Revista Brasileira de Farmacognosia 29 (2019) 152–161

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.elsevier.com/locate/bjp

Original Article

Simultaneous determination of five *N*-alkylamides in the root of *Anacyclus pyrethrum* by HPLC and profiling of components in its methanolic root extract by UPLC/Q-TOF-MS

Ruifang Ji, Qinghua Quan, Xiaoyu Guo, Jiamei Zhang, Yongli Song, Mengting Zhu, Peng Tan, Jing Han*, Yonggang Liu*

Beijing University of Chinese Medicine, Beijing 100102, China

ARTICLE INFO

Article history:
Received 12 March 2018
Accepted 7 December 2018
Available online 20 March 2019

Keywords:
N-alkylamides
Quantitative analysis
Qualitative analysis

ABSTRACT

The root of *Anacyclus pyrethrum* (L.) Lag., Asteraceae, is very widely used for treating various diseases in Traditional Uygur Medicine, particularly in the treatment of vitiligo. However, there have been few studies on the quality standards of *A. pyrethrum* in China. *A. pyrethrum* contains abundant *N*-alkylamides, which are considered to be the principal components. Therefore, based on the previous research in our group, six *N*-alkylamides were obtained by using column chromatography. We used ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry to determine the mass spectrometry cleavage mechanism of these six monomer components and established the mass spectrometry cleavage law of *N*-alkylamides. Then, we used the ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry method to rapidly identify and analyze the *N*-alkylamide components of the *A. pyrethrum* methanol extract. Finally, twenty *N*-alkylamides were identified, including eleven *N*-isobutylamides, two *N*-methyl isobutylamides, six 4-hydroxyphenylethyl-amide and one 2-phenylethylamide. Five of these compounds were identified as new compounds that have not been reported to date. Two of these compounds were identified for the first time in this herb. Therefore, this work provides an approach for the quality analysis of *N*-alkylamides in the root of *A. pyrethrum*. A search of the literature showed that the content determination in the *A. pyrethrum* quality standard is still a remaining problem. *N*-alkylamides are the main components of *A. pyrethrum*. Even though ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry has the advantages of lower time and higher efficiency compared to high-performance liquid chromatography, considering the ease of repeatability and universality of the quality control method, we chose to use high-performance liquid chromatography for content determination. In this experiment, high-performance liquid chromatography was used for the first time to establish a simple, rapid and accurate method for evaluating the *N*-alkylamide content in *A. pyrethrum* with five *N*-alkylamides used as the standards. Finally, this work provides a qualitative and quantitative method for the analysis of *N*-alkylamides in *A. pyrethrum*, improving the quality control standards for *A. pyrethrum*.

Introduction

Anacyclus pyrethrum (L.) Lag. belongs to the Asteraceae family, and it is a perennial, procumbent herb that is covered with long villous and reaches 15–45 cm in length, and is widely distributed in North Africa, Central Asia and the Xinjiang region of China (Pharmacopoeia Commission of the Ministry of Health, 1999; Annalakshmi et al., 2012; Kumar and Lalitha, 2012). In research carried out at The University of Macau, ZhiQiao et al. (2014) found that this Uyghur medicine not only was very widely used in Xinjiang but also had a profound impact in South Asia, Central Asia and Afghanistan. It is mainly used for the treatment of diseases such as tumors, knee osteoarthritis (Chinese Materia Medica Committee, 2005), and particularly for treatment of vitiligo (Mubarak and Aiguuli, 2004; Genaguri and Mairemu, 2011). Due to its remarkable role, it has received increasing attention, but its use faces problems such as insufficient research regarding pharmacodynamics and quality control. According to the literature (Yongmin, 1999), among these, *N*-alkylamides are considered to be the principal components of *A. pyrethrum*, and current research has focused on

* Corresponding authors.
E-mails: hanjing8585@163.com (J. Han), liuyg0228@163.com (Y. Liu).

https://doi.org/10.1016/j.bjp.2018.12.011

the separation and analysis of *N*-alkylamides. Boonen et al. (2011) isolated four *N*-alkylamides from *A. pyrethrum* while Boonen et al. (2012b) identified thirteen *N*-alkylamides in the ethanol extract of *A. pyrethrum.* *N*-alkylamide is an amide structure composed of an amine with a longer unsaturated acid at one end and an amine with a smaller substituent at the other end, and it is an important natural product widely present in plants such as Asteraceae and *Aristolochia* species. *N*-alkylamides are considered to be a promising group of bioactive compounds that possess multiple pharmacological activities, such as antimicrobial activities, tingling and related organoleptic effects, anti-inflammatory and immunomodulatory effects, and tyrosinase activation effect (Badhe et al., 2010; Boonen et al., 2012a; Sharma et al., 2013).

The research on quality control is highly significant for ensuring the safety of traditional Chinese medicine. Traditional Chinese medicine does not exert its effects through the action of one or even several components. Rather, its effectiveness is the result of the interaction of various components. As the commonly used medicine for the treatment of vitiligo in Uygur medicine, *A. pyrethrums* have been rarely studied for quality standards, particularly regarding the selection and content determination of the indicator components.

Currently, although some of the *N*-alkylamides in *A. pyrethrum* have been separated and analyzed, these results are still insufficient. Hence, further research on the composition of *A. pyrethrum* is highly necessary. Generally, the traditional techniques of extraction and separation of compounds are suitable. However, it is difficult to isolate the minor and trace constituents by using the traditional techniques. The previously reported methods for analyzing *N*-alkylamides in medicinal plants are based on High-Performance Liquid Chromatography coupled with Mass Spectra (HPLC-MS) (Boonen et al., 2010, 2012b; Sharma et al., 2013). While HPLC-MS has some advantages in medicinal herb component analysis, the sample separation process in HPLC is time-consuming and the low-resolution MS instrument does not allow the exact measurement of the masses of the precursor and fragment ions to obtain the highest reliability in structural identification.

