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ACCELERATED COMMUNICATION

Analysis of macamides in samples of Maca (*Lepidium meyenii*) by HPLC-UV-MS/MS

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The macamides are a distinct class of secondary metabolites that have so far been found only in *Lepidium meyenii* Walp. (Maca). Using HPLC-UV-MS/MS, the main macamides have been identified as *n*-benzylhexadecanamide, *n*-benzyl-(9*Z*)-octadecenamide, *n*-benzyl-(9*Z*, 12*Z*)-octadecadienamide, *n*-benzyl-(9*Z*, 12*Z*, 15*Z*)-octadecatrienamide and *n*-benzyloctadecanamide. The identities of *n*-benzyl-(9*Z*)-octadecenamide and *n*-benzyl-(9*Z*, 12*Z*)-octadecadienamide were confirmed by comparison of chromatographic and spectral properties with synthetic analogues. Total macamides have been quantified by HPLC-UV in plant material from different vendors using *n*-benzylhexadecanamide as an external standard. The amount of macamides in the dried plant material ranged from 0.0016 to 0.0123%. Copyright © 2005 John Wiley & Sons, Ltd.

Keywords: HPLC-UV-MS/MS; qualitative analysis; quantitative analysis; macamides; *Lepidium meyenii*.

INTRODUCTION

Maca (*Lepidium meyenii* Walp. synonym *L. peruvianum* Chacon, Brassicaceae), a root crop similar to radish (*Raphanus sativus* L.), has been consumed in Peru for thousands of years as food and medicine (Valentovà *et al*., 2001). The plant is used to increase human and livestock stamina and to ameliorate fertility problems associated with living at the high elevations where the plant grows (Leon, 1964). The reputation of Maca as a fertility enhancer has increased the popularity of the plant in the USA and other Western countries (Kilham, 2000). Recent studies (Zheng *et al.*, 2000; Cicero *et al.*, 2001; Gonzales *et al.*, 2003, 2004; Chung *et al*., 2005) have confirmed the effectiveness of Maca as a libido and fertility enhancer. Maca, however, has many other traditional therapeutic uses, e.g. as a laxative and for the treatment of rheumatism, respiratory problems, premenstrual discomfort, and menopausal symptoms (Kilham, 2000). Consumption of Maca is reputed to regulate hormone secretion, stimulate metabolism, improve memory and combat depression, anaemia, leukaemia, AIDS, cancer and alcoholism (Quiros and Aliaga, 1997). Even with the reputation of Maca as a panacea, scientific research has focused primarily on the sexually stimulating properties of the plant.

Dried Maca hypocotyls contain several classes of secondary metabolites of interest including alkaloids, amino acids, glucosinolates, fatty acids and macamides (Dini *et al*., 1994; Zheng *et al*., 2000; Ganzera *et al*., 2002; Piacente *et al*., 2002). Some of the aphrodisiac activities of Maca have been related to the lipidic fraction of Maca, which contains mainly fatty acids and macamides (Zheng *et al.*, 2000). To date, seven macamides have been characterised (Muhammed *et al.*, 2002; Zhao *et al*., 2004), but no quantitative information concerning the amounts of macamides in Maca extracts has been reported. Using HPLC-UV-MS/MS, macamides in root material of *Lepidium meyenii* derived from different sources have been characterised and the macamides have been quantified using synthetic *n*-benzylhexadecanamide (**1**) as standard.

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EXPERIMENTAL

Plant material. Whole *Lepidium meyenii* hypocotyls (voucher No. TOM02006) were obtained from a local market in Pisac, Peru and identified by Megan McCollom. Ground *Lepidium meyenii* hypocotyls were sourced from four different vendors: San Francisco Herb and Natural Food Co. (Vendor 1; Fremont, CA, voucher No. TOM02009), Imperio del Sol (Vendor 2; Cusco, Peru, voucher No. TOM02008), Maca Sanson (Vendor 3; Junin, Peru, voucher No. TOM02007), and Nature's Way Maca root capsules (Vendor 4; Springville, UT, lot No. 340164). The identity of the powdered plant material was confirmed by a comparison of the phytochemical profile with the whole Maca hypocotyls. All voucher specimens have been deposited at the Tom's of Maine herbarium.

