Fast Determination of the Bitter and Clove-like Flavor in Beer with the Agilent 1290 Infinity II LC

Application Note

Food Testing and Agriculture

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Abstract

The Agilent 1290 Infinity II LC was used to determine the bitter compounds iso-alpha-acids and reduced iso-alpha-acids, and the clove-like phenolic flavor 4-vinylguaiacol in bottled beer. A six-minute UHPLC method was developed to measure these important solutes in a single run. Diode array and fluorescence detectors (DAD and FLD) were placed in-series for quantification of iso-alpha-acids and reduced iso-alpha-acids, and of 4-vinylguaiacol, respectively. The fluorescence detector provided a selective, sensitive, and accurate determination of ppb amounts of 4-vinylguaiacol. Some figures of merit are presented together with the profiles of a series of different beers.

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Introduction

Hops (*Humulus lupulus L.*) or hop extracts contain alpha-acids or humulones, and are added to the boiling wort during beer brewing to impart a bitter flavor. The nearly tasteless humulones are converted into the bitter-tasting iso-alpha-acids or isohumulones (IAAs) in the boiling process. The acids also show bacteriostatic properties and play an essential role in enhancing the foam stability of beer as well as in the formation of off-flavors such as the light-struck flavor[1,2]. Foam and light-stability of beers are often improved by the addition of reduced IAAs such as tetrahydroiso-alpha-acids (tetrahydroisohumulones, THs). The addition of these nonnatural acids is not allowed in beers for the German market due to the *Reinheitsgebot*, which states that the only ingredients that can be used in the production of beer are water, barley, and natural hops. Figure 1 shows the compounds related to beer bitterness.

Another important flavor compound in some beers is 4-vinylguaiacol (4-VG). 4-VG is responsible for a clove-like or phenolic flavor, especially in wheat beers. The compound is produced through the conversion of ferulic acid by certain strains of yeast. While wheat and some other special beers contain significant amounts of 4-VG, most beers contain only trace amounts of this compound, that is, in the 0.05 to 0.25 ppm (mg/L) range. However, the flavor threshold of 4-VG is relatively low (about 0.1 to 0.2 ppm), and therefore a sensitive and robust method to quantify trace amounts of 4-VG is required.

From the above considerations, it is clear that accurate determination of IAAs, THs, and 4-VG is of utmost importance for beer quality control. This Application Note presents the development of a fast and sensitive UHPLC method for the analysis of all these compounds in bottled beers in a single run on the Agilent 1290 Infinity II LC using diode array and fluorescence detectors (DAD and FLD) in series.

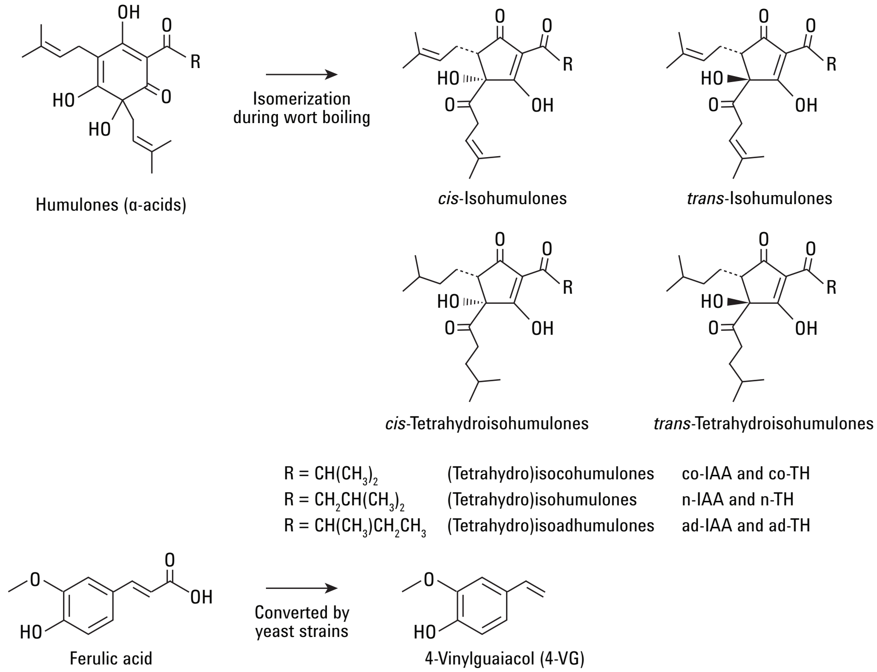

Figure 1. Target compounds.

