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RESEARCH ARTICLE

Discrimination of Synthetic and Natural Caffeine by Carbon and Nitrogen Stable Isotope Ratio Analysis Utilizing GC-C-IRMS

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ABSTRACT

Rationale: Caffeinated drinks and supplements are popular products, many of which claim to use naturally derived caffeine. The availability of less expensive synthetic caffeine can motivate adulteration. Additionally, the caffeine content of these products can be inaccurately labeled, raising health concerns. As a result, effective testing methods are required to distinguish synthetic and natural caffeine and to test caffeine content in complex matrixes. In this study, a stable isotope method for natural caffeine authentication is described and applied to commercial samples. The accuracy of labeled caffeine content of products is evaluated.

Methods: Samples containing exclusively natural caffeine (i.e., coffee, tea, guarana, yerba mate) and synthetic caffeine were selected. Commercial products claiming to contain natural caffeine including supplements, energy drinks, and guarana products were also tested. Caffeine was extracted by incubation with a water-ammonium hydroxide mixture and phase separation with dichloromethane. Its carbon and nitrogen stable isotope composition was determined using GC-C-IRMS. Caffeine content was determined by HPLC-DAD.

Results: Natural and synthetic caffeine could be distinguished, with some limitations, based on carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopes. Based on this distinction, 15 of the 75 commercial samples labeled as containing natural caffeine were adulterated. The rate of apparent adulteration was high in guarana products (~78%) and supplements (~22%). Caffeine content deviating from label claim by more than ±20% was observed in 46% of the 86 samples tested, ranging from 57% to 217% of the labeled content.

Conclusions: Carbon and nitrogen isotope ratio analysis by gas chromatography-combustion-isotope ratio mass spectrometry is an efficient method for distinguishing natural and synthetic caffeine in commercially available products. Such testing is important given the significant number of apparently mislabeled products on the USA market. The prevalence of inaccurate caffeine content labeling may raise health concerns, highlighting the need for better production quality control and increased testing.

1 | Introduction

Caffeinated products including drinks and supplements are widely consumed in many parts of the world [1], with the dietary supplement market estimated at roughly 30 billion dollars [2]. While caffeine occurs naturally in plants such as coffee, tea, guarana, and yerba mate, cheaper synthetic caffeine is often added to food and beverage products. Distinguishing between natural and synthetic caffeine is important for consumer preferences and for regulatory reasons, as current Food and Drug Administration regulations require labeling added caffeine but not naturally occurring caffeine, as it is Generally Regarded As Safe (GRAS) [3]. Following recent public concern over the safety of caffeinated products, legislative efforts are in progress in the

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United States to impose stricter labeling requirements on such products, including labeling of caffeine source [4]. Such legislation would increase the need for caffeine testing.

Of particular interest are pre-workout dietary supplements, roughly 86% of which contain caffeine [5]. These products may be at higher risk for adulteration and poor production quality control because the regulation of supplements differs from other food or medicinal products [3]. For example, investigation of popular pre-workout supplements sold in Australia found significant discrepancies between labeled and measured caffeine concentrations and variable concentrations between batches of the same product [6]. Given these findings, there is reason to believe that some products claiming to contain natural caffeine may also be mislabeled. These concerns highlight the need for efficient methods for ensuring that products are properly labeled.

Several methods have been used to determine the source of caffeine in food products. Discrimination of natural and synthetic caffeine has been demonstrated using both radiocarbon dating [7] and stable isotope ratio analysis [8–11]. Studies using elemental analyzer combustion/pyrolysis Isotope Ratio Mass Spectrometry (IRMS) measured carbon, nitrogen, oxygen, and hydrogen isotope ratios in caffeine finding distinct values for natural and synthetic samples [10]. Subsequently, carbon and oxygen isotope ratios were used to distinguish natural and synthetic caffeine in commercial products [8]. However, as with radiocarbon, the analytical method was time-consuming and labor-intensive. More recently, a faster liquid chromatography IRMS system has been used [9] and a significant difference between the carbon isotope composition of natural and synthetic samples was demonstrated. The method was particularly suitable for liquid samples but is limited to carbon isotope analysis. These studies demonstrated the potential of stable isotope ratio analysis as a tool for identifying natural and synthetic caffeine, and even for distinguishing different natural caffeine sources [10]. However, as new analytical techniques emerge and new products reach the market, an updated study is called for. Dietary supplements, for instance, were not previously examined.

Here, we used a compound specific Gas Chromatography-Combustion-IRMS method (GC-C-IRMS) for carbon and nitrogen stable isotope ratio analysis of caffeine. We first analyzed 92 caffeine samples extracted from purely natural or synthetic sources. Using the results, we established an isotopic range for natural and synthetic caffeine. In addition, we compared the isotopic composition of naturally occurring caffeine with that of the bulk plant material from which it was extracted. We then tested the label claims of 88 caffeinated products. Finally, we measured the caffeine concentrations of 86 products including pre-workout supplements, energy drinks, and guarana products and evaluated label claims.

2 | Methods

2.1 | Sample Collection and Preparation

We collected a wide range of caffeine containing samples to establish the isotopic composition of natural and synthetic caffeine. For natural caffeine, samples of coffee beans (whole green beans = 15, whole roasted beans $n = 1$, ground roasted beans $n = 3$), tea ($n = 21$), guarana seeds ($n = 2$), and yerba mate ($n = 4$) were used (Table 1). For synthetic caffeine, we analyzed pure caffeine powders purchased from Sigma Aldrich and the USGS (originally from Alpha Aesar) ($n = 2$) and caffeine extracted from supplement powders and energy drinks labeled as containing synthetic caffeine ($n = 44$) (Table 2).

In addition to samples containing distinctly natural or synthetic caffeine, we analyzed multi-ingredient products to assess the validity of label claims. These included solid samples claiming to contain tea powder or extracts ($n = 24$), coffee powder or extract ($n = 11$), and guarana powder or extract ($n = 9$). In addition, pre-workout supplements labeled as containing natural caffeine ($n = 26$) and mixtures of natural and synthetic caffeine ($n = 13$) were analyzed. Energy drink samples labeled as containing natural ($n = 5$) and mixed source ($n = 1$) caffeine were also analyzed. Besides isotope ratio analysis, the caffeine content of supplements, energy drinks, and guarana products was determined and compared with the labeled caffeine content.

Liquid samples and fine powders were analyzed directly using the method described below. Coarse samples such as loose-leaf tea, guarana seeds, and whole coffee beans were homogenized using a stainless-steel coffee grinder prior to extraction.

2.2 | Bulk EA-IRMS Isotope Analysis

The carbon and nitrogen stable isotope composition of bulk powders was determined by EA-IRMS. Powdered samples were weighed into tin capsules and analyzed using a Delta Plus XL IRMS (Thermo-Finnigan, Bremen, Germany) coupled to a Nitrogen Analyzer 1500 elemental analyzer (Carlo Ebra instrumentze, Milan, Italy) by a ConFlo III interface (Thermo-Finnigan, Bremen, Germany).

2.3 | Caffeine Extraction

All extractions and analyses were performed at IEH Analytical Laboratories. Caffeine was extracted from solid and liquid samples in the following manner. Between 0.45 and 0.55 g of each solid sample and 4–5 g of each liquid sample were placed in 50 mL centrifuge tubes. Five milliliters of 1:2 ammonium hydroxide: HPLC water solution was added to each sample followed by vortex mixing. Samples were then incubated for 5 min at 100°C in a dry heat block, removed from the heat block and allowed to cool to room temperature. If solid residue was present after incubation, samples were centrifuged at 2000–3000 rpm for 10 min after which the liquid was transferred to a clean 50 mL centrifuge tube. Five milliliters of dichloromethane (DCM) were subsequently added to each sample, and samples were vortexed for ≥ 20 s. After mixing, samples were centrifuged for 10 min at 3000–4000 rpm to achieve phase separation. The lower DCM phase was then transferred into 15 mL centrifuge tubes, and the samples were dried under nitrogen gas at 40°C. After drying, the caffeine was reconstituted in 1–2 mL of methanol, depending on the caffeine concentration of the sample. Samples were vortexed and 300 μL of each sample were transferred to 300 μL glass insert vials for analysis.

