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Quantitative Analysis of Amygdalin in Stone Fruit Pits Using HPLC

An Introduction to High Pressure Liquid Chromatography (HPLC)

Experiment Overview

You've all heard someone say, "Don't eat the apple seeds, you'll die of cyanide poisoning!" There is some truth to the statement; as various fruit seeds, pits, kernels, nuts, etc. do indeed contain amygdalin. Amygdalin is classified as a cyanogenic glycoside because an amygdalin molecule includes a nitrile group, which can be released as the toxic cyanide anion by the action of a beta-glucosidase that is present in the intestinal track. Thus eating amygdalin will cause the release of cyanide into the body. The cyanide concentration of seeds varies drastically by type, ranging typically between 1 – 1000 mg cyanide / kg of seed. For perspective, cyanide levels in the blood at levels of 0.5–1 mg/L are typically considered mild, 1–2 mg/L are typically considered moderate, 2–3 mg/L are typically considered severe, and concentrations greater than 3 mg/L generally result in death. In this experiment you will be provided a sample of fruit and tasked with quantifying the amygdalin concentration (and thus the cyanide concentration) in the seeds via high-pressure liquid chromatography (HPLC) outfitted with an ultra-violet (UV) light detector. While you are invited to search the vast research literature available on this topic, a couple of vetted publications have been posted for you on the D2L site to guide you. Note: the suggested references for this project discuss the epimers of amygdalin (D-amygdalin and neoamygdalin), but in this project you are only expected to be concerned with total amygdalin.

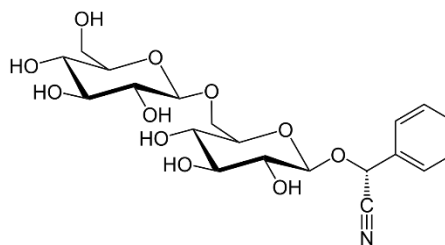


Figure 3.1: molecular structure of amygdalin

This 7-laboratory-session experiment consists of two milestones:

- Milestone 1 - analyze and optimize amygdalin extraction protocols using sunflower seed-based amygdalin standards. It is expected that this milestone will consume the first 3-5 of 7 laboratory sessions allotted for this experiment.
- Milestone 2 – employ optimized extraction and analysis protocol from Milestone 1 to the assigned fruit kernel sample

Milestone 1: Optimization of Amygdalin Extraction Procedure

Milestone 1 Assessment

- Milestone 1 Pre-laboratory assignment : 65 points
- Milestone 1 Experimental and Technical Report : 80 points

Milestone 1 Overview

In this first milestone you will analyze and optimize amygdalin extraction protocols – i.e. optimize sample preparation protocols for amygdalin extraction. The majority of publications discussing amygdalin extraction are concerned with maximizing extraction efficiency; because the goal of those works is typically to maximize the quantity of amygdalin collected. In this experiment, your goal is to accurately and precisely quantify the amygdalin present in fruit kernels, you are not actually concerned with maximizing the extraction efficiency. Thus your primary goal in this milestone is to select and optimize an extraction procedure that yields the most quantitatively precise, or consistent, extraction efficiency.

With even a cursory search of the amygdalin extraction literature, it becomes immediately obvious that a “best” extraction “sample-prep” procedure is a contentious topic. Numerous sources tout aqueous extraction methods over the use of organic solvents, and an equal number of sources tout the exact opposite. Thus based on your own review and analysis of the literature, you will choose to perform *either* an organic or aqueous extraction; and develop an optimized protocol which you must be able to defend with evidence (data). Subsequent to extraction, thorough sample cleanup, or sample preparation, is paramount for achieving success in this experiment. Any interferants or fouling agents present in the final solution, to be analyzed by HPLC, may limit resolution and cause variation (uncertainty) in chromatogram peak areas. Thus it is expected, that in addition to basic filtering, your sample preparation / cleanup plan include both a de-fatting step and solid phase extraction (SPE) step using C18 cartridges/columns. The use of SPE, if performed well, will serve to both remove potential interference and concentrate the amygdalin concentration in your final sample. Based on the reagents and labware available to you for this experiment, two publications are suggested for you to use to guide you in the development of your extraction and sample preparation protocol:

