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Volatile Components of Fermented Egg, an Animal Attractant and Repellent

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Since field tests have indicated that the volatile components of a fermented egg product (FEP) are attractive to coyotes and repellent to deer, it seems possible that a dual-purpose synthetic mixture could be prepared from the important compounds in this material. Both properties are potentially useful for controlling animal damage to agricultural and forest products. The quantities of 13 volatile fatty acids and eight amines were measured by gas–liquid chromatography. To identify other important compounds (alcohols, alkyl aromatics, esters, ketones, terpenes, and organosulfur compounds), we collected the headspace volatiles and analyzed them by combination capillary–column gas chromatography and mass spectrometry. The volatile constituents of FEP parallel those found in fermented food products. Also, some of the same volatile fatty acids and amines are found in anal gland secretions of canids. Thus, both food associated and chemical signaling aspects may be involved in coyote and deer responses.

A fermented egg product (FEP) recently patented as a bait for synanthropic flies (Mulla and Hwang, 1974) has been shown to be both repellent to deer and attractive to coyotes. The development of a material for either of these purposes has been a major research objective for many years.

The feeding activity of deer has become an increasingly important problem in agricultural crops, residential shrubbery, tree plantations, and reforestation areas. Reforestation areas are of special concern in the Pacific Northwest where protection of timber resources is particularly important. During the past two decades, reforestation efforts have been seriously hindered because deer and elk browse Douglas fir seedlings. If these seedlings could be protected, timber regeneration would be substantially accelerated. The approach toward finding methods to protect these resources from large game animals has generally been that of searching for nontoxic repellents which, when applied to the plants, will prevent browsing.

The coyote is currently one of the more frequent topics in discussions of animal damage control. Many stockmen are convinced that control of coyotes is necessary to remain in business, while others believe that claimed livestock losses to coyotes are exaggerated. Unfortunately, this dilemma cannot be resolved because not enough information is available on the effects of coyotes in natural biological systems, or their population distributions. Attractants can be used as a tool for gathering this information. For example, FEP was used from 1971 to 1975 to obtain indices of coyote population throughout the Western states (Linhart and Knowlton, 1975).

The dual potential of decomposed proteinaceous matter was recognized when a putrified fish formulation that had been used as a coyote lure was found to be an effective deer repellent (Campbell and Bullard, 1972). It was the best of 225 candidates (selected from over 4000 candidate chemicals in a screening program) that were evaluated in a standardized test for deer repellency (Dodge et al., 1967). Later, FEP was tested because it had some of the same properties. It also was effective in the deer repellent tests (unpublished data) and as an attractant for coyotes (Linhart et al., 1977). Since FEP is a manufactured item and can be readily purchased, we selected it for further development.

In the patented process for preparing FEP (Mulla and Hwang, 1974), a mixture of powdered whole egg and water is held in open contact with the air at room temperature for 7–14 days. Microorganisms from the air decompose the fat and protein. The egg–water mixture becomes a flowable slurry, which after aging is complete, is converted to a yellow powder (FEP) by freeze-drying.

Unfortunately, this method is subject to changing conditions, and batch-to-batch variation in quality occurs. Such variations in turn influence the behavioral response of animals and cause variable results. Another difficulty in the preparation of FEP is that pathogenic organisms may be cultured inadvertently, posing a health hazard to handlers.

Hwang and Mulla (1971) previously identified ten carboxylic acids in FEP, only three of which were considered to be key volatile components. Our objectives in this work were to identify other important volatile constituents, determine their relative concentrations, and formulate a synthetic preparation that duplicates the human odor panel response of FEP.

EXPERIMENTAL SECTION

It was apparent from the beginning of our work that information needed for the synthetic blending process must come from several analytical methods. For example, fatty acids were known to be predominant, and they could not be collected and analyzed under the same conditions as the other volatiles. Another procedure was required for the amines. However, most of the volatiles that were identified came from a cryogenic trapping procedure.

All analyses were conducted on samples taken from the FEP batches that had been used in the 1971–1975 surveys

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of coyote abundance. These samples were held at −12 °C until analysis.

The analyses were conducted on four aliquots of FEP. Each was treated by a specific procedure for the analysis of a specific class or group of compounds. The types of compounds and the modes of isolation and identification follow.

