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Assessing Calibration Methods for Fenuron Determination in Carrot Juice via GC-MS with Salt-Assisted Switchable Solvent Microextraction

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Abstract

An accurate and precise analytical method was established for the determination of fenuron in carrot juice samples. Salt-assisted switchable solvent-liquid phase microextraction (SA-SS-LPME) was performed to achieve a lower detection limit. Three different calibration methods were compared to quadruple isotope dilution (ID^4) in terms of accuracy and precision. Under the optimum SA-SS-LPME-GC-MS conditions, the linear range, coefficient of determination, limit of detection (LOD), and limit of quantitation (LOQ) were calculated as 0.12-2.85 mg/kg, 0.9988, 0.05 mg/kg and 0.15 mg/kg via the external standard calibration method, respectively. In addition, the internal calibration strategy was also implemented and the linear range, coefficient of determination, LOD and LOQ were found to be 0.12-5.13 mg/kg, 0.99996, 0.05 mg/kg and 0.17 mg/kg, respectively. Percent recovery results were recorded as 104.5%–118.1% (±1.9-7.2) via the external standard calibration, 75.1%–115.0% (±1.7-6.2) via the matrix–matching calibration, 89.7%–100.7% (±1.8-6.3) via the internal standard

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calibration and 99.6%–102.0% (±1.2-2.3) via the ID^4 strategy. The recovery results obtained using the ID^4 strategy demonstrate the advantage of integrating SA-SS-LPME-GC-MS with this approach.

Keywords: Fenuron, carrot juice, quadruple isotope dilution, switchable solvent, microextraction, gas chromatography-mass spectrometry

1. Introduction

Fenuron (1,1-dimethyl-3-phenylurea, Figure 1) is a member of the phenylurea class of herbicides and acts by interfering with photosynthesis in undesired plant weeds. This herbicide has a polar molecular structure and water solubility, which contribute to its occurrence in both surface and ground water [1]. It is reported that the phenylurea class of herbicides is commonly used in agricultural areas, but these chemicals also have high toxicity and carcinogenicity [2]. In addition, fenuron has adverse effects on human bodies, such as stomachache, nausea, diarrhea, vomiting, and even methemoglobinemia [3]. Considering these side effects of fenuron, the accurate and precise determination of fenuron is an important issue to save human health and the environment. In this study, an isotope dilution-based method using fenuron-d_6 (Figure 1) was established to improve the accuracy and precision of the analytical results.

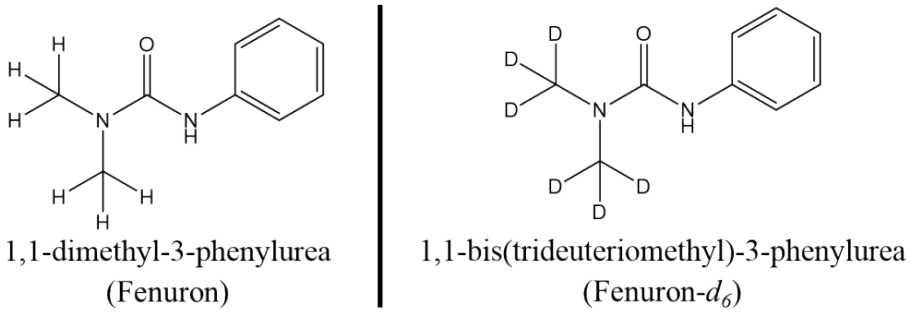


Figure 1. Molecular structures of fenuron and fenuron-d_6 with their IUPAC names.

Carrot (*Daucus carota*) is a frequently consumed vegetable in many countries and contains beneficial antioxidants and phytochemicals [4]. This

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vegetable can be consumed as carrot juice, canned, preserved, and pickled [5]. Based on the Food and Agriculture Organization (FAO), there were 1,147,155 ha of carrot plantations and 42,831,958 tons of carrot production in 2017 [6]. On the other hand, fenuron is one of the chemicals used for the protection of several vegetables, including carrot, pea, potato and corn [7]. For this reason, an analytical methodology was proposed for the determination of fenuron in carrot juice samples in this study.

In the literature, various analytical methods such as liquid chromatography-UV detection [8], capillary electrophoresis-diode array detector [9], GC-MS [10], and amperometry [11] have been used to detect fenuron in different samples. Among them, the GC-MS instrumentation technique has been utilized for the separation and determination of several herbicides [12–14]. It provides library-based analyte identification, which gives the name, molecular structure and elemental formula for the analyte(s) [15] and reproducible retention times and mass spectrums [16]. For these reasons, the GC-MS was preferred to qualify and quantify fenuron in carrot juice samples.

Green analytical chemistry offers the development of analytical methods with minimum effects on the environment and analysts. In this context, green sample preparation methods like microextraction have drawn significant attention due to low chemical usage and waste generation [17]. The usage of liquid phase microextraction (LPME) has gained momentum in recent years due to its good benefits, such as its compatibility with various instruments and analytes, simple operation and cost-effectiveness [18]. Switchable solvents have been employed as green solvents in microextraction in place of traditional extraction solvents such as dichloromethane and chloroform [19,20]. Amine-based switchable solvents are solubilized in water by protonation with the addition of a mineral acid or carbon dioxide (carbonic acid), followed by the addition of base, or bubbling nitrogen gas into the solution to reverse the carbonic acid formation. The neutralized amine forms a dispersion, extracting the analyte [21]. This process is a variant of dispersive liquid-liquid microextraction (DLLME) [22]. Switchable solvents have been frequently

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used during the sample preparation steps in analytical procedures. Pollutants [21], heavy metals [23], amino acids [24], drugs [25], pesticides [26,27] and herbicides [28] have been successfully extracted by the help of switchable solvents. In this study, salt-assisted switchable solvent-liquid phase microextraction (SA-SS-LPME) was carried out to extract fenuron from carrot juice samples.

