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Pyrazines as Interfering Substances in the Determination of Nitrosamines in Roasted Foods

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Distillates with nitrosamine-like properties as characterized by polarography were obtained from model systems of D-glucose and glycine subjected to more or less prolonged heating, and fractionated by means of gas chromatography.

In total seven fractions were obtained and the same fractions were found in a number of typical roasted foods. The fractions were identified as various simple alkyl substituted pyrazines by means of their NMR and mass spectra, and their UV absorption spectra.

Dimethylnitrosamine, a compound known to be carcinogenic, has been shown to be the toxic agent in fish meals produced from raw material preserved with sodium nitrite,[1,2] under circumstances where the sodium nitrite has not been allowed sufficient time to decompose before processing. As a part of the efforts to control the occurrence of dimethylnitrosamine in fish meal, a number of chemical methods for its determination were tried out, among them the polarographic method. SINTEF's department of analytical chemistry developed this method to the point where it proved itself sufficiently accurate, specific and sensitive for the present purpose, and correlated well with the results from feeding experiments.[3,4]

The limit of detection was estimated to be of the order of 0.01 mg/kg calculated as dimethylnitrosamine, corresponding to about 10^{-10} moles/kg.

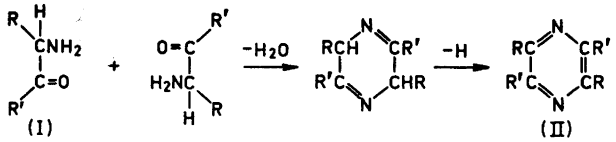
Consequently, a number of samples of various foods were assayed by this method, and the majority showed apparent dimethylnitrosamine contents at levels not higher than the estimated limit of detection (Lydersen and Nagy, *unpublished results*). Exceptions are some types of well ripened Emmenthal cheese, showing levels of about 0.05 mg/kg of apparent dimethylnitrosamine, and a number of roasted foods with estimated levels about two orders of magnitude higher. Constituents of the same general characteristics, and in similar concentrations were found in experiments with model systems in which representative amino acids and D-glucose adsorbed on starch were heated to roasting temperatures.[5]

The nitrosamine-like compounds could be reduced by Zn–HCl or by cuprous chloride,[7] but the presence of the corresponding simple aliphatic or cyclic amines or hydrazines could not be demonstrated by coupling with suitable reagents, *e.g.* *p*-nitrosalicylaldehyde [6] or 4'-nitroazobenzene-4-benzoylchloride.[8] A polarographic wave similar to the original one could, however, be produced by the action of sodium nitrite on solutions of reduced compounds.

On the other hand, the polarographic waves could not be suppressed by bisulphite, nor did the IR spectra of the concentrates show any absorption bands that could be ascribed to carbonyl groups.

At this stage, sufficient material was isolated by preparative gas chromatography to characterize the various fractions polarographically. The various fractions which were polarographically active, were characterized further by means of UV, IR, and mass spectrometry and by NMR and shown to be various alkyl substituted pyrazines.

The parent compound, pyrazine or 1,4-diazine, is a weak diacidic base (pK_a 0.65 and −5.78).[9] The various pyrazines are prepared by the self-condensation of α-amino-ketones (I) in neutral or alkaline conditions. Formally the formation of the pyrazine (II) requires oxidation, but the reaction seems to occur readily.[9]



Compounds of type I are found during the heating of sugars with amines, amino acids, or proteins, and various pyrazines have been isolated from the majority of roasted foods, *e.g.* coffee,[10] cocoa,[11] peanuts,[12] bread crumbs.[13] In the model systems referred to earlier,[5] the molar yield varied between 0.016 and 0.43 mole %, when recalculated as pyrazines.

In aqueous solutions of glucose and glycine heated to 95°C at pH 12.5 an average yield of 0.012 moles of pyrazines/mole glucose is obtained. Similar yields are obtained in reactions with D-ribose and L-arginine in water-ethyleneglycol systems.[14] In total, seven fractions that were polarographically active were obtained by gas chromatography from the above mentioned model systems of D-glucose and glycine.

Fig. 1 shows the UV spectra obtained from the various fractions. The molar extinction values of pyrazines and nitrosamines are similar, and the UV spectra of Fig. 1 give no indication of absorption maxima characteristic of simple aliphatic or cyclic nitrosamines (*cf.* Table 1).

Table 1 summarizes the average yield of the various fractions, their retention times relative to dimethylnitrosamine, their UV-absorption maxima and their registered polarographic half wave potentials. The same data are also presented for a number of nitrosamines with similar relative retention times.

