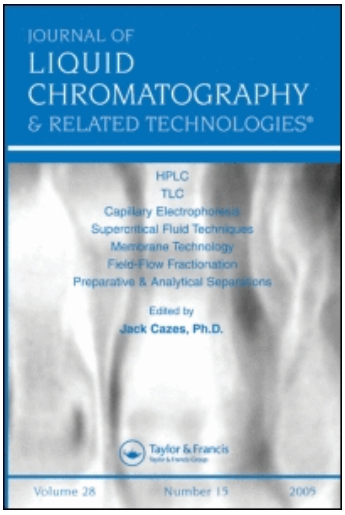

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Taking a Walk on the Wild Side with Planar Electrochromatography and Thin-Layer Electrophoresis: Of Peptides, Proteins, and Proteomics

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Taking a Walk on the Wild Side with Planar Electrochromatography and Thin-Layer Electrophoresis: Of Peptides, Proteins, and Proteomics

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Abstract: Planar electrochromatography (PEC) and thin-layer electrophoresis (TLE) are examined for their potential application to peptide and protein analysis, employing one-dimensional (1D) and two-dimensional (2D) separations, which could potentially be useful for proteomics applications. The PEC and TLE literature are reviewed because the two methods are fundamentally similar in mechanical operations and performance. The application of TLE to peptide mapping is discussed and approaches to extending the technique to proteins and proteomics are offered. Much of the discussion is prognostic or hopefully predictive, attempting to ascertain how PEC might evolve in the coming years for peptide, protein, and ultimately proteomics applications.

Keywords: Proteomics, Peptides, Proteins, Capillary electrochromatography (CEC), Thin-layer chromatography (TLC), Thin-layer electrophoresis (TLE), Planar electrochromatography (PEC)

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INTRODUCTION

Impetus for Developing a New Proteomics Separations Modality Based Upon Planar Electrochromatography

The human proteome is known to contain approximately 22,000 different genes. However, due to post-translational modifications and differential mRNA splicing, the total number of distinct proteins is most likely to be close to one million. The level of complexity, coupled with the relative abundances of different proteins, presents unique challenges in terms of separations technologies. Analytical methods for the simultaneous quantitative analysis of the abundances, locations, modifications, temporal changes, and interactions of thousands of proteins are fundamental to the field of proteomics. Two-dimensional (2D) or even multidimensional protein separations, based upon different physicochemical properties of the constituent proteins, are favored over single dimension separations in proteomics due to the increased resolution afforded by the additional dimensions of fractionation. 2D separation systems can be categorized by the type of interface between the dimensions. In "heart-cutting" methods a region of interest is selected from the first dimension and the selected region is subjected to second dimension separation. Systems that subject the entire first dimension to a second dimension separation, or alternatively, sample the effluent from the first dimension at regular intervals and fixed volumes for subsequent fractionation in the second dimension, are referred to as "comprehensive" methods. At this point in time, proteomics is dominated by a relatively few multidimensional separations technologies.[1–4] High resolution two-dimensional gel electrophoresis (2DGE) separates proteins in the first dimension according to their charge by isoelectric focusing and, in the second dimension, according to their relative mobility by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The technique is capable of simultaneously resolving thousands of polypeptides as a constellation pattern of spots, and is commonly used for the global analysis of protein expression levels. Polyacrylamide gels are mechanically fragile, being highly susceptible to stretching and breaking during handling. Other limitations include difficulty in automating the separation process, low throughput of samples, and difficulty in detecting low abundance, extremely basic, very high molecular weight or very low molecular weight proteins. The 2DGE technique is especially poorly suited to the fractionation of hydrophobic proteins, particularly proteins containing two or more alpha-helical transmembrane domains, mainly because the method is based upon fractionation in aqueous buffers using hydrophilic polymers. While detection of proteins directly in gels with labeled antibodies or lectins has been accomplished, the approach is not generally applicable to every antigen and is relatively insensitive. Consequently, proteins are usually electrophoretically transferred to polymeric membranes before specific targets are identified, considered by most a tedious process at best. The

polyacrylamide gel also poses difficulties in the identification of proteins by microchemical characterization techniques, such as mass spectrometry, since the gels must be macerated and rinsed, the proteins must be incubated with proteolytic enzymes, and peptides must be selectively retrieved and concentrated using a reverse-phase column prior to identification.

Two-dimensional liquid chromatography-tandem mass spectrometry (2D LC/MS/MS) has recently become a powerful alternative analytical approach for separation of complex proteomes.[5] In 2D LC/MS/MS, a proteolytic digest of a complex protein sample is loaded onto a microcapillary column that is packed with two independent chromatography phases, a strong cation exchanger and a reverse-phase material. Peptides are iteratively eluted directly into a tandem mass spectrometer and the spectra generated are correlated to theoretical mass spectra obtained from protein or DNA databases. This peptide based approach to proteomics generates large number of peptides from a specimen that exceeds the analytical capacity of the LC-MS system. Consequently, strategies have been devised that retrieve a small percentage (3–5%) of the peptides from a complex digest, such as tryptic peptides containing only cysteine residues or only histidine residues. The remaining 95–98% of the peptides are discarded, thus preventing a comprehensive analysis of the sample. Additionally, such procedures are unable to distinguish among the various protein isoforms exhibited in a proteome that arise from differential mRNA splicing and post-translational modification, due to a combination of poor sequence coverage and the sequence scrambling arising from the fragmentation process itself.

Considering the unprecedented need to develop newer analytical platforms for proteomics, this review article explores the possibility of developing an alternative separations approach, planar electrochromatography (PEC), also sometimes referred to as electroosmotic TLC, that embodies an amalgamation of some of the better features of the leading proteomics separations modalities, 2DGE and 2D LC/MS/MS. While aspects of PEC are well over forty years old, in terms of peptide and protein analysis, as well as implementation in proteomics workflows, the PEC technology should be considered only at an embryonic stage of development. There is much work to be done if PEC is ever to live up to its potential as an analytical separations technology for proteomics applications.

Generic Overview of TLC, HPTLC, HPLC, GC, HTLE, CEC, and PEC

The origins of thin-layer chromatography (TLC) can be traced back to 1938, when Ismalilov and Schreiber first described its use for the separation of medicinal preparations.[6] Comprehensive coverage of the state-of-the-art in TLC may be found in a trilogy of review articles authored by Dr. Colin Poole.[7–9] Additionally, an excellent monograph on the subject was

authored by Dr. Elke Hahn-Deinstrop.[10] TLC evolved into a mainstay laboratory technique for many decades, becoming first popularized in the late 1950s and early 1960s, beginning in Europe and subsequently taking root in the United States (US).[11–18] The technique has been especially useful for rapid, qualitative analysis of a wide range of analytes, and remains a routine laboratory tool in analytical chemistry environments, due to it being both highly economical and rapid to perform. TLC, however, is considered to provide poorer experiment-to-experiment reproducibility than many of the more modern separations techniques.

Chronologically, TLC followed the development of paper chromatography (PC),[19] and preceded introduction of high performance liquid chromatography (HPLC).[14–16,20–26] It was developed and optimized in about the same time period as gas chromatography (GC), but whereas GC has remained a mainstay analytical approach into the current century, TLC has suffered a serious decline in popularity and applications in the 1970s and thereafter, due in large part to HPLC taking hold and becoming the preeminent analytical (and preparative) separations technique(s).[27–32] The situation persists to the present day, wherein HPLC remains a major, perhaps the major, analytical (and preparative) separations technique for all types of analytes, organic and inorganic, as well as biochemical, but TLC still finds niche applications, mostly relating to rapid and simple quality control evaluations. Electrophoresis, was born from the Ph.D. thesis work of Arne Tiselius in 1930, and subsequently evolved in the earlier half of the last century into the now familiar flat bed format (FBE), again first in Europe, but has remained another mainstay for biochemical analysis, especially for macromolecules, such as nucleic acids and proteins/peptides or antibodies.[33–49] It may be considered, along with HPLC, perhaps one of the most prominent separations techniques, especially for the analysis of charged, high molecular weight (MW) biopolymers. In the late 1970s and early 1980s, slab gel electrophoresis was developed into a capillary format, becoming then known as high performance capillary electrophoresis (HPCE).[50–65] The new technique grew in popularity quite rapidly, but then its adoption reached a plateau and has not shown much growth in recent years, being utilized much less frequently than HPLC, even for charged, high MW macromolecules. Other separations techniques, such as field flow fractionation (FFF), countercurrent chromatography (CCC) or carrier-free continuous electrophoresis (CFCE), and others, have been introduced, but none of these have become nearly as popular as HPLC and FBE/HPCE, to the present day.[14–16,20,45,46]

High performance TLC (HPTLC) differs from TLC only in utilizing smaller particles ($\sim$5 μm as opposed to $\sim$11 μm) for the solid phase sorbent. Although TLC and then HPTLC were routinely used in the 1950s–1970s, they never gained sustained prominence for fractionation of peptides, or proteins and, of course, have made few inroads into the territory of proteomics. Most efforts in this realm were summarized several years ago,[66] though occasionally newer studies have been reported.[67,68]

There is scant literature on the development or application of TLC or HPTLC for any macromolecules, but especially literature is lacking for proteins of high MW.[67,69–72] Indeed, it would appear that the entire literature on HPTLC or TLC for peptides/proteins consists of fewer than fifty papers, total. TLC and HPTLC have been enlisted rather infrequently for peptide and protein separations because several other techniques with higher resolving capability have been routinely available for that purpose, including HPLC, IEC, CE, and MS. Examples of state-of-the-art 1D and 2D peptide separations achieved by HPTLC are depicted in Figures 1 and 2, from Dr. Heinz-Emil Hauck's Thin-Layer Chromatography Life Science and Analytics Laboratory at Merck KgaA in Darmstadt, Germany. Figure 1 shows a 1-D separation of several protein digests as bands on an HPTLC cellulose plate. Separation of tryptic peptide digests of phosvitin, myoglobin, cytochrome C, β-casein, and bovine serum albumin (BSA) was achieved by using a mobile phase consisting of 2-butanol/pyridine/acetic acid/water (30/20/6/24, v/v/v/v). The resolved peptides were sprayed with ninhydrin for visualization. A 2-D separation of BSA tryptic digest was also achieved on the cellulose HPTLC plate (Figure 2). In the latter case, the mobile phases used in the first and second dimension were 2-butanol/ammonia (25%)/pyridine/water (39/10/2/26, v/v/v/v) and 2-butanol/acetic acid/pyridine/water (30/6/20/24, v/v/v/v), respectively.

Conventional TLC or HPTLC, does not depend upon any electrical potential or electrophoresis, however, closely related techniques that do exploit these mechanisms are referred to as PEC or TLE, depending upon the mode of analyte separation. Operationally, for both of these related techniques, electrodes are attached to each end of a wetted TLC plate and an electric potential is applied across the plate to effect separations. We believe the earliest demonstration of the use of electroosmotic flow for driving a liquid on a thin-layer chromatographic plate was reported in the early 1950's.[73,74] Why have TLC, HPTLC, PEC, and TLE been marginalized to the present day? There are several possible explanations, but the most likely are the following. First, HPLC came along at about the same time that HPTLC was just starting to become popular, but it was very clear that HPLC would become a fully automatable approach with higher plate counts, improved efficiencies and resolutions, absolute quantification, simple interfacing with ultraviolet-, fluorescence-, photo diode array- and mass spectrometry based detection techniques, good reliability and reproducibility, and improved peak identification. HPTLC did not appear able to successfully compete with what HPLC might and then ultimately did offer the user, especially those wanting to perform protein/peptide analyses. Despite the much higher costs for HPLC instrumentation and apparatus, it rapidly overtook HPTLC with respect to biotechnology and proteomics applications and offerings. This has never changed, and HPTLC for proteins became less and less of a desirable approach as HPLC became more and more of just such an approach.

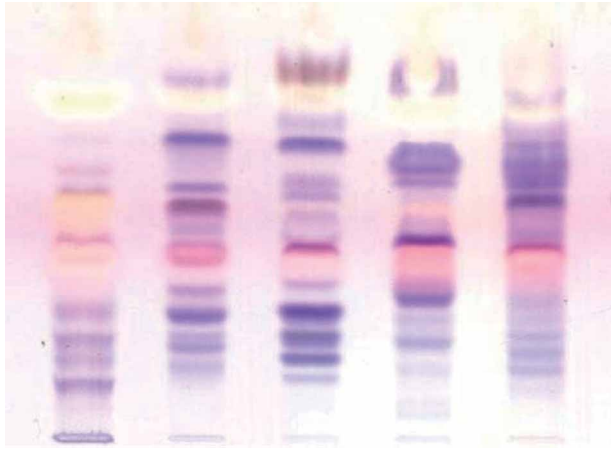

Figure 1. (Color). 1-D separation of tryptic peptide digests of, from left to right, phosvitin, myoglobin, cytochrome C, β-casein and bovine serum albumin (BSA) using a cellulose HPTLC plate. The mobile phase used for the separation consists of 2-butanol/pyridine/acetic acid/water (30/20/6/24, v/v/v/v). The resolved peptides were sprayed with ninhydrin for visualization. (Figure courtesy: Mr. Michael Schulz and Dr. Heinz-Emil Hauck, Merck KgaA, Darmstadt, Germany).

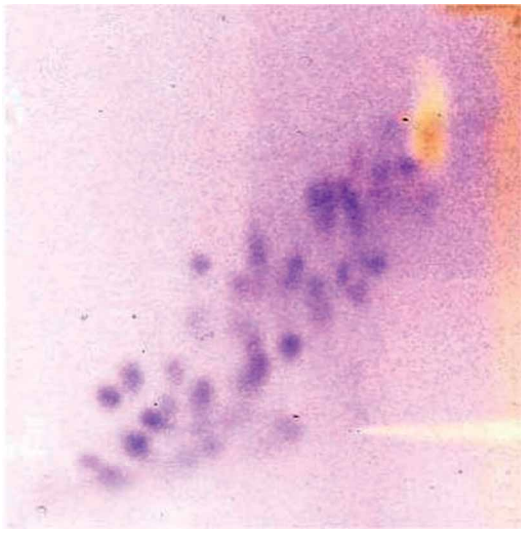

Figure 2. (Color). 2-D separation of BSA tryptic peptide digest using a cellulose HPTLC plate with mobile phases of 2-butanol/ammonia (25%)/pyridine/water (39/10/2/26, v/v/v/v)mL and 2-butanol/acetic acid/pyridine/water (30/6/20/24, v/v/v/v) in the 1st and 2nd dimensions, respectively. The resolved peptides were sprayed with ninhydrin for visualization. (Figure courtesy: Mr. Michael Schulz and Dr. Heinz-Emil Hauck, Merck KgaA, Darmstadt, Germany).

