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The establishment of metrological characteristics of the method "Ethanol as Internal Standard" for the direct determination of volatile compounds in alcoholic products

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Abstract: An extensive study concerning the problem of volatile compounds determination in alcoholic products by gas chromatography has been undertaken in the paper. A row of gravimetrically prepared standard solutions was analysed with three analytical methods: traditional method of internal standard, advanced method of internal standard and external standard method. The main analytical characteristics and metrological parameters of the "Ethanol as Internal Standard" method were compared with the traditional approaches. It was shown that this method leads to correct values of volatiles concentrations and the corresponding metrological characteristics are generally better.

Keywords: Alcoholic products; ethanol; gas chromatography; quantification; volatile compounds.

1. Introduction

Alcoholic beverage consumption is almost everybody's concern. The fact is that alcoholic products are foodstuff therefore the quality control tests are of top priority. Apart from the usual over-consumption there is another risk factor which poses a threat to human health. It is the high content of

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volatile compounds which are by-products of alcoholic beverages manufacturing process and the simplest organic molecules of primary alcohols, esters and aldehydes.

There are a lot of types of alcoholic beverages with different volatiles concentrations and alcohol by volume (ABV) content. The detection of volatiles in various alcoholic beverages is often performed with gas chromatography (GC) method [1]. There is also a challenge of quantitative determination of volatile compounds, i.e. the expression of their contamination in mg/mg, mg/L or mg/L of absolute alcohol (AA) units. The quantity of determined volatiles differs from beverage to beverage, for instance from few compounds [2-5] to more than 30 [6-10].

Speaking about differences in ethanol content in analysed samples it should be said that GC method is used worldwide for analysing beverages with small ethanol content [11-12] as well as with great one [13]. It is also necessary to note that according to European Pharmacopeia volatile impurities determination in ethanol (100% and 96%) also proceeds with GC [14].

2. Experimental

2.1. Analytical Methods

The main goal of the current study was to demonstrate the features and advantages of three methods of quantitative analysis of alcohol-containing products in order to make a comparison between them. Gravimetrically prepared standard solutions were used as analysed samples.

The first method taken into account is internal standard (IS) method. In accordance with the Commission Regulation (EC) No 2870/2000 [15], the official methods of the Association of Official Analytical Chemists (AOAC) [16-17] and the official methods of the International Organization of Vine and Wine (OIV) [18] the quantitative determination of volatile compounds in alcoholic products is performed with IS method. It means the artificial addition of IS compound into both standard solution and testing sample. In our study 2-pentanol was used as an IS compound. Relative response factors (RRF) of i-th analysed compound relatively 2-pentanol were calculated according to the formula:

$$RRF_i^{Pnt} = \frac{C_i^{st}}{A_i^{st}} \cdot \frac{A_{Pnt}^{st}}{C_{Pnt}^{st}}, \tag{1}$$

where A_i^{st} and A_{Pnt}^{st} are the values of detector response, for example, peak areas, for the i-th individual compound and 2-pentanol in standard solution, respectively; C_i^{st} and C_{Pnt}^{st} are the concentrations of the i-th analysed compound and 2-pentanol, correspondingly, in mg/kg units in standard solution. Then the obtained calibration coefficients were used for sample analysis:

$$C_i = RRF_i^{Pnt} \cdot \frac{A_i}{A_{Pnt}} \cdot C_{Pnt}, \tag{2}$$

where C_i is the concentration of i-th analysed volatile in mg/kg units; C_{Pnt} is the concentration of added 2-pentanol in mg/kg units; A_i and A_{Pnt} are the values of detector response for the i-th individual compound and 2-pentanol in analysed sample, respectively. The Eq. (2) slightly differs from the corresponding in [15], as internal standard compound was added in all standard solutions simultaneously with analysed compounds, so there was no need in coefficients which are responsible for dilution.

