Development and Validation of an Analytical Method for Quantitation of Alpha-pinene in Rodent Blood by Headspace GC-MS

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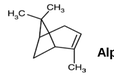
Abstract

Alpha-pinene (AP), produced by pine trees and other plants, is the main component of turpentine and is used as a fragrance and flavor ingredient. Exposure to AP occurs via use of personal care and household cleaning products and in the lumber industry. Despite widespread exposure, the toxicity data for AP are limited. The objective of this work was to develop and validate a method to quantitate AP in rat and mouse blood in support of the National Toxicology Program toxicokinetic and toxicology studies.



Alpha-pinene (AP)

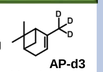
Standards were prepared by spiking a 100-µL aliquot of blood with 50 µL of spiking solution containing AP and internal standard (IS; AP-d3) in 50/50 ethanol/saline in a 2-mL headspace vial. The vial was sealed with a metal crimp-top cap, equilibrated for 10 min at 60°C, and a 500 µL headspace sample was analyzed by GC-MS using single ion monitoring [m/z 136 (AP) and 139 (IS)]. A DB-5MS column was used with oven temperature ramped from 40°C to 150°C in 9 min.

The method was successfully validated in male Sprague Dawley rat blood over the concentration range 5-500 ng/mL. Matrix standard curves were linear (r ≥ 0.99), and the percent relative error (%RE) values were ≤ ±15% for standards at all levels. Small background peaks were detected in the matrix and method blanks, but the response was < 30% of the response for the lowest standard and did not interfere with method performance. The limit of detection, determined from the standard deviation at the lower limit of quantitation (5 ng/mL), was 0.499 ng/mL. Absolute recovery was ≥ 67% at all concentrations.

Intra- and inter-day precision (% relative standard deviation, RSD) and accuracy (%RE) were ≤ 7% and ≤ ±13% respectively, for quality control (QC) standards prepared at 10, 100, and 250 ng/mL. Standards as high as 1500 ng/mL could be analyzed using a lower injection volume (150 µL), with %RE values ≤ ±19% and %RSD ≤ 1%. Loss of AP from blood occurred during overnight autosampler storage as well as frozen (-80°C) storage for 32 days, but incorporation of the IS prior to storage corrected for the loss such that determined concentrations were ≤ ±17% of fresh (Day 0) samples, with %RSD's ≤ 5%. The method was evaluated for male and female Harlan Sprague Dawley (HSD) rat blood and B6C3F1 mouse blood; %RE values were ≤ ±9% and %RSD ≤ 4%. These data demonstrate that the method is suitable for the analysis of AP in rodent blood generated from toxicokinetic and toxicology studies.

Materials & Methods

Materials



AP-d3

Alpha-pinene (AP; CAS No. 80-56-8): John D. Walsh Company, Inc., Ringwood, NJ
AP-d3 (Internal Standard, IS): AromaLAB GmbH, Planegg, Germany
Sprague Dawley (SD) and Harlan Sprague Dawley (HSD) rat blood; B6C3F1 mouse blood: BioIVT, Westbury, NY

Sample Preparation

Standards were prepared by spiking 100 µL blood with 50 µL AP spiking solution containing IS in 50/50 ethanol/saline. A cap was crimped onto each 2-mL vial, and the samples were analyzed by headspace GC-MS.

