Article

Development and Validation of a Stability-Indicating Liquid Chromatographic Method for Estimating Vilazodone Hydrochloride in Pharmaceutical Dosage Form Using Quality by Design

Sagar Suman Panda1,*, Venkata Varaha Bera Ravi Kumar1, Sarwar Beg2, Sunil Kumar Sahu1, and Swayamprabha Muni1

1Department of Pharmaceutical Analysis & Quality Assurance, Roland Institute of Pharmaceutical Sciences, Khodasingi, Berhampur-760010, Odisha, India and 2Department of Pharmaceutics, Roland Institute of Pharmaceutical Sciences, Khodasingi, Berhampur-760010, Odisha, India

*Author to whom correspondence should be addressed. Email: sagarguddu2002@gmail.com

Received 19 May 2015; Revised 24 May 2016

Abstract

A stability-indicating liquid chromatographic method was developed employing the principles of quality by design (QbD) to quantify vilazodone hydrochloride (VLN) in pharmaceutical dosage form. A Box–Behnken experimental design was employed to establish optimum conditions including method robustness by selecting organic phase proportion (%), mobile phase flow rate (mL/min) and pH of buffer as the factors, to study their effect on plate number as the response variable. Chromatography was performed on a C-18 column using methanol:phosphate buffer of pH 7.0 (85:15, v/v) as the mobile phase at a flow rate of 1.2 mL/min with photo diode array (PDA) detection at 285 nm. Calibration curve was linear over 5–80 µg/mL with values of accuracy within 99.4–100.8%. The limit of detection and quantification were found to be 1.5 and 5.0 µg/mL, respectively. The developed method revealed high specificity for VLN and its degradation products formed during forced degradation conditions. Furthermore, control strategies were developed based on system suitability test result. The QbD-based developed liquid chromatographic method was found suitable for routine analysis of VLN in bulk drug and pharmaceutical dosage form.

Introduction

Vilazodone hydrochloride (VLN), 5-[4-[4-[5-cyano-1H-indol-3-yl]butyl]-1-piperazinyl]-2-benzofuran carboxamide, hydrochloride (Figure 1) is a novel antidepressant agent used in treatment of major depressive disorders (1–3). It acts as a serotonin partial agonist and reuptake inhibitor (4). Few chromatographic methods have been developed for estimation of VLN in pharmaceutical dosage forms and biological samples including high performance liquid chromatography (HPLC) (5, 6) and liquid chromatography-mass spectrometry (LC-MS) methods (7). However, these reported chromatographic methods for estimation of VLN possess multiple drawbacks like non-stability indicating nature, lower plate number, complex mobile phase mixture, strict monitoring of critical method variables (CMVs) like mobile phase flow rate, injection volume, pH, etc. Moreover, there is no reported literature available describing a reliable stability-indicating analytical method for quantification of VLN and its degradation products in diverse pharmaceutical samples. This calls for development of a simple, sensitive, efficient and reliable stability-indicating liquid chromatographic method for quantification of VLN in bulk drug and pharmaceutical dosage form.
Quality by Design (QbD) is a systematic science and risk-based approach applicable to pharmaceutical development which begins with predefined objectives and intent for accomplishing high quality in the end product and/or process (8). Besides, it primarily facilitates rational understanding by establishing cause-effect relationship among the quality attributes. In the current practice for development of a highly effective and sensitive analytical liquid chromatographic technique, method development on the principles of QbD is quite popularized (9, 10). The QbD approach for analytical method development involves a multistep process, initiates with defining the quality target method profile and critical analytical attributes (CAAs), prioritization of the CMVs and their subsequent optimization using experimental design, followed by response surface analysis and desirability function to embark upon the optimal (11). Figure 2 explains the pictorial flow diagram depicting different steps involved in analytical QbD approach for development of analytical methods (12).

The Design of Experiments (DoE) is widely used for performing statistically well-designed experiments to evaluate the method superiority and for getting quality results. It provides knowledge about the design space to work within for achieving quality. The design space is an experimental safe zone where the method variables have no significant influence on the quality of product. Response surface methods like full factorial design, fractional factorial design, central composite design, Box–Behnken design (BBD), Dohlerlert designs and mixture designs are used for predicting and optimizing the responses for most of the analytical techniques (13–18). These designs help the analytical scientists to understand the relationship between different CMVs and their effect on the CAAs produced.

