Analysis of Vanilla Components and Their Common Adulterants in Liquid Nonbeverage Samples by UHPLC-PDA-QDa

Scope and Application

This method was validated to detect 13 chemicals, 10 of which can also be quantified in liquid nonbeverage samples (vanilla extracts, flavors, and other extracts), see **Table 1a** and **Table 1b** below. However, only six chemicals will be evaluated under the scope of the laboratory's ISO accreditation: vanillin, ethyl vanillin, maltol, and ethyl maltol, guaiacol, and eugenol.

Vanilla beans are the fruit of 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, vanilla acid, 4-hydroxybenzaldehyde, and vanillin. The concentrations of these flavor chemicals are dependent on the proportions of bean and ethanol used. This is called the "Fold" of the extract. See 21 CFR 169.175 for the standard of identity of a vanilla extract.

Historically, TTB subjects "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.

This method can be used for multiple purposes, one of which is to detect and determine the concentration of chemicals that are most abundant in vanilla extracts:

- 4-hydroxybenzoic acid
- Vanillic acid
- 4-hydroxybenzaldehyde
- Vanillin
- P-anisic acid (unique to Vanilla Tahitiensis or Tahiti Vanilla Extract)

The method can be used to determine the concentration of chemicals commonly found in liquid nonbeverage products so that the results can be used in the Flavor Unfitness Worksheet (FUW) rather than performing an organoleptic taste panel that is subjective. As of March 2025, these are the following chemicals in the FUW V2024.2:

- Vanillin (TTB limited ingredient if in artificial form)
- Ethyl vanillin (TTB limited ingredient if in artificial form)
- Maltol (TTB limited ingredient if in artificial form)
- Ethyl maltol (TTB limited ingredient if in artificial form)
- Guaiacol
- Eugenol

Also, this method can screen for adulteration of vanilla extracts by detecting chemicals that are not typically found in high abundance using liquid chromatography, but these chemicals can be added to imitate a vanilla extract flavor and aroma at a lower cost:

- Maltol
- Ethyl maltol
- Acetovanillone
- Guaiacol
- Ethyl vanillin
- Piperonal
- Coumarin
- Eugenol

Table 1a: Analytes for qualitative analysis by the method

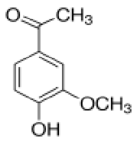
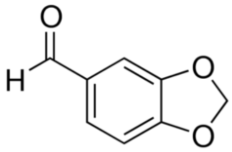
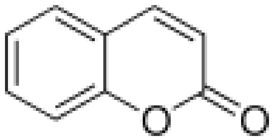
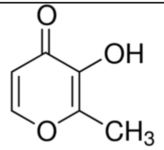
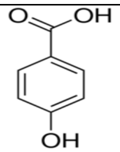
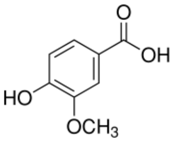
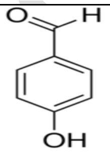
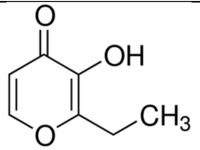
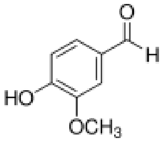
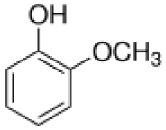
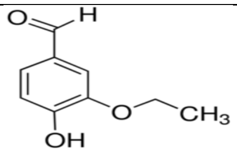
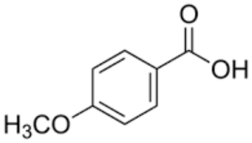
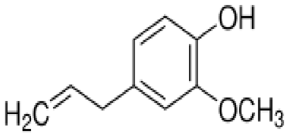
Analyte (Name, CAS #)	Molecular Weight and Formula	Structure
Acetovanillone 498-02-2	$C_9H_{10}O_3$ 166.17	
Piperonal 120-57-0	$C_8H_6O_3$ 150.13	
Coumarin 91-64-5	$C_9H_6O_2$ 146.14	

Table 1b: Analytes the method will quantitate.

Analyte (Name, CAS #)	Molecular Weight and Formula	Structure
Maltol 118-71-8	$C_6H_6O_3$ 126.11	
4-Hydroxybenzoic Acid 99-96-7	$C_7H_6O_3$ 138.12	
Vanillic Acid 121-34-6	$C_8H_8O_4$ 168.15	
4-Hydroxybenzaldehyde 123-08-0	$C_7H_6O_2$ 122.12	
Ethyl Maltol 4940-11-8	$C_7H_8O_3$ 140.14	
Vanillin 121-33-5	$C_8H_8O_3$ 152.15	
Guaiacol 90-05-1	$C_7H_8O_2$ 124.14	
Ethyl Vanillin 121-32-4	$C_9H_{10}O_3$ 166.17	
p-Anisic acid 100-09-4	$C_8H_8O_3$ 152.15	
Eugenol 97-53-0	$C_{10}H_{12}O_2$ 164.20	

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. 5111. 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.

27 CFR § 189.130 Coumarin:

(a) Coumarin is the chemical 1,2-benzopyrone, $C_9H_6O_2$. It is found in tonka beans and extract of tonka beans, among other natural sources, and is also synthesized. It has been used as a flavoring compound.

(b) Food containing any added coumarin as such or as a constituent of tonka beans or tonka extract is deemed to be adulterated under the act, based upon an order published in the Federal register of March 5, 1954 (19 FR 1239).

Levels and Limitations

This method is appropriate for liquid nonbeverage products that are highly concentrated and miscible in water; the solvent used for dilution is 90% water in acetonitrile. Samples should be diluted at least 1:4 to reduce matrix interferences and peak impurities in the chromatogram. It is recommended to start with a 1:100 dilution if quantity is unknown. Additional dilutions may be needed so that all chemicals fall within the linear range of the calibration.

There are compounds commonly found in vanilla extracts that co-elute with some chemicals in this method:

- P-anisic acid is unique to Vanilla Tahitiensis and co-elutes with coumarin, but in real vanilla extract samples, the presence of both p-anisic acid and coumarin should not occur.
- Maltol co-elutes with a naturally occurring compound found in vanilla extracts. Depending on the composition of the vanilla extract and concentration of maltol, this interference could impact the results.

The chemical guaiacol does not ionize in the QDa, but can be confirmed by the UV spectrum using the PDA detector.

The chemical eugenol has a noisy QDa spectrum, but can be confirmed by the UV spectrum using the PDA detector.

