

Introducing mass spectrometry to first-year undergraduates: Analysis of caffeine and other components in energy drinks using paper-spray mass spectrometry



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ABSTRACT

We describe the use of ambient ionization mass spectrometry in a freshman-level undergraduate class for the detection of caffeine and other components in several commercially available energy drinks (5-hour Energy, Starbucks Refreshers, Red Bull Sugarfree, and Coca Cola Classic). We use the technique of paper-spray mass spectrometry where a filter paper is used as a substrate on which a sample is directly applied and a high voltage is used to generate an electrospray of ions in ambient conditions which are then detected by a mass spectrometer. The purpose of this laboratory exercise is twofold: 1) to use the paper-spray method to determine the chemical composition of different types of commercially available energy drinks and 2) to perform tandem mass spectrometry (MS/MS) to demonstrate that the mass spectrometer can be used for structural analysis. This laboratory serves as an excellent means of introducing the beginning student to the concepts of charge, mass, and molecular structure. In addition to the freshmen general chemistry class, we also incorporated this lab into the introductory analytical chemistry class taught at the sophomore level with the inclusion of a third exercise where the usefulness of mass spectrometry for quantitative analysis is demonstrated by determining the concentration of caffeine in the energy drinks with deuterated caffeine as an internal standard.

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1. Introduction

The notion of atomic and molecular weights is one of the first concepts taught in a general chemistry class. Hence, what could be a better time to provide students with hands-on experience with mass spectrometry (MS), an instrument that provides a direct measurement of molecular weights by producing, separating, and detecting ions in the gas phase? We describe here a simple experiment that has been used as the first laboratory experience in a beginning general chemistry class at Stanford University. It is based on the use of paper-spray mass spectrometry (PSMS). The origin of this technique might be traced to what Fenn called wick spray [1,2] and was first introduced using a paper substrate by Ouyang, Cooks and co-authors [3,4]. Indeed, PSMS has been advocated before as an excellent pedagogical tool in upper-division undergraduate labs for the study of chemical reactions [5]. We believe that PSMS can be adapted for use earlier in the undergraduate chemistry

curriculum and allow students to appreciate mass spectrometry at the beginning of their study of chemistry.

In PSMS, the sample and spray solvent are directly loaded onto a piece of triangular-shaped filter paper (because of its high porosity) to which a high voltage is applied, generating a spray of ions that can be analyzed by a mass spectrometer. PSMS can be used to analyze a wide variety of compounds, including small organic molecules, monitoring therapeutic drugs in blood and illicit drug detection in urine samples [3,4]. Because of its simplicity, PSMS is a useful technique for teaching mass spectrometry to undergraduate students [5].

We describe here a simple laboratory exercise using PSMS, which involves the detection of different analytes present in commercially available energy drinks such as 5-hour Energy, Red Bull Sugarfree, Starbucks Refreshers, and Coca Cola Classic. Caffeine, a common component in these energy drinks, is used to further demonstrate the utility of a mass spectrometer for structural analysis. This lab is designed to introduce the technique of mass spectrometry to first-year undergraduate students in a laboratory setting wherein small groups of students work on the experiment with the guidance of a teaching assistant. The objective of this lab is to teach students that complex mixtures are made up of

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different molecules in different quantities where each molecule has a fingerprint in terms of molecular mass and structure. A mass spectrometer can be used to obtain this information by recording the mass-to-charge (m/z) ratio of different species in the mixture. We have conducted this laboratory exercise over two consecutive years, each time with a class of approximately 200 students. Students viewed this hands-on experience with a mass spectrometer as helping them to better understand the fundamental concept of molecular masses and structures. This laboratory makes vivid what they have learned about charge and mass in class.

We also describe here an additional exercise designed to introduce mass spectrometry as a quantitative analytical tool. This exercise involving determination of caffeine concentration in energy drinks was added to the introductory analytical chemistry laboratory taught at the sophomore level. We used this additional exercise in conjunction with the other two exercises for the upper undergraduate level students as they were better equipped with the knowledge of quantitative estimation compared to freshmen level students.

