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Inulin determination of yam bean tuber (*Pacyrrhizus erosus l.*) water extract from different altitude areas using TLC- Densitometry

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ABSTRACT

Inulin is a polysaccharide with a lot of potential in the field of food and pharmaceuticals. Inulin can be found in yam bean tuber. Chemical constituents of yam bean tuber are inulin, pachyrion, and rotenone. The levels of inulin could be influenced by differences in the altitude of the cultivation area. The objective of this study was to determine the inulin of yam bean tuber extract from several areas in East Java based on the altitude difference, they are low (Gresik), middle (Kediri), and high (Malang), using validated TLC Densitometry method. The samples were extracted by the infusion method. The samples were put to silica gel TLC plates and developed using ascending chromatography with a mobile phase of 0.5:7:2 glacial acetic acid:methanol:deionized water. Densitometry at 380 nm was used to assess the intensity of inulin spots dipped in Aniline: diphenylamine: phosphoric acid (5:5:1), and winCATS software version 1.3.0 was used to quantify the results. A standard curve meets the requirement of linearity ($r = 0.997$; $Vxo = 3.847\%$), sensitivity (LOD = 71.030 ng/spot, and LOQ = 236.766 ng/spot), selectivity and specificity, Precision (RSD ≤ 2.754 %), and accurate (% recovery ± RSD = 100.161 ± 1.839%). The results showed that the inulin content in yam tubers produced by areas with different altitudes had different inulin levels. The levels of inulin derived from the low altitude (Gresik) was 9.066 ± 1.218 %, middle (Kediri) was 7.776 ± 0.420 %, and high (Malang) was 6.796 ± 2.045 %. One-Way ANOVA test showed the significant difference between the levels of inulin from different altitude areas ($p < 0.01$), the highest inulin level comes from yam bean tuber from low altitude.

Keywords: inulin, yam bean tuber, altitude area, TLC-Densitometry

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INTRODUCTION

Inulin is a natural polysaccharide belonging to a class of carbohydrates that consists of a linear chain from D-fructose molecules and has a terminal glucose molecule (Karimi et al., 2015). Inulin has been found to have several benefits for the human body, which are as bifidogenic (it able to maintain the growth of Bifidobacterium in the large intestine), stimulating the immune system, reducing the number of pathogenic bacteria in the colon, relieving constipation, reduce the risk of osteoporosis by increasing the absorption of calcium, regulate the concentration of inulin and glucagon so it can control the metabolism of carbohydrates and fats by lowering blood glucose levels, and reduce the risk of colon cancer (Wilson & Whelan., 2016; Micka et al., 2017; Arcia et al., 2011).

Inulin is present excessively in tubers of dahlia (*Dahlia sp. L*); Chicory (*Chicoryum intybus L*), Dandelion (T*araxacum officinale Weber*), Jerusalem artichoke (*Helianthus tuberosus*); yacon tubers (*Samallanthus sanchifolius*), and small quantities in onion, garlic, asparagus, banana, and wheat (Redondo-Cuenca et al., 2021). Besides that, yam bean tuber is also contained inulin but there isn't use optimum as the source of inulin. Whereas, Indonesia especially east java has several areas that produce yam beans in huge production, such as Gresik, Malang, and Kediri where these three areas have different altitudes. Plant growth and development are influenced by the environment, particularly the microclimate element. Several previous research results show that the altitude area will affect the quality and major variations in the bioactive ingredients present in the plants (Herlina et al., 2017; Radácsi et al., 2016). Altitude is one of the most important physiographic elements that governs plant growth and development since functional traits could show great variance depending on the altitude level (Liu et al., 2015). Inulin as bioactive ingredients of yam tuber, its level is also suspected by the altitude where it grows, so research is needed on the effect of where the yam grows on its inulin content, to determine a good place for yam cultivation to produce quality inulin in large quantities. Currently, there is no research on the effect of altitude on inulin content in yam tubers, so this research is very important to do. The urgency of this study is to determine the effect of altitude on the inulin content of yam tubers. The result of this study can be used as a reference in determining the right cultivation area to produce quality inulin in larger quantities.