The BBD has been used in analytical sciences for optimization of extraction, derivatization, separation and robustness testing for chromatographic methods (19–22). BBD has several advantages than compared with other statistical design approaches such as ability for optimizing 3²-factorial designs, needs fewer numbers of total experiments, rotatability and possess less physical constraints for experimentation (23). Therefore, it can be effectively applied for navigating the design space for developing a robust liquid chromatographic method.

The objective of present research work, therefore, was to overcome the drawbacks of reported methods for developing a new stability-indicating liquid chromatographic method for quantification of VLN in bulk drug and pharmaceutical dosage form using QbD approach. The BBD was applied for systematic development and optimization of analytical method to ensure robustness. The validation studies were performed for detecting the linearity, accuracy, precision, specificity, limit of detection (LOD), limit of quantitation (LOQ) and system suitability test limits were derived for the optimized chromatographic method. Furthermore, the developed method was applied for estimation of VLN in pharmaceutical dosage form.

**Experimental**

**Instrumentation**

Chromatography was performed on a binary gradient ultrafast liquid chromatograph (UFLC) with two Shimadzu Prominance UFLC LC-20AD pumps, with a 20-μL sample injection loop (manual) and SPD M20A PDA detector. Signal was recorded and integrated using Shimadzu LC Solution Software. pH measurements were carried out using pH meter (Eutech, India). Ultrasonicator (Enertech, India) was used during mobile phase and sample preparation. Water bath (Thermolab, India) and UV Chamber (Jain Scientific Glass Works, Ambala, India) were used for forced degradation study of the drug. DoE, optimization and data analysis were performed by using trial version JMP software (SAS Institute, Inc., NC, USA).

**Materials and reagents**

Methanol (Merck Ltd., Mumbai, India) was of HPLC grade. Analytical grade potassium di-hydrogen orthophosphate, disodium hydrogen orthophosphate, sodium hydroxide, hydrochloric acid and hydrogen peroxide were purchased from S.D. Fine Chem. Ltd., Mumbai, India. The water for HPLC was obtained by using TKA GenPure Ultra Puriﬁcation System, Germany. VLN (purity >99.5%) was obtained as gift sample from Glenmark Pharmaceuticals Ltd., India. Purity of drug was assessed by testing melting point of drug. As the commercial formulation of VLN was not available in local market, in-house tablet formulation containing 40 mg of VLN was prepared using suitable excipients and analyzed.
Methods
Chromatographic conditions
An enable C18G column (250 × 4.6 mm i.d., 5-µm particle size) was used for chromatographic separation. Chromatography was performed at room temperature using the methanol:phosphate buffer pH 7.0 (85:15, v/v) as mobile phase at a flow rate of 1.2 mL/min in isocratic mode with injection volume of 20 µL. Buffer was prepared by dissolving 1.361 g of potassium dihydrogen orthophosphate in sufficient purified water to produce 100 mL and pH was adjusted to 7.0 using 3.5% w/v solution of disodium hydrogen orthophosphate. pH of buffer was monitored using a pH meter. Prior to use both the methanol and buffer were ultrasonicated up to 20 min for degassing. The mobile phase contents were separately filtered through a 0.45 µm filter. The run time was 7 min for all the solutions except the forced degradation samples where run time was extended up to 10 min to find out the presence of any possible degradation products.

Preparation of standard solution and calibration curve
A standard stock solution of VLN was prepared by taking 25 mg of drug in to 25 mL volumetric flask having 10 mL of methanol and was ultrasonicated for 5 min. Finally, the volume was made up with methanol, which gave 1000 µg/mL solution. A working stock solution was prepared by taking 2.5 mL of this solution and diluting it up to 25 mL with methanol, to get 100 µg/mL solutions. For preparation of calibration curve, suitable aliquots of the working stock solution were taken for concentrations 5, 10, 20, 30, 40, 50, 60, 70 and 80 µg/mL and finally diluted with mobile phase. Triplicate injections were performed for each standard solution and chromatograms were recorded. Calibration curve was plotted taking concentration (µg/mL) on x-axis and average peak areas on y-axis. The calibration curve data were further subjected to regression analysis to find out the regression statistics. Analysis of variance (ANOVA) was performed to check the appropriateness of linearity data.