Headspace Volatiles Fraction. A modified rotary evaporator was used in the collection of headspace volatiles from the 1972 batch of FEP. Bullard and Holguin (1977) described this apparatus and procedure in detail. A liquid nitrogen cold trap replaced the Friedrich condenser of the rotary evaporator. A 500-g sample of FEP was rotated in a 2-L round-bottom flask having three symmetrically located ribbed depressions (2 cm deep) for 6 h. This tumbling action facilitated release of the volatiles. An infrared lamp 10 cm from the flask produced a constant internal temperature of 50 °C that further enhanced volatile release while minimizing roasting reactions.

The interior of the trap was coated with 0.2 mL of dibutyl phthalate, which served as a "keeper" during the collection period and later provided a means for syringe injection into the gas chromatograph. Because dibutyl phthalate has a longer retention time than most of the important volatiles, it did not interfere with early emerging peaks, as would most other solvents. It is also odorless and consequently provided a good medium for evaluating odor quality. A 0.2-mL dibutyl phthalate solution of volatiles having a fruity-sulfurous odor was recovered from the trap and analyzed by gas chromatography–mass spectrometry.

Wire Loop Method of Headspace Analysis. Sully (1971) developed this method for the perfumer who is concerned with the composition of a liquid that produces a given vapor composition. The vapor composition can be brought into equilibrium with a suspended micro-drop of dibutyl phthalate to reproduce the original liquid composition. The slow step in this equilibrium process is the diffusion of vapor through the air space; consequently, the rate can be increased if the work is done under partial vacuum. Sully illustrated that the procedure was equally applicable for solid biological materials (rose and thyme). We chose this procedure because other methods of extraction and headspace analysis rarely produce quantitative values that could be used to formulate a synthetic essence having the same odor properties as the natural one.

A 250-mL Erlenmeyer flask containing 100 g of the 1972 batch of FEP was evacuated (10 mmHg) through a stopcock adapter. A 6 × 1 mm nichrome wire loop containing a drop of dibutyl phthalate was suspended in the vapor space for 2 days. The dibutyl phthalate solution was then removed by syringe and analyzed by gas chromatography–mass spectrometry. Since the instrumentation conditions were the same as those for the headspace volatiles fraction, the spectra and chromatograms could be directly compared. Known quantities of the identified compounds were then injected under the same conditions, thus allowing quantitative determinations to be made.

Analysis of Volatile Fatty Acids. A 10-g sample of FEP (in a paper thimble) was acidified with a mixture of 2 mL of 50% H_2SO_4 and 5 mL of acetone. This was allowed to stand for 15 min in a Soxhlet apparatus to assure complete wetting and then extracted for 2 h with Freon 11. Evaporation of Freon 11 from the system was minimized by passing cold tap water through a 60-cm section of dry ice before it entered the condenser.

The Freon 11 was removed by using low holdup distillation columns and a water bath temperature of 55 °C. The extract and three rinses were then transferred to a 10-mL volumetric flask and brought to volume with acetone for GLC analyses.

Analysis of Volatile Bases. A 20-g sample of FEP was Soxhlet extracted with Freon 11 for 2 h with the Soxhlet assembly. After the extract had cooled to room temperature, amine hydrochlorides were generated by adding 50 mL of 3 N HCl. The Freon 11 was removed from the flask as described in the previous section. The extract and two 5-mL water rinses from the flask were then transferred to a 250-mL separatory funnel and extracted three times with 50 mL of diethyl ether. The water layer was concentrated by rotary evaporation under reduced pressure. The residue and three 100-μL water rinses were then transferred to a 3-mL screw-cap septum vial and reduced to dryness under a stream of nitrogen.

The amines were regenerated by introducing 150 μL of 12 N NaOH through a Teflon–silicone disc septum. This procedure prevented loss of the highly volatile trimethylamine. Then 500 μL of $MeCl_2$ was injected, and this mixture was agitated in an ultrasonic bath for 10 min. Aliquots of the methylene chloride layer were then analyzed by gas–liquid chromatography.

Quantitative Analysis by Gas Chromatography. A Tracor Model 550 gas chromatograph with a flame ionization detector was used for the quantitative analysis. The columns for the two fractions were operated under the following conditions.

(1) Volatile fatty acid analyses were conducted on a 3 ft × 0.125 in. o.d. aluminum column packed with 100/120 mesh Chromosorb 101 porous polymer. The flow rate was 45 mL/min of nitrogen and the temperature was programmed from 90 to 190 °C at 4 °C/min and held at 190 °C until all components had emerged.

