Integrated strategy for the extraction and profiling of bioactive metabolites from *Passiflora mollissima* seeds combining pressurized-liquid extraction and gas/liquid chromatography–high resolution mass spectrometry

Diego Ballesteros-Vivas^{1,2a}, Gerardo Alvarez-Rivera^{2a}, Elena Ibáñez², Fabián Parada-Alfonso¹,
Alejandro Cifuentes^{2*}

¹ High Pressure Laboratory, Department of Chemistry, Faculty of Science, Universidad Nacional de Colombia, Carrera 30 #45-03, Bogotá D.C., 111321, Colombia.

² Laboratory of Foodomics, Institute of Food Science Research, CIAL, CSIC, Nicolás Cabrera 9, 28049 Madrid, Spain.

^aThese two authors contributed equally to this work.

*Corresponding author:

Prof. Dr. Alejandro Cifuentes, Laboratory of Foodomics, Institute of Food Science Research, CIAL (CSIC), Nicolás Cabrera 9, 28049 Madrid, Spain, e-mail: a.cifuentes@csic.es, Tel.: +34 910017955; fax: +34 910017905.

Keywords:

Passiflora mollissima; fruit by-products; Pressurized-liquid extraction; PLE; LC-Q-TOF; GC-Q-TOF; High-resolution mass spectrometry; MS data-mining strategies; metabolites profiling

ABSTRACT

An integrated analytical methodology based on pressurized-liquid extraction (PLE) in two steps, followed by *in vitro* assays and liquid chromatography/gas chromatography coupled to high-resolution mass spectrometry (HRMS), was developed and applied for the isolation and characterization of potential bioactive metabolites from *Passiflora mollissima* seeds. PLE was studied considering two sequential steps: 1) recovery of the lipidic fraction using nonpolar solvents, and 2) recovery of the phenolic fraction from the defatted seeds' residue using polar solvents. Cyclohexane was selected as the most suitable solvent for the seeds defatting process (20 min, 100 °C and 100 bar). PLE optimization by response surface methodology was carried out to obtain phenolic-rich extracts with the highest antioxidant activity. Optimal extraction yield (6.58 %), total phenolic content (29.99 mg GAE g⁻¹), total flavonoids content (0.94 mg Quer g⁻¹) and antioxidant activity (6.94 mM trolox g⁻¹ and EC₅₀ of 2.66 µg mL⁻¹) were obtained operating at 150 °C and 100% EtOH v/v. Untargeted and semi-targeted MS and MS/MS data-mining strategies were successfully implemented for the rapid and comprehensive profiling of the polar and lipidic PLE fractions analysed by UHPLC and GC, respectively, coupled to quadrupole time-of-flight mass spectrometry (q-TOF-MS/MS). Polyphenolic-rich extracts from *P. mollissima* seeds were characterized for the first time applying this approach, showing a broad variety of flavonoids, genuine flavanols (e.g. (epi)fisetinidol) and abundant proanthocyanidins dimers and trimers. This application can be considered a successful demonstration of the great potential of the proposed methodology to effectively characterize complex natural extracts with potential bioactivity, by making use of powerful integrated identification strategies to facilitate the challenging post-acquisition data processing of huge datasets generated by HRMS analysis.

1. INTRODUCTION

In recent years, the isolation of potential bioactive compounds from food by-products has become an emerging trend that largely contributes to the valorization of food residues containing valuable health-promoting compounds. In this regard, *Passiflora mollissima* (Kunth) L. H. Bailey (banana passion fruit) is getting more relevance as an exotic fruit in the international market [1]. Banana passion fruit is usually consumed as fresh food (juices), or processed products (jams, desserts, nectars and compotes), being seeds and peel the main by-products of the industrial processing; these by-products constitute around 40 % of the total product weight [2, 3]. Nutritional value, aromatic and phytochemical profile, as well as functional potential have been studied in the pericarp [4, 5] and in the pulp [6-8] of *P. mollissima* fruit. Chemopreventive properties of banana passion fruit have been observed against adenomatous polyps formation in female rat with neoplastic lesions induced [9]. Although *P. mollissima* seeds have been evaluated as oil source and as potential ingredient in nanoemulsions for pharmaceutical and cosmetic purposes [10], to the best of our knowledge, its content on potentially bioactive metabolites has not been comprehensively explored yet.

Integrated analytical methodologies involving efficient extraction techniques with low environmental impact, in combination with high resolution analytical techniques are essential to isolate and characterize metabolites of interest in complex matrices, such as fruit processing by-products. In this sense, extractive strategies based on compressed fluids, such as Pressurized Liquid Extraction (PLE), meet these conditions [11, 12]. Recently, the advantages of PLE have been reviewed [13-16]. Additionally, PLE provides fractionated extractions in sequential steps by modifying the extraction temperature, pressure and/or the solvent composition, leading to extracts or fractions with different chemical profiles and bioactive potential. This approach meets the new trends of *process intensification* and *biorefinery*, in which the same equipment can be used in a multipurpose way in order to exploit a biomass to obtain fractions with different potential [17].

High resolution mass spectrometry (HRMS) is playing an essential role in the phytochemical profiling of natural extracts, as can be seen in recent applications [18, 19] and review works [20, 21]. In this context, q-TOF-MS/MS instruments are being adopted as powerful tools for untargeted analysis of complex extracts, due to their capability of providing accurate mass data together with the structural information from HR-MS/MS fragmentation [22, 23]. Ultra-high performance liquid chromatography (UHPLC) coupled to q-TOF has become increasingly popular for the characterization of complex biological samples, combining high separation efficiency with high structural elucidation capacity, generating thousands of MS features per sample. Although to a minor extent, the coupling of q-TOF systems with gas chromatography (GC) can also be considered a powerful tool and the first choice for identification and structure elucidation of unknown volatile and semivolatile organic compounds [24-26]. GC-q-TOF(MS) provides high sensitivity in full-scan MS mode together with high resolving power and accurate-mass measurements. In addition, the standardized fragmentation using EI source (70 eV) allows systematic search in well-established MS databases (e.g. NIST MS database, Fiehn Lib, Wiley, etc.), making this technique very attractive in qualitative analysis, particularly for unknown's analysis in natural extracts [23].

On the other hand, the huge amount of datasets generated by HRMS measurements, as the current q-TOF instruments do, creates a great challenge due to the need of processing thousands of MS features to extract useful information. In this regards, several data mining strategies have emerged to facilitate the post-acquisition data processing. Thus, several systematic approaches such as mass defect filtering (MDF), diagnostic fragment ion filtering (DPIF), background subtraction filtering (BSF), or neutral loss filtering (NLF), have been reported [27-29]. The implementation of these filtering strategies allows to systematically screen HRMS data looking for families of compounds. Information provided by product ions or neutral loss fragments allow the detection of compounds sharing similar core substructures or functional groups.

Previously, the authors developed a multi-analytical approach for the valorization of goldenberry calyx, a promising source of health-promoting compounds [30, 31]. In these previous works a targeted strategy was proposed first to obtain withanolides-rich extracts with high antioxidant activity, followed by an untargeted profiling analysis of the selected extracts. Based on the previous achievements we propose herein an integrated analytical methodology including a sequential PLE for the fractionated extraction of phenolic and lipidic metabolites, and incorporating valuable post-acquisition HRMS data-mining procedures to greatly enhance data interpretation. Thus, a PLE extracts in two steps were optimized and subsequently characterized by LC-q-TOF-MS/MS and GC-TOF-MS, allowing tentative identification of the major phenolic components, fatty acids and terpenoids present in, up to now, practically unexplored *P. mollissima* seeds. This analytical strategy is proposed as a step further in the challenging task of agrifood by-products valorization.

2. MATERIAL AND METHODS

2.1 Samples of banana passion fruit

Banana passion fruit was purchased in the central supply market (www.corabastos.com.co) in Bogotá D.C., Colombia in June, 2017. About 30 kg of banana passion fruit were processed by blanching and pulping the fruit in order to separate the seeds and arils from the peels. After washing and drying (60 °C for 12 h), the seeds were ground in knife mill (Grindomix GM200-Retsch GmbH, Haan, Germany) using dry ice and then vacuum-packed and stored at -20 °C until their use.

