Journal of Chromatography, 170 (1979) 343–353

CHROM. 11,542

SEPARATION OF POTENTIAL FLAVORING COMPOUNDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

ASAF A. QURESHI, N. PRENTICE and W. C. BURGER

U.S. Department of Agriculture, SEA, Barley and Malt Laboratory, Madison, Wisc. 53705, and Department of Agronomy, University of Wisconsin, Madison, Wisc. 53706 (U.S.A.)

(Received October 17th, 1978)

SUMMARY

High-performance liquid chromatography separated successively and quantitatively the food flavoring agents pyrimidines, purines and nucleosides, followed by nucleotides, then by polyphenols and finally by pyrazines with a reversed-phase octadecylsilica (μBondapak C_{18}) column and various proportions of methanol, water, acetic acid and tetrabutylammonium phosphate (PIC A). The polar solvent (solvent A) was water–acetic acid–PIC A (97.5:1.5:1.0) and the relatively non-polar solvent (solvent B) was methanol–acetic acid–PIC A (97.5:1.5:1.0). Purines, pyrimidines, and nucleosides were eluted with solvent A. Nucleotides were eluted with a mixture of solvents A and B (9:1). Polyphenols were separated with a gradient starting at 10% solvent B and finishing at 25% solvent B, and finally the pyrazines were removed successively from the column with a gradient starting at 25% solvent B and finishing at 45% solvent B. The resolution and reproducibility were excellent for more than 50 compounds. By this method beverages could be analyzed directly, without solvent extraction, for flavoring compounds.

INTRODUCTION

Purines, pyrimidines, nucleosides, nucleotides, polyphenols, and pyrazines are among the common flavoring agents found in beverages and foods[1–4]. In particular, the pyrazines are flavorful, even at very low concentrations, and both desirable and undesirable characteristics of food have been related to them. Despite the importance of pyrazines, only limited work has been carried out towards developing a convenient chromatographic system with which to quantitate them. Flavor compounds have been separated by paper[5] and thin-layer chromatography[6], but these methods are

* Cooperative investigation between the Science and Education Administration, U.S. Department of Agriculture, and College of Agricultural and Life Sciences, University of Wisconsin, Madison. Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

laborious and quantitatively inaccurate. Gas chromatography has also been used, but it requires derivatization and operating temperatures that may cause thermal degradation of the compounds[7]. Gas chromatography also has molecular weight limitations. Some flavoring agents extracted from beverages have been separated by high-performance liquid chromatography (HPLC), but the columns and solvent systems differed for each group of compounds. For example, purines, pyrimidines, nucleosides and nucleotides were separated with a cation-exchange column[8], polyphenols and phenolic acids with an anion-exchange or an octadecylsilica (μBondapak C_{18}) column[9,10], and pyrazines were separated not by HPLC but by gas–liquid chromatography[11].

We report here the development of an HPLC method by which we quantitatively analyzed over 50 compounds consisting of purines, pyrimidines, nucleosides, nucleotides, polyphenols and pyrazines with a single column and appropriate combinations of water, methanol, acetic acid and tetrabutylammonium phosphate (PIC A).

MATERIALS AND METHODS

Reagents

Most of the purines, pyrimidines, nucleosides, nucleotides and polyphenols were obtained as a gift from Anheuser-Busch (St. Louis, Mo., U.S.A.). Others were purchased from Sigma (St. Louis, Mo., U.S.A.) and Aldrich (Milwaukee, Wisc., U.S.A.). Pyrazine and its derivatives were obtained from Pyrazine Specialties (Atlanta Ga., U.S.A.). None of the compounds exhibited any impurities when analyzed by HPLC. PIC A was purchased from Waters Assoc. (Milford, Mass., U.S.A.).

Instrumentation

The HPLC apparatus was obtained from Waters Assoc., and consisted of two Model 6000A solvent delivery systems, a Model 660 solvent programmer, a Model 440 absorbance detector which monitored the eluent at 280 and 254 nm, and a μBondapak C_{18} column (30 cm $\times$ 4 mm I.D.). Retention times and absorption counts at 254 and 280 nm were obtained with an Infotronics digital integrator (Beckman Instruments, Palo Alto, Calif., U.S.A.).

Standard solutions

Immediately before use, 10 mg of each compound were dissolved in 3 ml of water and aliquots were withdrawn and mixed for HPLC. Most of the purines, pyrimidines, nucleosides and nucleotides dissolved with warming, but those less soluble were dissolved by adding 10 μl of 4 *N* sodium hydroxide solution. The polyphenols were dissolved in 20–40% aqueous methanol. All of the pyrazines were readily soluble in water.

