High throughput analytical approach based on μQuEChERS combined with UHPLC-PDA for analysis of bioactive secondary metabolites in edible flowers

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ABSTRACT

Mallow blue (*Malva sylvestris* L.), hibiscus *(Hibiscus rosa-sinensis* L.) and nasturtium (*Tropaeolum majus* L.), are common edible flowers rich in bioactive secondary metabolites (BASMs) whose use in sophisticated gastronomy present currently as increasing trend. In this study the BASMs profile of these edible flowers was established using an emerging green extraction technique, μQuEChERS followed by ultra-high performance liquid chromatography coupled to a photodiode array detection system (UHPLC-PDA). After validation the μQuEChERS/UHPLC-PDA methodology allow to identify that apigenin and epigallocatechin gallate are the most abundant BASMs in mallow blue flowers, while catechin and dicaffeoylquinic acid are predominant in hibiscus flowers, and myricitrin and dicaffeoylquinic acid in nasturtium flowers. Total polyphenol content is the highest in the extract of hibiscus. Nasturtium shows the greatest radical scavenging activity. The results revealed that these flowers constitute a potential source of BASMs with different bioactive properties suggesting its use in design of new functional foods.

Keywords: BASMs; edible flowers; antioxidant activity; μQuEChERS; UHPLC-PDA

1. Introduction

Since ancient times, edible flowers have been part of the human diet. However, in recent years they began to be used in sophisticated dishes, with high added economic value, and appealing to the consumers due to their vibrant colors, exotic aromas, flavors and aesthetic value, providing freshness and refinement to dishes. Besides to its added value in high gastronomy, many species of edible flowers represent an important segment to expand food market, due to their suitable sensory and nutritional characteristics, as well as presence of bioactive secondary metabolites (BASMs) beneficial to human health. They are the primary sources of essential unsaturated fatty acids form the omega-3 and omega-6 families (Mikołajczak, Sobiechowska, & Tańska, 2020), present high concentration of phenolic compounds, such as flavonols, flavones, anthocyanins, phenolic acids, and flavanols, which express a high antioxidant activity (Zheng, Yu, Maninder, & Xu, 2018), carotenoids (mainly β-carotene, lycopene, lutein, and zeaxanthin) and tocopherols (α homolog dominated) (Fernandes, Ramalhosa, Pereira, Saraiva, & Casal, 2018) related to numerous health benefits such as anti-carcinogenic, antiproliferative, anti-inflammatory, anti-atherogenic, anti-ulcer, anti-thrombotic, immune modulating, anti-microbial, vasodilatory and analgesic effects (Chojnacka and Lewandowska, 2018; Wang et al., 2018; Barron et al., 2014; Piao et al., 2006; Sharma et al., 2017; Carmo et al., 2018; Joseph et al., 2016; Upadhyay and Dixit, 2015; Nayik and Nanda, 2016; Wang et al., 2006). In addition, BASMs are strongly related to the prevention or risk reduction of developing future chronic diseases such as cardiovascular diseases, diabetes, neurodegenerative diseases, and osteoporosis, as well as different types of cancer through the inhibition of their initiation and progression by modulating genes involved in key regulation processes (Scalbert et al., 2005; Fontana et al., 2016; del Río et al., 2013; Liu, 2013; Nicod et al., 2014; Anantharaju et al., 2016).

Several horticultural plants have been investigated as a source of bioactive compounds (Barros et al., 2020; Chen et al., 2015; Skrajda-Brdak et al., 2020). The interest in edible flowers has increased because in recent years a new trend has emerged in haute cuisine restaurants that has been received with

great acceptance by consumers and professional Chefs (Kelley et al., 2001a; Kelley et al., 2002). It is about the use of edible flowers as one more ingredient of the dish. The flowers are used in a wide range of culinary preparations with the aim of enhancing the sensory and nutritional qualities of dishes, providing color, aroma and flavor and a good visual appearance. Usually, these edible flowers are used to garnish gourmet dishes, and can be used in sauces, jellies, syrups, honey, oils, ice cubes, crystallized flowers, salads, soups or creams, desserts, teas and even in cocktails and other beverages (Barros et al., 2020; Koike et al., 2015a; Koike et al., 2015b; Kelley et al., 2001b). Although there are some works focused on the characterization of edible flowers, from the point of view of their nutritional value (e.g. phytochemicals, phenolic compounds), antioxidant properties, health benefits, and even their associated toxicology (Skrajda-Brdak et al., 2020; Fernandes et al., 2017; Lu et al., 2016; Pires et al., 2019), there are few publications related to edible flowers present and consumed on Madeira Island, such as mallow blue (*Malva sylvestris* L.), hibiscus *(Hibiscus rosa-sinensis* L.) and nasturtium (*Tropaeolum majus* L.), with the purpose of characterize them in terms of phenolic compounds and antioxidant activity (Barros et al., 2010; Salib, 2014; Garzón et al., 2015; Navarro-González et al., 2015). Barros et al. (2010) reported a comparative study of the composition in nutraceuticals (e.g., phenolics, flavonoids) and antioxidant properties of different parts of *Malva sylvestris* (leaves, flowers, immature fruits, and leafy flowered stems), while Navarro-González et al. (2015) established the phenolic profile of *Tropaeolum majus* in order to assess the relationship between the presence of phenolic compounds and the antioxidant capacity. The phenolic profile in edible petals of nasturtium flowers (*Tropaeolum majus*) of three colors was carried out by Garzón et al. (2015) using HPLC-MS/MS being identified several phenolic compounds including hydroxycinnamic acids, flavonoids (myricetin, quercetin, and kaempferol derivatives) and anthocyanins. On the other hand, the potential of *Hibiscus sabdariffa* to protect high-glucose-treated vascular smooth muscle cell was assessed by Huang et al. (2009). Therefore, the analysis of BASMs in food samples remains an analytical challenge, since it is not a simple and easy task either. To achieve the success of the analytical process, with efficient and

environmentally friendly analyses, it is crucial to apply an appropriate sample pretreatment method (Casado et al., 2018; Perestrelo et al., 2019). In this sense, the QuEChERS (quick, easy, cheap, effective, rugged and safe) strategy is considered a good extraction and purification technique that can be applied to various types of food samples. In addition, the QuEChERS procedure can be considered a sustainable analytical strategy, since it can be modified and miniaturized using smaller amounts of sample, organic solvents, cleaning sorbents and partition salts, thus complying with green analytical chemistry principles (Izcara et al., 2020). Therefore, the goal of this study was to identify BASMs responsible for edible flowers biological properties using an improved methodology based on the miniaturized QuEChERS (μQuEChERS) technique combined with ultra-high performance liquid chromatography equipped with a photodiode array detection system (UHPLC-PDA). In addition, the antioxidant activity, total phenolic and flavonoid content were also determined and compared among edible flowers considered in the current study. To the best of our knowledge, this is the first time that this procedure has been evaluated and applied for the analysis of BASMs in edible flowers.

2. Materials and methods

2.1. Chemicals, reagents and standard solutions

All chemicals and reagents were of analytical quality grade. HPLC grade acetonitrile (ACN), methanol (MeOH), ethyl acetate (EtAc) and formic acid (FA) were obtained from Fischer Scientific (Loughborough, UK). Ultrapure water (18 MΩ cm) was obtained from a Milli-Q water purification system (Millipore, Milford, MA, USA) and was used for preparing the mobile phase and other aqueous solutions. Anhydrous magnesium sulphate ($MgSO_4$), sodium citrate tribasic dihydrate, sodium citrate dibasic sesquihydrate and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{*}$, 98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Aluminium chloride ($AlCl_3$) and potassium chloride (KCl, > 99%) were acquired from Riedel-de Haën® (Seelze, Germany). Potassium persulfate ($K_2S_2O_8$, 99%) and sodium nitrite ($NaNO_2$) were purchased from Merck® (Buchs,

