases I and II metabolites formed during *in vivo* rat biotransformation have been analyzed using HPLC/MS/MS with electrospray ionization (ESI). Two main groups of polyphenolics are present in the hops, i.e., xanthohumol related compounds and so called α- and β-bitter acids (humulones and lupulones). In our study, hybrid quadrupole-time-of-flight (QqTOF) analyzer is used for the identification of both natural phenolics and their metabolites due to the possibility of accurate mass measurements in full scan and tandem mass spectra supported by MS^n data obtained with the ion trap analyzer. Both ESI polarity modes are used for the determination of molecular weights based on $[M+H]^+$ and $[M-H]^-$ ions in the full scan spectra and the structural information in subsequent tandem mass spectra. The emphasis is given on the elemental composition determination of individual metabolites based on accurate masses typically better than 5 ppm even with the external calibration. Advanced software tools are used for the metabolite identification using the comparison of the blank chromatogram with the real incubation sample together with the software prediction and detection of possible metabolites. Chromatograms of rat incubations are also compared with chromatograms of pure rat feed, rat feed enriched with hop extracts and the placebo experiment. More than ten compounds originating from the hops are identified in rat feces, two of them belong to phase I metabolites and five compounds are phase II metabolites.

1. Introduction

Natural bioactive compounds are important components of complex biological systems involving microbiological, plant, animal or human world. Many of them participate in reactions positively affecting the human health. One example is a group of polyphenolic compounds showing an antioxidative activity [1] and related effects on the reduction of oxidative stress of organism [2]. They are widely distributed in plants.

Female flowers of hops, used as the flavoring agent providing bitterness for beer, contain several important polyphenolic compounds, e.g., prenylflavonoids xanthohumol (XN), isoxanthohumol (IXN) and 8-prenylnaringenin (8-PN), α- and β-bitter acids (humulones and lupulones). Due to their cancer chemopreventive effects [4–6], anti-microbiological activity [7] or potential estrogenic properties of 8-PN [3], they are increasingly considered for the use in medicine, human diet and animal feeds. However, each xenobiotic compound present in the living organism gives rise to the formation of various phases I and II metabolites which necessitate their analytical characterization for a better understanding of their behavior in studied biological systems.

HPLC/MS/MS is usually a method of choice for the metabolic studies because of high sensitivity and structural information even for trace metabolites in highly complex matrices [8]. This technique has been already applied for the analysis of prenylated compounds [9–11] as well for their metabolites [12]. Mainly two ionization techniques, atmospheric pressure chemical ionization (APCI) for the identification of phase I metabolites and electrospray ionization (ESI) enabling the detection of both phase I or II metabolites, are used. The important issue in the positive identification of high number of metabolites is the use of advanced metabolite softwares and mass analyzers providing high resolution and high mass accuracy.

Two major sites of flavonoid biotransformation are the liver and the colon [13]. Several HPLC/MS *in vitro* biotransformation studies using rat and human liver microsomes have been performed in the field of prenylated chalcones. Various phase I metabolites formed by dehydrogenation, demethylation, dehydratation, hydroxylation or epoxidation reactions have been described for

* Corresponding author. Tel.: +420 46 6037087; fax: +420 46 6037068.
E-mail address: Michal.Holcapek@upce.cz (M. Holčapek).

0021-9673/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2010.02.041

XN, IXN or 8-PN [12,14,15]. Concerning phase II metabolites, the glucuronidation and sulfatation have been reported in the literature for *in vitro* experiments [16,17]. On the other hand, only few experiments involving *in vivo* biotransformation have been done so far, although these studies represent more realistic picture about the metabolism. Unfortunately, *in vivo* experiments are more complex, individual metabolites are present at lower concentration levels and many other compounds present in the whole metabolome unlike to *in vitro* experiments, which significantly complicates their identification. Only one publication dealing with *in vivo* biotransformation of XN has been published until now [18], where 22 metabolites are identified using HPLC/^{1}H NMR coupling in the stopped-flow mode and HPLC/MS.

This work is focused on the HPLC/MS/MS of biotransformation products of hop extracts obtained by colonic microflora of rats. The aim of this work is the identification of phases I and II metabolites with the support of high mass accuracy MS and MS/MS experiments providing elemental composition of observed ions and the use of metabolic softwares.

2. Experimental

2.1. Materials

Acetonitrile (HPLC gradient grade), formic acid and sodium formate were purchased from Sigma–Aldrich (St. Louis, MO, USA). De-ionized water was prepared with Demiwa 5-roi purification system (Watek, Ledeč nad Sázavou, Czech Republic) and by Ultra CLEAR UV apparatus (SG, Hamburg, Germany). Standards of XN, IXN and 8-PN were provided by Hop research institute (Žatec, Czech Republic). Bitter acids were purchased from Labor Veritas (Zurich, Switzerland). Hop extracts were provided by Favea Company (Kopřivnice, Czech Republic). The hop extract (HE) from hop cones (*Humulus lupulus* L., Agnus species, the crop in 2007) was obtained by the supercritical CO_2 extraction and tested in 1000 ppm concentration in the diet. The waste obtained after this extraction was again extracted with ethanol, isomerized (isomerized hop extracts, IHE), standardized to XN, IXN and 8-PN content in 100 and 1000 ppm concentration in the diet.

2.2. Animals

Male Wistar rats (240 ± 10 g) were purchased from BioTest (Konárovice, Czech Republic) and then 1 week acclimatized before the experiment. Rats were housed at temperature 23 ± 2 °C and moisture 30–70% in the controlled room, exposed to a controlled 12 h cycle of light and darkness. Experimental animals were separated into groups and orally dosed with the experimental diet during 90 days. The experimental diet for particular rat groups was the following:

(A) Group 1—100 mg of IHE mixed with the commercial diet (800 g), microcrystallic cellulose (190 g) and magnesium stearate (10 g);
(B) Group 2—1000 mg of IHE mixed with the commercial diet (800 g), microcrystallic cellulose (190 g) and magnesium stearate (10 g);
(C) Group 3—1000 mg HE mixed with the commercial diet (800 g), microcrystallic cellulose (190 g) and magnesium stearate (10 g);
(D) Placebo experiment—commercial diet (800 g), microcrystallic cellulose (190 g) and magnesium stearate (10 g).

Rats were allowed to free access to food and water. The amount of active compounds taken during the day depended on the feed consumption, which was controlled twice a week. The average feed consumption was 44 ± 3 g.

