IJPCE
International Journal of Physics and Chemistry Education, 15(2), 33-44

www.ijpce.org
ISSN: 2589-8876

RESEARCH ARTICLE

Teaching Food Analysis Through Unknowns: Study Case for Methylxanthines, Organic Acids, and Sugars Examination Over Liquid Chromatography

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Abstract

Three laboratory sessions are described, each one focused on a distinct group of analytes (i.e., 1. Methylxanthines, 2. Organic acids 3. Sugars). To study each analyte group, food profiling was achieved using liquid chromatography; where each analyte (n = 3, 15, and 5 analytes for methylxanthines, organic acids, and sugars, respectively) was identified and quantified. Different food samples (including beverages, powders, cereals, and dairy products) were given to students who within each class knew the possible identities of the group of samples given but had to pair, after examination, the resulting profiles obtained with each food sample as the food samples were unidentified (unknowns). Quali/Quantitative data were recollected from the resulting chromatograms after each food was subjected to analysis. For organic acids, solid phase extraction and potentiometry were used as tools to demonstrate separation science from colored drinks as sample pretreatment and as a classic alternative for instrumental analysis.

Keywords

food analysis, unknowns, liquid chromatography, methylxanthines, organic acids, sugars

◆ Received 20 May 2023 ◆ Revised 31 May 2024 ◆ Accepted 2 June 2024

Background

In food analysis courses that introduce the theory and application of prevalent methods available to characterize foods and their ingredients, students learn the principles behind analytical procedures and appraise their aptness for examining specific food products (Castro et al., 2021). Within these courses, laboratories present a student with a hands-on approach to learning food analysis; within this approach, we suspected that working with unknowns will help the student develop some of these abilities that they will need further down the road. Hence, we selected three different sets of analytes, which are routinely used in the food industry and research, for the students to examine.

State of the literature

- Most of the educational experiments are focused on methylxanthines, additionally, a lot of literature is available that tackles the analysis of methylxanthines including those using liquid chromatography.
- Some educational experiments focus on organic acid analysis, however, again, in literature, several papers use liquid chromatography to assess the organic acid profile in foods, especially in wine.
- Overall, few educational experiments focus on sugar analysis, most of the rest of the literature focuses on total sugar analysis rather than individual saccharide analysis using approaches such as liquid chromatography.
- The most recent educational experiments published based on liquid chromatography are for methylxanthines and organic acids.
- No experiments based on solving unknowns as teaching tools have been reported in food science laboratories.

Contribution of this paper to the literature

- Improving knowledge in food composition as complete profiles for several foods (including solid matrices/coffee and tea powders, fermentation products/beverages) are provided.
- Explores the subtle differences within foods and analyte profiles and highlights the type of information that is available after a liquid chromatography analysis.
- Teaching different principles of sample preparation and liquid chromatography with three other compounds extracted from diverse foods while encompassing the basis of food composition.
- The experiment will contribute to the gamut of liquid chromatography experiments available; few are available specifically tailored for food analysis.
- There is a vast array of samples/unknowns that can be used to highlight different aspects of food science and industry.
- This experiment is versatile enough to highlight concepts for grad and advanced undergrad courses.

Methylxanthines are a group of phytochemicals derived from the purine base xanthine and obtained from plant secondary metabolism which possess interesting biochemical and pharmacological properties (Monteiro et al., 2016; Monteiro et al., 2019; Jean-Marie et al., 2021). Plant species that generate considerable amounts of these compounds, and which are of interest to the food industry, include tea (*Camellia sinensis* L.), coffee (*Coffea* sp.), and cacao (*Theobroma cacao* L.), yerba mate (*Ilex paraguariensis* A. St.-Hil.) (Sanchez, 2017; González-Yépez et al., 2023).

From the teaching point of view, there are several educational papers focused on the determination of methylxanthines in food preparations mostly based on liquid chromatography (Novaki et al., 2021; Stitzel & Sours, 2013; Menguy et al., 2009). As with other research, methylxanthine analysis is usually focused on cocoa (see for example, Borja-Fajardo et al., 2022).

https://doi.org/10.51724/ijpce.v15i2.355

On the other hand, organic acids can prevent spoilage and improve food taste which enhances consumer acceptance and appeal (Bangar et al., 2022). Much attention has been given to these compounds, especially for the quality control of fermentation products (Cortés-Herrera et al., 2019). Chemical profiling has been used to assess lactic acid bacteria organic acid production (see Kuley et al., 2020, as an example).

There are available for the teacher/student a couple of interesting laboratory methods applied to food analysis that are very useful as instructions for organic acid quantification including one based on thin layer chromatography (analysis of fruit juices, Samarasekara et al., 2018) and HPLC (kombucha fermentation, Miranda et al., 2016).

Finally, as carbohydrates appear in virtually all food products, and with the increasing perception of high sugar levels in some foods, the interest in rapid, sensitive, and reliable methodologies for sugar analysis has increased (Ondrus et al., 1983). Hence, liquid chromatography has permitted automation and the identification of each sugar fraction in a specific food converting it into the most common approach for sugar analysis in foods (Cortés-Herrera et al., 2019).

There are some methods destined to teach sugar analysis including those using spectrophotometric analysis to assess total sugars (Bittman, 1974), HPLC (using a radially compressed column, Ondrus et al., 1983 and electrochemical detection, Luo et al., 1993), and NMR (Navarro et al., 2020).

From the chemical education standpoint, experiments requiring students to determine the identities of unknown compounds are common in chemical education. These experiments develop students' analytical thinking skills and their ability to apply these skills to real-world problems. The study by Dimidi et al. (1999) explores the impact of working with unknowns in chemical analysis courses on student success, showing the importance of such experiments in the learning process. Unknown analysis is already a common strategy in courses like analytical or organic chemistry (Vuilleumier, 1930; Liotta & James-Penderson, 2008).

Research has demonstrated that in chemistry (even in the laboratory setting) active learning requires improving both cognitive and psychomotor skills (Loppnow, 2018). Unknown analysis also improves problem-solving in chemistry and provides specific scaffolding for students who experience procedural difficulties (Yuriev et al., 2017). Similarly, several works by Bodner and coworkers (Bodner, 2015; Bodner & Bhattacharyya, 2018; Bodner & Domin, 2000) highlight the central role of problem-solving in learning chemistry, in this regard, unknown analysis may help as another strategy to highlight the challenges students face in the problem-solving process and how to surpass them making food analysis learning more effective.