Chromatographic procedure

The reservoir flask for a pump A contained water–acetic acid–PIC A (97.5:1.5:1.0) (solvent A), and the flask for pump B contained methanol–acetic acid–PIC A (97.5:1.5:1.0) (solvent B). The column was washed at 1 ml/min successively with at least 50 ml each of water and methanol, and was then equilibrated with solvent A. Individual compounds or mixtures in a volume of 10 μl were chromatographed at a solvent flow-rate of 0.5–1 ml/min and at a pressure of 400–1000 p.s.i. The detailed procedure for each group of compounds is described in the caption of the appropriate figure. Routinely the column was washed exhaustively with methanol after a maximum of four samples had been chromatographed. In all of the figures, A represents the percent full-scale absorbance at 280 nm and B that at 254 nm.

Calculations

The peak-area ratios at 280 nm and 254 and retention times were used as the criteria for purity. The absorbance counts at 280 and 254 nm were recorded under similar conditions for each compound, injected first individually and then in a mixture for each series of compounds. The amount of each compound in a mixture was calculated by the equation

$$A = \frac{bcd}{e} = \frac{10 \cdot 3.33d}{e}$$

where A (μg) = amount of each sample; b = volume injected (10 μl); c = total amount per microliter (3.33 μg); d (μl) = volume of the particular standard solution in the mixture of standard solutions; and e (μl) = total volume of the mixture of standard solutions. The absorbance counts per microgram for each compound were calculated by dividing the absorbance count by A.

Gallic acid (0.8–33 μg) was chromatographed to check the capability of the integrator to measure absorbance counts at different sensitivities of the detector. A linear response of absorbance counts to concentration was obtained for sensitivity settings of 0.02–2.0 absorbance units. The ratio of absorbance counts at 280 and 254 nm had a constant value of 1.2. These ranges of sample amounts and detector sensitivities were adequate for this work, as the smallest full-scale absorbance used was 0.1.

RESULTS AND DISCUSSION

Elution and separation of purines, pyrimidines and nucleotides

Eleven compounds consisting of purines, pyrimidines and nucleotides (Table I and Fig. 1) were chromatographed with solvent A. Fig. 1 shows that resolution was good, except between inosine (peak 8) and guanosine (peak 9). These two compounds could be resolved by eluting with 10% aqueous PIC A. This solvent, however, did not separate adenine and thymidine and changed the order of elution of the series. Table I gives the retention times and area counts at 280 and 254 nm, and shows that the combination of peak-area ratios at 280 and 254 nm and retention time was specific for each compound. This series of compounds was eluted in about 18 min. The 1.5% of acetic acid in solvent A sharpened the peak for each compound, but larger amounts of acetic acid impaired the resolution.

Separation of 2'-, 3'- and 5'-substituted nucleotides

The separation of sixteen nucleotides achieved isocratically with a mixture of solvents A and B (9:1) is shown in Fig. 2. The separation was accomplished

TABLE I

CHROMATOGRAPHY OF NUCLEOSIDES, PURINES, AND PYRIMIDINES

Elution with solvent A.

Peak No.	*Sample*	*Amount (µg)*	*Retention time (min*	*Absorbance counts*			*Counts per µg*	
				280 nm	*254 nm*	*Ratio, 280/254 nm*	*280 nm*	*254 nm*
0	Cytosine	0.89	4.7	31729	12296	2.58	35650	13815
1	Cytidine	1.11	5.1	30019	11727	2.56	27000	10547
2	Guanine	1.78	5.4	12358	35047	0.35	6907	19586
3	Adenine	0.89	6.3	21618	35642	0.61	24161	39836
4	Uracil	1.78	8.0	13550	74577	0.18	7612	41680
5	Uridine	4.47	9.2	20722	53904	0.38	4635	12059
6	Adenosine	4.47	10.1	26395	120971	0.21	5904	27042
7	Xanthine	4.47	11.0	73360	103493	0.70	16411	23152
8	Inosine	4.47	12.9	14710	88662	0.16	3288	19835
9	Guanosine	4.47	13.3	43841	76986	0.56	9807	17222
10	Thymidine	5.36	18.5	50873	68593	0.74	9477	12773

