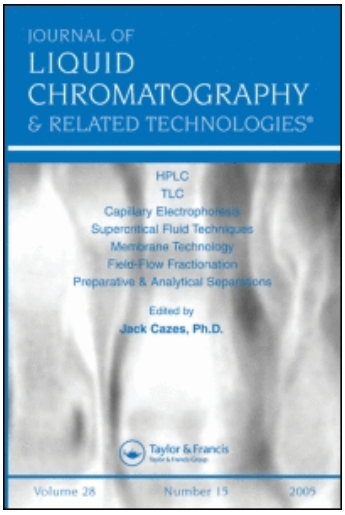

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Demian, Barbu A.(1993) 'Trace Analysis of Vanillin in Tobacco', Journal of Liquid Chromatography & Related Technologies, 16: 16, 3563 — 3574
To link to this Article: DOI: 10.1080/10826079308019709
URL: http://dx.doi.org/10.1080/10826079308019709

JOURNAL OF LIQUID CHROMATOGRAPHY, 16(16), 3563-3574 (1993)

TRACE ANALYSIS OF VANILLIN IN TOBACCO

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ABSTRACT

Vanillin is naturally occurring in a variety of products: vanilla, the primary source of natural vanillin, beer, grape brandy, roasted coffee, rum, strawberry, whiskey and tobacco. Vanillin, natural or synthetic, is frequently added to foods and beverages as an aroma chemical, and to tobacco products as well. The analytical method described allows quantitation of vanillin at ppm level in tobacco and tobacco products. The method is based on extraction, solid phase extraction clean-up, and high performance liquid chromatography with selective UV detection. The method was validated with respect to linearity, precision and recovery.

INTRODUCTION

Vanillin (3-methoxy-4-hydroxy-benzaldehyde, CAS 121-33-5, FEMA-GRAS 3107) is the "number one" aroma chemical, in diversity of application, and in tonnage [1]. Natural vanillin is extracted from vanilla pods. In smaller amounts, vanillin occurs naturally in a wide variety of products, among them beer, brandy and whiskey, roasted coffee, clove, lily, strawberry [2], and tobacco [3]. Synthetic vanillin, a high tonnage product, is almost a commodity chemical. Whether natural or synthetic, vanillin is

added as a flavorant to a large variety of foods and beverages, and to tobacco products.

The numerous methods described in the literature for vanillin determination are based on gas chromatography [3], liquid chromatography [4], thin layer chromatography [5], or UV-spectrophotometry [6]. E. Nesemann *et.al.* [7] developed a method specifically for tobacco and tobacco products, based on extraction, column chromatography clean-up, and packed column GC, but did not report any actual analytical data. J.N. Schumacher [3], using several extraction steps followed by GC and GC-MS, identified well over 100 compounds, including vanillin, in Maryland, Virginia and Burley tobaccos. Vanillin was found at concentrations from 0.3 to 0.7 μg/g. tobacco.

In the present paper, we describe a new analytical method for vanillin determination in tobacco and tobacco products.

MATERIALS

Equipment.

- BenchMate robotic sample preparation system (Zymark, Inc., Hopkinton, MA).
- HPLC system:
  - 3 Model 510 pumps (Waters, Milford, MA);
  - WISP 710 automatic injector (Waters, Milford, MA);
  - Model 481 variable wavelength UV-Vis detector (Waters, Milford, MA);
  - Model 990 diode array detector (Waters, Milford, MA);
  - Maxima 820 software for system control and data acquisition (Waters, Milford, MA).

Club 286 microcomputer (Everex Systems, Inc., Richmond, CA.).
Column heater (Jones Chromatography, Inc., Lakewood, CO).
Hypercarb 100 mm. x 4.6 mm. ID x 7 μm particle size column, and 1 cm.x 4 mm ID guard column (Keystone Scientific, Inc., Bellefonte, PA).

