Retention Behavior of Pyrazines in Reversed-Phase High Performance Liquid Chromatography

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Summary

Flavor is one of the most important constituents in cocoa products such as chocolates. Its precursors are developed during fermentation and drying of cocoa beans. The flavor precursors in cocoa beans, which include free amino acids, peptides and reducing sugars, develop into cocoa-specific flavor through Maillard reactions during roasting. Through the Maillard reactions, all of the cocoa flavor precursors interact to produce chocolate flavor components such as alcohols, ethers, furans, thiazoles, pyrones, acids, esters, aldehydes, imines, amines, oxazoles, pyrazines and pyrroles. The quantity of pyrazines in cocoa beans is important in the production of cocoa products such as chocolate, because their content is one of the key parameters in producing the desirable taste of these food products.

In this thesis, retention behavior of several pyrazines and their derivatives in reversed-phase liquid chromatography (RPLC) is described. When the column temperature was changed and acetonitrile (ACN) and methanol were used as the mobile phase component, a different retention behavior for pyrazines was observed.

In Chapter 1, a general introduction of this thesis including the aims and scope of the study is described along with the background of this work. Development of prediction models using multiple linear regression (MLR) and artificial neural networks (ANNs) that could accurately describe the retention

behavior of a group of pyrazine and alkylpyrazines in RPLC is described in Chapter 2. A better understanding of the mechanisms that govern the retention of solutes in the chromatographic system remains a major goal in separation science. For this perspective, the quantitative structure retention relationship (QSRR) method has become a popular tool for investigating the retention mechanism. The main objective of such QSRR methods is to find a mathematical model that relates the retention of a given analyte to physicochemical and structural parameters. Aside from their practical application in optimization strategies, QSRR models can help us to gain some insights into the separation mechanisms that occur at the molecular level. In this chapter, retention prediction models based on MLR and ANN were developed to describe and predict the retention behavior of pyrazines under reversed-phase conditions using octadecylsilica (ODS) as the stationary phase. MLR-derived models showed that the retention of the analytes can be attributed to the effect of the organic modifier in the mobile phase (ACN or MeOH) and log P of the analytes. A comparison between the MLR and the ANN models revealed that the predictive ability of the trained ANN was better than that of MLR, especially when applied to the ACN data set. The derived models can be used as tools for method development and optimization for the analysis of pyrazines and related compounds.

In Chapter 3, the retention behaviors of pyrazine and alkylpyrazines in

RPLC are examined, and an abnormal retention behavior in RPLC of pyrazines is reported. Concerning the change in the retention behavior, various factors such as the entropy changes could be considered. In this work, it was shown that alkylpyrazines tend to be in the stationary phase when the column temperature is elevated with ACN-based mobile phase solvent. In terms of the dependence of the retention on the content of organic solvent in the mobile phase at constant temperature, all of the pyrazines showed normal behavior, as found in most RPLC conditions. The results suggest that more efficient separation of pyrazines could be developed by tuning both the mobile phase composition and the column temperature, although further theoretical studies should be performed to interpret the abnormal dependence of the pyrazines' retention on the column temperature with ACN/water as the mobile phase solvent.

To clarify this phenomenon, the temperature effect on the retention of diazines, consisting of pyrazines, pyridazines, pyrimidines and their derivatives, in RPLC was further studied at various column temperatures in Chapter 4. For comparison, the retention behavior of pyrazole, imidazole and their methyl derivatives was also studied. From the RPLC separation of various nitrogen containing heterocyclic compounds performed at different temperatures and with mobile phase conditions, an abnormal temperature effect on retention of diazines (pyrazines, pyridazines and pyrimidines) —that is, increased retention

of these analytes when the column temperature was elevated—was confirmed with the mobile phases consisting of ACN and water. The enthalpy of solute transfer from mobile phase to stationary phase was calculated from van't Hoff plots, and enthalpy–entropy compensation effects were observed for diazines with ACN/water as the mobile phase.

Finally, the overall conclusions of this thesis are summarized in Chapter 5.

Contents

Chapter 1

General Introduction

General Introduction

In the last 20 years, the food market has evolved considerably. Food companies can no longer rely on producing a small number of strong brands to remain successful. A large number of consumers often demand a large variety of high-quality products at reasonable prices, and food companies must constantly innovate and launch new products to maintain profits and market share. Therefore, to develop new food products, a more precise characterization of raw food materials is necessary, such as flavor and texture. However, because of the significant complexity of raw food materials, most of the properties are not well characterized at this stage. For example, chocolate is a complex mixture of polymorphic confectionary fats, sugars and cocoa solids. Much research has been done to clarify such textures and flavors [1–4].

Flavor is one of the most important constituents in cocoa products such as chocolates. Its precursors are developed during fermentation and drying of cocoa beans. The flavor precursors in cocoa beans, which include free amino acids, peptides and reducing sugars, develop into cocoa-specific flavor through Maillard reactions during roasting. Through the Maillard reactions, all of the cocoa flavor precursors interact to produce chocolate flavor components such as alcohols, ethers, furans, thiazoles, pyrones, acids, esters, aldehydes, imines, amines, oxazoles, pyrazines and pyrroles [5,6]. The quantity of pyrazines in cocoa beans is quite important in the production of the cocoa products such as

chocolate because their content is one of the key parameters in producing the desirable taste of these food products.

Pyrazine and its derivatives can be regarded as an important group of compounds in the food industry because of their significant contribution to the flavor formed in various roasted, toasted and heated foods. They are a common constituent of foods and are generated primarily from a heat-induced condensation between amino acids and sugars through the Strecker degradation [7]. Pyrazine derivatives exhibit a wide variety of aromas in food [8]. Development of an efficient separation technique and the subsequent determination of pyrazines could be, therefore, an essential procedure for precise quality control of these food products.

Quantitative and qualitative analyses of pyrazines are usually achieved using gas chromatography (GC), gas chromatography–mass spectrometry (GC–MS) and GC–olfactometry [9]. Because alkylpyrazines are very volatile, the condensation of organic solvent extracts is so difficult that many researchers use head-space gas analysis directly or use the head space–SPME method [10–12]. In contrast to the analysis with these GC-based methods, however, high-performance liquid chromatography (HPLC) is not widely used in the separation and determination of pyrazines except for a few examples of pharmaceutical analysis [13,14]. This is probably because an accurate determination of alkylpyrazines in an aqueous matrix is rather difficult, although

a simple analytical method could be developed with HPLC based on an appropriate optimization of the experimental conditions [13,14].

A better understanding of the mechanisms that govern the retention of solutes in a chromatographic system remains a major goal in separation science. From this perspective, the quantitative structure–retention relationships (QSRR) method has become a popular tool for investigating the retention mechanism [15,16]. The main objective of such QSRR methods is to find a mathematical model that relates the retention of a given analyte to physicochemical and structural parameters. Aside from their practical application in optimization strategies, QSRR models can help us to gain some insights into the separation mechanisms that occur at the molecular level [17–19].

Liquid chromatography (LC) is one of the most widely used methods for the separation and isolation of various compounds, including pyrazines, because of the good separation performance and wide availability. In terms of the separation mechanism in LC, however, we still have to do a lot of work to obtain a comprehensive understanding because there are many parameters controlling the actual separation process of the analytes in LC. The retention and separation mechanism of aromatic compounds, including a variety of polycyclic aromatic hydrocarbons (PAHs) and pyrazines, have been studied [20–30].

In this thesis, prediction models using the QSRR methods such as MLR and ANN were developed to describe accurately the retention behavior of a

group of pyrazine and alkylpyrazines on an octadecylsilica (ODS) bonded phase operated in HPLC. An abnormal temperature dependence of pyrazines' retention is also studied, where the retention of pyrazines was systematically measured at various column temperatures.

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Chapter 2

Prediction of Chromatographic Retention of Pyrazine and Alkylpyrazines in RP-LC

Abstract

Retention prediction models for a group of pyrazines chromatographed under reversed-phase conditions were developed using multiple linear regression (MLR) and artificial neural networks (ANNs). Using MLR, the retention of the analytes was satisfactorily described by a two predictor model based on the logarithm of the partition coefficient of the analytes (log *P*) and the percentage of the organic modifier in the mobile phase (ACN or MeOH). ANN prediction models were also derived using the predictors derived from MLR as inputs and log *k* as outputs. The best network architecture was found to be 2-2-1 for both ACN and MeOH data sets. The optimized ANNs showed better predictive properties than the MLR models, especially for the ACN data set. In the case of the MeOH data set, the MLR and ANN models have comparable predictive performance.

Introduction

Pyrazine and its derivatives have been used as drugs and food flavors, and are some of the most important flavor compounds responsible for the cocoa taste. Typical examples of pyrazines are: native pyrazine, 2,5-dimethylpyrazine and tetramethylpyrazine in cocoa products as important flavor compounds [1], and 2-ethenyl-3,5-dimethylpyrazine and 2-ethenyl-3-ethyl-5-methylpyrazine in coffee products as earthy smelling compounds [2]. It is well known that aroma precursors in cocoa beans developed in fermentation are converted to cocoa-specific aroma during the roasting via the Maillard reaction [3,4]. The quantity of pyrazines in cocoa beans is important in the production of cocoa products such as chocolate, because their content is one of the key parameters in producing the desirable taste of these food products.

The solid phase microextraction (SPME) method has become a very widely used technique for sample preparation of food products containing pyrazines [5,6]. Quantitative and qualitative analyses of pyrazines are usually achieved using gas chromatography (GC), gas chromatography–mass spectrometry (GC–MS) and GC–olfactometry [7]. Because alkylpyrazines are very volatile, the condensation of organic solvent extracts is so difficult that many researchers use head-space gas analysis directly or the head-space–SPME method [5,6,8]. In contrast to the analysis with these GC-based methods, however, high performance liquid chromatography (HPLC) is not widely used in

the separation and determination of pyrazines except for a few examples of pharmaceutical analysis [9,10]. This is probably because an accurate determination of alkylpyrazines in an aqueous matrix is rather difficult, although a simple analytical method could be developed with HPLC based on an appropriate optimization of the experimental conditions [9,10].

A better understanding of the mechanisms that govern the retention of solutes in a chromatographic system remains a major goal in separation science. From this perspective, the quantitative structure–retention relationships (QSRR) method has become a popular tool for investigating the retention mechanism [11,12]. The main objective of this QSRR method is to find a mathematical model that relates the retention of a given analyte to physicochemical and structural parameters. Aside from their practical application in optimization strategies, QSRR models can help us to gain some insights into the separation mechanisms that occur at the molecular level [13–15].

Multiple linear regression (MLR) is the method most frequently used for the statistical treatment of QSRR multivariate data consisting of a set of observed retention values and descriptors for a given set of test molecules [16]. In recent years, artificial neural networks (ANNs) [17,18] have gained popularity as a powerful chemometric tool that can be used to solve chemical problems such as the optimization of chromatographic analysis [19–28]. Compared with classical statistical analyses, ANN-based modeling does not require any

preliminary knowledge of the mathematical form of the relationships between the variables. This makes ANN suitable for the analysis of data where a hidden nonlinearity or a complex interdependency among the variables is present.

The main objective of this study was to develop prediction models using MLR and ANN that could accurately describe the retention behavior of a group of pyrazine and alkylpyrazines on an octadecylsilica (ODS) bonded phase operated in reversed-phase liquid chromatography. The retention prediction models developed include both the effects of the molecular structure of the analytes, as expressed by suitable molecular descriptors, and the influence of the mobile phase, in terms of the organic solvent component, on the retention behavior. These models are useful not only for retention prediction but also for method development and for optimizing the chromatographic conditions used in the analyses of these analytes.

