Scientific Research Publishing

Food and Nutrition Sciences, 2023, 14, 90-100
https://www.scirp.org/journal/fns
ISSN Online: 2157-9458
ISSN Print: 2157-944X

Semi-Automated Enzymatic Determination of Ethanol in Beverages: Collaborative Study for RIDA®CUBE Ethanol

Markus Lacorn, Thomas Hektor

R-Biopharm AG, Darmstadt, Germany
Email: m.lacorn@r-biopharm.de

How to cite this paper: Lacorn, M. and Hektor, T. (2023) Semi-Automated Enzymatic Determination of Ethanol in Beverages: Collaborative Study for RIDA®CUBE Ethanol. *Food and Nutrition Sciences*, **14**, 90-100.
https://doi.org/10.4236/fns.2023.142007

Received: January 18, 2023
Accepted: February 21, 2023
Published: February 24, 2023

Abstract

Easy and quick methods to quantify ethanol reliably in beverages are always important. In 2022, the Enzytec™ *Liquid* Ethanol test kit was approved as AOAC Official Method℠ 2017.07 Final Action after a collaborative study was conducted with different beverages such as kombucha, juices, and beer. During set-up of this collaborative test, small sized companies asked to include the RIDA®CUBE Ethanol/RIDA®CUBE SCAN device since it is easy to use, suitable for a few samples only and contains the identical reagents as the Enzytec™ *Liquid* system. It is applicable to quantify ethanol in diluted kombucha, fruit juices, and alcohol-free beer samples around 0.5% alcohol-by-volume within 12 min. The overall relative reproducibility standard deviation across a wide concentration range for kombucha, was calculated to be 6.29%. Analysis of juices and beer showed an overall higher variation with an estimated overall RSD(R) value by regression of 14.4%. The data obtained by this collaborative study show that the RIDA®CUBE Ethanol in combination with the RIDA®CUBE SCAN device is suitable to quantify ethanol from matrices representing important alcohol-free liquid food categories.

Keywords

Ethanol, Enzymatic Analysis, Beverages, Automation, Reproducibility, Collaborative Test

1. Introduction

Precise and accurate ethanol determination is necessary due to the maximum allowed levels of ethanol in alcohol-free labelled beverages such as fruit juices, alcohol-free beers, and kombucha. Especially for the latter one, minimum performance requirements for ethanol determination in kombucha were set up by

AOAC Standard Method Performance Requirements (SMPRs®) 2016.001 [1]. Based on this SMPR, the Enzytec™ *Liquid* Ethanol (R-Biopharm, art. no. E8340) test kit for enzymatic ethanol quantification was approved as AOAC Official Method 2017.07 First Action in September 2017 [2]. In 2022, the kit was granted Final Action status after performing a collaborative study that not only included the classical manual format using 3 mL cuvettes but also investigated several bioanalytical automates with high throughput of samples [3]. During planning the above-mentioned collaborative test, R-Biopharm has received feedback from several users that requested the RIDA®CUBE Ethanol together with the measurement device, RIDA®CUBE SCAN (**Figure 1**) to be part of the collaborative test. The combination of test kit and device is widely used by small labs and kombucha producers for QC testing where small batch sizes are common and only a few analyses per day or week are performed. The reagents of the Enzytec™ *Liquid* Ethanol are identical to the reagents used to produce the RIDA®CUBE Ethanol test kit. Since the RIDA®CUBE system works technically differently from the Enzytec™ *Liquid* Ethanol, it was not included in the AOAC Official Method 2017.07. Here, we would like to present the performance characteristics obtained from the collaborative test for the RIDA®CUBE system to measure ethanol in beverages.

2. Materials and Methods

2.1. Sample Preparation before Measurement

2.1.1. Sample Treatment

For diluting sample solutions, it is advisable to pipette beneath the surface of the diluent. When filtering a sample solution, the filtrate shall not drop but rinse down the wall of the vial. Vials should be closed tightly before centrifugation.

Figure 1. The RIDA®CUBE SCAN device with a tablet on top.

