BECKMAN COULTER.

P/ACE Setter

Volume 3, Issue 1 • April 1999

The worldwide newsletter for capillary electrophoresis

Table of Contents

P/ACE Setter

The Worldwide Newsletter for Capillary Electrophoresis
Volume 3 • Issue 1 • April '99

Using cIEF to characterize recombinant human monoclonal antibodies

L.C. Santora[1], I.S. Krull[1] and K. L. Grant[2]
1. Department of Chemistry, Northeastern University, Boston, MA
2. Process Development, BASF Bioresearch Corp., Worcester, MA

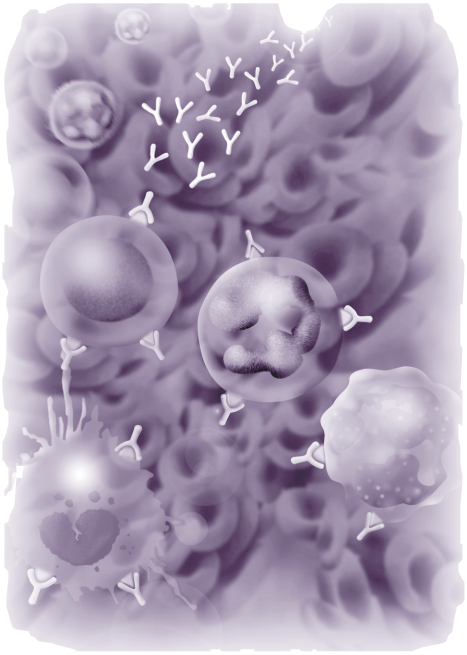
Biotechnology products, especially glycoprotein pharmaceuticals, provide many challenges to both the analytical and process chemist. Most of these challenges are due to the complexity and heterogeneity of proteins in general. The manufacture, release, and characterization of a recombinant biopharmaceutical product require many analytical tools.

Flatbed isoelectric focusing (IEF) has been used in biotechnology to monitor the consistency, stability and semi-quantitative levels of charged isoforms of protein products. Even though this technology is labor intensive, time consuming and semi-quantitative, it has become one of the most routine analytical methods in protein analysis[1-5].

Capillary isoelectric focusing (cIEF) extends the utility of IEF into an automated and quantitative format and clearly has an important role in analytical biotechnology. In our laboratory we have used cIEF in combination with cation ion-exchange chromatography and mass spectrometry not only to assess charge isoform heterogeneity but also to determine the identity and the quantity of isoforms.

Single amino acid variants

Isoforms generated from the differential processing of carboxy (C)-terminal lysine (Lys) and arginine (Arg) residues have been reported in proteins isolated from mammalian cell culture[6]. In this paper we



demonstrate the use of cIEF to separate and identify single amino variants of the recombinant human monoclonal antibody Ab1.

We also briefly examine the impact of different production methods on these variants by comparing Ab1 produced by the fermentation of Chinese hamster ovary cells (CHO) and by transgenic goats (G).

In addition to separating the isoforms by isoelectric point, we have employed several enzymatic treatments to assist us in determining the identity of the isoforms. By using enzymes which cleave at specific sites we can generate evidence to help identify these isoforms as C-terminal Lys variants, differing from 0 to 2 Lys residues. In this experiment carboxypeptidase C (CPB) was used to remove the C-terminal Lys while papain was used to digest the antibody into Fab and Fc fragments.

Experimental

Apparatus and chemicals

All separations were performed on the P/ACE™ MDQ System (Beckman Coulter, Inc., Fullerton, CA). The eCAP™ neutral capillary (Part No. 477441 Beckman Coulter), with dimensions of 50 µm (i.d.) x 31 cm (20 cm to detector), is used to eliminate electroosmotic flow. NaH_2PO_4, NaCl, hydroxypropylmethycellulose (HPMC) and tetramethylethylenediamine (TEMED) are from Sigma Chemical Co. (St. Louis, MO). All enzymes are from Boehringer Mannheim (Indianapolis, IN). CPB enzymatic reaction: 0.1 mg/mL protein was mixed with 0.05 mg carboxypeptidase B and incubated at 37° C for two hours. Anolyte, catholyte and mobilizer are from BioRad Laboratories (Hercules, CA). The Pharmalyte 8-10.5 is from Pharmacia Biotech (Piscataway, NJ), and Bio-Lyte 3-10 is from BioRad Laboratories. All recombinant human IgGs (CHO Ab1 and G Ab1 ▶

- BASF Bioresearch Corp., Worcester, MA) were diluted with HPLC-grade water to a concentration of 0.25 mg/mL for the cIEF analysis.

cIEF conditions

cIEF was performed with "normal" polarity (cathode nearest detector). Detection was with UV at 280 nm. The anolyte, catholyte, and mobilizer were 20 mM phosphoric acid, 40 mM sodium hydroxide and a zwitterionic solution, respectively. The ampholyte solution was a mixture of diluted Pharmalyte pH 8-10, Bio-Lyte 7-9 and Bio-Lyte pH 3-10 containing 0.4% TEMED (v/v) and 0.2% HPMC (w/v). The dilution factor was 20. The mixing ratio (v/v) was 8:1:1.

The pI standard markers (pI 10.1, 8.4 and 7.9) were diluted 1:20 with HPLC-grade water. Samples for cIEF consisted of 70 µL of 0.25 mg/ml mAb with 100 µL ampholyte solution plus 4 µL of each pI standard marker, as above.

CIEF capillary pre-conditioning consisted of rinsing first with HPLC-grade water for 2 minutes at 20 psi, followed by a rinse with 20 mM H_3PO_4 for 2 minutes at 20 psi, and then another water rinse for 2 minutes. Sample was introduced by filling the entire capillary using positive pressure at 20 psi. The focusing electric field was 580 V/cm for 8 minutes, followed by a mobilization field of 645 V/cm for 28 minutes or until all of the proteins of interest and standard markers migrated past the detector.

Results and discussion

The BASF human recombinant mAb (Ab1) has C-terminal Lys isoforms, 0-Lys, 1-Lys and 2-Lys. This antibody was first run on conventional gel IEF and three bands were observed (data not shown). The C-terminal 0-Lys isoform had the lowest pI, while the C-terminal 2-Lys isoform had the highest pI value.

The cIEF electropherogram of the CHO Ab1 has three peaks, which are due to Ab1 (Figure 1). The peak migrating at approximately 21 minutes represents the 2-Lys variant with a C-terminal lysine on both heavy chains. The second isoform, with

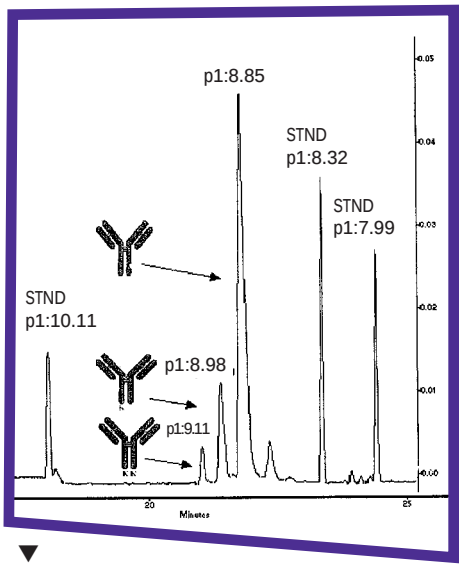


Figure 1: cIEF analysis of CHO Ab1 for the determination of isoelectric points (pI). The pIs are automatically calculated and annotated on the peak.

TABLES 1-3

Table 1: Summary of the overall reproducibility of cIEF analysis and pI determinations for the CHO derived antibody Ab1 from different injections on the same day. Also indicated are the average pI values of each peak in the sample and standard deviation (SD) and percent relative standard deviation (%R.SD). These three samples were all taken from the same lot No., 604.

Table I. The Reproducibility of Antibody D2E7 pI from Different Injections on the Same Day

Date	3/5/98	3/5/98	3/5/98	Lot# 604		
Sample ID	Is3-110-02	Is3-110-02a	Is3-110-02b	Avg pI	SD	%RSD
D2E7,unknown	8.669	8.678	8.662	8.670	0.008	0.1
D2E7,0Lys	8.862	8.859	8.848	8.856	0.007	0.1
D2E7,1Lys	8.982	8.980	8.972	8.978	0.005	0.1
D2E7,2Lys	9.112	9.116	9.108	9.112	0.004	0.1

Table 2: Summary of the overall reproducibility of cIEF analysis and pI determinations for the CHO derived antibody Ab1 from different days. These three samples were all taken from the same lot No., 802.