There are several quantitative determination methods such as external standard calibration [29], matrix–matching calibration [30], internal standard calibration [31] and isotope dilution (single, double, triple or quadruple) [32] methods. Among them, isotope dilution is one of the primary quantitative determination methods, which is known as a superior analytical method based on calibration against external reference materials [33]. In simple terms, it is based on the isotopic composition measurement of a mixture containing an aliquot of sample (A) and a known amount of isotopically enriched/labelled standard (B) [34]. The isotope dilution method is independent from instrumental fluctuations, analyte loss, matrix effects and interferences [35]. Isotope dilution has different experimental applications according to the known and unknown equation parameters. These applications are single (ID), reverse (ID^2), triple (ID^3) and quadruple (ID^4) isotope dilution methods [32,36]. Among all methods, the ID^4 method has three calibration blends (A^*B-1, A^*B-2, A^*B-3, where A^*: standard solution of analyte, B: standard solution of isotopic material) and one sample blend (AB, where A: sample solution, and B: standard solution of isotopic material). The mathematical formula of this method does not contain three important equation parameters (isotopic composition of sample (r_A), isotopic composition (r_B) and mass fraction (w_B) of isotopically labelled material) [32]. This strategy was used in this study to boost the accuracy and precision of the developed method.

This paper covers the determination of fenuron in carrot juice samples using a combination of the SA-SS-LPME and GC- ID^4-MS methods. No study has been reported for highly accurate and precise determination of fenuron at trace levels by the GC-ID^4-MS method after its preconcentration by the SA-SS-LPME method. First, important parameters for the SA-SS-LPME

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method were tested to determine their optimum values. Under the optimum SA-SS-LPME-GC-MS conditions, analytical performance parameters such as linear range, coefficient of determination, limit of detection (LOD) and limit of quantitation (LOQ) were evaluated using both external and internal standard calibration strategies. Recovery experiments were carried out employing the spiked carrot juice samples. Finally, the ID^4 method was performed and combined with the SA-SS-LPME-GC-MS method to enhance the accuracy and precision of the developed analytical method.

2. Materials and Methods

2.1 Standards and chemicals

Standard material of fenuron (purity ≥ 98.8%), which was used to prepare its standard solution in acetonitrile (990.7 mg/kg), was supplied by Dr. Ehrenstorfer GmbH (Augsburg, Germany). Dimethyl-d_6-amine hydrochloride (purity = 99%), toluene (purity = 99.8%), triethylamine (purity ≥ 99.5%) and phenyl isocyanate (purity ≥ 98%) were bought from Sigma–Aldrich (Darmstadt, Germany), while acetonitrile, N,N-dimethylbenzylamine (purity ≥ 98.0%), sodium hydroxide (purity ≥ 97.0%), sodium chloride (NaCl, purity ≥ 99.5%), potassium nitrate (KNO_3, purity ≥ 99.0%) and sodium sulfate (Na_2SO_4, purity ≥ 99.0%) were obtained from Merck (Darmstadt, Germany). Dry ice (as a source of CO_2) used during the synthesis of N,N-dimethylbenzylamine-based switchable solvent was purchased from a pastry shop (İstanbul, Türkiye). High purity helium gas employed as a carrier gas in gas chromatography–mass spectrometry (GC-MS) system was purchased from a local gas supplier in İstanbul, Türkiye. Ultrapure water having a resistivity of 18.2 MΩ.cm at 25 ºC, which was utilized during the synthesis of switchable solvent and the preparation of aqueous standard solutions, was generated using an ultrapure water purification system (Elga Flex 3 model, High Wycombe, United Kingdom). An N,N-dimethylbenzylamine-based switchable solvent was synthesized using the procedure found in our previous study [37].

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2.2 Instrumentation

An Agilent gas chromatograph (GC, 6890 model), which was integrated with an HP-5MS analytical column (possessing 30 m of length, 250 µm of internal diameter and 250 nm of film thickness) was employed to achieve chromatographic separation of fenuron. An Agilent mass spectrometer (MS, 5973 model) including an electron ionization source, a quadrupole mass filter and an electron multiplier tube was coupled with the GC system to qualify/quantify fenuron. During all analyses, a single ramp temperature program was used as follows; 1) The initial temperature was started from 130 ºC, 2) The temperature was increased to 280 ºC with 35 ºC/min of temperature ramp and 3) The temperature was held for 0.71 min at 280 ºC. Inlet temperature, flow rate of helium, transfer line temperature, quadrupole temperature, electron ionization temperature/energy, injection mode/volume and solvent delay were fixed to 280 ºC, 2.0 mL/min, 280 ºC, 150 ºC, 230 ºC/70 eV, splitless/1.0 µL and 2.0 min, respectively. In the quantitative determinations, 72 (m/z) and 78 (m/z) were used for fenuron and fenuron-d_6, respectively. Moreover, qualifier ions for fenuron and fenuron-d_6 were determined as 164/45 (m/z) and 170/49 (m/z), respectively. Selected ion monitoring (SIM) was acquired using the quantifier ions to determine the peak areas of fenuron and fenuron-d_6.

