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A Densitometric Method for Determination of Mangiferin, an Antioxidant Compound, with Thin Layer Chromatography in the Leaf Extracts of Coffee (*Coffea Arabica* L.)

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Abstract: Mangiferin is one of the antioxidants in Coffea arabica L. leaves that has many pharmacological effects. The content of secondary metabolites in the leaves including mangiferin can be affected by age. A Thin Layer Chromatography (TLC) method for the quantitative analysis of mangiferin in Coffea arabica L. leaves extract was developed and validated. The method was developed using a mobile phase prepared with analytical grade solvents: ethyl acetate, methanol, formic acid, and deionized water (8:2:1:1 v/v/v/v). Regression functions were established over the 199.98-600.00 ng/spot range with r=0.999. The limit of detection (LOD) and limit of quantification (LOQ) were 13.87 and 41.61 ng, respectively. The method was selective with a resolution value of more than 1.5 and specific with the spectra correlation value for purity and identity check of more than 0.99. The percentage RSD was found to be 2.43% for repeatability precision and 2.05% for intermediate precision. The method's accuracy was determined through the standard addition method by adding known quantities of standard mangiferin to the pre-analyzed test solution and the mean recovery was 101.69± 1,21%. This TLC Densitometry method was linear, sensitive, selective and specific, precise, accurate, and can be used for routine analysis of mangiferin. On the young Coffea arabica L. leaves, the concentration of mangiferin ± RSD was 0.830 ± 1.71% w/w, and on the old Coffea arabica L. leaves was 1.128± 1.59% w/w.

Keywords: *Coffea arabica L.*, mangiferin, validation, TLC-densitometry

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1. INTRODUCTION

Coffea arabica L. is the most developed Coffee in the world (1). *Coffea arabica L.* leaves contain highly antioxidant compounds. One of the antioxidants in *Coffea arabica L.* leaves is mangiferin (MGF). MGF is a natural xanthonoid with various biological activities they are antioxidant (2,3), anti-inflammatory (4,5), radioprotective, immunomodulatory (4), hypouricaemic (6), antimicrobial (7), anticancer (8), and antidiabetic (9,10,11). MGF has been isolated from various parts of *Mangifera indica*, but in 2008, a report that mangiferin was isolated from leaves of a wild *Coffea arabica L.*, *Coffea pseudozanguebariae* (11,12).

MGF is a C glucopyranoside of 1, 3, 6, and 7-tetrahydroxyxanthone. MGF features a highly condensed aromatic ring system coupled to a glucose moiety via an unusual C-C bond. The structure of MGF satisfies Lipinski's rules for druglike properties: molecular weight less than 500, cLogP = 2.73, fewer than 5 donor functions for hydrogen bonds; and fewer than 10 acceptor functions for hydrogen bonds (13). Some research results show that their age can affect the phenolic compound content in plant parts. MGF is also a phenolic compound whose content in *Coffea arabica L.* leaves is suspected to be affected by its age.

Literature review revealed that MGF can be determined by High-Performance Thin Layer

Chromatography (HPTLC) (14), High-Performance Liquid Chromatography- Ultra Violet (HPLC-UV) (15), High-Performance Liquid Chromatography (HPLC) and Mass spectrometry with Nuclear Magnetic Resonance (NMR) methods (12). The existing method for determining mangiferin levels has several drawbacks, including requiring expensive equipment, long analysis time, and requiring special skills. Thin-layer chromatography (TLC) is a chromatographic technique widely used for qualitative and quantitative analysis of organic compounds, isolation of the individual compound from multicomponent mixtures, and preparative-scale isolation (16). Conventional TLC is a quick, inexpensive, flexible, and portable method of surveying the composition of mixtures (16). With the development of modern precoated layers and the introduction of partially or completely automated equipment for the various stages of operation of TLC, not only are highly accurate quantitative determinations now possible but also the requirement that the work should comply with the Good Manufacturing Practice (GMP) and (Good Laboratory Practice (GLP) guidelines can be fulfilled (17). This study aimed to develop and validate the TLC-densitometry method to determine MGF levels in *Coffea arabica L.* leaves extracts of different ages, as an initial effort to utilize *Coffea arabica L.* leaves as a potential source of MGF.

