목재공학18(2) : 16~24(原著)
Mogjae Gonghak.18(2) : 16~24.1990

Gel Permeation Chromatography of Douglas-fir (*Pseudotsuga menziesii*(Mirb.) Franco) Bark Condensed Tannins*1

Young Soo Bae*2

美松 樹皮탄닌의 젤 침투 크로마토그래피*1

裵 映 壽*2

摘 要

아세틸化 高分子 縮合탄닌의 젤침투 크로마토그래피 分析을 위해 一般的으로 使用되고 있는 폴리스타이렌 標準物質은 不正確한 矯正값을 준다. 한편 유도체화 하지않은 축합탄닌의 젤침투 크로마토그래피는 폴리스타이렌 디비닐벤젠 코폴리머 컬럼과 디메틸 포름아미드를 溶媒로 使用하여 매우 만족스럽게 分析될 수 있었다. 그 컬럼의 교정은 유도처리 되지않은 高分子 縮合탄닌을 利用하여 수행되었으며 고분자 탄닌을 유도처리 할 필요가 없으므로 탄닌의 신속한 分子量 決定이 가능하고 컬럼 크로마토그래피로 分離되어지는 탄닌을 연속적으로 分析할 수 있는 長點을 가진다.

1. INTRODUCTION

Douglas-fir[*Pseudotsuga menziesii*(Mirb.) Franco] is the major commercial timber species of the Pacific Northwest, accounting for about three quarters of softwood inventory. In the process of harvesting and preparation of Douglas-fir logs for these industries a substantial amount of bark is also made available. For the most part this bark is burned for its fuel value.

Douglas-fir bark represents an abundant renewable resource that may be underutilized considering its chemical composition(8). Waxes, carbohydrates and polyphenols are the major components. Currently there is a great deal of interest in the potential utilization of renewable resources for the production of chemicals that can replace or supplement those derived from petroleum(4, 7). Extending this concept even further, perhaps new materials unlike those based on petrochemicals could also be produced by future generations.

In this regard it is very important to study the reaction by gel permeation chromatography(GPC) to better understand the molecular weight distribution of Douglas-fir bark condensed tannins for wood adhesive making. Standard techniques for estimating the molecular weights of condensed tannin polymers include ^{13}C NMR, vapour phase osmometry and GPC. GPC is perhaps the most convenient of these methods for many laboratories.

*1. 接受 1990年 3月 5日 Received March 5, 1990
*2. 江原大學校 林科大學 College of Forestry, Kangweon National University, Chuncheon 200-701, Korea.

2. EXPERIMENTAL

2.1 Equipments

The ^{13}C NMR spectrum was obtained from a Bruker AM 400 spectrometer with sample dissolved in methanol-d_4. GPC analyses were carried out using a Waters chromatography pump(model 6000 A) fitted with a Waters injector(model U6K) and a Gilson UV detector(model 111B). A wavelength of 280nm was used to detect the phenolic procyanidin polymers and the sensitivity of the detector was also set to 0.5 AUFS.

A bank of Waters μ-styragel columns(0.75×30 cm)(10^4, 10^3, 500 and 100Å) were connected in series for use with tetrahydrofuran(THF) as the eluting solvent. Polystyrene or oligomeric procyanidin peracetates were used to clibrate this system. The operational flow rate of this system was 2ml/min.

Polymer Labs PLgel precolumn(0.75×7.5cm) and 5μm polymer Labs PLGel columns(0.75×32.5cm) (10^3 and 100Å) connected in series for use with a N, N-dimethyl formamide(DMF) solvent system (1000ml of DMF was mixed with 5ml of 3M ammonium formate). A Waters temperature control system that consists of the temperature control module (TCM) and a column heater was connected to the GPC system to decrease the viscosity of the DMF solvent because it is highly viscous at room temperature and controlled to 50℃. The flow rate of the eluting solvent was 1ml/min.

A Spectra Physics computing integrator(model SP 4200) equipped with GPC+Prom was used to obtain the molecular weight profiles, calculations and graphs.

2.2 Collection of inner bark

The inner bark used in this study was taken from a freshly fallen 120 year old Douglas-fir [*Pseudotsuga menziesii*(Mirb.) Franco] tree in McDonald Forest, Benton County, Oregon in May of 1986. The tree was cut as part of a commercial harvesting operation. The inner bark was carefully stripped and immediately brought to the laboratory where it was cut into small strips and then immersed in methanol.