Experimental

Chemicals and reagents

All regents, solvents and sample solutes were of analytical grade and were used as purchased without further purification. Acetonitrile, uracil, 2-methylpyrazine, 2,3-dimethylpyrazine, 2,3,5-trimethylpyrazine, 2,3,5,6-tetramethylpyrazine, 2-ethylpyrazine, 2,3-diethylpyrazine, and 2,3-diethyl-5-methylpyrazine were purchased from Wako Pure Chemical Industries, Osaka, Japan and pyrazine was purchased from Tokyo Kasei Kogyo, Tokyo, Japan. Water was purified by a Milli-Q Water Purification System (Millipore, Tokyo, Japan).

Standard solution preparation

Stock solutions (1000 µg/L) of the standards were prepared by dissolving in water or methanol accurately weighed amounts of the standards. Standard solutions for the actual chromatographic analysis were prepared from the stock solutions by dilution with mobile phases to obtain solutions with a concentration of 20 µg/L. Then, t_0 marker (uracil) was spiked to the mixture.

HPLC measurement

The LC system consisted of a PU-980 pump, a model MD-910 photo diode array (PDA) detector (Jasco, Tokyo, Japan) and a Model 7125 injector

(Rheodyne, Cotati, CA, USA) with a 20 µL injection loop. For the data collection and processing, Borwin Chromatography Data Processing Software (Jasco, Tokyo, Japan) running on a personal computer was used. The separation the pyrazines was carried out on a Capcell Pak C18 column (5 µm, 250 mm x 4.6 mm i.d.) purchased from Shiseido (Tokyo, Japan).

HPLC measurements were performed using acetonitrile/water or methanol/water mobile phases at a flow rate of 0.6 mL/min. An isocratic elution was used for separation of these pyrazines. The mobile phase was flowed through the column for at least 20 min prior to the measurements to ensure a stable baseline. The detection of pyrazines was performed with the PDA detector set at a wavelength of 270 nm. Chromatographic analysis was performed at ambient temperature (25 °C).

Methods

Molecular structures and descriptors

The chemical structures of the pyrazines (Fig. 2-1) were generated using Chem3D Pro 7.0 (Cambridge Soft, Cambridge, MA, USA). All structures were subjected to energy minimization by the MM2 method using the MM2 interface in Chem3D. The default values of the MM2 force field parameters found in the software were used for all energy calculations. Molecular descriptors were calculated using the minimum energy structures for all of the analytes using the free online version of PreADMET. A total of 36 constitutional, geometric, electronic, topological and physicochemical descriptors were calculated. Because there is a possibility that some of the descriptors encode for the same molecular properties, the number of descriptors used in the actual regression analysis was reduced by a variable selection method described elsewhere [29,30].

The descriptors were selected based on a simple correlation analysis. Briefly, the descriptors were correlated with each other and then with the logarithm of the retention factor (log *k*). Descriptors that were not highly correlated *(R* value less than 0.9) were automatically used in building the models. For the descriptors that were highly correlated *(R* values 0.9 and above), the one with the highest correlation coefficient with log *k* was used for the actual analysis, discarding the descriptors that showed lower correlation with log *k*. After the

selection method, the number of molecular descriptors was reduced from 36 to seven. These descriptors are the logarithm of the partition coefficient (log *P*), energy of the highest occupied molecular orbital (HOMO), energy of the highest unoccupied molecular orbital (LUMO), maximum positive charge, maximum negative charge, topological index and fraction of hydrophobic area. The values of these descriptors are shown in Table 2-1.

Model parameters and data set

The log *k* values of the analytes were used as the dependent variables or target properties during model building. The seven molecular descriptors were used as the solute-related descriptors, while the percentages of the organic component of the mobile phase were used as the eluent related descriptors. Two organic modifiers were employed in this study; namely, acetonitrile (ACN) and methanol (MeOH). The concentrations of these modifiers were varied from 10% to 40% in increments of 10%.

The number of data points for each type of organic modifier is given by the product of the number of analytes and the number of mobile phase conditions in terms of %ACN or %MeOH (10 x 4 = 40 data points).

Multiple linear regression analysis

Multiple linear regression (MLR) were derived in the following form:

$$\log k = a_0 + a_1x_1 + \ldots + a_nx_n$$

where a_0 is the intercept and an are the regression coefficients of the predictors x_n. MLR is based on the method of least squares: the model is fit such that the sum of squares of differences of observed and predicted values is minimized. A forward-stepwise MLR procedure using SPSS Version 12 for Windows (SPSS, Chicago, IL, USA) was applied to derive the MLR models in this study. For each type of organic modifier, the log k values of the compounds were regressed with the seven molecular descriptors and with %ACN or %MeOH (10, 20, 30 and 40%).

The statistical parameters such as the square of the multiple correlation coefficient (R^2), the Fisher test value (F), the standard error of estimate (s), and the significance of the model and individual descriptors (p) where used as the bases for the selection of the optimum number of predictors and the best regression equations. The reliability and predictive ability of the derived MLR equations were also tested by two cross-validation methods; namely, leave-one-out cross-validation (LOO-CV) and ten-fold cross-validation (10-fold-CV).

Artificial neural network analysis

Details on the principle, functioning and application of ANNs can be found elsewhere [18,19]. ANN analyses were performed using the Neural

Network Toolbox of Matlab Student Version (The Mathworks, MA, USA).

ANNs are mathematical or computational models that function similar to the way biological nervous systems, such as the brain, process information. An ANN consists of an interconnected group of processing elements called neurons or nodes that are sorted into three different layers; namely, the input, hidden and output layers. The organization of neurons in the three layers and the linking of these layers of neurons by modifiable weighted interconnections make up an ANN topology or architecture [18,19].

Figure 2-2 shows a schematic representation of the ANN architecture used to model the MeOH data set. The network consisted of two input neurons corresponding to the two predictors derived from MLR. Two hidden neurons were found to be optimum, and an output neuron was used to represent the target variable, log k. This architecture can be written as 2-2-1 ANN.

The procedures for ANN training and prediction were discussed in our previous works [29–34]. Briefly, a training set was used to derive the optimum ANN architecture, and a test set was employed to evaluate the predictive property of the trained ANN. In this study, ANNs were trained using the Levenberg–Marquardt algorithm with mean square error (MSE) as a measure of the performance function. Before training the data, input and output variables were normalized to have zero mean and unit standard deviation. At the start of a training run, the biases and weights were initialized at random values in the

range between +1 and –1.

A common problem encountered during ANN training is overfitting or overtraining. This problem occurs when the trained ANN loses its ability to predict new or unseen data accurately. In this study, early stopping was used to avoid this problem. A requirement for early stopping is to divide the entire data set into training, validation and test sets. The validation set was obtained by randomly taking out 20% of the data points from the training set. Early stopping works as follows: during training, the error rate of the training set decreases, whereas the validation error first decreases and subsequently begins to rise again, revealing that overtraining of the network is occurring. When the validation error rate starts to deteriorate, the training process is stopped. After training the network, its ability to generalize was tested using the test compounds.

Results and Discussion

Prediction by MLR

An MLR prediction model for each organic modifier (ACN and MeOH) was derived by forward-stepwise MLR applied first to the whole data set without dividing them into training and test sets. For both ACN and MeOH data sets, a two-predictor model was obtained, containing the descriptors for the percentage of the organic modifier (%ACN or %MeOH) and the logarithm of the partition coefficient (log *P*). Table 2-2 summarizes these results. The relatively high values of R^2, as listed in Table 2-2, indicate that the derived models show adequate fits. The results of cross-validation procedures also showed that the MLR models were reliable and had good predictive properties. This is exemplified by the coefficients of determination (q^2) for both cross-validation techniques, which were very close to the calibration R^2, as shown in Table 2-2. Prediction by MLR was also conducted by splitting the data set into training and test sets. The training set consisted of the log *k* values of seven analytes (pyrazine, 2-methylpyrazine, 2,3-dimethylpyrazine, 2,3,5-trimethylpyrazine, 2,3,5,6-tetramethylpyrazine, 2,3-diethylpyrazine and 2,3-diethyl-5-methylpyrazine) at four different ACN or MeOH concentrations (28 data points). The test set, on the other hand, consisted of the log *k* values of three analytes (2,5-dimethylpyrazine, 2,6-dimethylpyrazine and 2-ethylpyrazine) at four different ACN or MeOH concentrations (12 data points). MLR analysis

was conducted using the training set to derive the equations that describe the retention of the analytes on the chromatographic system being studied.

The best equations for the ACN and MeOH data sets as well as the values of the statistical parameters R^2, *s*, *F* and *p* are listed in Table 2-3. The derived MLR equations using the training set contain the same predictors as those listed in Table 2-2. The MLR models in Table 2-3 also showed adequate fits, as indicated by their relatively high R^2 values. In addition, the R^2 derived from the training set is very close to the calibration R^2 in Table 2-2, indicating that models derived from both data sets have almost equal fits. The overall agreement between the experimental and observed log *k* values for the training compounds for both ACN and MeOH data sets is depicted in Figs. 2-3a and 2-3c.

The test compounds were used to check the validity and predictive property of the MLR models derived from the training set. The derived MLR equations were used to predict the log *k* values of the three test analytes at four different ACN or MeOH concentrations. Figures 2-3b and 2-3d show the predictive ability of the derived MLR models when applied to the test set. The R^2 values for the test sets for ACN and MeOH data sets were found to be 0.9175 and 0.9920, respectively. The closeness of these values to the R^2 values derived from the training sets suggests the good predictive ability of the derived MLR models. The R^2 values of the test sets are close to the q^2 values derived

by LOO-CV and 10-fold-CV, indicating that the models derived by splitting the data into separate training and test sets have comparable predictive properties to the models derived when all data points were used for calibration and then cross-validated..

Prediction by ANN

In some cases, the relationship between a chromatographic parameter such as log *k* with selected descriptors may not be accurately described by a linear relationship. Therefore, in this study, nonlinear modeling was conducted using ANN. The advantage of ANN over MLR is that ANN does not require knowledge of a mathematical model before the data are fitted. ANN can detect hidden nonlinearities among the variables that are not seen by MLR. One objective of this study is to compare the predictive performance of MLR- and ANN-derived models. Thus, the same set of training and test data were used for both MLR and ANN analyses. Also, the same predictors found in the MLR equations were used as inputs during the ANN modeling.

A three-layer feed-forward ANN [18,19] was used to derive retention prediction models for the studied analytes. A 2-2-1 network architecture was found to be the best in describing the retention for both ACN and MeOH data sets. This architecture generates nine adjustable connections: four connections link the two input neurons to the two hidden neurons, two

connections join the two hidden neurons to the output neuron, and two biases both of which have an activation level of +1 are connected to the hidden and output neurons by an extra three connections (Fig. 2-2). The important quantity in ANN modeling is not the overall number of connections but the ratio of the number of data points (training cases) to the number of model parameters or connections [35]. In addition, according to the recommended guidelines for determining the number of hidden neurons to be used [35], the ratio of the number of training cases to the number of model parameters should be approximately two. In the present case, this ratio is 3.11 for both the ACN and MeOH data sets.

Plots of the predicted versus the experimental log *k* values for the training and validation sets for all of the ACN and MeOH data sets are shown in Figs. 2-4a and 2-4c, respectively. The trained ANNs approximated the training set very well, as shown by the high R^2 values obtained for the ACN and MeOH data sets.

