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Factors Affecting the Concentration of Pyrazines in Cocoa Beans

Gary A. Reineccius,[1] Philip G. Keeney,* and Wendy Weissberger

Glc of the pyrazine fraction from roasted cocoa beans yielded nine well resolved peaks which could be quantitated. When beans from several producing countries were roasted under identical conditions, pyrazines generated varied between 142 μg/100 g of beans and 698 μg/100 g. The potential for generating pyrazines was greatest in samples from countries where beans are traditionally fermented. Tetramethylpyrazine, trimethylpyrazine, and pyrazines under a peak representing a mixture of 2-ethylpyrazine, 2,5-dimethylpyrazine, and 2,6-dimethylpyrazine were present in the highest concentrations. In fermented cocoa beans, pyrazine concentration increased rapidly during roasting to a near maximum value which did not change during extended roasting. Results indicate that fermentation influences the rate of formation and final concentration of pyrazines in roasted beans primarily through its effect on the free sugars. Ketoses dominated the sugars fraction (62% of total) in well fermented beans compared to 21% in nonfermented varieties. Tetramethylpyrazine was the only pyrazine detected in unroasted beans and then only in fermented samples.

Following the early patent reference (Reichstein and Staudinger, 1928) to pyrazines in coffee, Davison and Wiggens (1956) discovered pyrazine and pyridine bases in an abnormally treated batch of ammoniated molasses. Eight years later Dietrich *et al.* (1964) found 2,6-dimethylpyrazine and tetramethylpyrazine in chocolate liquor. Shortly thereafter pyrazines were identified in coffee (Reymond *et al.*, 1966; Viani *et al.*, 1965), potato chips (Deck and Chang, 1965), and peanuts (Mason *et al.*, 1966). This class of compounds is now recognized as an important contributor to flavor in certain highly heated food systems, especially when processing involves roasting (chocolate, coffee, and nut products). Since the original discovery, 30 pyrazines have been identified in chocolate. These include: Dietrich *et al.* (1964): 2,6-dimethyl; tetramethyl. Rizzi (1967): methyl; 2,3-dimethyl; 2,5-dimethyl; 2-methyl-5-ethyl; trimethyl; 2,5-dimethyl-3-ethyl; 2,6-dimethyl-3-ethyl. Marion *et al.* (1967): 2-methyl-6-ethyl. Flament *et al.* (1967): 2,5-dimethyl-3-propyl; 2,5-dimethyl-3-isoamyl; 2,3-dimethyl-5-isoamyl; 2,3-dimethyl-5-(2-methylbutyl); 2-ethyl-3,5,6-trimethyl; 2-isoamyl-3,5,6-trimethyl; 2-(2-methylbutyl)-3,5,6-trimethyl. van Praag *et al.* (1968): ethyl; 2,3-dimethyl-6-ethyl. van der Wal *et al.* (1968): 2-methyl-6-isoamyl; 2-methyl-6-(2-methylbutyl); 2,5-dimethyl-3,6-diethyl; 2,6-dimethyl-3,5-diethyl; 2,6-dimethyl-3-isoamyl. van der Wal *et al.* (1971): isopropyl; 2,5-diethyl; 2-methyl-6-(3-methylbutyl); 2,5-dimethyl-3-isobutyl; 2,5-dimethyl-3-(2-methylbutyl); 2,5-dimethyl-3-(3-methylbutyl); 2,6-dimethyl-3-(3-methylbutyl).

While a large number of pyrazines have been identified in food products, critical quantitative information is almost nonexistent. The only published study is that of Müggler-Chavan and Reymond (1967) who employed a peak ratio technique to reveal differences among several varieties of cocoa beans. This was strictly a comparison involving glc peak areas, and actual concentration data were not collected.

In the pyrazine study reported herein, quantitative aspects have been emphasized, especially as they relate to the different sources of cocoa beans, the fermentation process, and roasting practices.