2.3. Sample preparation

Samples of feed and feces (0.5 g) were homogenized in methanol (4 ml) using the homogenizer. The homogenate was further sonicated for 1 min and centrifuged at 3500 g for 3 min. Supernatants (3 ml) were evaporated at the atmospheric pressure with nitrogen gas (40 °C) followed by deep freeze at −80 °C.

2.4. HPLC/MS/MS

Particular samples of feed (Groups 1–3), feces (Groups 1–3) were quantitatively dissolved in 200 μl mixture of acetonitrile/water (1:1, v/v). HPLC was performed on a liquid chromatograph Agilent 1200 series (Agilent Technology, Waldbronn, Germany) using column Luna C18 250 mm × 2 mm, 5 μm (Phenomenex, Torrance, CA, USA) and linear gradient program. The mobile phase consisted of water (A) and acetonitrile (B) with the addition of 0.3% formic acid. The gradient program was: 0–5 min, 2% (B) isocratic; 5–10 min, the linear gradient from 2 to 5% (B); 10–40 min, the linear gradient from 5 to 35% (B); 40–66 min, the linear gradient from 35 to 95% (B); 66–80 min, 95% (B) isocratic; and finally washing and reconditioning of the column. The flow rate was 0.3 ml/min and the injection volume was 5 μl. The mass spectrometer (micrOTOF-Q, Bruker Daltonics, Germany) was used with the following setting of tuning parameters: capillary voltage 4.5 kV, drying temperature 210 °C, the flow rate and pressure of nitrogen were 9 l/min and 1.4 bar, respectively, the external calibration with sodium formate clusters before individual measurements. ESI mass spectra were recorded in the range of m/z 50–1000 both in positive- and negative-ion modes. The tandem mass spectra were measured according to the following conditions: the isolation width $\Delta m/z$ 6, the collision energy of 35 eV using argon as the collision gas. Ion trap analyzer (Esquire 3000, Bruker Daltonics, Germany) was used for the multistage mass spectra measurement.

The software package metabolite tool containing two algorithms Metabolite predict and Metabolite detect (Bruker Daltonics, Bremen, Germany) was used to help the metabolite detection.

3. Results and discussion

The complexity of *in vivo* experiments is illustrated by the total ion current chromatogram of rat *in vivo* biotransformation for the Group 2 (Fig. 1A). The presence of numerous compounds of different origin (e.g., from feed, plasma, metabolites) in studied samples complicates the chromatogram evaluation and several important steps are essential for the metabolite detection and subsequent identification. Possible metabolites can be predicted based on known structures of prenylated chalcones found in hop extracts. Various phases I and II metabolites can be expected in rat feces according to the literature [12,14,15,17]. The software Metabolite predict can be also used for the prediction of possible metabolites coming from the parent compound and defined metabolic rules. The most common reactions of prenylflavonoid metabolite formation are oxidation, dehydrogenation and demethylation for phase I metabolites or glucuronidation and sulfatation for phase II metabolites. The presence of proposed metabolites is tested using reconstructed ion chromatograms for given m/z values with the defined tolerance of mass error. The high mass accuracy of QqTOF analyzer enables an extraction of individual chromatograms in the narrow interval of m/z scale (±5 mDa), which is useful for the elimination of other compounds with similar masses. Moreover, the comparison with chromatograms of feed enriched with the hop

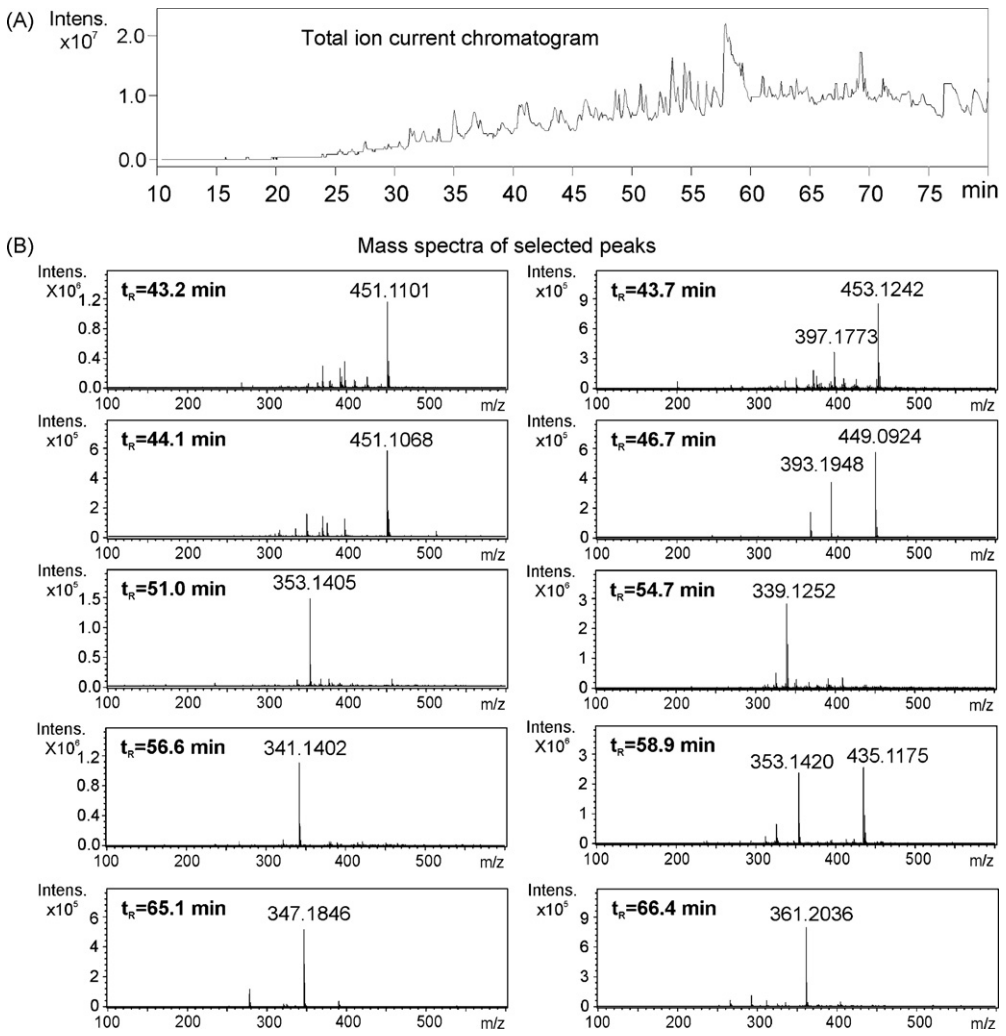

Fig. 1. HPLC/MS analysis of rat feces samples of Group 2 (see Section 2 for conditions). (A) Total ion current chromatogram in the negative-ion ESI mode; (B) negative-ion ESI mass spectra of selected peaks with their retention times.

extract and the placebo experiment is necessary to confirm the origin of detected compounds. For each detected peak, the match of retention times and accurate masses is checked. The following discussion concerns mainly to the Group 2 (see Section 2), for which the presence of XN metabolites is mainly expected.

