

# Determination of Glycols and Alcohols in Fermentation Broths by Ion-Exclusion Chromatography with Pulsed Amperometric Detection

Terri Christison and Jeffrey Rohrer, Dionex Corporation, Sunnyvale, CA, USA

## ABSTRACT

Development of an ion exclusion chromatography (ICE) method using pulsed amperometric detection (PAD) to determine glycol and alcohol metabolic byproducts in *S. cerevisiae* fermentation broths is discussed here. Glycols and alcohols were separated on an IonPac® ICE-AS1 4 mm column with 100 mM methanesulfonic acid at 0.20 mL/min, and detected by PAD using a three-potential waveform and a disposable platinum working electrode. This method demonstrated good retention time (RSDs < 0.1 [n = 250]) and peak area reproducibilities for glycerol and ethanol, (RSDs < 2), and propylene glycol (RSDs per day < 8), recoveries > 95%, and estimated LODs < 4 ng. Calibration from 25 to 6400  $\mu\text{M}$  was linear ( $r^2 > 0.999$ ) for ethanol. A quadratic calibration curve was used for *meso*-erythritol, glycerol, and propylene glycol with  $r^2 > 0.999$ . These results demonstrate this method to be suitable for monitoring glycols and alcohols in fermentation broths.

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## EXPERIMENTAL

### Equipment

Dionex ICS-3000 chromatography system consisting of:

- SP Single Pump module, gradient pump with degas option
- DC Detector/Chromatography module, single zone
- ED Electrochemical Detector
- AS Autosampler with sample tray temperature control option and 1.5 mL sample tray
- An electrochemical cell containing a combination pH/Ag/AgCl reference electrode and a disposable platinum (Pt) working electrode

Knitted reaction coil, 375  $\mu\text{L}$

Chromeleon® Chromatography Data System Software

Heated water bath (VWR Scientific 1200 series)

Shaker table (Lab Line)

Centrifuge (Eppendorf 5400 series)

## Consumables

- 1.5 mL glass sample vials, with caps and slit septa
- Sterile assembled micro centrifuge tubes with screw cap, 1.5 mL

## Reagents and Standards

- Deionized water, Type 1 reagent-grade, 18.2 M $\Omega$ -cm resistivity or better, freshly degassed by vacuum filtration for all reagent and sample preparation.
- Methanesulfonic acid
- Alditol and glycol standards (United States Pharmacopeia)
- pH 7 and pH 4 buffer solutions, NIST traceable

## Fermentation Samples

- Wyeast German Ale, Bohemian Lager, and American Wheat *Saccharomyces cerevisiae* samples (Hop Tech Home Brewing, Dublin, CA, USA)
- *S. cerevisiae* samples incubated in a Bacto YPD fermentation broth
- American, German, and British beer and American wine beverages

## Conditions

Column:	IonPac ICE-AS1, 4 × 250 mm
Flow Rate:	0.2 mL/min
Eluent:	100 mM Methanesulfonic acid
Column Temp.:	30 °C
Tray Temp.:	10 °C
Inj. Volume:	10 $\mu$ L
Detection:	PAD
Waveform:	See Table 1.
Reference Electrode:	pH/Ag/AgCl electrode in Ag/AgCl mode
Working Electrode:	Disposable platinum
Background:	60–90 nC
System Backpressure:	~800 psi
Noise:	< 10 pC
Typical pH:	1.0
Run Time:	30 min

## Preparation of Reagents and Standards

100 mM methanesulfonic acid eluent was prepared with high-purity methanesulfonic acid (Aldrich, St. Louis, MO, USA) and degassed deionized water.