Table II. The Reproducibility of Antibody D2E7 pI in Different Days

Date	3/13/98	3/25/98	5/1/98	Lot# 802		
Sample ID	Is3-118-01	Is3-124-08	Is3-145-06	Avg pI	SD	%RSD
D2E7,unknown	8.621	8.596	8.674	8.630	0.040	0.5
D2E7,0Lys	8.778	8.758	8.867	8.801	0.058	0.7
D2E7,1Lys	8.905	8.896	8.999	8.933	0.057	0.6
D2E7,2Lys	9.021	9.031	9.130	9.061	0.060	0.7

Table III. The Reproducibility of Antibody D2E7 pI in Different Lots

Date	3/5/98	3/13/98	3/5/98	3/13/98	3/13/98	3/5/98	3/5/98	3/5/98	3/13/98			
Lot #	604	608	703	704	706	707	751	801	802	Average	SD	%RSD
unknown	8.662	8.623	8.666	8.618	8.609	8.675	8.667	8.675	8.621	8.645	0.026	0.3
D2E7 0Lys	8.848	8.793	8.846	8.777	8.766	8.849	8.852	8.849	8.778	8.816	0.036	0.4
D2E7 1Lys	8.972	8.916	8.973	8.899	8.886	8.998	8.951	8.998	8.905	8.941	0.042	0.5
D2E7 2Lys	9.108	9.04	9.109	9.018	9.004	9.120	9.105	9.12	9.021	9.069	0.048	0.5

Table 3: Summary of the overall reproducibility of cIEF analysis and pI determinations for the CHO derived antibody Ab1 from different batches analyzed on different days. Ten separate batches are indicated (ID), all analyzed on different days, with average values for all lots reported.

only 1 C-terminal Lys, migrated a little later while the major component, having no C-terminal Lys residues, migrated third.

The "standard" markers act as internal calibrators to allow us to automatically calculate the isolectric points of our isoforms. We calculated the pI for 0 –C-terminal Lys to be pH 8.85, 1-C-terminal Lys to be pH 8.98 and the 2-C-terminal Lys to be pH 9.11. These isoelectric points are all in agreement with their expected values: the 2-Lys is the most basic with the greatest number of Lys residues, while the others have lower pI values, being less basic. The precision of this determination is very good, with same-day reproducibility of the analytes' iso-electric point being typically 0.1% R.S.D, and day to day and lot to lot reproducibility less than 1% R.S.D (Tables 1, 2 and 3).

cIEF is highly quantitative, unlike gel electrophoresis — which is only semi-quantitative. Quantitative analysis of the three isoforms based on peak area percent were as follows: 71.9% 0-Lys, 21% contained 1-Lys, while the 2-Lys isoform accounted for 7.1%. This data agreed very well with a cation exchange chromatography assay that we had developed in parallel. (Figure 2).

Compared to CHO Ab1 isoforms, there are more transgenic goat Ab1 (G Ab1) isoforms (Figure 3). The most significant difference is the increased acidic peaks, which we believe are due to sialic residues on the G Ab1.

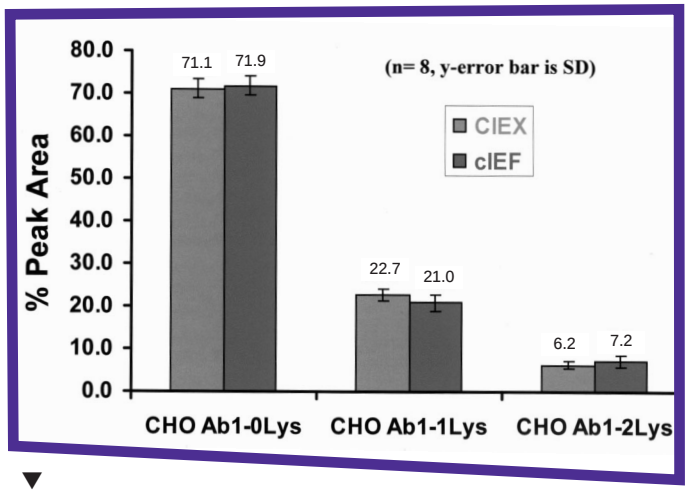


Figure 2: A plot of the percentages of antibody C-terminal lysine isoforms quantitated by both CIEX-HPLC and cIEF assays (n=8, y-error bar is SD).

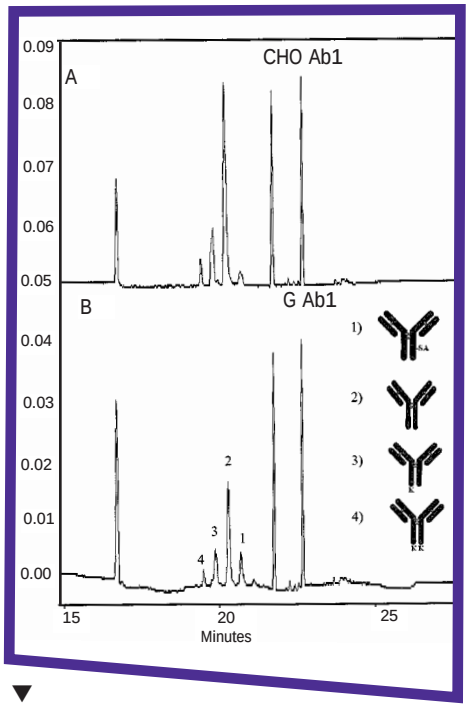


Figure 3: Comparison of the cIEF-charged isoform distribution in CHO Ab1 and G Ab1.

Enzymatic digestion

Papain is a proteolytic enzyme known to split antibody molecules into two separate and identical F_{ab} fragments, each with one antigen-binding site, and one F_c fragment. The fragments can be purified or separated by Protein A. If the isoforms are due to C-terminal Lys variation, then they should be present on the F_c portion of the antibody, rather than the F_{ab} fragments. Our results demonstrate that F_{ab} region shows one peak, while the F_c region migrates as three peaks (Figure 4).

CPB is known to cleave Lys and Arg residues from the C-termini of proteins. Therefore, if the isoforms are single C-terminal Lys variants, CPB treatment should collapse the three variants into one that co-migrates with the 0-Lys variant. Indeed, the three protein peaks collapse into the most acidic peak, which co-migrates with the 0-Lys variant (Figure 5).

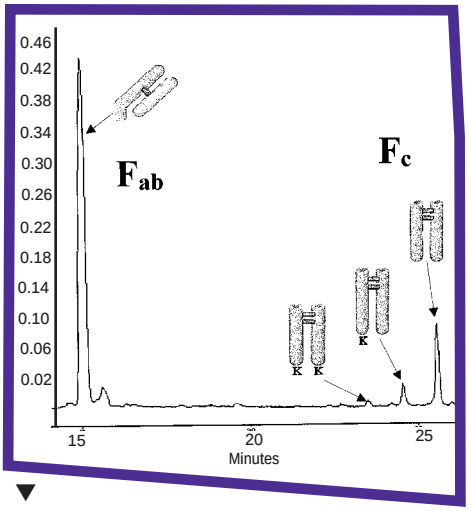


Figure 4: cIEF analysis of CHO Ab1 after digestion with the proteolytic enzyme Papain. The isoforms of Ab1 appear on the F_c region, not on the F_{ab} region.

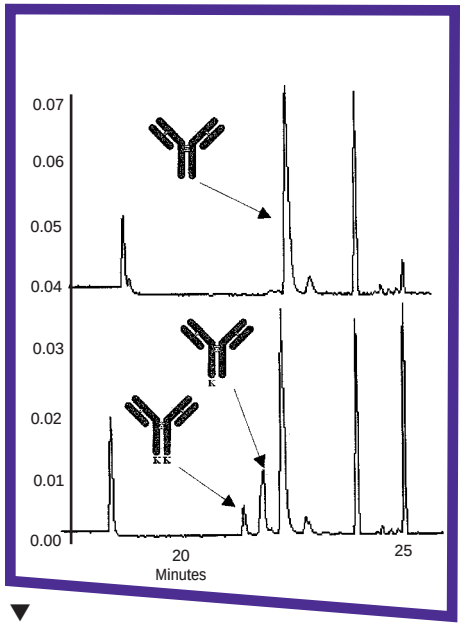


Figure 5: Comparison by cIEF analysis of CHO Ab1 before and after digestion with carboxypeptidase B (CPB). CPB removes the C-terminal Lysine residues, collapsing the three Ab1 peaks into one major peak.

Conclusions

We have been able to develop a very reproducible cIEF method for determining the pI values and quantifying the levels of charged isoforms present in any typical recombinant monoclonal antibody. In combination with protein sequencing, CIEX and LC/MS, we have been able to confirm that the charged isoforms seen here are indeed due to C-terminal Lys variants. Capillary electrophoresis (CE) is clearly a valuable tool for the automation of isoelectric focusing, providing much better reproducibility and quantitation than traditional gel methodology. We plan to extend the CE studies further by examining the microheterogeneity of antibody glycosylation, using APTs derivatization of the glycans and CE-LIF detection.

References

1) A.H. Gordon, Electrophoresis of Proteins in Polyacrylamide and Starch Gels, North-Holland/American Elsevier, Amsterdam, 1969; P.G. Righetti. Isoelectric Focusing: Theory, Methodology, and Applications. Laboratory Techniques in Biochemistry and Molecular Biology, General Editors, T.S. Work and R.H. Burdon, Elsevier Biomedical, Amsterdam, 1983.

