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Strategy for developing and optimizing liquid chromatography methods in pharmaceutical development using computer-assisted screening and Plackett–Burman experimental design

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Abstract

We describe a three-step method development/optimization strategy for HPLC assay/impurity methods for pharmaceuticals, which include multiple-column/mobile phase screening using a system equipped with a column-switching device, further optimization of separation by using multiple organic modifiers in the mobile phase, and multiple-factor method optimization using Plackett–Burman experimental designs. In the first two steps, commercially available chromatography optimization software, DryLab, was used to perform computer simulations. This allows the method developer to evaluate each condition (one column/mobile phase combination) with retention data from two scouting gradient runs. This approach significantly reduces the number of runs in method development. After a satisfactory separation was obtained, we used a method optimization step with Plackett–Burman experimental designs. The purpose of the 16-injection set experiments was to evaluate nine method factors with regard to method precision, accuracy, sensitivity and specificity. The results provided logical justifications in selecting method parameters such as column temperature, detection wavelength, injection volume, and sample solvent, etc. In data analysis, instead of the traditional mathematical manipulations, we used the graphical methods to examine and present data by creating the so-called main effect plots. Because replicates of design points were not run, the data did not allow the testing of statistical significance. However, it provided visual presentations in a way that is easy to understand for the method developer and end user alike.
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Keywords: Computer simulation; Plackett–Burman design; Method development; Optimization

1. Introduction

HPLC assay/impurity methods for active pharmaceutical ingredient (API) and impurities in drug product have to meet stringent regulatory requirements, must be transferable globally to different units, and remain operational over the lifetime of the product. They should not be too complicated and time-consuming to run, and should be as cost-effective as possible.

Strategies for method development have been discussed in the literature [1–3]. Different computer-assisted chromatography optimization methods have been developed. For example, DryLab from LC Resources uses retention data from scouting runs for

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subsequent retention and resolution prediction via simulation [4]. ChromSword, another optimization software, takes a somewhat different approach using structure fragments and dipole–dipole interactions to predict retention behavior [5,6]. Both of these methods work without any direct connection to the chromatographic apparatus. More sophisticated software utilizes artificial intelligence. An early example is the EluEx, which can suggest initial experimental conditions based on chemical structures [7–9]. The more recently introduced is the LabExpert software that can plan experiments, collect and evaluate results and adjust chromatographic conditions in real time according to the predefined decision schemes, until a satisfactory separation is achieved [10].

It should be pointed out that most of the method development strategies, as well as many types of chromatography software have been focused on achieving the optimized separation of a complex mixture. This is an important milestone in the development of an HPLC method but in addition to separation there are many other method parameters that also need to be optimized.

This paper describes a simple three-step strategy in HPLC method development and optimization. The first two steps involve preliminary screening of different column/mobile phase combinations and then further mobile phase optimization. These steps are referred to as the separation optimization steps. The third step is the optimization step using a Plackett–Burman design. These designs are very useful when a large number of factors are to be evaluated and they have a built-in ability to detect large main effects. They have been used previously in developing, optimizing, and validating methods [11–15]. One of the major disadvantages is that this type of scheme assumes that all interaction effects are negligible. If in cases those effects cannot be ignored, a full factorial design can be used assuming that the major factors have been identified through the Plackett–Burman design experiments.

2. Materials and methods

2.1. Chemicals and reagents

HPLC grade methanol and acetonitrile were purchased from EM Science (an affiliate of Merck KGaA, Darmstadt, Germany). HPLC grade equivalent water was obtained from an in-house Millipore Milli-Q-Gradient ultrapure water system (Millipore, USA). This study also involves nine proprietary Johnson & Johnson Pharmaceutical Research & Development (J&JPRD) compounds. Three actives are identified as API_1, API_2 and API_3. The other compounds are either process impurities or degradation products of the three actives. Two of them, API_2 and API_3, are mixtures of Syn and Anti structural isomers.

2.2. Instrumentation

A Hewlett-Packard (Palo Alto, CA, USA) 1100 HPLC system equipped with a diode array detector and a Waters (Milford, MA, USA) Alliance HPLC system equipped with a PDA (Models 2695/2996) and a column-switching device were used throughout the study. The Waters Millennium32 software was used to acquire, store, and process the chromatographic data and to report results.

2.3. HPLC columns

The HPLC columns used in this study include a Waters Nova-Pak® C18 column (3.9 mm × 150 mm, 5 μm particle size), a Phenomenex (Torrance, CA, USA) Luna C18 (2) column (4.6 mm × 150 mm, 5 μm particle size), and a Supelco (Bellefonte, PA, USA) Discovery® RP Amide C16 column (4.6 mm × 250 mm, 5 μm particle size).

2.4. Computer software

DryLab, the chromatography optimization software, was purchase from LC Resources Inc. (Walnut Creek, CA, USA). Minitab, the statistical software, was purchased from Minitab Inc. (State College, PA, USA).

2.5. Preparation of solutions

A test mixture containing API_1, API_2, and API_3, as well as the other six impurities, was prepared using 80/20 (v/v) methanol/water as sample solvent. This solution was used in the separation optimization steps.

A standard containing 38 μg/ml of API_1 and 0.3 mg/ml of API_3 was prepared and injected in each injection set for calibration and quantitative

calculations. A second standard solution was prepared by separate weighing. A sensitivity solution containing 0.5% of the nominal concentration relative to the standard was prepared and injected to check sensitivity of the method. In addition, a stressed sample containing API_1 and API_3 was prepared (a tablet form of API_1 and API_2, storage condition: 50 °C/2 months). These solutions were used in the method optimization experiments.

2.6. Chromatographic conditions

All chromatographic runs were performed using a flow rate of 1.0 ml/min, a column temperature of 40 °C, UV detection at 220 nm, and an injection volume of 25 μl, unless otherwise specified.

3. Results and discussion

3.1. Separation optimization step one—multiple-column screening

The screening experiments were conducted using the Waters Alliance HPLC system with a column-switching device, which can be programmed within the Millenium32 to control up to six columns. The HPLC pump has four reservoirs to accommodate four solvents. In this study, three columns were screened with methanol/water and acetonitrile/water as mobile phases. Because the compounds of interest are not ionic, pH-control of mobile phases was not necessary. For each column/mobile phase combination, a 20 min short scouting gradient and a 50 min gradient were run. The collected retention times were uploaded manually into DryLab for analysis. Based on retention data, the software allows computer simulation of separation through simultaneous changes in gradient, column dimensions and flow rate without running additional experiments. Fig. 1 illustrates chromatograms of the test mixture by using the Waters Nova-Pak® C18 column with water/acetonitrile as mobile phase. The chromatograms show that the structural isomers of API_2 were only partially separated, and so were API_1 and API_2 (Fig. 1A). The longer gradient time did not improve the separation (Fig. 1B). The Phenomenex Luna C18 (2) column had similar selectivity for the test mixture and similar results were obtained for both columns with water/methanol mobile phase.

