Pyrazine and Thiazole Structural Properties and Their Influence on the Recovery of Such Derivatives in Aroma Extraction Procedures

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This work aims to provide better knowledge of pyrazine and thiazole structural properties, exploitable in aroma extraction. An optimized extraction method including steam-vacuum distillation and liquid/liquid extractions of the aqueous distillate followed by gas chromatography−nitrogen−phosphorus detector analysis enabled us to quantify 50 ppt of such heterocycles in a food matrix such as beer. Lipophilicity appears as a determining parameter for vacuum distillation while basicity governs the liquid/liquid extractions.

Keywords: *Pyrazine; thiazole; aroma extraction; flavor; lipophilicity; NPD*

INTRODUCTION

The importance of the Maillard reactions in food quality, especially taste and aroma, has been recognized in many scientific papers and reviews over the years (Vernin et al., 1992). Among the volatiles produced, pyrazines and thiazoles have received much attention due to their pleasant flavors and extremely low odor threshold. In particular, pyrazines and thiazoles have been identified as the compounds directly contributing to the roasted or toasted flavors of cooked foods, such as bread, coffee, meats, and to the maltlike flavors of beer (Narziss et al., 1986; Jayalekshmy et al., 1987; Humbert and Sandra, 1988; Blank et al., 1992; Mermet et al., 1992). These odors of the "nutty class" are generally displayed by short-chain polyalkylheterocycles and by acetyl- and methoxy-substituted analogues (Fors, 1985; Shibamoto, 1986). Pyrazines and thiazoles have also been found in raw products, where they usually exhibit a green odor. The best-known example is 2-isobutyl-3-methoxypyrazine, the characteristic odorant of green bell pepper (Heymann et al., 1986; Harris et al., 1987; Allen et al., 1990, 1991; Lacey et al., 1991). This class of compounds is usually characterized by structures bearing longer hydrocarbon chains and, sometimes, methoxy groups (Hérent et al., 1995; Koehler et al., 1971; Buttery et al., 1976; Pelosi et al., 1983; Shibamoto, 1986). Due to their low sensory threshold, quantification of such compounds is a challenge for food analysts. Efficient techniques are necessary for investigating such nitrogen heterocycles usually found at very low concentration. The literature is poor in this field, and different authors report measured concentrations differing in order of magnitude from ppm (parts of compounds per 10^6 parts of water) to sub-ppb (parts of compounds per 10^9 parts of water) for the same food (Harding et al., 1978; Qureshi et al., 1979a,b; Narziss et al., 1986, 1988, 1989). Such differences could not be due to intrinsic variability of the material.

The purpose of this study was to accumulate better knowledge of pyrazine and thiazole structural properties to exploit them in aroma extraction. We have sought to correlate recovery factors for such heterocyclic compounds after liquid/liquid extractions or vacuum distillation with parameters such as lipophilicity (log k_w measured by RP-HPLC), steric hindrance (determined by theoretical geometry optimization), or electronic density. Finally, we have examined the potential of a specific chromatographic nitrogen−phosphorus detector (NPD) in quantifying about 50 ppt (parts of compounds per 10^{12} parts of water) of such heterocycles in a food matrix such as beer.

EXPERIMENTAL PROCEDURES

Materials. All pyrazines and thiazoles were commercially available (Sigma-Aldrich, except 2-butyl-3-methylpyrazine purchased from Extrasynthese) and are listed in Table 1. Dichloromethane and analytical grade methanol were obtained from Romil. γ-Morpholinopropanesulfonic acid (MOPS) and *n*-decylamine were purchased from Sigma.

Steam Vacuum Distillation. A 1 L aliquot of an aqueous solution containing pyrazine and thiazole derivatives (0.1 ppm) was poured into flask A (volume = 5 L) and heated in a 30 °C water bath (Figure 1). The mixture was stirred at 500 rpm. Valves 2−5 were opened; valve 1 was closed. Vacuum (1 mmHg) was applied to the system; traps B−D were cooled with liquid nitrogen. Vacuum was applied to flask A by gradually opening valve 1. After distillation for 1 h, the bath temperature was raised to 35 °C. When distillation was complete (5 h), the aqueous distillate was trapped in B.

