Vanillin and Ethyl Vanillin in Nonbeverage Products by Ultra High Performance Liquid Chromatography – Photodiode Array Detection (UHPLC-PDA)

Scope and Application

This method was developed to determine the concentration of chemicals found in liquid nonbeverage samples (vanilla extracts, flavors and other extracts): 4 hydroxybenzoic acid; vanillic acid; 4-hydroxybenzaldehyde; vanillin; ethyl vanillin; piperonal and coumarin. Piperonal, coumarin and ethyl vanillin are not commonly present in vanilla extracts but they were added to detect adulterated vanilla extracts. See **Table 1** for more information about the chemicals. The method was validated for all seven chemicals; however, only two are currently being evaluated under the scope of this method, and under the scope of the laboratory's ISO accreditation: vanillin and ethyl vanillin.

Regulatory Tolerances:

According to 27 CFR § 17.134: "The appropriate TTB officer has responsibility for determining whether products are fit or unfit for beverage purposes within the meaning of 26 U.S.C. 5131. This determination may be based either on the content and description of the ingredients as shown on TTB Form 5154.1, or on organoleptic examination". The intent of this method is to demonstrate fitness or unfitness based on a chemical verification of the information provided on TTB Form 5154.1.

Levels and Limitations

1. Vanilla Extracts
Vanilla beans are the fruit of the epiphytic orchids; Vanilla planifolia, and Vanilla tahitiensis. Vanilla extracts are made by grinding chopped vanilla beans and heating them in an ethanol/water solution. It is the glycosidase action on glycoside precursors present in the green vanilla beans that produce the flavorings of the vanilla extracts; among them are 4-hydroxybenzoic acid, vanillic acid, 4-hydroxybenzaldehyde, and vanillin. The concentration of these flavor chemicals are dependent on the proportions of bean and ethanol used. This is called the "fold" of the extract.

Historically, TTB has subjected 1 or 2 fold vanilla extracts to the organoleptic evaluation if they contain greater than 45% (by volume) alcohol. This is because the amount of alcohol is more than is necessary to extract all of the odorous and sapid materials from the vanilla beans" see TTB website: https://www.ttb.gov/scientific-services-division/drawback-tutorial.

Since the amount of chemicals present in the extracts is unknown, a dilution factor is not possible to calculate accurately. Since no estimation of the amount of flavor present is available, a starting dilution of 1/100 is recommended or a quick screen before quantitation (see "Dilution procedure for screening samples").

2. Liquid Nonbeverage Samples
This method will be applicable for the quantitation of the chemicals shown in **Table 1** for

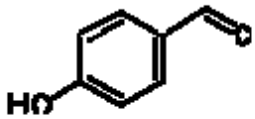
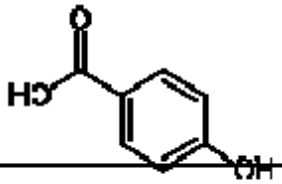
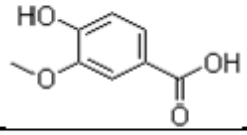
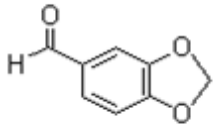
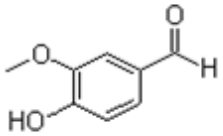
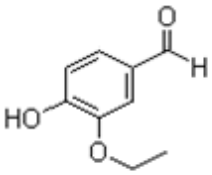
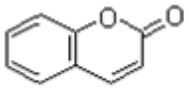
other nonbeverage samples besides vanilla extracts.

Important: it is possible that the 1/100 dilution is not the most appropriate. It is recommended that samples be screened first to determine the proper dilution factor. The sample should then be tested again so the concentrations of all chemicals fall within the linear range of the calibration curve.

