Maejo Int. J. Sci. Technol. **2025**, *19*(02), 122-132

Maejo International Journal of Science and Technology

ISSN 1905-7873
Available online at www.mijst.mju.ac.th

Full Paper

Simultaneous analysis of folate and derivatives in Thai rice grains using high performance liquid chromatography

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Received: 17 September 2024 / Accepted: 5 June 2025 / Published: 16 June 2025

Abstract: This study presents a simple and rapid method for the simultaneous determination of folate (FA), also known as vitamin B9 and its derivatives, namely 5-methyltetrahydrofolate (5-MTHF), tetrahydrofolate (THF) and 10-formylfolic acid (10-CHOFA), in Thai rice grains. Chromatographic separation was conducted using a C18 column with isocratic elution and a mixture of 0.1% formic acid and acetonitrile (85:15 v/v) as the mobile phase. The flow rate was maintained at 0.70 mL/min. and detection was performed using a photodiode array detector at a wavelength of 267 nm. The calibration curves for FA and its derivatives demonstrated excellent linearity (R > 0.9990) over the following concentration ranges: 0.50–50.0 µg/mL for FA, 0.50–200.0 µg/mL for 5-MTHF, 0.50–50.0 µg/mL for THF and 5.0–100.0 µg/mL for 10-CHOFA. The limits of detection ranged from 0.09 to 0.15 µg/mL while the limits of quantification were between 0.30 - 0.50 µg/mL. Recoveries at concentrations of 5, 20, 60 and 90 µg/mL were found to be between 91% and 104%. In the samples analysed the concentrations of FA and its derivatives ranged from 0.19 to 27.10 mg/100 g dry weight. This method demonstrates high reliability and practicality for the simultaneous determination of FA and its derivatives in rice.

Keywords: folate, tetrahydrofolate, 5-methyltetrahydrofolate, 10-formylfolic acid, Thai rice, high-performance liquid chromatography

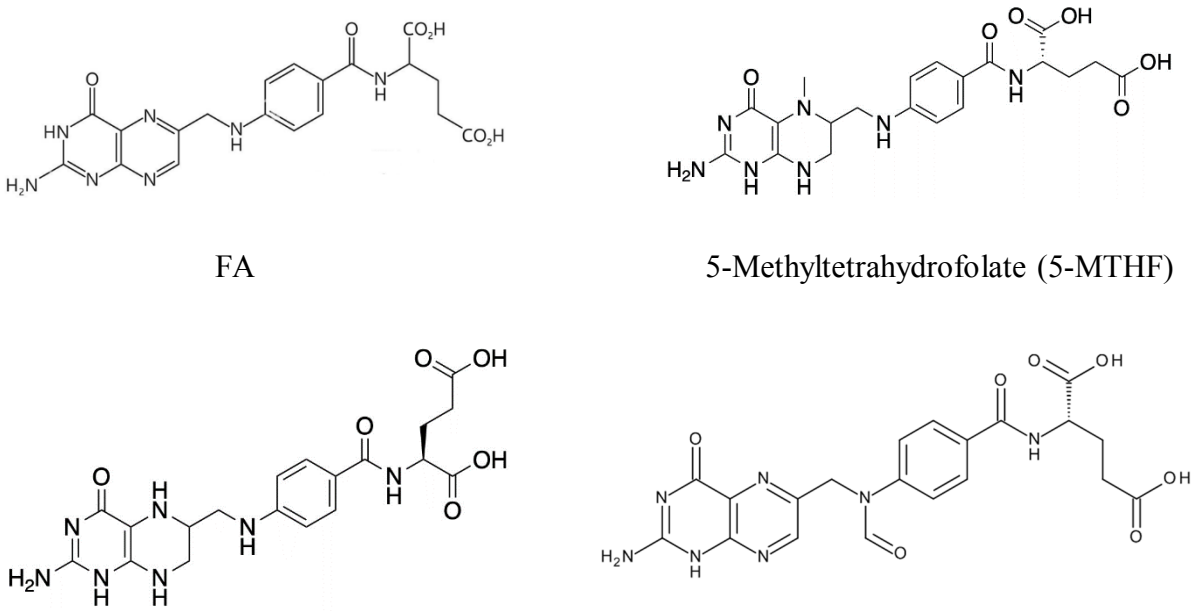
INTRODUCTION

Vitamins are essential nutrients that support various bodily functions and must be obtained from food or supplements since the body cannot produce them [1]. They are classified into fat-soluble (vitamins A, D, E and K) and water-soluble (vitamin C and B-complex) categories. Water-