EXPERIMENTAL

Source of Cocoa Beans. Cocoa beans from the major producing countries were supplied by several chocolate manufacturers. Included were beans from Brazil (Bahia), Ghana, Ecuador (Arriba), Mexico (Tabasco), the Dominican Republic (Sanchez), and Samoa.

Pyrazine Analysis. Cocoa beans (30 g) and 20 g of Celite 545 were pulverized 5 min in a Waring Blendor. This mixture

Division of Food Science and Industry, The Pennsylvania State University, University Park, Pennsylvania 16802.
[1] Present address: Department of Food Science and Industries, University of Minnesota, St. Paul, Minnesota 55101.

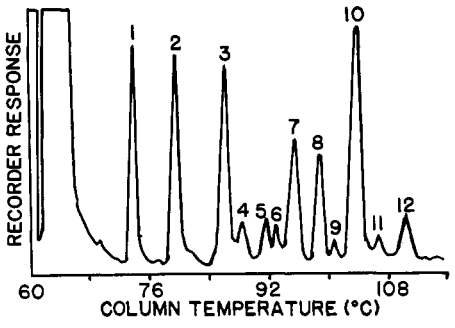

Figure 1. Gas chromatographic separation (adipate column) of alkylpyrazines in the basic fraction of Ghana cocoa beans (roasted at 150°C for 30 min). Key: 1) pyrazine (internal standard); 2) acetoin and methylpyrazine; 3) mixture of 2,5-dimethyl, 2,6-dimethyl, and ethyl pyrazines; 4) 2,3-dimethylpyrazine; 5) 2-ethyl-6-methylpyrazine; 6) 2-ethyl-5-methylpyrazine; 7) trimethylpyrazine; 8) 2,5-dimethyl-3-ethylpyrazine; 9) 2,3-dimethyl-6-ethylpyrazine; 10) tetramethylpyrazine; 11) and 12) contamination

Table I. Recovery of Pyrazines from Diethyl Ether

Quantity added, μg	Quantity recovered, μg		
	Methyl-pyrazine	Dimethyl-pyrazine	Tetramethyl-pyrazine
20	18	23	22
	23	23	18
40	38	44	40
	40	39	36
60	60	61	62
	60	58	57
80	78	82	80
	93	91	93
100	102	98	106
	105	100	101

was loosely packed into a $1^1/_{16}$-in. × 21.5-in. glass chromatographic column and extracted with 175 ml of distilled diethyl ether. The eluate was transferred to a 250-ml separatory funnel and 100 μg of pyrazine (internal standard) was added. The ether phase was extracted five times with 20-ml portions of pH 1.0 water (100 g of NaCl and 6 ml of concentrated HCl/l. of water). The aqueous extracts were pooled and washed twice with 40 ml of ethyl ether to free the extract of nonbasic organic material. After adjustment to pH 8.3 with 1 *N* KOH to make the pyrazines water-insoluble, the aqueous phase was extracted five times with 20-ml portions of distilled dichloromethane. The dichloromethane extract, which contained primarily basic compounds, was dried over anhydrous $MgSO_4$ and concentrated under nitrogen to about 150 μl.

A 2-μl aliquot of the basic fraction was analyzed for pyrazines using a Hewlett-Packard Model 5750B gas chromatograph equipped with a hydrogen flame detector. Two columns were used in this study: a 10 ft × $^1/_{16}$-in. i.d. stainless steel column packed with 15% Carbowax 20M on 80/100 mesh Gas Chrom Z, and an 8-ft × $^1/_{16}$-in. stainless steel column packed with 10% diethyleneglycol adipate (2% H_3PO_4) on 80/100 mesh Gas Chrom A. Both columns were temperature programmed from 60 to 190°C at 2°C per min using nitrogen as the carrier gas (30 ml/min). Injection port and detector temperatures were 280 and 260°C, respectively.

