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Validated TLC-Densitometry Method for Determination of Chlorogenic Acid In Coffee Leaves Extract.

Nia Kristiningrum[1]*, Yuni Retnaningtyas[1], and Ni Putu Pertiwi[1].

Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Jember University, Indonesia.

ABSTRACT

Thin Layer Chromatography method has been developed and validated for determination of chlorogenic acid in coffee leaves extract. The method was developed using a mobile phase prepared with analytical grade solvents: formic acid, ethyl acetate, aquabidest (1:8:1.5 v/v/v). Chlorogenic acid reference material and samples were chromatographed using pre-coated TLC silica gel 60 F_{254} plates and followed by densitometric measurements of their spots at 335 nm. Regression functions were established over the range of 5.02-194.58 ng/spot with r = 0.998. The limit of detection and limit of quantitation were 16.22 ng and 48.66 ng, respectively. The method was selective with resolution value more than 1.5 and specific with the spectra correlation value for purity and identity check more than 0.99. The percentage RSD was found 0.99% for repeatability precision and 1.75% for intermediet precision. The accuracy of the method was determined through standard addition method by adding known quantities of standard chlorogenic acid to the pre analyzed test solution and the mean recovery was 99.72% ± 1.58%. This TLC Densitometry method was linier, sensitive, selective, specific, precise, accurate and can be used for routine analysis of chlorogenic acid.

Keywords: Validation, TLC Densitometry, Chlorogenic Acid, Coffee Leaves Extract

Corresponding author

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INTRODUCTION

Coffee is one of antioxidants for human body. High content of antioxidants in this beverages is chlorogenic acid (CGA), which is one of polyphenolic compounds [1]. The green coffee bean contained 6-12% of chlorogenic acid [2]. In addition to the coffee bean, coffee leaves turns out to contain 25-46% of phenolic compounds [3]. Possibly one of the phenolic compounds contained in coffee leaves is chlorogenic acid.

CGA is an ester formed from trans-cinnamic acid and quinic acid having a hydroxyl group in the axial position on carbon 1 and 3 as well as equatorial hydroxyl at carbon 4 and 5 (fig. 1) [2]. CGA has antibacterial activity, antimutagenic, antitumor, antiviral, anticancer, analgesic, antipyretic, anti-inflammatory and antifungal [4].

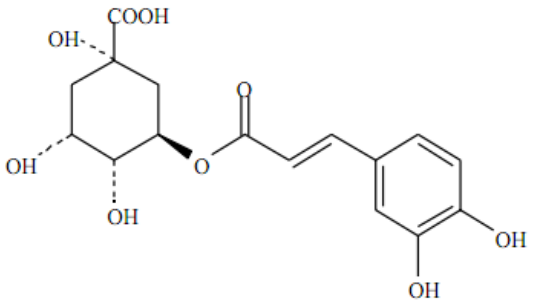

Figure 1: Structure of chlorogenic acid

Literature review revealed that CGA research mostly in coffee beans with a variety of methods such as High Performance Thin Layer Chromatography (HPTLC) and High Performance Liquid Chromatography (HPLC) [5], High Performance Gel Filtration Chromatography [6] and spectroscopy UV-VIS [7].

Determination of CGA content in coffee leaves is important to do in order to find out the natural antioxidant. The importance of this research is also supported by the lack of publications regarding the determination of CGA in coffee leaves.

The developed TLC method has several advantages over other available methods such as ability to analyze several samples simultaneously in parallel, as well as using small quantities of solvents as a mobile phase which reduces time and cost of analysis [8].

MATERIALS AND METHODS

Materials

Chlorogenic Acid working standard (Sigma-Aldrich), methanol, etyl acetate, formic acid, aquabidest. Sample of coffee leaves used were old and young arabica and robusta coffee leaves. Samples were taken from plantations of Indonesia Coffee and Cocoa Research Center.