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2.3 Synthesis and characterization of fenuron-d_6

The synthesis procedure of fenuron-d_6 was detailed in our previous study [38]. Briefly, the dissolution of triethylamine (1.2 equivalents) and dimethyl-d_6-amine hydrochloride (1.0 equivalent) was performed in 5.0 mL of toluene. Then, phenyl isocyanate (1.0 equivalent) was added into the reaction mixture. A mixing process with the help of a magnetic stirrer was applied to the mixture at 40 ºC for 6.0 h. After 6.0 h, the completion of the reaction was checked by thin layer chromatography (TLC), and it was observed that dimethyl-d_6-amine hydrochloride was completely consumed. Then, a cooling process was implemented to the mixture at room

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temperature. Next, the solvent was completely vaporized with the help of a rotary evaporator, and a white solid product was yielded. In the final step, the synthesized fenuron-d_6 was purified using a CombiFlash chromatography.

The characterization of synthesized fenuron-d_6 was carried out using Fourier transform infrared spectroscopy (FT-IR), nuclear magnetic resonance (NMR), and liquid chromatography-electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS) systems in our previous study [38]. In this study, the synthesized fenuron-d_6 was characterized by GC-MS possessing electron ionization. The SIM chromatograms (the chromatograms were plotted according to the major m/z values of fenuron and fenuron-d_6) belonging to the analyte and synthesized fenuron-d_6 can be seen in Figure S1.

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2.4 Procedure of switchable solvent liquid phase microextraction

A standard/sample solution (8.0 mL) was pipetted into a clean polypropylene centrifuge tube (15 mL) and then 2.0 mL of sodium sulfate solution (25.0% (*m/m)*) was added into the solution. Next, 0.75 mL of switchable solvent (a 1:1 (*mL/mL*) mixture of ultrapure water and N,N-dimethylbenzylamine) was pipetted into the solution. It should be noted that this volume refers to the total switchable solvent mixture rather than pure amine. After the addition of switchable solvent, 1.0 mL of NaOH solution (1.5 mol/L) was added into the solution, and a cloudy appearance was observed in the solution. Next, the solution was centrifuged for 2.0 min at 1800 *g* to attain efficient phase separation. In the final step, the upper analyte-rich phase (approximately 75 µL) was transferred to an insert vial to be analyzed by the GC-MS system. The synthesis of switchable solvent and the SA-SS-LPME-GC-MS are illustrated in Figure 1.

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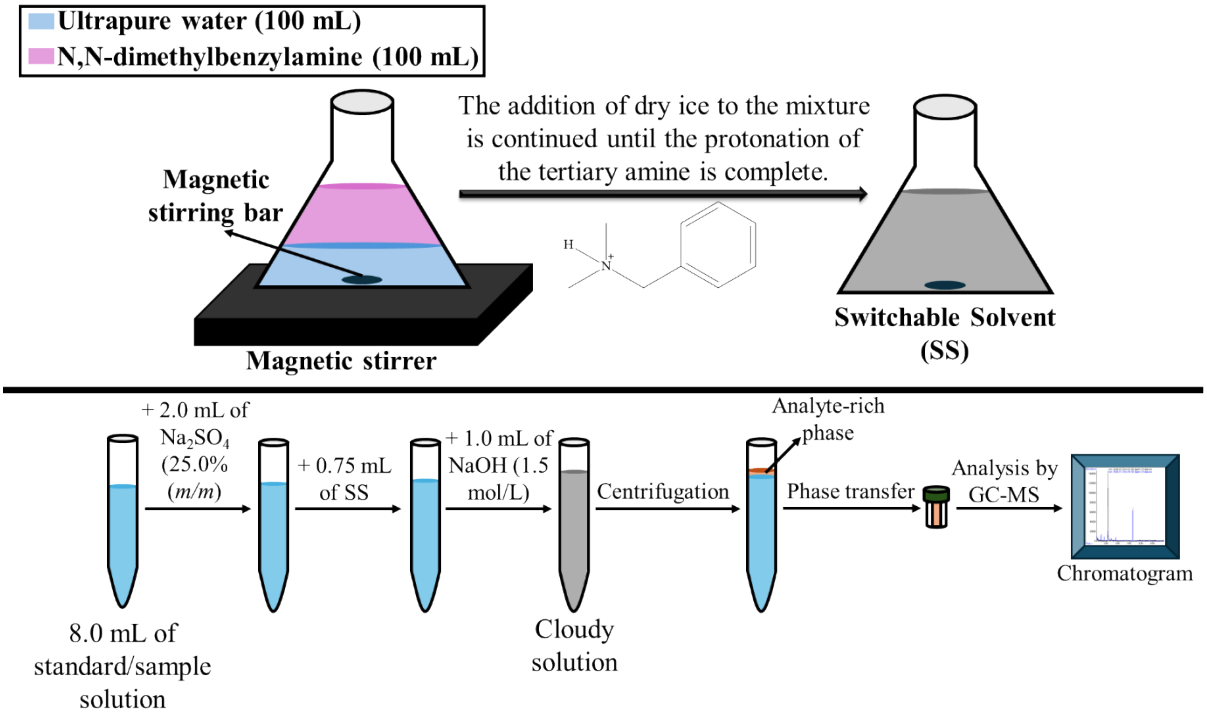


Figure 1. Graphical representation of the switchable solvent synthesis and SA-SS-LPME-GC-MS method.

