DEVELOPMENT OF CHROMATOGRAPHIC METHODS FOR GEOGRAPHICAL INDICATION IN THAI PINEAPPLES

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การพัฒนาสิ่งบ่งชี้ทางภูมิศาสตร์ของสับปะรดในประเทศไทยด้วยวิธีการทางโครมาโทกราฟี

น.ส.วรกานต์ ชูตะกูล

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สิ่งบ่งชี้ทางภูมิศาสตร์ (geographical indication, GI) หรือเครื่องมือทรัพย์สินทางปัญญาที่เทียบเคียงกัน มีการใช้งานอย่างแพร่หลายเพื่อระบุถึงลักษณะเฉพาะและเอกลักษณ์ของสินค้าอันมีความเกี่ยวพันกับแหล่งผลิต ดังเช่นในกรณีของสินค้าทางการเกษตร สภาพภูมิประเทศ ภูมิอากาศ ปริมาณธาตุอาหารในดินล้วนส่งผลต่อคุณลักษณะของสินค้าเหล่านั้น เช่น รสชาติและเนื้อสัมผัส เป็นต้น โดยในงานวิจัยนี้วิธีการทางเคมีได้ถูกนำมาใช้เพื่อพยายามแยกความแตกต่างของสับปะรดที่มาจากแหล่งเพาะปลูกต่างๆ ซึ่งกระบวนการดังกล่าวสำเร็จได้ด้วยการเก็บข้อมูลสารเคมีกลุ่มแคโรทีนอยด์ (carotenoids) ทั้งหมดในสับปะรดด้วยเทคนิคการแยกวิเคราะห์สารด้วยของเหลวสมรรถณะสูงที่ตรวจวัดด้วยตัวตรวจวัดหลายความยาวคลื่น (high-performance liquid chromatography with diode array detector, HPLC-DAD) ก่อนที่ข้อมูลทางเคมีนี้จะถูกประมวลผลด้วยวิธีการทางคีโมเมทริกซ์ (chemometrics) ในลำดับถัดไป เพื่อจัดจำแนกกลุ่มตัวอย่างสับปะรดตามแหล่งที่มา อีกประการที่สำคัญคือ ในการศึกษานี้ได้ถูกออกแบบให้เป็นส่วนหนึ่งของการทดลองในวิชาการเรียนปฏิบัติการด้วย โดยในการแยกแคโรทีนอยด์อันเป็นหมายหลักของการทดลองนี้ประกอบด้วยหลากหลายเทคนิค ได้แก่ คอลัมน์โครมาโทกราฟี การตรวจวัดสารในช่วงความยาวคลื่นอัลตร้าไวโอเล็ตจนถึงคลื่นแสงที่มองเห็น (UV-vis spectroscopy) โครมาโตกราฟีแบบแผ่นบาง (thin-layer chromatography, TLC) การแยกสารด้วยของเหลวสมรรถณะสูง (high-performance liquid chromatography, HPLC) การตรวจวัดมวล (mass spectrometry, MS) และการวิเคราะห์องค์ประกอบหลัก (principal component analysis, PCA) แต่ในทางกลับกันการทดลองย่อยที่กล่าวมานี้ถูกออกแบบในเป็นการเรียนการสอนแบบโมดูล (module) ดังนั้นจึงสามารถถูกเพิ่ม ลด หรือปรับปรุงได้ตามความเหมาะสม นั่นทำให้การทลดองนี้มีความเหมาะสมต่อการใช้งานในเหตุการณ์ที่ไม่แน่นอนต่าง ๆ เช่น การปิดทำการของมหาวิทยาลัยอันเนื่องมาจากเหตุการณ์การแพร่ระบาดของเชื้อไวรัส COVID-19 เป็นต้น ซึ่งในภาพรวมการทดลองที่มีความยืดหยุ่นสูงในแง่การเรียนการสอนนี้เป็นตัวอย่างการทดลองที่ใช้งานได้จริง สำหรับการชี้ให้เห็นว่าศาสตร์ทางเคมีสามารถช่วยแก้ปัญหาในชีวิตจริงได้อย่างไร และท้ายที่สุดนี้ การศึกษากระบวนการทางคีโมเมทริกซ์เชิงลึกที่เหนือไปกว่าการใช้งานในหลักสูตรการเรียนการสอนก็ได้ถูกศึกษาต่ออีกด้วย เพื่อที่กระบวนการที่พัฒนาขึ้นนี้จะสามารถกลายเป็นเครื่องมือที่มีประสิทธิภาพอีกวิธีหนึ่งสำหรับการจัดจำแนกแหล่งที่มาของสับปะรดไทยได้

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2565 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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Worakan Chutakool : DEVELOPMENT OF CHROMATOGRAPHIC METHODS FOR GEOGRAPHICAL INDICATION IN THAI PINEAPPLES. Advisor: Assoc. Prof. Dr. THANIT PRANEENARARAT

Geographical indication (GI), or its equivalent intellectual property tools, is widely used in many countries, including Thailand, to identify specific properties and uniqueness of a product that are related to its production origin. In the case of agricultural products, geographical location, weather, and soil nutrients affect their properties such as flavor and texture. In this study, a chemical method was conducted to attempt to distinguish pineapples based on their origins. This was done by collecting chemical profiles from carotenoids by high-performance liquid chromatography with diode array detector (HPLC-DAD). Thereafter, these chemical data were later processed by chemometric analysis to classify pineapples based on their origins. Importantly, this study was also designed to be a part of a teaching laboratory. Using carotenoid separation as a main goal, column chromatography, thin-layer chromatography (TLC), UV–vis spectroscopy, high-performance liquid chromatography (HPLC), mass spectrometry (MS), and principal component analysis (PCA) can be included in a cohesive laboratory experiment. On the other hand, these activities were also designed to be modular, thus allowing instructors to add, remove, or modify the contents in a highly customizable manner. This makes it highly versatile and amenable to uncertain situations like unexpected university closure due to COVID-19 related lockdown. Overall, this laboratory experiment serves as a practical example of how chemistry can help solve real-world problems while also allowing high flexibility in teaching management. Last but not least, deeper chemometric studies beyond the teaching lab version was also conducted – this can be developed further to become an effective tool to classify origins of Thai pineapple.

Field of Study:	Biotechnology	Student's Signature .....
Academic Year:	2022	Advisor's Signature .....

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CHAPTER I

INTRODUCTION

1.1 General information of pineapple and geographical indication

Pineapple (*Ananas comosus*) is one of the tropical fruits that consists of high variety of almost 70 cultivars, and their classification is divided into five groups based on their spininess, fruit shape and flower colors. This includes Spanish, Queen, Cayenne, Pernambuco and Perolera[1,2]. Thailand is known as a leader in global pineapple production[2], with two widely planted cultivars, including Cayenne (specifically 'Smooth Cayenne'), and Queen. Nevertheless, their representative or commercial names are different depending on their planting sources even though they are the same cultivar and have similar appearances. For example, while both are smooth Cayenne, Sriracha (**Figure 1**) pineapples are grown in the Chonburi province, while Ban Kha is from the Ratchaburi province.

Figure 1 Appearance of Sriracha pineapple[3].

According to the large number of pineapples distributed to the market, several trading organizations use GI (geographical indication) trade mark as an important tool to increase the market value of pineapples. Climate and geographical origins clearly affect the quality of plants. Thus, the marker is used to refer to the product's origin and its unique characters such as taste, juiciness, and texture. Nowadays, nine Thai pineapples have been registered as GI products[4] (**Table 1**).

Table 1 Registered Thai pineapples as geographical indication products and their origins

Cultivar group	GI name	Cultivation origin
Smooth Cayenne	Sriracha	Chonburi
	Hauymon	Uttaradit
	Tha Uthane	Nakhon Phanom
	Nanglae	Chaing Rai
	Ban Kha	Ratchburi
	Sri Chiang Mai	Nong Khai
Queen	Phuket	Phuket
	Trat Si Thong	Trat
	Rayong Golden	Rayong
	Chiangrai Phulae	Chiang Rai

Due to its rich chemical components, chemical profiling can be used as a part of product verification. During maturation, the fresh smell comes from aromatic chemicals while its shell and flesh change their shade of color because of chlorophylls degradation (green shade) and carotenoids production (yellow to red shade)[1]. In the same way, the taste and texture can be investigated by fiber, sugars content, organic acids, and vitamins. One of the interesting chemicals is antioxidants like vitamin C[5,6], flavonoids[7], and

carotenoids[8,9], which can be inspected by chromatographic techniques and also by spectroscopic techniques[10-14].

1.2 Previous study of chemical components in pineapples

Research using spectroscopy to analyze pineapple is an interesting study due to the highly complexed nature of the crude. ^{1}H NMR is used to identify metabolites in different tissues (**Figure 2**). Surprisingly, the researchers found that the crown was the part that contained the highest amounts of antioxidant compounds[15]. The found metabolites found only in the crown included catechin (20), protocathechuic acid (21), benzoic acid (22), vanillic acid (23), and 3-methylglutaric acid (31).

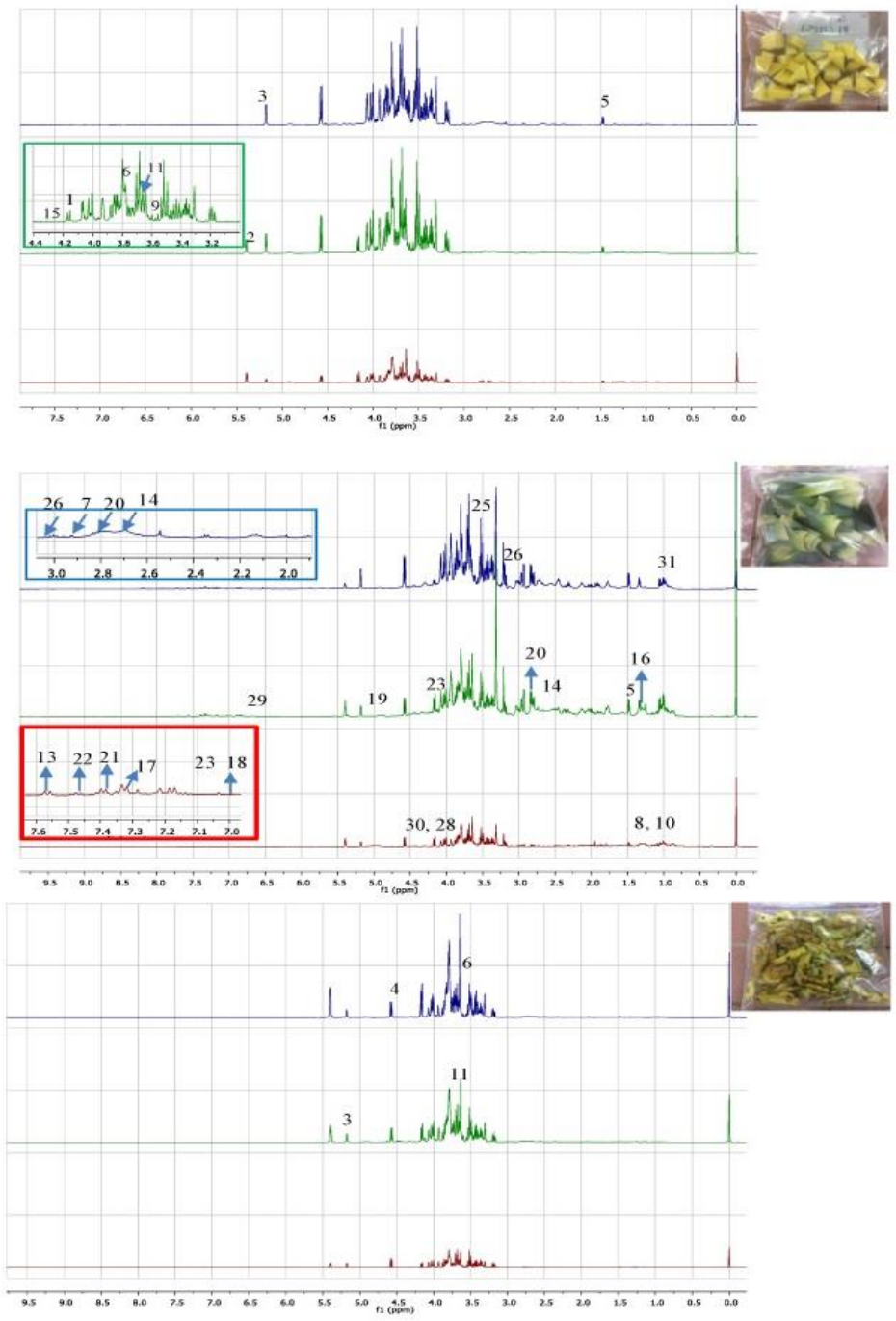


Figure 2 ^{1}H NMR Spectra of flesh, crown, and peel extracted with three ratios of ethanol; blue = 0% ethanol; green = 50% ethanol; and red = 100% ethanol.

