ESTIMATION OF WITHAFERIN – A FROM ASHWAGANDHADI LEHYA USING HPLC

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INTRODUCTION: Ashwagandhadi lehya (AL) is a polyherbal formulation, official in Ayurvedic formulary of India. AL is indicated in the treatment of piles, psychosis, as tonic, rejuvenating agents and in treatment of disorders related to male reproductive system. One of the major ingredients of AL is dried powdered roots of Withania sominfera (WS). It is reported to contain equal proportion, each of powdered roots of Smilax glabra, Hemidesmus indicus, dried powder of fruits of Cuminum cyminum and paste of Vitis vinifera. The standards to ensure the quality of AL is mentioned in Ayurvedic Pharmacopoeia of India 1. Amount of Withaferin -A (WA) was estimated from AL using HPTLC.

ABSTRACT: Ashwagandhadi lehya is an Ayurveda formulation, official in Ayurvedic Formulary of India and used as aphrodisiac, tonic, rejuvenating agents etc. It is one of the major components of Ashwagandhadi lehya is Withania sominfera which contain Withaferin –A. The study was undertaken to develop an analytical method using HPLC to estimate Withaferin - A, being a marker from, methanolic extract of Ashwagandhadi lehya as one of the quality parameters. Method development involved simple sample preparation without any purification, as well as isocratic separation of Withaferin-A. The method was subjected to validation involving establishing linearity, precision, accuracy, specificity, and robustness. Optimized mobile phase was acetonitrile: water (45: 55 v/v) contained 1% v/v glacial acetic acid. The column used was Phenomenex (250 x 4.6 mm, 5 µm) with flow rate set at 1 ml/min and chromatogram was recorded at 216 nm. The studies showed that peak corresponding to Withaferin –A within concentration range of 1-75 µg/ml was found to be correlated with respective concentration with correlation coefficient (r²) 0.99802 ± 0.0004. The developed analytical method was found precise, while accuracy was established using standard addition method. The average recovery was 95.59%, when determined at three different spiking levels (50%, 100% & 150%), in the pre-analyzed sample solution. LOD was found to be 0.3 µg/ml and LOQ was 0.1 µg/ml. Robustness study suggested that increase in acetonitrile proportion, increased Rf as well as increase in flow rate negatively impacted up on Rf. The content of Withaferin-A was estimated as 0.03 ± 0.001 mg % w/w. The results obtained from the studies showed that method was found to be accurate, precise and validated for estimation of Withaferin-A from Ashwagandhadi lehya.
libido, sexual performance, sexual vigor and penile erectile dysfunction in rats. The spermatogenic potential of WS was thought to be attributed to increased interstitial cells stimulating hormone and testosterone-like effects. Controlled clinical studies in oligospermia patients showed that WL root extract significantly improved deteriorated semen parameters, serum testosterone level and inhibited lipid peroxidation as compared to placebo-treated patients.

The roots of WS were found to contain Withanolides. Withanolides are ergostere type of steroids with atoms C-22 and C-26, bridged by a δ-lactone and an oxidized C-1 position. Withanolides include withanone, WA, withanolides, withasom-dienone. IUPAC name of WA is 4β, 27-dihyrox-5β-6-epoxy-1-oxowitha-2, 24-dienolide. Chemical structure of WA is shown in Fig. 1. WA isolated from WS was found to possess anti-inflammatory, anti-convulsive, anti-tumor and antioxidant activity. WA showed immunosuppressive action on blymphocyte proliferation too.

![FIG. 1: CHEMICAL STRUCTURE OF WA](image)

These compounds are specific for Solanaceae family, in particular, for the genus Withania, and thus they were used as marker compounds. WA was estimated from WS and different extracts thereof as well as from herbal formulations contained WS, as one of the ingredients, using TLC and HPLC, HPLC-MS/MS, UHPLC-MS/MS. Analytical methods involved HPTLC suffered from poor reproducibility. WA was also estimated using HPLC, from variety of matrices. The quantitative estimation procedure from plant drug or from other herbal formulations often found to possess disadvantages, including, either the acetylation of WA was required prior to analysis, or the separation time was long or the compounds were not baseline separated and eluted. Our efforts were concentrated particularly on developing an analytical method for quantitative estimation of WA from a complex matrix of AL using HPLC, which would include relatively simple sample preparation alongside, the developed method would robust, enough sensitive and reproducible.