According to the above-mentioned regulatory documents volatiles concentrations must be finally expressed in mg/L AA units. For this aim one should perform an ABV determination operation which lays in the density measurement and subsequent usage of international alcoholometric tables [19]. The presentation of concentration values of the examined compounds in the required mg/L AA units is performed according to the following expression:

$$C_i(mg/L\ AA) = \frac{C_i(mg/kg) \cdot \rho_{sample}}{ABV(^v/_v) \cdot 10^6}, \tag{3}$$

where ρ_{sample} is the density of the test sample in mg/L units. It should be noted that while testing alcohol-containing products with a noticeable content of volatile compounds (more than 800 mg/L AA totally), the usage of international alcoholometric water-ethanol tables will never provide the required accuracy in determination of ABV (0.1 % by volume) since these tables can be used only for binary water-ethanol solutions. Thus, there is a so-called phenomenon of "real" and "apparent" strength. This problem and one of its possible solutions were pointed out in [20].

Apart from IS method there is another well-known method of external standard (ES). Its main idea lies in the plotting of the calibration curve according to the data obtained from the subsequent measurement of a single (several) standard solution. In case of using ES method the response factors (RF) play the role of calibration parameters. There is no necessity in IS compound addition while applying this method. Subsequently the value of concentration of i-th compound in the analysed sample is defined by the following expression:

$$C_i = RF_i \cdot A_i = \frac{C_i^{st}}{A_i^{st}} \cdot A_i \ , \tag{4}$$

where the parameters C_i^{st}, A_i and A_i^{st} have the same meaning as in Eqs. (1-2).

Consequently the concentrations in mg/kg or mg/L units are determined. The conversion of obtained results into mg/L AA units proceeds as in the IS method. The ES method has its own application area, for instance, it was used in works [9, 11].

The last method taken into consideration is the method called "Ethanol as Internal Standard". It was proposed and discussed in publications [21-24] where its novel, innovative and up-to-date character was shown. The process of sample analysis is similar to that in the traditional IS method as RRFs are to be determined:

$$RRF_i^{Eth} = \frac{C_i^{st}}{A_i^{st}} \cdot \frac{A_{Eth}^{st}}{\rho_{Eth}}, \tag{5}$$

where C_i^{st} is a concentration of the i-th analysed compound in mg/L AA units ; ρ_{Eth} = 789300 mg/L is the ethanol density.

Oppositely to the generally adopted practices the advanced method uses the major volatile compound as the IS. As ethanol always presents in alcohol-containing products, there is no necessity in its artificial addition into analysed sample. Also its concentration in mg/L AA units in every ethanol containing product is known with a 100 % guarantee and is equal to ethanol density. So, this method eliminates the necessity of sample density measurement, as concentrations of volatiles in mg/L AA units are determined directly from GC data:

$$C_i(mg/L\ AA) = RRF_i \cdot \frac{A_i}{A_{Eth}} \cdot \rho_{Eth}. \tag{6}$$

Let us estimate the differences between uncertainties calculations for two IS methods. According to the guide [25] the total uncertainty of the method can be evaluated as the sum of uncertainties of its components. In our case these components are the values that are necessary for the calculation of volatiles concentrations in mg/L AA units. Thus, the formula for uncertainty calculation in case of the traditional internal standard method will include the uncertainties of all values from Eqs. (2) and (3):

$$\frac{u(C_i)}{C_i} = \sqrt{\left(\frac{u(RRF_i)}{RRF_i}\right)^2 + \left(\frac{u(A_i)}{A_i}\right)^2 + \left(\frac{u(A_{IS})}{A_{IS}}\right)^2 + \left(\frac{u(C_{IS})}{C_{IS}}\right)^2 + \left(\frac{u(\rho_{sample})}{\rho_{sample}}\right)^2 + \left(\frac{u(ABV(^{v}/_{v}))}{ABV(^{v}/_{v})}\right)^2}\,. \tag{7}$$

As "Ethanol as Internal Standard" method allows direct quantification of volatiles according to Eq. (6), the analogous formula will be written as follows:

$$\frac{u(C_i)}{C_i} = \sqrt{\left(\frac{u(RRF_i)}{RRF_i}\right)^2 + \left(\frac{u(A_i)}{A_i}\right)^2 + \left(\frac{u(A_{Ethanol})}{A_{Ethan}}\right)^2}. \tag{8}$$

It can be seen, that in these two formulas the first three terms under the square root have the same nature and the same order of magnitude. Also there are less additive components in Eq. (8) than in Eq. (7). These additional terms are responsible for the uncertainty in the determination of concentration of internal standard, density and ABV value of a test sample. The presence of these additional terms indicates a higher uncertainty during the determination of concentrations of volatile compounds by the traditional method than “Ethanol as Internal Standard” method.