2) Fundamentals of Protein Biotechnology, Edited by S.Stein, Marcel Dekker, NY, 1990.

3) A.S. Bhown. Protein/Peptide Sequence Analysis: Current Methodologies, CRC Press, Inc., Boca Raton, FL, 1988.

4) D.M. Bollag, M.D. Rozycki, and S.J. Edelstein. Protein Methods, Second Edition, Wiley-Liss, NY, 1996.

5) Protein Purification Protocols, Edited by S. Doonan, Humana Press, Totowa, NJ, 1996, Methods in Molecular Biology, Volume 59.

6) R.J. Harris. Processing of C-terminal lysine and arginine residues of proteins isolated from mammalian cell culture. J. Chromatogr., A, 705, 129 (1995).

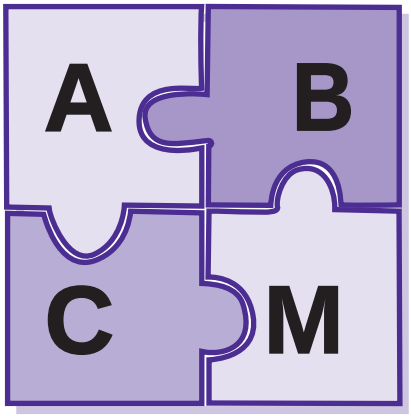
Factoring in the variables

Notes From the Lab

▼ *Mark Flocco*

In recent articles I have discussed sample screening with buffer arrays, carbohydrate analysis and the utility of capillary coatings. Over the next few articles I have chosen to take a step back and discuss CE fundamentals. From my personal observations and communication with our product support team, it is clear that many CE users are not considering all the variables associated with methods development and optimization. However, an important lesson that I learned very early was to "factor in the variables."

When I began my career I had excellent mentors. Each provided a unique perspective to the role of capillary electrophoresis and how to retrieve the best possible data. Today before sitting down at the instrument, I still use this simple chart provided by Richard Palmieri (Beckman Coulter, Inc). It's part of the paperwork one should think about before applying any voltage or installing a single capillary.



A = Analyte; B = Buffer;
C = Capillary; M = Sample matrix

All of these variables impact the performance of the others. Does the analyte have a charge? At which pH? How will the pH of the buffer affect my separation? What type of capillary should I consider using to avoid sample interaction with the wall and at which pH?

In this article I focus on the "M" in our table. The "M," or sample matrix, can have a profound effect on the efficiency, resolution and peak shape of our analysis. The counter ions in the sample matrix should be compatible with ions in the buffer in order to avoid tailing or skewing of peaks as they migrate through the buffer front. Ideally, the sample should be dissolved in water or a 1:10 dilution of the running buffer. This allows us to take advantage of the "stacking" capabilities of the buffer as well as concentrating the sample at that boundary. If your sample requires a higher salt concentration for solubility, then it will be necessary to increase the salt concentration in your buffer to continue to take advantage of the stacking. As you adjust this salt concentration remember to adjust the electric field strength in your method (Ohm's Law) and running time. You'll be glad you purchased a system in which the capillary is liquid cooled.

It is equally important that the pH in the matrix be compatible with the running buffer. If there are additives in the matrix to aid in the solubility, remember to add them to the buffer. The assay will be over very quickly if the sample precipitates in the capillary.

I stress running an Ohm's law plot on your buffer to optimize the electric field strength. If possible, inject the matrix and run the method to look for any system peaks or disturbances in the current profile. Maximum efficiency is directly proportional to the field strength. The higher the field strength the more efficient the separation. The higher the salt concentration in the buffer, the higher the current and therefore the higher the joule heating. The better the dissipation of the joule heat the better the reproducibility of the method — and the better the robustness. Now you will really appreciate your choice of liquid cooling.

In future articles we will discuss the other variables in our chart. Until then remember to consider all your options. Look over your variables and happy separations.

Mark Flocco is a Field Marketing Specialist with Beckman Coulter.

Micro Emulsion ElectroKinetic Chromatography

Application to pharmaceutical analysis

K. D. Altria
Pharmaceutical Development (Europe), Glaxo Wellcome Research & Development, UK

Microemulsion electrokinetic chromatography (MEEKC) is a CE technique in which solutes partition with moving oil droplets present in a microemulsion buffer. Ionized species will also separate by electrophoresis. In MEEKC the microemulsion droplets are often formed by mixing immiscible heptane or octane with water. SDS is added at relatively high concentrations to stabilize the emulsion. A co-solvent such as butan-l-ol is added to further stabilize the microemulsion.

High-pH buffers such as borate and phosphate are typically used, as these generate high electroosmotic flow. The droplet is negatively charged due to the SDS coating, and as such attempts to migrate against the EOF. Hydrophobic solutes favor partitioning into the oil droplet and, therefore, are highly retained. The migration time of a neutral solute in a MEEKC separation is therefore directly proportional to its hydrophobicity (log P). Figure 1 shows a schematic of this separation process.

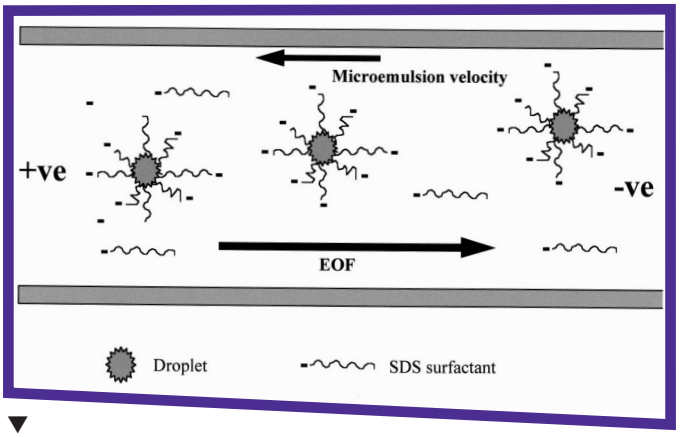


Figure 1: Principles of separation in MEEKC.

MEEKC has previously been shown[1-3] to be a powerful analytical technique for water-insoluble neutral compounds. However, the application of MEEKC to the separation of water-soluble ionic species had not been fully assessed. In this work a single set of optimized MEEKC separation conditions were applied[4-6] to a range of novel application areas. High separation efficiencies and selectivity are obtained for both water-soluble and insoluble compounds.

Experimental

P/ACE MDQ and P/ACE 5000 Series systems (Beckman Coulter, Inc., Fullerton, CA) were used for this work. Standard 50µm i.d. x 27cm or 30cm-long fused silica capillaries were used. Microemulsions were prepared by weighing 0.81% w/w octane, 6.61% w/w butan-1-ol, 3.31% w/w sodium dodecyl sulphate and 89.27% w/w 10mM sodium tetraborate buffer to a 100ml volumetric flask.

This was sonicated for 30 minutes, which produced an optically transparent microemulsion. Generally, samples were dissolved in the buffer — as this gave best peak efficiency — and injected for 3-5 seconds at 0.1 psi. Insoluble solutes were dissolved at 20 mg/ml in an appropriate solvent and then diluted to 0.5 mg/ml with the buffer. Detection was performed at 200 nm in all cases unless specified.

Analysis of neutral compounds

These compounds separate solely by partitioning effects. Figure 2 shows resolution of a range of neutral solutes. The more water-insoluble compounds are detected last, as they have the highest interaction with the microemulsions.

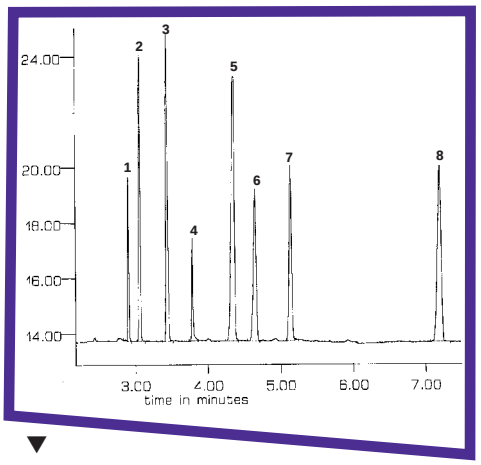


Figure 2: Separation of a range of Neutral Solutes.
1 = 1,4 benzenediol; 2 = 4 acetamidophenol; 3 = 1,3 benzenediol; 4 = Saligenin; 5 = Nitroacetophenone; 6 = 4 hydroxyacetophenone; 7 = Anisole; 8 = Benzophenone

Formulations of neutral and charged solutes

Figure 3 shows the separation of charged and neutral components of cold medicine liquid formulation. Table 1 gives some quantitative data from this analysis. Internal standards were used to obtain the required injection precision.