- ‘Amygdalin content of seeds, kernels and food products commercially-available in the UK’
- Quantification of Amygdalin in Nonbitter, Semibitter, and Bitter Almonds (*Prunus dulcis*) by UHPLC-(ESI)QqQ MS/MS’

- both posted on the course D2L site as ‘AmygdalinSamplePrep1 & 2’. The intention is that you utilize the relevant components from the ‘Experimental’ section of the first publication to guide either your aqueous or organic (ethanol) extraction protocols. It is intended that you utilize the ‘Materials and Methods’ section of the second publication to guide you in the SPE column conditioning, concentration / enrichment of amygdalin, and elution. Note: these two references are only intended to provide initial guidance; **it is expected that you experimentally determine (and defend!) an optimized sample preparation protocol.**

For this first milestone, you are to utilize the suggested literature to construct a plan for, carry out, and analyze the efficiency and precision of either an aqueous or organic extraction procedure. Note: in the development of your plan, keep in mind that in lieu of employing Soxhlet extract/reflux setups which are NOT available in this laboratory, you will utilize basic glassware, watch-glasses, stir bars, and hotplates.

This milestone is all about the planning, development, optimization, and analysis of an amygdalin extraction and quantitation protocol – i.e. **experimental design**. It is expected that you spend 3-5 laboratory sessions on this milestone; not just settling on a protocol **but actually optimizing one** – and obtaining evidence to demonstrate optimization. For this milestone you will develop and validate your entire amygdalin extraction and measurement protocol using sunflower seeds/kernels for which you can control the amygdalin content; as native sunflower seeds do not inherently contain amygdalin. Thus by adding a known amount of amygdalin to aliquots of homogenized seed samples – i.e. seed samples thoroughly ground with supplied coffee grinders - you have amygdalin standards in hand in which you can employ to develop and validate extraction and analysis procedures.

Subsequent to sample preparation – where the product is a homogenous amygdalin solution- amygdalin quantitation will be achieved using HPLC. HPLC is used (versus, for example, UV-Vis spectrophotometry) because even after thorough sample preparation, there will still be numerous compounds present that interact with light at the same wavelengths as amygdalin. Thus HPLC enables separation of these interfering compounds to enable amygdalin quantitation. Because multiple groups will be simultaneously utilizing/sharing the HPLC instruments, a single mobile phase of methanol and water (25:75 v:v) will be used in this experiment.

Milestone 1 Pre-lab Assignment

Recall that it is mandatory that your pre-lab assignment for the specific milestone be approved by your TA before you begin performing any experimental work. This is a rather lengthy pre-lab assignment which demands you to read the posted references and grasp an overall understanding of the entire experiment.

1. A critical component of quantitative chemical analysis is estimating lower-concentration limits of detection (LOD) and the resolution for a given method. It is essential to be aware of the minimum concentration that can be statistically resolved from a sample with zero analyte – i.e. the LOD. It is also essential to be aware of what the minimum difference in analyte concentration is that can be statistically resolved by the method employed – i.e. the resolution. Determining estimates for a method's LOD and resolution relies on two measurement parameters: i) a measure of the uncertainty, or variation, in the measured response obtained from repeated measurements of the same sample and ii) an estimate of the sensitivity of the method response to the analyte – e.g. the slope of the calibration curve. A common model used to estimate the LOD from chromatographic peak areas is:

$$\text{LOD} = \frac{3\sigma}{m} \quad (\text{equation 1})$$

where σ is the sample standard deviation in the measured response obtained from a statistically significant number of repeated trials with the same sample, and m is the sensitivity (i.e. the mean slope) of the calibration curve obtained. Similarly, one model for estimating the resolution for a given method when employing chromatograms is:

$$\text{resolution} = \frac{\sigma}{m} \quad (\text{equation 2})$$

Figure 3.2 is an example of a seven overlaid gas chromatograms obtained from a series of 2,3-dimethylpyrazine standards. The quantitative data from these standards is tabulated in Table 3.1

