Pharmaceutical Biology[1], University of Regensburg; Steigerwald Arzneimittelwerk GmbH[2], Darmstadt, Germany

Quantitative analysis of flavanones and chalcones from willow bark

A. Freischmidt[1], M. Untergehrer[1], J. Ziegler[1], S. Knuth[1], S. Okpanyi[2], J. Müller[2], O. Kelber[2], D. Weiser[2], G. Jürgenliemk[1]

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Dr. Guido Jürgenliemk, Pharmaceutical Biology, University of Regensburg, 93040 Regensburg, Germany
Guido.juergenliemk@ur.de

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Willow bark extracts are used for the treatment of fever, pain and inflammation. Recent clinical and pharmacological research revealed that not only the salicylic alcohol derivatives, but also the polyphenols significantly contribute to these effects. Quantitative analysis of the European Pharmacopoeia still focuses on the determination of the salicylic alcohol derivatives. The objective of the present study was the development of an effective quantification method for the determination of as many flavanone and chalcone glycosides as possible in *Salix purpurea* and other *Salix* species as well as commercial preparations thereof. As *Salix* species contain a diverse spectrum of the glycosidated flavanones naringenin, eriodictyol, and the chalcone chalconaringenin, a subsequent acidic and enzymatic hydrolysis was developed to yield naringenin and eriodictyol as aglycones, which were quantified by HPLC. The 5-*O*-glucosides were cleaved with 11.5% TFA before subsequent hydrolysis of the 7-*O*-glucosides with an almond β-glucosidase at pH 6-7. The method was validated with regard to LOD, LOQ, intraday and interday precision, accuracy, stability, recovery, time of hydrolysis, robustness and applicability to extracts. All 5-*O*- and 7-*O*-glucosides of naringenin, eriodictyol and chalconaringenin were completely hydrolysed and converted to naringenin and eriodictyol. The LOD of the HPLC method was 0.77 μM of naringenin and 0.45 μM of eriodictyol. The LOQ was 2.34 μM of naringenin and 1.35 μM for eriodictyol. The method is robust with regard to sample weight, but susceptible concerning enzyme deterioration. The developed method is applicable to the determination of flavanone and chalcone glycosides in willow bark and corresponding preparations.

1. Introduction

Willow bark extracts (Salicis cortex, *Salix spec*., Salicaceae) are used since ancient times for the treatment of fever, pain and inflammation (Hedner and Everts 1998). Recent research clearly revealed that not only salicylic alcohol derivatives but also the polyphenols significantly contribute to the pharmacological and clinical effects. Standardised extracts rich in polyphenols indicate significant clinical effects (for review: Vlachojannis et al. 2009) and extracts as well as fractions containing polyphenols also showed inhibitory activity *in vitro* on several molecular targets like transcription factors, pro-inflammatory cytokines (Bonaterra et al. 2010), cyclooxygenases and radical production (Khayyal et al. 2005). Consequently, the overall effect of willow bark extracts can be most likely explained by a multi-component/multi-target principle (Nahrstedt et al. 2007, Wagner and Ulrich-Merzenich, 2009).

Quantitative analyses of willow bark extracts with HPLC and HPTLC were mainly published with regard to the content of salicylic alcohol derivatives (Meier et al. 1988; Poblocka-Olech et al. 2007; Wagner et al. 2008), but also with the aim to determine simultaneously some flavonoids and salicylic alcohol derivatives (Meier et al. 1985; Julkunen-Tiitto and Sorsa 2001, Kammerer et al. 2005). Nevertheless, the exact HPLC quantification of several compounds of different groups has in general the disadvantage of long running times, long validation procedure, sometimes the necessity of special sample preparations, and the availability of every reference compound for the validation is a prerequisite. The presence of a broad flavonoid and chalcone spectrum with glycosidated and coumaroylated compounds in the genus *Salix* (Freischmidt et al. 2012) makes it very difficult to quantify the main compounds of only these two groups without those disadvantages (Krauze-Baranowska et. al. 2013).

