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Analysis of Piperaceae Germplasm by HPLC and LCMS: A Method for Isolating and Identifying Unsaturated Amides from *Piper* spp Extracts

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A method for extraction and high performance liquid chromatography−mass spectrometer (HPLC-MS) analysis of the medicinally important genus *Piper* (Piperaceae) was developed. This allows for a rapid and accurate measure of unsaturated amides, or piperamides, in black pepper, *Piper nigrum* L., and in wild species from Central America. Reflux extraction provided the highest recovery of piperine (>80%) from leaf and peppercorn material. HPLC analysis using a binary gradient of acetonitrile and water separated the major amide peaks between 5 and 12 min. Atmospheric pressure chemical ionization (APCI)-MS improved the detection limit to 0.2 ng, 10-fold below the 2 ng limit of the HPLC-diode array detector (DAD) based on linear standard curves between 0.1 and 250 μg/mL ($R^2 = 0.999$). The HPLC-MS method identified pellitorine, piperylin, 4,5-dihydropiperlonguminine, piperlonguminine, 4,5-dihydropiperine, piperine, and pipercide. The biological activity of six Costa Rican *Piper* species assessed by mosquito larval bioassays correlated well with piperamide content.

KEYWORDS: Piperaceae; *Piper nigrum*; piperamides; HPLC/MS

INTRODUCTION

The plant family Piperaceae is a source of many biologically active phytochemicals (*1, 2*) with great potential for medicinal (*3*) and agricultural use (*4*). Species in the genus *Piper* have a wide array of secondary compounds, principally alkaloids and amides (*1*). The most widely recognized species is black pepper, *Piper nigrum* L., a spice traded around the world for hundreds, if not thousands, of years. In India, the majority (90%) of *P. nigrum* is grown along with cardamom and ginger in the southwestern state of Kerala (*5*). Other tropical countries including Indonesia, Malaysia, Brazil, and Vietnam account for the remainder of the world pepper production. Use as a spice has been extended from simple culinary application in the home to industrial scale processing of essential oils and oleoresins, both as food additives, pharmaceuticals (*5*), and insecticides (*6, 7*). As such, there is an increasing requirement for improved extraction and analytical methods to ensure quality and consistency in the final product.

Three *Piper* species were chosen for use in the development of a separation and identification method that would be practical and applicable to many end users, especially those wishing to commercialize *Piper* species as a botanical insecticide. *Piper nigrum* along with the West African Guinea pepper, *P. guineense* Schum and Thonn, and the Central American *P. tuberculatum* Jacq., have recognized insecticidal activity (*8*−*10*). Several extraction methods to separate active compounds from the leaves, seeds, and roots of *P. nigrum* and *P. guineense* have been described previously (*11, 12*). High performance thin-layer chromatography (HPTLC) was used to isolate and identify the main *P. nigrum* constituent in peppercorns (*13*). HPLC techniques were used to fractionate *P. guineense*, and compounds were identified using ^{1}H NMR (*14*). HPLC methods have also previously been developed in order to measure the plasma and tissue levels of piperine in animals (*15, 16*).

One of the first active compounds isolated from *P. nigrum* was piperine (**Figure 1**A). Many other unsaturated amides have since been identified in *Piper* species from all tropical regions of the world (*1*). A systematic nomenclature for *Piper* compounds was proposed: the term piperamide was chosen to describe all compounds carrying an aromatic group and an amide group (*11*). The mixture of piperamides present in many *Piper* species demonstrates the diversity and apparent redundancy of phytochemicals within species (*17*). For example, the combination of four principal amides in *P. tuberculatum* was explained previously by our research group (*10*) as an example of analogue synergism and revealed phytochemical diversity at a variety of

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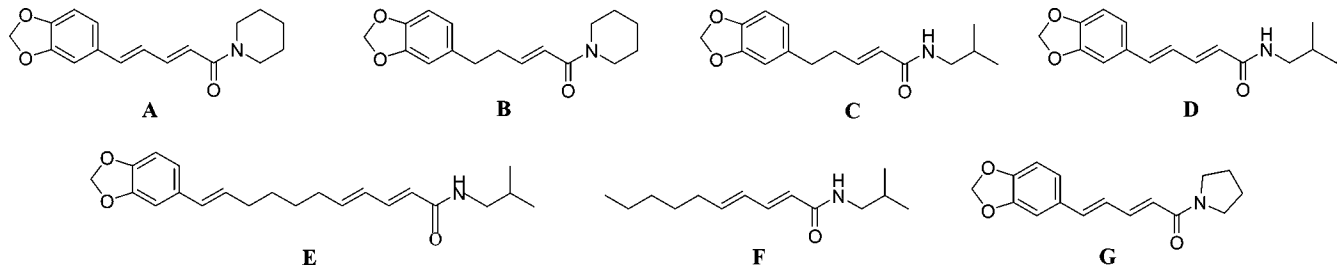

Figure 1. Structure of piperamides: piperine (A); dihydropiperine (B); dihydropiperlonguminine (C); piperlonguminine (D); pipercide (E); pellitorine (F); piperylin (G).

levels: different tissues of the same plant may exhibit different chemical profiles, and individuals may differ in their phytochemistry within a population.