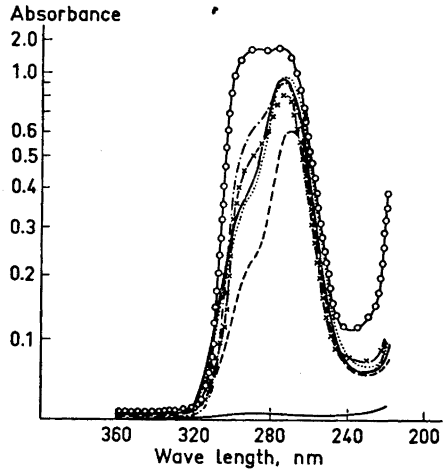


Fig. 1. UV absorption spectra of various fractions obtained by gas chromatography of steam distillates from a model system of D-glucose and glycine.

Fraction 2	(2,5-dimethylpyrazine)	(———),
3	(2-methyl-6-ethylpyrazine	(— — —),
4	(2-methyl-(?)-ethylpyrazine)	(· · ·),
5	(trimethylpyrazine)	(— · —),
6	(dimethylethylpyrazine)	(×),
7	(tetramethylpyrazine)	(○).

It is of interest to note that the polarographic reduction of pyrazines occurs in two steps; but when the pH is increased to 9.5, a single step is obtained.[14]

The observed mass spectra were in accordance with those to be expected for simple alkyl substituted pyrazines,[16] and with published values.[18,19] The most important fragments observed were M−HCN, M−CH_3CN, and CH_3CNH^+.

The NMR diagrams of the various pyrazines are fairly simple, the methyl substituted pyrazines of increasing degree of substitution exhibiting only the peaks characteristic of methyl and vinyl protons, with integral values of the various peaks proportional to the numbers of the respective protons.

Table 2 gives values of the shift of the various peaks observed for the fractions obtained by gas chromatography together with published values for 2,5-dimethylpyrazine,[20] for 2,5-dimethylpyrazine and 2,3-dimethylpyrazine synthesized in our laboratory.[17,21]

The identification of fractions 2, 3, 5, and 6 is based upon the observed NMR-spectra and the *m/e* values from mass spectrometry. Fraction 3 is identified as 2-methyl-6-ethylpyrazine because its relative position on gas chromatograms corresponds to what is observed by Marion *et al.*[11] in similar systems. In their observations, 2,3-dimethylpyrazine and 2-methyl-6-ethylpyrazine have similar retention times, and as fraction 4 shows no evidence

Table 1. Polarographic half wave potentials, UV absorption maxima and relative retention times observed for pyrazines isolated from model systems of D-glucose and glycine, together with similar data for typical nitrosamines.

Fraction No.	Compound	Percentage in distillate	UV [c] absorption maxima nm	Relative retention time [b]	Half wave potentials, V pH 1.3
	Pyrazines: [a]				
	Pyrazine	—		0.622	—
1	Methylpyrazine	1	265	0.970	−0.3; −0.75
2	2,5-Dimethylpyrazine	40	274	1.260	−0.3; −0.75
3	2-Methyl-6-ethylpyrazine	2.8	272	1.430	−0.31; −0.78
4	2-Methyl-?-ethylpyrazine	4.4	273	1.58	−0.30; −0.76
5	Trimethylpyrazine	40.0	(290) 274	1.74	−0.35; −0.78
6	2,5-Dimethyl-3-ethyl-pyrazine	4.0	(290) 274	2.00	−0.35; −0.76
7	Tetramethylpyrazine	7.2	(290) 274	2.22	−0.42; −0.77
	Nitrosamines:				
	N-Methyl-*N*-nitrosoacetamide		238	0.860	−0.47; −0.76 [d]
	Dimethylnitrosamine (DMNA)		(332) 230	1.000	−0.76
	Methylethylnitrosamine		(335) 230	1.26	−0.73
	Diethylnitrosamine		(340) 230	1.45	−0.71
	Methylnitrosourethane		237	1.63	−0.49; −0.83
	Ethylnitrosourethane		240	1.78	−0.42; −0.77
	Ethyl-*t*-butylnitrosamine		(342) 228	1.94	−0.75
	Dipropylnitrosamine		(339) 233	2.48	−0.65

[a] As identified by NMR and mass spectrography (v.i.).
[b] Relative to DMNA. Column: 25 % polyethyleneglycoladipate on diatomite 60—70 mesh, 150°C.
[c] UV-data for nitrosamines from Ref. 15.
[d] Lydersen *et al. Unpublished data.*

of the presence of a compound with a fragmentation pattern corresponding to the latter, it is concluded that fraction 3 consists of 2-methyl-6-ethylpyrazine.

