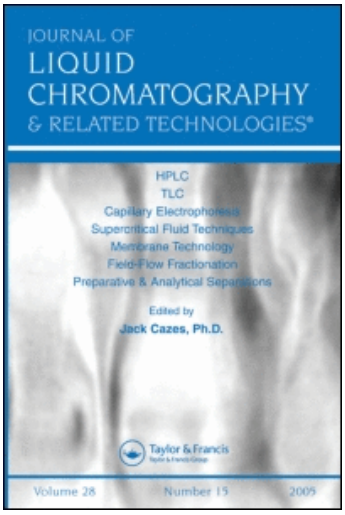

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PURIFICATION OF ANTI-HIV LIGNANS FROM *LARREA TRIDENTATA* BY pH-ZONE-REFINING COUNTERCURRENT CHROMATOGRAPHY

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ABSTRACT

Anti-HIV lignans were purified from extract of *Larrea tridentata* by high-speed countercurrent chromatography (CCC) using pH-zone-refining CCC. When a column filled with methyl t-butyl ether, containing trifluoroacetic acid at 25 mM, was eluted with aqueous NaOH, 10 to 20 g of the crude extract was separated into NDGA (nordihydroguaiaretic acid) and its monomethyl esters rectangular

peaks associated with their specific pH (pH zones). The method was also successfully applied to synthetic lignans, resulting in resolution of NDGA and its mono and dimethyl esters.

INTRODUCTION

It has been shown that a crude extract of creosote bush (*Larrea tridentata*) contains lignans which exhibit anti-HIV activity.[1-3] Initially, these lignans were isolated in our laboratory using the standard technique of high-speed countercurrent chromatography (CCC).[1]

The current studies describe separation of these lignans by pH-zone-refining CCC[4,5] using sodium hydroxide as an eluent. The method was applied to both crude plant extracts and a synthetic mixture derived from methylation of NDGA (nordihydroguaiaretic acid).

This new technique provides various advantages over the standard high-speed CCC technique, such as greater selectivity and efficiency, higher yields and shorter separation times.

EXPERIMENTAL

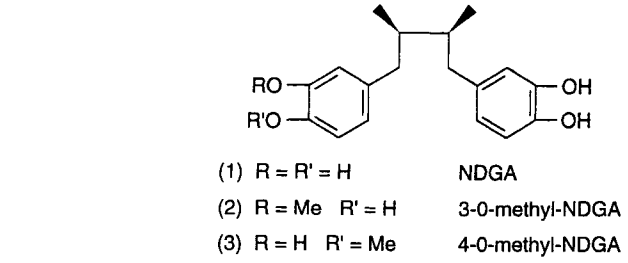
Reagents

Methyl t-butyl ether was obtained from Burdick and Jackson, Muskegon, MI and trifluoroacetic acid (TFA) from Pierce Chemicals, Rockford, IL, USA, both of glass-distilled HPLC grade. Sodium hydroxide (reagent grade) was obtained from J. T. Baker Chemical Co., Phillipsburg, NJ, USA, and NDGA (nordihydroguaiaretic acid) from Sigma Chemical Co., St. Louis, MO, USA.

Crude creosote bush extract was supplied by Professor R. C. C. Huang's laboratory, Johns Hopkins University, Baltimore, MD, USA.

Preparation of Crude Extract

Leaves of *L. tridentata* were collected in summer in Arizona and sun-dried for several weeks. The plant materials were ground to a powder and extracted by maceration with dichloromethane as previously described.[1]