In this study, sources of germplasm were assessed for the piperamide concentration in both *P. nigrum*, available commercially from wholesale spice suppliers, and *P. tuberculatum*, collected at two sites in Costa Rica. *Piper tuberculatum* is of particular interest because of the presence of piperamides in the leaf material, which has a greater potential than seed for large-scale production. Within different parts of *P. nigrum*, the fruit have the highest amount of piperine, but 2 to 10-fold differences can occur between samples (*12*). Geographically distinct populations of plants may also differ in phytochemical concentration, potentially as a result of selective pressures imposed by the different herbivores that attack the plant throughout its range (*18*) or selection by humans. Therefore, in assessing a source of germplasm as a potential supply of material, it is necessary to compare different geographical and ecological regions.

The objectives in the present study were (1) to develop methods in order to speed up the extraction of *Piper* materials, (2) to optimize the recovery of piperamides, and (3) to provide repeatable and quantitative analysis and identification of the principal active components.

MATERIALS AND METHODS

***Piper* spp. Germplasm Sources.** Kernels of *P. nigrum* were ordered through spice distributors in Canada, the United States, and Singapore. Peppercorns originated from four commercial sources in Malabar, India (accession nos. 1, 3, and 4) and Indonesia (accession no. 2). *Piper guineense* was obtained from only one source in Togo, West Africa. Two sites in Costa Rica were chosen as *P. tuberculatum* sources, Puntarenas and La Pacifica. Previous analyses (*10*) indicated that there was a difference in the levels of piperamides in leaves collected from these locations, La Pacifica having greater levels of the principal piperamide, 4,5-dihydropiperlonguminine. Other *Piper* species collected in Costa Rica included *P. nudifolium* C. DC., *P. cordulatum* C. DC., *P. aquale* Vahl, *P. biseriatum* C. DC., *P. pseudo-lindenii* C. DC., and a previously unknown species referred to as *Piper* species A. Leaf and peppercorn voucher specimens were deposited in the University of Ottawa Herbarium and Universidad National, Heredia, Costa Rica.

Chemicals. Four piperamide standards, piperine, 4,5-dihydropiperine, 4,5-dihydropiperlonguminine, and piperlonguminine (**Figure 1A**, **B**, **C**, and **D**), were synthesized as described previously (*10*). Pipercide (**Figure 1E**) was obtained from G. M. Strunz (University of New Brunswick, Fredricton, NB). Four piperamide derivatives (**Figure 2**A−**D**) were synthesized, and structures were confirmed with NMR (not shown). Pellitorine (**Figure 1F**) and piperylin (**Figure 1G**) were identified by MS (**Table 1**) and compared with literature reports (*11*, *24*).

***P. nigrum, P. guineense, P. tuberculatum,* and Other Costa Rican *Piper* spp. Extraction.** Two extraction processes for both *P. nigrum* and *P. guineense* peppercorns were chosen in order to compare extraction efficiency. The first was previously described (*10*) and the second, a modification of that technique, is as follows: *P. nigrum* or *P. guineense* peppercorns (50 g) were ground finely with a coffee grinder and then covered with ethyl acetate (125 mL) and refluxed, using a water-cooled condenser, at a boil for 20 min. The grounds and ethyl acetate slurry were shaken for 24 h followed by suction through a Buchner funnel with Whatman No. 1 filter paper to remove insoluble material. The filter cake was rinsed four times with 30 mL of ethyl acetate. The filtrate was transferred to a separatory funnel and washed twice with 75 mL of distilled water. The ethyl acetate fraction was separated and dried with anhydrous $MgSO_4$ and was refiltered as above. The filtrate was evaporated to dryness with a rotary evaporator and vacuum pump and the extract weighed. The filter cake was dried at 60 °C overnight in the drying oven and weighed.

Piper tuberculatum leaves (50 g) were finely ground with 125 mL of distilled water in a food blender. The aqueous slurry was sonicated for 15 min and transferred to a 500 mL flask containing 125 mL of ethyl acetate and shaken for 12 h. The leaf slurry was filtered by suction in a Buchner funnel to remove insoluble material and the filter cake was rinsed 3× with 30 mL of ethyl acetate. The filtrate was transferred to a separatory funnel to remove the aqueous phase, and the remaining ethyl acetate fraction was washed twice with 50 mL of distilled water. The extraction was repeated with the filter cake a second time, and the ethyl acetate was separated and washed with the same procedure and

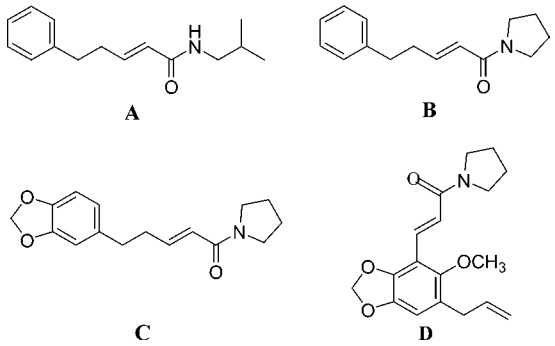

Figure 2. Structure of piperamide derivatives: EP7 (A); EP8 (B); EP6 (C); EP9 (D).