TABLE 1 | Isotopic composition of natural products.

ID	Country/Region	Brand	Bulk $\delta^{15}N$ AIR (‰)	Bulk $\delta^{13}C$ VPDB (‰)	Caffeine $\delta^{15}N$ AIR (‰)	Caffeine $\delta^{13}C$ VPDB (‰)
Coffee beans						
1764489-204842	Tanzania	C1	2.0	−27.4	1.4	−26.4
1764489-204843	Honduras	C1	2.1	−29.7	0.1	−29.5
1764489-204844	Indonesia	C1	2.9	−27.7	0.8	−28.2
1764489-204846	Dominican Republic	C1	1.4	−31.0	−0.3	−29.8
1764489-204845	Costa Rica	C1	2.0	−28.5	−0.2	−28.5
1764489-204847	Bali	C2	0.3	−29.7	−1.7	−29.2
1764489-204848	Ethiopia	C2	5.5	−26.5	3.7	−26.9
1764489-204849	Brazil	C2	2.6	−28.2	0.3	−28.1
1764489-204850	Dominican Republic	C2	2.2	−30.9	0.1	−30.3
1764489-204851	India	C2	2.9	−28.0	0.3	−27.8
1764489-204853	Colombia	C3	2.0	−29.7	0.4	−29.1
1764489-204852	Dominican Republic	C3	1.3	−28.6	−0.7	−29.6
1764489-204854	Nicaragua	C3	4.5	−28.4	2.4	−27.7
1764489-204856	Mexico	C3	1.7	−27.6	−1.2	−28.7
1764489-204855	Peru	C3	4.6	−29.0	1.7	−28.6
1761002-193821	Central America	C4	3.8	−29.0	0.9	−29.0
1764489-204859		C5	0.7	−29.5	−1.3	−29.9
1764489-204857	Ethiopia	C6	2.2	−27.2	0.9	−28.3
1764489-204858	Colombia	C6	4.2	−28.3	3.6	−29.4
Green tea						
1760678-192708	Japan	T1	1.7	−28.5	−0.7	−32.6
1760123-190532	Japan	T2	5.5	−29.4	2.8	−34.4

(Continues)

TABLE 1 | (Continued)

ID	Country/Region	Brand	Bulk $\delta^{15}N$ AIR (‰)	Bulk $\delta^{13}C$ VPDB (‰)	Caffeine $\delta^{15}N$ AIR (‰)	Caffeine $\delta^{13}C$ VPDB (‰)
1760123-190543	Japan	T3	7.8	−30.4	5.4	−36.2
1760123-190522	Japan	T4	7.7	−30.1	5.1	−35.4
1760123-190530	Japan	T5	7.2	−29.0	4.8	−34.3
1760123-190523	Japan	T6	4.6	−29.5	2.0	−34.8
1760123-190545	Japan	T7	6.1	−29.2	3.6	−34.4
1760863-193325	Japan	T8	3.7	−28.9	1.3	−34.4
1760863-193326	Japan	T8	6.5	−28.8	3.8	−33.9
1760863-193327	Japan	T8	2.1	−29.2	−0.8	−35.6
1760563-192123	Japan	T9	6.2	−28.6	4.1	−33.8
1760864-193354	Japan	T10	5.0	−30.6	2.6	−35.8
1760863-193324	Japan	T11	8.2	−28.2	6.3	−31.4
1763460-201695	Japan	T12	5.5	−29.4	2.7	−35.0
1764130-203588	Japan	T13	6.2	−29.5	3.9	−35.9
Other tea						
1764489-205012	Turkey	T14	1.2	−29.5	−2.0	−36.2
1764489-205009	India	T15	2.6	−28.6	−0.2	−30.2
1764489-209567	Taiwan	T16	3.5	−28.1	1.0	−33.8
1764489-209566	India	T17	1.6	−28.6	−0.5	−29.3
1764489-209564	India	T17	0.5	−28.0	−1.9	−29.8
1764489-209565	India	T17	1.1	−27.7	−1.3	−29.5
Guarana seeds						
1764489-208950		G1	5.0	−28.5	5.2	−28.0
1764489-208951	Brazil	G2	4.0	−28.4	4.2	−28.0

(Continues)

TABLE 1 | (Continued)

ID	Country/Region	Brand	Bulk $\delta^{15}N$ AIR (‰)	Bulk $\delta^{13}C$ VPDB (‰)	Caffeine $\delta^{15}N$ AIR (‰)	Caffeine $\delta^{13}C$ VPDB (‰)
Yerba mate						
1764489-205013	Argentina	Y1	1.9	−31.0	−0.7	−28.0
1764489-204862	Argentina	Y2	3.4	−30.2	0.6	−27.7
1764489-204861	Argentina	Y3	3.3	−30.8	0.3	−28.0
1764489-205010	Turkey	Y4	3.3	−30.3	0.7	−27.6

Between 20 and 30 samples were extracted in each batch. Quality control samples added to each batch included a blank (deionized water), spike (deionized water spiked with 1 mL of a caffeine solution with a known isotopic composition), a control sample (supplement powder containing caffeine with a known isotopic composition), and a duplicate of one of the samples in the batch.

2.4 | Caffeine Concentration Determination by HPLC-DAD

Prior to extraction, supplement powders were reconstituted in 250 mL HPLC water (×50 dilution). Caffeine was then extracted using the process described above excluding the DCM phase separation step. After incubation with 1:2 ammonium hydroxide: HPLC water, samples were diluted with HPLC water and loaded into 2 mL sample vials for analysis following AOAC method 979.11 [12]. Ten microliter samples were injected into an Agilent 1260 Infinity II Liquid Chromatograph (Agilent Technologies, United States) consisting of a quaternary pump, online degasser, column heater, autosampler, and diode array detector. Chromatographic separation was achieved using a ZORBAX Eclipse Plus C18 analytical column (4.6 × 150 mm, 5 μm particle size; Agilent Technologies, United States) preceded by a guard column with the same packing.

The mobile phase consisted of 25:75 (v/v) of methanol/water at a flow rate of 1.0 mL/min, and the column compartment was heated to 30°C. These conditions were held isocratically for 10 min, with caffeine eluting at around 6.7 min. The eluted caffeine was detected using a DAD set to a wavelength of 272 nm, following Agilent Application Note 5991-2851EN.

Two calibration curves for caffeine were used for quantification. One included seven levels ranging from 0.125 to 25 μg/mL (0.125, 0.50, 1.0, 2.5, 5.0, 12.5, 25 μg/mL) and the other included six levels ranging from 5 to 500 μg/mL (5.0, 12.5, 25, 100, 250, 500 μg/mL).

Caffeine content (concentration) is reported as percent caffeine. The deviation from label claim is calculated as:

$$\frac{\%\,\text{Caffeine(observed)}}{\%\,\text{Caffeine (labeled)}} * 100$$

A result of 100% indicates no deviation.

2.5 | Compound Specific GC-C-IRMS Isotope Analysis

A Trace GC 1310 with a TriPlus RSH autosampler coupled to an Isolink II device, Conflo IV interface and Delta V Plus isotope ratio mass spectrometer (Thermo Fisher) was used with a TG-5MS GC column, 30 m, 0.25 mm, 0.25 μm (Thermo Fisher). A liquid nitrogen trap was used to remove CO_2 during nitrogen isotope analysis.

For each analysis, 1 μL of sample was injected into the GC in splitless injection mode, with a splitless time of 1.5 min, a constant column flow rate of 1.2 mL/min, a split flow rate of 40 mL/min and a purge flow of 5 mL/min. The initial GC temperature of 80°C was held for 1 min, followed by an increase to the final temperature, 270°C, at a rate of 15°C/min. The final temperature was held for 6 min. The total GC run time was 20 min. The backflush was turned off between 500 and 1000 s, and the retention time was approximately 738 s. The isotope ratio of each sample was measured relative to a working reference gas. Three reference gas peaks were measured before and after each sample peak. Data was acquired by Isodat 3 software.