Standard Concentration [uM]	Peak Area [counts min ⁻¹]
138.	82.8
112.	68.8
98.	56.4
76.	44.5
43.	25.3
34.	17.9
11.	4.55

Table 3.1: Chromatographic peak areas obtained from gas chromatography of seven 2,3-dimethylpyrazine standards

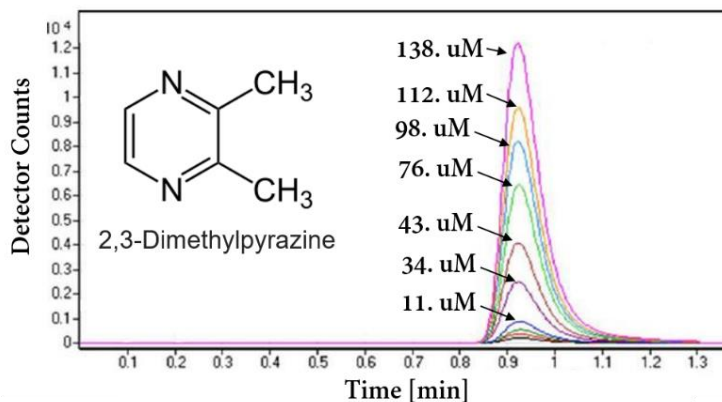


Figure 3.2 Overlaid gas chromatograms obtained with seven different 2,3-dimethylpyrazine standards

In order to obtain an estimate of the uncertainty in this method, which is necessary to estimate the LOD and resolution, 10 additional trials were performed, specifically with 34. uM 2,3-dimethylpyrazine standard, with the results tabulated in Table 3.2.

Use the data from Table 3.1 to construct a calibration curve and obtain an estimate for the method sensitivity, and the data from Table 3.2 to obtain an estimate for the uncertainty in the method to calculate and report both the LOD and resolution of the method

Trial #	Peak Area [counts min ⁻¹]
1	18.49
2	18.39
3	17.74
4	18.58
5	18.21
6	17.94
7	17.87
8	17.30
9	18.33
10	17.72

Table 3.2: Chromatographic peak areas obtained from gas chromatography with 10 trials of 34. uM 2,3-dimethylpyrazine standard

2. In the previous question, a table of chromatographic “peak areas” was provided for your analysis, but it is often necessary for you to numerically integrate the area under chromatographic raw-data yourself to obtain such peak areas. This is what you will do in this pre-laboratory question. A sample of “raw” chromatographic data, similar to what you will obtain with the HPLC instruments and software in this experiment, titled ‘NumericalIntegrationPeakAreas.xls’, is available for you to download from the D2L site. By graphing this data, you will observe two peaks, centered at ~2.0 and ~3.0 minutes respectively. **Your task for this question is to numerically integrate the area under each of**

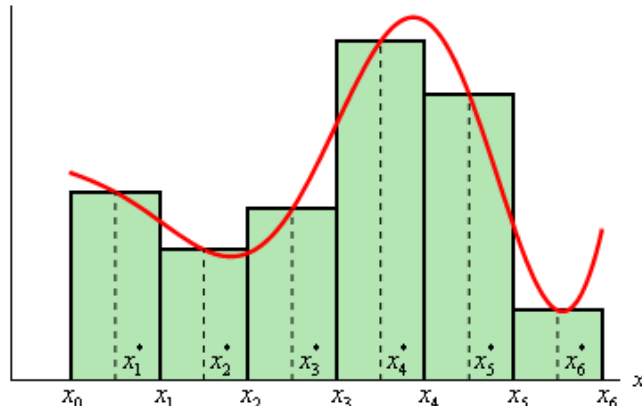


Figure 3.3 Illustration of a numerical 'rectangular' (also referred to as 'midpoint rule') integration

these two peaks. While there are many approaches to this challenge, for ease it is suggested that you employ Excel (or some spreadsheet program) and perform your numerical integration via rectangular integration (often referred to as the ‘midpoint rule’ for numerical integration), as illustrated in Figure 3.3, for the two chromatographic peaks. You can readily Google this topic or visit your introductory calculus text to refresh your memory on performing this. A couple of key points to consider in the process:

- Make sure to find the area under each peak **RELATIVE TO THE BASELINE**. If the baseline is not at 0.0, you should shift the baseline to zero or account for this offset in another way
- Make sure to include units associated with the absolute areas you calculate and report for each peak.
- You will need to estimate where each peak begins and ends – i.e. where to begin and end your numerical integration for each peak

Clearly report the areas – including units – under each of the two peaks. Make sure you are comfortable performing this process with Excel (or a similar software tool) as you will be doing this extensively with your HPLC data for this experiment.