Tainong No.4 and No.6, which are local Chinese pineapples and hybrids of “smooth cayenne” and “Shen wan”, showed distinguished profile of aromatic compounds of these pineapples by GC-MS. The samples showed different components of volatiles even though they were developed from the same parent[16]. The detail of characteristic odors in these sample is shown in **Table 2**.

Table 2 Aroma compounds found in pineapples from China[16]

Compound	Tainong No. 4	Tainong No.6	Odor Quality
3-(Methylthio)propanoic acid methyl ester	+	+	Meaty, oniony, fruity
3-(Methylthio)propanoic acid ethyl ester	+	+	Meaty, oniony, pineapple-like
Butanoic acid, 2-methyl-, methyl ester	-	+	Fruity, apple-like
Butanoic acid, 2-methyl-, ethyl ester	-	+	Fruity
Hexanoic acid, methyl ester	+	+	Fruity, ester-like
Hexanoic acid, ethyl ester	-	+	Apple peel-like, fruity
2,5-Dimethyl-4-hydroxy-3(2H)-furanone	+	-	Sweet, pineapple and caramel-like
Octanoic acid, methyl ester	+	+	Fruity, citrus-like
Decanal	-	+	
δ-Octalactone	+	-	Coconut-like
Decanoic acid, ethyl ester	-	+	

In addition, over 60 phenolic compounds were investigated in each part of pineapple (**Figure 3**), i.e., crown, peel, and flesh, by using high-performance liquid chromatography with diode array detection and electrospray ionization multiple-stage mass spectrometry (HPLC-DAD-ESI-MS^n) (**Figure 4**) and GC-MS[17]. The same research group also obtained their carotenoids profile with HPLC-DAD-APCI-MS^n[18].

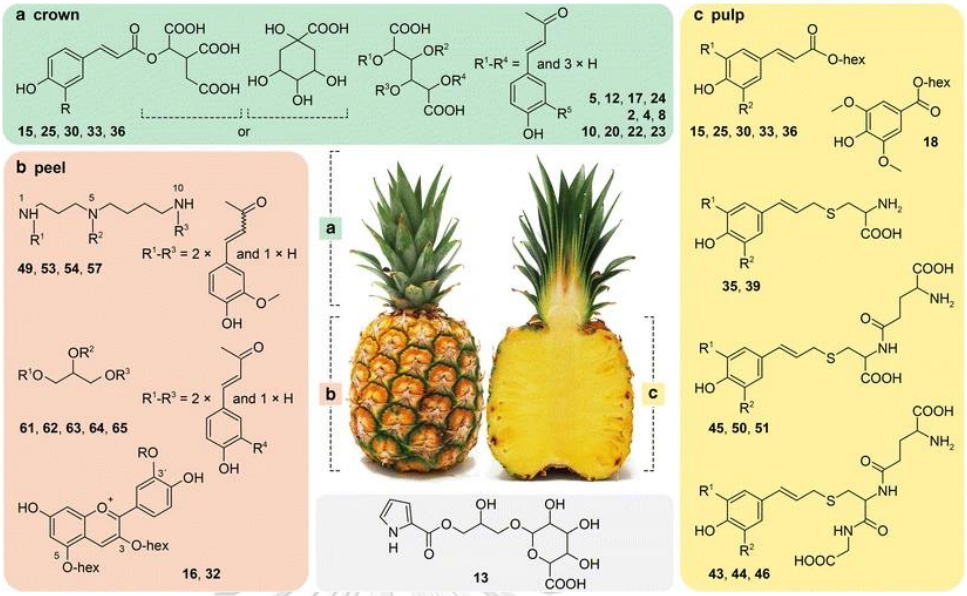


Figure 3 Basic structures of metabolites characterizing pineapple.

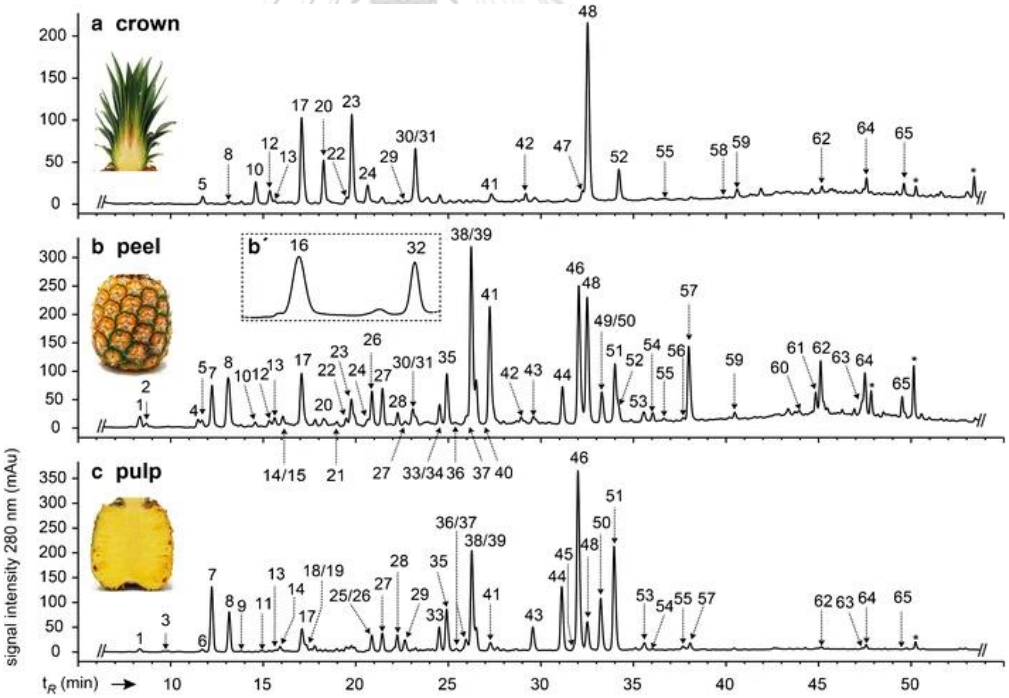


Figure 4 HPLC-DAD chromatogram (280 nm) of phenolic compounds in a methanolic extract from crown (a), peel (b) and pulp (c) tissues.

1.3 Carotenoids

One interesting class of compounds is carotenoids (**Figure 5**), the yellow-red pigments which relates to color changes of ripping fruits especially pineapple. Many structures of the terpenoids are divided into two subclasses regarding the presence of oxygen in their chemical structures. First, 'carotenes' refer to hydrocarbon carotenoids like **α**-carotene, **β**-carotene, and lycopene. Second, 'xanthophylls' or oxy-carotenoids contain oxygen atoms in their molecules in the form of alcohol (-OH), ketone (=O), and ester (-O-) such as lutein, zeaxanthin, and violaxanthin. The latter is more polar than the former[14,19,20].

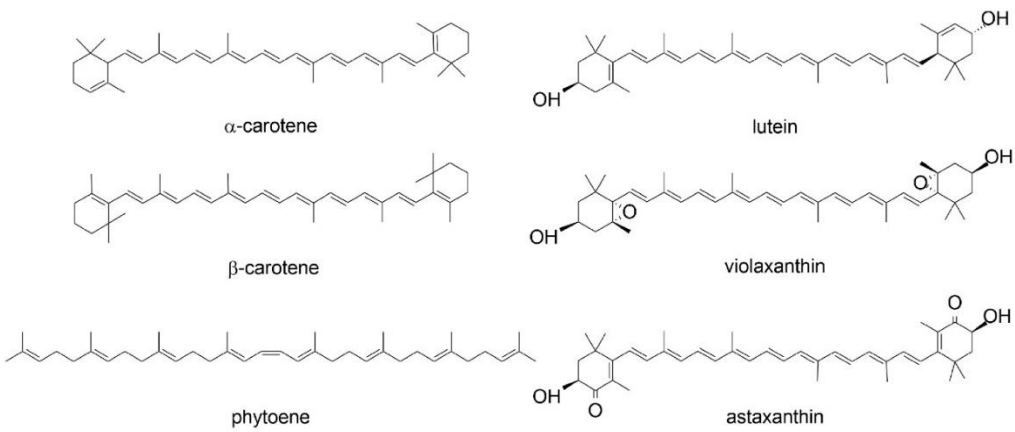


Figure 5 Some of carotenoids structure.

1.3.1 Extractions

The high-water content of fruits is an issue for efficient extraction of carotenoids. Besides, thermal-based dehydration methods can cause thermal degradation and isomerization of carotenoids. Hence, freeze-drying is conducted to protect losses of the substances but it significantly increases the extraction time and cost. Alternative extraction methods have been used (**Figure 6**) such as atmospheric liquid extraction with maceration or liquid-liquid extraction (LLE), Soxhlet extraction (SE), microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE),

pressurized liquid extraction (PLE), pulsed electric field (PEF) extraction, supercritical fluid extraction (SFE), and enzyme-assisted extraction (EAE)[20].

Extraction Method	Advantages	Disadvantages
Atmospheric liquid extraction with maceration	• High extraction yields without utilizing sophisticated instruments	• Requires large amounts of toxic solvents, thus increasing the cost of production
Soxhlet extraction	• Simple and conventional method providing the highest recovery of carotenoids • No sophisticated instruments required	• Time-consuming and also uses large amounts of solvents, which increases the cost of extraction • Can cause thermal degradation and *cis-trans* isomerization of carotenoids
Microwave-assisted extraction (MAE)	• Simple, fast and economical method	• Can cause thermal degradation and *cis-trans* isomerization of carotenoids
Ultrasound-assisted extraction (UAE)	• Rapid, non-thermal and efficient extraction	• Aging of the ultrasonic probe surface can change the extraction efficiency • Small particle size (≈50 μm) is required to achieve good extraction
Pressurized liquid extraction (PLE)	• Fast (few minutes), requires minimum amount of organic solvent • Highly applicable to a laboratory-scale context	• Difficult to apply to large volumes due to clogging caused by sugars and pectins of plant matrices
Pulsed electric field (PEF) extraction	• High extraction yield • Non-thermal process • Low energy usage	• High costs of instrumentation • Bubbles in the samples may cause technical problems • PEF parameters may differ with change in electrical conductivity of the sample
Supercritical fluid extraction (SFE)	• Uses non-flammable, non-toxic and recyclable solvent (CO2 and ethanol) • Continuous extraction process instead of batch processing • Useful for extraction of thermolabile compounds • Provide carotenoids with high purity	• Not suitable for samples containing high amounts of water • Low yield of polar carotenoids • High cost of instrumentation
Enzyme-assisted extraction (EAE)	• Rapid and efficient extraction with minimal usage of solvents	• High cost of the enzymes

Figure 6 Advantages and disadvantages of various carotenoids extraction methods[20].

Herein, LLE is the most preferred method due to the thermal sensitivity of carotenoids. (**Figure 7**) show standard steps of carotenoids extraction, which include 1) sample preparation, 2) extraction, 3) partitioning, 4) saponification, and 5) chromatographic separation[20].

Steps	Procedure	Important point to consider
Sample peroration	Preparation of homogenous samples	Volume reduction procedure should be followed to minimize the variation between sample samples
Extraction	Extraction of carotenoid in cold acetone	Minimum 1:10 ratio of sample and acetone should be used. Repeat the extraction until samples became colorless
Partitioning	Partitioned to the petroleum ether 40–60 °C containing 10% (v/v) diethyl ether and washed with water to remove the traces of acetone	Avoid micelle formation during addition of water
Saponification	Saponify overnight with 10% KOH (potassium hydroxide) in methanol (w/v), wash with water to remove the alkali, and dried in vacuum rotavapor (T ≤ 35 °C) (Optional)	Saponification step can be eliminated to routine chromatographic analysis. chances of artifact formation are more during saponification
Chromatographic separation	By RF-HPLC in C_{30} column	C_{30} carotenoid column can separate geometrical (-*cis* and -*trans*) isomers of carotenoids
Identification and quantification	Confirmation of carotenoids by appropriate standards, mass spectrometry and absorbance spectrum	Purified carotenoids are very sensitive to oxidation and light induced degradation, check the purity time to time

Figure 7 Common steps used in carotenoids extraction from fruits and vegetables[14].