Maejo Int. J. Sci. Technol. **2025**, *19*(02), 122-132

soluble B vitamins serve as enzyme cofactors, aiding metabolic processes and energy production [2]. They also offer significant health benefits including lowering of cholesterol levels and reduction of the risk of breast, colon and pancreatic cancers. Additionally, in pregnant women folate (FA) helps prevent birth defects and support fetal development [3].

FA, also known as folic acid or vitamin B9, is a water-soluble B vitamin essential for various physiological functions [4]. Like other B-complex vitamins, it serves as a cofactor in metabolic processes, particularly in conjunction with vitamin B12, of blood cell formation. Naturally occurring in both plant and animal sources, FA is crucial for DNA synthesis and cell division. Certain conditions, such as pregnancy, infections, anaemia and blood loss, increase the body's demand for FA. The recommended daily intake ranges from 300 to 600 μg [5]. The chemical structures of FA and its biologically active derivatives are illustrated in Figure 1.



FA | 5-Methyltetrahydrofolate (5-MTHF)

Tetrahydrofolate (THF) | 10-Formylfolic acid (10-CHOFA)

Figure 1. Structural formulas of FA and its derivatives

Dietary sources of FA include leafy greens, legumes and fortified grains [6] and whole grains such as red beans, soybeans and peanuts. Rice, especially in brown and unpolished form, is a notable source of vitamin B9 and essential minerals such as magnesium, phosphorus, zinc and iron [7, 8]. As one of the world's top rice exporters, Thailand is renowned for premium varieties such as Jasmine rice. Enhancing the nutritional quality of rice, particularly by increasing FA content, could strengthen Thailand’s global market position while addressing FA deficiencies in rice-consuming regions. Developing high-FA rice varieties would offer both the economic and public health benefits.

Traditionally, total FA measurement is conducted using microbiological assays with *Lactobacillus casei*, which has served as a standard method for FA analysis for several decades [9]. However, this method is labour-intensive, lacks specificity and cannot distinguish between individual FA derivatives. In addition to microbiological assays, other analytical techniques have been developed. For instance, Ramos developed liquid chromatography-mass spectrometry (LC-

MS) methods for FA profiling in rice and combined microbiological assays with LC-MS for more comprehensive analysis [10].

In a related study, Dong et al. [11] employed a tri-enzyme extraction method followed by microbiological assay to estimate total FA in rice germplasm. Similarly, Aiyswaraya et al. [12] used buffer extraction coupled with enzymatic treatments involving α-amylase and protease. However, their analysis using high-performance liquid chromatography (HPLC) produced broad chromatographic peaks, limiting the precision of FA quantification. De Brouwer et al. [13] leveraged more refined chromatographic techniques to profile FA content in wild rice, genetically modified strains and various rice cultivars. Although these methods provide reliable and accurate analysis, they often require complex sample preparation steps such as enzymatic digestion [11, 12, 14], dispersive solid-phase extraction [15] or chemical derivatisation [16]. These additional steps may limit the practicality of these methods for routine or high-throughput analysis. While the data generated from these studies are crucial for guiding rice biofortification and breeding programmes, few have focused on rice varieties cultivated in Thailand, one of the world's leading rice producers, or proposed simplified approaches that are feasible for use in standard laboratory settings.

FA consists of a pteridine ring, *p*-aminobenzoate and glutamic acid moieties, with its pterin-based structure exhibiting strong absorbance in the UV region, particularly at wavelengths around 280 nm and 360 nm. A spectrophotometric method utilising the wavelength at 285 nm, which is specific to FA, has been proposed for its determination in fortified salt [17]. Additionally, spectrophotometry is commonly used to analyse FA in pharmaceutical tablets, requiring pH adjustment to optimise the UV spectra [18]. However, direct UV-Vis spectroscopy often lacks specificity due to potential interference from other UV-absorbing compounds present in food or biological samples.

