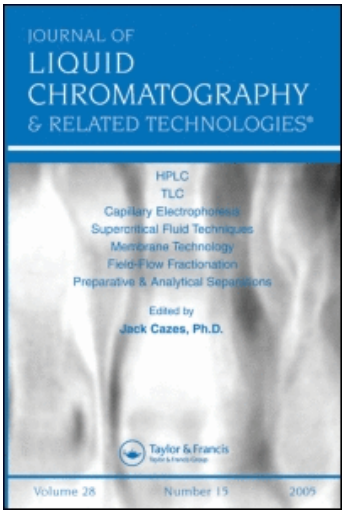

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MULTIDIMENSIONAL MULTIMODAL INSTRUMENTAL SEPARATION OF COMPLEX MIXTURES

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ABSTRACT

Presented here is a simple approach to the application of HPLC/CE for the separation of complex mixtures. A two-dimensional HPLC/CE instrumental setup was assembled from commercially available equipment. Fractions of the effluent from the HPLC system are collected into microtiter plates with the aid of a microfraction collector which allows the collection of samples by time, drops, or external signal (peaks). The fractions are then dried under vacuum at room temperature in a special unit, reconstituted, and analyzed by CZE. Any size or type of HPLC or CE column can be used with no limitation on the amount of sample injected into the HPLC. Any CE detection procedure, such as LIF, MS, UV, or other, can be used. This setup is practical, simple, robust, and allows the separation of complex mixtures. Preliminary results show the utility of this system for the analysis of a mixture of two protein digests, cytochrome C and myoglobin.

INTRODUCTION

Multidimensional multimodal separation methods have been used for the separation of mixtures that were hard to resolve by a single mode. Multimodal means that two or more different modes of separation which produce different selectivities: for example, ion exchange, size exclusion, adsorption, or partitioning for high performance liquid chromatography (HPLC) and thin layer chromatography (TLC); vapor pressure or molecule geometry for gas chromatography (GC); and size or charge for electrophoresis. Multidimensional, on the other hand, means the use of two different techniques such as HPLC and CZE, or HPLC and GC. A multimodal method can be performed using the same instrument (GC, HPLC or CE) or the same plate (TLC or slab gel electrophoresis).

The use of two different modes employing the same method has been reported. For example, multimodal thin layer chromatography, employing normal phase development in the first dimension and reversed phase (RP) development in the second dimension, is an excellent method for the separation of complex mixtures; it is simple, economical, and easy to use.[1-3] Multimodal slab gel electrophoresis has been used for the separation of complex protein mixtures based on two sequential steps: first by their charge and then by their mass.[4]

Gas chromatography has also been used in a multimodal format to resolve a mixture of polychlorinated biphenyl congeners, employing a heart cutting technique whereby a peak of interest eluting from the first column, DB-1, which resolves the mixture based on vapor pressure, is introduced into a second column of different chemistry, smectic liquid crystal, which resolves the mixture based on geometric shape, and produces different selectivities.[5] High performance liquid chromatography was used in our lab in a multimodal format to resolve a caffeine metabolite mixture. Two different chemistry columns were used: a reversed phase column for the first dimension and a Bio-Gel SEC20-XL column in the second dimension.[6]

Also, the combination of two on-line separation techniques, HPLC and CE, which resolve mixtures by two and three different modes of separation, has been reported.[7,8] Moore and Jorgenson used RP-HPLC/CZE[7] and size exclusion HPLC/RP-HPLC/CZE[8] for the separation of peptide mixtures. The main advantage of a multidimensional, multimodal method is the high peak capacity which results when two orthogonal methods are used on-line or off-line. Giddings[9] discussed the advantages of using two orthogonal techniques, i.e., methods that employ different separation mechanisms which result in different selectivities, and has shown that the peak capacities of the multidimensional method is the product of the peak capacities of its component one-dimensional method.

We present here a simple two-dimensional multimodal HPLC/CE method for the separation of complex mixtures of any kind. Our objective is to assemble a system from commercially available equipment and instrumentation, which is rugged and easy to use.

EXPERIMENTAL

Materials

Cytochrome C, myoglobin, and trypsin were purchased from Sigma (St. Louis, MO), acetonitrile and TFA from Fisher Scientific (Pittsburgh, PA), and ThinLids from Tomtec (Hamden, CT).

