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Review

Future potential of targeted component analysis by multidimensional liquid chromatography–mass spectrometry

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Abstract

Multidimensional liquid chromatography (MDLC) may be used in either (i) the profiling mode where it is the objective to fractionate all components in a mixture or (ii) the targeted component mode in which it is the objective to determine specific analytes. This paper focuses on targeted component analysis from complex mixtures, addressing the critical operations of analyte selection and transport from the first to the second dimension. Although the physical operation of switching a component into the second dimension with computer controlled valving is simple, it is shown that changes in analyte retention time and peak width with column age and fouling are a serious problem. The analyte moves out of the preselected time window for valve switching and quantitation is compromised in the second dimension. It is proposed that a solution to the "drifting peak" phenomenon in targeted component analysis is to use binary mobility elution in the first dimension. Binary mobility refers to those systems, such as affinity chromatography, in which analyte mobility is generally either 0 or 1 relative to mobile phase velocity. Coupling these binary changes in analyte mobility in the first dimension with valve switching eliminates the "drifting peak" phenomenon. In addition, it is shown that a wide time window may be used in affinity separations without compromising the separation or accumulating contaminants. Several cases are described in which immunosorbents were used with reversed phase columns to provide quantitative targeted component analyses from complex mixtures.

Keywords: Reviews; Multidimensional liquid chromatography; Mass spectrometry

Contents

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1. Introduction

Determining individual analytes in complex biological samples is a problem that frequently requires some type of separation as a prerequisite to the analytical measurement. The resolution phase of such analyses can be more difficult than the actual measurement of analytes. When samples contain several thousand components, even the highest resolution separation system may be incapable of resolving all the components. One approach to this problem is to use highly selective separation media, such as immunosorbents, that target structural properties of the analyte [1]. This approach is satisfactory when only one molecular species in the sample has the unique epitope against which the immunosorbent is targeted. When multiple species carry the same epitope, immunosorbents fail to be species specific. In this case, a small family of structural variants with the same epitope are captured [2]. This is the case in the analysis of protein biosynthesized by recombinant DNA technology. Expression errors, variations in post-translational modification, in-vivo proteolysis, in-vitro proteolysis, improper disulfide bridge formation and various forms of chemical degradation during isolation all contribute to the formation of a family of immunologically cross-reacting variants [3].

Since the late 1950s, life scientists have dealt with the problem of resolving single components from complex mixtures through multidimensional separations. The working concept being, that a variety of methods, each selected for a different property of the molecule being purified, would give the greatest differentiation. This strategy is still widely applied in protein purification. Ammonium sulfate and/or organic solvent precipitation, size exclusion chromatography (SEC), ion exchange chromatography (IEC), hydrophobic interaction chromatography (HIC), reversed phase chromatography (RPC), immobilized metal affinity chromatography (IMAC), immunoaffinity chromatography, isoelectric focusing and slab gel electrophoresis are among the most popular separation methods selected today to purify biological substances, particularly proteins. Virtually all known proteins have been purified and are analyzed by some combination of these methods. Multidimensional purification strategies have played a major role in the evolution of modern biochemistry.

Analyses by Giddings [4] and others [5–7] of the problem of resolving complex mixtures show that the power of multidimensional systems may be explained in several ways. One is, that multiple dimensions give more space within which to do the separation, i.e. the peak capacity of the system is the product of the peak capacities of the individual separation dimensions. Another is, that multidimensional systems apply so many more theoretical plates to the separation. The number of theoretical plates in a multidimensional chromatographic system is also equal to the product of plate counts in the individual dimensions. Despite their power, a recent report by Giddings points out that multidimensional systems are not always superior [8]. The nature of the sample, or "sample dimensionality" as he calls it, is also very important. Although a molecular species has many different properties that may be selected as a basis for fractionation, selecting molecular features that are the most different from other sample components will give the greatest resolution. Unfortunately, the dimensionality of complex samples is seldom known. The only way to determine which combination of separation methods will give maximum resolution is by trial-and-error.