In recent years, UPLC-Q-TOF-MS has been shown to be a rapid and convenient method, and it has started to play a significant role in the identification of complex components system, compensating for the shortcomings of the HPLC-MS method. The Q-TOF-MS has high MS/MS sensitivity, enabling accurate mass measurement of the precursor and fragment ions, and it reaches the lowest minimum detection limit of any other high resolution LC/MS method. Therefore, UPLC/Q-TOF-MS has become a powerful tool for rapid separation, screening and identification of complex systems of traditional medicine samples.

In our previous work, we isolated the dichloromethane fraction of *A. pyrethrum* with silica gel column chromatography and obtained six *N*-alkylamides. The cleavage mechanism of the *N*-alkylamide monomer compounds isolated from *A. pyrethrum* was analyzed and the regularity of mass spectrometry was established. UPLC/Q-TOF-MS was used to rapidly identify and analyze the *N*-alkylamides in the methanol extract of *A. pyrethrum.* Identification of *N*-alkylamides was completed based on the known fragmentation patterns and literature data. Finally, the five isolated *N*-alkylamide were selected as index components and quantitatively analyzed by HPLC. We sought to achieve quantitative and qualitative analysis of *N*-alkylamides from the root of *A. pyrethrum*. Our study can provide a basis for improving the quality standards of *A. pyrethrum*.

Materials and methods

Materials

The dried fruits of *Anaycclus pyerhturm* (L.) Lag., Asteraceae, were purchased from the Haozhou Medicinal Corporation, Anhui, China, with a batch number of 130704. Voucher specimens were identified by professor Muaitaer of Collece of Xinjiang Uyghur Medicine. Acetonitrile and methanol were obtained from Fisher Scientific (USA) and were of HPLC grade. The water used was Watsons distilled water, and all other reagents were of analytical grade. Standards of deca-2*E*,4*E*-dienoic acid 4-hydroxyphenylethylamide (**1**), deca-2*E*,4*E*-dienoic acid isobutyl-amide (**2**), dodeca-2*E*,4*E*-dienoic acid 4-hydroxyphenylethylamide (**3**), tetradeca-2*E*,4*E*,8*E*-trienoic acid 4-hydroxyphenylethylamide (**4**), tetradeca-2*E*,4*E*-dienoic acid 4-hydroxyphenylethylamide (**5**), and undeca-2*E*,4*E*-diene-8,10-diynoic acid 2-phenylethyl amide (**6**) were extracted and separated in our laboratory. The purity of each compound was not less than 98%, as specified by HPLC analysis.

Apparatus and method

Standard sample preparation through column chromatography

The air-dried powder of *A. pyrethrum* (2 kg) was extracted three times (each for 1.5 h) with 70% ethanol. The filtrate was concentrated under vacuum to obtain an ethanol extract (272 g). The ethanol extract was dissolved in water and extracted with petroleum ether and dichloromethane in turn. The dichloromethane extract (40 g) was obtained and then separated by silica gel column chromatography with a gradient elution of petroleum ether-ethyl acetate (10:1–1:1). After the further separation and purification using C_{18} and Sephadex LH-20 column chromatography, compounds **1**–**6** were obtained. Using ultraviolet, nuclear magnetic, and mass spectrometry, we identified these six components: deca-2*E*,4*E*-dienoic acid 4-hydroxyphenylethylamide (**1**, 22 mg), deca-2*E*,4*E*-dienoic acid isobutyl-amide (**2**, 20 mg), dodeca-2*E*,4*E*-dienoic acid 4-hydroxyphenylethylamide (**3**, 10 mg), tetradeca-2*E*,4*E*,8*E*-trienoic acid 4-hydroxyphenylethylamide (**4**, 5 mg), tetradeca-2*E*,4*E*-dienoic acid 4-hydroxyphenylethyl amide Tetradeca-2*E*,4E-dienoic acid 4-hydroxyphenylethyl amide (**5**, 5 mg) and undeca-2*E*,4*E*-diene-8,10-diynoic acid 2-phenylethyl amide (**6**, 10 mg). The six standard samples were stored for one month in the dark at a temperature below 4 °C. Among these six compounds, compound **4** was a novel compound and compounds **5** and **6** were isolated for the first time from this plant. The NMR spectrum of compound **4** is provided in the supplementary material.

Qualitative analysis of components by UPLC-Q-TOF-MS

Sample preparation. Methanol (approximately 25 ml) was added to *A. pyrethrum* (0.5 g), and the mixture was ultrasonicated (Kunshan Ultrasonic Instrument Co., Ltd., Kunshan, China, 100 kHz) and extracted for 1 h. The sample solution was filtered through a 0.45 μm filter membrane. The filtered solution was diluted 10-fold.

UPLC conditions. UPLC analysis was performed using an ACQUITY UPLC™ HSS C_{18} column (100 mm × 2.1 mm, 1.8 μm, Waters Co., Milford, MA, USA). The mobile phase consisted of 0.1% acid water (A) and acetonitrile (B) (v/v) with gradient elution separation as described in Table 1.

Q-TOF-MS conditions. The ACQUITY UPLC system was coupled to a hybrid quadrupole orthogonal time-of-flight (Q-TOF) mass

Table 1
Mobile phase composition in UPLC.

Time (min)	*A*% (0.1% acid water %)	*B*% (acetonitrile %)
0	40	60
1	40	60
10	5	95
12	5	95
12.1	40	60

Table 2
Mobile phase composition in HPLC.

Time (min)	*A*% (water %)	*B*% (methanol %)
0	30	70
12	30	70
14	20	80
40	20	80

spectrometer (Synapttm G2 HDMS, waters, Manchester, U.K.) equipped with electrospray ionization (ESI), and leucine-enkephalin with accurate mass was used as the correction fluid. The operating parameters were as follows: capillary voltage 3.0 kV (ESI+) or 2.2 kV (ESI−); sample cone voltage 40 V; extraction cone voltage 4 V, source temperature 100 °C, desolvation temperature 400 °C and desolvation gas flow 800 l/h. In the MSE mode, the trap collision energy of the low energy function was set to 6 eV, while the ramp trap collision energy of the high energy function was set at 10–40 eV. To ensure mass accuracy and reproducibility, the mass spectrometer was calibrated over the range of 100–1500 Da with sodium formate. Leucine-enkephalin (*m*/*z* 556.2771 in the positive ion mode; *m*/*z* 554.2615 in the negative ion mode) was used as the external reference of Lock-Spray™ infused at a constant flow of 5 μl/min. The data acquisition mode was 3D data acquisition under the Continuum mode. A V4.1 Mass Lynx data processing station was used for data analysis.