Supplemental Documents

1. SSD:WG:314 Startup and Shutdown Procedures for the Acquity UPLC I-Class with PDA and QDa Detectors
2. SSD:WG:313 Flavor Compounds (Vanillin and Ethyl Vanillin) in Nonbeverage Products by UHPLC-PDA using the Acquity UPLC I-Class
3. SSD:Form:313a-c Standard Preparation Calculations for SSD:TM:313 (Spreadsheet)
4. SSD:WG:316 Vanillin Logbook System Information

Table 1: Chemicals used in this study

Name	*CAS #*	*MW*	*Structure*	*Formula*
4-hydroxybenzaldehyde	123-08-0	122.12		$C_7H_6O_2$
4-Hydroxybenzoic acid	99-96-7	138.12		$C_7H_6O_3$
Vanillic acid	121-34-6	168.15		$C_8H_8O_4$
Piperonal	120-57-0	150.13		$C_8H_6O_3$
Vanillin	121-33-5	152.15		$C_8H_8O_3$
Ethyl Vanillin	121-32-4	166.17		$C_9H_{10}O_3$
Coumarin	91-64-5	146.14		$C_9H_6O_2$

Equipment

Instrumentation and Run Conditions:

UHPLC Waters Acquity or equivalent

Run Time	3.50 minutes
Mobile Phase A	Water with 0.1% Formic Acid (FA)
Mobile Phase B	Acetonitrile with 0.1% FA
Weak Wash	90% Water/10% Acetonitrile
Strong Wash	100% Acetonitrile
Column Temperature	65°C
Autosampler Temperature	10°C
Injection Volume	5.0 µL
Weak Wash Volume	600 µL
Strong Wash Volume	200 µL
Seal Wash Time	5.00 min
Injection Type	PLNO

Table 2. UHPLC Conditions.

PDA Waters Acquity or equivalent

Sampling Rate	20 points/sec
Range	210-400 nm
Resolution (3D Channel)	1.2 nm
Data Mode	Absorbance at 273 nm
Resolution (Channel 1)	4.8 nm

Table 3. PDA Conditions.

Gradient

	Time (min)	Flow Rate (mL/min)	%A	%B	Curve
1	Initial	0.700	90.0	10.0	
2	0.50	0.700	90.0	10.0	6
3	1.65	0.700	70.0	30.0	6
4	1.75	0.700	65.0	35.0	6
5	1.80	0.700	62.0	38.0	6
6	1.90	0.700	60.0	40.0	6
7	2.80	0.700	5.0	95.0	6
8	3.00	0.700	90.0	10.0	6
9	3.50	0.700	90.0	10.0	11

Table 4. Gradient Conditions.

Guard and Column VanGuard BEH C18 1.7 µL 1.0 x 100 mm, or equivalent
Acquity UPLC BEH C18 1.7 µL 1.0 x 100 mm, or equivalent

Analytical balance with accuracy to 4 decimal places, Sartorius 1602 MP8-1, or equivalent

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

- 2 mL autosampler vials and split septa caps, Waters part numbers for vials 186000848, and caps 186000305, or LCMS certified 600000669CV
- Graduated cylinders or volumetric flasks, 100 mL, 500 mL, 1 L, or as needed for solution preparation
- Amber bottles with 1 oz (30 mL) and 2 oz (60 mL) capacity, 02991993 and 02991912, or as needed
- Micropipettes and tips, 1000 µL and 2-200 µL, Eppendorf part numbers 022492055 and 022492039, or as needed
- Filters, PTFE, 0.2 µm syringe filters or filter vials, Fisherbrand PN 09-720-002 or Restek PN 25893

Reagent and Sample Preparation and Handling

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

- Deionized (DI) water, 18.0 megaohm or better
- Ethyl Alcohol, 200 proof, Pharmco 111000200
- Acetonitrile, HPLC Grade, Fisher Scientific A996-4
- Formic Acid, reagent grade ≥ 95%, Sigma Aldrich F0507
- Compounds in **Table 1**, Sigma Aldrich, purity at least 97%

Solutions: (Specific quantities can be adjusted, as needed, as long as the resulting solutions retain the specified concentrations.)

1) Water with 0.1% Formic Acid (FA)

   Prepare water with 0.1% FA, for example:
   1. Pipet 500 µL FA into water in a 500 mL container (graduated cylinder or volumetric flask) containing water.
   2. Bring to volume with water and mix well.

