Federal Aviation Administration

DOT/FAA/AM-07/23
Office of Aerospace Medicine
Washington, DC 20591

Intensity of the Internal Standard Response as the Basis for Reporting a Test Specimen as Negative or Inconclusive

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August 2007

Final Report

NOTICE

Technical Report Documentation Page

1. Report No.	2. Government Accession No.	3. Recipient's Catalog No.
DOT/FAA/AM-07/23		

4. Title and Subtitle	5. Report Date
Intensity of the Internal Standard Response as the Basis for Reporting a Test Specimen as Negative or Inconclusive	August 2007
	6. Performing Organization Code

7. Author(s)	8. Performing Organization Report No.
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9. Performing Organization Name and Address	10. Work Unit No. (TRAIS)
FAA Civil Aerospace Medical Institute P.O. Box 25082 Oklahoma City, OK 73125	11. Contract or Grant No.

12. Sponsoring Agency name and Address		13. Type of Report and Period Covered
[1]Department of Medical Technology Fooyin University Kaohsiung Hsien 831-01, Taiwan	[2]Department of Justice Sciences University of Alabama Birmingham, AL 35294	
[3]FAA Civil Aerospace Medical Institute P.O. Box 20582 Oklahoma City, OK 73125		14. Sponsoring Agency Code

15. Supplemental Notes

Work was accomplished under approved task AM-B-07-TOX-206

16. Abstract

Under normal circumstances, a test specimen is reported as "negative" when the response of the analyte is absent. However, if the intensity of the internal standard (IS) is low, indicating interference factors, the test could be considered "inconclusive." A quantitative hypothesis, A = (R×I×S)/L, serves as the "cutoff" for the acceptable signal-to-noise (S/N) ratio for the IS in making "negative/inconclusive" decisions, where A: acceptable S/N ratio for internal standard; R: relative response of the IS and the analyte (same concentration); I: concentration of the IS; S: (minimal S/N ratio); and L: limit of detection. The hypothesis was empirically tested using 9-carboxy-11-nor-Δ^9-tetrahydrocannabinol (THC-COOH) analyte, THC-COOH-d_3 IS, and ibuprofen and hydrogen peroxide (H_2O_2) as interference factors. Urine specimens containing 0–5 ng/mL of THC-COOH were spiked with various quantities of ibuprofen or H_2O_2, followed by liquid-liquid extraction, derivatization, and GC-MS analysis under selected-ion-monitoring mode. Among the "adulterated" test specimens evaluated, those with a S/N for the internal standard below the acceptable IS S/N "A," the quantitative criterion was indeed found to provide a useful guide for making negative/inconclusive decisions. This equation could be programmed into the instrument software to flag results as being inconclusive when they do not meet the criteria described in this paper.

17. Key Words	18. Distribution Statement
True Negative, Drug, Equation, Interpretation, Interfernce	Document is available to the public through the Defense Technical Information Center, Ft. Belvior, VA 22060; and the National Technical Information Service, Springfield, VA 22161

19. Security Classif. (of this report)	20. Security Classif. (of this page)	21. No. of Pages	22. Price
Unclassified	Unclassified	12	

Form DOT F 1700.7 (8-72) Reproduction of completed page authorized

ACKNOWLEDGMENTS

This work is partially supported by (Taiwanese) National Science Council (NSC 94-M-242-003). We thank the Federal Aviation Administration Office of Aviation Accident Investigation and the Office of Aerospace Medicine for sponsoring part of this research.

