1. Introduction

Reversed-phase liquid chromatography (RPLC) has been regarded as one of the most powerful separation techniques, and has been widely used for the separation and isolation of various compounds. The separation in RPLC is based on the partition of solute molecule between mobile phase (typically a mixture of organic solvent and water) and stationary phase (generally octadecyl functional groups bound to silica surface). The explanation of the retention mechanism and the corresponding experiments for understanding it have been carried out by many researchers for a long time, since RPLC was developed as a conventional separation technique [1,2]. However, the mechanism was not completely known due to the existence of many parameters controlling the separation process in typical RPLC conditions. Thus, it is quite important to understand the retention behavior in RPLC, because a good understanding of the retention mechanism that dominated the retention of solutes still remains as one of the major objectives in separation science [3,4].

Pyrazine and its derivatives comprise a group of heterocyclic nitrogen-containing and very volatile compounds that are useful and important in many industries [5,6]. They have been widely used as drugs and food flavors, and one of the most important flavor compounds responsible for the cocoa taste. Typical example of pyrazines is: pyrazine, 2,5-dimethylpyrazine and tetramethylpyrazine in cocoa products as important flavor compounds [7,8]. In pharmaceutical industry, they are found in natural medicines and
have anti-inflammatory and anti-thrombotic effects [9–11]. Thus, pyrazine and its derivatives are very important compounds in our daily life.

Based on the results in our previous investigations on the retention of pyrazines and its derivatives [12], a theoretical interpretation and the subsequent retention prediction were also studied, where the retention prediction models for pyrazines were developed using multiple linear regression and artificial neural networks [13]. In these previous investigations, the retention of pyrazines was systematically determined in various conditions, including a variety of mobile phase conditions and column temperatures. The results suggested the existence of abnormal temperature effect on the retention of several alkylpyrazines on a typical octadecylsilica (ODS) stationary phase with a mobile phase containing acetonitrile (ACN) as the organic component in reversed-phase (RP) conditions [12,13].

In this work, the temperature effect on the retention of diazines, consisted of pyrazines, pyridazines, pyrimidines and their derivatives, in RPLC was further studied at various column temperatures in the range from 0 to 50°C. For comparison the retention for pyrazole, imidazole and their methyl derivatives was also studied.

2. Experimental

2.1. Chemicals and Reagents

All reagents, solvents and sample solutes were of analytical grade and were used without further purification process. ACN, methanol (MeOH), uracil, 2-methylpyrazine, 2,3-dimethylpyrazine, 2,3,5-trimethylpyrazine, tetramethylpyrazine and 4,6-dimethylpyrimidimine were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan, and pyrazines, pyridazine, 3-methylpyridazine, pyrimidine, 4-methylpyrimidine, pyrazole, 3-methylpyrazole, imidazole, 4-methylimidazole were purchased from Tokyo Chemical Industry Co., Ltd., Tokyo, Japan. The chemical structures of compounds studied are shown in Figure 1. Water was purified by a Milli-Q Water Purification System (Millipore, Tokyo, Japan).

2.2. RPLC Measurements

The RPLC system was consisted of a PU–880 pump, a UV/Vis–990 detector (Jasco, Tokyo, Japan) and a Model 7125 injector (Rheodyne, Cotati, CA, USA) with 20 µL injection loop. Column temperature was controlled by a Model BF–61 Thermo Mate Bath (Yamato Scientific Co., Ltd., Tokyo, Japan), while a home–made water bath with a temperature–controlling system was also employed for the chromatographic measurements, if needed. For the data collection and processing, Borwin Chromatography Data Processing Software (Jasco) running on a personal computer was used.

Two types of commercially available columns packed with octadecylsilica stationary phases, ULTRON VX–ODS (150 mm × 4.6 mm i.d., Shinwa Chemical Industries Ltd., Kyoto, Japan) was employed, where octylsilica and triacontylsilica phases (Develosil C–8 and Develosil C–30, respectively; 250 mm × 4.6 mm i.d., Nomura Chemical Co., Ltd., Seto, Japan) were also used for comparison. As the mobile phase, mixtures of ACN/water and MeOH/water were used with compositions from 30/70 (v/v) to 50/50 (v/v). The mobile phase flowrate was set at 1.0 mL/min, and the column temperature was changed in the range between 0 to 50°C.

