Chemistry of Autumn Colors: Quantitative Spectrophotometric Analysis of Anthocyanins and Carotenoids and Qualitative Analysis of Anthocyanins by UPLC-MS/MS

Marianna Manninen[1], Veli-Matti Vesterinen[2] and Juha-Pekka Salminen[1]

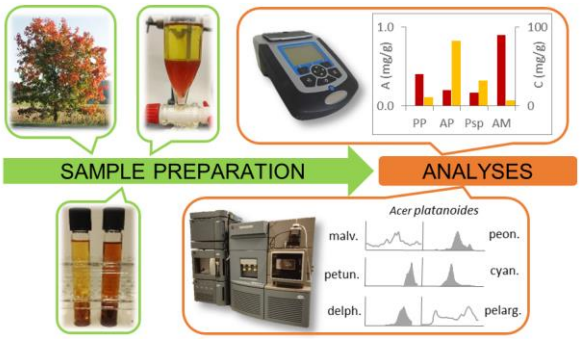
[1]Natural Chemistry Research Group, Department of Chemistry, University of Turku, FI-20014, Turku, Finland

[2]Department of Chemistry, University of Turku, FI-20014, Turku, Finland

ABSTRACT

As the autumnal leaf color change is familiar to most students living in temperate climate zones, the extraction and analysis of the pigments of the autumn leaves provides an engaging way to study both simple as well as more sophisticated analytical methods. In this laboratory experiment, students extract the red and yellow pigments i.e. anthocyanins and carotenoids from leaves and separate them from each other by liquid-liquid extractions. From the separated phases, anthocyanin and carotenoid concentrations can be evaluated visually or spectrophotometrically. The anthocyanins are analyzed by a rapid and simple UPLC-MS/MS method to discover which of the six most common groups of anthocyanins are present in the sample. The simplicity of the experiment setup allows it to be used as an introduction to mass spectrometry since the results can be easily interpreted without complicated data processing. The experiment provides opportunities for learning outside the classroom, as the samples can be collected from the nearby parks or forests and analyzed using more sophisticated methods on a student visit to a university or other research institution. The sample preparation followed by the visual analyses of the phases is however simple enough to be performed in a regular school laboratory.

GRAPHICAL ABSTRACT



KEYWORDS

Analytical Chemistry, Hands-On Learning, High School/Introductory Chemistry, Laboratory Instruction, Mass Spectrometry, Natural Products, Qualitative Analysis, Quantitative Analysis, UV-Vis Spectroscopy

INTRODUCTION

Autumnal leaf color change of most temperate deciduous trees is a beautiful phenomenon that can be utilized for teaching purposes from its chemical point of view. In this experiment, the yellow and red pigments are extracted from the autumn leaves and quantified spectrophotometrically. The red pigments are analyzed further by liquid chromatography combined with tandem mass spectrometry (UPLC-MS/MS) to classify them into the six common subgroups of natural pigments. This experiment combines chemistry and biology, and provides students out-of-classroom learning experiences, since the plant samples can be collected by the students from the nearby parks, forests or gardens, and can be analyzed in collaboration with university or any other institution with the suitable instrumentation.

In several laboratory experiments, anthocyanins and carotenoids, the red and yellow pigments in plants, are studied using different methods. In many experiments, these compounds are used to demonstrate the basic principles of chromatography[1–3] or studied utilizing different chromatographic methods such as thin layer chromatography (TLC)[4,5] and high-performance liquid chromatography (HPLC)[6,7]. Spectroscopy has also been used for quantitative and qualitative purposes in several laboratory experiments on anthocyanins and carotenoids.[4–10] All of this can be done with samples familiar to the students from everyday life, such as berries, fruits, flower petals and leaves. Working

with anthocyanins and carotenoids makes the experiments colorful and visually attractive, and therefore more interesting.

Until now, only few laboratory experiments published in this journal have utilized tandem mass spectrometry and the different tandem modes such as MRM or SRM (multiple/single reaction monitoring). The previously published studies have included analyses of perfluorinated surfactants in fish liver[11], amoxicillin in river waters[12] and drugs of abuse in paper currency[13] as well as the development of an MRM method for vanillin, ethyl vanillin and coumarin[14], or more technical overview of ESI-MS (electrospray ionization-mass spectrometry) instrumentation in different modes.[15] All of these experiments have been designed for undergraduate students, but a fast and simple method to demonstrate the use of mass spectrometry for younger students has not been previously described.

