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Analysis of dencichine in *Panax notoginseng* by gas chromatography–mass spectrometry with ethyl chloroformate derivatization

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Abstract

Dencichine (β-*N*-oxalyl-L-α,β-diaminopropionic acid) is a haemostatic agent present in well-known traditional Chinese medicinal herbs such as *Panax notoginseng*, as well as other *Panax* species. It is also a reported neurotoxic agent found in *Lathyrus sativus* (grass pea seed) and cycad seeds. A method was developed for quantitative determination of the non-protein amino acid, dencichine, in plant samples of *P. notoginseng* and the adventitious roots directly from the explants of *P. notoginseng* after derivatization with ethyl chloroformate (ECF) by gas chromatography–mass spectrometry (GC–MS). L-2-chlorophenylalanine was used as an internal standard. Calibration curves were linear ($r^2 = 0.9988$, $n = 6$) in the range of 10–800 μg/ml for dencichine. Limit of detection and quantification for dencichine were 0.5 μg/ml and 2 μg/ml, respectively. This rapid and specific method may be applied to the quantification of dencichine in complex medicinal plants and their products.

Keywords: Gas chromatography–mass spectrometry; Dencichine; *Panax notoginseng*; Adventitious roots; Ethyl chloroformate (ECF)

1. Introduction

Panax notoginseng (Burk.) F.H. Chen (commonly known as Tianqi or Sanqi) is a highly valued and important Chinese medicinal herb produced mainly in Yunnan Province, China. Some of its chemical constituents are similar to those present in two other well-known species in the same plant genus—*Panax ginseng* and *Panax quinquefolium*. For decades, the raw *P. notoginseng* has been highly valued for its wide ranging therapeutic properties which include the induction of blood clotting for bleeding conditions, promotion of blood circulation, relief of swelling, alleviation of pain. Currently, the roots of *P. notoginseng* are used to treat coronary heart disease, cardiac angina, apoplexy and atherosclerosis in clinics [1,2]. Recently, much of the research effort has been focused on the numerous bioactive saponins (such as ginsenosides, notoginsenosides) present in these *Panax* species.

Dietary non-protein amino acids have been implicated as potential factors interfering with fundamental biochemical processes and causing clinical disorders [3]. β-*N*-oxalyl-L-α,β-diaminopropionic acid (β-ODAP) (Fig. 1), a neuro-excitatory non-protein amino acid, was first isolated from the seeds of *Lathyrus sativus* (grass pea seeds) [4]. It was identified as the main cause of a neurological disorder known as neurolathyrism, a condition with typical and acute neurotoxic symptoms such as the inability to stand, head retraction, stiffening of the neck and extensor paralysis of the legs [5,6]. Besides being present in the seeds of *L. sativus*, β-ODAP was also found in cycad seeds [7]. The mechanisms whereby β-ODAP causes neurotoxicity have been researched by some investigators.

Interestingly, dencichine, a trivial name for β-*N*-oxalyl-L-α,β-diaminopropionic acid, was also found to be present in a very different *Panax* species, the only non-leguminous plants in which β-ODAP is known to be present [8]. Furthermore, it is a bioactive therapeutic amino acid component in *P. notoginseng*. Studies have reported dencichine as the compound responsible for the medicinal herb's main haemostatic and platelet-increasing properties in vivo [9] and found that the haemostatic effect was present at a low dose of dencichine, while neurotoxicity occurred at higher doses [9]. Therefore an accurate, sensitive, rapid and simple analytical method for detection of dencichine must be developed.

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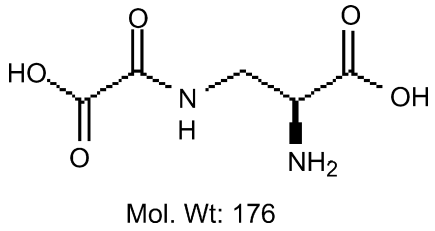

Fig. 1. Chemical structures of dencichine, i.e., β-*N*-oxalyl-L-α,β-diaminopropionic acid (β-ODAP).

