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REVIEW

Chemometric experimental design based optimization techniques in capillary electrophoresis: a critical review of modern applications

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Abstract A critical review of recent developments in the use of chemometric experimental design based optimization techniques in capillary electrophoresis applications is presented. Current advances have led to enhanced separation capabilities of a wide range of analytes in such areas as biological, environmental, food technology, pharmaceutical, and medical analysis. Significant developments in design, detection methodology and applications from the last 5 years (2002–2007) are reported. Furthermore, future perspectives in the use of chemometric methodology in capillary electrophoresis are considered.

Keywords Capillary electrophoresis · Chemometrics · Optimization · Experimental design · Response surface methodology

Introduction

Over the past two decades capillary electrophoresis (CE) has become the technique of choice in many analytical laboratories where analysis of small quantities of materials must be accurately, efficiently, and expeditiously assessed. CE is a powerful analytical separation technique that brings speed, quantitation, reproducibility, and automation to the inherently highly resolving but labor-intensive methods of electrophoresis [1–5]. It is an excellent tool in the analysis of biological materials and is an unparalleled experimental tool for examining interactions in biologically relevant media. CE differentiates charged species on the basis of mobility under the influence of an applied electric field gradient. Selectivity can be manipulated by the alteration of electrolyte properties such as pH, ionic strength, and electrolyte composition, or by the incorporation of electrolyte additives. CE offers a number of advantages as a separation technique: (1) it requires only small quantities of material; (2) it is applicable to water-soluble, nonvolatile, high molecular weight species in aqueous buffer solution; (3) it is readily automated and has good reproducibility; and (4) various separation modes make it applicable for the analysis of a variety of biological and nonbiological species.

CE also suffers from several weaknesses as an analytical technique. Adsorption of charged species to the capillary wall can occur in the absence of efforts to minimize adsorption and can change the magnitude of electroosmotic flow. The presence of Joule heating and other effects of using high voltage create variances in electroosmotic flow sometimes yielding irreproducible migration times for analytes, making comparison from run to run problematic. This disadvantage can be especially troubling in the pharmaceutical industry, where quality control is a priori and where method development is critical in product manufacture, analysis, and marketing. Hence, it is important to be able to determine optimal conditions in CE method development.

Fortunately, various chemometrics-based techniques including multivariate experimental design and response surface methodology (RSM) have been devised to aid in optimizing the performance of a system. The importance of, and theoretical concepts behind, experimental design and optimization methodology in research and development

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efforts have been thoroughly discussed in a number of informative publications [6–21] and are highlighted in subsequent sections. Previous reviews and informative research papers provided systematic studies on early development efforts of the use of experimental design methodology in CE [22–32].

In this paper, we present a critical review of applications of experimental design and optimization methodology within the last 5 years. The first section presents an overview of basic experimental design considerations. This is followed by a discussion of how recent applications have utilized such methodology for advanced separation and characterization in the areas of biological, environmental, food technology, pharmaceutical, and medical analysis. Moreover, future perspectives in the use of chemometric methodology in CE are considered.

Experimental design and optimization considerations

The main applications of experimental design include factor screening, response surface examination, system optimization, and system robustness. To properly implement such procedures the following steps should be considered:

1. Determine the overall goals and objectives of the experiment
2. Define the overall outcome (response) of the experiment
3. Define the factors (and their levels) that will influence the response
4. Choose a design that is compatible with the overall objectives, number of factors considered and required precision of measurements

Such an approach is opposite to the classical univariate method in which the response is investigated for each factor while all other factors are held at a constant level. Univariate methods are time-consuming and do not take interactive effects between factors into account. If the effects are additive in nature, then experimental designs are the optimum choice and require fewer measurements. This type of step-by-step approach ideally leads to a large number of independent analyses, which when considering the number of factors that may be varied to optimize CE methods, can be substantial. Factors typically varied include, but are not limited to, voltage, capillary length, injection time, buffer concentration, pH, and electrolyte composition. In addition, proper experimental design techniques are important for sample preparation procedures prior to CE analysis. Solid-phase extraction, for example, is commonly used as a preconcentration and cleanup technique prior to sample analysis.

Blocking is one of the fundamental principles of good experimental design and is employed when an analyst is aware of extraneous sources of variation that influence the response. The blocking process itself reduces the variability from the most important sources and hence increases the precision of experimental measurements. Essentially, experimental units are grouped into homogeneous clusters in an attempt to improve the comparison of treatments by randomly allocating the treatments within each cluster or "block." Randomization can then be used to reduce the variability from the remaining extraneous sources. The analyst can also employ the processes of replication and repetition for each factor combination, to allow a better estimate of experimental error, which helps determine whether observed differences in the data set are truly statistically different. Replication also allows an estimation of the true mean response for one or more factor levels, thus aiding in precisely defining the effect of a factor on the response.