1.4 Chemometrics

An outstanding tool used to discern the difference of sample groups is chemometrics. Clearly, this is useful for food traceability and geographical

location classification[21]. Chemometric analysis combined processed chromatographic or spectroscopic data to mathematic patterns[22,23]. The results from techniques such as LC, GC, MS, and UV-vis spectrophotometry can be processed with chemometric methods and result in meaningful data representations. Among the most important techniques are principal component analysis (PCA), self-organizing maps, (SOMs), and linear discriminant analysis (LDA).

Some examples of chemometric application for pineapples are the ripeness monitoring by analyzing aromatic compounds[24] (**Figure 8**), and by analyzing total soluble solid[25], quality control of processed pineapples[26], and product developments based on HPLC technique[27]. Moreover, the analysis method can be employed for genetic approach to identify pineapple cultivars[28,29].

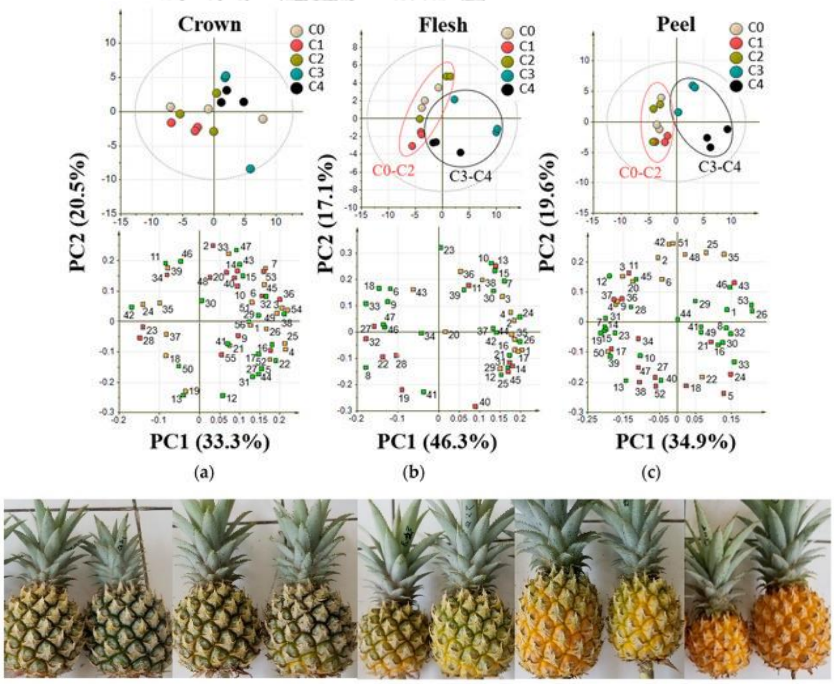


Figure 8 PCA result from flesh, crown and peel parts of pineapple from different ripening stages.

1.5 Research objective

According to the GI registration process of a product as set by the Division of Intellectual Property, required data include only physical and geographical information such as cultivar, description of physical appearances, areas of plantation location, and relations between the planted areas and goods production. In the case of pineapples, sugar content (in degree brix unit) and percentage of total acid were sometimes declared. This can imply that the registration process does not explicitly require chemical data to be included. On the other hand, European union intellectual property office (EUIPO) stated in protection and control of GIs for agricultural products in the EU member that *"the product must comply with detailed specifications including, at least, the raw materials and the principal physical, chemical, microbiological or organoleptic characteristics of the product. Moreover, the evidence of the geographical area of production must be provided, together with the production method, with all of these elements demonstrating that the product originates in the defined geographical area, and that it complies with the labelling method"* [30].

In this study, an alternative method was created for discovering GI in Thai pineapples by analyzing chemical profiles obtained from HPLC-DAD using chemometrics. The hypothesis is chemometric data can distinguish pineapples based on their chemical profiles, which in turn reflect their geographical origins. Techniques like principal component analysis (PCA) and linear discriminant analysis (LDA) provide visualized and numeric data that can help the differentiation without bias.

CHAPTER II

EXPERIMENTAL SECTION

2.1. Materials and chemicals

Different types of Thai pineapples, which are Sriracha, Suanpueng, Pattavia, Trat Si Thong, Phuket, and Ban Kha, were purchased from local markets for used in this study.

Chemical reagent and solvent were purchased from Carbo Synth, Carlo Erba, RCI Labscan (Thailand) and Merck. All chemicals are listed below:

- Hexanes (Hex)
- Acetone
- Diethyl ether (Et_2O)
- Methanol (MeOH)
- Ethyl acetate (EtOAc)
- Silica gel
- β-Carotene
- all-*trans*-Lutein
- all-*trans*-Violaxanthin
- Potassium hydroxide (KOH)
- Potassium permanganate ($KMnO_4$) reagent for TLC stain
- Sodium sulfate ($NaSO_4$)

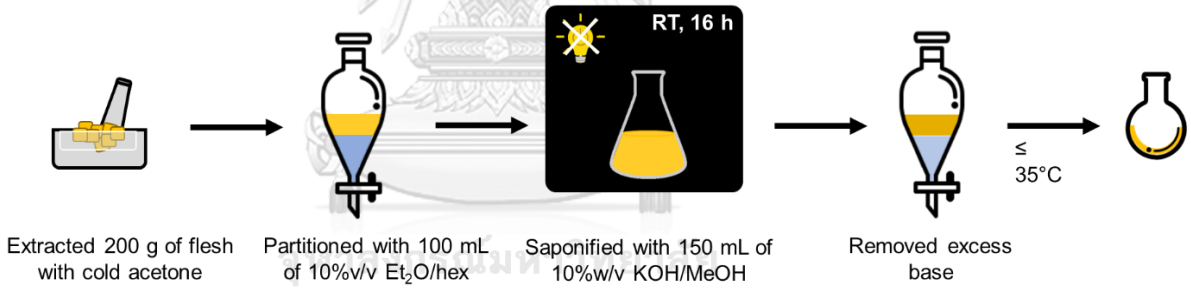
2.2. Sample extraction

200 g of frozen pineapple was blended with a laboratory blender and squeezed by using cloth filtration with acetone as a solvent. The liquid extract from manual squeezing was collected into a 500-mL beaker. The residue was done this step multiple times by repetitively adding new extraction solvent portion. At the end, all solid residue stayed in the cloth.

To describe, the cloth containing solid residue was transferred onto a mortar. After that, 50-mL chilled acetone was added and the residue was extracted further using a pestle to remove as much yellow substance as possible. The resulting solution was then poured into the same 500-mL beaker in the previous step. The addition of acetone and the extraction process was repeated for two more times. Then, all portions of yellow liquids were combined together.

2.3. Liquid-liquid extraction and saponification

All liquid from the previous step was transferred into a separatory funnel and liquid-liquid extraction with 100 mL of 10% Et_2O/Hex was performed. Subsequently, 150 mL of 10% KOH in MeOH was added into the flask. The flask is left shaken overnight on a magnetic stirrer under dark environment at room temperature. Thereafter, excess base in the mixture was neutralized with deionized water until the pH is neutral. The evaporated crude was kept in the dark until used.



Scheme 1 Pineapple preparation process

2.4. Column chromatography

Three separate fractions were expected from the following elution program through an 8-cm height of silica column. First, column was run with 100% Hex until the first visible yellow band is collected. Second, the mobile phase was switched to 30% EtOAc/Hex and the separation was continued until a new colored fraction is collected. Lastly, 50% EtOAc/Hex was used until the last colored fraction is collected.

2.4.1.Thin-layer chromatography (TLC)

TLC analysis was performed between crude (Cr), standard solution of β-carotene (B), first (Fr1), second (Fr2), and the last fractions (Fr3) using 50% EtOAc/Hex as a mobile phase. After developed, the TLC plate was taken a photo under different conditions: a) ambient light, b) UV light (254 nm), c) UV light (365 nm), and d) ambient light after the TLC plate is stained with $KMnO_4$ solution (1.5 g of $KMnO_4$, 10 g of K_2CO_3, and 1.25 mL of 10% NaOH in 200 mL of water).

2.4.2.Compound identification

2.4.2.1. UV-vis spectroscopy

The solutions of isolated β-carotene fraction, crude, and the standard solution of β-carotene were measured for their UV-vis properties by a NanoDrop 2000 spectrophotometer (in the range of wavelength of 350-600 nm).

2.4.2.2. Mass spectrometry (MS)

The model MS/MS experiment was conducted on a Thermo Scientific™ TSQ Quantum Ultra with the following parameters: mass range = 100-700 m/z; parent ion = 536.8 m/z; positive detection mode; flow rate = 15 µL/min; spray voltage = 3000 V; capillary temperature = 300 °C; collision energy (CE) = 10 V. Thermo Xcalibur software was used to process the data. Only isolated β-carotene was studied in this experiment.

2.4.2.3. High performance liquid chromatography (HPLC) analysis

Certain peaks in HPLC chromatogram were identified with standard solutions of carotenoids with the aforementioned HPLC

condition. This includes all-*trans*-lutein and all-*trans*-violaxanthin. The HPLC program was described below.

2.5. High performance liquid chromatography (HPLC)

The pineapple crude was dissolved with 1 mL of acetone and filtered through 0.45-μm syringe filter into an amber vial and kept in a fridge until analysis.

HPLC data were obtained from a Thermo Scientific™ UltiMate™ 3000 UHPLC with a diode array detector (detected at 450 nm). The injection volume was 5 μL. The column was ZORBAX Eclipses Plus C18 column (4.6 x 50 mm, 3.5 μm), with the following time program (**Table 3**).

Table 3 HPLC time program for ZORBAX Eclipses Plus C18 column

Retention (min)	Flow (mL/min)	Percentage of 9:1 MeOH:H_2O	Percentage of EtOAc
0.00	0.30	100	0
20.00	0.30	20	80
20.00	0.50	20	80
30.00	0.50	15	85
30.10	0.50	100	0
35.00	0.50	100	0

2.6. Chemometric analysis

Numerical data obtained from the areas of prominent peaks in HPLC chromatograms were used for chemometric analysis. These were converted to relative peak areas by taking the ratio between the peak area of interest over that of β-carotene. Chemometric analysis in the form of principal component analysis (PCA) was then performed by processing and dimensionally reducing data from normalized peak areas as explained above. All calculation was done with the MATLAB software.

CHAPTER III

RESULTS AND DISCUSSION

Since the development of methods for carotenoids extraction, separation, and characterization involve many basic techniques that are commonly learned by undergraduate students, one of the main tasks in this study was to design a lab that can be directly used for teaching experiments, specifically a laboratory course called integrated chemistry laboratory I. The purpose of this class is to allow students to utilize various branches of chemistry in solving more realistic problems. Thus, this experiment involving pineapples is clearly of relevance. Specifically, pineapple was used as a key model to allow for the incorporation of multiple important concepts into the laboratory. Using carotenoid separation as a main goal, column chromatography, high-performance liquid chromatography (HPLC), mass spectrometry (MS), and principal components analysis (PCA) can be induced in a cohesive laboratory experiment. The following sections highlight some key points regarding with the experiment.

3.1 Preparation of pineapple extract

This experiment commenced with the extraction of carotenoids from pineapples. In this case, standard protocol was applied where water and acetone were used for extraction. Polarity likely affects the obtained carotenoid profile, which can be clearly seen in subsequent HPLC experiment. Also, physical appearance of the liquid extracts of different cultivars of pineapples showed some differences (**Figure 9**). This is the first element for a possible introduction of chemometric concept that different colors could be due to different carotenoid profiles, which in turn may be used for cultivar classification.