Based on the complex flavonoid pattern in the genus *Salix* and the fact that several *Salix* species can be used for the drug Salicis cortex only depending on the content of salicylic alcohol derivatives (Ph. Eur. 8.0), we aimed to develop an easy, rapid and powerful HPLC method for the quantification of willow bark's flavanones and chalcones. Inspired by the quantification method of *Ginkgo biloba* leaves' flavonoids (Ph. Eur. 8.0), we established a quantitative enzyme and acid based hydrolysis to determine all abundant naringenin glycosides and corresponding chalcones as naringenin. Thus, the challenge of different λ_{max} and ε values in chalcones and flavanones due to their deviating mesomeric systems is circumvented. The method is also applicable for other *Salix* species with a higher content of eriodictyol derivatives (determined as eriodictyol) like eriodictyol-7-*O*-β-glucoside, which were not present in *S. purpurea* bark in higher amounts (Freischmidt et al. 2012). Determination of the predictive value of these compounds for the pharmacological action of willow bark extracts could now be a next step (Wuthold et al. 2004). From the analytical point of view, additional large scale testing in routine analytics and in a representative set of production scale batches would be an interesting topic as well.

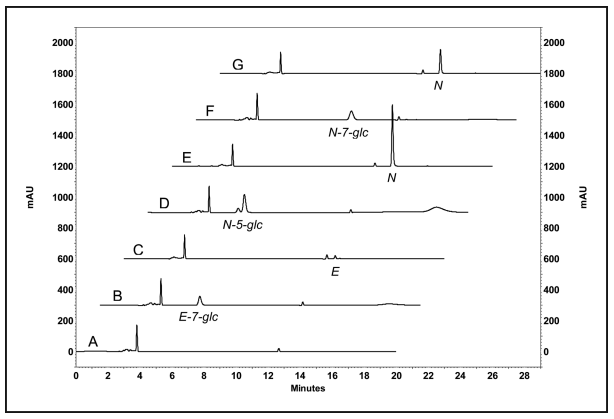

Fig. 1: Hydrolysis of naringenin-5-*O*-*β*-glucosides (N-5-glc) and -7-*O*-*β*-glucosides (N-7-glc) to naringenin (N) as well as eriodictyol-7-*O*-*β*-glucoside (E-7-glc) to eriodictyol (E), Method C; A. Solvent control after processing; B. Defined sample of E-7-glc (0.2 mg/mL) before hydrolysis; C. Defined sample of E-7-glc after hydrolysis; D. Defined sample of N-5-glc (0.6 mg/mL) before hydrolysis E. Defined sample of N-5-glc after hydrolysis; F. Defined sample of N-7-glc (0.3 mg/mL) before hydrolysis; G. Defined sample of N-7-glc after hydrolysis; (289 nm).

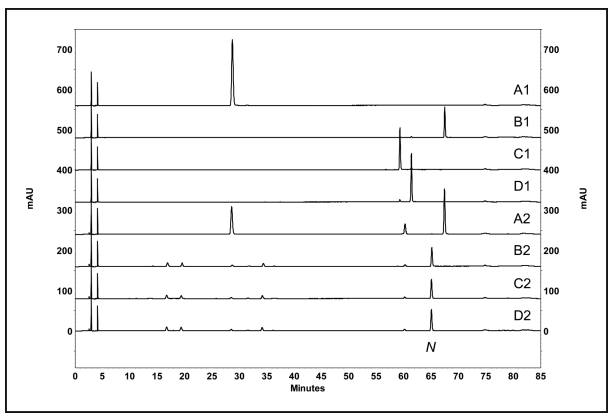

Fig. 2: Applicability of the hydrolysis to coumaroylated naringenin glucosides; coumaroylated naringenin glucosides (0.2 mg/mL) before (1) and after (2) hydrolysis (Method A); A. *trans*-p-coumaric acid (control);
B. 6”-*O*-*trans*-p-coumaroyl-isosalipurposide;
C. 6”-*O*-*trans*-p-coumaroyl-(2*R*)-naringenin-5-O-*β*-D-glucoside;
D. 6”-*O*-*trans*-p-coumaroyl-(2*S*)-naringenin-5-*O*-*β*-D-glucoside; N: arising naringenin; (289 nm).

