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Flavonol quantification and stability of phenolics in fermented extracts from fresh *Betula pendula* leaves

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ABSTRACT

An HPLC method, which allows reliable quantitation of flavonols and other phenolics in birch leaf extracts, was developed and validated. The method was applied to study the bioconversion of flavonols in fermented aqueous extracts. Almost 100% of the flavonols were converted during the 12 months observation period. The generated phenolics as well as consecutive conversion products were identified by HPLC–DAD, LC–MS and GC–MS techniques.

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1. Introduction

Quercetin and its glycosides are natural compounds displaying a broad variety of biological activities [1], such as antioxidant [2] and antiinflammatory [3] activity or tumor inhibiting properties [4–6]. They are widespread in nature, and occur in appreciable amounts in some medicinal plants, such as *Betula pendula* leaves [7]. Moreover, they are used as quality parameters for plant preparations. Here, we report a simple validated RP–HPLC method allowing reliable quantitation of quercetin glycosides and the corresponding aglycone in extracts prepared from birch leaves. We concentrated on fermented aqueous extracts which were investigated for the stability of phenolics including flavonols and caffeic acid derivatives over a period of 12 months. Lactic acid fermentation is known to prevent contamination of aqueous extracts with other pathogenic bacteria and results in microbiologically stable extracts with a pH below 4.5 [8]. As flavonoids are known to be enzymatically degraded by microorganisms [9,10], it was an interesting task to monitor the conversion of the predominant phenolics as well as subsequent decomposition products in the presence of *Lactobacillus* species and endogenous plant enzymes using HPLC–DAD, LC–MS and GC–MS.

2. Experimental

2.1. Chemicals

Hyperoside for quantification purposes was purchased from Extrasynthèse (Genay, France, >98%, HPLC). Acacetin, apigenin, caffeic acid and *p*-coumaric acid, isoquercitrin, rutin (all purum, TLC), quercetin-3-arabinopyranoside (without determination of α- or β-configuration; HPLC ≥99%), chlorogenic acid (>97%), quercitrin (≥98%), as well as methanol and acetonitrile HPLC grade, were all obtained from Roth (Karlsruhe, Germany). *m*-Hydroxyphenylpropionic acid (>98%) was purchased from Alfa Aesar (Karlsruhe, Germany). Catechol (99%), *p*-hydroxyacetophenone (>98%), *p*-hydroxyphenylethanol (97%), *p*-hydroxyphenylpropionic acid (98%) and quercetin (>98%, HPLC) were obtained from Sigma (Seelze, Germany), as well as acetic acid (99.8%), methanol and tetrahydrofurane p.a. grade. Quercetin 3-β-glucuronide (≥90%, HPLC) was isolated from *Arnica montana* [11]. Distilled water was purified with a Milli-Q Plus PF system (18.2 MΩ cm at 25 °C; Millipore, Billerica, Massachussetts, USA) and was further filtrated on a 4.5 μm filter for chromatographic use.

2.2. Fermented aqueous birch extracts

Samples from fermented aqueous *Betula pendula* extracts were provided by WALA Heilmittel GmbH (Bad Boll/Eckwälden, Ger-

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many). Birch leaves were collected on plantations in Bad Boll, Germany. The extracts were prepared according to an official specification [12] using fresh birch leaves, water and whey (whey according to [13]—as fermentation starter) in a ratio of 100:225:50 (w/w/w). Making use of the natural microbial flora of the plant and the whey, spontaneous fermentation was performed over 3.5 days at room temperature before removing the birch leaves. The resulting turbid filtrate was again fermented for 3.5 days at room temperature and then left for further storage at 15 °C over 12 months. Aliquots were taken after 24 h (*d1*), 3.5 days (*d3*) and 7 days (*d7*) of extraction as well as after 2, 4, 6, 9 and 12 months (*m2–m12*) of storage at 15 °C in the dark. Each sample was immediately frozen at −20 °C until analyses. Four batches of *B. pendula* extracts over a 3-year period from 2006 to 2008 were provided for analyses (batches 1-2006, 1-2007 and 1-2008). The validation procedure was carried out with a fermented extract from 2005 (batch 1-2005) taken on day 3.5 (*d3*). For quantification purposes, two aliquots from each fermented aqueous extract sample (*d3* to *m12*) were allowed to reach room temperature and diluted 1:3 (v/v) with water, then shortly vortexed and centrifuged. The supernatant was analyzed in duplicate by HPLC–DAD.

Sample 2008-*m6* of the fermented extract was separated on a Sephadex LH-20 column (Pharmacia–GE Healthcare, Sweden) with methanol as eluent. The isolated 3,4′-dihydroxypropiophenone was freeze-dried before further NMR and MS analyses.

