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Screening and quantification of the enzymatic deglycosylation of the plant flavonoid rutin by UV–visible spectrometry

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ABSTRACT

The enzymatic deglycosylation of the plant flavonoid rutin (quercetin-3-*O*-(6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside) is usually assessed by means of high performance liquid chromatography (HPLC). We have developed a spectrophotometric method for the quantification of the released quercetin. After the enzymatic reaction, quercetin is extracted with ethyl acetate, and subsequently oxidized under basic conditions. The absorbance of quercetin autooxidation products at 320 nm was correlated with the quercetin concentration by linear regression (molar extinction coefficient 23.2 (±0.3) × 10^3 M^{-1} cm^{-1}). With this method, rutin-deglycosylation activity in buckwheat flour and a commercial naringinase was measured, and showed no significant differences with the results obtained by HPLC. The convenience of this method resides on the enzymatic activity quantification using the natural substrate by UV–visible spectrometry. Moreover, the simplicity and speed of analysis allows its application for a large number of samples.

1. Introduction

Rutin consists in the flavonol quercetin 3-*O*-linked to the disaccharide rutinose. Rutin shows higher solubility in water than quercetin due to the hydrophilicity of the sugar moiety but a weaker antioxidant activity (Aherne & O'Brien, 2002; Dugas et al., 2000; Heim, Tagliaferro, & Bobilya, 2002). Rutin conversion to quercetin is based on the removal process of the glycosidic fraction, either chemically or enzymatically. Quercetin has several bioactive properties such as antioxidant, anti-inflammatory and anticarcinogenic activities, becoming an interesting compound to be incorporated into pharmaceutical, cosmetic or food products (Fahlman & Krol 2009a, 2009b; Gonçalves et al., 2015; Melo Branco de Araújo et al., 2013). Because its specificity and mild operational conditions the enzymatic approach is usually preferred (Giffhorn, Koper, Huwig, & Freimund, 2000; Van Rantwijk, Woudenberg-van Oosterom, & Sheldon, 1999).

The deglycosylation of rutin is the first step in the microbial degradative pathway (Surholt & Hosel, 1978). The aglycone released by this reaction may further be degraded by the so-called 'flavonol oxidases' (Barz & Koster, 1981). In the case of diglycosides, such as rutin, the disaccharidic moiety is removed either via two monoglycosidases acting in two sequential hydrolysis reactions or, more unusual, in one reaction. In the sequential mode, the α-L-rhamnosidase catalyzes the hydrolysis of terminal α-L-rhamnose residues and, subsequently, the β-D-glucosidase catalyzes the hydrolysis of terminal β-D-glucose residues (Sarry & Gunata, 2004; Yadav, Yadav, & Yadav, 2010). The one-reaction removal is performed by diglycosidases that have been discovered from eukaryotic organisms, specifically filamentous fungi and plants, and recently in the bacterium *Actinoplanes missouriensis* (Nam, Hong, Shin, & Oh, 2012; Narikawa, Shinoyama, & Fujii, 2000; Neher et al., 2016).

Tartary buckwheat (*Fagopyrum tataricum*) seeds are a major source of the flavonoid rutin (about 0.8–1.7% dry weight). It has been reported that Tartary buckwheat contains about 100-fold more rutin than common buckwheat (Fabjan et al., 2003). It is interesting to note that rutin is not found in any cereals and pseudocereals except buckwheat (Kreft, Fabjan, & Yasumoto, 2006). Tartary buckwheat is also a source of rutin-degrading enzymes (RDE). One of its RDE was isolated and characterized as specific rutin-hydrolyzing enzyme, providing a new enzymatic preparation method for quercetin (Cui & Wang, 2011). The use of commercially available preparations (e.g., naringinase, hesperidinase) containing α-L-rhamnosidase and β-D-glucosidase activities from fungal sources such as *Aspergillus* and *Penicillium* spp. is also effective but usually leads to a mixture of isoquercetin and quercetin

