Analysis of Coumarin, β-Asarone, and Thujone using GC/MS

Scope and Applications

This method will determine the concentration of the Coumarin, beta Asarone, and Thujone in alcoholic beverages (beers, wines and distilled spirits). The analytes are extracted from the sample by means of a liquid-liquid extraction. The analytes will partition from the aqueous sample to an organic layer consisting of methylene chloride. The analytes are separated and measured using a GC/MS instrument. The concentrations of the analytes are determined using an internal standard added to the sample.

Thujone (bicyclo[3.1.0]hexan-3-one) consists of two isomers, alpha and beta, found in different ratios depending on the plant. Common sources of thujone include *Artemisia* (wormwood), *Thuja occidentalis* (cedar leaves and twigs), *Tanacetum vulgare* (Tansy), *Achillea millefolium* (Yarrow). Food and Drug Administration's (FDA) regulation 21 CFR 172.510 states that a finished product that contains a natural flavoring substance or a natural substance in conjunction with a flavor that contains thujone, must be “Thujone Free”. TTB industry circular 2007-5 defines “Thujone Free” as less than 10 parts per million.

Food and Drug Administration's (FDA) regulation 21 CFR 189.130 states that food containing the direct addition of coumarin (1,2 benzo-pyrone), tonka beans, or tonka extract is deemed adulterated. However, naturally occurring coumarin is allowed. Some common sources of naturally occurring coumarin include: Hierochloe odorata (buffalo grass, vanilla grass, zubrovka), Galium odoratum (sweet woodruff), Trifolium pretense (Red clover), and Melilotus officinalis (yellow melilot, yellow sweet clover).

Beta asarone (trans-2,4,5-Trimethoxypropenylbenzene) is a primary ingredient of *Acorus calamus* (oil of calamus). Food and Drug Administration's (FDA) regulation 21 CFR 189.110 states that food containing any added calamus is deemed adulterated. Calamus is detected in foods by the presence of beta asarone. The limit of detection of the cited method is 0.5 g/L. Beverages containing 0.5 mg/L or greater beta asarone have detectable amounts of beta-asarone.

Levels and Limitations

Run the methylene chloride extracts within 24 hours of preparation.

Analyte	LOD (mg/L)	LOQ (mg/L)	Linear Range (mg/L)
Coumarin	0.05	0.14	0.5 – 30.0
β-Asarone	0.03	0.10	0.5 – 30.0
α- and β-thujone	0.05	0.16	1.0 – 60.0

Supplemental Documents

SSD:WG:203 Procedure for Reducing TAC Data

Equipment

Instrumentation and Equipment:

- GC/MS: Agilent 6890 Gas Chromatograph interfaced with a 5973 Mass Selective Detector or equivalent system.
- Shaker: Vortexer shaker or equivalent
- Centrifuge: Eppendorf Centrifuge 5702 or equivalent
- Dispensette S

Glassware and Supplies:

- Class A pipets and volumetric flask
- Pipettors various sizes
- 25 mL glass screw cap test tubes w/ Teflon resin lined caps
- 250 mL glass storage vials

GC/MS Instrument Parameters:

- Column: Stabilwax 30m x 0.25mm x 0.25 µm, or equivalent.
- Oven: 50 ºC for 1 min; ramp at 15 ºC/min to 250 ºC, hold for 1.67 min. Total Time 16.00 min.
- Mode: Constant Flow at 1 mL/min
- Inlet: Splitless at 250 ºC; Purge inlet after 1 min; carrier gas, Helium
- Injector: Injection volume, 1 µL; syringe size, 5 µL
- MSD Transfer: 270 ◦C
- MSD: mode, scan; solvent delay, at least 3.00 min; scan low mass, 50 amu; scan high mass, 300 amu; ionization mode, electron impact; calibration, autotune

	Quant Ion	Qualifying Ions
Thujone	110	81, 152
Cyclodecanone	111	94, 154, 125
Dihydrocoumarin	148	120, 91
Beta-Asarone	208	193, 165
Coumarin	146	118

Reagent and Sample Preparation and Handling

Reagents: (Specific vendors and product numbers are listed for convenience. Equivalent products may be used.)

1) Deionized Water: 18 megaΩ or better
2) Ethyl Alcohol (Pharmco, Connecticut, USA)
3) Alpha Thujone: (1*S*,4R)-1-Isopropyl-4-methylbicyclo[3.1.0]hexan-3-one,
   - **Primary source:** CAS #546-80-5; Sigma Aldrich product # 89231, purity ≥96.0%
   - **secondary source (CRM):** Alpha & Beta-Thujone solution, CAS #67-56-1; Sigma Aldrich product #CRM40909, 2000 ug/mL
4) Cyclodecanone: CAS # 1502-06-3, Sigma Aldrich product # 28698, purity ≥98.0%
5) Coumarin: 1,2-Benzopyrone
   - **Primary source:** CAS #91-64-5, Sigma Aldrich product # C4261, purity ≥99%
   - **Secondary source (CRM):** CAS #91-64-5, Sigma Aldrich product #72609
6) Beta Asarone: (E)-1,2,4-Trimethoxy-5-(1-propenyl)benzene
   - **Primary source:** CAS #2883-743-6, Chromadex product # 00011017, purity ≥96.0%)
   - **Secondary Source (pharm. ref. std.):** CAS #5273-86-9, Sigma Aldrich product #02890590
7) Dihydrocoumarin: 3,4-Dihydro-1-benzopyran-2-one (CAS# 119-84-6, Sigma Aldrich product # D104809, purity 99%)
8) Sodium Chloride
9) Methylene chloride (purity ≥99.5%)