MATERIALS AND METHODS: AL was manufactured by Sunder Pharmacy (GMP certified manufacturing unit associated with J.S Ayurveda College, Nadiad, Gujarat, India) on our request (batch number 131016). WA was obtained from Pharmanza Pvt., Ltd., Khambhat, Gujarat as gratis sample (purity 95% w/w) and it was used without any further processing.

Reagents and Chemicals: All HPLC grade solvents and chemicals (acetonitrile, glacial acetic acid) were purchased from Merck India Ltd. Water used for HPLC instrument was obtained from a Millipore water treatment system. Solvents utilized for HPLC were filtered through a 0.45 µm filter and degassed at room temperature for 20 min using laboratory sonicator (Remi Instruments, Mumbai, India), prior to use. The solvents used for extraction purpose were of analytical grade and procured from Ms. Loba Chemie, India.

Preparation of Standard Stock Solution: 100 mg WA was dissolved in 20 ml methanol using sonication. The solution was diluted up to 100 ml using methanol, to the produced standard stock solution (1000 µg/ml).

Preparation of Serial Dilutions: Different aliquots of standard stock solution of WA were diluted using methanol to produce different dilutions in the range of 1 µg/ml to 75 µg/ml.

Preparation of Sample Solution: 50 g AL was loaded on 100 g silica (Silica for Column Chromatography, 60-120). The mixture was placed in a thimble, prepared from filter paper, and extracted for 2 h at 60 ºC, using 200 ml hexane in a Soxhlet extractor. The hexane extract was discarded. Dried marc was extracted using Soxhlet extractor, with 200 ml methanol at 60 ºC, for 4 h. The methanol extract was collected, filtered and concentrated using rotary vacuum evaporator at 40 ºC. Residual solvent was evaporated from concentrated mass on a water bath. The semisolid, black-brown extract obtained was preserved in desiccator at room temperature.
Optimization of Chromatographic Parameters: Chromatographic separation was achieved using a Waters quadra gradient chromatographic system equipped with PDA detector. Optimized mobile phase was acetonitrile: water [contained 1% v/v glacial acetic acid] (45:55 v/v). The column used was Phenomenex (250 × 4.6 mm, 5 µm) with flow rate set at 1 ml/min. Injection volume was 20 µl and chromatogram was recorded at 216 nm. Data was collected, processed and integrated using Waters Empower software. The chromatogram of methanolic extract of AL with peak corresponded to WA is shown in Fig. 2b. The peak of WA from standard solution is shown in Fig. 2a.

System Suitability Parameters: Repeatability (from an area of peak corresponding to WA standard solution contained 30 µg/ml WA), capacity factor, number of plates, tailing factor and resolution factor were selected as system suitability parameters (SSP). These values were determined from chromatogram and compared with those recommended 37. The parameters were continuously monitored for each experiment. The value determined for selected parameters is shown in Table 1.

Validation of Developed Analytical Method: The developed analytical method was subjected to evolve various parameters by adopting the methodology proposed in ICH guideline 38.

Linearity: Linearity was established by injecting six different dilutions of WA solution covering the concentration range of 1-75 µg/ml (1, 15, 30, 45, 60, 75 µg/ml) individually, each 20 µl. The optimized chromatographic parameters were adopted to resolve the peak. Area and R_T of the peak corresponding to WA was noted. The experiment was repeated six times. Area and corresponded concentration of WA were processed mathematically to determine line equation using the least square regression analysis. The correlation coefficient (r^2) was also determined. Result of the studies is shown in Table 2, showing average value of slope, y-intercept, and r^2 with SD.