Generalization is the ability of an ANN not only to learn training data but also to perform well on unseen or new data. This property was assessed using the same test set as that used for MLR prediction. Plots of the predicted log *k* values obtained from ANN analyses versus the observed log k values for the test compounds are shown in Figs. 2-4b and 2-4d. The R^2 values for the test sets were found to be 0.9672 and 0.9866 for the ACN and MeOH data sets,

respectively. The high R^2 values, especially for the MeOH data set, indicate that the optimized ANNs generalized very well and that no overtraining occurred during the training process.

Comparison of MLR and ANN models

Graphical comparisons of the models derived from MLR and ANN are shown in Figs. 2-3 and 2-4. The performances of the models during the fitting or training stages are depicted in Figs. 2-3a, 2-3c and 2-4a, 2-4c while Figs. 2-3b, 2-3d and 2-4b, 2-4d show the predictive abilities of the derived models when applied to the test sets. The R^2 values and the root mean square error of prediction (RMSEP) were also used to quantify the predictive abilities of the models derived from MLR and ANN. The *RMSEP* is given by the equation:

$$RMSEP = \sqrt{\frac{\sum_{1}^{n} |\log k(observed) - \log k(predicted)|^2}{n}}$$

Table 2-4 summarizes these values for both the ACN and MeOH data sets. For the chromatographic system involving ACN as the organic modifier in the mobile phase, the ANN model showed a better predictive performance for both the training and the test sets. The performance of the MLR model derived for the ACN data set reveals both significant scattering and curvature of the

computed data points with respect to the ideal trend for both the training and the testing procedures. The ANN models, however, gave a better agreement between the predicted and the experimental log *k* values for both training and testing stages. The better predictive performance of the ANN model as compared with the MLR model is exemplified by the less scattered plots of the predicted and experimental log k values, as shown in Figs. 2-4c and 2-4d. The MLR model derived for the ACN data set was less accurate than the ANN model in predicting the log *k* values of the test analytes, as indicated by its lower R^2 and higher *RMSEP* values compared with those of the ANN models.

In the case of the MeOH data set, the derived MLR and ANN models showed comparable performance for both the training and testing stages. The plots shown in Figs. 2-3c, 2-3d and Figs. 2-4c, 2-4d that were derived from the MLR and ANN models, respectively, show good agreement between predicted and experimental log k values. The values of the *RMSEP* and R^2 derived for both MLR and ANN models, as listed in Table 2-4, are also close to each other, signifying that the models have almost the same predictive property. It can also be noted that prediction is more accurate in the MeOH data set than in the ACN data set.

For the ACN data set, addition of other solute-related descriptors to the MLR and ANN models did not significantly improve the performance of the models.

Retention prediction of extracted analytes from cocoa mass

The performances of the MLR- and ANN-derived models were also tested to predict the retention of five analytes in Table 2-5 extracted by steam distillation from cocoa mass. The calculated log *k* values from each model were compared with the experimental log *k* values obtained using mobile phases containing ACN of MeOH. Satisfactory separation of the analytes was achieved using 20% aqueous ACN and 40% MeOH, therefore, these mobile phases were used for comparison. Table 2-5 gives the comparison of the MLR and ANN predicted log *k* values and the observed log *k* values of the extracted analyte from the sample mixture. It is clearly seen in Table 2-5 that the log *k* values predicted by the trained ANN were much closer to the observed log *k* values of the sample mixtures compared with those of the MLR predicted values for both mobile phase conditions. In addition, the lower average percent deviation obtained from the ANN models demonstrates the superior predictive property of the ANN models over the MLR models.

Conclusions

In this chapter, retention prediction models based on MLR and ANN were developed to describe and predict the retention behavior of pyrazines under reversed-phase conditions using ODS as the stationary phase. MLR-derived models showed that the retention of the analytes can be attributed to the effect of the organic modifier in the mobile phase (ACN or MeOH) and log *P* of the analytes.

A comparison between the MLR and the ANN models revealed that the predictive ability of the trained ANN was better than that of MLR, especially when applied to the ACN data set. The derived models can be used as tools for method development and optimization for the analysis of pyrazines and related compounds.

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Table 2-1. Values of the molecular descriptors used in the forward-stepwise multiple linear regression.

Compounds	Molecular Descriptors						
	Maxpos	Maxneg	FHyphob	TopIndex	HOMO	LUMO	logP
Pyrazine	0.27	-0.52	0.88	3	-7.79	-0.88	-0.23
2-Methylpyrazine	0.28	-0.52	0.84	4	-7.66	-0.84	0.23
2,3-Dimethylpyrazine	0.27	-0.50	0.81	4	-7.54	-0.57	0.54
2,5-Dimethylpyrazine	0.28	-0.50	0.81	5	-7.53	-0.81	0.63
2,6-Dimethylpyrazine	0.28	-0.52	0.81	4	-7.56	-0.47	0.54
2,3,5-Trimethylpyrazine	0.28	-0.49	0.79	5	-7.42	-0.49	0.95
2,3,5,6-Tetramethylpyrazine	0.14	-0.47	0.77	5	-7.30	-0.16	1.28
2-Ethylpyrazine	0.28	-0.52	0.86	5	-7.63	-0.82	0.69
2,3-Diethylpyrazine	0.27	-0.50	0.85	5	-7.45	-0.37	1.51
2,3-Diethyl-5-methylpyrazine	0.28	-0.49	0.83	6	-7.33	-0.29	1.95

Maxpos – maximum positive charge,
Maxneg – maximum negative charge,
Fhyphob – fraction of hydrophobic area,
TopIndex – topological index,
HOMO – energy of highest occupied molecular orbital,
LUMO – energy of lowest unoccupied molecular orbital,
log *P* – logarithm of partition coefficient

Table 2-2 MLR-derived models using all cases in ACN and MeOH data sets.

Modifier	MLR Equation	Calibration R^2	Cross Validation	
			$q^2_{\text{LOO-CV}}$	$q^2_{\text{10-fold}}$
ACN	$\log k = 0.232 - 0.0292\ \text{ACN} + 0.691 \log P$	0.9244	0.9059	0.9062
MeOH	$\log k = 0.603 - 0.033\ \text{MeOH} + 0.892 \log P$	0.9723	0.9650	0.9639

Table 2-3 MLR models derived from the training sets of both ACN and MeOH data sets.

Organic Modifier	MLR equation	R^2	F	s	P
ACN	$\log k = 0.259 \pm 0.094 - (0.030 \pm 0.003)$ ACN + $(0.689 \pm 0.048) \log P$	0.9239	151.68	0.1796	$<10^{-4}$
MeOH	$\log k = 0.608 \pm 0.067 - (0.033 \pm 0.002)$ MeOH + $(0.898 \pm 0.036) \log P$	0.9708	398.32	0.1276	$<10^{-4}$

Table 2-4 Values of R^2 and RMSEP for the MLR and ANN models.

	ACN		MeOH	
	MLR	ANN	MLR	ANN
Training Set				
R^2	0.9239	0.9865	0.9708	0.9879
Test Set				
R^2	0.9175	0.9672	0.9920	0.0505
RMSEP	0.0979	0.0629	0.9866	0.0447

Table 2-5 Retention prediction of pyrazines in real sample matrices.

	Predicted log *k*		Observed log *k*	%DEV [a)]	
	MLR	ANN		MLR	ANN
20% ACN					
Pyrazine	-0.511	-0.592	-0.593	5.80	0.07
2-Methylpyrazine	-0.193	-0.312	-0.324	9.26	0.85
2,5-Dimethylpyrazine	0.083	-0.047	-0.074	11.10	1.91
2,3,5-Trimethylpyrazine	0.305	0.203	0.144	11.38	4.17
2,3,5,6-Tetramethylpyrazine	0.533	0.491	0.367	11.74	8.77
40% MeOH					
Pyrazine	-0.922	-0.763	-0.779	10.11	1.13
2-Methylpyrazine	-0.512	-0.435	-0.431	5.73	0.28
2,5-Dimethylpyrazine	-0.155	-0.130	-0.130	1.77	0.00
2,3,5-Trimethylpyrazine	0.130	0.110	0.120	0.71	0.71
2,3,5,6-Tetramethylpyrazine	0.425	0.367	0.382	3.04	1.06

a) Average percent deviation of each predicted log *k* from observed log *k*.

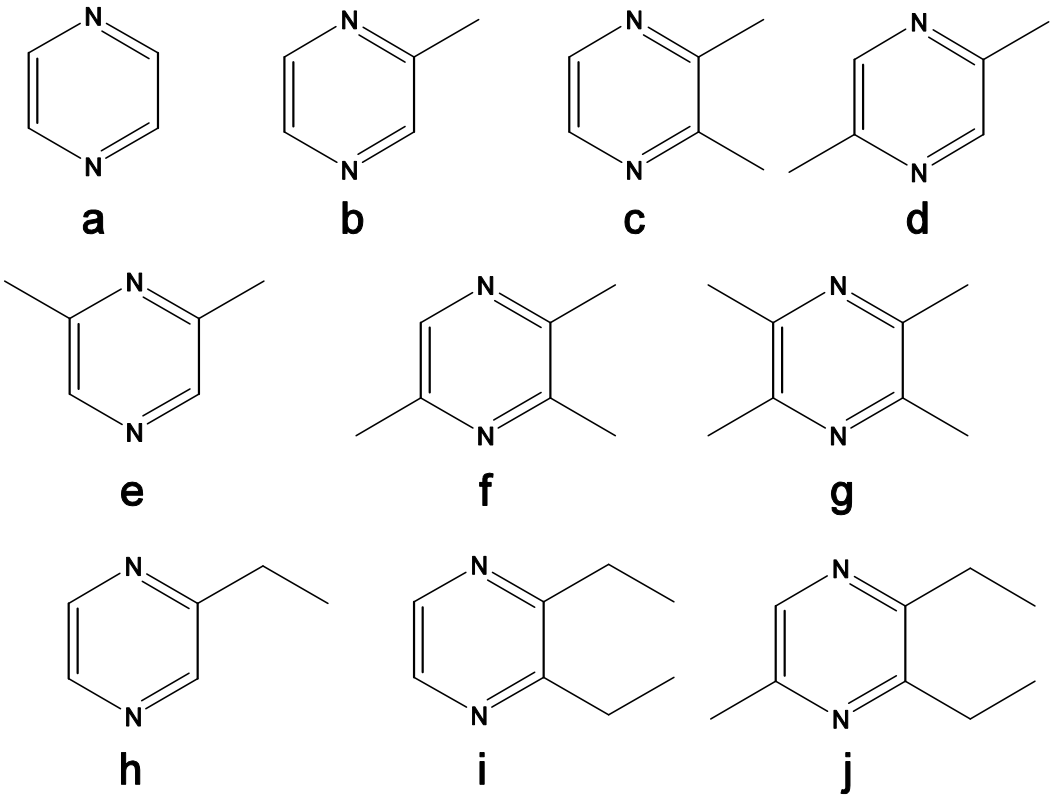

Figure 2-1 Structures of the analytes used in this study.
a) Pyrazine, b) 2-Methylpyrazine, c) 2,3-Dimethylpyrazine,
d) 2,5-Dimethylpyrazine, e) 2,6-Dimethylpyrazine, f) 2,3,5-Trimethylpyrazine,
g) 2,3,5,6-Tetramethylpyrazine, h) 2-Ethylpyrazine, i) 2,3-Diethylpyrazine and
j) 2,3-Diethyl-5-methylpyrazine.

