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A chemometric study: Automated flow injection analysis method for the quantitative determination of humic acid in Ilgın lignite

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Abstract A rapid, sensitive and provident flow injection analysis (FIA) method was developed within the framework of a chemometric approach for the quantification of humic acid (HA) in the lignite obtained from Ilgın, Konya, Turkey. The proposed method allows automatic determination of 60 samples per hour over a wide calibration range (0–2000 mg L⁻¹, R²: 0.9988) and needs only 10 µL of sample at a flow rate of mobile phase (X₁), 2 mL min⁻¹; pH of mobile phase (X₂), 8, and system temperature (X₃), 20 °C. The limits of detection (LOD) and quantification (LOQ) were calculated as 9.18 mg L⁻¹ and 30.60 mg L⁻¹, respectively, and the relative standard deviation (RSD) for 500 mg L⁻¹ HA was calculated as 3.44 (n: 9). It was revealed that the standard deviation (SD) values of the proposed FIA method are lower than those of the spectrophotometric method.

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1. Introduction

Turkey has substantial reserves of lignite; because of this, the Turkish energy system is based on lignite exploitation. However, high ash and high sulfur content limit the use of these coals for economical practice and in power generation cause serious environmental problems (Akgun et al., 1989;

Karaca et al., 1997; Lin et al., 1997). For these reasons, great interest has grown in the possible alternative use of lignites as a source of soil amendments in order to maintain and increase the content of natural organic matter (NOM) of the soil (Schobert, 1995; Peuravuori et al., 2006).

Humic substances (HSs) are the most dominant fraction of NOM in the soil. HSs are a series of different molecular weight, light-brown to black-colored complexes and heterogeneous organic polymers formed by secondary synthesis reactions (Stevenson, 1982). These substances can be classified into three main fractions based on their solubilities in alkaline and acidic extraction solutions. Humic acid (HA) fraction is soluble in alkaline solutions but insoluble in acidic solutions; fulvic acid (FA) fraction is soluble in both alkaline and acidic solutions; while humin is insoluble in both solutions (Stevenson, 1982; Schnitzer, 1982). Among these, the

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predominant fraction is HA, which is very active in interacting with organic and inorganic chemicals as compared to other fractions (Kishi, 1988; Senesi, 1993). This substance plays an important role in soil conservation, for water-holding capacity, and for the complexation of metals in terrestrial and aquatic systems (Swift, 1996; Hayes and Malcolm, 2001; Hayes and Graham, 2000).

Lignite that is another soil type is usually used for the production of HA which is in the form of alkali-soluble humate salts (Peuravuori et al., 2006). The characteristics (size, chemical composition and functional groups) may differ considerably, depending on the origin and age of the material (Stevenson, 1982; Fong and Mohamed, 2007). Because of these properties, determination of HA in lignite has great