Table 1. Retention Time and Mass Spectra for Piperamides Identified Using HPLC-DAD and APCI-MS Method (positive scan mode, 100 eV fragmentor)

piperamide	HPLC retention time (min)	mass spectra M^+ *m/z* (rel int)
pellitorine	6.00	223.1 (10); 182.0 (10); 181.1 (100)
piperylin	6.20	273.1 (15); 272.1 (100); 201.0 (40); 135.0 (10)
4,5-dihydropiperlonguminine	6.87	277.1 (15); 276.1 (100); 135.0 (45)
piperlonguminine	7.02	275.1 (15); 274.1 (100); 201.0 (40); 135.0 (25);
4,5-dihydropiperine	7.48	289.1 (18); 288.1 (100); 135.0 (10)
piperine	7.73	287.1 (20); 286.1 (100); 201.0 (35); 135.0 (5)
pipercide	12.12	357.1 (25); 356.1 (100); 255.1 (10); 234.1 (5); 135.0 (25)

then combined with the first extraction. The ethyl acetate phase was evaporated to dryness and the extract weighed. The filter cake was dried at 60 °C overnight in the drying oven and weighed.

Piperamide Recovery. Ground *Piper nigrum* peppercorns were spiked with piperine (25 mg/500 mg ground pepper) at twice the observed concentration in the seed material. *Piper tuberculatum* leaves (50 g fresh weight) were processed with 2, 3, 4, and 5 times the observed piperine content by spiking the leaves with 3, 6, 9, and 12 mg of piperine respectively at the beginning of the extraction process. The extraction of both *P. nigrum* and *P. tuberculatum* followed the methods described above.

HPLC Method Development. An HPLC method for separating the piperamides in *Piper nigrum*, *P. guineense*, and *P. tuberculatum* was revised from a method for *Echinacea* isobutyl amides (*19*) and further refined from the technique devised in this laboratory (*10*). A ten-point calibration curve was developed between 1 and 250 μg/mL for each amide. Piperine and piperlonguminine were measured at 340 nm, 4,5-dihydropiperine and 4,5-dihydropiperlonguminine were measured at 205 nm, and pipercide was measured at 275 nm. The four synthesized amides were measured at 205 nm. The optimized method used a binary gradient of acetonitrile (A) and water, beginning with 30% A, increasing to 70% in 10 min, 90% by 12 min, and back to 30% A at 15 min. Samples were injected at 2 μL and the flow rate was 0.4 mL/min. The instrument was a Varian Prostar model pump, model 330 UV/Vis photodiode array detector and model 410 autosampler (Varian Chromatography Systems, Walnut Creek, CA). The column was a Varian reverse-phase C18 (3 μm, 100 Å, 4.6 mm × 100 mm). The method was further optimized in order to improve resolution of *P. guineense* amides: the peaks were separated using a longer run time of 17 min with the same gradient.

LCMS Method Development. HPLC-MS analysis was conducted using an Agilent Technologies 1100 Series LCMS (Agilent Technologies Inc., Palo Alto, CA) attached to a G1315B DAD, G1322A degasser, G1311A Quatpump, G1313A ALS and G1316A Colcom. Separation was achieved using a Waters YMC ODS-AM reverse phase column (53 μm, 120 Å, 2.0 × 100 mm). The MS detector was equipped with APCI source and operated in positive ionization mode. The MS was set on Scan-mode with positive polarity and the following parameter settings: mass range = 100−370 ms; fragmentor = 100; gain = 1.0; threshold = 150; step size = 0.1. The N_2 gas flow rate = 6.0 L/min; temperature = 300−350 °C; Nebulizer pressure = 60 psig; vaporizer temperature = 400−500 °C; capillary voltage = 3000 V positive/3000 V negative; corona current = 4 μA positive/15 μA negative. A ten-point calibration curve was developed between 0.1 and 250 μg/mL for each amide.