Precision: Precision of the developed analytical method was determined by performing interday and intraday variation in the peak area of WA for three selected concentration (15 µg/ml, 30 µg/ml, 45 µg/ml) from linearity experiment. Intraday variation was determined by recording peak area of WA in standard solutions of WA, six times in a day while those were determined for six days, once in a day to determine interday precision. The data set obtained for each concentration was subjected to statistical treatment to determine mean and % RSD. The results of the studies are shown in Table 3.

Accuracy: Accuracy studies were performed by spiking WA standard, at three different levels (~50%, 100% & 150%), in pre-analyzed sample solution. The spiked sample solution was injected in HPLC system, and amount of WA in spiked sample was determined. Amount of WA recovered was calculated and recorded as % recovery in Table 4.

Selectivity: Selectivity of the analytical method was assessed by comparing the UV spectra of WA received for peak of WA from standard solution with that for WA from sample solution. UV spectra were recorded by scanning the eluents continuously in range of 400 -200 nm. Contour plots were developed for peaks, individually, from UV data and were compared. The contour plots for peak of WA in standard solution as well as in sample solution are shown in Fig. 3a and Fig. 3b, respectively.

LOD and LOQ: LOD and LOQ of the developed method were determined initially by visual observation by noting signal to noise ratio (S/N), while selecting concentrations for linearity experiments. S/N for LOD was considered as 3, while that for LOQ was considered as 10. Later, LOD and LOQ were determined from line equation, by adopting formula give below:

\[
\text{Limit of Detection (LOD): } 3.3 \times \sigma / S \\
\text{Limit of Quantification (LOQ): } 10 \times \sigma / S
\]

Where, \(\sigma\) = Standard deviation of the y-intercept, \(S\) = Slope of the calibration curve (m). The results of the studies are shown in Table 5.

Robustness: Robustness studies were performed by introducing deliberate alteration in selected important chromatographic parameters. The selected parameters were, the proportion of
The studies were performed by incorporation of alteration in one of selected chromatographic parameters. The chromatogram was recorded using altered chromatographic conditions. R_t for WA peak (Y1) and area of WA peak (Y2) were recorded for each experiment. Each experiment, for a set of condition, was performed thrice and mean was considered. The datasets were then compared, for statistical significance, with respective datasets obtained, following optimized chromatographic conditions.

**Studies on Effect of Chromatographic Parameters on Area and R_t:** Box–Behnken design was adopted as experiment design. The coding of the factors is shown in Table 6. The chromatograms recorded for each set of chromatographic conditions and response of dependent variables for a set of selected independent variables was noted. The mean value along with SD for both the dependent variables was determined. The data sets were processed using Design Expert™ software to generate regression equation and regression coefficient for each dependent parameter. The effect of independent variables on dependent variables was determined by calculating p-value for model as well as for each parameter. p ≤ 0.05 was selected as level of significance.

**Estimation of WA from AL:** Calibration range selected for the set of experiments was 1µg/ml to 75µg/ml. Each concentration was injected six times (n=6). The calibration curve was constructed by plotting peak area versus concentration. Optimized chromatographic parameters were adopted to resolve the peak of WA from other peaks in sample solution. The line equation was constructed using the least square regression analysis. Amount of WA present in AL was calculated from line equation using area of peak corresponded to WA.

**RESULTS AND DISCUSSION:** AL was intended to be evaluated for action on male reproductive system functions. This analytical method was developed as a part of evolving standards to ensure the quality of AL. AL was reported as a polyherbal formulation contained plants other than WS too. Amongst the plants incorporated in AL, WS was the only plant subjected to biological evaluation for action on male reproductive functions. WA was one of the major constituents of WS, thus selected as a chemical marker to standardize AL concerning the presence of WS. An analytical method based on HPLC was developed which could resolve peak of WA from peaks corresponding to nearby constituents in complex matrix of AL.