Solvents and chemicals. Acetonitrile, reagent alcohol and petroleum ether were of HPLC grade, and purchased from Fisher (Pittsburgh, PA, USA). Trifluoroacetic acid, diethyl ether, sodium hydroxide, benzylamine, oleoyl chloride (85% pure) and palmitoyl chloride, as well as the fatty acid standards, were obtained from Sigma (St Louis, MO, USA).

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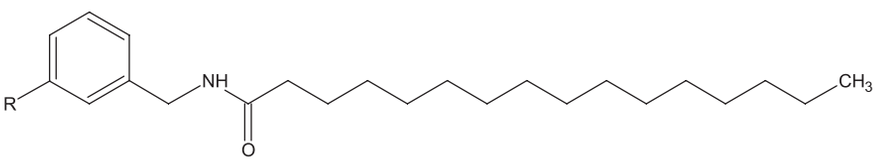
Extraction method. The petroleum ether extracts were prepared by extracting dried, ground *Lepidium meyenii* hypocotyls at a 1:5 (w/v) ratio of plant material to solvent. The plant material was extracted on an Innova 2000 platform shaker (New Brunswick Scientific, Edison, NJ, USA) for 24 h at 150 rpm. Subsequently, the extracts were filtered and evaporated to dryness. For quantitative analysis, three samples of each raw material were extracted as indicated above and quantified by HPLC-UV.

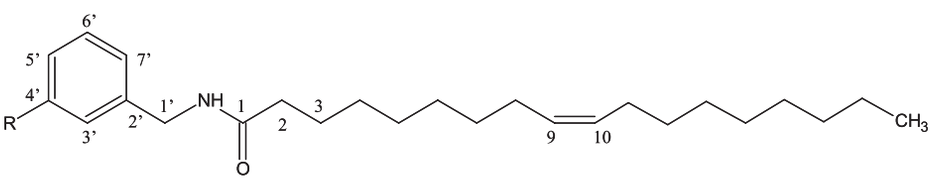
Preparation of *n*-benzylhexadecanamide (1). Palmitoyl chloride (2.70 mL, 0.008 mol) was placed in a 10 mL round bottom flask, which was attached to a Claisen adapter, and diethyl ether (10 mL) was injected through the Claisen adapter septum. The mixture was stirred until a homogeneous solution was obtained. Subsequently, anhydrous diethyl ether (1.33 mL) was added to a vial containing chilled benzylamine (1.772 mL, 0.016 mol). The cold benzylamine was injected drop-wise into the reaction vial through the septum of the Claisen adapter over a 15 min period, at the end of which a white precipitate was formed. The reaction mixture was stirred for 10 min, 7 mL of 10% sodium hydroxide was added, and the mixture stirred for an additional 15 min. The lower aqueous layer was discarded, and a further portion (7 mL) of 10% sodium hydroxide added and mixed for 5 min. The lower aqueous layer was discarded, and 1 mL of anhydrous diethyl ether was added to the mixture. The ether solution was extracted with 10% hydrochloric acid (2 mL). Finally, the precipitate (ca. 96% yield) was washed with ether, and the remaining solvent evaporated. The spectroscopic data (MS, ^{1}H- and ^{13}C-NMR) of the product corresponded to data previously published for *n*-benzylhexadecanamide (Muhammad *et al*., 2002).

Preparation of *n*-benzyl-(9*Z*)-octadecenamide (2). Oleoyl chloride (3.10 mL, 0.008 mol; 85% pure, with small amounts of linoleoyl chloride and stearoyl chloride) was placed in a 10 mL round bottom flask, which was attached to a Claisen adapter, and diethyl ether (10 mL) was injected through the Claisen adapter septum. The same reaction steps were followed as for the preparation of **1**. Finally, a light brown precipitate (2.86 g) was obtained in ether. After evaporation of the solvent, the product was analysed by HPLC and was found to consist mainly of **2** (85%) together with 7% of *n*-benzyl-(9*Z*, 12*Z*)-octadecadienamide (**3**) and 2% of *n*-benzyloctadecanamide (**4**). The identity of **2** was confirmed by ^{1}H and ^{13}C NMR analysis (the alkyl chain was identified based on literature data published for fatty acid esters (Gunstone *et al.*, 1977; Su *et al.*, 2002), and high-resolution MS data).