Experimental

Instrumentation

An Agilent 1290 Infinity II LC was used.

- Agilent 1290 Infinity II High-Speed Pump (G7120A)
- Agilent 1290 Infinity II Multisampler (G7167B)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B)
- Agilent 1290 Infinity II Diode Array Detector (G7117B)
- Agilent 1260 Infinity Fluorescence Detector Spectra (G1321B)

Standards and standard solutions

The following standards were used:

- 4-VG: 2-methoxy-4-vinylphenol, (Sigma-Aldrich, Diegem, Belgium)
- IAA: DCHA-Iso, ICS-I1, IAA content 64.5 % (Labor Veritas, Zürich, Switzerland)
- TH: Tetra, ICS-T2, TH content 99.4 % (Labor Veritas, Zürich, Switzerland)

Stock solutions were prepared in methanol. The stock solution of 4-VG had a concentration of 50 ppm. A mixed stock solution of the IAAs and the THs was made at a concentration of 800 and 200 ppm, respectively.

From these stock solutions, three different standard solutions were made with the following concentrations:

- **Standard 1**: 0.25 ppm 4-VG, 10 ppm IAA and 2.5 ppm TH in 20 % methanol
- **Standard 2**: 1 ppm 4-VG, 20 ppm IAA and 5 ppm TH in 20 % methanol
- **Standard 3**: 4 ppm 4-VG, 40 ppm IAA and 10 ppm TH in 20 % methanol

Sample preparation

Beer samples were degassed by sonication and filtered through an Agilent Captiva regenerated cellulose filter (pore size 0.45 µm, p/n 5190-5109) prior to injection.

Results and Discussion

One of the primary aims was to develop a method for simultaneous analysis of the bitter acids and of 4-VG without any sample preparation, that is, direct injection of degassed beer. The most challenging part of this analysis was the determination of 4-VG at sub-ppm levels in the complex beer matrix. A gradient method with an isocratic hold at the beginning of the run was developed. The isocratic part ensures preservation of chromatographic selectivity to reduce risk of interference from neighboring peaks with 4-VG. The gradient was started after the elution of 4-VG to sharpen the peaks of the IAAs and tetrahydroisohumulones (THIAAs) and reduce analysis time. 4-VG was detected with DAD and FLD; the IAAs and reduced IAAs with DAD only.

Method parameters

Parameter	Description
Column	Agilent ZORBAX Eclipse Plus C18 RRHD, 2.1 × 100 mm, 1.8 µm (p/n 959758-902)
Mobile phase	A) 1 % Triethylammonium phosphate solution* in acetonitrile/water (1/9 v/v) with 0.1 mM Na2-EDTA B) Acetonitrile
Flow rate	0.75 mL/min
Gradient	0 to 1.5 minutes, 25 %B 1.5 to 6 minutes, 25 to 85 %B
Posttime	2 minutes at 25 %B
Temperature	30 °C
Injection	Samples 4 µL Standards 3, 4, or 5 µL
Needle wash	Flush port, 5 seconds, water/methanol 1/1
Detection	DAD: 270/8 nm, ref. off, 40 Hz FLD: Ex 259 nm/Em 341 nm, 18.25 Hz

*Mobile phase reagent: triethylammonium phosphate solution, BioXtra, buffer solution 1 M pH 3.0, TEAP (Sigma-Aldrich, Diegem, Belgium)

The comparison of both signals for a standard solution is shown in Figure 2. It should be noted that the IAA standard contained only *trans*-IAAs, while the real beer sample contained both *cis*- and *trans*-IAAs (see below). Under the used analytical conditions, *cis*- and *trans*-IAAs and reduced *cis*- and *trans*-IAAs were separated, which enables the quantification of each important bitter solute.