Electrochemical sensors and biosensors have also been employed for FA analysis [19-22]. These sensors often require extensive chemical modifications involving various nanomaterials, FA-binding proteins or even salmon sperm double-stranded DNA, necessitating complex preparation and expertise to enhance selectivity and specificity for bioactive substances in complex matrices. Despite their advantages, these sensors have several limitations including poor reproducibility, problems in simultaneous detection and intricate fabrication processes. Furthermore, these instrumental approaches generally suffer from limited selectivity as they are unable to distinguish between different FA forms and are restricted to measuring only the total FA content in food samples.

Over the past decade, capillary electrophoresis has been utilised for the analysis of FA and its analogues in pharmaceuticals and food products to overcome selectivity limitations [23]. It offers advantages such as high resolution, rapid analysis and cost-effectiveness. However, its major drawbacks include poor reproducibility and low sensitivity due to its short optical path length and small sample volume, which can limit its applicability in complex food matrices.

Mahato et al. [24] reported an HPLC-UV method for estimating the total FA content in fortified rice and wheat flour. Since FA is sensitive to heat, oxygen and UV light, it readily degrades into inactive products. To prevent degradation and enhance detection accuracy, enzymatic extraction and immunoaffinity chromatographic enrichment were employed. However, these additional steps made the sample preparation process more complex. Building on this, Tornero et al. [25] introduced a method employing HPLC with fast-scanning fluorescence detection after post-column on-line photoderivatisation for analysing FA and its metabolites (5-MTHF and THF) in vegetables. This approach further improved sensitivity and selectivity in FA analysis. However, the

complex sample preparation and post-column derivatisation steps present challenges for routine analysis, limiting its practicality for high-throughput applications.

To the best of our knowledge, an in-depth study on FA content and its active forms in Thai rice has not yet been reported. Hence in this study we analyse FA and its three derivatives, namely 5-methyltetrahydrofolate (5-MTHF), tetrahydrofolic acid (THF) and 10-formylfolic acid (10-CHOFA), which are naturally occurring active forms in rice grains. Our method aims to overcome key analytical challenges and features a straightforward sample preparation process which, combined with high speed, cost-effectiveness and versatility, makes it well-suited for FA analysis in complex food matrices. The results of this study should contribute to the identification of native Thai rice varieties with enhanced nutritional qualities, offering valuable data for rice breeding programmes.

MATERIALS AND METHODS

Materials

Ultrapure water was obtained by a Milli-Q purification system (Millipore, USA). Methanol and acetonitrile, both HPLC-grade, were purchased from Merck (Germany). Acetic acid and ethyl acetate were reagent-grade and obtained from Merck (Germany). FA, 5-MTHF, THF and 10-CHOFA were purchased from Sigma-Aldrich (USA). Each compound was accurately weighed (10 mg) and dissolved in methanol to a final volume of 10 mL, yielding a stock concentration of 1.0 mg/mL. The stock solutions were stored and refrigerated at 4°C. Daily, these stock solutions were diluted to create a series of working standard solutions with concentrations ranging from 0.5 to 200 µg/mL.

Samples and Sample Preparation Procedure

The study involved cultivating ten different rice varieties, consisting of five native varieties (*Dok Payom white rice*, *Boonma Golden glutinous rice*, *Nang Nuan Pearl glutinous rice*, *Tom Black glutinous rice* and *Premium White Giant glutinous rice*) and five improved greenhouse varieties (*RD57 aromatic rice*, *RD7 white rice*, *Chainat 1 fragrant rice*, *Suphanburi 1 jasmine rice* and *Phitsanulok 2 high-yield rice*). The five native varieties consisted of one non-glutinous (white rice) variety and four glutinous rice varieties, while all five improved varieties were non-glutinous. Native or local rice varieties refer to parent strains (either paternal or maternal) traditionally cultivated in specific regions. In contrast, improved rice varieties are developed through hybridisation, a process in which local rice varieties are crossbred by combining paternal and maternal strains to enhance desirable traits. The rice samples were cultivated at a greenhouse located in Phatthana Saikaew sub-district, Chiang Mai, Thailand and collected during the 2022 rice cultivation season.