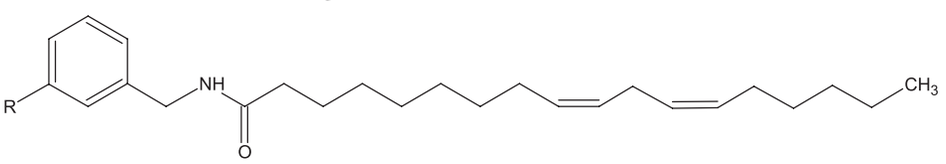
HPLC-UV and HPLC-MS/MS analysis of extracts. An 1100 series HPLC system consisting of an Agilent Technologies (Burlington, MA, USA) quaternary pump,



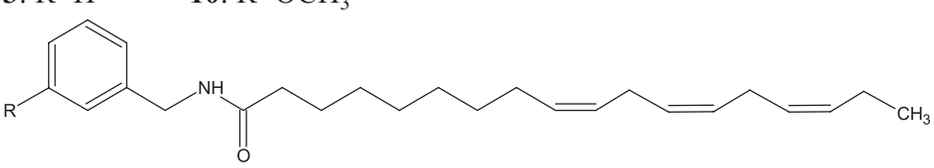
1: R=H **5**: R=OCH_3



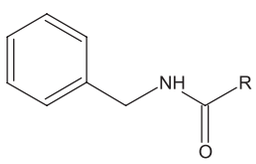
2: R=H **9**: R=OCH_3



3: R=H **10**: R=OCH_3



8: R=H **11**: R=OCH_3



4: R=$C_{17}H_{35}$ **6**: R= $C_{14}H_{29}$ **7**: R= $C_{16}H_{33}$

UV–vis detector (DAD), degasser, automatic sample injector and an Agilent 1100 series LC/MSD trap was used. Quantitative and qualitative analysis of macamides was performed using a Zorbax XDB C-18 column (250 × 4.6 mm i.d.; 5 μm particle size, Agilent Technologies, Burlington, MA, USA.). The solvent system consisted of (A) water containing 0.005% trifluoroacetic acid and (B) acetonitrile containing 0.005% trifluoroacetic acid, using a gradient of 20:80 (A:B) to 0:100 in 24 min, after which the column was washed with 100% B for 6 min. The flow rate was set at 0.8 mL/min, and the column temperature was 40°C. Macamides were quantified at 210 nm using an external standard method. Total macamides were calculated as the sum of compounds **1–5**, **7**, **8**, **10** and **11**. Compounds **6** and **9** were below the quantification level of the HPLC-UV method. The following parameters were used for the MS/MS analysis: high voltage capillary, 4500 V; capillary exit, 143.6 V; skimmer 1, 31.1 V; trap drive, 44.2; scan range (*m/z*), 150–500.

MS^n analysis of macamides. The MS^n analyses were performed using a hexane extract of Maca. The same ionisation conditions were used as for the HPLC-MS/MS analysis. For MS^2 experiments, ions of interest were fragmented using a fragmentation amplitude of 1.30 V. The cut-off was set at approximately 27% of the parent ion. For MS^3 and MS^4 experiments, the fragmentation amplitude was 1.00 V with the same settings for the fragmentation cut-off as for MS^2.

NMR analysis of macamides. ^{1}H- and ^{13}C-NMR experiments were recorded either on a Bruker (Billerica, MA, USA) Avance DPX 400 instrument at 400 and 100 MHz, respectively, or on a Bruker Avance DRX 300 instrument at 300 and 75 MHz, respectively. All spectra were recorded in $CDCl_3$ with the solvent used as an internal reference (δH 7.27; δC 77.3, 77.0, 76.7). High-resolution MS data was obtained using a Waters (Milford, MA, USA) Micromass LCT Classic instrument.