- The HPLC instruments you have access to in this experiment are outfitted with UV light sources-detectors. These detectors only monitor a single wavelength of light – one you must specify at the initiation of the experiment. Present a plan for how you will determine the wavelength of light that you will utilize to monitor amygdalin when performing HPLC. Hints: It is expected that you perform a cursory online/literature search to obtain estimates for standard concentrations and possible light wavelengths for monitoring – parameters you will experimentally confirm and optimize. You have access to UV-Vis spectrophotometers. In order to minimize waste and cost, make sure that any amygdalin standards you prepare will be used for subsequent parts of this experiment as well – consider this aspect in your pre-lab plans.
- Present a plan for how you will prepare a sunflower seed standard with a cyanide concentration in the range of 100 – 1000 mg cyanide / kg of seed. Based on your reading of the literature, what mass of sunflower seeds are you going to work with? Recognize that one molecule of amygdalin can result in a single molecule

of hydrogen cyanide release. You have access to pure D-amygdaline hydrate for this experiment. Note: it should always be one of your goals should be to use minimal reagents and materials in these experiments.

5. Present a plan for how you intend to identify amygdalin in the HPLC chromatograms – i.e. when you inject an extracted sample you may observe multiple peaks; which peak corresponds to amygdalin? Hint: based on published literature, what do you expect the retention time to be for amygdalin? And how will you confirm this for your experimental setup?
6. Present a plan for how you will determine estimates for the HPLC method sensitivity, LOD, and resolution. This plan should include details of any standard(s) you prepare.
7. Present a detailed plan for your aqueous or organic extraction of amygdalin – including optimization variables. This plan should include experimental apparatus, time, temperatures, conditions, volumes, filtering steps, centrifugation, estimates of masses and volumes, etc.
8. Part of your sample preparation / cleanup should include a de-fatting step (as described in the posted references). Present a plan for how you will de-fat your samples.
9. Present a plan for how you intend to employ SPE in the sample preparation / cleanup process. As a component in this first milestone is optimization of you sample preparation using SPE (i.e. achieving sufficient efficiency and best precision), make sure to discuss any variables you plan to employ and how you intend to justify the SPE protocol that you settle upon

Milestone 1 Experimental

(Note: these experimental bullet-points are not necessarily in the order that they must be completed, they are just a list of requirements for the experiment and pre-lab, and it is up to you to organize your time to complete them)

- Demonstrate that you can unambiguously identify amygdalin via the HPLC instrument – specifically you should acquire an estimate for its retention time and identify the optimum UV wavelength to be used in your experiments.
- Employ external standards and acquire sufficient data that enables you to determine the method LOD, sensitivity, and resolution.
- Prepare a sunflower seed standard with a cyanide concentration in the range of 100 – 1000 mg cyanide / kg of seed (i.e. a standard in the range of what you expect for your real seed kernel sample which is analyzed in Milestone 2). This milestone is the experimental design component. A significant objective of this milestone is determination of quantities of samples, solvents, and reagents necessary for performing quantitative analysis of amygdalin. An outcome of this milestone is development of a detailed procedure you will follow (and could provide to someone else, maybe through the “Experimental” section of a technical report) for the analysis in Milestone 2.
- Extract amygdalin from your sunflower seed standard via aqueous or organic extraction. Perform and optimize de-fatting, SPE, and general cleanup protocols you proposed in the pre-laboratory. Note: a significant objective in this milestone is the optimization of this extraction protocol – thus you must acquire evidence (i.e. DATA) to defend / justify the protocol that you settle upon as ideal.
- Employ HPLC to quantify efficiency and precision of your amygdalin extraction protocols.

