Non-targeted Metabolite Profiling to Evaluate the Drying Process Effect in the Peruvian Maca Actives Through Principal Component Analysis

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Abstract
This study aimed to evaluate the effect of the drying process on Peruvian maca flours. The analysis was done using a non-targeted metabolite profiling approach through principal component analysis (PCA) to evaluate the different molecular ions, detected by an ultra-high-performance liquid chromatography coupled to a hybrid quadrupole time-of-flight high-resolution mass spectrometer. The main advantage of this approach is to evaluate the whole set of ions, contemplating all the detectable substances. In this case, only the positive ions were considered. The spectra were acquired after methanolic extraction, and the data was organized in a matrix form. PCA allowed to find out the main substances responsible for differentiating between the drying processes, showing that oven drying mainly influences the amount of amino acids and glucosinolates. It was also possible to note that some macamides were still detectable after oven drying, and the main alkaloids also remained after heating. Therefore, this study offers a feasible method to interpret uHPLC-TOFMS/MS data for metabolomics in food authentication.

Keywords UHPLC-TOFMS/MS · PCA · Peruvian maca · Food analysis · Food authentication

Introduction
Popularly known as “Peruvian maca,” Lepidium meyenii Walp. is originally cultivated over the Peruvian Andes, and its characteristics have been described in several previous studies (Gonzales 2012; Sun et al. 2018). This plant is being commercialized worldwide due to several claims about health benefits and medicinal effects (Da Silva Leitão Peres et al. 2020), generating several studies such as neuroprotective effects (Gugnani et al. 2018), prevention of osteoporosis (Zhang et al. 2006), antifatigue effects (Choi et al. 2012), blood pressure regulation (Stojanovska et al. 2015), antiviral (del Valle Mendoza et al. 2014), prostate hyperplasia (Gasco et al. 2007; Vásquez-Velásquez et al. 2020), and mainly the effects related to sexual behavior such as spermatogenesis (Gonzales et al. 2006; Inoue et al. 2016), sexual dysfunction (Dording et al. 2008; Gonzales-Arimborgo et al. 2016), and fertility (Gonzales 2013).

Previous studies sustain the appeal of maca aphrodisiac and fertility-enhancing properties (Gonzalez et al. 2003; Gonzales et al. 2006), besides several studies suggesting that the consumption of this root is associated with multiple health effects (Kasprzak et al. 2018; Da Silva Leitão Peres et al. 2020). Nonetheless, claims regarding the lack of maca effects, as quoted in a previous study (Beharry & Heinrich, 2018), enforce the need for more studies on how the maca industrialization steps influence the product to be sold. One of the hypotheses for the claims, according to Esparza et al. (Esparza et al. 2015), is a consequence of the post-harvest drying practices, which can provide controversy over the maca expected effects, mainly due to the lack of regulations on the processing (Beharry and Heinrich 2018). The
traditional drying practices are related to the accumulation of monosaccharides, fatty acids, and amino acids due to slow drying and sun exposure prior to the milling process (Esparza et al. 2020).

According to several reports, the main bioactive compounds found in maca roots are the benzylalkamides (Esparza et al. 2015; Kasprzak et al. 2018; Carvalho and Ribeiro 2019; Da Silva Leitão Peres et al. 2020), known as macamides, which, according to Esparza et al., are products obtained from the condensation of benzylamine that originated from the hydrolysis of the glucosinolates, and as a consequence of the lipid hydrolysis, they are found as free fatty acids (Esparza et al. 2020). These compounds are present in fresh undamaged tissues, and they can be consumed or transformed during the processing. Despite that, Esparza et al. (2015) found out that when hypocotyls are dried by sun (environment) exposition (called the traditional Andean post-harvest practices) or through industrial oven drying, both present satisfactory amounts of the bioactive substances. On the other hand, the studies are generally made under controlled conditions, such as temperature and humidity, and do not necessarily reproduce the ideal conditions necessary to produce the Maca active substances. For example, in the study made by Pan et al. (Pan et al. 2016), they found that the content of total macamides was in the rank order of oven drying > air-drying > lyophilization > steaming, which is somehow intrigant considering that the traditional drying process takes from 8 to 12 weeks (Esparza et al. 2015), a period in which Maca rests exposed to the Andean weather, which means rarefied dry air in high ultra-violet radiation, besides a wide thermal amplitude (Instituto Nacional de Salud (Peru) et al. 2002).

