Research Article

Quantification of Some Herbal Distillates’ Methanol to Evaluate a New Diagnostic Kit

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Received 27 October 2019; Accepted 15 February 2020; Published 24 March 2020

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Methanol naturally exists in all plant products. In recent years, the consumption of certain kinds of herbal distillates has led to blurred vision, leading to neurotic blindness in Iran. The advanced methods needed for determination of methanol are not available in all parts of poor and developing countries. In this study, we evaluated some herbal distillates’ methanol with a new kit compared to gas chromatography for determining the efficacy of the kit. A gas chromatography apparatus was used to determine methanol concentration of 57 herbal distillates. At the same time, a spectrophotometer device was also used along with a newly designed kit based on the modified chromotropic acid method for the same purpose. All examined samples have different amounts of methanol from 21 to 770 mg/l. The methanol content of all samples was higher than the used kit’s limit of quantification (5 mg/l). The attained results by two used methods were very close together in both minimum (21 and 22 mg/l) and maximum (770 and 690 mg/l) amounts. The comparison of results was shown, and some of available herbal distillates in Iran have enough amounts of methanol to create chronic type of methanol poisoning. Our results suggested that the used kit had suitable efficacy for quantitative determination of herbal distillates’ methanol content. It was proved that the type of the herbal distillate did not affect the kit’s function. The new kit can be easily used with minimal equipment for quality control of herbal distillates in food industry area.

1. Introduction

Methanol is produced and stored in all land plants tissues liquid pools, especially in green stems and leaves [1–5]. Also, an important result of the land plants metabolism is emission of methanol to atmosphere (particularly during growth) [6–9]. Furthermore, methanol plays many physiologic roles in plant life including signaling, health, behavior, and defense in return to environmental factors. Therefore, existence of methanol in plant products including different kinds of juices and herbal distillates is completely logical. As, based on American Standard, existence of 120–460 mg/l (with mean 140 mg/l) methanol in fresh and canned juices is permitted [10]. Therefore, continued intake of enough amount of methanol by these products (like herbal distillates) can cause chronic methanol poisoning [5, 11–13].

Methanol is highly toxic for human body, and accidental intake of its enough amounts may cause severe intoxication due to accumulation of methanol toxic metabolites in different tissues [14–16]. Methanol is also quite toxic to the central nervous system, and permanent blindness is a major consequence of its toxicity in acute conditions [14, 15]. Moreover, prolonged intake of low amounts of methanol by herbal distillates can cause chronic type of methanol poisoning [11]. Herbaceous distillates are usually colorless liquids with mainly consistency of water and a variety of organic ingredients including drug compounds and essences which are used for therapeutic purposes in some countries including Iran [17]. However, there are some reports about existence of different contents of methanol in nearly all of these products [11–13, 17–21]. Therefore, determination of herbal distillate’s methanol content must be mandatory.
during their production process as a part of the quality control.

Several methods including high-performance liquid chromatography (HPLC) [22], selective flow injection [23], enzymatic method [4], Fourier-transform infrared spectroscopy (FT-IR) [24], gas chromatography-mass spectrometry (GC–MS) [25], and gas chromatography (GC) [26] are currently being applied to determine the methanol content in various samples. This is while the required pretreatment of the samples in the HPLC method, expensive needed apparatus, and high technical knowledge/experience have made them inapplicable in routine quantifications in developing countries [27].

Formaldehyde (HCHO) can react with chromotropic acid (CA) in hot concentrated sulfuric acid media. This specific reaction is adopted as a standard colorimetric method for determination of formaldehyde and formaldehyde-releasing compounds (methanol and formic acid) [26–28]. This method has also been recommended as an international reference method by AOAC (Association of Official Analytical Chemists) for measuring methanol in alcoholic drinks after its oxidization to HCHO [29].