2. MATERIAL AND METHOD

2.1. Sourcing and preparation of MGF extract from *Coffea arabica L.* leaves

Coffea arabica L. leaves as raw materials (Research Center for *Coffea arabica L.* and Cocoa, Jember, East Java). *Coffea arabica L.* leaves were separated from the peels, washed, and the washed leaves were then air-dried. The dried leaves were then blended and sieved with a B40 sieve to obtain *Coffea arabica L.* leaves powder with a uniform size. For sample extraction, 225 mg *Coffea arabica L.* leaves powder was extracted with petroleum ether (2 x 2 L, 6 h each time) to remove fatty matter, with cold acetone (4 x 2 L, 24 h each time) to remove tannins and finally with 70% ethanolic solvent (4 x 1 L, 6 h each time) (18). For sample preparation, 72 mg extract was diluted with 10 mL methanol and filtered with Whatman filter paper no.40.

2.2 Chromatographic condition

Planar chromatography was performed by spotting the sample on 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 a Camag Twin Through Chamber containing eluent ethyl acetate: formic acid: methanol: DI water (8:1:2:1 v/v/v/v) was saturated. The spot moves to a distance of 9 cm. Densitometric scanning in the absorbance 325 nm for all measurements. Quantitative evaluation was performed via peak areas by WinCats software (version 1.4.1.8154).2.3

2.3. Standard solution preparation

A Standard stock solution was prepared by dissolving 5.0 mg of MGF in methanol and transferred to a 10.0 mL calibration volumetric flask and 10.0 mL until a mangiferin stock solution was obtained with a concentration of 500.0 ug/mL. The standard stock solution of MGF was then diluted with methanol to obtain a standard solution with a concentration range of 30-100 ug/mL.

2.4. Construction of calibration curves

Calibration solutions were prepared by diluting the stock solution, so that the application of 6 µL volume gave a series of spots, covering the calibration range 199.98-600.00 ng of mangiferin. Sample application on 10 cm x 20 cm aluminum-backed silica gel 60 F254 TLC plates (E. Merck, Darmstadt, Germany) stored in a desiccator was 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 5.0mm. 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 30 min with the mobile phase ethyl acetate: formic acid: methanol: deionized water (8:1:2:1 v/v/v/v). Following the TLC running, the plates were dried with an air dryer. After elution, the plate densitometry scanning was performed at 25 nm on a Camag TLC scanner 3 operated by winCATS software version 1. .4.1.8154. The area under the peak was recorded and calibration curves relating the integrated area under the peak versus the corresponding concentrations as ng/band were then constructed, from which the polynomial regression equations were computed.

2.5. 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.

2.6. Determination of mangiferin

Young and old *Coffea arabica L.* leaf extracts were prepared as a sample preparation method. Determination of MGF levels in young and old Coffea arabica L. leaves extracts was carried out by spotting 6 µL of the sample on a silica gel 60 F254 TLC plates and analyzing it under optimum conditions and scanning with densitometry at a wavelength of 325. Assays were replicated (n=3) and spotted on plates.

2.7 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 MGF in an extract of *Coffea arabica L.* young and old leaves obtained. The analysis was conducted to test the normality and homogeneity. The normality test used the Shapiro-Wilk and the homogeneity test was performed using the Levene test.

2. RESULTS AND DISCUSSION

3.1 Optimation of the eluent and wavelength

Good separation in the TLC system can be produced at optimum separation conditions. The analytical conditions that greatly affect the separation process in TLC are the mobile phase selection (19). In addition to selecting the mobile phase, other analytical conditions optimized for determining MGF include solvent, test concentration, measurement wavelength, and development mode. The optimum separation conditions for MGF separation in *Coffea arabica L.* leaves extract are shown in (Table 1)

Table 1: Optimum condition for analysis of mangiferin.

Condition	optimum
Solvent	Methanol
Stationary phase	Silica Gel 60 F_{254}
Eluent	Ethyl acetate: methanol: formic acid: deionized water (v/v/v/v) = 8:2:1:1
Wavelength	325 nm
Method development	Ascending

(Table 1) showed the optimum conditions for the analysis of MGF using TLC densitometry. The selected mobile phase was a mixture of Ethyl acetate: methanol: formic acid: deionized water = 8:2:1:1(v/v/v/v) with the value of Rf is 0.8 (included in the range of 0.1-0.9), the value of N = 1024 and H = 0.0009. Selection of the maximum wavelength for MGF was done using the light of UV and UV Vis with a range between 200-700 nm, and the selected wavelength is 325 nm. Selected wavelength is the wavelength of the spectrum in high intensity. The results of standard MGF scanning spectra can be seen in (Figure 2).

