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Validation of Rutin Analysis in Cassava (*Manihot esculenta*) Leaves Using TLC-densitometer

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Introduction: Cassava leaf is the lesser used part of cassava compared to the root, which is a well known source of tapioca flour. Cassava leaves contain several compounds with health benefits, especially a flavonoid called rutin. The aim of this study was to develop and validate method for quantitative analysis of rutin in cassava leaf extract. **Methods:** All cassava leaves were extracted with 80% methanol and applied semi-automatically in TLC aluminum sheets, precoated with 0.2 mm thick layer of silica gel 60 F-254. The mobile phases were ethyl acetate-acetone – water [40:50:10] and ethyl acetate – formic acid- water [60:10:10]. The extracts were analyzed for rutin content using TLC densitometry. **Results:** The solvent system gave a compact spot for rutin with R_f value of 0.47. Linear regression analysis data for the calibration curve of rutin showed good linear relationship with r^2 of 0.999 in the concentration range from 200 – 1000 ng/spot. The method was validated for interday and intraday precision (overall % RSD < 6.91 %) and accuracy (% recovery = 97.69 -99.96 %). The limit of detection and quantification were 20 ng/spot and 100 ng/spot, respectively. The developed method was applied to determine rutin contents in cassava leaf extract from Kasetsart 50 cultivar and the contents was 12.30±0.61% dry weight. **Conclusions:** The developed TLC-densitometric methods provided a good accuracy and selectivity for the quantitative determination of rutin in cassava leaves. The rutin content analyzed by this method is effective and reliable.

Keywords: rutin, cassava leaf, TLC densitometer

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1. Introduction

Cassava (*Manihot esculenta*) is one of the major crops of Thailand, cultivated mainly for food and tapioca flour. Processed cassava products are available in several forms such as chip, pellets and also used in the ethanol industry (Nguyen TLT *et al*., 2007) for gasohol production. Cassava root is the main part-used of the plant where as cassava leaves are readily available in considerable amounts as a by-product at the time of harvesting. Cassava leaves are primarily used in animal feed as a source of protein. Cassava root is the good sources of some important minerals like zinc, magnesium, copper, iron, and manganese (Eggum RO, 1970; Adewusi SRA, Bradbury JH, 1993). All parts of cassava need to be handled and treated because unprocessed cassava plant contains potentially toxic levels of a cyanogens is glycoside called linamarin, which can be converted to cyanide. Even though the leaves are less commercially important than the root, they are rich in crude fiber, protein and also contain secondary metabolites such as alkaloids flavonoids and tannins, (Blagbrough IS *et al*., 2010). Cassava leaves are also the sources of the beneficial compound rutin. The flavonoid rutin (quercetion–3-O-Rutinoside :Figure 1) is a flavonol glycoside found in several medicinal plants, and is a well known as antioxidant used as a treatment for haemorrhoids (Prawat H *et al*., 1995). The current study aims to promote the use of cassava leaves focusing on the rutin content selected cassava cultivars. Chromatographic fingerprints are designed follow the Thai herbal pharma copoeia and the results will be useful to determine the characteristics and quality control of cassava leaves.

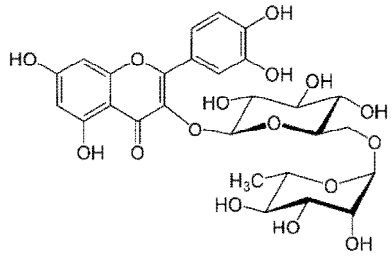

Figure 1: Chemical structure of rutin

2. Material and Methods

Plant material

11 cultivars of cassava leaves, named Rayong1, Rayong2, Rayong3, Rayong5, Rayong7, Rayong9, Rayong11 Rayong72, Rayong90, Huaipong60 and Kasetart50, grown in Khonkaen were provided by Khon Kaen Field Crops Research Center. The plants were collected when they were 6 month olds. Fresh cassava leaves were washed by water, air dried and ground to powder using a mortar and pestle with liquid nitrogen. The powder was sifted using sieve number 80 and the powder was stored at -20 °C. All 11 cultivars of cassava leaves were used for the TLC-chromatogram study and checked for pre sence of rutin. The representative cultivar selected for the analysis of rutin content was Kasetsart 50.

