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Investigation on the insecticidal limonoid content of commercial biopesticides and neem extract using solid phase extraction

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ABSTRACT

Neem extract is an alternative insecticide for organic farming and is an allowed component for commercial biopesticide in Korea. However, crop protection properties are not consistent in commercial products. In this report, quantitative analysis of commercial biopesticides for the four insecticidal limonoids of neem extract-azadirachtin A, azadirachtin B, deacetylsalannin and salannin, was conducted through solid phase extraction method with lipophilic-hydrophilic balanced material. The recoveries of the four limonoids ranged from 80.5% to 105%, and their limit of quantitation ranged from 0.028 mg/L to 0.356 mg/L. On the five imported neem extracts, the total contents of the four bioactive limonoids extracted were from 321 mg/L to 5810 mg/L, but there were big variations in the relative composition of the limonoids. The total limonoidal concentrations in 23 commercial bio-pesticides made from neem showed from below LOQ to 7190 mg/L with significant differences in the relative composition. These differences determine the biopesticide's efficacy on pests, therefore, tracking the active ingredients is necessary for the quality control of commercial bio-pesticides.

Keywords: Solid Phase Extraction; Azadirachitn; Salannin; Quantitative Analysis; Biopesticide

1. INTRODUCTION

Azadirachta indica A. Juss. (neem) of the family Meliaceae is native plant of Southeast Asia and tropical regions of the western hemisphere, and its oil is widely used for agricultural pest control as alternative insecticides in organic farming [1-6]. It is currently specified as allowable materials in the Environmentally-friendly Agriculture Promotion Act in Korea. Limonid was identified as the active component in neem extract. Some limonoidal compounds such as azadirachtin A, azadirachtin B, deacetylsalannin and salannin from neem were reported as the major bioactive metabolites, and their insecticidal [3,7-16], antifeedant [17] activities and putative antimicrobial property [18,19] had been reported. These studies recommended neem extract as a potent crop protector. However, commercialization is still a big problem due to the huge variation of azadirachtin content (0.01% - 0.9%) in neem extract by ecotype [10,20] and/or extraction conditions of the seeds [7,9,21,22].

The quantitative analysis of the limonoid from neem extract was intermittently reported for the pure neem extract by rough HPLC method [9,23-27], and the simple method was introduced for chemical pesticides which contain percent-level concentration of azadirachtin without messy plant metabolites [28]. But there was no report for the quantitation method in commercial neem biopesticides that had synergists or inert ingredients. The composition of commercial neem biopesticides in Korea was surveyed, and the ranges were as follow: 5% - 85% pure extract, 5.5% - 20% solvent, 0.1% - 35% surfactant and 5% - 80% others. Here in we investigated the quantitative analysis method of azadirachtin A, azadirachtin B, deacetylsalannin, and salanninas active ingredients of commercial biopesticides for quality control, and this is the first survey on limonoidal content in commercial biopesticide.

2. MATERIALS AND METHODS

2.1. Chemicals

The standard solution of azadirachtin A, azadirachtin B, deacetylsalannin and salannin were obtained from ChromaDex (Irvine, US), and the purchased standards were diluted to 100 μg/mL in methanol.

2.2. Solid Phase Extraction

To set up a simple purification method, solid phase extraction (SPE) method was applied with silica gel (SiOH) (500 mg, Macherey-Nagel Co., Duren, Germany), Florisil (500 mg, Macherey-Nagel Co., Duren, Germany) and hydrophilic-lipophilic balanced (HLB) material (60 mg, Oasis, Waters Co., Milford, USA), and the purification efficiency was compared.

First, the recovery was compared in distilled water. A 0.1 μg/mL limonoid-spiked 50 mL water was extracted with dichloromethane (20 mL × 3) and concentrated to 0.5 mL. There after the residue was loaded to SiOH or the Florisil cartridge that was preconditioned with *n*-hexane, and washed with 2.5 mL *n*-hexane then eluted with 10 mL dichloromethane/acetone (1:1, v/v) at 1 mL/min. The eluate was evaporated and redissolved with 1 mL methanol.