2. Materials and methods

The laboratory was carried out on an LTQ mass spectrometer (Thermo-Fisher Scientific, San Jose, CA). The paper used was grade 1 filter paper from Whatman (Maidstone, England) as it was readily available. Care is taken to use dry paper. The energy drinks (5-hour Energy, Starbucks Refreshers, Red Bull Sugarfree, Coca Cola Classic) were all purchased from supermarkets. Deuterated caffeine [Caffeine-(trimethyl-d₉), 99 at.% D, 98% (CP)] (Product-725625, Sigma-Aldrich, Milwaukee, WI) was used as the internal standard for quantitative analysis of caffeine. The methods section is divided into two parts: 1) the prelab and 2) the procedure. Each section is described below.

2.1. Prelab

This laboratory was a part of the general chemistry class taught to freshman undergraduates and was also introduced with an additional quantitative exercise to the sophomore level introductory analytical chemistry class. In both classes, the students were asked to read the handout that was provided explaining the concept of mass spectrometry, m/z ratio, various components of a mass spectrometer (ion source, mass analyzer, detector) and experimental procedure. For the prelab, they were asked to make a table representing the names, molecular weights (as well as the weights of $[M+H]^+$ and $[M+Na]^+$, the proton and sodium adducts, respectively, of the molecules of interest) and the chemical structures of the most abundant molecules present in the energy drinks listed above. The students were given the list of ingredients of each drink and asked to look for the above information by making an online search. The laboratory handout is provided in the supplemental material. For the analytical chemistry class, the students were also asked to look up the caffeine content in the above-mentioned drinks using an online link [6].

2.2. Procedure

For the freshmen class (200 students), This lab was conducted over a three-day period with a total of six 3-h sessions where each session had 30–35 students. In each session, students worked in groups of 5 under the instruction of a teaching assistant (TA). Two different laboratory exercises were conducted by each group: (1) direct analysis of the components of energy drinks and (2) structural identification of components of energy drinks by tandem mass spectrometry (MS/MS). The sophomore class was much smaller in comparison (a total of 30 students). Therefore, we divided the class

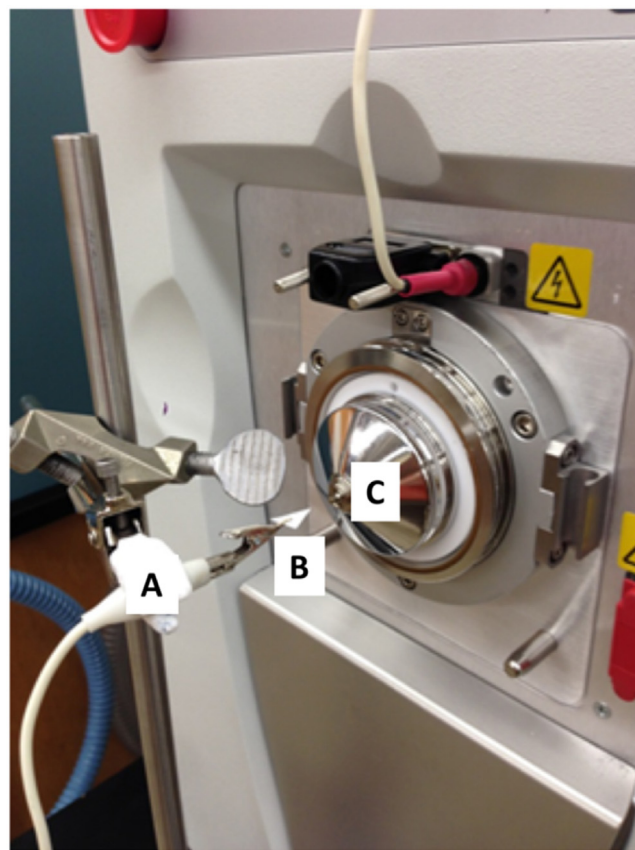


Fig. 1. Photo of the paper spray setup with ambient ionization mass spectrometry. A triangle-shaped filter paper (B) is attached to a flathead alligator clip (A) that is connected to the power cable of the instrument high-voltage power supply and positioned in line with the mass spectrometer inlet (C) at a distance of about 11 mm. Care must be taken while setting it up by switching off all voltages and avoid touching the capillary inlet.

into small groups of 5 students and a third exercise was added to this lab: quantitative determination of the concentration of caffeine in energy drinks. For both classes, the TAs were trained on the instrument prior to the class and were asked individually to run all parts of the laboratory exercise.

