Proceeding International Conference on Health Science
ocs.unism.ac.id/index.php/ICoHS
Vol. 1
October 2021

ANALYSIS OF TOTAL FLAVONOID CHEMICAL LEVELS IN HONEY CULTIVATION IN SOUTH BORNEO USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD

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Abstract

Honey has an antioxidant effect in certain levels or amounts that can react and neutralize free radicals. The antioxidant properties of honey are due to the presence of flavonoid compounds. Flavonoids are secondary metabolites of polyphenols, found widely in plants and food and have various bioactive effects including antioxidants. This study was an analytical observational study with a cross sectional design. The population and samples used in this study were cultivated honey found in South Borneo which were taken by purposive sampling technique. The purpose of this study was to determine the presence or absence of flavonoid compounds in cultivated honey in South Borneo using qualitative analysis methods and to find out the total flavonoid content in honey, besides knowing the difference in total flavonoid levels in cultivated honey based on location in South Borneo. The results obtained in this study are in the form of conclusions whether or not there is quercetin flavonoid content in the three samples tested and whether there is an effect of sampling location on the total flavonoid content obtained in honey.

Keywords: Cultivated honey, kelulut bees, HPLC, flavonoids, quercetin.

Introduction

Free radicals are atoms or molecules that have one or more unpaired electrons in their outer orbits, causing these molecules to be unstable and lead to highly reactive properties. To achieve stability, these molecules will react with surrounding molecules to obtain electron pairs. The reaction that continues to take place in the body if not stopped can cause various diseases such as cancer, heart disease, cataracts, premature aging, and other degenerative diseases. From these problems, the body requires an important substance called antioxidants that play a role in catching free radicals in the body, making it impossible to induce a disease (Pratama & Busman, 2020).Antioxidants are needed to

protect the body from free radical attack. Antioxidants are compounds or chemical components in certain levels or amounts capable of inhibiting or slowing down the damage caused by the oxidation process. The human body does not have an excessive amount of antioxidant reserves, so when a large number of radicals are formed, the body needs exogenous antioxidants. There are concerns regarding the unknown side effects of synthetic antioxidants, causing natural antioxidants to become a much-needed alternative (Budiarso et al., 2017).

Honey has antioxidant effects in certain levels or amounts that are able to react and neutralize free radicals (Legowo, 2016).Honey contains vitamin A, beta-carotene, vitamin B complex (complete), vitamins C, D, E, and K. Honey also contains many flavonoid components, such as luteolin, quercetin, apigenin, fisetin, kaempferol, ishoramnetin, acacetin, tamarixetin, chrystin, and galangin so that it can act as an antioxidant (Legowo, 2016)

The antioxidant properties of honey are due to the presence of flavonoid compounds. Flavonoids are secondary metabolites of polyphenols, found widely in plants and food and have various bioactive effects including antioxidants (Arifin & Ibrahim, 2018).The types of uflavonoids contained in honey are apigenin, pinocembrin, kaempferol, quercetin, galangin, chrysin and hesperetin compounds, and phenolic acids (such as ellagic, caffeic, p-coumaric, and ferulic acids (Mardhiati et al., 2020). has the capacity to reduce free radicals by modulating antioxidant activity enzymes such as catalase (Mardhiati et al., 2020).The correlation between flavonoids and antioxidants is known that the higher the uflavonoid content of an ingredient, the greater the antioxidant activity (Chotimah, 2019).

Indonesia is a tropical country that allows the growth of various plants that can produce nectar such as calliandra, rubber, randu, rambutan, mango, and others, thus allowing the availability of types of honey with different characteristics according to the origin of the plant nectar source. Different sources of nectar will make honey have a different composition, taste, aroma, and physical appearance. (Ustadi et al., 2017). However, the antioxidant activity value in honey followed the order of phenolic content and flavonoid content. This is because phenolic levels and flavonoid levels are antioxidant compounds present in honey, and indicate a strong correlation between the two types of compounds and antioxidant activity (Ustadi et al., 2017).