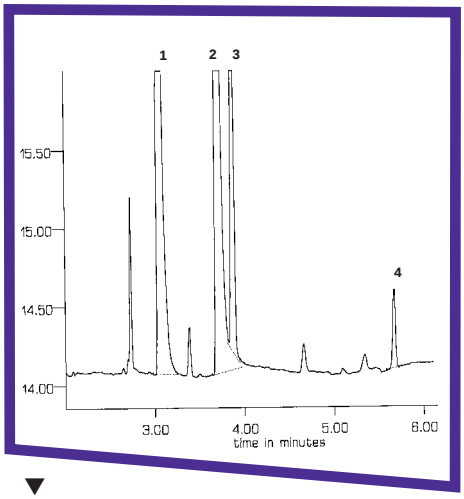


Figure 3: Separation of liquid cold medicine by the MEEKC method.
1 = Guaiphenisin; 2 = Pseudoephedrine 3 = Ethyl paraben; 4 = Propyl paraben

TABLE 1

Table 1 Quantitative application and repeatability of the MEEKC method.

Sudafed™ Expectorant assay results	Label claim	MEEKC assay results
Guaiphenisin	20mg/ml	20.95mg/ml
Pseudoephedrine	6mg/ml	5.86mg/ml
Methyl hydroxybenzoate	0.1%w/v	0.096w/v
Propyl hydroxybenzoate	0.01%w/v	0.01%w/v
Trogliazatone tablets	200mg/tablet	199.4mg/tablet

Injection precision measurements (peak area ratio , PAR)

Solute	Internal standard	No. injections	PAR (%RSD)
Methyl paraben	Ethyl paraben	10	0.31%
Propyl paraben	Ethyl paraben	10	0.63%

Water- and fat-soluble vitamins

Figure 4 shows resolution of a complex liquid multivitamin preparation of a range of water- and fat-soluble vitamins diluted 1 ml to 10 ml with buffer. Other peaks are due to excipients. The late migrating peaks at 9 minutes are the fat-soluble vitamins A and D.

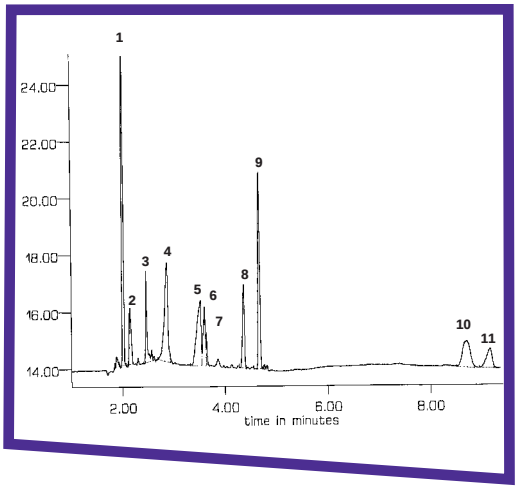


Figure 4: Analysis of a liquid vitamin formulation.
1 = Nicotinamide; 2 = 2.137; 3 = 2.465; 4 = Riboflavin; 5 = Ascorbic acid; 6 = 3.608; 7 = 3.642; 8 = Thiamine; 9 = Panthothenol; 10 = Vitamin A; 11 = Vitamin D.

Acidic drugs

Acidic compounds separate (Figure 5) based on both their electrophoretic migration and partitioning. The least water-soluble compounds, ibuprofen and troglitazone, migrate last.

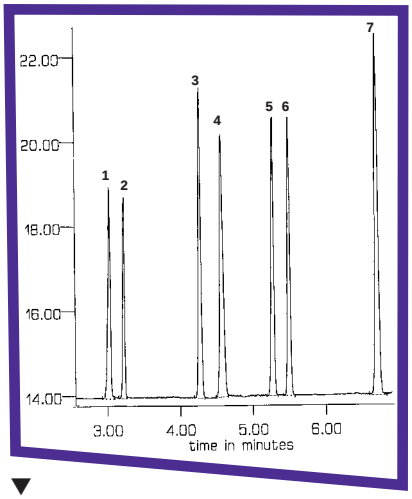


Figure 5: Separation of a range of water-soluble and insoluble acidic drugs.
1 = Warfarin; 2 = Barbitone; 3 = Indomethacin; 4 = Saccharin; 5 = Salicylic acid; 6 = Ibuprofen; 7 = Troglitazone.

Data was first presented at HPCE '99, in Palm Springs, Calif., Jan. 23-28, 1999.

Basic drugs

Figure 6 shows resolution of a range of basic drugs with a very different separation order to that obtained in CE.

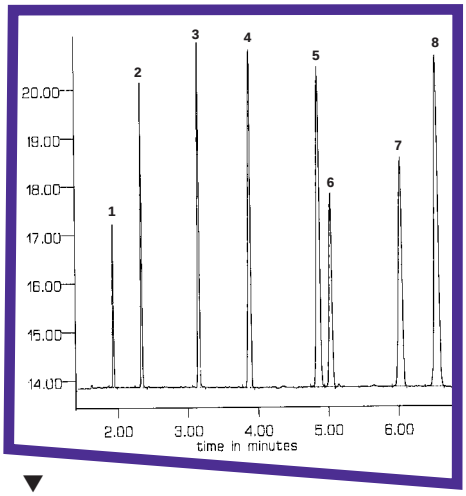


Figure 6: Analysis of basic drugs.
1 = Lamiduvine; 2 = Terbutaline; 3 = Sumatriptan; 4 = Alosetron; 5 = Clenbuterol; 6 = Lignocaine; 7 = Bupivacaine; 8 = Amitryptline.

Conclusions

The MEEKC method is applicable to a wide range of neutral and charged water-soluble and insoluble analytes. High separation efficiencies and peak capacities have been demonstrated even for complex samples, such as liquid formulations. The method is especially applicable to hydrophobic neutral solutes, which can present difficulties in free-solution CE, and MEEKC. The method was found to be highly reproducible in terms of migration time repeatability and repeatability of preparations of the microemulsions.

References

1. H.Watarai, J.Chromatogr.A, 780, 93 (1997)

2.S.Terabe, N.Matsubara,Y. Ishihama, Y.Okada, J. Chromatogr., 608, 23 (1992)

3. R.Szucs, S.Van Hove, Sandra P, JHRC, 19, 189 (1996)

4. M.Miola, M.Snowdon , K.D.Altria, J.Pharm. Biomed. Analysis 18, 807 (1998)

5. Altria KD, Chromatographia, accepted in press, March 1999

6. Altria KD J.Chromatogr.A, accepted in press, 1999.

Keep your P/ACE system clean and safe

CURRENT AFFAIRS

Harry Whatley

Electricity is amazing stuff. Properly handled it can light a lamp, turn a motor, or cause a mixture of molecules to separate into its components. Out of control it can create intense, local heat, punch holes through glass or plastic, and generally spoil your day.

Electricity is the movement of electrons from a source of high potential to a sink of low potential. The higher the potential (voltage) the more likely the electrons are to reach the sink (ground or earth). The quantity of electrons that are being moved is called the current. The currents encountered in capillary electrophoresis are generally small: 5-300 microamperes. By contrast, a 60 W incandescent light bulb may carry one-half of an ampere or 500,000 microamperes.

The voltage potentials used in CE are much greater than the 120-240 volts we work with every day. CE instrument power supplies can generate potentials of 30,000 volts. This is enough voltage to drive electrons through sizeable resistances on their way to ground. The designers of commercial CE instruments go to considerable effort to build systems that keep electrons flowing where they should flow — through the capillary — and away from the operator. However, if given the chance, electricity will take every opportunity to go where it should not. This article is about eliminating those opportunities.

Keeping electrons on track

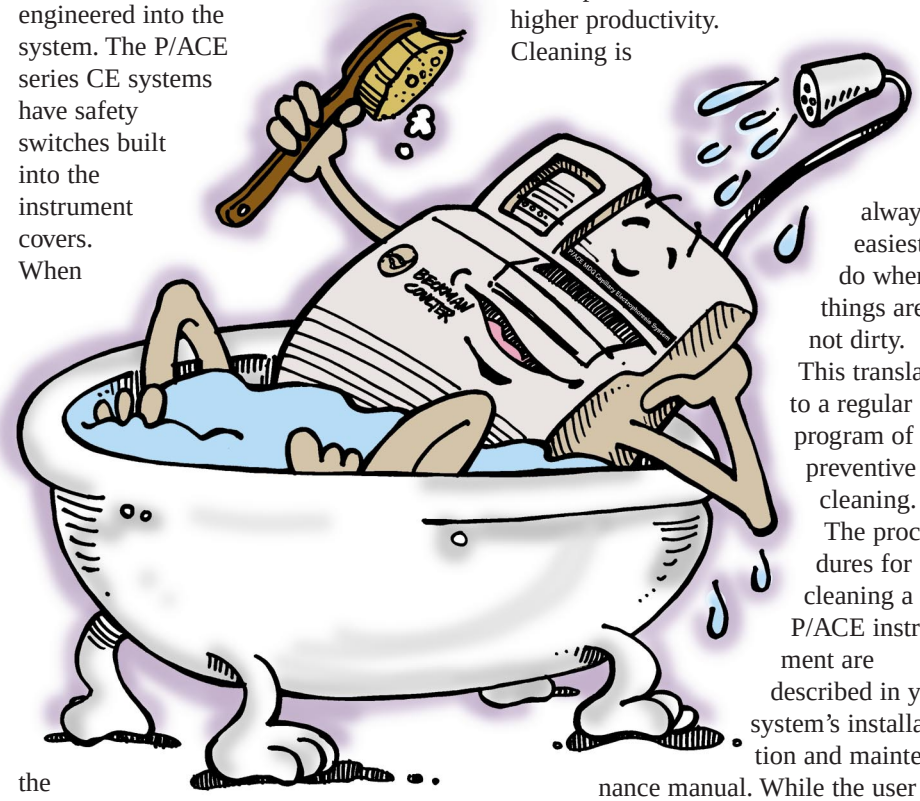
There are two major areas where the user can have an impact on keeping the electrons where they belong. The first of these is cleanliness. The buffers used in capillary electrophoresis are electrically conductive because they contain ionic components (salts). Over time these salts can be deposited on instrument surfaces. The most common area where salts accumulate is on the interface block. The interface block is the place where the wires from the power supply connect to the buffer and the capillary. Salt deposits here can create an excellent electrical pathway to the ground. When electrons take this pathway they can heat the surface sufficiently to create a carbon track, which can be a better conductor than the salts. Preventing and removing buffer deposits is covered later in this article.