Several methods have been developed to address the determination of β-*N*-oxalyl-L-α,β-diaminopropionic acid including a colorimetric method which utilized the reaction of *o*-phthalaldehyde (OPT) with α,β-diaminopropionic acid [10,11], high-performance thin-layer chromatography (HPTLC) [12], HPLC with pre-column derivatization methods [8,13–18] and hydrophilic interaction chromatography with positive electrospray ionization tandem mass spectrometry (HILIC/ESI-MS/MS) [19]. However, GC–MS with ethyl chloroformate (ECF) derivatization for the determination of dencichine from *P. notoginseng* has been not reported.

The aim of this study is to develop a GC–MS method for the quantification of dencichine with ethyl chloroformate derivatization in complex plant matrices of *P. notoginseng*. The method reported here is sensitive, reproducible and rapid, which is suitable for the determination of β-ODAP in *P. notoginseng*. Additionally, amino acids were also identified in the adventitious roots directly from the explants of the *P. notoginseng* by the developed method in order to examine whether the adventitious roots have dencichine also.

2. Experimental

2.1. Chemicals and materials

The dencichine standard was purified from *P. notoginseng* by our laboratory (Section 2.2), its purity was confirmed by thin layer chromatography (TLC), high performance liquid chromatography (HPLC) [20,21] and through ^{1}H NMR, ^{13}C NMR, gas chromatography (GC). The structure was confirmed as β-*N*-oxalyl-L-α,β-diaminopropionic acid. Ethyl chloroformate and all of the amino acids for confirmation and internal standard ware purchased from Sigma–Aldrich, including alanine, glycine, valine, leucine, sercine, isoleucine, threonine, proline, asparagine, aspartic acid, methionine, glutamine acid, phenylalanine, cysteine, glutamine, 2-chlorophenylalanine (IS), lysine, histamine, tyrosine, tryphtothan, cystine.

Analytical cation-exchange resin 732 was purchased from Huazhen science Co., Ltd., Shanghai, China. Analytical-grade reagents were from Shanghai Lin Feng chemical reagent Co., Ltd., China. HPLC water was prepared with the Millipore Milli-Q SP water purification system (18.2 MΩ, Milipore, Bedford, MA, USA). All aqueous solutions were prepared with HPLC water.

Genuine *P. notoginseng* was collected from Wenshan, Yunnan, China. They were verified by professor of Shanghai Jiao Tong University of China as the dried rhizoma of *P. notoginseng* (Burk.) F.H. Chen.

2.2. Standard dencichine preparation

The air dried roots of *P. notoginseng* (10 g) were powdered and then extracted with methanol (3 × 100 ml) at 40–50 °C with occasional stirring. After filtration, the residue was air dried and extracted with water (3 × 100 ml) at 40–50 °C with occasional stirring. The combined water extracts were concentrated under reduced pressure to about 10 ml. The concentrate was extracted with *n*-butanol (3 × 10 ml). The combined aqueous phase, after being freed of solvents was percolated through a 3 cm × 60 cm column of resin 732 (H^+), (200–400 mesh) and first eluted with Millipore water and then eluted with ammonium hydroxide (0.05 M). The pooled ninhydrin-positive fractions were concentrated to about 10 ml at 40–50 °C in a rotary evaporator and treated with an excess of acetone (400 ml) and stirred. The precipitated compound was collected and re-precipitated from water. Yield, 0.4–0.5 g. The compound was recrystallized from water; m.p. 207 °C, decomp $[\alpha]_D{}^{20} = -31.92^\circ$ (H_2O):

- ^{13}C NMR (D_2O) δ_C: 56.3 (CH), 42.1 (CH_2);
- ^{1}H NMR (D_2O) δ_C: 3.87 (1H, ABd, J = 15.0, 4.15 Hz), 3.96 (1H, ABd, J = 15.0, 4.15 Hz), 4.24 (1H, dd, J = 6.8, 4.2 Hz);
- ESI *m/z*: 176 (M) (100%).