Standards were prepared with high-purity reagents (Aldrich and Fisher, Hampton, NH, USA) and deionized water

Table 1. Waveform<sup>1</sup>

Time (sec)	Potential vs. Ag/AgCl (V)	Gain Region	Integration	Ramp
0.00	+ 0.30	Off	Off	Ramp
0.31	+ 0.30	On	Off	Ramp
0.32	+ 1.15	On	Off	Ramp
0.64	+ 1.15	On	On (Start)	Ramp
0.66	+ 1.15	On	Off (End)	Ramp
0.67	- 0.30	On	Off	Ramp
1.06	- 0.30	Off	Off	Ramp
1.07	+ 0.30	Off	Off	Ramp

## Preparation of Growth Media and Fermentation Broth Samples

Bacto Yeast Extract-Peptone-Dextrose (YPD) growth media, fermentation broth, and beverage samples were prepared according to Dionex Application Note 188.<sup>2</sup> The YPD growth medium was used for fermentation experiments and as a matrix blank. One mL aliquots were taken during sampling intervals and immediately heat-quenched in boiling water for 10 min, then centrifuged at 14,000 rpm for 10 min. The supernatant was transferred to another vial and diluted with deionized water.

The fermentation broth samples for growth experiments were prepared by incubating *S. cerevisiae* samples in Bacto YPD medium in a sterile 500 mL Erlenmeyer flask with an aeration tube at 37 °C in a shaking water bath (500–600 rpm) for 26–28 h. Sample aliquots were treated identically to growth media samples.

The beverage samples were centrifuged and the supernatant diluted prior to analysis.

## RESULTS AND DISCUSSION

### Separation

*meso*-Erythritol, glycerol, propylene glycol, ethanol, and other alcohols and glycols were separated using a Dionex IonPac ICE-AS1 (4 × 250 mm) ion exclusion column with 100 mM methanesulfonic acid at 0.2 mL/min and 30 °C for 30 min. Analytes were determined by PAD using a platinum working electrode and a three-potential waveform (Table 1).<sup>1</sup> ICE uses a fully sulfonated resin in conjunction with a strong acid eluent to exclude strongly ionic compounds by Donnan exclusion, and large compounds by steric exclusion. Small and neutral compounds are separated by adsorption partition.<sup>3</sup> Thus ICE excludes strongly ionic compounds, disaccharides, and polysaccharides which are typically present in fermentation broths. This allows better resolution of alcohols and glycols in the samples. See Application Note 188<sup>2</sup> for more detailed information.

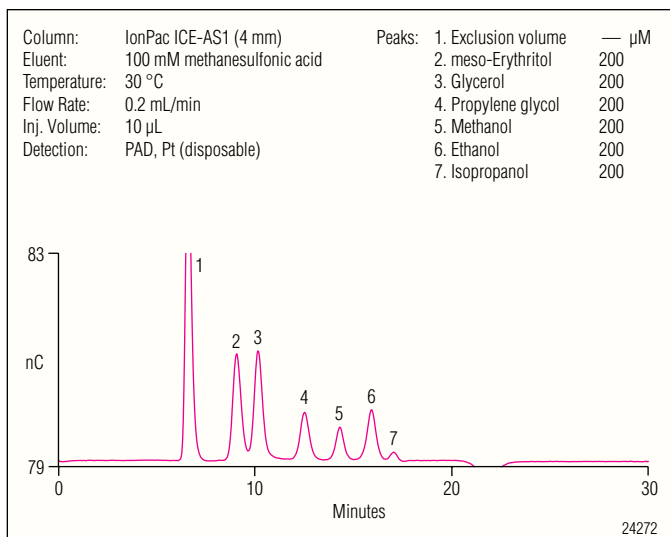


Figure 1. Separation of meso-erythritol, glycerol, propylene glycol, ethanol, methanol, and isopropanol.

Figure 1 shows separation and determination of glycol and alcohol standards in water using this method.

The resolutions of ethylene glycol from propylene glycol and diethylene glycol are thoroughly discussed in Application Note 188.<sup>2</sup>

### Method Qualification

Linearity, noise, and estimated limits of detection (LOD) were determined for meso-erythritol, glycerol, propylene glycol, and ethanol in water (Table 2).

