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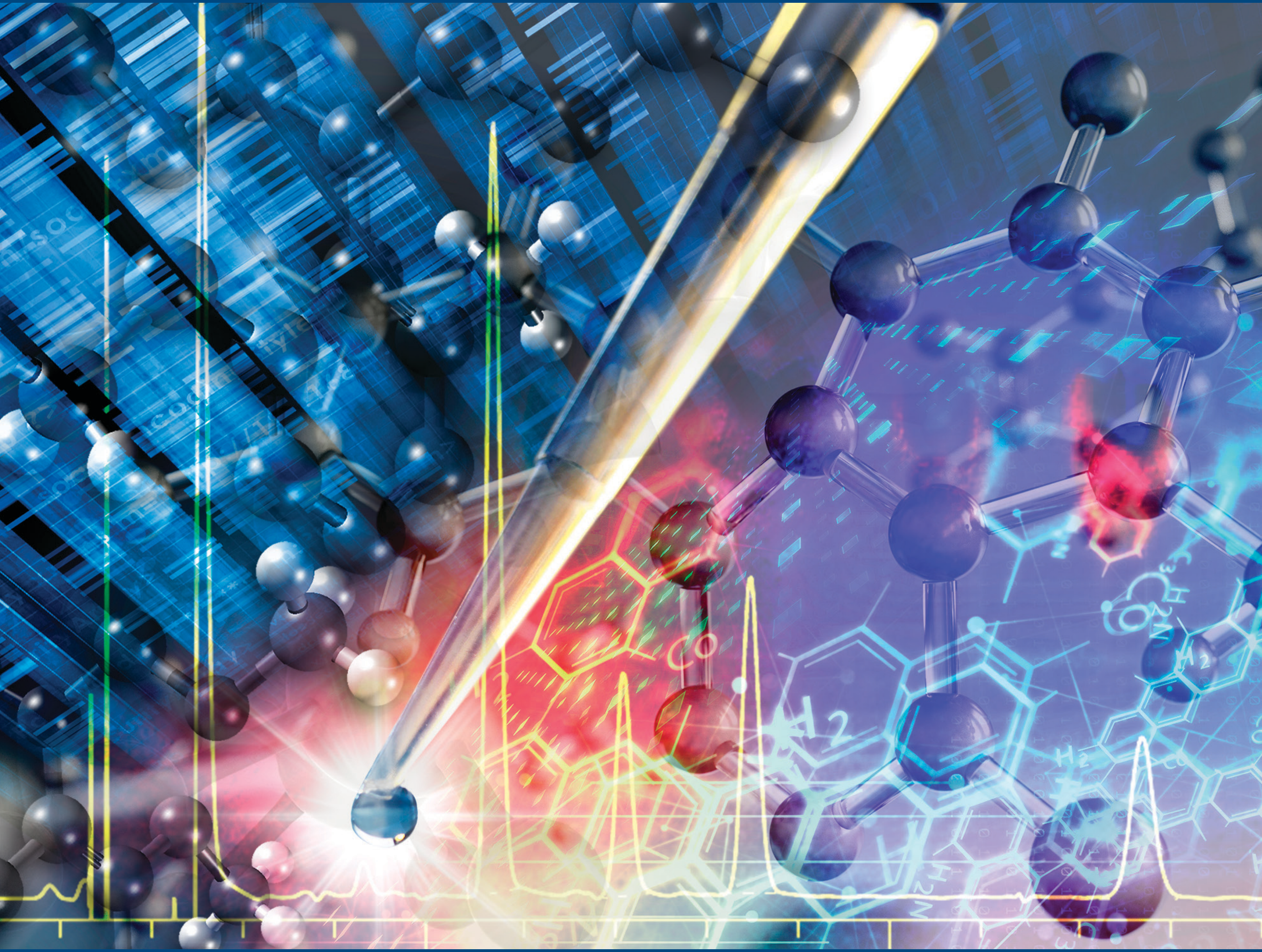

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RESEARCH ARTICLE

# Variability of internal standard method calibration factors estimated with a multifactorial Taguchi experiment in the analysis of alcoholic products by gas chromatography with flame ionization detection

