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Laboratory Experiment

Extraction and Purification of Coumarins in *Ipomoea cairica* by TLC and HPLC: An Integrative Experiment for Student Learning Chromatography

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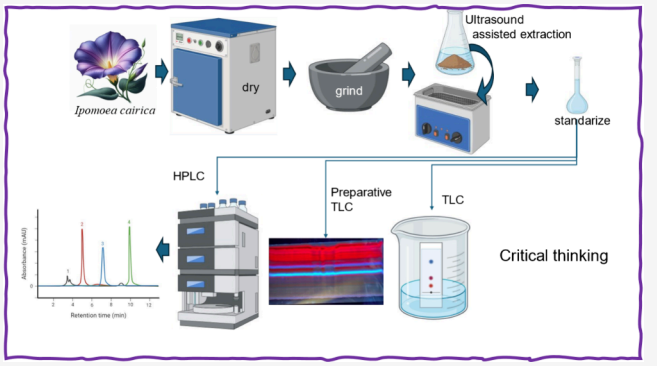
ABSTRACT: Chromatographic techniques played a major role in several separation processes. In this work, an integrative laboratory practice is proposed in which students are expected to isolate scopoletin and umbelliferone from raw *Ipomoea cairica*. Thin layer chromatography (TLC), preparative chromatography (PC), and high-performance liquid chromatography (HPLC) techniques are applied to isolate phytochemical bioactive compounds. Scopoletin and umbelliferone are coumarins that have been reported to possess anti-inflammatory and antioxidant properties. These metabolites are found in high concentrations in *Ipomoea cairica* stems, and their methanolic extracts have shown larvicidal properties against *Aedes aegypti*. The experimental procedure includes fundamental extraction techniques such as liquid extraction and ultrasonic extraction. Additionally, it incorporates more advanced methods, such as chromatographic separation on a thin-layer plate, analytical detection of coumarins using HPLC with a diode array detector, and the isolation of coumarins through preparative plate chromatography. This laboratory experiment allows students to integrate and compare, in two laboratory sessions, the basic principles of TLC and preparative chromatography with more advanced techniques such as HPLC.



KEYWORDS: *Chromatography, HPLC, TLC, Phytochemistry, Undergraduate education, Active Learning, Coumarins*

INTRODUCTION

Chromatography is a fundamental separation technique essential to any organic or analytical chemistry curriculum. The foundational principles of chromatography are usually introduced through thin-layer chromatography (TLC) and preparative-layer chromatography. These methods have been shown to be highly versatile and cost-effective for both analytical and preparative tasks, making them suitable for monitoring chemical reactions, purifying samples, and identifying compounds. Conversely, high-performance liquid chromatography (HPLC) offers a more sensitive and efficient approach to separation; although it is significantly a more expensive and time-consuming chromatographic technique, it relates to the core principles of TLC.[1−5] Numerous experiments involving both TLC and HPLC can be effectively incorporated into organic or analytical chemistry teaching laboratories, thus providing students with hands-on chromatographic skills as they are applied in industrial and research settings.[6,7]

Traditional chromatography laboratory sessions typically focus on mastering a single chromatographic technique, such as extracting and separating pigments from *Bixa orellana* or determining caffeine levels in soft drinks.[8,9] While these techniques are valuable for building foundational skills, integrative practices are thought to offer a deeper learning experience as well as a more efficient use of laboratory time, since they allow students to combine multiple techniques and apply them to comprehensive projects. When integrative experiments help students grasp fundamental concepts in fewer sessions, the extra time can be used to involve them in small research projects. These projects enable students to propose their own topics, conduct advanced literature reviews, and apply the knowledge gained earlier in the course to investigate real-world problems. This enhances their understanding and prepares them for future research and professional applications.

Ipomoea cairica (a species of morning glory) is a rather common ornamental plant in tropical regions; due to its rapid

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growth and spread, it is often employed to naturally cover fences and gates in gardens. The plant has been reported to contain various secondary bioactive metabolites with anticancer, antimicrobial, cytotoxic, and larvicidal properties.[10−17] This plant can be easily collected and used as a source of phytocompounds for a chromatographic laboratory experiment, offering students a practical lesson in natural products chemistry. The bioactivity of this plant is particularly relevant in tropical countries due to its potent larvicidal activity against *Aedes aegypti*, a mosquito species that serves as a vector for Dengue and Zika viruses.[18] Larvicidal activity of *I. cairica* ethanolic extracts is higher in stem (71.3%) compared to leaf (40.0%).[19] Simple TLC chromatographs typically reveal a higher concentration of phytochemicals in the stems compared with the leaves, allowing students to connect the reported bioactivity with their experimental findings. Among the major nonvolatile compounds reported in the ethanolic extract, the coumarins 7-hydroxy-6-methoxychromen-2-one (scopoletin, Figure 1(a)) and 7-hydroxychromen-2-one (umbelliferone, Figure 1(b)) are not directly responsible for the larvicidal activity but they play a synergistic role with other components of the ethanolic extract.[19] Those compounds were previously studied, but they have never been previously utilized for teaching purposes.

