Study on the application of chemically modified synthetic copolymer columns for liquid chromatographic food analysis

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Study on the application of chemically modified synthetic copolymer columns for liquid chromatographic food analysis

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Abbreviations

- ABEE, *p*-aminobenzoic ethyl ester
- CE, capillary electrophoresis
- CV, coefficient of variation
- DA, dopamine
- DOPAC, 3,4-dihydroxyphenylacetic acid
- DMF, *N,N*-dimethylformamide
- E, epinephrine
- ECD, electrochemical detection
- ESI, electrospray ionization
- FA, formic acid
- FLD, fluorescence detection
- GC, gas chromatography
- IEC, ion-exchange
- HILIC, hydrophilic interaction liquid chromatography
- HMD, hexamethylenediamine
- HPAEC-PAD , high-performance anion-exchange chromatography with pulsed amperometric detection
- HPLC, high-performance liquid chromatography

- HVA, homovanillic acid
- LC, liquid chromatography
- LOD, limit of detection
- log *k*, logarithmic retention factor
- LOQ, limit of quantification
- MHPG, 3-methoxy-4-hydroxyphenylglycol
- MS, mass spectrometry
- MS/MS, tandem mass spectrometry
- *m/z*, mass-to-charge ratio
- NMR , nuclear magnetic resonance
- NE, norepinephrine
- OLETF, Otsuka Long-Evans Tokushima Fatty
- OS, sodium 1-octanesulfonate
- PAD, pulsed amperometric detection
- PMP, 1-phenyl-3-methyl-5-pyrazolone
- PS, sodium 1-propanesulfonate
- RI, refractive index
- RIU, relative index unit
- Rs, resolution
- SEC, size-exclusion chromatography

- SFC, supercritical fluid chromatography
- *S/N*, signal to noise ratio
- TEA, tetraethylammonium hydroxide
- THF, tetrahydrofuran
- TLC, thin layer chromatography
- TOF, time-of-flight
- UV, ultra-violet

Chapter I

Introduction

1.1 Overview of chromatography

Chromatography is an analytical technique that was invented at the 20th century by Mikhail Tswett [1], a researcher for plant pigments, in which an original separation system by chalk-filled tube and organic solvent for the separation of a mixture of pigments was proposed. The separation technique was named as “chromatography” which is derived from Greek, chroma means “color”, and graphy means “to write”. Thereafter, a variety of chromatographic techniques have been exploited, including liquid chromatography (LC) [2], gas chromatography (GC) [3], thin layer chromatography (TLC) [4], supercritical fluid chromatography (SFC) [5], and so on. Although separation mechanisms are different in techniques, their extensive goal is to separate a mixture into individual components. In this study, LC separation technique was focused on, since it has become the most indispensable analytical technique in

food science, biotechnology, and pharmacy. Schematic diagram of LC system is represented in Fig. 1-1. In food science field, analytes for LC separation involve carbohydrates, nucleic acids, amino acids, and peptides for the evaluation of food quality, as well as the evaluation of physiological functions in preventing lifestyle-related diseases [6–9] and of bioavailability [10–12]. Commonly used LC detectors are ultra-violet (UV), refractive index (RI), fluorescence (FLD), and electrochemical (ECD) detectors [13], and mass spectrometry (MS) [14]. Considering high sensitivity and selectivity for target detection [14], MS detector is now growing in combination with LC system. In MS detector, compounds that are separated by LC are then ionized (in some cases fragmented) and separated according to their mass-to-charge ratio (*m/z*). Furthermore, each compound displays unique fragmentation patterns and mass spectra, being capable of identification of compounds.

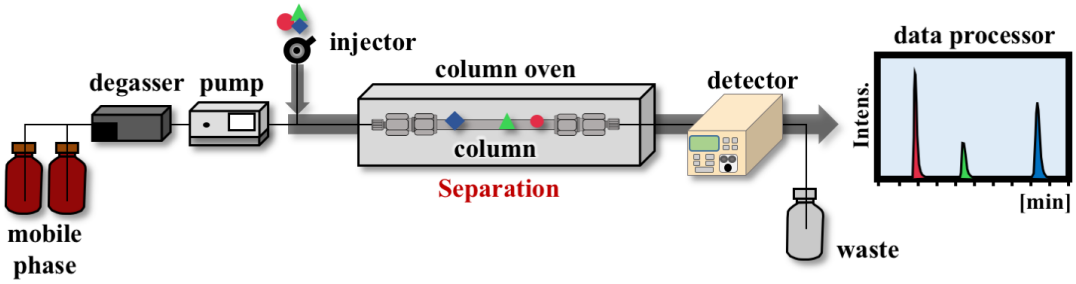


Fig. 1-1 Schematic diagram of LC system.

1.2 LC column characteristics

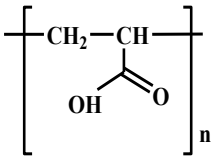
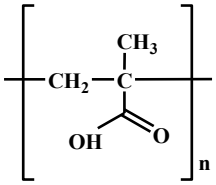
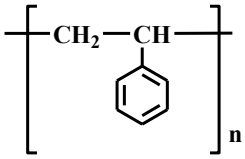
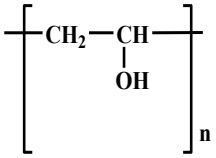
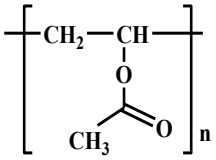
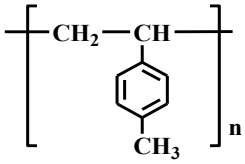
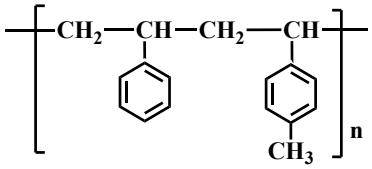
In LC separation technique, column characteristics may affect the separation profiles. LC separation is mainly categorized into normal-phase [15], reversed-phase [16,17], ion-exchange (IEC) [18], and size-exclusion (SEC) [19] modes, by interaction between analyte and stationary phase. In addition to the above-mentioned separation modes, Alpert *et al*. [20] proposed a new LC separation mode, named as hydrophilic interaction liquid chromatography (HILIC), which is characterized by the interaction of polar stationary phase with relatively nonpolar mobile phase. Yoshida [21] has applied an HILIC column for the separation of polar peptides with a linear gradient of 97 v/v% to 55 v/v% acetonitrile/0.1 v/v% trifluoroacetic acid. Li and Huang [22] achieved HILIC separation of epirubicin, a kind of antitumor polar antibiotics, under the elution of 20 mmol/L sodium formate buffer (pH 2.9)/90 v/v% acetonitrile. To date, a multiple LC separation modes have been proposed to achieve a simultaneous separation of analytes bearing diverse chemical properties such as size, polarity, and hydrophobicity in a single experimental run. The multiple column is called as a mixed-mode column, which is in combination with *e.g.*, reversed-phase/IEC, reversed-phase/hydrophilic, and hydrophilic interactions/IEC [23]. Yang and Geng [24] reported that a reversed-phased/IEC mixed-mode column was able to separate positive, negative, and neutral compounds in a single

high-performance LC (HPLC) assay. Another combinatory mixed-mode column, HILIC/strong cation-exchange column [25] or reversed-phase/strong cation-exchange column [26] was also useful for the separation of polar and small peptides according to their combinational interaction on stationary phase. In the case of IEC-based mixed-mode column, elution of analyte from the column is based on the removal of ionic interaction of target with IEC by pH in mobile phase. In general, silica gel is widely used for HPLC column, since silica gel possesses high resistance for pressure and high number of theoretical plates [22]. However, silica gel-based HPLC columns are restrictive for the separation in acidic mobile phase conditions due to their poor alkaline stability. Thus, the restrictive characteristics of silica gel-based columns may limit the development of mixed-mode column. In contrast, polymer-based HPLC columns could promote their widespread use in a variety of acid-alkaline mobile phases. In addition, polymer-based resin is capable of quantitative chemical modification (or regulation of the amount of IEC groups onto polymer units). As summarized in Table 1-1, polymers for HPLC resin are categorized into hydrophilic ones [polyvinylalcohol and polyvinylacetate (co)polymers] and hydrophobic ones [polystyrene and polyvinylbenzene (co)polymers] [27–39].

In this study, we evaluated two types of copolymer columns (HILIC and reversed-phase/cation exchange mixed-mode) for food analysis. Targeted analytes for each column separation were rare sugars and catecholamines, since both chemicals

may have difficulty in the separation on common columns owing to their structural similarity and ionic properties.

Table 1-1 Common polymer for liquid chromatography column

Polymer	Category	Monomer	Application [reference]
poly(acrylate) 	hydrophilic	acrylate	peptides [27]
poly(methacrylate) 	hydrophilic	methacrylate	pesticides [28] aliphatic amines [29] polyfluorocarboxylic acids [30] carboxylic acids [31]
poly(styrene) 	hydrophobic	styrene	phenols [32] amino acids [33]
poly(vinylalcohol) 	hydrophilic	vinylalcohol	opioid peptides [34]
poly(vinylacetate) 	hydrophobic	vinylacetate	amino acid [35]
poly(divinylbenzene) 	hydrophobic	divinylbenzene	anion compounds [36] polynuclear aromatic hydrocarbons [37]
poly(styrene-divinylbenzene) 	hydrophobic	styrene-divinylbenzene	protein and peptides [38,39]

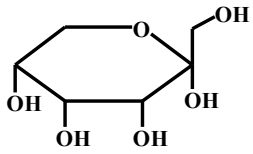
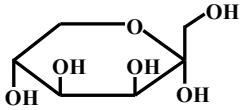
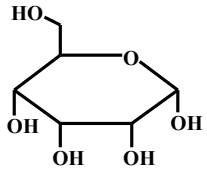
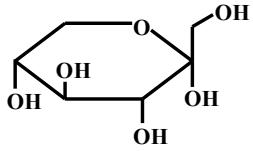
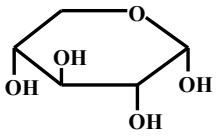
1.3 Rare sugars

Currently, the incidence of lifestyle-related diseases such as obesity, diabetes, hypertension, and hyperlipidemia have been increasing, owing to excess intake of high-fat and high-sugar diets. As summarized in Table 1-2, many rare sugars have been identified in nature so far [68]. Rare sugars, monosaccharides with low-calorie, have been paid in great attention for food researchers. Matsuo *et al*. [69] reported that D-psicose, a non-reducing rare sugar with sweet taste, showed anti-obestic effect in animal experiment. One gram of D-psicose produced 0.007 kcal, obviously lower than sucrose (2.29 kcal) and D-fructose (1.76 kcal) [69]. Previous studies have already reported that rare sugars themselves possessed physiological potentials such as anti-hyperglycemic (D-psicose [40–42]), anti-hyperlipidemic (D-psicose [40,41,44]; D-xylose [67]), anti-inflammatory (D-psicose [40,41,43]; D-allose [55,57,58]), neuroprotective (D-psicose [40,41,45]; D-allose [55,62]), anti-tumor (D-allose [55,56]), anti-cancer (D-allose [55,56]), anti-osteoporotic (D-allose [55,60]), anti-hypertensive (D-allose [55,61]), immunosuppressant (D-allose [55,63]), anti-atherosclerotic effects (D-psicose [40,41,47]), and anti-diabetic effect (D-psicose [40,41,48–50]).

The separation of monosaccharides has been encounting analytical challenges, because monosaccharides or rare sugars are highly hydrophilic and polar compounds. TLC [70], HPLC [71], GC [72], high-performance anion-exchange chromatography

with pulsed amperometric detection (HPAEC-PAD) [73], capillary electrophoresis (CE) [74], and nuclear magnetic resonance (NMR) [75] have been developed for monosaccharide detection. Chemical derivatization of monosaccharides using *p*-aminobenzoic ethyl ester (ABEE) [76–80] (Fig. 1-2) and 1-phenyl-3-methyl-5-pyrazolone (PMP) (Fig. 1-3) [76,77] seems to be acceptable for high sensitive detection using common reversed-phase column, but tedious derivatization procedures may cause the lack of rapidity and simplicity for assay. Therefore, a single column separation of monosaccharides is still desired, and a HILIC column separation may be a convenient assay with RI-HPLC. However, there was no report on simultanoues assay for rare sugars in a single HILIC column. Thus, in **Chapter II**, considering strong interaction of polar rare sugars with HILIC column, simultanoues assay for rare sugars was established on a copolymer HILIC column; the column used in this study was composed of ethyleneimine monomeric unit in polymer chain, causing higher affinity of rare sugars with resin by imine-induced strong base property.

Table 1-2 Physiological functions of rare sugars

Rare sugar	Structure	Physiological function [reference]
D-psicose		anti-hyperglycemic [40–42] anti-inflammatory [40, 41, 43] anti-hyperlipidemic functions [40, 41, 44] neuroprotective effect[40,41,45] ROS scavenging activity [40,41,46] therapeutic effect against atherosclerosis [40,41,48] anti-diabetic effect [40,41,48–50] anti-obesity activity [40,41, 50–52]
D-tagatose		anti-diabetic effect [53,54] obesity control [53,54] blood metabolite regulator [53] anti-aging property [53] anti-oxidation [53]
D-allose		anti-tumor [55,56] anti-cancer [55,56] anti-inflammatory effect [55,57,58] cryoprotective effect [55,59] anti-osteoporotic effect [55,60] anti-hypertension [55,61] neuroprotective effect [55,62] immunosuppressant [55,63] anti-oxidation [55,64]
D-sorbose		improving glucose metabolism [65] inhibitory effect of disaccharides activity [66]
D-xylose		alleviating adipogenesis and dyslipidemia, and improving lipid oxidation [67]

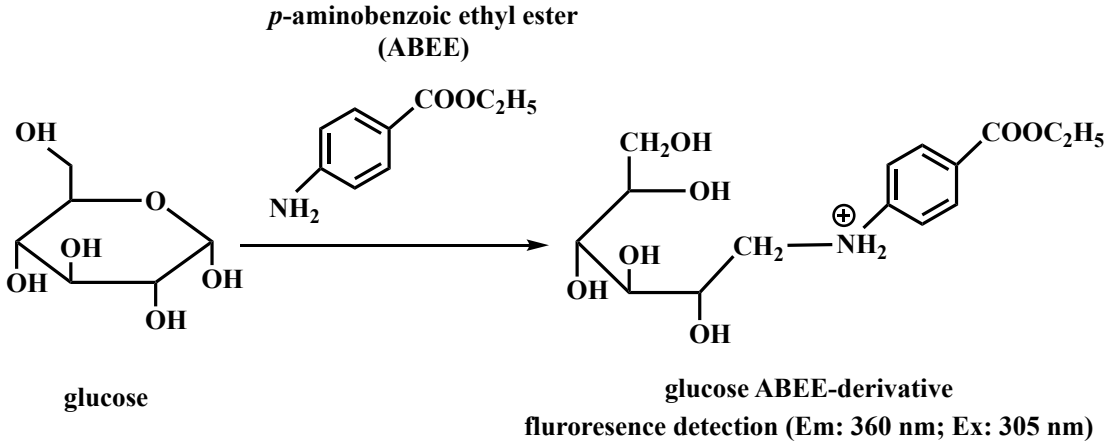


Fig. 1-2 Scheme of *p*-aminobenzoic ethyl ester (ABEE) derivatization reaction with glucose.

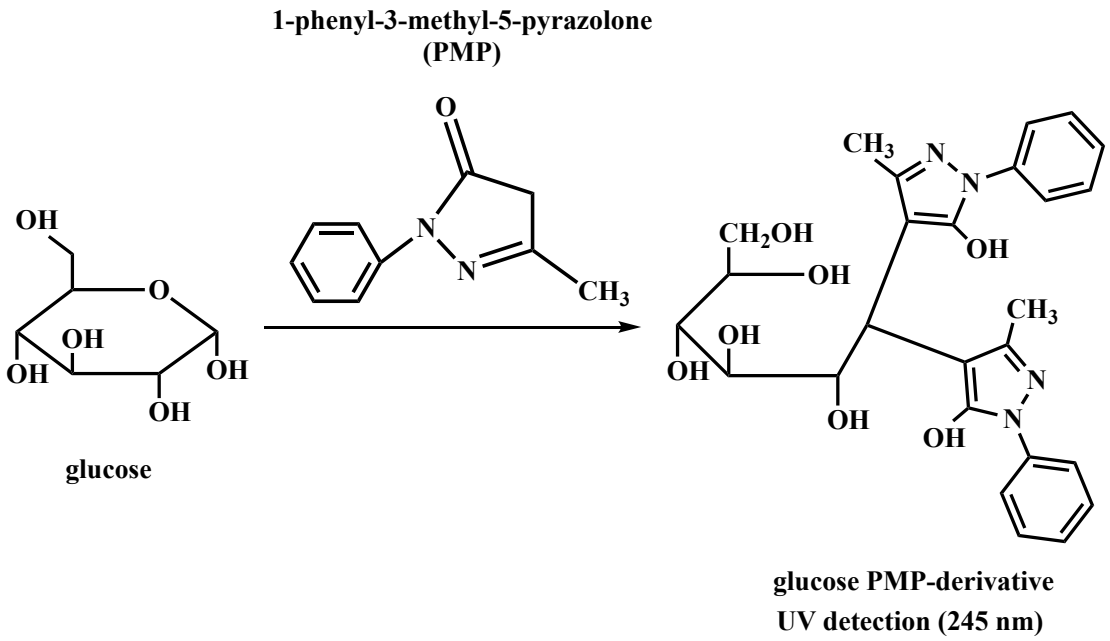


Fig. 1-3 Scheme of 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatization reaction with glucose.

