Video Article

Electroantennographic Bioassay as a Screening Tool for Host Plant Volatiles

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Abstract

Plant volatiles play an important role in plant-insect interactions. Herbivorous insects use plant volatiles, known as kairomones, to locate their host plant.[1,2] When a host plant is an important agronomic commodity feeding damage by insect pests can inflict serious economic losses to growers. Accordingly, kairomones can be used as attractants to lure or confuse these insects and, thus, offer an environmentally friendly alternative to pesticides for insect control.[3] Unfortunately, plants can emit a vast number volatiles with varying compositions and ratios of emissions dependent upon the phenology of the commodity or the time of day. This makes identification of biologically active components or blends of volatile components an arduous process. To help identify the bioactive components of host plant volatile emissions we employ the laboratory-based screening bioassay electroantennography (EAG). EAG is an effective tool to evaluate and record electrophysiologically the olfactory responses of an insect via their antennal receptors. The EAG screening process can help reduce the number of volatiles tested to identify promising bioactive components. However, EAG bioassays only provide information about activation of receptors. It does not provide information about the type of insect behavior the compound elicits; which could be as an attractant, repellent or other type of behavioral response. Volatiles eliciting a significant response by EAG, relative to an appropriate positive control, are typically taken on to further testing of behavioral responses of the insect pest. The experimental design presented will detail the methodology employed to screen almond-based host plant volatiles[4,5] by measurement of the electrophysiological antennal responses of an adult insect pest navel orangeworm (*Amyelois transitella*) to single components and simple blends of components via EAG bioassay. The method utilizes two excised antennae placed across a "fork" electrode holder. The protocol demonstrated here presents a rapid, high-throughput standardized method for screening volatiles. Each volatile is at a set, constant amount as to standardize the stimulus level and thus allow antennal responses to be indicative of the relative chemoreceptivity. The negative control helps eliminate the electrophysiological response to both residual solvent and mechanical force of the puff. The positive control (in this instance acetophenone) is a single compound that has elicited a consistent response from male and female navel orangeworm (NOW) moth. An additional semiochemical standard that provides consistent response and is used for bioassay studies with the male NOW moth is (Z,Z)-11,13-hexdecadienal, an aldehyde component from the female-produced sex pheromone.[6-8]

Video Link

The video component of this article can be found at http://www.jove.com/video/3931/

Protocol

1. Preparation of Volatiles Detected from the Host Plant for EAG Screening

1. After appropriate identification and authentication of all volatiles via GC-MS, perform EAG puff analysis of each available volatile. Initial screening can be a low replicate number of antennal responses (N=3-5) for each sex in order to achieve an indication of relative chemoreceptivity in a short amount of time (**Table 1**).
2. Prepare a solution of each volatile at a 5 mg/mL concentration in pentane. Tightly seal and refrigerate the sample until ready for immediate use (e.g., acetophenone, density = 1.03 g/mL, volume = (0.005 g/1.03 g/mL) × 1,000 = 4.85 µL of acetophenone into a 1.0 mL volumetric flask and dilute to 1.0 mL with pentane).
3. Just prior to EAG analysis, remove vials containing the 5 mg/mL concentration of volatiles to be tested and allow to warm to room temperature. Meanwhile, label the proper number of Pasteur pipettes to correlate with each volatile to be tested, and wrap a small piece of parafilm (ca. 15 × 15 mm) over the tip of the pipette. For the initial screening 10 stimulus pipettes (N=4 for each male and female) are loaded in the event of a bad prep or other unforeseen obstacle.
4. Using a pair of tweezers, gently fold the bioassay disc in half to facilitate placement into the pipette, then partially place the folded disc into the large end of the pipette so that ca. 2-3 mm of the disc are exposed. Align the labeled and prepped pipettes in a rack holder.
5. Using a pipettor or syringe, load 10 µL (50 µg) of volatile solution onto each disc and allow 2 minutes to pass before fully inserting discs into the pipette. Once loaded, immediately seal the end of the pipette with parafilm (ca. 15 × 30 mm). The amount of volatile stimulus to be loaded and puffed will most likely vary with insects species.
6. Following the same protocol noted above prepare controls to include in each analysis. For the negative control load 10 µL of just pentane onto each disc, wait 2 minutes, load into the labeled pipette, and seal. For the positive control, load 10 µL (50 µg) onto the disc, wait 2 minutes, load into the labeled pipette, and seal with parafilm.