2.3 Bark extraction

The bark(43% moisture content, 3kg) was allowed to stand in methanol for three days and then methanol solution was decanted, filtered(Whatman No.1 filter paper) to give a crude methanol extract. Each batch of bark was extracted three times in this way. The combined extractants were then concentrated on a rotary evaporator under reduced pressure at 35−40℃ to give a dark red-brown syrup(168g) which was then freeze dried.

2.4 Purification of polymeric procyanidins

A portion of the freeze dried powder(20g) was applied to a Sephadex LH-20 column(3×40cm). The column materials were composed of glass and glass frits with teflon tubing in order to avoid contact of the procyanidins with metal. The column was washed with methanol-water(1:1 v/v), until the eluent was almost colorless. This took 10 hrs at a flow rate of 2 ml/min. This fraction was evaporated to give 13.86g of carbohydrate material and low molecular weight procyanidins including dihydroquercetin.

Elution of the polymeric procyanidins was accomplished by washing the column with acetone-water(7:3 v/v) for 3 hrs at 2ml/min. The acetone was removed by evaporation using a rotary evaporator and the remaining aqueous phase was freeze dried to give the polymeric procyanidin as a powder(5.5g). This powder was then dissolved in methanol and extracted with petroleum ether to remove waxy material. The methanol soluble fraction was conce-

ntrated on a rotary evaporator and freeze dried again to give 5.48g of material. The ^{13}C NMR spectrum of this material showed it to be a homogeneous procyanidin polymer exhibiting broad peaks in the general region(Fig. 1).

2.5 Gel permeation chromatography(GPC)

2.5.1 GPC analysis of acetylated procyanidin polymers

2.5.1.1 Acetylation of procyanidin polymer

A sample of polymeric procyanidins(200mg) was dissolved in pyridin(10ml, dried over KOH) and acetic anhydride(10ml) was added. After standing at room temperature for 48hrs, the reaction mixture was poured into ice water(125ml) and extracted with chloroform(50ml, 2times). The chloroform fractions were combined together and washed with 2% dilute hydrochloric acid(25ml, 3times), 10% dilute sodium bicarbonate(25ml, 3times) and distilled water(25ml, 3times). After filtration through the anhydrous sodium sulfate, the solution was let stand overnight with the anhydrous sodium sulfate at room temperature. The solution was then filtered and the chloroform was evaporated to yield 107mg of a light yellow solid(9).

2.5.1.2 Calibration of GPC system

The polystyrene standard calibration kit(Polymer Laboratories Inc.) was used to obtain the GPC calibration report. Nine calibration standards(MW:162, 580, 1050, 1350, 1770, 2550, 3750, 5100) which 5mg of each standard was dissolved in 1ml THF were

