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Octopamine Levels in *Blattella Germanica* L. Tissues by Capillary Gas Chromatography with Electron Capture Detection

Canping Pan *, Weixi Li, Yongshan Wang and Shuren Jiang

College of Applied Chemistry, China Agricultural University, Beijing 100094, P. R. China
Tel.: (+86) 10 62732949, Fax: (+86) 10 62733620

*Author to whom correspondence should be addressed; E-mail: canpingp@cau.edu.cn; URL: http://www.cau.edu.cn/lxy/

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Abstract: Distribution and levels of octopamine (OA), one of the biogenic amines in the invertebrate nervous system, may have significant effects on insect physiological processes including growth, feeding and reproduction. In this paper capillary gas chromatography with electron capture detection (GC-ECD) and mass selective detection (GC-MS) were used to determine the content of OA in *Blattella germanica* L. central nervous system (CNS), and that of OA in cockroach stressed by kinds of insecticides, known octopaminergic agonists and some essential oils. A derivatization method for organic extracts via reaction with pentafluoropropionic anhydride (PFPA) was developed. The resulting OA derivatives were confirmed by GC-MS to be tris-pentafluoropropionyl-OA. The method was used to quantify the amount of OA in insect issues by capillary GC-ECD through an extraction-derivatization-liquid/liquid partition procedure. Average OA content in normal cockroaches was determined to be 68.49 ± 7.31 ng/g tissue (N=5 determinations). It was shown that insecticides including chlordimeform, methomyl, permethrin, chlorfluazuron, malathion, trichlorfon and some oxazolidine agonists, essential oils including eugenol, cinnamic alcohol, phenyl ethyl alcohol could led to significant increase of OA levels in the cockroach CNS comparing with which in insect treated by 1-butanone. Malathion, trichlorfon, chlorfluazuron and cinnamic alcohol were shown to be able to cause a 20- fold increase in OA levels.

Keywords: Octopamine; GC-ECD; trace level; chemical stressors; cockroach

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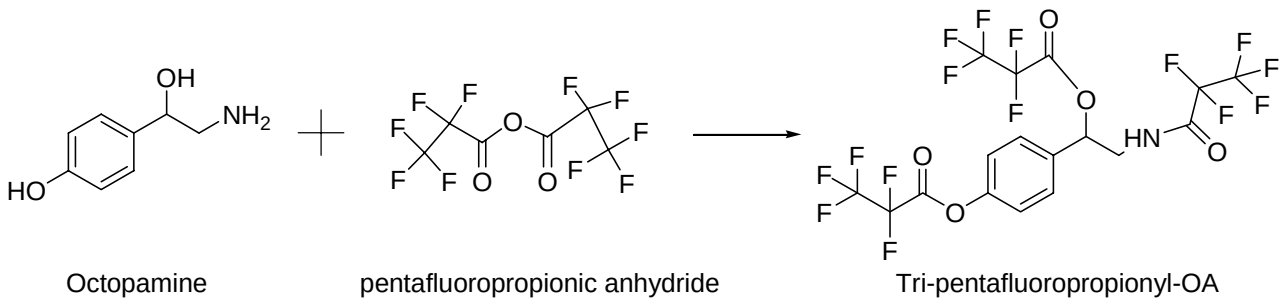
Introduction