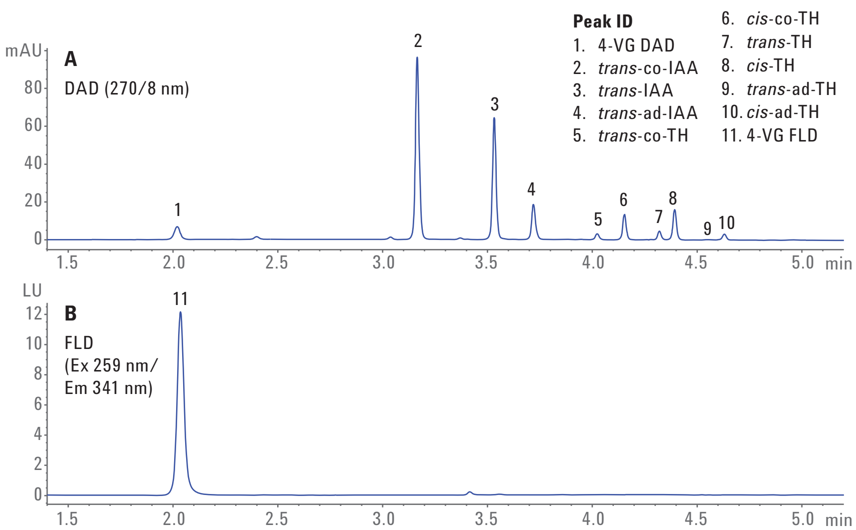

Figure 2. Analysis of a standard solution (standard 2, 4 µL injection volume) containing ca. 1 ppm 4-VG, 20 ppm IAAs, and 5 ppm THs. Detection: DAD 270 nm; FLD Ex 259 nm, Em 341 nm.

The standard solution was injected six consecutive times to determine the repeatability of injection. Results can be found in Table 1. The data were also used to determine the distribution of the individual acids and *cis/trans* ratios within the IAA and TH standards. The *cis/trans* ratio plays a role in the flavor stability since *cis*-compounds are known to be more stable compared to *trans*-compounds[3]. Higher ratios can be found in pre-isomerized hop products, which can be used in advanced brewing processes. Degradation of *trans*-compounds can also lead to high *cis/trans* ratios in aged beers. The bitterness quality differs among the various IAAs[4]. Whereas, n- and ad-IAAs lead to a more delicate bitterness, co-IAAs give a less desired flavor.

A calibration was carried out with only three standard solutions. Each standard solution was injected using three different injection volumes (3, 4, and 5 µL), resulting in a nine-level calibration. Peaks were integrated, and groups were created to report the concentration of the individual compounds as well as the grouped concentration of IAA and TH. Correlation coefficients were above 0.999 for all individual compounds.

Table 1. Data obtained from six replicate analyses of standard 2, 4 µL injection volume.

	Concentration (ppm)	Average area	Area RSD%	*cis/trans* ratio	% co/n/ad	co/n-ad ratio
4-VG (DAD)	1	14.31	0.31			
4-VG (FLD)	1	29.39	0.21			
IAA	20.25	231.31	0.21	Only *trans*		1.2
co-IAA		125.19	0.27		54.1 (53*)	
n-IAA		81.20	0.17		35.1	
ad-IAA		24.91	0.26		10.8	
TH	5.03	51.17	0.47	4.0		0.7
co-TH		20.97	0.38		41.0 (39*)	
n-TH		25.77	0.54		50.4	
ad-TH		4.43	0.97		8.6	