Chemical	Method Detection Limit (mg/kg)	Method Quantitation Limit (mg/kg)	Linear Range (mg/kg)
Maltol	0.05	10	1 – 50
4-Hydroxybenzoic Acid	0.05	10	0.1 – 50
Vanillic Acid	0.025	10	0.1 – 50
4-Hydroxybenzaldehyde	0.025	10	0.1 – 50
Ethyl Maltol	0.1	10	1 – 50
Vanillin	0.025	10	0.1 – 50
Acetovanillone	0.05	N/A	N/A
Guaiacol	0.25	10	0.1 – 50
Ethyl Vanillin	0.025	10	0.1 – 50
Piperonal	0.05	N/A	N/A
Coumarin	0.05	N/A	N/A
p-Anisic Acid	0.05	10	0.1 – 50
Eugenol	0.5	10	1 – 50

Note: Above concentrations are for the PDA detector.

Supplemental Documents

1. Form:SSD:313:001 Standard Preparation Calculations for SSD:TM:313
2. WG:SSD:313:001 Working Guideline for SSD:TM:313 (Formerly SSD:WG:313)
3. WG:LC:001 Waters Acquity I-Class System Maintenance Plan
4. WG:LC:002 Startup and Shutdown Procedures for the Acquity UPLC I-Class and I-Class Plus

Equipment

Note: Specific vendors, manufacturers, models, and product numbers listed in this Equipment section are for convenience. Equivalent products may be used.

Analytical balance (Sartorius, 1602 MP8-1) with accuracy to ± 0.1 mg
Microcentrifuge

Glassware and Supplies:

500 mL or 1 L solvent bottles (use amber bottle for aqueous mobile phase)
0.5 oz (15 mL) or 1 oz (30 mL) Amber Boston Round Bottles with Black Phenolic PolyCone Cap (GLC-04927 or GLC-01898)
15 mL or 50 mL conical centrifuge tubes with screw cap closure, if needed
Disposable Pasteur pipets, borosilicate glass (PN: 13-678-20C) and bulb
Micropipettes and disposable tips
Filters, PTFE, 0.22 µm or smaller syringe filters or filter vials (Fisherbrand PN 09-720-002 or Restek PN 25893)

Disposable luer-lock syringes
2 mL conical microcentrifuge tubes with snap cap closure, if needed
2 mL autosampler vials and pre-split septa caps (Waters vial PN: 186000848, Waters cap PN 186000305, or Waters LCMS certified PN 600000669CV)
Graduated cylinders
Class A volumetric flasks, if needed

Instrumentation:

Waters Acquity I Class UHPLC system equipped with:
  Waters Acquity CH-A (column heater with active pre-heater)
  Waters Acquity UPLC Sample Manager - FTN
  Waters Acquity UPLC Binary Solvent Manager
  Waters Acquity UPLC PDA Detector
  Waters Acquity UPLC Qda Detector

LC Run Conditions:

Column:	Acquity UPLC BEH C18 1.7 µm 2.1 x 100 mm Column (PN: 186002352)			
Guard Column:	VanGuard Acquity UPLC BEH C18 1.7 µm 2.1 x 5 mm Pre-Column (PN: 186003975)			
Column Temperature:	40 ºC			
Autosampler Temperature:	10 ºC			
Injection Volume:	2 µL			
Seal Wash:	At least 10% organic (e.g. methanol) in DI Water			
Seal Wash Time:	1 minute			
Sample Manager Purge:	10% Acetonitrile in DI Water			
Mobile Phases:	A: 0.1% v/v Formic Acid in DI Water B: 0.1% v/v Formic Acid in Acetonitrile			
Flow Rate:	0.4 mL/min			
Total Run Time:	10 minutes			
Gradient:	Time (min)	% A	% B	Curve
	0.00 (initial)	90	10	6
	1.25	90	10	6
	3.75	80	20	6
	5.00	80	20	6
	6.00	30	70	6
	6.10	0	100	6
	6.80	0	100	6
	7.00	90	10	6
	10.00	90	10	11
Software:	Empower 3 or equivalent			

PDA Method Parameters:

PDA Sample Rate:	20 points/sec
PDA Range:	210 – 400 nm
PDA Resolution (3D Channel):	1.2 nm
PDA Data Mode:	Absorbance at 273 nm
PDA Resolution (Channel 1):	4.8 nm

The expected PDA spectrums from 210 – 400 nm are shown in **Figure 2** for reference.

QDa Method Parameters:

The Waters QDa detector uses electrospray (ESI) and has limited settings that can be changed compared to other mass detectors:

Source Settings:	Probe Temperature: 600 °C Capillary Voltage: 0.8 kV for both Pos and Neg					
MS Scan Parameters:	Polarity	Cone Voltage	Start (min)	Stop (min)	Start Mass (m/z)	End Mass (m/z)
	Positive	50	1.10	1.70	40	130
	Negative	19	1.50	2.10	80	140
	Positive	25	2.00	5.60	70	170
	Positive	33	2.80	5.60	85	170
	Positive	17	6.40	6.80	120	170
Other Settings:	Sample Rate: 8.0 points/sec					

Note: The Waters QDa detector can be used as an extra confirmation of a chemical due to the complex nature of nonbeverage products. Unknown samples should be compared to recent injections of standards. The expected mass spectrums according to the QDa parameters above are shown in **Figure 3** for reference.

Reagent and Sample Preparation and Handling

Reagents:

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

Deionized (DI) water, 18 megaohm or better
Ethyl Alcohol, 200 proof, Pharmco 111000200
Acetonitrile, HPLC Grade or Optima LC/MS grade, CAS No. 75-05-8:
  Fisher Chemical, HPLC Grade Catalog No. A998-4 or,
  Fisher Chemical, Optima LC/MS Grade Catalog No. A955-4
Formic acid, Optima LC/MS grade:
  Fisher Chemical, Optima LC/MS Grade Catalog No. A117-50
Chemicals in **Table 1a** and **Table 1b** above, purity ≥ 97.0% from Sigma Aldrich or TCI America

Preparation of Solutions:

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

1. Mobile Phase A: 0.1% formic acid in DI water; solution is stable for 2 weeks at room temperature.
   For example:
   a. In a 500 mL graduated cylinder, pipet 500 µL of formic acid into the graduated cylinder.
   b. Add and mix DI water and mix to the 500 mL mark.
   c. Transfer to a LC solvent bottle (amber glass is recommended) and cap.
2. Mobile Phase B: 0.1% formic acid acetonitrile; solution is stable for 3 months at room temperature.
   For example:
   a. In a 500 mL graduated cylinder, pipet 500 µL of formic acid into the graduated cylinder.
   b. Add and mix acetonitrile to the 500 mL mark.
   c. Transfer to a LC solvent bottle and cap.
3. 10% Acetonitrile (ACN) in DI Water; solution is stable for 1 year at room temperature. This can be used for standard preparation, samples preparation, or as seal wash on the Acquity UPLC.
   For example:
   a. In a 1000 mL graduated cylinder, add 100 mL of acetonitrile
   b. Add DI water to the graduated cylinder and mix until 1000 mL mark on the graduated cylinder.
   c. Transfer to a LC solvent bottle and cap.

