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QUANTITATIVE ANALYSIS OF QUERCETIN IN MALPIGHIA EMARGINATA DRIED EXTRACT BY HPLC-DAD

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ABSTRACT

Acerola (*Malpighia emarginata*) is widely distributed in Brazil and has a large amount of flavonoids. Quercetin is a flavonoid present in this fruit and its protection is necessary due to its high antioxidant activity. Thus, encapsulation by spray drying is the most used technique for the protection of this compound. Furthermore, it is necessary to determine the biomarkers contained in the dry extract of acerola. In a sample of dry extract of acerola, a selective extraction was carried out for quercetin determination by chromatographic analysis. In HPLC-DAD the mobile phase was a 0.2% methanol and phosphoric acid solution (65:35). The flow rate was 1.0 ml min^{-1} at 30°C. and detection at 360 nm. The linear range of quercetin was 0.025–0.150 $mg.ml^{-1}$ (r2 = 0.9980) with a recovery rate of 97.50 - 103.30% (average 100.40%; RSD 2.28%). Separation of the baseline target component occurred within 4 min. An RSD repeatability of 1% was performed and the highest quercetin content in the dry extract was (average 0.025 mg g^{-1}). It was concluded that HPLC-DAD was sufficient for the determination of quercetin in the microencapsulated dry extract of Acerola and that the selective extraction was determinant for the quantification of this biomarker.

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INTRODUCTION

Acerola *(Malpighia emarginata)* is widely distributed in Brazil and the Northeast region is the largest producer of the fruit (MAZZA *et al.,* 2015). It is an excellent natural source of ascorbic acid (vitamin C) and has become notorious in the daily life of the consumers who are healthy-concious (HANAMURA *et al.,* 2008). In addition, its properties have been attributed to help the treatment of colds and flu, lung and liver diseases (SCHRECKINGER *et al.,* 2010). Phytochemical studies reveal that *Malpighia emarginata*contains a variety of secondary metabolites like carotenoids (MEZADRI *et al.,* 2005; FREITAS *et al.,* 2006; FILHO *et al.,* 2022), phenolic compounds (HANAMURA *et al*., 2005; SOUZA *et al*., 2014; BATAGLION *et al*., 2015; STAFUSSA *et al*., 2018) and flavonoids (OLIVEIRA *et al*., 2012; BATAGLION *et al*., 2015; STAFUSSA *et al*., 2018; FILHO *et al.,* 2022) were identified and isolated in the past few years. Some of the compounds were found to have potent antioxidant (BATAGLION *et al*., 2015; FILHO *et al.,* 2022), antimutagenic, anti-inflamatory (OLIVEIRA *et al*., 2012; TOMÁS-BARBERÁN & ANDRÉS-LACUEVA, 2012), antiallergic, antiviral activities (LEFFA *et al*., 2013; HORTA *et al*., 2016). The flavonoids isolated from *Malpighia emarginata*mainly include quercetin (quercetin 3-rhamnoside). Anthocyanin (cyanidin 3-O-α-rhamnoside and pelargonidin-3-α rhamnoside) and kaempferol (BATAGLION *et al*., 2015). Quercetin is present in Acerola fruit and has a variety of biological activities. Several studies demonstrate that this flavonoid may play an important role in the medicinal effects of cancer treatment, diabetes and immunological diseases (HANAMURA *et al*., 2005; SCHRECKINGER *et al.,* 2010; WAGNER *et al*., 2010). Nonetheless, flavonoids proved to be sensitive to pH, luminosity, temperature and oxygen concentration (AGUIAR *et al*., 2016; AGUIAR *et al*., 2017; GONÇALVES *et al*., 2017; AKBARBAGLU *et al*., 2021). In this sense, it is necessary to protect them so that they can remain stable and absorbable. One technique capable of such

action is encapsulation by spray dryer. Through this, standardized dry extracts with satisfactory amounts of the phytochemical of interest are produced (ERSUS & YURDAGEL, 2007; LOPES *et al*., 2007; BARRETTO *et al*., 2020). Thus, chromatographic studies in this project are necessary, as it is possible to confirm that quercetin was able to adhere to the wall material and was not degraded in the drying process. Therefore, we developed a fast, simple and selective high performance liquid chromatography with diode array detection (HPLC-DAD) method for the detection of quercetin content in dry encapsulated extracts of Acerola (*Malpighia emarginata*).