The present study was initiated because the polarographic measurements on steam distillates from alkaline suspensions of roasted foods could indicate presence of nitrosamines. Our results indicate that no such compounds can be demonstrated by the chemical methods used. The studies also indicate that the gas chromatographic and polarographic characteristics of pyrazines and nitrosamines overlap to such an extent that precise determinations of small amounts of nitrosamines by these methods will be difficult, unless improved methods of separation can be developed.

Until the stability of nitrosamines in the various organic solvents of sufficient polarity can be improved, the methods of separation should be based upon the use of aqueous systems. Our experience indicates that the present semiquantitative methods based upon reduction and formation of coloured benzamide derivatives [7,8] could be developed into quantitative methods.

Table 2. NMR peaks and relative integral values of various fractions obtained by gas chromatography of steam distillates from a model system of D-glucose and glycine.

Fraction No.	Compound	Type of proton	Shift ppm	Integral value relative to vinyl
—	2,5-Dimethylpyrazine	Methyl	2.52	3
		Aromatic	8.35	1
2	Same	Methyl	2.42	3
		Aromatic	8.35	1
—	2,3-Dimethylpyrazine	Methyl	2.48	3
		Aromatic	8.16	1
5	Trimethylpyrazine	Methyl	2.37	9
		Aromatic	7.94	1
7	Tetramethyl pyrazine	Methyl	2.38	—
3	2-Methyl-6-ethylpyrazine	Methyl	2.48	—
		Aromatic	8.22	—
		Ethyl (triplet)	1.32	—
4	2-Methyl-(?)-ethylpyrazine	Methyl	2.48	—
		Aromatic	8.20	—
		Ethyl (triplet)	1.29	—
6	2,5-Dimethyl-3-ethylpyrazine	Methyl	2.48	
		Aromatic	8.11	
		Ethyl (triplet)	1.28	—

Also, it would be of interest to develop methods to remove the interfering pyrazines by ion exchange, or to utilize polarography under alkaline conditions to estimate their level.[14]

METHODS

Preparation of steam volatile compounds from model systems. (a) 10 g of starch was moistened with an aqueous solution containing 0.01 gmole of glycine and 0.005 gmole of D-glucose, and air dried. The mixture was heated at 110°C overnight and subjected to vacuum distillation after addition of 3 N NaOH. The resulting distillate was extracted with methylene chloride and the solvent removed under vacuum at 30°C. (b) 0.2 gmole of glycine and 0.1 gmole of D-glucose were dissolved in about 200 ml of water and the pH adjusted to 12.5—13.0. After heating at 85°C overnight the isolation proceeded as under (a).

Gas chromatography was done on a Pye series 104 gas chromatograph equipped with a manually operated fractionating column in addition to the standard analytical one. For preparative purposes a 15′ × 3/8″ I.D. glass column was used, filled with 25 % PEGA (polyethyleneglycol-adipate) on siliceous diatomite 60—72 mesh. The carrier gas was Ar, the rate was 132 ml/min, of this 12 ml/min went to the flame ionization detector. Fractions were collected in water and stored as aqueous solutions.

Polarography was done according to Lydersen and Nagy.[3] The buffer solution contained 2 moles/l of $(NH_4)_2SO_4$, 2.5 moles/l of H_2SO_4 and 1 mole/l of KBr in water. The Hg-pool in the bottom of the cell was used as the one electrode. Temperature of the system was 25°C. Under these conditions, the potential of the Hg/HgBr electrode is 0.10 V against the saturated calomel electrode.

UV-absorption spectrometry was done with a Beckman DB spectrophotometer.

IR absorption spectrometry was done with a Beckman IR-5 spectrophotometer.

NMR spectra were obtained with a Varian A 60A at the Organic Chemistry Laboratories, NTH. Solution volume was about 0.5 ml, concentration 30—60 mg/ml, solvent CCl_4.

Mass spectra were determined directly on methylene chloride extracts dried over anhydrous Na_2SO_4 on a mass spectrometer, type MS−902 from A.E.I.

Synthesis of 2,3-dimethylpyrazine.[21] 5 g of diacetyl was condensed with 5 ml of ethylenediamine dissolved in ether, the solvent was removed and the concentrate refluxed for 5 h in alcoholic KOH. The acidified reaction mixture was evaporated under vacuum and the residue distilled after addition of 50 % KOH.

Synthesis of 2,5-dimethylpyrazine and 2,5-dimethyl-3-ethylpyrazine.[16] 20 ml of glycerol, 10 g of ammonium phosphate, and 10 g of ammonium chloride were distilled, and the pyrazines extracted from the distillate with methylene chloride. After removal of the solvent, the pyrazines were separated by preparative gas chromatography.

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