Preparation of Standards:

Note: Weights and container sizes may be adjusted as needed;

1. Prepare individual 1% by weight solutions in 200 proof ethanol for each chemical of interest, adjusted for purity.
   For example, instructions below are for using Form:SSD:313:001 (Tab A). See **Table 2** below for reference. The chemical reagent purities were obtained from the supplier (e.g. Certificate of Analysis):
   a. Place an empty 0.5 oz (15 mL) amber bottle and its cap onto balance.
   b. Tare the balance.
   c. Weigh about 0.1000 grams of a chemical into bottle and record the exact weight.
   d. Without taring the balance, add 200 proof ethanol until the balance reads about 10.0000 grams, carefully place the cap on the bottle, and record the exact weight.
   e. Mix the 1% solution so that the chemical reagent is fully dissolved.
   f. Each 1% stock solution can be stored in a refrigerator (set at a maximum of 5°C) for 3 years, except for the chemicals below have a shelf life of 1 year:
      i. Maltol
      ii. Ethyl Maltol

            iii. Guaiacol
            iv. Eugenol

2. Prepare an averaged 200 ppm (mg/kg) combined stock solution by mixing individual 1% chemical solutions in 200 proof ethanol.
    a. Create a combined stock solution of qualitative chemicals (**Table 1a**). The shelf life is 3 years when stored in refrigerator (set at a maximum of 5°C).
    b. Create a combined stock solution of quantitative chemicals (**Table 1b)**. The shelf life is 1 year when stored in refrigerator (set at a maximum of 5°C).
    For example, instructions below are for using Form:SSD:313:001 (Tab B). See **Table 3** of quantitative chemicals for reference:
        i. Place an empty 1 oz (30 mL) amber bottle and its cap onto balance. Tare the balance.
        ii. Add about 5 – 10 grams of 200 proof ethanol to the bottle, cap to prevent evaporation, and record the weight (do not tare the balance).
        iii. For each starting chemical, add the needed Target 1% Solution Weight (g) to the bottle, cap to prevent evaporation, and record the weight (do not tare the balance between chemicals).
        iv. Once all the expected 1% chemical solutions are added, add 200 proof ethanol to meet total stock solution goal weight to obtain an average 200 ppm in chemicals. Cap and record the weight.
3. Prepare quantitative working standards (WS) levels in 10% by volume ACN in DI Water using 200 ppm (mg/kg) combined stock solution of quantitative chemicals to target concentrations of 0.1, 1, 5, 10, 20, 35, and 50 ppm. The shelf life is 1 year, and can be stored in refrigerator (set at a maximum of 5°C).
For example, instructions below are for using Form:SSD:313:001 (Tab C). See **Table 4** for reference:
    a. Place an amber bottle and cap onto the balance, and then tare the balance.
    b. Add Target Amount (g) of 200 ppm Stock Needed as specified in the column of worksheet, cap to prevent evaporation, then record the weight in column, Actual Amount of 200 ppm Stock Added. Depending on the exact weight, the Target Balance Weight (g) to meet Target ppm will change.
    c. Add 10% by volume ACN in DI Water to Target Balance Weight (g) to meet Target ppm as specified in the column, cap to prevent evaporation, then record the weight into Final Total Weight (g) of Solution. Mix thoroughly.
4. Prepare qualitative working standards (WS) levels in 10% by volume ACN in DI Water using 200 ppm (mg/kg) combined stock solution of qualitative chemicals to target about 10 ppm. The shelf life is 3 years when stored in refrigerator (set at a maximum of 5°C).
    a. For example, see above instructions for the quantitative chemicals using Form:SSD:313:001 (Tab D) .

Preparation of Laboratory Control Sample (LCS):

1. The LCS is prepared by spiking a sample or determining a consensus value on a lab-prepared sample, as per lab policy.
    a. Suggested storage of lab-prepared LCS is up to 1 year in refrigerator (set at a maximum of 5°C).
2. The LCS sample should be diluted to fall within the linear range of this method and above the Method Quantitation Limit as specified in the Levels and Limitations section.
3. Dilute LCS with 10% ACN in DI on a balance, noting the weights to determine the dilution factor. For example, see Preparation of Samples below.

Preparation of Samples:

All samples should be diluted in 10% ACN in DI water so the concentrations of all chemicals of interest fall within the linear range of this method and above the Method Quantitation Limit as specified in the Levels and Limitation section. For example:

1. If available, determine the expected concentration of chemicals of interest in the sample. If the expected concentration of sample is unknown, a screen may be performed, such as starting at a 1:100 dilution.
2. If sample contains visible particulates, you may filter the sample or centrifuge the sample before diluting. For example:
    a. Transfer about 1 mL of sample into a microcentrifuge tube, and cap.
    b. Centrifuge the necessary samples for 5 minutes at 14,800 rpm.
3. Dilute samples on a balance, noting the weights to determine the dilution factor. Ensure that after diluting, there is at least 2.5 mL of sample. For example, a 1:100 dilution:
    a. Place a container that can hold 15 mL of liquid on the balance, and tare.
    b. Transfer about 0.1 grams of sample into the container, and record the exact weight.
    c. Without taring the balance, add diluent (10% ACN in DI water) into the container on the balance until it reaches about 10 grams. Record the exact weight. Cap the container and mix.
    d. If needed, a secondary dilution may be performed.
    e. Filter the sample through a 0.22 µm or smaller PTFE filter and then into a LC autosampler vial, for example:
        i. Attach a PTFE 0.22 µm syringe filter to a disposable luer-lock syringe.
        ii. Add enough sample so that the liquid reaches the 2 mL mark or more.
        iii. Dispense through the first 1 mL of filtered sample to a waste.
        iv. Using the same syringe, dispense about 1 mL of sample to a LC autosampler vial, and cap.
        v. Repeat steps above for more samples.
4. If needed, samples may be reinjected the following day.

