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Qualitative Screening for Limited and Prohibited Ingredients in Beverage Alcohol Products Using LC-HRMS

Scope and Application

This method will be used to identify and confirm the presence (or absence) of limited and prohibited ingredients (see table below) and other compounds of interest in alcohol beverage products, namely flavored spirits, using ultra-high performance liquid chromatography coupled with high-resolution tandem mass spectrometry (UHPLC-HRMS/MS). Additional ingredients may be added as the need arises so long as they are compatible with the validated LC-HRMS method.

Levels and Limitations

Ingredient	Approximate LOD	Tolerance	Reference
4-Hydroxybenzaldehyde	100 ppb	GRAS	
4-Hydroxybenzoic Acid	100 ppb	GRAS	
Acetovanillone	10 ppb	GRAS	
Asarone	10 ppb	1 mg/kg	Directive 88/388/EEC
Aspartame	10 ppb	GMP	21CFR172.804
Benzoic acid	10 ppb	0.1%	21CFR184.1021
BHA	1 ppm	0.02%	21CFR182.3169
BHT	1 ppm	0.02%	21CFR182.3173
Caffeine	10 ppb	0.02%	21CFR182.1180
Cinchonidine	1 ppb	83 ppm[a]	21CFR172.510
Cinchonine	10 ppb	83 ppm[a]	21CFR172.510
Coumarin	1 ppb	Prohibited	21CFR189.130
Ethyl Vanillin	1 ppm	GRAS[b]	21CFR182.60
Fumaric acid	1 ppm	GMP	21CFR172.350
Heptylparaben	10 ppb	12 ppm	21CFR172.145
Malic acid	1 ppm	0.7%	21CFR184.1069
Mannitol	100 ppb	2.5%	21CFR180.25
Methylparaben	10 ppb	0.1%	21CFR184.1490
Piperonal	1 ppm	GRAS	21CFR182.60
Propylparaben	100 ppb	0.1%	21CFR184.1670
Quinidine	10 ppb	83 ppm[c]	21CFR172.575
Quinine	10 ppb	83 ppm[c]	21CFR172.575
Shikimic Acid	100 ppb	GRAS	
Sorbitol	100 ppb	12%	21CFR184.1835
Stevioside	100 ppb	GRAS	
Sucralose	100 ppb	GMP	21CFR172.831
Vanillic Acid	1 ppm	GRAS	
Vanillin	100 ppb	GRAS[d]	21CFR182.60

[a] As combined total of cinchonidine and cinchonine
[b] TTB-specific limit of 40 ppm in alcohol beverages (www.ttb.gov)
[c] As combined total of quinidine (quinine stereoisomer) and quinine
[d] TTB-specific limit of 16 ppm in alcohol beverages for synthetic (www.ttb.gov)

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Identification Acceptance Criteria:[1,2]

Signal Intensity:

Peak has a signal-to-noise (S/N) ratio ≥ 3

Full Scan (MS^1):

Mass accuracy of precursor ion ≤ 5 ppm

Data-dependent acquisition MS/MS ($ddMS^2$):

Mass accuracy of product ion(s) ≤ 10 ppm (minimum 1)

Retention time (RT):

± 0.2 min relative to comparison standard

Isotopic Pattern (IP):

≤ 20% of expected pattern fit and mass accuracy of ≤ 10 ppm for all ions

Supplemental Documents

None

Equipment

Glassware and Supplies:

Graduated cylinders as needed
Auto pipettors capable of delivering 50 µL to 1000 µL
15 mL conical centrifuge tubes with screw cap closure
2 mL microcentrifuge tubes
2 mL autosampler vials with split-top caps
1 L amber solvent bottle (for aqueous mobile phases)
1 L clear solvent bottles

Instrumentation:

Thermo Scientific Vanquish UHPLC (or equivalent)
Thermo Scientific Q-Exactive High Resolution Mass Spectrometer (or equivalent)
Thermo Scientific Microcentrifuge (or equivalent)

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Instrument Parameters (LC):

Column:	Thermo Scientific Accucore C18 2.6 µM, 2.1 × 100 mm
Guard:	Thermo Scientific Accucore C18 2.6 µM, 2.1 × 10 mm Defender
Column Temperature:	40 °C
Injection Volume:	2 µL
Wash:	20 µL/s for 10 s
Flow Rate:	300 µL/min
Gradient:	Time (min) — Mobile Phase B Initial — 5% 8.0 — 100% 12.0 — 100% 13.0 — 5% 14.0 — 100% 15.0 — 5% 16.0 — 100% 17.0 — 5% 20.0 — 5%