Instrument and Conditions

GC-MS System; Software	Agilent 6890 GC / 5973 MSD; MSD Chemstation E.02.02 (Agilent Technologies, Santa Clara, CA)
Headspace Autosampler	Combipal Autosampler (CTC Analytics, Zwingen, Switzerland)
Vial Size	2 mL
Sample Cycle Time; Syringe Vol.	20 min.; 2.5 mL
Sample Temp.; Equil. Time	60°C; 10 min.; mixer on
Sample Volume	500 µL (also tested 150 µL)
Column	Agilent DB-5MS (30 m x 0.25 mm ID, 0.25-µm film)
Carrier Gas	Helium at 1.2 mL/min.
Oven Temp. Program	40°C for 5 min., ramp to 75°C at 5°C/min., ramp to 150°C at 37.5°C/min., hold for 1 min.; total time = 15 min.
Retention Time	~10.9 min (both AP and IS)
Injector Temp.; Injection Mode	270°C; Splitless
Auxiliary Temp.; MS Source Temp.	300°C; 150°C
Quad Temp.; MS Ionization Mode	150°C; Electron Ionization (70 eV)
Acquisition Mode	Single ion monitoring (SIM); *m/z* 136 (AP) and 139 (IS) [M+]

Validation Design

Linearity: 7-point calibration curve in male SD rat blood over the range 5-500 ng/mL on each of 3 days

Recovery: Compare a set of matrix standards to equivalent set of solvent standards

Selectivity: 6 method blanks (with IS) and 6 matrix blanks (without IS)

Sensitivity: 6 replicates at the lowest concentration level to define LLOQ and LOD

Intra- and Inter-Day Precision & Accuracy: Triplicate matrix standards at 3 levels on each of 3 days. Precision calculated as %RSD; Accuracy calculated as Relative Error (RE)

Instrument Drift: Matrix standards run at start and end with multiple samples in between

Carryover: 3 method blanks after high matrix standard

Method Extension: Triplicate matrix standards at 1500 ng/mL; analyzed with 150-µL injection

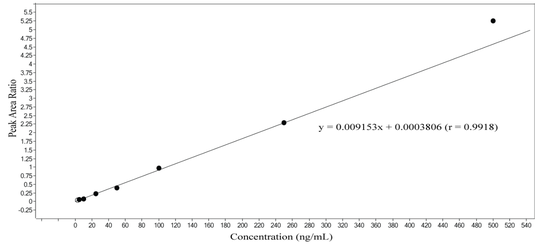
Autosampler Stability: Triplicate matrix standards at 2 levels; stored on autosampler overnight

Frozen Matrix Stability: Triplicate matrix standards at 2 levels; stored at -80 °C up to > 60 d

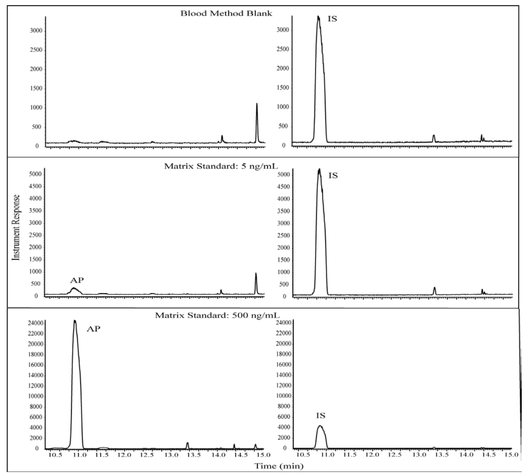
Secondary Matrix Evaluation: 6 method blanks, 6 matrix blanks, and 6 replicates at 2 x LLOQ in each secondary matrix; quantitated using primary matrix curve (male SD rat blood)

Results – Method Validation

The method was successfully validated over the concentration range 5-500 ng/mL in male SD rat blood (Primary Matrix). Small background peaks were detected in the matrix and method blanks, but the response was < 30% of the response for the lowest standard and did not interfere with method performance. The LOD, determined from the standard deviation at the LLOQ (5 ng/mL), was 0.499 ng/mL.