AL was semisolid material, which contained fatty substances, interfered in the extraction of AL from matrix. AL loaded on silica and was defatted using hexane, to remove fatty constituents present in formulation. Silica was used to avoid clumping and to increase the surface area. Dried marc was then extracted using methanol, being the solvent capable of dissolving WA. Cold continuous extraction using Soxhlet apparatus was opted to maximize the yield with utilization of comparatively less amount of solvent. The extraction procedure excluded the heating of sample mass; instead distilled solvent was passed through the bed of the marc. Time and temperature for extraction processes were set to get maximum yield of WA, which was ensured by performing co-TLC of AL extract and WA standard solution.

The analytical method was modified from the reported analytical method for estimation of WA from WS root powder using HPLC. The reported method could not resolve the peak of WA from nearby peaks in sample solution. Incorporation of GAA in mobile phase made the mobile phase acidic, which could resolve WA from sample solution. The concentration of GAA was set using trial and error.

The final concentration of GAA could resolve the peak of WA from nearby peaks, without altering the gaussian shape of WA peak appeared in chromatogram at R_t less than 10 min. UV spectra of the WA peak were recorded. Maximum absorbance was observed at 216 nm, which was selected as wavelength to record chromatogram using PDA detector. As shown in Fig. 2a and Fig. 2b, optimized mobile phase yielded well resolved, sharp, gaussian peak corresponding to WA, at 7.12 min.
Repeatability value was found less than 2.0. It ensured precision on the same operating conditions over a short interval of time. Theoretical plates were higher than 2000, ensured the sharp and Gaussian peak. Capacity factor value sufficiently high, to provide enough duration for analyte to interact with stationary phase. Resolution factor greater than 2.0 confirmed that peak of WA was separated properly from nearby peak, and could be used for quantitative purpose.

The developed analytical method was validated to ensure that the method would perform as per the standards set for the purpose. This could be achieved by showing observational and numerical data gathered and analyzed further, during a series of experiments, suggested for the parameters, as given in ICH guidelines 36.

The linearity of an analytical procedure is the ability of method, within the selected ranges, to obtain test results which are directly proportional to the concentration of the analyte. The linearity of analytical method was established by determining the correlation coefficient for the regression curve, prepared by plotting area of peak corresponded to different concentration of WA. The regression line was constructed by fitting the data in the least square regression model. The mean value of the regression coefficient was found to be 0.9980± 0.0004. Approaching the correlation coefficient value to 1.0, confirmed the linearity of the response in the selected concentration range.

Table: System Suitability Parameters for Peak Corresponding to WA (n=6)

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Parameter</th>
<th>Value determined</th>
<th>Recommended value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Reproducibility (n=10)</td>
<td>0.08% RSD less than 1%</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Capacity Factor</td>
<td>7.12 Greater than 2.0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Number of Plates</td>
<td>5427.85 Greater than 2000</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Resolution</td>
<td>3.71 Greater than 2.0</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Tailing Factor</td>
<td>0.916 Less than 1.5</td>
<td></td>
</tr>
</tbody>
</table>

The linearity of an analytical procedure is the ability of method, within the selected ranges, to obtain test results which are directly proportional to the concentration of the analyte. The linearity of analytical method was established by determining the correlation coefficient for the regression curve, prepared by plotting area of peak corresponded to different concentration of WA. The regression line was constructed by fitting the data in the least square regression model. The mean value of the regression coefficient was found to be 0.9980± 0.0004. Approaching the correlation coefficient value to 1.0, confirmed the linearity of the response in the selected concentration range.

Table: Linearity Data for WA

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Slope (m)</th>
<th>Confidence interval*</th>
<th>y-intercept (C)</th>
<th>Confidence interval*</th>
<th>Co-relation Co-efficient (r²) Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>WA</td>
<td>28053 ± 159.30</td>
<td>27925.53 - 28180.46</td>
<td>6356.70 ± 1392.76</td>
<td>5242.25 - 7471.14</td>
<td>0.99802 ± 0.0004</td>
</tr>
</tbody>
</table>

Table: Precision of Analytical Method (n=6)