Research in food science and technology is directly related to chemical education. Analyzing the chemical components of foods provides concrete examples of the applications of chemistry in everyday life (Franco-Mariscal, 2018; Md Noh et al., 2020; Naviglio & Gallo, 2020; Mondal et al., 2023). Food science is quite vital (He, 2019), understanding the chemical principles that govern it is essential for food scientists and technologists to manipulate and control the sensory qualities of food, optimize food preparation methods, and innovate (Boro, 2023). It also plays a role in ensuring food safety and quality throughout the food production and distribution chain. Hence, the three types of analytes selected here represent metabolites or ingredients that are routinely assessed in the food and beverage industry to evaluate quality (Nielsen, 2024). For students it also can provide a starting point into how food products are designed (McClements & Großmann, 2021) and even how we eat (McClements, 2019).

The main objective of this work was to assess if advanced food science students were able to guess the identity of selected food unknowns based solely on their chemical fingerprints and quantitative information. Students performed sample pretreatment according to general protocols for each analyte group and afterward, chromatograms obtained from each unknown were handed over. They were asked to analyze the information given and try to identify several foods based on their methylxanthine, organic acid, and sugar quantitative profile obtained during liquid chromatography analysis.

Using this approach, the students will actively i. Learn to problem-solve using complex food matrices ii. Familiarize themselves with several food profiles iii. Use LC (using two different types of detectors) to obtain profiles for routinely assessed metabolites in the food industry iv. Understand several extraction methods and approaches for sample pretreatment before LC analysis and v. Expand their knowledge regarding LC and proper chromatographic practices during analysis.

Experimental procedure

Duration of each laboratory session and unknowns

Students who participated in these laboratories were advanced food science students. During the duration of the course (the first half) they have already been working with HPLC analysis of other food components. During the lab sessions, the students were exposed to the LC systems available in the laboratory and were explained how each system was set up and how the data analysis works.

Each laboratory session lasted a total of four hours, a total of three sessions were used to complete all tasks (one session for each family of compounds and a round of unknowns). For the first laboratory session that involved unknowns, $n = 7$ solid samples were selected, for the second session only liquids ($n = 7$) were selected, and for the final session food samples were mixed (**Table 1**). Each unknown was given to a laboratory pair, in an unmarked 20 *mL* vial and just identified with letters A-G. Data analysis and a report were created individually for a total of $n = 10$ students. For each lab session, the students were asked to deliver a post-laboratory brief analysis with their results and final assignation for each unknown, to assess how they reach their conclusions.

Table 1. Unknowns were used during the laboratory sessions

	Methylxanthines (Lab session 1)	Organic acids (Lab session 2)	Sugars - (Lab session 3)
A	Decaffeinated instant coffee	Beer	Coca-Cola®
B	Pure roasted coffee	Apple cider	Apple juice
C	Black tea	Miso soup	Sangria-Type Wine
D	Green tea	Kefir	Cereal bar
E	Earl gray tea	Liquid yogurt	Lactose-free flavored milk beverage
F	Cocoa	Red wine	Beer
G	Yerba mate	Kombucha	

Methylxanthines

Caffeine is among the most broadly consumed central nervous system stimulants in the world, it is commonly added to some soft and energy drinks (Sanchez, 2017). However, methylxanthine analysis is paramount as some people require decaffeinated versions of these drinks as they may suffer from sensitivity or overstimulation effects. Then, the safety and recommended dosages of caffeine in healthy adults and vulnerable populations like children and pregnant women ingested through food and drinks should be monitored (Reddy et al., 2024).

In the case of methylxanthines, we selected only solid samples to force the students to use solid-phase extraction. Then, sample pretreatment was performed as described by Menguy et al. (2009). Briefly, 500 mg of the sample was crushed over 20 mL H_2O. Ultrapure water for all extraction and chromatographic steps (type I, 0.055 $\mu S\ cm^{-1}$ at 25 °C, 5 $\mu g\ L^{-1}$ TOC) was obtained using an A10 Milli-Q® Advantage system and an Elix® Advantage 10 system (Merck KGaA, Darmstadt, Germany). The solutions were placed in sealed tubes and heated in a water bath at 80 °C for 15 min. At this point, the students were told to also extract a second sample using mobile phase (see below) and ultrasound (AO-08895-91, Cole-Parmer, Vernon Hills, IL, USA). For methylxanthines, this step is critical since Chin et al. (2008) already demonstrated the relationship between steep-time and caffeine extraction. Sample extracts were obtained through sequential conditioning of the SPE tube (Sep-Pak® Vac, C_{18}, 3*cc*/500 *mg*, WAT020805, Waters Corporation, Milford, MA, USA), the addition of the sample, washing, and elution as follows: activation and equilibration were performed using 1 *mL* MeOH and H_2O, afterward, 0.5 *mL* of the sample extract was passed through the column and washed with H_2O. Elution of the compounds of interest was performed using 2× 2 *mL* MeOH. All steps pertaining to the extraction cartridge were performed by using a single SPE tube processor (Visi-1, 57080-U, SUPELCO, Bellefonte, PA, USA). The resulting eluate was evaporated to dryness under nitrogen at 40 °C and each residue was dissolved in 1000 μL of H_2O and then filtered by using a 0.45 μm syringe filter (18406, regenerated cellulose, 13 *mm* diameter, Sartorius®, Göttingen, Germany), 100 μL of the resulting filtrate were mixed with 900 μL of H_2O in an HPLC ready vial for injection (2 *mL*, borosilicate type I glass, screw cap, red PTFE/silicone septa, 8010-0542, Agilent, Santa Clara, CA, USA) resulting in a 10 fold dilution.

Table 2. Experimental data obtained during standard analysis for methylxanthines

Analyte	Retention time, min	Calibration curve equation
Theobromine	2.773	$y = 2.06\times10^{6}x + 1.46\times10^{5}$
Theophylline	4.217	$y = 3.20\times10^{6}x + 3.92\times10^{3}$
Caffeine	6.034	$y = 2.97\times10^{6}x + 1.69\times10^{4}$

Methylxanthines were assessed by a modified version of the method based on the work by Srdjenovic et al. (2008). The method was previously validated and ISO/IEC 17025 accredited using a Shimadzu system (Shimadzu, Kyoto, Japan) equipped with a photodiode array detector (SPD-M20AV), column compartment (CTO-20A), autosampler (SIL-20A HT) and a quaternary pump (LC-20AT). A Zorbax Eclipse C_{18} column (150 *mm* × 4.6 *mm*, 5 μm particle size; Agilent Technologies) was used to perform the separation. An isocratic method using MeOH-H_2O (24:76) at 1 $mL\ min^{-1}$ was used, and the column temperature was kept at 25 °C. Detection was performed at a wavelength of 272 *nm*. The injector volume was set to 20 μL. Under these conditions, all three major methylxanthines can be assessed in the same chromatographic run. To assess the concentration of caffeine, theobromine, and theophylline (**Figure 1A**, **Table 2**), 1 *mg* of each standard was weighted in a 2 *mL* vial and 1000 μL H_2O was added to the mixture to obtain a stock solution which was diluted accordingly to obtain an eight-point calibration curve was prepared from 2 to 160 $\mu g\ mL^{-1}$, of each analyte.