In addition, the availability of HPLC packing materials steadily increased in number and variety, with all types being commercialized for proteins in the 1980s and thereafter. A similar propagation of commercialized substrates never really occurred for HPTLC, and the number of pre-made plates for peptides was always rather limited when compared to HPLC packed columns. Though some vendors offered C_{18} hydrocarbon chain modified plates with particles of 60Å pore size for peptide separations and peptide mapping, very few ever marketed plates with 300Å pore size particles using any type of packing material optimized for protein separations. This remains true today, and thus, one is quite limited in terms of what commercial plates can be purchased for carrying out HPTLC of peptides and proteins. We have inquired of most current vendors of HPTLC plates (EMD Chemicals, Camag, Analtech, and others), and virtually none of these now routinely offer HPTLC plates of a suitable pore size for fractionation of proteins. Perhaps the only routinely available plates suitable for separation of peptides and proteins are 60Å C_{18} modified silica plates, which can be used for proteins, though they are not as valuable as would be 300Å C_{18} modified silica plates.[75] It is, of course, still possible to utilize cellulose (and variants thereof, such as DEAE cellulose) for proteins/peptides in an HPTLC format, though there are fairly few papers in the literature describing such approaches and applications. This would involve size exclusion chromatography (SEC) in the case of bare cellulose or ion-exchange chromatography (IEC) in the case of a substrate like DEAE cellulose based materials. It is apparent that the commercial HPTLC plates on the market today and perhaps for the past decade, have just never been optimized and successfully commercialized for separation of proteins and, consequently, proteomics applications. Whereas, 1DGE and 2DGE approaches for proteins have become *de rigueur* and widely adopted and practiced, this has clearly not become the situation for HPTLC alone. This situation is unlikely to change very much in the future, either. Until now, there has been no clear incentive for vendors to offer commercially prepared HPTLC plates for proteins, as there is clearly no developed market for them.

There may yet be a glimmer of hope for the use of HPTLC plates in a TLE or PEC format, and several recent publications attest to the general viability of such approaches, though almost never for fractionation of peptides or proteins, other than with cellulose supports.[70–72,76–79] There are, of course, some publications dealing with PEC utilizing various stationary phases, but virtually all of these have dealt with low MW species, never with proteins and rarely even with peptides.[80–84] Perhaps the very best review of PEC in recent times is that by Dr. Nurok.[80]

There are also numerous publications involving capillary electrochromatography (CEC) applied to the separation of peptides and proteins, though very few (if any) involving proteomics applications. However, operationally CEC is really quite a different approach when compared to PEC.[85–95] Whereas, very similar HPLC packing materials can be and have been employed in CEC for proteins/peptides, though much less so far for proteomics (this remains to

be explored, by and large), the same has not occurred for PEC or HTLE. We have already alluded to why this is so, as above, and will discuss it perhaps more fully below, but we have only been able to uncover a few concrete literature citations on the successful application of any form of PEC, with any packing material (e.g., silica gel), for the analysis of peptides or proteins (See Table 1). For these instances, the silanol groups would have to provide the ion exchange qualities of the solid phase support and could be deprotonated at a mobile phase pH value of about eight, leading to an electroosmotic driving force. However, at pH values below about three, there would be a reduction or elimination in electroosmosis and separations would be primarily electrophoretic in nature. The silica TLC plate approach was subsequently all but abandoned in favor of separations on cellulose based media, as popularized by the Hunter laboratory, though it is not clear that cellulose offers clear performance advantages over silica. While the HTLE work of Hunter does describe successful approaches for fractionating peptides and peptide mapping, strangely enough, it has not been employed for the analysis of proteins or application to proteomics, as yet.[70,78,79] HTLE has, thus far, only utilized a cellulose stationary phase or support, with a variety of aqueous buffers, but this does not really involve chromatography as much as it is a form of flat-bed electrophoresis on a cellulose or solid support, rather than conventional gel based, flat-bed electrophoresis (FBE). The cellulose supports are, by and large, really providing only a sieving material, as in size exclusion chromatography (SEC) or gel filtration chromatography (GFC), but do not provide adsorption, partitioning, ion-exchange, or any true stationary phase interactions with the peptides/proteins. The cellulose polymer is generally too hydrophilic to provide for significant binding of proteins to the solid phase surface. Thus, the proteins interact minimally with them in aqueous medium, and once the applied current is removed the separation pattern will begin to degrade due to diffusion.

DIFFERENCES BETWEEN PEC, AND HTLE/1DGE-SEMANTICS, OR NOT?

There is the need to differentiate for the reader what we believe are the key differences between performing PEC and TLE, especially for proteins and peptides, and where a line needs to be drawn in order to avoid reviewing the entire literature on FBE (1DGE). It is not the intent of this review to summarize all of 1DGE or 2DGE for proteins and/or proteomics, as clearly that is impractical. For an interesting review of the evolution of electrophoresis in general, readers can refer to a recent article by Righetti.[96] A number of terms have been used somewhat indiscriminately in the literature over the past forty or so years, including electrochromatography, chromatophoresis, chromatographic electrophoresis, and chromatoelectrophoresis. It is important in the context of this review article to discern between what is true

Table 1. 'Pre-Hunter' era TLE/PEC separation of amino acids, peptides and proteins

Stationary phase	Mobile phase (electrophoresis)	Operating conditions	Proteins/peptides/ amino acids
Silica gel G and alumina (99)	pH 2 or 6.5 (Appropriate mixture of acetic acid, formic acid and pyridine)	460 V, Detection using ninhydrin	Amino acids and peptide mixtures
Silica gel G (100)	pH 3.9 buffer (pyridine/ acetate/water)	1000 V, 30–80 mA, Detection with ninhydrin	Digests of protamine and myosin
Silica gel H and cellulose (101)	pH 3.9 buffer (pyridine/ acetate/water)	950 V, Detection with 0.2% ninhydrin in acetone	Tryptic digest of horse hemoglobin and various amino acids
Mixed cellulose/ silica layer (103)	pH 2.0 (17 mL 90% formic acid and 57 mL acetic acid per liter)	1 kV; 20–30 mA, 12–18°C, Detection using ninhydrin or autoradiography	Various amino acids from plant extracts
Cellulose and silica gel (104)	pH 2.5 buffer (0.15 M formic acid or pH 1.9 buffer, 0.5 M acetic acid, (5 mM KCl sometimes used) or pH 10.2 buffer, formic acid and NH_4OH)	1 kV, 6 mA or 4.5 kV, 45 mA	Various amino acids
Cellulose (105)	pH 2.0 buffer (glacial acetic acid, formic acid, water)	Same as above	Peptide digests of the B_I component of TYMV, globin from canine haemoglobin, c-lysine and argine labeled R_{17} bacteriophage coat protein, ribonulease B
Cellulose and Pevikon C-870 (106)	pH 2.5 (1 M acetic acid buffer and pH 10.0 ammonium bicarbonate buffer)	4200 V, 25 mA, or 3300 V, 20–25 mA, Detection using Pauly's reagent	Insulin, glucagon, chymotrypsinogen A, trypsin

(*continued*)

Table 1. Continued

Stationary phase	Mobile phase (electrophoresis)	Operating conditions	Proteins/peptides/ amino acids
Silica gel (107)	pH 6.5 buffer (pyridine/acetate)	300 V, 10 mA, 2 hr. room temperature. Detection with cadmium/ ninhydrin	Tryptic peptides from cytochrome c
Cellulose (108)	pH 1.9 buffer (formic acid/ acietic acid)	45 V/cm, 10 mA, 45 minutes, 15°C Detection with copper nitrate modified ninhydrin or Fluorescamine	Amino acids found in the peptide chains of actinomycins
Cellulose and silica (110)	pH 4.4 buffer (pyridine/ acetate/water)	Detection with ninhydrin, fluorescamine, and *o*-phthaldialdehyde	Various amino acids and peptides
Silica gel G, silica gel GHL (111)	pH 3.5 or 6.5 buffer (acetic acid and pyridine in H_2O)	1 kV; 40–45 min., 8–10°C. Fluorescence detection	Tryptic peptides from scallop and rabbit actin
Silica gel (109)	pH 3.5 or 6.5 buffer	500 V; 20 min., Cd-Ninhydrin detection	Tryptic digest of rabbit muscle akdolase
Cellulose (112)	pH 1.9 (mixture of formic acid and acetic acid)	1 kV; 30 min., Autoradiography and fluorography	^{3}H and ^{14}C-labeled α and β-tubulin peptide digest, rabbit and chicken muscle actin digest
Silica gel G (114)	pH 3.5 or 6.5 buffer	900 V; 60–120 min., Fluorescence detection	Alcohol dehydrogenase digest
Cellulose (115)	pH 3.5	Fluorescence detection	Rabbit or human triosephosphate isomerase digest

chromatography and what is purely or solely electrophoresis, without any chromatographic components. FBE is normally performed, either as in SDS-PAGE or IEF using a gel of some sort, usually a polymer of polyacrylamide type, with some degree of cross-linking, but that gel usually does not physically or chemically interact to a significant extent with the protein analytes, other than via sieving or size discrimination. Thus, in the absence of any true chromatographic interactions, which SEC and SDS-PAGE do

not really provide, FBE for proteins can not be considered as a part of PEC or HTLE. Sieving media/gels do not normally exhibit any degree of partitioning or hydrophobic interactions or ion-exchange separations, and thus should not really fall into the realm of chromatography or chromatographic separations. Once that sieving medium is changed, as in HTLE, perhaps by using a cellulose support on which the proteins/peptides are now separated under an applied voltage, the separation process might conceivably be considered at least analogous to PEC. This point is certainly debatable, but under conditions where the cellulose is really adsorbing the proteins or interacting with them in perhaps an ion-exchange mechanism, as well as functioning as a support for doing the FBE, this might really be classified as a form of PEC. Electrochromatography (EC) should be considered a hybrid separation technique that couples zonal electrophoresis with liquid chromatography (LC). In EC, both chromatographic and electrophoretic processes determine the magnitude of the overall migration rates of the analytes. The driving force of EC is electroosmotic flow (EOF), rather than hydraulic flow, the dominant force in LC or the electrophoretic mobility prevalent in simple FBE. Figure 3 illustrates the migration of an analyte by electrophoretic movement (A), in the absence of any interaction with the solid support as compared to EOF (B). Usually the EOF overrides the electrophoretic mobility of the individual analytes. The EC technique is unusual in that the separation mechanism is based upon both kinetic processes (electrokinetic migration) and thermodynamic processes (partitioning). This combination reduces band broadening and thus, allows for higher separation efficiencies compared with LC.

By applying the strictest requirement of an electroosmosis driven separation mechanism, the case for cellulose based HTLE as a form of PEC is weakened considerably. Nevertheless, the two technologies are operationally quite similar and thus, we have opted to then include HTLE in this review article, together with HPTLC and PEC, especially when the PEC references have used true stationary phases, similar to those used in TLC of proteins/peptides. Hence, this review has been limited to the literature relating to TLE employing a stationary phase other than a sieving gel, PEC with conventional stationary phases found in TLC, and modern HPTLC, all for applications to proteins and peptides.

Electroosmotic flow depends upon such factors as the surface charge density, the field strength, the thickness of the electric double layer, and the viscosity of the separation medium, which in turn depends upon the temperature. In practical terms, electroosmotic flow is highly dependent upon pH, buffer concentration (ionic strength), the organic modifier, and the type of stationary phase employed. One important advantage of EC relative to LC is that EC separations can be performed isocratically, thus dispensing with the requirement for gradient elution, which in turn simplifies instrumentation requirements, an especially attractive feature for possible implementation of PEC. The application of the CEC technique to protein and peptide analytical biochemistry has been attempted only relatively recently in the history of

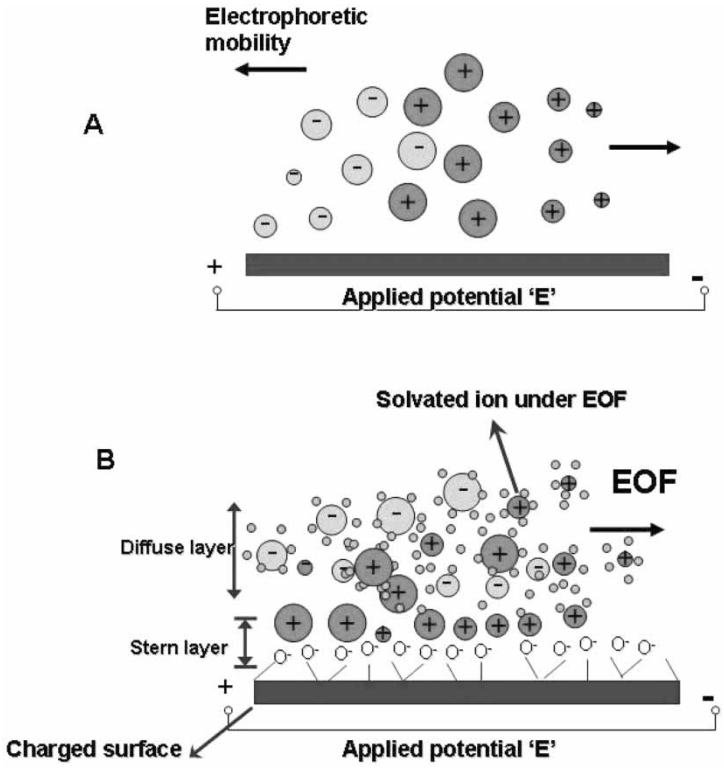

Figure 3. Schematic diagram illustrating electrophoretic- versus electroosmotic-driven separations. (A) Simple electrophoretic movement of analytes in the absence of any interaction with the solid support. (B) Migration of analytes under the influence of bulk electroosmotic flow (EOF).

separation sciences.[86] Consequently, the technology has not really followed the same evolutionary path as older electrophoretic techniques, such as isoelectric focusing and SDS-electrophoresis. The older technologies typically were first developed on cellulose based filter paper, then developed on gel media (starch, agarose, polyacrylamide), and finally adapted to capillaries and microfluidic devices. EC, however, was developed initially in capillaries and largely circumvented conscious implementation in planar media altogether.

