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Title: An innovative green extraction and re-use strategy to valorize food supplement by-products: Castanea sativa bud preparations as case study.

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Keywords: Castanea sativa Glyceric Macerates; Buds-derivatives waste valorisation; Pulsed Ultrasound-Assisted Extraction (PUAE); UV-Vis spectroscopy; Chemometrics; HPLC-phytochemical fingerprint.

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Abstract: This research takes place in the context of an Alcotra Italy-France trans-frontier project called FINNOVER, which includes among its objectives the "green" innovation of agro-industrial chains. Bud-derivatives are a category of natural products produced macerating meristematic tissues of trees and plants. They are quite expensive compared to other botanicals, since the collection period of their raw materials is extremely limited over the time. Consequently, the valorization of their by-products could have a significant economic impact for the producers. Castanea sativa buds have been selected as a case study.
Pulsed Ultrasound-Assisted Extraction (PUAE) has been employed to extract further valuable material from the buds by-products remaining after the production of C. sativa Glyceric Macerates. UV-Visible spectra coupled with chemometrics were employed, as untargeted phytochemical fingerprints, to quickly screen the best experimental conditions of extraction: a duty cycle of 80%, an extraction time of 15 minutes and a solvent/ratio of 1/10. Targeted phytochemical fingerprints by HPLC have been used to identify and quantify the main bioactive compounds of the most promising marcs extract comparing it with the corresponding commercial C. sativa Glyceric Macerate.
An innovative extraction and re-use strategy to obtain value-added products from botanicals by-products was developed in alternative to incineration or composting. It was applied to C. sativa buds production as case study, but it could be analogously applied for other herbal preparations.

Dear Editors,

This research takes place in the context of an Alcotra Italy-France trans-frontier project called FINNOVER (2017-2020) . The project involves several partners from France and Italy. The project aims at encouraging and developing new green supply chains, based on circular economy fundaments, to revamp the stagnant economy due to crisis of small and medium agricultural enterprises (SME) in France and Italy. In this context, we proposed a re-use strategy, based on a green extraction, to valorise botanicals by-products, which could have a significant economic impact for the producers.

In particular, bud-derivatives, that represent a category of natural products obtained by maceration of meristematic tissues of trees and plants such as buds and young sprouts, are rather expensive products compared to other botanicals, since the collection period of their raw materials is extremely limited over time. *Castanea sativa* buds were used as case study. Pulsed Ultrasound-Assisted Extraction (PUAE) has been employed to extract further valuable materials from the bud marcs.

The experimental effort to study the effect of the factors on the PUAE extraction was reduced considerably thanks to the use of the Design of Experiment (DOE).

UV-Visible spectra coupled with chemometrics were employed, as untargeted phytochemical fingerprints, to quickly screen the best experimental conditions.

Once the optimal conditions have been found, chromatographic methods (HPLC) were used, as targeted analytical methods, to identify and quantify the main bioactive compounds obtaining a phytochemical fingerprint in order to make a comparison with the corresponding commercial production of *C. sativa* Glyceric Macerate.

The procedure followed in this work could be considered as a promising and rapid method to manage marcs coming from small botanicals productions, as an eco-friendly alternative to incineration or composting, in order to obtain at the same time high added value products.

This paper is unpublished and has not been submitted for publication yet.

We hope that the paper is suitable for its publication in the Special issue "Conventional, Non-Conventional Extraction Techniques and New Strategies for the Recovery of Bioactive Compounds from Plant Material for Human Nutrition".

Sincerely yours,

Prof.ssa Raffaella Boggia

Highlights

- An example of agricultural waste management for small scale production was proposed
- PUAE was explored to recycle herbal supplements by-products
- Untargeted fingerprints by UV–VIS spectroscopy were coupled to chemometrics
- Targeted phytochemical fingerprints were obtained by HPLC

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An innovative green extraction and re-use strategy to valorize food supplement by-products: *Castanea sativa* bud preparations as case study.

An innovative re-use strategy to valorize food supplement by-products.

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Abstract

This research takes place in the context of an Alcotra Italy-France trans-frontier project called FINNOVER, which includes among its objectives the "green" innovation of agro-industrial chains. Bud-derivatives are a category of natural products produced macerating meristematic tissues of trees and plants. They are quite expensive compared to other botanicals, since the collection period of their raw materials is extremely limited over the time. Consequently, the valorization of their by-products could have a significant economic impact for the producers. *Castanea sativa* buds have been selected as a case study.

Pulsed Ultrasound-Assisted Extraction (PUAE) has been employed to extract further valuable material from the buds by-products remaining after the production of *C. sativa* Glyceric Macerates. UV-Visible spectra coupled with chemometrics were employed, as untargeted phytochemical fingerprints, to quickly screen the best experimental conditions of extraction: a duty cycle of 80%, an extraction time of 15 minutes and a solvent/ratio of 1/10. Targeted phytochemical fingerprints by HPLC have been used to identify and quantify the main bioactive compounds of the most promising marcs extract comparing it with the corresponding commercial *C. sativa* Glyceric Macerate.

An innovative extraction and re-use strategy to obtain value-added products from botanicals by-products was developed in alternative to incineration or composting. It was applied to *C. sativa* buds production as case study, but it could be analogously applied for other herbal preparations.

Keywords

Castanea sativa Glyceric Macerates; Buds-derivatives waste valorisation; Pulsed Ultrasound-Assisted Extraction (PUAE); UV-Vis spectroscopy; Chemometrics; HPLC-phytochemical fingerprint.

1. Introduction

FINNOVER (Innovative strategies for the development of cross-border green supply chains) is the name of an Interreg ALCOTRA Italy/France trans frontier project started in 2017 with the aim of innovating agro-industrial chains in terms of green circular economy. One of FINNOVER targets is the management of agricultural waste.

Food waste valorization and re-use strategies, rather than conventional food waste processing (i.e. incineration or composting), are becoming more and more popular and they are commonly named as "2nd generation food waste management" (Lin et al., 2013). Even if these strategies are particularly interesting for food processing companies, which usually generate waste on a large scale, whose transfer to landfill is very expensive, nevertheless there are small scale production, i.e. numerous herbal supplements productions, whose waste still represent an important source of botanicals to be valorized.