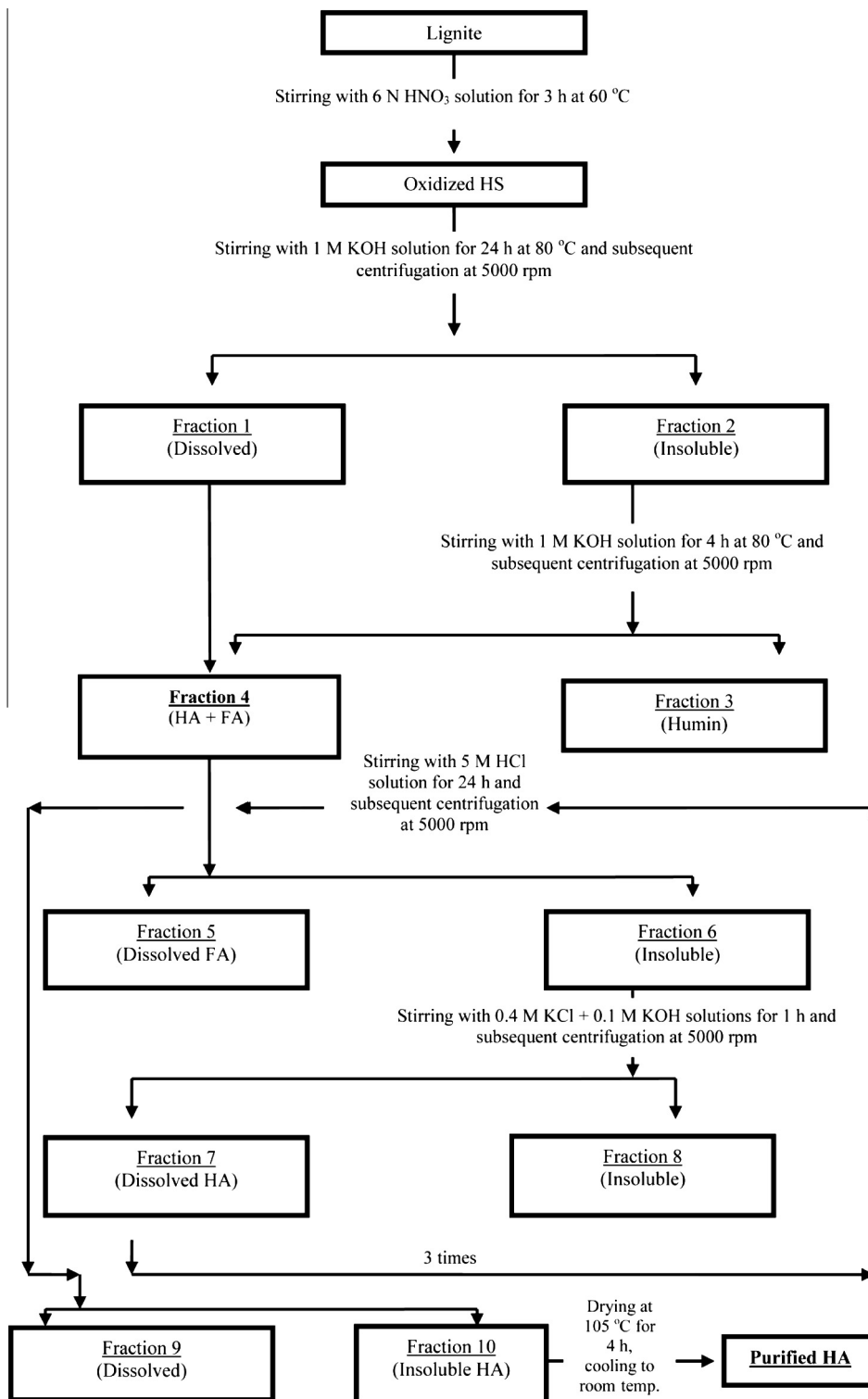


Figure 1 Extraction and purification procedure of lignite samples (Tarhan, 2011).

significance for conversion into products suitable for use in agriculture.

The colorimetric method suggested by Mehlich (19840) is currently used to estimate the HA and HA derivatives (i.e., extracts of humates). The method is based on the solubility of HA in dilute alkaline solutions and takes additional advantage of its precipitation when alkaline extracts are acidified. The method attempts to estimate the quantity of HS by comparing the intensity of sample color to the intensity of color produced by the extract of a standard HA (Lamar and Talbot, 2009). However, this method is not used for the determination of HA in lignite and is not applied in automated systems (e.g., FIA, HPLC). Because of this, to stimulate marketing and admittance of HA in lignite, it is important to have a rapid and reliable method to determine the organic matter of lignite.

This paper describes an automated FIA system for specific quantification of HA in lignite by modifying HPLC. In this way, a quantitative method having advantages of HPLC and FIA systems (high repeatability, accuracy, applicability, and rapidity) was created and the system parameters, which are X_1 , X_2 , and X_3 , were optimized. These optimizations were carried out within the framework of chemometric approaches by response surface methodology.