HTLE OF PEPTIDES AND PEPTIDE MAPPING

Hunter's Approach to Performing HTLE

There are several papers in the literature, mainly on peptides that deal with other stationary phases, using otherwise conventional TLE

apparatuses.[71,72,78,79,97,98] In fact, the earliest literature in TLE of peptides/proteins we could trace dates back to the early 1960's and implemented electrophoresis of protein digests in the first dimension, followed by thin-layer ascending chromatography in the second dimension on cellulose thin-layer plates.[99–115] Sporadic usage of this method occurred for several years before it was implemented using very similar experimental conditions by Dr. Tony Hunter's group at The Salk Institute, La Jolla, CA for phosphopeptide mapping, By and large, the majority of such papers from Hunter's group have utilized, almost exclusively, cellulose supports.[70,78,79] Much of the TLE work from the Hunter laboratory and the principle application supported by the commercialized TLE instrument, which is marketed by CBS Scientific, relies upon the digestion of ^{32}P labeled proteins with site specific proteases and separation of the digestion products in 1D or 2D on cellulose TLC plates using a combination of electrophoresis and chromatography or electrophoresis-electrophoresis. As Hunter's papers, and the literature based on related methodologies, never mention the term PEC or EC, it remains unclear whether on their cellulose TLC plates, chromatography is actually occurring along with electrophoresis during separations, or whether the cellulose simply acts as an anti-convective medium, much like conventional polyacrylamide. Unfortunately, there has been little to no discussion on the analytical figures of merit for this technique in any of the published articles. Table 1 compiles the early literature references in this area of separating amino acids, peptides and proteins on thin-layer plates coated with cellulose, silica and alumina as stationary phases.

Among the planar separations performed on TLC-like media, the Hunter approach to performing TLE appears to have received the widest attention and most extensive utilization. Perhaps the biggest disadvantage to the HTLE approach to doing peptide mapping or peptide analysis, for it has been but little applied to proteins and not at all to proteomics, is that it requires the use of radiolabeled ^{32}P for its detection. This radiolabel must be incorporated into all of the peptides that need to be identified and/or quantified. Though the radiolabeling method is quite sensitive, needing only a few hundred ^{32}P disintegrations per minute to obtain reproducible phosphopeptide maps, it still requires some sort of radioactivity counter and the assorted instrumentation. Additionally, the radiolabeling approach is somewhat limited with respect to the range of biological samples that can be analyzed, as evaluation of clinically derived samples requires *in vivo* labeling, which is not usually feasible. The hazardous nature of radiolabels, as well as the accompanying disposal costs for radioactive waste, also makes the radiolabeling approach less than ideal to perform.

Of course, using more extensive digestions than those employed for simple peptide map formation, it is also possible to use these same approaches for the analysis of the phosphoamino acid content of both intact phosphoproteins and individual phosphopeptides recovered from 1D or 2D separations using these HTLE approaches, as shown in Figure 4.[70] In this 2D TLE

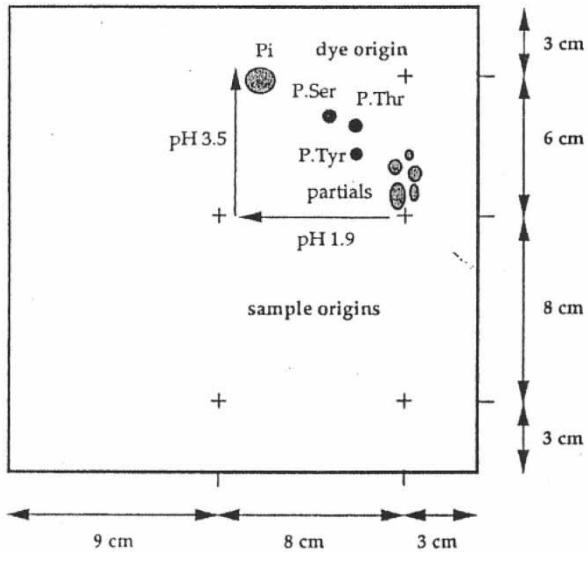

Figure 4. TLC plate marked for separation of phosphoamino acids in two dimensions. Four samples can be analyzed on one single plate. Marker dyes are spotted on the fifth origin, before electrophoresis in the first dimension at pH 1.9 (arrow towards anode). Electrophoresis in the second dimension is at pH 3.5 (arrow towards anode). The positions of phosphoserine (P.Ser), phosphothreonine (P.Thr), and phosphotyrosine (P.Tyr), free phosphate (Pi), and partially hydrolyzed phosphopeptides (partials) are shown for a sample spotted on the top right hand sample origin. (Reproduced with permission of the copyright holder, Electrophoresis Journal and the publisher, VCH Verlagsgesellschaft mbH, Weinheim, Germany).

separation of ^{32}P labeled amino acids, it was possible to have four separate samples electrophoretically separated at the very same time on the same plate, if desired. The crosses represent where each sample could be spotted, but in Figure 4, actually only one sample of phospho-amino acids was spotted. In this particular example, the first dimension separation was performed at pH 1.9, while the second dimension separation was done at pH 3.5, as indicated. The fully resolved phosphoamino acid standards and partially hydrolyzed phosphopeptides (partials) are shown for a sample spotted on the top right hand sample origin (+).

In TLC, two phases actively participate in the analytical separation, the stationary sorption layer (solid phase) and the liquid solvent (mobile phase). In the case of PEC, the laminar flow of mobile phase present in classical TLC is replaced with an electroosmotic flow. It is not clear whether electroosmosis is driving the actual peptide separations on cellulose. Cellulose has been used for many years in paper chromatography (PC), as well as in TLC or HPTLC, and it is generally considered an interactive stationary phase in such separations, as it has also been used in open column liquid

chromatography (LC). But, as we believe there is not much separation on cellulose that can be attributed to electroosmotic flow, it is preferable to term any separation on cellulose as HTLE and not a true variant of PEC. The major limitation of using cellulose as the stationary phase in both separation dimensions is, of course, that one is then limited to changes in pH or aqueous/organic content in order to bring about the separations. This should be contrasted with 2D LC or multidimensional HPLC (MDLC), which have become perhaps the classic approaches to performing proteomics today.[116–125] In such classic proteomics methods, one is able to interface IEC with RPLC, in a truly, online automated manner, injecting one sample and collecting numerous fractions from the second dimension.[126–128] At the same time, one is able to interface numerous other 2D LC modes, such as SEC-IEC or affinity-IEC-RPLC, all of which can then be online interfaced with ESI-MS or using fraction collection, with off-line MALDI-TOFMS for individual peak collection and identification.[128] Nevertheless, we believe that the existing literature on TLE of peptides may provide a foundation for true PEC approaches to peptide mapping, protein separations, and eventually, proteomics type applications, with some improvements on the apparatus, mobile phases, and stationary phases yet possible and desirable, and that such instrumental approaches, and similar ones, will prove useful not only for this class of phosphopeptides/phosphoproteins, but in general, for all classes of proteins, conjugated or not, glycosylated, acylated, aggregates, quaternary structures (hemoglobins, myoglobins), and so forth. At the present time, however, it appears that PEC or the HTLE approach, as first described by Hunter's group, have been too little applied to peptides/proteins, in general, and that most published work has involved phosphopeptides, much less even with phosphoproteins, to date.

Other Applications of TLE or PEC for Peptides

In a closely related application of the HTLE approach, Nagahara et. al. have described conducting 2-D phosphopeptide mapping using again a mixture of ^{32}P-labeled peptides (after digesting the intact ^{32}P-labeled proteins first). These were separated first on a cellulose TLC plate with electrophoresis (TLE) in the first dimension, followed by true TLC in an organic buffer in the second dimension.[72] The developed TLC plate was dried and then characterized using autoradiography film or a phosphor-imager screen. The specific locations of the now resolved ^{32}P-containing peptides were then defined. Individual phosphopeptides could be identified by their removal from the TLC plate in the second dimension, and analysis by hydrolysis to the amino acid mixture to identify the individual, phosphorylated residues present in each peptide. Alternatively, Edman based amino acid sequencing could also be performed on the individual, fully resolved peptides from the second dimension separation (TLC). The nature of the 2D separations

involving TLE and TLC, in a diagrammatic representation is provided by Nagahara,[72] a figure very similar to that provided by Hunter (Figure 4). Also, a diagram of the Multiphor II apparatus used to perform TLE in the first dimension is also given in this Nagahara paper, as well as an actual 2D chromatogram of now-resolved ^{32}P-phosphopeptides, as shown in Figure 5.

In a similar vein, Judd has reported in two separate methods papers on the use of 2D TLE-TLC for peptide mapping, now using ^{125}I labeled peptides.[71,98] In the earlier paper, Judd describes their separation of peptides by this 2D approach (termed 2D TLE-TLC), that results in very high resolution separation of the peptides, making subtle comparisons thus possible. As indicated in the papers, it was necessary to radiolabel the protein before enzymatic cleavage to individual peptides, in order to then permit for individual peptide location and determination. This is really another approach to accomplishing peptide mapping of individual proteins, since the protein's primary structure will always lead, with a specific protease, to the identical peptide fragments, unique for that specific protein. The 2D TLE-TLC separations were, in this instance, pursued via an immersion TLE chamber (Savant TLE 20 electrophoresis chamber or equivalent) with a 1200-V power pack, using a buffer of H_2O, glacial acetic acid, and pyridine. The TLC buffer used n-butanol, pyridine, H_2O, and glacial acetic acid. The TLE-TLC plate was a 0.1 mm Mylar backed cellulose sheet (E. Merck, MCB Reagents, Gibbstown, NJ, or its equivalent). The 2D TLE-TLC amino acid markers were Tyr, Ile, and Asp (1 mg/mL) in H_2O. A spray of 1% Methyl Green in H_2O (w/v) was

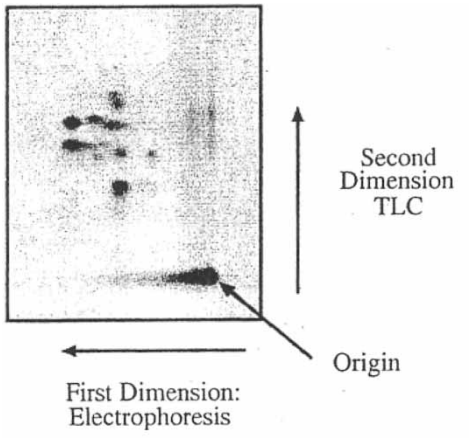

Figure 5. 2D phosphopeptide map of the retinoblastoma tumor-suppressor gene product (pRb) labeled with ^{32}P-phosphate in vivo. pRb contains 13 cyclin-dependent kinase (cdk) phosphorylation sites, hence the complexity of the phosphopeptide map. The origin, first- and second-dimension runs are as indicated.[72] (Reproduced with permission of the copyright holder, Humana Press, Inc., Totowa, NJ, USA).

also used to detect resolved peptides, with a standard laboratory sprayer. Another spray reagent used was 0.25% Ninhydrin in acetone. Examples of peptides separated by 2D TLE-TLC is given in Figure 6, using ^{125}I labeled proteins then cleaved with trypsin to generate the observed peptide maps. Indicated in Figure 6, are the directions of TLE and TLC for these trypsin digests.[71,98]. This approach to peptide mapping is actually given in two separate publications, but the results are basically identical.

The work of Stephens demonstrates that silica gel TLC plates are perfectly suitable for the 2D TLC/TLE separation of peptides from proteolytic digests, though cellulose plates have dominated for this application.[11] Silica gel G or GHL plates were employed to fractionate nanomole amounts of proteolytically digested protein. TLC using either chloroform-methanol-ammonium hydroxide (2 : 2 : 1, v/v/v) or n-propanol-ammonium hydroxide (7 : 3, v/v) solvent systems was followed by air- or oven-drying. Dried plates were then sprayed with either of two solvent systems, pyridine-acetic acid-water, pH 3.5 (2 : 20 : 978, v/v/v) or pyridine-acetic acid-water, pH 6.5 (100 : 3 : 897, v/v/v) and electrophoresis was performed perpendicular to the direction of the TLC separation at 1000 V potential, for 40–45 minutes. After completing the electrophoresis, plates were oven dried at 110°C and peptides visualized by spraying the plates with 0.025% fluorescamine in acetone (w/v). For peptides separated at pH 3.5, a subsequent neutralizing

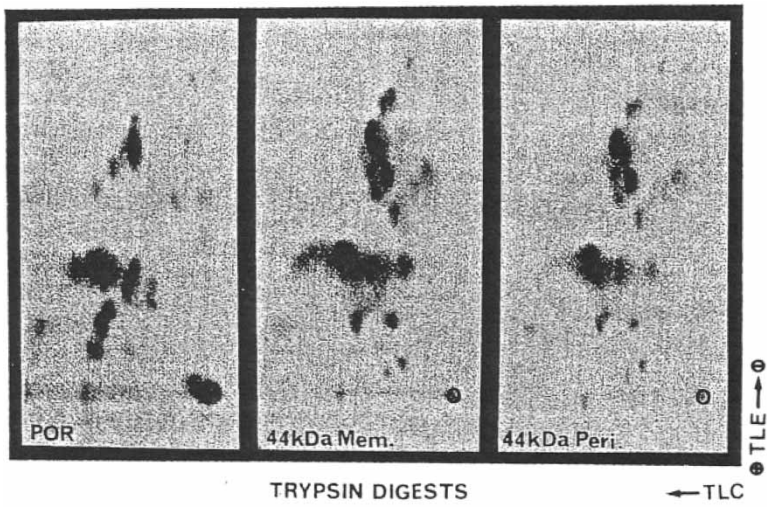

Figure 6. Examples of ^{125}I-labelled peptides separated by 2D TLE-TLC. Proteins were radiolabeled on nitro cellulose paper (NCP strips), and cleaved with trypsin. The peptides were spotted on a thin-layer cellulose sheet and subjected to 2D TLE-TLC. The origin (O) is at the lower right of each map. TLE = direction of thin-layer electrophoresis; TLC = direction of thin-layer chromatography. The ^{125}I-labeled peptides were visualized by autoradiography.[71,98]

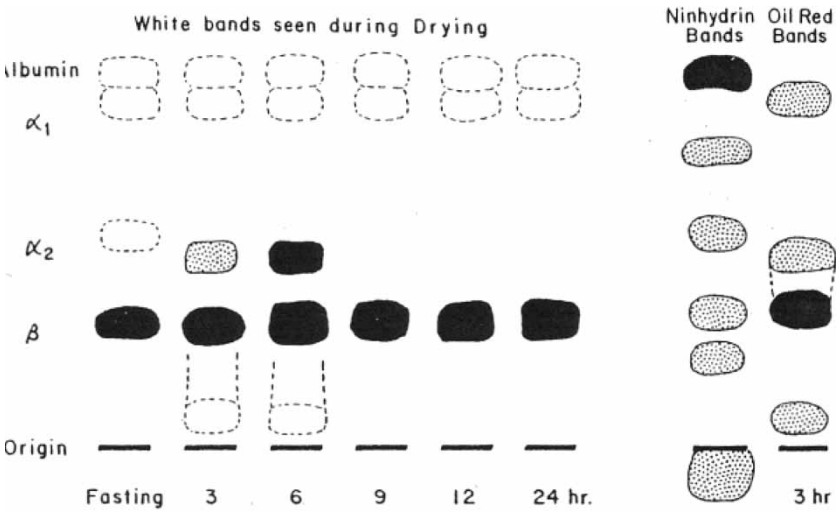

Figure 7. TLE of serum lipoproteins from samples taken as a function of time during a fat tolerance test or with a fasting subject. This separation was done on a starch granule thin-layer with a working voltage of 600 V, 18–20 mA, 2 hrs.[76] (Reproduced with permission of the copyright holder, J. Lipid Research, and the publisher).

spray was applied to plates using 5% triethylamine in acetone, in order to stabilize the fluorescent signal.