This is evidenced through previous research conducted by research (Ustadi et al., 2017) with the title "Bioactive Components of Rubber Honey (Hevea brasiliensis) Kaliandra Honey (Calliandra callothyrsus) and Randu Honey (Ceiba pentandra)" showing differences in flavonoid levels in each - each honey. The levels of flavonoids in the three types of honey tested ranged from 47.25-156.27.93 mg QE/100 g. Where calliandra honey occupies the first position with the highest flavonoid content of 156.27.93 mg QE/100 g, followed by rubber honey with a value of 63.40 mg QE/100 g, and maduurandu with a value of 47.25 mg QE/100 g. Meanwhile, in research (Mardhiati et al., 2020) with

the title "Characteristics and Some Nutrient Contents in Five Honey Samples Circulating in Supermarkets" this study found the total flavonoid content in five honey samples circulating in supermarkets had 4 different flavonoid levels ranging from 0.02-0.07% . In the research conducted (Sholekhah, 2018)with the title "Antioxidant Activity, Total Phenolic Levels and Total Flavonoid Levels of Calliandra Honey in Three Different Types of Bees (Apis Mellifera, Apis Cerana and Trigona Sp.)" stated that the highest total flavonoid levels of Calliandra honey were produced from three different types of bees, respectively. -includes Apis mellifera, Trigona sp. and Apis cerana, with total flavonoid values of 0.2664 mg QE/g, 0.2491 mg QE/g and 0.1176 mg QE/g.

The selection of HPLC (High Performance Liquid Chromatography) method in this study was because HPLC was a method with a fast and efficient substance separation system. HPLC (High Performance Liquid Chromatography) is a technique for separating a mixture of substances using a mobile phase and a stationary phase, the separation technique in HPLC occurs due to differences in adsorption power, solubility, partitioning, molecular size, ion size and vapor pressure on the components carried by the mobile phase. through the stationary phase (Aulia et al., 2016). Based on this explanation, researchers are interested in examining differences in total flavonoid levels in cultivated honey. The sample to be used is differentiated based on the location in South Borneo and the method used is the High Performance Liquid Chromatography method.

Materials and Methods

This research was conducted in Tapin Regency, Barito Kuala Regency, and Hulu Sungai Tengah Regency which was carried out in November 2020 to August 2021. The unit of analysis used in this study was cultivated honey in South Borneo. The data used is primary data collected by researchers with the observation method. The variables used in this study were total flavonoid variables and location variables by district. The analysis used is descriptive analysis and inferential analysis, descriptive analysis is carried out by conducting laboratory tests, the aim is to see a general description of the variables. Meanwhile, inferential analysis was carried out to see the effect of the location of the honey source on the total flavonoid content using linear regression.

Results and Discussion

The study was conducted in three areas in three districts in South Borneo. The three areas are in Tapin Regency, Barito Kuala Regency, and Hulu Sungai Regency. The reason for conducting research in these three districts is because each district has a kelulut bee cultivation farm. So that these three areas are used as sampling sites to be tested in this study.

Result

1. Sample Preparation

   Sample preparation is the process of preparing samples so that they are suitable for laboratory testing. The purpose of preparation in research is to prepare a substance to be analyzed in the laboratory. The basic principle in the sample preparation technique used in this study refers to the research procedure conducted by L.Sumarlin et al in 2018.

   Each honey sample was weighed as much as 25 g then added 100 mL of methanol, stirred for 3 minutes and then allowed to stand until a precipitate was formed. Filtered using Whatman No 42 filter paper to separate the filtrate and precipitate. The filtrate was then put into a separating funnel and partitioned with n-hexane gradually until the color of the filtrate was clear. This was done in order to form methanol extract and n-hexane extract perfectly. The methanol extract obtained was then concentrated using a rotary evaporator at a temperature of 65°C(L. Sumarlin et al., 2018).

Figure 1. Honey sample extract

2. Qualitative test

   The results of the extraction of each honey were tested qualitatively. The qualitative test in this study used the color reagent method, including the following:

   a. FeCl3 test

      The results obtained from the addition of FeCl3 in each test sample showed the results as shown in the table below:

Table 1 Test results for the addition of Mg and HCl

No.	Test sample	Research result	Information
1.	Tapin	Sample turns yellow	(-)
2.	Barabai	Sample turns yellow	(-)
3.	Tabunganen	Sample turns yellow	(-)

