Analysis of Nonbeverage Products Using Liquid Chromatography

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Abstract

The Department of the Treasury's Alcohol and Tobacco Tax and Trade Bureau (TTB) is responsible for regulating the use of ethanol in products manufactured in the United States[1], and collecting revenue generated from such use. One area that falls within TTB's jurisdiction is the regulation of nonbeverage products. Nonbeverage products are medicines, medicinal preparations, food products, flavors, flavoring extracts, and perfumes which are manufactured using tax-paid distilled spirits, and which are unfit for beverage purposes. Besides collection of revenue, TTB also has the mission of protecting the public.

For certain nonbeverage products such as flavoring extracts, the determination of unfitness is not simple due to the unknown chemical composition of the samples. For example, the exact chemical composition of a vanilla extract will depend on the country of origin of beans used, the moisture of the beans and the extract fold. Also, in certain products the presence and amount of limited and/or prohibited ingredients is unknown. For example, a woodruff extract may contain Coumarin (prohibited ingredient) but the presence and exact concentration is unknown unless the extract is analyzed.

In this study Ultra Performance Liquid Chromatography (UPLC) coupled with a Photo Diode Array (PDA) and Time of Flight (TOF) detectors were used to examine nonbeverage samples. The versatility of having two detectors coupled to the UPLC is examined and presence and quantitation of some unknown compounds is explored.

Preparation of Standard Solutions

5% wt/wt individual solutions of 7 compounds were prepared in 200 Proof alcohol. The analytes are: 4 Hydroxybenzoic Acid; Vanillic Acid; 4-hydroxybenzaldehyde; Vanillin; Ethyl Vanillin; Piperonal and Coumarin. (see Table 1). A combined stock solution of the seven compounds was prepared at ~750 ppm for each compound. Using the 750 ppm stock solution, five working standards were prepared with concentrations of : 1, 10, 50, 75, 125 ppm; they were prepared in 90/10 Deionized Water/Acetonitrile (r^2 values are reported in Table 1). The solvent of the working standards was chosen to match the initial conditions of the gradient mobile phase. Figure 1 shows the chromatogram of a 50 ppm standard. This is a faster method than the method published by AOAC[2] for analysis of vanilla extracts.

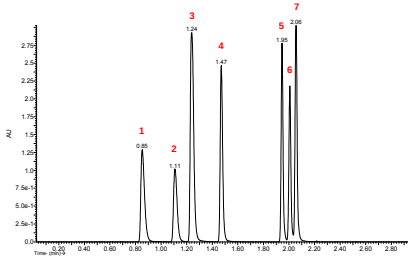


Figure 1. Chromatogram of 50 ppm standard at 273 nm
1) 4 Hydroxybenzoic Acid; **2)** Vanillic Acid; **3)** 4-hydroxybenzaldehyde; **4)** Vanillin; **5)** Ethyl Vanillin; **6)** Piperonal and **7)** Coumarin

Table 1 Compounds used in this study ǂ

Name	CAS #	RMF	Structure	Formula	r^2 (n=5)
4-hydroxybenzoic acid	123-08-0	172.12		$C_7H_6O_3$	0.999984
4-hydroxybenzaldehyde	99-76-7	138.12		$C_7H_6O_3$	0.999888
Vanillic acid	121-34-6	168.15		$C_8H_8O_4$	0.99957
Piperonal	120-57-0	150.13		$C_8H_6O_3$	0.999888
Vanillin	121-33-5	152.15		$C_8H_8O_3$	0.999954
Ethyl Vanillin	121-32-4	166.17		$C_9H_{10}O_3$	0.999954
Coumarin	91-64-5	146.14		$C_9H_6O_2$	0.999875

ǂ All chemicals purchased from Sigma Aldrich (St. Louis, MO). r^2 are for calibrations using PDA.