(2) The amine analyses were conducted on a 3 ft × 0.125 in. o.d. aluminum column packed with 100/120 mesh Chromosorb 103 porous polymer and a 5 ft × 0.125 in. o.d. aluminum column packed with 10% Carbowax 20M and 2% KOH on 80/100 mesh Chromosorb W acid washed. The flow rate of nitrogen through both columns was 45 mL/min. The temperature was programmed at 4 °C/min from 90 to 190 °C and held at 190 °C until all components had emerged.

The injection port and flame ionization detector were held at 220 and 270 °C, respectively, through all operations.

Gas Chromatography–Mass Spectrometry. The columns and operating conditions employed in GC–MS confirmation of peak identity for acid and basic fractions were the same as described in the previous section. The volatiles from the cryogenic trapping were separated on a 0.03 in. i.d. × 500 ft stainless steel open-tubular column coated with OV-101 containing 5% Igepal CO-880. This column was programmed from 50 to 185 °C at 2 °C/min and then held at 185 °C until the emergence of dibutyl phthalate. The column was then vented through a rotary valve to a bypass pump to prevent dibutyl phthalate from entering the ion source.

Analyses were conducted on an Aerograph 200 gas chromatograph interfaced with a Watson-Bieman separator to a Nuclide 1290G single-focusing mass spectrometer. The operating parameters of the mass spectrometer were: 70 eV ionizing potential; 200 °C source temperature; 200 °C separator temperature; 1.2×10^{-5} Torr source pressure; and 5000 V accelerating voltage.

Authentic compounds were purchased from reliable commercial sources, given to us, or synthesized in our laboratory. Spectra of tentatively identified FEP components were compared with those of the authentic compounds on the same system, under the same condi-

Table I. Mean Concentration in Parts per Million (± SD) of Volatile Fatty Acids in Fermented Egg Product

Acid	1972-1975 batches[a]	1975 batch[b]
Formic	Tr[c]	Tr
Acetic	141 (9)	290 (34)
Propionic	445 (155)	166 (9)
Isobutyric	168 (135)	124 (7)
Butyric	1619 (421)	2145 (236)
Isovaleric	190 (207)	311 (49)
Valeric	869 (169)	548 (63)
Isocaproic	231 (175)	297 (49)
Caproic	3264 (1504)	3129 (78)
Heptanoic	1353 (792)	2500 (172)
Caprylic	1253 (1090)	3764 (215)
Nonanoic	Tr	Tr
Capric	Tr	Tr

[a] Samples from each batch analyzed in triplicate. Each value represents the mean concentration and standard deviation for 12 samples. [b] Samples analyzed in quadruplicate to illustrate precision of the method. [c] Tr, trace.

Table II. Mean Concentrations in Parts per Million (± SD) of Volatile Amines in Fermented Egg Product[a]

Amine	Concentration
Trimethyl	335.6 (29.8)
Isobutyl	1.06 (0.24)
Butyl	3.38 (0.78)
Isoamyl	50.75 (5.42)
Amyl	28.19 (5.47)
Hexyl	1.62 (0.14)
Heptyl	5.12 (0.14)
Octyl	8.25 (0.58)
β-Phenethyl	35.75 (2.98)

[a] A composite consisting of equal quantities of the 1972 through 1975 batches was mixed and analyzed in quadruplicate.

tions. Retention times were compared by a peak enhancement technique described by Bullard and Holguin (1977).

RESULTS AND DISCUSSION

It soon became apparent that we were not dealing with a single, or even a few, "character-impact" (characteristic aroma) compounds. Instead, the activity resided in three distinct fractions, each of which required a special analytical procedure. The relative concentrations of these fractions were about 77% fatty acids, 13% bases, and 10% headspace (primarily neutrals).

The volatile fatty acid fraction received considerable attention because of the relatively high concentration of these compounds and their potential behavioral influence. A volatile fatty acid mixture had previously performed well as a coyote attractant (Linhart et al., 1977). The 13 acids and the variation of their concentrations over the four batches, compared to the procedural variation (Table I), illustrates the need mentioned earlier for a synthetic attractant of uniform composition.