2.2. Metrological Tests

Appropriate statistical tests should be applied to the received data sets in order to establish that one method is equivalent or better than the other. The Eurachem Guide [25] and series of ISO 5725 standards [26] were used as a theoretic base for metrological characteristics establishment. Inter-laboratory biases, repeatability limits and uncertainties were calculated for all prepared standard solutions except solution “WES-C” as it was used for calibration.

The value of limit of quantification (LOQ) appears to be the lowest level of volatile concentration at which the performance is acceptable for a typical application [25]. The determination of LOQ in GC method involves the measurement of standard solution with very low components concentrations that’s why the “WES-3” solution was used for this aim (see Table 1). Firstly, the s'_o value was determined as a ratio of standard deviation to the root of number of replicates according to the following formula:

$$s'_o(mg/L\ AA) = \frac{s_o}{\sqrt{N}} = \frac{\sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(x_i-\bar{x})^2}}{\sqrt{N}}, \tag{9}$$

where s_0 is a standard deviation, x_i is the value of concentration of a single measurement and $\bar{x}$ is the average value of concentration within *N* measurements. Finally LOQs were calculated as $k_Q \cdot s'_o$, where k_Q was set to 10 [25].

Inter-laboratory biases were calculated for each analysed compound as a characteristic of method accuracy [25]. Bias values were determined by the following equation:

$$bias, \% = \frac{|\bar{C}_i - C_i^{st}|}{C_i^{st}} \cdot 100\%\ , \tag{10}$$

where $\bar{C}_i$ is the average concentration value between laboratories.

Repeatability characterizes precision of the method as it shows the dispersion of the obtained values between each other. For within-laboratory evaluation of repeatability the relative standard deviations (RSD, %) were calculated for each used method by traditional practices. Repeatability limits of *n* measurements were calculated as $f\sqrt{n}$, where *f* was set equal to 1.96 for 95 % confidence level [26]. Repeatability limits describe inter-laboratory precision.

Uncertainty acts as one of the most important method’s performance. Expanded uncertainties were calculated for inter-laboratory results as reproducibility standard deviation multiplied by coverage factor equal to 1.96.

2.3. Materials and Preparation

All individual chemical compounds were purchased from Sigma-Aldrich (Berlin, Germany). High-purity ethanol was purchased from JSC "Dyatlovo Wine and Distillery Plant Algon" (Belarus). All standard solutions were prepared gravimetrically by the addition of individual chemical compounds into water-ethanol solution. Initial water-ethanol solution with the ABV equal to 40% was prepared by the dilution of pure ethanol with deionized water.

2.4. Instrumental Analysis

2.4.1. Laboratory of Analytical Research (LAR)

The samples were analysed using a Chromatec-Kristall 5000 gas chromatograph (JSC SDB Chromatec, Yoshkar-Ola, Russia) equipped with FID and an autosampler. Instrument control and data analysis were performed with UniChrom software (New Analytical Systems Ltd., Minsk, Belarus). The gas chromatograph was fitted with capillary column Rt-Wax, 60 m × 0.53 mm, with phase thickness of 1 µm (Restek, Bellefonte, PA, USA). The oven temperature was the following: the initial isotherm at 75 °C for 9 min was raised to 130 °C at a rate of 5 °C/min then raised to 180 °C at a rate of 10 °C/min with final isotherm of 155 °C for 5 min. The carrier gas was nitrogen (≥99.99 % purity); the gas flow was 6.9 mL/min; the injector temperature was 160 °C; the detector temperature was 200 °C; the injector volume was 1 µL; the split ratio was 1:7. Split ratio was selected so that ethanol and propanol-2 could be separated by GC, where in case of smaller split ratio there is a risk of overlapping of these two peaks. Analytic balance OHAUS PA-214C with a precision of 0.2 mg was used for gravimetric preparations.