A 1 mg/ml dencichine standard stock solution in water was prepared and kept at 4 °C for later use.

2.3. Sample preparation of P. notoginseng

The air dried roots of *P. notoginseng* (10 g) were powdered and then extracted with methanol (3 × 100 ml) at 40–50 °C with occasional stirring. After filtration, the residue was air dried and extracted with water (3 × 100 ml) at 40–50 °C with occasional stirring. The combined water extracts were concentrated under reduced pressure to about 10 ml. The concentrate was extracted with *n*-butanol (3 × 10 ml). The combined aqueous phase, after being freed of solvents, was dissolved with water by ultrasonication (250 W) and transferred to 25 ml volumetric flask. An aliquot of 50 μl of the solution was used for derivatization.

2.4. Sample preparation of adventitious roots

Adventitious roots were obtained by the methods of our laboratory [22]. The sample was prepared according to the procedure of Section 2.3. An aliquot of 200 μl of the sample solution was used for derivatization.

2.5. Ethyl chloroformate derivatization procedure

A 500 μl samples (balanced with water if sample volume was less than 500 μl) and 100 μl L-2-chlorophenylalanine internal standard, 0.1 μg/μl were placed in a screw glass tube (5 ml). A 400 μl alcohol, 100 μl pyridine and 50 μl ethylchloroformate

were added to the tube. After brief shaking, the mixture was sonicated for 30 s. To separate the derivatives from the reaction mixture, 300 μl of chloroform were added. The mixture was vortexed for 30 s and the upper aqueous layer was aspirated off after equilibrating for a few minutes; the organic layer was dried by adding a small portion of anhydrous sodium sulfate. The dry organic solution was transferred to a new vial and 1 μl was injected to the GC–MS by an auto sampler system [23,24].

2.6. GC–MS separation

A Perkin-Elmer GC–MS system consisting of a Model gas chromatograph and a Model TURBOMASS mass spectrometer was used. Samples were injected into a fused-silica capillary column (Agilent J&W DB-5MS, 30 m × 0.25 mm × 0.25 μm) in the split injection mode (3:1). The oven temperatures were as follows: the initial temperature was 80 °C, hold 2 min. It was raised to 140 °C at a rate of 10 °C min^{-1}, then to 240 °C at rate of 4 °C min^{-1} and followed by 10 °C min^{-1} to 280 °C hold for 3 min. Other instrumental parameters were as follows: the electron energy was set at 70 eV, the ion source temperature set at 200 °C and the injector temperature set at 260 °C. Helium, as a carrier gas, was set to a column flow rate of 1.0 ml min^{-1}.

All data were collected in the full scan mode (30–550 *m/z*). The dwell time for each scan was set at 100 ms and solvent delay at 2.5 min. Ions used for quantitation were 129 for dencichine and 102 for L-2-chlorophenylalanine (IS). Data were obtained and integrated with TurboMass software. The area ratio of these two selected ions was used for calculation.

Identification of structures/compounds of the peaks was supported by comparison to commercial mass spectral libraries in NIST98 format and/or mass spectral interpretation. In addition, 20 natural amino acids were confirmed by standards.

2.7. Method validation

To assess linearity, a series of the standard solutions of dencichine were tested between the standard concentration and the peak area ratio (analyte/internal standard). The limit of detection (LOD) was calculated by measuring dencichine standard of decreasing concentrations to establish the lowest concentration that the method can detect with a suitable response in the GC–MS (the ratio of the testing peak signal-to-noise, S/N = 3). On the other hand, the limit of quantification (LOQ), the lowest concentration that the method can quantify at acceptable GC–MS criteria with a S/N of 9. The instrument precision (calculated as relative standard deviation, R.S.D.), for a low and middle concentration of dencichine were determined by five consecutive injections. The intra-day and inter-day assay precision of the method was also evaluated using multiple preparations of the sample. Five replicate samples were extracted and analyzed in a single day and on three different days. The recovery experiments of dencichine were performed by adding dencichine standards of three different levels to the extract of *P. notoginseng*, which were treated according to the procedure described in Section 2.3 for five times.

