Sample purification for the analysis of caffeine in tobacco by gas chromatography–mass spectrometry

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Abstract

A commonly used additive to tobacco products is cocoa. A sensitive and selective method was developed to measure caffeine, a marker for cocoa, in tobacco by using gas chromatography–mass spectrometry (GC–MS). Tobacco components usually produce high background signals in GC–MS analysis. Therefore, a series of extraction steps were designed to effectively purify the tobacco extracts. The analytical recovery of caffeine was 100% when [trimethyl-$^{13}C_3$]caffeine was used as an isotope-dilution reference. A linear calibration curve was generated with caffeine concentration ranging from 0.01 to 20 μg/ml. The detection limit of caffeine was 0.02 μg/ml in the final solution. This method was applied to several commercial tobacco products, of which the corresponding caffeine levels varied from below the detection limit to 125 μg/g. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

Both nicotine (the major alkaloid in tobacco) and caffeine (the major alkaloid in coffee) affect the central nervous system (CNS), with the latter increasing anxiety levels [1,2] and the former having a calming effect [3]. Epidemiological studies have shown that the percentage of smokers who drink coffee, 86.4%, is higher than that of nonsmokers, 77.2% [4]. Because combined use of nicotine and caffeine accelerates caffeine metabolism [5], nicotine withdrawal may produce an increase in caffeine response along with nicotine withdrawal symptoms. Therefore, intake of caffeine might make quitting smoking more difficult.

Caffeine is mainly found in coffee, tea, chocolate, cocoa, soft-drinks, and drugs [6]. Caffeine and theobromine are two main constituents (0.2% and 2.2%, respectively) of cocoa prepared from ripe seeds of the *Theobroma* species [7]. Because manufacturers of cigarettes and other tobacco products sometimes add cocoa to the tobacco [8], smokers may take in caffeine from tobacco products as well as from other well-known caffeine sources.

In the Association of Official Analytical Chemists (AOAC) official method [9], caffeine and theobromine are separated from other compounds by high-performance liquid chromatography (HPLC) and are quantified by ultraviolet (UV) detection. However, the relatively lower levels of caffeine in tobacco products and the different matrix properties between tobacco and cocoa make this method impractical for

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tobacco caffeine analysis. HPLC has also been used as the separation tool for the measurement of caffeine in beverages [10], urine and plasma [11,12], and biological tissues [13,14]. Micellar electrokinetic capillary chromatography (MEKC) [15] using a glycine buffer containing sodium lauryl sulfate has been reported for fast screening and simultaneous determination of caffeine and other compounds in biological fluids. Separation by HPLC or MEKC is usually not as efficient as by gas chromatography (GC). Because tobacco contains numerous compounds with similar structures, the high separation efficiency of GC is required.

Most of the methods reported to measure caffeine take advantage of its UV absorbance. The detection limit of UV absorbance techniques typically is 0.5–2 ng on column. Detection with UV absorption is based upon the assumption that there are no other UV-active compounds coeluting with the target compound. This is not always true and, moreover, does not allow analysts to use a labeled reference standard. By using mass spectrometry (MS) as the detection method, analysts not only can obtain quantitation information, but they also can identify the target compound by its molecular ion and ionic fragmentation pattern. Because MS can monitor more than one ion at the same time, the use of a labeled reference standard becomes practical, which in principle can greatly diminish the system error caused by fluctuation of the sample preparation method and instrument performance.

Here we report a new method for the determination of caffeine in tobacco samples. It involves liquid–liquid and solid-phase extractions in the preparation of samples and uses GC–MS as the analytical tool. Labeled [trimethyl-$^{13}C_3$]caffeine was used as the reference standard for quantitation.

2. Experimental

2.1. Materials

Tobacco samples were purchased on the open market and stored in plastic zip-lock bags at $-70°C$. Before analysis, the cigarettes were thawed and used with or without conditioning. Tobacco conditioning was done by placing the tobacco sample in a

Relative Humidity Chamber (Hotpack, Philadelphia, PA, USA) maintained at 22.2°C and 60.0% humidity for 24 h. There was no measurable difference in the caffeine level between the samples with and without conditioning.

