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Horticulture Laboratory
University of Guam

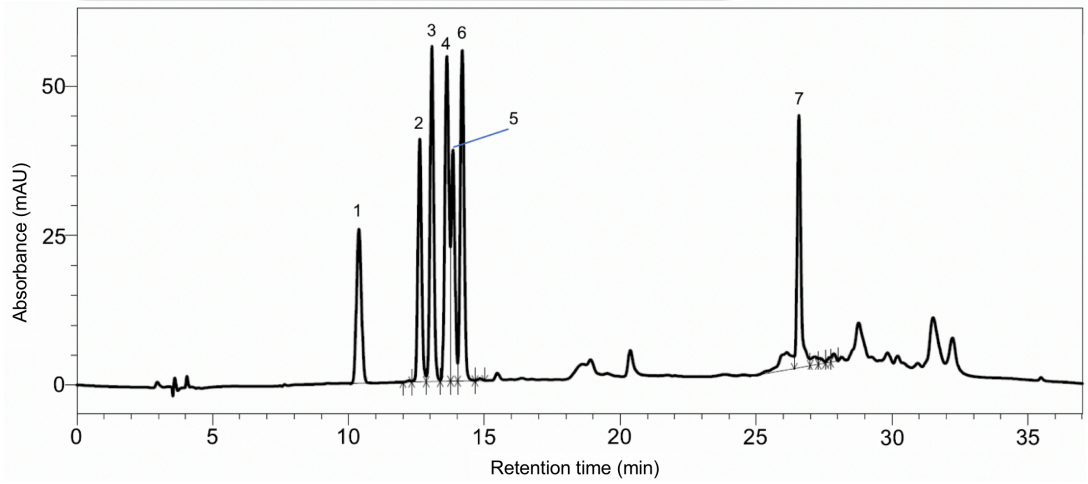
Agriculture and Life Science Division
College of Natural and Applied Sciences

Technical Report

Factsheet 2021-2
December 2021

Phytochemical Analyses of Horticultural Crops

Application of High Performance Liquid Chromatography (HPLC)



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INTRODUCTION

What are Phytochemicals?

Phytochemicals are naturally produced compounds in plants comprised of two main groups. Primary metabolites, such as amino acids and nucleotides, are found in all plants and are essential to basic plant growth and development. **Secondary metabolites** occur in specific groups of plant species and usually have an ecological function. These **natural secondary products** are often studied for their valuable properties as antibiotics, drugs, dyes, and flavors. Based on biosynthetic origin, there are three major groups of natural products: (a) **terpenoids** (Fig. 1), (b) **alkaloids** (Fig. 2)**,** and (c) **phenolic groups** (Fig. 3) (Croteau et al., 2000).

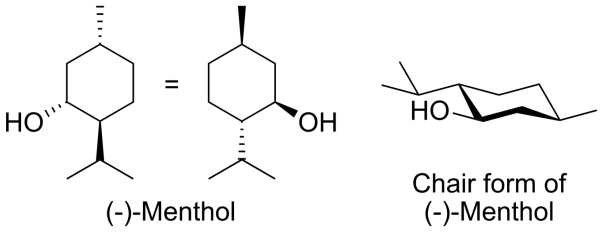


Figure 1. Menthol, a terpenoid, from mint plant (*Mentha* spp.).

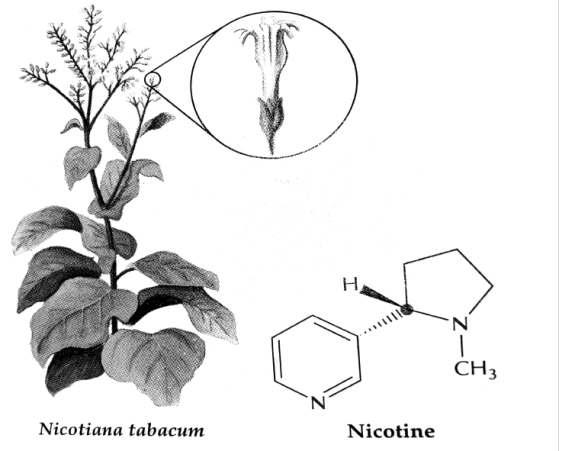


Figure 2. Nicotine, an alkaloid, from the tobacco plant (*Nicotiana tabacum*) (Croteau et al., 2000).

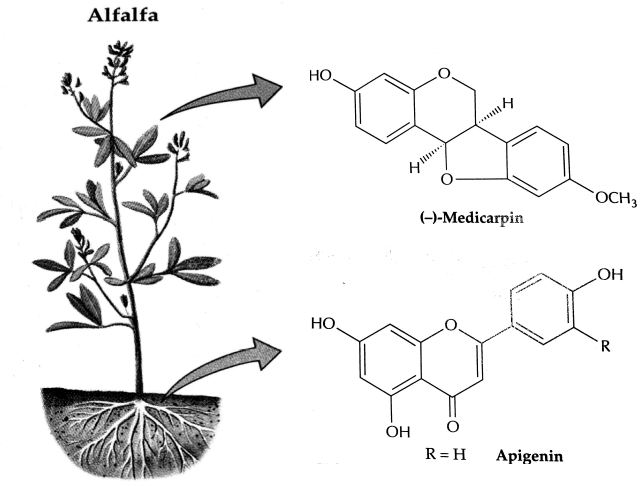


Figure 3. Phenolic flavonoids in alfalfa (*Medicago sativa*). Isoflavonoid medicarpin participates in inducible disease defense. Apigenin and luteolin facilitate the development of nitrogen-fixing root nodules (Croteau et al., 2000).

Among the phenolic groups, **capsaicinoids** are phenylpropanoid-derived phenolic compounds found in hot peppers (*Capsicum* spp.) and are responsible for their pungent flavor (Fig. 4). Another important phenolic compound are **flavonoids** (Fig. 5). Flavonoids are involved in many plant-animal interactions. Various flavonoids have also been studied for their role in human health and pharmaceuticals (Croteau et al., 2000).

Anthocyanins are a type of flavonoid found in the vacuoles of plant cells and are often responsible for the colors of flowers and fruits that attract pollinators and seed dispersers. Other flavonoids, such as flavonols, flavones, chalcones, and aurones, contribute to the color of plant tissues. **Quercetin** is one of the flavanols found in hibiscus plants (Croteau et al., 2000)

This report presents analyses of selected phytochemicals extracted from horticulture crops, hot peppers (*Capsicum* spp) and *Hibiscus* spp grown on Guam. Capsaicin was also examined from hot sauce made from local hot peppers.

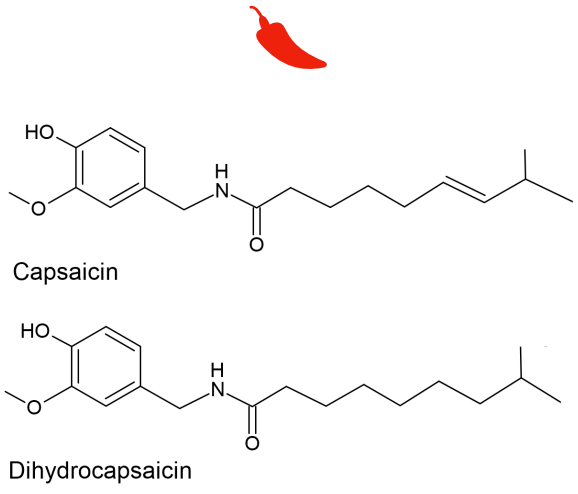


Figure 4. Chemical structures of capsaicin and dihydrocapsaicin from the fruits of hot pepper (*Capsicum* spp.).

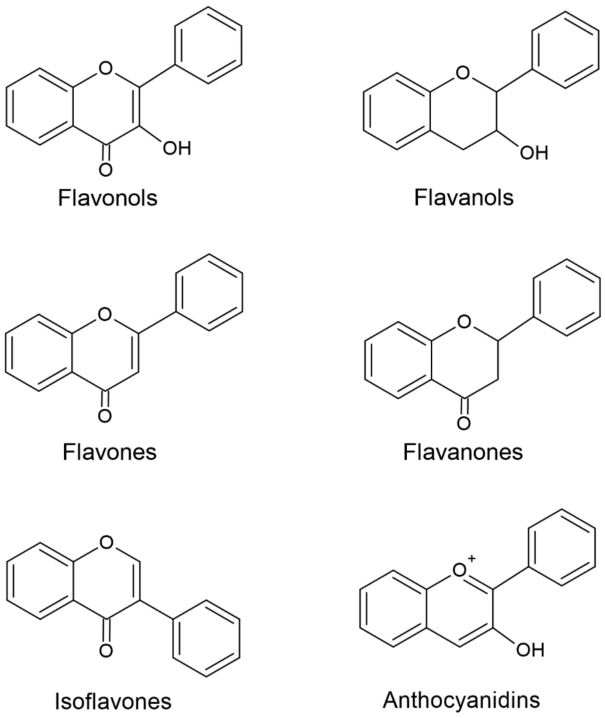


Figure 5. Chemical structures of six groups of flavonoids (Pietta, 2000).

