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Mixture-process variable approach to optimize a microemulsion electrokinetic chromatography method for the quality control of a nutraceutical based on coenzyme Q10

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ABSTRACT

In recent years, multivariate optimization has played an increasing role in analytical method development. ICH guidelines recommend using statistical design of experiments to identify the *design space*, in which multivariate combinations of composition variables and process variables have been demonstrated to provide quality results. Considering a microemulsion electrokinetic chromatography method (MEEKC), the performance of the electrophoretic run depends on the proportions of mixture components (MCs) of the microemulsion and on the values of process variables (PVs). In the present work, for the first time in the literature, a mixture-process variable (MPV) approach was applied to optimize a MEEKC method for the analysis of coenzyme Q10 (Q10), ascorbic acid (AA), and folic acid (FA) contained in nutraceuticals. The MCs (buffer, surfactant–cosurfactant, oil) and the PVs (voltage, buffer concentration, buffer pH) were simultaneously changed according to a MPV experimental design. A 62-run MPV design was generated using the I-optimality criterion, assuming a 46-term MPV model allowing for special-cubic blending of the MCs, quadratic effects of the PVs, and some MC-PV interactions. The obtained data were used to develop MPV models that express the performance of an electrophoretic run (measured as peak efficiencies of Q10, AA, and FA) in terms of the MCs and PVs. Contour and perturbation plots were drawn for each of the responses. Finally, the MPV models and criteria for the peak efficiencies were used to develop the design space and an optimal subregion (i.e., the settings of the mixture MCs and PVs that satisfy the respective criteria), as well as a unique optimal combination of MCs and PVs.

1. Introduction

Multivariate optimization is playing an increasing role in analytical method development. In the context of "quality by design" (in which quality is designed into products and methods), ICH guidelines [1] recommend using statistical experimental design, modeling, and optimization methods to identify a *design space*. The design space is the region of the experimental space where multidimensional combinations of mixture components (MCs) and process variables (PVs) have been demonstrated to provide assurance of quality. MCs are the ingredients in a mixture, typically expressed as proportions that sum to 1. PVs are factors in an experiment that do not form any portion of the mixture, but whose settings (when changed) can affect the responses.

Using the data resulting from a mixture-process variable (MPV) experimental design, MPV models are developed to represent the relationship of system performance with MCs and PVs [2]. Such data-based models provide an in-depth understanding of the problem and the basis for developing the design space and optimizing the quality of the analytical method. In this article, for the first time in the literature, a MPV approach was used to develop a microemulsion electrokinetic chromatography method (MEEKC) for the quality control of a nutraceutical based on coenzyme Q10 (Q10, CAS 303-98-0) and containing ascorbic acid (AA, CAS 50-81-7) and folic acid (FA, CAS 59-30-3). Several methods are described for the analysis of Q10 in pharmaceuticals and/or biological fluids [3–10]. However, to the best of our knowledge, no capillary electrophoresis method has been reported for the simultaneous quantitation of Q10, AA and FA in nutraceuticals. The goals of the MPV study were to identify the design space, an optimal subregion of the design space, and a desirable combination of MC proportions and PV settings within the optimal subregion.

Sometimes a two-stage approach is used to address MPV optimization problems [11–14]. In the first stage, a mixture experiment is performed, mixture models for the responses are fit to the experimental data, and then the mixture models are used to develop an optimum mixture at fixed settings of the PVs (e.g., at standard or central values of PVs). In the second stage, the

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PVs are investigated using a response surface experiment, response surface models are fit to the resulting data, and the models are used to develop optimal settings of the PVs for the optimal mixture developed in the first stage. Unfortunately, this two-stage approach to MPV experiments does not provide for investigating interactions between MCs and PVs. For example, the linear and/or nonlinear blending properties of the MCs may depend on the values of PVs. When there are interactions between MCs and PVs, the optimal MCs settings depend on the PVs settings, and vice versa. By not studying and taking advantage of interactions among MCs and PVs, the two-stage approach may lead to misidentifying the design space, optimal subregion, and selecting a sub-optimal solution of MC and PV settings. This problem is avoided by (1) using a MPV approach that varies both MCs and PVs simultaneously in one experiment, (2) developing MPV models from the resulting data, and (3) using the MPV models to identify the design space, optimal subregion, and desirable MC proportions and PV settings within the optimal subregion [2].

1.1. Microemulsion electrokinetic chromatography

Due to the different lipophilicity characteristics of the target compounds, exploratory tests were performed to select the operative mode for capillary electrophoresis. Using a suitable pseudostationary phase made it possible to take advantage of a double-separation mechanism based on the different electrophoretic mobilities of the solutes and their different partitioning in the retentive phase [15]. Reverse polarity MEEKC (where the anode is positioned at the outlet of the capillary and the background electrolyte (BGE) is constituted by a microemulsion) was found to be the most suitable operative mode. The electrophoretic runs were performed in short injection mode, where the detector is located at the inlet side of the capillary. In these operative conditions, the analytes and the negatively charged drops of the microemulsion migrated towards the anode. EOF was partially suppressed by using a high ionic strength buffer at low pH, as it would draw the analytes towards the cathode. In the exploratory tests, problems were encountered with the peak efficiency of Q10. For some experimental conditions, the Q10 peak was so broad that it could not be detected, as previously noticed [10]. For AA and FA peaks, only certain asymmetry and limited peak broadening were noticed.

In capillary electrophoresis, the operating conditions both in terms of BGE (composition, pH, concentration, viscosity of the buffer) and of instrumental parameters (capillary length, temperature, voltage applied) can practically influence efficiency [15]. Consequently, MC proportions and the PV settings are fundamental to the efficiency of a MEEKC system [16].

For the MEEKC study discussed in this article, the microemulsion composition was expressed in terms of three MCs (phases): buffer (B, acetate buffer), surfactant–cosurfactant (S, sodium dodecyl sulphate/*n*-butanol in 1:4.5 ratio), and oil (O, *n*-octane). The PVs investigated were applied voltage (V), buffer concentration (BC), and buffer pH (pH). Ranges of these variables studied in the experimental design were chosen based on a series of preliminary experiments and are shown in Table 1. Because the PVs can affect the blending properties of the MCs, the MCs and PVs were varied simultaneously in the MPV experimental design.

The MPV study was run considering as responses the efficiency values of Q10 (Eff.Q10), AA (Eff.AA) and FA (Eff.FA) in order to highlight potential similarity or differences of behavior of the peak efficiencies of three analytes. Resolution among analytes and analysis time were not used to represent the performances of the MEEKC method because good selectivity was obtained and analysis time was lower than five minutes in all the electropherograms of the MPV study.

1.2. Mixture-process variable models and experimental designs

MPV experimental designs and models (that approximate the true, unknown relationships between response variables and the MCs and PVs) can be very large as the number of MCs and/or PVs increases. For example, MPV designs and models are often formed by "crossing" separate mixture designs and models with separate PV designs and models. In the MEEKC study discussed in this article, previous experience [11–14] suggested that the three MCs may have special-cubic blending and the three PVs may have quadratic effects. The *special-cubic mixture model* [2] is

$$y_{MC} = \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{123} x_1 x_2 x_3 + \varepsilon \quad (1)$$

where the β_i, β_{ij}, and β_{123} represent, respectively, the linear, quadratic, and special-cubic blending properties of the three MCs, and the x_i represent proportions of the MCs (or pseudocomponent transformations thereof, as discussed subsequently). The MC proportions x_i satisfy the constraint $x_1+x_2+x_3=1$, as well as the lower and upper bounds in Table 1.