Due to solvent limitation, HLB SPE was applied to the different procedure of Florisil and SiOH SPE. The dichloromethane extract from the water sample was concentrated and redissolved with 0.1 mL methanol and 1.9 mL distilled water. The HLB cartridge was conditioned with 2 mL methanol and 2 mL water at a flow rate of 1 mL/min. The extracted sample was loaded and washed with 2 mL 5% methanol in distilled water then the cartridge was dried by vacuum. Thereafter the cartridge was eluted with 5 mL methanol, and evaporated to dry.

2.3. UPLC Analysis

The prepared sample was analyzed with ultra-performance liquid chromatography (UPLC) with BEH Phenyl column (1.7 μm, 3 mm × 100 mm, Waters, Dublin, Ireland) on Waters ACQUITY UPLC® System with PDA detector. The mobile phases were combined with 0.05% formic acid in distilled water (A) and acetonitrile (B) at 0.5 mL/min, and the gradient was programmed to gradually increase the solvent B content (initial, 5%; 5 min, 10%; 10 min, 50%; 15 min, 90%). The chromatogram was monitored at 217 nm. The retention times of azadirachtin A, azadirachtin B, deacetylsalannin and salannin were 10.40 min, 10.47 min, 11.97 min, and 12.60 min, respectively.

2.4. Method Validation

Limit of quatitation (LOQ), precision and recovery determination for each limonoid were followed as a reported method by Hansen *et al.* [29] with minor modification. Six 0.1 μg/mL and 20 μg/mL of each limonoid spikes were prepared and analyzed. Based on the standard deviation (σ) associated with the replicate analysis, a LOQ was calculated (LOQ = 10 σ/s, s was slope of calibration curve). Recovery was determined comparison of the responses of the spike and standard, and precision was determined repeatability RSD on the spiked concentrations in water, neem extract and biopesticide. The calibration linearity was tested on 0.25 - 25 μg/mL ranges of each limonoid.

3. RESULTS AND DISCUSSION

3.1. Optimization of SPE Method for Commercial Biopesticide

For these quantitations of azadirachthin A, azadirachtin B, deacetylsalannin and salannin, the calibration curves were ranged from 0.25 to 25 μg/mL, and the r^2 values were calculated over 0.998. A 0.1 mg/L limonoid-spiked 50 mL water sample was clean-up with SPE through the appropriate procedure that was described in the section 2.2 for Florisil, HLB and SiOH, and the final residue were dissolved with 2 mL methanol then analyzed by UPLC. The recovery of Florisil, HLB, and SiOH SPE were measured at 70.4%, 99.2%, and 66.5% respectively. Finally, the HLB SPE method was adopted for further method validation test.

The HLB SPE method was applied to a commercial biopesticide not containing neem extract but having a similar composition of synergists and inert ingredients. After 20-fold dilution of the sample with distilled water, 50 mL distilled water was added to the 1 mL diluted sample, and extracted with dichloromethane (20 mL × 3). This was followed by the HLB clean-up procedure. With this method, the recoveries of the four limonoids on 20 mg/L ranged from 80.5% to 105%, and the precision ranged from 1.7% to 4.2%. Limit of quantitation of the each limonoid ranged from 0.028 mg/L to 0.356 mg/L (**Table 1**). Additionally, the recovery was measured with 20 mg/L pinoresinol as an internal standard on the neem extract and its biopesticides, and the average recovery was 95.6% and their precision was calculated 8.7%. From the results, all the tested method validation values showed acceptable for the quantitation of the four limonoids in the extract and biopesticide.

3.2. Limonoidal Content of Crude Neem Extract

The optimized method was applied to the quantitative analysis of the limonoids on imported four commercial neem extracts (I-IV) and a neem oil (V). All the commercial extracts were collected from the domestic market in

Table 1. Recovery and limit of quantitation (LOQ) of azadirachitn A, azadirachtin B, deacetylsalannin and salannin in a commercial biopesticide.