The first steps of this experiment, i.e. sample collection, drying and extraction, are simple procedures. The following step, where the anthocyanins and carotenoids are separated by liquid-liquid extraction, is visually the most impressive phase of the experiment, and can also be carried out in a school laboratory. The last step is carried out using the state-of-the-art UPLC-MS/MS instrument and requires collaboration between schools and universities. Such collaboration can provide the students the opportunity to see and use cutting-edge methods in an authentic research environment, thus increasing the relevance of their studies and their understanding of nature of science.[16,17]

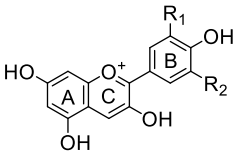
The main goal of this experiment is to introduce students to the basic principles of spectroscopy and the use of high-performance liquid chromatography and mass spectrometry in chemistry research. It is best suitable for an advanced chemistry course in upper secondary school, where the students have good understanding of basic chemical concepts such as solubility, concentration and polarity. During the experiment, students make observations in nature, prepare an extract from leaves, perform liquid-liquid extractions and use a spectrophotometer. By combining chemistry to a biological phenomenon, and giving out-of-classroom learning experiences in authentic locations, this experiment could help the students see chemistry in a different and more interesting way.

EXPERIMENTAL BACKGROUND

Anthocyanins are water-soluble pigments that give the red, purple and blue colors to many fruits, flowers and leaves. Lipid-soluble carotenoids are responsible of the yellow, orange and red colors in

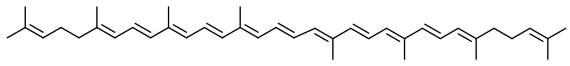
many kinds of plant organs, such as leaves and fruits, and they are essential components of photosystems. Carotenoids are tetraterpenes that consist of eight isoprene units; they are classified as carotenes if they are pure hydrocarbons, or as xanthophylls if they contain oxygen, most often in the form of hydroxyl or epoxy groups (Figure 1).[18] Anthocyanins consist of an aglycone (anthocyanidin), one or more sugar units and in many cases acyl groups. In general, there are six major groups of anthocyanidins that occur in nature, and they vary by the number of hydroxyl and methoxy groups in the B ring (Figure 1).[19] The structure and therefore the color of anthocyanins depends on pH. In acidic conditions, anthocyanins are in their red flavylium form.[19]

Basic structure of anthocyanidins

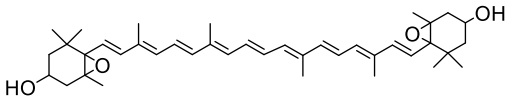


Anthocyanidin	R_1	R_2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

Examples of carotenoids



Lycopene



Violaxanthin

Figure 1. Examples of anthocyanidin and carotenoid structures.

In leaves, the intense green color of chlorophyll often masks the yellow and orange colors of the carotenoids. During the autumn coloration period, chlorophyll and carotenoids start to decay, but chlorophyll decays more rapidly than carotenoids, which leads to the yellow coloration of the foliage.[20] The carotenoid composition of leaves may also change as the growing season progresses; a study with 25 tree species showed, that the carotene content decreased and xanthophyll content increased towards the autumn.[21] Anthocyanins are however not necessarily present in summer green leaves, but mainly synthesized in the beginning of senescence.[22] Anthocyanin composition of autumn leaves has

not been studied in detail with modern methods, but the anthocyanins found in autumn leaves are known to be mainly cyanidin derivatives.[23,24]

MATERIALS AND METHODS

Chemicals

Analytical grade acetone used for extraction was from VWR Chemicals (France) and HPLC grade hexane from Rathburn Chemicals ltd (Great Britain). LC-MS grade acetonitrile from Sigma Aldric (Stenheim, Germany) and LC-MS grade formic acid was from VWR Chemicals (Finland). Water was purified by Millipore Synergy water purification system (Merck KGaA, Germany). The β-carotene (Type 1, approx. 95 % UV) used as a carotenoid standard was from Sigma Aldrich (USA) and the cyanidin-3,5-di-*O*-glucoside chloride (cyanidin chloride, purity (HPLC) ≥ 97 %) was from Extrasynthese (France). The plant samples were collected from Turku, Southwest Finland in September-October 2014 and October 2018.