2.4.2. Laboratory of Republican Centre for Hygiene, Epidemiology and Public Health (RCH)

The samples were analysed using an Agilent 6890 gas chromatograph (Agilent Technologies, Inc., USA) equipped with FID and an autosampler. Instrument control and data analysis were performed with GC ChemStation software chromatograph (Agilent Technologies, Inc., USA). The gas chromatograph was fitted with capillary column DB-Wax, 60 m × 0.53 mm with phase thickness of 1 µm (Agilent Technologies, Inc., USA). The oven temperature was the following: the initial isotherm at 65 °C for 9 min was raised to 155 °C at a rate of 7 °C/min with final isotherm of 155 °C for 2.6 min. The carrier gas was hydrogen (≥99.99 % purity); the gas flow was 2.7 mL/min; the injector temperature was 180 °C; the detector temperature was 250 °C; the injector volume was 1 µL; the split ratio was 1:10.

2.4.3. Laboratory of Faculty of Science of Charles University (CU)

The samples were analysed using a Shimadzu GC 2010 gas chromatograph (Shimadzu Corporation, Inc., Japan) equipped with FID and a split/splitless injector. Instrument control and data analysis were performed with GC Solution software (Shimadzu, ver. 2.41). The gas chromatograph was fitted with capillary column Supelcowax 10, 30 m × 0.25 mm with phase thickness of 0.5 µm (Sigma Aldrich, Inc., USA). The oven temperature was the following: the initial isotherm at 30 °C for 5 min was raised to 100 °C at a rate of 5 °C/min with final isotherm of 100 °C for 1 min. The carrier gas was nitrogen (≥99.999 % purity); the gas flow was 0.33 mL/min; the injector temperature was 280 °C; the detector temperature was 280 °C; the injector volume was 1 µL; the split ratio was 1:50.

2.5. Standard Solutions Preparation and Analysis

Six standard water-ethanol (with 40% ABV) solutions were prepared gravimetrically according to ASTM D 4307 [27] recommendations by addition of individual chemical substances or solutions into initial water-ethanol mixture. The concentrations of volatile compounds in all prepared standard solution are presented in the Table 1. In calculations it was considered that the following impurities were presented in the initial ethanol (rectified ethyl alcohol): acetaldehyde (1.84 mg/L AA); methanol (17.57 mg/L AA) and 2-propanol (1.15 mg/L AA).

All prepared standard solutions were measured by GC three times in each laboratory under repeatability conditions. The final concentration of each analysed compound considered to be the average value among three measurements. It was taken into consideration that standard solutions “WES-3” and “WES-A” were not measured in the CU laboratory.

Table 1. The concentrations of compounds in prepared standard solutions.

Compound/Solution	Concentration, mg/L AA					
	WES-3	WES-2	WES-1	WES-C	WES-B	WES-A
acetaldehyde	3.93 ± 0.12	11.0 ± 0.33	48.6 ± 1.5	223 ± 4.5	436 ± 4.4	4336 ± 43
methyl acetate	1.03 ± 0.02	9.74 ± 0.19	51.4 ± 1.0	242 ± 4.8	487 ± 4.9	5533 ± 55
ethyl acetate	1.11 ± 0.02	9.49 ± 0.19	49.7 ± 1.0	233 ± 4.7	476 ± 4.8	6813 ± 68
methanol	18.5 ± 0.56	27.7 ± 0.83	71.7 ± 2.2	271 ± 5.4	526 ± 5.3	5741 ± 57
2-propanol	2.75 ± 0.08	11.9 ± 0.36	55.6 ± 1.7	258 ± 5.2	512 ± 5.1	5774 ± 58
ethanol (adv. IS)	789300	789300	789300	789300	789300	789300
1-propanol	1.27 ± 0.03	10.7 ± 0.21	56.5 ± 1.1	265 ± 5.3	532 ± 5.3	6010 ± 60
isobutyl alcohol	1.45 ± 0.03	11.3 ± 0.23	58.9 ± 1.2	275 ± 5.5	553 ± 5.5	6214 ± 62
2-pentanol (trad. IS)	37.2 ± 0.74	75.8 ± 1.52	72.2 ± 1.4	70.0 ± 1.4	70.2 ± 1.4	91.6 ± 1.8
1-butanol	1.72 ± 0.03	11.0 ± 0.22	56.6 ± 1.1	264 ± 5.3	532 ± 5.3	5996 ± 60
isoamyl alcohol	1.95 ± 0.04	11.6 ± 0.23	59.2 ± 1.2	276 ± 5.5	556 ± 5.6	6244 ± 62

3. Results and discussion

The calibration coefficients RRFs and RFs were calculated on the basis of the preparatory and triplicate measurement data of standard solution "WES-C", so it was a "single-point calibration" approach. The established calibration coefficients in a single laboratory are presented in the Table 2. The others can be found in supporting materials (see Table 15). The typical chromatogram of standard solution "WES-C" is shown in the Figure 1 in logarithmic scale.