Superior selectivity for the structural isomers of API_2 and API_3 was observed on the Discovery® RP Amide C16 column. Fig. 2 shows the chromatograms of the test mixture on this column using water/acetonitrile as mobile phase. However, DryLab simulation indicated that a total separation of all components still could not be achieved with this column/mobile phase combination. One of the impurity co-eluted with the API_2-Syn peak, which made the condition unacceptable for a regulatory method. Further optimization would be needed. Nevertheless, after the above 12 scouting runs, the Discovery® RP Amide column was identified as the column of choice and the main goal of separating the two pairs of structural isomers was achieved. Also, sharp and symmetrical peaks were observed for all the components in the mixture. Accordingly, it was decided to stop the screening runs and proceeded to the step-two optimization.

It should be pointed out that water/tetrahydrofuran (THF) is another possible mobile phase choice, which sometimes demonstrates better selectivity for structural isomers [16]. However, based on internal policy this mobile phase is avoided due to safety and other concerns.

3.2. Separation optimization step two—gradient optimization

The more components that are present in a sample, the less likely it is that a satisfactory separation condition can be obtained through multiple-column/multiple mobile phase screening alone. Two different approaches can be considered for further optimization. One utilizes multiple organic modifiers in mobile phase, for example, a mixture of methanol and acetonitrile. The other is to modify pH of the mobile phase [17,18]. In this paper, the first approach was adopted since the analytes are neutral.

In the step-two experiments, the Waters Alliance HPLC system was programmed to run both the short and long scouting gradients with a mixture of methanol and acetonitrile as mobile phase B. The methanol to acetonitrile ratio was varied as 25/75, 50/50 and 75/25 (v/v). The best separations were obtained using the 50/50 mixture (Fig. 3). In both runs,

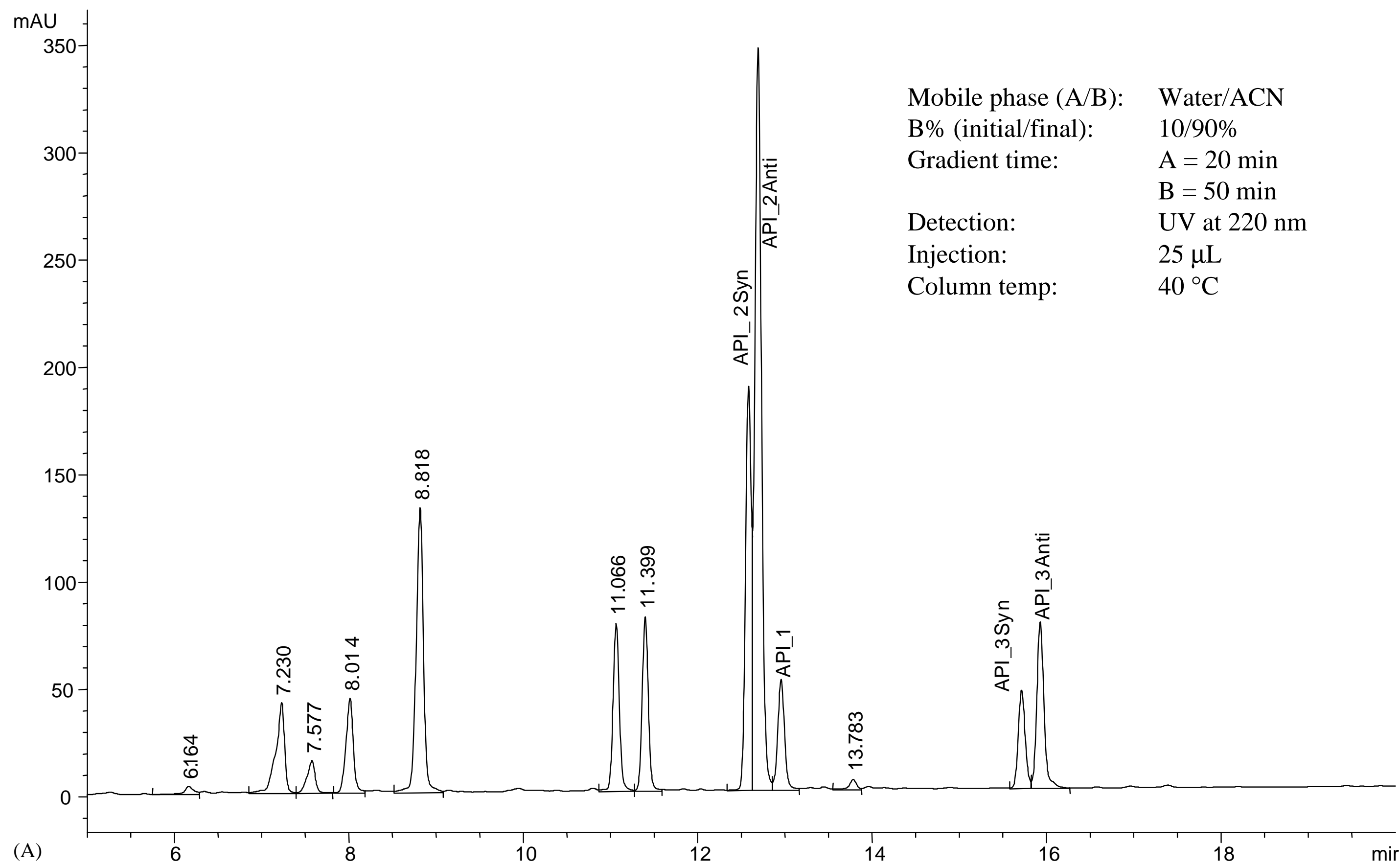

Fig. 1. Separation of the test mixture on a Waters Nova-Pak® C18 column.