7. The viability of the insect antennal prep will most likely vary with species, but we have found that *Amyelois transitella* antennae typically stay active and consistent for > 30 minutes after excision using the described protocol. This amount of time will generally allow for up to 4-10 volatile samples to be evaluated. **Table 2** provides an example of times and sequences.

2. Preparation of Insect Antennae for EAG Bioassay

1. The focus of this experiment is EAG analysis; thus, the assumption that each investigator will have access to appropriately reared insects.
2. For this experiment we will be studying 3-4 day old, mated male and female moths. Individual moths are transferred into a small, lidded, plastic container the day of analysis. Immediately prior to the bioassay, the moth to be tested is transferred head-first into a holding apparatus (i.e., made from various plastic pipettor tips) and secured from behind. Manipulation of the insect can be viewed under a low-power stereo-microscope to facilitate excision.
3. Antennae are teased out using a wire-tipped tool. The fork holder, with a small film of electrode gel, is placed in close proximity for quick transfer of antennae. The first antenna is excised using micro scissors and placed on the fork, ensuring the base of the antenna is placed on the non-red portion of the fork (the indifferent ground electrode). A timer set for 10 minutes is started and the second antenna is excised and placed next to the first antenna with both bases on the same side of the fork.
4. Alternatively, the antennae may be left secured within the holding tube, excised, then gently removed from between the tube and the insect using a wire-tipped tool with a dab of gel on the end.
5. Antennae are checked to ensure the base and tip are immersed in electrode gel and no bubbles are present in the gel. The ends/tips can be trimmed to guarantee full exposure to the electrode gel. Care should be used to ensure that the remainder of each antenna is not covered with gel, thus guaranteeing maximum antennal surface area is exposed to the puff.
6. The prepped fork is then inserted into the probe pre-amplifier and kept under a constant stream of humidified air at 200 mL/min for the remaining time of the 10 minute waiting period. The excise time and EAG initiation times are noted (**Table 2**) and 30 seconds prior to the first stimulus puff, the pipette containing the positive control is unsealed and placed in the holder which is adjusted to direct air/puff flow 2-3 mm from the prepped antennae.
7. After each experiment, the test moths are euthanized in a dry ice environment and properly disposed.
8. Female moths are dissected to check mating status. Cohabitating males are assumed to have mated.

3. EAG Protocol for Individual Components

1. Antennal responses are recorded via the AutoSpike software included with the Syntech EAG instrument. The configuration in the AutoSpike Properties tab for the channel with the EAG probe is set at a sampling rate of 106.7, NO to rectify, and a filter of 0-42 Hz. For the Filter tab, the EAG filter is ON and the low-cutoff is set to 0.1 Hz.
2. Stimulus puffs are 1-2 seconds in duration. A timer set for 1 minute is started after each puff.
3. The next test volatile pipette is unsealed and placed in the holder. The sequence of test volatiles, randomized between runs to ensure no test compounds consistently follow another test volatile, is then followed (e.g., **Table 2**) carefully allowing 1 minute between puffs.
4. To facilitate ease of reading EAG responses, portions of rulers are placed on the computer screen and the baseline level to the apex of the downward deflection signal is measured in mm and logged on a data sheet (i.e., for the 5 mV setting, 33 mm = 2.5 mV). The software has the capability of playback of the saved recordings for future analysis. Conversion of mm to either mV or µV amplitude is performed in later data analysis.
5. After the final positive control puff (e.g., record #12) is administered, the antennae are removed, the fork is cleaned with an ethanol-saturated wipe and allowed to dry before subsequent use.
6. To increase the throughput of assays, the excision of the next set of antennae can be started by a second laboratory personnel approximately 10 minutes before the end of current experiment - after the fourth puff; the second test volatile of the current experiment, as per **Table 2**. This will allow for the start of the next experiment directly after the current experiment ends.
7. Higher number of replications (on different antennae) can be performed on compounds of interest to provide further statistical validation.

4. An Example of EAG Analysis of Blends or Other Matrices (Table 3)

1. Next, the ratios and volumes for two tertiary blends (3-component mixtures) are calculated to provide an example of combining volatiles eliciting high EAG responses (**Table 4**). The calculations are for some basic ratios, a 1:1:1 molar ratio of α-humulene : 2-undecanone : 2-phenylethanol and then comparing to a second blend ratio of 1:2:4.
2. Using similar protocols described earlier, the mixtures are prepared and labeled Pasteur pipettes are loaded along with the necessary positive and negative controls.
3. The volatile samples are puffed across male and female antennae and the responses are measured and logged onto a data sheet (**Table 3**).