3.1. UV spectra

Individual groups of polyphenolic compounds occurring in hop extracts have characteristic chromophoric groups resulting in the possible group distinction using their UV spectra, e.g., XN group, IXN group and bitter acids group (Fig. 2). Fig. 3 demonstrates the application of this approach for the search of metabolites with common chromophors using UV chromatograms at two wavelengths (371 and 290 nm) for samples of placebo experiment (Fig. 3A), rat feed (Fig. 3B) and feces from the Group 2 (Fig. 3C). The wavelength 371 nm is the most important for the XN chromophoric group. Most prenylated chalcones and their metabolites provide a better signal at 290 nm, as evident from the number of peaks in the retention window between 40 and 65 min. 16 well pronounced peaks are detected in the Group 2 at 290 nm. Table 1 shows the full list of detected peaks compared with the feed sample and placebo experiment, which makes possible to verify the origin of individual detected compounds, i.e., compounds coming from feed, present in the placebo experiment or real metabolites. This approach is essential due to the enormous sample complexity, because the starting material for this metabolic study is not pure compound (e.g., drug) but the hop extract in complex matrix of rat feed. Six important peaks are recognized only in the feces sample, so the careful evaluation of their chromatograms and spectra is done to enable their identification. However, the data of remaining 10 peaks observed in

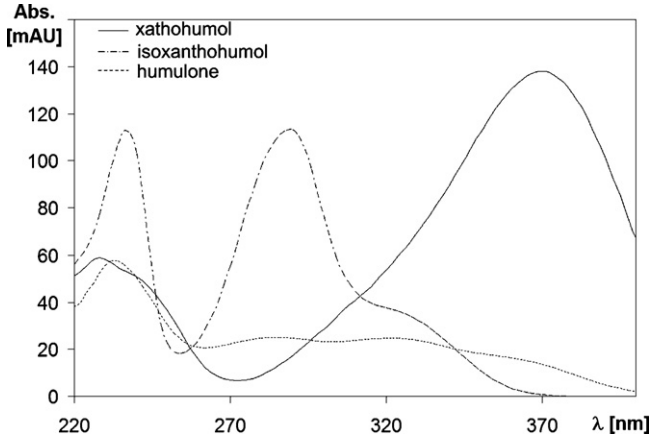

Fig. 2. UV spectra of xanthohumol (absorption maximum at 371 nm), isoxanthohumol (290 and 235 nm) and humulone (230, 280 and 325 nm).

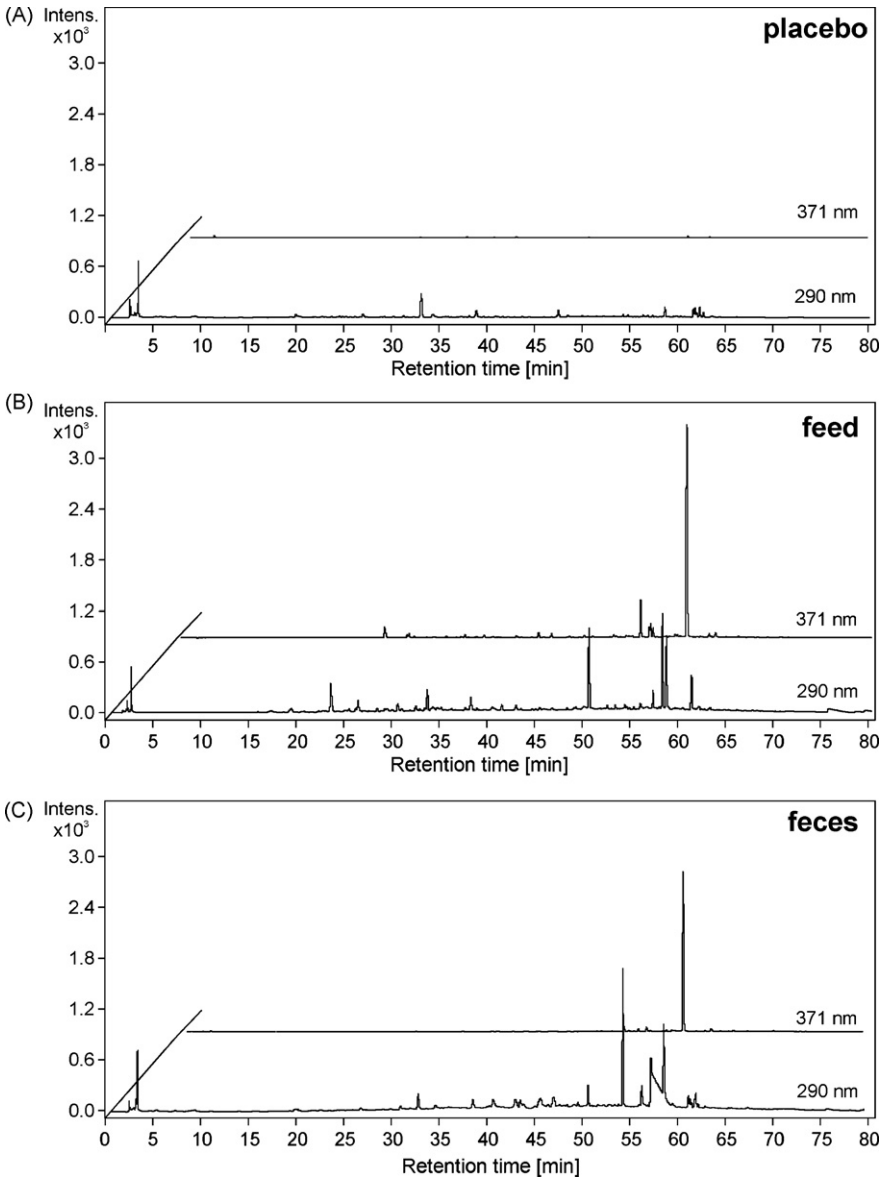

Fig. 3. UV chromatograms recorded at wavelengths 290 and 371 nm. (A) Placebo experiment; (B) rat feed; (C) rat feces sample of Group 2.

Table 1
Peak areas (in arbitrary units) of main peaks detected in the UV chromatogram at 290 nm.

