Journal of Chromatography A, 1218 (2011) 5995–6003

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Method development and validation for optimised separation of salicylic, acetyl salicylic and ascorbic acid in pharmaceutical formulations by hydrophilic interaction chromatography and response surface methodology

Nader Hatambeygi [a], Ghazaleh Abedi [b], Mohammad Talebi [a,*]

[a] Kimiafaam Pharmaceutical Co., Tehran 11458, Iran
[b] Novel Drug Delivery Systems Department, Iran Polymer and Petrochemical Institute, Tehran 1497713115, Iran

ARTICLE INFO

Article history:
Available online 12 June 2011

Keywords:
Hydrophilic interaction chromatography (HILIC)
Response surface methodology (RSM)
Derringer's desirability function
Box-Behnken
Optimisation
ICH guidelines

ABSTRACT

This paper introduces a design of experiments (DOE) approach for method optimisation in hydrophilic interaction chromatography (HILIC). An optimisation strategy for the separation of acetylsalicylic acid, its major impurity salicylic acid and ascorbic acid in pharmaceutical formulations by HILIC is presented, with the aid of response surface methodology (RSM) and Derringer's desirability function. A Box-Behnken experimental design was used to build the mathematical models and then to choose the significant parameters for the optimisation by simultaneously taking both resolution and retention time as the responses. The refined model had a satisfactory coefficient ($R^2 > 0.92$, $n = 27$). The four independent variables studied simultaneously were: acetonitrile content of the mobile phase, pH and concentration of buffer and column temperature each at three levels. Of these, the concentration of buffer and its cross-product with pH had a significant, positive influence on all studied responses. For the test compounds, the best separation conditions were: acetonitrile/22 mM ammonium acetate, pH 4.4 (82:18, v/v) as the mobile phase and column temperature of 28 °C. The methodology also captured the interaction between variables which enabled exploration of the retention mechanism involved. It would be inferred that the retention is governed by a compromise between hydrophilic partitioning and ionic interaction. The optimised method was further validated according to the ICH guidelines with respect to linearity and range, precision, accuracy, specificity and sensitivity. The robustness of the method was also determined and confirmed by overlying counter plots of responses which were derived from the experimental design utilised for method optimisation.

1. Introduction

The retention of polar compounds and the separation from their related impurities and degradants is an on-going challenge for chromatographers. Primarily defined by Alpert in 1990 [1], hydrophilic interaction chromatography (HILIC) provides an alternative approach to effectively separate especially small polar compounds on polar stationary phases such as bare silica. Similar to normal-phase liquid chromatography (NPLC), polar compounds are more strongly retained in HILIC, yet non-aqueous mobile phase in NPLC is replaced with an aqueous-organic mobile phase containing a mixture of an appropriate amount of water (typically at least 2.5 vol.%) and a less polar solvent (typically > 70% acetonitrile) with water being the strongest solvent [2–4]. This feature not only helps to overcome the drawbacks of poor aqueous solubility often encountered in NPLC, but also makes HILIC more amenable to use with MS and improves the MS sensitivity [5–8]. Moreover, derivatisation and the expensive ion-pair reagents are not required in the HILIC mode and no baseline artefacts are observed in contrast to ion-pair chromatography [9].

The retention mechanism in HILIC is believed to be predominantly hydrophilic partitioning of analytes between the bulk eluent and the water-rich layer that is partially immobilized on the surface of the stationary phase. It has also been found to be multimodal in nature and a combination of other interactions including electrostatic (ionic) interactions with positive or negative charges on the stationary phase, and/or hydrogen bonding or coulombic interactions with either the stationary phase or with tightly bonded water on the stationary phase may contribute to various degrees [2,4,10,11]. These interactions can potentially be altered in many ways, such as by changing the stationary phase, the type and concentration of organic modifier, buffer concentration, mobile phase pH and column temperature [10–12]. Consequently, these

* Corresponding author. Present address: Australian Centre for Research on Separation Science, School of Chemistry, University of Tasmania, Private Bag 75, Hobart TAS 7001, Australia. Tel.: +61 3 6226 1073; fax: +61 3 6226 2858.
E-mail addresses: mtalebi@utas.edu.au, talebi_md@yahoo.com (M. Talebi).

0021-9673/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2011.06.009

parameters can affect the performance characteristics of HILIC with different degrees of sensitivities.

While HILIC has been extensively used in various applications, few have studied in detail the effective parameters involved. The one-factor-at-a-time approach, which is a traditional method of optimisation, has been widely used through the previous studies, although it is well accepted that this method requires a relatively large number of experiments and frequently fails to predict optimal separation conditions [10,13,14]. It is generally time consuming, labour intensive, and can lead to misinterpretation of the results because of an inability to consider possible interactions between variables [13,14]. On the other hand, multivariate optimisation based on the statistical experimental design approach provides advantages including reduction in the number of experiments and improved statistical interpretation of the results that avoids misleading conclusions. Furthermore, the effect of a given factor can be determined at several levels of the other factors, so the conclusions are valid over a larger range of experimental conditions [15]. While experimental design-based procedures have been successfully applied to optimise other chromatographic methods such as RPLC and capillary electrophoresis, it is interesting to see that, to date, none of the HILIC methods found in the literature has been optimised using the experimental design approach [10]. While Guo et al. applied experimental design to study the effect of chromatographic conditions on the retention behaviour of some organic acids in HILIC mode, no optimisation was taken into account [12].

Acetylsalicylic acid (ASA) and ascorbic acid (AA) are widely employed in pharmaceutical formulations and are probably the major drugs consumed in the world [16]. In the last decade, various manufacturers have started to commercialise these substances together, in formulations that combine the action of the ASA for the relief of headache and fever with the power of the AA to increase the organism's resistance against microorganisms, as it participates in antibody formation [16]. Meanwhile, recent experiments have revealed the protecting effect of ASA on neuronal cells in a variety of pathophysiological situations including Alzheimer's disease and ischemic stroke, which could be promoted by the presence of AA [17,18].

The aims of this study were: (1) to explore the feasibility and utility of experimental design approach in identifying suitable separation conditions with a limited number of experiments for AA, ASA and its major impurity SA in pharmaceutical formulations; (2) to investigate the impact of various experimental parameters, such as organic modifier and its concentration, buffer pH, ionic strength and temperature on the chromatographic behaviour of these compounds and (3) by taking advantage of the proposed mathematical models, to gain more accurate insight into the retention mechanism involved using the phenomena occurring during the chromatographic process. To this end, a Box-Behnken response surface design was applied to fit the second-order model correlating the common chromatographic specifications, i.e., resolution and analysis time with significant independent parameters. Consecutively, optimum conditions for separation were predicted and corresponding models presented to describe the retention behaviour of studied compounds.

