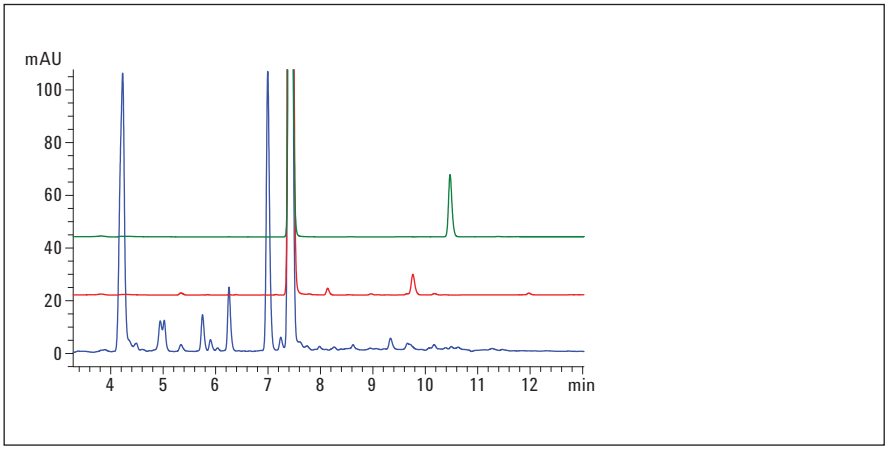
Agilent Application Solution

Analysis of natural and artificial vanilla preparations

Application Note

Food



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Abstract

Vanilla extract is widely used as a flavoring ingredient in foods and beverages. It contains approximately 200 substances. The main compounds are vanillin, 4-hydroxybenzaldehyde, vanillic acid and 4-hydroxybenzoic acid. Since natural vanilla extract is limited and prices are high, artificial vanilla flavorings are often used. Due to quality and price concerns, it is important to differentiate between these two.

In this Application Note, a conventional HPLC method was developed and validated for several natural and artificial vanilla flavorings using the Agilent 1260 Infinity LC system. In addition, a UHPLC method was developed using the Agilent 1290 Infinity LC System, saving time and solvent consumption.

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Introduction

Typical artificial vanilla flavorings contain synthetically produced vanillin, ethyl vanillin, guaiacol, vanillin mandelic acid, eugenol, and piperonal.

The vanillin compound used in these artificial flavorings is usually synthesized from cheap raw material, such as guaiacol, eugenol, or lignin. These products can be found in the final flavoring preparation at trace levels.[8]

Ethyl vanillin is another artificial produced vanilla compound which is three times more flavoring than vanillin and is also used in imitation products.[8]

Some vanilla extracts are adulterated with coumarin, a phytochemical, to increase the vanilla flavor perception since it has a sweet odor and is used as flavoring and fragrance enhancer.

Coumarin causes hepatoxicity in animals and has been banned for use as a food additive in the U.S. since 1956, especially since it was considered to be carcinogenic and genotoxic.[1-4] Meanwhile,coumarin was found not to be genotoxic or carcinogenic.[9]

Most countries have regulations to control the content of synthetic vanilla products and by-products such as the US Code of Federal Regulations from 1988, which requires that beverage alcohol products are labeled, if synthetic flavors are used.[1]

To determine vanilla flavoring compounds, several LC methods have been used.[5-9] Based on these publications, this method was developed.

The natural and artificial compounds shown in Figures 1 and 2 were analyzed.

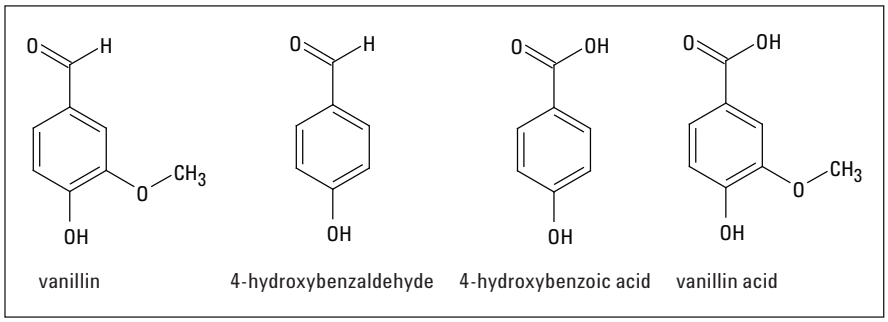


Figure 1
Natural flavoring compounds.

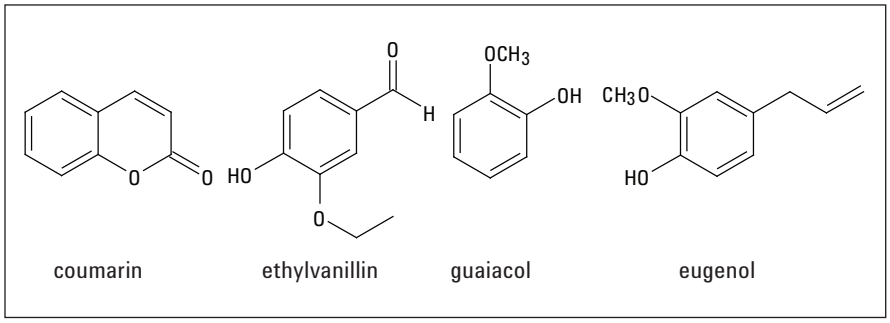


Figure 2
Artificial flavoring compounds and starting compounds for synthesis of vanillin.