Procedures

The following procedures are provided as a guide.

1. Equilibrate the column according to the Run Conditions listed above prior to beginning injections.
2. Begin the sequence with a blank (the diluent used to prepare samples, 10% ACN in DI water). Followed by the working standards. Bracket after every 8th sample with an intermediate check with a concentration near the middle of the linear range. For example:

Blank
Working standards
Second source intermediate check
LCS preparation #1
LCS preparation #2
Sample #1
Sample #2
Sample #3
Sample #4
Sample #5
Sample #6
Second source intermediate check

Quality Control

1. Linearity of each chemical of interest should have $R^2 \geq 0.995$ and calibrant residuals (% deviation) should be within ± 10% of the expected concentration. One point from the calibration range can be removed as long as all the samples analyzed fall within the remaining calibrants. In case of linearity failure, determine the potential cause (e.g. instrument issues, system equilibration). Resolve the issue and re-run. If problems persists, consult a POC.
2. The LCS is prepared in duplicate and injected. The consensus value on a lab-prepared sample must have results within the specified tolerances established. If applicable, the spiked sample LCS must have a 2.0% or less relative standard deviation. In case of LCS failure, determine the potential cause (e.g. dilution error, instrument issues, system equilibration). Resolve the issue and re-run. If problems persist, consult a POC. Refer to laboratory policy for initiating a corrective action report (CAR).
3. An intermediate check that falls around the middle of the calibration range is run after every 8th sample. The results of the intermediate check should be within ± 10% of the expected concentration. In case of an intermediate check failure, refer to the above LCS to determine the potential cause, and re-run. If problem persists consult a POC. Refer to laboratory policy for initiating a corrective action report (CAR).
4. A blank may be run after a sample if matrix interference is suspected.

Sources of Uncertainty

1. Preparation of the standards: weighing error.

2. Preparation of sample dilutions: weighing error.
3. Not a complete homogenized liquid sample: contains particulates
4. Filtering sample: incorrect filter material or not discarding the first ~ 0.5 – 1 mL.
5. Interfering peaks in the chromatogram: coelution.

Calculations

Instrument software is used to generate a 1/x weighted linear calibration curve to calculate the concentrations in ppm (mg/kg).

The raw result from the instrument software is multiplied by the dilution factor to determine the concentration of the original sample.

Reporting Results

This method will use the spectra from the PDA and may use the spectra from the QDa to confirm the presence of a chemical by comparing the sample to the standards. At a minimum, the raw data should include a chromatogram with peak retention time(s), and the PDA spectrum of the sample and/or standards.

All qualitative results (see **Table 1a**) are reported as either “detected” or “not detected”. Note that added coumarin is a prohibited ingredient according to 27 CFR § 189.130; consult your supervisor if coumarin is detected.

All quantifiable results (see **Table 1b**) are reported to the nearest whole number (XXX) as a concentration of ppm (mg/kg).

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. Electronic Code of Federal Regulations, Title 27. https://www.ecfr.gov/ (accessed 3/25/2025).
2. Jagerdeo ET AL, “Liquid Chromatographic Determination of Vanillin and Related Aromatic Compounds,” Journal of AOAC INTERNATIONAL Vol. 83, No. 1, 2000.
3. Takahashi ET AL, “Simultaneous Analysis of Guaiacol and Vanillin in a Vanilla Extract by Using Ultra High-Performance Liquid Chromatography with Electrochemical Detection,” Biosci. Biotechnol. Biochem, 77 (3), 595-600, 2013.

4. L.S. de Jager ET AL “Determination of Coumarin, Vanillin, and Ethyl Vanillin in Vanilla Extract Products: Liquid Chromatography/Mass Spectrometry Method Development,” J. Chromatogr. A. 2007;1145:83–88.
5. Kinton, Vanessa R. Laboratory Method Validation for Flavor Compounds in Nonbeverage Products by UPLC-PDA.
6. Flavor Unfitness Worksheet, SSD Nonbeverage Products Laboratory – The Drawback Tutorial. https://www.ttb.gov/scientific-services-division/drawback-tutorial (accessed 3/25/2025).

Required Training, Certification and Re-certification

1. Receive in house UHPLC training.
2. Initial certification is achieved by running 7 LCS replicates with results of precision and accuracy in agreement with the results of the validation package.
3. Periodically, chemists are retested for competency (e.g., every 5 years) and/or given proficiency test.

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.

Rev. 3 – Method revalidation requires major edits to method. No highlighting will be used to identify changes or additions.

Figure 1a: Sample chromatogram of a ~10 ppm qualitative working standard with chemicals in **Table 1a** at 273 nm.