3. Results and discussion

The main goal of this work was to develop a GC–MS method for the quantification of dencichine with ethyl chloroformate derivatization in complex plant matrices of *P. notoginseng*. With this method 21 amino acids were found and the content of dencichine in the plant *P. notoginseng* was measured. With the developed method dencichine was found in the adventitious roots directly from the explants of the *P. notoginseng*.

3.1. Extraction method development

P. notoginseng contains various saponins, amino acids, polysaccharides and flavonoids. The dammarane-type saponins, which include ginsenosides and notoginsenosides, account for more than 10% of the total root content. Dencichine only accounts for about 0.5% of the total root content. Dencichine easily dissolves in water but not in methanol, ethanol and acetone. In order to remove the saponins as much as possible and obtain maximum amount of dencichine, methanol was chosen as the first extraction solvent to separate saponins and then extracted with water. The water extract was then extracted with *n*-butanol to get rid of saponins further. The influence of the extraction time on the efficiency of extraction was also investigated. Powdered samples were extracted with methanol for 2 h, 4 h, 6 h and then with water for 2 h, 4 h, 6 h, respectively. It was found that the 6 h extraction gave a slightly higher amount of dencichine, so it was used in this study. Repeating the extraction process three times and concentrating to the same volume made a negligible difference to the amount of dencichine extracted.

3.2. Derivatization and extraction efficiencies of amino acids

To study the derivatization efficiencies of the amino acids, the aqueous solution of amino acids and samples were derivatized according to the method of Section 2.5. The derivatized amino acids were extracted with chloroform. The chloroform solution

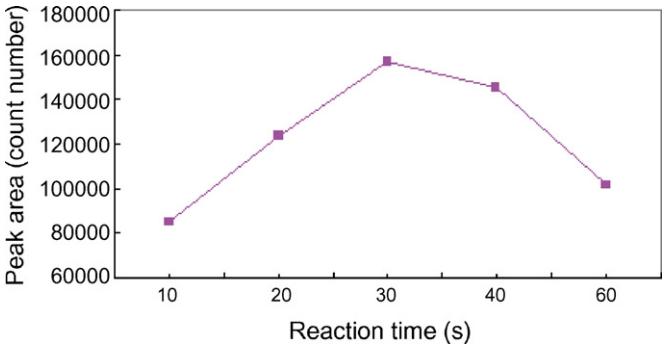

Fig. 2. Peak area vs. reaction time at 20 °C for the ECF derivatization of β-ODAP.

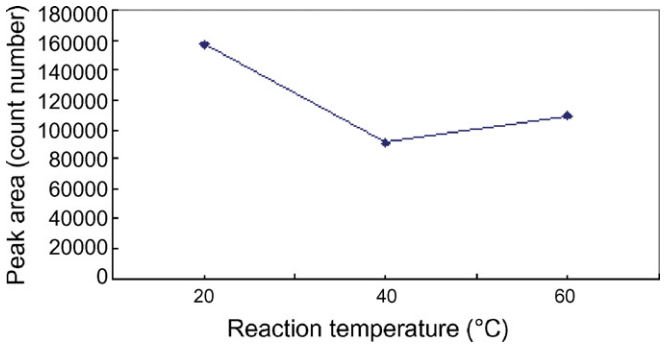

Fig. 3. Peak area vs. reaction temperature of 30 s for the ECF derivatization of β-ODAP.

was dried by adding a small portion of anhydrous sodium sulfate. The dry organic solution was transferred to a new vial and 1 μl was injected to the GC–MS by an auto sampler system. The extraction of the leftover aqueous solution by chloroform and subsequent GC–MS analysis of this chloroform layer showed no significant signal for derivatized amino acids, indicating that the extraction efficiency was nearly 100%.