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2.5 Samples

Carrots were purchased from two different local markets in İstanbul, Türkiye. In the first step, carrots were peeled off and then washed with tap water and ultrapure water to remove possible contaminants, respectively. Then, carrots (approximately 1 kg per batch) were pressed using a juice extractor to obtain carrot juice. In the final step, the carrot juices were transferred to separate clean centrifuge tubes and stored in a refrigerator at -20 ºC until their analyses.

During the first trials, 0.80 g of carrot juice bought from Market A was weighed in a clean centrifuge tube and then diluted to 40 g with ultrapure water. In the second step, the sample solution was centrifuged for 10 min to separate solid particles, and then the liquid part was decanted into a clean centrifuge tube. After, the developed SA-SS-LPME method was applied to the sample solution under the optimum conditions. After the application of the SA-SS-LPME method, it was observed that precipitates

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possessing an orange color completely covered the upper phase, and it was difficult to withdraw the upper organic phase due to the precipitates formed. Possibly, the precipitates observed in the upper organic phase contained mainly protein due to the relatively high content of protein in carrots [4,5]. In addition, carrots have high carbohydrate content [4,5,39]. It is known that acetonitrile is a good reagent for the precipitation of protein [40–43] found in the sample matrices and carbohydrates have poor solubility in traditional organic solvents such as acetonitrile, tetrahydrofuran and formaldehyde [44]. For this reason, 0.75 g of carrot juice bought from Market A was taken into a clean centrifuge tube and then completed to 1.50 g with acetonitrile. After, the solution was vortexed for 30 s and then centrifuged for 10 min. After the centrifugation process, a clear supernatant phase was obtained. Next, 0.72 g of supernatant was weighed into a clean centrifuge tube (50 mL) and then filled up to 18.0 g with ultrapure water. In the final step, the sample solution was treated with the developed SA-SS-LPME method, and a clear upper organic phase was observed. For this reason, the precipitation procedure with acetonitrile was implemented before the application of the SA-SS-LPME method to the carrot juice samples.

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2.5.1 Sample solutions used in external standard, matrix–matching and internal standard calibration methods

A carrot juice sample (0.75 g) was weighed in a clean centrifuge tube and then 0.04 g of fenuron-d_6 solution (prepared in acetonitrile, 911.5 mg/kg) was added into the sample. Then, the sample solution was filled up to 1.50 g with acetonitrile after spiking a suitable amount of fenuron standard solution (prepared in acetonitrile, 990.7 mg/kg). After, vortex mixing (for 30 s) and centrifugation (for 10 min at 1800 *g*) processes were applied to the sample solution, respectively. In the final step, 0.72 g of supernatant was taken into a clean centrifuge tube and then completed to 18.0 g with ultrapure water. Each sample solution prepared was pipetted 8.0 mL in two separate centrifuge tubes and the solutions were treated with the

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developed SA-SS-LPME method. After the implementation of the SA-SS-LPME method, each analyte-rich phase obtained were measured two times by the GC-MS system. Totally, the sample matrix was diluted approximately 50-fold after all sample preparation procedures.

2.5.2 Sample solutions used in ID^4 strategy

A carrot juice (0.75 g) was weighed in a clean centrifuge tube and then a proper amount of fenuron standard solution (990.7 mg/kg) was added into the sample solution. After, the total amount of sample solution was completed to 1.50 g with acetonitrile. The solution was vortexed for 30 s and then centrifuged for 10 min at 1800 *g*. In the final step, 0.80 g of supernatant was weighed in a clean centrifuge tube before filling up to 20.0 g with ultrapure water. An approximate 50-fold dilution of the sample was recorded at the end of the sample preparation procedure. Detailed information on the prepared sample solutions is provided in Table S1.

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2.6 Statistical test

Tukey's post hoc test, which was known as the honestly significant differences (HSD) method, comprises one-way analysis of variance (ANOVA) with pairwise comparison [45]. During the statistical evaluation among variables in optimization experiments, Tukey's post hoc test was implemented using the JASP software (version: 0.19.0.3). The error bars in the optimization figures were produced by triplicate measurements. Different lower-case letters found in the optimization figures present the statistical differences among variables.

3. Results and Discussion

3.1 Optimization of SA-SS-LPME parameters

Tertiary amines offer several advantages, including i) their hydrophobicity, which allows the formation of a biphasic system, ii) lower cost compared

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to primary amidines, and iii) their stability during hydrolysis, a property not observed in primary amidines due to their hydrolytic instability [46]. One study reported that N,N-dimethylbenzylamine is more effective to extract the analytes than other tertiary amines such as N,N-dimethylcyclohexylamine and N,N-diisopropylethylamine [47]. Furthermore, the extraction of fenuron into N,N-dimethylbenzylamine can be explained by π–π interactions between their benzyl rings as well as van der Waals forces that enhance solute–solvent affinity [48]. For this reason, N,N-dimethylbenzylamine was used to synthesize a switchable solvent in this study. In an SS-LPME method, there is an inverse relationship between switchable solvent volume and preconcentration factor. Hence, the selection of a suitable volume for the switchable solvent is a substantial parameter to record a high preconcentration factor [49]. For this reason, four different switchable solvent volumes were tested to enhance the preconcentration yield. As expected, peak areas belonging to the analyte decreased while increasing switchable solvent volume (Figure S2). However, 0.50 mL of switchable solvent did not provide an efficient phase separation to easily withdraw the analyte-rich phase. Therefore, 0.75 mL was selected as the optimum extraction solvent volume.