Bud-derivatives are a relatively new category of natural products, obtained macerating meristematic fresh tissues of trees and herbaceous plants, classified as plant food supplements in the European Community. Nowadays these products are still poorly studied, even if they are widely used for phytotherapy and homeopathy (Donno, Beccaro, Cerutti, Mellano, & Bounous, 2015; Donno, Mellano, Cerutti, & Beccaro, 2016a). Their use contributes to the birth of the so-called "Gemmotherapy", a fast-emerging branch of complementary medicine, which is expanding significantly in the market (Fowler M. W., 2006; Gurib-Fakim A., 2006).

Bud-derivatives are quite expensive compared to other botanicals, since the phenological stage of buds or young sprouts, necessary to obtain them, extremely limits their collection period over time. Consequently, the valorization of their by-products could have a significant economic impact for the producers and it could be an important innovation in this field.

This research describes a novel tool to enable manufacturers of bud extracts (gemmoderivatives) to evaluate a sustainable waste management option in order to increase their productivity. Nevertheless, the same strategy could be analogously applied also for other herbal preparations, becoming an example of "modus operandi" in a green economy strategy.

Castanea sativa buds were used as case study since this species is one of the most commonly used herbal medicines for its effects on stagnant and vascular fluids or against recurrent cystitis and for its curative, anti-oxidant and restorative properties against cardiovascular diseases (Donno, Beccaro, Mellano, Bonvegna, & Bounous, 2014).

Green procedures, namely those with complying with standards set by Environmental Protection Agency of USA (http://www.epa.gov/greenchem-istry/pubs/about_gc.html), compared to common conventional solid-liquid extraction techniques, have many advantages. Recently, Chemat F.et al. (2012) resumed these concepts in the following definition *"Green Extraction is based on the discovery and design of extraction processes which will reduce energy consumption, allows use of alternative solvents and renewable natural products, and ensure a safe and high-quality extract/product"* (Chemat, Vian, & Cravotto, 2012). Ultrasound-Assisted Extraction (UAE) is an efficient, green, relatively low-cost and sustainable procedure that presents many advantages with respect to conventional extractions. Furthermore, it can be used both on a small and large scale in the food extraction industry. The ultrasound waves (kHz range) are able to mechanically break the wall cells and thus extracting the intracellular liquids using several independent or combined mechanisms such as: fragmentation, erosion, capillarity, detexturation, and sonoporation (Kazemi, Karim, Mirhosseini, & Abdul Hamid, 2006).

In particular, high power ultrasonic probes usually operate at around 20 kHz and they are generally preferred for extraction applications respect to the ultrasonic bath, due to the direct delivery of ultrasounds in the extraction solvent with minimal ultrasonic energy loss. When UAE is used in pulsed mode (PUAE - Pulsed Ultrasound-Assisted Extraction), the ultrasound processor is turned on and off intermittently during pulsed extraction. This pulsed mode, if compared to the continuous one, is more suitable for the extraction of heat-sensitive biomolecules since heat generation is lower (Torres, Talavera, Andrews, Sánchez-Contreras, & Pacheco, 2017).

The solvent utilized in green extractions are bio-grade solvents produced from biomasses such as wood, starch, vegetables and fruits. In particular, ethanol and glycerol are both considered

bio-solvent, the first one is produced by the fermentation of sugar-rich materials, the second one is a by-product from the trans-esterification of vegetable oils. Both of them are used on a large scale because they are biodegradable, food grade, cheap and easily available in high purity.

Design of experiments (DOE), a well-established concept for planning informative experiments, has been very useful to optimize the PUAE extraction protocol with real advantages in terms of reduced experimental effort and in terms of increased quality of knowledge (Leardi R., 2009). It was applied using the whole UV–Vis spectrum of each extract, as multivariate response variable, and coupling chemometrics to quickly screen the best experimental conditions. This analytical shortcut has been already employed for a rapid untargeted identification of extracts of plants meristematic tissues (Boggia et al., 2017).

Finally, HPLC methods were used to identify and quantify the main bioactive compounds (in particular polyphenols, organic acids, and vitamins), selected as markers for their demonstrated health-promoting activity, obtaining a targeted chromatographic profile (phytochemical fingerprint) in order to assess the contribution of each single bioactive class to the total phytocomplex and to compare it with the commercial products: indeed, it is believed that synergistic or additive biological effects of different phytochemicals (phytocomplex), rather than a single compound or a group of compounds, contribute to disease prevention (Donno et al., 2014).

2. Materials and methods

2.1 Plant material

C. sativa buds (CBs) were collected from plants spontaneously grown in the valleys of Chisone, Pellice, Germanasca, Bronda, and Varaita (Turin, Italy) and authenticated by a botanist. In particular, the sampling sites were: Bobbio Pellice (N 44° 48' 33.84" E 7° 6' 43.919"), Bricherasio (N 44° 49' 10.2" E 7° 16' 31.079"), Perrero (N 44° 56' 27.96" E 7° 6' 10.439"), Pagno (N 44° 35' 54.24" E 7° 25' 28.2"; N 44° 35' 34.8" E 7° 25' 37.56"; N 44° 35' 47.76" E 7° 25' 37.199"), and Brondello (N 44° 35' 48.84" E 7° 25' 14.52"). Chestnut buds were used by an Italian commercial company of food supplements (Geal Pharma, Bricherasio, Turin) for the formulation of the corresponding Glyceric Macerates (GMs) in the year 2018 according to the European Pharmacopeia 8th edition (2014), following the procedure deriving from the French Pharmacopoeia (Ordre_National_des_Pharmaciens, 1965). The collection of the raw materials, in the meristematic phenological stage (buds), was performed over a limited period of time in March 2017, according to the different collection points. The fresh embryonic parts were immediately used in order to preserve their bioactive compounds. In this research original samples belonging to the same production batch were considered for analysis. Waste material obtained from the same herbal medicine production batch represented raw material for the following extraction steps assisted by pulsed ultrasounds.

2.2 Chemicals

All chemicals were purchased from Sigma-Aldrich (Steinheim, Germany) and from VWR Chemicals. High purity water produced (HPW) with Millipore Milli-Q system was used throughout.