Table 2. Calibration and Estimated Limits of Detection <sup>a</sup>			
Analyte	Linear Range	r <sup>2</sup>	Estimated Limit of Detection μM (ng)
meso-Erythritol	25–2000	0.9996	1.9 (2)
Glycerol	25–2000	0.9996	1.6 (2)
Propylene glycol	25–2000	0.9996	4.2 (4)
Ethanol	25–6400	0.9998	4.5 (2)

<sup>a</sup> Estimated limits of detection is defined as 3 times S/N

10 μL injection, n = 5.

Noise was determined over 60-min runs in 1-min intervals (from 5 to 60 min) without sample injection. Noise averaged  $8.0 \pm 2.7$  pC (n = 10) between three different disposable working electrodes.

Figure 2 shows a 300-fold dilution of the supernatant from American Wheat beer *S. cerevisiae* fermentation broth at time zero. The dilution factor was selected to provide the strongest response of ethanol and glycerol while minimizing column overload.

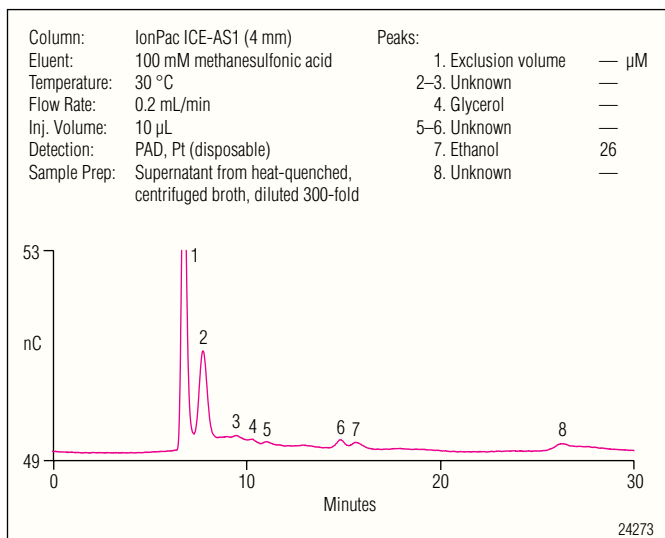


Figure 2. American wheat *S. cerevisiae* fermentation broth.

Figure 3 shows the American Wheat *S. cerevisiae* sample after incubating ~28 h with dilution identical to the time zero fermentation sample.

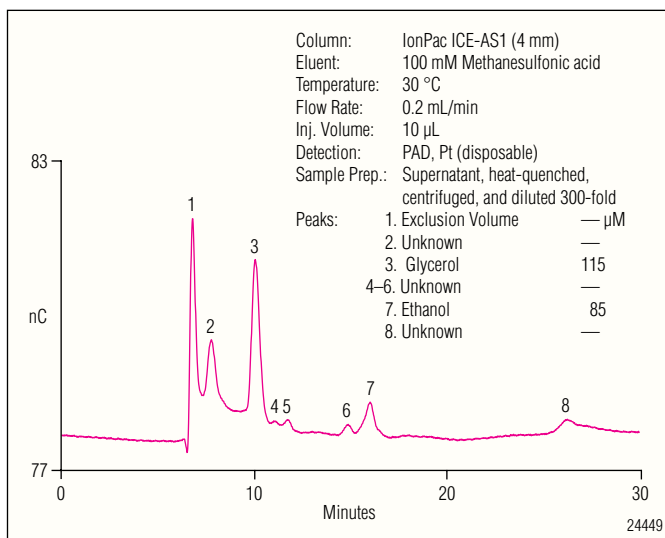


Figure 3. American wheat *S. cerevisiae* fermentation broth incubated for 28 h.

The retention time and peak area reproducibilities of glycerol, propylene glycol, and ethanol in 300-fold dilution of supernatant from American Wheat beer *S. cerevisiae* broth were determined over five days. Figure 4 shows that the retention times are stable.