*Theoretical % of co-isomers according to certificate

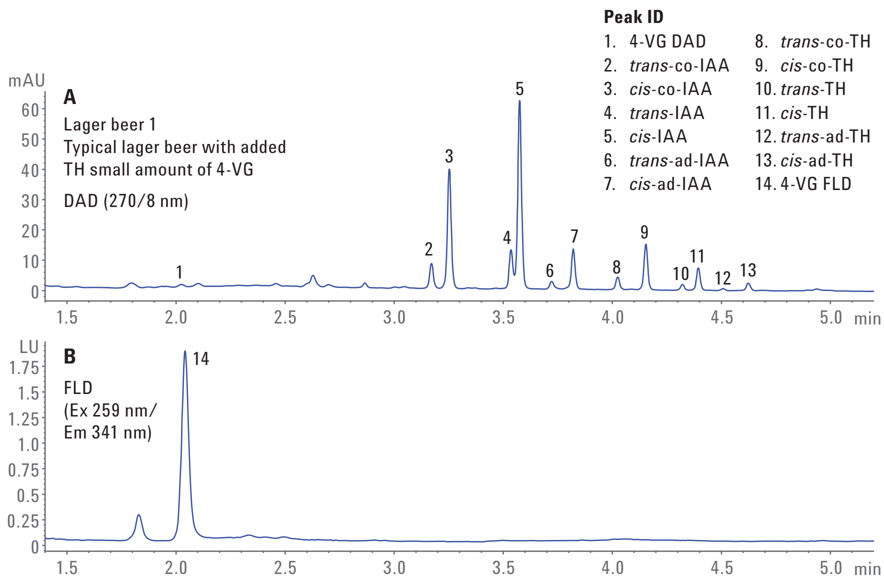

Figure 3. Analysis of typical pilsner-type beer (lager beer 1) containing (a small amount) 0.12 ppm of 4-VG. Detection: DAD 270 nm; FLD Ex 259 nm, Em 341 nm.

Beer samples were only degassed by sonication, and filtered with a syringe filter prior to analysis. Figure 3 shows the chromatogram for a typical Belgian lager beer containing all the bitter acids discussed above, together with a small amount of 4-VG (0.118 ppm). It is clear that detection and quantitation of this compound is difficult with DAD because of the low signal intensity and presence of numerous adjacent peaks (Figure 4). This is not the case when fluorescence detection is used. With the appropriate wavelength settings, 4-VG can easily be detected and accurately quantified in real beer samples.

A total of 14 various types of beer were analyzed for 4-VG, IAAs, and THs to demonstrate the applicability of the method. Table 2 summarizes the quantitative data together with ratios for the various acids. Some beers contain THs, which is common practice in modern brewing for improving the foam stability. As expected, the wheat beer contains a larger amount of 4-VG and smaller amounts of IAAs, making it less bitter. The abbey-style beer and Belgian strong pale ale also contain significant amounts of 4-VG. IAA *cis/trans* ratios are roughly between 2 and 6. Higher values indicate the use of special hop extracts. The fruit beer displays very low bitterness because of the low concentration of IAAs. The taste is masked by the characteristic fruit flavors present in this beer. The *cis/trans* ratio is extremely high because the amount of *trans*-IAAs is very low.

Figures 5–8 show the chromatograms for a number of selected beers.

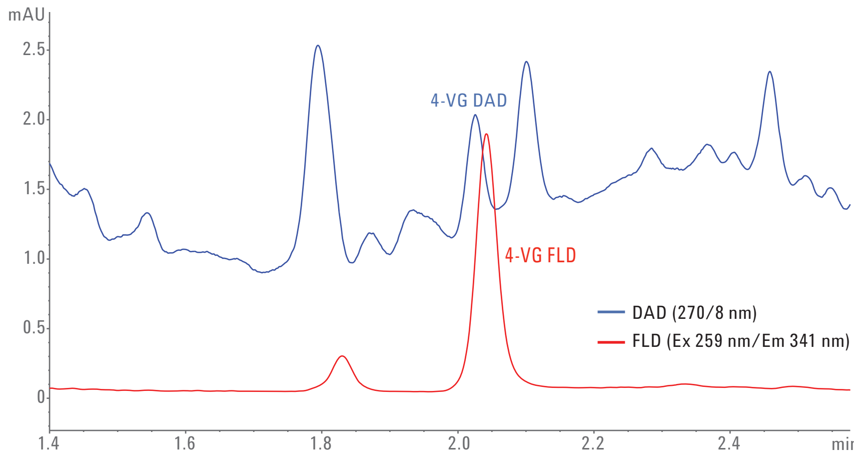

Figure 4. Detail of the analysis of typical pilsner-type beer (lager beer 1) containing a small amount of 4-VG. Detection: DAD 270 nm; FLD Ex 259 nm, Em 341 nm.

Table 2. Quantitative results for the selection of analyzed beers.