Lab setup is similar for both classes and is relatively simple and fast. As most LTQs come with a built-in ESI source, the first step involves removing the housing and defeating the interlocks with a jumper. Care must be taken while doing this as the capillary inlet is hot if the instrument has been running for a while. Fig. 1 shows a photograph of the PSMS setup. A clamp secured to the pole of a ring stand is placed at the same height as the MS capillary inlet. The filter paper is cut with conventional scissors into a sharply pointed triangular shape (isosceles triangle) whose length is 1 cm from back to tip. A toothless alligator clip is attached to the back end of the paper triangle. A volume of 5 μ L of drink solution was deposited about 3 mm from the tip of the paper triangle. The spray solvent mixture of 1:1 (v/v) methanol/water in 0.1% formic acid was added to the back end of the triangle before spraying. The alligator clip is attached to the MS instrument's high-voltage power supply cable. The tip of the wet filter paper triangle is placed 11 mm from the capillary inlet of the MS. The samples are analyzed in positive-ion mode, using a single spray voltage of 5 kV. The electrospray plume thus generated is directed to the MS capillary, which is set at a temperature of 275 °C.

It is important that the spray voltage is switched off while setting up the experiment. Care must be taken because of possible exposure to high voltage, but this danger is minimized by the fact

Table 1
Compounds detected from beverages by paper-spray MS: 5-hour Energy (1), Starbucks Refresher (2), Coca Cola Classic (3), and Red Bull Sugarfree (4).

Compounds (MW)	Ionic species	<i>m/z</i>	Drink
Aspartame	[M+H] ⁺	295.13	4
Caffeine	[M+H] ⁺	195.09	1, 3, 4
Fructose	[M+Na] ⁺ , [M+K] ⁺ , [2M+Na] ⁺	203.05, 219.03, 383.12	2
Fructose	[M-2H ₂ O+H] ⁺	145.04	2
Sucrose	[M+Na] ⁺ , [M+K] ⁺	365.17, 381.17	2
Pyridoxine	[M-HCl+H] ⁺	170.08	1, 4
<i>N</i> -acetyl-L-tyrosine	[M+H] ⁺ , [M+Na] ⁺	224.09, 246.07	1
Niacinamide	[M+H] ⁺	170.05	4
Phenylalanine	[M+H] ⁺	166.08	1

that the current is less than one microampere. At the end of each experiment, the spray voltage is set to zero and the instrument is set on standby mode. Indicators on the instrument front panel and in the software itself lets the operator know that the voltage is turned off. We kept the set up for paper spray for the entirety of three days so that the experiments are consistent for all groups. Most of the sample preparation, including cutting the filter paper into triangular shapes and preparing different solutions, was done by the TAs before the start of each lab period. Proper lab attire, including gloves, lab coats and safety glasses are required for both students and teaching assistants. The combination of spray voltage of 5 kV and heated capillary can cause burns and electric shocks. Each sample run required the use of a new filter paper triangle.

Because of the time constraint, each group of students used two types of drinks in their experiment. Later, data from different groups were shared such that the whole class had the data for all the energy drinks. Each set of experiments required two sets of paper changes: one for each drink. The TAs were asked to change the paper for each experiment to avoid potential accidents. Students were asked to place the solutions, turn on the voltage, and acquire data, thus providing them with a hands-on experience. Students should be advised not to stand very close to the paper while the experiment is running to avoid being exposed to charged aerosols. Safety considerations are important but are not very cumbersome.

In the same data acquisition file where the mass spectrum of any energy drink is recorded, MS/MS is performed by mass selecting one of the peaks, e.g., [caffeine+H]⁺ at *m/z* = 195.09 and fragmenting it by using a normalized collision energy of 25. If time permits, the normalized collision energy can be slowly changed to show the students how the fragmentation pattern (such as peak heights, different types of fragments) depends on the normalized collision energy. See the Supplemental Material for more details.