- DMS 100S/DS-15 UV-visible spectrophotometer (Varian, Inc., San Fernando, CA).
- RS1 software package (BBN, Inc., Cambridge, MA).
- PowerMate 386/20 microcomputer (NEC Corp., Boxborough, MA).

Supplies.

- Glass Fiber Acrodisc disposable filters (Gelman Sciences, Inc., Ann Arbor, MI).
- BondElut reverse phase C18 solid phase extraction cartridges, 3 cc. and 200 mg. packing (Varian, Inc., Sunnyvale, CA).

Chemicals and reagents.

- Sodium phosphate dibasic, certified ACS (Fisher Scientific Co., Inc., Pittsburgh, PA).
- Methanol, HPLC solvent (Burdick & Jackson, Inc., McGaw Park, IL).
- Methylene chloride, HPLC solvent (Burdick & Jackson, Inc., McGaw Park, IL).
- Tetra-propyl ammonium hydroxide, 1M in water (Aldrich Chem. Co., Inc., Milwaukee, WI).
- Sodium hydroxide, "Baker Analyzed" reagent (J.T. Baker Chem. Co., Phillipsburg, NJ).
- Hydrochloric acid, conc., certified ACS (Fisher Scientific Co., Inc., Pittsburgh, PA).
- Vanillin, 99% (Aldrich Chem. Co., Inc., Milwaukee, WI).

TABLE 1.

Solid Phase Extraction Procedure.

Step:	Action:
1	Filter 3 mL. sample into next tube.
2	Rinse filter holder with 2 mL. water.
3	Add 0.5 mL. HCl 1N to the second tube.
4	Vortex mix 20 sec. at speed 2.
5	Condition SPE cartridge with 3 mL. methanol.
6	Condition SPE cartridge with 3 mL. water.
7	Load 3 mL. sample onto cartridge.
8	Rinse with 1.5 mL. water.
9	Collect 1.5 mL. fraction using methanol/water 70/30 vol/vol .
10	END

Total time/sample: 19.6 min.

METHODS

Extraction.

Samples of 1-1.5 grams (0.1 mg. precision) of tobacco leaf or tobacco filler are extracted with 20 mL. of 0.05M disodium phosphate in water solution for 1 hr., in a 50 mL. Erlenmeyer flask, on wrist-action shaker. The suspension is filtered through qualitative filter paper. An aliquot of 4 mL. filtrate is transferred to a test tube for the solid phase extraction clean-up.

Solid phase extraction clean-up.

The solid phase extraction (SPE) is performed by the BenchMate robotic system. The steps of the procedure are listed in Table 1.

Chromatography.

The chromatographic parameters are listed in Table 2.

Data reduction.

For data reduction, an interactive RS1 procedure was written in RPL language. The main program, **VANILLIN**, can call two subroutines: **VAN_CAL** for calibration, and

TABLE 2.

Chromatographic Parameters.

Mobile phase.	A: 40 vol. methanol, 60 vol. water, 0.05N NaOH, 0.05N tetra-propyl ammonium hydroxide. B: methanol. C: methylene chloride.
Mobile phase program.	0-15 min.: 100% A. 15-20 min.: 0% B to 100% B. 20-25 min.: 100% B to 100% C 25-30 min.: 100% C. 30-35 min.: 100% C to 100% B. 35-40 min.: 100% B to 100% A 40-55 min.: 100% A. All gradients linear.
Flow rate.	1.6 mL/min.
Injection volume.	50 µL.
Column temperature.	50° C.
Detection.	UV, 352 nm.
Data acquisition time.	15 min.

PRTTABLE for report printing. The procedure is calling as input for the number of samples and the number of replications per sample, sample name, sample weight, SPE eluate weight, peak area, primary extract volume, and HCl 1N addition volume. The output is the vanillin concentration, Mean and SEM in ppm units for each sample.