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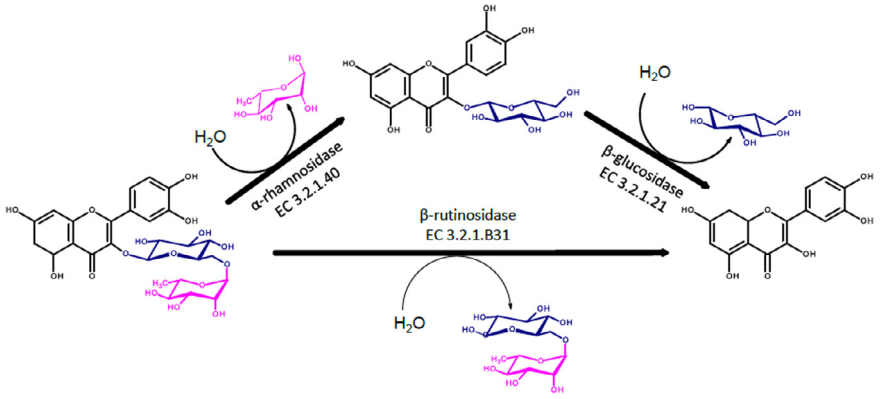

Fig. 1. Different pathways for enzymatic deglycosylation of rutin.

(Fig. 1). Other possibility would be the application of the recently heterologous expressed β-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, Kubáň, & 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-chromen-4-one) and rutin (quercetin 3-*O*-(6-*O*-α-L-rhamnopyranosyl-β-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 dimethylformamide 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 μM quercetin and 840 μM isoquercetin 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 μM. 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:

$$A = \varepsilon bC$$

where A, absorbance; ε, 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 (15,600*g*, 5 min, 4 °C) and the supernatant was collected and stored at 4 °C. The reaction contained 100 μl enzyme solution extracted from Tartary buckwheat flours, 200 μl 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 μl of naringinase (20 mg/ml in 5 mM sodium citrate buffer pH 5.0), 300 μl 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 (15,600*g*, 2 min) to separate the aqueous and the organic phase. One hundred μl 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 μmol 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 μm, 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. Results and discussion

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 autooxidation, 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).

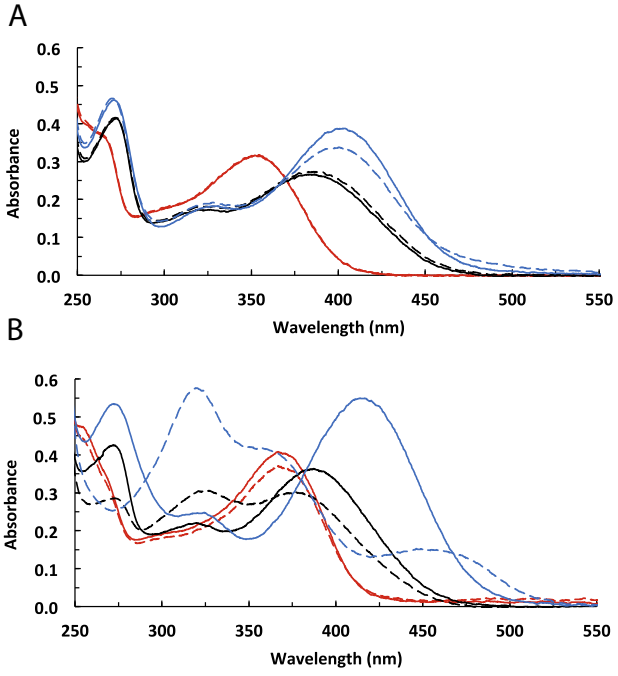

Fig. 2. UV–visible spectra of 18 μM rutin (A) and 18 μM quercetin (B) at pH 5.0 (50 mM sodium citrate buffer) after — 0 min and - - 30 min, pH 8.0 (50 mM sodium phosphate buffer) after — 0 min and - - 30 min and pH 10.0 (50 mM sodium carbonate solution) after — 0 min and - - 30 min.

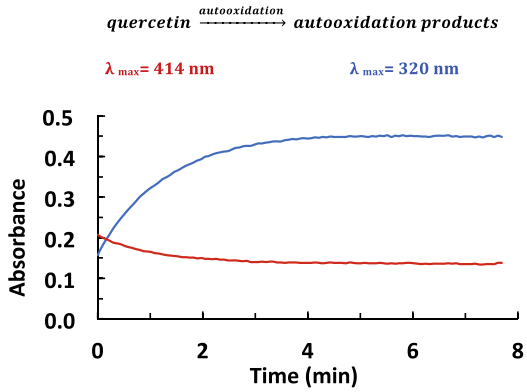

Fig. 3. Quercetin autooxidation (50 mM sodium carbonate solution pH 10.0) as evidenced by changes in the absorbance intensity at — 414 nm and — 320 nm.