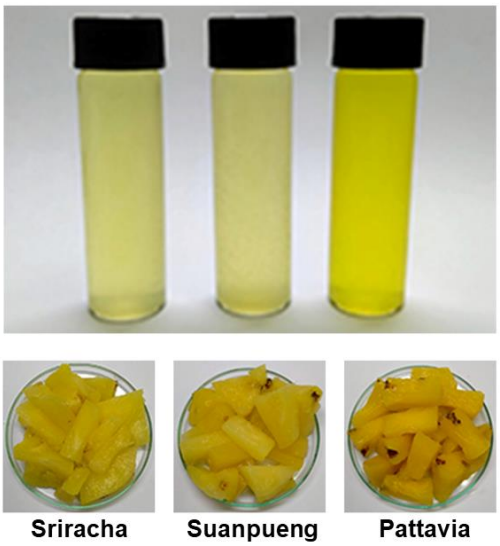
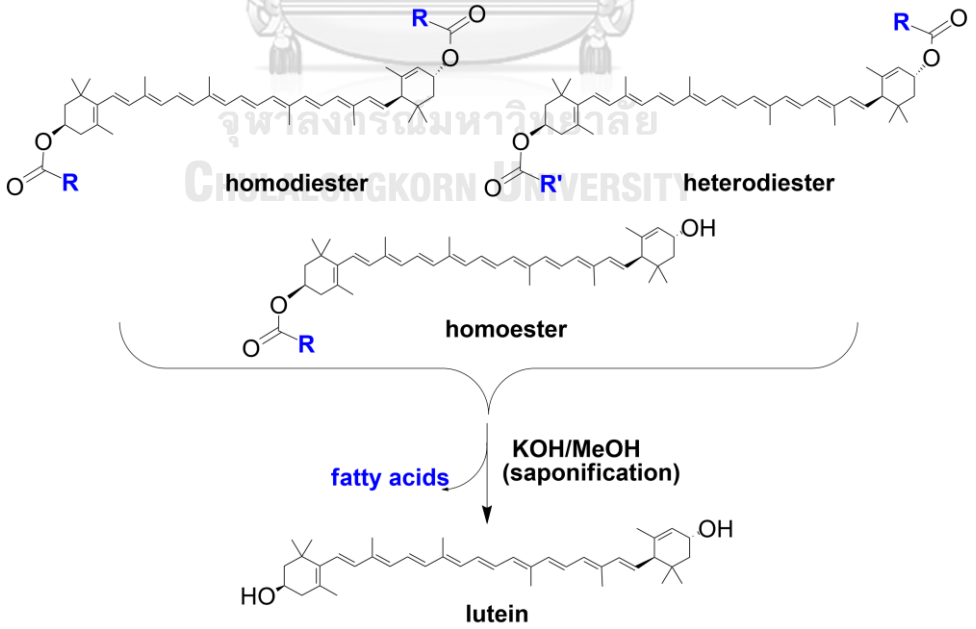


Figure 9 Photographs of the liquid extracts of three cultivars of pineapples; (Left) Sriracha; (Middle) Suanpueng; (Right) Pattavia.

The last step in the preparation phase (item 2.3 in chapter II) deals with a saponification reaction (**Scheme 2**). This was required to reduce the complexity of chromatograms obtained from subsequent HPLC analysis by removing carotenoid esters, chlorophylls, and lipids[18,20].



Scheme 2 A representative set of saponification reactions of carotenoid substrates commonly found in pineapple.

3.2 Column chromatography and thin layer chromatography (TLC)

In this study, the crude separation aims to separate colored compounds into three groups, which can be well separated from the change in the mobile phase composition. Additionally, to allow for connected discussion with HPLC, extensive data collection on TLC was performed (**Figure 10**). Herein, four sets of data for TLC were acquired. Apart from colors from ambient light, UV light at different wavelengths was used to demonstrate how various compounds can appear under different conditions. The concept of chromophore can be introduced when explaining the dark spot in 254 nm light, while 365 nm is suitable for showing fluorescent compounds present in the sample. Also, a well-known TLC staining agent, $KMnO_4$, was used to educate students on how reducing properties of carotenoids can be used as a means to detect them in TLC analysis. These expanded data sets can be beneficial for deeper analysis on structure–property relationships.

Mobile phases and stationary phase are related to carotenoids elution. Firstly, eluted yellow band (Fr1 in **Figure 10**) with 100% Hex is a low polar carotenoid, which was confirmed to be **β**-carotene by TLC (R_f = 0.93) and HPLC (**Figure 12**, bottom.). Then, the higher polar compounds were eluted with 30% EtOAc/Hex as the second isolated fraction (Fr2 in **Figure 10**, R_f = 0.79). Finally, the highest polar carotenoid group, the third isolated fraction (Fr3 in **Figure 10**), were eluted with 50% EtOAc/Hex. This finding can be compared later with HPLC results, allowing another important point for students' discussion.

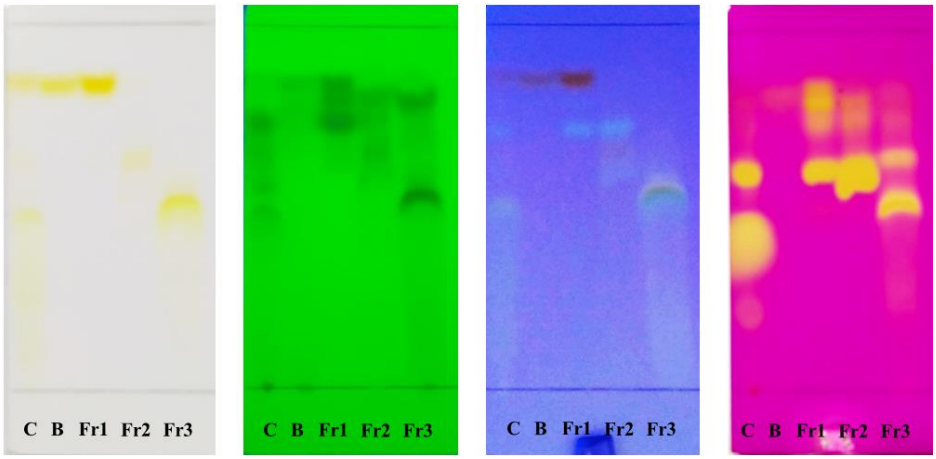


Figure 10 A set of TLC data (mobile phase = 50% EtOAc/Hex) under four conditions including (left to right) white light, 254 nm light, 365 nm light, and $KMnO_4$ staining; B = β-carotene standard; Cr = crude mixture; Fr1 = first; Fr2 = second; Fr3 = third isolated fraction.

3.3 UV-vis spectroscopy and HPLC analysis

These spectroscopic experiments are an important tool to identify the isolated solution with the conventional techniques. Here, nanodrop spectrophotometer was used due to little consumption of sample (only 3 μL per analysis), which is suitable for the amount of sample obtained in this experiment. UV-vis spectrum (**Figure 11**) demonstrates the similarity in features of spectra of standard of β-carotene solution and the first fraction, both of which show the maximum absorption at 450 nm. On the other hand, crude spectrum shows some difference, with the lambda max shifting to shorter wavelength. Nevertheless, the highest absorbed region is still around 450 nm, which match the expected color of yellow in carotenoids.

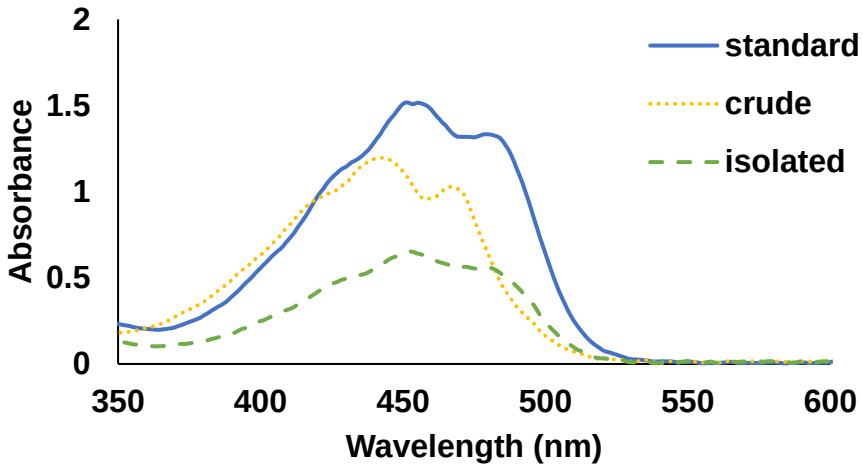


Figure 11 UV-vis spectra obtained from a Nanodrop 2000 spectrophotometer of a standard β -carotene solution, crude extract prior to column chromatography, and firstly isolated fraction containing β -carotene.

HPLC with UV detector or DAD is a standard tool in modern analytical science for separation and analysis of samples with some UV–vis absorption. Perhaps what is most interesting, in terms of connection to the introductory level of chromatography teaching, is the dominance of reversed-phase HPLC in frontier research. With hydrocarbon moiety capped on the surface, this type of column leads to an opposite elution order where more polar compounds are eluted out first, along with the reversed definition of solvent strength (less polar = stronger elution power). Importantly, having both normal-phase column chromatography and reversed-phase HPLC, *i.e.,* ZORBAX Eclipses Plus C18 column, in the same experiment is a great opportunity whereby the concept of chromatography can be discussed in-depth. Specifically, the top of **Figure 12** shows an HPLC chromatogram for a crude pineapple extract where multiple peaks from carotenoids are shown. These major peaks allow for some thoughtful discussion regarding with the relationship of the polarities of compounds and their elution orders. For instance, β-carotene appeared at about 20.8 min, which is a relatively long retention time. This corroborates well with its very low polarity, hence being retained the most with hydrophobic stationary phase in reversed-phase HPLC. On the contrary, there is a group of carotenoids whose retention times

are significantly shorter than that of β-carotene (**Figure 12**, top). Comparison with some standards and previous literature[18] suggests the presence of violaxanthin and lutein that were eluted in approximately 8.35 and 11.32 min, respectively. These compounds are expected to be more polar than β-carotene based on their functionalities. Hence, these data permit the discussion and comparison about the relationship of polarity and elution in both normal (column chromatography and TLC) and reversed-phase chromatography (HPLC), thereby enhancing the learning experience about chromatography. Also, the bottom of **Figure 12**, which shows a chromatogram after column separation, is a good indicator of student's performance in conducting column chromatography. This particular example shows good purification efficiency, whereby only β-carotene peak is visible. Not only does this open up discussion about the separation efficiency (by comparing the results between the top and the bottom of **Figure 12**) but also it can allow comparison with other techniques, for example, comparison with TLC result (**Figure 10**). All in all, this can be viewed as an opportunity for students to gain skills in chromatography.

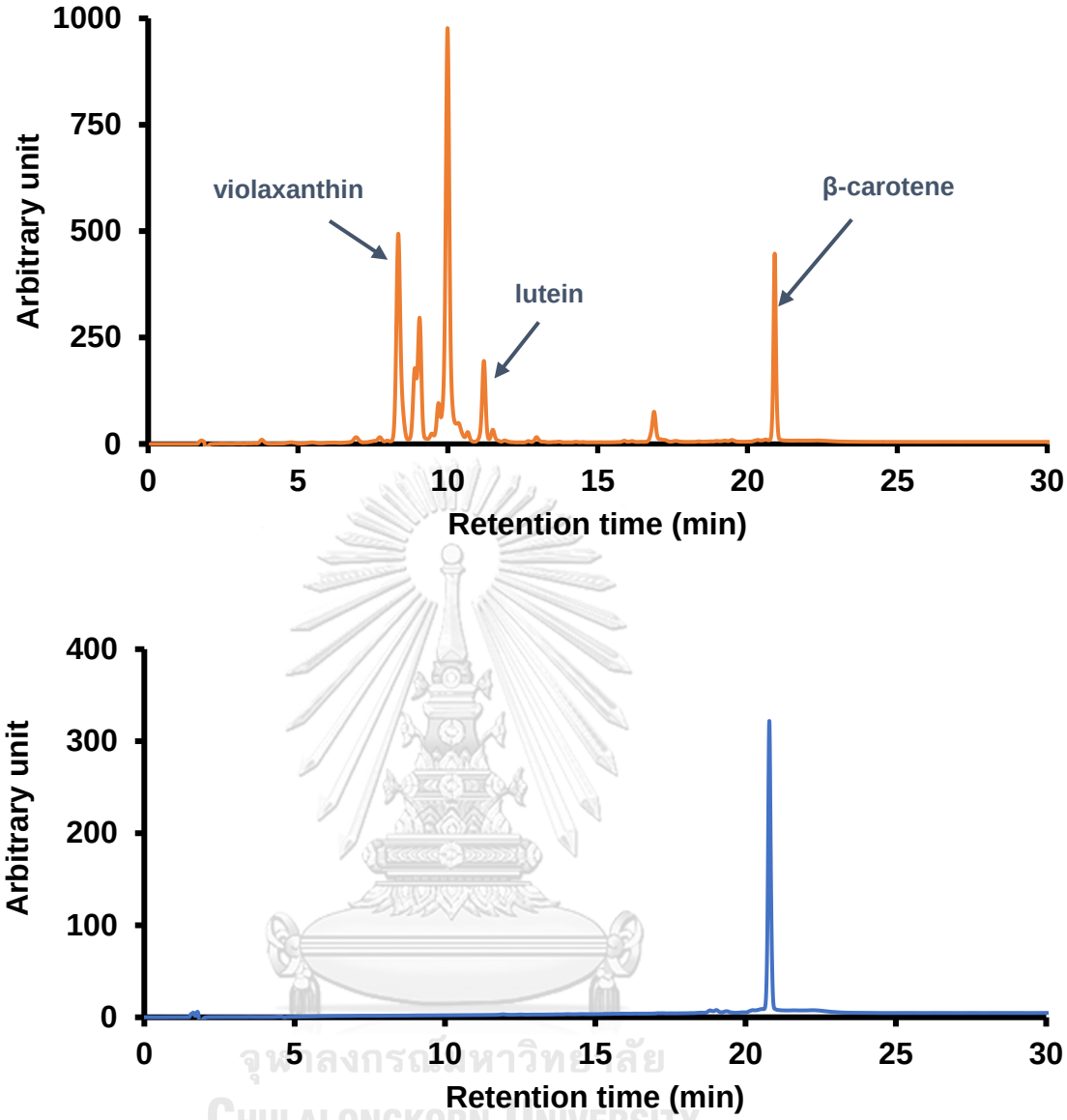


Figure 12 A representative set of HPLC chromatograms of (top) crude extract and (bottom) first fraction.