Table 2. The concentrations, average detector response and the calculated calibration coefficients for three analytical methods obtained in LAR for standard solution "WES-C".

Compound	Concentration			Average peak area, nA·min	Average calibration coefficient		
	mg/kg	mg/L AA	mg/L		2-pentanol as IS	Ethanol as IS	External standard, mg/L AA··(nA·min)$^{-1}$
acetaldehyde	94.1	223	89.2	2.420	2.214	1.309	92.15
methyl acetate	102	242	96.8	2.327	2.498	1.477	104.0
ethyl acetate	98.3	233	93.2	3.060	1.829	1.082	76.15
methanol	114	271	108	3.043	2.139	1.264	89.02
2-propanol	109	258	103	4.546	1.363	0.806	56.73
ethanol	333038	789300	315720	11210	1.692	1.000	70.41
1-propanol	112	265	106	5.483	1.161	0.686	48.32
isobutyl alc.	116	275	110	6.784	0.975	0.576	40.57
2-pentanol	29.5	70.0	28.0	1.682	1.000	0.591	41.62
1-butanol	112	264	106	6.041	1.051	0.621	43.76
isoamyl alc.	117	276	111	6.837	0.971	0.574	40.42

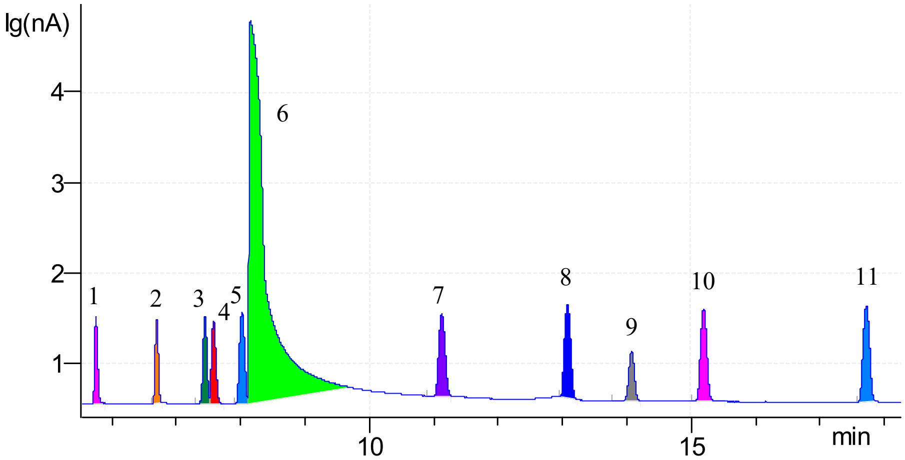

Figure 1. The typical chromatogram of standard solution "WES-C" obtained in LAR.
1- acetaldehyde, 2 – methyl acetate, 3 – ethyl acetate, 4 – methanol, 5 – 2-propanol, 6 – ethanol, 7 – 1-propanol, 8 – isobutyl alcohol, 9 – 2-pentanol, 10 – 1-butanol, 11 – isoamyl alcohol.

Closed circular charts were chosen for the demonstration of obtained results as they allow to visualise great amount of data. Lower percentage characteristics of a method cause smaller area of a corresponding polygon so it becomes easy to compare the chosen methods. As the whole statistical report would include lots of charts only few of them are presented in this paper for the solution "WES-1" as an example. The others can be found in supporting materials in tabular forms (see Tables 16-18).