RESULTS

n-Benzyl-(9*Z*)-octadecenamide (2)

^{1}H-NMR ($CDCl_3$) δ (ppm): 7.30 (5H, m, H-3′ to H-7′), 5.74 (1H, s, N-H), 5.35 (2H, m, H-9, H-10), 4.44 (2H, d, J = 5.7 Hz, H_2-1′), 2.21 (2H, t, J = 7.7, H_2-2), 2.00 (4H, m, H_2-8, H_2-11), 1.66 (2H, m, H_2-3), 1.29 (20H, m, H_2-4 to H_2-7, H_2-12 to H_2-17), 0.88 (3H, t, J = 6.6 Hz, H_3-18); ^{13}C-NMR ($CDCl_3$) δ (ppm): 172.89 (C-1), 138.45 (C-2′), 129.99 (C-10), 129.73 (C-9), 128.68 (C-4′, C-6′), 127.80 (C-3′, C-7′), 127.46 (C-5′), 43.58 (C-1′), 36.78 (C-2), 31.88 (C-16), 29.12-29.75 (C-4 to C-7 and C-12 to C-15), 27.21 (C-11), 27.16 (C-8), 25.74 (C-3), 22.65 (C-17), 14.07 (C-18); HRMS (TOF) *m/z* 372.3248 (calculated for $C_{25}H_{42}NO$, $[M + H]^+$ 372.3266).

The identifications of *n*-benzyloctadecanamide (**4**), *n*-(3-methoxybenzyl)-hexadecanamide (**5**) *n*-benzylpentadecanamide (**6**) and *n*-benzylheptadecanamide (**7**) in the extract were based on the results from the MS^n experiments. The main fragments obtained for molecules containing a saturated alkyl-moiety are shown in Figs 1 and 2, and are in agreement with fragmentation patterns observed for capsaicinoids (Reilly *et al*., 2003). The position of the methoxyl-substituent on the aromatic moiety was based on the reported presence of *n*-(3-methoxybenzyl)-hexadecanamide in Maca (Zhao *et al.*, 2004). Peaks with molecular weights of *m/z* 372, 370 and 368, and the presence of large amounts of oleic, linoleic and linolenic acids in Maca, suggested that the plant used the main fatty acids to form *n*-benzyl-(9*Z*)-octadecenamide (**2**), *n*-benzyl-(9*Z*, 12*Z*)-octadecadienamide (**3**) and *n*-benzyl-(9*Z*, 12*Z*, 15*Z*)-octadecatrienamide (**8**). Unfortunately, the MS^n experiments (Figs 3 and 4) did not permit the establishment of the exact position of the double bond(s) in the fatty acid moiety. Finally, the synthesis of *n*-benzyl-(9*Z*)-octadecenamide [containing a small amount of *n*-benzyl-(9*Z*, 12*Z*)-octadecadienamide] allowed the correct identification of the two compounds