3.4 Mass spectrometry (MS)

Even though undergraduate students are usually not allowed to operate MS instruments, an idealized MS spectrum (**Figure 13**), obtained by a student's sample, can be useful for students' discussion. This is because MS interpretation is usually a learning element in spectroscopic courses focusing on organic chemistry. For example, fragmentation pattern causing a loss of *m/z* 92 is attributed to the loss of toluene

(**Scheme 3**). This can spark many more discussion involving writing arrow-pushing mechanism, which is a very important skill in learning organic chemistry.

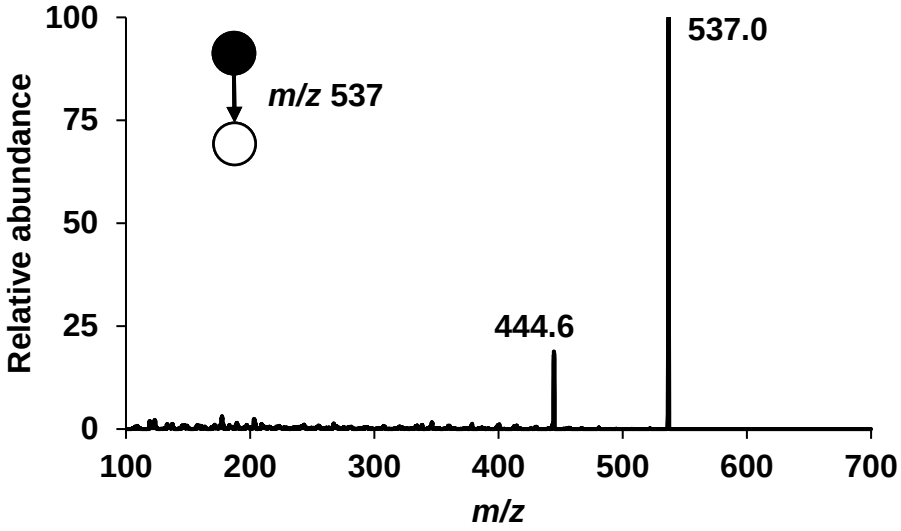
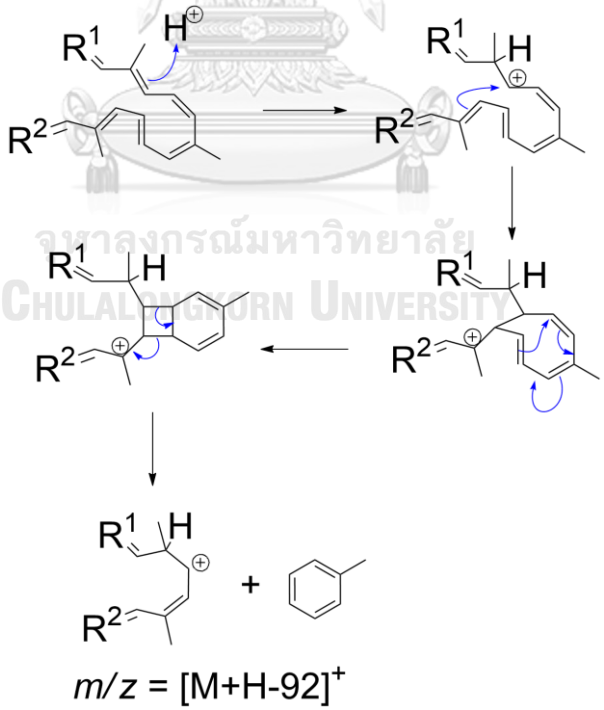


Figure 13 Idealized MS/MS spectrum of an isolated fraction containing β-carotene using a triple quadrupole MS.



Scheme 3 Fragmentation pathway of β-carotene to lose toluene (m/z =92)

3.5 Chemometric analysis

Chemometrics is a worth-mentioning topic in chemistry that can be incorporated as a hands-on activity. This discipline utilizes statistical science to uncover meaningful interpretation of multidimensional data, which can then be further exploited in a variety of ways. For example, data in the forms of peak area and retention time (R_t = 8.3, 8.9, 9.6, 9.9, 11.1, 16.8, and 20.8 min) obtained from HPLC analyses of various cultivars of pineapples were processed, dimensionally reduced, and presented in a form of PCA (**Figure 14**). Interestingly, while there was significant variability in students' data due to some human errors, PCA, along with data pre-processing, was sufficiently robust to still provide decent discrimination among various pineapple cultivars. Overall, incorporating chemometrics not only demonstrates the power and the robustness of the discipline, but it also serves to connect to a topic of wide impact, *i.e.,* geographical indication, which is a form of intellectual properties of increasing importance. Thus, this approach can greatly help demonstrate the impact and the practicality of chemical sciences to students.

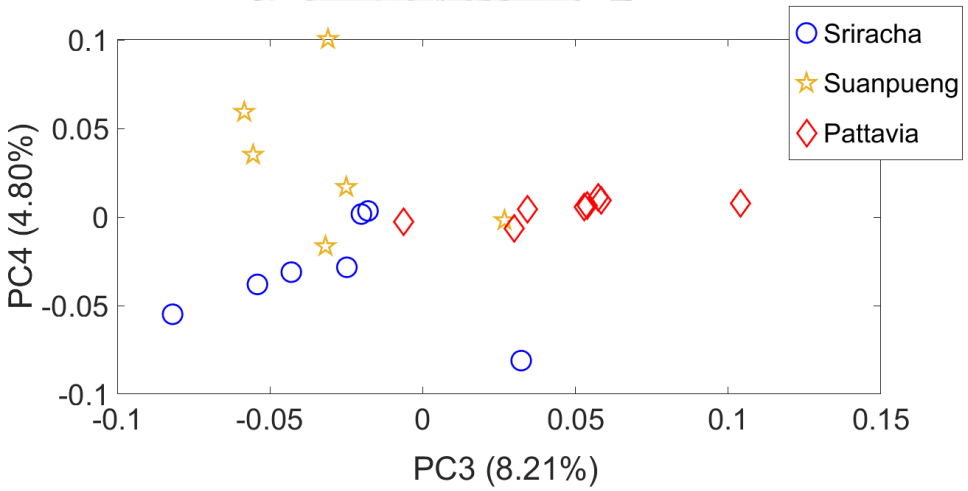


Figure 14 PCA of three cultivars of pineapples created from students' HPLC data. It should be noted that the students were not aware of the difference of obtained samples, nor were they introduced to chemometrics in this particular course. Also, PC3&4 gave the best outcome, which can be explained by the fact that minor, but

more unique compounds are likely responsible for discrimination, as compared to major, but more commonplace components.

3.6 Details about teaching design

Table 4 highlights different versions of the teaching laboratory that allows instructors to add, remove, or modify the contents in a highly customizable manner. This makes this experiment highly versatile and can fit different needs or abrupt changes like school closure due to pandemic. Also, it offers hands-on experience on pineapple, which helps connect students to real-world usage of chemistry.

Table 4 Different versions of the experiment

Day	**Activity**
Standard version (This plan is suitable for courses with limited time and/or having limited resources especially rotary evaporators. The instruction for students is based on this version.)	
1	Sample preparation including pineapple extraction, liquid-liquid extraction, and saponification.
2	Stopping saponification reaction
3	Column chromatography (using 100% Hex only as a mobile phase), UV, and HPLC analysis Note: UV, HPLC, and MS analysis are performed by the instructor's team.
Advanced version (This plan may be applied to students with more experience and skills, without significantly increasing the overall required time.)	
1	Exactly the same as the standard version.
2	Exactly the same as the standard version.
3	Column chromatography, UV, and HPLC analysis Note: UV, HPLC, and MS analysis are performed by the instructor's team.

Table 5 Different versions of the experiment (continue)

The fully integrated version (This plan is enhanced even further with hands-on experience on instruments and chemometric analysis.)	
1	Exactly the same as the standard version.
2	Exactly the same as the standard version.
3	Column chromatography
4	UV and HPLC analysis by student Note: MS analysis is not allowed to hands-on operation.
5-6	Chemometric analysis

3.7 Extension of chemometric study

To increase the chance of successful classification of more pineapple samples, HPLC method was further modified for more detailed separation of carotenoid components. Three GIs pineapple were employed as the representative of the big data with 45 data points per GI product. The newly developed HPLC program, a YMC C30 carotenoid S-3 µm (150 x 4.6 mm) column and the methanol (MeOH)-water-methyl diethyl ether (MTBE) system were used as the stationary phase and the mobile phase, respectively. The chromatogram shows better separation of carotenoids and now about 14 peaks were detected (**Figure 15**).

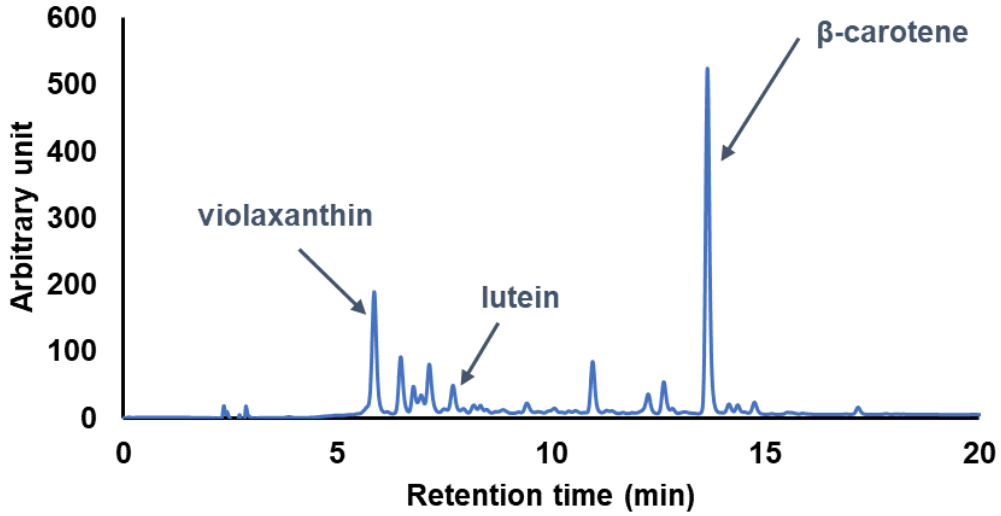


Figure 15 A represent of HPLC chromatogram of pineapple separation by using YMC C30 column.

After the HPLC program was developed, PCA was re-optimized to suit the new set of chromatographic data (**Figure 15**). The two well-known cultivars of Thai pineapples that are Queen and Smooth Cayenne were used in this study. PC2 and PC4 were chosen for the PCA plot (**Figure 16**) according to correctly classified (CC) values (**Figure 17**). Linear discrimination analysis (LDA) was used to help explain the overlapping data points in the PCA plot caused by a large number of samples. LDA indicates that Queen and Smooth Cayenne reached approximately 80% of correct classification by coupling 4 PCs together (**Figure 18**).