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This study compares the variability of relative response factors (RRFs) using Taguchi's multifactorial analysis for two internal standard (IS) methods in gas chromatography (GC) for quality control of alcoholic products. Methods where either ethanol or pentan-1-ol was used as an IS were compared. For ten volatile substances prescribed by legislation, the RRFs of both methods were compared under 27 different experimental conditions. The influence of parameters (control factors) such as ethanol content in the matrix, analyte concentration, injected volume, injector temperature, split ratio, and flame ionization detector temperature was evaluated. The selected control factors had values at one of the three levels to cover the commonly used ranges of their settings in the measuring system and to characterize the majority of alcoholic products commonly analyzed in practice. The obtained results showed that the biggest differences in the variability of the results between the two methods were found for the most strictly controlled substances in alcoholic products, acetaldehyde, and methanol, where the application of ethanol as an IS provides clearly better results. For both methods, the way control factors affect the repeatability of GC measurements expressed in the form of relative deviation was also evaluated.

KEYWORDS
alcoholic products, calibration, internal standard method, relative response factors, Taguchi

## 1 | INTRODUCTION

An unavoidable step in the routine use of any quantitative instrumental analytical method is calibration, which can be performed in many different ways. However, it is usually time- and material-intensive, which calls for an effort to implement it effectively. Gas chromatography (GC) with flame ionization detection (FID) is one of the most popular techniques for the quantification of both major and minor sample components and is therefore used in a large number of different analyses worldwide.

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**Artilce related Abbreviations:** AA, absolute alcohol; ABV, alcohol by volume; ECN, effective carbon number; FID, flame ionization detection; GC, gas chromatography; IS, internal standard; MS, mass spectrometry; RRF, relative response factor; RSD, relative standard deviation.

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Some studies regarding GC-FID response and calibration factors have attempted to predict or correlate relative response factor (RRF) values with combustion enthalpies of compounds based on the fact that the FID is actually a small burner [1–5]. In other studies, the effective carbon number (ECN) model was used for this purpose [3, 6, 7]. However, in general, using ECN is difficult to justify when corresponding standards are available.

The way in which GC system parameters affect the resulting calibration factors has already been studied by different research teams. For example, El-Naggar [8] has demonstrated how GC-FID response changes with different carrier and fuel gas flow rates, sample volume, etc. Cicchetti et al. [9] described RRF variation for two compounds, polar (benzyl benzoate) and non-polar (limonene) under a variation of 10 experimental parameters. The proactive goal was to create a reliable database of calibration factor values that could be used for GC-FID instruments from different manufacturers. We were therefore interested in continuing such a challenging investigation. Technically, the creation of a calibration factors database would enhance the entire calibration process. Understanding the extent to which calibration factors change their values is also of great interest and potential value. While it is unlikely that a specific validated GC method would undergo extensive changes, it is highly probable that the same analytes are analyzed under different GC and matrix conditions, especially in different laboratories.

In view of previous studies, we wondered whether a similar database of RRF values could be created, especially for the purpose of quality control of alcoholic products, where at least 10 volatile substances need to be determined. These are mostly determined by GC-FID with the internal standard (IS) method, which is prescribed in the legislation [10–12]. The analysis of alcoholic products includes the measurement of distillates and distillation by-products, various spirits, pure alcohol, etc. The need for optimization and development of efficient analytical methods arises due to the large production volumes and corresponding analyses of alcoholic products. Therefore, the “Ethanol as IS” method has been proposed, developed, and validated as an efficient tool for the quality analysis of any alcoholic product by GC-FID or GC-mass spectrometry (GC-MS) [13–15]. The method allows for the use of ethanol as an IS compound, even without any information about its content in the sample and the need to artificially add it, as is typically done with conventional IS compounds.

This paper is a logical continuation of our previous investigations [16, 17], where we have focused on the study of the variation of RRF values for both the proposed (ethanol) and the traditional (pentan-1-ol) IS method with changes in matrix composition, analyte concentration, and GC-MS parameters. The obtained results showed that due to the complexity and sensitivity of the GC-MS instrument, tabulating calibration factors would only be possible within a narrow range of variable parameters. The method using ethanol as an IS showed inferior results compared to the traditional IS method with pentan-1-ol. This work focuses on the GC-FID instrument, which has a different detection principle compared to GC-MS, potentially resulting in different results. Moreover, in comparison with the previous study, an additional sixth control factor of the FID detector temperature was included in the multifactorial experimental design.