MATERIAL AND METHODS

Samples: The fruits of *Malpighia emarginata* were procured from Niterói, Rio de Janeiro, Brazil (22º55'55.89" S 43º04'13.45" W) during the months of January, February and March, 2022). The fruits were selected according to the uniformity and color of the skin. In the laboratory, the specimens were sanitized in running water with subsequent disinfection in a 2.5% sodium hypochlorite solution for 15 min and freezed for further use.

Instrumentation: Analytical balance (AUW 2200 MarteCientífica) Rotary Evaporator (QUIMIS®), Agilent 1100 Series High-Performance Liquid Chromatography system (Agilent Technologies, Santa Clara, CA, USA), J16 Moisture Analyzer (Mettler Toledo) and Mini Spray Dryer LM MSDI 1.0 (LABMQ).

Chemical and Reagents: Standard quercetin was obtained from Santa Cruz Biotechnology (Catalogue number: sc-203225A, Batch number: B0722), Methanol used was of HPLC-specific grade (Supelco®), Ethanol P.A Perfyl Tech (São Paulo, Brazil), Capsul® (Quimis, Brazil), Petroleum Ether P.A 30/70 (ÊxodoCientífica) and Ethyl Acetate P.A (DinâmicaQuímicaContemporâneaLtda).

Preparation of the Liquid Extract: 704g of frozen Acerola were weighed in a 1L beaker and 704 mL of solvent (95% Ethanol) were added to this same container in an adapted 1:1 ratio (REZENDE *et al.,* 2018). Then, this mixture was placed in a crusher (Juicer Fun Kitchen) to obtain the raw Acerola juice. The process was repeated twice. An ultrasonication was performed for 15 minutes at a temperature of 40ºC on all the material obtained. After this step, the crude material was obtained and it was filtered, conferring a total of 800mL that was called Acerola Crude Extract (ACE). The final yield of ACA was 75%.

Wall agent ratio and Dry residue: Firstly 2 g of ACE sample were weighed in a J16 Moisture Analyzer (Mettler Toledo) for the determination of the dry weight in accordance with Brazilian Pharmacopoeia. Then, Capsul® was used as a wall material due to its microencapsulation capacity (CARVALHO *et al*., 2019) in the following ratios: (1 capsul:1 dry residue). At the end the wall agent and ACE were mixed by magnetic stirring for 20 minutes, before starting the drying procedure.

***Spray Drying*:** A spray dryer model Mini Spray Dryer LM MSDI 1.0 (LABMQ) with a capacity of 1.0 $L.h^{-1}$ was used. The drying parameters are in accordance with (REZENDE *et al*., 2018; BARRETTO *et al.*, 2020) inlet air temperature (150°C), air flow (2,01 m^3/min) and feed flow (0,5 L/h). The operating conditions were independently controlled according to the equipment used. The Acerola Dry Crude Extract (ADCE) was packed in a dark package at a temperature of -2°C until the beginning of the HPLC analysis.

HPLC Sample Preparation: An adaptation of the method described by (LIU *et al.,* 2011) was done. The ADCE was reflux-extracted 4 times (Soxhlet), using 200 mL de methanol for 1 hour each time in a water bath at 60 °C. The pooled extract was evaporated under vacuum to dryness (50/60 °C). The residue was dissolved in 30 mL of distilled water and extracted using 30 ml of petroleum ether. The petroleum ether extraction liquid was discarded and 5 ml of HCL (10%) was added to water liquid extract which was extracted for 30 min in a water bath at 50/60 °C. After rapid cool off, the liquid was extracted using 30 ml of ethyl acetate each time. The ethyl acetate extraction was collected, evaporated (50/60 °C) to dryness, re-dissolved in methanol, transferred to a 25 mL measuring flask and filtered with 0.45-µm Millipore membrane filter before use.

HPLC-DAD: Quercetin contents in ADCE were analyzed by using HPLC-DAD, high pressure liquid chromatography with diode ray detector (LIU *et al*., 2011). At first, three different buffers including methanol buffer (buffer A), acetonitrile buffer (buffer B), and 0.4% phosphoric acid buffer (buffer C), were mixed at three different ratios such as 60:20:20; 65:20:15; and 70:15:15. It was found that the whole separation course required almost 21 min and the peak of quercetin was obtained at the 15th minute and was asymmetric. Thus, we opted to use 0.2% phosphoric acid buffer (buffer D) and mixed buffer A and buffer D at the ratios of 70:30; 60:40; and 65:35. The quercetin peak time now shifted to the 4th minute point and the whole separation course required 14th min while the peak obtained was symmetric. Since quercetin is reported to have the maximum absorption at 250 and 360 nm wavelengths. Therefore, it was compared the shapes of quercetin peaks at these wavelengths and found that the shape of the peak was more symmetric and higher at the detection wavelength of 360 nm. An Agilent 1100 system with TC-C^{18} column (250 x 4.6 mm, 5 µm) was used. The mobile phase was methanol-0.2% phosphoric acid (65:35) solution. The flow rate was 1.0 ml min^{-1}. The column temperature was 30 °C and the detection wavelength was 360 nm. The quercetin peaks were identified and quantified against the external reference standards. Quercetin standard was purchased from the Santa Cruz Biotechnology United States. The solvents used were of spectra analytical grade and were filtered through 0.45 µm filters before use.