Preparation of standard solution and samples

Standard solution was always freshly prepared by dissolving 10 mg of CGA in methanol p.a up to 10 ml. the standard solution of CGA (1000 ppm) was diluted to get solutions in concentration range of 30-100 ppm. For sample extraction, 250 mg coffee leaves powder was extracted using 70% of methanol and then concentrated to form a thick extract. For sample preparation, 80 mg extract transferred to a weighing bottle and added with 5 ml methanol p.a. The bottle was placed in ultrasonic cleaner until the extract was soluble. The solution transferred to 25 ml volumetric flask and then diluted up to the mark with the same solvent.

Chromatographic condition

Planar chromatography was performed by spotting the sample on precoated TLC silica gel F_{254} (20 x 10 cm) using 2.0 µl glass capillaries. A Camag Twin Through Chamber containing a mixture of formic acid:ethyl

acetate:aquabidest (1:8:1.5 v/v/v) was saturated. The spots move to a distance of 9 cm. Densitometric scanning was performed on camag TLC Scanner 3 in the absorbance mode at 335 nm for all measurements. CGA was detected at Rf 0.47. Quantitative evaluation was performed via peak areas by WinCats software (version 1.4.1.8154).

Method Validation

The method of analysis was validated for the parameters like linearity, Limit of Detection (LOD), Limit of Quantification (LOQ), selectivity & specificity, precision and accuracy.

Linearity

The evaluation of the calibration curve's linearity was done by spotting 59.02, 78.69, 88.52, 98.36, 129.72, 151.34, 172.96 and 194.58 ng/spot of standard solutions. Peak area was record for each concentration and a calibration curve was obtained by plotting peak area vs concentration.

Limit of detection and quantification

Standard solution were prepared at the concentration 17.98, 20.88, 23.78, 26.68 and 29.58 µg/ml. The 2 µl of each solutions was spotted on the TLC plate. Peak area was recorded for each concentration. Limit of detection (LOD) and Limit of Quantification (LOQ) were determined using software validation method version 1.03.

Selectivity and Specifity

Selectivity of this method was determined by analyzing sample. Selectivity was showed by resolution that calculated from CGA peak to unknown peak in sample chromatogram. Specificity of this method was determined by analyzing standard and sample. Specificity was showed by purity and identity test that 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 of standard and A is spectrum of sample and purity checks were from r(S.M) and r(M.E) where S is start, M is center; and E is end of spectrum.

Precision

The precision of this method was performed by repeatability and intermediate precision studies. Repeatability studies was performed by analyzing one concentration of the sample for six times on same day. The intermediate precision was checked by repeating repeatability studies on three different days.

Accuracy

The accuracy of this method was evaluated through recovery experiments by adding three different amounts of CGA standards i.e. 30, 45 and 60% of the concentration samples. Each concentration were replicated (n=3).

Analysis of coffee leaves extract

Old and young leaves extract of robusta and arabica coffee were prepared as sample preparation method. Each of samples were replicated (n=3) and spotted on TLC plates. The analysis was done in the same way as described earlier.

Data analysis

Data analysis purposed to determine whether or not significant differences of CGA levels in Old and young leaves extract of robusta and arabica coffee using SPSS 16.0 software with the level of confidence was 99%.

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RESULTS AND DISCUSSION

Table 1 showed the optimum conditions for analysis of CGA using TLC densitometry. The mobile phase of formic acid:ethyl acetate:aquabidest (1:8:1.5 v/v/v) give the efficient chromatogram. Efficiency of chromatogram was evaluated by the value of Number of Theoritical Plate (N), Height Equivalent to a Theoritical Plate (H) and resolution (Rs). The Rf of analyte is 0.47 (Fig. 2).

