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# Validation of an analytical method for fast determination of IAA in wild marjoram seeds by HPLC

## INTRODUCTION

The Department of Vegetable and Medicinal Plants for several years carried out a detailed study on increasing the value of medicinal plants seeds. These works concern the biology of flowering as well as determination of seed conditioning effect on germination, seedling vigor, yield and quality of seeds of the species belonging to the *Lamiaceae* family. To be able to fully assess the impact of the seed treatment, determination of the changes in plant growth regulators content, such as gibberellin GA3, abscisic acid, zeatin, brassinosteroids acid, and indole-3-acetic acid (IAA, auxin, Fig. 1) in the plant material is necessary. A high concentration of auxins in plant tissue causes attraction of nutrients and other hormones. It is one of the essential functions of auxins. Several regulators of plant growth and development can operate only (or mainly) in the presence of auxins.

The validation of analytical method aims at documented and consistent with assumption confirmation that procedures, processes, equipments, materials, operations and systems actually lead to the expected results. The validation requires experimental documentation of reliability of method and demonstration that it is suitable for particular analytical problem solution. Validation requirements of analytical method comprise parameters such as precision, repeatability, reproducibility, accuracy, linearity, range of detection, limit of detection (LOD), limit of quantification (LOQ), selectivity, robustness, ruggedness, and recovery.

In this work the parameters and basic validation of indole-3-acetic acid (IAA) determination method in wild marjoram (*Origanum vulgare* L.) seeds by high pressure liquid chromatography (HPLC) with fluorescence detector (FLD) were presented.

## MATERIAL AND METHODS

The work was carried out by Prominence HPLC Shimadzu Scientific Instruments consisting of two LC-20AD Prominence pumps, DGU-20A3 Prominence degasser, SIL-20AC HT Prominence autosampler, CTO-10AS VP oven, RF-10A XL fluorescence detector and CBM-20A Prominence system controller. The devices were controlled by Schimadzu LCsolution v. 1.21 SP1 software. Separation of standards solutions and real samples were carried out using a modern C-18 reversed-phase column with core-shell technology (Phenomenex Kinetex® 2.6 µm, C18, 100A, 100×4.60 mm i.d.).

Binary gradient of mobile phase A (deionized water obtained in the laboratory using WCA R03 DP ECO water purification system by Cobrabid Aqua) and mobile phase B (acetonitrile [ACN] CHROMASOLV® gradient grade, for HPLC, ≥ 99,9% Sigma-Aldrich) was used (Table 1). Both solvents were acidified with ortho-phosphoric acid (85%, Fluka) to a concentration of 0.1%. The following conditions were applied: injection volume: 1 µl, flow rate 1.0 ml×min$^{-1}$, oven temperature 40 °C, average back pressure 14.5 MPa, total time of analysis 10 min. The optimal excitation (280 nm) and emission wavelength (355 nm) for IAA was checked and confirmed experimentally by stopping the flow of the mobile phase and run the scan mode (Table 2).

The determination of IAA was performed using an external standard method. Six-point calibration curve in six replicates was built (Table 3). The standard of indole-3-acetic acid (67330, 5g, ≥ 98%) was purchased from Fluka Analytical and dissolved in methanol for HPLC CHROMASOLV ® ≥99.9% from Sigma-Aldrich.

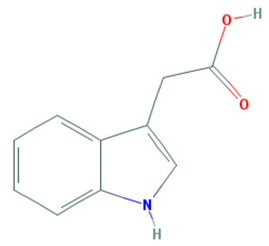

**Fig. 1. Chemical structure of IAA**

**Table 1. The binary gradient of mobile phase A and B**

| Minute | % A | % B |
|---|---|---|
| 0.01 | 75 | 25 |
| 3.50 | 45 | 55 |
| 4.00 | 45 | 55 |
| 4.01 | 75 | 25 |
| 10.00 | stop | stop |