Equipment

The quantitative measurements were done with a portable spectrophotometer (DR1900, Hach, Loveland, CO, USA) using micro cuvettes from Hellma Analytics (volume 700 µl, path length 10 mm, optical glass, black walls). The qualitative analysis of anthocyanins was carried out with an Acquity UPLC system (Waters Corp., Milford, MA, USA) coupled with a Xevo TQ triple-quadrupole mass spectrometer (Waters Corp.). The UPLC system consisted of a sample manager, a binary solvent manager, a column (Acquity UPLC BEH Phenyl 30 × 2.1 mm, 1.7 µm, Waters Corporation, Ireland), and a diode array detector. Acetonitrile (A) and 0.1 % aqueous formic acid (B) were used as solvents and following elution profile was used: 0-0.1 min 10 % A in B (isocratic); 0.1-2.0 min 10-50 % A in B (linear gradient); 2.0-2.1 min 50-90 % A in B (linear gradient); 2.1-3.2 min column wash and stabilization. The flow rate was 0.65 ml/min and the injection volume 5 µl. Mass analyzes were performed using ESI source and positive ionization. ESI conditions were chosen so, that the anthocyanins are fragmented into anthocyanidins already in the ion source: capillary voltage, 3.4 kV, source temperature, 150 °C, desolvation temperature, 650 °C, desolvation and cone gas (N_2), 1000 and 100 l/h, respectively, and collision gas, argon. The SRM parameters are described in Table 1.

Table 1. Single Reaction Monitoring (SRM) parameters for the six groups of anthocyanidins and their derivatives

Compound class	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
Pelargonidin	271	121	70	30
Cyanidin	287	213	80	31
Peonidin	301	286	60	25
Delphinidin	303	257	55	30
Petunidin	317	302	60	25
Malvidin	331	315	55	30

Experimental Procedure

The plant samples were either freeze-dried or dried with a household vegetable dryer, shredded in a plastic bag into as fine powder as possible, and 200 mg samples were weighed into test tubes. 8 ml of extraction solvent (80/20 acetone water, *V/V*) was added into the tubes, and the tubes were transferred into a fridge for maceration at least overnight. The extracts were filtered into beakers using a qualitative filter paper and evaporated to dryness in a fume hood. Images of two samples in different phases of the extraction are presented in figure 2. The dried extracts were liquid-liquid extracted with 5 ml of 4 % aqueous formic acid and 8 ml of hexane (figure 3). Aqueous phase was analyzed by spectrophotometer at 520 nm for presence of anthocyanins and hexane phase was analyzed at 450 nm for carotenoids. Anthocyanin and carotenoid concentrations were calculated using predefined calibration curves of cyanidin (5, 10, 20 and 40 µg/ml) and β-carotene (0,25; 0,5; 1,0 and 2,0 mg/ml) . A three-fold dilution was prepared from the aqueous anthocyanin phase for qualitative LC-MS measurements. Detailed instructions and tips for the instructor can be found in the Supporting Information.

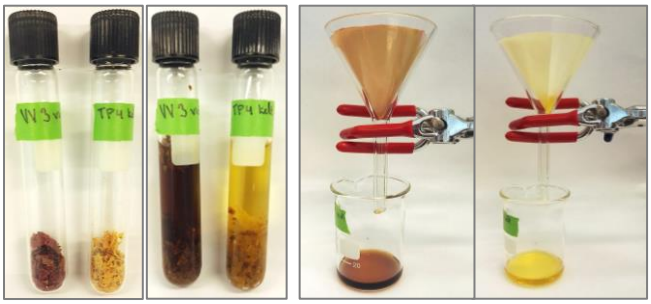


Figure 2. Different phases of the extraction protocol. (A) 200 mg of ground red and yellow leaves weighed into test tubes; (B) the test tubes after the addition of the extraction solvent; (C) filtration of the extracts.