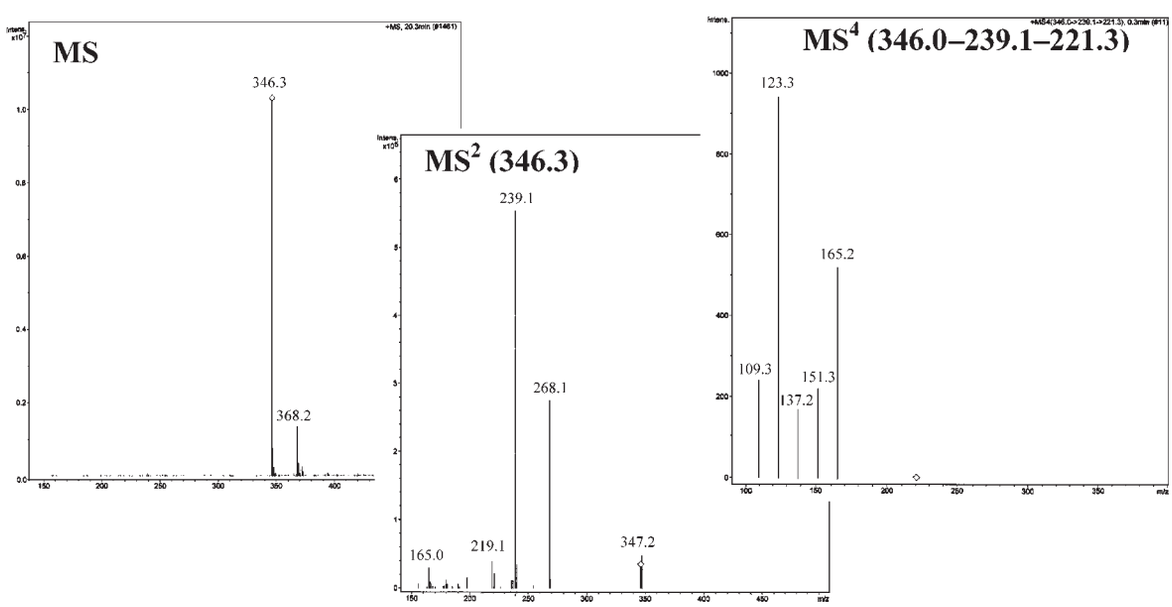


Figure 1. MS, MS^2 and MS^4 spectra obtained for **1**.

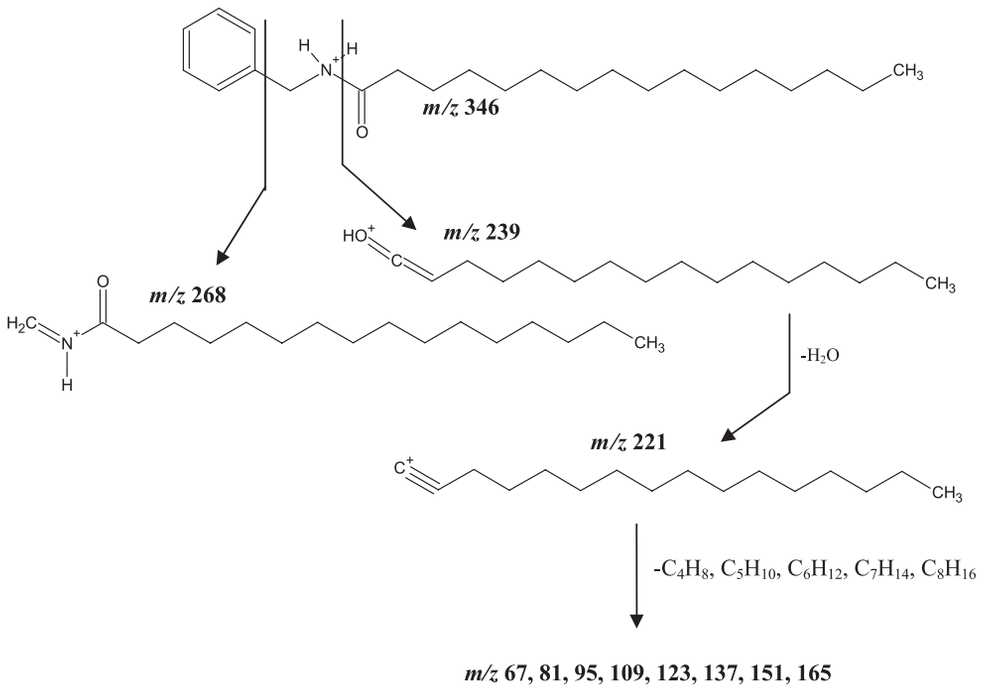


Figure 2. Proposed fragmentation pattern of **1** based on established fragmentation patterns of capsaicinoids.

in the extract. It could also be inferred that the main free fatty acids are precursors of the macamides, and the structure of **8** was therefore proposed as *n*-benzyl-(9*Z*, 12*Z*, 15*Z*)-octadecatrienamide. The remaining minor compounds (**9–11**), with molecular weights of *m/z* 402, 400 and 398, were identified as 3-methoxy-derivatives of **2**, **3** and **8**. Compounds **2–4** and **6–11** are new natural products.

There has been one previous report on the synthesis of a macamide, *n*-benzyl-15*Z*-tetracosenamide, using *cis*-15-tetracosenoic acid, (dimethylamino)pyridine and dicyclohexyl carbodiimide (Zhao *et al.*, 2004). This synthesis is also rapid, but the product requires purification by CC over silica gel. The one-step synthesis of **1** and **2** presented in the present study is faster and gives higher yields; however, the commercial availability of fatty acid chlorides is limited.

A recent paper (Ganzera *et al.*, 2002) described an analytical HPLC method for the determination of the main macamide and macaenes, the marker compounds of *L. meyenii.* The authors obtained a good separation within 35 min using a C-12 stationary phase, an acidic mobile phase comprising acetonitrile and water, and by raising the column temperature to 40°C. We opted for a similar system, although a C-18 stationary phase was used with a run time of 30 min (Fig. 5).