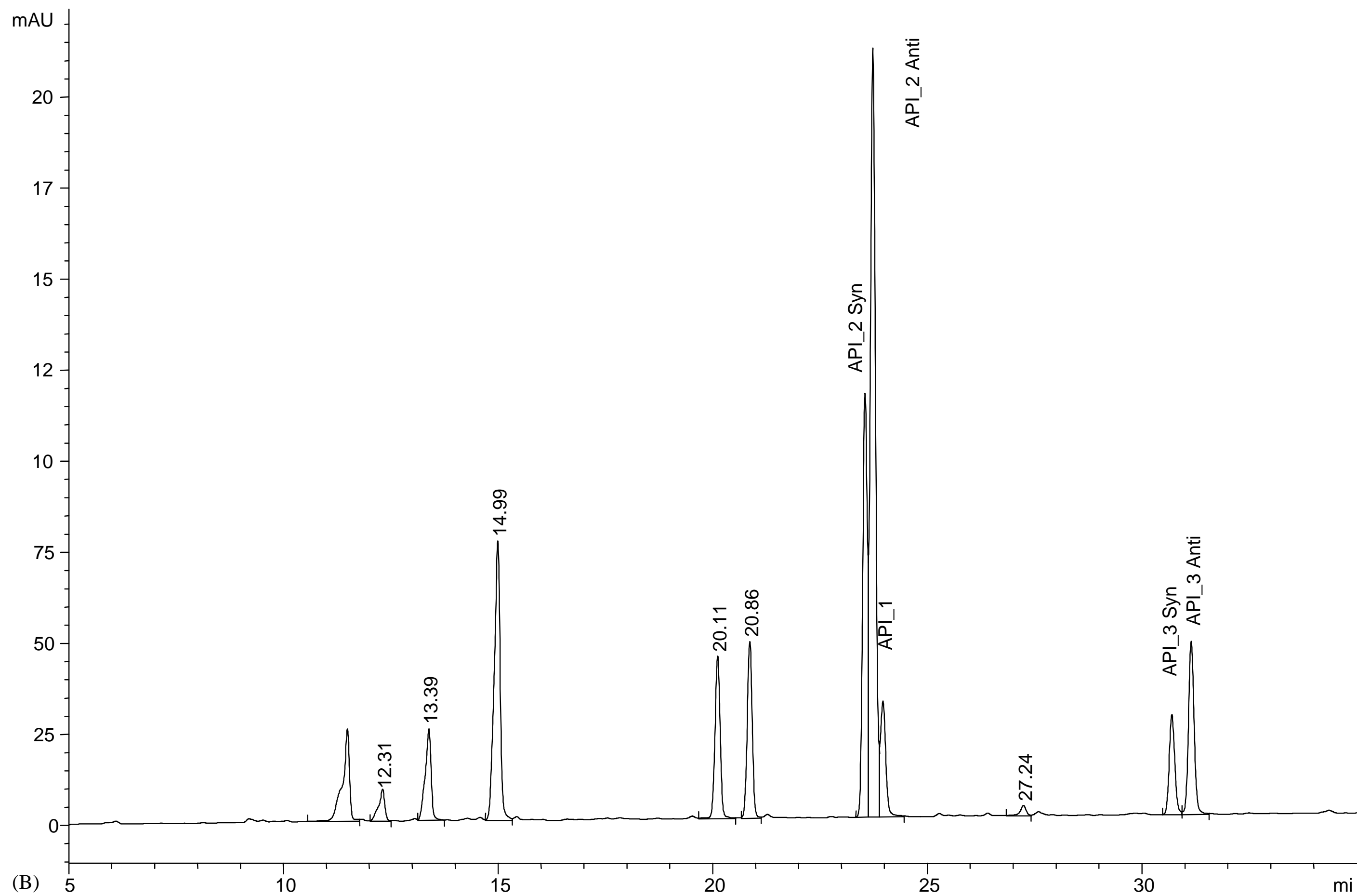

Fig. 1. (*Continued*).

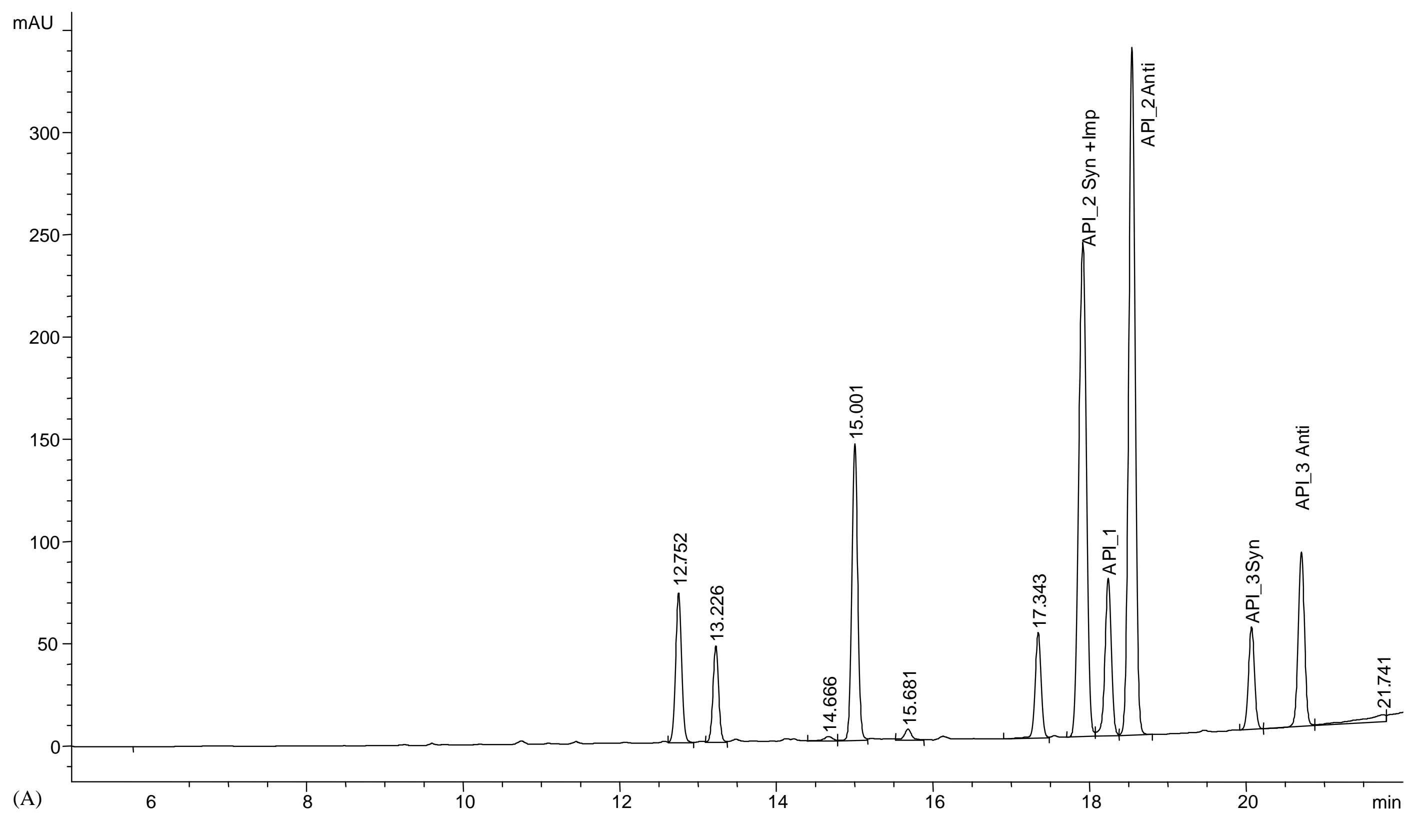

Fig. 2. Separation of the test mixture on a Supelco Discovery® RP Amide C16 column (HPLC conditions: same as Fig. 1).