Quantitative analysis of N*-alkylamides by HPLC*

Sample preparation. Standards stock solutions were prepared by accurately dissolving each component in 5 ml of methanol and storing at 4 °C. Mixed standard solutions of the five *N*-alkylamides were obtained at concentration of 10, 29.3, 8.8, 0.86, and 3.4 g/ml, respectively. This solution was used for method development and validation.

Anaycclus pyrethrum (approximately 0.5 g) was extracted by ultrasound (Kunshan Ultrasonic Instrument Co., Ltd., Kunshan, China, 100 kHz) with methanol (25 ml) for 1 h. The sample solution was filtered through a 0.45 μm filter membrane. The filtered sample solution (10 μl) was directly injected into the HPLC system.

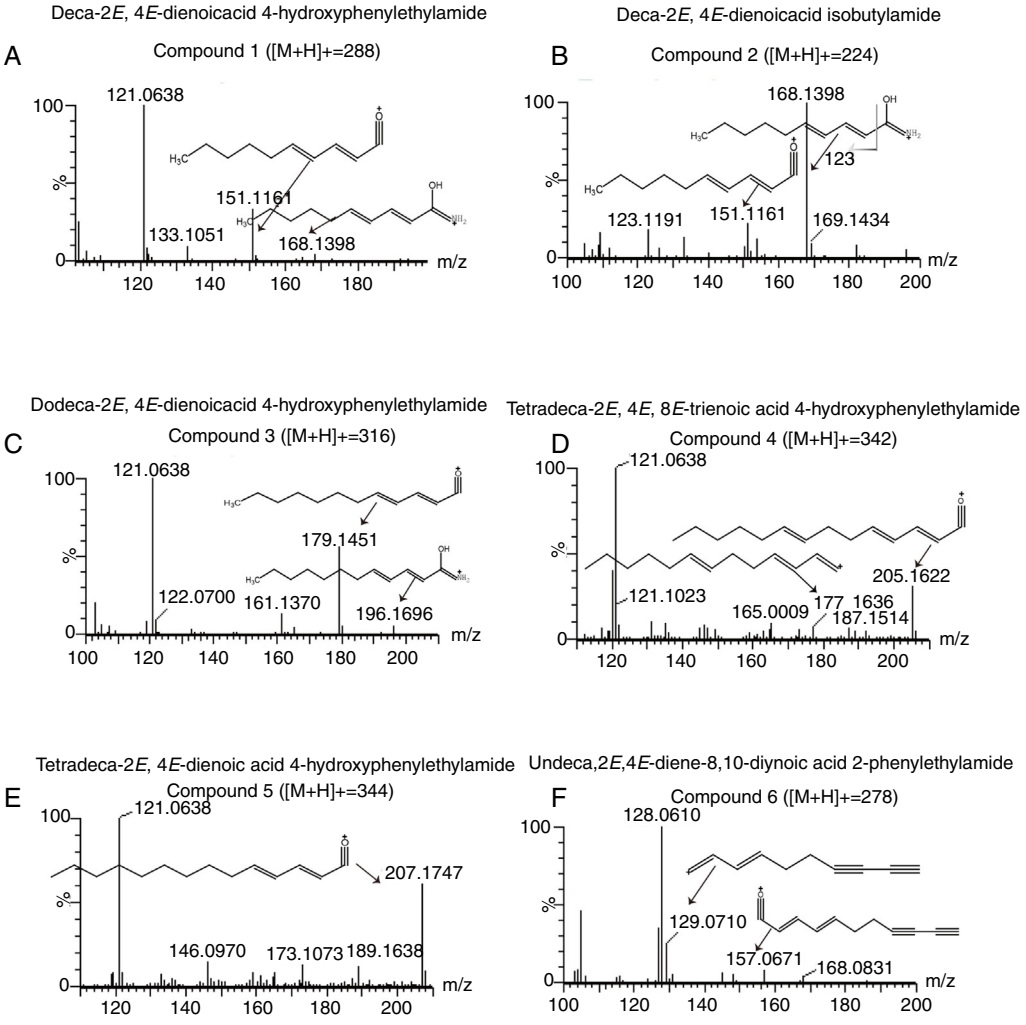


Fig. 1. MS^2 fragmentation spectra of the six *N*-alkylamides separated in *Anacyclus pyrethrum*. (A) Deca-2*E*,4*E*-dienoic acid 4-hydroxyphenylethylamide (**1**); (B) Deca-2*E*,4*E*-dienoic acid isobutyl-amide (**2**); (C) Dodeca-2*E*,4*E*-dienoic acid 4-hydroxyphenylethylamide (**3**); (D) Tetradeca-2*E*,4*E*,8*E*-trienoic acid 4-hydroxyphenylethylamide (**4**); (E) Tetradeca-2*E*,4E-dienoic acid 4-hydroxyphenylethyl amide (**5**); and (F) Undeca-2*E*,4*E*-diene-8,10-diynoic acid 2-phenylethyl amide (**6**).

Table 3
Compounds **1–13** identified in *Anacyclus pyrethrum* extract by UPLC/Q-TOF-MS.