114 Switzerland), whilst sodium chloride (NaCl), disodium phosphate dihydrate ($Na_2HPO_4.2H_2O$, 99%),
115 potassium dihydrogen phosphate (KH_2PO_4, 99%) and anhydrous sodium carbonate were acquired from
116 Panreac (Barcelona, Spain). Sodium hydroxide (NaOH, 98%) and sodium carbonate (Na_2CO_3, > 99%)
117 were obtained from Eka Chemicals AB (Amsterdam, The Netherlands) and Labsolve® (Lisboa,
118 Portugal), respectively. Folin-Ciocalteu reagent (FR, 2 N), 2,2-diphenyl-1-picrylhydrazyl (DPPH•) and
119 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox, 98%) were from Fluka (Buchs,
120 Switzerland). Sorbents (50 μm particle size) for dSPE, including primary-secondary amine (PSA), and
121 anhydrous $MgSO_4$, were obtained from Waters (Milford, MA, USA).
122 Gallic acid monohydrate (98%), ferulic acid (98%), *p*-coumaric acid (99%), *o*-coumaric acid (≥
123 97%), cinnamic acid (≥ 99%), sinapic acid (≥ 98%) and 4-hydroxybenzoic acid (≥ 99%) were purchased
124 from Fluka (Buchs, Switzerland). Kaempferol (≥ 97%), catechin (≥ 99%), epicatechin (≥ 95%) and
125 protocatechuic acid (98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA), whereas
126 quercetin (99%) was from Acros Organics (Geel, Belgium).
127
128 *2.2. Preparation of standard solutions*
129 Individual stock standard solutions (1000 μg/mL) were prepared in MeOH and stored at -20 °C in
130 darkness conditions. From the individual solutions, a multicomponent standard solution containing all
131 the 12 analytes at 100 μg/mL (each of them) was prepared in MeOH. This multicomponent solution was
132 used to achieve working standard solutions at different concentration levels (prepared daily) by
133 appropriate dilution with MeOH to carry out the analytical performance of the method. All the standard
134 solutions were stored at -20 ºC.
135
136 *2.3. Edible flowers*
137 Hibiscus (*Hibiscus rosa-sinensis* L.) and nasturtium (*Tropaeolum majus* L.) were fresh collected
138 from different geographical origins in the Madeira Island (Ribeira Da Janela, located in the GPS

coordinates: 32° 50′ 52.55″ N and 17° 9′ 9.85″ W, and Funchal, located in the GPS coordinates: 32° 38′ 30.05″ N and 16° 55′ 29.51″ W), while mallow blue (Malva sylvestris L.) was grown by a local farmer and was purchased from a local market in Funchal (Madeira Island, Portugal). Before extraction, the edible flowers were freeze-dried, milled to a fine powder with a grinder (A11 Basic analytical mill, IKA, Staufen, Germany) and stored at room temperature until their analysis.

2.4. µQuEChERS procedure

The µQuEChERS procedure, a miniaturization of the original QuEChERS (Anastassiades et al., 2003) was adapted from the methodology reported by Casado et al. (2018): 0.5 g of sample were directly weighted into a 2 mL centrifuge tube with screw cap. Then 0.4 g of the partitioning salts mixture (anhydrous $MgSO_4$, NaCl, sodium citrate tribasic dihydrate and sodium citrate dibasic sesquihydrate keeping the original QuEChERS proportion 4:1:1:0.5) and 2 mL of ACN:EtAc (1:1, v/v) containing 0.1% FA were added. The tube was vortexed for 15 s, followed by ultrasound agitation for 5 min and centrifuged 5 min at 5000 rpm. An aliquot (1.4 mL) from the upper part of the extract was transferred into a 2 mL PTFE dSPE clean-up tube containing 150 mg of $MgSO_4$ and 25 mg of PSA. This mixture was vortexed for 30 s and centrifuged at 4000 rpm for 5 min and the supernatant (1 mL) of the purified extract was filtered through a 0.22 µm PTFE filter membrane and evaporated under a gentle nitrogen stream to dryness. Finally, the residue was reconstituted in 100 µL of MeOH for subsequent analysis on the UHPLC-PDA system. The schematic µQuEChERS procedure is shown in Figure 1 and each edible flower was analyzed in triplicate.

2.5. Evaluation of bioactive secondary metabolites and antioxidant activity in µQuEChERS extracts by spectroscopic assays

2.5.1. Total phenolic content (TPC)

164 The TPC of edible flowers was determined by the widely used Folin-Ciocalteu's colorimetric method
165 (Singleton et al., 1999) with some modifications. Briefly, the μQuEChERS extracts were diluted in water
166 up to 3 mL final volume and were mixed with 300 μL of Folin-Ciocalteu reagent, 1200 μL of 20 % (w/v)
167 Na_2CO_3, and 1500 μL of distilled water. The resulting mixture was vortexed for 1 min and incubated for
168 30 min in the dark at room temperature. After that, the absorbance was measured by a UV-Vis
169 spectrophotometer (Perkin Elmer Lambda 25, ILC-Instrumentos de Laboratório e Científicos, Lda.,
170 Portugal) at 765 nm. A calibration curve obtained with gallic acid (from 0.5 to 15 mg/L) was used to
171 determine the TPC in the extracts, and the results were expressed as mg of gallic acid equivalents (GAE)
172 per 100 g of dry sample. The TPC assays were performed in triplicate.
173
174 *2.5.2. Total flavonoid content (TFC)*
175 The TFC of edible flower sample extracts was determined using the aluminum chloride colorimetric
176 assay described by (Figueira, Porto-Figueira, Pereira, & Câmara, 2020), with slightly modifications.
177 Briefly, the extracts were diluted in methanol (70 %) up to 3 mL final volume and 120 μL of 5 % $NaNO_2$
178 were added. The mixture was kept in darkness during 5 min, and then 120 μL of 10 % $AlCl_3$ were added
179 and rested another 5 min in the dark before the addition of 800 μL of NaOH (1 M), which was followed
180 by 2 min rest in the dark. Finally, added 960 μL of methanol (70 %) and the acid-stable complexes
181 formed by the $AlCl_3$ with flavones and flavanols were measured at 510 nm. In order to determine the
182 TFC of the sample extracts, a calibration curve of gallic acid (from 5 to 600 mg/L) was properly
183 prepared. The results were expressed as mg of GAE per 100 g of dry sample. The TFC assays were
184 carried out in triplicate for all edible flowers.
185
186 *2.5.3. DPPH scavenging capacity*
187 The DPPH (2,2-diphenyl-1-picrylhydrazyl) method (Woraratphoka et al., 2007) was performed to
188 determine free radical scavenging activity (RSA) of the sample extracts. The stock DPPH solution was

prepared by dissolving 24 mg of DPPH in 100 mL of MeOH. The working DPPH solution was then obtained by diluting the stock solution in a 1:6 proportion in methanol. 100 µL of the diluted extracts were added to the reaction tube, followed by 3.9 mL of the DPPH working solution. The free radical reduction was measured at 515 nm after an incubation time of 45 min in the dark at room temperature. The free radical scavenging capacity ($A_{AR(DPPH)}$) against DPPH was calculated as follow: $\ln(\%\Delta A_{515}) = 0.7631 \times \ln(A_{AR(DPPH)}) - 0.0765$, where $\%\Delta A_{515} = [(A_{515(0)} - A_{515(45)})/ A_{515(0)}] \times 100$, $A_{515(0)}$ is the absorbance value measured at the beginning of the reaction and $A_{515(45)}$ is the absorbance value measured after 45 min of reaction. A calibration curve was obtained using a Trolox standard solution at different concentrations (from 5 to 400 mg/L). The results were expressed as mg of Trolox equivalents (TE) per 100 g of dry flower. The DPPH scavenging capacity assays were carried out in triplicate.

