Research Article

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Development of a green and rapid ethanol-based HPLC assay for aspirin tablets and feasibility evaluation of domestically produced bioethanol in Thailand as a sustainable mobile phase

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Abstract: Despite the growing trend of using ethanol as a greener alternative to hazardous solvents like methanol and acetonitrile in high-performance liquid chromatography (HPLC) analysis, no ethanol-based assay has been developed for aspirin tablets, nor has the potential of domestically produced bioethanol as a mobile phase been explored – until now. This study introduces a novel ethanol-based HPLC assay for aspirin and assesses the feasibility of using locally produced bioethanol. The optimized method, featuring a 40% (v/v) ethanol–water mobile phase adjusted to pH 3.6 with glacial acetic acid, with a 15-cm C18 column at 40°C and UV detection at 237 nm, successfully separated aspirin and its degradation product, salicylic acid, in 5 min. It demonstrated excellent linearity (0.1–0.6 mg·mL^{-1}, r^2 = 0.9997), sensitivity (LOD = 0.01 mg·mL^{-1}, LOQ = 0.03 mg·mL^{-1}), accuracy, and precision, with no interference from excipients. The method's performance on commercial aspirin tablets aligned with official pharmacopoeial methods. In a case study using fuel-grade bioethanol (>99.5% purity) derived from sugarcane molasses or cassava from local manufacturers in Thailand, chromatographic and analytical results matched those obtained with imported ethanol, validating its effectiveness. This innovative approach not only reduces costs and dependence on imported solvents but also supports local bio-circular-green economies and promotes greener, more sustainable analytical practices.

Keywords: ethanol, HPLC, aspirin, domestic, sustainability

1 Introduction

High-performance liquid chromatography (HPLC) is a versatile and extensively used analytical technique for the identification, quantification, and purity assessment of compounds across various fields, including pharmaceuticals, environmental analysis, and food safety. Currently, most HPLC separations are conducted using the reversed-phase mode (RP-HPLC) [1,2]. However, the traditional solvents commonly used as mobile phases in RP-HPLC – such as acetonitrile, methanol, and tetrahydrofuran – pose significant environmental and health concerns. For instance, methanol is toxic if inhaled, absorbed through the skin, or ingested [3]. High exposure to tetrahydrofuran can cause unconsciousness and death [4]. The disposal of acetonitrile requires safe and specialized treatment processes, as improper combustion produces highly toxic hydrogen cyanide gas [5]. In response to the increasing demand for sustainable and green practices, the development and utilization of green solvents in HPLC have become a focal point of research.

Among green solvents, recent studies have highlighted ethanol's feasibility and effectiveness as a green and sustainable alternative to traditional solvents like acetonitrile and methanol due to its numerous advantages [6]. Ethanol's lower vapor pressure reduces the toxic effects of inhalation, making it safer for human health. Environmentally, ethanol is biodegradable and has a lesser negative

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impact compared to acetonitrile and methanol [7] since methanol is highly toxic, especially to aquatic organisms, and can cause serious ecological harm if it contaminates water sources [8]. Acetonitrile is also toxic to both mammals and aquatic life, with potential chronic effects like central nervous system damage [9]. In terms of biodegradation, acetonitrile persists longer than ethanol and methanol in the environment before being broken down. For HPLC use, ethanol's UV cutoff is acceptable, at around 210 nm [10]. Regarding disposal, ethanol's lower toxicity also reduces waste management costs, ultimately lowering expenses in pharmaceutical analysis. Although ethanol has higher viscosity, which can lead to increased column backpressure, this can be managed by using higher column temperatures and/or lower flow rates [7].

Ethanol is produced via two primary routes: petrochemical synthesis and biomass refining [11]. Petrochemical synthesis relies on natural gas, coal, and ethylene (a petroleum by-product), making it an unsustainable option. In contrast, biomass refining uses renewable sources such as corn, sugarcane, and cellulosic materials, including forest and agricultural residues, offering a greener and more sustainable alternative [12,13]. Moreover, the domestic production of bio-based ethanol or bioethanol utilizing locally available agricultural feedstocks provides numerous socioeconomic benefits. These include stimulating investment in agriculture, reducing reliance on fossil fuel imports, driving industrial development, creating jobs, and boosting rural incomes, all of which contribute to overall economic expansion [14,15].

While ethanol is emerging as a greener alternative solvent for HPLC in pharmaceutical analysis [6,7,16], no studies have yet utilized domestically produced bioethanol as a mobile phase. To date, all published research studies have relied on ethanol sourced from globally recognized chemical suppliers such as Merck, Sigma-Aldrich, Fisher Scientific, and Honeywell. While many countries can produce bioethanol with a purity exceeding the HPLC-grade standard of 99.5%, this product is predominantly used as an additive in fuel for transportation, industrial solvent, chemical feedstock, beverage ingredient, or antiseptic [17,18]. Even in Thailand, the world's seventh-largest fuel ethanol producer [19], there is currently no domestically produced bioethanol specifically marketed for chemical analysis purposes.

Aspirin (acetylsalicylic acid) is a drug widely used to relieve pain, reduce fever, and decrease inflammation. Its antiplatelet properties also help prevent blood clots, thereby reducing the risk of heart attacks and strokes. Current United States Pharmacopeia (USP) and European Pharmacopoeia assays of aspirin tablets rely on HPLC techniques using acetonitrile in the mobile phases [20,21], although methanol is also employed in some published methods [22–25]. Notably, ethanol-based HPLC methods for the assay of this formulation are unavailable. To address the environmental and health concerns associated with current assays and to leverage domestic bioethanol production, this study aimed to develop an HPLC assay for aspirin tablets using ethanol as both mobile phase and diluent (solvent used to prepare standard solutions and sample solutions). The method was then used to evaluate the analytical performance of Thai fuel additive-grade bioethanol samples derived from sugarcane molasses and cassava, produced by four local manufacturers, in comparison to an HPLC-grade corn-based ethanol (EMSURE®) from Merck. The findings of this research not only introduced a new greener assay applicable for the pharmaceutical quality control of aspirin tablets but also revealed the potential of Thai bioethanol as an effective HPLC solvent, with benefits for both environmental and economic sustainability.

