Shodex™

HPLC Columns

Shodex™ NH2P-50 series columns

Analysis of saccharides in food industry

Technical notebook
No. 2

SHOWA DENKO EUROPE

Contents

1. Basic characteristics and features of NH2P-50

1-1. What is the NH2P-50 series?

Methods of analysis of monosaccharides and oligosaccharides using high performance liquid chromatography (HPLC) include normal phase, ligand exchange, size exclusion, and ion exchange chromatography. As amino columns, which are used for normal phase chromatography, provide high resolution of saccharides under simple analytical conditions, they are widely used in areas such as the food industry. Conventional silica-based amino columns, however, have a problem with chemical instability, which leads to (i) declines in retention power with time, and (ii) shorter column life.

Shodex Asahipak NH2P-50 series of columns are greatly improved amino columns which not only maintain the high separation power of the conventional silica-based amino columns, but also solve the problem of declines in retention over time. This is due to stable chemical bonding of polyamine with hydrophilic polymer gel.

<Features of NH2P-50 series>

* They are new amino columns in which polyamine has been bonded to a hydrophilic polymer gel (polyvinyl alcohol gel).
* They have the inherent chemical stability of a polymer gel, solving the problem of deterioration over time that plagues conventional silica-based amino columns.
* Stable chromatograms can be obtained for a long period of time by using these columns.
* Analysis under moderate conditions (around pH 7 and room temperature) is possible.

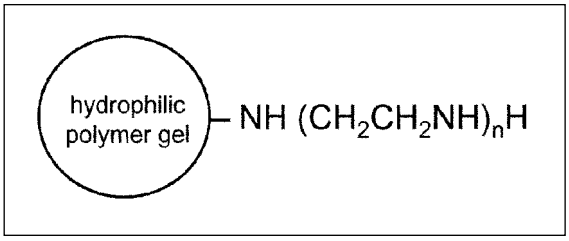


Fig. 1-1 Schematic drawing of NH2P-50 gel

* Sharp, near-symmetric peaks can be obtained for a wide variety of saccharides.
* Accurate quantitative determination can be made.
* A wide range of eluents, such as various buffer solutions, alkaline solutions, or acidic solutions can be used.
* Alkaline washing of columns is possible.

Table 1-1 Specifications for Shodex Asahipak NH2P series

Product code	Product name		Column size ID x L (mm)	Average particle size (μm)	Theoretical plate number (TP/column)	Usable pH range
F7630002	Analytical Scale Columns	Asahipak NH2P-50 4D	4.6 x 150	5	≥ 5,500	2~13
F7630001		Asahipak NH2P-50 4E	4.6 x 250	5	≥ 7,500	2~13
F6710016		Asahipak NH2P-50G 4A	4.6 x 10	5	guard	2~13
F7630006		Asahipak NH2P-50 2D	2.0 x 150	5	≥ 3,500	2~13
F6713000		Asahipak NH2P-50G 2A	2.0 x 10	5	guard	2~13
F6830001	Preparative Scale Columns	Asahipak NH2P-50 10E	10.0 x 250	5	≥ 10,000	2~13
F6830031		Asahipak NH2P-90 20F	20.0 x 300	9	≥ 10,000	2~13
F6830007		Asahipak NH2P-130 28F	28.0 x 300	13	≥ 1,000	2~13
F6710017		Asahipak NH2P-130G	7.5 x 50	13	guard	2~13
F6710100	Line filter	Asahipak NH2P-LF	8.0 x 75	5	line filter	2~13

Semi-micro (2.0, 1.0mmID) and micro column (0.8, 0.5, 0.3mmID) with 35, 50, 150, 250 mm length are also available.

1-2. Separation mechanism of NH2P-50

(a) Elution characteristics of amino columns

Amino columns, such as NH2P-50, are packed with material having high polarity, when compared with other partition/adsorption columns. (Fig. 1-2) With amino columns, saccharides elute in order of increasing polarity due to the function of normal phase chromatography.

Usually, a mixed solvent of acetonitrile and water is used as the eluent. When the mixing ratio of acetonitrile is increased, the polarity of the eluent becomes lower. This results in a stronger interaction between saccharides and the column and a larger elution volume.

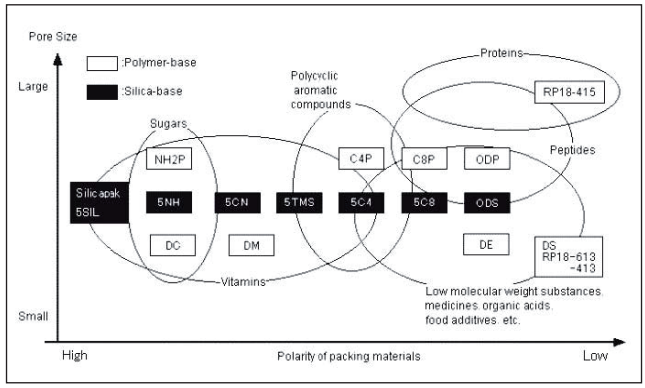


Fig. 1-2
Characteristics of packing material in partition/ adsorption columns and areas suited to use of these columns.

(b) Ratio of non-protonated to protonated amino groups and theoretical plate number

As with conventional amino columns, columns of the NH2P-50 series are packed with ion exchange resin which terminate in anion exchange groups (amino groups) introduced in it. Due to the pH and ion composition of the eluent, there is equilibrium between the protonated and non-protonated amino groups. (Fig. 1-3) The ratio of non-protonated to protonated amino groups has a great influence on the elution characteristic of saccharides.

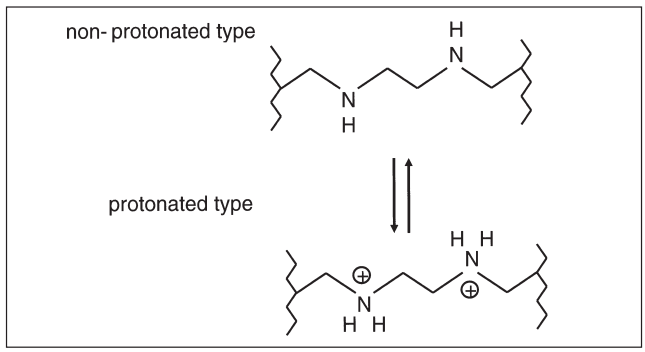
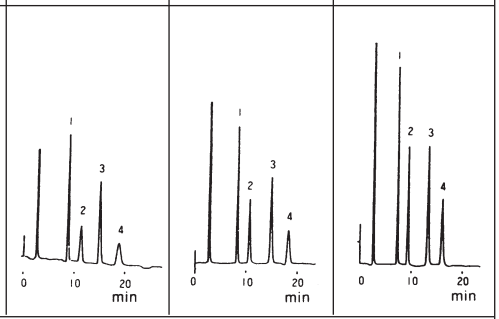


Fig. 1-3 Type of amino group

An NH2P-50 4E column was equilibrated using an aqueous ammonium acetate solution at three different pH values to produce different non-protonated/protonated ratios. Analysis was conducted with these columns holding all other conditions the same. The results showed the larger the non-protonated/protonated ratio, the smaller the elution volume for each saccharide and the sharper its peak. (Fig. 1-4, 1-5)

pH	2.84	8.50	9.30
ratio	32%	82%	93%



Sample : 5mg/mL each, 10μL
1. Fructose, 2. Glucose, 3. Sucrose, 4. Maltose

Fig. 1-4
Relationship between ratio of non-protonated to protonated amino groups and elution time (Equilibrium is achieved by passing a 100 mM aqueous ammonium acetate solution through the column.)

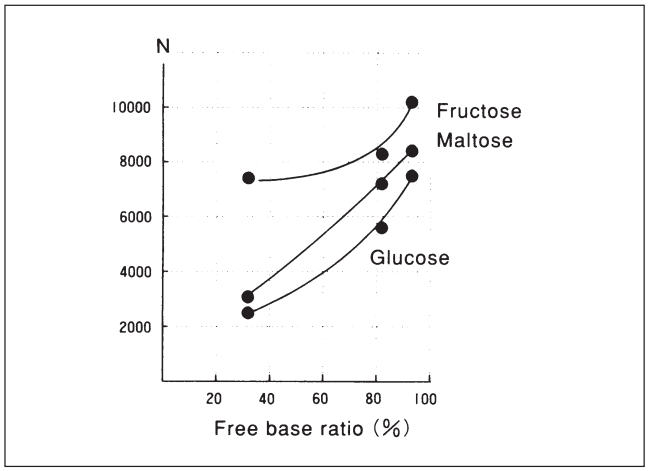


Fig. 1-5
Relationship between ratio of non-protonated to protonated amino groups and theoretical plate number of saccharides

Fig. 1-4 & 1-5 Column : Shodex Asahipak NH2P-50 4E(4.6 x 250mm)
Eluent : CH_3CN/H_2O=75/25
Flow rate : 1.0mL/min
Detector : Shodex RI
Column temp. : 30℃

1-3. Problem with anomer separation of saccharides

Reducing saccharides and saccharides with reducing terminals have α and β anomers. These anomers are in equilibrium in the solution. (Fig. 1-6)

Under the conditions in which the conversion rate between the anomers is low, α and β anomers are separated by the column causing the peak tops to split or widen. Measures to prevent these problems include the following:

* Analysis at high temperature
* Analysis under strong alkaline conditions

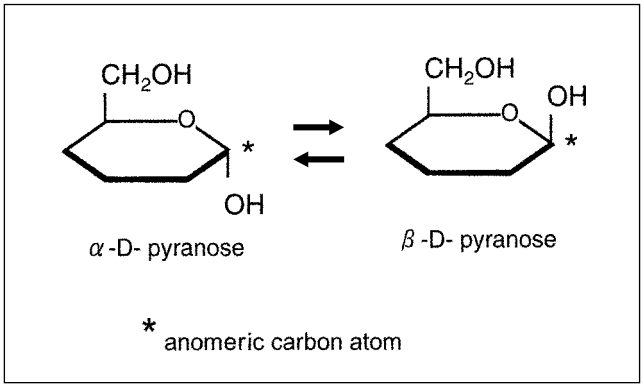


Fig. 1-6 Structural formula of α and β aldohexapyranose

As NH2P-50 columns have weak alkaline amino groups, the condition inside the column is alkaline. This enables saccharides to be analyzed without causing separation of anomers even at room temperature.

There are columns, called amide columns, which are used for analysis of saccharides under the same elution conditions as those for amino columns. Although amide columns have acrylamide groups introduced, analysis has to be made at high temperature because the acrylamide group is not basic.

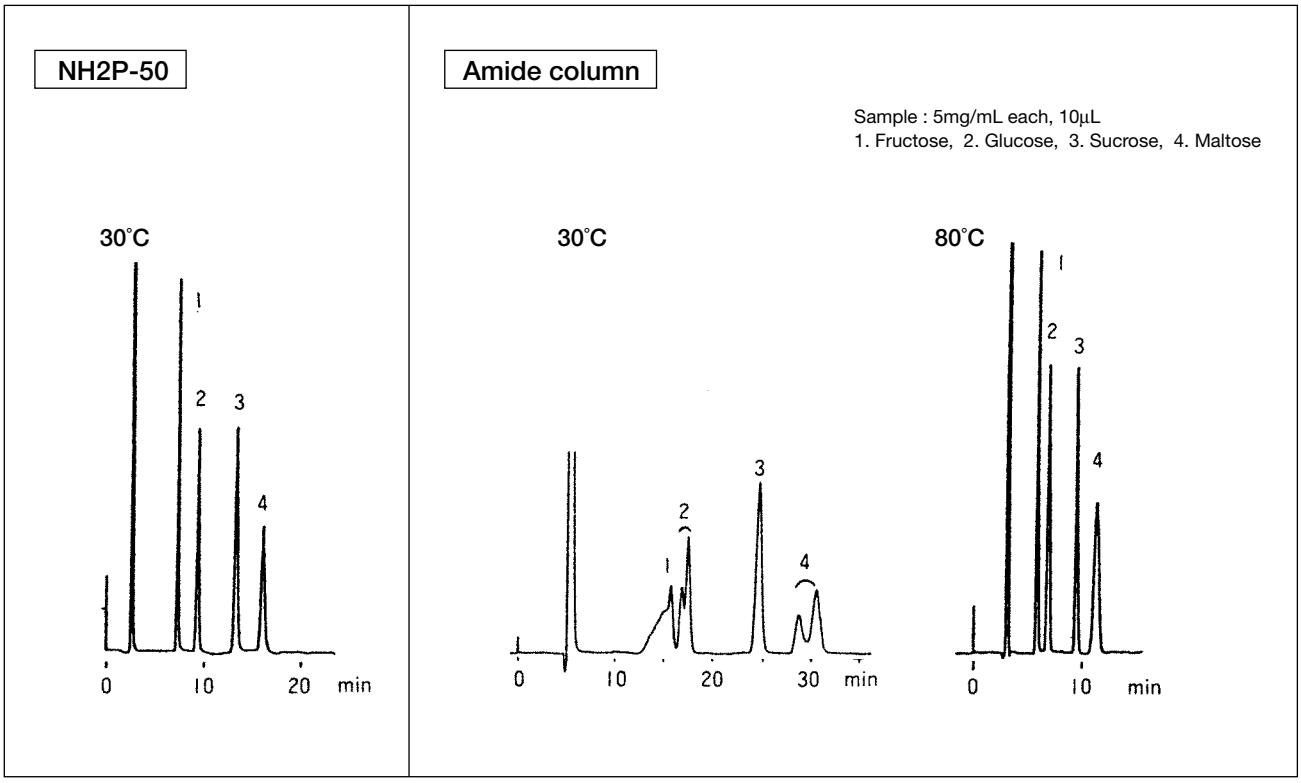


Fig. 1-7 Effects of column temperature on elution patterns (comparison with amide column)

Column : Shodex Asahipak NH2P-50 4E (4.6 x 250mm)
amide column from company-A (4.6 x 250mm)
Eluent : CH_3CN/H_2O=75/25
Flow rate : 1.0mL/min
Detector : Shodex RI

1-4. Mixing ratio of acetonitrile and elution time

It is possible to achieve proper separation of saccharides with the NH2P-50 series by adjusting the mixing ratio of acetonitrile to water. It is possible to obtain near-symmetric, sharp peaks for many saccharides using the NH2P-50 series.