Screening designs

Screening techniques such as factorial designs allow the analyst to select which factors are significant and at what levels. Such techniques are vital in determining initial factor significance for subsequent optimization. They are especially important in CE method development, where the most influential factors, their ranges, and interactions are not yet known. The most general (two-level design) is a full factorial design and described as 2^k designs, where the base 2 stands for the number of factor levels and k is the number of factors each with a high and a low value [17, 33, 34]. The lower level is indicated with a minus sign; the higher level with a plus sign. The combination of two factor levels is termed a treatment or run. With two factors this defines a square in the factor space (Fig. 1) and with three factors this ultimately defines a cube (Fig. 2). Fractional factorial

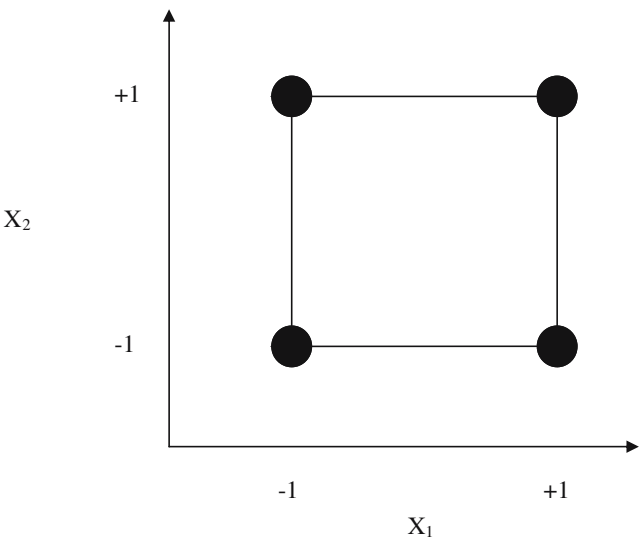

Fig. 1 Two factor two-level factorial design (2^2 design)

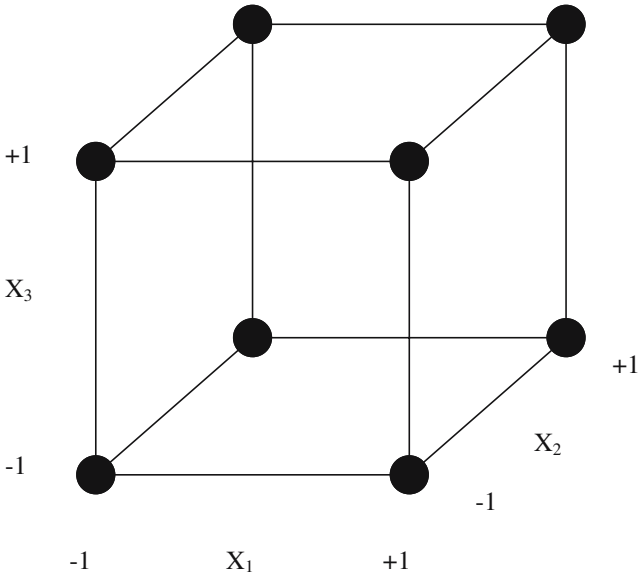

Fig. 2 Three-factor two-level factorial design (2^3 design)

designs are good alternatives to a full factorial design, especially in the initial stage of a project, and are considered a representative subset of a full factorial design [33]. In fractional factorial designs, the number of experiments is reduced by a number p according to a 2^{k-p} design. As an extension to factorial and fractional factorial designs, first-order Pareto charts can be used in the determination of statistical significance and to provide easier response visualization through the use of specialized versions of histograms.

Response surface methodology

RSMs are multivariate techniques that mathematically fit the experimental domain studied in the theoretical design through a response function [7, 8]. Two key early papers on the topic of response surfaces were written by Box and Wilson [35] and Box and Hunter [36]. Two of the most common designs generally used in response surface modeling of CE applications are central composite and Box–Behnken designs. Central composite designs contain imbedded factorial or fractional factorial designs with center points that are augmented with a group of axial (star) points that allow estimation of curvature [7, 8]. A central composite design always contains twice as many star points as there are factors in the design. The star points then represent new extreme values (minus and plus) for each factor in the design.

The Box–Behnken design is considered an efficient option in RSM and an ideal alternative to central composite designs [7]. It has three levels per factor, but avoids the corners of the space, and fills in the combinations of center and extreme levels. It combines a fractional factorial with incomplete block designs in such a way as to avoid the extreme vertices and to present an approximately rotatable design with only three levels per factor. This design is appropriate for situations where the experimenter is not interested in predicting response at extremes. A less common, but effective method is the Doehlert design. Like the Box–Behnken design, Doehlert designs require lower numbers of experiments than the central composite design. Another advantage of the Doehlert design over the central composite approach is its higher efficiency value, ultimately determined by dividing the coefficient number of the quadratic equation by the number of experiments required for the design.

Recently, artificial neural networks (ANNs) have been incorporated, either separate or in combination with many of the experimental design techniques discussed above, into CE optimization methods [37–40]. ANNs are computational models based on biological neural networks consisting of an interconnected group of artificial neurons and process information using a connectionist approach to represent the strengths (weights) of the connections [37, 38].

Overall, the chemometric approaches presented above provide enhanced design and optimization strategies for a variety of CE methods. Additionally, they can lead to improved robustness evaluations, but often within narrower ranges. This helps ensure that the quality of the data generated is independent of minor variations during the analytical procedure.

