Headspace Gas Chromatography Isotope Ratio Mass Spectrometry (HS-GC-IRMS) Determination of Stable Carbon Isotope Ratios of Ethanol in Alcohol Beverages

Scope and Application

This method is used to determine the stable carbon isotope ratio of ethanol in alcohol beverages with an alcohol content at or between ~5 – 100 % alcohol by volume, and may also be used for any other product that contains ethanol (flavors, gels, denatured alcohol, etc.). ^{13}C abundances in the ethanol may be used to determine the agricultural source of the ethanol in products containing ethanol.

For example,
Sake: Sake which contains alcohol produced solely by fermentation is taxed as a malt beverage. Sake which contains any alcohol produced by distillation is taxed as a distilled spirit. Sakes which are produced from rice (a C3 plant) fortified with alcohol not derived from a C3 plant type (e.g. sugar cane or brewer's alcohol) may have a relative ^{13}C abundance that is distinct from rice and discernable by HS-GC-IRMS.
Enforcement Refill Samples: This method may be useful for suspected refills involving distilled spirits. When contrasted to a commercially available reference sample, the ^{13}C abundance of a suspected refill may be used as a probe of the agricultural source and therefore the authenticity of the product.

Regulatory Tolerances:
When sake is fortified with distilled spirits, the tax classification of the product changes from a beer to a distilled spirit.

Levels and Limitations

This method can be used to distinguish between the plant type groups, but cannot distinguish a positive identification of one particular plant within the plant type groups [please refer to Table 1]. For example, this method can detect the addition of rum made from cane sugar to sake, but it cannot detect the addition of vodka made from potatoes to sake.

Table 1

Plant Type	δ ^{13}C values (‰)*	Examples
C3 (95% of plants are C3)	-30 to -23	grape, rice, barley, sugar beet, molasses made from sugar beet, coconut, palm, peanut, plum, potatoes, rye, soybean, sunflower, wheat
C4 (1% of plants are C4)	-10	sugar cane, corn, millet, sorghum, molasses made from sorghum
CAM	-18 to -12**	vanilla, agave, cactus, pineapple

* These ranges are approximations.
**Results in this range can also indicate a mixture of C3 and C4 plant origin alcohols.

C3, C4, and CAM refer to distinct photosynthetic pathways used by certain types of plants. The name C3 comes from the fact that CO_2 is first incorporated into a 3-carbon compound. Likewise, the name C4 comes from the fact that CO_2 is first incorporated into a 4-carbon compound. CAM stands for Crassulacean Acid Metabolism, and its name comes from the first plant family in which the pathway was discovered. In the CAM pathway, the CO_2 is first stored as an acid before use. The differences among the photosynthetic pathways lead to the different $^{13}C/^{12}C$ ratios essential to this method.

The results of carbon analysis are the ratios of $^{13}C/^{12}C$, referred to as “delta 13 C” and reported as ‰ “per mil”, which are normalized against standard ratios. Therefore, different concentrations of alcohol in samples and/or different injection volumes do not make a difference in the ratio obtained for samples.

The international standard for $^{13}C/^{12}C$ is Vienna-Pee Dee Belemnite (VPDB), which has a $^{13}C/^{12}C = 1.1237x10^{-2}$. The final $\delta\ ^{13}C$ (‰) results produced by BAL have been normalized against isotope reference standards that have been characterized in relation to VPDB.

