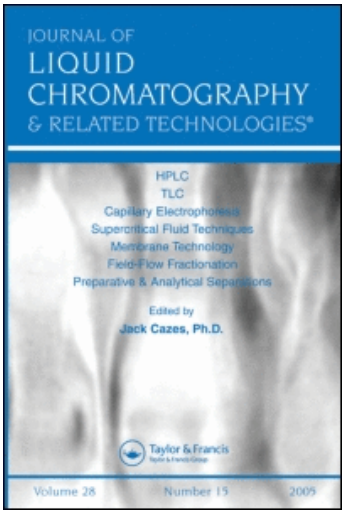

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PREPARATIVE SEPARATION OF CURCUMINOIDS FROM CRUDE CURCUMIN AND TURMERIC POWDER BY pH-ZONE-REFINING COUNTERCURRENT CHROMATOGRAPHY

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PREPARATIVE SEPARATION OF CURCUMINOIDS FROM CRUDE CURCUMIN AND TURMERIC POWDER BY pH-ZONE-REFINING COUNTERCURRENT CHROMATOGRAPHY

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ABSTRACT

Turmeric powder and its main component, curcumin, have a wide range of medicinal and culinary uses. The purpose of this study was to separate and purify curcumin from demethoxycurcumin and bis-demethoxycurcumin which are present in both turmeric powder, and commercial crude curcumin.

The separation and purification of curcumin was accomplished through standard high-speed countercurrent chromatography (CCC) as well as pH-zone-refining CCC. The pH-zone-refining CCC technique was able to separate multi-gram quantities of curcumin and other curcuminoids from crude curcumin and turmeric powder, while maintaining a high level of purity.

INTRODUCTION

Curcumin [CAS 458-37-7, 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is the major active principle in the rhizome of turmeric plant (*Curcuma longa*). Turmeric powder (dried rhizome of *Curcuma longa*) has been used in India from time immemorial for several medicinal purposes. It has been found useful in the treatment of inflammation,[1,2] as an anti-hepatotoxic agent,[3-5] and anticancer agent by inhibiting the activation of cancer-causing chemicals.[6-10] Free radicals which are, to some extent, responsible for many diseases including cancer are scavenged by curcumin.[11] Curcumin has been proven to be the main anti-oxidant in turmeric.[12] In addition, turmeric powder is extensively used in the preparation of various types of food and is an essential ingredient in curry powder.

Even though a number of medicinal properties have been attributed to turmeric by its content of curcumin, it is not clear whether all the biological activities reported for turmeric are due to curcumin or other curcuminoids present in turmeric. Rhizome of *Curcuma longa* contains three major curcuminoids: curcumin, demethoxycurcumin, and bis-demethoxycurcumin.

The main purpose of this study has been to separate these three curcuminoids present in crude curcumin samples as well as in turmeric powder, both of which are commercially available. Previous methods have used preparative thin layer chromatography for the separation of these curcuminoids, but have not been efficient in separating more than 10-20 milligram quantities.[13] In the present method, we have used high-speed countercurrent chromatography preparation with or without pH-zoning in order to separate all three curcuminoids from 2 g of curcumin or 20 g of turmeric powder. These have been characterized by MALDI-MS, NMR, and thin layer chromatography.

EXPERIMENTAL

Apparatus

The apparatus used in the present study is a multilayer coil planet centrifuge for performing high-speed CCC (P.C. Inc., Potomac, MD, USA). It contains a column holder and a counterweight in symmetrical positions at a distance of 10 cm from the central axis of the centrifuge. The detailed description of the apparatus was reported earlier.[14,15] The separation column was prepared in our laboratory from a single piece of Tefzel tubing (Zeus Industrial Products, Raritan, NJ, USA) of 1.6 mm ID and 130 m in length by winding it directly onto the column holder hub making multiple coiled layers between a pair of flanges spaced 2 inches apart. The total capacity of the column is about 320 mL. A pair of flow tubes of the column is first led through the hollow column holder shaft downward, and then makes an arc to enter the side hole of the central pipe,

finally exiting the centrifuge at the top where they are tightly supported by a pair of clamps.