Liquid/Liquid Extraction. The aqueous distillate was adjusted to pH 0.1 with concentrated hydrochloric acid. The acidified distillate was extracted 4 times with bidistilled dichloromethane (1/3, 1/5, 1/5, 1/5 (v/v)); the resulting organic phases were collected in AN (acid and neutral fraction). The distillate was then adjusted to pH 12 with sodium hydroxide (1 M), after which the distillate was extracted 3 times with bidistilled dichloromethane (1/3, 1/5, 1/5 (v/v)). The three organic phases were collected in B (basic fraction). The extractions were performed under vigorous stirring (1000 rpm) for 10 min.

Concentration. The AN and B phases were allowed to evaporate under atmospheric pressure through a Kuderna Snyder column until the volume was reduced to about 0.5 mL (about 6 h). Then 20 μL (1000 ppm in bidistilled dichloromethane) of 2-ethoxythiazole (NPD experiments) or undecane (FID experiments) was added.

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Table 1. Total Recovery Factors (%) of Pyrazines and Thiazoles after Vacuum Distillation, Four Liquid/Liquid Extractions at pH 0.1 and Three Others at pH 12, and Concentration

		recovery factor (%)			
code	name	B	AN	log k_w	boiling point (°C)
P1	pyrazine	42	7	−0.339	116
P2	2-methylpyrazine	64	4	0.199	135
P3	2-ethylpyrazine	63	14	0.680	153
P4	2-methoxypyrazine	26	47	0.648	155
P5	2-acetylpyrazine	1	81	0.232	
P6	2,5-dimethylpyrazine	74	2	0.640	
P7	2-isopropyl-3-methoxypyrazine	2	76	2.116	
P8	2-methyl-3-propylpyrazine	61	15	1.520	190
P9	2,3-dimethylpyrazine	80	1	0.583	156
P10	2,3-diethylpyrazine	65	19	1.456	182
P11	2-methyl-3-methoxypyrazine	62	14	1.124	
P12	2,6-dimethylpyrazine	74	2	0.586	
P13	2-isobutyl-3-methoxypyrazine	2	88	2.507	
P14	2-acetyl-3-methylpyrazine	3	90	0.763	90
P15	2,3,5,6-tetramethylpyrazine	79	0	1.273	190
P16	2-butyl-3-methylpyrazine	47	58	2.011	
P17	pyrazinylethanethiol	0	18	1.876	220
P18	2,3,5-trimethylpyrazine	71	1	0.848	172
T1	thiazole	59	0	0.373	118
T2	4-methylthiazole	71	2	0.858	134
T3	2-ethyl-4-methylthiazole	74	2	1.798	162
T4	4-methyl-5-vinylthiazole	69	7	1.835	188
T5	2-isopropyl-4-methylthiazole	63	3	2.207	
T6	5-(2-hydroxyethyl)-4-methylthiazole	26	1	0.387	282
T7	2-acetylthiazole	0	72	0.720	210
T8	2-ethoxythiazole	43	29	1.568	
T9	2,4,5-trimethylthiazole	74	1	1.746	

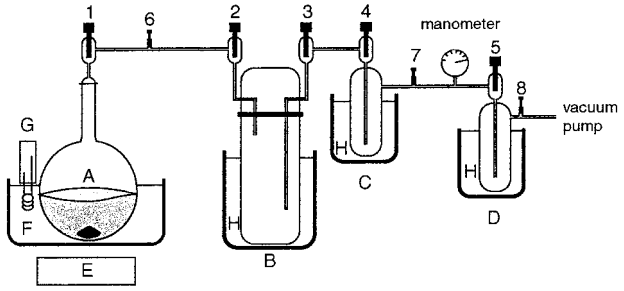

Figure 1. Vacuum distillation apparatus: A, 5 L flask; B, trap for collection of nitrogen heterocyclic compounds; C and D, traps for protection of the vacuum pump; E, magnetic stirrer; F, water bath; G, heater; H, liquid nitrogen vessels.

