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Article

Optimization of Acid Hydrolysis of Myricetin-3-*O*-rhamnoside Using Response Surface Methodology

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This study aimed to optimize the acid hydrolysis of glycosylated flavonols, to apply the optimum conditions for hydrolyzing *Inga edulis* extract (IEE), rich in glycosylated flavonols, and evaluating its impact on the antioxidant capacity. To evaluate the influence of three independent variables on the aglycone obtained after the reaction, the response surface methodology was applied using myricetin-3-*O*-rhamnoside (M3R) as a pure compound. The phenolic compound profile and antioxidant capacity were determined by high-performance liquid chromatography (HPLC) and the Trolox-equivalent antioxidant capacity (TEAC) assay, respectively. The maximum content of the aglycone myricetin (81.15%) occurred with 2.5 M HCl at 75 °C for 60 min. Under these conditions, the IEE produced the aglycones myricetin, quercetin and cyanidin. The TEAC values of the M3R samples and IEE before and after acid hydrolysis did not show significant differences ($p > 0.05$). These results suggest that the hydrolytic process is effective to produce aglycone flavonoids from leaf extracts of *I. edulis*, and probably for other plant extracts rich in M3R.

Keywords: phenolic aglycone, antioxidant capacity, hydrolytic process, *Inga edulis*

Introduction

In food preservation, oxidative processes are the main cause of quality loss. Faced with this problem, food manufacturers add antioxidants to prevent the formation of oxidation products. The interest in finding natural antioxidants for use in foods, cosmetics and pharmaceuticals has increased considerably, with the aim of substituting the synthetic antioxidants, which have recently been reported to be potentially dangerous to human health.[1-3]

Natural antioxidants are formed by a large variety of compounds, including phenolics, carotenoids, ascorbic acid and tocopherols.[4] Among the phenolic compounds, flavonoids have been shown to be potent antioxidants in foods preventing oxidative stress in aerobic conditions, which is defined as an imbalance in reactive oxygen species.[4-6]

Flavonoids are polyphenolic compounds with two aromatic rings connected by a three-carbon bridge, C_6–C_3–C_6. The basic flavonoid skeleton can have numerous substituents. Sugars are very common, with the majority of flavonoids existing naturally as glycosides, which increases their water solubility.[7] *Inga edulis* Mart. (Leguminosae) is an Amazon plant that it can actively fix nitrogen.[8] The leaves of this plant are rich in polyphenolic compounds and myricetin-3-*O*-rhamnoside (M3R) is one of the major phenolics of this plant and is responsible for 88.6% of the flavonol family concentration.[9,10]

Recent efforts have been concentrated on the structural modification of phenolic compounds, by chemical or enzymatic catalysis, to alter their properties and expand the range of their application to more diverse systems, e.g. nanoencapsulation, adsorption onto macroporous resins.[11-15] Lipophilic derivatives of phenolic compounds generally maintain their antioxidant activity and, in some cases, exhibit novel bioactivities that are not found in the original phenolic compounds.[16-18]

To potentiate the use of polyphenolic antioxidants from natural sources, in addition to the extraction and purification of these compounds, it is necessary to use a process that separates the aglycones (more apolar) from the carbohydrates, such as acid hydrolysis. Acid hydrolysis is a chemical reaction in which an organic molecule undergoes decomposition caused by water, where the acid acts as a catalyst.[17,19]

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Thus, the aims of this paper are to (*i*) optimize the hydrolysis of a flavonol, M3R, by response surface methodology (RSM) and determine the experimental conditions (acid concentration, temperature and reaction time) that maximize the aglycone yield; (*ii*) evaluate the applicability of the optimized conditions on a partially purified extract of *Inga edulis* and (*iii*) evaluate the effect of hydrolysis on the antioxidant capacity of the extract by the Trolox-equivalent antioxidant capacity (TEAC).

Experimental

Preparation of flavonol standard solutions

Myricetin, cyanidin, quercetin and M3R (Sigma, St. Louis, MO, USA) were of HPLC grade. Stock solutions of standards were prepared in methanol at a concentration of 1000 μg mL^{-1} and stored under a nitrogen atmosphere at –20 °C.