Octopamine (OA), a biogenic monoamine structurally related to noradrenaline, acts as a neurohormone, a neuromodulator and a neurotransmitter in invertebrates [1]. Recently much interest has been drawn on the octopamine and its receptors in the invertebrate nervous system [2-5]. It has been suggested that distribution and levels of OA have greatly significant effect on insect physiological processes including growth, feeding and reproduction. Thus, more and more investigations have been focused on trace level determination methodology and factors that affect the levels of OA in insect tissues [6-8]. Davenport *et al*. [9] reported the OA presence in *Periplaneta Americana* and *Schistocerca Americana* by a radioenzymatic method. Goudey-Perriere [10] evaluated the changes of OA, Dopamine and 5-HT in *Blaberus craniifer* by a HPLC-ECD method, which was utilized by many other researchers including Grosclaude [11] and Hiripi *et al*. [12]. Hirashima *et al*. also examined the OA contents in *Periplaneta americana*, emphasizing the chemical stressors effects [8]. Kamal and MacFarlane [13] developed the derivatization method for detecting OA and synerphrines in urine by NCI-GCMS. Nusrut reported [14] that the GC-NICI-MS method could detect at least 100pg OA in CNS of *Periplaneta Americana*. Amendola *et al*. [15] have demonstrated that alcohol and amine groups could be simultaneously reacted with pentafluoropropionic anhydride (PFPA) to form thermo-stable products.

In this study a simple and sensitive method was developed based upon the reaction of OA with pentafluoropropionic anhydride, a strong electrophoric reagent (Figure 1). The resulting derivative is highly responsive to an electron-capture detector (ECD) and detectable at sub-pmol levels. Though the derivation was already well established for bioamines in urine for GCMS [14], we here report the OA levels in insect tissues by GC-ECD quantization and GCMS confirmation. This derivatization-capillary GC-ECD method was developed to determine the content of OA in cockroach (*Blattella germanica* L.) central nervous system (CNS), and that of OA in cockroach stressed by representative insecticides, known octopaminergic agonists and some essential oils. Relationship between the levels of OA and mode of action of the above chemicals was discussed.

Figure 1. Chemical structure of Octopamine and its possible reaction product with PFPA.