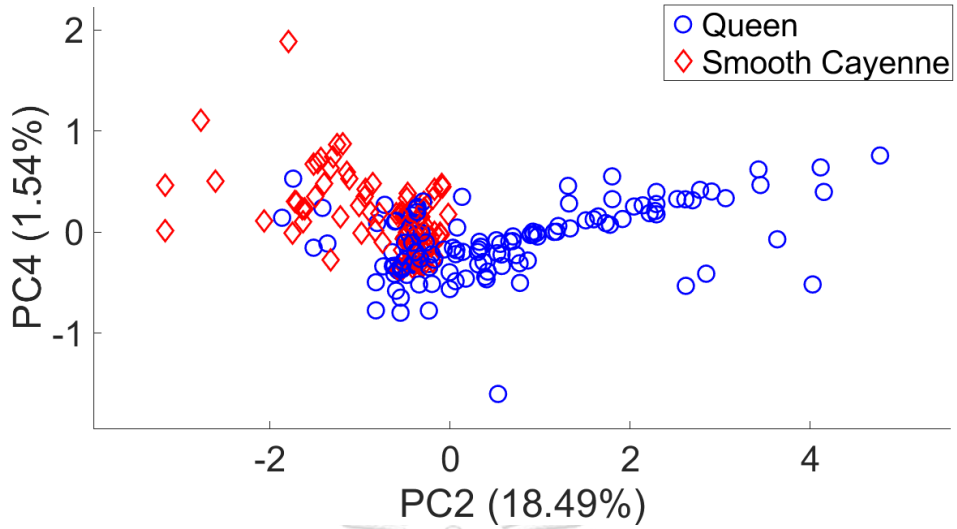


Figure 16 PCA of pineapple cultivars classification.

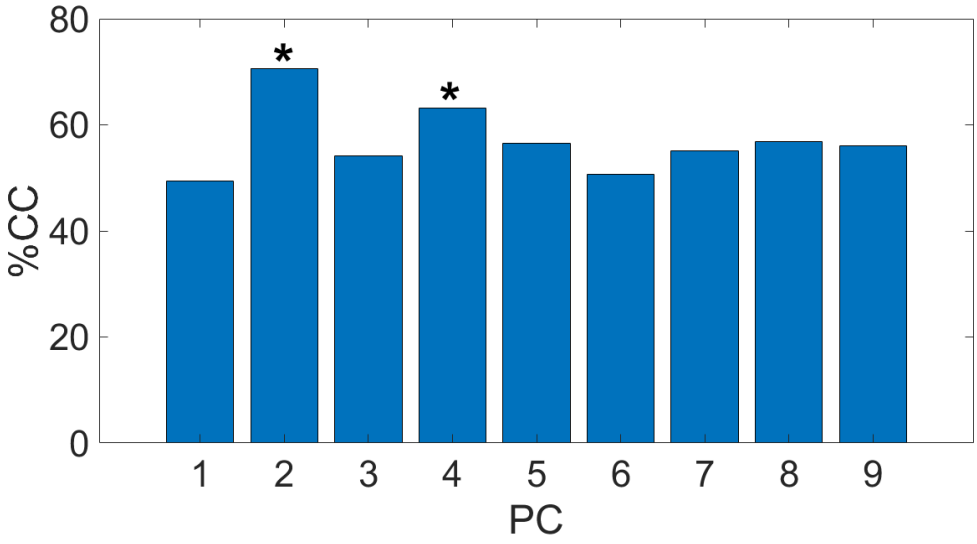


Figure 17 PC Discrimination of Queen and Smooth Cayenne.

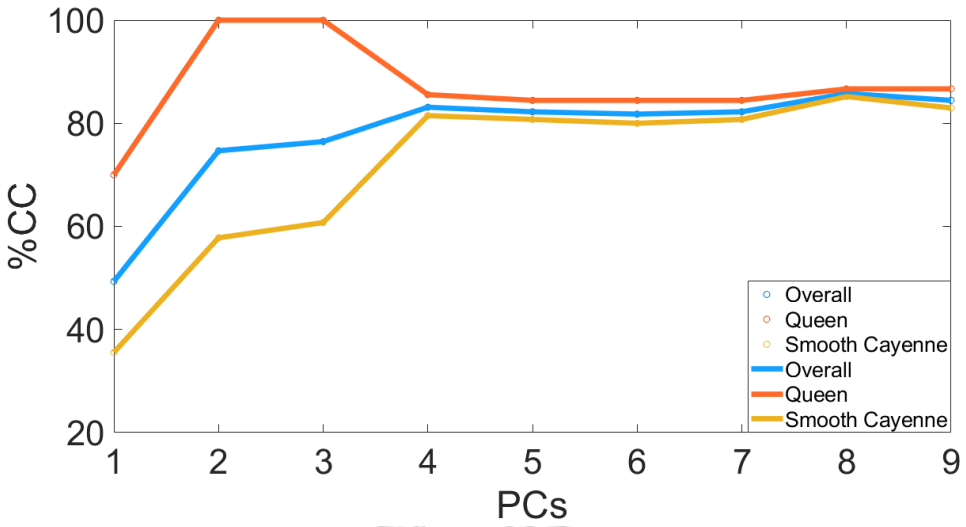


Figure 18 Linear discrimination of Queen and Smooth Cayenne.

To get more insight in the methodology, PCA was also performed on pineapple samples based on their geographical locations (Trat Si Thong, Phuket (both as Queen), and Ban Kha (Smooth Cayenne)). **Figure 19** shows the better PCA projection on PC1-3 of the representative GI Thai pineapples. As a result, the PCA plot corresponded with LDA plot (**Figure 20**), where it is clear that combining 2 - 3 PCs can already achieve about 80% correct classification of these three GIs Thai pineapple. This confirms that the improved HPLC protocol may be amenable to the analysis of larger groups of samples.

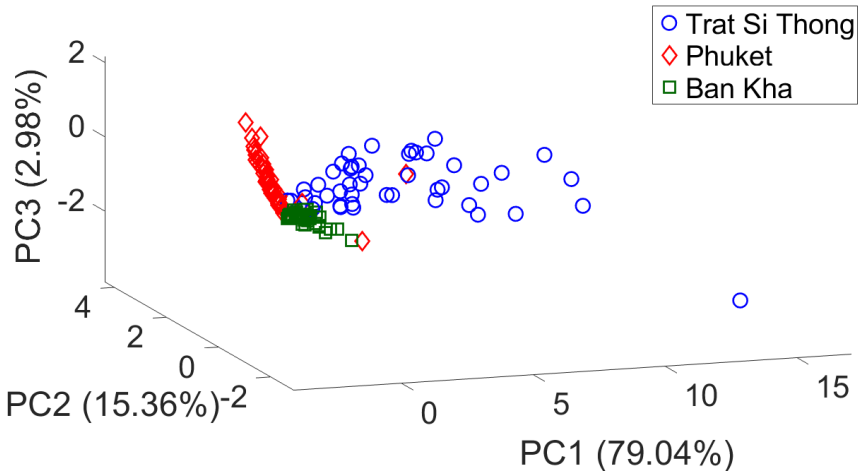


Figure 19 PCA plot of three GIs pineapple.

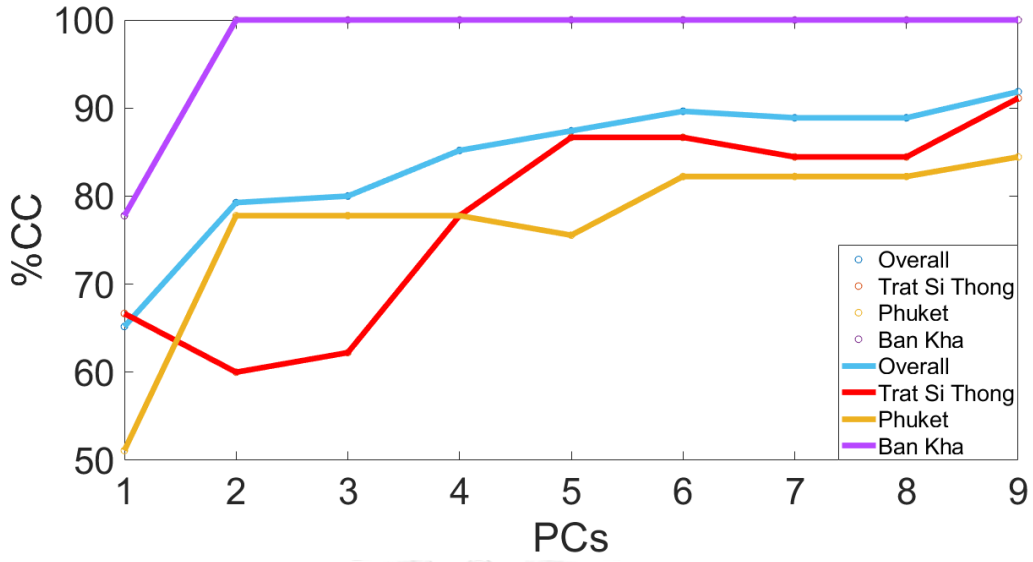


Figure 20 Linear discrimination of three GIs pineapple.

CHAPTER IV
CONCLUSION

To conclude, this study demonstrated that the carotenoids fingerprints, one of the dominant elements in pineapples, can be combined with chemometric analysis to distinguish their geographical origins. Notably, the relatively short analysis time (35 min) in separating carotenoids with C18 column was enough for data processing. Even though the developed method cannot completely separate all compounds, but it is sufficient to classify the pineapple varieties, *i.e.,* Sriracha, Pattavia, and Suanpueng.

Herein, the method for geographical indication was also adapted to a teaching laboratory, which centered around the concept of chromatography for pineapples. The combination of multiple techniques, such as liquid–liquid extraction, column chromatography, and HPLC, along with possible expansion to chemometrics or MS allows students to fully appreciate how multiple branches of chemistry are really necessary for solving modern real-world challenges. Importantly, while these sub-experiments appear to be related to each other, the laboratory was designed in such a way that they can be easily combined, modified, or removed while maintaining the core goal of the experiment. This not only resolves the need for abrupt adjustment in the pandemic era but it is also useful in broader terms where core experiments can be constantly modified to suit laboratories with different resource availability and different levels of students.

Moreover, the last developed method to get the intensive carotenoids profile, which performed on Trat Si Thong, Phuket, and Ban Kha, confirms that the improved HPLC protocol may be amenable to the analysis of larger groups of samples.

APPENDIX

This study was published as a laboratory experiment article in the title of "Characterization of Carotenoids from Pineapples: An Integrated and Modular Experiment for Practical Learning of UV–vis Spectroscopy, Chromatography, Mass Spectrometry, and Chemometrics" in Journal of Chemical Education since April 5, 2022. The published article is shown below.

JOURNAL OF CHEMICAL EDUCATION

pubs.acs.org/jchemeduc Laboratory Experiment

Characterization of Carotenoids from Pineapples: An Integrated and Modular Experiment for Practical Learning of UV−vis Spectroscopy, Chromatography, Mass Spectrometry, and Chemometrics

Worakan Chutakool and Thanit Praneenararat*

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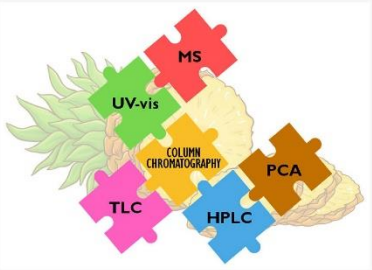
ABSTRACT: In the past few decades, chemistry has evolved to interact with various disciplines to synergistically help tackle global challenges. This, in turn, requires that newer generations of chemistry students are trained to be more flexible in accepting and coordinating new concepts. In this experiment, pineapple was used as a key model to allow for the incorporation of multiple important concepts into the laboratory. Using carotenoid separation as a main goal, column chromatography, UV−vis spectroscopy, thin-layer chromatography, high-performance liquid chromatography, mass spectrometry, and principal component analysis can be included in a cohesive laboratory experiment. On the other hand, these activities were also designed to be modular, thus allowing instructors to add, remove, or modify the contents in a highly customizable manner. This makes it highly versatile and amenable to uncertain situations like unexpected university closure due to COVID-19 related lockdown. Overall, this laboratory experiment serves as a practical example of how chemistry can help solve real-world problems while also allowing high flexibility in teaching management.



