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UPLC-TQD-MS/MS Method Validation for Quality Control of Alkaloid Content in *Lepidium meyenii* (Maca)-Containing Food and Dietary Supplements

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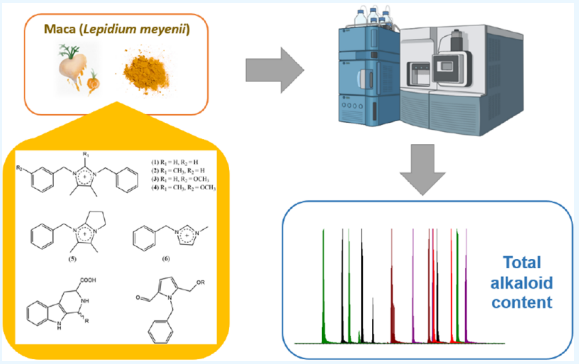
ABSTRACT: *Lepidium meyenii* Walp. (Brassicaceae), also known as Maca or Peruvian ginseng, is a common ingredient in food supplements with many claimed health benefits, such as improved endurance, increased energy level, and enhanced sexual properties. Due to potential toxicity of its chemicals, including alkaloids, some regulatory authorities, e.g., in Belgium, Germany, the United States, expressed concerns about the safe consumption of Maca root. However, due to the lack of commercial standards, no established analytical method currently exists for this purpose. The current project focuses on the quantitative determination of potentially toxic alkaloids from Maca. The current study presents the first analytical method for quality control of alkaloid content in Maca-containing food and dietary supplements, assessing the presence of 11 major compounds belonging to three different classes, i.e., imidazole, β-carboline, and pyrrole alkaloids. An accurate, rapid, and sensitive UPLC-TQD-MS/MS method is reported, which was fully validated according to the International Council for Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and SANTE/11312/2021 guidelines. To ensure the method's applicability and practicability in the absence of primary standards, validation of secondary standards (SSs) alongside primary standards (PSs) was also conducted for imidazole alkaloids. As a result, in Maca raw powder, total alkaloid content was found to vary from 418 to 554 ppm (mg/kg). Furthermore, all quantified imidazole alkaloids were ascertained to be the major alkaloids with the total content from 323 to 470 ppm in Maca raw powder, followed by the β-carboline and pyrrole alkaloids. It was also observed that the commercial preparation of finished products affects the total alkaloid content, evidenced by the large variation from 56 to 598 ppm. Ultimately, from a regulatory point of view, it seems advisible not to request the complete absence of the alkaloids but to impose a maximum level based on safety considerations. In addition to the analytical method, a low-cost, simple, and scalable synthetic scheme of macapyrrolins A, C, and G was reported for the first time.



1. INTRODUCTION

Lepidium meyenii Walp. (Brassicaceae) or 'Maca' is an herbaceous plant native to the high plateaus of the Andes of Peru.[1] The climatic and environmental conditions in this region are exceptionally distinctive, characterized by high altitudes, low temperatures, humidity, strong winds, abundant rainfall and sunlight, low atmospheric pressure, as well as high levels of UV and cosmic radiation.[2,3] Growing and thriving in these harsh living conditions are considered a significant evolutionary factor of Maca, forcing the production of protective and biologically active compounds. Nowadays, Maca is grown not only in Peru but also in some other South American countries, as well as in China.[3] Maca is primarily used as a food supplement due to its claimed medicinal properties and nutritional value, such as improved endurance and energy level, increased memory, enhanced fertility and sexual performance, and protection against UV radiation.[4,5] Many studies have indicated that the distinctive presence of macamides and macaenes, which are fatty acid derivatives, in Maca likely plays a crucial role in the purported medicinal properties.[5] Glucosinolates and isothiocyanates, widely distributed in the Brassicaceae family, are two other major classes of compounds in Maca, which recently have been considered as new plant-derived antimicrobial agents.[6]

In the early 2000s, the imidazole alkaloids lepidilines A and B were reported, as well as the β-carboline alkaloid 1-methyl-

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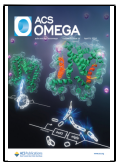

1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTCA) and macaridine.[7,8] However, in 2021, the structure of macaridine was revised as macapyrrolin C, a pyrrole alkaloid.[9,10] Since 2016, discovery of minor yet distinctive agents containing nitrogen and sulfur from Maca has emerged as an increasingly captivating research topic, disclosing novel classes of compounds including lepipyrrolins, meyeniins, hydantoins, thiohydantoins, and macathioureas.[1,11,12]

Concerns have been raised about the safety of Maca as food supplements, e.g., in Belgium, Germany, and the United States. More specifically, this has led to the legal requirement that no detectable amounts of alkaloids may be present in food supplements containing Maca (Belgian Royal Decree of 21/08/2021).[13] According to the German Federal Institute for Risk Assessment (published on 03/04/2007), insufficient information and concrete evidence for safety and quality control of Maca-containing food supplements were available, and therefore no safe intake of Maca could be derived.[14] Besides, no report for Maca from the European Food Safety Authority (EFSA) was found at the present time. On the other hand, in the United States Pharmacopeia (USP) safety review of Maca (published in 2012), 50−100 g of dried hypocotyls per day as food and a dose range of 1.5−3.0 g of dried hypocotyls (corresponding to about 10 g of fresh Maca) as a dietary supplement were suggested. However, the USP also documents numerous nonserious adverse effects of Maca consumption in humans, including rash, syncope, burning sensation, nausea, abdominal pain, vomiting, diarrhea, dehydration, impaired concentration, headache, epistaxis, anxiety, insomnia, tachycardia, and thirst.[15]

Several advancements in the development of analytical methods for macamides, macaenes, glucosinolates, and isothiocyanates in *L. meyenii* and some plant species were already made.[16−19] In 2017, Chen et al. reported HPLC and UHPLC-ESI-Q-TOF-MS/MS analyses of main macamides and macaenes from Maca.[17] Two years later, macamides and macaenes were the targeted compounds of an HPLC-DAD method published by Xia et al.[16] The authors applied a chemometric method to distinguish Macas of different origins. Also in 2019, an UHPLC-Q-Orbitrap-MS method was reported for the simultaneous determination of 15 glucosinolates in eight Brassicaceae plant species, including Maca.[18] Most recently, Theunis et al. reported an HPLC-DAD methods for the quantification of glucosinolates and isothiocyanates in *Nasturtium officinale* and *Brassica oleracea*.[19] Up until now, no analytical method has been reported for determining the alkaloid content from Maca. From an analytical perspective, the main challenge lies in the scarcity of commercially available standards of Maca alkaloids that can be used as primary standards, which hampers the progress of analytical method development. Therefore, our study systematically addresses the issue in the following manner: (1) conducting a phytochemical and metabolomic investigation to assess the alkaloidal composition of Maca and to isolate appropriate primary standards − this research was previously published by our group,[10] (2) devising a straightforward synthetic scheme for generating pyrrole-containing standards, (3) developing and validating an analytical method employing secondary standards, thus enabling the quality control of Maca even in the absence of primary standards, and (4) as the last step, analyzing some commercial products available on the Belgian market using the method developed and validated hereinafter.

