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Research Article

Investigation and Health Risk Assessment of Nitrate ion, Nitrite ion, and Phthalate Esters in Herbal Distillates Produced by Iranian Companies

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Abstract: The concentrations of nitrate ion, nitrite ion, nitrosamines, and phthalate esters in the ten herbal distillates, including rose, caraway seed, cumin seed, fumitory, carum, fenugreek, chicory, salix aegyptian, and blessed, produced by four companies were determined. The results were evaluated for each species by statistical methods. For this purpose, the normality test, one-way analysis of variance (ANOVA), and post hoc test were applied to compare the obtained results of four companies. The Shapiro-Wilk test was used to study the normality of the results. Also, the control chart was plotted to investigate and compare the quality of herbal distillates produced by these companies. The results indicated that the concentrations of nitrate ion and nitrite ion in all samples were in the ranges of 5.11-7.84 mg L^{-1} and 0.10-0.97 mg L^{-1}, respectively. All herbal distillate samples produced by these companies were free of nitrosamines. The phthalate esters with low concentrations (0.14-0.47 µg L^{-1}) were presented in the herbal distillate samples produced in the second week after their production. The concentrations of nitrate ions and phthalate esters followed a normal distribution, while the nitrite ion concentrations didn't. The concentrations of phthalate esters in the samples were also determined in the sixth, tenth and fourteenth weeks after producing the herbal distillate samples to investigate the time effect and rate of phthalate ester release in the samples. The concentrations of nitrate ions, nitrite ions, and phthalate esters were lower than their maximum permissible limits in drinking water declared by the World Health Organization (WHO).

***Keywords*:** nitrate ion, nitrite ion, nitrosamine, phthalate esters, herbal distillates

1. Introduction

The growing use of medicinal plants and herbal medicines has attracted particular attention to these herbals such as Echinacea, Ginseng, Ginkgo, Rose, Chicory, Mint, Caraway seed, and Cumin seed.[1] According to the World Health Organization (WHO), 25% of conventional medicines are plant origins. Traditionally, the medicinal effects of 74% of herbal medicines used in a new form have long been known.[2] Due to the unique Geographical and climate state, Iran

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has more than 7,500 herbal species, which is 2-3 times more than the vegetation of the whole continent of Europe. It is predicted that there are more than 750 herbal medicinal species in the vegetation of Iran.[3] Herbals form a large part of nature and have long been considered by humans for food consumption and treatment of diseases. Human attention to plants decreased with the production of synthetic drugs because of their easy use and fast effects. Today the use of plants as drugs and foods has spread in Iran and around the world due to their low side effects on humans and animals. The growing trend of using plants as raw materials to produce herbal medicines and health foods requires a proper and standard quality control procedure, complying with international standard guidelines.[4] Herbal medicines provide a natural alternative to conventional pharmaceutical drugs, often with fewer side effects.[5,6] They have been used for centuries in cultures around the world and offer a wealth of knowledge and traditional wisdom.[7] However, plants are exposed to various contaminants before their applications as medicine or food. Therefore, the safety, efficiency, and effectiveness of plants must be adequately monitored to avoid their side effects, especially allergic reactions and toxicities.

The presence of nutrients in the soil solution is essential for plant growth.[8] Nitrogen is one of the most critical nutrients in plant growth. This element is present in the structure of amino acids, nucleic acids, purine bases, alkaloids, chlorophyll, etc. Besides, nitrogen deficiency reduces photosynthetic efficiency, plant dry weight, leaf area index, protein content, and delays in plant vegetative and reproductive growth.[9] The plants' needs for nitrogen depend mainly on the type of species and soil conditions. Plants in two forms absorb nitrogen, including ammonium and nitrate ions. The nitrate ion is the predominant source for plants in soil.[10] This ion is available for plants through chemical and animal fertilizers and the decomposition of plant residues and other organic residues. Due to the increasing use of synthetic nitrogen fertilizers, the concentration of nitrate ions in vegetables and drinking water has sharply increased.[11] Not all nitrate ions in fertilizers, a large amount of its surface pollutes, and groundwater, through leaching and soil erosion, are absorbed by plants.[12] In the adult human body, nitrate ion is first converted to nitrite and then to nitrosamines through combination with amines, which in nitrosamines increase the risk of cancer.[13] Nitrate accumulation in plants is affected by many environmental and genetic factors.[14] According to previous studies, leafy vegetables are more disposed to nitrate accumulation than other plant products.[15] Investigations in Europe show that nitrate ions accumulate in vegetables in the winter more than in summer due to low light intensity and limited hours of sunshine during the day.[16] Factors that lead to a decrease in the nitrate reductase enzyme activity in the plant are somehow associated with the accumulation of nitrate ions in the plant's shoots.[17] Low light, high temperatures, and humidity stress reduce nitrate reductase enzyme activity, increasing nitrate accumulation. Also, low temperatures affect growth reduction, in which a decrease in nitrate reductase enzyme activity and an increase in nitrate accumulation are displayed.[18] The nitrate ion is non-toxic, but various products of its reaction, including nitrite ions, nitric oxide, and nitrous compounds, are essential to nutritionists because of their detrimental effects on human health, such as meth-hemoglobinemia and carcinogenicity.[19] Various methods were developed for the determination of nitrate and nitrite ions in meat,[20] baby food,[21] and fruit and vegetable samples.[22-24]

Nitrosamine compounds as contaminants are produced by reacting the nitrite ions with secondary amines under certain conditions, including strongly acidic conditions, high temperatures, and so on.[25] The nitrite ions are a product of reducing nitrate ions in the stomach of infants, in which these ions can oxidize iron(II) ions of hemoglobin to iron(III) ions in the bloodstream, leading to meth-hemoglobin pigments, disruption of the oxygen delivery into the infant organs, and eventually suffocation and death of the infant.[13] The reactions for the formation of nitrosamine in two steps are presented as follows:

Step 1: Nitrate to Nitrite Conversion

$$NO_3^- + \text{Reducing agent} \rightarrow NO_2^- + \text{Oxidized product}$$

Step 2: Nitrite and Amine Reaction to Form Nitrosamines

$$NO_2^- + \text{Primary or secondary amine} \rightarrow \text{Nitrosamine} + \text{Byproduct}$$

Phthalate esters (PAEs) were added to plastics to increase the durability, longevity, flexibility, and transparency of

plastics as inexpensive and suitable additives.[26,27] The high amount of plastics was widely utilized due to their low price and proper properties, leading to an increase in the concentration of PAEs in water sources of environment and food production. In other words, PAEs were released into nutria water and food packed with plastics because PAEs as the additives have not formed the chemical bonding in the plastic composition. Occurrence and risk assessment of PAEs as pollution in various samples were investigated, indicating that PAEs are known as a critical problem that affects human health.[28-31] PAEs cause adverse effects on the reproductive system, pancreatic beta cells, immune system, birth weight, and endocrine glands in humans.[32-34] Therefore, the determination of PAEs is necessary for evaluating their risk assessment on human health.

Due to the health concern of nitrate, and its derivatives, much attention has been paid to nitrate ion accumulation in plants. In fact, nitrate ion accumulation is considered one of the qualitative biological indicators for the pollution of plants. With the increasing consumption of herbal distillates in Iran, it is necessary to measure the concentration of nitrate and its toxic derivatives and PAEs released in these real samples to control their adverse effects on humans. Therefore, the present study was conducted to determine nitrate, nitrite, nitrosamines, and PAEs in the herbal distillates produced in Iran. For this purpose, several samples of herbal distillates (10 herbal distillates types from four companies) were randomly selected and analyzed to determine the concentration of nitrate, nitrite, nitrosamines, and PAEs. A statistical strategy, including the normality test, one-way analysis of variance (ANOVA), and post hoc test, was used to investigate the obtained results a 95% confidence interval. The control charts were plotted for each analyte to compare the quality of herbal distillates produced by these companies. Graphic representation of contamination of distillates due to irrigation with contaminated water or improper packaging is presented in Figure 1.

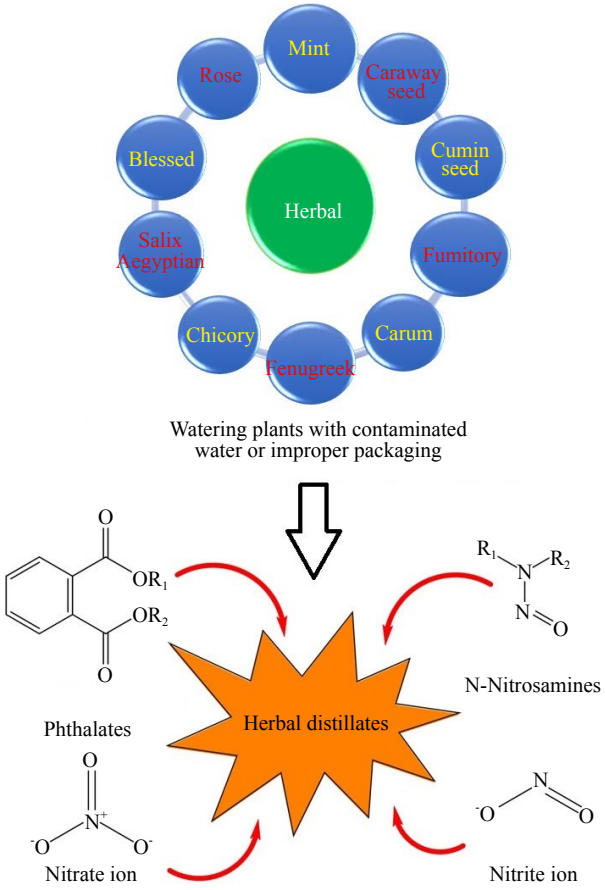

Figure 1. Graphic representation of contamination of herbal distillates due to irrigation with contaminated water or improper packaging

2. Experimental

2.1 *Sampling*

The samples of herbal distillates, including Rose, Mint, Caraway seed, Cumin seed, Fumitory, Carum, Fenugreek, Chicory, Salix Aegyptian, Blessed, were purchased from sales agents of different factories in Sabzevar, Iran. For this purpose, five packages (each pack containing 100 bottles with a volume of 1,000 ± 5 mL) were selected from each herbal distillate with a difference in the production time of 7 ± 1 day. Three bottles from each package were randomly chosen (fifteen bottles in total) and mixed as a reference sample for measuring the analytes. The production and expiration dates of all five samples were the same. It was refrained from mentioning the names of the factories, and English letters, including Za, Na, Af, and Re, were used instead of factory names to observe ethical issues.