The reagents for preparation of standard or sample solutions were used as purchased. Citric acid, sodium hydrogenphosphate, sodium phosphate ($Na_3PO_4 \cdot 12H_2O$), ascorbic acid, and sodium sulfate were purchased from Aldrich (Milwaukee, WI, USA); sodium hydroxide and concentrated hydrochloric acid from J.T. Baker (Phillipsburg, NJ, USA); ethyl acetate and acetone from Burdick and Jackson (Muskegon, MI, USA); [methyl-$^2H_3$]nicotine (minimum 99 atom%) and [trimethyl-$^{13}C_3$]caffeine (minimum 99 atom% ^{13}C) from Isotec (Miamisburg, OH, USA); and caffeine from United States Biochemical Corporation (Cleveland, OH, USA).

Caffeine, [trimethyl-$^{13}C_3$]caffeine, and [methyl-$^2H_3$]nicotine solutions were prepared in ethyl acetate. The solutions of caffeine and [trimethyl-$^{13}C_3$]caffeine were prepared initially at concentrations of 100 µg/ml; less concentrated standard solutions were prepared through dilution. The final concentration of [trimethyl-$^{13}C_3$]caffeine in caffeine standard solutions was 5 µg/ml, as was the final concentration of the [methyl-$^2H_3$]nicotine in the analysis samples.

Recovery flasks and separatory funnel (Kontes, Vineland, NJ, USA) were washed with deionized water and dried in an oven at 150°C, and were rinsed with ethyl acetate solution before each use. Analyses of blanks carried through the sample preparation system indicated that contamination of samples does not occur.

2.2. Sample preparation

Individual cigarettes were removed from their packaging material, and the tobacco was placed in a pre-weighed 125-ml Erlenmeyer flask after the cigarette wrapping paper and filter were opened and discarded. Usually tobacco from one entire cigarette was analyzed. When the caffeine level in one cigarette was found to be greater than the highest calibration standard, another analysis was conducted by reducing the mass of tobacco used.

The sample-preparation protocol involved liquid–liquid and solid-phase extraction to purify the tobac-

co extracts for GC–MS analysis. Tobacco from one cigarette was spiked with 10 μl of [trimethyl-$^{13}C_3$]caffeine solution (100 μg/ml) and was shaken at 130 rpm by an orbital shaker (Lab-Line Instruments, Melrose Park, IL, USA) in 25 ml of citrate buffer (5 mM ascorbic acid in 100 mM citrate–phosphate buffer, pH 4.5) at room temperature for 24 h. The dark brown citrate buffer solution was filtered through filter paper (S&S Filter Paper, Keene, NH, USA), and the tobacco residue was rinsed with 2 ml of deionized water. The combined solution was adjusted to pH 5 with 1 M NaOH and extracted successively with 25, 15, and 10 ml of ethyl acetate in a separatory funnel. An emulsion formed and changed into two layers within a few minutes. The yellow ethyl acetate extracts were combined and then extracted successively with 40, 20, and 20 ml of 1 M HCl. No emulsion was formed during this extraction step. The slightly yellow acid extracts were combined and carefully adjusted to pH 5 with 10 M NaOH and partitioned successively with 60, 40, and 20 ml of ethyl acetate. The almost clear organic extracts were combined and dried with sodium sulfate. The dried ethyl acetate solution was concentrated to about 1 ml with a rotary evaporator (Rotavapor, Büchi, Switzerland).

The slightly yellow concentrated ethyl acetate solution was purified through a 2-g Sep-Pak Silica solid-phase extraction (SPE) cartridge (Waters, Milford, MA, USA) that had been conditioned with 20 ml of ethyl acetate. After the sample solution was quantitatively transferred into the cartridge, it formed a narrow yellow band at the top of the silica column. A 10-ml volume of ethyl acetate was used to wash the cartridge, and caffeine was eluted with 10 ml of acetone. The clear acetone fraction was dried in a nitrogen evaporator (Organomation, Berlin, MA, USA) with a nitrogen stream, and the dried residue was redissolved in 200 μl of ethyl acetate or 200 μl of [methyl-$^2H_3$]nicotine solution (5 μg/ml).