Applications

The applications presented in the following sections combine many of the screening and optimization techniques highlighted in the previous sections. A common approach among these studies is to first screen for significant factors, followed by full optimization using, for example, a proven RSM technique. Table 1 lists examples of experimental design and optimization applications in the last 5 years (2002–2007). This list has been selected to provide a representative coverage of such techniques in biological, environmental, food, and pharmaceutical analyses. Upon examination, the reader will find that the myriad of factors present in CE methods have proven significant across the wide range of applications presented. More in-depth discussions and critical evaluation of selected studies in each area are discussed in the following sections.

Biological/biomedical

The ability to separate complex mixtures of DNA is a major goal of any separation technique. During the past decade CE has been increasingly used to separate DNA fragments owing to its inherent resolving power, speed, and variable separation conditions. Predominantly in the capillary gel electrophoresis

Table 1 Selected chemometric experimental design and optimization techniques and related capillary electrophoresis (*CE*) applications (2002–2007)

Application	Analytes	Detection scheme	Design and optimization strategy	Reference
Biological/ biomedical	DNA	CE with laser-induced fluorescence detection with excitation from an argon laser (λ_{exc}=488 nm)	3-factor simplex optimization method	[41]
Agriculture	Streptomycin, oxytetracycline	CZE with UV detection at 195 nm	2^3 factorial screening design followed by a central composite response surface design	[42]
Biological/ biomedical	Carbonic anhydrase B	Affinity CE with UV detection at 200 nm	Box–Behnken response surface design	[46]
Biological/ biomedical	3′-Azido-2′,3′-dideoxythymidine	CE with UV detection at 268 nm	Plackett–Burman design with 2 levels	[47]
Biological/ biomedical	DNA	CE with laser-induced fluorescence detection with excitation from an argon laser (λ_{exc}=488 nm)	6-factor simplex optimization method	[48]
Biological/ biomedical	Adenine, uracil, adenosine, guanosine, uridine, inosine	CE with UV detection at 254 nm	Central composite response surface design	[49]
Biological/ biomedical	α-casein, β-casein, κ-casein	CE with UV detection at 214 nm	Desirability functions combined with a central composite design	[50]
Biological/ biomedical	Quinolones	CZE–tandem mass spectrometry	Fractional factorial screening design (2^{6-1} plus 3 central points) and a Doehlert response surface design	[51]
Biological/ biomedical	Ascorbic acid, isoascorbic acid	CZE with UV detection at 254 nm	Full factorial screening design, Box–Behnken, and central composite response surface designs	[52]
Environmental	2,4-D, dicamba, 2,4,5-T	CE with UV detection at 200 nm	Orthogonal experimental design	[53]
Environmental	Zn, Na, Ca, Mg	CE with UV detection at 214 nm	2^6 factorial screening design	[58]
Environmental	1-*n*-Butyl-3-methylimidazolium bis (trifluoromethanesulfonyl) amide	CE with UV detection at 254 nm	4-factor D-optimal experimental design	[59]
Food	Sucralose	CZE with indirect UV detection at 238 nm	Central composite response surface design	[60]
Food	Tartaric, malic, succinic, acetic, and lactic acids	CE with indirect UV detection at 214 nm	2^3 factorial screening design and a central composite response surface design	[61]
Food	Biogenic amines	CE with multichannel mode monitoring at 230, 305, 360, and 480 nm	2-factor central composite response surface design with the use of Derringer's desirability function	[62]
Pharmaceutical	Tryptophane and tyrosine methylesters	CE with multichannel mode monitoring at 218, 236, and 254 nm	3-factor central composite response surface design	[64]
Pharmaceutical	Thiazinamium, promazine, promethazine	CZE with UV detection at 254 nm	Face-centered Draper–Lin small composite design	[66]
Pharmaceutical	Pentosan polysulfate	CZE with UV detection at 320 nm	Central composite response surface design	[69]
Pharmaceutical	Mizolastine	CZE with UV detection at 210 nm	4-factor Doehlert response surface design	[70]
Pharmaceutical	Tetracycline, chlortetracycline, oxytetracycline, doxycycline	CZE with UV detection at 270 nm	2^{4-1} fractional factorial and central composite surface response design	[71]

Table 1 (continued)

Application	Analytes	Detection scheme	Design and optimization strategy	Reference
Pharmaceutical	*Gingko biloba* extracts	CE with UV detection at 270 nm	Experimental design (5 deliberate and 4 random experiments) and artificial neural network	[72]
Pharmaceutical	Brompheniramine, chlorpheniramine, cyproheptadine, diphenydramine, doxylamine, hydroxyzine, loratadine	CZE with UV detection at 214 nm	3^2 full factorial design	[73]
Pharmaceutical	*Salvia officinalis* plant extracts	CE with UV detection at 280 nm	Central composite response surface design and artificial neural network	[74]

CZE capillary zone electrophoresis

(CGE) format, a myriad of separations methods have been developed to separate base pair (bp) fragments of large variation. Unfortunately, realizing optimal experimental conditions via standard univariate approaches has been time-consuming and the results questionable owing to false optimal points. Catai et al. [41] used a simplex optimization method to maximize the correlation coefficient (r^2) of a logarithmic plot of electrophoretic mobility (μ) versus bp in the analysis of a large DNA size range by CGE. Six CE separation variables (sample buffer concentration, injection time, electric field strength of injection, temperature, matrix concentration, and electric field strength of separation) were simultaneously varied by the simplex, leading to an optimal point. With use of DNA fragments ranging between 75 and 4,072 bp, three vertexes were achieved with a correlation coefficient of more than 0.98. Vertex 21 showed the highest resolution and signal intensity and it was these conditions that were used for the study. The value of r^2 increased to 0.992 with a relative standard deviation less than 0.02% using vertex 21. To determine the precision of the method in determining the size of DNA fragments, a 1-kbp DNA ladder was evaluated. The 517-bp fragment showed the highest error (13%); still, the simplex was successful in minimizing other separation mechanisms. We disagree with the authors' statement that the simplex method is the most efficient and easily employed optimization procedure and believe it can be situation-specific. If there are active upper-limit constraints on the components, for example, then designs like the D-optimal design would be more appropriate. This work, however, does highlight the successful use of a simplex design to optimize separation conditions for DNA fragments.

