Thermal Degradation Kinetics of Flavonoid Derived From
*Orthosiphon stamineus*

J. Gimbun¹²*, S.F. Pang¹, S.J. Lau¹, M.M. Yusoff³, L. Chuah⁴

¹Faculty of Chemical & Natural Resources Engineering, ²Centre of Excellence for Advanced Research in Fluid Flow (CARIFF), ³Faculty of Industrial Sciences & Technology, Universiti Malaysia Pahang, 26300 Gambang, Pahang, MALAYSIA.
⁴Dept. Chemical Engineering, Faculty of Engineering, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, MALAYSIA.

*Corresponding author. Email: jolius@ump.edu.my, Tel: +6095492899, Fax: +6095492889

**Abstract**

This paper presents a thermal degradation study of total flavonoid content and two main types of flavonoid contained in *Orthosiphon stamineus*. Detailed analyses of individual targeted flavonoid were examined using high performance liquid chromatography. Thermal degradation study was performed using a sealed metal tube immersed in a water bath. The flavonoid degradation (sinensetin and eupatorin) fitted well to Hinrich-Redemacher kinetics with an error of about 6%. The degradation rate constant for eupatorin (859.18/sec) is much higher than sinensetin (195.06/sec), indicating a faster rate of degradation. Eupatorin is more susceptible to thermal degradation (48.2%) compared to sinensetin (26.3%).

**Keywords**

*Orthosiphon stamineus*, thermal degradation, flavonoid, misai kucing, eupatorin, sinensetin

1. Introduction

*Orthosiphon stamineus* or Misai Kucing, are traditionally used in Malaysia for treatment of eruptive fever, urinary lithiasis, edema, hepatitis, jaundice, hypertension, diabetes mellitus, gout, rheumatism, diuretic, anti-inflammatory and influenza [1,2]. Previous studies revealed that extracts of *O. stamineus* had many useful bioactive compounds that poses a diuretic [3] and antidiabetic properties [4-5]. *O. stamineus* have been reported to possess anti-inflammatory, anti-hypertensive, and hypoglycemic activity [6].

Bioactive compounds such as flavonoid, vitamins, protein and antioxidant are known to suffer from a thermal degradation process when exposed to high temperature over a long period, which is often the case during the extraction and powder making process. Anandharamakrishnan et al. [7] for instance, reported more than 60% protein denaturation in a tall pilot scale spray dryer at operating temperature above 100 °C (outlet), commonly used condition for a spray drying operation. Thermal degradation of other bioactive compounds such as Vitamin E and Vitamin A were also reported by many authors [8-9]. The thermal degradation is undesirable because the degraded product is of low nutritional value and consequently, hampers the intention to produce a nutraceuticals product. Therefore, it is very important to understand the degradation kinetics of bioactive compound from *O. stamineus*, which is vital for development of high quality product. Thermal degradation kinetics of flavonoid from *O. stamineus* has never been studied before and hence this is the aim of this work.

2. Materials and Methods

2.1 Chemicals and Plant Material

Sodium nitrite, methanol, isopropanol, sodium hydroxide, and HPLC grade acetonitrile were obtained from Merck (Darmstadt, Germany). HPLC grade methanol, trifluoroacetic acid and quercetin were obtained from Fisher Scientific (Pittsburgh, PA). HPLC grade dimethyl sulfoxide, aluminium hexachloride, eupatorin, and sinensetin were obtained from Sigma Aldrich (St. Louis, MO). Leaves were collected in Gambang, Pahang, Malaysia from a white flowered *O. stamineus* similar to one that has been deposited at the Forest Research Institute, Malaysia (voucher no.
The freshly collected leaves were washed and dried in an oven at 35 °C for 4 days before grounding to powder form and kept in an air tight plastic bag at room temperature to prevent moisture absorption.

2.2 Ultrasonic Assisted Extraction
O. stamineus extracts were prepared using ultrasonic assisted extraction in an ultrasonic bath (CREST P1800D, US) at 50 °C for 90 min at 45 kHz. Total of 8 g of powdered O. stamineus leaves was added to 100 ml of 50% methanol (8 wt.%) in a 250 ml sealed Erlenmeyer flask. The supernatant was then separated from the residue by filter through 0.22µm membrane by vacuum filtration. Extract was concentrated by evaporating out excessive methanol from the extract by rotary evaporator at 40 °C under vacuum.