<table>
<thead>
<tr>
<th>WA Concentration</th>
<th>Intra-day variation in peak area of WA (% RSD)</th>
<th>Interday variation in peak area of WA (% RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 µg/ml</td>
<td>0.71</td>
<td>0.96</td>
</tr>
<tr>
<td>30 µg/ml</td>
<td>0.81</td>
<td>0.97</td>
</tr>
<tr>
<td>45 µg/ml</td>
<td>0.73</td>
<td>1.58</td>
</tr>
</tbody>
</table>
Accuracy studies were performed by adopting the standard addition method. WA was spiked at three different levels in pre analyzed sample solution. The amount of WA recovered from spiked sample solution was determined. The result of the studies, as reported in Table 3, showed average recovery was 95.59%. The closeness of recoveries nearer to 100% assured the accuracy of developed analytical method for the purpose. As, the amount of WA was low and matrix was complex, recoveries in range of 90%-110% was acceptable to ensure accuracy.

The concentration of WA equivalent to reported value LOD and LOQ was injected in HPLC and chromatogram was recorded. S/N ratio was determined manually, to ensure the results. Finally, 0.3 µg/ml and 1.0 µg/ml were accepted as LOD and LOQ for developed method from manual observations and calculating S/N of the recorded chromatogram.

The selectivity of an analytical method is ability of the analytical method to measure accurately analyte in presence of different interfering compounds. It was to be proved for the purpose, that nothing except WA was co-eluted with WA.

UV spectra of peak WA were compared and found comparable. Contour was received (generated in range of 400 nm to 200 nm) for the peak of WA obtained in sample solutions as well as in standard solution.

The contours were found to be matched in shapes, as shown in Fig. 3a and Fig. 3b. These two observations suggested that nothing was co-eluted with WA, confirmed specificity.

Robustness of the analytical method was ascertained by incorporating deliberate alterations in the optimized chromatographic conditions, and chromatogram was recorded for a selected concentration of WA. The studies showed that, peak area and $R_T$ for WA peak, was not affected statistically as compared to standard, when flow rate was altered by ± 0.1 ml, % of GAA in water was altered by ± 0.05% v/v and wavelength was altered by ± 1 nm, while composition of

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**TABLE 4: ACCURACY STUDIES (n=3)**

<table>
<thead>
<tr>
<th>S. no.</th>
<th>% WA Spiked</th>
<th>Conc. of WA (µg/ml) determined in Solution Average ± SD</th>
<th>% Amount Recovered ± SD</th>
<th>% Average Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>1.86 ± 0.01</td>
<td></td>
<td>(---)</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>2.78 ± 0.09</td>
<td>96.03 ± 2.89</td>
<td>95.59%</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>3.82 ± 0.13</td>
<td>97.05 ± 3.12</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>150</td>
<td>4.66 ± 0.06</td>
<td>93.70 ± 1.15</td>
<td></td>
</tr>
</tbody>
</table>

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**TABLE 5: LOD AND LOQ**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>LOD</td>
<td>0.3 µg/ml</td>
</tr>
<tr>
<td>LOQ</td>
<td>1.0 µg/ml</td>
</tr>
</tbody>
</table>

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**FIG. 3A: CONTOUR OF WA IN STANDARD SOLUTION**

**FIG. 3B: CONTOUR OF WA IN METHANOLIC EXTRACT OF AL**

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**TABLE 6: CODING OF DEPENDENT VARIABLES**

<table>
<thead>
<tr>
<th>Selected</th>
<th>Coded Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor A: % v/v glacial acetic acid in water</td>
<td>0.8 1 1.2</td>
</tr>
<tr>
<td>Factor B: % v/v of ACN in Mobile Phase</td>
<td>43 45 47</td>
</tr>
<tr>
<td>Factor C: Flow Rate of Mobile Phase (ml/min)</td>
<td>0.8 1 1.2</td>
</tr>
<tr>
<td>Factor D: Wavelength selected for recording chromatogram</td>
<td>213 216 219</td>
</tr>
</tbody>
</table>
acetonitrile was altered by ± 1% in mobile phase. It must be noted that only one parameter was altered at a time while performing the studies.