Stock solutions (1000 µg/L) of the standard analytes were prepared with either water or MeOH as the sample solvent. By diluting these stock solutions with the mobile phase solvents, all the working solutions of 20 µg/L concentration were prepared. As the dead–volume maker uracil was used, and at least five injections
were carried out for the all the chromatographic measurements. The detection wavelength for pyrazoles and imidazoles having a five–membered ring was set at 254 nm, while for pyrazines, pyridazines and pyrimidines consisted of six–membered ring, it was set at 270 nm on the basis of preliminary experiments.

3. Results and Discussion

Typical chromatograms for the separation of pyrazines on an ODS phase are shown in Figure 2. With a mobile phase consisted of MeOH and water (having the ratio from 30/70 to 50/50), all the retention factors of pyrazines were decreased with increasing the column temperature in over all the temperature range studied. This is the same trend as normally found for most of the analytes, such as polycyclic aromatic hydrocarbons (PAHs), on typical ODS phase in RPLC conditions [14−16]. However, as typically found in Figure 2 B, where ACN/water = 70/30 was used as the mobile phase, an increased retention factors for pyrazines was observed when the column temperature was elevated. A similar trend was also observed for all the ACN–based mobile phases studied. The results have a good agreement with previous reports [12,13], suggesting an abnormal temperature effect on the retentions of pyrazines with ACN/water as the mobile phase.

Figure 3 shows the van’t Hoff plots of pyrazines in the above cases with MeOH/water and ACN/water as the mobile phase, where logarithmic retention factor was plotted against the inverse absolute column temperature. As can be found in Figure 3, logarithmic retention factors for pyrazines are increasing linearly with the decrease of the column temperature using MeOH as the mobile phase component, showing a normal temperature effect found in most of the column temperature studies in RPLC [14−16]. On the other hand, with ACN/water as the mobile phase (Figure 3 B), the retention factors are logarithmically increasing with the increase of

![Figure 2](image2.png)

**Figure 2.** Typical chromatograms for the separation of pyrazines at different column temperatures. Mobile phase: (A) MeOH/water = 30/70; (B) ACN/water = 30/70. As dead volume marker, uracil (U) was used. Other conditions: column, ULTRON VX–ODS (5 µm, 150 mm × 4.6 mm i.d.); flowrate, 1.0 mL/min; detection, UV at 270 nm.

![Figure 3](image3.png)

**Figure 3.** van’t Hoff plots for pyrazines. Mobile phase: (A) MeOH/water = 30/70; (B) ACN/water = 30/70. Other conditions are the same in Figure 2; and the assignments are the same as in Figure 1.
the column temperature. During the above temperature study, no hysteretic effect was observed for the comparison of retention factors measured when the column temperature was sequentially increased and decreased to reach the target temperature.

In order to further investigate this abnormal temperature effect of pyrazines with ACN/water as the mobile phase, the separation of the same pyrazines mixture was carried out on other type of stationary phases, including octyl− and triacontyl−silica phases (Develosil C−8 and Develosil C−30, respectively), at various column temperatures from 0 to 50°C. The results clearly demonstrated that a similar trend was also observed for these stationary phases in terms of the abnormal temperature effect on the retentions of pyrazines with a mixture of ACN/water as the mobile phase. That means, for all the measurements with ACN/water as the mobile phase, the retention of pyrazines increased with elevated the column temperature, suggesting an abnormal temperature effect on the retention of these analytes with ACN as the organic solvent in the mobile phase.