2. Experimental

2.1. Materials and reagents

SA, ASA and AA were of analytical grade from Merck (Darmstadt, Germany), Across (New Jersey, NJ) and AppliChem (Darmstadt, Germany), respectively. The common tablet excipients were provided by Kimiafaam Pharmaceutical Co. (Tehran, Iran). HPLC grade acetonitrile and methanol were obtained from Scharlau (Barcelona, Spain). All other reagents and chemicals were of analytical or HPLC grade obtained from Merck. Water purified via Milli-Q system, Millipore Corp. (Bedford, MA), was used for all purposes.

Table 1
Coded and actual experimental factors and levels used in the Box-Behnken design.

Factor	Level (−)	Level (0)	Level (+)
x_1: Acetonitrile content (% v/v)	70	75	80
x_2: Buffer concentration (mM)	10	30	50
x_3: Buffer pH	3.0	4.5	6.0
x_4: Column temperature (°C)	25	30	35

2.2. Apparatus and procedures

All experiments were conducted on an Agilent 1200 series liquid chromatograph (Waldbronn, Germany) equipped with a vacuum degasser (G 1379B), binary pump (G 1312A), manual injector (G 1328B), thermostated column compartment (G 1316A) and diode array detector (G 1315D). Instrument control and data acquisition and analysis were performed through ChemStation (Rev. B.02.01) software.

Separation was achieved on a Zorbax RX-SIL silica column 4.6 mm × 250 mm, 5 μm with a 4.6 mm × 12.5 mm, 5 μm guard column (Agilent Technologies, Wilmington, DE). The injection volume was 20 μl with UV detection at 285 nm. The elution was isocratic with mobile phase of acetonitrile–acetate buffer at specific compositions. The buffer was prepared by adjusting the ammonium acetate solution in water with acetic acid to the required pH. Buffer concentration and pH values were varied and refer only to the aqueous portion of the mobile phase before the addition of organic solvent. Like other variables, column compartment temperature was also varied and dependent on the experimental conditions (see Table 1).

2.3. Data analysis

The data analysis was performed using Design-Expert Version 6.0.6 statistical software (Stat-Ease Inc., Minneapolis, MN, USA). Factor significance was calculated using the statistical tool of analysis of variance (ANOVA) that was estimated and run up to the first order interaction terms. For all the calculations it was assumed that higher order interaction terms did not contribute significantly to the behaviour of the selected statistical model, since the chances of these interactions happening are low.

2.4. Method validation

The optimised method was validated by following the International Conference on Harmonisation (ICH) guidelines for analytical method validation [19]. System suitability testing (SST) was investigated with respect to tailing factor and resolution of analytes as well as instrument precision (repeatability) in terms of retention time and peak area. The following validation characteristics were addressed: robustness, selectivity, linearity and range, precision, accuracy, and limit of detection and quantitation.

3. Results and discussion

3.1. Preliminary experiments and factor selection

In previous studies in HILIC, the level of organic solvent in the mobile phase has probably been the factor with the greatest influence on retention. Typically, acetonitrile has been selected over methanol because methanol is considered to be stronger than acetonitrile, leading to poor retention [6,10]. In addition,

the hydrogen-bonding interaction between methanol and analytes may introduce extra resonance structures and potentially cause broad or tailing peaks [11]. Consequently, the effect of acetonitrile content was investigated over the concentration range of 70–80% (v/v).

As in RPLC, the ionic strength of the mobile phase can affect the retention and selectivity of polar compounds in HILIC. However, many salts typically used in RPLC are not suitable for HILIC due to their poor solubility in a mobile phase containing high levels of acetonitrile. Ammonium acetate buffer, which has already afforded promising results in HILIC retention of acidic compounds [2,5,12], was selected as a compromise between reasonable solubility and technically suitable pH range over which an available buffer capacity can be achieved. Accordingly, the effect of buffer concentration and pH on the retention was investigated by varying the ammonium acetate concentration from 10 to 50 mM and pH from 3 to 6, respectively.

Column temperature is another parameter that has been shown to influence the retention behaviour of polar compounds in the HILIC mode [5,11]. In general, temperature increases the diffusion coefficient which results in narrower peaks and higher separation efficiency [11]. By considering the technical restrictions imposed by the column manufacturer, the temperature effect on retention was evaluated by varying the column compartment temperature from 25 to 35 °C. Table 1 summarises the four factors and three corresponding levels of each variable selected in this study.

In addition to the chromatographic conditions, the type of stationary phase also has a marked influence on the retention and selectivity of organic acids [3,5,12]. Based on our preliminary experiments and the prior success of others, a bare silica column, which continues to be the most popular stationary phase for HILIC separation [2–4], was selected for further studies.

3.2. Response surface methodology

The objective of a design of experiment (DOE) approach is to provide enough tests to fit the second-order equations correlating the response function with independent parameters. A Box-Behnken response surface design was employed for this purpose followed by mapping the mathematical models corresponding to each response.

Box-Behnken designs are a class of rotatable or nearly rotatable second-order designs, with all points lying on a sphere of radius $\sqrt{2}$ [15,20]. By combining a fractional factorial with incomplete block designs, each factor requires only three levels instead of the five required for central composite designs (unless alpha is equal to one), which results in fewer design points. In addition, Box-Behnken designs do not have axial points, thus all design points fall within the safe operating zone [15,21]. Although this avoids factor-level combinations that are prohibitively expensive or technically impossible to perform, Box-Behnken designs are not suitable when one wishes to know the responses at the extremes, i.e., where all the factors are at their highest or lowest levels. Moreover, they are less economical than Doehlert designs, especially when the number of factors increases [20]. Nevertheless, Box-Behnken designs are considered as efficient options in response surface methodology and ideal alternatives to central composite design [15,22]. The design matrix for the Box-Behnken study was generated using four factors at three levels resulting in a total of 27 analytical experiments. Evidently, a larger number of experiments would have been necessary if the method were optimised by the conventional univariate approach. The design matrix as well as the corresponding retention time and resolution response values is shown in Table 2. All experiments were performed in randomised order to minimise the effects of uncontrolled variables that may introduce a bias in the measurements. Three centre point experiments, numbers 9, 18 and 27, were incorporated to estimate the experimental error that does not depend on the fitted model. As running the whole set of experiments in one session was impossible, the design was blocked to ensure randomisation and consistency from run to run. The retention data was analysed to describe the retention mechanism involved in the HILIC separation of the studied compounds, whereas the retention data of the first eluting peak (T_1), which corresponds to SA, and resolution data were considered to fully optimise the separation conditions.