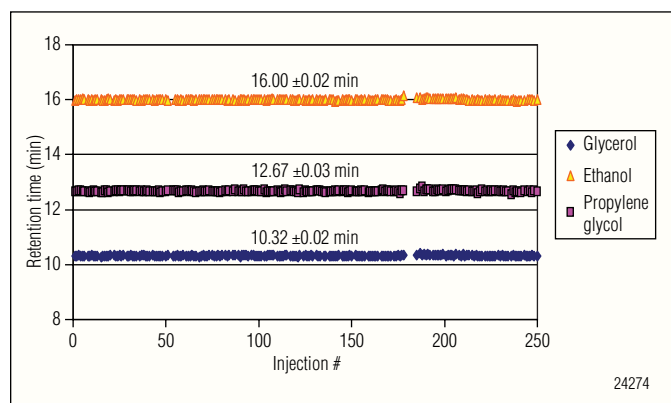


Figure 4. Retention time stability of 50  $\mu\text{M}$  glycerol, propylene glycol, and ethanol spiked into a 300-fold dilution of the supernatant from heat-quenched and centrifuged American Wheat *S. cerevisiae* incubated in Bacto YPD.

Figure 5 and Table 3 show the peak area stabilities on a daily basis. Glycerol and ethanol had peak area reproducibilities of RSD < 2 per day. The peak area reproducibilities of propylene glycol peak areas had RSDs < 8% per day.

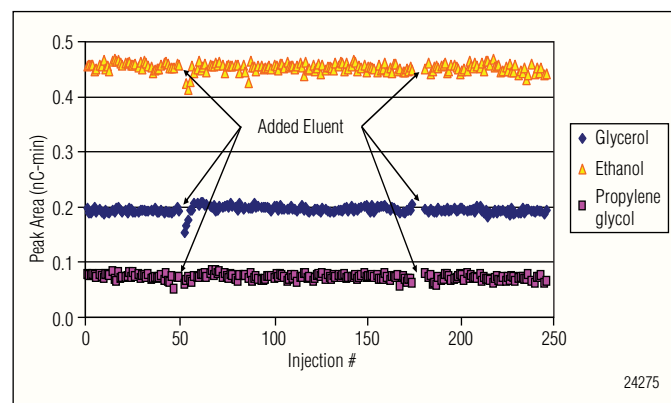


Figure 5. Peak area stability of 50  $\mu\text{M}$  glycerol, propylene glycol, and ethanol spiked into a 300-fold dilution of the supernatant from heat quenched and centrifuged American Wheat *S. cerevisiae* incubated in Bacto YPD.

Table 3. Peak Area Reproducibilities for Glycerol, Propylene Glycol, and Ethanol Spiked into 300-Fold Dilute Sample <i>S. cerevisiae</i> Incubated in Bacto YPD Fermentation Broth			
Day	Glycerol (nC-min)	Propylene Glycol (nC-min)	Ethanol (nC-min)
1	0.193 $\pm$ 0.003	0.073 $\pm$ 0.005	0.457 $\pm$ 0.006
2	0.200 $\pm$ 0.004	0.074 $\pm$ 0.005	0.453 $\pm$ 0.008
3	0.196 $\pm$ 0.003	0.073 $\pm$ 0.004	0.454 $\pm$ 0.006
4	0.195 $\pm$ 0.004	0.071 $\pm$ 0.006	0.452 $\pm$ 0.006
5	0.193 $\pm$ 0.004	0.070 $\pm$ 0.005	0.451 $\pm$ 0.006

### Disposable Platinum Working Electrodes

Peak responses of 50  $\mu\text{M}$  glycerol, propylene glycol, and ethanol standard were compared during the course of the experiments, using three disposable platinum working electrodes from the same lot. The working electrode was replaced with a new electrode when the peak response fell to 80% of the original response measured (1 h after installation). The three working electrodes had similar initial responses for glycerol (0.193  $\pm$  0.004, 0.189  $\pm$  0.013, and 0.194  $\pm$  0.002 nC-min), propylene glycol (0.072  $\pm$  0.005, 0.073  $\pm$  0.003, and 0.072  $\pm$  0.003 nC-min), and ethanol (0.456  $\pm$  0.007, 0.456  $\pm$  0.007, and 0.454  $\pm$  0.006 nC-min [n = 5]). The electrodes showed good reproducibility within the same lot (RSDs < 1.4). All three electrodes exceeded the two-week lifetime specification.