High Performance Liquid Chromatography (HPLC)

What is HPLC?

High performance liquid chromatography (HPLC) is used to quantify, identify, and isolate chemicals according to the size of their molecules and their polarity.

The HPLC system consists of a "pressure pump", "mobile phase", "sample injection/ auto-sampler", "stationary phase", "UV/VIS detector", "data recorder/chromatogram", and "waste reservoir". Solvent (mobile phase) is pumped from a solvent reservoir and mixed with the liquid sample being studied. The solvent-sample mixture passes through a HPLC column (stationary phase) and into a detector, where an electronic output is given as chromatograph data. After solvent passes through the system, the waste is collected in the waste reservoir.

The HPLC column (stationary phase) is the separation component of the system. HPLC columns contain "packing material" with various pore sizes. As the solvent sample mixture passes through the pores of the column, the larger molecules become trapped by the smaller pores, allowing the smaller molecules to elute faster. This is the isolation and separation process of chemical compounds.

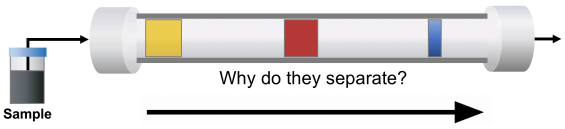
What is in the sample?

Individual compounds in a sample are isolated and determined by their unique retention time, the time it takes for the compound to pass through the HPLC column and reach the detector. The retention times are measured against known standards, allowing for the molecules in the sample to be identified. The internal pressures on the mobile phase can also reach up to 400 atmospheres. Compounds present in higher concentrations transmit stronger signalsto the detector that result in higher peaks.

Normal phase vs Reverse phase

HPLC chromatography has two main types: normal-phase and reversed-phase. For normal-phase chromatography, polar compounds are strongly attracted to the polar column (stationary phase) while non-polar compounds move fast through the column with a non-polar mobile phase solvent. On the other hand, in reversed-phase chromatography, non-polar compounds are strongly attracted to the non-polar C-18 column (stationary phase) while more polar compounds are pushed out faster by the solvent (mobile phase).

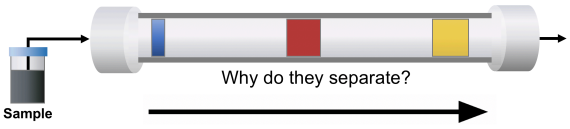
Stationary Phase: POLAR (Silica)



***Mobile Phase**:* NON-POLAR

Figure 6. Normal-phase chromatography. The **blue** non-polar compound moves fastest while the **yellow** compound is polar and moves slower in the column. (Source: www.discoveryscientificsolutions.com)

Stationary Phase: NON-POLAR (C-18)



***Mobile Phase**:* POLAR

Figure 7. Reversed-phase chromatography. The **yellow** polar compound moves fastest while the **blue** compound is non-polar and moves slower in the column. (Source: www.discoveryscientificsolutions.com)

How Does Reversed-Phase HPLC Work?

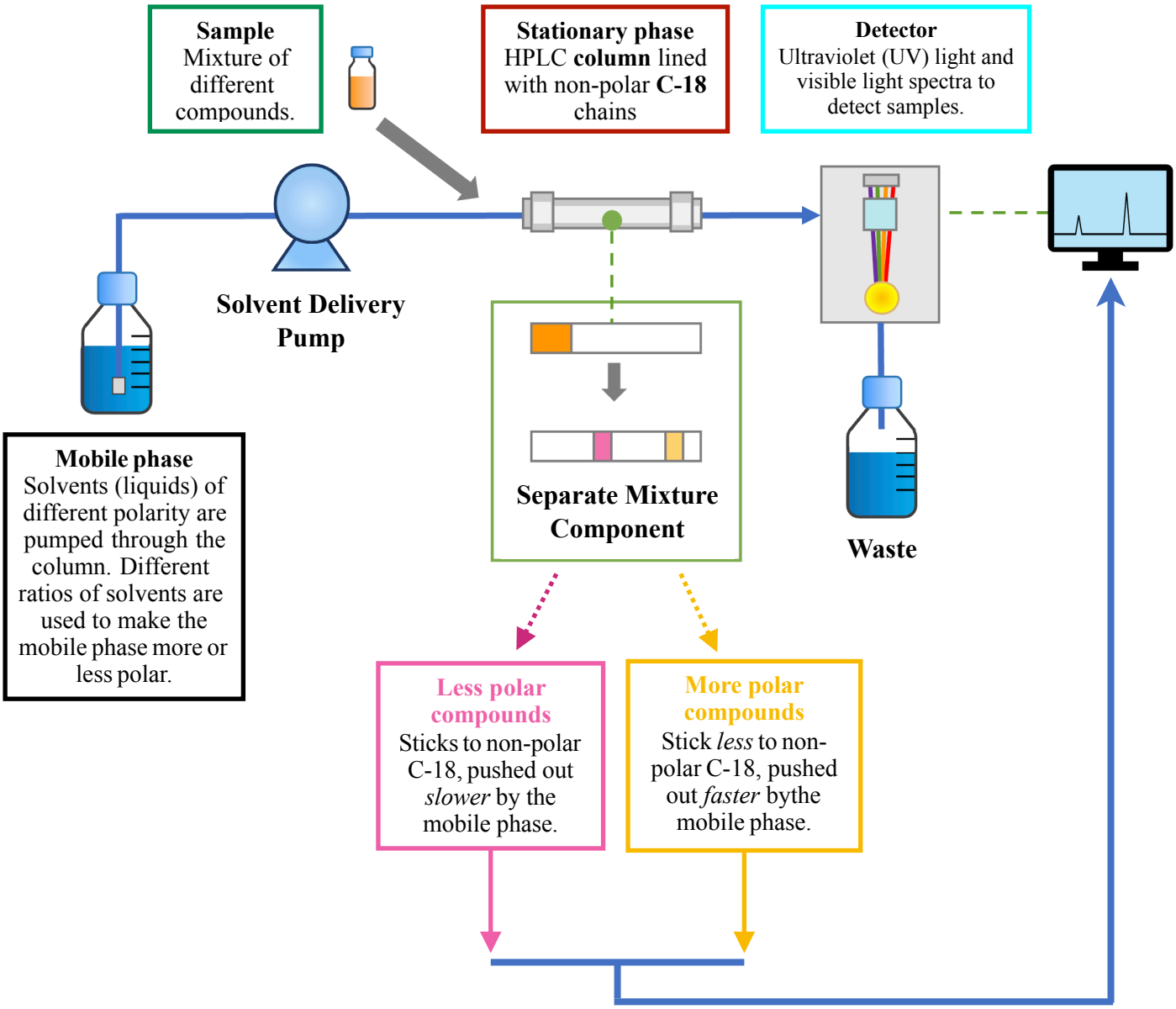


Figure 8. Separation of **flavonoids** and **capsaicinoids** using **reversed-phase (C-18)** chromatography.

Study 1:

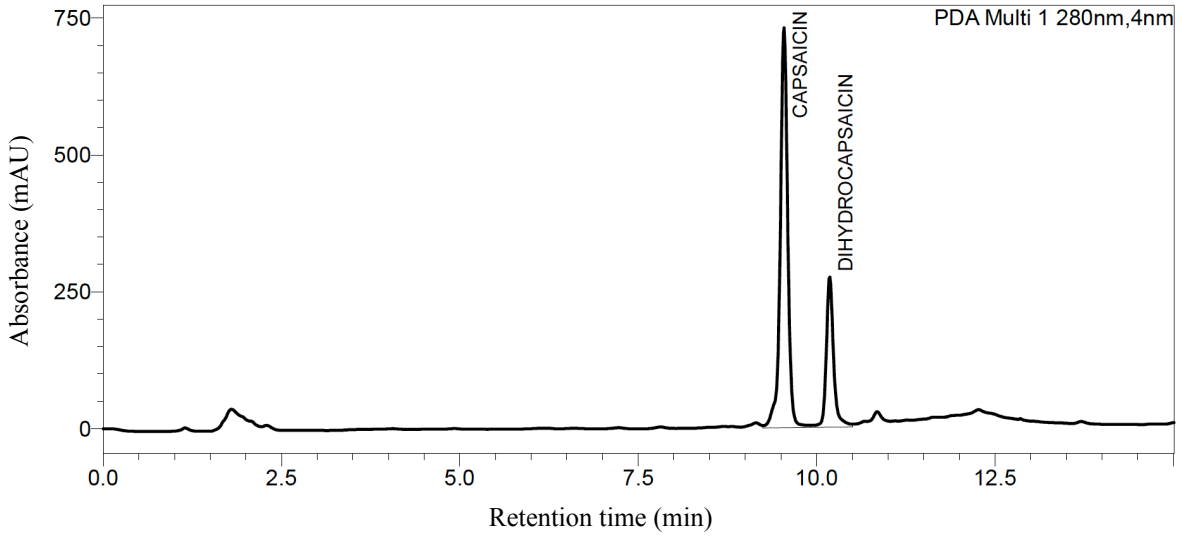
Capsaicinoids and Scoville Heat Unit of Hot Peppers

Hot peppers (*Capsicum* spp.) of the family Solanaceae originated from Central and South America. Hot peppers are now widely grown throughout the world and have become an important economic crop globally (González-Zamora, 2013).

