Fig. 1). Other possibility would be the application of the recently heterologous expressed b–rutinosidase) (Šimčíková et al., 2014). (Fig. 1). The measurement of rutin deglycosylation activity relies on the quercetin quantification by high performance liquid chromatography (HPLC) (Cho, Howard, Prior, & Clark, 2004; Vojtíšková, Kmentová, Kubán, & Kráčmar, 2012). In this work, we developed a spectrophotometric method for the screening and quantification of rutin–deglycosylation activity based on the spectral properties of the substrate and products of the reaction. 2. Materials and methods 2.1. Chemicals and enzymes Quercetin (2–(3,4–dihydroxyphenyl)–3,5,7–trihydroxy–4H–chro– men–4–one) and rutin (quercetin 3–O–(6–O–a–L–rhamnopyranosyl– b–D–glucopyranoside) were purchased from Sigma Chemical (St. Louis). HPLC grade methanol LiChrosolv was obtained from Merck (Darmstadt). Naringinase from Penicillium decumbens was obtained from Sigma Chemical (St. Louis) and store at 20 °C. Rutin–degrading enzymes were obtained from commercial Tartary buckwheat flours from local market. 2.2. UV–visible spectra of flavonoids Rutin and quercetin (180 mM) were solubilized in dimethylfor– mamide and diluted in water as stock solutions for spectrophotometric assays. To adjust pH, 50 mM sodium citrate pH 5.0, 50 mM sodium phosphate pH 6.0–8.0, 50 mM Tris–glycine pH 8.0, 50 mM Tris–HCl pH 9.0, and 50 mM sodium carbonate pH 10.0 were used. The spectra (250–550 nm) of the analytes were obtained using a USB4000 spectrophotometer (Ocean Optics). 2.3. Calibration curve For the calibration curve, 840 lM quercetin and 840 lM iso– quercetin were solubilized in ethyl acetate and the standard solutions were prepared in 50 mM sodium carbonate solution pH 10.0. Triplicates of quercetin and isoquercetin standards were used in the range 0–40 lM. The intensities of quercetin and isoquercetin peak maxima at 320 and 400 nm were plotted against the concentration. Data were fitted using the least–squares method according to Beer–Lambert equation: A ¼ ebC where A, absorbance; e, molar extinction coefficient (L mol−1 cm−1); b, path length (cm); and C, molar concentration (M). 2.4. Enzyme assays Two grams of three commercial Tartary buckwheat flours obtained from the local market were extracted with 30 ml of 0.2 M sodium acetate buffer pH 4.0 at 4 °C for 3 h. The buckwheat slurry was centrifuged (15,600g, 5 min, 4 °C) and the supernatant was collected and stored at 4 °C. The reaction contained 100 ll enzyme solution extracted from Tartary buckwheat flours, 200 ll substrate (0.11% w/v rutin in 50 mM sodium citrate buffer pH 5.0), and was performed for 1 h at 40 °C. For the commercial enzyme preparation, the reaction mixtures containing 12 ll of naringinase (20 mg/ml in 5 mM sodium citrate buffer pH 5.0), 300 ll substrate (0.11% w/v rutin in 50 mM sodium citrate buffer pH 5.0), and was performed for 1 h at 40 °C. Control of the reactions were conducted in the absence of enzyme. At the end of the reaction, one volume of ethyl acetate was added and mixed. Then the tubes were centrifuged (15,600g, 2 min) to separate the aqueous and the organic phase. One hundred ll of the upper organic phase were added to 1.9 ml sodium carbonate 50 mM pH 10.0 and incubated at room temperature for 5 min. The absorbance (320 and 400 nm) was measured and the concentration of quercetin was calculated using the Lambert Beer equation. One unit of RDE activity was defined as the amount of enzyme required to release 1 lmol quercetin per min. 2.5. HPLC quantification of flavonoids Hydrolysis of rutin was quantified by measuring the released quercetin by HPLC using a KONIK–500–A series HPLC system attached to a KONIK UVIS 200 detector. The column was a reversed–phase LiChroCART 125–4 MERCK (12.5 cm length, 4 mm internal diameter) LiChrospher 5 lm, RP 18 (pore.