Example Matrix Calibration Curve (Experiment 1)



Representative GC-MS Ion Chromatograms from Selected Ion Monitoring (SIM) of Alpha-pinene (left; m/z 136) and Alpha-pinene-d3 (right; m/z 139) in Male Rat Blood

Results (cont'd)

Recovery						
Nominal Conc. (ng/mL)	**Matrix Standard Response**	**Solvent Standard Response**	**Absolute Recovery[a] (%)**	**Matrix Standard PAR**	**Solvent Standard PAR**	**Relative Recovery[b] (%)**
5.00	2878	3782	76.1	0.0501	0.0495	104
10.0	2622	3597	72.9	0.0810	0.0805	98.8
25.0	9438	11952	79.0	0.2206	0.2272	99.5
50.0	20675	28736	71.9	0.3911	0.3878	103
100	43121	58945	73.2	0.9783	0.9880	99.2
250	122006	155249	78.6	2.3153	2.3585	101
500	266732	397726	67.1	5.2607	5.4407	102
		Mean Recovery =	**74.0**	**Mean Recovery =**		**101**
		Variation[c] =	**11.9**	**Variation[c] =**		**4.8**

Solvent standards prepared same as matrix standards, except water used instead of blood.
PAR = peak area ratio
[a] Absolute Recovery = (Matrix Standard Response / Solvent Standard Response) x 100
[b] Relative Recovery = (Matrix Standard PAR / Solvent Standard PAR) x 100
[c] Variation = Highest % Recovery – Lowest % Recovery

- Absolute recovery ≥ 67% at all concentrations.

	Intra-Day Precision & Accuracy[a]		Inter-Day Precision & Accuracy[b]	
Nominal Conc. (ng/mL)	**Mean Found Conc. (ng/mL) (%RSD)**	**Mean RE (%)**	**Mean Found Conc. (ng/mL) (%RSD)**	**Mean RE (%)**
10.0	8.66 (0.78%)	-13.4	9.04 (5.9%)	-9.6
100	107 (0.68%)	6.9	102 (7.1%)	1.8
250	253 (0.15%)	1.3	253 (0.3%)	1.2
1500 (lower inj. volume)	1780 (0.32%)	18.7	N/A	

[a] n = 3 (within calibration curve no. 1)
[b] n = 9 (across calibration curves no. 1, 2, and 3)

- Intra- and inter-day precision and accuracy ≤ 7% and ≤ ±13%, respectively, for QC standards prepared at 10, 100, and 250 ng/mL.
- Standards as high as 1500 ng/mL could be analyzed using a lower injection volume (150 µL), with %RE values ≤ ±19% and %RSD ≤ 1%.

Stability				
Stability Condition	**Nominal Conc. (ng/mL)**	**Mean Response vs. Day 0**	**Mean Found Conc. (ng/mL) (%RSD)**	**Mean % of Day 0**
Autosampler, overnight	10.0 250	76% 67%	9.25 (0.13%) 276 (0.93%)	99.4 98.9
-80 °C, 18 days	10.0 250	35% 38%	10.6 (5.4%) 280 (2.5%)	114 100
-80 °C, 32 days	10.0 250	39% 44%	11.5 (4.2%) 285 (0.72%)	117 105
-80 °C, 74 days	10.0 250	35% 48%	14.8 (2.2%) 343 (1.2%)	147 126

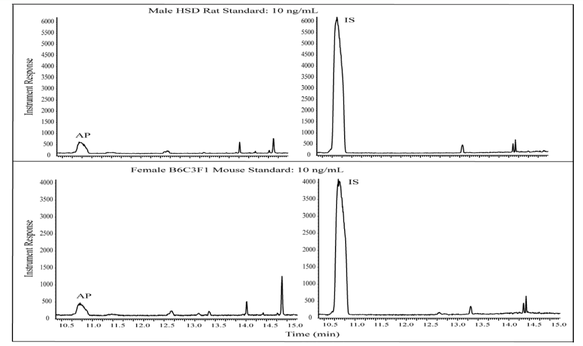
% of Day 0 = (Found Stored Conc. / Found Day 0 Conc.) x 100

- Loss of AP from blood occurred during overnight autosampler storage as well as frozen storage for 32 days, but incorporation of the IS prior to storage corrected for the loss (Mean % of Day 0 ≤ 117%; %RSD ≤ 5%).
- Day 74 results did not meet the acceptance criterion for accuracy.