### Fermentation Samples

#### Method Applied to Fermentation Samples and Wine and Beer

Figures 6–7 show the concentrations of ethanol and glycerol determined in sampling intervals of Bohemian lager and German ale with *S. cerevisiae* incubated in YPD fermentation media. Growth patterns show an exponential increase of ethanol during the growth of *S. cerevisiae*, and a decrease at ~25 h as the yeast begin to die. Glycerol appears late in the incubation process, likely as a result of anaerobic fermentation.

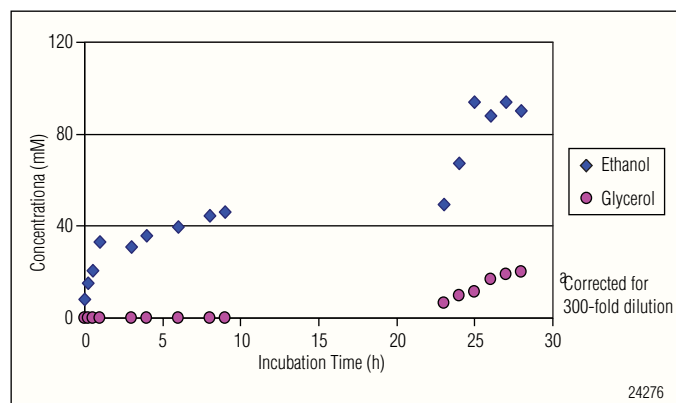


Figure 6. Ethanol and glycerol concentrations during incubation of German ale *S. cerevisiae* in Bacto YPD growth medium.

## 4 Determination of Glycols and Alcohols in Fermentation Broths by Ion-Exclusion Chromatography with Pulsed Amperometric Detection

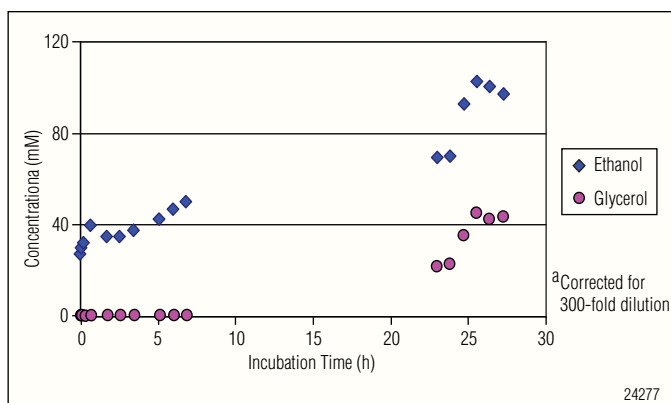


Figure 7. Ethanol and glycerol concentrations during incubation of Bohemian lager *S. cerevisiae* in Bacto YPD growth medium.

Figure 8 shows the Bohemian lager *S. cerevisiae* incubated for 27 h unspiked and spiked with 60  $\mu\text{M}$  glycerol, and 100  $\mu\text{M}$  propylene glycol and ethanol.