2. MATERIALS AND METHODS

2.1. Isolation and Purification of Imidazole Alkaloids. Isolation of imidazole standards was reported before.[10] Lepidilines A−E (**1**−**5**) were obtained in sufficient amounts (10−20 mg). Structure elucidation was performed by means of extensive spectroscopic and computational techniques using the published protocol of the authors.[20] Standard purity was determined by means of the total intensity method applied to the ^{1}H NMR spectrum of the purified compounds. Briefly, the total integration (area) of all ^{1}H signals belonging to a lepidiline is compared to the total integration of all signals observed in its ^{1}H NMR spectrum.

2.2. Synthesis of Pyrrole Alkaloids. Macapyrrolin C (**12**) was synthesized by a modified procedure reported by Adhikary et al.[21] The condensation of β-D-glucose and benzyl amine in an acidic medium created by oxalic acid allows the formation of macapyrrolin C (yield ∼40%). The use of readily available starting materials allowed a multigram scale synthesis of macapyrrolin C, which was later on used as the starting material for synthesizing macapyrrolins A (**13**) and G (**14**) by alkylation. The synthetic scheme is demonstrated in Figure S25.

Gram-scale synthesis of macapyrrolin C (modified Maillard reaction of carbohydrate and amine) was carried out as follows: in a 500 mL round-bottom flask containing 90 mL of DMSO, 33 g of β-D-glucose, 20 mL of benzyl amine (1 equiv), and 16.5 g of oxalic acid (1 equiv) were mixed, and then the mixture was heated to 80 °C for 5 h. The reaction was monitored by UPLC-MS and quenched by water. Macapyrrolin C (**12**): brownish oil (18 g); UV λ_{max} 210, 274, 294 nm; ^{1}H and ^{13}C NMR ($CDCl_3$, 400 and 100 MHz): δ_H 9.53 (s, −CHO, H-6), 7.28 (overlapped, H-4′, H-6′), 7.25 (m, H-5′), 6.99 (overlapped, H-3′, H-7′), 6.96 (d, 3.9, H-3), 6.31 (d, 3.9, H-4), 5.75 (s, H-1′), 4.56 (H-7); δ_C 180.1 (−CHO, C-6), 142.4 (C-5), 138.1 (C-2′), 133.0 (C-2), 129.0 (C-4′, C-6′), 127.7 (C-5′), 126.4 (C-3′, C-7′), 124.7 (C-3), 111.1 (C-4), 56.9 (C-7), 48.8 (C-1′); positive HRESIMS *m*/*z* 216.1016 $[M + H]^+$ (calcd for $C_{13}H_{14}NO_2$, 216.1025).

2.2.1. Synthesis of macapyrrolin A. 200 mg of macapyrrolin C was first added into a 50 mL flask, and then 5 mL of THF was added. The solution was stirred in an ice bath until it cooled. 0.11 g of NaH (5 equiv) was added, and the solution was stirred in the ice bath for 20 min. Then, 0.069 mL of iodomethane (2 M solution in *tert*-butyl methyl ether) was added, followed by stirring for 5 h. The reaction was monitored by UPLC-MS and quenched by water. Macapyrrolin A (**13**): brownish oil (109 mg); UV λ_{max} 210, 274, 294 nm; ^{1}H and ^{13}C NMR ($CDCl_3$, 400 and 100 MHz): δ_H 9.57 (s, −CHO, H-6), 7.28 (overlapped, H-4′, H-6′), 7.25 (m, H-5′), 6.99 (overlapped, H-3′, H-7′), 6.96 (d, 3.9, H-3), 6.31 (d, 3.9, H-4), 5.75 (s, H-1′), 4.35 (H-7), 3.33 (6-OCH_3); δ_C 180.1 (−CHO, C-6), 139.5 (C-5), 138.1 (C-2′), 133.2 (C-2), 128.9 (C-4′, C-6′), 127.5 (C-5′), 126.5 (C-3′, C-7′), 124.3 (C-3), 112.2 (C-4), 66.1 (C-7), 58.3 (7-OCH_3), 48.9 (C-1′); positive ESIMS *m*/*z* 230.1179 $[M + H]^+$ (calcd for $C_{14}H_{16}NO_2$, 230.1181).

2.2.2. Synthesis of macapyrrolin G. 200 mg of macapyrrolin C was first added into a 50 mL flask, and then 5 mL of THF was added. The solution was stirred in an ice bath until it cooled. 0.11 g of NaH (5 equiv) was added, and the solution was stirred in the ice bath for 20 min. Then, 0.069 mL of iodoethane was added, followed by stirring for 5 h. The

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reaction was monitored by UPLC-MS and quenched by water. Macapyrrolin G (**14**): brownish oil (88 mg); UV λ_{max} 210, 274, 294 nm; ^{1}H and ^{13}C NMR ($CDCl_3$, 400 and 100 MHz): δ_H 9.57 (s, −CHO, H-6), 7.28 (overlapped, H-4′, H-6′), 7.25 (m, H-5′), 6.99 (overlapped, H-3′, H-7′), 6.96 (d, 3.9, H-3), 6.31 (d, 3.9, H-4), 5.75 (s, H-1′), 4.40 (H-7), 3.48 (q, 7.0, CH_2 of 7-OCH_2CH_3), 3.48 (t, 7.0, CH_3 of 7-OCH_2CH_3); δ_C 180.0 (−CHO, C-6), 140.0 (C-5), 138.2 (C-2′), 133.2 (C-2), 128.9 (C-4′, C-6′), 127.5 (C-5′), 126.4 (C-3′, C-7′), 124.4 (C-3), 112.1 (C-4), 66.2 (C-7), 64.2 (CH_2 of 7-OCH_2CH_3), 48.8 (C-1′), 15.3 (CH_3 of 7-OCH_2CH_3); positive ESIMS m/z 244.1330 $[M + H]^+$ (calcd for $C_{13}H_{14}NO_2$, 244.1338).