Retention time [min]	Sample of feces	Sample of feed	Placebo experiment
32.9	1445	n.d.[a]	2071
38.7	1420	1655	728
40.9	1664	813	n.d.[a]
43.2	1941	667	n.d.[a]
43.7	1124	n.d.[a]	n.d.[a]
44.1	942	n.d.[a]	n.d.[a]
45.9	2575	n.d.[a]	n.d.[a]
46.7	340	n.d.[a]	n.d.[a]
47.4	2566	n.d.[a]	652
51.0	2532	6258	n.d.[a]
54.7	16381	n.d.[a]	n.d.[a]
56.6	2480	n.d.[a]	n.d.[a]
57.6	18171	1481	n.d.[a]
58.9	9148	6510	n.d.[a]
61.6	929	2778	1391
62.4	910	546	780

[a] n.d.—not detected.

placebo and/or feed samples are also important to detect possible coelutions with more absorbing compounds.

3.2. HPLC/MS

ESI full scan and tandem mass spectra in both polarity modes are measured for all studied samples. Negative-ion ESI mode provides a better sensitivity and higher information content for the metabolite identification. The external calibration with sodium formate clusters developed in our previous work [19] is applied for the whole mass range. The time shift between UV and MS record is determined with standards of XN, IXN and 8-PN and the measured delay of 0.2 min is then used for the exact alignment of both records. Peaks corresponding to retention times of these standards are found for both feed and feces samples but not for the placebo sample. Relative amounts of XN and IXN decrease in feces samples in comparison to feed, which confirms that both XN and IXN are metabolized in the rat body. On contrary, the concentration of 8-PN is significantly increased during the biotransformation, therefore it has to be formed by metabolism, as already reported previously [20,21]. The reconstructed ion current chromatograms are used not only

Table 2
List of main peaks detected by HPLC/MS with their retention times, *m/z* values of their $[M-H]^-$ ions, elemental composition of deprotonated molecules, mass accuracies and the description of metabolite formation.

Retention time [min]	*m/z* of $[M-H]^-$			Elemental composition	Mass accuracy [ppm]		Description of metabolite formation
	Theoretical	Experimental (feed)	Experimental (feces)		Feed	Feces	
41.6	369.1344	369.1329	n.d.[a]	$C_{21}H_{21}O_6$	−4.1	–	Initial compound (XN + O)
43.2	451.1068	n.d.[a]	451.1101	$C_{21}H_{23}O_9S$	–	7.3	IXN + O + H_2 + SO_3 (phase II)
43.7	453.1224	n.d.[a]	453.1242	$C_{21}H_{25}O_9S$	–	4.0	IXN + O + 2H_2 + SO_3 (phase II)
44.1	451.1068	n.d.[a]	451.1068	$C_{21}H_{23}O_9S$	–	0	IXN + O + H_2 + SO_3 (phase II)
46.7	449.0912	n.d.[a]	449.0924	$C_{21}H_{21}O_9S$	–	2.7	IXN + O+ SO_3 (phase II)
51.0	353.1394	353.1389	353.1405	$C_{21}H_{21}O_5$	−1.4	3.1	Initial compound (IXN)
53.4	369.1344	369.1332	n.d.[a]	$C_{21}H_{21}O_6$	−3.3	–	Initial compound (XN + O)
54.7	339.1238	n.d.[a]	339.1252	$C_{20}H_{19}O_5$	–	4.1	8-PN (phase I)
54.9	369.1344	369.1305	n.d.[a]	$C_{21}H_{21}O_6$	−10.6	–	Initial compound (XN + O)
56.6	341.1383	n.d.[a]	341.1402	$C_{20}H_{21}O_5$	–	5.6	IXN-CH_2 + H_2 (phase I)
57.6	435.1119	n.d.[a]	435.1188	$C_{21}H_{23}O_8S$	–	15.8	IXN + H_2 + SO_3 (phase II)
58.9	353.1394	353.1383	353.1420	$C_{21}H_{21}O_5$	−3.1	7.4	Initial compound (XN)
65.1	347.1864	347.1857	347.1846	$C_{20}H_{27}O_5$	−2.0	−5.2	Initial compound (cohumulon)
66.4	361.2020	361.2019	361.2036	$C_{21}H_{29}O_5$	−0.3	4.4	Initial compound (humulon)

[a] n.d.—not detected.

for the confirmation of retention times but also for obtaining exact regions for signal averaging and baseline subtraction, which yields good quality mass spectra even for trace and partially overlapping peaks (Fig. 1B).

The different strategy in the metabolite detection is targeted screening, which can provide complementary information to the untargeted metabolite detection described in the previous text. The targeted screening is based on the prediction of possible metabolites for a given parent compound (e.g., XN) using the software Metabolite tools. First, the Metabolite predict algorithm is applied for the prediction of possible XN metabolites in two generations allowing all metabolic rules defined for mammals, which generates 431 possibilities. After the automatic removal of duplicated structures and sorting this extensive list, only 20 different molecular weights are obtained (340, 356, 368, 370, 384, 386, 388, 420, 434, 450, 514, 516, 528, 530, 546, 587, 610, 659, 677 and 706) with numerous positional isomers. These data are downloaded into the Metabolite detect software, where the difference chromatogram is generated as a subtraction of feed sample from biotransformation sample of Group 2. 14 compounds originated from the hop extract are found using a combination of targeted and untargeted metabolite search described above, whereof 3 compounds are observed only for feed, 7 only for feces (i.e., metabolites) and 4 compounds are found in both samples (i.e., initial compounds). The right elemental composition of individual ions (Table 2) is determined with the help of so called sigma parameter combining the accurate determination of *m/z*, relative abundances of all isotopic peaks and distances among individual isotopic peaks [22,23]. No compound associated with the hop extract is found in the placebo experiment, as expected. It is noteworthy to mention that compounds eluted at 43.2 and 57.6 min in the UV chromatogram are found to be metabolites due to the interpretation of mass spectra, although the UV signal in this time region is also apparent in case of feed sample.