2.3 Heating Experiment
The heat treatments were performed using O. stamineus extract and carried out at different temperatures (70, 90 and 120 °C) for 0–30 min. O. stamineus extract was enclosed in seal metal tubes (outer diameter = 16 mm; inner diameter = 13 mm, length = 150 mm) and placed in the oil bath for different treatment times, depending on the temperature. The temperatures were measured by a thermometer which is sealed with PTFE. For all treatment, preheating time was taken into account for the extract to reach at the isothermal phase. After treatment, the tubes were immediately cooled in an ice bath to stop further thermal degradation. The sample was analysed using high-performance liquid chromatography (HPLC) to determine the degradation of individual compound, meanwhile the total flavonoid content was determined via colorimetric assay using UV-Vis.

2.5 Total Flavonoid Content
Total flavonoid content (TFC) was measured by the aluminum chloride colorimetric assay [10]. Diluted of heat treated extract (0.2 ml) or standards solution of quercetin (0.0025- 0.5 mg/ml) was added to centrifuge tube containing 4.8 ml ultrapure water. NaNO₂ (0.3 ml, 5%) was added and mixed using a vortex mixer. After 5 min, 0.3 ml 10% AlCl₃ was added. At the 6th min, 2 ml 1M NaOH solution was added and the total volume was made up to 10 ml with ultrapure water. The solution was mixed well and the absorbance was measured against prepared reagent blank at λ = 414 nm using a calibrated UV-Vis (Chang et al., 2002). Total flavonoid content of the leaves was expressed as mg quercetin equivalents per gram dry weight (mg QE/g DW) by comparing with the calibration curve for quercetin.

![Figure 1: Typical HPLC chromatogram of O. stamineus leaves extracts](image)

2.6 HPLC Analysis of Polyphenols
HPLC analysis was performed according to Akowuah et al. [11] using Agilent Technologies 1200 system (Agilent Technologies, USA) equipped with an automatic injector, a column oven, and a UV detector. A Zorbax Eclipse plus C18 (Agilent Technologies, USA) column (5 µm, 250mm×4.6mm i.d.) was used for the chromatographic separation. The temperature was maintained at 25 °C, with injection volume of 10 µl and flow rate of 1 ml/min. All markers were separated under isocratic condition with methanol–water–tetrahydrofuran (45:50:5 v/v) as mobile phase. The peaks were detected at 340 nm (see Figure 1) and verified by comparison with standards i.e. eupatorin (Eup) and sinensetin (Sin). HPLC quantification was carried out using external standards method and the results reported as mg marker/g DW sample. The calibration curves of the studied flavonoid compounds showed good
linearity ($r^2 > 0.997$) in the range of 0.08 – 500 µg/L concentration. Limit of detection was determined by setting the signal to noise ratio of 3:1.

2.7 Mathematical Model for Thermal Degradation Kinetics

Thermal degradation mechanism of bioactive content (sinensetin, eupatorin and total flavonoid content) in *O. stamineus* extract was considered to follow either the Arrhenius type kinetics model [12]:

\[
\ln \left( \frac{c_t}{c_0} \right) = -A_0 \exp \left( -\frac{E_a}{RT} \right) t
\]

or Hinrichs-Rademacher denaturation kinetics approach [13]:

\[
\frac{dc}{dt} = -k_{o,T} C^n
\]

\[
\frac{c_t}{c_0} = \left[ 1 + (n - 1)k_{o,T}C_0^{n-1}t \right]^{\frac{1}{n}}
\]

The variable $C$, $A_0$, $k_{o,T}$, $n$, $R$, $E_a$ and $T$ are the concentration of bioactive compound content, prefactor, rate constant, holding time, order of reaction, universal gas constant, activation energy and temperature, respectively. Applying equation (2) and (4) to fit experimental data using nonlinear regression (generalized reduced gradient within Microsoft Excel Solver) allows for determination of $k_{o,T}$, $n$, and $E_a$ values.

2.8 Statistical Analysis

Each experiment was repeated in triplicates. Analysis of variance (ANOVA) was performed by using the data analysis tools in Microsoft Excel 2010, and a least significant difference (LSD) test was used to compare the means with a confidence interval of 95%.

3. Results and Discussion

3.1 Influence of Heat Treatment on Total Flavonoid Content

Figure 2 shows the graph of total flavonoids content of extracted solution after heat treatment at 90 °C. The result suggests that total flavonoids content did not decreased much (2.2%) after the heat treatment at 90 °C. According to Murakami et al. [14] flavonoid such as rutin, luteolin and luteolin-7-glucoside is stable even at 100 °C. However, findings from previous work on effect of thermal treatment to flavonoid degradation are tinged with confusion. For instance, 80s pasteurization at 95 °C lead to decreases of naringin, rutin, quarcetin and naringenin content from grapefruit juices [15], meanwhile increasing temperature from 40°C to 70°C during apple juice processing increases flavonoid content (50%) [16]. Thermal pasteurization treatments (90°C, 60s) for strawberry juices have no effect on quercetin and kaempferol contents [17]. Thus, the minimal decreases in total flavonoid content from *O. stamineus* extract in this work are consistent with findings from previous work. Furthermore, the tube containing sample was sealed during heat treatment experiment, hence limiting flavonoid degradation. Earlier, Buchner et al. [18] showed the absence of oxygen highly reduces quercetin degradation and prevents rutin breaking up during heating. Both the Arrhenius and Hinrich-Rademacher equation showed good fitting of flavonoid degradation from *O. stamineus* extract with error of 0.28% and 0.13%, respectively.