2.3 Preparation protocol of *Castanea sativa* buds extracts (commercial product)

C. sativa buds extracts were prepared following the traditional protocol of GMs (Ordre_National_des_Pharmaciens, 1965). using a mixture of water/glycerol/ethanol (50/30/20 by weight) as extraction solvent and with a 1:20 weight ratio between plant and solvent. About 1 kg of fresh plant was treated. Bioactive compounds were extracted through a cold maceration process for 21 days, followed by a first filtration (Whatman filter paper, hardened ashless circles, 185 mm diameter), a manual pressing and, after 2 days of decanting, a second filtration (Whatman filter paper, hardened ashless circles, 185 mm diameter). The obtained extracts, which represent the commercial products were stored in dark bottles at normal atmosphere (N.A.), at 4 °C and 95% relative humidity until commercialization. At the same time, the wet marcs obtained after the 2nd filtration, which represent the solid by-products, were stored frozen at -20 °C until further treatments

2.4 Waste management: Pulsed Ultrasound-Assisted Extraction

The wet marcs remaining after the formulation of the commercial GMs, were milled in a Grindomix (Grindomix GM200, Retsch, Haan, Germany) for 20 s at 5000 rpm and sifted obtaining a powder of homogeneous size. Their moisture content (relative humidity) were determined to be 52.0% ± 0.3 by a Sartorius moisture analyzer (Massachusetts, USA). All measurements were made in triplicate and average results reported.

Pulsed Ultrasound-Assisted Extraction (PUAE) was performed directly using a sonicator with an operating frequency of 26 kH, effective output of 200 Watts, equipped with a titanium (7 mm i.d.) sonotrode (Hielscher Ultrasonics UP200 St, Germany). The pulse duration and pulse interval refer to ‘‘ON’’ time and ‘‘OFF’’ time of the sonochemical reactor. The total time of a pulse duration period plus a pulse interval period is the cycle time. A duty cycle (expressed as %) is the proportion of the pulse duration period to the cycle time.

The extraction operations were carried out directly under the pulsed mode, keeping the temperature under control always below 70°C. The extraction solvent, water/ethanol/glycerol

(50/20/30 by weight), was the same solvent used in the traditional protocol to produce the GMs commercial products.

Process conditions of the PUAE have been optimized by Design of Experiment (DOE) using a 2^{4-1} fractional factorial design. Four parameters (i.e. variables under study) such as: the amplitude level, the duty cycle, the extraction time and the sample/solvent ratio, at two levels were investigated. Eight experiments plus three replicates of central point were planned. The experimental plan (i.e. the conditions of the selected experiments) is reported in Table 1. As response variable the score on PC1 of the PCA (Principal Component Analysis) performed on the UV-Vis spectra, as described in the next paragraph, was taken into account.

2.5 UV-Vis Spectroscopy coupled to Chemometrics

Absorption spectra in the ultraviolet and visible regions were recorded in the range 190–1100 nm using an Agilent 8453 spectrophotometer with 1 nm resolution. The cells were rectangular quartz cuvettes with 1 cm path length. Before being analysed, the marcs extracts were filtered under vacuum through filter paper (Macherey-Nagel MN 615 70 mm) centrifuged at 3500 rpm for 10 minutes and properly diluted using a blank mixture of water/ethanol/glycerol (50/20/30). For each of the eleven extracts, obtained according the experimental plan, the total spectrum was collected at room temperature in triplicate, against blank solution, and the results were averaged.

The eleven spectra (training set) were organized into a data-matrix named $A_{11,271}$ consisting in how many rows as the number of samples (11) and how many columns as the recorded absorbance (the 271 absorbances at different wavelengths in the range 230–500 nm, since the two intervals 190–230 nm and 500–1100 nm were preliminary removed because the signals were saturated or without interesting absorptions respectively). Then PCA was performed on the column centred data, after the use of Standard Normal Variate (SNV) (Barnes, Dhanoa, & Lister, 1989) as preprocessing techniques with the goal of removing light scattering or other interfering phenomena

(Weeranantanaphan, & Downey, 2010). The scores on the first PC (explaining 86.8 % of the total variance) were used as response of the DOE.

Analogously the *C. sativa* GM, formulated by GealPharma using the same raw materials (buds) whose by-products are under study, were tested at two different dilutions in the already mentioned blank mixture (namely *commercial product_d80 and commercial product_d100*: diluted 1:80 and 1:100, respectively). Their spectra were subsequently used as external test set.

Data analysis was performed by an R-based chemometric software developed by the Group of Chemometrics of the Italian Chemical Society, freely downloadable from gruppochemiometria.it/index.php/software (2018).

2.6 Chromatographic analysis of bioactive compounds

In this study, effective HPLC–DAD methods were used for fingerprint analysis and phytochemical identification of bud preparations. Five polyphenolic classes were considered: benzoic acids, catechins, cinnamic acids, flavonols, and tannins. Organic acids and vitamin C (as sum of ascorbic and dehydroascorbic acids) were also considered to obtain an analytical fingerprint: total bioactive compound content (TBCC) was determined as the sum of the most important bioactive compounds with positive effects on human organism ("multimarker approach") (Mok & Chau, 2006).By single bioactive compound profile, phytochemicals were grouped into different bioactive classes to evaluate the contribution of each class to the total phytocomplex composition.

An Agilent 1200 High-Performance Liquid Chromatograph coupled to an Agilent UV-Vis diode array detector (Agilent Technologies, Santa Clara, CA, USA) was used for the chromatographic analysis. Four chromatographic methods were used to separate the bioactive molecules on a Kinetex C18 column (4.6 × 150 mm, 5 μm, Phenomenex, Torrance, CA, USA).

Several mobile phases were used for bioactive compound identification and UV spectra were recorded at different wavelengths, based on HPLC methods previously tested and validated for herbal medicines (Donno et al., 2016b): a solution of 10 mM KH_2PO_4/H_3PO_4 and acetonitrile with a

flow rate of 1.5 mL·min^{-1} (method A - analysis of cinnamic acids and flavonols, gradient analysis: 5% B to 21% B in 17 min + 21% B in 3 min + 2 min of conditioning time); a solution of methanol/water/formic acid (5:95:0.1 v/v/v) and a mix of methanol/formic acid (100:0.1 v/v) with a flow rate of 0.6 mL·min^{-1} (method B - analysis of benzoic acids, catechins, and tannins, gradient analysis: 3% B to 85% B in 22 min + 85% B in 1 min + 2 min of conditioning time); a solution of 10 mM KH_2PO_4/H_3PO_4 and acetonitrile with a flow rate of 0.6 mL·min^{-1} (method C - analysis of organic acids, gradient analysis: 5%B to 14%B in 10 min + 14%B in 3 min + 2 min of conditioning time); a solution of methanol–water (5:95, v/v) containing 5 mM cetrimide and 50 mM KH_2PO_4/H_3PO_4 with a flow rate of 0.9 mL·min^{-1} (method D - analysis of ascorbic and dehydroascorbic acids, isocratic analysis: 10 min + 5 min of conditioning time). UV spectra were recorded at 330 nm (A); 280 nm (B); 214 nm (C); 261 and 348 nm (D).