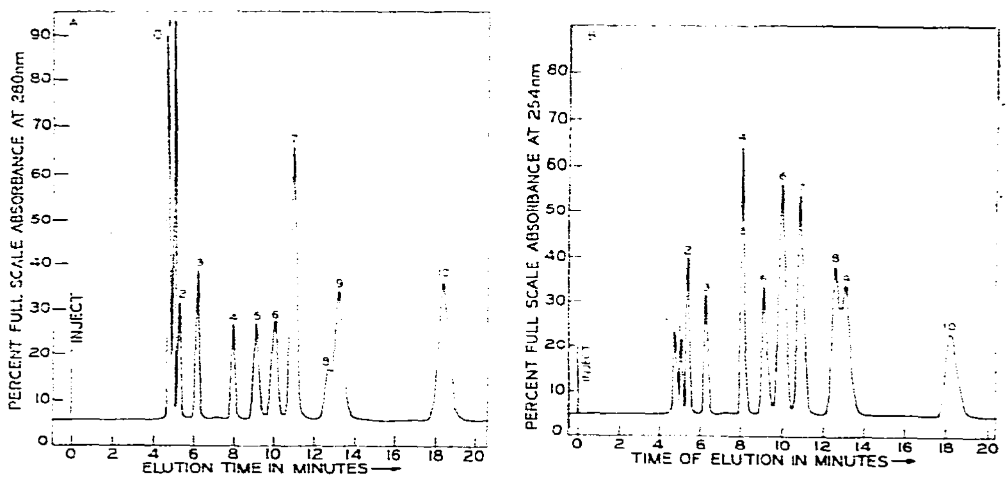

Fig. 1. Separation of purines, pyrimidines and nucleosides with solvent A at 1 ml/min and 500 p.s.i. See Table I for identification of peaks. A 10-mg amount of each compound was dissolved in 3 ml water, and from these solutions 20, 25, 40, 20, 40, 100, 100, 100, 100, 100, and 120 µl for peaks 0–10, respectively, were combined and 10 µl of this mixture were injected. Full-scale absorbance was 2.0 and 1.0 for 254 and 280 nm, respectively. Chart speed, 0.5 cm/min.

within 36 min, but peaks 18 and 19 were not resolved. Table II shows the retention times, ratio of counts, and counts per microgram at 280 and 254 nm for each compound. Compounds 18 and 19 (2′-AMP and 3′-UMP) were separable with a linear gradient of 0–10% solvent B (Fig. 3), but the elution time for the series was 50 min. These two compounds by themselves could be separated from each other with either elution system.

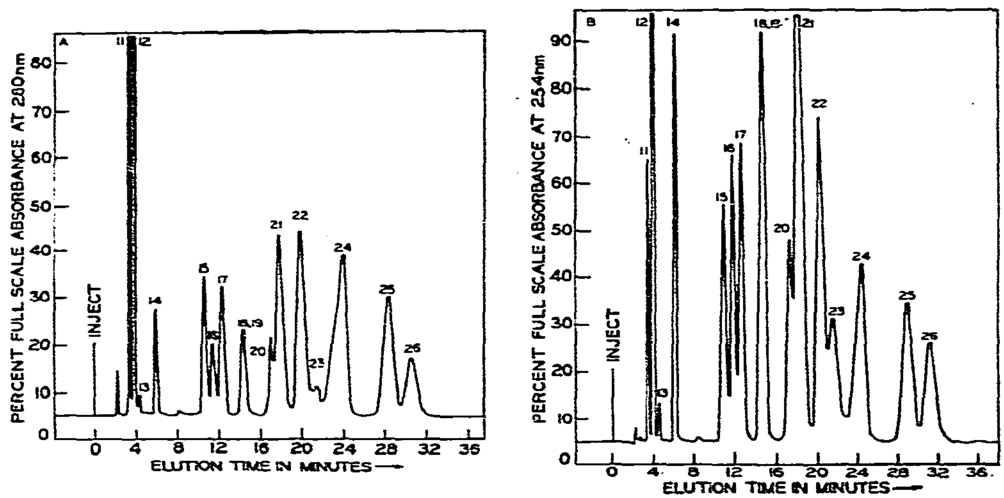

Fig. 2. Separation of nucleotides with a mixture of solvents A and B (9:1) at 1 ml/min and 800 p.s.i. See Table II for identification of peaks. A 10-mg amount of each compound was dissolved in 3 ml of water and from these solutions 30, 30, 30, 20, 30, 20, 40, 20, 50, 200, 50, 100, 50, 100, 100 and 20 μl for peaks 11–26, respectively, were combined and 10 μl of this solution were injected on the column. Full-scale absorbance was 0.1 for 280 and 254 nm, respectively. Chart speed, 0.5 cm/min.

TABLE II

CHROMATOGRAPHY OF NUCLEOTIDES

Elution with a mixture of solvents A and B (9:1).