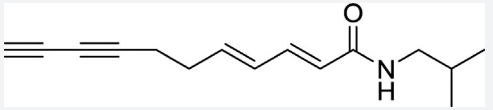
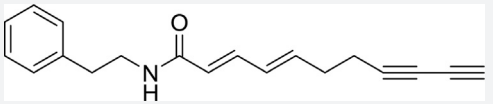
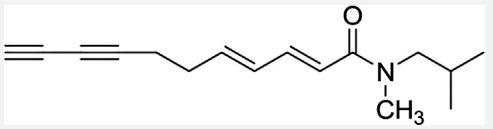
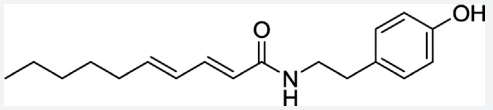
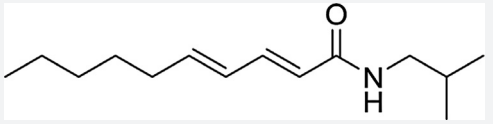
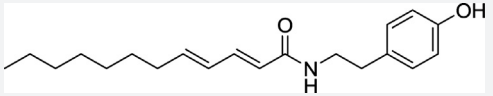
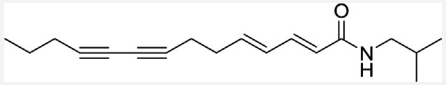
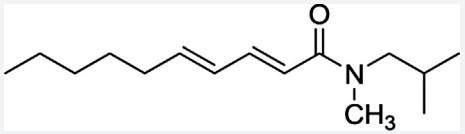
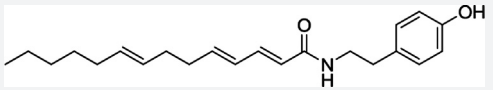
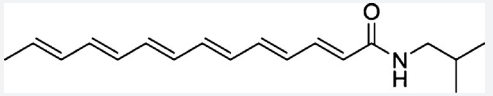
Compound	Rt (min)	Precursor ion (*m*/*z*)	Cal. (M+H)	Productions (*m*/*z*)	Structural formula	Identification	Structure
7	2.07	230.1589	230.1545	174, 157, 131, 129, 116	$C_{15}H_{19}NO$	Undeca-2*E*,4*E*-diene-8,10-diynoic acid IBA	
6	2.41	278.1564	278.1545	157, 141, 131, 129, 103	$C_{16}H_{29}NO$	Undeca-2*E*,4*E*-diene-8,10-diynoic acid 2-phenylethyl amide	
8	2.51	244.1706	244.1701	180, 174, 157, 166, 131, 129, 114, 103	$C_{16}H_{21}NO$	Undeca-2*E*,4*E*-diene-8,10-diynoic acid *N*-Me IBA	
1	2.59	288.2002	288.1964	168, 151, 133, 123, 121, 119, 105	$C_{18}H_{25}NO_2$	Deca-2*E*,4*E*-dienoic acid 4-hydroxyphenylethylamide	
9	3.76	224.2031	224.2014	168, 151, 133, 123, 119,	$C_{14}H_{25}NO$	Deca-2*E*,4*E*-dienoic acid IBA (pellitorine)	
3	4.17	316.2322	316.2277	196, 179, 161, 133, 119	$C_{20}H_{29}NO_2$	Dodeca-2*E*,4*E*-dienoic acid 4-hydroxyphenylethylamide	
10	4.39	272.2027	272.2014	216, 173, 171, 131,	$C_{18}H_{25}NO$	Tetradeca-2*E*,4*E*-diene-8,10-diynoic acid IBA (anacycline)	
11	4.58	238.2027	238.2171	182, 168, 151, 133, 123, 119, 109	$C_{15}H_{27}NO$	Deca-2*E*,4*E*-dienoic acid *N*-Me IBA	
4	4.82	342.2451	324.2433	205, 187, 177, 173, 145, 131, 121,	$C_{22}H_{31}NO_2$	Tetradeca-2*E*,4*E*,8*E*-trienoic acid 4-hydroxyphenylethylamide	
12	5.3	276.2373	276.2327	220, 203, 173, 152, 135,	$C_{18}H_{29}NO$	Tetradeca-2*E*,4*E*, X*E*/*Z*, Y*E*/*Z*-tetraenoic IBA	

Table 3 (*Continued*)

Compound	Rt (min)	Precursor ion (*m*/*z*)	Cal. (M+H)	Productions (*m*/*z*)	Structural formula	Identification	Structure
2	5.79	252.2324	252.2327	196, 161, 179, 119	$C_{16}H_{29}NO$	Dodeca-2*E*,4*E*-dienoic acid isobutyl-amide	
5	6.18	344.2594	344.259	224, 207, 189, 172, 147, 133, 121, 119	$C_{22}H_{33}NO_2$	Tetradeca-2*E*,4*E*-dienoic acid 4-hydroxyphenylethyl amide	
13	8.05	280.265	280.264	224, 207, 182, 154, 147, 133, 141	$C_{18}H_{33}NO$	Tetradeca-2*E*,4*E*-dienoic acid IBA	

HPLC conditions. HPLC analysis was performed using a Shimadzu LC-20A system (Shimadzu, Corporation, Kyoto, Japan), configured with a PDA detector, a quaternary gradient pump and an autosampler. The data analysis was performed using the Shimadzu "LC Lab-Solution" software (Shimadzu Corporation, Kyoto, Japan). Separation was performed using an Agilent Extend C_{18} column (4.6 mm × 250 mm, 5 μm) at a flow rate of 0.8 ml min^{-1}. The mobile phase consisted of water (A) and methanol (B) (v/v). A gradient program was adopted as described in Table 2.

Accuracy, reproducibility, stability and recovery rate tests. The accuracy test included intra-day and inter-day analyses that were used to evaluate the method precision. Mixed standards solution (10 μl) were precisely drawn, injected continuously six times over three days, and their peak areas were measured.

Stability tests were further performed to analyze the variations in the sample solutions over 10 h. Sample solutions (10 μl) were precisely drawn, injected continuously at 0, 2, 4, 6, 8, and 10 h, and their peak areas were measured.

Reproducibility tests were further performed to test the repeatability of the method. Six test solutions (10 μl) were continuously injected into a high-performance liquid chromatography instrument, and their peak areas were measured.

Recovery tests were also performed to validate the accuracy of the developed method by adding a known amount of the reference marker compounds into accurately weighed samples. The spiked samples were extracted using the sample-preparation method, and then the developed HPLC method was used for analysis.