The second way that the user can keep electrons inside the CE instrument is to respect the safety interlocks that are engineered into the system. The P/ACE series CE systems have safety switches built into the instrument covers. When the covers over the high voltage areas are opened, these switches shut down the high-voltage supply.

P/ACE systems also incorporate devices that monitor the current. When things are operating properly the current leaving one terminal of the high-voltage power supply should equal the current returning to the other terminal. Any difference indicates that current may be flowing where it should not. The current monitoring devices recognize this difference and shut off the high voltage (generating an Error 18 message in the process). Although no one likes to see an error 18, it's better that the instrument recognize it and react to it. This protects both you and your instrument.

Regular system cleaning

To increase the reliability of a CE system there is nothing more important than system cleaning. The time spent on cleaning the instrument will be more than repaid in reduced downtime and higher productivity.



Cleaning is always easiest to do when things are not dirty. This translates to a regular program of preventive cleaning. The procedures for cleaning a P/ACE instrument are described in your system's installation and maintenance manual. While the user should refer to that manual for specific details, the major steps are as follows:

- Turn off the instrument power. Unplug it.
- Remove the capillary cartridge and set it aside.
- Remove the covers from the elec- ▶

trode area. Refer to the manual for details.

• Place a pad of absorbent material (paper towels) under the area to be cleaned.

• Using water-soaked swabs, gently clean the area around the electrodes.

• Rinse with water. Rinse with methanol (to accelerate drying).

• Allow drying before reassembling.

This procedure is most conveniently done when the instrument will not be used for a period of time; Friday afternoon, for example, if the instrument will not be run over the weekend. This allows plenty of time for all parts to dry. If this is not possible, the system can be dried with clean compressed air. The canned air used to remove dust from electronics is ideal for this purpose, as opposed to laboratory compressed air lines, which often deliver dirt and oil along with the air.

Use care around the electrodes. CE electrodes are made of platinum, a soft metal that is easily bent. Bent electrodes can cause a host of other problems. Badly damaged electrodes should be replaced.

At the same time the instrument is cleaned it should be inspected. Look for discolored, cracked, or broken parts that may indicate chemical, thermal, mechanical, or electrical damage has occurred. Check that all covers are in place and all interlocks function properly. While the cartridge is out of the instrument, check it, too. Use a magnifying glass to check the ends of the capillary for irregularities, and trim if necessary. Examine the window area for dust or debris.

How often to clean depends on how the instrument is being used. If the system is only run a few times a week with a "clean" buffer system, a once-a-week schedule is enough. Operating 24 hours a day demands more frequent cleaning. High-viscosity buffers, such as the gels used for DNA and protein analyses, are sticky and particularly prone to leave residues. Buffers that contain urea sometimes seem to be able to crawl out of vials and across surfaces. Clean more frequently when using these buffer systems.

Good operating practices can simplify cleaning. Never fill buffer vials above the base of the neck. With sticky buffers use a little less. The use of higher-pressure rinses (>20 psi) on the P/ACE MDQ can lead to splashing when the pressure is released suddenly, particularly if the vials are overfilled. With P/ACE MDQ (software and firmware versions 1.6 and above) pressure is vented gradually, which reduces splashing.

Working with high voltage is a lot like the game of baseball. The object is to complete the circuit and still get home safe. A clean CE instrument with all safety interlocks functioning will keep high voltage inside the instrument — where it belongs.

From eternity to here

Reduction of DNA analysis run time from 40 to 15 minutes

Judith A. Miller and Jill M. Kolesar
School of Pharmacy, University of Wisconsin-Madison

Capillary electrophoresis with laser induced fluorescence (CE-LIF) is used in our laboratory to separate and quantitate dsDNA fragments, particularly PCR products such as HIV-1, HBV, CMV and HCV. As we analyze a large quantity of samples every day we are always looking for ways to decrease the run time. This is especially true with our calibration standard, which takes 40 minutes per run and a total of six runs for each calibration. Furthermore, as mutation analyses require very high resolution, we are always looking for ways to improve the efficiency and resolution of our assay.

The purpose of this communication is to share our observations on the evaluation of a new sieving polymer formulation recommended to us by Beckman Coulter, Inc. We found that we could significantly decrease our run time, while improving the resolution of our dsDNA products.

Methodology

Reagents

All reagents were molecular biology grade. Our calibration standard, pGEM, consists of DNA fragments from 36 to 2,645 base pairs and present in a concentration of 1mg/mL (Promega, Madison, WI). Stock concentrations were diluted 1/100 with ddH_2O and analyzed as described.

Instrumentation

Separations were performed on a P/ACE 2050 CE system with the temperature held constant at 20ºC. Detection of samples was achieved using laser-induced fluorescence in the reversed-polarity mode (anode at the detector side) with excitation of 488 nm and emission of 520 nm. Post-run analysis of data was performed using the System Gold® HPLC system (Beckman Coulter, Inc., Fullerton, CA).

Method 1

Samples were introduced hydrodynamically by 10 s injections at 0.34 Pa across a 47 cm x 100 µm i.d. coated eCAP dsDNA capillary filled with the dsDNA 1000 linear polyacrylamide. The capillary was conditioned with eCAP dsDNA 1000 gel buffer, which contained 60 µL of EnhanCE intercalator per 20 mL of gel. Separations were performed under constant voltage at 7.0 kV for 30 min. The capillary was rinsed with gel buffer for 3 minutes prior to each injection.

Method 2

The new sieving polymer was kindly provided by Beckman Coulter for purposes of our evaluation. The polymer

solution consisted of 25 mM Mops-Tris pH 7.55 containing 0.5% PEO (mol. wt. 4 million) and 0.4% PEO (mol. wt. 0.9 million). The anode buffer was made up of the polymer solution as defined; the cathode buffer was made of the polymer solution with the addition of 4 µL of EnhanCE intercalator per 10 mL of gel. The capillary was rinsed with the polymer solution (with EnhanCE addition) for 2 minutes prior to each injection. Samples were introduced hydrodynamically by 10 s injections at 0.34 Pa across a 27 cm x 75 µm ID capillary pretreated with the polymer solution. A 10-second water plug was injected prior to each sample. Separations were performed under constant voltage at 5.4 kV for 15 minutes.

Results

With this new formulation and methodology, the largest fragment of our calibration standard (2,645 bp) migrated at approximately 14 minutes, allowing us to decrease our run time from 40 to 15 minutes while realizing a net improvement in resolution (Figure 1).

There were several important differences that we noted between our previous method and this new one. These include:

- decreased capillary length
- decreased capillary internal diameter
- change in separation voltage
- introduction of an injection water plug
- change in EnhanCE concentra tion
- use of a different sieving poly mer.

These differences are summarized in Table 1.

Table 1

Factor	Method 1	Method 2
Capillary length	37-47 cm	27 cm
Capillary id	100 micron	75 micron
Capillary characteristics	Coated	Pretreated
Separation voltage	7.0 kV	5.4 kV
Water plug	Absent	Present
EnhanCE concentration	60µL/20mL	4 µL/10mL
Gel/Buffer	Linear polyacrylamide	Polyethylene Oxide Formulation

Comparison of current dsDNA method to the new recommended procedure.