Peak No.	*Sample*	*Amount (μg)*	*Retention time (min)*	*Absorbance counts*			*Counts per μg*	
				280 nm	*254 nm*	*Ratio, 280/254 nm*	*280 nm*	*254 nm*
11	5'-CMP	1.11	3.5	119595	47824	2.50	106876	42738
12	2'-CMP	1.11	4.0	224729	169081	1.32	210830	151100
13	3'-CMP	1.11	4.5	71700	143530	0.49	64075	128260
14	5'-AMP	0.74	6.3	36660	155808	0.23	49142	208857
15	5'-GMP	1.11	10.8	59670	144915	0.41	53324	129504
16	3'-AMP	0.74	11.5	43009	98307	0.50	57652	131779
17	5'-UMP	1.49	14.0	54086	213088	0.25	36250	142820
18	2'-AMP*	0.74	15.4	154802	355627	0.43	207510	476713
19	3'-UMP*	1.86	17.0	55900	156306	0.35	29973	83917
20	2'-UMP	7.45	19.0	265174	637953	0.42	35594	85527
21	3'-GMP	1.86	21.1	211532	625399	0.33	113422	335333
22	5'-TMP	3.72	23.1	34810	58767	0.59	9335	15759
23	5'-IMP	1.86	24.4	70351	608233	0.14	37722	273243
24	3'-IMP	3.72	27.2	181778	242371	0.75	48747	64996
25	3'-TMP	3.72	31.9	176260	203378	0.86	47267	54539
26	2'-GMP	0.74	34.5	101260	155787	0.64	27155	41777

* Values reported after recycling the fraction collected at 16 min.

Separation of polyphenols

Fig. 4 and Table III illustrate the separation of eighteen substituted polyphenols that are often present in foods and beverages. The elution of seventeen

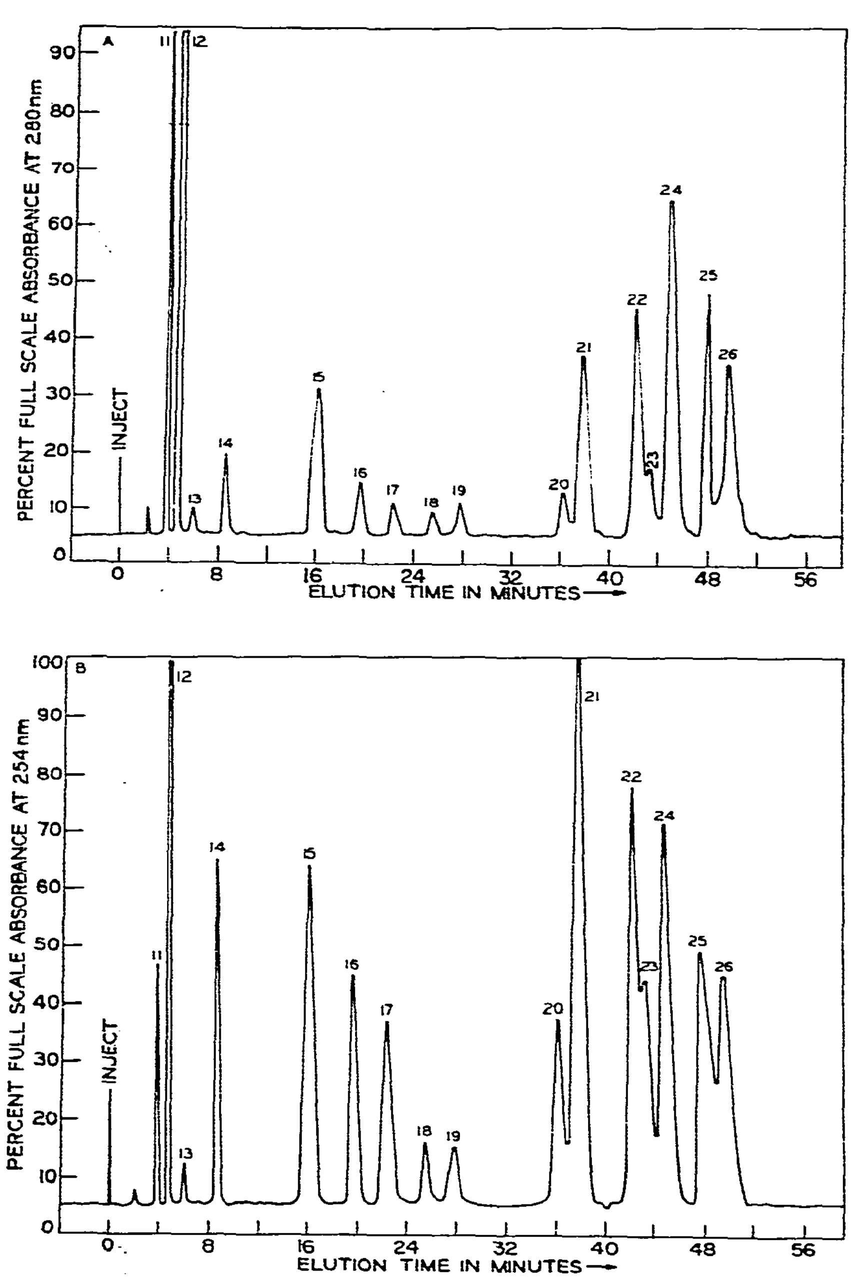

Fig. 3. Chromatography of nucleotides with a linear gradient (gradient curve 6) of 0–10% of solvent B in solvent A. Time: 40 min. Other conditions as in Fig. 2.