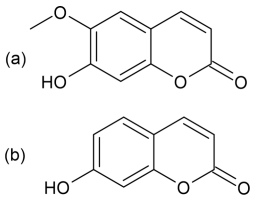

Figure 1. Chemical structures of main coumarins isolated from *Ipomoea cairica*: (a) scopoletin; (b) umbelliferone.

Purified scopoletin and umbelliferone can be easily isolated from *Ipomoea cairica* using straightforward laboratory methods such as liquid−liquid extraction and ultrasonication. Their separation and identification are efficiently accomplished through thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). These compounds can also be incorporated into other experiments, serving, for example, as standards in Total Phenolic Content (TPC)[19−22] determination or as reference materials in spectroscopy laboratory exercises. Some of the key concepts of each technique are summarized in Table 1.

Table 1. Key Concepts of Each Chromatographic Technique

General concepts	HPLC	TLC
Mobile phase	Elution time	Rf
Stationary phase	Column	Chromatographic plates
Normal and reversed phase	Elution order	Elution order
Standards	Quantification	Identification

Simplicity, wide availability, high yield, and complex structure make these coumarins compelling molecules for designing a laboratory that may include analytical and preparative TLC, and HPLC into an undergraduate course that trains students in the following learning goals: (i) to integrate several concepts regarding natural products and bioactivities; (ii) to help them to connect a basic chromatographic method like TLC and an advanced technique such as HPLC; (iii) to develop a wide range of laboratory skills, including sample preparation, liquid−liquid extraction, ultrasound-assisted extraction (UAE), TLC, HPLC, data interpretation, and decision-making; (iv) to allows them to extract, isolate, and analyze compounds out of complex mixtures collected from natural sources. This procedure aims to provide a straightforward laboratory experience for undergraduates, helping them connect both chromatographic techniques in separating metabolites from a simple plant matrix, which are relevant for other practical applications.

IMPLEMENTATION IN THE LABORATORY COURSE

This experiment was carried out typically in groups of two students within a class of 11 to 14 students. Initially, it was used as a postpandemic leveling class during the summer of 2021 and 2022 for students who had taken virtual organic chemistry laboratories during the lockdown. Since then, the experiment has been incorporated into the regular sophomore-level curriculum (2023−2024). Students were provided with the instructions (see Supporting Information) at the start of the semester.

EXPERIMENTAL PROCEDURE

Laboratory Activity Outline

This laboratory practice requires two 4 h experimental sessions, including theoretical explanations. In the first session, students prepare a methanolic extract from *Ipomoea cairica* stems through drying, grinding, and liquid extraction. Ultrasound-assisted extraction (UAE) is recommended to ensure a suitable number of coumarins. The extract is then analyzed by TLC on a fluorescence plate and revealed under ultraviolet light.

In the second laboratory session, the isolation of both coumarins is carried out by preparative TLC, analyzing the results of both work sessions with an HPLC system equipped with a diode array detector (DAD). Students then identify the scopoletin and umbelliferone peaks at 280 and 350 nm (maximum absorbance for coumarins), expecting these to be the major components present in the methanolic extract collected from the first laboratory session. Additionally, they compare the retention times of these major analytes with the isolated compounds from the preparative TLC. Students correlate the chromatographic results of the HPLC, analytical, and preparative TLC extracts, discussing the similarities and differences observed.