In order to validate the method, accuracy (recovery), instrument precision, intermediate precision, repeatability, linearity and limit of detection were determined. A summary of the method validation data can be found

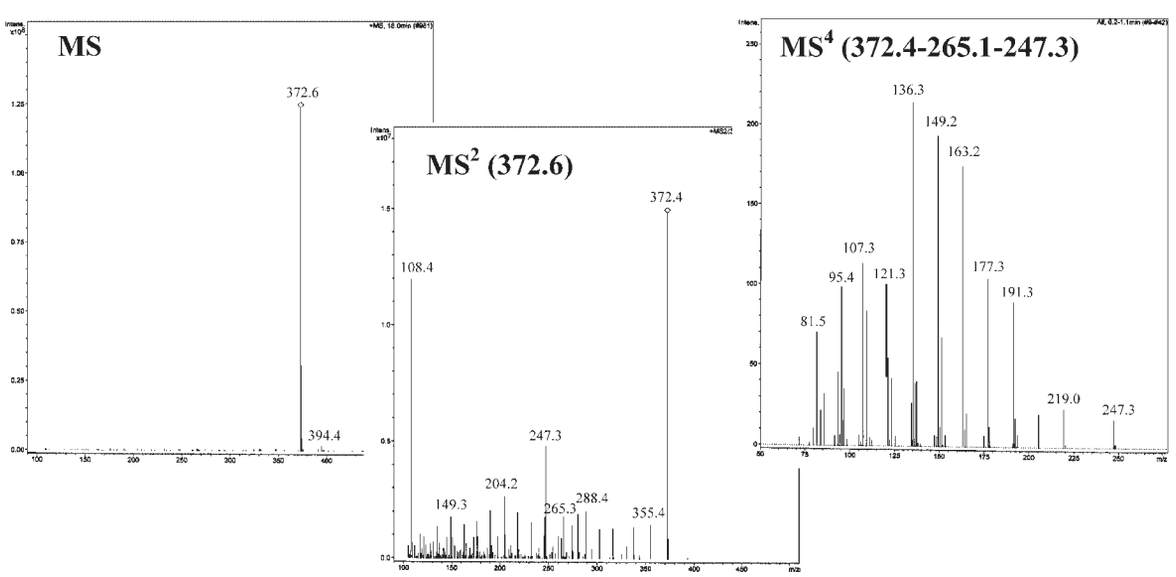


Figure 3. MS, MS^2 and MS^4 spectra obtained for **2**.

