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Alkaloids analysis using an off-line two-dimensional supercritical fluid chromatography × ultra-high performance liquid chromatography

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Abstract

In this study, an off-line two-dimensional (2-D) supercritical fluid chromatography (SFC) × ultra-high performance liquid chromatography (UHPLC) method with high orthogonality was developed to analyze the practical amide alkaloids fraction from *P. longum* L.. The effects of SFC parameters such as column, organic modifier, temperature and back pressure on separation were systematically evaluated. Different selectivity among columns (BEH, BEH 2-EP, XAmide and CSH FP) was observed. Then, investigation on orthogonality of different columns and systems was performed by geometric approach with a set of amide alkaloid samples. The orthogonality between CSH FP column and BEH column reached to 50.79%, which was much higher than the other columns. While the orthogonality between SFC and UHPLC based on XAmide column and HSS T3 column reached to 69.84%, which was the highest of all combinations. At last, the practical amide alkaloids fraction was analyzed on the off-line two-dimensional (2-D) chromatography SFC × UHPLC system. In total, at least 340 peaks were detected by this method. Rapid separation on these two dimensions and easy post treatment of SFC facilitated this 2-D system for the separation of complex samples.

**Key words:** supercritical fluid chromatography (SFC); ultra-high performance liquid chromatography (UHPLC); two-dimension; amide alkaloids; *Piper Longum* L.

1. Introduction

During the last periods, high performance liquid chromatography (HPLC) and ultra-high performance liquid chromatography (UHPLC) had been widely used for the rapid analysis of complex samples to some extent\(^1\). Compared with HPLC and UHPLC, the application of supercritical fluid chromatography (SFC) is not so widely, but it is also a powerful strategy for the analysis of complex samples. It is known that supercritical fluid (SF) has the properties between those of gas and liquid. Thus, SFC had lower mass transfer resistance between stationary phase and mobile phase compared with HPLC. Meanwhile, SFC facilitates lower pressure drop at relatively high flow rate. Moreover, the mobile phase in SFC has a solvating power like that in HPLC. Thus, high resolution and high speed separations could also be achieved under
SFC mode\textsuperscript{2,3}.

As it is well known, in SFC, stationary phase\textsuperscript{4}, organic modifier, backpressure and temperature modifications induce great effects on the retention. The effects of flow rate or column length on retention time changes were also interesting to some extent and samples can be analyzed in only a few minutes or even seconds with appropriate column dimensions and flow rates\textsuperscript{5}. The analytical range of SFC was broadened because various types of columns could be used. Non-polar\textsuperscript{6,7}, polar ionizable compounds\textsuperscript{6,8} and even peptides\textsuperscript{9} have been analyzed by SFC. Though supercritical CO\textsubscript{2} has high solvation ability, it is not enough for some polar analytes. Luckily, its polarity and solvation ability can be changed by adding a polar organic modifier such as methanol, ethanol, isopropanol and so on. In addition, close to the critical point, the densities and solvating power of SF can change sharply with a slight change in pressure. Thus, separation can be achieved by regulating the pressure of SFC.

Nowadays, 2-D chromatography technique is becoming an effective method to separate complex samples, such as polymers, metabolites, proteins and natural products\textsuperscript{10-14}. Orthogonal separation based on different separation mechanisms could improve separation selectivity and peak capacity\textsuperscript{15-17}. Sandra et al.\textsuperscript{18} developed an automated off-line SFC×SFC system using octadecyl silicagel (ODS) and silver-loaded stationary phases in the first and second dimensions, respectively. The first dimension effluent was captured, concentrated and re-injected on the secondary column in a completely automated manner. In previous research, retention behavior of up to 46 solutes of varying molecular properties have been studied under reversed-phase liquid chromatography (RPLC), SFC, gas-liquid chromatography (GLC), and micellar electrokinetic capillary chromatography (MECC) modes, respectively. The orthogonality of different 2-D systems combined with these modes were evaluated by the 2-D chromatographic plots of their ranged-scaled retention date\textsuperscript{19}. Thanks to the different separation mechanism between SFC and RPLC, the complementary separation could be obtained, demonstrating that 2-D chromatography based on these two modes will be a promising method for the analysis of complex components.
In this study, the goal was to explore the application of SFC system in separation of amide alkaloids from *Piper* species. Most of earlier works on *Piper* species suggested that the major bioactive constituents were amide alkaloids\textsuperscript{20-22}. However, the structures of amide alkaloids in *P. longum* L. are so similar that resulting in a similar retention behavior on single column. Furthermore, some alkaloids with the same molecular weight complicate MS identification. Therefore, new 2-D chromatography methods should be developed to separate amide alkaloids from *Piper Longum* L., which is beneficial for further characterization and bioactive research. Firstly, the effects of column selectivity, organic modifiers, temperature and back pressure on SFC separation were investigated. Then, orthogonality of different stationary phases and systems were evaluated using selected 25 amide alkaloid compounds. Based on the evaluation results, an off-line 2-D SFC × UHPLC method was developed to separate alkaloids fraction of plant origin.