Preparation of Standards and LCS:

1) Stock Standards: Prepare a stock standard solution consisting of 1000 mg/L thujone and 500 mg/L each of asarone and coumarin, and the internal standards stock solution consisting of 1000 mg/L cyclodecanone and 500 mg/L dihydrocoumarin. Store the stock solutions in the refrigerator for up to 12 months.
   a. Standards Stock Solution

i. Weight 0.2000 g (±0.5%) of thujone and 0.1000 g (±0.5%) each of coumarin and asarone into a 200 mL volumetric flask [Note: account for purity if the compound is less than 98% pure].
ii. Bring to volume with 100% ethanol.
iii. Due to the limited nature of CRM material, the second source stock preparation may not be identical to the primary stock solution. The second source stock may be individual analytes or combination of analytes.

b. Internal Standards Stock Solution:
i. Weight 0.1000 g (±0.5%) of cyclodecanone and 0.0500 g (±0.5%) of dihydrocoumarin into a 100 mL volumetric flask [Note: account for purity if the compound is less than 98% pure].
ii. Bring to volume with 100% ethanol.

2) Working Standards: Store the working solutions in the refrigerator for up to 6 months.
a. **Calibration Working Solutions**:
i. Level 5 (40.0 mg/L Thujone, 20.0 mg/L each of Asarone and Coumarin): e.g., Pipet 8.0 mL of the Standards Stock Solution into a 200 mL volumetric flask. Q.S. with 40% Ethanol by volume.

ii. Level 4 (20.0 mg/L Thujone, 10.0 mg/L each of Asarone and Coumarin): e.g., Pipet 4.0 mL of the Standards Stock Solution into a 200 mL volumetric flask. Q.S. with 40% Ethanol by volume.

iii. Level 3 (10.0 mg/L Thujone, 5.0 mg/L each of Asarone and Coumarin): e.g., Pipet 2.0 mL of the Standards Stock Solution into a 200 mL volumetric flask. Q.S. with 40% Ethanol by volume.

iv. Level 2 (2.0 mg/L Thujone, 1.0 mg/L each of Asarone and Coumarin): e.g., Pipet 10.0 mL of the Level 5 into a 200 mL volumetric flask. Q.S. with 40% Ethanol by volume.

v. Level 1 (1.0 mg/L Thujone, 0.5 mg/L each of Asarone and Coumarin): e.g., Pipet 10.0 mL of the Level 4 into a 200 mL volumetric flask. Q.S. with 40% Ethanol by volume.

b. Second Source Level 3 Check Solution (10.0 mg/L Thujone, 5.0 mg/L each of Asarone and Coumarin): Make up solution with the same analyte concentration of the level 3 standard using the appropriate second source materials available.

3) Saturated Sodium Chloride Solution:
    a. Vigorously dissolve sodium chloride into DI water until crystals no longer go into solution.

4) Laboratory Control Sample:
    a. The LCS is prepared by spiking a non-flavored vodka at the 20 ppm level for thujone and at the 10 ppm level for both coumarin and beta asarone. To prepare the LCS, pipet 4.0 mL of the Standards Stock Solution into 200 mL volumetric flask and bring to volume with a non-flavored vodka. Transfer the LCS to a storage vial and store in the refrigerator for up to 6 months.

Procedure

Liquid-Liquid Extraction of Standards and Samples:

1) Pipet 5 mL of standard, LCS, or sample into a **glass** test tube. A class pipet or a pipettor may be used.
2) Pipet 5 mL of saturated sodium chloride solution into the test tube. A class A pipet or a pipettor may be used.
3) Pipet 50.0 µL of internal standard into each test tube. The concentration of the internal standard in the sample is 10 mg/L cyclodecanone and 5 mg/L dihydrocoumarin.
4) Pipet 5.0 mL of methylene chloride into the test tube using a **glass** volumetric pipet or bottle top dispensette.
5) Cap the test tubes and place in the shaker for approximately 10 minutes (the level of shaking is to be high enough to agitate the whole sample in the tube).
6) After shaking allow the organic layer and aqueous layer to separate. If the layers do not easily separate use a centrifuge to separate the layers.
7) Transfer 1.5 – 2.0 mL of the organic (usually the bottom layer) layer into a GC vial.