3.3. Interpretation of tandem mass spectra

3.3.1. MS/MS of initial compounds and their phase I metabolites

The fragmentation behavior is compared with the knowledge obtained with standards of XN, IXN and 8-PN. Important fragment ions are formed by the retro-Diels-Alder (RDA) reaction yielding the pair of complementary product ions at *m/z* 233 (A ring) and 119 (B ring) [12]. If the collision energy is increased from 25 eV [12] to 30 eV, then the neutral loss of C_4H_8 from XN leads to the formation of *m/z* 295, which is further followed by RDA reaction yielding the pair of product ions at *m/z* 175 and 119. Moreover, fragment ions at *m/z* 217 $[233\text{-}CH_4]^-$, 189 $[233\text{-}CO_2]^-$, 163 $[233\text{-}C_4H_6]^-$ originated from the ion *m/z* 233 are also present in the spectrum. This fragmentation pattern is verified by MS^n measurements with the ion trap. Considering the summary formula of precursor ion used as upper boundary in the elemental composition determination, only one (see Fig. 4B) is suggested for individual fragment ions even for a rather wide range of 5 mDa. The similar approach is used for the interpretation of other tandem mass spectra in Fig. 4, which confirms the suggested elemental composition for all detected compounds.

3.3.2. MS/MS of phase II metabolites

Sulfatation, hydrogenation and oxidation are dominant metabolic reactions in our study. Due to the identical skeleton with phase I metabolites, similar fragment ions are expected also for phase II metabolites. Their MS/MS spectra are shown in Fig. 5. The neutral loss of $\Delta m/z$ 82 ($SO_3 + H_2$) leads to the formation of ions at *m/z* 371 (Fig. 5A), 369 (Fig. 5B and C) and 353 (Fig. 5D and E). These fragment ions are observed already in the full scan mass spectra in accordance with a low stability of sulfate functional group. Fig. 5C shows the spectrum with the reduced value of collision energy from 30 to 20 eV, which leads to the significant increase of relative abundance of this loss (*m/z* 369 is the base

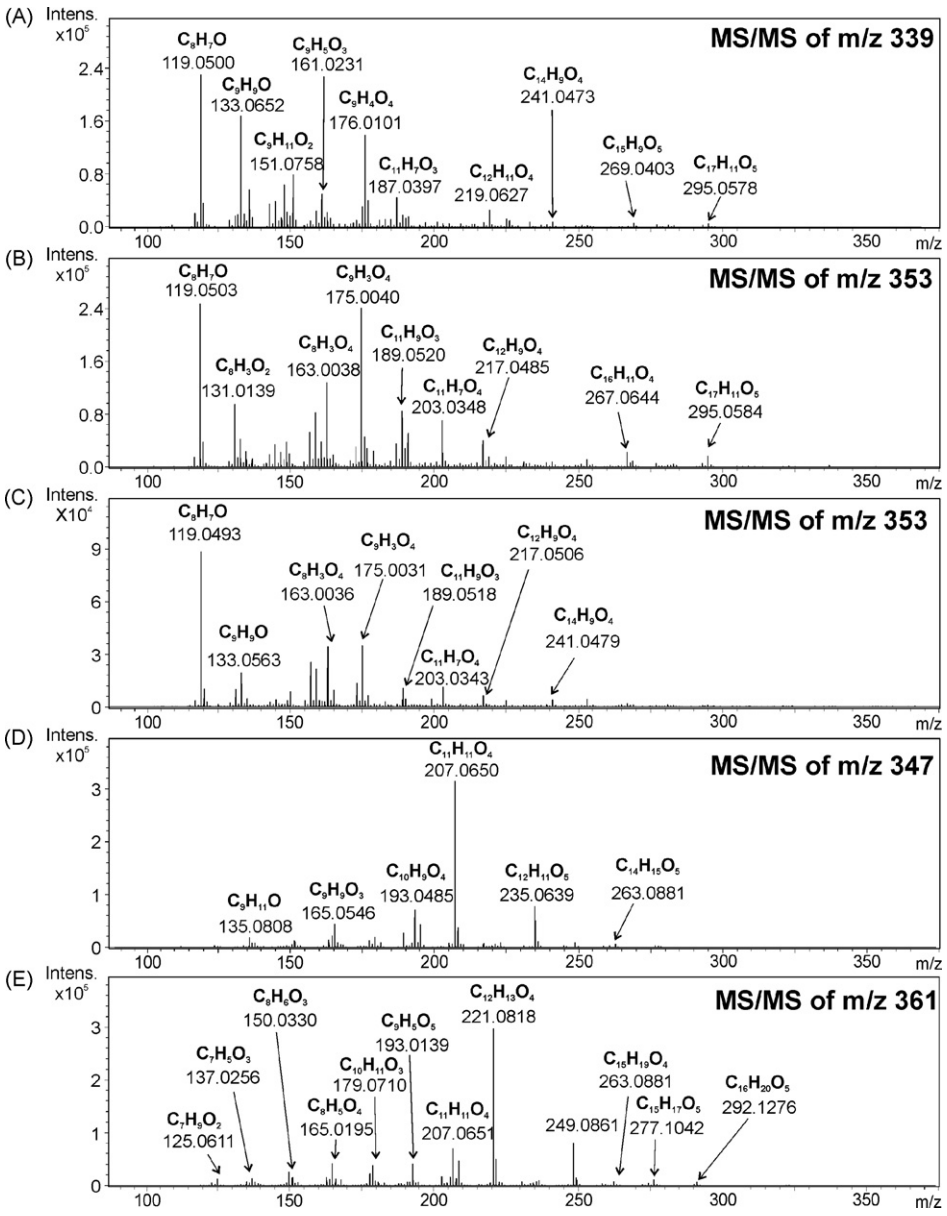

Fig. 4. MS/MS spectra of $[M-H]^-$ ions measured for the sample Group 2. (A) 8-Prenylnaringenin (m/z 339); (B) xanthohumol (m/z 353); (C) isoxanthohumol (m/z 353); (D) cohumulone (m/z 347); (E) humulone (m/z 361).

peak) confirming the labile character of sulfate group. The neutral losses $\Delta m/z$ 82 ($SO_3 + H_2$, see Fig. 5) and $\Delta m/z$ 100 ($H_2SO_4 + H_2$, low abundant ion at m/z 351 in Fig. 5C) for sulfates are rather unusual, because the common neutral losses for sulfates are $\Delta m/z$ 80 (SO_3) and $\Delta m/z$ 98 (H_2SO_4) or fragment ions m/z 80 $[SO_3]^-$ and m/z 97 $[HSO_4]^-$, as known for sulfated drugs [24], oligosacharides [25], peptides [26] and organic dyes [27]. The reason, why neutral losses of $\Delta m/z$ 82 and 100 occur instead of more common SO_3 or H_2SO_4 [8], is unknown. The change in relative abundances of M + 2 isotopic peaks is in accordance with the presence of one sulfur atom present in sulfates. Sulfated metabolites do not provide any response in the positive-ion ESI mode, as expected.