<table>
<thead>
<tr>
<th>TFC</th>
<th>Model</th>
<th>$A_0$ (s$^{-1}$)</th>
<th>$E_a$ (J/mol)</th>
<th>$n$</th>
<th>$k_{90^\circ C}$ (s$^{-1}$)</th>
<th>% error</th>
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<tbody>
<tr>
<td>TFC</td>
<td>Arrhenius</td>
<td>0.00002</td>
<td>163.93</td>
<td></td>
<td>60.47</td>
<td>0.13</td>
</tr>
<tr>
<td>TFC</td>
<td>Hinrich-Rademacher</td>
<td>0.00043</td>
<td>147.09</td>
<td>4.21</td>
<td>859.18</td>
<td>5.84</td>
</tr>
<tr>
<td>Eupatorin</td>
<td>Arrhenius</td>
<td>0.000035</td>
<td>2504.46</td>
<td></td>
<td>195.06</td>
<td>7.91</td>
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</tbody>
</table>
3.2 Influence of Heat Treatment on Sinensetin and Eupatorin Content

It is not possible to study the thermal degradation of individual component from *O. stamineus* extract by measuring the total flavonoid content. The proximate analysis using a colorimetric assay (total flavonoid content) shows only a minimal degradation (2.2%) but they are not an accurate indicator for an individual target compound such as sinensetin and eupatorin. Detailed analysis using HPLC is needed to determine accurately single component degradation. Therefore, the sample was analysed using HPLC to determine the concentration of individual target compound before and after heating experiment. Figures 3 and 4 showed the degradation of sinensetin and eupatorin at 90 °C. The results suggest that eupatorin is more susceptible to thermal degradation (48.2%) compared to sinensetin (26.3%). This is due to presence of two-hydroxyl group in the C-ring of eupatorin molecular structure but no hydroxyl group in sinensetin. Flavonoid with more hydroxyl group is known to be more susceptible to thermal degradation [18]. Hinrichs-Rademacher model provides a better fit to experimental data for both sinensetin and eupatorin degradation with error of 5.95% and 5.84%, respectively. The degradation rate constant \( k_{o,T} \) for eupatorin (859.18 s\(^{-1}\)) is much higher than sinensetin (195.06 s\(^{-1}\)), indicating a faster rate of degradation. There is no significant difference between the order of degradation \( n \) for both sinensetin (4.33) and eupatorin (4.21). Meanwhile, Arrhenius model provides a fair fit for sinensetin and eupatorin 7.91% and 11.29%, respectively. The prefactor \( A_0 \) for sinensetin (0.00035 s\(^{-1}\)) is much smaller than those of eupatorin (0.00045 s\(^{-1}\)), and thus resulting in a slower degradation for the former. The activation energy \( E_a \) which represents the amount of energy required for degradation to occur is also much bigger for sinensetin (2504.46 J/mol) than eupatorin (147.09 J/mol). As discussed earlier, eupatorin consists of 3 hydroxyl group while no hydroxyl group exists on sinensetin, contributing to higher heat stability to the latter. This heat stability is reflected on the calculated kinetics parameters for both sinensetin and eupatorin thermal degradation.

![Figure 3: Thermal degradation kinetics of sinensetin at 90 °C](image)
4. Conclusions
Heat treatment of *O. stamineus* extract in a sealed metal tube for 40 minutes at 90 °C caused 26.3% and 48.2% degradation of sinensetin and eupatorin, respectively. The proximate analysis using a colorimetric assay (total flavonoid content) shows only a minimal degradation (2.2%) but they are not an accurate indicator for an individual target compound such as sinensetin and eupatorin. Detailed analysis using HPLC is needed to determine accurately single component degradation. Thermal degradation of sinensetin and eupatorin from *O. stamineus* extract is better fitted with Hinrich-Rademacher kinetics model with error of less than 6%. The degradation rate constant for eupatorin was 859.18/sec is much higher than that for sinensetin which was 195.06/sec, this indicate that the former falls under a faster rate of degradation. However, the order of degradation for both sinensetin (4.33) and eupatorin (4.21) shows minimal difference.

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