2. Materials and methods

2.1. Samples and reagents

All chemicals and solvents used were of analytical or HPLC grade and were obtained from Merck® (Darmstadt, Germany) and Sigma–Aldrich® (St. Louis, MO) and were used without further purification. Deionized water (DI) was used to prepare all solutions. The three lignite samples, L_1 , L_2 , and L_3 , were obtained from Turkey Coal Enterprises, Ilgin, Konya. Standard HA used for optimization of the proposed FIA method was isolated from L_1 . Stock solution (2000 mg L^{-1}) of this standard was dissolved daily in 1000 mL of $1.10^{-8} \text{ mol L}^{-1}$ KOH with the use of an ultrasonic bath, and was used to prepare other standard HA solutions (ranging from 0 to 2000 mg L^{-1}).

2.2. Colorimetric method

HSs may vary greatly in molecular-weight distribution, degree of condensation, carbon content, and degree of aromaticity to aliphaticity either obtained from the same or different sources (Peuravuori et al., 2006). Thus, the accuracy of the colorimetric technique can be developed by creating standards for each source of raw humate or HA derivative (Lamar and Talbot, 2009). The technique can also be made more accurate if the FA and other compounds spectrally active at the wavelength employed were removed prior to analysis so that only HA remained in the extraction solution. This could be easily done by acidifying the extraction solution, separating the precipitated HA by centrifugation, and redissolving it in new prepared extraction solution prior to colorimetry.

2.3. Extraction and purification procedure of HA

The HA fraction was isolated from lignite using the pre-oxidation alkaline extraction method and was purified

according to the method schemed in Fig. 1 (Tarhan, 2011). Air-dried and 0.5-mm sieved lignite samples were extracted by solution 6 N HNO_3 and 1 M KOH using a sample extractant ratio of 1:10. Mixtures were shaken mechanically under N_2 gas in capped plastic bottles for 24 h at 80°C . The alkaline supernatant solution was then separated from the residues by centrifugation at 5000 rpm for 30 min. The extraction procedure was repeated two times on the residues, which were finally discarded. The combined alkaline supernatants (HA + FA) were acidified with 5 M HCl to pH 1, allowed to stand for 24 h in a refrigerator to permit coagulation of the HA fraction, and then centrifuged at 5000 rpm for 30 min. The HA precipitates were purified by dissolving them in a minimal volume of 0.4 M KCl and 0.1 M KOH under N_2 gas, centrifugation at 5000 rpm for 30 min to remove the residues, and acidification of the alkaline supernatants with 5 M HCl to pH 1. Suspensions were left for 24 h at room temperature (RT), and then centrifuged at 5000 rpm for 30 min. The purification steps were repeated three times. The precipitated HA fractions were then recovered with distilled water, dialyzed using a membrane made from natural cellulose having a molecular weight cutoff of 12,000–14,000 Da until free of Cl^- ions, dried at 105°C , and stored at RT in plastic vials placed in a desiccator containing P_2O_5 .

2.4. Flow injection analysis (FIA)

In order to conduct FIA procedures, the Agilent 1200 HPLC system was modified by removing the analytical column. The FIA system, shown schematically in Fig. 2, consisted of a quaternary pump with degasser, thermostated column compartment, diode array detector (DAD), and auto sampler. The DAD was operated at 410 nm, and the detector response was recorded on a personal computer with a Chemstation data processor (Ver. B.03.02).

HA solutions before injection were filtered with the use of $0.45 \mu\text{m}$ nylon syringe filter and injected into the FIA system. The volume of injection was $10 \mu\text{L}$. The standard HA solutions used for optimization were injected into the system for each experimental condition so that parameters of the proposed system be optimized. After the optimization, HAs isolated from L_2 , and L_3 , were dissolved in $1.10^{-8} \text{ mol L}^{-1}$ KOH solution and were injected into the system. The HA content was estimated on the basis of the absorbance at 410 nm.