Milestone 1 Technical Report

Download and use the “Technical Report Template” to construct a concise, neat, and well formatted technical report for this Milestone 1. This Technical Report for Milestone 1 must be submitted no later than 7 days after the day that you begin the experimental laboratory component of Milestone 2. Your report must include:

- Abstract - A brief statement about the optimization of the extraction protocol that you settled upon including the extraction efficiency and precision achieved.
- Sample HPLC chromatogram identifying the retention time for amygdalin - this figure should be professionally formatted, include a caption that lists the mobile phase, amygdalin concentration, mobile phase flow rate, stationary phase, and wavelength used for detection.
- Calibration curve figure illustrating the method response vs. amygdalin concentration. The figure must include a caption that lists the mobile phase, amygdalin concentrations, mobile phase flow rate, stationary phase, and wavelength used for detection. The calibration curve figure should include the best-fit linear model.
- A table summarizing estimates for the HPLC method LOD, sensitivity, and resolution obtained using amygdalin external standards.
- Table and/or figure summarizing the results from your extraction optimization protocols. It should present the initial concentration in the “spiked” sample, the measured amygdalin concentration after sample prep, and thus an extraction efficiency and precision for each protocol. This table/figure should communicate to the reader which extraction method is optimal (based on precision) for this analysis.
- A brief discussion (defense) that references your DATA in order to justify the extraction method that settled upon as best and will be employing in the next milestone. It should include some optimization variable where the graph clearly illustrates the optimization of some parameter of your choosing.
- Experimental: detailed procedure for the optimal extraction protocol that you have settled upon
- Experimental: explain you prepared the sunflower seed-based amygdalin standards.
- Reference section – you must reference any works from which you obtained experimental procedures, precedents and/or justifications. This includes the laboratory manual. Science progresses by standing upon the shoulders of those who have gone before us, and the least one can do is give credit to those. Nobody enjoys writing the references section, but think of it as being respectful to the works that have assisted you. And it is unethical to not reference.
- The carbon copies of your notebook pages used for this Milestone 1 should be attached to the back of the hard-copy of this report when you submit it

Milestone 2: Amygdalin Quantitation in Fruit Kernels

Milestone 2 Assessment

- Milestone 2 Pre-laboratory assignment : 45 points
- Milestone 2 Experimental and Technical Report : 70 points

Milestone 2 Overview

In this second milestone you will employ the protocol you developed in Milestone 1 for quantifying the amygdalin (also enabling cyanide quantitation) in provided fruit kernels. Go!

Milestone 2 Pre-lab Assignment

Recall that it is mandatory that your pre-lab assignment for the specific milestone be approved by your TA before you begin performing any experimental work.

1. What should we use: peak heights or integrated peak areas for quantitative analysis of chromatograms?

A chromatogram, such as illustrated in Figure 3.4, is a time-based graphical representation of a detector response to various chemical species within the eluted matter from a chromatographic separation. In order to perform quantitative chemical analysis, some mathematical model relating the detector response and analyte concentration (e.g. calibration or response curve) is typically constructed through the use of standards. A common inquiry about quantitative analysis of chromatograms is, ‘should I use the peak *heights* or peak *areas*?’ If chromatograms yielded ideal peaks – i.e. perfectly symmetric and constant width “normal” peaks – the decision would be inconsequential as both options would yield comparable and sufficient results. But in typical “real” chromatographic experiments, dispersion, loading effects, column non-idealities, non-linear detector responses, etc. all rear their ugly heads to some degree, resulting in peaks exhibiting broadening, splitting, fronting, and tailing as illustrated in Figure 3.5. Thus the choice between utilizing the peak area or peak height for baseline resolved peaks is *not* immaterial and can result in significantly different quantitative results.