Extract of *Inga edulis* leaves

The extract of *I. edulis* leaf was kindly donated by Amazon Dreams S/A, a company located in Belém (Pará, Brazil) that manufactures purified plant extracts. The extract was obtained after applying the appropriate techniques for the extraction and purification (adsorption/desorption) of phenolic compounds using synthetic macroporous resin.[20,21]

Preliminary study

A preliminary study was conducted to determine the impact that the type and amount of alcohol present in the reaction medium have on the percentage of aglycone obtained. Two alcohols were chosen, methanol and ethanol; the former is frequently used for hydrolysis reactions, and the second is suitable for food-grade use. The aglycone yield was determined after the hydrolysis of M3R (25 μg mL^{-1}) under the same conditions of temperature (75 °C), HCl concentration (2.5 M), time (120 min), and alcohol:water ratio (60:40, v/v). After defining the type of alcohol, a second experiment was carried out with different percentages of alcohol (0-80%, v/v) to determine the optimal percentage to obtain the highest concentration of aglycone.

Experimental design

The optimization was done through RSM, using a central composite rotational design with six replicates at the central point. The influences of three variables, hydrochloric acid concentration (HCl), hydrolysis time (time) and temperature (T), were evaluated on the dependent variable, myricetin yield (%). The experimental domain used in this work is presented in Table 1.

The experimental design runs were conducted in tubes with a screw cap and Teflon septum. In all runs, 25 μg mL^{-1} of M3R was used for the hydrolysis in alcoholic medium and was acidified with HCl under heating in a water bath. A multiple linear regression analysis was performed on the yield data. Mathematical modeling of data was done using a second-order polynomial model (equation 1):

$$Y(\%) = \beta_0 + \beta_H H + \beta_T T + \beta_t t + \beta_H H^2 + \beta_T T^2 + \beta_t t^2 + \beta_{HT} HT + \beta_{Ht} Ht + \beta_{Tt} Tt + \beta_{HTt} HTt \quad (1)$$

where H ([HCl]), T (reaction temperature) and t (reaction time) are the independent variables affecting the response variable, Y (yield, in percentage), and β_0, β_i (i = H, T and t), β_{ii}, and β_{ij} (j = H, T and t) are the coefficients for the intercept, linear, quadratic and interaction parameters, respectively.

Determination of optimum conditions and model validation

The choice of optimum conditions was performed according to the values of desirability (D). D values can vary from 0 to 1 and usually have a directly proportional relationship with the dependent variable in experiments that aim to maximize the product.[22,23] Thus, the optimized conditions for the HCl concentration, temperature and reaction time would be those in which the D value corresponds to the maximization of the concentration of aglycones produced (reaction yield), i.e., D = 1.

Table 1. Coded and actual levels of the three independent variables

Variable	Coded and original value of variable				
	–1.68	–1	0	+1	+1.68
Hydrochloric acid concentration / M	0.22	1.1	2.5	3.9	4.78
Hydrolysis time / min	22.2	60	120	180	217.8
Reaction temperature / °C	50.5	60	75	90	99.5

For validation, hydrolysis was performed in triplicate under the optimum conditions. The values of the aglycone flavonol yield obtained experimentally were compared with those predicted by the model to verify the applicability of the empirical model. After validation, the optimized conditions for the hydrolysis of glycosylated flavonol were applied to the extract of *I. edulis*, a plant that contains a high concentration of M3R. The concentration of M3R was adjusted to the standard concentration, and the percentage of ethanol was the same as that used for the standards.

Evaluation of hydrolysis by high-performance liquid chromatography coupled to diode array detector (HPLC-DAD)

The HPLC system employed was a Shimadzu LC-10Avp (Tokyo, Japan) equipped with a diode array detector (SPD-M20A). The analysis was performed on a Gemini C18 reversed-phase column (3 µm i.d., 150 × 4.6 mm; Phenomenex, Torrance, CA) maintained at 30 °C. The methodology used to analyze the flavonols by HPLC was based on a previous study.[9] The mobile phase for the elution of myricetin and M3R standards was composed of ultrapure water (solvent A) and acetonitrile (solvent B), both of which were acidified with 1% formic acid. The elution of standards was performed under a flow rate of 1 mL min^{-1} with the following gradient: 25-35% B for 5 min, 35-25% B for 1 min, and 25% B for 2 min. The elution of the extract of *I. edulis* followed the elution gradient 7-35% B for 26 min, 35-7% B for 6 min and 7% B for 3 min.