The quadratic polynomial model in the PVs is

$$y_{PV} = \alpha_0 + \alpha_1 z_1 + \alpha_2 z_2 + \alpha_3 z_3 + \alpha_{12} z_1 z_2 + \alpha_{13} z_1 z_3 + \alpha_{23} z_2 z_3 + \alpha_{11} z_1^2 + \alpha_{22} z_2^2 + \alpha_{22} z_3^2 + \varepsilon. \quad (2)$$

Crossing the models in (1) and (2) involves multiplying all terms in one model by all terms in the other model, yielding a (special-cubic mixture) x (quadratic PV) MPV model of the form

$$\begin{aligned} y_{SC-Q} = {} & g_1^0 x_1 + g_2^0 x_2 + g_3^0 x_3 + g_{12}^0 x_1 x_2 + g_{13}^0 x_1 x_3 + g_{23}^0 x_2 x_3 + g_{123}^0 x_1 x_2 x_3 \\ & + (g_1^1 x_1 + g_2^1 x_2 + g_3^1 x_3 + g_{12}^1 x_1 x_2 + g_{13}^1 x_1 x_3 + g_{23}^1 x_2 x_3 + g_{123}^1 x_1 x_2 x_3) z_1 \\ & + (g_1^2 x_1 + g_2^2 x_2 + g_3^2 x_3 + g_{12}^2 x_1 x_2 + g_{13}^2 x_1 x_3 + g_{23}^2 x_2 x_3 + g_{123}^2 x_1 x_2 x_3) z_2 \\ & + (g_1^3 x_1 + g_2^3 x_2 + g_3^3 x_3 + g_{12}^3 x_1 x_2 + g_{13}^3 x_1 x_3 + g_{23}^3 x_2 x_3 + g_{123}^3 x_1 x_2 x_3) z_3 \\ & + (g_1^{12} x_1 + g_2^{12} x_2 + g_3^{12} x_3 + g_{12}^{12} x_1 x_2 + g_{13}^{12} x_1 x_3 + g_{23}^{12} x_2 x_3 \\ & + g_{123}^{12} x_1 x_2 x_3) z_1 z_2 + (g_1^{13} x_1 + g_2^{13} x_2 + g_3^{13} x_3 + g_{12}^{13} x_1 x_2 + g_{13}^{13} x_1 x_3 \end{aligned}$$

Table 1
Ranges of original and coded microemulsion mixture components and process variables.

Mixture component	Original variables				Coded variables		
	Units	Notation	Lower bound	Upper bound	Notation	Lower bound	Upper bound
Buffer	Proportion	B	0.900	0.948	x_1	0	1
Surfactant:CoSurfactant	Proportion	S	0.050	0.098	x_2	0	1
Oil	Proportion	O	0.002	0.020	x_3	0	0.375
Process variable							
Applied voltage	kV	V	22	27	z_1	−1	1
Buffer concentration	mM	BC	95	105	z_2	−1	1
Buffer pH	pH	pH	4.5	5.5	z_3	−1	1

$$+g_{23}^{13}x_2x_3+g_{123}^{13}x_1x_2x_3)z_1z_3+(g_1^{23}x_1+g_2^{23}x_2+g_3^{23}x_3+g_{12}^{23}x_1x_2$$
$$+g_{13}^{23}x_1x_3+g_{23}^{23}x_2x_3+g_{123}^{23}x_1x_2x_3)z_2z_3+(g_1^{11}x_1+g_2^{11}x_2+g_3^{11}x_3$$
$$+g_{12}^{11}x_1x_2+g_{13}^{11}x_1x_3+g_{23}^{11}x_2x_3+g_{123}^{11}x_1x_2x_3)z_1^2$$
$$+(g_1^{22}x_1+g_2^{22}x_2+g_3^{22}x_3+g_{12}^{22}x_1x_2+g_{13}^{22}x_1x_3+g_{23}^{22}x_2x_3$$
$$+g_{123}^{22}x_1x_2x_3)z_2^2+(g_1^{33}x_1+g_2^{33}x_2+g_3^{33}x_3+g_{12}^{33}x_1x_2+g_{13}^{33}x_1x_3$$
$$+g_{23}^{33}x_2x_3+g_{123}^{33}x_1x_2x_3)z_3^2+\varepsilon \quad (3)$$

In models (1), (2) and (3), the ε term at the end of the model represents experimental and measurement error (uncertainty) in the response variable. When models are fit to data using ordinary least squares (OLS) regression, the ε errors are typically assumed to be statistically independent and identically distributed (i.e., with the same mean and variance). When the errors have a normal (Gaussian) distribution, many standard statistical data analysis methods can be applied.

The MPV model (3) contains $7\times10=70$ terms, a large number. An experimental design to support fitting this model would require at least 70 data points to fit the model, plus extra data points to quantify "pure error" (experimental and measurement uncertainty) and to assess model lack-of-fit (LOF). A separate mixture design that supports fitting (1) consists of the four vertices, four edge centroids, and overall centroid of the irregular polyhedral region defined by the lower and upper bounds on the MCs (in Table 1). A separate central composite design [17] in the three PVs that supports fitting (2) contains 8 vertices, 6 axial points, and the overall center point of the cube defined by the PV lower and upper bounds. Crossing these designs would yield a MPV design with $9\times15=135$ points. Crossing MC and PV models and designs yielded models and designs that were too large for the MEEKC problem discussed in this article.

An alternative for generating MPV designs containing fewer points is to use an *optimal experimental design* approach [18]. With this approach, the researcher specifies a MPV model form that he/she believes will adequately approximate the relationships between the response variables and the MCs and PVs. Then, software (e.g., Design-Expert [19], JMP [20], Minitab [21], or SAS [22]) is used to generate a MPV design (containing a specified number of points) that optimizes a mathematical design criterion. Atkinson et al. [18] discuss several optimal design criteria, including D-optimality, I-optimality, and others. D-optimality focuses on minimizing the uncertainties of the model coefficients, while I-optimality focuses on minimizing the average uncertainty of model predictions over the experimental region. The I-optimal design approach was used for the MEEKC study discussed in this article.

The number of points in an optimal MPV design must be at least as large as the number of terms in the MPV model selected for use with the optimality criterion chosen. Further, the design should have at least 5 replicates to estimate experimental and measurement uncertainty for each response and 10 additional (non-replicate) points to assess the adequacy of the models fitted to the response data.

In summary, care must be taken in (1) selecting a MPV model that will adequately approximate the relationships between responses and the MCs and PVs and (2) generating a MPV experimental design that will support fitting the selected MPV model to experimental data and assessing model adequacy. After MPV models for the response variables have been developed and assessed using the experimental data, the fitted models can then be used to develop equations that specify the design space and determine the settings of the MCs and PVs to optimize the responses [23].

2. Experimental

2.1. Chemicals

All chemicals and reagents used were of analytical-reagent grade with no further purification. Methanol (HPLC grade), ethanol (HPLC grade), acetic acid, sodium dodecyl sulphate (SDS), sodium hydrogen carbonate, *n*-butanol, *n*-octane, coenzyme Q10 (Q10), L-ascorbic acid (AA), folic acid (FA), fumaric acid (FUM), and naproxen (NAP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Proxeed NF® sachets (labeled to contain 20 mg Q10, 90 mg AA and 200 μg FA) (Sigma Tau, Roma) were purchased locally in pharmacies and the excipient acesulfame K (ACE) was obtained from LabService Analytica (Bologna, Italy).

Ultrapure water used for the preparation of solutions was provided by a Simplicity 185 system (Millipore, Billerica, MA, USA) after an electrodeionisation treatment using an Elix system (Millipore).