Determination of inulin can be done by several methods such as High-Performance Liquid Chromatography (HPLC) with RI Detector (Retnaningtyas, 2012). In this study, HPLC was used to determine inulin levels in multivitamin syrup samples. Determination of inulin levels can also be done using the Thin Layer Chromatography (TLC) method (Li et al., 2011; Nisa et al., 2015).TLC densitometry method The TLC method is preferred in this study because it has several advantages, which are high specificity, reliability, relatively easy and quick, relatively low operating costs, the polarity of the solvent or solvent mixture can be changed in a short time, use less solvent and it does not require a special detector such as an RI detector (Liu et al., 2015; Chavhan et al., 2017). The purpose of this study was to determine the effect of altitude on the inulin content in yam tubers by determining the inulin content of water extract of yam tubers from three regions in East Java that have different altitude areas using the TLC-Densitometry method. A validation approach was carried out on the following characteristics to confirm the method's application and effectiveness: sensitivity (LOD & LOQ), selectivity and specificity, precision, and accuracy.

MATERIALS AND METHOD

Materials

Inulin standard was purchased from Fluka, yam bean from Kediri, Gersik, and Malang which has been determined by the Faculty of Mathematics and Natural Sciences, the University of Jember.

TLC Instrumentation

Chromatographic separation was performed on a TLC plates Silica Gel 60 F254 (10 cm x 10 cm with 250 µm thickness, E. Merck, Germany). Linear ascending development was carried out in the

Camag Twin Trough TLC chamber. Densitometric scanning was performed on Camag TLC scanner at λ 380 nm for all measurements and operated by winCATS software version 1.3.0.

Methods

Extraction of inulin from Yam Bean tuber

Yam bean tubers were separated from the peels, washed, and cut into small pieces. After that, blended with hot water at the temperature of 90ºC for 1 hour. The small yam bean tubers were heated over a water bath ± 1 hour with stirring frequently, then filtered. The filtrate was deposited at a temperature of 20ºC for 18 hours, followed by storage for 42 hours at a temperature of 8ºC. The concentrate was centrifuged at 1500 rpm for 15 minutes. The precipitate obtained was taken, dried at 60 °C in an oven, and refined using a mortar and Stemper (Winarti et al., 2011).

Solution

A Standard stock solution containing 6 and 3 mg/mL of inulin was prepared in deionized water (60 ºC) : ethanol 96% v/v (3:1). 180 mg of samples were weighed and was transferred to a 25mL calibration volumetric flask, extracted with 19 mL deionized water for 15 min by shaking mechanically, diluted to volume with ethanol 96%, and filtered with Whatman filter paper no.40 (manufactured by Whatman International Ltd. Maidstone, England).

Construction of calibration curves

Calibration solutions were prepared by diluting the stock solution, so that application of 2µL volume gave a series of spots, covering the calibration range 1200 - 4800 ng of inulin. Sample application on 10 cm x 20 cm aluminum backed silica gel 60 F254 TLC plates (E. Merck, Darmstadt, Germany) stored in a desiccator were used for the stationary phase. The sample application was in the form of bands with a band length of 5 mm and the distance between the bands was 5.0 mm. Bands were applied 10 mm apart and 10 mm from the bottom edge. The linear ascending development of plates was performed to a distance of 8 cm in a twin-trough chamber (20 · 10 cm) previously saturated for 25 min with the mobile phase glacial acetic acid: methanol pa: deionized water = 0.5:7:2 mixture as the mobile phase. Following the TLC running, the plates were dried with an air dryer. After elution the plate was dipped in Aniline: diphenylamine: phosphoric acid (5:5:1) for detection (Wimala et. al 2015), the staining techniques for the plate of TLC was immersed and Densitometry scanning was performed at 380 nm on a Camag TLC scanner 3 operated by winCATS software version 1.3.0. The area under the peak was measured, and calibration curves linking the integrated area under peak to the appropriate concentrations in ng/band were created, from which the polynomial regression equations were calculated.

Validation method

The validation parameters tested in this study include linearity, sensitivity (LOD & LOQ), selectivity and specificity, precision, and accuracy. The determination of all the validation parameters is carried out under the conditions of the analysis of the optimization results.