Lipid Separation Procedure. To further concentrate piperamides, a method was devised to saponify the *Piper* extracts. Crude extract of *Piper* species (0.5 g) was dissolved in 5 mL of methanol. A 10% methanolic KOH solution was prepared by adding 500 mg of KOH to the 5 mL of methanol. The mixture was refluxed for 30 min with a water-cooled condenser. The solution was cooled and 5 mL removed, and approximately 80% of the solvent of this fraction (*1*) was evaporated while refluxing was continued with the other fraction (*2*) for another 30 min. Diethyl ether (20 mL) and distilled water (20 mL) were added to fraction 1 and transferred to a separatory funnel and shaken gently in order to reduce foaming. After the aqueous layer was withdrawn, 20 mL of 10% aqeuous NaOH was added to the ether layer still in funnel and then shaken. The aqueous extracts were combined and retained. The ether fraction was dried with anhydrous $MgSO_4$ and then filtered by suction. The ether layer was evaporated to dryness by rotary evaporator and the extract weighed. Concentrated (10 M) HCl was added dropwise to the aqueous layer until the solution was acidic by litmus paper. Ether (20 mL) was added to the aqueous solution, and then the two phases were separated. The ether layer containing fatty acids was dried with anhydrous $MgSO_4$ and filtered by suction until dry and weighed. The ether and aqueous fractions were then analyzed for piperamide content by the previously described HPLC method.

Assessment of Biological Activity. The Costa Rican *Piper* species were screened for activity using a mosquito larval bioassay (*10*). All leaf extracts were prepared in 99% ethanol and dissolved in dechlorinated tap water at 0.1, 1, and 10 mg/100 mL (1, 10, and 100 ppm, respectively). Twenty 2nd instar *Aedes atropalpus* L. (Diptera: Culicidae) larvae were added to one replicate at each concentration level. After 24 h the number of surviving larvae was assessed, using a probe in order to elicit the characteristic twisting movement. *Piper* extracts producing 100% mortality at 10 mg/100 mL or less were retested in a narrower concentration range. Three replicates of 20 2nd instar larvae were tested, and the 24-h survival was assessed.

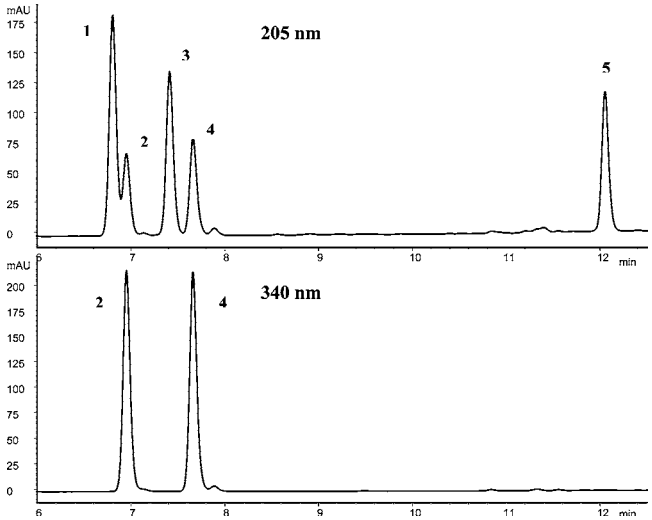

Figure 3. HPLC chromatographs with piperamide standards at 205 (top) and 340 nm (bottom): 4,5-dihydro-piperlonguminine (1); piperlonguminine (2); 4,5-dihydropiperine (3); piperine (4) and pipercide (*5*).

Statistical Analyses. Comparison between extraction techniques and germplasm sources was analyzed using one-way ANOVA with Tukey's multiple range means test (*20*). Anaylses of pre- and post-lipid extraction results were compared with a one-sample *t*-test and Bonferroni's comparison of means test and between aqueous and ether extraction by one-way ANOVA (*20*).

RESULTS

HPLC Separation and Piperamide Identification, Extraction, and Recovery. The chromatogram of the principle piperamides found in *P. nigrum*, 4,5-dihydropiperlongumine, piperlonguminine, 4,5-dihydropiperine, piperine, and pipercide at 250 μg/mL, demonstrates the separation and resolution obtained with the binary method (**Figure 3**). The 4,5-dihydopiperamides have a peak absorbance at 205 nm, while piperine and piperlonguminine have a peak absorbance at 340 nm. The HPLC and LCMS detection limits were 2 and 0.2 ng respectively based on linear standard curves between 0.1 and 250 μg/mL (R^2 = 0.999). The mass spectra (**Table 1**) for all piperamides were used to confirm their identity.

Piper nigrum (**Figure 4**) and *P. tuberculatum* (**Figure 5**) were analyzed using the same method with a 15 min run time while *P. guineense* was analyzed with the 17 min run time (**Figure 6**). Piperine is the principal amide identified in *P. nigrum* extracts at 340 nm. The coefficient of variance on replicate samples (n = 2) was <5.5%. Pelletorine was identified in *P. tuberculatum* (**Figure 5**) and piperylin in *P. guinense* (**Figure 6B**) confirmed by published MS results (**Table 1**). The synthetic piperamide derivatives (**Figure 2A**, **B**, **C**, and **D**) were found to have optimal absorption spectra at 205 nm, and peaks were separated with retention times at 6.0, 6.2, 6.5, and 8.1 min respectively using the HPLC method.