Preparation of test solution
Tablet powder equivalent to 25 mg of VLN was transferred into a 25-mL volumetric flask, containing 10 mL of methanol and ultrasonicated for 20 min to facilitate complete dissolution and subsequent extraction of drug into the solvent. The volume was made up and mixed well. Solution was filtered by 0.45 µm filter to remove particulate matter, if any. The filtered solution was further properly diluted for analysis, to get a test concentration within linear range. All the solutions were stored at 2–8°C temperature up to 2 days for future use.

Establishment of cause-and-effect relationship
Ishikawa fish-bone diagram being one of the simplest tools is helpful in understanding the overall cause-and-effect relationship among the potential method factors, which may affect the method performance. It also addresses selection of CMVs, which can affect the method performance adversely and helps in carrying out an effective risk assessment. In the present studies, a fish-bone diagram was drawn by highlighting diverse method variables plausibly influencing the method attributes and assessed. The CMVs were identified from assessment of the fish-bone diagram and then subjected to response surface methodology employing BBD to create a design space for developing a robust and reliable chromatographic method.

Method optimization according to the BBD
Based on the prior knowledge and prioritization, three CMVs were decided to have significant influence on method performance. Fifteen experimental runs with three center points were performed as per the three-factor three-level obtained according to the BBD, to assure method robustness and to demarcate the optimal design space where the influence of CMVs such as methanol proportion (%), flow rate and buffer pH were found to be critically affecting the plate number as the response variable. A standard concentration 30 µg/mL was used for all the experimental runs performed as per the selected experimental design.

DoE optimization data analysis
The obtained data for each experimental run were fitted to a suitable mathematical model by multiple linear regression analysis using JMP software (SAS Institute, Inc., NC, USA). The developed model was allowed to study both the main effects and interaction effects among the studies. Exhaustive data analysis was performed by comparing the actual versus predicted plot, fit summary analysis, ANOVA followed by analysis of parameters like coefficient of correlation ($R^2$), adjusted and predicted $R^2$, predicted residual sum of squares (PRESS), respectively. Besides, other vital tools like prediction profiler, interaction profiler and 3-D response surface profiler were used to decide aptness of the adopted model. Optimal chromatographic solution was identified by numerical desirability function by trading-off the studied factors for the responses followed by demarcation in the design space region.

Method validation
Specificity
Specificity of the method was determined by checking interference of any of the possible degradation products produced during forced degradation study of VLN. PDA detection was applied to determine peak purity of degraded samples. Forced degradation of the drug was carried out using 0.1 mol/L HCl, 0.1 mol/L NaOH, 3% v/v H2O2, thermal (80°C) and photolysis (365 nm) for discovering the stability nature of the drug. Degradation samples were prepared by taking suitable aliquots of the drug solution, and then undertaking the respective stress testing procedures for each solution. After the fixed time period, the stressed test solutions were diluted up to 10 mL with mobile phase. For every stress condition, a solution of concentration 30 µg/mL of VLN was prepared. The specific stress conditions are described as follows.

Acidic degradation was carried out by adding 1 mL of 0.1 mol/L HCl, and after 45 min neutralizing the mixture by adding 0.1 mol/L NaOH. Alkali degradation was carried out by adding 1 mL of 0.1 mol/L NaOH, and after 45 min neutralizing the mixture by adding 0.1 mol/L HCl. Oxidative degradation was performed by exposing the drug to 1 mL of 3% (v/v) H2O2 for 45 min. Thermal degradation was performed by heating the drug content at 80°C on a thermostatically controlled water bath for 45 min. Photolysis was carried out by exposing the drug content to UV light (365 nm) inside an UV chamber for 1 h.

Accuracy and precision
To find out accuracy of the method, recovery studies were carried out at 80, 100 and 120% of the test concentration (20 µg/mL) of VLN by standard addition method. Recovery solutions were prepared by taking 1.6, 2.0 and 2.4 mL aliquots of working standard
solution (100 µg/mL) into three separate 10 mL volumetric flasks each containing 2.0 mL (100 µg/mL) of sample solution. Volume was made up using mobile phase to obtain total concentrations of 36, 40 and 44 µg/mL for 80%, 100% and 120% solutions, respectively. The recovery study was performed thrice at each level. VLN standard drug present in the recovery solutions were calculated using the calibration curve.