The students were quick to observe that unknown F has a high content of theobromine and cataloged it as cocoa (**Table 3**). In addition, unknowns A and B were cataloged correctly as decaffeinated and regular coffee, respectively, as A had a negligible amount of alkaloids and B was the only sample that showed only caffeine as the sole alkaloid (**Table 3**). For these three cases, all students correctly assigned the identity (n = 10/10, 100%, correctness). Yerba mate (unknown G) was correctly assigned by n = 7/10 students. From the samples tested, yerba mate has been reported to exhibit the content of the three alkaloids (Gawron-Gzella et al., 2021) (**Figure 1B-H**).

Considering Earl Grey is the result of a mixture of black tea and bergamot oil (Jankech et al., 2019), some confusion is expected among unknowns C and E, as expected were correctly assigned by only 60% and 30% of students, respectively. Notwithstanding, the students did effectively recognize that these two samples were characterized by having higher concentrations of caffeine than other types of tea (i.e., unknown D, green tea; Jankech et al., 2019) (**Figure 1B-H**).

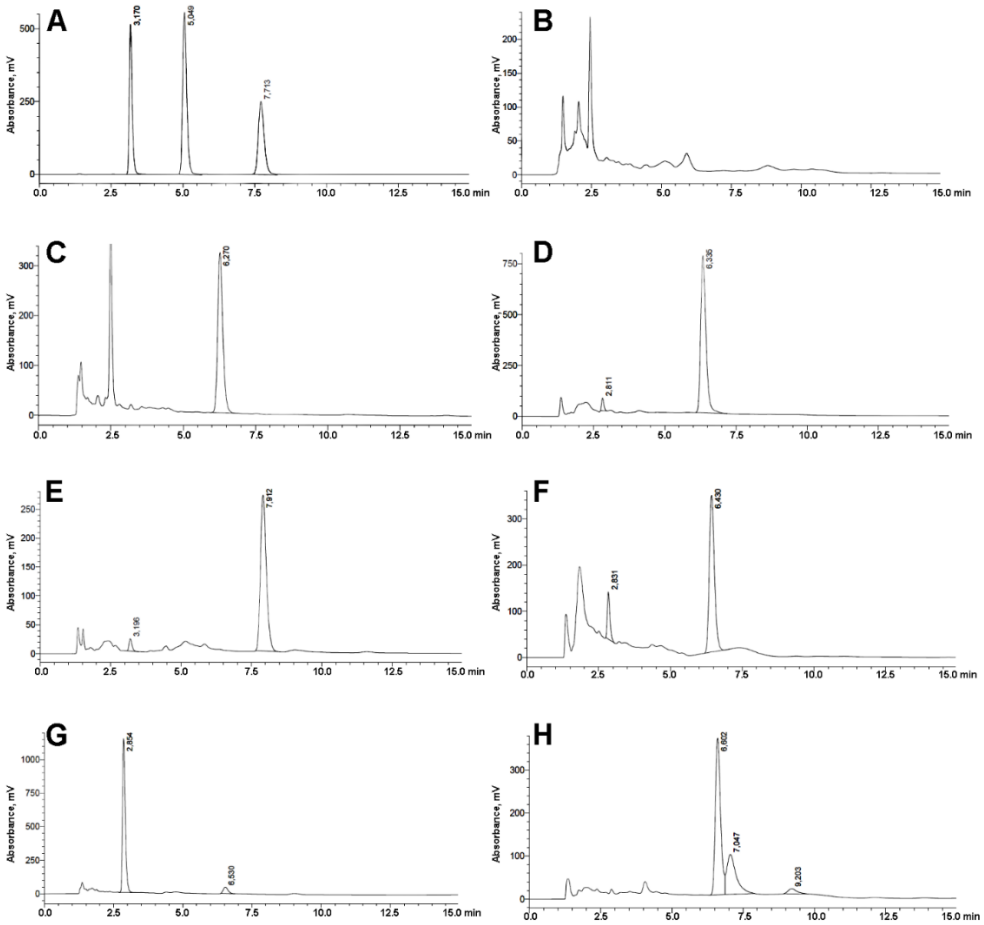

Figure 1. Methylxanthine chromatography-based analysis for A. Standard for theobromine, theophylline, and caffeine in order of elution. B. Unknown "A" (decaffeinated coffee), where there are no relevant peaks. C. Pure roasted coffee, where the signal for caffeine is evident D. Black tea D. Green tea, E. Earl Grey tea, F. Cocoa where the main alkaloid is theobromine instead of caffeine, and H. Yerba mate with a more complex profile (two unidentified signals with retention times above caffeine).

Table 3. Experimental data were obtained during unknown analysis

Sample[a]	Retention time, min	Identified alkaloid	Area, mAU	Interpolated concentration, μg mL^{-1}
A_1	None detected	None detected	None detected	None detected
A_2	6.233	Caffeine	2.51×10^4	18.02
B_1	6.270	Caffeine	3.97×10^5	269.02*
B_2	6.296	Caffeine	2.85×10^5	193.08*
C_1	2.811	Theobromine	3.49×10^4	19.67*
	6.335	Caffeine	9.98×10^5	673.41*
C_2	2.814	Theobromine	2.64×10^4	11.42*
	6.381	Caffeine	5.98×10^5	404.03*
D_1	2.896	Theobromine	1.55×10^4	21.80*
	6.312	Caffeine	3.96×10^5	267.77*
D_2	2.861	Theobromine	2.66×10^4	11.64*
	6.377	Caffeine	2.77×10^5	187.64*
E_1	2.831	Theobromine	6.64×10^4	50.30*
	6.430	Caffeine	7.19×10^5	486.78*
E_2	2.836	Theobromine	3.94×10^4	24.09*
	6.477	Caffeine	5.30×10^5	358.75*
F_1	2.854	Theobromine	7.95×10^5	758.85
	6.530	Caffeine	5.46×10^4	37.92
F_2	2.855	Theobromine	6.50×10^5	617.50*
	6.463	Caffeine	5.25×10^4	36.52
G_1	4.074	Theophylline	1.97×10^4	12.10
	6.302	Caffeine	4.68×10^5	316.22
G_2	4.120	Theophylline	1.95×10^4	13.57
	6.342	Caffeine	4.70×10^5	317.88

[a]Samples coded with a subscripted number "1" represent extractions made with a water bath, meanwhile, letters coded with a subscripted "2" represent those samples extracted using ultrasound. *Represent values significantly different among 1 and 2, with $p < 0.05$.