   The shelf life of this solution is 2 weeks when stored at room temperature in a UHPLC mobile phase bottle.

2) Acetonitrile with 0.1% Formic Acid (FA)

   Prepare acetonitrile with 0.1% FA, for example:
   1. Pipet 500 µL FA into acetonitrile in a 500 mL container (graduated cylinder or volumetric flask) containing acetonitrile.
   2. Bring to volume with acetonitrile and mix well.

   The shelf life of this solution is 3 months when stored at room temperature in a UHPLC mobile phase bottle.

3) 90% Water/10% Acetonitrile

Prepare 90% Water/10% Acetonitrile, for example:

1. Measure 900 mL water and add to a bottle.
2. Measure 100 mL acetonitrile and add it to the same bottle.
3. Cap and shake to mix well.

The shelf life of this solution is 1 year when stored at room temperature in a UHPLC mobile phase bottle or other closed laboratory bottle (to be used as sample diluent).

Calibrants:

1) Individual-5% solutions wt/wt in 200 proof ethanol

Prepare 5% solutions of all chemicals of interest in ethanol, for example:

1. Weigh 1.0000 (± 0.0001) g of each compound of interest into individual amber bottles with a volume capacity of 1 oz (30 mL) and record the exact weight.
2. Add ~19.0 g of 200 proof ethanol to each bottle, and record exact weight.
3. Account for exact weights and purity to calculate exact concentration as ppm wt/wt.

The shelf life of these solutions is 3 years. They should be stored in amber bottles in a refrigerator (set at a maximum of 5°C).

Standard #	Analyte Name	Starting Material ID	Solvent ID	Amount of Ethanol (gr)	Final Mass (g)	Amount Chemical (g)	Conc (% wt/wt)
SB-NPL-11-23	Vanillin	2010-186	2011-098 (EtOH)	18.5069	19.4845	0.9776	5.0173
SB-NPL-11-24	Ethyl Vanillin	2010-185	2011-098 (EtOH)	18.8034	19.8549	1.0515	5.2959
SB-NPL-11-25	Coumarin	2010-003	2011-098 (EtOH)	18.9797	19.9409	0.9612	4.8202
SB-NPL-11-26	4-Hydroxy benzaldehyde	2011-004	2011-098 (EtOH)	18.7676	19.7819	1.0143	5.1274
SB-NPL-11-27	4-Hydroxy benzoic Acid	2009-185	2011-098 (EtOH)	18.8853	19.8913	1.006	5.0575
SB-NPL-11-28	Vanillic Acid	2009-188	2011-098 (EtOH)	19.0067	20.0143	1.0076	5.0344
SB-NPL-11-29	Piperonal	2009-186	2011-098 (EtOH)	18.8077	19.8095	1.0018	5.0572

Table 5. Example preparation of 5% wt/wt chemical solutions.

2) Stock solution, combined solution of 750 ppm of each chemical in 200 proof ethanol

   Once exact concentrations of all the 5% solutions are calculated, determine the amount needed for each compound in order to obtain a combined 750 ppm stock solution. For example:
   1. Deliver the grams needed of each 5% solution into a single amber bottle with a volume capacity of 50 mL, record each weight
   2. Add enough ethanol to obtain a final weight of approximately 750 ppm wt/wt of each chemical.

   The shelf life of the stock solution is 3 years. It should be stored in an amber bottle in a refrigerator (set at a maximum of 5°C).

Solution Identification Number	Name	Concentration (ppm) [Accounting for Purity]	Target Grams	Balance weight	Actual Grams	Calculated PPM
	Ethanol~10 mL			8.0085		
SB-NPL-11-023	Vanillin	50072.8682	0.7489	8.7620	0.7535	754.5015
SB-NPL-11-024	Ethyl Vanillin	52694.4230	0.7117	9.4754	0.7134	751.7478
SB-NPL-11-025	Coumarin	48202.4382	0.7780	10.2593	0.7839	755.6211
SB-NPL-11-026	4 Hydroxy benzaldehyde	49992.2909	0.7501	11.0341	0.7748	774.5814
SB-NPL-11-027	4-Hydroxy benzoic Acid	50322.0001	0.7452	11.7832	0.7491	753.8277
SB-NPL-11-028	Vanillic Acid	50293.6600	0.7456	12.5288	0.7456	749.8831
SB-NPL-11-029	Piperonal	50521.1237	0.7423	13.2766	0.7478	755.4972
		SUM Chemicals:	5.2217			
	Ethanol -Balance		36.7698			
			Total weight:	50.0064		
					Average Con	756.5228

Table 6. Example preparation of ~750 ppm wt/wt solutions in ethanol.