Instrument Parameters (HESI Source Settings):

Spray Voltage:	3.5 kV (-2.5 kV in negative mode)
Capillary Temperature:	250 °C
Aux Gas Heater Temperature:	400 °C
Aux Gas Flow Rate:	10
Sheath Gas Flow Rate:	50
Sweep Gas Flow Rate:	2
S-lens RF level:	50

Instrument Parameters (MS^1, dd-MS^2, and dd Settings):

MS^1 Settings	Resolution:	70,000
	AGC Target:	1e6
	Maximum IT:	100 ms
	Scan Range:	75 to 1,000 m/z
dd-MS^2 Settings	Resolution:	35,000
	AGC Target:	1e6
	Maximum IT:	100 ms
	Isolation Window:	1.5 m/z
	Stepped (N)CE:	10, 30, 60
dd Settings	Inclusion List Tolerance:	± 5 ppm
	Minimum AGC Target:	2.5e3 (Intensity Threshold = 2.5e4)
	Apex Trigger:	3 to 10 s
	Dynamic Exclusion:	5 s

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Reagent and Sample Preparation and Handling

Reagents:

(Vendors and product numbers are listed for convenience. Equivalent products may be used.)

Reagent	CAS #	Grade/Purity	Vendor	Solvent
Methanol	67-56-1	LCMS	Fisher Scientific	N/A
Water	7732-18-5	LCMS or 18 MΩ	Fisher Scientific	N/A
Formic Acid	64-18-6	LCMS	Fisher Scientific	N/A
Ammonium Formate	540-69-2	LCMS	Fisher Scientific	N/A
Positive Analytes				
Acetovanillone	498-02-2	100%	TCI America	Methanol
Asarone	2883-98-9	98%	Sigma Aldrich	Methanol
Aspartame	22639-47-0	96.6%	Sigma Aldrich	Water
BHA	25013-16-5	97.3%	Sigma Aldrich	Methanol
BHT	128-37-0	100%	Supelco	Methanol
Caffeine	58-08-2	100%	Sigma Aldrich	Methanol
Cinchonidine	485-71-2	96%	Sigma Aldrich	Methanol
Cinchonine	118-10-5	98%	Sigma Aldrich	Methanol
Coumarin	91-64-5	99%	Acros Organics	Methanol
Piperonal	120-57-0	99%	Sigma Aldrich	Methanol
Quinidine	56-54-2	96%	Sigma Aldrich	Methanol
Quinine	130-95-0	98%	Sigma Aldrich	Methanol
Vanillin	121-33-5	99%	Sigma Aldrich	Water
Negative Analytes				
4-Hydroxybenzaldehyde	123-08-0	98%	Sigma Aldrich	Methanol
4-Hydroxybenzoic Acid	99-96-7	99%	Sigma Aldrich	Methanol
Ethyl Vanillin	121-32-4	99%	Sigma Aldrich	Water
Fumaric Acid	110-17-8	99%	Sigma Aldrich	Water
Heptyl 4-Hydroxybenzoate	1085-12-7	(unknown)	Sigma Aldrich	Methanol
Malic Acid	6915-15-7	99%	Sigma Aldrich	Water
Mannitol	69-65-8	98%	Sigma Aldrich	Water
Methyl Paraben	99-76-3	100%	Sigma Aldrich	Methanol
Potassium Sorbate	24634-61-5	73.6%	Sigma Aldrich	Water
Propyl Paraben	94-13-3	100%	Sigma Aldrich	Methanol
Shikimic Acid	138-59-0	99%	Sigma Aldrich	Water
Sodium Benzoate[a]	532-32-1	83.6%	Sigma Aldrich	Water
Sorbitol	50-70-4	99%	Sigma Aldrich	Water
Stevioside Hydrate	57817-89-7	98%	Sigma Aldrich	Water
Sucralose	56038-13-2	98%	Sigma Aldrich	Water
Vanillic Acid	121-34-6	97%	Sigma Aldrich	Water

[a] Substitute for Benzoic Acid

Preparation of Solutions:

1) Mobile phase A: 5 mM ammonium formate and 0.1% formic acid in water
   For example, for 1 L (stable for 3 months at room temperature):
   i. To a 1 L graduated cylinder add approximately 250 mL of water
   ii. Add 1.0 mL formic acid and swirl to mix
   iii. Add 0.315 ± 0.005 g ammonium formate and swirl to mix and dissolve

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iv. Add water to the 1 L mark
v. Decant into a LC solvent bottle and swirl (amber glass recommended)