There are certain differences among the recovered concentrations of methylxanthines when comparing the extraction made with boiling water vs the ultrasound-assisted ones. In general, the latter tends to exhibit lower recoveries. However, both approaches for active compounds have been optimized previously for tea (Ayyildiz et al., 2018). Data from **Tables 3 and 6** also gives the teacher an occasion to explain some fundamentals of statistical analysis when presented/compared with two discrete variables. Finally, methylxanthines are a perfect opportunity to compare quantification techniques so the teacher is strongly suggested using the same standards and extracts used above and quantifying them using thin-layer chromatography as well. Chromatographic separations can be easily performed on the silica gel F254 plates (usually

Table 4. Standard analysis for organic acid determination

Organic acid	Concentration[a], µg mL^{-1}	Retention time, min	Area, mAU	Relative response factor
		Mix 1		
Oxalic	200	7.787	905927	4.53×10^{3}
Maleic	6	8.600	73157	1.22×10^{4}
Citric/Isocitric	250	8.704	124801	4.99×10^{2}
Tartaric	200	8.976	404034	2.02×10^{3}
Gluconic	200	9.868	40948	2.05×10^{2}
Malic	50	10.278	82714	1.65×10^{3}
Succinic	200	12.886	161591	8.08×10^{2}
Fumaric	7	13.505	931100	1.33×10^{5}
		Mix 2		
Lactic	200	6.770	206316	1.03×10^{5}
Formic	300	14.090	148499	4.95×10^{2}
Adipic	80	15.259	237227	2.97×10^{3}
Acetic	70	16.761	83516	1.19×10^{3}
Propionic	200	19.975	88387	4.42×10^{2}
Isobutyric	200	22.436	102064	5.10×10^{2}
Butyric	200	24.168	89238	4.46×10^{2}

[a]Concentration for each organic acid is determined previously by the sensitivity (response) of the detector for each analyte.

available in the lab) developed with chloroform–dichloromethane–isopropanol (4:2:1) and revealed under a UV lamp at 254 *nm* (Cimpoiu et al., 2010).

Organic acids

Organic acids in foods have several uses including improving sensory characteristics, as preservation agents, acidity modulators, and indicators of fermentation (or lack thereof). The composition of organic acids varies in different foods. They are also energy sources and are related to several health benefits (Shi et al., 2022). Fruits and vegetables often contain citric acid, creatine is a unique organic acid found in meat, fermented foods have a high content of acetic acid, and seasonings have a wide range of organic acids (Shi et al., 2022). Hence, the determination of the organic acid contents among different food matrices allows us to monitor the sensory properties, origin identification, and quality control of foods and further provides a basis for food formulation design (Shi et al., 2022).

For the organic acids analysis, each pair of students was given a 20 mL vial with ca. 15 mL of each unknown beverage. In this case, samples were selected from foods that were subjected to fermentation at some point during their processing (Dimidi et al., 2019). Only beverages were selected as they are easy to manipulate and (sample treatment-wise) to process. Most food

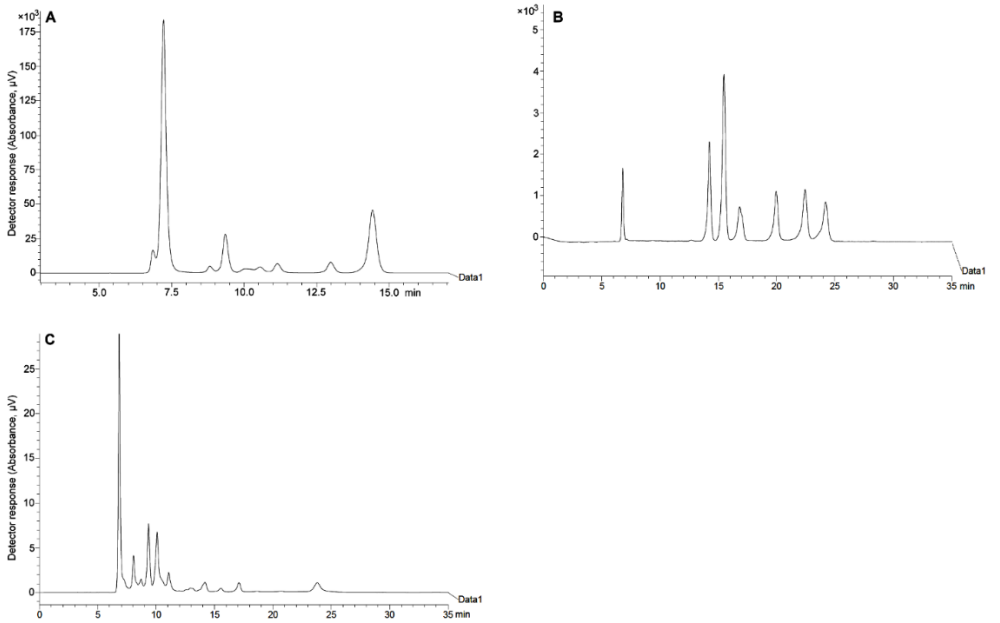

Figure 2. Chromatograms were obtained during the organic acid analysis, all measured at 210 nm. A. Mix 1 containing oxalic, citric, tartaric, gluconic, quinic, succinic, fumaric, and malic acids, in order of elution B. Mix 2 which contains lactic, formic, acetic, propionic, isopropionoic, and butyric acids, in order of elution C. Profile obtained for organic acids sample F (red wine). Solvent peak evident at ca. 7.5 min.

samples used herein can be just degassed and passed through a 0.45 μm syringe filter, however, colored beverages were processed through solid phase extraction prior to LC analysis (see below).

In this case, a total of n = 15 organic acids were assessed using two independent solution mixes (**Table 4**, **Figure 2A-B**). Considering the sheer number of analytes and the additional difficulty and time-consuming that constructing individual calibration curves represents, the students were asked to calculate concentrations using response factors for each analyte. Hence,

Response Factor = Peak Area (or Height)$_{standard}$/Concentration$_{standard}$ (in this case $\mu g\ mL^{-1}$); Sample/Unknown Concentration = Peak Area (or Height)$_{sample}$/Response Factor.