The addition of NaOH or purging with an inert gas led to the conversion to the nonionic form of the switchable solvent. A two-phase system (including aqueous and organic phases) is obtained with the help of these deprotonation processes in an SS-LPME method [50–52]. In this study, NaOH was used as a deprotonation reagent for the synthesized switchable solvent. Therefore, the effect of concentration and volume of NaOH on the preconcentration yield were examined to augment the signal intensity. While 1.5 mol/L and 2.0 mol/L of NaOH solution ensured efficient phase separation, insufficient phase separation for transferring to an insert vial was obtained from 1.0 mol/L of NaOH. As can be seen in Figure S3 (A), 1.5 mol/L of NaOH solution gave about 13% higher analytical signals than 2.0 mol/L of NaOH solution. Additionally, 0.75, 1.0, 1.25 and 1.50 mL of NaOH solutions (1.5 mol/L) were compared to each other (Figure S3 (B)). It was difficult to withdraw the analyte-rich phase at 0.75 mL of NaOH solution.

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For this reason, 1.0 mL was chosen as the optimum NaOH volume to avoid difficulties in terms of the phase withdrawal in real samples.

Fenuron has high water solubility in water (3.85 g/L at 25 ºC) [53], therefore; it is difficult to extract/preconcentrate fenuron from water media [10]. The extraction/preconcentration efficiency can be increased with the help of salt addition by reducing the solubility of organic analytes in aqueous medium [54–56]. For these reasons, the solutions with sodium chloride, potassium nitrate and sodium sulfate solutions were compared to the solutions without salt addition. As can be seen in Figure S4 (A), the solutions salted with sodium chloride and potassium nitrate solutions gave similar analytical results statistically. Among the tested variables, sodium sulfate solution (25% (*m/m*)), which was used in further experiments, provided the highest signal-to-noise ratio with a statistically significant difference. The average hydration enthalpies for Na^+, K^+, Cl^-, NO_3^- and SO_4^{2-} have been experimentally reported as –409 kJ/mol, –322 kJ/mol, –381 kJ/mol, –314 kJ/mol and –1059 kJ/mol, respectively [57]. Therefore, the superior efficiency of sodium sulfate can be attributed to the high hydration energy of sulfate ion compared to the other ions. In addition, extraction performance is strongly influenced by the ionic strength of the sample matrix since it can induce both salting-out and salting-in effects [58]. The ionic strength (μ) was calculated using the expression "$\mu = \frac{1}{2} \bullet \sum m_i \bullet z_i^2$", where m_i represents the molality (mol/kg) of each ion and z_i denotes its charge [59]. Based on these calculations, the ionic strengths of NaCl, KNO_3 and Na_2SO_4 were determined to be 0.48, 0.27 and 0.49, respectively. During the calculation of ionic strengths, the dilutions were taken into account after the addition of salt solution into the standard solution. Consequently, the increment in ionic strength could enhance the salting-out effect, thereby improving the preconcentration performance. Furthermore, five different sodium sulfate volumes were tested and a linear increment in peak area values was observed from 0.75 mL to 2.0 mL. In addition, peak area values reached the plateau region after 2.0 mL (Figure S4 (B)). Thus, 2.0 mL of sodium sulfate solution (25.0% (*m/m*)) was determined as the optimum condition.

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It is well-known that initial sample volume affects the extraction/preconcentration yield in a microextraction method [60]. Hereby, 4.0, 6.0, 8.0 and 10.0 mL of sample volumes were tried to record high signal intensity. Relatively, a linear increment was observed from 4.0 mL to 8.0 mL, yet similar analytical results were obtained for 8.0 mL and 10.0 mL according to the mean value of peak area values (Figure S5). The reason why the analytical signals did not increase at 10 mL might be due to the high-water solubility of fenuron [53]. As a result of this optimization experiment, 8.0 mL was selected as the optimum initial sample volume.

A mixing process can increase the analyte's mass transfer to the target organic phase because mixing can enhance the diffusion of analyte(s) to achieve equilibrium between aqueous and organic phases [61]. For this reason, the solutions stirred with three different mixing modes after the addition of NaOH and were compared to the solutions without any mixing process. According to the post hoc test (Figure S6), similar analytical results were obtained for all mixing modes and the solutions without any mixing process. In line with the results obtained, the solutions were not stirred in further studies.

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3.2 Dynamic range, LOD and LOQ

To evaluate the dynamic range, limit of detection (LOD) and limit of quantitation (LOQ), standard solutions ranging from 0.12 mg/kg to 17.25 mg/kg were prepared. Additionally, fenuron-d_6 was added into all standard solutions. The solutions prepared were treated with the developed SA-SS-LPME method prior to introducing the GC-MS system. During the evaluation of dynamic range, LOD and LOQ, both external and internal standard calibration plots were constructed. Dynamic range was determined according to LOQ value and correlation of determination (R^2). The external standard calibration method provided a dynamic range between 0.12 and 2.85 mg/kg while a linearity in the range of 0.12–5.13 mg/kg was recorded from the internal standard calibration strategy. Furthermore, the LOD/LOQ values were found to be 0.05/0.15 mg/kg and

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0.05/0.17 mg/kg via the external and internal standard calibration strategies, respectively. The LOD/LOQ values were determined based on the relationship between the standard deviation of analytical response at the lowest concentration and slope of the constructed calibration plot [62]. These values were calculated employing related expressions (Eq. (1–4)). Additionally, the constructed external and internal standard calibration plots and LOQ signal belonging to the analyte can be seen in Figure 2.

$$LOD = 3 \bullet \frac{SD_{LC}}{m_{External\ Standard\ Calibration\ Plot}} \quad \text{Eq. (1)}$$

$$LOQ = 10 \bullet \frac{SD_{LC}}{m_{External\ Standard\ Calibration\ Plot}} \quad \text{Eq. (2)}$$

$$LOD = 3 \bullet \frac{SD_{LR}}{m_{Internal\ Standard\ Calibration\ Plot}} \bullet C_{IS} \quad \text{Eq. (3)}$$

$$LOQ = 10 \bullet \frac{SD_{LR}}{m_{Internal\ Standard\ Calibration\ Plot}} \bullet C_{IS} \quad \text{Eq. (4)}$$

SD_{LC}: Standard deviation of the lowest concentration found in the constructed external standard calibration (n=6).