1.4 Catecholamines

Catecholamines are a group of *o*-dihydroxylbenzene bearing amine groups. As shown in Fig. 1-4, in the brain, tyrosine (or phenylalanine) can be metabolized to form dopamine (DA). DA is then converted to produce norepinephrine (NE) and epinephrine (E) as neurotransmitter. In the catecholamine metabolism in the brain, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 3-methoxy-4-hydroxyphenylglycol (MHPG) are thought to be an end-product of the catecholamine metabolic pathways [81,82]. They also play a role as a mediator for neurophysiological signaling regarding memory, recognition, learning, and hormone release [83]. Also, polyphenol structures such as catechins, and caffeoyl compounds, showing diverse preventive effects against lifestyle-related diseases contain catechol moiety. Therefore, convenient and reliable analytical assay system for catecholamines and their analogues in a single chromatographic run would allow us to understand the overall metabolism and bioavailability.

Thus far, ECD-HPLC has been widely used for catecholamine assay with high sensitivity [84], since a target catechol moiety is electrochemically active. However, limited HPLC conditions (isocratic elution) to maintain the stability of ECD detector or stable baseline often cause the restrictive chromatographic separation of analytes. LC-MS assay may be an alternative catecholamine assay, because of its high

selective property on the basis of target mass unit (*m/z*). However, successful *m/z*-based MS detection of catecholamines may be closely associated with adequate LC separation, since targeted catechols have high similarity in structure and *m/z*. Thus, in **Chapter III**, we propose a simultanoues assay system for catecholamines and the metabolites in a single LC-MS run by using a mixed-mode column for complete separation.

In compliance to the aforementioned explanations and viewpoints, the proposing new analytical methods using chemically modified mixed-mode copolymer columns can be applied in the future to the analyses of not only the targets in this study, but also a variety of food compounds and metabolites in diverse matrices in one single HPLC and/or LC-MS run.

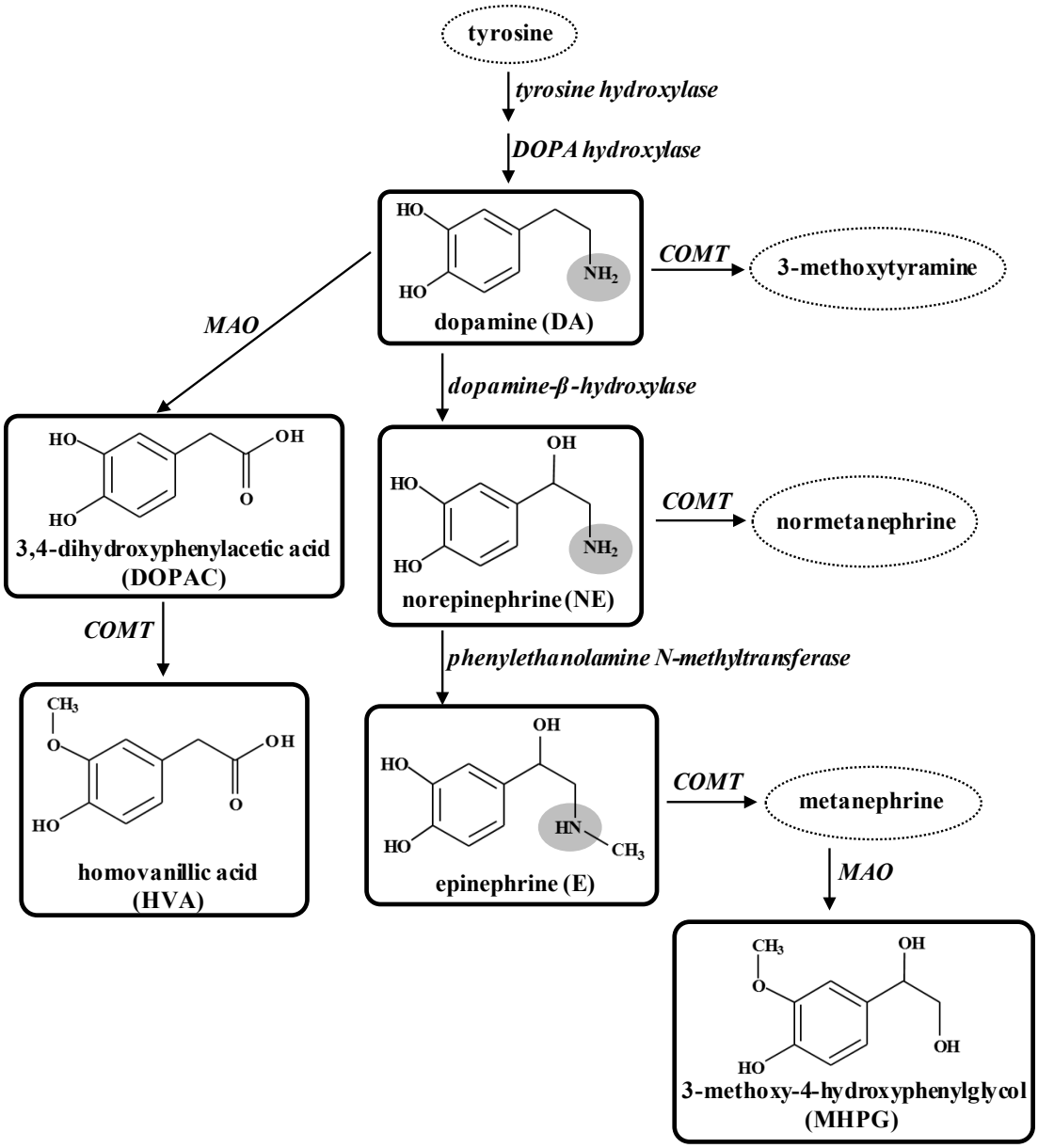


Fig. 1-4 Metabolism of catecholamines

Chapter II

Discriminant analysis of reducing and non-reducing monosaccharides on a polyethyleneimine-attached polymer-based hydrophilic interaction liquid chromatography copolymer column

2.1 Introduction

Rare sugars and monosaccharides, the simplest units of carbohydrates, play a crucial role in providing energy in our body. It has been reported that their effective health benefits by intake concern anti-oxidant [85,86], anti-hyperlipidemic, and anti-hyperglycemic effects [87,88]. For example, long-term intake of 5% D-psicose to Otsuka Long-Evans Tokushima Fatty (OLETF) rat, a model of type 2 diabetes, improved the impaired insulin sensitivity or glucose tolerance [48]. Hence, to date, research interest of rare sugars has grown in the prevention and improvement of

lifestyle-related diseases.

Analytical evaluation (qualification and quantification) of rare sugars or monosaccharides seems to be challenging owing to their similar structure and hydrophilicity (*e.g.* no retention on reversed-phase HPLC column [89]). Also, the lack of chromophoric or fluorophoric moiety in structure causes the poor detection by UV and FLD [90]. Additionally, MS analysis of monosaccharides also suffers from the low ionization efficiency of neutral chemical property [91]. To solve these problems for rare sugar detection, various techniques have been developed so for. Derivatization techniques by anthranilic acid and 1-pheny-3-methyl-5-pyrazolone (Figs. 1-2 and 1-3) have been proposed for successful UV-HPLC detection of common monosaccharides [92]. Meyer *et al*. [93] reported that *p*-aminobenzoic acid-derivatization successfully allowed the fluorescence detection of sugars at >50 ng/mL by HPLC. However, derivatization aided-sugar analysis still has problems in terms of rapidity due to tedious procedures. Recently, a HILIC separation has been developed as a powerful methodology for carbohydrate assay [94]. Although aforementioned HPLC derivatization assay on reversed-phase column has a high sensitivity, RI-HPLC using a HILIC column may be convenient for the analysis of sugars, because of its rapidity in LC run. A selective evaporative light scattering detection (ELSD) -HPLC assay of a rare sugar, D-psicose, in various food products has been proposed on a carbamoyl-silica HILIC column with an isocratic elution of 5

mmol/L ammonium formate/82 v/v% acetonitrile (pH 5.5) [95]. However, no studies have been reported on simultaneous separation of rare sugars in a single HPLC assay owing to their similar chemical and structural properties. Thus, in this Chapter, by considering strong base property of imine group, an imine-induced polymer-based HILIC column was attempted to simultaneous separate rare sugars by RI-HPLC. In this study, a polyethyleneimine-induced glycidyl methacrylate-ethylene glycol dimethacrylate resin column was applied for the separation of seven monosaccharides, including five rare sugars, i.e., D-xylose, D-allose, D-psicose, D-sorbose, and D-tagatose.

2.2 Material and Methods

2.2.1 Materials

D-Glucose, D-xylose, tetrahydrofuran (THF), hexamethylenediamine (HMD), and sodium 1-octanesulfonate (OS) were purchased from Nacalai Tesque Inc. (Kyoto, Japan). D-Fructose was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). D-Psicose, D-sorbose, and sodium 1-propanesulfonate (PS) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). D-Allose and D-tagatose were obtained from Sigma-Aldrich (St. Louis, MO, USA). Pyridine was obtained

from Hayashi Pure Chemical Ind.,Ltd. (Osaka, Japan). Acetonitrile and *N,N*-dimethylformamide (DMF) were purchased from Kanto Chemical Co. (Tokyo, Japan). Deionized water was prepared using a Milli Q system (Millipore, Tokyo, Japan).

2.2.2 HPLC analysis

An LC-10AD HPLC system (Shimadzu Co., Kyoto, Japan) connected with an RI-930 detector (JASCO, Tokyo, Japan) was used in this study. A polyethyleneimine-induced polymer-based HILIC column (4.6 mm I.D × 150 mm, 5 μm) was a product of Mitsubishi Chemical Co. (Tokyo, Japan). The column was packed with glycidyl methacrylate-ethylene glycol dimethacrylate copolymer attached to 8 wt% polyethyleneimine. A polyamine-attached silica-based HILIC column was purchased from Shodex Co. (Asahipak NH2P-50 4D, Tokyo, Japan; 4.6 mm I.D × 150 mm, 5 μm).

Chromatographic run was carried out at 40°C in an isocratic elution mode. Other conditions were discussed in the text later. The concentration of target monosaccharides dissolved in deionized water was set at 1.0 wt%. Sample solution was injected to RI-HPLC system with an injection volume of 10 μL. RI detection was performed in positive signal mode; detection range, 4×10^{-5} relative index unit (RIU);

temperature, 40°C. The retention of analyte on column was calculated as a logarithmic retention factor (log k): log k = log [($t - t_0$)/t_0], where t is a retention time of analyte. t_0 was a retention time of injection peak. Resolution (Rs) value was calculated as Rs = ($t_{R2} - t_{R1}$)/0.5 ($W_1 + W_2$), where t_{R1} and t_{R2} represent a retention time of target peaks ($t_{R2} > t_{R1}$). W_1 and W_2 are a peak width of each peak.

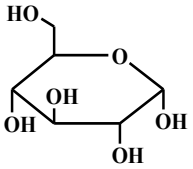
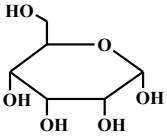
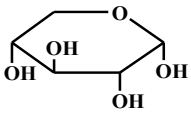
2.3 Results and Discussion

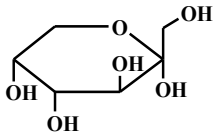
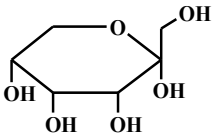
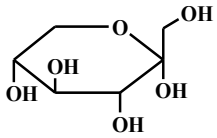
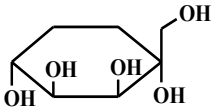
2.3.1 Elution behavior of D-fructose on polyethyleneimine-attached HILIC column

Seven monosaccharides (Table 2-1), including five rare sugars (reducing aldoses: D-glucose, D-allose, and D-xylose; non-reducing ketoses: D-fructose, D-psicose, D-sorbose, and D-tagatose) were used to investigate the elution characteristics of the polyethyleneimine-attached HILIC column. To get an insight of the HILIC column characteristics, D-fructose (a typical non-reducing ketose) was used as typical monosaccharide. The effect of acetonitrile concentration on the retention of D-fructose was primarily investigated, since organic mobile solvents may be a crucial retention factor on a HILIC column. As shown in Fig. 2-1, elution of D-fructose on the imine-induced HILIC column was greatly influenced by acetonitrile concentration at a flow rate of 0.75 mL/min; obtained peak shapes were broadened with increasing

acetonitrile concentration (70–90 v/v%), together with the decrease of peak intensity. The logarithmic retention factor (log *k*) was also increased with increasing acetonitrile concentration; log *k* values of D-fructose on the HILIC column were 0.07, 0.18, 0.71, and 0.99 at 70 v/v%, 75 v/v%, 85 v/v%, and 90 v/v%, respectively. Fig. 2-1 also shows that at 95 v/v% acetonitrile, D-fructose could not be detected, owing to its high retention or broadened peak on the column. It was reported that a common silica-based HILIC column resulted in a higher retention of analyte by increasing the hydrophilicity of mobile solvent [96]. This indicates that the polyethyleneimine-attached HILIC column used in this study may possess the same elution characteristics as a common HILIC column.

Table 2-1 Chemical structure and properties of seven monosaccharides and rare sugars used in this study

Category	Reducing aldoses		
Monosaccharide	**D-glucose**	**D-allose**	**D-xylose**
Structure			
Index of refrection	1.573	1.573	1.544
Density (g/cm^3)	1.6	1.6	1.5

Non-reducing ketoses			
D-fructose	**D-psicose**	**D-sorbose**	**D-tagatose**
			
1.574	1.574	1.574	1.574
1.6	1.6	1.6	1.6

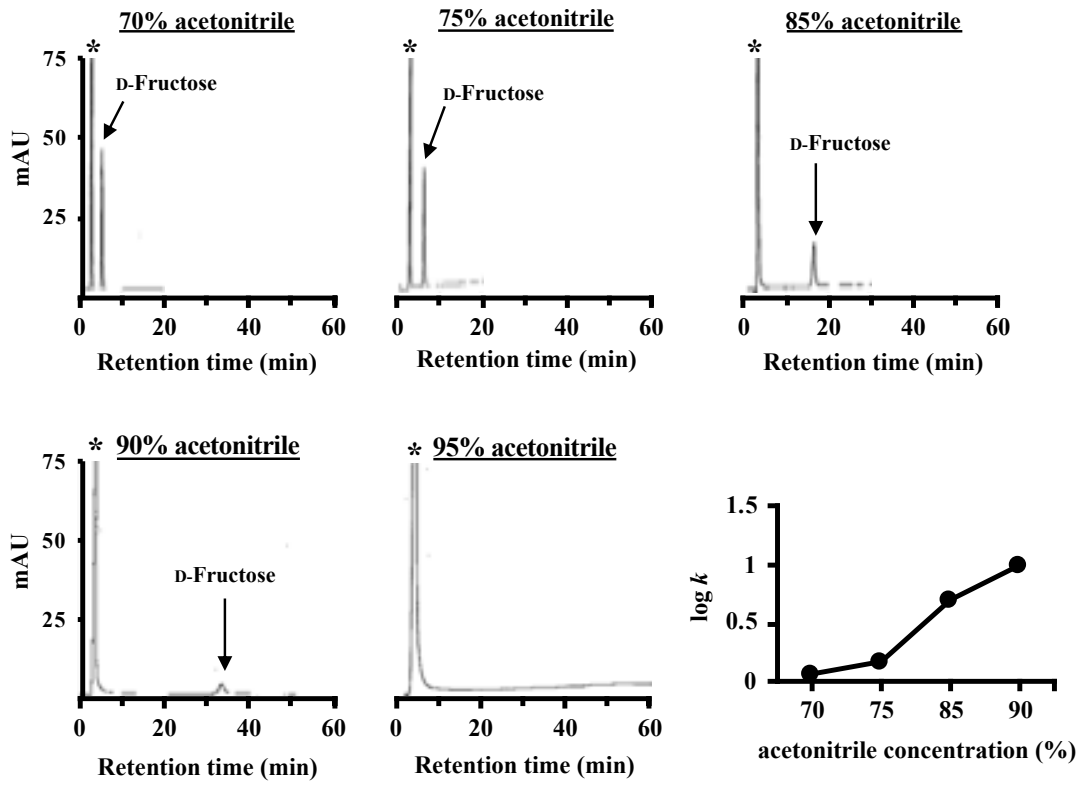


Fig. 2-1 RI-HPLC elution profiles of D-fructose on polyethyleneimine-attached HILIC column.