Secondary Matrix Evaluations: Precision & Accuracy (n = 6)			
Matrix	**Nominal Conc. (ng/mL)**	**Mean Found Conc. (ng/mL) (%RSD)**	**Mean RE (%)**
Male HSD rat blood	10.0	10.0 (2.4%)	0.2
Female HSD rat blood	10.0	9.89 (4.3%)	-1.2
Male B6C3F1 mouse blood	10.0	9.61 (2.4%)	-3.9
Female B6C3F1 mouse blood	10.0	9.09 (1.7%)	-9.2

- The method was evaluated for male and female HSD rat blood and B6C3F1 mouse blood; %RE values were ≤ ±9% and %RSD ≤ 4%.

Results (cont'd)



Representative Chromatograms of AP (left) and AP-d3 (right) in Male HSD Rat Blood (Top) and Female B6C3F1 Mouse Blood (Bottom) [Secondary Matrix Evaluation]

Method Validation Summary

Validation Parameter	Acceptance Criteria	Results
Linearity	*r* ≥0.99 and %RE ≤ ±15% (≤ ±20% at LLOQ)	**Passed**: *r* ≥ 0.99 and %RE ≤ ±15% for all calibration standards
Recovery	Absolute Recovery >50% at each level, with variation ≤20% across levels	**Passed**: Mean absolute recovery 67.1 – 79.0%
Selectivity	Method blanks ≤30% of LLOQ response	**Passed**: Mean method blank response ≤ 29.8% of LLOQ
Sensitivity (LLOQ and LOD)	LLOQ: %RE ≤20% and %RSD ≤ ±20%; LOD = 3xSD for LLOQ replicates	**Passed**: %RE ≤ ±8.3% and %RSD ≤ 3.2 % at **5 ng/mL (LLOQ) LOD = 0.499 ng/mL**
Intra- and Inter-day Precision & Accuracy	Mean %RE ≤ ±15% and %RSD ≤15%	**Passed**: Mean %RE ≤ ±13.4% and %RSD ≤ 7.1%.
Instrument Drift	%Diff ≤15%; ≤ ±20% at LLOQ	**Passed**: %Diff ≤ ±8.0%
Carryover	N/A	**Carryover present** after high standard, but cleared after 2nd blank
Method Extension	Mean %RE ≤ ±20% and %RSD ≤20%	**Passed**: Mean %RE = 18.7% and %RSD = 0.32%.
Autosampler Stability	Mean % of Day 0 = 100 ± 20% and %RSD ≤20%	**Stable**: Mean % of Day 0 = 98.9 - 99.4% and %RSD ≤ 0.93%
Frozen Matrix Stability	Mean % of Day 0 = 100 ± 20% and %RSD ≤20%	**Stable (32 Days)**: Mean % of Day 0 = 100 - 117% and %RSD ≤ 5.4%
Secondary Matrix Evaluations - Male, female HSD blood - Male, female B6C3F1 blood	Mean %RE ≤ ±15% and %RSD ≤15%; Method blanks ≤30% of LLOQ response	**Passed**: Mean %RE ≤ ±9.2% and %RSD ≤ 4.3%; Mean method blank response ≤29.0% of LLOQ, except male mouse (31.5%)

Conclusions

Alpha-pinene (AP) can be quantitated in male and female rat and mouse blood using this simple headspace GC-MS method.

The method was successfully validated over the range 5-500 ng/mL in whole blood. Validation parameters included linearity, recovery, selectivity, sensitivity, precision, accuracy, and stability. It was also demonstrated that blood concentrations as high as 1500 ng/mL could be analyzed using a lower injection volume.

The validated method is currently being applied for the analysis of AP in rodent blood samples from toxicokinetic and toxicology studies.

Acknowledgement

This research was supported by the National Toxicology Program, National Institute of Environmental Health Sciences, NIH under Contract No. HHSN273201400022C.