One, perhaps final representative report on the use of 2D TLE for proteins and related materials is that by Keler-Bacoka and Pucar.[77] In this report, the authors resolved a mixture of protein-hemoglobin complexes in plasma from patients and controls, using filter paper sheets at pH 7.0. The 2D TLE/TLC was done discontinuously on an apparatus for continuous electrophoresis. The experimental conditions used Whatman No. 1 filter paper; sheets, 30 × 40 cm, with a supporting electrolyte of 0.013 M phosphate buffer at pH 7.0, with a DC voltage of 400 V, an electrical field strength of 13.3 V/cm, an electrical current of 12 mA, a duration of 6 h, and a volume of plasma loaded with hemoglobin of 80–100 μL. Identification of the protein complexes, as well as free hemoglobin, was performed on filter paper sheets using a modified o-anisidine visualization method. The determination of individual protein spots on the same 2D electrochromatograms was done by additional staining of proteins with bromphenol blue (Figure 8).

Applications of TLE for Proteins (not Peptides)

Only a relatively few descriptions of TLE for proteins can be found, and they may be gleaned from the very earliest attempts at developing protein

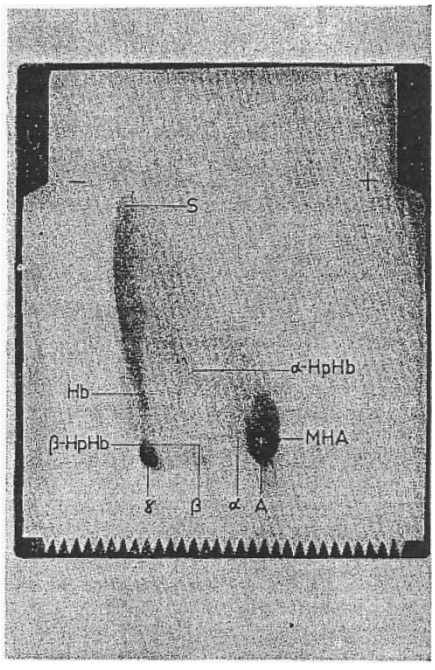

Figure 8. 2D electrophoretic separation on filter paper of hemoglobin-loaded plasma from an individual with hypohaptoglobinemia (30 mg% haptoglobin HBC, 60 mg% Hb). Staining was first performed for hemoglobin and then for proteins. Abbreviations: A = albumin; alpha = alpha-globulins; beta = beta-globulins; gamma = gamma-globulins; Hb = free hemoglobin; MHA = methemalbumin; alpha-HpHb = alpha-haptoglobin-hemoglobin complex; beta-HpHb = beta-haptoglobin-hemoglobin complex; gamma-GHb = hemoglobin bound to gamma-globulins; S = starting point.

separations.[76] For example, in one report, Reissell et. al. described the use of TLE on thin-layers of starch granules (not, apparently, a real TLC plate) to separate a series of serum lipoproteins under electrophoretic conditions, without pressurization of the plate. After separation, the lipids were extracted from the starch segments of the plate and then subjected to conventional TLC on a different stationary phase, and then quantified colorimetrically. This was then really a form of 2D TLE-TLC, though at that time (1966), the authors did not refer to this as a multidimensional separation, as we would today. Figure 7 then represents the TLE plate after development and spraying with Ninhydrin and Oil Red O spray reagents, often used in conventional TLC separations of serum lipoproteins. These samples represent fasting of the donor along with samples from a fat tolerance test as a function of time (hrs).[76] The individual serum lipoproteins are indicated in the leftmost column under the heading, albumin. Note that the band (spot) shapes in Figure 7 are perhaps less than ideal, in that they are quite broad and diffuse, not as found in

modern HPTLC spots. They appear more like FBE bands for the same lipoproteins, using SDS-PAGE as the separation medium. It is not entirely clear how the starch granules are functioning in this approach to TLE, similar to cellulose plates, perhaps in an SEC mode? Little is said in this or other, related papers (Hunter's) about the actual mechanism(s) of separation involved in TLE of proteins or peptides, whether it is SEC, IEC, HIC, or other modes?

HTLE/PEC INSTRUMENTATION

Overview of Instrument Requirements

The basic instrumentation required for performing HTLE or PEC is relatively simple. Unlike with HPLC, there are no specific requirements for pumps and plumbing relating to performing gradient elution of analytes. All that is required is a chamber having at least bottom and side walls defining an area for performing the separation, two regions within the chamber for containing a liquid mobile phase, a planar stationary phase (TLC plate) positioned between the first and second regions within the chamber and in contact with the liquid mobile phase, a pair of electrodes capable of electrical contact with opposing sides of the planar stationary phase, and a power source capable of generating an applied electric potential between the electrodes. The electrodes are typically made of non-reactive metals or graphite. Exemplary non-reactive metals include platinum, palladium, or gold. The electrodes may be in the shape of rectangular bars, wires, rods, or any other shape with sufficient length to substantially span the width of the stationary phase. Typically, the electrodes and the planar stationary phase are in contact with a planar wick. A wick is a solid or semisolid medium used to establish uniform electrical paths between the planar solid phase and the electrodes of a horizontal electrophoresis apparatus. The wick may consist of cellulose-based filter paper, rayon fiber, buffer-impregnated agarose gel, or even moistened paper towel. Additionally, a device for applying down pressure to the planar stationary phase seems to be a key aspect to performing PEC. The reason for applying the pressure is to minimize mobile phase evaporation, resulting from Joule heating caused by the passage of electrical current through the mobile phase.[81,129,130] The applied potential that drives separations is usually supplied by a high voltage DC power supply. The power supply may be controlled by a computer, a programmable controller, a microprocessor, a timer, or the like, in order to precisely control the separation conditions for more reproducible results.

Specific Instrument Setups

As mentioned earlier, a prototype instrument developed by the Hunter group is commercially available and this version of analysis has been referred to as

'Hunter's thin-layer electrophoresis'[70,78,79] (Model #HTLE-7002, 2005), Figures 9–11. This instrument seems to be a modification of the older versions previously described in the literature.[99,100,104] A safety interlock is present on this system to cut off the voltage in the absence of water circulation or to prevent electrical shock if opened inadvertently.[104] Figure 9 illustrates the placement of the power cords and coolant flow interlock, since the apparatus works best when the separation plate is water cooled during the electrophoresis. Figure 10 illustrates the apparatus actually ready to perform conventional, pressurized, and water cooled 1D or 2D type TLE separations, with the inflatable plastic bag like device used for pressurizing with air and the water cooled base acting as a heat sink. A cellulose plate spotted with peptides and pre-wetted according to the instructions in the manual is placed under pressure with two wicks overlapping the plate from either side. The wicks are immersed in the buffer contained in chambers that also come equipped with electrode circuitry to enable the application of an electric potential across the plates. Typically, an electrical potential of 1000 V is applied and a water circulator envelopes the surface where the plate rests and prevents any heating caused by the application of high potential.

In yet another instrumentation set up, aimed at the separation of phospho amino acids, a pre-coated silica sheet spotted with the analyte is placed in a gel casting tray and electrophoresis is performed in a Bio-Rad electrophoresis

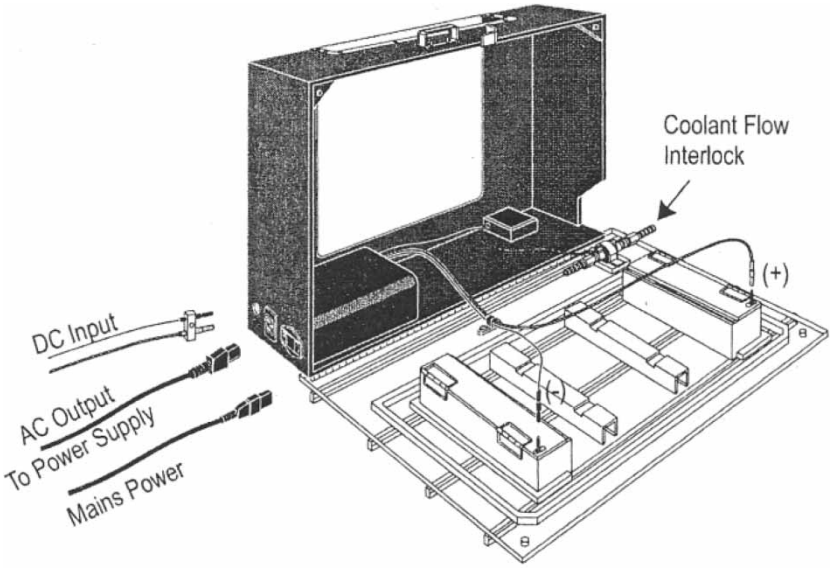

Figure 9. Schematic diagram displaying the base of the HTLE apparatus upon initial unpacking, prior to actual use, providing guidance regarding the basic unit set-up (CBS Scientific, Instruction Manual). Power cord and coolant attachment sites are indicated in the diagram.

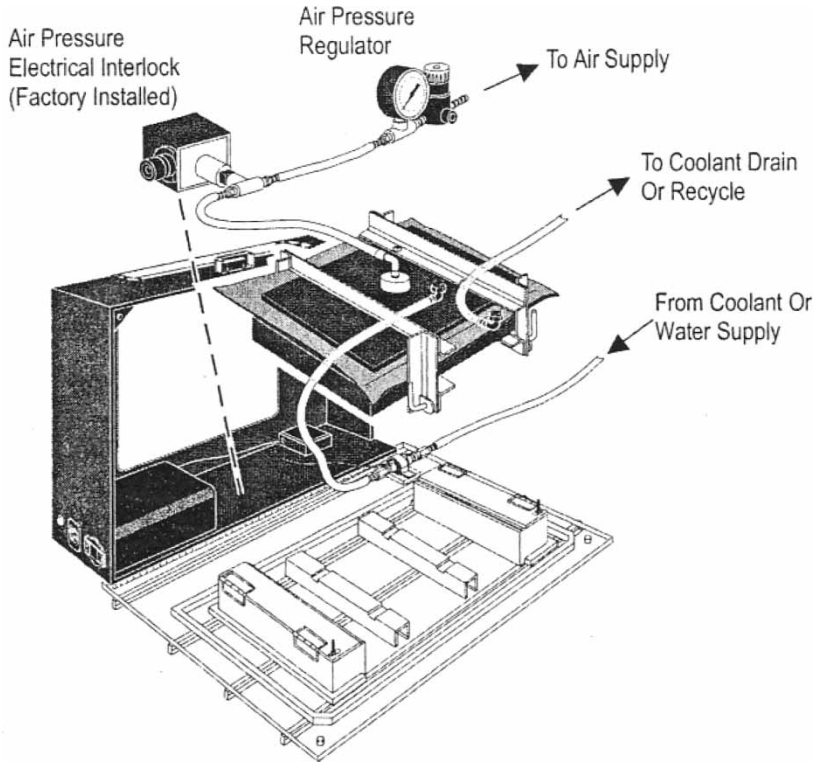

Figure 10. Schematic diagram of the HTLE apparatus fully assembled and ready for initial use (CBS Scientific, Instruction Manual). The inflatable nylon air bag assembly with black neoprene rubber cushion, teflon insulator sheet, and polyethylene protector sheets, for TLE plate pressurization, is visible in this diagram (pictured as suspended above the base unit). Ancillary equipment, such as air-pressure regulator and coolant hoses are also shown.

chamber.[131] In this case, buffer is added to the two electrode chambers and wicks from the buffers are in contact with the ends of the plate. A "low voltage" of 500 V or less is applied and the authors refer to this method as low voltage thin-layer electrophoresis (LV-TLE). Unlike the HTLE, the authors claim that no pressurization of the plate is required.