RI-HPLC detection of 1.0 wt% D-fructose was performed on HILIC column packed with 8 wt% polyethyleneimine-attached copolymer resin with an isocratic elution of 70, 75, 85, 90, or 95 v/v% acetonitrile at a flow rate of 0.75 mL/min at 40°C. The logarithmic retention factor (log k) of D-fructose was calculated by log k = log [(t – t_0)/t_0], where t is the retention time of analyte and t_0 is the retention time of injection peak. * indicates t_0.

2.3.2 Elution behavior of reducing and non-reducing monosaccharides on polyethyleneimine-attached HILIC column

To understand the elution characteristics of reducing and non-reducing monosaccharides on the polyethyleneimine-attached HILIC column, chromatographic runs of monosaccharides (Fig. 2-1) were performed with an elution of 90 v/v% acetonitrile at 0.75 mL/min, at which an appropriate retention of D-fructose was observed (Fig. 2-2). As a result, an individual injection of non-reducing ketoses (D-fructose, D-psicose, D-sorbose, and D-tagatose) was successfully detected by RI-HPLC on the HILIC column with log *k* values of 0.91, 0.67, 1.00, and 0.89 for D-fructose, D-psicose, D-sorbose, and D-tagatose, respectively. In contrast, reducing aldoses (D-glucose, D-allose, and D-xylose) could not be detected on the column, as seen in Fig. 2-2. As expected from the individual injection of non-reducing ketoses (Fig. 2-2), a single injection of five non-reducing ketoses revealed an appropriate separation when the elution was performed at 90 v/v% acetonitrile at 0.75 mL/min, compared to poor retention at 80 v/v% and 85 v/v% acetonitrile (Fig. 2-3). Further elution experiments were performed to improve the retention of non-reducing monosaccharides on the HILIC column as a function of flow rate at 90 v/v% acetonitrile. Fig. 2-4 shows the effect of flow rate (0.75, 0.8, 0.85, and 0.9 mL/min) on the retention of non-reducing monosaccharides. It was clear that an improved

resolution (Rs: D-psicose/D-tagatose, 7.51; D-tagatose/D-fructose, 0.91; D-fructose/D-sorbose, 1.81) was obtained at a flow rate of 0.9 mL/min; e.g., 0.75 mL/min: Rs of D-psicose/D-tagatose, 3.73; D-tagatose/D-fructose, 0.44; D-fructose/D-sorbose, 1.19). At the flow rate of 0.9 mL/min, the elution of the four non-reducing ketoses was completed within 40 min on the present imine-attached HILIC column. Taken together, the polyethyleneimine-induced copolymer HILIC column could provide a sufficient separation and RI-detection of non-reducing ketoses when elution was conditioned in 90 v/v% acetonitrile at 0.9 mL/min and 40°C. In contrast, no peaks of reducing aldoses were observed when the elution was done at >80 v/v% acetonitrile at a flow rate of 0.75 mL/min (Fig. 2-3). On the other hand, a commercially available Shodex HILIC column coated with polyamine group revealed an individual detection or separation within 8–20 min when a mixture solution of either non-reducing or reducing monosaccharides was injected to the RI-HPLC (Fig. 2-5); in turn, the narrow retention times of targets failed to achieve the complete separation of both non-reducing and reducing monosaccharides in a single polyamine-attached HILIC column. In addition, it was found that the polyethyleneimine-attached HILIC column has chromatographic advantage allowing the discriminant detection of only non-reducing ketoses at 90 v/v% acetonitrile at 0.9 mL/min (Fig. 2-4).

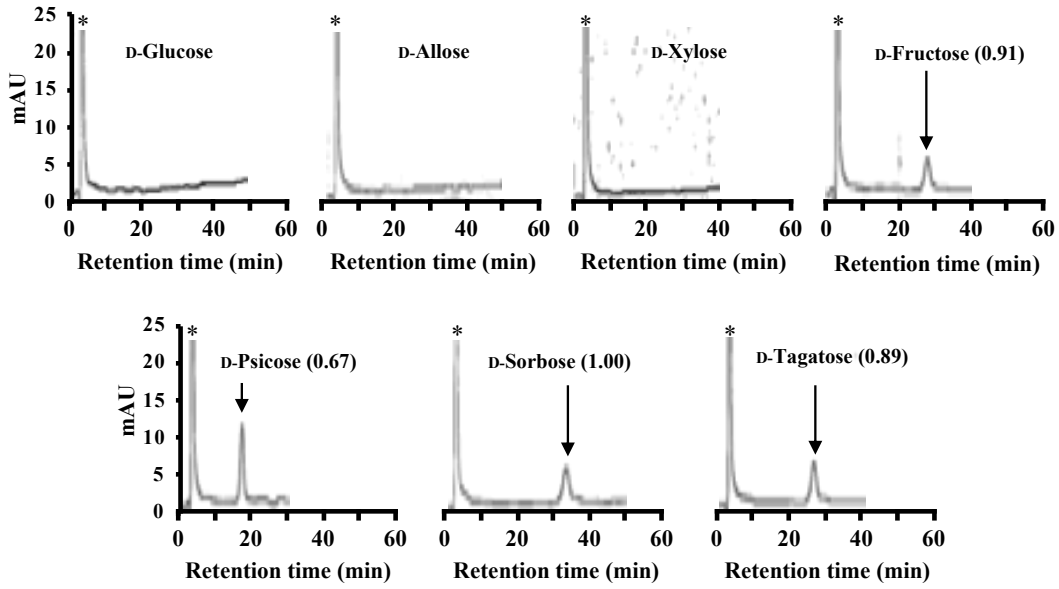


Fig. 2-2 HPLC elution profiles of reducing and non-reducing monosaccharides on polyethyleneimine-attached HILIC column.

RI-HPLC detection of 1.0 wt% monosaccharides by their individual injection was performed on the column with 90 v/v% acetonitrile elution at 0.75 mL/min at 40°C. Monosaccharides (reducing aldose: D-glucose, D-allose, D-xylose; non-reducing ketose: D-fructose, D-psicose, D-sorbose, D-tagarose) were individually injected (10 μL) to RI-HPLC system. A number in parentheses indicates the log *k* value of each monosaccharide. * indicates t_0.

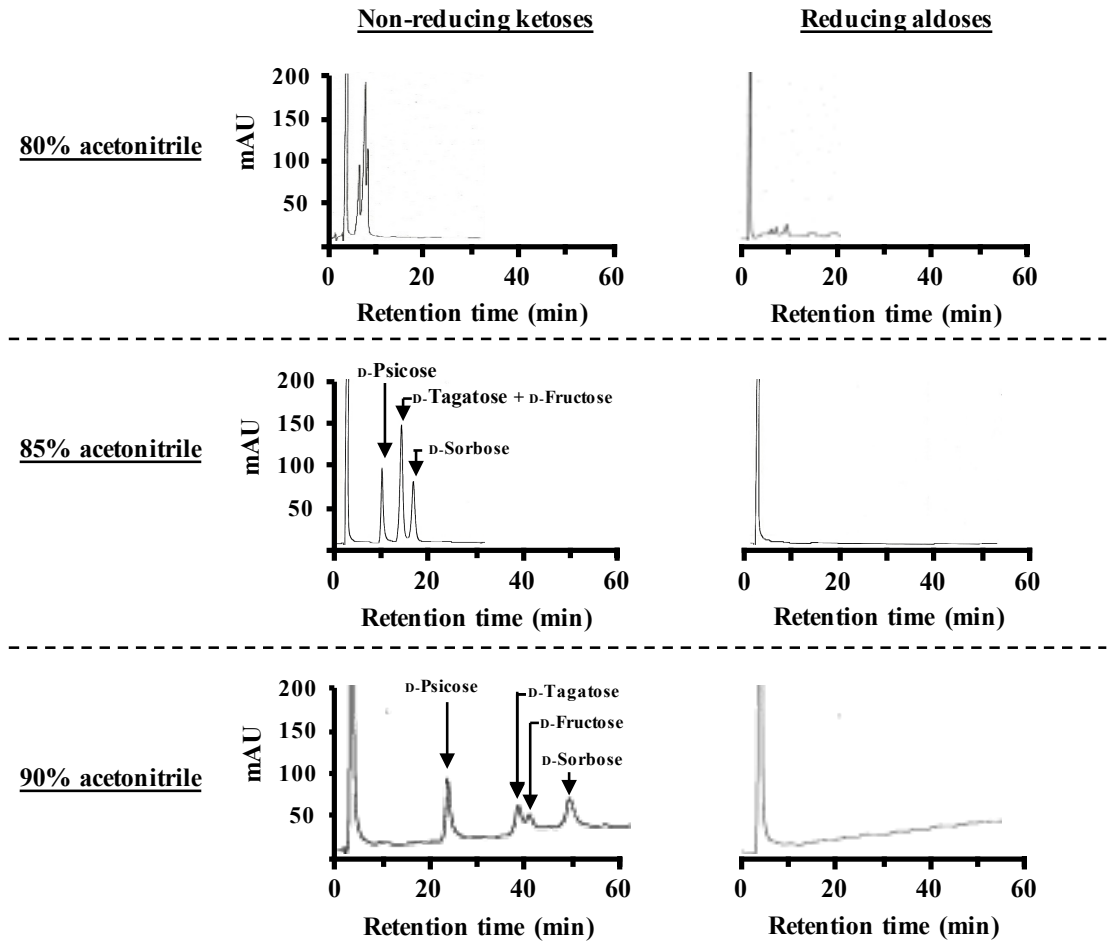


Fig. 2-3 Effect of acetonitrile concentration on HPLC elution of either a mixture of reducing aldoses or non-reducing ketoses on polyethyleneimine-attached HILIC column.

A mixture of each 1.0 wt% reducing aldose (D-glucose, D-allose, D-xylose) or a mixture of each 1.0 wt% non-reducing ketose (D-fructose, D-psicose, D-sorbose, D-tagarose) was injected to RI-HPLC with an elution of 80, 85, or 90 v/v% acetonitrile at a flow rate of 0.75 mL/min and a temperature of 40°C.

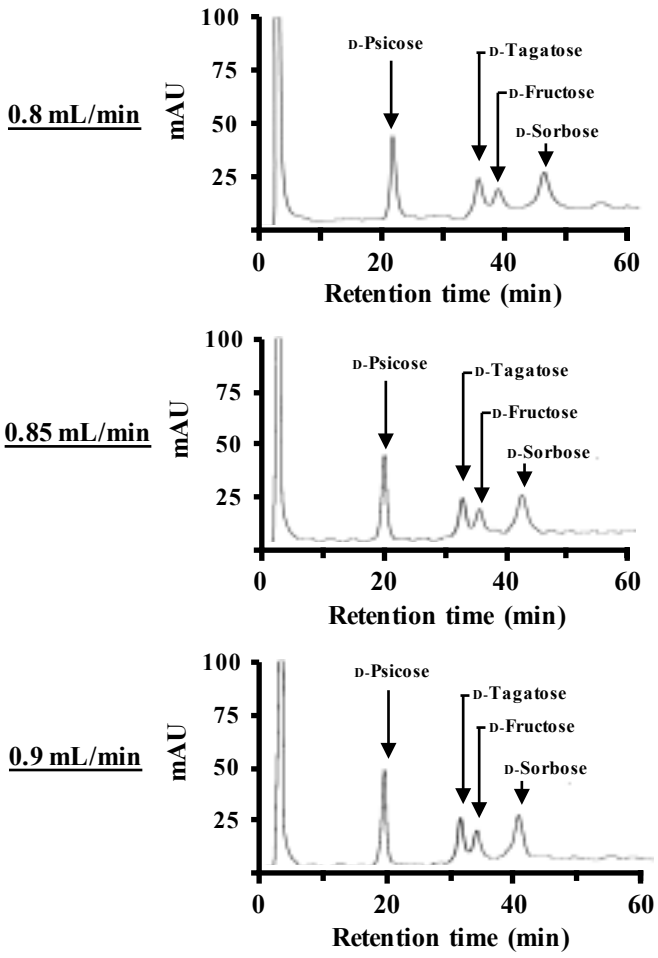


Fig. 2-4 Effect of flow rate on the chromatographic separation of a mixture of non-reducing ketoses on polyethyleneimine-attached HILIC column.

A mixture of each 1.0 wt% non-reducing ketose (D-fructose, D-psicose, D-sorbose, D-tagarose) was injected to RI-HPLC with an elution of 90 v/v% acetonitrile a flow rate of 0.8, 0.85, or 0.9 mL/min and a temperature of 40°C.

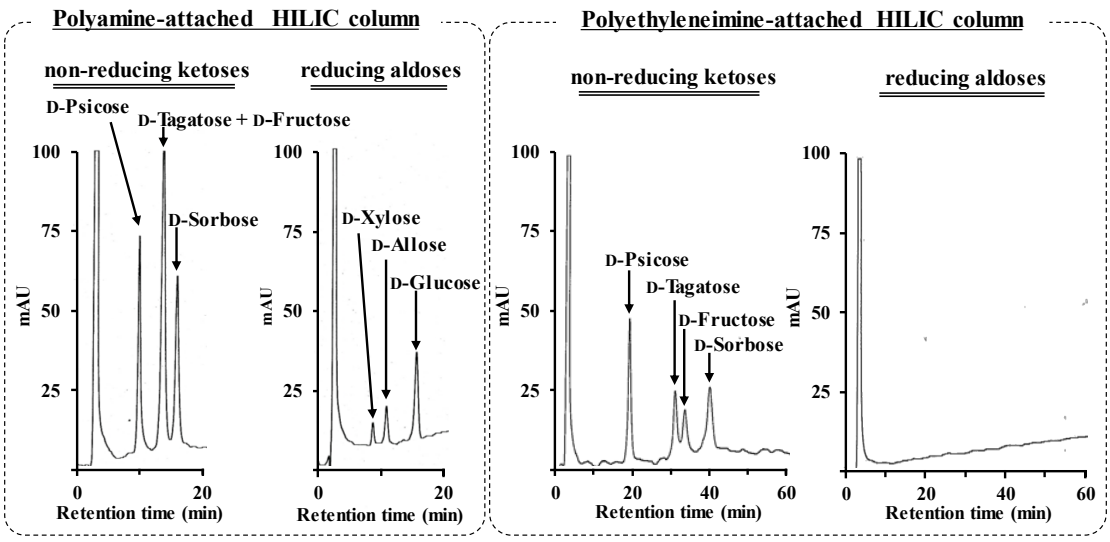


Fig. 2-5 HPLC profiles of reducing aldoses or non-reducing ketoses on polyamine-attached or polyethyleneimine-attached HILIC column.

A mixture solution of 1.0 wt% non-reducing ketose (D-fructose, D-psicose, D-sorbose, D-tagarose) or a mixture solution of 1.0 wt% reducing aldoses (D-glucose, D-allose, D-xylose) was injected to RI-HPLC with an elution of 90 v/v% acetonitrile at a flow rate of 0.9 mL/min and a temperature of 40°C.

