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## Analysis of Coumarin, $\beta$ -Asarone, and Thujone using GC/MS

### Scope and Applications

This method will determine the concentration of the Coumarin, B-Asarone, and Thujone in alcoholic beverages (beers 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), *Salvia officinalis* (Sage), *Thymus* (thyme), and *Rosmarinus officinalis* (rosemary). 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.

Coumarin (1,2 benzo-pyrone) is added directly to beverages or added through the addition of flavoring materials. Common sources of coumarin include: *Hierochloe odorata* (buffalo grass, vanilla grass, zubrovka), *Galium odoratum* (sweet woodruff), *Dipteryx odorata* (tonka beans), *Trifolium pretense* (red clover), *Melilotus officinalis* (yellow melilot, yellow sweet clover), and *Aesculus hippocastanum* (horse chestnut). Food and Drug Administration's (FDA) regulation 21 CFR 189.110 states that food containing the direct addition of coumarin, tonka beans, or tonka extract is deemed adulterated.

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.

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## 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
$\beta$ -Asarone	0.03	0.10	0.5 – 30.0
$\alpha$ - and $\beta$ -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

### Glassware and Supplies:

- Class A pipets and volumetric flask
- Pipettors: 5000  $\mu$ L, 1000  $\mu$ L, 10-100  $\mu$ L
- 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  $\mu$ m, or equivalent.
- Oven: 50  $^{\circ}$ C for 1 min; ramp at 15  $^{\circ}$ C/min to 250  $^{\circ}$ C, hold for 1.67 min. Total Time 16.00 min.
- Mode: Constant Flow at 1 mL/min
- Inlet: Splitless at 250  $^{\circ}$ C; Purge inlet after 1 min; carrier gas, Helium
- Injector: Injection volume, 1  $\mu$ L; syringe size, 5  $\mu$ L; sample wash, 2; sample pumps, 2; pre wash solvent A and B (methylene chloride), 2; post wash solvent A and B, 2; plunger speed, fast
- MSD Transfer: 270  $^{\circ}$ 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

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-	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 mega $\Omega$  or better
- 2) Ethyl Alcohol (Pharmco, Connecticut, USA)
- 3) Alpha Thujone: (1*S*,4*R*)-1-Isopropyl-4-methylbicyclo[3.1.0]hexan-3-one, (CAS# 546-80-5; Sigma Aldrich product # 89231, purity  $\geq$ 96.0%)
- 4) Cyclodecanone: (CAS# 1502-06-3, Sigma Aldrich product # 28698, purity  $\geq$ 98.0%)
- 5) Coumarin: 1,2-Benzopyrone (CAS # 91-64-5, Sigma Aldrich product # C4261, purity  $\geq$ 99%)
- 6) Beta Asarone: (E)-1,2,4-Trimethoxy-5-(1-propenyl)benzene (Cas # 2883-743-6, Chromadex product # 00011017, purity  $\geq$ 96.0%)
- 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  $\geq$ 99.5%)

### Preparation of Standards and LCS:

- 1) Stock Standards: Prepare the standards stock 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 ( $\pm$ 0.5%) g of thujone and 0.1000 ( $\pm$ 0.5%) g each of coumarin and asarone into a 200mL volumetric flask [Note: account for purity if the compound is less than 98% pure].
    - ii. Bring to volume with 100% ethanol.
  - b. Internal Standards Stock Solution:
    - i. Weight 0.1000 ( $\pm$ 0.5%) g of cyclodecanone and 0.0500 ( $\pm$ 0.5%) g of dihydrocoumarin into a 100mL volumetric flask [Note: account for purity if the compound is less than 98% pure].
    - ii. Bring to volume with 100% ethanol.

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- 2) Working Standards: Store the stock solutions in the refrigerator for up to 6 months.
  - a. Level 5 (*40.0 mg/L Thujone, 20.0 mg/L each of Asarone and Coumarin*): Pipet 8.0 mL of the Standards Stock Solution into a 200 mL volumetric flask. Q.S. with 40% Ethanol by volume.
  - b. Level 4 (*20.0 mg/L Thujone, 10.0 mg/L each of Asarone and Coumarin*): Pipet 4.0 mL of the Standards Stock Solution into a 200 mL volumetric flask. Q.S. with 40% Ethanol by volume.
  - c. Level 3 (*10.0 mg/L Thujone, 5.0 mg/L each of Asarone and Coumarin*): Pipet 2.0 mL of the Standards Stock Solution into a 200 mL volumetric flask. Q.S. with 40% Ethanol by volume.
  - d. Level 2 (*2.0 mg/L Thujone, 1.0 mg/L each of Asarone and Coumarin*): Pipet 10.0 mL of the Level 5 into a 200 mL volumetric flask. Q.S. with 40% Ethanol by volume.
  - e. Level 1 (*1.0 mg/L Thujone, 0.5 mg/L each of Asarone and Coumarin*): Pipet 10.0 mL of the Level 4 into a 200 mL volumetric flask. Q.S. with 40% Ethanol by volume.
- 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 10ppm level for both coumain 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.

Note: The LCS may be combined with the vanillin and ethyl vanillin LCS in method SSD:TM:204.

## Procedure

### Liquid-Liquid Extraction of Standards and Samples:

- 1) Pipet 5 mL of standard, LCS, or sample into a **glass** test tube. A class A 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.

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- 3) Pipet 50.0  $\mu$ L 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.
- 5) Cap the test tubes and place in the shaker for 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 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:  $\alpha$ -Thujone,  $\beta$ -Thujone, Cyclodecanone, Dihydrocoumarin,  $\beta$ -Asarone, Coumarin.

Note: Run the  $\text{MeCl}_2$  extracts within 24 hours from the time they are prepared.

#### **Calculating Results:**

Chemstation 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  $\alpha$ -thujone in relation to cyclodecanone as the internal standard at 10mg/L. The calibration curves for  $\beta$ -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,  $\beta$ -asarone, or coumarin in the sample is confirmed by comparing the retention time with those in the standards.

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## 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 sample/s for accuracy and a duplicate sample for precision. The values for accuracy and precision are to be within the prescribed limits.
- 3) Re-run the level 3 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.

## 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

- 1) OMA AOAC, 15<sup>th</sup> ed.; 1990; 997.09, 976.12, 940.16
- 2) Dyer, R.H., Martin, G.E., and Buscemi, P.C., "Gas-Liquid Chromatographic Determination of  $\beta$ -Asarone in Wines and Flavors", *JAOAC*, **1976**, 59, 675-677.

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- 3) Dyer, R.H., "Gas-Liquid Chromatographic Determination of  $\beta$ -Asarone in Wine: Collaborative Study", *JAOAC*, **1977**, 60, 1041-1043.
- 4) Dyer, R.H., Martin, G.E., Butts, B.B., "Gas-Liquid Chromatographic Determination of Coumarin (1,2 Benzopyrone) in May Wine", *JAOAC*, **1975**, 58, 140-142.
- 5) Dyer, R.H., and Martin, G.E., "Collaborative Study of the Gas-Liquid Chromatographic Determination of Coumarin (1,2, Benzopyrone) in Wine", *JAOAC*, **1976**, 59, 780-782.
- 6) TTB Industry Circular 2007-5
- 7) 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 the results of the validation package.
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 – Correction to column type, change low mass amu from 40 to 50, addition of related documents section (12/4/2014)