Chemicals and instruments

All solvents were of analytical grade as follows: acetone (QRec, New Zealand), ethyl acetate (BDH PROLAB, France), methanol (BDH PROLAB, France), 98% formic acid (Merck, Germany) and glacial acetic acid (Carlo Erba, Spain).

Standard rutin hydrate and linamarin were purchased from Sigma Aldrich, USA. A Camag TLC system (Switzerland) composed of an automatic TLC sampler (Linomat IV), TLC scanner and CATS 4 software was used for sample application and quantitative evaluation.

TLC-chromatogram study of rutin in cassava leaf extract

A TLC chromatogram was performed prior to the analysis of rutin content. All the 11 cultivar were spotted on a TLC plate and were compared for the fingerprint of each cultivar. The dried leaf powder of cassava from each cultivar (250 mg) was weighed in 25 mL volumetric flask and extracted with methanol (20 mL) using sonication method for 30 minutes. The extract was then filtered and adjusted to 25 mL with methanol. Samples were filtered through a Whatman filter paper no 1 and analyzed immediately after extraction. All assays of samples were performed in triplicate. Three mobile phase are applied as follows: Ethyl acetate–Acetone-Water (40:50:10) Ethyl acetate-Formic acid-Water (60:10:10) and Ethyl acetate-Formic acid-Glacial acetic acid-Water (100:10:10:12). After the TLC plates were fully developed to the distance of 80 mm, each plate was sprayed with 2% Aniline-2% Phenylalanine-15% Phosphoric acid, Anisaldehyde-Sulfuric acid Ammonia T.S and Picric Acid. The sprayed TLC plate was photograph and recorded the Rf value of rutin.

Validation of TLC-densitometric method

TLC-densitometric method was validated in terms of linearity, accuracy, precision, limit of detection, limit of quantification and inter-day/intra-day precision. A stock solution of the standard rutin (0.1 mg/mL) was made in methanol, and subsequently diluted to provide a series of the standard ranging from 200,400, 600, 800 and 1000 ng/spot for use in constructing a calibration curve for rutin. The data of peak area versus rutin concentration was treated by linear least square regression analysis to obtain the linearity. Accuracy was evaluated by preparing the known amounts of rutin (400, 500 and 600 ng/band, n=5), area under the curve was analyzed and then % recovery was calculated. The precision of the method was studied by analyzing aliquots of three different concentrations of standard solutions of rutin on the same day (intraday-precision) and on three different days (interday-precision) and % RSD values were calculated. In order to obtain and estimate of the LOD/LOQ, the series of concentrations of the rutin solution (range from 1-100 ng) were spotted on the TLC plate and analysed to determine LOD and LOQ by considering (S/N) ratio. LOD was considered as (Signal/noise ratio) = 3:1 while LOQ as S/N = 10:1.

Determination of rutin content

Kasetsart 50 cultivar was selected as the representative of cassava of Thailand due to its abundance. The dried leaf powder of cassava (250 mg) was weighed into a 25 mL volumetric flask and extracted with methanol (20 mL) using sonication method for 30 minutes. The extract

was then filtered and adjusted to 25 mL with methanol. Samples were filtered through a Whatman filter paper no 1 and analyzed immediately after extraction. All assays of samples were performed in triplicate. Rutin analysis was performed using the validated TLC-densitometry.

3. Results

TLC-chromatogram study of rutin in cassava leaf extract

Three mobile phases mentioned earlier in material and methods yielded the TLC chromatogram in Figure 2. Linamarin was not detected in the TLC chromatogram whereas rutin was detected in all 11 cultivars at Rf = 0.47.