2) Mobile phase B: 5 mM ammonium formate and 0.1% formic acid in methanol
For example, for 1 L (stable for 3 months at room temperature):
i. To a 1 L graduated cylinder add approximately 250 mL of methanol
ii. Add 1.0 mL formic acid and swirl to mix
iii. Add 0.315 ± 0.005 g ammonium formate and swirl to mix and dissolve
iv. Add methanol to the 1 L mark
v. Decant into a LC solvent bottle and swirl

3) Needle/Seal wash: 0.1% formic acid and 10% methanol in water
For example, for 1 L (stable for 6 months at room temperature):
i. To a 1 L graduated cylinder add 100 mL of methanol
ii. Add 1.0 mL formic acid and swirl to mix
iii. Add water to the 1 L mark
iv. Decant into a LC solvent bottle and swirl

4) Dilution solvent: 10% methanol in water
For example, for 100 mL (stable for 6 months at room temperature):
i. To a 100 mL graduated cylinder add 10 mL of methanol
ii. Add water to the 100 mL mark
iii. Decant into a glass solvent bottle and swirl

Preparation of Stock/Working Standards:

1) Individual stock standards (1 mg/mL in methanol or water)
For example, for 10 mL (stable for 12 months, stored refrigerated:
i. Weigh approximately 10 mg standard in a 15 mL centrifuge tube
ii. Add methanol (or water) up to the 10 mL mark and shake
2) Working standard mixes (1 µg/mL each in methanol)
For example, for 10 mL (stable for 12 months, stored refrigerated:
i. Add approximately 2 mL of methanol to a 15 mL centrifuge tube
ii. Add 10 µL of each positive stock standard solution
iii. Fill to the 10 mL mark with methanol and shake
iv. Repeat steps 1 – 3 for negative stock standard solutions

Preparation of Samples:

1) Transfer 100 µL of sample to a 2 mL centrifuge tube
2) Add 900 µL of dilution solution
3) Centrifuge at 13,000 rpm for 5 min
4) Transfer to an autosampler vial and inject

Procedures

1) Prepare all solutions and standards as described above and as needed.
2) Prepare samples as described above.
3) Prime the LC pumps and equilibrate the system to the initial method conditions.
4) Inject blanks, samples, and QC in the following order:
- Solvent blank
- QC (Positive and negative standard mixes)
- Samples

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- QC (Positive and negative standard mixes)
- Solvent blank
- Column flush

5) Shut down system and properly store LC column.
6) Process sequence in TraceFinder using the targeted screening method.
7) Report results as described below.

Quality Control

1) QC samples (positive and negative standard mixes) are injected with each sequence and spot-checked for true positive results using the identification acceptance criteria to ensure acceptable instrument performance.

Sources of Uncertainty

Relevant Definitions:

True Positive:
A test result which correctly indicates the presence of a compound.

True Negative:
A test result which correctly indicates the absence of a compound.

False Positive:
A test result which incorrectly indicates the presence of a compound.

False Negative:
A test result which incorrectly indicates the absence of a compound.

Sources for False Positive/False Negative Occurrences:

1) Matrix interferences
2) Improperly calibrated MS system
3) Contaminated reagents

Reporting Results

The following table outlines the three conclusions that are possible for the confirmation of identity of a compound at or above its limit of detection:

Confirmed	Detected	Not Detected
All 4 criteria met: • RT ± 0.2 min • MS^1 ≤ 5 ppm • MS^2 ≤ 10 ppm • IP ≤ 10 ppm and ≤ 20% expected fit	2 (or 3) of 4 criteria met: • RT ± 0.2 min • MS^1 ≤ 5 ppm (MS^2 and IP not required)	All other scenarios where both RT and MS^1 are not met

Note: False positives and/or false negatives may be observed due to unforeseen complications (see Sources of Uncertainty). True positives and true negatives are ultimately determined by the analyst after reviewing the data.

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If a compound is confirmed or detected, it will be quantitatively analyzed with a different method (if available).

Safety Notes

The anticipated waste volume for each sample preparation and analysis is approximately 5 mL consisting primarily of aqueous organic solvent (methanol, acetonitrile) with water. The waste will potentially contain up to percent levels of some of the compounds of interest.

References

1) U.S. Food & Drug Administration, Office of Foods and Veterinary Medicine: Acceptance Criteria for Confirmation of Identity of Chemical Residues using Exact Mass Data for the FDA Foods and Veterinary Medicine Program (September 2015).

2) Codex Alimentarius, International Food Standards: Guidelines on Performance Criteria for Methods of Analysis for the Determination of Pesticide Residues in Food and Feed (2017).