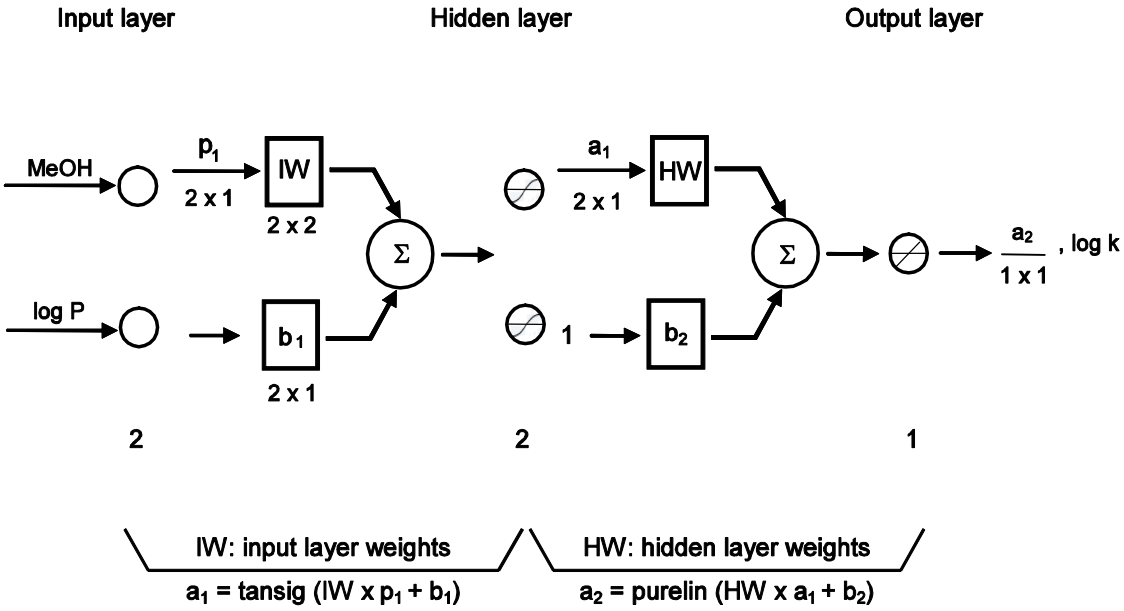

Figure 2-2 Schematic representation of the ANN architecture used for training the MeOH data set. The input layer consists of two neurons corresponding to the two predictors derived from MLR. The hidden layer contains two neurons, while the output layer has one neuron representing the dependent variable log *k*. The input neurons are connected via four adjustable connections to the hidden neurons, which are further linked to the output neuron via two connections. A bias is linked to the hidden and output neurons by an extra three connections. In this neural network, the Tan-Sigmoid (tansig) and the linear (purelin) transfer functions are used in the hidden and output layers, respectively.

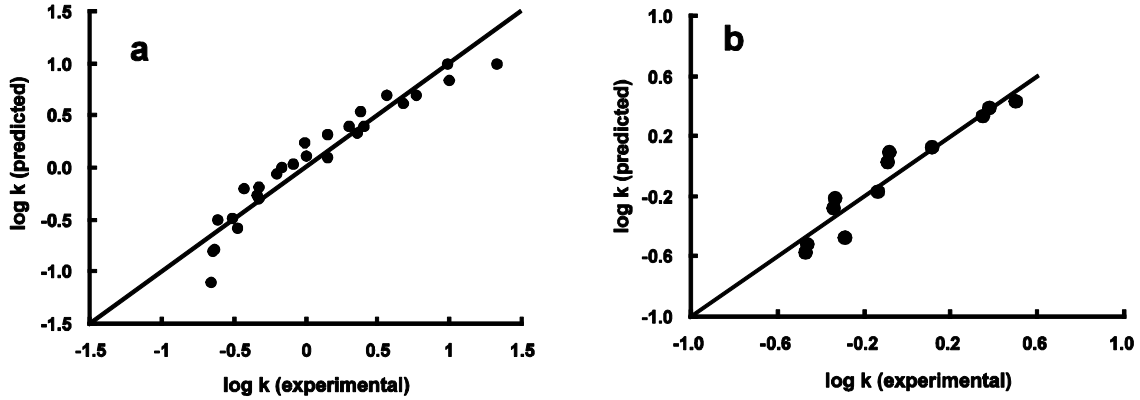

Figure 2-3-1 Plots of the predicted vs. observed log *k* using the derived MLR models. (a) and (b) are for the training and test sets of the ACN data set, respectively. The data points for the training set represent the log *k* values of 7 compounds at 4 different %ACN (10, 20, 30 and 40%). Points on (b) is predicted log *k* values of the 3 test compounds at 4 different %ACN (10, 20, 30 and 40%). The regression line represents the line when predicted log *k* values are equal to experimental log *k* values.

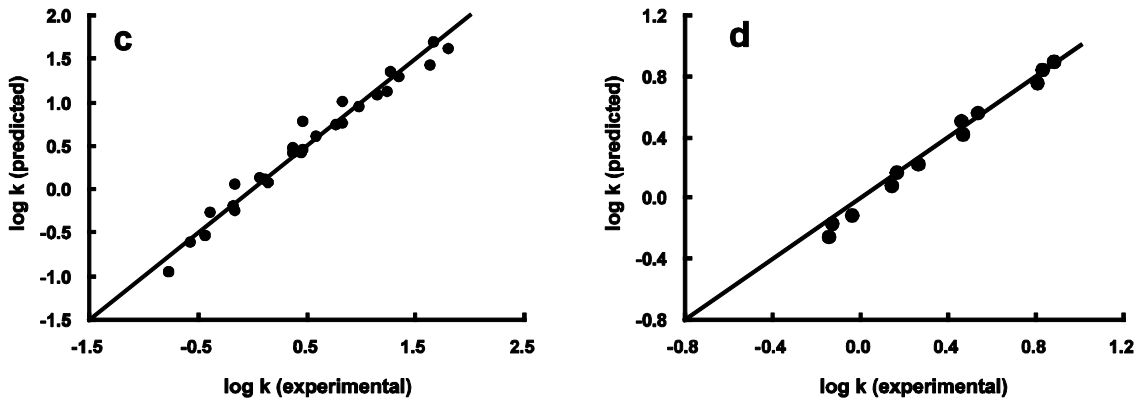

Figure 2-3-2 Plots of the predicted vs. observed log *k* using the derived MLR models. (c) and (d) are for the training and test sets of the MeOH data set, respectively. The data points for the training set represent the log *k* values of 7 compounds at 4 different %MeOH (10, 20, 30 and 40%). Points on (d) is predicted log *k* values of the 3 test compounds at 4 different %MeOH (10, 20, 30 and 40%). The regression line represents the line when predicted log *k* values are equal to experimental log *k* values.

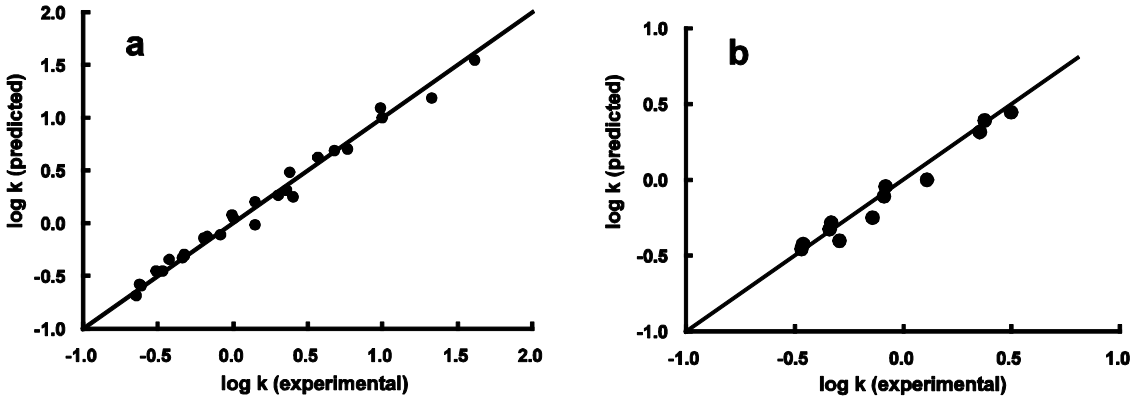

Figure 2-4-1 Plots of the predicted vs. observed log *k* using the derived ANN models. (a) and (b) are for the training and test sets of the ACN data set, respectively. Other conditions are the same as in Fig. 2-3-1.

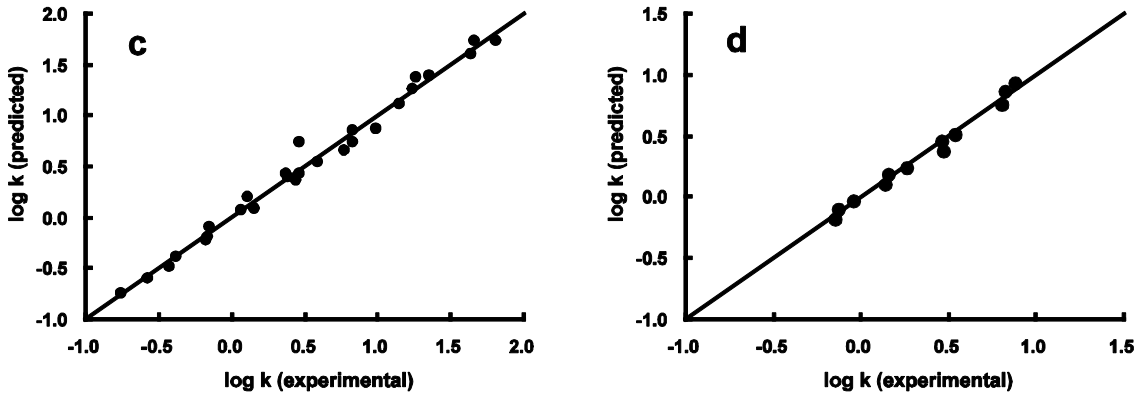

Figure 2-4-2 Plots of the predicted vs. observed log *k* using the derived ANN models. (c) and (d) are for the training and test sets of the MeOH data set, respectively. Other conditions are the same as in Fig. 2-3-2.

Chapter 3

An Abnormal Temperature Dependence of Alkylpyrazines' Retention in Reversed-Phase Liquid Chromatography

Abstract

Retention behaviors of pyrazine and alkylpyrazines on various stationary phases in reversed-phase liquid chromatography were examined. An abnormal temperature effect on the retention of alkylpyrazines with a mobile phase consisting of acetonitrile and water was observed when changing the column temperature. In contrast, no similar trend was found with a methanol–water mobile phase. For all of the columns investigated in this work, the above tendency of the temperature dependence was consistently observed, suggesting that the abnormal temperature effect on the retention of alkylpyrazines could be mainly induced by an acetonitrile-based mobile phase.

Introduction

Pyrazine and its derivatives are among the most important components in processed foods. Pyrazines are important contributors to the flavor of various roasted, toasted or similarly heated foods. They are a common constituent of foods and are thought to arise primarily from a heat-induced condensation between amino acids and sugars, through the Strecker degradation [1]. Pyrazine derivatives exhibit a wide variety of aromas in food. For instance, 2,5-dimethylpyrazine and tetramethylpyrazine in cocoa products are important flavor compounds [2], and 2-ethenyl-3,5-dimethylpyrazine and 2-ethenyl-3-ethyl-5-dimethylpyrazine are present in coffee products as earthy smelling compounds [3].

Liquid chromatography (LC) is one of the most widely used methods for the separation and isolation of various chemical compounds because of its good separation performance. In contrast to a number of LC applications in various scientific fields, such as pharmaceutical, agricultural and medicinal sciences as well as separation science, the separation mechanism in the chromatographic process has not been clarified yet. This is because there are many parameters controlling the recognition process of solute molecules in LC. To clarify the molecular recognition mechanism, polycyclic aromatic hydrocarbons (PAHs) as the sample probe have been introduced in reversed-phase (RP) LC using an octadecylsilica (ODS) stationary phase [4,5].