Dr. Nurok and colleagues describe a chamber used to perform pressurized planar electrochromatography (PPEC).[80,81] In the prototype described, the sorbent layer is covered by a material which acts as a thermal conductor and electrical insulator to prevent the heating of the layer. In order to apply pressure to the layer and reduce the accumulation of liquid on the surface, a hydraulic ram is used to press a die block covered with aluminum nitride onto the layer covered by PTFE. The long edges of the plate are coated with silicone rubber to prevent the movement of liquid off the plate and a

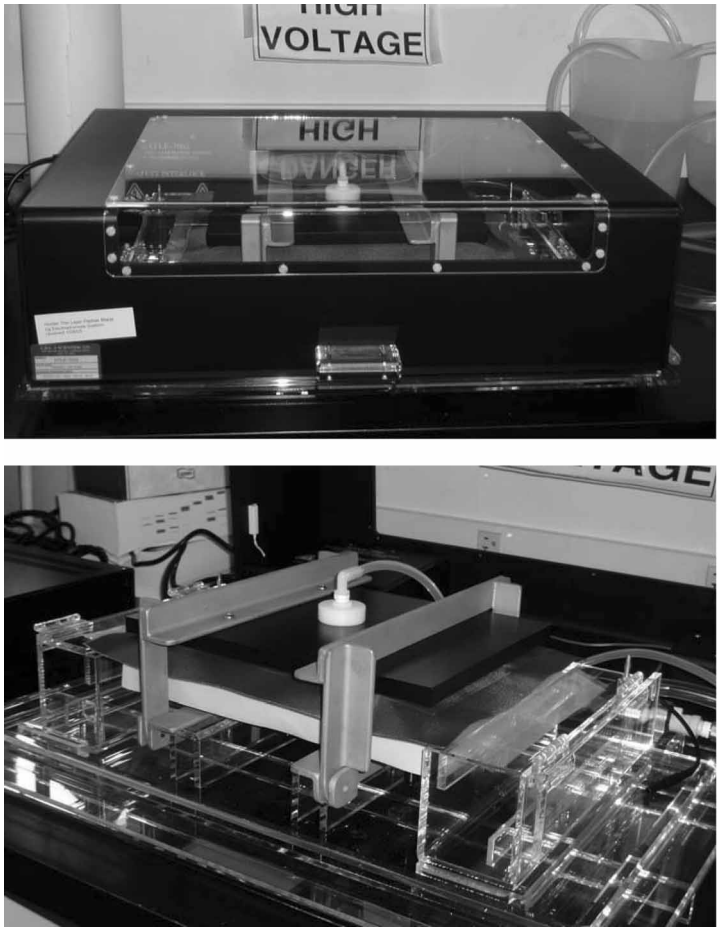

Figure 11. Photographs of the fully assembled HTLE apparatus. (A) Unit with safety interlock cover closed and ready for application of the electrical potential. (B) Unit with safety interlock cover opened in order to view instrument components. The TLC plate is located underneath the inflatable plastic bag, which is clamped to pressurize the plate.

filter paper wick removes liquid that accumulates on the surface. An electrode is placed along one side of the plate, unlike the other instruments whose electrodes lie in buffer reservoirs with wicks overlapping the plates. The high pressure and high voltage applied in PPEC provide high quality, rapid separations. Although this technique has yet to be utilized in the separation of proteins or peptides, there is no apparent reason why it could not be extended to these macromolecules.

Drs. Tate and Dorsey describe yet another instrument for PEC.[130] In this prototype, the TLC plate lies on a PVC covered aluminum block used to cool

the plate. Pressure is applied to the plate through the use of an external weight (~27 kg), providing about 1.0 atmosphere of applied pressure. Electrodes are located on the instrument base on opposite sides of the plate. Buffer is first placed in the positive reservoir and capillary action between a glass plate and reservoir wall brings the buffer into contact with the plate until the plate becomes completely wet. Buffer is then placed in the negative reservoir where the glass plate has the same effect. At this point, the power supply is turned on and data can be recorded using a unique cover grid where voltage is monitored at eight points between the reservoirs. Experimental data suggests after plate equilibration, the voltage and flow characteristics stay reasonably constant over time and across the plate. The investigators indicate that their instrument is somewhat limited, being unable to achieve electrical potentials higher than 2000 volts. They considered the Nurok instrument design superior, with 50 to 100 atmospheres of applied pressure allowing good separations with an applied potential of 7000 volts.[130]

Finally, Berezkin and coworkers present an alternative setup for PEC that does not require applied pressure, but simply a covered sorption layer and standard horizontal electrophoretic cell.[129] They argue that it is simply necessary to prevent mobile phase evaporation during PEC. Thus, according to their procedure, a thin polyethylene film is softened and applied to the absorption layer, resulting in strong adhesion between the film and the surface layer of absorbent particles on the plate. Separation of various organic dye mixtures has been demonstrated with this system, using applied electrical potentials of up to 4000 volts. The investigators determined that PEC according to their protocols resulted in higher rates of separation and less band broadening than obtained using conventional TLC with the same covered sorption layer. Using an applied electrical potential of 1000 volts, PEC separations were found to be 1.9 to 6.4 fold faster than comparable separations with TLC, depending upon the mobile phase and solid phase sorbent used for the actual fractionation.

EXPERIMENTAL CONSIDERATIONS

A number of key experimental parameters have been discussed in the TLE and PEC literature, while others may be inferred from the extensive literature on HPLC and CEC of peptides and proteins.[80,81,129,130] Key parameters have been identified as crucial to optimization efforts in order to minimize diffusion and obtain high resolution separations. These parameters can be summarized as follows:

Mobile Phase Compositions

The isoelectric point or net charge of the peptides/proteins at a given pH value and the extent of hydrophobicity/hydrophilicity determine the optimum

mobile phase to be used in the analytic separation. As with other chromatographic methods, the liquid mobile phase can be a purely aqueous or an aqueous mixture containing a water miscible organic liquid. For example, the liquid mobile phase may be a methanol-aqueous buffer; acetonitrile-aqueous buffer; ethanol-aqueous buffer; isopropyl alcohol-aqueous buffer; butanol-aqueous buffer; isobutyl alcohol-aqueous buffer; carbonate-aqueous buffer, or any of a wide range of other buffer systems found suitable for separation of peptides and proteins by HPLC or CEC. By utilizing different mobile phases in the first and second dimension, maximum analyte resolution can be obtained. As with CEC, mobile phases rich in organic modulators will exhibit relatively little chromatographic retention and in mobile phases low in organic modulator, chromatographic retention will tend to dominate the separation process. Different cathode and anode buffers can certainly be used as a discontinuous buffer system for the separation of peptides and proteins by PEC. In fact, the stationary phase could be incubated in a buffer that is compositionally different from either electrode buffer. Additives, such as carrier ampholytes may also be included in the buffer in which the stationary phase is incubated. Finally, the composition of the mobile phase may be altered temporally to provide a composition gradient that facilitates separation of peptides and proteins. In 2D separation of peptides and proteins by PEC, the sample may be applied to the center of the TLC plate (dry or pre-wetted with mobile phase) or elsewhere on the plate, should certain knowledge regarding extent of migration and direction already be available. The stationary phase may then be incubated in a mobile phase and an electrical potential applied. Once the proteins are separated in one direction, the planar stationary phase may be washed (or allowed to evaporate) and incubated in a second mobile phase, and then separated in a direction perpendicular to the first direction. Joule heating occurs as current passes through the plate surface and heat is generated due to the resistivity of the plate material. Nurok et. al. describe the fine balance that exists between evaporation of the accumulating buffer caused by Joule heating and overheating the plate.[80,81] Ionic strength diminishes the electrical double layer at the plate interface and increases Joule heating, which helps prevent flooding by increasing evaporation. A cooling jacket around the plate circulating water helps prevent any significant heating. Excessive cooling could also accumulate or condense water on the plate surface. The liquid mobile phases can be adjusted to different pH values, concentrations of organic solvent, and ionic strengths to facilitate 2D separations of peptides or proteins by PEC.

Planar Stationary Phase

To date, PEC and HTLE of peptides and proteins have been performed almost exclusively on silica- and cellulose based solid phase media, respectively. As with HPLC and CEC, it is anticipated that other planar stationary phases,

including silica based thin-layer chromatography resin derivatized with alkyl groups (e.g., C_3 to C_{18} surface chemistry), aromatic groups, or cyanoalkyl groups will find application in PEC. For separation of proteins in the 10 and 100 kDa range using a silica based stationary phase, it is expected that derivitization with C_8 and C_4 groups, respectively, will be useful. Phenyl functionalities are slightly less hydrophobic than C_4 functionalities and may be advantageous for the separation of certain polypeptides. The ideal planar stationary phase for PEC should include pores or connected pathways of a dimension that permits unimpeded migration of the proteins. For particulate stationary phases, such as silica TLC plates, the stationary phase should consist of particles that form pores of about 30–100 nanometers in diameter, although for some smaller peptides with molecular weights of 2,000 daltons or less, 10 nanometers pores may be acceptable. Typical adsorbants commercially available for thin-layer chromatography are made of particles that form pores sizes of only 1–6 nm. The particles may have a diameter of about 5–50 microns, with the smaller diameter particles expected to produce higher resolution protein separations. For higher protein loads, large particle absorbents are preferable. This is particularly advantageous for the preparative scale isolation of proteins. The size distribution of the particles should be relatively narrow and particles are preferably spherical, rather than irregularly shaped. While the base material of the particles can be silica, synthetic polymers, such as polystyrene-divinylbenzene are also expected to be appropriate. Besides particulate thin-layer chromatography substrates, large pore mesoporous substrates, grafted gigaporous substrates, gel-filled gigaporous substrates, nonporous reversed phase packing material, and polymeric monoliths should be applicable to PEC of peptides and proteins.

Preconditioning of TLC/HPTLC plates has been well documented and is routinely followed in QA/QC laboratories for separation of a variety of analytes. In order to obtain reproducible results, precoated plates should be heated to $>100^{\circ}C$ and stored in a desiccating chamber before using them. This will ensure uniform moisture content and reproducibility. General applicability of plate preconditioning to PEC is not fully defined as of yet and cellulose plates are not typically subjected to a preconditioning step.

It should be stressed that as no commercially available TLC plates are currently available with the specifications listed above, no published work on protein separations by PEC can be looked to for guidance. Instead, the authors have provided their best estimates of appropriate media based upon years of experience in the separations sciences, especially borrowing heavily from the HPLC and CEC fields.

Sample Preparation, Loading, and Handling

Peptide or protein samples should typically be prepared by first dissolving the materials in the mobile phase or a weaker solvent of lower ionic

strength. Often, "biological buffers", such as Good's buffers (eg., TRIS, HEPES, MES), are useful for sample preparation. These biological buffers produce lower currents than inorganic salts, thereby allowing the use of higher sample concentrations and higher field strengths. If salts are used to facilitate extraction and isolation of the protein specimen, desalting of protein samples may be performed using reverse phase resins followed by organic solvent based protein precipitation or sample dialysis prior to sample fractionation by PEC. It is anticipated that protein samples may be prepared by first dissolving the proteins in HPLC solvent systems, thereby avoiding the use of detergents, chaotropes, and strong organic acids for protein dissolution.[132,133] HPLC solvent systems may include buffered solutions containing organic solvents, such as methanol or acetonitrile. For example, 60% methanol or acetonitrile, 40% water containing 0.1% formic acid or 60% methanol or acetonitrile, 40% 50 mM ammonium carbonate, pH 8.0 may be suitable sample solubilization buffers. Typically, final protein concentration in the solubilization buffer should be from about 0.4 mg/mL to about 0.6 mg/mL. Extraction and solubilization of proteins can be facilitated by intermittent vortexing and sonication. Surfactants are well known to suppress peptide ionization in mass spectrometry and also to interfere with chromatographic separations, particularly with reversed-phase liquid chromatography. Buffered solutions containing organic solvents are more compatible with liquid chromatography and mass spectrometry and, thus, should facilitate characterization of the proteins after PEC. Another important advantage of the buffered organic solvent extraction procedure is that it facilitates solubilization, separation, and identification of integral membrane proteins, including proteins containing transmembrane-spanning α-helices.

Various spot volumes, sizes, and shapes, as used in TLC, can also be used in HTLE/PEC.[10] TLC plates are usually dried with nitrogen after spotting, and spots confined to a minimum size ($\sim$2 mm in diameter) tend to give better resolution. Streaking occurs if the sample is overloaded. Typically, cellulose plates can separate up to 100 μg of sample material. A variety of devices designed for dispensing a sample on to TLC plates have been devised over the years. These dispensers can be manual or automated. For example, the manual dispenser can be a pipette, piezo-electric dispensing tip, solid pin, or quill pin. Automated dispensing may be achieved using general purpose liquid handling robotics or dedicated liquid handlers developed specifically for the task, such as the Automatic TLC Sampler (ATS 4; Camag, Muttenz, Switzerland). Care has to be taken to wet the plate so that there is no flooding and the spotted area does not spread out. For example, when wetted correctly, the cellulose plate appears dull gray, while a plate that is overly wet will appear glossy. Whatman 3MM or equivalent filter paper, devoid of any impurities, transfers the buffer at a nominal rate, minimizing diffusion that can lead to band broadening and streaking. Also, it was observed that if the size of the wick extends beyond the plate

area or overlaps the plate more than a couple of centimeters, buffer tends to accumulate at the edges of the plate causing diffusion.

Pressurization vs. Atmospheric Pressure PEC/HTLE

As the TLC sheets have a very thin coating of the stationary phase, the mobile phase has a tendency to rise up to the surface due to capillary action.[60] Pressurizing the plate counteracts this and leads to a better resolution. In HPTLC, decades ago researchers realized that separations were much more reproducible and of higher plate counts when the plates were somehow pressurized. Attempts to perform PEC without plate pressurization are, in general, less efficient and of lower resolution than when pressure is applied to the plate during the electrophoretic/electroosmotic stages of these separations. Without pressurization, there is some degree of solvent evaporation and it also appears that with pressurization, there is a more constant level of solvent permeation throughout the cellulose or silica based TLC plates, just as occurs in pressurized HPTLC (as discussed elsewhere in this review). However, as discussed earlier, simply using a covered sorption layer may be sufficient to ameliorate problems associated with evaporation. The evaporation of the mobile phase during PEC can result in decreased current, drying of the surface, and subsequent degradation in the quality of the separation, leading to overall poor reproducibility of the method. The degree of pressurization can be varied from run-to-run, if so desired, until optimum resolution and spot shapes are realized. This is sometimes optimized by a trial-and-error approach, but recommended pressures to be applied when beginning with the CBS Scientific HTLE apparatus are suggested by the manufacturer.[8,79] Figures 10–11 show the commercialized HTLE apparatus that provides for a constant pressurization of the TLE plates.

DETECTION OF PROTEINS AND PEPTIDES

Peptides and proteins may be detected after PEC or TLE using a variety of modalities borrowed from the 1DGE and 2DGE literature.[134] Exemplary strategies employed for protein and peptide detection include organic dye staining, silver staining, radio-labeling, fluorescent staining (pre-labeling, post-staining), chemiluminescent detection, mass spectrometry based approaches, negative-imaging approaches, contact detection methods, direct measurement of the inherent fluorescence of proteins, evanescent wave, label free mass detection, optical absorption and reflection. Historically, ninhydrin, ninhydrin-cadmium, dansyl chloride, fluorescamine, and o-phthalaldehyde have been regularly employed to detect peptides from protein digests.[111]

In negative imaging approaches, the peptides or proteins remain unlabeled, but the planar surface itself contains a fluorescent indicator that

is detected. The protein or peptide is visualized as a shadow against the fluorescent background. Ultraviolet light-excitable F_{254} and F_{366} fluorescent TLC plates are commercially available. We have found that ninhydrin stained peptides may readily be imaged from cellulose TLC plates through negative imaging of the low fluorescence background of the plates. Typically, the plates are excited using a xenon-arc lamp source with 480 nm excitation bandpass filter and fluorescent signal is collected with a 530 nm emission bandpass filter. The ProXPRESS® 2D Proteomic Imager (PerkinElmer) provides the requisite capabilities for this type of imaging.