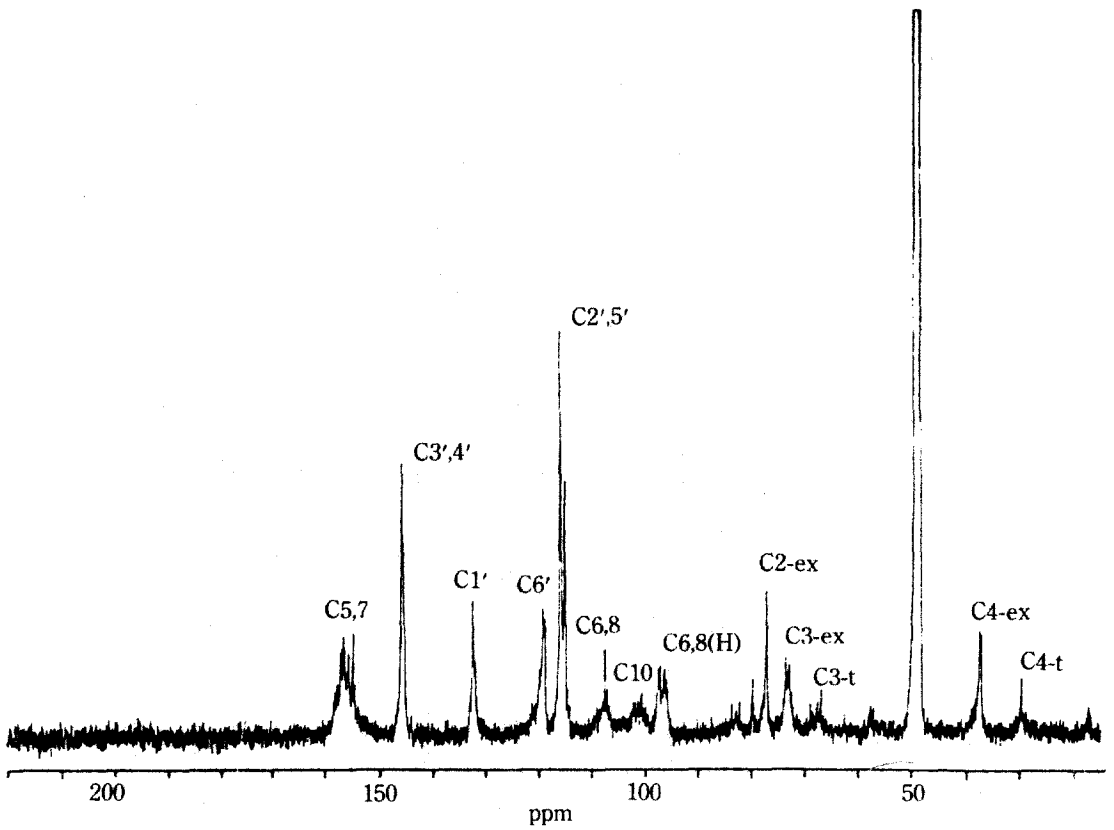


Fig. 1. ^{13}C NMR spectrum of purified polymeric procyanidins.

used to as standard solutions. Injection volume was 20μl. The elution time(ET) of the standards was decided by the average value of three to five injections.

The calibration report, the regression analysis and the calibration curve obtained by the integrator are shown in Table 1, 2 and Fig. 2. According to the calibration report(Table 1), the molecular weight values by the cubic regression analysis were quite similar to the real molecular weights of polystyrene standard and the cubic method(Table 2) had the highest significance. Therefore, the cubic regression analysis method was used to calculate the molecular weight profile of procyanidin peracetate.

2.5.1.3 GPC analysis of acetylated procyanidin polymers

For analysis, 5mg procyanidin peracetate was di-

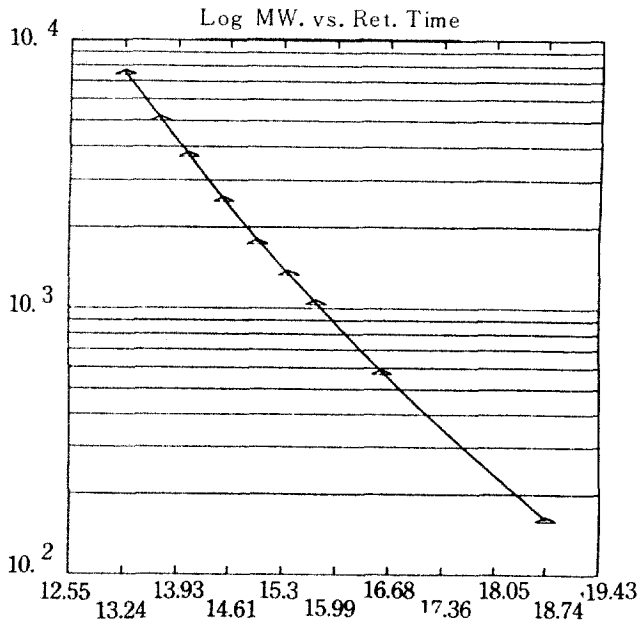


Fig. 2. Calibration curve of GPC system for procyanidin peracetate analysis.