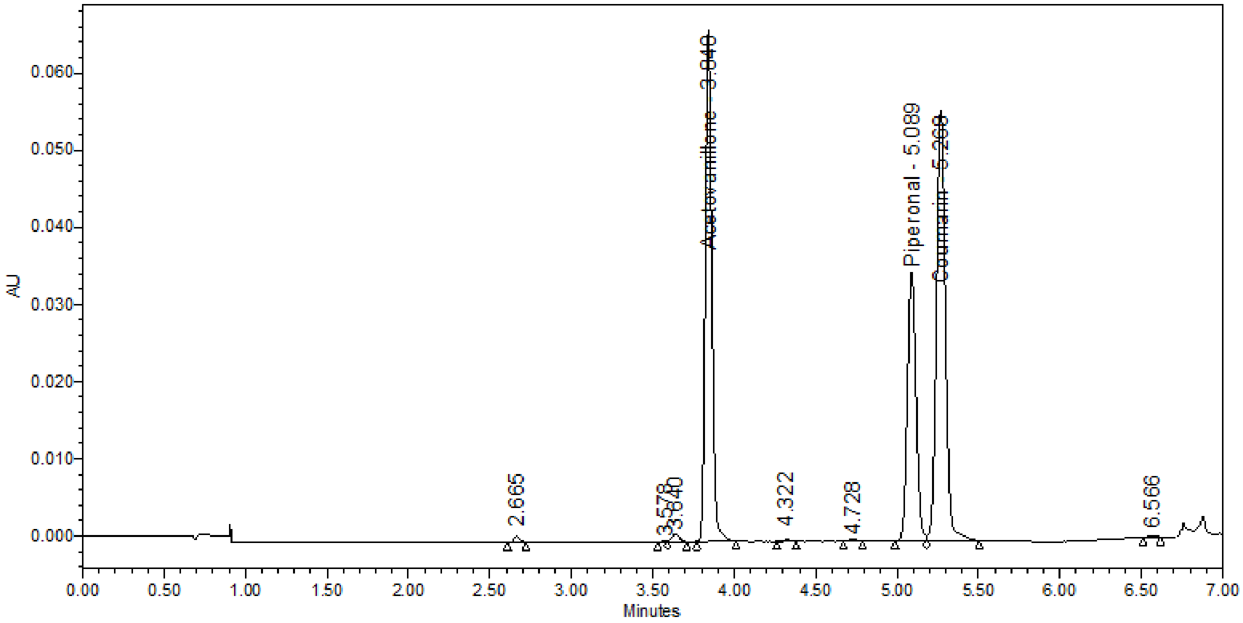


Figure 1b: Sample chromatogram of a ~10 ppm quantitative working standard with chemicals in **Table 1b** at 273 nm.

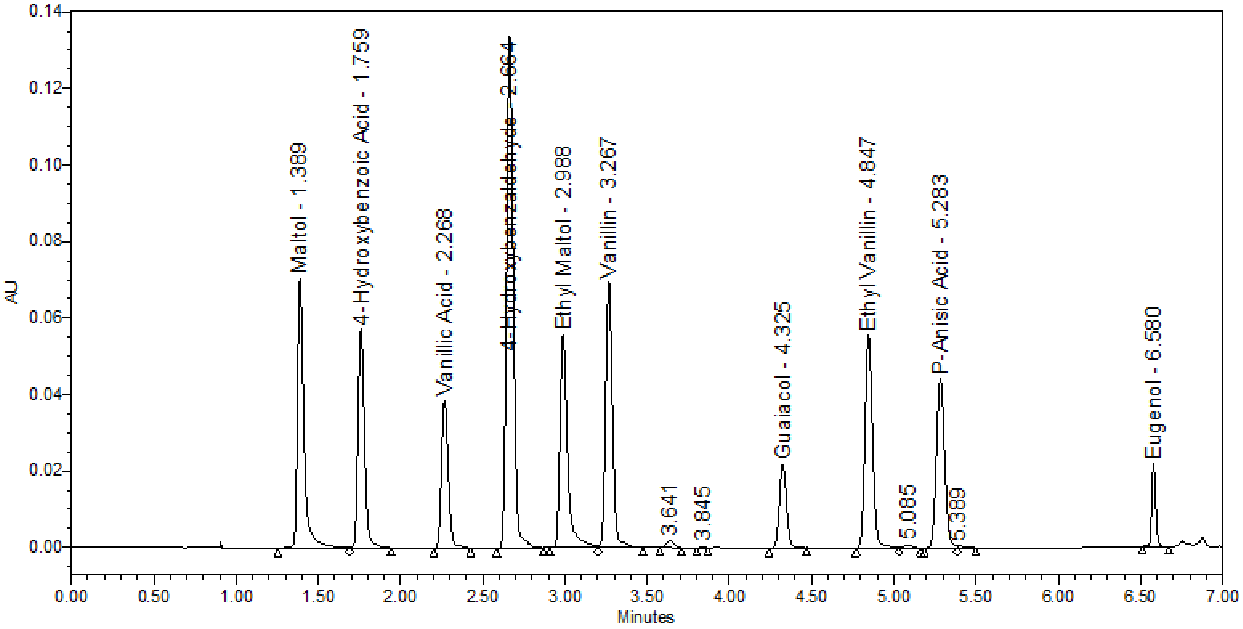


Figure 2: Sample UV spectrum from the PDA detector of all chemicals.