2.2 Reagents and materials

The phenolic standards gallic acid, quercetin, catechin, vanillic acid, and caffeic acid; the antioxidant assay reagents trolox, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH); as well as formic acid, ammonium acetate, potassium acetate, sodium carbonate, potassium persulfate, and aluminium chloride, were obtained from

Sigma-Aldrich (Madrid, Spain). Folin-Ciocalteu phenol reagent was acquired from Merck (Darmstadt, Germany). HPLC-grade solvents such as methanol, ethanol and acetonitrile were purchased from VWR Chemicals (Barcelona, Spain), ethyl acetate from Scharlau (Barcelona, Spain) and dichloromethane from Fluka AG (Buchs, Switzerland). Ultrapure water was obtained from a Millipore system (Billerica, MA, USA). MS grade acetonitrile and water from LabScan (Dublin, Ireland) were employed for UHPLC-q-TOF-MS.

2.3 Pressurized liquid extraction (PLE)

A Dionex accelerated solvent extractor (ASE 200, Sunnyvale, CA, USA) with 11 mL stainless steel extraction cell was used for PLE procedure in two steps. Dried samples of banana passion fruit seeds were mixed with sea sand in a proportion of 1:2 w/w and loaded into the extraction cell. The extractions were performed at constant pressure (100 bar) on static mode. The solvent was evaporated from the extract under N₂ gas until completely dry. Extraction yield was expressed as g of extract/100 g dry weight basis of sample (mean of two replicates).

2.3.1. PLE-first step evaluation

In the first step, PLE process was tuned to achieve the maximum defatting of banana passion fruit seeds. Different nonpolar solvents were studied at constant extraction pressure (100 bar) and temperature (100°C): n-heptane, n-cyclohexane, (+)-limonene and n-hexane. Kinetics of the defatting step was assessed considering extraction times from 10 to 60 min with sample collection every 10 minutes.

2.3.2. PLE-second step optimization

A central composite design (CCD) with response surface methodology (RSM) were employed in order to optimize the extraction of phenolic compounds from banana passion fruit seeds after the

defatting process. Two factors were considered at 3 levels: solvent composition (percentage of EtOH in the mixture EtOH/EtOAc: 0, 50 and 100 % v/v) and temperature (50, 100 and 150 °C). Response variables studied were extraction yield, TPC, TFC and antioxidant activity (EC₅₀ and TEAC). The quadratic model proposed for each response variable Y_i was:

$$Y_i = \beta_0 + \beta_1 SC + \beta_2 T + \beta_{1,2} T \times SC + \beta_{1,1} SC^2 + \beta_{2,2} T^2 + error \quad \text{Eq. (1)}$$

where SC is the solvent composition (percentage of ethanol in the mixture), T is the temperature, β_0 is the intercept, β_1 , β_2 are the linear coefficients, $\beta_{1,2}$ is the linear-by-linear interaction coefficient, $\beta_{1,1}$, $\beta_{2,2}$ are the quadratic coefficients, and $error$ is the error variable.

The software STATISTICA 12 (Stat Soft, Inc., Tulsa, OK 74104, USA) was used for experimental design, data analysis and multiple response optimization. The analysis of variance (ANOVA), standardized Pareto charts, coefficient of determination (R^2), p values for the model and lack-of-fit testing were performed [32].

2.4 Conventional extractions

Bligh & Dyer (B&D) method for total lipid determination and dynamic maceration (DM) were selected as conventional extraction techniques for comparison. B&D method was applied with some modifications [33, 34]. Briefly, to a ~0.5 g of banana passion fruit seed sample, 2.0 mL of MeOH and 1.0 mL of CHCl₃ were added and the mixture was vortexed for 2 min. Later, 1.0 mL of CHCl₃ were added a second time and the mixture was shaken vigorously for 2 min. Then, 1.8 mL of distilled water were added and the mixture was vortexed again for 2 min. The layers were separated by centrifugation for 10 min at 2000 rpm. The lower layer was transferred to a vial with a Pasteur pipette. A second extraction cycle was done with 2.0 mL of MeOH (10% v/v in CHCl₃) by vortexing for 2 min. After centrifugation, the chloroform phase was added to the first extract. Finally, the solvent was evaporated under a stream of nitrogen at 25 °C. On the other hand, DM was performed using EtOH, EtOAc and EtOH/EtOAc (50:50 v/v) as solvents. Samples of banana

passion fruit seeds (~1 g) were agitated at 750 rpm, 20 °C for 24 h (Thermomixer R Eppendorf AG, Hamburg, Germany). The extract was centrifuged and the solvent was eliminated by evaporation with nitrogen at 25 °C.

2.5 Determination of total phenolic content (TPC)

The TPC of the extracts was measured by Folin-Ciocalteu method [35]. An aliquot (10 µL) of extract solution was mixed with 600 µL of H₂O and 50 µL of Folin-Ciocalteu reagent (0.2 M). After mixing for 5 min, 150 µL of Na₂CO₃ (20% w/v) and 190 µL of H₂O were added. After 120 min of incubation at 25 °C, the absorbance was measured at 760 nm in a microplate spectrophotometer reader (Synergy HT, BioTek Instruments, Winooski, VT, USA). A calibration curve was constructed with gallic acid (0–100 µg gallic acid/mL EtOH), and the sample TPCs (mean of three replicates) were expressed as gallic acid equivalents (mg GAE/g) on dry extract weight basis (DB).

2.6 Determination of total flavonoid content (TFC)

In order to determine the TFC for the extracts, the method described by Hosu et al. [35] was followed with some modifications. In a microcentrifuge tube, 100 µL of extract solution, 30 µL of potassium acetate (1 M), 300 µL of EtOH absolute and 540 µL of H₂O were mixed. After 30 min of incubation at 25 °C, the absorbance was measured at 415 nm in a microplate spectrophotometer reader (Synergy HT, BioTek Instruments, Winooski, VT, USA). A calibration curve was constructed with quercetin (0–100 µg quercetin/mL EtOH), and the sample TFCs (mean of three replicates) were expressed as quercetin equivalents (mg QE/g) on dry extract weight basis (DB).

2.7 Antioxidant capacity assays

2.7.1 DPPH assay

Extract antiradical capacity by the DPPH radical scavenging method [36] was measured. An aliquot (25 µL) of the different concentrations of the extracts (0.3–5.0 mg mL⁻¹ MeOH) was added to 975

μL of DPPH solution (60 μM in MeOH). After 4 h of incubation the absorbance was measured at 516 nm in a microplate spectrophotometer reader (Synergy HT, BioTek Instruments, Winooski, VT, USA). A DPPH methanol solution was used as the reference sample. The effective concentration of the extracts to decrease the initial DPPH concentration by 50% (EC₅₀) was calculated and expressed as μg/mL of extract solution.

2.7.2 TEAC assay

TEAC assay was performed according to Pellegrini et al. [37]. The aqueous ABTS^{•+} solution was diluted with 5 mM phosphate buffer (pH 7.4) to an absorbance of 0.7 at 734 nm. The extracts were diluted with 5 mM phosphate buffer at concentrations between 0.3 and 5.0 mg mL⁻¹. An aliquot (10 μL) of the different concentrations of the extracts was added to 990 μL of ABTS^{•+} solution. Afterward, 300 μL of the mixture were transferred into a 96-well microplate and the absorbance was measured at 734 nm every 5 min for 45 min in a microplate spectrophotometer reader (Synergy HT, BioTek Instruments, Winooski, VT, USA). Trolox (0.25-2.0 mM) was used for calibration and results expressed as TEAC values (mM trolox g⁻¹ extract). All analyses were performed in triplicate.