Statistical Analysis: The data were acquired and analyzed by Agilent 1100 HPLC software. The standard curve of quercetin was obtained by plotting concentrations against peak area and the regression equation and correlation coefficient (r^2) were determined. The sample analyses were performed in triplicate and the mean values were calculated. RSD < 3% was considered as significant for the precision (repeatability) and the accuracy (recovery) of the method used.

RESULTS AND DISCUSSION

HPLC-DAD Optimization Conditions: The best stage of the gradient mobile phase was methanol-0.2% phosphoric acid (65:35) solution, the flow rate was 1.0 ml min^{-1}. For detection was performed with DAD, wavelength 360 nm. Quercetin standard presents in 4 to 5 min maximum peak.

Validation, Linearity and Recovery: Six samples, in triplicate, were processed and analyzed by HPLC-DAD. Comparing the standard validation and recovery rate. The relative standard deviation (RSD) was 1.77%. The recovery rate was 0.99, recovering most part of the standard, but part of the solution was lost during purification. The standard solutions of quercetin were prepared at the concentrations of 0.025, 0.050, 0.075, 0.100, 0.125 and 0.150 mg ml^{-1}. The least-square regression analysis of the quercetin calibration graph was: y = 6.0046 x 108 ***x*** + 41710.9 (r^2 = 0.9980) where, y represents peak area, x represents the concentration in mg ml^{-1}. By adding different concentrations of standard quercetin (e.g., 4.05, 2.70, and 1.35 mg g^{-1}) into samples with known (2.69 mg g^{-1}) quercetin content, the recovery rate of the method was determined at the reaction conditions used. As shown in Table 1, the recovery rate ranged from 97.50 to 103.30% (average 100.40%; RSD 2.28%; n = 6). The linearity of the method was found to be from 0.025 to 0.150 mg ml^{-1}. The standard solutions of quercetin were prepared at the concentrations of 0.025, 0.050, 0.075, 0.100, 0.125 and 0.150 mg ml^{-1}. The least-square regression analysis of the quercetin calibration graph was: y = 6.0046 x 108 □ + 41710.9; r^2 = 0.9980 where y represents peak area, x represents the concentration in mg ml^{-1}.

Quercetin quantification in dry extracts of Malpighia emarginata fruit: The average quercetin contents in the whole fruit were 0.027 mg g^{-1}, RSD = 0.01, and a standard concentration of 2.69 mg g^{-1}.

Table 1. Recovery Rate (n= 36)

Background (mg g^{-1}) *	Added (mg g^{-1})	Detected (mg g^{-1})	Recovery rate (%)	Average recovery rate (%)	RSD (%)
2.68	0.01350	2.6935	100		
2.68	0.01010	2.6901	100		
2.68	0.00675	2.6746	99.55		
2.68	0.00338	2.6699	99.50	99.67	0.01
2.68	0.00135	2.6692	99.55		
2.68	0.00067	2.6672	99.50		

* Each sample represents quantification of 3 extractions evaluated

Table 2. Quercetin Quantification Dry Extract of *Malpighia emarginata* Fruit (n = 6)

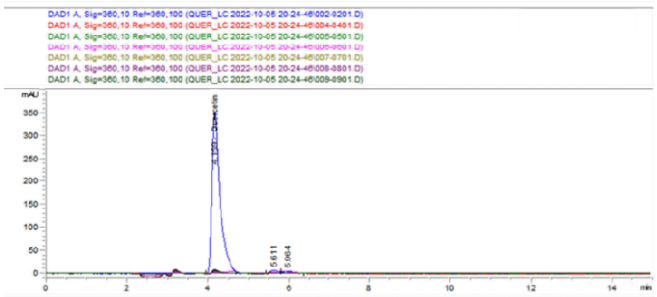
No	Quercetin Content (mg g^{-1})	Reference Data*	Average	RSD (%)
1.1	0.001414			
1.2	0.001399	0.027	0.001406	0.053382
1.3	0.001406			
2.1	0.002755			
2.2	0.002570		0.002755	4.737688
2.3	0.002822			
3.1	0.007323			
3.2	0.007075		0.007256	1.768090
3.3	0.007256			
4.1	0.014568			
4.2	0.014535		0.014554	0.113804
4.3	0.014554			
5.1	0.018237			
5.2	0.018236		0.018236	0.463826
5.3	0.018090			
6.1	0.025701			
6.2	0.025480		0.025654	0.453820
6.3	0.025654			