Organic acids were assessed by a previously validated method using a Shimadzu system (Shimadzu, Kyoto, Japan) equipped with a photodiode array detector (SPD-M20AV), column compartment (CTO-20A), autosampler (SIL-20A HT) and a quaternary pump (LC-20AT). The system was equipped with a Hi-Plex H column (7.7 × 300 *mm*, 8 μm particle size; PL1170-6830,

Agilent Technologies) which was used to perform analyte separation. An isocratic method using 100 *mmol* L^{-1} H_2SO_4 at 0.6 *mL* min^{-1} was used, and the column temperature was kept at 50 °*C*. Detection was performed at a wavelength of 210 *nm*. The injector volume was set to 10 μL. Under these conditions, the major food-related organic acids can be assessed in the same chromatographic run (**Figure 2C**). Solutions of each organic acid were prepared from 1 mg of each standard into 2 mL of H_2O and appropriate dilutions thereof (**Table 4**). Analytical standards were acquired from a commercially available organic acids kit (catalog 47264, Sigma-Aldrich, St Louis, MO, USA).

Is evident that based on organoleptic properties alone the students can suspect which food sample was assigned. However, the general idea is that even if they have some notion, the organic acid profile will confirm or reinforce such an idea.

Students quickly noticed that samples A and B presented a translucent yellowish color, and that sample A also presented a malty aroma, so it was associated with beer. This was verified by comparing the acids present in the sample, since previous studies report that beer contains malic, citric, acetic, and succinic, among others (Li & Liu, 2015). For its part, sample B, due to its high content of malic and citric acid, in addition to the lactic acid presence is associated with apple cider. Malic acid is predominant in apples and during fermentation, lactic acid and carbon dioxide are produced (Zhang et al., 2008).

Considerable concentrations of lactic acid are sample D directed the students toward a milk-based beverage. Sample E was not analyzed with the HPLC, so the unknown is identified according to the results obtained for sample D (**Table 5**). In yogurt, the main acid is lactic, but it also has acetic, butyric, pyruvic, formic, citric, uric, hippuric, and orotic (Vénica et al., 2014); the fermentation occurs by lactic bacteria. Kefir fermentation is produced by both lactic/acetic bacteria and yeast (Güzel-Seydim et al., 2000). Since formic acid is predominant, followed by maleic and then citric, the unknown is associated with the kefir sample, since lactic acid is not predominant.

Both unknowns F and G have a similar organic acid profile (**Table 5**) and they are both red-hue-colored gas-containing beverages. However, students quickly identified F as wine and G as kombucha.

All students were able to identify the totality of the unknowns correctly. The only exception was Kefir and Yogurt (unknowns D and E) which seem to generate a little confusion. Nevertheless 81.81% of the students guessed these samples correctly.

Table 5. Organic acids determined in unknown food samples

Sample	Retention time, min	Identified organic acid	Area, mAU	Concentration, µg mL^{-1}
A	8.717	Citric	244388	489.56
	9.374	Gluconic	1559144	7615.24
	10.132	Malic	1170398	707.50
	12.554	Succinic	2912830	3605.19
	14.154	Formic	955778	1930.88
	15.181	Adipic	748238	252.33
	16.300	Acetic	652038	546.51
	18.711	Propionic	1320139	2987.18
	21.580	Butyric	398644	893.44
B	7.551	Lactic	61056	59.16
	8.743	Citric	3174618	6359.36
	9.444	Gluconic	116604	569.52
	10.203	Malic	5451500	3295.39
C	8.731	Citric	1897786	3801.62
	10.160	Malic	11988034	7246.68
	12.560	Succinic	5789783	7165.97
	17.174	Acetic	642658	538.65
	18.747	Propionic	5038653	11401.34
D	7.238	Lactic	1390578	1348.01
	8.072	Maleic	3799603	311.63
	8.835	Citric	2178588	4364.12
	9.360	Gluconic	599853	2929.83
	14.243	Formic	7911875	15983.69
	15.418	Adipic	993941	335.19
	17.085	Acetic	80612	67.57
	23.530	Butyric	440628	987.53
F	8.722	Citric	181414	363.41
	9.290	Gluconic	4192199	20475.72
	10.467	Malic	296861	179.45
	12.902	Succinic	2721479	3368.35
	14.222	Formic	735746	1486.37
	17.102	Acetic	205526	172.26
	18.706	Propionic	361458	817.90
	22.682	Isobutyric	6030470	11817.04
G	7.549	Oxalic	1788735	394.90
	8.274	Citric	330225	661.50
	8.696	Tartaric	18595	9.20
	9.345	Gluconic	1261497	6161.46
	11.057	Malic	796249	481.33
	12.918	Succinic	121734	150.67
	14.106	Formic	350164	707.41
	17.085	Acetic	892765	748.28
	22.787	Isobutyric	1723605	3377.50

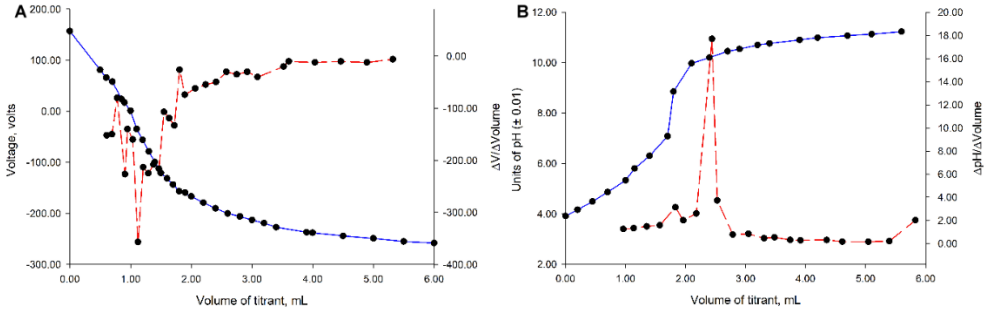

Figure 3. Potentiometric curves for unknowns A (beer) and B (apple cider). Titration with sodium hydroxide ca. 0.1 mol L^{-1}. The inflection point (endpoint) is also visualized (red dotted lines) using the second derivative calculated as $\delta_{pH}/\delta_{volume}$ and $\delta_{voltage}/\delta_{volume}$.