2.5.4. ABTS assay

The *in vitro* antioxidant activity was also measured based on the ABTS radical cation decolorization assay. The ABTS method performed in this work was adapted from the procedure reported by Paixão et al. (2007). Briefly, a stock solution of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS*) (20 mM) was prepared in 50 mL of phosphate-buffered saline (PBS, pH 7.4) and added 200 µL of potassium persulfate solution (70 mM) to the ABTS* solution. The mixture was stored in darkness at room temperature for 16 h to form stable radical cation. The ABTS* solution was diluted with PBS in a proportion of 1:6. Then, 12 µL of the diluted sample solution were added to 3 mL of the diluted ABTS* solution. The absorbance was measured at 734 nm after20 min in the dark at room temperature. The decolorization of mixed solution indicates that antioxidant compounds in the extract quenched ABTS radical cations so, there is a quantitative relationship between the reduction of absorbance at 734 nm and the concentration of antioxidants present in the sample. The free radical scavenging capacity against ABTS ($A_{AR(ABTS)}$) was calculated using the following formula: $I = 0.0448 \times A_{AR(ABTS)} + 0.7094$, and $I = [(A_B - A_A)/ A_B] \times 100$, with I being the percentage of inhibition of ABTS*,

where A_B is the absorbance of a blank sample (t = 0 min) and A_A the absorbance after 20 min of adding the sample extracts. A standard curve (10-600 mg/L) was performed by plotting the concentrations of Trolox against the percentage of inhibition of ABTS* (*I*). The value of antioxidant capacity of each sample can be calculated out as TEAC (Trolox equivalent antioxidant capacity). The results were expressed as mg of TE per 100 g of dry flower, and presented as average value of three replicates.

2.6. Analysis of bioactive secondary metabolites in edible flowers by UHPLC-PDA analysis

An UHPLC system (Waters Ultra-High Performance Liquid Chromatographic Acquity H-Class system) (Milford, MA, USA) equipped with a Waters Acquity quaternary solvent manager (QSM), a column heater, a Waters Acquity sample manager (SM), a 2996 PDA detector and a degassing system was used for the chromatographic analysis of BASMs. The chromatographic separation was achieved using an Acquity HSS T3 analytical column packed with a trifunctional C18 alkyl phase (2.1 mm x 100 mm, 1.8 µm particle size, Waters, Milford, MA, USA) at 40 ºC and a gradient elution, according to Aguiar et al. (2020) and Casado et al. (2018). The mobile phase included water containing 0.1% FA (solvent A) and ACN (solvent B). The gradient conditions were: 80% A (0 min), 60% A (3 min), 55% A (6 min), 30% A (8–10 min), and 80% A (12–14 min), followed by a re-equilibration of 2 min to initial conditions prior to next injection, yielding a total analysis time of 16 min. The flow rate was 0.250 mL/min, the injection volume was 2 µL and samples were kept at 20 ºC during the analysis. The UV detection wavelength was set to the maximum of absorbance for the target analytes (Table 1) and the Empower 2 software (Milford, MA, USA) was used for chromatographic data gathering and integration of chromatograms. The identification of BASMs was based on the comparison of the retention times (Table 1) and UV spectrum obtained for each sample with pure standards using the same instrumental conditions.

2.7. µQuEChERS/UHPLC-PDA method validation

The proposed μQuEChERS/UHPLC-PDA methodology for the identification and quantification of BASMs in edible flowers was properly validated in terms of selectivity, linearity, detection and quantification limits, intra-day and inter-day precision and trueness (expressed as recovery %) (Table 1).

The selectivity of the method was assessed by checking the PDA spectra, retention time and purity of the peaks obtained for the target BASMs from the edible flowers analyzed, comparing with standards when available. Linearity was assessed by constructing a calibration curve for each analyte with six calibration points (n = 6) with concentrations ranging from 0.1 to 120 mg/L. Furthermore, Mandel's test was investigated to complement linearity of the method. The concentration ranges were selected according to the sensitivity of the UHPLC-PDA system towards each target analyte and the range of BASM concentrations commonly found in edible flowers. Calibration curves were obtained by plotting the average peak area of each analyte against the analyte concentration in standards prepared and analyzed under the proposed μQuEChERS procedure and were fitted by linear least-square regression. The limits of detection (LOD) and quantification (LOQ) were calculated for each phenolic compound considering the concentration that produced a signal-to noise ratio (S/N) equal or higher than 3 and 10, respectively, using the lowest concentration of the calibration curve. The trueness (extraction efficiency) of the method, expressed as recovery percentage (%), was assessed by spiking the edible flowers in triplicate at three concentration levels, low (LL), medium (ML) and high (HL), corresponding to linear range of each BASM and subjecting them to the μQuEChERS procedure. The recovery values were determined by comparing the areas obtained for spiked samples with those obtained for simulated samples (samples spiked at the same concentration levels but at the end of the extraction process, prior to their chromatographic analysis). Regarding the precision of the method, expressed as relative standard deviation (RSD %), was evaluated in terms of intra-day (repeatability) and inter-day (reproducibility) using the same fortification levels used in the trueness assays. Six replicates (n = 6) of the whole procedure for each sample were performed on the same day, by the same analyst to obtain intra-day

264 precision. For inter-day precision, six replicates of each level were analyzed daily through three different
265 days (n = 18). Each assay was analyzed in triplicate.
266
267 *2.8. Statistical analysis*
268 The statistical analysis of the samples was performed using the MetaboAnalyst 5.0 web-based tool
269 (Pang et al., 2021). The data obtained were normalized (data transformation by cubic root and data
270 scaling by autoscaling) and subjected to one-way analysis of variance (ANOVA) followed by Fisher's
271 test for post-hoc multiple comparisons of means from three edible flowers varieties data at p-value <
272 0.001 to identify significant differences. Besides, principal component analysis (PCA) and partial least
273 squares-discriminant analysis (PLS-DA) were used to provide insights into the separations among the
274 edible flowers under study and to detect the BASMs that may indicate differences among the samples
275 sets. Hierarchical cluster analysis (HCA) was performed using the BASMs identified in edible flowers
276 and was generated through Ward's algorithm and Euclidean distance analysis, with the aim of identify
277 clustering patterns that can help in the characterization of the edible flowers analyzed.
278
279 **3. Results and discussion**
280 *3.1. Total phenolic content and antioxidant activity of edible flowers*
281 Edible flowers have a wide range of phytochemical compounds, among which BASMs stand out for
282 their high antioxidant and free radical scavenging activities. In this context, the TPC, TFC and
283 antioxidant activity of the investigated edible flowers were determined, and results are presented in Table
284 2. The TPC found in edible flowers was in the range between 8.6 mg GAE/100 g dry weight (DW) for
285 nasturtium flower to 12.7 mg GAE/100 g DW for hibiscus flower. There was no statistically significant
286 difference ($p \leq 0.05$) between the TPC values obtained for mallow blue and nasturtium samples. These
287 results were correlated with flowers color and therefore the difference in TPC values may be associated
288 with the supposed higher anthocyanin content of hibiscus flowers (reddish coloration) compared to

mallow blue (purple-blue coloration) and nasturtium (yellow-orange coloration) flowers. It was found that nasturtium flowers had the highest TFC values (5616 mg GAE/100 g DW), following by hibiscus and mallow blue flowers with very similar TFC values, 1750 and 1475 mg GAE/100 g DW, respectively. A higher TFC was observed in relation to the TPC in the three edible flowers analyzed. This may be since flavonoid levels can increase during the growth process in the generative organs of plants, such as flowers. These results agree with the obtained by Maina et al. (2021). However, comparing our results with those reported in the literature, it was observed that hibiscus and nasturtium flowers investigated in this work presented a lower TPC and TFC than those obtained by Mak et al. (2013) and Garzón and Wrolstad (2009). Several factors can influence with the observed differences in TPC and TFC, such as agronomic and environmental conditions, geographical region and harvest time.

The antioxidant activity of edible flowers was evaluated by DPPH and ABTS assays (Table 2). The obtained DPPH values ranged from 133 to 259 mg TE/100 g DW, being the lowest value for mallow blue flowers and the highest for nasturtium flowers, respectively. In the case of ABTS results, these varied from 455 mg TE/100 g DW for hibiscus flowers to 801 mg TE/100 g DW for nasturtium flowers. Therefore, in both antioxidant activity assays, nasturtium flowers showed significantly higher values than those obtained for mallow blue and hibiscus flowers (Table 2, $p \leq 0.05$). It was also observed that the antioxidant activity provided by the nasturtium flowers investigated in this work was higher than that reported by Garzón and Wrolstad (2009). In addition, the antioxidant activity showed a good correlation to TFC values, since malva and hibiscus flowers had very similar antioxidant activity values in both assays, as in TFC, while nasturtium flowers reached higher antioxidant activity values in agreement with higher TFC value. In fact, mallow blue and hibiscus flowers presented very similar values in terms of TFC and antioxidant activity, while the results obtained for nasturtium flowers showed statistical significance, being higher. Therefore, good correlation between TFC values and antioxidant activity for nasturtium flowers could indicate that the higher antioxidant activity associated to nasturtium flowers is due to its high flavonoid content.