Briefly, this method consists of three steps. (1) Methanol is oxidized to HCHO and subsequently to formic acid using potassium permanganate in acidic media. (2) The potassium permanganate color (dark violet) is faded by administration of sodium hydrogen sulfite by transformation of violet Mn⁷⁺ (Manganese⁷⁺) to colorless mn²⁺ (Manganese²⁺). (3) Formic acid is changed to HCHO to react with its specific color indicator (CA) in vicinity of heated concentrated sulfuric acid that is associated with appearance of violet complex, whose color intensity depends on the methanol concentration [27, 29]. This reference method has some limitations including long operation time and the painstaking process to treat formic acid, which was formed during the methanol oxidation process [30]. However, consumption of a large volume of hot concentrated sulfuric acid is the major drawback of this method, which is potentially hazardous and corrosive [27].

Furthermore, Rafizadeh et al. [11, 12, 18] and Saadat and Rafizadeh [13] had shown, application of the AOAC recommended CA method for determination of methanol in ethanol-free liquids (including herbal distillates) can lead to erroneous results. They announced that such a situation is plenty visible in the recent Iranian researchers’ results and conclusions [11, 13]. Therefore, it seems, having access to a precise, accurate, low-cost and efficient kit that can easily quantify the different kinds of herbal distillates’ methanol contents is highly required [11–13, 18]. So, we had two aims in this study. In opposition to the other researchers’ investigations, we determined some different kinds (57) of herbal distillates’ methanol content using a newly designed kit for quantification of these products’ methanol for the first time. This kit is designed based on the traditional CA reference method. We quantified the samples’ methanol contents with the GC method as the gold standard. Then, the gained results by both methods were compared together to evaluate the efficacy of the used kit.

2. Materials and Methods

Herbal distillates produced by six different companies were purchased for evaluation. Each company was given an alphabet letter (A–F) to be identified. Methanol content of 57 herbal distillates was examined by two modified CA and GC methods. After approving the kit function (Tables 1 and 2), the methanol contents of samples were compared with GC results (Table 3).

2.1. Apparatus. A GC apparatus (YL 6100 GC model, South Korea) was used for determination of methanol. The GC system was equipped with a flame ionization detector (FID) and Tr25. The length and inner diameter of Si column was 30 m and 0.53 mm, respectively. Helium carrier gas (flow rate = 6 ml/min) was used for methanol separation. A spectrophotometer (6405 UV/VIS Jenway, England) was also used to perform the chemical (kit) method.

2.2. Chemicals. Methanol and ethanol needed for preparation of standard and control solutions were purchased from the Merck Company (Merck KGaA, Darmstadt, Germany) with analytical grade and used without more purification. A newly designed kit produced by Arya Mabna Tashkhis Co., Tehran, Iran, was used to measure the methanol content of the samples. This kit contains 5 reactants (shown by A, B, C, D, and E), 5 standards with 0, 12.5, 25, 50, and 100 mg/l concentrations of methanol, and an instruction brochure available in the pack. Fifty five different herbal distillates (including Mentha L., Anethum graveolens L., Alhagi maurorum L., Medicago sativa L., Cichorium intybus L., Salix alba L., Urtica dioica L., Carum carvi L., and Fumaria officinalis L.) were totally purchased from different local commercial stores. The manufacturing and expiring dates of each product were checked, as well. To prepare the samples, one mL of each sample was diluted in four mL of distilled water (D. W) to reach a dilution of 1: 5. In the GC method, ethanol was added to 10 mL of each sample to obtain a 100-mg/l concentration of ethanol as an internal standard.

2.3. Standard Solutions. Five standard solutions containing 0, 12.5, 25, 50, and 100 mg/l of methanol with 100 mg/l ethanol as an internal standard were prepared by the serial method to be used for GC. Three samples containing 10, 50, and 100 mg/l of methanol without ethanol were also prepared to be tested as controls for analytical quality assurance of the kit.

2.4. Procedure of the Kit. According the brochure of the kit, 0.2 mL of each standard and all diluted samples (1: 5) were poured into previously labeled separate test tubes with 50 μL of reactants A and B (sulfuric acid and potassium permanganate, respectively) and well shaken. Five minutes later, reactant C (sodium hydrogen sulfite) was added, and the mixture was shaken hardly until became colorless. Fifty μL of reactant D (chromotropic acid) and one mL of reactant E (concentrated sulfuric acid) were then added to
the tubes and shaken. After cooling down, absorbance of their content was read at 575 nm length. Finally, the methanol content of each sample was calculated in comparison to the standard curve by multiplication of the result into the dilution factor (5).