RESULTS AND DISCUSSION

The analytical procedure described in the present paper is based on extraction, solid phase extraction clean-up, and HPLC quantitation. In each of these steps, a key

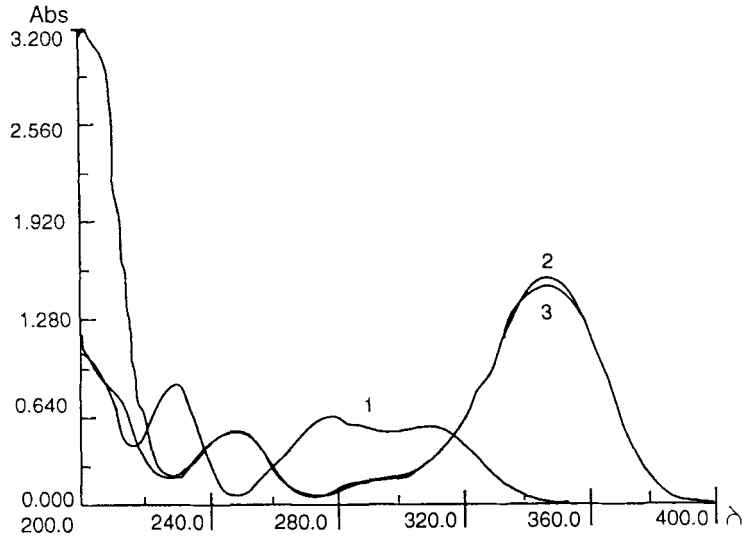

FIGURE 1. Vanillin UV spectra, C = 0.0452 mg/ml. **1:** HCl 0.1N aq. **2:** NaOH 0.1N aq. **3:** Na_2HPO_4 0.05M aq.

element is the control of the vanillin acid-base equilibrium:

$$HO(H_3CO)\text{-}C_6H_3\text{-}CHO + H_2O = {}^-O(H_3CO)\text{-}C_6H_3\text{-}CHO + H_3O^+ \qquad 1$$

The phenolic group of vanillin is relatively strong acidic, due to the inductive and mesomeric effects of the -CHO group in the para position. The pK of the phenolic group is 7.38 in water at 25° C [8]. The UV spectra of vanillin in acidic (1), basic (2), and 0.05M disodium phosphate aq. (3) solutions are presented in Fig.1. In disodium phosphate solution, vanillin is extracted from the tobacco matrix in its phenolate anion form, with a significantly higher water solubility, to ensure a complete extraction.

In the solid phase extraction clean-up stage, the pH is reverted to acidic, and vanillin is retained on and eluted from the reverse phase packing of the SPE cartridge

TABLE 3.

Statistics of SPE Final Extract Weight Data.

	n, number of samples	Mean	SEM, standard error of the mean	%CV, coefficient of variation
Within-day	8	1.2629	0.0030	0.72
Day-to-day	8	1.2647	0.0031	0.54
	8	1.2652	0.0055	1.06

as a phenol. In addition to the rather obvious advantage of automation, performing the clean-up with the BenchMate system is highly reproducible. The BenchMate system provides an audit trail of all the important parameters of the SPE procedure for each sample, i.e., filtrate weight, HCl addition weight, vortex loss, SPE load weight, and final extract weight. Statistics of representative final extract weight data are listed in Table 3. The one-way analysis of variance (ANOVA) of the data in Table 3 did not identify any significant day-to-day variation.

The column used in the chromatographic separation of the final extract, Hypercarb® has a graphitized carbon packing, developed by J. H. Knox and B. Kaur [9]. This packing, which behaves like a reverse phase type, has an important property from the standpoint of this application, namely it can withstand extreme pH values of the mobile phase. The mobile phase used has a 0.05N NaOH concentration, in which the vanillin will be again in its phenolate anion form. The long wavelength UV absorption band of vanillin, associated with the aromatic system, undergoes a major bathochromic and hyperchromic shift when the phenolic group is converted to phenolate anion due to the extension of the conjugated system. The detection at 352 nm., the λ_{max} of the phenolate anion, provides a significant gain in sensitivity. The 3D chromatogram of a sample extract, collected with the diode array detector, is