Intensity of the Internal Standard Response as the Basis for Reporting a Test Specimen as Negative or Inconclusive

INTRODUCTION

Interference factors such as adulteration, substitution, or badly decomposed or contaminated postmortem specimens may result in poor recovery of the analyte and internal standard (IS), causing false-negative or inconclusive results in toxicology tests. Even under normal analytical conditions where the IS is poorly recovered, the substance may escape detection if it is present at or near the limit of detection (LOD). As the most commonly abused substance in the United States, marijuana (in the form of the commonly targeted metabolite, 9-carboxy-11-nor-Δ^9-tetrahydro-cannabinol, or THC-COOH) constitutes a substantial portion of positive specimens reported by forensic urine drug testing laboratories [1]. Therefore, specimen adulteration (and substitution) activities have most commonly been associated with urine specimens derived from marijuana users. Approaches proven to be effective for the adulteration of THC-COOH-containing specimens include the addition of oxidants, such as hydrogen peroxide (H_2O_2) and pyridinium chlorochromate [2-4], bleach (hypochlorite) [5], and nitrite or ammonia-containing substances [6]; and ibuprofen, which may interfere with the derivatization of the analyte in the sample preparation process [7].

The most common analytical approach is to incorporate an isotopically-labeled analogue of the analyte as the IS, which serves well to identify false negative test results when the IS is absent and the analyte's signal is absent. An unexpected low response from the IS, even in the absence of the analyte's signal, is indicative of abnormal specimen conditions and should alert the analyst to investigate further before issuing a "negative" or "inconclusive" report. However, lacking a quantitative tool, the analyst's decision to issue a negative or inconclusive finding is often subjective in nature.

The objective of this study was to develop a quantitative criterion that would be helpful in making "negative/inconclusive" decisions. Parameters that are included in formulating the proposed acceptable (A) IS signal-to-noise "cutoff" are: (a) the relative response (R) of the analyte and the IS when they are present at the same concentration; (b) the concentration of the IS (I) added into the test specimen; (c) the minimal signal-to-noise ratio (S) for an acceptable signal; (d) the limit of detection (L) of the adapted analytical protocol. In developing an equation to quantitatively analyze the response of the IS for determining the reliability of the procedure to see the analyte at the LOD we considered the following variables:

1. the amount of IS added to the specimen. The IS is normally added in much higher concentrations than the LOD of the procedure. Therefore, detection of the IS cannot be used to indicate that the analyte is truly negative at the LOD of the procedure. The response of the IS would be detected well after the analyte response as the LOD of the procedure dropped below detectable limits.
2. (R) the relative response of the analyte to the IS. If the analyte has a greater response than the IS, the acceptable signal-to-noise ratio (S/N) of the equation would need to be adjusted lower to account for the lower response of the IS. If the analyte has a lower response than the IS, the acceptable IS S/N should be adjusted higher.
3. (S) the minimum acceptable S/N for designating a real response. Raising the minimum acceptable S/N for the procedure results in the acceptable IS signal-to-noise being adjusted higher.
4. (L) the LOD for the procedure takes into account the concentration of the analyte that is detectable. A low LOD results in a higher acceptable IS signal-to-noise.

Laboratory studies were conducted adapting THC-COOH and THC-COOH-d_3 as the analyte and IS [4,8] with ibuprofen and H_2O_2 serving as the interference factors.

EXPERIMENTAL

Chemicals and reagents

Standard (THC-COOH, 100 µg/mL in ethanol) and deuterated IS (THC-COOH-d_3, 100 µg/mL in methanol) were obtained from Research Triangle Institute (Research Triangle Park, NC, US) and Radian (Cerillant) Corporation (Austin, TX, US), respectively. The chemical derivatization reagent (iodomethane) was purchased from Ferak Berlin GmbH (Berlin, Germany). The sources of the interference reagents and extraction solvents were as follows: ibuprofen: Sigma-Aldrich (Steinem, Germany); H_2O_2: Niohon Shiyaku Industries (Osaka, Japan); tetramethylammonium hydroxide (TMAH): ACROS (Fair Lawn, NJ, US); *n*-hexane and ethyl acetate: Mallinokrodt

Chemical (Phillipsburg, PA, US); dimethyl sulphoxide (DMSO and glacial acetic acid: Panreac Quimica SA (Barcelona, Spain); potassium hydroxide: Katayama Chemical (Osaka, Japan). Other solvents were analytical or HPLC grade.