2.5. Procedure of GC Method. Helium carrier gas (at a linear flow of 6 mL/min) was used to separate methanol. Two μL of all standards and samples were directly injected to GC apparatus with split 1:20. The samples were first incubated at 50°C for one minute and then increased to 80°C in three minutes (10-centigrade degree increase in every minute). The results were corrected according to the internal standard peak, and a mean of three results for each sample was considered as the final result.

2.6. Statistical Analysis. Statistical analysis was done by statistical package for social sciences (SPSS) version 24 (IBM Corporations, Chicago, Ill, USA). To perform analytical quality assurance of the kit, mean results of the control was compared with the real methanol concentrations using relative standard deviation (RSD) and relative mean error (RME). Also, after computation of tests’ results, all attained data by both methods were analyzed using paired t-test with the signification level of p value less than 0.05 (p < 0.05). Linear regression analysis was done to compare mean changes of each herbal distillate measured by gold standard (GC method) versus the used kit. Also, the mean of results were compared with gold standard ones. The study was conducted in accordance with the Basic and Clinical Pharmacology and Toxicology policy for experimental and clinical studies [31].

3. Results and Discussion

A GC chromatogram has shown in Figure 1. In this figure, the picks of methanol (the standard with 12.5 mg/l of methanol content) and ethanol (the internal standard with

| Table 1: Parameters of chemical method validations. |
|-----------------------------------------------|
| Analyte | Calibration curve | $R^2$ | LOD (mg/l) | LOQ (mg/l) |
|---------|-------------------|------|-----------|-----------|
| Methanol| $Y = 0.0047x + 0.017$ | 0.9994 | 3 | 5 |
| LOD: limit of detection |
| LOQ: limit of quantification |

| Table 2: The kit’s precision and accuracy data. |
|-----------------------------------------------|
| Methanol concentration (mgL$^{-1}$) | Intraday (n = 5) | Interday (n = 5) |
|-----------------------------------|---------------|---------------|
|                                  | RME (%) | RSD (%) | RME (%) | RSD (%) |
| 10                                | 2.9     | 2.1     | 3.3     | 4.5     |
| 50                                | 0.5     | 0.6     | 1.7     | 2.1     |
| 100                               | 0.3     | 0.4     | 1.1     | 2.2     |

| Table 3: The average of gained results by both kit and GC methods. The results were shown based on mg/l. |
|-----------------------------------------------|
| Name of herbal distillate | A | B | C | D | E | F | Mean* | Difference (%) |
|---------------------------|---|---|---|---|---|---|-------|----------------|
| Mentha                    | MCA | 479 | 293 | 375 | 208 | 243 | 365 | 327 | 9 (2.83) |
|                           | GC  | 477 | 294 | 375 | 200 | 202 | 361 | 318 |       |
| Anethum graveolens L.     | MCA | 277 | 306 | 690 | 26  | 113 | 478 | 315 | 8 (−2.54) |
|                           | GC  | 259 | 294 | 770 | 26 | 112 | 479 | 323 |       |
| Alhagi maurorum L.        | MCA | 215 | 433 | 74  | 238 | 387 | 329 | 279 | 3 (−1.06) |
|                           | GC  | 202 | 433 | 84  | 244 | 397 | 333 | 282 |       |
| Medicago sativa L.        | MCA | 252 | 142 | 126 | 386 | 282 | 276 | 244 | 9 (3.83) |
|                           | GC  | 204 | 149 | 126 | 370 | 283 | 280 | 235 |       |
| Cichorium intybus L.      | MCA | 229 | 232 | 107 | 238 | 205 | 278 | 215 | 6 (2.87) |
|                           | GC  | 205 | 236 | 103 | 234 | 199 | 275 | 209 |       |
| Thymus serpyllum L.       | MCA | N. S | 94  | 74  | 155 | 45  | 201 | 114 | 7 (5.79) |
|                           | GC  | N. S | 87  | 71  | 157 | 41  | 247 | 121 |       |
| Salix alba L.             | MCA | 53  | 93  | 73  | N. S | N. S | 106 | 81  | 8 (10.96) |
|                           | GC  | 44  | 90  | 71  | N. S | N. S | 86  | 73  |       |
| Urtica dioica L.          | MCA | 327 | 261 | 124 | 514 | 283 | 252 | 294 | 8 (2.80) |
|                           | GC  | 289 | 268 | 117 | 525 | 271 | 243 | 286 |       |
| Carum carvi L.            | MCA | 102 | 100 | 127 | 22  | 43  | 104 | 83  | 8 (10.67) |
|                           | GC  | 70  | 95  | 126 | 21  | 40  | 95  | 75  |       |
| Fumaria officinalis L.    | MCA | 131 | 193 | 66  | 204 | 122 | 225 | 157 | 7 (4.67) |
|                           | GC  | 102 | 181 | 62  | 205 | 125 | 225 | 150 |       |