$m_{External\ Standard\ Calibration\ Plot}$: Slope of external standard calibration plot.

SD_{LR}: Standard deviation of the lowest concentration ratio found in the constructed internal standard calibration (n=6).

$m_{Internal\ Standard\ Calibration\ Plot}$: Slope of internal standard calibration plot.

C_{IS}: Concentration of internal standard (1.01 mg/kg) found in the lowest concentration (0.12 mg/kg).

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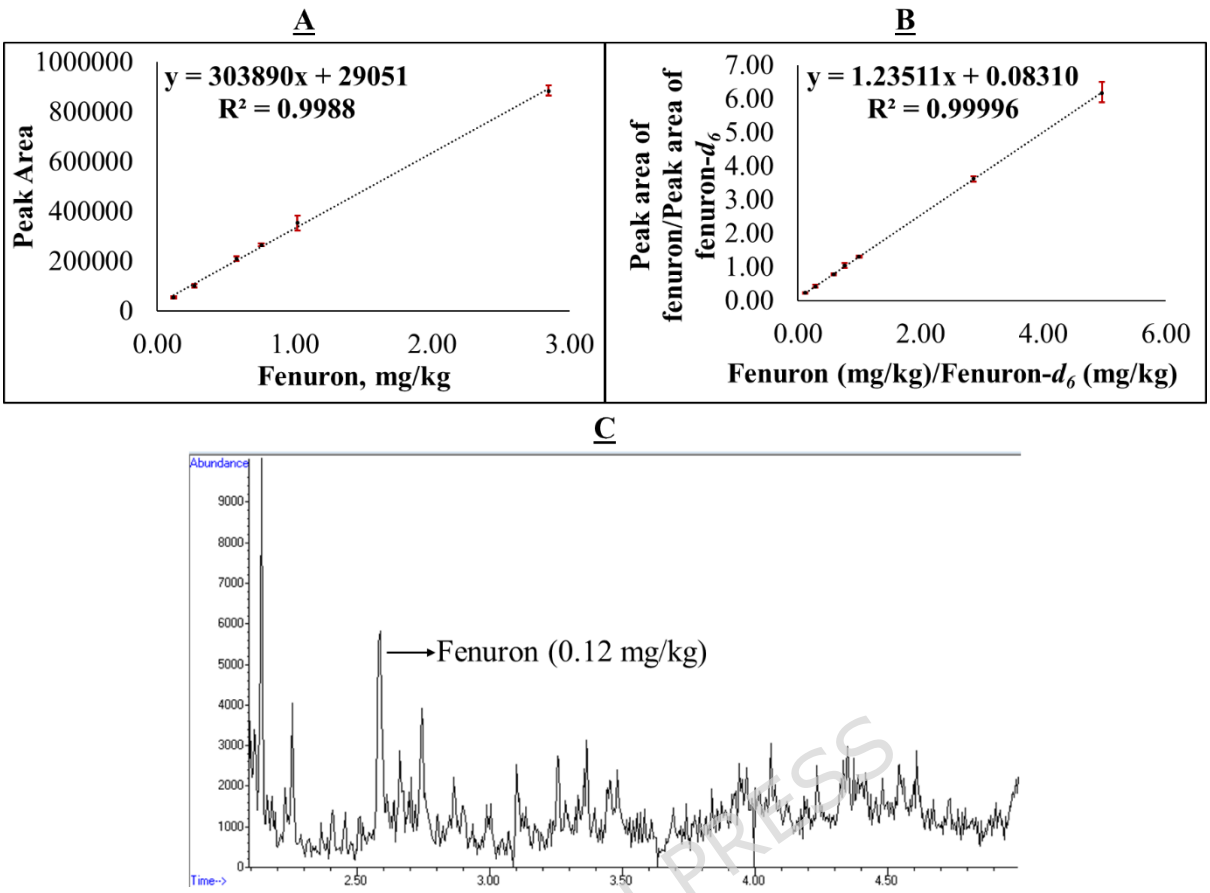


Figure 2. External standard calibration plot (A), internal standard calibration plot (B) and LOQ signal.

According to the European Union (EU), the maximum residue level (MRL) for fenuron in carrots and their products is not specified; therefore, a default MRL of 0.01 mg/kg can be applied [63,64]. Although the sensitivity of the developed method is insufficient to accurately quantify fenuron at such low concentrations, it remains suitable for qualitative and quantitative determination at higher residue levels.