Multidimensional separation systems may be used in either the profiling [9–11] or target mode [12,13]. As noted above, the objective in the profiling mode is to fractionate all components in the mixture. Two dimensional thin-layer chromatography, 2-D electrophoresis [9] and more recently capillary electrophoresis-reversed phase chromatography [10] or ion exchange-reversed phase chromatography [11] are examples. Every component from the first dimension is fractionated and reported in the second dimension. In contrast, the objective in the targeted mode of multidimensional separation is to isolate a single or small group of components. Enzyme purification from a biological extract is an example of a targeted separation.

Multidimensional separations are generally referred to as being complex, slow and difficult to automate, unless GC–MS and LC–MS are included. For this reason, they are not widely applied in routine analytical determinations. It is unfortunate that such a powerful technique is not customarily

used in analytical chemistry. The rest of this paper will deal with possible reasons in the case of liquid chromatography–liquid chromatography (LC–LC) systems and begin to address the problems associated with targeted component analysis by column liquid chromatography.

2. The problems associated with LC–LC

A critical operation in column LC–LC is the selection and transport of eluent fractions from the first to the second dimension. This process has been both lengthy and labor intensive in classical systems, such as those used in protein purification. Less manual, more rapid methods are needed if LC–LC is to become a routine analytical tool. It has been shown that computer controlled valving, which switches column effluent directly into the second dimension can provide a solution to the sample transport problem. This approach eliminates the intermediate steps of fraction collection and manual transfer between dimensions as practiced in classical column LC–LC systems.

In the profiling mode, it is generally the case that the first column is operated at either very low flow-rate [11] or the flow is interrupted intermittently [14], while the second column is operated at very high velocity to analyze column fractions as they elute from the first column. Eluent is directly transferred between columns at regular time intervals without fraction collection. Analysis time (t_a) in such a profiling system is given by the expression $t_a = Pnt_c$; where P is the peak capacity of the column in the first dimension, n is the number of fractions taken from each peak in the first dimension and t_c is the cycle time of the column in the second dimension. Assuming that P, n, and t_c are 150, 2 and 1 min, respectively, the analysis time would be 5 h. Obviously, the rate limiting step in profiling is analysis time in the second dimension. Even when t_c is one min the total analysis time (t_a) is still too long.

Targeted component analysis has a very different problem, i.e. selection of the component to be transferred into the second dimension. Selection is generally achieved by time based valve switching, assuming that analyte retention time and peak width are constant. This assumption may not be valid in many high throughput analytical systems. As columns age, retention time will drift with changes in the phase ratio or fouling and peak width will increase from column degradation. This means that the analyte will move either partially or totally out of the preselected time window for valve switching and quantitation will be compromised in the second dimension. The problem in targeted component analysis is how to deal with this "drifting peak" phenomenon.

3. Binary mobility, high selectivity systems for targeted component analysis

A major difference between bioaffinity chromatography and other forms of liquid chromatography is that analyte mobility is generally binary, i.e. the substance is either adsorbed or unbound. This means that relative to mobile phase velocity, analyte mobility is either 0 or 1. The binary mobility of analytes in affinity chromatography systems where a protein is either (i) the stationary phase, (ii) the analyte, (iii) or both, is due to the fact that elution is achieved in a single step to a mobile phase that at least partially denatures proteins. This causes a rapid decrease in the binding constant between the receptor–ligand complex with concomitant dissociation. Elution of analytes in this way makes retention time insensitive to properties of the chromatography system, such as stationary phase density, phase ratio, chemical properties of the support and column age. Furthermore, the binary nature of bioaffinity chromatography enormously reduces the significance of column efficiency. The major impact of column efficiency is on sensitivity, i.e. a very narrow peak is easier to detect at low concentration than a broad peak.

These unique features of bioaffinity chromatography, and in this context any other form of binary mobility chromatography, are ideally suited to targeted component analysis. The fact that retention time is highly reproducible in binary mobility systems and that peak width is unimportant in high selectivity forms of chromatography, eliminates the drifting peak problem in time-based peak selection. When a time window has been set for switching a component from the first to the second dimension,

the analyte will remain in that window for hundreds of runs [15].

4. Some examples

There are at least two types of analyses where LC–LC has been used as part of a routine analytical method. One is in the case of protein analysis in complex biological samples. The other is drug analysis in serum. Both will be examined below.