2.3. Instrumental analyses

HPLC–DAD: HPLC analyses were carried on a HP-1090 system (Hewlett-Packard, Palo Alto, CA), equipped with autosampler, tertiary pump and photodiode array-detector. The analysis software was HP ChemStation (Agilent, version A.10.02). A Symmetry RP-column (C18; 3.5 μm; 150 mm × 4.6 mm), was connected to a Nova-Pak precolumn (C18; 4 μm; both from Waters, Eschborn, Germany). In addition, the following columns were tested during the optimization: Hypersil ODS (5 μm, 125 mm × 4.6 mm, Bischoff, Leonberg, Germany), Nova-pak (4 μm, 250 mm × 4.6 mm, Waters, Eschborn, Germany) and Spherisorb ODS (5 μm, 125 mm × 4.6 mm, Waters, Eschborn, Germany). Detection was performed at 280 and 351 nm, respectively. The following eluents were used: (A) tetrahydrofurane, acetonitrile, water and acetic acid (>99.8%) at a ratio of 4:1:90:5 (v/v/v/v); (B) methanol (HPLC grade). The elution profile was: 0–5 min, 10% B; 5–35 min, 10–20% B; 35–37 min, 20–60%; 37–46 min, 60% B; 46–49 min, 60–100% B; 49–55 min, 100% B; 55–56 min, 100–10% B; 56–60 min, 10% B. The injection volume was 20 μl and the flow rate 1.2 ml/min.

LC–MS: The HPLC–system was an Agilent HPLC series 1200 (Agilent, Waldbronn, Germany) equipped with a degasser G1322A, a binary gradient pump G1312A, an auto sampler G1329A, a column oven G1316A, and a diode array-detector G1315B. Separation was achieved on a Zorbax RP-column (C18; 3.5 μm; 250 mm × 4.6 mm, Agilent, Waldbronn, Germany), connected with a Security Guard Cartridge (C18; 4 mm × 2.0 mm, Phenomenex, Aschaffenburg, Germany). Solvent A was 0.05% formic acid, solvent B methanol. The elution system was: 0–3 min, 15% B; 3–6 min, 15–30% B; 6–18 min, 30–35% B; 18–25 min, 35–70% B; 25–35 min, 70% B; 35–40 min, 70–100% B; 40–42 min, 100% B; 42–45 min, 100–15% B; 45–50 min, 15% B. The injection volume was 20 μl and the flow rate 1.0 ml. Monitoring was performed at 280 and 351 nm, respectively. The LC system was coupled to an HCT ultra ion trap (Bruker Daltonic GmbH, Bremen, Germany) with an ESI source operating in the negative mode. Full scan mass spectra of the HPLC eluates were recorded during the chromatographic separation yielding $[M-H]^-$ ions. To gain further structural information, these ions were trapped and fragmented to yield the precursor product patterns of the analytes. MS^n data were acquired in the auto MS/MS mode with a compound stability and trap drive level of 100%. The instruments were controlled by an Agilent Chemstation and EsquireControl Software (6.1).

GC–MS: GC–MS analyses were carried out as previously described [14]. The temperature profile started at 60 °C, followed by a rate of 10 °C/min to 120 °C, which was held for 10 min. The injector and detector temperatures were set to 290 °C, the injection volume was 1.0 μl and the split was 10:1. Mass spectra were recorded over the *m/z*-range from 40 to 400. Chromatograms were monitored in total ion mode. For the analyses of trace level compounds, 1 ml of each original sample was freeze-dried, 1 ml methanol was added, mixed during 10 min in an ultrasonic bath and centrifuged before the supernatant was injected in the GC–MS system.

NMR: NMR spectra were recorded on a Bruker DRX 400 MHz Nuclear Magnetic Resonance Spectrometer instrument (Bruker, Bremen, Germany) at 400 MHz (^{1}H) and 100 MHz (^{13}C).

2.4. Validation procedure

The quantification method was validated according to the FDA [15] and ICH guidelines [16,17]. The guidelines were compared according to [18,19]. The validation included tests on specificity, linearity, precision, accuracy and robustness. Standard deviations of 5% for the reference solutions but of 10% for the birch leaves extracts were tolerated because of the higher instability of flavonols in natural extracts.

2.4.1. Linearity

Stock solutions of 200 μg/ml hyperoside and 10 μg/ml quercetin in methanol (HPLC grade) were separately prepared in 10 ml volumetric flasks. Calibration samples were prepared by dilution of the stock solutions with methanol (HPLC grade), to obtain hyperoside and quercetin solutions ranging from 2 to 200 μg/ml and 1 to 10 μg/ml, respectively. Each concentration was analyzed twice (Fig. 1).

2.4.2. Precision

The precision of the system was established by injecting one quercetin sample (5 μg/ml) and one hyperoside sample (50 μg/ml) six times on the same day.

The repeatability and the inter-day intermediate precision were determined by analyzing three samples of different concentrations covering the range of the calibration curve (1, 5 and 10 μg/ml quercetin and 2, 50 and 200 μg/ml hyperoside) three times a day at three different days (Tables 1 and 3). Repeatability and inter-day intermediate precision were also monitored for a fermented aqueous extract (2005-*d3*).

2.4.3. Accuracy of the mean

Accuracy was determined by spiking the sample 2005-*d3* with different concentration levels of hyperoside (Table 2) and quercetin. For this purpose, 44 and 150 μl of a 205 μg/ml hyperoside stock solution as well as 1 μl of a 10 μg/ml quercetin stock solution were added to 50 μl of 2005-*d3*. Each sample thus obtained was further diluted with water to reach a final volume of 200 μl.