Each extraction batch was analyzed in an individual analytical sequence. An initial concentration run was conducted to estimate the caffeine concentration of the samples, which included a single injection of each sample and each standard. The caffeine peak amplitude was used to estimate concentration. If necessary, samples were then diluted with methanol to produce peak amplitudes approximately equal to those of the standards.

After the concentration was adjusted, two calibration runs were performed, one for carbon isotope composition and one for nitrogen isotope composition. In these runs, samples were analyzed in duplicate. Standard caffeine solutions with accepted $\delta^{13}C$ and $\delta^{15}N$ values (prepared from USGS61, USGS62, USGS63 caffeine powders) were analyzed at the beginning and end of each run and after every five samples (every 10 injections). A pure caffeine solution (prepared from Sigma-Aldrich C0750 ReagentPlus) was run alongside the standard solutions as a quality control.

TABLE 2 | Isotopic composition of caffeine and comparison of observed caffeine content to label claims in caffeinated products. A deviation from label claim of 100% indicates no deviation. Samples with isotope values in bold were labeled as containing natural caffeine but appear to contain synthetic caffeine. Samples with caffeine content in bold had deviation from label claim larger than ±20%, the acceptable FDA limit.

r	brand	Labeled caffeine type	Caffeine $\delta^{15}N$ AIR (‰)	Caffeine $\delta^{13}C$ VPDB (‰)	Total caffeine claimed (%)	Total caffeine found (%)	Deviation from label claim (%)
Coffee products							
1760678-192719	CP1	Natural	0.5	−29.2			
1760123-190539	CP2	Natural	0.8	−28.6			
1760123-190520	CP3	Natural	2.8	−27.5			
1760123-190519	CP3	Natural	2.7	−27.5			
1760123-190521	CP3	Natural	1.8	−27.2			
1760123-190528	CP4	Natural	0.6	−28.6			
1760565-192140	CP5	Natural	2.1	−26.9			
1760565-192141	CP5	Natural	2.3	−26.5			
1760565-192139	CP5	Natural	2.5	−26.5			
1760865-193393	CP6	Natural	2.3	−26.9			
1760864-193366	CP7	Natural	2.1	−26.9			
Green tea products							
1760865-193390	TP1	Natural	0.8	−34.7			
1760864-193370	TP2	Natural	2.0	−35.0			
1760123-190529	TP3	Natural	4.8	−34.8			
1760678-192700	TP4	Natural	0.5	−34.5			
1760678-192699	TP4	Natural	0.8	−34.3			
1760563-192125	T9	Natural	4.0	−33.9			
1760563-192124	T9	Natural	3.8	−33.8			
1760123-190547	T9	Natural	2.6	−35.1			
1760864-193353	T10	**Natural**	**−3.4**	**−37.0**			
1760865-193352	T10	**Natural**	**−4.2**	**−37.2**			
1760865-193355	T10	Natural	2.4	−35.7			

(Continues)

TABLE 2 | (Continued)

r	brand	Labeled caffeine type	Caffeine $\delta^{15}N$ AIR (‰)	Caffeine $\delta^{13}C$ VPDB (‰)	Total caffeine claimed (%)	Total caffeine found (%)	Deviation from label claim (%)
1760864-193356	T10	Natural	−0.8	−34.9			
1760864-193358	TP5	Natural	3.2	−34.0			
1760121-190468	TP6	Natural	4.3	−34.2			
1760123-190538	TP7	Natural	2.1	−35.3			
1760864-193357	TP8	Natural	2.4	−32.1			
1760123-190525	TP9	Natural	0.7	−34.8			
1760864-193363	TP10	Natural	0.1	−34.5			
1760864-193362	TP10	Natural	0.2	−35.4			
1760865-193388	TP11	Natural	1.2	−34.4			
Black tea products							
1760678-192706	TP12	Natural	−0.7	−32.2			
1760123-190527	TP9	Natural	−1.2	−34.7			
1760122-190508	TP13	Natural	0.0	−30.5			
1760123-190524	TP14	Natural	1.2	−29.4			
Guarana products							
1764489-205011	GP1	Natural	4.9	−27.1		1.07	
1764489-205008	GP2	**Natural**	**−3.6**	**−33.3**	22	24	109
1764489-207237	GP3	**Natural**	**−2.2**	**−42.1**		3.82	
1764489-209205	GP4	**Natural**				< 0.025	
1764489-204860	GP5	**Natural**	**−1.1**	**−34.0**	22	25.1	114
1764489-209206	GP6	**Natural**	**−0.3**	**−33.8**	22		
1764489-209563	GP7	**Natural**	**−0.5**	**−33.7**	**16.92**	**9.61**	**57**
1764489-209562	GP8	Natural	1.2	−28.8	**22**	**15**	**68**
1764489-209204	GP9	**Natural**	**−1.3**	**−31.3**	**22**	**13**	**59**
Energy drinks							
1764489-208240	E1	**Natural**	**−2.0**	**−32.4**	**0.033**	**0.071**	**217**

(Continues)

TABLE 2 | (Continued)

r	brand	Labeled caffeine type	Caffeine $\delta^{15}N$ AIR (‰)	Caffeine $\delta^{13}C$ VPDB (‰)	Total caffeine claimed (%)	Total caffeine found (%)	Deviation from label claim (%)
1764489-208241	E2	Natural	0.3	−28.2	**0.034**	**0.042**	**124**
1764489-208245	E3	Natural	−1.1	−31.2	**0.024**	**0.031**	**132**
1764489-208948	E4	Natural	−0.1	−28.6			
1764489-208949	E5	Natural	2.0	−26.6			
1764489-208242	E6	Mix	−3.6	−31.6	0.056	0.046	82
1764489-208243	E7	Synthetic	−2.6	−31.5	0.032	0.036	114
1764489-208947	E8	Synthetic	−4.0	−33.1	0.032	0.037	116
Caffeine chemicals							
CR1	Sigma Aldrich	Synthetic	−2.3	−32.1			
CR2	USGS	Synthetic	−2.9	−35.1			
Supplements							
1760685-192781	S3	Natural	0.8	−31.1	**1.53**	**2.71**	**177**
1760685-192782	S3	Natural	0.3	−31.1	1.64	1.82	111
1762963-200035	S4	Natural	0.6	−30.4	3.33	3.59	108
1762963-200037	S6	Natural	0.6	−31.0	**4.00**	**3.01**	**75**
1763591-202119	S8	Natural	−0.7	−30.7	**0.96**	**1.71**	**178**
1760685-192772	S10	**Natural**	**−1.6**	**−41.1**	**1.53**	**1.89**	**124**
1764329-204284	S11	Natural	0.9	−28.7	**1.25**	**1.55**	**124**
1762734-199212	S15	Natural	0.9	−27.0	2.19	1.78	81
1764844-205884	S16	**Natural**	**−1.9**	**−31.5**	0.78	0.91	116
1761427-195098	S23	**Natural**	**−0.5**	**−40.6**	1.01	1.20	119
1761094-194177	S27	Natural	0.8	−27.0	**1.71**	**2.12**	**124**
1760678-192714	S28	Natural	0.5	−31.1	**1.04**	**1.30**	**125**
1762734-199228	S38	Natural	0.3	−28.1	1.74	1.40	80
1763259-200980	S39	Natural	0.3	−28.7	**6.25**	**4.54**	**73**
1763591-202131	S40	Natural	0.5	−27.2	**1.88**	**2.47**	**132**

(Continues)