Gas Chromatography. A Hewlett-Packard Model 5890 gas chromatograph equipped with a Hewlett-Packard Model 7673 automatic sampler, a split/splitless injector, a nitrogen−phosphorus detector (NPD) or an ionization flame detector (FID), and a Shimadzu CR3A integrator was used for quantification. Analyses of heterocyclic nitrogenous compounds were carried out on a 50 m × 0.32 mm, wall-coated, open tubular (WCOT) CP-SIL5 CB capillary column (film thickness, 1.2 μm). The splitless injection time was 0.5 min, and the volume injected was 1 μL. The oven temperature was programmed to rise from 36 to 80 °C at 20 °C/min, then to 105 °C at 0.5 °C/min, and to 250 °C at 5°/min. The carrier gas was helium at a flow rate of 1.5 mL/min, and the detector temperature was 250 °C. The potential applied to the active element of the NPD detector was gradually raised until the amperage reached 25 pA. Optimization of the NPD detector was performed by injection of a model solution (pyrazines and thiazoles in dichloromethane, 0.5 ppm) under various gas pressures, detector temperatures, and splitless injection times in order to obtain the best possible resolution.

Mass Spectrometry. For GC-MS analysis, the same column was directly connected to an HP 5988 quadrupole mass spectrometer. Electron impact mass spectra were recorded at 70 eV. Spectra were recorded automatically throughout elution with HP59970C software.

Geometry Optimization. Ab initio theoretical calculations were done within the 3-21G basis set using the Gaussian 92 program which includes a geometry optimization procedure. C−H and C−C bond lengths were initially fixed at 1.09 and 1.54 Å, respectively. Dihedral C−C−H and H−C−H were estimated at 109.0 and 120° for saturated chains and aromatic rings, respectively. The Gaussian 92 program was also used for molecular electrostatic potential calculations.

Lipophilicity Measurements. Lipophilicity was measured by RP-HPLC. The column was prepacked with LiChrosorb RP-18, and the mobile phase consisted of methanol (varying in concentration from 30 to 70% (v/v)) and a solution containing MOPS buffer (0.01 M) and *n*-decylamine (0.2% (v/v)). The complete protocol has been described in a previous paper (Hérent et al., 1995). The capacity factor was defined as $k = (t_r - t_o)/t_o$, where "t_r" is the compound retention time and "t_o" the column dead time. log k for 100% water (log k_w values) was linearly extrapolated from results obtained for different mobile phase compositions.

RESULTS AND DISCUSSION

In most references, pyrazines are extracted by steam distillation followed by successive pH 0.7−1 and pH 8.3−9 liquid/liquid extractions (van Praag et al., 1968; Jayalekshmy et al., 1987). When tested on a model system (18 pyrazines and 9 thiazoles, 10 ppm; detailed structures in Table 1), this method gave us recovery factors below 45% for all compounds except some thiazole derivatives. Such nitrogen heterocycles are present in very low concentration in food and thus require a high-performance analytical method. To improve recovery, we optimized the liquid/liquid extraction step and replaced steam distillation with a more efficient vacuum distillation.

Liquid/Liquid Extraction Optimization. Extractions were performed at different pH values below or equal to 1 (1, 0.7, 0.5, 0.3, and 0.1). As shown in Table 2, recovery was significantly higher in the final B phase when the pH was lowered to 0.1 before acid extraction. Other optimization experiments revealed that four acidic extractions and three basic extractions are necessary to improve yields (Figure 2). On the other hand,

Table 2. B Phase Recovery Factors (%) Obtained by Decreasing the pH before Acid Extraction of a 10 ppm Model Solution

	value for given pH						value for given pH				
	1.0	0.7	0.5	0.3	0.1		1.0	0.7	0.5	0.3	0.1
P1	21	30	37	44	53	P15	80	83	85	85	85
P2	29	45	53	59	66	P16	1	4	11	33	51
P3	11	27	41	61	73	P17	0	0	0	0	0
P4	1	3	7	24	42	P18	65	74	78	78	82
P5	0	0	0	1	3	T1	58	65	69	70	73
P6	47	63	69	73	78	T2	63	68	70	73	74
P7	0	0	0	2	6	T3	63	68	69	70	71
P8	6	23	38	57	67	T4	15	34	46	62	66
P9	57	72	76	81	85	T5	53	64	68	68	69
P10	0	13	16	55	67	T6	16	18	19	19	31
P11	10	27	42	60	69	T7	0	0	1	5	7
P12	47	63	69	73	78	T8	1	6	15	38	53
P13	0	0	0	2	5	T9	69	72	73	74	78
P14	0	0	0	2	9						