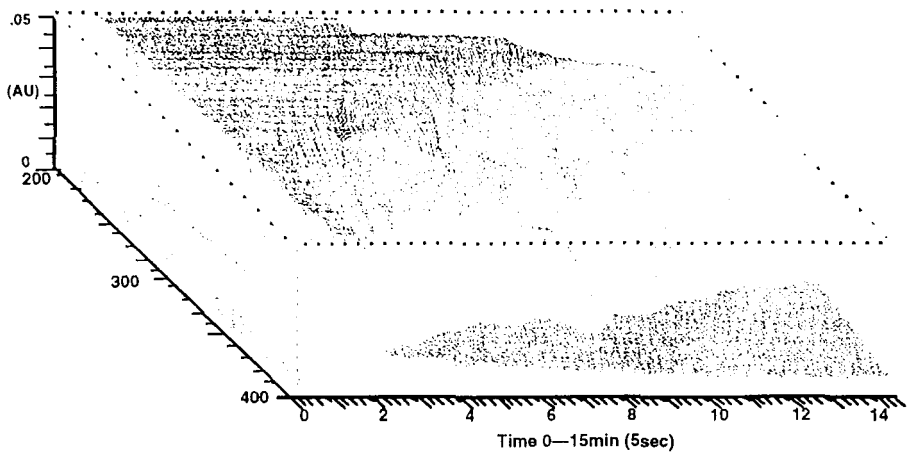

FIGURE 2. 3D chromatogram of an American blend cigarette filler extract.

presented in Fig.2. From the examination of Fig.2, it is evident that the 352 nm. detection provides also a second and more important advantage: selectivity.

In order to increase the retention of the vanillin phenolate anion on the stationary phase, tetra-propyl ammonium hydroxide is added to the mobile phase as an ion-pairing reagent. Vanillin phenolate anion is retained on and eluted from the column as an ion pair with $^{+}N(C_3H_7)_4$. The elution of ion-pairs is strongly dependent on the column temperature, and this is one more useful parameter to control the chromatography. At the 50° C column temperature, the vanillin peak elutes in a retention time window practically free of interference.

The diode array detector was used at the method development stage to check the vanillin peak purity. The UV spectra of a vanillin standard, eluting at 8.8 min. retention time, and of the corresponding peak in a sample run, are presented in Fig.3. The main features of the vanillin spectrum are recognizable in the sample spectrum. If the background (essentially featureless spectrum) just before the RT=8.8 min. peak is

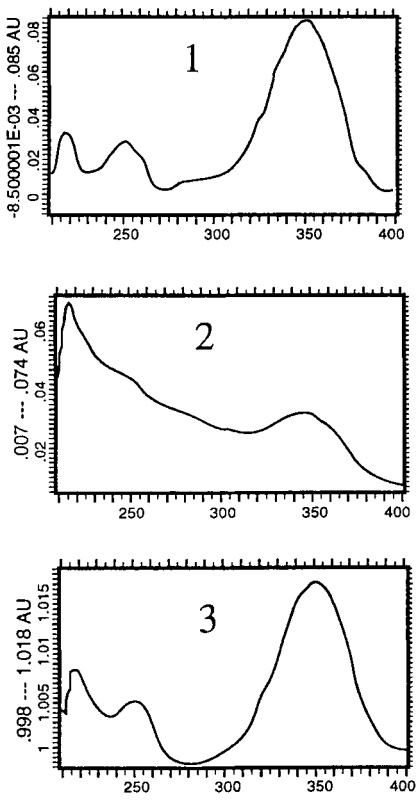

FIGURE 3. UV spectra extracted from DAD data matrix. **1:** Vanillin, standard. **2:** Vanillin, sample.**3:** Vanillin, sample, background subtracted.

TABLE 4.

Statistics of the Calibration Line.