2.2 *Determination of nitrate content measuring of herbal distillates*

Nitrate ion concentrations were determined using a spectrophotometric method presented in a previous paper.[35] Potassium nitrate (Sigma-Aldrich, Purity ≥ 99.99%) at 45 °C for 24 h in an oven in a vacuum was dried and used to prepare the standard solution of nitrate ions. The standard solution of nitrate ions (100 mg L^{-1}) was prepared by dissolving 0.163 g of dried potassium nitrate and 1.0 mL of chloroform in distilled water and diluted to 1.0 L with distilled water. This solution is stable for at least 6 months. The daily standard solutions of nitrate ions with concentrations of 1.0, 2.0, 5.0, and 10.0 mg L^{-1} were individually prepared by diluting the stock standard solution of nitrate ions in distilled water. A solution of resorcinol (2.0% w/v) was prepared from dissolving 2.0 g of resorcinol (Sigma-Aldrich, Purity ≥ 99.0%) in 100.0 mL of distilled water. The calibration curve was linear in the concentration range of 0.03-25.3 mg L^{-1} with an R^2 of 0.9979 and a limit of detection (LOD) of 0.08 mg L^{-1}. The intra-day and inter-day relative standard devation (RSD, n = 3) for the nitrate ion determination with a concentration of 2 mg L^{-1} was determined for three measurements of nitrate ion on one day and three consecutive days and were 1.26% and 1.39%, respectively. Certified reference material (CRM) of the nitrate ion (Cat. No. 132240, Merck Millipore, Germany) was used to study the method's accuracy. The concentration of nitrite ion in CRM was determined with the procedure and was 0.98 ± 0.02 mg L^{-1}, indicating the process has a suitable accuracy for the nitrite ion measurement.

The nitrate ions were determined based on the standard addition methods due to the unknown matrix of herbal distillate samples. Briefly, 2.5 mL of the herbal distillate sample and 2.5 mL of each nitrate ion standard solution (0.5, 1.0, 2.5, and 5.0 mg L^{-1}) were poured into a suitable vial, followed by adding 0.6 mL of resorcinol solution (2.0% w/v) and concentrated sulfuric acid (0.5 mL). The vials were vigorously shaken for 5 min, remaining at room temperature in the dark for 30 min. The absorbance of each solution was measured at 505 nm versus the blank sample (distilled water) using a UV-Vis spectrophotometer (Varian-Cary 50, Australia). A typical Uv-Vis spectrum for determining the nitrate ions are shown in Figure 2a. The nitrate ion concentration of each herbal distillate sample was determined by drawing the standard addition plot.

2.3 *Determination of nitrite content measuring of herbal distillates*

Nitrite ions are one of the pollutants containing nitrogen in soil and water samples. The nitrite ion concentration in herbal distillates was determined by a spectrophotometric method by forming azo reddish-purple color at a pH of about 1.5 in the presence of two solutions, including sulfanilamide a and N-(1-naphthyl)-ethylene diamine hydrochloride solutions.[36]

Sodium nitrite (Sigma-Aldrich, Purity of 99.999%) was utilized to prepare nitrite ion standard solution. The sodium nitrite was dried in an oven at 80 °C for 10 h before use. The stock standard solution of nitrite ions (100 mg L^{-1}) was prepared by dissolving 0.177 g of dried sodium nitrite and 1.0 mL of chloroform in 1.0 L of distilled water. The daily standard solutions of nitrite ions with concentrations of 10, 15, 20, and 25 mg L^{-1} were individually prepared by diluting the stock standard solution of nitrite ions in distilled water. The sulfanilamide solution (2.0% w/v) was prepared by dissolving 2.0 g of sulfanilamide (Sigma-Aldrich, Purity ≥ 99.0%) in hydrochloric acid (1.2 mol L^{-1}) in a volumetric flask (100 mL). The N-(1-naphthyl)-ethylenediamine dihydrochloride solution (0.1% w/v) was prepared by dissolving 0.1 g of N-(1-naphthyl)-ethylenediamine dihydrochloride (Sigma-Aldrich, Purity ≥ 98.0%) in distilled water in a volumetric flask (100 mL). The calibration curve was linear in the concentration range of 0.09-16.8 mg L^{-1} with an R^2 of 0.9969 and

a LOD of 0.19 mg L^{-1}. The intra-day and inter-day RSD (n = 3) for the nitrate ion determination with a concentration of 2 mg L^{-1} was determined for three measurements of nitrite ion on one day and three consecutive days and were 1.47% and 1.65%, respectively. The nitrite ion concentration was measured in a CRM of nitrite ion (Cat. No. 125041, Merck Millipore, Germany) to evaluate the method's accuracy. The nitrite ion concentration was 0.64 ± 0.01 mg L^{-1} (or NO_2-N content of 0.195 mg L^{-1}) and was in a cooperative agreement with the certified nitrite ion concentration.

The standard addition procedure was applied to measure nitrite ions. 25.0 mL of the herbal distillate sample and 25.0 mL of each nitrite ion standard solution (0.5, 1.0, 2.5, and 5.0 mg L^{-1}) were poured into an Erlenmeyer flask (100 mL). The sulfanilamide solution (1.0 mL) was added to the solution, followed by dropwise adding 1.0 mL of the N-(1-naphthyl)-ethylenediamine dihydrochloride solution (0.1% w/v) for 2 to 6 min. The obtained solution remained for 10 min at room temperature, and its absorbance was determined at 543 nm using the spectrophotometer versus the blank sample (distilled water). A typical Uv-Vis spectrum for determining the nitrite ions are shown in Figure 2b. The nitrate ion concentration of each herbal distillate sample was determined by drawing the standard addition plot.

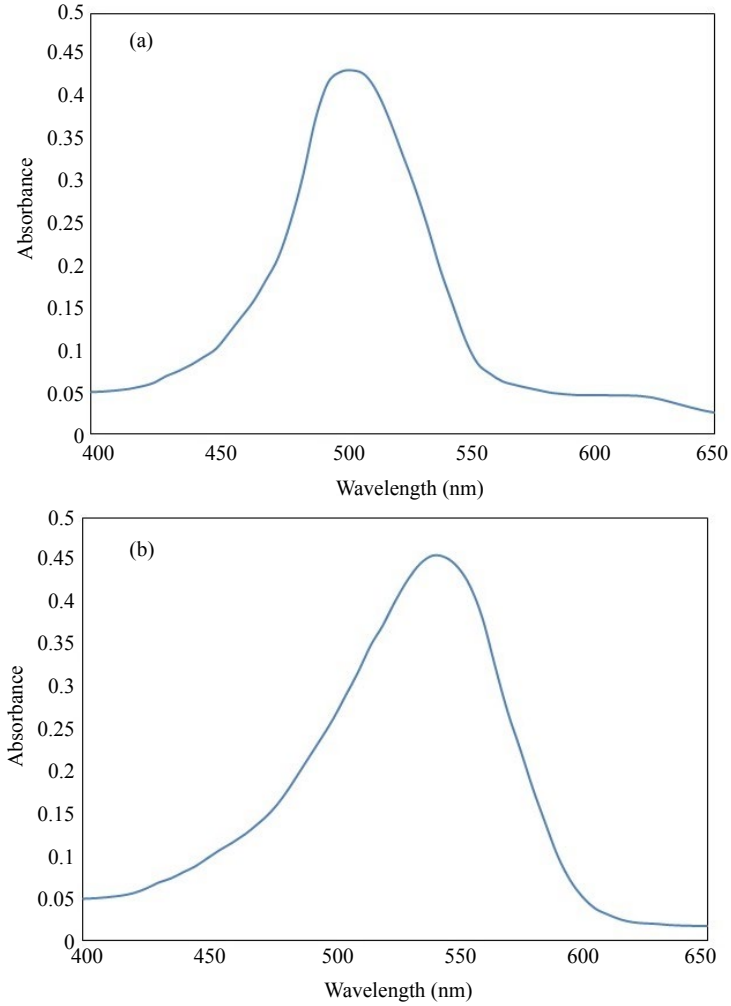

Figure 2. A typical Uv-Vis spectrum for determining the nitrate ions (a) and nitrite ions (b)

2.4 *Determination of nitrosamines and PAEs content measuring of herbal distillates using dispersive liquid-liquid microextraction/GC-MS*

2.4.1 *Dispersive liquid-liquid microextraction*

The sample solution (5.0 mL) was poured into a conical tube (10 mL), and a solution containing carbon tetrachloride (41 µL) as the extraction solvent and acetonitrile (0.75 mL) as the dispersive solvent for extraction of PAEs or methanol (1.5 mL) for extraction of nitrosamines was injected into it. The suspension was sonicated for 30 s and then centrifuged for 5 min at 6,000 rpm. The carbon tetrachloride phase was segmented at the bottom of the conical tube and withdrawn using a gas chromatography (GC) syringe, and 1 µL of it was injected into gas chromatography/mass spectrometer (GC/MS) for analysis.[37,38]

2.4.2 *Sample preparation*

Two reagents, including Carrez solution I and Carrez solution II, were prepared for the sedimentation of proteins in the samples. Carrez solution I was prepared by dissolving 10.6 g of ferrocyanide in 100.0 mL distilled water. Carrez solution II was also prepared by mixing 21.9 g of zinc acetate and 3.0 mL of acetic acid and adjusting the solution to 100.0 mL with distilled water.

To precipitate the proteins in all samples, 1.0 mL of the carrez solution (I) and 1.0 mL of the carrez solution (II) were added to the vessel containing 20.0 mL of herbal distillates. The suspension was centrifuged at 4,000 rpm for 5 min, and the solution was separated.[39] Biphenyl (Merck, Germany) at a concentration of (100 mg L^{-1} in methanol) as an internal standard in the analysis of nitrosamines and PAEs was employed.

2.4.3 *Instrumentation*

The nitrosamines and PAEs were determined using a gas chromatography-mass spectrometer (GC-MS), equipped with a 7890A GC system from Agilent Technologies (Palo Alto, CA, USA), with a split/splitless injection port, as well as a 5975C inert mass selective detector (MSD) network. The nitrosamines and PAEs were separated using a HP-5MS capillary column (30 m × 0.25 mm, ID; 0.25 µg, film thickness). The temperature program was adjusted as follows; the first temperature was 70 °C and held for 15 min. The temperature was increased to 76 °C with a ramp of 3 °C min^{-1} and held for 1 min. Finally, the temperature was increased to 280 °C with a ramp of 30 °C min^{-1}. A helium flow rate of 0.8 mL min^{-1} in the split mode (1:50 ratio) was used for the nitrosamines and PAEs analysis. The analysis was performed for 25 min. The sample volume of 3 µL was injected into the split mode to GC-MS. The temperatures of the auxiliary and injector were fixed at 280 °C.[40] In the selected ion monitoring (SIM) mode, one qualifier ion was chosen for each quantified compound. The mixture components were separated using a Centrifuge (Hettich ROTOFIX 32A) with a speed of 4,000 rpm. Accelerating of alkaline hydrolysis as well as primary extraction was performed by applying a microwave oven (Delonghi type MW 602).

3. Results and discussion

3.1 *Investigated nitrate ion level in the herbal distillates*

The obtained nitrate ion concentrations in the herbal distillate samples for four companies were statistically evaluated. The nitrate ion concentrations in the various samples were determined from three repeated measurements of each sample under the same conditions. Figure 2a displays a representative UV-Vis spectrum used for the determination of nitrate ions, exhibiting a peak absorbance at 505 nm.The results are shown in Table 1. The normality of the results was checked using the Shapiro-Wilk test. The statistical description of the results and the study of their normality are reflected in Table 2.

