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Laboratory Experiment

Identification of Tree Species by Their Defense Compounds: A Study with Leaf Buds of White and Silver Birches

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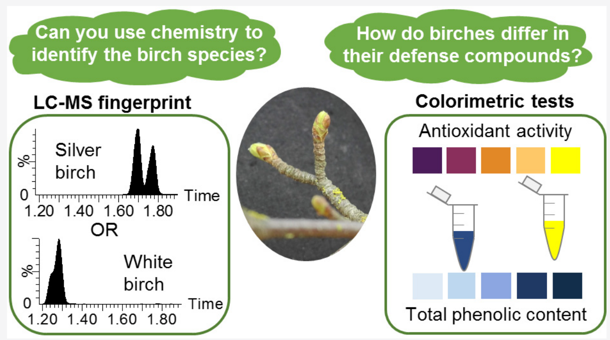
ABSTRACT: Plants encounter several different threats that affect their well-being during the spring. With chemistry, plants may defend themselves from, for example, excess UV-radiation and herbivores. The defense compounds between plant species vary, which makes it possible to utilize chemistry in identifying the plant species. In this laboratory experiment, students extracted the defense compounds from the surface of leaf buds, estimated the total phenolic content of the extract, and determined its antioxidant activity. In addition, the chemical fingerprints of the leaf buds were analyzed by liquid chromatography combined to mass spectrometry to identify the species as white birch, silver birch, or some other tree species. The laboratory experiment was performed with secondary school and university students in one approximately 3 h laboratory session. Pre- and post-tests done by the university students showed that the experiment provided students a basic understanding of how the instruments function and what they are used for. Their mind maps of the chemistry of plants were concentrated on the primary metabolites, but the experiment widened their views of specialized metabolites and their functions in plants, thus encouraging the students to combine chemical and biological information.



KEYWORDS: *Analytical Chemistry, Hands-On Learning/Manipulatives, High School/Introductory Chemistry, Interdisciplinary/Multidisciplinary, Mass Spectrometry, Natural Products, Plant Chemistry, UV–Vis Spectroscopy*

INTRODUCTION

White birch and silver birch can be hard to separate from one another during the winter and spring time. However, chemistry can reveal the difference easily. In fact, the epicuticular leaf surface flavonoids have successfully been utilized before in distinguishing *Betula pendula* and *Betula pubescens* type birch species.[1] Flavonoids are found on the leaf surface of various plants,[2−6] and they function as an important part of the chemical defense of plants by protecting plants from excess solar radiation[7] and herbivores,[8−10] and act as antioxidants.[11] In addition to flavonoids, other compounds such as terpenoids[12] and coumarins[13] are involved in the chemical defense of plants. Looking into the chemistry of leaf buds offers therefore an interdisciplinary opportunity to learn about the biology of the plant as well.

Interdisciplinary approaches have been widely utilized in chemistry education. Previous publications have described laboratory experiments and activities in the context of, for example, forensic science,[14] herbicides,[15] cell and molecular biology,[16] archeology,[17] and music.[18] Even entire courses have been created around an interdisciplinary theme, such as beer brewing,[19,20] pigments,[21] and plants.[22] Interdisciplinary teaching may help in engaging students, increasing meaningful learning, and connecting chemistry to real-world problems.[16,19,21,23] Heinrich et al. describe how an interdisciplinary approach may also promote the "authentic big picture" of scientific research to students.[23] This laboratory experiment shares the same goal by giving an example of an interdisciplinary research problem.

In addition to the interdisciplinary topic, authenticity of the laboratory experiment described here is supported by utilizing modern methods and instruments. Use of authentic practices has been seen to contribute to perceived relevance and to evoke interest, as long as students are sufficiently familiar with the chemical concepts and methods involved.[24] As instrumentation plays a huge role in chemical research and practice, there is a need to familiarize students with the use and role of modern research methods and instruments on all levels of education.[25,26] Hands-on exposure to the state-of-the-art

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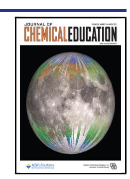