2. Investigations, results and discussion

As the principle concept of the method is based on the hydrolysis of flavanones and chalcones with different glycosidation positions and their conversion to naringenin and eriodictyol, the first step was the development of suitable hydrolysis conditions to enable a quantitative cleavage to the corresponding aglycone. Naringenin-5-*O*-*β*-glucoside, naringenin-7-*O*-*β*-glucoside, eriodictyol-7-*O*-*β*-glucoside and isosalipurposide were selected as model compounds. Markham (1982) reported a different susceptibility of naringenin glucosides to acid and enzymatic hydrolysis. Whereas naringenin-5-*O*-*β*-glucoside is quantitatively hydrolyzed with diluted HCl within 30 min, the corresponding 7-*O*-*β*-glucoside undergoes degradation prior to quantitative hydrolysis. On the other hand, the flavanone-7-*O*-*β*-glucosides can be easily hydrolyzed by *β*-glucosidases (Markham et al. 1978). Therefore, a subsequent acidic and enzymatic hydrolysis was developed to quantitatively cleave all model flavanone glucosides which were controlled by HPLC analysis (Fig. 1). As expected, the chalcone isosalipurposide is also quantitatively converted to the corresponding naringenin after hydrolysis (data not shown). In a second step, the applicability of the method was proven on coumaroylated naringenin glucosides (Fig. 2), revealing an

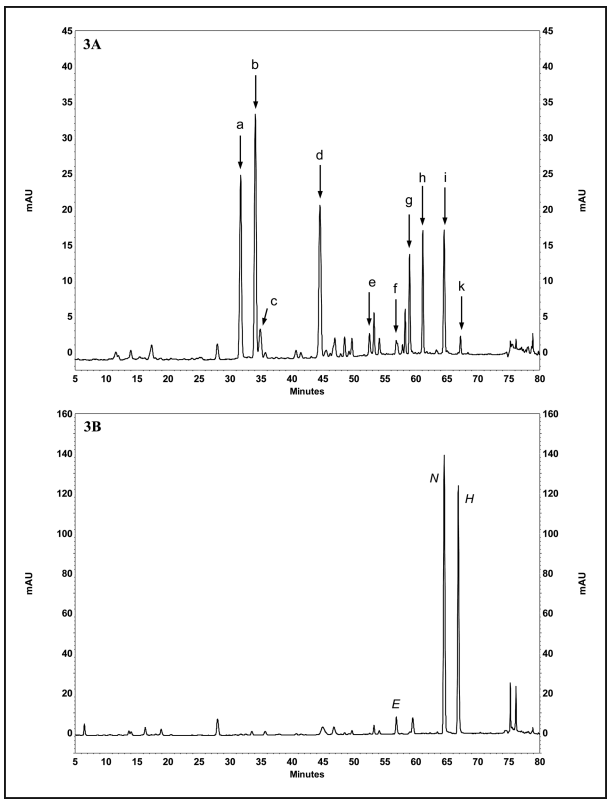

Fig. 3: **A.** HPLC section of an unhydrolyzed willow bark extract STW 33-I (5.00 mg/mL; detection wavelength 289 nm), (Method A): a and b: (2*R*) and (2*S*)-naringenin-5-*O*-*β*-glucoside; c: eriodictyol-7-*O*-*β*-glucoside; d: naringenin-7-*O*-*β*-glucoside; e: isosalipurposide; f: eriodictyol; g and h: 6”-*trans*-*p*-coumaroyl-(2*R*)- and (2*S*)-naringenin-5-*O*-*β*-glucoside; i: naringenin; k: 6”-*trans*-*p*-coumaroyl-isosalipurposide; **B.** HPLC section of the corresponding hydrolyzed willow bark extract (method A): naringenin (N) and eriodictyol (E); internal standard: hesperetin (H).

almost quantitative cleavage of the glucosidic ester also. An HPLC analysis for the separation of the entire flavanone glycoside spectrum in the commercially available extract STW 33-I was developed and revealed the presence of several flavanone glucosides (Fig. 3A). After application of the hydrolysis to the extract, the chalcone- as well as the naringenin- and eriodictyol-glucoside signals disappeared and the chromatogram showed the corresponding naringenin and eriodictyol peaks (Fig. 3B). As an internal standard, the flavanone hesperetin was selected. Then, the HPLC method was shortened and optimized regarding the separation of the aglycones naringenin, eriodictyol and the internal standard. Willow bark extract (STW 33-I) was quantitatively analysed and revealed a total naringenin content of $8.12 \pm 0.52\%$ and a total eriodictyol content of $0.54 \pm 0.01\%$ (Fig. 4). Concluding a total flavanon value of $8.65 \pm 0.53\%$ was shown in STW 33-I (n = 6).