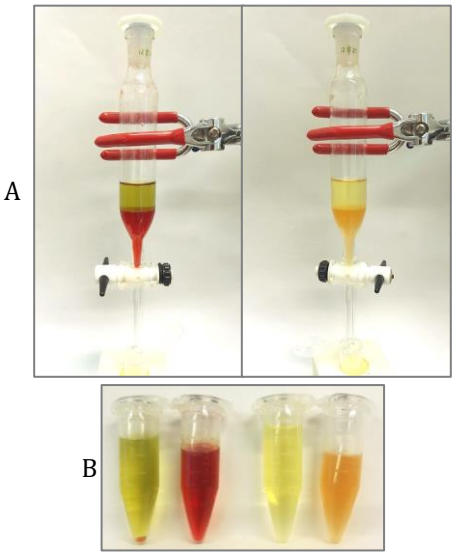


Figure 3. (A) Liquid-liquid extractions for the extracts from figure 2; (B) on the left are the hexane and aqueous phase of the extract from red leaves, on the right are the hexane and aqueous phase of the extract from the yellow leaves.

Participants and setting

A total of 34 students performed this experiment in three groups: students in groups A (8 students) and B (10 students) were upper secondary school students from a chemistry course, and group C (16 students) was a group of university students from a chemistry education course for BSc students. Since groups A and B did not have the possibility to collect their own plant samples, they were given freeze-dried plant samples that they extracted at their school in two < 1 h sessions. In group A, each student had two samples. In group B, each student had one sample. The samples were analyzed with the spectrophotometer by the students themselves and the UPLC-MS instrument was operated by the instructor. All analyses were done during a 4 h visit to the Chemistry Department at the University of Turku. Group C performed the experiment in four sessions: plant sampling and starting the drying of the samples (1 h), starting the extraction of the samples (1 h), filtration of the extracts and drying them (done independently during the day, less than 30 min), and spectrophotometric analysis of one sample per student (3 h).

SAFETY HAZARDS

Suitable gloves and eye protection should be used throughout the experiment and all the solvents and formic acid should be manipulated in a fume hood. Hexane and acetone are highly flammable liquids and vapors should be kept away from sources of ignition. Hexane and acetone are harmful if ingested, inhaled or in contact with the skin. Vapors may cause drowsiness and dizziness. Acetone

causes serious eye irritation. There is a danger of serious damage to health by prolonged exposure through inhalation of hexane, including a possible risk of impaired fertility. Hexane may be fatal if swallowed and enters airways and may cause skin irritation. Even when diluted, acid solutions can cause severe irritation. Hexane solutions must be disposed in a hazardous organic waste container. Formic acid solutions can be diluted and discarded as aqueous waste.

RESULTS AND DISCUSSION

In the present study, each student prepared and analyzed at least one sample. The students were successful in extracting their samples, and they succeeded in the liquid-liquid extractions. The anthocyanin and carotenoid phases were used as such for spectrophotometric measurements without dilution. UPLC-MS results were obtained from the extracts of groups A and B. The instrument and the basic idea of the method was demonstrated to the students, and the chromatograms were interpreted together. The instructor operated the analysis software to keep the students' focus on the results instead of the mechanical performance of how to use the software. All of the results were combined in a shared Excel sheet. In group A, the students filled in the absorbances and the Excel template calculated the concentrations in mg/g (mg of compounds per gram of plant dry weight). Group B added also the concentrations (in μg/ml or mg/ml) that they had calculated by themselves. Group C calculated the concentrations in mg/g.

Altogether, students analyzed 42 extracts from nine different plant species in this experiment. The anthocyanin contents varied from 0 to 2.8 mg/g with the average of 0.6 mg/g, and carotenoid contents from 0 to 83 mg/g with the average of 26 mg/g. The variation within the extracts obtained from the same plant species was relatively high. For example, the average carotenoid concentration in yellow Norway maple (*Acer platanoides*) leaves was 34 mg/g and the standard deviation was 23 mg/g. However, the pigment concentrations change during the senescence, so it is expected to have varying results even from the same plant species. Cyanidin or its derivatives were found in all of the plant samples that had anthocyanins, and none of them had malvidin derivatives. In addition to cyanidin derivatives, most of the plant samples had pelargonidin and/or peonidin derivatives. Bird cherry (*Prunus padus*) samples had most diverse anthocyanidin contents, as they contained derivatives of all

other types of anthocyanidins except malvidins. Examples of the MS/MS results are presented in figure 4.