2.8 Phytochemical profiling of banana passion fruit seeds extracts

2.8.1 Liquid chromatography-tandem mass spectrometry (UHPLC-q-TOF-MS/MS)

An Agilent 1290 UHPLC system coupled to an Agilent 6540 quadrupole-time-of-flight mass spectrometer (q-TOF MS) and equipped with an orthogonal ESI source was employed for the phytochemical profiling of banana passion fruit seeds extracts. Chromatographic separation was conducted using a Zorbax Eclipse Plus C18 column (2.1 × 100 mm, 1.8 μm particle diameter, Agilent Technologies, Santa Clara, CA) at 30 °C. The mobile phase was composed of water (0.01% formic acid, solvent A) and acetonitrile (0.01% formic acid, solvent B). A 5 μL aliquot of the sample

was injected at a flow rate of 0.5 mL/min during gradient elution. The gradient program was as follows: 0 min, 0% B; 7 min, 30% B; 9 min, 80% B; 11 min, 100% B; 13 min, 100% B; 14 min, 0% B. The mass spectrometer was operated in MS and MS/MS modes for the structural analysis of all compounds. MS parameters were the following: capillary voltage, 4000 V; nebulizer pressure, 40 psi; drying gas flow rate, 10 L/min; gas temperature, 350 °C; skimmer voltage, 45 V; fragmentor voltage, 110 V. The MS and Auto MS/MS modes were set to acquire m/z values ranging between 50-1100 and 50-800, respectively, at a scan rate of 5 spectra per second.

2.8.2 Gas chromatography-mass spectrometry (GC-q-TOF-MS)

Oil composition corresponding to the first step-PLE was studied using GC-q-TOF-MS after derivatization according to Fiehn method, with some variations [38]. The derivatization process was performed by methoxyamination and silylation reactions. For this purpose, 10 µL of methoxyamine hydrochloride solution in pyridine (40 mg/mL) were added to 5 µL of oil sample and the mixture was vortexed for 1 min and subsequently shaken at 750 rpm for 60 min and 30 °C (Thermomixer R Eppendorf AG, Hamburg, Germany). Then 90 µL of MSTFA (*N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide) with 1% TMCS (trimethylchlorosilane) and 2 µL of d27-myristic acid were added to mixture and shaken again (750 rpm for 30 min and 37 °C). Derivatized samples were transferred to 2 mL glass autosampler vials and analysed by GC-q-TOF-MS after two hours. The analysis was performed employing an Agilent 7890B GC system coupled to an Agilent 7200 quadrupole time-of-flight (q-TOF), equipped with an electronic ionization (EI) interface. The separation was carried out using an Agilent Zorbax DB5- MS + 10 m Duragard Capillary Column (30 m × 250 µm × 0.25 µm). Helium was used as carrier gas at a constant flow rate of 0.8 mL min⁻¹. The injection volume was 1 µL. Split mode was used for injection (split ratio of 10:1 and a split flow of 8.4 mL min⁻¹), keeping the injector temperature at 250 °C. The GC oven was programmed at 60 °C for 1 min, then increased at a rate of 10 °C min⁻¹ to 325 °C, and held at this temperature

for 10 min. MS detector was operated in full-scan acquisition mode at a m/z scan range of 50–600 Da (5 spectra per second). The temperatures of the transfer line, the quadrupole, and the ion source were set at 290, 150, and 250 °C, respectively.

2.8.3 Post-acquisition MS data processing software

Agilent Mass Hunter Qualitative analysis software (B.07.00) was used for post-acquisition data processing, making use of filtering tools by fragments features based on diagnostic product ions (DPIs) and or neutral loss of interest, or compounds search by MS or MS/MS features, that greatly enhances data interpretation. MDF was achieved using the Find Compound by Molecular Features tool available in the Agilent's Software. Mass defect is calculated from the molecular formula of the target core molecular structure, based on the difference between compound's exact mass and its nominal mass. The mass defect tolerance was set at 0.02 Da, working at different mass defect windows (mass range: 150-600 Da) depending on the target family of compounds. For GC-MS analysis, systematic mass spectra deconvolution of chromatographic signals and tentative identification of unknowns was performed using the Agilent Mass Hunter Unknown Analysis tool and mass spectral databases (i.e. NIST MS Search v.2.0 and Fiehn Lib).

3. RESULTS AND DISCUSSION

3.1 Analytical set-up and post-acquisition data-mining strategy

The global proposed workflow for the isolation and characterization of compounds of interest from food by-products is depicted in Figure 1. The analytical strategy set-up involves sequential PLE and subsequent LC- and GC-q-TOF-MS analysis followed by post-acquisition data processing. As illustrated in Figure 1, the developed PLE process for banana passion fruit seeds is divided in two sequential steps to first recover oils and then polar compounds. In the first step, seeds were defatted evaluating different non-polar solvents under PLE conditions. *n*-hexane was employed as reference

solvent, whereas *n*-heptane, *n*-cyclohexane and (+)-limonene were used as “alternative and greener” solvents [39]. The defatted seeds’ residue was subsequently submitted to the second PLE step to obtain polyphenolic-rich extracts; this step was optimized using an experimental design and considering extraction temperature and solvent composition as independent variables. Extraction yield (g of extract/100 g dry weight basis of sample), total phenolic content (mg Gallic acid equivalents/g dry weight basis of sample), total flavonoid content (mg Quercetin equivalents/g dry weight basis of sample) and antioxidant capacity (EC_{50} μ g/mL extract and TEAC mM Trolox/g extract) were investigated as responses variables.

A comprehensive profiling analysis of the optimized polar and non-polar PLE extracts was carried out based on the integrated combination of LC-, GC-, MS and MS/MS with post-acquisition data-mining. Full-scan MS data was firstly screened looking for potential molecular ions with significant contribution to the chromatographic peak signal (intensity threshold value of 5,000 counts). Intense precursor ions not related to the sample were removed applying background subtraction filtering (BSF) in order to discard interferences from the blank. A preliminary peak list (*m/z* values) of tentative precursor ions was used as MDF template to screen structurally related compounds including isomer or conjugate derivatives, based on the difference between a compound’s exact mass and its nominal mass. MDF results allowed the chromatographic peak detection of unknown compounds through high-resolution extracted ion chromatograms (HREIC), followed by the calculation of plausible molecular formulae based on accurate mass data. Further structural information was obtained from MS/MS data acquired by data-dependent scan (DDS), which was subjected to a double filtering approach; neutral loss filtering (NLF) and diagnostic product ions filtering (DPIF). NLF was implemented to screen characteristic neutral loss fragments (e.g. glycosyl, methoxyl groups), whereas typical product ions systematically present in MS/MS fragmentation spectra were used as diagnostic fragment ions template to conduct DPIF of compounds with similar base structure (e.g. flavonoid derivatives and procyanidins). Compounds

belonging to the same family exhibit similar core substructures and substitution patterns, thus exhibiting similar fragmentation behaviour by sharing the same product ions and neutral loss fragments in the MS/MS spectra. Finally, tentative identification of the detected compounds was proposed comparing MS and MS/MS data with information reported in MS databases, and in literature; identity was confirmed by using commercial standards, when available.

3.2 Selection of solvent for the first step of the PLE procedure

In order to achieve good extraction efficiency of the lipidic content from seeds, the selection of an appropriate extraction solvent was evaluated. For this purpose, *n*-heptane, *n*-cyclohexane, (+)-limonene and *n*-hexane (reference solvent in PLE process) were tested. The extraction time (10 to 60 min) was studied through extraction yield curves for each one of the solvents at 100 bar and 100°C. On the other hand, B&D method for total lipid determination was employed as reference. Results are shown in the kinetic curves of Figure 2. As can be seen, B&D method (7.34 %) and *n*-hexane (7.61 %) provided similar yield values, whereas the highest yield (7.85 %) was obtained using cyclohexane. *n*-heptane and (+)-limonene exhibited the lowest performance (7.13 and 3.31 %, respectively) and were discarded. As shown in the kinetic behaviour, the recovery of the lipid content was faster at the beginning of the extraction process, reaching the equilibrium after 10 min of extraction. The rapid increase in the extraction yield during the initial stage of the process can be mainly attributed to the extraction of nonpolar components located on the external surface of the matrix particles [40]. After this initial step, when a decrease in the extraction rate is observed, the increase in the concentration of the compounds in the extract phase was mainly controlled by solute diffusion inside the broken particles [41]. For this reason, 20 min were considered as appropriated extraction time. Cyclohexane was finally selected for operational reasons as suitable solvent for the first PLE, although *n*-heptane could also used, since no significant differences among cyclohexane and *n*-heptane performances ($p > 0.05$) were observed. Both solvents are considered as usable to

replace more toxic non-polar solvents such hexane, due to their favorable scoring in terms of safety, health and environmental impact (air, water and waste) [39].