The solutions used as the mobile phase and the samples were previously filtered through a 0.22-µm membrane. The sample volume that was manually injected in both methods was 20 µL, and the wavelength used for the detection of the flavonols was 370 nm. Both phenolic compounds were quantified through a calibration curve by the injection of standards at different concentrations, expressed in mg L^{-1}. Subsequently, the percentage of myricetin yield was calculated according to equation 2:

$$Y(\%) = \left[\frac{C_M 100}{Ci_{M3R}\ 0.6853} \right] \quad (2)$$

where C_M and Ci_{M3R} are the concentrations of myricetin and M3R, respectively. The factor 0.6853 is related to the ratio between the molecular weights of myricetin (318.235 g mol^{-1}) and M3R (464.379 g mol^{-1}).

Evaluation of antioxidant capacity

The TEAC assay used was a method adapted by our team[10] from the original protocol[24] to perform an analysis using transparent microplates. The reaction mechanism of this method involves transferring electrons and measuring the scavenging of the radical $ABTS^{+\bullet}$.[24] The analysis was performed in triplicate, and the results are expressed in micromol of Trolox equivalents *per* liter (µmol TE L^{-1}).

Results and Discussion

Preliminary studies

Initially, two studies were conducted to determine the type of alcohol (methanol or ethanol) and the percentage of alcohol (v/v) required to solubilize both M3R and its aglycone form, myricetin. In Figure 1, it is observed that both alcohols allow the complete conversion of the flavonol glycoside to aglycone because no peak (retention time (t_R) = 3.8 min) of M3R could be detected after the elution of the hydrolysates containing methanol or ethanol. Interestingly, a higher amount of myricetin was present when ethanol was used in the reaction.

This behavior is probably because ethanol is more apolar than methanol, thus favoring the solubility of the aglycone produced during the acid treatment. In fact, the results obtained for the solubility of quercetin (another flavonol) in water, water-methanol, and water-ethanol showed that the solubility of quercetin is higher when a water-ethanol mixture is used as the solvent.[25]

Another experiment was conducted to investigate the best percentage of ethanol to solubilize and preserve the myricetin during the reaction (Figure 2). The aglycone concentration increased when the ethanol proportion varied from 20-60%, and the concentration then decreased significantly, probably due to the lack of solubility of both M3R and intermediate form before cleavage. Thus, the percentage of 60% was established as suitable for hydrolysis.

Optimization of acid hydrolysis by RSM

To optimize the acid hydrolysis, RSM was used to evaluate the impact of the HCl concentration ([HCl]), reaction time (time) and temperature (T) on the percentage yield of the reaction. Table 2 shows the different experimental conditions and their experimental results of aglycone yield.

The yield values were subjected to an analysis of variance (ANOVA) and lack of fit test on a 2nd-order polynomial model. The ANOVA (Table 3) showed that the model was statistically significant ($p < 0.05$), indicating that the experimental variation of the response (reaction yield)

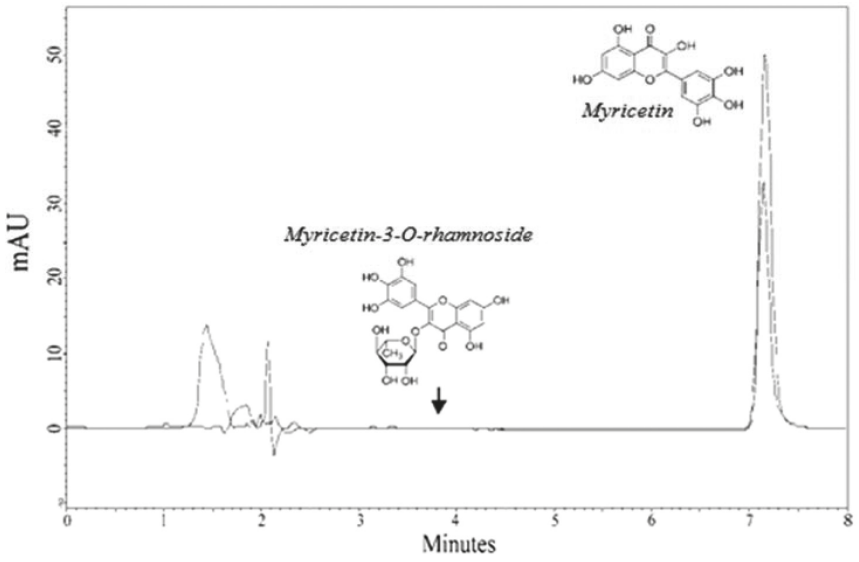

Figure 1. HPLC chromatogram of the acid hydrolysis of myricetin-3-*O*-rhamnoside in hydroalcoholic solutions with 60% methanol (continuous line) and 60% ethanol (dotted line) in the reaction medium.