Instrumentation

HPLC: Hewlett Packard Model 1090 HPLC System (Rockville, MD). CE: Beckman (Fullerton, CA) P/ACE System MDQ capillary electrophoresis equipped with an autosampler that accepts two 96- or 48-well microtiter plates and a UV detector. Fraction collector: microfraction collector, model Foxy Jr from Isco (Lincoln, NE), which collects samples by time, drops, or external signal. Concentrator: Universal SpeedVac System by Savant (Bethesda, MD), equipped with model MPTR8-210 rotor which accommodates 2-12 microtiter plates, depending on the size of the plates.

Procedure

Protein Digestion

Cytochrome C and myoglobin were dissolved in 50 mM Tris-Cl (pH 8.5) as 10 mg/mL solutions. Trypsin was made up in water as 10 mg/mL solution. 200 μL of protein solution was mixed with 10 μL of trypsin solution and the digestion was carried out at 37°C for 18 hours. The digestion mixture was diluted to 4 mg/mL and 1 mg/mL of total protein digest with water before HPLC and CZE analyses, respectively.

HPLC Conditions

HPLC was performed with a 250 mm x 4.6 mm, 5 μm silica particle Vydac ODS C_{18} column from Resolution Systems (Holland, MI). The injection volume was 100 μL and the flow rate was 1 mL/min. Buffer A was 0.1% TFA in water

and buffer B was 0.1% TFA in acetonitrile. The tryptic peptides were eluted from the column with a 50 minute gradient from 5-50% B and were detected by UV absorption at 214 nm.

CZE Conditions

CZE was performed with a 31 cm x 50 μm fused-silica capillary. The separation buffer was 50 mM phosphoric acid titrated to pH 2.1 with sodium hydroxide. The capillary was flushed with the separation buffer for one minute between runs. 1-4-dimethyl amino pyridine (0.1 mg/mL) was used as a marker to account for the migration change (if any) between runs. The marker and protein digests or HPLC fractions were sequentially injected by vacuum at 5s x 0.1 psi and 20s x 0.5 psi, respectively. The peptides were detected by UV absorption at 214 nm.

Method

The procedure used in this study is as follows: A sample solution is injected into the HPLC, and column effluent is collected with the aid of the fraction collector into 2 mL volume wells microtiter plates every minute (i.e., 1 mL/well). The microtiter plates are placed in the SpeedVac and dried at room temperature under vacuum; after drying, 70 μL of water is added, the plates are covered, shaken, removed from the SpeedVac, and placed on the autosampler of the MDQ CE system and analyzed under the experimental conditions described above. In order to prevent sample evaporation, a drop of paraffin oil is placed in the microtiter plate wells, or they can be covered by the ThinLid which provides a means of sealing the microtiter plate, prevents evaporation of the solvent, and allows the capillary and electrode to penetrate through the seal by way of a small cross hair slit located over each of the 96-wells. In the future we plan to use a computer program to collate the data from all the fractions to produce a final electropherogram.

RESULTS AND DISCUSSION

The setup presented here meets our objectives of assembling a working system from commercially available equipment and instrumentation which is robust and easy to use. The fractions eluting from the HPLC column, Figure 1, are collected into 96-well microtiter plates with the aid of a micro sample collector which accepts two microtiter plates, Figure 2. If needed, the samples are dried in the Speed Vac, which is equipped with a rotor that accepts four microtiter plates, Figure 3. The CE system used in this study, the Beckman MDQ capillary electrophoresis instrument, Figure 4, can accommodate two 96-well microtiter plates**,** which means that up to 192 samples can be loaded and analyzed overnight**.**

Figure 1. An HPLC instrument, HP-1090 by Hewlett Packard.

The idea of using microtiter plates appeals to us because we are interested in developing a multimodal system which is amenable to high throughput analysis. The introduction of array capillary electrophoresis**,** up to 96 capillaries, by few manufacturers in the last year, makes this setup amenable to automation and high throughput, since all the commercially available array capillary electrophoresis instruments use the microtiter plate as a platform for sample loading and introduction. Unfortunately, at present all these array electrophoresis instruments are made to sequence DNA; they are not equipped to be high throughput instruments for the simultaneous multi-sample analysis.

We are hopeful that in the future the instrument companies will manufacture a multi-purpose array electrophoresis instrumentation. Then the separation scientist will be able to collect as many fractions as needed and analyze 96 of them simultaneously in a few minutes.