3.3 Optimization of the second step of the PLE procedure

In order to optimize the extraction of phenolic compounds, a central composite design (CCD) was applied. For this purpose, the effect of solvent composition (percentage of EtOH in the mixture EtOH/EtOAc: 0, 50 and 100 % v/v) and temperature (50, 100 and 150 °C) were studied on extraction yield, TPC, TFC and antioxidant activity (EC_{50} and TEAC). The experimental data were fitted to linear, interaction and quadratic regression models. The significance level (F -values) of regression models was analysed using an ANOVA (see supplementary material, Table S1). Most of the terms in the designed quadratic model were found to be statistically significant ($p < 0.05$). The R^2 values indicated that the model was adequate. For all terms, the lack of fit values was non-significant ($p > 0.05$), so that the models turned out to be adequate to describe the observed data. The experimental data of CCD are summarized in Table 1. In order to determine the factors that influence each of the response variables, Pareto charts and surface responses were constructed (see Figure 3). The highest extraction yield (6.58 %) was obtained at the higher temperature evaluated (150 °C) and 100 % EtOH as extraction solvent; Pareto chart for extraction yield (Figure 3A) shows that both, solvent composition and temperature significantly contribute to the final yield, being the linear effect of temperature the most important. However, the extraction yield obtained by DM at 25 °C and 50 % EtOH was higher (10.16 %) than the yield obtained by PLE extractions. This difference can be explained by the longer extraction time in DM (24 h), which allows higher contact between the sample and the solvent. As for TPC, TFC and EC_{50} responses, the best results (29.99 mg GAE g⁻¹ Db, 0.94 mg Quer g⁻¹ Db and 2.66 µg mL⁻¹, respectively) were obtained at 100 % EtOH and 150 °C. TPC and TFC variables were principally influenced by the linear effect of the temperature and by the solvent composition (see Figure 3B and 3C, respectively). This behaviour

may be explained by the mass transfer, which is favoured by temperature, as well as by the thermal stability and medium polarity of the phenolic compounds extracted at the evaluated conditions. Whereas, EC₅₀ response was affected by temperature (negative and linear) and the quadratic effect of solvent composition (Figure 3D). On the other hand, as for TEAC, the best value (12.64 mM trolox g⁻¹) was observed using 100 % EtOH at 100 °C and it was influenced principally (Figure 3E) by effect of solvent composition (linear) and by temperature (quadratic and negative). To the best of our knowledge, there are no reports about phenolic and antioxidant activity from banana passion fruit seeds. Contreras-Calderon and coworkers [42], evaluated the phenolic content and antioxidant activity in different edible and non-edible parts of some exotic fruits, including banana passion fruit. However, those authors studied an extract (acidic methanol-water 50:50 v/v pH 2) of the mixture of pulp and seed of banana passion fruit. The TPC and TEAC values reported by those authors (635 mg GAE 100 g⁻¹ fresh weight and 131 µmol Trolox g⁻¹ fresh weight, respectively) were significant, but given the differences between the biomasses and calculation basis, it is difficult to compare with the results obtained in the present study. In another research, Simirgiotis and coworkers [4] studied the phenolic content and antioxidant activity of banana passion fruit pulp. According to the authors, the methanolic extract (ultrasound at 25 °C, 40 Hz for 1 h) presented important values of TPC (22.57 mg GAE 100 g⁻¹ DB), TFC (77.16 mg Quer 100 g⁻¹ DB) and EC₅₀ (12.89 µg mL⁻¹). Comparatively, the best TPC and EC₅₀ values obtained in this work were significantly higher than Simirgiotis and coworkers results. This difference was probably caused not only by banana passion fruit material, but also by physical conditions of the extraction technique, such as temperature and pressure. The notable antioxidant activity of the obtained banana passion fruit seed extract can be explained by the broad variety of phenolic compounds identified (See section 3.4). Flavan-3-ols and proanthocyanidins oligomers were the most abundant polyphenolic compounds in the PLE extracts, being reported in literature as strong antioxidant compounds due to the electronic configuration [43]. In this sense, a recent structure-activity

relationship study revealed that the 3-OH group at ring C of flavonoids is fundamental in the reactions with free radicals [44].

Due to the notably high phenolic content as well as antioxidant power of banana passion fruit seeds' extracts of the current work, PLE extraction was optimized respect to all variables evaluated. To achieve this purpose, desirability function combining yield, TPC, TFC, EC₅₀ and TEAC responses was calculated in the range from 0 to 1, where 1 represents the most desirable response. Response surface and profiles for predicted values estimated by desirability function are shown in Figure 4. Optimal conditions were 150 °C and 100% EtOH v/v at 0.82 desirability value, corresponding to experiment 10 of the experimental design. Predicted response values obtained by desirability approach under optimum conditions were checked with those of experiment 10 (Table 2). As can be seen, the observed and predicted data were close and they were within the confidence intervals. Since optimum conditions were at the experimental region limit, an additional experiment increasing the temperature (200 °C) was performed (Experiment 11, Table 1). Only the extraction yields increased (10.65 %) due to the high mass transfer. The TPC (1.99 mg GAE g⁻¹), TFC (0.04 gm Quer g⁻¹), EC₅₀ (28.36 µg mL⁻¹) and TEAC (1.01 mM trolox g⁻¹) responses were negatively affected by the high temperature. This phenomenon is probably related to the thermal decomposition of phenolic compounds with antioxidant activity [40] or to the chemical transformations (i.e. hydrolysis, condensation or browning reactions) at elevated temperatures [45]. These results confirmed that selected RSM model was successfully applied for PLE of banana passion fruit seeds in order to obtain extracts with maximum phenolic content and antioxidant activity.

3.4 UHPLC-q-TOF-MS/MS profiling analysis of *P. mollissima* seeds extracts

According to data reported in literature for other *Passiflora* species, flavonoids are the major phytoconstituents in pulp and peel matrices of this genus [46]. However, limited information is

available about the phenolic composition of *P. mollissima* seeds. Results obtained in this work by UHPLC-ESI(-)-q-TOF-MS/MS analysis revealed the presence of numerous phenolic compounds. Namely, based on the proposed MS and MS/MS data processing strategy included in the integrated analytical approach developed in this work (see Figure 1), a total of 61 phytochemicals, mainly phenolic acids, flavanols, flavanones and other flavonoid derivatives, including abundant proanthocyanidins (PACs), were detected and tentatively identified in the polar fraction of PLE extract from *P. mollissima* seeds. The tentatively identified compounds, exact mass, generated molecular formulae, calculated mass defect and MS/MS diagnostic product ions are summarized in Table 3.

3.4.1 Identification of phenolic acids and flavonoids

A mass defect filtering (MDF) template for deprotonated molecular ions of phenolic compounds commonly found in natural extracts was applied to screen MS and MS/MS data, allowing the detection of fifteen phenolic acids and twenty six flavonoids. The MDF window was set between 10 and 60 mDa for phenolic acid (mass range: 130-250 Da), and between 50 and 110 mDa for flavonoids (mass range: 270-570). Three major phenolic acids in terms of relative abundance (peaks 15, 13 and 30), sharing the same deprotonated molecular ion $[M-H]^-$ at m/z 137.0246 $[C_7H_5O_3]^-$, were tentatively identified as hydroxybenzoic acid isomers. The identity of the most intense phenolic acid (peak15) as 4-hydroxybenzoic acid was confirmed by commercial standard injection. Two dihydroxylated benzoic acid (peaks 3 and 9) were also detected at m/z 153.0193 $[C_7H_5O_3-H+OH]^-$. Metabolite 9 was confidently identified as protocatechuic acid (3,4-dihydroxybenzoic acid) on the basis of characteristic DPIs and the positive match of the MS/MS fragmentation pattern with on-line MS database (Metlin). Metabolites 4 and 22 exhibited the same deprotonated molecular ion m/z 167.0355 $[C_7H_5O_3-H+OCH_3]^-$, corresponding to methoxylated isomers of hydroxybenzoic acid. The identity of peak 22 (vanillic acid), along with gallic acid (peak 1) and

426 caffeic acid (peak 23), was confirmed comparing retention time and MS data with those of
 427 commercial standards. In addition, dimethoxylated isomer of hydroxybenzoic acid and
 428 hydroxycinnamic acid were tentatively identified at m/z 197.0453 $[C_7H_5O_3 - 2H + 2 \times OCH_3]^-$
 429 (peak 29) and m/z 223.0611 $[C_9H_7O_3 - 2H + 2 \times OCH_3]^-$ (peak 11), respectively.