	Concentration (ppm)			co/n-ad ratio		*cis/trans* ratio	
	4-VG (FLD)	IAA	TH	IAA	TH	IAA	TH
Lager beer 1	0.118	15.56	3.98	0.53	1.52	4.84	3.72
Lager beer 2	0.119	22.27		0.51		2.68	
Lager beer 3	0.103	19.13		0.48		2.81	
Lager beer 4	0.126	23.34		0.58		3.31	
Lager beer 5	0.289	15.98		0.70		3.57	
Lager beer 6	0.124	9.91		0.54		2.69	
Saison ale	0.474	25.37	4.04	0.61	0.96	3.09	3.97
Belgian strong pale ale	1.041	27.99	2.27	0.64	1.07	3.44	3.84
Blonde abbey-style beer	1.984	16.02	3.48	0.58	1.69	5.70	3.77
Stout	0.117	35.53		0.62		5.03	
Wheat beer	1.687	11.00		0.84		5.84	
Belgian pale ale 1	0.116	18.32		0.44		2.66	
Belgian pale ale 2	0.036	12.74	2.09	0.58	0.73	2.78	4.28
Fruit beer		3.06		0.67		17.57	

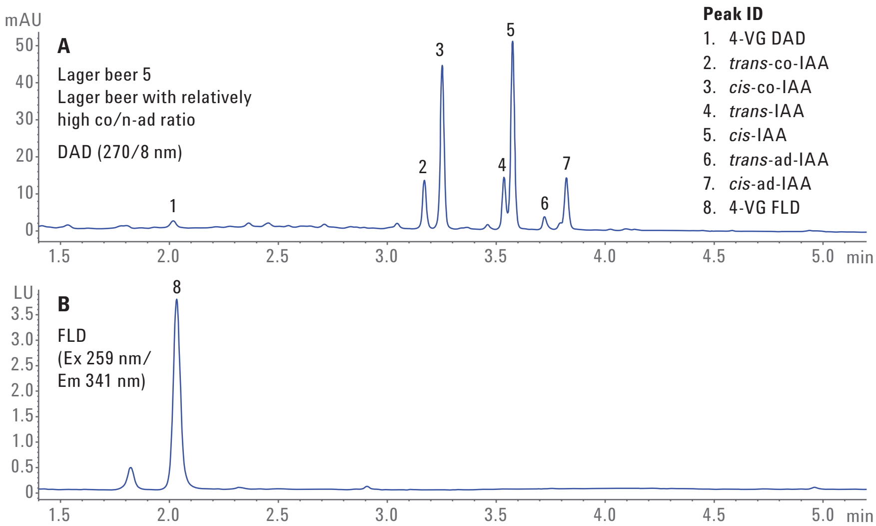

Figure 5. Analysis of lager beer 5 in a clear bottle. Detection: DAD 270 nm; FLD Ex 259 nm, Em 341 nm.

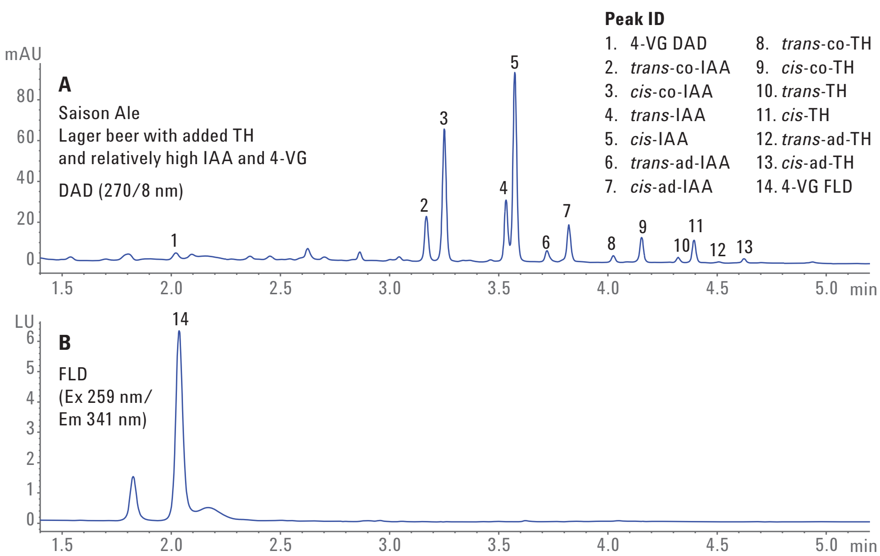

Figure 6. Analysis of Saison ale. Detection: DAD 270 nm; FLD Ex 259 nm, Em 341 nm.