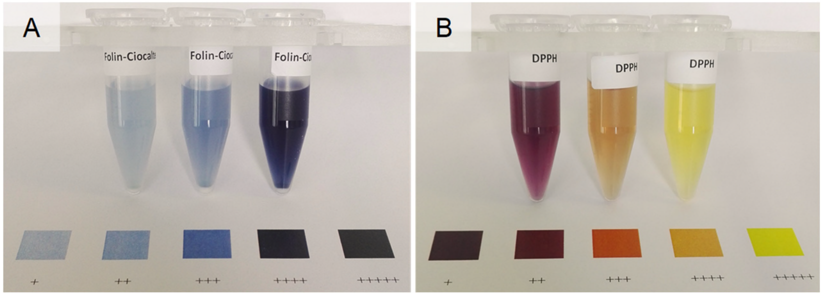


Figure 1. Examples of the range of colors observed in (A) the Folin−Ciocalteu assay for the total phenolic content and (B) the DPPH assay for the antioxidant activity.

research instrumentation is seen especially valuable for university chemistry education.[27]

Analytical techniques combining liquid chromatography (LC) to mass spectrometry (MS) are routinely used in chemistry research, and numerous laboratory experiments utilizing LC-MS have been published in this *Journal*.[28−36] However, only a small number of them provide a context that students would be familiar with from everyday life. In order to introduce this modern technique to students, a familiar context could make the abstract theory more interesting and worth understanding.[34] Even smaller number of the experiments have been designed for an audience outside of university.[36] Yet, they would benefit the most from having a glance into the world of chemistry research by trying out the instruments used by scientists, since students outside university do not usually have a possibility to use modern methods and instruments.

Here we describe a laboratory experiment where students extract the epicuticular compounds of leaf buds, identify the birch species by ultrahigh performance liquid chromatography combined with mass spectrometry (UHPLC-MS), and determine the antioxidant activity (AOA) and total phenolic content (TPC) of the leaf bud extract. It can be completed in one less than 3 h laboratory session and is suitable for secondary school students and introductory chemistry courses in university. The main goals for the experiment are (I) to demonstrate the chemical differences between plants and thus make the students combine chemical and biological knowledge, and (II) to introduce students to modern analytical methods, that is, spectrophotometry and especially LC-MS. Students' understanding of the chemistry of plants was tested with mind maps that the students created at the beginning of the laboratory session, and which they could supplement at the end of the laboratory session. Pre- and post-tests were used to assess how well students understood the methods before and after the laboratory work.

■ EXPERIMENTAL OVERVIEW

Laboratory Procedure

The experiment was tested with university students as well as secondary school students. The university students used freeze-dried leaf bud samples chosen for them beforehand. The secondary school students collected their samples by themselves and stored the samples in a fridge (4 °C). The leaf buds were extracted by dropping one leaf bud into an Eppendorf tube containing 2 mL of ethanol−water (95/5, *v*/*v*) for 30 s. After that, the extract was filtered with a syringe and 0.20 μm PTFE filter into a new Eppendorf tube. A volume of 500 μL of the extract was pipetted into a glass vial for the LC-MS analysis. The total phenolic content was estimated with the modified Folin−Ciocalteu assay,[37] where 150 μL of the plant extract was pipetted into a tube containing 1 mL of the Folin−Ciocalteu reagent. After mixing gently, 2 mL of 20% Na_2CO_3 (*m*/*v*) solution was added to the tube. The color of the solution was recorded by comparing the color to a color chart after 20 min (Figure 1A). A modification of the DPPH assay[38] was used to determine the antioxidant activity of the extract. A volume of 450 μL of the plant extract was pipetted into a tube containing 3 mL of DPPH solution, and the color was compared to a color chart after 15 min (Figure 1B).

The TPC was measured at 730 nm with a spectrophotometer, and the result was presented as milligrams per liter using a preinstalled calibration curve with gallic acid as the standard. The AOA was measured at 517 nm, and the students calculated the AOA from the absorbance reading with the calibration curve (gallic acid as the standard) that was given in the instructions. The LC-MS analysis was carried out at the same time with the Folin−Ciocalteu and DPPH assays, and the instrument was operated by the instructor. The instrument used in the experiment was an Acquity UPLC system (Waters Corp., Milford, MA, USA) coupled with a Xevo TQ triple-quadrupole mass spectrometer (Waters Corp.). Gradient elution and selected ion recording (SIR) were utilized in the novel method that had 3.5 min analysis time per sample. The LC-MS parameters and other details for the instructor are given in the Supporting Information.