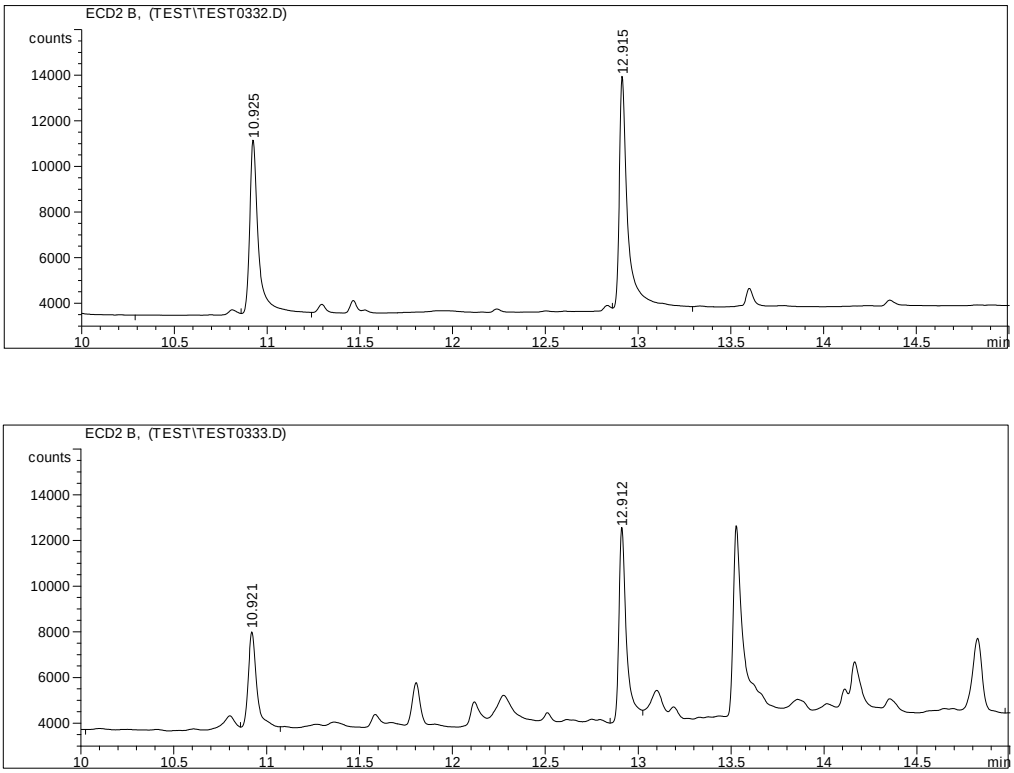
Results and Discussion

Derivatization conditions and GC-ECD qualitative confirmation

The extraction of OA from homogenizing buffer was conducted in 1M hydrochloric acid solution. Bioamines may be transformed to their hydrochloride salts and dissolved in aqueous buffer. Extraction efficiency was compared with organic solvent including ethyl acetate, acetone, methanol and common physiological phosphate buffers. The method of transformation was proven to be stable and highly efficient. The derivatization reaction was optimized by selecting from several potential derivatization reagents including TMSiCl-PFPA, TFPA and PFPA. PFPA was found to be a mild and suitable reactant for OA, and its products could be separated from the substrates on the GC column. TPFA derivatives could be formed under milder temperature conditions, like 40°C for 30 min, but were not easy to separate on the GC column. The TMS-PFPA method, according to the literature, was tedious, unstable and less sensitive. Thus, the optimized derivative condition chosen were 30 μL PFPA at 60°C for 1 hr.

With high concentration of OA standard in pure solvent for the derivatization reaction, its GC-ECD chromatogram showed a single peak (Figure 2, top, R_t =10.9 min), which was consequently confirmed by GC-MS to be the tris-PFPA-OA form. Bis-PFPA-OA form was only observed in situations of derivatization at lower temperatures (<40°C) or for shorter time (<15 min). Figure 2 (bottom) shows the GC-ECD chromatogram of fortified OA at a concentration of 500ng/g tissue (basal OA level was 68.49ng/g head tissue for controlled cockroach untreated with chemicals) .

Figure 2. GC-ECD chromatogram of 0.5μg/ml OA standard (top); OA spiked at a concentration of 0.5μg/g tissue (bottom).



Identification of derivative products by GC-MS

A relatively high concentration of OA derivative (1μg/mL) was injected onto a GC-MS for confirmation of product structures. Figure 3 shows the TIC of a typical injection of OA derivative reference solution. One main peak could be found in the TIC (R_t = 10.6min), and the mass spectrum of this compound showed no parent ion at 591(M^+), which may be fragmented upon electron ionization. Rather, ion at m/z 428 was found to be the most heavy mass, which is assumed to be $[M\text{-}CF3CF2COO]^+$. The m/z 267 peak could be $[M\text{-}CF3CF2COO\text{-}CF3CF2CON)]^+$. This information clearly indicated that the two hydroxyls and one amine group on OA had reacted with PFPA to produce tri-pentafluoropropionyl-OA (Tris-PFPA-OA) (Figure 1). No tetrakis-pentafluoropropionyl-OA (tetra-PFPA-OA) products were observed in the TIC.

In another derivatization reaction with PFPA at 40°C for 15 min, the bis-pentafluoropropionyl-OA (bi-PFPA-OA) derivative was obtained. As shown in Figure 4, a R_t = 7.4 min compound was eluted, which displayed ion fragments of m/z 427, 264 and 159 (B), etc. The m/z 427 peak was obviously $[M\text{-}OH]^+$ of bis-PFPA-OA and the m/z 264 was the $[M\text{-}OH\text{-}CF3CF2COO]^+$ peak. OA levels in real insect tissue samples was determined by GC-ECD and confirmed by GC-MS in SIM mode.

Figure 3. TIC (upper) and Mass (lower) Spectra of OA derivative products (60°C, 1hr with 30μL PFPA). Oven temperature program: see Section 2.4.2. (R_t = 10.6min, tris-PFPA-OA).