Drug-free urine and "adulterated" urine test specimens

Drug-free urine specimens were collected from laboratory co-workers, who signed an institutional consent form for their urine to be used in this experiment. This urine was confirmed negative for THC-COOH by GC-MS. Urine specimens were filtrated with 55-mm filter paper (Toyo Roshi Kaisha: Taito-Ku, Tokyo, Japan) upon collection and refrigerated.

Two categories of adulterated urine specimens were prepared by adding various amount of ibuprofen or H_2O_2 to arrive at desired concentrations of the adulterants (further described in the Result and Discussion section).

Sample preparation and extraction

Analytical procedures reported in the literature for the analysis of THC-COOH were slightly revised and adapted in this study. Specific procedures are described as follows. To a clean 16 × 100-mm borosilicate glass tube (Kimble: Vineland, NJ, US) was added 1-mL of urine sample (calibration standard or test specimen) containing a predetermined concentration of THC-COOH. The IS was added to each tube in the analytical batch using 15-µL working stock solution (1 µg/mL) with final concentration of 15 ng/mL for the standards and test samples. The mixture was made alkaline with 200 µL of 10-N KOH and incubated for 20 min in a heating block device set at 60° C. The mixture was cooled to room temperature and adjusted to pH 3.5±0.5 by adding 2-mL glacial acetic acid, followed by the addition of 2-mL *n*-hexane/ethyl acetate (9:1, v/v) mixture. The mixture was vortex-mixed and centrifuged at 2500 rpm (r = 16.5 cm) for 5 min. The organic phase was removed and evaporated to dryness under a stream of nitrogen at 50° C [4,8–12]. The extract residue was suspended in 100 µL of TMAH/DMSO (1:20) and vortex-mixed for 2 min, followed by the addition of 100-µL diluted iodomethane (1:50, v/v, in DMSO). The mixture was incubated at room temperature for 5 min, then acidified with 200 µL of 0.1-N HCl, and extracted with 1-mL *n*-hexane. The organic phase was removed and evaporated to dryness under a stream of nitrogen at 50° C. Finally, the residue was reconstituted with cyclohexane. Typically, the reconstitution volume was100 µL, while 1 µL was injected for GC-MS analysis [4,13,14].

GC-MS analysis

GC-MS analysis was performed on an Agilent 6980 GC interfaced to an Agilent 5975 MS (Agilent: Palo Alto, CA, US). A 12-m HP-5 crosslinked 5% phenyl methyl siloxane capillary column (200-µm ID, 0.33-µm film thickness) from Agilent (Wilmington, DE, US) was used for this study. Helium carrier gas flow rate was set at 1.0 mL/min. The injector and GC-MS interface temperatures were maintained at 260 and 280° C, respectively. The sample was introduced into the gas chromatograph in the splitless mode. The initial oven temperature was held at 150° C for 1 min, then raised to 270° C at 30° C /min, and held for 5 min. The final temperature was set at 300° C and held for 4 min to clean up the system before recycling back to the initial temperature for next injection. The following ions were selected to monitor methyl-derivatives of the analyte and the IS: *m/z*, 313, 357, and 372 for THC-COOH; and 316, 360, and 375 for THC-COOH-d_3. The first ion listed for each compound was used for quantization [4,13–14].

Calculation of Signal-to-Noise Ratio (S/N)

The IS peak height and average noise peak height were used to calculate the S/N (Fig. 1). The average noise peak height is the average of the max noise + minimum noise peak height divided by 2 measured in the region adjacent to the IS peak. The noise peak height is affected by many variables including dirty detector, column degradation, specimen matrix, carrier gas purity, and putrefaction of the specimen.

RESULT AND DISCUSSION

Under normal circumstances, a test specimen will be reported as "positive" when the analyte's signal is above the detection limit, and "negative" when the analyte's response is absent or at the noise level. However, if the intensity of the IS is critically low, indicating the presence of interference factors, the test should be considered "inconclusive," even in the absence of the analyte's signal. A quantitative criterion is developed to serve as the "cutoff" for making "negative/inconclusive" decisions when the response of the IS falls in the critical range. Specifically, the hypothesis states that when

$$\text{Signal-to-noise IS} > (R \times I \times S)/L \qquad \text{Eq. (1)}$$

the specimen can be reported as "negative"; otherwise, the test should be considered "inconclusive," where R: relative response of the IS and the analyte (when present at the same concentration); I: concentration of the IS;

S: 3 (the minimal S/N for an acceptable signal); and L: limit of detection of the analytical protocol.