2.4.4. Robustness

The sample stability in the autosampler was evaluated by analyzing 4 samples (1, 5 and 10 μg/ml quercetin and aliquot 2005–*d3*) three times a day on three different days (Table 4). The samples stayed in the autosampler (at around 20 °C) over the whole period

Table 1
Validation data: repeatability and intermediate precision for hyperoside in standard solutions with different concentration, in the fermented extract (diluted 1:3 (v/v) in water) and in a methanolic extract.

Sample conc. (μg/ml)	Mean hyperoside conc. exp. determined (μg/ml)	RSD (%) of repeatability (within-day precision)	RSD (%) of intermediate precision (3 days)	Recovery (%)
Hyperoside (2)	1.69	0.50–4.30	3.23	81.00–86.80
Hyperoside (50)	49.13	0.25–2.78	1.14	96.89–100.90
Hyperoside (200)	199.60	0.66–2.14	1.77	98.36–100.61
Ferm. extract	3.18	2.71–6.70	1.13	–
MeOH-extract	196.30	1.97–4.65	1.50	–

of analysis and the three series of injection were started with 21 h interval (each beginning from the lowest concentration).

The freeze and thaw stability of the reference solutions was evaluated by analyzing two samples (57 μg/ml hyperoside and 5 μg/ml quercetin) three times a day on three different days (Table 5). These samples were kept at −20 °C and allowed to reach room temperature prior to each injection series.

3. Results and discussion

3.1. Method development

The analytical method for quantitation of hyperoside was optimized based on the study by Dallenbach-Tölke et al. [20]. A gradient was developed with methanol as solvent B resulting in the best