2. **Experiment**

2.1 **Reagents and materials**

Methanol (MeOH), ethanol (EtOH), isopropanol (IPA) and acetonitrile (ACN) of HPLC grade were purchased from J&K (Beijing, China). The water used in this study was purified with a Milli-Q water purification system (Millipore, Bedford, MA, USA). Liquid CO\textsubscript{2} (food grade) was purchased from Zhenxin Gaisi (Shanghai, China) *P. Longum* L. was purchased from Anguo Herb Market, Hebei province (China). The herb was authenticated by the Institute of Medication, Xiyuan Hospital of China Academy of Traditional Chinese Medicine.

2.2 **Sample preparation**

The amide alkaloids fraction and twenty-five compounds (shown in Table 1) investigated in this study were prepared by our lab\textsuperscript{23}. The pKa, log P, H-bond donor and acceptor values of these compounds were shown in support information. All samples were stored at -20°C before used.

2.3 **Instruments and columns**

The Waters Acquity UPC\textsuperscript{2} TM system (which stands for Acquity Ultra Performance Convergence Chromatography) was equipped with a binary manager, an autosampler
manager-FL, a column manager, a PDA detector and a convergence with an automatic
backpressure regulator (ABPR).

The RPLC analysis was performed on a Waters ACQUITY UHPLC® H-Class
system including a quaternary solvent manager, a sample manager-FTN, a column
manager and a PDA detector.

Data acquisition and processing of UPC² and UHPLC were conducted using
Waters Empower 3 software.

The columns used in SFC were as follows: Acquity UPC²™ CSH Fluoro-Phenyl
(50 × 2.1 mm i.d., 1.7 µm), Acquity UPC²™ BEH 2-EP (50 × 2.1 mm i.d., 1.7 µm),
Acquity UPC²™ BEH (50 × 2.1 mm i.d., 1.7 µm) were purchased from Waters
(Milford, MA, USA), abbreviated as CSH FP, BEH 2-EP, BEH in this paper,
respectively. XAmide column (150×4.6 mm i.d., 5 µm) was purchased from Acchrom
(Beijing, China).

The column used in H-Class was Acquity UHPLC HSS T3 (100 × 2.1 mm i.d.,
1.8µm, Waters, USA), abbreviated as HSS T3.

2.4 Chromatographic conditions

2.4.1 Evaluation of retention and selectivity of four columns under SFC
conditions

A mixture of eight amide alkaloids compounds (No.1-8 shown in Table 1) were
dissolved in a mixture of n-hexane/isopropanol (7:3, v/v) with different
concentrations. The linear velocity was adjusted the same and the CO₂/MeOH
gradient conditions were adjusted to the same gradient steepness irrespective of the
column dimension. For CSH FP, BEH 2-EP and BEH columns, the gradient condition
was 1-5% MeOH in CO₂ from 0-10 min, the flow rate was 0.8 mL min⁻¹, the injection
volume was 1 µL. For XAmide column, the gradient condition of 1-7% MeOH in
CO₂ from 0-30 min were used, the flow rate was 3.8 mL min⁻¹ and the injection
volume was 5 µL. The column temperature and the ABPR were set at 40 °C and 1800
psi, respectively. The UV detection wavelength was 254 nm.

2.4.2 Evaluation of the effect of organic modifiers, temperature and
backpressure in SFC
The XAmide column and the same mixture as described in section 2.4.1 were assessed to evaluation of the effect of organic modifiers, temperature and backpressure on amide alkaloid compounds' separation. The conditions were set as follow: the isocratic condition was set at 4% MeOH in CO$_2$; the flow rate was 3.0 mL min$^{-1}$; the injection volume was 5 µL. The organic modifiers investigated in this study were MeOH, EtOH, IPA and ACN. The temperature was set at 35, 40, 45 and 50 °C when the effect of temperature was investigated. The backpressure was set at 1600, 1800, 2000 and 2200 psi when the effect of backpressure was investigated.