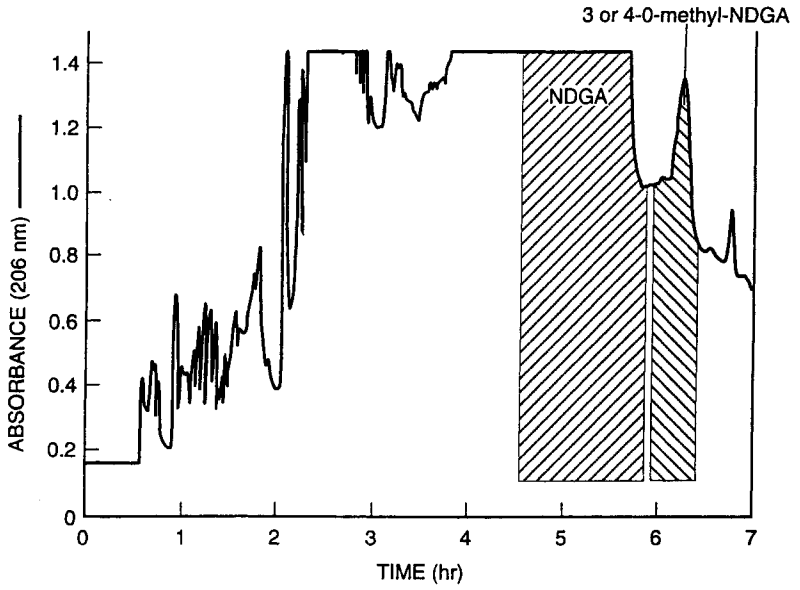


Figure 1. pH-Zone refining separation of crude extract of *Larrea tridentata* using a high-speed CCC centrifuge equipped with a semi-preparative column. The experimental conditions are as follows: Apparatus: commercial multilayer coil planet centrifuge (Ito Multilayer Coil Separator/Extractor, P.C. Inc.); column: multilayer coil prepared from 1.6mm ID Tefzel tubing with 310 mL capacity; sample: 10g of crude extract of *L. tridentata*; Solvent system: methyl t-butyl ether/water, upper organic mobile phase contained 25 mM TFA and the lower aqueous mobile phase, NaOH at 0, 50, and 100 mM in stepwise elution; flow rate: 3 mL/min; revolution: 800 rpm.

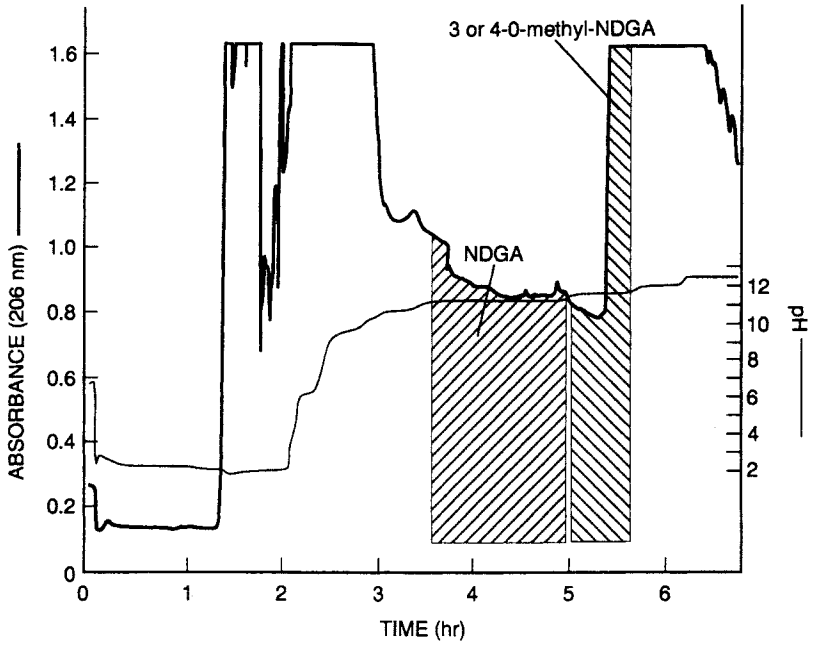


Figure 2. pH-Zone-refining CCC of crude extract of *Larrea tridentata* using a cross-axis coil planet centrifuge. Experimental conditions are: apparatus: cross-axis coil planet centrifuge with 10 cm radius; column; a pair of preparative multilayer coil, 2.6 mm ID and 570ml capacity; sample: 10g of crude extract of *L. tridentata*; solvent system: methyl *t*-butyl ether/water, upper organic mobile phase contained 25 mM TFA and the lower aqueous mobile phase, 100 mM NaOH; flow rate: 4 ml/min; revolution: 700rpm.