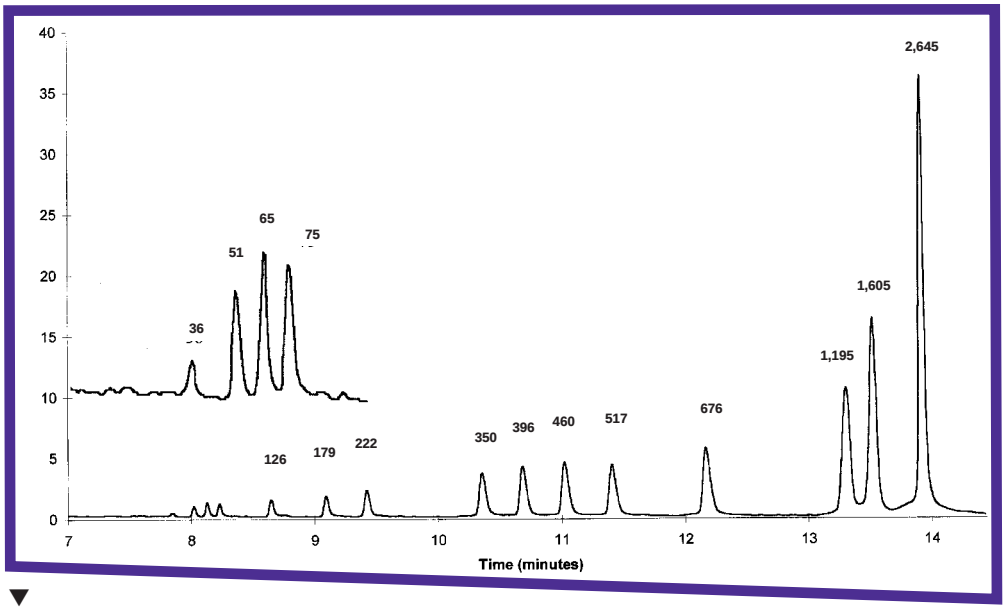


Figure 1: Method 2, using a 27 cm capillary.

Submit manuscripts — exchange ideas

Providing a forum for the exchange of ideas, we invite you to submit articles or manuscripts describing novel uses of capillary electrophoresis in your laboratory.

Content: You decide. If the subject is of interest to you, it may be interesting to others as well.

Length: Please keep your article to less than 1,200 words.

Use appropriate referencing, where copywrite and trademarks are involved.

Beckman Coulter reserves the right to edit for length/clarity.
Send a hard-copy version of your article and electronic version (on disk) to:

Beckman Coulter, Inc.
P/ACE Setter Review Group
M/S D-31-E
4300 Harbor Blvd. / P.O. Box 3100
Fullerton, CA 92834-3100
or send your article via email to:
jdchapman@beckman.com

HPCE '99: Truly putting CE to work

Beckman Coulter participated in the 12th International Symposium on High Performance Capillary Electrophoresis and Related Microscale Techniques (HPCE), Jan. 23-28 in Palm Springs, Calif.

The meeting opened Sunday evening with an excellent plenary session, consisting of three presentations. Leroy Hood from the University of Washington spoke on "Functional Genomics – From the Genome to the Physiome." Fred McLafferty from Cornell University presented "The Optimum CE detector for Biomolecules: High-Resolution 2-D Mass Spectrometry." Peter Gill from Priory House Forensic Science Service provided "A Review of DNA Profiling in Forensic Science – Implications for the Future."

The rest of the week was then split into parallel sessions, including: microscale MS, DNA analysis, DNA sequencing , protein analysis, applications in bioanalysis, fundamental concepts, advances in detection, CEC column technology, CEC fundamentals and applications, single-cell analysis, microfabricated devices and affinity CE

Does it sound like a long week? It sure was; but it was a very informative week and by all accounts a very successful scientific meeting. There was literally something for every one of the 629 scientists in attendance. The most notable part of the meeting, however, was the poster session, with more than 311 posters being presented. These posters were of very high caliber and most were applied to solving every-day analytical problems. It was nice for a change to see CE being used simply as a tool to get a job done, and of course it was nice to see so many P/ACE systems actively being used.

As the acknowledged leader in capillary electrophoresis, Beckman Coulter takes a strong interest in the HPCE meeting with a focus on accomplishing three major goals:

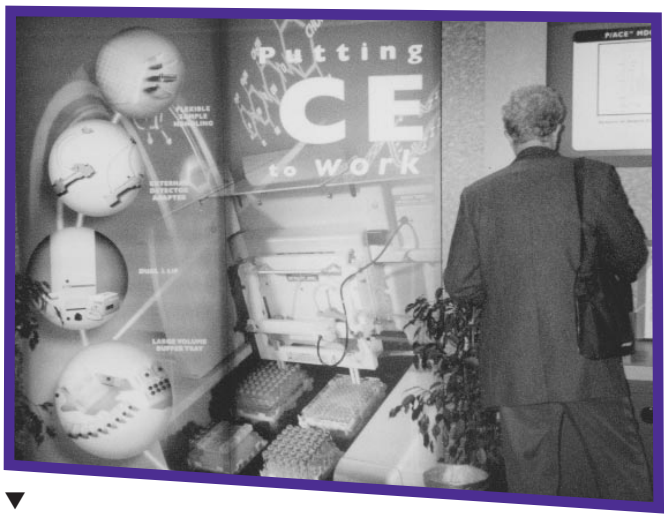
1) Support the scientific exchange of information on CE.

2) Present our latest enhancements to the P/ACE product line.

3) Thank our current P/ACE users for their support.

Exchange of information

The No. 1 goal of the HPCE meeting is in the exchange of scientific information. To promote this exchange, we once again invested in being a major HPCE sponsor. We sent more than 45 delegates, exhibited with a 30-foot display, and participated in the scientific sessions with the following presentations:



The centerpiece of the Beckman Coulter booth was an eye-catching graphic featuring the P/ACE MDQ with new LIF detector.

Industrial seminars

1) Implementing Capillary Zone Electrophoresis Screening Strategies in a Forensic Toxicology Laboratory. John C. Hudson, Royal Canadian Mounted Police.

2) Recent Developments in Capillary Electrochromatography: Advanced Monolithic Column Technology. Chitra K. Ratnayake, Michael P. Henry and Chan S. Oh, Beckman Coulter.

3) Advances in Fully Automated DNA Sequencing and dsDNA Analysis. Ming-Sun Liu, Fu-Tai A. Chen and Michael W. Clark, Beckman Coulter.

Session lecture

Multi-Capillary CE/LIF Using a Galvometric Scanner: Application to DNA Analysis. Stephen Pentoney, Clarence Lew and David Yang, Beckman Coulter.

Poster sessions

Poster 1) Optimized Conditions for Sequencing a Variety of DNA Templates using the CEQ™ 2000 DNA Analysis System. Mark Dobbs, Doni Clark, Keith Roby and Scott Boyer, Beckman Coulter.

Poster 2) The CEQ 2000: An Automated Multi-Capillary DNA Analysis System. David Yang, Beckman Coulter.

Poster 3) Multi-segmented Column Performance in Capillary Electrochromatography. Chitra Ratnayake and Michael P. Henry, Beckman Coulter.

Poster 4) Detection of Aberrant Paraproteins by Capillary Zone Electrophoresis. Cynthia R. Blessum, Marina Andres and Neeta Khatter, Beckman Coulter.

Poster 5) Detection of Urinary Bence Jones Proteins by Capillary Zone Electrophoresis. Cynthia R. Blessum, Zara Safarian, Marina Andres and Neeta Khatter, Beckman Coulter

Poster 6) Detection of Non-Protein Components in Serum by Capillary Zone Electrophoresis. Cynthia R. Blessum, Marina Andres, and Neeta Khatter, Beckman Coutler.

Poster 7) Characterization of Highly

Sulfated Cyclodextrins and their Applications for Enantiomeric Separation of Chiral Species. Fu-Tai A. Chen, Ramon Evangelista and Gene Shen, Beckman Coulter.

Poster 8) Profiling N-linked Oligosaccharides of Glycoproteins by Capillary Electrophoresis. Fu-Tai Chen, Isabel Flores and Ramon Evangelista, Beckman Coulter Inc.

P/ACE enhancements

The theme at the Beckman Coulter booth this year was "Putting CE to Work," a focus on applying CE technology to solving real world analytical challenges. At the center of this focus was the preview of our new dual-wavelength laser-induced fluorescence detection system, designed as a modular detector for the P/ACE MDQ platform. This detection system brings carbohydrate analysis, gene expression, genotyping and high-affinity assay capability to a platform designed for the pharmaceutical/biotechnology laboratory. Whether it's research, drug discovery, process development, analytical methods development or quality control — a single platform can be configured to address all demands.

Beckman Coulter also launched a new M-Grade APTS manufactured to a purity level required for monosaccharide analysis. Although APTS has been used successfully for the analysis of glycans, not until now has a commercially available product been available at the purity required for monosaccharides. Data on this analysis was presented during the poster session.

Finally, Beckman Coulter displayed the new CEQ 2000 DNA Analysis System. The eight-capillary CE-based genetic analysis system fully automates the sequencing process, from sample denaturation and loading to sample separation and DNA base calling.

Customer appreciation

The 12th Annual International HPCE meeting gave us the opportunity to host our seventh annual P/ACE Users Appreciation Event. This year Beckman Coulter treated more than 160 P/ACE users to an evening of "Desert Discoveries." The event was held at Palapas of Araby Cove – a unique hands-on artists' garden.

Experimenting with pottery.

Participants select "dream sticks" that will be used to predict their future.