Liquid Extraction and UAE Procedure

Approximately 5 g of *Ipomoea cairica* stems is predried at 40 °C in an oven for 48 h or through lyophilization (freeze-drying) and then ground to 1 mm particle size in a blade mill. About 1 g of the pulverized material is placed in a 125 mL Erlenmeyer flask. Fifteen mL of methanol is added, and the mixture is stirred into a vortex to ensure that all the liquid comes into contact with the solid. Subsequently, the flask is placed in an ultrasonic bath for 10 min and the extract is filtered by gravity, first using cotton and then with a No. 42 filter paper. The solid is discarded. The liquid extract is then transferred to a 125 mL separatory funnel and extracted with a 15 mL hexane volume. The liquid−liquid extraction is performed, keeping the methanol phase and discarding the hexane phase. A second extraction is carried out on the methanolic phase using 10 mL of hexane.[17,19,22]

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A portion of the methanolic extract may be refrigerated and employed to evaluate antilarval activity,[15−17] TPC, or antioxidant activity.

TLC and Preparative Chromatography Procedure

A microcapillary tube is used to apply a spot of the methanolic extract onto two silica gel 60 (200 μm tickness) chromatographic HPTLC plates containing fluorescence indicators (F_{254}), from Merck chemicals (Darmstadt, Germany). A chromatography chamber is saturated with vapors of a mobile phase composed of toluene:ethyl ether (1:1) saturated with 10% acetic acid (aqueous). Once the chamber is set up, both plates are placed inside, allowing the mobile phase to ascend nearly to the top of the plates. The plates are then removed from the chamber and dried in a fume hood for 5 min. One of the plates is treated with a spray of 10% KOH in ethanol. KOH intensifies coumarin bands under UV light (see the Notes for Students). Subsequently, the plates are observed under ultraviolet light at 254 and 365 nm.[19] The presence of different groups can be analyzed by determining whether bands are visible and what color they present under white light and the two UV wavelengths. Rf values for each spot are calculated for comparison with HPLC results.

Preparative TLC uses the same stationary and mobile phases employed in analytical TLC but with a higher sample volume. Bands of interest are scraped off, extracted with dichloromethane, and evaporated to dryness to obtain the isolated compounds. Alternatively, dichloromethane can be substituted with ethyl ether.[19] Approximately 200 μL of extract were applied for preparative TLC.

HPLC Procedure

For the HPLC analysis, an instrument with a quaternary pump, a 50 μL loop manual injector, a C18 column (250 mm × 4.6 mm, particle size 5 μm), and a diode array detector (DAD) (with 280 and 350 nm wavelengths) is required. A gradient of 0.05% trifluoroacetic acid in water (A) is used as mobile phase A, and methanol is used as mobile phase B. The gradient starts with a 85% mobile phase A and ends with a 100% mobile phase B, over a 20 min run, and a flow rate of 1 mL/min, as shown in Table 2.[19] Once the HPLC run ends, the obtained signals are then compared with those obtained from the TLC analysis.

Qualitative Additional Test for Coumarin Detection

Additionally, the following test may be conducted for the detection of coumarins: 100 μL of the methanolic extract is dissolved in 1 mL of 10% NaOH, generating a yellow coloration. The disappearance of the color upon acidification indicates a positive result for the test.[23]

Table 2. Gradient Used for Reversed-Phase HPLC Separation of Scopoletin and Umbelliferone from *I. cairica* Methanolic Extract

Time (min)	%A (H_2O)	%B (CH_3OH)	Flow rate (mL/min)
0.01	85	15	1.00
3.00	65	35	1.00
7.00	60	40	1.00
10.00	55	45	1.00
13.00	50	50	1.00
15.00	50	50	0.50
16.00	50	50	0.50
18.00	15	85	0.50
18.00	15	85	1.25
19.00	0	100	1.25
20.00	85	15	1.00

HAZARDS

The hazards involved in this laboratory practice are minimal. However, certain precautions must be observed when handling methanol and hexane to minimize skin contact during the extraction procedure. Similarly, precautions are necessary when toluene and hexane are handled during the TLC procedure. Methanol is a flammable liquid that can cause a central nervous system depression. Hexane and toluene are highly flammable and volatile and capable of causing drowsiness, dizziness, and skin irritation upon contact. Dichloromethane is toxic, and its utilization is regulated in some countries. Students must wear safety glasses and gloves when handling these solvents, and manipulation must also be carried inside a fume hood.

RESULTS AND DISCUSSION

TLC Section

Olga O. de A Lima and Raimundo Braz-Filho[24] extracted compounds from the *Ipomoea cairica* plant using column chromatography and showed the presence of various types of coumarins, as well as other compounds of interest that may be obtained from this plant. The thin layer chromatography (TLC) separation of the methanol extract is shown in Figure 2.