2.3. Instrumentation

Chromatography was carried out on a Hewlett-Packard (HP) (Avondale, PA, USA) 6890 GC system equipped with a J&W (Folsom, CA, USA) 30 m DB-5ms column with 0.25 μm film thickness. The column was connected through a heated interface to an HP 5973 mass-selective detector equipped with an electron ionization source and a single-stage quadrupole. A 1-m J&W precolumn was installed in the front of the column. The helium carrier gas (Air Products and Chemicals, Allentown, PA, USA) was maintained at a velocity of 40 cm/s. Sample injection was done with the HP autosampler. The 10-μl syringe was washed three times with ethyl acetate before and after use and rinsed with 8 μl of sample solution before 1 μl of solution was injected in the splitless mode. The temperatures of the GC–MS instrument were set as 250°C at the injector, 260°C at the transfer line, 230°C at the ion source, and 106°C in the quadrupole. The GC oven was programmed as follows: the temperature was initially held at 40°C for 1 min, increased at 20°C/min to 160°C and held for 1 min, increased at 4°C/min to 200°C and held for 1 min, and increased at 15°C/min to 260°C and held for 1 min; the post-run period was at 260°C for 20 min. Mass analysis parameters were set for positive electron impact and selected ion monitoring (SIM) for m/z 111, 193, 194, and 197 ions. The identity of the caffeine was confirmed by comparing retention times and mass spectral ion ratios with those of a known caffeine standard and those found in the National Institute of Standards and Technology (NIST) MS database.

3. Results and discussion

3.1. Calibration curve

The peak intensities of mass spectra of the caffeine in the standard solutions were in agreement with those in the NIST library database. The strong molecular ion peak of caffeine at m/z 194 was selected as the quantitation signal. The m/z 193 ion was selected as the confirmation ion even though its intensity was lower than that of the m/z 109 ion, because the m/z 193 ion has lower background interference in extracted tobacco samples.

[Methyl-$^2H_3$]nicotine (quantitation ion m/z 165, confirmation ion m/z 87) was used as an external standard to determine analyte recovery, and [trimethyl-$^{13}C_3$]caffeine (quantitation ion m/z 197, confirmation ion m/z 111) was used as an isotopic reference for quantitation. The mass spectrum of

[trimethyl-$^{13}C_3$]caffeine does not have signal at m/z 194 or at m/z 193, and that of caffeine does not have signal at m/z 197 or at m/z 111. Thus, there are no contributions between the caffeine and [trimethyl-$^{13}C_3$]caffeine masses. The calibration curve with [trimethyl-$^{13}C_3$]caffeine as the labeled isotope compound is linear for all the standard solutions within the concentration range of 0.01 μg/ml to 20 μg/ml, which is equivalent to the content of caffeine in tobacco from 0.011 μg/g to 4.4 μg/g, assuming 0.9 g of tobacco in each cigarette. The slope of the least-squares linear regression fit of the calibration curve is 0.1934, the intercept is 0.0003632, and the correlation coefficient is 0.9998.

3.2. SPE: tobacco extract elution profile

Before SPE of tobacco samples, caffeine was analyzed by thin-layer chromatography (TLC) using a silica TLC plate; results showed that the R_F value of caffeine in ethyl acetate is around 0.33. For a better separation, the ethyl acetate extract of tobacco was concentrated to a small volume before being loaded onto an SPE cartridge because a large extract volume resulted in a wide caffeine band. After less

polar impurities were washed out of the SPE cartridge by ethyl acetate, caffeine was eluted with acetone.