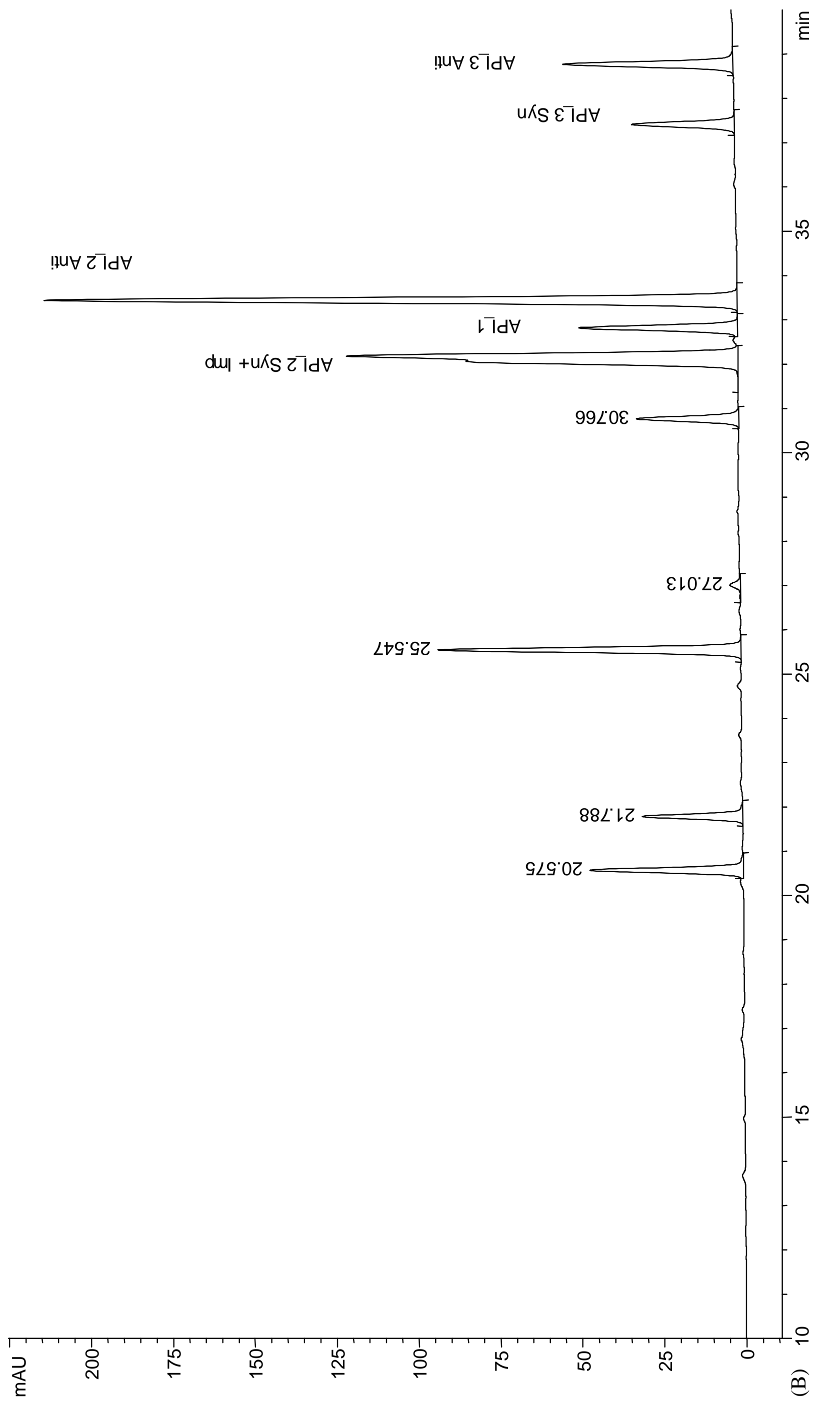

Fig. 2. (*Continued*).

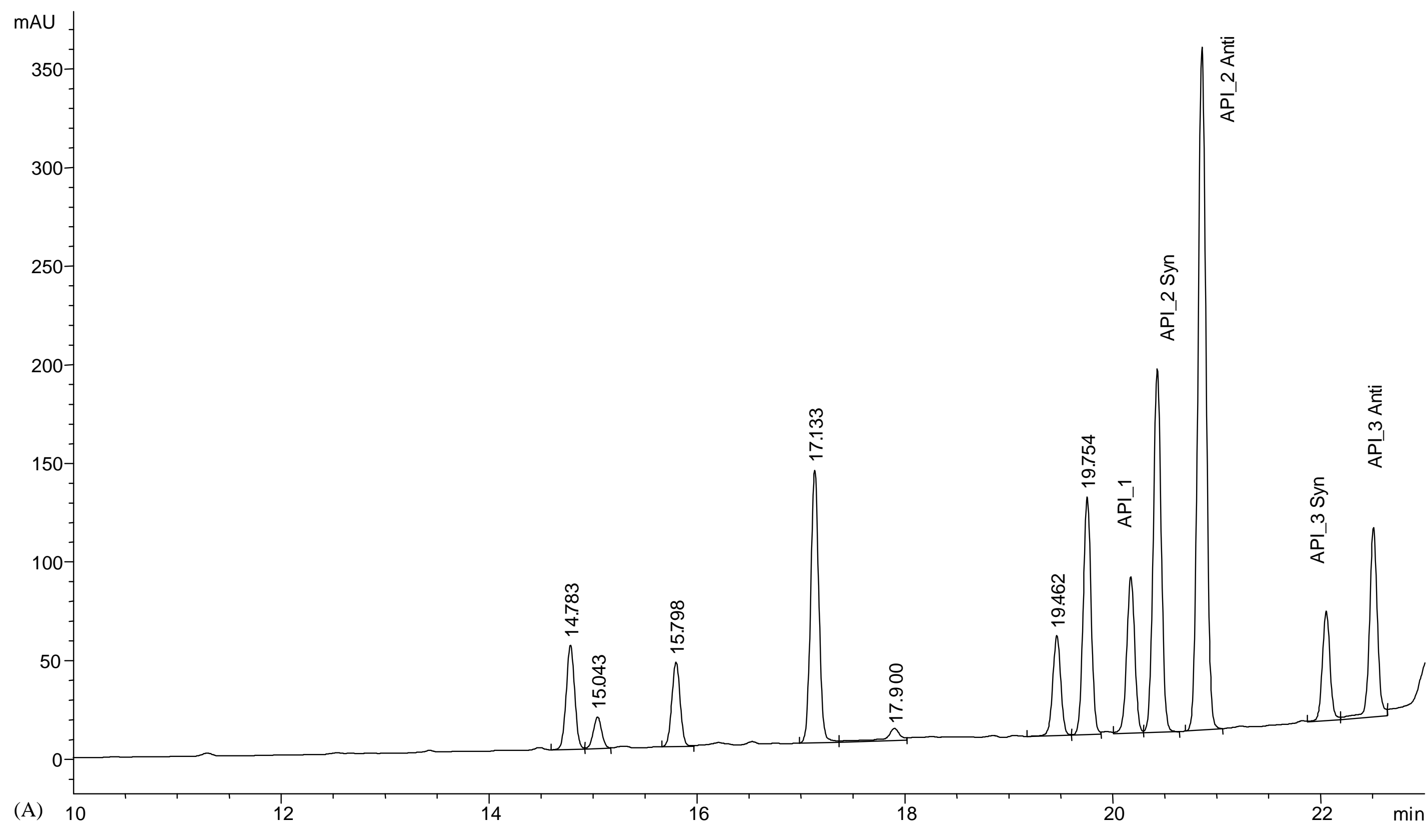

Fig. 3. Separation of the test mixture on a Supelco Discovery® RP Amide C16 column (mobile phase B: methanol/acetonitirle 50/50 (v/v); other conditions same as Fig. 1).