The aqueous solution remaining from the above derivatization process was rederivatized and extracted with chloroform. GC–MS analysis of the chloroform layer showed no significant signal for derivatized amino acids and at the same time, examination of the remaining aqueous solution after the second derivatization process by direct insertion probe indicated that the amount of remaining underivatized amino acids was insignificant. That is, after one derivatization processes, almost 100% of the amino acids were derivatized.

The maximum percentage of reaction between β-ODAP and ECF was reached for 30 s at 20 °C (Figs. 2 and 3).

3.3. Method validation

The limit of detection (LOD, S/N = 3) and limit of quantification (LOQ, S/N = 9) for dencichine were found to be 0.5 μg/ml and 2 μg/ml, respectively. Calibration curves of peak area ratio (dencichine/internal standard) (y) versus concentration (x) were constructed. These curves showed good linearity over the concentration range 10–800 μg/ml with correlation coefficient $r^2 = 0.9988$ ($y = 0.0088x - 0.1398$). The calibration range

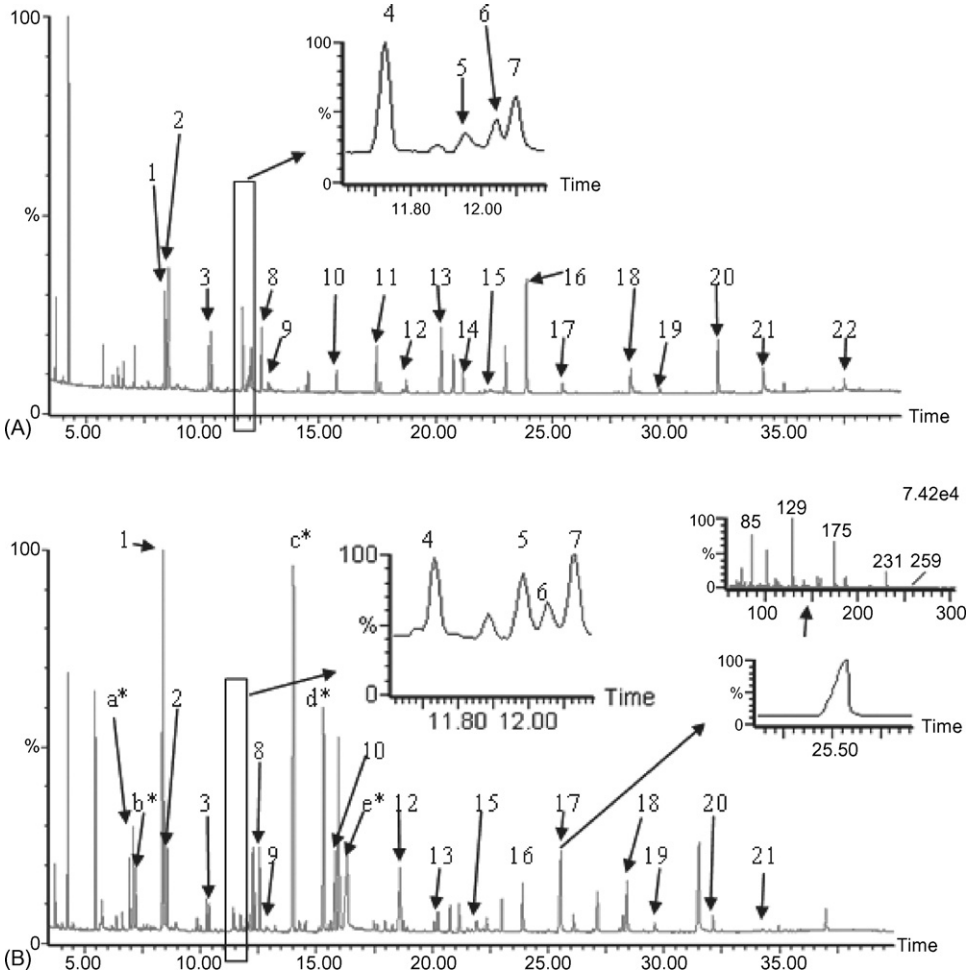

Fig. 4. Chromatograms of (A) amino acid standards and (B) water extract of *P. notoginseng*. Peaks—1: L-alanine; 2: L-glycine; 3: L-valine; 4: L-leucine; 5: L-sercine; 6: L-isoleucine; 7: L-threonine; 8: L-proline; 9: L-asparagine; 10: L-aspartic acid; 11: L-methionine; 12: L-glutamine acid; 13: L-phenylalanine; 14: L-cysteine; 15: glutamine; 16: L-2-chlorophenylalanine (IS); 17: dencichine; 18: L-lysine; 19: L-histamine; 20: L-tyrosine; 21: L-tryphtothan; 22: L-cystine. a*: Succinic acid; b*: fumaric acid; c*: malate; d*: γ-amino butyrate; e*: citric acid.

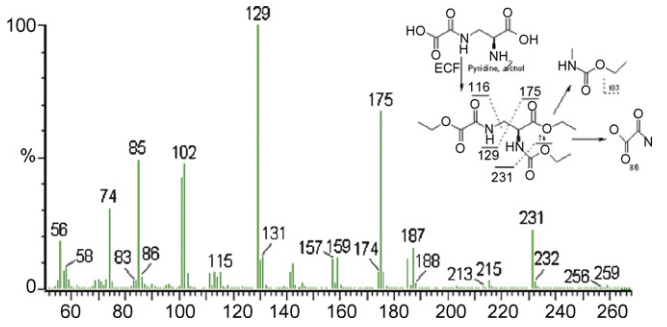

Fig. 5. Mass spectrum of ECF derivative of dencichine (peak 17, M_r 176) from *P. notoginseng*.