Precision study was performed in terms of system, interday and intraday precision. Six replicates of a fixed concentration (30 µg/mL) drug solution were injected onto the column for determining the system precision. Intraday precision of the method was determined from the peak areas obtained by injecting six separately prepared solutions of VLN at a fixed concentration (30 µg/mL). Interday precision was found out using the same procedure by a different analyst on different days under similar experimental conditions. The percent relative standard deviation (RSD) values for precision studies were calculated.

Solution stability
Stability of drug solution stored under refrigeration (2–8°C) was determined by the developed chromatographic method. Drug solutions of concentration 30 µg/mL were prepared and injected onto the column in triplicate after every 24 h to determine the drug content. The obtained results were compared with those obtained from freshly prepared standard solutions.

LOD and LOQ
The LOD and LOQ were determined based on visual detection of signal-to-noise (S/N) ratio. For LOD, the S/N ratio was taken as 3:1. For LOQ, the S/N ratio was taken as 10:1.

Results
Method development and optimization
The early liquid chromatographic method development needs enough prior knowledge about various chromatographic parameters and physico-chemical properties of the analyte. Various chromatographic parameters including mobile phase composition, pH of aqueous phase, stationary phase, mobile phase flow rate, etc. were changed one at a time to find desired response. Selection of a suitable buffer was accomplished as the analyte pKa value can significantly affect the chromatographic separation the analyte. The selected candidate VLN is reported to exhibit pKa value of 7.1, thus it was assumed to be well separated employing mobile phase with pH closer to its pKa value (± 2 pH units). Hence, a phosphate buffer of pH 7.0 was selected to obtain optimum selectivity for the analytical target. A C-18 column was used for separation of the compound as it was found suitable based on the analytes chemical properties. Various preliminary experimental trials conducted at ambient temperature using different mobile phase compositions like methanol: water, methanol:10 mmol/L TBAHS (tetra butyl ammonium

Figure 3. Typical chromatograms of VLN (A) in standard drug and (B) tablet dosage form.
hydrogen sulfate), methanol:phosphate buffer pH 7.0 at varying ratios (i.e., 50:50, 60:40, 70:30, 80:20 and 85:15 v/v) and flow rates (0.8, 1.0 and 1.2 mL/min). Among these, methanol:phosphate buffer pH 7.0 (85:15 v/v) at a flow rate of 1.2 mL/min produced a symmetrical peak at retention time of 4.3 min with PDA detection at 285 nm. The representative chromatogram of VLN in standard drug and in-house tablet formulation is shown in Figure 3A and B, respectively.

**Establishment of method robustness employing DoE**

In order to study the factors affecting separation of VLN and to optimize the method robustness, QbD approach was implemented employing experimental design. Methanol proportion, flow rate and pH were selected as the CMVs, which needed further study to test the method robustness (Table I).

The BBD was used to evaluate the effect of CMVs on response plate number. Response obtained for each of the experiments according to BBD (Table II) was further subjected to statistical analysis. A P-value below 0.05 was set as a criterion for accepting the null hypothesis ($H_0$). A thorough analysis of the BBD model using different statistical analysis tools was carried out and inferences were drawn to address risk assessment (through ANOVA, parameter estimates, pareto plot and prediction profiler), risk reduction (through observation of response behavior) and to develop method control strategies for obtaining the desired quality of the analytical method.

Figure 4 illustrates the baseline model (blue line) in actual versus predicted plot, where the obtained line for the experimental data was found to be well within the limit or boundaries of confidence intervals. This rejects the null hypothesis, as the model effectively describes variation in data where the predicted and observed data were quite analogous. A higher value for $R^2$ (0.9916) with mean response of 4593.53 for 15 observations indicates the model capability to explain 99.16% of the variation. Furthermore, ANOVA in Table III suggested the P-value is <0.0001, indicating the model appropriateness to address the variability and suggests rejecting the null hypothesis. Besides, the lower value for PRESS also ratified the model suitability.