Participants and Setting

This study was conducted in spring 2019 and 2020 with 125 students: 13 lower secondary school students (age 14−15 years), 59 upper secondary school students (age 15−18 years), and 53 university students of a first-year laboratory course on experiments in general chemistry. The students performed the experiment in one approximately 3 h session. Each university student analyzed one sample and measured the TPC and AOA spectrophotometrically. In the other groups, the number of samples that the students analyzed varied, and some groups evaluated the TPC and/or AOA only visually. Details of the different student groups are given in the Supporting Information.

Feedback was collected from all student groups. To evaluate the learning outcomes of this laboratory experiment, the university students had several pre- and post-tasks. Two weeks before the laboratory exercise, they answered to a pretest measuring their knowledge of the chemical research instruments. The students were given 15 min time to answer the following four questions:

- Describe how a spectrophotometer works.
- Describe how a liquid chromatograph works.

- Explain what an ion source is.
- Describe how a mass spectrometer works.

One week before the experiment, the university students received the instructions for the experiment and an online assignment to calculate the AOA with the given information. At the beginning of the laboratory practical lesson, the students were given 15 min to create a mind map with the topic "Compounds in plants and their functions". They could supplement their mind maps with a different colored pencil at the end of the lesson, which took 5 min. One week after the experiment, the students answered for the second time the four questions about the instrumentation with a 15 min time limit and calculated the TPC in an online assignment.

■ SAFETY HAZARDS

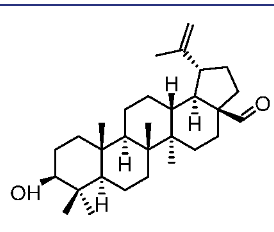
Protective clothing, eyewear, and gloves should be used when handling chemicals. Aqueous ethanol is flammable. Folin–Ciocalteu reagent is an aqueous solution of inorganic compounds. It is corrosive to metals and may cause severe skin burns and eye damage. Sodium carbonate causes serious eye irritation. DPPH is dissolved in ethanol. The powder may cause allergy or asthma symptoms or breathing difficulties if inhaled, and it may cause an allergic skin reaction.

■ RESULTS AND DISCUSSION

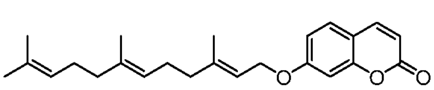
LC-MS Fingerprints and Defense Compounds of White Birch and Silver Birch

Altogether, 103 leaf bud samples were analyzed in this study. The LC-MS analyses were carried out during the laboratory sessions, and students were able to identify their samples as white birch, silver birch, or some other tree species. Here, 42% of the samples were white birches, 43% were silver birches, and the plant species of the rest of the samples remained unknown. The university students analyzed samples that the instructor had already identified, but the species was not revealed to the students beforehand. All students were able to identify their samples correctly with the LC-MS fingerprints.

The LC-MS fingerprints were presented as the combination of SIR traces of ions with *m*/*z* of 365.0 and 439.6. Possible structures are presented in Figure 2, and detailed information about the compounds can be found in the Supporting Information. The SIR chromatograms of theses ions showed small variation in the shapes of the peaks, but all white birch samples had one clear peak at 1.3 min (corresponding *m*/*z* 365.0), whereas silver birch samples had two peaks at 1.7 and 1.8 min (corresponding *m*/*z* 439.6) (Figure 3). However, the method was not able to separate white birch from its subspecies mountain birch (*Betula pubescens* ssp. *czerepanovii*) that was included in the university students' samples, as they had identical fingerprints. In practice, that is a minor problem since mountain birches have a narrow geographical distribution. The SIR chromatograms of samples that were not identified as white birch or silver birch showed background noise at low intensity.