The retention time which NH2P-50 columns have for each saccharide increases as the percent of acetonitrile increases, just as in the case with silica-based amino columns. However, with NH2P-50 columns, galactose elutes earlier than glucose, and lactose than maltose. Thus, there are cases in which the elution order is reversed from that with silica-based amino columns. (Fig. 1-8 and Table 1-2)

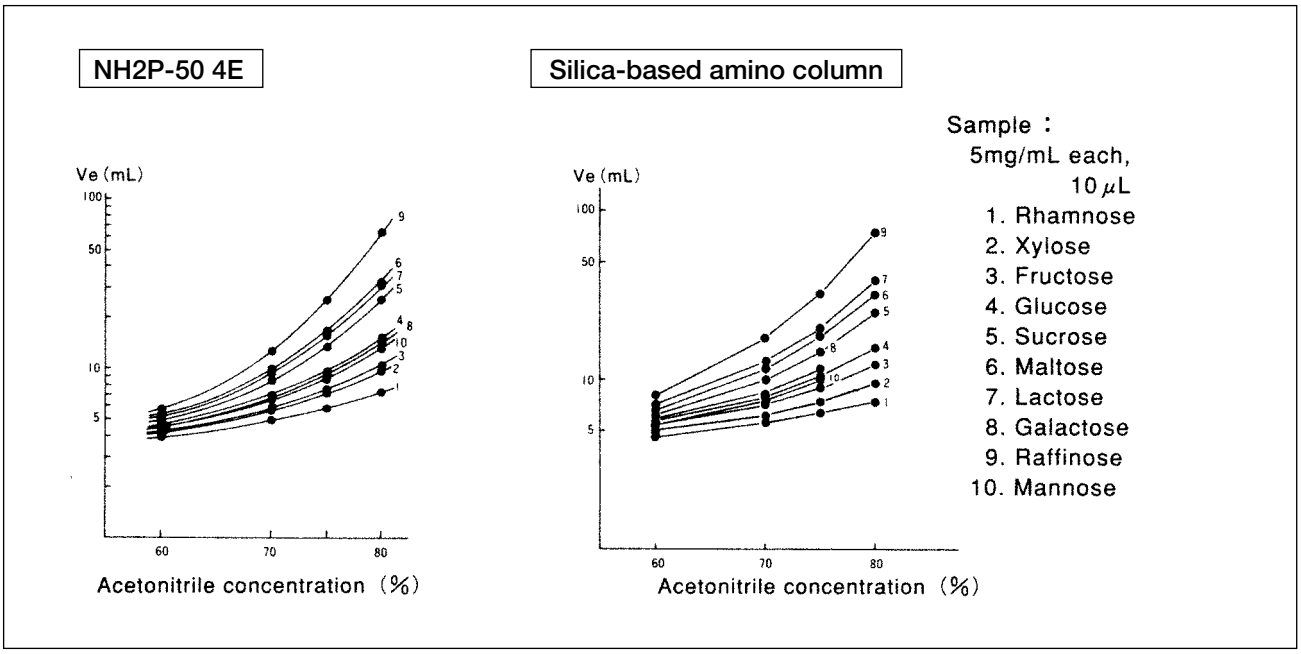


Fig. 1-8 Relationship between acetonitrile concentration and elution volume

Table 1-2 Comparison of elution of monosaccharide, disaccharide and sugar alcohol

	NH2P-50 4E		silica-based amino column	
	Ve (mL)	k'	Ve (mL)	k'
meso-Erythritol	5.40	1.13	8.32	1.49
Xylitol	6.11	1.42	10.51	2.15
Fructose	6.72	1.66	11.36	2.40
Sorbitol	7.14	1.82	13.29	2.98
Sorbose	7.37	1.91	12.05	2.61
Mannitol	7.42	1.94	13.64	3.08
Galactose	8.14	2.22	15.17	3.54
Glucose	8.62	2.41	14.85	3.45
Xylobiose	9.08	2.59	14.89	3.46
Sucrose	11.92	3.72	21.94	5.57
Lactose	13.45	4.32	29.70	7.89
Maltose	14.33	4.67	27.54	7.25

Sample : 5mg/mL each, 20μL
Column : Shodex Asahipak NH2P-50 4E (4.6 x 250mm)
silica-based amino column from company-A (4.6 x 250mm)
Eluent : CH_3CN/H_2O=75/25
Flow rate : 0.6mL/min (NH2P-50 4E)
1.0mL/min
(silica-based amino column)
Detector : Shodex RI
Column temp. : 30℃

k' = (Ve-Vo)/Vo Vo : Elution volume of H_2O Ve : Elution volume

1-5. Mixing ratio of acetonitrile and theoretical plate number

With NH2P-50 columns, as with silica-based amino columns, when the mixing ratio of acetonitrile in the eluent becomes high, the retention of saccharide gets stronger. In addition, with NH2P-50 columns, when the retention of saccharide gets stronger, the theoretical plate number increases for saccharides and sugar alcohols.

In contrast, with silica-based amino columns, the detection sensitivity and theoretical plate number vary greatly depending on the kind of saccharides. Saccharides, such as galactose, that have an abnormally low detection sensitivity have been observed with such columns. (Fig. 1-9, 1-10)

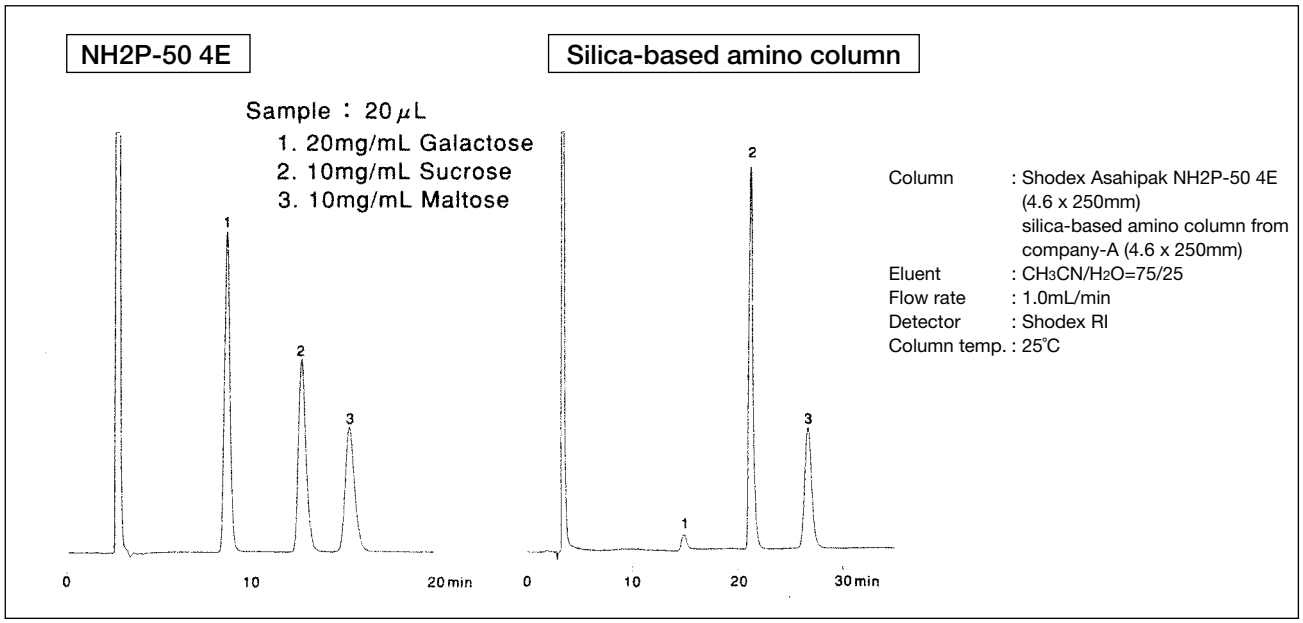


Fig. 1-9 Analysis of saccharides using NH2P-50 4E column and silica-based amino column

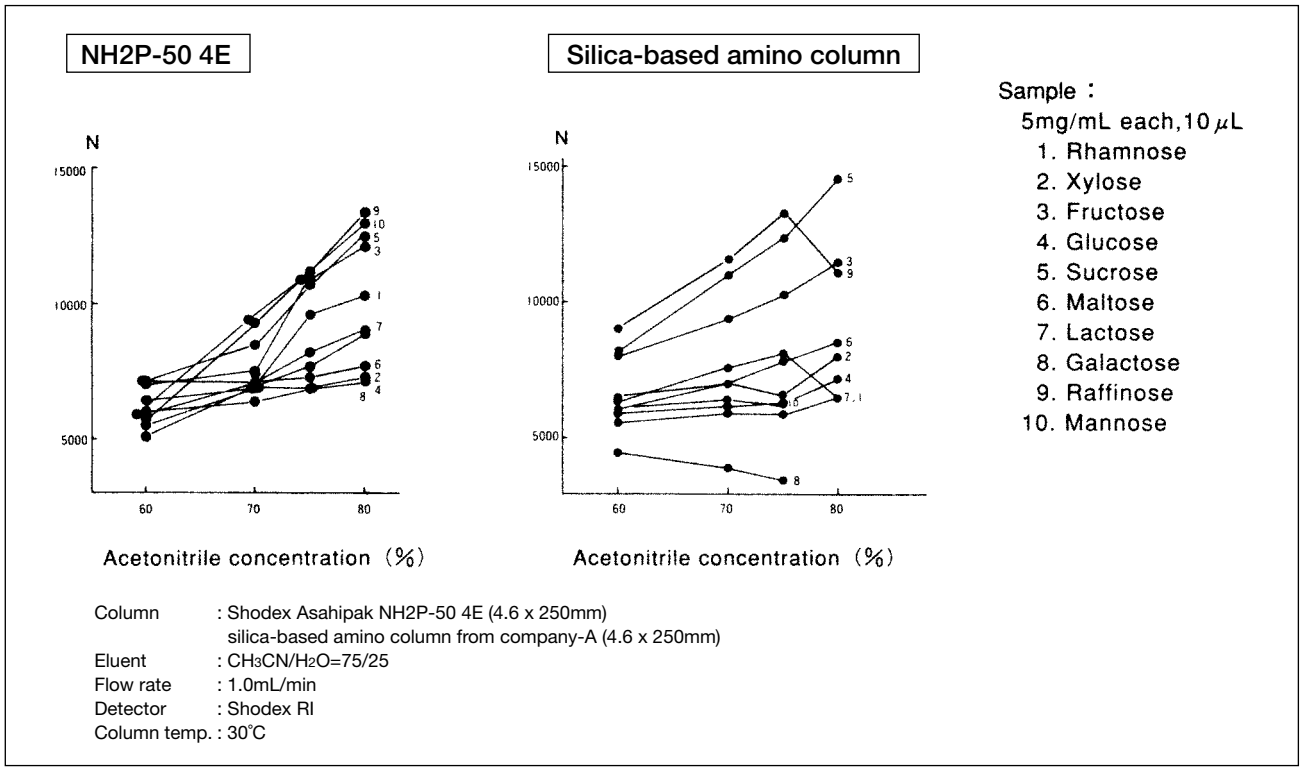


Fig. 1-10 Relationship between acetonitrile concentration and theoretical plate number

1-6. Separation of sugar alcohols

Recently, sugar alcohol has attracted attention as one of the foods with specific functions. NH2P-50 columns can separate saccharides from their sugar alcohols effectively. This cannot be easily achieved by silica-based amino columns. (For example, separation of glucose from sorbitol, and lactose from lactitol.) With NH2P-50 columns, the elution volume of sugar alcohols increases as the ratio of acetonitrile increases, following the same trend as saccharides. (Fig. 1-11, 1-12)

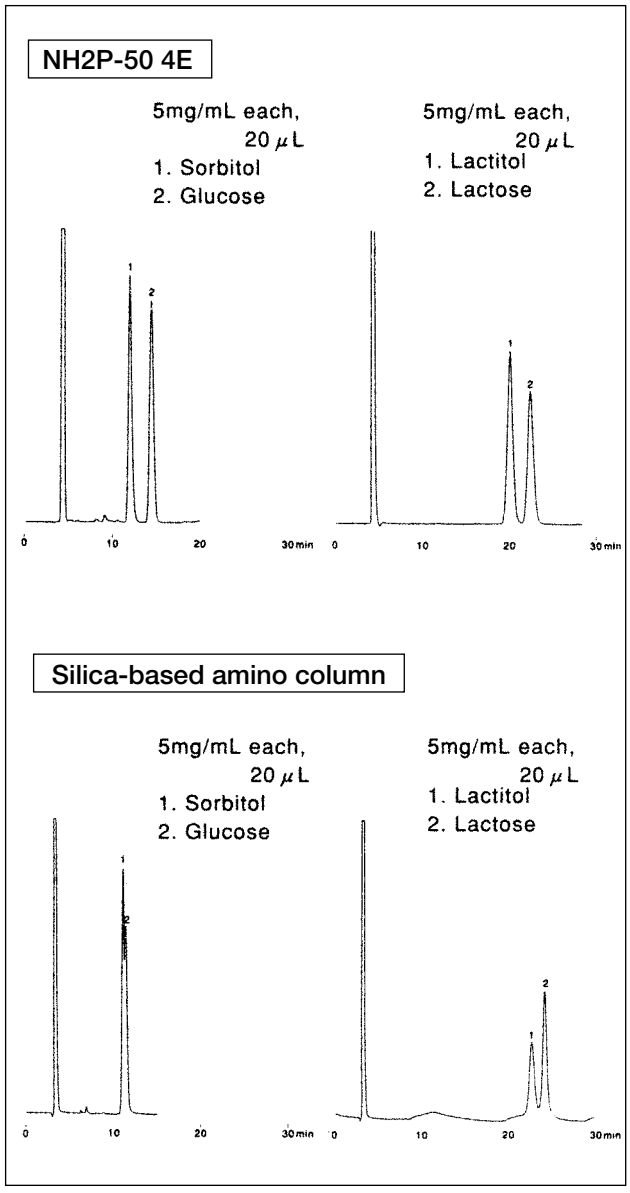


Fig. 1-11
Analysis of saccharides and sugar alcohols with NH2P-50 4E column and silica-based amino column