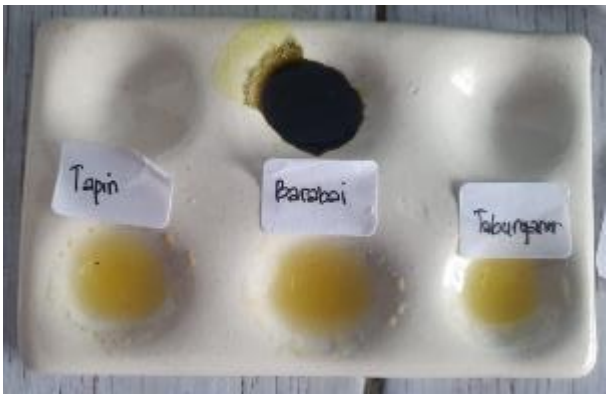

Figure 2. Test results for the addition of FeCl3

b. NaOH Uji test

The results obtained from the addition of NaOH solution in each test sample showed the results as shown in the table below:

Table 2. Test results for adding NaOH solution

No.	Test sample	Research result	Information
1.	Tapin	Sample no color change	(-)
2.	Barabai	Sample no color change	(-)
3.	Tabunganen	Sample no color change	(-)

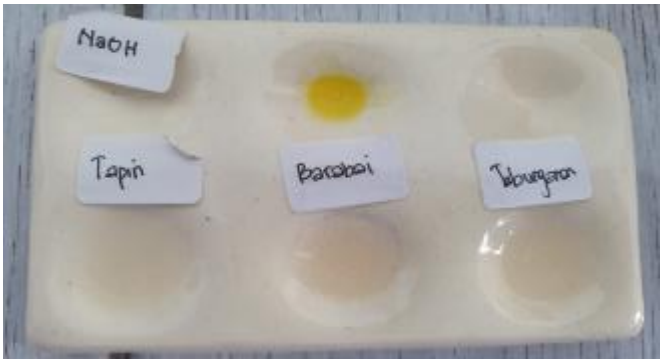

Figure 3. Test results for the addition of NaOH

c. AlCl3 . test

The results obtained from the addition of AlCl3 powder to each test sample showed the results as shown in the table below:

Table 3 Test results of adding AlCl3 powder

No.	Test sample	Research result	Information
1.	Tapin	Sample no color change	(-)
2.	Barabai	Sample no color change	(-)
3.	Tabunganen	Sample no color change	(-)

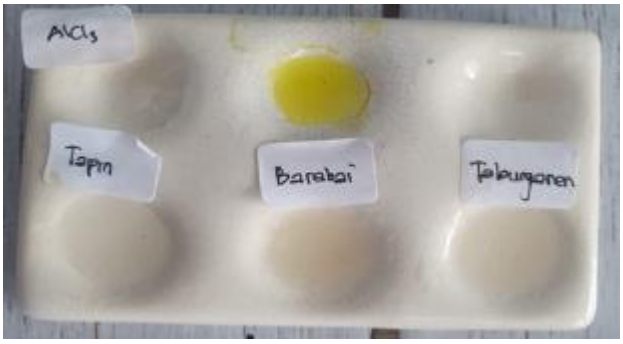

Figure 4. AlCl3 addition test results

d. NH4OH test

The results obtained from the addition of NH4OH solution in each test sample showed the results as shown in the table below:

Table 4. Test results for addition of NH4OH solution

No.	Test sample	Research result	Information
1.	Tapin	Sample no color change	(-)
2.	Barabai	Sample no color change	(-)
3.	Tabunganen	Sample no color change	(-)

Figure 5. Test results for the addition of NH_4OH

Quantitative test

a. Flavonoid standard solution

The flavonoid standard solution used is quercetin which has a maximum wavelength of 369.11 nm. previously run using a UV-Vis spectrophotometer at a wavelength range of 300-700 nm. The results of the wavelength measurement using a standard solution were then made with several concentrations, namely 6 ppm, 8 ppm, 10 ppm and 12 ppm using the mobile phase of methanol: aquabides (59: 41). The standard solution and the standard curve of the quercetin solution can be seen in the Image.