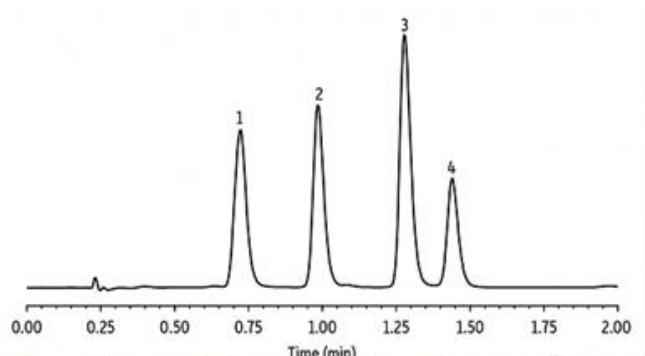


Figure 3.4 : Sample chromatogram in which the x-axis is time and the y-axis is a transduced signal form a detector in the chromatography

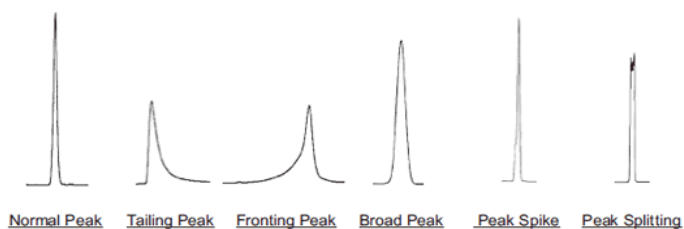


Figure 3.5 : Illustration of ideal "normal" chromatographic peak as well as common non-ideal peaks typically observed in chromatograms

Since integrated peak areas tend to be less sensitive than peak heights to non-idealities, quantitative chromatogram peak analysis utilizing peak areas will generally yield calibration curves with less uncertainty than those constructed from peak heights. In this pre-lab assignment you are provided the opportunity to apply and compare both methods to a set of chromatographic data. Figure 3.6 is a series of overlaid gas chromatograms obtained from a set of 2,3-dimethylpyrazine standards in ethanol. 2,3-dimethylpyrazine is the volatile compound responsible for the distinct aroma of roasted sesame seeds.

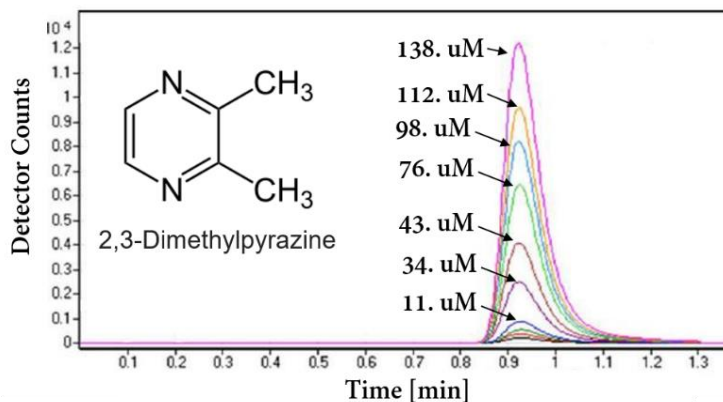


Figure 3.6 Overlaid gas chromatograms obtained with seven different 2,3-dimethylpyrazine standards

The data presented in Table 3.3 corresponds to the overlaid chromatograms presented in Figure 3.6. Your task is to utilize the data provided in Table 3.3 and produce two calibration curves:

- 1) Peak height vs. concentration
- 2) Peak area vs. concentration.

Your calibration curve models that you construct (and present as a product of this pre-laboratory question) to quantitate the concentration of unknown solutions based on a measured response **must include estimates for the uncertainty in both fitted parameters – i.e. uncertainty in the slope and y-intercept** - of the form:

$$\text{concentration } (\pm \text{uncertainty}) = \frac{(\text{detector response}) - (\text{calibration curve } y\text{-intercept} \pm \text{uncertainty})}{\text{calibration curve slope} \pm \text{uncertainty}}$$

In order to obtain estimates for uncertainty quantities associated with the best-fit slope and y-intercept values for your calibration curves, you should employ the LINEST function in Excel and consider the standard uncertainty provided from LINEST as the uncertainty associated with each parameter. If the LINEST function is new to you, refer to the document provided on D2L and/or Google the topic which will lead you a number of webpages dedicated to describing this function.