Column : Shodex Asahipak NH2P-50 4E (4.6 x 250mm)
silica-based amino column from company-A (4.6 x 250mm)
Eluent : CH_3CN/H_2O=75/25
Flow rate : 0.6mL/min (NH2P-50 4E)
1.0mL/min (silica-based amino column)
Detector : Shodex RI
Column temp. : 25℃

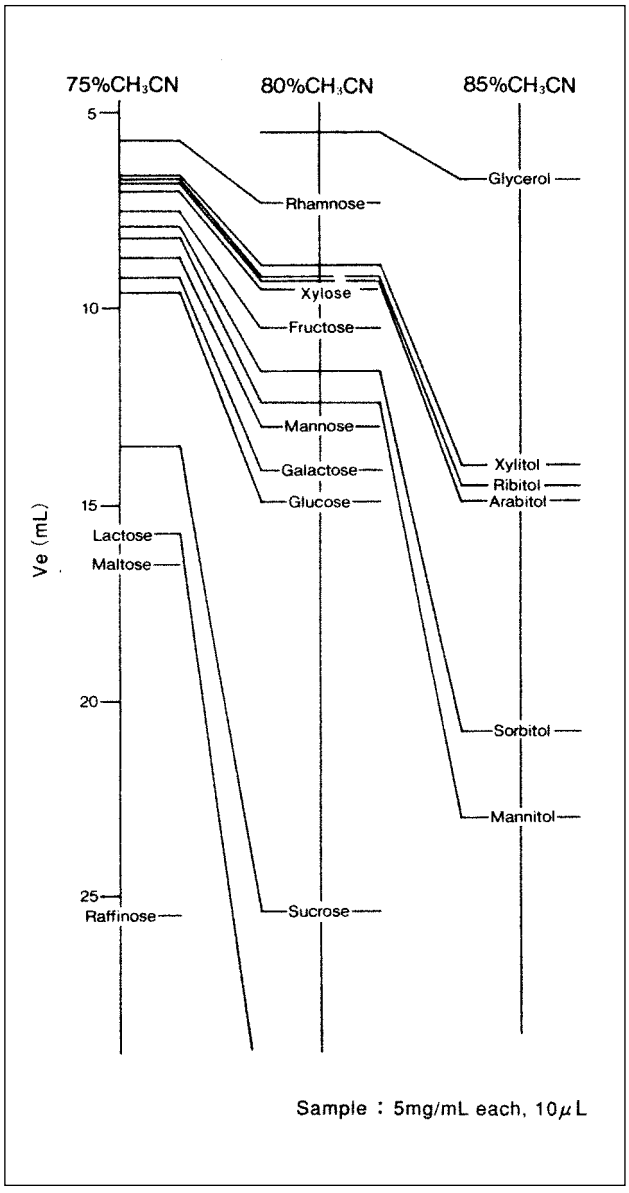


Fig. 1-12
Relation between acetonitrile concentration and elution volume of sugar alcohols

Column : Shodex Asahipak NH2P-50 4E (4.6 x 250mm)
Eluent : CH_3CN/H_2O=75/25
Flow rate : 1.0mL/min
Detector : Shodex RI
Column temp. : 30℃

1-7. Analysis of actual samples

Here is an example of analysis of saccharides in yogurt using an NH2P-50 column and a silica-based amino column. With the silica-based amino column, no peak of fructose or galactose was detected. Judging from the lactose peak obtained with the silica-based amino column being smaller, it can be said that a less amount of the saccharide was adsorbed by the NH2P-50 column, making it possible to detect even a trace amount. (Fig. 1-13).

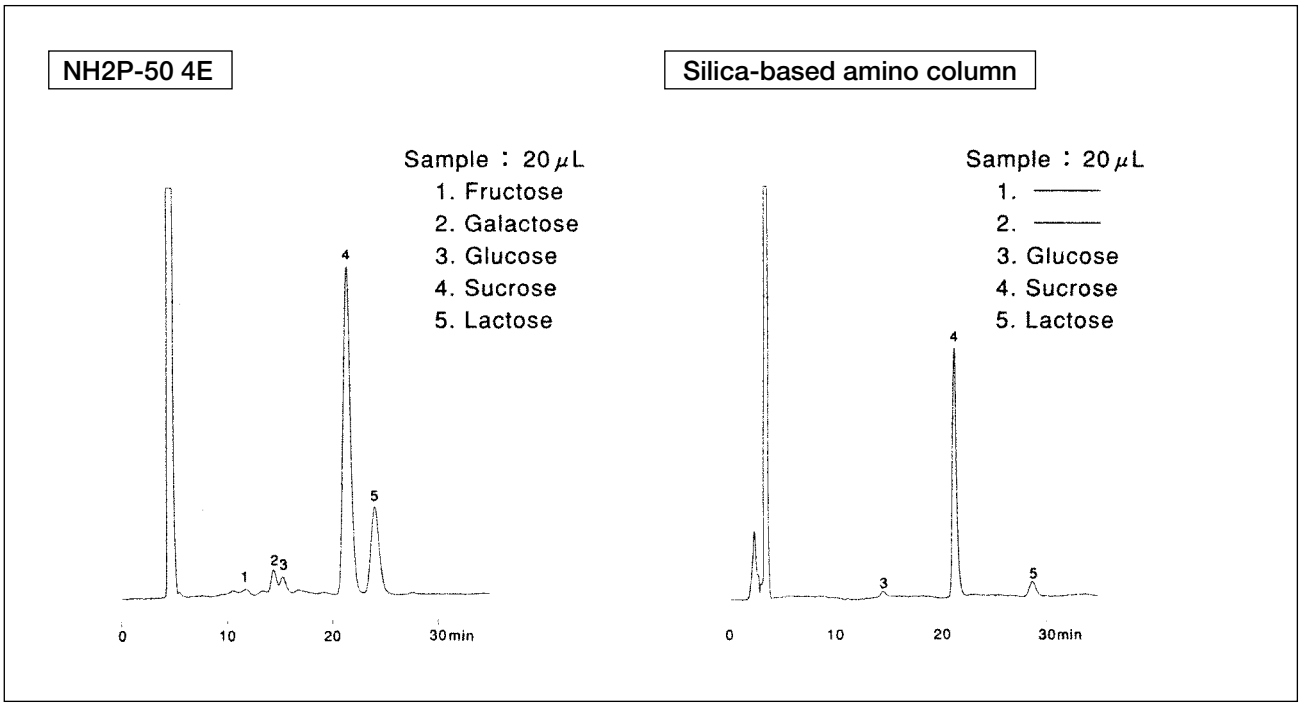


Fig. 1-13 Analysis of saccharides in sugar-added yogurt

Column :Shodex Asahipak NH2P-50 4E (4.6 x 250mm)
silica-based amino column from company-A (4.6 x 250mm)
Eluent : CH_3CN/H_2O=75/25
Flow rate : 0.6mL/min (NH2P-50 4E)
1.0mL/min (silica-based amino column)
Detector : Shodex RI
Column temp. : 25℃

(Pretreatment of sample)

(1) Measure 5 g of yogurt into a beaker.
(2) Add 30 mL of pure water to the yogurt. After having stirred the mixture, neutralize it with a 10 w/v% sodium hydroxide aqueous solution.
(3) After 30 min of ultrasonic extraction, add pure water until total volume becomes 50 mL.
(4) Pass the solution (3) through a No. 5B filter paper. To 3 mL of the filtrate, add the same volume of acetonitrile and stir this mixture.
(5) Pass the mixture through a membrane filter (0.45μm) to make it an HPLC test solution.

1-8. Reproducibility

Chromatograms with good reproducibility over a long period of time can be obtained with the NH2P-50 series, because the chemical structure of the packing material is stable.

With silica-based amino columns, retention of saccharide weakens with passage of time, resulting in significant widening of every peak. This clearly indicates deterioration of the column.

However, with the NH2P-50 columns, columns show little deterioration with time, achieving stable retention time and maintaining sharp peak for every saccharide. (Fig. 1-14)

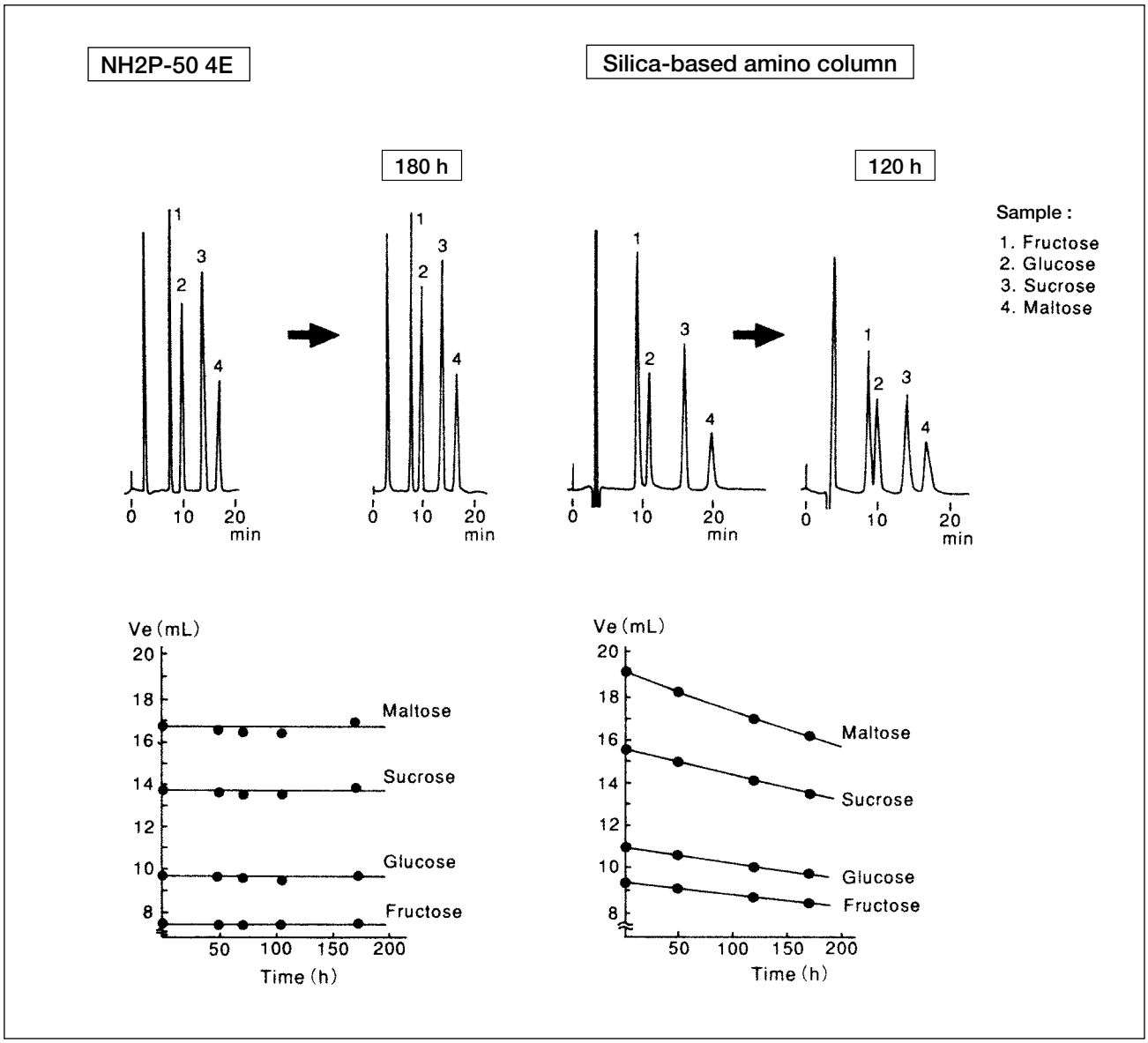


Fig. 1-14 Reproducibility of chromatograms with NH2P-50 4E column and silica-based amino column

Column : Shodex Asahipak NH2P-50 4E (4.6 x 250mm)
silica-based amino column from company-A (4.6 x 250mm)
Eluent : CH_3CN/H_2O=75/25
Flow rate : 1.0mL/min
Detector : Shodex RI
Column temp. : 30℃

1-9. Durability

(a) Durability under acidic conditions

Because of their high chemical stability, NH2P-50 columns are more durable under acidic conditions than silica-based amino columns. (Fig. 1-15 and Table 1-3)

<Test method>
(1) Test with eluent passing through the column (63 h)
Eluent : 0.05M H_2SO_4
Flow rate : 0.1 mL/min
Column temp. : 25℃
(2) Replacement with pure water (1 h)
Eluent : H_2O
Flow rate : 0.5 mL/min
Column temp. : 25℃
(3) Equilibration of column (1 h)
Eluent : 0.1M NaOH
Flow rate : 0.5 mL/min
Column temp. : 25℃

(Conditions for analysis: Refer to Fig. 1-14)

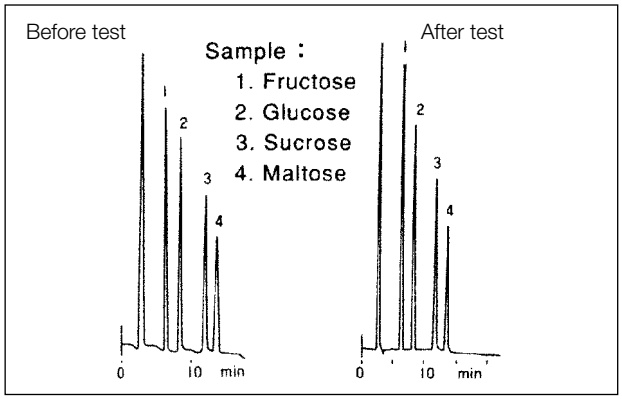


Fig. 1-15 Comparison of chromatograms before and after test with eluent passing through the column

Table 1-3
Comparison of column performance before and after test with eluent passing through the column

	Before test		After test	
	Elution volume (mL)	Theoretical plate number	Elution volume (mL)	Theoretical plate number
Fructose	6.73	9600	6.70	8400
Glucose	8.59	7900	8.58	8500
Sucrose	11.88	9600	11.93	9300
Maltose	14.24	8500	13.90	8600

(b) Durability under alkaline conditions

Unlike silica-based amino columns, NH2P-50 columns are packed with polymer gel as the base material and, therefore, exhibit good durability under alkaline conditions as well. (Fig. 1-16 and Table 1-4)