Data Analysis

The data were analyzed using a One-Way ANOVA test with a 99% confidence level to determine whether there were significant differences among the three levels of data average inulin in water extract of yam tubers obtained. The analysis was conducted to test the normality and homogeneity. The normality test was used the Shapiro-Wilk and the homogeneity test was performed using the Levene test.

RESULT AND DISCUSSION

Optimation of the eluent and wavelength

Better separation in chromatography is determined by the optimum separation conditions. In Thin Layer Chromatography, one of the most important things in separation is the determination of the eluent (Rollando et al., 2019). In this study, the optimization of the eluent was a mixture of acetic acid, methanol, and distilled water with a variety of comparisons and the selected eluent was a mixture of glacial acetic acid:methanol:deionized water = 0.5:7:2 with the value of Rf is 0.89 (included in the range of 0.1-0.9), the value of N = 356.790, and H = 0.009. Selection of the maximum wavelength for inulin was done using the light of UV and UV Vis with a range between 200-700 nm. Selected wavelength is the wavelength of the spectrum in high intensity. The results of standard inulin scanning spectra can be seen in (Figure 1).

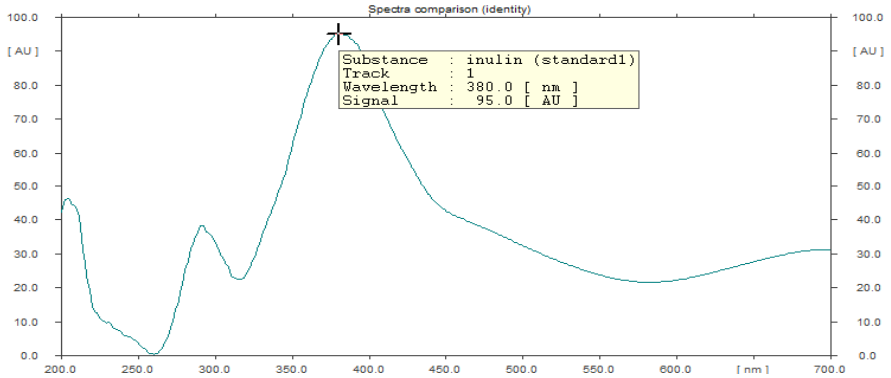


Figure 1. Spectra of inulin standard at a wavelength of 200-700 nm

Based on the spectra, it can be seen that the highest spectra intensity is reached when the wavelength at 380 nm with absorbance signal of inulin is 95.0 AU.

Validation of analysis method

Linearity

Linearity is the ability of the analytical methods to respond directly or with the help of a good mathematical transformation, proportional to the concentration of analyte in the sample (Michael et al, 2017). Data obtained standard curve equation corresponding (Figure 2) below.

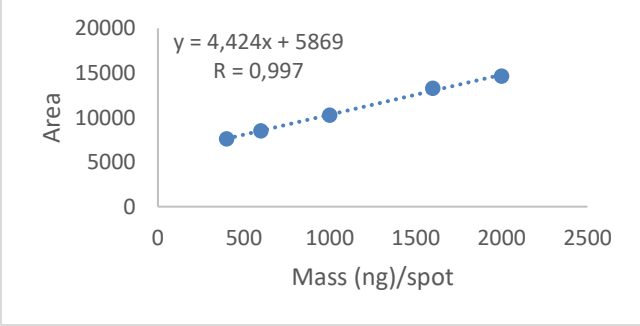


Figure 2. Graph of equation curve the correlation between area and mass (ng)/spot

Figure 2 shows the graph of the equation standard curve with 5 standard inulin concentrations in the range of 400-2000 ng/spot. The equation obtained from 5 standard concentration measurements is

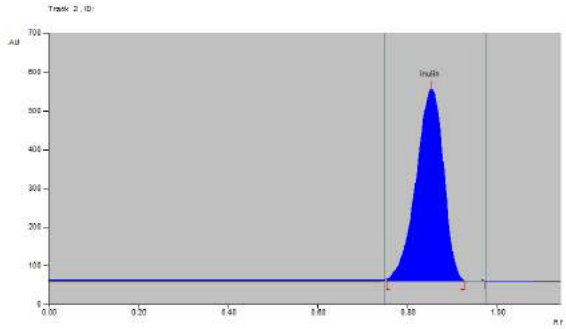
y=4.424x+5869 with a value of r = 0.997, Vxo = 3.847%, Based on the value, which is known to have met the requirements of the r value > 0.99, Vxo < 5%, (AOAC International, 2016) then the curve said to be linear.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