Table 1. Nitrate ion concentration levels (mg L^{-1}) in the analyzed herbal distillates (n = 3)

Sample	Za	Na	Af	Re
Rose	7.35 ± 0.11	7.84 ± 0.13	7.81 ± 0.12	6.52 ± 0.10
Mint	6.36 ± 0.12	6.83 ± 0.11	5.14 ± 0.11	6.21 ± 0.12
Caraway seed	7.33 ± 0.10	6.42 ± 0.11	6.75 ± 0.14	6.25 ± 0.13
Cumin seed	7.69 ± 0.11	5.11 ± 0.10	5.55 ± 0.12	6.41 ± 0.15
Fumitory	7.40 ± 0.13	5.22 ± 0.11	5.52 ± 0.10	6.34 ± 0.14
Carum	6.60 ± 0.09	6.42 ± 0.13	7.14 ± 0.11	6.58 ± 0. 12
Fenugreek	7.52 ± 0.11	5.52 ± 0.12	6.67 ± 0.13	6.20 ± 0.11
Chicory	7.23 ± 0.10	5.39 ± 0.10	5.16 ± 0.11	6.31 ± 0.11
Salix Aegyptian	7.65 ± 0.12	6.71 ± 0.12	6.12 ± 0. 12	6.75 ± 0.13
Blessed	7.19 ± 0.10	5.14 ± 0.12	5.25 ± 0.10	6.22 ± 0.12

Table 2. Description of the results and test of normality for investigating the nitrate ion concentration in the herbal distillates

Herbal distillates	Mean	Median	Standard Deviation	Minimum	Maximum	Range	Shapiro-Wilk
							Significant
Za	7.1320	7.2800	0.44030	6.36	7.69	1.33	0.196
Na	6.0600	5.9700	0.92123	5.11	7.84	2.73	0.160
Af	6.1110	5.8350	0.93802	5.14	7.81	2.67	0.216
Re	6.3790	6.3250	0.18490	6.20	6.75	0.55	0.157

A normal Q-Q plot is a graphical tool used to assess the normality of a dataset by comparing the observed data distribution to the expected distribution under the assumption of normality. It is significant because it can visually detect departures from normality by plotting quantiles of observed data against quantiles of a theoretical normal distribution. If the points on the normal Q-Q plot form a straight line, it suggests the data follows a normal distribution, which is important for statistical techniques. However, if the points deviate from the straight line, it indicates non-normal characteristics, requiring alternative analysis methods. The results have a normal distribution when the significant value of groups is higher than 0.05 at a 95% confidence interval. The significant values of 0.196, 0.160, 0.216, and 0.157 were obtained for nitrate ion concentrations in Za, Na, Af, and Re herbal distillates, indicating that the results follow a normal distribution. Normal Q-Q plots of nitrate ion concentrations for each company are shown in Figure 3. A one-way ANOVA test was utilized to compare the means of the groups' results at a 95% confidence interval.[41] The results indicated a significant difference in the mean of groups due to a lower significant value than 0.05 (Table 3). The post poc test was necessary in the studies to determine if there were any significant differences among the groups or conditions being compared, after the initial analysis of variance was conducted. It helps to identify which specific group(s) differ significantly from each other when the overall ANOVA result indicates a significant difference. In other words, it allows for a more detailed examination of the data to determine the specific sources of differences or similarities between the groups. Multiple comparisons of the means were carried out using the post hoc tests (Table 4), indicating that the mean of nitrate ion concentrations for Za with Na, Za with Af, Na with Za, Af with Za have a significantly different and other means of nitrate ion concentrations are not significant. The control charts of mean and range were drawn to compare the quality of the herbal distillate samples for four companies in terms of nitrate ion concentration (Figure 4). The control chart of the mean showed that the Za products do not have suitable quality compared with other company products. In contrast, the control chart of the range indicated that all company products have the same quality without any significant difference. Finally, the investigation of nitrate ion concentration in all samples showed that all samples have no higher nitrate ion concentration than the maximum permissible limit of nitrate ion in drinking water by WHO (10 mg L^{-1}). So all herbal distillate samples produced by their companies could be used without any side effects caused by nitrate ions.

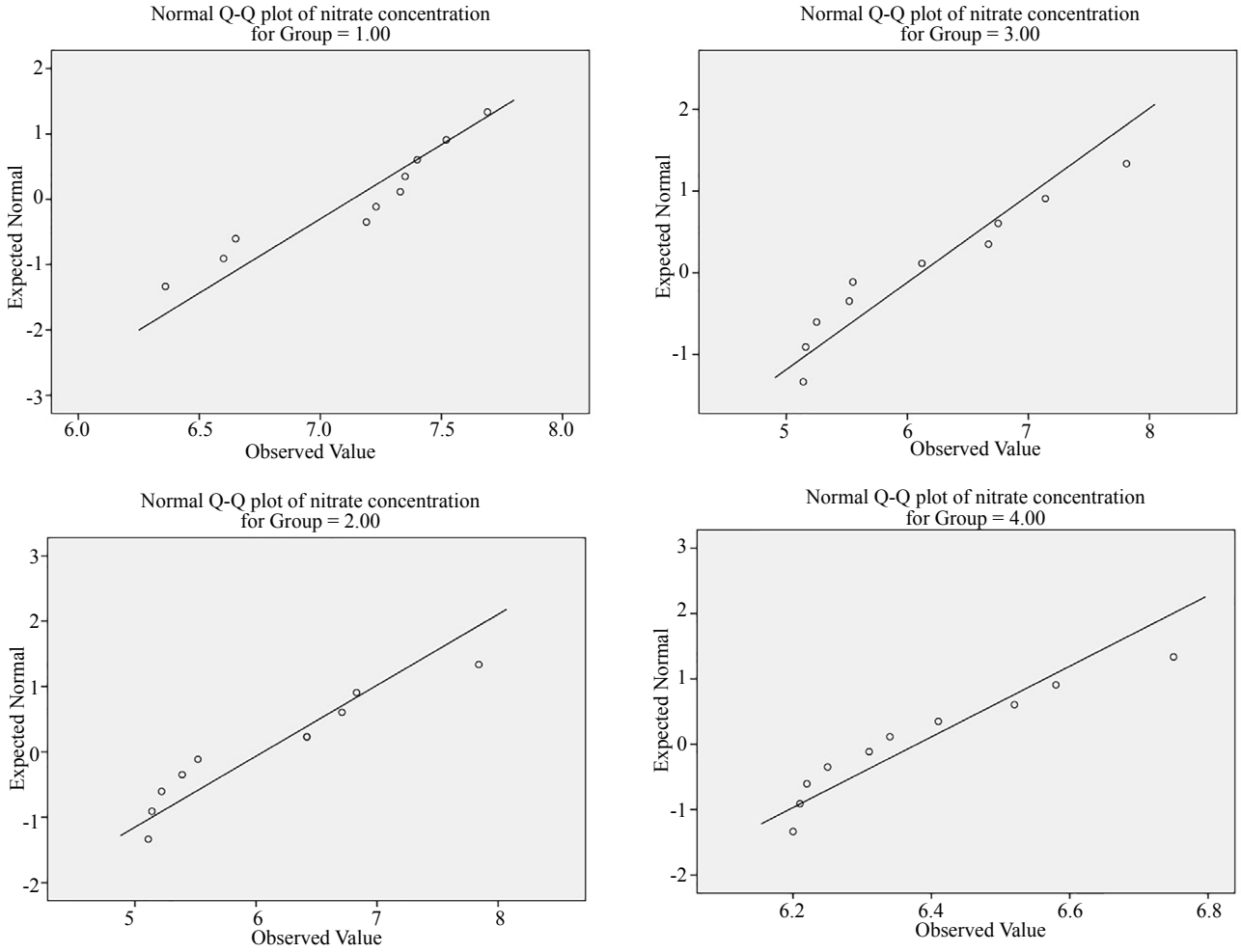

Figure 3. Normal Q-Q plots for the nitrate ion concentration; Group 1.00 (Za), Group 2.00 (Na), Group 3.00 (Af), and Group 4.00 (Re)

Table 3. One-way ANOVA test for the comparison of the nitrate ion concentration means

ANOVA	Sum of Squares	df	Mean Square	F	Significant
Between Groups	7.337	3	2.446	5.000	0.005
Within Groups	17.609	36	0.489	-	-
Total	24.946	39	-	-	-

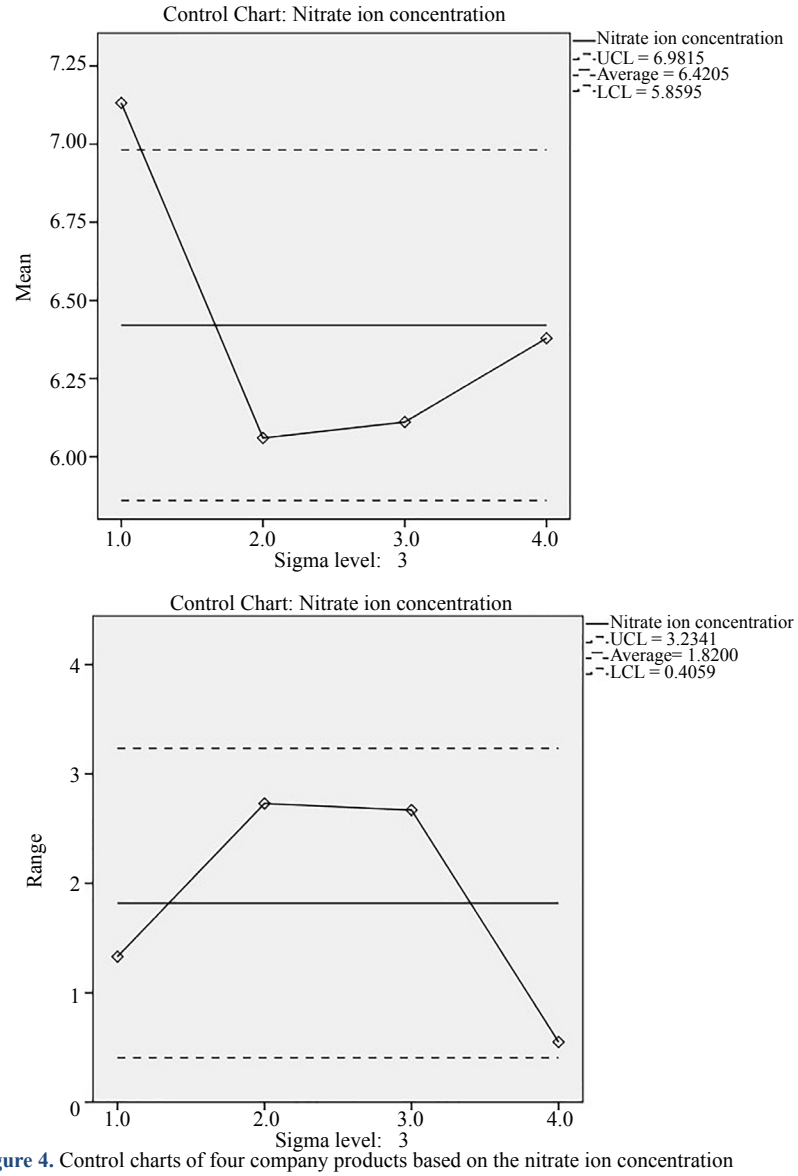

Figure 4. Control charts of four company products based on the nitrate ion concentration