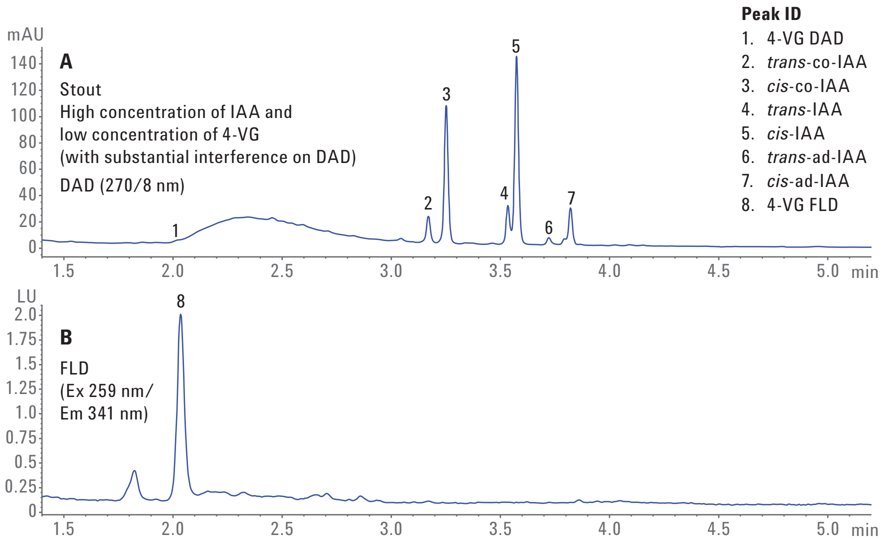

Figure 7. Analysis of stout. Detection: DAD 270 nm; FLD Ex 259 nm, Em 341 nm. The hump is reproducible and typical for this beer. The DAD completely masks the 4-VG content.

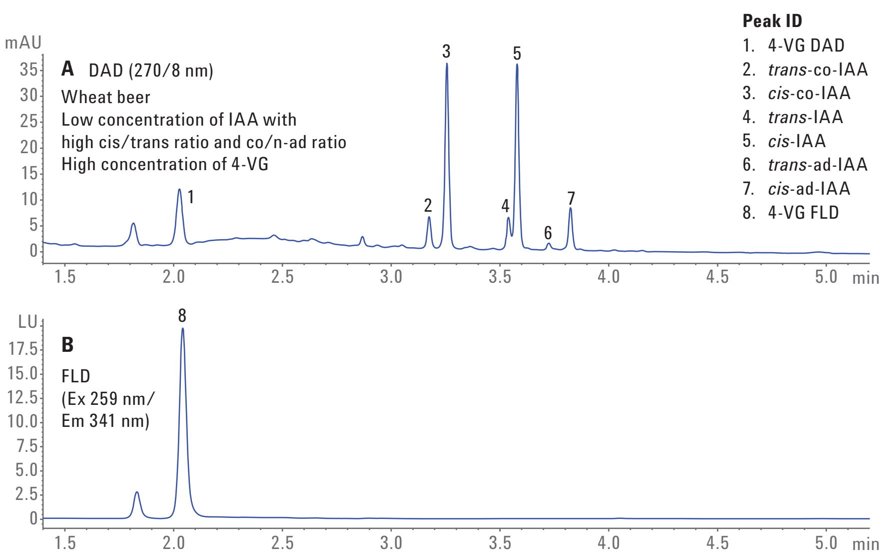

Figure 8. Analysis of wheat beer. Detection: DAD 270 nm; FLD Ex 259 nm, Em 341 nm.

Conclusion

The developed method enables the fast and accurate determination of IAAs, THIAAs, and 4-VG in beers with minimal sample preparation. Beers are only degassed and filtered prior to analysis with a six-minute UHPLC method. The use of an FLD compared to a DAD for the detection of 4-VG facilitates the quantification at the threshold value of this important flavor compound. The Agilent 1290 Infinity II LC was successfully used to quantify the compounds in a selection of beers, demonstrating the applicability of the method.

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