size 100 Å). As mobile phase, an isocratic flow of methanol 20 mM disodium phosphate (40:60 v/v) (pH adjusted to 3.0 with phosphoric acid) at a flow rate of 1.0 ml/min at 25 °C was applied. To prepare stocks solutions (180 mM) for HPLC, the flavonoids were solubilized in dimethylformamide. The standard solutions were prepared by diluting the stock solutions in mobile phase. Triplicates of quercetin standard were used in the range 0–40 µM. Quercetin calibration curve was calculated from chromatograms of authentic standard solutions (triplicates) detected at 285 nm. The retention times for rutin and quercetin were 1.6 and 2.4 min, respectively. The samples of the enzymatic reaction (500 µl) were deproteinized by adding 1.5 ml methanol previous to HPLC assays (Contin, Mohamed, Albani, Riva, & Baruzzi, 2008).

3.1. UV–visible spectra of rutin and its aglycone, quercetin To characterize the substrate of the enzymatic reaction rutin and the product quercetin, the UV–visible spectra of these compounds at different pH values (5–10) and 40 °C were done. Rutin spectrum at pH 5.0 shows a maximum absorbance peak at 351 nm whereas at alkaline pH (8.0 and 10.0) the maximum absorbance peaks shift towards longer wavelengths, 380 and 403 nm, respectively, with a shoulder at 327 nm (Fig. 2A). After 30 min incubation, no significant changes occurred in the spectra of rutin at different pH values. On the other hand, quercetin shows maximum absorbance at 367 nm under acidic conditions (pH 5.0). At alkaline pH, the maximum absorbance for quercetin was found at 387 nm at pH 8.0 and 414 nm at pH 10.0. After 30 min under slightly acidic conditions (pH 5.0 and 6.0), the wavelength of the maximum absorbance peak did not change, while at pH 7.0 the maximum absorbance peak of quercetin barely changes. At pH 8.0, the intensity of the peak at 387 nm corresponding to quercetin decreased along with the development of a new absorption peak at 320 nm. This change was previously reported to be due to quercetin autoxidation, which is favored by alkaline pH, presence of metal ions and high temperatures (El Hajji, Nkhili, Tomao, & Dangles, 2006; Fahlman & Krol, 2009a, 2009b; Jungbluth, Ruhling, & Ternes, 2000; Krishnamachari, Levine, & Pare, 2002; Sokolová et al., 2012). In order to avoid autoxidation, the buffer composed of Tris and the antioxidant glycine at pH 8.0 was used. In this case, no changes in the absorption spectrum were observed after 30 min. At pH values around 10, the autooxidation rate increases, as evidenced by the almost complete disappearance of the peak at 414 nm after 30 min (Fig. 2B).