For the quantitative estimation of caffeine concentrations in the energy drinks, deuterated caffeine (caffeine-d₉) was used as an internal standard. This part of the lab was only performed in the sophomore-level class. The students were asked to prepare the solutions in the following manner. An aliquot of energy drink is taken and diluted 10 times in methanol. Using the stock solution of deuterated caffeine (1 mg/mL), five to ten 50- μ L solutions were prepared containing the concentrations between 1 and 0.1 mg/mL in a methanol. These concentrations were chosen such that they fall in the range of the caffeine concentrations reported in these energy drinks [6]. In a small centrifuge tube, 10 μ L of the diluted energy drink were mixed with 10 μ L of each of the deuterated caffeine solution. A 5 μ L aliquot of each of the five solutions was placed on separate paper triangles and a spray solvent mixture of 1:1 (v/v) methanol/water in 0.1% formic acid was added to the back end of the triangle before spraying. Data were acquired for a minute in each case for consistency, using the same conditions as mentioned above.

The mechanism of paper spray has been discussed in detail [4,7]. Unlike ESI [8], paper spray does not require the presence of a sheath gas. A filter paper is ideal to use in these PSMS experiments: it

is inexpensive, commercially widely available, and requires small amounts of sample (in terms of sample loading on paper). Paper also represents a disposable substrate. Multiple sets of experiments can be performed before the instrument needs to be cleaned, and therefore, this technique is quite suitable for teaching a laboratory class of several hundred. At the end of the lab period, the MS is cleaned with a solution containing 1:1 (v/v) methanol/water until a relatively clean background is seen. If required, a further cleaning can be performed by sonicating the inlet capillary and then running a blank afterwards.

3. Results and discussion

In the postlab analysis part of the laboratory exercise, the freshmen-level students were asked (1) to assign as many peaks as possible in their mass spectra and (2) to propose a possible fragmentation pattern for caffeine. The sophomore-level class, students were asked to plot the concentration of deuterated caffeine versus the ratio of the deuterated caffeine-to-caffeine signal intensities in combination with the two above-mentioned postlab exercises. The correct answers were provided after grading the students' lab reports. We present here a few representative spectra and brief discussion for each part of the lab results.

3.1. Direct analysis of chemical composition of energy drinks

In total, four different energy drinks (5-hour Energy, Starbucks Refresher Blueberry Acai, Red Bull Sugarfree, and Coca Cola Classic) were analyzed. Fig. 2A shows the mass spectrum of 5-hour Energy, and Fig. 2B shows the mass spectrum for Starbucks Refresher Blueberry Acai that has been directly loaded on the paper. The spectrum is averaged for about 1 min in each case. Energy drinks contain multiple functional ingredients, and in positive-ion mode most of the peaks seen in the mass spectra corresponds to either H⁺ or Na⁺ adducts of the chemical species, [M+H]⁺ or [M+Na]⁺, respectively. Table 1 lists the ingredients detected with high abundances for each energy drink. In the case of Starbucks Refresher Blueberry Acai, the [caffeine+H]⁺ peak usually observed at 195.09 *m/z* is not observed, although fructose and sucrose are observed as their H⁺ and Na⁺ adducts. We suggest that the high concentration of sugars (about 20 g) in the Starbucks drink (355 mL) saturates the ion signal, hence suppressing the signal of caffeine, which is comparatively lower in concentration (about 40–55 mg) according to the amounts advertised on the label of the drink container.

3.2. Tandem MS

Structural identification of compounds is at the core of chemistry. While PSMS preserves the molecule in its intact form, and gives information on molecular weights, it is important to introduce students to methods that allow for structural analysis. One of the best ways to determine the chemical structure of any molecule is to break it apart into its constituent parts and then to reassemble these