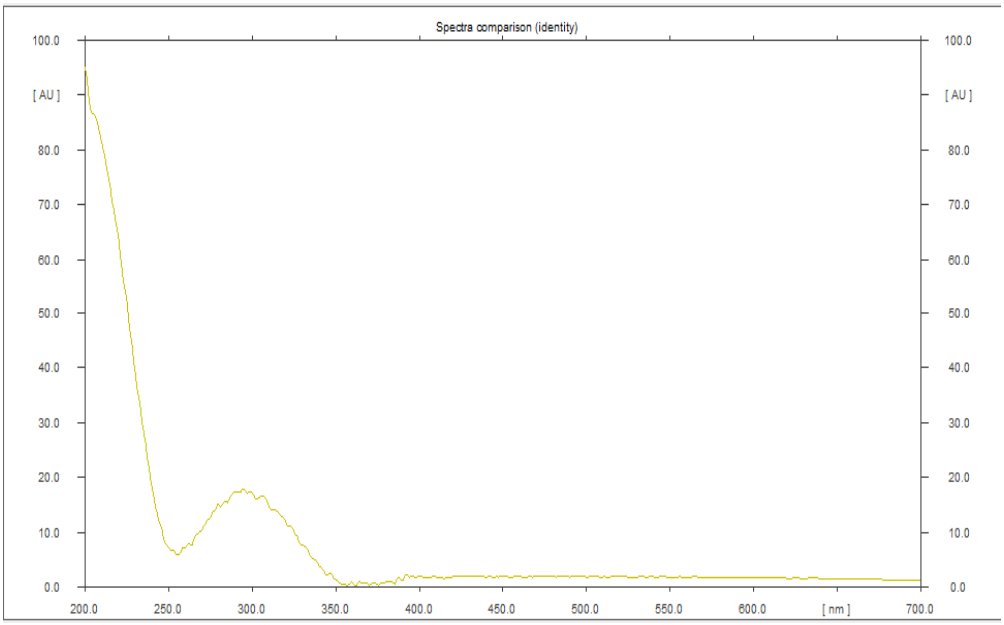

Figure 1: Spectrum of MGF standard at a wavelength of 200-700 nm.

Based on the spectra, it can be seen that the highest spectra intensity is reached when the wavelength at 325 nm with an absorbance signal of MGF is 49.9 AU. The chromatogram of the results of the MGF analysis at optimum conditions is shown in (Figure 3)

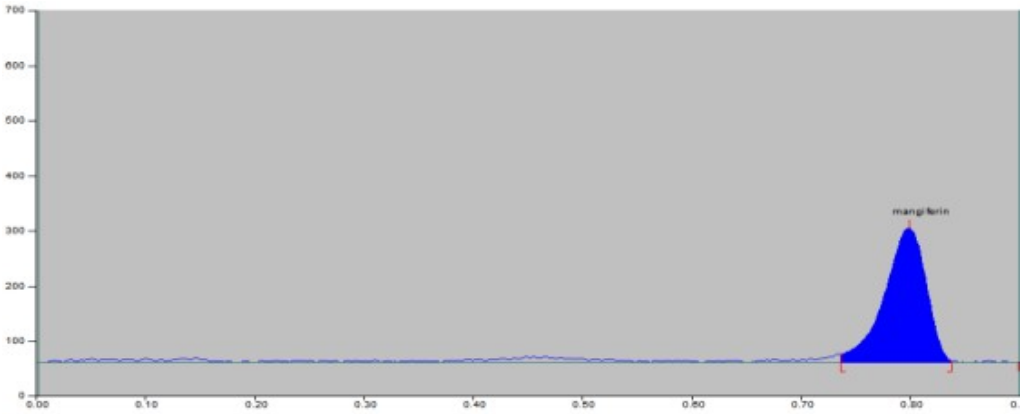

Figure 2: Chromatogram of mangiferin standard.

The chromatogram shown in (Figure 3) proves that under optimum conditions the developed TLC-Densitometri method is capable of separating and detecting MGF properly.

3.2. Validation of analysis method

3.2.1. Linearity

Linearity is the capacity of an analytical technique to produce an outcome that is directly related to the concentration of an analyte in the sample (20). The standard linearity curve for MGF at a series of concentrations in this study is shown in Figure (Figure 4) below.