2 Materials and methods

2.1 Chemicals and reagents

Aspirin (purity of 99.0%) and salicylic acid (purity of 99.0%) chemicals were sourced from Sigma Aldrich (Missouri, USA). Commercial enteric-coated (delayed-release) aspirin tablets, each containing 81 mg of the active ingredient, were obtained from a hospital in Thailand. Bioethanol was generously supplied by local producers: two samples derived from sugarcane molasses were provided by Mitr Phol Biofuel Co., Ltd. and ES Power Co., Ltd., while two samples derived from cassava were supplied by Ubon Bio Ethanol Co., Ltd. and Rajburiethanol Co., Ltd. All these bioethanol samples were produced by fermentation and further purification to meet the specifications for use as fuel additives, with a purity exceeding 99.5% as verified by gas chromatography (Table 1). For assay method development and comparison with domestic bioethanol, commercial EMSURE® ethanol (purity, >99.9%) from Merck (produced from corn) was utilized. Other reagents and chemicals used were of analytical grade, and deionized water was employed.

2.2 Preparation of aspirin standard solutions and sample solutions

In the proposed ethanol-based method, a standard curve of aspirin was constructed over a concentration range of 0.1 to 0.6 $mg{\cdot}mL^{-1}$. For this purpose, a 1.0 $mg{\cdot}mL^{-1}$ aspirin

Table 1: Properties of bioethanol used in this study

Specification	Manufacturer/supplier				
	Merck*	Mitr Phol Bio Fuel	Rajburi Ethanol	ES Power	Ubol Bio Ethanol
Purity (%)	99.9	99.97	99.90	99.83	99.94
Water content (%)	<0.1	nd	0.1123	0.25	0.1
Density 20°C	0.791	0.7894	nd	0.7901	0.7896
Density 15°C	nd	nd	0.7940	nd	0.7938
Acidity as acetic acid (ppm)	≤30	26.15	17.55	8.60	15.36
pH	nd	nd	7.55	7.45	7.92
Electrical conductivity (μS/m)	nd	nd	11	22	27

*EMSURE®; nd = not determined.

stock solution was initially prepared by dissolving aspirin in a diluent composed of 10% glacial acetic acid in ethanol. Working standards within the specified concentration range were subsequently obtained through appropriate dilutions of the stock solution using the same diluent.

For tablet analysis, twenty tablets were accurately weighed and ground. A quantity of powdered tablets containing 160 mg of aspirin was transferred to a 100 mL volumetric flask. Fifty milliliters of diluent were added, and the mixture was sonicated for 20 min or until complete tablet disintegration. The volume was adjusted to 100 mL with diluent, and the resulting solution was filtered through a 0.45-μm nylon filter. This filtrate served as the stock sample solution. To achieve a working sample concentration of 0.4 $mg \cdot mL^{-1}$, an appropriate aliquot of the stock sample solution was diluted with diluent. Prior to HPLC injection, the sample was filtered through a 0.45-μm nylon filter to remove any residual particulate matter.

2.3 Instrumentation and chromatographic conditions

Chromatographic analysis was performed using an Agilent 1100 Infinity system (Agilent Technologies, USA). The ethanol-based method, developed in this work, employed a C18 column (4.6 mm × 15 cm, particle size of 5 μm; VertiSep^{tm} GES, Vertical Chromatography Co., Ltd., Bangkok, Thailand). Isocratic elution with 40% (v/v) ethanol in water as a mobile phase, adjusted to pH 3.6 with glacial acetic acid, was performed at a flow rate of 1.0 $mL \cdot min^{-1}$. Ten microliter samples were injected using an autosampler. The column temperature was maintained at 40°C. Peak detection was performed using a diode array detector set at 237 nm since this wavelength gave more sensitivity (higher peak area) than that at 280 nm.

For comparison, two pharmacopeial methods were used. The USP method was conducted using a C18 column (4.6 mm × 25 cm, particle size of 5 μm). The mobile phase consisted of a 2 $g \cdot L^{-1}$ sodium 1-heptanesulfonate solution in acetonitrile:water (15:85 v/v), adjusted to pH 3.4 with glacial acetic acid. The flow rate was set to 2 $mL \cdot min^{-1}$ with a 10 μL injection volume. Detection was performed at 280 nm. In the BP method, a C18 column (4.6 mm × 25 cm, particle size of 5 μm) was employed. The mobile phase consisted of o-phosphoric acid:acetonitrile:water (2:40:60 v/v). The flow rate was set to 1 $mL \cdot min^{-1}$ with a 20-μL injection volume. Detection was performed at 237 nm. The column temperature was maintained at 25°C.

2.4 Method development

Three chromatographic parameters were investigated to optimize the separation. These included modifying the pH of the mobile phase between 2.8 and 3.6, altering the ethanol content in the mobile phase from 30% to 50%, and varying the column temperature from 25°C to 50°C. The goal was to achieve peaks with good symmetry (USP tailing factor of less than 2), high theoretical plate number (a minimum of 2,000), adequate resolution between the drug and its impurity, namely, salicylic acid (at least 3), short analysis time (within 5 min), and acceptable backpressure (below 200 bars). Additionally, the composition of the diluent used for the preparation of standard solutions and sample solutions was investigated to maintain the stability of aspirin in the solution. This involved testing various concentrations of glacial acetic acid in ethanol.

2.5 Method validation

The proposed ethanol-based HPLC method was validated in accordance with the USP ⟨1225⟩ Validation of Compendial

Procedures [21]. To assess linearity and range, aspirin standard solutions of different concentrations in the range of 0.1 to 0.6 mg·mL^{-1} were injected into the HPLC system. The peak area was plotted against the concentration. The regression equation along with the r^2 was reported. While not strictly required for the quantitation of the active ingredient in pharmaceutical products, the method's sensitivity was also demonstrated in this work by determining the limit of detection (LOD) and limit of quantitation (LOQ). These parameters were calculated using the standard deviation of the response (σ) and the slope of the calibration curve (S), according to the formulas LOD = 3.3 × σ/s and LOQ = 10 × σ/s.

Accuracy was assessed by spiking standard aspirin at three concentration levels (0.3, 0.4, and 0.5 mg·mL^{-1}) into a matrix composed of common tablet excipients: corn starch, calcium hydrogen phosphate, povidone K30, colloidal silicon dioxide, sodium starch glycolate, hypromellose phthalate, and methacrylic acid. The percentage recovery of aspirin from these spiked samples was determined by HPLC.