	Recovery* (average, %)	Precision (RSD, %)	LOQ (mg/L)	Linearity (r^2)
Azadirachtin A	82.2	3.8	0.138	0.9994
Azadirachtin B	82.3	1.7	0.028	0.9988
Deacetylsalannin	80.5	4.2	0.356	0.9999
Salannin	105	3.1	0.345	0.9999
Pinoresinol (IS)¶	99.3	2.3	NT†	NT

*Recovery was tested on 20 μg/mL standards and the chemical composition of the tested sample: 80% sophora extract, 10% solvent, 10% surfactant; †NT means not tested; ¶IS means internal standard.

Korea, and the neem oil was diluted with 5% Tween 20 in water before the clean-up. Total content of the limonoids ranged from 321 mg/L to 5810 mg/L, and the ratios of the four limonoids showed big variation among the five neem extracts. For example, the neem oil (V) showed only deacetylsalannin among the four limonoidal compounds, but the neem extract I and IV showed below 10% of deacetylsalannin contents in **Figure 1**.

3.3. Limonoidal Content of Commercial Biopesticide

The commercial biopesticides of neem extract were investigated for their concentrations of the active ingredients. Twenty-three commercial neem biopesticides (A-W) manufactured in Korea were collected from the local market in 2012. Total concentration of the limonoid ranged from below LOQ to 7190 mg/L in commercial biopesticides, with an average of 961 mg/L. Azadirachtin A and azadirachtin B were found in only eight commercial products (A-B and Q-V), and their concentrations ranged from 43.1 mg/L to 5620 mg/L. Deacetylsalannin or salannin was found in most samples, and their concentration ranged from 14.6 mg/L to 3440 mg/L (**Table 2**). However, the four limonoids were not found in three samples (J, N and W). Esparza-Diaz *et al.* [9] reported that the azadirachtin contents could be related to the insecticidal activities. Thus, the concentration of this major bioactive ingredient should be considered for the guarantee of crop protection properties in commercial neem biopesticides.

4. CONCLUSION

The quantitative analysis method was introduced for commercial biopesticide through HLB SPE method with UPLC analysis. From the established method, the concentration and the composition ratio of the bioactive limonoids in commercial biopesticide and neem extracts were determined and results showed big differences between the products. These big variations of the limonoidal content might be due to physiological and ecological variations, and the manufacturing process of the product. It might also affect the crop protection properties such as toxicity and bioactivity of the biopesticide. Therefore, tracking the active ingredients would be needed for the quality control of commercial biopesticide.

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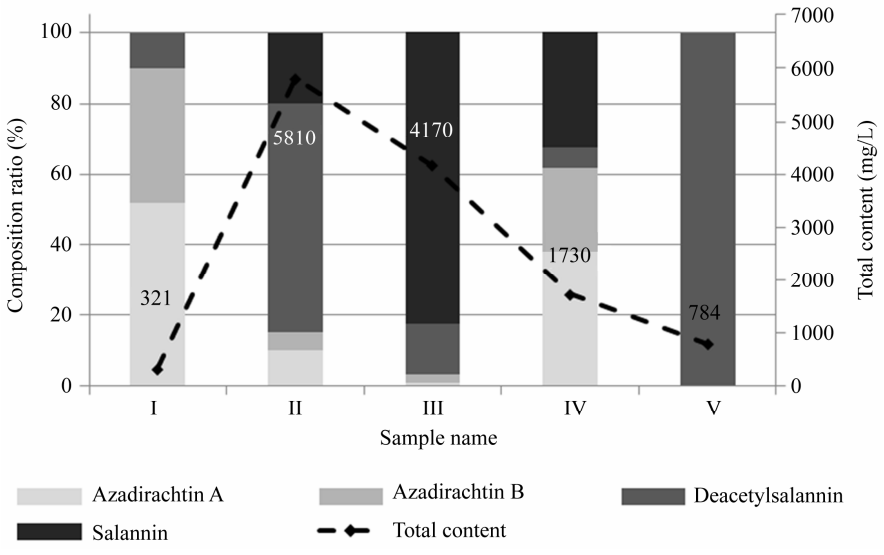

Figure 1. Composition ratio (%) of azadirachtin A, azadirachtin B, deacetylsalannin and salannin, and total content (mg/L) of the bioactive limonoids on imported neem extracts.