Agriculture

The dramatic increase in the human population over the past 50 years has put great stress on increasing crop yield. Crop biotechnology, for example, has been offered as a solution to overcome the stagnation in crop yield potential, particularly in those areas of the world where yields are low and malnutrition and starvation are constant threats. Another way to increase crop yield is through the use of antimicrobials such as fungicides and/or herbicides in the agriculture sector to aid in the extermination of insect pests. Maia et al. [42] have described the development of a CE method to determine and separate streptomycin and oxytetracycline. The method was validated using six performance criteria: linearity and linear range, sensitivity, selectivity, intraday and interday precision, detectability, accuracy, and ruggedness. Initially, the authors first carried out a simple factorial plan (2^3) to distinguish the significant parameters by the analysis of the effects. The resulting design was used to conduct a higher-order design with central composite features. The use of the initial 2^3 factorial is questioned as central composite designs contain an embedded factorial or fractional factorial design with center points. However, the method described herein is complete and may, in the future, be an alternative to time-consuming microbial assays or high-performance liquid chromatography (HPLC) requiring much larger sample volumes.

Affinity capillary electrophoresis

Affinity CE (ACE) is a versatile analytical technique that has been used extensively to measure affinity parameters between biological species. Since the first paper in 1992 detailing the use of ACE to estimate binding and dissociation constants, a host of receptor–ligand interactions have been probed, including protein–ligand, peptide–peptide, antibody–antigen, and enzyme–drug [43]. ACE uses the resolving power of CE to distinguish between free and bound forms of a receptor as a function of the concentration of free ligand in the electrophoresis buffer. In a typical form of ACE, a sample of receptor and standard

(s) is exposed to an increasing concentration of ligand in the running buffer, causing a shift in the migration time of the receptor relative to the standard(s). In earlier work, we developed more specialized forms of ACE called partial filling ACE (PFACE) and flow-through PFACE (FTPFACE) [44, 45]. In the former, the capillary is partially filled with ligand and a sample plug of receptor is introduced into the capillary and electrophoresed. During electrophoresis the zones of samples overlap within the capillary and equilibrium is established prior to the point of detection. In FTPFACE, an even smaller zone of ligand is used in the assay. Following up on this work, we used a univariate approach to optimizing conditions for FTPFACE [44]. However, this approach was time-consuming and did not take interactive effects into account. In order to address such concerns, Hanrahan et al. [46] used a Box–Behnken design to predict the significance of capillary length, voltage, and injection time on the binding of a small ligand to carbonic anhydrase B (EC 4.2.1.1). In their work, the design was an efficient option in RSM and an alternative to central composite designs. By combining a fractional factorial with incomplete block designs the approach avoided the extreme vertices, yielding a rotatable design with only three levels per factor. In this approach, statistical analysis results were used to create a mathematical model for response surface prediction at the target response (K_d= 1.19×10^{-6} M). Figure 3 shows the response surface of the main interaction: injection time/capillary length. The adequacy of the model was validated by experimental runs with the predicted model solution. Here, a K_d of 1.29×10^{-6} M was obtained (an 8.4% discrepancy difference from the target response). In conclusion, the approach yielded a large amount of information while minimizing the number of experimental runs.

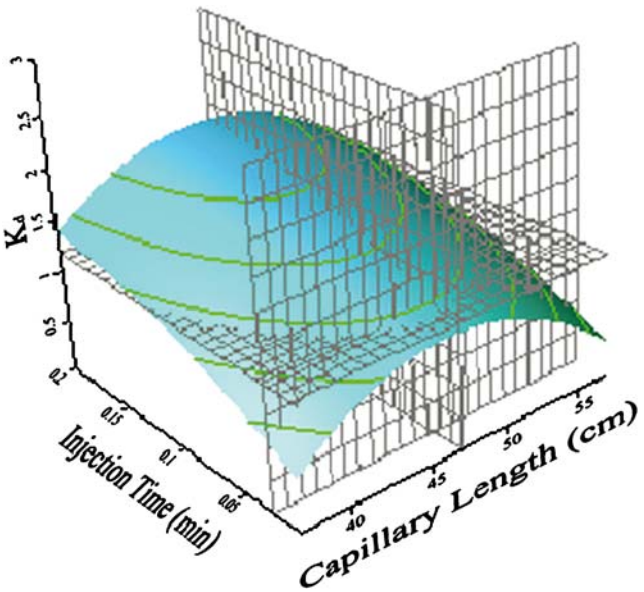

Fig. 3 Response surface generated plot showing main interaction injection time versus capillary length (from [42] with permission from Wiley-Interscience)