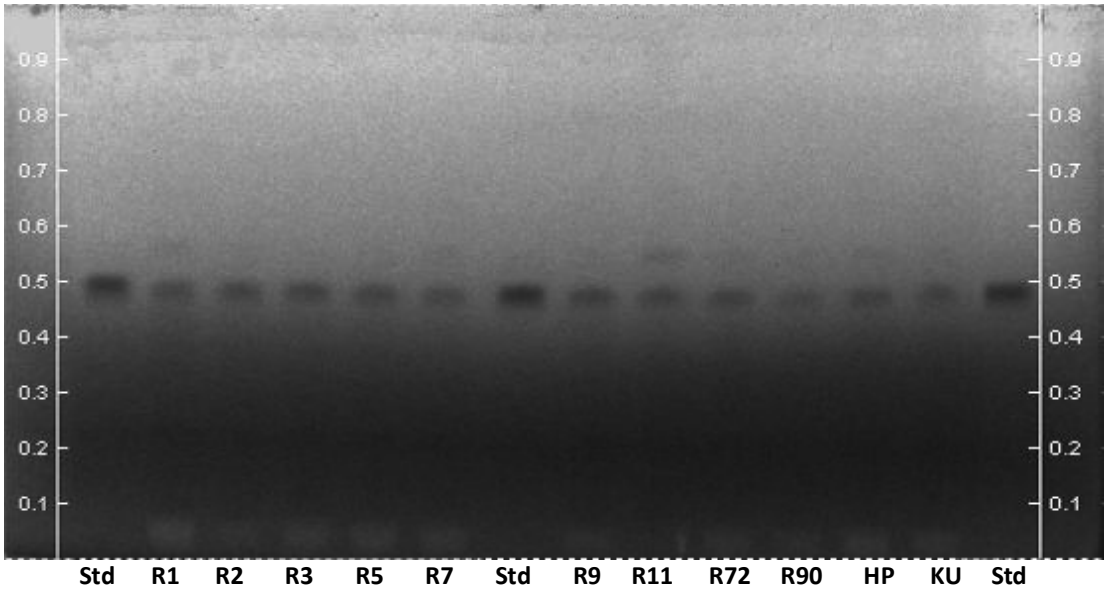

Figure 2: TLC chromatogram of 11 cultivars of cassava leaf extract.
(Rutin was detected in all cultivars at Rf = 0.47)
Std = Standard rutin
R1 = Rayong1 R2 = Rayong2 R3 = Rayong3 R5= Rayong5
R7 = Rayong7 R9 = Rayong9 R11 = Rayong11 R72 =Rayong72
R90=Rayong90 HP = Huaipong60 KU = Kasetsart50

Validation of TLC-densitometric method

For the validation method, the linear regression data for respective calibration curves showed a good linearity for rutin analysis with r = 0.999 (Figure 3). The relative standard deviation (RSD) of the inter-day and intra-day analysis ranged from 5.20-6.90% and 3.10 - 6.46% respec tively (acceptable range < 10% RSD). The estima tion of rutin in ethanolic solution afforded recovery of 99.82, 99.96 and 97.69%, respectively (accept able range [95-105%]). Limit of detection and limit of quantification were found to be 20 ng/band and 100 ng/band respectively.

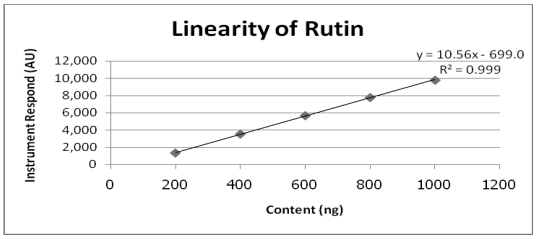

Figure 3 Calibration curve of rutin obtained by TLC-densitometer

Determination of rutin content

Kasetsart 50 was the selected cultivar of cassava for the determination of rutin content in leaf extract. The average rutin content in the leaf was 12.30±0.61% (Table 1). UV spectra of standard rutin and rutin in the extract completely overlapped (Figure 4) which indicated the uniformity of the rutin in the leaves and confirmed the suitability of TLC-densitometric method.

Table 1: Rutin contents from ethanolic cassava extracts (Kasetsart 50) using TLC-densitometry

Sample Kasetsart 50 (KU50)	Content of rutin (%dry weight of leaf) Means± SD
1	11.85±0.45
2	13.21±0.91
3	11.84±0.46
Average	12.30±0.61