Equation	C(μg/ml) = K x Area (a.u.)
K, slope	3.075×10^{-6}
Standard error of the slope, K	1.801×10^{-8}
F value	29,150
Significance level	0.0001
r^2, correlation coefficient squared	0.999828

TABLE 5.

Statistics of the Precision Test.

n, number of replications	8
Vanillin, ppm, MEAN	5.65
SEM, standard error of the mean	0.08
%CV, coefficient of variation	1.42
Significance level, sample-to-sample + extraction	0.028
Significance level, HPLC	0.638

TABLE 6.

Recovery Test.

Sample #	Vanillin addition, μg.	Vanillin found, μg.	Recovery, %
1	7.52	7.82	104.0
2	15.04	15.53	103.3
3	6.76	6.00	92.6
4	13.52	13.32	98.5
5	20.28	20.40	100.6
6	6.56	7.00	106.7
7	6.50	7.15	108.9
		MEAN:	102.1
		SEM:	2.1

subtracted, as presented in the third spectrum in Fig.3, the match with the vanillin standard spectrum is almost perfect, and confirms the peak purity.

The method was validated with respect to linearity, precision and recovery. The linearity was checked with vanillin in the concentration range from 0.9 to 4.4 μg/ml, triplicate standards in duplicate runs each. The statistics of the calibration line are listed in Table 4.

The precision of the method was tested by running four replications of an American blend cigarette filler, in duplicate HPLC runs each. This experimental setup will partition the total variance in one term associated with sample-to-sample and

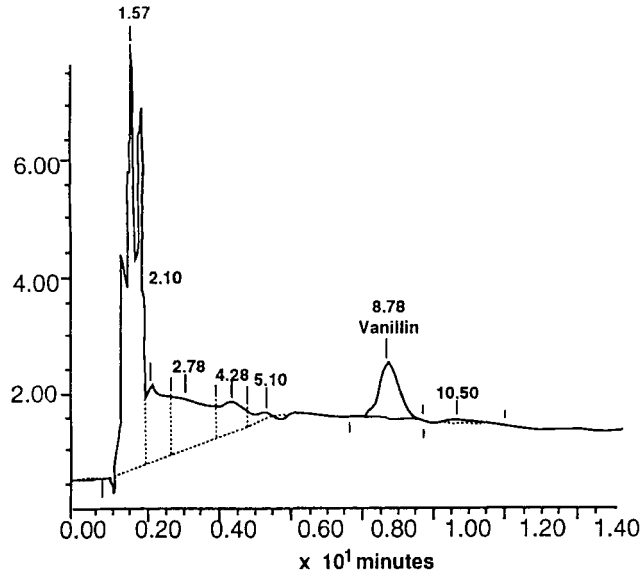

FIGURE 4. Chromatogram of an American blend cigarette filler extract.

TABLE 7.

Vanillin in Tobacco Leaf Samples.

Tobacco leaf type.	Vanillin, ppm, Mean	SEM
Virginia	1.78	0.03
Burley	1.51	0.04
Oriental	0.89	0.06

extraction, and a second term associated with the HPLC analysis. The statistics of the precision test are listed in Table 5. The two-way analysis of variance (ANOVA) identifies a significant sample-to-sample + extraction variation.

The recovery test was performed on seven independent samples of tobacco filler. The results are listed in Table 6. The mean recovery determined is statistically undistinguishable from 100%.

A representative chromatogram of an American blend cigarette filler extract is presented in Fig. 4. Several samples of tobacco leaf were analyzed with this method. The results are listed in Table 7.

In summary, a new analytical method for determination of vanillin in tobacco and tobacco products was developed. The precision of the method is better than 2 %CV. The naturally occurring vanillin content found in tobacco leaf samples is significantly higher than that reported by J. N. Schumacher [3].

ACKNOWLEDGMENTS

I thank Mr. K. F. Heidsieck for the tobacco leaf samples.

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Received: January 31, 1993
Accepted: March 3, 1993