Table 2. Concentration of azadirachtin A, azadirachtin B, deacetylsalannin and salanninin commercial biopesticides containing neem extracts (mg/L).

	Azadirachtin A	Azadirachtin B	Deacetylsalannin	Salannin	**Total**
A	72.0 ± 1.12	189 ± 2.68	32.3 ± 0.58	281 ± 5.86	**574** ± 10.3
B	43.1 ± 0.78	83.3 ± 1.50	<LOQ	<LOQ	**126** ± 2.18
C	<LOQ	<LOQ	32.9 ± 0.59	71.3 ± 1.58	**104** ± 1.98
D	<LOQ	<LOQ	33.2 ± 0.60	62.7 ± 1.13	**95.9** ± 1.73
E	<LOQ	<LOQ	332 ± 6.28	158 ± 2.84	**490** ± 9.82
F	<LOQ	<LOQ	<LOQ	69.4 ± 1.25	**69.4** ± 1.25
G	<LOQ	<LOQ	41.5 ± 0.65	<LOQ	**41.5** ± 0.65
H	<LOQ	<LOQ	52.5 ± 1.85	<LOQ	**52.5** ± 1.85
I	<LOQ	<LOQ	89.6 ± 1.61	38.9 ± 0.70	**129** ± 2.31
J	<LOQ	<LOQ	<LOQ	<LOQ	**<LOQ**
K	<LOQ	<LOQ	34.3 ± 0.62	14.6 ± 0.26	**48.9** ± 0.79
L	<LOQ	<LOQ	184 ± 3.01	<LOQ	**184** ± 3.01
M	<LOQ	<LOQ	124 ± 2.23	<LOQ	**124** ± 2.23
N	<LOQ	<LOQ	<LOQ	<LOQ	**<LOQ**
O	<LOQ	<LOQ	3440 ± 61.9	413 ± 7.43	**3850** ± 60.1
P	<LOQ	<LOQ	<LOQ	22.4 ± 0.40	**22.4** ± 0.40
Q	418 ± 4.52	192 ± 2.96	32.7 ± 0.59	598 ± 10.7	**1240** ± 22.3
R	214 ± 3.85	84.6 ± 1.82	<LOQ	<LOQ	**298** ± 5.37
S	5620 ± 82.5	1370 ± 24.7	32.4 ± 1.18	171 ± 3.08	**7190** ± 109
T	3000 ± 54.9	663 ± 10.5	104 ± 1.98	578 ± 11.4	**4360** ± 70.2
U	344 ± 6.19	72.4 ± 1.30	<LOQ	342 ± 5.15	**758** ± 10.8
V	<LOQ	161 ± 2.98	221 ± 3.98	1960 ± 38.4	**2340** ± 42.2
W	<LOQ	<LOQ	<LOQ	<LOQ	**<LOQ**

ricultural Science, Rural Development Administration, Republic of Korea.

REFERENCES

[1] Ascher, K.R.S. (1993) Nonconventional insecticidal effects of pesticides available from the neem tree, *Azadirachta indica*. *Archives of Insect Biochemistry and Physiology*, **22**, 433-449. http://dx.doi.org/10.1002/arch.940220311

[2] Singh, S. and Sagar, S.K. (2001) Evaluation of deoiled-neem (*Azadirachta indica* A. Juss) seed kernel extracts and neem leaf suspension as growth regulators for *Aedesa egypti* (L.) (*Diptera culicidae*). *Indian Journal of Entomology*, **63**, 60-65.

[3] Liang, G.M., Chen, W. and Liu, T.X. (2003) Effects of three neem-based insecticides on diamondback moth (*Lepidoptera plutellidae*). *Crop Protection*, **22**, 333-340. http://dx.doi.org/10.1016/S0261-2194(02)00175-8

[4] Lynn, O.M., Song, W.G., Shim, J.K., Kim, J.E. and Lee, K.Y. (2010) Effect of azadirachtin and neem-based formulations for the control of sweet potato whitefly and root-knot nematode. *Journal of the Korean Society for Applied Biological Chemistry*, **53**, 598-604. http://dx.doi.org/10.3839/jksabc.2010.092