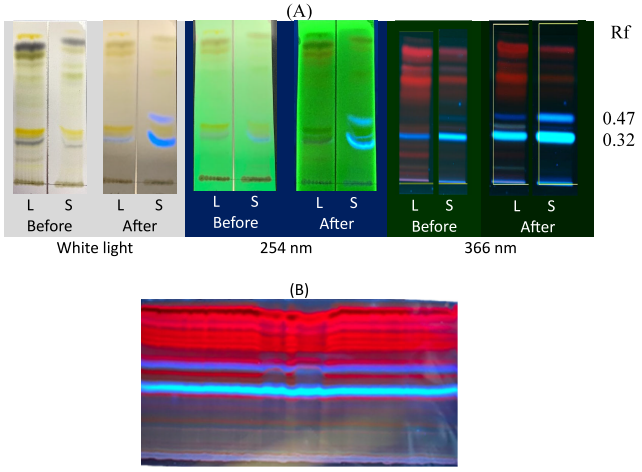

Figure 2. TLC plate of *I. cairica* extract eluted with toluene:ether (1:1) saturated with 10% acetic acid in water. (A) TLC plates before and after derivatization with 10% KOH in ethanol and observed under white light + UV (366 nm), 254 nm light, and 366 nm light. Letters L and S at the bottom of chromatographs represent leaves and stems. (B) Preparative plate used to isolate the compounds revealed under 366 nm. Rf values for coumarins main are indicated (see Notes for Students for additional information). Note: Pictures were recorded from different experiments.

Two blue bands are readily observed at 365 nm when the plates are developed under UV light. These bands increase in intensity on the sample sprayed with 10% KOH in ethanol (plate on the right side of Figure 2), which is an indication of the presence of coumarins. Derivatization also makes visible the blue bands when they are observed at 254 nm and a combination of white and UV light. The band with the lower Rf corresponds to scopoletin, due to its greater interaction with the stationary phase, because of the additional oxygen in its

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methoxy group. The absence of the methoxy group in umbelliferone results in a lower affinity for the stationary phase and therefore shows a higher Rf.

Once the Rf values of the coumarins are established based on the analytical TLC results, both compounds may be isolated using preparative TLC in the second laboratory session. After the compounds are isolated, they are further analyzed using high-performance liquid chromatography (HPLC) to confirm their identities and purities.

HPLC Section

The analytical HPLC chromatogram displaying the separation of the two coumarins in the methanolic extract is shown in Figure 3. The reading was taken at 280 nm, a typical wavelength for the absorption of molecules with aromatic rings, and 350 nm, which is used to detect a few secondary metabolites, including coumarins. The peaks at 11.8 and 14.9 min correspond to scopoletin and umbelliferone, respectively, with scopoletin being the compound with the highest concentration. The students should relate the inverse elution order to the reversed-phase chromatographic conditions. Understanding these retention times helps students connect the interactions between the compounds and the chromatographic phases, thereby reinforcing their grasp of the principles underlying reversed-phase HPLC.

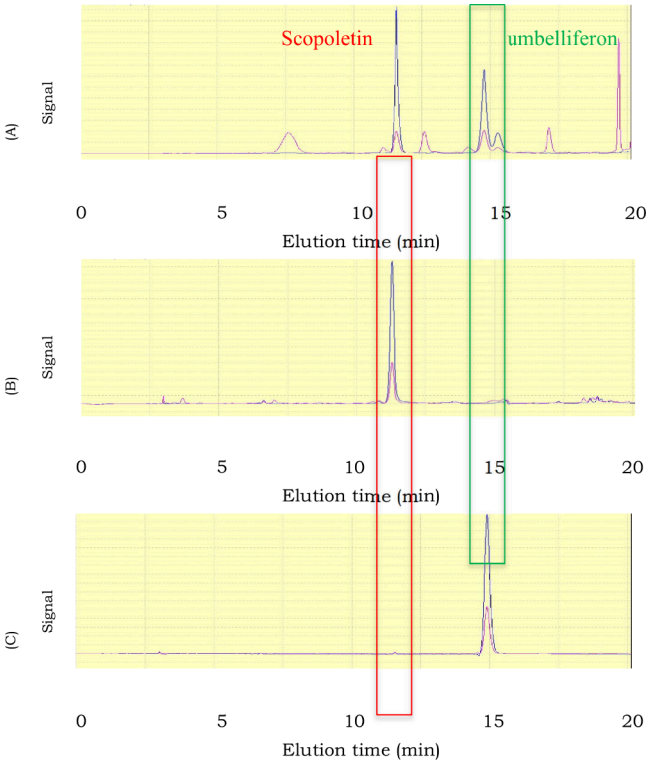

Figure 3. Reverse-phase HPLC-DAD chromatogram of the methanolic extract obtained at 280 (purple line) and 350 nm (blue line). (a) *I. cairica* extract, (b) scopoletin, isolated by preparative TLC, and (c) umbelliferon, isolated by preparative TLC.