*All p values were not significant comparing two methods. MCA: modified chromotropic acid, GC: gas chromatography.
100 mg/l of ethanol concentration) are gained under the mentioned condition. As it is visible/as demonstrated, as for the trace methanol concentration of methanol in this standard solution, the GC detector can be sufficiently quantified methanol content.

Also, one of the used standard curves to calculate the test values is depicted in Figure 2. As it is visible, this curve is fully linear and has good slope and equation.

Analytical quality assurance of the method indicated a good linearity with high coefficient of correlation (more than 0.99) (Table 1).

According to Table 1, the LOQ of proposed kit is 5 mg/l that as for usual concentration of methanol in different herbal distillates, it seems to be completely suitable. Also, for investigation of precision and accuracy of proposed chemical method, three different concentration of methanol (10, 50 and 100 mg L⁻¹) were analyzed for five times (n = 5) in one day (intraday) and over 3 days (interday) that the gained results (Table 2) were confirmed the chemical method reproducibility [32].

In Table 2, the reproducibility of the method is acceptable, because intraday and interday variations are less than 5%. The gained results using both GC and the proposed kit were compared in Table 3.

As Table 3, the methanol content of the all (57) samples were successfully determined that varied from 21–22 mg/l (GC/kit) to 690–770 mg/l (kit/GC). In other words, all samples have more methanol than the kit LOQ. Comparison of the two used methods results indicates their high similarity together. This similarity is easily deductible due to their mean (217.14 and 213.33 mg/l), standard deviations (138.053 and 144.739 mg/l) and very little difference between them (3.81 and 6.686 mg/l). This indicates that there is no significant difference between the results of the kit and GC method (Table 4).

Likewise, these findings confirmed that the organic ingredients including drug compounds and essence concentrations did not affect the kit function since its sensitivity was not affected by them. Their methanol content was independently determined in aqueous media containing these compounds. Therefore, this method had enough validity and could be applied in similar examinations.

Methanol plays many important roles in plant physiology, and it is a natural ingredient in all land plant tissues with more concentrations in green leaves and stems. Based on this, varying concentrations of methanol are usually present in herbal distillates. Based on our results, all samples included varying amounts of methanol, a result in accordance with previous results in the literature [11–13, 17–21]. Determination of methanol content of these products is important due to the hazardous effects of methanol on human body. Thus, it should be a major step in quality control of their production process. There are more than 4000 small traditional and large industrial herbal distillates’ producers in Iran, which do not consider this observation due to unavailability of any easy, low-cost, and suitable tool for controlling these products’ methanol content. Quantification of methanol requires expensive and complicated equipment, and therefore, having access to easier and less complicated techniques such as mentioned kit is warranted.

To the best of our knowledge, in spite of many valuable reports on applications and benefits aspects of herbal foods, distillates, and medicines, no study has been performed for new easy and cheap methods of methanol determination. Therefore, complete comparison of our results with previous studies is practically impossible, and it was only probable to compare this with the previous Iranian researchers’ studies about the presence of methanol in the herbal distillates. They have made great efforts to provide an easy method for measuring methanol in recent years.