It has been noted that structural variants of proteins may be produced during biosynthesis and purification. When the protein is to be used for therapeutic purposes, federal regulatory agencies are proposing that these variants be monitored as part of quality control and batch release [16]. The analytical objective is to confirm that the protein has the target structure, ideally at the earliest stage of production, to provide information for process control. As noted above, variants differ from the native protein in amino acid composition, conformation, carbohydrate composition, or glycosylation pattern must be detected; generally in the presence of large amounts of both impurities and target protein of correct structure. To achieve this goal, analytical data would be required in a time frame ranging from a few seconds, in the case of collecting fractions from a high speed preparative chromatography column, to a few hours, in the case of a fermenter [17]. This is orders of magnitude faster than is possible with the normal structure elucidation techniques used in research. Application of powerful instrumentation, such as amino acid analyzers, protein sequencers, X-ray crystallographic instrumentation and nuclear magnetic resonance spectrometers, are not useful until the target protein has been purified and digested in the case of amino acid and sequence analysis. Moreover, these instruments are slow, differentiate between variant mixtures with difficulty and are not suitable for rapid, repetitive determinations. It is apparent that a very different approach must be applied to the problem of rapid structure confirmation in the process monitoring and quality control environment.

The problem of drug analysis in serum as part of metabolism and pharmacodynamic studies is only slightly different. Because of the large number of samples that must be analyzed and the complexity of serum, it is highly desirable that minimal sample work-up is required, the analytical methods must be of high selectivity, and high throughput is essential [18]. Analytical methods must also be sufficiently robust that minimal down-time is required to maintain the analytical system.

4.1. LC–LC–MS

With the advent of electrospray [19] and matrix assisted laser desorption ionization [20] techniques, mass spectrometry (MS) has emerged as a powerful analytical tool for protein structure analysis [21,22]. The technique is both rapid and of high selectivity. Data may be acquired in a time frame of 1–60 s on molecules exceeding 100 kD with resolution in the range of 1000 to 15 000. The problem is that samples must be at a relatively high degree of purity. When dealing with complex mixtures, this means that MS analyses must be preceded by extensive sample pretreatment.

There are now multiple cases in which LC–LC has been used in the sample pretreatment mode. One of the most successful is in the case of drug analysis [18,23]. Immunosorbent columns in which the antibody is either covalently immobilized or adsorbed on a protein G column have been used in the first dimension to capture the analyte. When non-binding species have been eluted from the immunosorbent, a switching valve is used to couple the column in tandem with a reversed phase column and acidic mobile phase introduced into the system to dissociate the immunological complex. The analyte, and antibody in the case of the protein G column, is transported into the reversed phase column in the second dimension where they were concentrated at the column inlet. The analyte was then chromatographed in the second dimension and introduced directly into the mass spectrometer in the third dimension through an electrospray interface. Mass spectral analyses have been achieved in both the MS and MS–MS modes of operation. Cycle times were in minutes.

An almost identical procedure has been applied to the analysis of insulin variants [24]. An immunosorbent directed against human insulin was used to capture all insulin species (the antibody used in this