We identified and quantified eight amines in the volatile bases fraction (Table II). Of the several procedures tried, only this one permitted analysis of the highly volatile trimethylamine along with those of lesser volatility while retaining adequate precision. All of the identified amines could be separated and measured quantitatively on at least one of the two GC columns.

A gas chromatogram (total ion current monitor) of the headspace volatiles fraction of FEP is shown in Figure 1. Table III lists the compounds identified in order of retention time. Forty-nine esters were identified; ethyl esters predominated in both numbers and concentration. The

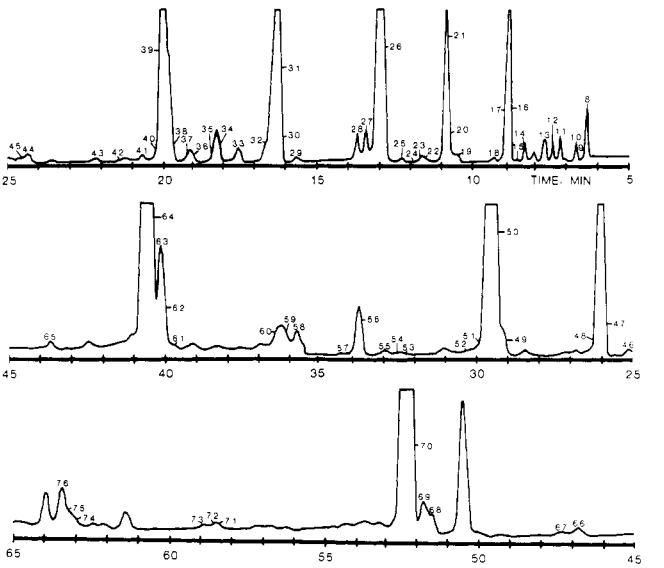

Figure 1. Chromatogram of large bore open tubular column gas chromatography–mass spectrometry analysis of headspace volatiles from fermented egg.

relatively high abundance of the esters explains the fruity character of the fraction. The sulfur components in this fraction, because of their low olfactory threshold, introduce a sulfurous overtone to the basic fruity aroma of the esters.

Sufficient quantities of 54 volatiles were collected by the wire loop method for mass spectral identifications. The identification was easy because spectra and retention times had been obtained earlier for the headspace volatiles fraction. Quantitative determinations were made for the resolved peaks and good estimates could be made of the unresolved ones by injecting appropriate combinations of known compounds. The compounds and their relative concentrations are listed in Table III.

Mulla and Hwang (1974), besides developing FEP, were also the first to report on its chemical composition (Hwang and Mulla, 1971; Hwang et al., 1976b) and to attempt development of a synthetic fly attractant (Hwang et al., 1975; 1976a). They identified acetic, propionic, butyric, isovaleric, and isocaproic acid, and trimethylamine. Because we were concerned only with volatiles we did not attempt to identify 10 of the nonvolatile compounds which they reported.

There is some evidence about the mechanism by which the volatile compounds are formed. Whole fresh eggs contain about 11.5% protein and 10.2% fat (Adams, 1975). Both of these sources could be considered about equally in any degradation scheme. The composite product could arise from several possibilities: products of microbial enzymes (bacteria, molds, and yeast), products of egg enzymes, atmospheric oxidation products or unchanged components of fresh eggs.

The fermentation process brings substantial changes to the composition of egg volatiles. MacLeod and Cave (1975) have conducted the most extensive investigation of the volatile flavor components of fresh eggs. They positively identified 65 and tentatively identified five compounds. Weurman and de Rooij (1961) identified four amines in whole fresh eggs. Sato et al. (1968) identified ten volatile compounds in egg white. Only ten of these compounds identified in fresh eggs were found in fermented egg. Atmospheric oxidation also appears to be of minor influence, judging by the extremely low aldehyde content of FEP. The oxidation of fats usually begins with the formation of hydroperoxides which are subsequently converted into aldehydes, some of which are oxidized further