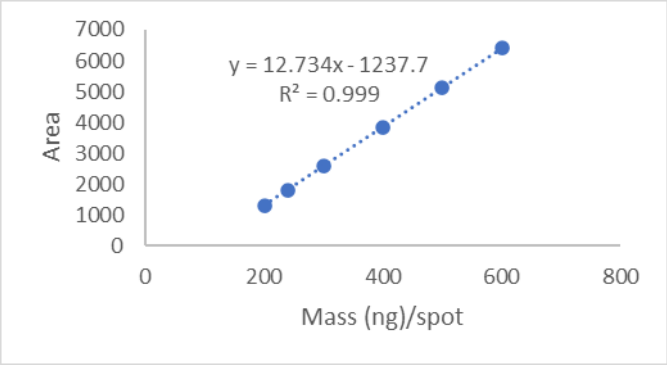

Figure 4: Graph of equation curve the correlation between area and mass (ng)/spot of MGF.

The linearity curve shown in (Figure 4) is a linearity curve of 6 standard concentrations of MGF with concentration ranges of 200-600 ng/spot. The equation obtained from 6 standard concentration measurements is y=12.73x–1237.66 with correlation coefficient (r) 0.999, Vx0=0.15%, and Xp=3.72 ng. The result showed that the MGF compound has a proportional correlation between mass and area indicated with an r value more than 0.99 (21), which is known to have met the requirements of the r value > 0.99, Vxo <5% (21), (22) then the curve said to be linear.

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

The determination of the Limit of Detection (LOD) and Limit of Quantification (LOQ) in this study was to determine the sensitivity of the TLC-Densitometry method for determining the levels of MGF in *Coffea arabica L.* leaves extract.

The LOD value is the minimum concentration of an analyte that can be detected and LOQ is the minimum amount of an analyte that can be quantified (23). Determination of LOD and LOQ values in this study was carried out by making a standard calibration curve for MGF in the concentration range of 36-132ng/spot. then the LOD and LOQ values are determined using software of validation method version 1.03. The LOD and LOQ were found to be 13.87 ng and 41.61 ng with correlation coefficients 0.998, Vx0= 2.5%, and Xp=13.87 ng. This result indicates that the resulting method is sensitive because the LOQ value was ≤ 400 ng/spot (22)

3.2.3. Selectivity/Specificity

The selectivity/ specificity of an analytical method is its ability to measure accurately an analyte in the presence of interferences that may be expected to be present in the sample matrix (24). Selectivity was determined by analyzing the sample. Selectivity was shown by the resolution that was calculated from the MGF peak to the unknown peak in the sample chromatogram. The results of separation with TLC show that no interferences were observed, meaning that it can separate MGF from other components in the sample, this is evidenced by the resulting resolution value (Rs) > 2, as shown in (Figure 5.)

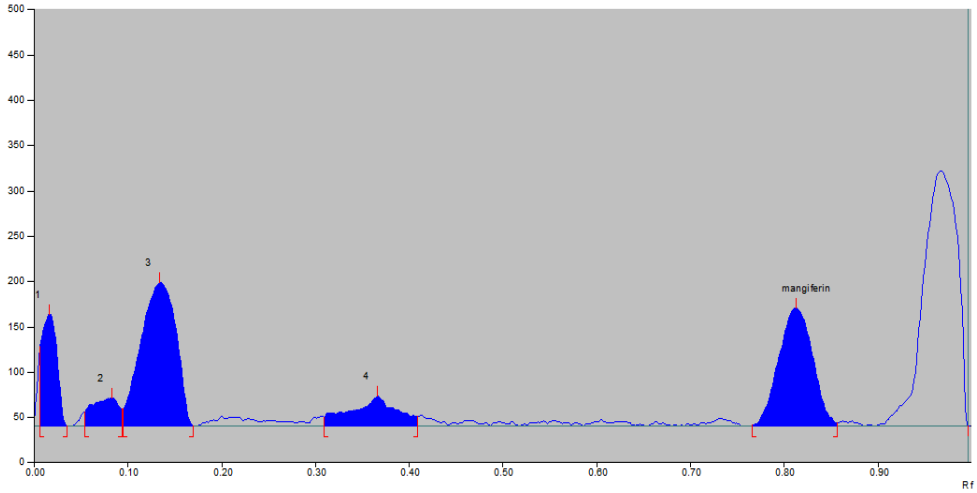

Figure 4: TLC-chromatogram of MGF in ethanol extract of *Coffea arabica L.* leaves using a selected solvent.