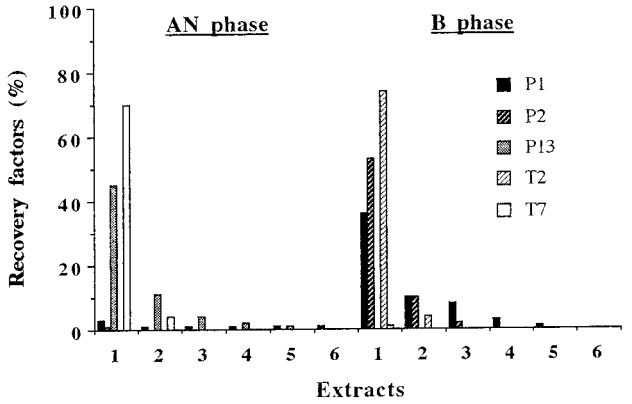

Figure 2. Recovery factors (%) obtained by increasing the number of extractions of a 10 ppm model solution in both AN and B phases. Each extract is separately analyzed.

a stirring time of 10 min seems sufficient for extraction of the bulk of the compounds of interest. Table 3 shows the comparison of recoveries after 10 and 30 min of stirring.

Table 1 lists the B phase recovery factors obtained under these conditions. Recovery factors exceeding 50% are obtained for 16 heterocycles. On the other hand, less basic compounds such as P5 (2-acetylpyrazine), P7 (2-isopropyl-3-methoxypyrazine), P13 (2-isobutyl-3-methoxypyrazine), P14 (2-acetyl-3-methylpyrazine), and T7 (2-acetylthiazole) are still mainly extracted in the AN phase. Intermediate behavior is observed for P4 (2-methoxypyrazine) and P16 (2-butyl-3-methylpyrazine), recovered in both AN and B phases. Last, very polar compounds such as P17 (pyrazinylethanethiol) and T6 (5-(2-hydroxyethyl)-4-methylthiazole) are not satisfactorily extracted by a nonpolar solvent like dichloromethane. Two essential properties of the nitrogen heterocycles seem to influence their partition between the aqueous and organic phases: basicity and, to a lesser extent, lipophilicity. The higher basicity of the thiazole moiety, as shown by a pK_a value of 2.44 compared to 0.65 for the pyrazine nitrogen atoms (Peppard and Halsey, 1980), increases the capacity of the former to be protonated and hence its recovery in the B phase. The hydrophilicity of pyrazine (log k_w = −0.339, compared to +0.373 for unsubstituted thiazole) further decreases its B phase recovery factor. Alkyl-substituted pyrazines are more basic, which leads to better recovery factors in the B phase: P15 (79%) > P18 (71%) ≈ P6 (74%) > P2 (64%) > P1 (42%). Even at pH 0.1, recovery is high in the AN phase for acetyl derivatives (81% for P5, 90% for P14, 72% for T7) and methoxy derivatives (47% for P4, 76% for P7, 88% for P13). Since the methoxy and acetyl derivatives stay in the AN phase, we can conclude that no protonation occurs because of too low basicity. In these cases, the low basicity is mainly due to the fact that nitrogen lone pair availibility is decreased by steric hindrance. This masking effect was demonstrated by ab initio geometry optimization of 2-isopropyl-3-methoxypyrazine and 2,5-dimethyl-3-methoxypyrazine (Figure 3). The methoxy group coplanar with the aromatic ring shows a C1−C2−O3−C4 torsion angle of 180.0°.

