Application of ASCA as a Multivariate Statistical Tool for Identification of Critical Parameters for Spectroscopic Determination of Dexamethasone

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ABSTRACT: *The main goal of this study was to apply chemometrics techniques such as (ANOVA)-Simultaneous Component Analysis (ASCA), Response Surface Methodology (RSM), and Central Composite Design (CCD) to identify important factors in Dexamethasone Sodium Phosphate (DSP) microextraction from plasma samples. This work proposes the pre-concentration and determination of DSP using a Dispersive Liquid-Liquid Microextraction (DLLME) and spectrophotometry in combination with chemometrics approaches. ASCA as a multivariate statistical tool was used to more thoroughly analyze the influencing factors on DLLME and their interactions. By ASCA the diversity of the data matrix was divided into five levels for four variables: the major impact of each experimental component (dispersive and extraction solvent volume, amount of salt, and incubation time), followed by the impact of each second-order interaction. The significance of each factor or interaction effect was determined by a permutation test. The outcomes were compared with the results of the ANOVA approach to determine the ideal circumstances for measuring the trace amount of DSP. Under optimal conditions, a linear calibration curve with a detection limit of 0.071 µg/mL in the 0.1-5 µg/mL range was obtained.*

KEYWORDS: *Dexamethasone sodium phosphate; Dispersive liquid-liquid microextraction; Response surface methodology; ANOVA; ANOVA simultaneous component analysis.*

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INTRODUCTION

Dexamethasone sodium phosphate (DSP) (Fig. 1) is a potent anti-inflammatory and immunosuppressant drug that is used to treat allergic, endocrine, rheumatic, dermatologic, and other inflammation-related diseases [1]. Dexamethasone mimics the action of natural compounds produced by the body to reduce inflammation. It is approximately 25 times more effective than other corticosteroid compounds [2]. Most chemotherapy patients utilize it as well [3] and it was the first drug to show life-saving efficacy in COVID-19-infected patients [4, 5]. DSP is rapidly absorbed after oral administration, and up to 65% of a dose is excreted in urine in 24 h. The plasma concentration of it was found to be the highest (3 mg/L) at 4 h, declining rapidly to about 0.5 mg/L at 24 h. Dexamethasone treatment can decrease deaths by 35% in ventilated patients and 20% in oxygen therapy patients [6].

DSP has been determined in plasma and other biological samples using a variety of methods, including high-performance liquid chromatography [7-9], capillary electrophoresis [10-12], and electrochemical approaches [13-15]. Most of these methods take a long time or require expensive equipment. As a result of their simplicity, sensitivity, accuracy, and low cost, spectrophotometric approaches have become increasingly appealing for DSP studies [16-18]. A pre-concentration step is typically required prior to analysis by each reported method due to the complexity of the biological sample medium which is incompatible with the method instrumental or lacks enough sensitivity to identify trace amounts of DSP in the biological samples.

Although there have been reported a lot of extraction methods for sample preparation, a number of microextraction methods have found a place in modern analytical labs including Solid-Phase MicroExtraction (SPME) and the other most common method is liquid-liquid microextraction [19, 20]. Dispersive Liquid-Liquid MicroExtraction (DLLME) is a modified form of liquid-liquid microextraction that allows the simultaneous extraction and preconcentration of analytes into a micro-volume of extracting solvent based on a ternary solvent system involving an aqueous phase, a nonpolar water immiscible high-density solvent that acts as extraction phase, and a disperser solvent, which is often polar and water miscible. DLLME can overcome the limitations of simple liquid-liquid extraction due to its advantages, such as

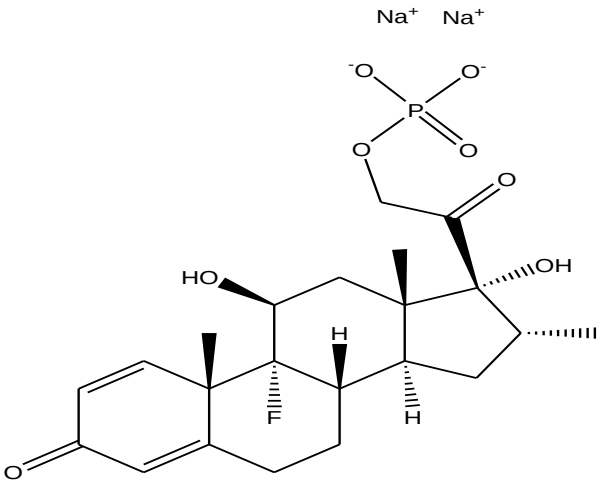


Fig. 1: Structure of DSP

ease of use, high recoverability, low cost, quickness, and high enrichment factor [21].