Table 4. The post hoc tests for investigating multiple comparisons of the obtained means of nitrate ion concentration

(I) Herbal distillates	(J) Herbal distillates	Mean Difference (I-J)	Standard Error	Significant
Za	Na	1.07200*	0.31278	0.008
	Af	1.02100*	0.31278	0.012
	Re	0.75300	0.31278	0.094
Na	Za	-1.07200*	0.31278	0.008
	Af	-0.05100	0.31278	0.998
	Re	-0.31900	0.31278	0.739
Af	Za	-1.02100*	0.31278	0.012
	Na	0.05100	0.31278	0.998
	Re	-0.26800	0.31278	0.827
Re	Za	-0.75300	0.31278	0.094
	Na	0.31900	0.31278	0.739
	Af	0.26800	0.31278	0.827

3.2 *Investigated nitrite ion level in the herbal distillates*

The nitrite ion concentrations were determined using the presented procedure in section 3.2 based on three times each sample analysis under the same conditions. Figure 2b depicts a typical UV-Vis spectrum employed for determining nitrite ions, exhibiting a peak absorbance at 543 nm. The results and standard deviation are shown in Table 5. The normality of the results was checked using the Shapiro-Wilk test. The statistical description of the results and the study of their normality are reflected in Table 6, indicating the results do not follow a normal distribution due to the obtained significant values lower than 0.05 at a 95% confidence interval. Data were normalized using the Ln function, and the significant values for Ln (nitrite ion concentration) of Za, Na, Af, and Re herbal distillates were equal to 0.119, 0.130, 0.608, and 0.071, respectively.

Table 5. Nitrite concentration levels (mg L^{-1}) in the analyzed herbal distillates (n = 3)

Sample	Za	Na	Af	Re
Rose	0.10 ± 0.005	0.10 ± 0.004	0.10 ± 0.01	0.19 ± 0.01
Mint	0.91 ± 0.08	0.96 ± 0.09	0.81 ± 0.03	0.97 ± 0.14
Caraway seed	0.32 ± 0.03	0.36 ± 0.02	0.28 ± 0.02	0.76 ± 0.12
Cumin seed	0.28 ± 0.02	0.11 ± 0.01	0.37 ± 0.02	0.26 ± 0.007
Fumitory	0.76 ± 0.01	0.89 ± 0.56	0.91 ± 0.05	0.51 ± 0.02
Carum	0.93 ± 0.11	0.35 ± 0.04	0.45 ± 0.02	0.96 ± 0.13
Fenugreek	0.87 ± 0.09	0.80 ± 0.07	0.27 ± 0.02	0.34 ± 0.008
Chicory	0.16 ± 0.01	0.32 ± 0.02	0.19 ± 0.01	0.31 ± 0.02
Salix Aegyptian	0.36 ± 0.027	0.28 ± 0.02	0.23 ± 0.01	0.95 ± 0.09
Blessed	0.10 ± 0.006	0.10 ± 0.005	0.10 ± 0.007	0.93 ± 0.11

Table 6. Description of the results and test of normality for investigating the nitrite ion concentration in the herbal distillates

Herbal distillates	Mean	Median	Standard Deviation	Minimum	Maximum	Range	Shapiro-Wilk
							Significant
Za	0.4790	0.3400	0.34786	0.10	0.93	0.83	0.042
Na	0.4270	0.3350	0.33257	0.10	0.96	0.86	0.039
Af	0.3710	0.2750	0.28037	0.10	0.91	0.81	0.046
Re	0.6180	0.6350	0.32724	0.19	0.97	0.78	0.039

Table 7. One-way ANOVA test for the comparison of the means of Ln (nitrite ion concentration)

ANOVA	Sum of Squares	df	Mean Square	F	Significant
Between Groups	2.158	3	0.719	1.138	0.347
Within Groups	22.751	36	0.632	-	-
Total	24.909	39	-	-	-

The normal Q-Q plots for the Ln (nitrite ion concentration) of each company are shown in Figure 5. A one-way ANOVA test and the post hoc tests were applied to compare the means of the results of the groups at a 95% confidence interval, indicating that there is no significant difference between the means obtained for the Ln (nitrite ion concentration) in the herbal distillate samples produced by the different companies due to a higher significant value (0.347) than 0.05 in the ANOVA test and between companies based on the post hoc tests (Table 7 and 8). The control charts of mean and range were plotted for four companies to compare the quality of the herbal distillate samples in terms of nitrite ion concentration (Figure 6). The results indicated no significant difference between the quality of products of

different companies in terms of nitrate ion concentration. The nitrite ion concentrations in all herbal distillate samples were compared with the maximum permissible limit of nitrite ion in drinking water by WHO (1.0 mg L^{-1}), showing that the nitrite ion concentrations in all samples are lower than the maximum permissible limit of nitrite ion. The results confirm the samples are not contaminated and can be consumed.

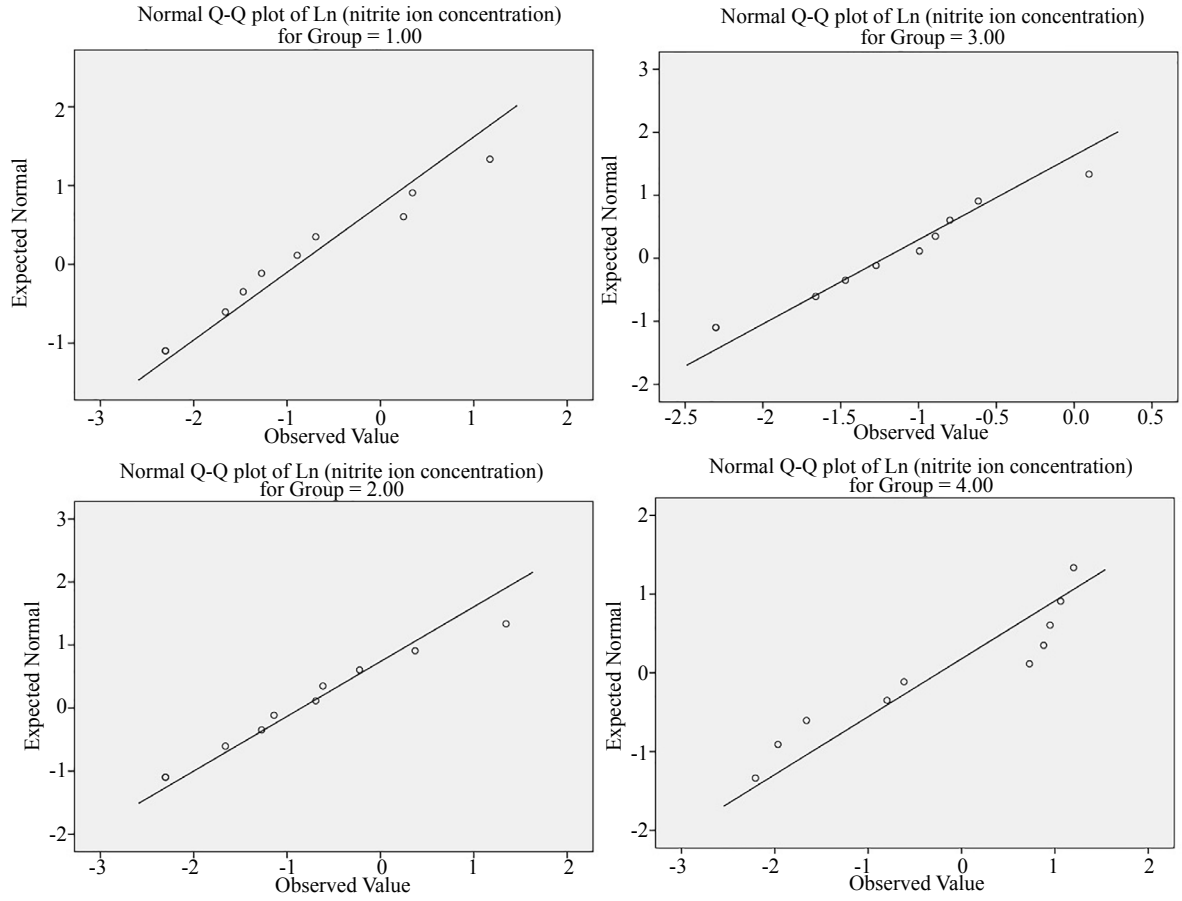

Figure 5. Normal Q-Q plots for the Ln (nitrite ion concentration) of each company; Group 1.00 (Za), Group 2.00 (Na), Group 3.00 (Af), and Group 4.00 (Re)

Table 8. The post hoc tests for investigating multiple comparisons of the obtained means of nitrite ion concentration

(I) Herbal distillates	(J) Herbal distillates	Mean Difference (I-J)	Standard Error	Significant
Za	Na	0.12244	0.35552	0.986
	Af	0.19633	0.35552	0.945
	Re	-0.40517	0.35552	0.668
Na	Za	-0.12244	0.35552	0.986
	Af	0.07389	0.35552	0.997
	Re	-0.52761	0.35552	0.457
Af	Za	-0.19633	0.35552	0.945
	Na	-0.07389	0.35552	0.997
	Re	-0.60149	0.35552	0.343
Re	Za	0.40517	0.35552	0.668
	Na	0.52761	0.35552	0.457
	Af	0.60149	0.35552	0.343