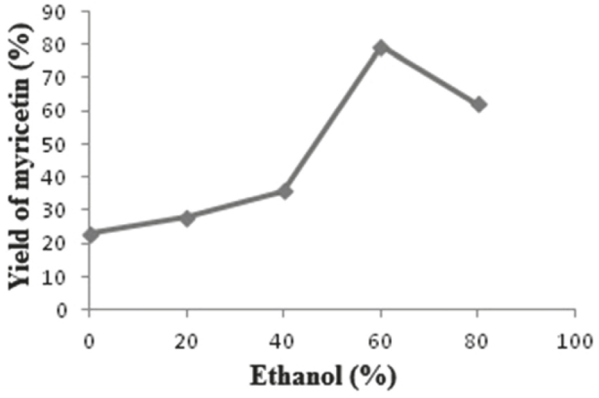

Figure 2. Effect of ethanol:water ratio (v/v) on the concentration of myricetin recovered after the hydrolysis of myricetin-3-*O*-rhamnoside.

can be explained by at least one of the model parameters.[16] The lack of fit test was not statistically significant ($p > 0.05$), which indicated that the model proposed for hydrolysis is adequate to fit the observed experimental data. The model achieved a coefficient of determination (R^2) value of 0.8269; i.e., most of the variability in the response can be explained by the model.

The regression coefficients of the 2nd-order polynomial model are presented in Table 4. The results indicate that the yield of myricetin depends strongly on the linear terms of HCl and T, on the quadratic term of HCl and on the interactions between HCl and T. The positive linear coefficient for HCl indicates that the yield of myricetin globally increases with the concentration of the catalyst, although the negative quadratic effect for HCl shows that there is a maximum yield, and further increases in the HCl concentration decrease the yield of the reaction. The presence of acid significantly accelerates the M3R hydrolysis by the protonation of the reaction medium until a maximum point, beyond which the yield decreases, probably due to myricetin degradation. The same behavior was observed by Wang *et al.*[26] when the concentration of

Table 2. Experimental data obtained during the study on the acid hydrolysis of myricetin-3-*O*-rhamnoside

Run	Experimental condition			Myricetin
	HCl / M	T / °C	time / min	Yield / %
1	1.1	60	60	40.18
2	1.1	90	180	77.34
3	3.9	60	180	85.59
4	3.9	90	60	63.75
5	2.5	75	120	84.93
6	2.5	75	120	82.81
7	1.1	60	180	48.42
8	1.1	90	60	73.75
9	3.9	60	60	88.14
10	3.9	90	180	40.43
11	2.5	75	120	77.66
12	2.5	75	120	77.49
13	0.22	75	120	37.27
14	4.78	75	120	89.52
15	2.5	50.5	120	90.31
16	2.5	99.5	120	59.04
17	2.5	75	22.2	86.11
18	2.5	75	217.8	71.20
19	2.5	75	120	72.40
20	2.5	75	120	85.69

Table 3. Analysis of variance for the response surface quadratic model of apparent myricetin released after the acid hydrolysis of myricetin-3-*O*-rhamnoside

	Degrees of freedom	Sum of squares	Mean of squares	F^a
Model	9	4934.40	4934.40	146.45[b]
Lack of fit	5	830.48	166.09	4.93[c]
Pure error	5	168.46	33.69	
R^2		0.8269		

[a]Fisher test; [b]significant at $p < 0.05$; [c]not significant ($p > 0.05$). R^2: coefficient of determination.