**Table 2. The parameters of the fluorescence detector RF-10A XL**

| Parameter | Value | Unit |
|---|---|---|
| RF | ON | |
| Sampling | 1.43 | Hz |
| Start Time | 0.00 | min |
| End Time | 10.00 | min |
| Response | 1.5 | sec |
| Ex Wavelength | 280 | nm |
| Em Wavelength | 355 | nm |
| Gain | 16 | |
| Sensitivity | medium | |
| Recorder Range | 1 | |

**Table 3. Preparation of calibration sol.**

| IAA concentration [µg×L$^{-1}$] | IAA concentration after taking into account the standard purity [µg×L$^{-1}$] | The volume of stock solution [µL] | The final volume [mL] |
|---|---|---|---|
| 10 | 9.8 | 10 | 50 ± 0.040 |
| 50 | 49.0 | 10 | 10 ± 0.025 |
| 250 | 245.0 | 50 | 10 ± 0.025 |
| 500 | 490.0 | 100 | 10 ± 0.025 |
| 1000 | 980.0 | 200 | 10 ± 0.025 |
| 2500 | 2450.0 | 500 | 10 ± 0.025 |

Real samples of seed were prepared using sonication-assisted extraction. Seed samples were extracted twice with 10 ml of methanol. 0.5000 g of seeds was crushed in a mortar, transferred to a flask and poured over the first portion of the solvent, after which a certain amount of IAA solution was added (10, 50, 100, 200 and 500 µl). Flask was placed in an ultrasonic bath and extracted for 15 minutes. The obtained extract was filtered into another flask, the residue was poured over the second portion of methanol and extracted again for 15 minutes. Both extracts were combined and concentrated at reduced pressure. The obtained residue were transferred into volumetric flasks 10 ± 0,025 ml and filtered with Supelco Iso-Disc™ Syringe Tip Filter Unit, PTFE membrane, diameter 25 mm, pore size 0.20 µm and subjected to HPLC.

Statistical evaluation of the received data was based on determining the specific parameters according to their definitions and relationships. Calculations were performed using Shimadzu LCsolution v. 1.21 SP1 software, Microsoft Excel and e-stat services - Statistical Analysis on-line (http://www.chem.uw.edu.pl/stat/e-stat/ by Wojciech Hyk).

## RESULTS

For all standard solutions clear, accurate analytical signals in the form of a chromatographic peaks were recorded (Fig. 2, Fig. 3, Table 4). The coefficient of variation does not exceed 3%, so it can be assumed that the precision of method is good (Table 5). Dixon's Q test, performed before determining the linear regression, showed a lack of outliers, at 95% confidence; it was also proved that the population of the results had a normal distribution (data not shown). It has been shown that the method has sufficient sensitivity, linearity and good precision (Table 6). Linearity was also evaluated by analyzing the residues. The uniform and random scattering of residuals around zero shows a linear relationship between the measured signal and the content of the analytical standard in the calibration solution (Fig. 5). The value of the standard uncertainty for the lowest concentration (9.8 mg × L$^{-1}$) is 11.721 mg × L$^{-1}$, fot the highest (2450.0 mg × L$^{-1}$) is 17.97 mg × L$^{-1}$. The detection limit (LOD) and quantitation limit (LOQ) were calculated based on a signal-to-noise ratio (Table 7). The recovery of standard from spiked samples of seeds was presented in Table 8.