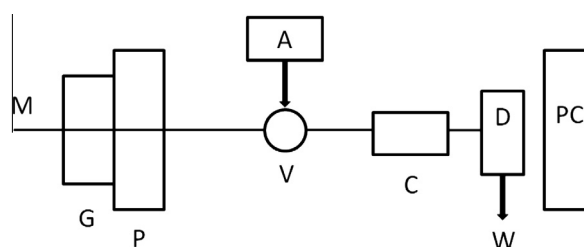


Figure 2 The FIA system used for determination of HA in lignite: M: mobile phase, G: degasser, P: quaternary pump, A: autosampler, V: injection valve, C: column oven, D: DAD detector, W: waste, PC: personal computer.

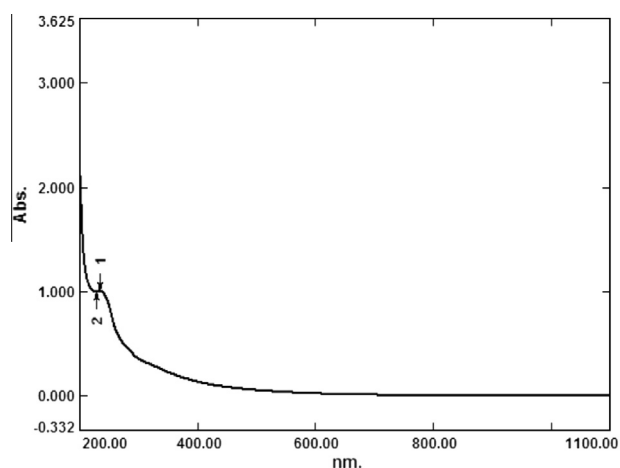


Figure 3 The complete UV-Vis spectrum of the specific standard HA isolated from L₁.

2.5. UV-Vis spectroscopic analysis

In order to evaluate results of the proposed FIA method, measurements of the lignite samples were performed on a Lambda 25 model Perkin Elmer double beam spectrophotometer using 1 cm quartz cells (Schnitzer and Khan, 1972). The complete UV-Vis spectrum of the specific standard HA isolated from L₁ can be seen in Fig. 3.

2.6. Statistical analysis

Optimization has been carried out by monitoring the influence of one factor at a time on an experimental study using a

technique called one variable at a time. Its few disadvantages are that it does not include the interactive effects among the variables or the increase in the number of experiments, which leads to an increase of time, expenses, reagents and materials (Bezerra et al., 2008). For this reason, the optimization of the proposed FIA method has been carried out in this study using multivariate statistical techniques.

One of the most relevant multivariate techniques used in analytical optimization is response surface methodology (RSM). RSM is a collection of mathematical and statistical disciplines based on the fit of a polynomial model to the experimental data, which must describe the behavior of a data set with the objective of making statistical predictions. In this study, the RSM has been used in order to optimize the parameters to attain the best system performance simultaneously.

The normal distribution plot (NDP) is a fast and simple technique to get a symptom very quickly if any of the estimated variables are diverging significantly from the normal distribution. If a variable has a large deviation from the normal distribution, it probably exhibits the most effect when compared with other variables (Box et al., 1978; Montgomery, 1984; Davies, 1956).

There are three types of polynomial models. These are linear, second order interaction, and quadratic models (Lundstedt et al., 1998). RSM and NDP are suitable for quadratic models, as they require fewer data and provide interaction effects on the response in addition to variable effects compared to the classical methods (Mehlich, 1984; Lundstedt et al., 1998).

In order to optimize parameters of the proposed FIA method, a calibration design set was used (Brereton, 1997). The difference vector [0231] and cyclical generator -2, 1, 2,

Table 1 The variable values used in the RSM design and their respective levels used in the NDP with FFD (X_1 : flow rate of mobile phase, mL min⁻¹; X_2 : pH of mobile phase; X_3 : the system temperature, °C; Y : response R^2).