314

315 *3.2. Analysis of bioactive secondary metabolites in edible flowers*

316 *3.2.1. Validation of µQuEChERS/UHPLC-PDA methodology*

317 The performance of the proposed µQuEChERS/UHPLC-PDA method was validated in terms of
318 selectivity, linearity, LOD, LOQ, trueness, intra- and inter-day precision to demonstrate its feasibility
319 and practicability for quantification of BASMs in edible flowers. Good analytical performance of the
320 method was achieved, and the validation parameters are shown in Table 1. The selectivity was assessed
321 by checking the PDA spectra, retention time and the purity of the peaks observed for the target BASMs
322 from the edible flowers analyzed. The least-squares linear regression analysis of the data provided
323 excellent linearity with correlation coefficients (R^2) values higher than 0.991 in the concentration range
324 studied (Table 1). Nevertheless, the R^2 value is unsuitable as a test model fit. Consequently, it was
325 required to assess the fit of the model using an alternative method, namely, by measuring the
326 homoscedasticity of data through the F-test and the residual plot. In the F-test, the data are uniformly
327 distributed when the tabulated F-value ($F_{tab,}$ 10.13) is higher than the experimental F-value (F_{exp}) ($F_{tab} >$
328 F_{exp}). Analyzing the data reported in Table 1, it was obvious that the data were homoscedastic, since the
329 condition of homoscedasticity ($F_{tab} > F_{exp}$) was verified for all BASMs. From the analysis of the residual
330 graphs, the residues were randomly distributed around the axis of concentrations. In general, the obtained
331 LODs values are low for all analytes, ranging from 0.001 mg/L for kaempferol to 0.220 mg/L for
332 catechin, while LOQs ranged between 0.003 and 0.735 mg/L for both BASMs, respectively (Table 1).
333 Therefore, the sensitivity of the method is adequate for the determination of low levels of BASMs in
334 edible flowers.

335 Trueness and precision were evaluated at three fortification levels (low (LL), medium (ML) and high
336 (HL) within the linear range of each BASM), and the results are shown in Table 1. Recovery values
337 range from 76% (quercetin) to 118% (kaempferol), and the precision for all BASMs showed RSD values
338 lower than 10% (Table 1). Therefore, these results suggest that this procedure is a good microextraction

339 and purification procedure for BASMs in edible flowers. Indeed, to the best of our knowledge, the
340 determination of BASMs in edible flowers under μQuEChERS conditions is the first time it has been
341 carried out.
342
343 *3.2.2. Quantitative determination of BASMs of edible flowers*
344 The BASMs profile of studied edible flowers were established and the results are shown in Table 3.
345 The quantification of the validated analytes (Table 1), clearly identified by their PDA spectrum and
346 retention time, was performed by direct interpolation of the area BASM in the corresponding calibration
347 curve. In addition, the tentative quantification of other BASMs found in the chromatogram recorded of
348 edible flower extracts, but not validated, was also carried out by interpolating their areas in the
349 calibration curves of those BASMs with the most similar chemical structure (Table 3). Contents below
350 the LOD were considered as not detected (n.d.), whereas contents between the LOD and the LOQ were
351 included as <LOQ. Figure 2 shows the chromatograms obtained for three edible flower analyzed extract
352 recorded at 280 nm showing slight differences in the qualitative and quantitative pattern. The statistical
353 significance for a specific analyte among edible flowers analyzed was registered in Table 3. Therefore,
354 and in a general way, we can say that the BASMs profile was significantly different among the edible
355 flowers analyzed in this work, in terms of presence and abundance of BASMs. A total of 15 BASMs
356 were quantified in the mallow blue flowers, while 14 BASMs were quantified in the hibiscus and
357 nasturtium flowers. Not all BASMs were detected in each edible flower analyzed, while cinnamic acid,
358 for example, was detected in nasturtium flowers only at concentrations below the LOQ so it
359 quantification was not performed. Apigenin, in all flowers, epigallocatechin gallate, in mallow blue
360 flowers, followed by catechin, in hibiscus sample, were the major constituents of all identified BASMs
361 in the target edible flowers investigated. Even though apigenin was identified in all investigated edible
362 flowers, only the mallow blue flower presented a high concentration. On the other hand, gallic, 4-
363 hydroxybenzoic, sinapic, *m*-coumaric and cinnamic acids were identified at low concentrations.

Quercetin, apigenin, apigenin glucoside, luteolin, *p*-coumaric, *m*-coumaric, *o*-coumaric and dicaffeoylquinic acids were detected and quantified in all the analyzed flowers, to a greater or lesser extent. The main BASMs quantified in mallow blue flowers were apigenin (2105 µg/100 g DW), epigallocatechin gallate (1769 µg/100 g DW), *trans*-resveratrol, ferulic acid, apigenin glucoside, and kurarinone. Apigenin and epigallocatechin gallate contributed significantly to the total BASMs content, while dicaffeoylquinic acid and quercetin were quantified in much fewer amounts. Barros et al. (2012) have already reported the presence of some BASMs in mallow blue flowers, although BASMs, such as epigallocatechin gallate, *trans*-resveratrol, ferulic acid, kurarinone, luteolin, dicaffeoylquinic, *p*-coumaric, chlorogenic, o-coumaric, protocatechuic, m-coumaric and cinnamic acids have been identified in mallow blue flowers for the first time in this work. In hibiscus flowers, the most dominant BASMs were catechin (1344 µg/100 g DW), dicaffeoylquinic acid (309 µg/100 g DW), epicatechin, luteolin and apigenin. Other analytes such as myricitrin and quercetin were also quantified at lower concentrations. Some BASMs such as catechin and quercetin had already been identified in other previous works in hibiscus flowers (Purushothaman et al., 2016; Huang et al., 2009), but to our knowledge, it is the first time that dicaffeoylquinic acid, epicatechin, luteolin, apigenin, myricitrin, apigenin glucoside and ferulic, o-coumaric, *m*-coumaric, *p*-coumaric, cinnamic and 4-hydroxybenzoic acids have been identified in this edible flower. Finally, nasturtium flower constitutes a rich source of myricitrin (236 µg/100 g DW), dicaffeoylquinic acid (208 µg/100 g DW) mainly, in addition to kaempferol, apigenin glucoside and luteolin, while chlorogenic acid, apigenin, quercetin, sinapic and gallic acids were also detected and quantified but in low concentrations. The nature and abundance of BASMs found in nasturtium flowers was very similar to that reported in the literature (Pires et al., 2019) highlighting the presence of hydroxycinnamic acids like dicaffeoilquinic and chlorogenic acids, and flavonols such as myricitrin, kaempferol or quercetin. These most prominent BASMs that have been identified in nasturtium flowers (caffeoylquinic acid, kaempferol and myricetin derivatives) are in agreement with those reported in the literature (Garzón et al., 2015; Navarro-González et al., 2015).