Table 2
Box-Behnken experimental design matrix of coded variables[a] and studied responses.

Block	Run	Coded variable				Response				
						Resolution		Retention time[b]		
		x_1	x_2	x_3	x_4	R_1	R_2	T_1	T_2	T_3
1	1	70	10	4.5	30	3.3	1.3	2.4	2.8	3.0
	2	80	10	4.5	30	5.0	2.9	2.4	3.0	3.5
	3	70	50	4.5	30	4.0	3.1	2.8	3.2	3.6
	4	80	50	4.5	30	6.5	7.5	2.8	3.6	4.8
	5	75	30	3.0	25	2.0	3.8	2.8	3.0	3.5
	6	75	30	6.0	25	3.9	4.3	2.5	3.0	3.6
	7	75	30	3.0	35	2.1	2.8	2.8	3.0	3.5
	8	75	30	6.0	35	3.8	3.6	2.5	3.0	3.4
	9	75	30	4.5	30	5.1	3.9	2.7	3.3	3.7
2	10	70	30	4.5	25	4.0	2.5	2.7	3.1	3.5
	11	80	30	4.5	25	6.5	5.7	2.7	3.5	4.4
	12	70	30	4.5	35	4.1	2.2	2.7	3.1	3.4
	13	80	30	4.5	35	6.0	5.2	2.7	3.4	4.2
	14	75	10	3.0	30	2.6	3.4	2.6	3.0	3.4
	15	75	50	3.0	30	1.9	4.0	2.9	3.0	3.5
	16	75	10	6.0	30	2.5	1.3	2.2	2.5	2.7
	17	75	50	6.0	30	5.0	5.5	2.9	3.2	4.0
	18	75	30	4.5	30	5.1	3.9	2.7	3.2	3.7
3	19	70	30	3.0	30	1.5	2.8	2.8	3.0	3.3
	20	80	30	3.0	30	2.6	4.8	2.8	3.0	3.5
	21	70	30	6.0	30	3.0	2.2	2.5	2.9	3.1
	22	80	30	6.0	30	5.7	6.1	2.5	3.2	4.0
	23	75	10	4.5	25	5.1	2.0	2.4	2.9	3.2
	24	75	50	4.5	25	5.5	5.7	2.8	3.4	4.1
	25	75	10	4.5	35	4.0	1.8	2.4	2.9	3.1
	26	75	50	4.5	35	5.0	5.0	2.8	3.4	4.0
	27	75	30	4.5	30	5.2	3.8	2.7	3.2	3.7

[a] x_1: acetonitrile content (% v/v); x_2: buffer concentration (mM); x_3: buffer pH; x_4: temperature (°C).
[b] T_1, T_2, and T_3 corresponds to the retention times of SA, ASA, and AA, respectively.

By postulating a fitted full quadratic model described in Eq. (1), the mathematical models were obtained for each response (Y_i) in terms of coded factors after fitting Eq. (1) by the least square regression, see Table 3.

$$Y_i = b_0 + \sum_{i=1}^{k} b_i x_i + \sum_{1 \le i \le j}^{k} b_{ij} x_i x_j + \sum_{i=1}^{k} b_{ii} x_i^2 \quad (1)$$

In this equation, k is the number of factors (variables); b_0 is the intercept parameter; and b_i, b_{ij} and b_{ii} are the regression parameters for linear, interaction and quadratic effects of each factor x_i, respectively.

Because of the potential problems associated with the normality assumption, unequal error variance by treatment or block, and block-treatment interaction, the adequacy of the assumed model needs to be examined. In this study, adequacy checking of final refined models was carried out using the adequate precision statistic tool and normal probability plots of the studentised residuals. The adequate precision values, which correspond to the signal-to-noise ratios, were found to be more than the ratio limit of 4 (Table 3) and all normal probability plots revealed a nearly constant variance over the studied ranges (not shown). Also a fitted model is usually assessed with the coefficient of determination, R^2. A concern with this statistic is that it always increases as terms are added to the

Table 3
Refined regression equations[a] and statistical parameters for studied responses from the Box-Behnken experimental design.

Response	Regression equations	C.V. (%)[b]	Adjusted R^2	Predicted R^2	Adequate precision
R_1	$4.96 + 1.03x_1 + 0.45x_2 + 0.93x_3 - 1.92x_3^2 + 0.4x_1x_3 + 0.8x_2x_3$	7.83	0.9548	0.9114	25.54
R_2	$3.74 + 1.51x_1 + 1.51x_2 + 0.12x_3^c - 0.28x_4 + 0.70x_1x_2 + 0.47x_1x_3 + 0.9x_2x_3$	8.88	0.9583	0.9042	29.81
T_1	$2.67 + 0.22x_2 - 0.13x_3 - 0.057x_2^2 + 0.1x_2x_3$	1.12	0.9755	0.9269	46.50
T_2[c]	$3.25 + 0.13x_1 + 0.23x_2 - 0.017x_3^c - 0.099x_2^2 - 0.24x_3^2 + 0.075x_1x_3 + 0.18x_2x_3$	2.27	0.9169	0.7280	23.37
T_3	$3.79 + 0.38x_1 + 0.43x_2 + 0.008x_3^c - 0.12x_2^2 - 0.29x_3^2 + 0.17x_1x_2 + 0.17x_1x_3 + 0.30x_2x_3$	3.34	0.9338	0.8090	25.51

[a] Obtained by applying backward elimination tool to remove nonsignificant ($P > 0.05$) terms from the full models.
[b] Coefficient of variation.
[c] The nonsignificant term x_3 was included in the equation to maintain model hierarchy.

model, even if the added terms are not significant. Consequently, this statistic is usually smaller for the refined model in comparison to the corresponding full model. To overcome the drawback associated with the use of R^2, the adjusted coefficient of determination, adjusted R^2, is used. This is a statistic adjusted for the "size" of the model; that is, the number of factors. The adjusted R^2 can actually decrease if nonsignificant terms are added to a model [15]. Therefore, to obtain a simple yet more accurate model, the nonsignificant terms ($P > 0.05$) were removed from the models through the "backward elimination" process. While the main effect x_3 was not a significant term in the models corresponding to R_2, T_2 and T_3, to comply with the model hierarchy it was included in the resulting equations. As can be seen in Table 3, the adjusted R^2 of the refined models are higher than those of the full second-order models, implying that it is very unlikely that nonsignificant terms were included in the revised model. Also, the values are in reasonable agreement with the "predicted R^2" and are well within the acceptable limits of $R^2 \geq 0.80$, which reveals that the experimental data show a good fit with the second-order polynomial equations [15]. It is also worth mentioning that the difference between predicted R^2 and adjusted R^2 for response T_2 is still not unreasonable by considering the fact that the corresponding model accounts for about 92% (adjusted $R^2 = 0.9169$) of the variability in the response (Table 3). The model F values imply that all five models are significant, and there is only 0.01% probability that the obtained level of fit could occur due to random chance. In addition, the values achieved for the coefficient of variation (C.V.) percent, which is a measure of reproducibility of a model and should generally be less than 10%, are satisfactory for the models.

