Table III. Ranking of Amino Acids in Order of Decreasing Variability Based on F Ratios Obtained from the Analysis of Variance of the Cacao Protein Groups

Ranking order	Amino acid	F ratio
1	Glu	87.59
2	Ala	60.20
3	Arg	55.96
4	Phe	36.97
5	Ser	31.13
6	Val	26.71
7	Thr	24.90
8	Asp	20.37
9	Ile	20.22
10	Gly	18.43
11	Leu	9.25
12	Tyr	4.61
13	Pro	2.94
	$p < 0.05$	
14	Lys	2.52
15	His	1.43
16	Cys	1.06
17	Met	0.41

Amino Acid Analyses. Statistical analyses using Duncan's least significant difference test ($p < 0.05$) showed all protein groups to be different with respect to amino acid composition. Six groups had at least one amino acid significantly different in mole percent when compared to every other group (Table II). Group 5 had 6 amino acids in this category. This may reflect its unusually high glutamic acid content. Neither group 4 nor 7 had an amino acid at a level which was significantly different from all other groups. These two groups were quite similar, varying only in serine and isoleucine contents. Histidine and especially cysteine and methionine were present only in trace amounts in all instances.

In Table III the amino acids are ranked in descending order based on F ratios. A high F ratio indicates a wide distribution for the mole percent of an amino acid among protein groups, while taking into consideration the effect of experimental error on the distribution. Based on this method, 13 amino acids vary sufficiently to warrant further study, since there is a 95% chance that the mole percent of at least one of them in a protein group will be different than the levels in the remaining groups. If the p value of the F ratio were decreased to 0.005, differences would still be expected for 12 of the amino acids in Table III. Lysine, histidine, cysteine, and methionine have F ratios below the value required for $p < 0.05$, and it is unlikely that any real differences between groups would be found.

Essentially all of the cacao grown throughout tropical regions of the world evolved from two types, Criollo and Forastaro, both of which are indigenous to and about the western Amazon basin. Protein content of the latter is higher, and recovered protein fractions are less contaminated and more soluble compared to Criollo (Zak and Keeney, 1976). These differences reflect in part protein reactions with oxidized polyphenols.

It would seem that differences in amino acid profiles between Criollo and Forastaro would also be involved in explaining why Criollo is more easily and extensively tanned, and why electrophoresis patterns are not similar. However, the data of Zak and Keeney (1976) are inconclusive in this regard. Improvements in methodology described herein, especially the introduction of ion exchange chromatography on Sephadex SP-25, resulted in the recovery of several protein fractions from a single cacao type, and with an amino acid pattern different from the other groups. Using these methods we anticipate that amino acid differences among protein groups of Criollo and Forastaro Cacao and varieties derived from them will be found. Hopefully, this information might serve as a chemical index in evaluating wild genotypes and classifying cacao types currently in use.

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Determination of Biphenyl by Gas and Liquid Chromatography

Citrus fruits in shipping cartons are protected from decay with biphenyl-impregnated pads. Two methods of analyzing the biphenyl content of the pads, by gas and liquid chromatography, were compared and both were satisfactory. Analysis of extracts of whole pads rather than of representative areas is suggested. A method of analysis of biphenyl vapor by gas chromatography was developed. This entailed multiple injections from a heated sampling syringe to overcome the tendency of biphenyl to adhere to the glass. This method should be useful in determining relationships between vapor concentration and fungistatic activity and to atmospheric monitoring.

For the analysis of biphenyl, a widely used fungistatic agent, infrared (Newhall et al., 1954), ultraviolet following cleanup by thin-layer chromatography (Norman et al., 1966, 1968, 1969), gas chromatography of solutions (Beernaert, 1973; Morries, 1973; Wells et al., 1963), and liquid chromatography of residues in citrus products (Reeder, 1975) and of vapors sampled with a loop (Sharma and Palmer, 1974) have been used. Within cartons of packed citrus fruit, biphenyl is usually applied by insertion of two kraft paper pads impregnated with the fungistat

Table I. Comparison of Repeated Analyses of Standard Ethanol Solutions of Biphenyl by Gas and Liquid Chromatography

	GC	LC
No. of analyses	10	10
Retention time	6 min, 40 s	1 min, 45 s
Column	Silicone gum rubber	Micro-Bondapak C_{18}
Sample size	2 μl	5 μl
Sample concn	0.02%	0.01%
Mean peak height (div)	66.8	84.7
Std dev	1.135	1.337
Std error of mean	0.395	0.423
Coeff of variation	0.0170	0.0158

at a rate of about 2 g/pad. Biphenyl vapor, rather than residue content in or on the peel, primarily prevents growth and development of fungi (Norman et al., 1968, 1969). Thus, it is essential to know the quantity of biphenyl vapor existing, or potentially available, within cartons. These vapor quantities are dependent upon initial amounts on the pads, exposure of the pads prior to use, temperature, and ventilation. This paper presents a comparison of methods of biphenyl determination.