2.2.3. Isolation and purification of macapyrrolins C, A, and G. The reaction mixture was extracted three times with EtOAc. The EtOAc extracts were combined and subsequently washed with water (2×) and brine solution before drying over Na_2SO_4. Then, the extract was dried under reduced pressure to obtain a dark brown residue. Purification of macapyrrolin A was performed by flash chromatography on a normal phase silica gel. Solvents comprised *n*-heptane and EtOAc, and the separation was obtained by gradually increasing the proportion of EtOAc in the gradient. The following instruments were used for fractionation, isolation, and structure elucidation: for flash chromatography, a Reveleris iES system from Grace (Columbia, MD, USA) using the Reveleris Navigator software and commercially packed Claricep flash columns containing 40 g of irregular deactivated silica gel (Agela Technologies, Wilmington, DE, USA); for analytical purposes, an Agilent 1200 series HPLC-DAD system (Agilent Technologies, Santa Clara, CA, USA) equipped with OpenLAB v.A.01.05 software, together with a Phenomenex Kinetex EVO C18 (250 mm × 4.6 mm, 5 μm) column. NMR spectra were recorded on an Avance Nanobay III instrument (Bruker BioSpin, Rheinstetten, Germany). NMR data processing was performed with TopSpin v.4.0.6 from Bruker. Finally, accurate mass measurements were conducted for all isolated compounds using a Xevo G2-XS QToF mass spectrometer (Waters, Milford, MA, USA) coupled to an Acquity UPLC system; an Acquity BEH UPLC column (100 mm × 2.1 mm, 1.7 μm) was used.

2.3. Sample Collection. Method development was conducted using Maca root powder ('Bio-Maca Pulver,' 1 kg), batch number 87619, supplied by Herbis Natura GmbH (Berlin, Germany). Seven representative commercial products available on the Belgian market were purchased and were coded as CP-1 to CP-7. Of these, CP-1, CP-6, and CP-7 were capsule formulations containing Maca powder/extract, conservatives, and additives; CP-2, and CP-3 were tablets; CP-4 a pure powder; and CP-5 a hydroglycerin flour. Among them, CP-2 was the sole finished product in which Maca was mixed with other plant species.

2.4. Instrumentation. *2.4.1. Chromatographic Conditions.* An Acquity ultraperformance liquid chromatography (UPLC) system, consisting of an autosampler and a binary pump (Waters, Milford, MA) equipped with a 10 μL loop, was used. Compounds were separated on an Acquity BEH C18 column (2.1 × 100 mm, 1.7 μm; Waters, Milford, MA). The column and autosampler were maintained at temperatures of 40 and 7 °C, respectively. 5 μL was injected using full loop injection. A flow rate of 0.5 mL/min was chosen, and the following gradient was used: solvent A = water containing 0.1% FA; solvent B = ACN containing 0.1% FA; gradient: 0−1 min (5% B), 1−3 min (from 5 to 15% B), 3−9 min (from 15 to 95% B), 9−10 min (95% B), 10−10.5 min (from 95 to 5% B), 10.5−12 min (5% B).

2.4.2. Mass Spectrometric Conditions. The UPLC system was coupled to a triple quadrupole (TQD) mass spectrometer (Waters, Milford, MA) equipped with an electrospray ionization (ESI) source operated in positive ionization mode. The quantification of the molecular ions (imidazole alkaloids) and the protonated adducts (for all other compounds) was performed by using the multiple-reaction monitoring (MRM) mode to increase sensitivity and selectivity. A quantifier and a qualifier were determined for every compound using direct infusion in the combined mode. The optimal conditions were as follows: capillary voltage 3.5 kV, extractor voltage 3 V, source temperature 140 °C, desolvation temperature 500 °C, RF lens 0.1 V, desolvation gas flow 900 L/h, and cone gas flow 20 L/h. The quadrupole was set to the maximum resolution. All data were recorded and processed using the Quanlynx package in Masslynx software, version 4.1 (Waters, Milford, MA).

2.5. Sample Preparation. The quantification of alkaloids in Maca powder was carried out using the following optimized procedure. Initially, approximately 100 mg of dried powder with internal and secondary standards added was sonicated in 10 mL of 75% methanol (v/v) for 30 min. Concentrations of added standards at the final solution were 50 ng/mL ETCA, 5 ng/mL BMI, and 5 ng/mL DMI. After centrifugation at 9000 rpm (RCF or *G*-force = 9050) for 5 min, the supernatant was transferred to a round-bottom flask. This extraction process was repeated two more times, and the three extracts were combined and subsequently dried under reduced pressure. The resulting dried sample was then reconstituted in 3 mL of water and loaded onto a SPE HLB cartridge (200 mg − 3 mL, Waters, Milford, MA) that had been preconditioned with methanol and water. The column was rinsed with 6 mL of water. Finally, the alkaloids were eluted from the column using 12 mL of methanol. The eluent was subsequently dried under a vacuum before being transferred into a 25 mL volumetric flask. For the first solution, the sample was transferred and brought to the mark using a 5 mM $HCOONH_4$ pH 3 in 10% MeOH buffer. The sample was then further diluted 50-fold to obtain the second dilution. The quantification of macapyrrolin A, macapyrrolin G, and β-carbolines took place in the first dilution, while all lepidilines and macapyrrolin C were quantified in the second dilution.

2.6. Method Validation. Validation was performed according to the ICH guidelines for physicochemical method validation and SANTE/11312/2021 guideline for LC-MS related specifications issued by the European Union. The following validation criteria were investigated: selectivity, linearity, LOD, LOQ, extraction recovery, accuracy, precision, matrix effect, and robustness.

2.6.1. Selectivity. Selectivity was determined using the optimal UPLC-TQD-MS/MS conditions, and the obtained signals were compared with those of the standard mixture in terms of retention time, precursors, and product ions. The evaluation of selectivity involved identifying both quantitative and qualitative transitions for each compound. The selectivity of a compound is determined by the ratio between the quantifier and the qualifier. This ratio was examined for every compound in the primary standard solutions and processed samples.

2.6.2. Linearity, Response Factor, and Calibration Model. Linearity was first examined in a wide concentration range for

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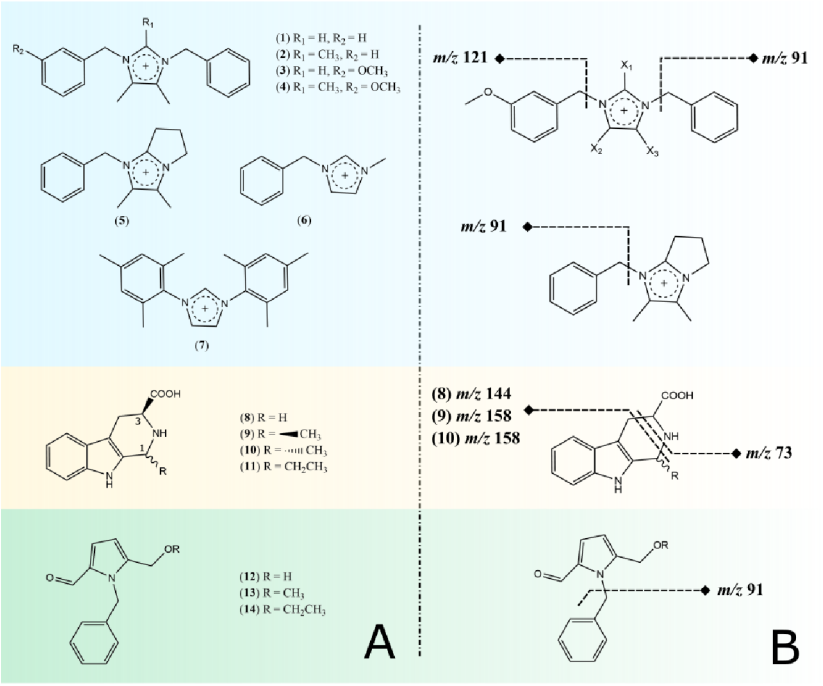