Each variety was planted in three separate pots, with identical amounts of soil, fertiliser and watering. The pots were arranged randomly, with three replicates for each set-up. When the rice plants matured, the seeds from each variety were harvested and milled into brown rice for further analysis. After harvesting, the rice grains were dried at 50 °C for five days and then stored in brown paper bags inside a desiccator at room temperature.

One gram of whole grain rice was ground in a porcelain mortar with a small amount of liquid nitrogen and then sieved through an 80-mesh sieve. The use of liquid nitrogen during

grinding prevents the rise in temperature that can accelerate the degradation of folic acid and minimises oxidative degradation by reducing the sample's exposure to air. The extraction of the resulting rice flour and bran was performed in duplicate, following the method of Kothakota et al. [26] with slight modifications, using a smaller amount of rice, extraction volume and time. To minimise folic acid degradation during sample preparation, special precautions were taken: all steps following grinding were performed under dim light to reduce UV exposure and the extracted solutions were handled in amber microtubes to limit light and oxygen contact. Additionally, all sample tubes were tightly sealed to prevent oxidation and the extraction process was conducted at room temperature (25 °C) rather than elevated temperatures.

Firstly, 0.5000 g of each rice sample powder was soaked in 1 mL of water and homogenised for 1 min. using a vortex mixer. The sample was then shaken at room temperature for 3 hr in a shaking water bath. After shaking, the sample was centrifuged at 3500 rpm for 15 min. The resulting supernatant was immediately transferred to an amber vial and filtered through a 0.22 µm nylon membrane filter to remove any remaining suspended particulates. The filtered extract was analysed on the same day to avoid prolonged storage. An aliquot 20 µL of the filtered solution was manually injected into the HPLC system for analysis.

HPLC Equipment and Conditions

Analyses were carried out on an HPLC– diode array detector (DAD) model HP 1100 system (Agilent Technologies, USA) . Separations were carried out under isocratic conditions using a Hypersil C18 column (250×4 mm I.D., 5 µm particle size) (Thermo Quest, Germany) coupled with a guard column. A mobile phase consisting of 85% acetonitrile and 15% aqueous 0.1% formic acid (v/v) was used. The flow rate was 0.70 mL $min.^{-1}$ with a total run time of 15 min. The quantification of FA and its derivatives was based on the absorption peak heights measured using a photodiode array detector set at a wavelength of 267 nm, slightly modified from a previous method by Rodriguez et al. (wavelength of 268 nm and a flow rate of 0.90 mL/min.) [27].

Validation and Quantification

The method validations were performed following AOAC guidelines [28], which assessed its linearity, accuracy, precision, limit of detection (LOD) and limit of quantitation (LOQ). The LOD and LOQ were determined based on signal-to-noise ratios of 3 and 10 respectively. The accuracy was assessed by calculating the recovery of samples spiked with mixed standards. The precision was expressed as relative standard deviation. The precision of the analyses was investigated by performing the tests five times on the same day and five times on three different days.

RESULTS AND DISCUSSION

Calibration and Validation Method

A chromatogram of FA, 5-MTHF, THF and 10-CHOFA standards is shown in Figure 2. The retention times for 5-MTHF, THF, 10-CHOFA and FA standards are observed at 4.89, 5.36, 7.29 and 9.16 min. respectively. Under the optimum condition, the chromatographic results show linear calibration curves in the concentration ranges of 0.50-200.00 µg/mL for 5-MTHF, 0.50-50.00 µg/mL for THF, 0.50-100.00 µg/mL for 10-CHOFA and 0.50-50.00 µg/mL for FA. The correlation

Maejo Int. J. Sci. Technol. **2025**, *19*(02), 122-132

coefficients of standard solutions range between 0.998-0.999. A summary of the method validation is presented in Table 1.