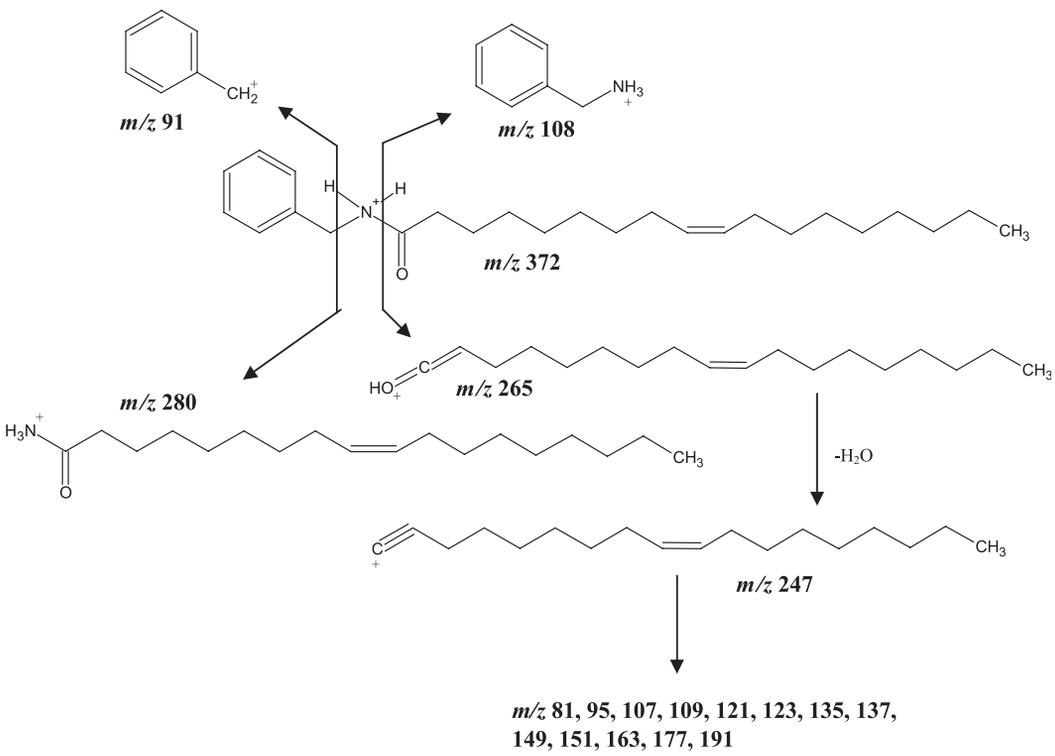


Figure 4. Proposed fragmentation pattern of **2** based on established fragmentation patterns of the capsaicinoids

in Table 1. A good linear response (curve equation: $y = 1971\ x + 0.064$; $r^2 = 0.9999$) was obtained with 0.28–1.76 μg of **1**. The limit of quantification was determined to be 15 ng, which was the amount of **1** at which an obvious change in the response factor was observed (signal–noise ratio of 18). The limit of detection was established at a signal–noise ratio of 3, which was obtained with a 2.8 ng injection of **1**. In order to evaluate the specificity, which means the ability to unequivocally attribute a peak to a compound, a peak purity evaluation, as is often done

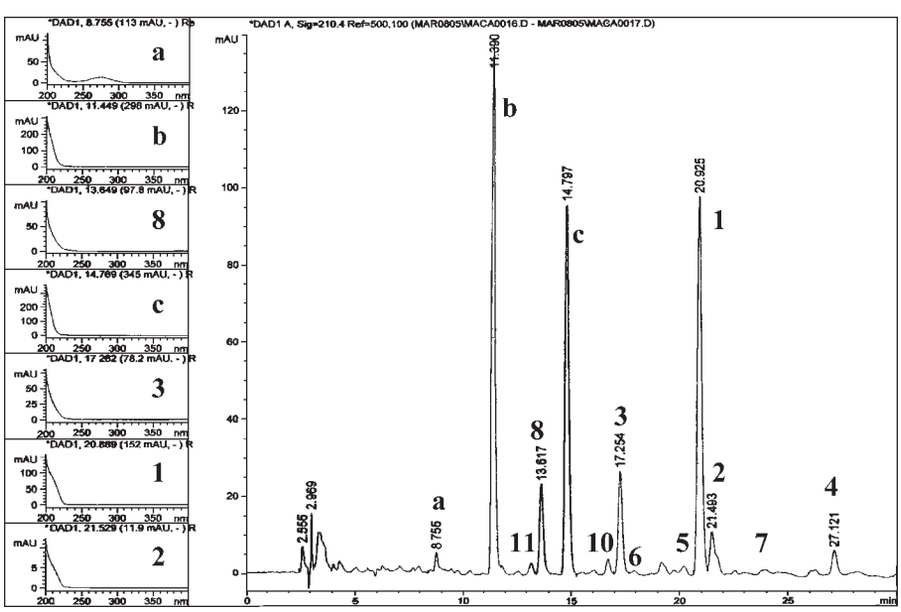


Figure 5. HPLC-UV/DAD chromatogram of a petroleum ether extract of ground Maca hypocotyls (vendor 1) showing the separation of macamides **1**–**8**, **10** and **11**: peak **a** is associated with an unidentified macamide ($m/z = 383$), whilst peaks **b** and **c** correspond to linolenic acid and linoleic acid, respectively. The UV-DAD spectra of **1–3** and **8**, and **a–c** are shown as inserts. (For chromatographic protocol see Experimental section: UV detection at 210 nm with baseline subtraction to account for the absorption of acetonitrile at this wavelength.)

