APPLICATION NOTE

Gas Chromatography
High-Throughput Analysis

AUTHORS

Nick D.M.Z. Wilton
Thomas Dillon

PerkinElmer, Inc.,
Shelton, CT USA

HS-GC-FID: Survey of Ethanol Content in Fermented Food and Drink

Introduction

Alcohol fermentation is the metabolic process by which organisms, particularly yeasts, convert sugars (e.g., glucose, fructose, & sucrose) into ethanol and CO_2 under anaerobic conditions. In addition to their inebriating effects, fermented foods are prized for their complex flavor profile—a result of the combination of yeast metabolome with feedstock biomass. Once bottled and packaged, many foods, especially unpasteurized foods and probiotics, may continue to ferment, increasing alcohol content with time.

In the United States, beverages are considered non-alcoholic if they possess an ethanol concentration ≤ 0.5% vol/vol[1], while in Canada this value is ≤ 1.1%[2]. In the European Union, beverages exceeding 1.2% vol/vol alcohol are labeled differently from those beneath this value[3], while in the Muslim world naturally-fermented foods ≤ 1.0% ethanol can be considered Halal[4]. These thresholds are important, as the sale of alcoholic beverages is regulated and taxed differently than non-alcoholic ones. Other food products, such as fruits and sauces, may contain alcohol as well from natural fermentation.

PerkinElmer®
For the Better

The PerkinElmer GC 2400™ System with flame ionization detector and PerkinElmer HS 2400™ Headspace Sampler (HS-GC/FID) is an ideal workflow solution for the rapid, sensitive quantification of ethanol in food. The reliable HS 2400 HS-GC/FID is capable of queuing up samples in advance of injection, and the precise pneumatics of the HS 2400 and GC 2400 means that ethanol can be accurately quantitated over a wide range of concentrations. The Elite-BAC1 Advantage column offers low noise thresholds for sensitive, robust quantitation in complex food matrices. This application note will expand upon the tried-and-true, high-throughput ethanol in food method first developed at PerkinElmer[5], analyzing for alcohol content in a variety of commercially available beverages and foods, including fruit juice, soy sauce, and kombucha. Data acquisition and processing was performed with PerkinElmer SimplicityChrom™ Chromatography Data System (CDS) Software. The detachable touchscreen interface allows for intuitive, high-throughput laboratory workflows and the real-time monitoring of data, anywhere the operator is connected to the VPN.

Experimental

Table 1. Instrument operating conditions.

System		Part Numbers
Gas Chromatograph	PerkinElmer GC 2400 System with HS 2400 Headspace Sampler	--
Injector	Capillary Split/Splitless (CAP)	--
	Advanced Green Inlet Septum	N9306218
	1 mm Ultra Deactivated Straight Inlet Liner, no Wool	N6502037
Detector	Flame Ionization Detector (FID)	--
	Grade 5 Hydrogen, 30 ml/min	--
	Grade 5 Air, 400 ml/min	--
	Grade 5 Nitrogen, 25 ml/min	--
Gas Filters	Triple Filter (Hydrogen & Nitrogen)	N9306110
	Moisture/Hydrocarbon Trap (Air)	N9306117
Analytical Column	Elite-BAC1 Advantage Capillary Column; 30 m x 0.32 mm x 1.80 µm	N9315071
Software	SimplicityChrom CDS Software	
Headspace Conditions		
Temperatures	60 °C Oven, 110 °C Needle, 120 °C Transfer Line	
Pressure	Grade 5 Nitrogen Gas, 16 psi Pressure	
Timings	12 min Thermostat, 1 min Pressurizing, 0.04 min Injection, 0.3 min Withdraw	
Transfer Line	2.0 m 0.32 mm I.D. Fused Silica	
Options	Operative Mode: Constant, Inject Mode: Time	

Table 1. Instrument operating conditions. Continued...

System		Part Numbers
GC Conditions		
Carrier	Grade 5 Hydrogen, 12 psi	
Septum Purge	3 ml/min	
Split	5 ml/min	
Detector Temp	250 °C	
Oven	45 °C Isothermal	

The PerkinElmer GC 2400 System with HS 2400 Headspace Sampler.

Stock Standard Preparation

200 proof ethanol standard and pure t-butanol internal standard (IS) were both purchased from Millipore Sigma (Burlington, MA). Deionized water diluent was obtained from a filtration system within the laboratory. Stock ethanol standard was prepared by diluting 1.0 ml of ethanol in DI water volumetrically. Similarly, t-butanol IS was prepared by volumetrically diluting 20.0 ul of pure t-butanol into 100 ml of DI water. Weights were used when measuring and these values were converted to volume using the density of ethanol at room temperature (0.789 g/ml). Thus, stock standards of exactly 0.994% vol/vol ethanol, and 0.199% vol/vol t-butanol were prepared.