Synthesis of 3 or 4-O-Methyl-NDGA

3 g of NDGA (10 mmol) was dissolved in 20 ml of 1M NaOH solution and cooled in an ice-bath. Dimethylsulfate (1.25g or 10 mmol) was gradually added to the solution over a period of 30 min. The solution was constantly stirred at room temperature for 1 hour and then acidified with concentrated HCl until the pH dropped to 2-3.

The solution was then eluted with 50 mL of water and extracted with methyl t-butyl ether. The organic phase was washed with water and dried over sodium sulfate. After solvent evaporation, 3.5 g of an oily material was obtained.

Apparatus

Two different models of the coil planet centrifuge (CPC) were used: A commercial multilayer CPC and the cross-axis CPC. These planetary centrifuges are equipped with a rotary-seal-free, flow-through system which permits continuous elution of the mobile phase through the rotating column without using a conventional rotary seal device. Detailed designs of these instruments have been described elsewhere.[6]

Two models of the multilayer coil high-speed CCC centrifuge were employed: a semipreparative unit with 310 mL capacity (P. C. Inc., Potomac, MD, USA) and a preparative unit with 850 mL capacity (Pharma-Tech Research Corporation, Baltimore, MD, USA). The semipreparative multilayer coil was prepared in our laboratory by winding a single piece of 160m, 1.6mm ID Tefzel tubing directly around the holder hub, making 11 layers of tight coils between a pair of flanges spaced 5 cm apart.

Each terminus of the column was connected to a 0.85-mm ID Teflon tubing.

The cross-axis CPC (model X-1.5L) used in the present study is our most recent prototype fabricated at the machine shop of the National Institutes of Health, Bethesda, MD, USA. It holds a pair of multilayer coils symmetrically on the rotary frame at 10 cm from the central axis of the centrifuge. Each column holder undergoes a synchronous planetary motion in such a way that the holder rotates about its horizontal axis and simultaneously revolves around the vertical axis of the centrifuge at the same angular velocity.

The rotary frame can accommodate the column holders in two different locations either in the central position or off-center position. In the present study, the column holders were mounted at the off-center position. Each multilayer coil was prepared by winding a single piece of 2.6mm ID Teflon tubing around the holder hub making multiple layers of left-handed coils between the flanges. Two identical columns were connected in series on the rotary frame to make a total capacity of 560 mL. The apparatus was rotated at an optimum speed of 650 rpm. During the operation a bag of dry ice was placed over the top plate of the centrifuge to maintain the ambient temperature at 23-25°C.

Preparation of Solvent System and Sample Solution

A binary solvent system composed of methyl t-butyl ether and water in an arbitrary volume ratio was equilibrated in a separatory funnel and the two phases

were separated. The upper organic phase was acidified with TFA (25 mM) and used as the stationary phase. The lower aqueous phase was basified by adding various amounts of sodium hydroxide (0 - 100mM) and used as the mobile phase.

The sample solution of the crude creosote bush extract was prepared by dissolving 10 g of dried powder in 70 mL of the CCC solvent system . This solvent consisted of 50 mL of the upper stationary phase containing 25 mM TFA and 20 mL of the lower mobile phase. The sample solution of the methylated NDGA was prepared by dissolving 3.5 g of the dimethyl sulfate reaction product in 20 mL of solvent consisting of equal volumes of each phase.

General Separation Procedure

The separation column was first completely filled with the acidified organic stationary phase and the sample solution was injected through the sample port. The aqueous mobile phase containing sodium hydroxide at a desired concentration was then pumped into the column while the column was rotated. The effluent from the outlet of the column was continuously monitored with a UV monitor (LKB Uvicord S, LKB Instruments, Stockholm, Sweden) and passed through a pH cell (Sensorex, Stanton, CA). The eluent was collected into test tubes using a fraction collector (Ultrorac, LKB Instruments). After the separation, the column contents were pushed out by nitrogen gas into a graduated cylinder to determine the volume of the stationary phase retained in the column and the percent retention.