The HPLC/CE system in this study was evaluated using a mixture of two protein digests, cytochrome C, and myoglobin, which produced a complex peptide mixture. When analyzed by RP-HPLC and by CE, 40 and 36 peaks were observed simultaneously. However, when the fractions from the HPLC column were analyzed separately by CE, a total of 109 peaks was observed.

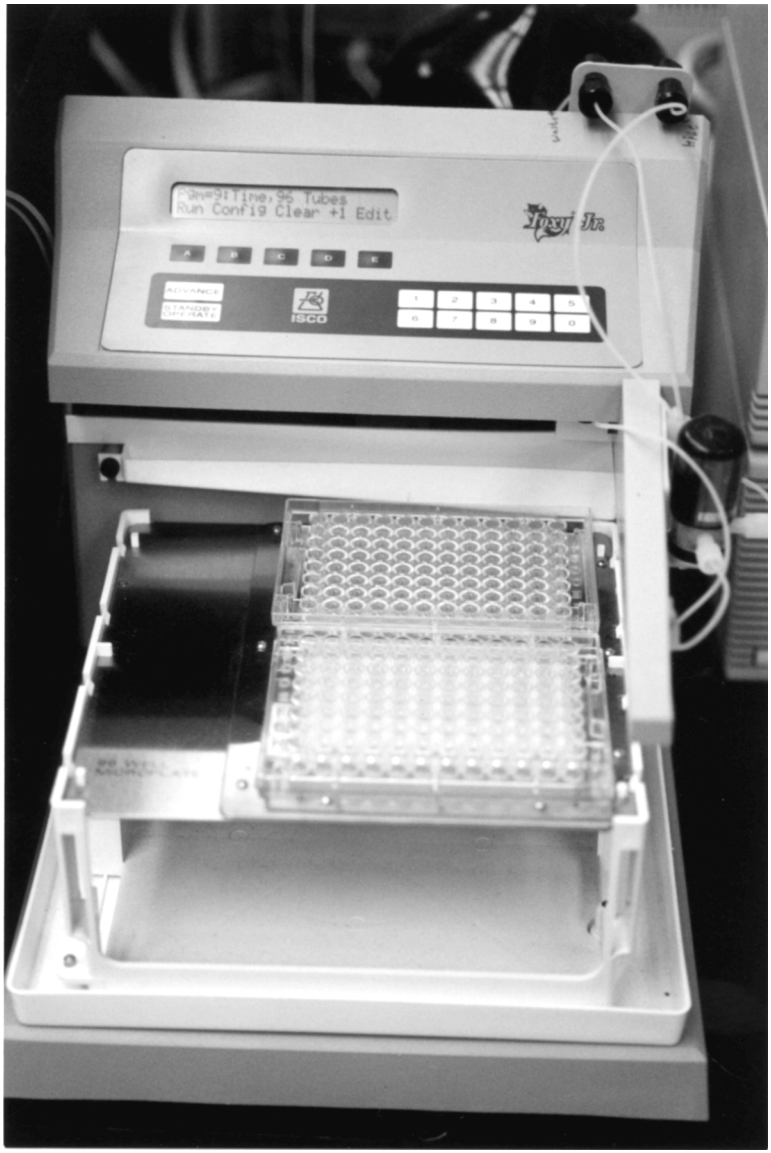

Figure 2. Microsample collector, Foxy, Jr., by ISCO.