2.4.3 Evaluation of Orthogonality on different SFC × SFC systems and SFC × UHPLC systems

The orthogonality among three SFC columns and one UHPLC column were investigated by using twenty-five amide alkaloids compounds as probes (listed in Table 1).

On SFC system, the gradient conditions were set from 1-5% MeOH in 10min for CSH FP and BEH columns. The flow rate was 0.8 mL min$^{-1}$, the injection volume was 1 µL; 1-7% MeOH in 15 min for XAmide column, the flow rate was 3.0 mL min$^{-1}$. The injection volume was 5 µL. The column temperature, backpressure and UV detection wavelength were same as section 2.4.1.

On UHPLC system, the mobile phases were H$_2$O (A) and ACN (B). The gradient condition was as follows: 0-10 min, A/B, 40/60-20/80; 10-20 min, A/B, 20/80-5/95; 20-30 min, A/B, 5/95. An HSS T3 column was used in this system. The flow rate, injection volume, column temperature and UV detection wavelength were set at 0.2 mL min$^{-1}$, 2 µL, 25 °C and 254 nm, respectively.

2.4.4 2-D chromatography analysis of practical amide alkaloids fraction from *Piper Longum* L.

In the 2-D chromatography separation, the XAmide column was employed in the first dimension SFC system. The corresponding mobile phase A was CO$_2$ and mobile phase B was MeOH. The linear gradient elution condition was as follows: 0-8 min, A/B, 98/2; 8-13 min, 98/2-97/3; 13-15min, 97/3-92/8; 15-20 min, 92/8. The flow rate was 3.0 mL min$^{-1}$. The column temperature and the ABPR were set at 40 °C and 1800
psi, respectively. The UV detection wavelength was 254 nm. About 125 mg crude alkaloids fraction were dissolved in 1 mL n-hexane/isopropanol (7:3, v/v). The injection volume was 5 µL. The fractions were split into two parts. One part flowed into the detector while the other part was collected. Fractions were collected manually from 2 to 18 min at 0.5 min interval, and they were denoted as Fraction 1 to Fraction 32 orderly. All fractions were dried with nitrogen and re-dissolved in H₂O/ACN (3:7, v/v). The samples were stored at 4 °C until use.

The HSS T3 column was used as the second dimension RPLC column. The mobile phases were H₂O (A) and ACN (B). The elution condition in this dimension was as follows: 0-10 min, A/B, 40/60-20/80; 10-20 min, A/B, 20/80-5/95; 20-30 min, A/B, 5/95. The flow rate was 0.2 mL min⁻¹. 5 µL of each fraction collected from the first dimension was injected into the second dimension, respectively.

2.5 Data analysis

Orthogonality was evaluated according to a reported method²⁴. The retention times of 25 compounds on each column in single-dimension chromatography setup were normalized according to Eq. (1),

\[ t_{R,normal}^{(i)} = \frac{t_{R}^{i} - t_{R}^{min}}{t_{R}^{max} - t_{R}^{min}} \]  

(1)

in which \( t_{R}^{max} \) and \( t_{R}^{min} \) represented the retention times for the peaks showing greatest and least retention among all the second dimension runs, respectively. The retention times \( t_{R}^{i} \) were converted to normalized \( t_{R,normal}^{(i)} \) values that range from 0 to 1. Then the normalized retention data were plotted into a 2-D separation space which was divided into 5×5 bins. The orthogonality O% was calculated according to Eq. (2),

\[ O\% = \frac{\sum bins - \sqrt{P_{max}}}{0.63P_{max}} \times 100 \]  

(2)

in which \( \sum bins \) was the number of bins containing data points in the 2-D plot. \( P_{max} \) was the sum of all bins, which represented the total peak capacity in this evaluation system.

3. Result and discussion
3.1 Effect of column’ selectivity in SFC

In order to investigate the effect of column’s selectivity on the retention behavior of amide alkaloids under SFC conditions, four different columns including three SFC columns and one hydrophilic interaction liquid chromatography (HILIC) column were employed. The results were shown in Fig. 1. For the purpose of preserving the gradient steepness irrespective of the column dimension, the linear velocity was converted into the same and the gradient conditions were adjusted. Thus, the retention and selectivity can be directly compared on these chromatograms. At first, the peak shapes were acceptable for eight amide alkaloids on all columns in SFC. In term of retention time, the XAmide column was longer than the others.