HPLC has long been the method of choice in diastereomeric separations on both achiral and chiral stationary phases. For example, proteins, cyclodextrins, and macrocyclic antibiotics have been widely used in separation techniques, the majority of this work involving chiral ligands immobilized on silica material. Unfortunately, HPLC is limited by slow diffusional mass transfer and large void volumes between the packed particles. Over the past decade, CE has become more the technique of choice in diastereomeric separations given its inherent advantages, including small sample volumes, high efficiency, and the variety of chiral selectors available. Given the immense volume of work on the use of CE in chiral separations, the use of chemometrics in methodological development of diasteromeric separations is appropriate. Perrin et al. [47] examined the separation of pronucleotide diastereomers of 3′-azido-2′,3′-dideoxythmidine (AZT) using the chiral additive carboxymethyl β-cyclodextrin. The aim of the work was to identify a set of conditions whereby a satisfactory separation would be achieved and within the shortest analysis time. Here, an experimental design strategy was used to examine the influence of cyclodextrin and phosphate buffer concentration, methanol content of the electrolyte, injected volume, capillary length, electric field, and separation temperature on the separation of AZT derivatives. A 12-experiment Plackett–Barman design with two levels was used to test seven potential critical factors against four dummy factors. Selectivity, resolution, and analysis time were obtained in the study and the effects of each of the seven factors on their effects were examined via Pareto plots. Satisfactory results were obtained in terms of linearity, accuracy, and repeatability.

In CE, hydrodynamic injection is generally used owing to negligible sample bias and ease of use. Small molecules and proteins, for example, are generally separated in aqueous media and, hence, in buffers of high viscosity. In the case of DNA, separation matrices of low viscosity have traditionally been used requiring electrokinetic injection. Catai and Carrilho [48] have studied electrokinetic injection of DNA fragments in order to maximize the signal-to-noise ratio and to improve the resolution of a pair of DNA fragments. Using a simplex optimization, they studied the influence of the sample composition and electrokinetic injection conditions on the reproducibility and the quality of the DNA optimization results. To quantitate the quality of the separation, the resolution and the signal-to-noise ratio of the 506- and 517-bp fragments from a 1-kbp DNA ladder were examined. Separation of the 1-kbp ladder intercalated with ethidium bromide using a poly(vinyl alcohol)-coated capillary filled with 0.5% hydroxyethyl cellulose in 100 mM tris(hydroxymethyl)aminomethane/*N*-

[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid/EDTA (TTE) buffer did not realize baseline resolution of either of these bp fragments. To optimize the separation while controlling the injection parameters, the Nelder and Mead simplex method was used. Optimal conditions for injection (buffer concentration, injection time, and electric field injection) of the 1-kbp DNA ladder was determined to be 1 mM, 20 s, and 55.00 V/cm, respectively. With use of these conditions, the resolution of the 506-and 517-bp pair was improved and signal strength was sufficient for detection of all smaller DNA fragments. Analysis by a univariate approach would have required at minimum 15 experiments. In the present work, nine experiments were sufficient to reach the optimized condition.

The separation and identification of biopolymers (DNA, proteins, and complex carbohydrate molecules) is of particular interest in analytical biochemistry. Consequently, separation of the smaller fragments which constitute these larger species is also critical given their importance in, for example, DNA sequencing and genotyping, protein identification, and carbohydrate analysis for the determination of posttranslational modifications. Gong et al. [49] described a simple method to determine six main nucleosides (adenine, uracil, adenosine, guanosine, uridine, and inosine) in *Cordyceps* by CE. To find the optimum resolution chemometric optimization based on central composite design was employed. Initial experiments were run in which the effects of five factors were examined. Three (buffer concentration, pH, and proportion of acetonitrile) factors were chosen which displayed the most pronounced effect on the separation expressed as resolution (voltage and temperature were determined to be insignificant within the range studied). AMP was used as an internal standard to examine the linearity, regression, reproducibility, and recovery of the six nucleosides. Of particular novelty in this study was the use of hierarchical cluster analysis to discriminate between natural and cultured *Cordyceps*. Here, analysis was based on 32 peak characteristics from electrophoretic profiles of both natural and cultured *Cordyceps*. It was deduced that characteristics of peaks, in particular, adenosine and inosine, from such profiles of nucleosides could be used as markers for discrimination and quality control of natural and cultured *Cordyceps*.

The safety of the world's food supply has been in the headlines given the potential for bioterrorism and the effects of environmental factors. Small quantities of a biological agent could cause havoc in the world's food supply and kill many people. Similarly, mass changes in weather patterns or mutations in the genetic make-up of crops could drastically reduce the availability of certain food products. Hence, there is a great need to develop analytical techniques with the ability to identify and quantify small quantities of material both accurately and expeditiously. Proteins, for example, are a major component of most food products, yet their variety in any food is quite large, making their analysis sometimes problematic. CE is a powerful separation technique owing to its small sample requirements, efficiency, selectivity, and ease of use. The low limits of detection and high-throughput capability make it a viable alternative to traditional techniques (for example, HPLC and gas chromatography) to analyze components of food. Rodriguez-Nogales [50] investigated the susceptibility of goat's milk proteins to cross-linking with the enzyme transglutaminase using CE. Transglutaminase improves the rheological properties of the milk proteins. In this work an optimization strategy based on desirability functions and experimental design was used to optimize the preheating conditions (temperature and time) of goat's milk that maximized the cross-linking reactions. A total of 12 experiments were performed and the center point of the design was repeated four times to estimate the experimental error and to provide additional information about the central composite response surface curvature. There was no clear indication of a screening design to justify their limited choice of only two variables (temperature and time). The analysis of additional variables could have revealed interactive effects even if they had no single effect on the response. Nevertheless, the results of their analysis showed that the optimum preheating treatment was obtained at 90 °C for 60 min.