2.3.4 RI-HPLC detection of reducing aldoses on polyethyleneimine-attached HILIC column

It has been reported that an amino group-induced resin may form the Schiff-base with reducing aldoses, which may cause high retention or no elution on column [89]. To solve the issue, additives and buffers as a modifier of mobile phase are often applied to improve insufficient elution profile of reducing aldoses by ion exchange capacity or pH [97]. Therefore, in order to accomplish the compete detection of the three reducing aldoses (D-glucose, D-allose, and D-xylose) on the imine-induced HILIC column, the effect of additives into acetonitrile was then investigated to overcome the no RI-detection of aldoses in this study (Figs. 2-2, 2-3, and 2-5). Six additives were used, including 0.1 v/v% pyridine, 0.1 v/v% tetrahydrofuran (THF), 0.1 v/v% *N,N*-dimethylformamide (DMF), 0.1 v/v% hexamethylenediamine (HMD), 1 mmol/L sodiumn 1-propanesulfonate (PS), and 1mmol/L sodium 1-octanesulfonate (OS). The mobile phase of 80 v/v% acetonitrile at 0.75 mL/min was used in this study, since a significant peak of reducing aldoses was observed only at the above elution conditions, though their RI intensity was still low (Fig. 2-3). Six cationic and anionic additives elected for this study (Fig. 2-6) were expected to suppress the strong Schiff-base interaction of analyte with polyethyleneimine moiety of the HILIC column by reducing ion pairing or the Schiff-base formation. As shown in Fig. 2-6,

however, cationic ion-pair reagents (pyridine, THF, DMF, and HMD) did not show any significant improvement of poor RI-detection of the three targeted aldoses. This indicated that pyridine, THF, DMF, and HMD had less ability to reduce the strong interaction of aldoses with the imine-induced HILIC column (Fig. 2-6). In contrast, a significant enhancement of RI-detection intensity and retention of reducing aldoses was obtained by the addition of 1 mmol/L PS and 1 mmol/L OS anionic ion-pair reagents (pH 4.8, adjusted with 5 mmol/L NaH_2PO_4), both of which are potent ion-pair reagents for solid phase extraction [98]. This indicates that anionic reagents weakened the interaction or high retention of aldoses with the imine-induced HILIC column by strong counter-ionic effect. The improvement of Rs by OS was greater than that by PS on the polyethyleneimine-attached HILIC column (Rs of PS: D-xylose/D-allose: 1.00, D-allose/ D-glucose: 1.57; Rs of OS: D-xylose/D-allose: 1.63, D-allose/ D-glucose: 1.37). The chain length of OS (C8) is longer than PS (C3), possibly providing strong ion-paring interaction [99]. The present finding on OS was in good agreement with the report by Fan *et al*, [100], who achieved an improved separation of nucleoside reverse transcriptase inhibitors by 8 mmol/L OS addition. The pH of solution may also affect the elution characteristics of ion-pair reagents [101], as discussed in the next section.

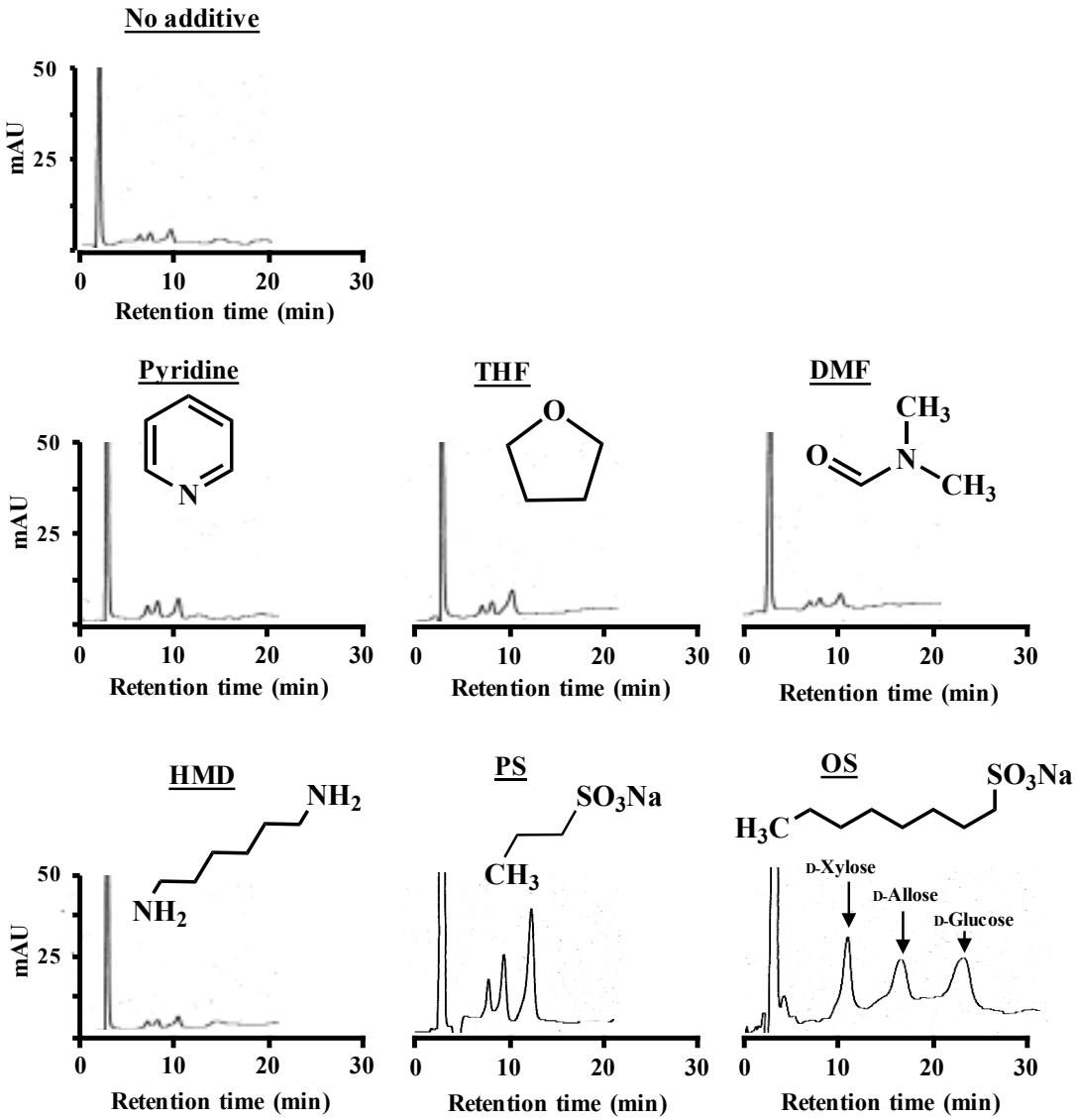


Fig. 2-6 Effect of ion-pair additives on the detection and separation of reducing aldoses on polyethyleneimine-attached HILIC column.

A mixture of 1.0 wt% reducing aldose (D-glucose, D-allose, D-xylose) was injected to RI-HPLC with an elution of 80 v/v% acetonitrile containing ion-pair reagent at 0.75 mL/min at 40**°C**. Ion-pair reagents used in this experiment were 0.1 wt% pyridine, 0.1 wt% THF, 0.1 wt% DMF, 0.1 wt% HMD, 1 mmol/L PS (pH 4.8), and 1 mmol/L OS (pH 4.8).

2.3.5 Simultaneous detection of reducing and non-reducing monosaccharides on polyethyleneimine-attahced HILIC column

The elution of 80 v/v% acetonitrile/1 mmol/L OS (pH 4.8) as mobile phase enabled a successful detection and separation of the three reducing aldoses on the imine-induced HILIC column at the flow rate of 0.75 mL/min (Fig. 2-6). However, all the monosaccharides (i.e., a mixture of three reducing aldoses and four non-reducing ketoses) were not separated individually at any elution conditions in this study (Fig. 2-7). Thus, further experiments to achieve simultaneous separation and detection of both reducing and non-reducing sugars on the imine-induced HILIC column (flow rate: 0.75 mL/min, 40°C) were attempted. As shown in Fig. 2-7, the increase in acetonitrile concentration (70 v/v% to 85 v/v%) caused a great improvement of OS-aided separation of the seven monosaccharides on the polyethyleneimine-attached HILIC column, while a simultaneous separation of each analyte was not fully achieved at 85 v/v% acetonitrile containing 1 mmol/L OS (pH 4.8). By increasing OS concentration to 5 mmol/L, a complete separation of all the seven monosaccharides was obtained as shown in Fig. 2-7. Considering the limited solubility of 5 mmol/L OS in >90 v/v% acetonitrile solution, the elution composed of 85 v/v% acetonitrile containing 5 mmol/L (pH 4.8) was thought to be the optimal separation conditions for both non-reducing and reducing monosaccharides when the imine-induced HILIC

column was used at 0.75 mL/min. Fig. 2-7 also revealed that at the elution condition of 90 v/v% acetonitrile without additives, only the four non-reducing monosaccharides were eluted on the HILIC column when the mixture of reducing and non-reducing monosaccharides was injected, in good agreement with the results in Fig. 2-3. Next, the effect of pH on the separation of the mixture of seven monosaccharides under the aforementioned optimal elution conditions was examined. At alkaline pHs of 7.5 and 9.5, each peak was broadened and/or disappeared, as compared to the elution at pH 4.8 (Fig. 2-8). It was known that the ability of ion-pair reagents vary with pH [101], so that the cationic status of the (poly) ethyleneneimine group at pH 4.8 (pKa 7.0 [102]) may lead to the ion-pair interaction with anionic OS to prevent the Schiff-base formation of the reducing aldoses with the polyethyleneimine-attached HILIC column.

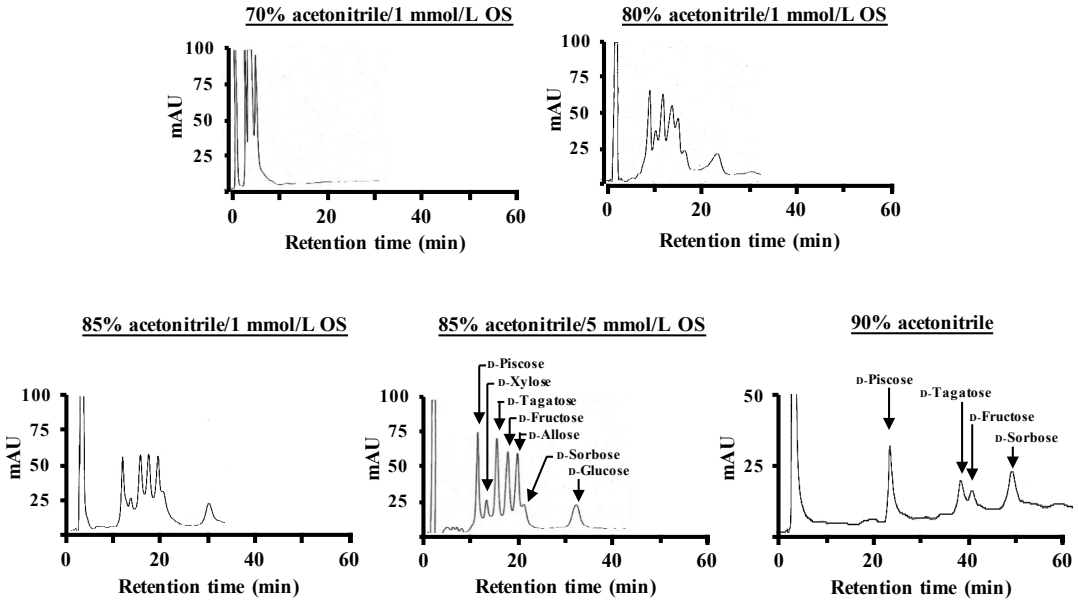


Fig. 2-7 Simultaneous detection of both reducing aldoses and non-reducing ketoses on polyethyleneimine-attached HILIC column. Each 1.0 wt% reducing aldose (D-glucose, D-allose, D-xylose) and non-reducing ketose (D-fructose, D-psicose, D-sorbose, D-tagarose) were mixed and injected to RI-HPLC at 0.75 mL/min at 40**°C**. Elution experiments were performed at each isocratic elution condition of 70, 80, or 85 v/v% acetonitrile containing 1 mmol/L OS (pH 4.8), or 85 v/v% acetonitrile containing 5 mmol/L OS (pH 4.8). Elution with 90 v/v% acetonitrile without additives was also performed.

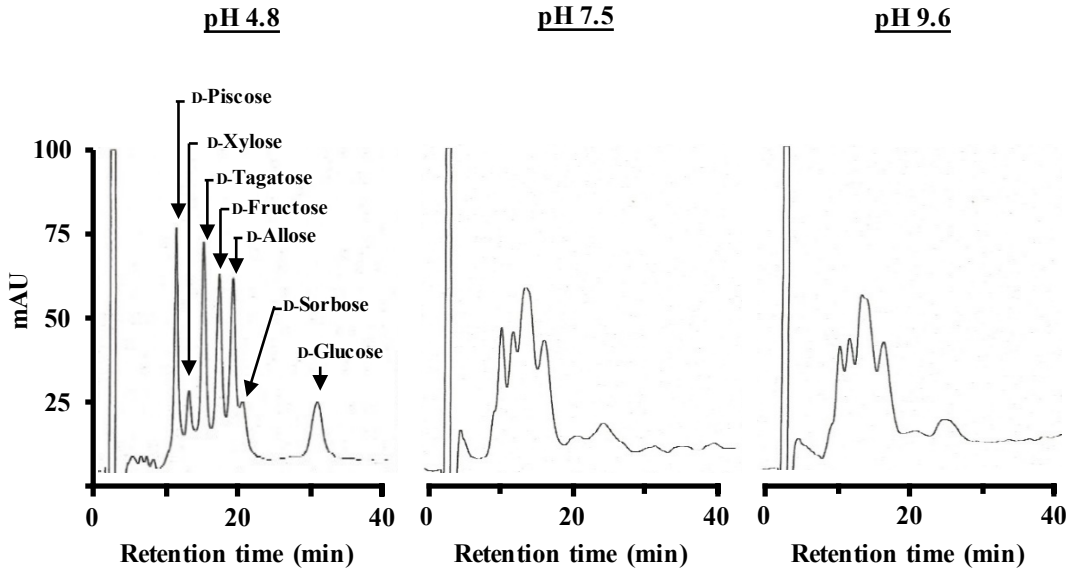


Fig. 2-8 Effect of pH on the separation of a mixture of four non-reducing ketoses and three reducing aldoses on polyethyleneimine-attached HILIC column.

HPLC profiles of a mixture of seven monosaccharides (D-psicose, D-xylose, D-fructose, D-tagatose, D-allose, D-sorbose, and D-glucose; each concentration, 1.0 wt%) were obtained at the following elution conditions: 85 v/v% acetonitrile containing 5 mmol/ L OS (pH 4.8, 7.5, or 9.6) at a flow rate of 0.75 mL/min. The chromatogram at pH 4.8 was the same as Fig. 2-5.

2.4. Summary

In this **Chapter II**, it was demonstrated that a polyethyleneimine (8 wt%) attached copolymer-based HILIC column enables the discriminant RI-HPLC analysis of non-reducing ketoses at the elution with 90 v/v% acetonitrile at a flow rate at 0.9 mL/min at 40°C without any derivatization. Alternatively, elution with 85 v/v% acetonitrile containing 5 mmol/L OS (pH 4.8) at 0.75 mL/min allowed the simultaneous detection of both reducing and non-reducing monosaccharides on the imine-induced HILIC column. Thus, we can choice the chromatographic way for the detection of either non-reducing or non-reducing/reducing monosaccharides on a single imine-induced HILIC column by changing the composition of mobile phase.

Chapter III

Simultaneous analysis of catecholamines and the metabolites on a sulfonated mixed-mode copolymer column

3.1 Introduction

Catecholamines, including dopamine (DA), norepinephrine (NE), and epinephrine (E), together with their metabolites, 3,4-dihydroxy phenylacetic acid (DOPAC), homovanillic acid (HVA), and 3-methoxy-4-hydroxyphenylglycol (MHPG), are recently highlighted as diagnostic and therapeutic metabolites in neurobiology [103,104]. UV-, FLD-, ECD-, MS- or tandem-mass spectrometry (MS/MS)-HPLC has been a trend in technique for catecholamine assay [105]. Among the techniques, ECD-HPLC is the most common technique for catecholamine assay, since an ECD detection is highly sensitive for electrochemically active catecholamines [106].

However, ECD-HPLC assay for catecholamines is limited for complete chromatographic separation due to a restrictive isocratic elution [105,107,108]. Although Sasaki *et al.* [109] proposed a simultaneous ECD-HPLC assay of seven catecholamine metabolites in human urine by a gradient elution mode of methanol, a severely controlled ECD condition required a long elution time of >100 min. Thus, an LC-MS or LC-MS/MS has been currently applied for catecholamine assay instead of the use of ECD-HPLC [110], although the limited volatile mobile phase for MS-electrospray ionization (ESI) may cause poor chromatographic separation on common reversed-phase column. A challenging separation of catecholamines and their metabolites using a cation-exchange/reversed-phase (mixed-mode) column was performed by the aid of fluorescence derivatization [111]. The mixed-mode column with pH gradient elution achieved an adequate LC separation, while the imidazole-derivatization of catecholamines still involved tedious analytical procedures [111]. Thus, to establish a convenient and direct assay for catecholamines and their metabolites, an LC-MS-based assay, using a cation-exchange/reversed-phase mixed-mode column was newly developed in this study. It was reported that the separation of catecholamines may affected by reversed-phase chromatography and cation exchange chromatography [112]. A strong cation-exchange resin coated with sulfonyl groups may allow the separation of catecholamines by ion-exchange property. A column packed with ethylstyrene-divinylbenzene copolymer resin attached sulfonyl

groups was used for catecholamine assay in this study, since the column was found to be sufficient for the separation of basic dipeptides [113] .