Validation of the HPLC method was done with regard to intraday, interday and injection precision, reproducibility, stability, linearity, specificity**,** limit of detection (LOD), and limit of quantification (LOQ) (Table 1). Although the samples were processed so that the glycosides were cleaved in a two-step hydrolysation prior to HPLC analysis, the inter- and intraday precision revealed a relative standard deviation (RSD) of only 2% for the aglycones. This is in accordance with Zhao et al. (2014) who found similar values for flavonol-glycosides from *Salix bordensis* but without processing of the samples. Due to the low sensitivity of the UV detector, total peak purity of eriodictyol was less satisfying than the peak purity of naringenin in the relevant concentration range. Despite the less chromophore of the quantified flavanones, the achieved LOQ and LOD are comparable to values found for flavonols by Zhao et al. (2014) and are more sensitive than values

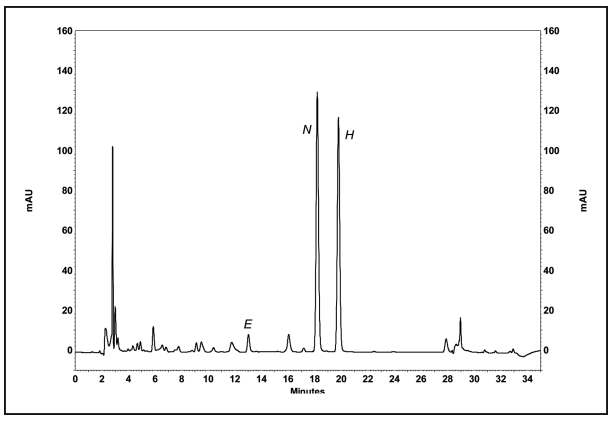

Fig. 4: HPLC of the completely hydrolyzed willow bark extract for quantification (method B, containing the internal standard hesperetin (5.17 mg/mL STW 33-I, 15.70 µg/mL hesperetin (H); 16.40 µg/mL naringenin (N); 1.11 µg/mL eriodictyol (E)).

Table 1: Validation parameters of the HPLC method (Method B): regression equation, correlation coefficient, limit of detection and quantification, total peak purity as well as inter- and intraday precision for naringenin (N) and eriodictyol (E)

Analyte	N	E
Regression equation N	y = 403989 x - 268	
Regression equation E	y = 393436 x - 17072	
Correlation coefficient r^2	1.0000	0.9997
Limit of detection (µM)	0.77	0.45
Limit of quantification (µM)	2.34	1.35
Total point peak purity (n = 10), (%)	95.01	67.69
Intraday precision (n = 6), RSD (%)	2.08	2.16
Interday precision (n = 6), RSD (%)	1.88	1.96

for flavonols determined by Dubber and Kanfer (2004). The recovery of reference substances in solvent was 89 to 99%. The recovery of standards by adding them to the extract sample was 93 to 102%. Thus, naringenin- and eriodictyol glucosides can be determined accurately as naringenin and eriodictyol, respectively (Table 2). Investigated robustness parameters were time of hydrolysis and differences in sample weight (data not shown). There were no significant differences at sample concentrations 2 - 8 mg/mL. At higher concentrations, less amounts of naringenin were found, probably caused by a lack of enzyme activity. This powerful and effective validated method is applicable to quantify flavanone and chalcone glucosides from willow bark and corresponding preparations.

With minor modifications, the simultaneous determination of flavanonols like dihydrokaempferol or taxifolin derivatives should be also possible. As several willow species have been reported to produce also significant amounts of flavones and flavonols (Meier et al. 1988; Nyman and Julkunen-Tiitto 2005), the application of the method to the reported apigenin, luteolin, myricetin and quercetin derivatives should be an interesting challenge. Additional large scale testing of this method in routine analytics and in a representative set of production scale batches would be a promising topic in the future.