5. Representative Results

For female navel orangeworm the following settings are used: 2 second puffs, 10 second recording times, 10 second window, and 5 mV scale. A negative deflection is the typical response, yet the absolute value is recorded (e.g., -3,400 µV deflection is recorded as 3,400 µV). A relatively weak response of the prep to the positive control is discarded. **Figure 1** provides a graphical representation of a poor response to the positive control by navel orangeworm.

For example of a poor control result, the average female antennal response to acetophenone is typically ca. 2,600 µV (**Figure 2**), if the prep only gave a response of ca. 1,300 µV it would be discarded and another pair of antennae prepped. Similarly, the average male response to (*Z*,*Z*)-11,13-hexadecadienal was typically 3,000 µV; thus, any response less than 1,500 µV was typically discarded.

The positive control at the start and end of each experiment also provides information regarding the condition of the antennae. A rule of thumb we follow for rapid screening is if the antennal response to the puff of the post-control (record #12, **Table 2**) is either less than 75% of the 1st puff of the pre-control (record #1, **Table 2**) or less than the 2nd puff of the pre-control (record #2, **Table 2**) then the experiment is not used in the data analysis due to possible degradation of the prep (**Figure 3**). An example of the first rule of thumb would be record #1 = 2,730 µV and record #12 = 1,680 µV; and the second rule of thumb if record #2 = 2,350 µV and record #12 = 1,680 µV, In each of these cases, the prep and experiment's results would be discarded.

A representative example of correcting the EAG response values as measured to the positive control would be as follows.

Run #	EAG (μV)	Run #2	EAG (μV)	Run #3	EAG (μV)
(+) Ctrl	2800	(+) Ctrl	2420	(+) Ctrl	3030
Cmpnd A	3000	Cmpnd A	2500	Cmpnd A	3440
(-) Ctrl	530	(-) Ctrl	755	(-) Ctrl	910
Cmpnd B	2400	Cmpnd B	2000	Cmpnd B	2560
(+) Ctrl	2770	(+) Ctrl	2400	(+) Ctrl	3020

Using the values above for an N=3 experiment, the negative control response is subtracted from every value within each experiment under the assumption the negative control is the baseline antennal response to the mechanical puff and residual solvent.

Run #1	EAG (μV)	Run #2	EAG (μV)	Run #3	EAG (μV)
(+) Ctrl	2270	(+) Ctrl	1665	(+) Ctrl	2120
Cmpnd A	2470	Cmpnd A	1745	Cmpnd A	2530
(-) Ctrl	0	(-) Ctrl	0	(-) Ctrl	0
Cmpnd B	1870	Cmpnd B	1245	Cmpnd B	1650
(+) Ctrl	2240	(+) Ctrl	1645	(+) Ctrl	2110

The positive controls for each experiment would then be averaged and corrected to 1,000 μV, noting the ratio for correction to 1,000 μV. A data sheet (e.g., Excel) can easily be manipulated to convert responses to usable data.

Run #1	Avg (μV)	Run #2	Avg (μV)	Run #3	Avg (μV)
(+) Ctrl	2255	(+) Ctrl	1655	(+) Ctrl	2115
(+) Ctrl adj.	1000 (0.443)	(+) Ctrl adj.	1000 (0.604)	(+) Ctrl adj.	1000 (0.473)

Multiplying by the correction ratio within each experiment, the values for compound A and compound B are then adjusted.

Run #1	Adj. EAG(μV)	Run #2	Adj. EAG(μV)	Run #3	Adj. EAG(μV)
Cmpnd A	1094	Cmpnd A	1054	Cmpnd A	1197
Cmpnd B	828	Cmpnd B	752	Cmpnd B	780

The averages (means) for each compound are then determined along with other relevant statistical data and the EAG responses for the compounds tested can then be evaluated for candidacy for further investigation.