The importance of each term in the mathematical models was assessed using ANOVA. The magnitudes of the coefficients in the regression equations were utilized as the basis for judging statistical significance and illustrating the relative effects of linear, quadratic and cross product interactions between the parameters (Table 3). The analysis shows that the concentration of buffer (x_2) and its cross-product with buffer pH (x_3), x_2x_3, had a significant, positive influence on all studied responses. For resolution R_1 (SA and ASA), the square term x_3 had the most significant effect on the separation, as a natural consequence of its meaningful effect on retention time T_2. However, the significant factors were totally different for resolution R_2 (ASA and AA); only buffer concentration (x_2) and acetonitrile content (x_1) had equally important effects on the response. Similarly, the retention time of AA (T_3) was positively influenced by these two factors, but to a lesser extent than R_2. Finally, the column temperature neither individually nor as an interactive factor with the others was found to be significant over the studied range, possibly due to the limited operating range employed.

3.3. Retention mechanism

In order to better understanding the effects of independent variables and their interaction on the separation, and consequently, realising the retention mechanism of studied compounds, the three-dimensional response surface images and corresponding contour plots were visualised with retention time as a function of the main interactions among acetonitrile content, buffer concentration and pH. Accordingly, we could assess how the predicted responses change with respect to changing two of the factors simultaneously, while keeping the third one constant at the middle level. The results generally revealed very similar response surface plots for ASA and AA yet different from those obtained for SA.

Fig. 1a and b demonstrates the effect of acetonitrile content and buffer concentration on the retention of SA and AA with the buffer pH and column temperature kept constant at their centre points 4.5 and 30 °C, respectively. As can be seen, and reported previously, increasing the acetonitrile content over the studied range resulted in a gradual increase in retention of both ASA (not shown) and AA (Fig. 1b), while the retention time of SA remains essentially unchanged. However, a pronounced increase in retention was obtained by increasing the buffer concentration. The trends are also consistent with the largest magnitude of this factor (x_2) in the regression equations (Table 3). This can be explained by considering the fact that the ions in the mobile phase form ion pairs with charged groups on the surface of the stationary phase, shielding charged solutes from electrostatic repulsion induced by a surface of the same charge [23]. The concentration and polarity of the counterions (ammonium ions in this case) thus has a major effect on the selectivity.

Fig. 1c and d represents the effects of buffer concentration and pH, while keeping the acetonitrile content and temperature at their middle points, on the retention of SA and ASA, respectively. From the plots, retention is increased by increasing the buffer concentration and is decreased as the buffer pH increases. This confirms that the hydrophilic partitioning was probably not the only mechanism involved in retention. The observed trends can be ascribed to the effect of pH on the ionisation of solutes and also surface silanols [4,5,7,13]. Meanwhile, the extent of ionisation of both surface silanols and analytes increases as the buffer pH increases from 3 to 6, thus leading to an increase in electrostatic repulsion between the negatively charged silica surface and the analyte anions and consequently a decrease in retention [5,7].

In addition to an increase in hydrophilic partitioning, higher salt concentrations should contribute to the retention increase of solutes by weakening the assumed electrostatic repulsion [4,5,7,12]. However, depending upon the pK_a of the studied compounds, the strength of the buffer concentration effect on retention could vary across the selected pH range. As shown in Fig. 1c, and depicted from the corresponding regression equation, retention of SA was not significantly affected by the interaction between buffer concentration and pH, because there was no significant change in ionisation of SA ($pK_a \sim 3$) in the pH range of 3–6. Therefore, by increasing buffer concentration, there seemed to be no marked change on the curvature of the response surface over the studied pH range. Nevertheless, due to the direct correlation between buffer capacity and concentration, the decrease in retention is more obvious at lower buffer concentrations, perhaps because silanol