Piperine, using the ethyl acetate extraction/sonication method, was recovered with an average efficiency of 80%. Piperine recovery by this method from *P. tuberculatum* leaves was 83%. The reflux extraction method was much more efficient for

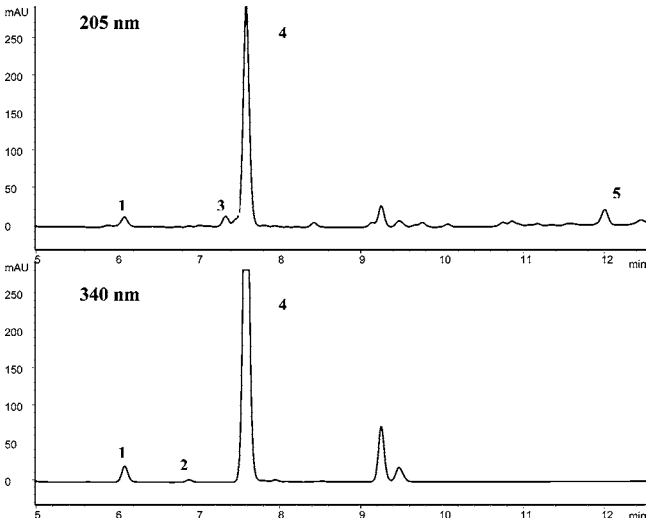

Figure 4. HPLC chromatographs at 205 (top) and 340 nm (bottom) for *P. nigrum*. Peaks identified are piperylin (1), piperlonguminine (2), dihydropiperine (3), piperine (4), and pipercide (5).

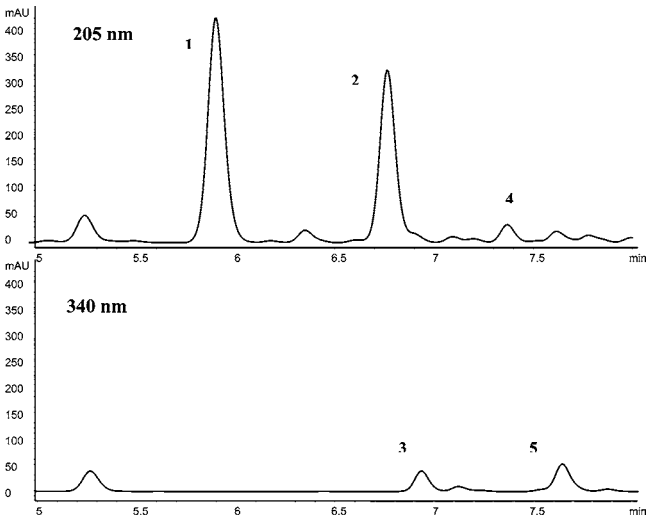

Figure 5. HPLC chromatograph at 205 (top) and 340 nm (bottom) for *P. tuberculatum*. Peaks identified are pellitorine (1), dihydropiperlonguminine (2), piperlonguminine (3), dihydropiperine (4), and piperine (5).

extracting compounds from *P. nigrum* than the sonication method as shown by the significantly greater recovery of piperine from one of two *P. nigrum* accession numbers (F = 6.48; df = 3,8; P = 0.016) (**Table 2**).

Germplasm Analyses. HPLC analyses of four available *P. nigrum* germplasm sources showed that three commercial sources had similar levels of piperine per g dried seed material. There was only a significant difference between the highest, accession no. 2, and the lowest, no. 4 (F = 5.640; df = 3,8; P = 0.023) (**Table 3**) When the total of all amides was considered, no. 2 again had greater amounts than 4 (F = 5.438; df = 3,8; P = 0.025) but was no different from 1 or 3 (Tukey's multiple range test, P > 0.486). *Piper tuberculatum* leaves collected at La Pacifica had greater levels of dihydropiperlonguminine (F = 19.906; df = 1,4; P = 0.011) and piperlonguminine (F = 11.110; df = 1,4; P = 0.029) than at Puntarenas, whereas dihydropiperine (F = 31.840; df = 1,4; P = 0.005) and piperine (F = 17.469; df = 1,4; P = 0.014) were significantly lower in La Pacifica leaves. However, when the total of all amides was determined there was no significant difference between the two sites (F = 0.073; df = 1,4; P = 0.800).