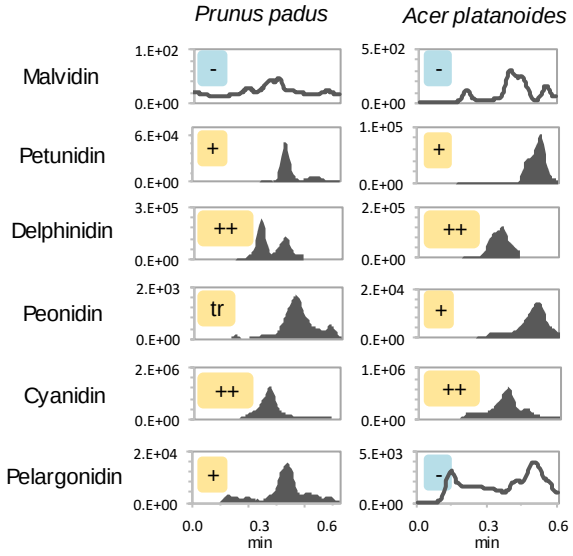


Figure 4. Examples of the SRM chromatograms for the six groups of anthocyanidins and their derivatives of two plant species. Peaks with intensity of 10^3 or under were considered as background noise and were not integrated. The symbols in the yellow boxes correspond to the intensity of the signal: ++, high intensity of the signal (10^5 or 10^6); +, low intensity of the signal (10^4); tr, traces; -, not detected

To test the reliability of the sample preparation protocol and the quantitative methods, a trained chemist (the first author) prepared and analyzed some of the same plant samples that were used by the students. As can be seen from figure 5, the results from the same plant sample were quite similar between the students as well as with the results determined by a trained chemist. Only Student F's carotenoid concentration was significantly different from others, but his/hers anthocyanin concentration was similar to the other results. Too detailed conclusions about the anthocyanin and carotenoid concentrations in autumn leaves cannot be drawn from the results due to the deviation in the results. However, students could draw conclusions, whether the anthocyanin and carotenoid concentrations were high or low in their sample.

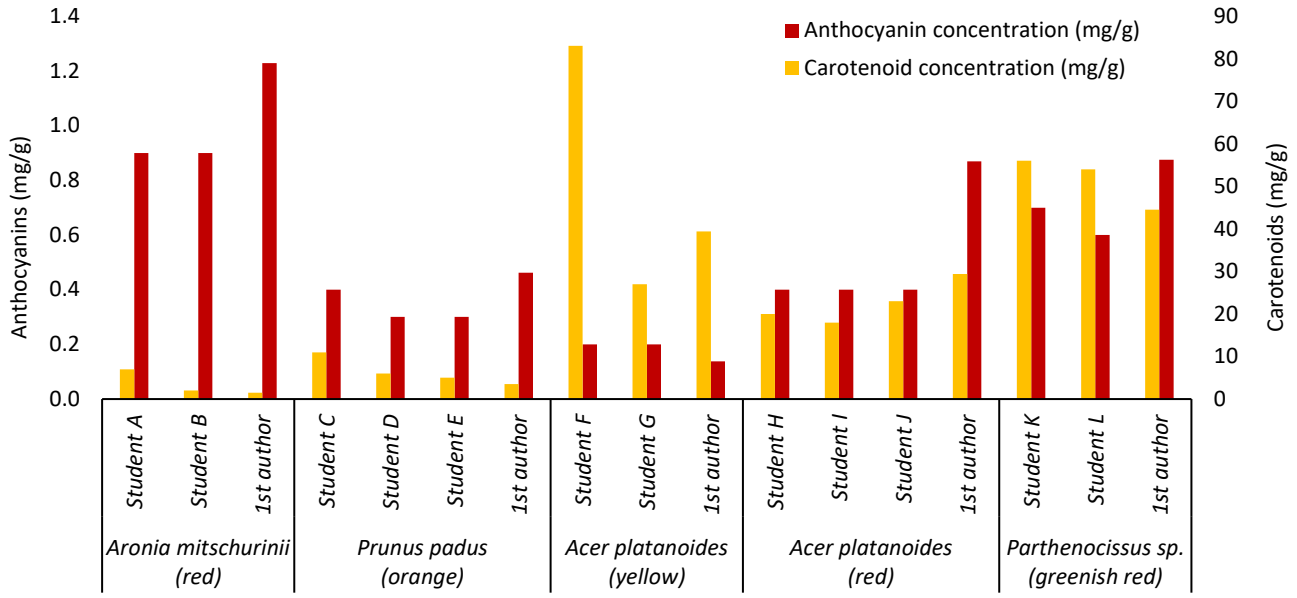


Figure 5. Anthocyanin and carotenoid concentrations determined by the first author and students from the same plant samples. In parentheses is the color of the plant sample.