3.3 Literature comparison in terms of system analytical performance values

In a previous study published, a differential pulse voltammetry method was used to quantify fenuron in environmental water samples. The proposed analytical method ensured 0.34 µmol/L (~55.8 µg/L) of LOD with a linearity ranged from 0.4 µmol/L (~65.7 µg/L) to 12.0 µmol/L (~1970 µg/L) [1]. Another study found in the literature proposed a direct laser photoinduced fluorescence (DL-PIF) for the determination of fenuron in river and

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seawater samples. A relatively wide linear range between 0.02 mg/L and 10 mg/L, 0.0015 mg/L of LOD and 0.005 mg/L of LOQ value were reported [65]. Furthermore, Mou et al. introduced a high-performance liquid chromatography-fluorescence detection (HPLC-FL) for the quantitation of fenuron in rice and corn samples. Dynamic range, LOD and LOQ values for the developed method were reported as 0.01–1.0 mg/L, 0.003 mg/kg (for rice)/0.003 mg/kg (for corn) and 0.010 mg/kg (for rice)/0.009 mg/kg (for corn), respectively [2]. In our previous study, LC-ID^4-ESI-MS/MS method was developed to determine fenuron with high accuracy and precision in environmental water and fruit juice samples. Dynamic range, LOD and LOQ values for the analyte via the LC-ESI-MS/MS system were calculated as 0.11–492.23 µg/kg, 27.4 ng/kg and 91.4 ng/kg, respectively [38]. In another of our published studies, the GC-MS system was combined with a simultaneous derivatization and preconcentration method to quantify fenuron in domestic wastewater samples. The proposed method generated a linearity in the range of 0.51–24.50 mg/kg, 0.15 mg/kg of LOD and 0.49 mg/kg of LOQ [10]. Especially, the developed SA-SS-LPME-GC-MS method gave higher LOD and LOQ values than the LC-ESI-MS/MS and HPLC-FL methods. However, the developed SA-SS-LPME-GC-MS method exhibited similar or lower LOD and LOQ values compared to other literature studies published. In addition, the developed SA-SS-LPME method can easily be coupled with a GC-MS/MS system to determine the analyte at lower limits with the help of multi-reaction monitoring acquisitions.

3.4 Recovery experiments

During the recovery experiments, four different strategies, including external standard, matrix–matching calibration, internal standard calibration, and ID^4 were compared to each other in order to evaluate the superior quantitative determination strategy in terms of accuracy and precision. For this purpose, the sample solutions used in the external standard, matrix–matching, and internal standard calibration strategies were prepared according to the procedure detailed in *Section 2.5.1*. In addition, both sample solutions without any spiking procedure were

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analyzed by the developed SA-SS-LPME-GC-MS method, but no detectable signals were monitored.

The external standard calibration method is a basic quantitative determination strategy using standard solutions of analyte(s) prepared in a related solvent [29]. However, the elimination of possible positive/negative matrix effects cannot be acquired in the external standard calibration method [66]. During the calculation of percent recovery results, the external standard calibration given in Figure S7 was used. The external standard calibration method gave acceptable recovery results between 104.5 and 118.1% (Table 1).

The matrix–matching calibration strategy comprises a series of standard solutions prepared in an analyte–free sample matrix for the elimination of possible matrix effects in order to boost the accuracy of an analytical method [30]. During the application of the matrix–matching calibration method, the matrix–matching calibration plot built by the spiked Market A carrot juice samples (Figure S8 (A)) was used to figure out recovery results of the spiked Market B carrot juice samples. Furthermore, recovery results of the spiked Market A carrot juice samples were calculated via the matrix–matching calibration plot constructed by the spiked Market B carrot juice samples (Figure S8 (B)). The recovery results were found to be between 75.1 and 115.0% (Table 1) for the spiked Market A and B carrot juice samples. Based on these percent recovery results, matrix-matching calibration strategy did not compensate for the heavy matrix of carrot juice samples.

In the internal standard calibration method, a substance or an isotopically labelled material of the analyte(s) is utilized as an internal standard to eliminate possible matrix effects and instrumental errors [31]. An internal standard calibration plot constructed by the standard solution containing fenuron–d_6 (Figure S9) was employed to calculate recovery results for the spiked carrot juice samples. The internal standard calibration method provided the recovery results in the range of 89.7%–100.7% (Table 1) with acceptable standard deviation (≤6.3).

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The ID^4 is a superior analytical methodology, which consists of calibration blends possessing three different isotope ratios and a sample blend to reach highly accurate and precise analytical results [32,38,67]. Before the implementation of the ID^4 strategy, the isotopes should equilibrate in the solution to obtain stable isotope ratios [68,69]. For this reason, a blend (containing 0.98 mg/kg of fenuron and 1.00 mg/kg of fenuron-d_6) was preconcentrated with the developed SA-SA-SS-LPME method and then analyzed by the GC-MS system within the periods of 0–240 min. According to the results given in Figure S10, the ratio of fenuron and fenuron-d_6 reached equilibration after 24 min according to the mean value of peak area ratios and the post hoc test. Therefore, the blends prepared were kept at room temperature at least 24 min before their analysis. The recovery results were calculated via the ID^4 formula reported in the previous publications [32,37,38]. The recovery results were figured out between 99.6%–102.0% (Table 1) with low standard deviation values (≤2.3). This experimental result showed that the matrix effect of carrot juice samples was overcome by the combination of SA-SS-LPME and GC-ID^4-MS methods. Additionally, representative SIM chromatograms obtained from the calibration and sample blends are shown in Figure S11.

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Table 1. Recovery results obtained from external standard, matrix–matching and internal standard calibration methods, and ID^4 strategy.