In contact detection methods, a membrane or filter paper that has been imbibed with a substrate is placed in contact with the planar surface, and protein species resident on the planar stationary phase interact with the substrate molecules to generate a product. In direct measurement of the inherent fluorescence of proteins, solid-phase supports of low inherent fluorescence are used and detection is based upon the innate fluorescence of tryptophan and tyrosine residues. Detection methods suitable for revealing protein post-translational modifications include methods for the detection of glycoproteins, phosphoproteins, proteolytic modifications, S-nitrosylation, arginine methylation, and ADP-ribosylation. Methods for the detection of a range of reporter enzymes and epitope tags include methods for visualizing β-glucuronidase, β-galactosidase, oligohistidine tags, and green fluorescent protein. These analytical approaches have recently been reviewed extensively and most should be applicable to TLE and PEC.[134] Peptide and protein samples that have undergone PEC appear as discrete spots on the TLC plate that are accessible to staining or immunolabeling, as well as to analysis by various detection methods. Other detection methods suitable for TLE and PEC include mass spectrometry, Edman-based protein sequencing, or other micro-characterization techniques. Proteins bound to the TLC plate should be highly accessible to a variety of labeling reagents, such as, antibodies, peptide antibody mimetics, oligonucleotide aptamers, or even quantum dots. The TLE and PEC format should be particularly amenable to chemiluminescence based detection of peptides and proteins. For example, proteins could be biotinylated and then detected using horseradish peroxidase conjugated streptavidin and standard Western blotting chemiluminescence kits. The TLC plate itself serves as a mechanically strong support, allowing archiving of the separation profiles without the need for vacuum gel drying, as required with conventional polyacrylamide gels.

As alluded to earlier, the primary application of HTLE to date has been for the evaluation of phosphorylated peptides derived from ^{32}P-labeled proteins using autoradiogarphic film or storage phosphor plates. Other approaches to performing phosphopeptide and phosphoprotein analysis are indeed possible today, not requiring the use of radiolabels or their emission counters. For example, the recently commercialized Pro-Q® Diamond phosphoprotein stain (Molecular Probes) detects phosphoproteins in polyacrylamide slab gels, on polymeric membranes used for electroblotting, and on

protein microarrays through a mechanism that combines a fluorescent metal ion-indicator dye and a trivalent transition metal cation titrated to acidic pH value.[135–137] The stain has also recently been adapted to phosphate based quantification of phosphoproteins and phosphopeptides from solution and detection of phosphopeptides by high performance liquid chromatography. The staining technique is rapid, simple to perform, readily reversible, and fully compatible with analytical procedures such as MALDI-TOF mass spectrometry. Figure 12A shows the separation of β-casein digest on silica 60 plate using 1D–PEC/TLE followed by selective staining with Pro-Q® Diamond phosphoprotein stain. Separation of the 3 μL peptide spot on the silica 60 HPTLC plate was achieved with pH 4.7 buffer (n-butanol/pyridine/glacial acetic acid/water, 50 : 25 : 25 : 900, v/v/v/v). Figure 12B shows all the separated peptides derivatized by fluorescamine, indicating that the phosphopeptides migrated to the left of the origin. Further resolution can be achieved using a second dimension of electrochromatography, with an appropriate solvent system.

Alternatively, detection of phosphorylated peptides should readily be performable by standard immunostaining procedures using phosphoamino acid and phosphorylation state-specific antibodies. Analogous immunostaining procedures have already been devised for the detection of specific oligosaccharides, phospholipids, and glycolipids after TLC.[138–141] Finally, based upon successful direct detection of phosphoproteins on electroblot membranes, it is likely that laser ablation inductively-coupled plasma mass spectrometry (ICP-MS) can be employed to directly measure phosphorous as an m/z 31 signal liberated from phosphoproteins or phosphopeptides displayed on PEC or TLE plates, without the use of radiolabels or surrogate dyes and antibodies.[142] Apparently, neither the Hunter group nor CBS

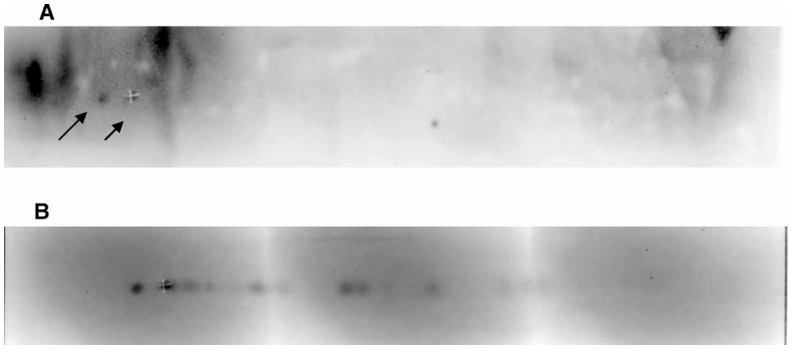

Figure 12. Separation of a β-casein peptide digest on silica 60 plate using 1D–PEC, followed by selective staining with Pro-Q® Diamond phosphoprotein stain (A). The separated peptides were subsequently derivatized using fluorescamine in order to visualize all the peptides (B). Comparison of (A) and (B) indicates that the phosphopeptides migrated to the left of the origin.

Scientific have yet to develop simpler, alternative methods for detecting the phosphopeptides or phosphoproteins that can be resolved using their HTLE apparatus and approaches.

POTENTIAL PROTEOMICS APPLICATIONS FOR PEC

Proteomics studies are often based upon the comparison of different protein profiles. The primary motive for conducting differential display proteomics experiments is to increase the information content of proteomics studies through multiplexed analysis. Currently, two gel based approaches to differential display proteomics are widely employed, difference gel electrophoresis (DIGE) and Multiplexed Proteomics (MP).[134] Once fully developed, PEC could potentially be used with difference gel electrophoresis (DIGE) to increase the information content of proteomics studies through multiplexed analysis. Succinimidyl esters of the cyanine dyes (e.g., Cy2, Cy3, and Cy5) can be employed to fluorescently label as many as three different complex protein populations prior to mixing and running them simultaneously on the same 2D gel using DIGE. Images of the 2D gels are acquired using three different excitation/emission filter combinations, and the ratio of the differently colored fluorescent signals is used to find protein differences among the samples. DIGE allows two to three samples to be separated under identical electrophoretic conditions, simplifying the process of registering and matching the gel images. DIGE can be used to examine differences between two samples (e.g., drug treated Vs-control cells or diseased Vs-healthy tissue). The principle benefit that PEC might offer with respect to DIGE is that protein separations could be achieved more quickly and samples could more readily be evaluated by MS after profile differences are determined. One requirement of DIGE is that from about 1% to about 2% of the lysine residues in the proteins be fluorescently modified, so that the solubility of the labeled proteins is maintained during electrophoresis. It is likely that very high degrees of labeling could be achieved when separations are performed by the PEC technique, due to the fact that organic solvents can be employed in the mobile phase and sample buffers. High degrees of labeling should, in turn, dramatically improve detection sensitivity using the DIGE technology.

PEC might also be used with Multiplexed Proteomics to increase the information content of proteomics studies through multiplexed analysis. The Multiplexed Proteomics (MP) platform is designed to allow the parallel determination of protein expression levels, as well as certain functional attributes of the proteins, such as levels of glycosylation, levels of phosphorylation, drug binding capabilities, or drug metabolizing capabilities. The MP technology platform utilizes the same fluorophore to measure proteins across all gels in a 2DGE database, and employs additional fluorophores with different excitation and/or emission maxima to accentuate specific functional attributes

of the separated species. With the MP platform, a set of 2D gels is fluorescently stained and imaged to reveal some functional attribute of the proteins, such as drug-binding capability, or a particular post translational modification. Then, protein expression levels are revealed in the same gels using a fluorescent total protein stain. However, in MP, the gels must be serially stained and imaged, as succeeding stains mask their predecessors in polyacrylamide gels. It is expected that PEC can be used to assist MP in simultaneous imaging of multiple signals on profiles generated. We have observed that fluorescent dyes do not have the same strong tendency to mask one another on solid phase supports, compared with polymeric gels.

PEC should be highly compatible with MALDI-TOF MS for direct analysis of peptides and proteins.[143] After separation, the proteins are displayed on solid phase supports in a manner that is amenable to direct probing with a MALDI-TOF laser. For example, the analytes might be probed with an orthogonal MALDI-TOF mass spectrometer (e.g., prOTOF 2000® PerkinElmer, Boston, MA, USA/MDS Sciex, Concord, ON, Canada). The prOTOF 2000® MALDI O-TOF mass spectrometer is a MS MALDI with orthogonal time of flight technology. The prOTOF's novel design provides improved instrument stability, resolution, and mass accuracy across a wide mass range compared with conventional linear or axial based systems. The more accurate and complete protein identification achieved with the prOTOF instrument's prOTOF 2000® instrument reduces the need for peptide sequencing using more complicated tandem mass spectrometry techniques such as Q-TOF and TOF-TOF. The instrument is particularly well suited for PEC because the MALDI source is decoupled from the TOF analyzer. As a result, any discrepancies arising from the solid phase surface topography or differential ionization of the sample from the surface are eliminated before the sample is actually delivered to the detector. The presentation of the peptides or proteins bound to a solid phase surface facilitates removal of contaminating buffer species and exposure to protein cleavage reagents (e.g., trypsin) prior to analysis by MS. The use of HPLC based buffers in the fractionation process minimizes the potential for downstream interference by detergents and chaotropes during MS based analysis. "Virtual" 2D profiles could be generated by 1D PEC separations, followed by desorbing proteins directly from the planar substrate using MALDI-TOF mass spectrometry, in effect substituting mass spectrometry for SDS polyacrylamide gel electrophoresis. Analytical data obtained could be presented as a computer generated image with 2D gel type appearance. While a similar approach has already been taken with immobilized pH gels, the procedure is currently quite slow, requiring a day to run the gel, two days to dry it down, and two days to acquire spectra.[144,145] The accessible nature of the PEC support offers a definite opportunity to streamline the analytical process substantially.

In an analogous manner as DIGE based imaging of fluorescent signal, peptides and proteins could be detected by mass tagging approaches, using

MALDI-TOF mass spectrometry. For example, Isotope coded affinity tag (ICAT) peptide labeling is a mass tagging approach useful for distinguishing between two populations of proteins using isotope ratios. ICAT reagent employs a reactive functionality specific for the thiol group of cysteine residues in proteins and peptides. In the classic ICAT method, two different isotope tags are generated by using linkers that contain either eight hydrogen atoms (d0, light reagent) or eight deuterium atoms (d8, heavy reagent). A reduced protein mixture from one protein specimen is derivatized with the isotopically light version of the ICAT reagent, while the other reduced protein specimen is derivatized with the isotopically heavy version of the ICAT reagent. The combined sample could then be fractionated by PEC. The gel separated proteins would then be treated with protease and identified by peptide mass profiling. The ratio of the isotopic molecular weight peaks that differ by 8 daltons, as revealed by MS, would provide a measure of the relative amounts of each protein from the original samples. 2D PEC could be combined with mass tag labeling into a single platform for differential display proteomics. PEC would conceivably provide much faster separations and the proteins would be more amenable to downstream mass spectrometry-based analysis. Already, there have been several reports regarding the interfacing of TLC with MALDI-TOF MS.[143,146–149]

SUMMARY AND CONCLUSIONS

PEC could potentially provide a high resolution protein and peptide separation system that employs a chromatographically based solid phase support and simple combinations of organic and aqueous mobile phases to facilitate the fractionation of biological species by a combination of electrophoretic and/or chromatographic mechanisms. Features of the envisioned separation system include mechanical stability of the separating medium, accessibility of the analytes to post separation characterization techniques (immunodetection, mass spectrometry), ability to fractionate hydrophobic analytes and large molecular complexes, as well as minimal sample consumption, and number of manual manipulations and timelines for performing the actual fractionation. As such, PEC may offer clear advantages over the well established proteomics technologies, 2DGE and 2D LC/MS/MS. The future implementation of PEC as an analytical approach for peptide and protein separations depends upon a critical mass of scientists investing their time, energy, and intellect in developing the fledgling methodology.

GLOSSARY

CCC	counter current chromatography
CE	capillary electrophoresis

CFCE	carrier-free continuous electrophoresis
DEAE	diethylaminoethyl
1D	one dimensional
1DGE	one dimensional gel electrophoresis
2D	two dimensional
2DGE	two dimensional gel electrophoresis
DIGE	difference gel electrophoresis
2D LC/MS/MS:	two-dimensional liquid chromatography-tandem mass spectrometry
EC	electrochromatography
FBE	flat bed electrophoresis
FFF	field flow fractionation
GFC	gel filtration chromatography
GC	gas chromatography
HEPES	2-(4-(2-Hydroxyethyl)-1-piperazinyl)ethanesulfonic Acid
HPCE	high performance capillary electrophoresis
HPLC	high performance liquid chromatography
HTLE	Hunter thin-layer electrophoresis
HPTLC	high performance thin-layer chromatography
IEC	ion exchange chromatography
LC	liquid chromatography
MES	2-(N-Morpholino)ethanesulfonic Acid
MP	Multiplexed Proteomics
MS	mass spectrometry
MW	molecular weight
MDLC	multidimensional liquid chromatography
PAGE	polyacrylamide gel electrophoresis
PEC	planar electrochromatography
PC	paper chromatography
SDS	sodium dodecyl sulfate
SEC	size exclusion chromatography
PKI	PerkinElmer Corporation
TLC	thin-layer chromatography
TLE	thin-layer electrophoresis
TRIS	Tris(hydroxymethyl)aminomethane

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REFERENCES

1. Patton, W.F.; Schulenberg, B.; Steinberg, T.H. Two-dimensional gel electrophoresis; better than a poke in the ICAT? Curr. Opin. Biotechnol. **2002**, *13* (4), 321–8.
2. Monteoliva, L.; Albar, J.P. Differential proteomics: an overview of gel and non-gel based approaches. Brief Funct. Genom. Proteom. **2004**, *3* (3), 220–39.
3. Righetti, P.G.; Campostrini, N.; Pascali, J.; Hamdan, M.; Astner, H. Quantitative proteomics: a review of different methodologies. Eur. J. Mass Spectrom. **2004**, *10* (3), 335–48.
4. Resing, K.A.; Ahn, N.G. Proteomics strategies for protein identification. FEBS Lett. **2005**, *579* (4), 885–9.
5. Aebersold, R.; Mann, M. Mass spectrometry-based proteomics. Nature **2003**, *422* (6928), 198–207.
6. Berezkin, V.G. The discovery of thin-layer chromatography. J. Planar Chromatogr. **1995**, *8*, 401–401.
7. Poole, C.F. Planar chromatography at the turn of the century. J. Chromatogr. A **1999**, *856* (1–2), 399–427.
8. Poole, C.F.; Dias, N.C. Practitioner's guide to method development in thin-layer chromatography. J. Chromatogr. A **2000**, *892* (1–2), 123–42.
9. Poole, C.F. Thin-layer chromatography: challenges and opportunities. J. Chromatogr. A **2003**, *1000* (1–2), 963–84.
10. Hahn-Deinstrop, E. *Applied Thin-Layer Chromatography; Best Practices and Avoidance of Mistakes*; Wiley-VCH: NY, 2000.
11. Stahl, E.; Ed. *Thin-Layer Chromatography, A Laboratory Handbook*; Springer-Verlag: Berlin, Germany, 1965.
12. Getz, M.E. *Paper and Thin-Layer Chromatographic Analysis of Environmental Toxicants*; Heyden & Son Ltd.: London, 1980.
13. Touchstone, J.C.; Ed. *Quantitative Thin Layer Chromatography*; J. Wiley & Sons: NY, 1973.
14. Issaq, H.J.; Cazes, J.; Eds. Special Issue of J. Liquid Chromatogr. *Forty Years of Chromatography*; Marcel Dekker: NYC, 1987; 10/8–9.