TABLE 2 | (Continued)

r	brand	Labeled caffeine type	Caffeine $\delta^{15}N$ AIR (‰)	Caffeine $\delta^{13}C$ VPDB (‰)	Total caffeine claimed (%)	Total caffeine found (%)	Deviation from label claim (%)
1761094-194170	S43	Natural	1.0	−26.9	2.50	2.76	110
1760682-192746	S47	Natural	0.5	−28.2	**2.00**	**2.44**	**122**
1762735-199239	S49	Natural	0.6	−28.3	2.50	2.94	118
1764131-203620	S50	**Natural**	**−1.3**	**−38.4**	1.82	2.02	111
1760685-192774	S55	Natural	0.6	−29.2	**1.94**	**1.38**	**71**
1763459-201671	S61	**Natural**	**−3.0**	**−32.5**	1.11	1.21	109
1763460-201684	S62	Natural	0.7	−27.3	2.08	2.16	104
1761537-195421	S63	**Natural**	**−0.7**	**−40.6**	**2.70**	**3.49**	**129**
1763258-200948	S65	Natural	0.6	−28.0			
1763259-200986	S67	Natural	0.6	−27.3	1.97	2.21	112
1760563-192127	S68	Natural	0.1	−27.5	**1.35**	**1.71**	**126**
1763259-200973	S2	Mix	−2.6	−37.2	1.33	1.51	113
1763460-201681	S14	Mix	−2.2	−33.2			
1762734-199201	S18	Mix	−2.8	−31.3			
1762735-199250	S19	Mix	−1.5	−31.3	**3.61**	**2.19**	**61**
1762901-199876	S22	Mix	−1.2	−30.8	7.86	7.06	90
1763460-201696	S24	Mix	−1.8	−34.8			
1760121-190482	S30	Mix	−3.4	−33.2	1.52	1.69	111
1762734-199221	S34	Mix	−1.0	−32.7	2.18	2.08	95
1763259-200979	S39	Mix	−1.6	−30.6	2.13	2.19	102
1763259-200976	S48	Mix	−1.4	−31.0	**1.07**	**1.88**	**176**
176083-193331	S52	Mix	−3.5	−33.2	2.72	2.56	94
1760685-192777	S55	Mix	−3.5	−32.8	2.61	2.53	97
1764132-203642	S64	Mix	−1.9	−36.8	**0.87**	**1.20**	**138**
1763459-201677	S1	Synthetic	−3.5	−32.4	1.25	1.36	109
1763459-201676	S1	Synthetic	−4.4	−33.0	1.54	1.80	117

(Continues)

TABLE 2 | (Continued)

r	brand	Labeled caffeine type	Caffeine $\delta^{15}N$ AIR (‰)	Caffeine $\delta^{13}C$ VPDB (‰)	Total caffeine claimed (%)	Total caffeine found (%)	Deviation from label claim (%)
1763259-200974	S2	Synthetic	−2.2	−32.2	9.09	8.13	89
1760863-193335	S5	Synthetic	−2.2	−30.7	**1.16**	**1.44**	**124**
1763460-201692	S7	Synthetic	−2.9	−32.0	**2.14**	**2.60**	**121**
1763460-201691	S7	Synthetic	−3.2	−32.9	**2.41**	**1.90**	**79**
1763258-200945	S9	Synthetic	−3.1	−32.1	1.43	1.58	111
1762734-199207	S12	Synthetic	−2.3	−30.4	**1.19**	**1.49**	**125**
1760565-192158	S13	Synthetic	−3.9	−32.9	2.54	2.97	117
1763460-201682	S17	Synthetic	−2.5	−31.3	**1.05**	**1.35**	**129**
1762735-199249	S20	Synthetic	−1.4	−30.9			
1762734-199204	S21	Synthetic	−1.6	−31.7	2.36	2.80	119
1763459-201675	S25	Synthetic	−3.0	−33.2	**1.36**	**1.64**	**120**
1760121-190485	S26	Synthetic	−2.9	−33.2	**2.71**	**3.49**	**129**
1760121-190478	S29	Synthetic	−1.3	−33.5	**1.35**	**1.76**	**130**
1760121-190479	S29	Synthetic	−1.4	−33.1			
1760121-190486	S31	Synthetic	−3.8	−33.1	1.94	2.11	109
1760121-190483	S31	Synthetic	−3.2	−31.8	3.50	3.74	107
1764130-203571	S32	Synthetic	−4.1	−32.1	**2.11**	**2.59**	**123**
1760566-192177	S33	Synthetic	−3.3	−33.4	2.00	2.39	119
1760565-192154	S35	Synthetic	−1.5	−39.9	**0.98**	**1.19**	**122**
1762735-199231	S36	Synthetic	−3.7	−32.5	1.79	1.93	108
1762735-199241	S37	Synthetic	−2.1	−30.6	1.70	1.91	112
1760682-192732	S41	Synthetic	−3.3	−33.1	1.97	2.17	110
1763459-201673	S42	Synthetic	−3.3	−32.1	**1.80**	**2.19**	**122**
1761094-194168	S43	Synthetic	−1.5	−31.3	2.45	2.32	95
1763460-201700	S44	Synthetic	−3.3	−32.3	2.00	2.37	119
1762735-199233	S45	Synthetic	−2.6	−32.8	**1.67**	**2.49**	**149**

(Continues)

TABLE 2 | (Continued)

r	brand	Labeled caffeine type	Caffeine $\delta^{15}N$ AIR (‰)	Caffeine $\delta^{13}C$ VPDB (‰)	Total caffeine claimed (%)	Total caffeine found (%)	Deviation from label claim (%)
1760682-192743	S46	Synthetic	−2.9	−32.7	**2.29**	**2.85**	**125**
1760685-192773	S51	Synthetic	−3.9	−32.8	**1.20**	**1.83**	**152**
1760863-193330	S52	Synthetic	−3.4	−32.6	4.48	5.07	113
1760863-193328	S52	Synthetic	−4.1	−33.2	**1.89**	**2.30**	**122**
1762901-199885	S53	Synthetic	−3.1	−31.2	1.88	2.11	112
1763259-200982	S54	Synthetic	−3.9	−32.5	1.06	1.24	117
1760685-192784	S56	Synthetic	−2.1	−41.6	1.07	1.20	113
1760685-192785	S56	Synthetic	−2.3	−42.5	1.29	1.38	107
1760685-192787	S56	Synthetic	−2.7	−33.3	**2.07**	**2.59**	**125**
1764131-203605	S57	Synthetic	−1.4	−31.0	2.24	2.65	118
1760682-192755	S58	Synthetic	−3.2	−33.2	**1.78**	**2.19**	**123**
1763459-201658	S59	Synthetic	−3.8	−32.3	1.75	1.84	105
1761093-194143	S60	Synthetic	−3.2	−31.8	**1.72**	**2.11**	**122**
1762736-199270	S66	Synthetic	−3.4	−32.3	1.00	1.10	110

2.6 | Isotope Ratio Calculation and Calibration

Carbon and nitrogen isotope ratios were calculated using accepted conventions and reported in permil notation using the equation:

$$\delta^{13}C = \left[\frac{[^{13}C/^{12}C]\text{sample}}{[^{13}C/^{12}C]\text{standard}} - 1 \right] * 1000$$

and an equivalent equation for $^{15}N/^{14}N$ ratios. $\delta^{13}C$ values are reported in the Vienna Pee Dee Belemnite scale (VPDB) and $\delta^{15}N$ value are reported in relative to air nitrogen (AIR).