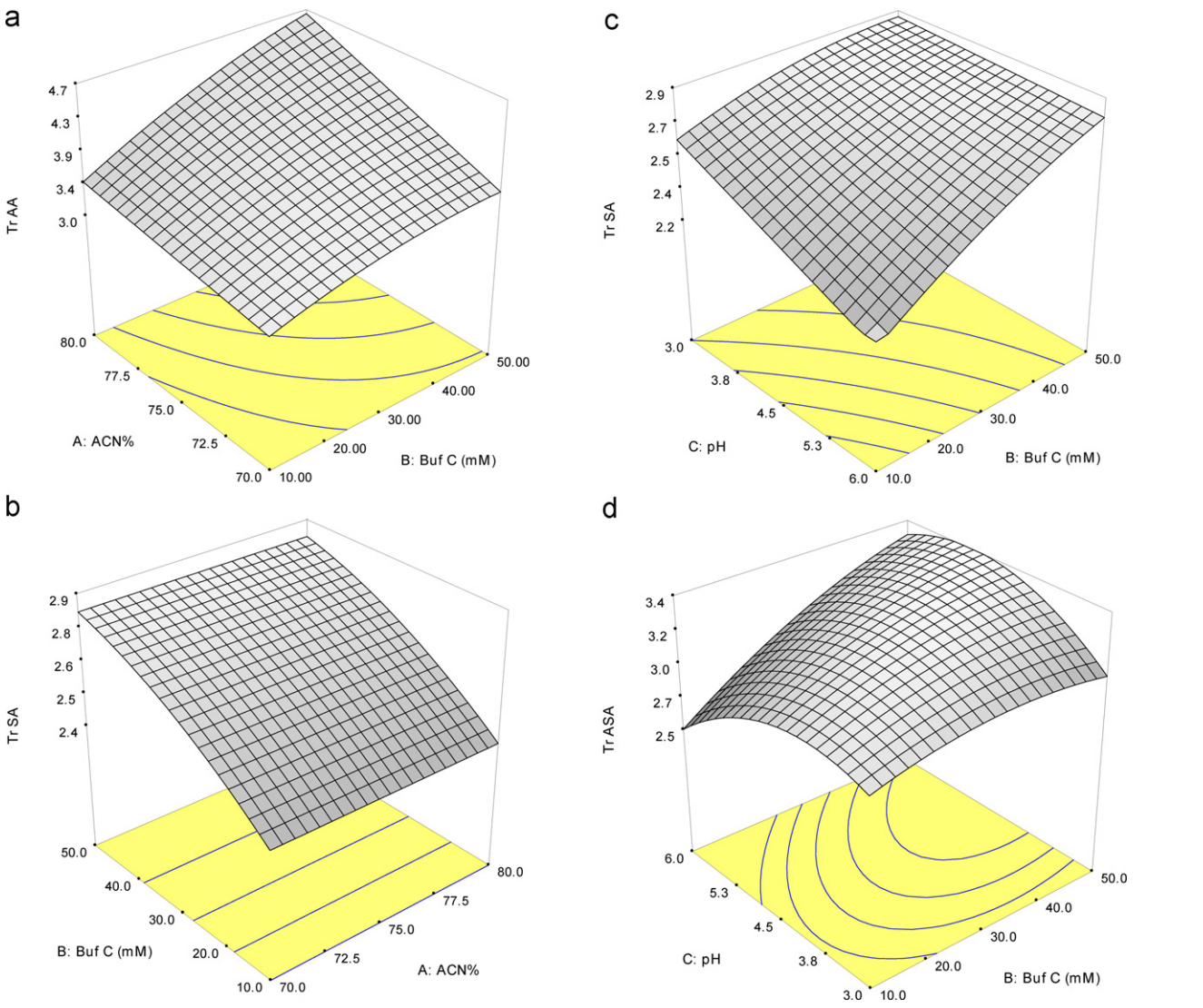

Fig. 1. Response surface plots representing the retention time as a function of acetonitrile content and buffer concentration for SA (a) and AA (b) by keeping the buffer pH and temperature constant at 4.5 and 30 °C, respectively; and the buffer concentration and pH for SA (c) and ASA (d) by keeping the acetonitrile content and temperature constant at 75% and 30 °C, respectively.

dissociation is somewhat suppressed by increasing the buffer capacity in a pH range bellow the pK_a of silanols.

In contrast however, buffer pH (in combination with buffer concentration) is presumed to have a more significant impact on the retention of the less acidic compounds ASA and AA. As discussed earlier, there was a significant ion-interaction effect on the bare silica surface. When the buffer pH dropped below its pK_a (~3.5), ASA was protonated significantly, thus the retention was governed by the hydrophilicity of the undissociated form of the compound and remained relatively unchanged (Fig. 1d). However, a factor of comparable, or perhaps greater significance, is the lack of buffering capacity of the buffer employed in that pH range. On the other hand, when the buffer pH increased above its pK_a, ASA became more deprotonated. The electrostatic repulsion induced from the similarly charged silanol groups contributed significantly to the overall retention, thus leading to a progressively decrease in retention with an increase in the buffer pH from ~4.5 to 6.0. Meanwhile, because of the positive interaction existing between buffer concentration and pH (x_2x_3) for all studied compounds (Table 3), increasing buffer concentration, when the buffer pH was high enough, contributed significantly to offsetting the undesirable effect of pH on the retention of ionised compounds by forming ion pairs with the surface charged residues which resulted in weakening electrostatic repulsion as a major contributor to decreasing retention (Fig. 1c and d). With a pK_a ~ 4.2 in water [24], very similar trends to those obtained for ASA were also observed in AA retention (data not shown).

3.4. Multi-criteria decision making

When a simple response is being analysed, model analysis indicates areas in the design region where the process is likely to give desirable results. Many response surface problems involve the

Table 4
Settings for multi-criteria optimization of the individual factors and responses.

Factor/response	Goal	Lower limit	Upper limit	Importance
x_1	in range	70.0	85.0	3.0
x_2	minimize	10.0	50.0	4.0
x_3	in range	3.0	6.0	3.0
x_4	in range	25.0	35.0	3.0
T_1	target = 2.6	2.2	2.9	4.0
R_1	target = 6.2	6.0	6.5	4.0
R_2	in range	2.0	5.0	3.0

Global desirability (D) = 0.804.

Table 5
Method validation characteristics for studied compounds.

Parameter	SA	ASA	AA	Limits
SST				
Asymmetry factor (A_s)	0.729	1.624	0.771	$A_s \leq 1.5$
Resolution (R)	–	7.6	5.8	$R_1 \geq 6.2$; $R_2 \geq 2.0$
Repeatability, T (% RSD)	0.70	0.35	0.21	RSD < 1.0%
Repeatability, A (% RSD)	1.11	0.28	0.13	RSD < 1.0%
Validation				
Intra-day precision (% RSD)	3.30	1.75	1.26	RSD < 5.0%
Inter-day precision (% RSD)	5.1	3.7	1.7	RSD < 5.0%
Mean recovery (%), % RSD	105.8, 3.9	93.9, 2.5	96.1, 1.4	100 ± 5, 5.0%
LOD (μg/ml)	0.03	N.D.	N.D.	–
LOQ (μg/ml)	0.09	2.50	1.50	–

Intra-day precision (repeatability), and inter-day precision (reproducibility) and accuracy (mean recovery) were determined for the drug substances by analyzing two replicates QC standards and samples prepared at three levels of ~50%, 100% and 150% of the LC concentration, and for the impurity SA at a single level of ~4.0% (7 μg/ml) of the LC concentration of ASA.
Inter-day precision and accuracy were assessed by duplicate preparation of QC standards and samples (spiked placebos) on three consecutive days.

analysis of several responses. Meanwhile, simultaneous consideration of multiple responses involves first building an appropriate response surface model for each response and then trying to find a set of operating conditions that in some sense optimises all responses or at least keeps them in a desired range. As it is not an easy task, there is practical interest in more formal optimisation strategies for multiple responses. One useful approach is to use the Derringer's desirability function, which allows for compromise among the various responses. This function searches for a combination of factor levels that jointly optimise a set of responses by satisfying the requirements for each response in the design. The optimisation is accomplished by: converting each response Y_i ($i = 1, 2, \ldots, m$) into a dimensionless desirability scale that defines a partial desirability function (d_i), combining the individual desirabilities to obtain the composite or global desirability function (D), and finally maximizing the D and identifying the optimal factor settings [15]. The scale of the desirability function ranges between $d = 0$, for a completely undesirable response, to $d = 1$ for a fully desired response above which further improvements would have no importance [22,25]. The individual desirabilities (d) for each response are obtained by specifying the goals, i.e., minimise, maximise or target the response, and boundaries required for each one (Table 4). A weight factor, which defines the shape of the desirability function for each response, is then assigned. Weights must be between 0.1 and 10, with larger weights corresponding to more important responses. A weight factor of 1 was chosen for all individual desirabilities in this work.