2.2. Solutions, Microemulsions, and sample preparation

Standard stock solutions of Q10 (0.5 mg mL^{-1}) and NAP (1 mg mL^{-1}) were prepared in ethanol and a standard stock solution of FA (1 mg mL^{-1}) was prepared in 0.1 M $NaHCO_3$. Standard stock solutions of AA (10 mg mL^{-1}), ACE (0.1 mg mL^{-1}), and FUM (1 mg mL^{-1}) were prepared in water. All solutions were stored at 4 °C and used within one week and the Q10 and FA solutions were protected from light. Standard working solutions were obtained daily adding the appropriate volume of each of the stock solutions directly in a vial and filling up to 500 μL with water. The test concentration values were: Q10 (0.04 mg mL^{-1}), AA (0.2 mg mL^{-1}), FA (0.02 mg mL^{-1}), ACE (0.015 mg mL^{-1}), FUM (0.1 mg mL^{-1}), and internal standard NAP (0.02 mg mL^{-1}).

Microemulsions were prepared on a w/w basis by sequentially mixing in a beaker proper amounts of buffer phase, cosurfactant (*n*-butanol), surfactant (SDS) and finally oil (*n*-octane). Each component was added only after reaching a complete dissolution of the previously mixed compounds. The buffer phase of the microemulsion system was constituted by pH 4.5–5.5 acetate buffer in the concentration range 95–105 mM and was prepared by mixing an adequate volume of 0.1 M acetic acid, adjusting pH with 1 M sodium hydroxide and then filling up to volume with water. The proportions of the microemulsion components considered during the optimization phase were 0.900 – 0.948 for buffer, 0.002 – 0.020 for oil, and 0.050 – 0.098 for the surfactant/cosurfactant mixture in 1:4.5 ratio.

With regard to sample preparation, a sachet containing 5 g of powder was opened and 125 mg of the powder was dissolved in 1 mL ethanol to obtain a solution of 0.5 mg mL^{-1} for Q10, 2.25 mg mL^{-1} for AA, and 0.005 mg mL^{-1} for FA. The sample was shaken vigorously, sonicated for 10 min, shaken again, centrifuged and the supernatant was analyzed.

2.3. Equipment and capillary electrophoretic conditions

An ultrasonic bath (300 UltraSonik, Ney Company, Bloomfield, USA) and a centrifuge (5415D, Eppendorf, Hamburg, Germany) were used to sonicate and centrifuge solutions, respectively. A Metrohm 691 pH Meter (Metrohm, Herisau, Switzerland) was used to measure pH values.

An Agilent Technologies ^{3D}CE system (Agilent Technologies, Waldbronn, Germany), equipped with an on-column UV–visible diode-array detector and an air thermostating system, was used for all separations. The system was controlled by ^{3D}CE ChemStation software (Rev.A.09.01, Agilent Technologies). The fused-silica capillaries (inner diameter 50 μm, outer diameter 375 μm) were

purchased from Composite Metal Services (Ilkley, UK) and had a total and effective length of 48.5 and 40.0 cm, respectively. The detection window was built-in by burning off the polyimide coating on the capillary using The WindowmakerTM (MicroSolv, postnova Analytica, Landsberg/Lech, Germany).

The detection wavelength was 215 nm and temperature was set at 20 °C. The hydrodynamic injection of the sample was performed from the detector side of the capillary (short injection) and from the cathode to the anode, which was positioned at the outlet side of the capillary (reverse polarity) at 50 mbar for 20 s.

Table 2
62-point I-optimal MPV design and peak efficiency data for Q10, ascorbic acid, and folic acid.

Design point #	Replicate pairs	Mixture components			Process variables			Response variables		
		Buffer (B)	Surfactant: cosurfactant (S:CoS)	Oil (O)	Applied voltage (V)	Buffer conc. (BC)	Buffer pH (pH)	Eff.Q10[a]	Eff.AA	Eff.FA
1		0.9350	0.0630	0.0020	27.00	100.80	5.50	10	3705	7092
2		0.9024	0.0776	0.0200	22.00	105.00	4.50	1000	9768	5254
3		0.9000	0.0889	0.0111	24.46	95.00	5.46	11187	3471	4364
4		0.9000	0.0898	0.0102	22.89	99.35	4.50	9899	1459	2200
5		0.9180	0.0620	0.0200	26.50	100.15	4.98	2930	3472	4106
6		0.9206	0.0670	0.0124	22.00	105.00	4.89	1000	4653	10595
7	1a	0.9480	0.0500	0.0020	22.00	95.00	4.50	10	6914	8371
8		0.9186	0.0792	0.0022	22.00	95.00	4.90	10	5474	12871
9		0.9000	0.0848	0.0152	24.90	105.00	4.95	21444	9097	3778
10	1b	0.9480	0.0500	0.0020	22.00	95.00	4.50	10	5822	3868
11		0.9480	0.0500	0.0020	27.00	95.00	5.50	10	3796	6265
12		0.9141	0.0672	0.0187	23.58	97.50	4.50	10	6894	10011
13	2a	0.9189	0.0693	0.0118	22.00	95.00	5.43	10	3122	5282
14		0.9143	0.0657	0.0200	25.63	95.00	5.50	4722	3428	3411
15		0.9090	0.0890	0.0020	24.40	105.00	4.50	10	1706	3795
16		0.9372	0.0500	0.0128	25.04	95.70	5.42	10	3277	6320
17	2b	0.9189	0.0693	0.0118	22.00	95.00	5.43	10	3552	3244
18		0.9323	0.0657	0.0020	22.00	99.95	4.93	10	5210	11562
19		0.9377	0.0500	0.0123	23.23	100.00	4.50	10	6375	2751
20		0.9102	0.0878	0.0020	23.20	105.00	5.18	20538	4677	14453
21		0.9336	0.0500	0.0164	22.00	102.15	5.50	10	3266	3199
22		0.9115	0.0685	0.0200	23.40	102.15	5.39	2190	4706	9466
23		0.9000	0.0980	0.0020	25.15	100.50	5.40	21771	5977	6808
24		0.9000	0.0968	0.0032	27.00	103.05	4.50	1000	5990	5466
25		0.9225	0.0755	0.0020	22.00	104.15	5.50	10	3938	6022
26		0.9000	0.0980	0.0020	26.63	105.00	5.50	23832	6414	5137
27		0.9377	0.0500	0.0123	27.00	101.74	5.01	1000	3748	8605
28		0.9258	0.0722	0.0020	23.68	100.70	4.50	9497	7739	11935
29	3a	0.9000	0.0800	0.0200	27.00	96.55	4.50	1000	8977	13127
30		0.9069	0.0731	0.0200	24.78	97.65	5.12	5187	4828	11108
31		0.9173	0.0705	0.0122	27.00	95.00	4.67	10	4810	8234
32		0.9313	0.0667	0.0020	27.00	95.00	4.55	3156	6250	8359
33		0.9000	0.0980	0.0020	22.00	95.00	5.50	18239	5629	12335
34		0.9480	0.0500	0.0020	25.15	98.50	4.90	10	4849	11894
35		0.9456	0.0500	0.0044	24.73	105.00	5.46	1000	5836	1489
36		0.9480	0.0500	0.0020	27.00	105.00	4.50	10	6720	9577
37		0.9000	0.0980	0.0020	25.35	95.00	4.50	19336	1252	2352
38	4a	0.9300	0.0500	0.0200	27.00	95.00	4.50	1000	6753	6040
39		0.9196	0.0661	0.0143	27.00	105.00	4.50	10	6717	8752
40	5a	0.9000	0.0800	0.0200	27.00	101.25	5.50	1000	4842	2484
41		0.9000	0.0800	0.0200	22.00	95.00	5.05	3990	5519	10397
42		0.9319	0.0500	0.0181	22.00	95.00	4.92	10	3670	4616
43	5b	0.9000	0.0800	0.0200	27.00	101.25	5.50	7531	4319	8889
44		0.9480	0.0500	0.0020	22.00	104.85	4.80	1000	3967	8121
45	6a	0.9159	0.0727	0.0114	27.00	103.50	5.50	10	4122	8962
46		0.9480	0.0500	0.0020	24.55	100.15	5.50	4385	3013	4482
47		0.9254	0.0726	0.0020	24.60	95.00	5.50	10	3161	7042
48		0.9013	0.0967	0.0020	22.00	105.00	4.50	12273	1023	1890
49	6b	0.9159	0.0727	0.0114	27.00	103.50	5.50	10	4198	7829
50		0.9480	0.0500	0.0020	22.00	98.10	5.42	8185	2996	8555
51		0.9000	0.0980	0.0020	24.10	95.05	4.89	18990	6582	10060
52		0.9000	0.0968	0.0032	27.00	97.40	5.22	16829	5650	3135
53	3b	0.9000	0.0800	0.0200	27.00	96.55	4.50	1000	9026	6709
54		0.9000	0.0892	0.0108	22.00	103.70	5.50	36057	7743	3529
55		0.9113	0.0867	0.0020	23.25	98.50	5.50	4337	3677	8299
56		0.9300	0.0500	0.0200	24.20	105.00	4.87	1000	4751	9983
57		0.9000	0.0980	0.0020	23.80	100.90	5.04	24261	6600	18379
58		0.9266	0.0534	0.0200	27.00	105.00	5.50	1000	4109	5808
59	4b	0.9300	0.0500	0.0200	27.00	95.00	4.50	1000	6854	4674
60		0.9480	0.0500	0.0020	27.00	99.30	4.62	2244	5823	7018
61		0.9150	0.0650	0.0200	25.69	101.00	4.50	1000	8638	11098
62		0.9202	0.0778	0.0020	25.88	105.00	4.92	4583	5583	12551