The activity requires two 4 h laboratory sessions of each and a HPLC unit for a group of 10 students. For larger groups, it is recommended that the student body be divided into smaller groups so that at the beginning of the second laboratory session, some students work on the TLC separation, while the others focus on the HPLC analysis. After a set period, the roles are switched. If a second HPLC unit is available, then it is recommended to conduct a UV−vis run prior to the HPLC analysis to confirm the absorption maxima. This preliminary step helps optimize the HPLC analysis by ensuring that the correct absorption wavelengths are used

■ ASSESSMENT OF LEARNING OUTCOMES

Learning outcomes are assessed by a short quiz during the first 10 min of class, the laboratory notebook preparation, a visual evaluation of the instructor during the laboratory session, and a laboratory report to be sent a week after the session by each group of two students. The quiz is designed to assess the prerequisite concepts needed to safely perform and understand the experiment. General concepts about chromatography (theoretical and experimental) and questions about safety and chemical handling are assessed in the quiz (see Notes for Instructors).

The notebook evaluated the general comprehension of the laboratory procedure before the session. The students are required to prepare a schematic diagram of the procedure. This simple task is essential for evaluating the students' understanding of the written procedure. Also, the students are required to include a table with all the physical and chemical properties of the materials utilized as well as all the relevant hazard information.

Quizzes and notebook preparation are designed to mainly assess learning objective ii. The results (Figure S3) show a good general understanding of the basic concepts. Around 70% of the students correctly answered at least 95% of questions.

During the laboratory session, the instructor evaluates if the students can perform basic operations such as extraction, use of ultrasound bath, and preparation of TLC chamber, etc. Students may make mistakes, but they are expected to actively participate in and collaborate with each other during the process. Learning objectives iii and iv are evaluated during the lab session. Most students complete the tasks, but still many of them need some sort of guidance. For objective number iv is recommended to keep the *I. cairica* plants on campus, so students can collect, dry and grind them 1 week before the lab session.

During the laboratory report, the students had time to think and analyze the results, so a deeper understanding of the process is expected. For instance, students follow the isolation of a single compound from a natural source (vegetal material) utilizing at least two techniques. Also, the students are learning about derivatizing or revealing techniques and their chemical fundamentals. Also, the concepts of mobile and stationary phases and their relationship with elution times and affinity. For example, the TLC experiment utilizes silica gel plates (normal phase), eluting the least polar coumarin first, but HPLC is performed utilizing a C18 column, inverting the elution order of the coumarins. At this stage, students should recognize that the coumarin eluting first on silica elutes second on C18, and vice versa, due to the polarity differences of the stationary phases. Furthermore, based on the chemical structures of the two identified coumarins, students have all the necessary clues to identify both peaks without requiring a standard, as they already know the two main coumarins of *I. cairica* and their chemical properties. Also, learning objective I is evaluated during lab reports. Students are requested to do some research about previous publications, other reports, natural products, etc. A helpful strategy to guide the students to successfully achieve this objective consists of a double

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review step: the first one just qualitative, pointing out any mistake and/or missing information.

CONCLUSION

The *Ipomoea cairica* stem and leaves contain coumarins that may be extracted and separated using simple laboratory techniques. This procedure provides students with an activity that not only introduces them to new analytical skills but also integrates these with previously acquired skills. The methods detailed in this paper first engage students in extracting coumarins from the raw plant and then in the chromatographic separation of the main compounds. During this procedure, students apply various basic techniques such as grinding, liquid−liquid extraction and ultrasonic-assisted extraction, as well as more skilled techniques such as analytical and preparative TLC, plate development, and analytical HPLC. Additionally, the process may be complemented with UV−vis spectrophotometry to further characterize the extracts. The products and extracts obtained from these activities may be used in subsequent sessions of the program, providing a comprehensive and continuous learning experience.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available at https://pubs.acs.org/doi/10.1021/acs.jchemed.4c01131.

Notes for students (PDF, DOCX)
Notes for Instructors (PDF, DOCX)

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Notes

The authors declare no competing financial interest.

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