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

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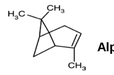
Abstract

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, 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 mammary tissue, a potential target tissue, in support of the National Toxicology Program toxicokinetic and toxicology studies.



Alpha-pinene (AP)

Standards were prepared by spiking a ~100 mg aliquot of mammary tissue with 100 µL of spiking solution containing AP and internal standard (IS; AP-d3) in 50/50 ethanol/saline in a 2-mL headspace vial containing 18 stainless steel beads. The vial was sealed and homogenized for 30 sec for 2 cycles at 1000 rpm. Each vial was equilibrated for 10 min at 60°C and a 200 µL headspace sample was analyzed by GC-MS using single ion monitoring [m/z 136 (AP); 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 female Sprague Dawley rat mammary tissue over the concentration range 100-5000 ng/g. Matrix standard curves were linear (r ≥ 0.99), and the percent relative error (%RE) values were ≤ ±12% for standards at all levels. Small background peaks were detected in the matrix and method blanks, but the response was low and did not interfere with method performance. Absolute recovery was low (2%) likely due to high lipophilicity of AP. However, the limit of detection, determined from the standard deviation at the lower limit of quantitation (100 ng/g), was 17.7 ng/g, demonstrating adequate sensitivity. Recoveries incorporating the IS were ≥90% at all concentrations.

Intra- and inter-day precision (% relative standard deviation, RSD) and accuracy (%RE) were ≤ 5.7% and ≤ ±6.3%, respectively, for quality control standards prepared at 250 and 2500 ng/g. Standards as high as 20,000 ng/g could be analyzed using a lower injection volume (20 µL) or by extrapolating the calibration curve beyond 5000 ng/g, with mean %RE ≤ ±1.4% and %RSD ≤ 2.2%. Smaller sample sizes (~50 mg) could also be analyzed, with mean %RE ≤ ±2.0% and %RSD ≤ 1.9%. The method was evaluated for female Harlan Sprague Dawley rat and B6C3F1 mouse mammary tissues; %RE values were ≤ ±3.8% and %RSD ≤ 2.1%. These data demonstrate that the method is suitable for the analysis of AP in rodent mammary tissues 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 mammary tissue; B6C3F1 mouse mammary tissue: BioIVT, Westbury, NY

Sample Preparation

Standards were prepared by spiking ~100 mg mammary tissue with 100 µL AP spiking solution containing IS in 50/50 ethanol/saline in a 2-mL headspace vial containing 18 stainless steel beads. The vial was sealed and homogenized for 30 sec for 2 cycles at 1000 rpm. Each vial was equilibrated for 10 min at 60°C and a 200 µL headspace sample was analyzed by 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.; 1 mL
Sample Temp.; Equil. Time	60°C; 10 min.; mixer on
Sample Volume	200 µL (also tested 10, 20, 50, 100 and 500 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 Times	~10.6 min (IS) and 10.7 min (AP)
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: 6-point calibration curve in female SD rat mammary tissue over the range 100-5000 ng/g 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)

Carryover: 3 method blanks after high matrix standard

Method Extension: Triplicate matrix standards at ~20,000 ng/g; analyzed with 20-µL injection

Curve Extrapolation: Triplicate matrix standards at ~20,000 ng/g; analyzed by extrapolating beyond the curve

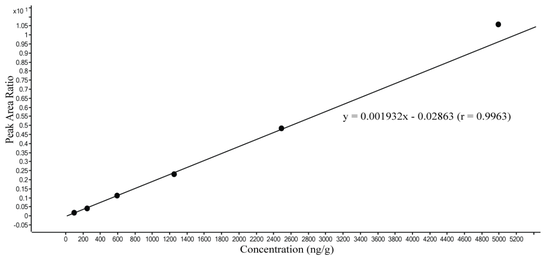
Smaller Sample Size: Triplicate matrix standards prepared using 50 mg tissue