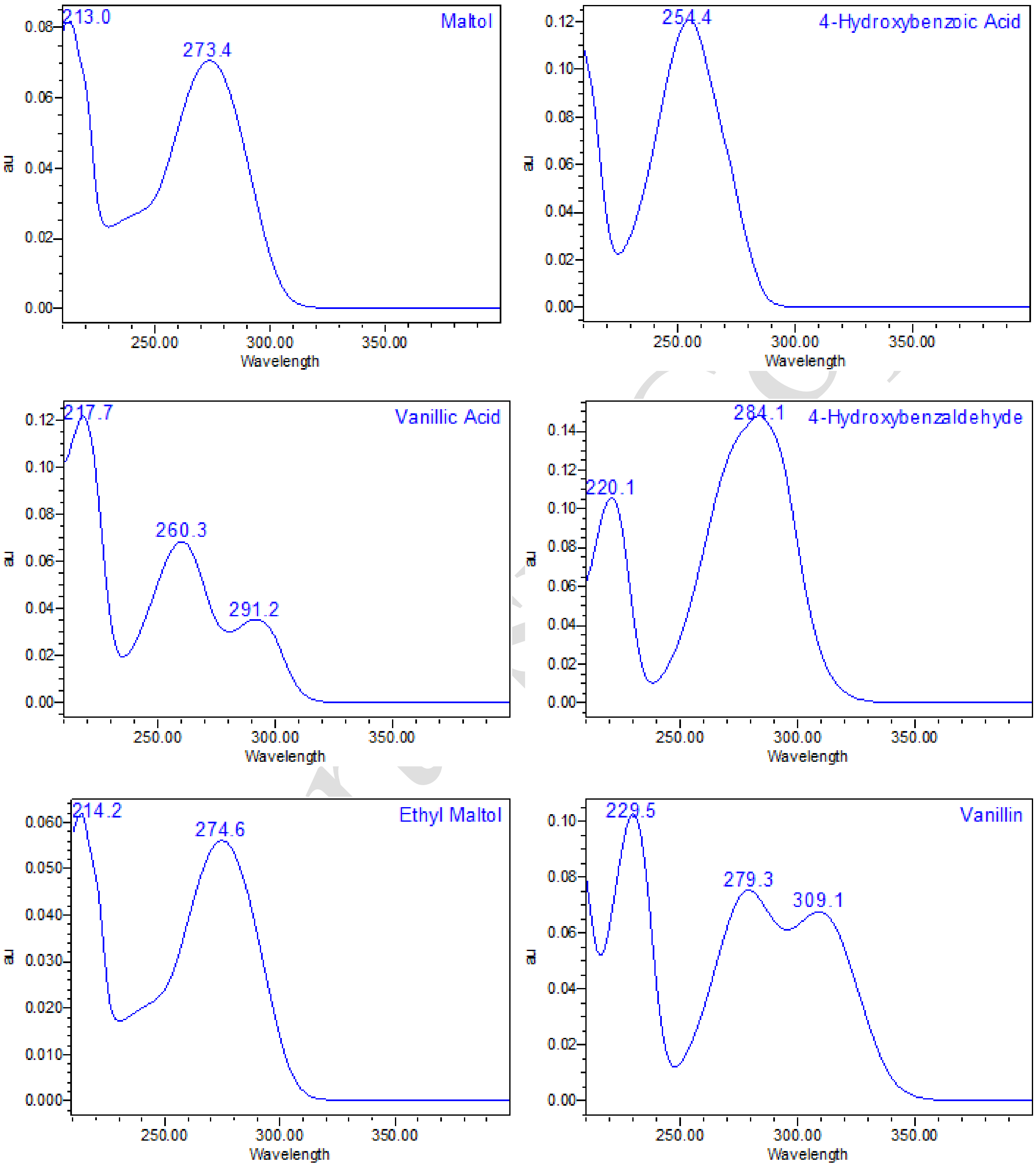


Figure 2 continued: Sample UV spectrum from the PDA detector of all chemicals.

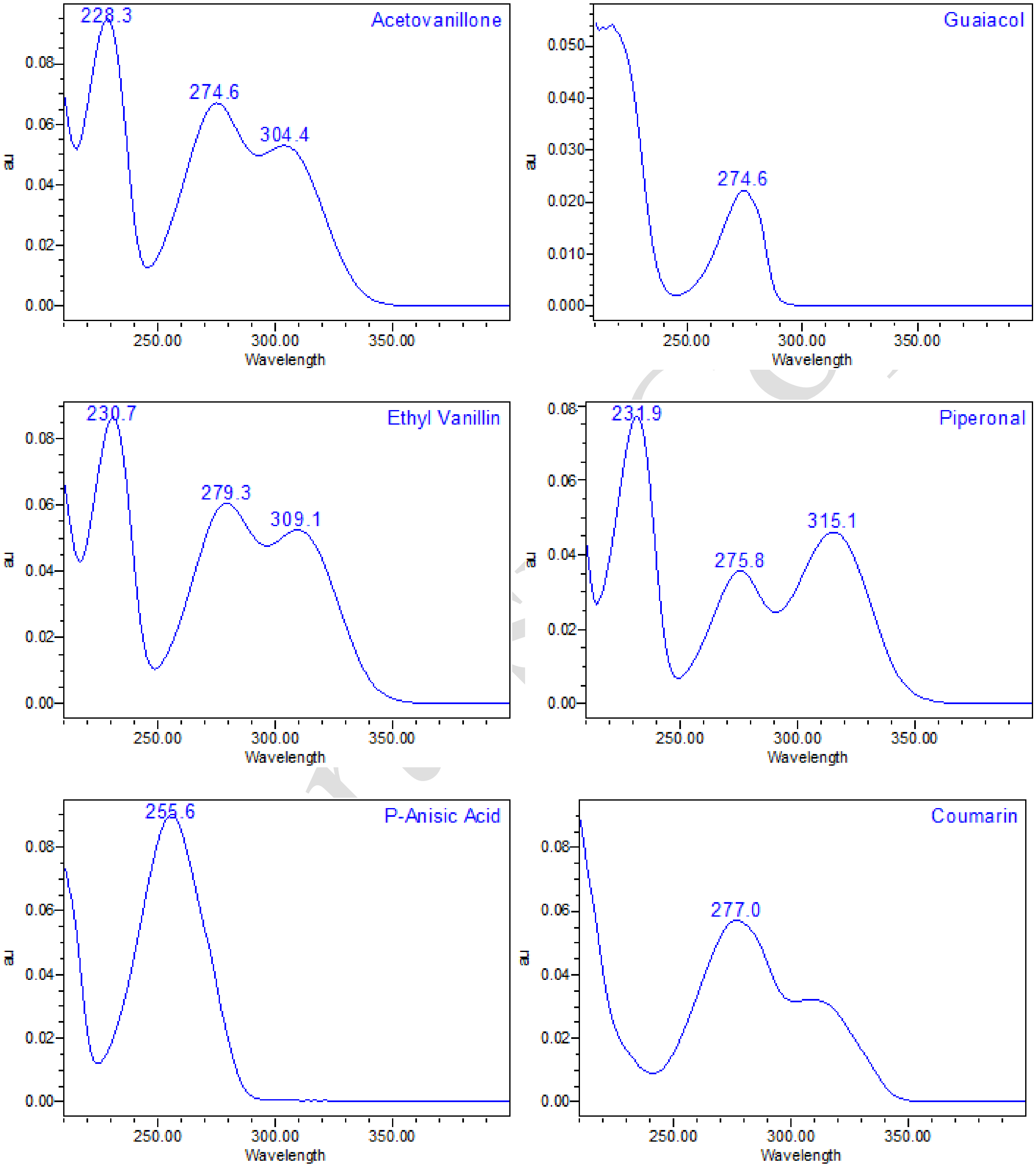


Figure 2 continued: Sample UV spectrum from the PDA detector of all chemicals.

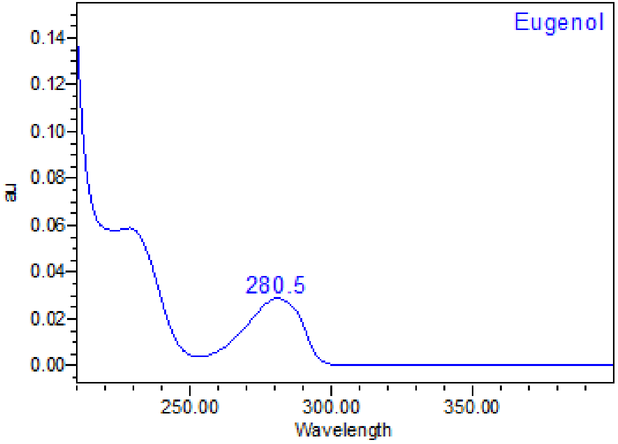


Figure 3: Sample mass spectrum from the QDa detector of all chemicals, minus guaiacol that does not ionize.

