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Recieved for review December 13, 1976. Accepted February 25, 1977.

Transfer of Exogenously Applied and Endogenous Alkaloids and Sterols from Tobacco to Its Smoke Condensate

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Exogenously applied alkaloids were transferred from treated cigarette tobacco to its smoke condensates in quantities relative to that of endogenous alkaloids, although there was a linear trend toward reduced transfer percentage as alkaloid levels were increased. In contrast to the alkaloids, exogenously applied sterols were not transferred to the condensate in quantities relative to the endogenous sterols. Transfer percentages were higher for the increased levels of added sterol. The alkaloid × sterol interaction was significant for sterol recovered from the cigarette tobacco and that transferred to the condensate, but not transfer percentage. This would indicate that the added alkaloid resulted in greater quantities of sterol being adhered to the shredded leaf surface. There were no significant differences among treatments for total particulate matter. These results emphasize the need for quantification of chemical constituents in the tobacco and its smoke condensate in chemical additive studies.

The major alkaloids and sterols that occur in the leaf have been identified in tobacco smoke condensate (Stedman, 1968; Tso, 1974). The influence of total alkaloid and smoking variables on their delivery into smoke condensate has been examined more extensively than any other tobacco constituent (Bogen, 1929; Newsome and Keith, 1957; Wynder and Hoffmann, 1967; Kaburaki et al., 1965; Stedman, 1968; Bush et al., 1972; Housemann, 1973; Jenkins et al., 1975). Sterol transfer from the leaf to condensate has been examined by several investigators (Johnstone and Plimmer, 1959; Kallianos et al., 1963; Stedman, 1968; Grunwald et al., 1971; Cheng, 1973; Schmeltz et al., 1975). The information on quantitative transfer of specific compounds needs to be expanded to include exogenously applied compounds. The results would be valuable in determining the effect of changes in levels of leaf components on condensate levels especially in view of the possible use of additives to nontobacco materials. Also, the use of additives could provide a practical means of study for biological activity if levels in condensate could be altered by adding compounds to tobacco prior to smoking. For the latter studies to be meaningful, the transfer of the exogenously applied compounds should be similar to that in the leaf. We studied alkaloids and sterols, major groups of compounds which are present as natural products in tobacco. The major objectives were: (1) to ascertain the influence of exogenously applied alkaloids and sterols on smoke condensate levels of these compounds and (2) to further examine the transfer of endogenous quantities of these two groups of compounds into the smoke condensate.

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MATERIALS AND METHODS

Chemicals. The alkaloids, nicotine (99%) and anabasine (70%) with 30% anatabine contamination, used in this research were obtained from K & K Laboratories, Inc.,

Plainview, N.Y. The sterol reference standards cholesterol (99+%), campesterol (99%), stigmasterol (99.5%), and sitosterol (98+%) were purchased from Applied Science Laboratories, State College, Pa. Cholestane (99%) (Sigma Chemical Co., St. Louis, Mo.) was used as the internal standard for sterol analysis. Sitosterol, campesterol, and cholesterol additives were obtained from Sigma Chemical Co. All solvents used were of reagent grade.

Alkaloid and Sterol Analysis. The cigarette tobacco was ground in a mill equipped with a 40 mesh screen. Constant moisture was achieved and a 1-g sample was extracted with benzene–chloroform (9:1, v/v) for alkaloid analysis. The benzene–chloroform was removed and the extract made up to volume with methyl acetate. Individual alkaloids were quantified by gas chromatography using an acid-washed, dimethyldichlorosilane-treated 60–80 mesh Chromosorb W column coated with 10% DC 550 (Bush et al., 1972). GLC conditions and quantification of individual alkaloids were as described previously (Bush, 1972).

Five-gram samples were weighed directly in a Soxhlet for the sterol determination. Sterols were determined by a gas chromatographic modification (Grunwald, 1970) of the gravimetric method by Stedman and Rusaniwskyj (1959). For total values, the glycosides and esters were hydrolyzed. The sterol–digitonide precipitate was broken using a 12-h treatment with 2 mL of pyridine containing the internal standard, cholestane. Digitonin was precipitated with diethyl ether and component sterols analyzed by gas chromatography. Calculations by computer were conducted using the internal standard for quantification and external standards to correct for flame ionization response.