While the adipate column provided the best overall separation of pyrazine homologs, it failed to separate acetoin (a constituent of raw and roasted cocoa) from methylpyrazine. However, they could be separated on the Carbowax column which was employed specifically for determining the quantity of methylpyrazine in the basic extract.

Pyrazines were identified from mass spectral data and glc retention times in comparison to authentic reference samples and the literature (Bondarovich *et al.*, 1967; van Praag *et al.*, 1968). Mass spectra were obtained using an LKB Model 9000 combined gas chromatograph–mass spectrometer. Pyrazine homologs were separated on a 6-ft × $^1/_8$-in. i.d. glass column packed with the above described DEGA material. Ion source and separator temperatures were 290 and 220°C, respectively. Helium was the carrier gas (25 ml/min).

Quantitation of the pyrazines present in cocoa beans was accomplished using glc. A standard curve of peak area (relative to pyrazine, the internal standard) *vs.* concentration was derived by adding 100 μg of internal standard and from 20 to 100 μg of each of the available reference pyrazines to 120 ml of diethyl ether. The pyrazines were isolated from the ethyl ether solution and analyzed by the described procedure for cocoa beans. A standard curve was constructed which related concentration of several pyrazines to the internal standard and was used to calculate the quantity of each pyrazine in ether extracts of cocoa beans.

Analyses for Free Sugars. Procedures used in the analysis of free sugars were those of Reineccius *et al.* (1972). The sugars were isolated by grinding whole beans with water:methanol (80:20), precipitating the polyphenols with lead acetate, removing contaminating substances by ion exchange, and then freeze-dehydrating the sample. The sugars residue was silylated prior to glc analysis.

Amino Acid Analysis. Free amino acids were extracted from cocoa beans by the method of Rohan and Stewart (1966). Qualitative and quantitative data were obtained using a Beckman Model 120C amino acid analyzer.

RESULTS AND DISCUSSION

Recovery of Pyrazines. Following the exact procedures described for the isolation of pyrazines from cocoa beans, solutions containing 100 μg each of pyrazine, 2-methylpyrazine, dimethylpyrazine, and tetramethylpyrazine in 120 ml of diethyl ether were used to test for recovery efficiency. Less than 5 μg of each pyrazine remained in the pooled diethyl ether phases after extraction with pH 1.0 water. A continuous ethyl ether extraction of the pH 8.3 aqueous phase after removal of the pyrazines with dichloromethane yielded equally small amounts of each pyrazine. Extraction losses, concentrating and drying, and the repeated transfers involved in the procedure resulted in an overall loss of approximately 30% of the pyrazine internal standard, 20% of the methylpyrazine, and 15% of other pyrazine standards.

The precision of the procedures employed was determined using solutions which contained variable amounts of selected pyrazine standards. Results are presented in Table I. Based on the amount added, values varied ±15% for the recovery of 20 μg of standards to ±6% for 100 μg of standards.

Identification of Pyrazines in Roasted Cocoa Beans. A typical chromatogram (adipate column) of the pyrazine fraction recovered from roasted cocoa beans is presented in Figure 1. Peaks 2 through 10 represent the pyrazines isolated from the beans, peak 1 is the internal standard, and peaks 11 and 12 are nonpyrazine contaminants. Each compound identified in the pyrazine fraction had been found previously in cocoa beans and its products by other investigators. Mass spectral scanning revealed that peaks 4 through 10 were relatively pure in respect to the assigned pyrazine homolog. Peak 3 was a mixture of 2,5-dimethyl, 2,6-dimethyl, and ethyl

Table II. Concentrations of Pyrazines in Several Varieties of Roasted Cocoa Beans[a]