Method	Sample	Actual spiked concentration (Fenuron), mg/kg	Actual added concentration (Fenuron-d_6), mg/kg	%Recovery ± SD
External standard calibration	Carrot juice bought from Market A	0.60	–	114.0%±4.3*
		0.77		111.7%±7.2*
		1.95		115.3%±1.9*
		2.62		107.7%±4.0*
	Carrot juice bought from Market B	0.56		104.5%±4.7*
		0.90		114.0%±2.2*
		1.80		118.1%±6.0*
Matrix–matching calibration	Carrot juice bought from Market A	0.60	–	102.7%±3.7*
		0.77		100.0%±6.2*
		1.95		101.5%±1.7*
		2.62		94.6%±3.5*
		3.45		88.3%±4.0*

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		4.79		87.7%±5.5*
	Carrot juice bought from Market B	0.56		75.1%±4.8*
		0.90		96.7%±2.3*
		1.80		111.1%±6.2*
		3.49		107.6%±2.0*
		4.57		115.0%±6.2*
Internal standard calibration	Carrot juice bought from Market A	0.60	1.02	100.4%±1.8*
		0.77	0.96	97.6%±6.3*
		1.95	1.01	97.9%±2.0*
		2.62	0.97	92.3%±2.5*
		3.45	1.00	93.9%±4.3*
		4.79	0.95	89.7%±3.9*
	Carrot juice bought from Market B	0.56	1.01	96.9%±3.6*
		0.90	0.93	100.7%±5.1*
		1.80	0.97	97.2%±2.9*
		3.49	0.94	92.1%±3.1*
		4.57	0.95	92.3%±4.4*
ID^4 strategy	Carrot juice bought from Market A	1.05	Concentration found in the blends prepared can be seen in Table S3 and S4.	99.6%±2.3**
		2.55		102.0%±1.3**
	Carrot juice bought from Market B	1.01		101.7%±1.7**
		2.51		101.4%±1.2**

**±SD: Standard deviation obtained by quadruplicate measurements.*
***±SD: Standard deviation obtained by 81-combination.*

Additionally, the matrix matching and internal standard calibration strategies gave wider linear ranges than the external standard calibration method. Although the recovery results obtained from the external standard, matrix–matching and internal standard calibration methods were acceptable, the ID^4 strategy provided superior recovery results compared to other calibration methods implemented. The recovery results obtained from the ID^4 strategy showed that the developed analytical methodology (SA-SS-LPME-GC-ID^4-MS) can be employed as a reference methodology due to its high accuracy and precision.

3.5 Comparison of greenness evaluations

Many analytical metrics such as AGREEprep [70], GAPI [71], ComplexGAPI [72], AMGS [73] and others [74,75] have been presented to the literature to evaluate the greenness of chemical procedures. Wojnowski et al. introduced an AGREEprep tool in 2022 to assess the greenness of analytical methods based on ten green analytical chemistry principles. It is an open-source program that figures out greenness factors for analytical

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methods [76]. It includes several criteria such as material usage, solvent selection, energy consumption, waste production, sample throughput and sample size. The scores are between zero (lowest) and one (highest) [77]. In the presented study, the AGREEprep tool was employed to assess the greenness of the proposed SA-SS-LPME-GC-ID^4-MS method and to compare it with our previously reported methods (spray-based fine droplet formation–liquid phase microextraction (SFDF-LPME)-GC-MS [10] and LC-ID^4-ESI-MS/MS [38]). As can be seen in Figure S12, the LC-ID^4-MS/MS method appears to be the greenest among the compared methods since it does not require a preconcentration step, uses a low initial sample volume, and involves non-hazardous reagents. However, the LC-ESI-MS/MS system is not commonly available in all laboratories, and it generally consumes more energy than the GC-MS systems. Furthermore, the SFDF-LPME-GC-MS method showed a lower central score compared to the proposed method, mainly due to the use of multiple hazardous chemicals and a higher initial sample volume.

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4. Conclusion

Herein, an SA-SS-LPME-GC-ID^4-MS method was developed to quantify fenuron in carrot juice samples with high accuracy and precision. To the best of our knowledge, this is the first study about the combination of SA-SS-LPME and GC-ID^4-MS methods for determination of fenuron in carrot juice samples. The substantial parameters of the SA-SS-LPME method were optimized to increase the signal-to-noise ratio of the analyte. After, the assessment of system analytical performance for the developed SA-SS-LPME-GC-MS method was performed, and the LOD/LOQ values were figured out to be 0.05/0.15 mg/kg and 0.05/0.17 mg/kg via the external and internal standard calibration methods, respectively. Additionally, the internal standard calibration method provided wider dynamic range than the external standard calibration method. Furthermore, three different univariate calibration methods, including the external standard, matrix–matching and internal standard, were compared to the ID^4 strategy in

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terms of accuracy and precision. For this purpose, recovery experiments were conducted with the spiked carrot juice samples, and the best analytical results (99.6%–102.0%) were obtained from the ID^4 strategy in terms of accuracy and precision. In line with the results attained, the proposed SA-SS-LPME-GC-ID^4-MS method, which provided highly accurate and precise analytical results, can be employed as a reference method to quantify fenuron in carrot juice samples. Contrary to the positive aspects of the developed method, the SA-SS-LPME-GC-MS method does not ensure detection/quantitation limits at ultra-trace levels, which can be said to be a disadvantage of the developed method. On the other hand, the developed SA-SS-LPME method can be readily integrated with a GC-MS/MS system to determine fenuron at ultra-trace levels. Furthermore, the presented GC-ID^4-MS method can be coupled with a more efficient microextraction method to be developed in the future to achieve highly accurate and precise analytical results at trace concentrations. Additionally, the developed analytical methodology can be used to determine fenuron in other fruit/vegetable juices after the proper sample preparation procedure.

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Declarations

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CRediT authorship contribution statement

Süleyman Bodur: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft.

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