Clear, slightly colored and pH-neutral liquid samples can be used directly, or after dilution into the relevant measurement range of 20 to 500 mg/L ethanol. If the sample is slightly acidic or alkaline it may be used directly after dilution. It is advisable to check strong acidic sample solution for recovery. Samples containing carbon dioxide have to be de-gassed by a short burst of ultrasound at 0˚C (ultrasonic device filled with ice cubes and distilled water). Clear kombucha, alcohol-free beer, and juice samples should be diluted with water (if necessary). Turbid kombucha, alcohol-free beer, and juice samples have to be centrifuged before dilution with water (if necessary).

2.1.2. Dilution

Samples with 0.6 g/L up to 6 g/L ethanol (0.076 - 0.76% ABV) should be diluted 1 + 19 with water e.g. 100 µL sample is pipetted into 1900 µL of distilled water. Samples with 3 g/L up to 30 g/L ethanol (0.38% ABV - 3.8% ABV) should be diluted 1 + 99 e.g. 100 µL sample is pipetted into 9.90 mL of distilled water. Other dilutions as e.g. 1:50 or 1:10 are possible if the ethanol concentration of the diluted samples lies within the measurement range. Dilution of ethanol containing samples with water is very susceptible to pipetted volumes used for dilution. Therefore, pipette at minimum 100 µL ethanol containing sample into the respected volume of water; lower volumes e.g. 20 µl will result in higher CVs.

Use diluted sample solutions within three days for ethanol measurement (storage temperature 2˚C - 8˚C).

2.2. Test kit and Measurement Device

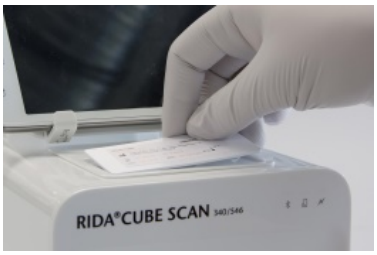
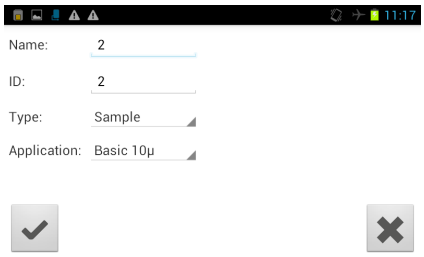
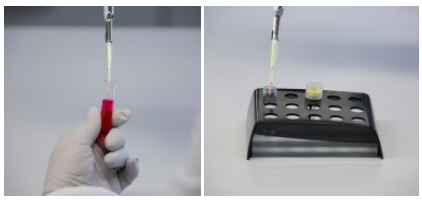
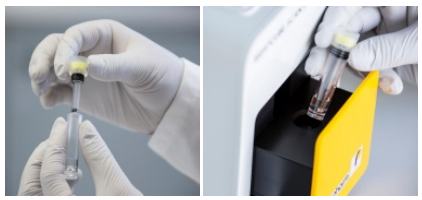
The quantification is based on the catalytic activity of alcohol dehydrogenase, which oxidizes ethanol to acetaldehyde and converts nicotinamide-adenine dinucleotide (NAD) to its reduced form, NADH. Measurement is performed at 340 nm in the RIDA®CUBE SCAN device (**Figure 1**) and calculated against a calibration function stored on a lot-specific RFID card (see **Table 1**).

The test kit RIDA®CUBE Ethanol (R-Biopharm, art. no. RCS4340) consists of 32 tubes (containing 800 µL reagent 1), 32 caps (200 µL of reagent 2) and one lot-specific RFID card. For measurement, the RFID card is placed on the RIDA®CUBE SCAN (R-Biopharm, art. no. ZRCS0546 or ZRCS0580) device as shown in **Table 1**. Next, information about the sample is entered into the tablet app. One tube (see also **Figure 2**) is opened and 20 µL of diluted/undiluted sample is added. The tube is closed with the cap and inserted in the scan device. After closing the door of the device, the analysis is finished automatically and a concentration for the analyzed sample is given on the tablet. A dilution step prior to measurement needs to be taken into account for calculation. If the result should be given as % alcohol-by-volume (%ABV) calculate as follows: % ABV = Ethanol [g/L]/7.8924 [1].