430 Besides phenolic acids, a broad variety of flavonoid metabolites were also identified. Thus, the two
 431 main peaks observed in the TIC (see Figure 5) at 4.9 min (peak 28) and 5.7 min (peak 34),
 432 corresponding to the major phenolic metabolites in the obtained PLE extracts, exhibited the same
 433 $[M-H]^-$ ion at m/z 273.0768 $[C_{15}H_{13}O_5]^-$. The calculated molecular formula for this compounds is
 434 consistent with trihydroxylated flavan-3-ols. In addition, DPIs at m/z 149.0218 and m/z 123.0452
 435 generated after retro-Diels–Alder (RDA) dissociation of C-ring indicated a single hydroxyl group
 436 in A-ring (most probably at C7 position) and hydroxylation in C4' and C5' in B-ring. This
 437 assumption is confirmed by heterocyclic ring fission (HRF) fragmentation, leading to the DPI m/z
 438 109.0303 (1,3-dihydroxybenzene). Therefore, majority peaks 28 and 34, along with other two
 439 minor compounds (peaks 42 and 48) sharing the same molecular ion were confidently assigned as
 440 (+/-)(epi)fisetinidol diastereoisomers. Similar identification strategy was applied for the structural
 441 elucidation of catechin (peak 19, m/z 289.0720 $[M-H]^-$), one of the most abundant flavan-3-ols,
 442 whose identity was confirmed by commercial standard. O-methylated and di-O-methylated
 443 catechin derivatives were identified at m/z 303.0874 and m/z 317.1031, respectively, whereas
 444 galocatechin diastereoisomers (peaks 5, 10 and 16), were detected at m/z 305.0667 $[289.0720 - H$
 445 $+ OH]^-$.

446 Metabolites 14, 31, 33, 58, 45, 60 and 50 exhibited molecular ions and MS/MS fragmentation
 447 spectra consistent with flavanone structures. These compounds were tentatively identified as
 448 hydroxylated and methoxylated flavanones, as calculated molecular formulae from exact mass
 449 revealed an additional unsaturation degree compared to flavanols. Thus, peaks 45 and 60 were
 450 identified as trihydroxyflavanones with $[M-H]^-$ at m/z 271.0621 $[C_7H_5O_3]^-$. The subtle exact mass

shift between the two major DPIs m/z 135.0450 [$C_8H_7O_2$] $^-$ and m/z 135.0088 [$C_7H_3O_3$] $^-$ generated after RDA fragmentation indicates monohydroxylation in A-ring (C7) and dihydroxylation in B-ring. On the other hand, metabolites 14, 31 and 33 exhibited an additional OH group (m/z 287.0562 [$271.0621-H+OH$] $^-$), whereas metabolite 50 showed an additional methylene group (m/z 285.0772 [$271.0621-H+CH_2$] $^-$). Therefore, these compounds were identified as tetrahydroxyflavanones and dihydroxymethoxyflavanone, respectively. The DPIs at m/z 109.0303 (1,3-dihydroxybenzene) in metabolites 31 and 33 suggested monohydroxylation in A-ring (C7) and trihydroxylation in B-ring, as explained by HRF fragmentation mechanism. Peak 58 (hydroxymethoxyflavanone) was shown to be the lowest molecular weight flavanone, exhibiting a 16 Da less deprotonated molecular ion (m/z 269.0819) compared to peak 50.

The molecular formulae obtained from exact mass of metabolites 51, 46, 55 and 57 revealed an additional unsaturation degree compared to flavanones. Therefore, according to their deprotonated molecular ions and MS/MS spectra, the identity of these metabolites was assigned as hydroxylated/O-methylated derivatives of flavones and flavonols. Metabolites 51 and 46 were identified as trihydroxyflavone (m/z 269.0455, [$M-H$] $^-$) and diosmetin (5,7,3'-trihydroxy-4'-methoxyflavone, m/z 299.0561 [$M-H+OCH_3-H$] $^-$), respectively, showing deprotonated molecular ions that differed in a methoxy group (30.0106 Da difference). Further hydroxylation degree was observed in metabolites 55 and 57, whose identity was confidently assigned to quercetin (5,7,4',3,3'-trihydroxyflavone) and isorhamnetin (3'-methoxyquercetin) based on characteristic DPIs found in Metlin database.

Glycosylated derivatives were detected applying a NLF of 162.0525 Da for glucose (Glu) and 308.1107 Da for rutin (Rut). Thus, DPIs at m/z 152.0112 [$M-H-Glu$] $^-$ (peak 6), m/z 153.0191 [$M-H-Glu$] $^-$ (peak 2), 137.0243 [$M-H-Rut$] $^-$ (peak 8) and their MS/MS spectra indicated O-glycosylation metabolites of vanillic acid, protocatechuic acid and hydroxybenzoic acid, respectively. A dihydroxymethoxyflavone glucoside (peak 61) was also identified at m/z 445.0929

m/z with major DPI at m/z 283.0601 [M-H-Glu]⁻. Further MS/MS data screening, applying NLF of 132.042260 Da (pentose), 146.0580 Da (deoxyhexose), 130.0630 Da (dideoxyhexose) revealed the low presence of glycosylated derivatives in *P. mollissima* seeds, compared to their abundance in peel or pulp, in agreement with that reported in literature [4].

3.4.2 Identification of proanthocyanidins

Proanthocyanidins are polymers of flavan-3-ol subunits characterized by a single interflavan linkage through C4(A-ring)→C8(C-ring) or C4(A-ring)→C6(C-ring) bonds, which are referred to as B-type procyanidins. Occasionally, A-type polymers with an additional ether linkage between the A-ring OH at C7 and C2 of the A-ring (C2→O→C7) can also be found. As these oligomeric structures share a common skeleton based on flavanols such as (epi)catechin (m/z 289.0718), (epi)afzelechin/(epi)fisetinidol (m/z 273.0768) or (epi)gallocatechin (m/z), oligomeric forms of these subunits are expected. Full-scan MS data screening analysis of PLE phenolic extracts revealed different types of deprotonated proanthocyanidins dimers at m/z 575.1195 [C₃₀H₂₃O₁₂]⁻, m/z 559.1246 [C₃₀H₂₃O₁₁]⁻, m/z 593.1301 [C₃₀H₂₆O₁₃]⁻, m/z 577.1352 [C₃₀H₂₅O₁₂]⁻, m/z 561.1402 [C₃₀H₂₅O₁₁]⁻, m/z 575.1559 [C₃₁H₂₈O₁₁]⁻, with a Δ m/z below 5 ppm, as determined by HRMS (See Table 3). Using these [M-H]⁻ molecular ions as MDF templates and applying a MDF window between 0.1 and 0.2 Da (mass range: 540-840), 21 major PACs were detected as illustrated in the TIC and HREICs of Figure 5.

Additional structural information was obtained applying DPIF and NLF to MS/MS fragmentation data, which allowed the differentiation and structural elucidation of the flavanol subunits. Thus, PAC dimers exhibiting m/z 289.0718 [C₁₅H₁₃O₆]⁻ as major product ion were determined to contain an (epi)catechin subunit, generated after quinone methide (QM) cleavage of the interflavan bond (See fragmentation mechanism in Figure 6). The second subunit of these PAC dimers, and the interflavan-type bond was elucidated on the basis of (M-H-289.0718) Da neutral loss and

characteristic DPIs in the MS/MS spectrum.

According to the proposed strategy, metabolites 21 and 25 with $[M-H]^-$ ion at m/z 577.1352 were tentatively identified as isomeric B-type procyanidins dimer, that is (epi)catechin-(epi)catechin oligomers linked through C4-C8 bonds. This assignation is supported by typical DPIs explained by well described fragmentation pathways in literature [46]. According to these mechanisms, DPI at m/z 425.0875 ($[M-H-C_8H_8O_3]^-$, 152.0473 Da neutral loss) is generated by RDA reaction. A subsequent loss of a water molecule, leads to a more stable product ion at m/z 407.0074 $[M-H-152-H_2O]^-$. Additionally, a 126.0320 Da neutral loss yields DPI at m/z 451.1034 through a HRF mechanism, as illustrated in Fig 6.