Calibration Standard Preparation

Calibration was performed using static headspace methodology. The stock standard of ethanol was serially diluted into 2.0 ml autosampler vials at a 1:1 ratio with DI water until a total of 10 standards were produced ranging from ~1.0% to ~0.0020% vol/vol. Headspace calibration standards were made in 22.0 ml headspace vials by adding 0.750 ml IS solution to 0.075 ml of each of the ten aforementioned serial dilutions. After analyzing each ethanol standard, linear regression was assessed as well as the response factor (RF) according to the equation:

$$RF = \frac{Area_{Ethanol} / Concentration_{Ethanol}}{Area_{IS} / Concentration_{IS}}$$

Sample Preparation

Fruit juices, i.e., apple, orange, and pomegranate juice, were purchased from a local market. Two brands of kombucha were also obtained, as well as a soy sauce from the local market. Sample preparation was straightforward and analogous to calibration standard preparation. In brief, 0.750 ml IS and 0.075 ml of each liquid sample were added to a 22 ml headspace vial. Kombucha samples were allowed to effervesce before pipetting to maintain volumetric accuracy. If a sample was too concentrated for our calibration curve it was diluted with DI water and the resultant concentration multiplied by this volumetric dilution factor.

Method Detection Limit (MDL) Study

Calibration standard 10 was made and analyzed a total of 7 times. Its concentration was determined in each analysis and the standard deviation was multiplied by the one-tailed Student's t value at 6 degrees of freedom to empirically determine the MDL. The upper (UCL) and lower confidence limits (LCL) were determined by multiplying the MDL by 0.64 and 2.2 respectively.

Results & Discussion

System Performance

Calibration was exceptionally linear over the concentration range analyzed. Figure 1 presents the curve for ethanol by HS-GC/FID. Linear regression begat a R^2 of 0.9999, indicating high system performance. The response factor (RF) also showed good precision as represented by the relative standard deviation (RSD) of 3.44% shown in Table 2. Chromatograms are presented for Standard 1 (most concentrated) in Figure 2A and Standard 10 (least concentrated) in Figure 2B. Figure 2C shows an inset of Standard 10, highlighting the acceptable signal/noise ratio at the lowest concentration analyzed.

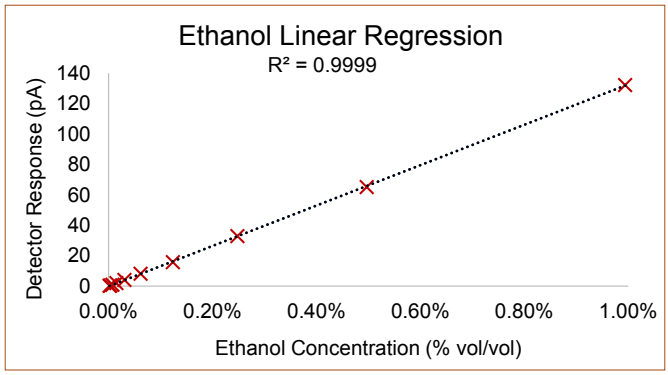

Figure 1. Calibration curve for ethanol by headspace GC/FID.

Table 2. Response Factor and system performance parameters for ethanol calibration against t-butanol internal standard.

Ethanol:t-Butanol RF	RF Standard Deviation	RF RSD
0.234	0.0080	3.44%

The ethanol method detection limit (MDL) was determined from analysis of calibration standard 10 performed 7 times on different headspace vials. Table 3 lists the MDL, LCL and UCL of ethanol. These results illustrate that ethanol quantitation using the PerkinElmer GC 2400™ with Elite-BAC1 Advantage capillary GC column is sensitive, in addition to being highly robust.

Table 3. MDL, LCL, and UCL for ethanol by HS-GC/FID.