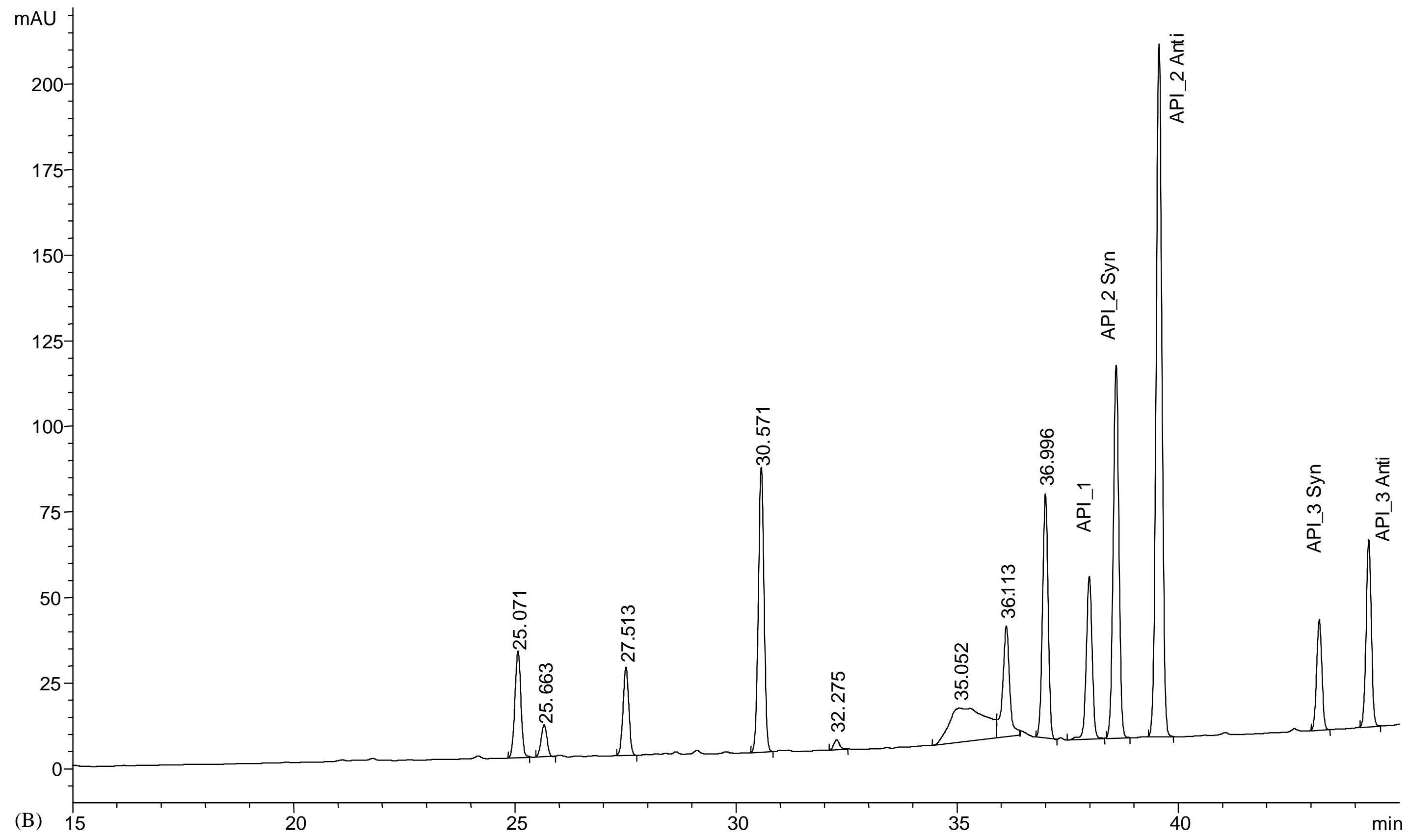

Fig. 3. (*Continued*).

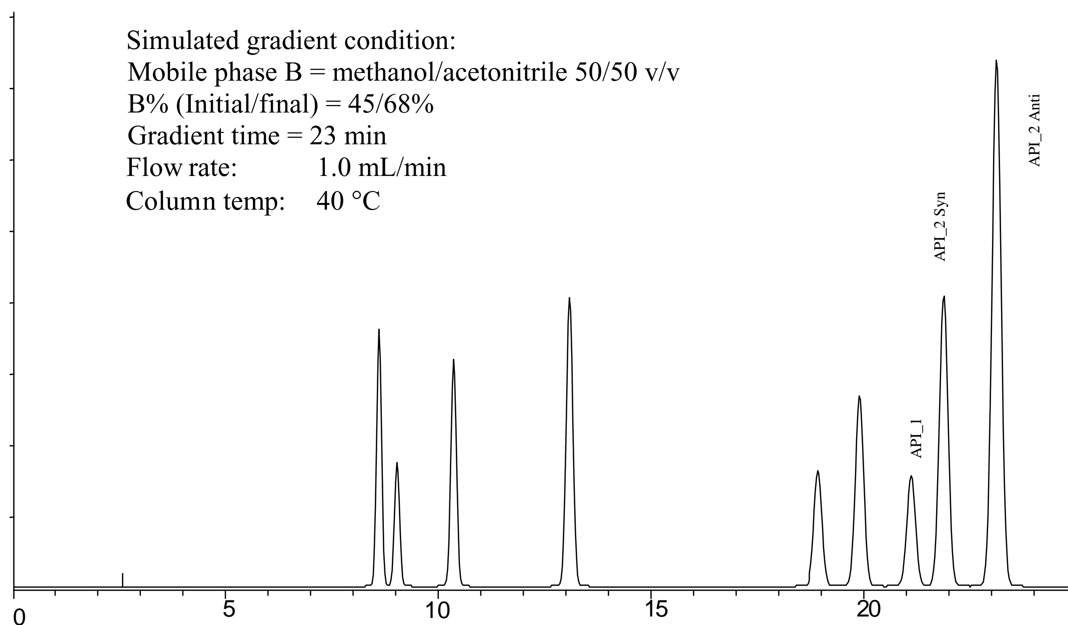

Fig. 4. Computer simulation of separation based on retention data in Fig. 3.

all the components, including the structural isomers, were well separated. The retention data was uploaded into DryLab for computer simulation. By adjusting the gradient profile within the software, a simulated chromatogram, illustrated in Fig. 4, was obtained. Based on the conditions from the simulation, a confirmation run was made, generating the chromatogram in Fig. 5. The separation conditions were deemed suitable for further optimization and validation.