Figure 6. Preparation of a standard solution of quercetin

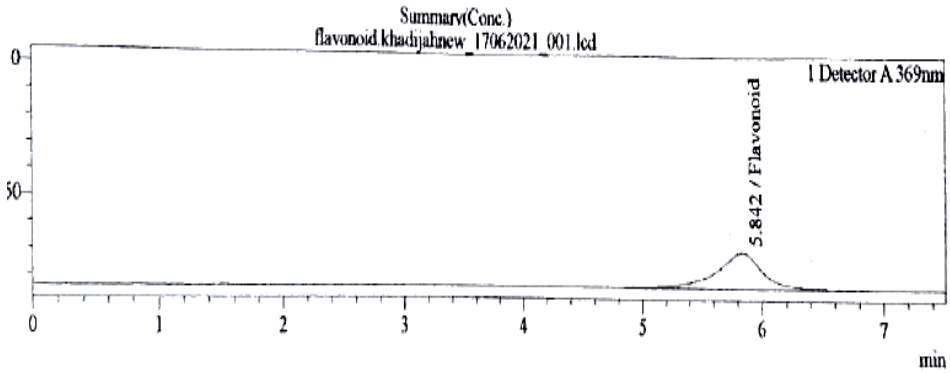

Figure 7. Standard solution of quercetin 6 ppm

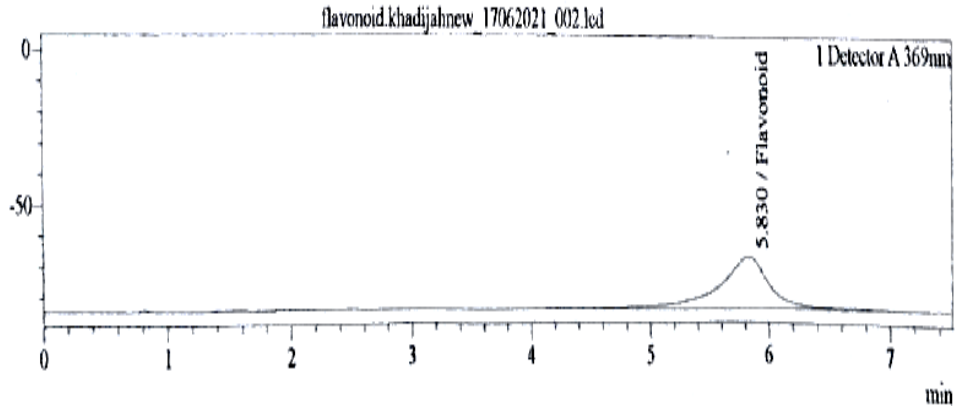

Figure 8. Standard solution of quercetin 8 ppm

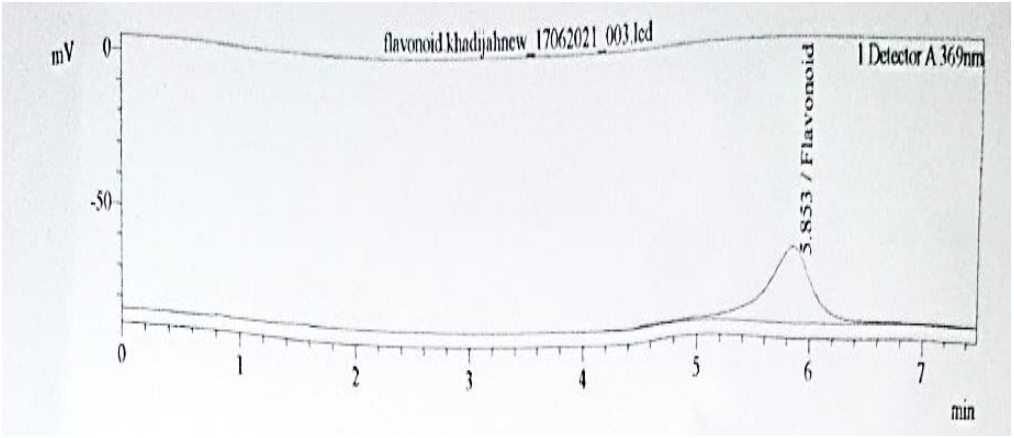

Figure 9. Standard solution of quercetin 10 ppm

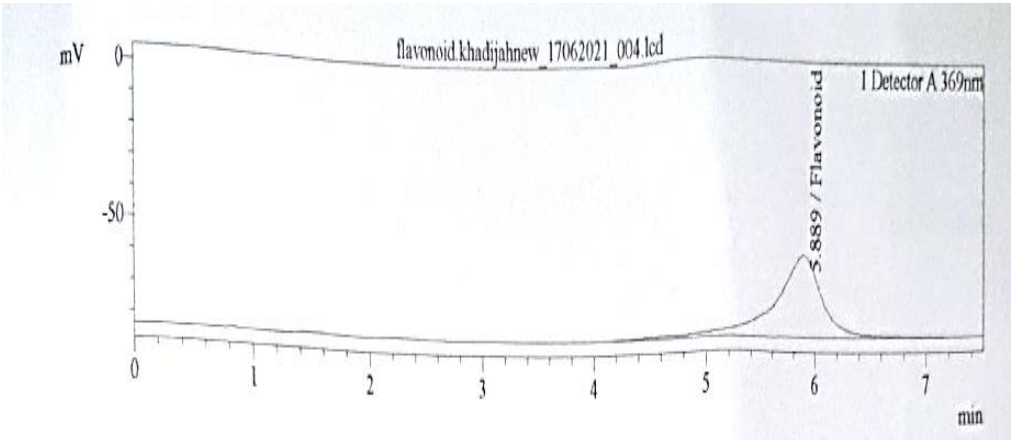

Figure 10. Standard solution 12 ppm

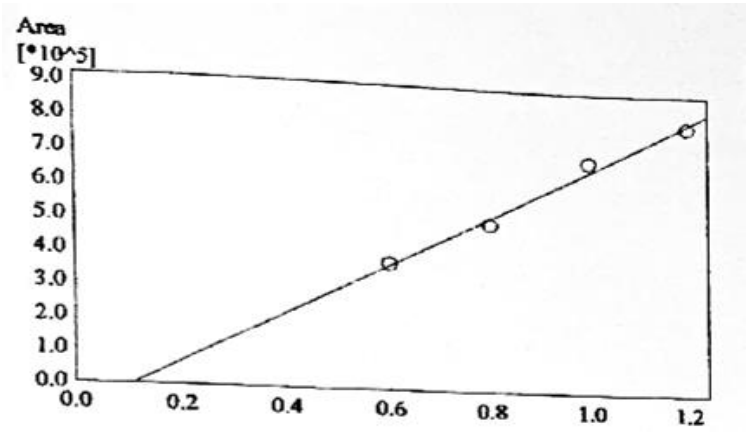

Figure 11. Standard curve of quercetin solution

Table 5. Results of standard measurements of quercetin

No	standard	Area
1	6 ppm	381310
2	8 ppm	510497
3	10 ppm	711408
4	12 ppm	825124

The data from the standard curve is made a linear regression equation by connecting the concentration and the area of the chromatogram. The data can be seen in Image 4.10 The calibration curve is obtained by the equation of the line y = 76617.7 x – 82474.5, the value of r = 0.994. The function of the standard curve is usually used to show the concentration of the sample solution from the measurement results so that the concentration of the sample solution can be obtained easily through the standard curve. The standard curve shows the correlation between the concentration of the solution (x axis) and the area (y axis) of the standard curve, resulting in a regressed equation, namely the equation y = a + bx where y = dependent variable, x = independent variable, a = intercept and b = regression coefficient / slope. The standard curve is said to be good if the value of r 0.98 is obtained.

b. Determination of quercetin content in honey

In the quantitative test research, testing was carried out using the HPLC method (high performance liquid chromatography) with a wavelength of 369.11 nm. The results obtained using this method can be seen in Image 4.12, Table 4.13 and Table 4.14