The revolution speed of the apparatus is regulated with a speed controller (Bodine Electric Co., Chicago, IL, USA) at 800 rpm.

Reagents

Hexane, ethyl acetate, methyl-*tert*-butyl ether, and water, all of chromatographic grade, were purchased from Fisher Scientific Co., Fair Lawn, New Jersey, USA; ethanol from Warner-Graham Company, Cockeysville, MD, USA; acetonitrile from Burdick & Jackson in Muskegon, Michigan, USA; trifluoroacetic acid purchased from Pierce Company, Rockford, Illinois, USA; and sodium hydroxide from Mallinckrodt Company, Paris, Kentucky, USA. Curcumin and turmeric samples were acquired, respectively, from Aldrich Chemical Company, Inc., Milwaukee, WI, USA and Keshav Exports PVT, Ltd., Bangalore, India.

Preparation of Solvent Systems and Sample

For the standard CCC, one liter of solvent system composed of hexane, ethyl acetate, ethanol, and water (1:1:1:1) was equilibrated in a separatory funnel and the two phases were subsequently separated. For pH-zone-refining CCC[16,17] methyl-*tert*-butyl ether, acetonitrile, and water (4:1:5) was prepared and the two phases were separated. Then, sodium hydroxide was added to the mobile lower phase to obtain a final concentration of 30 mM which served as an eluter, while trifluoroacetic acid was added to the stationary upper phase to obtain a final concentration at 20 mM which served as a retainer.

The sample solution of curcumin was prepared by dissolving 5 mg to 2 g of crude curcumin in 5 to 50 mL of the solvent consisting of about equal volumes of each phase. Turmeric powder 100 mg to 20 g was extracted with 10 to 50 mL of the upper organic phase and the particulates were eliminated by centrifugation (2000 g x 15 min).

CCC Separation Method

The upper stationary phase was loaded into the multilayer coil followed by sample injection through the sample port. Then, the lower mobile phase was introduced to the column at a flow rate of 3 mL/min while the column was rotated at 800 rpm. The effluent was continuously monitored at 280 nm and collected into test tubes at 1 to 2 min intervals.

Analysis of CCC Fractions

In pH-zone-refining CCC, the pH of each fraction was measured using a portable pH meter (Accumet AP61, Fisher Scientific Co., Fair Lawn, NJ, USA). After acidifying with HCl, the curcuminoid fractions were then extracted with 2 mL of ethyl acetate.

The peak fractions were analyzed by MALDI-MS, NMR and TLC. Thin layer chromatography was performed using silica gel plates (DC-Alufolien, Kieselgel, $60F_{254}$, E. Merck, D-6100 Darmstadt, Germany) with a solvent system consisting of hexane, ethyl acetate, and ethyl alcohol (8:4:1) with 0.5% trifluoroacetic acid.

RESULTS AND DISCUSSION

High-speed countercurrent CCC[14,15] is the most advanced form of CCC which eliminates the use of a solid support. Recently, an efficient preparative