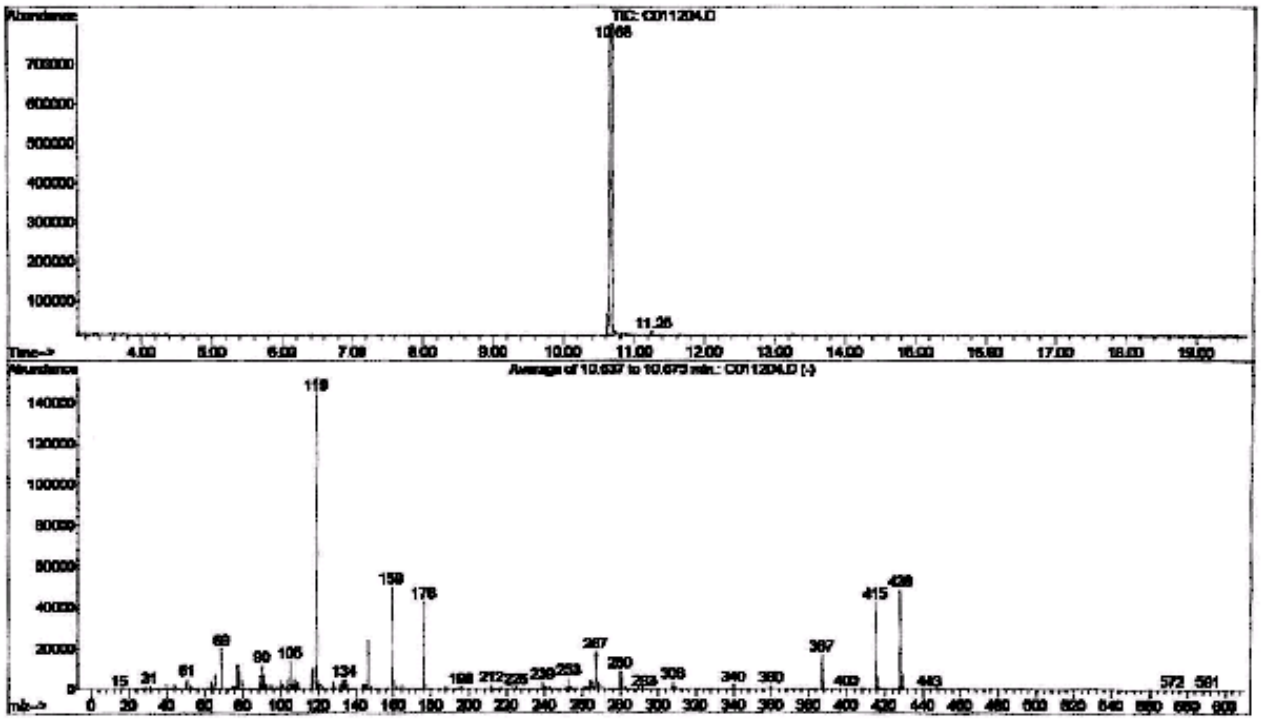
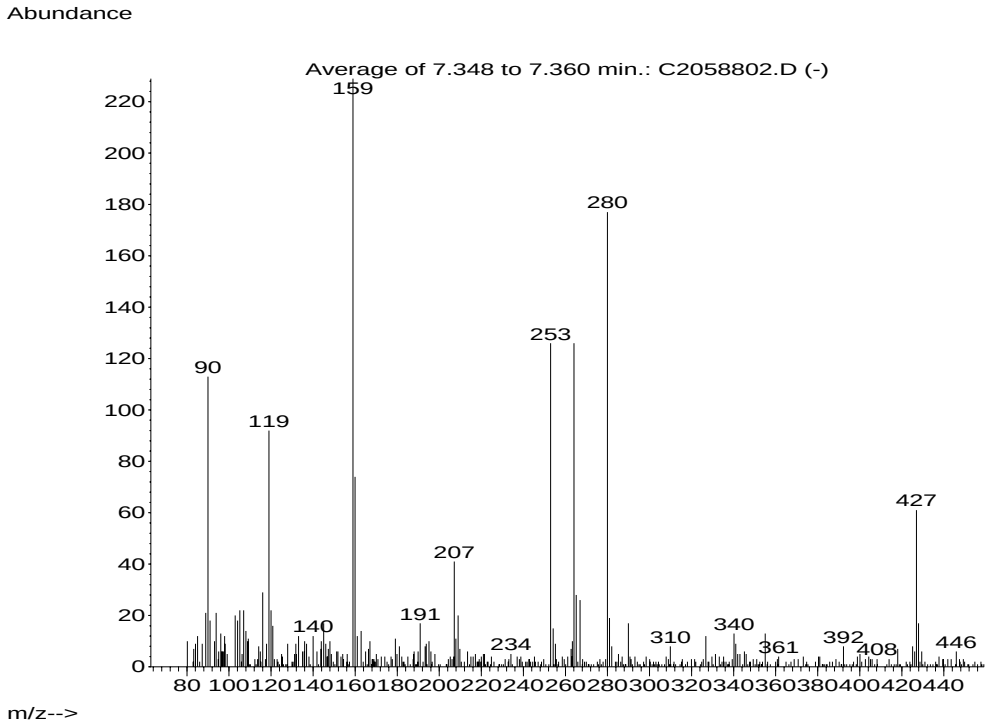