Figure 12. Samples of honey from Tapin, Barabai and Tabunganen daerah areas

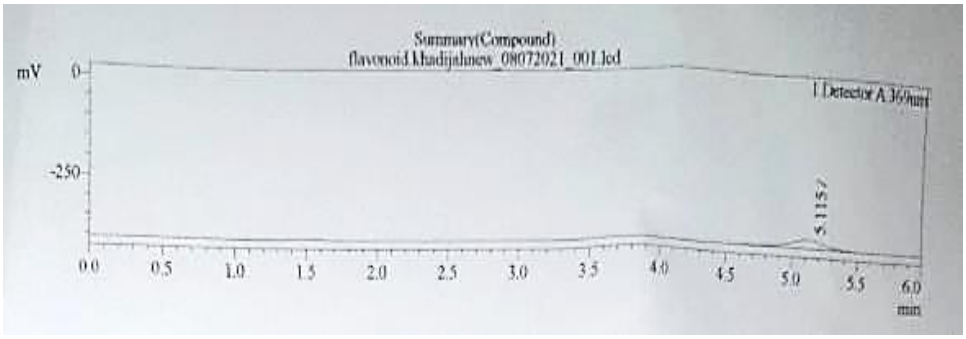

Figure 13. Quercetin content of honey cultivated in tapin area

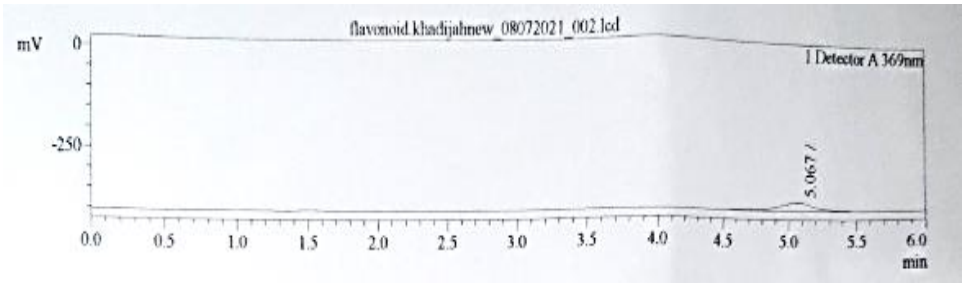

Figure 14. Levels of quercetin honey cultivated in the Barabai area

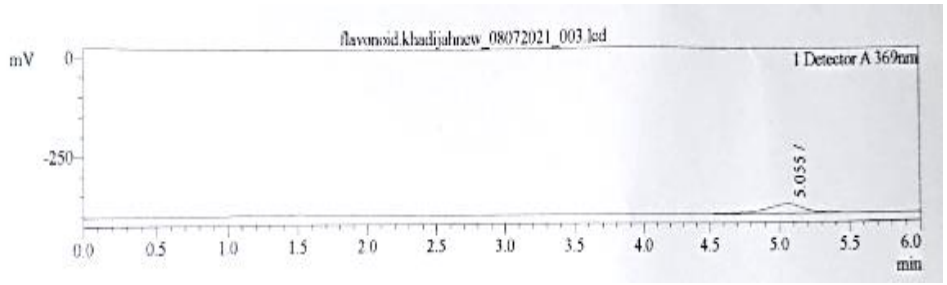

Figure 15. Quercetin content of honey cultivated in the Tabunganen area

The retention time produced by each honey sample is close to the retention time of the standard solution. But the retention time which is not the same as the standard solution produces a negative result. So it can be concluded that the three negative samples contained Quercetin.

Discussion

The analysis of flavonoid chemical compounds in cultivated honey in South Borneo was carried out in two stages of testing, namely qualitatively and quantitatively. Before the test, sample extraction was carried out and then continued with qualitative testing using color reagents on the drip plate. Meanwhile, quantitative testing uses HPLC (high performance liquid chromatography) method.

1. Sample extraction

Prior to testing, honey samples were extracted. Extraction is carried out in the form of liquid-liquid extraction. This liquid-liquid extraction under certain conditions has several advantages including being able to operate at room conditions, being able to separate systems that have sensitivity to temperature, being able to separate systems with relatively small differences in boiling points and relatively small energy requirements.(Mirwan & Ariono, 2018). Liquid-liquid extraction is carried out because flavonoid compounds are a group of compounds that are not heat resistant and easily oxidized at high temperatures.

The solvent used in the extraction of each honey sample used methanol and n-hexane as solvents. The solvent is one of the determining factors in the extraction process, so many factors must be considered in the choice of solvent. There are two main considerations in choosing the type of solvent, namely the solvent must have a high solubility and the solvent must be harmless or non-toxic. The solvent used in the extraction must be able to dissolve only the desired extract, have a large solubility, not cause chemical changes to the extract components, and the boiling points of the two ingredients should not be too close. The most commonly used solvents are water, methanol, ethyl acetate, petroleum ether, chloroform, and hexane.(Arsa & Ahmad, 2020)

Flavonoids are a phenol group which is a polar compound because it has a number of hydroxyl groups so that it will dissolve in polar solvents such as methanol, ethanol, butanol, acetone, and dimethylsulfoxide. The use of n-hexane because n-hexane is a straight chain alkane hydrocarbon which has 6 carbon atoms with the molecular formula C6H14. The isomer of n-hexane is unreactive and is widely used as an inert solvent in organic reactions because n-hexane is nonpolar. N-hexane is obtained from the distillation of crude oil where the industrial product is the fraction that boils at a temperature of 65-70 °C. N-hexane is commonly used to extract oils and fats that have the same polarity.