Biomarkers were selected for their demonstrated positive healthy properties and antioxidant activity by literature in relation to the use of this plant material and derived herbal preparations. All single compounds were identified in samples by comparison and combination of their retention times and UV spectra with those of authentic standards in the same chromatographic conditions. All analysis were triplicated, the results were averaged and reported as mg/g of fresh weight marcs. The statistical significance was determined using V-PARVUS 2010 (Forina et al., 2010) and the Excel Data Analysis Tool (Microsoft Corporation, Seattle,WA, US).

3. Results and Discussion

The whole recovery process, starting from the fresh raw materials of *C. sativa* buds to produce the corresponding commercial product and ending with the corresponding bagasse processed by PUAE, is resumed in the Graphical abstract.

Eight extracts (namely from CA01 to CA08), obtained according to the experimental plan described in Table 1, plus three extracts (namely from CA09 to CA11), obtained replicating the

experimental condition at the central point of the DOE, were prepared and spectrophotometrically analyzed.

At the top of Figure 1 there are the UV-Vis spectra of these eleven extracts opportunely diluted in the blank solvent, after filtration and centrifugation to clarify them. Since the vector of UV-Vis absorptions of each extract has been proven to be strictly correlated to the whole phytocomplex[12], it has been used as multivariate non-targeted signal and elaborated as response for each experiment of the DOE. PCA was used to elaborate the multivariate signals. Before chemometric analysis, SNV was used as data pre-treatment.

The corresponding score plot on the first two PCs of $A_{11,271}$ after column centering, whose explained variance is 98.6%, is reported at the bottom of Figure 1. The projections of the two spectra of the *C. sativa* GM commercial product, diluted 1:100 and 1:80 in the blank solution respectively (as external test set in red), in the score plot were also reported in Figure 1.

These dilutions of the commercial product were necessary to avoid signal saturation. The first PC, explaining 86.8%, retained all the useful information of the 271 original variables. The corresponding scores were reported in Table 1 and used as "global" response (to be maximized). The following model has been obtained:

$$\mathbf{Y=-9.1\ e\text{-}11 - 0.4\ X_1 + 0.1\ X_2 + 0.1\ X_3 + 0.8\ X_4 + 0.7\ X_1X_2 + 0.5\ X_1X_3 - 0.1\ X_1X_4}$$

remembering that the following interaction terms X_1X_2, X_1X_3, X_1X_4 are confused with X_3X_4, X_2X_4, X_2X_3 respectively (MacNamara, Leardi, & McGuigan, 2009). The linear term X_4 ($p < 0.01$) and the first two interaction terms ($p < 0.05$) are the only significant coefficients, as highlighted in Figure 2, and they should be increased. It has anyway to be considered that the linear model is not validated (the predicted value at the center point is significantly different from the experimental values) and therefore is not suitable for predictions.

The projection of spectra obtained from *C. sativa* GM commercial product gave high positive scores on PC1, not far from the "best" samples from the design.

Analyzing Figure 1, the experimental condition named CA08, whose details are reported in Table 1, seemed to be the most suitable to the aim of the research. In fact, the corresponding extract provides a spectrum having a score on PC1 similar to that of both the commercial products (*commercial product_d80, commercial product_d100*).

Thus, with the aim to obtain something still useful from the buds bagasse, this experimental condition was chosen as the best one among those tested by DOE.

Since X_4 resulted the most important variable in building the model, further experiments were planned setting it to 1/20, 1/15 and 1/10 (experiments: CA_R20, CA_R15, CA_R10) hoping both to improve the extraction yield and to save extraction solvent. The corresponding extracts were prepared, and the corresponding spectra were plotted in Figure 3 together with CA08 extract all at the same dilution (1:50).

The extract corresponding to CA_R10 resulted the most promising, since it seems more similar to the already mentioned *commercial product_d80* and thus deserving of further HPLC compositional investigation.

Figure 4 shows the HPLC-fingerprint of the commercial product (*C. sativa* GM), obtained by the fresh buds, and the extract obtained from the corresponding marcs by PUAE, namely CA_R10. The phytochemical analysis was focused on flavonols, phenolic acids expressed as benzoic and cinnamic acids, catechins, tannins as polyphenolic markers, also providing information on organic acids and vitamin C. In particular, it is known that dehydroascorbic acid has an important biological activity and can be easily converted to ascorbic acid by humans with positive antioxidant effects on human health-status. Thus, the sum of dehydroascorbic acid and ascorbic acid was considered for the evaluation of vitamin C. Table 2 resumes the content (expressed as mg/100 g of Fresh Weight buds/marcs) in the phytochemical classes both for the commercial products and for the marc extracts respectively, in order to make a comparison. It is important to point out that about the 12% of the *C. sativa* GM total bioactive compound content (TBCC) was preserved in the marc extracts and could be recovered. Particularly, the cinnamic acids (20.18 $\pm$ 0.01 mg/100 g_{FW} for

commercial product and 14.51 ± 0.01 mg/100 g_{FW} for CA_R10) and the vitamin C (18.08 ± 0.01 mg/100 g_{FW} for commercial product and 11.71 ± 0.01 mg/100 g_{FW} for CA_R10) contents followed by the flavonols (64.22 ± 0.04 mg/100 g_{FW} for commercial product and 18.10 ± 0.02 mg/100 g_{FW} for CA_R10) were more preserved in the marcs extract if compared to the other classes as highlighted in Table 2 and Figure 4. Many mechanisms have been proposed to explain biological protective effects of polyphenols, which, for several years, have been often ascribed mainly to their antioxidant capacity as vitamin C. Studies have demonstrated that, besides antioxidant and anti-inflammatory capacities, phenolics may engage with cellular signalling flow, controlling the action of transcription factors and subsequently affecting the expression of those genes involved in cellular metabolism and cellular survival (Donno, Mellano, Prgomet, & Beccaro, 2018). In this research, cinnamic acids, mainly represented by chlorogenic acid, and vitamin C in the marcs extract were respectively the 71.92% and 64.76% of the correspondent *C. sativa* GM content. The other bioactive compound classes, as benzoic and organic acids, catechins, and tannins were identified and quantified in the marc extracts, but they showed lower values (about 5-15%) than the relative commercial products.