Supplemental Documents

WG:SSD:210:001 Storage and Handling of Reference Standards and LCS for IRMS (Formerly NLC:WG:210-2)
WG:SSD:210:002 How to Reduce Data for Headspace Ethanol Analysis in TM-210
WG:IRMS:001 Special Instructions - How to Put the IRMS Back into Service (Formerly BAL:WG:577)
WG:IRMS:002 IRMS Shutdown and Startup Procedures (Formerly BAL:WG:578)
WG:IRMS:003 Changing Gas Cylinders for IRMS (Formerly NLC:WG:210-1)
WG:IRMS:004 IRMS On/Off and Linearity Instructions using ConFlo (Formerly BAL:WG:605)
WG:IRMS:005 Regeneration of the Combustion Reactor (IRMS) (Formerly BAL:WG:604)
Form:SSD:210:001 IRMS Single-Injection Spreadsheet (Formerly BAL:Form:210g)
Form:SSD:210:002 IRMS QC Control Chart Table (Formerly BAL:Form:210e)
Form:IRMS:001 Gases Log for IRMS Instruments (Formerly BAL:Form:210f)
Form:IRMS:002 IRMS CARBON Daily Preparation (Formerly BAL:Form:210b)
Form:IRMS:003 IRMS On/Off Standard Deviations (Formerly BAL:Form:210d)
Form:IRMS:004 IRMS 2 Daily Log Page (Formerly BAL:Form:210a)
Form:IRMS:005 IRMS Maintenance Sheet (Formerly BAL:Form:210h)

Equipment

Instrumentation (equivalents may be used):

GC:	Thermo Trace GC Ultra		
Autosampler:	CTC Analytics PAL System	or	Thermo TriPlus
Syringe:	Gastight 100µL syringe for CTC autosampler, MicroLiter Analytical Supplies, Inc. product number CL-G10026S-AS	or	Gastight 100µL syringe for Thermo TriPlus autosampler, Thermo part number 36520050 (Fisher part number 03-170-520, side hole in needle) or 36500495 (Fisher part number 03-170-509, cone tip needle)
IRMS:	Thermo Delta V Plus		
Interface:	Thermo GC Combustion III and ConFlo IV	or	Thermo GC Isolink and ConFlo IV
Mode:	Continuous Flow, CO_2 configuration		
GC Conditions:	**DB-5**	or	**WAXETR ("FO")**
Column:	J&W Scientific DB-5 30m, 0.25mm ID, 0.25µm film		J&W Scientific DB-WAXETR, 30m x 0.53mm x 1µm film
Carrier Gas:	Helium, constant flow, 1.2 mL/min		Helium, constant flow, 5.9 mL/min
Temperature:	35°C for 2 min, 30°C/min to 100°C, ~4.5 min total FID** 250°C, air 350mL/min, hydrogen 35 mL/min, makeup 30 mL/min		40°C for 5 min, 5°C/min to 100°C, ~17 min total FID** 250°C, air 375mL/min, hydrogen 40 mL/min, makeup 30 mL/min
Injector:	Ethanol retention time ~150 seconds*** (~1.1 relative retention time between ethanol peak and ambient peak) Inlet: split, split flow 18mL/min, split ratio 15, 250°C		Ethanol retention time ~440 seconds*** (~3.2 relative retention time between ethanol peak and ambient peak) Inlet: split, split flow 30mL/min, split ratio 5, 220°C
	100 µL syringe, ambient air as “wash solvent”, 1 wash cycle before injection, three wash cycles after injection.		
Other Gases:	Oxygen, to regenerate the oxidizing reactor		
	Hydrogen and nitrogen and house air*, for the FID**		
Injection Volume:	90 µL of headspace		

*House air is also used for the pneumatic valves.

**While the FID is a useful source of information (for troubleshooting, for example), it is not required for this analysis.

***Retention time will vary (for example, as the column is clipped, with the autosampler's timing of the injection relative to reference pulses, etc.).