Because PAHs possess relatively simple molecular structures compared with real samples that are normally separated in LC applications, studying their retention behavior is suitable for the systematic analysis of the separation mechanism based on molecular shape recognition. Fullerene separation with monomeric and/or polymeric ODS phases found that the bonded phase association and the configuration change with temperature are important factors controlling the retention of fullerenes in LC [6–9]. The stationary phase needs to have enough depth and space to catch and hold the bulky molecules within the space between ligands attached to the silica gel. Therefore, it is reasonable to assume that a bonded phase having long alkyl chains may interact more effectively with bulky molecules.

In terms of the separation mechanism in LC, however, we still have to do a lot of work to obtain a comprehensive understanding because there are many parameters controlling the actual separation process of the analytes in LC. The retention and separation mechanism of aromatic compounds including a variety of polycyclic aromatic hydrocarbons (PAHs) and pyrazines have been studied [4–17].

On the basis of actual retention measurements for pyrazines in several experimental conditions, a theoretical interpretation and the subsequent retention prediction were also studied in our previous work [17], where the retention prediction models for pyrazine and alkylpyrazines were developed

using multiple linear regression (MLR) and artificial neural networks (ANNs). During the study, the retention of these compounds was carefully determined in various conditions, including a variety of mobile phase conditions and column temperatures. The results suggested the existence of an abnormal temperature dependence in the retention of several alkylpyrazines on a typical ODS stationary phase with a mobile phase containing ACN as the organic component in RP conditions.

In this chapter, the temperature effect on the retention of pyrazines was further studied over the temperature range from 10 °C to 60 °C using various types of alkyl-bonded stationary phases and mobile phase compositions in RPLC.

Experimental

Materials and methods

All regents, solvents and sample solutes were of analytical grade and were used as purchased without further purification. ACN, methanol (MeOH), uracil, 2-methylpyrazine, 2,3-dimethylpyrazine, 2,3,5-trimethylpyrazine and 2,3,5,6-tetramethylpyrazine were purchased from Wako Pure Chemical Industries, Osaka, Japan and pyrazine was purchased from Tokyo Kasei Kogyo, Tokyo, Japan.

The chemical structures of the alkylpyrazines used in this work are shown in Fig. 3-1. Water was purified by a Milli-Q Water Purification System (Millipore, Tokyo, Japan).

LC measurements

Stock solutions (1000 μg/L) of the standards were prepared by dissolving in water or methanol, and were used for preparing standard working solutions in the actual chromatographic analysis, where these stock solutions were diluted with mobile phase to obtain a concentration of 20 μg/L. For the dead time measurement, the peak of uracil added to all samples solutions was used.

The retention behavior of pyrazines was studied with the following commercially-available columns: Inertsil ODS-3, C8-3 and C4 columns (GL

Sciences, Tokyo, Japan), Capcell Pak C18 UG120 and C1 UG120 columns (Shiseido, Tokyo, Japan), a Sunrise C28 column (ChromaNic Technologies, Osaka, Japan), where all the column dimensions and the particle sizes are the same: 150 mm x 4.6 mm i.d. and 5 µm.

The LC system consisted of a PU-980 pump, a Model MD-910 photodiode array detector (Jasco, Tokyo, Japan) and a Model 7125 injector (Rheodyne, Cotati, CA, USA) with a 20 µL injection loop. The column temperature was mainly controlled using a Model CO-2060 column oven (Jasco, Tokyo, Japan), while a home-made water bath with a temperature-controlling system was employed for the chromatographic measurements at subambient temperatures, as described earlier [6,7]. For the data collection and processing, Borwin Chromatography Data Processing Software (Jasco, Tokyo, Japan) running on a personal computer was used.

LC measurements were performed with ACN/water or MeOH/water mobile phase at a flow rate of 0.6 mL/min. An isocratic elution was employed in all of the chromatographic runs. The mobile phase was equilibrated for at least 20 min prior to the measurements. The detection of pyrazines was done with the detector set at a wavelength of 270 nm. Spectrum measurements to make sure of the identification of analytes were carried out, if necessary. Measurements were performed at least five times for each set of separation conditions.

Results and Discussion

Temperature is one of the major factors influencing the retention ability of the stationary phase. The separation of a standard mixture containing five pyrazines (Fig. 3-1) was carried out on all of the stationary phases described above at different column temperatures. Figure 3-2 shows typical chromatograms for the separation of pyrazines at two different column temperatures, 30 °C and 60 °C, on an ODS phase. When using methanol in the mobile phase, decreasing the column temperature induces a slight increase in retention of pyrazine solutes. This is normal chromatographic behavior [18–22].

In contrast, with ACN as a component of the mobile phase, all of the retention factors for these alkylpyrazines were increased when the column temperature was elevated, suggesting an abnormal temperature dependence of the retention factor, as found in previous reports [23,24]. Similar trends were also observed for all other alkyl-bonded stationary phases employed in this work. To explain this phenomenon, the result of measuring the partition coefficient of an organic solvent (the solvent chosen for the stationary phase) and mobile phase solvent that dissolves tetramethylpyrazine at each temperature is shown in Figure 3-3. Here, the aspect of the organic solvent is chosen in the stationary phase, and water/acetonitrile and water/methanol solutions are chosen for the mobile phase. The phase in which tetramethylpyrazine was

distributed easily was confirmed. For the results, when existing on the mobile phase side is steadier than on the stationary phase side and acetonitrile is used or the column temperature is very low, tetramethylpyrazine is especially remarkable. From the result, tetramethylpyrazine is more stable in the acetonitrile/water mobile phase than in the methanol/water mobile phase at low temperature. It is thought that is one of the reasons that retention of alkyl pyrazines decreases as the column temperature decreases.

For a better understanding of the abnormal temperature effect on the retention of pyrazines with ACN as the organic modifier in the mobile phase, van't Hoff plots were prepared, as shown in Fig. 3-3. In RPLC with a typical nonpolar stationary phase, such as an ODS phase, the retention can be expressed by the following equation:

$$\ln k = -\Delta H/RT + \Delta S/R + \ln \Phi, \quad (1)$$

where k is the solute retention factor, ΔH and ΔS are the enthalpy and the entropy of solute transfer from the mobile phase to the stationary phase, respectively. R is the gas constant, T is the absolute temperature and Φ is the volume phase ratio of the stationary phase and mobile phase. Equation (1) clearly shows a linear relationship between $\ln k$ and $1/T$, known as the van't Hoff plot. Therefore, it is quite natural that a linear plot could be obtained in typical RPLC conditions, if the retention mechanism is constant over the temperature

range studied [8–12].

As can be seen in the plots in Fig. 3-3, with a mobile phase consisting of MeOH and water, a good linear relationship was observed for all alkylpyrazines on both octyl- and octadecyl-bonded (i.e., ODS) stationary phases. Logarithmic retention factors for all of the analytes increase linearly with the decrease in the column temperature, as confirmed in most of the column temperature studies in RPLC. For all of the measurements with ACN as the organic solvent in the mobile phase, however, the retention factors are logarithmically decreasing with the decrease in the column temperature, clearly showing an abnormal temperature effect on the retention with a mixture of ACN/water as the mobile phase. In addition, a similar trend was also observed for all other stationary phases, including C1 and C28 phases, studied in this work.

During the above temperature study, all of the retention factors at a particular column temperature were measured at least five times, and no hysteretic effect was observed for the comparison of retention factors measured when the column temperature was sequentially increased or decreased to reach the desired temperature. As can be expected, for all of the stationary phases studied, the retention of pyrazines was decreased with increasing content of organic solvent, MeOH or ACN, at constant column temperature, indicating that the dependence of the retention on the volume fraction of organic solvent in the

mobile phase could be the same tendency as found in conventional RPLC separations [4,5].

Table 3-1 summarizes the calculated enthalpy of solute transfer from the mobile phase to an ODS stationary phase. Although all of the analytes showed a negative enthalpy of transfer from the mobile phase to the stationary phase with MeOH/water as the mobile phase, as can be expected from Fig. 3-3, the values of enthalpy obtained with certain mobile phase compositions consisting of ACN and water are positive. In addition, when increasing the content of ACN in the mobile phase, the value of the enthalpy will be decreased, showing a relatively more thermodynamically stable state of pyrazines in the stationary phase.

Similar studies were also carried out for pyridine and triazine; however, the behavior could be classified as a normal temperature dependence in conventional RPLC conditions. The logarithmic retention factors increase linearly with the decrease in the column temperature, and there were no observable differences in the temperature dependence of the retention between the mobile phases of MeOH/water and ACN/water.

Similar to the extensive studies on the retention behaviors of other aromatic compounds such as PAHs [4–7], more precise and comprehensive considerations, including theoretical interpretation of this phenomenon, should be considered for the analysis of this unusual temperature dependence. At this

stage, however, it can be said that the increased retention of pyrazines at elevated column temperatures might be induced by the abnormal thermodynamic behavior during the partition between these stationary phases and the mobile phase containing ACN at a certain content. This abnormal temperature dependence might be useful for the development of a more efficient, but environmentally friendly, separation protocol of alkylpyrazines in RPLC.

Conclusions

In this chapter, an abnormal temperature effect on the retention of pyrazines was confirmed with mobile phases consisting of ACN and water in this work. In terms of the dependence of the retention on the content of organic solvent in the mobile phase at constant temperature, all of the pyrazines showed normal behavior, as found in most RPLC conditions. The results suggest that more efficient separation of pyrazines could be developed by tuning both the mobile phase composition and the column temperature, although further theoretical studies should be performed to interpret the abnormal dependence of the pyrazines' retention on the column temperature with ACN/water as the mobile phase solvent.

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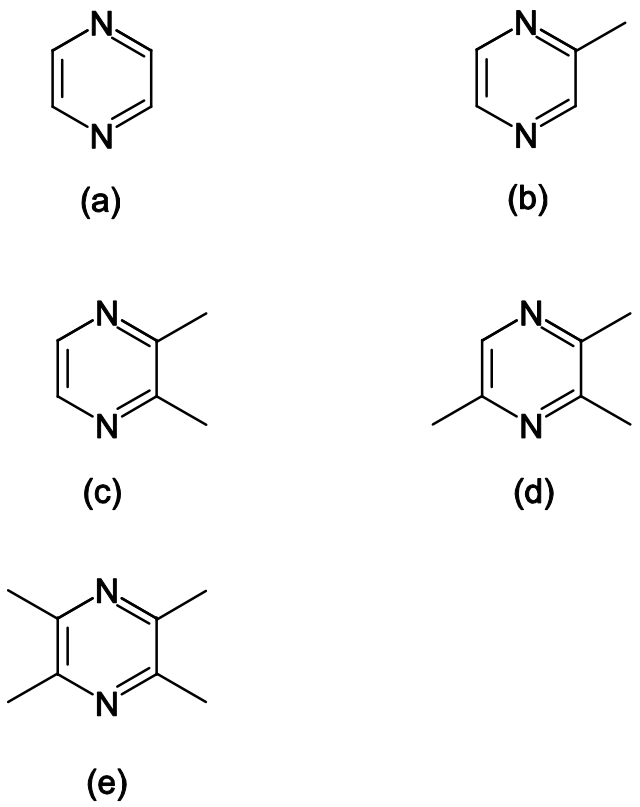

Figure 3-1 Chemical structures of pyrazine and alkylpyrazines used in this work.