compounds with good baseline separation was accomplished with a linear gradient of 10–25% solvent B within 60 min and cinnamic acid appeared after elution for a further 35 min with 25% solvent B. A linear gradient of solvent B in solvent A (program curve 7) was equally effective, but cinnamic acid did not appear until 110 min. The resolution of gentisic acid (2,5-dihydroxybenzoic acid, peak 36), syringic

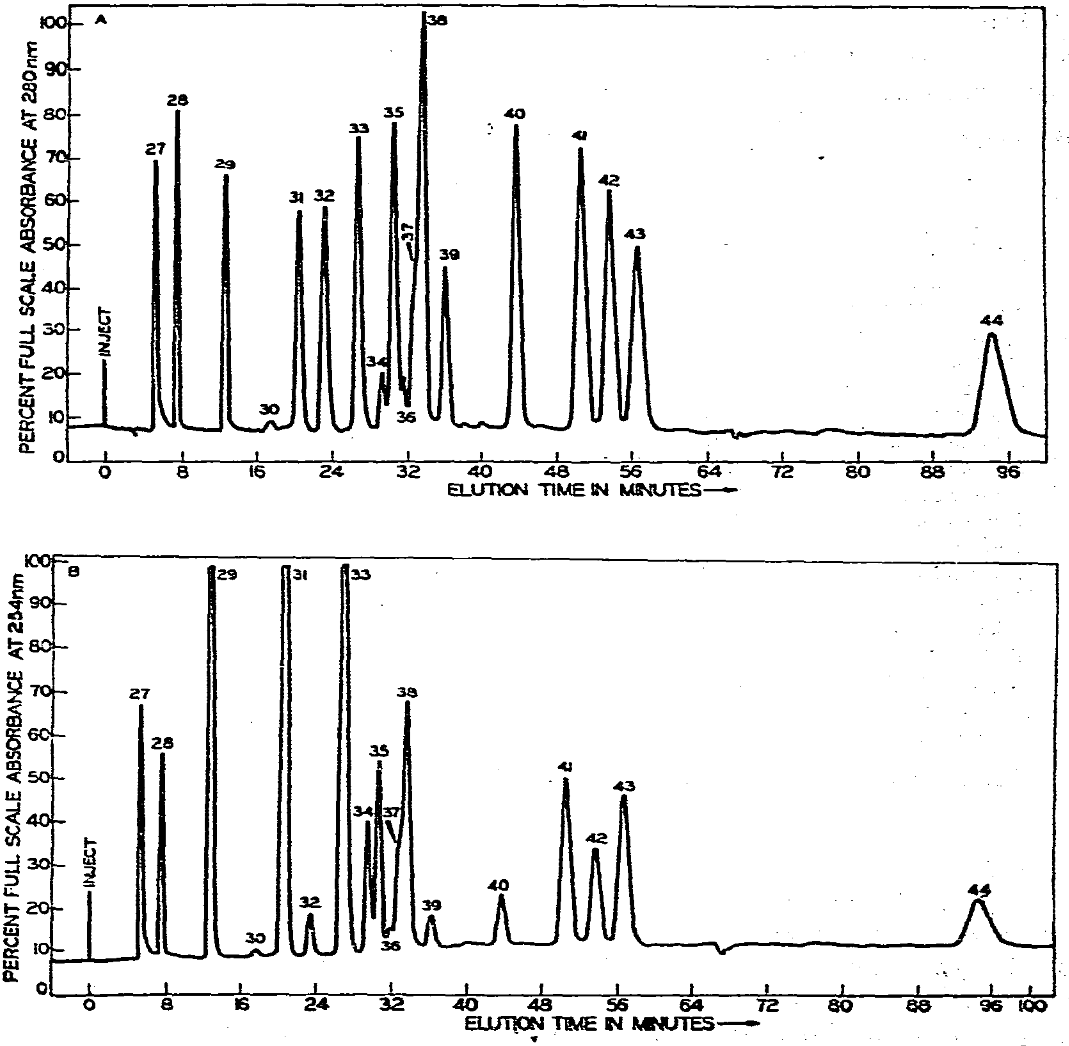

Fig. 4. Chromatography of polyphenols with a gradient of 10–25% of solvent B in solvent A at 1.0 ml/min and 800 p.s.i. with program curve 6 for 40 min. The column was eluted for a further 60 min with 25% of solvent B in solvent A. See Table III for identification of peaks. A 10-mg amount of each compound was dissolved in 3 ml of water and from these stock solutions 20, 15, 30, 40, 30, 100, 25, 30, 40, 200, 30, 50, 80, 20, 40, 80, 70 and 15 μl for peaks 27–44, respectively, were combined and 10 μl of this solution were injected on the column. Full-scale absorbance was 0.1 for both wavelengths. Chart speed, 0.5 cm/min.