Required Training, Certification and Re-certification

1) Receive in-house LC-HRMS training.

2) Initial certification is achieved by running a sequence which includes a QC and 3 samples that contain known ingredients and the analyst is able to interpret the data and report results with precision and accuracy in agreement with the validation package.

3) Periodically, chemists are retested for competency (e.g. every 5 years).

Revision History

Rev. 1 – initial revision

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Compound Database:

Ingredient	Molecular Formula	Retention Time (min)	Precursor (m/z)	Product 1 (m/z)	Product 2 (m/z)	Product 3 (m/z)	Product 4 (m/z)
(Positive Ions)							
Caffeine	$C_8H_{10}N_4O_2$	3.27	195.08765	138.06619	110.07127	83.06037	135.04271
Cinchonine	$C_{19}H_{22}N_2O$	3.56	295.18049	277.16993	130.06513	168.08078	81.06988
Vanillin	$C_8H_8O_3$	3.66	153.05462	111.04406	125.05971	93.03349	65.03858
Cinchonidine	$C_{19}H_{22}N_2O$	3.76	295.18049	277.16993	130.06513	168.08078	81.06988
Quinidine	$C_{20}H_{24}N_2O_2$	3.86	325.19105	160.07569	307.18049	172.07569	81.06988
Aspartame	$C_{14}H_{18}N_2O_5$	3.87	295.12885	120.08078	180.10191	235.10772	88.0393
Acetovanillone	$C_9H_{10}O_3$	3.99	167.07027	109.06479	125.05971	89.05971	-
Quinine	$C_{20}H_{24}N_2O_2$	4.11	325.19105	160.07569	307.18049	172.07569	81.06988
Coumarin	$C_9H_6O_2$	4.57	147.04406	103.05423	91.05423	77.03858	53.03858
Piperonal	$C_8H_6O_3$	4.59	151.03897	111.00441	123.0441	89.05968	107.08559
BHA	$C_{11}H_{16}O_2$	5.85	181.12231	135.11691	163.11177	121.10121	107.08559
Asarone	$C_{12}H_{16}O_3$	6.87	209.11722	194.09375	181.08592	179.07027	151.07536
BHT	$C_{15}H_{24}O$	7.65	221.18999	161.09616	203.17955	88.97816	57.07014
(Negative Ions)							
Mannitol	$C_6H_{14}O_6$	0.73	181.07176	89.02442	71.01385	59.01385	101.02442
Sorbitol	$C_6H_{14}O_6$	0.73	181.07176	89.02442	71.01385	59.01385	101.02442
Shikimic Acid	$C_7H_{10}O_5$	0.79	173.04555	93.03459	73.0295	137.02442	111.04515
Malic Acid	$C_4H_6O_5$	0.80	133.01425	115.00368	71.01385	72.99312	82.02442
Fumaric Acid	$C_4H_4O_4$	0.98	115.00368	68.9982	71.01385	73.0295	59.01385
4-Hydroxybenzoic Acid	$C_7H_6O_3$	2.80	137.02442	93.03459	108.02168	65.03967	94.04241
4-Hydroxybenzaldehyde	$C_7H_6O_2$	3.25	121.02950	108.02168	94.04241	91.01894	95.01385
Benzoic Acid	$C_7H_6O_2$	3.26	121.02950	108.02184	93.03471	94.02998	91.01913
Vanillic Acid	$C_8H_8O_4$	3.26	167.03498	108.02168	152.01151	123.04515	-
Sucralose	$C_{12}H_{19}Cl_3O_8$	3.55	395.00727	165.42444	157.12222	59.01251	79.99095
Sucralose (+HCO_2H)	$C_{12}H_{19}Cl_3O_8$	3.55	441.01275	395.00727	359.02878	295.20532	220.68443
Ethyl Vanillin	$C_9H_{10}O_3$	4.40	165.05572	136.01659	108.02168	95.01385	81.03459
Methyl Paraben	$C_8H_8O_3$	4.66	151.04007	136.01659	95.01385	108.02168	131.01366
Propyl Paraben	$C_{10}H_{12}O_3$	6.21	179.07137	93.03459	136.01659	108.02168	95.01385
Stevioside	$C_{38}H_{60}O_{18}$	6.87	803.37069	641.3205	317.21133	255.55858	101.02461
Stevioside (+HCO_2H)	$C_{38}H_{60}O_{18}$	6.87	849.37617	803.37069	641.31708	317.21133	255.55858
Heptyl Paraben	$C_{14}H_{20}O_3$	8.03	235.13397	93.03459	136.01659	108.02168	95.01385

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