was based on the concentration of dencichine in the real samples to be analyzed, and its upper limit (800 μg/ml) proved to be sufficient for analyzing the real samples. The injection precision (R.S.D.) for a low concentration was 4.5% ($n = 6$) and for a middle concentration was 3.2% ($n = 6$). The overall intra- and inter-day variations were less than 5.0% ($n = 5$) and 8.0% ($n = 3$), respectively. The precision at low, middle and high concentrations was less than 4.5%. The accuracy ranged from −0.3% to 4.1% for each concentration level. To investigate the accuracy, recovery studies were performed by spiking *P. notoginseng* samples with different amounts of dencichine. The recoveries of dencichine at three spike concentration levels ranged from 92% to 103% ($n = 3$ for each concentration).

3.4. Quantification of real samples

The developed GC–MS assay method was subsequently applied to the determination of dencichine in the *P. notoginseng* and the adventitious roots directly from the explants of *P. notoginseng*. The typical chromatograms of the extraction are shown in Figs. 4b and 7.

The calculated contents of dencichine are given in Table 1.

3.5. Results analysis

The ECF derivatives of a mixture of standard dencichine and other amino acid standards together with extract of *P. notoginseng* were analyzed. All ECF derivatives of amino acid standards showed excellent resolution on GC–MS. Because of the mild reaction conditions, dencichine (β-ODAP) could be easily derivatized and analyzed by this method.

Table 1
Contents of dencichine in the powder of *Panax notoginseng* and the adventitious roots ($n = 3$)

Sample	Content (%)	R.S.D. (%)
P. notoginseng	0.59	2.35
Adventitious roots	0.13	2.92

The reconstructed ion chromatograms of the ECF derivatives of an amino acid standard mixture, standard and non-standard amino acids from *P. notoginseng* are present in Fig. 4. Based on the retention times and mass spectra, the ECF derivatives of amino acids were identified by comparison with the standards. Dencichine and other known amino acids such as Ala, Glu, Ser, Thr, Asp, Gln, Leu, His and Lys were identified. In addition, several organic acids (marked with an asterisk) were also identified in extracts of *P. notoginseng*.