PACs with $[M-H]^-$ ion at m/z 561 were tentatively identified as a dehydroxylated procyanidin dimer, considering the 16 Da less compared to the homologous m/z 577 procyanidins. Major DPI at m/z 289 and minor conjugate ion at m/z 271 confirmed the connection sequence of the dimer as trihydroxy(iso)flavanol – (epi)catechin (top unit– base unit). The identity of top subunit could be tentatively elucidated based on the DPIs m/z 409 $[M-H-152]^-$, m/z 451 $[M-H-110]^-$, and m/z 495 $[M-H-152-H_2O]^-$. The proposed fragmentation mechanism in Figure 6, supports the assignation of metabolites 24, 26, 27, 32, 36 as (iso)fisetinidol–(epi)catechin diastereoisomers. The presence of (epi)fisetinidol subunits in the oligomeric PACs is not surprising considering the remarkable presence of this flavan-3-ol in the phenolic fractions of PLE extract, as discussed above (section 3.4.1).

Metabolites 7, 12 and 17, shared similar diagnostic ions m/z 425, 407, 289 with A-type procyanidins dimers. According to the proposed fragmentation pathway given in Figure 6, the presence of this fragment ions indicate the presence of (epi)catechin at the bottom subunit. In addition, 425 m/z ion revealed dihydroxylation in A-ring of the top subunit. This assumption is supported by the 126 Da (1,3,5-trihydroxybenzene) neutral loss to yield the m/z 467 $[M-H-C_6H_6O_3]^-$ fragment ion. Since this DPI is 16 Da higher compared to the analogous fragment found in B-type procyanidin dimer,

an additional OH group at C3' of the B-ring is expected. Hence, proanthocyanidins 7, 12 and 17 were tentatively assigned as (epi)gallocatechin - (epi)catechin diastereoisomers.

On the other hand, peaks 18 and 20 with $[M-H]^-$ ion at m/z 575.1195 were tentatively identified as A-type procyanidins dimers, on the basis of the structural information obtained from the accurate mass shift (2.01565 Da less) exhibited by the molecular ion and DPIs m/z 423 $[M-H-C_6H_6O_3]^-$, compared to B-type procyanidins (m/z 577 and m/z 425). This m/z shift along with the presence of DPI with m/z 289 revealed an additional interflavan bond, linking two (epi)-catechin subunits.

Another A-type PAC was detected at 7.09 min (peak 49) showing m/z 559.1246 as $[M-H]^-$ ion. The MS/MS spectrum of peak 49 exhibited the DPIs m/z 289 ((epi)catechin) and a trihydroxy(iso)flavanol conjugate (m/z 269). Two major DPIs (m/z 410 and m/z 423) coming from the HRF and RDA reactions allowed the tentative identification of the target compound as an A-type (iso)fisetinidol-(epi)catechin dimer.

Although peaks 37, 39 and 47 shared molecular ions at the same nominal mass (m/z 575) compared to previously described peaks 18 and 20, they showed differences in the exact mass value and DPIs that revealed a different family of PCAs. The absence of a m/z 289 fragment in MS/MS spectra indicates the lack of an (epi)catechin subunit. A NLF of 32.0262 Da (CH_3OH), corresponding to the difference between the two major conjugate DPIs at m/z 303.0880 $[M-H-C_{16}H_{16}O_6]^-$, and m/z 271.0618 $[M-H-C_{15}H_{12}O_5]^-$, allowed the tentative assignation of peak 37, 39 and 47 as (epi)fisetinidol-O-methyl(epi)catechin diastereoisomers. The presence of an (epi)fisetinidol monomer in the top subunit is supported by the DPIs m/z 465 and m/z 423 generated by HRF and RDA mechanisms, respectively. The neutral loss of 110 Da during HRF indicated the monohydroxylation on A-ring, whereas the loss of a 152 Da neutral fragment suggested dihydroxylation in B-ring of top unit.

Additionally, flavan-3-ols oligomers of three subunits were also identified (peaks 35 and 41). Structural elucidation of MS/MS spectra corresponding to the fragmentation of m/z 833.2087

[M-H]⁻ precursor ion revealed two B-type trimers tentatively identified as (epi)fisetinidol- (epi)fisetinidol- (epi)catechin isomers. This sequence can be confirmed by the DPIs at m/z 681, 561, 409, 289, following the proposed DPI and NFL strategy discussed above.

The presence of oligomeric flavan-3-ols up to trimeric proanthocyanidins in *P. mollissima* has been reported in pulp and peel [7], although the characterization of PACs in *P. mollissima* seeds is described herein for the first time.

3.5 GC-q-TOF-MS profiling analysis of *P. mollissima* seeds extracts

A semi-targeted strategy for the profiling analysis of untargeted GC-q-TOF-MS data was applied for characterizing the lipid fraction of *P. mollissima* PLE extracts (corresponding to PLE-step one). As illustrated in the proposed analytical workflow (see Figure 1), PLE extracts were derivatized to improve the detectability of fatty acids (FA), unsaturated fatty acids (UFA) and polyunsaturated fatty acids (PUFA), as well as other lipidic metabolites (e.g. phytosterols, tocopherol and other terpenoids). Using the derivatization procedure described in section 2.9.2, trimethylsilyl (TMS) and tertbutyl dimethyl (TBDMS) derivatives were identified applying an automated full-scan MS data-mining process, which integrates peak detection, deconvolution and MS database search. Following this profiling strategy, 32 major metabolites, including FAs, (P)UFAS and relevant terpenoids were tentatively identified. Table 4 summarizes the GC-identified metabolites, including their corresponding characteristic GC-HRMS parameters (e.g. retention time, match factor values given by NIST database, molecular formula, monoisotopic mass, calculated mass defect ($\Delta m/z$) and main HR-MS/MS fragments), that confirm their unambiguous identification.

The MS/MS fragmentation spectrum of the FA-TMS derivatives is mainly characterized by intense fragments below m/z 150, and characteristic [M-H+TMS-CH₃]⁺ ions, which exact mass was used in this work as an additional qualitative parameter for improving tentative identification reliability. Thus, long chain saturated FAs such as nonanoic acid, lauric acid, pentadecanoic acid, palmitic

acid, heptadecanoic acid, stearic acid, arachidic acid, behenic acid, lignoceric acid, pentacosanoic acid and hexacosanoic acid, were positively identified. In addition, highly valuable PUFAS such as trans-palmitoleic acid, (E/Z)-oleic acid, ricinoleic acid, (E/Z)-linoleic acid, α -linolenic acid, (Z)-13-eicosenoic acid and eicosapentaenoic acid. were confidently identified based on commercial standards. This FA composition is reported in literature as that of a premium-grade edible oil, with remarkable applications for food, cosmetics, and pharmaceutical purposes [47].

On the other hand, *P. mollissima* seeds lipid extracts also contained valuable terpenoids such as squalene, tocopherol (α , γ , δ), cholesteneol derivative, ergosterol, campesterol methyl ether, stigmasterol and β -sitosterol. Unlike fatty acids, the $[M-H+TMS]^+$ molecular ion of the terpenoid-TMS derivatives can be clearly detected, whereas terpenoids with blocked OH groups did not undergo derivatization, exhibiting a highly fragmented $[M]^+$ ion.

4. CONCLUSIONS

The proposed analytical strategy was successfully developed and applied for the isolation and characterization of a wide variety of metabolites of interest from *P. mollissima* seeds. The developed methodology involved PLE in two steps for the fractionated extraction of phenolic and lipidic metabolites. An experimental design based on response surface methodology was carried out to obtain phenolic-rich extracts with the highest antioxidant activity, according to in vitro DPPH (EC_{50} 2.66 μ g mL⁻¹) and ABTS-TEAC (6.94 mM trolox g⁻¹) assays.