In this study a preliminary phytochemical fingerprint was described: adding other markers with demonstrated biological activity would be a necessary step for a better identification of the chromatographic pattern in further fingerprint studies together with a mass spectrometry detection of unknown peaks using liquid chromatography coupled to mass/mass spectrometry (LC–MS/MS) as a very effective technique for complex plant extract analysis.

4. Conclusions

The valorisation of bud marcs remaining after the production of GMs, in this case study of *C. sativa*, could have a significant economic impact for the commercial producers, representing an important innovation in this sector.

For these reasons, an innovative and eco-compatible strategy to recycle these by-products, which proved to be still rich in bioactive compounds, was develop as an alternative to incineration or composting, in order to obtain high added value products. Pulsed Ultrasound-Assisted Extraction (PUAE), using the same solvent of GMs, have allowed to rapidly obtain an extract with a content in secondary metabolites of 160.42 mg/g of fresh weight marcs, which represents about the 12% of the corresponding commercial GM (1276.17 mg/g of fresh weight buds).

The procedure followed in this work could be considered as a promising and rapid tool to manage marcs coming from herbal preparations and it could be applied to other botanical productions.

Conflict of interest

The authors declare that they have no conflict of interest.

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Captions.

Figure 1.

UV-Vis averaged spectra (230 – 500 nm) of the eleven experiments selected by the DOE (A) and the score plot on the first two PCs selected by PCA using the vector of UV-Vis absorptions of each extract as multivariate untargeted signal (B). The projections of the two averaged spectra of the *Castanea sativa* Glyceric Macerate diluted 1:100 and 1:80 in the blank solution respectively (commercial product_d80, commercial product_d100), were also reported as external test set in the plot (in red).

Figure 2.

Coefficients plot of the DOE: the coefficients of the models of Y (PC1_scores) obtained by the DOE (X1: amplitude; X2: cycle; X3: time; X4: sample/solvent ratio) are reported. * = $p < 0.05$, **= $p < 0.01$.

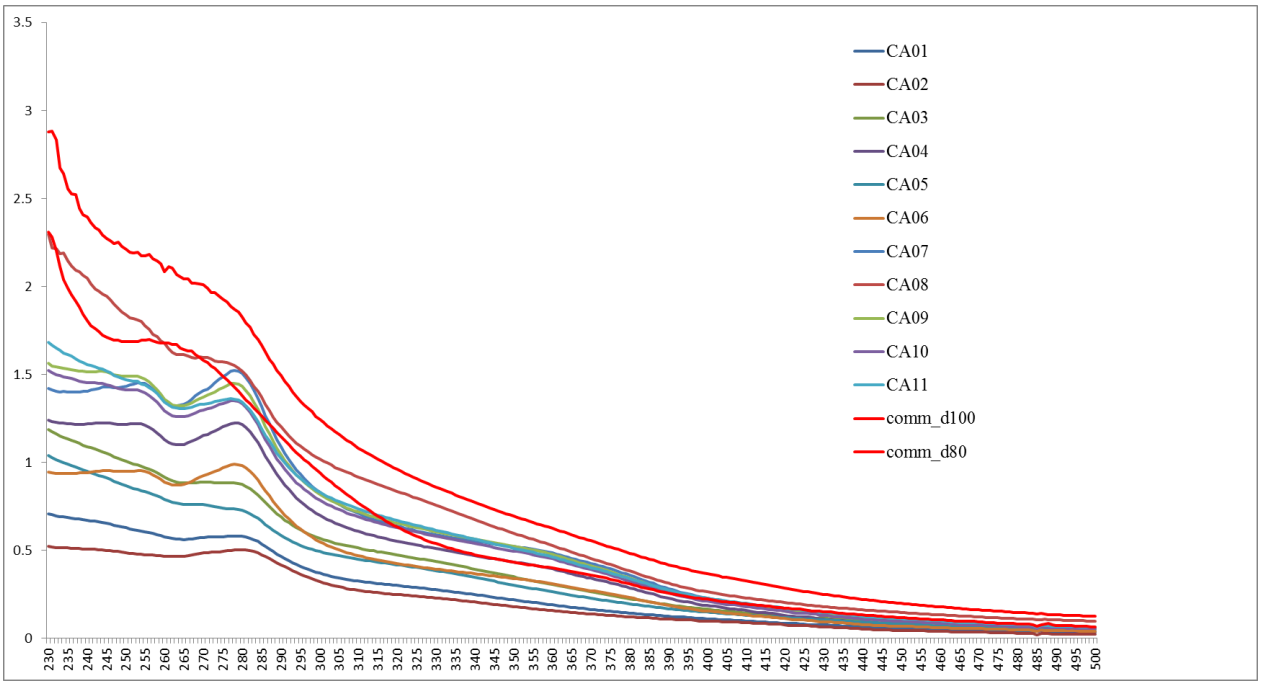
Figure 3.

UV-Vis averaged spectra (230 – 500 nm) of the following experiments: CA_R20, CA_R15, CA_R10 performed increasing the sample/solvent ratio (X4) are reported together with both CA08 spectrum and the commercial Glyceric Macerate spectra.

Figure 4.

HPLC-fingerprint of the *Castanea sativa* Glyceric Macerate and the most promising extract obtained by PUAE from the corresponding marcs, namely CA_R10. Results are expressed as mg/100 g of Fresh Weight buds/marcs. Mean values and error bars are reported.