Figure 1. (A) Structures of compounds **1**−**14**: (1) lepidiline A, (2) lepidiline B, (3) lepidiline C, (4) lepidiline D, (5) lepidiline E, (6) 1-benzyl-3-methylimidazolium chloride (BMI) (SS), (7) 1,3-dimesitylimidazolidinium chloride (DMI) (IS), (8) 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (TCCA), (9) 1-(*R*)-methyl-1,2,3,4-tetrahydro-β-carboline-3-(*S*)-carboxylic acid (RS-MTCA), (10) 1-(*S*)-methyl-1,2,3,4-tetrahydro-β-carboline-3-(*S*)-carboxylic acid (SS-MTCA), (11) 1-ethyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (ETCA) (IS), (12) macapyrrolin C, (13) macapyrrolin A, and (14) macapyrrolin G. (B) Characteristic fragmentation patterns of all compounds.

all compounds (0.1−1000 ng/mL) to identify linear ranges. Regression analysis of the concentration and peak area was performed with and without log−log transformation. Analysis of variance, significance test of regression coefficient, and homoscedasticity were examined and compared between the two models.

After obtaining linear ranges, response factors between the lepidilines (**1**−**5**) and BMI (**6**) were established as the ratio between a compound of interest and BMI at the same concentration, calculated from two independent linearity data of 2 days.

2.6.3. Limit of Detection (LOD) and Limit of Quantification (LOQ). LODs and LOQs were identified at the concentrations that provided signal-to-noise ratios higher than 3 and 10, respectively, from three consecutive injections.

2.6.4. Matrix Effect. Matrix effects were first investigated using dilution tests. A series of five dilutions (dilution factor of 3) were analyzed, and calibration curves of lepidilines were compared. The procedure was carried out for a sample prepared from 500 mg of Maca powder.

2.6.5. Extraction Recovery. Recovery of the extraction procedure was examined for BMI (**6**) at two different spike concentrations, each in triplicate. Known amounts of BMI were spiked to Maca powder before performing the adapted extraction procedure. Recovery was calculated based on the obtained BMI compared to the spiked amount.

2.6.6. Precision. To determine the intermediate precision, six independently prepared samples weighing 100% (100 mg) were analyzed by using the method described above. This procedure was repeated on three different days. To assess repeatability across different concentration levels, six samples weighing 50% (50 mg) and six samples weighing 200% (200 mg) of the nominal mass were also analyzed. Standard mixture solutions were prepared daily: imidazole alkaloids (0.015−250 ng/mL), β-carbolines (0.060−1000 ng/mL), and pyrrole alkaloids (0.030−500 ng/mL). The standard mixture solutions were injected twice: once before the samples for calibration and once at the end of the sequence to assess the stability of the MS signal.

As for macapyrrolin G, the same set of samples above was used, and samples were spiked at three concentration levels (50%, 100%, and 200%). Among these levels, the 50% concentration was specifically chosen to align with the LOQ level.

2.6.7. Accuracy. To investigate the accuracy of the method, a recovery experiment was performed. To 50% of the plant material (50 mg) was added a known amount of the standards until a total amount of around 75%, 100%, and 125% was reached. For each level, samples were prepared in triplicate according to the described procedure.

2.6.8. Robustness. Three independent samples weighing 100% (100 mg) were prepared for this investigation. The robustness of the validated method was examined for a minor variation of column temperature (38−42 °C) and a different column batch. Quantitative results between the optimal and changed methods were compared. The method was considered robust if deviation between them was within ±10%.

2.7. Statistical Analysis. Statistical analyses were performed by Excel 2016 with the add-in Analysis ToolPak. Design of the experiment (central composite design) was modeled by JMP Pro 16.0.0 (JMP Statistical Discovery LLC).

3. RESULTS AND DISCUSSION

3.1. Method Development. *3.1.1. Optimization of Mass Spectrometric Parameters.* Initially, all compounds were analyzed in both positive and negative electrospray ionization

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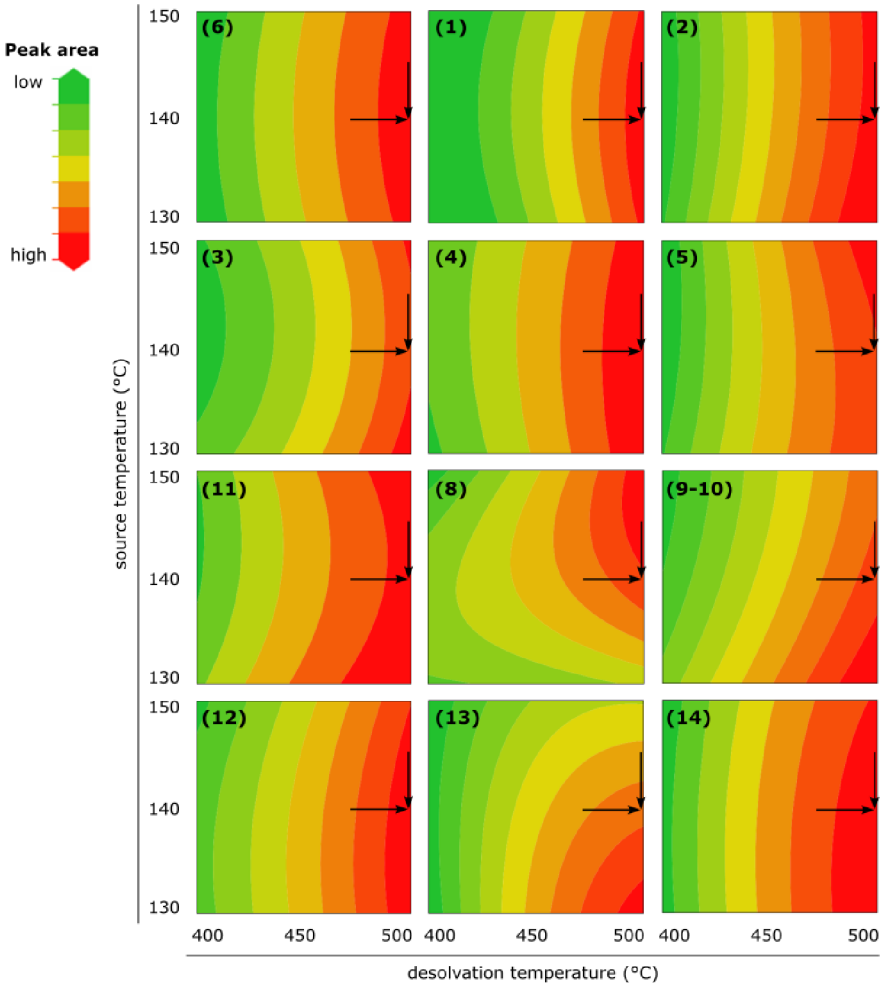

Figure 2. Contour plots of all target compounds obtained from the central composite design for the optimization of the source temperature (130−150 °C) and desolvation temperature (400−500 °C). Color gradient from green to red represents the increase of peak area as the optimized instrumental response.