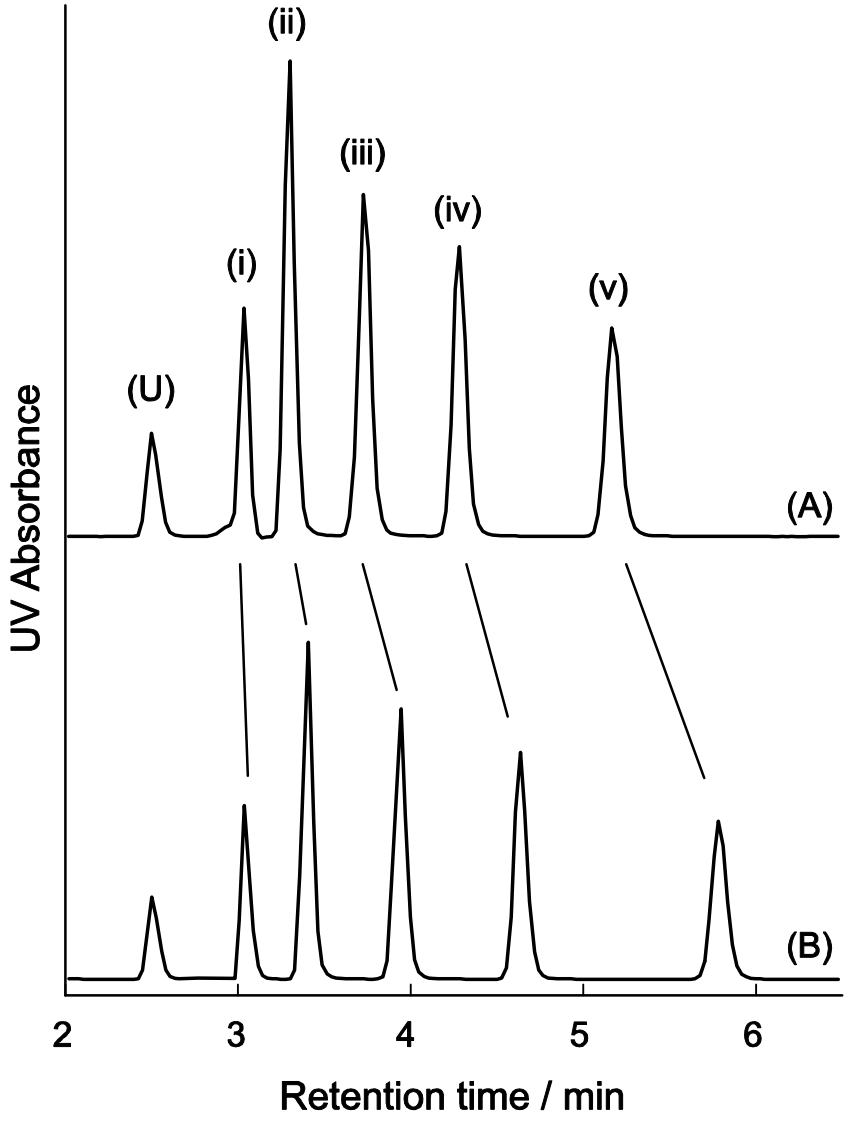

Figure 3-2 Typical chromatograms for the separation of pyrazine and alkylpyrazines at different column temperatures. (A) 30ºC and (B) 60ºC. Other conditions: column, Inertsil ODS-3 (5 µm, 150 mm x 4.6 mm i.d.); mobile phase, ACN/water = 30/70, flowrate, 0.6 mL/min; detection, UV at 270 nm. Peaks: (U) uracil, (i) pyrazine, (ii) 2-methylpyrazine, (iii) 2,3-dimethylpyrazine, (iv) 2,3,5-trimethylpyrazine, (v) 2,3,5,6-tetramethylpyrazine.

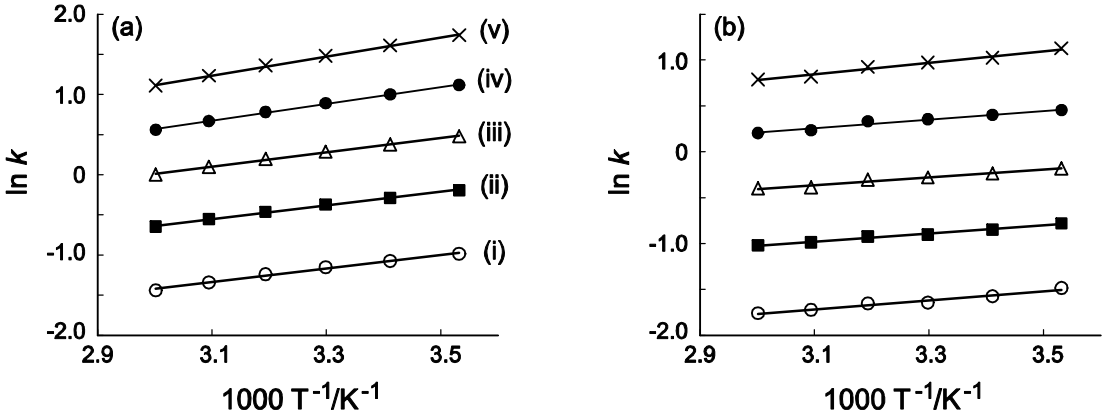

Figure 3-3-1 van't Hoff plots for pyrazines with MeOH/water = 40/60 as the mobile phase on two types of bonded stationary phases. Stationary phase: (a) Inertsil ODS-3 (5 µm, 150 mm x 4.6 mm i.d.), (b) Inertsil C8-3 (5 µm, 150 mm x 4.6 mm i.d.). Other chromatographic conditions and the peak assignments are the same as in Fig. 3-2.

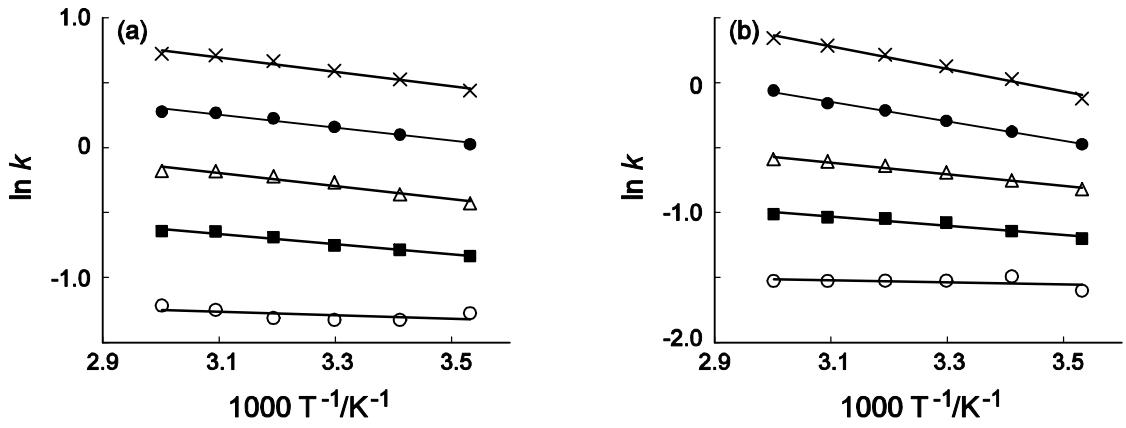

Figure 3-3-2 van't Hoff plots for pyrazines with (B) ACN/water = 30/70 as the mobile phase on two types of bonded stationary phases. Stationary phase: (a) Inertsil ODS-3 (5 µm, 150 mm x 4.6 mm i.d.), (b) Inertsil C8-3 (5 µm, 150 mm x 4.6 mm i.d.). Other chromatographic conditions and the peak assignments are the same as in Fig. 3-2.

Table 3-1 Standard enthalpy (kJ mol^{-1}) of transfer for pyrazines from various mobile phases to the bonded phase of the ODS column

Analyte[a]	Mobile phase					
	MeOH/water			ACN/water		
	30/70	40/60	50/50	30/70	40/60	50/50
i	-4.66	-4.07	-3.99	0.67	-1.25	-5.99
ii	-4.49	-3.66	-2.24	2.91	-1.08	-3.24
iii	-5.57	-3.49	-2.49	3.74	2.00	-1.08
iv	-5.99	-3.91	-2.58	6.32	4.49	2.16
v	-6.98	-5.24	-3.33	7.23	6.15	3.82

a. All the assignments are the same as in Fig. 3-1.

Chapter 4

Effect of Temperature on Retention of Diazines in Reversed-Phase Liquid Chromatography

Abstract

The retention behavior of diazines, consisting of pyrazines, pyridazines and pyrimidines, was studied at various column temperatures using reversed-phase liquid chromatography (RPLC). An increased retention of pyrazines was observed on several types of stationary phases, including octadecylsilica (ODS) phases, when the column temperature was elevated, suggesting an abnormal temperature effect. A similar abnormal temperature effect on the retention was also observed for pyridazines and pyrimidines with an acetonitrile/water mobile phase at elevated column temperatures.

In contrast, no similar trend was found for these analytes with a methanol/water mobile phase. In addition, the retention for pyrazole, imidazole and their methyl derivatives decreased with increasing column temperature regardless of the type of organic modifier in the mobile phase. The results suggest that the abnormal temperature effect on the retention of diazines could be mainly induced by an unusual thermodynamic behavior between the stationary phase and the mobile phase containing acetonitrile.

Introduction

Reversed-phase liquid chromatography (RPLC) is 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 molecules between a mobile phase (typically a mixture of organic solvent and water) and a stationary phase (generally octadecyl functional groups bound to a silica surface).

The explanation of the retention mechanism and corresponding experiments to understand the mechanism 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 is not completely known because of 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 dominates 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 are one of the most important flavor compounds responsible for the cocoa taste. Typical examples of pyrazines are: pyrazine, 2,5-dimethylpyrazine and

tetramethylpyrazine in cocoa products as important flavor compounds [7,8]. In the 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 an 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 chapter, the temperature effect on the retention of diazines, consisting 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.

Experimental

Chemicals and Reagents

All regents, 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-dimethylpyrimidine were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan, and pyrazines, pyridazine, 3-methylpyridazine, pyrimidine, 4-methypyrimidine, pyrazole, 3-methylpyrazole, imidazole, 4-methylimidazole were purchased from Tokyo Chemical Industry Co., Ltd., Tokyo, Japan.

The chemical structures of the compounds studied are shown in Figure 4-1. Water was purified using a Milli-Q Water Purification System (Millipore, Tokyo, Japan).

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. The 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 all the chromatographic 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 x 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 x 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 50ºC.

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.

Results and Discussion

Typical chromatograms for the separation of pyrazines on an ODS phase are shown in Figure 4-2. With a mobile phase consisting of MeOH and water (having the ratio from 30/70 to 50/50), all of the retention factors of pyrazines decreased with increasing column temperature over all of the temperature range studied. This is the same trend as normally found for most of the analytes, such as polycyclic aromatic hydrocarbons (PAHs), on a typical ODS phase in RPLC conditions [14–16].

However, as typically found in Figure 4-2B, where ACN/water = 70/30 was used as the mobile phase, increased retention factors for pyrazines were observed when the column temperature was increased. A similar trend was also observed for all of the ACN-based mobile phases studied. The results show good agreement with previous reports [12,13], suggesting an abnormal temperature effect on the retention of pyrazines with ACN/water as the mobile phase.

Figure 4-3 shows the van't Hoff plots of pyrazines in the above cases with MeOH/water and ACN/water as the mobile phase, where the logarithmic retention factor was plotted against the inverse absolute column temperature. As can be seen in Figure 4-3, the logarithmic retention factors for pyrazines increase linearly with decrease in the column temperature using MeOH as the mobile phase component, showing the normal temperature effect found in most

column temperature studies in RPLC [14–16].