2.7 | Method Validation

The method for caffeine quantitation by HPLC-DAD was validated in terms of linearity, reproducibility, recovery, selectivity, matrix effect, and limits of detection and quantitation. Linearity was evaluated across both stated calibration ranges. Both demonstrated acceptable linearity: the low-level calibration range (0.125–12.5 μg/mL) demonstrated a correlation coefficient (R^2) of 0.9993 and the high-level range (5.0–500 μg/mL) demonstrated an R^2 of 0.9999. Intra-day repeatability was assessed by extracting and analyzing six replicate preparations of an energy drink mix, yielding an RSD of 0.26%. Inter-day repeatability was evaluated using replicate extractions performed by two separate analysts on two separate days and yielded an RSD of 1.03%. Recovery was evaluated using representative complex matrices, including pre-workout and drink mix powders. Recoveries ranged from 96.3% to 98.0%, demonstrating good accuracy of the method across these representative sample matrices. Matrix effect was evaluated by calculating the matrix factor using the peak areas of caffeine spiked into pure solvent and an extract of caffeine-free pre-workout powder at three different concentrations (1.0, 5.0, and 25 μg/mL). The average matrix factor of the three concentrations was 1.01, indicating minimal matrix effect. The limit of detection (LOD) was calculated by performing seven low-level spikes at 6.25 μg/g. The standard deviation of the replicates was multiplied by 3.14, corresponding to the Student's *t*-value for seven replicates where $1-\alpha = 0.99$. The limit of quantitation (LOQ) was calculated by multiplying the standard deviation of the replicate measurements by 10. From these calculations, the LOD is 2.4 μg/g and the LOQ is 7.7 μg/g.

A method validation study was also conducted to verify the suitability of the GC-C-IRMS method. First, a single supplement powder was extracted 30 times and analyzed to evaluate reproducibility. The 30 extractions had a $\delta^{13}C$ standard deviation of 0.08‰ and a $\delta^{15}N$ standard deviation of 0.15‰. These standard deviations were equal to or lower than the standard deviation of caffeine standard solutions analyzed in the same run, demonstrating that the extraction was highly reproducible.

Second, a set of blank and spike samples were extracted to rule out contamination and isotopic fractionation during the extraction process. Six blanks did not produce measurable caffeine peaks, indicating no contamination during the extraction. In addition, 18 spike samples were prepared by adding a 4-mg/mL caffeine-standard solution with a known isotopic composition to deionized water. Spikes were added at three different volumes, 0.5, 1.0, 1.5 mL. The caffeine was then extracted and samples were analyzed. All samples had $\delta^{13}C$ values between +0.04‰ and +0.40‰ of the known value (average absolute difference of 0.2‰) and $\delta^{15}N$ values between −0.37‰ and +0.52‰ (average absolute difference of 0.2‰) of the known value, demonstrating that caffeine did not significantly fractionate during the extraction process.

To further verify the lack of fractionation during the extraction a caffeine recovery test was conducted. Three solutions of caffeine in deionized water were prepared at concentrations of 0.043, 0.106, and 0.137 mg/mL. Caffeine was extracted in duplicate from 0.5 g aliquots of the solutions and the caffeine content was measured by HPLC-DAD. The caffeine content of the initial solutions was also measured by HPLC-DAD and compared with the extracts, such that the instrument response was directly comparable. During the extraction, 4 of the 5 mL of DCM were collected to avoid inadvertent mixing of the aqueous phase. As the DCM layer is well mixed, this should not induce fractionation. Adjusting for this partial collection factor, the recovery rates were 87.6%–90.9% (average 89.4%). These high rates further demonstrate the suitability of the method for isotope analysis. As discussed above, any caffeine loss did not result in significant fractionation.

3 | Results

3.1 | Isotopic Composition of Natural Samples

Caffeine extracted from coffee beans had $\delta^{13}C$ and $\delta^{15}N$ values of −26.4‰ to −30.3‰ and −1.7‰ to 3.7‰, respectively (Table 1, Figure 1). The $\delta^{13}C$ values of bulk coffee beans and extracted caffeine were within 1.2‰ of each other, showing no systematic offset (Figure 2), indicating little to no fractionation. Caffeine extracted from tea had $\delta^{13}C$ and $\delta^{15}N$ values of −29.3‰ to −36.2‰ and −2.0‰ to 6.3‰, respectively. The difference in $\delta^{13}C$ values between bulk tea and caffeine extracted from the same sample was variable, but caffeine from Japanese green tea had consistently lower values relative to the bulk material compared with other plants, averaging 5.3‰ lower. Caffeine from ground guarana seeds had $\delta^{13}C$ and $\delta^{15}N$ values of −28.0‰ and 4.1‰ to 5.2‰, respectively, with nearly identical values for the bulk material. Yerba mate derived caffeine had $\delta^{13}C$ and $\delta^{15}N$ values of −28‰ to −27.6‰ and 0.7‰ to −0.7‰, respectively. The $\delta^{13}C$ values of yerba mate derived caffeine were 2.6‰–3.0‰ higher than bulk yerba mate. Interestingly, all natural samples showed a similar, strong correlation between bulk and caffeine $\delta^{15}N$ values (Figure 2). In all plants, except for guarana, caffeine $\delta^{15}N$ values were ~2.1‰ lower than the those of the bulk material.

The isotopic values of bulk coffee, yerba mate, guarana, and caffeine extracted from these samples are generally consistent with the results of previous studies [8, 9, 11]. However, the values of caffeine extracted from green tea, particularly Japanese matcha, are lower than previously reported for tea-derived caffeine despite having bulk isotopic values consistent with previous studies.

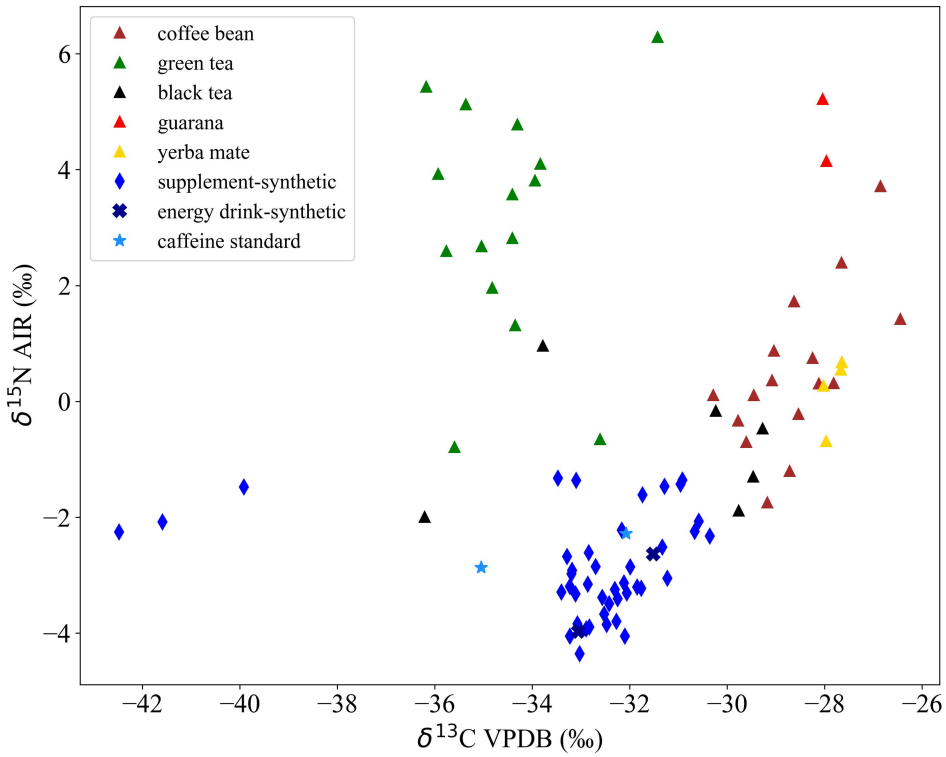


FIGURE 1 | Isotopic composition of natural and synthetic caffeine. Triangles are caffeine extracted from natural products. Diamonds, X symbols and stars are pure caffeine chemicals and caffeine extracted from supplements and energy drinks labeled as containing synthetic caffeine.