3. Experimental

3.1. Chemicals and reagents

Naringenin (purity ≥ 99%, HPLC), naringenin-7-*O*-*β*-glucoside (purity ≥ 90%, HPLC), eriodictyol (purity ≥ 99%, HPLC) and eriodictyol-7-*O*-*β*-glucoside (purity ≥ 99%, HPLC) were purchased from Extrasynthese (Genay Cedex, France). (2*R*)/(2*S*)-naringenin-5-*O*-*β*-glucoside, isosalipurposide, 6′′-*O*-*trans*-*p*-coumaroylisosalipurposide, 6′′-*O*-*trans*-*p*-coumaroyl-(2*R*)-naringenin-5-*O*-*β*-D-glucoside and 6′′-*O*-*trans*-*p*-coumaroyl-(2*S*)-naringenin-5-*O*-*β*-D-glucoside were isolated from *S. purpurea* (Freischmidt et al., 2012, purity ≥ 99%, HPLC). *p*-Coumaric acid (purity ≥ 98%, GC) and hesperetin (purity ≥ 95%, HPLC) was obtained from Roth (Karlsruhe, Germany). Willow bark dry extract (STW33-I) was a kind gift of Steigerwald Arzneimittelwerk GmbH (Darmstadt, Germany). Acetonitrile and methanol (LiChroSolv®) in HPLC-grade were purchased from Merck (Darmstadt, Germany). Trifluoracetic acid (99%, ReagentPlus®) was obtained from Sigma-Aldrich (Taufkirchen, Germany). Formic acid (98 - 99%, p.a.), NaH_2PO_4 x H_2O, Na_2HPO_4 x 2 H_2O, NaOH, (p.a.) were obtained from Merck (Darmstadt, Germany). *β*-Glucosidase (≥ 1000 U/mg *ex amygdale*) was obtained from Roth (Karlsruhe, Germany). Wheaton V-Vials (Manufacturer Part No: W986299NG, 5 mL) were obtained from VWR International GmbH (Arlington Heights, USA).

3.2. Sample preparation

3.2.1. Single compounds

Naringenin-5-*O*-*β*-glucoside (6 mg), 3 mg of naringenin-7-*O*-*β*-glucoside, 3 mg of isosalipurposide, 2 mg of eriodictyol-7-*O*-*β*-glucoside, 2 mg of 6”-*O*-*trans*-*p*-coumaroylisosalipurposide, 2 mg of 6”-*O*-*trans*-*p*-coumaroyl-(2*R*)-naringenin-5-*O*-*β*-D-glucoside, and 2 mg of 6”-*O*-*trans*-*p*-coumaroyl-(2*S*)-naringenin-5-*O*-*β*-D-glucoside were dissolved in 10.0 mL of degassed methanol/water 7/3 (V/V).

3.2.2. Samples

STW 33-I extract (50 mg) was dissolved in 10.0 mL methanol/water 7/3 (V/V) and sonicated for 15 min. After filtration, 1.0 mL of the sample were mixed with 1.0 mL TFA solution (11.5% in degased methanol/water 3/7 (V/V)) in a Wheaton V-Vial and heated for 30 min at 120 °C in a drying oven. After cooling, 0.6 mL of NaOH solution (2 M NaOH in H_2O) was added and the sample was transferred to a 5.0 mL volumetric flask. After the addition of 1.0 mL phosphate buffer (1 M NaH_2PO_4 x H_2O / Na_2HPO_4 x 2 H_2O in H_2O, pH 6.4), the flask was filled with water. A pH value between 6 and 7 is mandatory and should be controlled. 1.0 mL were taken and mixed with 0.6 mL solution of *β*-glucosidase (2 mg/mL *β*-glucosidase in water) and 1.0 mL H_2O. The mixture was kept for 30 min at 37 °C in a water bath. After cooling down to room temperature, 1.0 mL of the internal standard (7.5 mg hesperitin in 10.0 mL MeOH and diluted 1/10 with MeOH/H_2O 1/1 (V/V)) was added and filled to 5.0 mL with MeOH. After filtration through a 0.20 µm membrane filter (WICOM, Heppenheim, Germany), 30 µL were injected by HPLC. Internal standard solution was injected three times. The ratio of the area of analyte and internal standard was multiplied with the mean reference area of internal standard and calculated against a naringenin calibration curve, or an eriodictyol calibration curve respectively.

3.3. Instrumentation

Analytical HPLC was performed using a VWR Hitachi Elite LaChrom, equipped with an L-2455 DAD, L-2200 autosampler, L-2130 pump, L-2350 column oven and EZ LaChrom software. As column a 250-4 Purospher® Star RP-18e (5 µm) with pre-column Purospher®star RP-8e (5 µm) was used (Merck).

3.4. HPLC chromatography

Mobile phase for hydrolysis control of the extract and the esterified reference glycosides (Method A): Solvent A, 0.1% formic acid in millipore water (pH 4), solvent B, acetonitrile (95%), flow: 0.75 mL/min, gradient: 0-35 min 10% B → 20% B, 35-45 min 20% B → 25% B, 45-70 min 25% B → 45% B followed by washing and recalibration.