In this **Chapter III**, the mixed-mode of cation exchange/reversed-phase copolymer column packed with ethylstyrene-divinylbenzene resin coated with sulfonyl groups was used to establish a simultaneous assay for catecholamines and their metabolites by a single LC-MS technique without any derivatization techniques.

3.2 Experimental

3.2.1 Reagents and chemicals

L-Norepinephrine hydrochloride (NE), (±) epinephrine hydrochloride (E), and 3-methoxy-4-hydroxyphenylglycol hemipiperazinium salt (MHPG) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). 3,4-Dihydroxyphenylacetic acid (DOPAC) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Dopamine hydrochloride (DA), homovanillic acid (HVA), ammonium hydroxide, ammonium acetate, and ammonium formate were purchased from Nacalai Tesque Inc., (Kyoto, Japan). Tetraethylammonium hydroxide (TEA) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). LC-MS grade formic acid (FA), acetonitrile, and water as LC-MS grade were

obtained from Merck Millipore (Darmstadt, Germany). LC-MS grade methanol was purchased from Kanto Chemical Co. (Tokyo, Japan). Other chemicals used in this study were of analytical reagent grade.

3.2.2 LC-TOF/MS analysis

A standard mixture of six catecholamine metabolites (DA, NE, E, DOPA, HVA, and MHPG) was prepared in LC-MS grade water at each concentration of 50.0 μmol/L. Twenty microliter of the standard mixture was injected into an LC-TOF/MS system. LC chromatography was performed on an Agilent 1200 series HPLC (Agilent Technologies, Waldbronn, Germany). Chromatographic separation was performed on an ethylstyrene-divinylbenzene copolymer column packed with 0.81 wt% sulfonyl group (MCl GEL^{TM} CHK40/C04, 2.1 mm I.D. × 100 mm, 4 μm, Mitsubishi Chemical Co., Tokyo, Japan) or a column without sulfonyl group (MCl GEL^{TM} CHP20/C04, 2.1 mm I.D. × 100 mm, 4 μm) at 40°C. A linear gradient elution of mobile phase A to B (20 min) was performed at 0.20 mL/min. Optimization of mobile phase for LC separation is discussed in the latter section. A time-of-flight (TOF)/MS analysis was performed on a micrOTOF-Ⅱ mass spectrometer (Burker Daltonics, Bremen, Germany) in negative ESI mode. MS spectral data were collected at a range of 50 to 500 *m/z*. The ESI-MS conditions were as follows: dry N_2 gas flow, 8.0 L/min; drying

temperature: 200°C; nebulizer pressure: 1.6 bar; capillary voltage: 3,800 V; capillary exit: -100 V; and hexapole RF: 100 Vpp. Targeted DA, NE, E, DOPAC, HVA, and MHPG were identical at *m/z* 152.0706, *m/z* 168.0655, *m/z* 182.0812, *m/z* 167.0339, *m/z* 181.0495, *m/z* 183.0652, respectively, by Bruker Data Analysis version 3.2 software.

3.2.3 Linearity and validation

Under the optimized LC-TOF/MS conditions the linearity, coefficient of variation (CV), lower limit of detection (LOD), and lower limit of quantification (LOQ) were validated for catecholamines and their metabolites. LOD and LOQ were defined as the concentration yielding to the signal to noise ratio (*S/N*) of 3 and 10, respectively. Samples were analyzed in triplicate for individual concentrations.

3.2.4 Preparation of mouse brain tissues

Brain tissues used in this study were obtained from three 9-week old male ICR mice with 30–40 g body weight [Crl:CD1(ICR), Charles River Japan, Kanagawa, Japan]. All mice were housed for 1 week under controlled temperature at 21 ± 1°C, humidity at 55 ± 5%, and lightening from 8:00 to 20:00. The mice were fed rodent

diet CE-2 (CLEA Japan, Tokyo, Japan) and water *ad libitum*. Animal experiments were handled in accordance with the Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the competence of the Ministry of Education, Culture, Sports, Science, and Technology in Japan. The Ethics Committee on Animal Experiments at Fukuoka University approved all experimental protocols (permit number: 1715152). Mice fasted for 16 h were sacrificed after anesthetization with 25% urethane (Sigma-Aldrich Co.). Whole brain was then removed from the mice by decapitation and weighed. Brain samples were immediately frozen in liquid N_2 and stored at -80°C until LC-TOF/MS analysis. The brain sample was mashed with a Bio-masher (Nippi Inc., Tokyo, Japan), and lyophilized to dryness. Brain powder (50 mg) was accurately weighted, and dissolved in 3 mL of 0.1 v/v% FA solution. The solution was subjected to sonication with a Sonifier 250 (Branson Ultrasonics, CT, USA) for 3 cycles of 30 s at output control 3, followed by homogenization for 2 cycles of 60 s at 20,000 rpm with a Polytron PT2500E homogenizer (Kinematica, Luzern, Switzerland) on ice. The obtained homogenate was then centrifuged at 14,000 × *g* for 15 min at 4°C. The supernatant was filtered through Amicon 3K (Merck Millipore), followed by the evaporation to dryness. The dried brain sample was dissolved in LC-MS grade water prior to LC-TOF/MS analysis.

3.3 Results and Discussion

As depicted in Fig. 1-4, DA derived from tyrosine (or phenylalanine) is a precursor of monoamine neurotransmitters (NE and E), and it also enters the DOPAC and HVA (an end-product of DA metabolism) production pathway [81]. MHPG is also known to be an end-product of the NE/E metabolic pathway [82]. Therefore, the simultaneous determination of catecholamines and the metabolites including the two end-products, would allow us to better understand the overall brain homeostasis.

3.3.1 Application of sulfonated mixed-mode copolymer column for the separation of catecholamines and the metabolites

To get the chromatographic characteristics of polymer-based reversed-phase HPLC columns, two types of column packed with ethylstyrene-divinylbenzene copolymer resin were used for catecholamine assay (Fig. 3-1). LC-TOF/MS experiments were primarily performed using a MCl GEL^{TM} CHP20/C04 column (no attached sulfonyl groups) with a linear elution gradient ranging from 0 to 50 v/v% methanol containing 0.1 v/v% FA. As shown in Fig. 3-2, all the six metabolites (50.0 μmol/L) were detected according to their corresponding *m/z* value in a negative ESI-ionization mode. However, the elution profiles observed in Fig. 3-2 were clearly

divided into two groups. DA, NE, and E, bearing sulfonyl groups were not retained on the reversed-phase copolymer column attached no sulfonyl groups (retention time of <2 min); while DOPAC, HVA, and MHPG, bearing no amino groups exhibited a significant retention on the column (retention time of DOPAC, 24.0 min; HVA, 30.9 min; MHPG, 22.0 min). Therefore, the reversed-phase copolymer (ethylstyrene-divinylbenzene) column was suitable for the retention of neutral catecholamines of DOPAC, HVA, and MHPG by their hydrophobic interaction with copolymer column. In contrast, no retention of catecholamines bearing amino groups on the reversed-phase copolymer column (Fig. 3-2A) may be due to their polar cationic property in structure. Ion-exchange interactions on the column were, thus, expected to be crucial in achieving the retention of catecholamines bearing amino groups. Tsunoda and Imai [114] have reported the advantage of cation-exchange column for pre-extraction of catecholamines from blood. Thus, further separation experiments were performed using a partially sulfonated (0.81 wt%) ethylstyrene-divinylbenzene copolymer column (MCl GEL^{TM} CHK40/C04). The mixed-mode cation-exchange/reversed-phase copolymer column was reported to show an appropriate retention of basic dipeptides by both ionic and hydrophobic interactions [113]. As shown in Fig. 3-2B, a good separation and retention of the three metabolites (DOPAC, HVA, and MHPG) were obtained on the mixed-mode column, as similar to the non-sulfonated column (MCl GEL^{TM} CHP20/C04) (Fig. 3-2A). This

indicates that the partially sulfonated ethylstyrene-divinylbenzene copolymer column still possessed the similar hydrophobic interaction characteristic as common reversed-phase column. In contrast, the mixed-mode column used in this study could not separate catecholamines. Considering the reported strong ionic interaction of small amines with cation-exchange stationary phase [115], no detection of catecholamines (Fig. 3-2B) may be caused by their high retention onto the resin.

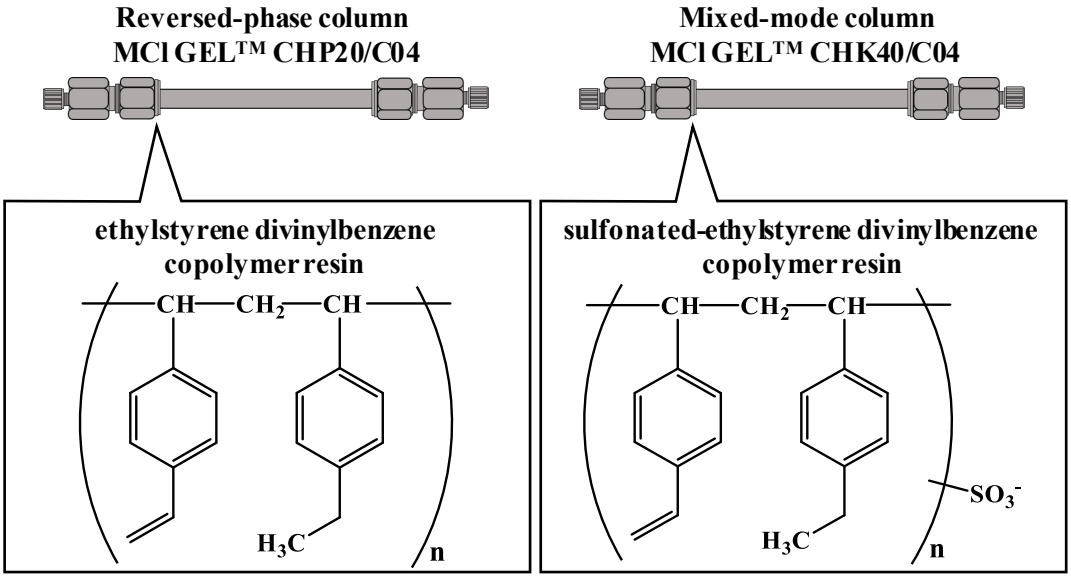


Fig. 3-1 Ethylstyrene-divinylbenzene copolymer columns used in this study.

MCl GEL™ CHP20/C04) as no sulfonated column (2.1 mm I.D. × 100 mm, 4 μm)

MCl GEL™ CHK40/C04 as 0.81 wt% sulfonated column (2.1 mm I.D. × 100 mm, 4 μm).

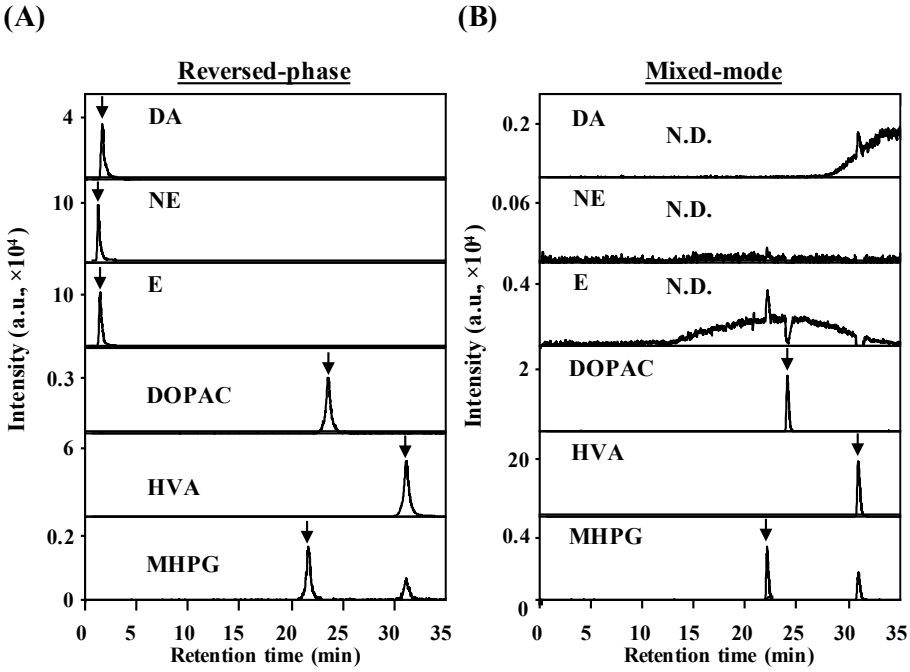


Fig. 3-2 Elution profiles of catecholamines and the metabolites on either copolymer column containing (A) no or (B) 0.81 wt% sulfonyl groups by negative ESI-LC-TOF/MS.

Identical mixtures of six catecholamine metabolites, each at a concentration of 50.0 μmol/L, were assayed on either copolymer column with or without sulfonyl groups. Linear elution was performed using 0 to 50 v/v% methanol in 0.1 v/v% FA at a flow rate of 0.20 mL/min at 40°C. Other LC-MS conditions were described in the Experimental section.

3.3.2 Elution of catecholamines on a sulfonated mixed-mode copolymer column

Provided that no LC-TOF/MS detection of catecholamines (DA, E, and NE) on sulfonated mixed-mode column (Fig. 3-2) were caused by their high retention in an elution condition of methanol containing 0.1 v/v% FA, further elution experiments were performed using volatile cationic additives (ammonium hydroxide and TEA) and volatile ion-pairing MS reagents (ammonium acetate and ammonium formate) in 50 v/v% methanol as mobile phase B. The concentration of each additive in the methanol mobile phase was 10 mmol/L, except for 1 mmol/L TEA. As shown in Fig. 3-3, cationic additives (ammonium hydroxide and TEA) and ammonium acetate failed to elute the three catecholamines on the sulfonated mixed-mode column. In contrast, the addition of 10 mmol/L ammonium formate into mobile phase B resulted in a successful elution of catecholamines from the sulfonated mixed-mode column possibly by reducing the ionic interaction with ammonium acetate. It is known that in negative ESI-MS a volatile ion-pairing reagent may form additive ion complex such as $[M + CH_3COO]^-$ and $[M + HCOO]^-$, causing the reduction of MS intensity [116]. However, in this study no $[M + NH_4COO]^-$ ion complex was observed when 10 mmol/L ammonium formate was added into 50 v/v% methanol (data not shown). Therefore, the low MS signal intensities of catecholamines observed in Fig. 3-3 suggested that some catecholamines still retained on or were not completely eluted

from the sulfonated mixed-mode column.

3.3.3 Optimization of LC-TOF/MS assay for catecholamines and the metabolites on a sulfonated mixed-mode copolymer column

To improve the low MS signal intensity of catecholamines in 50 v/v% methanol containing 10 mmol/L ammonium formate (Fig. 3-3D), effect of the concentration of ammonium formate (1, 10, and 50 mmol/L in 50 v/v% methanol) on MS signal intensity was investigated. As shown in Fig. 3-4A to 3-4C, increasing concentration of ammonium formate in mobile phase B slightly improved of MS intensity. In 50 mmol/L ammonium formate in 50 v/v% methanol, each catecholamine (DA, NE, and E) was successfully eluted, while the corresponding MS signal intensities were still weak on the sulfonated mixed-mode column. This indicated that the high retention (no or less elution) of catecholamines and the metabolites on the sulfonated mixed-mode column was cleaved by ammonium formate-aided ionic/acetonitrile-aided hydrophobic properties. Considering the strong solvent effect of acetonitrile compared to methanol [117], acetonitrile was then used as mobile phase B containing 50 mmol/L ammonium formate. As a result of the use of acetonitrile, the inferior MS signal intensity observed in Fig. 3-4C was markedly improved on the sulfonated mixed-mode column (Fig. 3-4D). To confirm the complete separation and detection of

all the six catecholamines and the metabolites at the optimized LC-TOF/MS conditions, the mixture of the six targets were injected to the system. As a result, all the six catecholamies and the metabolites were successfully separated and simultaneously detected on the sulfonated mixed-mode column within 25 min (Fig. 3-5A). The retention times of DA, NE, E, DOPAC, HVA, and MHPG at 0.20 mL/min and 40°C on the sulfonated mixed-mode column were 24.2, 20.6, 22.8, 17.6, 20.0, and 16.6 min, respectively. As summarized in Table 3-1, MS detection of each metabolite gave a linear regression at concentrations between 0.1–50.0 μmol/L of DA, NE, E, and HVA or 2.5–25.0 μmol/L of DOPAC and MHPG (r > 0.991). LOD was ranged from 12.6 nmol/L for NE to 3.2 μmol/L for MHPG. CV was ranged from 1 to 15 % (at each concentration of 50.0 μmol/L). These results strongly demonstrated that the proposed LC-TOF/MS assay using sulfonated mixed-mode copolymer column can be used for simultaneous catecholamine detection with high sensitivity and reproducibility.