15. Issaq, H.J.; Ed. *A Century of Separation Science*; Marcel Dekker: NYC, 2002.
16. Cazes, J.; Ed. *Encyclopedia of Chromatography*; Marcel Dekker: NYC, 2001.
17. Zlatkis, A.; Kaiser, R.E.; Eds. *HPTLC, High Performance Thin-Layer Chromatography*; Elsevier Publishing Co.: Amsterdam, 1977.
18. Fried, B.; Sherma, J. *Thin-Layer Chromatography, Techniques and Applications*; Marcel Dekker: NYC, 1982.
19. Hais, I.M.; Macek, K.; Eds. *Paper Chromatography, A Comprehensive Treatise*; Academic Press: London, 1963.
20. Heftsman, E.; Ed. *Chromatography, Fifth Edition*; Elsevier Science Publishing Co: Amsterdam and New York, 1992; Parts A and B.
21. Brown, P.R.; Hartwick, R.A.; Eds. *High Performance Liquid Chromatography*; Wiley-Interscience: New York, 1989.
22. Hancock, W.S.; Ed. *High Performance Liquid Chromatography in Biotechnology*; Wiley & Sons: NY, 1990.
23. Bidlingmeyer, B.A. *Practical HPLC Methodology and Applications*; Wiley & Sons: NY, 1992.
24. Neue, U. *HPLC Columns, Theory, Technology, and Practice*; Wiley-VCH Publishers: New York, 1997.
25. Snyder, L.; Kirkland, J.J.; Glajch, J. *Practical HPLC Method Development*; Wiley-Interscience Publishers: New York, 1997.
26. Lunn, G.; Schmuff, N.; Eds. *HPLC Methods for Pharmaceutical Analysis*; Wiley-Interscience Publishers: New York, 1997 (CD-ROM).
27. Freeman, R.R.; Ed. *High Resolution Gas Chromatography*; Hewlett-Packard Company, 1981.
28. Jennings, W. *Gas Chromatography With Glass Capillary Columns*, Second Edition; Academic Press: San Diego, CA, 1980.
29. Jennings, W. *Analytical Gas Chromatography*; Academic Press: San Diego, CA, 1987.
30. Grob, R.L.; Ed. *Modern Practice of Gas Chromatography*, Second Edition; Wiley & Sons: NY, 1985.
31. Lethard, D.A.; Shurlock, B.C. *Identification Techniques in Gas Chromatography*; Wiley-Interscience: NY, 1970.
32. Jaeger, H.; Ed. *Glass Capillary Chromatography in Clinical Medicine and Pharmacology*; Marcel Dekker: NY, 1985.
33. Aboul-Enein, H.Y.; Ed. *Analytical and Preparative Separation Methods of Biomacromolecules*; Marcel Dekker: New York, 1999.
34. Bhown, A.S.; Ed. *Protein/Peptide Sequence Analysis: Current Methodologies*; CRC Press: Boca Raton, FL, 1987.
35. Hancock, W.S.; Ed. *New Methods in Peptide Mapping for the Characterization of Proteins*; CRC Press: Boca Raton, FL, 1996.
36. Karger, B.L.; Hancock, W.S.; Eds. *High Resolution Separation and Analysis of Biological Macromolecules, Part A, Fundamentals, Methods in Enzymology*; Academic Press: San Diego, CA, 1996; Vol. 270.
37. Robyt, J.F.; White, B.J. *Biochemical Techniques, Theory and Practice*; Waveland Press: Prospect Heights, IL, 1987.
38. Janson, J.-C.; Ryden, L.; Eds. *Protein Purification, Principles, High Resolution Methods, and Applications*, Second Edition; John Wiley & Sons: New York, 1998.
39. Doonan, S.; Ed. *Protein Purification Protocols*; Humana Press: Totowa, NJ, 1996.

40. Bollag, D.M.; Rozycki, M.D.; Edelstein, S.J. *Protein Methods*; John Wiley & Sons: New York, 1996.
41. Gordon, A.H. *Electrophoresis of Proteins in Polyacrylamide and Starch Gels*; North-Holland American Elsevier: Amsterdam, 1969.
42. Everaerts, F.M.; Mikkers, F.E.P.; Verheggen, Th.P.E.; MVacik, J. Electrophoresis. In *Chromatography: Fundamentals and Applications of Chromatographic and Electrophoretic Methods: Part A, Fundamentals and Techniques*; Elsevier Science Publishing Co.: Amsterdam, 1983.
43. Righetti, P.G. *Isoelectric Focusing: Theory, Methodology, and Applications*; Elsevier: Amsterdam, 1983.
44. Ehrambach, X. *The Practice of Quantitative Gel Electrophoresis*; VCH Publishers: Germany, 1985.
45. Heftmann, E.; Ed. *Chromatography: Fundamentals and Applications of Chromatographic and Electrophoretic Methods, Parts A and B, Journal of Chromatography Library*; Elsevier: Amsterdam, 1983, 1984; Vol. 22A and 22B.
46. Tracy, R.P. *High-Resolution, Two-Dimensional, Gel Electrophoresis of Proteins: Basic Concepts and Recent Advances in Chromatography and Separation Chemistry, Advances and Concepts*; Ahuja, S., Ed.; ACS Symposium Series No. 297; American Chemical Society: Washington, DC, 1984.
47. *Protein Electrophoresis, a Technical Manual*; Amersham Pharmacia Biotech: San Francisco, CA, 1999.
48. *2-D Electrophoresis Using Immobilized pH Gradients, Principles and Methods*; Amersham Pharmacia Biotech: San Francisco, CA, 1998.
49. *2-D Electrophoresis for Proteomics, A Methods and Product Manual*; Bulletin 2651, Bio-Rad Corporation: Hercules, CA, 2002.
50. Jorgenson, J.W.; Phillips, M.; Eds. *New Directions in Electrophoretic Methods*; ACS Symposium Series,, American Chemical Society: Washington, D.C., 1987; Vol. 335.
51. Horvath, Cs.; Nikelly, J.G.; Eds. *Analytical Biotechnology: Capillary Electrophoresis and Chromatography*; ACS Symposium Series, American Chemical Society: Washington, DC, 1990; Vol. 434.
52. Li, S.F.Y. *Capillary Electrophoresis: Principles, Practice and Applications*; Elsevier Science Publishers: Amsterdam, 1992.
53. Grossman, P.D.; Colburn, J.C.; Eds. *Capillary Electrophoresis-Theory and Practice*; Academic Press: San Diego, CA, 1992.
54. Guzman, N.; Ed. *Capillary Electrophoresis-Technology*; Marcel Dekker: New York, 1993.
55. Weinberger, R. *Practical Capillary Electrophoresis*; Academic Press: San Diego, CA, 1993.
56. Landers, J.P.; Ed. *CRC Handbook of Capillary Electrophoresis: Principles, Methods, and Applications*; CRC Press: Boca Raton, FL, 1994.
57. Baker, D.R. *Capillary Electrophoresis, Techniques in Analytical Chemistry Series*; J. Wiley & Sons Inc.: New York, 1995.
58. Righetti, P.G.; Ed. *Capillary Electrophoresis in Analytical Biotechnology*; CRC Series in Analytical Biotechnology; CRC Press: Boca Raton, FL, 1996.
59. Camilleri, P.; Ed. *Capillary Electrophoresis, Theory and Practice*; CRC Press: Boca Raton, 1993.
60. Mosher, R.A.; Thormann, W. *The Dynamics of Electrophoresis*; VCH Publishers: Weinheim, FRG, 1992, Chapter 7.
61. Altria, K.D.; Ed. *Capillary Electrophoresis Guidebook: Principles, Operation, and Applications*; Humana Press: Totowa, NJ, 1996.

62. Weinberger, R.; Lombardi, R. *Method Development, Optimization and Troubleshooting for High Performance Capillary Electrophoresis*; Simon & Schuster Custom Publishing: Needham Heights, MA, 1997.
63. Cunico, R.L.; Gooding, K.M.; Wehr, T. *Basic HPLC and CE of Biomolecules*; Bay Bioanalytical Laboratory: Richmond, CA, 1998.
64. Wehr, T.; Rodriguez-Diaz, R.; Zhu, M. *Capillary Electrophoresis of Proteins*; Marcel Dekker: New York, 1999.
65. Weinberger, R. *Practical Capillary Electrophoresis*, Second Edition; Academic Press: San Diego, CA, 2000.
66. Bhushan, R.; Mahesh, V.K.; Mallikharjun, P.V. Thin layer chromatography of peptides and proteins: a review. Biomed. Chromatogr. **1989**, *3* (3), 95–104.
67. Luo, Q.; Andrade, J.D.; Caldwell, K.D. Thin-layer ion-exchange chromatography of proteins. J. Chromatogr. A **1998**, *816* (1), 97–105.
68. Baczek, T.; Marszall, M.P.; Kaliszan, R.; Walijewski, L.; Makowiecka, W.; Sparzak, B.; Grzonka, Z.; Wisniewska, K.; Juszczyk, P. Behavior of peptides and computer-assisted optimization of peptides separations in a normal-phase thin-layer chromatography system with and without the addition of ionic liquid in the eluent. Biomed. Chromatogr. **2005**, *19* (1), 1–8.
69. Marek, M. Determination of molecular weights of proteins, esterases, and basic phospho-monoesterases in the hemolymph of Galleria mellonella L. Prepupa and pupa using a thin-layer chromatography. Experientia **1969**, *25* (2), 136–137.
70. van der Geer, P.; Hunter, T. Phosphopetide mapping and phosphoamino acid analysis by electrophoresis and chromatography on thin-layer cellulose plates. Electrophoresis **1994**, *15*, 544–554.
71. Judd, R.C. *Peptide Mapping by Two-Dimensional Thin-Layer Electrophoresis-Thin Layer Chromatography, The Protein Protocols Handbook*, Second Edition; Walker, J.W., Ed.; Humana Press: Totowa, NJ, 2000; 543.
72. Nagahara, H.; Latek, R.R.; Ezhevsky, S.A.; Dowdy, S.F. *2-D Phosphoteptide Mapping, The Protein Protocols Handbook*, Second Edition; Walker, J.W., Ed.; Humana Press: Totowa, NJ, 2000; Chapter 96.
73. Mould, D.L.; Synge, R.L. Separations of polysaccharides related to starch by electrokinetic ultrafiltration in collodion membranes. Biochem. J. **1954**, *58* (4), 571–85.
74. Smith, N.W.; Carter-Finch, A.S. Electrochromatography. J. Chromatogr. A **2000**, *892*, 219–255.
75. Niessen, G. Personal communication, December 2004.
76. Reissell, P.K.; Hagopian, L.M.; Hatch, F.T. Thin-layer electrophoresis of serum lipoproteins. J. Lipid Res. **1966**, *7*, 551–557.
77. Keler-Bacoka, M.; Pucar, Z. Two-dimensional electrochromatography of human haptoglobin and plasma protein-hemoglobin complexes. Clin. Chim. Acta **1969**, *23*, 431–436.
78. Meisenhelder, J.; Hunter, T.; van der Geer, P. *Analysis of Protein Phosphorylation by Phosphopeptide Mapping*; CBS Scientific: Del Mar, CA (www.cbsscientific.com).
79. Hunter, T. *Thin Layer Peptide Mapping Electrophoresis System, Model #HTLE-7002, Instruction Manual*; CBS Scientific Company: Del Mar, CA, 2004.
80. Nurok, D. Planar electrochromatography. J. Chromatogr. A **2004**, *1044*, 83–96.
81. Nurok, D.; Koers, J.; Novotny, A.L.; Carmichael, M.A.; Kosiba, J.J.; Santini, R.E.; Hawkins, G.L.; Replogle, R.W. Apparatus and initial results for pressurized planar electrochromatography. Anal. Chem. **2004**, *76* (5), 1690–1695.