2.3. Study Design

Following AOAC guidelines, which are published as Appendix D [4], an inter-

Table 1. Handling steps of the RIDA®CUBE Ethanol.

Place the RFID-card on the instrument	
Enter sample data into tablet app: - identification - volume (20 µL)	
Pipette the sample into the test-tube (reagent 1)	
Close the tube with the cap (reagent 2), insert into the instrument and close the door	

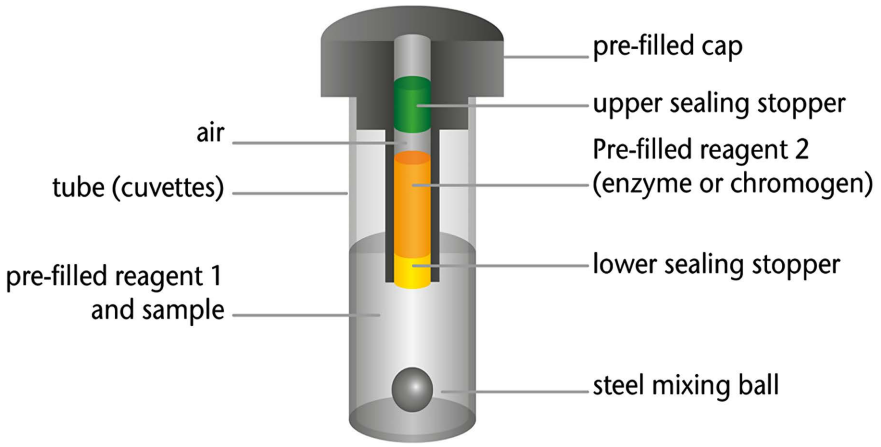


Figure 2. Detailed scheme of the test tube with the tube filled with reagent 1 and the cap filled with reagent 2.

national collaborative study was set up. Detailed instructions for collaborators and data return sheets were provided to all participants. In order to qualify participants, we asked each laboratory to analyze two aqueous reference materials with known ethanol concentrations (0.1 g/L and 2.0 g/L). Results were for-

warded to the study director before each lab could continue with the analysis of the matrix samples.

2.4. Collaborators

Eight laboratories participated: One each from France, Canada, United States of America, and Argentina, two from Italy and two from Germany. To mimic the typical user of such a measurement device, it was decided not to look for the best-trained specialist in analysis but in analysts that will use the method after reading the instruction for use and a short training. The participants were advised to ensure that no alcoholic vapors (ethanol or propanol) e.g. from cleaning solutions or beverages with high ethanol content were present during sample preparation and measurement of the extracts. Samples were shipped to the laboratories in September 2020, and all results were received by the end of December 2020.

2.5. Samples and Sample Preparation

Three matrices (kombucha, orange juice, and beer) were tested at different concentrations (Table 2) up to 9 g ethanol per L (equivalent to 1.14% alcohol by volume; % ABV).

Each of the 14 different samples was analyzed as blind duplicates by each participant. For spiking of the juice and beer, EMSURE® Ethanol (absolute for analysis; Merck, 1.00983.1000) was used. For preparation, a 100 mL volumetric flask was filled with about 50 mL distilled water. Twenty mL of absolute ethanol was pipetted into the flask, mixed and filled up with water up to 100 mL. This solution was used for spiking the matrices designated as spiked in Table 2.

Table 2. Samples for the collaborative test.

Sample	Concentration	
Control A	0.10 g/L	Order no. AQ01-015[a]
Control B	2.00 g/L	Order no. AQ20-015[a]
Kombucha I	<0.5 g/L	naturally contaminated
Kombucha II	2 g/L	naturally contaminated
Kombucha III	5 g/L	naturally contaminated
Kombucha IV	6 g/L	naturally contaminated
Kombucha V	9 g/L	naturally contaminated
Orange juice with pulp I	0.1 g/L	naturally contaminated
Orange juice with pulp II	2 g/L	spiked
Orange juice with pulp III	4 g/L	spiked
Beer I	2 g/L	naturally contaminated
Beer II	4 g/L	naturally contaminated
Beer III	6 g/L	spiked

[a]both from ACQ Science, Rottenburg-Hailfingen, Germany; volume is 1.5 mL.