Table 3. Recovery Factors (%) Obtained by Increasing the Stirring Time of the Liquid/Liquid Extraction of a 10 ppm Model Solution

	AN phase		B phase	
codes	10 min	30 min	10 min	30 min
P1	5	4	62	60
P2	2	2	70	71
P3	9	7	74	75
P4	39	31	43	48
P5	68	67	2	3
P6	1	1	80	77
P7	63	56	8	12
P8	7	6	61	65
P9	1	1	80	77
P10	10	8	58	66
P11	8	6	66	68
P12	1	1	80	77
P13	66	62	4	6
P14	69	63	0	1
P15	0	0	75	73
P16	24	21	45	52
P17	46	41	0	0
P18	0	0	73	70
T1	0	0	78	71
T2	0	0	77	68
T3	1	1	69	70
T4	1	1	66	70
T5	2	2	62	69
T6	0	0	39	35
T7	60	57	8	9
T8	19	19	52	57
T9	1	1	72	73

Vacuum Distillation Optimization. A distillation step before liquid/liquid extraction is usually required for real samples to eliminate all heavier constituents (lipids, polyphenols, etc.) which would interfere with GC analysis. We thus optimized this step on a model solution by gradually increasing the distillation time: 1 h, 1 h 30 min, 2 h, 3 h, or 5 h (complete distillation). Figure 4 presents the total recovery factors (R_{tot}) for the entire process including distillation, pH 0.1 and pH 12

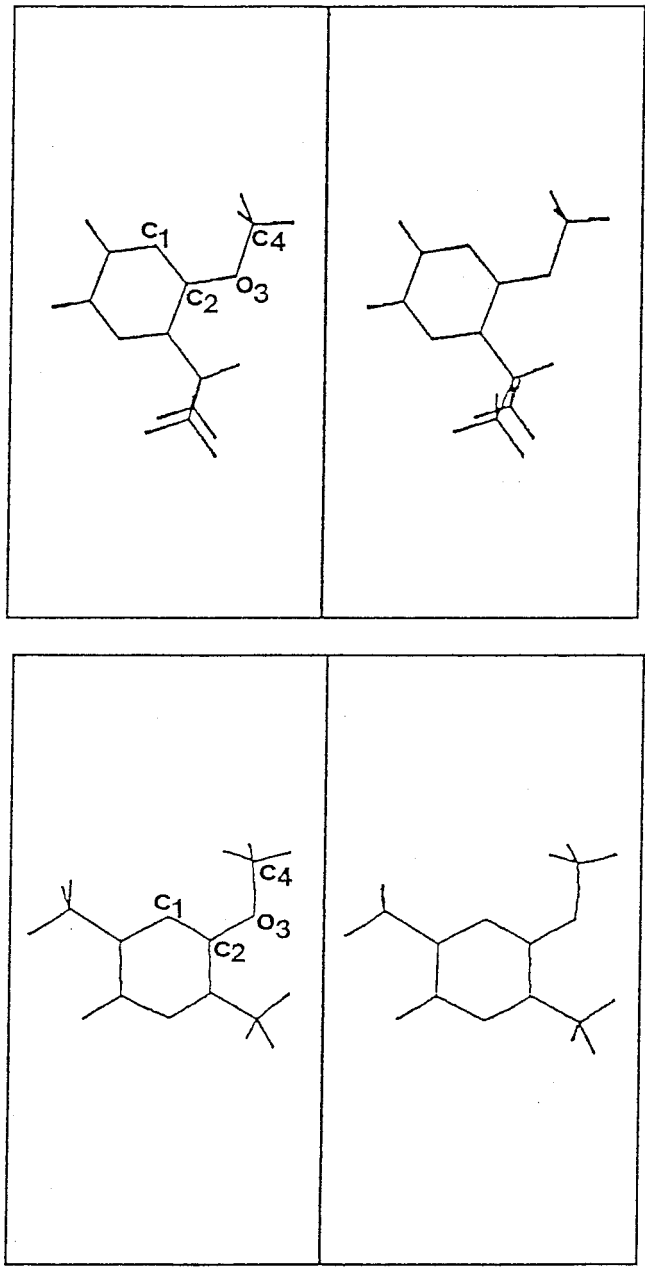

Figure 3. Stereoview of 2-isopropyl-3-methoxypyrazine (top) and 2,5-dimethyl-3-methoxypyrazine (bottom) after geometry optimization.