3.3. Method optimization step three—factor optimization using Plackett–Burmann design

To this point, it has been demonstrated that a complicated separation problem can be solved by multiple-column screening and additional mobile phase optimization. However, additional critical parameters/procedures, such as column temperature, detection wavelength, sample solvent, injection volume, and sample preparation procedure, etc. must also be chosen prior to validation. The choice of these parameters/procedures is frequently based on trial-and-error experiments and can become a time-consuming process. More importantly, this approach may lead to non-optimized methods. The use of Plackett–Burman experimental designs allows for the study of multiple method factors in a systematic and logical way and leads to the identification of optimized conditions.

To apply this approach to the separation of API_1 and API_3 and the related impurities and degradation products, nine factors were selected for the designed experiments (Table 1). A relatively wide range of experimental conditions was chosen for each of the factors. In addition, a center point was added for the factors that can have numerical values. Based on these factors, a plan for the 12-injection set (plus 4 pseudo-center) Plackett–Burman design was generated using Minitab (Table 2). The advantage of this design is that it allows a quick screening of all the factors through limited number of experiments. The weakness of the design is that replicates of design points are not included to permit the calculation of error terms.

As an output of this experiment, it is desirable to examine the fundamental attributes of the method, including precision, accuracy, sensitivity, and specificity, as well as an evaluation of peak tailing, column efficiency, resolution of critical pairs, baseline noise, and baseline shifting. To achieve these goals, the samples were injected in following sequence: (1) five injections

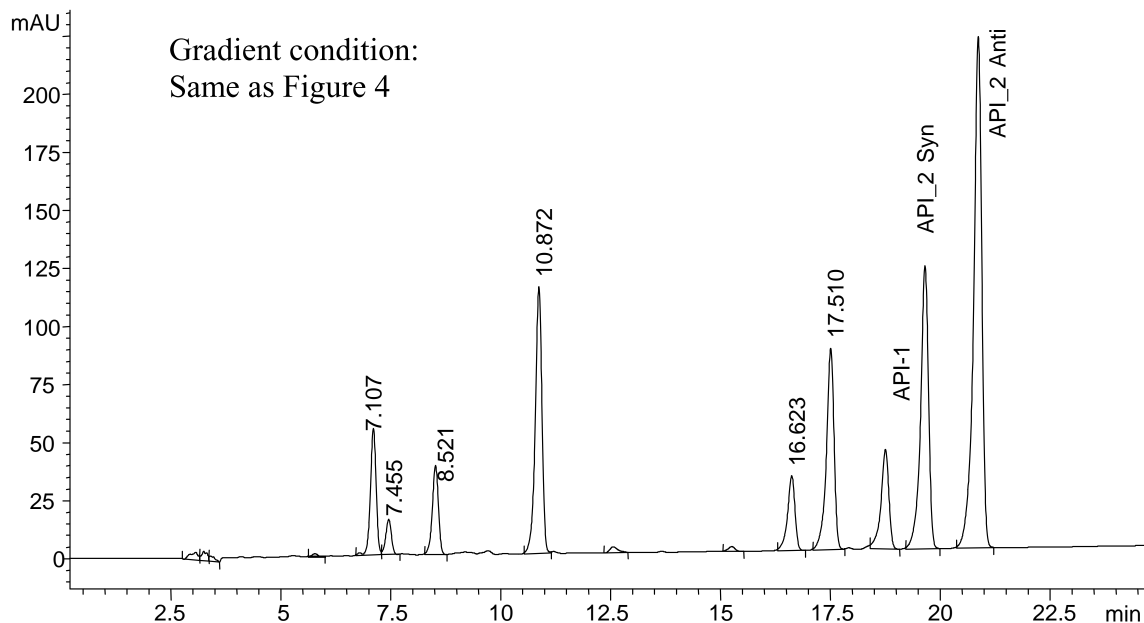

Fig. 5. Separation of a test mixture on a Supelco Discovery® RP Amide C16 column.

of standard one; (2) one injection of standard two; (3) the sensitivity solution; (4) the test mixture; (5) the stressed sample; and (6) another injection of standard one. In each of these injection sets, a total of 10 injections were made. Results from the experiments are shown in Table 3.

3.3.1. Precision

R.S.D.% of the peak areas from five injections of the same standard solution is presented in Table 3 (Responses A–C for the three major peaks, respectively). In 10 out of the 16-injection sets, the R.S.D.% for all three major peaks was less than 0.3%. For the other six sets, the R.S.D.% was between 0.05 and 1.63%. A very interesting conclusion can be made from these results. We assume the measured standard deviation (σ_M) is the sum of individual standard deviations related to independent chromatographic factors and is expressed by the equation:

$$(\sigma_M)^2 = (\sigma_{Inj})^2 + \sum(\sigma_i)^2$$

where σ_{Inj} is the contribution from the injector and σ_i represents each factor other than σ_{Inj}, then for the 10

Table 1
The factors examined in the method optimization and their levels

Factor	Factor ID	Low	Middle	High
Column temperature (°C)	A	30	40	50
Injection volume (μl)	B	40	50	60
Methanol in mobile phase (%) (v/v)	C	40	50	60
Initial gradient organic total (%)	D	40	45	50
Gradient slope (min)	E	15	20	25
Detection wavelength (nm)	F	210	220	230
Columns of different lots	G	Column no. 1		Column no. 2
Different instrument	H	HP		Waters
Methanol in sample solvent (%)	I	60	70	80

Table 2
List of experiments in the Plackett–Burman experimental design for method optimization

Run order	Factor ID								
	A	B	C	D	E	F	G	H	I
	Column temperature (°C)	Injection volume (μl)	MeOH in mobile phase	Initial gradient (B%)	Gradient slope (min)	Wave-length (nm)	Column	Instrument	MeOH in sample solvent
1	50	60	40	50	25	210	1	HP	60
2	40	50	50	45	20	220	1	HP	70
3	30	60	60	50	15	230	1	HP	80
4	30	60	40	40	15	230	1	Waters	60
5	40	50	50	45	20	220	1	Waters	70
6	30	40	40	40	15	210	2	HP	60
7	50	40	60	40	15	210	1	Waters	80
8	30	40	60	50	25	210	1	Waters	60
9	50	40	40	40	25	230	1	HP	80
10	40	50	50	45	20	220	2	Waters	70
11	40	50	50	45	20	220	2	HP	70
12	50	60	40	50	15	210	2	Waters	80
13	30	60	60	40	25	210	2	HP	80
14	50	60	60	40	25	230	2	Waters	60
15	50	40	60	50	15	230	2	HP	60
16	30	40	40	50	25	230	2	Waters	80

sets that had low R.S.D.%, it can be concluded that $\sigma_{\text{Inj}} \leq \sigma_{\text{M}}$ and the other method factors did not significantly contribute to the measured standard deviation. For the sets that had larger R.S.D.%, the other factors became dominant, assuming that σ_{Inj} is a constant. In other words, the precision of the method depends upon two major factors, the mechanical and electronical design of the injector and selection of the separation/