389 However, other BASMs, such as apigenin, apigenin glucoside, luteolin, and sinapic, gallic, *p*-coumaric,
390 *m*-coumaric, 4-hydroxybenzoic and *o*-coumaric acids that had not been analyzed so far until now in
391 nasturtium flowers, were also determined. In general, flavonoids were the family of BASMs quantified
392 in the large amount in the three edible flowers analyzed, standing out compounds such as apigenin,
393 epigallocatechin gallate, catechin, epicatechin, luteolin and quercetin. Regarding the composition of
394 phenolic acids found in the studied edible flowers, dicaffeoylquinic acid was undoubtedly the most
395 dominant, as well ferulic acid in mallow blue flowers.
396
397 *3.2.3. Bioactive potential of BASMs identified*
398 The phytochemical composition of edible flowers is very important due to the bioactive properties
399 (e.g., anticancer, cytotoxic, anti-diabetic, anti-obesity, anti-inflammatory, microbial) attributed to them
400 which contribute positively and in various ways to human health and the prevention of various types of
401 chronic and degenerative diseases (Kumari et al., 2021; Takahashi et al., 2020; Dias et al., 2019; Skrajda-
402 Brdak et al., 2020). The biological activities of BASMs found in the studied edible flowers have been
403 evaluated *in vitro* on pure enzymes, cultured cells, or isolated tissues (Manach et al., 2004). In this sense,
404 flavonols like myricitrin, kaempferol and quercetin quantified in the analyzed edible flowers were found
405 in numerous species of edible flowers (Kumari et al., 2021), and have anti-inflammatory, genotoxic and
406 antioxidant potential, as also effects against cardiovascular, Alzheimer's and Parkinson's diseases (Dias
407 et al., 2019). Flavones like as apigenin, apigenin glucoside and luteolin, Table 3, showed several
408 biological properties, such as anticancer, cytotoxic, hepatoprotective, antidiabetic, anti-inflammatory,
409 antiviral and anti-ageing properties (Dias et al., 2019). The main flavanols found in the studied edible
410 flowers were catechin, epicatechin, epicatechin gallate, and epigallocatechin gallate derivatives (Zheng,
411 Meenu, & Xu, 2019). Huang et al. (2009) reported that catechin and epigallocatechin gallate were the
412 main flavanols in the hibiscus flowers, which agrees the data obtained in this study (Table 3). A
413 significant concentration of epicatechin was also found in hibiscus flowers (Table 3). On the other hand,

mallow blue flowers presented a high concentration of epigallocatechin gallate, being the second most abundant BASMs and the main flavanol quantified in these flowers (Table 3). Ostrowska et al. (2004) reported that epigallocatechin gallate significantly reduced the lipid peroxidation markers and increased the glutathione peroxidase activity. In addition, epigallocatechin gallate reduce oxidation-induced neuronal necrotic-like cell death by around 40% and apoptosis by around 30%, thereby preventing neurodegeneration (Pogačnik et al., 2016). Catechin is strong antioxidant that can quench reactive oxygen species (ROS) such as super oxide radical, singlet oxygen, hydroxyl radical, peroxyl radical, nitric oxide, nitrogen dioxide, and peroxynitrite (Feng, 2006). Among the bioactive properties of flavanols, the most important are related to cardiovascular protection and anticancer agents (Dias et al., 2019; Vlachojannis et al., 2016; Teodor et al., 2020). Phenolic acids constitute a very important group of phytochemical compounds in edible flowers. Caffeoylquinic acids have been quantified in relevant amounts in hibiscus and nasturtium edible flowers, among others (Jabeur et al., 2017; Navarro-González at el., 2015). In fact, the data found in the literature agrees with the results obtained in our work, where dicaffeoylquinic acid was the phenolic acid quantified in all the samples and stood out in higher quantities compared to other phenolic acids. Caffeoylquinic acid derivatives from protects cells against hydrogen peroxide (H_2O_2) injury by reducing oxidative stress, increasing intracellular glutathione peroxidase, and superoxide dismutase levels, reducing the phosphorylation of MAPK signaling pathways (ERK1/2, JNK, and p38) and increasing phosphorylation of AKT (Jiang et al., 2017). Phenolic acids are present in almost all plants and therefore their presence in the diet is linked to their antioxidant effects and to the prevention of diseases related to oxidative stress such as cancer and cardiovascular and neurodegenerative diseases (Zheng, Meenu, & Xu, 2019; Butts-Wilmsmeyer et al., 2018; Kucekova et al., 2013). It is known that hibiscus and nasturtium flowers have anti-inflammatory and anti-obesity properties that they carry out through different mechanisms and thanks to the action of the BASMs present in their composition (Bayani et al., 2018; Kim et al., 2017). Similarly, the flavonoids found in hibiscus flowers have cytotoxic effects against some breast cancer cells (Kaulika and Febriansah, 2019;

439 Nguyen et al., 2019). Loizzo et al. (2016) have reported the antioxidant activity and hypoglycemic
440 effects provided by some flavonols present in mallow blue flowers. *Trans*-resveratrol has been also
441 identified in mallow blue flowers and clinical studies have demonstrated the beneficial effects on
442 neurological and cardiovascular disorders (Berman, Motechin, Wiesenfeld, & Holz, 2017). Therefore,
443 in this sense, the characterization of these edible flowers in terms of their composition and abundance in
444 phytochemical compounds is a very interesting challenge, which can help to understand the mechanisms
445 of action that cause certain health benefits and in the prevention of many diseases thanks to the
446 consumption of these edible flowers.
447
448 3.3. *Statistical analysis*
449 A statistical analysis of the data matrix, samples (n = 12) and variables (n = 22), was performed using
450 MetaboAnalyst 5.0 web-based tool (Pang et al., 2021), where PCA and PLS-DA were applied as
451 multivariate analysis. PCA is an unsupervised method that was performed to visualize the
452 difference/similarity among samples profile and that allows the determination of the significant variables
453 (BASMs) that contribute to the most for such differences / similarities. Figure 3a and b shows PCA score
454 plot and PLS-DA, respectively, from the investigated edible flowers. The PC1 and PC2 variances were
455 57.3 and 32.5%, respectively, representing 89.8% of the total BASMs variability of data, allowing a
456 good differentiation of the edible flowers. The nasturtium (*Tropaeolum majus* L.), projected in PC1 and
457 PC2 negative quadrants, was chiefly characterized by gallic acid (GA), kaempferol (K) and sinapic acid
458 (SA), whereas hibiscus flowers projected in PC1 negative and PC2 positive by catechin (C), epicatechin
459 (EC) and dicaffeoylquinic acid (DCA). Mallow blue flowers in PC1 positive and PC2 negative were
460 characterized by quercetin (Q), kurarinone (Ku), epigallocatechin gallate (EPGG), *trans*-resveratrol (T-
461 R) and protocatechuic acid (PA). PLS-DA was used as a supervised clustering method and in accordance
462 with PCA, their results also showed good discrimination among edible flowers (Figure 3b). A total
463 variance of 89.8 % was obtained by the first two principal components obtained from PLS-DA.

Moreover, HCA was carried out using all identified BASMs in edible flowers analyzed. The dendogram associated with heat map was performed by Euclidean distance through Ward´s clustering method (Figure 3c), providing intuitive visualization of the data set which, in complementarity with the statistical analysis carried out previously, also allows a better identification of the inherent clustering patterns between each edible flower.

4. Conclusions

In this work, the simultaneous determination of 12 BASMs in different samples of edible flowers, namely mallow blue (*Malva sylvestris* L.), hibiscus (*Hibiscus rosa-sinensis* L.) and nasturtium (*Tropaeolum majus* L.) flowers, was carried out. In addition, other 10 BASMs were tentatively identified and quantified in the mentioned samples was also carried out through a sensitive and improved analytical method based on the miniaturization of the QuEChERS extraction technique combined with UHPLC-PDA analysis was developed. This methodology allowed the reduction of the amounts of sample, organic solvents, clean-up sorbents and partitioning salts, leading to an improved cost-effective and environmentally friendly microextraction strategy, which meets the Green Analytical Chemistry principles. In addition, the little requirement for sample preparation and the adequate clean-up that facilitates the detection and quantification of the analytes, means that this method also reduces the time and cost of the analysis. Satisfactory figure of merit was achieved for selectivity, linearity, LOD, LOQ, trueness, intra- and inter-day precision were achieved, which demonstrate the feasibility and practicability of the method for quantification of BASMs in edible flowers, which in turn allows to characterize the abundance of the selected BASMs in these foodstuffs. In addition, the phytochemical composition and antioxidant activity of edible flowers was also evaluated. Therefore, this work represents a first step to determine, evaluate and characterize, in terms of abundance and content of BASMs, the nutritional quality of these edible flowers. Moreover, their consumption contributes to the intake of BASMs in the diet, being these edible flowers also proposed as a good source of bioactive

compounds with potential benefits on human healthy design of novel functional foods with improved benefits and application on cosmetic and pharmaceutical formulation.

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Figure Captions

Fig. 1 Schematic representation of the μQuEChERS/UHPLC-PDA analysis.