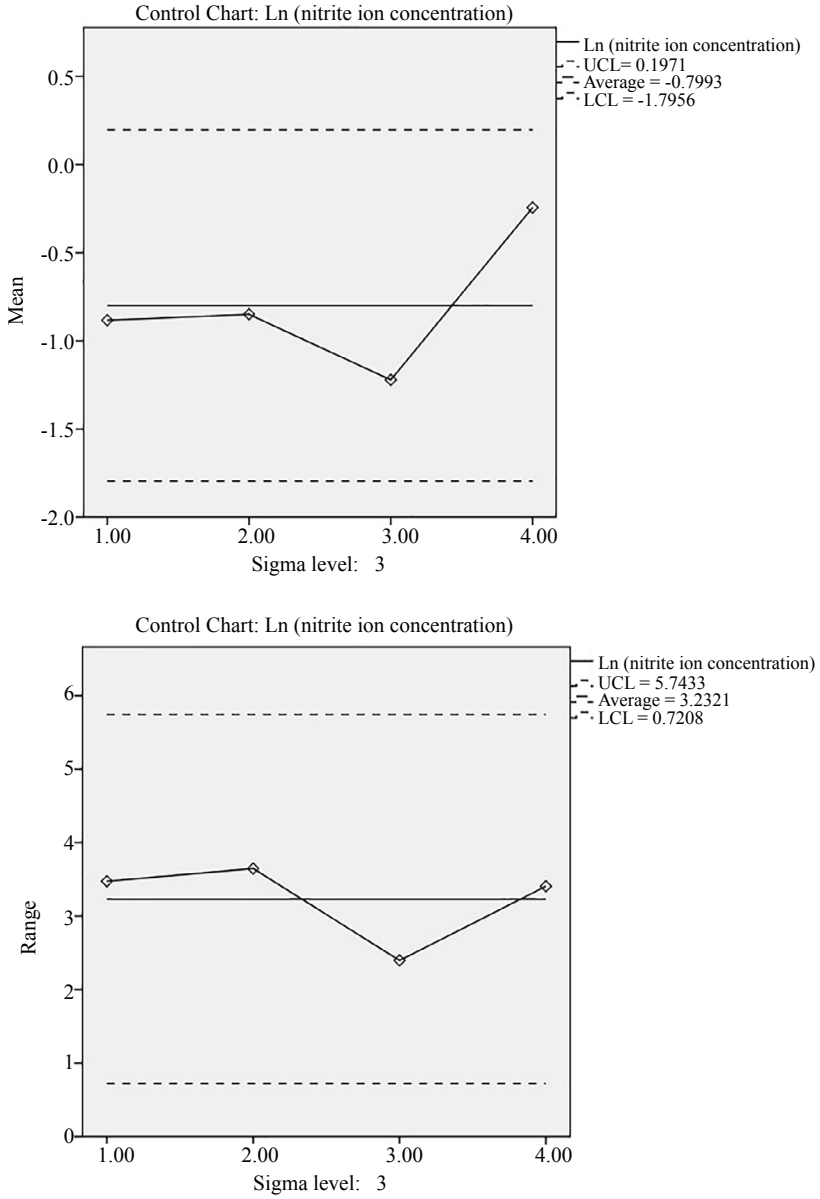

Figure 6. Control charts of four company products based on the Ln (nitrite ion concentration)

3.3 *Investigated nitrosamine and PAE levels in the herbal distillates*

All samples were analyzed for nitrosamine and PAE concentrations in the second week of production of the herbal distillates. A typical GC-MS chromatogram is shown in Figure 7, indicating the presence of diethyl phthalate in the herbal distillate sample. The results showed that the concentration of nitrosamines was not detectable in any of the herbal distillate samples, indicating the reaction between nitrite ion and secondary amines has not been performed meaningfully. The low concentration of nitrite ions in these herbal distillates and the low rate of degradation of proteins into secondary amines can be the most important reasons for the non-detectability of nitrosamines in the samples.

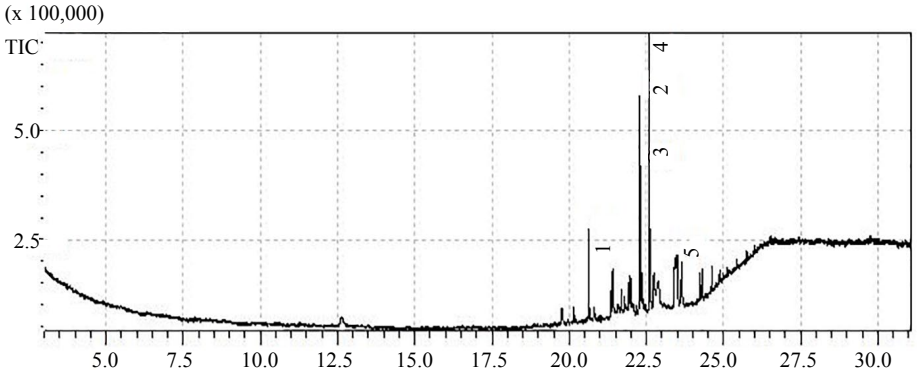

Figure 7. A typical GC-MS choromatogram for determining nitrosamine and PAEs (Peak No. 1: Glycine, Peak No. 2: 2-Cyclopenten-1-one, Peak No. 3: 12-Oxabicyclo[9.1.0]dodeca-3, 7-diene, Peak No. 4: Imidazole, Peak No. 5: Diethyl Phthalate)

Analysis of the samples to determine the concentration of PAEs in the herbal distillate samples also showed that PAEs with a low concentration in the range of 0.14-0.47 μg L^{-1} are detected in all herbal distillate samples. Diethyl phthalate and Di-n-butyl phthalate have the highest concentrations in the samples, respectively. The total concentrations of all PAEs determined in each sample are summarized in Table 9. The Shapiro-Wilk test was utilized to investigate the normality of the results (Table 10), indicating that the results follow a normal distribution because the significant values of the Shapiro-Wilk test for products of four companies are higher than 0.05 at a 95% confidence interval. Normal Q-Q plots of concentrations of PAEs for each company are shown in Figure 8. The means of the groups' results were compared using a one-way ANOVA test at a 95% confidence interval (Table 11). A p-value of 0.575 and more than 0.05 (at a 95% confidence interval) showed that there is no significant difference between the mean concentration of PAEs in the products of these companies.

Table 9. Concentrations of PAEs (C (μg L^{-1}) ± Standard deviation) in the second week after producing the herbal distillate samples (n = 3)

Sample	Za	Na	Af	Re
Rose	0.46 ± 0.019	0.19 ± 0.010	0.32 ± 0.019	0.39 ± 0.020
Mint	0.21 ± 0.011	0.42 ± 0.020	0.48 ± 0.023	0.37 ± 0.019
Caraway seed	0.16 ± 0.008	0.45 ± 0.021	0.29 ± 0.018	0.14 ± 0.009
Cumin seed	0.31 ± 0.016	0.22 ± 0.011	0.41 ± 0.023	0.29 ± 0.018
Fumitory	0.19 ± 0.009	0.29 ± 0.016	0.37 ± 0.021	0.31 ± 0.019
Carum	0.26 ± 0.010	0.33 ± 0.019	0.32 ± 0.018	0.28 ± 0. 016
Fenugreek	0.34 ± 0.018	0.41 ± 0.019	0.28 ± 0.014	0.35 ± 0.020
Chicory	0.29 ± 0.012	0.47 ± 0.023	0.16 ± 0.009	0.32 ± 0.021
Salix Aegyptian	0.42 ± 0.019	0.26 ± 0.016	0.26 ± 0. 018	0.21 ± 0.019
Blessed	0.37 ± 0.017	0.37 ± 0.021	0.25 ± 0.019	0.18 ± 0.009

Table 10. Description of the results and test of normality for investigating the PAEs concentration in the second week after producing the herbal distillate samples (n = 3)

Herbal distillates	Mean	Median	Standard Deviation	Minimum	Maximum	Range	Shapiro-Wilk
							Significant
Za	0.264	0.30	0.099	0.16	0.46	0.30	0.913
Na	0.304	0.35	0.098	0.19	0.47	0.28	0.614
Af	0.289	0.31	0.090	0.16	0.48	0.32	0.941
Re	0.284	0.30	0.083	0.14	0.39	0.25	0.567

Table 11. One-way ANOVA test for the comparison of the means of PAEs concentration in the second week after producing the herbal distillate samples (n = 3)

ANOVA	Sum of Squares	df	Mean Square	F	Significant
Between Groups	0.017	3	0.006	0.672	0.575
Within Groups	0.310	36	0.009	-	-
Total	0.327	39	-	-	-

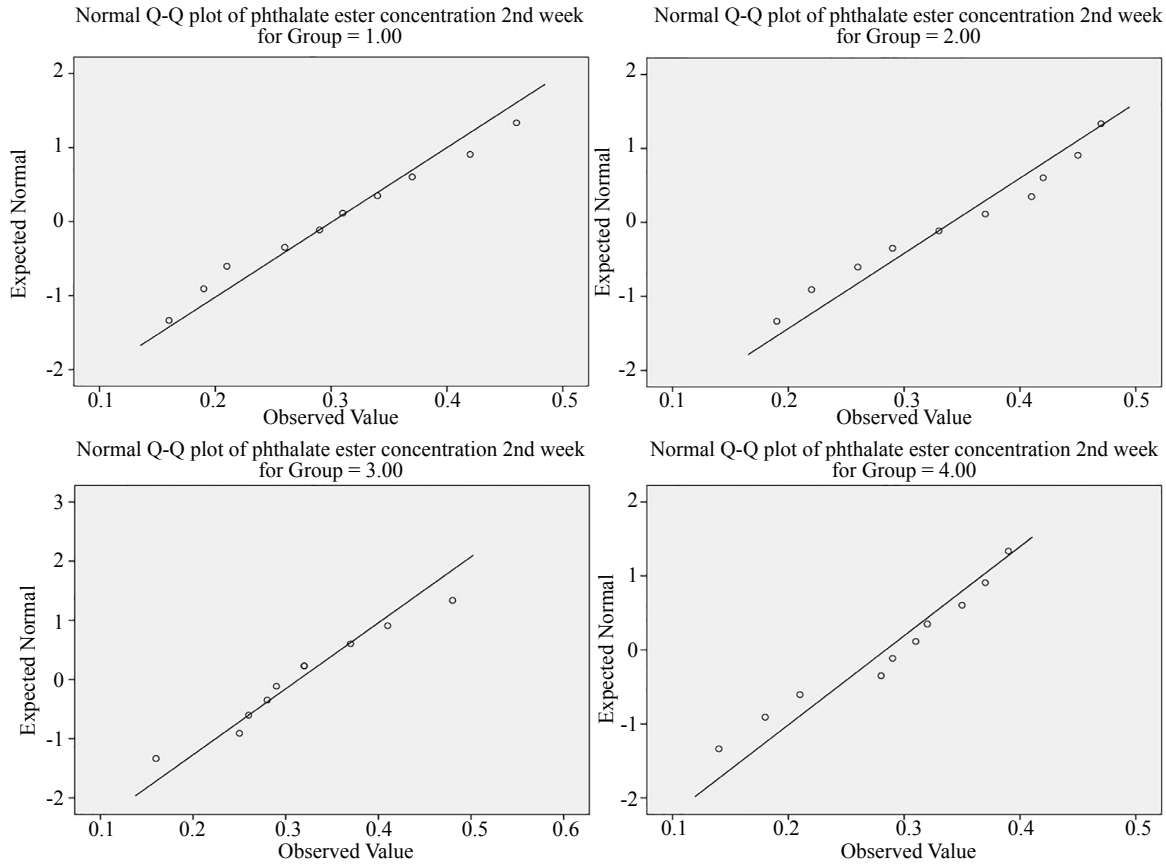

Figure 8. Normal Q-Q plots for the PAEs concentrations of each company in the second week after their production: Group 1.00 (Za), Group 2.00 (Na), Group 3.00 (Af), and Group 4.00 (Re)

Besides, the post hoc test was performed for multiple comparisons of the mean concentration of PAEs between the products of these companies, showing that they have a non significantly difference because the significant values are higher than 0.05 for all comparisons (Table 12). The control charts of mean and range were plotted for four companies to compare the quality of the herbal distillate samples in terms of concentration of PAEs (Figure 9). There is no significant difference between the quality of products of different companies. The total concentration of PAEs in all samples was much less than the maximum permissible limit of PAEs (8×10^{-3} mg L^{-1}) in drinking water declared by WHO,[42] confirming the samples are not contaminated with nitrosamines and PAEs and can be consumed.