The same temperature studies were also carried out for pyridazines, pyrimidines, pyrazoles and imidazoles. van’t Hoff plots for pyridazine and 3−methylpyridazine are illustrated in Figure 4, along with the corresponding plots for pyrimidines in Figure 5. From these plots, an abnormal temperature effect of these analytes could be observed with ACN as the organic component in the mobile phase. The results are quite consistent with that obtained for pyrazines. In contrast to above trend, the abnormal temperature effect was not observed for pyrazoles and imidazoles having a five−membered ring in their molecular structure, as shown Figure 6. For pyrazoles and imidazoles, the retention was logarithmically decreased with increasing the column temperature regardless the organic component in the mobile phase. The behaviors of this group of solutes have normal temperature dependence that was identical with the trend in the case using MeOH as the mobile phase component.

By calculating the enthalpy of solute transfer from mobile phase to stationary phase (Table 1), it was confirmed the abnormal temperature dependence that usually associated with enthalpy−entropy compensation effects as reported previously [17−20]. As can be seen in Table 1, all the analytes, except for pyridazines and pyrimidines, showed negative enthalpy of transfer from mobile phase to stationary phase with MeOH/water (30/70) as the mobile phase, while the values of enthalpy obtained with ACN/water (30/70)
70) are positive. At this stage, it can be said that the increased retention of diazines (pyrazines, pyridazines and pyrimidines) at elevated column temperature might be induced by the abnormal thermodynamic behavior during the partition between these stationary phase and the mobile phase containing ACN at a certain content. As similar to the extensive studies on the retention behavior of other class of compounds at various column temperatures [21−28], more comprehensive considerations should be scheduled to interpret this unusual temperature dependence.

### 4. Conclusions

From the RPLC separation of various nitrogen-containing heterocyclic compounds performed at different temperatures and with mobile phase conditions, an abnormal temperature effects on retention of diazines (pyrazines, pyridazines and pyrimidines), that is, the retention of these analytes were increased when the column temperature was elevated, was confirmed with the mobile phases consisting of ACN and water. Enthalpy of solute transfer from mobile phase to stationary phase were calculated from van’t Hoff plots, and enthalpy−entropy compensation effects were observed for diazines with ACN/water as the mobile phase. Further theoretical studies should be made to interpret the abnormal dependence of these solutes retention on the column temperature with ACN/water as the mobile phase solvent. However, the results suggest that more efficient separation of diazines could be developed by tuning up both the mobile phase and column temperature conditions.

### Acknowledgements

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### References


### Table 1. Standard enthalpy (kJ mol⁻¹) of transfer from the mobile phase to the bonded phase.

<table>
<thead>
<tr>
<th>Mobile phase</th>
<th>MeOH/water</th>
<th>ACN/water</th>
</tr>
</thead>
<tbody>
<tr>
<td>pyrazine</td>
<td>-5.33</td>
<td>1.92</td>
</tr>
<tr>
<td>2−methylpyrazine</td>
<td>-5.07</td>
<td>3.30</td>
</tr>
<tr>
<td>2,3−dimethylpyrazine</td>
<td>-5.99</td>
<td>4.29</td>
</tr>
<tr>
<td>2,3,5−trimethylpyrazine</td>
<td>-6.41</td>
<td>6.13</td>
</tr>
<tr>
<td>tetramethylpyrazine</td>
<td>-7.40</td>
<td>7.28</td>
</tr>
<tr>
<td>pyridazine</td>
<td>-2.69</td>
<td>5.25</td>
</tr>
<tr>
<td>3−methylpyridazine</td>
<td>-4.24</td>
<td>0.50</td>
</tr>
<tr>
<td>pyrimidine</td>
<td>-6.48</td>
<td>2.63</td>
</tr>
<tr>
<td>4−methylpyrimidine</td>
<td>-5.93</td>
<td>3.48</td>
</tr>
<tr>
<td>4,6−dimethylpyrimidine</td>
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<td>4.53</td>
</tr>
<tr>
<td>pyrazole</td>
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<td>4.49</td>
</tr>
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<td>-10.93</td>
<td>-2.69</td>
</tr>
<tr>
<td>imidazole</td>
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<td>-15.99</td>
</tr>
<tr>
<td>4−methylimidazole</td>
<td>N/A</td>
<td>-11.06</td>
</tr>
</tbody>
</table>

N/A: not measured.