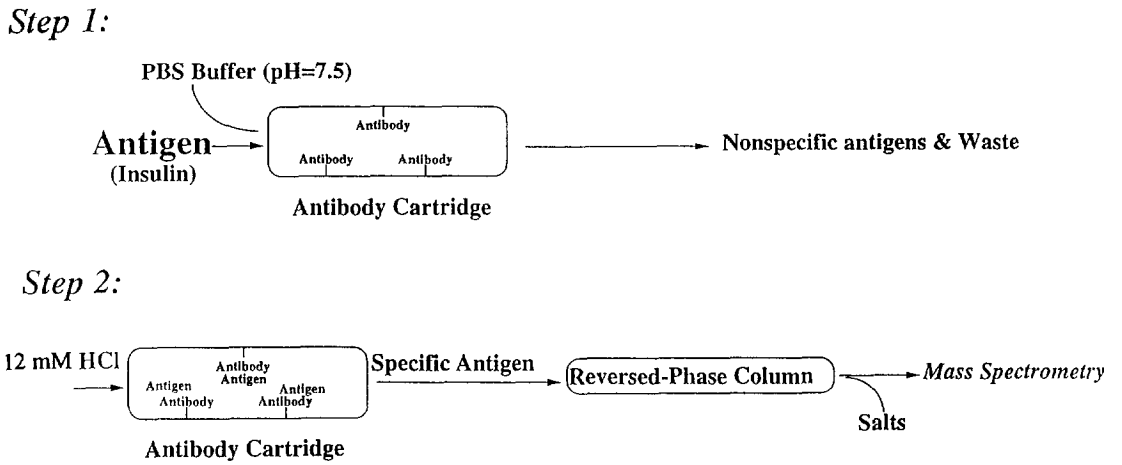

Fig. 1. Affinity LC–LC–MS for identifying and quantitating antigen (adapted from Ref. [24]).

case cross-reacted with both bovine and porcine insulin). Insulin was then desorbed from the immunosorbent and transferred to the second dimension where it was chromatographed on a reversed phase column (Figs. 1 and 2). Again, the analyte was transported to the MS in the third dimension through

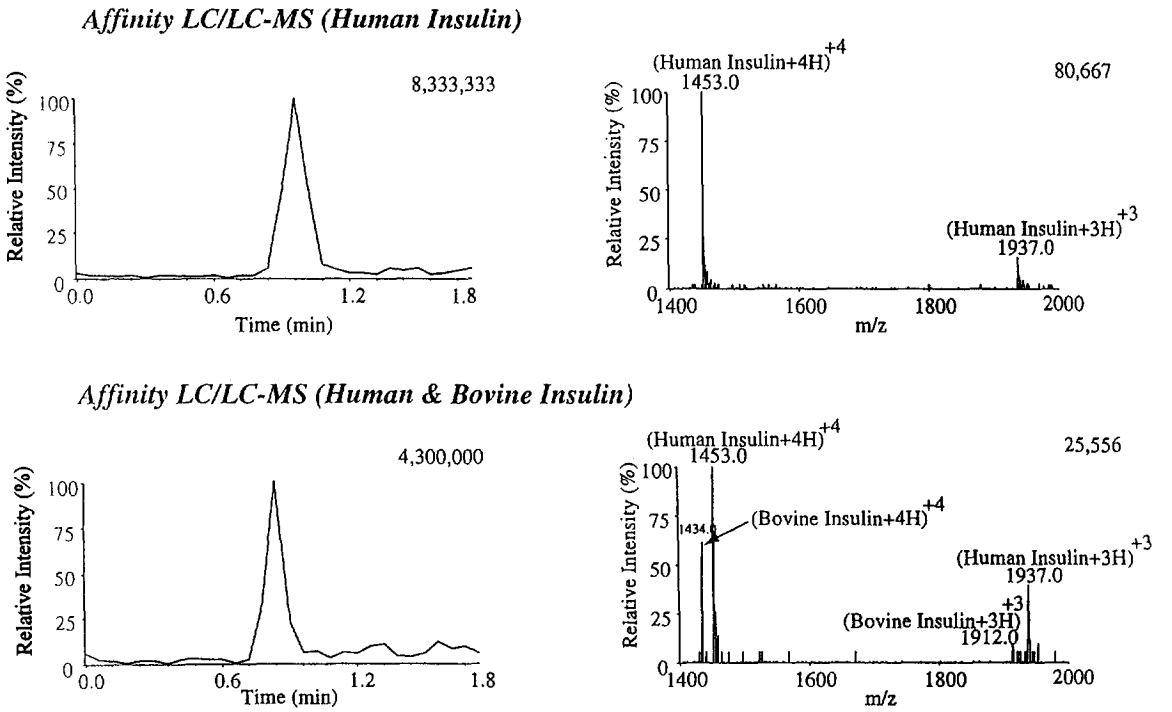

Fig. 2. Affinity LC–LC–MS of insulins (adapted from Ref. [24]).

an electrospray interface. Contamination of human insulin with bovine insulin was detected in 90 s in this LC–LC–MS system.