Numerical optimisation will optimise any combination of one or more desired goals. The goals may apply to either factors, responses or both [25]. The criteria for the optimisation of each individual response are shown in Table 4. By taking into consideration the BP monograph for ASA [26], a target value of 6.2 was assigned to the resolution between ASA and its major impurity SA (R_1). In order to shorten the analysis time while separating the first eluting peak SA from the solvent front, the retention time of SA (T_1) was targeted to 2.6; that is the immediate retention after the void time (t_o) of the column. Design-Expert also enables us to set criteria for each factor. Although higher buffer concentrations provide increased buffer capacity, it can potentially cause a solubility problem in the mobile phase with a high percentage of organic solvent. Buffer concentration (x_2) was therefore assigned to be minimized in the studied concentration range. However, to offset the potential drawback of minimizing x_2 on the targeted responses, the upper limit of acetonitrile content (x_1) was extended to 85. The "importance" of a goal can be changed in relation to the other goals. It can range from 1 (the least important) to 5 (the most important), which gives emphasis to the goal. The default is for all goals to be equally important at a setting of 3. Accordingly, a high importance value of 4 was assigned for desirability indices (d_i) of two responses R_1 and T_1 as well as for the independent variable x_2 (Table 4).

After the individual desirabilities are calculated for each response, they are combined to provide a measure of the composite desirability of the multi-response system [15,22]. There are many ways in which the individual desirabilities can be combined. If the composite desirability is the geometric average of the partial desirabilities of m responses (Eq. (2)), it is referred to as the Derringer's desirability function.

$$D = (d_1 \times d_2 \times \cdots \times d_m)^{1/m} \quad (2)$$

One advantage of this function is that if any of the responses or factors falls outside its desirability range, the overall function becomes zero [15,22,25]. Thus, the goals were combined employing this strategy into an overall desirability function and the global desirability (D) for the optimal solution was determined to be 0.804. Following the conditions and restrictions discussed, the optimal calculated parameters were obtained as: acetonitrile content 81.54%, buffer concentration 22.16 mM, buffer pH 4.44 and column compartment temperature 27.85 °C. Accordingly, the following experimental parameters were set as the operating conditions: acetonitrile content 82%, buffer concentration 22 mM, buffer pH 4.4 and column temperature 30 °C.

3.5. Method validation

The HILIC method is developed for the routine analysis of batch products. Therefore, the suitability of the method for its intended purpose has to be demonstrated in a series of experiments to assess particular aspects of the method, e.g., selectivity, linearity and range, accuracy, precision, sensitivity and robustness.

3.5.1. System suitability testing (SST)

Working standard solutions of each analyte were injected to determine the individual retention time (T_1, T_2, and T_3) and UV spectrum as well as the chromatographic purity. A mixed standard solution was then injected and the retention time, UV spectrum and chromatographic purity for each of the studied compounds confirmed. We considered relative standard deviation (RSD) in retention time (T) and peak area (A) for five consecutive injections ≤2%, asymmetry factor (A_s) ≤ 1.5, resolution between ASA and its major impurity, SA, (R_1) ≥ 6.2, and between ASA and AA (R_2) ≥ 2, and retention time of SA (T_1) ≥ 2.6 as acceptable values [19,26]. As in reversed-phase chromatography, the sample diluent can strongly influence the peak shape of analytes in HILIC [6]. It should be as close to the mobile phase composition as possible. However, polar analytes often have low solubilities in organic

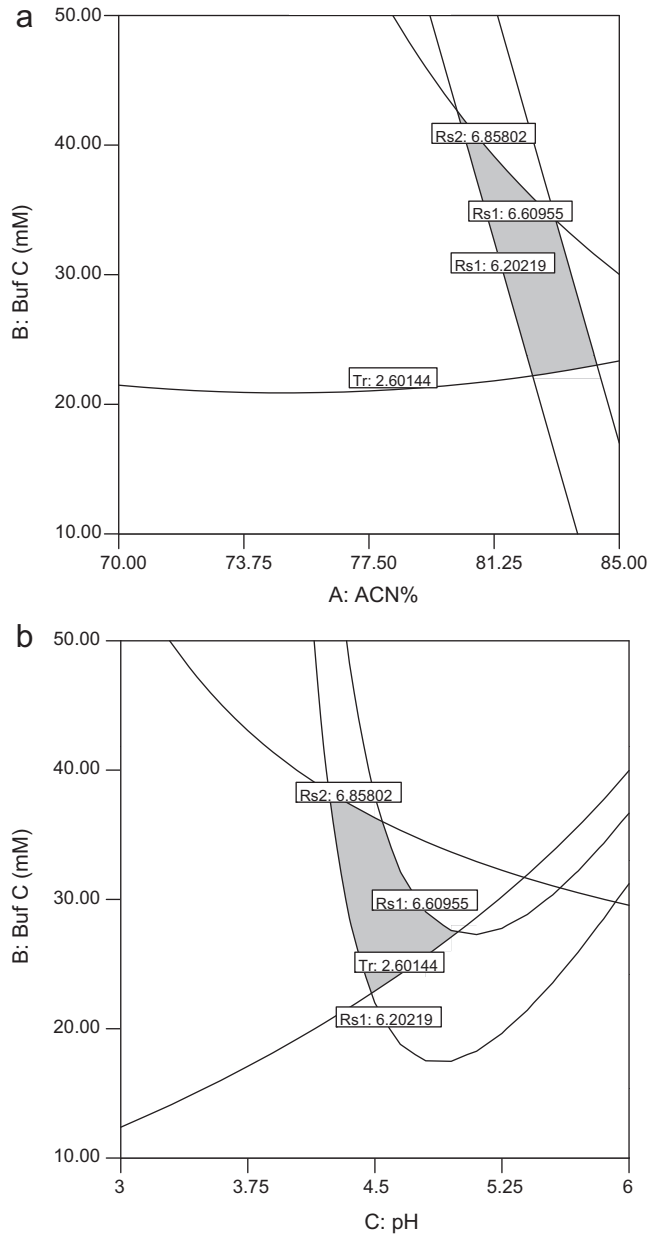

Fig. 2. Regions of the optimum found by overlying response contours for resolution R_1 (SA and ASA), R_2 (ASA and AA), and retention time T_1 (SA) as functions of (a) acetonitrile content vs. buffer concentration by keeping the pH and temperature constant at 4.4 and 28 °C, respectively; and (b) buffer concentration vs. pH by keeping the acetonitrile content and temperature constant at 82% and 28 °C, respectively.

solvents. It was determined that the best compromise for solubility and peak shape was achieved by initially dissolving analytes in water before mixing with a proportion of acetonitrile equivalent to the mobile phase composition. As can be seen in Table 5, a system suitability test confirmed the adequacy of the chromatographic system operated under the optimised conditions for the purpose of this study.