| Experiment No. | Values and levels of three variables | | | | | | Y |
|----------------|--------------------------------------|-------|-------|-------|-------|-------|--------|
| | X_1 | Level | X_2 | Level | X_3 | Level | |
| 1 | 1 | 0 | 8 | -2 | 20 | -2 | 0.9480 |
| 2 | 0.75 | -1 | 8 | -2 | 25 | -1 | 0.5316 |
| 3 | 1.5 | 1 | 8 | -2 | 30 | 0 | 0.9985 |
| 4 | 2 | 2 | 8 | -2 | 35 | 1 | 0.9988 |
| 5 | 0.5 | -2 | 8 | -2 | 40 | 2 | 0.6219 |
| 6 | 1.5 | 1 | 9 | -1 | 20 | -2 | 0.9956 |
| 7 | 1 | 0 | 9 | -1 | 25 | -1 | 0.8554 |
| 8 | 0.5 | -2 | 9 | -1 | 30 | 0 | 0.8898 |
| 9 | 0.75 | -1 | 9 | -1 | 35 | 1 | 0.5105 |
| 10 | 2 | 2 | 9 | -1 | 40 | 2 | 0.9981 |
| 11 | 0.75 | -1 | 10 | 0 | 20 | -2 | 0.6387 |
| 12 | 2 | 2 | 10 | 0 | 25 | -1 | 0.9989 |
| 13 | 1 | 0 | 10 | 0 | 30 | 0 | 0.6635 |
| 14 | 0.5 | -2 | 10 | 0 | 35 | 1 | 0.7572 |
| 15 | 1.5 | 1 | 10 | 0 | 40 | 2 | 0.9988 |
| 16 | 0.5 | -2 | 10.5 | 1 | 20 | -2 | 0.5661 |
| 17 | 1.5 | 1 | 10.5 | 1 | 25 | -1 | 0.9983 |
| 18 | 2 | 2 | 10.5 | 1 | 30 | 0 | 0.9986 |
| 19 | 1 | 0 | 10.5 | 1 | 35 | 1 | 0.7729 |
| 20 | 0.75 | -1 | 10.5 | 1 | 40 | 2 | 0.8439 |
| 21 | 2 | 2 | 11 | 2 | 20 | -2 | 0.9986 |
| 22 | 0.5 | -2 | 11 | 2 | 25 | -1 | 0.5872 |
| 23 | 0.75 | -1 | 11 | 2 | 30 | 0 | 0.1101 |
| 24 | 1.5 | 1 | 11 | 2 | 35 | 1 | 0.9987 |
| 25 | 1 | 0 | 11 | 2 | 40 | 2 | 0.6540 |

Table 2 The values of NDP.