Since the introduction of antimicrobial therapy in the 1940s, antibiotics have been extensively used to treat bacterial infections of either the Gram-positive or the Gram-negative type. Unfortunately, the growth of resistance to antibacterial agents is increasing worldwide and is threatening the chemical effectiveness of drugs used in the treatment of many infectious diseases. Hence, the development of novel analytical techniques to examine the physicochemical properties of antibiotics is warranted. Lara et al. [51] has developed a new method based on CE–tandem mass spectrometry (MS/MS) to identify and quantify eight quinolones (danofloxacin, sarafloxacin, ciprofloxacin, marbofloxacin, enrofloxacin, difloxacin, oxolinic acid, and fluequine) in bovine raw milk. Separation buffer composition and electrospray conditions were optimized to obtain an adequate CE separation and high sensitivity using experimental design methodology to determine the interactions among the variables. In this work it was critical to develop an analytical technique that would achieve unambiguous identification of the antibiotic residues but at low limits of detection. A half-fraction factorial screening design 2^{6-1} in two block plus three central points per block was carried out to optimize the CE–electrospray ionization–MS/MS. The selected response was the signal-to-noise ratio of danofloxacin. The different factors studied included nebulizer pressure, dry gas flow, dry gas temperature, sheath liquid flow rate, percentage of

2-propanol in the sheath liquid, and percentage of formic acid in the sheath liquid. This is the first study of these variables under CE separation conditions. A Pareto chart was obtained in which neither the percentage of formic acid nor the dry gas temperature was found to be significant. To optimize the significant factors a Doehlert design was employed. Here, the percentage of 2-propanol in the sheath liquid and the sheath liquid flow were studied at seven levels, the nebulizer pressure at five levels, and the dry gas flow at three levels. The MS/MS mode was then optimized by monitoring the intensities of the fragment ions in order to reach a maximum. Finally, the analytical method was validated in terms of linearity, limits of detection, limits of quantification, repeatability, intermediate precision, and trueness. Chemometric analysis allowed for the determination of the most efficient ionization below the established maximum residue limits set by the European Union.

There are several variables that play a role in the separation efficiency, migration time, and resolution of separations in CE, including voltage, buffer composition, capillary length, temperature, and injection time. Most research has focused on employing a univariate form of study whereby a single parameter is modified while the other parameters are kept constant. This approach, though, is both tedious and time-consuming and does not provide information on interactive effects between parameters. Gong et al. [52] examined the separation of ascorbic and isoascorbic acids in CE by comparing three RSMs, specifically the Box–Behnken design, central composite face-centered design, and full fractional design. For the optimization designs, pH, the concentration of the buffer, and the voltage were chosen as the variables to be examined. In this work the resolution response and migration time were simultaneously optimized. It was found that the central composite design and the full fractional design provided better agreement between the observed and predicted responses. We applaud this comparative design approach and feel it revealed valuable information for investigators struggling with similar separation issues.

Environmental

Analysis of potential pollutants and overall quality of the environment is largely based upon the quantitative analysis of abiotic matrices such as air, soil, sediment, and water. The heterogeneity of these differing and complex matrices raises difficulties in regards to the ability of investigators to detect potential pollutants of interest and extrapolate analytical results for management purposes. Proper experimental design and optimization techniques thus prove useful in maximizing the ability of CE to separate, characterize, and fully determine environmental analytes of interest.

Liu et al. [53] developed a novel, nonaqueous separation method for residue analysis of 2,4-D, dicamba, and 2,4,5-T in tobacco leaves, herbicides typically analyzed in water by more traditional chromatographic methods [54–56]. Plant species, such as tobacco examined in this study, provide a more complex matrix for herbicide determination and thus require properly designed and executed methods of analyses. Liu et al. first concentrated on the development of efficient tobacco pretreatment and extraction protocols involving ultrasonication with ethyl acetate followed by gel permeation chromatography.