Tobacco contains thousands of compounds, many of which are soluble in ethyl acetate. Polar compounds are especially troublesome for GC analysis because they may deposit in the injector or the column and, therefore, affect the GC performance. Analysis of SPE fractions by GC–MS in full-scan mode (m/z 50–400) indicated that caffeine was not present in the ethyl acetate washes, which contained some less polar compounds. The first 5 ml of acetone-eluting solution contained only about 1% of total caffeine loaded, whereas the second 5 ml of acetone contained 99% of the total caffeine (Fig. 1). No caffeine was detected in the third 5 ml of acetone and afterwards. The elution profile of theobromine, another major alkaloid of cocoa, was also monitored, and it showed that theobromine began eluting with the third 5 ml of acetone and that the majority of theobromine was present in the fourth 5-ml acetone fraction. Pure theobromine has little solubility in ethyl acetate, but the presence of other tobacco compounds increases its solubility. The retention time of theobromine is approximately 0.5 min longer
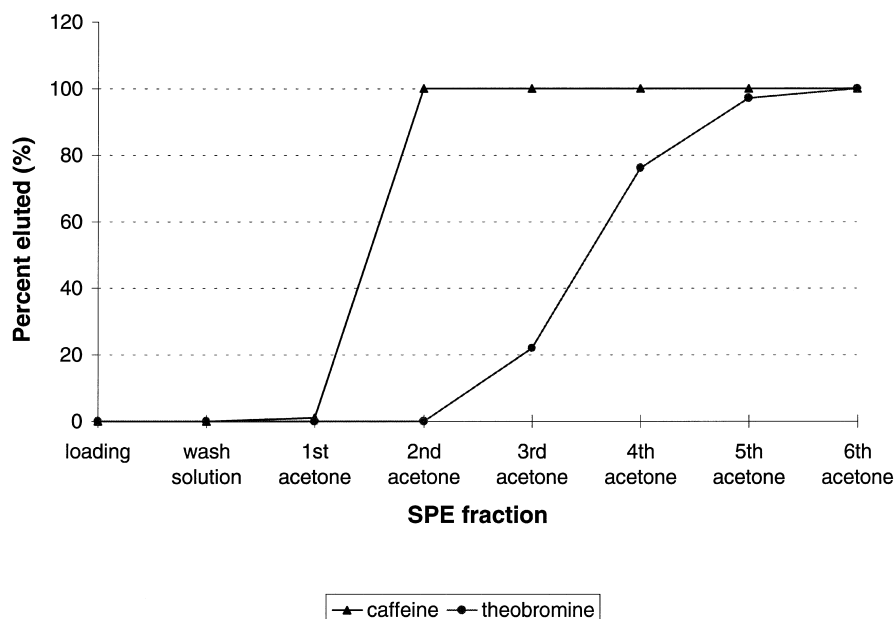

Fig. 1. Eluting profile of caffeine and theobromine from a 2-g silica SPE cartridge. The washing solution was 10 ml ethyl acetate. The eluting solution was acetone and was collected in every 5-ml interval.

than that of caffeine in the GC chromatogram, but it presents as a broad peak in the chromatogram and can affect the GC performance. More polar compounds, such as nicotine and cotinine started to elute from the SPE cartridge even later. Some very polar yellow compounds remained on the upper region of the cartridge. Thus, by collecting the first 10 ml of the acetone fraction, we were able to obtain caffeine nearly quantitatively and to leave more polar compounds on the cartridge.

3.3. Chromatogram of tobacco extraction

In Fig. 2 a full-scan GC chromatogram of a tobacco extract is shown. The retention time of labeled nicotine is 8.14 min, and that of caffeine and labeled [trimethyl-^{13}C$_3$]caffeine 15.51 min. No other compounds were observed at the retention time of nicotine, and the caffeine peak was also well separated from other tobacco component peaks. Even after purification by liquid–liquid extraction and SPE, the tobacco extract is still a mixture of many compounds. Bipyridine (M_r 156) at 10.39 min; megastigmatrienone isomers (M_r 190) at 10.91 min, 11.45 min, and 11.66 min; and scopoletin (M_r 192) at 17.76 min were identified. Because of the com-

plexity of the tobacco compound, we did not attempt to identify all the peaks in the GC chromatogram. The other compounds that we observed in the GC chromatogram suggest that this method could also be modified and applied to quantitation of additional components.