The method's precision was evaluated through intra- and inter-day studies. For intra-day precision, six replicate analyses of commercial tablet samples were performed on a single day. Inter-day precision was assessed by analyzing six replicate samples on three consecutive days. Precision was expressed as the percentage relative standard deviation (% RSD) of the assay results.

Specificity was assessed by inspecting chromatograms for potential interfering peaks that might co-elute with aspirin, including those from tablet excipients and salicylic acid. Additionally, peak purity was evaluated using a diode array detector to confirm that the aspirin peak represented a single compound.

Robustness was assessed by deliberately varying chromatographic conditions, including flow rate, column temperature, and detection wavelength, within predefined limits. The effects of these variations on assay results were measured by calculating the % RSD and testing with ANOVA. Additionally, the stability of both the standard solution and the sample solution was tested over periods of 12 h at ambient temperature.

2.6 Comparison of the proposed method to pharmacopeial methods

The content of aspirin (expressed as percentage labeled amount) in commercial tablet samples (n = 6) was determined using three methods: the proposed method employing ethanol (EMSURE®, Merck) and the two pharmacopeial methods described in the USP and BP. The percentage labeled amounts obtained from the analyses were compared across these methods using one-way ANOVA at a significance level of p = 0.05.

2.7 Study on the applicability of Thai domestic bioethanol as an alternative solvent

To investigate the potential and applicability of domestically produced bioethanol as an alternative HPLC solvent to imported products, four Thai fuel additive-grade bioethanol samples were tested. These solvents were used to prepare both diluent and mobile phase which were subsequently employed for the assay of commercial aspirin tablets following the proposed HPLC method. The assay results were compared to those obtained using EMSURE® ethanol (Merck) using one-way ANOVA at a significance level of p = 0.05.

3 Results and discussion

3.1 The optimal condition for the proposed ethanol-based method

To address the absence of an ethanol-based HPLC assay of aspirin and to establish a model analytical method used for evaluating the potential of bioethanol as a mobile phase, a novel HPLC assay using ethanol was developed. In this study, a 15-cm C18 column was selected for the analysis instead of the longer columns used in the USP (30 cm) and BP (25 cm) methods. The shorter column offers several advantages: it contains less packing stationary phase material, resulting in lower flow resistance and reduced back pressure, especially when using ethanol in the mobile phase. Furthermore, the USP method requires approximately 16 min to separate aspirin and salicylic acid, while the BP method takes around 10 min (Figure 1), thereby a shorter column was chosen with the expectation of reducing the analysis time. For peak detection, a wavelength of 237 nm, the same as that used in the BP method, was employed due to its greater sensitivity compared to the wavelength of 280 nm which is used in the USP method (Figure 2). This wavelength also stayed above the UV cutoff of ethanol which is 210 nm. In addition to these parameters, the effects of mobile phase pH, ethanol concentration in the mobile phase, and column temperature were

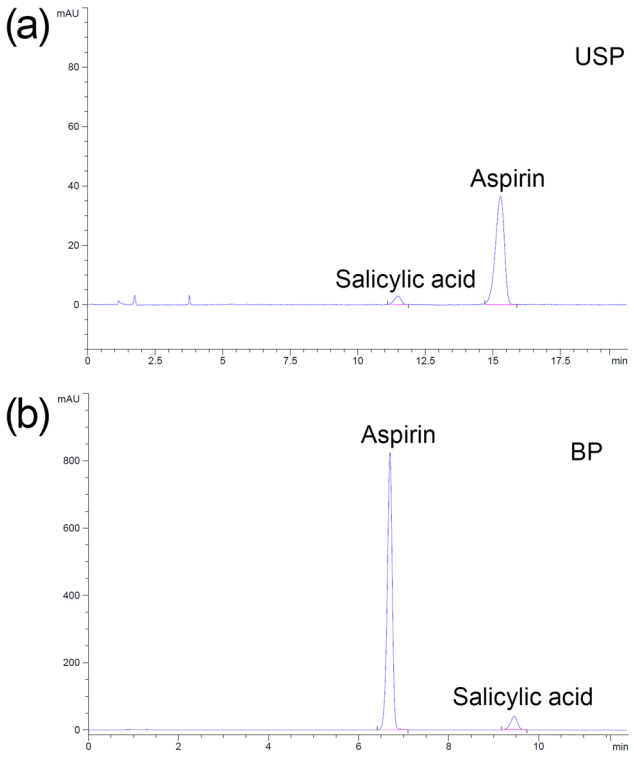


Figure 1: The chromatograms of aspirin assay following (a) USP method and (b) BP method.

investigated and optimized. The goal was to achieve a separation with the desirable peak characteristics outlined in Section 2.4 Method Development, while also maintaining acceptable backpressure and using minimal column temperature to extend the column's lifespan.

To evaluate the effect of the pH, three levels – pH 2.8, 3.2, and 3.6 – were tested by adjusting the mobile phase with glacial acetic acid to achieve the desired values. These pH levels were selected to be below the pK_a values of salicylic acid (2.97) [26], between the pK_a of salicylic acid and aspirin (3.49) [27], and above the pK_a of aspirin. This adjustment aimed to achieve the optimal ionization states for both compounds, allowing for effective separation and analysis. In addition, aspirin is most stable in a pH range of 2–3 [28]. In this study, raising the pH of the mobile phase beyond 3.6 was avoided, and it is not recommended due to aspirin's susceptibility to hydrolytic degradation at pH levels higher than 4 [28]. It was observed that when the ethanol concentration (40%) and column temperature (40°C) were kept constant, increasing the pH of the mobile phase significantly shortened the retention time of salicylic acid while slightly affecting that of aspirin (Figure 3 and Table 2). This effect occurred because the carboxylic groups of both compounds became more ionized at higher pH, facilitating their elution from the reversed-phase column. In terms of the concentration of ethanol which served as an organic modifier in the mobile phase, it was clearly observed that a higher percentage of ethanol led to more rapid elution (Figure 3 and Table 2). Specifically, using 40% and 50% ethanol resulted in separation within 5 min. The effect of column temperature was similar to that of ethanol content in the mobile phase; higher temperatures decreased the retention times of the analytes, resulting in faster separations (Figure 3 and Table 2). This can be attributed to several factors, including increased solubility of the analytes, decreased viscosity of the mobile phase, enhanced mass transfer of analytes