Table 1. Calibration report for procyanidin peracetate

ET	Molecular weight(MW) of standard	Linear MW	Quadratic MW	Cubic MW
13.71	5100	4750	5064	5061
14.07	3750	3690	3750	3740
14.54	2550	2654	2566	2557
14.99	1770	1935	1808	1804
15.39	1350	1462	1339	1338
15.74	1050	1144	1039	1040
16.59	580	630	579	582
18.74	162	139	162	162

Table 2. Regression analysis for procyanidin peracetate

Coefficient	Linear	Quadratic	Cubic
Ka	7.8531029	11.359391	12.982746
Kb	-.304624	-.749493	-1.0596454
Kc		0.0139421	0.0335389
Kd			-.0004093
Correlation(r ↑ 2)	0.9930758	0.99989	0.999941
Standard error	0.0457097	0.0062232	0.0049918

ssolved in 1ml freshly redistilled THF. This solution was filtered with a Millipore filter(millipore corp., type FH, 0.5μm) and 20μl of the solution was injected into the GPC system. The number average molecular weight(Mn) of the procyanidin peracetate was 2000 and the weight average molecular weight (Mw) was 3127.

2.5.2 GPC analysis of procyanidin polymers

2.5.2.1 Calibration of GPC system

For the calibration, five procyanidin polymers (oligomers of epicatechin)(MW:290, 578, 702, 866, 1154, 1442) were used as a standard:epicatechin (monomer), epicatechin-(4β→8)-epicatechin(B2) (dimer), epicatechin-(4β→8)-epicatechin-(4β→1)-phloroglucinol, epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin (trimer), epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin (tetramer), epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin(pentamer).

Of these, trimeric to pentameric procyanidin standards including the dimeric phloroglucinol adduct were given by Dr L. Y. Foo(Chemistry Division, DSIR, Petone, New Zealand) who isolated these compounds.

1mg of each standard was dissolved in 1ml DMF and the 20μl solution was injected into the injector after Millipore filtration. The elution time(ET) of the standards was decided by the average value after three to five time injection.

The calibration report, the regression analysis and the calibration curve obtained by the integrator are shown in Table 3, 4 and Fig. 3. The calibration report(Table 3) represented that the quadratic and cubic regresson method gave the similar molecular weight value to the real molecular weight values of the procyanidin polymer standards and these two analytical method had the same significance values(Table 4). The cubic method was also applied to this GPC system in order to compare the result of this system with that of polystyrene standards and to make the analytical condition just like that of procyanidin peracetate.

2.5.2.2. GPC analysis of procyanidin polymers

1mg procyanidin polymers was dissolved in 1ml DMF and the solution was filtered with a Millipore filter. The injection volume was 20μl. The number average molecular weight($\bar{M}n$) and the weight average molecular weight($\bar{M}w$) of the polymeric procyanidins were 1604 and 2706 respectively.

3. RESULTS AND DISCUSSION

The purpose of this work was to obtain the molecular weight profiles of procyanidin polymers by GPC using THF and DMF as eluting solvents, to compare the GPC results from DMF solvent with the result from THF and to try a new method for the estimation of the molecular weight profile of procyanidin polymers without derivatization such as acetylation or methylation.

Physical characteristics of flavanoids, such as volatility, solubility and ease of crystallization may be altered considerably by derivatives formation. Derivatives are also often required for analytical purposes, for spectroscopic studies and occasionally for the conversion of one flavonoid to another(9). In this study, the acetylation was used to increase the solubility of procyanidin polymers when THF is used as eluting solvent in GPC analyses.

Many natrually occurring polymers consist of molecules with different molecular weights and are said to be polydisperse. Since typical small molecules and large molecules with moleculr weights less than a critical value(Z) required for chain entanglement are weak and are readily attacked by approp-

Table 3. Calibration report for procyanidin polymers

ET	Molecular weight(MW) of standard	Linear MW	Quadratic MW	Cubic MW
12.75	1442	1407	1434	1434
12.99	1154	1165	1170	1170
13.39	866	850	841	841
13.58	702	732	722	722
13.87	578	583	574	574
14.78	290	285	290	290

Table 4. Regression analysis for procyanidin polymers

Coefficient	Linear	Quadratic	Cubic
Ka	7.55034546	10.273189	9.3830421
Kb	-.3415852	-.7446944	-.5501414
Kc		0.0146302	0.0004786
Kd			0.0004326
Correlation(r ↑ 2)	0.998008	0.9988252	0.9988189
Standard error	0.01222914	0.0108996	0.01333849