3.4. Effects of HCl extraction

During sample preparation, the liquid–liquid extraction is the most time-consuming step. We compared the samples prepared by only one ethyl acetate extraction with the samples from combined ethyl acetate/HCl/ethyl acetate extractions as described in the Experimental section. The GC–MS chromatograms are shown in Fig. 3. Even with SPE purification, the sample prepared without the HCl extraction step has a very high background in the chromatogram. The high background affects the baseline–baseline separation between the peaks, interferes with the ionization efficiency of the compounds, and decreases the GC performance rapidly after analysis of a few samples. The sample prepared with the HCl extraction shows a much lower background in the chromatogram, and most of the peaks are baseline
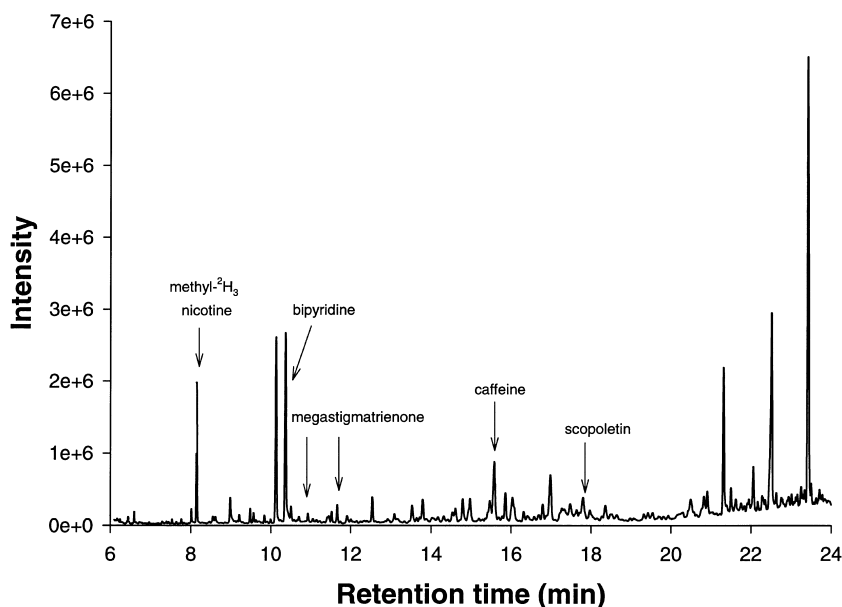

Fig. 2. An example of a GC–MS chromatogram of a tobacco extract after sample purification.
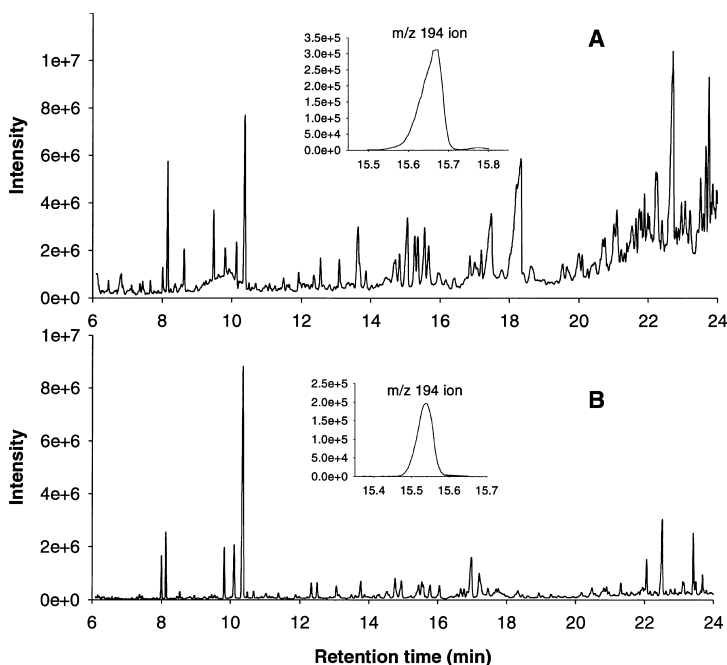
Fig. 3. GC–MS full-scan (m/z 50–350) chromatogram comparison of tobacco extract (A) without HCl extraction and tobacco extract (B) with HCl extraction. Inset displays the intensity of the m/z 194 ion.

separated. The HCl extraction step eliminates acidic and neutral compounds that are more soluble in ethyl acetate at low pH. For example, most of the scopoletin was eliminated in this step.