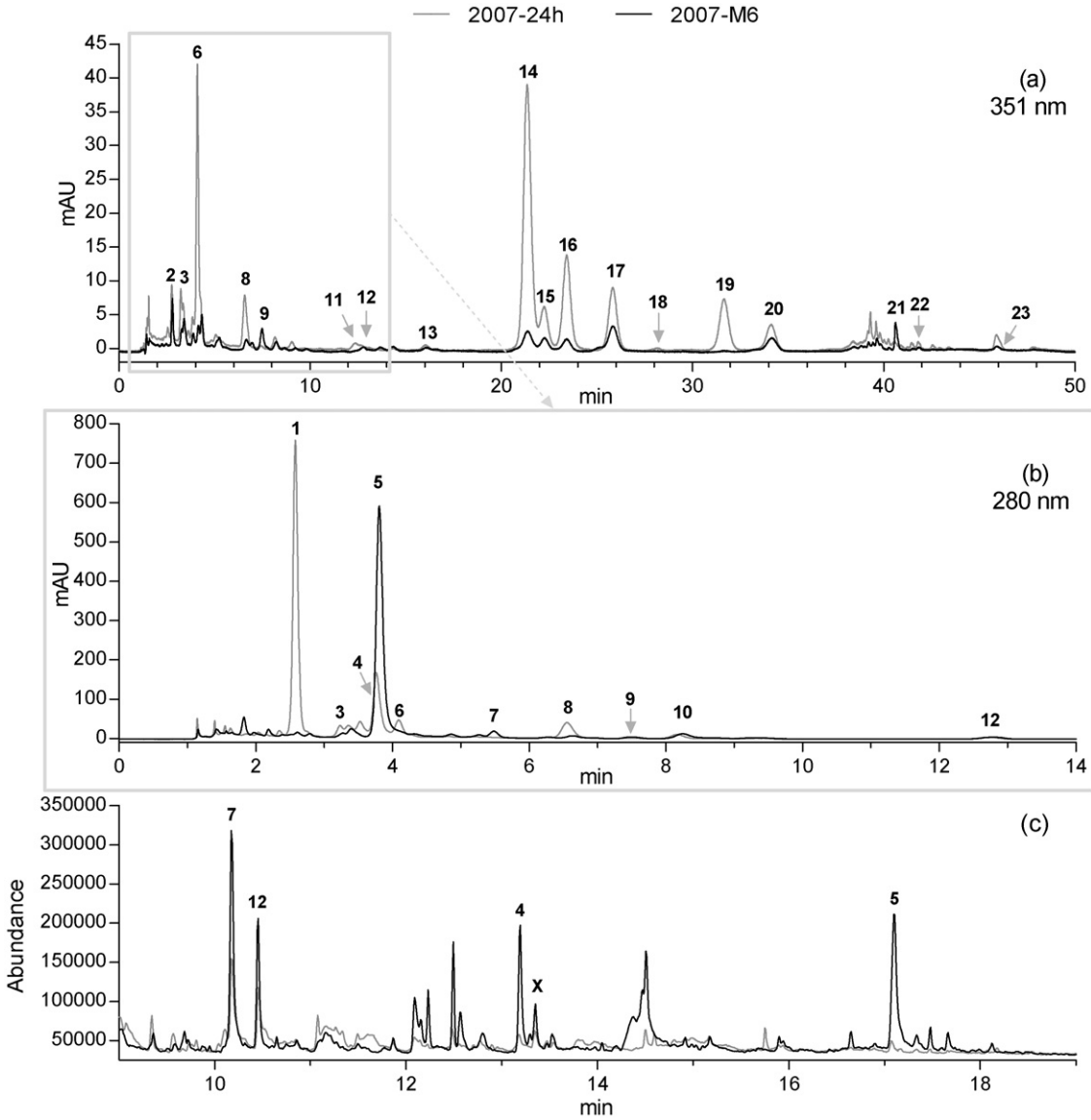

Fig. 1. Superimposed chromatograms of two samples of fermented aqueous extract from fresh birch leaves (year 2007). Gray: extract after 24 h; black: extract after 6 months. (a) HPLC–UV at 351 nm; (b) HPLC–UV, most polar part at 280 nm; (c) GC–MS. Peak assignment: (1) 3,4′-dihydroxypropiophenone 3-β-glucopyranoside; (2) neochlorogenic acid; (3) 3-*p*-coumaroylquinic acid; (4) *p*-hydroxyphenylethanol; (5) 3,4′-dihydroxypropiophenone; (6) chlorogenic acid; (7) catechol; (8) 5-*p*-coumaroylquinic acid; (9) caffeic acid; (10) *p*-hydroxyphenylpropionic acid; (11) myricetin 3-β-galactoside + myricetin 3-β-glucuronide; (12) *p*-coumaric acid; (13) myricetin 3-β-glucoside; (14) hyperoside; (15) rutin + isoquercitrin; (16) quercetin 3-β-glucuronide; (17) quercetin 3-arabinopyranoside*; (18) kaempferol 3-glycoside; (19) quercetin 3-α-arabinofuranoside; (20) quercitrin; (21) quercetin; (22) apigenin; (23) acacetin; (X) *p*-hydroxyacetophenone. * α or β configuration not determined.

Table 2
Validation data: repeatability and recovery by spiking the fermented extract with hyperoside.

Hyperoside conc. spiked (μg/ml)	Mean hyperoside conc. determined (μg/ml)	RSD (%) of repeatability ($n = 3$)	Recovery hyperoside (%)	Recovery flavonol glycosides (%)
45.10	46.41	0.76	96.13	97.23
153.75	161.49	0.05	102.90	104.91

separation. A C18 Symmetry column turned out to afford the optimum results compared to the other columns, such as Hypersil ODS, Nova–pak, and Spherisorb ODS.

3.2. Validation

Peak assignments in the HPLC–DAD chromatogram (Fig. 1) were performed by comparison with literature data [21–24], by analyzing UV–spectra and LC–MS data as well as by spiking with authentic reference substances where available. Peak purity of flavonols was verified using the ChemStation peak purity test confirming the specificity of the method. The validation procedure was carried out for hyperoside and quercetin representatively. All flavonol glycosides were quantified as hyperoside equivalents. A sample of fermented aqueous extract (from year 2005, taken after 3.5 days = 2005–*d3*) was used for all validation steps.

3.2.1. Hyperoside: limit of quantification, precision, accuracy

The limit of quantification (LOQ) was determined for hyperoside to be 2 μg/ml. The signal/noise ratio was 11.3:1 (ICH requires a value >10:1), the relative standard deviation was <4% (see Table 1) and the smallest recovery was 81% (FDA requires a recovery from 100 ± 20% [15]). Calibration was performed by analysis of hyperoside reference solutions in duplicate at six concentration levels between 2 and 200 μg/ml. As flavonol glycoside content varied significantly between extracts, calibration curves had to cover a wide range. The best correlation between concentration (C_H) and peak area (A_H) was non-linear (Fig. 2, left). Calibration function and correlation coefficient were calculated as given below:

$$C_H\ [\mu g/ml] = \left(\frac{A_H + 0.7293}{20.64}\right)^{1/1.014}; \quad R^2 = 0.9999 \qquad (1)$$

The plotted residual was randomly scattered, justifying the non linear model (Fig. 2, low panel).

A standard deviation of 0.41% (50 μg/ml hyperoside) was calculated by the investigation of the system precision. This was not significant, confirming the injector stability.

Repeatability and intermediate precision analysis was performed for hyperoside at three concentration levels (2, 50 and 200 μg/ml) (Table 1), for a fermented aqueous extract from fresh leaves (2005–*d3*) and a methanolic extract from dried birch leaves (25 mg/ml, w/w). All standard deviations were <4% for the standard solutions and <7% for the plant extracts.

The accuracy of the mean was deduced from the repeatability data at different concentration levels. The recovery values for the higher concentration range (50 and 100 μg/ml) were all in the range of 100 ± 2%.

3.2.2. Quercetin: limit of quantification, precision, accuracy, stability

For quercetin, the LOQ was determined to be 1 μg/ml, with a relative standard deviation of <5% (Table 3) and a recovery in the range of 100 ± 3%. Signal/noise ratio was 10.6:1. The LOQ was set in both cases as the lowest concentration for the calibration.