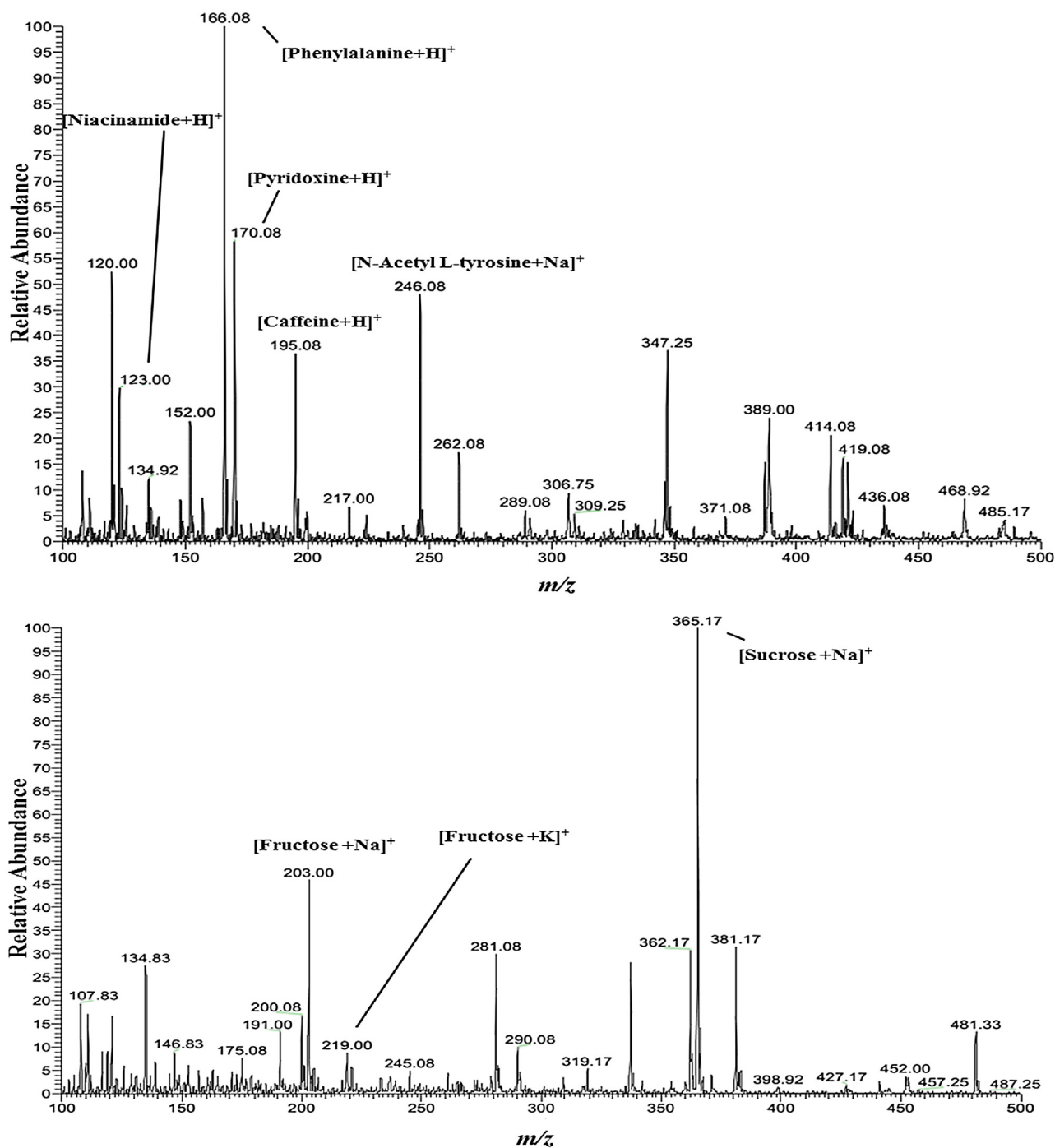


Fig. 2. Mass spectra of 5-hour Energy drink in spray solvent (A) and Starbucks Refresher Blueberry Acai in spray solvent (B). Spray solvent is 1:1 (v/v) methanol/H₂O with 0.1% (v/v) formic acid. Spray voltage is 5 kV. The data is collected on the LTQ-ion trap.

parts to see what kind of structure can give rise to the observed fragmentation pattern. Fortunately, modern mass spectrometers (an LTQ-ion trap, in this case) are designed to use methods such as MS/MS in conjunction with PSMS, allowing us to deduce structural information through interpretation of fragment mass spectra. MS/MS is a method where the gaseous ions are subjected to two or more stages of mass analysis [9]. Of the multiple ions present in a complex mixture, the desired precursor ion can be mass selected then fragmented via gas-phase chemical reaction in a collision cell. We have used the method of collision induced dissociation (CID) where collision with an inert gas molecule, such as He or Ar, (we used He here) leads to an increase in the internal energy

of the precursor ion ultimately causing its dissociation. The fragment ions are then detected via a mass analyzer. Fig. 3 shows representative fragment ion mass spectra of [caffeine+H]⁺ (Fig. 3A) and [phenylalanine+H]⁺ (Fig. 3B). Students were provided with the phenylalanine fragmentation pattern and its structural analysis as an example to help them understand the process of interpreting fragment spectra, and to enable them to do the same for caffeine. It is expected that freshmen-level students will not be able to predict the correct fragment structures, as it often involves molecular rearrangements that they are not familiar with. However, they did seem to understand how and where the bonds need to be broken to generate a particular fragment.