Alkylpyrazine	Variety					
	Ghana[b]	Bahia[b]	Samoa[c]	Arriba[b]	Sanchez[c]	Tabasco[c]
	Relative %					
Methyl	7.1	5.4	7.5	10.0	10.3	36.0
Dimethyl and ethyl[d]	22.3	14.5	19.0	25.2	35.2	36.0
2,3-Dimethyl	5.3	4.9	6.7	8.8	6.0	7.0
2-Ethyl-6-methyl	3.5	2.0	3.9	3.8	3.0	2.1
2-Ethyl-5-methyl	4.4	2.6	3.9	3.8	3.0	2.1
Trimethyl	14.2	17.0	14.2	14.3	16.3	4.9
2,5-Dimethyl-3-ethyl	7.8	4.4	7.5	7.1	7.3	7.0
2,3-Dimethyl-6-ethyl	2.4	3.2	1.9	2.1	1.3	0.0
Tetramethyl	32.8	45.8	35.2	24.8	17.6	4.9
Total (μg/100 g)	698	587	358	238	233	142

[a] Roasted at 150°C for 30 min. [b] Average of three lots. [c] Average of two lots. [d] Mixture of 2,5-dimethyl, 2,6-dimethyl, and 2-ethyl.

Table III. Concentrations of Pyrazines in Roasted Cocoa Beans—Different Lots of Same Variety[a]

Alkylpyrazine	Ghana			Bahia			Arriba		
Lot	1	2	3	1	2	3	1	2	3
	Relative %								
Methyl	6.8	4.8	9.5	5.0	2.6	1.9	7.5	5.6	22.1
Dimethyl and ethyl[b]	35.0	18.1	25.0	21.4	19.3	8.2	22.5	32.5	27.2
2,3-Dimethyl	5.6	4.8	5.5	6.0	5.0	3.6	8.2	7.9	12.5
2-Ethyl-6-methyl	4.0	1.2	5.0	2.5	3.1	1.3	3.1	5.5	5.1
2-Ethyl-5-methyl	4.8	3.0	5.0	2.5	5.0	1.3	3.1	5.5	5.1
Trimethyl	16.0	14.5	11.6	15.4	16.9	20.6	15.7	15.9	7.3
2,5-Dimethyl-3-ethyl	8.0	6.0	9.5	6.0	5.0	3.6	6.8	5.5	9.7
2,3-Dimethyl-6-ethyl	3.2	1.2	2.3	1.8	3.1	4.6	3.1	0.0	0.0
Tetramethyl	28.4	46.3	26.6	39.4	39.4	55.0	30.0	21.4	9.7
Total (μg/100 g)	850	564	613	544	592	744	450	126	136

[a] Roasted at 150°C for 30 min. [b] Mixture of 2,5-dimethyl, 2,6-dimethyl, and 2-ethyl.

pyrazines, while peak 2 contained acetoin in addition to methylpyrazine.

Influence of Cocoa Bean Variety upon Pyrazine Concentration after Roasting. Pyrazine formation during roasting varied, depending upon the geographical origin of the cocoa beans. Of special significance is the finding that substantially more pyrazines were formed during roasting of the traditionally well-fermented beans (Ghana and Bahia) than with the lightly fermented (Arriba) and unfermented (Sanchez and Tabasco) varieties (Table II). The same pyrazines were present in all samples, but in different proportions. Major quantitative differences involved primarily the dimethyl, trimethyl, and tetramethyl pyrazine peaks.

Pyrazine content after roasting varied considerably, even among lots of the same type of bean (Table III). These differences reflect the variableness of fermentation processes upon the production of reducing sugars (Rohan and Stewart, 1967a; Reineccius *et al.*, 1972), and free amino acids (Rohan and Stewart, 1967b), the suspected precursors of pyrazines.

Influence of Roasting Time Upon Pyrazine Formation. As revealed in Figure 2 the pyrazines in Ghana cocoa beans were generated quite rapidly and linearly during the first 30 min of roasting at 150°C. The rate of accumulation of the pyrazines was much less thereafter, indicating a depletion of precursors or their involvement in other reactions, or a volatilization of pyrazines nearly equal to their rate of formation.