3.5.2. Robustness

For an analytical method to be robust, it must be able to demonstrate that it can produce quantitative results despite small changes in the experimental parameters, which may occur in a typical testing laboratory [19]. Fig. 2 presents the optimum operating region visualised by overlying counter plots of responses as a function of two variables by keeping the third one constant. Optimisation criteria were fulfilled in the dark section of each plot. As can be seen, the buffer concentration can be changed over a relatively wide range from 20 to about 40 mM, whereas pH is more sensitive and needs to be maintained within a narrow range of 4.5–5.0. Also, acetonitrile content can be changed from about 81 to 85%, depending upon the applied buffer concentration.

These results mean the values of the three responses in this region are stable. The optimised method is therefore robust and meets the criteria set for responses. This also demonstrates that the experimental design utilised for method optimisation could be further employed to assess the method robustness, and therefore, made it unnecessary to construct another individual design.

3.5.3. Selectivity

Selectivity of the method was evaluated by analysing a placebo sample containing a mixture of the three drug product excipients and verified by peak purity analysis. Solutions of each individual drug product as well as samples containing ASA, its main degradation product, SA, and AA were also injected. Fig. 3(a) represents a typical chromatogram obtained by spiking each analyte in a placebo sample to obtain a final concentration of 0.05, 3.5 and 1.5 μg/ml for SA, ASA and AA, respectively. Generated from impurities or excipients, a few small peaks are observed in the chromatogram but they are either well separated from compounds of interest or have insignificant areas in comparison to co-eluting analytes.

3.5.4. Linearity and range

After appropriate dilution, the label claim (LC) concentration ranges of ASA and AA in the studied drug products were 160–250 and 20–96 μg/ml, respectively. If the typical detection limits for drug related impurities were considered to be 0.1% or lower [27], the corresponding concentration range of SA would be ~0.1–0.25 μg/ml.

Standard calibration curves were prepared with six calibrators over a concentration range of 2.5–500 μg/ml (~1.5–200% LC) for ASA, 1.5–300 μg/ml (~7–300% LC) for AA and 0.09–15 μg/ml (~0.05–6.0% LC of ASA) for SA. The correlation coefficients (R^2) better than 0.999 (RSD < 1.5%) were achieved for all compounds studied. The residual sum of square values were also to be found no greater than 0.00025.

3.5.5. Precision and accuracy

Precision and accuracy of the method were determined for the drug substances by analysing quality control (QC) standards prepared at three levels of ~50%, 100% and 150% of the LC concentration, and for impurity SA at a single concentration of 7 μg/ml (~4.0% of the LC concentration of ASA). The method precision was established by injecting four QC standards, each in three replicates, for intra-day precision (repeatability) and on three consecutive days for the intermediate precision (reproducibility). Precision was expressed by the RSD (%) of the analyte peak area. Results for all studied compounds (Table 5) met the proposed requirement RSD ≤ 5% [19]. For accuracy studies, QC samples were prepared by appropriate spiking placebos to obtain the above-mentioned QC concentrations. Each compound was prepared in duplicate at each level. As shown in Table 5, mean recoveries obtained ($n = 6$) met the ICH criteria for recovery (100 ± 5) and percent RSD (≤5%).

3.5.6. Sensitivity

The limit of detection (LOD) was only determined for the impurity SA. By implementing the visual evaluation method [19], LOD was determined by successive dilution of standard solutions until no signal was reliably detected (Table 5). Accordingly, LOQ was considered as the lowest concentration of analyte in a standard that can

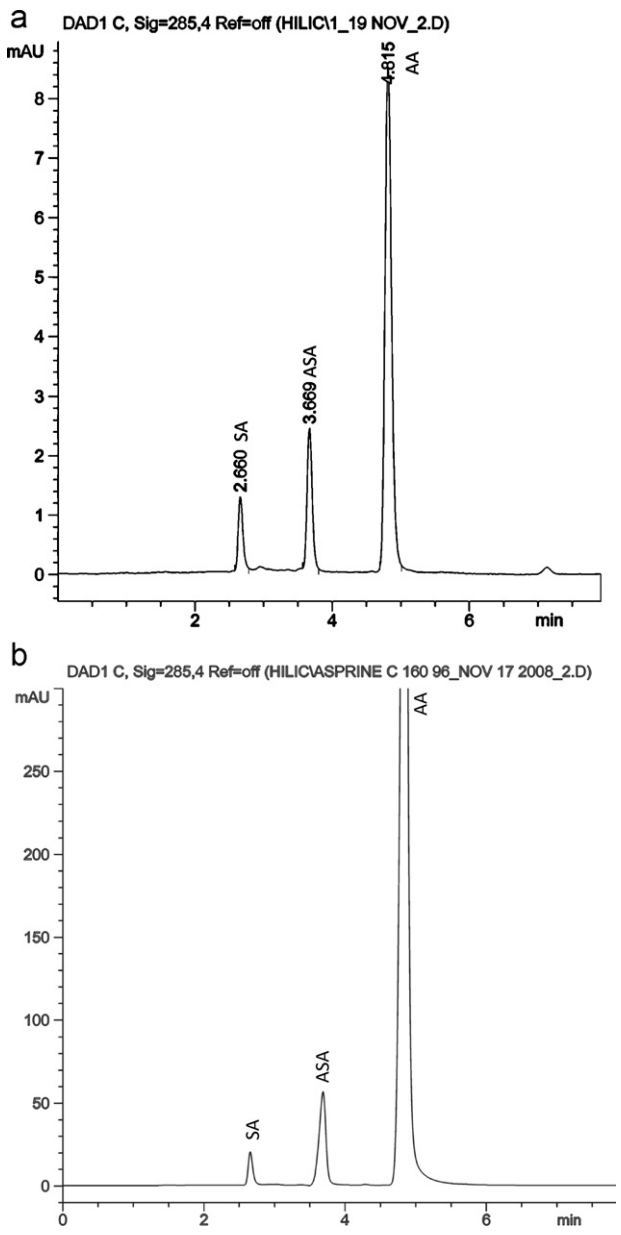

Fig. 3. Representative chromatograms of (a) a typical QC standard prepared by spiking placebo to a final concentration of 0.05, 3.5 and 1.5 μg/ml for SA, ASA and AA, respectively; and (b) a real sample of effervescent tablet (Aspirine®-C) containing 160 μg/ml ASA ($t_R \sim 3.7$ min) and 96 μg/ml AA ($t_R \sim 4.8$ min).

be reproducibly measured with acceptable accuracy and precision (RSD ≤ 2%).