*(Bataglion et al., 2015)

The range has a maximum value of 0.025701 and a minimum of 0.001399 value. The results are shown in Table 2. HPLC is the technique of choice recommended for the separation, identification and quantification of food, pharmaceutical, and medicinal analytes. Thus, the objective of this experiment was to validate the methodology of identification and quantification of quercetin present in the microencapsulated dry extract of Acerola *Malpighia emarginata* according to analytical methods, the guidelines from the Internationalconference on the harmonization of technical requirements for the human use (SINGH, 2015). Flavonoids are sensitive, thus demanding a high level of research on protecting them and increasing their bioavailability. Encapsulation is an effective alternative as it protects them against isomerization, oxidation and degradation (POOL *et al*., 2013). In addition, quercetin is also capable of increasing the commercial shelf life of the industrialized product (NATHIYA *et al*. 2014; TAVARES *et al.* 2014).

In the present study, it was possible to observe that the encapsulation by spray drying was efficient in maintaining the quercetin attached to the wall material Capsul®, since it was identified and quantified by HPLC in a simple and fast way. This result is similar to that found by Gatak and Iyyaswami (2019) who encapsulated quercetin from onion skins with casein particles by emulsion. Thus, it can be said that HPLC-DAD is sufficient to isolate and validate micrograms of natural compounds through an analytical column that allows them to be identified and quantified. However, if it is desired to purify and prepare specific amounts of a particular analyte, it will be necessary to associate a mass spectrometer or other optimization technique. One way of concentrating and optimizing the identification and quantification of bioactive compounds of plant origin is to perform their previous extraction in a way that complements encapsulation (CASTRO-ALATORRE *et al*., 2021; PATTNAIK *et al.*, 2021). In the encapsulation of red grapes (*Vitis vinifera* L.), ethanol extraction was performed to increase the concentration of anthocyanins and phenolic compounds, consequently increasing their concentration in the microencapsulated material (BOONCHU AND UTAMA-ANG, 2015).

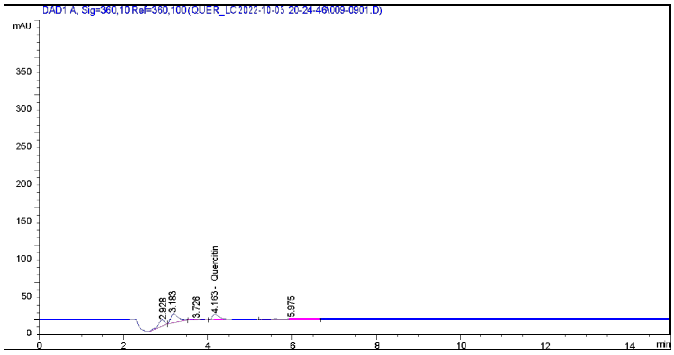
This fact was similar to that found in the present study, as the extraction step also complemented the spray dryer and helped in the purification and identification of quercetin when the extract was resuspended in the HPLC analysis. Quercetin concentrations (Table 2) found in the present study are close to the values found by Bataglion *et al*., (2015) who validated the methodology of analysis of phenols present in Acerola pulp through the use of ultra-high performance liquid chromatography-tandem mass spectrometry UHPLC-MS/MS. The maximum value found by the researchers was 0.027 mg/g^{-1} and the one obtained by us by HPLC-DAD was 0.025 in the encapsulated powder extract of Acerola *Malpighia emarginata*. This result demonstrates that HPLC-DAD was as efficient as UHPLC-MS/MS and that the encapsulation process satisfactorily preserved quercetin. The efficiency of the extraction method and quantification can be observed in the chromatograms (Figures 1, 2 and 3).