Figure 4. Mass spectra of OA derivative product bis-PFPA-OA (40°C, 15min with 30μl PFPA). Oven temperature program: see section 2.4.2. R_t = 7.4 min, bis-PFPA-OA).



Method validation for GC-ECD

For validation of the method for determining OA levels in cockroach by GC-ECD, calibration curves and data of specificity, precision and accuracy of the method were examined. Analysis of blank cockroach head tissue samples (butanone treated, n=5) showed that the average basal OA level among the individuals was about 0.69±0.07 ng/head, equally 68.49 ± 7.31 ng/g tissue. Spiked head tissue samples with OA were measured and the recovery rates were calculated by exclude the average base value. Five calibration curves (n=5 levels, 2*2 injections) were determined in the range of 0.05-5 μg/g for OA, indicating a good linear relationship with r^2>0.99. Quantification was performed using the peak area of tri-PFPA-OA by the external standard curve method. Precision (%, RSD) within day ranged from 1.6 to 6.4% for a OA concentration of 0.1μg/g (n=10). Precision (%, RSD) between days ranged from 3.5 to 7.6% at a OA concentration of 0.1μg/g (n=10). Analytical recoveries were tested at three levels of 0.05, 0.5 and 5 μg/g for a head tissue blank in which the OA content was known. Analytical recoveries ranged from 86 to 117%. Limit of quantitation (LOQ) for this method is about 0.12 ng/g tissue.

Application of method: OA levels found in homogenizing tissue of Blattella germanica L. under control and various chemical stress conditions

Seventeen chemicals were applied to the cockroach as the method described in section 2.1. OA levels were measured in 5 duplicates, and the results are displayed in Table 1. It is very interesting that various chemicals exhibit different effects on the OA levels. Imidacloprid, avermectin, lindane and

phenyl methyl alcohol were found to be statistical ineffective (*t* test, P=0.05). Essential oil compositions vary, with phenyl ethyl alcohol being slightly effective, eugenol moderately effective and cinnamic alcohol the highest. Organophosphorus insecticides including trichlorfon and malathion were among the most effective ones. The explanation for this phenomenon may come from the mode of action of phosphorus compounds, namely excitation and convulsion. OA receptor agonists including the synthesized compounds BAO50, BAO118, AIO12 and chlorfluazuron and CDM significantly affected the OA levels in cockroach CNS.

Table 1. OA levels found in homogenizing head tissue of *Blattella germanica* L. under control conditions and chemical stress.