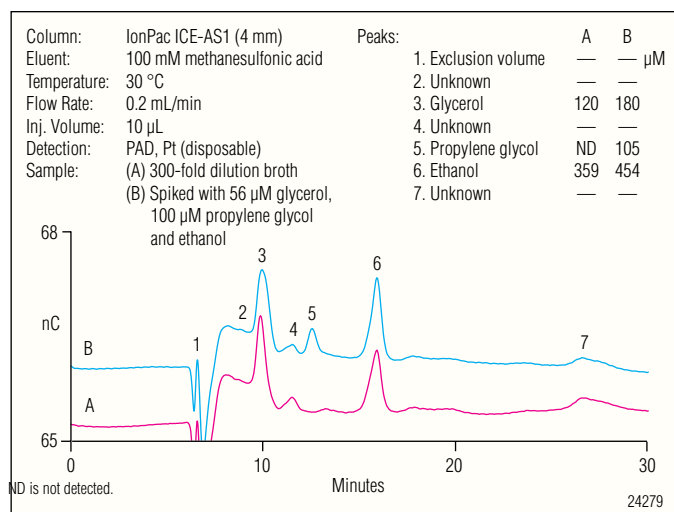


Figure 8. Bohemian lager *S. cerevisiae* incubated in Bacto YPD medium for 27 h.

Table 4 shows recoveries for glycerol (99.8–101.3 %), propylene glycol (95.0–98.0%), and ethanol (94.5–100.6%) in the fermentation broth samples.

Glycerol and ethanol concentrations were determined in 400-fold dilutions of American, British, and German beer samples and American wine samples. Figure 9 shows the chromatogram of 400-fold dilution of a German lager spiked with glycerol and ethanol.

Figure 10 shows the chromatogram of 400-fold dilution of an American white wine (Chardonnay) sample.

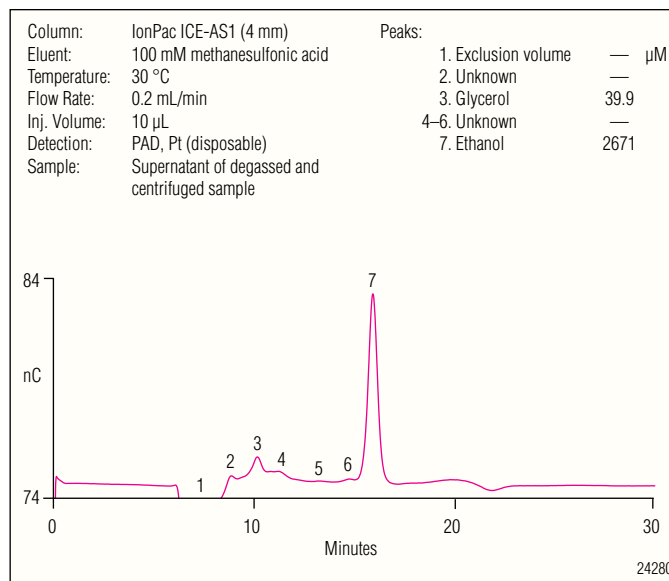


Figure 9. 40  $\mu\text{M}$  glycerol and 400  $\mu\text{M}$  ethanol spiked into a 400-fold dilution of German lager.

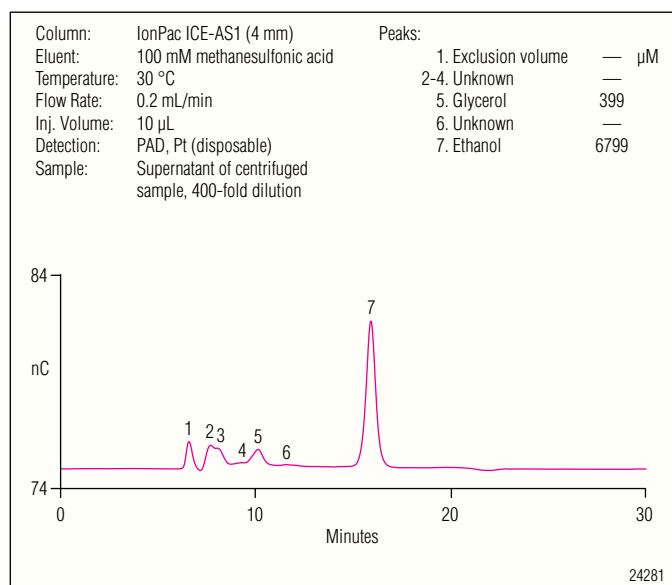


Figure 10. Glycerol and ethanol in Chardonnay.