Results and discussion

Rapid analysis of methanolic root extract by UPLC/Q-TOF-MS

Establishment of MS fragmentation pathways of N-*alkylamides*

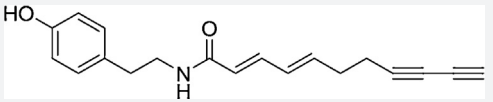
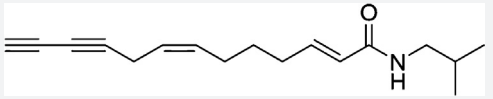
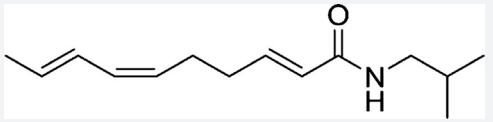
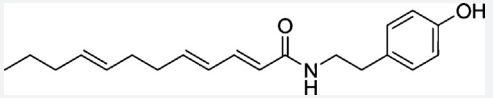
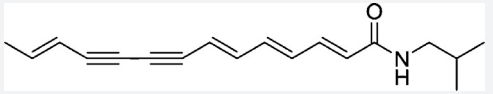
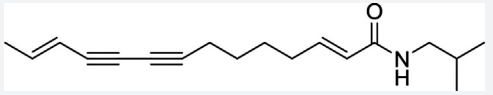
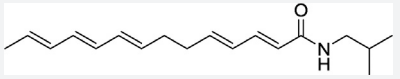
It was reported that there are four main types of *N*-alkylamides in *A. pyrethrum*, namely, isobutylamide (IBA), *N*-methyl isobutylamide (*N*-methyl isobutylamide), 2-phenylethylamide (2-PEA) and 4-hydroxyphenylethylamide (4-OH PEA). However, there have been few reports on the fragmentation pathways of *N*-alkylamides, and the corresponding pathways are often presented by only one reference standard (Boonen et al., 2011; Sharma et al., 2013). Therefore, to obtain accurate and comprehensive information about the fragmentation pathways of the *N*-alkylamides, compounds **1**–**6** were analyzed by Q-TOF-MS. The characteristic fragment ions were identified and the patterns were deduced.

In the positive ionization mode, the $[M+H]^+$ peak is prone to α-cracking, both in the *N*-position and the carbonyl-position, and different types of *N*-alkylamides produce different characteristic fragment ions. Furthermore, most *N*-alkylamides contain polyunsaturated aliphatic fatty acid chain that produces one or more fragmentation ions with the loss of *m*/*z* 14 (CH_2). The IBA presents the characteristic fragment losses of 56, 73, and 101 (Fig. 1B). The 2-PEA presents the characteristic fragment losses of 121, 133, and 151 (Fig. 1F). The 4-OH PEA has the characteristic fragmentation losses of 120, 137, 155, and 165 (Fig. 1B–E). The MS^2 fragmentation spectra of the six *N*-alkylamides separated from *A. pyrethrum* were elucidated (Fig. 1, Table 3).

Rapid analysis of the compounds in Anacyclus pyrethrum

The identification of *N*-alkylamides in the *A. pyrethrum* extract required two steps. First, a preliminary identification was performed by combining the precise molecular weight of each compound that was obtained by high-resolution mass spectrometry; second, based on the determination of the MS fragmentation pathways of the *N*-alkylamides, a further structural inference was

Table 4
Compounds **14**–**20** detected in *Anacyclus pyrethrum* extract by UPLC/Q-TOF-MS.

Compound	Rt (min)	Precursor ion (*m*/*z*)	Cal. (M+H)	Productions (*m*/*z*)	Structural formula	Identification	Structure
14	1.59	294.1532	294.1494	246, 163, 157, 129, 121	$C_{19}H_{19}NO_2$	Undeca-2E,4E-diene-8,10-diynoic acid 4-hydroxy-phenylethylamide	
15	2.81	258.1892	258.1858	202, 172, 157, 131, 117, 105	$C_{17}H_{23}NO$	(2E,7Z)-N-isobutyl-2,7-tridecadiene-10,12-diynamide	
16	2.93	222.1874	222.1858	172, 166, 149, 123, 121,	$C_{14}H_{23}NO$	(2E,6Z,8E)-N-isobutyl-2,6,8-decatrienamide	
17	3.16	314.2166	314.212	190, 177, 151, 149	$C_{20}H_{27}NO_2$	Dodeca-2E,4E, nE- trienoic acid 4-hydroxyphenylethylamide	
18	3.48	270.1894	270.1858	214, 197, 171, 169, 129, 115, 128, 113	$C_{18}H_{23}NO$	Tetradeca-2E,4E, nE-trienoic-8,10-diynoic acid IBA	
19	4.29	274.2163	274.2171	201, 173, 159	$C_{18}H_{27}NO$	Tetradeca-2E-diny-8,10-diynoic acid IBA	
20	6.55	278.2511	278.2484	222, 205, 167, 152, 141	$C_{18}H_{31}NO$	Tetradeca-2E, 4E, nE-trienoic acid IBA	

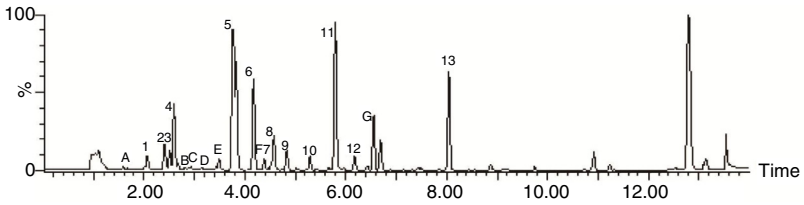


Fig. 2. Base peak intensity of *Anacyclus pyrethrum* in the positive ionization mode.