Experimental

Instruments and Software

An Agilent 1260 Infinity Binary LC system consisting of the following modules was used:

- Agilent 1260 Infinity Binary Pump (G1312B)
- Agilent 1260 Infinity Vacuum Degasser (G1379B)
- Agilent 1260 Infinity Autosampler and Thermostat (G1367E, G1330B)
- Agilent 1260 Infinity Thermostatted Column Compartment (G1316A)
- Agilent 1260 Infinity Diode Array Detector (G4212B) with 10-mm Max-Light flow cell

The UHPLC analysis was developed and performed using an Agilent 1290 Infinity LC System consisting of the following modules:

- Agilent 1290 Infinity Binary Pump (G4220A)
- Agilent 1290 Infinity Autosampler and Thermostat (G4226A, G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1290 Infinity Diode Array Detector (G4212A) with 10-mm Max-Light flow cell

Columns:

- Agilent ZORBAX Eclipse Plus C18, 4.6 × 150 mm, 5 µm (p/n 95993-902)
- Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 50 mm, 1.8 µm (p/n 959757-902)

Software:

- Agilent ChemStation B.04.02

Reagents and materials

All chemicals and solvents used were HPLC grade, and highly purified water from a Milli Q water purification system was used. Acetonitrile gradient grade was purchased from Merck (Darmstadt, Germany). All standards were ordered from Sigma Aldrich, Germany.

The standards contained a lot of other trace compounds, see the carry over experiment. The peak height of these trace compounds was between 0.04 and 2 mAU whereas the main compounds had peak heights between 105 (Eugenol) and 2330 mAU (4-hydroxybenzaldehyde) for dilution 0 (for dilutions see Table 1). Typically, this will not significantly influence the quantitation of the main compounds.

Preparation of standards

To perform the validation tests, a dilution series was set up, see Table 1. For the stock solution and all other dilutions, acetonitrile was used as solvent. The stock solution was stored at 4 °C and was stable for at least 2 months.

The concentration range of the analyzed compounds is quite different, depending whether it is a main flavoring compound, a minor flavoring compound, or a by-product from synthesis that is present at trace levels, for example:

- Typical calibration range for coumarin: 1–25 µg/mL
- For vanillin: 20–400 µg/mL

Chromatographic conditions

Parameter	Conventional method	UHPLC method
Column:	Agilent ZORBAX Eclipse Plus C18, 4.6 × 150 mm, 5 µm	Agilent ZORBAX Eclipse Plus C18, 2.1 × 50 mm, 1.8 µm
Mobile phase:	Water + 0.1% TFA (A), acetonitrile + 0.09% TFA (B)	Water + 0.1% TFA (A), acetonitrile + 0.09% TFA (B)
Gradient:	At 0 min 5% ACN At 12 min 65% ACN At 14 min 90% ACN At 16 min 90% ACN At 16.01 min 5% ACN At 21 min 5% ACN Stop at 21 min	At 0 min 5% ACN At 0.93 min 65% ACN At 1.08 min 90% ACN At 1.24 min 90% ACN At 1.25 min 5% ACN At 1.62 min 5% ACN Stop at 1.62 min
Flow rate:	1 mL/min	0.9 mL/min
Column temp:	30 °C	30 °C
DAD:	280/10 nm, 260/10 nm, 230/10 nm Ref: 400/60 nm, 10 Hz	280/10 nm, 260/10 nm, 230/10 nm Ref: 400/60 nm, 40 Hz
Injection volume:	3 µL with 6 s needle wash	0.5 µL with 6 s needle wash

Sample preparation

Sample preparation is as follows:

1. Dilute 10 µL vanilla preparation with 990 µL acetonitrile to make a 1:100 dilution of the vanilla extracts and the synthetic aroma preparations.
2. Filtrate 1 mL of diluted sample through a syringe filter (p/n 5061-3365) for the vanilla extract (no further sample preparation was needed).
3. Inject 3 µL of the filtered solution.

Procedure

The following steps were taken to develop and validate the method on the Agilent 1260 Infinity LC system:

- **Method Development**: Standards were injected to elute all peaks in a reasonable time (~20 minutes) with an Agilent 1260 Infinity LC using a standard-bore 4.6 mm id columns.
- **Method validation**: Area and RT precision, LOD/LOQ, linearity (relevant range), robustness (column temperature, flow, gradient steepness, wavelength, injection volume) were evaluated.