The Fig. 3 inset shows the comparison of the single ion chromatogram of m/z 194. The intensity of the m/z 194 ion for the sample prepared by HCl extraction is about 52% as high as that of the sample solution without HCl extraction. Because caffeine is soluble both in water and in ethyl acetate, it is difficult to extract caffeine completely from one liquid phase to another. Although adjusting the pH affects the distribution of the ionic forms of caffeine and thus its solubility in different solvents, recovery is not 100% after each extraction. Additional extraction steps result in lower recovery. Since extraction is essential to purification of the sample for GC–MS analysis, the problem of sample loss during the extraction steps was eventually solved by using [trimethyl-^{13}C$_3$]caffeine as the reference standard, which was added into the sample before the citrate buffer extraction.

3.5. Recovery study for caffeine

From the above discussion, it is clear that caffeine was not 100% recovered in each extraction step. It is conceivable that sample loss might also occur during solution transfer and other steps. The recovery study was carried out by spiking a 100% tobacco (no-additive) cigarette sample. A 200-μl volume of each standard caffeine solution (3, 5, 10, and 20 μg/ml) was spiked into this tobacco sample. Because the spiking caffeine standard solution contained [trimethyl-^{13}C$_3$]caffeine, this reference compound also went through the shaking, extraction, and SPE steps with the caffeine. The chemical similarity between labeled and unlabeled caffeine enabled us to compensate for sample loss during each step and to correct the analytical recovery. The final dried sample residue was redissolved in 200 μl of a [methyl-^{2}H$_3$]nicotine solution. The external standard [methyl-^{2}H$_3$]nicotine added at the final redissolving step allowed us to determine the actual recovery of caffeine. After the GC–MS analysis, we calculated

Table 1
The calculated caffeine recoveries when [trimethyl-$^{13}C_3$]caffeine is used as the labeled isotope reference (analytical recovery) and [methyl-$^2H_3$]nicotine is used as an external reference (actual recovery) for spiked tobacco samples

Sample	Spiked caffeine Conc. (μg/ml)	Analytical recovery (%)	Actual recovery (%)
1	3.0	110	30.0
2	5.0	99.2	31.6
3	10.0	103	30.3
4	20.0	100	34.8

the amount of caffeine using both [methyl-$^2H_3$]nicotine and [trimethyl-$^{13}C_3$]caffeine as the references. We determined the caffeine level in the 100% tobacco cigarette sample itself at the same time as in the spiked samples, assuming a 100% recovery using the [trimethyl-$^{13}C_3$]caffeine reference.

Recoveries were calculated by dividing the calculated levels (caffeine in the cigarette itself was subtracted) with the spiked levels. In Table 1 the calculated recoveries when [methyl-$^2H_3$]nicotine and [trimethyl-$^{13}C_3$]caffeine were used as the references are listed. With [trimethyl-$^{13}C_3$]caffeine as the labeled isotope reference, the calculated recovery ranges from 98% to 110%; with [methyl-$^2H_3$]nicotine as the reference, the calculated recovery ranges from 25% to 34%. These results demonstrate the necessity of using an isotope-labeled reference.