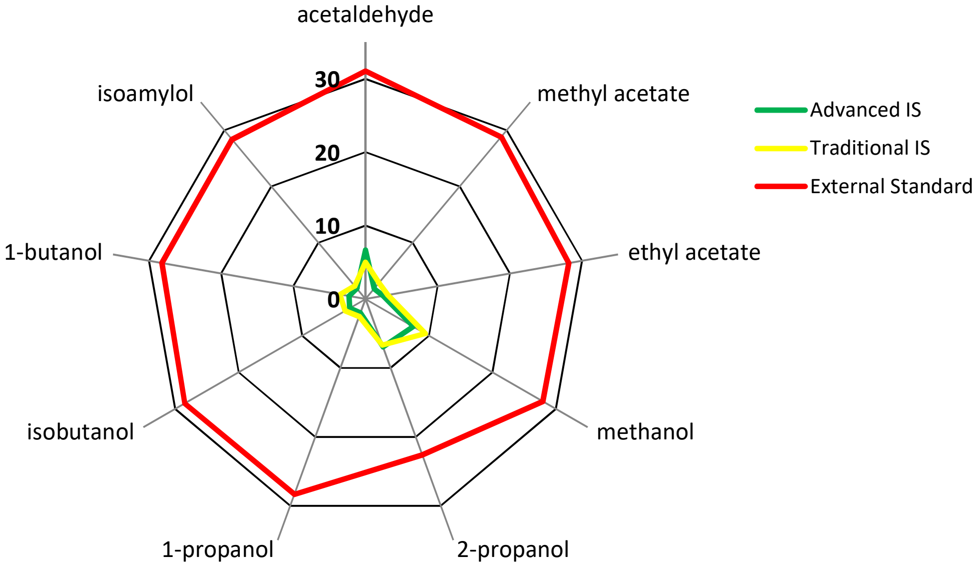

Figure 2. The histogram of repeatability limits (%) comparison for the solution "WES-1"

Eventually in spite of different histograms shapes from various measurements the two IS methods showed better results and great similarity in characteristics values relatively to each other. Oppositely, ES method charts appeared to be much bigger than corresponding charts of the above mentioned IS methods.

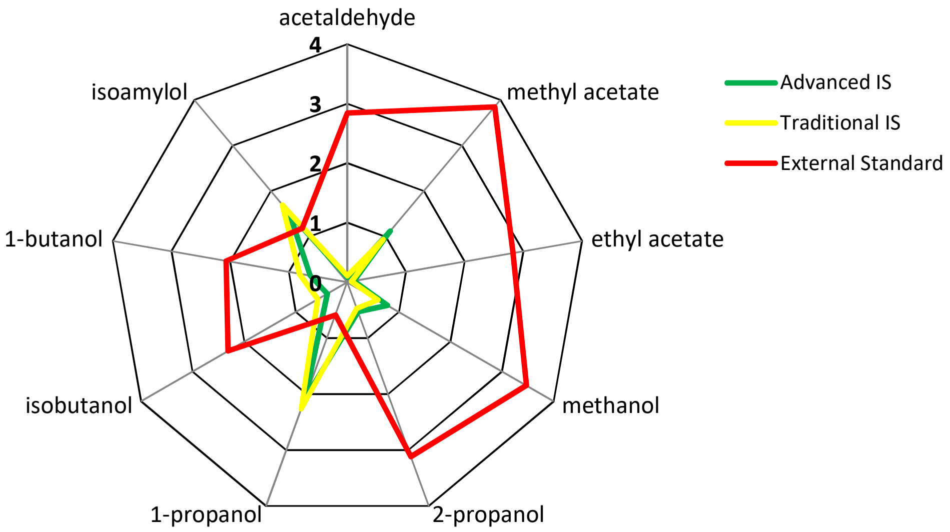

Figure 3. The histogram of biases (%) comparison for the solution “WES-1”