Compound	EAG (μV)	No. Runs, N=
A	1115	3
B	787	3

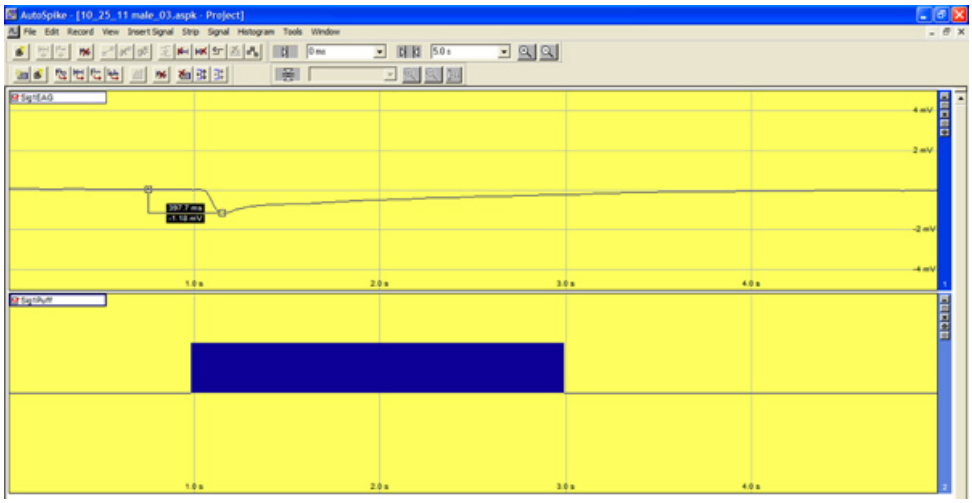

Figure 1. Representative EAG for male antennal response (1,180 μV) to the pre-control (1st positive control puff) that would be discarded due to poor antennal response after ensuring the antennae have good contact with the gel. Blue bars in bottom windows represent the two second puff of volatile. Click here to view larger figure.

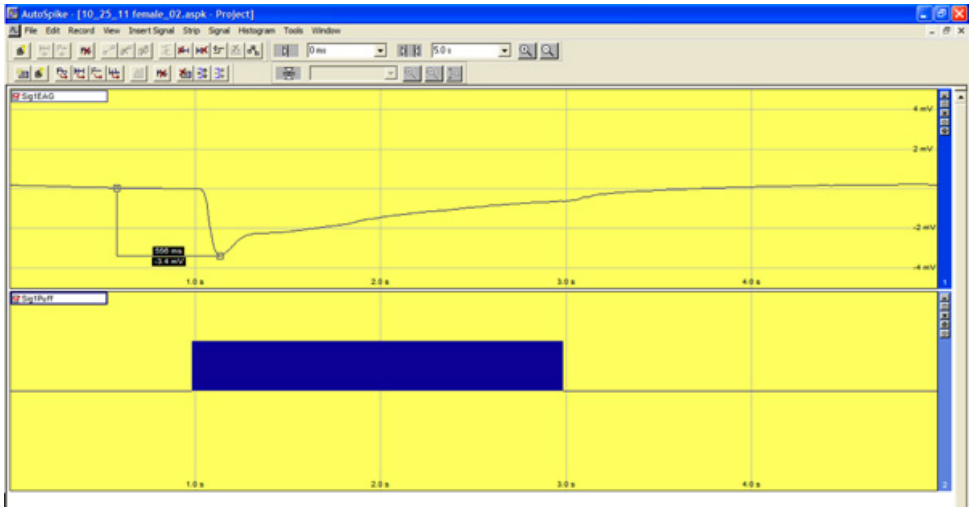

Figure 2. Representative EAG for the female antennal response (3,400 µV) to the 1st puff pre-control that would be considered appropriate. Click here to view larger figure.