Table 1. Statistical data for recovery, instrument precision, repeatability and intermediate precision of the described HPLC method for the quantitative determination of macamides in extracts of Maca

Validation parameter	*n*-Benzylhexadecanamide (**1**)	Total macamides[a]
Recovery (% ± RSD)	100.2 (± 4.7)	Not determined
Instrument precision[b] (% ± RSD)	0.73 (± 0.2)	1.32 (± 0.3)
Repeatability[c] (% ± RSD)	0.043 (± 2.5)	0.180 (± 3.4)
Intermediate precision[d] (% ± RSD)	0.044 (± 1.0)	0.183 (± 2.2)

[a] Total macamides expressed as the sum of compounds **1–5**, **7**, **8**, **10** and **11**.
[b] Ten consecutive injections of the same sample.
[c] Six preparations of the same sample (note: the samples used for instrument precision, and repeatability and intermediate precision were different).
[d] Six preparations of the same sample, analysed by different operators on different days using different instruments.

Table 2. Amounts of *n*-benzylhexadecanamide and total macamides in *Lepidium meyenii* obtained from different sources (± RSD)

	Amount sample[b]	
Maca sample[a]	*n*-Benzylhexadecanamide (**1**)	Total macamides
Vendor 1	7.11×10^{-3} (± 15.7)	1.23×10^{-2} (± 17.0)
Vendor 2	6.51×10^{-4} (± 1.4)	2.10×10^{-3} (± 1.5)
Vendor 3	5.37×10^{-4} (± 5.5)	1.61×10^{-3} (± 5.5)
Vendor 4	1.83×10^{-3} (± 17.3)	5.50×10^{-3} (± 15.4)

[a] For details of sample analysed see Experimental section.
[b] Mean values (± RSD; $n = 3$) expressed as percentage of dry hypocotyls.

with HPLC-UV methods, was considered insufficient due to the similarity of the chromophores of macamides and fatty acids. Every peak was therefore evaluated by HPLC-MS in order to determine if there were any overlapping peaks. This approach was limited by the fact that the fatty acids do not ionise well under the given acidic conditions of the mobile phase. None of the major fatty acids found in the extract overlapped with the macamides, as determined by injection of commercially available standards (linoleic, linolenic, oleic, palmitic and stearic acids). The HPLC-MS analysis, however, indicated a co-elution of **1** and **9**, but the amount of **9** in all of the samples was too low to be quantified (based on MS data) and therefore did not interfere with the outcome of the results.

The quantitative results for the four samples of *L. meyenii* are given in Table 2. There is a significant difference in the total amount of macamides between the samples, ranging from 0.0016 to 0.0123% of the dried plant material. In all extracts, **1** was the predominant macamide, but there was a difference in the macamide pattern. For example, the material from vendor 2 contained a much larger amount of **7**, but almost no **4**, whilst all the other plant materials contained **4** in much larger quantities than **7**. It should be noted that there were small amounts of four unidentified macamides, which eluted between 7 and 10 min. Interestingly, the main peak for these macamides in the MS spectra was the $[M+Na]^+$ ion rather than the $[M+H]^+$ ion. This is probably due to the presence of a Keto group on the alkyl chain. The molecular weight information for these four molecules does agree with (12*Z*)-*n*-benzyl-9-oxooctadec-12-enamide, (12*Z*, 15*Z*)-*n*-benzyl-9-oxooctadec-12,15-dienamide, (9*E*, 11*E*)-*n*-benzyl-13-oxooctadec-9,11-dienamide and (6*E*, 8*E*)-*n*-benzyl-5-oxooctadec-6,8-dienamide, which have been isolated recently from *L. meyenii* (Zhao *et al.*, 2004). These compounds were not included in the quantification of macamides due to the low amounts present in the extracts and due to the fact that the identity of the peaks could not be established with certainty.

Two studies on testicular function and spermatogenesis in rats (Gonzales *et al.*, 2004; Chung *et al.*, 2005) performed with aqueous extracts that contain only trace amounts of macamides (M.M. McCollom, unpublished results), showed a positive outcome. Based on this information, compounds other than the macamides must contribute to the fertility enhancing activities of Maca. The macamides, however, are a distinct class of secondary metabolites that have not been found in any other plant species so far. Therefore, these compounds are useful marker compounds for the quality control of *Lepidium meyenii* products.

Acknowledgements

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