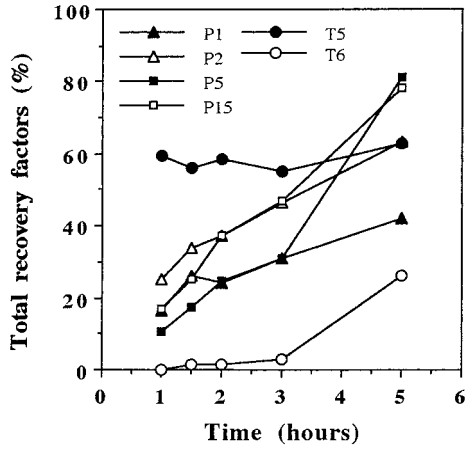

Figure 4. Total recovery factors in relation to distillation time.

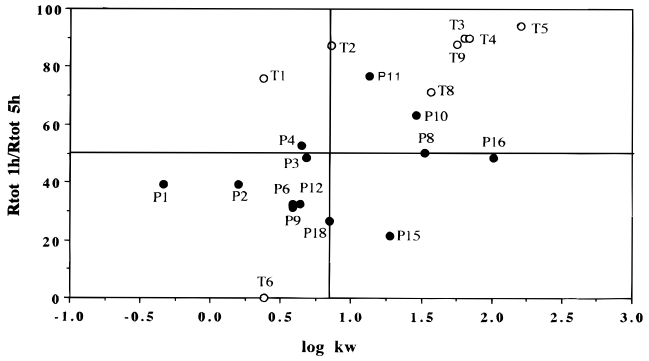

Figure 5. Relation between volatility (R_{tot} (1 h)/R_{tot} (5 h)) and lipophilicity (log k_w) of heterocyclic compounds extracted in the B phase.

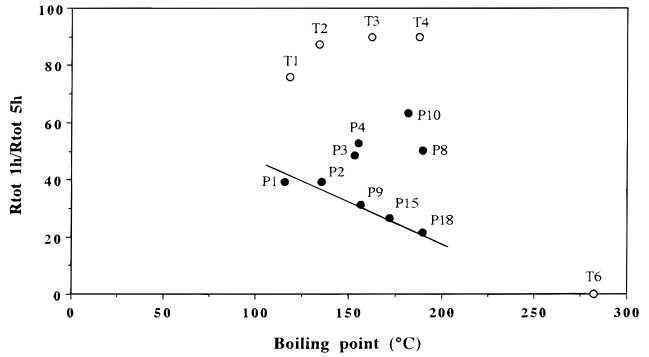

Figure 6. Relation between the volatility (R_{tot} (1 h)/R_{tot} (5 h)) and the boiling point (bp) of heterocyclic compounds extracted in the B phase.

successive dichloromethane extractions, and concentration. For all compounds, recovery is much better (average recovery improvement = 40%) after complete distillation (5 h) than after a 3 h distillation. Complete distillation greatly reduces the loss of heterocyclic compounds during the distillation step. In fact, the distillation yield can be estimated at 100%, since it gives rise to total recovery factors (R_{tot} (5 h)), similar to those obtained without distillation after liquid/liquid extraction and concentration ($R_{ext}R_{cc}$).

Hence, the ratio of the total recovery factors obtained after 1 and 5 h of distillation represents the recovery related to the first hour of distillation (R_{tot} (1 h)/R_{tot} (5 h) = R_{dist} (1 h)). This yield gives interesting information as to volatility, which can be discussed in terms of both lipophilicity and boiling temperature.

Figure 5 reveals two distinct behaviors. Less lipophilic compounds (log k_w < 0.85) are poorly recovered after 1 h of distillation while analogues with a log k_w exceeding 0.85 display recovery factors above 50%. This suggests that high lipophilicity is favorable to volatility. Due to a higher concentration at the liquid/gas interface, such highly lipophilic compounds are logically extracted more easily. For most compounds, the boiling temperature does not seem to be a crucial parameter, as shown by Figure 6. There appears to be no correlation between volatility and boiling temperature, except for homologous methylpyrazines (represented by the straight line), in which case addition of a methyl group on the ring raises the boiling temperature and lowers the distillation yield. Boiling temperature can also explain why, as shown in Figure 5, T1 recovery is high despite this compound's low log k_w value (0.373) (the boiling point