The aim of this study was to perform a multifactorial experiment according to Taguchi’s design and to reveal to what extent the calibration factors change their values when the matrix composition and different GC-FID parameters are varied. The obtained results were compared for both ISs, pentan-1-ol, and ethanol.

## 2 | MATERIALS AND METHODS

### 2.1 | Standard solution preparation

The following common volatile compounds stated in European Commission protocols [10] were analyzed: 2-methylpropan-1-ol (isobutanol), 3-methylbutan-1-ol (isoamylol), acetal (1,1-diethoxyethane), acetaldehyde, butan-1-ol, butan-2-ol, ethyl acetate, methanol, methyl acetate, and propan-1-ol. Pentan-1-ol was used as a common IS as stated in [10]. All chemicals mentioned above and ethanol with a concentration of 96.4% alcohol by volume (ABV) were of more than 99.5% purity (GC-FID; Sigma-Aldrich or Merck). Deionized water (18 MΩ cm) was used for the preparation of all calibration solutions.

In the current study, nine standard solutions were prepared. These were solutions with a combination of three different ABV values (20%, 40%, and 96% ABV) and three different concentrations of volatile compounds (2000, 600, and 300 mg/L AA), which are presented in regulatory required units of mg/L of absolute alcohol (AA). The only exception was acetaldehyde. As we have shown earlier [16, 17] acetaldehyde is very reactive and tends to form secondary undesirable products such as its own oligomers (paraldehyde and metaldehyde) and acetal. Solutions with high acetaldehyde and ethanol concentration tend to these reactions. To impede such unwanted processes target acetaldehyde concentrations of 800, 240, and 120 mg/L AA were used in the prepared standard solutions. The detailed algorithm of standard solutions preparation, following the estimation of the volatile concentrations and their uncertainties is presented in the supplementary material. To increase the accuracy of the calculated concentrations we took into account and considered the presence of some

**TABLE 1** Selected control factors and their values.

| Control factor level | ABV, % | Concentration, mg/L AA | Injected volume, µL | Injector Temperature,°C | Split ratio | FID temperature,°C |
|---|---|---|---|---|---|---|
| **1** | 20 | 300 | 0.4 | 180 | 1:20 | 220 |
| **2** | 40 | 600 | 0.8 | 210 | 1:40 | 250 |
| **3** | 96 | 2000 | 1.2 | 240 | 1:80 | 280 |

impurities in the initial ethanol 96.4% ABV. These compounds were acetaldehyde, methanol, and acetal. The calculations were performed with the successive iteration method prescribed in the supplemental material.

## 2.2 | Instrument parameters

A Shimadzu GC-2010 equipped with FID was used for chromatographic measurements. Injections were performed with a standard 10 µL syringe (Shimadzu, Japan) set in the AOC-20i auto-injector (Shimadzu, Japan). Rxi-624 Sil MS (6% cyanopropylphenyl/94% dimethyl polysiloxane) capillary column (20 m length, 0.18 mm ID, and 1.0 µm phase thickness) was used for separation (Restek, USA). The chosen column provided sufficient separation of all the tested volatile compounds, ethanol, and pentan-1-ol. Hydrogen obtained from the NM-600 hydrogen generator (VICI DBS) was employed as the carrier gas (99.9999% purity) at a 35 cm/sec speed; column flow was 1.89 mL/min, septum purge flow was 4.1 mL/min. The following oven temperature program was used: 35°C for 3 min, rising by 30°C/min to 185°C, hold for 4 min. FID was used at different temperatures following the Taguchi design (see section 2.3). Hydrogen, used as a carrier gas, also served as a burner fuel at a flow rate of 30 mL/min; an airflow of 300 mL/min was provided by an air compressor. Nitrogen was used as a make-up flow gas at 30 mL/min. Chromatograms were obtained and processed with GC-MS Solution (Shimadzu) and UniChrom (New Analytical Systems) software.