In Guam, hot peppers are grown to make traditional sauces, such as *finadene'* and *dinanche',* which provide a spicy kick in local dishes. They are also consumed either fresh, dried, and pickled. Guam's local peppers come in a variety of different shapes, sizes, colors, and pungency. The most notable local pepper is d*onne' såli*, which is characterized by its very high pungency and small size.

What gives hot peppers their remarkable pungency is the presence of **capsaicinoids**, which are predominantly found as **capsaicin** and **dihydrocapsaicin** (Figure 4). Capsaicinoids have strong pharmacological effects, which may be used for pain relief, cancer prevention, and weight reduction, besides providing gastrointestinal and cardiovascular benefits (Basith et al., 2016).

In this study, capsaicin and dihydrocapsaicin of hot peppers were investigated using HPLC. Pungency was calculated based on the concentration of capsaicin and dihydrocapsaicin shown in Fig. 9. (Todd et al. 1977; ASTA 1985 &1998; de Aguiar et al. 2016; and Dong, 2006).



Scoville Heat Unit (SHU) = [(Capsaicin conc. in ug/g) x 16 + (Dihydrocapsaicin conc. in ug/g) x 16]

Figure 9. HPLC chromatogram of capsaicin and dihydrocapsaicin standards and equation to determine Scoville Heat Unit (SHU) according to ASTA (1985 & 1998).

Sample preparation

Hot pepper fruits were cut into small pieces and dried at 52 °C (125°F) for 24 hrs using a food dehydrator. Dried peppers were then ground into a fine powder using a coffee grinder. One gram of the resulting powder was dissolved in 10 mL of HPLC grade acetonitrile to create a 10 times dilution. Pepper-acetonitrile solutions were then vortexed for 30 sec. Aliquots of 1.5 mL were transferred into separate glass vials for HPLC analysis.

HPLC Instrument Parameters and Conditions

Column: Shimadzu C-18 dimension 150 x 4.6 mm. Column temperature: 40°C. Injection sample volume: 25 µl. Flow Rate: 1.50 mL/min. UV Detector: 280 nm. Solvents:

Mobile Phase A: Water
Mobile Phase B: HPLC-grade Methanol

Run Time Total: 15 min

Time (min)	Mobile Phase A	Mobile Phase B	Flow Rate (mL/min)
0	60	40	1.5
8	15	85	1.5
10	1	99	1.5
15	60	40	1.5

Figure 10. Hot peppers collected from flea market and farms in Guam.

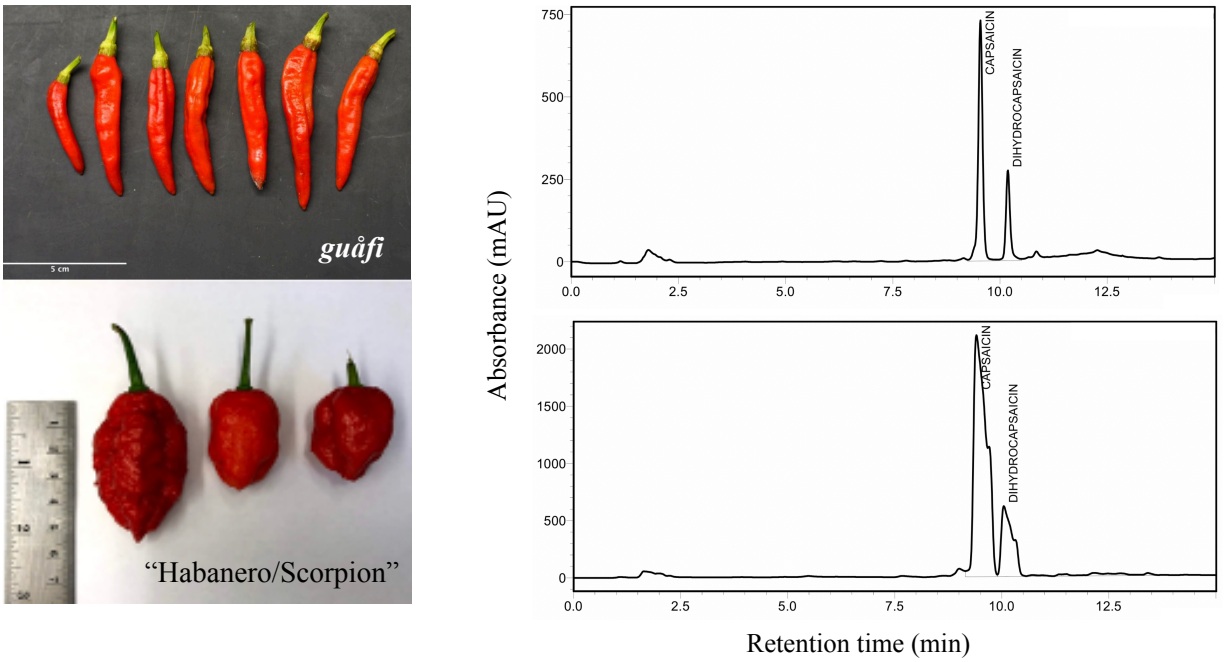


Figure 11. Hot peppers *guåfi* (upper left) and "Habanero/Scorpion" (lower left) and their HPLC chromatograms (280 nm) showing concentrations of capsaicin and dihydrocapsaicin (right).

The HPLC chromatogram in Fig. 11 shows the concentration of capsaicin and dihydrocapsaicin in extracts made from dry hot peppers of *guåfi* and "Habanero/Scorpion" pepper. Scoville Heat Unit (SHU) was determined using an equation based on the concentrations of the two capsaicinoid compounds (ASTA, 1998). The highest SHU was found in "Habanero/Scorpion'"with 1,700,659 SHU. It was sold at a local flea market as a hybrid of "Habanero" and "Scorpion." Its origin is unknown. The SHU of "Ghost Pepper", *donne' såli*, and *kika* was also compared between red and green colored fruits. Red fruits of "Ghost Pepper" and *kika* had higher SHU than green fruits. Interestingly, green fruits of *donne' såli* had a higher SHU than red fruits. SHU differed among the hot pepper varieties and among different parts of fruits or fruit maturity.

Table 1. Scoville Heat Unit (SHU) of Dry Hot Peppers

Hot Pepper Variety Name	Source	Fruit Color	SHU
Habanero/Scorpian	Flea Market	Orange	1,700,659
Ghost Pepper (Red)	Flea Market	Red	1,013,434
Ghost Pepper (Green)	Flea Market	Green	853,022
Carolina Reaper	Flea Market	Red	539,833
Dededo/Local	Flea Market	Red	334,257
Donne' Sali (Green)	Flea Market	Green	218,787
Guafi	Mangilao	Red	218,316
Dragon	Flea Market	Red	218,017
Kika (Red)	Talofofo	Red	210,795
Donne' Sali (Red)	Flea Market	Red	197,701
Vietnamese	Rota	Red	147,846
Thailand	Flea Market	Red	78,078
Kika (Green)	Talofofo	Green	66,896

Study 2:

Scoville Heat Unit of Hot Pepper Sauce Products

The spiciness of hot sauce or other food products is expressed with the **Scoville Heat Unit (SHU).** Originally, an American pharmacist, Wilbur Scoville, developed the Scoville scale to determine the pungency of peppers for use as a heat-producing ointment. Scoville developed the **Scoville Organoleptic Test** in which he prepared one grain (~65 mg) of ground peppers into alcohol and then added it to sweetened water to create a diluted solution. The diluted solutions were given to individual tasters and dilutions were continuously made in specific proportions until a distinct but weak pungency was detected by the tongue. A single unit of dilution was equivalent to one Scoville Heat Unit (SHU). (Scoville, 1912).

The Scoville Organoleptic Test had some limitations due to physiological differences in the tasters' senses and sensitivity to the pepper's heat. When modern chemical analytic instruments such as HPLC were developed, threshold pungencies of individual capsaicinoids that contribute to the pungency of peppers were more accurately determined (Todd, 1977).

Later, the American Spice Trade Association (ASTA) adopted and modified the Scoville Heat Test to create a standard method for analyzing capsicum pungencies (ASTA, 1985). In the original 21.0 method of ASTA, pure samples of individual capsaicinoids were prepared and their pungencies were determined by panels of tasters. The pungency data was obtained by tasting the same capsaicinoid at five dilution levels.