[a] For not detectable peaks efficiency values were set to 10, and for measured values lower than 2000 efficiency values were all set to 1000.

During the MPV study, the values of voltage were set according to the experimental design (discussed subsequently).

The capillary was treated prior to its first use by flushing with 1 M NaOH for 5 min, 0.1 M NaOH for 5 min and water for 5 min. Between runs, a rinse-cycle was applied, consisting of 1 M NaOH, 0.1 M NaOH, and water for 1 min each, and run buffer for 4 min.

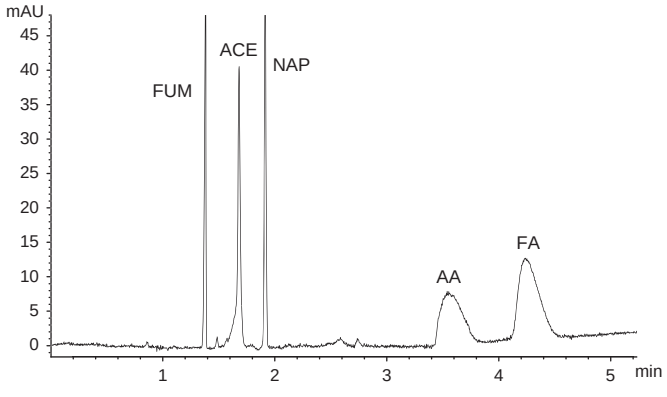

Fig. 1. Electropherogram for design point #15 (in Table 2). Symbols as in Sec. 2.1. Q10 peak is not detectable.

2.4. Calculations and software

The efficiency of the peak for each of Q10, AA, and FA was calculated according to the formula

$$N = 5.54(t_m/w_{1/2})^2 \quad (4)$$

where N is the number of theoretical plates, t_m is the migration time and $w_{1/2}$ is the peak width at half height [24].

Design-Expert Version 8 (DX8) [19] was used to generate the experimental design, fit two response models, and perform other data analyses and graphics. Matlab [25] was used to fit one response model.

3. Results and discussion

The following subsections present and discuss (1) the experimental design, (2) the MPV models for Eff.Q10, Eff.AA. and Eff.FA, (3) interpretation of MC and PV effects on the three responses by contour plots and perturbation plots and (4) developing the design space and an optimal subregion, and selecting desirable settings of the MCs (buffer, surfactant–cosurfactant, and oil) and the PVs (applied voltage, buffer concentration, and buffer pH) within the optimal subregion.

Table 3
Reduced MPV models obtained using backward elimination, starting with the 46-term MPV model (5) used as the basis for the experimental design. Presenting MPV model coefficients in this two-way layout provides for assessing both how the MC blending properties depend on the PVs and how the PV effects depend on the MCs.

Process-variable terms		Mixture terms						
		Buffer (B) x_1	S:CoS (S) x_2	Oil (O) x_3	B∗S x_1x_2	B∗O x_1x_3	S∗O x_2x_3	B∗S∗O $x_1x_2x_3$
ln(Eff.Q10)								
1	1	9.278***	10.023***	10.057***	0.037 ns	−7.234***	−4.391***	−19.537***
V[b]	z_1	−2.362***	−0.580***	21.124***	10.698***	−7.787***	−30.402***	−46.761***
BC[b]	z_2	1.797***	0.047***	−0.110***	–	–	–	–
pH	z_3	−1.007***	0.203***	6.344***	–	–	−6.740***	–
V∗BC	z_1z_2	−1.412***	–[c]	–				
V∗pH	z_1z_3	−2.820***	–	–		# Terms	30	
BC∗pH	z_2z_3	–	0.766***	–		R^2	0.911	
V^2	z_1^2	−2.497***	0.642***	−5.921***		R^2_{Adj}	0.830	
BC^2	z_2^2	−4.598***	–	3.937***		LOF p	< 0.01	
pH^2	z_3^2	–	−0.776***	–				
Eff.AA								
1	1	4530.895[a]	6772.840[a]	17396.809(a)	−912.128 ns	−26430.624***	−17091.561*	–
V[b]	z_1	92.249 ns	683.481**	–	–	–	–	–
BC[b]	z_2	928.153**	898.219**	−898.728 ns	−5205.808***	–	8247.271**	–
pH	z_3	−1577.229***	1762.482***	−23896.288***	−5588.994***	34298.718***	25472.302**	–
V∗BC	z_1z_2	–	–	–				
V∗pH	z_1z_3	939.128**	−1686.862***	–		# Terms	26	
BC∗pH	z_2z_3	–	–	–		R^2	0.879	
V^2	z_1^2	–	–	–		R^2_{Adj}	0.795	
BC^2	z_2^2	−1131.011*	–	2768.183*		LOF p	0.013	
pH^2	z_3^2	1559.750**	−2988.315***	3672.356**				
$(Eff.FA)^{0.5}$								
1	1	98.539[a]	118.174[a]	323.101[a]	54.235**	−433.213***	−443.817***	178.107 ns
V[b]	z_1	–	−28.359***	−554.544***	71.623**	847.693***	937.476***	−767.868***
BC[b]	z_2	−8.668 ns	8.872*	37.743 *	–	–	−140.074***	425.292**
pH	z_3	−9.131**	5.982 ns	−12.346 ns	–	–	–	–
V∗BC	z_1z_2	–	14.236**	–				
V∗pH	z_1z_3	–	−15.894***	–		# Terms	29	
BC∗pH	z_2z_3	−14.873***	14.167**	–		R^2	0.804	
V^2	z_1^2	–	–	–		R^2_{Adj}	0.638	
BC^2	z_2^2	–	−17.091**	–		LOF p	0.971	
pH^2	z_3^2	−24.427**	−38.029***	60.689***				

[a] Asterisks *, **, and *** denote coefficients that are statistically significant with 90%, 95%, and 99% confidence, respectively. An "ns" denotes coefficients that are not statistically significant with at least 90% confidence. It is inappropriate to consider the statistical significance of the three linear mixture terms [Cornell 2002].