Fig. 2 Extracted chromatograms of the target BASMs at 280 nm in edible flowers analyzed by the μQuEChERS/UHPLC-PDA methodology. Peak number identification: 1 – kurarinone, 2 – catechin, 3 – epicatechin, 4 – Epigallocatechin gallate, 5 – p-coumaric, 6 – ferulic acid, 7 – sinapic acid, 8 – quercetin, 9 – apigenin, 10 – kaempferol, 11 – dicaffeoylquinic acid, 12 – luteolin, 13 – *trans*-resveratrol.

Fig. 3 Principal component analysis (a) and partial least squares discriminant analysis (b) score plots of the BASMs profile of edible flowers. Hierarchical cluster analysis (HCA) performed using the polyphenols identified in three edible flowers (mallow blue, hibiscus and nasturtium flowers). The dendrogram associated with heat map (c) was generated by Ward´s algorithm and Euclidean distance analysis. The abbreviations of BASMs were reported in Table 3.

Table 1. Retention time (RT), maximum absorbance (λ_{max}) and validation parameters of the μQuEChERS/UHPLC-PDA methodology for the determination of the target BASMs in edible flowers.

RT (min)	BASMs	λ_{max} (nm)	Linear range (mg/L)	Calibration curve		Trueness Recovery (% ± RSD)		%Bias	Precision (RSD %)		LOD[d] (mg/L)	LOQ[e] (mg/L)
									Intra-day	Inter-day		
1.501	Gallic acid	271	0.1 – 12.0	Equation	y = 13889× + 2994	LL[a]	106 ± 5	5.61	2.57	3.24	0.016	0.054
				R^2	0.995	ML[b]	101 ± 2	4.13	1.98	4.13		
				Adjusted R^2	0.994	HL[c]	106 ± 3	0.15	1.58	2.97		
				F value	8.02							
1.787	Protocatechuic acid	259	0.1 – 4.0	Equation	y = 82323× - 11832	LL	92 ± 3	1.2	2.41	9.74	0.003	0.01
				R^2	0.998	ML	99 ± 4	-4.17	1.78	5.76		
				Adjusted R^2	0.997	HL	95 ± 1	5.21	0.98	1.96		
				F value	6.63							
1.867	Catechin	278	2.0 – 60.0	Equation	y = 10983× - 7927	LL	91 ± 3	-5.92	6.72	4.57	0.22	0.735
				R^2	0.992	ML	101 ± 5	2.66	0.75	3.96		
				Adjusted R^2	0.992	HL	91 ± 2	0.10	0.25	4.97		
				F value	9.41							
2.116	Epicatechin	279	2.0 – 100.0	Equation	y = 15383× - 2045	LL	92 ± 4	-3.75	0.68	4.92	0.03	0.101
				R^2	0.997	ML	87 ± 3	-1.21	0.73	2.85		
				Adjusted R^2	0.997	HL	84 ± 6	3.64	0.34	1.97		
				F value	8.76							
2.323	4-hydroxybenzoic acid	255	0.1 – 4.0	Equation	y = 159489× - 6059	LL	113 ± 2	3.51	2.97	2.74	0.003	0.008
				R^2	0.999	ML	107 ± 4	-3.66	0.94	1.95		
				Adjusted R^2	0.999	HL	103 ± 2	0.8	1.56	1.97		
				F value	6.66							
3.3	*p*-coumaric acid	309	0.1 – 8.0	Equation	y = 243307× - 40839	LL	116 ± 1	7.69	1.89	2.85	0.005	0.017
				R^2	0.997	ML	108 ± 5	-1.98	0.87	1.59		
				Adjusted R^2	0.996	HL	117 ± 3	1.92	0.43	0.87		
				F value	8.91							
3.612	Ferulic acid	323	0.1 – 20.0	Equation	y = 57422× - 17914	LL	109 ± 2	3.03	3.95	3.78	0.023	0.078
				R^2	0.991	ML	102 ± 1	-2.59	1.62	2.75		
				Adjusted R^2	0.990	HL	86 ± 0	4.23	3.91	4.82		
				F value	7.91							
3.655	Sinapic acid	323	0.1 – 4.0	Equation	y = 128863× - 9539	LL	109 ± 2	5.63	0.91	3.76	0.002	0.007
				R^2	0.999	ML	93 ± 1	-2.52	1.85	2.68		
				Adjusted R^2	0.998	HL	86 ± 0	0.11	0.83	0.99		
				F value	3.10							

4.314	*o*-coumaric acid	276	0.1 – 4.0	Equation R^2 Adjusted R^2 F value	y = 135966× - 8072 0.997 0.997 5.69	LL ML HL	105 ± 4 102 ± 2 116 ± 3	1.04 -2.78 2.93	2.97 0.76 0.82	3.85 1.58 3.97	0.012	0.039
5.224	Quercetin	372	0.5 – 120.0	Equation R^2 Adjusted R^2 F value	y = 102428× - 606816 0.998 0.997 7.37	LL ML HL	111 ± 5 89 ± 2 76 ± 4	-3.20 -1.78 2.18	2.58 2.61 0.63	2.93 4.75 0.78	0.03	0.101
5.717	Cinnamic acid	277	0.1 – 4.0	Equation R^2 Adjusted R^2 F value	y = 208823× - 23903 0.996 0.995 4.98	LL ML HL	103 ± 9 106 ± 3 93 ± 1	1.04 2.10 -0.98	2.67 1.86 0.83	2.83 3.75 3.67	0.003	0.008
6.247	Kaempferol	366	0.1 – 12.0	Equation R^2 Adjusted R^2 F value	y = 87463× + 21798 0.999 0.998 7.90	LL ML HL	119 ± 4 112 ± 6 118 ± 1	3.14 5.24 -0.79	5.68 3.83 0.65	6.57 5.74 0.84	0.001	0.003

[a] LL: Low concentration level (mg/L) corresponding to linear range of each phenolic compound; [b] ML: Medium concentration level (mg/L) corresponding to linear range of each phenolic compound; [c] HL: High concentration level (mg/L) corresponding to linear range of each phenolic compound; [d] LOD: limit of detection; [e] LOQ: limit of quantification.

Table 2. Results obtained for total phenolic content (TPC), total flavonoid content (TFC) and radical scavenging activity (RSA) determined by DPPH and ABTS assays of edible flowers. Values expressed as mean ± SD per 100 gram dry weight (DW) (n = 3).

Sample	TPC	TFC	DPPH	ABTS
	mg GAE/100 g DW	mg GAE/100 g DW	mg TE/100 g DW	mg TE/100 g DW
Mallow blue	9.7 ± 0.8^{a}	1475 ± 94^{a}	133 ± 14^{a}	502 ± 98^{a}
Hibiscus	12.7 ± 0.7^{b}	1750 ± 206^{a}	145 ± 3^{a}	455 ± 72^{a}
Nasturtium	8.6 ± 1.0^{a}	5616 ± 585^{b}	259 ± 32^{b}	801 ± 27^{b}

GAE: Gallic acid equivalents; TE: Trolox equivalents

Different superscript letters in the same column indicate statistical significance ($p \leq 0.05$) among edible flower samples.

Table 3. Concentration of BASMs found in three edible flower samples analyzed by the μQuEChERS/UHPLC-PDA methodology.