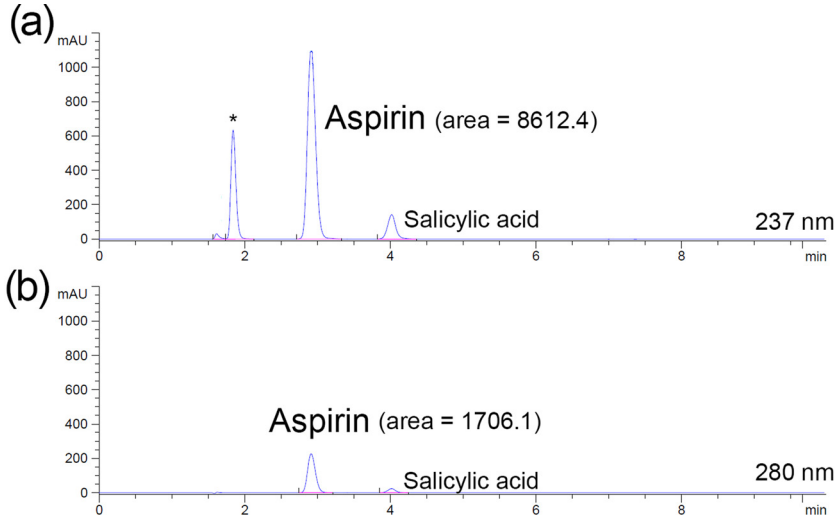


Figure 2: Comparison of the chromatograms from the proposed assay, setting the detection wavelength at (a) 237 nm and (b) 280 nm. (*peak of acetic acid in diluent).

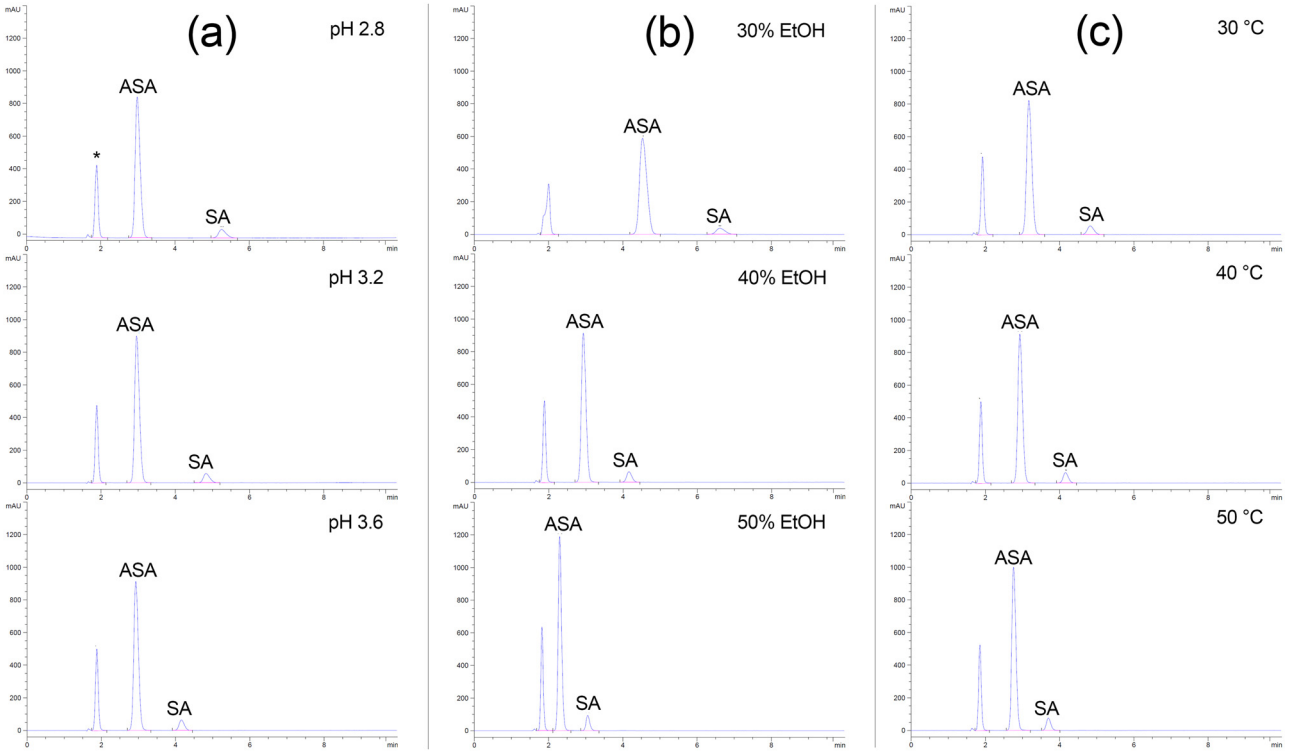


Figure 3: Effect of (a) pH of the mobile phase (tested using 40% ethanol at 40°C), (b) percent of ethanol in the mobile phase (tested at pH 3.6, 40°C), and (c) column temperature (tested using 40% ethanol, pH 3.6) on the separation of aspirin (ASA) and salicylic acid (SA). (*peak of acetic acid in diluent).

through the column, and altered solute retention due to weakened interactions between the analytes and the stationary phase [29,30].

Based on these results, several conditions tested were found to satisfactorily met the selection criteria mentioned above, in terms of the quality of separation and acceptable backpressure and column temperature, demonstrating the flexibility of the analysis condition. In this work, one of the most effective conditions – using 40% ethanol in water, pH 3.6 as the mobile phase, with a column temperature of 40°C – was selected for further studies. This protocol not only optimized the assay performance but also minimized ethanol use, making the assay greener and more cost-effective. However, other conditions may be employed depending on specific

Table 2: Effect of chromatographic parameters on the separation of aspirin and salicylic acid and backpressure generated

Parameter	Aspirin			Salicylic acid	Resolution between aspirin and salicylic acid	Backpressure (bar)
	Retention time (min)	Plate number	USP tailing	Retention time (min)		
pH						
2.8	2.98	2,396	1.26	5.25	7.53	155
3.2	2.96	2,533	1.27	4.83	6.76	156
3.6	2.93	2,671	1.20	4.16	5.06	156
% (v/v) Ethanol						
30	4.53	2,399	1.23	6.61	5.10	140
40	2.93	2,671	1.20	4.16	5.06	156
50	2.30	3,134	1.21	3.05	4.36	164
Temperature (°C)						
30	3.17	2,421	1.17	4.82	5.71	189
40	2.93	2,671	1.20	4.16	5.06	156
50	2.76	2,928	1.15	3.70	4.40	129

needs and available equipment. For instance, running the separation using 50% ethanol at 50°C is possible if a shorter analysis time is desired and HPLC columns available in the laboratory are resistant to high pressure and temperature.