Table 3
List of responses from the Plackett–Burman design experiments

Run order	Response ID										
	A	B	C	D	E	F	G	H	I	J	K
	R.S.D. API_1	R.S.D. API_3-Syn	R.S.D. API_3-Anti	Baseline slope	*S/N* API_1	*S/N* API_3-Syn	*S/N* API_3-Anti	Assay API_1	Separation	Tailing	Recovery API_1
1	0.06	0.14	0.08	1.28	84	62	86	107.5	Y	1.05	100.0
2	0.09	0.07	0.07	0.39	75	229	400	105.9	Y	1.03	100.3
3	0.31	0.05	0.05	0.27	56	444	923	107.0	Y	1.01	101.2
4	0.36	0.57	0.80	0.47	148	1036	2007	107.0	N	1.01	101.0
5	0.13	0.14	0.15	0.99	144	466	789	107.0	Y	0.97	100.5
6	0.13	0.04	0.10	2.18	139	114	176	106.6	N	0.97	100.1
7	0.09	0.19	0.07	5.88	229	212	328	107.2	Y	0.96	100.3
8	0.18	0.21	0.85	2.96	147	148	215	108.0	Y	0.98	100.1
9	0.12	0.09	0.05	0.12	21	153	298	114.5	Y	1.03	100.1
10	0.12	0.10	0.08	0.98	101	376	600	108.5	Y	0.97	100.3
11	0.08	0.14	0.09	0.47	71	225	384	107.9	Y	0.97	100.5
12	1.63	0.93	1.16	4.26	0	277	287	107.1	N	0.96	99.7
13	0.03	0.09	0.07	1.66	135	123	173	107.0	Y	0.97	100.3
14	0.23	0.81	0.77	0.49	100	704	1202	111.9	Y	0.96	100.5
15	0.06	0.20	0.03	0.27	44	313	617	106.3	Y	1.00	100.2
16	0.06	0.09	0.10	0.30	17	123	243	112.4	N	0.98	100.2

method conditions. Based on the data, it is shown that injectors are capable of injecting sample with very high precision (R.S.D.% less than 0.3%). However, the correct chromatographic conditions must be developed to fully achieve this precision.

3.3.2. Accuracy

Accuracy of the method was estimated by calculating the recovery of the second standard. The results (Response K in Table 3) show that recovery was not affected by the factors within the tested range.

3.3.3. Sensitivity

To evaluate sensitivity of the method, a solution containing 0.5% of the actives relative to the standard solution was prepared and injected. The data were collected as signal-to-noise ratios for the peaks and listed in Table 3 (Responses E–G). Large variation of the data is due to the fact that API_1 has stronger UV absorbance at 210 nm, whereas for API_3 the maximum absorbance is at 230 nm. Another factor is methanol as a modifier in mobile phase. Although methanol has an UV cut-off at 205 nm, baseline shifting should be expected in the 210–230 nm wavelength ranges due to its large quantity in mobile phase. Main effect plots were generated by Minitab for these responses (Figs. 5 and 6). None of the factors has shown a dominant effect on sensitivity. But these plots show interesting trends. For example, an increase in initial organic solvent percentage in the mobile phase will result a decrease in signal-to-noise (Factor D). A longer gradient time will also result in a decrease in signal-to-noise.

In addition to signal-to-noise, another factor that can significantly affect impurity detection and quantitation is baseline shifting. Baseline slope is defined as:

$$\text{Baseline slope} = (B_e - B_b)/Gt$$

where B_e = baseline reading at the end of gradient
B_b = baseline reading at the beginning of gradient
Gt = gradient time

and has a unit of mV/min. When the value is between 0 and 1 mV/min, the baseline shifting will not significantly affect the integration of low-level impurity peaks. When it is >1, the higher the baseline slope value, the more difficult it becomes for the chromatographic data system to perform automatic integration of peaks. Main effect plots revealed that Factor F, wavelength, was the dominant factor that affected the baseline slope.

3.3.4. Specificity

Method specificity was evaluated against the separation of known impurities and degradation products, as well as unknown impurities. Response I in Table 3 is a summary of all the separations obtained in the 16-injection set experiments. A “yes” (Y) indicates all the peaks were separated from each other in that set while a “no” (N) means some peaks co-eluted. In 12 out of the total 16 sets, all the known components were separated from each other. In addition to known impurities, unknown impurities in the stressed sample were evaluated. Response H in Table 3 represents the assay results for API_1 in the stressed tablet sample (storage condition: 50 °C/2 months). In three of the injection sets, the assay values were significantly higher than the average. Examination of the chromatograms shows that there was an unknown impurity co-eluting with the major peak. This finding implies that the experimental design approach may be another way to check method specificity, which is usually verified using peak purity analysis or an orthogonal method.

3.3.5. Selection of optimized condition

After the above discussions, the selection of optimized method condition becomes relatively easy. In Table 2, each injection set corresponds to a set of method conditions. In Table 3, each set of responses corresponds to a specific injection set. Therefore, the optimized method condition can be chosen based on the responses that meet the requirements. For example, if the criteria is set as follows: method precision: R.S.D.% <0.3%; sensitivity: *S/N* >50; baseline slope <1; and specificity: known and unknown peaks separated; the conditions that meet these requirements are achieved in injection sets 2, 3, 11, and 15. Among these sets, sets 2 and 11 have the same method condition except that two different columns were used.