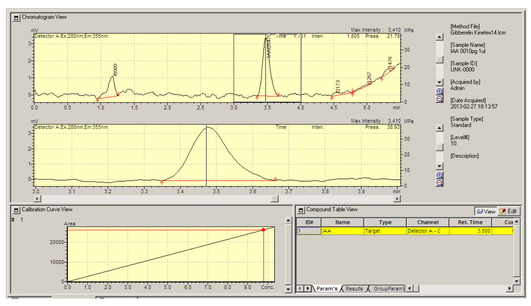

**Fig. 2. The chromatogram of indole-3-acetic acid standard at 10 µg × L$^{-1}$ obtained with a fluorescence detector RF-10A XL and LCsolution v.1.21 SP software**

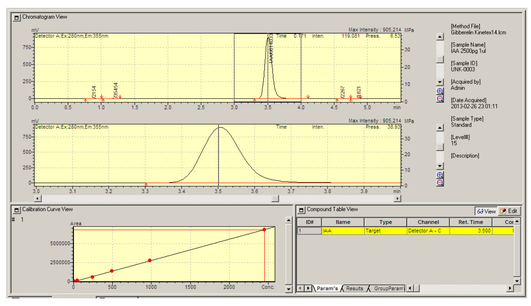

**Fig. 3. The chromatogram of indole-3-acetic acid standard at 2500 µg × L$^{-1}$ obtained with a fluorescence detector RF-10A XL and LCsolution v.1.21 SP software**

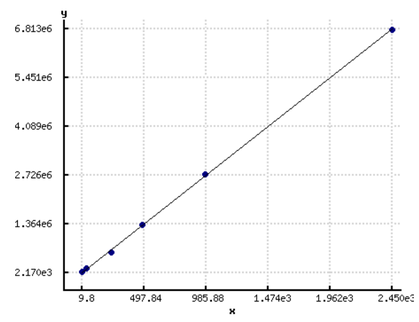


**Fig. 4. Calibration curve, the graph of y = 2791,0307 x – 25182, calculated by e-stat**

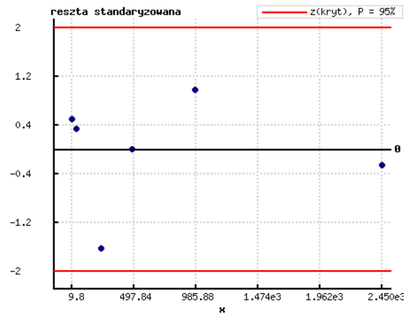


**Fig 5. Standardized residuals, calculated by e-stat**

**Table 4. Results obtained for 2500 µg × L$^{-1}$ indole-3-acetic acid using fluorescence detector RF-10A XL and LCsolution ver. 1.21 SP software**

| Compound Table View | | |
|---|---|---|
| **Param's** | **Results:** | |
| Name: IAA | Name: IAA | Tailign F: 1.247 |
| Ret. Time: 3.500 | Ret. Time: 3.502 | Tailign F(10%): 1.199 |
| Conc. 1: 9.8 | Conc.: 2504.59204 | Resolutions: 5.804 |
| Conc. 2: 49.0 | Area: 6818639 | k': 24.606 |
| Conc. 3: 245.0 | Height: 907201 | Separation: 2.405 |
| Conc. 4: 490.0 | Area%: 99.1508 | Height%: 99.1845 |
| Conc. 5: 980.0 | T.Plate#: 5148.389 | USP Width: 0.215 |
| Conc. 6: 2450.0 | HETP: 19.424 | Width (10%): 0.215 |

**Table 5. Peak area obtained for each calibration solution**

| IAA concentration (µg × L$^{-1}$) | Average peek area | Standard deviation | Coefficient of variation (%) |
|---|---|---|---|
| 9.8 | 27192.83 | 634.64 | 2.33 |
| 49.0 | 128427.00 | 1929.09 | 1.50 |
| 245.0 | 579517.67 | 6639.27 | 1.15 |
| 490.0 | 1343228.00 | 15816.13 | 1.18 |
| 980.0 | 2758551.50 | 69792.80 | 2.53 |
| 2450.0 | 6800746.00 | 65915.47 | 0.97 |