Analysis of CCC Fractions

The CCC fractions were analyzed by TLC using chloroform/ methanol/water (100:15:1) with cerium sulfate spray as previously described.[1] NDGA and 3-O-methyl-NDGA served as standards for monitoring the eluent.

RESULTS AND DISCUSSION

Separation of the Anti-HIV Lignans from *Larrea tridentata*

The lignans obtained from the crude extract of creosote bush represent a new class of anti-HIV agents which inhibit HIV gene transcription and replication.[2] Isolation of these lignans was successfully performed in our laboratory using the standard high-speed CCC technique.[1] However, several problems emerged in the early study with the standard CCC technique. The need for a large quantity of the

material required for clinical trial prompted the development of a new pH-zone-refining CCC method which provides over a 10-fold increase in sample loading capacity compared to the standard CCC technique.

This improved method also shortened the separation times by a factor of 2 for equal sample size. Since weakly acidic phenol groups in these lignans do not form stable salts with ammonium hydroxide, they could not be eluted with the ammonium hydroxide mobile phase used in the ordinary pH-zone-refining system. This problem was overcome by using a stronger base, such as NaOH, as an eluent in the aqueous mobile phase.

Figure 1 shows a typical chromatogram of the crude creosote bush extract using a 310 mL capacity column. The separation was performed as follows: The column was filled with methyl *t*-butyl ether containing TFA at 25 mM followed by injection of the sample solution containing 10 g of the crude extract in 50 mL of the stationary phase and 20 mL of water. Then the column was eluted, first with 180 mL of water, then with 180 mL of 50 mM NaOH, and finally with 900 mL of 100 mM NaOH. The lignans formed highly concentrated fused rectangular peaks (shaded) while most of the impurities are eluted earlier.

TLC analysis of fractions corresponding to each pH zone revealed that the NDGA (1.9 gram) and 3 or 4-O-methyl-NDGA (1.4 gram) were eluted at retention times of 4.5-5.8 hr and 5.9-6.4 hr, respectively. In this separation, a stepwise elution by increasing the NaOH concentration in the mobile phase was required in order to obtain a satisfactory retention of the stationary phase, although this considerably increased the separation time. This problem was overcome by using the cross-axis CPC or the high-speed CCC centrifuge with a preparative column which provides better retention of the stationary phase in the column.

Figure 2 shows a similar chromatogram of 10 g of the same sample by pH-zone-refining CCC using the cross-axis CPC with a 560 mL column capacity. After loading the stationary phase and sampie solution, the column was isocratically eluted with a mobile phase of 100 mM NaOH at 700 rpm. In this case, NDGA (2.0 g) and 3 or 4-O-methyl-NDGA (1.5 g) were each eluted with a distinct flat pH-zone at retention times of 3.6-4.9 hr and 5.0-5.7 hr, respectively.

The sample size can be increased by use of the preparative high-speed CCC centrifuge with 2.6mm ID and a 850ml capacity. Figure 3 shows the chromatogram obtained from 20 g of the crude extract by pH-zone-refining CCC. NDGA (3.7 g) and 3 or 4-O-methyl-NDGA (2.8 g) were eluted at 3.8-4.7 hr and 4.8-5.2 hr,