[b] V=applied voltage, BC=buffer concentration.

[c] A dash (–) denotes that the term is not included in the model.

3.1. MPV experimental design and associated model

Based on the experimental region specified by the MC and PV ranges (in Table 1) and preliminary testing, a MPV model was postulated for use to generate an I-optimal MPV experimental design. Previous experience indicated that the MCs may have up to special-cubic blending, and that the PVs may have up to quadratic (interaction and curvature) effects [11–14]. Crossing these two models results in the 70-term MPV model (3), which would have required too large of a MPV experiment for this study. Also, the 70-term MPV model (3) includes higher-order interactions between MCs and PVs that were not anticipated to be significant. Hence, a smaller 46-term MPV model

$$\begin{aligned} y = f(x,z) &= g_1^0x_1 + g_2^0x_2 + g_3^0x_3 + g_{12}^0x_1x_2 + g_{13}^0x_1x_3 + g_{23}^0x_2x_3 + g_{123}^0x_1x_2x_3 \\ &+ (g_1^1x_1 + g_2^1x_2 + g_3^1x_3 + g_{12}^1x_1x_2 + g_{13}^1x_1x_3 + g_{23}^1x_2x_3 + g_{123}^1x_1x_2x_3)z_1 \\ &+ (g_1^2x_1 + g_2^2x_2 + g_3^2x_3 + g_{12}^2x_1x_2 + g_{13}^2x_1x_3 + g_{23}^2x_2x_3 + g_{123}^2x_1x_2x_3)z_2 \\ &+ (g_1^3x_1 + g_2^3x_2 + g_3^3x_3 + g_{12}^3x_1x_2 + g_{13}^3x_1x_3 + g_{23}^3x_2x_3 + g_{123}^3x_1x_2x_3)z_3 \\ &+ (g_1^{12}x_1 + g_2^{12}x_2 + g_3^{12}x_3)z_1z_2 + (g_1^{13}x_1 + g_2^{13}x_2 + g_3^{13}x_3)z_1z_3 \\ &+ (g_1^{23}x_1 + g_2^{23}x_2 + g_3^{23}x_3)z_2z_3 + (g_1^{11}x_1 + g_2^{11}x_2 + g_3^{11}x_3)z_1^2 \\ &+ (g_1^{22}x_1 + g_2^{22}x_2 + g_3^{22}x_3)z_2^2 + (g_1^{33}x_1 + g_2^{33}x_2 + g_3^{33}x_3)z_3^2 + \varepsilon \end{aligned} \quad (5)$$

was selected as the basis for generating the experimental design, where y denotes the peak efficiency of Q10, AA, or FA; x_i denotes the L-pseudocomponent transformation of the ith MC; and z_j denotes the $[-1, 1]$ coding of the jth PV. The codings of the MCs and PVs are given in Section 3.2. The various "g" model coefficients in (5) are to be fitted using experimental data. A subscript on a "g" coefficient denotes the MCs involved in that model term, while the superscript denotes the PVs involved in that model term. The model terms in the first row of (5), with zero superscripts, represent the linear, quadratic, and special-cubic blending effects of the MCs when all PVs are at their zero coded values (i.e., their middle values). The terms in the remaining lines of (5) represent the effects of PVs on MC blending properties. Hence, the 46-term model in (5) allows for (1) linear effects of all three PVs on the linear, quadratic and special-cubic blending properties of the MCs, and (2) linear blending properties of the MCs to be affected by the two-variable interactions and/or quadratic curvature effects of the three PVs. The 46-term MPV model (5) was selected for generating the MPV experimental design with the belief that it would adequately approximate the true, unknown relationships between the response variables (Eff.Q10, Eff.AA, and Eff.FA), the MCs, and the PVs.

To support estimating the coefficients of the MPV model (5), to quantify the experimental and measurement uncertainty, and to assess model LOF for each response, it was decided that a MPV experimental design containing 62 runs would be generated. The basis for 62 runs was the 46 terms in the MPV model, 10 extra points to assess model LOF, and 6 replicated points. The 62-run MPV design was generated by DX8 [19] using the "best optimal design" algorithm with the I-optimality criterion. The "best optimal design" algorithm generates designs using both (1) a point-exchange algorithm to select a design from specified candidate points, and (2) a coordinate-exchange algorithm that does not require specifying candidate points [18]. The "best" I-optimal design ended up being one that was generated by the coordinate-exchange algorithm. The settings of the MCs and PVs for the 62-point MPV design are shown in Table 2.

3.2. Response values and MPV models

The responses selected to measure the method performance were peak efficiencies of Q10 (Eff.Q10), ascorbic acid (Eff.AA), and folic acid (Eff.FA). However, significant problems in measuring peak efficiency were observed for Q10, and to lesser degrees for AA and FA. In particular, there was a high variability of Eff.Q10 depending on different experimental conditions, leading sometimes to such a peak broadening that Q10 could not be detected. For such cases, Eff.Q10 was assigned a value of 10. In addition, it was troublesome to measure Eff.Q10 when lower than 2000, so for such cases it was assigned a value of 1000. Fig. 1 shows one electropherogram that illustrates for one of the design points (#15) the low efficiency of the method, which otherwise had good selectivity and low analysis time.

The values of the Eff.Q10, Eff.AA, and Eff.FA responses for the 62-run experimental design, including six replicate pairs of points, are shown in Table 2. Based on the replicates, estimates of the experimental and measurement standard deviations in the Eff.Q10, Eff.AA, and Eff.FA responses are 1885, 373, and 3025, respectively. Note that all but one of the replicate pairs for Eff.Q10 had identical assigned values of 10 or 1000. Hence, the 1885 estimate of experimental and measurement uncertainty may not be representative of what the uncertainty is in higher values of Eff.Q10.

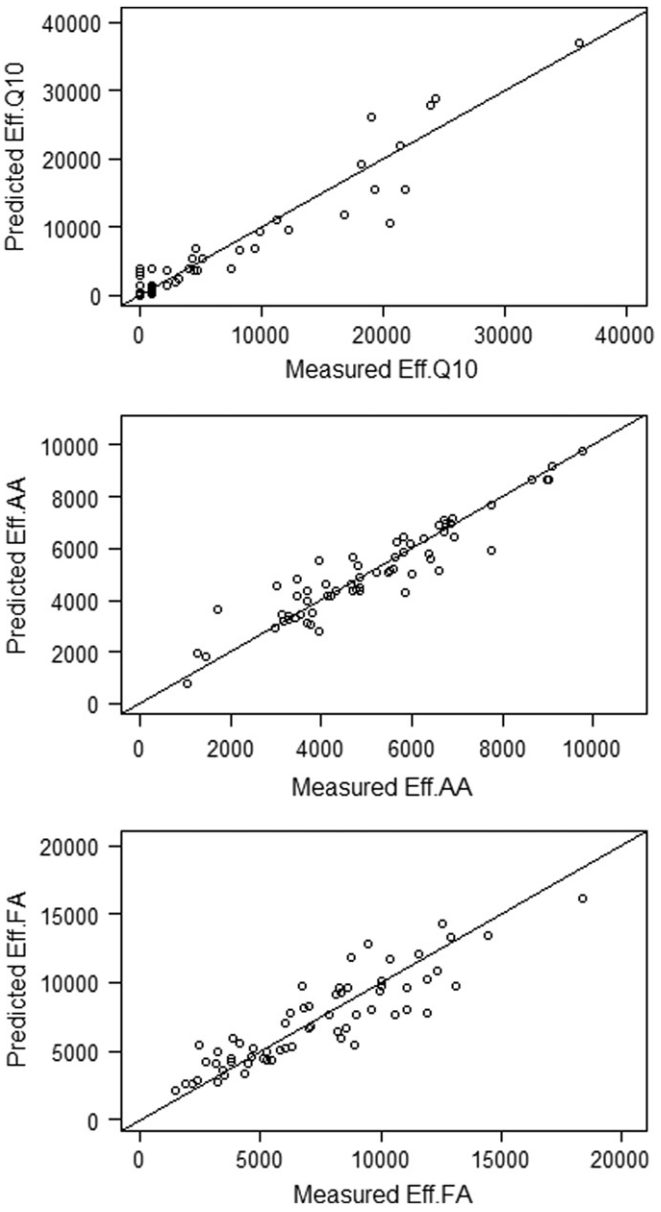

Fig. 2. Predicted versus measured plots for the Eff.Q10, Eff.AA, and Eff.FA models in Table 3.