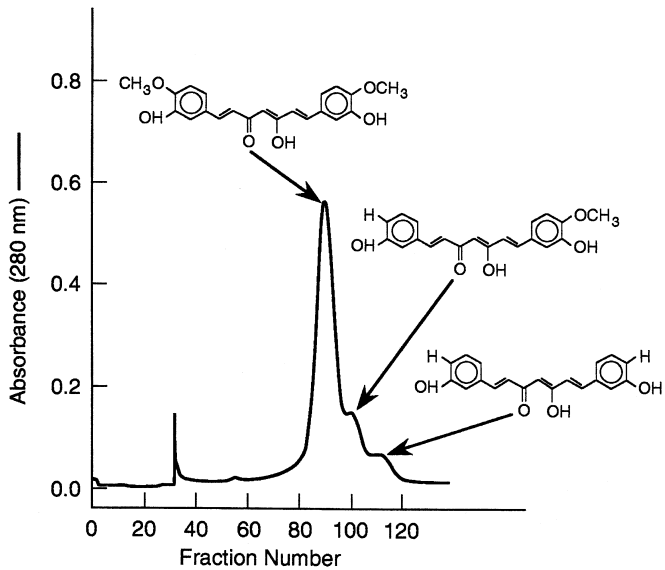

Figure 1. Separation of curcumin by the standard HSCCC technique. Experimental conditions: Apparatus: multilayer CPC with 10 cm revolution radius; column: multilayer coil, 1.6 mm ID, 160 m long with 320 mL capacity; Sample: curcumin 5 mg; Solvent system: hexane, ethyl acetate, ethanol, water (1:1:1:1); Mobile phase: aqueous phase; Flow-rate: 3 mL/min; Revolution: 800 rpm; Retention: 64%; Pressure: 30-80 psi.

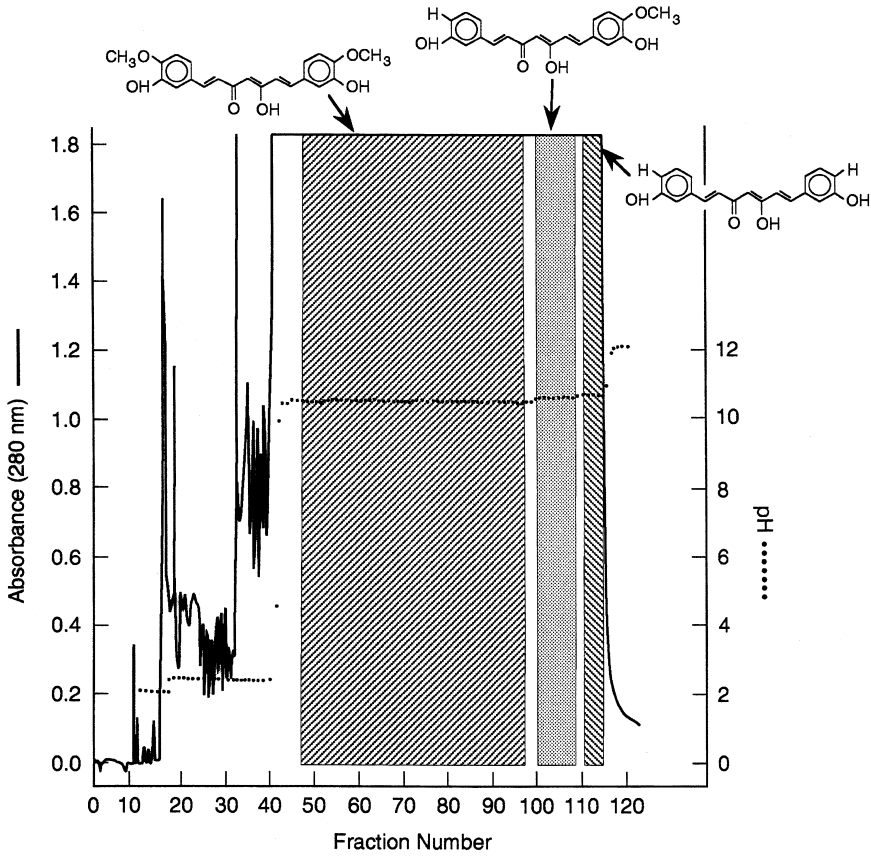

Figure 2. Separation of crude curcumin by pH-zone-refining CCC technique. Chromatogram: Sample: crude curcumin 2 g; Solvent system: methyl-*tert*-butyl ether, acetonitrile, water (4:1:5); Stationary phase: upper organic phase containing 20 mM TFA; Mobile phase: lower aqueous phase containing 30mM NaOH; Flow-rate: 3 mL/min; Revolution: 800 rpm; Retention: 53%; Pressure: 30-120 psi. Other conditions are described in the Figure 1 legend.

separation method called pH-zone-refining CCC has been developed from high-speed CCC. It produces a characteristic rectangular peak which contains highly concentrated fractions of ionized compounds with minimum overlap.[15,16] In the present study, both standard high-speed CCC and pH-zone-refining CCC were used to separate curcuminoids from commercial crude curcumin and turmeric powder.