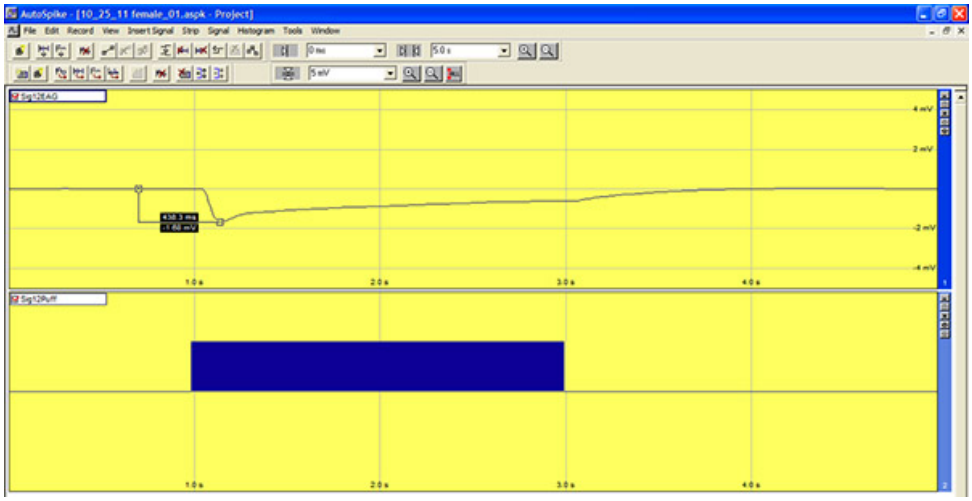

Figure 3. Representative EAG for the female antennal response (1,680 µV) to the post-control (last positive control for each experiment) that would be considered poor, and suggestive of antennae degradation (<75% of 1st pre-control or < 2nd puff value of pre-control). For this example the 1st pre-control puff was 2,730 µV and 2nd pre-control puff was 2,350 µV. Click here to view larger figure.

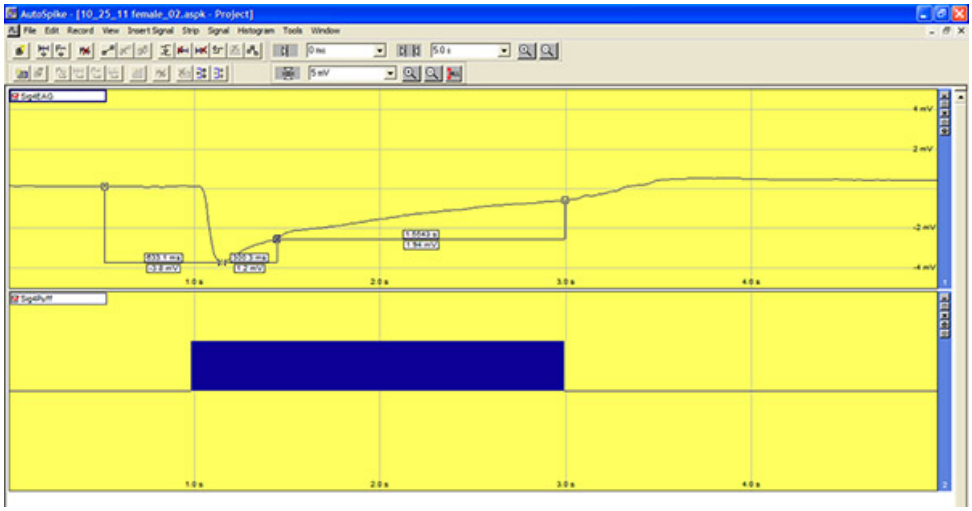

Figure 4. Representative EAG for the female antennal response (3,800 µV) to the puff of a candidate volatile blend and the subsequent measurements of the maximum initial deflection (3,800 µV), the initial slope during the puff duration (0.3 s/1.2 mV = 0.25 s/mV), and the slope for the remaining puff duration (1.6 s/1.9 mV = 0.84 s/mV). Click here to view larger figure.

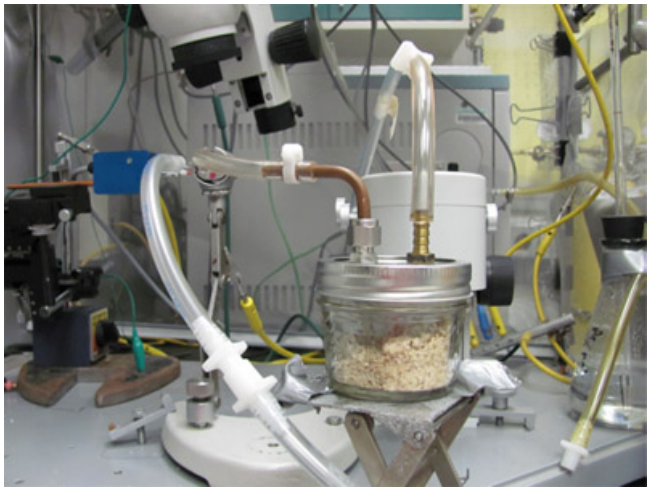

Figure 5. Small modified vessel containing a sample matrix and associated volatiles to be puffed across *A. transitella* antennae.