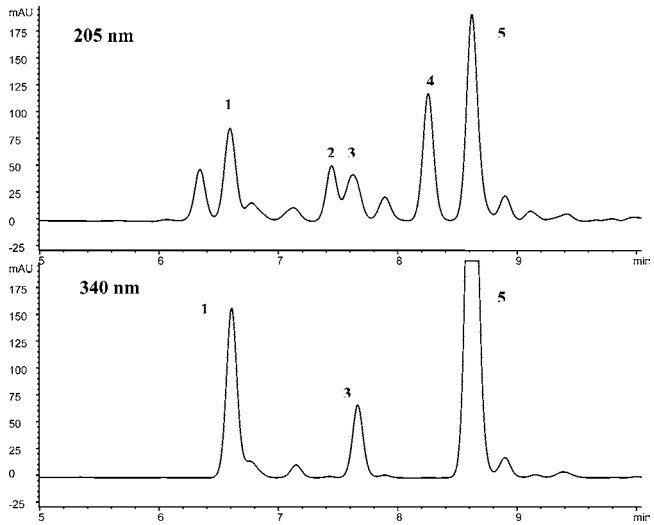

Figure 6. HPLC chromatographs at 205 (top) and 340 nm (bottom) for *P. guineense*: piperamide peaks identified are piperylin (1), 4,5-dihydropiperlonguminine (2), piperlonguminine (3), 4,5-dihydropiperine (4), and piperine (5).

Table 2. Average Piperine Concentration (μg/mg) + Standard Error (SE) in Extracts from Two Sources of *P. nigrum* Peppercorn and Two Extraction Methods (n = 3)[a]

Piper nigrum peppercorn accession no.	sonication extraction	reflux extraction
1	340 (10) a	540 (70) b
2	390 (30) ab	510 (40) b

[a] Piperine concentrations followed by the same letter are not significantly different either between extraction methods or accession nos. (Tukey's P > 0.05).

Lipid Separation. Lipid separation of *P. guineense* and *P. tuberculatum* extracts produced significantly higher amounts of dihydropiperlonguminine (F = 138.449; df = 3,8; P < 0.001) and piperine (F = 247.497; df = 3,8; P < 0.001) in the ether compared to the aqueous fraction (**Table 4**). The most significant concentration of piperamides from saponification of *P. tuberculatum* extract is demonstrated by the 6-fold increase in 4,5-dihydropiperlonguminine levels (one sample t-test, P = 0.048) and 5-fold increase in piperlonguminine levels (one sample t-test, P = 0.005). Piperine concentration was doubled for both *P. tuberculatum* and *P. guineense* (one sample t-test, P < 0.04) and 4,5-dihydropiperine concentrations were 50% greater for *P. guineense* (one sample t-test, P = 0.022).

Assessment of Biological Activity in a Convenient Mosquito Larvae Bioassay. In addition to *P. nigrum* and *P. guineense*, four of seven Costa Rican *Piper* species tested were acutely toxic to *Aedes atropalpus* larvae at 100 μg/mL (**Table 5**). When *P. aequale*, *P. cordulatum*, *P. tuberculatum*, and *Piper* species A were retested, the most active was *P. tuberculatum* producing 50% mortality between 2.5 and 10 ppm (**Figure 7**). *Piper* species A was similarly active between 12.5 and 25 ppm, while *P. cordulatum* and *P. aequale* were only active at >50 and >100 ppm, respectively. The HPLC analysis of *Piper* species A and *P. cordulatum* (**Figure 8**) indicates the lower concentrations of piperamides in those species compared to *P. tuberculatum*. *Piper* species A was not identified but the major peak was analyzed by LCMS and determined to have a MW of 205.2. Based upon the MS data, the molecular formula is predicted to be $C_{13}N_2H_{19}$, a compound not recognized from the *Piper* literature to date. Unfortunately no further extract material was available to isolate the compound by column chromatog-

Table 3. Average Concentration of Piperamides + Standard Error (SE) of $n = 3$ Different Samples in *P. nigrum* (μg or mg/g dried peppercorn) from Four Commercial Suppliers and *P. tuberculatum* (μg or mg/g dried leaves) from Two Distinct Ecological Areas in Costa Rica

pepper accession	DHPLG, μg/g (SE)[a]	PLG, μg/g (SE)[b]	DHPip, mg/g (SE)[c]	Pip, mg/g (SE)[d]	total piperamides, mg/g
P. nigrum no. 1	203 (51) a	222 (29) c	2 (0.3) e	51 (2) ghi	53 (3) jk
P. nigrum no. 2	223 (124) a	112 (31) d	3 (0.1) f	55 (3) h	58 (2) j
P. nigrum no. 3	0 b	81 (2) d	2 (0.2) e	43 (6) ghi	45 (7) jk
P. nigrum no. 4	250 (16) a	95 (13) d	3 (0.3) ef	34 (3) i	37 (3) k
[f] P. tuber. no. 1	2 (0.1) a	351 (20) c	209 (16) e	168 (9.0) g	2.7 (0.3) i
P. tuber. no. 2	1 (0.1) b	224 (33) d	743 (93) f	433 (63) h	2.7 (0.1) i

[a] 4,5-Dihydropiperlonguminine. [b] Piperlonguminine. [c] 4,5-Dihydropiperine. [d] Piperine. [e] Individual piperamide concentrations for each *Piper* spp. followed by the same letter are not significantly different (Tukey's $P > 0.05$). [f] *P. tuberculatum* accession no. 1 and no. 2 were from La Pacifica and Puntarenas, Costa Rica, respectively.