3) Working standards, 1, 10, 50, 75, 125 ppm in 90/10 DI water/acetonitrile

Prepare 5 working standards with the above concentrations using the 750 ppm stock and 90/10 DI water/acetonitrile solution. For example:

1. Weigh the amount of stock solution for each standard as shown in **Table 7**, and record the exact weight.
2. Add 90/10 DI water/acetonitrile solution to each standard as shown in **Table 7**, and record exact weight.

The shelf life of these solutions is 1 year. They should be stored in amber bottles in a refrigerator (set at a maximum of 5°C).

Solution Identification Number	Target PPM	Grams Required to Meet Target	Actual Grams Added†	Total Weight (g)	Calc. PPM
WS-NPL-11-045	0	0.0000	0.0000	20.0113	0
WS-NPL-11-046	1	0.0264	0.0264	20.0054	0.9983
WS-NPL-11-047	10	0.2644	0.2651	20.0002	10.0276
WS-NPL-11-049	50	1.3218	1.3066	20.0211	49.3715
WS-NPL-11-050	75	1.9828	1.9511	20.0211	73.7248
WS-NPL-11-052	125	3.3046	3.2540	20.0077	123.0389

Table 7. Example preparation of working standards in 90% Water/10% Acetonitrile

L0	L1	L2	L3	L4	L5	Chemical Name
0	1.00	10.00	49.24	73.53	122.71	Vanillin
0	0.99	9.96	49.06	73.26	122.26	Ethyl Vanillin
0	1.00	10.02	49.31	73.64	122.89	Coumarin
0	1.02	10.27	50.55	75.48	125.98	4 Hydroxy benzaldehyde
0	0.99	9.99	49.20	73.46	122.60	4-Hydroxy benzoic Acid
0	0.99	9.94	48.94	73.08	121.96	Vanillic Acid
0	1.00	10.01	49.30	73.62	122.87	Piperonal

Table 8. Example of actual concentrations of individual chemicals.

Preparation of Samples:

The samples should be diluted so the concentrations of all chemicals of interest fall within the linear range of this method. For example, a dilution of 1/100 with 90/10 DI water/acetonitrile:

1. Weigh 0.2 grams of the filtered extract into an amber bottle, and record exact weight.
2. Add 90/10 DI water/acetonitrile for a final weight of 20 grams.

   Analysis of these samples should be carried out right away after dilution, otherwise, store the solutions in a refrigerator (set at a maximum of 5°C).

Dilution procedure for screening samples. This is used to determine the appropriate dilution factor.

1. Determine the density of the sample.
2. Filter sample.
3. Pipet the necessary volume of filtered sample into the UPLC vial to obtain the desired dilution factor (see **Table 9**).

   If this procedure is followed, use the density to convert the concentration found to a weight basis. Since this procedure is faster, it could be used to find the approximate concentration of the chemicals in the samples without wasting solvents. **Table 9** shows the dilution factors obtained by pipetting different volumes of samples.

Extract Amount	MP	Total Volume	Dilution Factor
10	1190	1200	120
50	1150	1200	24
100	1100	1200	12
300	900	1200	4
500	700	1200	2.4

All volumes are in µL. MP = initial mobile phase (90/10 DI water/Acetonitrile).

Table 9. Dilution factors according to sample volume in UHPLC vial.

Preparation of Laboratory Control Sample (LCS):

The LCS is prepared by spiking a sample or determining a consensus value on a lab-prepared sample, as per lab policy.

Procedures

Analyze standards and unknowns in a UHPLC with the parameters listed in the Instrument and Run Conditions section above.