## 2.3 | Taguchi design

For the aim of this study, the following six control factors were selected for the Taguchi-designed experiment: ABV value of the matrix, the concentration of volatile compound, injected volume of the sample, GC injector temperature, split ratio, and FID temperature. Each control factor value took one from three levels. The latter were selected after the broad literature survey of various papers dealing with the GC-FID analysis of different alcoholic products. The values of the control factors were chosen to extend the range of their commonly used values. For example, a literature search revealed that the most widely used injection volumes were 0.5 and 1.0 µL thus in the current study injection volumes of 0.4, 0.8, and 1.2 µL were used. The exact values assigned to selected control factors are presented in Table 1.

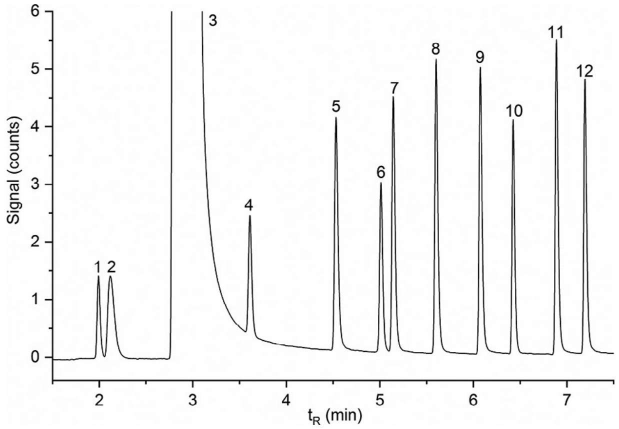

**FIGURE 1** Chromatogram of the prepared standard solution under the conditions of row #5. 1 – acetaldehyde, 2 – methanol, 3 – ethanol (IS #1), 4 – methyl acetate, 5 – propan-1-ol, 6 – ethyl acetate, 7 – butan-2-ol, 8 – 2-methylpropan-1-ol, 9 – butan-1-ol, 10 – acetal, 11 – 3-methylbutan-1-ol, and 12 – pentan-1-ol (IS #2).

The plan of the Taguchi experiment which included 27 different combinations of control factors values was created in Minitab 16 (LEAD Techn.). The “Nominal is best” type of loss function was selected, as our aim was to reduce variability around a specific target. The exact description of the designed experiment is presented in Table S2 of the supplementary material. Each of the 27 positions was measured four times under the repeatability conditions. The chromatogram obtained from measuring the standard solution under the conditions of row #5, where all control factor values were set to “2”, is presented in Figure 1.

## 2.4 | Relative response factors calculation

Relative Response Factors which are the calibration factors for the Internal Standard method were calculated

**TABLE 2** Taguchi conditions that required minor changes in system configuration to separate coeluting compounds.

| No. of rows | Coeluting compounds | Split ratio |  | Injected volume |  |
|---|---|---|---|---|---|
|  |  | Initial | Modified | Initial | Modified |
| 7 | Acetaldehyde and methanol | 1:20 | 1:30 | 1.2 | 1.0 |
| 19 | Methyl acetate and ethanol |  |  | 1.2 | 1.0 |
| 25 |  |  |  | 0.8 | 0.6 |

according to the generally employed equations:

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

where $C_i^{st}$ and $C_{IS}^{st}$ are concentrations of the *i*-th analyzed compound and IS, correspondingly; $A_i^{st}$ and $A_{IS}^{st}$ are the detector responses for the *i*-th analyzed compound and IS, correspondingly. In the case of using ethanol as an IS compound, its concentration in each and every prepared standard solution equals 789300 mg/L AA [13–15].

Equation (1) was used for calculating RRF values from a single GC measurement. These values of single-measured calibration factors were then used for the evaluation of the precision within four replicates. However, the final RRF values of each of 27 Taguchi's conditions among $n = 4$ repeated measurements were calculated according to the following equation [18]:

$$RRF_i^{IS} = \frac{C_i^{st}}{C_{IS}^{st}} \cdot \frac{\sum_{j=1}^{N} \frac{A_{ij}^{st}}{A_{IS,j}^{st}}}{\sum_{j=1}^{N} \left( \frac{A_{ij}^{st}}{A_{IS,j}^{st}} \right)^2}, \tag{2}$$

where *j* is one from $n = 4$ repeated measurements.