Table 12. The post hoc tests for investigating multiple comparisons of the obtained means of PAEs concentration in the second week after producing the herbal distillate samples (n = 3)

(I) Herbal distillates	(J) Herbal distillates	Mean Difference (I-J)	Standard Error	Significant
Za	Na	-0.040	0.041	0.770
	Af	-0.013	0.041	0.989
	Re	0.017	0.041	0.976
Na	Za	0.040	0.041	0.770
	Af	0.027	0.041	0.915
	Re	0.057	0.041	0.523
Af	Za	0.013	0.041	0.989
	Na	-0.027	0.041	0.915
	Re	0.030	0.041	0.887
Re	Za	-0.017	0.041	0.976
	Na	-0.057	0.041	0.523
	Af	-0.030	0.041	0.887

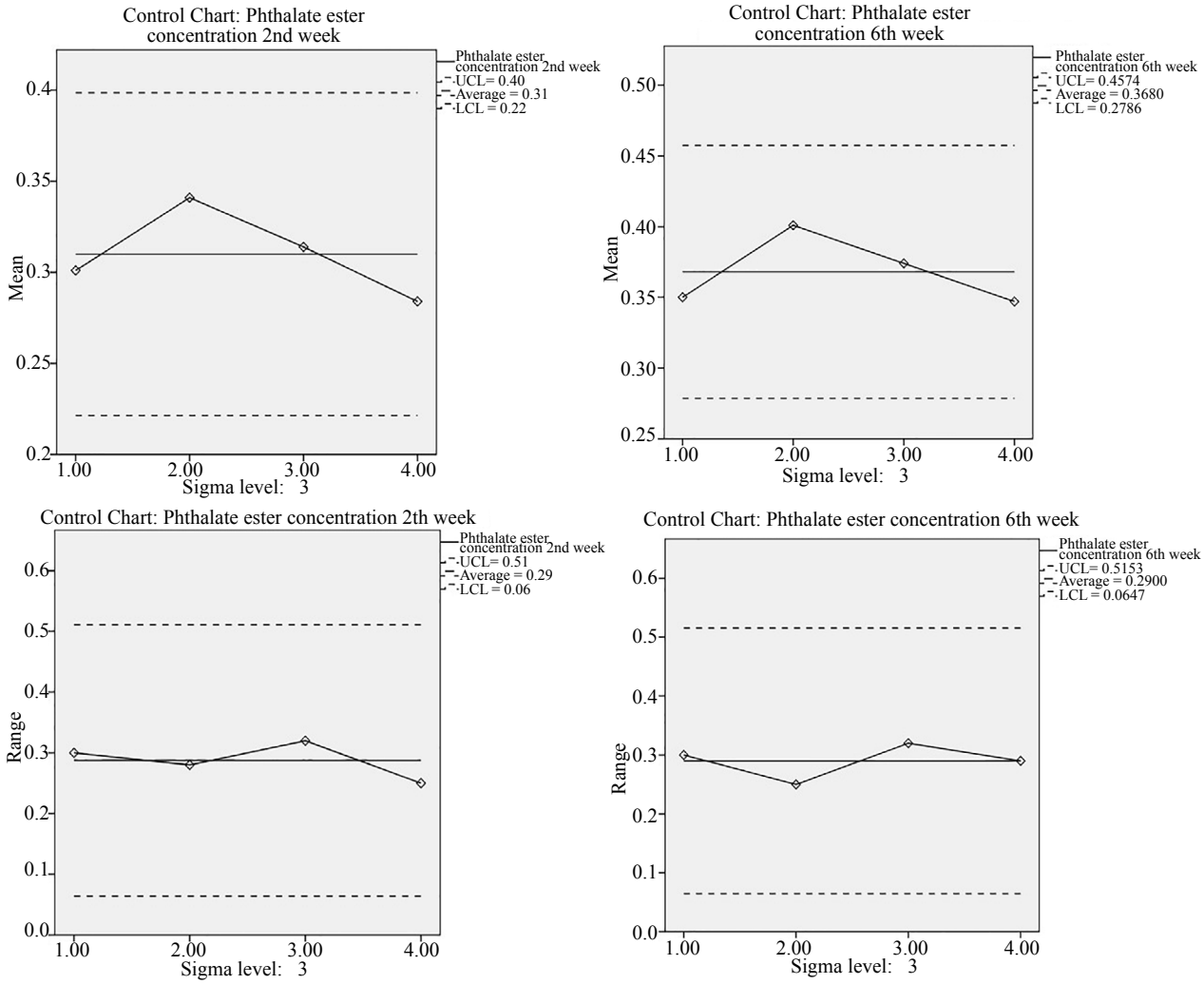

Figure 9. Control charts of four company products based on the PAEs concentration in the second and sixth weeks

3.4 *Effect of time on concentration of PAEs*

All analyzed samples were stored in a room at 23 ± 4 °C for four months from production. The concentrations of PAEs in the samples were determined in the sixth, tenth and fourteenth weeks after producing the herbal distillate samples to investigate the time effect (Table 13). Each sample was analyzed three times under the same conditions to determine the concentrations of PAEs using the DLLME-GC/MS procedure. In the first stage, the normality of the results in the sixth, tenth and fourteenth weeks was individually evaluated using the Shapiro-Wilk test (Table 14). All evaluations were carried out at a 95% confidence interval in which the α-level is 0.05. The statistical description of the results and the study of their normality indicated that all results obtained for four companies follow a normal distribution due to significant values higher than 0.05. Normal Q-Q plots of concentrations of PAEs for each company in different weeks are shown in Figure 10, 11, and 12. One-way ANOVA test was used to evaluate the means of the groups' results in different weeks, indicating that the significant differences between the mean concentration of PAEs in the products of these companies in different weeks were not displayed (Table 15) due to the obtained significant values were more than α-level (0.05).

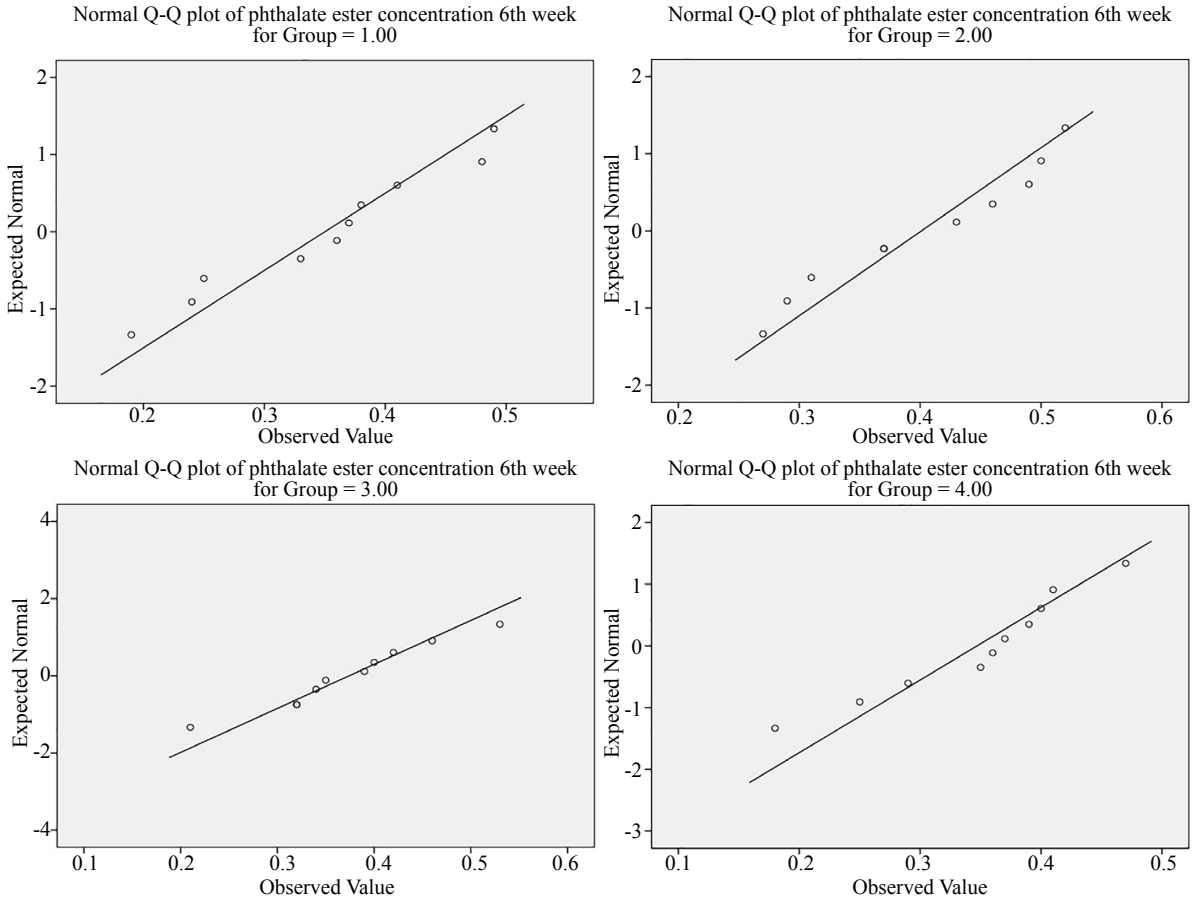

Figure 10. Normal Q-Q plots for the PAEs concentrations of each company in the sixth week after their production: Group 1.00 (Za), Group 2.00 (Na), Group 3.00 (Af), and Group 4.00 (Re)