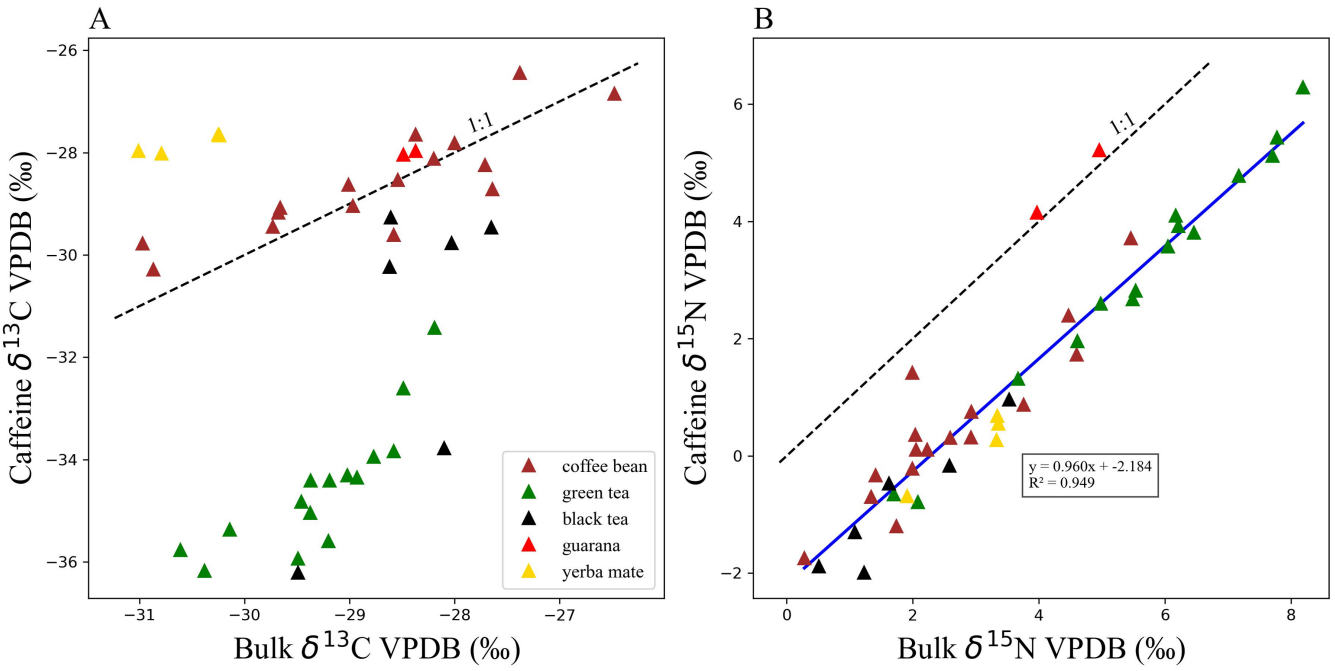


FIGURE 2 | Correlation between the isotopic composition of bulk leaves and beans and caffeine extracted from the same natural samples. (A) Carbon isotope values. (B) Nitrogen isotope values. Dashed lines represent equal values indicating no fractionation between the bulk material and caffeine. The blue line in (B) is a least squares regression on all data points except for guarana seeds. The line's equation and coefficient of determination (R^2) are below the line.

3.2 | Isotopic Composition of Synthetic Samples

The two pure synthetic caffeine samples had $\delta^{13}C$ values of −32.07‰ and −35.05‰ (value from USGS) and $\delta^{15}N$ values of −2.28‰ and −2.87‰ (value from USGS) (Table 2). Supplements and energy drinks labeled as containing only synthetic caffeine had $\delta^{13}C$ values between −42.5‰ and −30.4‰ and $\delta^{15}N$ values of −4.4‰ to −1.1‰. The $\delta^{13}C$ values of synthetic caffeine samples

had a bimodal distribution, with three samples returning distinctly low $\delta^{13}C$ values (< −39‰), lower than all other synthetic and natural samples (Figure 1).

3.3 | Isotopic Composition of Caffeinated Products

Caffeine extracted from samples labeled as containing coffee powder returned $\delta^{13}C$ and $\delta^{15}N$ values of −29.2‰ to −26.5‰ and 0.5‰ to 2.8‰, respectively (Table 2), entirely within the range of coffee bean-derived caffeine (Figure 3). Similarly, caffeine extracted from samples labeled as containing black tea powder or extract (not including supplements) had $\delta^{13}C$ and $\delta^{15}N$ values of −34.7‰ to −29.4‰ and −1.2‰ to 1.2‰, respectively. The values are in the range of caffeine extracted from black tea and other natural samples. Of the 20 samples labeled as containing green tea powder, 18 had $\delta^{13}C$ and $\delta^{15}N$ values of −35.7‰ to −32.1‰ and −0.8‰ to 4.8‰, respectively, consistent with the values of pure green tea samples. The two remaining samples had $\delta^{13}C$ values ≤ −37.0‰ and $\delta^{15}N$ values ≤ −3.4‰, lower than any green tea caffeine sample or any other natural caffeine in the dataset (Figure 3).

Supplement powders claiming to contain natural caffeine or mixtures of synthetic and natural caffeine had $\delta^{13}C$ and $\delta^{15}N$ values of −41.1‰ to −26.1‰ and −3.5‰ to 1.0‰, respectively (Table 2). Of the 26 pre-workout samples labeled as containing only natural caffeine, six returned isotopic values overlapping those of synthetic caffeine (Figure 3). The energy drinks labeled with natural or mixed caffeine had $\delta^{13}C$ and $\delta^{15}N$ values of −26.6‰ to −31.6‰ and −3.6‰ to 2.0‰, respectively. One sample labeled with natural caffeine had values in the range of synthetic caffeine.

Of the nine guarana samples analyzed, two returned plausible values for guarana caffeine, with $\delta^{13}C$ values of −28.8, −27.1‰ and $\delta^{15}N$ values of 1.2, 4.9‰. The rest returned much lower $\delta^{13}C$ values (−42.1‰ to −31.3‰) and $\delta^{15}N$ values (−3.6‰ to −0.3‰). Six of the products were labeled as pure guarana powder or extract and were additionally analyzed for bulk isotope composition. A single product, which also returned plausible caffeine isotopic ratios, had bulk values of −27.9‰ and 4.5‰ for $\delta^{13}C$ and $\delta^{15}N$, respectively. The remaining five had $\delta^{13}C$ values of −12.6‰ to −17.9‰ and $\delta^{15}N$ values of −3.3‰ to −0.43‰, incompatible with guarana or any C3 plant. One of these had no measurable caffeine or nitrogen.

3.4 | Caffeine Content of Supplements, Energy Drinks, and Guarana Products

Significant deviations from label claims were found in all caffeinated product types (Figure 4, Table 2). Caffeine content in supplements was between 60.6% and 178% of the labeled content and 33 of the 75 samples tested (44%) had caffeine content not within ±20% of the labeled content. Half of the energy drinks tested ($n = 6$) had a caffeine content not within ±20% of the labeled content, one sample containing more than double the labeled concentration (217%). Several guarana product labels were also inaccurate, with three of five samples containing only