For assessing the variability, risk from different variables evaluation of parameter estimates is crucial. An observed P-value <0.05 suggest a non-zero value of slope. Flow rate × flow rate (mL/min) and methanol proportion (%) were found to be the most influencing method variables (Figure 5). In fact the earlier one has more significant effect on plate number than methanol (%), which is supported by a Pareto plot. The longest horizontal bar in the Pareto plot (Figure 5) describes flow rate × flow rate (mL/min) is most influencing variable on response plate number for this model. The prediction expression explains the relationship among the method variables and response. It also helps in predicting the response values within range of levels studied. The expression for adopted model developed by JMP software is given below:

$$\text{Plate Number} = 4746.6667 + [-187.375 \times \% \text{Methanol}] + [-143.375 \times \text{Flow rate}] + [-62.25 \times \text{pH}] + [69.25 \times (\% \text{Methanol} \times \text{Flow rate})] + [-39.5 \times (\% \text{Methanol} \times \text{pH})] + [-167.5 \times (\text{Flow rate} \times \text{pH})] + [-79.9383 \times (\% \text{Methanol} \times \% \text{Methanol})] + [-317.4583 \times (\text{Flow rate} \times \text{Flow rate})] + [110.29167 \times (\text{pH} \times \text{pH})]$$

| Table I. Chromatographic Variables and Range Studied During Robustness Study |
|------------------|-------------|-------------|-------------|
| Variable         | Unit        | Level (−1)  | Level (+1)  | Nominal (0) |
| Methanol (%)     | %           | 83          | 87          | 85          |
| Flow rate (mL/min)| mL/min     | 1.1         | 1.3         | 1.2         |
| pH               | –           | 6.8         | 7.2         | 7.0         |

| Table II. Experimental Design Matrix for Robustness Study and Obtained Response |
|------------------|-------------|-------------|-------------|
| Run no.          | Methanol (%)| Flow rate (mL/min)| pH  | Plate number |
| 1                | 87          | 1.2         | 7.2       | 4492         |
| 2                | 87          | 1.1         | 7.0       | 4239         |
| 3                | 87          | 1.3         | 7.0       | 4057         |
| 4                | 85          | 1.3         | 7.2       | 4193         |
| 5                | 85          | 1.3         | 6.8       | 4633         |
| 6                | 85          | 1.1         | 6.8       | 4551         |
| 7                | 83          | 1.2         | 7.2       | 4918         |
| 8                | 85          | 1.2         | 7.0       | 4782         |
| 9                | 85          | 1.2         | 6.8       | 4983         |
| 10               | 85          | 1.2         | 7.0       | 4765         |
| 11               | 83          | 1.3         | 7.0       | 4321         |
| 12               | 85          | 1.1         | 7.2       | 4781         |
| 13               | 87          | 1.2         | 6.8       | 4715         |
| 14               | 83          | 1.1         | 7.0       | 4780         |
| 15               | 85          | 1.2         | 7.0       | 4693         |

| Table III. ANOVA Results for Response Surface Model |
|------------------|-------------|-------------|
| Source of data   | Plate number |
| Mean of squares  | 118,816     | 65.6918     | <0.0001   |
| Fisher ratio     | 1809        |             |            |

Figure 4. Actual by predicted plot for plate number.
The interaction effect analysis was carried out employing interaction plots depicted in Figure 6, which explains a nonlinear pattern among all the studied factors on the response variable. The higher interaction effect was observed between the factors, that is, flow rate and pH, as evident from the factors lines crossing with each other. The response surface analysis was carried out employing 3D plot, which showed higher values for the response (i.e., plate number) at higher levels of methanol proportion and intermediate levels of flow rate, as illustrated in Figure 7. The response surface between methanol concentration (%) and flow rate showed a curvilinear relationship, where higher value for plate number was observed at intermediate levels of both the factors (Figure 7A). Likewise, a twisted response surface plot shown in (Figure 7B) was observed between the concentration of methanol (%) and pH of mobile phase, where higher number of plate number was observed at low levels of both the factors followed by decrease in the plate number at their increasing levels. On the contrary, a complex relationship was observed between the factors, that is, flow rate and pH depicted in (Figure 7C), where the increasing levels of both the factors showed the value of response approaching towards each other to form “saddle point”. Furthermore, the data analysis employing the prediction profiler plot (Figure 8) revealed a nonlinear type trend for all the factors, that is, methanol proportion, flow rate and pH, suggested significantly influential nature of all the method variables with prominent effect of flow rate as evident from the steepest nature of the curve. Furthermore, the desirability plot along with prediction profiler (Figure 8) suggested a linear increase in values of the response at all the levels of studied factors.