After UHPLC-ESI(-)-q-TOF-MS/MS analysis, a comprehensive post-acquisition MS data-mining strategy based on mass defect, neutral loss and diagnostic product ion filtering approaches, allowed the characterization of the unexplored phenolic fraction of *P. mollissima* seeds for the first time. Flavonoids, genuine flavan-3-ols (e.g. (epi)fisetinidol) and abundant proanthocyanidins oligomers were the major secondary metabolites identified. Complementary untargeted analysis of the oily

600 fraction by the GC-q-TOF-MS platform revealed the FAs and PUFAs profile of the target seeds
601 extracts, as well as the presence of relevant terpenoids, including tocopherols and phytosterols.
602 The different analytical approaches integrated in the proposed methodology, represent a powerful
603 strategy for food by-products valorization, combining a green and efficient extraction procedure
604 with accurate and sensitive HRMS-based analytical methodologies followed by systematic and
605 comprehensive strategies for detection and elucidation of highly valuable secondary metabolites.

606

607 **Acknowledgements**

608 This research was supported by COOPA20145, project from CSIC (Programa de Cooperación
609 Científica para el Desarrollo “i-COOP+”). G.A.-R. would like to acknowledge the Ministry of
610 Economy and Competitiveness for a “Juan de la Cierva” postdoctoral grant. The authors also thank
611 the support from the AGL2017-89417-R project.

References

- [1] A. Salazar, C. Ramírez, Fruit maturity stage and provenance affect seed germination of *Passiflora mollissima* (banana passion fruit) and *P. ligularis* (sweet granadilla), two commercially valuable tropical fruit species, *Seed Science and Technology*, 45 (2017) 383-397.
- [2] J. Aular, Y. Rodríguez, S. Roa, P. Iade, M. Antolínez, Características del fruto de cuatro pasifloras de la zona andina venezolana, *Bioagro*, 16 (2004) 137-142.
- [3] F. Casierra-Posada, J. Lancheros-Olmos, J. Cutler, Fruit growth and fruit drop in banana passion fruit plants (*Passiflora tripartita* var. *mollissima*), *Revista Colombiana De Ciencias Hortícolas*, 11 (2017) 237-246.
- [4] M. Simirgiotis, G. Schmeda-Hirschmann, J. Bórquez, E. Kennelly, The *Passiflora tripartita* (Banana Passion) Fruit: A Source of Bioactive Flavonoid C-Glycosides Isolated by HSCCC and Characterized by HPLC–DAD–ESI/MS/MS, *Molecules* (Basel, Switzerland), 18 (2013) 1672-1692.
- [5] S.M. Zucolotto, C. Fagundes, F.H. Reginatto, F.A. Ramos, L. Castellanos, C. Duque, E.P. Schenkel, Analysis of C-glycosyl flavonoids from South American *Passiflora* species by HPLC–DAD and HPLC–MS, *Phytochemical Analysis*, 23 (2012) 232-239.
- [6] N. Conde-Martínez, D.C. Sinuco, C. Osorio, Chemical studies on curuba (*Passiflora mollissima* (Kunth) L. H. Bailey) fruit flavour, *Food chemistry*, 157 (2014) 356-363.
- [7] A. García-Ruiz, A. Girones-Vilaplana, P. León, D.A. Moreno, C.M. Stinco, A.J. Meléndez-Martínez, J. Ruales, Banana Passion Fruit (*Passiflora mollissima* (Kunth) L.H. Bailey): Microencapsulation, Phytochemical Composition and Antioxidant Capacity, *Molecules* (Basel, Switzerland), 22 (2017) 1-12.
- [8] D. Chassagne, J.C. Crouzet, C.L. Bayonove, R.L. Baumes, Identification and Quantification of Passion Fruit Cyanogenic Glycosides, *Journal of agricultural and food chemistry*, 44 (1996) 3817-3820.

- 637 [9] D.C. Chaparro, M.E. Maldonado-Celis, L.A. Urango, B.A. Rojano, Propiedades
638 quimiopreventivas de *Passiflora mollissima* (Kunth) L. H. Bailey (curuba larga) contra cáncer
639 colorrectal, *Revista Cubana de Plantas Medicinales*, 20 (2015) 62-74.
- 640 [10] J.A. Hernández-Rivera, J.A. Martínez-Ramírez, M. Rojas-Cardozo, D.M. Aragón-Novoa,
641 Evaluation of *Passiflora tripartita* var. *mollissima* seed oil as potential nanoemulsion excipient,
642 *Journal of Excipients and Food Chemicals*, 9 (2018) 16-27.
- 643 [11] M. Herrero, A.d.P. Sánchez-Camargo, A. Cifuentes, E. Ibáñez, Plants, seaweeds, microalgae
644 and food by-products as natural sources of functional ingredients obtained using pressurized liquid
645 extraction and supercritical fluid extraction, *TrAC Trends in Analytical Chemistry*, 71 (2015) 26-
646 38.
- 647 [12] S. Armenta, S. Garrigues, M. de la Guardia, The role of green extraction techniques in Green
648 Analytical Chemistry, *TrAC - Trends in Analytical Chemistry*, 71 (2015) 2-8.
- 649 [13] S. Armenta, S. Garrigues, M. de la Guardia, The role of green extraction techniques in Green
650 Analytical Chemistry, *TrAC Trends in Analytical Chemistry*, 71 (2015) 2-8.
- 651 [14] B. Gilbert-López, J.A. Mendiola, E. Ibáñez, Green foodomics. Towards a cleaner scientific
652 discipline, *TrAC Trends in Analytical Chemistry*, 96 (2017) 31-41.
- 653 [15] E. Ibáñez, A. Cifuentes, Green extraction techniques 2015, *TrAC Trends in Analytical*
654 *Chemistry*, 71 (2015).
- 655 [16] M. Herrero, M. Castro-Puyana, J.A. Mendiola, E. Ibáñez, Compressed fluids for the extraction
656 of bioactive compounds, *TrAC Trends in Analytical Chemistry*, 43 (2013) 67-83.
- 657 [17] M. Palmeros Parada, P. Osseweijer, J.A. Posada Duque, Sustainable biorefineries, an analysis
658 of practices for incorporating sustainability in biorefinery design, *Industrial Crops and Products*,
659 106 (2017) 105-123.
- 660 [18] G. La Barbera, A.L. Capriotti, C. Cavaliere, S. Piovesana, R. Samperi, R. Zenezini Chiozzi,
661 A. Lagana, Comprehensive polyphenol profiling of a strawberry extract (*Fragaria x ananassa*) by

662 ultra-high-performance liquid chromatography coupled with high-resolution mass spectrometry,
 663 Analytical and bioanalytical chemistry, 409 (2017) 2127-2142.

664 [19] R. Zenezini Chiozzi, A.L. Capriotti, C. Cavaliere, F. Ferraris, G. La Barbera, S. Piovesana, A.
 665 Lagana, Evaluation of column length and particle size effect on the untargeted profiling of a
 666 phytochemical mixture by using UHPLC coupled to high-resolution mass spectrometry, Journal of
 667 separation science, 40 (2017) 2541-2557.

668 [20] G. Alvarez-Rivera, D. Ballesteros-Vivas, F. Parada-Alfonso, E. Ibañez, A. Cifuentes, Recent
 669 applications of high resolution mass spectrometry for the characterization of plant natural products,
 670 TrAC Trends in Analytical Chemistry, 112 (2019) 87-101.

671 [21] G. La Barbera, A.L. Capriotti, C. Cavaliere, C.M. Montone, S. Piovesana, R. Samperi, R.
 672 Zenezini Chiozzi, A. Lagana, Liquid chromatography-high resolution mass spectrometry for the
 673 analysis of phytochemicals in vegetal-derived food and beverages, Food Res Int, 100 (2017) 28-52.

674 [22] A. Zhang, H. Sun, G. Yan, X. Wang, Recent developments and emerging trends of mass
 675 spectrometry for herbal ingredients analysis, TrAC Trends in Analytical Chemistry, 94 (2017) 70-
 676 76.

677 [23] M.Z. Zhu, G.L. Chen, J.L. Wu, N. Li, Z.H. Liu, M.Q. Guo, Recent development in mass
 678 spectrometry and its hyphenated techniques for the analysis of medicinal plants, Phytochemical
 679 analysis : PCA, 29 (2018) 365-374.