In contrast, with ACN/water as the mobile phase (Figure 4-3B), the retention factors logarithmically increase with the increase in 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.

To investigate further 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 types of stationary phases, including octyl- and triacontylsilica 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 retention 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 increase in the column temperature, suggesting an abnormal temperature effect on the retention of these analytes with ACN as the organic solvent in the mobile phase.

Similar 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 4-4, along with the corresponding plots for pyrimidines in Figure 4-5. 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 the above trend, an abnormal temperature effect was not observed for pyrazoles and imidazoles having a five-membered ring in their molecular structure, as shown Figure 4-6. For pyrazoles and imidazoles, the retention logarithmically decreased with increasing column temperature, regardless of the organic component in the mobile phase. This group of solutes has a normal temperature dependence that is identical with the trend in the case using MeOH as the mobile phase component.

By calculating the enthalpy of solute transfer from the mobile phase to the stationary phase (Table 4-1), the abnormal temperature dependence was confirmed that is usually associated with enthalpy–entropy compensation effects, as reported previously [17–20]. As can be seen in Table 4-1, all of the analytes, except for pyridazines and pyrimidines, showed a negative enthalpy of transfer from the mobile phase to the stationary phase with MeOH/water (30/70) as the mobile phase, while the values of enthalpy obtained with ACN/water (30/70) are positive. At this stage, it can be said that the increased retention of diazines (pyrazines, pyridazines and pyrimidines) at elevated column temperatures 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. Similar to the extensive studies on the retention behavior of other classes of compounds at various column temperatures [21–28], more comprehensive considerations should be considered to interpret this unusual temperature dependence.

Conclusions

From the RPLC separation of various nitrogen-containing heterocyclic compounds performed at different temperatures and with mobile phase conditions, an abnormal temperature effect on retention of diazines (pyrazines, pyridazines and pyrimidines)—that is, the retention of these analytes increased when the column temperature was increased—was confirmed with the mobile phases consisting of ACN and water. The enthalpy of solute transfer from the mobile phase to the stationary phase was 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 performed to interpret the abnormal dependence of the retention of these solutes 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 both the mobile phase and column temperature conditions.

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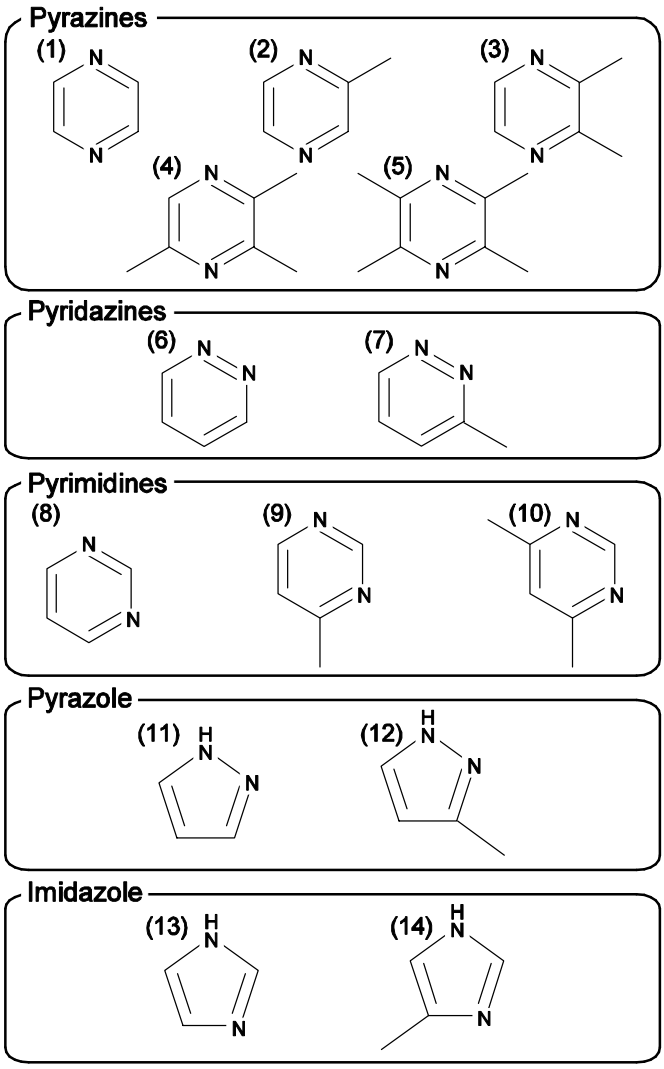

Figure 4-1 Chemical structures of analytes used in this study. (1) pyrazine; (2) 2-methylpyrazine; (3) 2,3-dimethylpyrazine; (4) 2,3,5-trimethylpyrazine; (5) tetramethylpyrazine; (6) pyridazine; (7) 3-methylpyridazine; (8) pyrimidine; (9) 4-methylpyrimidine; (10) 4,6-dimethylpyrimidine; (11) pyrazole; (12) 3-methylpyrazole; (13) imidazole; and (14) 4-methylimidazole.

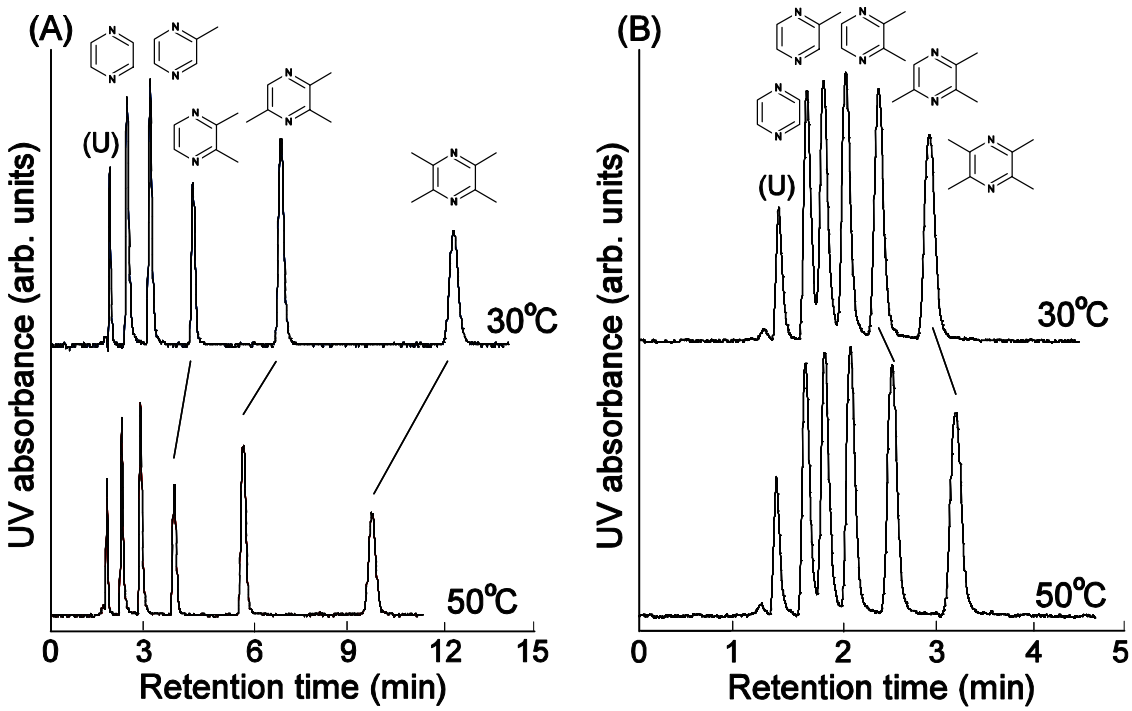

Figure 4-2 Typical chromatograms for the separation of pyrazines at different column temperatures. Mobile phase: (A) MeOH/water = 30/70; (B) ACN/water = 30/70. As dead volume marker, uracil (U) was used. Other conditions: column, ULTRON VX-ODS (5μm, 150 mm x 4.6 mm i.d.); flowrate, 1.0 mL/min; detection, UV at 270 nm.

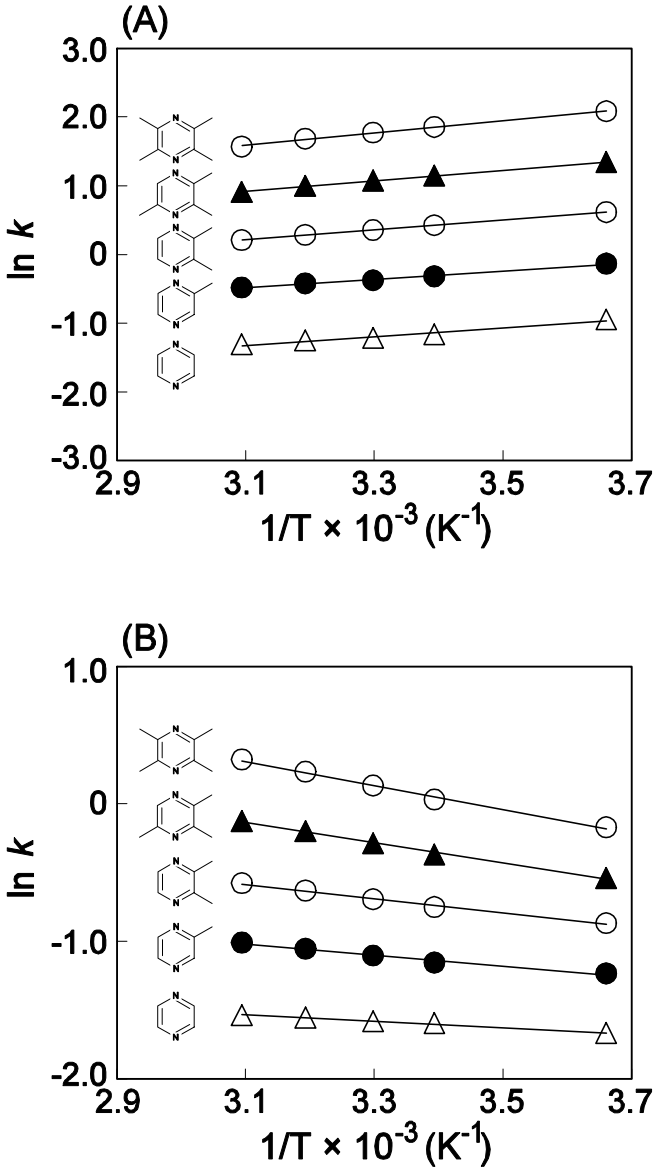

Figure 4-3 van't Hoff plots for pyrazines. Mobile phase: (A) MeOH/water = 30/70; (B) ACN/water = 30/70. Other conditions are the same in Figure 4-2; and the assignments are the same as in Figure 4-1.