2. Qualitative analysis

Qualitative analysis was carried out first on the honey extract using a color reagent test. Qualitative analysis is a test by determining the presence or absence of a compound but not calculating its mass or concentration. This test aims to determine the presence or absence of secondary metabolites (flavonoids) contained in the cultivated honey extract. The color reagent test in the qualitative test used a test with the addition of reagents consisting of FeCl3, NaOH, AlCl3 and NH4OH. Color test using FeCl3 reagent will form a color change that is green(Aminah et al., 2017). Color test using NaOH, AlCl3 and NH4OH reagents will form a color change to yellow(Nurmila et al., 2019)

Based on the results of research conducted on the three samples, namely samples of kelulut honey from Tapin, Barabai and Tabunganen areas, organoleptic test observations were carried out first. The kelulut honey sample from the tapin area has a brownish yellow color with a characteristic honey aroma and has a sweet, sour taste. The kelulut honey sample from the Barabai area has a dark brown color with a characteristic honey aroma and a slightly sweet and sour taste. Meanwhile, the kelulut honey sample from the Tabunganen area has a brownish yellow color and a brighter color than the other samples and has a distinctive honey aroma with a sweet and sour taste.

The organoleptic test on the three honeys was in accordance with the theory that honey had the form of a thick liquid, clear or pale yellow to brownish in color. It tastes sweet with a delicious and fresh aroma(Legowo, 2016). The taste of honey in the three samples has a characteristic sweet to sweet acidity. The taste of honey is determined by the content of organic acids, carbohydrates, and nectar sources. The sweetness of honey is determined by the ratio of carbohydrates contained in the nectar of the plant that is the source of honey. The taste of honey can change if it is stored under unsuitable conditions and high temperatures(Syuhriatin, 2019)

Tests using color reagents showed that all samples did not change color to FeCl3, NaOH, AlCl3 and NH4OH reagents. The negative results in the three samples showed that this was not in accordance with the theory because according to research by L.Sumarlin et al in the journal "Antioxidant Activity of Methanol Extracts of Liquid Honey and Indonesian Local Honey Powder" stated that samples of longan honey contained flavonoid compounds where the test was carried out by qualitative analysis. According to research by dzomba et al in the journal L. sumarlin mentions the presence of phenolic, flavonoid and saponin content in honey from Chinyani village, Murewa area, Mashonaland East Zimbabwe.(L. Sumarlin et al., 2018)

According to the journal "Antioxidant Activity of Monoflora Honey Combination with Namnam Leaf Extract (Cynometra cauliflora L.)" on phytochemical testing of samples of commercial packaged rambutan honey, rambutan honey without commercial packaging and rubber honey showed negative

results containing flavonoids in phytochemical testing because honey contains high total phenolic content. low (0.298-0.588 mg GAE/g) so it was not detected in the qualitative test(LO Sumarlin et al., 2018). The discrepancy between the study and the literature is due to the different physical and chemical characteristics of honey depending on internal and external factors. Internal factors include the type of flower while external factors are season, soil conditions or geographical location, processing and storage(Evahelda et al., 2018).

3. Quantitative analysis

Quantitative analysis of flavonoid compounds was carried out using high performance liquid chromatography (HPLC) method. High performance liquid chromatography (HPLC) is an advanced separation method in pharmaceutical analysis that can be used as identity test, purity test and assay(Susanti & Dachriyanusus, 2016). The working mechanism of HPLC is a technique for separating a mixture of substances using a mobile phase and a stationary phase, where the separation occurs due to differences in adsorption power, solubility, partitioning, molecular size, ion size and vapor pressure on the components carried by the mobile phase through the stationary phase.(Aulia et al., 2016). The superiority of the HPLC method compared to other separation methods lies in the accuracy of the analysis and high sensitivity and is suitable for separating nonvolatile compounds that are not resistant to heating.

