LC-MS/MS Method Development for Undergraduate Lab Curriculum by Analysis of Caffeine and Theobromine in Chocolate

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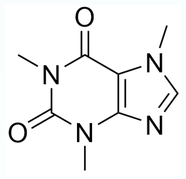
Abstract

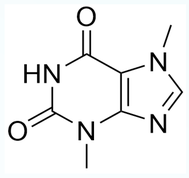
Most modern research labs utilize liquid chromatography-tandem mass spectrometry, or LC-MS/MS, to analyze mixtures in both qualitative and quantitative capacities. This makes it imperative to teach students how to operate and understand these instruments at an undergraduate level before reaching industry. The two compounds that are the focus of this study are caffeine and theobromine. These are most commonly known as the stimulants in coffee and chocolate respectively. These natural products are ideal for demonstrating the power of this instrument because they differ by a single methyl making the compound very difficult to separate using standard chromatography, but easy to distinguish using mass spectrometry. It is rare in analytical instruments that clean peak resolution is not a necessity, but this is the case using tandem mass spectrometry as the instrument can be set to recognize individual compounds. As both compounds are frequently found at varying ratios in the same products it is important to be able to look at these compounds together. This project will address the development of a method to train undergraduate students as well as instructors how to apply LC-MS/MS to various applications.

Introduction

The power of the LC-MS/MS is that it can be used to detect several ranges of concentration on a single instrument with compounds exhibiting a broad range of polarities, essentially enabling a larger dynamic range.Compounds that are similar in concantration and polarity can also be decerned and analyzed as seen with the caffeine and theobromine in Figure 1. One of the major challenges is setting the activity up in such a way that students gain meaningful understanding of the instrument and the broad range of its capabilities while being very mindful of the time constraint and skill level of the students. This project involves quantification of caffeine and theobromine. These purine alkaloids are part of a very important group of natural products today known as methylxanthines. These compounds are produced by more than 60 plants and can be found in the leaves, stems and seeds.[1] The medicinal uses of these compounds have been known for centuries and are still relevant today. Chocolate has been reported for its medicinal properties since the Aztec and Mayan civilizations.



CAFFEINE
1,3,7-TRIMETHYLXANTHINE



THEOBROMINE
3,7-DIMETHYLXANTHINE

Caffeine is a central nervous system stimulant used medicinally to enhance the effectiveness of analgesics such as acetaminophen in products like Excedrin. Growing popularity of caffeine containing products has raised the level of awareness related to the health risks associated with this stimulant. This is especially true among teenagers with the growing popularity of energy drinks in recent years. Theobromine is a diuretic and smooth muscle relaxant but is no longer used in commercial medication. Studies have shown that theobromine increase lipid and glucose metabolism contributing to increased weight loss.[2] Variations of methylxanthines show up together at various ratios in many products, but more importantly caffeine can break down to theobromine as well as several other alkaloid metabolites making it important to be able to not only test for both but to be able to differentiate between them effectively.[3]

Methodology

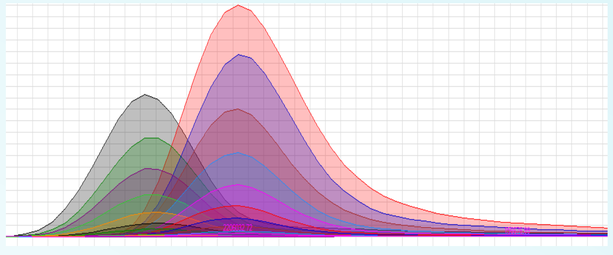


Figure 1: *The overlapping chromatograms of caffeine and theobromine respectivly at various concnetrations*

A mixture of caffeine and theobromine were analyzed using Scan, SIM and MRM methods to create calibration curves. The compounds were prepared in 95% H_2O with 0.1% formic acid, 5% ACN with 0.1% formic acid, both Fischer Optima LC/MS grade. The analyses ware performed on an Agilent Technologies 6410 Triple Quad LC/MS, using an Agilent Technologies 1260 Infinity LC. A single pump gradient was established and used for all three of the analysis modes shown in Table 1. For both the SIM and MRM methods the collision energy was 10%. The precursor ions were set at 181 m/z for theobromine, and 195 m/z for caffeine on the SIM mode. While the transitions for the MRM were set at 181-> 138.1 m/z for theobromine and 195-> 138.1 m/z for caffeine. This primary fragmentation pattern is the result of a retro-Diels Alder rearrangement corresponding to the loss of methyl isocyanate for caffeine. The same portion of the ring structure is lost for theobromine in the form of isocyanic acid resulting in the same 138.1 m/z fragment shown in Figure 2.[4]