**Table 6. Regression coefficients and coefficients of significance, calculated by e-stat**

| Regression coefficients | Coefficients of statistical significance |
|---|---|
| Slope ratio with a confidence interval<br>a ± t (95%, 4)$s_a$: 2791.0307 ± 65.888571 | Critical Student-t distribution (two-sided variant)<br>$t_{kryt}$ (95%, 4): 2.7764476 |
| Coefficient of the line intersection with the axis Y<br>b ± t (95%, 4)$s_b$: -25182.1 ± 72504.987 | Coefficient of significance a<br>$t_a$: 117.60994 (a ≠ 0) |
| Standard error of the slope coefficient<br>$s_a$: 23.73125 | Coefficient of significance b<br>$t_b$: 0.96430306 (b = 0) |
| Standard error of intersection coefficient<br>$s_b$: 26114.3 | Coefficient of significance r<br>$t_r$: 117.60971 (r ≠ 0, variables correlated) |
| Residual standard deviation<br>$s_{y/x}$: 49164.985 | |
| Standard error of the method<br>$s_m$: 17.615351 | |
| Coefficient of variation<br>$v_m$: 2.502299% | |
| The coefficient of determination<br>$R^2$: 0.9997109 (r: 0.99985544) | |

**Table 7. LOD and LOQ**

| | IAA concentration [µg×L$^{-1}$] | Average peek area |
|---|---|---|
| Signal | 9.8 | 32998.33 |
| Noise | 0.7 | 2438.17 |
| LOD | 2.2 | |
| LOQ | 7.2 | |

**Table 8. Recovery of standard spiked samples**

| The average concentration of the matrix (µg × L$^{-1}$) | The average concentration of standard (µg × L$^{-1}$) | Concentration of spiked sample (µg × L$^{-1}$) | Recovery (%) |
|---|---|---|---|
| 71.49 | 245 | 326.65 | 104.15 |
| | | 329.28 | 105.22 |
| | | 328.85 | 105.05 |
| | | 315.80 | 99.72 |
| | | 326.61 | 104.13 |
| | | 327.33 | 104.43 |
| | 490 | 634.08 | 114.81 |
| | | 608.99 | 109.69 |
| | | 598.03 | 107.46 |
| | | 618.53 | 111.64 |
| | | 616.48 | 111.22 |
| | | 608.34 | 109.56 |
| | 980 | 1028.45 | 97.65 |
| | | 1067.81 | 101.67 |
| | | 1007.80 | 95.54 |
| | | 1016.77 | 96.46 |
| | | 1016.77 | 96.46 |
| | | 1093.85 | 104.32 |
| | 2450 | 2519.60 | 99.92 |
| | | 2502.98 | 99.24 |
| | | 2520.40 | 99.96 |
| | | 2508.95 | 99.49 |
| | | 2514.95 | 99.73 |
| | | 2515.31 | 99.75 |

## CONCLUSIONS

**The result of the study showed that the presented method is reliable and useful for the determination of indole-3-acetic acid in wild marjoram seeds.**

| Parameter | Obtained value | Required |
|---|---|---|
| Precision - the coefficient of variation [%] | 0.95 – 2.53 | ≤ 3% |
| Linearity | r = 0.99986 | r > 0.999 |
| Slope Factor $t_a$ sensitivity | 117.611 | $t_{kryt}$ (95%, 4) = 2.776 |
| Slope Factor $t_b$ sensitivity | 0.964 | $t_{kryt}$ (95%, 4) = 2.776 |
| Coefficient of variation of the method | $V_m$ = 2.5% | ≤ 3% |
| The measuring range | 9.8 ÷ 2450.0 µg×L$^{-1}$ | |
| Uncertainty in linear regression | 17.97 µg×L$^{-1}$ | |
| LOD | 2.2 µg×L$^{-1}$ | |
| LOQ | 7.2 µg×L$^{-1}$ | |
| Recovery | 103.22% | 80% ÷ 120% |

The participation in the Mini-Symposium is financed from EU "FP7 - Capacities" REGPOT project 2011-1-286093-WULS Plant Health