Quality Control

1. The LCS or a sample should be run in duplicate to test for precision (as per lab policy).

2. Determine the linearity of the curve. Data should not be reported unless the correlation coefficient is greater than 0.99.

3. After every 8th sample, re-run the middle standard (second source L3 check) as a control and check for drift. The concentration of L3 should be 50 ppm $\pm$ 10%, or between 45-55 ppm. If L3 is not within $\pm$ 10%, repeat the injections that were not bracketed by successful standard analyses.

4. If one of the control samples (LCS or L3 check) falls outside the accuracy range, then re-prepare the one that failed and re-run the sequence. If all quality data of the re-run passes, then report the data and initiate a CAR (will be tracked as an incident report). If any of the quality data of the re-run fails, then tag the instrument out of service and initiate a CAR.

Sources of Uncertainty

1. Use of incorrect filter material and/or not discarding the first ~1/2 mL of filtered sample.

2. Preparation of sample dilutions (pipet usage, etc.).

3. Preparation of standards and calibrants (balance usage, etc.).

Calculations

Instrument software can be used to generate linear equations using the absorbance of the standard solutions. Calculations can also be done using Excel software or in a Laboratory Information Management System (LIMS).

Reporting Results

Results are reported in ppm to the nearest whole number (XX).

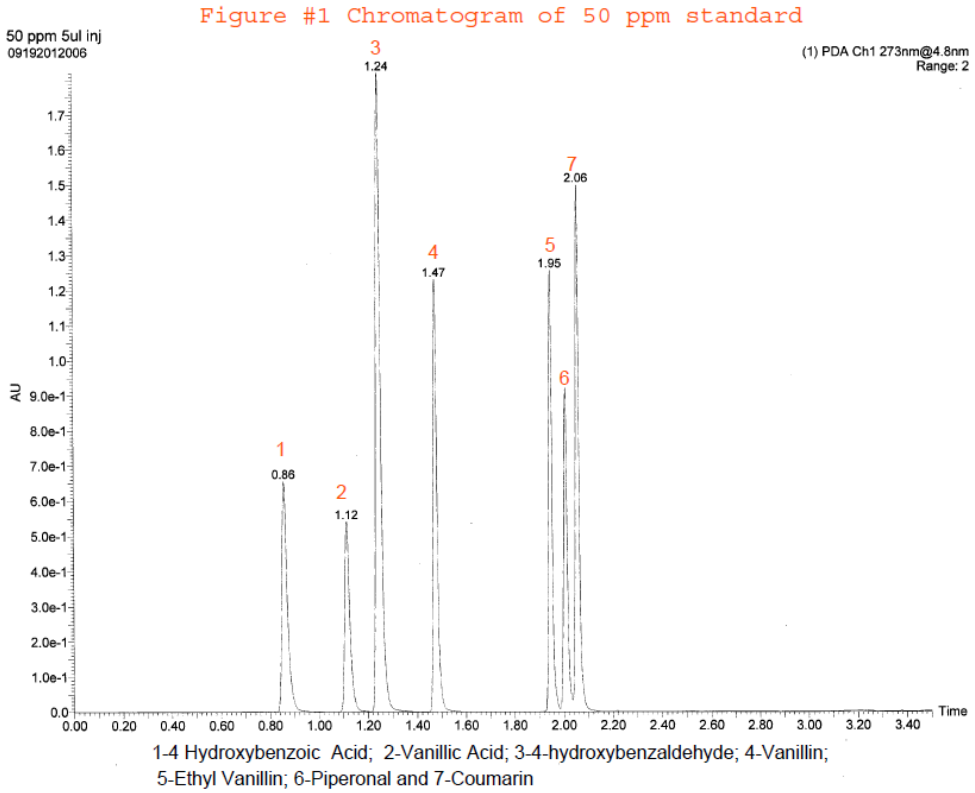


Figure 1. Chromatogram of 50 ppm standard.