Cigarette Sample. The tobacco selected for these studies was shredded University of Kentucky 1R1, 2-69 reference cigarette tobacco. This tobacco has as additives 5.5% (w/w) corn syrup and 2.9% (w/w) glycerine. In addition to a reference cigarette check, which was analyzed for alkaloids and sterols, the following five treatments were added to shredded tobacco prior to cigarette manufacture: (1) low alkaloid, 20 mg of nicotine + 0.3 mg of anabasine per g of 1R1 tobacco; (2) high alkaloid, 40 mg of nicotine + 0.6 mg of anabasine per g of 1R1 tobacco; (3) low sterol, 0.5 mg of cholesterol + 1.0 mg of sitosterol + 0.5 mg of campesterol per g of 1R1 tobacco; (4) high sterol, 1.0 mg of cholesterol + 2 mg of sitosterol + 1.0 mg of campesterol per g of 1R1 tobacco; and (5) high alkaloid–high sterol, a combination of treatments 2 and 4. The alkaloids and sterols were applied in aerosol spray with ethyl acetate. The resulting material was conditioned in an Aminco Unit for 48 h at 60% relative humidity and 20 °C prior to being manufactured into cigarettes by the University of Kentucky Cigarette Preparation Lab. A Hauni-Baby (Nr 45, Werke-Kober and Co., Hamburg, West Germany) was used for the cigarette manufacture. The cigarettes were selected for a weight of 1.06 ± 0.04 g and a pressure drop of 6.35 ± 1.5 cm. The cigarettes were conditioned as above for an additional 48 h prior to being smoked.

Smoking and Condensate Collection. For the alkaloid transfer analyses, six replications of five cigarettes from each treatment were smoked on a Philip Morris automated smoking machine to a 23-mm butt length using a standard 35-mL puff of 2-s duration and 1 puff/min. The alkaloids were collected on a Cambridge filter. Total particulate matter (TPM) and puffs per cigarette were determined for the various treatments. Cigarettes used for the quantification of sterol transfer were smoked on a J. Borgwaldt apparatus with the same smoking parameters as for the alkaloids. The smoke condensate was collected directly into an acetone trap and stored in a freezer until analyzed for sterols. The cigarettes were smoked in lots of 30 with the condensate from 60 being collected in each of the three replications.

Statistical Analysis. The alkaloid data were analyzed by a multivariate profile analysis of the four components as described by Morrison (1967) and modified to include tests of five mutually orthogonal comparisons among treatments. These comparisons were: the linear effect of added alkaloid, the quadratic effect of added alkaloid, two increments of added sterol vs. no added sterol, the quadratic effect of added sterol when no alkaloid was added, and the interaction of added alkaloid by added sterol.

The profile analysis was accomplished by carrying out two multivariate analyses of variance, the first with the four-component alkaloids as Y variables and the second three linearly independent contrasts among the component alkaloids as Y variables. The first analysis provides a simultaneous test for treatment effects on any of the four components. In the second analysis, the hypothesis that the mean vector is zero was tested. This is a test for the existence of any differences among component means because the test statistic does not depend upon which set of linearly independent contrasts is chosen. For the same reason, the test of a treatment comparison in the second analysis is a test for interaction of the comparison by components. Since all of the effects tested in these multivariate analyses have only one degree freedom, the significant tests were based on Hotelling's T^2 statistic.

If a significant interaction with components were found for any treatment comparison, the data were subjected to univariate analyses of variance of the components separately as well as for total alkaloids. Due to large differences in the relative magnitudes of the four components, such interactions were expected (and found for at least one comparison) in the tobacco and condensate analyses. However, the major purpose of the profile analyses was to study the effects of treatments on recovery percentage and to determine whether these effects differed for the four components.

The sterol data were analyzed similarly, with the exception that the five comparisons among treatments were redefined by interchanging sterol and alkaloid in the above definitions and the elements of the Y vector in the multivariate analyses were component sterols (or contrasts among them) rather than alkaloids.