Compound Information						EAG	
DB-Wax		DB-1 RI		DB-Wax RI		Response (μV)	
r.t.	Identity	Lit	Calcd	Lit	Calcd	M	F
11.50	styrene	871	872	1252	1253	0	0
12.05	hexyl acetate	995	993	1268	1272	0	0
13.43	(*Z*)-3-hexenyl acetate	986	987	1312	1317	0	0
14.58	farnesane	1379	1378	1358	1354	45	0
15.64	(*Z*)-3-hexenol	834	838	1381	1388	0	0
15.77	nonanal	1082	1081	1389	1392	0	50
15.93	tetradecane	1400	1398	1400	1397	0	25
17.89	(*Z*)-3-hexenyl butyrate	1167	1168	1458	1461	238	75
18.77	α-copaene	1374	1374	1490	1489	30	20
19.56	β-bourbonene	1382	1381	1516	1515	-	-
20.08	2-nonenal	1134	1132	1532	1533	183	200
20.56	linalool	1083	1084	1546	1549	58	200
21.88	caryophyllene	1415	1413	1594	1593	0	190
21.99	2-undecanone	1273	1271	1596	1597	387	275
21.99	hexadecane	1600	1596	1600	1597	74	40
22.84	butyrolactone	855	859	1623	1627	0	25
23.95	α-humulene	1449	1455	1666	1666	187	250
24.42	pentadecane	1500	1499	1500	1489	0	150
25.59	2-undecanol	1286	1284	1721	1724	0	25
28.93	decyl acetate	1393	1392	1679	1678	264	75
29.10	geranylacetone	1429	1428	1853	1855	88	200
29.76	germacrene-D	1474	1473	1707	1713	20	115
30.57	2-phenylethanol	1081	1082	1910	1912	54	320
38.32	methyl anthranilate	1302	1301	2238	2232	33	65

Table 1. *In situ* volatile emission of Nonpareil almonds (2007) and EAG responses determined by a different and less sensitive configuration in the Autospike program.

EAGs of volatiles from *in situ* almonds							
					Date	11/21/2011	
			EAG Time		Notes		
Saved As...	Compound[a]	M/F	Prep	EAG	Record #	Record #	Comments:
11_21_11_Female_01	Positive Ctrl (ACEP)	F	8:57	9:07	1	2	
	HUMU				3		
	UDON				4		
	C16 Ald				5		
	2PEA				6		
	Negative Ctrl (C5)				7		
	Cmpnd 1				8		
	Cmpnd 2				9		
	Cmpnd 3				10		
	Cmpnd 4				11		
	Positive Ctrl (ACEP)			9:18	12		
11_21_11_Male_01	Positive Ctrl (ACEP)	M	9:10	9:20	1	2	
	HUMU				3		
	UDON				4		
	C16 Ald				5		
	2PEA				6		
	Negative Ctrl (C5)				7		
	Cmpnd 1				8		
	Cmpnd 2				9		
	Cmpnd 3				10		
	Cmpnd 4				11		
	Positive Ctrl (ACEP)			9:31	12		

[a] ACEP = acetophenone; HUMU = α-humulene; UDON = 2-undecanone; 2PEA = 2-phenylethanol
C5 = pentane; C16 Ald = (*Z*,*Z*)-11,13-hexadecadienal

Table 2. Example of a form for recording male and female antennal responses to individual volatile components.

EAGs of volatile blends

Date 11/21/2011

Saved As...	Compound[a]	M/F	EAG Time Prep	EAG Time EAG	Notes Record #	Notes Record #	Notes Comments:
11_21_11_Female_02	Positive Ctrl (ACEP)	F	9:33	9:43	1	2	
	Blend A				3		
	Blend B				4		
	Ground Almonds				5		
	Ground Pistachios				6		
	Negative Ctrl (C5)				7		
	Blend C				8		
	Blend D				9		
	Almond Meal				10		
	C16 Ald				11		
	Positive Ctrl (ACEP)			9:54	12		
11_21_11_Male_02	Positive Ctrl (ACEP)	M	9:56	10:06	1	2	
	Blend A				3		
	Blend B				4		
	Ground Almonds				5		
	Ground Pistachios				6		
	Negative Ctrl (C5)				7		
	Blend C				8		
	Blend D				9		
	Almond Meal				10		
	C16 Ald				11		
	Positive Ctrl (ACEP)			10:17	12		

[a] ACEP = acetophenone; C5 = pentane; C16 Ald = (Z,Z)-11,13-hexadecadienal

Table 3. Example of a form for recording male and female antennal responses to volatile blends and/or bouquets.