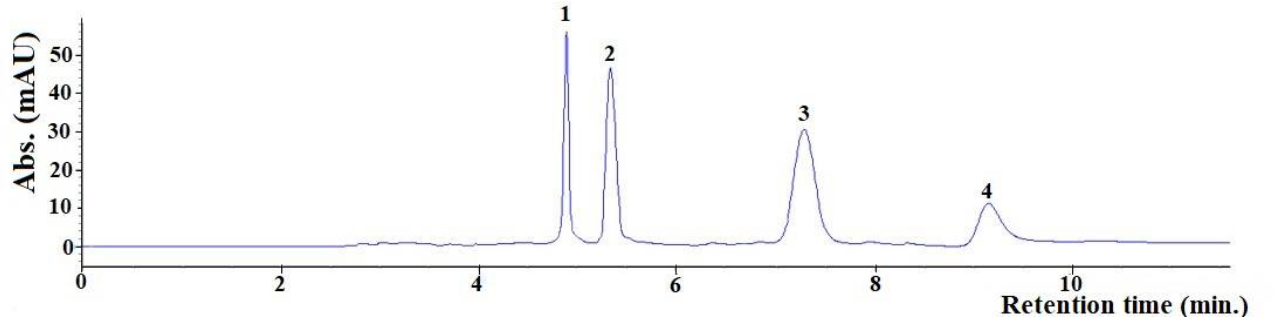


Figure 2. Chromatogram of standard mixture of 5-MTHF (1), THF (2), 10-CHOFA (3) and FA (4)

Table 1. Linear range, LOD and LOQ of FA and its derivatives determined by HPLC

Analytical parameter	5-MTHF	THF	10-CHOFA	FA
Linear range (µg/mL)	0.50-200.00	0.50-50.00	0.50-100.00	0.50-50.00
Calibration equation	y=12.64x +1.57 r = 0.999	y=17.84x +4.53 r = 0.998	y=63.32x + 10.05 r = 0.999	y=19.3x - 4.61 r = 0.999
LOD (µg/mL)	0.13	0.12	0.15	0.09
LOQ (µg/mL)	0.43	0.40	0.50	0.30

The values obtained from the validated methods, such as accuracy reported as per cent recovery and precision reported as per cent relative standard deviation, were compared with AOAC standard criteria to confirm the reliability of the analytical method. The samples were then directly spiked with standard FA and derivatives at concentrations of 5-90 µg/mL to determine the accuracy. As shown in Table 2, the recoveries of FA and its derivatives range between 95.0-113%. According to AOAC guidelines [28], the acceptable recovery range at a 10-ppm concentration is 60-115%, thus verifying the applicability of the method.

Table 2. Concentrations of FA and its derivatives in spiked samples obtained by HPLC

Compound	Added (µg/mL)	Found (µg/mL)	RSD* (%)	Recovery (%)
5-MTHF	0	32.0		
	60	54.7	1.3	91.2
	90	86.4	0.8	95.9
THF	0	2.80		
	5	5.18	2.9	103.7
	20	20.2	2.8	101.1
10-CHOFA	0	5.35		
	5	5.22	0.9	104.4
	20	20.1	4.2	100.3
FA	0	2.52		
	5	4.74	4.4	94.8
	20	20.0	1.6	100.2

* relative standard deviation (n=3)

Maejo Int. J. Sci. Technol. **2025**, *19*(02), 122-132

The analysis precision was also evaluated. The intra-day precision (5 determinations within the same day) ranges between 0.46-0.75%, while the inter-day precision (5 determinations over 3 different days) ranges between 0.51-1.35%. The method's precision, calculated as per cent relative standard deviation, was below 6%, adhering to AOAC guidelines [28].

Analysis of FA and Derivatives

The HPLC chromatograms for the ten rice samples are displayed in Figure 3. The amounts of FA and its derivatives in the rice samples were calculated and are presented in Table 3. The amounts ranged from 0.19 to 27.10 µg/100 g dry weight. Among the native rice varieties, *Tom Black glutinous rice* contains the highest amounts of 5-MTHF and 10-CHOFA; *Dok Payom white rice* and *Nang Nuan Pearl glutinous rice* have the highest levels of THF, and *Nang Nuan Pearl glutinous rice* exhibits the highest amount of FA. In the improved rice varieties, *RD57 aromatic rice* has the highest levels of 5-MTHF, THF and 10-CHOFA, whereas *RD7 white rice* contains the highest amount of FA.