In this empirical study, ibuprofen and H_2O_2 were adapted as the interference factors and added into THC-COOH-containing urine specimens to create circumstances under which the signal levels of the analyte (THC-COOH) and the IS (THC-COOH-d_3) are at the critical levels, requiring a quantitative guide for making negative/inconclusive decisions. Ibuprofen and H_2O_2 interfere with the GC-MS assay of THC-COOH with different mechanisms. Specifically, ibuprofen competes with the analyte (and the IS) at the derivatization step, while H_2O_2 destroys the analyte (and the IS) through an oxidation reaction.

Analytical parameters

For the purpose of this study, the concentration of the IS (I) was set at 15 ng/mL, as commonly adapted by urine drug testing laboratories, while the S/N for an acceptable signal (S) is set at 3. A series of preliminary experiments (data not shown) were performed to establish the limit of detection (LOD, L) of the adapted analytical protocol as 3 ng/mL and the relative response (R) of the analyte (*m/z* 313) and the IS (*m/z* 316), when they are present at the same concentration, as 0.85. Additional series of preliminary studies indicated the best analyte (THC-COOH) concentration suitable for this study is at the 0–5 ng/mL range, while the amounts of the interference factors, ibuprofen and H_2O_2 (35% solution), are 350–450 µg/mL and 75–200 µL, respectively.

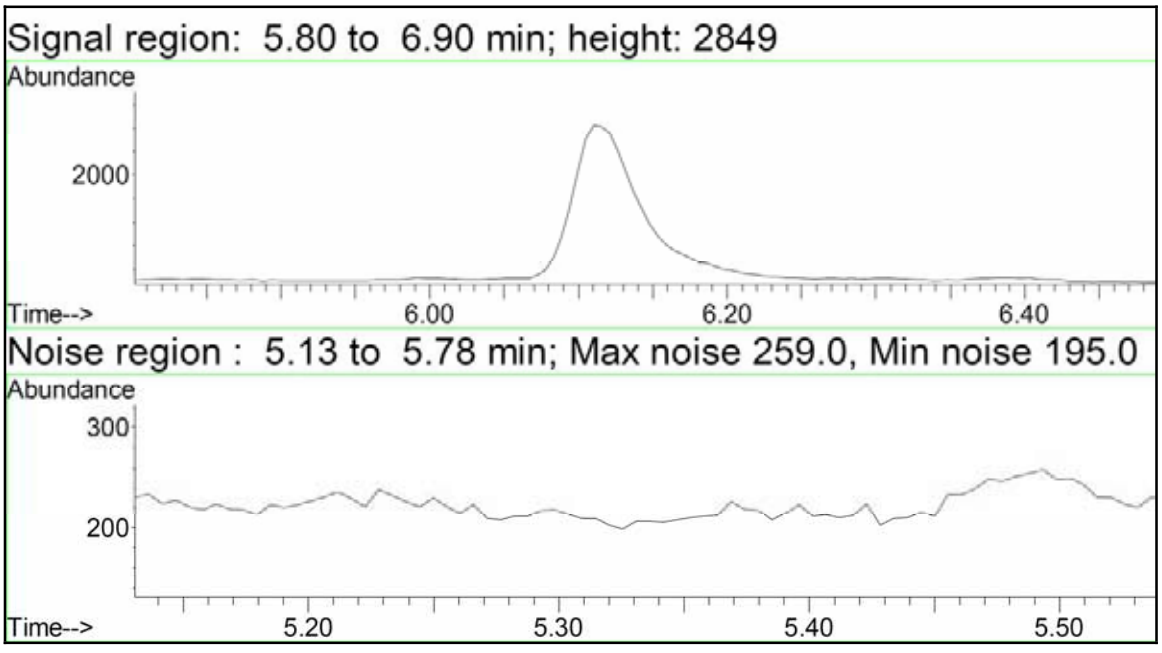
When the amounts of the interference factors are below the lower limits, the signals of the IS and the analyte are not significantly interfered; thus, a specimen containing the analyte above LOD can be properly reported as being positive or negative, using the normal ion intensity ratio criteria. On the other hand, when the amounts of the interference factors are above the higher limits, the signal for the IS becomes absent, resulting in the test being listed as inconclusive, again without requiring any additional criterion in the decision-making process.