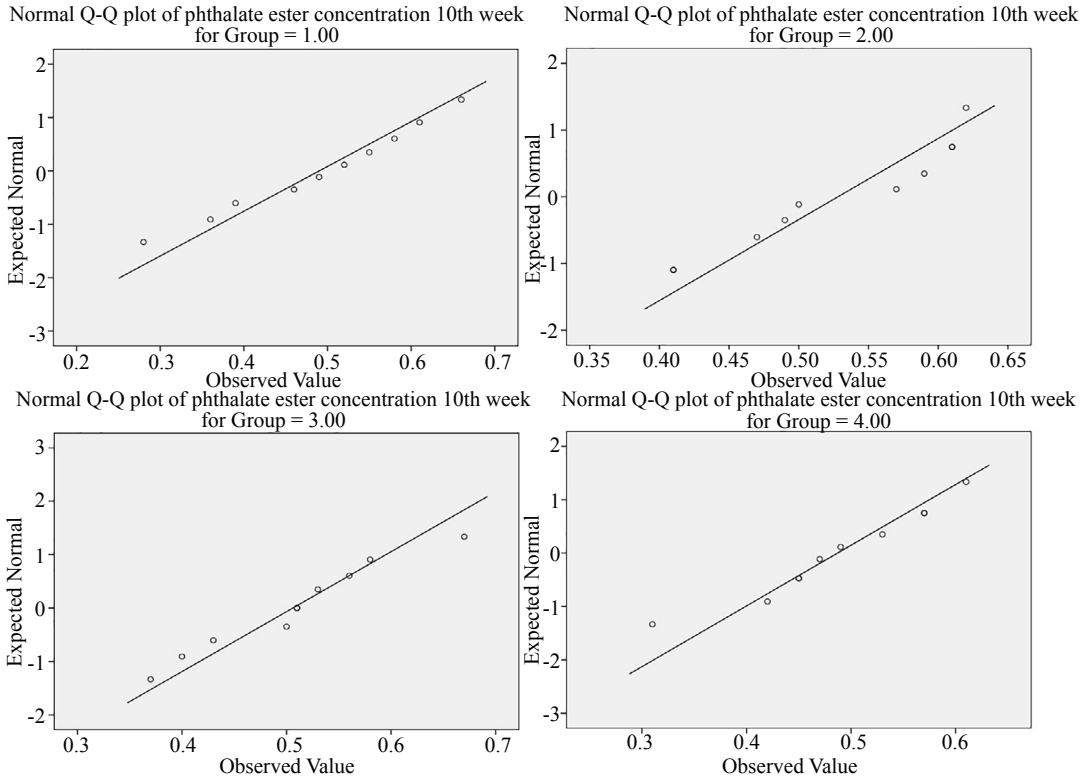

Figure 11. Normal Q-Q plots for the PAEs concentrations of each company in the tenth week after their production: Group 1.00 (Za), Group 2.00 (Na), Group 3.00 (Af), and Group 4.00 (Re)

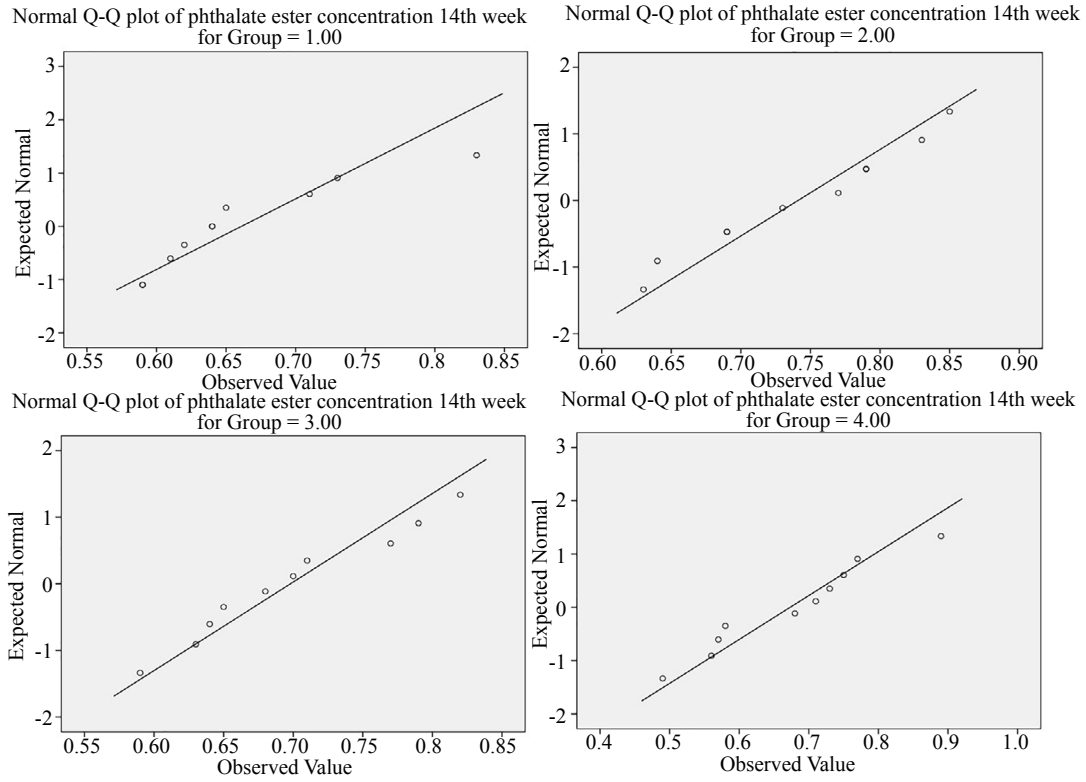

Figure 12. Normal Q-Q plots for the PAEs concentrations of each company in the fourteenth week after their production: Group 1.00 (Za), Group 2.00 (Na), Group 3.00 (Af), and Group 4.00 (Re)

Table 13. Concentrations of PAEs (C (µg L^{-1}) ± Standard deviation) in the sixth, tenth and fourteenth weeks after producing the herbal distillate samples (n = 3)

Sample	Za			Na			Af			Re		
	6th week	10th week	14th week	6th week	10th week	14th week	6th week	10th week	14th week	6th week	10th week	14th week
Rose	0.49 ± 0.021	0.61 ± 0.024	0.83 ± 0.038	0.27 ± 0.013	0.41 ± 0.014	0.69 ± 0.029	0.39 ± 0.020	0.53 ± 0.026	0.65 ± 0.027	0.47 ± 0.018	0.61 ± 0.021	0.77 ± 0.031
Mint	0.25 ± 0.017	0.36 ± 0.019	0.65 ± 0.027	0.49 ± 0.022	0.62 ± 0.025	0.85 ± 0.031	0.53 ± 0.025	0.67 ± 0.024	0.82 ± 0.034	0.41 ± 0.021	0.57 ± 0.022	0.71 ± 0.029
Caraway seed	0.19 ± 0.011	0.28 ± 0.013	0.59 ± 0.027	0.50 ± 0.021	0.61 ± 0.024	0.79 ± 0.029	0.32 ± 0.019	0.40 ± 0.023	0.63 ± 0.028	0.18 ± 0.010	0.31 ± 0.014	0.49 ± 0.021
Cumin seed	0.38 ± 0.019	0.52 ± 0.021	0.64 ± 0.029	0.29 ± 0.014	0.47 ± 0.015	0.63 ± 0.023	0.46 ± 0.021	0.58 ± 0.026	0.79 ± 0.035	0.36 ± 0.017	0.47 ± 0.021	0.58 ± 0.025
Fumitory	0.24 ± 0.010	0.39 ± 0.013	0.59 ± 0.025	0.37 ± 0.017	0.49 ± 0.020	0.69 ± 0.027	0.42 ± 0.022	0.51 ± 0.024	0.70 ± 0.029	0.35 ± 0.022	0.45 ± 0.021	0.89 ± 0.035
Carum	0.33 ± 0.013	0.46 ± 0.013	0.62 ± 0.032	0.37 ± 0.021	0.50 ± 0.024	0.73 ± 0.028	0.40 ± 0.021	0.56 ± 0.021	0.77 ± 0.031	0.37 ± 0.019	0.49 ± 0.024	0.73 ± 0.029
Fenugreek	0.37 ± 0.019	0.55 ± 0.022	0.73 ± 0.034	0.46 ± 0.020	0.59 ± 0.024	0.79 ± 0.03	0.35 ± 0.016	0.51 ± 0.019	0.71 ± 0.026	0.40 ± 0.021	0.53 ± 0.019	0.68 ± 0.027
Chicory	0.36 ± 0.014	0.49 ± 0.015	0.71 ± 0.029	0.52 ± 0.026	0.61 ± 0.026	0.83 ± 0.034	0.21 ± 0.011	0.37 ± 0.012	0.59 ± 0.024	0.39 ± 0.026	0.57 ± 0.026	0.75 ± 0.030
Salix Aegyptian	0.48 ± 0.021	0.66 ± 0.022	0.61 ± 0.026	0.31 ± 0.017	0.41 ± 0.021	0.64 ± 0.025	0.34 ± 0.017	0.50 ± 0.020	0.68 ± 0.029	0.29 ± 0.021	0.45 ± 0.025	0.56 ± 0.024
Blessed	0.41 ± 0.018	0.58 ± 0.021	0.64 ± 0.027	0.43 ± 0.026	0.57 ± 0.028	0.77 ± 0.029	0.32 ± 0.020	0.43 ± 0.024	0.64 ± 0.023	0.25 ± 0.011	0.42 ± 0.016	0.57 ± 0.027

Table 14. Description of the results and test of normality for investigating the PAEs concentration in the herbal distillates in the second week after production

week	Herbal distillates	Mean	Median	Standard deviation	Minimum	Maximum	Range	Shapiro-Wilk
								Significant
6th	Za	0.350	0.365	0.09978	0.19	0.49	0.3	0.680
	Na	0.401	0.40	0.09183	0.27	0.52	0.25	0.376
	Af	0.374	0.37	0.08771	0.21	0.53	0.32	0.945
	Re	0.347	0.365	0.08499	0.18	0.47	0.29	0.614
10th	Za	0.490	0.51	0.119	0.28	0.66	0.38	0.943
	Na	0.528	0.54	0.082	0.41	0.62	0.21	0.124
	Af	0.526	0.51	0.089	0.37	0.67	0.3	0.865
	Re	0.487	0.48	0.088	0.31	0.61	0.3	0.725
14th	Za	0.661	0.64	0.07534	0.59	0.83	0.24	0.065
	Na	0.741	0.75	0.07695	0.63	0.85	0.22	0.574
	Af	0.698	0.69	0.07525	0.59	0.82	0.23	0.730
	Re	0.673	0.695	0.12139	0.49	0.89	0.40	0.784

Table 15. One-way ANOVA test for comparing the means of PAEs concentration in the sixth, tenth and fourteenth weeks after producing the herbal distillate samples (n = 3)

	PAEs concentration 6th week				
	Sum of Squares	df	Mean Square	F	Significant
Between Groups	0.019	3	0.006	0.757	0.526
Within Groups	0.300	36	0.008	-	-
Total	0.319	39	-	-	-
	PAEs concentration 10th week				
	Sum of Squares	df	Mean Square	F	Significant
Between Groups	0.011	3	0.004	0.385	0.764
Within Groups	0.330	36	0.009	-	-
Total	0.340	39	-	-	-
	PAEs concentration 14th week				
	Sum of Squares	df	Mean Square	F	Significant
Between Groups	0.038	3	0.013	1.564	0.215
Within Groups	0.288	36	0.008	-	-
Total	0.325	39	-	-	-

The quality of the herbal distillate samples in terms of concentration of PAEs in different weeks was evaluated using the control charts of mean and range for four companies (Figure 13), showing the quality of all productions in the different weeks were the same. Also, multiple comparisons of the mean concentration of PAEs between the products of each company with other companies were individually performed using the post hoc test. There was no significant difference in the mean concentration of PAEs over time in the products of these companies. However, an increase in the mean concentration of PAEs over time in the products of all companies was shown (Table 16, 17 and 18). The post hoc test was performed to multiple comparisons of the mean concentration of PAEs between the products of these companies, showing that they have a non significantly difference because the significant values are higher than 0.05 for all comparisons.