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

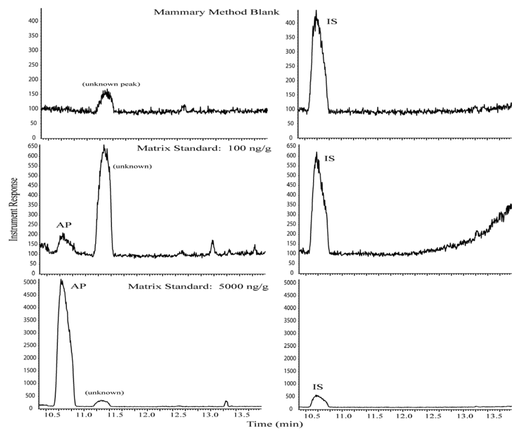
Secondary Matrix Evaluation: 3 method blanks, and 6 replicates at 2 x LLOQ in each secondary matrix; quantitated using primary matrix curve (female SD rat mammary tissue)

Results – Method Validation

The method was successfully validated in female SD rat mammary tissue (Primary Matrix) over the concentration range 100-5000 ng/g. Matrix standard curves were linear (r ≥ 0.99) and %RE ≤ ±12% for standards at all levels. Small background peaks were detected in the matrix and method blanks, but the response was low and did not interfere with method performance.



Example Matrix Calibration Curve



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 Rat Mammary Tissue

Results (cont'd)

Recovery						
Nominal Conc. (ng/g)	**Matrix Standard Response**	**Solvent Standard Response**	**Absolute Recovery[a] (%)**	**Matrix Standard PAR**	**Solvent Standard PAR**	**Relative Recovery[b] (%)**
99.5	580.35	35622	1.63	0.1504	0.1696	88.7
250	2204.3	59538	3.70	0.5103	0.4995	102
597	4560.1	223594	2.04	1.0350	1.0948	94.5
1250	7321.0	544741	1.34	2.5922	2.8129	92.2
2490	14619	1157242	1.26	4.8511	5.3350	90.9
4990	52899	2335171	2.27	10.7339	11.9410	89.9
		Mean Recovery =	**2.04**		**Mean Recovery =**	**93.1**
		Variation[c] =	**2.4**		**Variation[c] =**	**13.3**

Solvent standards prepared same as matrix standards, except water used instead of mammary tissue.
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 was low (2%) likely due to high lipophilicity of AP. However, the LOD, determined from the standard deviation at the LLOQ (100 ng/g), was 17.7 ng/g, demonstrating adequate sensitivity.
- Recoveries incorporating the IS ≥ 90% at all concentrations.

	Intra-Day Prec. & Accuracy[a]		Inter-Day Prec. & Accuracy[b]	
Nominal Conc. (ng/g)	**Mean Found Conc. (ng/g) (%RSD)**	**Mean RE (%)**	**Mean Found Conc. (ng/g) (%RSD)**	**Mean RE (%)**
250 2490	238 (2.0%) 2650 (0.8%)	-4.9 6.3	252 (5.7%) 2500 (4.6%)	0.9 0.5
20,000 (lower injection volume)	20,300 (2.2%)	1.4	N/A	
20,000 (extrapolation)	20,600 (1.3%)	-0.3	N/A	
250 (50 mg sample) 2500 (50 mg sample)	255 (1.9%) 2460 (1.6%)	2.0 -1.5	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 ≤ 5.7% and ≤ ±6.3%, respectively, for QC standards prepared at 250 and 2500 ng/g.
- Standards as high as 20,000 ng/g could be analyzed using a lower injection volume (20 µL) or by extrapolating the calibration curve beyond 5000 ng/g (mean RE ≤ ±1.4%; RSD ≤ 2.2%).
- Smaller sample sizes (~50 mg) could also be analyzed (RE ≤±2.0%; RSD ≤1.9%).