Herein, a DLLME technique coupled with a spectrophotometry method was developed for the determination of DSP. For this purpose, the impacts of various experimental parameters were investigated, and the improved approach was used to DLLME with success. Optimization strategies aim to improve a system's, process's, or product's performance and maximize its benefits. Chemometric tools have frequently been used to solve problems with the optimization of analytical methods. One of their benefits is that they reduce the number of required tests, resulting in decreased reagent usage and significantly less laboratory work. As a result, they are more cost-effective and faster to implement than standard univariate techniques [22]. The Response Surface Methodology (RSM) is a set of statistically valid methods for organizing experiments, simulating outcomes, and assessing the impact of independent variables on response. It can be used to evaluate the relative value of a variety of important factors. RSM's main purpose is to choose the best operational conditions for desired results [23]. The Central Composite Design (CCD) is a type of RSM that is useful for improving a well-known operation, such as DLLME, in which just a few of the participating factors are crucial to the outcome [24, 25]. The analysis of data from an experimental design using ANOVA is a prominent method. Because it is a univariate method, it cannot adjust for covariance between variables [26, 27]. To understand more about the behavior of the parameters that affect a procedure, one can use the (ANOVA)-simultaneous

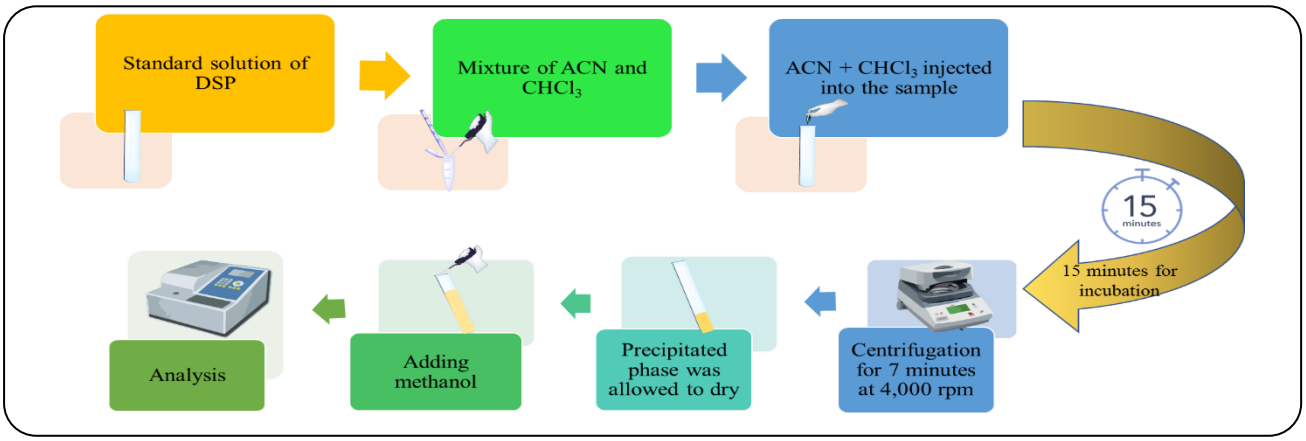


Fig. 2: Procedure for DLLME of DSP

component analysis (ASCA) chemometric technique. ASCA as a new experimental method for multivariate data analysis with an underlying experimental design was recently introduced [28-32]. In comparison to prior methods, ASCA is a user-friendly way of extracting more information from a multivariate dataset in a single experiment [33]. ASCA is a hybrid of analysis of variance (ANOVA) and Principal Component Analysis (PCA) that solves the shortcomings of both methods separately. When there are more variables than samples or the dependent variables are correlated, ANOVA fails. PCA, on the other hand, ignores the experimental design, resulting in a combination of the experimental design's multiple contributors to variation [32-34]. The inference of factor values of evaluated variables using loadings analysis is one of ASCA's major advantages [35]. Herein, with an unbalanced set of varieties obtained through CCD, we utilized ASCA to investigate the effects of dispersive solvent volume, extraction solvent volume, amount of salt, and incubation period on the DLLME of DSP. Briefly, the main aim of this research was to use chemometrics approaches such as ASCA, RSM, and CCD to find essential parameters in DSP analysis using the microextraction-spectrophotometry method and give details about each method.

EXPERIMENTAL SECTION

Materials and reagents

All of the chemicals and reagents utilized in this study were analytical reagent grade. The DSP injection sample was provided by Darupakhsh, Iran. Chloroform, tetrachloroethylene, carbon tetrachloride, and dichloromethane which were utilized as investigated extraction solvents were provided by Merck (Germany). Acetone, methanol, and acetonitrile as investigated disperser solvents were also provided by Merck. All aqueous solutions were prepared in double-distilled water.

Preparation of plasma samples

Drug-free plasma samples (donated by the blood transfusion organization of Eastern Azerbaijan, Tabriz, Iran) were stored in polypropylene microtubes at -20 ºC. 100 µL concentrated HCL was added to 500 µL plasma spiked with the proper amount of DSP in the range of 0.1-5 µg/mL DSP. Then the mixtures were centrifuged at 10,000 rpm for ten min and the supernatant liquid was used for analysis.