In term of selectivity, both stationary phases and solute’s structure played important roles for the separation. The elution order was very similar on BEH, BEH 2-EP and XAmide columns while that was great difference on CSH FP column (Fig. 1). Compared with other columns, compounds 4 and 5 were eluted before other compounds and the retention of compound 6 was stronger than compounds 7 and 8 on CSH FP column. Although the same elution order was observed between BEH and BEH 2-EP, the resolution was some different (Fig. 1 B and C). Compounds 4 and 5 which cannot be separated on BEH were effectively separated on BEH 2-EP. Compounds 7 and 8 that were co-eluted on BEH 2-EP were effectively resolved on BEH. Compounds 1 and 2, as well as compounds 4 and 5 (as shown in Table 1), were all similar combinations except the difference of length of the carbon chain. However, only compounds 1 and 2 with piperidine ring could be separated effectively on these four columns at the same time. Difference in the selectivity of these columns indicated their potential application in the design of 2-D system for complex samples’ separation.

3.2 Effect of organic modifier, temperature and backpressure in SFC

Comparing with the other three SFC column (CSH FP, BEH, BEH 2-EP), the XAmide column was usually used on HILIC mode\textsuperscript{25,26}. In this study, the XAmide column was used for the separation of amide alkaloids under SFC conditions and good result was obtained. It can be speculated that HILIC stationary can also be used
on SFC in addition to traditional SFC columns. Thus, the effect of organic modifier, temperature and backpressure in SFC system was investigated on XAmide column. As shown in Fig. 2, the selectivity was not apparent changed as the polarity of organic modifiers increased, while the retention time of eight compounds were greatly decreased. It illustrated that the elution mode of amide alkaloids was similar to normal-phase liquid chromatography (NPLC) on XAmide column under SFC conditions. Both the best resolution and the relative weakest retention were observed when using MeOH as organic modifier\textsuperscript{27}.

Furthermore, the retention time was increased with the increase of temperature (shown in Fig. 3) or the reduction of backpressure (shown in Fig. 4). As temperature increased or backpressure decreased, the density of CO\textsubscript{2} was decreased, leading to the elution capacity diminished. As shown in Fig. 3 and 4, the resolution of compounds 1 and 2, as well as compounds 4 and 5 was increased when temperature increased or backpressure decreased. On the contrary, the resolution of compounds 7 and 8 was decreased with the increase of temperature or the reduction of backpressure.

Compared with compounds 1, 2, 4, and 5, only compounds 7 and 8 had pepper ring structures (as shown in Table 1). Thus, the effect of temperature and backpressure on selectivity might be different for amide alkaloids with pepper ring from others.

### 3.3 Evaluation of the orthogonality of SFC × SFC system and SFC × UHPLC system

According to the result described in section 3.1, there was good retention and separation for amide alkaloids on SFC system. Because of the different selectivity among various stationary phases, as well as different separating mechanism between SFC and UHPLC, good orthogonality could be obtained both on SFC × SFC system and SFC × UHPLC system. In this research, the geometric approach developed by Gilar et al.\textsuperscript{28} was used to evaluate the orthogonality between different 2-D chromatography systems. A series of amide alkaloids purified from \textit{Piper Longum} L. was selected as test compounds for orthogonality evaluation (listed in Table 1). Structures of these compounds were similar and some of them were isomers. Hence, some of the test compounds were not fully resolved in uni-dimensional separation.
The BEH, CSH FP and XAmide columns, operated under SFC conditions, were used to develop 2-D SFC × SFC systems. A RPLC column, HSS T3, was chosen to generate the basic set of retention time data under UHPLC conditions. Three 2-D SFC × UHPLC systems were developed using HSS T3 column and three SFC columns. The normalized retention time plots for six different 2-D systems were shown in Fig. 5.

On SFC × SFC system, the orthogonality between CSH FP column and the other two columns was relatively high, which reached to 50.79% (Fig. 5 B) and 44.44% (Fig. 5 A). It may relate to the different functional groups between CSH FP column and the other two columns. The orthogonality of BEH and XAmide was 25.39%, which suggested the separation selectivity was similar between them.