**Table 4. Recoveries of Glycerol, Propylene Glycol, and Ethanol Spiked into 300-Fold Diluted Samples of *S. cerevisiae* Incubated in Bacto YPD Fermentation Medium**

<i>S. cerevisiae</i> Sample	Incubation (h)	Glycerol			Propylene Glycol			Ethanol		
		Unspiked ( $\mu\text{M}$ )	Spiked ( $\mu\text{M}$ )	Recovered (%)	Unspiked ( $\mu\text{M}$ )	Spiked ( $\mu\text{M}$ )	Recovered (%)	Unspiked ( $\mu\text{M}$ )	Spiked ( $\mu\text{M}$ )	Recovered (%)
Brand A, German Ale	5	ND	55.8 $\pm$ 1.8	99.8	ND	105.9 $\pm$ 2.5	96.2	291.4 $\pm$ 0.7	375.4 $\pm$ 3.1	94.5
Brand A, German Ale	26	55.3 $\pm$ 0.1	112.4 $\pm$ 1.0	101.1	ND	107.9 $\pm$ 1.6	98.0	293.8 $\pm$ 6.3	402.3 $\pm$ 0.6	100.6
Brand A, Bohemian Lager	7	ND	56.1 $\pm$ 0.5	100.4	ND	105.2 $\pm$ 2.7	95.5	347.9 $\pm$ 1.3	442.9 $\pm$ 1.3	97.6
Brand A, Bohemian Lager	27	120.3 $\pm$ 2.3	178.5 $\pm$ 2.4	101.3	ND	104.6 $\pm$ 1.0	95.0	358.6 $\pm$ 2.6	456.3 $\pm$ 1.4	97.4

Table 5 shows recoveries of glycerol (93.3–101.3%) and ethanol (94.5–100.6%) from the beverage samples.

Table 5. Recovery of 40 $\mu\text{M}$ Glycerol and 400 $\mu\text{M}$ Ethanol Spiked into 400-Fold Diluted Samples of Wine and Beer						
400-fold Diluted Beverage Sample	Glycerol			Ethanol		
	Unspiked ( $\mu\text{M}$ )	Spiked ( $\mu\text{M}$ )	Recovered (%)	Unspiked ( $\mu\text{M}$ )	Spiked ( $\mu\text{M}$ )	Recovered (%)
Domestic Chardonnay	399 $\pm$ 4	430 $\pm$ 5	97.9	6799 $\pm$ 67	7183 $\pm$ 51	99.8
Domestic Cabernet Sauvignon	374 $\pm$ 6	409 $\pm$ 6	98.8	6391 $\pm$ 49	6801 $\pm$ 114	100.1
German Lager	ND	39.9 $\pm$ 2	99.8	2257 $\pm$ 32	2671 $\pm$ 26	100.5
Domestic Hefeweizen	ND	39.6 $\pm$ 1	100.5	2365 $\pm$ 78	2765 $\pm$ 14	100.0
British Brown Ale	ND	40.5 $\pm$ 1	101.3	1924 $\pm$ 34	2296 $\pm$ 43	98.9

## CONCLUSION

- The chromatography method demonstrated here can be used to determine and quantify glycols and alcohols in beverages in complex matrixes such as fermentation broths.
- This method offers a sensitive direct detection of alcohols and glycols without the need for sample preparation other than dilution and centrifugation.
- For additional information regarding applications related to fermentation broths see Dionex Application Notes 117, 122, 123, 150 and 188.<sup>2,4</sup>

## REFERENCES

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2. Dionex Corporation. Application Note 188; LPN 1947. Sunnyvale, CA, USA 2007.
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\* Corresponding author. Terri T. Christison, Senior Applications Chemist, Dionex Corporation, 1214 Oakmead Parkway, Sunnyvale, CA, 94088-3603, USA; Phone: (408) 481-4217, Email: terri.christison@dionex.com

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### Dionex Corporation

1228 Titan Way  
P.O. Box 3603  
Sunnyvale, CA  
94088-3603  
(408) 737-0700

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