Experimental procedure

The samples obtained from the previous section were taken, and their pHs were adjusted to 3.0. Using a 5 mL syringe, a dispersive and extraction solvent mixture of acetonitrile (2 mL) and chloroform (150 mL) was rapidly injected into the sample. DSP was extracted into the droplets of a cloudy solution containing small distributed chloroform drops. Allow 15 minutes for incubation and after centrifugation for 7 minutes at 4,000 rpm, the tiny chloroform drops precipitated at the bottom of the conical tube. The upper aqueous solution was withdrawn, and the precipitated phase was allowed to dry before being mixed with 500 µL of methanol and transported to a microcell for spectrophotometric analysis (Fig. 2).

Instrumentation and software

The spectra were obtained using the Windows 7 operating system and a UV-1800 spectrophotometer (Shimadzu, Japan) with a 1 mL quartz microcell connected to a PC. A centrifuge was used to accelerate the phase

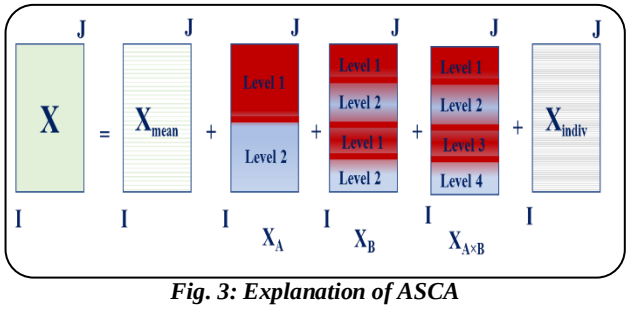


Fig. 3: Explanation of ASCA

separation process. A 780 Metrohm digital pH meter with a mixed glass calomel electrode was used to determine the pH. DesignExpert version 12 was used to run the CCD and RSM. The ASCA calculation programs were built in MATLAB 2013 with PLS-toolbox 7.8 and run on a PC with the Windows 7 operating system.

ASCA

In an ANOVA-like method, ASCA decomposes the centered data matrix (X) into the sum of several arrays, containing the variance attributable to each factor's main effects and interactions, as well as a residuals matrix. The partition of X is carried out in Fig. 3.

Where X_A, X_B, and X_{AB} account for the variability induced by the effect of factor *A*, factor *B*, and factor A/factor B interaction, respectively, while E carries the residuals not explained by the ANOVA model. As a result, the effect of each design element is calculated as the sum of squares of the matrix components.

$$Effect_k = SSQ_k = \| X_k \|^2 \quad (1)$$

Where the individual matrices X_k represent the main effects of the factors. Effect matrices are created using the centered mean spectra corresponding to the different values of the variable or interaction involved.

The related sum of squares, as determined by Eq. (1) to establish the statistical significance of each design factor is compared to the null distribution, which is commonly approximated non-parametrically using permutation tests. Simultaneous Component Analysis (SCA) can be used to interpret the corresponding matrix X_k, which is divided into scores and loadings if the effect of a factor (or interaction) is significant which under the ANOVA restrictions is equivalent to PCA. Additionally, projecting the residual matrix Xresiduals onto the SCA model allows for the construction of the modified SCA score plots, which provide a graphical depiction of the variability between and within levels. Bootstrapping, on the other hand, can be used to discover the spectral regions most influenced by the researched factor's effect by obtaining confidence intervals for loadings (or interaction) [29, 36]. According to *Vis et al*. [37] work, bootstrapping is not the most reliable method for estimating the standard deviation of the difference between level means without making any additional assumptions. On the other hand, permutation tests randomly permute the factor levels, typically by rearranging the rows of D and recalculating the level-mean differences each time. As a result, this approach produces null distributions of a specific metric for each factor or interaction, which can be compared to the actual values [38]. The statistical significance of controlled factors and interactions in this study was determined using permutation tests with 10,000 randomizations.

RESULTS AND DISCUSSIONS

Some parameters that influenced the DPS enrichment factor in the DLLME process include extraction and the dispersive solvent type and volume, salt amount, and extraction time. At first, one variable at a time optimization method was utilized to analyze influencing parameters such as extraction types and dispersive solvents on extraction efficiency while constantly keeping the rest of the analytical parameters. CCD was then used to optimize the appropriate levels of variables in the DSP extraction, and RSM was utilized to evaluate the relative relevance of numerous influencing factors by analyzing the effect of the independent factors on the response.

One-at-a-time optimization for the types of extraction solvent and dispersive solvent

The type of extraction solvent and dispersive solvent were optimized using the One-Variable-at-a-Time (OVAT) method. The OVAT design is a standard univariate strategy that explores each factor's response while controlling for all other variables.

Dichloromethane (DCM), tetrachloroethylene (CCl_4), chloroform ($CHCl_3$), and carbon tetrachloride (TCE) were investigated as an extraction solvent for DSP and according to the results (Fig. 4), DSP was more extracted into chloroform than the other four studied extraction solvents.