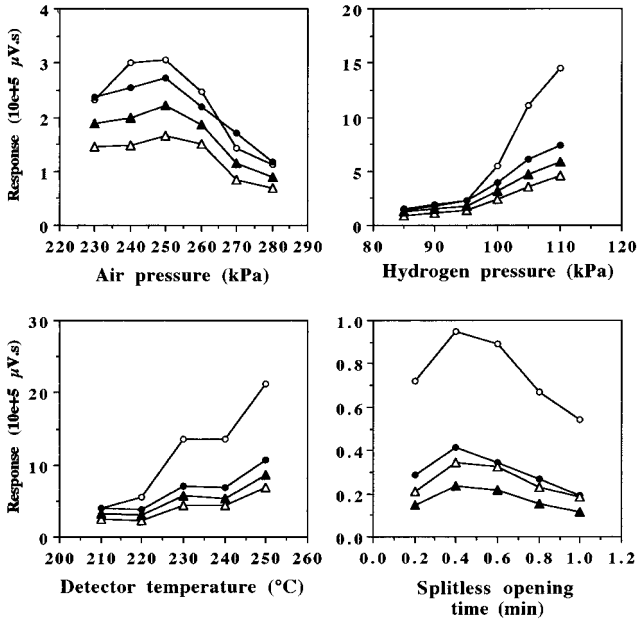

Figure 7. Steps in optimizing the splitless injector and NPD detector operating conditions (filled circles. P7; open circles, P1; filled triangles, T8; open triangles, T2).

Table 4. Effect of Ethanol on the Total Recovery Factors (%) after 2 h Distillation, Extraction, and Concentration of a 0.1 ppm Model Solution

	AN phase		B phase	
code	water	water + 5% EtOH	water	water + 5% EtOH
P1	7	8	24	35
P2	4	5	37	45
P3	18	19	40	51
P4	46	53	11	23
P5	24	34	0	1
P6	2	2	41	52
P7	57	76	1	5
P8	17	18	44	40
P9	1	1	45	54
P10	23	23	51	59
P11	29	20	50	57
P12	2	2	41	52
P13	70	82	7	3
P14	43	50	1	0
P15	0	0	37	43
P16	58	51	28	38
P17	11	38	0	2
P18	1	0	37	43
T1	1	1	41	40
T2	1	3	53	60
T3	1	2	62	72
T4	10	10	56	67
T5	10	4	59	65
T6	1	0	2	3
T7	42	62	0	0
T8	35	33	23	37
T9	1	2	59	87

is low: only 118 °C) and why, on the contrary, recovery of P15 is unexpectedly low despite its high lipophilicity (log k_w = 1.273 but boiling point = 190 °C).

Similar experiments with model solutions containing 5% ethanol showed no negative effect of this alcohol on the extraction efficiency at the concentration used (Table 4).

NPD Detector and Splitless Injector Optimization. Air and hydrogen pressures, NPD detector temperature, and splitless opening time were optimized. As shown in Figure 7, the best responses were obtained when these parameters were respectively 250 kPa, 110 kPa, 250 °C, and 0.5 min. In the case of a real sample concentrated 1000-fold (from 500 mL to 0.5 mL), 25 ppt pyrazine and 50 ppt thiazole could be detected. In beer samples, we recommend adding P13 and P11 as internal standards for phases AN and B, respectively. Both pyrazines are never encountered in this matrix and have an average recovery factor among the studied nitrogen heterocycles. Relative recovery factors can easily be deduced from Table 1. When this method was applied to a wort sample mashed with special malts, the variation coefficient was lower than 10% for alkylpyrazines and alkylthiazoles (mass spectrometry identification, concentration range: 100 ppt to 2 ppm).

CONCLUSION

An optimized extraction method including total steam vacuum distillation, liquid/liquid extraction of the aqueous distillate with a first step at pH 0.1, and NPD selective detection enabled us to quantify 50 ppt of pyrazines and thiazoles in aqueous or water/ethanol solutions. Similar concentrations of such heterocycles have been calculated in beer by using the here-published recovery factors, in comparison with the traditional method with tidious elaboration of calibration curves for increasing concentration in the initial sample. Of course, isotope dilution assay constitutes another alternative when standards are available.

For other protocol opimizations, we have clearly demonstrated that lipophilicity is the determining parameter for vacuum distillation, while basicity governs the liquid/liquid extraction.

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