At a 1:1:1 ratio

	HUMU	UDON	2PEA		
MW	204.35	170.29	122.16		
density	0.89	0.83	1.02		
Ratio	1	1	1		
(MW of each)*ratio*x	204.35	170.29	122.16	0.00010	
x value	0.00010	0.00010	0.00010		
moles of each	0.00010	0.00010	0.00010		
g (MW*moles)	0.021	0.017	0.012	0.050	
mL	**0.023**	**0.021**	**0.012**	0.06	9.94

At a 1:2:4 ratio

	HUMU	UDON	2PEA		
MW	204.35	170.29	122.16		
density	0.89	0.83	1.02		
Ratio	1	2	4		
(MW of each)*ratio*x	204.35	340.58	488.64	0.00005	
x value	0.00005	0.00005	0.00005		
moles of each	0.00005	0.00010	0.00019		
g (MW*moles)	0.010	0.016	0.024	0.050	
mL	**0.011**	**0.020**	**0.023**	0.05	9.95

Sample of equations to set up for volume of components to add

	A	B	C	D	E		
1		**Cmpd A**	**Cmpd B**	**Cmpd C**			
2	MW	B2	C2	D2			
3	density	B3	C3	D3			
4	Ratio	B4	C4	D4			
5	(MW of each)*ratio*x	=B2*B4	=C2*C4	=D2*D4	=(total g desired)/SUM(B5:D5)		
6	x value	=E5	=E5	=E5			
7	moles of each	=B4*B6	=C4*C6	=D4*D6			
8	g (MW*moles)	=B2*B7	=C2*C7	=D2*D7	=SUM(B8:D8)	*This is a double check of the calculations*	
9	mL	=B8/B3	=C8/C3	=D8/D3	=SUM(B9:D9)	=Total volume-E9	

Table 4. Examples of preparation of 10 mL of a 5 mg/mL solution for two different ratios of blends.

EAGs of volatiles from *in situ* almonds

Date 11/21/2011

Saved As...	Compound[a]	M/F	EAG Time Prep	EAG	Notes Record #	Record #	Comments:
11_21_11_Female_03	Positive Ctrl (ACEP)	F	10:19	10:29	1	2	
	HUMU				3	4	
	UDON				5	6	
	Negative Ctrl (C5)				7	8	
	2PEA				9	10	
	C16 Ald				11	12	
	Positive Ctrl (ACEP)			10:42	13	14	
11_21_11_Male_03	Positive Ctrl (ACEP)	M	10:44	10:54	1	2	
	HUMU				3	4	
	UDON				5	6	
	Negative Ctrl (C5)				7	8	
	2PEA				9	10	
	C16 Ald				11	12	
	Positive Ctrl (ACEP)			11:07	13	14	

[a] ACEP = acetophenone; HUMU = α-humulene; UDON = 2-undecanone; 2PEA = 2-phenylethanol
C5 = pentane; C16 Ald = (*Z*,*Z*)-11,13-hexadecadienal

Table 5. Example of form for recording two consecutive puffs of single volatile components across male and female antennae.

Discussion

Use of electroantennogram recordings as a bioassay to determine chemoreception responses of a target insect is fairly common and numerous studies utilizing EAG as a detector for effluent from a gas chromatogram (GC-EAD) can be found in the literature.[9,10] The method demonstrated will provide a rapid screening of equivalent amounts of volatile components with high replications for confident assignment of the relative responsiveness. The AutoSpike program in the Syntech software is a good program for screening volatiles since it is able to provide the maximum deflection amplitude signal from the antennae (**Figures 1-3**), which we present here as the "screening" value. Additionally, other basic information for semi-advanced use (see **Figure 4**) can be obtained with AutoSpike depending on the configuration settings and what the researcher wants to derive from the antennal response. The GcEAD or EagPro Syntech programs are appropriate for more advanced experiments or for scientists familiar with electrophysiological responses since resultant traces provide greater detail of the time-course of antennal depolarization response.

Prior to the screening process of the compounds detected from a host plant, the proper identification of the volatiles is important and should follow strict protocols. If possible, two GC columns of differing polarity (i.e., DB-Wax and DB-1) should be used for initial component identification via matching of retention indices (RIs, see **Table 1**). The best method is to verify the identity of each volatile with an authenticate standard on two columns.[11] If the identity of some of the compounds is not possible, elucidation of their bioactivity can still be achieved by a use of GC-EAD.[12] However, replication of the bioassay may be limited depending on the volatile collection method, the amount of the analyte will not be readily available without an internal standard, the compound's identity will not be immediately known, and subsequent testing in blends would not be possible.