RESULTS AND DISCUSSION

Alkaloids. Total alkaloids, nicotine, nornicotine, anabasine, and anatabine levels were determined in reference cigarette tobacco as well as cigarette tobaccos which had exogenously applied alkaloids and sterols and the smoke condensates from these cigarettes. Total alkaloid levels were 21.54, 31.63, and 42.13 mg per g of cigarette tobacco for the reference and low, and high levels of added alkaloids, respectively (Table I). On a milligram per gram of tobacco smoked basis, the corresponding levels in the condensates were 2.41, 3.53, and 3.83 mg for the three treatments. Nicotine, the predominant alkaloid, was at 20.27, 29.86, and 39.52 mg per g in the cigarette tobaccos for these respective treatments and 2.31, 3.40, and 3.72 mg per g of tobacco smoked in their respective condensates. It should be noted that substantial losses in nicotine, a volatile compound, occurred during the addition to the tobacco and cigarette manufacture; however, since the levels of all the cigarettes were determined prior to smoking the actual transfer percentages can be compared.

Table I. Total Alkaloid and Nicotine in Cigarette Tobacco and Its Condensate with a Control and Two Levels of Added Alkaloid and Sterol

		Alkaloids					
		Reference		Low		High	
Sterol level		Total	Nicotine	Total	Nicotine	Total	Nicotine
Reference	Tobacco	21.54[a]	20.27	31.63	29.86	42.13	39.52
	Condensate	2.41[b]	2.31	3.53	3.40	3.83	3.72
	Transfer %	11.18	11.40	11.16	11.38	9.18	9.40
Low	Tobacco	21.83	20.52				
	Condensate	3.14	3.06				
	Transfer %	14.41	14.93				
High	Tobacco	22.74	21.42			40.99	38.34
	Condensate	2.66	2.57			4.53	4.36
	Transfer %	11.70	11.99			11.06	11.40
Average	Tobacco	22.03	20.74	31.63	29.86	41.56	38.93
	Condensate	2.74	2.65	3.53	3.40	4.20	4.04
	Transfer %	12.43	12.77	11.16	11.38	10.12	10.40

[a] Milligrams per gram dry weight. [b] Milligrams per gram of tobacco smoked. Total alkaloid $LSD_{0.05}$ for tobacco = 1.66, for condensate = 0.66, for transfer % = 2.07. Nicotine $LSD_{0.05}$ for tobacco = 1.62, for condensate = 0.63, for transfer % = 2.09.

Table II. Nornicotine, Anabasine, and Anatabine in Cigarette Tobacco and Its Smoke Condensate with a Control and Two Levels of Alkaloid and Sterol

		Alkaloids								
		Reference			Low			High		
Sterol level		Nornic.	Anab.	Anat.	Nornic.	Anab.	Anat.	Nornic.	Anab.	Anat.
Reference	Tobacco	0.612[a]	0.114	0.536	0.705	0.305	0.767	1.120	0.540	0.954
	Condensate	0.073[b]	0.007	0.018	0.096	0.013	0.025	0.104	0.022	0.027
	Transfer %	11.90	6.13	3.36	13.79	4.29	3.22	9.33	4.14	2.79
Low	Tobacco	0.649	0.100	0.558						
	Condensate	0.061	0.007	0.018						
	Transfer %	9.37	3.39	3.29						
High	Tobacco	0.631	0.108	0.580				1.123	0.567	0.958
	Condensate	0.067	0.004	0.021				0.108	0.025	0.029
	Transfer %	10.86	3.39	3.60				9.75	4.47	3.06
Average	Tobacco	0.631	0.107	0.558	0.705	0.305	0.767	1.121	0.554	0.956
	Condensate	0.067	0.006	0.019	0.096	0.013	0.025	0.106	0.024	0.028
	Transfer %	10.71	4.30	3.42	13.79	4.29	3.22	9.54	4.31	2.93

[a] Milligrams per gram dry weight. [b] Milligrams per gram of tobacco smoked. Nornicotine $LSD_{0.05}$ for tobacco = 0.128, for condensate = 0.035, for transfer % = 4.81. Anabasine $LSD_{0.05}$ for tobacco = 0.030, for condensate = 0.004, for transfer % = 2.13. Anatabine $LSD_{0.05}$ for tobacco = 0.088, for condensate = 0.008, for transfer % = 0.94.