Table 3. FA and its derivatives in brown rice samples

Sample	Amount of FA and its derivatives ± SD* (µg/100 g dry weight)			
	5-MTHF	**THF**	**10-CHOFA**	**FA**
Dok Payom white rice[a]	23.02 ± 1.26	1.85 ± 0.10	0.48 ± 0.05	0.46 ± 0.10
Boonma Golden glutinous rice[a]	20.55 ± 0.14	0.44 ± 0.21	2.68 ± 0.10	0.43 ± 0.21
Nang Nuan Pearl glutinous rice[a]	23.69 ± 0.71	1.85 ± 0.11	3.70 ± 0.09	0.51 ± 0.10
Tom Black glutinous rice[a]	**27.10 ± 1.63**	0.50 ± 0.05	**10.09 ± 0.16**	0.19 ± 0.02
Premium White Giant glutinous rice[a]	25.28 ± 1.63	0.76 ± 0.10	8.67 ± 0.34	0.19 ± 0.04
RD57 aromatic rice[b]	23.46 ± 1.36	**3.43 ± 0.31**	3.57 ± 0.25	0.46 ± 0.01
RD7 white rice[b]	23.09 ± 1.34	0.75 ± 0.05	2.76 ± 0.35	**0.79 ± 0.21**
Chainat 1 fragrant rice[b]	15.88 ± 0.30	1.41 ± 0.05	1.55 ± 0.31	0.53 ± 0.05
Suphanburi 1 jasmine rice[b]	23.37 ± 1.65	1.86 ± 0.21	3.03 ± 0.08	0.48 ± 0.10
Phitsanulok 2 high-yield rice[b]	17.86 ± 0.95	0.77 ± 0.16	1.42 ± 0.19	0.29 ± 0.06

* Standard deviation from triplicate measurements; [a] Local rice; [b] Improved rice

Based on these findings, *Nang Nuan Pearl glutinous rice* and *Tom Black glutinous rice* are identified as suitable candidates for hybridisation to develop rice varieties with higher FA content. These local varieties, which naturally exhibit high FA levels, can be selectively bred to enhance their nutritional value. Meanwhile, improved varieties of *RD57 aromatic rice* and *RD7 white rice* should be promoted among farmers to encourage widespread cultivation.

A comparative summary of four analytical methods used for FA determination is presented in Table 4. Our method, based on HPLC-DAD, is simple and requires minimal sample preparation, making it especially suitable for routine and high-throughput analysis in general laboratory settings.