Dilutions close to the estimated threshold pungency values were changed in small increments to determine the threshold pungency value for the specific capsaicinoid (Todd, 1977). Pungencies of a capsicum sample were calculated by multiplying the individual capsaicinoid content to the corresponding value of threshold pungency.

ASTA updated the standard procedure for determining the Scoville Heat Unit (SHU) of capsicums using HPLC techniques (ASTA, 1985). The outlined 21.3 methods provided by ASTA utilizes HPLC analysis of individual capsaicinoids (capsaicin, dihydrocapsaicin, and nordihydrocapsaicin) to determine the SHU of capsicums. These methods compared the capsaicinoids to standard solutions to determine the parts per million of heat, which would then be multiplied by the accepted heat factors to obtain the SHU of the capsaicinoid. The total SHU was then calculated by taking the sum of the SHUs of the individual capsaicinoids (ASTA, 1998). The HPLC methods and calculations outlined by ASTA are still commonly used to determine the SHU of capsicums.

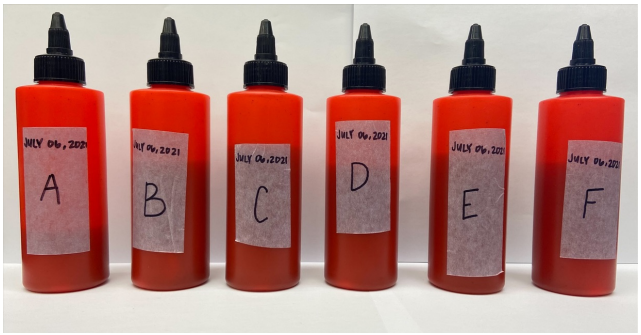


Figure 12. Six hot sauce products tested in the study.

Standards preparation preparation

Standards of capsaicin and dihydrocapsaicin in concentrations of 10, 25, 50, and 100 ppm were prepared. Analytical standard (2.5 mg) of each chemical was placed in a 25 mL volumetric flask and diluted in ethanol to make a 100 ppm stock solution. Stock solution of 2.5 mL, 6.25 mL, and 12.5 mL was added to separate volumetric flasks and diluted with ethanol to create 10, 25, and 50 ppm standard solutions, respectively. Three 1.5 mL aliquots of each standard solution was transferred into glass vials for HPLC analysis and creation of a calibration curve.

Sample preparation

Six different hot sauces marketed as the same product were tested. Sample preparation was performed using the extraction method of Batchelor and Jones with a few modifications (2000). Each sauce bottle was shaken vigorously, and 15g of hot sauce was placed in a 125 mL Erlenmeyer flask. HPLC grade ethanol was added to the hot sauce until the volume reached 50 mL. The flask was then heated to a low boil on a hot plate for 30 min with constant stirring using a magnetic stir bar. The extract was then filtered through a Whatman 90 mm grade 1 filter paper. 2.5 mL of the filtered extract was added to a 5 mL volumetric flask and diluted with HPLC grade ethanol. The diluted extract (2x) was further filtered through a 0.45 um pore size filter cartridge and syringe. Aliquots of 1.5 mL of the extract were then placed into glass vials for HPLC analysis.

HPLC Instrument Parameter and Condition

Column: Shimadzu C-18 dimension 150 x 4.6 mm. Column temperature: 50°C. Injection sample volume: 20 µl. Flow Rate: 1.0 mL/min. UV Detector: 280 nm. Solvents:

Mobile Phase A: 0.1% Phosphoric Acid
Mobile Phase B: Acetonitrile

Run Time Total: 22 min

Time (min)	Mobile Phase A	Mobile Phase B	Flow Rate (mL/min)
0	100	0	1.0
5	75	25	1.0
10	45	55	1.0
15	10	90	1.0
20	10	90	1.0
22	100	0	1.0

Calculations of Scoville Heat Unit (SHU)

SHU = [(Capsaicin conc. in ug/g) x 16 + (Dihydrocapsaicin conc. in ug/g) x 16]

Concentration of capsaicinoids was taken from HPLC data programs and multiplied by a dilution factor of 2 to determine the concentration in ppm. Concentration in ppm was multiplied by 16 since pure capsaicin has a pungency of about 16,000,000 Scoville Units (ASTA, 1985).

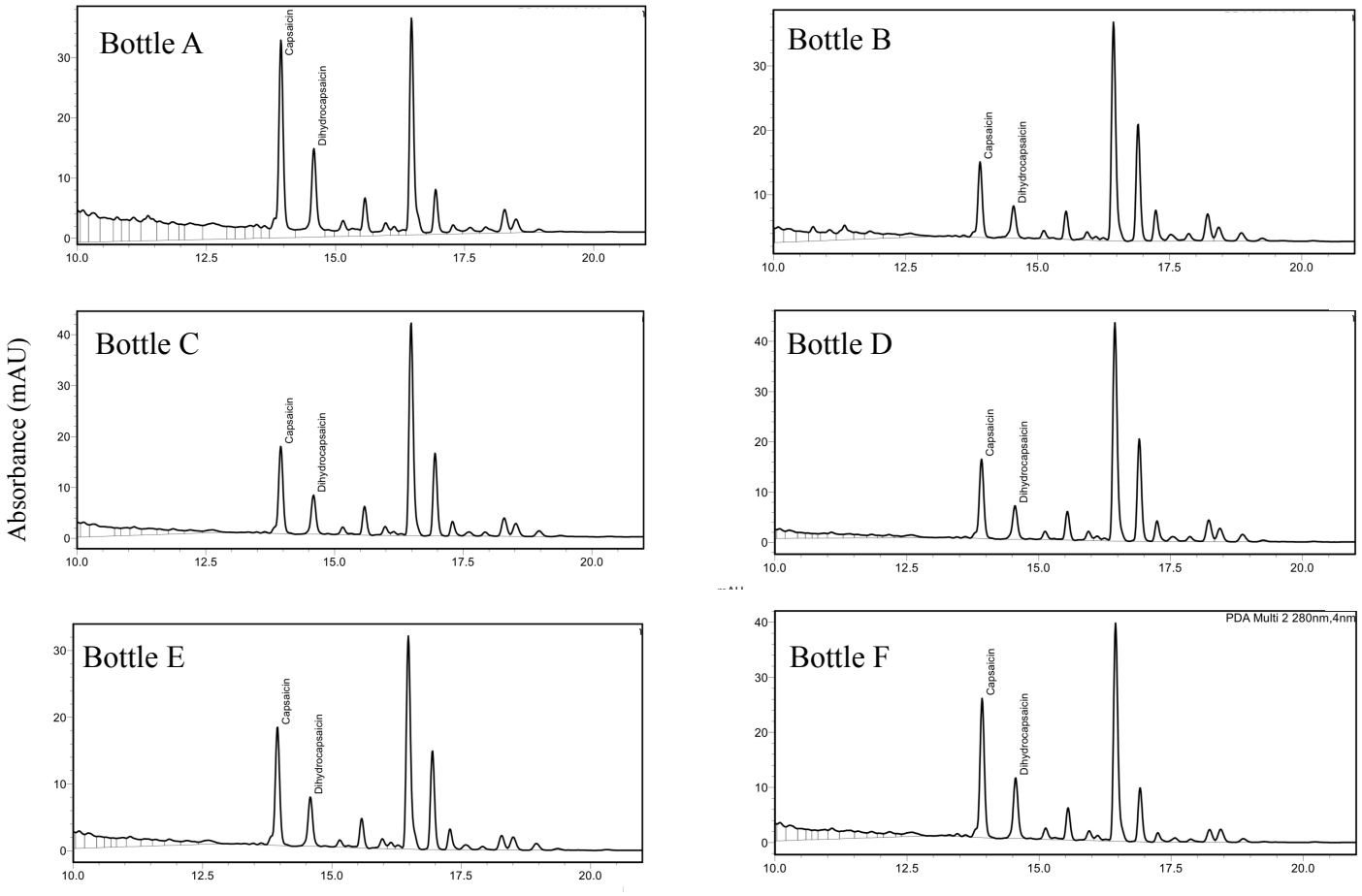


Figure 13. HPLC chromatograms taken at 280 nm detecting capsaicin and dihydrocapsaicin to determine Scoville Heat Unit of hot sauces in Figure 12.

In this study, our sample preparation and extraction method of capsaicinoids for HPLC analysis successfully determined the pungency level of hot sauces. Six hot sauce products showed similar HPLC chromatograms with slightly different amounts of capsaicin and dihydrocapsaicin (Fig. 14). Based on the HPLC analysis, Scoville Heat Unit (SHU) ranged from 1,108 (Sample B) to 3,280 (Sample A) SHU based on the weight of the hot sauce. This procedure can be applied to other hot sauce products to provide information on quality control in order to maintain consistency of pungency of locally manufactured hot pepper products.