GC/MS Operating Procedures:

1) Analyze the samples and standards using the previously mentioned GC/MS parameters.
2) Example sequence order:
    1. Methylene Chloride Blank
    2. Level 1 Standard
    3. Level 2 Standard
    4. Level 3 Standard
    5. Level 4 Standard
    6. Level 5 Standard
    7. Methylene Chloride Blank
    8. LCS Sample
    9. LCS Sample (or duplicate sample, see Quality Control section)

10. Unknown Sample
11. …

3) The elution order of the compounds in the standards is as follows: α-Thujone, β-Thujone, Cyclodecanone, Dihydrocoumarin, β-Asarone, Coumarin.

Note: Run the $MeCl_2$ extracts within 24 hours from the time they are prepared.

Quality Control

1) The correlation coefficient for the standards calibration curve is to be greater than or equal to 0.995. If the correlation coefficient is less than 0.995, employ traditional troubleshooting techniques (change liner/septa, check for leaks, dirty MS source) to solve the problem and rerun the samples.

2) Run the LCS samples for accuracy and a duplicate sample for precision. The values for accuracy and precision are to be within the prescribed limits.

3) Run the second source level 3 check standard at least once every 8 samples. The measured value is to be within 1 mg/L of the expected value. If the value is not within 1 mg/L of the expected value rerun the standards and the previous eight samples.

Sources of Measurement Uncertainty

- Pipetting errors
- Impure and/or contaminated standards or reagents.
- Poor instrument performance (column condition, dirty liner, …)
- Instability of chemicals in methylene chloride.

Calculations

Agilent MassHunter software or an equivalent software package is used to determine the peak areas of the quant ions from 5 standards. A linear curve is generated from the ratio of the target compound to the appropriate internal standard. The Total Thujone calibration curve is determined using the peak area of α- and β-thujone in relation to cyclodecanone as the internal standard at 10 mg/L. The calibration curves for β-asarone and coumarin are determined using the peak areas of the selected compound in relation to dihydrocoumarin as the internal standard at 5 mg/L.

The presence of thujone, β-asarone, or coumarin in the sample is confirmed by comparing the retention time and ion ratios with those in the standards.

Reporting Results

The analytes are calculated in mg/L and reported as ppm to the tenth decimal place (XX.x).

Note: ppm and mg/L are interchangeable units for this method.
Report the specific analyte

Safety Notes

Methylene chloride is a National Toxicology Program suspect carcinogen and a known carcinogen in the State of California. Methylene chloride extractions must be performed inside the fume hood. Care should be taken to reduce exposure. Wear appropriate personal protective equipment such as safety glasses, gloves (Viton gloves are recommended), and lab coats. A respirator must be worn whenever workplace conditions warrant to reduce exposure (e.g., fume hood not operating sufficiently to keep exposure below limit). Review MSDS for exposure limits and first aid responses.

Dispose of Methylene chloride into the appropriate waste container.

References

Official Methods of Analysis (2019) 21st Ed., AOAC INTERNATIONAL, Rockville, MD, **Method 976.12** (*Coumarin in Wines*). www.eoma.aoac.org [accessed on March 25, 2021]

Official Methods of Analysis (2019) 21st Ed., AOAC INTERNATIONAL, Rockville, MD, **Method 977.09** (*ß-Asarone in Wines*). www.eoma.aoac.org [accessed on March 25, 2021]

Official Methods of Analysis (2019) 21st Ed., AOAC INTERNATIONAL, Rockville, MD, **Method 983.16** (*Benzoic Acid and Sorbic Acid in Food*). www.eoma.aoac.org [accessed on March 25, 2021]

Dyer, R.H., *et al.* (1975) *J. AOAC Int.* **58**, 1, 140-142.
Title: *Gas-Liquid Chromatographic Determination of Coumarin (1,2-Benzopyrone) in May Wine.*

Dyer, R.H. & Martin, G.E. (1976) *J. AOAC Int.* **59**, 4, 780-782.
Title: *Collaborative Study of the Gas-Liquid Chromatographic Determination of Coumarin (1,2-Benzopyrone) in Wine*.
TTB Industry Circular 2007-5

Code of Federal Regulations.

Location of Validation Package

Quality system files.

Required Training, Certification

1. GC/MS operation training.
2. In-house training on sample preparation and extraction techniques is required.
3. Initial certification by running 7 LCS samples with results of precision and reproducibility in agreement with mean and standard deviation acceptance criteria.
4. Periodically, chemist are retested for competency (e.g. every 5 years) and/or given proficiency testing.

Revision History

Rev. 1 – Deleted qualifying ion at 89 from coumarin (3/1/2011)

Rev. 2 – Minor updates in Instrument parameters

Rev. 3 – Updates to references in the Scope and Application. Added that the organic layer is the bottom layer.

Rev. 4 – Generalized working standard make-up and included second source standard. Added additional reagent examples. Updated Quality Control for the inclusion of second source standard. Updated calculating results section.

Rev. 5 – Updated to allow for use of bottle top dispensette for transferring methylene chloride; added statement secondary source stock preparation may differ from primary stock solution due to the limited nature of CRMs.

Rev. 6 – Reference section was edited as part of the web review project.

Rev. 7 – Removed injector parameters. Removed reference to retired SSD:TM:204.

Rev. 8 – Clarified shaking time is approximate, removal of notes with references, and some minor formatting changes.