Table 3
Precursor ions and two product ions for observed *retro Diels-Alder* reactions in the spectra.

Precursor ion		Product ion 1		Product ion 2	
m/z	Elemental composition	m/z	Elemental composition	m/z	Elemental composition
371	$C_{21}H_{23}O_6$	251	$C_{13}H_{15}O_5$	119	C_8H_7O
369	$C_{21}H_{21}O_6$	249	$C_{13}H_{13}O_5$	119	C_8H_7O
353	$C_{21}H_{21}O_5$	233	$C_{13}H_{13}O_4$	119	C_8H_7O
339	$C_{20}H_{19}O_5$	219	$C_{12}H_{11}O_4$	119	C_8H_7O
297	$C_{17}H_{13}O_5$	177	$C_9H_5O_4$	119	C_8H_7O
295	$C_{17}H_{11}O_5$	175	$C_9H_3O_4$	119	C_8H_7O
283	$C_{16}H_{11}O_5$	163	$C_8H_3O_4$	119	C_8H_7O
281	$C_{16}H_9O_5$	161	$C_9H_5O_3$	119	C_8H_7O

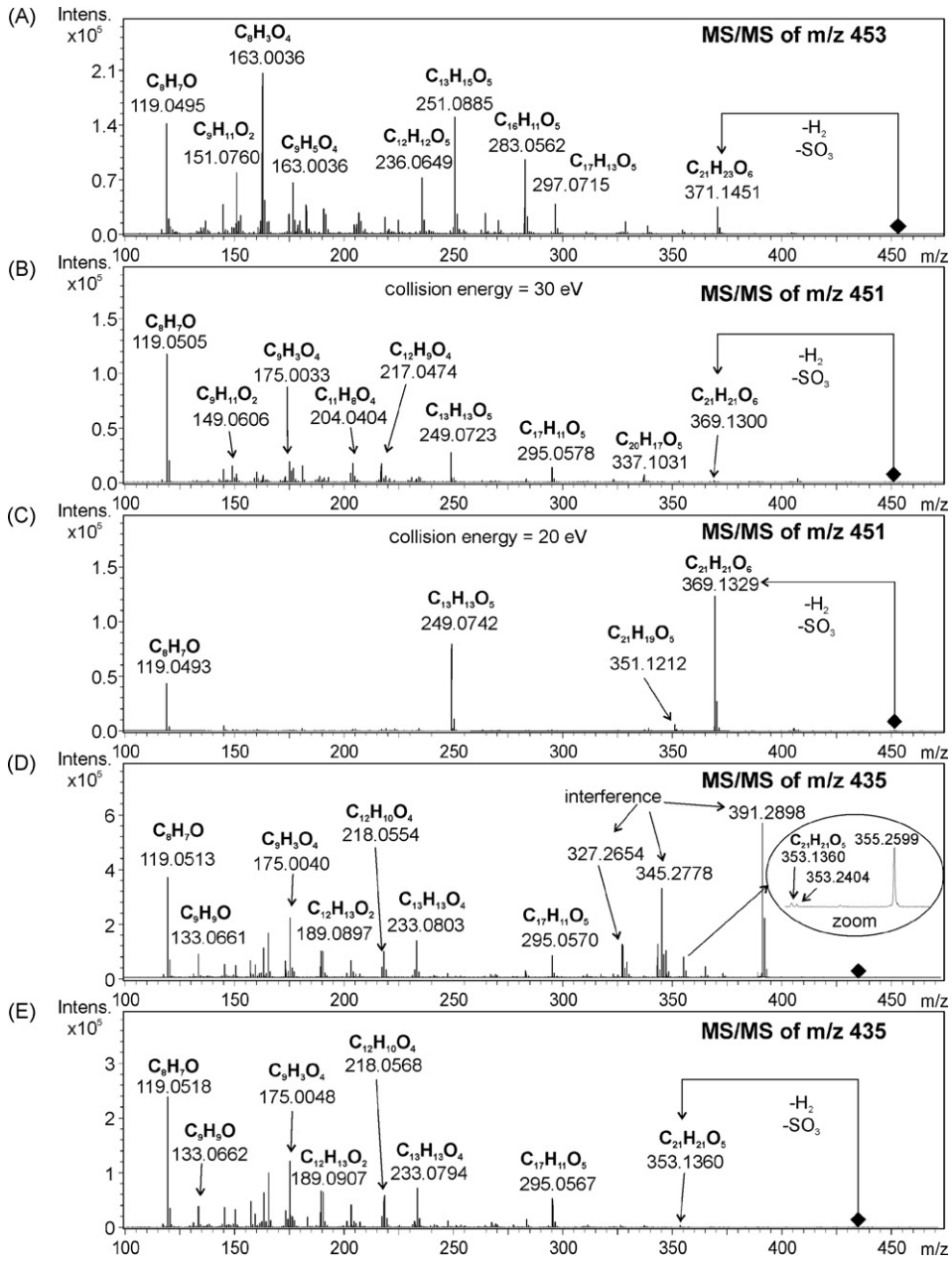

Fig. 5. MS/MS spectra of $[M-H]^-$ ions for phase II metabolites with the collision energy 30 eV for (A), (B), (D), (E) and the reduced value 20 eV for (C). (A) *m/z* 453 (Group 2); (B) *m/z* 451 (Group 2); (C) *m/z* 451 (Group 2); (D) *m/z* 435 (Group 2); (E) *m/z* 435 (Group 1).

The RDA mechanism is typical for metabolites derived from XN and other prenylflavonoids, as discussed in the previous paragraph. The survey of ions formed according to the RDA mechanisms is listed in Table 3. In all cases, one product of RDA is the ion *m/z* 119 with the elemental composition C_8H_7O and the complementary product ion has *m/z* value depending on the structure of precursor ion (see Table 3). This simple rule enables to determine the position of oxidation of some metabolic products, e.g., for ions *m/z* 453 and 451. The fragmentation pattern of *m/z* 451 is shown in Fig. 6A, and Fig. 6B–D shows potential positional isomers of these sulfates. MS data does not enable to determine the position of sulfate conjugation, because the sulfate moiety is the first neutral loss occurring during the fragmentation. In case of sulfate with MW = 452 Da, two positional isomers (t_R 43.2 and 44.1 min) are observed, while other sulfates with MW = 454 and 450 Da provide only one peak each.