IRMS Conditions:

Gas Configuration:	CO2				
Integration Time:	0.200 sec				
Peak Center Pre-delay:	15 sec				
Peak Center Post-delay:	15 sec				
Cup:	3				
Autodilution Fixed Reference:	6000 mV				
Peak Detection:	checked				
Background Detection:	checked				
Detection on Mass:	44				
Background Parameter:	Individual BGD				
Perform Timeshift:	checked				
Acquisition Start:	Immediately				
Time Events:	**DB-5**		or	**WAXETR ("FO")**	
Time Seconds:	**Reference:**	**Backflush:**	**sec:**	**Reference:**	**Backflush:**
0		Green	**0**		Green
10	Green		**10**	Green	
30	Red		**30**	Red	
60	Green		**60**	Green	
80	Red		**80**	Red	
100		Red	**100**		Red
300		Green	**800**		Green
310	Green		**810**	Green	
330	Red		**830**	Red	
360	Green		**860**	Green	
380	Red		**880**	Red	
Acquisition End:	420 sec		or	900 sec	
Calculate on Peaks:	80, 330, 380 (sec)			80, 830, 880 (sec)	
Oxidize (if shown):	Red				
Switch Gas (if shown):	Blank				

Glassware and Supplies:

Standard laboratory glassware for dilutions
2 mL autosampler vials with Teflon-lined septa (silicone recommended over rubber, as silicone is easier for the needle to penetrate), National Scientific C4000-192W, or equivalent
200 µL micropipette and tips, VWR numbers 53513-408 and 47745-104, or equivalent

Reagent and Sample Preparation and Handling

Reagents: (Specific vendors and product numbers are listed for convenience. Equivalent products may be used.)

Standards normalized to an internationally accepted standard, at least one in the C4 range, one in the C3 range, and one Mix in between.
Deionized (DI) water, 18 megaohm or better, if needed for dilutions
Ethanol, 200 Proof, Pharmco-Aaper part number 111000200 (agricultural source) and 11100020SCSGG (synthetic source), optional for LCS preparation

Preparation of Stock, Intermediate, and Working Standards:

Dilute the stock standard to approximately 10-15% alcohol by volume.

For example, if the C4 standard is at 86% ABV, diluting 0.872 mL to 5 mL would be appropriate. Other appropriate dilutions may be used, depending on analyst discretion.

Pipet 200 µL standard into 2 mL autosampler vials for analysis.

The stock standards should be kept refrigerated (approximately 4 ºC) in amber bottles. Dilutions of standards can be kept pre-aliquoted into autosampler vials (also refrigerated). The $\delta\ ^{13}C$ values (‰) of the standards should not change over time; therefore, the standards should be suitable for use indefinitely; however, an expiration date needs to be assigned to satisfy ISO requirements.

Preparation of Laboratory Control Sample:

The Laboratory Control Sample (LCS) should have a value between those of the C4 and C3 standards, and the percent alcohol by volume should be ~15%. The LCS could be tequila, a mixture of other beverage alcohol samples, or a mixture of diluted absolute ethanol reagents, etc.; as long as the conditions in the previous sentence are satisfied.

It is preferable to store the LCS in multiple small bottles to limit any possible cross-contamination during the period of use. The LCS should be characterized and implemented according to ISO procedures.

The LCS should be kept refrigerated (approximately 4 ºC) in amber bottles. The LCS should be aliquoted, in duplicate, along with samples for analysis. The LCS may be used directly from the refrigerator, as the method is robust in regards to aliquot volume. Also, the ethanol in the aliquots comes to equilibrium in the headspace within ten minutes. The $\delta\ ^{13}C$

values (‰) of the LCS should not change over time; therefore, the LCS should be suitable for use indefinitely; however, an expiration date needs to be assigned to satisfy ISO requirements.

Preparation of Samples:

Note: Samples for which added alcohol produced by distillation is declared in the method of manufacture are not analyzed.

Pipet 200 µL neat sample into autosampler vials for analysis.

If required, dilute the sample to ~15% alcohol before aliquoting for analysis. It is also acceptable to dilute directly into the autosampler vial (for example, add 125 µL DI water and 75 µL 40% ABV sample directly into an autosampler vial, cap, and mix by shaking or vortex). For example, five shakes by hand is sufficient for mixing. Other dilution ratios are acceptable but the total aliquot in the vial after dilution should be 200 µL.