The latter was determined by analyzing quercetin reference solutions at five concentration levels between 1 and 10 μg/ml in duplicate. Again, a non-linear response from the area (A_Q) to the concentration (C_Q) was obtained (Fig. 2, right), and calibration function and correlation coefficient were calculated as given below:

$$C_Q\ [\mu g/ml] = \left(\frac{A_Q + 2.658}{12.18}\right)^{1/0.9451}; \quad R^2 = 0.9996 \qquad (2)$$

The plotted residual was randomly scattered, justifying the non-linear model (Fig. 2, low panel).

Confirmation of the system precision was achieved for quercetin (5 μg/ml), with a standard deviation of 0.85%.

Precision analyses were also carried out for quercetin at three concentration levels (1, 5 and 10 μg/ml) and for the fermented extract (2005-*d3*). Standard deviations were <5% for all standard solutions and <8% for the extract (Table 3). The recovery data for quercetin were in the range of 100 ± 5% for all concentration levels (Table 3).

Some recovery experiments were also done for quercetin by spiking the fermented extract (6.3 μg/ml) with different concentrations of the standards. For a concentration of 1 μg/ml the calculated mean concentration was 6.8 g/ml with a recovery of 97.06% and a relative standard deviation of 4.87%. Higher levels of quercetin spiked gave results outside the calibration range.

An autosampler stability test (Table 4), performed over 3 days for three quercetin concentrations and for the fermented extract, showed instability for all samples when left overnight in the autosampler, with a much higher instability at small concentration levels. There from a recovery rate of 40.12% was calculated for the 1 μg/ml quercetin sample after 3 days, but this data was below the quantification range. 6.3 μg/ml of the fermented extract gave a recovery rate of 19.79% after a 3 days period. Consequently, reference solutions should be left only a short time in the autosampler (particularly for low concentration).

3.2.3. Robustness determination of hyperoside and quercetin solutions

The robustness was studied by means of a freeze and thaw stability test (Table 5). Even though the standard deviation remained below 5% in the "within" as well as in the "inter-day" precision, the recovery slightly decreased over 3 days from 101.19 to 99.45% for hyperoside and from 97.57 to 95.71% for quercetin, respectively. Hence, reference solutions should not be frozen and thawed more than three times.

3.2.4. System suitability

System suitability tests were conducted with a couple of HPLC–DAD runs (from standard solutions and extract samples). For all of them, the capacity factor was >40. The resolution factor was >8 for quercetin and >2 for all flavonol glycosides except for hyperoside and isoquercitrin which were not completely separated. The number of theoretical plates was >8000. The peak shape was also evaluated with the tailing factor, which was between 1.0 and 1.8. As shown at the beginning of Section 3.2, the standard deviation of the injection precision test was <1%. All data met the FDA requirements [25].

3.3. Stability of flavonols in the fermented aqueous birch extracts

Calculated concentrations of flavonol glycosides and of quercetin (%) in the fermented aqueous extracts (years 2006–2008) are given in the Supplementary Information (Table S1). Flavonol

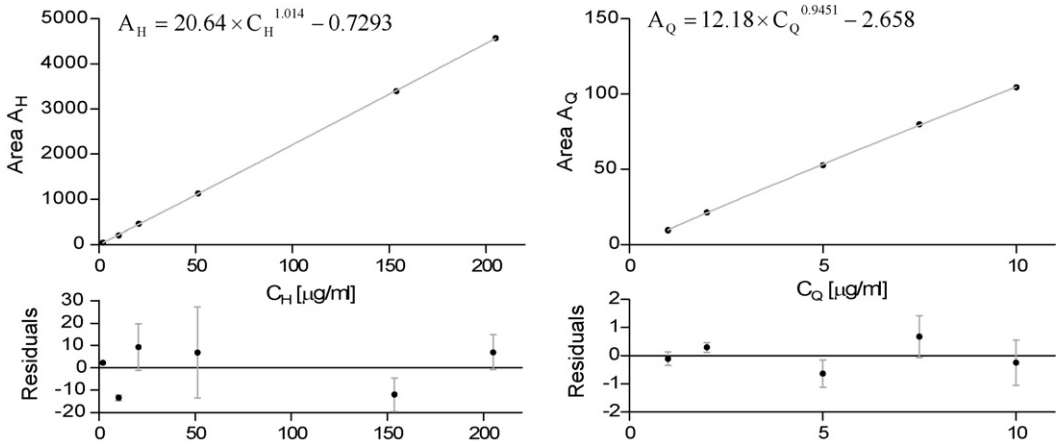

Fig. 2. Calibration curves and residual plots for reference solutions of hyperoside at six different concentrations (left) and quercetin at five concentrations (right), each injected in duplicate.

Table 3
Validation data: repeatability and intermediate precision for quercetin in standard solutions with different concentrations and in the fermented extract (diluted 1:3 (v/v) in water).