Maejo Int. J. Sci. Technol. **2025**, *19*(02), 122-132

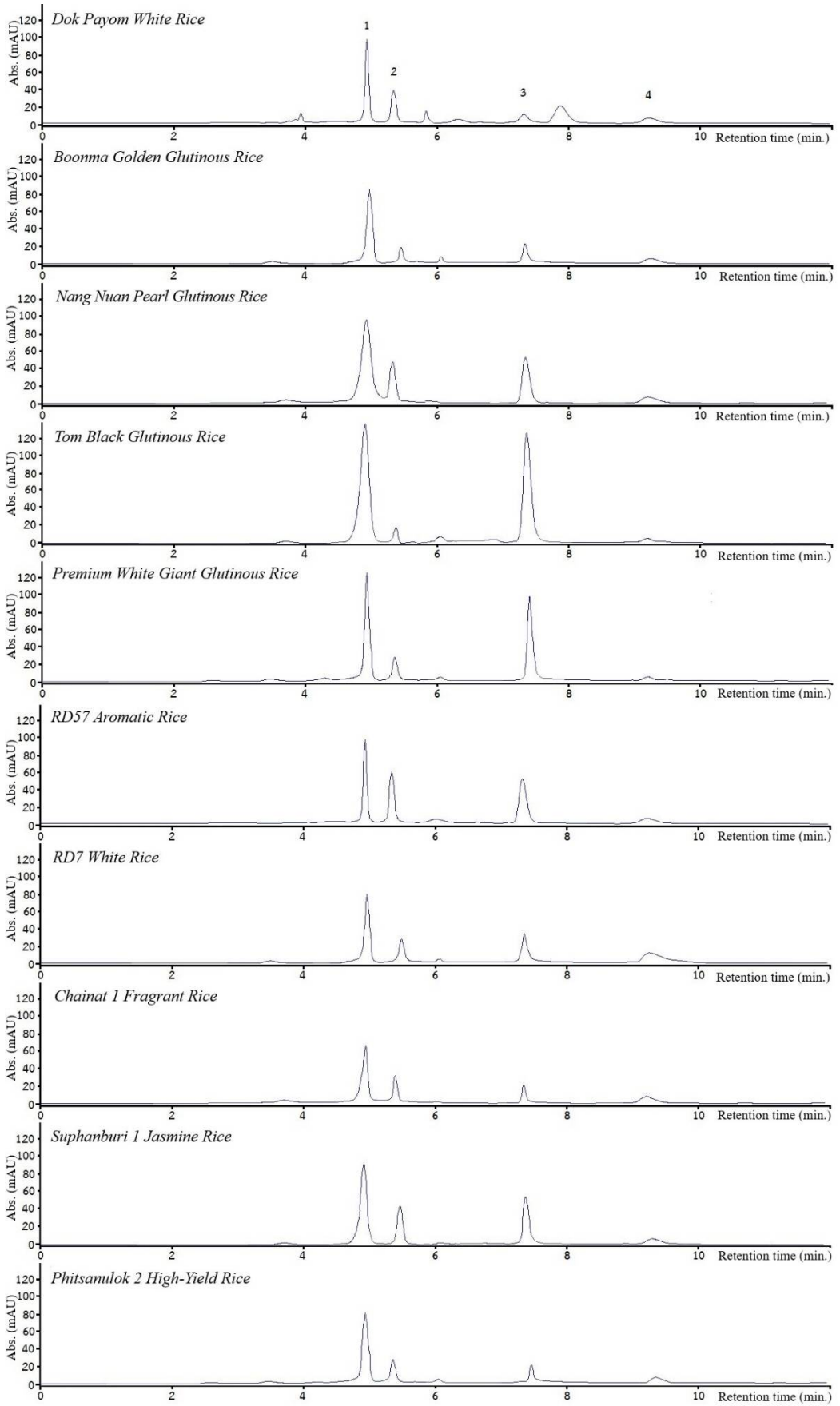


Figure 3. Chromatograms of brown rice samples: native types and improved types. The retention times of 5-MTHF (1), THF (2), 10-CHOFA (3) and FA (4) are 4.89,5.36, 7.29 and 9.19 min. respectively.

Maejo Int. J. Sci. Technol. **2025**, *19*(02), 122-132

Table 4. Comparison of five analytical methods for FA determination in rice

Method	Accuracy	Sample Preparation Complexity	FA Form Differentiation	Suitability for General Laboratories
Microbiological assay [9]	Moderate	High	No	Not suitable
UV-Vis spectrometry [17]	Moderate	Moderate	No	Suitable
LC- enzymatic extraction [12]	Moderate	High	No	Suitable
LC-MS/MS [14]	High	High	Yes	Requires specialised equipment
HPLC-DAD (this study)	High	Low	Yes	Highly suitable

CONCLUSIONS

HPLC-DAD has been successfully employed as a simple and rapid method for the simultaneous determination of FA and its derivatives, namely 5-MTHF, THF and 10-CHOFA, in ten rice cultivars. The HPLC-DAD method demonstrates superior performance, achieving low LOD and LOQ values of 0.09 μg/mL and 0.30 μg/mL respectively, as well as excellent linearity. The optimised HPLC conditions offer excellent selectivity and specificity, allowing for a clear separation of FA and its derivatives.

Accurate measurement of FA content in rice can help identify cultivars that are particularly rich in this essential vitamin, aiding in dietary planning and nutritional improvements. The findings reveal significant variation in FA content among different rice types, with some cultivars providing substantially higher levels of FA and its derivatives. This information is valuable for both consumers and producers, underscoring the value of specific cultivars in contributing to dietary FA intake and improving overall public health. Moreover, the data obtained from such analysis can be further utilised for the selection of rice varieties in breeding programmes aimed at enhancing nutritional quality, as well as for quality control purposes within the rice industry.

ACKNOWLEDGEMENTS

This work was partially supported financially by the Program in Chemistry, Faculty of Science, Maejo University. The authors also wish to thank the Program in Genetics for providing the rice samples.

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