Chemicals applied	OA level（ng/head）(n=5 *)	Percentage compared to baseline value (%)
solvent (butanone)	0.69±0.07	100
imidacloprid	0.85±0.25	123
avermectin	1.09±0.37	158
phenyl methyl alcohol	1.21±0.53	175
lindane	1.27±0.48	184
allethrin	2.03±0.06	294
permethrin	2.28±0.52	330
phenyl ethyl alcohol	3.65±0.75	529
BAO50	3.97±0.75	575
methomyl	4.52±0.91	655
BAO118	5.43±0.81	787
eugenol	5.83±1.08	845
CDM	6.75±1.23	978
AIO12	6.99±0.49	1013
trichlorfon	14.52±2.73	2104
chlorfluazuron	17.33±3.69	2512
cinnamic alcohol	17.47±4.37	2532
malathion	19.48±5.14	2823

* Average OA levels were determined in 5 tests, values were given in average with SD.

Conclusions

A specific, sensitive GC assay for the determination of OA in cockroach is reported. Offline derivatization-capillary gas chromatography with electron capture detector (GC-ECD) was developed to determine the content of OA in *Blattella germanica* L. central nervous system (CNS), and that of OA in cockroach stressed by representative insecticides, known octopaminergic agonists and some essential oils. A derivatization method involving reaction with PFPA in an aliquot of buffer was developed. The OA derivatives prepared under optimized conditions were confirmed by GC-MS to be tri-pentafluoropropionyl-OA. The method was characterized by adequate sensitivity for determining OA levels in cockroach individuals. This method should be a good alternative and economic way for

laboratories which only have gas chromatographic instrumentation available. Average OA content in normal cockroach was determined to be 68.49 ± 7.31 ng/g tissue (N=5 determinations). It demonstrates that chemicals including chlorfluazuron, malathion, trichlorfon, chlordimeform, methomyl and some oxazolidine agonists, essential oils including eugenol, cinnamic alcohol etc could led to significant increases of OA levels in the cockroach CNS compared with those treated by 1-butanone alone. Among them, malathion, trichlorfon, chlorfluazuron and cinnamic alcohol caused up to 20-fold increases in OA levels. These interesting results with various insecticides with different mode of action may have implications for further research in this field. Not only octopamine, but also other biogenic amines like dopamine, 5-HT, etc., may also be future target molecules in proposing a relationship between stressors and bio-amine levels. The above preliminary tests might be valuable in interpreting synergism and guiding the field application of insecticides.

Acknowledgements

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Experimental

Materials and reagents

Chemicals: Octopamine hydrochloride (OA-HCl, purity 99%), Pentafluoropropionic anhydride (PFPA, purity 99%), Trifluoropropionic anhydride (TFPA, purity 99%), Tetramethylsilicone chloride (TMSiCl, purity 99%) was purchased from Sigma-Aldrich Co. Chlordimeform (CDM, purity 97%), methomyl (purity 98%), lindane (purity 99%), permethrin (purity 96%), allethrin (purity 97%), imidacloprid (purity 99%), chlorfluazuron (purity 98%), Avermectin (B1a+B1b, purity 96%), malathion (purity 95%) and trichlorfon (purity 96%) were purchased from ICAMA (P.R. China). Eugenol (purity 98%), cinnamic alcohol (purity 95%), phenyl methyl alcohol (purity 96%) and phenyl ethyl alcohol (purity 98%) were obtained from Beijing Chemical and Reagents Co. Ltd. 2-(3,4-dichlorophenylimino)oxazoline (BAO50, purity 96%), 2-(2-methyl-4-bromobenzylimino) oxazolidine (AIO12, 98%) and 2-(3,4-diflurophenylimino) oxazolidine (BAO118, purity 96%) were synthesized in our previous study [10]. All solvents and other reagents were analytical grade obtained from commercial sources. Solvents were dried and re-distilled. Water was de-ionized and purified through a Millipore purification system.