Specificity was determined by analyzing standards and samples. The purity and identity test showed specificity, which was determined by scanning at 200 nm–700 nm. Calculations for identity checks were from r(S.S) and r(S.A) where S is spectrum standard and A is spectrum sample and purity checks were from r(S.M) and r(M.E) where S = start, M = center; and E = end of the spectrum. The result of the specificity test is shown in (Figure 6); (Tables 2 and 3).

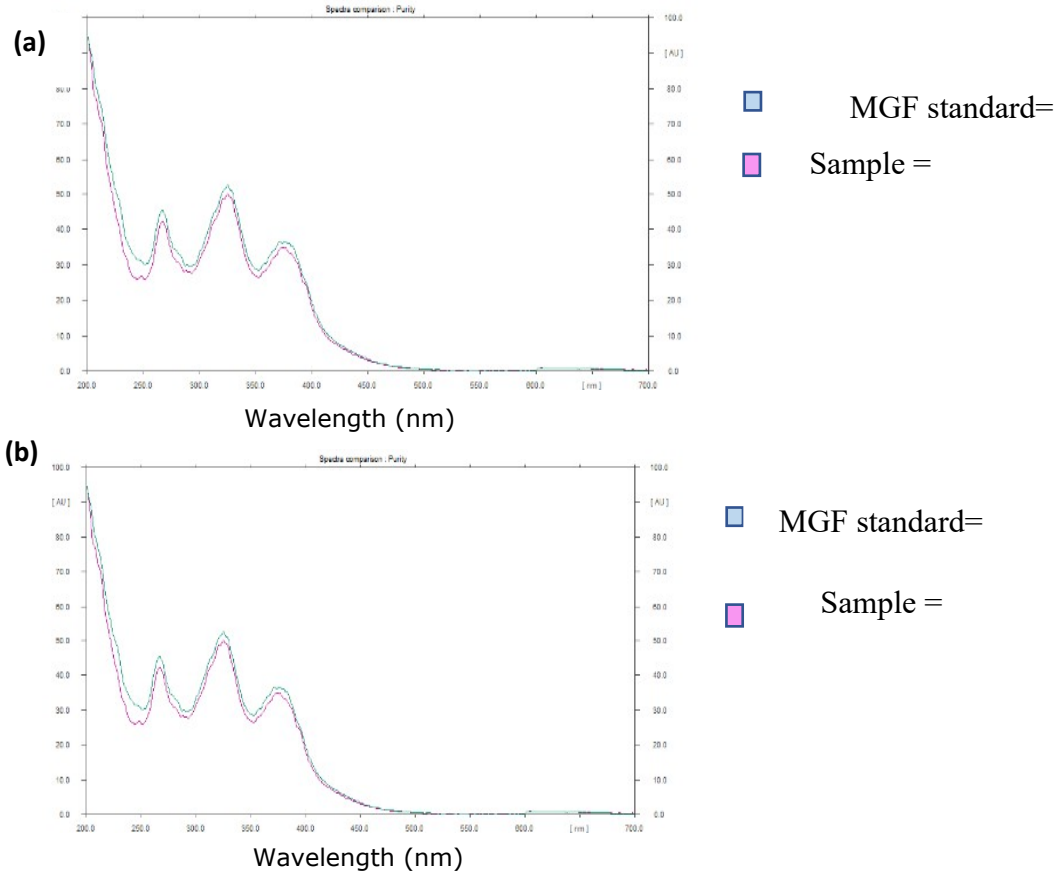

Figure 6: Spectra of standard and MGF samples in the **(a)** identity test; and **(b)** purity test.

Table 2: Purity Test of the Proposed Method.

Test	Track	Rf	r(s,m)[a]	r(m,e)[b]	Conclusion
Purity	Standard	0.81	0.998642	0.991039	*Pure*
	Sample	0.82	0.997896	0.993178	*Pure*

[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 3. Identity test of the proposed method.