If sealed properly and refrigerated, solutions of volatiles in pentane can typically be stored for about 1 week. If the sealed pipets containing the loaded disc are placed in a zip-lock bag and refrigerated they can be stored for about 24 hours. However, we found it best to load the discs the morning of the EAG analyses and properly dispose of leftover pipets at the end of the day. Dosages for the standardized puffs should be determined experimentally for each insect species if related literature is not readily available.

Formulation of blends is typically an arduous process. Shown here are some relatively simple approaches, albeit not comprehensive. Researchers are encouraged to do further reading regarding various techniques. After evaluation of individual component responses from the host plant volatiles has been performed, studies of blends can be undertaken. One example is using the volatiles eliciting the higher relative responses from the screening. Other examples are: combinations by relative amounts emitted, ratios of relative responses versus relative amounts, sorting by class of compounds, or volatile differences in phenological stages or various states of the matrices (damaged vs. undamaged).[13]

The blends demonstrated are simple combinations of these various approaches. The 1:1:1 is a tertiary mixture based on the relative high responses in the initial screening, but also represents various classes of compounds. Humulene is a sesquiterpene, 2-undecanone is a fatty acid breakdown product, and 2-phenylethanol is a benzenoid. These compounds represent the major classes of volatiles typically seen in plant emissions. The 1:2:4 ratio in the second blend incorporates the relative ratios of volatiles detected during the GC-MS analysis.[4] However, the use of SPME and GC-MS provide only relative ratios and the use of GC-FID analysis in conjunction with calibration curves of the classes of compounds is recommended for a more accurate starting point for ratios based on volatiles detected.

The fork electrode EAG technique is one of the more simple methods employed in electrophysiological experimentation.[14] The reader is encouraged to perform further literature research for advanced applications beyond this method. Additionally, the screening of *ex situ* matrices can be performed using the method demonstrated, but utilizing small (60 mL) modified vessels (**Figure 5**) containing plant parts (e.g., ground almonds). When using larger containers (e.g., 120 mL lidded vessel with special adapters for use with the EAG puffer) it is recommended to increase the flow rate to ensure proper evacuation of the container. An experiment should be performed where a positive control is placed in the container to ensure the proper stimulation is achieved at the necessary flow rate. The use of a two second puff for the individual components is not absolutely necessary and puffs on the order of 0.5 to 1.0 seconds are more typical. However, it does allow for easier future comparisons with puffs of containerized volatile bouquets since these typically require a longer puff at higher flow rates. Our labs utilize the two second puff in order

to compare single component and/or blend responses directly to puffs using matrices in small vessels (see **Figure 5**). The two second puff on these small vessels ensures complete evacuation of the container when the appropriate flow rate is set.

Further, acquisition of a second puff can be performed, however the second puff is not absolutely necessary for screening since the amount of the component volatilized is no longer kept at a strict standard (**Table 5**). However, this information may be valuable for any subsequent dose-response experiments.[15] A much lower response may indicate a diminished response to lower concentrations while a consistent response may indicate the dose is near the threshold for a high response. It should be noted there are other physiological explanations for the change in responses[14] to the second puff, but the information does assist in guidance for future experiments. If high-throughput is not absolutely critical, the use of a second puff of each component can be informative.

If virgin female moths are the targeted specimen, NOW larvae in the last instar or pupae can be sexed and segregated[16] to allow female emergence to occur in separate containers.

Adjustment of the scale may be necessary to accommodate the antennal response if it exceeds the current scale. The scales on the EAG software screen can assist in determining how many mm per µV response. Other insects may vary in their sensitivity.

The method demonstrated provides an easy to learn, rapid, reliable, and high-throughput screening protocol to reduce the number of volatiles for bioactivity consideration from the complex composition of host plant volatiles. Provided the antennae of the specimen are suitable, the fork EAG method allows for the quick assessment of numerous volatile components or blends of components, and comparison of the responses to that of a standard. Ultimately, a bioassay that assesses the activity of the component or blend of components in a field setting is the most valid method. However, field studies often are very time and labor consuming, expensive, and require multiple months to obtain proper results.

Disclosures

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