3.2. Calibration curve

Because of the correlation between the quercetin concentration and the absorbance of its autooxidation 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 ($\varepsilon_{320,Q}$) was calculated to be 26.2 (±0.3) × 10^3 M^{-1} cm^{-1} (Fig. S1). Using regression analysis, strong linear relationship between the absorbance and concentration was obtained with a correlation coefficient value (R^2) 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 (σ) and the slope of calibration curve (s) using the following equation (Arayne, Sultana, & Tabassum, 2013):

$$LOD = 3.3\sigma/s \qquad LOQ = 10\sigma/s$$

LOD, the lowest concentration that could be detected was estimated at 0.91 μM quercetin, while LOQ, the lowest concentration that could be quantified was 2.64 μM 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 μM quercetin, and a 92% and 89% extraction with 10 μM and 15 μM quercetin, respectively. Finally, 100 μl 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.

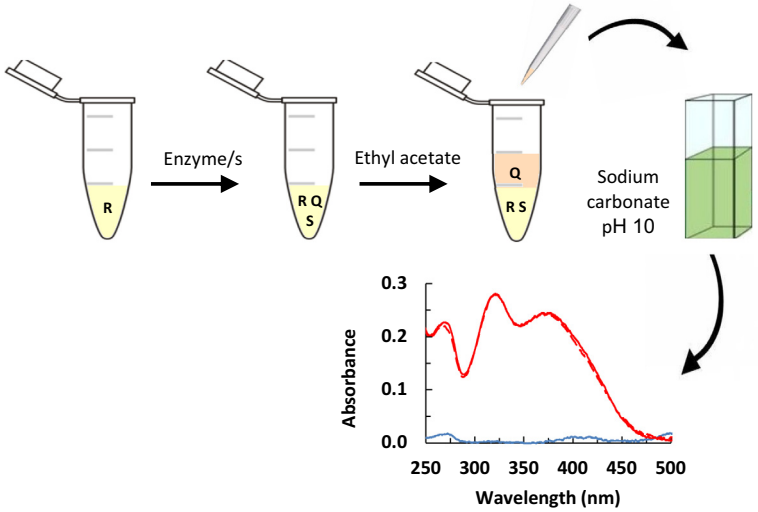

Fig. 4. Spectrophotometric method for rutin-deglycosylation activity quantification. R: Rutin, Q: Quercetin, S: Sugar.

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 μM quercetin (Q) and 2.5 μM isoquercetin (I) in 50 mM sodium carbonate pH 10.0 was carried out. A comparison was performed with the spectra of 2.5 μM quercetin and 2.5 μM isoquercetin, which were mathematically added. The additivity of the spectra was tested according to the following equation:

$$A_{Q+I} = A_Q + A_I$$

for each wavelength. The spectra confirm that the absorbance of isoquercetin and quercetin at 320 and 400 nm are additive (Fig. 5). Therefore, the concentration of quercetin can be estimated from the following equation system that considers the molar extinction coefficients of quercetin and isoquercetin at 320 and 400 nm:

$$A_{320} = \varepsilon_{320,Q} b C_Q + \varepsilon_{320,I} b C_I$$

$$A_{400} = \varepsilon_{400,Q} b C_Q + \varepsilon_{400,I} b C_I$$

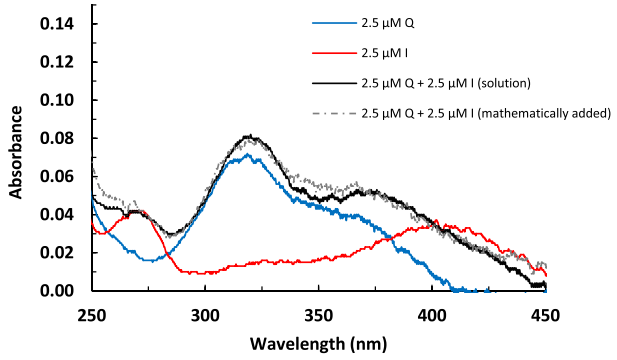

Fig. 5. Absorbance additivity of isoquercetin (I) and quercetin (Q) solutions.