Total acidity using potentiometry

In addition to HPLC analysis, students were asked to titrate 5 mL of each unknown. Briefly, 20 mL of H_2O was added to the 5 mL aliquot of the sample and mixed in a 50 mL beaker. Thereafter a combined pH glass electrode (6.0233.100, Metrohm, Herisau, Switzerland) was introduced into the solution, pH/Voltage was measured using a 781 pH Ion meter (Metrohm). Then, a 10 mL burette was filled with titrant [NaOH (0.0971 ± 0.0025) *mol* L^{-1}] and the resulting solution was titrated dropwise until a brink in potential or pH was observed. Titrant volume consumption is graphed in real-time versus change in potential or pH (**Figure 3**). To visualize more easily the end-point Concentration is calculated considering that the reaction that occurs (using acetic acid as a model) $CH_3COOH_{(aq)} + NaOH_{(aq)} \rightarrow CH_3COO^- Na^+_{(aq)} + H_2O_{(l)}$. In this case, the mole relationship among reagents and products is 1:1 but the students must remember that some of these acids are polyprotic (e.g., for sample A the most abundant acid is gluconic acid (a monoprotic acid, $HOCH_2(CHOH)_4COOH$. Meanwhile, in sample B the most abundant acid is citric, a tricarboxylic acid). This part of the experiment should also help the students recognize that while HPLC can determine each organic acid individually and, hence, the input of each acid can be determined, the potentiometric analysis will account for all acid species present in the sample. For data treatment for the potentiometric analysis please see Villela et al. (2015). Finally, each sample concentration was expressed as the mg of the majoritarian organic acid, based on the profile obtained by HPLC analysis.

Solid phase extraction (SPE)

Both methylxanthine and organic acid analysis are great opportunities to teach the students techniques such as solid phase extraction (SPE). For example, the students were quite surprised to see how a C_{18} cartridge retains grape juice anthocyanins (see below, Ferreiro-González et al., 2014). The students used SPE as a cleanup tool. An SPE cartridge is equilibrated using 5 *mL* H_2O and 5 *mL* CH_3OH and 2 *mL* of grape juice (296 *mL*, Welch's, Concord, Massachusetts, USA) or a tropical fruit juice mix (330 *mL*, Florida Ice and Farm Company, Heredia, Costa Rica) was passed through the SPE cartridge (Sep-Pak® Vac, C_{18}, 3cc/500 mg, WAT020805, Waters Corporation, Milford, MA, USA). All the cartridges were processed by applying direct positive pressure using a single SPE tube processor (Visi-1, 57080-U, SUPELCO, Bellefonte, PA, USA). This will result in a clear and colorless extract ready to inject into the LC system which is directly recovered in an LC vial (2 *mL*, borosilicate type I glass, screw cap, red PTFE/silicone septa, 8010-0542, Agilent, Santa Clara, CA, USA). The students were asked to compare the concentrations obtained both with or without SPE treatment and the general differences in signal appreciation (i.e., a cleaner chromatogram after SPE) (**Figure 4**). Additionally, if the instructor so desires, the pigments retained in the SPE cartridge could be eluted with an appropriate solvent and used afterward in polyphenol analysis and used as yet another teaching tool (see for example, Brenneman & Ebeler, 1999).

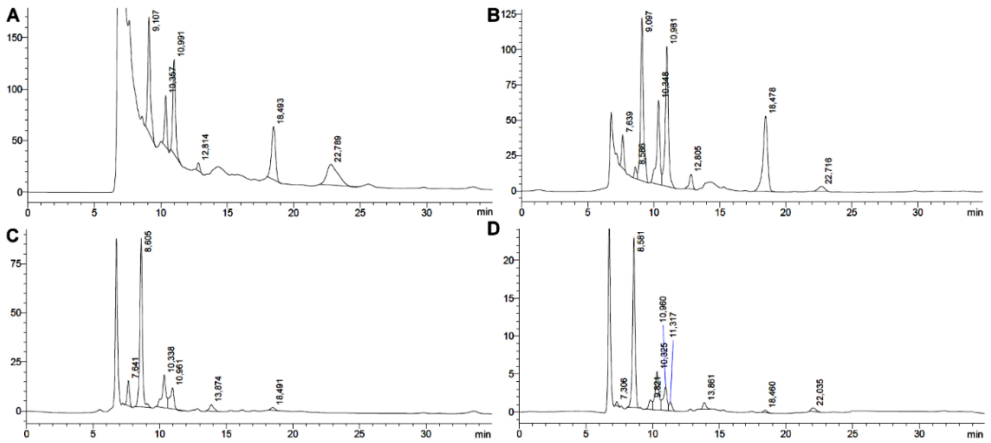

Figure 4. Organic acid profile for grape (A and B) and tropical fruit juice (C and D) before and after treatment using SPE, respectively.

Sugars

There seems to be an association between sucrose consumption and body weight gain. Given the association between excess body weight and type 2 diabetes occurrence, there is rationale to promote a reduction of sugar intake related to diabetes occurrence and substitution of sugar-sweetened beverages (including fruit juices) with water or no/low-calorie beverages as much as possible (Reynolds & Mitri, 2024). On another hand, fructose is a naturally occurring monosaccharide found in fruits, some vegetables, and honey, and high fructose corn syrup is an

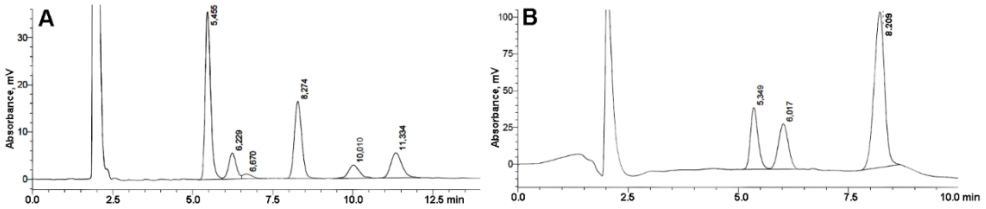

Figure 5. Chromatograms for A. Solution of sugar standards, a mixture of 10 mg L^{-1} of fructose, glucose, sucrose, galactose, and lactose. B. Chromatogram of sugar unknown A (Coca-Cola®). Signal at ca. 2.4 min corresponds to the solvent front/solvent peak due to the differential in the composition of the mobile phase and injection solvent (for a primer in solvent behavior in HPLC the work of Buszewski et al., 2012 is suggested).

abundantly used sweetener in processed foods (as a less expensive alternative to sucrose). Fructose consumed in naturally occurring foods such as fruit, (that also contain fiber) may result in better glycemic control compared with isocaloric intake of sucrose or fructose added to food and is less likely to have detrimental effects on triglycerides if intake is limited (Reynolds & Mitri, 2024). Considering this, sugars must be quantitatively verified including those available naturally in foods and sugars added through formulation (Louie et al., 2015).

Sample treatment for sugars was performed according to Ondrus et al. (1983). Briefly, For soft drinks, wines, and fruit juices. These products were degassed (for the case of carbonated beverages) and filtered using a 0.45 μm regenerated cellulose membrane filter (Sartorius). Some juices that contain a considerable deal of pulp may require centrifugation. Though some products may restrict the flow through the filter very rapidly, due to saturation, very few drops of filtrate are sufficient for injection into the liquid chromatograph.