To develop MPV models for Eff.Q10, Eff.AA, and Eff.FA, the MCs were coded using L-pseudocomponent transformations [2]:

$$x_1 = (B-0.9)/(1-0.9-0.05-0.002) = (B-0.9)/(0.048)$$
$$x_2 = (S-0.05)/0.048$$
$$x_3 = (O-0.002)/0.048 \quad (6)$$

and the PVs were coded to have ranges $-1 \le z_i \le 1$, using the transformations

$$z_1 = (V-24.5)/2.5$$
$$z_2 = (BC-100)/5$$
$$z_3 = (\mathrm{pH}-5)/0.5 \quad (7)$$

The L-pseudocomponent coding of the MCs reduces collinearity that results from the small ranges of the components [2]. The $-1 \le z_i \le 1$ coding of the PVs provides for easier interpretation of the MPV model in (5). The portion of a MPV model containing only MC terms represents the blending behavior of the mixture components when the coded PVs=0 (i.e., middle values of the PVs). The terms with both MCs and PVs represent the effects of changing the PVs (from their middle values) on the blending properties of the MCs. Table 1 shows the lower and upper bounds of the coded MCs and PVs.

For each response, the full 46-term MPV model in coded variables (as shown in (5)) was fit to the data from the 62-point experimental design using ordinary least squares (OLS) regression implemented in DX8 [19]. However, many model terms were statistically nonsignificant, and so a statistical variable selection method (backward elimination) was used to develop reduced

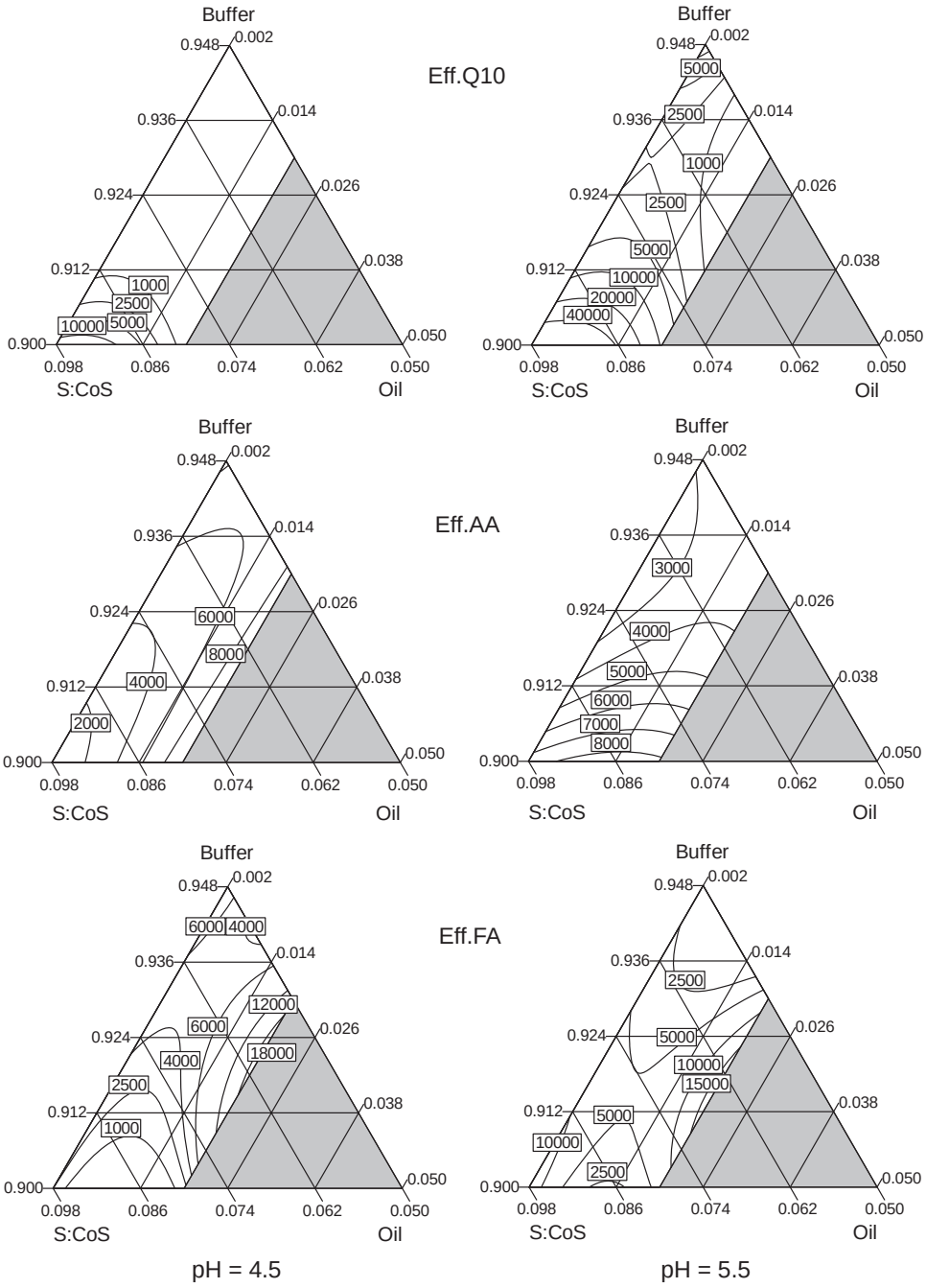

Fig. 3. Contour plots using the Eff.Q10, Eff.AA, and Eff.FA MPV models over the constrained mixture region for two combinations of the process variables (pH=4.5 and 5.5, with BC=105 mM and V=22 mV).

forms of the 46-term MPV model. Acceptable models were obtained for Eff.AA and sqrt(Eff.FA) using this approach. The square root transformation of Eff.FA was selected based on a Box-Cox transformation analysis of the data [17], as implemented in DX8. The results of the Eff.AA and Eff.FA models are shown in Table 3.

The preceding approach to MPV modeling did not yield an acceptable model for Eff.Q10. The most likely reason for this is the difficulty in determining Q10 peaks and because unmeasurable and smaller Eff.Q10 values were set to either 10 or 1000 (for 36 of the 62 design points). Such grouping and assignment of values for two subsets of the Eff.Q10 data can be expected to cause problems for OLS approaches to regression modeling. Hence, a generalized linear model (GLIM) approach was used to model Eff.Q10 [17]. A variety of link and error distributions were investigated as part of the model selection process. A logarithm link with Poisson error distribution were found to produce the best model results, which are shown in Table 3.