(ESI) modes. The imidazole compounds (**1**−**7**) exclusively exhibited MS responses in positive mode, whereas the *β*-carbolines (**8**−**11**) and pyrroles (**12**−**14**) displayed signals in both modes (see Figure 1). The absence of MS signals in the negative mode for the imidazoles can be attributed to the positively charged nature of the quaternary imidazole moiety. Interestingly, this phenomenon also explains the exceptional sensitivity of imidazoles to mass spectrometry as these compounds are inherently charged and do not require additional ionization for detection. Molecular ions $[M]^+$ can be detected for the imidazoles, while the pyrroles and *β*-carbolines showed the highest intensity as protonated adducts $[M + H]^+$.

For the optimization of cone and collision voltages to be used in a multiple-reaction monitoring (MRM) method, each standard was directly infused into the mass spectrometer in positive ESI mode. A quantifier (transition with the highest intensity) and a qualifier (transition used for identification) were identified for each compound. Table S1 presents the optimal cone and collision energies for obtaining the transitions of interest. Characteristic fragmentation patterns were identified for all three classes of compounds, and Figure 1 demonstrates the stable and most intense fragments formed. The m/z 91 fragment was observed for lepidilines **1**−**6** and macapyrrolins **12**−**14**, corresponding to the benzylic moiety, while the m/z 121 fragment was observed only for lepidilines C and D (**3** and **4**), corresponding to the methoxylated benzylic moiety. The neutral loss of 73 Da is characteristic for the *β*-carbolines, resulting in the major fragments of m/z 144 for TCCA (**8**) and m/z 158 for RS-MTCA (**9**) and SS-MTCA (**10**).

In the next step, the final experimental conditions for instrument operation, including capillary voltage, RF lens, source temperature, desolvation temperature, and desolvation flow, were optimized using a design-of-experiment approach. Initially, a screening study was performed with the aim of selecting the most influential parameters and reducing the complexity of the study. To achieve this, a standard mixture consisting of target compounds and internal and secondary standards was screened by applying the established MRM method and the optimized chromatographic conditions described in Section 2.4.1, which later revealed the significant influence of temperature on the peak area. For this reason, a central composite design was employed to optimize the two critical parameters: source temperature and desolvation temperature. The remaining parameters were fixed as follows: capillary voltage (3.5 kV), RF lens (0.1 V), and desolvation gas flow (900 L/h). As can be seen in the contour plots in Figure 2, the variation of the peak area obtained for target compounds is presented based on the modification of the two influential

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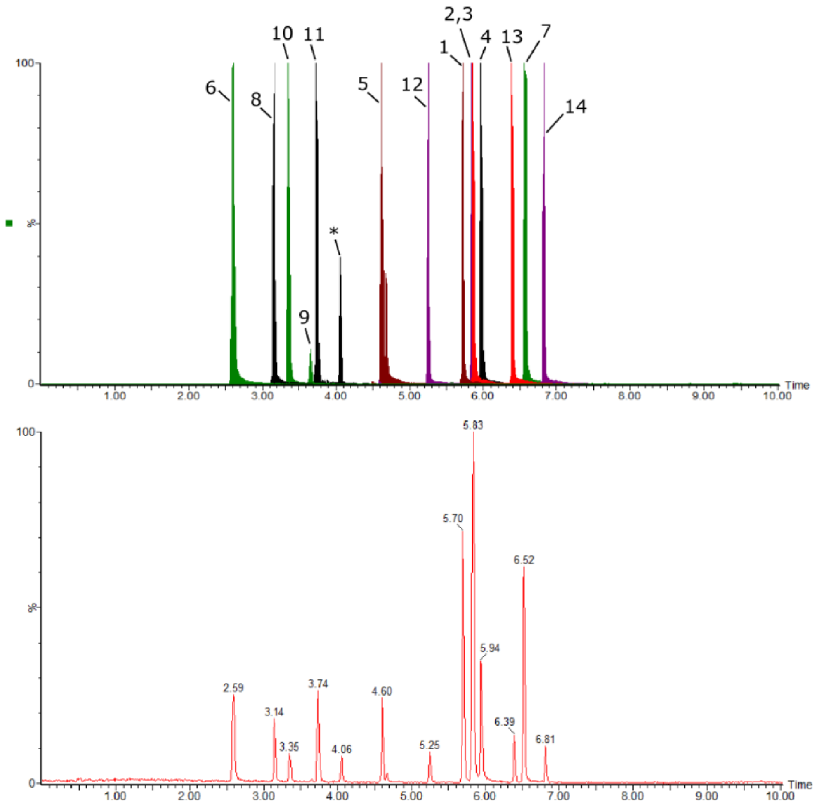

Figure 3. Normalized EICs (top) and MRM chromatogram (bottom) of the optimal separation achieved for all compounds under the chromatographic conditions described in Section 2.4.1. Co-elution occurs for lepidilines B and C (**2** and **3**). Commercial ETCA used in this study is a mixture of RS-ETCA and SS-ETCA. SS-ETCA, denoted as * on the chromatogram, is not included in the method.

factors, i.e., the source temperature (130−150 °C) and desolvation temperature (400−500 °C). The desolvation temperature displayed a much greater impact on the area than the source temperature under the tested conditions. The higher the desolvation temperature, the greater the area, whereas the influence of source temperature was solely observed for TCCA (**8**), MTCA (**9** and **10**), and macapyrrolin A (**13**). The optimal point (source temperature of 140 °C and desolvation temperature of 500 °C) provided the largest peak area for most of the target compounds (red zones) and reasonably high instrumental responses for quantifying TCCA (**8**), MTCA (**9** and **10**), and macapyrrolin A (**13**) (red-orange zones).

3.1.2. Optimization of Chromatographic Conditions. At first, preliminary screening of chromatographic conditions was conducted for acidic (FA 0.1%) and basic (NH_4OH 0.1%) mobile phases with three different columns (BEH C18, BEH C18 Shield, and HSS C18). The choice of 0.1% FA was straightforward, since peak splitting was observed for the β-carbolines when 0.1% NH_4OH was used as an additive. This can be explained by the presence of a carboxylic group in the structures of the β-carbolines. On the other hand, FA could provide sharp peaks as none of the target compounds possesses strong basic properties. In all cases, the nitrogen is involved in an aromatic ring system where its free electron pair participates in the conjugated system instead of playing an electron-donating role. For the selection of the column, no significant difference was found between the tested ones. The BEH C18 was chosen due to its ubiquity.