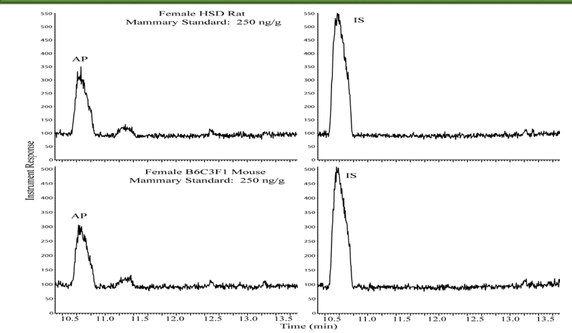
Stability				
Stability Condition	**Nominal Conc. (ng/g)**	**Mean Response vs. Day 0**	**Mean Found Conc. (ng/g) (%RSD)**	**Mean % of Day 0**
-80 °C, 15 days	250 2490	110% 47%	276 (1.6%) 2780 (7.0%)	107 106
-80 °C, 30 days	250 2490	91% 37%	338 (18.9%)* 2590 (6.3%)	142* 106
-80 °C, 60 days	250 2490	73% 36%	203 (3.0%) 2230 (4.1%)	99.2 84.7
% of Day 0 = (Found Stored Conc. / Found Day 0 Conc.) x 100				

- Loss of AP from mammary tissue occurred during frozen storage, but incorporation of IS prior to storage corrected for the loss (Mean % of Day0 ≤107%; %RSD ≤ 7%).

Secondary Matrix Evaluations: Precision & Accuracy (n = 3)			
Matrix	**Nominal Conc. (ng/g)**	**Mean Found Conc. (ng/g) (%RSD)**	**Mean RE (%)**
HSD rat mammary tissue	250 2490	242 (2.1%) 2390 (1.3%)	-3.4 -3.8
B6C3F1 mouse mammary tissue	250 2490	242 (2.0%) 2430 (1.1%)	-3.2 -2.6

- The method was evaluated for female HSD rat and B6C3F1 mouse mammary tissues; %RE values were ≤ ±3.8% and %RSD ≤2.1%.

Results (cont'd)



Representative Chromatograms of AP (left) and AP-d3 (right) in HSD Rat Mammary Tissue (Top) and B6C3F1 Mouse Mammary Tissue (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 ≤ ±12% for all calibration standards
Recovery	Relative Recovery >80% at each level, with variation ≤20% across levels	**Passed**: Mean relative recovery 88.7 – 102%. Absolute recovery only 2.0%
Selectivity	Method blanks ≤30% of LLOQ response	**Passed**: Mean method blank response ≤ 7.6% of LLOQ
Sensitivity (LLOQ and LOD)	LLOQ: %RE ≤20% and %RSD ≤ ±20%; LOD = 3xSD for LLOQ replicates	**Passed**: %RE ≤ ±7.6% and %RSD ≤ 5.9% at **100 ng/g (LLOQ) LOD = 17.7 ng/g**
Intra- and Inter-day Precision & Accuracy	Mean %RE ≤ ±15% and %RSD ≤15%	**Passed**: Mean %RE ≤ ±6.3% and %RSD ≤ 5.7%.
Carryover	N/A	**Carryover present** after high standard, but cleared after 1st blank
Method Extension	Mean %RE ≤ ±20% and %RSD ≤20%	**Passed**: Mean %RE ≤ ±1.4% and %RSD ≤ 2.2%.
Curve Extrapolation	Mean %RE ≤ ±20% and %RSD ≤20%	**Passed**: Mean %RE ≤ ±0.3% and %RSD ≤ 1.3%.
Smaller Sample Size	Mean %RE ≤ ±15% and %RSD ≤15%	**Passed**: Mean %RE ≤ ±2.0% and %RSD ≤ 1.9%.
Frozen Matrix Stability	Mean % of Day 0 = 100 ± 20% and %RSD ≤20%	**Stable (60 Days)**: Mean % of Day 0 = 84.7 - 107% and %RSD ≤ 7.0%
Secondary Matrix Evaluations - HSD mammary tissue - B6C3F1 mammary tissue	Mean %RE ≤ ±15% and %RSD ≤15%; Method blanks ≤30% of LLOQ response	**Passed**: Mean %RE ≤ ±3.8% and %RSD ≤ 2.1%; No peaks detected in blanks.

Conclusions

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

The method was successfully validated over the range 100-5000 ng/g in mammary tissue. Validation parameters included linearity, recovery, selectivity, sensitivity, precision, accuracy, and stability. It was also demonstrated that mammary tissue concentrations as high as 20,000 ng/g could be analyzed using a lower injection volume or by extrapolating beyond the curve.

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

Acknowledgement

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