Owing to the complex nature of separations in this study an orthogonal experimental design investigating four factors (electrolyte concentration, proportion of organic solvent, applied voltage, and buffer pH) at three levels (low, moderate, and high) was employed with separation resolution (R) and analysis time (T_m) evaluated as responses. Such designs have been previously successful in pesticide separation studies [57]. The interval range for each factor and responses achieved are reported in the nine-experiment design matrix (Table 2). The corresponding electropherograms for the nine experiments are shown in Fig. 4. As displayed, experiments 4 and 9 produced better resolutions, with experiment 4 featuring a longer analysis time. It appears that the investigators were successful in determining the factors (and levels) that influenced the response appropriately. Model conditions were validated appropri-

Table 2 Orthogonal design matrix with responses (modified from Liu et al. [49] with permission from Wiley-VCH)

Experiment	Electrolyte concentration (mmol L^{-1})	Proportion of acetronitrile (%)	Applied voltage (-kV)	Buffer pH	R_1	R_2	T_m (min)
1	20	70	20	9.2	–	–	–
2	20	80	25	9.7	11.41	0	20.68
3	20	90	30	10.2	11.47	1.5	11.01
4	40	70	25	10.2	14.37	3.43	29.35
5	40	80	30	9.2	11.78	0	12.50
6	40	90	20	9.7	7.34	1.11	10.33
7	60	70	30	9.7	9.82	1.46	8.62
8	60	80	20	10.2	7.06	0	12.41
9	60	90	25	9.2	10.45	1.9	16.85

R_1 resolution between peaks 1 and 2, R_2 resolution between peaks 2 and 3

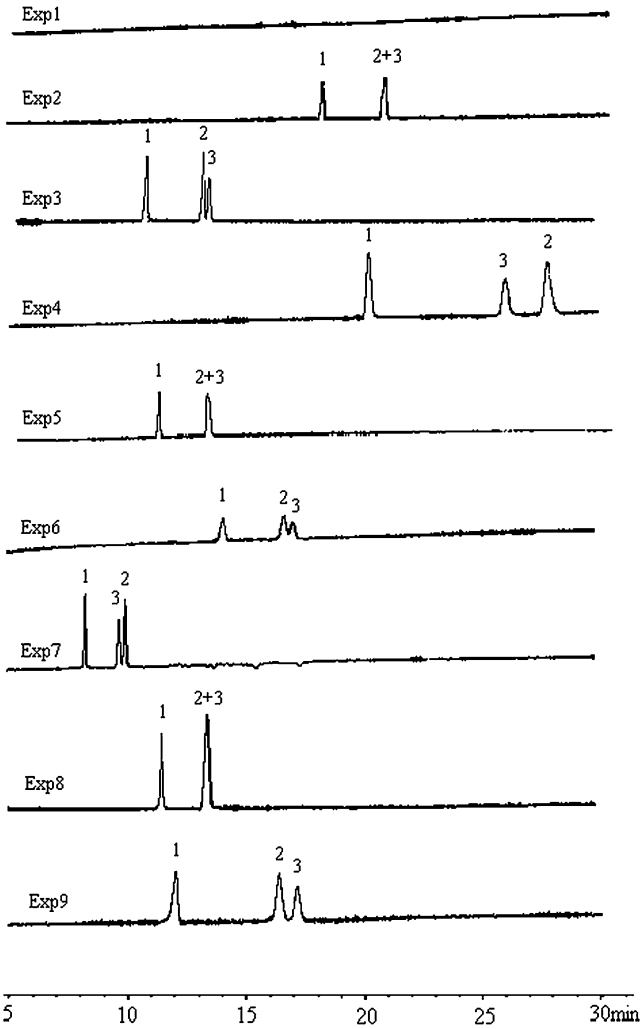

Fig. 4 Representative electropherogram for the nine-experiment separation of 2,4-D, dicamba, and 2,4,5-T in tobacco leaves (from [49] with permission from Wiley-VCH)

ately by experimental runs and limits of detection calculated at 0.40–0.60 μg mL^{-1} for the three herbicides with overall recoveries ranging from 80.8–84.1%.

Jurardo-Gonzáles et al. [58] studied the influence of both chemical and instrumental variables on the separation and analysis of Ca^{2+}, Mg^{2+}, Na^{+}, and Zn^{2+} in natural water samples by CE after complexation with 1,10-phenanthroline. Simultaneous determination of a variety of metals such as this is often problematic owing to matrix complexity and the concentration ratios between analytes. In an attempt to overcome these limitations, the authors employed the use of a 2^6 factorial (64 experiments) screening design assessing the following factors: 4-methylbenzylamine concentration, sample introduction time, run voltage, 1,10-phenanthroline concentration, pH, and capillary length. We question the sole use of a screening design in determining optimum conditions as a three-level surface design (e.g., central composite, Box–Behnken designs) would provide greater prediction capabilities. The authors, however, did report the establishment of a set of conditions suitable for simultaneous determination of all metals with good separation efficiency and reasonable analysis times. Such conditions were applied to real samples with satisfactory results.

Food

Food technology has grown tremendously in recent years owing to demands such as increased health and safety awareness, the desire for innovative products, and the development of modern and sustainable production practices. Advances in experimental design and optimization would thus prove beneficial in helping satisfy these demands. McCourt et al. [60] used a central composite design to screen, optimize, and validate a capillary zone electrophoresis (CZE) method for the determination of sucralose in still, carbonated, and alcoholic beverages, yoghurt, and hard-boiled sweets. A total of five experimental factors (electrolyte pH, electrolyte concentration, injection time, applied potential, and capillary temperature) were initially screened and their effect on resolution was examined. Screening studies revealed that the principal factors affecting sucralose resolution were capillary temperature and applied voltage. An additional 11 experiments were performed to further examine the ranges for each parameter investigated, with optimum values determined to be 10±1.0 kV and 22±1.0 °C for applied voltage and column temperature, respectively. The optimized method allowed for the detection of sucralose at more than 30 mg kg^{-1} with a linear range of 50–500 mg kg^{-1}.