Insects: Investigations were done on German cockroaches (*Blattella germanica* L.) of both sexes. The insects were reared under crowded conditions in our laboratory at 27°C with a photoperiod of 12 hr/12hr dark and at a relative humidity of 65-70%. They were provided with an artificial diet and water.

Application of stressors: Cockroaches were stressed by topically applying insecticides and OA agonists as described by Davenport and Evans [4]. The thorax of individual insects was treated with chemical solutions (100μg in 10μL butanone) and the tissues were sampled 30 minutes after application. Negative control insects were treated with butanone only.

Sample homogenates

Head tissues of German cockroaches (10 individuals, weighed) were homogenized in cold 100mM hydrochloric acid solution (1 mL), using a glass-Teflon homogenizer. Then the whole mixture was centrifuged at 4,500 rpm for 30 min at 4°C. The supernatant was collected and keep under cold conditions for further determination. OA levels are expressed in ng/g tissue or ng/head.

Derivatization

A 500 μL aliquot of reference OA·HCl at various concentrations (0.05, 0.5, 5μg/mL) or 500 μL sample extraction in homogenizing buffer was pipetted into a 5mL capped test tube containing potassium hydroxide (5 M, 100μL) and potassium phosphate (pH 7.2, 800 μL, 40 mM). The mixture was extracted twice with ethyl acetate (2 mL) in the presence of anhydrous sodium sulphate (500mg) by vortexing for 1 min. After centrifuging at 2,500 rpm for 5 min, the organic layer was evaporated to 500μL under dried nitrogen gas. PFPA (30 μL) was then added to the solution, which was incubated at 60 °C for 1 h in a water bath. After derivatization, it was washed with ammonia (1M, 500 μL) to remove excess PFPA, free acid and water soluble substances to thus minimize possible column contamination. The upper layer was separated and gently evaporated to dryness under a nitrogen gas flow. The resulting residue was reconstituted with hexane-ethyl acetate (4:1) and was injected into the GC–ECD (sample size, 1μL). For optimized conditions, derivatization of OA at 60 °C for 1 h was selected.

Gas chromatography

GC-ECD

An Agilent 4890D gas chromatograph equipped with an ECD was used (manual injection with split/splitless port). The capillary column was 30m x 0.25mm i.d. with 0.25μm film of HP-35MS (Agilent, USA). The operation temperatures were 250, and 280 °C, respectively, for injector and detector. The carrier gas (nitrogen) flow was set to 1mL/min. Makeup gas was adjusted at 60mL/min. The split/splitless injection with an inlet split ratio of 1/20, purge time of 0.7 min was applied. Oven temperature program was as follows: initial temperature 105°C for 1 min, then 10°C/min to 190°C and kept 1 min, then ramped at 20°C to 250°C and held for 10 min.

GC-MS

A Hewlett-Packard 6890 gas chromatograph coupled with a 5973 quadrupole mass spectrometric detector (MSD) with a HP-5 capillary column (30 m x 0.25 mm I.D. with 0.25μm film thickness). Helium was used as carrier gas at flow 1.0 mL/min. Injection port and transfer zone temperatures were maintained at 250 °C. A 1 μL sample volume was injected in the split mode (30:1) split port. Samples was carried out on a temperature programmed column, namely initial oven temperature 80°C for 1 min, then ramped at 15°C/min to 150°C and kept there for 3 min, then at 20°C/min to 250°C and held for 10 min. MSD was run in a scan mode (mass range 70–600) with electron impact ionization (70 eV).

Method validation

The intra-assay precision and accuracy were determined by analyzing five samples spiked with OA·HCl at concentrations of 0.05, 0.5 and 5.0 μg/mL, respectively. The inter-assay precision and accuracy were determined by analyzing the spiked samples on different days. Method validation parameters included linearity, precision and accuracy, lower limit of quantitation (LOQ), selectivity and recovery.

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