acid (3,5-dimethyoxy-4-hydrozybenzoic acid, peak 37) and chlorogenic acid (peak 38) was poor, but when these three compounds were recycled a good separation was achieved. The elution time of cinnamic acid could be shortened when less PIC A was used, but the resolution of some of the other compounds was then decreased. The retention time (Table III) for each acid was the same whether in a mixture or chromatographed individually. The absorbances of these compounds were higher at 280 nm than at 254 nm. The absorbance of quercetin at both wavelengths was very low.

TABLE III

CHROMATOGRAPHY OF POLYPHENOLS

Elution with a gradient of 10–25% of solvent B in solvent A.

Peak No.	*Sample*	*Amount (μg)*	*Retention time (min)*	*Absorbance counts*			*Counts per μg*	
				280 nm	*254 nm*	*Ratio, 280/254 nm*	*280 nm*	*254 nm*
27	Kojic acid	0.72	5.3	122495	123157	0.99	168124	169032
28	Gallic acid	0.54	7.4	139011	95570	1.45	254413	174908
29	3,4-Dihydroxybenzoic acid	1.09	12.5	161004	171391	0.93	147318	156822
30	Quercetin	1.45	17.4	5415	4809	1.12	3716	3300
31	*p*-Hydroxybenzoic acid	1.09	19.9	211190	1135899	0.18	193238	1039343
32	Catechin	3.64	23.0	206020	44849	4.59	56552	12311
33	Vanillic acid	0.91	26.3	332607	762826	0.43	365221	837626
34	Ferulic acid	1.09	27.2	18895	43864	0.43	17289	40135
35	Caffeic acid	1.45	29.8	384687	280845	1.36	263991	192729
36	Gentisic acid*	7.28	31.3	86616	220871	0.39	11888	30314
37	Syringic acid*	1.09	32.7	361175	316272	1.14	330474	289388
38	Chlorogenic acid*	1.82	33.8	350239	251581	1.39	192281	138117
39	Epicatechin	2.91	36.1	218815	62096	3.52	75081	21306
40	*p*-Coumaric acid	0.72	43.5	363532	70296	5.17	498946	96481
41	Rutin	1.45	50.7	432136	251542	1.71	296552	172619
42	Salicylic acid	2.91	53.9	396186	189110	2.09	135941	64886
43	Sinapic acid	2.55	57.1	343554	286970	1.19	134722	112532
44	Cinnamic acid	0.54	95.3	476018	247926	1.92	871190	453749

* Values reported after recycling the fraction collected at 30 min.

Separation of pyrazines

Pyrazines are not easily separated by the usual organic techniques, but the thirteen we examined were separated by the proposed HPLC system. Fig. 5 shows that chromatography was accomplished within 36 min. For pyrazines, as for the polyphenols, absorbance at 280 nm shows distinctions (see peaks 51 and 52) that are not evident in the absorbance at 254 nm. These compounds are aromatic with nitrogen atoms *para* with respect to each other, and the aromatic character accounts for the higher absorbance and better resolution at 280 nm. Although the separation of 2,3- and 2,6-dimethylpyrazine (peaks 48 and 49) injected individually or together was very good, when injected as a mixture of thirteen compounds, both of these compounds appeared in one peak (Table IV, Fig. 5). The ratio of absorbance counts for these two compounds was obtained by collecting this peak and recycling through the column. The identification of 2-ethyl-6-methylpyrazine and 2-ethyl-5-methylpyrazine (peaks 54 and 55) and 2-ethyl-3,6-dimethylpyrazine and 2-ethyl-3,5-dimethylpyrazine (peaks 56 and 57) is tentative and remains to be accomplished by NMR spectroscopy, because these compounds are available commercially only as mixtures. Table IV also shows the retention times and absorbance counts per microgram at 280 and 254 nm.