Standard Concentration [uM]	Peak Height [counts]	Peak Area [counts min ⁻¹]
138.	1.21 x 10 ⁴	82.8
112.	9.63 x 10 ³	68.8
98.	7.34 x 10 ³	56.4
76.	5.88 x 10 ³	44.5
43.	4.13 x 10 ³	25.3
34.	1.93 x 10 ³	17.9
11.	1.10 x 10 ³	4.55

Table 3.3: Chromatographic peak heights and peak areas obtained from gas chromatography of seven 2,3-dimethylpyrazine standards

Now that you have your response curve models – including estimate for uncertainty associated with them - you run an aliquot of a solution with an unknown concentration of 2,3-dimethylpyrazine present which results in a chromatogram for which the analyte peak can be characterized by:

- Peak area = 31.70 counts min⁻¹
- Peak height = 4452 counts

Use the models you constructed from the calibration data to calculate and report a concentration, and corresponding uncertainty, of 2,3-dimethylpyrazine in this solution. Consider these reported values and provide a short (~25 words) summary of the implications of the results of quantifying analyte via peak area versus peak height. Also make sure that your pre-lab submission clearly presents the models you employed - including the uncertainty associated with each term – to estimate the concentration of the unknown.

2. This pre-lab assignment is actually the “Experimental” section required for your “technical report” for Milestone 1. Thus it is expected that his pre-laboratory assignment be typed – not hand written. Based on what you did in Milestone 1 – present a detailed plan of how you are going to perform your sample preparation (e.g. sample collection, sample mass, drying, cleanup, de-fatting, concentration, homogenization, etc.) and quantitative analysis. This should include mobile phase used, HPLC settings, calibration standards – in other words a plan at a detailed level where you could ideally hand it off to someone else to perform. Note: this “plan” is the entire pre-lab and worth a significant fraction of the points allotted for this entire experiment – so it is in your best interest to put forth sufficient effort on this.

Milestone 2 Experimental

(Note: these experimental bullet-points are not necessarily in the order that they must be completed, they are just a list of requirements for the experiment and pre-lab, and it is up to you to organize your time to complete them)

- Employ the optimized extraction method, you developed in Milestone 1, to the fruit kernels
- Obtain a calibration curve from standards, using a single batch of mobile phase, during the same session that you will also run your unknown.
- Perform a statistically significant number of trials with your amygdalin extract and determine a mean peak area obtained with your unknown

Milestone 2 Technical Report

Download and use the “Technical Report Template” to construct a concise, neat, and well formatted technical report for this Milestone 2. This Technical Report for Milestone 2 must be submitted no later than 7 days after the last session allotted for this experiment.

- Abstract - A brief statement of what you analyzed, the technique, and the result (with uncertainty)
- Sample HPLC chromatogram from a sample – identifying the peak(s) of amygdalin.
- Calibration curve figure illustrating the instrument response vs. amygdalin concentration – plus inclusion of the data points for the response from amygdalin extracted from your sample on this

same graph. The figure must include a caption that lists the mobile phase, amygdalin concentrations, mobile phase flow rate, stationary phase, and wavelength used for detection.

- Results/Discussion : clearly present the model – including estimates of uncertainty associated with each term in your fitted model (as you did in the pre-laboratory assignment) – that will be used to determine the amygdalin concentration of your sample
- Table and/or figure summarizing the results from your various HPLC trials with your sample – this table should report results in terms of peak areas (mean, standard deviation) from your various trials as you will employ your model (as you did in the pre-lab) to estimate the actual concentration.
- Table and/or figure: Clear presentation of the amygdalin concentration in your sample (with uncertainty) and extrapolation to the mass of amygdalin per kilogram of seed (including uncertainty).
- Experimental: detailed experimental section for this experiment.
- Reference section – you must reference any works from which you obtained experimental procedures, precedents and/or justifications. This includes the laboratory manual. Science progresses by standing upon the shoulders of those who have gone before us, and the least one can do is give credit to those. Nobody enjoys writing the references section, but think of it as being respectful to the works that have assisted you. And it is unethical to not reference.
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