Compound	Stock µg/mL	Dil. 0 (1:10) µg/mL	Dil. 1 (1:10) µg/mL	Dil. 2 (1:2) µg/mL	Dil. 3 (1:2) µg/mL	Dil. 4 (1:2) µg/mL	Dil. 5 (1:2) µg/mL	Dil. 6 (1:2) µg/mL
4-hydroxybenzoic acid	4550	455	45.5	22.75	11.375	5.6875	2.84375	1.421875
vanillic acid	4510	451	45.1	22.55	11.275	5.6375	2.81875	1.409375
4-hydroxybenzaldehyde	4590	459	45.9	22.95	11.475	5.7375	2.86875	1.434375
vanillin	4620	462	46.2	23.1	11.55	5.775	2.8875	1.44375
guaiacol	9050	905	90.5	45.25	22.625	11.3125	5.65625	2.828125
ethylvanillin	4760	476	47.6	23.8	11.9	5.95	2.975	1.4875
coumarin	3180	318	31.8	15.9	7.95	3.975	1.9875	0.99375
eugenol	1730	173	17.3	8.65	4.325	2.1625	1.08125	0.540625

Table 1
Dilution series.

- **Sample preparation**: A relevant matrix of natural and artificial vanilla preparations were chosen.
- **Analysis**: Injection of real-life sample with quantification and identification through UV spectra was performed.

Having developed and validated the conventional method, the analysis was then transferred to the Agilent 1290 Infinity LC System for developing a UHPLC method:

- **Method transfer to UHPLC**: An UHPLC method was developed with increased speed and sensitivity using the 1290 Infinity LC and a short sub-2 µm column.
- **Proof of UHPLC method performance**: Precision of area and RT, LOD and LOQ was evaluated.

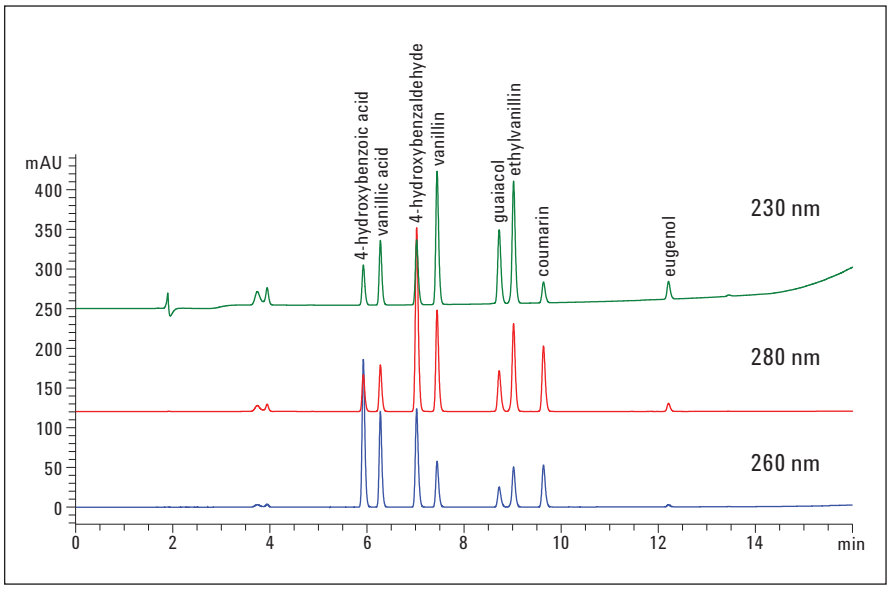


Figure 3
Analysis of vanilla compounds at three different wavelengths.

Results and Discussion

Separation and detection

A conventional method was developed using an Agilent ZORBAX Eclipse Plus C-18, 4.6 × 150 mm, 5 µm particles column. A gradient from 5% to 90% organic was used at a flow rate of 1 mL/min, and gave an excellent separation (see Figure 3). Water and acetonitrile acidified with TFA were used to achieve the separation of the eight compounds. Resolution was > 2 for all compounds. During the optimization process, the eight compounds were analyzed at three different wavelengths, 230, 260, and 280 nm, see Figure 3.

Even though 280 nm was not the absorbance maximum for all compounds, it was selected for all further experiments due to the very good selectivity, when real life samples had to be determined.

The spectra (Figure 4) of the eight compounds are very characteristic. Therefore, they were used to create a UV spectral library, which allowed identification of compounds in the vanilla preparations in addition to the retention times.

Method validation

Precision of retention times (RT) and areas

RT RSD values for all compounds across the eight linearity levels were calculated. The precision of retention times for six consecutive runs was typically < 0.05% RSD. The precision over the complete sequence was < 0.19% RSD, over 68 runs within 23 hours.

The precision of areas was tested over the complete dilution series. The results are combined in Figure 5. The RSD of dilution 7 is < 3.1% which is very good. All other RSD values were < 2% over the complete dilution series and typically < 1% from dilution 0 to 5.