2.6. Presentation of Samples to Participants

Following the collaborative test guidelines of AOAC Appendix D [4], a set of blind duplicates for each sample was provided to each participating laboratory. The samples were marked with a laboratory-specific letter (B to N) and a randomized number. Each laboratory obtained its own coding (different randomized numbers for each laboratory).

2.7. Methods and Measurement of Samples

The method protocol was provided to each laboratory with the instruction to follow the method as written with no deviations. The final data from the laboratories were sent to the study director. The participants were advised to analyze all samples with an initial dilution of 1:20. After identification of diluted samples with concentrations lower than 20 mg/L, the participants were advised to run these samples undiluted.

3. Results and Discussion

3.1. Pre-collaborative Study

A total of eight data sets from eight participants were obtained for the pre-collaborative study using two aqueous solutions with known ethanol concentrations (Table 2 and Table 3). Lab K and lab F only analyzed one of the solutions while lab N analyzed the high-concentrated solution only once.

Table 3. Results for the analysis of two aqueous solutions with known ethanol concentrations by eight participants; performance characteristics on precision, RSD(r) and RSD(R), are given.

Participant	Reference solution			
	0.100 g/L	0.100 g/L	2.00 g/L	2.00 g/L
B	0.108	0.108	2.22	2.15
C	0.110	0.110	2.24	2.24
G	0.100	0.100	2.04	2.03
N	0.122	0.105		2.22
H	0.109	0.108	2.16	2.10
J	0.107	0.104	2.15	2.11
K			1.95	2.14
F	0.090	0.105		
No. laboratories	7		7	
No. of replicates	14		13	
Grand mean, g/L	0.106		2.134	
s(r), g/L	0.006		0.063	
s(R), g/L	0.007		0.089	
RSD(r), %	5.81		2.94	
RSD(R), %	6.65		4.17	

Relative repeatability standard deviation RSD(r) and relative reproducibility standard deviation RSD(R) are within the expected range and nearly fulfil the requirement RSD(R) of ≤6% laid down in AOAC SMPR 2016.01 [1]. Consequently, all participants were allowed to proceed with the analysis of matrix samples.

3.2. Outlier Detection and Performance Characteristics

Data sets were checked for outliers (Tables 4-6) according to AOAC Appendix D [4]. In total, four out of eight data sets showed no outliers at all. Six pairs of duplicates out of a total of 112 pairs were outliers according to Cochran marked with a small type c in Tables 4-6. Outliers according to the Cochran test indicate higher differences between the two replicates compared to the other labs. There is no tendency towards a specific proportion of outliers due to a participant or a sample. The pattern looks quite random.

Table 4. Data sets for the method after identification of outlying values; small type c means a Cochran outlier; participant J obtained a result for sample V higher than 10 g/L and did not repeat the run at a higher dilution.

Participant	Kombucha									
	I	I	II	II	III	III	IV	IV	V	V
B	0.03	0.03	2.16	2.27	5.20	4.99	6.52	6.16	8.94c	9.54c
C	0.03	0.03	2.45	2.46	5.65	5.56	6.68	6.87	9.33	9.30
G	0.03	0.04	2.69	2.59	5.91	5.96	6.81	7.63	10.46	10.52
N	0.04	0.04	2.72	2.78	6.22	5.60	6.66	6.98	9.32	9.36
H	0.02	0.03	2.23	2.38	5.36	5.49	6.57	6.52	9.79	9.75
J	0.13c	0.14c	2.56	2.58	5.83	6.14	6.73	6.99	>	>
K	0.04	0.03	2.31	2.21	4.85	5.07	5.79	5.80	8.66	8.67
F	0.03	0.03	2.23	1.98	3.00c	5.30c	6.01	6.36	9.62	9.52

Table 5. Data sets for the method after identification of outlying values; small type c means a Cochran outlier.