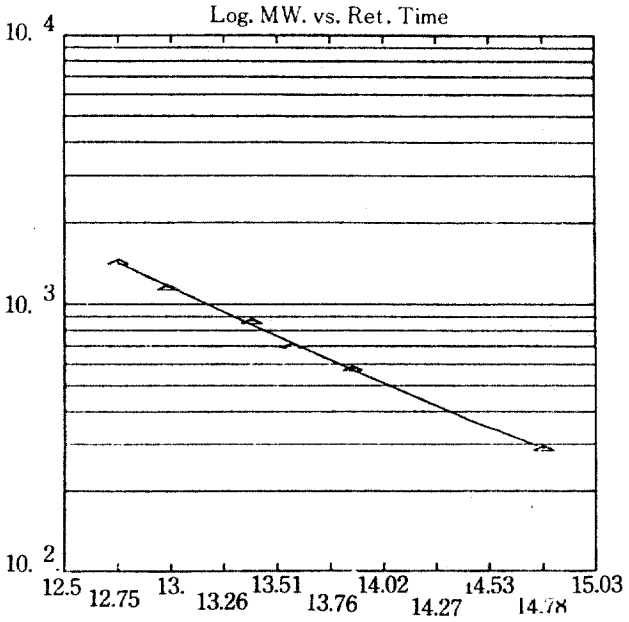


Fig. 3. Calibration curve of GPC system for polymeric procyanidin analysis.

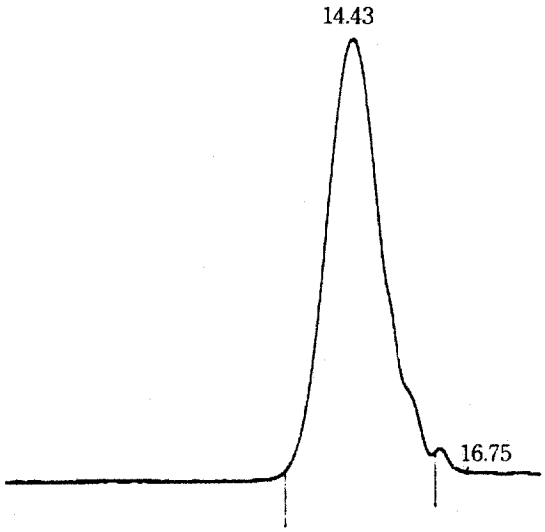
riate reactants, it is apparent that these properties are related to molecular weight. The number average molecular weight($\bar{M}n$) value is the number of molecules that are present in a given weight of sample and is calculated by dividing the sum of the individual molecular weight values by the number of molecules. The $\bar{M}n$ values are highly sensitive to samll molecules present in the mixture. Therefore, this value is used to calculate the average molecular weight of a polymers. The weight average molecular weight($\bar{M}w$) value is determined from experiments in which each molecules or chain makes a contribution to the measured result and is dependent on the total number of particles (11).

Both $\bar{M}n$ and $\bar{M}w$ values were reported in this research, as well as dispersity which is $\bar{M}w/\bar{M}n$.

3.1 GPC analysis of acetylated procyanidin polymers

The number average molecular weight($\bar{M}n$) and the weight average molecular weight($\bar{M}w$) of procyanidin peracetates(Fig. 4) were 2000 and 3172 res-

pectively. The $\bar{M}n$ value means that the procyanidin peracetates consisted of the molecular weight of tetramer and that the highest molecular weight of the polymers was hexamer(molecular weight of monomeric unit is 500). The polydispersity index was 1.56. Fig. 5 showed the molecular weight distribution curve of the procyanidin peracetate which



Wt. Avg MWt=3127. No. Avg MWt=2000.
Z Avg MWt.=4512. Z+1 Avg MWt=6830.
Polydisp. Index=1.5632183 Fit Type (Ft)=3.

Fig. 4. GPC chromatogram of procyanidin peracetates.

most of molecules have the molecular weight in the range of 2000-4000.

Polystyrene calibration standards are useful for the estimation of apparent molecular weights of polymers whose structures are not known or where standards with the appropriate structures are not available.