When the amounts of the interference factors are in the critical range (75–200 µL for H_2O_2 and 350–450 µg/mL for ibuprofen), the signals of the IS and the analyte fall in the critical range, requiring a quantitative criterion to make a negative/inconclusive interpretation of the test results. Specifically, the analytical parameters discussed above allow for the calculation of the acceptable S/N for the IS.

$$A = (R \times I \times S)/L = (0.85 \times 15 \times 3) / 3 = 13.$$

Thus, when the S/N of the IS is greater than 13, the test result can be interpreted as N (negative), otherwise, as I (inconclusive). The following two sections will focus on the discussion of data collected when the amounts of the interference factors are in the critical range.

Figure 1. Chromatograph showing example of Signal-to-Noise measurement.



Ibuprofen study

Data from Table I clearly demonstrates the concentration of ibuprofen that may cause interference to the analytical protocol fall between 350–450 ng/mL. With this information in mind, four sets of a five-concentration series of standards containing 0, 1, 2, 3, and 5 ng/mL THC-COOH (each with 15 ng/mL IS) were spiked with 0, 350, 400, and 450 ng/mL of ibuprofen. Data derived from this batch of the experiment are shown in Sections I-1 to I-4 in Table I. Intensity data shown in the table are the peak heights for the IS (*m/z* 316).

The S/N of the 0, 1, and 2 ng/mL standards in Sets 1 and 2 are larger than the critical value, (R×I×S)/L = 13); thus, they are correctly designated as "N." For the 3 and 5 ng/mL standards, the three ions monitored for the analyte are at higher intensity level. The two ion intensity ratios are within ±20% of corresponding ratios observed in the calibration standard; thus, they are designated as "P." Set I-3 concentrations all fell below the value calculated with the proposed equation and are designated as inconclusive. For the set of standards (Set I-4) containing 450 ng/mL ibuprofen, the ion intensities of the analyte and the IS are all at the noise level; thus, they were designated as "I." These designations are based on the intensity of the IS; however, the value calculated by the equation would have come to the same conclusion.

Data derived from Sets I-3 and I-4 clearly indicate the truly critical concentration of ibuprofen falls within the 400–450 ng/mL range. Thus, a second batch of standards containing 420 and 440 ng/mL of ibuprofen was prepared, and the experiment was conducted on a different day. The resulting data shown in Sets II-1 and II-2 (Table I) demonstrate the expected trend, i.e., as the concentration of ibuprofen increases, the standards with higher analyte concentrations are designated as "I."

To further confirm the observed phenomenon, a third set of standards with ibuprofen concentrations at 300, 340, 360, 380, and 400 ng/mL was studied on yet another day. Data shown in Sets III-1, III-2, III-3, and III-4 (Table I) nicely agree with the data observed in Set I, except the data from set III-1 at 0 ng/mL THC. It appears that, in that specimen, unknown factors caused a significant decrease in the IS response relative to all of the other specimens in set III-1. The equation did, however, correctly identify this specimen as inconclusive, because the IS was not significantly recovered to ensure that the analyte of interest would have been detected if it had been present at the LOD.

Sets I-3 and III-5 had the same concentration of interfering substance (400 ug/mL ibuprofen); however, Set III-5 had a much lower S/N This demonstrates the importance of taking into consideration the S/N in calculating the acceptable recovery of the IS for the identification of the analyte of interest at the LOD of the procedure.