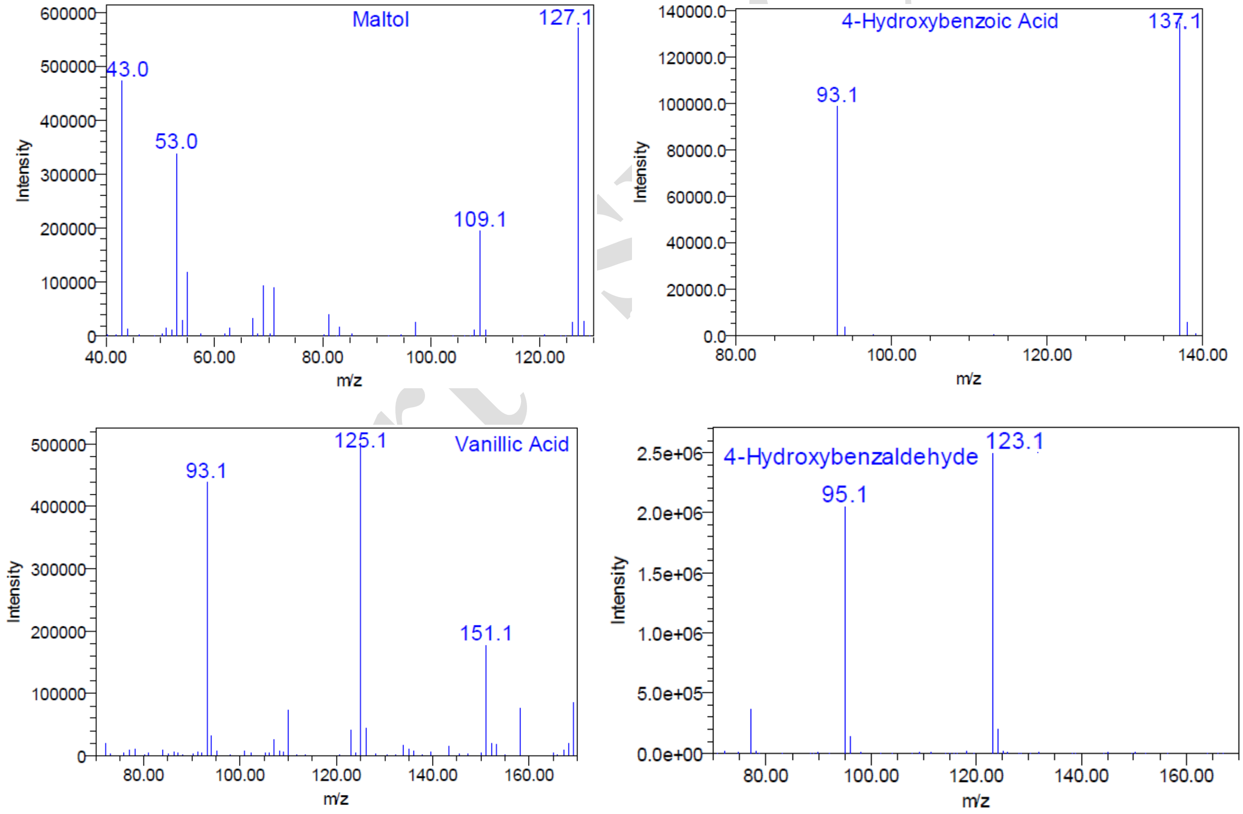


Figure 3 continued: Sample mass spectrum from the QDa detector of all chemicals, minus guaiacol that does not ionize.