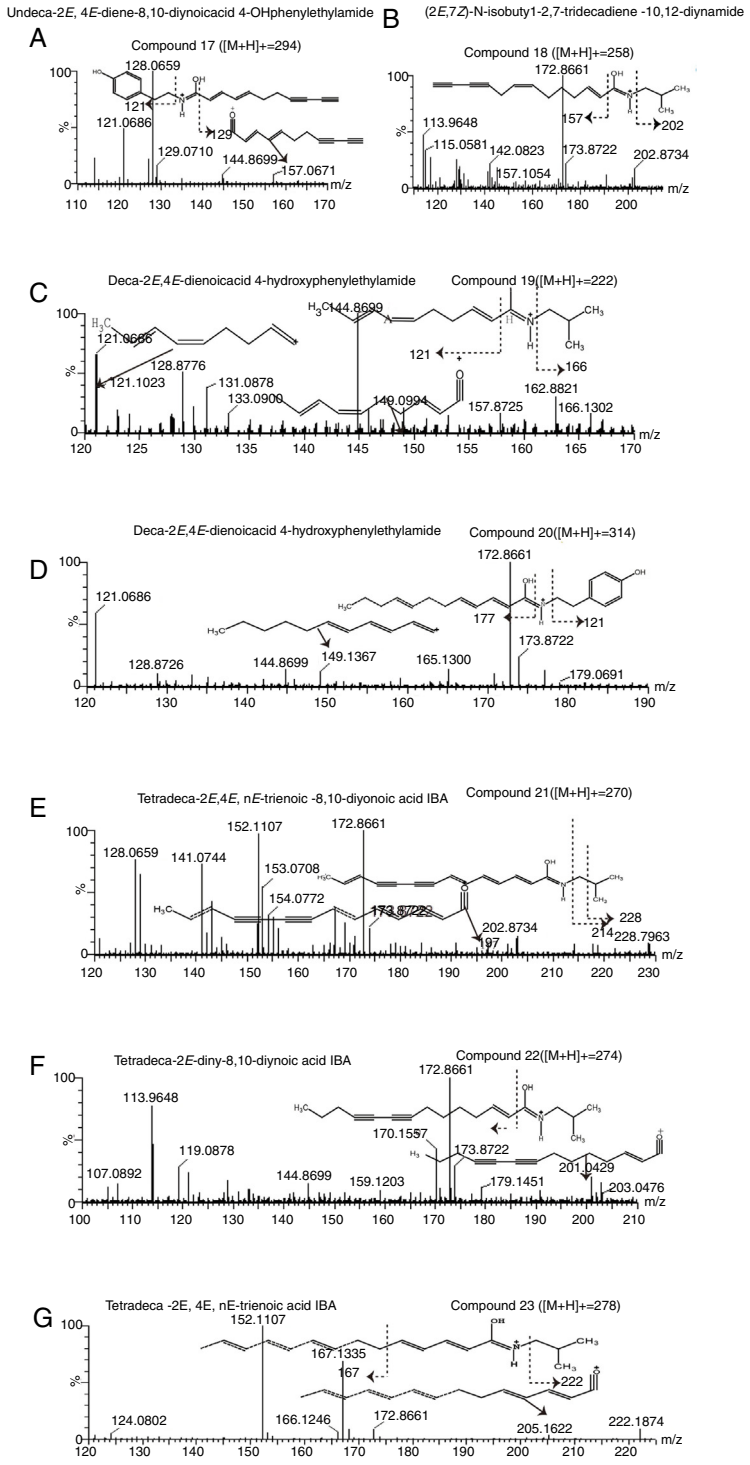


Fig. 3. MS^2 fragmentation spectra of compounds **14**–**20** identified in *Anacyclus pyrethrum* extract.

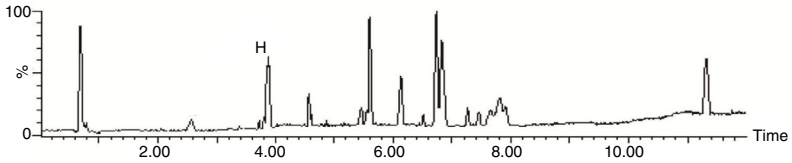


Fig. 4. Base peak intensity of *Anacyclus pyrethrum* in the negative ionization mode.

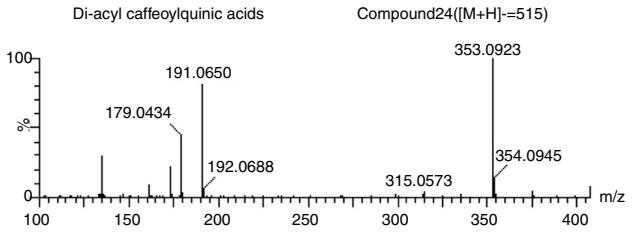


Fig. 5. MS^2 fragmentation spectra of compound **21**.

made. In the positive ionization mode, 20 *N*-alkylamides were identified in the *A. pyrethrum* extract: eleven IBA, two *N*-Me IBA, six 4-OH PEA and one 2-PEA. The base peak intensity chromatogram of the main components is presented in Figs. 1, 2, and Table 4 illustrate the seven novel *N*-alkylamides identified in the *A. pyrethrum* extract.

UPLC/Q-TOF-MS detected twenty *N*-alkylamides in the *A. pyrethrum* extract. Compounds **1**–**13** have been reported in the literature and were unambiguously identified based on their fragmentation patterns and literature data. Table 3 presents their retention time, precursor ion and fragmentation information, as well as their structural information. Another seven compounds found in *A. pyrethrum* that have not been reported previously were obtained.

Table 4 and Fig. 2 present their retention times (Rt), precursor ions, calculated mass, produced ions, and proposed structures. Compound **14** displayed ion peaks at *m*/*z* 157, *m*/*z* 129 and *m*/*z* 103, which are the characteristics of an undeca-2*E*,4*E*-diene-8,10-diynoic acid moiety, respectively. Compounds **15** (*m*/*z* 258) and **16** (*m*/*z* 222), two known *N*-alkylamides, were first discovered in *A. pyrethrum* and identified as (2*E*,7*Z*)-*N*-isobutyl-2,7-tridecadiene-10,12-diynamide and (2*E*,6*Z*,8*E*)-*N*-isobutyl-2,6,8-decatrien-amide, respectively. Compound **17** was identified as a 4-OH PEA (*m*/*z* 314) and had typical ion peaks at *m*/*z* 177 and *m*/*z* 149, consistent with dodec-dienoic acid 4-hydroxyphenylethyl-amide. Compounds **18** and **19** were identified as IBA based on their fragmentation patterns that correspond to anacycline (*m*/*z* 272). The structural formulae of compounds **18** and **19** with *m*/*z* 270 and 274 are $C_{18}H_{23}NO$ and $C_{18}H_{27}NO$, respectively. The 4-OH PEA possesses typical fragmentation losses of −120, −137 and −165. Compound **20** showed the characteristic fatty acid fragmentation signal at *m*/*z* 205 that was consistent with the tetradeca-trienoic acid moiety. The structural formulae of **17** and **20** were $C_{20}H_{27}NO_2$ and $C_{18}H_{31}NO$, respectively, but the location of the third saturated bond could not be assigned under the LC–MS conditions (Fig. 3).