Table 1: Optimum condition for analysis of chlorogenic acid

Parameters	Data
Solvent Stationary phase Eluent	Methanol p.a Silika Gel 60 F_{254} Asam format : etil asetat : aquabides (v/v/v) = 1:8:1,5
Wavelength	335 nm
concentration Method development	50 ppm Ascending

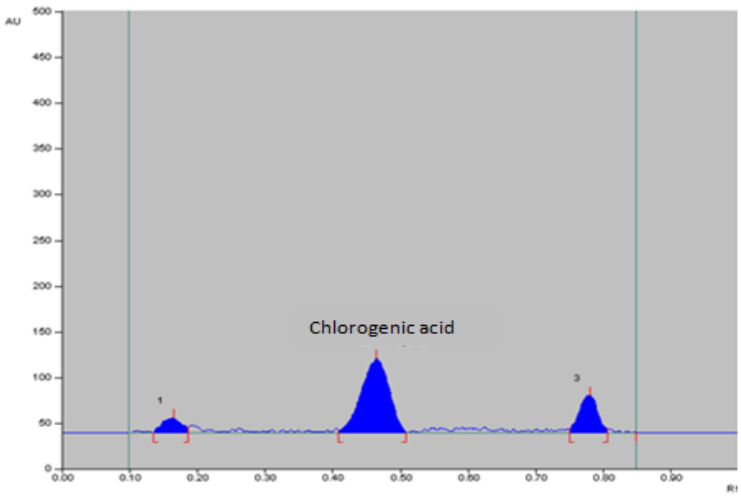

Figure 2: Chromatogram of robusta coffee leaves extract

Method validation

Linearity

The linear regression data for the calibration curves showed good linear relationship over the concentration range 59-194 ng/spot. Linear regression equation was found to be y=24.71x–200.88 with correlation coefficient (r) 0.998, Vx0=2.60% and Xp=16.99 ng.

Limit of detection and quantification

The LOD and LOQ were found to be 16.22 ng and 48.66 ng. This indicates that adequate sensitivity of the method.

Selectivity and Specifity

TLC Densitometry data showed the method was selective with resolution value more than 1.5. Purity check of the analyte spots using winCATS software showed that analyte spots were pure. The values of rS,M and rM,E were more than 0.99. Identity check showed that analyte spots in samples were identical with standard CGA. This purity and identity assay demonstrating that proposed TLC Densitometry method is highly specific.

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Precision

All the values of the repeatability and intermediate precision evaluation were less than 2.7% [9], showed in Table 2. The three measurements were perfomed within one laboratory by same analyst on different plates and different days.

Table 2: Intra-day and inter-day precision of TLC method

Measurements[a]	RSD value (n=6)[b]
1	0,99 %
2	2,15 %
3	2,12 %
Average RSD	1,75 %

[a]Each measurement was perfomed by the same analyst and on a different plate and different days
[b]Evaluted by one analyst on one plate (repeatability)

Accuracy

The accuracy of the proposed method were 99.72%±1.58% (Table 3). This result had met the requirements that were in the range 97-103% [10]. The summary of data validation parameters as listed in Table 4.

Table 3: Accuracy of TLC method

Analyte	Added (%)	Recovery ±RSD (%)
Chlorogenic acid	30	102.12±1.97
	45	98.28±1.16
	60	98.75±1.61
Average recovery±RSD		99.72±1.58

Table 4: Summary of Validation Parameter

Parameters	Value
Linierity	r=0.998; Vx0=2.60%; Xp=16.99 ng
Sensitivity	LOD=16.22 ng LOQ=48.66 ng
Selectivity Spesificity Precision Accuracy	Resolution >1.5 Purity test≥0.99 Identity test≥0.99 RSD=1.75% 99.72%±1.58%

Analysis of coffee leaves extract

Table 5 showed the concentration of chlorogenic acid in coffee leaves extract . All samples were significantly different ($p<0.01$).

Table 5: Analysis of Chlorogenic acid in coffee leaves extract

Leaves extract	%w/w±RSD (%)
Old arabica	2.79±1.87
Young Arabica	1.89±2.15
Old robusta	1.46±0.83
Young robusta	1.05±1.19

CONCLUSION

The TLC-Densitometry method for the determination of chlorogenic acid in coffee leaves extract was linier, sensitive, selective, specific, precise and accurate, so can be used for routine analysis of chlorogenic acid

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