In the following 1 mL of methanol, acetonitrile, and acetone mixed with 500 mL of chloroform were rapidly injected into aqueous samples to find the optimum disperser,

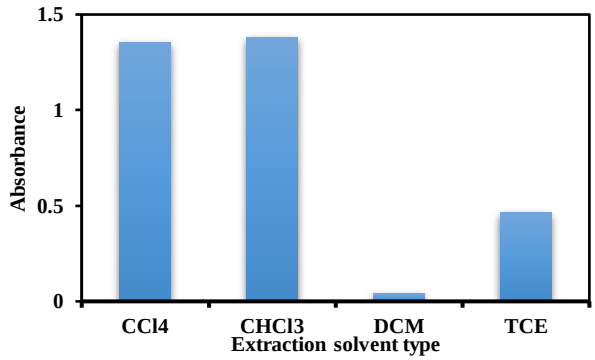


Fig. 4: Effect of extraction solvent type on the extraction efficiency. Extraction conditions: extraction solvent (500 µL) chloroform ($CHCl_3$), tetrachloroethylene (TCE), carbon tetrachloride (CCl_4), dichloromethane (DCM); disperser solvent: methanol (1 mL); rate and time of centrifugation 3500 rpm for 5 min; DSP concentration, 2.0 µg/mL

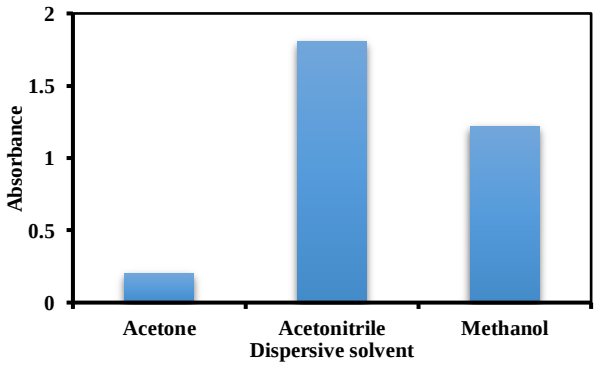


Fig. 5: Effect of disperser type on the extraction efficiency of DLLME. Extraction conditions: extraction solvent, chloroform (500 µL); rate and time of centrifugation 3500 rpm for 5 min; DSP concentration, 2.0 µg/mL

and as shown in Fig. 5 the maximum response was seen when acetonitrile was used as a dispersant.

Multivariate optimization for other variables

Experimental design by CCD

The fundamental drawback of the one-variable-at-a-time strategy is that it overlooks the interacting effects of the model's variables. As a result, the effect of all variables on the response is not obtained by this method. Another downside of one-variable optimization is the increased number of experiments needed to complete the work, which increases time and costs as well as reagent and material usage.

The CCD approach was used to design the tests in this work for the extraction of DSP. The main parameters for these experiments were dispersive solvent volume (X_1), extraction solvent volume (X_2), salt amount (X_3), and incubation duration (X_4). The independent components are coded (-1, +1), and the low and high levels are coded -2 and +2. The experiments were designed and analyzed by the Design-Expert12. The ranges and levels of the variables are presented in Table 1.

RSM studies

In RSM section, a model was constructed according to data obtained in the experimental design procedure, describing the link between factors (independent parameters) and response (dependent parameters) [39]. RSM as a strategy for designing experiments aids in the development of models, the evaluation of the effects of various variables, and the achievement of optimal conditions for intended responses, and reduces the number of tests [40]. RSM is used to optimize for biodiesel production and environmental assessment of produced biodiesel [41], extraction conditions for the extraction of phenolic compounds from Moringa oleifera leaves [42], leaching parameters for ash reduction from low-grade coal [43], for separation and preconcentration of quercetin in wine and food samples [44] and so on.

The ideal DLLME conditions for DSP are determined using RSM and based on the performed experimental design by CCD. 3D response surfaces are shown in Fig.6 and schematically show the determined optimum condition for experimental. The optimum values for each parameter were, 2 mL for dispersive solvent (acetonitrile), 150 µL chloroform, 0.03 g NaCl, and 15 min incubation time. Also, in Table 2, the optimum value for each parameter has been shown.

ANOVA studies

The acquired results were examined using ANOVA to choose a suitable response surface model and model terms. A *P*-value of less than 0.05 for a variable indicates that it has a significant influence. As can be seen in Table 3, the leading terms of X_1, X_2, X_3, X_{13}, and X_{23} are significant according to the ANOVA (2FI model). The model's aptitude was evaluated using the determination coefficient (R^2), which was 80 percent. Fig. 7 shows that the model indicated a good correlation between the experimental and predicted responses. The trained equation as stated by

Table 1: Coded and actual levels of the variables

Original variable	levels				
	-2	-1	0	1	2
Dispersive solvent volume (mL): X_1	0.5	1	1.5	2	2.5
Extraction solvent volume (µL): X_2	50	150	250	350	450
Amount of salt (mg): X_3	0.01	0.03	0.05	0.07	0.09
Incubation time (min): X_4	5	15	25	35	45

Table 2: Optimized parameters for extraction of DSP

Original variable	Coded optimized values	Actual optimized values
Dispersive solvent volume (ml): X_1	1	2
Extraction solvent volume (µl): X_2	-1	150
Amount of salt (mg): X_3	-1	0.03
Incubation time (min): X_4	1	15