Figure 1.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3
2.5
2
1.5
1
0.5
0
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CA01
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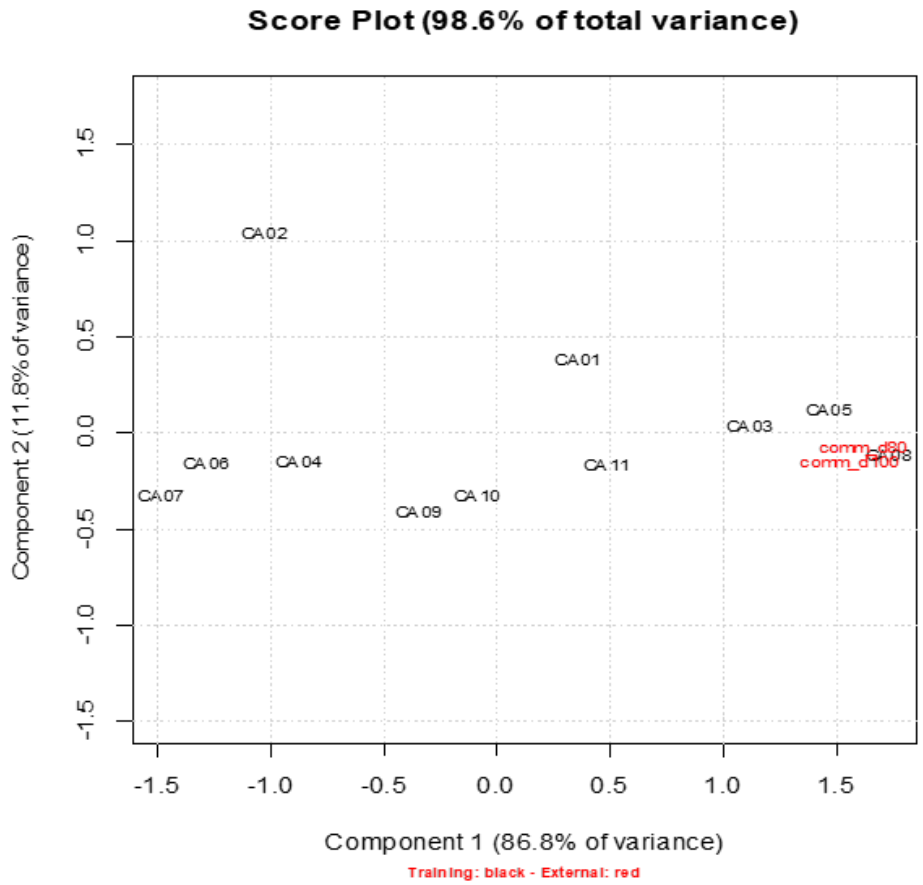
CA09
CA10
CA11
comm_d100
comm_d80

A

B


Score Plot (98.6% of total variance)
Component 2 (11.8% of variance)
Component 1 (86.8% of variance)
Training: black - External: red
CA 01
CA 02
CA 03
CA 04
CA 05
CA 06
CA 07
CA 09
CA 10
CA 11
comm_d80
comm_d100
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-0.5
0.0
0.5
1.0
1.5

Figure 2.

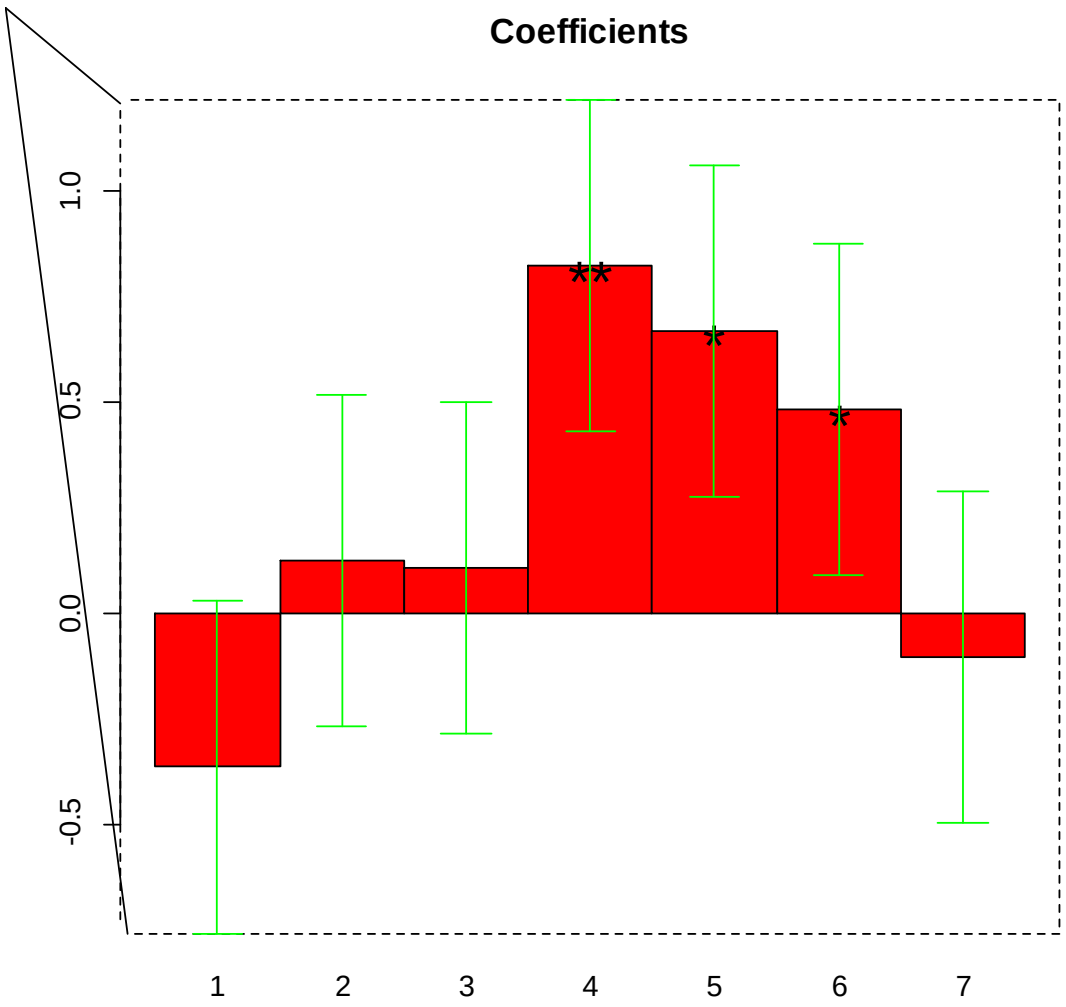


Figure 3.