Though, the robustness studies showed the developed analytical method was robust within the limits proposed for the selected factors, an extended set of studies were performed to evaluate relative impact of each selected factor on area and \( R_T \) of WA peak. Box - Behnken design, including four independent variables set at three different levels (-1, 0, +1) for their effect on two dependent variables, as shown in Table 6, was evolved and implemented. Multiple regression analysis equations was constituted for both the selected variables.

The regression equation is shown as equation a,

\[
R_T (Y_1) = (1.52) X_1 + (-0.83) X_2 + (-1.72) X_3 + (-0.012) X_4 + 7.02 \quad \text{(Analogy to AX}_1 + BX_2 + CX_3 + DX_4)\]

Where, \( A, B, C, D \) are the numbers associated with factors).

The model was reported to be significant, with regression coefficient 0.0028. The studies also showed that alteration was not significant statistically, in case of dependant variable \( Y_1 \) for factor \( D \) as well as combination of selected factors- \( AB, BC, CD, AD, BC, BD, CD \) (\( p>0.05 \)).

The reduced regression equation, as shown in b,

\[
R_T (Y_2) = (1.52) X_1 + (-0.83) X_2 + (-1.72) X_3 + 7.02
\]

Thus, it was suggested from studies that, wavelength selected to record peak of WA, did not affect \( R_T \) while and increase in acetonitrile proportion and flow rate decreased \( R_T \) as well as increase in % glacial acetic acid increased \( R_T \) of WA peak.

Similarly, the regression equation for second dependant variable, area of WA peak was constructed, shown as equation B.

\[
\text{Area (}Y_3\text{)} = (7441.83)X_1 + (-3683.67)X_2 + (-3805.50)X_3 + (-14334.83)X_4 \quad \text{(Analogy to } A X_1 + BX_2 + CX_3 + DX_4)\]

Where, \( A, B, C, D \) are the numbers associated with factors). The model was found significant with regression coefficient 0.7770. The results showed that effect of factor \( D \), combined effect of factor AC and BC was statistically significant on dependant variable \( Y_2 \). The reduced regression equation, as shown in c, was

\[
Y_2 = (-14334.83) X_4 + (-1.86 \times 10^5) X_1 X_3 + 1.801 \times 10^5 X_2 X_4.
\]

The mathematical treatment, thus, showed that effect of alteration in % GAA, mobile phase ratio or flow rate did not significantly affect the area of WA; though wavelength affected area only. The combined effect of % GAA and flow rate as well as of acetonitrile proportion and wavelength might affect the area of WA peak, in a statistically significant manner. It was observed in robustness studies that, the range for selected chromatographic parameters, were narrow; to get statistically unaltered mean value for area of WA peak, as compared to optimized chromatographic system. The above mathematical treatment yielded the relative impact of alterations in selected chromatographic parameters on area and \( R_T \) of WA.

Methanolic extract of AL was used to estimate WA in AL. This sample solution was chromatographed using optimized chromatographic conditions. Area of the guassian peak \( (y) \) obtained at \( R_T \) value in the chromatogram of sample solution, corresponded to WA peak received for standard solution, was noted. The concentration of WA in sample solution \( (x) \) was determined using regression equation obtained for linearity. The experiment performed thrice. The amount of WA present in AL was found to be \( 0.037 \pm 0.001 \text{ mg % W/W} \).

**CONCLUSION**: An analytical method using HPLC was developed for estimation of WA from AL. The amount of WA was estimated to be \( 0.03 \pm 0.001 \text{ mg % w/w} \) in sample of AL taken. The optimized chromatographic parameters might serve as a tool to record chromatographic fingerprint for AL. The developed analytical method included simple sample preparation. The resolution was achieved without gradient set in proportions of mobile phase as well as without any pre chromatographic treatment using PDA detector.

Results from validation studies confirmed that, developed method had linear response in selected concentration ranges. The method was found to be precise and accurate too. The robustness studies suggested that, an increase in acetonitrile
proportion, increased $R_T$ and an increase in flow rate negatively impacted $R_T$. Area of WA peak was not statistically affected by alteration in wavelength selected or by altering concentration of glacial acetic acid in mobile phase.

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CONFLICTS OF INTEREST: Nil

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