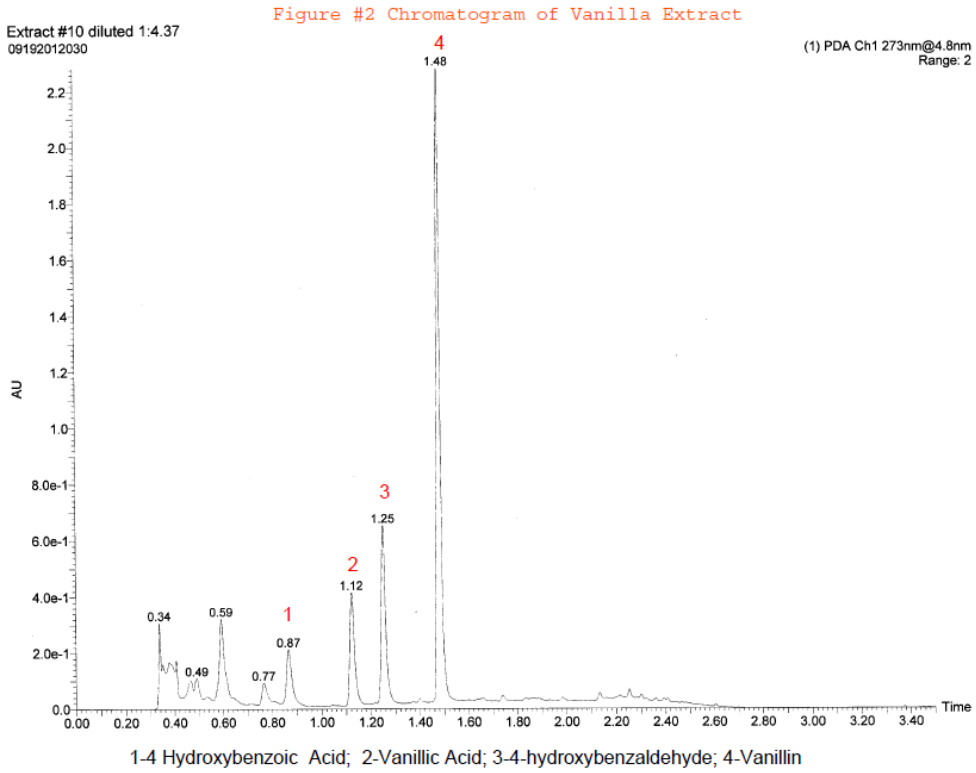


Figure 2. Chromatogram of Vanilla Extract.

Safety Notes

Normal laboratory safety protocol should be followed. Personnel should follow good laboratory practices such as wearing protective eye wear, gloves, and a lab coat.

Consult the MSDS for any chemicals used that are unfamiliar. All chemicals shall be considered hazardous - avoid direct physical contact.

High proof alcohol products are flammable. Ethanol burns with an almost invisible blue flame.

References

1. Code of Federal Regulations (2007) Title 27. U.S. Government Printing Office, Washington, DC 20404-001

2. AOAC Official Method 990.25. Vanillin, Vanillic Acid, p-Hydroxybenzaldehyde, p-Hydroxybenzoic Acid, and Ethyl Vanillin in Vanilla Extract and Artificial Vanilla Flavor Liquid Chromatographic Method. Scalese, J. M. Ed. Flavors. In AOAC Official Methods of Analysis Vol. 2; Horwitz, W.; AOAC International; Gaithersburg, MD, 2000; Chapter 36, pp 2-4.

3. Flavor Unfitness Worksheet, SSD Nonbeverage Products Laboratory—The Drawback Tutorial. https://www.ttb.gov/scientific-services-division/drawback-tutorial (accessed November 27, 2019)

Location of Validation Package

Quality System Files

Required Training, Certification and Re-certification.

1. In-house training by a certified chemist in UHPLC operation.

2. Periodically, chemists are re-tested for competency (e.g., every 5 years). A successful proficiency test will qualify as re-testing for competency.

Revision History

Rev. 1 – initial revision

Rev. 2 – Minor formatting. Updated link to drawback tutorial, supplement documents, and part numbers for supplies. Added what to do if intermediate check and LCS fails.