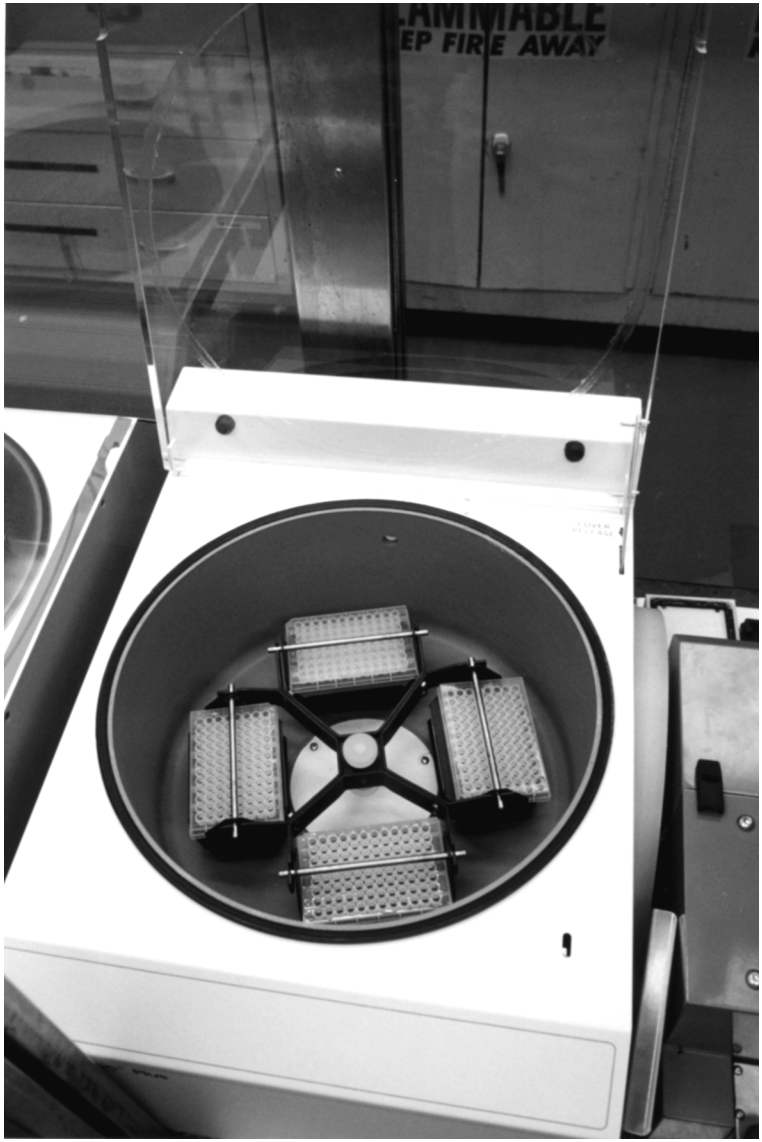

Figure 3. SpeedVac sample concentrator by Savant.

Figure 4. MDQ capillary electrophoresis by Beckman.

Construction of an electropherogram from the individual fractions' electropherograms produced an electropherogram similar to that obtained when the mixture digest was analyzed by CE, Figure 5. These results reveal that in both methods certain peptides coeluted. Also, the advantages and resolving power of using two multidimensional multimodal systems, which resolve mixtures based on different mechanisms of separation, in this case by hydrophobicity (HPLC) and electrophoretic mobility or charge density (CE), are obvious.

When a multidimensional system is used to separate a complex mixture, the first dimension possesses less resolving power than the second dimension. This is true in the present study and in earlier studies[7,8] because CE has a higher efficiency, plates/meter, than HPLC.

An on-line multidimensional HPLC/CZE system[7,8] is faster than the off-line system presented here. However, an on-line system has requirements that are not required by an off-line system, which may limit the on-line system's usefulness. The main requirement of an on-line 2D-HPLC/CE system is that the second dimension (CE) must be much faster than the first dimension (HPLC), to accommodate an on-line sampling, where part of the HPLC effluent is introduced into the capillary.[7,8]

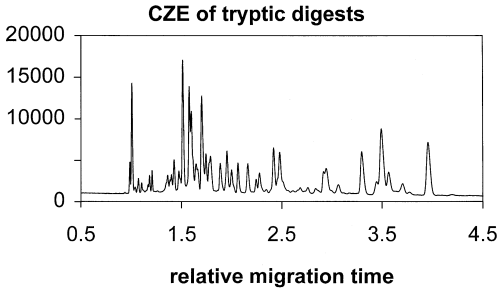

Figure 5. A reconstructed electropherogram of the sum of CZE peaks of HPLC collected fractions. Experimental conditions as in text.

The injection and detection require the use of a laser and the derivatization of the peptides with a fluorescent tag. This system[7] produced promising results, but it is not commercially available and requires special parts and special computer programs for data acquisition and presentation.

The 2D off-line system described here is made of commercially available parts and eliminates the above limitations. It is versatile, easy to use and has unlimited possibilities. Any HPLC column chemistry and size can be used. Any sample mixture type, size, and concentration can be analyzed. The CE system accepts the microtiter plate as sample holder. Any column type (coated, uncoated or packed) and dimension (up to 80 cm long, and 75 um i.d.) can be used. Although the setup is an off-line system, in the future it will be easy to automate with the aid of robotics. The use of array capillary electrophoresis, as mentioned above, will give the system the high throughput capabilities for the fast analysis of a large number of samples.

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