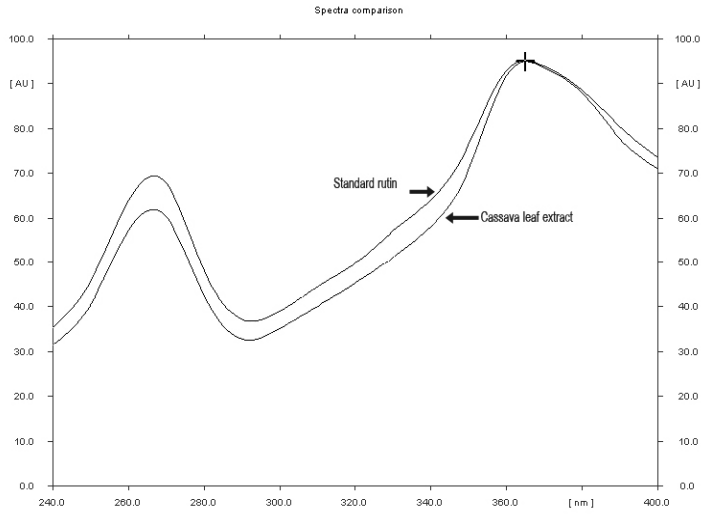

Figure 4: UV spectra of standard rutin overlayed with rutin in methanol extract of cassa leaves [Kasetsart 50] (Absorbance are 254 nm and 365 nm)

4. Conclusion

The proposed TLC-densitometric method was simple and precise. TLC-densitometry is an attractive alternative for the simultaneous deter mination of rutin in various cultivars of cassava. The optimum conditions were silica gel as station ary phase with two mobile phase systems as indicated in material and method to yield a good separation chromatogram. The existence of rutin, an antioxidant compound with capillary-protective action, in cassava leaves is particularly encouraging. The rutin content in cassava leaf extract (Kaset sart 50) was 12.30 ± 0.61 % dry weight. This amount is comparable to the buckwheat plant in Family Polygonaceae, the most prominent source of rutin [15 % dry weight] (Suzuki T *et al*., 2005). Buckwheat is not a domestic plant of Thailand whereas cassava leaves are cheaper and highly available as a by-product during the harvesting of cassava root. Rutin were found in all 11 cultivar of cassava leaf extract with the Rf value = 0.47. According to the overall result, cassava leaves can be use a new source of rutin and may be developed for them to the health food supple-ments in the future.

5. Acknowledgments

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References

Adewusi SRA, Bradbury JH. Carotenoids in cassava: Comparison of open-column and HPLC methods of analysis. *J Sci Food*. 1993;62(4):375-83.

Afshar D, Delazar A. Rutin from Ruta graveolens L. DARU *Journal of Pharmaceutical Sciences*. 1994;4(1-2):1-12.

Blagbrough IS, Bayoumi SAL, Rowan MG, Beeching JR. Cassava: An appraisal of its phy tochemistry and its biotechnological prospects. Phytochemistry. 2010;71 (17–18):1940-51.

Bodart P, Penelle J, Angenot L, Noirfalise A. Direct Quantitative Analysis of Linamarin in Cassava by High-Performance Thin Layer Chromatography. *J Planar Chromat*. 1998;11(January/February):38-42.

Communities CotE, editor Validation of Analytical Procedures. Proceedings of the International Conference on Harmonization (ICH); 1996; Geneva, Switzerland.

Eggum RO. The protein quality of cassava leaves. British Journal of Nutrition. 1970; 24:761-8.

Galand N, Pothier J, Viel C. Plant drug analysis by planar chromatography. *J Chromat Sci*. 2002;40(10):585-97.

Gupta N, Sharma SK, Rana JC, Chauhan RS. Expression of flavonoid biosynthesis genes vis-à-vis rutin content variation in different growth stages of Fagopyrum species. *J Plant Physiol*. 2011;168(17) :2117-23.

Ihme N, Kiesewetter H, Jung F, Hoffmann KH, Birk A, Müller A, *et al*. Leg oedema protection from a buckwheat herb tea in patients with chronic venous insufficiency: A single-centre, randomised, double-blind, placebo controlled clinical trial. *Eur J Clin Pharmacol*. 1996;50(6):443-7.

Nguyen TLT, Gheewala SH, Garivait S. Energy balance and GHG-abatement cost of cassava utilization for fuel ethanol in Thailand. Energy Policy. 2007;35(9):4585-96.

Prawat H, Mahidol C, Ruchirawat S, Prawat U, Tuntiwachwuttikul P, Tooptakong U, *et al*. Cyanogenic and non-cyanogenic glycosides from Manihot esculenta. Phytochemistry. 1995;40(4):1167-73.

Suzuki T, Honda Y, Mukasa Y. Effects of UV-B radiation, cold and desiccation stress on rutin concentration and rutin glucosidase activity in tartary buckwheat (Fagopyrum tataricum) leaves. Plant Science. 2005; 168(5):1303-7.