## 3 | RESULTS AND DISCUSSION

According to the planned Taguchi experiment, 27 different combinations of six control factors at three value levels were measured four times under repeatability conditions. Three of these 27 combinations led to a situation when not all the compounds were satisfactorily separated; in these cases, measuring conditions were slightly changed in order to register them. Because unsatisfactory separation was caused by excessive amounts of volatile compounds, two control factors, explicitly split ratio and injection volume, were changed. After repeated measurements under new conditions, only RRF values of coeluted compounds were recalculated. The rest of the compounds' RRFs were calculated under the initial system conditions. The list of changes made is presented in Table 2.

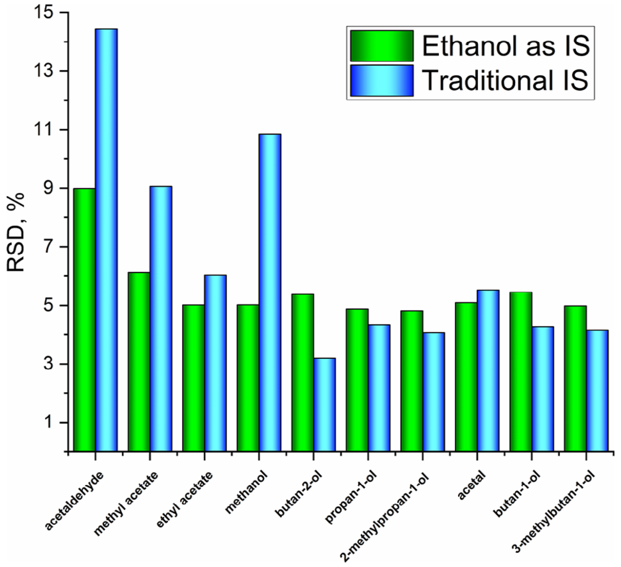

**FIGURE 2** Variation of relative response factor (RRF) values for both internal standard (IS) methods.

### 3.1 | General variation

For all analyzed volatile compounds relative standard deviations (RSD) of obtained 27 RRF values (see Equation (2)) were calculated first. Then, RRF variation for each of the two used IS methods was estimated as an average RSD value of obtained 10 individual RSD values for all volatile compounds. The confidence interval for that average value was calculated by the following common equation:

$$\delta = t_{0.05} \cdot \frac{SD}{\sqrt{10}}, \tag{3}$$

where $t_{0.05}$ is Student's *t*-criterion on a significance level of $\alpha = 0.05$, *SD* is the standard deviation of ten RSD values.

The average RSD value for the "Ethanol as IS" and traditional IS method was 5.6 ± 0.9 % and 6.6 ± 2.6 %, consequently. Individual RSD values are presented in Figure 2.

The obtained data clearly indicates that the variation of RRF values was significantly lower for the most volatile analytes (acetaldehyde, methanol, and methyl acetate) when the "Ethanol as IS" method was employed. These compounds eventually caused overall slightly worse