General appraisal of the method

To our knowledge, there has been no previous separation of purines,

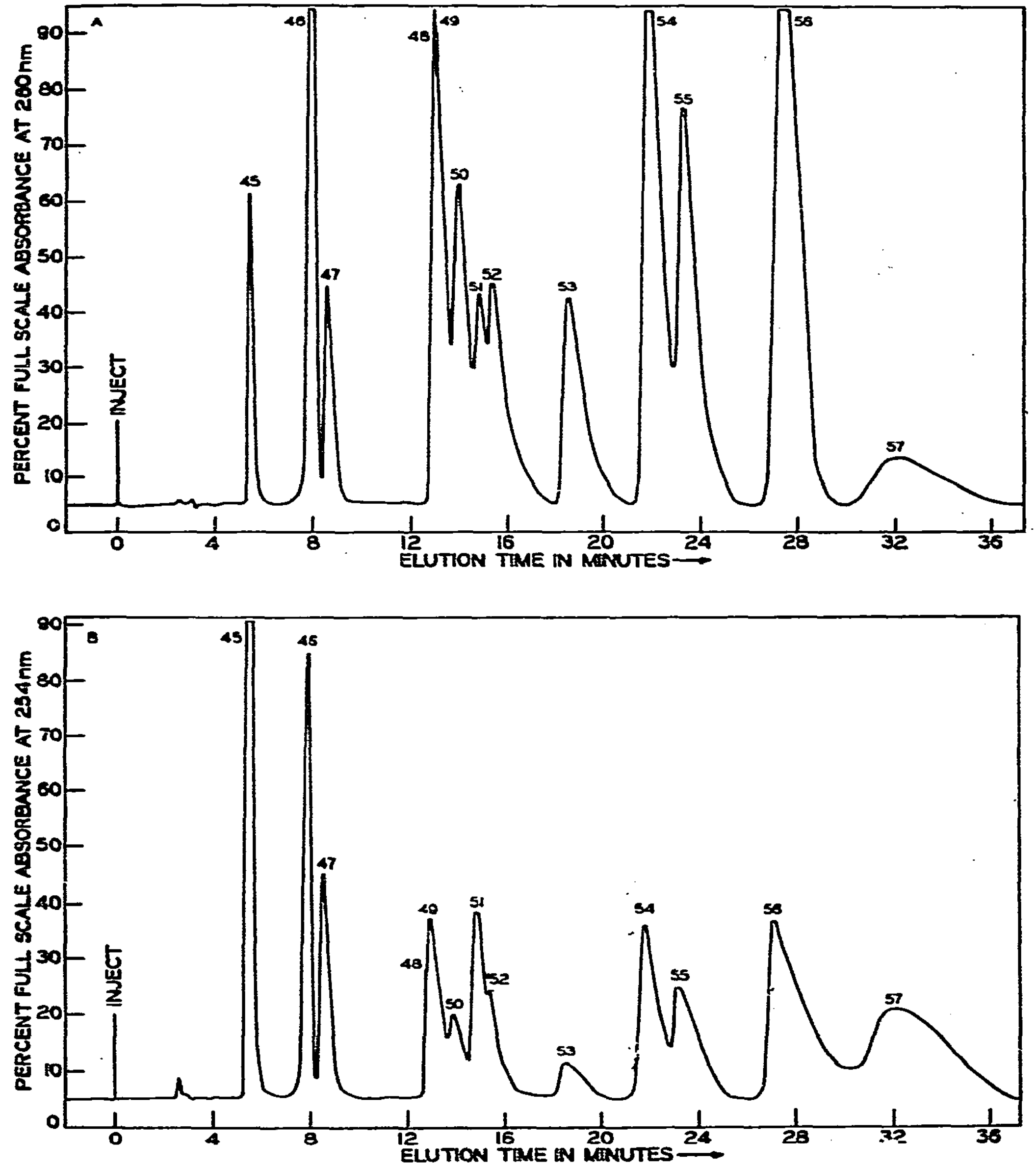

Fig. 5. Separation of pyrazines with a gradient starting with 25% and finishing with 45% of solvent B in solvent A at 1 ml/min and 1000 p.s.i. with program curve 8 for 25 min followed by elution with 45% of solvent B in solvent A for 11 min. A 10-mg amount of each compound in Table IV was dissolved in 3 ml water and from these solutions 100, 50, 50, 40, 40, 50, 50, 40, 50, 25, 25, 100 and 100 μl for peaks 45–57, respectively, were combined and 10 μl of this solution were injected on the column. Full-scale absorbance was 0.2 for each wavelength. Chart speed, 1.0 cm/min.

pyrimidines, nucleosides, nucleotides, polyphenols and pyrazines with the simplicity of our system. We separated 58 compounds with this system. The chromatography of each of the four series of compounds was replicated at least eight times, and the peak areas agreed to within 2%.

TABLE IV

CHROMATOGRAPHY OF PYRAZINES

Elution with gradient of 25–45% of solvent B in solvent A (program 8).