1 Betulin aldehyde, [M–H]⁻ ion at *m/z* 439.6



2 Umbelliprenin, [M–H]⁻ ion at *m/z* 365.0

Figure 2. Possible structures for the marker compounds used in the LC-MS method.

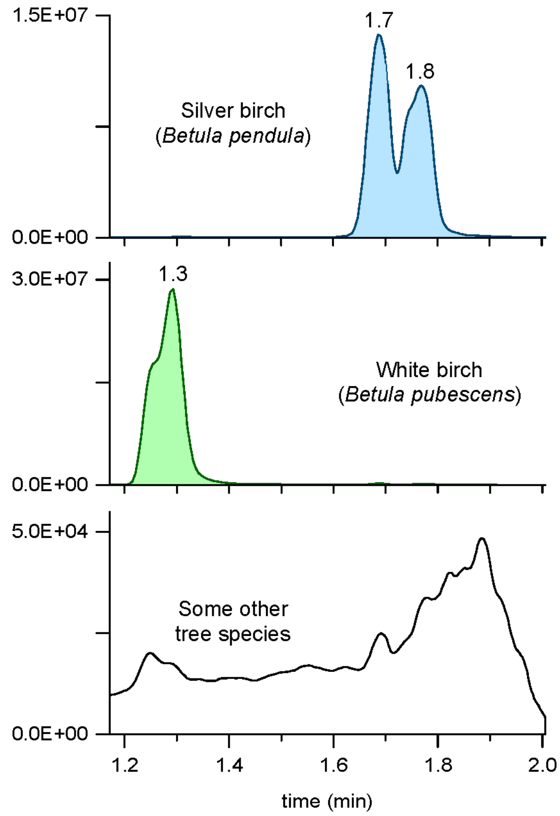


Figure 3. Examples of LC-MS fingerprints of silver birch and white birch leaf buds and an example of a sample that is neither. The LC-MS fingerprint is the combination of two selected ion recording traces of ions with *m*/*z* of 365.0 and 439.6.

Generally, the visual and spectrophotometric results for AOA and TPC were lower for other leaf bud samples than for white birch or silver birch (Figure 4). There was greater variation in the results among the birch samples. In the university students' results, mountain birch samples (N = 5) showed on average lower AOA and TPC than those of white birch and silver birch samples. Silver birch samples (N = 17) showed moderate bioactivity compared to that of mountain birch and white birch samples. The two types of white birch samples were from different trees at different times of the year. The first type of white birch samples (N = 15) had higher TPC and AA compared to the second type of white birch samples (N = 13). The results from the colorimetric tests should be considered only preliminary due to the substantial deviation between the students' results of samples from the same plant. However, some of the deviation may be explained by biological reasons, as, for example, the leaf buds are not all the same size. The visual evaluations were in line with the spectrophotometrically measured values.

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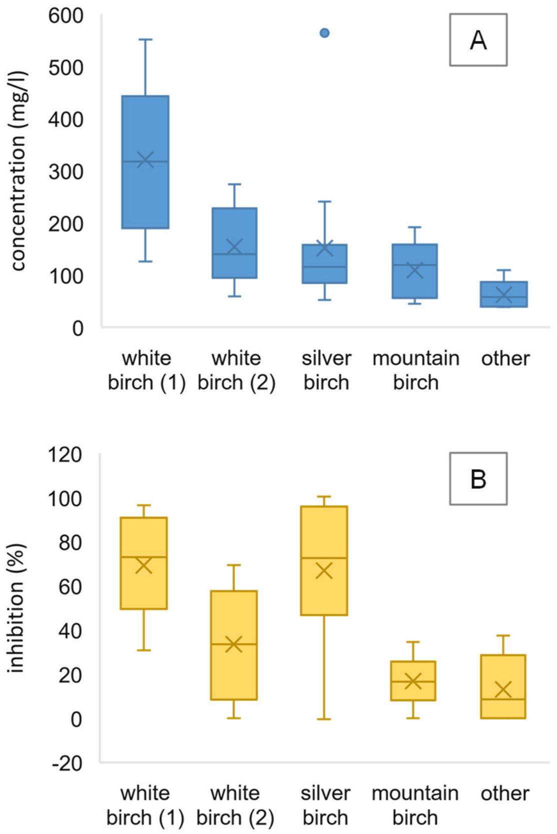

Figure 4. University students' results of the (A) total phenolic contents and (B) antioxidant activity assays.