The post-harvest drying process is the most important step to achieving the Maca bioactive compounds, such as non-starch polysaccharides, polyphenols (flavonolignans), glucosinolates, alkaloids, essential amino acids, and minerals (Zhu et al. 2020). Generally, the studies made over Maca composition are targeted to one or more bioactive groups of substances, most of them dedicated to investigations regarding glucosinolates (Yábar et al. 2011; Xu et al. 2021). One of the reasons for investigating the glucosinolates is that the β-thioglucoside-N-hydroxysulfate combination together with macamides is unique to the Peruvian maca and has been considered a chemotaxonomic marker (Fahey et al. 2001; Xu et al. 2021). In a study made by Yábar et al. (Yábar et al. 2011), the glucosinolate content was evaluated during pre-harvest, harvest, and post-harvest drying in yellow, red, and black Maca hypocotyls, finding six glucosinolates that were in a similar amount to each Maca variety. In this case, it is important to remark that the authors made the glucosinolate extraction, and so, they found a gradual and significant increase in the amount of this substance 90 days before harvest. They also reported that after 15–30 days of post-harvest drying at −10 to 15 °C and 70–90% relative humidity, all the Maca types showed an increase of total and aromatic glucosinolates. Nonetheless, after 45 to 90 days post-harvest, the Maca extracts displayed losses from 20 to 50% of total glucosinolates, which were correlated to the decrease in a specific enzymatic (myrosinase) activity. This indicates that traditional drying influences the amount of glucosinates after 45 days of the drying process. On the other hand, glucosinolates are biosynthetic precursors of macamides, macaenes, and other substances (Huang et al.; Clarke 2010; Hanschen et al. 2014; Xu et al. 2021). Therefore, reactions such as fermentation may take place after environment rest-drying, which is not possible to be reproducible in oven drying conditions.

In this sense, analytical chemistry plays an important role in determining possible differences between samples related to the drying process by a non-targeted metabolite profiling to allow for a description of the small molecules. The importance of using the non-target approach is related to the possibility of allowance to check for the macamide presence together with other glucosinolates, which is controversial regarding the drying process after 30 days, which drives to the lowering on the glucosinolate amount. Therefore, this study was driven by an ultra-high-performance liquid chromatography coupled to a hybrid quadrupole time-of-flight high-resolution mass spectrometer (UHPLC-MS/MS) to evaluate Peruvian maca samples from certified origin compared to commercial ones using the strategy of non-targeted metabolite profiling to be evaluated through principal component analysis approach, allowing for further understanding the influence of the drying process.

Materials and Methods

Sample

The certified maca flours were composed of 5 packages containing 1 kg each. These 1-kg packages resulted from a mix of plants obtained from the same harvest period, but produced in different sites. The packages comprised 2 samples from the red variety, 2 of the black, and 1 yellow type. Cultivation took place in the community of Santiago de Huari, in the state of Sebastián Pagador, Departamento de Oruro, at the Cordillera de Azanaques over 4000 m above sea level (Bolivia), being harvested in June 2019. Maca tubers were randomly taken from 20 plants of each variety to produce approximately 1 kg of maca flour. The post-harvest handling followed the traditional Andean post-harvest practices, spreading the maca tubers in beds of 10-cm height, being wamble each day to allow it for drying the samples by sun (environment) exposure for 8 weeks. During this period, the temperature ranged from −20 to 14 °C.
Other 7 samples of different brands commercialized in the Paraná State (south of Brazil) were purchased in different cities and produced through an oven drying process. There was not much information regarding the oven drying conditions on the labels. Moreover, it is important to remark that there was no specification regarding the maca variety in any of these commercial maca samples, besides no information regarding the drying temperature, harvest period, or cultivation conditions. Nonetheless, due to the availability and color, it is believed that all of the commercial flours came from the yellow maca type.

**Sample Preparation**

The samples were prepared by weighing 100 mg of each sample, added with 2 mL of an extraction solution composed of H₂O:MeOH 1:1 (v:v), mixed in a vortex for 2 min, and centrifuged for 5 min. Afterward, the supernatant was filtered through a syringe filter (hydrophobic PTFE membrane, 0.22 µm) and transferred to a 1-mL vial to be analyzed in UHPLC-MS/MS.