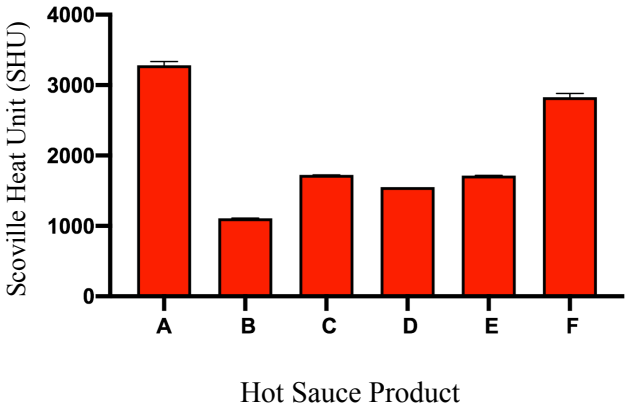


Figure 14. Scoville Heat Unit (SHU) of six hot sauce products showing mean and standard deviation (n=3).

Study 3:

Flavonoids of Roselle, Hibiscus sabdariffa L.

Hibiscus sabdariffa L., commonly called "roselle" or "sorrel" is commonly grown for its calyxes and leaves to make herbal tea, jellies, and other food additives. Roselle plants and their derivatives contain the flavonoid group anthocyanin which are responsible for the reddish pigment of the leaves and calyxes and are also known to possess antioxidant properties. Figure 15 a. shows a roselle plant growing in Guam with red calyxes. These calyxes are harvested and dried (Fig. 15 b. & c.) and commonly prepared as tea (Fig. 16). Pigmented leaves also contain anthocyanins (Fig. 17) and are used to make tea. HPLC chromatography can be used to separate these flavonoids to determine how much of each anthocyanin and other flavonoid compounds are present in the leaves and calyxes of different roselle varieties. This biochemical study will aid in the selection of roselle varieties for the production of high quality food and medicinal products on Guam.

Figure 15. A roselle plant with red calyxes (a). Close-up of fresh red calyxes (b) and dried calyxes (c).

Figure 16. Roselle tea made from red calyxes.

Figure 17. Two types of roselle plant with red leaves and green leaves**.**

Standards preparation

A standard curve was prepared from four concentrations (10, 25, 50, 100 ppm) for each flavonoid to determine the concentration of each compound in each sample. A 100 ppm stock solution was prepared by mixing 2.5 mg of the standard in a 25 mL volumetric flask with HPLC grade methanol. Aliquots of the following amounts of stock solution were diluted in a 25 mL volumetric flask with water to prepare the four different concentrations; 12.5 mL (50 ppm), 6.25 mL (25 ppm), and 2.5 mL (10 ppm). All standard solutions were analyzed with HPLC. Peak area and retention time for each component were recorded and used to estimate concentrations in unknown plant samples.

Sample preparation

Leaf and calyx tissues were placed in a freezer at -20°C. Frozen samples were freeze-dried for 24 hrs at -58°C (4.00 mbar) using a freeze-dryer (Buchi Lyovapor L-200). Samples were ground into a fine powder with a mortar and pestle, then stored at -20°C until extraction. 0.25 g of each sample was placed into a 15 mL centrifuge tube with 2 mL of an acidified ethanol solvent which contained 95% ethanol: 2% acetic acid [80:20 (v/v)]. The aqueous mixture was stirred via an orbital shaker (Hotech Instrument Corp.) at 50 rpm for 15 min. The mixture was then centrifuged at 14,000 rpm for 10 min. Aqueous extracts were stored at -20°C until further HPLC analysis.

HPLC Instrument Parameters and Conditions

Column: Shimadzu C-18 dimension 150 x 4.6 mm. Column temperature: 25°C. Injection sample volume: 10 µl. Flow Rate: 0.5 mL/min. UV Detector: 280 nm. Solvents:

Mobile Phase A: 10% Formic Acid
Mobile Phase B: Acetonitrile

Run Time Total: 35 min

Time (min)	Mobile Phase A	Mobile Phase B	Flow Rate (mL/min)
0	100	0	0.5
13	80	20	0.5
20	70	30	0.5
25	20	80	0.5
28	20	80	0.5
30	100	0	0.5
35	100	0	0.5

Seven flavonoid compounds have been identified in roselle plants in previous studies (Pietta, 2000) (Fig. 18). HPLC chromatogram (Fig. 19) shows the retention time and peak area of the standards of each compound when detected at 280 nm. The structures of the se seven compounds are also shown in Fig. 18. An anthocyanin, cyanidin 3-glycoside has the shortest retention time at 11.0 min while quercetin has the longest retention time of 26.5 min. Four anthocyanins, protocatechuic acid, delphinidin 3-sambubioside, delphinidin 3-glucoside, and chlorogenic acid (Fig. 19 1-4) are responsible for the red and pink color of calyxes and leaves.

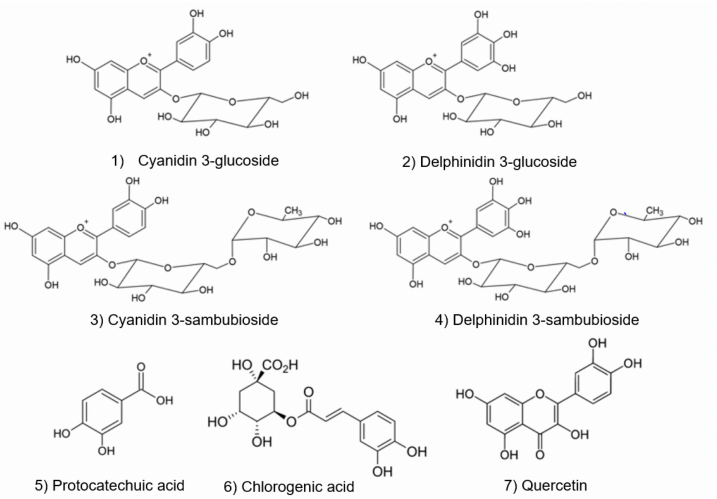


Figure 18. Chemical structures of compounds identified in roselle (PubChem, Pietta, 2000).

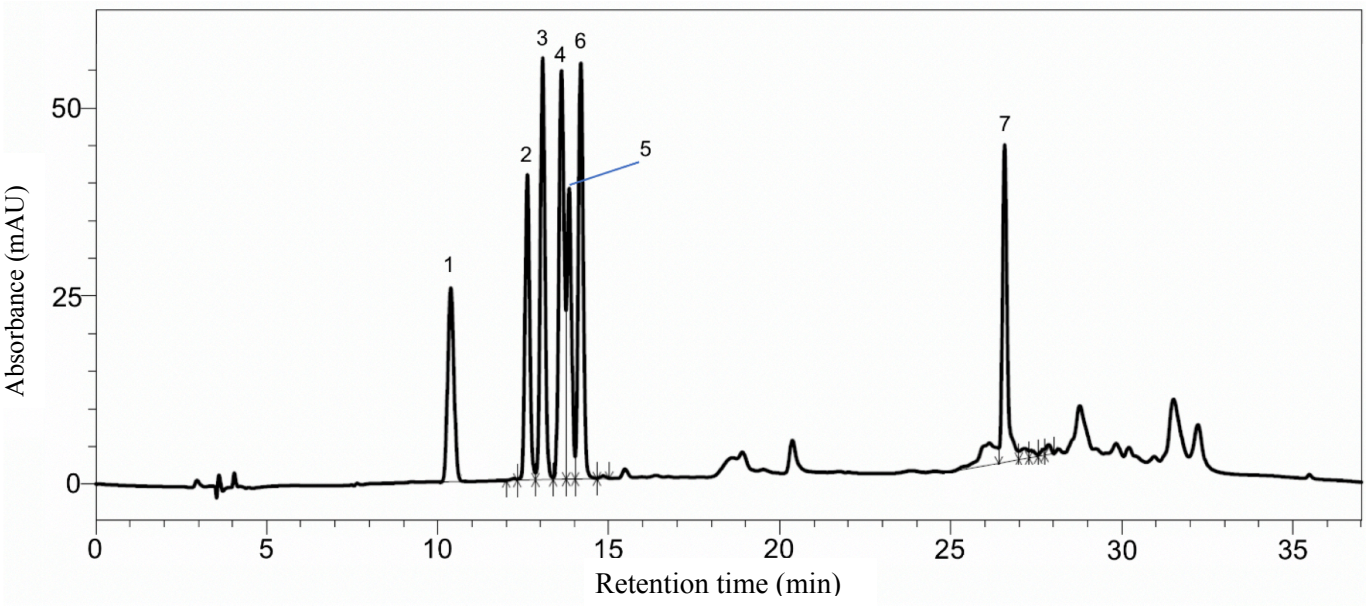


Figure 19. HPLC chromatogram of a 100 ppm mixture of standards detected at 280 nm, having a p -associated peaks: (1) protocatechuic acid, (2) delphinidin 3-sambubioside, (3) delphinidin 3-glucoside, (4) chlorogenic acid, (5) cyanidin 3-sambubioside, (6) cyanidin 3-glucoside, and (7) quercetin.