The average number of puffs per cigarette was 9.1 with only slight differences among treatments. Also, there was no significant difference for TPM which averaged 29.51 mg per cigarette. There was a trend for treatments with higher levels of added sterols and alkaloids to have higher TPM values. The transfer of nicotine from the reference cigarette sample was slightly lower than previously reported for similar smoking conditions (Bush et al., 1972). In the previous report the cigarettes were manufactured by a commercial company, whereas the cigarettes for the present study were manufactured on a Hauni-Baby machine. Nornicotine, anabasine, and anatabine occurred in much smaller quantities than nicotine (Table II). In general, the transfer percentages were lower for anabasine and anatabine than for the other two alkaloids. The transfer of anabasine was much lower compared to the previous report. Anatabine had the lowest transfer percentage of the alkaloids in both studies.

The addition of a higher level (4 mg/g) of sterol did not significantly alter the total alkaloid in the leaf or in the condensate (Table I). Also, the alkaloid transfer percentage was not influenced by 4 mg/g added sterol. However, the multivariate analysis did indicate a quadratic effect of added sterol on the alkaloid transfer percentage when no alkaloid was added. The univariate analyses indicated this was due mainly to an increased level of transferred nicotine at the low level of added sterol (Table I).

There was a highly significant linear effect of added alkaloid for the tobacco and condensate samples and a significant linear effect on alkaloid recovery percentage. The univariate analyses showed a highly significant linear effect, for nicotine, nornicotine, anabasine, and anatabine in the tobacco and condensate. Added nicotine resulted in a linear trend toward lower nicotine transfer percentage. This may occur as a result of a larger proportion of the added and possibly more volatile nicotine going into the sidestream and less into the mainstream or greater pyrolysis of the added nicotine. Housemann (1973), using ^{14}C-labeled nicotine, found a high percentage of the ^{14}C activity in the sidestream TPM.

The quadratic effect of added alkaloid was found to be significant only for the nornicotine and anabasine in the cigarette tobacco. When high alkaloid vs. no added alkaloid was compared, it was found that a significant difference occurred for nicotine in the condensate. In the multivariate analysis the alkaloid × sterol interaction was not significant; however, the univariate tests indicated significant alkaloid × sterol interaction effects on nicotine in the tobacco and anabasine in the condensate and transfer percentage.

These results would indicate that the total quantity of exogenously applied alkaloids is transferred to the smoke condensate in a manner similar to the endogenous alkaloids; however, at high levels there would be a reduction in transfer percentages and there may be variation in the

Table III. Total Sterol and Sitosterol in Cigarette Tobacco and Its Smoke Condensate with a Control and Two Levels of Added Alkaloid and Sterol

		Sterols					
		Reference		Low		High	
Alkaloid level		Total	Sitosterol	Total	Sitosterol	Total	Sitosterol
Reference	Tobacco	1.948[a]	0.829	2.992	1.352	3.816	1.786
	Condensate	0.237[b]	0.090	0.375	0.162	0.517	0.233
	Transfer %	12.20	10.92	12.58	12.08	13.56	13.09
Low	Tobacco	1.874	0.698				
	Condensate	0.211	0.079				
	Transfer %	11.43	11.63				
High	Tobacco	1.840	0.736			4.369	2.084
	Condensate	0.230	0.90			0.567	0.257
	Transfer %	12.59	12.27			13.00	12.37
Average	Tobacco	1.887	0.754	2.992	1.352	4.093	1.935
	Condensate	0.226	0.086	0.375	0.162	0.542	0.245
	Transfer %	12.07	11.61	12.58	12.08	13.28	12.73

[a] Milligrams per gram dry weight. [b] Milligrams per gram of tobacco smoked. Total sterol $LSD_{0.05}$ for tobacco = 0.307, for condensate = 0.048, for transfer % = 3.08. Sitosterol $LSD_{0.05}$ for tobacco = 0.188, for condensate = 0.028, for transfer % = 3.06.