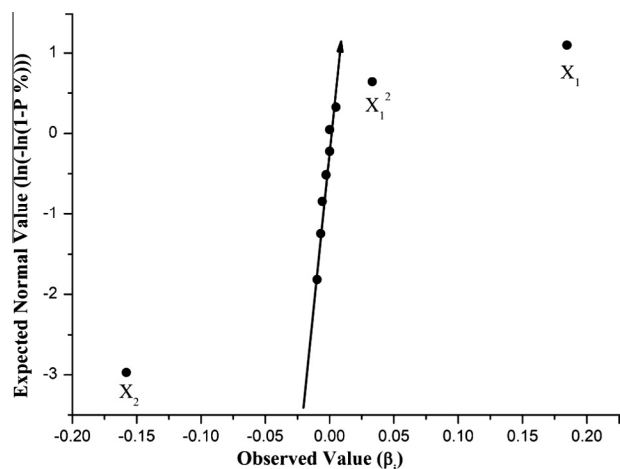
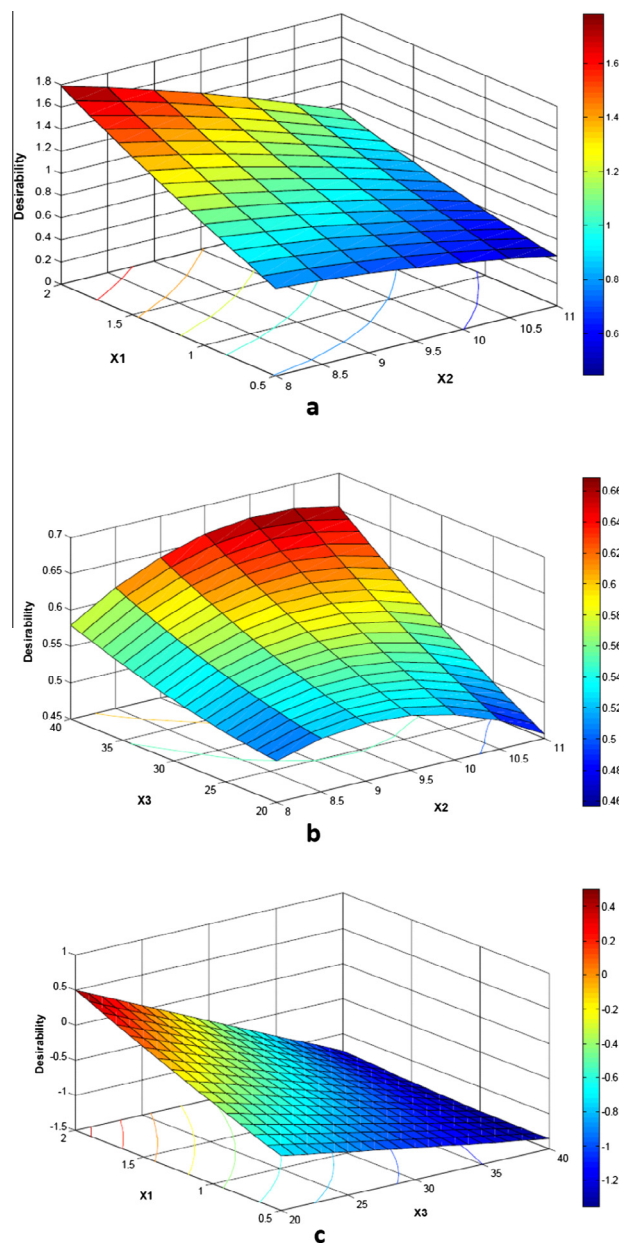
| Rank (from small to big) | β_i (from the above equation) | $P\%$ ((Rank - 0.5)/10) | $\ln(-\ln(1-P\%))$ |
|--------------------------|-------------------------------------|-------------------------|--------------------|
| 1 | -0.1581 (X_2) | 0.05 | -2.9702 |
| 2 | -0.0097 (X_2^2) | 0.15 | -1.8170 |
| 3 | -0.0069 (X_1X_2) | 0.25 | -1.2460 |
| 4 | -0.0057 (X_3) | 0.35 | -0.8422 |
| 5 | -0.0028 (X_1X_3) | 0.45 | -0.5144 |
| 6 | 0.0001 ($X_1X_2X_3$) | 0.55 | -0.2250 |
| 7 | 0.0001 (X_3^2) | 0.65 | 0.0486 |
| 8 | 0.0049 (X_2X_3) | 0.75 | 0.3266 |
| 9 | 0.0332 (X_2^2) | 0.85 | 0.6403 |
| 10 | 0.1846 (X_1) | 0.95 | 1.0972 |

1, -2 were used in the calibration design matrix. The calibration design, three variables including X_1 , X_2 , and X_3 , were used based on fractional factorial designs (FFD) by employing a five-level, three-variable calibration design from RSM (Table 1). The HA standards (0–2000 mg/L) were injected to the modified FIA system nine times (n : 9) for each experimental condition, and the calibration graphs (peak area versus concentration of HA standards) were drawn. The correlation coefficients (R^2) obtained from the calibration graphs were taken as the response, Y , which reflects the degree of linearity. Statistical analysis was performed, based on the experimental data, and was adapted into a quadratic polynomial model as shown in the equation below (1).

$$Y: \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 + \beta_{12}X_1X_2 + \beta_{23}X_2X_3 + \beta_{13}X_1X_3 + \beta_{123}X_1X_2X_3 \quad (1)$$

where Y represents the response value, β_0 is a constant, β_i , β_{ii} , and β_{ij} are the linear, quadratic, and interactive coefficients, respectively.