Along with optimizing the mobile phase, we also investigated the appropriate diluent used for preparing the standard and sample solutions by varying the concentration of acetic acid in ethanol. As illustrated in Figure 4, 10% acetic acid in ethanol significantly reduced the degradation of aspirin, likely due to the enhanced stability of the drug in an acidic environment. Therefore, this mixture was selected as the optimal diluent.

3.2 Validation results of the proposed method

The developed HPLC method underwent rigorous validation in accordance with USP guidelines, confirming its reliability and suitability for routine use. The summary of the results is shown in Table 3. Linearity was evaluated over a concentration range of 0.1–0.6 mg·mL^{-1} for aspirin, with the method demonstrating an excellent linear relationship, evidenced by an r^2 value of 0.9997 (Figure 5). The LOD was determined to be 0.01 mg·mL^{-1}, and the LOQ was established at 0.03 mg·mL^{-1}, demonstrating the method's high sensitivity for pharmaceutical samples. Accuracy was assessed through recovery studies at three distinct concentration levels, with recoveries ranging from 98% to 102%, confirming the method's accuracy. Specificity was demonstrated by the method's ability to clearly resolve the aspirin from potential impurities, such as salicylic acid, as well as tablet excipients, with no interference peaks observed in the chromatograms (Figure 6). Additionally, a high peak purity factor of 999.996, determined by a diode array detector, further confirmed the method's specificity (Figure 6).

The precision of the method was validated through both intra-day and inter-day studies, yielding RSDs below 2%, indicating the method's repeatability and reproducibility. Robustness was tested by deliberately varying the method parameters, including detection wavelength (±3 nm), flow rate (±0.1 mL·min^{-1}), and column temperature (±2°C). It was found that these variations had minimal impact on the assay results, with all changes remaining within acceptable limits,

Table 3: Summary of the method validation results

Parameter	Result
Regression equation	y = 19,146x − 276.89 when y is peak area and x is concentration of aspirin (mg·mL^{-1})
r^2	0.9997
Range	0.1–0.6 mg·mL^{-1}
LOD	0.01 mg·mL^{-1}
LOQ	0.03 mg·mL^{-1}
Accuracy	
% Recovery (n = 3, for each level)	101.14 ± 0.62% (low; spiked with 0.3 mg·mL^{-1}) 99.42 ± 0.37% (medium; spiked with 0.4 mg·mL^{-1}) 99.78 ± 0.96% (high; spiked with 0.5 mg·mL^{-1})
Precision	
% RSD for intra-day precision (n = 6)	1.20%
% RSD for inter-day precision (n = 18)	1.12%

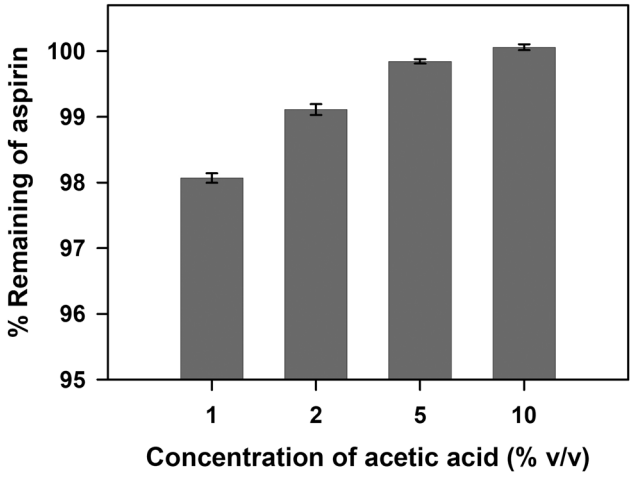


Figure 4: % Remaining of aspirin in ethanol containing different percentages of glacial acetic acid, after being kept at room temperature after 12 h (n = 3).

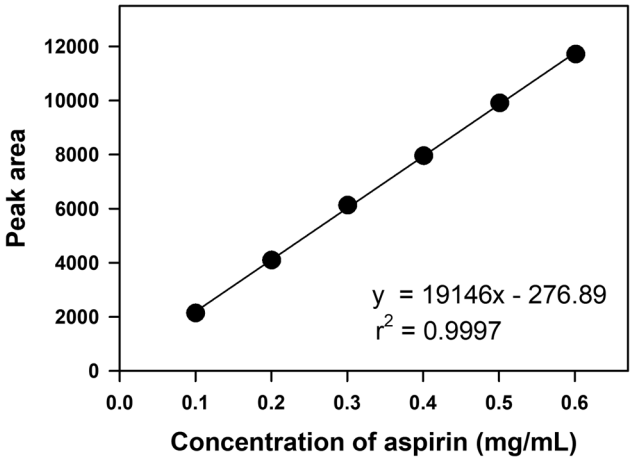


Figure 5: Standard curve of aspirin in the proposed ethanol-based HPLC method.

as evidenced by the overall % RSDs within 2% and no significant difference tested by ANOVA (p = 0.05) (Table 4). These findings confirmed the robustness of the method.

Finally, a stability study was conducted, revealing that aspirin remained stable in the diluent (10% acetic acid in ethanol) for up to 12 h at room temperature, with the remaining concentrations of 100.3 ± 0.7% and 99.6 ± 0.8% (n = 3) of the initial value for both standard solutions and sample solutions, respectively. All of the validation results confirmed that the proposed method was linear, sensitive, accurate, precise, specific, robust, and stable, making it well-suited for the reliable quantification of aspirin in tablet formulations.