<Test method>
(1) Equilibration of column (1h) Condition before test
Eluent : 100mM ammonium acetate (pH 9.3)
Flow rate : 1.0 mL/min
Column temp. : 25℃
(2) Test with eluent passing through column (160h)
Eluent : 5mM NaOH (pH 11.4) / CH_3CN = 25 / 75
Flow rate : 1.0 mL/min
Column temp. : 25℃
(3) Replacement with pure water (30 min)
Eluent : H_2O
Flow rate : 0.5 mL/min
Column temp. : 25℃
(4) Equilibration of column (1 h)
Eluent : 100mM ammonium acetate (pH 9.3)
Flow rate : 1.0 mL/min
Column temp. : 25℃

(Conditions for analysis: Refer to Fig. 1-14)

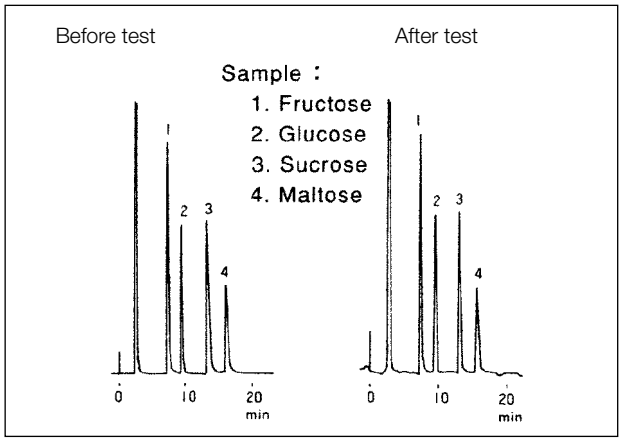


Fig. 1-16 Comparison of chromatograms before and after test with eluent passing through the column

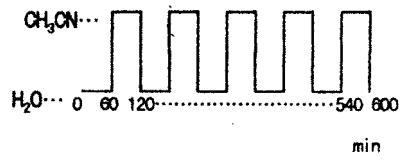
Table 1-4
Comparison of column performance before and after test with eluent passing through the column

	Before test		After test	
	Elution volume (mL)	Theoretical plate number	Elution volume (mL)	Theoretical plate number
Fructose	7.52	7100	7.62	8100
Glucose	9.63	6100	9.68	7700
Sucrose	13.14	8400	13.40	9700
Maltose	16.16	7200	16.08	7400

(c) Durability under changes in acetonitrile concentration

As NH2P-50 columns are designed so that the packing material swells or contracts less when the mixing ratio of the eluent is changed, mixed acetonitrile solution of any given ratio can be used. (Fig. 1-17 and Table 1-5)

<Test method>
Test with eluent passing through the column



Flow rate : 1.0mL/min
Column temp. : 25 ℃

(Conditions for analysis: Refer to Fig. 1-14)

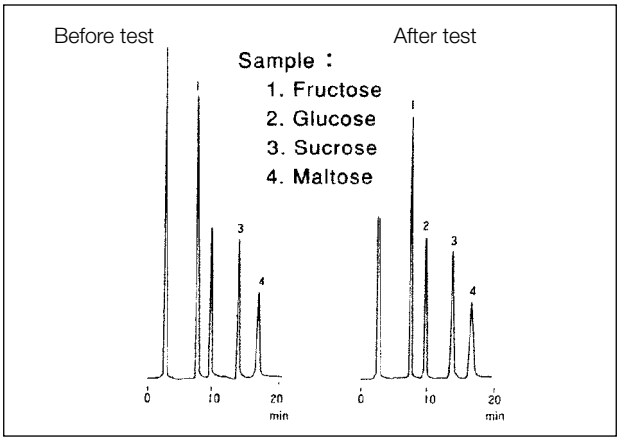


Fig. 1-17 Comparison of chromatograms before and after test with eluent passing through the column

Table 1-5
Comparison of column performance before and after test with eluent passing through the column

	Before test		After test	
	Elution volume (mL)	Theoretical plate number	Elution volume (mL)	Theoretical plate number
Fructose	7.61	11600	7.56	9800
Glucose	9.80	7500	9.72	6200
Sucrose	13.86	11700	13.75	9300
Maltose	16.78	8300	16.66	6700

1-10. Quantitative analysis

For typical saccharides contained in foods, calibration curves were drawn from sample concentrations and peak heights. For every saccharide, the calibration curve showed high linearity passing through the origin with a correlation factor of 0.999 or higher. (Fig. 1-18 and Table 1-6). Even when peak areas are used instead of peak heights, highly linear calibration curves can also be obtained.

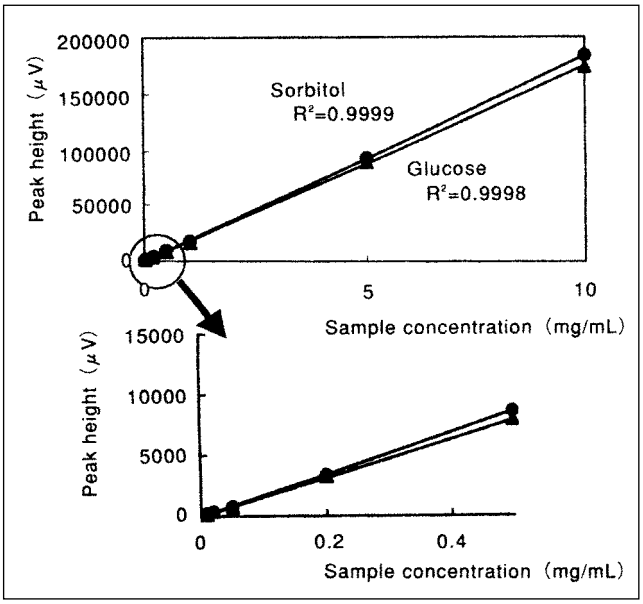


Fig. 1-18 Calibration curves with NH2P-50 4E

Table 1-6 Correlation for other saccharides (R^2)

Fructose	0.9998
Sucrose	0.9999
Lactose	0.9997
Maltose	0.9998
Meso-Erythritol	0.9999
Xylitol	0.9999
Mannose	0.9999
Maltitol	0.9997

(0.01~10mg/mL)

Condition of Fig. 1-18 and Table 1-6

Column : Shodex Asahipak NH2P-50 4E (4.6 x 250mm)
Eluent : CH_3CN/H_2O=75/25
Flow rate : 1.0mL/min
Detector : Shodex RI
Column temp. : 25℃

2. Analysis of saccharides contained in foods

2-1. Sample pretreatment (Nutrient indication criteria)

There are cases in which foods contain large amounts of proteins and lipids in addition to saccharides. To analyze saccharides using HPLC, therefore, the saccharides need to be extracted from the food by removing components other than saccharides. This process of removing such components is called pretreatment. Here is an example of pretreatment, it was described in the Nutrient Labeling Standards in Japan.

(a) Preparation of sample

(1) Solid samples should be ground using a coffee mill or the like.

(b) Preparation of test solution

(b-1) Basic operation

(1) Measure a sample of 0.5 to 5 g and place it in a 50 mL beaker.
(2) Add about 30 mL of pure water to the sample and conduct ultrasonic extraction for 30 min.
(If the liquid is acidic, neutralize it with 10 w/v% NaOH solution.)
(3) Add pure water to the above solution to make it up to 50 mL.
(If any undissolved matter remains, pass it through a No. 5B filter paper.)
(4) Pass the solution through a membrane filter (0.45μm) and prepare a sample solution for HPLC by diluting the solution accordingly.

(b-2) For foods containing large amounts of proteins or polysaccharides,
follow the same procedures as (b-1) above using 50 v/v% ethanol instead of pure water.

(b-3) For foods containing large amounts of salts,
Desalt, using an electric dialyzer (or ion exchange resin), 5 to 10 mL of the test solution prepared in accordance with (b-1) or (b-2) above.

(b-4) For foods containing large amounts of lipids,

(1) Measure a sample of 0.5 to 5 g and place it in a 50 mL centrifuging tube.
(2) Add 40 mL of petroleum ether to the above measured sample and leave the mixture to stand for 15 min, stirring it from time to time.
(3) After centrifuging the solution (at 2,000 rpm for 10 min), pour off the supernatent liquid.
(4) Repeat the above operations (2) and (3), and let remaining petroleum ether evaporate completely in a nitrogen gas stream or by submerging the beaker in a water bath (40℃).
(5) Then do the same operations as (b-1) or (b-2).

Note: The above procedure is an excerpt taken from the Nutrient Labeling Standards compiled by the Environmental Health Bureau, the Ministry of Health and Welfare in Japan.

2-2. Sample pretreatment (Other)

Use of trichloroacetic acid solution or solid phase extraction is a simple, yet effective method to remove proteins. Below is a brief description of the method:

(a) Use of trichloroacetic acid to remove proteins

(1) Add trichloroacetic acid solution (20 w/v%) to the sample solution so that the final concentration of the trichloroacetic acid becomes 5 to 10 %. Then stir the mixture.

(2) After leaving the mixed solution to stand until no further precipitation occurs, remove the precipitates by a centrifugal separator.

(3) Add ether to the above solution and shake it to remove the water layer. Repeat this operation three times to remove the trichloroacetic acid. In this way, obtain the water layer as the sample to be analyzed.

Note: Excerpt from the "new method of food analysis" compiled by Japanese Society for Food Science and Technology.

(b) Solid phase extraction to remove protein

(1) Using methanol and pure water, perform conditioning of a cartridge (for solid phase extraction) packed with polymer-based gel and another cartridge packed with C18 gel.

(2) Pass the sample solution through and collect the un-adsorbed portion.

(c) Dairy products

Ultrafiltration (molecular weight limit for filtration: 10,000) is also an effective measure to filter dairy products which cannot be easily filtered.

2-3. Example of analysis

(a) Standard samples

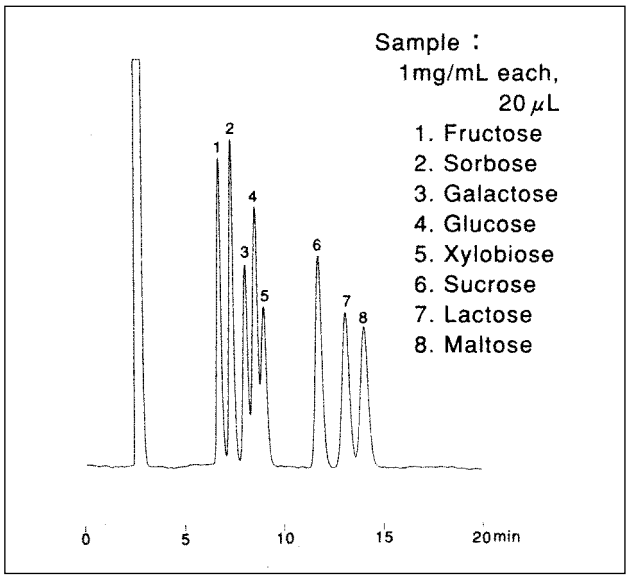


Fig. 2-1 Analysis of monosaccharides and disaccharides

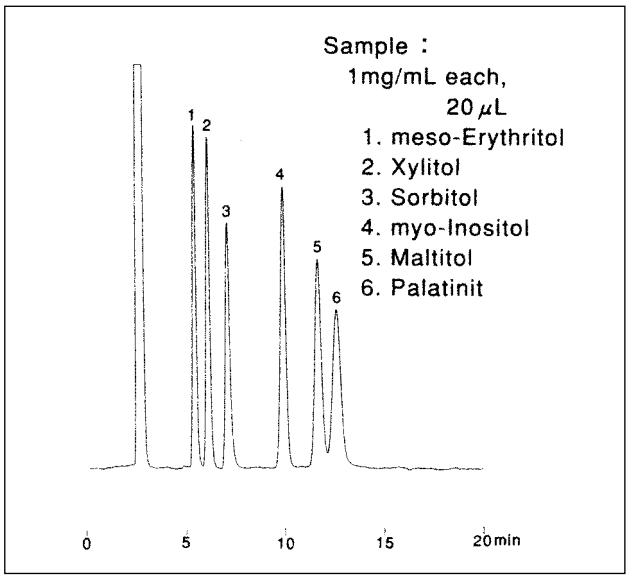


Fig. 2-2 Analysis of sugar alcohols

Condition of Fig. 2-1 and 2-2

Column : Shodex Asahipak NH2P-50 4E (4.6 x 250mm)
Eluent : CH_3CN/H_2O=75/25
Flow rate : 1.0mL/min
Detector : Shodex RI
Column temp. : 25°C

(b) Saccharides in foods

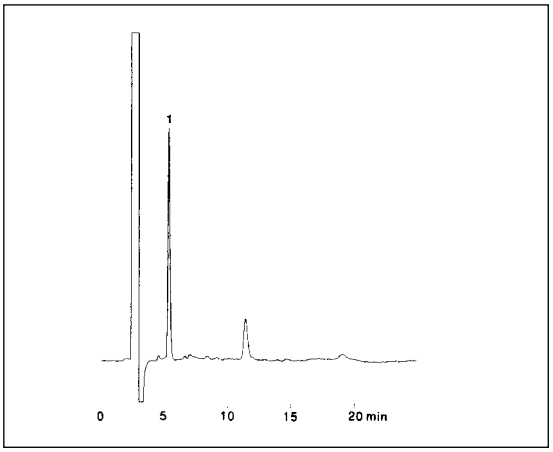


Fig. 2-3 Carbonated drink containing erythritol

Injection of 20μL	Concentration of peak (mg/mL)	Content (g) in 100 g sample
1. meso-Erythritol	1.15	2.3

Nutrient components (100 mL) indicated by manufacturer			
Calorie	0 kcal	Carbohydrate	0 g
Protein	0 g	Sucrose	0 g
Lipid	0 g	Vitamin B6	1.0 mg
Sugar	2.9 g	Vitamin C	1.0 mg
Sodium	7.0 mg	Vitamin E	0.3 mg