The setup of this experiment can be used to answer several different research questions by choosing the plant samples wisely. By having several different plant species or leaves with variable colors, the levels of anthocyanins and carotenoids in autumn leaves can be screened, as well with the diversity of anthocyanidin derivatives in them. Another approach is to collect samples from the same species but from different locations, and see whether it has an effect especially on the concentrations. A specimen from a selected plant species can also be monitored as a function of time by collecting samples from the beginning of senescence to the end of it, and see how the pigment composition and/or concentration changes. In all of the cases, it should be taken into consideration, that the leaf samples should not contain chlorophylls, since they will be extracted alongside the carotenoids and increase the UV-Vis response, although the wavelength 450 nm is relatively selective for carotenoids over the chlorophylls.

This experiment can be altered depending on the time and equipment available. The extraction and liquid-liquid extraction are easy to perform with school laboratory equipment with plant samples that are dried with a household plant dryer. These steps could be used as such to demonstrate how compound groups can be separated based on their chemical properties. If there is no

spectrophotometer available, the pigment concentrations can be evaluated visually to compare which samples have the highest and the lowest amounts of anthocyanins and carotenoids based on the color.

EVALUATION OF LEARNING OUTCOMES

The experiment was designed to give students an example of application of chemistry in a real-life scientific context. Rather than concentrating on specific chemical concepts, the focus was on providing the students with the experience of using chemistry to study plants. Therefore, the emphasis with the test groups was on observing how well and reliably the methods used in the experiment work, when carried out by students. Both upper secondary school students as well as the undergraduate university students were able to successfully complete each step of the experiment in the time allocated to it. As can be seen by comparing the data obtained by the students with the data obtained by a trained chemist (see figure 5), the students could also obtain reliable results with the instructions provided. Thus, the main goal for the development of the experiment was successfully achieved.

To assess the amount of background information students should be provided, a postlab assignment was used to evaluate students' understanding of the some of the key chemical concepts related to the experiment. The assignment included following questions:

- How is absorbance related to the concentration of a compound?
- What happens to the compounds of a sample in the column of a liquid chromatograph?
- Why can different anthocyanin classes be identified using a mass spectrometer?

According to the answers of 28 students, all of them understood—or at least had correct elements in their answers on—how absorbance is related to concentration. 15 out of 28 answers stated that the higher the absorbance the higher the concentration is. In seven answers, the absorbance was even related to the color of the extract or to how the light goes through the extract. Examples of these responses are provided in Supporting Information, P. 13-14. As expected, questions related to liquid chromatography and mass spectrometry proved to be more difficult. 12 students from 28 described in a coherent way that compounds are separated from each other in the column (see Supporting Information, P. 13-14). Rest of the answers were incorrect or non-committal. Four students answered that the compounds are degraded, heated or ionized which suggest that they confused column with the ion source. As for the identification of different anthocyanidin derivatives, only one high school

student and eight university students had adequately understood that it is based on the different masses (or mass to charge ratios) of the molecules. Majority of the answers were superficial (9), for example: “different anthocyanins react differently”, or had the wrong chemical property (4). To prevent confusion related to liquid chromatography and mass spectrometry, the students should probably be familiarized with the methods beforehand. If this is not possible, the instructor should at least thoroughly explain what happens in each phase of the experiment.

To measure student interest and satisfaction, the students were also asked which phase of the experiment was most pleasant and why, as well as which phase of the experiment was the most difficult or least pleasant and why. 13 students out of 28 mentioned extraction or liquid-liquid extraction as one of the nicest parts of the work because either it was easy to do, new to them or visually attractive. Half of the students in group C had liked collecting their samples by themselves because they got to go outside. As expected, these steps seem to support situational interest of the students and thus their motivation to carry out the experiment. Most difficult or least pleasant parts of the experiment were the ones that demanded precision and accuracy, such as diluting and filtrating (7) or calculations (3).