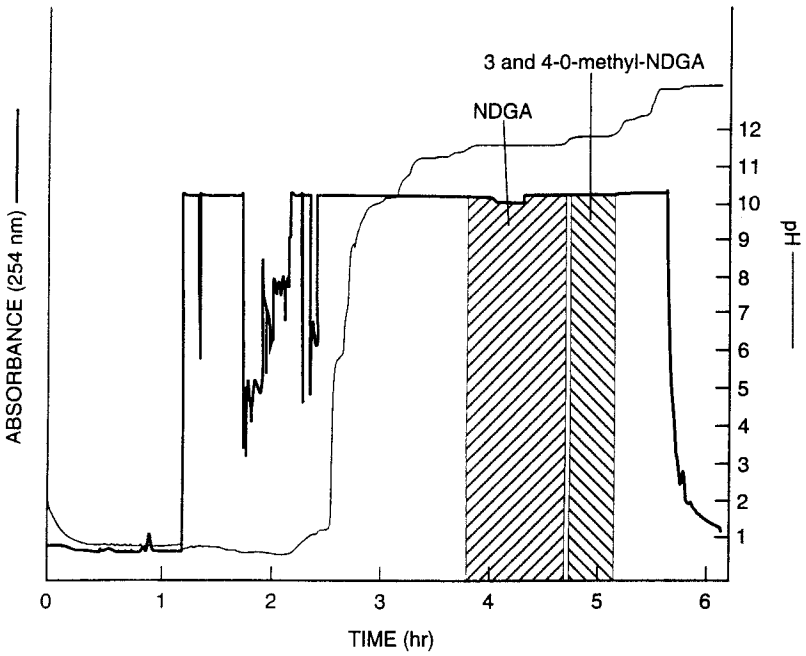


Figure 3. pH-Zone-refining CCC of crude extract of *Larrea tridentata* using a commercial high-speed CCC centrifuge equipped with a preparative column. Experimental conditions are: apparatus: triplet multilayer coil planet centrifuge with 10 cm revolution radius; column: serially connected three multilayer coils of 2.6 mm ID and total capacity of 800 ml; sample: 20g of crude extract of *L. tridentata*; Solvent system: methyl *t*-butyl ether/water, upper organic mobile phase contained 25 mM TFA and the lower aqueous mobile phase, 100 mM NaOH; flow rate: 5ml/min; revolution: 1000rpm.

respectively, each associated with a distinct pH plateau. This preparative high-speed CCC centrifuge provides a greater centrifugal force (1000 rpm) than the semipreparative unit (800 rpm), thus allowing more stable retention of the stationary phase even at a higher flow rate of 5 ml/min.

Separation of Anti-HIV Lignans from Synthetic Mixture

Similar lignans with various degrees of methylation were synthesized by methylating NDGA. The crude reaction mixture was subjected to pH-zone-refining CCC following the same procedure applied to the crude plant extract.

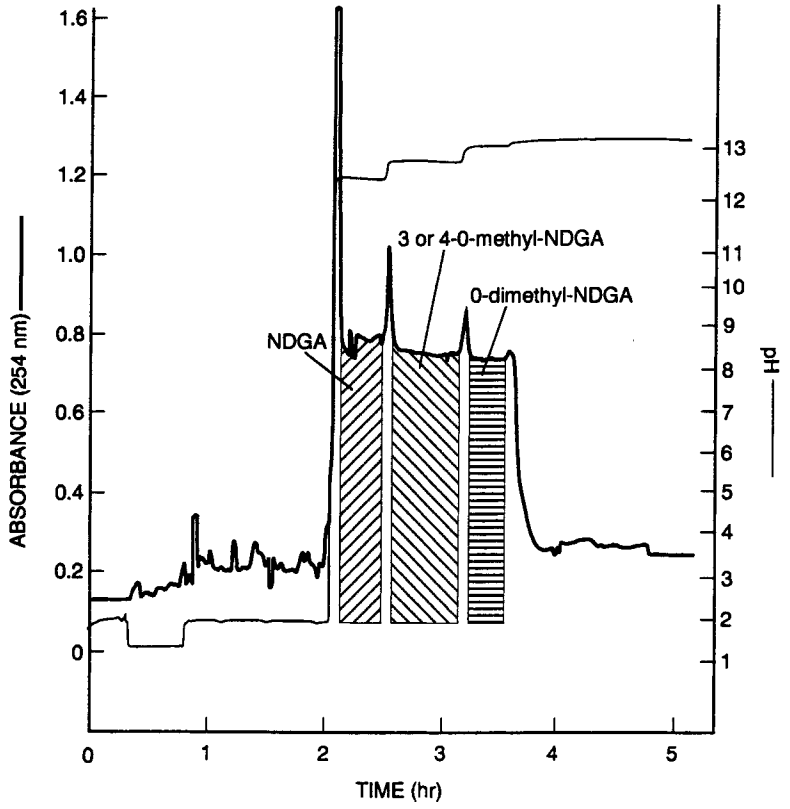


Figure 4. pH-Zone-refining CCC of synthetic lignans using the high-speed CCC centrifuge equipped with a semipreparative column. Experimental conditions: sample: 3 g of crude methylation mixture of NDGA; Solvent system: methyl *t*-butyl ether/water, upper organic mobile phase contained 25 mM TFA and the lower aqueous mobile phase, 100 mM NaOH; flow rate: 3 ml/min; revolution: 800 rpm.