RT (min)	Code	λ_{max} (nm)	BASMs	Concentration (μg/100 g DW)±SD		
				Mallow blue	*Hibiscus*	Nasturtium
1.112	Ku	288	Kurarinone[a,e]	155.2 ± 1.4	n.d.	n.d.
1.501	GA	271	Gallic acid	n.d.	n.d.	29.1 ± 1.6
1.787	PA	259	Protocatechuic acid	41.2 ± 1.0	n.d.	n.d.
1.867	C	278	Catechin	n.d.	1343.5 ± 26.3	n.d.
2.116	EC	279	Epicatechin	n.d.	241.4 ± 19.1	n.d.
2.323	4-HA	255	4-hydroxybenzoic acid	n.d.	2.7 ± 0.1[a]	5.8 ± 0.1[b]
2.413	CHA	326	Chlorogenic acid[b]	91.5 ± 2.1[b]	n.d.	53.0 ± 0.9[a]
2.654	EPGG	274	Epigallocatechin gallate[c]	1768.6 ± 31.9	n.d.	n.d.
3.108	M	261	Myricitrin[a,e]	n.d.	30.0 ± 0.1[a]	236.3 ± 1.4[b]
3.300	p-CA	309	p-coumaric acid	94.2 ± 0.3[c]	6.49 ± 0.02[a]	17.6 ± 0.2[b]
3.612	FA	323	Ferulic acid	256.7 ± 1.5[b]	15.4 ± 0.4[a]	n.d.
3.655	SA	323	Sinapic acid	n.d.	n.d.	29.6 ± 0.2
3.969	m-CA	278	m-coumaric acid[d]	18.6 ± 0.1[c]	7.2 ± 0.1[b]	5.9 ± 0.1[a]
4.314	o-CA	276	o-coumaric acid	48.2 ± 0.8[c]	12.3 ± 0.5[b]	3.0 ± 0.1[a]
5.224	Q	372	Quercetin	102.7 ± 0.7[c]	27.4 ± 0.4[b]	25.9 ± 0.1[a]
5.717	CA	277	Cinnamic acid	9.9 ± 0.1[b]	3.27 ± 0.04[a]	< LOQ
6.023	A	336	Apigenin[a]	2105.0 ± 7.7[c]	104.3 ± 0.5[b]	32.8 ± 0.1[a]
6.247	K	366	Kaempferol	n.d.	n.d.	172.5 ± 1.0
8.734	AG	336	Apigenin glucoside[a,e]	227.1 ± 12.7[c]	23.45 ± 0.04[a]	127.5 ± 4.4[b]
9.346	DCA	237	Dicaffeoylquinic acid[b,e]	136.4 ± 4.3[a]	309.4 ± 3.9[c]	208.0 ± 3.2[b]
10.225	L	356	Luteolin[a]	59.4 ± 2.3[a]	123.3 ± 5.9[c]	83.1 ± 0.6[b]
10.988	T-R	304	*Trans*-Resveratrol[a]	298.2 ± 11.3	n.d.	n.d.

Values were expressed as mean ± standard deviation of three replicates (n = 3). n.d.: not detected.

[a] Expressed in equivalents of quercetin; [b] Expressed in equivalents of ferulic acid; [c] Expressed in equivalents of epicatechin;

[d] Expressed in equivalents of p-coumaric; [e] BASMs tentatively identified.

Different superscript letters in the same row indicate significant differences ($p < 0.05$) among edible flower samples in each phenolic compound.

Fig. 1

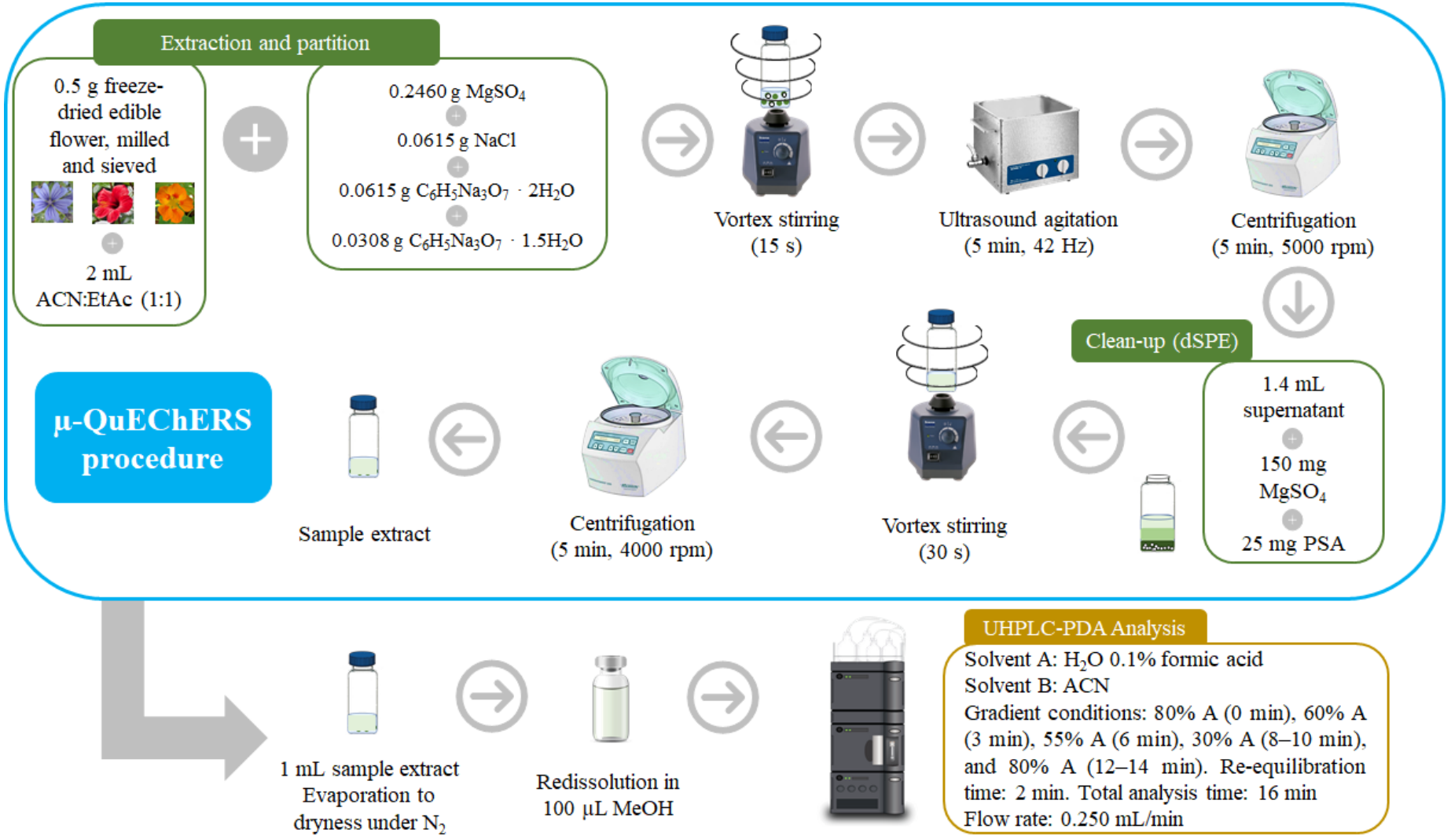
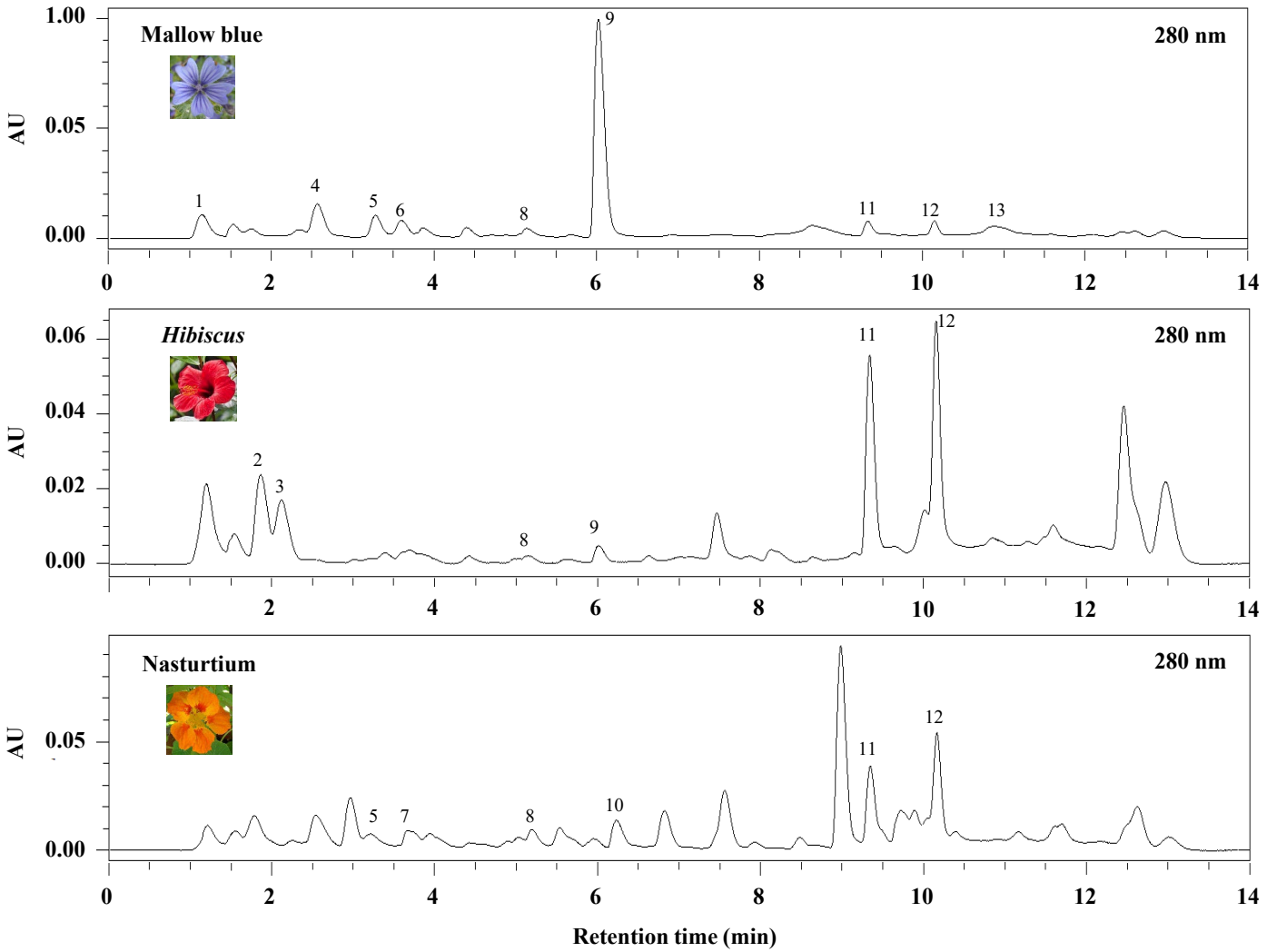