Test	Track	Rf	r(s,s)[a]	r(s,a)[b]	Conclusion
Identity	Standard	0.81	0.998947	0.99778	MGF
	Sample	0.82	0.998947	0.99275	MGF

[a]: the correlation coefficient between the two tracks' standard spectra(s,s) in 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 5) ;(Tables 2 and 3) it can be seen that the spectra of standard and inulin samples have identical spectra. It can be concluded that the sample was containing MGF. The purity check of the analyte spots using winCATS software showed that the analyte spots were pure. The values of r (s, m) and r (m, e) were >0.99 in the identity check showing that analyte spots in samples were identical with standard mangiferin. This purity and identity assay demonstrates that the proposed TLC Densitometry method is highly specific (Figure 5).

3.2.4. Precision

The precision was performed by repeatability and intermediate precision studies. Repeatability studies were performed by analyzing the 100% concentration of the sample six times on the same day. The intermediate precision was checked by repeating repeatability studies on three different days (25) In this research, the precision that was used is repeatability and intermediate precision. Repeatability test, measured test analyte concentration in the sample with 6 replications. To determine the intermediate precision, performed the same experiment as the repeatability test, but performed on three different days. Precision is measured by the value of RSD / CV (relative standard deviation / relative standard deviation). The result of repeatability and intermediate precision can be seen in (Table 4).

Table 4: The precision of MGF in ethanol extract of Coffea arabica L. leaves

Weight of samples (mg) (Mean ±SD)	Intra-day		Inter-day		
	Conc. % (Mean±SD)	RSD (%)	Day	Conc.% (Mean ± SD)	RSD (%)
73.517 ± 1.141	1.028 ± 0,025	2.43	1	1.028 ± 0.025	2.43
			2	0.993 ± 0.016	1.57
			3	1.051 ± 0.023	2.16

Based on the results shown in (Table 4), the RSD values for repeatability and inter-day precision of the analysis method are respectively 2.43% and 1.57-2.43%. Based on the RSD value obtained, it can be concluded that the method is precise and meets the precision requirements of AOAC, which is 2.7% (22)

3.2.5. Accuracy

The accuracy of the analysis method is expressed as the nearness of agreement between the values found and values that are already available. It can also be defined as the closeness between the true value and observed value, which is determined based on the value of % recovery (20). The accuracy test is done by calculating the % recovery 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 replications at each level (26). The accuracy test results of the analytical method expressed as % recovery are shown in (Table 5).

Table 5: Accuracy of the proposed method.

Inulin added to the analyte (%)	**Weight of samples addition (mg)**	**Theoretical content (mg)**	**Concentration found (mg)**	***Recovery* (%)**	**RSD (%)**
30	72.25	961.8 10^{-3}	983.0 10^{-3}	101.3	0.6
45	72.25	1072.8 10^{-3}	1067.0 10^{-3}	102.23	1.13
60	72.25	1184.0 10^{-3}	1213.0 10^{-3}	101.47	1.91

Test requirements for accuracy concentration of 1% MGF are 97-103% with RSD ≤ 2.7% (26), (22). The mean recoveries obtained should be included in that range. From (Table 5), it can be seen that the analytical method produced % recovery ± RSD = 101,3-102.23% ± 0.6-1.91%, so it can be concluded that this analytical method generated accurate data.

3.3. MGF assay in ethanol extract of coffee arabica leaves in different age

The result of the determination of MGF concentration in the extract coffee arabica leaves with three replications at each age is shown in (Table 6).

Based on the results in (Table 6), show that there are differences in the MGF content in *Arabica coffee* leaves of different maturities. Old leaves have a mangiferin content of 1.128±1.59 % while young leaves have an MGF content of 0.830 ±1.71 These results are also by previous research conducted by Campa et al where the highest MGF content was found in leaves with the highest maturity (27).

A homogeneity test was performed using the Levene test which obtained a significance value is 0.56 > 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, the obtained significance value is 0.001<0.05, meaning there are at least MGF levels that were significantly different in the two groups.

Table 6: Result of MGF assay in ethanol extract of coffee arabica leaves

Sample	Level of MGF (%)	RSD (%)
Young leaves	0.830	1.71
Old Leaves	1.128	1.59

4. CONCLUSION

Determination of MGF in the ethanol extract of coffee arabica leaves can be achieved by TLC densitometry and the result analysis was Linear, sensitive, persistent, and accurate. Based on the results of the assay, it can be concluded that the MGF content in Arabica coffee leaves is affected by age, where the older the age, the higher the MGF content.

5. CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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