MS/MS spectrum of the ion at *m/z* 435 shown in Fig. 5D contains some fragment ions (*m/z* 327.2654, 345.2778, 355.2599 and 391.2898) unmatched with the precursor ion. The explanation is evident from Fig. 7A with a detailed zoom of the ion at *m/z* 435, where two other ions of low intensity are present next to the ion at *m/z* 435.1188 or its isotopes. The presence of isobaric impurities is mainly reflected in mentioned tandem mass spectrum (Fig. 5D), where high abundant interfering product ions are observed as well. Their origin is also confirmed by tandem mass spectrum of *m/z* 391 (Fig. 7B). A wide tailing peak is observed in the interval of retention time between 57.6 and 60.0, which is caused by the insufficient separation of positional isomers of sulfate metabolites. All ions related to sulfate have identical profiles of reconstructed ion currents, which confirm the proposed interpretation. On the other hand, no overlay between peaks of XN and its sulfate metabolite (MW = 436) is observed for the sample Group 1 and also no

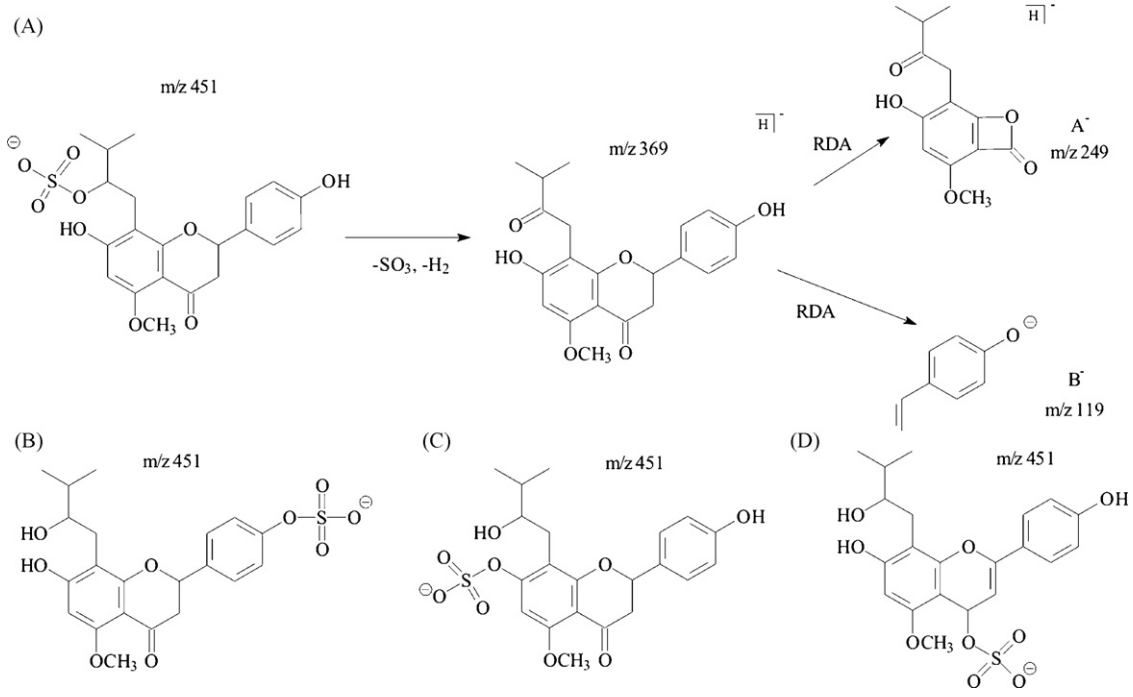

Fig. 6. (A) Proposed fragmentation scheme of sulfate metabolite including retro-Diels-Alder (RDA) mechanism. (B) Other possible structures of sulfate positional isomers with $[M-H]^-$ at m/z 451.

interfering ions neighboring with the ion at m/z 435 are found in comparison with the previous Group 2 sample so that no interfering fragment ions are observed in its tandem mass spectrum (Fig. 5E).

UV spectra of sulfate metabolites (Fig. 8) clearly proof that XN chromophoric groups is not retained for these metabolites due to the absence of absorption maximum around 371 nm. Sulfates with MW = 452 and 454 Da have very similar UV spectra with IXN which suggests that these metabolites are derived from IXN. The unambiguous confirmation of sulfate structures can be done with nuclear magnetic resonance spectroscopy, but it would require either the isolation of sufficient amount of pure metabolite from this complex mixture (almost impossible) or the synthesis and purification

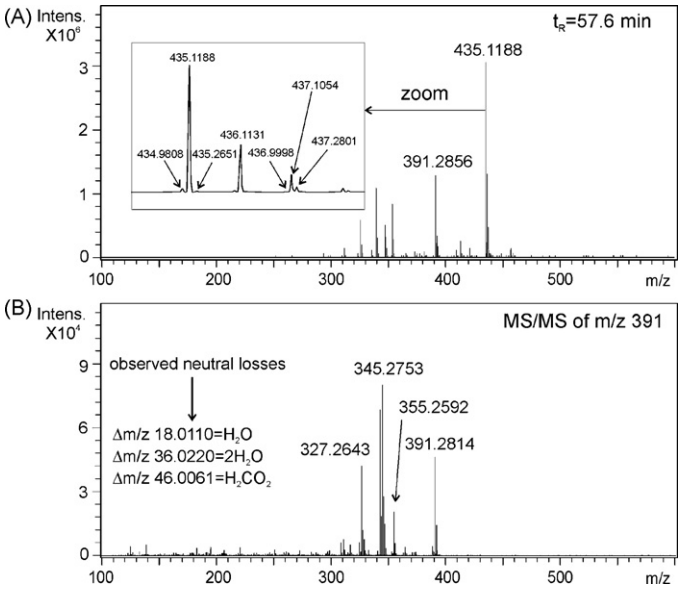

Fig. 7. Mass spectra of the peak at 57.6 min. (A) Full scan mass spectrum with the zoomed region around the ion at m/z 435. (B) MS/MS spectrum of the ion at m/z 391.