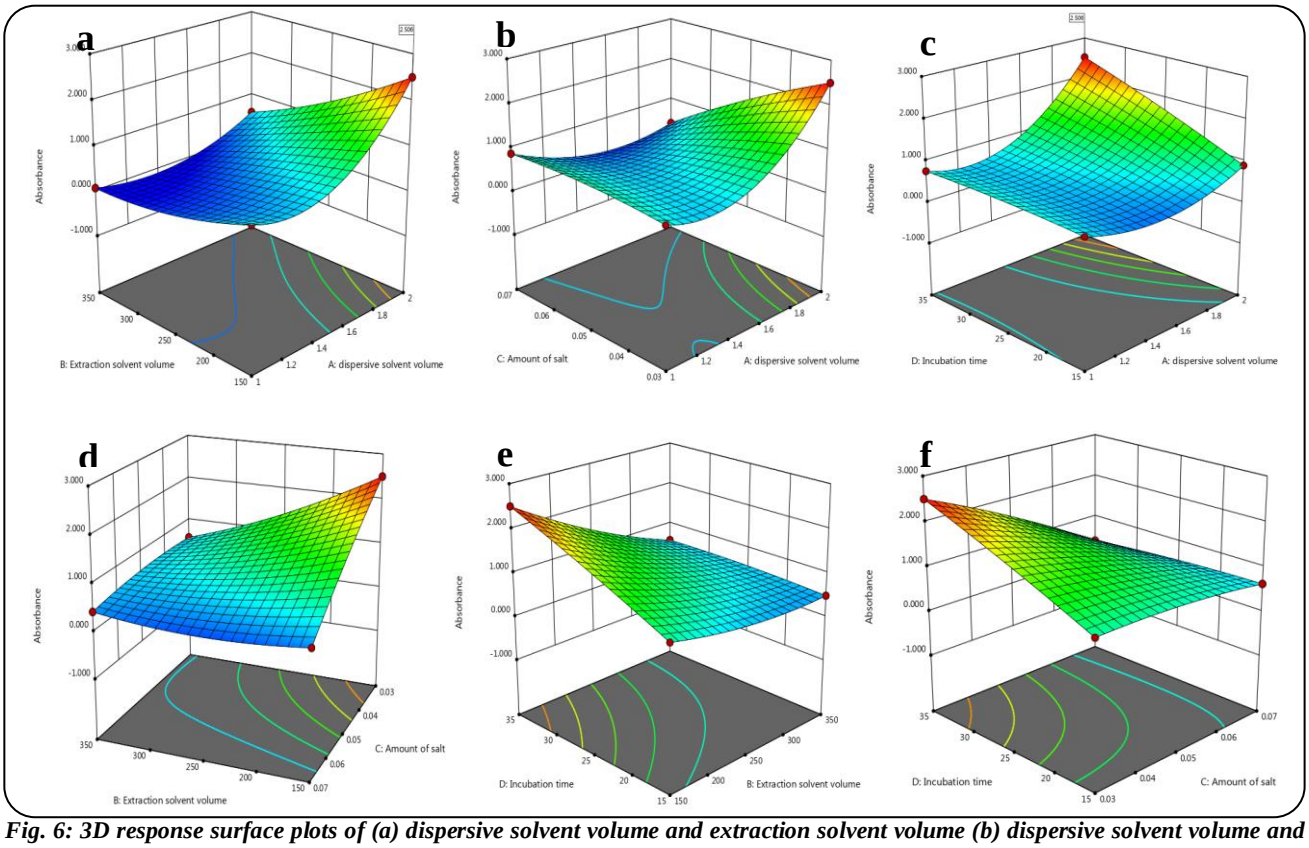


Fig. 6: 3D response surface plots of (a) dispersive solvent volume and extraction solvent volume (b) dispersive solvent volume and amount of salt, (c) incubation time and dispersive solvent volume, (d) amount of salt and extraction solvent volume, (e) incubation time and extraction solvent volume, and (f) incubation time and amount of salt

directed experiments programmed by CCD in uncoded units is described below:

$$Y = 1.6503 + -0.325275\ X_1 + 0.202515\ X_2 + -0.277081\ X_3 + 0.334106\ X_{13} + -0.344035\ X_{23} \quad (2)$$

ASCA Studies: Significance and Effect Estimation

ASCA combines the benefits of both experimental designs and multivariate exploratory analysis, allowing researchers to see if individual experimental parameters or factors (or the interplay of several of them) have a substantial impact on the collected data and how they change under their influence. According to the ANOVA technique, ASCA decomposes the centered data matrix (X) into the sum of many arrays mathematically. UV spectra were imported into Matlab 2013a (The Mathworks Inc., Natick, MA, USA) for chemometric analysis using

Table 3: Analysis of variance (ANOVA) for the response surface 2FI model obtained from CCD