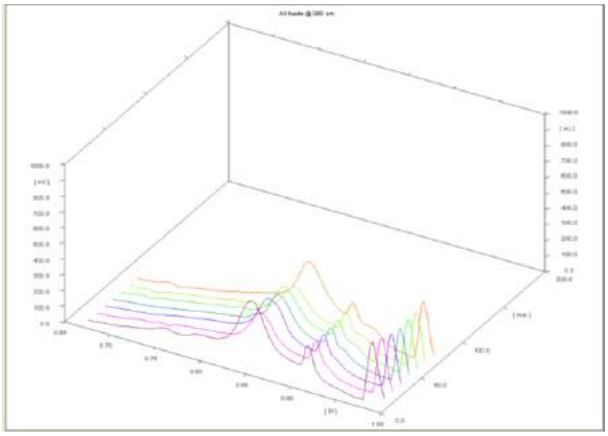
The concentration range that is used for LOD and LOQ is below the concentration of linearity. In the results of LOD and LOQ, we found 71,030 ng/spot and 236,766 ng/spot respectively. This result indicates that the resulting method is sensitive because the LOQ value was ≤ 400 ng/spot (AOAC International, 2016).

Specificity

The specificity of the method showed by how accurately and specifically the analytes of interest were determined in the presence of other components in the samples (Abdelaleem & Abdelwahab, 2013). The TLC–densitograms (Figure 3) prove method specificity. The specificity of a method also can be determined by testing identity (identity) and purity (purity). This method of analysis can be selective because it can respond to the compound inulin which can be clearly distinguished from the other responses.



(a) Two dimensions



(b) Three dimensions

Figure 3. TLC-densitogram of inulin in water extract of yam bean tuber using a selected solvent

Test specificity of the method also can be seen in the identity and purity of the test in Figure 4; Table 1 and Table 2.