There are some factors that restrict the variable values. These factors were affected by materials and conditions of the FIA system. The main factor is the flow rate of the system. In order to decrease the consumption of chemicals, the values of X_1 were chosen in the range of 0.5–2 mL min⁻¹. The other restricting factor is the pH range of the system. Due to pH values over 11 having a corrosion effect on the system, X_2 values

**Figure 4** Determination of the significant factors by means of NDP.**Figure 5** The 3D profiles of RSM of detection responses affected by the FIA parameters; (a) X_1 (flow rate of mobile phase) and X_2 (pH of mobile phase), (b) X_2 and X_3 (the system temperature), (c) X_1 and X_3 .

were selected between pH 8 and 11. The last factor is the temperature range of the system. As the suitable operating temperature range of the system is ranked from 20 to 40 °C, this range was chosen for X_3 values.

3. Results and discussion

3.1. Optimization of the proposed FIA method parameters

In order to determine of optimum values of the proposed FIA method parameters, the variables given in Table 1 were processed to obtain a regression equation that allowed deducing NDP and RSM plots.

For determination of significant variables, the NDP was utilized. For this purpose, the quadratic polynomial Eq. (2) created from the level values given in Table 1 was calculated and the NDP was generated using Eq. (2) (Table 2 and Fig. 4).

$$Y: 0.8526 + 0.1846X_1 - 0.1581X_2 - 0.0057X_3 + 0.0332X_1^2 - 0.0097X_2^2 + 0.0001X_3^2 - 0.0069X_1X_2 + 0.0049X_2X_3 - 0.0028X_1X_3 + 0.0001X_1X_2X_3 \quad (2)$$

The NDP has indicated that significant variables are linear X_1 and X_2 , and quadratic X_1^2 at the level of P : 0.01, also X_1 was found to be most significant variable compared to others (Fig. 4). This result was also supported by the RSM plots (Fig. 5).

For the determination of optimum values of the proposed FIA method parameters, the RSM was utilized. These plots provide a method to visualize the relationship between responses and experimental levels of each variable and the type of interactions between two test variables as reported in the literature (Mehlich, 1984; Box et al., 1978). For this purpose, Eq. (3) was calculated from the variable values given in Table 1 and the RSM plots were generated using this equation (Fig. 5).

$$Y: -2.0905 + 1.3307X_1 + 0.7763X_2 - 0.0531X_3 + 0.1035X_1^2 - 0.0470X_2^2 + 0.0007X_3^2 - 0.1158X_1X_2 - 0.0482X_2X_3 + 0.0019X_1X_3 + 0.0045X_1X_2X_3 \quad (3)$$

Fig. 5a displays the effect of X_1 and X_2 on desirability (correlation coefficient R^2). With a given X_1 value, desirability increased rapidly with decreasing X_2 . Maximum response for X_1 was observed as 2 mL min⁻¹ and for X_2 at pH 8 under these conditions.

The interaction between X_2 and X_3 is shown in Fig. 5b. This figure presents a saddle point as the critical point. The saddle point is an inflexion point between a relative maximum and a relative minimum. As our purpose is to obtain a maximum response to our studied system, the saddle point coordinates were not used. Same approach was used by Mehlich, 1984. Therefore, optimum region was searched through visual inspection of the surfaces. There is an increase of desirability, with the increase of X_2 and X_3 . The maximum response obtained from these levels was observed for X_2 , at pH 11 and for X_3 , 40 °C.

Fig. 5c shows the interaction between X_1 and X_3 . With a given X_1 value, desirability increased rapidly with decreasing X_3 and the maximum response obtained from these levels was observed for X_1 , 2 mL min⁻¹ and X_3 , 20 °C.

According to the results, effects of X_2 and X_3 on desirability were not important as in the case of X_1 . Moreover, these results are compatible with the NDP because the most significant variable in the NDP was X_1 (Fig. 4) and the value of 2 mL min⁻¹ of X_1 has already received the highest desirability when compared with other level values of X_1 (Fig. 5a and c).