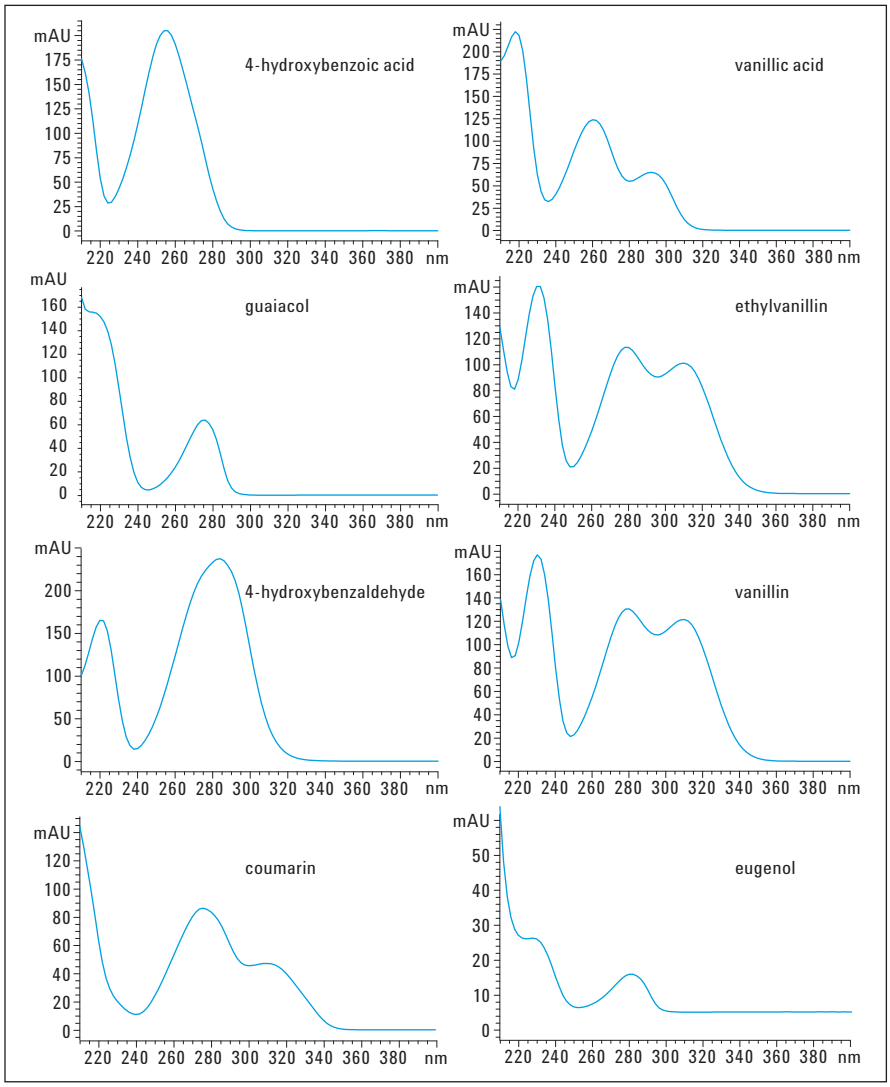


Figure 4
Spectra of analyzed compounds.

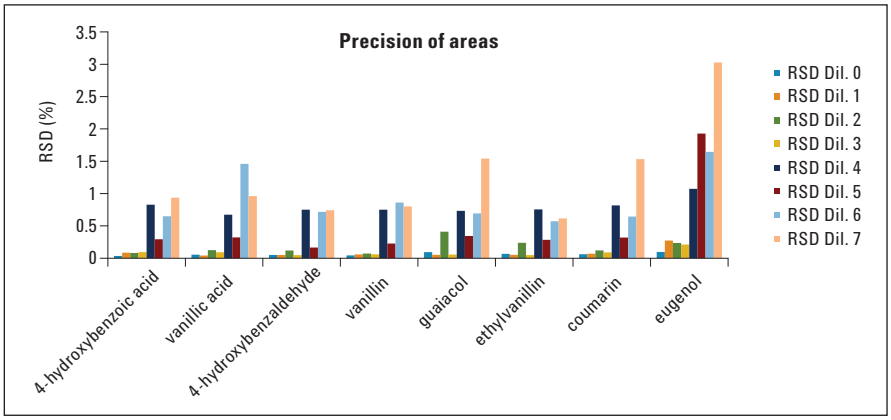


Figure 5
Precision of area over the complete dilution series.

Limit of detection (LOD) and Limit of quantitation (LOQ)

The limit of detection and quantitation was evaluated using dilution 7. In Figure 6, the chromatogram for this concentration is shown. The injected amount is in the low ng range. The analyte concentration that provides a signal to noise ratio (S/N) of > 3 was considered as LOD and compound concentration with 10*LOD was considered as LOQ. Only coumarin and starting material for synthesis like eugenol and guaiacol have to be measured at low levels. All other compounds are present in rather high concentration.

Overall the LOD is < 0.1 µg/mL and the LOQ (10*LOD) is < 1 µg/mL.

Linearity

To test the linearity dilution 0 to 7 was used. Each linearity solution was injected six times to measure and evaluate linearity. The linearity over the complete dilution series was determined by calculating the detector response factors (amount/area). For a wide concentration ranges, this is typically more accurate and meaningful than regression curves. All response factors were within the ± 5% range, which is accepted to provide linearity, see Figure 7. The linearity experiments were used to create a multi level calibration and this multi level calibration was used to evaluate the amounts of the real life samples. The multi level calibration was based on dilution 0 to 7.