The absorbance change at 414 and 320 nm was recorded as a function of time during the incubation of quercetin (Fig. 3). A B Fig. 2. UV–visible spectra of 18 µM rutin (A) and 18 µM quercetin (B) at pH 5.0 (50 mM sodium citrate buffer) after 0 min and 30 min, pH 8.0 (50 mM sodium phosphate buffer) after — 0 min and — 30 min and pH 10.0 (50 mM sodium carbonate solution) after 0 min and 30 min. Fig. 3. Quercetin autoxidation (50 mM sodium carbonate solution pH 10.0) as evidenced by changes in the absorbance intensity at 414 nm and 320 nm. 3.2. Calibration curve Because of the correlation between the quercetin concentration and the absorbance of its autoxidation products at 320 nm, a calibration curve was performed. Quercetin standards were incubated (pH 10.0, 25 °C at room temperature) for 5 min according to the results shown in Fig. 3. The 320 nm absorbance was plotted against the original quercetin concentration. The molar extinction coefficient (ε320,Q) was calculated to be 26.2 (±0.3) 103 M−1 cm−1 (Fig. S1). Using regression analysis, strong linear relationship between the absorbance and concentration was obtained with a correlation coefficient value (R2) above 0.99. The
sensitivity of the method was assessed by the limit of detection (LOD) and limit of quantification (LOQ) using the standard deviation of responses (r) and the slope of calibration curve (s) using the following equation (Arayne, Sultana, & Tabassum, 2013): LOD ¼ 3:3s/r LOQ ¼ 10s/r, the lowest concentration that could be detected was estimated at 0.91 IM quercetin, while LOQ, the lowest concentration that could be quantified was 2.64 IM quercetin. 3.3. Design of a protocol to quantify rutin deglycosylation activity We designed a protocol to quantify quercetin, based on the spectral characteristics of rutin, quercetin and its autooxidation products (Fig. 4), as follows: The enzymatic reaction can be carried out in the range of pH between 5 and 7 using a chosen buffer, however, if the reaction needs to be performed at higher pH values, e.g. pH 8.0, the antioxidant buffer Tris-glycine should be used. Since quercetin autooxidation products could affect the stability of the enzyme, alkaline pH values should be avoided. At alkaline pH, rutin spectrum shows a maximum absorbance peak close to 400 nm with a shoulder at 327 nm, which overlaps with the maximum absorbance of quercetin. To avoid the spectra overlapping of both compounds close to 320 nm, a liquid-liquid extraction with ethyl acetate is necessary after the enzymatic reaction. Quercetin is extracted in the organic phase, while the unconverted rutin remains in the aqueous phase. One volume ethyl acetate was demonstrated to be enough to produce a quantitative extraction up to 5 IM quercetin, and a 92% and 89% extraction with 10 IM and 15 IM quercetin, respectively. Finally, 100 ll of the upper phase, containing the aglycone, is added to 1.9 ml 50 mM sodium carbonate (pH 10.0) and the absorbance at 320 nm – corresponding to the oxidation products of quercetin – is measured. G. Weiz et al. / Food Chemistry 229 (2017) 44–49 47 Enzyme/s Ethyl acetate R R Q S Q Sodium RS carbonate pH 10 0.3 0.2 0.1 0.0 250 300 350 400 450 500 Wavelength (nm) Fig. 4. Spectrophotometric method for rutin-deglycosylation activity quantification. R: Rutin, Q: Quercetin, S: Sugar. 3.4. Isoquercetin as the intermediate product of rutin deglycosylation When the biological rutin deglycosylation follows the sequential mode, i.e. with two monoglycosidases, and the limiting step corresponds to the glucosidase, an accumulation of the intermediate product isoquercetin can occur (Fig. 1). By performing the ethyl acetate extraction, a partition of isoquercetin between the two phases is observed, overlapping quercetin spectrum. At pH 10.0, isoquercetin spectrum shows a maximum at 400 nm with a shoulder at 320 nm. The spectrum remains unmodified over the time, in contrast to quercetin. To overcome the interference of isoquercetin, the spectrum of a solution formed by 2.5 IM quercetin (Q) and 2.5 IM isoquercetin (I) in 50 mM sodium carbonate pH 10.0 was carried out. A comparison was performed with the spectra of 2.5 IM quercetin and 2.5 IM isoquercetin, which were mathematically added. The additivity of the spectra was tested according to the following equation: The molar extinction coefficients were 14.4 (± 0.18) 103 M—1 cm—1 (ε320,I), 5.8 (± 0.14) 103 M—1 cm—1 (ε400,I), and 5.2 (± 0.13) 103 M—1 cm—1 (ε400,Q). For a reaction rendering 5 IM quercetin, which has been co-extracted with a 5% isoquercetin, the ratio between the absorbance at 320 and 400 nm would be 4.1. Hence, for ratios higher than 4.1, an error