In the present work, both polystyrene standards and acetylated procyanidin oligomers were used to calibrate the GPC system. A system calibrated with polystyrene standards did not however give the same molecular weight data for acetylated proc-

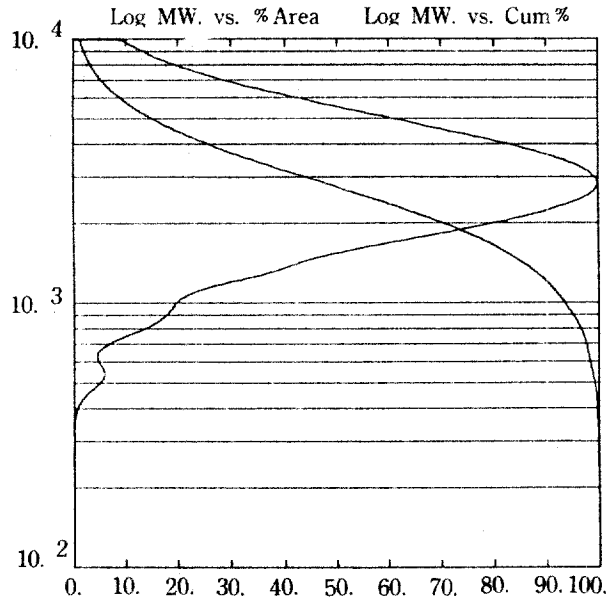


Fig. 5. Distribution curve representing the molecular weight profile of procyanidin peracetates.

yanidin polymers as did the same system calibrated with oligomeric procyanidin acetates. The reason is apparent when one compares the calibration curve for polystyrene against the curve for procyanidin oligomers peracetates(Fig. 6). Therefore polystyrene standards may not correctly estimate molecular weight of polymeric procyanidin acetates. This finding explains the discrepancies observed when comparing the molecular weights of tannins that have been done as acetates(with polystyrene calibration) and as free phenols(with phenol calibration). This situation points out dramatically that GPC is an indirect method of molecular weight analysis and its accuracy depends on the structural similarity of the standards and compounds analyzed.

3.2 GPC analysis of underivatized procyanidin polymers

The number average molecular weight($\bar{M}n$) and the weight average molecular weight($\bar{M}w$) of the

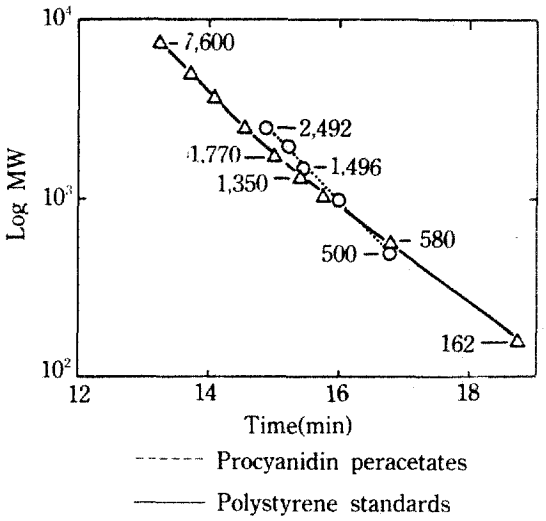
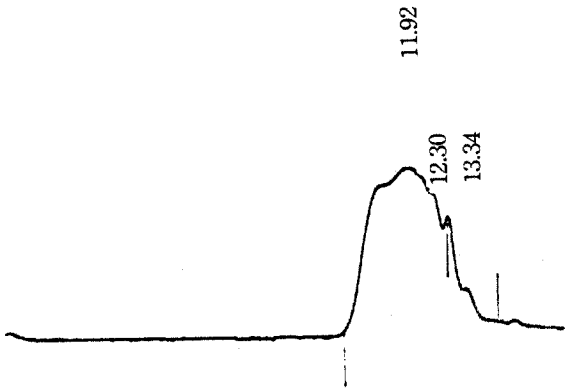


Fig. 6. Comparison of the calibration curve between polystyrene and procyanidin oligomer peracetate standards.

procyanidin polymers(Fig. 7) were 1604 and 2706. In this result, The $\bar{M}n$ value was equivalent to the molecular weight of pentamer to hexamer(molecular weight of monomeric unit is 290) and the $\bar{M}w$ was equal to nine to ten monomeric units. The



Wt. Avg MWt.=2706. No. Avg MWt.=1604.
Z Avg MWt.=4057. Z+1 Avg MWt.=5307.
Polydisp. Index=1.6866869 Fit Type (Ft)=3.