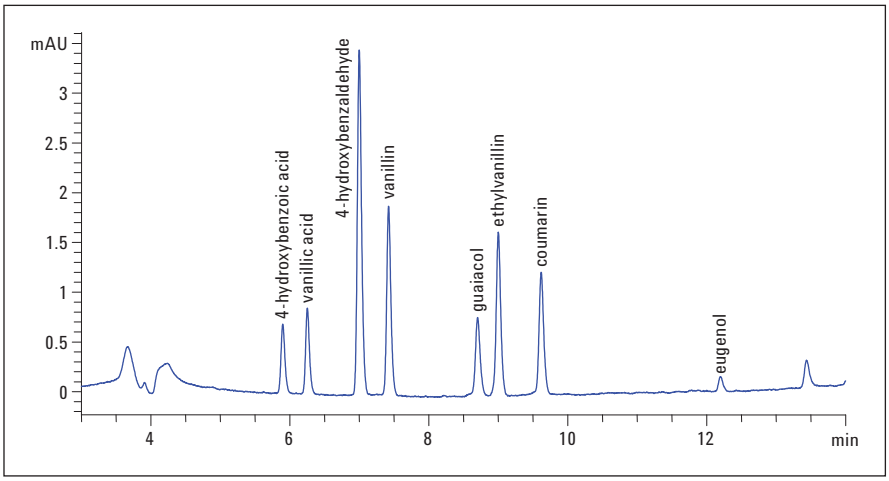


Figure 6
Chromatogram of dilution 7.

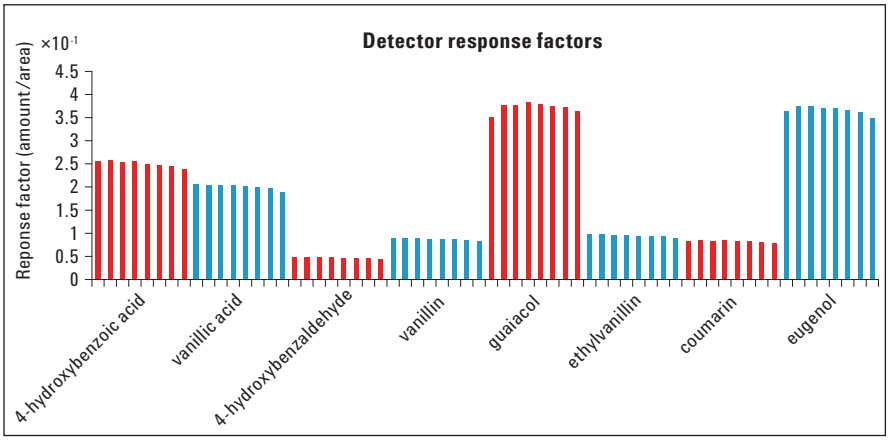


Figure 7
Response factors for dilution 0 to 7 for each compound.

Carry over

To test the carry over behavior dilution 0 was injected 10 times followed by the injection of 3 µL of acetonitrile, see Figure 8. No carry over was observed.

Robustness test

Five critical parameters were changed and data collected in 10 replicate injections. Values from the last six replicates were used for the analysis. Allowed deviation for retention time and area was set to ± 3.0% and ± 6% respectively. Robustness of the method was tested using dilution 1. The results showed that retention time shifts > 3% have to be expected for gradient slope differences of ± 10% and changes for the flow rate changes especially for 4-hydroxybenzoic acid and vanillic acid, see Table 2.

Regarding robustness related to compound areas, changes of the wavelength and injection volume is critical, see Table 3.