Make sure that all vial caps are finger-tightened (not loose) prior to analysis.

Procedures

Instrument Startup Procedures:

Please refer to the Working Guidelines, which can be found in the logbooks, for important instrument startup and shutdown information.

CAUTION: The IRMS is running at 3000V, and so is very dangerous for an untrained user.

Sample Analysis Procedures:

Reference standards are injected in singlet at the beginning and end of each run (bracketing standards), and after no more than 12 samples (also injected in singlet) within the same sequence. The LCS aliquots are treated as samples. The LCSs may be placed in any position in the sequence that is appropriate for a sample.

In order to add samples to a sequence once it has been started, the current run must be terminated. It is acceptable to add samples within 24 hours from the end of the last successful on/off and linearity test. If the run is terminated for any reason (house air outage, for example) after 24 hours from the last successful on/off and linearity test, a new on/off and linearity test must be performed before analyses can continue. Samples analyzed within bracketing standards may be reported if they meet the Quality Control parameters.

The analysis of sequences may last longer than 24 hours. A planned and uninterrupted sequence may continue as long as needed, and samples results may be reported if they meet the Quality Control parameters.

If samples are above the linear range, dilute into range and re-inject.

If samples are below the linear range (i.e., containing ~5% ABV or lower of ethanol), refer to WG:IRMS:004 for procedures on adjusting the ConFlo pressure.

Quality Control

1. An on/off test should be performed before analysis begins. Refer to the IRMS Logbook for instructions for on/off testing and appropriate results. Proceed only if logbook guidelines are met. Figure 1 shows a typical on/off chromatogram.

2. A linearity test should be performed after an on/off test and before analysis begins. Refer to the IRMS Logbook for instructions for linearity testing and appropriate results. Proceed only if logbook guidelines are met. Figure 2 shows a typical linearity chromatogram. Only those sample values resulting from peaks with amplitudes (44 mV) between the lowest and highest in the linear range may be reported.

3. Regression analysis of the entire run (or portion of the run to be used) must result in a standard error ≤ 1.5, the R Squared ≥ 0.99, and the difference between known and normalized values of standards ≤ 1.00 for the data to be acceptable.

4. Two aliquots of LCS are analyzed in each data set. The results must fall within the acceptable limits as described in the IRMS Logbook. Proceed according to laboratory procedures and IRMS Logbook instructions if a violative result occurs.

5. On occasion, the IRMS may misinject. If the chromatogram shows an obvious lack of a peak or very small peak, this will cause the result to be faulty and the sample will be re-injected in duplicate. Alternatively, if the replicates have a standard deviation >1.00, the sample will be re-injected in duplicate.

6. If results are unstable or unsatisfactory, GC and IRMS troubleshooting techniques should be employed. Figure 3 shows a typical analysis chromatogram using the DB5 column and conditions. Figure 4 shows a typical analysis chromatogram using the WAXETR column and conditions.

Sources of Uncertainty

1. Ethanol cross-contamination. Any ethanol residue from another source will alter the result of a sample analysis. Take care to ensure that all supplies are uncontaminated. (For example, store syringe tips and vials in closed containers, and aliquot away from the containers of tips and vials to avoid dripping on them.)

2. Room temperature fluctuations. For the IRMS to perform optimally, the room temperature must not be greater than 28°C, and the room temperature must not fluctuate more than 1°C per hour. Additionally, fluctuations in the room temperature affect the amount of alcohol in the headspace of the vials, thus changing the height of the alcohol peak while not changing the height of the reference peaks, which may lead to poor results.

3. Gastightness of syringe. If the syringe loses gastightness, the alcohol peaks may be too low to quantitate correctly, may fluctuate between being present and being absent, or may not be present at all.