Participant	Orange Juice					
	I	I	II	II	III	III
B	0.32	0.30	2.19	2.29	3.85	4.18
C	0.31	0.33	2.16	2.34	4.82	5.10
G	0.31	0.32	2.46	2.23	4.90	4.49
N	0.42	0.36	2.62	2.48	5.78	5.24
H	0.32	0.30	2.03	2.02	4.00	4.17
J	0.38	0.37	2.19	2.11	5.71	5.12
K	0.27	0.30	1.55c	2.24c	4.13	3.44
F	0.32	0.31	1.98	2.12	4.24	4.15

Table 6. Data sets for the method after identification of outlying values; small type c means a Cochran outlier.

Participant	Beer					
	I	I	II	II	III	III
B	2.08	2.08	2.90	2.63	6.05	5.52
C	2.05	2.35	3.72	4.03	6.07	6.37
G	2.23	2.18	3.89	3.86	6.77	6.81
N	2.14	2.20	3.86	4.00	6.76	7.20
H	1.87	1.95	3.75	3.83	5.96	5.68
J	2.43c	4.57c	4.39	4.54	6.54	4.68
K	2.03	1.55	2.26c	3.48c	5.28	4.39
F	1.85	1.44	3.38	3.40	5.93	6.08

After elimination of outliers, the performance characteristics repeatability standard deviation s(r) and reproducibility standard deviation s(R) were calculated using the AOAC International Interlaboratory Study Workbook for Blind (Unpaired) Replicates (version 2.0). Table 7 shows the results of these calculations for the kombucha samples.

Table 8 compiles these calculations for orange juice and beer. In the past, it was common to characterize each s(r) or s(R) individually for each sample. Here we would like to do an additional characterization for all kombucha sample at a time as described by Lacorn *et al.* [3]. In an effort to interpret observed RSD values across a wide concentration range, we have modeled reproducibility standard deviation s(R), as a function of mean observed concentration.

We analyzed the relationship by simple least squares linear regression (Figure 3). The procedures used for modeling reproducibility by mean are taken from ISO 5725-2 [5]. The coefficient of regression is 0.964, and the slope of the regression line is 0.0629, which corresponds to an overall RSD(R) of 6.29%. As expected, the estimated relative standard deviation will rise as the concentration approaches zero (Table 6). Kombucha I with a mean ethanol concentration of 0.031 g/L is close to the LoQ of the method and the higher relative standard deviation was in the expected range.

Other methods for determination of ethanol in Kombucha are OMA 2019.08 [6] and OMA 2016.12 [7]. OMA 2019.08 is also based on an enzymatic principle but was not studied collaboratively until today [6]. OMA 2016.12 is a gas-chromatographic method with RDR(R) values between 10% for low-concentrated kombucha samples and around 5% for samples with higher ethanol concentrations [7].

Analysis of orange juice and beer showed an overall higher variation as can be seen in Table 5. RSD(R) range from 8.7% up to 15.2% depending on the concentration of the samples. The estimated overall RSD(R) value by regression is 14.4% (Figure 4). It is obvious that one sample deviates from the others for unknown reasons, but the coefficient of regression is still at 0.961.

Table 7. Performance characteristics s(r), s(R), RSD(r), and RSD(R) for the method in case of kombucha.

	Kombucha				
	I	II	III	IV	V
Target (g/L)	0.04	2.25	5.04	6.02	9.02
No. laboratories	7	8	7	8	6
No. of replicates	14	16	14	16	12
Grand mean, g/L	0.031	2.41	5.56	6.57	9.53
s(r), g/L	0.003	0.09	0.21	0.27	0.04
s(R), g/L	0.005	0.24	0.44	0.49	0.60
RSD(r), %	8.36	3.64	3.72	4.08	0.42
RSD(R), %	14.5	9.78	7.96	7.42	6.33

Table 8. Performance characteristics s(r), s(R), RSD(r), and RSD(R) for the method in case of orange juice and beer.