[5] Morgan, E.D. (2009) Azadiracthin, a scienctific gold mine. *Bioorganic and Medicinal Chemistry*, **17**, 4096-4105. http://dx.doi.org/10.1016/j.bmc.2008.11.081

[6] Seljasen, R. and Meadow, R. (2006) Effects of neem on oviposition and egg and larval development of *Mamestra brassicae* L: Dose response, residual activity, repellent effect and systemic activity in cabbage plants. *Crop Protection*, **25**, 338-345. http://dx.doi.org/10.1016/j.cropro.2005.05.007

[7] Boursier, C.M., Bosco, D., Coulibaly, A. and Negre, M. (2011) Are traditional neem extract preparations as efficient as a commercial formulation of azadirachtin A? *Crop Protection*, **30**, 318-322. http://dx.doi.org/10.1016/j.cropro.2010.11.022

[8] Cherry, R. and Nuessly, G. (2010) Repellency of the biopesticide, azadirachtin, to wireworms, coleopteran: Elateridae). *Florida Entomologist*, **93**, 52-55. http://dx.doi.org/10.1653/024.093.0107

[9] Esparza-Diaz, G., Lopez-Collado, J., Villanueva-Jimenez, J.A., Osorio-Acosta, F., Otero-Colina, G. and Camacho-Diaz, E. (2010) Azadirachtin concentration, insecticide efficacy and phytotoxicity of four neem *Azadirachta indica* A. Juss extracts. *Agrociencia*, **44**, 821-833.

[10] Kumar, J. and Parmar, B.S. (1996) Physicochemical and chemical variation in neem oils and some bioactivity leads against *Spodoptera litura*. *Journal of Agricultural and Food Chemistry*, **44**, 2137-2143. http://dx.doi.org/10.1021/jf950283s

[11] Nathan, S.S., Kalaivani, K., Chung P.G. and Murugan, K. (2006) Effect of neem limonoids on lactate dehydrogenase (LDH) of the rice leaffolder, *Chaphalocrocis medinalis* (Guenee) (Insecta: Lepidoptera: Pyralidae). *Chemosphere*, **62**, 1388-1393. http://dx.doi.org/10.1016/j.chemosphere.2005.07.009

[12] Naumann, K. and Isman, M.B. (1996) Toxicity of neem (*Azadirachta indica* A. Juss.) seed extracts to larval honeybees and estimation of dangers from field application. *American Bee Journal*, **136**, 518-520.

[13] Pineda, S., Martinez, A.M., Figueroa, J.I., Schneider, M.I., Del Estal, P., Vinuela, E., Gomez, B., Smagghe G. and Budia, F. (2009) Influence of azadirachtin and methoxyfenozide on life parameters of *Spodoptera littoralis* (Lepidoptera: Noctuidae). *Journal of Economic Entomology*, **102**, 1490-1496. http://dx.doi.org/10.1603/029.102.0413

[14] Riba, M., Marti, J. and Sans, A. (2003) Influence of azadirachtin on development and reproduction of *Nezara viridula* L. (*Het. Pentatomidae*). *Journal of Applied Entomology*, **127**, 37-41. http://dx.doi.org/10.1046/j.1439-0418.2003.00684.x

[15] Simmonds, M.S., Jarvis, A.P., Johnson, S., Jones, G.R. and Morgan, E.D. (2004) Comparison of anti-feedant and insecticidal activity of nimbin and salannin photo-oxidation products with neem (*Azadirachta indica*) limonoids. *Pest Management Science*, **60**, 459-464. http://dx.doi.org/10.1002/ps.834

[16] Tome, H.V.V., Martins, J.C., Correa, A.S., Galdino, T.V.S., Picanco, M.C. and Guedes, R.N.C. (2013) Azadirachtin avoidance by larvae and adult females of the tomato leafminer. *Tutu absoluta*. *Crop Protection*, **46**, 63-69. http://dx.doi.org/10.1016/j.cropro.2012.12.021

[17] Mordue, A.J. and Blackwell, A. (1993) Azadirachtin: An update. *Journal of Insect Physiology*, **39**, 903-924. http://dx.doi.org/10.1016/0022-1910(93)90001-8