Study 4:

Quercetin in Hibiscus spp.

Hibiscus rosa-sinensis L. and *Hibiscus tiliaceus* L. are widely distributed in the tropics and subtropical regions. The flowers of these plants are traditionally consumed as a tea because of the therapeutic effects of their flavonoids. A few of these therapeutic effects include antioxidant activity, anti-inflammatory, antimicrobial, and anti-ulcer activity. Both species are commonly found on Guam; however, the content of anthocyanins and flavanols have not been determined. Among flavonoids, quercetin, and its derivatives, a yellow pigment, flavanol, has been found to treat illnesses including anti-cancer activity in colon cells, neuroprotective effects, ovarian cancer, diabetes, and inflammation. In this study, quantitative analysis of quercetin aglycone concentration was determined for-dried calyx, petals, and carpel/stamen of three varieties of *Hibiscus rosa-sinensis* and a yellow native hibiscus grown commonly in Guam, *Hibiscus tiliaceus,* using HPLC.

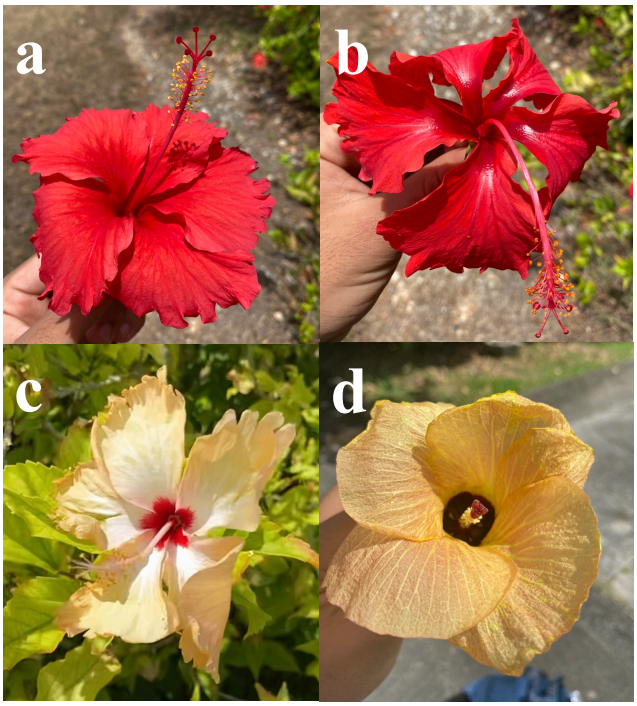


Figure 20. Flowers of *Hibiscus rosa sinensis* "Dark-Red" (a), "Red-White" (b), "Peach" (c), and *Hibiscus tiliaceus* (d).

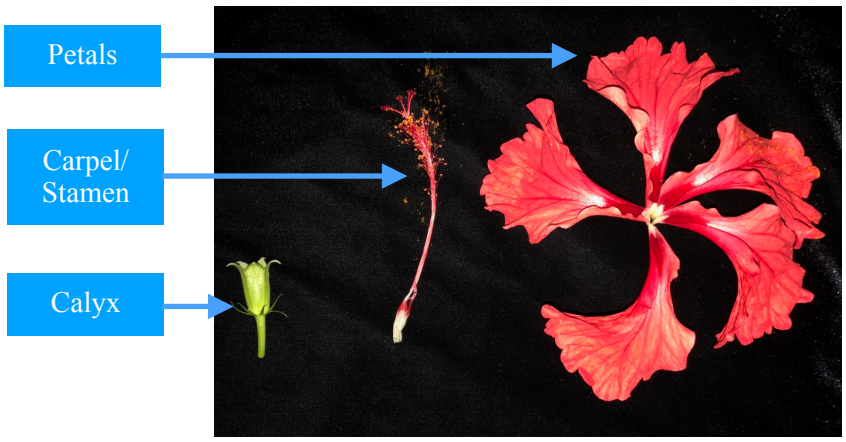


Figure 21. Petals, carpel/stamen and calyx of a hibiscus flower examined for concentration of quercetin.

Standards preparation

A standard curve was prepared from six concentrations (10, 25, 50, 75, 100, 200 mg/L) of quercetin standard in HPLC grade methanol. The calibration equation for the quercetin standard was y = 61477.3x - 70685.2. Coefficient of determination (R^2) was 0.9947831 and correlation coefficient (R) was 0.9973881. Limit of detection (LOD) and limit of quantification (LOQ) were determined from the standard solutions and slope of the calibration curve. LOD was 18.350 mg/L and LOQ was 55.605 mg/L.

Sample preparation

Hibiscus flowers were dissected into three parts: calyx, carpel/stamens, and petals. Plant material was frozen at -20°C prior to freeze drying. Sample tissues were freeze-dried for 24 hrs with the freeze drier condenser temperature stabilized at -58°C and pressure stabilized at 4.00 mbar (Buchi Lyovapor L-200). After freeze drying, 0.5 g of plant material was hydrolyzed in 25 mL of 2.8M HCl and methanol (60:40 v/v) solution at a temperature of 80°C for 30 min according to the extraction protocol by Desmiaty & Alatas (2008). The acid hydrolysis of quercetin glycosides resulted in the quercetin aglycone. While hot, the extract solution was filtered directly through a Whatman 90 mm grade 1 filter paper. Aliquots of 1.5 mL of the filtered extract were taken and centrifuged for 5 min. Then 1 mL of the resulting supernatants were then placed into glass vials for HPLC analysis.

HPLC Instrument Parameters and Conditions

Column: Shimadzu C-18 dimension 150 x 4.6 mm. Column temperature: 40°C. Injection sample volume: 15 µl. Flow Rate: 1.5 mL/min. UV Detector: 372 nm.

Solvents (Isocratic):

Mobile Phase: Acetonitrile:Water (97:3)

Run Time Total: 5 min

Time (min)	Mobile Phase	Flow Rate (mL/min)
0	100	1.5
5	100	1.5

The simplest form of quercetin (center) and its derivatives (outer) are shown in Fig. 22. Six concentrations of quercetin standard were analyzed via HPLC to determine the area of each peak corresponding to the quantity of quercetin (Fig. 23). The calibration curve of these quercetin standards is shown in Fig. 24.

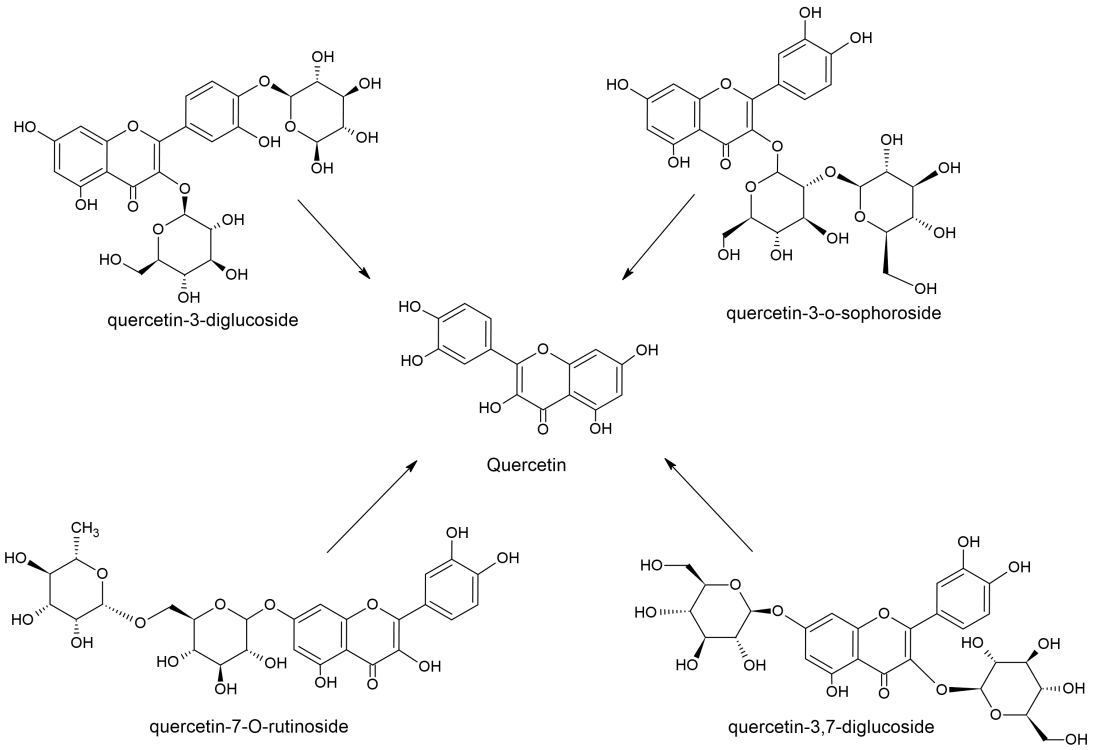


Figure 22. Acid hydrolysis of quercetin derivatives found in *Hibiscus rosa-sinensis* and *Hibiscus tiliaceus.*

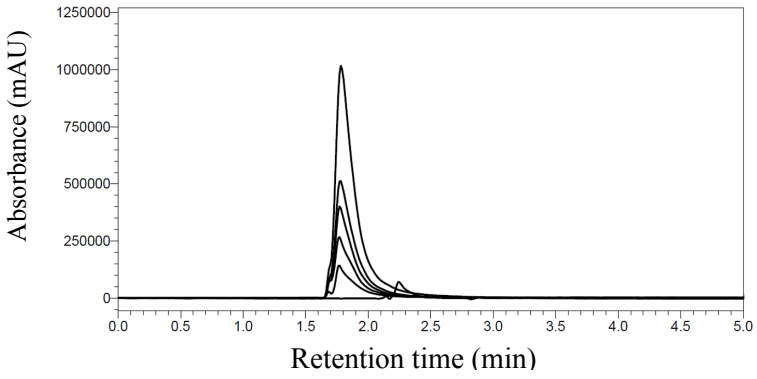


Figure 23. Chromatogram of six quercetin standards detected at 372 nm.