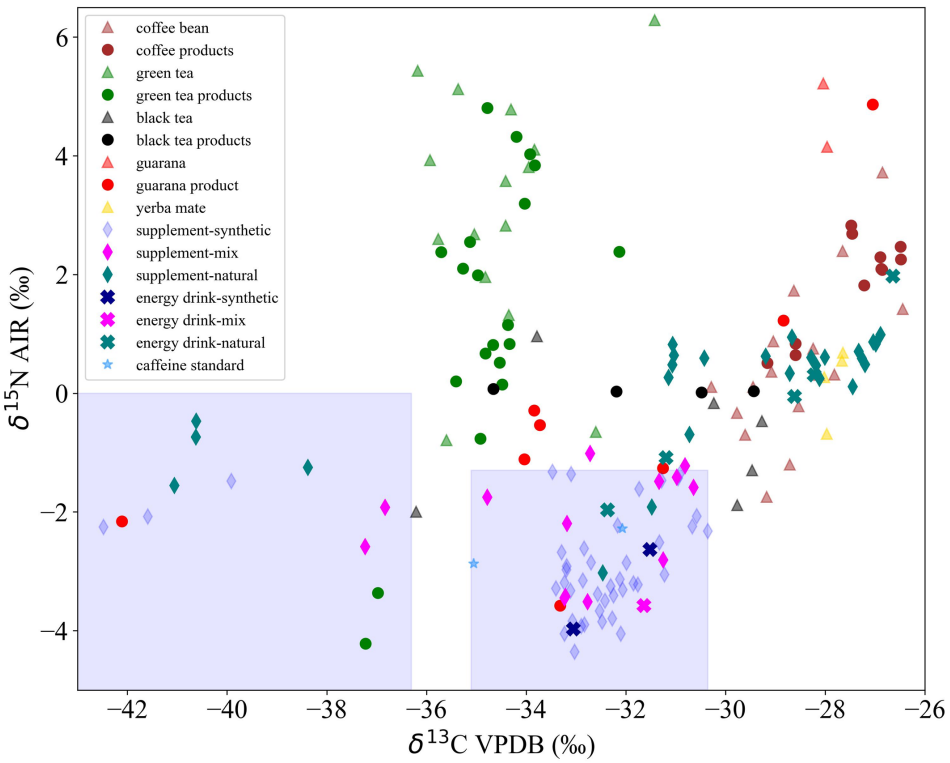


FIGURE 3 | Isotopic composition of caffeine extracted from caffeinated products, natural samples and synthetic caffeine. Blue shaded areas are ranges of isotopic compositions likely reflecting synthetic caffeine. The upper limit of the low $\delta^{13}C$ shaded area (bottom left) is cut off arbitrarily as 0‰ for clarity. Samples labeled as natural but falling within the blue shaded regions are likely adulterated. Supplement and energy drinks labeled "mix" are products labeled as containing a mixture of synthetic and natural caffeine.

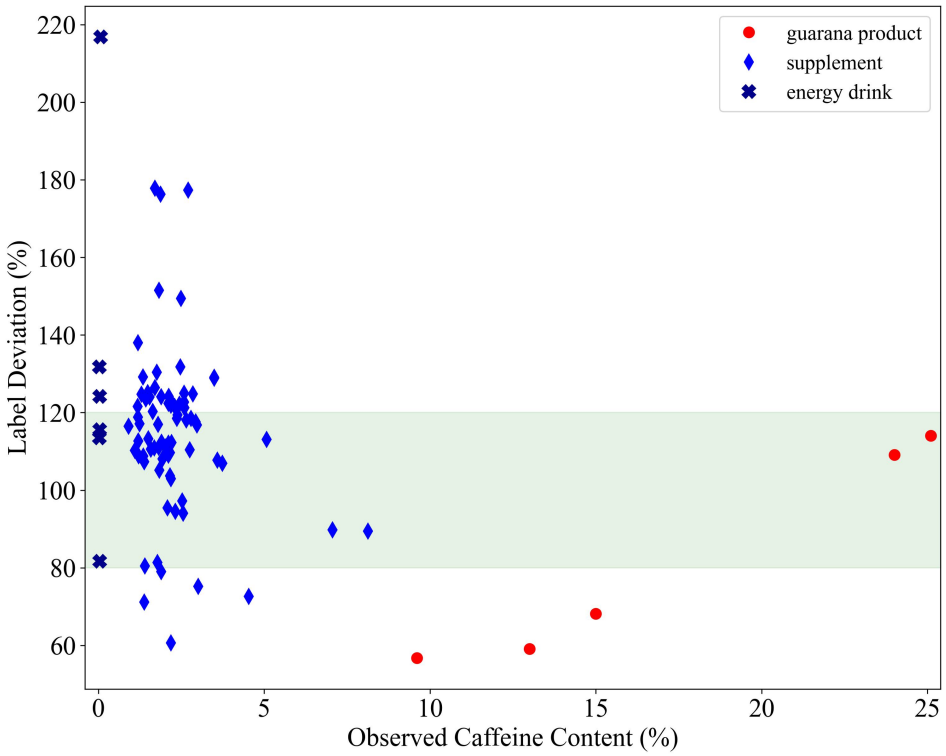


FIGURE 4 | Caffeine content comparison to label claims. The deviation is calculated as the ratio of measured percent caffeine to labeled percent caffeine, multiplied by 100, such that 100% indicates no deviation. The green shaded area is a ±20% deviation range considered acceptable by the FDA.

56.8%–68.2% of the labeled concentration. One product contained no caffeine, but its label did not explicitly state caffeine as an ingredient, so it is not technically mislabeled.

4 | Discussion

4.1 | Isotopic Discrimination of Natural and Synthetic Caffeine

The main objective of this study was to evaluate the use of carbon and nitrogen isotope ratios to distinguish natural and synthetic caffeine. Our results indicate that such discrimination is possible but is not as clear-cut as suggested by previous studies. We find that $\delta^{13}C$ values alone are nearly sufficient for distinguishing caffeine extracted from coffee beans, guarana, and yerba mate from synthetic caffeine, as previously argued [9]. However, synthetic caffeine can have higher $\delta^{13}C$ values than previously reported ($> -33‰$) and tea derived caffeine can have lower $\delta^{13}C$ values than previously reported ($< -32‰$), overlapping with those of synthetic caffeine. Considering the lowest measured $\delta^{13}C$ value of caffeine from tea, only samples with values $< -36.2‰$ can be identified as synthetic based on carbon isotopes alone.

The combined use of carbon and nitrogen isotopes provides a more robust method of discrimination. The $\delta^{15}N$ values of synthetic caffeine are generally lower than those of natural caffeine ($< -1.3‰$), as previously reported [10]. However, tea and coffee-derived caffeine samples returned values as low as $-2.0‰$, so only samples with $\delta^{15}N$ values $< -2.0‰$ can be identified as synthetic based on nitrogen isotopes alone. Despite the overlap in values when carbon or nitrogen isotopes are considered separately, we find no overlap between natural and synthetic caffeine in combined $\delta^{15}N$–$\delta^{13}C$ space (Figure 1). The few natural samples with low $\delta^{15}N$ values have $\delta^{13}C$ values outside of the ranges of synthetic samples, which have a bimodal carbon isotope distribution. For example, a coffee bean sample with a $\delta^{15}N$ value of $-1.7‰$ could be mistakenly identified as synthetic based on nitrogen alone, but it is $\delta^{13}C$ value of $-29.2‰$ is inconsistent with synthetic caffeine. This separation allows identification of synthetic caffeine but limits the ability to detect adulteration of natural caffeine with small amounts of synthetic caffeine. Also, certain combinations of natural caffeine can, theoretically, produce values that could be interpreted as synthetic and vice versa.

These observations lead to a somewhat complicated framework for distinguishing natural and synthetic caffeine. Based on this dataset, caffeine can be classified as synthetic based on $\delta^{15}N$ values $< -2.0‰$, $\delta^{13}C$ values $< -36.2‰$ or $\delta^{15}N$ values $< -1.3‰$ and $\delta^{13}C$ values between $-35.1‰$ and $-30.4‰$ (Figure 3), the latter option being more susceptible to incorrect interpretation in case of mixtures of caffeine sources. It is difficult, based on our dataset, to determine the exact maximum possible $\delta^{15}N$ value for synthetic caffeine. As with any classification, it is only as comprehensive as the dataset it is based on, so these values may change with additional data. For example, caffeine $\delta^{15}N$ values as low as $-3‰$ have been reported for coffee [10], lower than any natural sample in our dataset. Despite these limitations, we found that most of the products we analyzed fell clearly within the natural or synthetic ranges (Figure 3), allowing classification while taking a conservative approach. It is possible that