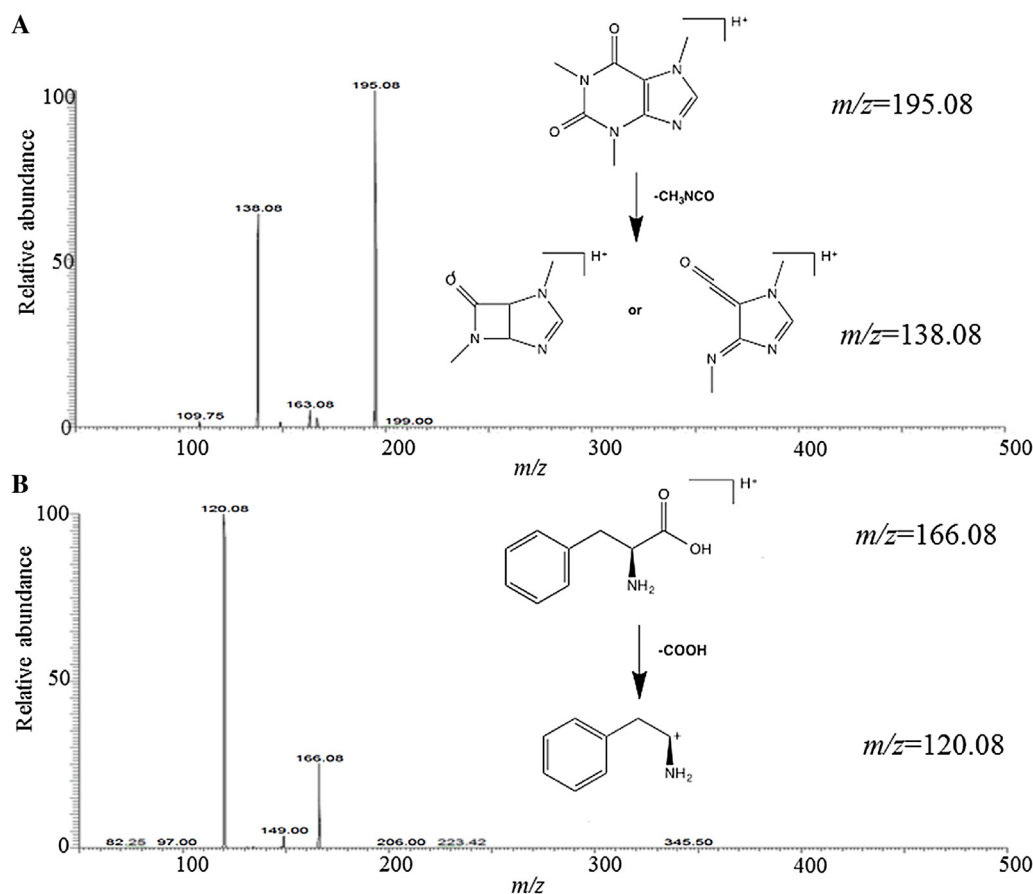


Fig. 3. Fragmentation ion spectrum of (A) [caffeine+H]⁺ and (B) [L-phenylalanine+H]⁺ using CID as collected on a LTQ-ion trap. A detailed fragmentation pattern for caffeine is shown in [11].

3.3. Quantitative estimation of caffeine using deuterated caffeine as an internal standard

Determination of the concentration of a component of a complex mixture can be achieved in this PSMS experiment by using an internal standard. The internal standard used is generally an isotopically labelled counterpart of the analyte or any other molecule that is structurally similar to the analyte of interest [10]. The reason behind this choice is that the intensities of different analyte peaks are highly sensitive to the ionization efficiency, the sensitivity of detection, and matrix effects. Hence, using the molecule that is almost identical or has a similar structure to that of the analyte of interest essentially eliminates these effects and provides a reliable way of measuring concentrations. The idea is to measure the concentration of caffeine in the energy drinks and to compare these measurements to that of the reported value. Caffeine is chosen for this analysis because it is the common ingredient in all these energy drinks and its deuterated counterpart (caffeine-d₉) is commercially available. One can also use theobromine, which is structurally similar to caffeine as an internal standard, although the results are not as good as those obtained using the deuterated isotopic counterpart.