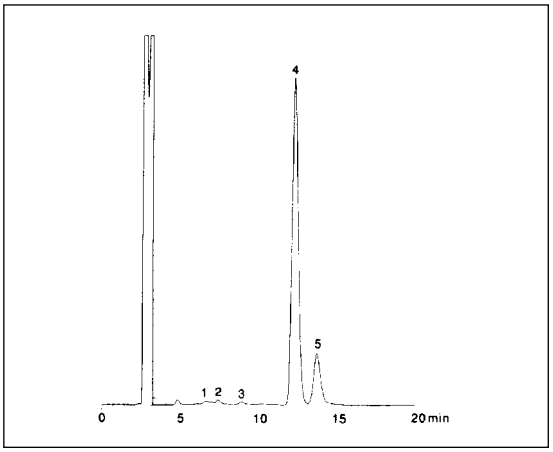


Fig. 2-4 Chocolate Cake

Injection of 20μL	Concentration of peak (mg/mL)	Content (g) in 100 g sample
1.Fructose	0.12	0.2
2.Sorbitol	0.20	0.4
3.Glucose	0.12	0.2
4.Sucrose	17.84	35.7
5.Lactose	3.70	7.4
Total		44.2

Indicated value by Producer Components in 100g					
Calorie	557 kcal	Saccharides	52.8 g	Protein	8.3 g
Sodium	75 mg	Lipids	34.8 g		

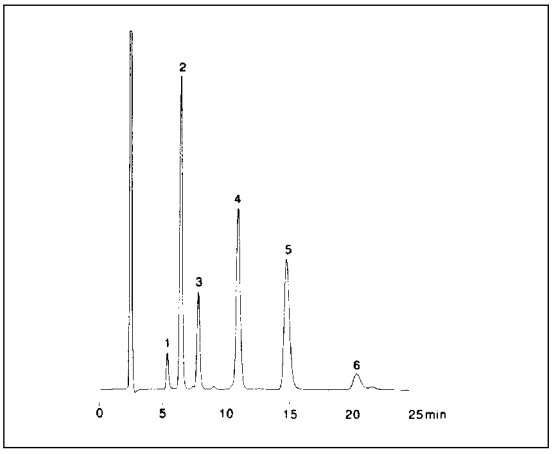


Fig. 2-5 Fructo-oligosaccharide Syrup

Injection of 20μL	Concentration of peak (mg/mL)	Content (g) in 100 g sample
1. Fructose	0.71	2.8
2. Glucose	5.11	20.5
3. Sucrose	2.03	8.1
4. 1-Kestose	5.78	23.1
5. Nystose	5.91	23.7
6. 1-Fructofuranosyl -D-nystose	1.95	7.8
Total		86.0

Fig. 2-3 and 2-4

Column : Shodex Asahipak NH2P-50 4E (4.6 x 250mm)
Eluent : CH_3CN/H_2O=75/25
Flow rate : 1.0mL/min
Detector : Shodex RI
Column temp. : 25°C

Fig.2-5

Column : Shodex Asahipak NH2P-50 4E (4.6 x 250mm)
Eluent : CH_3CN/H_2O=70/30
Flow rate : 1.0mL/min
Detector : Shodex RI
Column temp. : 25°C

3. Recommended use of NH2P-50

This section lists important points to note for lengthening the effective lifetime of NH2P-50 columns.

3-1. Mixture of acetonitrile

A mixed liquid of acetonitrile and water is usually used as the eluent for NH2P-50 columns. As the elution volume varies with changes in the ratio of the acetonitrile mixed (refer to Section 1), stable eluent needs to be supplied to guarantee reproducibility of analysis.

(a) Preparation of mixture of acetonitrile (75%) and water (25%)

Mixing 750 mL of acetonitrile and 250 mL of pure water does not become 1,000 mL of mixture. This is because molecules of water are absorbed into molecules of acetonitrile resulting in a decline in the total volume.

Therefore, when measuring one of the two liquids in a measuring cylinder and adding the other to the liquid in the cylinder to obtain a given volume of the mixture, solution with a different mixing ratio will be obtained by choosing either of the two liquids (acetonitrile or pure water) to add to obtain a given volume of the mixture. Furthermore, a reduction in the total volume when mixing can be affected by the temperature. Thus, even if the same mixing procedures are taken, a solution with a different mixing ratio could be obtained.

Therefore, we have adopted a method in which each of the two liquids to be mixed is measured and they are then mixed together. For example, to prepare a mixed liquid of acetonitrile and water at a mixing ratio of acetonitrile 75 to water 25, we measure 750 mL of acetonitrile and 250 mL of pure water respectively and mix them.

(b) Filter and degass the eluent

Contamination, suspended foreign matter, and air bubbles are the main factors which cause problems for columns. When the eluent has been prepared, pass it through a membrane filter (0.45μm). Even if not visible to the naked eyes, eluent often contains some of foreign matter. Subsequently, remove air dissolved in the eluent (degassing).

One method of degassing is to depressurize the eluent using an aspirator while applying ultrasonic vibration to the eluent. For mixed liquids, such as a mixture of acetonitrile and water, however, use of the aspirator for a long period of time causes the ratio of water to acetonitrile to become higher than the set value because acetonitrile evaporates more easily than pure water. Therefore, we recommend that degassing under the depressurizing condition be limited to a short period and equipment (DEGASSER) which enables the eluent to be degassed with the column and piping connected on line be installed upstream of the pump.

(c) Buffer precautions

As explained in Section 1, when using NH2P-50 columns, the elution volume of saccharides increases and peaks widen with rises in the portion of protonated amino groups. Furthermore, it has been confirmed that anomer separation is caused to occur.

Use of alkaline aqueous buffer solution as eluent is effective in maintaining good reproducibility and high separation power for a longer period of time. Below are the conditions that the buffer solution has to meet:

(1) It should behave as a buffer under the alkaline conditions.
(2) It should exhibit adequate basicity in highly concentrated acetonitrile.
(3) It should be adequately soluble in highly concentrated acetonitrile.

10mM tetrapropylammonium hydroxide · acetic acid (pH 10.0) of quarternary alkylamine (hereinafter called 10 mM TPA · acetic acid) is recommended to use for analysis of saccharides with NH2P-50 columns. This eluent shows the same elution characteristics as that of the acetonitrile/water elution with no reversion of the elution order occurring.

(d) Use of line filter

One solution for good chromatography is using a line filter placed between the pump and injector. It removes impurities in the eluent and improves reproducibility. NH2P-LF is the appropriate line filter for the NH2P-50 series and it may be regenerated.

3-2. Prior to column connection

If the foreign matter in the flow line moves to the column, it could lead to deterioration of the column. Therefore, before connecting the flow line to the column, wash the flow line of HPLC thoroughly. Make sure the flow line of the injector is washed at the same time.

Washing method (1) **[When a mixture of polar organic solvent and water is used as eluent]**

Wash the flow line with 100 mL of the eluent.

Washing method (2):**[When a buffer solution is used as eluent]**

Wash the flow line with the following liquids (1) through (6) in that order:

(1) 40 mL of pure water
(2) 100 mL of 50 mM phosphoric acid aqueous solution
(3) 40 mL of pure water
(4) 100 mL of 50 mM NaOH aqueous solution
(5) 100 mL of pure water
(6) 100 mL of eluent

3-3. Preparation of sample

(a) Advance removal of polysaccharides

If samples containing polysaccharides are injected into amino columns, those polysaccharides are adsorbed by the columns causing peaks to broaden and separation power to deteriorate. Figure 3-3 shows the effect of allowing pullulan with a molecular weight of 200,000 to be adsorbed by an NH2P-50 4E column and observing the changes in the elution of saccharides, almost no change in the elution volume of saccharides is found, but it can be seen that the theoretical plate number gradually declines and the peaks have a tailing in the chromatograms. (Figs. 3-1, 2 and 3)

When samples are expected to contain polysaccharides, these should be removed in advance during the pre-treatment process . This is because it is difficult to remove these polysaccharides completely once they have been adsorbed by the columns. (Refer to Section 2)

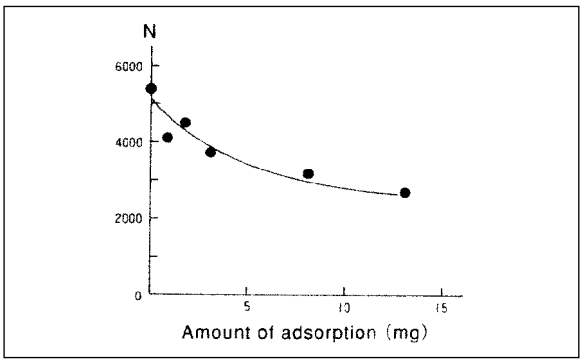


Fig. 3-1 Changes in amounts of polysaccharides adsorbed and theoretical plate number for sucrose

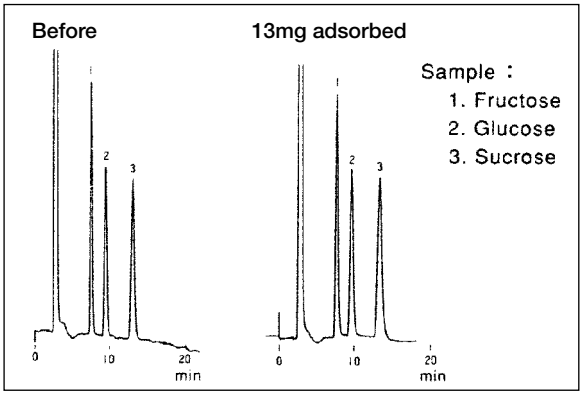


Fig. 3-3 Effects of polysaccharide adsorption on chromatograms

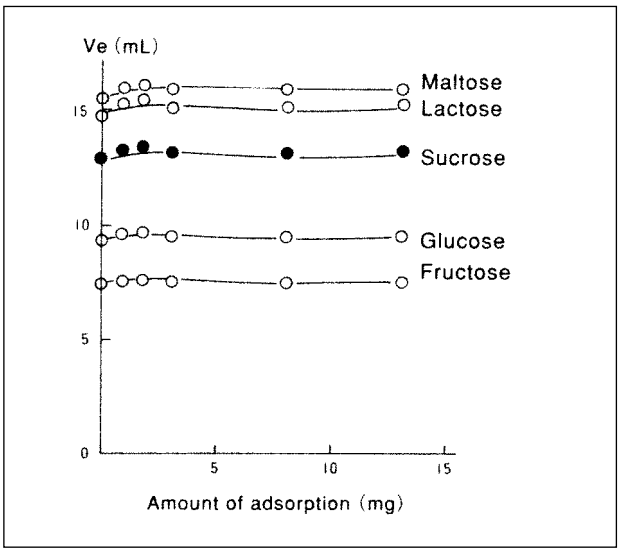


Fig. 3-2 Changes in amounts of polysaccharides adsorbed and elution volume of saccharides

Condition of Fig. 3-1, 3-2 and 3-3

Column	: Shodex Asahipak NH2P-50 4E (4.6 x 250mm)
Eluent	: CH_3CN/10mM TPA · acetic acid (pH 10.0)=75/25 *
Flow rate	: 1.0mL/min
Detector	: Shodex RI
Column temp.	: 30℃

* TPA: tetrapropylammonium hydroxide

(b) Closely match the sample solvent to the eluent

In preparing a sample solution, make the composition of the solvent which is to dissolve samples as close to that of the eluent as possible. If a sample is hard to dissolve in the eluent, first dissolve the sample in water in which it can dissolve easily. Then from the solution prepare an acetonitrile aqueous solution with a 50 % or higher mixing ratio.

If there is no choice but to inject a sample solution with a high water content, make the injected amount as small as possible. The same is true for when a sample is dissolved in 50 v/v% ethanol. (Fig. 3-4 and 3-5)

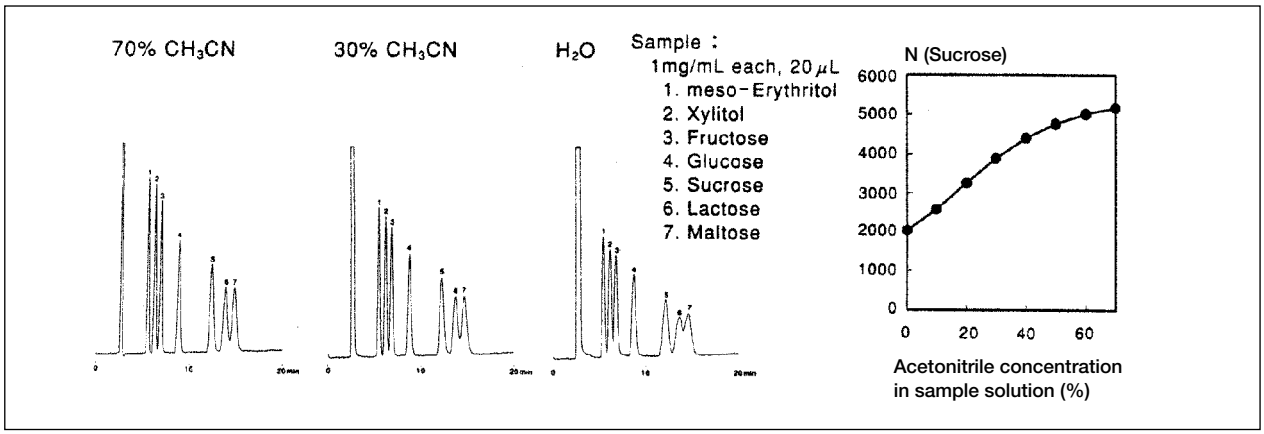


Fig. 3-4 Effects of sample solvent on separation

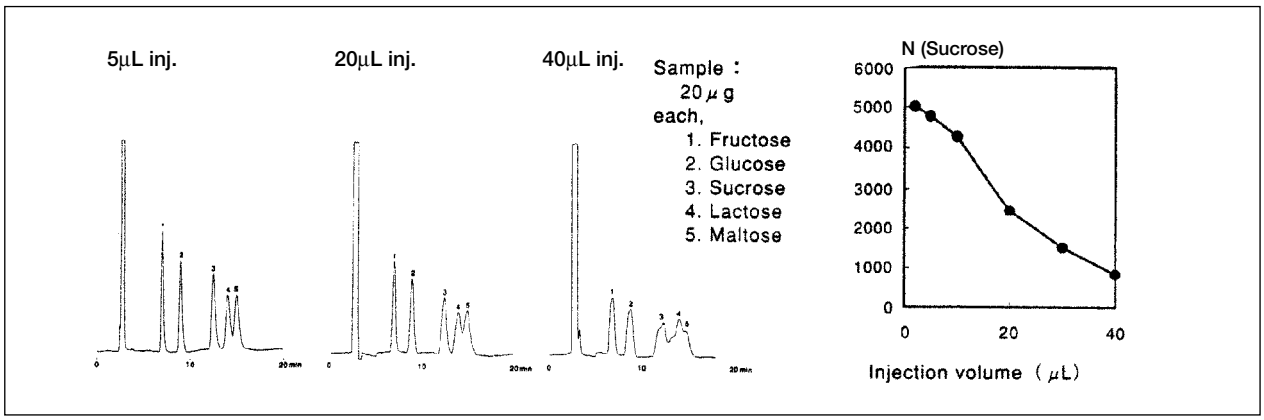


Fig. 3-5 Effects of injection amount on separation when sample is dissolved in pure water