Method conditions can also be optimized using the Response Optimizer, a function within the Minitab software, which will help identify the combination of factor settings that jointly optimize a single response or a set of responses. An example is given in Table 4, which contains the target values for five responses. The

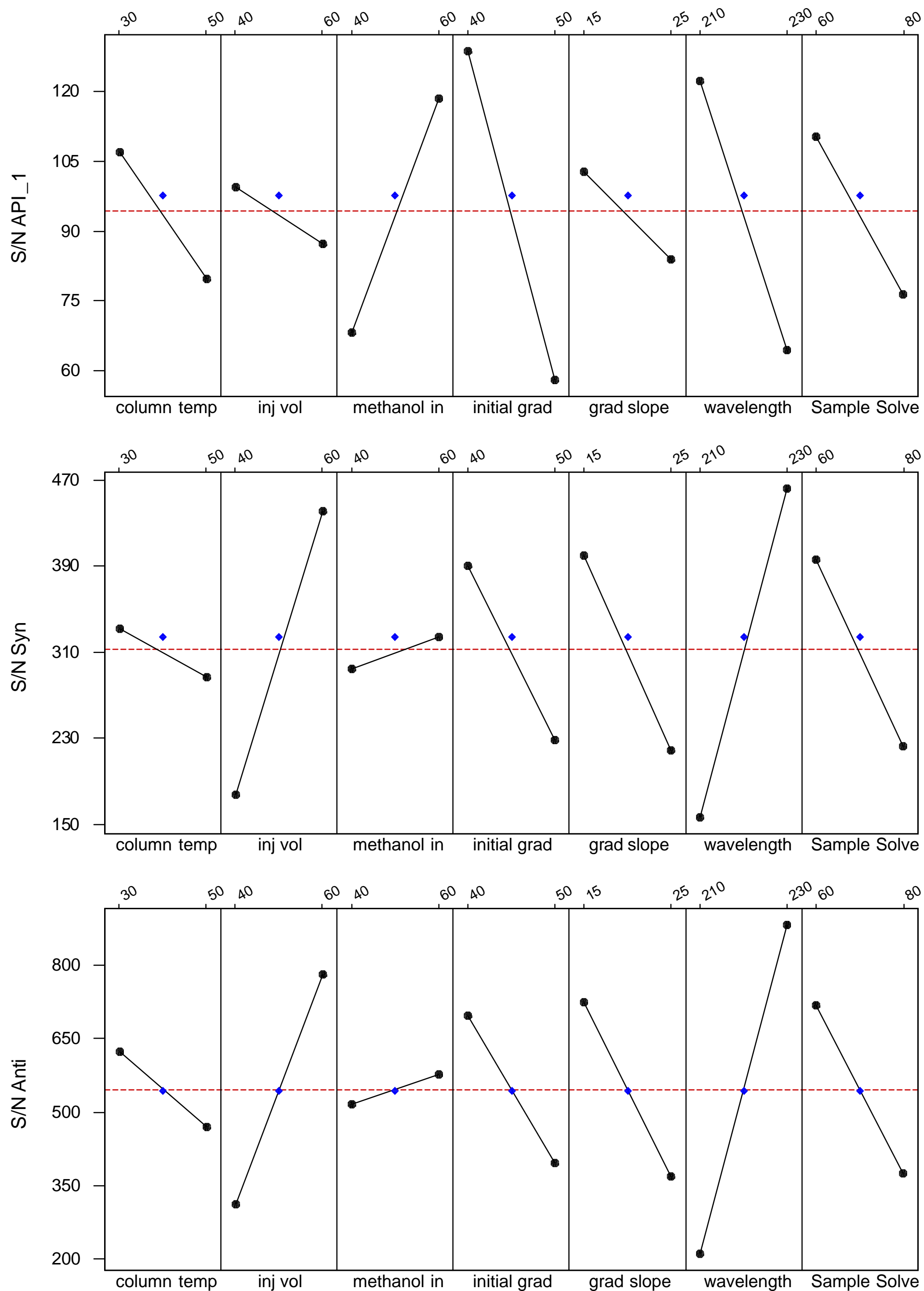

Fig. 6. Main effect plots.

Table 4
Factors and targeted criteria used in Minitab optimization

Response	Response ID	Goal	Lower	Target	Upper	Weight
R.S.D. API_1	A	Target	0.1	0.3	1	1
R.S.D. API_3-Anti	C	Target	0.1	0.3	1	1
Baseline slope	D	Target	0.3	0.5	0.7	1
S/N API_1	E	Target	100	200	300	1
S/N API_3-Anti	G	Target	200	300	400	1

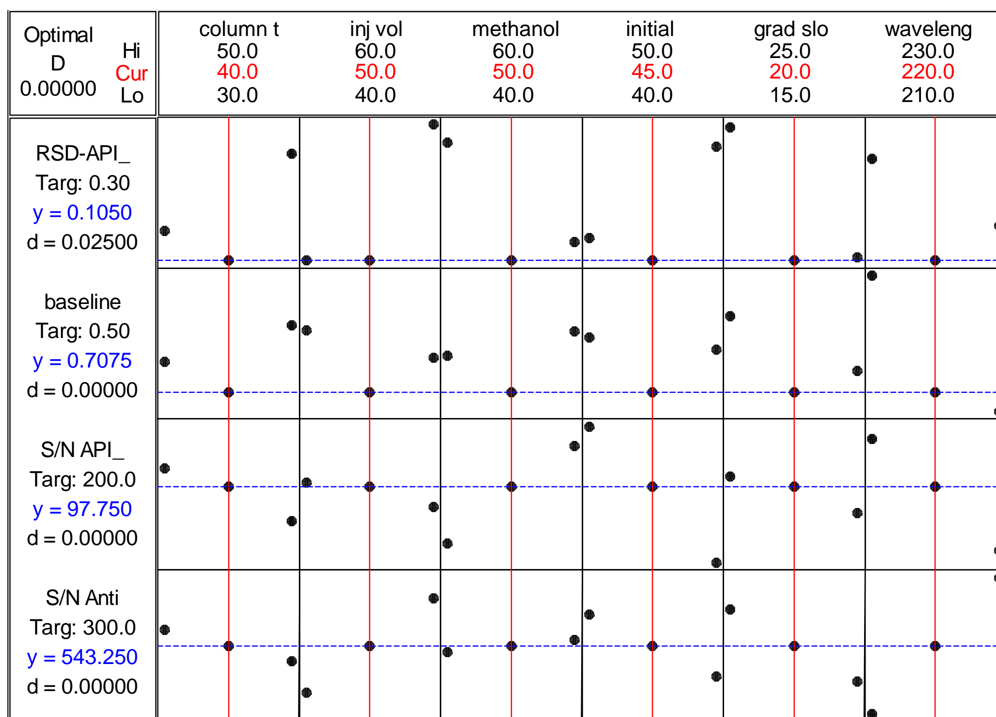

Fig. 7. Factor optimization plots generated using Minitab.

Response Optimizer generated the factor optimization plots (Fig. 7) along with the optimized method condition, which is the same as that of sets 2 and 11. It was not surprising that both optimizing approaches reached the same method condition.

4. Conclusion

A three-step strategy for method development and optimization has been proposed. Three columns were screened with two mobile phases to lead to the rapid identification of a promising separation condition for nine compounds, two of them structural isomers. Subsequent optimization of the separation conditions was done by using multiple organic modifiers. An alternative is to modify mobile phase pH to achieve different selectivity. Final optimization was achieved using a Plackett–Burman design and evaluating nine factors through 16-injection sets. Graphical methods were used to analyze the results by generating the main effect plots. Optimized method conditions were obtained

by analyzing the response data and with the help of the Response Optimizer.

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