MATERIALS AND METHODS

Biphenyl was extracted from commercially impregnated pads with 95% ethanol. Other solvents, such as isooctane, were found to be equally effective, but ethanol has the advantages of lower cost and ready availability. Extracts were analyzed by gas and liquid chromatography (GC and LC). The GC was a Microtek GC-2000R with a 15% silicone gum rubber column, SE-30 (methyl), 3 m × 6 mm, an oven temperature of 175 °C, an inlet temperature of 200 °C, the flame ionization detector at 250 °C, and the N_2 carrier gas at 90 ml/min. Extracts were sampled by drawing 1 μl of isooctane, 1 μl of air, and then 2 μl of sample into a 10-μl syringe. The LC analyses were made with a Waters Associates instrument ALC-202/401, Bondapak C_{18} column, 0.25 in. × 30 cm, redistilled ethanol as solvent at 2 ml/min, UV detector set at 254 nm, universal injector, and 5-μl samples.

No simple method for measurement of small samples of biphenyl vapor was known to us. As noted by Wells et al. (1963), biphenyl has a strong affinity for glass, metal, and plastic. Preliminary experiments showed that samples of vapors taken with a glass syringe gave low results from loss of sample by adsorption on the syringe itself. To circumvent this, a small heating coil which surrounded the syringe during injection was positioned outside the injection port. The temperature inside the coil was 80 °C. The syringe was left in place and, after the first injection, was allowed to slowly refill with carrier gas and then the gas was reinjected. Because the retention time was a little over 6 min, the reinjections could be repeated at least three times, at 1-min intervals, without producing overlapping peaks. Peak heights for all injections were added for a "total" peak height. Two replicate samples of 5 g of biphenyl crystals in 60-ml bottles fitted with septums were placed at 11, 27, and 35 °C. To minimize errors from dilution and adsorption during sampling, 2 ml of air was introduced into the first bottle containing crystals and mixed by pumping the syringe several times. Then 2 ml of the headspace vapors from the first bottle was transferred to the second bottle and, using a clean syringe, a 2-ml sample of headspace vapors was taken for analysis by GC.

RESULTS AND DISCUSSION

Standard Solutions. A comparison of ten analyses of standard biphenyl solutions in ethanol with each instrument indicated that either GC or LC is capable of producing valid results (Table I). The amounts injected were 0.0004 and 0.0005 mg per sample, and the attenuations ×64 and ×32 for GC and LC, respectively. For analysis of liquid extracts of biphenyl, LC had a slight advantage because retention time was shorter: 1 min, 45 s, compared to 6 min, 40 s for GC. Otherwise, the choice between gas or liquid chromatography would depend upon instrument availability because the speed, accuracy, and reliability of the methods were so similar.

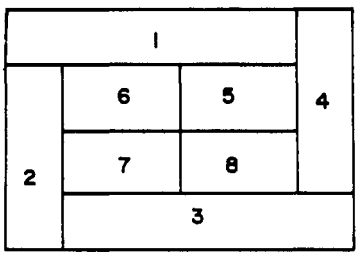

Figure 1. Arbitrary sectioning of commercially impregnated pads for subsequent extraction and analysis.

Table II. Variation of Biphenyl on Sections of Impregnated Pads, Analyzed by Gas and Liquid Chromatography

Section no.	Pad 1			Pad 2		
	Area, cm^2	GC,[a] mg/cm^2	LC,[a] mg/cm^2	Area, cm^2	GC,[a] mg/cm^2	LC,[a] mg/cm^2
1	215	1.87	1.88	204	2.36	2.28
2	120	1.75	1.79	122	2.18	2.05
3	215	1.62	1.63	215	1.83	1.82
4	124	1.78	1.75	124	2.25	2.09
5	104	1.74	1.67	106	2.16	2.32
6	103	1.96	1.99	106	2.09	2.06
7	102	1.52	1.52	106	2.30	2.26
8	104	1.75	1.78	104	2.16	2.12

[a] Each figure an average of two aliquots of each ethanol extract.