On SFC × UHPLC system, it was exciting to find that the orthogonality between XAmide and HSS T3 was as high as 69.84%, which was much higher than the other combinations. It can be easily explained by the different separation mechanism of SFC and UHPLC. It could be seen that many co-eluted compounds in one dimension could be well-separated by the orthogonal methods. Taking XAmide × HSS T3 system (Fig. 5 F) as an example, the data points in the dashed lines a and b, which represented the normalized retention times of the six solutes in the XAmide × HSS T3 systems. Compounds 18, 15, 5 and 20 (line a) could not be separated on the XAmide column, but good resolution can be obtained on the HSS T3 column. The case was just reverse for the compounds 18, 10 and 1 (line b). Supposing a sample contains these seven compounds, good separation of them could not be achieved with any column system in one dimension separation, but this problem could be resolved by the combination of these two complementary column systems. In addition, some of the positional isomers could also be well separated under two-dimensional system in this research. For example, compounds 15 and 16 could not be separated on HSS T3 column, but good resolution could be obtained on XAmide column.

3.4 Off-line 2-D chromatography separation of the amide alkaloids fraction from *Piper Longum* L.

It was well known that the orthogonality was dependent not only on the separation
mechanism, but also on the properties of the compounds and the separation conditions\textsuperscript{29}. More validation in practical analysis needed to be investigated. Thus, an off-line 2-D SFC × UHPLC system was attempted to be developed for the analysis of practical samples based on the previous experiments in this research. At first, one-dimensional SFC and UHPLC analysis of amide alkaloids fraction obtained from \textit{Piper Longum} L. were done on XAmide and HSS T3 column, respectively. As shown in Fig. 6 A and B, selectivity of the sample on these two columns were significant different, which suggested that good orthogonality for the separation of amide alkaloids could be obtained on these two dimensions. Therefore, it was promising for the construction of an off-line 2-D chromatography system based on SFC and UHPLC. Here, the XAmide column under SFC conditions was used on the first dimension. The post treatment of fractions eluted from the SFC could be easily evaporated, which benefit for the re-dissolving of the second dimension. The HSS T3 column under UHPLC conditions was used on the second dimension in order to obtained better separation.

Here, the 2-D chromatography analysis showed excellent separation results and good orthogonality. Taking Fraction 20 (eluting from 11.5 to 12 min in Fig. 6 A) as an example, re-analysis of it on the XAmide column revealed a simple composition which was only one main peak with a purity of more than 90% (Fig. 7 A). However, after the separation in the second dimension on HSS T3, more than 13 peaks dispersed throughout the chromatogram and more information could be got (Fig. 7 B). On the other word, the low-abundance alkaloids that were always covered up by the major component in a uni-dimensional separation could be identified by the 2-D chromatography system.

In order to illustrate the orthogonality of this SFC × UHPLC system, A three-dimensional chromatogram was also constructed (Fig. 8), which could not only indicate good orthogonality on this two dimensions, but also could show the high resolving power of this system. After deleted the same retention in the adjacent fractions, at least 340 peaks could be separated by the 2-D chromatography, while less than 50 peaks (Fig. 6B) could be separated by one-dimensional liquid...
In summary, this 2-D SFC × UHPLC system based on XA mide and HSS T3 had the following advantages: firstly, high orthogonality could be obtained between two dimensions thanks to their different separation mechanism, which could lead to higher resolving ability and simple operation during experiments; secondly, both of these two dimensions could separate amide alkaloids fraction with high-speed and high-efficiency and the post treatment of the first dimension was easy. Thus, it could obtain amide alkaloids as many as possible if this method enlarged to preparation scale using corresponding preparative SFC and preparative HPLC. Finally, many low-abundance compounds were detected in this system, which was greatly benefit to in-depth understanding the composition of *P. longum* L.

4. Conclusions

In this research, an off-line 2-D SFC × UHPLC method with high orthogonality was developed to analyze the practical amide alkaloids fraction from *P. longum* L.. Separation of amide alkaloids was systematically investigated on SFC. The main conclusions were as follows:

1. The effects of column’s selectivity, as well as organic modifier, temperature and back pressure on separation of amide alkaloids were systematically evaluated. Different separation selectivity were exhibited among different columns, which provided the potential probability to build a 2-D chromatography method. When using MeOH as the organic modifier, the retention times of amide alkaloids were weaker than the others but the resolution was the highest. In addition, the retention time was increased with the increasing of temperature or the reduction of backpressure. It may relate that the elution capacity of supercritical CO$_2$ was influenced by the density of CO$_2$ which was significantly affected by the temperature and backpressure.

2. Orthogonality evaluation with 25 amide alkaloids compounds were performed on different 2-D chromatography systems. The orthogonality between CSH FP and BEH reached to 50.79%, which was much higher than the other SFC × SFC systems. Thanks to the different separation mechanism of SFC and UHPLC, the
orthogonality between XAmide and HSS T3 was as high as 69.84%, which was
the highest of all combinations.