Table IV. Cholesterol, Campesterol, and Stigmasterol in Cigarette Tobacco and Its Smoke Condensate with a Control and Two Levels of Alkaloid and Sterol

		Sterols								
		Reference			Low			High		
Alkaloid level		Chol.	Camp.	Stig.	Chol.	Camp.	Stig.	Chol.	Camp.	Stig.
Reference	Tobacco	0.179[a]	0.309	0.632	0.390	0.537	0.714	0.553	0.755	0.723
	Condensate	0.023[b]	0.039	0.085	0.051	0.072	0.089	0.082	0.108	0.094
	Transfer %	13.04	12.64	13.48	13.15	13.61	12.54	14.84	14.30	13.02
Low	Tobacco	0.198	0.358	0.620						
	Condensate	0.022	0.036	0.074						
	Transfer %	11.36	10.60	12.09						
High	Tobacco	0.190	0.321	0.593				0.702	0.937	0.646
	Condensate	0.023	0.039	0.079				0.095	0.133	0.081
	Transfer %	12.13	12.23	13.34				13.53	14.25	12.70
Average	Tobacco	0.189	0.329	0.615	0.390	0.537	0.714	0.628	0.846	0.685
	Condensate	0.022	0.038	0.079	0.051	0.072	0.089	0.089	0.121	0.088
	Transfer %	12.18	11.82	12.97	13.15	13.61	12.54	14.19	14.28	12.86

[a] Milligrams per gram dry weight. [b] Milligrams per gram of tobacco smoked. Cholesterol $LSD_{0.05}$ for tobacco = 0.032, for condensate = 0.007, for transfer % = 2.27. Campesterol $LSD_{0.05}$ for tobacco = 0.087, for condensate = 0.008, for transfer % = 3.60. Stigmasterol $LSD_{0.05}$ for tobacco = 0.085, for condensate = 0.001, for transfer % = 3.12.

transfer of individual alkaloids. Also, the addition of other compounds such as sterols might influence the levels of individual alkaloids in the smoke condensate. This emphasizes the need to do a chemical analysis of the condensate as well as of the tobacco leaf, if such additives are used in studies to test biological activity of selected compounds.

Sterols. Total sterol levels were 1.95, 2.99, and 3.82 mg/g of cigarette tobacco for the reference and low and high level treatments, respectively (Table III). On the basis of milligrams of sterol transferred per gram of tobacco smoked, the corresponding levels in the condensates were 0.24, 0.38, and 0.52 mg/g for these three treatments. Sitosterol, stigmasterol, campesterol, and cholesterol accounted for 0.83, 0.63, 0.31, and 0.18 mg/g of untreated reference cigarette tobacco, respectively (Tables III and IV).

Transfer percentages from the reference tobacco to smoke condensate were 10.9, 13.5, 12.6, and 13.4 for sitosterol, stigmasterol, campesterol, and cholesterol, respectively. These percentages are similar to those previously reported (Grunwald et al., 1971) with the exception of a lower transfer for stigmasterol. As expected, the high level of added alkaloid did not significantly alter the sterol extracted from the tobacco that had no sterol added, nor did it significantly influence the condensate levels or transfer percentages for the endogenous sterols (Tables III and IV). However, when the high added sterol plus alkaloid and high added sterol treatments were compared, the high added sterol plus alkaloid sterol level was significantly greater than for high added sterol for all sterols except stigmasterol, a sterol not included in the added sterols except as a trace contaminant. Condensate levels of total sterol, cholesterol, campesterol, and stigmasterol were also influenced by the added alkaloid in the presence of added sterol. The condensate level of stigmasterol was lowered by added alkaloid. When the sterol profile analysis was examined, a significant linear effect was found for the added sterol for the tobacco condensate and transfer percentage. There was a quadratic effect of added sterol for the condensate. This resulted from the transfer of campesterol. The alkaloid × sterol interaction was significant for both the sterol recovered from the cigarette tobacco and that transferred to the condensate. This was a result of the added alkaloid causing the sterols to adhere in greater quantities to the shredded leaf surface. The sterols differed significantly in their transfer percentages (Tables III and IV). Transfer percentages increased with increasing levels of the nonvolatile sterols, which is in contrast to the volatile alkaloids. Schmeltz et al. (1975), using a ^{14}C-labeled internal standard, found about 20% of the leaf sterol was transferred as sterol to the smoke and that the sidestream smoke contained lesser amounts of sterol than did the mainstream whereas, nicotine was reported to be transferred predominately to the sidestream smoke (Housemann, 1973). The multivariate test for