The effect of roasting temperature on the formation of alkylpyrazines was also investigated. Ghana beans yielded 80, 150, 280, and 710 μg of pyrazines per 100 g when roasted for 30 min at 70, 100, 125, and 150°C, respectively. This is in marked contrast to the model systems study of Koehler and Odell (1970) in which essentially no pyrazine compounds were formed at temperatures below 100°C, and at temperatures up to 150°C many hours of heating were required before alkylpyrazine concentration reached a maximum. In their study, the specific pyrazine produced and its yield varied, depending on the carbon and nitrogen source and on pH. This indicates that in a complex natural product such as cocoa beans these and other variables, both chemical and physical, influence the rate at which the pyrazines accumulate during roasting.

Of particular interest was the discovery of trace amounts (~20 μg/100 g) of tetramethylpyrazine in unroasted Ghana beans. This prompted a survey to determine the frequency of the occurrence of pyrazines in unroasted samples. Tetra-

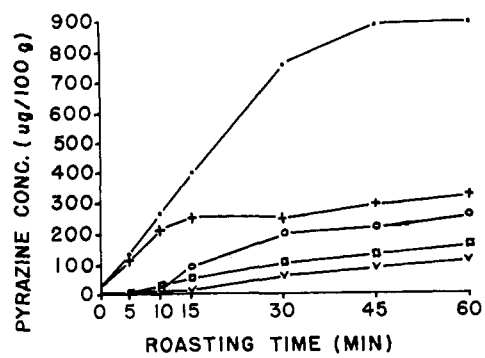

Figure 2. Influence of roasting time upon the formation of pyrazines in Ghana cocoa beans (150°C). ●—●, total pyrazines; +—+, tetramethylpyrazine; □—□, trimethylpyrazine; ○—○, dimethylpyrazine; V—V, methylpyrazine

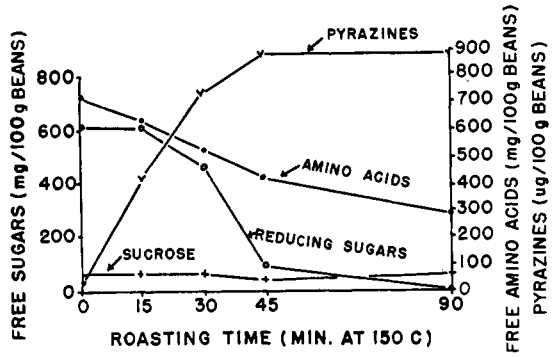

Figure 3. Relationship between pyrazine formation and the destruction of free amino acids and sugars during roasting of Ghana cocoa beans

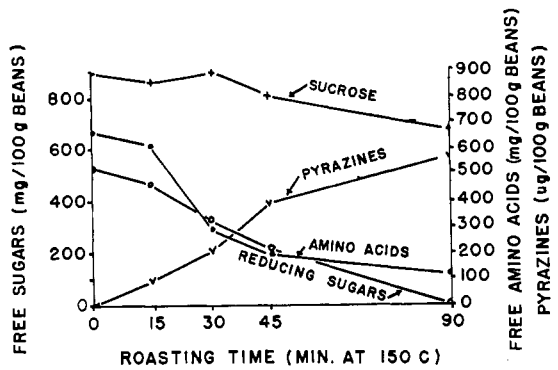

Figure 4. Relationship between pyrazine formation and the destruction of free amino acids and sugars during roasting of Sanchez cocoa beans

methylpyrazine was found in all well-fermented beans but was absent in the lightly and nonfermented varieties (Arriba, Sanchez, and Tabasco). No other pyrazine was detected in unroasted cocoa beans.

The tetramethylpyrazine in fermented beans might arise during fermentation through thermally initiated reactions or by microbial synthesis. It was readily formed during roasting at 150°C (Figure 2) and it accounted for almost all of the alkylpyrazine content of beans roasted for 30 min at 70°C. Since the center of a fermenting mass of beans may reach 50°C (Rohan, 1963), heat generation of tetramethylpyrazine would seem plausible.