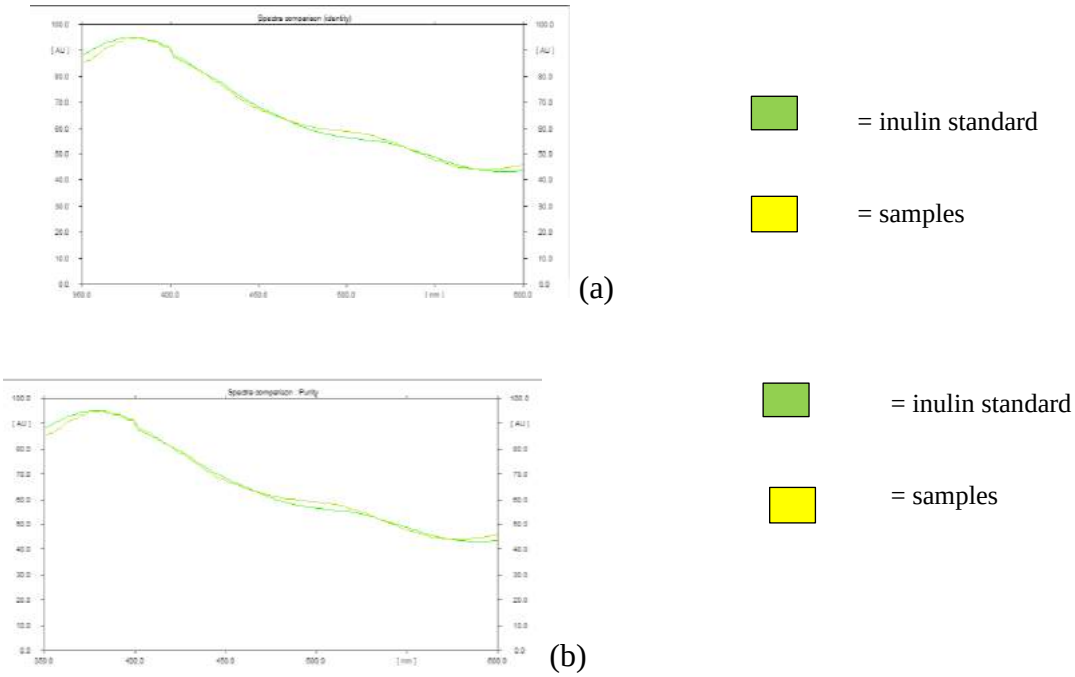


Figure. 4 (a): Spectra of standard and inulin samples in the identity test; (b) Spectra of the standard and inulin samples from Gresik in the purity test

Table 1. Purity test of the proposed method

Test	Track	Rf	r(s,m)[a]	r(m,e)[b]	Conclusion
Purity	Standar	0.89	0.99912	0.99922	*Purity*
	Sample	0.91	0.99858	0.99999	*Purity*

[a]: the correlation coefficient between the start position (s) and the top position (m) of the peak
[b]: the correlation coefficient between the top position (m) and the end position of the peak (e)

Table 2. Identity test of the proposed method

Test	Track	Rf	r(s,s)[a]	r(s,a)[b]	Conclusion
Identity	Standar	0.89	0.99979	0.99778	Inulin
	Sample	0.91	0.99979	0.99275	Inulin

[a]: the correlation coefficient between the two tracks' standard spectra(s,s) that have the same concentration
[b]: the correlation coefficient between the spectra of the standard track (s) and the track of the analyte (a) in the sample

Based on Figure 4; Table 1 and Table 2 it can be seen that the spectra of standard and inulin samples have identical spectra. It can be concluded that the sample was containing inulin. The purity of the analyte in the sample is based on the value of r (s, m) and r (m, e). The value of the correlation coefficient

of r(s,m) and r (m, e) of inulin spectra is over 0.990. It means that the analyte in the sample is pure. The identity test analyte is indicated by the value of r (s, s) and r (s, a). The correlation coefficient values were obtained more than 0.990 which means that the analyte in the sample is identical to the standard inulin. Based on the assessment of selectivity and specificity parameters described above, it can be concluded that this analytical method is selective and specific (Mohammad & Moazzam, 2012).

Precision

Precision is measured by the value of RSD/CV (relative standard deviation/relative standard deviation). In this study, the Precision that was used is repeatability and intermediate precision. The repeatability test, measured test analyte concentration in the sample with 6 replication. that is 1000 ppm. The requirement of the precision test at inulin concentration 1000 ppm is RSD ≤ 3% (Sonia et al., 2017). To determine the intermediate precision, performed the same experiment as the repeatability test, but performed on three different days. The result of repeatability and intermediate precision can be seen in Table 3.

Table 3. Precision of inulin in water extract of yam bean tuber

Weighing of samples (mg) (Mean ±SD)	Intra-day		Inter-day		
	Conc. % (Mean±SD)	RSD (%)	Day	Conc.% (Mean ± SD)	RSD (%)
180.88±0.55	23.89±0.66	2.754	1	23.880 ± 0.660	2.754
			2	24.004 ± 0.364	1.512
			3	24.330 ± 0.369	1.538

Based on Table 3 it can be seen that the value of RSD in the repeatability test is 2.754 % and the inter-day repeatability was in the range of 1.512–2.754%. Based on the RSD value, it can be concluded that this analysis method can provide precision in the results of the analysis and has complied with the requirements of AOAC, which is 3.7% (AOAC International, 2016).

A**ccuracy**

Accuracy of the analysis indicates the degree of closeness result to the actual analyte concentration, which is determined based on the value of % recovery. Accuracy test is done by calculating the % recovery by resulting from the addition of standard as much as 30%, 45%, and 60% of the analyte concentration in a sample obtained from the precision test with 3 replication at each level (Sonia et al., 2017). The accuracy of the test results can be seen in Table 4.