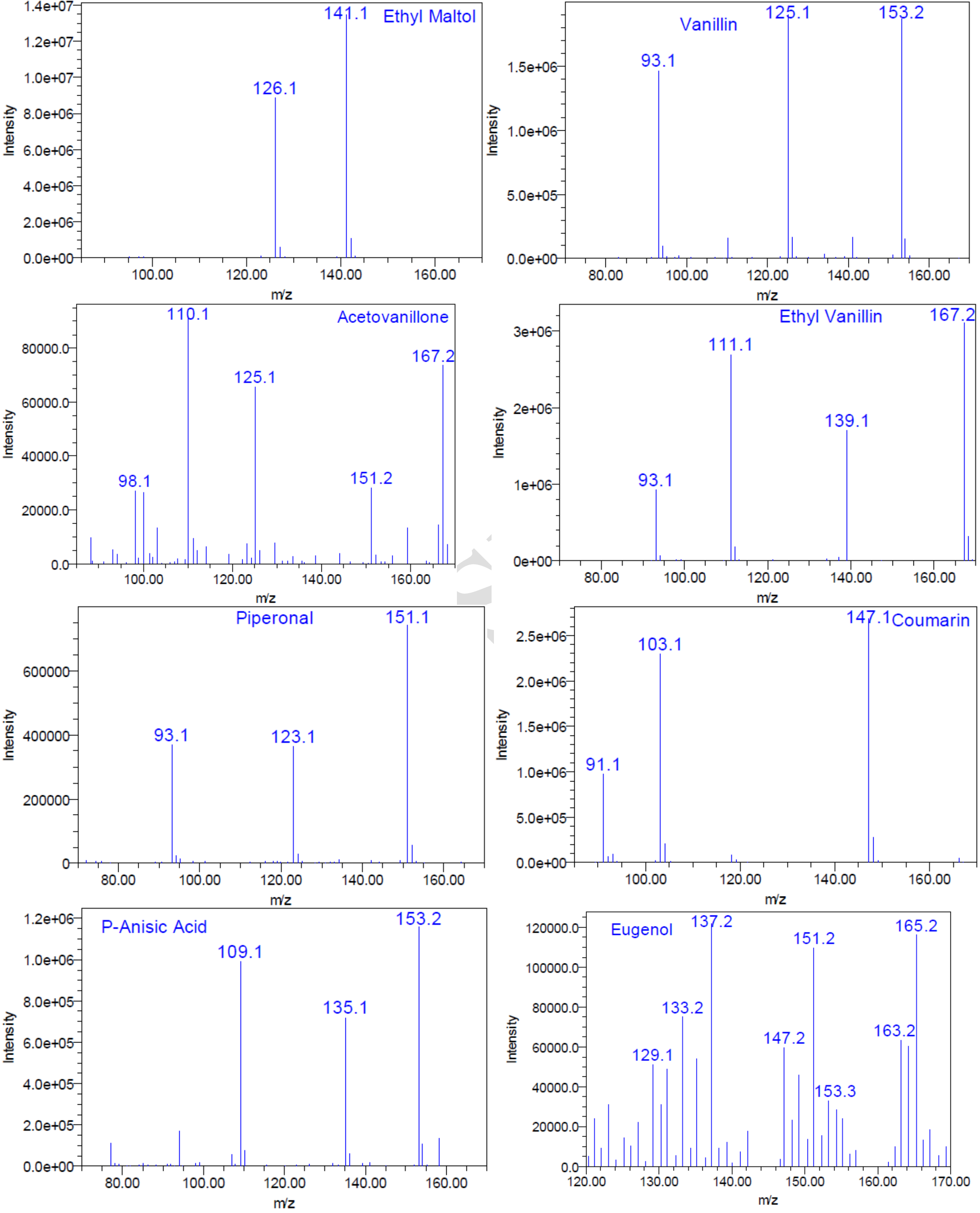


Table 2: Example preparation of 1% chemical solutions in 200 proof ethanol that accounts for purity. Note certain columns from Form:SSD:313:001 (Tab A) are excluded in example to fit.

Starting Chemical Name	Starting Chemical Purity (%)	Amount of Starting Chemical Added (g)	Actual Final Solution Weight (g)	Conc. ppm (mg/kg)	Conc. ppm (adjusted for purity)
Maltol	99.9	0.1283	12.6729	10124.0	10113.8
4-Hydroxybenzoic Acid	99.9	0.1303	12.7400	10227.6	10217.4
Vanillic Acid	99.3	0.1275	12.7368	10010.4	9940.3
4-Hydroxybenzaldehyde	99.9	0.1214	12.2621	9900.4	9890.5
Ethyl Maltol	99.9	0.1251	12.5295	9984.4	9974.5
Vanillin	100.0	0.1216	12.1881	9976.9	9976.9
Acetovanillone	98.0	0.1204	11.8088	10195.8	9991.9
Guaiacol	98.0	0.1299	12.7325	10202.2	9998.2
Ethyl Vanillin	99.9	0.1261	12.6338	9981.2	9971.2
Piperonal	99.8	0.1203	12.0089	10017.6	9997.5
Coumarin	100.0	0.1286	12.2752	10476.4	10476.4
p-Anisic Acid	99.0	0.1230	12.1771	10100.9	9999.9
Eugenol	99.1	0.1272	12.5241	10156.4	10065.0

Table 3: Example preparation of 200 ppm combined standard stock solution in 200 proof ethanol using information from **Table 2**. Note certain columns from Form:SSD:313:001 (Tab B) are excluded in example to fit.

Target 200 ppm Combined Stock Solution goal (g): 20.0000

Chemical Name	Starting Concentration (mg/kg) [Accounting for Purity]	Target 1% Solution (Starting) Weight (g) to add	Actual Amount (g) of Starting Material Added	Target Balance Weight (g) to meet 200 ppm	Final Balance Weight (g)	Calculated ppm (mg/kg)
200 Proof Ethanol	N/A	N/A	11.9310	N/A	11.9310	N/A
Maltol	10012.3	0.3995	0.4063	12.3305	12.3373	203.3808
4-Hydroxybenzoic Acid	9999.9	0.4000	0.3992	12.7373	12.7365	199.5791
Vanillic Acid	9989.2	0.4004	0.3995	13.1369	13.1360	199.5149
4-Hydroxybenzaldehyde	10000.5	0.4000	0.4098	13.5360	13.5458	204.8910
Ethyl Maltol	9982.3	0.4007	0.4021	13.9465	13.9479	200.6752
Vanillin	10014.1	0.3994	0.3987	14.3473	14.3466	199.6126
Guaiacol	9998.2	0.4001	0.4045	14.7467	14.7511	202.1943
Ethyl Vanillin	9953.0	0.4019	0.4065	15.1530	15.1576	202.2749
p-Anisic Acid	10102.0	0.3960	0.3957	15.5536	15.5533	199.8482
Eugenol	9954.4	0.4018	0.4028	15.9551	15.9561	200.4634
				QS with 200 Proof ETOH	20.0019	

Table 4: Example preparation of different levels of working standards (WS) in 10% by volume ACN in DI water using information from **Table 3**. Note certain columns from Form:SSD:313:001 (Tab C) are excluded in example to fit.

Target total amount of WS Solution Goal (g): 30.0000

Target PPM (mg/kg)	Target Amount (g) of 200 ppm Stock Needed	Actual Amount (g) of 200 ppm Stock added	Target Balance Weight (g) to Meet Target ppm	Final Total Weight (g) of Solution	Average Calculated ppm (mg/kg)
0.1	0.0149	0.0300	60.3990	50.5267	0.1195
1	0.1490	0.1570	31.6088	30.0345	1.0524
5	0.7450	0.7450	29.9982	30.0073	4.9985
10	1.4901	1.4997	30.1935	30.2085	9.9950
20	2.9802	2.9852	30.0505	30.1408	19.9401
35	5.2153	5.2384	30.1328	30.2444	34.8708
50	7.4505	7.4589	30.0340	30.2225	49.6882

Total Stock Needed (g): 18.0988

Table 5: Example expected working standard level concentrations (mg/kg) of each chemical for using information from **Table 2** through **Table 4**.

Chemical Name	**Calculated ppm (mg/kg)**	**L1**	**L2**	**L3**	**L4**	**L5**	**L6**	**L7**
		0.1	**1**	**5**	**10**	**20**	**35**	**50**
Maltol	203.3808	0.121	1.063	5.049	10.097	20.143	35.226	50.194
4-Hydroxybenzoic Acid	199.5791	0.118	1.043	4.955	9.908	19.767	34.568	49.256
Vanillic Acid	199.5149	0.118	1.043	4.953	9.905	19.760	34.556	49.240
4-Hydroxybenzaldehyde	204.8910	0.122	1.071	5.087	10.172	20.293	35.488	50.567
Ethyl Maltol	200.6752	0.119	1.049	4.982	9.963	19.875	34.757	49.527
Vanillin	199.6126	0.119	1.043	4.956	9.910	19.770	34.573	49.264
Guaiacol	202.1943	0.120	1.057	5.020	10.038	20.026	35.021	49.901
Ethyl Vanillin	202.2749	0.120	1.057	5.022	10.042	20.034	35.034	49.921
p-Anisic Acid	199.8482	0.119	1.045	4.962	9.921	19.793	34.614	49.322
Eugenol	200.4634	0.119	1.048	4.977	9.952	19.854	34.721	49.474