In the case of ice milk and related dairy products, 1.00 g of melted or liquid product was transferred to a 25 mL Erlenmeyer flask using a dropper. Afterwards, 5.00 mL of H_2O was added followed by 9.00 mL of isopropanol. The mixture was placed on a shaker-stirrer and mixed gently for at least an hour, then transferred to a conical tube (15 mL centrifuge tubes, PET, CLS430055, Corning®, NY, USA) and centrifuged (75004240, Sorvall™ ST 16 Centrifuge Series, Thermo Scientific, Waltham, MA, USA). The clear supernatant solution was filtered through a 0.45 μm syringe filter (Sartorius).

For breakfast cereals, 1.00 g of dry cereal was weighed in a small beaker, and 20 mL H_2O was added. The mixture was stirred for 15 minutes using a vortex (Vortex-Genie 2, Scientific Industries Inc., Bohemia, NY, USA). The resulting mixture was centrifuged and then filtered through a membrane filter.

In this case, a mixture with a total of $n = 5$ sugars [i.e., fructose (catalog number PHR1002), glucose (PHR1000), galactose (PHR1206), sucrose (PHR1001) and lactose (PHR1024)] was used to determine their identity and concentration (Sigma-Aldrich). Again, 40 mg of each standard was used to prepare a 2 mL stock solution and diluted to obtain concentrations ranging from 0.5 $g/100$ mL to 2 $g/100$ mL (**Figure 5A**).

Sugars were assessed by a previously validated and ISO 17025 accredited method using a Shimadzu system (Shimadzu, Kyoto, Japan) equipped with a refractive index detector (RID-20A), column compartment (CTO-20A), autosampler (SIL-20A HT) and a quaternary pump (LC-20AT). The system was equipped with a Zorbax (NH_2) Amino (Pore Size: 70 $Å$, Particle Size: 5.0 μm, Inner Diameter: 4.6 mm, Length: 250 mm; AG880952-708, Agilent Technologies) which was used to perform analyte separation. An isocratic method using 75:25 CH_3CN and H_2O at 1.4 mL min^{-1} was used, and the column temperature and the detector were set to 30 °C. The injector volume was set to 10 μL.

In the case of sugars, guaranteed labels for each of the food samples were shared with the students. Once the identity of the unknown was established, they were asked to compare the total sugar amount found experimentally with that of the label (**Table 6**).

Sugars in a beer sample (unknown F) should be low as sugars, especially simple ones are expected to be consumed during fermentation (Jurková et al., 2018). In lactose-free dairy (as is the case for unknown E), the lactose is predigested into glucose and galactose. Consequently, the lactose content may be very low (i.e., < 0.1 g/L) (Dekker et al., 2019). In the case of unknown D, students quickly recognize it as the cereal bar as the labeling indicates it might contain lactose (milk derivatives). Interestingly, from the three remaining samples, apple juice was the one that exhibited a higher content of sugars (**Table 6**). In this case, unknown B is identified by the higher proportions of glucose and fructose (provided by the fruit, Cywińska-Antonik et al., 2023) compared to cola (**Figure 5B**) and wine.

In the case of sugars not only does the student have to calculate interpolated concentrations for each sugar but must recognize that to compare with the guaranteed label they must i. return each concentration to the original sample (using density for liquid samples or by considering the mass and volume used to process each sample) and ii. as each sugar is quantified individually in HPLC, the sum of all sugars present must be calculated (**Table 6**, **Figure 5B**).

As stated previously there are other methods available for the determination of sugars in foods (Magwaza & Opara, 2015; Al-Mhanna et al., 2018). However, most of these approaches, because of their lack of specificity, have considerable restraints, depending on the complexity of the matrix. For example, spectrophotometric approaches may overestimate sugar content when based on the redox ability of reducing sugars as any compound with similar structural properties

Table 6. Sugars were determined in unknown food samples

Sample	Retention time, min	Identified sugar	Area, mAU	Concentration, g/100 g	Total sugars, g/100 g	Total sugars according to guaranteed label, g/100 g
A	3.820	Fructose	741298	1.11	5.11*	7.5*
	4.142	Glucose	1215035	2.05		
	4.941	Sucrose	1279664	1.95		
B	3.803	Fructose	3361650	13.86	30.91	28
	4.101	Glucose	2744655	12.76		
	4.926	Sucrose	809212	3.38		
C	3.818	Fructose	483899	2.92	14.28	13.1
	4.138	Glucose	816578	5.52		
	4.934	Sucrose	960537	5.84		
D	3.832	Fructose	188482	1.90	7.32	7
	4.155	Glucose	738202	0.83		
	4.954	Sucrose	1880795	1.91		
	6.357	Lactose	233421	2.68		
E	3.092	Fructose	4608	0.13	10.13	11
	4.215	Galactose	63997	2.53		
	4.381	Glucose	69105	1.68		
	4.995	Sucrose	258206	5.92		
F	3.805	Fructose	326857	0.49	1.06	0
	4.083	Glucose	260860	0.44		
	4.917	Sucrose	86342	0.13		

*Represent amounts significantly different among the experimental value and the guaranteed label, with $p < 0.05$.

will respond to the color-forming reagents (e.g., other aldehydes). Moreover, for total sugar concentration, those foods with sucrose or other non- must suffer inversion before measurement (the same goes for polarimetric methods). HPLC sugar analysis can be performed at relatively low temperatures (avoiding possible thermal decomposition), can assess each fraction of the sugar present in the food sample, and can determine simultaneously several simple/complex and reducing/non-reducing sugars in a single analysis.

Quality Control and Statistical Analysis

All assays were performed and measured once. Chromatographic data treatment was performed using the proGamma LabSolutions Lite software (version 5.82 Shimadzu Corporation). Fapas® FCFA29-DRN14 (Taurine, Caffeine, Total Sugars, and Citric Acid in Energy Drink Proficiency Test, Sand Hutton, York, UK) was used also as a standard for HPLC analysis. Meanwhile, FCOH4-DRA13QC [Total Acidity (expressed as tartaric acid)] was used as quality control during the titrimetric analysis. The coefficient of determination (r) was used to corroborate the association between methylxanthine concentrations and detector response. A value of $r \sim 0$ was deemed as a lack of correlation. Calibration curves were constructed each time an analysis was to be performed. This data set was evaluated using Sigmaplot 15.0 software (Systat Software Inc., San Jose, CA). Fisher's exact tests were performed to assess sugar concentration using the initial nominal concentration (indicated in the label) as a reference. For methylxanthines results obtained from boiling water were compared to ultrasound-assisted extractions. For any statistical analysis $\alpha < 0.05$ is considered the threshold for significant difference, all performed with SPSS® Statistics (IBM®, version 29.0 Armonk, NY, USA).