Sample conc. (µg/ml)	Mean quercetin conc. determined (µg/ml)	RSD (%) of repeatability (within-day precision)	RSD (%) of intermediate precision (3 days)	Recovery (%)
Quercetin (1)	1.0	1.48–4.34	3.51	97.03–100.36
Quercetin (5)	4.8	0.91–1.63	1.36	95.30–96.49
Quercetin. (10)	10.0	0.65–1.19	1.09	99.34–101.01
Ferm. extract	6.3	3.26–7.99	1.23	–

Table 4
Validation data: repeatability and intermediate precision for the autosampler stability of quercetin in standard solutions with different concentrations and in the fermented extract (diluted 1:3 in water). Concentration is given in µg/ml of diluted sample. Mean concentration and recovery data are given for 1d/2d/3d in the autosampler.

Sample conc. (µg/ml)	Mean quercetin conc. determined (µg/ml)	RSD (%) of repeatability (within-day precision)	RSD (%) of intermediate precision (3 days)	Recovery (%)
Quercetin (1)	0.9/0.7*/0.5*	0.75–7.21*	29.61	80.91/58.87*/40.12*
Quercetin (5)	4.8/4.7/4.3	1.46–3.41	4.96	95.89/93.77/86.68
Quercetin (10)	9.6/9.4/8.1	0.06–1.58	8.03	96.48/94.47/81.11
Ferm. extract (6.3)	6.3/2.1/1.2	5.39–11.15	87.19	100.42/32.66/19.79

* Data outside of the validated quantification range.

glycoside contents significantly differed between the three studied leaf batches, with higher concentrations in the samples from 2007. This variability is not surprising, as it is known that the content of flavonoids is influenced by various parameters [26], such as UV light [27], CO_2 concentration, temperature [28], or stage of the leaves development [29]. Fig. 3 shows the decrease of the total amount of flavonol glycosides, quercetin and of some single flavonol glycosides in the fermented extract. After 12 months, only 0–12% of the initial concentration of the total flavonol glycosides remained in the fermented aqueous extract. Flavonols were rapidly converted between the first and the third day of extraction (50–80% conversion rate). Interestingly, the different flavonol glycosides did not behave the same way. While the predominant compound hyperoside decomposed most rapidly, quercitrin displayed the best stability among the flavonol glycosides monitored in this study. Hein et al. [10] reported the same sugar dependent metabolism when studying decomposition kinetics of flavonol glycosides by gut microflora fermentation.

Whereas the concentration of flavonoid glycosides decreased, quercetin levels increased, but a significant correlation was not observed, since quercetin was generated as well as degraded at the same time. Highest levels of quercetin ranged from 168% (*d3*, 2006) to 763% (*m2*, 2007) of the initial concentration (Fig. 3, Table S1). The difference in the increase of quercetin between the three extracts cannot yet be explained. The pH-value or protein concentrations are not involved, as similar values were obtained for the three extracts (data not shown).

3.4. Conversion of phenolics in fermented aqueous extracts

As described in Section 3.3, quercetin was the most common deglycosylation product which itself was subsequently further degraded (see Fig. 3). In samples taken later during fermentation (*m6*, 2007 and 2008) traces of hydroxyphenylpropionic acid (peak 10, Fig. 1), an oxidation product of quercetin, was detected.

Table 5
Validation data: robustness—freeze and thaw stability of two reference solutions.

Sample (conc. µg/ml)	Mean conc. exp. determined (µg/ml)	RSD (%) of repeatability (within-day precision)	RSD (%) of intermediate precision (3 days)	Recovery (%)
Hyperoside (57)	57.18	0.19–0.94	0.95	101.19/100.29/99.45
Quercetin (5)	4.82	1.61–3.39	2.51	97.57/95.93/95.71