In the negative ionization mode, one compound was identified. The BPI of *A. pyrethrum* is presented in Fig. 4. Compound **21** was identified as di-acyl caffeoylquinic acid. The MS^2 fragmentation spectrum of compound H is shown in Fig. 5. The spectrum of compound H showed the precursor ion of *m*/*z* 515, and some mass fragments at *m*/*z* 353, 191, 179, and 135 that had a good match with di-acyl caffeoylquinic acids, but the specific structure of the compound could not be confirmed under the LC–MS conditions.

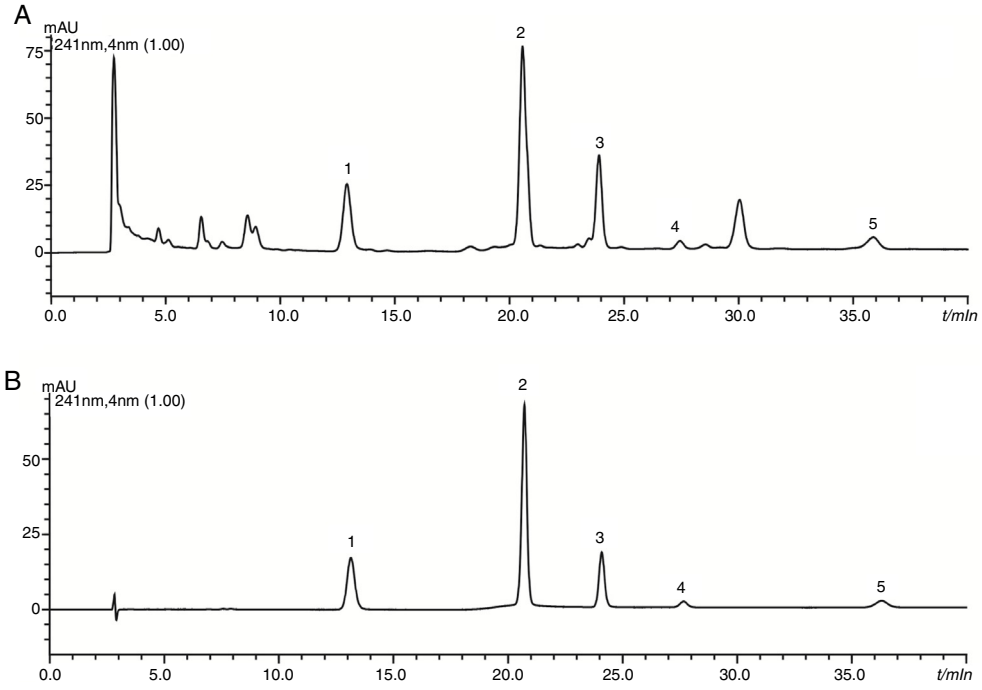


Fig. 6. HPLC chromatograms of the methanol extract of the root of *Anacyclus pyrethrum* (A) and a mixed standard solution of the five *N*-alkylamides (B). Deca-2*E*,4*E*-dienoic acid 4-hydroxyphenylethylamide (**1**); Deca-2*E*,4*E*-dienoic acid isobutylamide (**2**); 3. Dodeca-2*E*,4*E*-dienoic acid 4-hydroxyphenylethylamide (**3**); 4. Tetradeca -2*E*,4*E*,8*E*-trienoic acid 4-hydroxyphenylethylamide (**4**); 5. Tetradeca-2*E*, 4E-dienoic acid 4-hydroxyphenylethyl-amide (**5**).

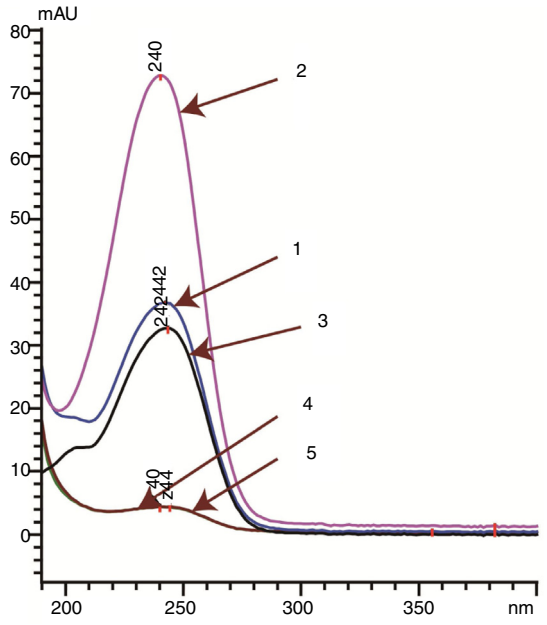


Fig. 7. Absorption spectra of the five *N*-alkylamides: Deca-2*E*,4*E*-dienoicacid 4-hydroxyphenylethylamide (**1**); Deca-2*E*, 4*E*-dienoic acid isobutylamide (**2**); Dodeca-2*E*, 4*E*-dienoicacid 4-hydroxyphenylethylamide (**3**); Tetradeca-2*E*,4*E*,8*E*-trienoic acid 4-hydroxyphenylethylamide (**4**); Tetradeca-2*E*,4E-dienoic acid 4-hydroxyphenylethyl amide (**5**).