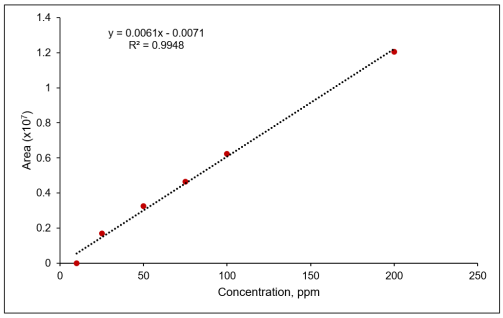


Figure 24. Calibration curve of quercetin standards.

Quercetins were isolated from all flower parts (calyx, carpel/stamens, and petals) for each of the hibiscus plants studied (Fig. 25). Previous phytochemical studies showed that *Hibiscus rosa-sinensis* contained quercetin-3-diglucoside, quercetin-3,7-diglucoside, and quercetin-3-sophoroside. *H. tiliaceus* contained quercetin-7-*O*-rutinoside shown in Fig. 22 (Kristi & Patel 2017; Shen et al. 2021; Shimokawa et al. 2015).

In this study, the concentration of quercetin aglycones, resulting from the acid-hydrolysis of quercetin glycosides, were analyzed via HPLC. The highest concentration of quercetin based on the dry weight of plant material was isolated from petals of *H. tiliaceus* (Figure 26). The yellow to deep yellow-colored petals of *H. tiliaceus* are suggestive of higher amounts of quercetin.

Figure 26. *Hibiscus tiliaceus* growing along a roadside in Yona, Guam.

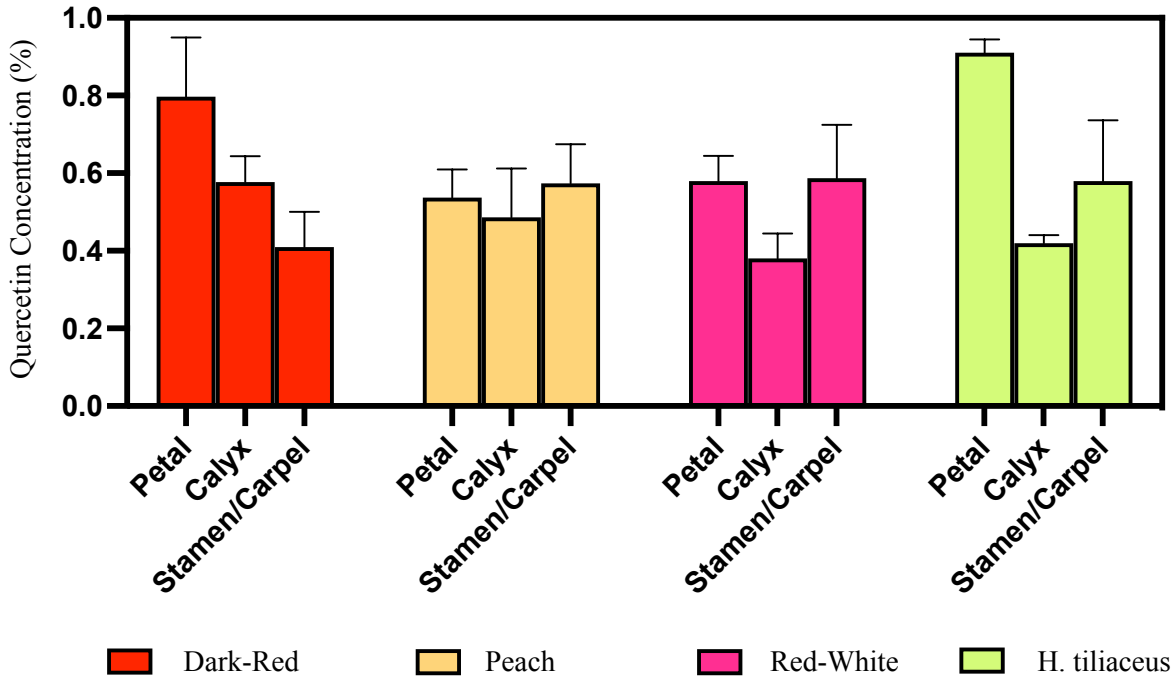


Figure 25. Average concentrations of quercetin aglycones in the petals, calyxes, and stamens/carpels of *Hibiscus rosa-sinensis* "Dark-Red", "Peach", and "Red-White", and *Hibiscus tiliaceus* (mean ± SE, n=3).

Study 5:

Anthocyanins in Hot Pepper Fruits

Anthocyanins are responsible for the color of hot pepper fruits (*Capsicum annuum*). Five local hot peppers were examined including *guåfi*, "Toves", "Saipan", "Barcinas", and *håchon* (Fig. 27) for two anthocyanins studied and found in previous research: delphinidin-3-p-coumaroyl rutinoside-5-glucoside (nasunin) (Fig. 28) and cyanidin-3-glucoside (chrysanthemin) (Fig. 29) (Aza-Gonzalez, 2012). Nasusin is responsible for the purplish color in hot pepper fruits and chrysanthemin is responsible for the magenta, red, and crimson color.

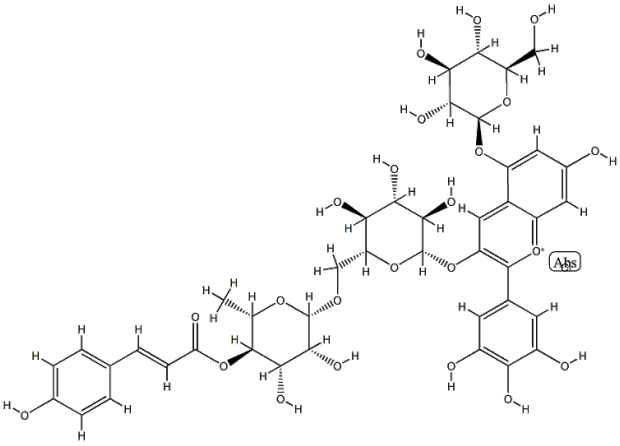


Figure 28. The major anthocyanin, nasunin**,** delphibidin-3-p-coumaroylrutinoside-5-glucoside, is responsible for the purplish color in hot pepper fruits.

Figure 27. The hot pepper fruits studied: *guåfi* (a), "Toves" (b), "Saipan" (c), "Barcinas" (d), and *håchon* (e).

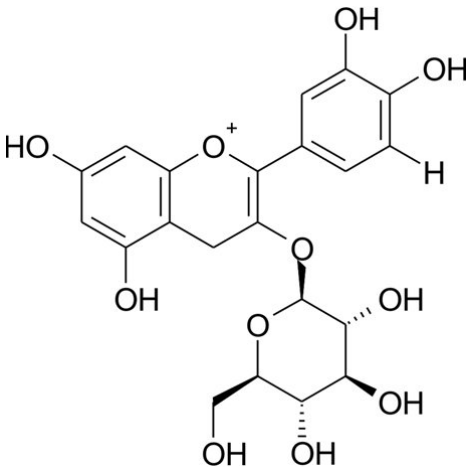


Figure 29. The anthocyanin, chrysanthemin, cyanidin-3-glucoside, is responsible for the magenta, orange, and crimson color in hot pepper fruits.

Sample preparation

Mature fruits were harvested, cleaned, and freeze-dried. Freeze-dried peppers without seeds were ground into a fine powder and stored at -20°C until further analysis. Pepper powder (10 g) was mixed with acidified methanol (50 mL of 2% HCl) and anthocyanins extracted using a magnetic stir plate and bar for 15 min. Crude extracts were filtered using filter paper and a 0.2 µm syringe filter to remove solid particulates.