Developing method control strategy
Based on the results of statistical analysis by BBD model, the method was established at the selected nominal values for the different CMVs. Furthermore, the system suitability was defined based on
system suitability test limits, to set up the method control strategies. These limits can be efficiently derived from the experimental results of robustness study (24). Experimentally, these limits can be determined from the result of one or several experiments performed at the optimized conditions. For a replicated experiment, the system suitability limits can be described as the upper or lower limit from one-sided 95% confidence interval around a response mean. For method performance parameters such as retention time and plate number, the lower limit can be chosen, while for tailing factor it would be the upper limit. In the present study, number of plate number was selected as the system suitability test parameter for establishing control strategy. Equation (1) can be used to determine the system suitability test limit for plate number. The result of system suitability test limit is shown in Table IV, which is well within the acceptance limit.

\[
\left\{ \bar{Z} - t_{\alpha, n-1} \cdot \left( \frac{s}{\sqrt{n}} \right) \right\}
\]

where \( \bar{Z} \) is the average of three observations \( t_{\alpha, n-1} = t_{\text{critical}}(\alpha = 0.05, n-1 = \text{degrees of freedom}) \), \( s \) the standard deviation of three observations and \( \sqrt{n} \) the square root of number of observations. Statistical interpretation of experimental design data suggested a controlled method operable range for the most influential method variables such as methanol proportion (±2%) and flow rate (±0.1 mL/min).

Figure 7. 3-D Response surface for plate number against flow rate vs. methanol proportion (A); pH vs. methanol proportion (B) and pH vs. flow rate (C).
Method validation
The method was validated according to ICH guidelines for specificity, linearity, accuracy, precision, LOD, LOQ and system suitability (25).

Specificity
Specificity was determined by a PDA detector to calculate peak purity of chromatographic peaks obtained after forced degradation. The overlaid chromatograms and result for forced degradation of VLN are shown in Figure 9 and Table V, respectively. VLN undergoes severe degradation when subjected to 0.1 mol/L HCl and 0.1 mol/L NaOH for 45 min. So the stress conditions were optimized by reducing the strength to 0.01 and 0.001 mol/L of HCl and NaOH, respectively, with decrease in exposure time. Optimizing the stress conditions did not decrease the degradation behavior of VLN in both acidic and alkaline stress conditions. However no co-eluting peaks were noticed in both stress conditions. VLN showed degradation in the order of acid > alkali > H2O2 > photolysis > thermal. Run time was extended up to 10 min to detect presence of any co-eluting peaks of possible degradation products along the analyte peak. Few well-separated (resolution >2.0) extra peaks were noticed in chromatograms of different stress conditions. Peak purity values >0.999 suggested absence of co-eluting or hidden peaks along with drug peak. Hence the method is specific and stability-indicating in nature.

Linearity
The calibration curve was found to be linear over a concentration range of 5–80 µg/mL for VLN. The linear regression equation was $y = 19909x - 3996.8$. The values obtained for statistical parameters by regression analysis such as multiple $R$, $R^2$, Adjusted $R^2$ and standard error were 0.9997, 0.9995, 0.9994 and 12231.5, respectively. ANOVA suggested appropriateness ($P$-value <0.05) of the linearity data.

Accuracy and precision
The results obtained for accuracy and precision are summarized in Table VI and were found satisfactory.

Solution stability
The average ($n = 3$) recoveries for solution stability were 100% (RSD = 0.5%) and 100% (RSD = 0.9%), for Days 1 and 2,
The developed analytical method for estimation of VLN was found reliable and robust. Implementing QbD work ensured quality of developed method. The results of validation study were satisfactory. Hence, the method is capable of analyzing VLN in bulk and pharmaceutical dosage form.

**Acknowledgments**

The authors are thankful to Glenmark Pharmaceuticals Ltd. for kindly providing the standard drug of Vilazodone hydrochloride and M/S Roland Institute of Pharmaceutical Sciences, Berhampur, Odisha, India for providing the research facilities.

**References**

quantification of sulindac and its related impurities; *Journal of Pharmaceutical and Biomedical Analysis*, (2011); 54: 694–700.


19. Gfrerer, M., Lankmayr, E.; Screening, optimization and validation of microwave-assisted extraction for the determination of persistent organochlorine pesticides; *Analytica Chimica Acta*, (2005); 533: 203–211.