Bianchi et al. [61] utilized both a 2^3 factorial and central composite design for the rapid determination of water-soluble organic acids in wine using CZE with UV detection and hexadimethrinebromide as a coating agent. We applaud the use of an additional star design in the 2^3 factorial to help confirm the presence/absence of relevant quadratic effects. Key to this study was the ability to separate citric and succinic acids by investigating the following experimental factors: temperature, applied voltage, and percentage of methanol. Stepwise regression analysis revealed optimum conditions at a voltage of −10 kV and 26% (v/v) methanol addition to the buffer solution. Separation of citric acid and succinic acid was achieved, but failure to separate acetic acid and lactic acid was observed.

Pharmaceutical

Experimental design and optimization techniques are becoming increasingly involved in the statistical planning of pharmaceutical experiments, including such processes as preformulation, formulation, scale-up, and robust testing. They also have the potential to help relieve economic

constraints for more efficient and productive formulation development. The enantioselective separation of chiral molecules has become an intensely studied area of pharmaceutical research owing to the fact that different stereoisomers of a compound may result in completely different pharmokinetic effects [63].

Elek et al. [64] studied the enantioselective CE separation of tryptophane and tyrosine methylesters in a dual selector system employing a cyclodextrin derivative and a novel tetraoxadiaza crown ether derivative. Earlier work by Iványi et al. [65] showed that newly synthesized diaza crown ethers individually did not have the ability to achieve chiral separation. The objective of the study of Elek et al. was therefore twofold: (1) to study the influence of experimental parameters on the separation of tryptophane- and tyrosine-methylesters and (2) to optimize and improve the separation conditions used by Iványi et al. [65], who utilized a three-factor central composite design. To help achieve this, a modified three-factor (five-level) (cyclodextrin concentration, *N*-[2(1,4,10,13-tetraoxa-7,16-diazacyclooctadeca-7-yl)propanoyl]glycine (*RS*-1) concentration and buffer concentration) central composite design based on the conditions utilized by Iványi et al. [65] was employed. They found that the dual system approach allowed better chiral separation of the amino acid derivatives. The modified central composite design allowed for improved separation conditions than the design used in the previous study [65]. It would have been interesting, however, to see what effects, if any, other experimental parameters (e.g., applied voltage, capillary length) would have had on separation conditions.

Lara et al. [66] developed a novel CZE method for the simultaneous analysis of thiazinamium methylsulfhate (TMS), promethazine (PMT), and promazine (PMH) in pharmaceutical preparations. Optimization was performed by the use of a face-centered Draper–Lin small composite design examining pH, buffer concentration, percentage of acetonitrile, voltage, and separation temperature and their potential effects on separation efficiency. Careful examination of this paper revealed limited discussion on how the range for each factor was chosen. We assume this was based on prior experience and chromatographic intuition. Greater discussion on separation efficiency (response) would also have been helpful along with the inclusion of this response for each experimental run in the design matrix table. Nevertheless, the method proved useful and allowed proper separation in 5.0 min at the following optimized conditions: 100 mM tris(hydroxymethyl)aminomethane buffer, pH 8.0, 15% acetonitrile, 25 °C, and a voltage of 30 kV. Method validation was performed with limits of detection of 2.8 μg mL^{-1} for TMS and 3.3 μg mL^{-1} for both PMH and PTH achieved. The method was successfully applied in the analysis of pharmaceutical preparations.

Full separation of high molecular mass oligosaccharide species by CE has traditionally been difficult to achieve owing to fragments migrating at similar retention times [67, 68]. However, through proper experimental design procedures it is likely that the parameters can be altered in such a way to achieve acceptable resolution. Prochazka et al. [69], for example, optimized the reverse-polarity CE separation of the oligosaccharide sodium pentosan polysulfate with the aid of a central composite design. The authors investigated injection pressure, injection time, and voltage and the effect of the conditions on retention time, peak areas, separation efficiency, and method sensitivity. The authors did a superlative job in justifying the levels chosen and presented the results of the response surface model in detailed fashion. The model produced optimum conditions as follows: injection pressure (35 mbar), injection time (44 mbar), and voltage (−20 kV). Increased resolution was achieved with decreased retention times and high reproducibility of repeated method runs. In addition, the robustness of the method was established with the use of a five-factor Plackett–Burman design.

Conclusions and future considerations

Advances in, for example, molecular biology, combinatorial chemistry techniques, and pharmaceutical preparations have hastened the need for high-throughput CE methods that can effectively screen and resolve numerous compounds in a short period of time. In addition, since many of these compounds are available in only limited quantities, lower limits of detection are a must in any new analytical technique. In the past 5 years, chemometric experimental design and optimization techniques have been instrumental in separating multicomponent environmental samples, DNA fragments, soluble organic acids and chiral molecules that otherwise proved troublesome. An exhaustive literature search on the use of chemometric experimental design and optimization techniques in CE has revealed 141 research papers and reviews for the period 2002–2007. These numbers will continue to increase as new developments in data processing based on genetic algorithms, ANNs and fuzzy logic emerge to meet the high analytical demands of modern research. The interface between effective method development, optimization, and real-world applications is currently a strong focus of researchers, including the authors, and will no doubt be a major area of investigation in CE for years to come.

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