Attendees were allowed to wander through the gardens enjoying sumptuous food, and participate with artisans tending to their crafts. Some had their fortunes told by the Native Dream Stick Reader, while others made their own pottery. This truly was a magical place under the mystical desert skies.

Of course, the event would not be complete without the CEparation of the Year Contest. All attendees were invited to judge the contest entries. This year's winners were:

First Place: Ken K.C. Yeung, Department of Chemistry, University of Calgary, Alberta Canada. The CEparation was titled: "Ultra High Resolution CE": Separation of Isotopes

Ken K.C. Yeung

Second Place: Kevin Altria, Pharmaceutical Methods Development, Glaxo Wellcome, Ware, Herts, U.K. The CEparation was titled: Application of Microemulsion Electrokinetic Chromatography (MEEKC) to the Analysis of a Wide Range of Solutes.

Kevin Altria

Third Place: Mark P. Richards, U.S. Department of Agriculture, Beltsville, MD. The CEparation was titled: Leaping over Leptin Levels. A QC-RT-PCR Assay for Leptin Gene Expression.

Mark P. Richards

And, of course, an honorable mention to all of those who participated. The CEparations submitted were of high quality and were all consistent with truly "Putting CE to Work." Next year's CEparation contest will be held in Saarbrucken Germany. We encourage all of you who plan to ▶

attend to consider submitting an entry into the contest. It's not about "rocket-science," but instead simply "Putting CE to Work" in your laboratory. What's important to you is most often important to others as well. So in addition to having some fun and sharing good prizes, it's an opportunity to communicate with your peers and share good science.

Beckman Coulter also provided a contest at the booth, giving away a pH meter a day! As we have just launched a whole new line of pH meters and since pH measurement is critical to CE, we took the opportunity to thank our patrons with this contest.

Congratulations are extended to the winners of the new Φ® 265 waterproof pH meters:

Monday
Danilo Corradini
CNR Institute Chromatography
Monterotonao Station, Italy.

Tuesday
Scott Waite
Huntsman
Austin, Texas.

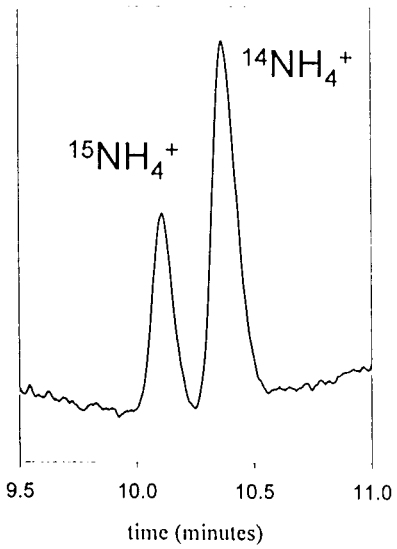
Danilo Corradini

Wednesday
Hideki Horie
NIVOT
Kanaya, Shizuoka, Japan.

Overall, HPCE '99 was a very successful scientific meeting, and for the first time there really was a sense of the maturing of CE technology. This is no longer a technology looking for an application, but rather simply an analytical tool being put to work to solve complex analytical separations.

CEparation of the Year

"Ultra High Resolution CE" : Separation of Isotopes



- isotopic separation was demonstrated as an example of "Ultra High Resolution" CE Separation
- separation based on the small isotope effect on acid dissociation, pKa
- small mobility difference was accentuated by an opposing EOF; *i.e.*, "*mobility counterbalance*"
- an anodic EOF was precisely controlled by a mixture of cationic surfactant (CTAB) and zwitterionic surfactant (Rewoteric AM CAS U, Witco) as buffer additives

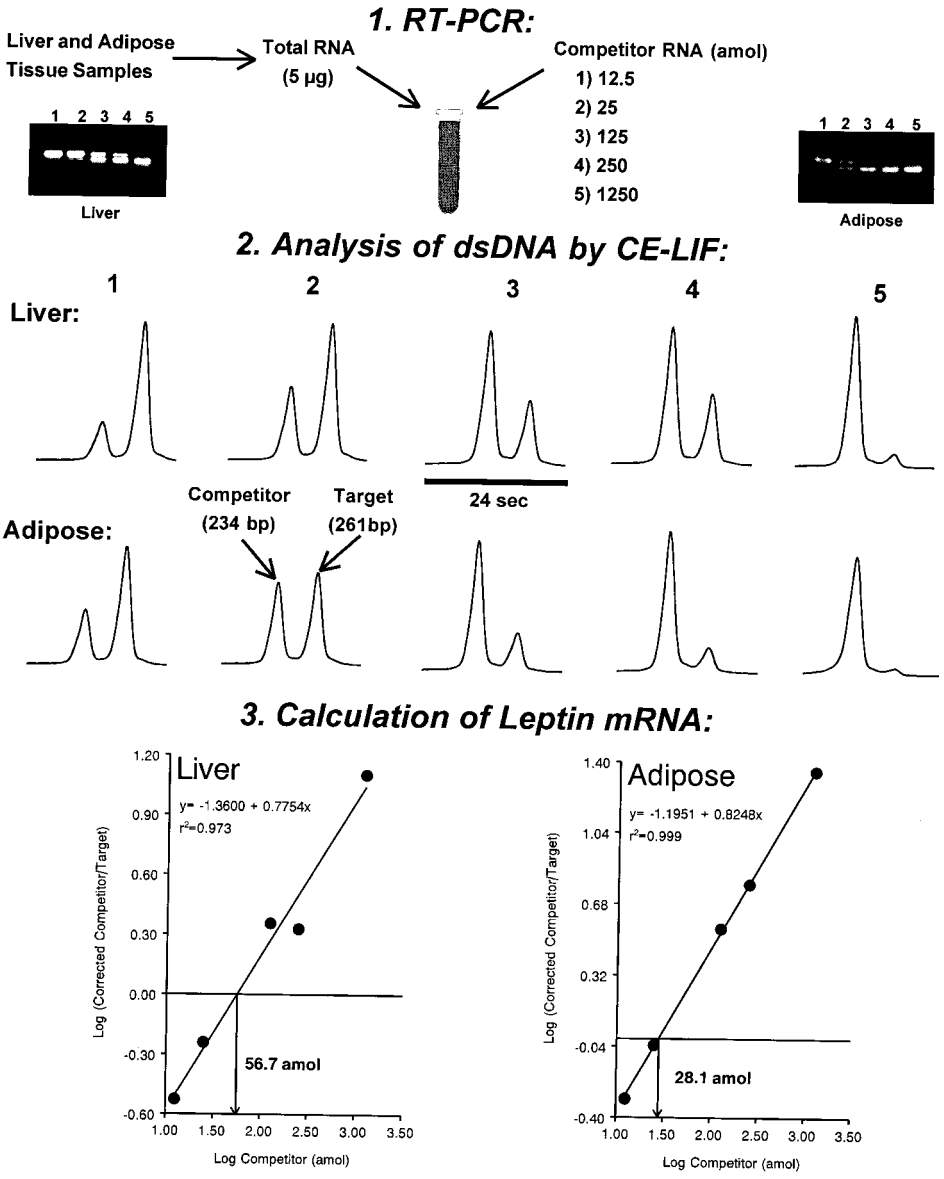
Conditions: sample, 0.1 mM $^{15}NH_4^+$ and 0.2 mM $^{14}NH_4^+$, buffer, 20 mM benyzltributylammonium borate at pH 9.5; additives, 0.5 mM CAS U and 15 μM CTAB; voltage +20kV; capillary length 40/47 cm; detection, indirect at 214 nm. Instrument, Beckman P/ACE 2100

CEparation of the Year, first-place poster, Ken K.C. Yeung, Department of Chemistry, University of Calgary, Alberta Canada.

For second-place winner, see article by Kevin Altria, page 5.

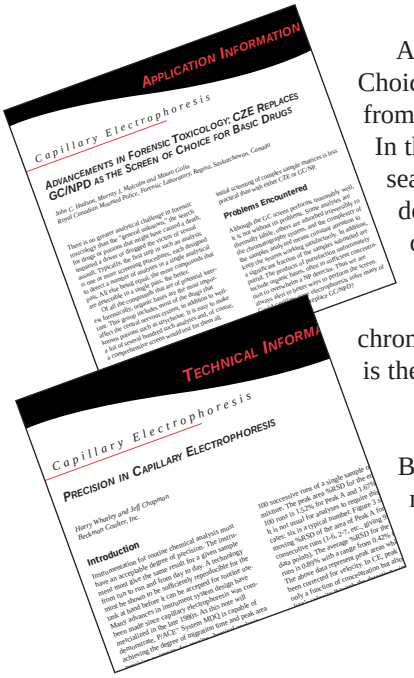
QC-RT-PCR Assay for Leptin Gene Expression

Leptin is a peptide hormone encoded by the obesity (ob) gene that functions in the regulation of feeding behavior, energy balance and reproduction in humans and animals. In mammals, leptin is expressed in adipocytes and its level in blood increases in direct proportion to body fat mass. Circulating leptin signals the brain about the status of body energy (fat) stores, and the brain, in turn, activates specific neural pathways that modulate food intake and energy expenditure to help maintain energy stores at a set level. The brain (primarily the hypothalamus) expresses a specific receptor for leptin that allows it to sense changes in blood leptin levels. Thus, a negative feedback loop exists between adipose tissue and the central nervous system, with leptin serving as an afferent signal to the brain. Recessive mutations in the genes encoding leptin and its receptor have been identified and linked with the pathological conditions of morbid obesity, diabetes and infertility. In light of this, it is important to accurately monitor leptin expression at the levels of nucleic acid (mRNA) and protein.