Figure 4 shows a chromatogram of a crude reaction mixture derived from 3 g of NDGA obtained by the semipreparative high-speed CCC apparatus. All components were eluted as a train of highly concentrated rectangular peaks with three distinct pH plateaus at retention times of 2.2-2.5, 2.6-3.2 and 3.3-3.6 hr, while impurities were concentrated at the boundaries of the major peaks. TLC analysis of fractions corresponding to each pH zone revealed that these lignans were resolved and eluted in an increasing order of methylation, i.e. NDGA, 3 or 4-O-methyl NDGA and O-dimethyl NDGA.

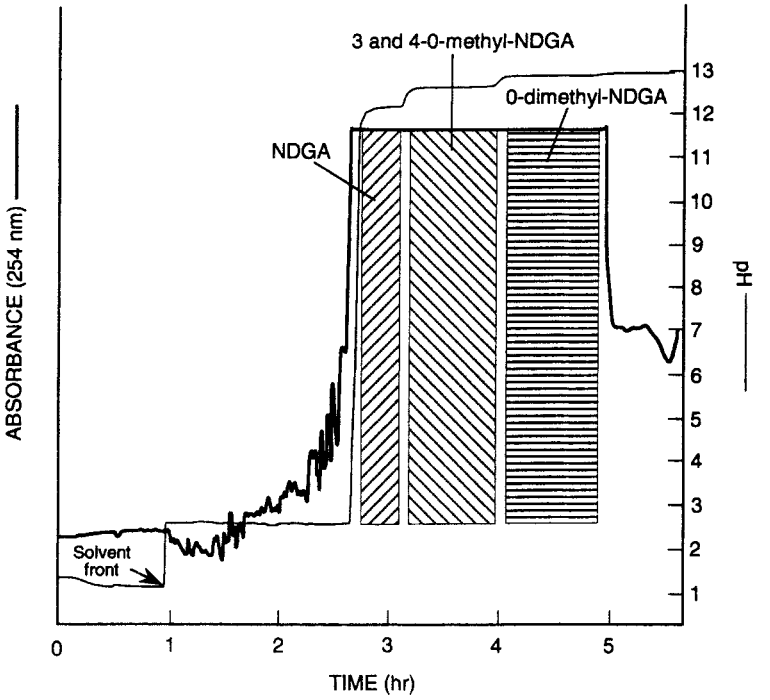


Figure 5. pH-Zone-refining CCC of synthetic lignans using the high-speed CCC centrifuge equipped with a preparative column. Experimental conditions: sample: 10.5 g of crude methylation mixture of NDGA; Solvent system: methyl *t*-butyl ether/water, upper organic mobile phase contained 25 mM TFA and the lower aqueous mobile phase, 100 mM NaOH; flow rate: 5 ml/min; revolution: 800 rpm.

Figure 5 shows a similar separation of 10.5 g of a synthetic mixture by the preparative high-speed CCC unit. The lignans were resolved in three major components with distinct pH plateaus. However, in these chromatograms there is no evidence of the separation among their isomers.

SUMMARY

A pH-zone-refining CCC system has been developed for preparative-scale isolation of methylated lignans present in creosote bush, *Larrea tridentata*. The method uses sodium hydroxide in the mobile phase to elute the lignans according to the order of their pK_a and hydrophobicity. This method has several advantages over the conventional high-speed CCC technique:

1. The sample loading capacity is increased over 10-fold for a given column.
2. The fractions are highly concentrated to a near saturation level.
3. The method yields a higher degree of purity of fractions.
4. Separation times are considerably shorter and more selective for a given compound.

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