Table 4: Robustness of the method (n = 3)

Parameter	% Labeled amount of aspirin	% RSD	Statistical test results
Detection wavelength (nm)			
234	101.0 ± 1.8	1.63	No difference (F_{cal} = 0.3350, F_{crit} = 5.1433)
237	100.2 ± 1.8		
240	99.8 ± 1.8		
Flowrate (mL·min^{-1})			
0.9	100.2 ± 2.1	1.69	No difference (F_{cal} = 0.0543, F_{crit} = 5.1433)
1.0	100.2 ± 1.8		
1.1	100.6 ± 1.9		
Temperature (°C)			
38	100.6 ± 1.9	1.61	No difference (F_{cal} = 0.0565, F_{crit} = 5.1433)
40	100.2 ± 1.8		
42	100.6 ± 1.9		

3.3 Application to analysis of the real samples

The proposed HPLC method was effectively applied for analyzing aspirin content in commercially available tablets. As detailed in Table 5, the assay results indicated that the aspirin contents in the tablet samples were within the labeled range, with the average aspirin content of 101.9 ± 1.5% of the stated amount. These results aligned with those obtained using the USP (101.7 ± 1.2%) and BP (101.0 ± 1.1%) methods, with no significant differences observed between the methods (ANOVA; F_{cal} = 0.6912, F_{crit} = 3.6823, p > 0.05).

The proposed method effectively separated the peaks of aspirin and its main degradation product, salicylic acid, making it a viable stability-indicating assay for assessing aspirin tablets over extended storage periods. This method also showed promise for adaptation into a limit test for quantifying salicylic acid impurity in aspirin formulations. In terms of analysis speed, the method provides a significant improvement, with both aspirin and salicylic acid being eluted within 5 min – a substantial reduction from

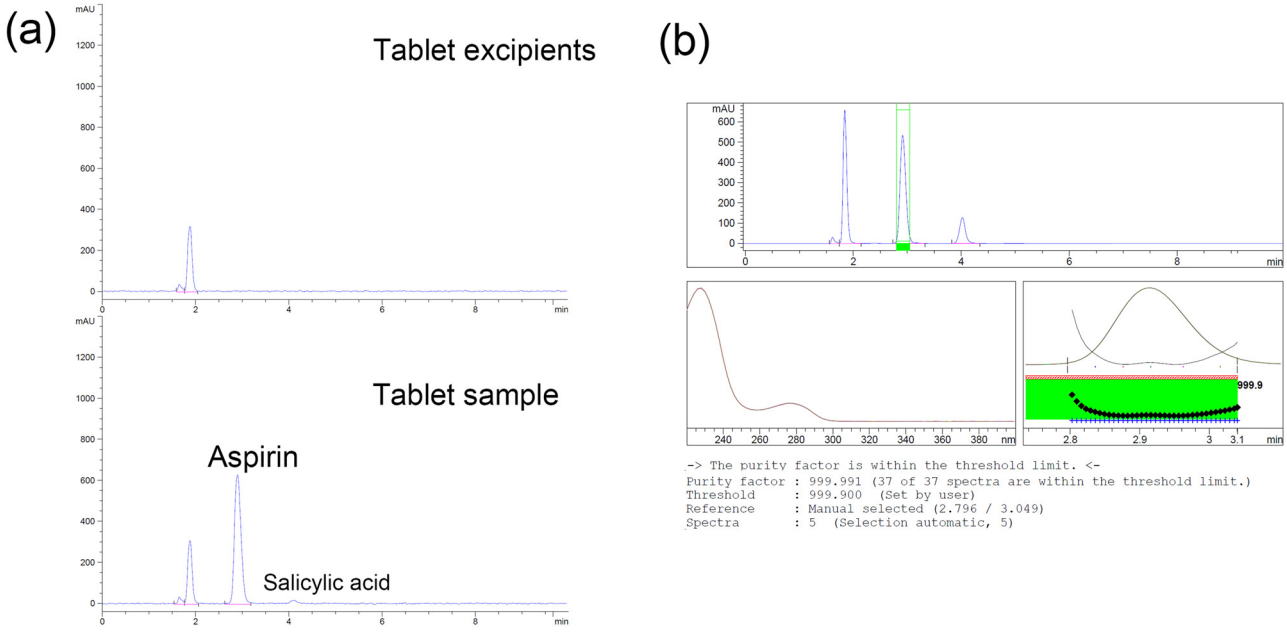


Figure 6: Specificity of the method shown by (a) chromatograms with separated peaks of aspirin and (b) peak purity factor analyzed by DAD.

Table 5: Chromatographic parameters and assay results for commercial tablets obtained from the proposed method and the pharmacopeial methods

Method	Chromatographic parameter				Assay result
	Retention time of aspirin (min)	USP tailing	Resolution from salicylic acid	Backpressure (bar)	% Labeled amount
Proposed method	2.919	1.154	5.47	160	101.9 ± 1.5
USP Method	16.650	0.898	7.04	257	101.7 ± 1.2
BP Method	6.697	0.888	11.86	120	101.0 ± 1.1

the 17 and 10 min required by the USP and BP methods, respectively (Figure 1). Additionally, by using a more environmentally friendly solvent compared to acetonitrile in conventional pharmacopeial methods, the proposed approach offers improved user safety and reduced environmental impact. Overall, the proposed ethanol-based HPLC method not only matched the performance of current methods in accuracy, precision, and specificity but also offered improvements in speed and environmental impact. This makes it a viable alternative for routine quality control of aspirin in pharmaceutical formulations.

3.4 Feasibility of domestically produced bioethanol for HPLC

As noted, while many countries are capable of producing high-quality bioethanol from various agricultural feedstocks, there has been no study investigating the feasibility of using these products for HPLC or analytical purposes. Therefore, bioethanol produced in Thailand from sugarcane molasses or cassava was used as a case study in this work to explore its potential. These products, currently used as fuel additives, possess a purity in the range of 99.83–99.97%, conforming to the standard rubric (>99.5%) for absolute ethanol of HPLC/spectrophotometric grade [31] (Table 1). The study revealed that using four bioethanol products as both the diluent and mobile phase in the developed HPLC method for analyzing aspirin tablets resulted in chromatograms and separation outcomes similar to those obtained using imported EMSURE® ethanol from Merck. These similarities included the retention time of the analyte, peak symmetry, resolution, baseline smoothness, and generated backpressure (Figure 7 and Table 6). Importantly, no significant difference in the assay results, in terms of the % labeled number of aspirin in the tablets, was observed among the different ethanol samples. Hence, it can be concluded that domestically produced bioethanol in Thailand can be an effective alternative solvent for HPLC applications.