CONCLUSION

The experiment described here gave students hands-on experience with separation and analysis of natural compounds from plant leaves. It introduced the students to the quantitative analysis of the pigments by a spectrophotometer. It provided also a chance to utilize modern methods and the UPLC-MS instrument to reveal more details of the pigment composition of a plant sample, giving thus an introduction to high-performance liquid chromatography and mass spectrometry.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available on the ACS Publications website at DOI: 10.1021/acs.jchemed.XXXXXXX.

- Instructions with additional information to the instructor, summary of the students’ results, examples of the students’ responses to the postlab assignment, examples of the SRM chromatograms (DOCX, PDF)

- Student instructions (DOCX, PDF)

AUTHOR INFORMATION

Corresponding Author

*E-mail: mailma@utu.fi

ACKNOWLEDGMENTS

We thank Marika Suovanen and her students for the participation in the testing of the experiment and the university students who participated in the experiment. Maarit Karonen is acknowledged for comments and assistance with the manuscript and Suvi Vanhakylä is acknowledged for the collection of the plant samples. The study was funded by Turku University Foundation. The Strategic Research Grant (Ecological Interactions) enabled the purchase of the UPLC–DAD–MS/MS instrument.

REFERENCES

(1) Dias, A. M.; Ferreira, M. L. S. "Supermarket Column Chromatography of Leaf Pigments" Revisited: Simple and Ecofriendly Separation of Plant Carotenoids, Chlorophylls, and Flavonoids from Green and Red Leaves. *J. Chem. Educ.* **2015**, *92* (1), 189–192. https://doi.org/10.1021/ed500299j.

(2) Curtright, R. D.; Emry, R.; Markwell, J. Student Understanding of Chromatography: A Hands-On Approach. *J. Chem. Educ.* **1999**, *76* (2), 249. https://doi.org/10.1021/ed076p249.

(3) Du Toit, M. H.; Eggen, P.-O.; Kvittingen, L.; Partali, V.; Schmid, R. Normal- and Reverse-Phase Paper Chromatography of Leaf Extracts of Dandelions. *J. Chem. Educ.* **2012**, *89* (10), 1295–1296. https://doi.org/10.1021/ed200851w.

(4) McCullagh, J. V.; Ramos, N. Separation of the Carotenoid Bixin from Annatto Seeds Using Thin-Layer and Column Chromatography. *J. Chem. Educ.* **2008**, *85* (7), 948. https://doi.org/10.1021/ed085p948.

(5) Ayudhya, T. I.; Posey, F. T.; Tyus, J. C.; Dingra, N. N. Using a Microscale Approach To Rapidly Separate and Characterize Three Photosynthetic Pigment Species from Fern. *J. Chem. Educ.* **2015**, *92* (5), 920–923. https://doi.org/10.1021/ed500344c.

(6) Silveira, A.; Koehler, J. A.; Beadel, E. F.; Monroe, P. A. HPLC Analysis of Chlorophyll a, Chlorophyll b, and Beta-Carotene in Collard Greens. *J. Chem. Educ.* **1984**, *61* (3), 264. https://doi.org/10.1021/ed061p264.

(7) Sirvent, T. M.; Miller, A. L.; Vaughan, P. Effects of Storage Conditions on Lycopene Stability in Tomato Extracts. An Undergraduate Experiment. *J. Chem. Educ.* **2009**, *86* (11), 1304. https://doi.org/10.1021/ed086p1304.

(8) Galloway, K. R.; Bretz, S. L.; Novak, M. Paper Chromatography and UV–Vis Spectroscopy To Characterize Anthocyanins and Investigate Antioxidant Properties in the Organic Teaching

Laboratory. *J. Chem. Educ.* **2015**, *92* (1), 183–188. https://doi.org/10.1021/ed400520n.

(9) Soares, M. H. F. B.; Ramos, L. A.; Cavalheiro, É. T. G. Spectrophotometric Determination of Total Sulfite in White Wine Samples Using Crude Extracts from Flowers. *J. Chem. Educ.* **2002**, *79* (9), 1111. https://doi.org/10.1021/ed079p1111.