The optimal conditions obtained using RSM were as follows: flow rate of the mobile phase, 2 mL min⁻¹; pH of the mobile phase, 8; the system temperature, 20 °C. The determination of HA using the proposed FIA method was achieved under these optimal conditions and the correlation coefficient was 0.9988 (n : 9). This value revealed that the experimental data were in good agreement with the predicted values of correlation coefficient (R^2). As a result, these level values were selected as optimum conditions of the proposed FIA method parameters.

3.2. Calibration parameters of the proposed FIA method and comparison with the spectrophotometric method

The HA standard solutions, ranging from 0 to 2000 mg L⁻¹, were injected into the proposed FIA system under the optimal conditions. It was seen that all of the HA standards were given

Table 3 Some important parameters of the proposed FIA method with the spectrophotometric method.

| Parameter | The proposed FIA method Value | The spectrophotometric method (Schnitzer and Khan, 1972) Value |
|-----------------------------|---|---|
| Calibration range | 0–2000.0 mg L ⁻¹ , $y: 1.701x + 1.513$, $R^2: 0.9988$ (n : 9, $P < 0.01$) | 0–1000.0 mg L ⁻¹ , $y: 0.004x + 0.076$, $R^2: 0.9828$ (n : 9, $P < 0.01$) |
| LOD ^a | 9.18 mg L ⁻¹ | 8.34 mg L ⁻¹ |
| LOQ ^b | 30.60 mg L ⁻¹ | 27.80 mg L ⁻¹ |
| RSD ^c (n : 9) | 3.44% (500.0 mg L ⁻¹ HA) | 2.89% (500.0 mg L ⁻¹ HA) |
| Sample analyzed per hour | 60 | 20 |
| Sample amount needed | 10 µL | 3 mL |
| Reagent consumed per hour | 120 mL | 350 mL |
| Waste volume per hour | 120 mL | 400 mL |

^a Limit of detection.

^b Limit of quantification.

^c Relative standard deviation.

Table 4 Results of determination of HA in lignite samples by the proposed FIA and spectrophotometric method ($n: 9$).

| Sample | Concentration of HA (mg L^{-1}) | | | |
|-----------------------------|--|--|--------------------|--|
| | The proposed FIA method (main value \pm SD) | The spectrophotometric method (Schnitzer and Khan, 1972) (main value \pm SD) | Relative error (%) | F test ($P: 0.01, F_{\text{cri}}: 6.03$) |
| L ₂ ^a | 39.0 \pm 0.2 | 40.3 \pm 0.4 | -3.2 | 4.0 |
| L ₃ ^a | 27.7 \pm 0.2 | 28.6 \pm 0.3 | -3.2 | 2.3 |

^a It was obtained from Turkey Coal Enterprises (Ilgın, Konya).

linear response over this operating range with correlation coefficient 0.9988 ($n: 9, P < 0.01$), but the spectrophotometric method has lost linear responses after 1000 mg L^{-1} of HA. Some important parameters of the proposed FIA method are provided in Table 3. Results of FIA and spectrophotometric methods of lignite samples were compared statistically and shown in Table 4.

Calculated (F) and critical F (F_{cri}) values indicated that both methods are comparable, because of $F < F_{\text{cri}}$ and the null hypothesis cannot be rejected at the $P: 0.01$ level of confidence (Table 4).

4. Conclusion

In this study, quantitative determination of HA in lignite has been successfully obtained over a wide calibration range by the proposed FIA method. The method is rapid, sensitive, provident, and simple as well as avoiding the use of many chemical reagents. It also has great potential for the routine analysis of HA in lignite samples and certification of the commercial HA products obtained from Ilgın lignite. As a result, the proposed FIA method could be used as an alternative to the spectrophotometric method for quantitative determination of HA in lignite. Furthermore, proposed method is capable of making a fully automatic analysis when it is programmed.

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