Table 4. Parameters estimated for the predicted second-order model for the response variable (apparent myricetin yield)

Model parameter	Coefficient of regression	Standard error
Intercept	80.24[a]	2.36
[HCl] / M	9.26[b]	1.59
$[HCl]^2$	–7.68[b]	1.60
T / °C	–4.36[c]	1.59
T^2	–3.46[d]	1.60
time / min	–2.88[d]	1.59
$time^2$	–1.96[d]	1.60
[HCl] × T	–16.50[a]	2.05
[HCl] × time	–4.71[d]	2.05
T × time	–3.17[d]	2.05

[a,b,c]Significant at $p < 0.001$, $p < 0.01$ and $p < 0.05$, respectively; [d]not significant ($p > 0.05$). T: temperature.

phosphoric acid used in the hydrolysis of rutin was higher than 2.5%. This effect might be due to the instability of aglycone at relatively high temperatures in the presence of a strong acid.

The negative effect of temperature was more pronounced when the HCl concentration was higher, as indicated by the negative interaction term (Table 4). This result clearly suggests that a maximum yield can be achieved at a lower temperature and higher HCl concentration (Figure 3). This effect could also be observed in the optimization of the acid hydrolysis of soybean isoflavones[27] and of red wine anthocyanin.[28]

Determination of optimum conditions and model validation

To verify the validity of the model, optimum conditions were sought using a maximum desirability (D) for the yield. The maximum value found for D was 0.82, indicating that the hydrolysis process can be optimized at 2.5 M HCl and under heating at 75 °C for 60 min. In this condition, the predicted yield is 81.2 ± 6.68%. After performing three hydrolyses of M3R under these optimal conditions, we observed a yield of 81.15 ± 9.97%. Similar results were reported for the hydrolysis of the flavones of *Flos chrysanthemi* extract (2.4 M HCl, 80% methanol, 80 °C for 120 min) to produce maximum concentrations of luteolin, apigenin and diosmetin.[29] The optimal conditions found for the hydrolysis of the glycosides of myricetin of *Malpighia glabra* (cherry) were 0.6 M HCl and 90 °C for 40 min.[30]

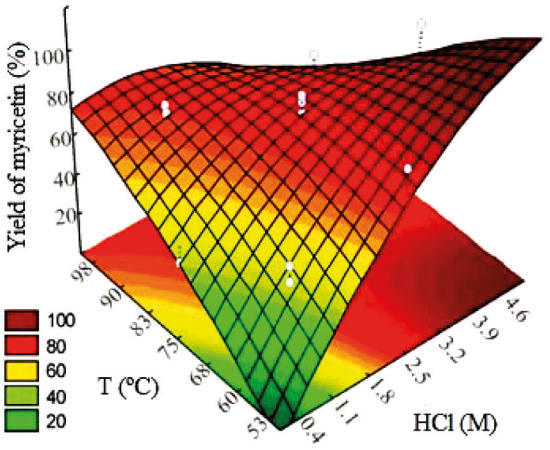

Figure 3. Response surface and contour plot of the yield percentage of myricetin formed after 120 min of hydrolysis as a function of the interaction between the temperature (T) and concentration of HCl of the reaction medium.

Application of optimum hydrolysis conditions to purified extract of *Inga edulis*

We used the optimum experimental conditions for the hydrolysis of the flavonol M3R present in the leaf extract of *I. edulis*. The effect of hydrolysis was qualitatively evaluated by comparing the chromatographic profiles of the extract before and after hydrolysis, as shown in Figure 4.

In the chromatogram of Figure 4a, there is a large peak of M3R (370 nm) that, after hydrolysis, was converted into myricetin. Quercetin could also be detected after the treatment (Figure 4b). This aglycone was probably derived from the hydrolysis of the quercetin glycosides that are present in the *I. edulis* leaf extract.[16] After hydrolysis, the *I. edulis* extract had a red color due to the presence of delphinidin and cyanidin (Figure 4b), which are derived from the acid cleavage of the proanthocyanidins present in the extract.[10]

Evaluation of antioxidant capacity

We evaluated the effect of the optimized acid hydrolysis on the antioxidant capacity of M3R (4567 ± 189 μmol TE L^{-1})