Condition of Fig. 3-4 and 3-5

Column	: Shodex Asahipak NH2P-50 4E (4.6 x 250mm)
Eluent	: CH_3CN/H_2O=75/25
Flow rate	: 1.0mL/min
Detector	: Shodex RI
Column temp.	: 25℃

3-4. Column cleaning

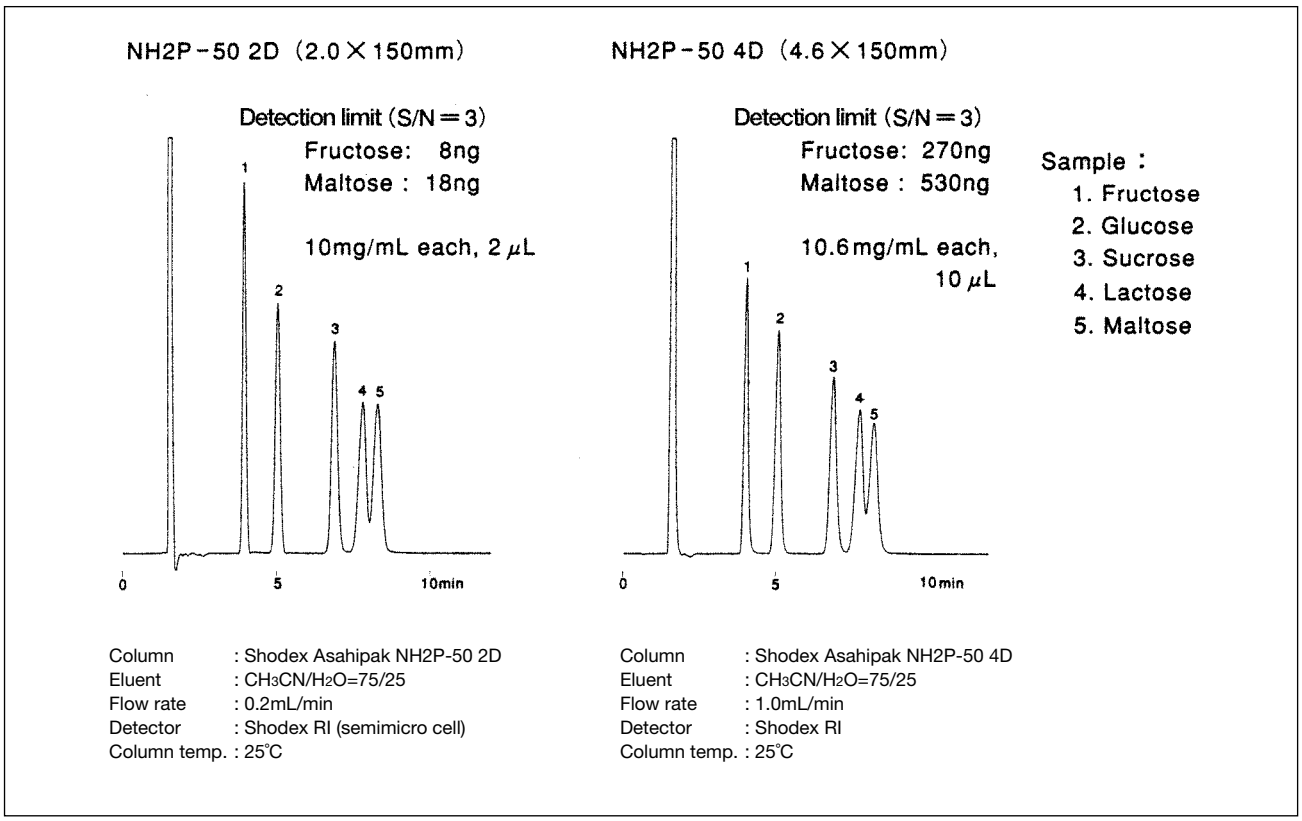
The polymer gel packed in NH2P-50 columns is chemically stable, so alkaline washing is possible. In some cases, the column performance is recovered with column cleaning. A sample washing method for NH2P-50 4E (4.6 x 250 mm) is described. Reverse the flow direction of the column and set the flow rate 0.5mL/min. Flow the following series of solvents, (1) 5mL of pure water, (2) 60mL of 0.1M $HClO_4$, (3) 5mL of pure water, (4) 60mL of 0.1M NaOH and (5) 10mL of pure water. Return to the analytical eluent and check performance.

3-5. Column protection

We recommend that guard columns be used to extend the column life of NH2P-50 series.

4. Application

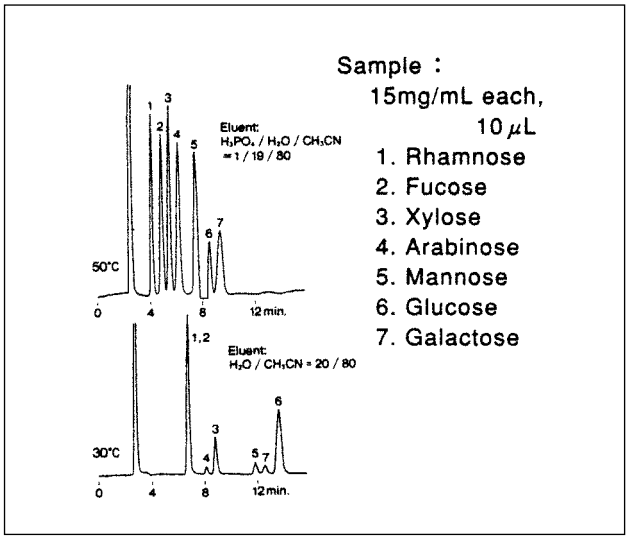
Fig. 4-1. High sensitivity analysis with semi-micro columns



Column	: Shodex Asahipak NH2P-50 2D
Eluent	: CH_3CN/H_2O=75/25
Flow rate	: 0.2mL/min
Detector	: Shodex RI (semimicro cell)
Column temp.	: 25℃

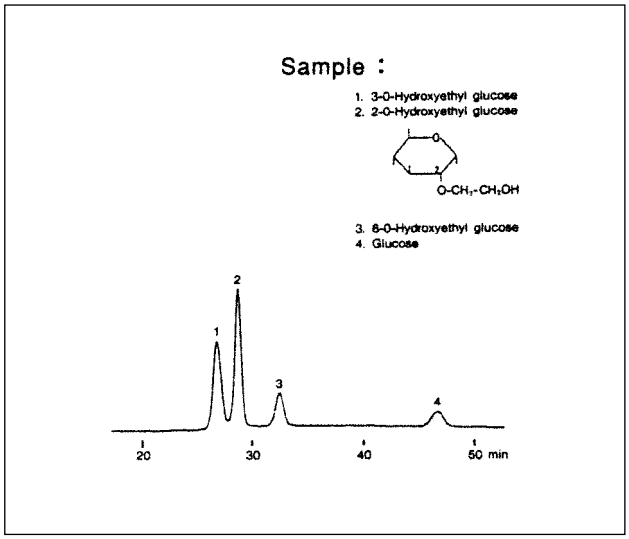
Column	: Shodex Asahipak NH2P-50 4D
Eluent	: CH_3CN/H_2O=75/25
Flow rate	: 1.0mL/min
Detector	: Shodex RI
Column temp.	: 25℃

Fig. 4-2. Monosaccharides (1)



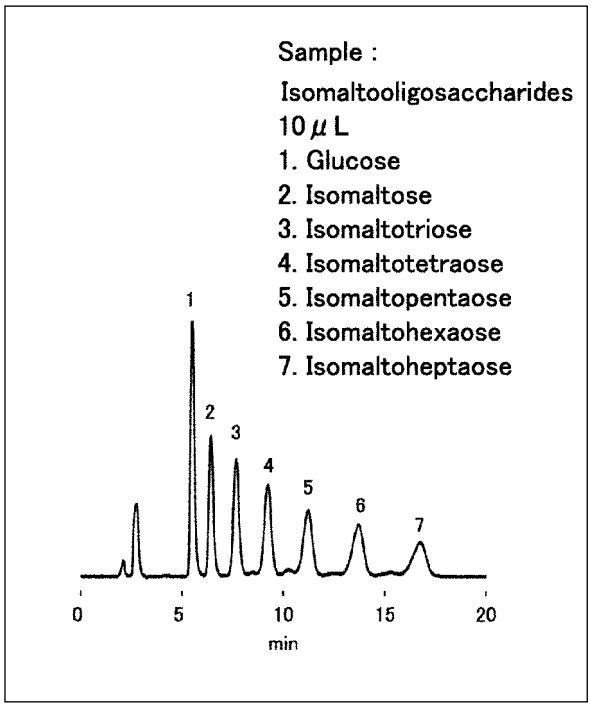
Column	: Shodex Asahipak NH2P-50 4E (4.6 x 250mm)
Flow rate	: 1.0mL/min
Detector	: Shodex RI

Fig. 4-3. Glucose derivatives



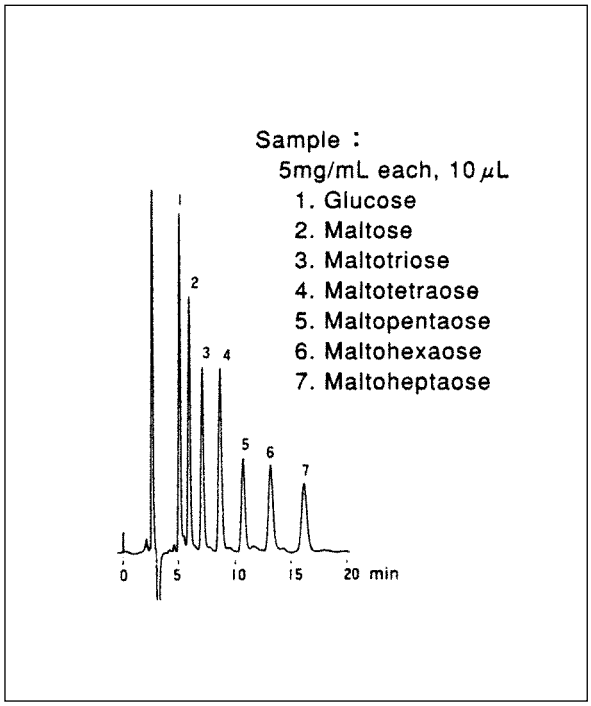
Column	: Shodex Asahipak NH2P-50 4E (4.6 x 250mm)
Eluent	: CH_3CN/H_2O=85/15
Flow rate	: 0.6mL/min
Detector	: Shodex RI
Column temp.	: 30℃

Fig. 4-4. Isomalto-oligosaccharides



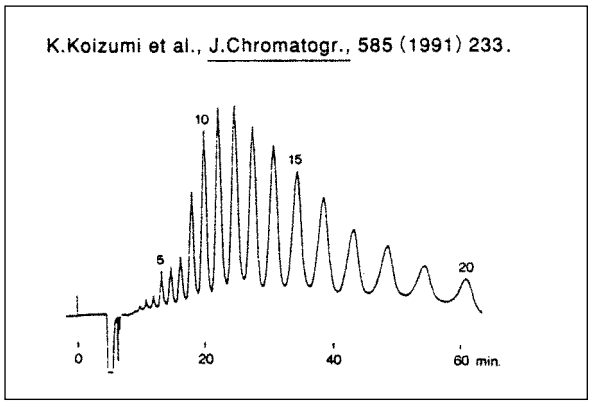
Column : Shodex Asahipak NH2P-50 4E (4.6 x 250mm)
Eluent : CH_3CN/H_2O=60/40
Flow rate : 0.8mL/min
Detector : Shodex RI
Column temp. : 30°C

Fig. 4-5. Malto-oligosaccharides



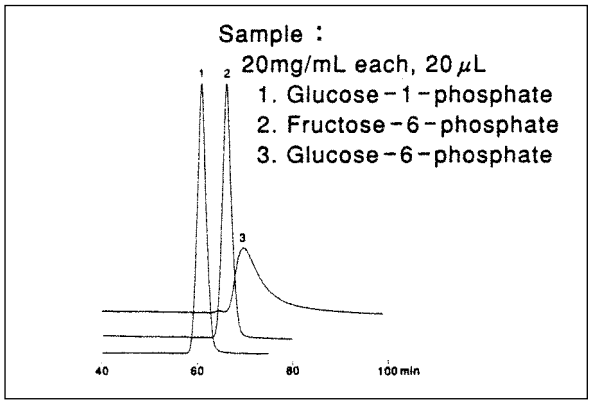
Column : Shodex Asahipak NH2P-50 4E (4.6 x 250mm)
Eluent : CH_3CN/H_2O=60/40
Flow rate : 1.0mL/min
Detector : Shodex RI
Column temp. : 30°C

Fig. 4-6. Short-chain amylose



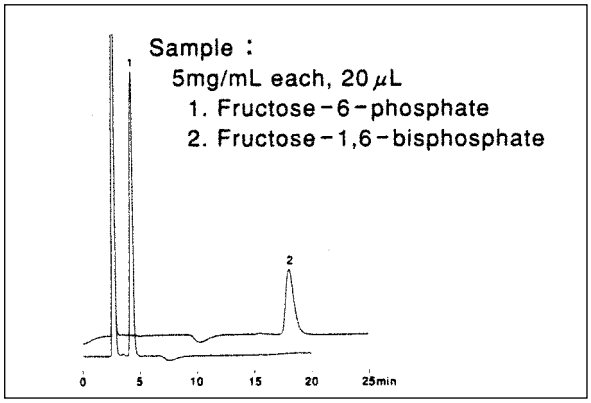
Column : Shodex Asahipak NH2P-50 10E (10.0 x 250mm)
Eluent : CH_3CN/H_2O=55/45
Flow rate : 2.0mL/min
Detector : RI
Column temp. : 33°C