Hydrogen peroxide study

Several sets of standards containing 0, 1, 3, and 5 ng/mL of THC-COOH included in this study were spiked with 0, 75, 100, 150, 200, and 300 µL of H_2O_2 solution (35%). During the preliminary phase of this study, H_2O_2 was added into these standard solutions immediately following their preparation. These "adulterated" standards were then stored for later analysis. The IS was added when the analytical batches were assembled as practiced in a normal analytical protocol. Data derived from this standard protocol (in terms of the timing in adding the IS) were found sporadic. It was concluded that the reaction between the analyte and H_2O_2 had occurred prior to the addition of the IS; thus, it was not suitable for the intended study.

An alternate approach was then adapted in which H_2O_2 was added into the standard solution after the addition of the IS, allowing the analyte and the IS to react with the adulterant competitively. Data shown in Table II demonstrate that, as the amount of the H_2O_2 solution increases from 75 µL (Set 2) to 200 µL (Set 5), the standards containing increased concentrations of the analyte are designated as "I." These designations are based on the quantitative criterion proposed in Eq. (1).

Further considerations and concluding remarks

The validity of the proposed quantitative criterion for designating a test result as "negative" or "inconclusive" in the event of the poor recovery of the IS has been demonstrated by the ibuprofen and H_2O_2 studies. In the absence of an interfering substance, the equation correctly identified all true negatives and positives. The equation did properly identify several false negative results for concentrations at or above the 3 ng/mL LOD of the procedure when the interfering substance was in the critical range. It is interesting to note in data sets III (concentrations 0 to 1 ng/mL), where the equation showed true negative results, that an analyst using subjective judgment may have incorrectly designated these true negative results as inconclusive due to the poor recovery of the IS. It could be incorrectly argued in some of the data presented that some of the 0 ng/mL – 2 ng/mL concentrations were incorrectly identified as inconclusive, when they were, in fact, negative. However, it is important to realize that the analyst does not know the starting concentration and can only rely on the IS response in making a decision as to whether the test would have detected the analyte at the LOD of the procedure. The equation provided has

Table I. Ibuprofen Interference on GC-MS Analysis of THC-COOH

Exp.	THC-COOH	Ibuprofen	Responses of the IS & its background noise					
set #	conc. (ng/mL)	conc. (µg/mL)	IS	max	min	mean	Ratio	(N/I/P) †
I-1	0	0	3576	192	156	174	20.6	N
	1	0	3131	242	163	203	15.5	N
	2	0	2685	208	146	177	15.2	N
	3	0	2910	220	163	192	N/A‡	P
	5	0	4019	252	180	216	N/A‡	P
I-2	0	350	3982	256	201	229	17.4	N
	1	350	3562	242	185	214	16.7	N
	2	350	3490	247	196	222	15.8	N
	3	350	3255	236	184	210	N/A‡	P
	5	350	3248	247	193	220	N/A‡	P
I-3	0	400	2698	250	191	221	12.2	I
	1	400	2945	299	230	265	11.1	I
	2	400	2807	296	230	263	10.7	I
	3	400	2014	246	189	218	9.26	I
	5	400	2849	259	195	227	12.6	I
I-4	0	450	255	286	211	249	1.03	I
	1	450	252	277	206	242	1.04	I
	2	450	289	367	261	314	0.920	I
	3	450	238	282	205	244	0.977	I
	5	450	233	255	187	221	1.05	I
II-1	0	420	2401	473	244	359	6.70	I
	1	420	925	126	98	112	8.26	I
	2	420	566	100	89	95	5.99	I
	3	420	821	100	87	94	N/A‡	P
	5	420	484	97	86	92	N/A‡	P
	0	430	831	99	88	94	8.89	I
	1	430	521	112	95	104	5.03	I
	2	430	145	101	89	95	1.53	I
	3	430	889	99	89	94	9.46	I
	5	430	143	102	90	96	1.49	I
II-2	0	440	794	98	87	93	8.58	I
	1	440	541	105	93	99	5.46	I
	2	440	240	99	89	94	2.55	I
	3	440	118	97	87	92	1.28	I
	5	440	160	141	104	123	1.31	I