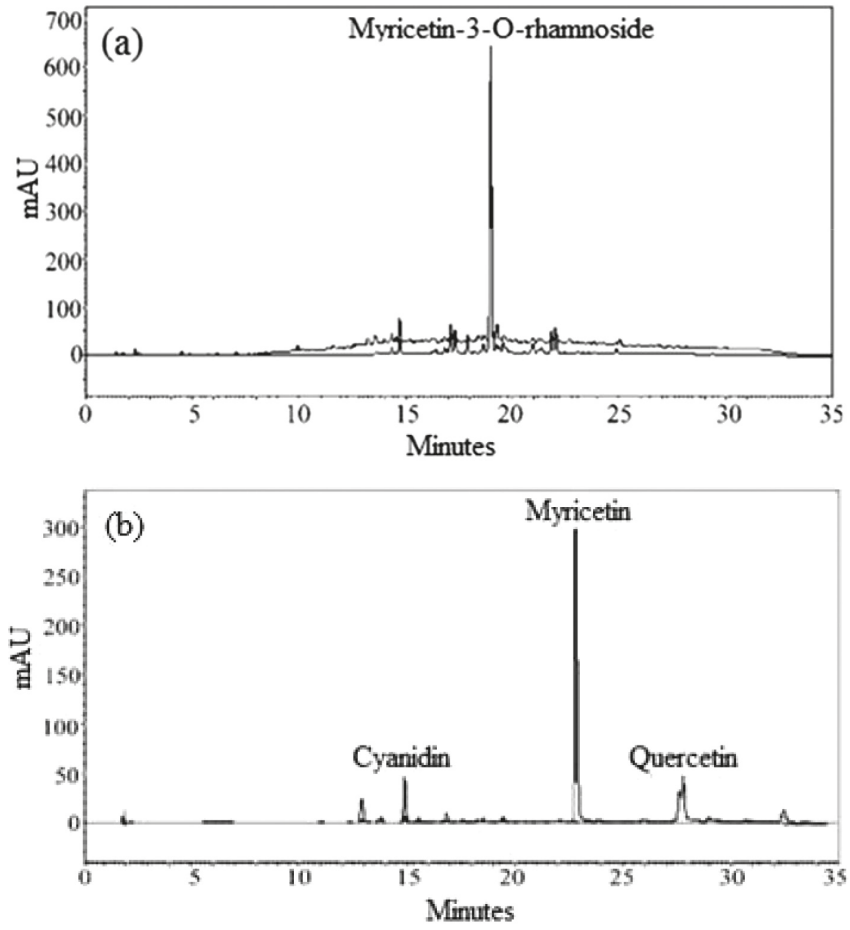

Figure 4. Chromatogram of the extract of *Inga edulis* leaves before (a) and after (b) acid hydrolysis.

and the extract of *I. edulis* leaf (2345 ± 142 μmol TE L^{-1}) using the TEAC assay. The TEAC results after the hydrolysis of M3R (5227 ± 472 μmol TE L^{-1}) and *I. edulis* extract (2283 ± 57 μmol TE L^{-1}) showed that the acid treatment had not modified the antioxidant capacity ($p > 0.05$). This result confirms that acid hydrolysis can be employed to reduce the polarity of the phenolic compounds without reducing their antioxidant capacity.

Conclusions

By using RSM, it is possible to propose an optimized process for the hydrolysis of M3R and to assess the effect of this process on the antioxidant capacity for Trolox equivalent antioxidant capacity assay. The optimum conditions for acid hydrolysis were achieved. Under these conditions, the conversion of glucoside to aglycone presented a yield of 81.15%, thus enabling the conservation of the TEAC antioxidant activity of M3R (now in the form of myricetin) and the extract of *I. edulis* leaf. This control of the lipophilicity/hydrophilicity balance of naturally occurring antioxidants serves as a good approach to develop novel antioxidants with expanded application in more diverse systems, e.g., fats and oils, lipid-based foods, cosmetic formulas, emulsions and many biological environments.

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References

1. Halliwell, B.; Murcia, M. A.; Chirico, S.; Aruoma, O. I.; *Crit. Rev. Food Sci. Nutr.* **1995**, *35*, 7.
2. Lobo, V.; Patil, A.; Phatak, A.; Chandra, N.; *Pharmacogn. Rev.* **2010**, *4*, 118.
3. Sindhi, V.; Gupta, V.; Sharma, K.; Bhatnagar, S.; Kumari, R.; Dhaka, N.; *J. Pharm. Res.* **2013**, *7*, 828.