The mass spectra of ECF-derivative of dencichine together with the proposed fragmentation pathway for dencichine are presented in Fig. 5.

The measured useful *m/z* ions for dencichine, in order of abundance, are the following: *m/z* $M^+ = 231$, $(M^+ - 15) = 216$, the base peak 129 for quantification, 175, 103, 86. From these *m/z* assignments, the quantification and confirming ions were the following: 129 for quantification, 231, 216, 175, 103 and 86. In Fig. 6, a scheme of fragmentation for dencichine is proposed.

With the developed method, dencichine and other amino acids were also found in the adventitious roots directly from the explants of *P. notoginseng* and the content is shown in Table 1. Fig. 7 shows the reconstructed ion chromatograms of the ECF derivatives of amino acids and dencichine of the adventitious roots.

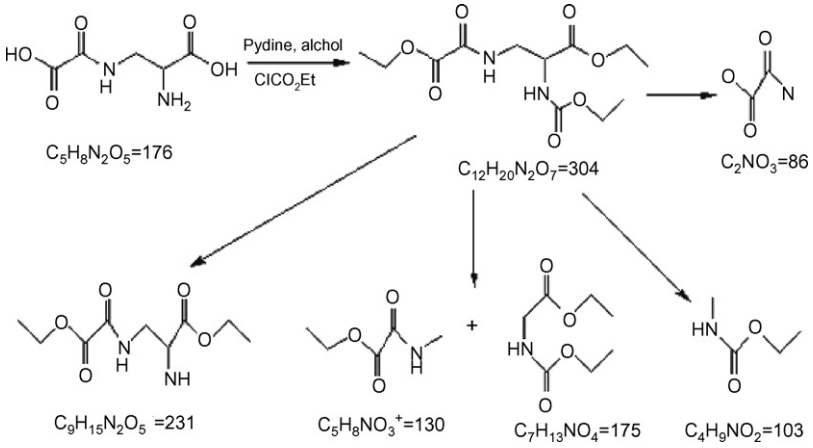

Fig. 6. Scheme of fragmentation proposed for dencichine.

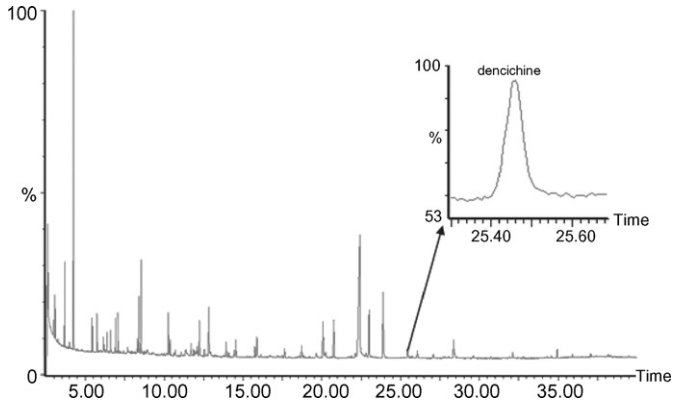

Fig. 7. Chromatogram of water extract of adventitious roots directly from the explants of *P. notoginseng*.

4. Conclusion

ECF reagent is a useful alternative derivatizing reagent for determination of dencichine or other amino acids of *P. notoginseng*. In this study, a novel GC–MS method has been developed for the analysis of dencichine in *P. notoginseng* and the adventitious roots from the explants of *P. notoginseng*. Compared to other conventional methods, the technique is simple, rapid, sensitive and accurate. The relatively short time for analysis and good sensitivity for dencichine make this method preferable to earlier methods in the studies of dencichine evaluation in *P. notoginseng*. With this method, dencichine was also found and determined in the adventitious roots directly from the explants of *P. notoginseng*. From the chromatograph of amino acid standard mixture and the extract sample, it can be seen that it is possible to simultaneously determine other amino acids as well as dencichine.