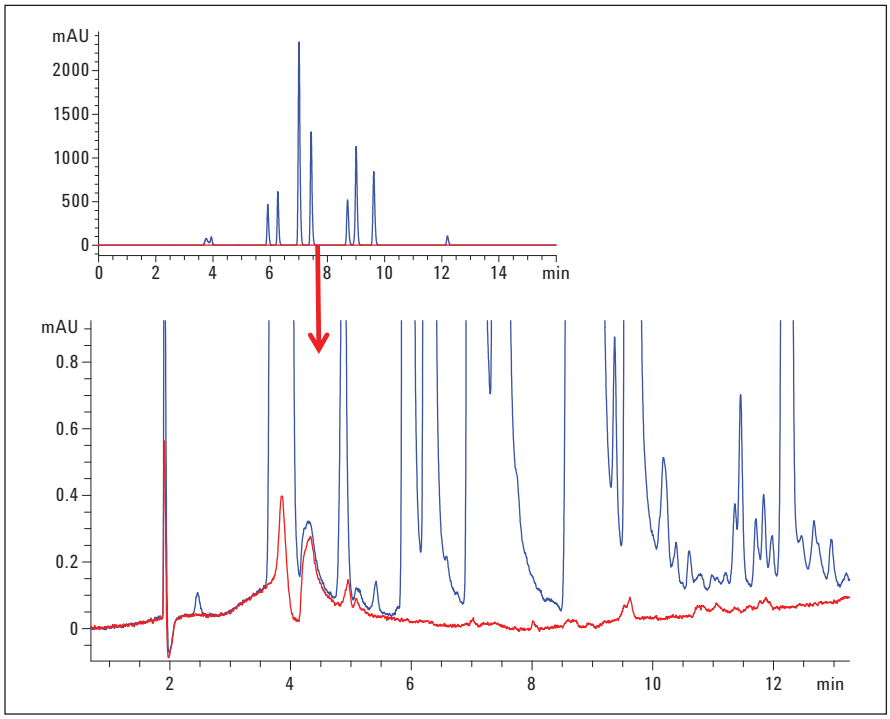


Figure 8
Overlay of chromatogram for dilution 0 followed by the chromatogram of the blank injection of pure solvent, blue trace chromatogram of dilution 0, red trace blank injection of 3 µL acetonitrile.

	Changes	4-Hydroxy benzoic acid % deviation for RT	Vanillic acid % deviation for RT	4-Hydroxy benzaldehyde % deviation for RT	Vanillin % deviation for RT	Guaiacol % deviation for RT	Ethylvanillin % deviation for RT	Coumarin % deviation for RT	Eugenol % deviation for RT	% deviation limits
Flow 2% Standard: 1 mL/min	High: 1.04 mL/min	+ 3.1	+ 8.9	– 2.7	– 2.5	– 2.3	– 2.2	– 2.1	– 1.6	± 3%
	Low: 0.96 mL/min	+ 2.9	+ 2.7	+ 2.7	+ 2.5	+ 2.3	+ 2.1	+ 2.1	+ 1.6	± 3%
TCC ± 5% Standard: 30 °C	High: 31.5 °C	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	± 3%
	Low: 28.5 °C	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	± 3%
Inj ± 5% Standard: 3 µL	High: 3.15 µL	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	± 3%
	Low: 2.85 µL	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	± 3%
Gradient slope ± 10%	In 12 min 71% + 10%	– 2.9	– 3.3	– 3.4	– 3.7	– 4.1	– 4.5	– 4.6	– 5.6	± 3%
	In 12 min 59% – 10%	+ 3.2	+ 3.7	+ 3.7	+ 4.2	+ 4.6	+ 5.1	+ 5.3	+ 6.6	± 3%
Wavelength ± 3 nm	High DAD 283 nm	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	± 3%
	Low DAD 277 nm	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	± 3%

Table 2
Robustness tests related to retention times.

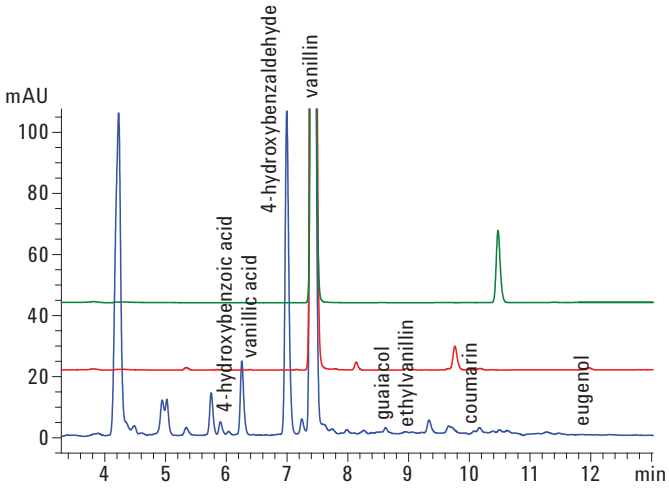
	Changes	4-Hydroxy benzoic acid % deviation for area	Vanillic acid % deviation for area	4-Hydroxy benzaldehyde % deviation for area	Vanillin % deviation for area	Guaiacol % deviation for area	Ethylvanillin % deviation for area	Coumarin % deviation for area	Eugenol % deviation for area	% Deviation limits
Flow 2% Standard: 1 mL/min	High: 1.04 mL/min	– 0.6	– 2.4	– 3.0	– 3.1	– 3.1	– 3.3	– 3.0	– 3.3	± 6%
	Low: 0.96 mL/min	+ 7.3	+ 5.5	+ 4.9	+ 4.7	+ 5.0	+ 4.8	+ 4.5	+ 4.5	± 6%
TCC ± 5% Standard: 30 °C	High: 31.5 °C	+ 1.2	– 0.6	– 1.2	– 1.3	+ 0.9	– 1.2	– 1.1	– 0.5	± 6%
	Low: 28.5 °C	+ 1.0	– 0.7	– 1.2	– 1.3	+ 1	– 1.3	– 1.1	– 0.1	± 6%
Inj ± 5% Standard: 3 µL	High: 3.15 µL	– 6.5	+ 4.7	+ 4.1	+ 3.8	+ 6.0	+ 3.9	+ 3.8	+ 3.6	± 6%
	Low: 2.85 µL	– 3.4	– 5.1	– 5.6	– 5.8	– 3.6	– 5.8	– 5.8	– 5.5	± 6%
Gradient slope ± 10%	In 12 min 71% + 10%	+ 1.7	+ 0.1	– 0.5	– 0.7	– 0.3	– 0.7	– 0.9	– 0.9	± 6%
	In 12 min 59% – 10%	+ 1.7	– 0.2	– 0.9	– 0.9	– 0.1	– 0.8	– 0.9	– 0.9	± 6%
Wavelength ± 3 nm	High DAD 283 nm	– 34.2	– 1.9	+ 0.3	– 2.3	– 9.5	– 2.8	– 3.3	– 1.3	± 6%
	Low DAD 277 nm	+ 50.1	+ 8.3	– 5.1	– 2.5	+ 15.6	– 2	+ 1.5	– 6.9	± 6%

Table 3
Robustness tests related to areas.