Despite the deviation in the results, the colorimetric and spectrophotometric assays presented here are suitable for rapid and simple screening of leaf bud samples. If the reagents would be prepared and divided into tubes in advance, the extraction and visual estimations of TPC and AOA could be performed in the nature immediately after sample collection. The assays showed that there are differences in the chemistry between plant species and even between individuals. In addition, they proved that going into the molecular level in plant chemistry might be necessary if one wants to identify the plant species utilizing chemistry. At the end of the laboratory session, students were asked to compare their results with the information on the identity of the plant species. They concluded that the TPC and AOA values alone were not sufficient information for identification. By identifying suitable marker compounds from other tree species, the concept could be expanded to other species as well.

University Students' Understanding of the Instrumentation

Different concepts regarding spectrophotometry, liquid chromatography, and mass spectrometry in the university students' answers in the pre- and post-test were classified and quantified, and a summary can be found in the Supporting Information. The answers were examined from two perspectives: whether they described (i) what the instrument measures or what is the purpose of the instrument, and (ii) how it functions. The goal was to estimate the students' level of knowledge before the laboratory experiment and how the experiment changed students' understanding of the instrumentation and methods used.

When comparing the pre- and post-test answers, the answers improved in terms of what the instruments are used for, and how the measurements are performed. The students connected correct concepts with the instruments more often (Table 1). Many of those concepts mentioned in the answers were rather practical in nature, and showed the effect of the hands-on experience of the laboratory work. For example, the students mentioned a cuvette almost three times more often in the post-test when describing the functioning of a spectrophotometer. The post-test answers got somewhat more specific as well. Instead of the general expression "produces ions", the students could describe in the post-test that an ion source converts molecules into ions. Another practical improvement was seen in how the students learned to utilize calibration curves. In the prelab assignment, only 52% of the university students calculated the AOA correctly. During the laboratory session, 98% of the students calculated the AOA of their own sample correctly. In the postlab assignment, 82% of the students calculated the TPC correct.

Table 1. Number of the Most Common Correct Concepts in the Pre- and Post-test Answers

Instrument	What It Does	How It Functions	Pretest (N = 53)	Post-test (N = 53)
spectrophotometer	measures absorbance/ability to absorb light		13	19
		cuvette is used	7	20
		light goes through the sample	14	23
liquid chromatograph	separates substances		5	11
	”organizes” the particles, they come out at different times		0	7
		polarity	0	8
ion source	converts molecules into ions		1	15
	a part of a mass spectrometer		1	17
mass spectrometer	separates particles of different masses		7	14
	detects a certain molecule		3	16
		mass or m/z value of a particle	12	21
		may function in many different ways	0	10
		quadrupole	2	14
		particles with the correct mass pass through, others do not	4	16

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Table 2. Number of Different Misconceptions in the Pre- and Post-test Answers

Instrument	What It Does	How It Functions	Pretest (N = 53)	Post-test (N = 53)
spectrophotometer	measures the refractive index		9	8
		is based on the refraction/scattering of light	6	2
	measures the wavelength		3	1
	LC described		0	3
liquid chromatograph	separates liquids/phases		5	3
	measures density		1	0
	TLC described		12	14
	centrifuge described		3	0
	spectrophotometer described		2	1
	refractometer described		2	3
ion source		a certain substance does the ionization	5	2
		ionization is based on radiation	4	2
		ionization is performed through an ion beam	0	2
		ionization happens in the quadrupole	0	3
mass spectrometer	no clear misconceptions detected			

The pre- and post-tests also revealed, that the students had several misconceptions regarding especially spectrophotometry and liquid chromatography in terms of what they are used for, while the ion source was linked to misconceptions about how it functions (Table 2). In the pretest, 17% of the students thought that a spectrophotometer is used to measure the refractive index of a substance. Liquid chromatography was confused with thin layer chromatography (TLC) by 23% of the students, and additional 13% confused it with some other instrument such as a centrifuge or a spectrophotometer. In the post-test, some misconceptions were still present, but the number of the most glaring misconceptions had decreased. A refractometer or the refractive index was still mentioned in the spectrophotometry answers, but only two students had made the same mistake in both tests. In contrast, 83% of the students that had described TLC instead of LC in the pretest answered similarly in the question of LC in the post-test. Since these students already had some basic knowledge of chromatography, they could have benefited from comparing LC to TLC, which might have improved their learning outcomes.