The chromatographic separation was investigated with respect to the resolution of the peaks and time effectiveness. The target compounds exhibit a wide range of polarities: the β-carbolines are the most hydrophilic, while the lepidilines and the macapyrrolins are more hydrophobic. Therefore, the gradient slope is gradually increased in the first half of the chromatogram, allowing an adequate retention of the β-carbolines and steadily increased in the second half, allowing the elution of the lepidilines and the macapyrrolins. Results indicated a chromatographic separation of all analytes, except for lepidilines B (**2**) and C (**3**) (Figure 3). Separation of these two compounds cannot be obtained with FA as the additive regardless of the gradient slope and the column used. While our previous study reported the use of a mixture of ammonium acetate and formic acid as an additive for isolation of the two compounds, we opted for FA for reasons of simplicity and routine analytical application, considering the fact that chromatographic separation is not mandatory for an MRM method. Also, FA is commonly used in positive ionization mode to enhance the instrument response.

3.1.3. Optimization of Sample Preparation Procedure. The sample preparation included extraction and purification. Sonication-assisted extraction was initially tested due to its high extraction efficiency and cost effectiveness. Since the stability of the compounds of interest remained unaffected by sonication, this extraction method was adopted. As no marked changes of ultrasonic extraction efficiency were observed when the extraction time exceeded 30 min, the optimal extraction conditions were determined as 30 min ultrasonic extraction with 2−3 time manual shaking (repeated three times). Knowledge obtained from our reported phytochemical study indicated the difference in polarity of the target compounds: the imidazoles and the pyrroles were isolated from the

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chloroform extract (lipophilic) and the β-carbolines from the *n*-BuOH extract (relatively hydrophilic). Therefore, optimization of the extraction solvent was required. To this end, six ratios of the aqueous/methanolic extraction solvents were evaluated with an increasing percentage of MeOH i.e., 0−20−40−60−80−100% MeOH. As a result, it was observed that the amount of β-carbolines was low when pure MeOH was used, indicating the necessity of adding a certain amount of water in the extraction solvent, while an obvious decline in the amount of the lepidilines was observed when H_2O, 20% MeOH, and 40% MeOH were used, implying the need of a high proportion of organic solvent. Considering the practicality and the fact that lepidilines are the major components among the alkaloids, 75% MeOH was selected as the optimal extraction solvent.

The impact of the plant sample matrix on the intensity of the signal was assessed through SPE purification and dilution experiments. For solid-phase purification, several types of SPE cartridges were evaluated, including Oasis HLB, Oasis MCW, Chromabond C18, Chromabond HR-XC, and Chromabond HR-XCW. All wash and elution solutions were analyzed using MS full scan mode with an *m*/*z* range of 100−900. Among them, the Oasis HLB demonstrated a straightforward procedure and satisfactory efficacy in removing impurities, particularly polar ones (see Tables S6 and S7 and Figures S22−S24). It appears that a larger linearity range was obtained by removing very polar impurities and some highly lipophilic components, evidenced by the results of the dilution test (Figures S22 and S23) and the MS scan chromatogram of the wash solution from the Oasis HLB (Figure S24). Furthermore, the matrix effect was examined on finished product matrices (commercial products analyzed at the end of the study), with the Oasis HLB cartridge exhibiting superior cleaning capabilities. Consequently, the use of Oasis HLB was adopted for samples encompassing a wide variety of matrices. As a final step, the extraction recovery of the adapted protocol was examined for BMI (**6**) at two concentrations, yielding an average recovery of 94%.

The optimal amount of Maca powder, designated as 100% level, was established at 100 mg following an evaluation of different powder weights ranging from 50 to 500 mg. This quantity represents the minimum amount of plant material at which the presence of minor compounds (**9**, **10**, **13**) can be accurately quantified without any interference from the plant sample matrix. Compound **14**, while previously identified in Maca, was not even detected during the analysis of the 500 mg powder. Thus, this compound was spiked to Maca sample when validating the method.

At this point, screening results showed that the concentrations of imidazoles (**1**−**5**) and macapyrrolin C (**12**) in pure Maca powder are approximately 10−50 times higher than those of the minor compounds. Additionally, the imidazoles exhibit a much higher sensitivity, roughly 3−5 times more than that of the other compounds, which poses challenges in accurately and plausibly quantifying all compounds within a single sample. Hence, we adopted the sample preparation procedure as depicted in Figure 4, wherein two dilutions were analyzed for each sample: one for quantifying the minor compounds (first dilution in a 25 mL volumetric flask; compounds **8**−**11**, **13**, **14**) and another for quantifying the major compounds (second dilution = 50-fold diluted solution; compounds **1**−**7**, **12**). After drying, a buffer (5 mM $HCOONH_4$ in 10% MeOH (pH 3)) was used for reconstituting and diluting samples for the following reasons:

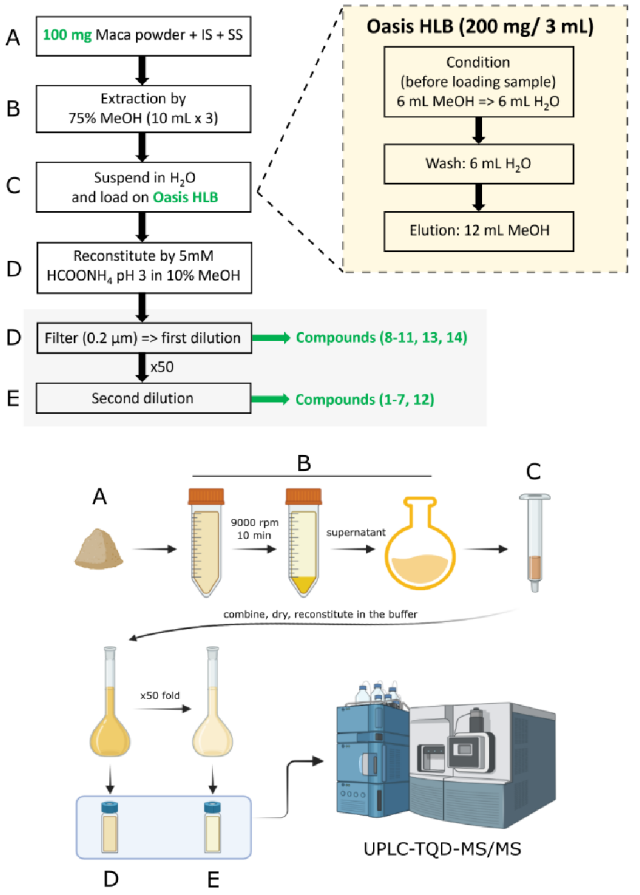

Figure 4. Sample preparation procedure applied in this study with a double-dilution strategy.