The rate of PAEs release over time based on the mean concentration of PAEs is shown in Figure 14. The obtained equations and R-squared are summarized in Table 19, indicating that the rate of PAEs release production for Za, Na, Af, and Re were 0.0327, 0.034, 0.0333 and 0.036 μg L^{-1} $week^{-1}$, respectively. However, the rate of PAEs release was highest and lowest for Za and Re companies.

Table 16. The post poc tests for investigating multiple comparisons of the obtained means of PAEs concentration in the sixth, tenth and fourteenth week after producing the herbal distillate samples (n = 3)

(I) Herbal distillate samples	(J) Herbal distillate samples	Mean Difference (I-J)	Standard Error	Significant
1.00	2.00	-0.05100	0.04081	0.600
	3.00	-0.02400	0.04081	0.935
	4.00	0.00300	0.04081	1.000
2.00	1.00	0.05100	0.04081	0.600
	3.00	0.02700	0.04081	0.911
	4.00	0.05400	0.04081	0.555
3.00	1.00	0.02400	0.04081	0.935
	2.00	-0.02700	0.04081	0.911
	4.00	0.02700	0.04081	0.911
4.00	1.00	-0.00300	0.04081	1.000
	2.00	-0.05400	0.04081	0.555
	3.00	-0.02700	0.04081	0.911

Table 17. The post hoc tests for investigating multiple comparisons of the obtained means of PAEs concentration in the tenth week after producing the herbal distillate samples (n = 3)

(I) Herbal distillate samples	(J) Herbal distillate samples	Mean Difference (I-J)	Standard Error	Significant
1.00	2.00	-0.038	0.043	0.811
	3.00	-0.016	0.043	0.982
	4.00	0.003	0.043	1.000
2.00	1.00	0.038	0.043	0.811
	3.00	0.022	0.043	0.955
	4.00	0.041	0.043	0.774
3.00	1.00	0.016	0.043	0.982
	2.00	-0.022	0.043	0.955
	4.00	0.019	0.043	0.970
4.00	1.00	-0.003	0.043	1.000
	2.00	-0.041	0.043	0.774
	3.00	-0.019	0.043	0.970

Table 18. The post hoc tests for investigating multiple comparisons of the obtained means of PAEs concentration in the fourteenth week after producing the herbal distillate samples (n = 3)

(I) Herbal distillate samples	(J) Herbal distillate samples	Mean Difference (I-J)	Standard Error	Significant
1.00	2.00	-0.08000	0.04000	0.207
	3.00	-0.03700	0.04000	0.792
	4.00	-0.01200	0.04000	0.990
2.00	1.00	0.08000	0.04000	0.207
	3.00	0.04300	0.04000	0.707
	4.00	0.06800	0.04000	0.338
3.00	1.00	0.03700	0.04000	0.792
	2.00	-0.04300	0.04000	0.707
	4.00	0.02500	0.04000	0.923
4.00	1.00	0.01200	0.04000	0.990
	2.00	-0.06800	0.04000	0.338
	3.00	-0.02500	0.04000	0.923

Table 19. The rate of PAEs release over time

Company	Mean concentration of PAEs				Equation	R^2	Slope (µg/L day)
	2nd week	6th week	10th week	14th week			
Za	0.264	0.350	0.490	0.661	Y = 0.0051x + 0.2059	0.9671	0.0051
Na	0.304	0.401	0.528	0.741	Y = 0.0048x + 0.1751	0.9797	0.0048
Af	0.289	0.374	0.526	0.698	Y = 0.0049x + 0.195	0.9699	0.0049
Re	0.284	0.347	0.487	0.673	Y = 0.0047x + 0.1864	0.9571	0.0047

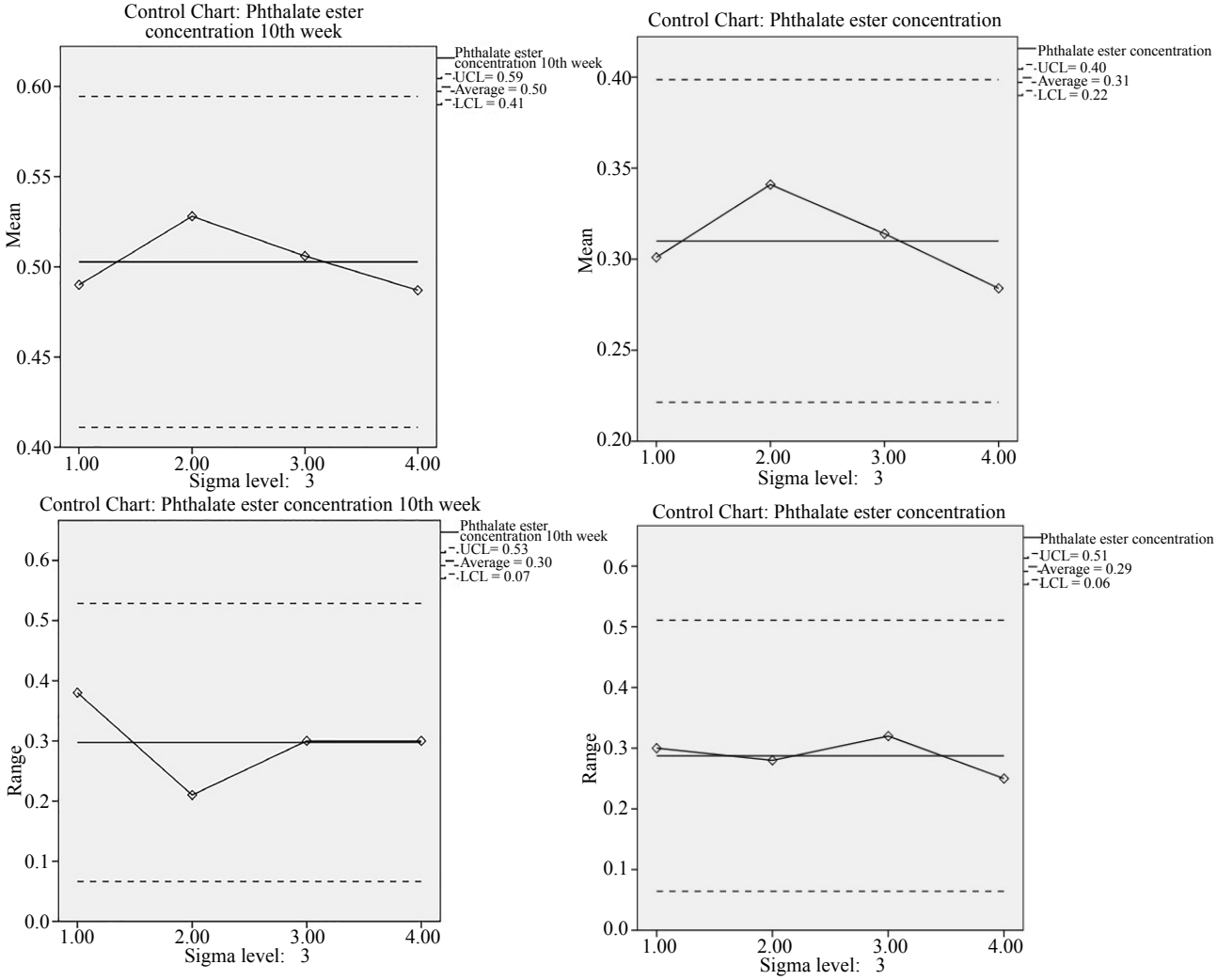

Figure 13. Control charts of four company products based on the PAEs concentration in the tenth and fourteenth weeks

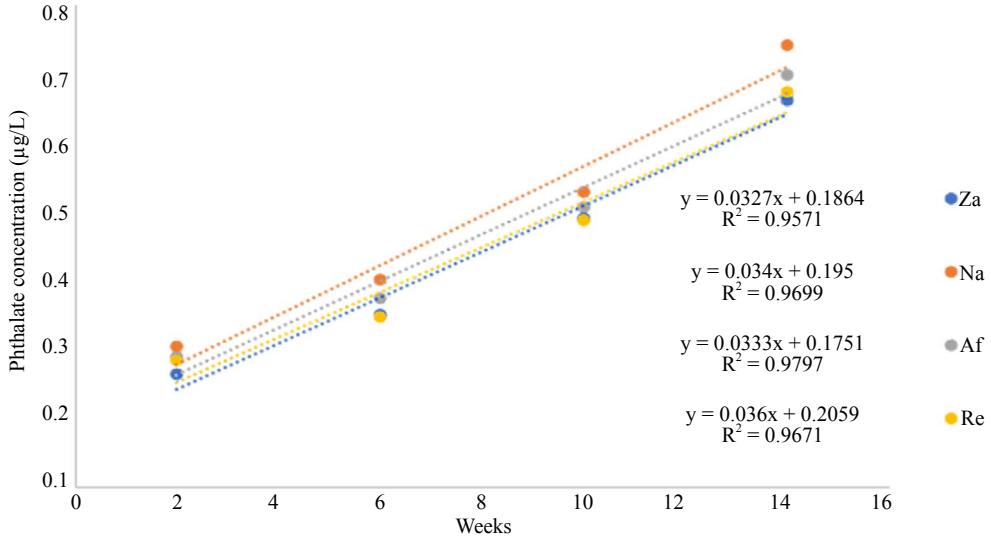

Figure 14. The rate of PAEs release over time in the herbal distillate samples

4. Conclusion

Ten herbal distillate samples produced by four companies were randomly selected in the study, and concentrations of nitrate ion, nitrite ion, nitrosamines, and PAEs in the samples were determined. The results indicated that the nitrate ion concentration in the samples followed the normal distribution while the nitrite ion concentration didn't follow. A comparison of the means of the results was shown that the means of nitrate ion concentrations obtained for four companies have a significant difference together, while the mean of nitrite ion concentrations does not have a significant difference. The control chart also indicated that the average nitrate concentration for herbal distillate samples prepared in ZA is significantly higher than other companies' products.

There was no significant difference in the mean concentration of nitrite ions in the herbal distillate samples of the four companies. However, the nitrate ion and nitrite ion concentrations in all herbal distillate samples were lower than the maximum permissible limit of these ions in drinking water by WHO. All herbal distillate samples were free of nitrosamines, but PAEs were observed in the herbal distillate samples produced with low concentrations. The concentrations of PAEs in the samples were studied in the sixth, tenth and fourteenth weeks after producing the herbal distillate samples to investigate the time effect. The concentrations of PAEs followed the normal distribution and the means of their concentrations have no significant difference based on ANOVA and post hoc test. Also, there is no significant difference between the quality of products of different companies in different weeks. The total concentration of PAEs in all samples was much less than the maximum permissible limit in drinking water declared by WHO.

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Data sharing policy

The data that supports the findings of this study are available in the supplementary material of this article.

Compliance with ethical standards

This article does not contain any studies with human or animal subjects.

Conflict of interest

The authors declare no competing financial interest.

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