Legend: The standard peak of quercetin and six samples. Quercetin content in *Malpighia Emarginata* was determined by HPLC-DAD using the Agilent 1110 system. The mobile phase of methanol-0.2% phosphoric acid (65:35) solution and a flow rate of 1.0 ml min^{-1} and a detection wavelength of 360 nm. The sample solutions of quercetin at the concentrations of 0.025, 0.050, 0.075, 0.100, 0.125 and 0.150 mg ml^{-1}. The least-square regression analysis of the quercetin calibration graph was: y = 6.0046 x 108 ***x*** + 41710.9 (r^2 = 0.9980). Where, y represents peak area, x represents the concentration in mg ml^{-1} and r represents the correlation coefficient.

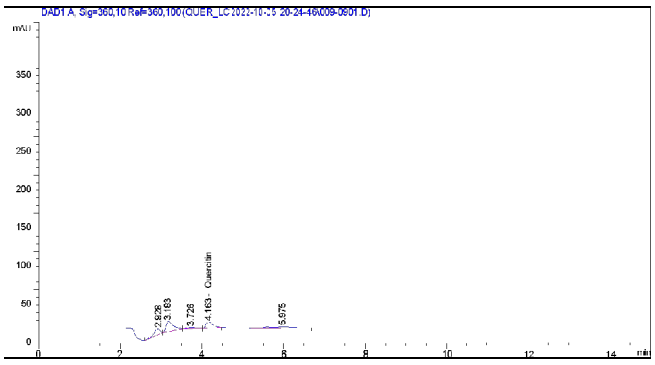
Figure 1. HPLC chromatograms of quercetin standard and of six samples of the acerola dried extracts

The natural sources of quercetin are diverse. Fruits such as açaí, apple, blueberry, blackberry, orange, lemon and Acerola are rich in this flavonoid (STAFUSSA *et al*., 2018; DABEEK AND MARRA, 2019). Acerola stands out because it is a fruit widely distributed in Latin America that contains a high amount of phenolic compounds such as cyanidin, quercetin, and catechins in all its stages of maturation and in its different species (OLIVEIRA *et al.*, 2012; PRAKASHI AND BASKARAN, 2018; FERREIRA *et al*., 2021).



Legend: The sample peak. Quercetin content in *Malpighia Emarginata* was determined by HPLC-DAD using the Agilent 1110 system. The mobile phase of methanol-0.2% phosphoric acid (65:35) solution and a flow rate of 1.0 ml min^{-1} and a detection wavelength of 360 nm. The sample solution of quercetin at the concentration of 0.150 mg ml^{-1}. The assay was performed in triplicate and average quercetin content (mg g^{-1}) was calculated.

Figure 2. HPLC chromatogram at concentration 0.150 mg ml^{-1}



Legend - Sample peak of quercetin. Quercetin content in *Malpighia Emarginata* was determined by HPLC-DAD as described before. Quercetin sample peaks were identified and quantified against the external reference standards. The assay was performed in triplicate and average quercetin content (mg g^{-1}) was calculated. The standard solutions of quercetin at the concentrations of 0.025, 0.050, 0.075, 0.100, 0.125 and 0.150 mg ml^{-1}. The least-square regression analysis of the quercetin calibration graph was: y = 6.0046 x 108 $\boldsymbol{x}$ + 41710.9; (r^2 = 0.9980). Where, y represents peak area, x represents the concentration in mg ml^{-1} and r represents the correlation coefficient.

Figure 3. HPLC chromatogram standard of quercetin

When compared to fruits that are also easily distributed throughout Brazil, such as persimmon, pineapple, guava, melon, mango, papaya, and watermelon, its concentration of phenolic compounds is much higher and it also has a very high amount of Vitamin C with a content 50-100 times greater. then orange and lemon (PRAKASHI AND BASKARAN, 2018). Acerola was chosen for the present study because of the facts mentioned above and because Brazil is the largest producer, consumer, and distributor of the fruit in the world, and its climate, mainly in the Northeast region, favors the growth of the fruit throughout the year (FERREIRA *et al*., 2021). Vendramini and Trugo (2004) have also studied and detected quercetin by HPLC in the Acerola fruit *Malpighia punicifolia, L*. Its retention time was in the 8th min, different from that presented by us with *Malpighia emarginata* which occurred in the 4th min. Demonstrating that the present study was better as an analytical methodology, saving costs with the solvents used.

CONCLUSION

The results show that the HPLC-DAD method was viable and successfully developed for the determination of quercetin levels in dry encapsulated extracts of Acerola *Malpighia emarginata*. Selective extraction was crucial to obtain definitive amounts of Acerola biomarkers, particularly quercetin. Furthermore, the wall agent Capsul® was able to keep the bioactive compound quercetin stable in the dry extract produced by the spray dryer.

Declaration of Competing Interest: The authors have no conflicts of interest to declare.

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