4. Brewer, M. S.; *Compr. Rev. Food Sci. Food Saf.* **2011**, *10*, 221.
5. Núñez-Sellés, A. J.; *J. Braz. Chem. Soc.* **2005**, *16*, 699.
6. Crozier, A.; Jaganath, I. B.; Clifford, M. N. A.; *Nat. Prod. Rep.* **2009**, *26*, 965.
7. Michotte, D.; Rogez, H.; Chirinos, R.; Mignolet, E.; Campos, D.; Larondelle, Y.; *Food Chem.* **2011**, *129*, 1228.
8. Nichols, J. D.; Carpenter, L.; *For. Ecol. Manage.* **2006**, *233*, 344.
9. Souza, J. N. S.; Silva, E. M.; Silva, M. N.; Arruda, M. S. P.; Larondelle, Y.; Rogez, H.; *J. Braz. Chem. Soc.* **2007**, *18*, 1276.
10. Dias, A. L. S.; Souza, J. N. S.; Rogez, H.; *Quim. Nova* **2009**, *15*, 1.
11. Shahidi, F.; Zhong, Y.; *Eur. J. Lipid Sci. Technol.* **2010**, *112*, 930.
12. Pompeu, D. R.; Moura, F. G.; Silva, E. M.; Rogez, H.; *Sep. Sci. Technol.* **2010**, *45*, 700.
13. McClements, D. J.; *Adv. Colloid Interface Sci.* **2015**, *219*, 27.
14. Parekh, I.; Khanvilkar, A.; Naik, A.; *J. Food Process. Preserv.* **2017**, *41*, e13244.
15. Hasnat, M. A.; Pervin, M.; Kim, D. H.; Kim, Y. J.; Lee, J. J.; Pyo, H. J.; Lee, C. W.; Lim, B. O.; *Food Sci. Biotechnol.* **2015**, *24*, 689.
16. Mbatia, B.; Shanker, S.; Mattiasson, B.; Mulaa, F.; Adlercreutz, P.; *J. Agric. Food Chem.* **2011**, *59*, 7021.
17. Chambi, F.; Chirinos, R.; Pedreschi, R.; Betalleluz-Pallardel, I.; Debaste, F.; Campos, D.; *Ind. Crops Prod.* **2013**, *47*, 168.
18. Pereira, D.; Pinheiro, R. S.; Heldt, L. F. S.; de Moura, C.; Bianchin, M.; Almeida, J. F.; dos Reis, A. S.; Ribeiro, I. S.; Haminiuk, C. W. I.; Carpes, S. T.; *Food Sci. Technol.* **2017**, *37*, 17.
19. Kim, T. J.; Silva, J. L.; Jung, Y. S.; *Food Chem.* **2011**, *126*, 116.
20. Silva, E. M.; Rogez, H.; Larondelle, Y.; *Sep. Purif. Technol.* **2007**, *55*, 381.
21. Silva, E. M.; Rogez, H.; da Silva, I. Q.; Larondelle, Y.; *Food Bioprod. Process.* **2012**, *91*, 558.
22. Maricán, A.; Ahumada, I.; Richter, P.; *J. Braz. Chem. Soc.* **2012**, *23*, 267.
23. Mang, D. Y.; Abdou, A. B.; Njintang, N. Y.; Djiogue, E. J. M.; Loura, B. B.; Mbofung, M. C.; *J. Food Meas. Charact.* **2015**, *9*, 495.
24. Re, R.; Pelligrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. A.; *Free Radical Biol. Med.* **1999**, *26*, 1231.
25. Razmara, R. S.; Daneshfar, A.; Sahraei, R.; *J. Chem. Eng. Data* **2010**, *55*, 3934.
26. Wang, J.; Zhao, L.; Sun, G.; Liang, Y.; Wu, F.; Chen, Z.; Cui, S.; *Afr. J. Biotechnol.* **2011**, *10*, 1460.
27. Chiang, W.; Shih, C.-J.; Chu, Y.-H.; *J. Food Chem.* **2001**, *72*, 409.
28. Pinho, C.; Melo, A.; Mansilha, C.; Ferreira, I. M. P. L. V. O.; *J. Agric. Food Chem.* **2011**, *59*, 50.
29. Li, F. G.; Zhou, H.; Sun, D. L.; Wang, R. W.; Zeng, S.; Jiang, H. D.; *J. Chin. Pharm. Sci.* **2009**, *18*, 55.
30. Ribani, R. H.; Amaya, D. B. R.; *Quim. Nova* **2008**, *31*, 1378.

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