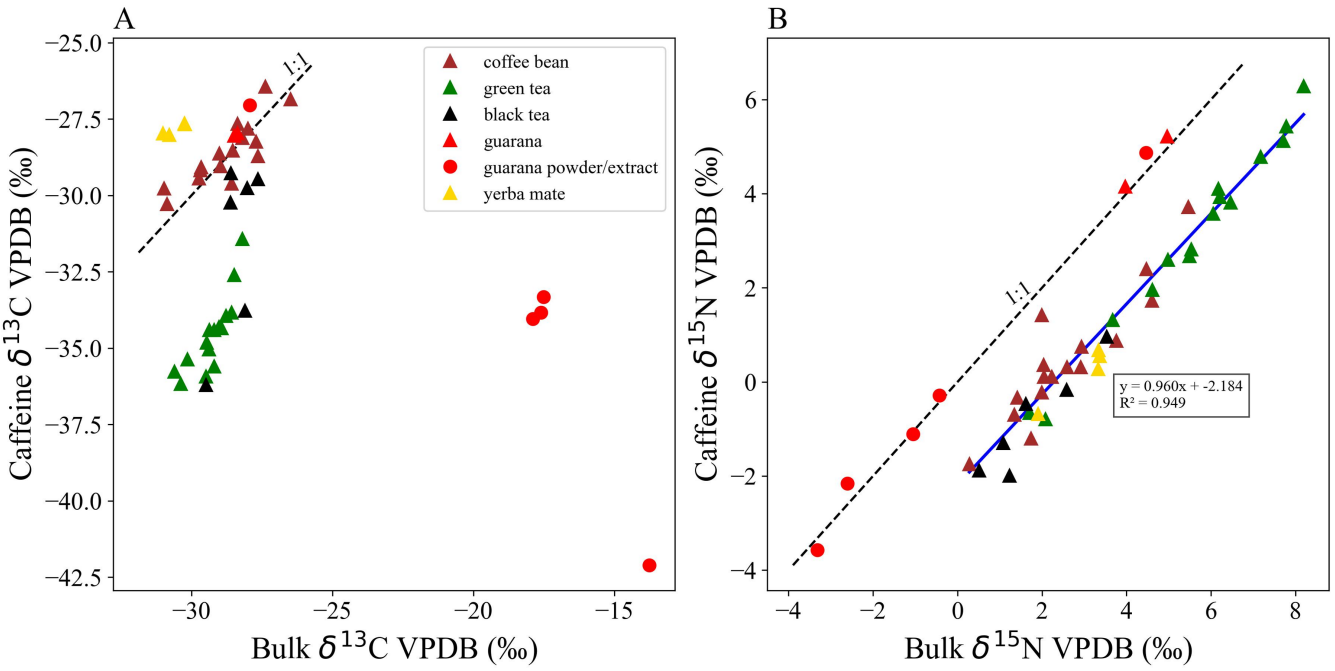


FIGURE 5 | Correlation between bulk and caffeine isotopic composition for natural samples and guarana products. Guarana products significantly deviating from the correlations observed for guarana seeds are likely adulterated. The blue and dashed lines are the same as in Figure 3.

measuring oxygen isotopes, as well as position specific isotope analysis in addition to carbon and nitrogen could enable better discrimination of synthetic and natural caffeine, as previously suggested [8, 10, 11].

It is often desirable to establish thresholds for the percent adulterant that can be added to an unadulterated sample before the adulteration is detectable by a given method. However, the similarity in isotopic composition between natural and synthetic caffeine in our dataset makes such a calculation largely impractical. The two most similar natural and synthetic caffeine samples have δ^{13}C values of −29.8‰ and −30.4‰ and δ^{15}N values of −1.9‰ and −2.3‰, respectively. Given the small differences, a relatively large proportion of synthetic caffeine could be added to a natural caffeine product before it was detected. In a worst-case scenario, where the pure natural product has a δ^{13}C value of −26.9‰ and a δ^{15}N value of 3.7‰ (sample 1764489-204848) and the synthetic caffeine had the composition described above, ~83% synthetic caffeine could be added before the δ^{13}C value was outside the natural range and ~93% could be added before the δ^{15}N value would be outside the natural range. As such, the method is more appropriate for distinguishing purely natural and purely synthetic caffeine. Adulteration of specific types of natural caffeine that have a narrower δ^{13}C value range, such as caffeine derived from coffee, yerba mate, or guarana, may be more readily detected.

The correlation between the isotopic composition of bulk coffee beans, tea leaves, yerba mate leaves, and guarana seeds and caffeine extracted from these materials provides a secondary authentication tool for single ingredient products labeled as powders or extracts of these plants. In such samples, the isotopic composition of the bulk powder should correlate with the composition of caffeine. A large deviation from the correlations observed in this study (Figure 2) could indicate adulteration. For example, the isotopic composition of bulk guarana seeds and caffeine extracted from the same seeds is nearly identical (Figure 2). Of the four samples labeled as pure guarana powders and extracts analyzed in this study, one follows this expected trend while the other three have very different bulk and extracted caffeine δ^{13}C values, supporting the interpretation that they are adulterated (Figure 5). Interestingly, the δ^{15}N values of bulk powder and caffeine extracted from these sample are nearly identical. This is likely because caffeine is the only significant nitrogen source in these samples. They appear to be C4 plant powders, such as corn cellulose, to which synthetic caffeine has been added.

4.2 | Prevalence of Adulteration in the USA Market

Using the combined carbon–nitrogen isotope approach described above, we assessed the authenticity of samples labeled as containing naturally derived caffeine. All products labeled as containing coffee or black tea powder (and other ingredients, $n = 15$) returned caffeine isotopic values consistent with the label claim (Figure 3). Two of the 20 samples labeled as containing Japanese matcha green tea powder, both from the same brand, had caffeine isotopic values indicating adulteration. Overall, these results suggest a low prevalence of fraud for samples containing coffee and tea powders.

Given that supplement powders and energy drinks often have high concentrations of purified caffeine, they may be more susceptible to adulteration. Based on our results, ~22% of the supplement samples labeled as containing only natural caffeine appeared synthetic (Figure 3), constituting a substantial adulteration rate. The labeled caffeine content of these products was also frequently inaccurate, indicating an overall lacking

quality control system in the production of many of them. One of the five energy drinks labeled as containing natural caffeine had isotopic values inconsistent with natural caffeine and its caffeine content was more than twice the labeled content. Two others contained significantly higher caffeine than labeled. The energy drink dataset may be too small to assess the overall prevalence of adulteration in the United States market but does suggest some mislabeling of natural caffeine and common mislabeling of caffeine content.

Previous studies found a high rate of fraud in guarana products [8]. Our results strongly support this finding. Only two of the nine samples of guarana powder or extract had isotopic values consistent with guarana seeds, implying an alarming ~78% fraud rate. In addition, three of the samples contained significantly less caffeine than labeled, one containing no caffeine at all. A larger dataset is needed to more accurately evaluate the prevalence of mislabeled guarana products on the market, but our findings and those of previous researchers point to a long-standing, serious adulteration problem with these products. The inaccurate caffeine content labeling is particularly concerning in these products considering the very high caffeine concentration, especially in guarana extract samples.

5 | Conclusions

The results of the study demonstrate that a combined carbon–nitrogen isotope approach can distinguish natural and synthetic caffeine, with some limitations. For pure powder or extract products, comparison of bulk and extracted caffeine isotopic composition may also be useful for identifying adulterated products. Using this isotope analysis approach, we found that a significant number of products including supplements, energy drinks, and guarana products appear to contain mislabeled synthetic caffeine. Nearly half of the products tested also had significantly mislabeled caffeine content. These results suggest that better production quality control and more rigorous testing are required to ensure the authenticity and safety of caffeinated products.

Author Contributions

Nitzan Yanay: conceptualization, formal analysis, investigation, visualization, writing – original draft. **Dana Rothwein:** conceptualization, formal analysis, project administration, writing – review and editing. **Damien Gadomski:** conceptualization, resources, project administration, supervision, writing – review and editing. **Sheel Prajapati:** investigation, writing – review and editing. **Raquel Angulo Morales:** investigation, writing – review and editing.

Data Availability Statement

All data is available in the manuscript tables.

Peer Review

For transparency, the peer review documents associated with this article are available at https://doi.org/10.1002/rcm.70201.

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