There were no clear misconceptions related to the mass spectrometer in the pretest, but the answers were mainly concentrated on mass analyzers. Since the question was not defined to a specific mass analyzer, many students described a sector instrument. Mass spectrometry is not included in the first-year chemistry studies, so the students were not expected to have consistent knowledge about mass spectrometry. Therefore, the instructor accentuated during the laboratory session that there are many different types of mass analyzers that function in different ways. The basic functioning of both the sector instrument and the triple-quadrupole instrument was discussed with the students. In the post-test, magnetic field and a sector instrument were mentioned as well. Unfortunately, some answers were unclear in whether the instrument described was a sector instrument or a triple-quadrupole mass spectrometer. This observation highlights the importance of recognizing students' previous knowledge in mass spectrometry. It might have been be difficult for the students to understand the operating principle of a quadrupole mass spectrometer (utilizes electric fields) if the primary mass spectrometer for them is a sector instrument which utilizes magnetic field as well.

In summary, the laboratory experiment provided university students basic understanding of how the instruments function and what they are used for. The students were rather unfamiliar with the instruments beforehand and they would have needed more time to develop a deeper understanding of the chemistry and mechanisms behind the functioning of the instruments. The pretest revealed some misconceptions about the instruments, but the number of the most significant misconceptions decreased in the post-test. However, the issues about mass spectrometry and liquid chromatography in post-test underlines the importance of recognizing students' previous knowledge when introducing them new methods and instruments.[24,27]

University Students' Comprehension of the Chemistry of Plants

University students' mind maps contained on average 7.4 compounds and compound groups, and 5.2 biological functions at the beginning of the laboratory session. The occurrence of different compound groups and biological functions connected to them are summarized in the Supporting Information. The majority of the compounds were related to photosynthesis (on average 3.0 per mind map). Other primary metabolites were rarer (only 1.7 per mind map). Specialized metabolites such as phenolic compounds, pigments and toxins were present in small numbers before the laboratory experiment. Figure 5 shows the frequency of different compound groups in the students' mind maps.

The most common compounds were water and sugars, which were present in 64% and 74% of the answers, respectively. The functions of these compounds were typically connected to photosynthesis. Out of other primary metabolites, cellulose and nutrients were mentioned most frequently (38% and 32% of the mind maps in total, respectively). The task of cellulose was most often related to the physical structure of plants and in 17% of the mind maps more specifically even to the cell walls. Nutrients, however, were the most often left without functions. After the laboratory session, only few modifications or additions were done in primary metabolites either as new compounds or new functions. Only water and glucose were connected with new functions. This was an expected result, since the primary metabolites or photosynthesis were not discussed during the laboratory experiment.

On average, students added 1.6 new compounds and 1.6 new biological functions to their mind maps, usually regarding phenolic compounds and antioxidants. Before the laboratory

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Figure 5. Different compound groups in the university students' mind maps before the laboratory experiment. The size of the slice is proportional to the frequency of the compound in the mind maps.

session, these types of compounds were mentioned in 23% and 19% of the mind maps, respectively. After the laboratory session, the numbers were 89% and 60%. Also, the number of functions for these compounds increased markedly. Before the laboratory experiment, the function connected to phenolic compounds was defense mechanism. After the laboratory experiment, students described more specifically that phenolic compounds protect plants from herbivores (53% in total) and UV radiation (34% in total). The most common function connected to antioxidants before and after the laboratory experiment was protection from oxidation.