Table 2: Recovery of standards and recovery by addition method of standards to the sample (method B, mean ± SD, n = 6)

	N-5-glc 20%	N-5-glc 40%	N-7-glc 40%	N-7-glc 80%	E-7-glc 20%	E-7-glc 40%
References	97.09 ± 2.72	99.15 ± 3.47	93.95 ± 6.00	94.76 ± 8.87	91.22 ± 9.08	89.41 ± 11.53
Addition method	92.59 ± 6.89	97.06 ± 6.24	99.55 ± 5.64	93.49 ± 7.84	101.94 ± 6.04	99.63 ± 7.39

Mobile phase for quantification after hydrolysis (Method B): Solvent A, 0.1% formic acid in millipore water (pH 4), solvent B, acetonitrile (95%), flow: 1.0 mL/min, gradient: 0-25 min 25% B → 45% B followed by washing and recalibration.
Mobile phase for stepwise hydrolysis of unesterified reference-glycosides (Method C): Solvent A, 0.1% formic acid in millipore water (pH 4), solvent B, acetonitrile (95%), flow: 0.75 mL/min, gradient: 0-7 min 25% B, 7-9 min 25% B → 70% B, 9-14 min 70% B, 14-16 min 70% B → 25% B followed by washing and recalibration.
The injection volume was always 30 µL, column temperature 30 °C, the autosampler temperature was set to 20 °C, and detection wavelength was 289 nm.

3.5. Method validation

3.5.1. Intraday, interday and injection precision

Samples were hydrolysed six times a day (intraday precision) respectively on six different days. The relative standard deviations (RSD) for intraday and interday precision are shown in Table 1. For injection precision, one sample was hydrolysed and injected six times. The RSD was 0.28%.

3.5.2. Reproducibility

Three different persons investigated individually the flavanon content of STW 33-I (n = 6). RSD was 4.86%.

3.5.3. Stability

Six samples were processed and quantified by HPLC. The same samples were quantified again after 8 h. Mean naringenin concentrations were 17.54 ± 0.36 µg/mL at t = 0 h and 17.31 ± 0.34 µg/mL at t = 8 h. Mean eriodictyol concentrations were 0.93 ± 0.05 µg/mL at t = 0 h and 0.93 ± 0.05 µg/mL at t = 8 h. No significant differences (t-test, $p < 0.05$) could be observed between t = 0 and 8 h.

3.5.4. Linearity

Stock solutions of the standards naringenin (70 µg/mL) and eriodictyol (50 µg/mL) both in methanol were diluted to obtain 9 calibration concentrations. Naringenin was calibrated within the range of 0.5 - 70 µg/mL and eriodictyol within the range of 0.2 – 10 µg/mL (n = 6). The internal standard was added to each calibration concentration and also injected 3 times to obtain a mean reference area. The calibration curves were calculated by plotting the analyte concentrations (in µg/mL) towards the ratio area of analyte to the area of internal standard multiplied with the mean reference area of internal standard (Table 1).

3.5.5. Specificity

The total peak purity of naringenin and eriodictyol after hydrolysis was calculated by means of the HPLC software EZ EliteLaChrom (Table 1).

3.5.6. Limit of detection (LOD)/Limit of quantification (LOQ)

Limit of detection (LOD) and quantification (LOQ) were calculated based on the standard deviation of the response (σ) and the slope of the calibration curve (S) (ICH 2005). LOD was determined as 3.3 x σ/S and LOQ as 10 x σ/S (Table 1).

3.5.7. Accuracy and recovery

Accuracy is reported as the percent recovery of the known amount of analyte added to a sample (Table 2). Recovery was investigated by hydrolyzing 80% and 40% of the estimated content of naringenin-7-*O*-*β*-glucoside in the extract, as well as 40% and 20% of naringenin-7-*O*-*β*-glucoside and eriodictyol-7-*O*-*β*-glucoside. The ratio of the measured amount and the calculated amount of the reference-glucosides was expressed as recovery of reference substances in Table 2. In order to examine matrix effects these defined amounts of references were added to the extract sample and hydrolyzed. A sample without adding references served as base value. The ratio of the measured amount and the calculated amount (base value plus calculated reference value) was expressed as recovery by addition method in Table 2.

3.5.8. Robustness

In order to examine robustness of the method, the influence of the sample weight in regard to the enzyme quantity was tested. Therefore, different concentrations of STW 33-I (2 - 32 mg/mL) were hydrolysed. The highest naringenin concentration after hydrolysis was set as 100%. Furthermore the influence of TFA hydrolysis time was investigated. Samples were hydrolysed with TFA for 10, 20, 30 and 60 min at 120 °C and processed as described above.

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