Table 4. Concentration of Piperamides (μg/g) + Standard Error (SE) in *Piper tuberculatum* and *P. guineense* Ethyl Acetate (EtOAc) Extracts and Post-Saponification Aqueous and Ether Fractions and the Ratio of the Ether Fraction Concentration to EtOAc Extract Concentration

Piper species/extraction type		DHPLG, μg/g (SE)[a]	DHP, μg/g (SE)[b]	PLG, μg/g (SE)[c]	piperine, μg/g (SE)
P. tuber.	EtOAc	[d] 38 a	15	6 d	3 g
aqueous	post	6.6 (0.8) b	N.A.	1.5 (0.2) e	0.6 (0.1) h
ether	post	237 (45) c	N.A.	32 (1.9) f	10 (1.0) i
ratio: ether/EtOAc		6.2	N.A.	5.3	3.2
P. guineense	EtOAc	8.4 a	38 d	0	45 g
aqueous	post	17 (0.3) b	28 (0.3) e	N.A.	25 (4.8) g
ether	post	32 (0.5) c	56 (0.5) f	N.A.	98 (11) h
ratio: ether/EtOAc		3.8	1.5	N.A.	2.2

[a] 4,5-Dihydropiperlonguminine. [b] 4,5-Dihydropiperine. [c] Piperlonguminine. [d] Individual piperamide concentrations for each *Piper* spp. followed by the same letter between pre and post extracts are not significantly different (Bonferroni's, $P > 0.05$) and piperamide concentrations with the same letter between aqueous and ether extractions are not significantly different (Tukey's $P > 0.05$). Note that all extracts were evaporated to dryness before analysis.

Table 5. Survival of 20 *Aedes atropalpus* 2nd Instar Larvae after 24-h Exposure to Costa Rican *Piper* Species at 1, 10, and 100 μg/mL

	number of survivors		
Piper species	1 (μg/mL)	10 (μg/mL)	100 (μg/mL)
P. aequale	20	20	0
P. biseriatum	20	20	18
P. cordulatum	20	20	0
P. nudifolium	20	20	19
P. pseudo-lindenii	19	20	19
Piper species A	19	16	0
P. tuberculatum	19	0	0
P. nigrum	20	0	0
P. guineense	20	0	0

raphy, but the method shows the utility of HPLC/MS for identifying new amides for selection studies.

DISCUSSION

The extraction and HPLC methods described provided rapid and accurate analysis for assessing the level of the active components found in the three species, *P. nigrum*, *P. guineense*, and *P. tuberculatum*. The addition of the LCMS improved on resolution and sensitivity from the method previously used (*10*). Although several reports of HPLC separation for piperamides exist (*14*−*16*), this is the first report that provides a method including recoveries and detection limits. The present method uses small diameter columns and the present run time was half that used by a similar method (*21*). *Piper nigrum* has been analyzed using a high performance thin-layer chromatography method (*13*); however, the HPLC method has better resolving power and can separate coeluting unknowns.

The determination by the present study that reflux extraction increases piperamide yield confirms values previously reported (*11*), and our piperine recoveries per peppercorn compares favorably with those of 50.8 mg/g (*13*). High extraction efficiencies have also been achieved with near-critical carbon dioxide extraction (SCO_2) (*22*) and with microwave-assisted extraction (MAE) (*23*). Supercritical CO_2 extraction of pepper requires high pressures and temperatures but can reduce the amount of solvent required. However, the levels of piperine extracted were about half that obtained with nonpolar solvents such as acetone (*22*). Although MAE is a more rapid extraction technique for piperine compared to conventional extraction techniques, it provides the same level of piperine extraction efficiency and may only be possible for small scale screening of raw pepper (*23*).

Saponification of the extracts was shown to concentrate the piperamides from the original extract and further separate less active components (**Table 4**). The physical appearance of the three extracts was as follows: *P. nigrum* was the driest, followed by *P. tuberculatum*, while *P. guineense* was the most fluid. This might help to explain why removal of lipid material greatly increases the amide profile of the latter two versus black pepper. Therefore, the efficacy of a botanical insecticide based on either *P. guineense* or *P. tuberculatum* material will benefit more from a saponification step in the production process.

All of the *Piper* species could be analyzed between 0.1 and 1 mg/mL ethanol and the principal active compounds identified by LCMS based on five piperamide standards and literature MS values. The method also provided clear separation of the derivatized amides, influenced by lipophilicity and steric effects. For example, the MDP group present on EP6 (MW = 277, **Figure 2A**) creates a more polar compound compared to EP7 (MW = 231, **Figure 2B**) and EP8 (MW = 230, **Figure 2C**) where it is absent. Similarly the presence of a pyridine ring moiety on the opposite end of the amide chain creates a more polar molecule, while a conjugated diene chain decreases the polarity of the molecule. For example, 4,5-dihydropiperine (MW = 287, **Figure 1B**) has a lower retention time compared to piperine (MW = 285, **Figure 1A**), as observed in **Figure 4** top.