4. Oxidizing the reactor. The reactor needs to be recharged with oxygen for the conversion of ethanol carbon to CO_2 which is detected by the IRMS. Quickly changing per mil values or quickly disappearing peaks can be indicative of lack of oxygen in the reactor. Instructions for oxidizing the reactor can be found in the instrument logbook.

5. IRMS agitation. The IRMS is very sensitive to physical contact while it is running. A peak may not be detected at all if the IRMS is agitated during analysis.

Calculations

Summary:

Isodat software calculates the δ 13C values (‰) relative to VPDB as follows:

$\delta\ ^{13}C\ (‰) = (R_{sample} – R_{VPDB} / R_{VPDB}) \times 1000$

where R = the isotope ratio $^{13}C/^{12}C$.

Data is exported to Excel for further manipulations. Please refer to the Working Guidelines, which can be found in the logbook, for step by step calculation instructions.

Reporting Results

The $\delta\ ^{13}C$ value (‰) is reported to two decimal places.

Results below the low end of the linear range are reported as Not Detected.

Only the data resulting from peaks with amplitudes that fall within the daily linear range should be reported. Please refer to the Quality Control section above.

Safety Notes

Normal laboratory safety protocol should be followed. Personnel should follow good laboratory practices.

Consult the MSDS for any chemicals used that are unfamiliar. All chemicals shall be considered hazardous - avoid direct physical contact.

High proof alcohol products are flammable. Ethanol burns with an almost invisible blue flame.

High voltage. Do not touch any parts of the instrument that are unfamiliar. Do not attempt any repairs if untrained to do so.

Helium can act as a simple asphyxiant by displacing air. Helium is colorless, odorless, and tasteless at normal temperature and pressure; therefore, adequate ventilation should be provided and cylinders should be protected against and checked for possible damage.

References

An Overview of Isotopic Analysis for the Control of Alcoholic Drinks and Spirits (A background and future perspective for the use of stable isotopes for the characterization of alcoholic beverages and alcohol in the European Union), European Commission Joint Research Centre and Institute for Health and Consumer Protection, 2005, p. 1-22.

Internal Revenue Code (2007) Title 26 section 5002, http://uscode.house.gov, accessed on 01/30/2023.

Saké Resources | TTB: Alcohol and Tobacco Tax and Trade Bureau, accessed on 11/28/2025.

http://wwwrcamnl.wr.usgs.gov/isoig/projects/fingernails/results/interpretdata.html, accessed on 1/30/2023.

Location of Validation Package

Quality System Files

Required Training, Certification and Re-certification.

1. In-house training by a certified chemist in IRMS and Isodat operation. The IRMS should only be operated by a well-trained person.

2. Initial competency is obtained by running 7 LCS with results in agreement with mean and standard deviation acceptance criteria.

3. Periodically, chemists are re-tested for competency (e.g., every 5 years) and/or given proficiency testing.

Revision History

Rev 1 – Initial Revision
Rev 2 - Added option for additional sample dilution to sample vial preparation and sample analysis procedure sections; added that all vial caps be finger-tightened prior to analysis; changed from performing triplicate injections to duplicate injections to sample analysis procedure section; made correction to standard error criteria and edits to standard deviation criteria in Quality Control section.
Rev 3 – Changed from performing duplicate to singlet injections for standards and samples and changed to re-inject standards after no more twelve samples (previously ten samples) in the sample analysis procedure section
Rev 4 - Updated related documents, reagents, % ABV range of samples without dilution, standards preparation example, reference links, and training certification requirements

Rev 5 – Changed document IDs to the new ID structure. Added a new WG. Updated preparation of standards. Fixed broken link.

Figure 1. Typical On/Off Test Chromatogram.