3.6. Sample analysis precision

The reproducibility of the method was measured by triplicate analysis of tobacco from three different cigarette packs. Tobacco of three cigarettes from each pack was combined, spiked with 30 μl of a 100

μg/ml [trimethyl-$^{13}C_3$]caffeine solution, and extracted with 75 ml of citrate buffer. After filtration, the citrate extract was adjusted to pH 5 and divided equally into three portions. Each portion, equivalent to citrate extract of one cigarette spiked with 10 μl of 100 μg/ml [trimethyl-$^{13}C_3$]caffeine solution, was analyzed in accordance with the sample preparation and quantitation procedures as described in the Experimental section, including purification by liquid–liquid extractions and SPE, and analysis by GC–MS. The amount of caffeine in each portion was determined, and the results are listed in Table 2. In triplicate analysis, the first cigarette pack yielded an average caffeine content of 0.116 μg/g with 0.5% relative standard deviation (RSD); the second cigarette pack yielded an average of 0.211 μg/g with 1.2% RSD; and the third cigarette pack yielded an average of 0.422 μg/g with 1.9% RSD. These results indicate that the sample purification and GC–MS analysis procedure is very reproducible.

3.7. Detection limit

The detection limit of this method was determined by using a 100% tobacco (no-additive) cigarette sample. Although this cigarette contained a low level of caffeine, its contribution was removed by allowing a non-zero intercept in the calculation of detection limit. To minimize the effect of the variation in the tobacco matrix, we pooled the tobacco citrate extracts from nine cigarettes (0.8 g tobacco mass for each cigarette) and then divided the pool into nine samples. We then used a spiking standard caffeine solution in triplicate at three levels (0.05, 0.1, and 0.25 μg/ml) and spiked 200 μl of one of these caffeine solutions into each citrate extract. After sample purification and GC–MS analysis under SIM

Table 2
The calculated caffeine levels from triplicate runs for three cigarette packs

Cigarette sample	First aliquot caffeine (μg/g)	Second aliquot caffeine (μg/g)	Third aliquot caffeine (μg/g)
Pack 1	0.116	0.116	0.117
Pack 2	0.214	0.211	0.216
Pack 3	0.436	0.424	0.420

mode, the integrated intensity data were analyzed by least-squares linear regression.

The calculated caffeine concentrations for the three triplicate runs are listed in Table 3. The plot of response factor vs. caffeine concentration for this analysis yields a slope that is the same as that of caffeine standards, showing there is no matrix effect. Variation in the response factor for these analysis was used to calculate the standard deviation of the calculated caffeine concentration and the standard deviation at zero concentration. The standard deviation of the calculated caffeine concentration is listed in Table 3, and the standard deviation at zero concentration is 0.0054. The limit of detection, equal to three times the standard deviation at zero concentration [16], is 0.02 μg/ml (equivalent to 0.005 μg/g tobacco) for caffeine.

To evaluate the reagent blank, we spiked [trimethyl-$^{13}C_3$]caffeine into citrate buffer and treated the solution as usual through all the sample purification steps. Results of this analysis were below the lowest caffeine standard (0.01 μg/ml) and the detection limit.

3.8. Ion ratio quality control

The caffeine peak in the sample chromatogram was identified and further confirmed by the confirmation ion/quantitation ion ratio. As the integration results were reviewed, peak integration parameters, such as width and baseline, were manually adjusted if necessary. To establish an acceptable ratio range, we analyzed the ion ratios for the spiked tobacco sample runs. The ion ratio increased slightly with the caffeine concentration and leveled off at a mean of 7.46 when the caffeine concentration was above 0.2 μg/ml. Validity criteria required that the ion ratio for an unknown had to be within ±10% of the spiked sample ion ratio for the results to be considered

acceptable. This analysis resulted in a lower validity limit of approximately 0.03 μg/g. Results near or below this value with invalid ion ratios were considered below the detection limit and assigned a value of $\frac{1}{2}$ of the validity limit for statistical purposes. Results above this limit with invalid ion ratios were considered not reportable.