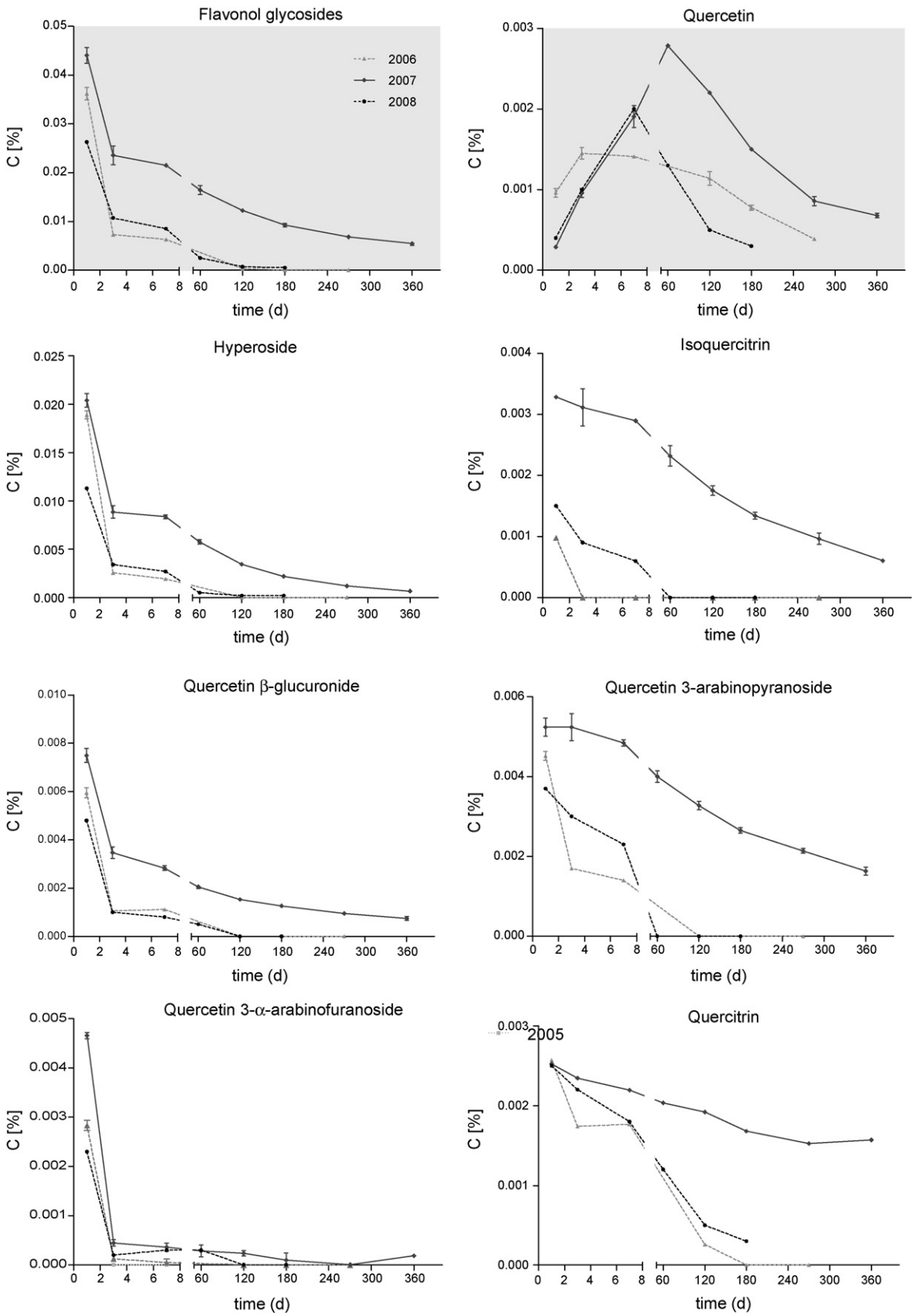

Fig. 3. Flavonol concentration (%, w/v) in three fermented aqueous extracts (years 2006–2008) over 12 months. Error bars show the standard deviation including two independent analyses.

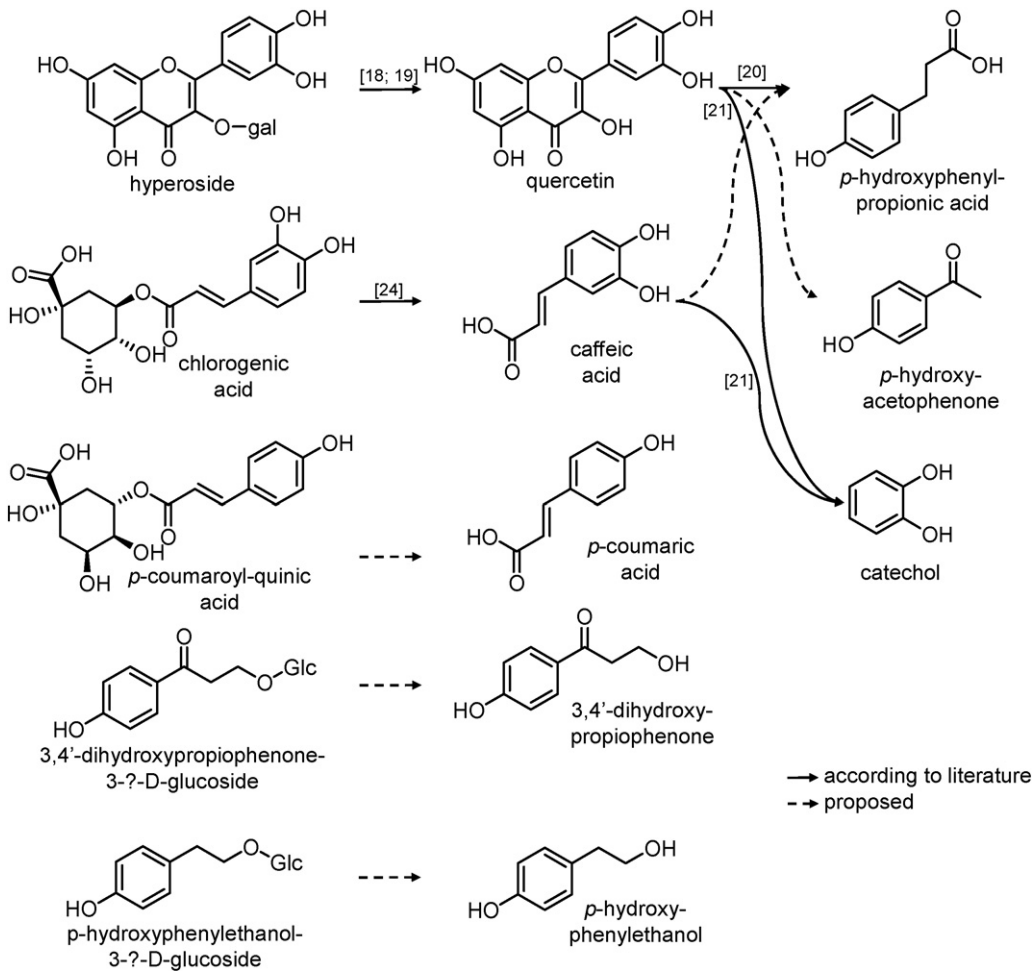

Fig. 4. Proposed pathways for phenolic compound conversion in fermented aqueous extracts from fresh birch leaves.