4.2. LC–LC–LC–LC–MS–MS

Even higher orders of multidimensionality have been achieved in the analysis of protein primary structure. Polypeptide primary structure analysis is generally achieved by using proteolytic enzymes to generate small peptide fragments which are more amenable to sequencing. Structure analysis of protein samples by tryptic mapping with combination liquid chromatography–mass spectrometry (LC–MS) has typically been achieved in a series of non-integrated steps including (i) protein purification by multiple chromatographic or electrophoretic operations, (ii) several desalting or buffer exchange steps, (iii) tryptic digestion, (iv) reversed phase chromatography and (v) mass spectrometry [25]. Other than LC–MS, all the steps in this process are executed manually. It has recently been shown [26] with a multidimensional LC–LC–LC–LC–MS–MS system, that sickle cell hemoglobin (Hb S) in serum may be (i) purified by immunoaffinity chromatography, (ii) desalted and buffer exchanged on a mixed bed strong ion exchange column, (iii) digested in a column of immobilized trypsin, (iv) the trypsin digest fractionated by reversed phase chromatography and (v) the separated peptides analyzed by electrospray ionization mass spectrometry (Fig. 3). Through MS–MS it was possible to show that Hb S varied at position 122 with a valine for glutamic acid substitution. The procedure was totally automated with a cycle time of 90 min/sample. It is significant that even chemical reactions and the equivalent of dialysis were achieved in a column format using binary mobility; relative mobility on the immobilized enzyme column was 1, whereas a combination of 0 and 1 was used in desalting. The ease with which higher orders of dimensionality were achieved was largely due to exploitation of binary mobility in all the LC dimensions except the last. Binary mobility in the final LC dimension is optional. In the case of tryptic mapping, it is critical that shallow gradients are applied to separate the large number of peptide fragments formed by proteolysis.

5. The future of hyphenated techniques

The growing list of hyphenated techniques used by analytical chemists attests to the power and utility derived from the integration of analytical systems and/or methods. This is a trend that will grow, not so much in terms of new types of hyphenated systems, but in going from two dimensional integration to multiple dimensions. As in the six dimensional case cited above, it is possible to go far beyond two dimensional, hyphenated techniques. It is to be expected that operations such as sampling, dialysis, chemical reactions, separations and multiple levels of detection, including multidimensional MS, will be incorporated into single analytical systems. Because (i) a wide variety of unit operations may be carried out in the autosampler, columns and detectors of available LC–MS instrumentation, (ii) LC–MS systems may be easily reconfigured to adapt to a number of different multidimensional analyses involving either direct analyte determination, structure analysis, or both, (iii) analytes may be analyzed directly from complex mixtures, (iv) analyses may generally be achieved automatically from complex mixtures in an hour or less, and (v) analytical method development takes no longer in the integrated mode, integrated multidimensional liquid chromatographic methods will become widely accepted. These attributes of integrated multidimensional analysis will be particularly useful in biotechnology, where it is necessary to identify product variants that differ (i) by single amino acids in a total of 100–300 amino acids, (ii) in conformation, (iii) by small differences in glycosylation or (iv) through chemical alterations in structure during biosynthesis and isolation. Each of these different levels of macromolecular structure must be examined by a different analytical procedure and will require a different configuration of the integrated multidimensional analytical system.

This brings us to the final aspect of the future potential of multidimensional analytical systems. It has been noted above that in the case of human therapeutic proteins, confirmation of structure and use regulation by federal agencies is based on multiple analytical methods which examine different features of the molecule. This means that, as opposed to small molecules, structure confirmation with large molecules will always involve multiple analytical