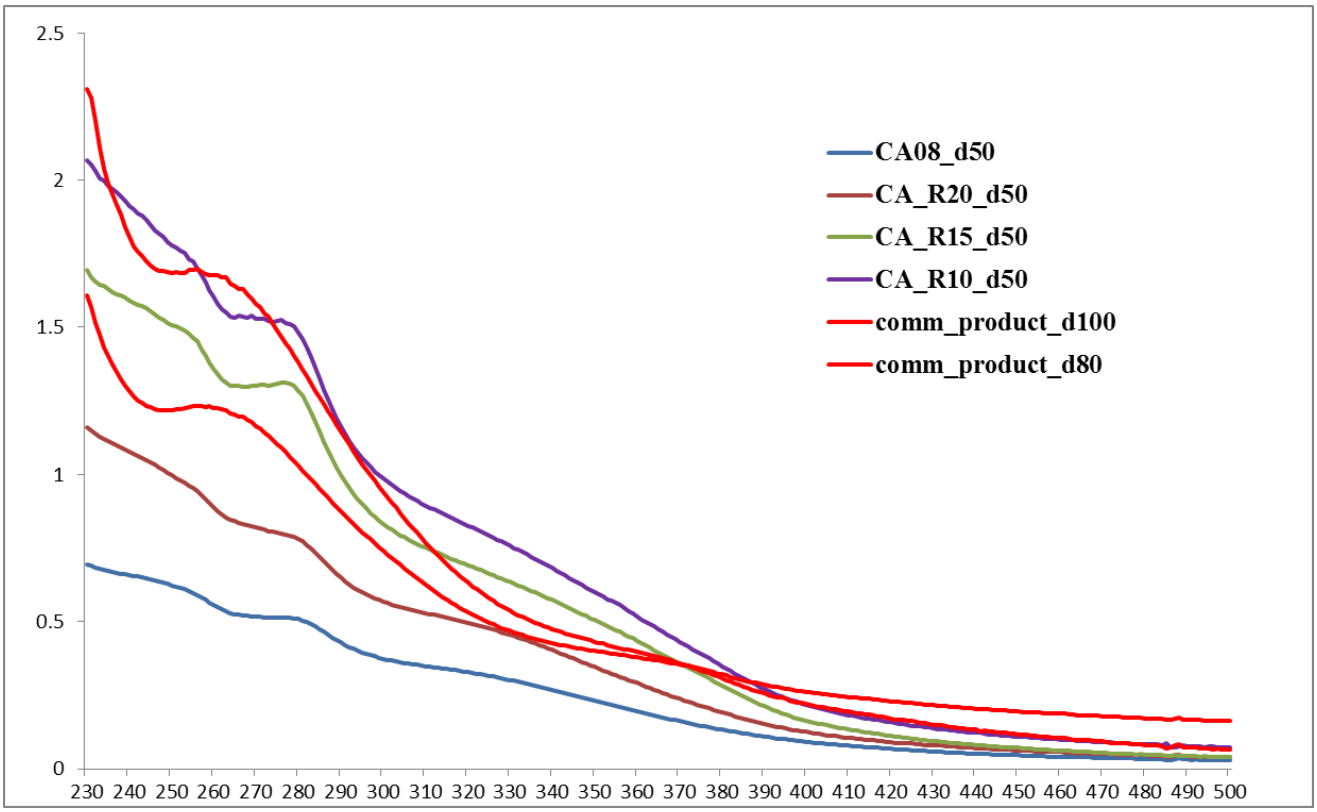
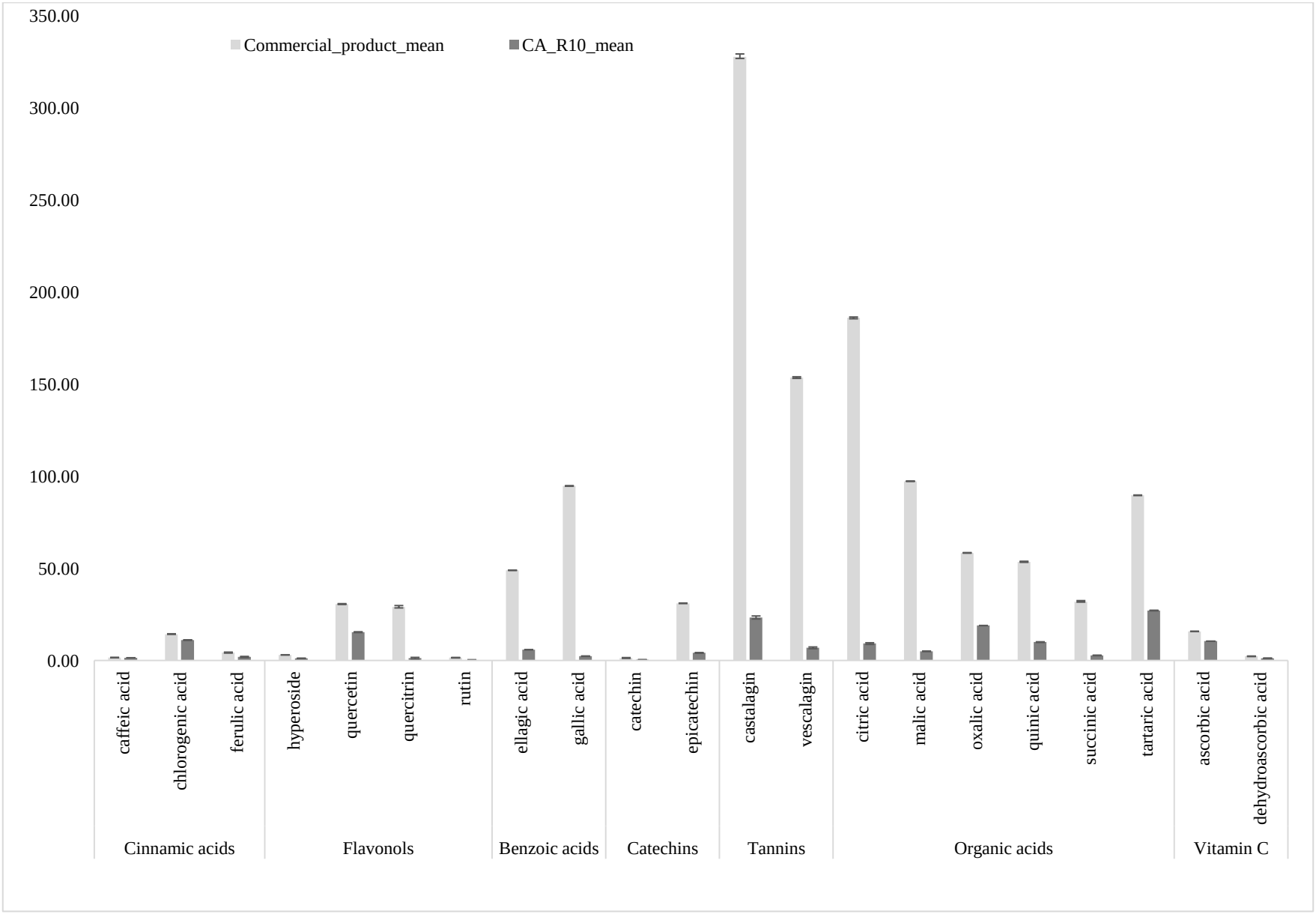

2.5
2
1.5
1
0.5
0
230 240 250 260 270 280 290 300 310 320 330 340 350 360 370 380 390 400 410 420 430 440 450 460 470 480 490 500
CA08_d50
CA_R20_d50
CA_R15_d50
CA_R10_d50
comm_product_d100
comm_product_d80

Figure 4.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300.00
250.00
200.00
150.00
100.00
50.00
0.00
Commercial_product_mean
CA_R10_mean
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chlorogenic acid
ferulic acid
hyperoside
quercetin
quercitrin
rutin
ellagic acid
gallic acid
catechin
epicatechin
castalagin
vescalagin
citric acid
malic acid
oxalic acid
quinic acid
succinic acid
tartaric acid
ascorbic acid
dehydroascorbic acid
Cinnamic acids
Flavonols
Benzoic acids
Catechins
Tannins
Organic acids
Vitamin C

Table 1. The experimental plan of the 2^{4-1} fractional factorial design and the corresponding response variable (Y).