(3) An off-line 2-D chromatography system based on SFC and UHPLC using
XAmide and HSS T3 was developed to separate alkaloids fraction effectively due
to their high orthogonality and fast analysis speed. It could not only separate the
non-separated peaks in one-dimensional separation, but also could detect more
low abundant components covered up by the major component in a
uni-dimensional separation. The development of this 2-D chromatography system
would be an effective tool for the separation of complex samples.

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32, 1459-1464.


1299-1306.

263-272.

1218, 8551-8560.


FIGURE CAPTION

Table 1 Structure characterization of isolated compounds from *Piper Longum* L.

Fig. 1. Selectivity of different stationary phases for 8 amide alkaloids. Experimental conditions were described in section 2.4.1.

Fig. 2. Separation of 8 amide alkaloids in different organic modifiers. Experimental conditions were described in section 2.4.2.

Fig. 3. Separation of 8 amide alkaloids in different temperature. Experimental conditions were described in section 2.4.2.

Fig. 4. Separation of 8 amide alkaloids in different backpressure. Conditions were the same as section 2.4.2.

Fig. 5. Normalized retention time plots for SFC × SFC systems of “CHS FP × BEH” (A), “CSH FP × XAmide” (B), “BEH × XAmide” (C), and SFC × UHPLC systems of “CSH FP × HSS T3” (D), “BEH × HSS T3” (E) and “XAmide × HSS T3” (F). Compounds numbers were listed as in Table 1. Experimental conditions were described in section 2.4.3.

Fig. 6. SFC and UHPLC chromatogram of amide alkaloids fraction. (A) SFC, XAmide column (150 mm × 4.6 mm i.d., 5 µm). (B) UHPLC, ACQUITY UHPLC HSS T3 (100 mm × 2.1 mm i.d., 1.7 µm). Experimental conditions were described in section 2.4.4.

Fig. 7. SFC and UHPLC chromatogram of fraction 20. Experimental conditions were described in section 2.4.4.

Fig. 8. Three-dimensional chromatogram of amide alkaloids fractions 1-32 obtained from the first dimension analyzed on HSS T3 column.
Fig. 5. Normalized retention time plots for SFC × SFC systems of “CHS FP × BEH” (A), “CHS FP × XAmide” (B), “BEH × XAmide” (C), and SFC × UHPLC systems of “CSH FP × HSS T3” (D), “BEH × HSS T3” (E) and “XAmide × HSS T3” (F). Compounds numbers were listed as in Table 1. Experimental conditions were described in section 2.4.3.
Fig. 6. SFC and UHPLC chromatogram of amide alkaloids fraction. (A) SFC, XAmide column (150 mm × 4.6 mm i.d., 5 µm). (B) UHPLC, ACQUITY UHPLC HSS T3 (100 mm × 2.1 mm i.d., 1.7 µm). Experimental conditions were described in section 2.4.4.

60x44mm (300 x 300 DPI)
Fig. 8. Three-dimensional chromatogram of amide alkaloids fractions 1-32 obtained from the first dimension analyzed on HSS T3 column.  
119x83mm (300 x 300 DPI)
Table 1 Structure characterization of isolated compounds from *Piper Longum* L.

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<th>Name</th>
<th>Structure</th>
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<td>N-isobutyl-2E,4E-octadecadienamide</td>
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14 Pipercallosine

15 (2E,4E,12Z)-N-Isobutylloctadeca-2,4,12-trienamide

16 1-[9-(3',4'-methylenedioxyphenyl)-4E,6E,8E-nonatrienoyl]piperidine

17 dehydropipernonaline

18 Pipernonatine

19 (2E,4E,14Z)-N-Isobutyleicosa-2,4,14-trienamide

20 N-isobutyl-2E,4E-decyldecadienamide

21 1-[(2E,4E,14Z)-1-oxo-2,4,14-eicosatrienyl]-piperidine

22 (2E,4E,14Z)-N-Isobutyldocosa-2,4,14-trienamide

23 (2E,4E,12E)-13-(benzo[d][1,3]dioxol-6-yl)-1-(piperidin-1-yl)trideca-2,4,12-trien-1-one

24 (2E,4E,13E)-14-(benzo[d][1,3]dioxol-6-yl)-N-isobutyltetradeca-2,4,13-trienamide

25 brachyamide B