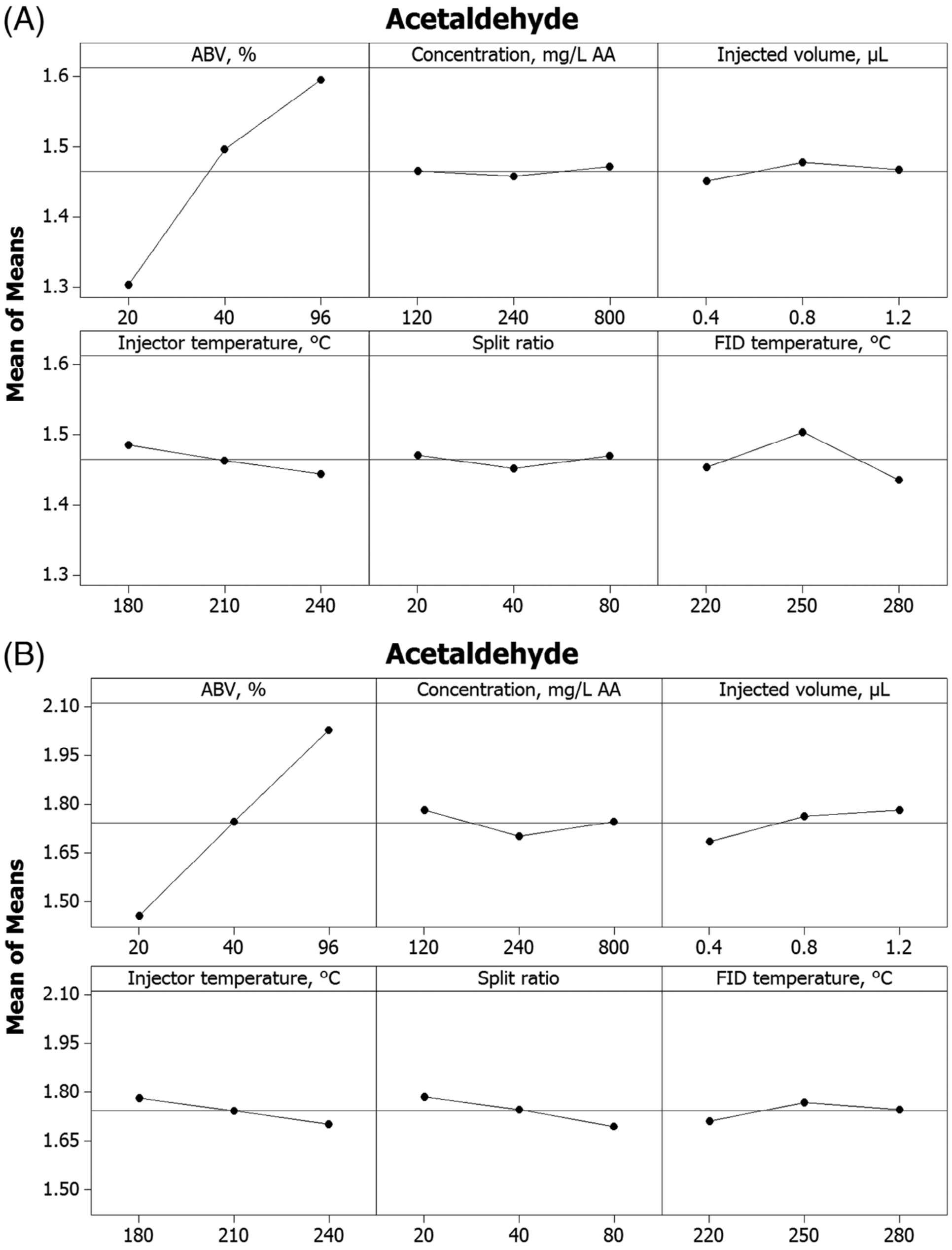

**FIGURE 3** Taguchi graphs obtained for acetaldehyde for the "Ethanol as IS" method (A) and the traditional IS method (B). The mean of means refers to the average relative response factor (RRF) value calculated from 27 sets of averages. Each set is derived from four repeated measurements of 27 distinct systems. configurations.

stability of RRFs for the traditional IS method. Acetaldehyde showed the biggest variation for both used IS methods.

## 3.2 | Taguchi analysis

Taguchi graphs showing the influence of each control factor on RRF value for the two used methods are presented in Figures S1 and S2. The example of obtained graphs is presented in Figure 3 for acetaldehyde for both IS methods.