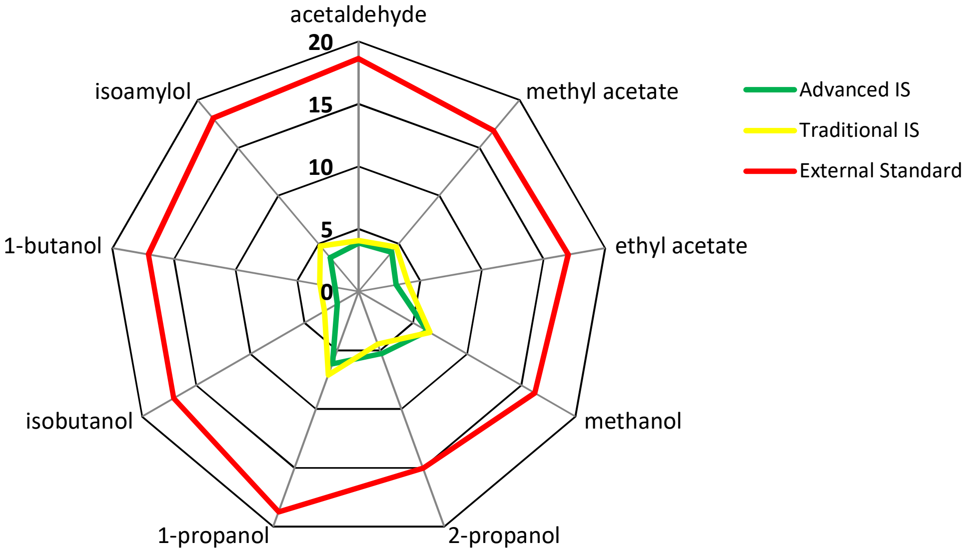

Figure 4. The histogram of the uncertainties (%) comparison for the solution “WES-1”

The obtained LOQ values are presented in the Table 3. The great LOQ value for acetaldehyde in CU laboratory seems to be the result of incorrect storage conditions during transportation. Thus, acetaldehyde is the most sensitive component of all and it can be easily involved into chemical reactions of oxidation and ester formation even at room temperatures. Speaking about relative divergence in LOQ among methods it should be pointed out that the ES method generally lead to much higher values. This can characterize ES method as the worst method among all used under the represented conditions. The two IS methods showed very similar LOQ values in all laboratories, as expected. 2-propanol couldn’t be separated at CU laboratory, that’s why there is no data in corresponding cells.

Table 3. Limits of quantification of the three analytical methods.

Compound	LOQ, mg/L AA								
	LAR			RCH			CU		
	Trad IS	Novel IS	ES	Trad IS	Novel IS	ES	Trad IS	Novel IS	ES
acetaldehyde	0.26	0.20	0.39	0.99	1.18	3.73	14.1	14.0	14.1
methyl acetate	0.34	0.34	0.39	0.45	0.41	0.30	1.49	1.72	2.09
ethyl acetate	0.17	0.18	0.21	0.21	0.27	0.98	1.33	1.02	0.92
methanol	0.61	0.44	1.84	2.03	1.13	11.4	2.64	3.19	4.05
2-propanol	0.61	0.59	0.35	0.28	0.25	1.87	N/A	N/A	N/A
ethanol	—	—	—	—	—	—	—	—	—
1-propanol	0.79	0.79	0.69	0.14	0.20	0.99	1.47	1.35	1.12
isobutyl alc.	0.65	0.66	0.57	1.14	1.09	0.34	3.51	3.85	3.79
2-pentanol	—	—	—	—	—	—	—	—	—
1-butanol	1.39	1.39	1.22	0.83	0.78	1.04	1.79	1.85	2.39
isoamyl alc.	2.12	2.12	1.85	0.44	0.55	1.81	0.76	1.03	1.36

4. Conclusions

The analysis of obtained data shows that the advanced method “Ethanol as Internal Standard” is comparable with the traditional IS method from analytical point of view. It was showed that this method is robust and can be applied at a wide range of volatiles concentrations: from few mg/L of AA to more than 5000 mg/L of AA. The calculated values of metrological characteristics obtained during the inter-laboratory experimental tests show the great similarity between traditional and novel internal standard methods. Here the question about the possibility of using ethanol as IS compound can be answered positively. It was shown, that in spite of the fact that ethanol concentration is 10^5 more than volatiles, modern GC systems allow to do correct quantification procedures.

In addition the “Ethanol as Internal Standard” method can be validated according to the inter-laboratory test of prepared standard solutions as it was done in [15]. No more additional measurements and operations required except taking ethanol peak area into the calculations.

It should be also pointed out that method “Ethanol as IS” is the best from the traditional ones technically as there is no need to add IS compound in each analysed sample and measure the density of the sample in order to establish its ABV value. As a result the correct quantification of volatile compounds in alcoholic products becomes cheaper, faster and easier when the advanced method is applied.

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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