Pad Analyses. Biphenyl-impregnated pads have been analyzed by extracting disks from representative areas of the pads. This method may lead to erroneous results because of variability on the pad. To test the uniformity of distribution of biphenyl on commercially impregnated pads, pads were arbitrarily marked and cut as shown in Figure 1. Individual pad areas, ranging from 102 to 215 cm^2, were cut into 4-cm^2 pieces and placed in glass-stoppered Erlenmeyer flasks to which 100 ml of ethanol was then added. Preliminary experiments indicated that a single extraction for 1 h at room temperature yielded 99.8% or more of the biphenyl from commercial pads. The results of typical analyses (Table II) showed that some variation did exist, which might be expected. Although the variation was small, 0.5 mg/cm^2 or less, subsequent pad analyses were conducted on whole pads cut into small pieces and extracted with 500 ml of ethanol. Since pads frequently have eight ventilation holes, this procedure also eliminated errors in determining the exact total area and percentage area extracted.

With the whole-pad extraction method, we found that individual pads obtained from nine different packing-houses, but originally prepared by a single manufacturer, contained from 1.56 to 2.30 g/pad. This variation could have resulted from several factors, such as initial amount impregnated, age, and ventilation and temperature conditions at which pads were stored. Storage temperature had a marked effect on retention of biphenyl on exposed pads (Table III). These results suggest that pads in

Table III. Effect of Temperature on Biphenyl Content of Exposed Pads

		10.0 °C, g/pad	15.5 °C, g/pad	21.0 °C, g/pad
Initial	1.79 g/pad[a]			
After 1 week exposure		0.59	0.20	0.12
After 2 weeks exposure		0.26	0.15	<0.01
After 3 weeks exposure		<0.01	<0.01	<0.01

[a] Each figure an average of two aliquots of ethanol extracts from two pads analyzed by GC.

Table IV. Loss of Biphenyl from Whole Pads during a 4-Week Simulated Grapefruit Shipping Test at 10 °C

Treatment	Pads/box	g/pad[a] LC	GC
Vent holes covered with tape	2	1.19	1.11
	3	1.23	1.31
Vent holes open, covers removed for 2 min each day	2	0.48	0.49
	3	0.51	0.51

[a] Each figure an average of two aliquots of ethanol extracts. Initial concentration was 1.94 g/pad (average of eight pads).

Table V. Analysis of Biphenyl Vapor by Multiple Injection. Samples from Saturated Atmospheres of Bottles Containing Biphenyl Crystals

	11 °C	27 °C	35 °C
No. of analyses	10	10	10
Mean, μg/l.	4.4	52.1	77.0
Std dev	0.43	3.5	3.2
Std error of mean	0.14	1.1	1.0
Coeff of variation	0.10	0.07	0.04
VP ($\times 10^3$; cm Hg)[a]	0.05	0.63	1.38

[a] Calculated from $P = (g/MV)RT$.

packinghouses be kept well wrapped and at low temperatures.

As part of a simulated grapefruit export shipping experiment conducted at this laboratory, pads to be analyzed were removed from boxes that had either two or three pads per box and had either vent holes covered with tape, or open vent holes, and, in addition, were aired by removing the covers for 2 min each day (Table IV). Pads from boxes with covered vent holes retained over one-half the original amount of biphenyl, whereas the others retained about one-fourth.

Vapor Analyses. The measurement of biphenyl vapor was somewhat less precise than measurement of liquid (Table V), but the precision probably is adequate for many biological experiments. The first injection of the vapor sample usually accounted for about 80% of the total (Figure 2), and the response to the fourth injection was usually less than one chart division.

As pointed out by Bradley and Cleasby (1953), there are surprising discrepancies among values in the literature for vapor pressures of aromatic ring compounds. The calculated vapor pressures for biphenyl reported herein are lower than those found by Bradley and Cleasby (1953), near those of Sharma and Palmer (1974), and higher than those of Bright (1951). It was not our intention to establish

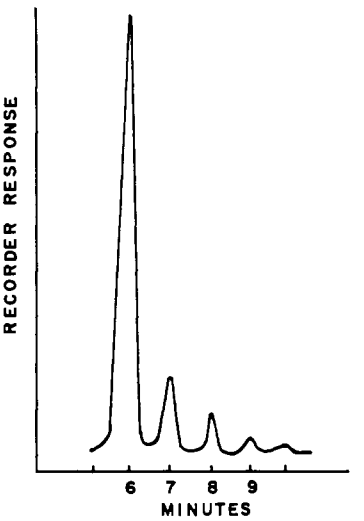

Figure 2. Trace of typical detector response to multiple injection of biphenyl vapor.

exact vapor pressure data, and our calculations were based on atmospheres assumed to be saturated. Because the values are within the range of those previously reported, we feel that the method of multiple injections is suitable for the determination of vapor concentrations as they exist in experimental conditions. The relation of vapor concentration to fungistatic activity has been hampered by difficulties inherent in analysis of vapor samples; however, the method described herein may alleviate these difficulties. In addition, should inhalation of biphenyl or similar aromatic ring compounds of low vapor pressure prove hazardous to humans, atmospheric monitoring, which may be carried out by this new method, may be necessary.

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