Interestingly, antioxidants were presented as separate nodes in some mind maps (Figure 6A). Generally, all antioxidants are not phenolic compounds, but in the context of this laboratory experiment the observed antioxidant activity is presumably caused by the phenolic compounds.[11] The separate nodes with different functions suggested that some students perceived antioxidants and phenolic compounds as separate groups of compounds, and this issue should be considered when discussing the results with the students. In contrast in Figure 6B, antioxidants and phenols share the same node and the same function. Moreover, it showed how these specialized metabolites differ from primary metabolites: they may be different in different plant species.

The results suggested that the laboratory experiment was able to broaden students' view of the chemistry of plants by adding a new class of compounds, that is, specialized metabolites. Both mind maps in Figure 6 are excellent examples of that. The experiment also made students connect the chemistry to the biology of plants, that is, the functions of the different compounds in plants. Thus, the use of an interdisciplinary approach seemed to support student in creating a more "authentic big picture" of scientific research.[23]

Feedback

University students estimated their understanding and interest by rating how strongly they agreed or disagreed with the statements of the feedback survey on a five-point Likert scale (Supporting Information). The statements were categorized into five main themes:

(1) statements that measured students' interest in the instruments,

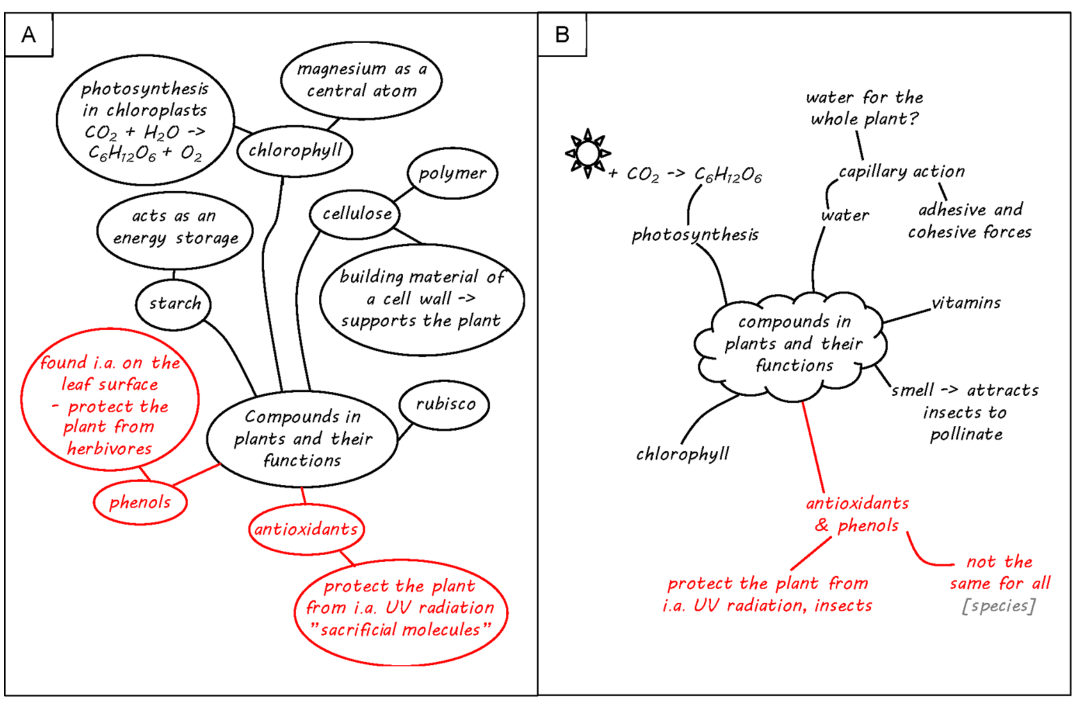

Figure 6. (A) Example of a mind map where phenolic compounds and antioxidants have been presented as separate nodes. (B) Typical mind map with mostly primary metabolites, but antioxidants and phenols presented in the same node. Students were asked to use red color in the markings they made after the laboratory experiment.

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(2) interest toward the research aspects (for example, "I am interested in how research in chemistry is carried out"),
(3) interest toward the topic of the experiment,
(4) enjoyment of the concrete and practical aspects of the experiment, and
(5) understanding of the different phases of the experimental procedure.