The Taguchi plots in Figure 3 show how each selected control factor affects the change in the outcome value, which in our case is the RRF. The figures show that in the case of acetaldehyde, five of the six control factors (concentration, injection volume, injector temperature, split ratio, FID temperature) had almost no effect on the resulting RRF value for both methods. Conversely, the ABV, that is, the ethanol content of the matrix, plays a major role in the variation of the RRF. For both methods, ABV had a direct effect on the resulting RRF value of acetaldehyde. This phenomenon can be explained by the high reactivity

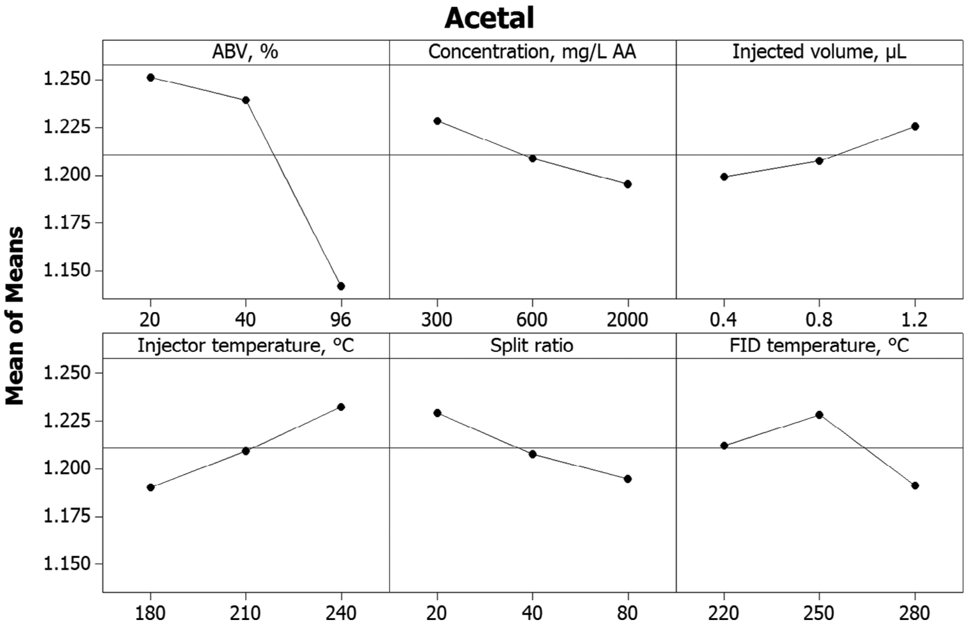

**FIGURE 4** Taguchi graph obtained for acetal using the “Ethanol as IS” method.

of acetaldehyde, which tends to form acetal and other by-products, even when its concentration is reduced compared to the other components. However, the significant excess of ethanol molecules, which rises from 20% to 96% ABV, shifts the equilibrium toward acetal formation from acetaldehyde and ethanol. This effect is clearly visible on the Taguchi graph (see Figure 4) generated for acetal using the “Ethanol as IS” method.

In addition, a more detailed assessment of the effect of control factors on RRF was performed using the response tables provided by Minitab software. As shown in Figure 3, each Taguchi plot is a three-point plot, where each point corresponds to the average value of the RRF using the exact value of the chosen control factor. To compare the effects of each factor on RRF variability, we calculated the average relative span defined as the difference between the largest and the smallest RRF value (obtained for the selected control factor) divided by its average value and multiplied by 100 (%). The relative values of the RRF span for all 10 volatiles analyzed for the same control factor were then averaged and the resulting value was used to evaluate the effect of the control factor on the variation in RRF values. The results obtained are shown in Figure 5. The Grubbs test of the relative span values of 10 analytes, which were averaged to create a graph, revealed no outliers for all 6 control factors.

As can be seen from Figure 5, ABV can be considered the control factor that influences the RRF value to the greatest extent of all the 6 factors examined. For the two IS methods used, the average relative range of the RRF value for the ABV factor is close to 9%. The second parameter that most influenced the variability of the calibration factors is concentration, where the average relative range values were 6.8% and 4.5% for the traditional IS method and the “Ethanol as IS” method, respectively. We assume that in the case of concentration of volatile compound traditional IS method showed worse results because of higher uncertainty caused by additional operations. The other control factors affect the RRF to a much lesser extent and their rankings were different for the two methods.