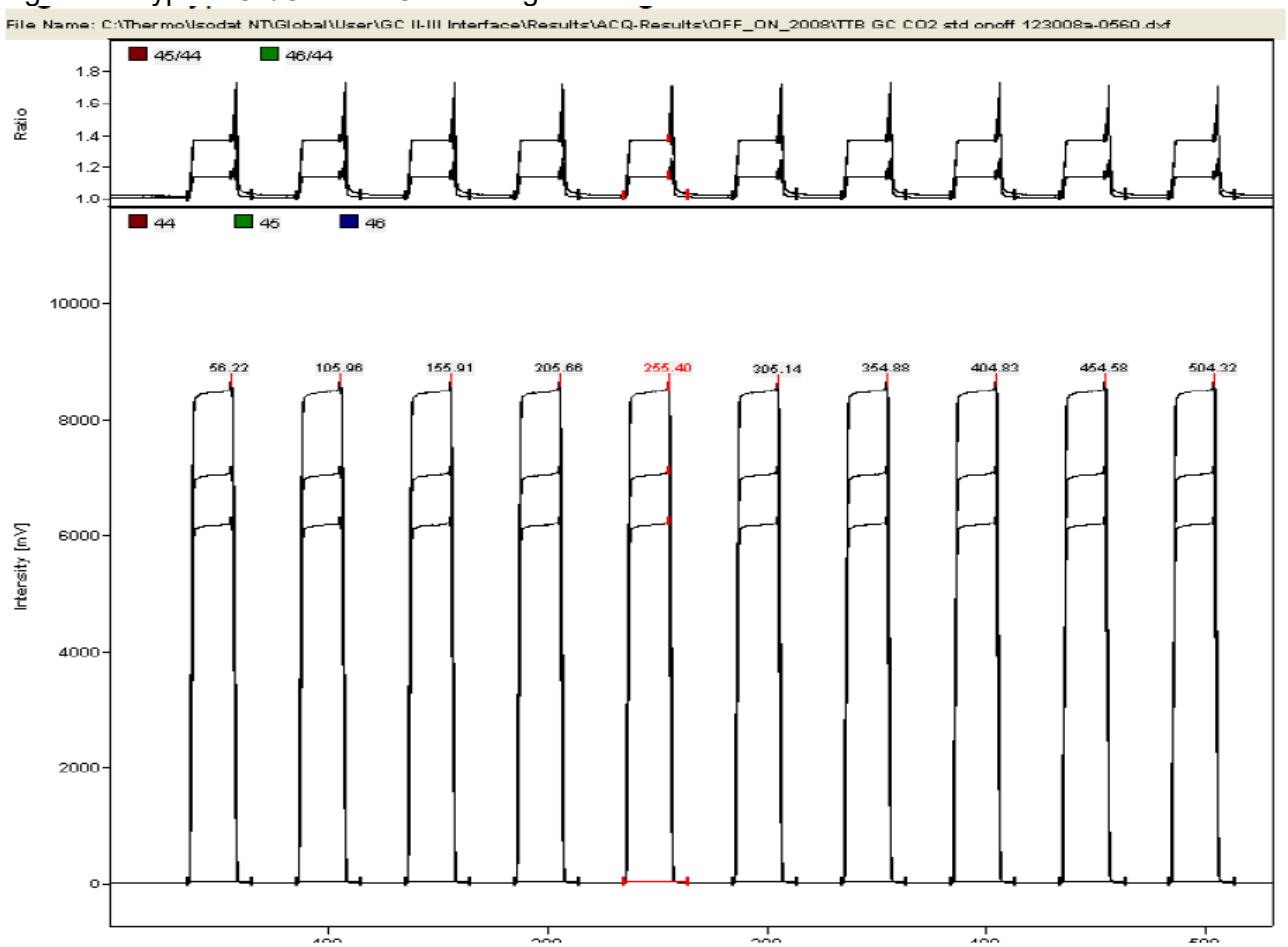

Figure 2. Typical Linearity Test Chromatogram.