In view of the increasing popularity of botanical medicines, the control of their quality is very important for the administration dosage and to safeguard the health of patients. Therefore, this method will be useful for the determination of dencichine for the quality control of *P. notoginseng*, as well as other food crops and medicinal plants that may contain this compound.

References

[1] Y.P. Zhu, Chinese Material Medica, Harwood Medicine Association, Australia, 1998.
[2] The State Pharmacopoeia Commission of PR China, Pharmacopoeia of the People's Republic of China, vol. I, Chemical Industry Press, Beijing, 2005, p. 10.
[3] E. Rubenstein, Medicine 79 (2000) 80–89.
[4] S.L.N. Rao, P.R. Adiga, P.S. Sarma, Biochemistry 47 (1964) 432–436.
[5] P.R. Adiga, S.L.N. Rao, P.S. Sarma, Curr. Sci. 32 (1963) 153–155.
[6] G. Wu, S.B. Bowlus, K.S. Kim, B.E. Haskell, Phytochemistry 15 (1976) 1257–1259.
[7] M. Pan, T.J. Mabry, P. Cao, M. Moini, J. Chromatogr. A 787 (1997) 288–294.
[8] Y.H. Kuo, F. Ikegami, F. Lambein, Phytochemistry 62 (2003) 1087–1091.
[9] G.Q. Zhao, X.X. Wang, Chin. Trad. Herbal Drugs 17 (1986) 34–36.
[10] S.L.N. Rao, Anal. Biochem. 86 (1978) 386–395.
[11] C.J. Briggs, N. Parreno, C.G. Campbell, Planta Med. 47 (1983) 188–190.
[12] G.D. Zhang, J.F. Cui, H.Y. Liu, Chin. J. Pharm. Anal. 10 (1990) 209–212.
[13] J.K. Khan, Y.H. Kuo, N. Kebede, F.J. Lambein, J. Chromatogr. A 687 (1994) 113–119.
[14] J.K. Khan, N. Kebede, Y.H. Kuo, F. Lambein, A. De Bruyn, Anal. Biochem. 208 (1993) 237–240.
[15] A. Geda, C.J. Briggs, S.J. Venkataram, J. Chromatogr. A 635 (1993) 338–341.
[16] F. Wang, X. Chen, Q. Chen, X.C. Qin, Z.X. Li, J. Chromatogr. A 883 (2000) 113–118.
[17] X. Chen, F. Wang, Q. Chen, X.C. Qin, Z.X. Li, J. Agric. Food Chem. 48 (2000) 3383–3386.
[18] Y. Zhang, X.Y. Chen, X.Y. Li, D.F. Zhong, Anal. Chim. Acta 566 (2006) 200–206.
[19] H.L. Koh, A.J. Lau, E.C.Y. Chan, Rapid Commun. Mass Spectrom. 19 (2005) 1237–1244.
[20] T.T.X. Dong, X.M. Cui, Z.H. Song, K.J. Zhao, Z.N. Ji, C.K. Lo, K.W.K. Tsim, J. Agric. Food Chem. 51 (2003) 4617–4623.
[21] X.M. Cui, L.S. Xu, Q. Wang, Z.J. Chen, T.X. Deng, K.W.K. Tsim, Chin. Pharm. J. 40 (2005) 1017–1019.
[22] X.F. Gao, C.B. Zhu, W. Jia, W.Y. Gao, M.F. Qiu, Y.Y. Zhang, P.G. Xiao, Biotechnol. Lett. 27 (2005) 1771–1775.
[23] P. Hušek, FEBS Lett. 280 (1991) 354–356.
[24] A. Namera, M. Yashiki, M. Nishida, T. Kojima, J. Chromatogr. B 776 (2002) 49–55.