[18] Dillio, V., Pasquariello, N., van der Esch, A.S., Cristofaro, M., Scarsella, G. and Risuleo, G. (2006) Cytotoxic and antiproliferative effects induced by a non terpenoid polar extract of *A. indica* seeds on 3T6 murine fibroblasts in culture. *Molecular and Cellular Biochemistry*, **287**, 69-77. http://dx.doi.org/10.1007/s11010-005-9062-x

[19] Harikrishnan, R., Balasundaram, C., Dharaneedharan, S., Moon, Y.G., Kim, M.C., Kim, J.S. and Heo, M.S. (2009) Effect of plant active compounds on immune response and disease resistance in *Cirrhina mirgala* infected with fungal fish pathogen *Aphanomyces invadans*. *Aquaculture Research*, **40**, 1170-1181. http://dx.doi.org/10.1111/j.1365-2109.2009.02213.x

[20] Sidhu, O.P., Kumar, V. and Behl, H.M. (2003) Variability in neem (*Azadirachta indica*) with respect to azadirchtin content. *Journal of Agricultrual and Food Chemistry*, **51**, 910-915. http://dx.doi.org/10.1021/jf025994m

[21] Jadeja, G.C., Maheshwari, R.C. and Naik, S.N. (2011) Extraction of natural insecticide azadirachtin from neem, *Azadirahta indica* A. Juss) seed kernels using pressurized hot solvent. *Journal of Supercritical Fluids*, **56**, 253-258. http://dx.doi.org/10.1016/j.supflu.2011.01.004

[22] Ramesh, A. and Balasubramanian, M. (1998) Rapid preconcentration method for the determination of azadirachtin-A and -B, nimbin and salannin in neem oil samples by using graphitized carbon solid phase extraction. *Analyst*, **124**, 19-21. http://dx.doi.org/10.1039/a806527f

[23] Govindachari, T.R., Sandhya, G. and Raj, S.P.G. (1990) Simple method for the isolation of azadirachtin by preparative high-performance liquid chromatography. *Journal of Chromatography*, **513**, 389-391. http://dx.doi.org/10.1016/S0021-9673(01)89462-0

[24] Johnson, S. and Morgan, E.D. (1997) Comparison of chromatographic systems for triterpenoids from neem (*Azadirachta indica*) seeds. *Journal of Chromatography A*, **761**, 53-63. http://dx.doi.org/10.1016/S0021-9673(96)00796-0

[25] Silva, J.C.T., Jhama, G.N., D'arc, R., Oliveria, L. and Brown, L. (2007) Purification of the seven tetranortriterpenoids in neem (*Azadirachta indica*) seed by countercurrent chromatography sequentially followed by isocratic preparative reversed phase high-performance liquid chromatography. *Journal of Chromatography A*, **1151**, 203-210. http://dx.doi.org/10.1016/j.chroma.2007.03.086

[26] Thejavathi, R., Yakkundi, S.R. and Ravindranath, B. (1995) Determination of azadirachtin by reversed-phase high performance liquid chromatography using anisole as internal standard. *Journal of Chromatography A*, **705**, 374-379. http://dx.doi.org/10.1016/0021-9673(95)00314-D

[27] Yamasaki, R.B., Klocke, J.A., Lee, S.M., Stone, G.A. and Darlington, M.V. (1986) Isolation and purification of azadirachtin from neem (*Azadirachta indica*) seeds using flash chromatography and high-performance liquid chromatography. *Journal of Chromatography*, **356**, 220-226. http://dx.doi.org/10.1016/S0021-9673(00)91483-3

[28] Kim, Y.J. (2003) Azadirachtin. In: Ryu, G.H., Ed., *Pesticide Analysis Method*, Korea Pesticides Analytical Council (KOPAC), Gyeonggi, 38-39.

[29] Hansen, K.J., Clemen, L.A., Ellefson, M.E., Johnson, H.O. (2001) Compound-specific, quantitative characterization of organic fluorochemicals in biological matrices. *Environmental Science and Technolology*, **35**, 766-770. http://dx.doi.org/10.1021/es001489z