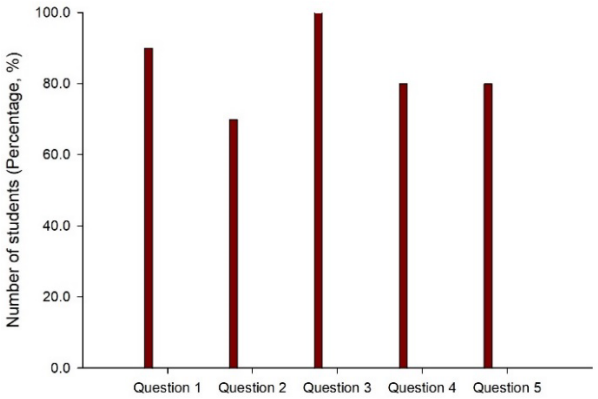

Figure 6. Results from the opinion questionnaire for Food Science students after the course. Percentages correspond to participants responding "YES" to "YES/NO" queries.

Queries to target audience and discussion points for post-experiment analysis

A total of $n = 5$ questions (structured, dichotomous questions) were asked to students who participated in these activities after the finalization of the course. That is *Question 1*. Did you find the unknown laboratories more interesting/engaging vs. regular sessions? *Question 2*. Did you find the unknowns laboratory to be more difficult/challenging than regular lab sessions? *Question 3*. As the course progressed, did you feel that working with the unknowns became easier/you were more knowledgeable? *Question 4*. Do you think you gained abilities you did not have/learned new concepts through the unknown lab sessions? *Question 5*. Do you feel the analyses selected for the laboratory session were pertinent for your formation/will help you in your career as a Food Scientist? The results of are summarized in **Figure 6**.

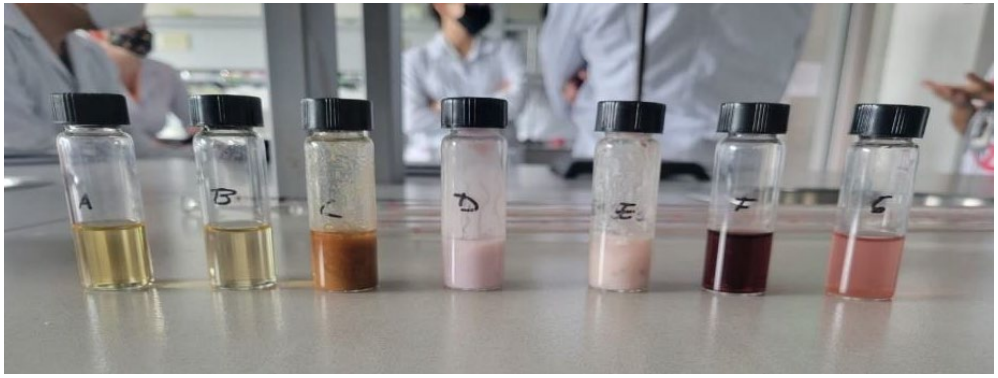

Figure 7. The visual aspects of the unknowns were selected and distributed among the student groups for analysis of organic acids.

Many students considered the experience helpful and learned tools they until then did not yet possess or honed abilities they already had. Overall, the above experiment can be categorized as a successful exercise in problem-solving (Bodner, 2015; Bodner & Bhattacharyya, 2018; Bodner & Domin, 2000).

As discussed, for each laboratory session students were asked to submit a report summarizing their findings. In fact, the conclusion reached for each analyte type is a synopsis of their information. As guidelines of each report should discuss at least the following (as mere suggestions):

Which data can be drawn from the preliminary sensory analysis (odor, aspect, color, viscosity)? (**Figure 7**).

A. Which particularities of the sample pretreatment were observed for each of the analyte families? Or, how the analytes of interest are isolated prior to their actual analysis? For example, the principle of how the C_{18} SPE cartridge works in separating organic acids from a drink. What happens to the interaction if organic acids are protonated? If not?

B. How does the sample treatment relate to analyte structure?

C. What type of chromatography was used to assess the analytes? Column selection? For example, why does sugar analysis use an amino (-NH_2) functionalized column? Which solvents are used during each separation and why? For example, why does the solvent in organic acid analysis have to be acidified?

D. For each chromatography, how does the elution order of the compounds relate to the analyte structure? How do you know which analytes are present and which ones are not in the chromatogram?

E. Drawbacks and benefits of using instrumental (HPLC) analysis versus more classical techniques (potentiometry). In which cases do you select one over the other during routine food analysis?

F. Calculations or at least calculation samples. How was each analyte concentration determined quantitatively?

G. Justified observations and elucidation process flowchart. What conclusion do they obtain from each result and how that leads them to the unknown? A proper workflow as a step-by-step guide on how they tackle each unknown.

Conclusions

Student improvement with each laboratory session is evident as during the last laboratory all students were able to determine the identity of the unknowns without fail, this speaks toward their increase in abilities. Guaranteed labels present a unique opportunity to explain to the student the difference between total sugars and added sugars which is now an FDA requirement (Huang 2019; Jiyoon Kim, 2021). Finally, most of the analytes studied herein are the result of plant metabolism so there is an additional opportunity to introduce the food science student to chemical fingerprinting of foods. As demonstrated with these experiments, food analysis requires analytical acuity as determinations demand accuracy, and organic chemistry as the structure of the analytes is the basis for the quantification approach. Furthermore, as an applied cumulative science, to assess properly the identity of these unknowns, some basic principles must be governed by the student such as acid-base chemistry, and principles of instrumental and compositional analysis. In general terms, the clearest limitation of the approach recommended herein is that by using traditional detectors despite it being demonstrated that a useful profile for different foods can be attained, no information is recollected regarding possible unknown substances that could appear during the routine analysis for food matrices with complex formulations which could be paramount for food quality, safety, and adulteration assessment (Knolhoff et al., 2016; Chen et al., 2024). Finally, an additional hindrance to reproducing the approach relies on possessing the specific chromatographic analysis (i.e., equivalent columns may force teachers to adjust before attempting the analysis).

Acknowledgments

The author would like to thank all students from course TA-0215/PF-6736 (I semester 2022) for their curiosity, dedication, and interest in solving the assignments. Graciela Artavia is acknowledged for her help in selecting the food samples. Special thanks to María Sabrina Sánchez for her suggestions, revising the manuscript, and language editing.

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