Table 4. Accuracy of the proposed method

Inulin added to analyte (%)	Weighing of samples adition (mg)	Theoritical content (mg)	Concentration found (mg)	*Recovery* (%)	RSD (%)
30	180.03	$4.172.\ 10^{-3}$	$4.174.\ 10^{-3}$	100.04	0.243
45	180.03	$4.505.\ 10^{-3}$	$4.513.\ 10^{-3}$	100.193	3.641
60	180.07	$4.814.\ 10^{-3}$	$4.826.\ 10^{-3}$	100.249	1.635

Test requirements for accuracy concentration of inulin in 1000 ppm are 95-105% with RSD ≤ 3.7% (Sonia et al., 2017; AOAC International, 2016). The mean recoveries obtained should be included in that range. From Table 6, it can be seen that the inulin produced % recovery ± RSD = 100.161% ± 1.839%, so it can be concluded that this analytical method generated accurate data.

Inulin assay in some of Yam bean centra in different altitude area

The result of the determination of inulin concentration in the extract of yam bean tuber with three replication at each level can be seen in Table 5.

Tabel 5. Result of inulin assay in water extract of yam bean tuber

Sample	Level of Inulin (%)	RSD (%)
Gresik	9.066	1.218
Kediri	7.776	0.420
Malang	6.796	2.045

Based on the results obtained from Table 5 it is known that the water extract of yam bean tubers from low altitude areas (Gresik), showed the greatest levels of inulin 9.066%±1.218%. Next is inulin concentration in the water extract of yam bean tubers from middle areas (Kediri) 7.776%±0.420%. Yam tuber water extract of the high altitude (Malang), has the smallest levels of inulin in the amount of 6.796%±2.045%. These results meet the required range of RSD ≤ 2.7% for the actual content of an analyte in a sample of ≥ 1% (Sonia et al., 2017; AOAC International, 2016).

The results obtained indicate that the difference in height affects the content of inulin in the yam tubers. This difference is due to the difference in the height of sea level caused a real difference in environmental conditions such as air temperature, relative humidity, solar radiation, and precipitation. The higher the surface area of the sea, the lower the temperature, the lower the solar radiation, the higher the relative humidity, and rainfall is higher (Hilman, 2014). Some things that affect groundwater levels in an area can lead to differences in levels of inulin in the tubers of yam from regions with different heights. For example, a study on the effect of water availability on growth and active ingredients of Summer savory (*Satureja hortensis L.*). Happen to mention that the low availability of water provides the highest levels of essential oil. The higher the level of availability of water, the levels of essential oil will decrease (Radácsi et al., 2016).

Another study shows that the total dissolved solids on onion bulbs with the location, varieties, and different soil moisture showed that total dissolved solids, which can be used to observe the content of soluble solids in onion bulbs and including the sugar content, the less in the higher areas. Inulin assay yam tuber water extract showed the same result, namely the higher areas of the sea surface, the lower the levels of inulin. Conversely, the lower area of the sea surface, the higher the levels of inulin (Mohammad & Moazzam., 2012). Inulin is a polysaccharide produced from the process of photosynthesis in cells that contain chlorophyll. The process of photosynthesis will be influenced by the intensity of light. The intensity of this light will also be influenced by the height of the place to grow where the higher the place to grow, the intensity of the light will be smaller (Safrina & Priyambodo, 2018). The low light intensity causes the photosynthesis process that is produced cannot to run optimally so that less inulin is produced.

A homogeneity test was performed using the Levene test which obtained a significance value is 0.258 > 0.01 which indicates that the data variance is homogeneous. Because the data is the same variant, the ANOVA test is valid. In the ANOVA test obtained significance value is 0.001 < 0.05 which means there are at least inulin levels that were significantly different in the three groups. Such differences can be identified by further testing using the One-Way ANOVA test, post hoc analysis. The analysis of the results is as follows: Gresik with Kediri, p = 0.000; Gresik with Malang, p = 0.000; Malang and Kediri, p = 0.000; by 99% confidence level. Thus, differences in levels of inulin in all sample groups were significant with a significance value of 0.000.

CONCLUSION

Determination of inulin in the water extract of yam bean tubers can be achieved by TLC densitometry and the result analysis were Linear, sensitive, persist and accurate. There is an effect of altitude on the levels of inulin in yam tubers where the inulin content in yam tubers from lowlands has higher levels of inulin than medium and high altitude areas.

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