The test was carried out on samples of cultivated honey in South Borneo which aimed to see the levels of flavonoids in honey. The standard used is quercetin with concentrations of 6 ppm, 8 ppm, 10 ppm and 12 ppm using methanol as a mobile phase: aquabides (59: 41). The wavelength used in the quantitative test of the flavonoid Quercetin is 369.11 nm. The wavelength was obtained by running using a UV-Vis spectrophotometer in the wavelength range of 300-700 nm. A spectrometer produces light from a spectrum with a certain wavelength and a photometer is a device for measuring the intensity of light that is transmitted or absorbed. So a spectrophotometer is used to measure the relative energy of light if the energy is transmitted,

The use of quercetin as a standard solution is because honey generally contains flavonoid compounds such as quercetin, kaempferol, myricetin, luteolin, apigenin and naringenin.(L. Sumarlin et al., 2018). *Quercetin*itself is one of the best flavonols. Quercetin, received a lot of attention because Quercetin represents a flavonol subclass that exhibits nutritional and pharmaceutical properties. Its ring structure and aglycon configuration of the hydroxyl group, make it one of the most potent flavonoids in terms of antioxidant ability. Quercetin is used as a standard because quercetin is a flavonoid that has high reactivity compared to rutin, daflon, diosmin and morin.(Dewi et al., 2018).

The samples tested using the high performance liquid chromatography (HPLC) method amounted to 3 samples, namely samples from the Barabai, Tapin and Tabunganen areas. Where, the three areas are included in the South Borneo region. Sampling of cultivated honey was carried out by proposive sampling using inclusion criteria and exclusion criteria.

Quercetin levels in the honey sample have very little concentration so that the peak that appears is also very low, so the addition method is used, where the addition method is the addition of a standard solution to the sample solution with the aim of ensuring the compounds contained in the sample, which are marked by increasing peak after measurement again on the sample of 1000 ppm which has been added 1 mL of standard solution with a concentration of 50 ppm.

Based on the results of research conducted on standard solutions of Quercetin, positive retention time (RT) containing flavonoids can be seen in Images 4.6, 4.7, 4.8 and 4.9. The results are positive if the standard and sample solutions show the same retention time (RT). The results of the research on the three samples using the HPLC method obtained quercetin levels as follows:

Table 6. levels of quercetin using HPLC method

No	Sample	*Retention time* (RT)
1	Barabai	5,115 minutes
2	Tapin	5,067 minutes
3	Tabunganen	5,055 minutes

The results obtained in this study were that there was no Quercetin flavonoid content in the three samples tested. The retention time produced by each honey sample is not the same as the retention time of the Quercetin standard solution. So that the flavonoid levels in the three samples were not detected. This does not resemble the journal literature entitled "Bioactive Components of Rubber Honey (Hevea brasiliensis), Kaliandra Honey (Calliandra callothyrsus) and Randu Honey (Ceiba pentandra)". Where Calliandra honey has the highest phenolic content of 557.93 mg GAE/100 g, followed by rubber honey with a value of 385.63 mg GAE/100 g, and randu honey of 309.12 mg GAE/100 g.

Research conducted by L. sumarlin et alstated that the highest total flavonoid content was in the sample of powdered longan honey extract with a total flavonoid of 0.079 mg QE/g sample, while commercial powdered honey (EMBK) had the lowest total flavonoid of 0.006 mg QE/g sample. The discrepancy between the study and the literature is due to the different physical and chemical characteristics of honey depending on internal and external factors. Internal factors include the type of flower while external factors are season, soil conditions or geographical location, processing and storage(Evahelda et al., 2018).

The absence of flavonoid content can also be caused by the storage factor of honey from the time of production until the honey is analyzed. This is according to research by Saric et al in the journal L. Sumarlin et al (2018) mentioned that the total flavonoid content in acacia honey stored for 6 months decreased from 82.26 to 28.94 mg GAE/kg.

Based on the results of this study, (Ha) cannot be related to the research hypothesis, which means that the hypothesis is rejected, because the results of the study show that there is no influence of the sampling location on the levels of flavonoid chemical compounds in cultivated honey in South Borneo. As for (Ho) the hypothesis is accepted because Ho≠ 0 indicates that there is no effect of sampling location on levels of flavonoid chemical compounds in cultivated honey in South Borneo.

Conclusion

From the results of the lab which was carried out with color testing, it was found that honey from the three locations did not contain flavonoid compounds. The levels of the positive quercetin standard solution containing flavonoids were at a different retention time from the retention time of the tested sample. The results obtained in this study were that there was no quercetin flavonoid content in the three samples tested. The retention time produced by the sample is not the same as the retention time of the standard quercetin solution. So that the flavonoid levels in the three samples were not detected. The results of the formation of the regression model are known that there is no influence of location on the levels of flavonoid compounds according to the location of the honey source.

Declaration of Interest Statement

The authors declare that they have no conflict of interests.

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