82. Lapizco-Encinas, B.H.; Pinto, N.G. On the potential of electrochemically modulated liquid chromatography of proteins in a micro open parallel plate separator. J. Sep. Sci. **2004**, *27*, 667–674.
83. Nurok, D.; Frost, M.C.; Chenoweth, D.M. Separation using planar electrochromatography with electroosmotic flow. J. Chromatogr. A **2000**, *903*, 211–217.
84. Nurok, D.; Koers, J.M.; Carmichael, M.A. Role of buffer concentration and applied voltage in obtaining a good separation in planar electrochromatography. J. Chromatogr. A **2003**, *983*, 247–253.
85. Stevenson, R.; Mistry, K.; Krull, I.S. *CEC '98: The State-of-the-Art*; American Laboratory, 1998; 16A.
86. Krull, I.S.; Sebag, A.; Stevenson, R. Analysis of peptides, proteins, and antibodies by capillary electrochromatography. J. Chromatogr. A **2000**, *887*, 137–163.
87. Krull, I.S.; Stevenson, R.; Mistry, K.; Swartz, M.E. *Capillary Electrochromatography (CEC) and Pressurized Flow CEC (PEC) or Electro-HPLC, An Introduction*; HNB Publishers: New York, 2000.
88. Kazmi, S.; Krull, I.S. Application of CEC to Biopolymers and Pharmaceuticals. In *Encyclopedia of Chromatography*; Cazes, J., Ed.; Marcel Dekker: New York, 2001; 49–58.
89. Mistry, K.; Cortez, H.; Meunier, D.; Schmidt, C.; Grinberg, N.; Krull, I.S. Nonaqueous capillary electrochromatographic separation of synthetic, neutral polymers by size exclusion chromatography using polymeric stationary phases. Anal. Chem **2002**, *74*, 617–625.
90. Mistry, K.; Grinberg, N.; Krull, I.S. CEC: an alternative to HPLC and CE? J. Sepn. Sci. **2002**, *25* (15–17), 935–958.
91. Mistry, K.; Krull, I.S.; Grinberg, N. Capillary electrochromatographic separation of polysacchaides by size exclusion chomatography using polymeric stationary phases. Electrophoresis. **2003**, *24*, 1753–1763.
92. Zhang, S.; Zhang, J.; Horvath, Cs. Rapid separation of peptides and proteins by isocratic capillary electrochromatograpy at elevated temperature. J. Chromatogr. A **2001**, *914* (1–2), 189–200.
93. Bandilla, D.; Skinner, C.D. Protein separation by monolithic capillary electrochromatography. J. Chromatogr. A **2003**, *1044* (1–2), 167–179.
94. Ericson, C.; Hjerten, S. Reversed-phase electrochromatography of proteins on modified continuous beds using normal-flow and counterflow gradients. Theroetical and practical considerations. Anal. Chem. **1999**, *91* (8), 1621–1627.
95. Bedair, M.; El Rassi, Z. Capillary electrochromatography with monolithic stationary phases. III. Evaluation of the electrochromatographic retention of neutral and charged solutes on cationic stearyl-acrylate monoliths and the separation of water-soluble proteins and membrane proteins. J. Chromatogr. A **2003**, *1013* (1–2), 47–56.
96. Righetti, P.G. Electrophoresis: The march of pennies, the march of dimes. J. Chromatog. A **2005**, *1079*, 24–40.
97. Reissell, P.K.; Hagopian, L.M.; Hatch, F.T. Thin-layer electrophoresis of serum lipoproteins. J. Lipid Res. **1966**, *7*, 551–557.
98. Judd, R.C. Peptide mapping by two-dimensional thin-layer electrophoresis-thin-layer chromatography. In *The Protein Protocols Handbook*; Walker, J.M., Ed.; Humana Press: Totowa, NJ, 1996; Vol. 437.
99. Honegger, C.G. Thin-film ionophoresis and thin-film ionophoresis-chromatography. Helv. Chim. Acta **1961**, *44*, 173–179.

100. Ritschard, W.J. Thin-layer techniques for making peptide maps. J. Chromatog. **1964**, *16*, 327–333.
101. Stegemann, H.; Lerch, B. Fast fingerprinting on thin layers with polyacrylamide as diaphragm. Anal. Biochem. **1964**, *9*, 416–422.
102. Bieleski, R.L. Separation of phosphate esters by thin-layer chromatography and electrophoresis. Anal. Biochem. **1965**, *12*, 230–234.
103. Bieleski, R.L.; Turner, N.A. Separation and estimation of amino acids in crude plant extracts by thin-layer electrophoresis and chromatography. Anal. Biochem. **1966**, *17*, 278–293.
104. Katz, S.; Lewis, A. Apparatus for high-voltage thin-layer electrophoresis: application to amino acid analysis. Anal. Biochem. **1966**, *17*, 300–309.
105. Burns, D.J.W.; Turner, N.A. Peptide mapping on cellulose thin layers. J. Chromatog. **1967**, *30*, 469–475.
106. Tometsko, A.M.; Delihas, N. The application of high-voltage thin-layer electrophoresis to the separation of peptides, nucleotides, and proteins. Anal. Biochem. **1967**, *18*, 72–80.
107. Sargent, J.R.; Vadlamudi, B.P. Electrophoresis of peptides on thin layers of silica gel. Anal. Biochem. **1968**, *25*, 583–587.
108. Bogdansky, F.M. The two-dimensional characterization of amino acids in actinomycin hydrolysates. J. Chromatogr. Sci. **1975**, *13*, 567–569.
109. Bates, D.L.; Perham, R.N.; Coggins, J.R. Methods for obtaining peptide maps of proteins on a subnanomole scale. Anal. Biochem. **1975**, *68*, 175–184.
110. Schiltz, E.; Schnackerz, K.D.; Gracy, R.W. Comparison of ninhydrin, fluorescamine, and o-pthaldialdehyde for the detection of amino acids and peptides and their effects on the recovery and composition of peptides from thin-layer fingerprints. Anal. Biochem. **1977**, *79*, 33–41.
111. Stephens, R.E. Fluorescent thin-layer peptide mapping for protein identification and comparison in the subnanomole range. Anal. Biochem. **1978**, *84*, 116–126.
112. Nelles, L.P.; Bamburg, J.R. Thin-layer mapping of peptides labeled with 2H or ^{14}C via reductive methylation. Anal. Biochem. **1979**, *94*, 150–159.
113. Aromatorio, D.K.; Parker, J.; Brown, W.E. High-resolution, analytical and preparative mapping by a combination of ion-exchange and thin-layer chromatographies. Anal. Biochem. **1980**, *103*, 350–358.
114. Fishbein, J.C.; Place, A.R.; Ropson, I.J.; Powers, D.A.; Sofer, W. Thin-layer peptide mapping: quantitative analysis and sequencing at the nanomole level **1980**, *108*, 193–201.
115. Oray, B.; Jahani, M.; Gracy, R.W. High-sensitivity peptide mapping of triosephosphate isomerase: A comparison of high-performance liquid chromatography with two-dimensional thin-layer methods. Anal. Biochem. **1982**, *125*, 131–138.
116. Anderson, N.L.; Anderson, N.G. Proteome and proteomics: new technologies, new concepts and new words. Electrophoresis **1998**, *19*, 1853.
117. Hatzimanikatis, V.; Choe, L.H.; Lee, K.H. Proteomics: theoretical and experimental considerations. Biotechnol. Prog. **1999**, *15*, 312.
118. Westermeir, R.; Naven, T. *Proteomics in Practice, A Laboratory Manual of Proteome Analysis*; Wiley-VCH Publishers: Weinheim, Germany, 2002.
119. Dunn, M.J.; Ed. *Proteomics Reviews, 2001*; Wiley-VCH Publishers: Weinheim, Germany, 2001.
120. Pennington, S.R.; Dunn, M.J.; Eds. *Proteomics, From Protein Sequence to Function*; BIOS Scientific Publishers, Ltd., Springer-Verlag Publishers: New York, 2001.

121. Lieber, D.C. *Introduction to Proteomics, Tools for the New Biology*; Humana Press: Totowa, NJ, 2002.
122. Wilkins, M.R.; Williams, K.L.; Appel, R.D.; Hochstrasser, D.F.; Eds. *Proteome Research: New Frontiers in Functional Genomics*; Springer-Verlag Publishers: New York, 1997.
123. Kellner, R.; Lottspeich, F.; Meyer, H.E. *Microcharacterization of Proteins*, Second Edition; Wiley-VCH Publishers: New York and Weinheim, 1999.
124. Kazmi, S.; Krull, I.S. Proteomics and the current state of protein separation science, Part I. PharmaGenom. Mag. **2001** (August), 14.
125. Kazmi, S.; Krull, I.S. Proteomics and the current state of protein separation science, Part II. PharmaGenom. Mag. **2001** (October/November), 16.
126. Millea, K.; Krull, I.S. Approaches to subproteomics involving chromatography. J. Liq. Chromatogr. & Rel. Technol. **2003**, *26* (14), 2195–2224.
127. Millea, K.; Kass, I.J.; Krull, I.S.; Gelber, J.C.; Berger, S.J. Evaluation of multidimensional (ion exchange/reversed phase) complex protein separations using linear and step gradients in the first dimension. J. Chromatogr. A **2005**, *1079*, 287–98.
128. Berger, S.J.; Millea, K.M.; Krull, I.S.; Cohen, S.A. In *Middle-Out Proteomics: Incorporating Multidimensional Protein Fractionation and Intact Protein Mass Analysis as Elements of a Proteomic Analysis Workflow, Separations in Proteomics*; Smejkal, G., Lazarev, A., Eds.; Taylor and Frances Publishers: NYC, 2005, in press.
129. Berezkin, V.G.; Balushkin, A.O.; Tyaglov, B.V.; Rozhilo, Y.; Malinovska, I. Electroosmotic thin layer chromatography with a covered sorption layer. Doklady Phys. Chem. **2004**, *399*, 287–289.
130. Tate, P.A.; Dorsey, J.G. Characterization of flow and voltage profiles in planar electrochromatography. J. Chromatogr A **2005**, *1079* (1–2), 317–27.
131. Hardin, S.C.; Wolniak, S.M. Low-voltage separation of phosphoamino acids by silica gel thin-layer electrophoresis in a DNA electrophoresis cell. Biotechniques **1998**, *24*, 344–346.
132. Blonder, J.; Goshe, M.B.; Moore, R.J.; Pasa-Tolic, L.; Masselon, C.D.; Lipton, M.S.; Smith, R.D. Enrichment of integral membrane proteins for proteomic analysis using liquid chromatography-tandem mass spectrometry. J. Proteom. Res. **2002**, *1* (4), 351–60.
133. Blonder, J.; Conrads, T.P.; Veenstra, T.D. Characterization and quantitation of membrane proteomes using multidimensional MS-based proteomic technologies. Expert Rev. Proteom. **2004**, *1* (2), 153–63.
134. Patton, W.F. Detection technologies in proteome analysis. J. Chromatogr. B **2002**, *771* (1–2), 3–31.
135. Schulenberg, B.; Aggeler, R.; Beechem, J.M.; Capaldi, R.A.; Patton, W.F. Analysis of steady-state protein phosphorylation in mitochondria using a novel fluorescent phosphosensor dye. J. Biol. Chem. **2003**, *278* (29), 27251–5.
136. Steinberg, T.H.; Agnew, B.J.; Gee, K.R.; Leung, W.Y.; Goodman, T.; Schulenberg, B.; Hendrickson, J.; Beechem, J.M.; Haugland, R.P.; Patton, W.F. Global quantitative phosphoprotein analysis using multiplexed proteomics technology. Proteomics **2003**, *3* (7), 1128–44.
137. Schulenberg, B.; Goodman, T.N.; Aggeler, R.; Capaldi, R.A.; Patton, W.F. Characterization of dynamic and steady-state protein phosphorylation using a fluorescent phosphoprotein gel stain and mass spectrometry. Electrophoresis **2004**, *25* (15), 2526–32.
138. Higashi, H.; Hirabayashi, Y.; Ito, M.; Yamagata, T.; Matsumoto, M.; Ueda, S.; Kato, S. Immunostaining on thin-layer chromatograms of oligosaccharides

released from gangliosides by endoglycoceramidase. J. Biochem. (Tokyo) **1987**, *102* (2), 291–6.
139. Sorice, M.; Griggi, T.; Circella, A.; Garofalo, T.; d'Agostino, F.; Pittoni, V.; Pontieri, G.M.; Lenti, L.; Valesini, G. Detection of antiphospholipid antibodies by immunostaining on thin layer chromatography plates. J. Immunol. Meth. **1994**, *173* (1), 49–54.
140. Ishikawa, D.; Taki, T. Thin-layer chromatography immunostaining. Meth. Enzymol. **2000**, *312*, 157–9.
141. Saito, M.; Yu, R.K. Planar Chromatography in Life Sciences. In *TLC-Immunostaining of Glycolipids*; Touchstone, J.C., Ed.; John Wiley & Sons: NY, 1995.
142. Wind, M.; Feldmann, I.; Jakubowski, N.; Lehmann, W.D. Spotting and quantification of phosphoproteins purified by gel electrophoresis and laser ablation-element mass spectrometry with phosphorus-31 detection. Electrophoresis **2003**, *24* (7–8), 1276–80.
143. Ivleva, V.B.; Sapp, L.M.; O'Connor, P.B.; Costello, C.E. Ganglioside analysis by thin-layer chromatography matrix-assisted laser desorption/ionization orthogonal time-of-flight mass spectrometry. J. Am. Soc. Mass Sepctrom. **2005**, *16*, 1552–1560.
144. Loo, R.R.; Cavalcoli, J.D.; VanBogelen, R.A.; Mitchell, C.; Loo, J.A.; Moldover, B.; Andrews, P.C. Virtual 2-D gel electrophoresis: visualization and analysis of the E. coli proteome by mass spectrometry. Anal Chem. **2001**, *73* (17), 4063–70.
145. Walker, A.K.; Rymar, G.; Andrews, P.C. Mass spectrometric imaging of immobilized pH gradient gels and creation of "virtual" two-dimensional gels. Electrophoresis **2001**, *22* (5), 933–45.
146. Ivleva, V.B.; Elkin, N.Y.; Budnik, B.A.; Moyer, S.C.; O'Connor, P.B.; Costello, C.E. Coupling thin-layer chromatography with vibrational cooling matrix-assisted laser desorption/ionization fourier transform mass spectrometry for the analysis of ganglioside mixtures. **2004**, *76*, 6484–6491.
147. Mehl, J.T.; Hercules, D.M. Direct TLC-MALDI coupling using a hybrid plate. Anal Chem. **2000**, *72* (1), 68–73.
148. Sommerer, D.; Suss, R.; Hammerschmidt, S.; Wirtz, H.; Arnold, K.; Schiller, J. Analysis of the phospholipid composition of bronchoalveolar lavage (BAL) fluid from man and minipig by MALDI-TOF mass spectrometry in combination with TLC. J. Pharm. Biomed. Anal. **2004**, *35* (1), 199–206.
149. Crecelius, A.; Clench, M.R.; Richards, D.S.; Parr, V. Quantitative determination of Piroxicam by TLC-MALDI TOF MS. J. Pharm. Biomed. Anal. **2004**, *35* (1), 31–9.

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