Microbial formation of tetramethylpyrazine in fermented beans is even more likely. Kosuge and Kamiya (1962) found tetramethylpyrazine as a metabolic product of *Bacillus subtilis* grown on certain types of media. Of significance is the fact that Ostovar (1971) identified several species of this organism in a fermenting mass of Trinidad cocoa beans.

Relationship between Pyrazine Precursors and Pyrazine Production. The data presented graphically in Figures 3 and 4 represent the relationship found between the consumption of assumed precursors (sugars and amino acids) and the production of total pyrazines in well-fermented Ghana and unfermented Sanchez cocoa beans. Only a small portion of the amino acids and sugars consumed during roasting ended up as pyrazines (~0.2%). For example, in Ghana beans 190 mg of reducing sugars and 200 mg of free amino acids were destroyed during a 30-min roast at 150°C, but only 720 μg of pyrazines were generated.

Analyses of Ghana cocoa beans removed from the roaster after various periods of time at 150°C revealed that pyrazine concentration increased rapidly during roasting to a near maximum value which remained relatively unchanged during the remainder of the roasting period (Figure 3). Pyrazines in Sanchez beans were generated much more slowly and never reached the concentration developed in the Ghana sample even after 90 min of roasting (Figure 4).

As expected, both the reducing sugars and the free amino acids, especially the former, decreased in concentration during roasting. Since free amino acids were still present in Ghana (286 mg/100 g) and Sanchez (135 mg/100 g) beans even after prolonged heating, their depletion would not be the factor limiting the amount of pyrazines produced.

Data concerning changes in individual amino acids during roasting are presented in Table IV. The quantity of free amino acids consumed by roasting Sanchez beans for 30 min was 84% greater than in Ghana beans, but only one-third as many pyrazines were produced. This is a further indication that factors other than amino acid content limit the amount of pyrazines that can be generated. Changes in individual amino acids during roasting were quite similar to those reported by Pinto and Chichester (1966).

As revealed in Figures 3 and 4, the reducing sugars were consumed during roasting at a rate roughly paralleled to the buildup of pyrazines. However, the curves for the reducing sugars and pyrazines in Ghana beans were quite different from those obtained for the Sanchez beans, even though both samples had about the same reducing sugar content prior to

Table IV. Consumption of Free Amino Acids in Ghana and Sanchez Cocoa Beans During Roasting for 30 Min at 150°C

	Ghana			Sanchez		
Amino acid	Unroasted, mg/100 g	Consumed, mg/100 g	%	Unroasted, mg/100 g	Consumed, mg/100 g	%
Lysine	58.3	21.9	48	31.9	18.4	58
Histidine	7.6	1.2	16	6.4	1.1	17
Arginine	41.3	11.8	29	34.3	22.7	66
Aspartic acid	55.2	15.6	28	26.2	10.9	42
Threonine	21.7	6.9	32	26.9	14.2	52
Serine	55.6	20.4	37	41.7	19.5	47
Glutamic acid	64.0	32.6	51	43.4	29.6	68
Proline	32.2	7.3	23	43.4	11.4	26
Glycine	7.9	0.4	5	11.9	6.4	54
Alanine	57.0	14.4	25	65.4	30.6	47
Valine	46.2	10.3	22	57.5	29.2	51
Isoleucine	30.1	4.3	14	29.5	6.8	22
Leucine	103.5	29.9	29	108.1	72.0	67
Tyrosine	43.4	7.5	17	65.7	39.9	61
Phenylalanine	95.7	22.1	23	88.7	61.2	69
Total	719.7	199.3	26	674.6	367.5	55

Table V. Effect of Roasting on the Composition of the Sugars Fraction of Cocoa Beans