Pepper Powder Samples *Sample Extracts*

Figure 30. Freeze dried hot pepper sample (left) and preparation of extracts (right).

HPLC Instrument Parameters and Conditions

Column: Shimadzu C-18 dimension 150 x 4.6 mm. UV Detector: 520 nm.

(1) For **nasunin (delphinidin-3-p-coumaroylrutinoside-5-glucoside)**

Column temperature: 40°C. Injection sample volume: 10 µL. Flow Rate: 0.5 mL/min.

Solvents:

Mobile phase A: 10% formic acid

Mobile phase B: 100% acetonitrile

Run Time Total: 37 min

Time (min)	Mobile Phase A	Mobile Phase B	Flow Rate (mL/min)
0	100	0	0.5
13	80	20	0.5
20	70	30	0.5
25-30	1	99	0.5
30-37	100	0	0.5

HPLC Instrument Parameters and Conditions

(2) For **chrysanthemin (cyanidin-3-glucoside)**

Column temperature: 25°C. Injection sample volume: 10 µL. Flow Rate: 0.7 mL/min.

Solvents (Isocratic):

Mobile Phase: 100% Methanol: 2% acetic acid (65:35, v/v)

Run Time Total: 5 min

Time (min)	Mobile Phase	Flow Rate (mL/min)
0	100	0.7
5	100	0.7

The HPLC chromatogram of 'Arbol' pepper fruit shows isomers of nasunin (delphinidin-3-p-coumaroylrutinoside-5-glucoside) at retention times of 32.2 min (trans-isomer) and 34.3 min (cis-isomer) (Fig. 31) (Aza-Gonzalez, 2012).

The chromatogram was used to determine isomers of nasunin in local hot peppers in the study. Figure 32 presents the chromatogram of a local pepper. Two peaks at the retention times of 32.299 min and 34.374 min were trans- and cis- isomers of nasunin, respectively. The larger peak area of the cis-isomer indicates a higher concentration than the trans-isomer.

In order to determine the total amount of nasunin in pepper fruits, the quantities of the two isomers were combined and shown as the amount of nasunin to compare among the local hot peppers in this study.

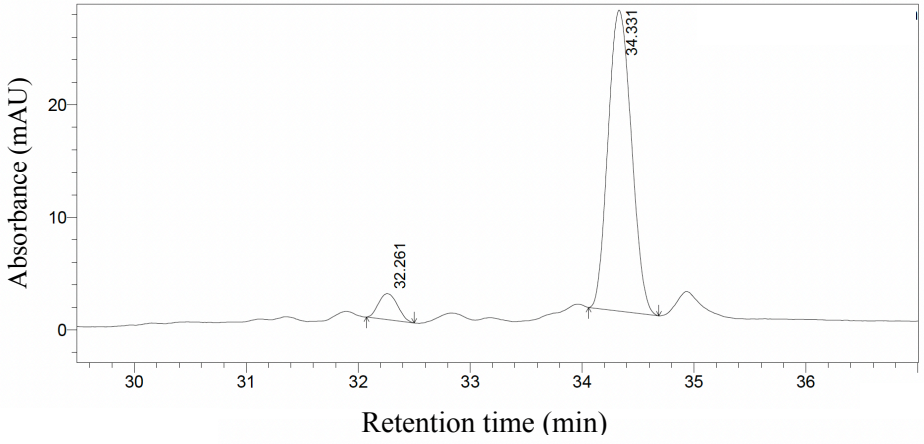


Figure 31. HPLC chromatogram of trans- and cis-isomers of anthocyanin delphinidin-3-p-coumaroylrutinoside-5-glucoside (nasunin) from extract of dried hot pepper 'Arbol' detected at 520 nm.

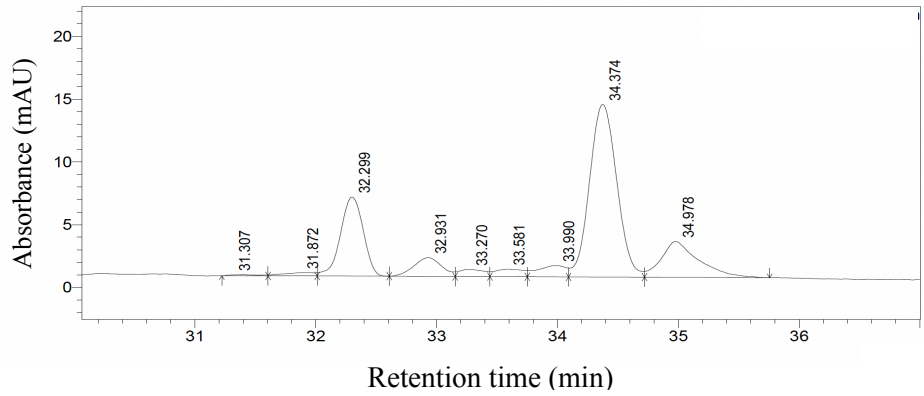


Figure 32. HPLC chromatogram of trans- and cis-isomers of anthocyanin delphinidin-3-p-coumaroylrutinoside-5-glucoside (nasunin) from extract of a local dried hot pepper detected at 520 nm.

The major anthocyanin isolated from red hot pepper fruits was delphinidin-3-p-coumaroylrutinoside-5-glucoside (nasunin). The total area of the two peaks representative of the two isomers of nasunin in the HPLC chromatograms (Fig. 33) represent the total amount of nasunin in the hot pepper fruits examined. Red fruits of *guåfi* had the greatest amount of nasunin, followed by "Toves", "Saipan", and "Barcinas." Nasunin was not detected from the orange fruits of *håchon* (Fig. 34).

The other anthocyanin detected from hot pepper fruits was chrysanthemin (cyanidin-3-glucoside) at very low concentrations in all varieties (Fig. 35). The concentration of chrysanthemin of hot peppers was quantified based on a chrysanthemin standard. "Saipan" had the greatest amount of chrysanthemin than the other three red pepper varieties. The pigment was also detected at a very low concentration in *håchon*. Chrysanthemin concentrations of hot peppers from Guam overall were lower than reported in previous literature (Arnnok, 2012).

In conclusion, this study found that nasunin, delphinidin-3-p-coumaroylrutinoside-5-glucoside, was the major anthocyanin in red pepper varieties from Guam. Furthermore, this anthocyanin appeared as trans- and cis- isomer having two different retention times with two different concentrations. By comparing the concentration of the nasunin standard, we can determine the concentration of this major anthocyanin extracted from local hot peppers.

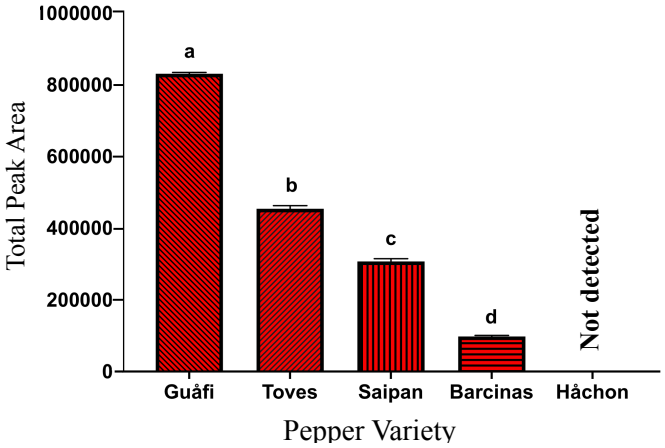


Figure 33. Nasunin identified in local hot pepper varieties of peak areas in HPLC chromatograms (mean +SEM), (n=3). Nasunin was not detected in orange Håchon. Bars with same letter are not significantly different at P<0.05.

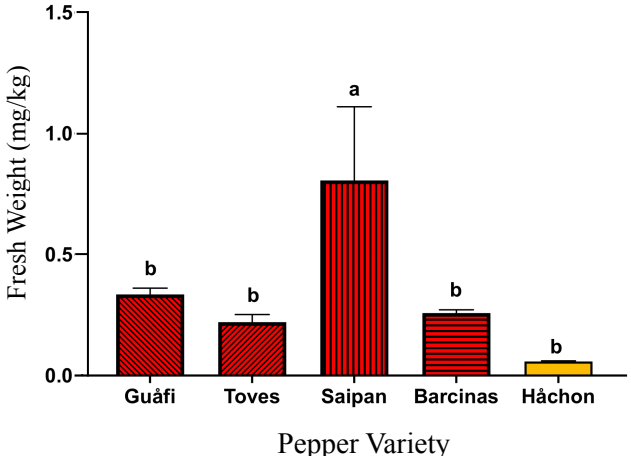


Figure 34. Concentration of chrysanthemin (cyanidin-3-glucoside) isolated from fruits of local hot pepper varieties detected in HPLC chromatograms (mean ±SEM), (n=3). Bars with the same letter are not significantly different at *P*<0.05.

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