Extraction and partition
0.5 g freeze-dried edible flower, milled and sieved
2 mL ACN:EtAc (1:1)
0.2460 g MgSO4
0.0615 g NaCl
0.0615 g C6H5Na3O7 · 2H2O
0.0308 g C6H5Na3O7 · 1.5H2O
Vortex stirring (15 s)
Ultrasound agitation (5 min, 42 Hz)
Centrifugation (5 min, 5000 rpm)
Clean-up (dSPE)
1.4 mL supernatant
150 mg MgSO4
25 mg PSA
Vortex stirring (30 s)
Centrifugation (5 min, 4000 rpm)
Sample extract
µ-QuEChERS procedure
1 mL sample extract
Evaporation to dryness under N2
Redissolution in 100 µL MeOH
UHPLC-PDA Analysis
Solvent A: H2O 0.1% formic acid
Solvent B: ACN
Gradient conditions: 80% A (0 min), 60% A (3 min), 55% A (6 min), 30% A (8–10 min), and 80% A (12–14 min). Re-equilibration time: 2 min. Total analysis time: 16 min
Flow rate: 0.250 mL/min

Fig. 2


Mallow blue
280 nm
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14
Hibiscus
280 nm
AU
0.06
0.04
0.02
0.00
2
3
8
9
11
12
Nasturtium
280 nm
AU
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5
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8
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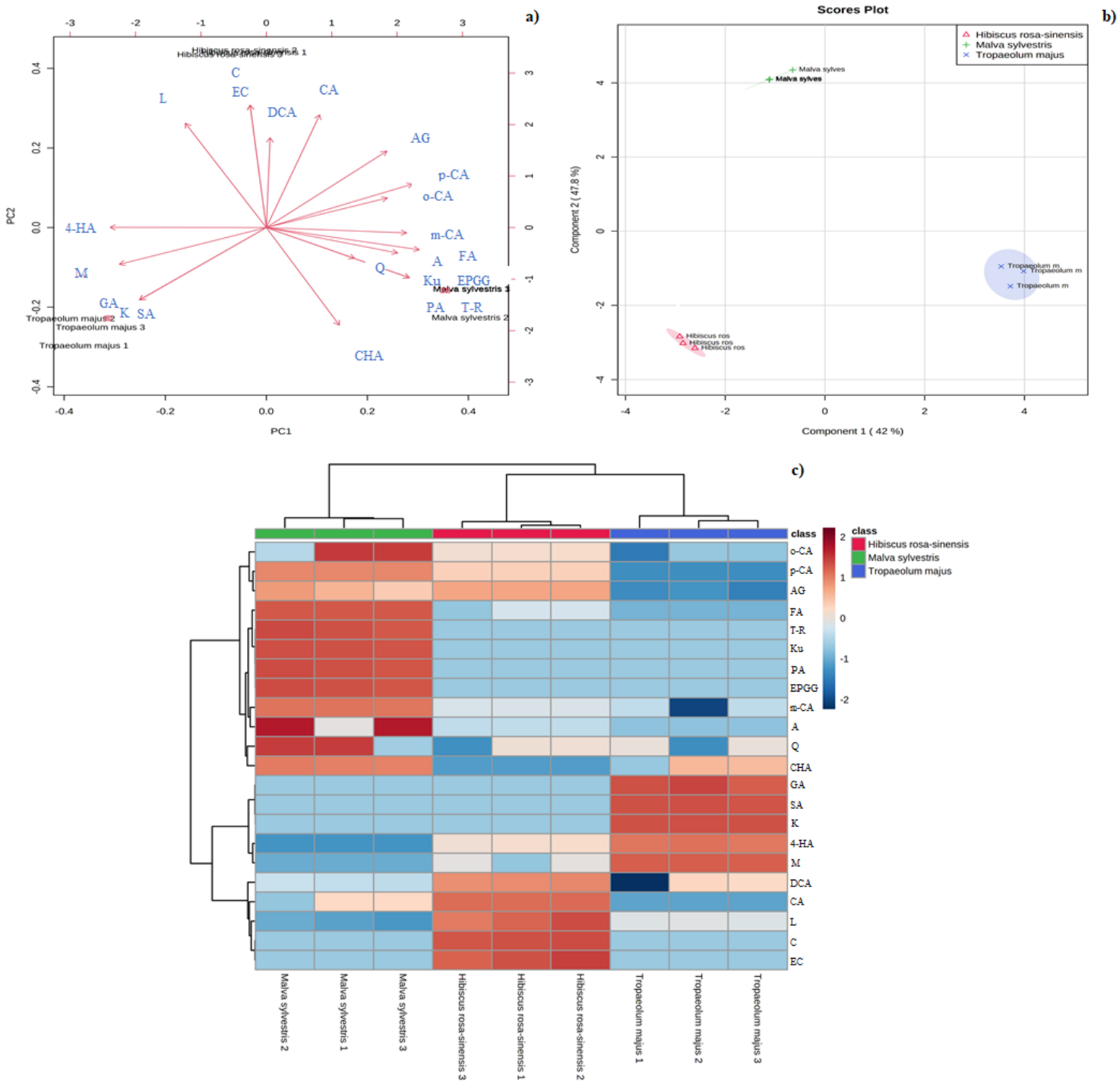
11
12
Retention time (min)

Fig. 3


a)
PC1
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EPGG
GA
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SA
PA
T-R
CHA
Tropaeolum majus 2
Tropaeolum majus 3
Tropaeolum majus 1
Malva sylvestris 1
Malva sylvestris 2
b)
Scores Plot
Hibiscus rosa-sinensis
Malva sylvestris
Tropaeolum majus
Component 1 (42 %)
Component 2 (47.8 %)
c)
class
Hibiscus rosa-sinensis
Malva sylvestris
Tropaeolum majus
o-CA
p-CA
AG
FA
T-R
Ku
PA
EPGG
m-CA
A
Q
CHA
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4-HA
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Malva sylvestris 2
Malva sylvestris 1
Malva sylvestris 3
Hibiscus rosa-sinensis 3
Hibiscus rosa-sinensis 1
Hibiscus rosa-sinensis 2
Tropaeolum majus 1
Tropaeolum majus 2
Tropaeolum majus 3