Table III. Identified Volatile Components from Fermented Egg

Compound	Rel concn of WL[a] volatiles, %	Compound	Rel concn of WL[a] volatiles (%)
1. Acetaldehyde		39. Ethyl valerate	5.56
2. Methanol		40. o-Xylene	0.32
3. Ethanol		41. Butyl propionate	0.09
4. Acetone		42. Isobutyl butyrate	0.14
5. Propanal		43. Methyl caproate	0.40
6. Methyl acetate		44. α-Pinene	0.07
7. 1-Propanol		45. Propyl valerate	0.06
8. Ethyl acetate		46. Butyl isobutyrate	0.08
9. Methyl propionate		47. Ethyl isocaproate	0.73
10. Propyl formate		48. 1,3,5-Trimethylbenzene	
11. 2-Pentanone		49. Butyl butyrate	0.97
12. Pentanal		50. Ethyl caproate	15.00
13. Benzene		51. 1,2,4-Trimethylbenzene	
14. 2-Mercaptoethanol		52. Octanal	0.14
15. Methyl isobutyrate		53. Methyl heptanoate	0.17
16. Ethyl propionate	2.00	54. 1,2,3-Trimethylbenzene	
17. Propyl acetate	0.07	55. p-Cymene	
18. Methyl butyrate	0.07	56. Limonene	0.07
19. Dimethyl disulfide	0.13	57. Butyl isovalerate	0.07
20. Isoamyl alcohol		58. Amyl butyrate	0.16
21. Ethyl isobutyrate	0.33	59. Propyl isocaproate	0.18
22. Isobutyl acetate		60. Isoamyl butyrate	0.16
23. Toluene	0.18	61. Acetophenone	
24. Methyl isovalerate	0.44	62. Isobutyl valerate	0.78
25. 2-Hexanone	0.86	63. Propyl caproate	0.74
26. Ethyl butyrate	5.87	64. Ethyl heptanoate	33.00
27. Propyl propionate	0.29	65. Methyl caprylate	0.40
28. Butyl acetate	0.37	66. Isobutyl caproate	0.16
29. Isopropyl butyrate	0.07	67. Butyl isocaproate	0.23
30. Methyl valerate	0.53	68. Butyl caproate	0.72
31. Ethyl isovalerate	0.16	69. Propyl heptanoate	2.10
32. Propyl isobutyrate	0.09	70. Ethyl caprylate	19.50
33. Ethyl benzene	0.13	71. Amyl caproate	0.27
34. Isoamyl acetate	0.36	72. Isobutyl heptanoate	0.56
35. p-Xylene	0.29	73. Isoamyl caproate	0.18
36. m-Xylene	0.12	74. Butyl heptanoate	1.12
37. 2-Heptanone	0.71	75. Propyl caprylate	1.03
38. Propyl butyrate	1.07	76. Ethyl nonanoate	0.56

[a] Relative concentration of headspace volatiles as collected by the wire loop method.

to give acids. Egg enzymes are undoubtedly influential but far less important than bacterial enzymes. This conjecture is supported by the fact that the odor of the contents of a spoiled egg with an intact shell (no contamination by airborne microorganisms) is completely different from that of FEP. Thus, it appears that microbial enzyme activity is the primary source of volatile compounds in FEP.

Dougan and Howard (1975) reached a similar conclusion for the origin of the components of fermented fish sauces. They attributed the source of most of the volatile fatty acids to the bacterial decomposition of a lipid substrate primarily composed of straight chain acids. The fatty acid composition of hen's eggs is also primarily straight chains. Another similarity was their attribution of the aroma of fermented fish sauce to three distinct "notes": cheesy from volatile fatty acids; ammoniacal from amines; and meaty. The aroma of FEP is composed of four distinct notes: cheesy from volatile fatty acids; ammoniacal from amines; fruity from esters; and sulfurous from organosulfur compounds (Bullard et al., 1978).

There are close similarities between FEP and some of the cultured dairy products, especially cheeses. In dairy products, selective lactic-acid-producing bacteria are introduced under conditions which produce exacting flavors, whereas the microflora of FEP are only cursorily understood. Four genera of bacteria, two bacilli and two micrococci, have been identified in FEP cultures by microbiologists at McLaughlin, Gormley, and King Company in Minneapolis, Minn. (Priess, 1977).