	Orange Juice			Beer		
	I	II	III	I	II	III
Target (g/L)	0.28	2.07	4.03	1.93	3.61	5.54
No. laboratories	8	7	8	7	7	8
No. of replicates	16	14	16	14	14	16
Grand mean, g/L	0.33	2.23	4.58	2.00	3.73	6.01
s(r), g/L	0.020	0.10	0.31	0.19	0.13	0.56
s(R), g/L	0.038	0.19	0.70	0.26	0.53	0.78
RSD(r), %	6.26	4.48	6.68	9.56	3.37	9.24
RSD(R), %	11.5	8.74	15.2	13.0	14.3	13.0

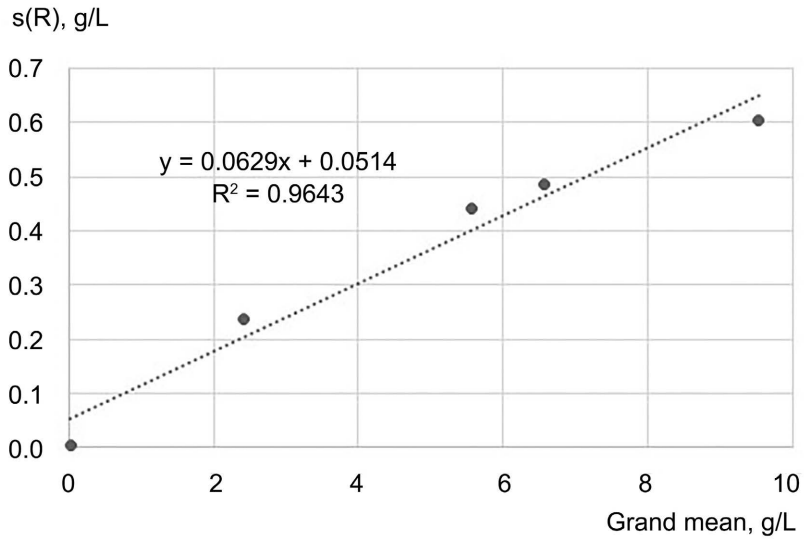


Figure 3. Reproducibility standard deviation modeled by mean concentration of the samples for all kombucha samples; the slope of the linear regression line is an estimate of the overall reproducibility relative standard deviation.

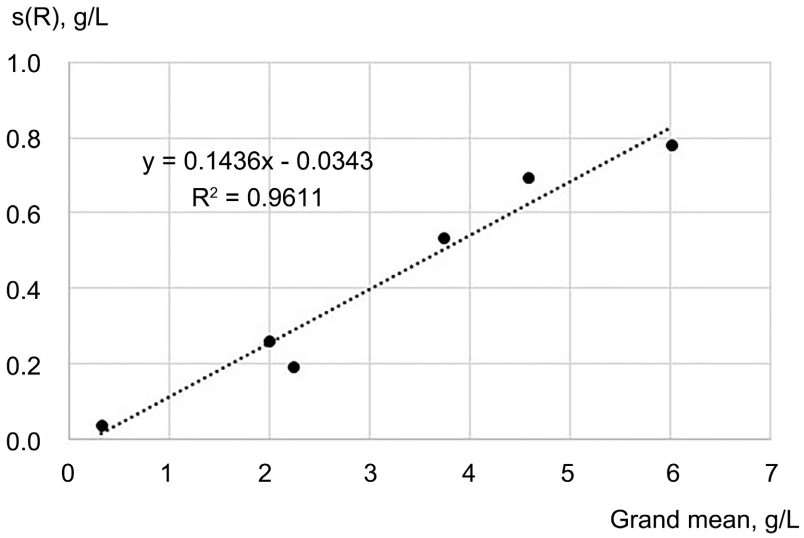


Figure 4. Reproducibility standard deviation modeled by mean concentration of the samples for the non-kombucha samples; the slope of the linear regression is a measure of the mean reproducibility relative standard deviation.

It was not possible to calculate recovery for kombucha since all samples were naturally contaminated. Nevertheless, recovery data on reference materials is provided in Table 4.