Simultaneous determination of five N*-alkylamides in the root of* A. pyrethrum *by HPLC*

Optimization of quantitative analysis of five N*-alkylamides*

To achieve the maximum extraction efficiency of the five *N*-alkylamides, three parameters including extraction methods, the amount of extraction solvent and extraction time were compared individually. The results showed that for the extraction methods, ultrasonic extraction had a higher efficiency than reflux extraction. Using methanol (25 ml) and 1 h extraction allowed the full extraction for analysis. Therefore, we adopted ultrasonic extraction for 1 h by methanol (25 ml) for the analysis.

HPLC conditions were optimized by changing the HPLC gradient program. The selected program described in the HPLC conditions section above was shown to have high separation performance on C_{18} column. Fig. 6 shows the typical HPLC chromatogram of the root of *A. pyrethrum*.

Methodological inspection

Calibration curves, limit of detection (LOD), and limit of quantification (LOQ). Six different concentrations of standard solutions were injected to determine the calibration curve. Quantification of the test compounds was performed using the external standard method. The detector response was set at 241 nm, where each compound presented its maximum absorption (Fig. 7). The correlation coefficients (r) of all standard components (within the range of 0.9997–0.999) showed good linearity. By comparing the signal-to-noise ratios of threefold (3σ) and tenfold (10σ) variations, the limit of detection (LOD) and limit of quantification (LOQ) of the five analytes were calculated. The LOD and LOQ ranges were 0.44–3.73 and 1.19–12.44 ng, respectively. The obtained results are described in detail in Table 5.

Accuracy, reproducibility, stability and recovery rate tests. The RSD was used as a measure of the accuracy, repeatability, stability and recovery rate. The RSD value of the accuracy tests was less than 1.67%. Therefore, it was determined that the instrument has good precision and meets the experimental requirements. The RSD value of the stability tests was less than 1.43%. Therefore, it was determined that the sample solution is stable within 10 h. The RSD value of the reproducibility tests was smaller than 1.81%. Therefore, it was determined that the method is reproducible and meets the experimental requirements. The RSD value of the recovery tests was less than 2.79%. The results of the four experiments are listed in Table 6, confirming that this method was appropriate for analysis.

Determination of sample content

Anaycclus pyrethrum samples were prepared in triplicate and were injected into the HPLC system for content determination. The results are shown in Table 7.

Conclusion

UPLC/Q-TOF-MS was applied for qualitative profiling of the components of *A. pyrethrum* root extract. *N*-alkylamides present explicitly characteristic fragmentation pathways that can be used as a basis for the identification of the components in *A. pyrethrum*. Using this approach, 21 compounds were identified, including twenty *N*-alkylamides and one organic

Table 5
Regression data, limit of detection (LOD), and limit of quantification (LOQ) of the five analytes by HPLC.

Analyte	Regression equation	*r*	Linear range (μg)	LOD (ng)	LOQ (ng)
1	$y = 83176x - 4896.8$	0.9999	10.00–400.00	0.44	1.46
2	$y = 205274x + 143461$	0.9999	29.33–1173.20	3.73	12.44
3	$y = 62833x - 2853.5$	0.9997	8.80–352.00	0.37	1.24
4	$y = 9436.1x - 1749$	0.9998	0.86–34.40	0.35	1.19
5	$y = 16982x - 4020.1$	0.9997	3.40–136.00	1.40	4.68

Table 6
Precision, repeatability, stability and recovery rate of the five analytes by HPLC.

Analyte	Precision (RSD, %)		Repeatability (RSD, %, *n* = 6)	Stability (RSD, %, *n* = 6)	Recovery rate (*n* = 6)	
	Intraday (*n* = 6)	Interday (*n* = 3)			Mean	RSD (%)
1	0.21	0.63	0.90	0.71	99.69%	1.57
2	0.55	0.69	0.64	0.59	96.23%	2.79
3	0.43	0.46	1.59	1.06	96.04%	2.28
4	1.67	1.28	1.81	1.43	101.12%	2.03
5	0.65	0.59	1.72	0.81	105.06%	2.46

Table 7
Contents of five *N*-alkylamide based on HPLC method in the root of *Anacyclus pyrethrum* samples ($n = 3$).

Analyte	1	2	3
1	0.040%	0.042%	0.040%
2	0.128%	0.132%	0.131%
3	0.049%	0.050%	0.049%
4	0.003%	0.003%	0.003%
5	0.018%	0.019%	0.018%

acid. (2*E*,7*Z*)-*N*-isobutyl-2,7-tridecadiene-10,12-diynamide and (2*E*,6*Z*,8*E*)-*N*-isobutyl-2,6,8-decatrienamide were reported for the first time in this plant. Undeca-2*E*,4*E*-diene-8,10-diynoic acid 4-OH-phenylethylamide, dodeca-2*E*,4*E*,*E*-trienoic acid 4-hydroxyphenylethylamide, tetradeca-2*E*,4*E*,n*E*-trienoic-8,10-diynoic acid, tetradeca-2*E*-diny-8,10-diynoic acid and tetradeca-2*E*,4*E*,n*E*-trienoic acid were identified as new compounds under the UPLC-Q-TOF-MS conditions. Based on the literature and the analysis of the mass spectrometry results, it was determined that the *N*-alkylamides are the main chemical components in *A. pyrethrum*. Therefore, five *N*-alkylamides were selected as the index components for HPLC determination. In this study, a simple and accurate HPLC method for the simultaneous separation and quantitative analysis of five *N*-alkylamides in the root of *A. pyrethrum* was established for the first time. This method has excellent linearity, precision, stability, and accuracy, and can be employed for the quality evaluation of *A. pyrethrum*. In conclusion, our study should be valuable for qualitative and quantitative analysis of *A. pyrethrum* and can improve the quality control standards.

Authorship

RJ, determination of sample solution and wrote the article; QQ and XG, separation of compounds; JZ and YS, extraction of sample solution; MZ, literature search; PT, instructor and guidance of the experiments; JH, instructor and corrections in the article; YL, instructor and design of the experimental plan.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

This work was supported by Natural Science Foundation of Xinjiang (No. 201318101-5).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2018.12.011.

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