Plots showing predicted values (using the models in Table 2) versus measured values of the design points are shown in Fig. 2. The R^2 values of 0.913, 0.879, and 0.804 for the ln(Eff.Q10), Eff.AA, and $(\text{Eff.FA})^{0.5}$ models, respectively, are not as high as is desirable (e.g., >0.95). However, this was expected given the mentioned difficulties in determining peak efficiencies. The R^2 values are high enough that a substantial fraction of the variability in the peak efficiencies are accounted for by the MPV models in Table 3. Hence, the models can be used (remembering their uncertainties) to interpret effects of the MCs and PVs on the responses, based on the statistically significant terms included in the models. A term is considered statistically significant if the confidence that its coefficient is different from zero is at least 90%. It is worthwhile to point out that there is some danger in interpreting MCs and PVs as having effects (e.g., MC and PV interactions, PV interactions, linear vs. curvature effects of PVs) corresponding to terms remaining in (or missing from) a MPV model. This is because high correlations among model terms could lead to terms remaining in a model in a certain combination when in fact it is some other combination of variable effects that are the true explanations of response dependence on the MCs and PVs. However, when terms involving special-cubic blending of MCs appear or do not appear in models, it is safe to conclude the presence/absence of special-cubic blending. Also, terms of the form $\text{MC}*(\text{PV})^2$ being present or absent support safe conclusions about whether the PV has or does not have a curvature effect on the linear blending property of MC. Further, graphical assessments of the models considered in the following subsection can be used as another basis for assessing the effects of MCs and PVs on the response variables. The models were subsequently employed to identify the design space and the optimal subregion of settings for the MCs and PVs.

3.3. Contour plots and perturbation plots

Contour plots of Eff.Q10, Eff.AA, and Eff.FA over the MC constrained experimental region for each of eight lower-and-upper-bound combinations of the PVs were generated, but for

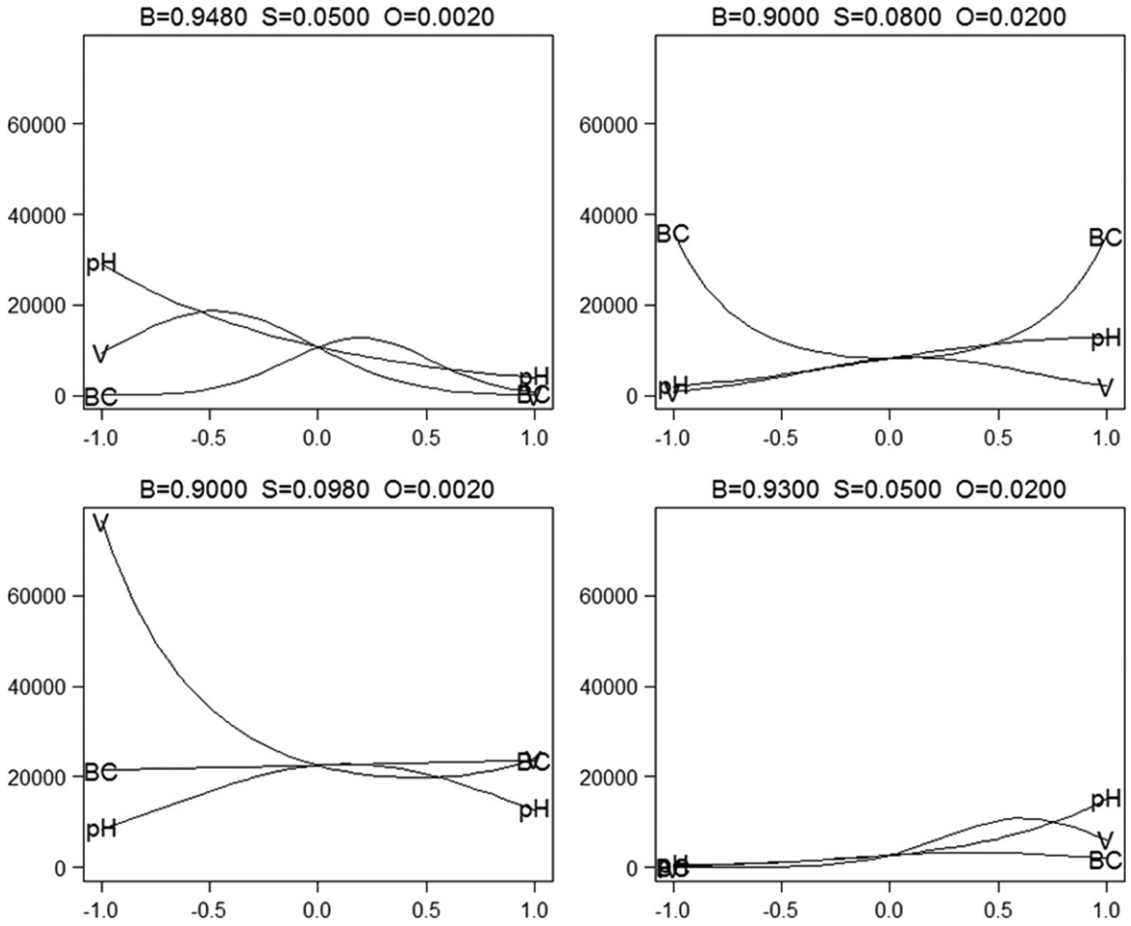

Fig. 4. Perturbation plots using the MPV model for Eff.Q10 to predict the effect of varying each process variable from its lower bound (coded −1.0) to its upper bound (coded 1.0), with the other process variables at their middle values (coded 0.0), for the four vertices of the constrained mixture experiment region. The x-axis represents the differences in PVs from their middle values.

space reasons they are provided as Supplementary Figures S1, S2 and S3, respectively. These contour plots show how changing the MC proportions (microemulsion composition) affects each response for the eight combinations of the PVs. For each of Eff.Q10, Eff.AA, and Eff.FA, the contour plots differ significantly over the eight combinations of the PVs. This indicates that the PVs have significant effects on the blending properties of the MCs (i.e., the MCs and PVs have interactive effects). Fig. 3 shows two of the eight contour plots (pH=4.5 and 5.5 with BC=105 mM and V=22 kV) for each of Eff.Q10, Eff.AA, and Eff.FA.

Perturbation plots of how variations in the PVs affect Eff.Q10 for each of the four vertex combinations of the MC constrained experimental region are displayed in Fig. 4. Similar plots for Eff.AA and Eff.FA are available as Supplementary Figures S4 and S5, respectively. A given perturbation plot shows three curves that display the effects of varying each of the three PVs from its lower bound to its upper bound, with the other PVs at their middle values. Fig. 4 shows that some PVs have relatively small effects on Eff.Q10, with some exceptions now discussed. Eff.Q10 increases as (1) pH decreases when B (=0.948) is at its highest level, and S (=0.050) and O (=0.002) are at their lowest levels, (2) BC moves from its middle value to either its lowest or highest values when B (=0.900) is at its lowest level, S (=0.08) is at a relatively high value, and O(=0.02) is at its highest value, (3) V moves to its lowest value, when B (=0.900) and O (=0.002) are at their lowest values and S (=0.098) is at its highest value. At the ME formulation B=0.93, S=0.05, and O=0.02, none of the PVs have much effect on Eff.Q10. The different effects of the PVs for different ME formulations is because of the interactive effects of MCs and PVs on Eff.Q10.