Liebich et al. (1970) in attempting to simulate cheese flavor mixtures, concluded that free fatty acids are the basis of any cheese flavor. The aroma of blue cheese, which has a closer similarity to FEP than many of the others, is believed to be primarily related to the composition of fat and the specificity of the lipase systems (Day, 1967). Alcohols, which are found in both FEP and cheeses, react with the volatile fatty acids to form esters. A rather extensive list of fatty acid esters was reported for cheeses (Day and Anderson, 1965; Day and Libbey, 1964; Liebich et al., 1970; Langler et al., 1967). As in FEP, ethyl esters predominate, with ethyl butyrate and ethyl caproate generally being the most abundant. The flavor properties and their relatively large contribution are very important to overall flavor. For example, the delicacy of the balance of cheddar flavor components is best illustrated by the development of a fruity defect in the cheese when esters exceed some undetermined concentration (Day, 1967).

Many of the miscellaneous compounds are common to both FEP and cheeses. Methyl ketones are found in both. It is generally accepted that in cheese the methyl ketones result from β-oxidation of fatty acids; in FEP they probably have the same origin. The terpenes, α-pinene and limonene, have been reported in cheddar cheese, and several alkyl benzenes have been identified in both blue cheese and cheddar cheese and are believed to make important contributions to the flavor (Day, 1967; Liebich et al., 1970).

Proteolysis in cheddar cheese has been studied extensively; amines are products of these reactions (Silverman

and Kosikowski, 1956). Because few of the extraction processes are designed for isolating these compounds, the literature reports on these products are scarce. However, it is a good assumption that most of the amines reported in FEP are products of proteolysis.

Finally, the sulfur compounds found to be important odor components in FEP are also found in cultured dairy products. Dimethyl disulfide is in most of these products and 2-mercaptoethanol is in butter (Lindsay et al., 1965). These, too, are believed to be products of proteolysis derived from sulfur-containing amino acids, principally cysteine. Of relevance to the present discussion was Walkup's (1975) report that some coyote trappers use 2-mercaptoethanol to enhance the odor of trap lures.

The biological significance of fermented egg compounds may be complex indeed. The attractancy of smelly decomposed proteinaceous matter for canids is well known. However, in this case there is perhaps an added dimension. Trimethylamine and all of the volatile fatty acids found in anal sac secretions of red foxes (Albone and Fox, 1971) and dogs and coyotes (Preti et al., 1976) are also present in FEP. Although biological functions of anal gland secretions have not been definitely established, most authors suggest some form of pheromonal activity.

Numerous tubules and sebaceous glands in the tissue lining of the anal sacs of dogs secrete proteins, carbohydrates, and lipids (Montagna and Parks, 1948). Microbial action on these substrates can produce short-chain aliphatic acids, trimethylamine, ethanol, and acetone (Preti et al., 1976). The C_2–C_6 acids in red fox anal sac secretions are products of the resident microflora (Albone and Perry, 1976). In addition, Gorman et al. (1974) have shown that a series of C_2–C_5 acids in the anal sacs of the Indian mongoose are the products of bacterial breakdown of sebum and apocrine secretion deposited within the sac.

These same volatile fatty acids are widely distributed in the scents of many mammals. Other sources include the anal sacs of lions (Albone et al., 1974) and weasels (Gorman, 1976); the vaginas of rhesus monkeys (Michael et al., 1971) and humans (Michael et al., 1974); perineal glands of guinea pigs (Berüter et al., 1974); and subauricular glands of male pronghorns (Müller-Schwarze et al., 1974). Gorman (1976) has suggested that a unique combination of six carboxylic acids in the anal sac of the Indian mongoose give a distinctive odor to the animal that serves as the basis of individual recognition. Albone and Perry (1976) believed there was insufficient evidence to reach this conclusion for red foxes. However, they advanced a fermentation hypothesis of group chemical recognition. By a process of cross-infection, a group of animals living together would be expected to share a common microflora. Therefore, a member of a group which has been apart for some time may no longer be recognized by the group, and an alien animal forcibly introduced to a group may eventually become accepted (Albone et al., 1974).

Although the biological implications of these volatile fatty acids and amines are not yet clearly understood, the presence of compounds in fermented egg which occur as glandular substances in social mammals raises some interesting questions. Thus, coyotes could be attracted to this material for a variety of reasons; such as curiosity, pheromonal activity, or palatability of decomposed natural materials. Deer, being herbivores, are not attracted to decomposed natural materials. Another factor possibly related to their response to FEP may be an association of volatile fatty acids and amines with the odor of canid predators. We believe that much of the activity of FEP like that reported earlier for rat attractants (Bullard and Shumake, 1977) is attributable to the high response of animals to familiar natural materials in their environment.

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