**UHPLC-MS/MS Analysis**

A 0.2 µL of each extract was injected and analyzed by ultra-high-performance liquid chromatography (Shimadzu, Nexera X2, Japan) using an Acquity UPLC HSS T3 C18 column (Waters, USA, 1.7 µm, 2.1 ×100 mm) coupled to a hybrid quadrupole time-of-flight high-resolution mass spectrometer (Impact II, Bruker Daltonics Corporation, Germany) equipped with an electrospray ionization source operating in positive mode and adjusted to 4500 V, with a potential plate end of −500 V. The dry gas parameters were set to 8 L min⁻¹ at 180 °C with a nebulization gas pressure of 4 bar. Chromatographic separation was performed using a gradient mixture of solvents A (H₂O with 0.1% formic acid, v:v) and B (methanol with 0.1% formic acid, v:v). The instrument was calibrated using a solution of sodium formate (10 mmol L⁻¹; isopropanol:water; 1:1; v:v) containing 50 µL of concentrated formic acid. The ionization source was operated in the positive ionization mode. The data were obtained in a range of m/z 50 to 1300 with an acquisition rate of 5 Hz. The 5 most intense ions were selected for automatic fragmentation (Auto MS/MS).

**Compound Identification**

The compounds were putatively identified based on a manual comparison of their fragmentation spectra with mass spectra libraries and literature. The mass error was calculated using a tolerance of less than 5 ppm.

**Data Treatment**

The matrix was composed of 12 lines (samples) and 10,627 columns (positive values of mass/charge). These lines, representing the samples, were ordered with the first 7 being the data from the commercial samples, followed by 5 lines assigned to the data from the samples of traditional drying. The matrix was mean-centered, and data was scaled through the Pareto approach to be evaluated by principal component analysis (PCA), which is a relatively simple tool to work with a large variable dataset (Wold et al. 1987; Bro and Smilde 2014). PCA was employed using the software Matlab 2017®, and the pls toolbox. PCA, the most popular tool in chemometrics, is a pattern recognition method that is a powerful way to overview complex multivariate datasets, allowing for the observation of patterns between samples and between variables. To find out detailed information regarding the method, it is recommended to look at the tutorial made available by Bro and Smilde (2014).

**Results and Discussions**

After acquiring the mass spectra, the PCA loadings indicated that the values of mass/charge (m/z) higher than 500 were not essential to differentiate between samples. So, the spectra were cut to be used from 50 to 510 (m/z), diminishing it to a 5845 variable dataset. A new PCA was applied to this new set, providing the separation projected in Fig. 1. As it was a non-targeted analysis, PC1 provided a satisfactory pattern distinction between the samples regarding the process. To check which compounds (ions) were responsible for the differentiation, the loadings from PC1, presented in Fig. 1 Scores of PC1 vs PC2 of the non-targeted metabolite analysis of Peruvian maca produced using traditional and industrial oven drying process
Fig. 2, were evaluated. These loadings were used to putatively identify the ions through manual comparison of their fragmentation spectra with mass spectra libraries and literature. Table 1 presents the most important ions identified in this study as responsible for differentiating samples.

The loadings indicated that the drying process influences the content of the sample on the amino acids (arginine, tryptophan, proline, and phenylalanyl-alanine), glucosinolates (glucotropaeolin), hydroxycinnamic acids (coumaric and ferulic acids), and in the n-benzyl-(9Z,12Z)-octadecadienamide (which is one of the macamides), besides the 12(13)-epoxy-9Z-octadecenoic acid, which is a polyunsaturated fatty acid. It can be said that it is somehow expected, once the high temperature used in the industrial ovens can degrade thermosensitive compounds such as these. On the other hand, one of the macamides, the n-benzyl-13-oxo-9E,11E-octadecadienamide, is suggested to resist oven temperatures. Moreover, most of the alkaloids are present in these samples, indicating that there are structures that are maintained after the heating.