Economically, using domestically produced bioethanol provides considerable cost savings by reducing import costs, which in turn lowers the final market price. For example, while bioethanol in Thailand is not yet marketed specifically for laboratory use, its current price is as low as 1.2 USD per liter, compared to around 30 USD per liter for imported ethanol. This substantial price difference makes domestic bioethanol an attractive option for laboratories.

3.5 Greenness, practicality, and sustainability features of the proposed method

The ethanol-based method presents several advantages in terms of greenness and sustainability over the USP and BP methods, including reduced hazards and lower environmental impact, as ethanol is a safer alternative to acetonitrile. Additionally, the faster analysis provided by this method allows for higher sample throughput (i.e., more samples analyzed per hour) and reduced waste generation. Moreover, ethanol derived from bio-based sources can effectively replace acetonitrile or methanol, both of which are typically produced through petrochemical processes. These characteristics align the proposed method with the principles of green analytical chemistry, making it a greener option compared to pharmacopeial methods. To support the environmental friendliness features, the analytical greenness metric (AGREE) [32] tool was used to evaluate the “greenness” of an analytical method by assessing its environmental impact and alignment with sustainable practices. It uses 12 criteria based on the principles of green chemistry to provide a comprehensive analysis of a method’s eco-friendliness. Through a color-coded score, AGREE highlights areas where the method is environmentally sustainable and identifies opportunities for improvement. In addition, the green analytical procedure index (GAPI) [33] was employed to assess the environmental impact of analytical methods by examining their life cycle, from sample collection to waste management. It evaluates key factors like energy use, reagent consumption, and waste

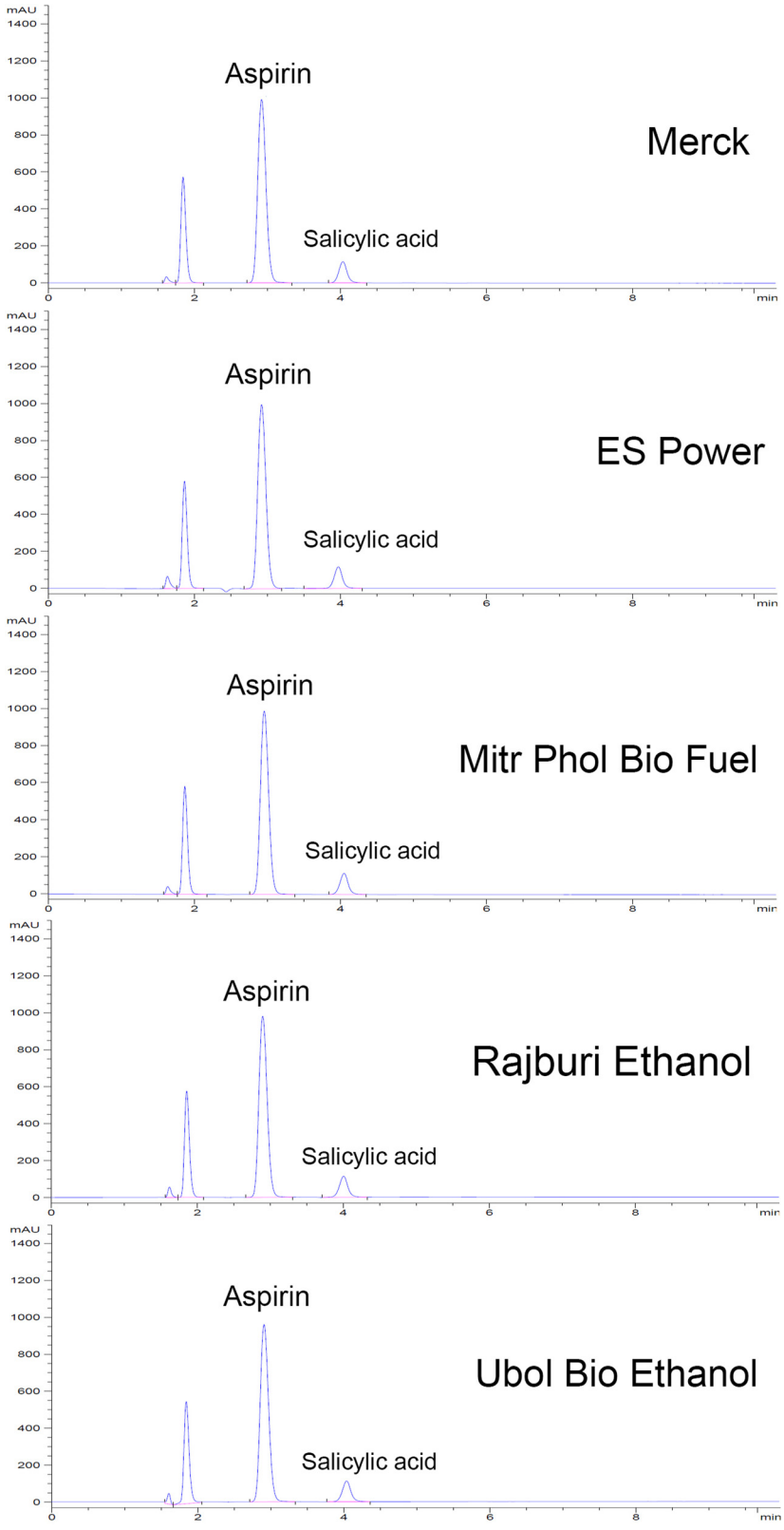


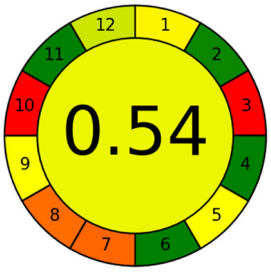
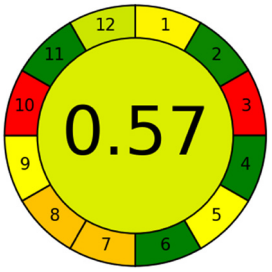
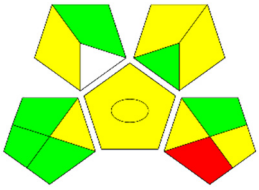
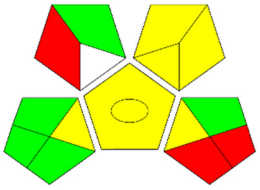
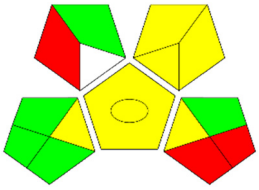
Figure 7: The chromatograms obtained from the assays using different bioethanol.