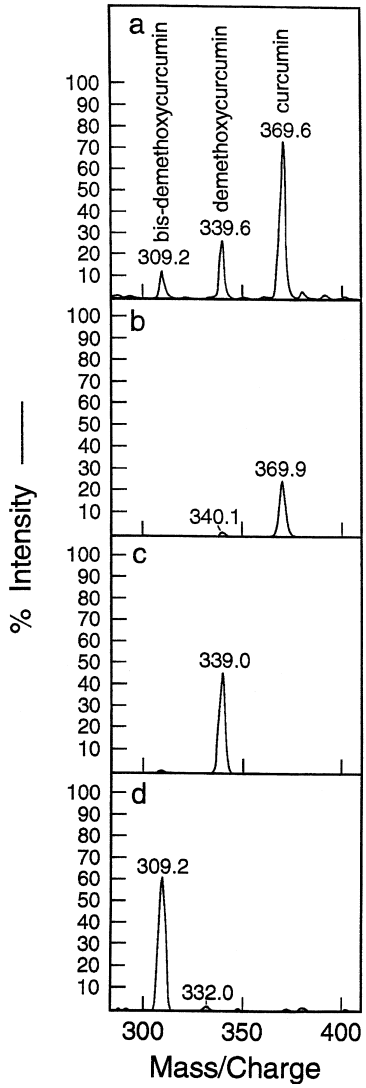

Figure 3. MALDI-mass spectrometric analysis of crude curcumin sample (a) and CCC fractions from each pH plateau (b, c, and d). Curcumin: MW 368, $(M + H)^+$ 369; Demethoxycurcumin: MW 338, $(M + H)^+$ 339; Bis-demethoxycurcumin: MW 308, $(M + H)^+$ 309.

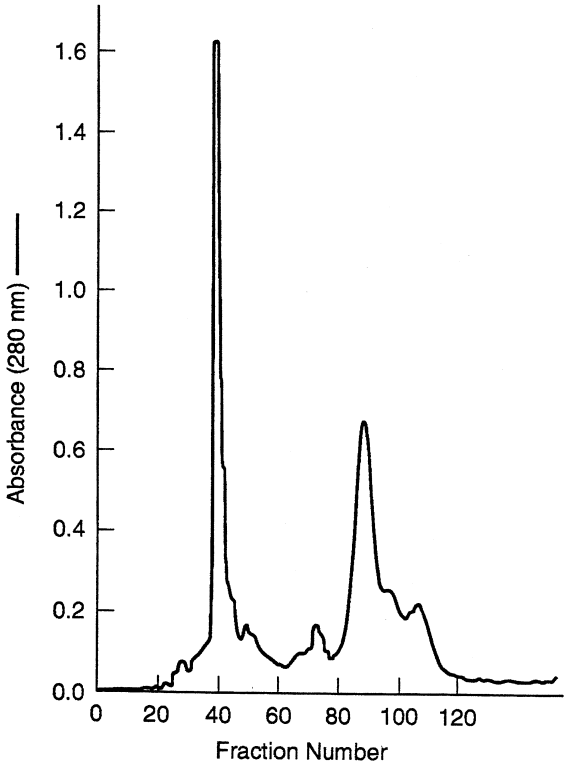

Figure 4. Separation of turmeric extract by the standard HSCCC technique. Sample: turmeric 100 mg; Solvent system: hexane, ethyl acetate, ethanol, water (1:1:1:1); Mobile phase: aqueous phase; Flow-rate: 3 mL/min; Revolution: 800 rpm; Retention: 58%; Pressure: 30-90 psi. Other conditions are described in the Figure 1 legend.