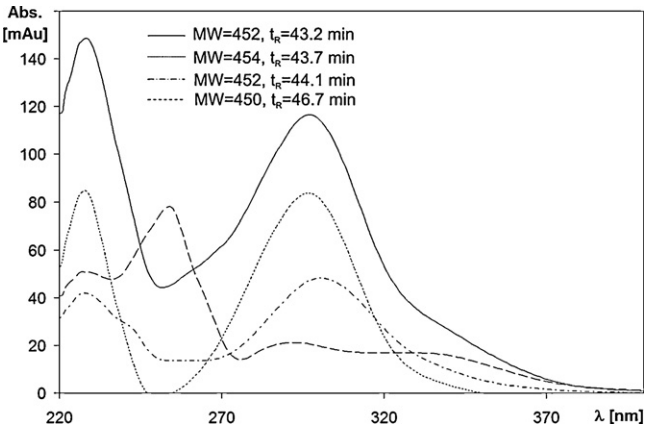

Fig. 8. UV spectra of sulfate metabolites: MW = 452 at t_R = 43.2 (absorption maxima at 297 and 228 nm); MW = 454 at t_R = 43.7 (297 and 228 nm); MW = 452 at t_R = 44.1 (301 and 228 nm) and MW = 450 at t_R = (254, 292 and 337 nm).

of expected metabolites (quite difficult task). It is interesting to note that the sulfate with MW = 450 Da has quite different UV spectrum with absorption maxima at 254, 292 and 337 nm, which can be tentatively explained by the presence of additional unsaturation (one more double bond or cycle) with a significant effect on the chromophoric system (bathochromic shift). In contrary to conclusions stated in Ref. [17], we have identified only sulfate metabolite but no glucuronides.

4. Conclusions

The present work shows a great potential of high mass accuracy HPLC/ESI-MS/MS measurements in the metabolic study of phases I and II metabolites for polyphenolic compounds present in hop extracts. The results obtained based on the comparison of feces chromatograms with referent chromatograms of rat feed and placebo experiment, the support of UV chromatograms at suitable

wavelengths, extracted ion chromatograms for expected masses of metabolites together with the information about characteristic neutral losses are summarized and applied for the metabolite identification. In total, two phase I metabolites and five phase II metabolites are detected in the rat *in vivo* biotransformation. The oxidation, demethylation, hydration and sulfatation reactions are among observed mechanisms of the metabolite formation.

Acknowledgements

This work was supported by the grant project Nos. MSM0021627502 (RJ and MH) and MSM6198959216 (EV, JU and VŠ) sponsored by the Ministry of Education, Youth and Sports of the Czech Republic and the project No. 203/08/1536 sponsored by the Czech Science Foundation. Authors would like to thank to Dr. K. Krofta (Hop Research Institute, Žatec, Czech Republic) for providing hop samples.

References

[1] K. Krofta, A. Mikyska, D. Haskova, J. I. Brewing 114 (2008) 160.
[2] C.S. Yang, J.M. Landau, M.T. Huang, H.L. Newmark, Annu. Rev. Nutr. 21 (2001) 381.
[3] S. Possemiers, A. Heyerick, V. Robbens, D. De Keukeleire, W. Verstraete, J. Agric. Food Chem. 53 (2005) 6281.
[4] C. Gerhauser, A. Alt, E. Heiss, A. Gamal-Eldeen, K. Klimo, J. Knauft, I. Neumann, H.R. Scherf, N. Frank, B. Bartsch, H. Becker, Mol. Cancer Ther. 1 (2002) 959.
[5] M.C. Henderson, C.L. Miranda, J.F. Stevens, M.L. Deinzer, D.R. Buhler, Xenobiotica 30 (2000) 235.
[6] S. Vogel, S. Ohmayer, G. Brunner, J. Heilmann, Bioorg. Med. Chem. 16 (2008) 4286.
[7] P.J. Magalhaes, D.O. Carvalho, J.M. Cruz, L.F. Guido, A.A. Barros, Nat. Prod. Commun. 4 (2009) 591.
[8] M. Holčapek, L. Kolářová, M. Nobilis, Anal. Bioanal. Chem. 391 (2008) 59.
[9] P.J. Magalhaes, L.F. Guido, J.M. Cruz, A.A. Barros, J. Chromatogr. A 1150 (2007) 295.
[10] X. Zhang, X. Liang, L. Xiao, Q. Xu, J. Am. Soc. Mass Spectrom. 15 (2004) 180.
[11] L. Česlová, M. Holčapek, M. Fidler, J. Drštičková, M. Lísa, J. Chromatogr. A 1216 (2009) 7249.
[12] D. Nikolic, Y.M. Li, L.R. Chadwick, G.F. Pauli, R.B. van Breemen, J. Mass Spectrom. 45 (2005) 289.
[13] P.C. Hollman, M.B. Katan, Arch. Toxicol. Suppl. 20 (1998) 237.
[14] M. Yilmazer, J.F. Stevens, M.L. Deinzer, D.R. Buhler, Drug. Metab. Dispos. 29 (2001) 223.
[15] D. Nikolic, Y.M. Li, L.R. Chadwick, S. Grubjesic, P. Schwab, P. Metz, R.B. van Breemen, Drug. Metab. Dispos. 32 (2004) 272.
[16] M. Yilmazer, J.F. Stevens, D.R. Buhler, FEBS Lett. 491 (2001) 252.
[17] C.E. Ruefer, C. Gerhauser, N. Frank, H. Becker, S.E. Kulling, Mol. Nutr. Food Res. 49 (2005) 851.
[18] A. Nookandeh, N. Frank, F. Steiner, R. Ellinger, B. Schneider, C. Gerhäuse, H. Becker, Phytochemistry 65 (2004) 561.
[19] R. Jirásko, M. Holčapek, E. Rosenberg, Int. J. Mass Spectrom. 280 (2009) 198.
[20] D. Nikolic, Y.M. Li, L.R. Chadwick, G.F. Pauli, R.B. van Breemen, J. Mass Spectrom. 40 (2005) 289.
[21] S. Possemiers, S. Rabot, J.C. Espın, A. Bruneau, C. Philippe, A. Gonzalez-Sarrıas, A. Heyerick, F.A. Tomas-Barberan, D. de Keukeleire, W. Verstraete, J. Nutr. 138 (2008) 1310.
[22] S. Ojanperä, A. Pelander, M. Pelzing, I. Krebs, E. Vuori, I. Ojanperä, Rapid Commun. Mass Spectrom. 20 (2006) 1161.
[23] R. Jirásko, M. Holčapek, L. Kolářová, M. Nádvorník, A. Popkov, J. Mass Spectrom. 43 (2008) 1274.
[24] S.T. Wu, K. Cao, S.J. Bonacorsi, H. Yhang, M. Jemal, Rapid Commun. Mass Spectrom. 23 (2009) 3107.
[25] Y. Zhang, T. Iwamoto, G. Radke, Y. Kariya, K. Suzuki, A.H. Conrad, J.M. Tomich, G.W. Conrad, J. Mass Spectrom. 43 (2008) 765.
[26] K. Imami, Y. Ishihama, S. Terabe, J. Chromatogr. A 1194 (2008) 237.
[27] M. Holčapek, K. Volná, D. Vaněrková, Dyes Pigments 75 (2007) 156.