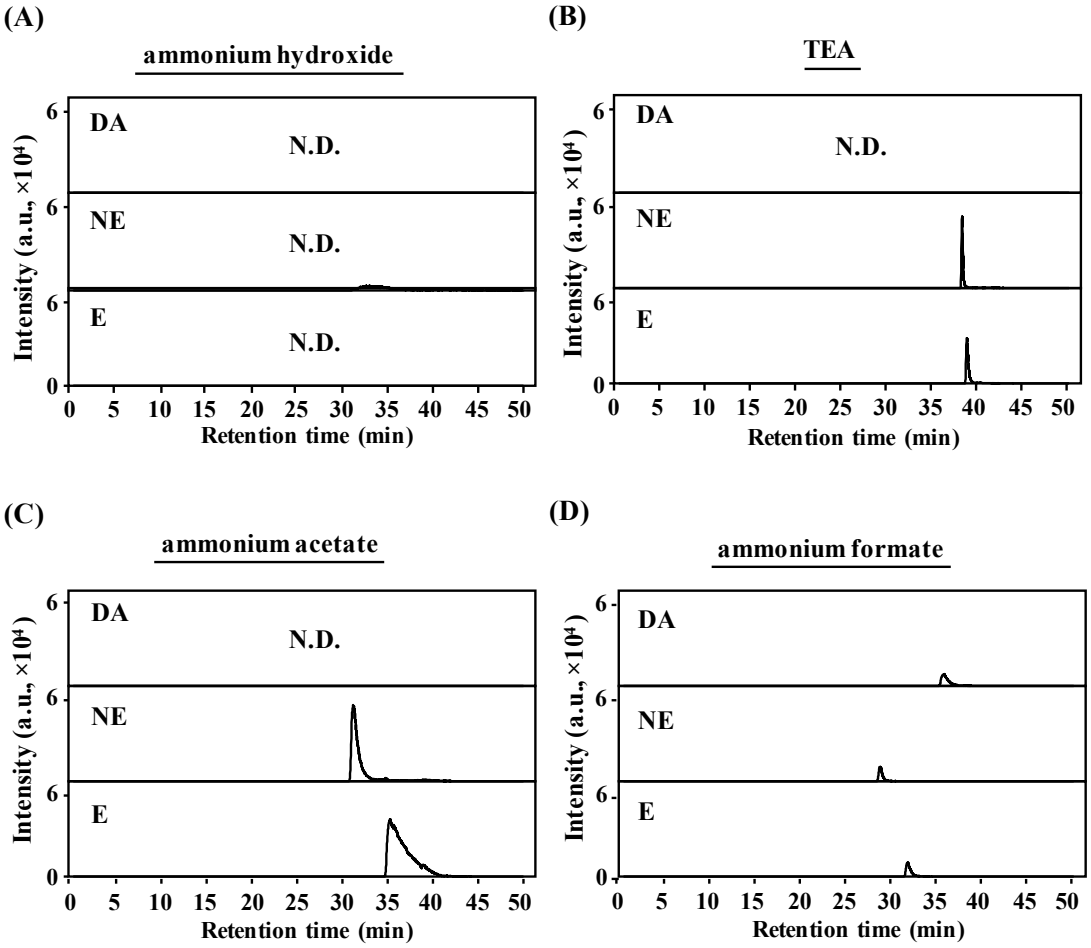


Fig. 3-3 Effect of volatile additives on elution of catecholamines on a sulfonated mixed-mode column by negative ESI-LC-TOF/MS.

Elution of DA, NE, and E was performed using water containing 0.1 v/v% FA to 50 v/v% methanol containing either (A) 10 mmol/L ammonium hydroxide, (B) 1 mmol/L TEA, (C) 10 mmol/L ammonium acetate or (D) 10 mmol/L ammonium formate at a flow rate of 0.20 mL/min and at 40°C over 20 min. Other LC-MS conditions were described in the Experimental section. N.D., not detected.

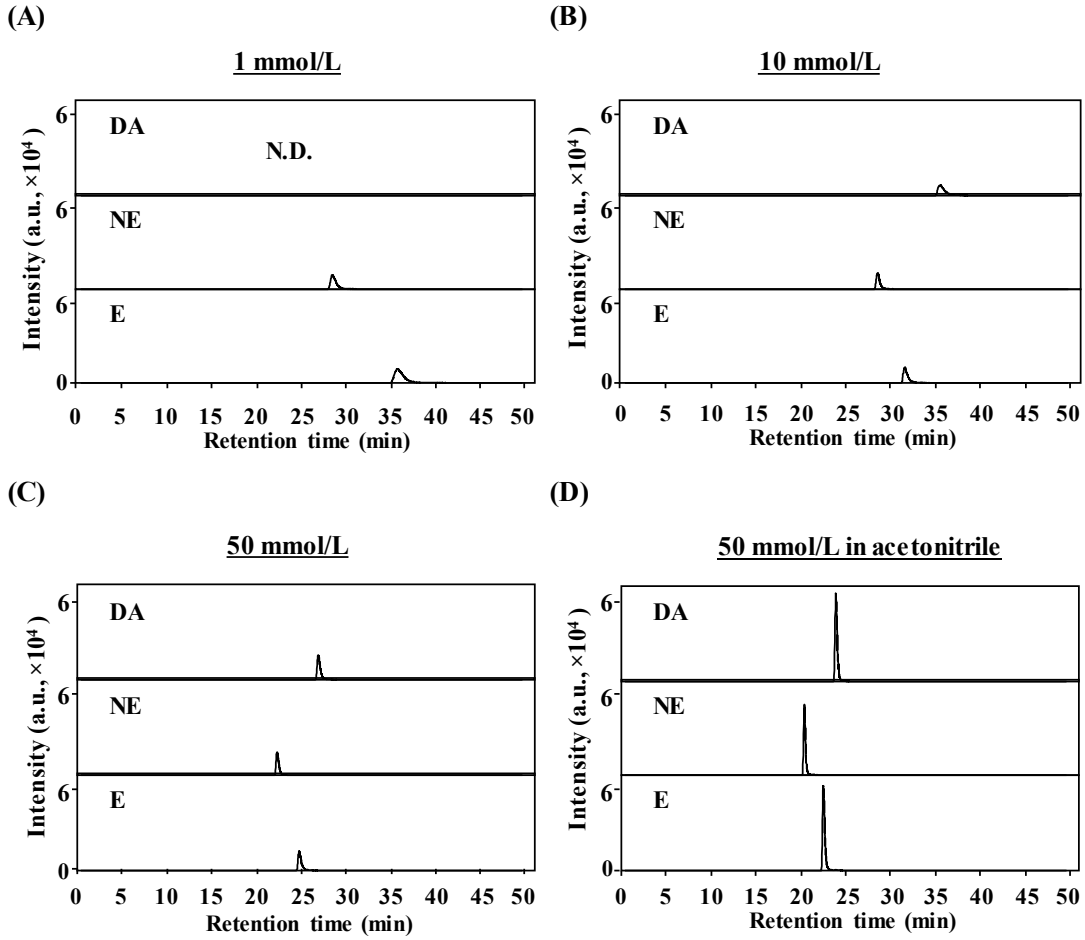


Fig. 3-4 Effect of concentration of ammonium formate on the elution of catecholamines on a sulfonated mixed-mode column by negative ESI-LC-TOF/MS.

Elution of 50.0 μmol/L DA, NE, and E was performed using water containing 0.1 v/v% FA to 50 v/v% methanol containing (A) 1 mmol/L, (B) 10 mmol/L, and (C) 50 mmol/L ammonium formate or (D) water containing 0.1 v/v% FA to 50 v/v% acetonitrile containing 50 mmol/L ammonium formate at a flow rate of 0.20 mL/min at 40°C. Other LC-MS conditions were described in the Experimental section. N.D., not detected.

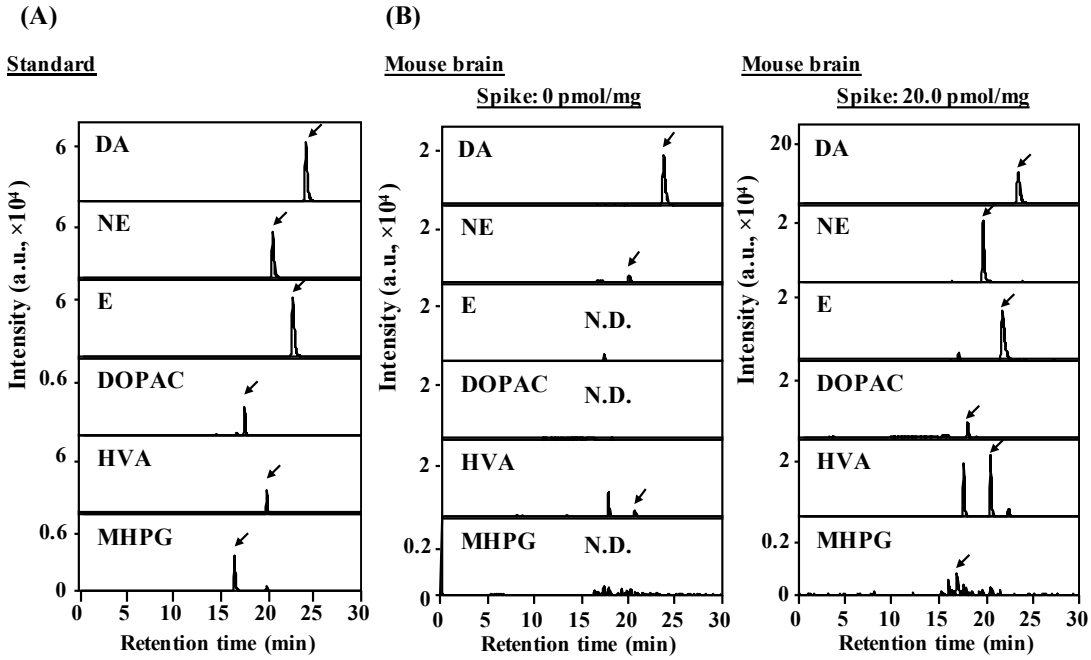


Fig. 3-5 Typical LC-TOF/MS chromatograms of six catecholamine metabolites on a sulfonated mixed-mode copolymer column in (A) a standard solution (50.0 μmol/L) and (B) mouse brain samples.

Brain samples spiked with 0 and 20.0 pmol/mg of each metabolite were subjected to an LC-TOF/MS assay. Linear elution was performed using water containing 0.1 v/v% FA to 50 v/v% acetonitrile containing 50 mmol/L ammonium formate at a flow rate of 0.20 mL/min at 40°C. Other LC-MS conditions were described in the Experimental section. N.D., not detected.

Table 3-1 Quantitation of catecholamines and the metabolites using LC-TOF/MS on a sulfonated mixed-mode column[a]

Analyte	RT (min)	Linearity[b] correlation coefficient (r)	Linear range (μmol/L)	LOD		LOQ		CV (%) at 50 μmol/L (n=3)
				nmol/L	pmol/injection	nmol/L	pmol/injection	
DA	24.2	$y = 6380.3x - 8017.6$ $r = 0.9975$	0.1–50.0	20.7	0.41	62.6	1.3	5.6
NE	20.6	$y = 6446.3x - 247.28$ $r = 0.9999$	0.1–50.0	12.6	0.25	38.1	0.76	1.1
E	22.8	$y = 8070.3x - 5015.9$ $r = 0.9995$	0.1–50.0	74.6	14.9	226	4.5	1.3
DOPAC	17.6	$y = 79.151x - 389.46$ $r = 0.9909$	2.5–25.0	1110	22.2	3365	67.3	9.4
HVA	20.0	$y = 4146.6x - 7358.7$ $r = 0.9974$	0.1–50.0	18.7	2.4	56.7	1.1	13.1
MHPG	16.6	$y = 27.728x - 29.821$ $r = 0.9999$	2.5–25.0	3196	63.9	9686	194	7.2

[a]A mixture of six catecholamines and the metabolites was assayed using a linear elution of water containing 0.1 v/v% FA to 50 v/v% acetonitrile containing 50 mmol/L ammonium formate over 20 min at 0.20 mL/min and 40°C. [b]y is an MS signal peak, and x is a concentration of target. Other LC-MS conditions were described in the Experimental section.

3.3.4 Assay for endogenous catecholamines and the metabolites in mouse brain

The proposed LC-TOF/MS assay on the sulfonated mixed-mode copolymer column was applied for endogenous catecholamine assay in organs. Thus far, although an ECD-HPLC assay for DA, NE, and E [84], and an immunoassay for HVA as inflammatory marker [118] have been proposed, no reports have been published on a simultaneous assay for all the six metabolites. In this study, male ICR mice with no stress by injection and with stable situation by urethane anesthetization were used to take whole brain. As shown in Fig. 3-5B, among the six targets, DA, NE, and HVA were detected as endogenous catecholamine metabolites in the ICR normal mouse brain samples, whereas other three metabolites were not detected (Fig. 3-5 and Table 3-1). The present LC-TOF/MS assay determined the endogenous DA, NE, and HVA as 21.6 to 29.0, 9.0 to 9.2, and 4.3 to 5.7 pmol/mg, respectively (Table 3-2). As shown in Fig. 3-5B, each of the six targets was successfully detected in the brain sample spiked at 20.0 pmol/mg of each metabolite. This suggested that the proposed assay can be applicable for simultaneous detection of catecholamines and the metabolites without any interferences from brain matrices.

Table 3-2 Levels of catecholamines and the metabolites in brain of ICR mouse by LC-TOF/MS on a sulfonated mixed-mode column

Analyte	Amount (pmol/mg brain)		
	mouse **A**	mouse **B**	mouse **C**
DA	21.6	29.0	22.4
NE	9.2	9.1	9.0
E	N.D.[a]	N.D.	N.D.
DOPAC	N.D.	N.D.	N.D.
HVA	5.7	5.4	4.3
MHPG	N.D.	N.D.	N.D.

Endogenous catecholamines and the metabolites in ICR mouse brain were assayed using a linear elution of water containing 0.1 v/v% FA to 50 v/v% acetonitrile containing 50 mmol/L ammonium formate over 20 min at 0.20 mL/min at 40°C. Other LC-MS conditions were described in the Experimental section. [a]N.D., not detected.

3.4 Summary

The present study demonstrated that a partially sulfonated (0.81 wt%) ethylstyrene-divinylbenzene mixed-mode copolymer column could be a useful simultaneous catecholamine assay by negative ESI-LC-TOF/MS. The successful elution or detection of catecholamines and the metabolites on the cation-exchange/reversed-phase mixed-mode column were achieved by a gradient elution of water containing 0.1 v/v% FA to 50 v/v% acetonitrile containing 50 mmol/L ammonium formate as volatile ion-pairing reagent. Using the proposed simultaneous LC-TOF/MS assay, endogenous DA, NE, and HVA were detected in ICR mouse brain at concentrations of >4 pmol/mg. Cai *et al.* reported that DA, NE, and HVA were detected from schizophrenic patients' urine at 5.9 nmol/L, 0.09 nmol/L, and 6.2 nmol/L, respectively, as tumor marker [119]. In conclusion, LC-MS assay combined with sulfonated mixed-mode column can allow the simultaneous analysis of not only catecholamines, but also fluids such as urine. The present assay can also open a new diagnostic science regarding neurotransmitters and related diseases.

Chapter IV

Conclusion

Great interests were grown in revealing the health benefits of foods. Various bioactive food compounds were analyzed to explore physiological functions and to provide more reliable evidences on physiological functions, involved in the improvement or prevention of lifestyle-related diseases. Besides, to understand and to clarify further physiological functions and their underlying mechanisms of bioactive compounds, reliable and appropriate separation techniques have been still required. LC is the most indispensable and common analytical technique for the separation and identification of compounds from complex foodstuffs to evaluate food quality and physiological contribution. Among a variety of LC separation columns such as reversed-phase, ion-exchange, HILIC and multi-functional mixed-mode columns were attempted to achieve simultaneous separation of analytes with diverse chemical properties (polar, non-polar, or hydrophobic) in a single LC chromatographic run in this study. Commonly, silica gel is used for LC column, but silica gel-based LC

columns are restrictive for the separation in acidic mobile phase conditions due to their poor alkaline stability. In contrast, polymer-based HPLC columns could open their widespread use in a variety of acid-alkaline mobile phases. In addition, polymer-based resin is capable of diverse chemical modifications.