Fig. 4-7. Sugar phosphates (1)



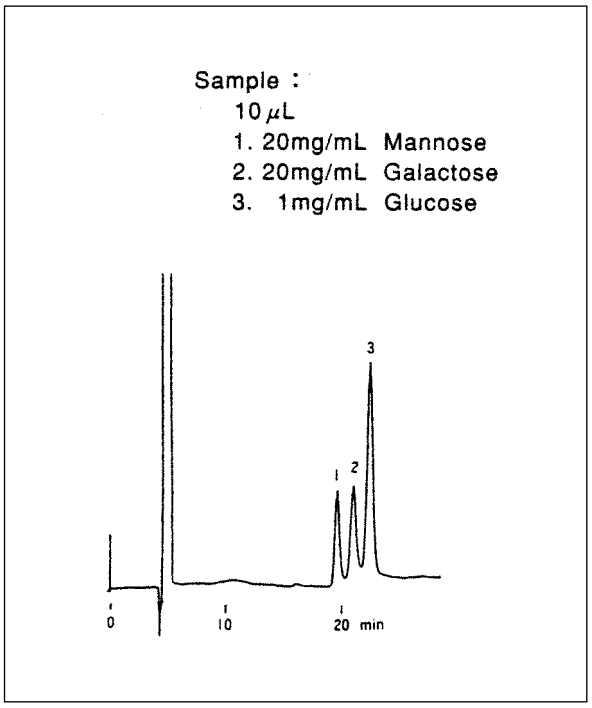
Column : Shodex Asahipak NH2P-50 4E (4.6 x 250mm)
Eluent : 10mM Sodium phosphate buffer (pH 4.4)
Flow rate : 1.0mL/min
Detector : Shodex RI
Column temp. : 40°C

Fig. 4-8. Sugar phosphates (2)



Column : Shodex Asahipak NH2P-50 4E (4.6 x 250mm)
Eluent : 0.3M Sodium phosphate buffer (pH 4.4)
Flow rate : 1.0mL/min
Detector : Shodex RI
Column temp. : 40°C

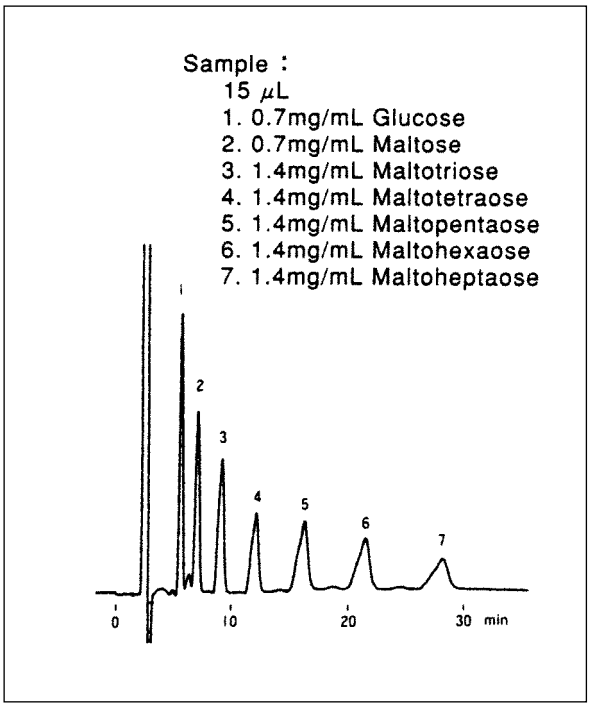
Fig. 4-9. Monosaccharides (2)



Column : Shodex Asahipak NH2P-50 4E (4.6 x 250mm)
Eluent : CH_3CN/10mM TPA · acetic acid (pH10.0)=80/20 *
Flow rate : 0.6mL/min
Detector : Shodex RI
Column temp. : 30℃

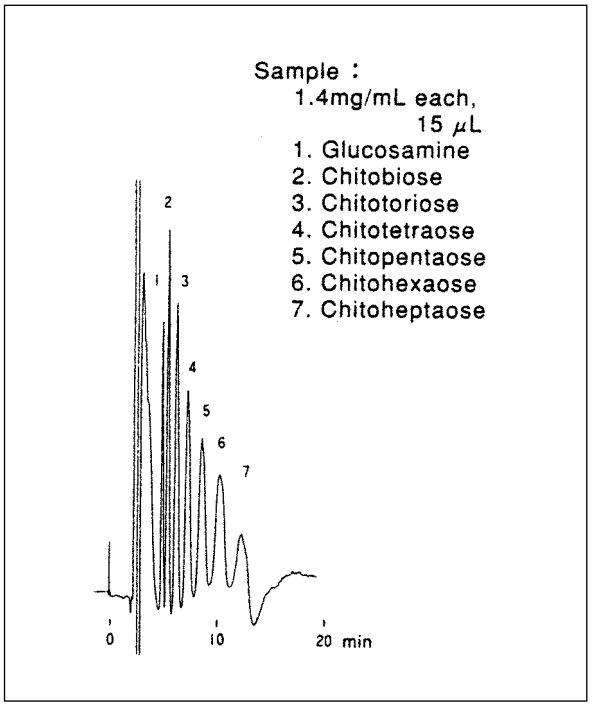
* TPA: tetrapropylammonium hydroxide

Fig. 4-10. Malto-oligosaccharides



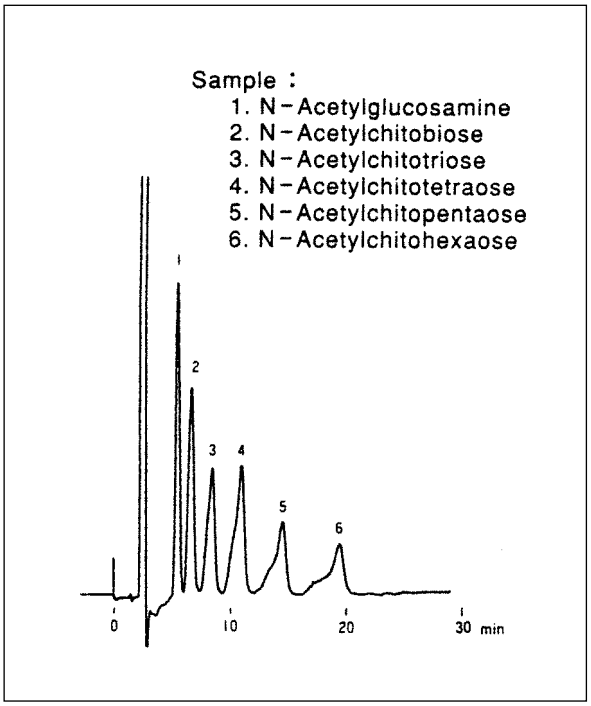
Column : Shodex Asahipak NH2P-50 4E (4.6 x 250mm)
Eluent : CH_3CN/10mM TPA · acetic acid (pH10.0)=65/35 *
Flow rate : 1.0mL/min
Detector : Shodex RI
Column temp. : 30℃

Fig. 4-11. Chito-oligosaccharides



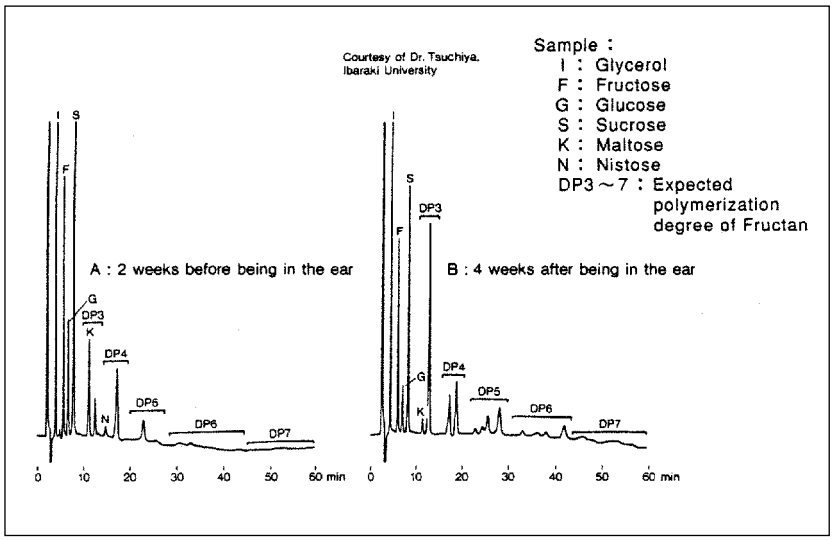
Column : Shodex Asahipak NH2P-50 4E (4.6 x 250mm)
Eluent : CH_3CN/10mM TPA · acetic acid (pH10.0)=70/30 *
Flow rate : 1.0mL/min
Detector : Shodex RI
Column temp. : 30℃

Fig. 4-12. N-Acetylchito-oligosaccharides



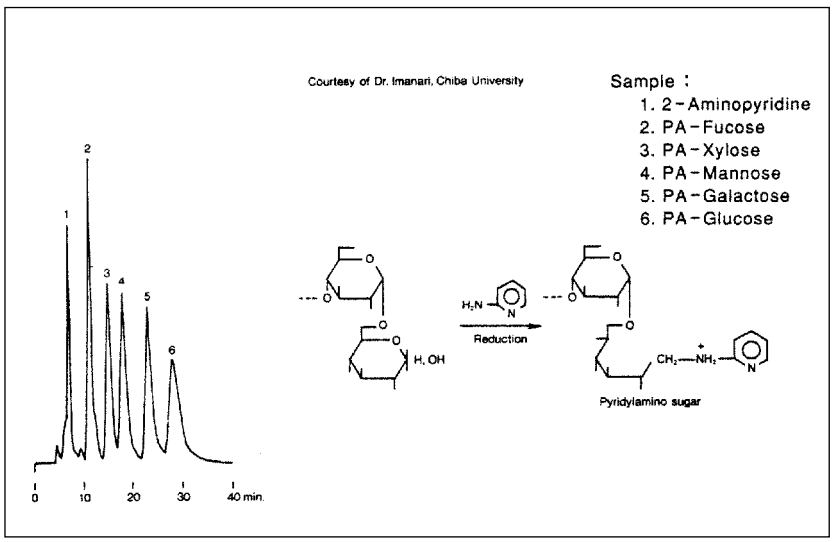
Column : Shodex Asahipak NH2P-50 4E (4.6 x 250mm)
Eluent : CH_3CN/10mM TPA · acetic acid (pH10.0)=70/30 *
Flow rate : 1.0mL/min
Detector : Shodex RI
Column temp. : 30℃

Fig. 4-13. Liquid extract of wheat rod



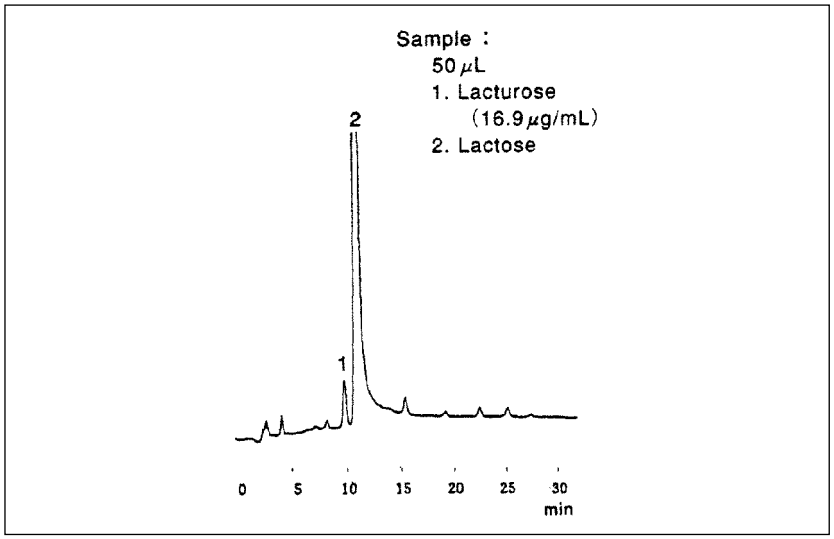
Column : Shodex Asahipak NH2P-50G 4A (4.6 x 10mm) + NH2P-50 4E (4.6 x 250mm)
Eluent : CH_3CN/50mM Ammonium acetate =70/30
Flow rate : 1.0mL/min
Detector : Shodex RI
Column temp. : 40℃

Fig. 4-14. Neutral saccharides as pyridylamino-derivatives



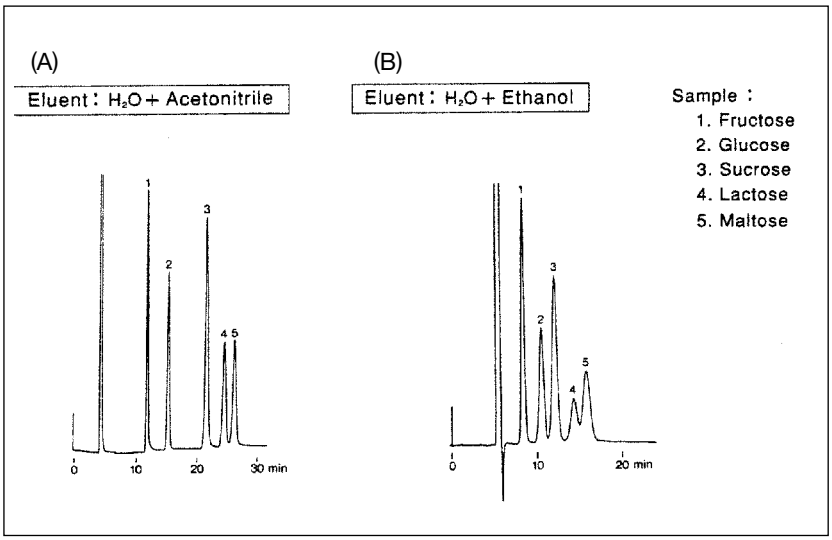
Column : Shodex Asahipak NH2P-50 4E (4.6 x 250mm)
Eluent : CH_3CN/[50mM Tris - HCl Buffer + 25mM Boric acid (pH8.5)]=100/20
Flow rate : 0.5mL/min
Detector : Fluorescence Detector, Ex 320nm, Em 400nm
Column temp. : 50℃