Table I. Ibuprofen Interference on GC-MS Analysis of THC-COOH (continued)

Exp. set #	THC-COOH conc. (ng/mL)	Ibuprofen conc. (µg/mL)	Responses of the IS & its background noise					
			IS	max	min	mean	Ratio	(N/I/P) †
III-1	0	300	375	69	62	66	5.73	I
	1	300	1396	72	65	69	20.38	N
	3	300	1331	73	66	70	N/A‡	P
	5	300	1391	74	66	70	N/A‡	P
III-2	0	340	1364	73	67	70	19.49	N
	1	340	1384	71	64	68	20.50	N
	3	340	1344	71	65	68	N/A‡	P
	5	340	1422	71	66	69	N/A‡	P
III-3	0	360	1327	70	63	67	19.95	N
	1	360	1194	69	63	66	18.09	N
	3	360	1299	69	62	66	N/A‡	P
	5	360	1144	70	64	67	N/A‡	P
III-4	0	380	1205	70	63	67	18.12	N
	1	380	991	68	62	65	15.25	N
	3	380	1124	69	62	66	N/A‡	P
	5	380	1102	67	61	64	N/A‡	P
III-5	0	400	963	66	61	64	15.17	N
	1	400	1159	68	62	65	17.83	N
	3	400	1025	67	60	64	N/A‡	P
	5	400	918	67	61	64	N/A‡	P

* Signal level (peak height).

† N: negative; I: inconclusive; P: positive.

‡ Intensities of the ions designating the analyte and the IS were abundant and meet the ion intensity ratio required for "positive" designations.

Table II. Interference of H_2O_2* on GC-MS Analysis of THC-COOH

Exp.	THC-COOH	H_2O_2*	Responses of the IS & its background noise Result					
set #	conc. (ng/mL)	µL	IS	max	min	mean	Ratio	(N/I/P) †
1	0	75	1855	903	625	764	2.43	I
	1	75	698	154	95	125	5.61	I
	3	75	2759	2734	1304	2019	1.37	I
	5	75	1696	703	206	455	N/A‡	P
2	0	100	1445	392	155	274	5.28	I
	1	100	3436	910	491	701	4.91	I
	3	100	2095	1989	1022	1506	1.39	I
	5	100	1996	772	413	593	N/A‡	P
3	0	150	1444	518	198	358	4.03	I
	1	150	1344	259	156	208	6.48	I
	3	150	1588	1666	862	1264	1.26	I
	5	150	1682	831	485	658	2.56	I
4	0	200	555	517	188	353	1.57	I
	1	200	619	349	174	262	2.37	I
	3	200	687	1006	618	812	0.846	I
	5	200	172	238	152	195	0.882	I
5	0	300	225	402	175	289	0.780	I
	5	300	195	185	133	159	1.23	I

* Sample size: 1 mL urine; amount of H_2O_2 (35%) added (in microliter)

† Signal level (peak height).

‡ N: negative; I: inconclusive; P: positive.

§ Intensities of the ions designating the analyte and the IS were abundant and meet the ion intensity ratio required for "positive" designations.

proven very reliable in quantitatively establishing whether the recovery of the IS was sufficient to call a specimen a true negative.

This same equation would prove extremely valuable in the analysis of postmortem specimens where the specimens are contaminated or putrefied, leading to large backgrounds and poor recovery of the IS.

The equation was developed to take into consideration the use of isotopic IS or non-isotopic IS. However, the authors' experiments were limited to isotopic IS, and further research would be needed to prove how effective the proposed equation would be using a non-isotopic IS. If a non-isotopic IS is used, this equation should still be applicable for evaluating the probability of seeing the analyte of interest at the LOD of the procedure, given a known R relative response for the IS and analyte. It is very important, when using a non-isotopic IS, that R be calculated after the specimen has been extracted to take into consideration differences in extraction efficiency for the IS and analyte.

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