3.6. Analysis of the marketed products

The validated method was used in the analysis of three pharmaceutical products from different manufacturers, as three different dosage forms and two different dosage strengths. These included effervescent tablets (each contains 400 mg ASA and 240 mg AA), effervescent powder (contains 250 mg/g ASA and 20 mg/g AA) and syrup (declared to contain 250 mg/ml ASA and 20 mg/ml AA). Several wavelengths were examined for quantitative purpose and 285 nm was chosen as a good compromise between selectivity (examined by peak purity test) and sensitivity for all but one of the examined samples. A typical chromatogram is shown in Fig. 3(b).

4. Conclusion

The major contribution of this work is applying an established strategy based upon experimental design approaches for optimising the separation conditions in the HILIC mode. The mathematical models developed for relating resolution and retention time to the composition of the mobile phase proved to be an efficient strategy for optimisation of the chromatographic method. A significant good fit with the models was found between predicted and observed data, which means the method was suitable for the analysis of compounds investigated. In addition, the predictive nature of a validated experimental design in determining the significant factors and their interactions enabled us to gain more accurate understanding of the interaction existed and mechanisms involved in HILIC separation. Accordingly, buffer pH and ionic strength and their interaction were found to be influential. The analytes were protonated significantly when the buffer pH dropped below their pK_a, thus the retention was governed by the hydrophilicity of the undissociated form of the compounds and also influenced by the lack of buffering capacity in that pH range. On the other hand, when the buffer pH increased above the pK_a, analytes became more deprotonated and the electrostatic repulsion induced from the similarly charged silanol groups affected the overall retention significantly. Meanwhile, because of the positive interaction existing between buffer concentration and pH for all studied compounds, increasing buffer concentration, when the buffer pH was high enough, contributed significantly to offsetting the undesirable effect of pH on the retention of ionised compounds by forming ion pairs between the mobile phase cations and the surface negatively charged residues which resulted in lessening the electrostatic repulsion and increasing retention. The described method also enabled the determination of the optimum conditions in a minimum number of experiments that simultaneously fulfilled the ICH monograph requirements for studied compounds and contributed to conditions for longer column life time by using an acceptable lower buffer concentration at an appropriate pH. The method validation indicated that the optimised HILIC method has suitable performance characteristics for the analysis of compounds studied. The data obtained from the utilised Box-Behnken design was further employed to evaluate the robustness of the optimised method, obviating the need to build an independent design or implement further experiments. The optimisation strategy presented together with the attractive features of separation under HILIC mode could be applied in optimising separation of similar polar compounds as the method is flexible, inexpensive and efficient.

Acknowledgements

The authors wish to gratefully thank Prof Emily F. Hilder (University of Tasmania) and Dr. Andrew J. Alpert (PolyLC Inc.) for English revision and their invaluable comments.

References

[1] A.J. Alpert, J. Chromatogr. 499 (1990) 177.
[2] P. Hemström, K. Irgum, J. Sep. Sci. 29 (2006) 1784.
[3] T. Ikegami, K. Tomomatsu, H. Takubo, K. Horie, N. Tanaka, J. Chromatogr. A 1184 (2008) 474.
[4] D.V. McCalley, J. Chromatogr. A 1217 (2010) 3408.
[5] Y. Guo, S. Gaiki, J. Chromatogr. A 1074 (2005) 71.
[6] E.S. Grumbach, D.M. Wagrowski-Diehl, J.R. Mazzeo, B. Alden, P.C. Iraneta, LCGC North Am. 22 (2004) 1010.
[7] M. Liu, E.X. Chen, R. Ji, D. Semin, J. Chromatogr. A 1188 (2008) 255.
[8] G. Jin, Z. Guo, F. Zhang, X. Xue, Y. Jin, X. Liang, Talanta 76 (2008) 522.
[9] M. Yang, R. Thompson, G. Hall, J. Liq. Chromatogr. Rel. Technol. 32 (2009) 628.
[10] B. Dejaegher, D. Mangelings, Y. Vander Heyden, J. Sep. Sci. 31 (2008) 1438.
[11] Z. Hao, B. Xiao, N. Weng, J. Sep. Sci. 31 (2008) 1449.
[12] Y. Guo, S. Srinivasan, S. Gaiki, Chromatographia 66 (2007) 223.
[13] A.M. Siouffi, R. Phan-Tan-Luu, J. Chromatogr. A 892 (2000) 75.

[14] R. Gheshlaghi, J.M. Scharer, M. Moo-Young, P.L. Douglas, Anal. Biochem. 383 (2008) 93.
[15] D.C. Montgomery, Design and Analysis of Experiments, Fifth ed., John Wiley & Sons Inc., New York, 2001.
[16] M.M. Sena, J.C.B. Fernandes, L. Rover Jr., R.J. Poppi, L.T. Kubota, Anal. Chim. Acta 409 (2000) 159.
[17] B.L. Fiebich, K. Lieb, N. Kammerer, M. Hüll, J. Neurochem. 86 (2003) 173.
[18] E. Candelario-Jalil, R.S. Akundi, H.S. Bhatia, K. Lieb, K. Appel, E. Muñoz, M. Hüll, B.L. Fiebich, J. Neuroimmunol. 174 (2006) 39.
[19] ICH Quality Guidelines, Topic Q2 (R1): Validation of Analytical Procedures: Text and Methodology, 2005. Available from: http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html.
[20] S.L.C. Ferreira, R.E. Bruns, E.G.P. da Silva, W.N.L. dos Santos, C.M. Quintella, J.M. David, J.B. de Andrade, M.C. Breitkreitz, I.C.S. Fontes Jardim, B.B. Neto, J. Chromatogr. A 1158 (2007) 2.
[21] R. Ragonese, M. Macka, J. Hughes, P. Petocz, J. Pharm. Biomed. Anal. 27 (2002) 995.
[22] A. Gonzalez, K.L. Foster, G. Hanrahan, J. Chromatogr. A 1167 (2007) 135.
[23] A.J. Alpert, Anal. Chem. 80 (2008) 62.
[24] A.C. Moffat, M.D. Osselton, B. Widdop (Eds.), Clarke's Analysis of Drugs and Poisons, Third ed., Pharmaceutical Press, London, 2005, Electronic version.
[25] Design-Expert Version 6.0.6 Statistical Software, User's Guide, Stat-Ease Inc., Minneapolis, MN, USA, 2003.
[26] British Pharmacopoeia 2007 (BP 2007), TSO Press, London, 2006, Electronic version 11.0.
[27] ICH Quality Guidelines, Topic Q3B (R2): Impurities in New Drug Products, 2003. Available from: http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html.