In our study, all samples included varying amounts of methanol is similar to previous results in the literature. Also, regardless of some details, the CA method recommended by AOAC has been applied in all of these studies for this purpose [11–13, 17–21]. Some of them used the same recommended AOAC CA method with a little modification for their experiments [17, 19–21]. The type and amount of changes were not sufficient to enable this particular method.

![Chromatogram](https://example.com/chromatogram.png)

**Figure 1:** The GC chromatogram of standard 12.5 mg/l of methanol.
of alcoholic beverages, getting applied for measuring amount of methanol in ethanol-free samples (herbal distillates). While, Saadat and Rafizadeh [13] and Rafizadeh et al. [11, 12, 18] used two completely modified chromotropic acid methods for quantitative determination and qualitative detection of herbal distillates’ methanol in their studies that were led to different conclusions [11–13, 18]. So, the results of this study are completely matched with their previous findings.

Furthermore, GC was used as gold standard method for evaluation and validation of kit results. As deductible from Table 1 and 2, the suggested kit has high accuracy and precision to detect 5 mg/l of methanol in herbal distillates which lies below the safe content of methanol in these products. Also, according to Table 3, the kit yielded accurate results in comparison to the gold standard test of GC. The minimum methanol determined by both methods was 22 and 21 mg/l in Carum carvi L. distillate of company D, and the maximum content was 690 and 770 mg/l in Anethum graveolens L. distillate of company C. Also, as observed, the least content means were obtained in Salix alba L. distillate using both chemical and GC methods with 81 and 73 mg/l methanol concentration with 8 mg/l (10.96%) difference, while the highest were detected in Mentha domestica L. (3 mg/l (1.06%)) and Medicago sativa L. (9 mg/l (3.83%)), respectively. All these comparisons show great similarity of the results obtained by both the kit and GC methods.

On the other hand, as seen in Table 4, the chemical and GC methods minimum (22 and 21 mg/l) and maximum (690 and 770 mg/l) amounts are very similar and in both cases, slight differences (1 and 80 mg/l respectively) exist between them. The computed means and standard deviations of the two methods gained results (217.14 ± 138.053 and 213.33 ± 144.739 mg/l) are very close and have very low differences (3.81 and 6.686 mg/l respectively), as well. These results have shown that the type of the herbal distillate did not affect the kit’s function. In other words, the various organic compounds of these products do not interfere with methanol. Such high accuracy has proved that this kit can successfully be applied to determine the methanol content of any type of herbal distillate.

In this test, the specificity of the proposed CA method is statistically confirmed by comparing attained results with gold standard ones with a p value less than 0.05 (p < 0.05). Existence of a high linear regression factor (0.993) with sig = 0.000 shows a significant relationship between used kit and gold standard variables that proves some previous conclusions. On the other hand, the sig = 0.118 of paired t-test (more than 0.05) can indicate lack of any significant relationship between colorimetric and GC (gold standard) methods, which means the average obtained by used chemical method in each case is similar to the one attained by the GC method. Hence, paired t-test and linear regression confirmed the ability of the proposed kit, as well. Using these tests, it was revealed that there was no significant difference between the results withdrawn in applying the modified CA method and GC in different herbal distillates.

4. Conclusions

Our results show that all herbaceous distillates contain varying amounts of methanol which can be dangerous
enough to induce chronic methanol poisoning. The newly designed kit can be easily and rapidly applied with high accuracy and minimum laboratory equipment, professional knowledge, and low-cost. In general, the designed kit has the validity and efficacy to determine the methanol content of the herbal distillates.

Data Availability

The average of gained results by both kit and GC methods used to support the findings of this study is included within the article.

Conflicts of Interest

The authors (Farshid Saadat, Hossein Hassanian-Moghadam, Nasim Zamani, Ali Rafizadeh) declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

The authors (Farshid Saadat, Hossein Hassanian-Moghadam, Nasim Zamani, Ali Rafizadeh) of this paper would like to thank the Deputy of Research & Technology of Guilan medical sciences university for financial and official support.

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