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	10.55	10	1.06	6.49	0.0005	Significant
A-dispersive solvent volume	3.45	1	3.45	21.24	0.0003	Significant
B-Extraction solvent volume	1.16	1	1.16	7.12	0.0168	Significant
C-Amount of salt	1.71	1	1.71	10.53	0.0051	Significant
D-Incubation time	0.0011	1	0.0011	0.0069	0.9346	Not significant
AB	0.0839	1	0.0839	0.5158	0.4830	Not significant
AC	2.90	1	2.90	17.84	0.0006	Significant
AD	0.0881	1	0.0881	0.5419	0.4723	Not significant
BC	1.12	1	1.12	6.86	0.0186	Significant
BD	0.0104	1	0.0104	0.0637	0.8040	Not significant
CD	0.0336	1	0.0336	0.2069	0.6553	Not significant
Residual	2.60	16	0.1626			
Lack of Fit	2.56	14	0.1827	8.47	0.1104	Not significant
Pure Error	0.0431	2	0.0216			
Cor Total	13.16	26				
R^2						0.8023
Adjusted R^2						0.6787
Predicted R^2						0.4566

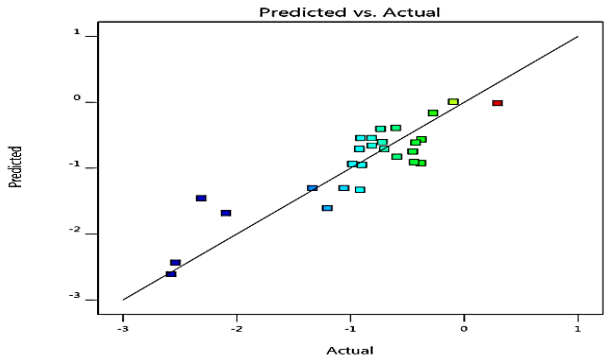


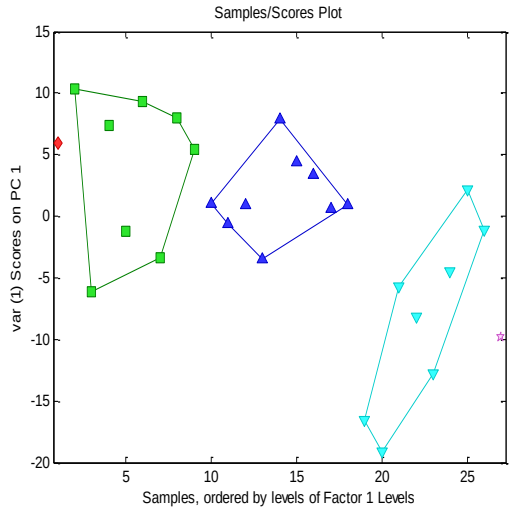
Fig. 7: Comparison of the predicted and actual responses

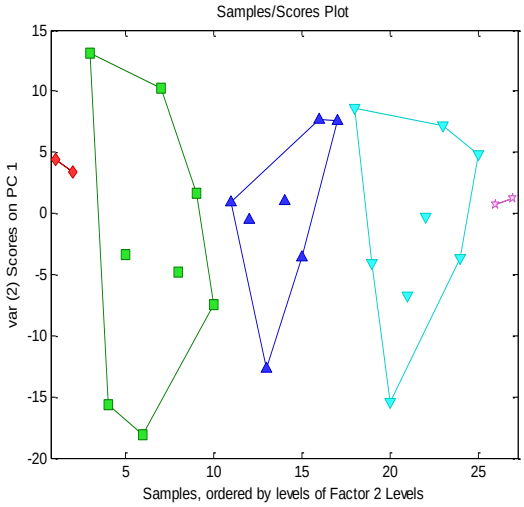
the PLS Toolbox 7.8 in this investigation (Eigenvector Research Inc., Wenatchee, WA, USA). Mean-centering preprocessing was used on the mean center to reduce undesired multiplicative effects from dataset spectra. After determining the importance of the influence of factors, smaller data subsets were investigated to compare the effects of individual components and their interactions. ASCA divides the data into the contributions of each factor and interaction as a first step. In this work, the variety of the data matrix was separated into five levels for four factors: the main influence of each experimental factor (dispersive solvent and extraction solvent volume, amount of salt, and incubation period) and then the effect of each second-order interaction. A permutation test was used to determine the significance of the factors' or interactions' effects. Table 4 shows the results of ASCA modeling on dividing total variance into individual terms related to main effects and interactions as well as significance tests. Even though the effect of the factor "dispersive solvent volume" and its interactions with other factors had a higher explained variance than other effects. In contrast, the effect of the factor of "incubation time" explained just 8% of the overall variation, implying that incubation time has a smaller effect than the other three primary components. This is consistent with the results of the ANOVA. The fact that second-order interactions of the components "dispersive solvent volume and amount of salt" explain about 92% of the overall variance. According to a significant interaction, the effect of extraction solvent volume was dependent on the dispersive solvent volume during microextraction.