(1) in the case of MeOH proportions higher than 20%, the peak shape of the early eluting peaks was affected; (2) an acidic medium allows the dissolution of the alkaloids in 10% MeOH; (3) the use of the buffer allows a relatively matrix-matched quantification when samples are adequately diluted.

3.1.4. Selection of Internal and Secondary Standards. Internal and secondary standards were chosen with respect to their structural similarity with the target compounds and commercial availability. A secondary standard is a substance whose content has been compared and validated against a primary standard and is mainly used in case of the absence of the primary standard for routine analytical analysis. Since the quaternary imidazole nucleus determines the behavior of the lepidilines in the TQD (triple quadrupole detector), it is required that this moiety is maintained. BMI, DMI, and DDI possess this moiety and are readily commercially available. After preliminary screening with the optimal operational conditions, BMI was selected as the secondary standard and DMI as the internal standard (IS) for the lepidilines, based on fragmentation pathways and chromatographic behavior. Only the lepidilines require a secondary standard since they are not commercially available. Primary standards are commercially available for TCCA (**8**) and MTCA (**9** and **10**), and the macapyrrolins (**12**−**14**) were obtained in gram scale following a two-step synthetic scheme (see Section 2.3). A synthetic β-carboline, ETCA, was also used as the IS after checking for its absence in Maca raw powder. To compromise with the two-dilution strategy, as described in Section 2.5, ETCA was considered the IS of the first dilution and DMI of the second

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dilution. Macapyrrolins A and G were identified in the first dilution, and their contents were corrected based on ETCA. Similarly, macapyrrolin C (**12**) was quantified in the second dilution and, therefore, its recovery was calculated according to DMI.

3.2. Method Validation. The method was validated in terms of selectivity, linearity, LOD, LOQ, repeatability, recovery, and robustness. Identification of target compounds was confirmed: the same retention time, quantifier, and qualifier were observed for each compound in both processed samples and standard mixture, except for macapyrrolin G (**14**), which was not detected in commercial Maca samples.

For validation, concentrations of the target compounds were not equal in the standard mixture and were adjusted according to their sensitivity to avoid overloading the MS detector and contaminating the instrument. Specifically, in all dilutions of the standard mixture, concentrations of the β-carbolines doubled those of the macapyrrolins and were four times higher than those of the lepidilines. Logarithmic transformation of concentration and peak area prior to performing regression analysis provided extended linear ranges and fulfilled homoscedasticity. The 11 analytes demonstrated good linearity ($R^2 \geq 0.9991$) in a wide concentration range (see Table S2). In parallel, response factors were established for the lepidilines from linearity data of two different days by comparing instrumental responses of each compound with BMI (**6**) at the same concentrations (Table 1). The response

Table 1. Response Factors Established for Lepidilines Compared to BMI (109−56 250 pg/mL)

	SS-day1	SS-day2	mean-SS
lepidiline A (**1**)	2.01	2.24	2.12
lepidiline B (**2**)	2.31	2.44	2.38
lepidiline C (**3**)	0.57	0.60	0.59
lepidiline D (**4**)	0.55	0.66	0.60
lepidiline E (**5**)	0.58	0.61	0.60

factors were stable in the concentration range of 109−56 250 pg/mL. It is noteworthy that the response factors slightly varied on a day-to-day basis but still remained comparable.

The LOD and LOQ of all compounds were determined, revealing varying sensitivity. The lepidilines were the most sensitive (LODs < 0.05 ppm = pg/mL scale), while the β-carbolines were the least sensitive (LODs ≈ 1 ppm). Lepidilines A−D, due to their high content in samples and outstanding sensitivity, always appeared as the most intense peaks in any sample chromatogram in this study. Compared to major compounds reported from Maca, the total content of lepidilines A−E (around 400 ppm in this study) is relatively close to those previously reported for macamides and macaenes (80−3000 ppm),[22,23] thus providing a new insight into the chemical composition of Maca. Notably, it was reported that macamides and macaenes are byproducts of air-drying process according to Xia and Esparza, while freeze-dried Maca contains solely around 80 ppm of macamides and macaenes,[23,24] which is significantly lower than the lepidiline content. Based on this result, we suggest considering the lepidilines as analytical chemical markers for the identification of Maca. Our previous phytochemical study already confirmed the exclusive existence of lepidilines in Maca, together with macamides and some macaenes.[1,10] Literature research supports this finding. More specifically, in *Lepidium sativum*, a known species in the *Lepidium* genus, no quaternary imidazole alkaloid is reported so far.[25] Furthermore, regarding the sensitivity to MS detection, lepidilines are much more sensitive to MS detection than macaenes and macamides (fatty acid derivatives), allowing the detection of very small amounts.

The intraday precision (run-to-run precision) showed RSDs < 8% for all compounds. For the interday precision (day-to-day precision), RSDs of all compounds were lower than 15%, except for macapyrrolin A (Table S3). Nonetheless, since macapyrrolin A appears to be a minor compound in comparison with the others, it is not deemed necessary to increase the amount of plant material for analysis, risking the impact of matrix effects and possible contamination of the MS instrument. Accuracy was evaluated by a recovery study. The mean recovery of all analytes at three spiked concentration levels ranged from 82.4 to 122.4% (Table S3), meaning the method was fully validated.

Minor shifts of retention time (±0.3 min) were observed with slight changes of column temperature or when another BEH C18 column (of a different production batch) was used. Quantitative results, however, remained within an RSD of 5%. Thus, the applicability of the method when the column was changed or with minor temperature variations in the column chamber was ensured.

3.3. Application of the Validated Method on Commercial Food Supplements. Although in general the use of Maca is considered as safe, cytotoxicity has been reported for some of the imidazole alkaloids, including lepidilines A and B.[5] In addition, some local publications from Peru document the toxicological effects of Maca on cell cultures and on mice.[2] Among all alkaloids, the toxicity and pharmacological activities of β-carbolines have been studied extensively. On the one hand, some β-carbolines exhibited potent neuropharmacological, antitumor, antioxidant, anti-inflammatory, and antimicrobial effects.[26,27] On the other hand, some β-carbolines were reported as comutagens or precursors of mutagens, neurotoxin, and carcinogens, such as norharman, harman, 1-trichloromethyl-1,2,3,4-tetrahydro-β-carboline, and aminophenyl-β-carboline derivatives.[27,28] Although not directly related to Maca, a case of β-carboline alkaloid intoxication was reported, following the digestion of *Peganum harmala* seed extract.[29]

A range of food supplements, available on the Belgian market, were analyzed for its alkaloidal constituents with the newly developed and validated method. The total alkaloid content was expressed as the overall content of the target compounds. As seen in Figure 5, the lepidilines (**1**−**5**) and macapyrrolin C (**12**) were the major alkaloids.