Experiment	X1 Amplitude (%)	X2 Duty cycle (%)	X3 Extraction time (min)	X4 Sample/solvent ratio	Y (Response variable) PC1_scores
CA1	30	20	5	1/60	0.355196
CA2	50	20	5	1/40	-1.03264
CA3	30	80	5	1/40	1.114245
CA4	50	80	5	1/60	-0.88268
CA5	30	20	15	1/40	1.462808
CA6	50	20	15	1/60	-1.28896
CA7	30	80	15	1/60	-1.48396
CA8	50	80	15	1/40	1.723962
CA9	40	50	10	1/50	-0.35296
CA10	40	50	10	1/50	-0.09684
CA11	40	50	10	1/50	0.481829

Table 2. HPLC-fingerprint of the *Castanea sativa* commercial GM (Commercial_product) and the most promising extract obtained by PUAE from the corresponding marcs (CA_R10). Results are reported as mg/100 g of Fresh Weight buds/marcs and expressed as mean value ± interval confidence 95%.

		Commercial_product_mean	**CA_R10_mean**
Cinnamic acids	(mg/100 g_{FW})	20.18 ± 0.01	14.51 ± 0.01
Flavonols		64.22 + 0.04	18.10 ± 0.02
Benzoic acids		143.66 + 0.01	8.23 ± 0.01
Catechins		32.30 ± 0.01	4.53 ± 0.01
Tannins		481.22 ± 0.07	30.27 ± 0.05
Organic acids		516.51 ± 0.01	73.06 ± 0.01
Vitamin C		18.08 ± 0.01	11.71 ± 0.01
TBCC		1276.17 ± 0.13	160.42 ± 0.09

*Graphical Abstract

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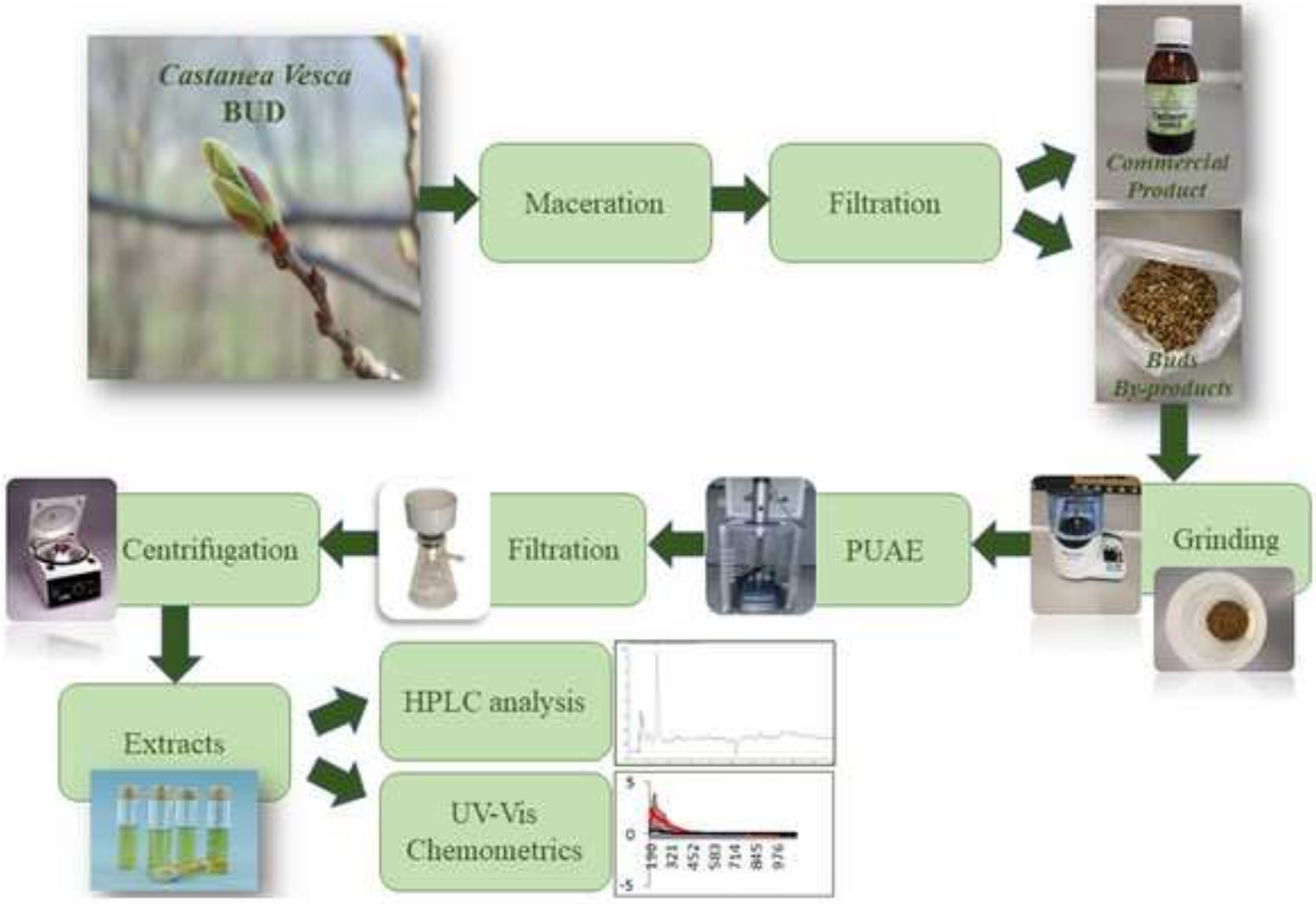

Data Statement

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