Table 6: Chromatographic parameters and assay results of commercial tablets obtained from the proposed method using different bioethanol

Manufacturer	Chromatographic parameter				Assay result
	Retention time of aspirin (min)	USP tailing	Resolution from salicylic acid	Backpressure (bar)	% Labeled amount
Merck, USA[a]	2.919	1.154	5.47	160	101.9 ± 1.5
ES Power, Thailand[b]	2.919	1.132	5.19	157	101.4 ± 1.4
Mitr Phol Bio Fuel, Thailand[b]	2.956	1.129	5.34	158	101.3 ± 1.3
Rajburi Ethanol, Thailand[c]	2.896	1.160	5.39	160	101.3 ± 0.9
Ubol Bio Ethanol, Thailand[c]	2.906	1.166	5.49	162	101.9 ± 1.2

[a]from grain or sugar cane, [b]from sugarcane molasses, and [c]from casava.

Table 7: Greenness and practicality and applicability of the proposed method and pharmacopeial methods assessed by Analytical GREEnness (AGREE), Green Analytical Procedure Index (GAPI), and Blue applicability grade index (BAGI)

Assessment of greenness	Proposed method	USP method	BP method
AGREE*			
GAPI			
BAGI			

*1 = Sample treatment, 2 = Sample amount, 3 = Device positioning, 4 = Sample preparation Stages, 5 = Automation, miniaturization, 6 = Derivatization, 7 = Waste, 8 = Analysis throughput, 9 = Energy consumption, 10 = Source of reagents, 11 = Toxicity, 12 = Operator's safety.

production, summarizing the results in a five-segment pictogram. This visual output allows users to quickly identify both the greener aspects of a method and areas where sustainability could be enhanced. It was found that the proposed method had an AGREE score of 0.65 which was higher than the scores of 0.54 and 0.57 for the USP and BP methods, respectively (Table 7). Additionally, the GAPI highlights more green sections and fewer red sections, underscoring the method's superior greenness profile (Table 7).

Apart from AGREE and GAPI, the Blue applicability grade index (BAGI) [34]. Index assesses the practicality and applicability of an analytical method by evaluating ten criteria to generate a pictogram and a score, which reflect the method's applicability and functionality. The score, ranging from 25 to 100 points, is represented on a blue color scale, with scores nearer to 100 and darker blue shades indicating higher performance in applicability. In this study, the proposed method achieved a BAGI score of

77.5 (Table 7), highlighting its strong practicality and applicability and aligning with environmental sustainability principles.

In line with the United Nations Sustainable Development Goals (SDGs) [35], the effective analytical method for aspirin drugs supports SDG #3: promoting healthy lives and well-being for all at all ages. Aspirin is widely used not only to relieve pain, reduce fever, and decrease inflammation but also to prevent heart attacks and strokes, especially in elderly individuals at higher risk of these conditions. Effective quality test of aspirin products guarantees their safety and efficacy for consumers, contributing to overall public health. In terms of using ethanol as a mobile phase, it aligns closely with SDG #12: responsible consumption and production. As drug analysis is a crucial step in the pharmaceutical manufacturing industry, employing safer solvents like ethanol in laboratories helps minimize the release of toxic waste into the environment. This further helps protect ecosystems and biodiversity, both in aquatic and terrestrial environments, fitting SDG #14: Life Below Water and SDG #15: Life on Land. In the aspect of socio-economy, using domestically produced bioethanol supports local bioethanol production, thereby stimulating job creation and economic growth within the country [15], aligning with SDG #8: promoting sustained, inclusive, and sustainable economic growth, along with full and productive employment for all.

Environmentally, domestically produced bioethanol typically has a lower carbon footprint because it is derived from sustainable, renewable resources such as agricultural residues or crops. This aligns with sustainability goals and reduces the environmental impact of laboratory operations. Moreover, local bioethanol ensures a consistent and reliable supply, crucial for maintaining uninterrupted analytical processes and tailored to meet specific local standards and needs. It is important to note that this study used only four domestically produced bioethanol products. To strengthen the findings, additional bioethanol sources should be investigated to confirm their broader applicability. Based on the feasibility demonstrated in this study and the highlighted benefits, there is a promising opportunity to introduce high-purity, domestically produced bioethanol to the market as a laboratory-grade reagent for analytical applications. In summary, leveraging domestically produced bioethanol for broader applications, such as HPLC, not only enhances the environmental friendliness of analytical practices but also supports both economic and environmental sustainability. By adopting this greener alternative, countries can reduce reliance on imported solvents, lower costs, and contribute to a more sustainable future.

4 Conclusion

This study introduces two groundbreaking innovations. First, it offers a novel green HPLC assay for aspirin tablets, utilizing ethanol in the mobile phase, effectively addressing a gap in current analytical techniques for this drug. Additionally, ethanol has a similar eluting capability to methanol and acetonitrile, it could serve as a greener solvent alternative in reversed-phase HPLC for various drugs and analyses. Second, while ethanol-based HPLC assays are increasingly adopted, this work is the first to demonstrate the potential of domestically produced bioethanol, marking a significant advance in sustainable analytical practices. The developed assay not only meets the required analytical performance, adhering to method validation guidelines, but also enhances efficiency by shortening analysis time to less than 5 min. By utilizing ethanol, which is less toxic and more biodegradable, the method aligns with the growing demand for environmentally friendly analytical practices. A comparison between fuel-grade bioethanol produced in Thailand and imported commercial HPLC-grade ethanol demonstrates that local bioethanol offers comparable chromatographic performance and analytical results, confirming its viability as a sustainable alternative. Additionally, the use of domestic bioethanol results in significant cost-savings, supports local economies, and reduces carbon emissions. Overall, integrating locally produced bioethanol into HPLC represents a significant advancement toward more sustainable, cost-effective, and eco-friendly analytical methodologies.

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Rojanarata: conceptualization, validation, writing – review & editing, supervision, project administration, funding acquisition.

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