3.4. Design space, optimal subregion, and desirable settings

The objectives of this study were to (1) identify the design space and optimal subregion (see Section 1), and (2) select a desirable microemulsion formulation and PV settings that yield optimum values of Eff.Q10, Eff.AA, and Eff.FA. Based on the visual inspection of the electropherograms obtained in the MPV design, an acceptable lower limit and optimum lower limit for each response were specified:

Eff.Q10 : Acceptable lower limit = 11000,
Optimum lower limit = 20000
Eff.AA : Acceptable lower limit = 3500,
Optimum lower limit = 6000
Eff.FA : Acceptable lower limit = 5000,
Optimum lower limit = 8500 (8)

The design space is specified using the acceptable lower limits in the equations

$$M(\text{Eff.Q10}) \geq 11000,\ M(\text{Eff.AA}) \geq 3500,\ \text{and}\ M(\text{Eff.FA}) \geq 5000, \quad (9)$$

while the optimal subregion within the design space is given by the equations

$$M(\text{Eff.Q10}) \geq 20000,\ M(\text{Eff.AA}) \geq 6000,\ \text{and}\ M(\text{Eff.FA}) \geq 8500, \quad (10)$$

where in (9) and (10) the notations M(Eff.Q10), M(Eff.AA), and M(Eff.FA) represent the MPV models in Table 3. The design space and optimal subregion are five-dimensional regions specified by coded values of MCs and PVs ((x_1, x_2, x_3, z_1, z_2, z_3), where $x_1+x_2+x_3=1$) used in the models. Because these regions are five dimensional, they cannot be illustrated in their entirety. Instead, Fig. 5 illustrates the formulation portion (in terms of x_1, x_2, and x_3) of the design space and optimal subregion for two selected combinations of PV values. Fig. 5(a) is for the combination of PV values (V, BC, pH)=(23.8, 100.9, 5.04) that corresponds to design point #57, the only one that satisfied all the optimal subregion constraints. Fig. 5(b) is for the combination of PV values (23, 101, 5.3) discussed in the following paragraph.

Specific desirable settings of the MCs and PVs within the optimal subregion (shown in terms of the coded and uncoded variables)

x_1=0 B=0.900
x_2=1.000 S=0.098
x_3=0 O=0.002
z_1=-0.6 V=23
z_2=0.2 BC=101
z_3=0.6 pH=5.3

were chosen inside the optimal subregion, based on practical considerations. In particular, the low level of O made it possible to

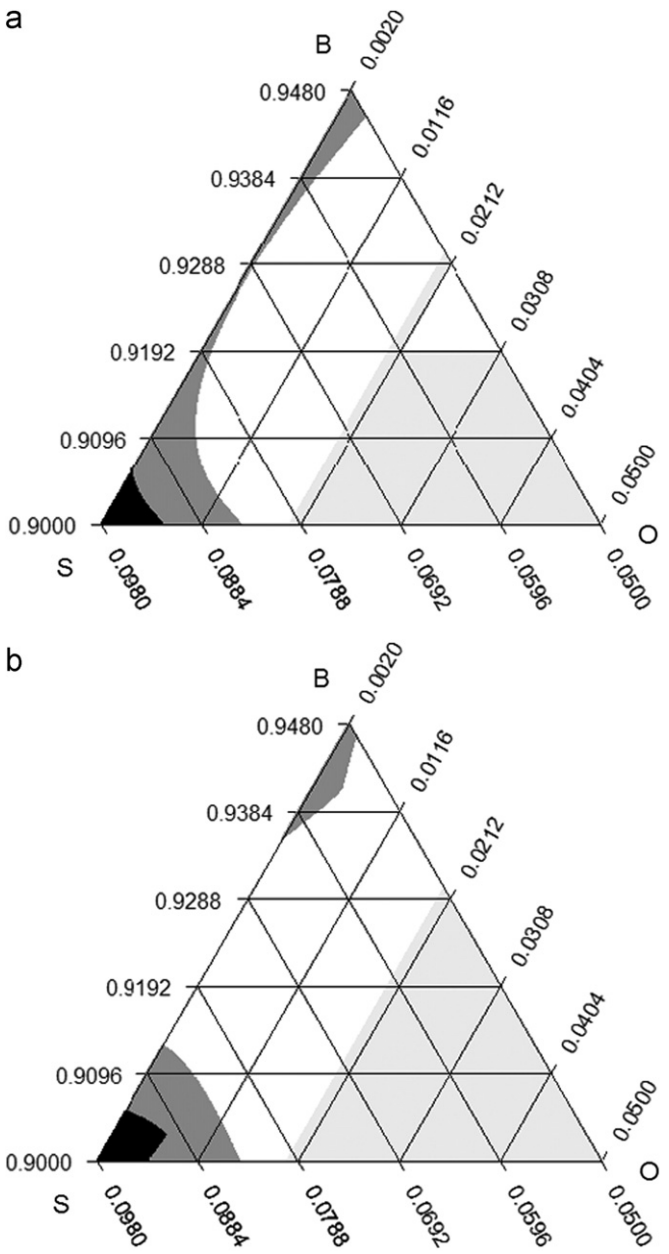

Fig. 5. Formulation portions of the design space (shaded medium gray) and optimal subregion (shaded black) for two selected combinations of PV conditions (V, BC, pH): (a) Combination (23.8, 100.9, 5.04) corresponding to design point #57, (b) combination (23, 101, 5.3) selected as desirable within the optimal subregion. The portion of the ternary shaded light gray is outside the experimental region.

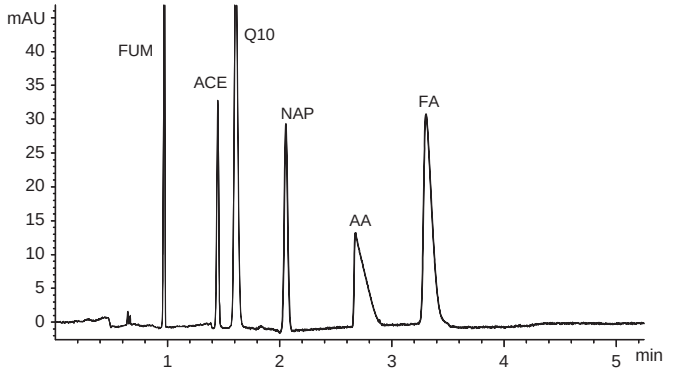

Fig. 6. Electropherogram for the optimal MEEKC conditions given by proportions of the microemulsion components (buffer=0.9000, surfactant/cosurfactant=0.098, oil=0.002) and settings of the process variables (voltage=23 kV, buffer concentration=101 mM, buffer pH=5.3). Symbols as in Sec. 2.1.

reduce the cleaning procedure of the capillary and to prepare the microemulsion in less time. A high value of S and a low level of B were preferred in order to increase the draining power of the negative microemulsion droplets towards the outlet of the capillary. A low value of voltage allowed the generated current to be kept lower, and finally a high value of pH was preferred to increase the electrophoretic mobility of Q10, directed towards the anode.

The electropherogram for the selected optimal conditions is shown in Fig. 6, evidencing good efficiencies for all the analyte peaks (Eff.Q10=21018, Eff.AA=6443, Eff.FA=10166), which were baseline separated in about 3 min.

4. Conclusions

For the first time in the literature, a MPV approach was used to simultaneously optimize formulation (mixture) and process factors during the development of a MEEKC method, where the peak efficiencies of three analytes (Q10, FA, AA) were used as responses. The MPV approach made it possible to identify the design space, optimal subregion, and desirable MC proportions and PV settings within the optimal subregion.

For all the considered responses, contour plots showed that PVs have significant effects on the blending properties of the MCs, and perturbation plots showed the different effects of the PVs for different microemulsion formulations. Finding interactive effects of MCs and PVs demonstrated that the MPV approach is a very powerful tool for method optimization, and that in general the classical two-step optimization of mixture and process variables should be avoided. In fact, the latter approach does not provide for investigating interactions between MCs and PVs, thus possibly leading to misidentifying the design space, optimal subregion, and selecting a sub-optimal solution of MC and PV settings. On the other side, the main drawback of the MPV approach is the high number of experiments required. However, in this case the experimental runs were fast and economical, thus not representing a practical problem. Both these aspects should be carefully considered by the researcher when planning a multivariate optimization strategy, making it necessary to take into account the goal of the optimization and the easiness or difficulty in finding acceptable solutions from preliminary experiments.

Appendix A. Supporting material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.03.064.

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