The molar extinction coefficients were 14.4 (± 0.18) × 10^3 M^{-1} cm^{-1} ($\varepsilon_{320,I}$), 5.8 (± 0.14) × 10^3 M^{-1} cm^{-1} ($\varepsilon_{400,I}$), and 5.2 (± 0.13) × 10^3 M^{-1} cm^{-1} ($\varepsilon_{400,Q}$). For a reaction rendering 5 μM 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 <5% in the activity quantification considering the quercetin calibration curve at 320 nm is expected.

3.5. Assessment of bulk enzyme (naringinase) and commercial buckwheat flours

The developed method was applied to measure the enzymatic activity of a commercial preparation of naringinase, which possess α-L-rhamnosidase and β-D-glucosidase activities (Fig. 6A). Rutin-degrading activity present in buckwheat flour was also measured in four commercial samples. The assessment with the HPLC quantification of the released quercetin showed a correlation of 0.98 (Fig. 6B). The data of buckwheat flour I, II and III were analyzed using a *t*-test for comparison of the means of paired samples with a 95% confidence (Massart et al., 1997). There were not significant differences between the quantification by the spectrophotometric method and HPLC method. A recent paper reported a spectrophotometric method to detect the content of quercetin in samples of Tartary buckwheat seeds at isoabsorptive wavelengths of rutin (Chen & Gu, 2011). Although it is useful for the high-throughput screening of samples, its accuracy is not enough for the activity quantification. The method developed in our work can be likewise used for the high-throughput screening of samples. Moreover, the enzyme activity quantification does not differ from the quantification by HPLC. The developed method considers a possible accumulation of the intermediate isoquercetin and its interference can be easily corrected.

The substrate specificity of glycosidases regarding the sugar moiety can be easily assessed by means of the commonly employed *p*-nitrophenyl-derivatives. However, several flavonoid-hydrolyzing glycosidases show aglycone recognition and/or regioselectivity concerning the linkage between the sugar moiety and the aglycone (Mazzaferro et al., 2010). In this work, we have developed a spectrophotometric method for the study of rutin-hydrolyzing enzymes using the natural substrate and rutin-deglycosylation activity was successfully measure in commercial available buckwheat flour as well as the mixture of microbial glycoside

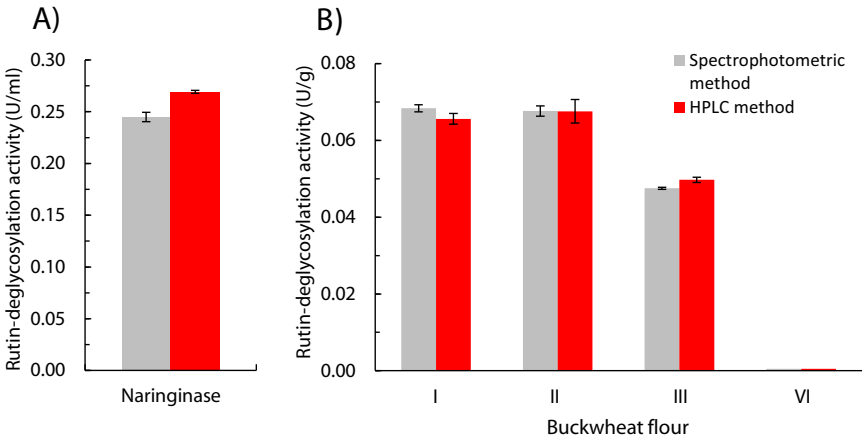

Fig. 6. Rutin deglycosylation activity of naringinase (A) and buckwheat flour samples (B) measured by different methods. The error bars correspond to the standard deviation of two replicate samples.

hydrolases (naringinase). Because of its simplicity and speed of analysis in comparison to the traditionally used HPLC method, it is applicable in testing a large number of samples and can be adapted to a microplate reader.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.02.029.

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