Overall, students' responses in all categories were very positive. Students found especially the topic and research in chemistry interesting (Figure 7). They also appreciated the concrete hands-on parts of the experiment. The lower average value for interest toward the instruments is mainly due to the statement "I would have wanted to use the LC-MS instrument independently." Here, 32% of the students agreed with the statement, 42% of the students disagreed, and the rest of the students chose the option in between. However, 79% of the students found it interesting to be able to analyze their own sample with the LC-MS instrument. For the majority of the students, it seemed that giving a short introduction and the possibility to analyze their own sample was enough when they utilized LC-MS for the first time. According to the students' evaluation, they understood well what happened during the experiment. The hardest part to understand was how the fingerprint LC-MS method works.

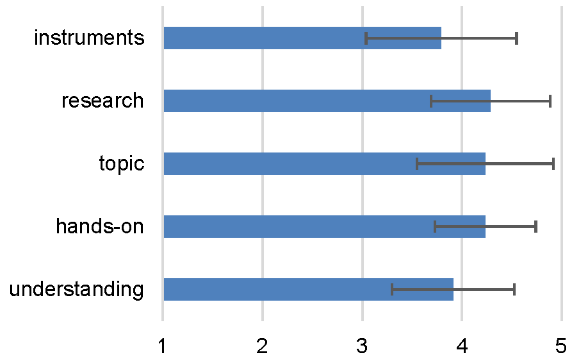

Figure 7. Average grades and standard deviations of students' (N = 53) responses in the feedback survey of how interesting they found the different aspects of the laboratory exercise and how well they understood the protocols; 1 = disagree totally; 5 = agree totally.

Secondary school students were asked to choose their favorite part of the experiment. The majority (59%, $N = 69$) of the students chose the Folin–Ciocalteu and DPPH assays as their favorite part. They enjoyed mixing the solutions, seeing the colors change, and using pipettes. The LC-MS analysis was the favorite part for 26% of the students either because it revealed the birch species, they found the instrument "cool" or they enjoyed hearing how it works. Overall, the students enjoyed working with the laboratory facilities, from syringes and filters to pipettes and actual measuring equipment. These are such tools that are not used regularly at school laboratories, which might explain why the students got excited about them. It also suggested that this laboratory experiment was successful in providing an authentic laboratory experience to them. For example, one of the students wrote the following feedback: "It was cool to work at the university, we got to learn what people actually do at the university and we learned new things."

In summary, the results showed that both the university and the secondary school students appreciated the opportunity to work hands-on with state-of-the-art research instruments. To support students' interest in chemistry and their understanding of the role of instrumentation in chemical research,[25,26] there might be a need for creating even more opportunities for gaining hands-on experiences on the use of modern instruments both in university and secondary education.[27] Also, the students found the topic interesting as in other publications utilizing interdisciplinary contexts.[14,18,21]

CONCLUSIONS

The laboratory experiment was successfully conducted with lower and upper secondary school students as well as with first year university students. It was modified to fit the different time slots booked by the groups by altering the number of samples that the students analyzed, and whether a spectrophotometer was used in addition to the visual evaluations. It provided tools to show how birches differ from each other chemically. With the novel LC-MS method, the birch species could be identified in less than 4 min. With the colorimetric tests, students evaluated the TPC and AOA of their extracts and were able to calculate the AOA and TPC using calibration curves. With these methods they could also make conclusions of the chemical defense of plants. As the mind maps showed, students were able to connect the defense compounds (chemistry) to their functions in plants (biology). The experiment provided students a basic understanding of how a spectrophotometer and UHPLC connected to a triple-quadrupole mass spectrometer work. Finally, the students enjoyed the colorful TPC and AOA tests, and although the mass spectrometric part was the hardest to understand, students appreciated the possibility to analyze their own samples and explore the instrument.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available at https://pubs.acs.org/doi/10.1021/acs.jchemed.0c00589.

Background information, materials and protocols, additional information on the marker compounds, pre- and postlab tests and assignments, and summaries of students' responses (PDF, DOCX)

Student instructions (PDF, DOCX)

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Notes

The authors declare no competing financial interest.

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