As expected for the group of participants, especially precision of reproducibility is sometimes quite high for orange juice and beer. It should bear in mind that these samples had to be centrifuged or degassed whereas kombucha could be used directly. From our experience, more training of some participants will decrease this un-precision. Since the analysis of aqueous solutions during the pre-collaborative test resulted in much better precision in any case, it is clear that not the measurement per se is the main contributor to uncertainty but the handling and preparation of matrix samples. As stated above, pipetting ethanol-containing solutions is also a critical point that needs to be accounted for. The combination of the RIDA®CUBE Ethanol and the RIDA®CUBE SCAN was especially developed for users with a low sample throughput, limited laboratory facilities, and basic knowledge of analytical chemistry. If needed just one sample can be analyzed and due to high stability of the reagents in the test tubes, there is no issue if only a few samples per month have to be analyzed.

4. Conclusion

The data obtained by this collaborative study show that the RIDA®CUBE Ethanol together with the measurement device RIDA®CUBE SCAN is suitable to quantify ethanol from matrices representing important alcohol-free liquid food categories.

Acknowledgements

We would like to thank Patricia Meinhardt for proofreading of the manuscript

and for her helpful contribution to this successful study.

The participation of the following labs in the collaborative study is greatly acknowledged.

Claus Patz, FH Geisenheim University, Geisenheim, Germany.

Michaela Hessler-Noll, R-Biopharm, Darmstadt, Germany.

Ryan Malone, Trilogy Analytical Lab, Lujan, Argentina.

Ezio Messina, Birra Peroni Asahi Europe, Roma, Italy.

Pasquale Ferranti, Univeristy of Naples, Naples, Italy.

William Davis, VitaliTea Group LLC, Kihei, HI, US.

Elsa Herault, Brasserie Licorne, Saverne, France.

Jeremie Theolier, Université Laval, Quebec, Canada.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Konings, E.J.M., Barrett, W.B., Beshore, M., Beshore, T., Buscher, J., Crum, H., *et al.* (2016) AOAC SMPR® 2016.001 Standard Method Performance Requirements for Determination of Ethanol in Kombucha. *Journal of AOAC International*, **99**, 1120-1121. https://doi.org/10.5740/jaoacint.SMPR2016.001

[2] Lacorn, M. and Hektor, T. (2018) Determination of Ethanol in Kombucha, Juices, and Alcohol-free Beer by Enzytec™ Liquid Ethanol: Single-Laboratory Validation, First Action 2017.07. *Journal of AOAC International*, **101**, 1101-1111. https://doi.org/10.1093/jaoac/102.1.316

[3] Lacorn, M., Hessler-Noll, M., Wehling, P. and Hektor, T. (2022) Determination of Ethanol in Food by Enzymatic Method Enzytec™ Liquid Ethanol, Collaborative Study for OMA 2017.07. *Journal of AOAC International*. https://doi.org/10.1093/jaoacint/qsac145

[4] AOAC International (2023) Appendix D: Guidelines for Collaborative Study Procedures to Validate Characteristics of a Method of Analysis. In: Latimer Jr, G.W., ed., *Official Methods of Analysis of AOAC International*, 22nd edition, Oxford University Press, Oxford.

[5] International Organisation for Standardisation (2019) ISO 5725-2:2019-12: Accuracy (Trueness and Precision) of Measurement Methods and Results Part 2: Basic Method for the Determination of Repeatability and Reproducibility of a Standard Measurement Method. https://www.iso.org/standard/69419.html

[6] Ivory, R., Delaney, E., Mangan, D. and McClearly, B.V. (2012) Determination of Ethanol Concentration in Kombucha Beverages: Single-Laboratory Validation of an Enzymatic Method, First Action Method 2019.08. *Journal of AOAC International*, **104**, 422-430. https://doi.org/10.1093/jaoacint/qsaa122

[7] Liu, Y., Chan, M., Ebersole, B., Sy, H. and Brown, P.N. (2019) Determination of Ethanol Content in Kombucha Products by Gas Chromatography with Flame Ionization Detection: A Multilaboratory Study. *Journal of AOAC International*, **102**, 878-882. https://doi.org/10.5740/jaoacint.18-0190