In Maca raw powders (CP-4, CP-5, and CP-8), the total alkaloid content varied from 418 to 554 ppm (mg/kg), of which the lepidiline content (**1**−**5**) ranged from 323 to 470 ppm, and the macapyrrolin C content (**12**) varied between 50 and 75 ppm, thus representing the major alkaloids in Maca. Macapyrrolin A (**13**) was a minor alkaloid with a content of approximately 1 ppm. Macapyrrolin G (**14**) was not detected in any sample. Detailed quantitative results are tabulated as Supplementary data in Table S4. As for the other commercial preparations (finished products) (CP-1, CP-2, CP-3, CP-6 and CP-7), the total alkaloid content varied from 50 to 600 ppm. CP-2 contained the lowest alkaloid content, which could be expected since CP-2 was a mixture of several plant species. CP-7 was low in lepidilines, indicating a possible influence of different processing and preparation steps of the finished

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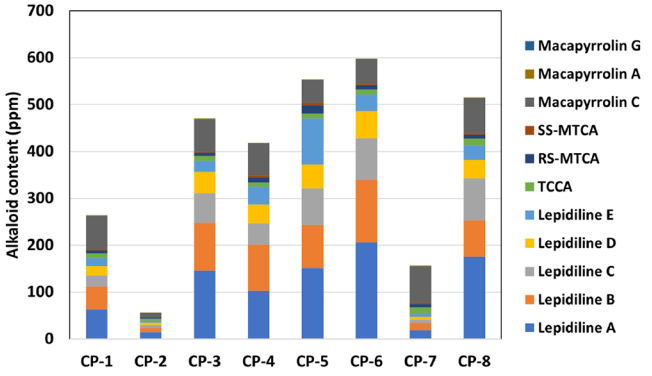

Figure 5. Alkaloid content in commercial products (ppm, mg/kg) estimated by primary standards (n = 3).

products. Influence of different harvest times and locations might also be responsible for the observed differences in the quantitative alkaloid content of the products.

The toxicity and pharmacological properties of β-carbolines were the most well-studied among all alkaloid classes, as mentioned in Section 1. The current investigation of Maca-containing products revealed that the total β-carboline content ranged from 20 to 30 ppm in all samples. TCCA (**8**) exists as a single isomer, while the two diastereoisomers of MTCA (RS-MTCA (**9**) and SS-MTCA (**10**)) were found in all samples. It also appears that the RS-MTCA content is roughly three times higher than the SS-MTCA content.

Table S5 shows the alkaloid content obtained from the established response factors using BMI (**6**) as a secondary standard. Most quantitative errors were within 15% in comparison with those calculated by primary standards. Hence, the use of BMI as a secondary standard for the lepidilines could be considered as a valid alternative to the primary standard. With the obtained results in our analysis, the use of the averaged response factors as shown in Table 1 in all cases led to quantitative errors of less than 15%.

4. CONCLUSIONS

In recent years, Maca-containing food supplements have become ubiquitous in the European market. This raises a need for quality control of these products since some alkaloids isolated from Maca were reported as potentially cytotoxic agents. However, the lack of commercial standards for lepidilines and macapyrrolins hindered the development of an analytical method. The present work is the first report of an accurate, rapid, sensitive, and comprehensive method for quality control of the most abundant alkaloids in raw material and Maca-containing food supplements. Eleven alkaloids, belonging to three different classes (imidazole, pyrrole, and β-carboline alkaloids), were simultaneously determined, resulting in the total alkaloid content in Maca raw powder (418−554 ppm (mg/kg)). Nine alkaloids were identified as the main alkaloids (the lepidilines, the β-carbolines, and macapyrrolin C), accounting for approximately 95% of the total alkaloid content, and two others as minor components (macapyrrolins A and G), accounting for less than 5%. This method was fully validated and offered good linearity, accuracy, repeatability, precision, and robustness. More importantly, we also validated in parallel the use of a secondary standard (BMI) to quantify lepidilines. In addition, a simple, easy-to-scale-up synthetic scheme of pyrrole alkaloids was also proposed. Thus, this validated UPLC-TQD-MS/MS method represents the first and most valuable method for both qualitative and quantitative assays of major alkaloids in Maca. The authenticity and adulteration of Maca can also be investigated by using the current validated method. The exclusive existence of lepidilines in Maca and the permanently positively charged nature of the quaternary imidazole ring leads to an excellent sensitivity of the lepidilines toward MS detection in positive ion mode. Therefore, the lepidilines represent excellent chemical markers for characterization of Maca in all types of commercial preparations (raw material, mixtures, monopreparations, poly preparations). From a regulatory point of view and based on safety concerns about the alkaloidal constituents of *L. meyenii*, it may be advisable to establish a maximum level of particular alkaloids or alkaloid classes in food supplements containing Maca, rather than to request their total absence.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c09356.

Validation results, the synthesis scheme of macapyrrolins, and full NMR data; representative chromatograms of the standard mixture (Figure S1) and CP-1−CP-8 (Figures S2−S9); calibration curves of lepidiline A (**1**), lepidiline B (**2**), lepidiline C (**3**), lepidiline D (**4**), lepidiline E (**5**), BMI (**6**), TCCA (**8**), RS-MTCA (**9**), SS-MTCA (**10**), macapyrrolin C (**12**), macapyrrolin A (**13**), macapyrrolin G (**14**) (Figures S10−S21); representative linear regression of lepidiline A in the dilution test with SPE (Figure S22), without SPE (Figure S23); ms scan of wash and elution solutions when the Oasis HLB was tested (Figure S24); synthetic scheme of macapyrrolins C, A, and G (**12**−**14**) (Figure S25); ^{1}H, ^{13}C, DEPT135, DEPT90, COSY, HMBC spectra of macapyrrolin C (**12**) in $CDCl_3$ (Figures S26−Figure S29); ^{1}H, ^{13}C, DEPT135, DEPT90, COSY, HMBC spectra of macapyrrolin A (**13**) in $CDCl_3$ (Figures S30−Figure S33); ^{1}H, ^{13}C, DEPT135, DEPT90, COSY, HMBC spectra of macapyrrolin G (**14**) in $CDCl_3$ (Figures S34−Figure S37) MS/MS transitions, cone and collision energies selected for the target compounds, internal and secondary standards (Table S1); method validation parameters (linearity), for quantitative and qualitative determination and (precision, accuracy) for quantitative determination of target compounds (Tables S2 and S3); alkaloid content in commercial products (ppm) (Table S4); lepidiline content in commercial products (CP) in ppm (Table S5); ratios of peak area obtained for a series of 3-fold dilutions without SPE and with SPE (Tables S6 and S7); extraction recovery of BMI at two final concentrations (Table S8) (PDF)

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N.-T.-H.L. contributed to conceptualization, performing all experiments, data analysis, and writing the manuscript; K.F., M.T., and T.N. contributed to data analysis and proofreading the manuscript; M.B. and P.V.D.V. contributed to synthesis work; L.P. and E.T. contributed to supervision and proofreading the manuscript.

Notes

The authors declare no competing financial interest.

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