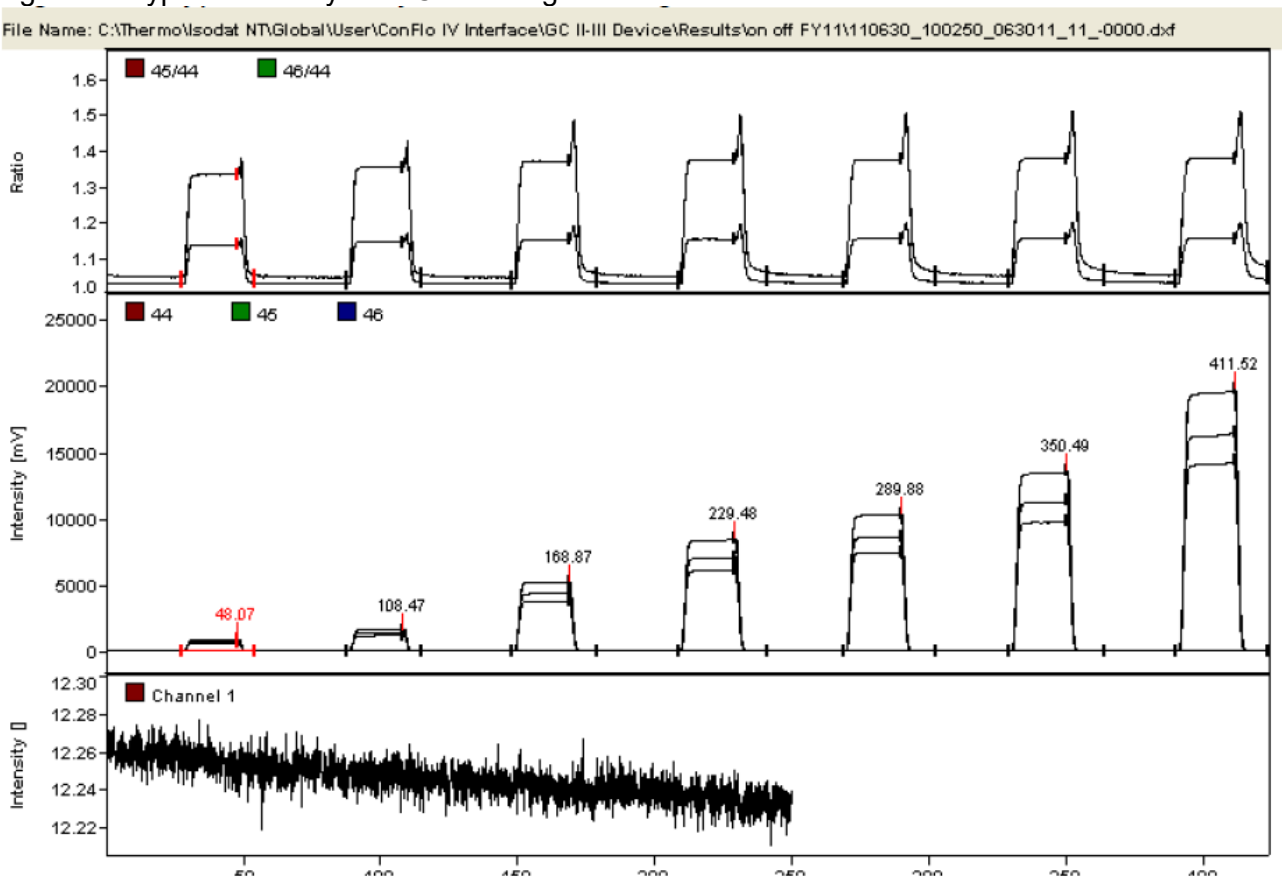

Figure 3. Typical DB5 Column and Conditions Chromatogram (15% ABV Vodka Standard).