KEYWORDS: *Upper-Division Undergraduate, Interdisciplinary/Multidisciplinary, Hands-On Learning/Manipulatives, Chemometrics, Chromatography, Food Science, HPLC, Mass Spectrometry, Thin Layer Chromatography, UV−vis Spectroscopy*

Interdisciplinary approaches in chemical research have become a mainstay in solving global challenges. This in turn has influenced chemistry teaching where more emphasis into real-world problems is encouraged. Topics like food chemistry,[1] natural products,[2] and plant sciences[3] are among the most popular topics to be utilized by instructors due to their close connections to everyday life.[3−7] This is especially true in organic chemistry where studies related to organic matters are the core of the whole discipline. One specific example is the utilization of vegetables,[8,9] or plant leaves[10] as a starting mixture for the learning of the concept of chromatography, which is one of the most important practical concepts in chemistry curricula.[11−13] These typically involve column chromatography and thin-layer chromatography (TLC). Some emphasized on the improvement of existing methods in terms of simplicity,[10] while others promoted greener methods of separation.[9,10]

In this report, a modular design of an experiment related to chromatographic separation of pineapples is highlighted. This design was driven by the uncertainty in course planning due to the recent COVID-19 pandemic. Consequently, nationwide lockdown was implemented, thus disrupting teaching activities in schools. With the learning outcome being the abilities to understand various types of chromatographic separation and to evaluate the separation efficiency, this modular lab design (Scheme 1) allows instructors to adjust the length of the experiment to match with individuals' situations. Apart from the core chromatography experiment, expansion can involve plant extraction, liquid−liquid extraction with a revisit on the concept of saponification, UV−vis analysis, high-performance liquid chromatography (HPLC) experiments, mass spectrometry (MS) experiments, and even an introduction to more advanced topics like chemometrics or principal component analysis (PCA).

Overall, this experimental design seeks to maintain students' interest by using a real-world subject, that is, pineapples, while still allowing for flexible planning in various circumstances, for example, the adaptation to occasional closure of schools and universities. Some parts of this design have been used with 24 students in an advanced undergraduate course called integrated chemistry laboratory. Indeed, not all lab modules were conducted because the spike of COVID-19 cases forced

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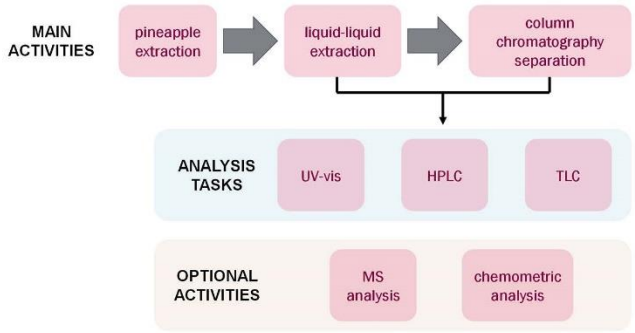
Scheme 1. Modular Design of Pineapple Experiment Centering on Chromatographic Separation[a]



[a]When circumstances permit, longer and more comprehensive experiments can be added up.

the university to prematurely close its operation for the semester. Despite this abrupt change, the learning process and enthusiasm of students were not heavily impacted, thanks to the modular nature of the experiment that allowed us to quickly remove some modules without confusion.

■ MATERIALS AND METHODS

General Information

This experiment was designed to be highly modular by allowing modules of activities to be easily included or removed. In the standard version (mainly discussed in this report), the experiment is meant to be a single-person experiment with simpler setup for column chromatography separation. The students were required to analyze data from UV and HPLC experiments that were conducted by a teaching assistant. Additional activities including (1) more complex column chromatography (the advanced version and the fully integrated version in the Notes for Instructors) and TLC; (2) PCA (the fully integrated version); and (3) MS (the fully integrated version) can be incorporated for more advanced students with extended lab time. Details and suggested scheduling of these additional activities are included in the Notes for Instructors.

Sample Preparation

Pineapples were purchased from local markets. If chemometric analysis (see Chemometric Analysis subsection) is to be included, there should be more than one type of samples with differing attributes such as cultivars, maturity, or locations of growing. Sample preparation includes (1) blending to break pineapple solid matters into smaller pieces, (2) simple squeezing using cloth filtration with acetone as a solvent, (3) liquid−liquid extraction to obtain carotenoids that are soluble in 10% diethyl ether in *n*-hexane, and (4) overnight saponification with 10% KOH in MeOH. The final step before column chromatography was solvent evaporation by a rotary evaporator. Once the crude carotenoid is concentrated, care should be taken to avoid heat and light. More details on the protocol can be found in the Supporting Information.

Column Chromatography and TLC

Column chromatography was performed to collect β-carotene in pineapples. *n*-Hexane was used as a mobile phase, which rapidly eluted the nonpolar β-carotene out within 15 min. This was determined by simply observing the first yellow band to come down by naked eyes. It is worth noting that isolation of more than one carotenoid is possible, and a suggested protocol is discussed in the Discussion section, along with the Notes for Instructors.

TLC analysis was done with 50% ethyl acetate/*n*-hexane as the mobile phase. To maximize available data for analysis, ambient light, UV light (254 and 365 nm), and $KMnO_4$ staining were used with digital images recorded for each condition, totaling four images.

UV−vis Spectroscopy and HPLC Analysis

Samples for subsequent analyses in UV−vis, HPLC, and MS experiments were prepared by dissolving evaporated crudes or purified compounds with acetone. HPLC and MS experiments require syringe filtration prior to injections to the instruments. Details can be found in the Instruction for Students.

UV−vis spectra were recorded from a NanoDrop 2000 spectrophotometer (350−600 nm). This is required due to the small amount of sample obtained after the workup process of saponification step. Alternatively, a conventional UV−vis spectrophotometer can be used to analyze on the solution from the first extraction step with acetone, or simple naked-eye observation may suffice for obvious cases (clearly different colors).

HPLC analysis was performed on a Thermo Scientific UltiMate 3000 UHPLC with a diode array detector (DAD). The column used was ZORBAX Eclipses Plus C18 column (4.6 × 50 mm, 3.5 μm). The time program is outlined in the Notes for Instructors. Also, some calculation examples using quantitative data obtained from HPLC are demonstrated in the Notes for Instructors.

Chemometric Analysis

Chemometric analysis in the form of PCA, as a model for expanded versions of experiment, was performed by processing and dimensionally reducing data from normalized peak areas of HPLC experiments. All calculation was done with the MATLAB software. Full detail regarding with data preprocessing can be found in the Notes for Instructors.

■ HAZARDS

Safety goggles, gloves, and closed-toe shoes must be worn all the time. Various organic solvents were used in many steps, so care should be taken when handling these flammable liquids. Also, it would be of interest to teach students regarding with

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proper waste disposal. Specific hazard information can be found in the Supporting Information.

RESULTS AND DISCUSSION

Preparation of Pineapple Extract

The experiment commenced with an extraction of carotenoids from pineapples. In this case, standard protocol[14] was applied where water and acetone were used for extraction. While the process itself is a simple mechanical squeezing, it offers a point of discussion regarding with extraction efficiency. For example, students can be asked about what would happen if other solvents were used instead of water and acetone in this experiment. Polarity likely affects the obtained carotenoid profile, which can be clearly seen in subsequent HPLC experiments. Also, physical appearance of the liquid extracts of different cultivars of pineapples showed some difference (Figure 1). This is the first element for a possible introduction of chemometric concept to students (see below).

Figure 1. Photographs of the liquid extracts of three cultivars of pineapples; (Left) Sriracha; (Middle) Suanpueng; (Right) Pattavia.

The last step in the preparation phase deals with a saponification reaction (Scheme 2). This was required to reduce the complexity of chromatograms obtained from subsequent HPLC analysis by removing carotenoid esters, chlorophylls, and lipids.[15] Also, the inclusion of this reaction allows students to relate a common reaction covered in introductory chemistry[16] to real-world examples that involve more complex chemical structures.

Column Chromatography and TLC Analysis

As alluded to above, the use of plant-based samples as a starting mixture for column separation has been well adopted by chemistry teachers in introducing the concept of column chromatography.[8−10] Here, pineapples, a very common type of fruits, were analyzed in detail. While the comparison of totally different compound classes, for example, anthocyanins versus carotenoids, is typically used in demonstrating the concept of chromatography, subclasses of carotenoids including carotenes and xanthophylls can also be used. Although their structures appear to be quite similar (Figure 2), these subtle differences, that is, the presence of hydroxy, carbonyl, or epoxide groups, were sufficient to cause differing rates of compound migrations, as evident in both TLC and HPLC analyses (see below). This can be considered a more advanced level in polarity analysis that can be utilized with higher-level undergraduate students.

Notably, column chromatography and TLC experiments can also be designed to be modular. The standard version (used with students in this course) aims to separate only β-carotene, which is the least polar compound of significant abundance. Hence, this is eluted first in column chromatography and thus requires a relatively short amount of time (<15 min of elution). On the other hand, the advanced version (detail in the Notes for Instructors) aims to separate colored compounds into three groups, which can be well separated from the change in the mobile phase composition. In this version, deeper discussion and evaluation on skills can be expanded at the expense of longer required time.

To allow for connected discussion with HPLC, extensive data collection on TLC was performed (Figure 3). This was designed so that students performing column separation that aims for isolation of only β-carotene can still observe multiple compounds for discussion. In this experiment, four sets of data for TLC were acquired. Apart from colors from ambient light, UV light at different wavelengths was used to demonstrate how various compounds can appear under different conditions. The concept of chromophore can be introduced when explaining the dark spot in 254 nm light, while 365 nm is suitable for showing fluorescent compounds present in the sample. Also, a well-known TLC staining agent, $KMnO_4$, was used to educate students on how reducing properties of carotenoids can be used as a means to detect them in TLC analysis. These expanded data sets can be beneficial for deeper analysis on structure−property relationships. Specifically, a mobile phase composition (50% ethyl acetate/*n*-hexane) that was able to reveal multiple compounds of higher polarity was employed (Figure 3). This gave a clear spot of β-carotene at R_f = 0.93 (spot I). Besides, this condition can uncover an interesting blue fluorescent spot at R_f = 0.79 (spot II), which should well attract students' attention. If the HPLC experiment is not quite amenable, instructors may opt to rather have deep discussion about structure−property relationship at this stage by providing some more standards of different polarity to be spotted together in TLC analysis. This will then allow the comparison of structural features of carotenoids in relation to their observed polarities, i.e., R_f values. In the advanced version (see the Notes for Instructors), three fractions were to be obtained, and students could be extensively evaluated on their skills of column chromatography. Improper setup of column chromatography, for example, poor crude loading, can cause band broadening and affect the separation, which should be clearly reflected in the TLC experiment. Similar to the standard version, each fraction can be subjected to individual HPLC (similar to Figure 5B below), hence permitting the creation of even more advanced experiments. This should facilitate students in achieving the learning outcome of understanding how to evaluate the performance of column separation even further.

UV−vis Spectroscopy and HPLC Analysis

UV−vis spectroscopy is an important tool in encouraging students to think about electromagnetic spectrum in relation to the color of a sample. In this case, yellow−orange color of carotenoids results in an absorption maximum around 450 nm

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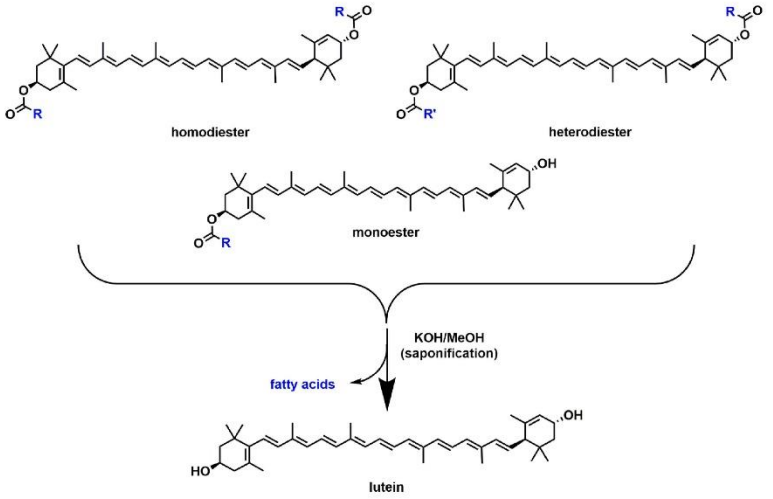
Scheme 2. A Representative Set of Saponification Reactions of Carotenoid Substrates Commonly Found in Pineapple



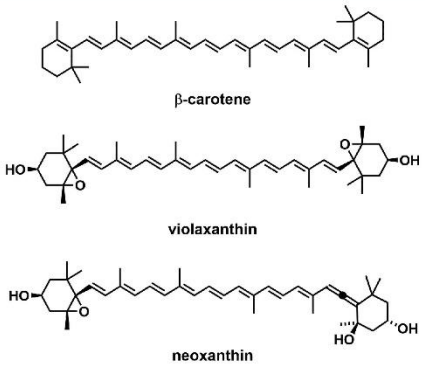


Figure 2. Examples of carotenes and xanthophylls.