MDL	LCL	UCL
0.000273%	0.00017%	0.00060%

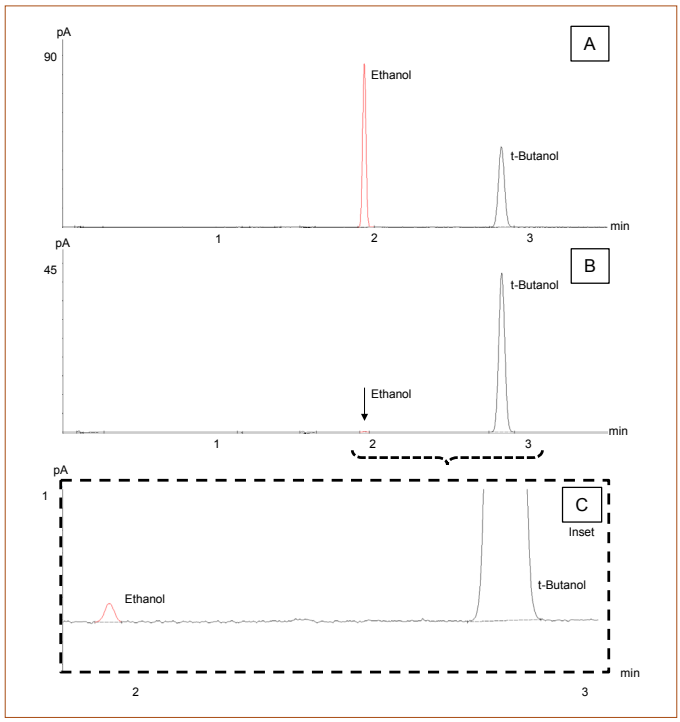

Figure 2. Calibration standard chromatograms of (A) ~1.0% vol/vol standard, (B) ~0.0020% vol/vol standard, and (C) inset of low-level standard highlighting signal/noise at low end of calibration curve.

Sample Quantitation

Three commercial juices, two kombucha brands, and one soy sauce were each measured for alcohol content. Table 4 offers the results for these items. Pomegranate juice contained a barely-detectable quantity of ethanol, while soy sauce contained the most. Since soy sauces' concentration was above the calibration curve limit, it was diluted volumetrically in DI water at a ratio of 1:4 soy sauce:water and the measured value was multiplied by this dilution factor. Interestingly, despite being purchased within the United States, one of the two kombucha brands exceeded the US regulatory limit for ethanol by 0.22% vol/vol, nearly 50% over the limit of 0.50% vol/vol. This is possibly due to post-bottling fermentation from the active microbial colony.

Table 4. Results of the survey of foods from a local market for ethanol content.

Sample	Ethanol % vol/vol
Apple Juice	0.045%
Orange juice	0.005%
Pomegranate Juice	< Limit of Quantitation
Kombucha Brand 1	0.719%
Kombucha Brand 2	0.140%
Soy Sauce	3.162%

A Note About Ionic Strength

A sample with a large salt content possesses high ionic strength. This strength has the effect of increasing the gas-phase partitioning of organic compounds from solution. The technique of adding salt to solution is a common method of increasing sensitivity in headspace analysis[6]. This can reduce quantitative accuracy for organic compounds in especially salty samples when no salt was used in calibration.

For example, t-butanol internal standard response was remarkably consistent in every standard and nearly every sample except for soy sauce, which is very salty. The average of 16 calibration standards (including 7 MDL tests of calibration standard 10) was precise, producing a t-butanol response of 113.8 ± 1.7 pA, with values ranging from 111.0 to 116.1 pA. Fruit juice and kombucha samples, too, produced internal standard responses within this range.

On the other hand, the undiluted soy sauce sample is very salty (960 mg/tbsp) and produced a t-butanol response of 125.8 pA, or 10.5% above the aforementioned average. If one uses RF to quantitate ethanol, this ionic strength deviation will skew the resultant ethanol concentration. When soy sauce was diluted at a 1:4 ratio with DI water, its ionic strength lowered and the diluted sample produced an internal standard response of 115.9—within the range of expected values. When quantifying high ionic strength samples, be sure to account for deviations in partition coefficient by comparing sample internal standard response against those from the calibration curve. Diluting a salty sample with DI water may be an appropriate remediation even when the sample concentration falls within the calibration curve.

Conclusion

The PerkinElmer GC 2400 System is the ideal choice for fast, robust, and sensitive analysis of alcohol in food. Calibration from ~1.0% vol/vol to ~0.0020% vol/vol obtained excellent linearity (R^2 = 0.9999), with method sensitivity down to 0.00027% vol/vol. A variety of liquid samples were successfully analyzed for alcohol composition such as fruit juices, kombucha, and soy sauce. In addition to aiding quantitation, t-butanol internal standard acted as an indicator of the impacts of ionic strength on the static headspace partitioning of organics.

References

1. United States Code of Federal Regulations. 27 C.F.R. § 25.11
2. Government of Canada. Labelling Requirements for Alcoholic Beverages. 2021.
3. European Union. Regulation No. 1169/2011. 2011.
4. Jawad Alzeerab and Khaled Abou Hadeed. Ethanol and its Halal Status in Food Industries. Trends Food Sci. & Technol. 2016. 58: 14-20.
5. Nathan Kuffel and Timothy Ruppel. A Method for the Quantification of Ethanol Content in Consumable Fruit Juices by Headspace Injection. PerkinElmer Inc.
6. Emma Hollender and Edward W. Hammersley. Ionic Liquids: Sensitivity Enhancement in Headspace Gas Chromatography. Chromatography Today. 2011. 30-33.

PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com