Table 2A- Experimental conditions-UPLC-PDA

Run Time	3.50 minutes
Mobile Phase A1	Deionized Water with 0.1% Formic Acid (FA)
Mobile Phase B1	Optima Grade Acetonitrile with 0.1% FA
Weak Wash	90% DI water/10% Optima Acetonitrile
Strong Wash	100% Optima Grade Acetonitrile
Column Type	Acquity UPLC® BEH C18 1.7uL 1.0 x 100mm
Column Temp.	65°C
Autosampler	10°C
Injection Volume	5.0 uL
PDA	273 nm
Strong Wash Vol	200 uL
Seal Wash Time	5.00 min
Injection Type	PLNO

Table 2B-Experimental conditions-Gradient

	Time (min)	Flow Rate	%A	%B	Curve
1	Initial	0.700	90.0	10.0	
2	0.50	0.700	90.0	10.0	6
3	1.00	0.700	70.0	30.0	6
4	1.75	0.700	65.0	35.0	6
5	1.80	0.700	10.0	90.0	6
6	2.30	0.700	10.0	90.0	6
7	2.80	0.700	1.0	99.0	6
8	3.00	0.700	90.0	10.0	6
9	3.50	0.700	90.0	10.0	11

Table 2C QTOF Conditions

Polarity	ES+
Capillary (kV)	3.3
Source Temperature (°C)	150
Desolvation Temperature (°C)	500
Detector Voltage (V)	2010
Lock Mass	Leucine Enkephalin

Filtering Experiments

Figure 1 shows that using a column with 1.7 um particle size results in fast analysis, for this application the runs are 3.5 minutes. Since the particle size of the column is very small, a standard procedure for samples preparation is to filter them. Three filters were tested: 1) PALL filter Gx/F/0.45 µm (P/N AP-4425) was recommended by PALL technical support due to the difficulty of filtering vanilla extracts, 2) Restek filter PTFE 0.45 µm (Restek P/N 26145), and 3) Puradisc 13 Syringe Filter, 0.45 µm, nylon. As seen in Figure 2 using PTFE filter provided the most accurate area counts. Similar reuslts were found for the other 6 compunds.

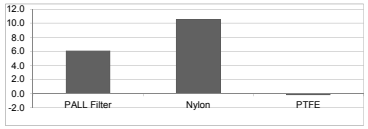


Figure 2. Percent difference in area counts for Piperonal peak between unfiltered and filtered standards using 3 types of filters

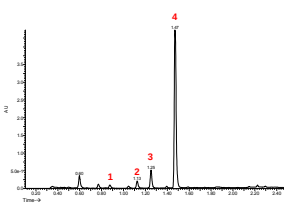
Vanilla Extracts

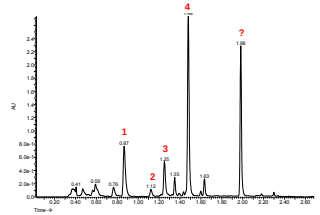
Twenty in-house vanilla extracts were prepared using beans from different countries of origin. These extracts were tested and the results are shown in Table 3. Extracts from Tahitian origin contained what originally was thought was high amounts of Piperonal, but after several spiking studies, it was concluded that none of these Tahitian extracts contained Piperonal. Using the Time-of-Flight detector, the unknown compound was determined to have a structure of $C_8H_8O_3$. Figure 5 indicates that the retention time and pattern fragmentation indicates that the unknown compound is *p*-Anisic Acid. All of the Tahitian extracts contained high amounts of p-Anisic Acid.

Table 3 Concentrations (ppm) found in in-house prepared extracts

Sample #	ID	Year Prepared	Fold	4 Hydroxybenzoic Acid	Vanillic Acid	4 Hydroxy-Benzaldehyde	Vanillin	Ethyl Vanillin	Piperonal	Coumarin
Extract #1	Indonesia*	2010	2	30	61	24	224	0	0	0
Extract #2	Bourbon	2010	2	59	112	102	1274	0	0	0
Extract #3	Madagascar	2010	2	83	168	86	1225	0	0	0
Extract #4	Madagascar	2010	2	59	188	97	1406	0	0	0
Extract #5	Madagascar	2010	2	58	109	123	1794	0	0	0
Extract #6	Bourbon	2010	2	66	149	162	2369	0	0	0
Extract #7	PNG	2010	2	354	60	87	736	0	0	0
Extract #8	Indonesia	1998	1	36	107	61	616	0	0	0
Extract #9	Tahitian	1998	1	411	31	84	298	0	0	0
Extract #10	Indonesia	1998	1	59	143	71	382	0	0	0
Extract #11	Madagascar	1998	1	35	98	80	1067	0	0	0
Extract #12	Indonesia	2010	2	65	203	89	682	0	0	0
Extract #13	PNG	2010	2	501	111	124	1257	0	0	0
Extract #14	Bourbon	2011	2	79	143	226	2363	0	0	0
Extract #15	Indonesia	2011	2	81	191	127	1915	0	0	0
Extract #16	India	2011	2	77	207	166	2683	0	0	0
Extract #17	PNG	2011	2	600	49	131	1138	0	0	0
Extract #18	Madagascar	2011	2	48	127	150	2072	0	0	0
Extract #19	Bourbon	2011	2	63	233	153	3220	0	0	0
Extract #20	Uganda	2011	2	61	159	161	2278	0	0	0