Comparison of the GC–MS data with the respective *m*- and *p*-hydroxylation samples confirmed a *para*-hydroxylation which is in contrast to literature data. Previously, mostly *meta*-hydroxylation of quercetin metabolites has been reported [9], and this was verified with GC–MS in case of *in-vitro* metabolism with colonic microflora [30]. Only Justesen and Arrigoni [31] described *p*-hydroxyphenylpropionic acid as degradation product of quercetin following fermentation with human faecal flora, also via GC–MS analyses. Additionally, traces of catechol could be detected by GC–MS and HPLC in the late stage of the fermentation (peak 7, Fig. 1; *m*/*z* 110, 81 and 64; λ_{max} = 277 nm). Catechol was already reported as a metabolite of quercetin and caffeic acid through fermentation by the human gut microflora [32].

Interestingly, no other quercetin metabolites, such as protocatechuic acid, a reported fermentation product from quercetin [30,31,33] or thermal processing [34] could be found in the present work.

Chlorogenic and *p*-coumaroylquinic acids were also decomposed (highest amount at *d1*; after *m6* only trace levels were measured) to caffeic and *p*-coumaric acids (peaks 9 and 10, Fig. 1; traces by *d1*, highest amount in the last samples). This may be due to hydrolysis in the aqueous solution. Under *in vivo* conditions, several different human colon bacteria are known to be able to hydrolyze chlorogenic acid [35].

Most interestingly, a further compound was detected as the main decomposition product. It was present in all samples especially after 6 months (peak 5, Fig. 1). This metabolite was isolated from sample 2008-*m6* on a Sephadex LH-20 column and identified by mass and UV spectra as well as ^{1}H and ^{13}C NMR analyses. In line with data from an earlier report [36], compound 5 was confirmed as 3,4′-dihydroxypropiophenone (DHPP). It appears plausible that DHPP is a deglycosylation product of 3,4′-dihydroxypropiophenone 3-β-glucoside, which itself has been reported as one of the main phenolics from birch leaves [22,23]. This glucoside, with *m*/*z* 327 and *m*/*z* 147 in the negative ionization mode [23] was present in high amounts in the earliest samples (*d1–d7*; peak 1, Fig. 1) and was afterwards degraded fast (detected as traces in sample *m2*). While the aglycone was isolated from *B. platyphylla* [37] earlier, no report exists on its presence in *B. pendula* extracts.

Further conversion products such as *p*-hydroxyacetophenone and *p*-hydroxyphenylethanol could be detected in traces in all samples from a late stage of the fermentation by GC–MS (peak X and 4, Fig. 1). *p*-Hydroxyacetophenone has yet not been described as a flavonol metabolite and could also be derived from the degradation and oxidation of other phenolics. *p*-Hydroxyphenylethanol was probably formed by deglycosylation of salidroside. The latter was earlier isolated from leaves [28] and winter-dormant twigs [38] of *B. pendula*. Fig. 4 summarizes all pathways of phenolic compound conversion in the course of aqueous birch leaf fermentation.

The reactions of deglycosylation can be assigned to birch leaves' endogenous β-glycosidases, released through pestling and non-denaturating extraction. In addition, accompanying microbiological screening on the fermented extracts showed the presence of lactobacilli in the samples (data not shown). A comparison between extracts made with different amounts of whey indicated a direct correlation between whey quantity (equivalent to *Lacto-*

bacilli quantity) and the speed of flavonols degradation (data not shown).

4. Conclusion

A simple and robust method for the qualitative and quantitative study of the flavonol glycosides in extracts from birch leaves was developed, which turned out to be suitable also to detect lower molecular weight phenolics formed during fermentation. High instability of flavonol glycosides was observed. The conversion of quercetin derivatives and phenolics was time dependently monitored. Quercetin glycosides were deglycosylated. The rapidity of conversion strongly depended on the glycosylation pattern: Quercetin 3-β-galactoside (hyperoside) was the most unstable, quercetin 3-α-rhamnoside (quercitrin) the most stable flavonol glycoside. The common deglycosylation product quercetin was subsequently further transformed. The resulting phenolics were identified, some of which have not yet been reported. Degradation was most likely due to microorganisms from the plant tissue, endogenous enzymes of birch leaves as well as to the whey. Therefore, it is not possible to differentiate which activity mainly contributes to the formation of the respective transformation product. Nevertheless, these findings broaden our knowledge on the stability and pathways of phenolic compound conversion in fermented aqueous plant extracts.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2010.02.001.

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