interaction of linear effect of added sterol × sterols analyzed was not quite significant at the 5% level. These results indicate that exogenously applied sterols are not transferred to the smoke condensate in the same quantities as endogenous sterols and that other added compounds such as alkaloids influence the amount recovered from the tobacco leaf, as well as that transferred to the condensate.

ACKNOWLEDGMENT

This investigation (No. 76-3-134) is in connection with a project of the Kentucky Agricultural Experiment Station and is published with the approval of the Director.

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Received for review August 31, 1976. Accepted March 3, 1977. Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable. The work was supported by U.S. Department of Agriculture–Agricultural Research Service Contracts 12-14-100-100317(34) and 12-14-100-11041(34).

Determination of Methyl Anthranilate in Grape Beverages by High-Pressure Liquid Chromatography and Fluorescence

Alun T. Rhys Williams and Walter Slavin

Methyl anthranilate has been determined to less than 0.05 μg/mL in grape beverages with no sample preparation beyond dilution. Using a fluorescence detector to provide very high sensitivity and selectivity for methyl anthranilate, the other products in commercial grape beverages produced no interferences in the method. The fluorescence detector was also shown to be useful in identifying other compounds in the samples. As little as 300 pg of methyl anthranilate was detected by this method.

Methyl anthranilate, a methyl ester of *o*-aminobenzoic acid, is the principal flavorant of Concord grape beverage products. A very recent AOAC method uses steam extraction, finishing with fluorescence (Casimir et al., 1976). In this paper we have applied reversed-phase liquid chromatography to the separation of methyl anthranilate from the other compounds in the commerical grape beverages. To enhance the sensitivity for this compound and provide selectivity from the many other compounds available in the commercial products, we have used a new fluorescence detector (Slavin et al., 1977).

We believe that considerable analytical time is saved by use of this method compared with any of the current procedures for methyl anthranilate because no sample preparation is required. In addition, this procedure appears to be more sensitive than the method of Casimir et al. (1976).

METHOD AND EQUIPMENT

The Perkin-Elmer Model 601 liquid chromatograph was used with a reversed-phase, ODS Sil-X-1 column used in an isocratic mode. The Perkin-Elmer Model LC-1000 was used as the fluorescence detector. A Model LC-55 variable-wavelength UV detector was coupled between the fluorescence detector and the column to provide a general indication of the presence of peaks that could not be detected by fluorescence. Model 56 recorders were used with both detectors. The Perkin-Elmer Model 200 UV spectrophotometer was used to determine the optimum UV absorbance.

Table I. Chromatographic Conditions

Column	ODS Sil-X-1, 0.26 × 25 cm
Mobile phase	3% acetonitrile in water, buffered to pH 6 in 35 mM PO_4, 2 mL/min
Temperature	60 °C
Detectors	LC-1000 Fluorescence Detector Excitation: 330 nm Emission: 430 nm X50M, 5mV, Fast LC-55 Variable Wavelength UV 217 nm, 0.02 AUFS

In Table I we show the general chromatographic conditions that were used for this analysis. About 3% acetonitrile is necessary in the aqueous eluent. The desired concentration of acetonitrile was achieved by using 10% acetonitrile in pump A and water in pump B. The mixing dial was set to 30%. This was done because it was occasionally useful to vary the acetonitrile content. The

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