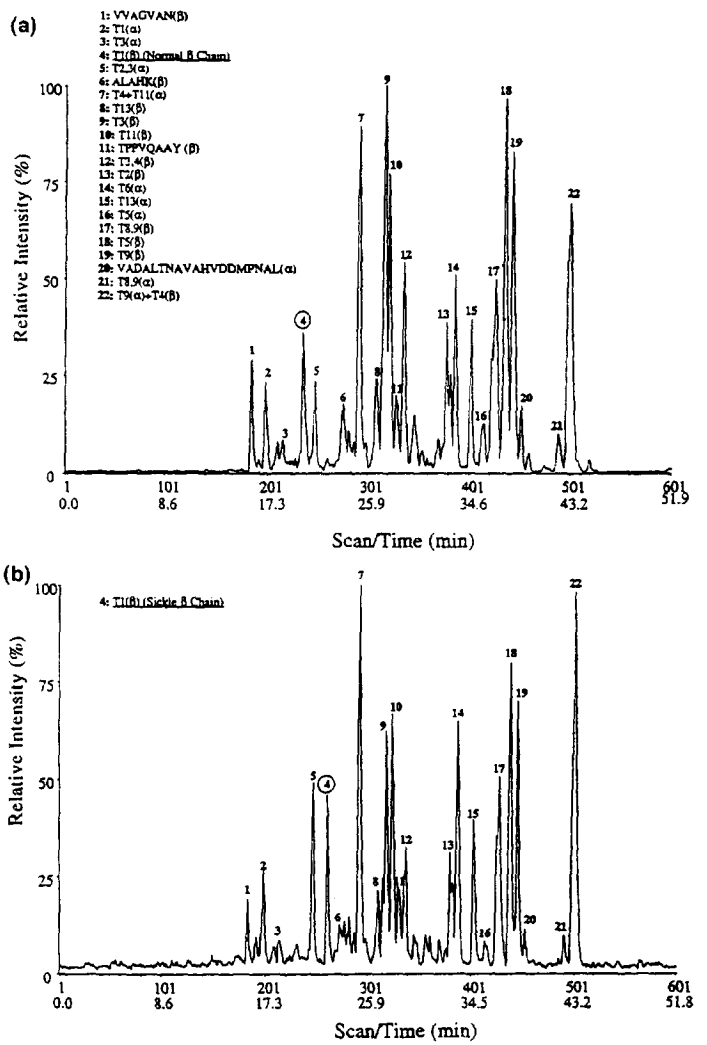

Fig. 3. Total ion chromatograms for (a) normal-cell hemoglobin (Hb A_0) and (b) sickle-cell hemoglobin (Hb S) produced from the five-column chromatography mass spectrometric analysis system. The peak numbers correspond to the peptide sequences listed in the upper left of (a). Peptides identified which correspond to expected tryptic peptides are labelled T#, with the number corresponding to the sequential position of the tryptic peptide in the protein sequence. The α and β refer to the α- and β-chains of hemoglobin. The only peak differing in retention time and mass of the observed peptide is peak 4 in chromatogram (b) (adapted from Ref. [26]).

determinations. It is conceivable that instruments will be developed which are capable of automatic reconfiguration to execute the multiple, multidimensional methods required to confirm structures of the complexity found in proteins biosynthesized by recombinant DNA technology. This would favorably impact the production and regulation of human therapeutic proteins.

6. Conclusions

It has been shown that targeted component analysis of both drugs and proteins by multidimensional LC, either alone or in conjunction with MS, can be a powerful tool when the analyte of interest is present in a complex mixture. The problem with the technique, and the probable reason it is not currently

used in routine analyses, is that of selecting the target analyte for transport from one dimension to another. Peak drift with column ageing in all forms of partitioning based separations has been identified as the major issue yet to be solved.

In contrast, bioaffinity chromatography, immobilized enzyme columns and other forms of chromatography which allow binary mobility adapt well to multidimensional analysis. It has been shown that by exploiting binary mobility, up to four chromatographic dimensions of selectivity may be achieved using time based analyte transfer between dimensions. Because these multidimensional analytical systems are easily automated and apply broadly in biotechnology and the pharmaceutical industry, it is concluded that target component analysis by multidimensional LC will become a routine analytical tool in the manufacture, release and regulation of therapeutic substances for human use.

7. Notation

CE	capillary electrophoresis
GC–MS	gas chromatography–mass spectrometry
Hb S	sickle hemoglobin
HIC	hydrophobic interaction chromatography
IEC	ion exchange chromatography
IMAC	immobilized metal affinity chromatography
kD	kilodalton
LC	liquid chromatography
LC–LC	liquid chromatography–liquid chromatography
LC–MS	liquid chromatography–mass spectrometry
MDLC	multidimensional liquid chromatography
MS	mass spectrometry
n	number of fractions taken through a peak in a two dimensional chromatographic analysis directed at sample profiling
P	peak capacity of a chromatography column
RPC	reversed phase chromatography
SEC	size exclusion chromatography
t_a	analysis time
t_c	chromatographic cycle time including loading, elution, recycling and recalibration

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