680 [24] I. Spanik, A. Machynakova, Recent applications of gas chromatography with high-resolution
 681 mass spectrometry, Journal of separation science, 41 (2018) 163-179.

682 [25] T. Kind, H. Tsugawa, T. Cajka, Y. Ma, Z. Lai, S.S. Mehta, G. Wohlgemuth, D.K. Barupal,
 683 M.R. Showalter, M. Arita, O. Fiehn, Identification of small molecules using accurate mass MS/MS
 684 search, Mass spectrometry reviews, 37 (2018) 513-532.

685 [26] L. Bell, N.D. Spadafora, C.T. Müller, C. Wagstaff, H.J. Rogers, Use of TD-GC-TOF-MS to
686 assess volatile composition during post-harvest storage in seven accessions of rocket salad (*Eruca*
687 *sativa*), *Food chemistry*, 194 (2016) 626-636.

688 [27] D. Ren, L. Ran, C. Yang, M. Xu, L. Yi, Integrated strategy for identifying minor components
689 in complex samples combining mass defect, diagnostic ions and neutral loss information based on
690 ultra-performance liquid chromatography-high resolution mass spectrometry platform: *Folium*
691 *Artemisiae Argyi* as a case study, *Journal of chromatography. A*, 1550 (2018) 35-44.

692 [28] Z. Shang, W. Cai, Y. Cao, F. Wang, Z. Wang, J. Lu, J. Zhang, An integrated strategy for rapid
693 discovery and identification of the sequential piperine metabolites in rats using ultra high-
694 performance liquid chromatography/high resolution mass spectrometry, *Journal of Pharmaceutical*
695 *and Biomedical Analysis*, 146 (2017) 387-401.

696 [29] W. Zhao, Z. Shang, Q. Li, M. Huang, W. He, Z. Wang, J. Zhang, Rapid screening and
697 identification of daidzein metabolites in rats based on uhplc-ltq-orbitrap mass spectrometry coupled
698 with data-mining technologies, *Molecules (Basel, Switzerland)*, 23 (2018).

699 [30] D. Ballesteros-Vivas, G. Alvarez-Rivera, E. Ibanez, F. Parada-Alfonso, A. Cifuentes, A multi-
700 analytical platform based on pressurized-liquid extraction, in vitro assays and liquid
701 chromatography/gas chromatography coupled to high resolution mass spectrometry for food by-
702 products valorisation. Part 2: Characterization of bioactive compounds from goldenberry (*Physalis*
703 *peruviana* L.) calyx extracts using hyphenated techniques, *Journal of Chromatography A*, 1584
704 (2019) 144-154.

705 [31] D. Ballesteros-Vivas, G. Álvarez-Rivera, A. del Pilar Sánchez-Camargo, E. Ibáñez, F. Parada-
706 Alfonso, A. Cifuentes, A multi-analytical platform based on pressurized-liquid extraction, in vitro
707 assays and liquid chromatography/gas chromatography coupled to high resolution mass
708 spectrometry for food by-products valorisation. Part 1: Withanolides-rich fractions from

goldenberry (*Physalis peruviana* L.) calyces obtained after extraction optimization as case study, Journal of Chromatography A, 1584 (2019) 155-164.

[32] J. Živković, K. Šavikin, T. Janković, N. Čujić, N. Menković, Optimization of ultrasound-assisted extraction of polyphenolic compounds from pomegranate peel using response surface methodology, Separation and Purification Technology, 194 (2018) 40-47.

[33] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Canadian Journal of Biochemistry and Physiology, 37 (1959) 911-917.

[34] P. Manirakiza, A. Covaci, P. Schepens, Comparative Study on Total Lipid Determination using Soxhlet, Roese-Gottlieb, Bligh & Dyer, and Modified Bligh & Dyer Extraction Methods, Journal of Food Composition and Analysis, 14 (2001) 93-100.

[35] A. Hosu, V.-M. Cristea, C. Cimpoiu, Analysis of total phenolic, flavonoids, anthocyanins and tannins content in Romanian red wines: prediction of antioxidant activities and classification of wines using artificial neural networks, Food chemistry, 150 (2014) 113-118.

[36] W. Brand-Williams, M.E. Cuvelier, C. Berset, Use of a free radical method to evaluate antioxidant activity, LWT - Food Science and Technology, 28 (1995) 25-30.

[37] R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans, Antioxidant Activity Applying an Improved ABTS Radical Cation Decolorization Assay, Free Radical Biology & Medicine, 26 (1999) 1231-1237.

[38] T. Acunha, V. García-Cañas, A. Valdés, A. Cifuentes, C. Simó, Metabolomics study of early metabolic changes in hepatic HepaRG cells in response to rosemary diterpenes exposure, Analytica chimica acta, (2018) 1-12.

[39] F.G. Calvo-Flores, M.J. Monteagudo-Arrebola, J.A. Dobado, J. Isac-Garcia, Green and Bio-Based Solvents, Top Curr Chem (Cham), 376 (2018) 18.

- [40] D.C.G. Okiyama, I.D. Soares, M.S. Cuevas, E.J. Crevelin, L.A.B. Moraes, M.P. Melo, A.L. Oliveira, C.E.C. Rodrigues, Pressurized liquid extraction of flavanols and alkaloids from cocoa bean shell using ethanol as solvent, *Food Research International*, 114 (2018) 20-29.
- [41] J. Xi, L. Yan, L. He, Pressure-dependent kinetic modeling of solid-liquid extraction of the major green tea constituents, *Separation and Purification Technology*, 133 (2014) 155-159.
- [42] J. Contreras-Calderón, L. Calderón-Jaimes, E. Guerra-Hernández, B. García-Villanova, Antioxidant capacity, phenolic content and vitamin C in pulp, peel and seed from 24 exotic fruits from Colombia, *Food Research International*, 44 (2011) 2047-2053.
- [43] P.M. Aron, J.A. Kennedy, Flavan-3-ols: nature, occurrence and biological activity, *Mol Nutr Food Res*, 52 (2008) 79-104.
- [44] X. Chen, Z. Deng, C. Zhang, S. Zheng, Y. Pan, H. Wang, H. Li, Is antioxidant activity of flavonoids mainly through the hydrogen-atom transfer mechanism?, *Food Research International*, (2018).
- [45] M.D.A. Saldaña, C.S. Valdivieso-Ramírez, Pressurized fluid systems: Phytochemical production from biomass, *The Journal of Supercritical Fluids*, 96 (2015) 228-244.
- [46] F. Cuyckens, M. Claeys, Mass spectrometry in the structural analysis of flavonoids, *Journal of Mass Spectrometry*, 39 (2004) 1-15.
- [47] G.C. Silva, C.B.G. Bottoli, Analyses of Passiflora Compounds by Chromatographic and Electrophoretic Techniques, *Critical Reviews in Analytical Chemistry*, 45 (2015) 76-95.

753 **Figure captions**

754 **Figure 1.** Proposed workflow for the isolation and characterization of valuable bioactives from *P.*
755 *Mollissima* seeds.

756 **Figure 2.** Kinetic behaviour of the extraction yield employing different solvents during the
757 defatting step. PLE extractions performed at 100 °C and 100 bar.

758 **Figure 3.** Standardized Pareto charts for the response variables studied and their corresponding
759 response surfaces.

760 **Figure 4.** A) Desirability surface to maximize all response variables and B) Profiles for predicted
761 values at estimated optimal point, namely, solvent composition of 100% EtOH v/v at 150 °C.

762 **Figure 5.** TIC (A) and HREICs with +/- 10 ppm mass defect (B-D), corresponding to the phenolic
763 fraction of *P. mollissima* seeds analyzed by UHPLC-ESI(-)-q-TOF-MS/MS. (B) Phenolic acid and
764 derivatives; (C) Proanthocyanidins; (D) Flavonoids.

765 **Figure 6.** (A) Fragmentation pathways of A-type proanthocyanidins, along with diagnostic product
766 ions and neutral loss fragments used as DPIF and NLF template. (B) MS/MS product ion spectra
767 of different A-type proanthocyanidins identified by UHPLC-ESI(-)-q-TOF-MS/MS analysis