A concentration curve was prepared by plotting the concentrations of caffeine-d₉ to the peak intensity ratios of caffeine to caffeine-d₉. In this plot, when the ratio of caffeine to caffeine-d₉ is 1, the amount of caffeine in the sample approximately equals the amount of caffeine-d₉ added. A representative curve is shown in Fig. 4 for 5-hour Energy, the concentration of caffeine detected was 2.8 mg/mL, which is closer to that of the reported value of 3.39 mg/mL by the company. For reference, the caffeine concentrations reported for these drinks are 3.39 mg/mL, 0.32 mg/mL,

0.14 mg/mL and 0.096 mg/mL for 5-hour Energy, Red Bull Sugarfree, Starbucks Refreshers Blueberry Acai, and Coca Cola Classic, respectively [6]. The students were encouraged to look up these values, and were provided with the link mentioned in ref. 6. Most undergraduates are quite interested in caffeine content, which helps make this lab appealing and relevant.

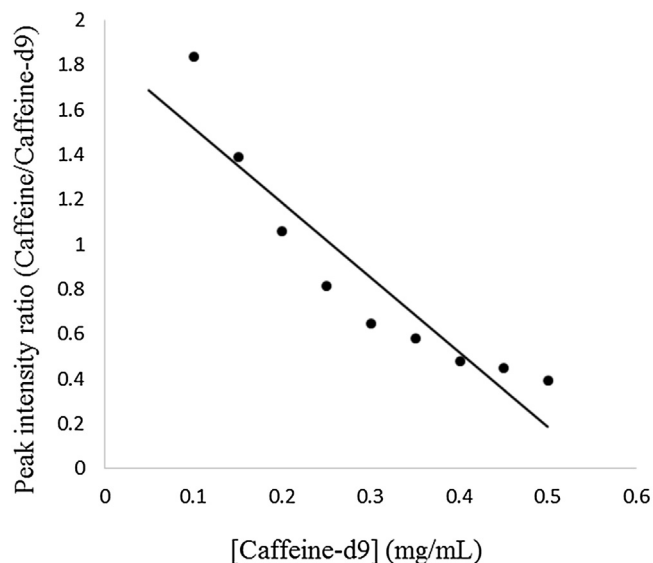


Fig. 4. Concentration curve of Caffeine in 5-hour Energy ($R=0.86$). The caffeine concentrations are shown in ten times dilution.

4. Conclusions

We have shown that paper spray mass spectrometry can be used to devise a laboratory exercise that can be used to introduce mass spectrometry early in the undergraduate curriculum. We have proposed a short lab involving two steps that introduce students to the analytical power of the mass spectrometer. We have generated the mass spectra for different drinks by directly loading the samples on the paper substrate. By choosing caffeine as one of the analyte ions at a given m/z , we used CID to fragment it and demonstrated how through analyzing the fragment mass spectra, one can deduce structural information of molecules. In addition, for an upper level undergraduate class, we have also included the exercise that shows how MS can be used to determine the concentrations of different analytes present in complex mixtures when a structurally appropriate internal standard is used.

Our main goal in this article is to introduce a simple MS laboratory exercise that can be conducted for classes of several hundred students within a given laboratory time period while giving important information on various aspects of mass spectrometry. The resources used in this experiment are mostly inexpensive and readily available with the exception of the mass spectrometer. Most institutions have a mass spectrometer available for research purposes and because this experiment is fast, and requires little sample volume, it is relatively easy to perform the experiment and clean the instrument afterwards. However, care must be taken because of possible exposure to high voltage, but this danger is minimized by the fact that the current is less than one microampere. Early introduction of the use of a mass spectrometer in an undergraduate laboratory curriculum can enhance the student's understanding of matter and its composition and properties. We hope that teachers across the world will be able to use this experiment or a part of it to provide students with hands-on experience in mass spectrometry and the importance of instrumentation in chemical analysis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ijms.2016.09.019>.

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