Fig. 7. GPC chromatogram of procyanidin polymers.

polydispersity index was 1.68. Fig. 8 represented that the molecular weight profile of the procyanidin polymers had the range of 2000-5000.

This GPC system consisted of two Polymer Labs PLgel 5μm columns(100 and 10^3Å) and used a DMF solvent at 50℃ for elution. Calibration was accomplished with authentic procyanidin oligomers. This result demonstrated for the first time that polymer procyanidins can be analyzed by GPC in their free phenolic form. This means that GPC analyses can be carried out much faster than before and losses due to derivatization can be avoided.

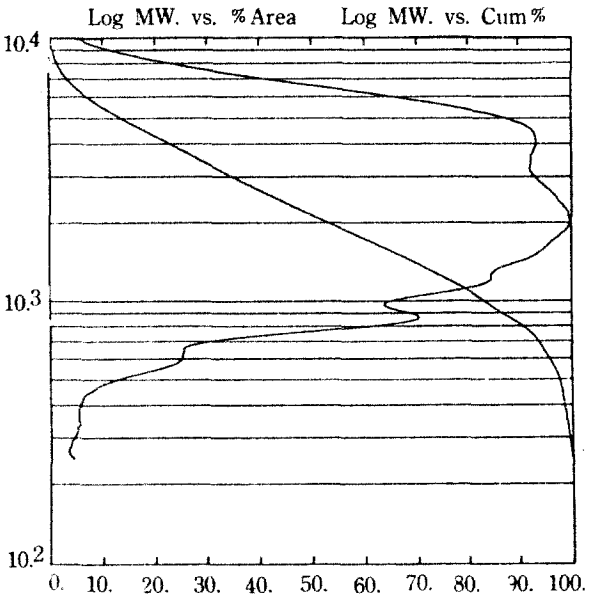


Fig. 8. Distribution curve representing the molecular weight profile of procyanidin polymers.

LITERATURE CITED

1. Brandon, M. J., L. Y. Foo, L. J. Porter, and P. Meredith. 1982. Proanthocyanidins of barely and sorghum: composition as a function of maturity of barely ears. Phytochem., 21(12):2953-2957.
2. Czochanska, Z., L. Y. Foo, R. H. Newman, and

L. J. Porter. 1980. Polymeric proanthocyanidins. Sterochemistry, structural units and molecular weight. J. chem. soc., perkin trans I, 2278-2286.

3. Foo, L. Y., W. T. Jones, L. J. Porter, and V. M. Williams. 1982. Proanthocyanidin polymers of fodder legumes. Phytochem., 21(4):933-935.
4. Hemingway, R. W., A. H. Conner, and S. J. Branham. 1989. Adhesives from renewable resources. ACS Symp. Seri. 385, Amer. Chem. Soc., Washington DC.
5. Jones, W. T., R. W. Broadhurst, and J. W. Lyttleton. 1976. The condensed tannins of pasture legume species. Phytochem. Vol. 15 1407-1409.
6. Karchesy, J. J., and R. W. Hemingway. 1980. Lobloly pine bark polyflavanoids. J. agri. food chem., 28:222-228.
7. Koch, G. S., F. Klareich, and B. Exstrum. 1987. Adhesives for the composite wood panel industry. Noyes Data Corp., Park Ridge, NJ.
8. Laver, M. L., H. H-L. Fang, P. M. Loveland, J. V. Zerrudo, C. H. Chen, and Y-C. L. Liu. 1977. Chemical constituents of Douglas-fir bark: a review of more recent literature. Wood Sci., 10(2): 85-91.
9. Markham, K. R., 1982. Techniques of flavonoid identification. Academic Press, N. Y.
10. Samejima, M., and T. Yoshimoto. 1979. Procyanidins from the inner bark of sugi(*Cryptomeria japonica* D. Don). Mokuzai Gakkaishi, 25(10): 671-677.
11. Seymour, R. B., and C. E. Carraher, Jr. 1988. Polymer chemistry, An introduction(second edition). Marcel Dekker, Inc. New York 91-121.
12. Williams, V. M., L. J. Porter, and R. W. Hemingway. 1983. Molecular weight profiles of proanthocyanidin polymers. Phytochem., 22(2)569-572.