Robustness results indicate that the method is reliable for normal usage and to a great extent the performance remains unaffected by deliberate change in parameters. However, some parameters like wavelength are critical and must be carefully controlled.

Analysis of real life sample

Three different vanilla preparations were analyzed, one natural vanilla extract and two artificial vanilla preparations, see Figure 9. The blue trace is the natural vanilla extract. Obviously, a lot of compounds are present whereas in the artificial preparations (red and green trace) only vanillin in high concentration and two or three other compounds are present. The compound amounts were calculated based on the previously described multi level calibration, see table in Figure 9.



Compound	Natural extract (blue) µg/mL	Artificial preparation 1 (red) µg/mL	Artificial preparation 1 (green) µg/mL
4-hydroxybenzoic acid	4.5	-	-
vanillic acid	18.5	trace?	trace?
4-hydroxybenzaldehyde	20.8	-	-
vanillin	271	386.8	131.7
guaiacol	-	-	-
ethylvanillin	trace?	trace?	-
coumarin	trace?	-	-
eugenol	trace?	-	-

Figure 9
Overlay of true vanilla extract (blue) and two artificial vanilla preparations (red and green).

The created UV library was used to identify compounds in addition to retention times, see Figure 10. Even at low peak height like for 4-Hydroxybenzoic acid (Peak height = 4 mAU), identification through the UV spectrum is possible with a match factor >990.

Method transfer to UHPLC method

An UHPLC method with diode array detection was established for the separation of flavoring compounds using the Agilent Method Translator. This tool enables to easily convert methods from either binary or quaternary pump systems to optimized methods for the Agilent 1290 Infinity LC System.

To demonstrate the usability of faster methods, the described conventional method was transferred to an UHPLC method using an Agilent 1290 Infinity LC System. The analysis time was decreased to 1.62 minutes using a short sub-2 µm column, see Figure 11.

The benefits of the ultra fast analysis are:

- Time savings: 92.3%
- Solvent savings: 93.1%

The disadvantage is that the resolution for ethylvanillin is only 1.7, for all other compounds the resolution is > 2.

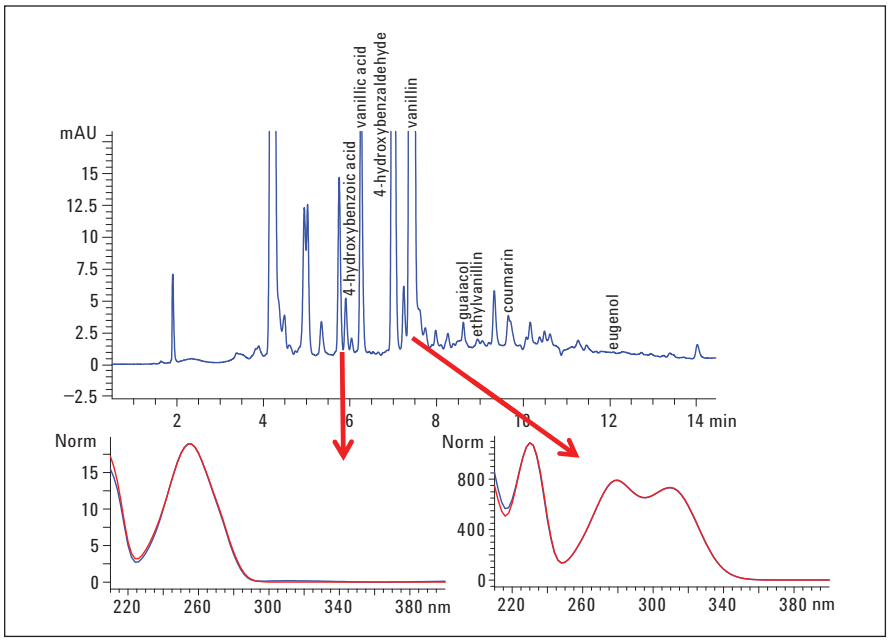


Figure 10
Identification of flavoring compounds in a true vanilla extract by UV spectra.