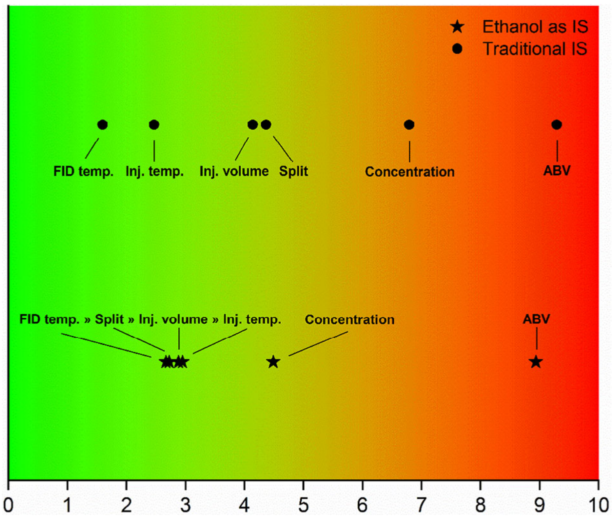

**FIGURE 5** Control factors influence on relative response factor (RRF) variation for both methods. X-axis—percentage calculated as [($RRF_{max}$ − $RRF_{min}$)/$RRF_{aver}$ × 100%].

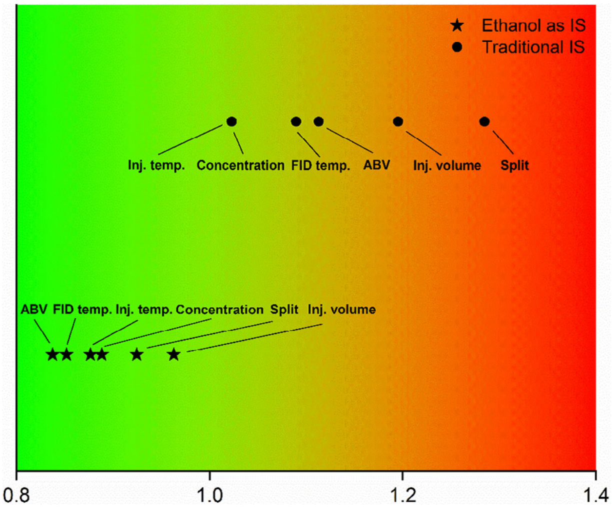

**FIGURE 6** Control factors influence on relative response factor (RRF) variation within four repeated measurements for both methods. X-axis—RSD calculated as [$SD_{max}/RRF_{aver}$•100%].

### 3.3 | Repeatability of repeated measurements

Similar to Figure 5, the average relative standard deviations (RSDs) were obtained from the Taguchi plots for each control factor. However, in contrast to the previous approach, only maximum values of SD were considered instead of calculating the span between the extrema. The resulting plot of how the various control factors affect the repeatability of the measurements of the two methods is shown in Figure 6.

As can be seen in Figure 6, the "Ethanol as IS" method showed slightly better results than the traditional method using pentan-1-ol, with the average RSD value among the 4 replicate measurements not exceeding 1 %. The traditional IS method gave slightly worse RSD values ranging from 1.0 to 1.3 %. For both IS methods tested, injection volume and split ratio were the factors affecting RSD to the greatest extent. This phenomenon can probably be explained by the non-ideality of the GC injection and splitting system.

## 4 | CONCLUSION

In this work, the variability of the RRFs of 10 analytes for two IS methods for the analysis of alcoholic products by GC was evaluated. The effects of experimental conditions (factors) such as ethanol content in the sample matrix, analyte concentration, injected sample volume, injector temperature, split ratio, and FID temperature were considered. A multi-factorial analysis using Taguchi methodology was planned and performed and the results obtained showed that the "Ethanol as IS" method provides less variability in RRF compared to the traditionally used IS method. The overall variability of RRF values was 5.6 ± 0.9 % for the "Ethanol as IS" method and 6.6 ± 2.6 % for the traditional method. The relative standard deviations of the RRF values obtained in the four replicate measurements confirmed that the "Ethanol as IS" method is more reliable than the traditional one. It should also be pointed out that the traditional IS method showed noticeably worse (60% and 110% bigger RSD values) results in the variability of RRF values for acetaldehyde and methanol, respectively, the two most important and most strictly controlled volatile compounds in all alcoholic products. Given the obvious advantages of the "Ethanol as IS" method, it can be highly recommended for routine use as a robust and reliable tool for quality and safety control of alcoholic products by GC-FID instrumentation.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the Supporting Information of this article.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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