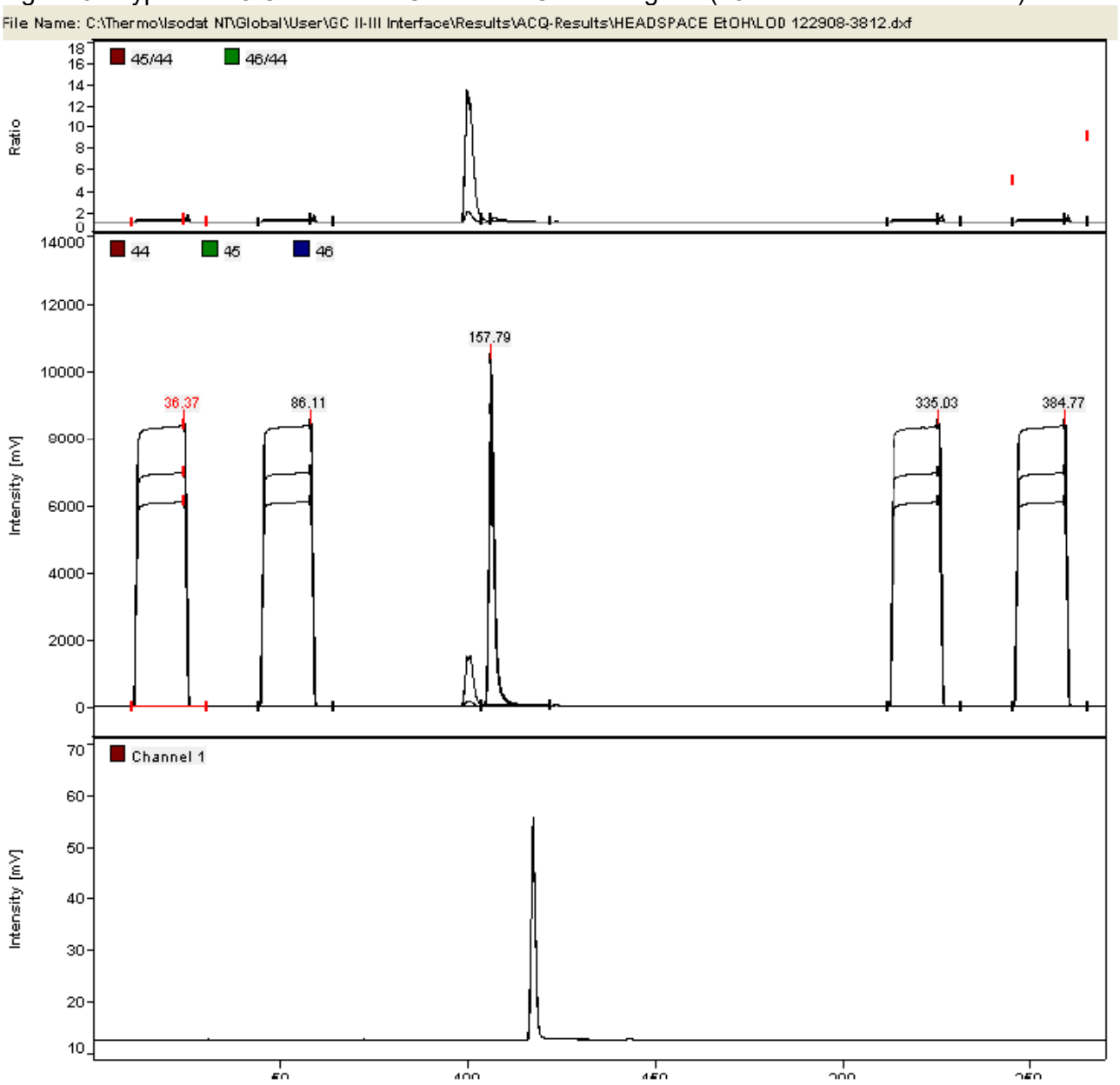

Figure 4. Typical WAXETR Column and Conditions Chromatogram (15% ABV Rum Standard).