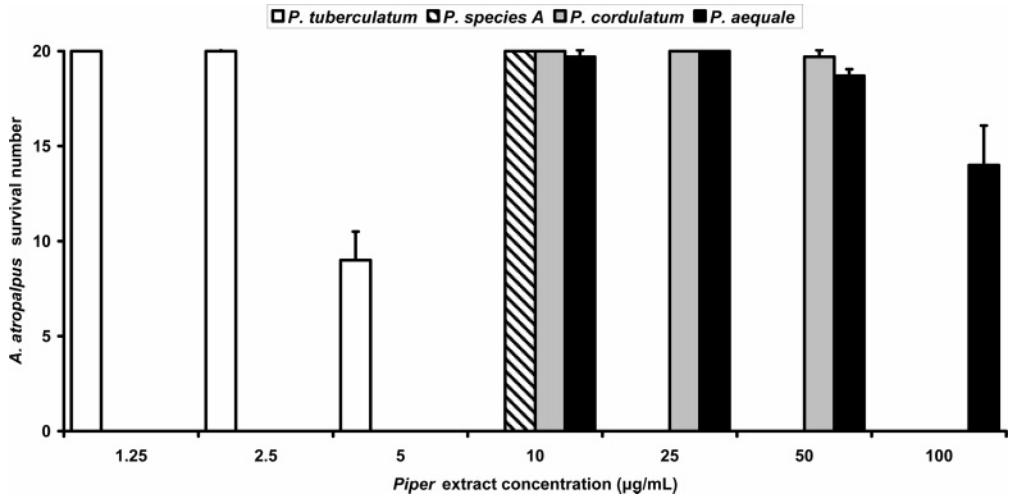

Figure 7. Survival of *Aedes atropalpus* 2nd instar larvae after 24-h exposure to *Piper tuberculatum* between 1 and 10 μg/mL, *Piper* species A, *P. cordulatum*, and *P. aequale* between 10 and 100 μg/mL ($n = 20$/treatment level).

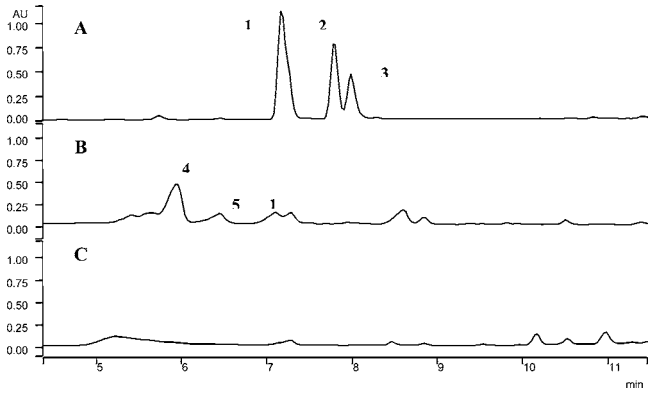

Figure 8. HPLC chromatogram at 205 nm showing (A) piperamide standards: dihydropiperlonguminine (1), dihydropiperine (2) and piperine (3); (B) *Piper* species A with new compound (4), pellitorine (5), and dihydropiperlonguminine; and (C) *Piper cordulatum*, no peaks identified.

In confirmation of our previous study (*10*), separate *P. tuberculatum* populations in Costa Rica have significantly different piperamide profiles. In the present study, leaves collected from plants at the La Pacifica site had levels of 4,5-dihydropiperlonguminine almost twice (2.02 versus 1.26 mg/g dried leaves) those found in leaves from Puntarenas (**Table 3**); however, when the total amount of piperamides is considered, the difference between leaves from the two sites was no different (2.7 mg/g). This suggests the amount of protection afforded by the secondary compound profile is similar between populations, as observed previously (*10*), regardless of ecological differences between the two sites.

In the case of *P. nigrum*, our relatively small selection of germplasm did affect the level of piperamides present: Accession no. 2 was black peppercorns from Indonesia, while no. 4 was from India (**Table 3**). The main purpose of the four accessions was to show the utility of the extraction and chromatographic methods. Future work should examine much larger and diverse pepper varieties as explored in other studies (*25*, *26*).

The lack of identifiable peaks in chromatographs for one of the Costa Rican species (**Figure 8C**) correlated well with the lack of biological activity in the insect bioassay (**Figure 7**). This is not to say that these species do not contain biologically active secondary compounds, but in comparison to the recognized *Piper* species, they do not provide a useful material for a fast-acting botanical insecticide.

The continued culinary and medicine application of *P. nigrum*, and the potential for insect-control usage in the future, will require increasing sophistication in analytical assurance. LCMS analysis will ensure that levels of piperamides in mixtures can be reported so that commercial sources of either oleoresin or formulated extracts can have an assured range of active components.

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