To develop convenient separation techniques without tedious procedures, in the present study, thus, two types of chemically modified synthetic copolymer columns, i.e., HILIC column, and mixed-mode (cation exchange/reversed-phase) column, were evaluated for the simultaneous detection of monosaccharides (**Chapter II**) and catecholamines (**Chapter III**), respectively, which may contribute to open a new insight of LC separation techniques in food analysis.

Rare sugars have been paid in great attention in promoting health-benefits, since they evidentially possess various physiological functions against lifestyle-related diseases. However, the simultaneous separation of rare sugars or monosaccharides has been encountering analytical challenges, since they are highly hydrophilic and polar molecules. Considering strong interaction between polar rare sugars and HILIC column, simultaneous assay for rare sugars was established on a copolymer-based HILIC column by RI-HPLC. Seven monosaccharides (reducing aldoses: D-xylose, D-allose, and D-glucose; non-reducing ketoses: D-fructose, D-psicose, D-sorbose, and D-tagatose) were targeted in this study. The used column was composed of ethyleneimine monomeric unit in polymer chain, showing a higher affinity of rare

sugars with the column by imine-induced strong base property. In **Chapter II**, the polyethyleneimine-attached copolymer HILIC column was attempted to achieve simultaneous analysis of reducing and non-reducing monosaccharides, or discriminant analysis of non-reducing monosaccharides only by changing mobile solution containing anionic ion-pair reagent.

Since catecholamines and the metabolites are highlighted as diagnostic and therapeutic metabolites in neurobiology, a convenient and reliable analytical method in a single chromatographic run may allow us to rapidly understand the overall metabolism in brain. In **Chapter III**, simultaneous separation of catecholamines and the metabolites was challenged in a single MS assay on a mixed-mode copolymer HPLC column. A successful LC-TOF/MS detection of catecholamines and the metabolites was achieved by using a partially sulfonated (0.81 wt%) ethylstyrene-divinylbenzene copolymer HPLC column, with a gradient elution of water containing 0.1 v/v% FA to 50 v/v% acetonitrile containing 50 mmol/L ammonium formate as volatile ion-pairing reagent at 0.20 mL/min at 40°C within 25 min. The proposed LC-TOF/MS assay allowed the detection of endogenous DA, NE, and HVA in ICR mouse brain samples at concentrations of > 20, 9, and 4 pmol/mg, respectively. Therefore, the proposed LC-TOF/MS assay on a partially sulfonated mixed-mode copolymer column can allow a new diagnostic assay regarding neurotransmitters and related diseases.

In conclusion, the present study demonstrated that the two types of chemically modified synthetic copolymer columns used in this study effective for simultaneous separation and detection of monosaccharides and catecholamines. The results provide a new insight of analytical separation science by using a single chemically modified synthetic copolymer columns.

Reference

[1] L. S. Ettre, Chromatography: The separation technique of the 20th century. *Chromatographia*, **2000**, *51*, 7–17.

[2] J. H. Knox, Practical Aspects of LC Theory, *J. Chromatogr. Sci.*, **1977**, *15*, 352–364.

[3] K. D. Bartle, and P. Myers, History of gas chromatography. *Trends Analyt. Chem.,* **2002**, *21*, 547–557.

[4] M. Santiago, and S. Strobel, Thin layer cjromatography. *Meth. Enzymol.*, **2013**, *533*, 303–324.

[5] M. Saito, History of supercritical fluid chromatography: Instrumental development. *J. Biosci. Bioeng.*, **2013**, *115*, 590–599.

[6] T. Matsui, and K. Matsumoto, Antihypertensive peptides from natural resources. *Adv. Phytomed.*, **2006**, *2*, 255–271.

[7] F. P. J. Martin, I. Montoliu, S. Collino, M. Scherer, P. Guy, I. Tavazzi, A. Thorimbert, S. Moco, M. Rothney, D. L. Ergun, M. Beaumont, F. Ginty, S. D. Qanadli, L. Favre, V. Giusti, and S. Rezzi, Topographical body fat distribution links to amino acid and lipid metabolism in healthy non-obese women. *PLoS ONE*, **2013**, *8*, e73445–e73458.

[8] K. Erdmann, B. W.Y. Cheung, and H. Schröder, The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. *J. Nutr. Biochem.*, **2008**, *19*, 643–654.

[9] P. M. K. Etherton, K. D. Hecker, A. Bonanome, S. M. Coval, A. E. Binkoski, K. F. Hilpert, A. E. Griel, and T. D. Etherton, Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. *Am. J. Med.* **2002**, *113*, 71–88.

[10] M. J. Rein, M. Renouf, C. C. Hernandez, L. A. Goretta, S. K. Thakkar, and M. D. S. Pinto, Bioavailability of bioactive food compounds: A challenging journey to bioefficacy. *J. Clin. Pharmacol*., **2012**, *75*, 588–602.

[11] K. Oehlke, M. Adamiuk, D. Behsnilian, V. Gräf, E. M. Miebach, E. Walz, and R. Greiner, Potential bioavailability enhancement of bioactive compounds using food-grade engineered nanomaterials: a review of the existing evidence. *Food Funct.*, **2014**, *5*, 1341–1359.

[12] J. M. C. Capella, M. Buniowska, F. J. Barba, M. J. Esteve, and A. Frígola, Analytical methods for determining bioavailability and bioaccessibility of bioactive compounds from fruits and vegetables: A review. *Compr. Rev. Food Sci. Food Saf.*, **2014**, *13*, 155–171.

[13] R. D. Conlon, Liquid chromatography detectors. *Anal. Chem.*, **1969**, *41*, 107A–114A.

[14] T. R. Covey, E. D. Lee, A. P. Bruins, and J. D. Henion, Liquid chromatography/mass spectrometry. *Anal. Chem*., **1986**, *58*, 1451A–1461A.

[15] J. J. Kirkland, C. H. J. Dilks, and J. J. DeStefano, Normal-phase high-performance liquid chromatography with highly purified porous silica microspheres. *J. Chromatogr. A*, **1993**, *635*, 19–30.

[16] B. L. Karger, J. R. Gant, A. Martkopf, and P. H. Weiner, Hydrophobic effects in reversed-phase liquid chromatography. *J. Chromatogr. A*, **1976**, *128*, 65–78.

[17] L. R. Snyder, J. W. Dolan, and J. R. Gant, Gradient elution in high-performance liquid chromatography: I. Theoretical basis for reversed-phase systems. *J. Chromatogr. A*, **1979**, *165*, 3–30.

[18] A. Jungbauer, and R. Hahn. Ion-exchange chromatography. In: *Methods Enzymol*., **2009**, *463*, 349–371.

[19] H. G. Barth, C. Jackson, and B. E. Boyes, Size exclusion chromatography. *Anal. Chem.*, **1994**, *66*, 595–620.

[20] A. J. Alpert, Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds. *J. Chromatogr. A*, **1990**, *499*, 177–196.

[21] T. Yoshida, Peptide separation by hydrophilic-interaction chromatography: A review. *J. Biochem. Bioph. Methods*, **2004**, *60*, 265–280.

[22] R. LI, and J. Huang, Chromatographic behavior of epirubicin and its analogues

on high-purity silica in hydrophilic interaction chromatography. *J. Chromatogr. A*, **2004**, *1041*, 163–169.

[23] L. Wang, W. Wei, Z. Xia, X. Jie, and Z. Z. Xia, Recent advances in materials for stationary phases of mixed-mode high-performance liquid chromatography. *Trends Analyt Chem*., **2016**, *80*, 495–506.

[24] Y. Yang, and X. Geng, Mixed-mode chromatography and its applications to biopolymers. *J. Chromatogr. A*, **2011**, *1218*, 8813–8825.

[25] C. T. Mant, and R. S. Hodges, Mixed-mode hydrophilic interaction/cation-exchange chromatography (HILIC/CEX) of peptides and proteins. *J. Sep. Sci.*, **2008**, *31*, 2754–2773.

[26] J. Guo, T. Saiki, K. Thanutchaporn, W. Liu, A. Shimura, and T. Matsui, Elution profile of di-peptides on a sulfonated ethylstyrene–divinylbenzene copolymer resin column by high-performance liquid hromatography. *Anal. Sci.*, **2005**, *31*, 45–50.

[27] B. Çelebi, B. Özen, Ç. Kip, and A. Tuncel, A new stationary phase for hydrophilic interaction chromatography: Polyacrylate-based hydrophilic, monosized-porous beads with zwitterionic molecular brushes. *Chromatographia*, **2014**, *77*, 1511–1520.

[28] M. Hiemstra, and A. Kok, Determination of propamocarb in vegetables using polymer-based high-performance liquid chromatography coupled with

electrospray mass spectrometry. *J. Chromatogr. A*, **2002**, *972*, 231–239.

[29] M. Mori, M. I. H. Helaleh, Q. Xu, W. Hu, M. Ikedo, M. Y. Ding, H. Taoda, and K. Tanaka, Vacancy ion-exclusion/adsorption chromatography of aliphatic amines on a polymethacrylate-based weakly basic anion-exchange column. *J. Chromatogr. A*, **2004**, *1039*, 129–133.

[30] T. Abe, H. Baba, I. Soloshonok, and K. Tanaka, Novel way of separating polyfluorocarboxylic acids by ion-exclusion chromatography. *J. Chromatogr. A*, **2000**, *884*, 93–103.

[31] K. Tanaka, H. Chikara, W. Hu, and K. Hasebe, Separation of carboxylic acids on a weakly acidic cation-exchange resin by ion-exclusion chromatography. *J. Chromatogr. A*, **1999**, *850*, 187–196.

[32] S. Cheng, H. Tang, and H. Yan, Effects of multiple weak interactions on the binding of phenolic compounds by polymeric adsorbents. *J. Appl. Polym. Sci.*, **2006**, *102*, 4652–4658.

[33] S. Cheng, H. Yan, and C. Zhao, The synergistic effect between hydrophobic and electrostatic interactions in the uptake of amino acids by strongly acidic cation-exchange resins. *J. Chromatogr. A*, **2006**, *1108*, 43–49.

[34] G. Q. Zhang, M. Kai, M. Nakano, and Y. Ohkura, Pre-column fluorescence derivatization high-performance liquid chromatography of opioid peptides in rat brain and its use for enzymatic peptide characterization. *Chem. Pharm. Bull.*,

1991, *39*, 126–129.

[35] T. Yamabe, N. Takai, and H. Nakamura, Analysis of Dns-amino acids by liquid chromatography: I. Selection of optimum mobile phase composition for separation of Dns-amino acids on polyvinyl acetate gel. *J. Chromatogr.*, **1975**, *104*, 359–365.

[36] L. M. Nair, B. R. Kildew, and R. S. Nordhaus, Enhancing the anion separations on a polydivinylbenzene-based anion stationary phase. *J. Chromatogr. A*, **1996**, *739*, 99–110.

[37] G. H. Dodo, and M. M. Knight, Application of polydivinylbenzene liquid chromatography columns to remove lipid material from fish tissue extracts for the analysis of semivolatile organics. *J. Chromatogr. A*, **1999**, *859*, 235–240.

[38] C. G. Huber, G. Kleindienst, and G. K. Bonn, Application of micropellicular poly-styrene/divinylbenzene stationary phases for high-performance reversed-phase liquid chromatography electrospray-mass Spectrometry of proteins and peptides. *Chromatographia*, **1997**, *44*, 438–448.

[39] Y. B. Yang, K. Harrison, and J. Kindsvater, Characterization of a novel stationary phase derived from a hydrophilic polystyrene-based resin for protein cation-exchange high-performance liquid chromatography. *J. Chromatogr. A*, **1996**, *723*, 1–10.

[40] W. Mu, W. Zhang, Y. Feng, B. Jiang, and L. Zhou, Recent advances on applications and biotechnological production of D-psicose. *Appl. Microbiol. Biotechnol.*, **2012**, *94*, 1461–1467.

[41] W. Zhang, S. Yu, T. Zhang, B. Jiang, and W. Mu, Recent advances in D-allulose: physiological functionalities, applications, and biological production. *Trends Food Sci. Technol.*, **2016**, *54*, 127–137.

[42] N. Hayashi, T. Iida, T. Yamada, K. Okuma, I. Takehara, T. Yamamoto, K. Yamada, and M. Tokuda, Study on the postprandial blood glucose suppression effect of D-psicose in borderline diabetes and the safety of long-term ingestion by normal human subjects. *Biosci. Biotechnol. Biochem.*, **2010**, *74*, 510–519.

[43] D. Moller, and J. Berger, Role of PPARs in the regulation of obesity-related insulin sensitivity and inflammation. *Int. J. Obes.*, **2003**, *27*, S17–S21.

[44] T. Matsuo, Y. Baba, m. Hashiguchi, K. Takeshita, K. Izumori, and H. Suzuki, Dietary D-psicose, a C-3 epimer of D-fructose, suppresses the activity of hepatic lipogenic enzymes in rats. *Asia Pac. J. Clin. Nutr.*, **2001**, *10*, 233–237.

[45] M. K. Takata, F. Yamaguchi, K. Nakanose, Y. Watanabe, N. Hatano, I. Tsukamoto, M. Nagata, K. Izumori, and M. Tokuda, Neuroprotective effect of D-psicose on 6-hydroxydopamine-induced apoptosis in rat pheochromocytoma (PC12) cells. *J. Biosci. Bioeng.,* **2005**, *100*, 511–516.

[46] S. Suna, F. Yamaguchi, S. Kimura, M. Tokuda, and F. Jitsunari, Preventive effect of D-psicose, one of rare ketohexoses, on di-(2-ethylhexyl) phthalate (DEHP)-induced testicular injury in rat. *Toxicol. Lett.,* **2007**, *173*, 107–117.

[47] K. Murao, X. Yu, W. M. Cao, H. Imachi, K. Chen, T. Muraoka, N. Kitanaka, J. Li, R. A. M. Ahmed, K. Matsumoto, T. Nishiuchi, M. Tokuda, and T. Ishida, D-Psicose inhibits the expression of MCP-1 induced by high-glucose stimulation in HUVECs. *Life Sci.*, **2007**, *81*, 592–599.

[48] M. A. Hossain, S. Kitagaki, D. Nakano, A. Nishiyama, Y. Funamoto, T. Matsunaga, I. Tsukamoto, F. Yamaguchi, K. Kamitori, Y. Dong, Y. Hirata, K. Murao, Y. Toyoda, and M. Tokuda, Rare sugar D-psicose improves insulin sensitivity and glucose tolerance in type 2 diabetes Otsuka Long-Evans Tokushima Fatty (OLETF) rats. *Biochem. Biophys. Res. Commun.*, **2011**, *405*, 7–12.

[49] M. Ochiai, Y. Nakanishi, T. Yamada, T. Iida, and T. Matsuo, Inhibition by dietary D-psicose of body fat accumulation in adult rats fed a high-sucrose diet. *Biosci. Biotechnol. Biochem.*, **2013**, *77*, 1123–1126.

[50] M. Y. Chung, D. K. Oh, and K. W. Lee, Hypoglycemic health benefits of D-psicose. *J. Agric.Food Chem.*, **2012**, *60*, 863−869.

[51] Y. M. Chung, J. H. Lee, D. Y. Kim, S. H. Hwang, Y. H. Hong, S. B. Kim, S. J. Lee, and C. H. Park, Dietary D-psicose reduced visceral fat mass in high-fat diet-induced obese rats. *J. Food Sci.,* **2012**, *77*, H53–H58.

[52] T. Iida, T. Yamada, N. Hayashi, K. Okuma, K. Izumori, R. Ishii, and T. Matsuo, Reduction of abdominal fat accumulation in rats by 8-week ingestion of a newly developed sweetener made from high fructose corn syrup. *Food Chem.*, **2103**, *138*, 781–785.

[53] S. Roy, J. Chikkerur, S. C. Roy, A. Dhali, A. P. Kolte, M. Sridhar, and A. K. Samanta, Tagatose as a potential nutraceutical: production, properties, biological roles and applications. *J. Food Sci.,* **2018**, *83*, 2699–2709.

[54] Y. Lu, G. V. Levin, and T. W. Donner, Tagatose, a new antidiabetic and obesity control drug. *Diabetes, Obes. Metab.,* **2008**, *10*, 109–134.

[55] Z. Chen, J. Chen, W. Zhang, T. Zhang, C. Guang, and W. Mu, Recent research on the physiological functions, applications, and biotechnological production of D-allose. *Appl. Microbiol. Biotechnol.*, **2018**, *102*, 4269–4278.