(10) Edionwe, E.; Villarreal, J. R.; Smith, K. C. How Much Cranberry Juice Is in Cranberry–Apple Juice? A General Chemistry Spectrophotometric Experiment. *J. Chem. Educ.* **2011**, *88* (10), 1410–1412. https://doi.org/10.1021/ed2002237.

(11) Stock, N. L.; Martin, J. W.; Ye, Y.; Mabury, S. A. An Undergraduate Experiment for the Measurement of Perfluorinated Surfactants in Fish Liver by Liquid Chromatography–Tandem Mass Spectrometry. *J. Chem. Educ.* **2007**, *84* (2), 310. https://doi.org/10.1021/ed084p310.

(12) Homem, V.; Alves, A.; Santos, L. Development and Validation of a Fast Procedure To Analyze Amoxicillin in River Waters by Direct-Injection LC–MS/MS. *J. Chem. Educ.* **2014**, *91* (11), 1961–1965. https://doi.org/10.1021/ed500482j.

(13) Parker, P. D.; Beers, B.; Vergne, M. J. What Is in Your Wallet? Quantitation of Drugs of Abuse on Paper Currency with a Rapid LC–MS/MS Method. *J. Chem. Educ.* **2017**, *94* (10), 1522–1526. https://doi.org/10.1021/acs.jchemed.7b00196.

(14) Betts, T. A.; Palkendo, J. A. Teaching Undergraduates LC–MS/MS Theory and Operation via Multiple Reaction Monitoring (MRM) Method Development. *J. Chem. Educ.* **2018**, *95* (6), 1035–1039. https://doi.org/10.1021/acs.jchemed.7b00914.

(15) Stock, N. L.; March, R. E. Hands-On Electrospray Ionization-Mass Spectrometry for Upper-Level Undergraduate and Graduate Students. *J. Chem. Educ.* **2014**, *91* (8), 1244–1247. https://doi.org/10.1021/ed500062w.

(16) Hofstein, A.; Kesner, M. Industrial Chemistry and School Chemistry: Making Chemistry Studies More Relevant. *Int. J. Sci. Educ.* **2006**, *28* (9), 1017–1039. https://doi.org/10.1080/09500690600702504.

(17) Vesterinen, V.-M.; Aksela, M. A Novel Course of Chemistry as a Scientific Discipline: How Do Prospective Teachers Perceive Nature of Chemistry through Visits to Research Groups? *Chem. Educ. Res. Pr.* **2009**, *10* (2), 132–141. https://doi.org/10.1039/B908250F.

(18) Britton, G. Structure and Properties of Carotenoids in Relation to Function. *FASEB J.* **1995**, *9* (15), 1551–1558. https://doi.org/10.1096/fasebj.9.15.8529834.

(19) Andersen, Ø. M.; Jordheim, M. Anthocyanins. *Encycl. Life Sci.* **2010**, No. October. https://doi.org/10.1002/9780470015902.a0001909.pub2.

(20) Sanger, J. E. Quantitative Investigations of Leaf Pigments from Their Inception in Buds through Autumn Coloration to Decomposition in Falling Leaves. *Ecology* **1971**, *52* (6), 1075–1089. https://doi.org/10.2307/1933816.

(21) Czeczuga, B. Autumn Carotenoids in the Leaves of Some Trees. *Biochem. Syst. Ecol.* **1986**, *14* (2), 203–206. https://doi.org/10.1016/0305-1978(86)90063-3.

(22) Lee, D. W.; O'Keefe, J.; Holbrook, N. M.; Feild, T. S. Pigment Dynamics and Autumn Leaf Senescence in a New England Deciduous Forest, Eastern USA. *Ecol. Res.* **2003**, *18* (6), 677–694. https://doi.org/10.1111/j.1440-1703.2003.00588.x.

(23) Ishikura, N. Anthocyanins and Other Phenolics in Autumn Leaves. *Phytochemistry* **1972**, *11* (8), 2555–2558. https://doi.org/10.1016/S0031-9422(00)88535-0.

(24) Ji, S. B.; Yokoi, M.; Saito, N.; Mao, L. S. Distribution of Anthocyanins in Aceraceae Leaves. *Biochem. Syst. Ecol.* **1992**, *20* (8), 771–781. https://doi.org/10.1016/0305-1978(92)90036-D.