Previous studies postulated that maca exhibits many biological activities being most of it due to the macamides, glucosinolates, saponins, alkaloids, and glucosinolates (Wang and Zhu 2019). Amino acids are also important nutrients and are susceptible to break depending on the heating temperature. According to Wang and Zhu (2019), the most abundant amino acid found in yellow maca is arginine. In this study, the PCA indicated that arginine was the most important compound for the traditional dried maca, suggesting that it is influenced by maca processing.

The main alkaloids are imidazole, which has shown biological activities related to protection against cytoxicity and anti-microbial action. Two of the imidazole alkaloids were previously reported as being present in maca (Cui et al. 2003), being lepidiline A (1,3-dibenzyl-4,5-dimethylimidazolium chloride) and lepidiline B (1,3-dibenzyl-2,4,5-trimethylimidazolium chloride). As found by Jin et al. (Jin et al. 2016), two of the rare imidazole alkaloids, lepidiline C and D, were also putatively identified. In this case, even though lepidiline B has not been identified, PCA indicates that the alkaloids lepidiline A, C, and D are influencing the separation of the maca oven-dried. Nonetheless, according to a previous study (Wang and Zhu 2019), the total content of alkaloids is influenced by the area of cultivation and the maca variety (color). In this way, there was no specification to the commercial maca regarding its type.

Glucosinolates found in maca are anionic sulfur-rich secondary metabolites, and they are said to act in the plant defense system, imposing a pungent flavor on this food. In a maca compositional study, it was found that this compound is present in about 1% of fresh maca (Wang and Zhu 2019), and most of it is composed of glucotropaeolin (benzyl glucosinolate). In this study, the PCA results indicate that it is present in maca dried by traditional methods and is an important compound to differentiate between the drying process. Therefore, it is dependent on post-harvest processing.

The macamide identified as n-benzyl-(9Z,12Z)-octadecadienamide was found in maca traditionally dried. Nonetheless, the macamide n-benzyl-13-oxo-9E,11E-octadecadienamide was found in maca oven-dried. Therefore, it is suggested that oven processing does not eliminate macamides. Also, one substance identified by its m/z 307.1903 with molecular formula C18H26O4 as a polyunsaturated fatty acid (PUFA), besides the 5-oxo-6E,8E-octadecadienoic acid (macaene) or isomer, was found for the maca oven-dried. Nonetheless, the PUFA 12(13)-epoxy-9Z-octadecenoic acid was found in the traditional dried maca. Therefore, the application of oven drying does not result in the thermal degradation of fatty acids.

![Fig. 2](https://example.com/fig2.png)

**Fig. 2** PC1 loadings of the non-targeted metabolite analysis of Peruvian maca produced using traditional and industrial oven drying process.
Conclusion

The non-targeted metabolomic profiling approach allowed for a feasible evaluation of the substances responsible for differentiating between the samples, with the advantage of evaluating the majority of molecular ions for all the groups detected in the samples and not only the glucosinolates, as happens in the majority of the studies. PCA provided enough information to differentiate maca according to the drying process, evidencing differences in the products. Considering that the processing of Peruvian maca influences the substances available to the consumer, it is important to establish protocols for maca production to achieve the whole plant’s potential. Moreover, the study indicates that industrial oven processing does not seem to lead to most of the plant bioactive thermal degradation, even though it drives changes in the substances profile.

Since non-targeted metabolomics generally uses few samples and deals with a large set of variables, this study shows a promising approach to food authentication analysis since it simplifies the search for the substances responsible for differentiating between samples.

Author Contribution Heloísa de Carvalho Rodrigues: data curation, formal heating analysis, investigation, methodology, writing—original draft. Luíza Mariano Leme: uHPLC-MS analysis, methodology, investigation, writing—original draft. Hellen Fernanda da Silva Paulino: investigation, heating analysis, methodology. Eduardo Jorge Pilau: uHPLC-MS analysis supervisor, methodology, investigation, methodology, writing—original draft. Patrícia Valderrama: conceptualization, investigation, writing—review and editing. Paulo Henrique Março: conceptualization, supervision, formal analysis, investigation, data curation, writing—review and editing.

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Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors declare no competing interests.

Ethical Approval This article does not contain any studies with human participants or animals performed by the authors.

Informed Consent Not applicable.

Conflict of Interest All the authors in this research declare they have no conflict of interest.

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