[56] C. Noguchi, K. Kamitori, A. Hossain, H. Hoshikawa, A. Katagi, Y. Dong, L. Sui, M. Tokuda, and F. Yamaguchi, D-Allose inhibits cancer cell growth by reducing GLUT1 expression. *Tohoku J. Exp. Med.*, **2016**, *238*, 131–141.

[57] T. Huang, D. Gao, Y. Hei, X. Zhang, X. Chen, and Z. Fei, D-Allose protects the blood brain barrier through PPARγ-mediated anti-inflammatory pathway in the mice model of ischemia reperfusion injury. *Brain Res.*, **2016**, *1642*, 478–486.

[58] N. Shinohara, T. Nakamura, Y. Abe, T. Hifumi, K. Kawakita, A. Shinomiya, T. Tamiya, M. Tokuda, R. F. Keep, T. Yamamoto, and Y. Kuroda, D-Allose attenuates overexpression of inflammatory cytokines after cerebral ischemia/reperfusion injury in gerbil. *J. Stroke Cerebrovasc. Dis.*, **2016**, *25*, 2184–2188.

[59] L. Sui, R. Nomura, Y. Dong, F. Yamaguchi, K. Izumori, and M. Tokuda, Cryoprotective effects of D-allose on mammalian cells. *Cryobiology*, **2007**, *55*, 87–92.

[60] K. Yamadaa, C. Noguchia, K. Kamitoria, Y. Donga, Y. Hirataa, M. A. Hossaina, I. Tsukamotob, M. Tokudaa, and F. Yamaguchi, Rare sugar D-allose strongly induces thioredoxin-interacting protein and inhibits osteoclast differentiation in Raw264 cells. *Nutr. Res.*, **2012**, *32*, 116–123.

[61] S. Kimuraa, G. X. Zhanga, A. Nishiyamaa, Y. Nagaib, T. Nakagawab, H. Miyanakab, Y. Fujisawab, A. Miyatakeb, T. Nagaia, M. Tokudac and Y. Abe, D-Allose, an all-cis aldo-hexose, suppresses development of salt-induced hypertension in Dahl rats. *J. Hypertens*., **2005**, *23*, 1887–1894.

[62] D. Gao, N. Kawai, T. Nakamura, F. Lu, Z. Fei, and T. Tamiya, Anti-inflammatory effect of D-allose in cerebral ischemia/reperfusion injury in rats. *Neurol Med Chir (Tokyo)*, **2013**, *53*, 365–374.

[63] M. A. Hossain, H. Wakabayashi, F. Goda, S. Kobayashi, T. Maeba, and H. Maeta, Effect of the immunosuppressants FK506 and D-allose on allogenic orthotopic liver transplantation in rats. *Transplant. Proc.*, **2000**, *32*, 2021–2023.

[64] Y. Ishihara, K. Katayama, M. Sakabe, M. Kitamura, M. Aizawa, M. Takara, and K. Itoh, Antioxidant properties of rare sugar D-allose: Effects on mitochondrial reactive oxygen species production in Neuro2A cells. *J. Biosci. Bioeng.*, **2011**, *112*, 638–642.

[65] T. Yamada, N. Hayashi, T. Iida, S. Takamine, K. Okuma, and T. Matsuo, Dietary D-sorbose serum insulin levels in growing Sprague-Dawley rats. *J. Nutr. Sci. Vitaminol.*, **2014**, *60*, 297–299.

[66] T. Oku, Y. M. Takenoshita, Y. Yamazaki, F. Shimura, and S. Nakamura, D-Sorbose inhibits disaccharidase activity and demonstrates suppressive action on postprandial blood levels of glucose and insulin in the rat. *Nutr. Res.,* **2014**, *34*, 961–967.

[67] E. Lim, J. Y. Lim, J. H. Shin, P. R. Seok, S. Jung, S. H. Yoo, and Y. Kim, D-Xylose suppresses adipogenesis and regulates lipid metabolism genes in high-fat diet–induced obese mice. *Nutr. Res.,* **2015**, *35*, 626–636.

[68] K. Beerens, T. Desmet, and W. Soetaert, Enzymes for the biocatalytic production of rare sugar, *J. Ind. Microbiol. Biotechnol.*, **2012**, *39*, 823–834.

[69] T. Matsuo, H. Suzuki, M. Hashiguchi, and K. Izumori, D-Psicose is a rare sugar that provides no energy to growing rats. *J. Nutr. Sci. Vitaminol.,* **2002**, *48*, 77–80.

[70] I. S. Menziex, J. N. Mount, and M. J. Wheeler, Quantitative estimation of clinically important monosaccharides in plasma by rapid thin layer chromatography. *Ann. Clin. Biochem.*, **1978**, *15*, 65–76.

[71] Y. Lv, X. Yang, Y Zhao, Y. Ruan, Y. Yang, and Z. Wang, Separation and quantification of component monosaccharides of the tea polysaccharides from Gynostemma pentaphyllum by HPLC with indirect UV detection. *Food Chem.*, **2009**, *112*, 742–746.

[72] G. O. Guerrant, and C. W. Moss, Determination of monosaccharides as aldononitrile, *O*-methyloxime, alditol, and cyclitol acetate derivatives by gas chromatography. *Anal. Chem*. **1984**, *56*, 633–638.

[73] T. R. I. Cataldi, and D. Nardiello, Determination of free proline and monosaccharides in wine samples by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). *J. Agric. Food Chem*. **2003**, *51*, 3737–3742.

[74] A. Guttman, Analysis of monosaccharide composition by capillary electrophoresis. *J. Chromatogr. A*, **1997**, *28*, 1–2.

[75] J. M. Harvey, and M. C. R. Symons, The hydration of monosaccharides–an NMR study. *J. Solution Chem.*, **1978**, *7*, 57 –586.

[76] F. N. Lamari, R. Kuhm, and N. K. Karamanos, Derivatization of carbohydrates for chromatographic, electrophoretic and mass spectrometric structure analysis. *J. Chromatogr. B*, **2003**, *793*, 15–36.

[77] D. J. Harvey, Derivatization of carbohydrates for analysis by chromatography; electrophoresis and mass spectrometry. *J. Chromatogr. B*, **2011**, *879*, 1196–1225.

[78] D. B. Gomis, D. M. Tamayo, and J. M. Alonso, Determination of monosaccharides in cider by reversed-phase liquid chromatography. *Anal. Chim. Acta*, **2001**, *436*, 173–180.

[79] H. Kwon, and J. Kim, High performance liquid chromatography of mono- and oligosaccharides derivatized with *p*-aminobenzoix ethyl ester on a C18-bonded silica column. *J. Liq, Chromatogr.*, **1995**, *18*, 1437–1449.

[80] S. Yasuno, T. Murata, K. Kokubo, T. Yamaguchi, and M. Kamei, Two-mode analysis by high-performance liquid chromatography of p-aminobenzoic ethyl ester-derivatized monosaccharides. *Biosci. Biotechnol. Biochem.*, **1997**, *61,* 1944–1946.

[81] J. Meiser, D. Weindl, and K. Hiller, Complexity of dopamine metabolism. *Cell Commun. Signal.*, **2013**, *11*, 34–51.

[82] G. Eisenhofer, I. J. Kopin, and D. S. Goldstein, Catecholamine metabolism: A contemporary view with implications for physiology and medicine. *Pharmacol. Rev.,* **2004**, *56*, 331–349.

[83] K. Kobayashi, Role of catecholamine signaling in brain and nervous system functions: new insights from mouse molecular genetic study. *J. Invest. Dermatol.*, **2001**, *6*, 115–121.

[84] A. M. V. S. Pedersen, S. N. Hansen, and P. T. N. J. Lykkesfeldt, Simultaneous quantification of monoamine neurotransmitters and their biogenic metabolites intracellularly and extracellularly in primary neuronal cell cultures and in sub-regions of guinea pig brain. *J. Chromatogr. B*, **2016**, *1028*, 222–230.

[85] A. Hossain, F. Yamaguchi, K. Hirose, T. Matsunaga, S. Li, Y. Hirata, C. Noguchi, A. Katagi, K. Kamitori, Y. Dong, I. Tsukamoto, and M. Tokuda, Rare sugar D-psicose prevents progression and development of diabetes in T2DM model otsuka long-evans tokushima fatty rats. *Drug Des. Devel. Ther.*, **2015**, *9*, 525–535.

[86] A. Hossain, F. Yamaguchi, T. Matsuo, I. Tsukamoto, Y. Toyoda, M. Ogawa, Y. Nagata, and M. Tokuda, Rare sugar D-allulose: Potential role and therapeutic

monitoring in maintaining obesity and type 2 diabetes mellitus. *Pharmacol. Ther.*, **2015**, *155*, 49–59.

[87] Y. Sun, S. Hayakawa, M. Ogawa, and K. Izumori, Antioxidant properties of custard pudding dessert containing rare hexose, D-psicose. *Food Control*, **2007**, *18*, 220–227.

[88] T. Matsuo, and K. Izumori, Effects of supplemental D-psicose on glucose tolerance and serum adipocytokine levels in rats fed a high-fat diet or a low-fat diet. *J. Oleo Sci.*, **2014**, *53*, 453–460.

[89] J. E. Young, J. J. Pesek, and M. T. Matyska, Robust HPLC- Refractive Index Analysis of Simple Sugars in Bev erages Using Silica Hydride Columns. *Curr. Nutr. Food Sci.*, **2016**, *12*, 125–131.

[90] J. Yan, S. Shi, H. Wang, R. Liu, N. Li, and Y. Chen, Neutral monosaccharide composition analysis of plant-derived oligo- and polysaccharides by high performance liquid chromatograpgy. *Carbohydr. Polym.*, **2016**, *136*, 1273–1280.

[91] L. P. Hermes, J. Venkatraman, Z. Sylvia, and K. Molly, Development of a multi-sugar LC-MS/MS assay using simple chemical derivatization with acetic anhydride. *Anal. Methods, **2016**, **8***, 3023–3033.

[92] H. Stepan, and E. Staudacher, Optimization of monosaccharide determination using anthranilic acid and 1-phenyl-3-methyl-5- pyrazolone for gastropod analysis. *Anal. Biochem.*, **2011**, *418*, 24–29.

[93] A. Meyer, C. Raba, and K. Fischer, Ion-pair RP-HPLC determination of sugars, amino sugars, and uronic acids after derivatization with p-aminobenzoic acid. *Anal. Chem.*, **2001**, *73*, 2377– 2382.

[94] B. Buszewski, and S. Noga, Hydrophilic interaction liquid chromatography (HILIC)—a powerful separation technique. *Anal. Bioanal. Chem*., **2012** *402*, 231–247.

[95] G. Karlsson, S. Winge, and H. Sandberg, Separation of monosaccharides by hydrophilic interaction chromatography with evaporative light scattering detection. *J. Chromatogr. A,* **2005**, *1092*, 246–249.

[96] S. Eksborg, P. O. Lagerström, R. Modin, and G. Schill, Ion- pair chromatography of organic compounds. *J. Chromatogr*., **1973**, *83*, 99–110.

[97] R. L. Bruttoa, A. Jonesa, Y.V. Kazakevicha, and H. M. M. Nair, Effect of the eluent pH and acidic modifiers in high-performance liquid chromatography retention of basic analytes. *J. Chromatogr. A*, **2001**, *913*, 173–187.

[98] M. C. Carson, Ion-pair solid-phase extraction. *J. Chromatogr. A*, **2000**, *885*, 343–350.

[99] J. H. Knox, and R. A. Hartwick, Mechanism of ion-pair liquid chromatography of amines, neutrals, zwitterions and acids using anionic hetaerons. *J. Chromatogr.*, **1981**, *204*, 3–21.

[100] B. Fan, and J. T. Stewart, Determination of zidovudine/lamivudine/nevirapine in human plasma using ion-pair HPLC. *J. Pharm. Biomed. Anal.*, **2002**, *28*, 903–908.

[101] C. Brons, and C. Olieman, Study of the high-performance liquid chromatographic separation of reducing sugars, applied to the determination of lactose in milk. *J. Chromatogr. A*, **1983**, *259*, 79–86.

[102] J. Suh, H. J. Park, and B. K. Hwang, Ionization of poly(ethyleneimine) and poly(allylamine) at various pH's. *Bioorg. Chem.*, **1994**, *22*, 318–327.

[103] H. L. Cai, R. H. Zhu, H. D. Li, J. Zhang, and L. F. Li, MultiSimplex optimization of chromatographic separation and dansyl derivatization conditions in the ultra performance liquid chromatography–tandem mass spectrometry analysis of risperidone, 9-hydroxyrisperidone, monoamine and amino acid neurotransmitters in human urine. *J. Chromatogr. B*, **2011**, *879*, 1993–1999.

[104] G. Eisenhofer, I. J. Kopin, and D. S. Goldstein, Catecholamine metabolism: a contemporary view with implications for physiology and medicine. *Pharmacol Rev.*, **2004**, *56*, 331–349.

[105] J. Bicker, A. Fortuna, G. Alves, and A. Falcão, Liquid chromatographic methods for the quantification of catecholamines and their metabolites in several biological samples-A review. *Anal. Chim. Acta,* **2013**, *768*, 12–34.

[106] B. Si, and E. Song, Recent Advances in the Detection of Neurotransmitters. *Chemosensors*, **2018**, *6*, 1–24.

[107] N. Unceta, E. Rodriguez, Z. G. d. Balugera, C. Sampedro, M. A. Goicolea, S. Barrondo, J. Sallés, and R. J. Barrio, Determination of catecholamines and their metabolites in human plasma using liquid chromatography with coulometric multi-electrode cell- design detection. *Anal. Chim. Acta*, **2011**, *444*, 211–221.

[108] M. Cosentino, R. Bombelli, M. Ferrari, F. Marino, E. Rasini, G. J. M. Maestroni, A. Conti, M. Boveri, S. Lecchini, and G. Frigo, HPLC-ED measurement of endogenous catecholamines in human immune cells and hematopoietic cell lines. *Life Sci*., **2000**, *68*, 283–295.

[109] T. Sasaki, T. Fukushima, M. Ohishi, and T. Toyo'oka, Development of a 6-hydroxychroman-based derivatization reagent: application to the analysis of 5-hydroxytryptamine and catecholamines by using high-performance liquid chromatography with electrochemical detection. *Biomed. Chromatogr.,* **2008**, *22*, 888–899.

[110] Y. Gu, Q. Li, V. Melendez, and P. Weina, Comparison of HPLC with electrochemical detection and LC–MS/MS for the separation and validation of

artesunate and dihydroartemisinin in animal and human plasma. *J. Chromatogr. B*, **2008**, *867*, 213–218.

[111] M. Tsunoda, C. Aoyama, H. Nomura, T. Toyada, N. Matsuki, and T. Funatsu, Simultaneous determination of dopamine and 3,4-dihydroxyphenylacetic acid in mouse striatum using mixed-mode reversed-phase and cation-exchange high-performance liquid chromatography. *J. Pharm. Biomed. Anal.*, **2010**, *51*, 712–715.

[112] P. Hjemdahl, Catecholamine measurements by high-performance liquid chromatography. *Am. J. Physiol.*, **1984**, *247*, E13–E20.

[113] J. Guo, T. Saiki, K. Thanutchaporn, W. Liu, A. Shimura, and T. Matsui. Elution profile of di-peptides on a sulfonated ethylstyrene-divinylbenzene copolymer resin column by high-performance liquid chromatography. *Anal. Sci.*, **2015**, *31*, 45–50.

[114] M. Tsunoda, and K. Imai, Development of highly selective and sensitive determination methods for catecholamines. *Bunseki Kagaku*, **2003**, *52*, 1081–1089.

[115] M. Rey, and C. Pohl, Novel cation-exchange column for the separation of hydrophobic and/or polyvalent amines. *J. Chromatogr. A*, **2003**, *997*, 199–206.

[116] R. Kostiainen, and T. Kauppila, Effect of eluent on the ionization process in liquid chromatography–mass spectrometry. *J. Chromatogr. A*, **2009**, *1216*, 685–

699.

[117] S. T. Uribe, L. Montero, L. L. Giraldo, E. Ibáñez, and M. Herrero, Characterization of secondary metabolites from green cocoa beans using focusing-modulated comprehensive two-dimensional liquid chromatography coupled to tandem mass spectrometry. *Anal. Chim. Acta*, **2018**, *1036*, 204–213.

[118] M. Yoshioka, Development of immunoassays of catecholamines and their metabolites. *J. Arab. Soc. Med. Res*., **2016**, *11*, 37–42.

[119] H. L. Cai, R. H. Zhu, H. D. Li, J. Zhang, and L. F. Li, *J. Chromatogr. B*, **2011**, *879*, 1993–1999.

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