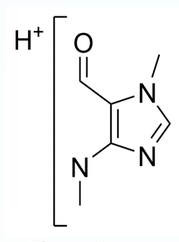


Figure 2: *Fragment for both caffeine and theobromine*

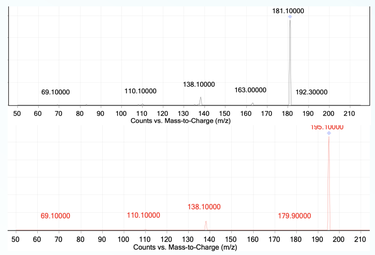
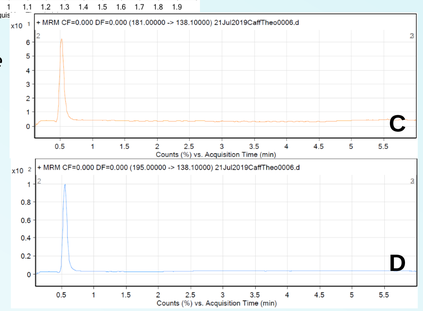


Figure 3: *Mass spectra for theobromine and caffeine showing fragmentation at 10% collision energy*

Figure 4: *The same mixture of theobromine and caffeine at 0.25 ppm while only changing the acquisition method showing decreased noise moving from Scan (A) theobromine and (B) caffeine, to MRM mode (C) theobromine and (D) caffeine*



The Rip Current Brewing Java Storm Coffee Imperial Porter was directly filtered without dilution. Cocoa samples were Almen dra Blanca, El Carmen and Asochivite from Goodnow Farms, and Nutell a spread. All cocoa sample were prepared by adding approximately 50 mg into 5.0 mL of water, warmed in a water bath to dissolve, then diluted. Nutella spread was prepared by doing a liquid-liquid extraction with approximately 0.1 g of the sample, 5.00mL of each Fischer HPLC grade isooctane and Fischer LCMS grade methanol. Bottom layer of methanol was used for injection after diluting. The isooctane extraction ensured the C-18 column would not be adversely affected. All food samples filtered using 0.45 µm PTFE eXtre me filter vial from Thomson Instrument Company.

Table 1: *Final pump gradieant A= water, B= ACN*

Time (min)	% A	% B
0	95	5
1	95	5
3	5	95
4	95	5
5	5	95
6	95	5

Results

Linearity of the calibration curves were established between 0.1-0.7 mg/mL in Scan mode, and below 0.1-0.7µg/mL using MRM mode (see Figure 3), with the overall detection limit at just over 1 pg/mL. Students were able to operate instrument and use both qualitative and quantitative analysis programs. The coffee containing beer was shown to have127 µg of caffeine, and 26.6 µg of theobromine per can. The cocoa samples are listed in Table 2.

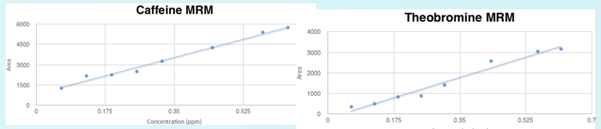


Figure 5: *Calibration curves for theobromine and caffeine for MRM method*

Table 2: *Resutls for cocoa samples*

	Caffeine	***Theobromine***	***Ratio***
	mg/g sample	***mg/g sample***	***Theo.:Caffeine***
Goodnow Farms: Almendra Blanca	9.09	55.6	6.12
Goodnow Farms: El Carmen	22.5	81.5	3.62
Goodnow Farms: Asochivite	14.8	86.7	5.86
Nutella	10.5	97.2	9.26

Conclusion

There are several factors that make the LC-MS/MS a unique and powerful piece of lab equipment. It is rare in analytical instruments that clean peak resolution is not a necessity, but this is the case using tandem mass spectrometry as the instrument can be set to recognize individual compounds. As both caffeine and theobromine are frequently found at varying ratios in the same products or biological samples it is important to be able to look at these compounds together. This experiment effectively demonstrates to students the advantages of this instrument and increased their knowledge base moving into the workforce.

References

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