* This extract was prepared with old beans





1) 4 Hydroxybenzoic Acid; **2)** Vanillic Acid; **3)** 4-hydroxybenzaldehyde; **4)** Vanillin

Figure 3. Chromatogram Madagascar Extract **Figure 4.** Chromatogram Tahitian Extract

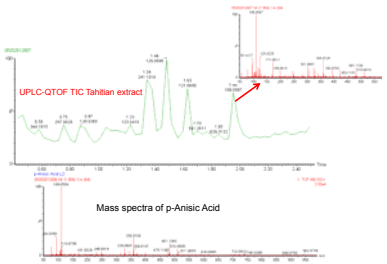


Figure 5. Identification of unknown compound using UPLC-QTOF

Flavors/ Flavoring Extract

Part of TTB's mission is to protect the public.In the sample below, a banned compound was found. Coumarin was found at 1748 ppm and according to 21 CFR 189.130 "*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)*".

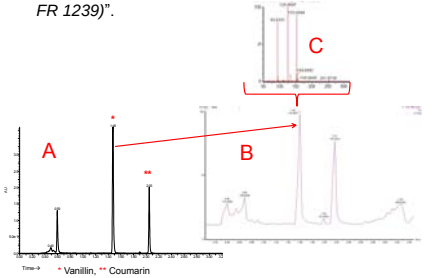


Figure 6 Chromatogram of "*vanilla essence*" A) UPLC-PDA; B) UPLC-QTOF ; C) Mass Spectra Vanillin

Dietary Supplement

Another type of samples analyzed by the Nonbeverage Products Laboratory include dietary supplements, Figure 7 shows the TIC of a wormwood extract. Only traces of coumarin were detected in this sample.

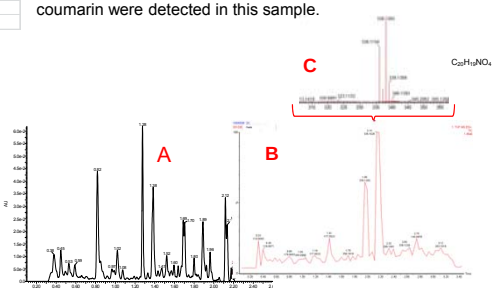


Figure 7 Chromatogram dietary supplement A) UPLC-PDA; B) UPLC-QTOF ; C) Mass Spectra $C_{20}H_{19}NO_4$

Conclusions and future work

- Excellent correlation coefficients were obtained with current method for all 7 compounds.
- This method has faster overall run time (3.50 min) than the AOAC method (20.04 min). Therefore, new method uses less mobile phase.
- This method is capable of detecting Piperonal. This compound was not present in any of the Tahitian type beans.
- Using a time-of-flight detector, all the Tahitian extracts were found to contain p-Anisic Acid.
- This method is suitable for analysis of vanilla extracts, flavoring substances and dietary supplements in which compounds from Table 1 could be present.
- Future work includes analysis of more extracts to build a database and comparison of concentrations against the Flavor Unfitness Worksheet.[3]

References

1. Code of Federal Regulations (2007). Title 21 Part 169. U.S. Government Printing Office, Washington, DC 20402-001
2. AOAC Official Method 990.25 Vanillin, Vanillic Acid, p-Hydroxybenzaldehyde, and 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. http://www.ttb.gov/ssd/drawbacktutorial.shtml#_Toc134863875 (accessed March 2013)