Fig. 4-15. High sensitivity analysis of saccharides in dry milk



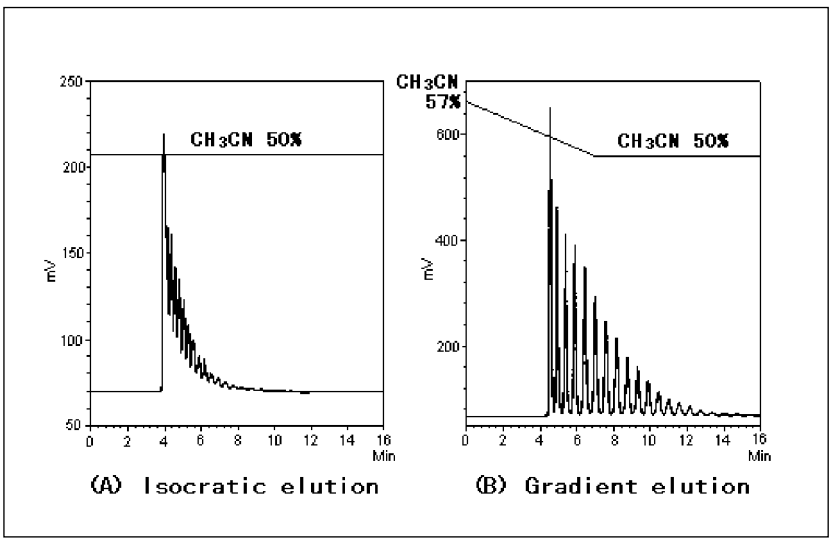
Column : Shodex Asahipak NH2P-50 4E (4.6 x 250mm)
Eluent : A) CH_3CN/H_2O=80/20
B) H_2O
5%B (0min) → 40%B (45min)
→ 40%B (45min)
Flow rate : 1.0mL/min
Detector : Pulsed amperometry
System : Agilent 1050
Reagent : 0.6M LiOH, 0.8mL/min
Working electrode : Gold
Potential : Pot 1=0.65V, Pot 2=-0.95, Pot=0.15V
Column temp. : 40℃

Fig. 4-16. Analysis using ethanol (low toxicity eluent)



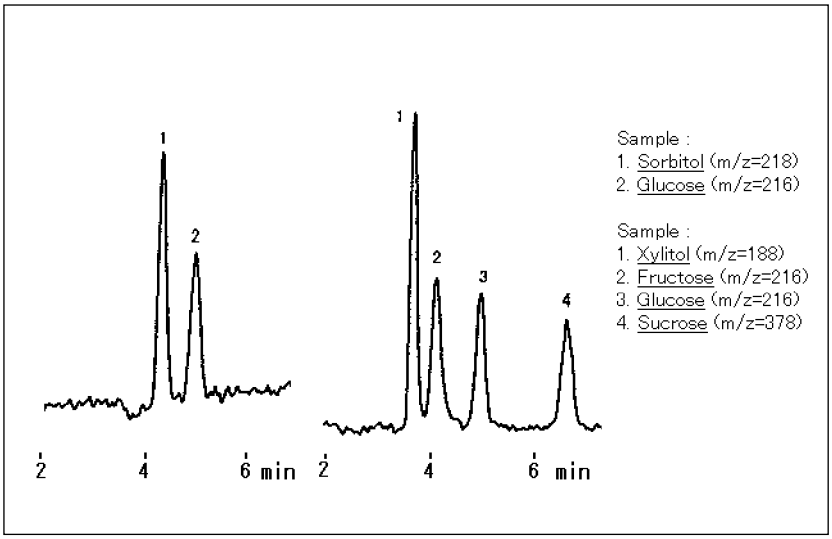
Column : Shodex Asahipak NH2P-50 4E (4.6 x 250mm)
Eluent : (A) CH_3CN/H_2O=75/25
(B) C_2H_5OH/H_2O=90/10
Flow rate : 0.6mL/min
Detector : Shodex RI
Column temp. : 30℃

Fig. 4-17. Hydrolyzed dextran with ELSD



Column : Shodex Asahipak NH2P-50 4E (4.6 x 250mm)
Eluent : (A); CH_3CN/H_2O=50/50
: (B); Linear gradient:
0min to 7 min,
CH_3CN/H_2O=57/43 to 50/50
7min to 16 min,
CH_3CN/H_2O=50/50
Flow rate : 1.0mL/min
Detector : ELSD
Column temp. : 30℃

Fig. 4-18. Saccharides and Sugar Alcohols



(HPLC)
Instrument : Agilent 1100
Column : Shodex Asahipak NH2P-50 2D (2.0 x 150mm)
Eluent : CH_3CN/H_2O=75/25
Flow rate : 0.2mL/min
Column temp. : 40℃
(Post column)
Eluent : $CH_3CN/CHCl_3$=50/50
Flow rate : 0.2mL/min
(MS)
Instrument : Agilent 1100MSD
Mass range : 100-500(m/z)
Ionization : APCI
Mode : SIM(M + Cl)
Polarity : Negative
Fragmentor : 20V
Nebulizer : N_2(40psi)
Drying gas : N_2(10L/min , 350℃)
Corona current : 30μA
Vaporizer temp. : 400℃

5. Appendix

List of elution volume of saccharides

Substances	Elution Volume (mL)						
	Asahipak NH2P-50 4E	SUGAR Series					Asahipak GS-220 HQ x2
		SP0810	SC1011	KS-801	SZ5532	SC1211	
		Pb^{2+}	Ca^{2+}	Na^{+}	Zn^{2+}	Ca^{2+}	
N-Acetyl-α-D-glucosamine	6.66	8.86	7.75	6.68	—	4.10	
D(+)-Arabinose	6.18	10.42	8.91	8.21	5.11	5.56	
D-Arabitol	6.29	15.86	11.33	7.63	7.27	8.16	
Aspartame	—	—	—	—	—	34.02	
2-Deoxy-D-glucose	6.02	8.83	7.58	7.15	4.34	4.02	
Difructose anhydride III	7.77	7.07	6.30	5.81	4.30	*	
Dulcitol	7.45	20.18	12.76	7.40	9.46	11.28	
meso-Erythritol	5.43	12.70	10.09	7.86	5.73	6.27	17.38
Ethanol	—	11.13	11.33	10.09	—	4.27	
1-Fructofuranosyl-D-nystose	—	6.05	5.27	4.76	31.43	*	14.24
D(-)-Fructose	6.75	11.05	8.85	7.71	5.37	5.90	17.05
D(+)-Fucose	5.43	10.48	8.84	8.09	4.50	4.96	
D(+)-Galactose	8.10	9.74	7.98	7.58	6.46	4.98	16.60
4'-Galactosyllactose	21.66	7.42	6.02	5.40	19.02	*	
α-D-Galacturonic acid	—	—	6.28	4.36	—	5.63	
Gentiobiose	16.36	7.22	6.08	5.75	10.50	*	
Glucose	8.61	8.63	7.30	7.17	5.87	4.76	16.75
Glycerol							17.83
Glycyrrhizic acid	—	—	—	—	—	2.71	
myo-Inositol	9.96	12.77	8.86	7.99	12.63	7.87	
Isomaltose	15.18	7.68	6.26	5.95	10.57	*	15.57
Isomaltotriose	27.55	7.09	5.75	5.34	21.17	*	14.72
1-Kestose	20.11	6.79	5.75	5.26	13.09	*	15.31
Kojibiose	14.82	7.56	6.21	5.88	9.65	*	
Lactitol	11.82	13.27	8.09	6.13	16.35	6.67	15.50
Lactose	13.27	8.05	6.51	5.99	10.12	4.07	15.71
Lactosylfructoside	18.98	7.12	5.88	5.29	14.69	*	
Lactulose	10.72	9.13	6.99	6.19	9.16	4.65	
Maltitol	11.82	12.23	8.26	6.03	13.04	6.77	15.82
Maltoheptaose							14.82
Maltohexaose							14.56
Maltopentaose							14.31
Maltose	14.24	7.85	6.34	5.94	8.67	*	16.11
Maltotriose	24.96	7.48	5.89	5.38	13.79	*	15.56
Mannitol	7.39	15.80	11.10	7.23	8.75	9.03	
D-Mannose	7.84	10.72	8.17	7.64	5.83	5.01	
D(+)-Melezitose	19.27	6.94	5.79	5.24	13.60	*	
Melibiose	14.70	8.16	6.45	5.98	11.69	4.23	
Methyl-α-D-mannopyranoside	4.71	11.13	8.87	7.78	3.99	4.39	

Substances	Elution Volume (mL)						
	Asahipak NH2P-50 4E	SUGAR Series					Asahipak GS-220 HQ x2
		SP0810	SC1011	KS-801	SZ5532	SC1211	
		Pb^{2+}	Ca^{2+}	Na^{+}	Zn^{2+}	Ca^{2+}	
Nystose	31.90	6.38	5.45	4.93	20.05	*	14.72
Palatinit	12.73	2peaks	2peaks	5.90	2peaks	2peaks	
Palatinose	12.12	7.84	6.45	5.89	8.08	3.99	
Panose	25.60	7.14	5.78	5.32	16.87	*	
L-Phenylalanine	—	—	—	—	—	31.58	
D(+)-Raffinose	20.25	7.14	5.78	5.29	16.36	*	15.08
D(+)-Rhamnose	5.52	9.77	8.23	7.37	3.93	4.43	
D(-)-Ribose	5.45	19.35	13.66	9.04	4.82	8.64	
Rutinose	10.87	7.81	6.49	5.80	6.65	*	
Saccharin sodium	5.68	6.96	5.44	4.33	6.77	*	
D(-)-Sorbitol	7.09	21.61	13.31	7.42	9.79	11.88	16.60
D(+)-Sorbose	7.35	9.67	8.03	7.38	5.12	4.92	
Stachyose	36.22	6.82	5.57	4.97	—	*	14.33
Stevioside	6.07	—	—	—	4.14	*	
Sucrose	11.87	7.54	6.29	5.87	7.91	*	16.26
α-D-Talose	6.47	21.33	12.59	8.76	5.69	8.51	
Theanderose	2peaks	2peaks	2peaks	2peaks	2peaks	*	
Trehalose	13.25	7.62	6.27	5.78	10.85	*	
Trehalulose	11.68	8.92	6.95	6.10	9.54	4.78	
Xylitol	6.10	19.87	13.14	7.94	7.77	10.16	16.88
Xylobiose	9.05	8.16	6.68	6.40	5.65	*	
D(+)-Xylose	6.58	9.21	7.09	7.71	4.55	4.48	17.46
D-Xylulose	5.41	10.64	9.02	8.04	4.06	5.07	

(—) → cannot be detected
(*) → cannot be separated from solvent peak

Column : Shodex Asahipak NH2P-50 4E (4.6 x 250mm)
Eluent : H_2O/CH_3CN=25/75
Flow rte : 1.0mL/min
Detector : Shodex RI
Column temp. : 30℃

Column : Shodex SUGAR SZ5532 (6.0 x 150mm)
Eluent : H_2O/CH_3CN=25/75
Flow rte : 1.0mL/min
Detector : Shodex RI
Column temp. : 60℃

Column : Shodex SUGAR SC1211 (6.0 x 250mm)
Eluent : H_2O/CH_3CN=65/35
Flow rte : 1.0mL/min
Detector : Shodex RI
Column temp. : 70℃

Columns : Shodex SUGAR SP0810, SC1011, KS-801 (8.0 x 300mm each)
Eluent : H_2O
Flow rte : 1.0mL/min
Detector : Shodex RI
Column temp. : 80℃

Column : Shodex SUGAR SC1211 (6.0 x 250mm)
Eluent : H_2O/CH_3CN=65/35
Flow rte : 1.0mL/min
Detector : Shodex RI
Column temp. : 70℃

Kinds of saccharides

Monosaccharide (n=1)	glucose (glc), galactose (gal), fructose (fru), xylose, arabiose, mannose, ribnose etc.
Monosaccharides are carbohydrates in the form of simple sugars. Monosaccharides are usually colorless, water soluble crystalline solids. Some monosaccharides have a sweet taste.	

Disaccharide (n=2)	sucrose (glc+fru), lactose (glc+gal), maltose (glc+glc), cellobiose, trehalose etc.
A disaccharide is a sugar (a carbohydrate) composed of two monosaccharides. The two monosaccharides are bound via a condensation reaction. Like monosaccharides, they are crystalline, water soluble, and sweet tasting.	

Oligosaccharide (n=3~6)	gulacto-oligo saccharide, soybean-oligo saccharode, xylo-oligo saccharide, lactoducrose etc.
An oligosaccharide is a saccharide polymer typically containing three to six component sugars. They are generally found either O- or N-linked to compatible amino acid side chains in proteins or to lipid moieties. Oligosaccharides are often found as a component of glycoproteins or glycolipids and as such, are often used as chemical markers, frequently for cell recognition.	

Polysaccharide	starch, amylase, glycogen, cellulose, pectin, lignin etc.
Polysaccharides are relatively complex carbohydrates. They are polymers made up of many monosaccharides joined together by glycosidic linkages. They are therefore very large, often branched, molecules. They tend to be amorphous, insoluble in water, and have no sweet taste. Some polysaccharides work as energy resource, and some form the primary structural component of plants.	

Sugar alcohol	sorbitol, xylitol, maltitol, lactitol, dulcitol, arabitol, mannitol etc.
A sugar alcohol is a hydrogenated form of carbohydrate, whose carbonyl group (aldehyde or ketone, reducing sugar) has been reduced to a primary or secondary hydroxyl group. The sugar alcohols are not as sweet as sucrose, but they are also lower in calories than sucrose. In addition sugar alcohols are not metabolized by oral bacteria, and so they do not contribute to tooth decay. They are commonly used for replacing sucrose in foodstuffs, often in combination with high intensity artificial sweeteners to counter the lower sweetness.	

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Notice

1. Please read the instruction manual accompanying the product in its entirety before using the NH2P-50 column.
2. The specifications for the products are subject to change without notice for purposes of improvement.
3. No guarantee is offered to figures in this technical paper; those figures should be used just as reference.
4. Even if no precautions are given in the instruction manual as to the safety or danger of reagents and chemical products, make sure that in handling the products, the usual precautions are taken.
5. The products described herein are not designed for use in clinical examinations in the medical area.

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