3.9. Cigarette sample analysis

After the above characterizations, the present method was applied to the analysis of tobacco samples, including snuff and commercial cigarettes. Cigarette samples included no-additive cigarettes and full-flavor cigarettes. Each sample run was followed by an ethyl acetate blank run before the next sample run to prevent sample carry over. One sample solution showed a caffeine level of 380 μg/ml, but none of the ethyl acetate blank analyses showed any detectable caffeine. The caffeine levels for the samples are listed in Table 4. For some cigarette tobacco, multiple measurements were done on different cigarettes in the same pack or on different packs with the same brand. The wide range of standard deviations is due to the varied caffeine levels in individual cigarettes.

The snuff sample had a caffeine content of 1.1 μg/g. No-additive cigarettes had caffeine levels from nondetectable to 0.2 μg/g, whereas most cigarettes without the 'no-additive' label had higher caffeine levels, from 1.2 μg/g to 16 μg/g. One full-flavor cigarette without the 'no-additive' label had a caffeine level of 0.043 μg/g, which is very low, at the level of no-additive cigarettes. The highest caffeine level, 120 μg/g, was found in a tobacco sample used for self-wrapping. Caffeine levels in reference cigarettes were 0.023 μg/g in 1R3F (produced in 1974), 0.13 μg/g in 1R4F (produced in 1983), 3.9 μg/g in 1R5F (produced in

Table 3
The calculated caffeine concentrations in final solutions for spiked tobacco samples

Spiked caffeine conc. (μg/ml)	1st Analysis (μg/ml)	2nd Analysis (μg/ml)	3rd Analysis (μg/ml)	Standard deviation
0.05	0.182	0.156	0.159	0.0030
0.1	0.223	0.222	0.215	0.0009
0.25	0.393	0.385	0.363	0.0032

Table 4
Caffeine levels in some commercial tobacco products

Tobacco type	Measurements	Caffeine level (μg/g) $\pm$standard deviation
Filters No-additives Hard Box (carton 1)	2	ND[a]
Filters No-additives Hard Box (carton 2)	7	0.17$\pm$0.08
Filters No-additives Hard Box (Brand 2)	3	0.096$\pm$0.095
Filters No-additives Lights Hard Box	2	0.20$\pm$0.030
No-additives Hard Box	2	ND[a]
Filter 100s No-additives Soft Pack (carton 1)	3	ND[a]
Filter 100s No-additives Soft Pack (carton 2)	3	0.031$\pm$0.0094[b]
100s Full-flavor Soft Pack	6	1.2$\pm$0.27
Full-flavor Hard Box (Brand 1)	5	0.043$\pm$0.011
Full-flavor Hard Box (Brand 2)	1	9.9
Full-flavor Soft Pack (Brand 1)	2	16$\pm$1.6
Full-flavor Soft Pack (Brand 2)	1	11
Snuff	1	1.1
Self-wrap leaves	1	120
CM2	1	0.019
1R3F	1	0.023
1R4F	1	0.13
1R5F	1	3.9

[a] Results below the detection limit.
[b] Some results did not meet validity limits due to levels close to the detection limit. For statistical analysis purposes these values were set equal to 0.015 μg/g, $\frac{1}{2}$ of the approximate limit for meeting validity criteria.

1989), and 0.019 μg/g in CM2 (produced in Sweden).

4. Conclusion

The method developed previously in this laboratory for the measurement of nitrosamines in tobacco samples has been successfully modified for caffeine measurement. The recovery of caffeine is around 30%, but application of the isotope-dilution method yields an analytical recovery of nearly 100%. Although the recovery of caffeine after the sample-preparation procedure is not as high as that of nitrosamines, the sample-preparation procedure is effective in eliminating troublesome compounds and therefore produces a clean sample solution for GC–MS analysis. When operated in the selected ion monitoring mode, and when [trimethyl-^{13}C$_3$]caffeine is used as the labeled isotope reference, the mass-selective detector provides a linear calibration curve starting from the caffeine concentration of 0.01 μg/ml (0.01 ng on column) to 20 μg/ml. The method's low detection limit of 0.005 μg/g enables analysts to measure caffeine in tobacco samples using only one cigarette. The present sample-preparation and analysis protocol should be applicable to the measurement of other related compounds in tobacco products and to the analysis of other complicated systems.

Acknowledgements

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