Subjecting 5 mg of crude curcumin to standard CCC produced only a partial separation of the sample into its components: curcumin, demethoxycurcumin, and bis-demethoxycurcumin (Figure 1). However, when 2 g of crude curcumin were subjected to pH zone-refining CCC, the separation was greatly improved giving three plateaus representing three different compounds each with distinct pH value measuring 10.5, 10.6, and 10.7 (Figure 2). The fractions from this pH-zoning trial were then acidified with HCl and extracted with 2 mL of ethyl acetate, and analyzed by MALDI-MS (Figure 3), NMR, and TLC; these three forms of analysis gave the following results. The middle fraction from the pH plateau of 10.5 (fraction 73) contained mostly pure curcumin but had a trace of demethoxycurcumin; the fraction from the middle of pH plateau 10.6 (frac-

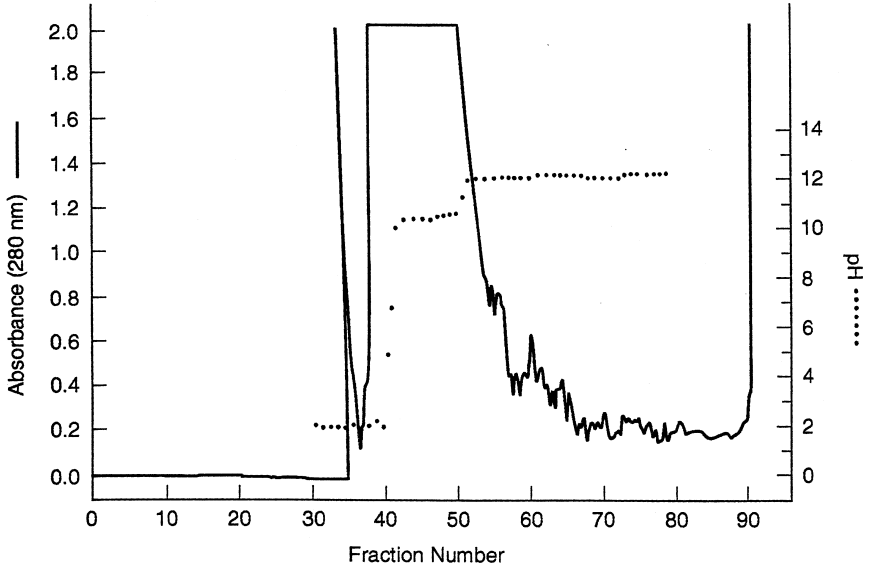

Figure 5. Separation of turmeric extract by pH-zone-refining CCC technique. Sample: turmeric 20 g; Solvent system: methyl-*tert*-butyl ether, acetonitrile, water (4:1:5); Stationary phase: upper organic phase containing 20mM TFA; Mobile phase: lower aqueous phase containing 30mM NaOH; Flow-rate: 3 mL/min; Revolution: 800 rpm; Retention: 12%; Pressure: 30-70 psi. Other conditions are described in the Figure 1 legend.

tion 107) consisted of solely demethoxycurcumin; and the fraction from the middle of pH plateau of 10.7 (fraction 113) contained only bis-demethoxycurcumin. The yield of curcumin, demethoxycurcumin and bis-demethoxycurcumin in these plateaus was 1.85 g, 258 mg, and 50 mg, respectively.

Results from the standard CCC as well as the pH-zone-refining CCC of turmeric extract were quite similar to those of crude curcumin. As with crude curcumin, the standard CCC of the turmeric extract produced similar separations of curcuminoids (Figure 4). The pH-zone-refining CCC separation of turmeric extract also gave three pH plateaus (Figure 5), although the second and the third plateaus are much shorter due to a much smaller sample size. It is important to note, that despite the crude nature of turmeric, the results from the separation of turmeric extract were quite similar to those of crude curcumin.

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