Peak No.	*Sample*	*Amount (μg)*	*Retention time (min)*	*Absorbance counts*			*Counts per μg*	
				280 nm	*254 nm*	*Ratio, 280/254 nm*	*280 nm*	*254 nm*
45	Pyrazine	4.62	5.4	129325	301354	0.42	27968	65171
46	2-Methylpyrazine	2.31	7.9	257744	160970	1.60	11481	69623
47	2-Acetylpyrazine	2.31	8.5	98292	102444	0.95	42514	44309
48	2,3-Dimethylpyrazine*	1.84	13.1	180869	165502	1.55	97819	89508
49	2,6-Dimethylpyrazine*	1.84	13.4	190870	75101	2.54	103228	40617
50	2,5-Dimethylpyrazine	2.31	14.1	236043	57323	4.11	102094	24793
51	2-Ethylpyrazine	2.31	14.9	111427	124550	0.89	48195	53871
52	Tetramethylpyrazine	1.84	15.5	229708	73416	3.12	124233	39705
53	Trimethylpyrazine	2.31	18.5	226453	46393	4.88	97946	20066
54	2-Ethyl-6-methylpyrazine	1.15	21.9	209463	161467	1.29	181196	139677
55	2-Ethyl-5-methylpyrazine	1.15	23.4	169174	121513	1.39	146344	105115
56	2-Ethyl-3,6-dimethylpyrazine	4.62	27.3	452503	202826	2.23	97859	43863
57	2-Ethyl-3,5-dimethylpyrazine	4.62	31.3	332503	598370	0.55	71908	129405

* Values reported after recycling the fraction collected at 10–18 min.

Compounds 0–26 (Tables I and II, purines, pyrimidines, nucleosides and nucleotides) could be separated by an alternative method. Instead of using solvent A for compounds 0–10 (Table I) and the mixture of solvents A and B (9:1) for peaks 11–26 (Table II), a 0–10% linear gradient of solvent B in solvent A (program curve 6) could be used over a 2-h period for the separation of compounds 0–26. However, although gradients of 10–25% and 25–45% of solvent B in solvent A could separate compounds 27–44 and 45–57, respectively, a gradient of 0–45% of solvent B cannot be used to separate all 58 compounds. Further, if a linear gradient of 0–45% of solvent B is used, peak broadening occurs for most of the nucleotides, polyphenols and pyrazines, and fewer baseline separations are obtained. If program curve 8 is used from 0 to 45% solvent B, a separation time of 4–5 h is needed, and peak broadening still occurs.

The first members of a succeeding group are separable from the last members of the preceeding group with the solvent used for the preceeding group. For example, peaks 27–30 (polyphenols, Fig. 4), if present in the group of nucleotides (Figs. 2 and 3), would be eluted and separated with the solvent used for the nucleotides and would appear after the last of the nucleotides.

By the technique described, beverages can be analyzed directly for flavoring compounds of different types. The method eliminates the need for solvent extractions, which are laborious and are seldom quantitative. The chromatographic data described in this paper were obtained with one column, but essentially the same separations were made with two other columns of the same type from the same manufacturer. After continuous use for 2 months, the elution times of all the compounds decreased but the resolution remained unchanged.

The use of PIC A allows weak bases to be separated by an ionic suppression mechanism. Normally 10% aqueous PIC A would be used. However, this solution did not separate adenine from thymidine and eluted the series of compounds we tested only very slowly from the column. We therefore used solvents that contained 1% of PIC A. The application and the advantage of stepwise elution to the four series of compounds presented here will be reported in subsequent papers.

REFERENCES

1 J. A. Maga and C. E. Siza, *J. Agr. Food Chem.*, 21 (1973) 22.
2 R. Timmer, R. ter Heide, H. J. Wobben and P. J. de Valos, *J. Food Sci.*, 36 (1971) 462.
3 G. Charalambous, K. J. Bruckner, W. A. Hardwick and T. J. Weatherby, *Tech. Quart., Master Brew. Ass. Amer.*, 12 (1975) 203.
4 G. Charalambous, K. J. Bruckner, W. A. Hardwick and A. Linnebach, *Tech. Quart., Master Brew. Ass. Amer.*, 11 (1974) 150.
5 R. K. Ibrahim and G. H. N. Towers, *Arch. Biochem. Biophys.*, 87 (1960) 125.
6 R. B. Saha, J. E. Middlekauff and W. A. Hardwick, *Amer. Soc. Brew. Chem. Proc.*, (1971) 206.
7 E. S. Keith and J. J. Powers, *J. Food Sci.*, 31 (1966) 971.
8 B. Bakay, E. Nissinen and L. Sweetman, *Anal. Biochem.*, 86 (1978) 65.
9 L. W. Wulf and C. W. Nagel, *J. Chromatogr.*, 116 (1976) 271.
10 J. B. Murphy and C. A. Sturte, *Anal. Biochem.*, 86 (1978) 220.
11 R. J. Harding, H. E. Nursten and J. J. Wren, *J. Sci. Food Agr.*, 28 (1977) 225.