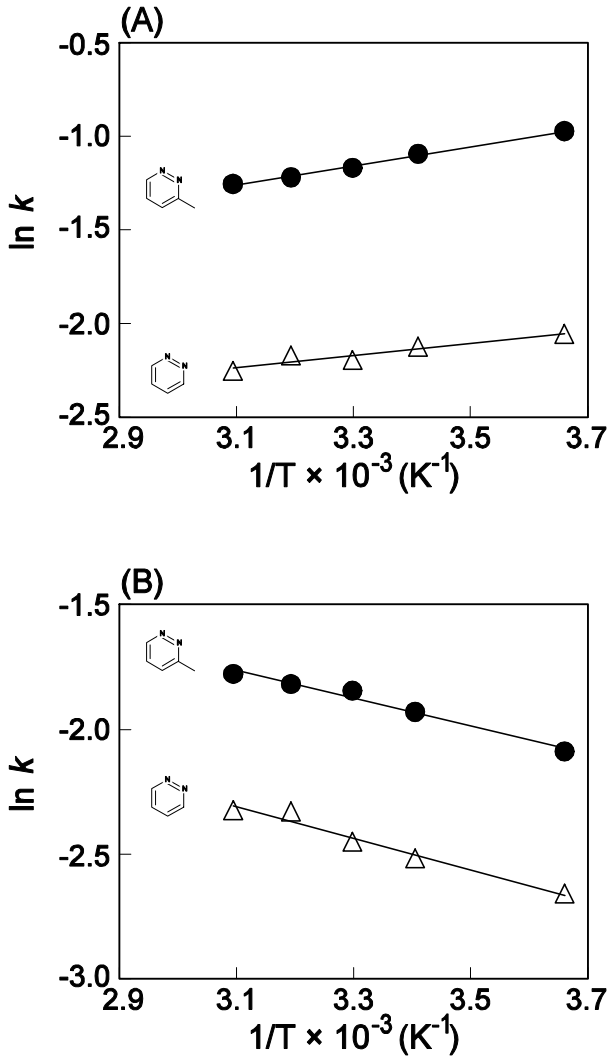

Figure 4-4 van't Hoff plots for pyridazine and 3-methylpyridazine. Mobile phase: (A) MeOH/water = 30/70; (B) ACN/water = 30/70. Other conditions are the same in Figure 4-3.

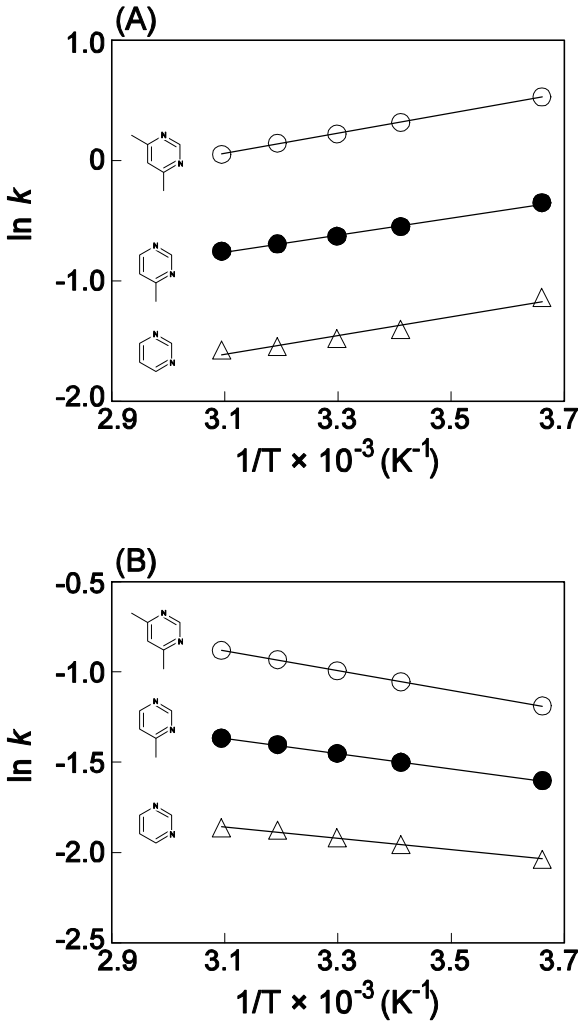

Figure 4-5 van't Hoff plots for pyrimidine, 4-methylpyrimidine and 4,6-dimethylpyrimidine. Mobile phase: (A) MeOH/water = 30/70; (B) ACN/water = 30/70. Other conditions are the same in Figure 4-3.

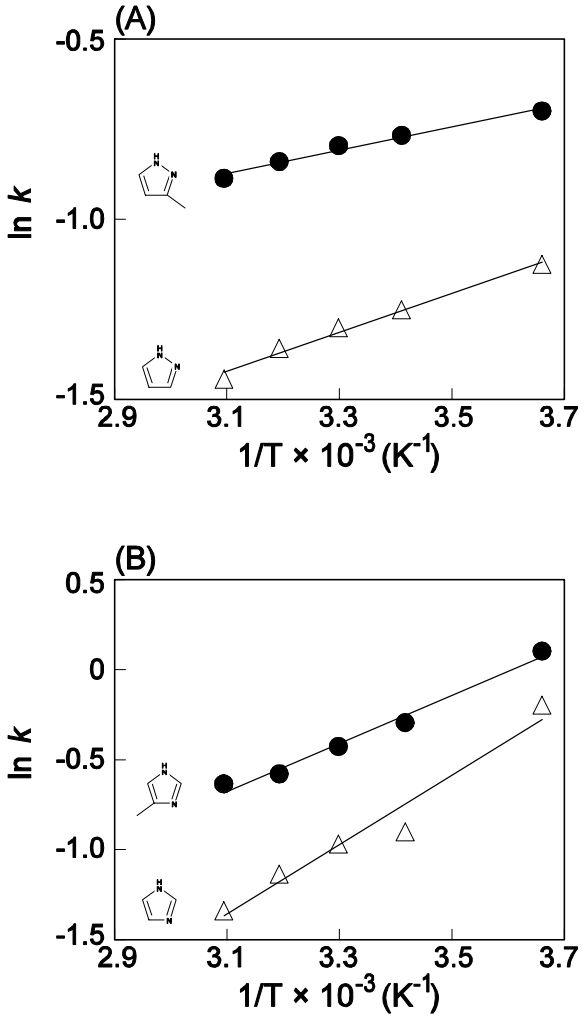

Figure 4-6 van't Hoff plots for pyrazoles and imidazoles. Mobile phase: ACN/water = 30/70. Analyte: (A) pyrazoles and (B) imidazoles. Other conditions are the same in Figure 4-3.

Table 4-1 Standard enthalpy (kJ mol^{-1}) of transfer from the mobile phase to the bonded phase.

Analytes	Mobile phase	
	MeOH/water	ACN/water
	30/70	30/70
pyrazine	-5.33	1.92
2-methylpyrazine	-5.07	3.30
2,3-dimethylpyrazine	-5.99	4.29
2,3,5-trimethylpyrazine	-6.41	6.13
tetramethylpyrazine	-7.40	7.28
pyridazine	-2.69	5.25
3-methylpyridazine	-4.24	0.50
pyrimidine	-6.48	2.63
4-methylpyrimidine	-5.93	3.48
4,6-dimethylpyrimidine	-6.95	4.53
pyrazole	-9.42	-4.49
3-methylpyrazole	-10.93	-2.69
imidazole	N/A	-15.99
4-methylimidazole	N/A	-11.06

Chapter 5

General Conclusion

General Conclusions

In this thesis, the retention behavior of various types of pyrazines in RP-HPLC was studied because the characterization of raw food materials is regarded as one of the most important objectives of food manufacturers. The analysis of pyrazines could be quite important in precisely determining odor components in processed foods such as cocoa and chocolate.

To clarify the retention behavior of the pyrazines in HPLC, some QSRR methods were used [1,2]. Retention prediction models based on MLR and ANN were developed to describe and predict the retention behavior of pyrazines under reversed-phase conditions using an ODS stationary phase in Chapter 2. The MLR-derived models showed that the retention of the analytes can be attributed to the effect of the organic modifier in the mobile phase (ACN or MeOH) and log *P* of the analytes. A comparison between the MLR and the ANN models revealed that the predictive ability of the trained ANN was better than that of MLR, especially when applied to the ACN data set.

The derived models can be used as tools for method development and optimization for the analysis of pyrazines and related compounds. It was able to be used effectively for the quality of each pyrazine, and quantification, without using expensive apparatus, such as a mass spectrometer, was possible using these prediction models.

In Chapter 3, the temperature effect on the retention of pyrazines was

further studied at various column temperatures using several types of alkyl-bonded stationary phases and mobile phase compositions in RPLC. An abnormal retention behavior was shown also in previous research [3–5]. During the consideration of the prediction model, it was found that there was a possibility suggesting unusual retention behavior of pyrazines. The retention behavior of the pyrazines was examined using various types of columns and mobile phase conditions.

An abnormal temperature effect on the retention of pyrazines was confirmed with mobile phases consisting of ACN and water. In terms of the dependence of the retention on the content of organic solvent in the mobile phase at constant temperature, all of the pyrazines showed normal behavior, as found in most RPLC conditions. The results suggest that more efficient separation of pyrazines could be developed by tuning both the mobile phase composition and the column temperature, although further theoretical studies should be performed to interpret the abnormal dependence of the pyrazines' retention on the column temperature with ACN/water as the mobile phase solvent.

To investigate further the tendency described in Chapter 3, the temperature effect on the retention of diazines, consisting of pyrazines, pyridazines, pyrimidines and their derivatives in RPLC, was further studied at various column temperatures shown in Chapter 4. From these van't Hoff 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 the above trend, an abnormal temperature effect was not observed for pyrazoles and imidazoles having a five-membered ring in their molecular structure. For pyrazoles and imidazoles, the retention decreased logarithmically with increasing column temperature regardless of the organic component in the mobile phase. The enthalpy of solute transfer from mobile phase to stationary phase was calculated from van't Hoff plots, and enthalpy–entropy compensation effects were observed for diazines with ACN/water as the mobile phase.

As can be seen from these studies, understanding the retention behavior of pyrazines in RP-HPLC is still developing. However, the results obtained in this thesis could be further applied to a more efficient and precise analysis of pyrazines in food materials, especially to characterize adequately the important odor components in the food industry.

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Publications

Publications

1. Papers Related to this Dissertation

[1] **Kentaro Yogo**, Noel Samson Quiming, Yoshihiro Saito and Kiyokatsu Jinno,
"Prediction of Chromatographic Retention of Pyrazine and Alkylpyrazines in RP-LC"
Chromatographia, **70**, 677-684 (2009).

[2] **Kentaro Yogo**, Chiharu Takemura, Yoshihiro Saito and Kiyokatsu Jinno,
"An Abnormal Temperature Dependence of Alkylpyrazines' Retention in Reversed-Phase Liquid Chromatography" (Notes)
Analytical Sciences, **27**, 1257-1260 (2011).

[3] Chiharu Takemura, **Kentaro Yogo**, Ikuo Ueta, Kiyokatsu Jinno and Yoshihiro Saito,
"Effect of Temperature on Retention of Diazines in Reversed-Phase Liquid Chromatography,"
Chromatography, **34**, 97-102 (2013).

2. Other Publications

[1] Yoshihiro Saito, Ikuo Ueta, Mitsuhiro Ogawa, Akira Abe, **Kentaro Yogo**, Shingoro Shirai, and Kiyokatsu Jinno,
"Fiber-Packed Needle-Type Sample Preparation Device Designed for Gas Chromatographic Analysis"
Analytical and Bioanalytical Chemistry, **393**, 861-869 (2009).

[2] Ikuo Ueta, Yoshihiro. Saito, Nadia Binti Abdul Ghani, Mitsuhiro Ogawa, **Kentaro Yogo**, Akira Abe, Shingoro Shirai and Kiyokatsu Jinno,
"Rapid Determination of Ethylene Oxide with Fiber-Packed Sample Preparation Needle"
Journal of Chromatography A, **1216**, 2848-2853 (2009).

[3] Kazuya Sasaki, **Kentaro Yogo**, Kenzo Yamaguchi, Yoshihiro Saito and Mitsuo Fukuda,
"Non-destructive Detection of Vitamin B_2 in Plant Leaves Using Fluorescence Monitoring" (JAPANESE)
Journal of Society of High Technology in Agriculture, **23**, 152-158 (2011).