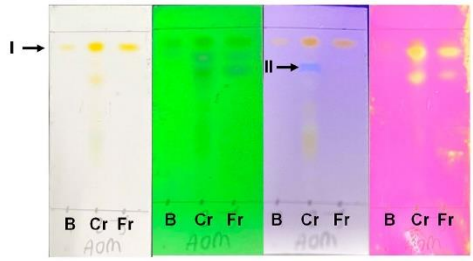


Figure 3. A set of student's TLC data (mobile phase = 50% ethyl acetate/*n*-hexane) under four conditions including (left to right) white light, 254 nm light, 365 nm light, and $KMnO_4$ staining; B = *β*-carotene standard; Cr = crude mixture; Fr = isolated fraction.

(Figure 4). This property should be clearly seen and slight variance of colors (due to the use of different samples as suggested above) may be sufficient to exert different

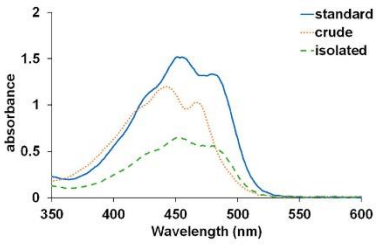


Figure 4. UV−vis spectra obtained from a Nanodrop 2000 spectrophotometer of a standard *β*-carotene solution (2 mg/mL), crude extract prior to column chromatography, and an isolated fraction containing *β*-carotene.

absorption maxima, leading to the possibility for more discussion.

HPLC with UV detector or DAD is a standard tool in modern analytical science for separation and analysis of samples with some UV−vis absorption. Perhaps what is most interesting, in terms of connection to the introductory level of chromatography teaching, is the dominance of reversed-phase HPLC in frontier research. With hydrocarbon moiety capped on the surface, this type of column leads to an opposite elution order where more polar compounds are eluted out first, along with the reversed definition of solvent strength (less polar = stronger elution power). Importantly, having both normal-phase column chromatography and reversed-phase HPLC in the same experiment is a great opportunity whereby the concept of chromatography can be discussed in-depth. Specifically, Figure 5A shows an HPLC chromatogram for a crude pineapple extract where multiple peaks from carotenoids are shown. These major peaks allow for some thoughtful discussion regarding with the relationship of the polarities of compounds and their elution orders. For instance, *β*-carotene appeared at about 20.8 min, which is a relatively long retention time. This corroborates well with its very low polarity, hence

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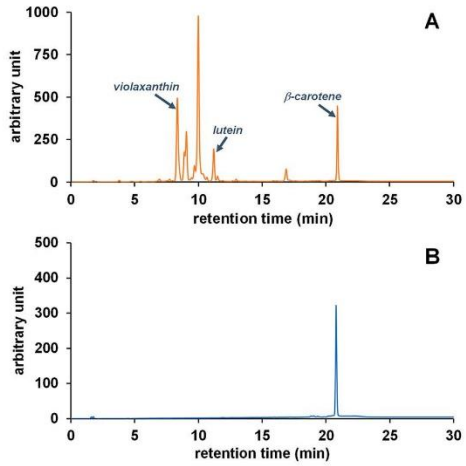

Figure 5. A representative set of HPLC chromatograms (A, crude extract; B, isolated material after column chromatography) from a student's sample.

being retained the most with hydrophobic stationary phase in reversed-phase HPLC. On the contrary, there is a group of carotenoids whose retention times are significantly shorter than that of β-carotene (Figure 5A). Comparison with some standards and previous literature[17] suggests the presence of violaxanthin and lutein. These compounds (structures shown in Scheme 2 and Figure 2) are expected to be more polar than β-carotene based on their functionalities. Hence, these data permit the discussion and comparison about the relationship of polarity and elution in both normal (column chromatography and TLC) and reversed-phase chromatography (HPLC), thereby enhancing the learning experience about chromatography. Also, Figure 5B, which shows a chromatogram after column separation, is a good indicator of student's performance in conducting column chromatography. This particular example shows good purification efficiency, whereby only β-carotene peak is visible. Not only does this open up discussion about the separation efficiency (by comparing the results of Figure 5A vs B) but also it can allow comparison with other techniques, for example, comparison with TLC result (Figure 3). All in all, this can be viewed as an opportunity for students to gain skills in chromatography.

Discussion

Students' reports allow for the evaluation of the fulfillment of the main pedagogical goal, which is to develop a deep understanding on the concept of chromatographic separations in various instrumental setups (column chromatography, TLC, and HPLC). The evaluation of reports provided some interesting points to be shared. First, almost all students had no problem in calculation if the whole methodology was provided. In our case, the definition of retinol equivalence[18] (see Instruction for Students) as a real-world use cases in nutritional science was included. Thus, this section of calculation, which is straight conversion of micrograms of material to retinol equivalence (the last equation in the example of calculation method for retinol equivalence in the Notes for Instructors), was not found to be an issue for students. On the contrary, the derivation of the original amount of β-carotene from HPLC analysis (the first part of the calculation method) was done correctly only by about 25% of students. While this number seems to be low, it may not be much surprising due to various factors. For instance, in our case, no review on this part of stoichiometric calculation was provided in the briefing period. Since the calculation requires clear understanding of how many steps of dilution and aliquot taking were involved, it is possible that average students may not fully comprehend the necessary steps required to perform a correct calculation. Also, there were about 40% of students who were able to provide sufficiently deep discussion about the relationship between various chromatography techniques, the obtained results, and the structural features of relevant carotenoids. This moderate percentage is not surprising given the guided-inquiry nature of the experiment where not all information was provided.

Also, another worth mentioning insight from this experiment is in the variation of β-carotene amounts calculated from HPLC data. In this analysis, we compared the amounts of the β-carotene peak in HPLC data from students' crude mixtures. As shown in the Notes for Instructors (section "summary of HPLC quantification results"), generally large variation (% relative standard deviation of 19−46%) was observed, which is not unexpected due to the differences in students' skill levels. Most importantly, the mistake of not fully evaporating the solvent was a main contributing factor for vastly different calculated amounts. Nevertheless, by comparing the β-carotene amount between different cultivars, Pattavia (9.9 mg/100 g fresh pineapple) was found to show significantly higher β-carotene amount than did Sriracha (3.5 mg/100 g fresh pineapple) and Suanpueng (2.7 mg/100 g fresh pineapple). While the comparison of only one peak is not sufficient for distinguishing geographical origins, this can spark interest and discussion in many aspects, for example, being a prelude to the concept of chemometrics (see below).

Last but not least, some more techniques/activities can be included to further improve students' learning on the connection of various chemical tools. For example, nuclear magnetic resonance (NMR), an essential tool in organic chemistry research, may be used to analyze samples in this experiment. Given some symmetry in the related carotenoids, NMR data obtained from purified samples may be simple enough to be used as practice problems for NMR interpretation. In this study, we also demonstrated two potential experiments/activities (details in the Notes for Instructors) that can enhance the learning experience. First, MS, one of the most widely used analytical tools in a variety of modern applications,[19,20] was used to analyze the isolated fraction (β-carotene) of an idealized (instructor-purified) sample from column chromatography (Figure 6). From pedagogical aspect, the concept of MS is interesting in that it contains great detail to be studied in both theory and instrumentation. Therefore, having a proper learning tool for MS can be very beneficial for achieving firm understanding on the concept, thereby benefiting the students in the long term. Here, various practical topics can be discussed such as the concept of tandem MS (typically covered in analytical chemistry), and the elucidation of fragmentation pattern to cause a loss of m/z 92 in Scheme 3 (typically covered in organic chemistry).[21]

Second, this real-life topic also offers another worth-mentioning topic in chemistry, namely chemometrics,[22]

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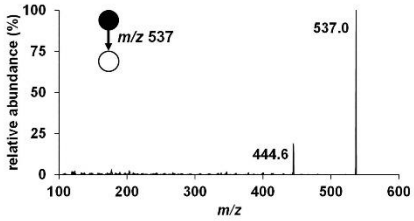
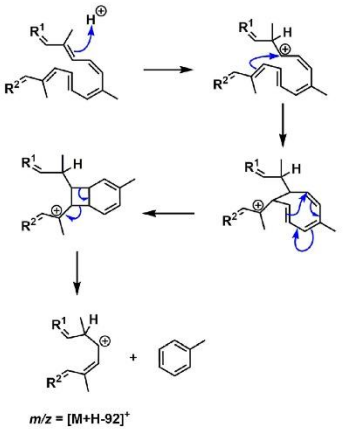


Figure 6. Idealized MS/MS spectrum of a column-purified sample of β-carotene using a triple quadrupole MS.

Scheme 3. Fragmentation Pathway of β-Carotene to Lose Toluene (m/z = 92)



which can be incorporated as a hands-on activity (see the Notes for Instructors for suggested schedules and experimental detail). This discipline utilizes statistical science to uncover meaningful interpretation of multidimensional data, which can then be further exploited in a variety of ways. For example, data in the forms of signal intensity and retention time obtained from HPLC analyses of various cultivars of pineapples were processed, dimensionally reduced, and presented in a form of PCA (Figure 7). Interestingly, while

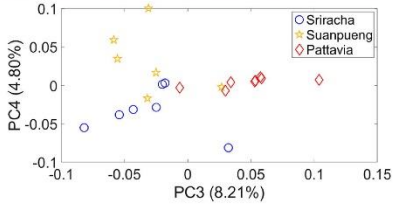


Figure 7. PCA of three cultivars of pineapples created from students' HPLC data. It should be noted that the students were neither aware of the difference of obtained samples nor were they introduced to chemometrics in this particular course. Also, PC3 and 4 gave the best outcome, which can be explained by the fact that minor but more unique compounds are likely responsible for discrimination, as compared to major but more commonplace components.

there was significant variability in students' data due to some pitfall discussed below, PCA, along with data preprocessing (details in the Notes for Instructors), was sufficiently robust to still provide decent discrimination among various pineapple cultivars. Overall, incorporating chemometrics not only demonstrates the power and the robustness of the discipline but also serves to connect to a topic of wide impact, that is, geographical indication, which is a form of intellectual properties of increasing importance.[23,24] Thus, this approach can greatly help demonstrate the impact and the practicality of chemical sciences to students.

Nevertheless, in pursuing quantitative treatment of data, care must be taken to ensure that students perform all transferring steps properly. In our case, observation on the obtained weights in various steps and also the peak areas from HPLC runs showed noticeable variability. Investigation later revealed that the key step was rotary evaporation. Some students who were not familiar with the concept did not wait long enough for complete evaporation, thus leaving substantial amount of solvent in the sample and significantly distorting the obtained weight. Some other execution mistakes added up to the final variability found in the students' data. While this was proved to only partly distort PCA analysis (partly due to our preprocessing step in PCA), instructors should make a decision about whether specific warning regarding to sample handling should be emphasized. On one hand, it would help reduce the variability and thus any confusion in subsequent introduction to chemometrics. On the other hand, this possible pitfall may be a good discrimination tool in terms of technical proficiency of students.

■ CONCLUSION

Herein, a modular laboratory experiment centered around the concept of chromatography in real food samples, that is, pineapples, is demonstrated. The combination of multiple techniques, such as liquid−liquid extraction, column chromatography, and HPLC, along with possible expansion to chemometrics or MS allows students to fully appreciate how multiple branches of chemistry are really necessary for solving modern real-world challenges. It should be noted that the use of pineapples serves merely as a guideline example, and instructors can adopt any fruit/vegetable that is widely available in their regions. Importantly, while these subexperiments appear to be related to each other, the laboratory was designed in such a way that they can be easily combined, modified, or removed while maintaining the core goal of the experiment. This not only resolves the need for abrupt adjustment in the pandemic era but it is also useful in broader terms where core experiments can be constantly modified to suit laboratories with different resource availability and different levels of students.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available at https://pubs.acs.org/doi/10.1021/acs.jchemed.1c01021.

Notes for Instructors: technical details about experiments, hazards of chemicals, safety, tips and notes regarding the laboratory, suggested schedules, calibration plot of β-carotene standard, calculation example for retinol equivalence, summary of HPLC quantification results, details on additional activities (PDF)

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Instruction for students: example of instruction manual for students, exemplar lab report (PDF, DOCX)

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Notes

The authors declare no competing financial interest.

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