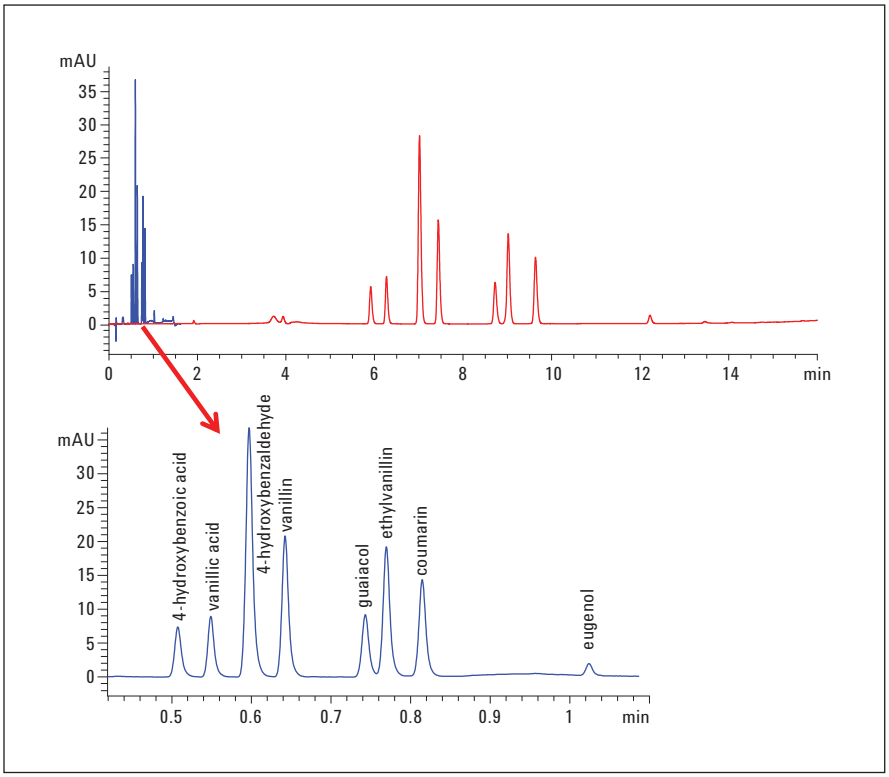


Figure 11
Transfer of conventional method to an ultra fast method, the red trace represents the conventional analysis and the blue trace the ultra fast analysis.

Partial validation of ultra fast method

The following parameters were tested:

- Limit of detection and quantitation using dilution 7 (Figure 12)
- RSD of retention times and areas for dilution 4

As a result, it was found that the performance for the UHPLC method is comparable to the performance of a conventional method, except for the resolution for ethylvanillin.

Performance data

RSD of RT:	Typically < 0.1% RSD for dilution 4
RSD of Areas:	Typically < 1.1% RSD for dilution 4 with an injection volume of 0.5 µL
LOD:	< 0.14 µg/mL (dilution 7)
LOQ (10*LOD):	< 1.4 µg/mL (dilution7)

The LOD/LOQ is slightly higher than for the conventional method. This is mainly due to the higher noise level at 40 Hz versus 10 Hz. The noise (peak to peak) for the conventional method is 0.01897 mAU. For the UHPLC method, the noise level is 0.02706 mAU.

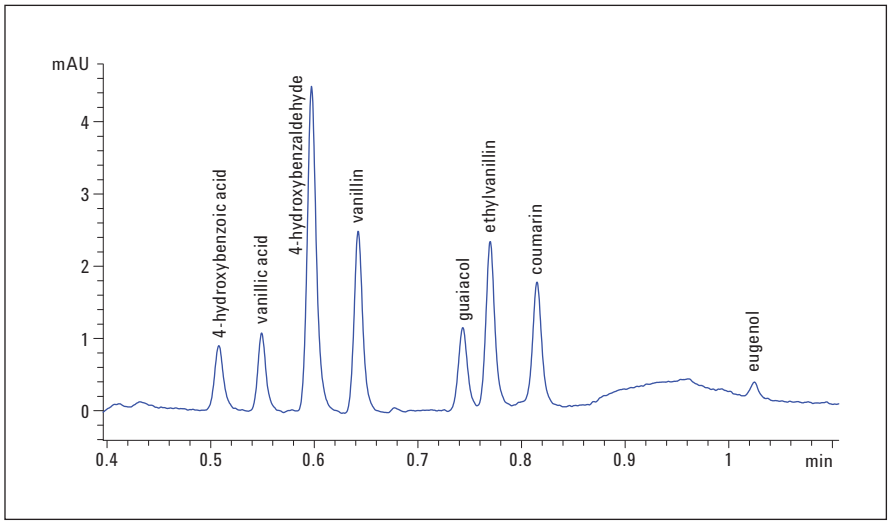


Figure 12
Chromatogram of dilution 7, 0.5 µL injection volume.

Conclusion

A conventional method for the analysis of vanilla compounds in a natural vanilla extract and two artificial vanilla preparations was developed and validated using the Agilent 1260 Infinity Binary LC system. The method is robust and suitable for the quantitation within a concentration range > 400 to < 1 µg/mL. The extraction method is based on a simple dilution and filtration step. The LOD is typically < 0.1 µg/mL injected amount. Faster results with significant decrease in solvent consumption and time can be achieved by applying an UHPLC method using the Agilent 1290 Infinity LC System.

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