	Sanchez			Ghana		
		Time at 150°C			Time at 150°C	
Sugar	Raw	30 min	45 min	Raw	30 min	45 min
	Percentage composition of sugars fraction					
Pentitol	1.3	1.4	1.8	2.4	3.5	10.0
Fructose	17.5	14.7	12.8	57.0	52.5	22.2
Sorbose	1.3	0.7	0.0	7.6	5.2	3.4
Glucose	15.3	9.9	7.0	9.2	10.1	10.1
Mannitol	1.5	3.5	4.1	14.0	14.6	34.5
Unknown	1.3	1.4	1.0	1.2	1.2	2.7
Inositol	1.5	2.1	1.8	2.8	2.1	3.4
Sucrose	60.7	66.2	73.2	7.1	10.5	20.1

roasting. Pertinent to the subject is the work of Reineccius *et al.* (1972) who reported substantially higher concentrations of ketoses in unroasted, fermented Ghana beans compared to the unfermented Sanchez variety.

The percentage compositions of the free sugars fractions of Ghana and Sanchez cocoa beans roasted for varying periods of time at 150°C are recorded in Table V. Ketoses dominated the sugars fraction of Ghana beans and were responsible for most of the total weight of sugars consumed during roasting. This is in contrast to the Sanchez sample in which glucose and fructose were present in comparable amounts after various periods of roasting. Extrapolation of the quantitative data used in Figures 3 and 4 indicated that Ghana beans contained 80% more fructose and sorbose than Sanchez beans, but total sugars was 50% less. Kato *et al.* (1969) reported that the ketoses were about three times more reactive than aldoses in forming volatiles, and Koehler and Odell (1970) obtained a greater yield of pyrazines in heated model systems when fructose was substituted for glucose. The greater reactivity of the ketoses might account for the more rapid and greater generation of pyrazines in fermented Ghana beans.

Hydrolysis of sucrose during roasting was negligible. This indicates that the sucrose concentration in unroasted cocoa beans is not an important variable in the generation of pyrazines during roasting. The rapid and nearly complete hydrolysis of sucrose during the roasting of coffee (Feldman *et al.*, 1969), negligible hydrolysis in roasted peanuts (Newell *et al.*, 1967), and our finding of minor hydrolysis in roasted cocoa is interesting to note.

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Characterization of Some Volatile Constituents of Roasted Pecans

Pao-Shui Wang* and George V. Odell

The volatiles from roasted pecans were separated into carbonyl, basic, acidic, and noncarbonyl oxygenated fractions and qualitatively investigated. Carbonyls were converted to their 2,4-dinitrophenylhydrazones and identified by analysis of the regenerated carbonyls using combined gas chromatography–mass spectrometry, or direct probe mass spectrometry of each crystalline hydrazone isolated by column and thin-layer chromatography. Basic, acidic, and neutral noncarbonyl oxygenated fractions were characterized by gas chromatography and mass spectrometry. Nineteen carbonyls, pyridine, eight pyrazines, seven acids, five alcohols, and one lactone were positively identified by comparison of their mass spectral and gas chromatographic retention data with those of authentic compounds. Pecan oil may be considered as one of the main sources of roasted pecan carbonyls. The possible precursors of the pyrazines are discussed. Compounds believed to contribute to the flavor of roasted pecans are also briefly discussed.

Roasted pecans possess a characteristic pleasant aroma but little is known about the compounds responsible for the aroma. This paper deals with results of a qualitative investigation of volatile compounds from roasted pecans. The origin of the carbonyls and pyrazines identified was also studied in model systems and is discussed.

Oklahoma State University, Biochemistry Department, Stillwater, Oklahoma 74074.

EXPERIMENTAL

Materials. Good quality, air-dried, shelled pecans of the Stuart Variety were obtained from the Oklahoma State University Horticulture Department. On identification of components isolated from roasted pecan volatiles, the authentic compounds were obtained from reliable commercial sources or were gifts.

Sample Preparation. About 400 g of raw pecans were dry roasted at 170° C to a deep brown color. The roasted pecan