CEparation of the Year, third-place poster, Mark P. Richards, U.S. Department of Agriculture, Beltsville, MD.

New CE literature from Beckman Coulter



A-1859A, Advancements in Forensic Toxicology: CZE Replaces GC/NPD as the Screen of Choice for Basic Drugs, focuses on the work of John Hudson, Murray Malcolm and Mauro Golin from the Royal Canadian Mounted Police Forensic Laboratory, Regina, Saskatchewan, Canada. In this bulletin, Hudson stresses the greatest analytical challenge in forensic toxicology: the search for drugs that may have caused injury or death. For five years Hudson's lab has been developing capillary zone electrophoresis (CZE) on the P/ACE 5500 to analyze drugs in various matrices. Most recently, they have used a P/ACE MDQ with diode array detection (DAD). His lab found that the combination of mobility and UV spectral data generated by CZE-DAD is much stronger evidence than the combination of retention index (RI) and gas chromatography (GC). Hudson concludes that the future is truly bright for CZE; that CZE-DAD is the screen of choice for basic drugs by toxicologists in their lab.

T-1860A, Precision in Capillary Electrophoresis, by Harry Whatley and Jeff Chapman of Beckman Coulter, illustrates that the P/ACE MDQ is capable of achieving the degree of migration time and peak area precision needed for routine chemical analysis. The basic requirements to reproducibility are a quality instrument that is properly operated, calibrated, and equilibrated.

Course descriptions/Training schedule

Introduction to Capillary Electrophoresis **P/N 149844** **$395 (US)**

This one-day lecture course provides a sound theoretical basis for capillary electrophoresis and an overview of current chemistries and approaches to methods validation. *8:30 a.m. to 4:30 p.m.*

1999 training schedule:
Somerset, NJ: **Sept. 7**
Schaumburg, IL: **May 11, Nov. 2**
Palo Alto, CA: **Aug. 17**

P/ACE™ Station Software Operation **P/N 149846** **$395 (US)**

This is a one-day class covering advanced software functions and instrument operation. Hands-on exercises at PC workstations utilize practice data sets provided by the instructor. *8:30 a.m. to 4:30 p.m.*

NOTE: To ensure maximum benefit for all students, this course requires that all attendees complete the P/ACE Station Software Tutorial Workbook prior to attendin and bring exercise results with them.

1999 training schedule:
Somerset, NJ: **Sept. 8**
Schaumburg, IL: **May 12, Nov. 3**
Palo Alto, CA: **March 24, Aug. 18**

Troubleshooting CE Methods and Systems **P/N 149845** **$395 (US)**

In this one-day course the student learns to troubleshoot method and electropherogram problems as well as common system problems. This class includes a troubleshooting workshop and hands-on system maintenance practice. *8:30 a.m. to 4:30 p.m.*

1999 training schedule:
Somerset, NJ: **Sept. 9**
Schaumburg, IL: **May 13, Nov. 4**
Palo Alto, CA: **Aug. 20**

To enroll, call 1-800-742-2345 (USA) • 1-800-387-6799 (CANADA)
or contact your local Beckman Coulter office

Quality Control of Drugs by CE: Practical Approach

June 14-17, 1999, Montpellier, France

European training program in microseparation techniques, promoted by the European Union, Leonardo da Vinci project.

Objective

Capillary electrophoresis (CE) is becoming a routine method for the quality control of drugs. This training course is mainly focused on the quantitative aspects of the technique. It is aimed to show from practical how it is possible to optimize the criteria required for validating the assay method of an active drug and its degradation products.

Intended participants

Technical executives and technical staff of development and quality control laboratories from pharmaceutical, chemical, agro-chemical, cosmetic industries and laboratories concerned by the quantitative aspects of the technique. People wishing to acquire a training in specialized analytical techniques for quality control.

Program

- Principle; instrumentation; background review of applications (impurities, assay, identification, chiral separations and small ions)
- Optimization of selectivity, repeatability and sensitivity
- Quantitative procedures
- Good working practices
- Method ruggedness and validation, method transfer
- Chiral separations
- Analysis of small ions (stoichiometry)
- Good laboratory practice

Similar lectures given concurrently in English Dr. Kevin Altria, Glaxo Wellcome, and in French by Prof. Huguette Fabre, Faculté de Pharmacie.
Course will include lectures and practical. Participants can bring samples for testing.

Cost

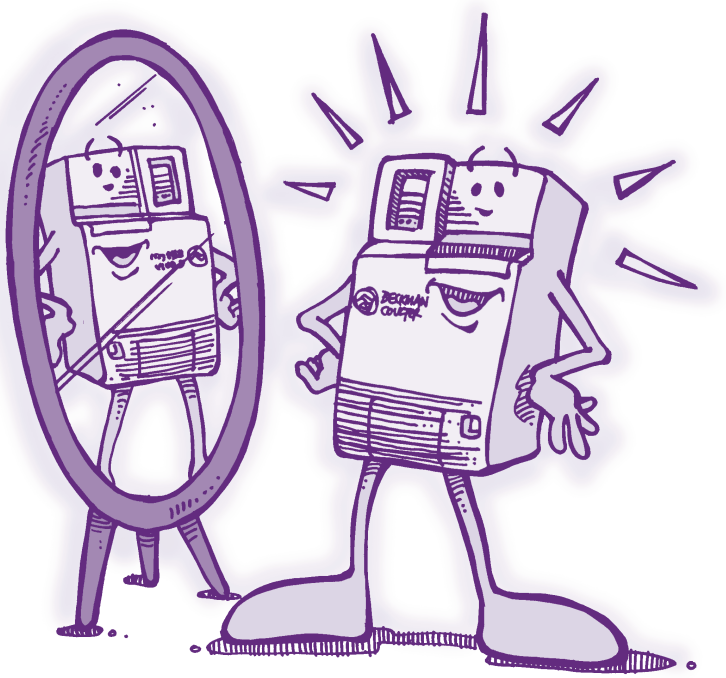
1300 (EUR) for registration before May 15, 1450 (EUR) after May 15

For more information

Contact: Prof. H. Fabre or Dr. M.D. Blanchin
Laboratoire de Chimie Analytique, Faculté de Pharmacie
F-34060, Montpellier Cedex 2
France
Tel +33.4.67.54.45.20
Fax: +33.4.67.52.89.15
E-mail hfabre@pharma.univ-montp1.fr

P/ACE Setter

What's new with Dr. Q



MDQ Laser-Induced Fluorescence Detector

- Dual λ excitation
- Dual λ emission
- Universal laser input
- Rapid analysis (10-cm capillaries)
- Automated calibration
- Ultra-high sensitivity

The new LIF detector — available as an upgrade module for existing MDQ users — is also integrated into application-specific CE systems designed for:

- Oligosaccharide and monosaccharide analysis
- DNA-protein interactions
- Quantitative gene expression
- DNA fragment analysis
- CE-based immunoassays

Part No. 144954: P/ACE MDQ Dual λ LIF Detector
Part No. 144901: P/ACE MDQ Single λ LIF Detector
Contact your local sales representative for upgrade information

For more information on the new LIF Detector, ask for Data Sheet No. DS-8284A

BECKMAN COULTER™

Developing innovative solutions in genetic analysis, drug discovery, and instrument systems.

Innovate SIMPLIFY Automate

Beckman Coulter, Inc. • 4300 N. Harbor Boulevard, Box 3100 • Fullerton, California 92834-3100
Sales: 1-800-742-2345 • Service: 1-800-551-1150 • Telex: 678413 • Fax: 1-800-643-4366 • www.beckmancoulter.com

Worldwide Bioresearch Division Offices:
Australia (61) 2 9844-6000 **Canada** (905) 819-1234 **China** (86) 10 6515 6028 **Eastern Europe, Middle East, Africa** (41) 22 994 07 07
France 01 49 90 90 00 **Germany** (89) 35870-0 **Hong Kong** (852) 2814 7431 / 2814 0481 **Italy** 02-953921 **Japan** 03-5404-8359
Mexico 525-559-16-35 **Netherlands** 0297-230630 **Singapore** (65) 339 3633 **South Africa** (27) 11-805-2014/5 **Spain** 91 728 7900
Sweden 08-98 53 20 **Switzerland** 0800 850 810 **Taiwan** (886) 2 2378 3456 **U.K.** 01494 441181 **U.S.A.** 1-800-742-2345.

B98-3134-CB-8K

Printed in U.S.A. on recycled paper.