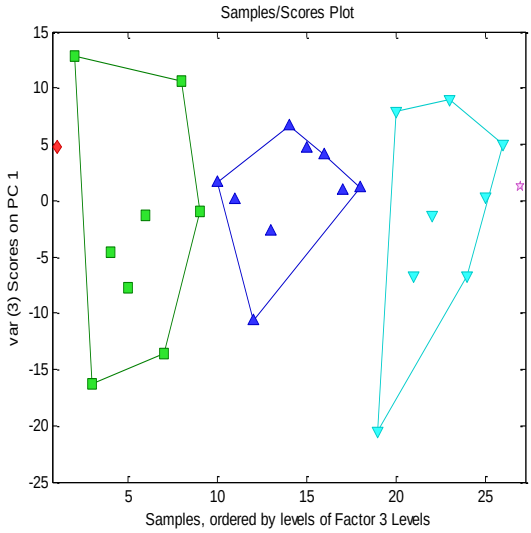
To provide a visual presentation of results, Fig. 8 shows the PC1 diagrams on the samples for 5 levels of the investigated factors and their binary interaction. This method can be considered as a compromise between the interpretability of a typical PCA and the level of a single interaction model. Depending on the specific aspect being

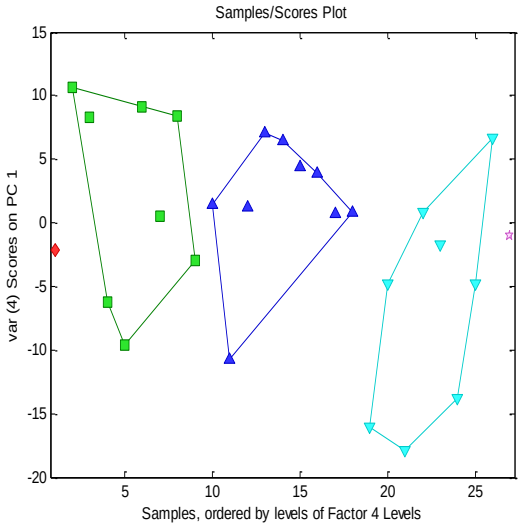
Table 4: ASCA modeling: effect significance and partitioning total variance into individual terms corresponding to main effects and interactions

Effect	Significance (p-value)	Explained variance (%)
Dispersive solvent volume (mL): X_1	0.105	13.11
Extraction solvent volume (µL): X_2	0.04	22.08
Amount of salt (mg): X_3	0.21	13.90
Incubation time (min): X_4	0.09	8.13
$X_1 \times X_2$	0.05	92.19
$X_1 \times X_3$	0.105	87.55
$X_1 \times X_4$	0.078	55.17
$X_2 \times X_3$	0.621	42.17
$X_2 \times X_4$	0.126	60.21
$X_3 \times X_4$	0.496	39.35









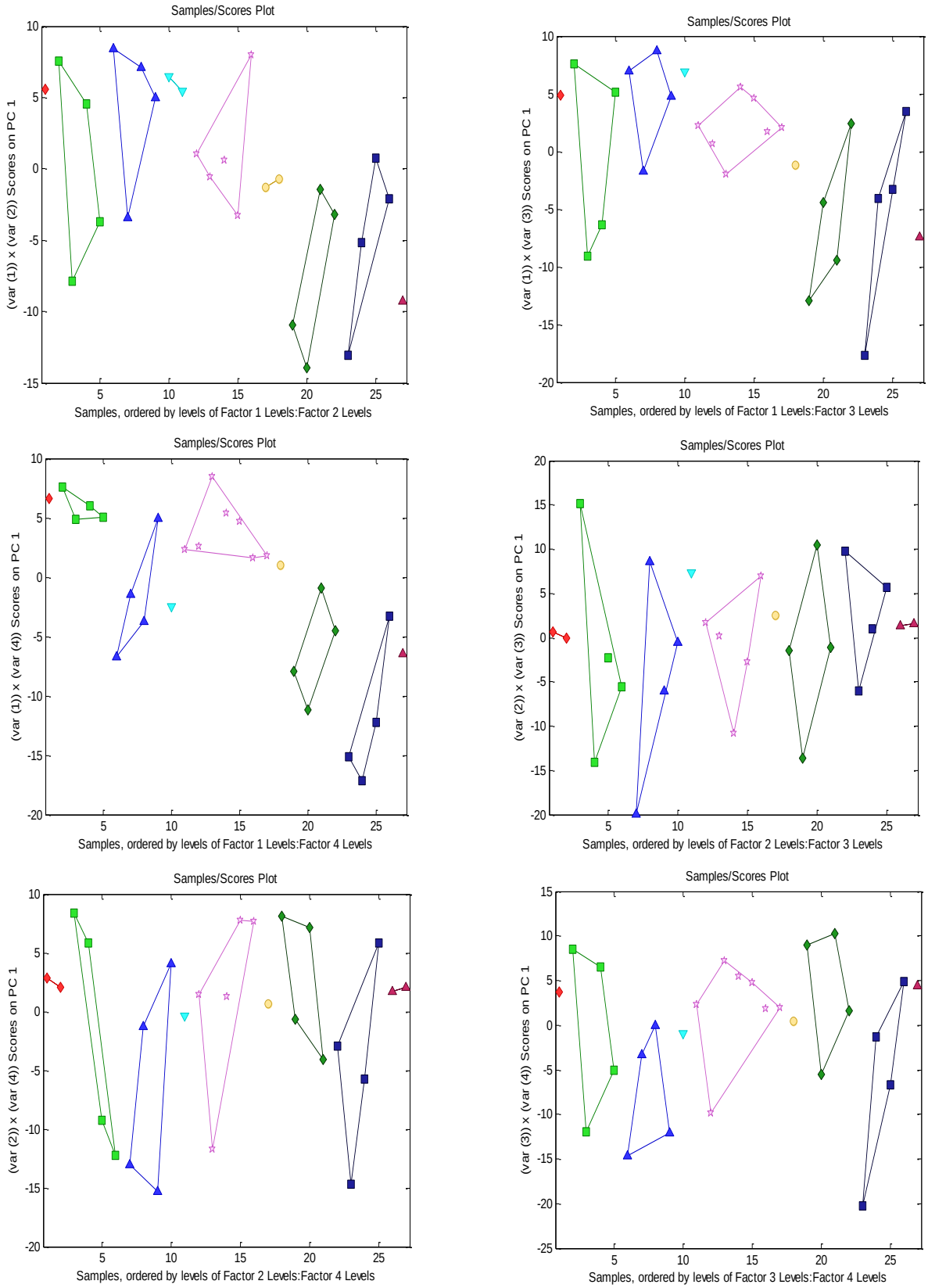


Fig. 8: ASCA model for scaled data. Sample score plot for PC1 on each factor and interactions between factors

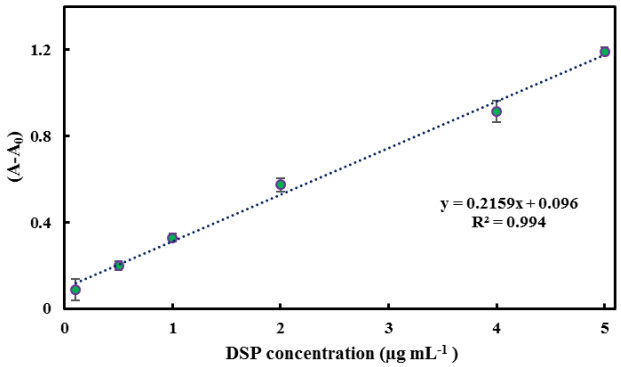


Fig. 9: Calibration plot for the extracted DSP in the concentration range of 0.1–5.0 µg·mL^{-1}

studied, other factors and interactions may also be included in these types of models. The results show that all 5 levels of the factors are significant because the group of each level in all factors have no interaction with each other and were the right choice in the design of the experiment.

Comparison between ASCA and ANOVA results

ASCA is a method that extract more information from a multivariate dataset from a defined experiment than existing methods. The focus of this study is on how to choose the right model and how to merge or separate distinct design factors and interactions in the ASCA model. As can be seen from the results, ASCA gives the effect of each factor on the liquid-liquid extraction rate of dexamethasone, which one could not measure in ANOVA. One can also use ASCA to find which level of each factor is significant. As a result, ASCA as a multivariate method gives us a wider view of the test design results.

Analytical figures of merit

The figures of merit of the proposed DLLME method were acquired under the ideal conditions. The calibration curve was linear in the range of 0.1-5.0 µg/mL with a correlation coefficient of 0.9948 (Fig. 9) and, 0.071 µg/mL was determined to be the detection limit (LOD) for the used method. For five replicate assays using 2.0 µg/mL DSP, the relative standard deviations (RSDs) for intra-days and inter-days were 1.8% and 2.8%. A pre-concentration factor of 4.5-fold corresponds to an extraction recovery of 90.0% achieved for DSP DLLME. The recovery test was indicated as a percent of the spiked drug found and calculated from the calibration equation. For this purpose, three different levels (in low, middle, and high points selected from the calibration range) of DSP were added to blank plasma samples and subjected to the proposed technique. The recoveries were calculated in the range of 88.0 and 103.0%. These results indicated the reliability of the method for DSP analysis in plasma samples.

CONCLUSIONS

In the current study, DSP was used as a model chemical, and a DLLME method coupled with spectrophotometry was used for its extraction. The main aim of this investigation is to identify important factors in dexamethasone microextraction from plasma samples. For this purpose, the chemometrics approaches such as ASCA, RSM, and CCD were applied to the results obtained from spectrophotometry. We use experimental design data and permutation testing to see how effectively both approaches detect experimental effects and present a quantitative comparison of ASCA and ANOVA. ASCA was found to be more discriminatory between experimental factor levels than ANOVA, however, it is prone to overfitting level differences. These investigations show that ASCA's visually obvious improved discriminating capability is quantitatively validated.

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