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Dispersive solid phase extraction and quadruple isotope dilution–mass spectrometry combination for the accurate and sensitive quantification of capsaicin in pepper, domestic wastewater and human saliva samples by GC–MS system

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ABSTRACT

In the presented study, reduced graphene oxide/Fe_3O_4 (rGO/Fe_3O_4) nanocomposites based dispersive solid phase extraction (DSPE) – gas chromatography–mass spectrometry (GC–MS) method was developed for the determination of capsaicin in domestic wastewater (DW), pepper (PP) and human saliva (HS) samples. All important parameters of the DSPE method affected the preconcentration factor were carefully optimized to achieve high signal to noise ratio for the analyte. After the optimization studies, the system analytical performance of DSPE-GC–MS system was evaluated using the aqueous standard solution of capsaicin. Limit of detection (LOD), limit of quantitation (LOQ) and dynamic range were figured out to be 0.54 μg/kg, 1.80 μg/kg and 2.66 – 487.35 μg/kg, respectively. Under the optimum experimental conditions, recovery studies were conducted with the spiked DW, PP and HS samples, and percent recovery results were recorded between 52.6 % and 183.6 % via matrix matching calibration strategy. After the implementation of ID^4 strategy, percent recovery results for the spiked DW, PP and HS samples were calculated as 98.2 %–99.3 %, 99.7 %–100.7 % and 99.4 %–99.8 %, respectively. In addition, capsaicin content in Sivri (S)-PP, Kıl (K)-PP and Samandağ (SA)-PP samples were found to be 309.5 ± 11.8 mg/kg, 873.7 ± 26.7 mg/kg and 165.3 ± 5.1 mg/kg via DSPE-GC-ID^4-MS method, respectively. As a result, the combination of quadruple isotope dilution (ID^4) strategy and the DSPE-GC–MS method were successfully performed to boost the accuracy and precision of developed DSPE-GC–MS method.

1. Introduction

Capsaicin, which is an alkamide, has been gained traction by scientific authorities due to its potential applications in therapeutic fields and food industry [1]. Capsicum family naturally contains capsaicin which gives hot taste to chili peppers (PPs) [2]. It is known that capsaicin has anti-cancer, anti-oxidant, anti-inflammatory, anti-obesity and analgesic properties according to the previous publications [3–5]. Contrary to these beneficial features of capsaicin, the consumption of excess capsaicin poses harmful effects on human health [6,7]. In addition, PPs containing low capsaicin content are inconvenient for commercial purposes due to the increment in extraction cost [8]. Furthermore, there are effects of capsaicin on saliva secretion [9,10] and aroma perception [11,12] which are associated with human oral health. With respect to a report, capsaicin concentration in wastewater was reported between 0.65 ng/L and 1023 ng/L due to high consumption of PPs [13]. Therefore, the determination of capsaicin is important issue in wastewater (to evaluate consumption habit), saliva (to protect human health) and PP

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(to decrease the cost of capsaicin extraction) samples.

In literature, electrochemical methods [14], gas chromatography–mass spectrometry (GC–MS) [7,15–17], high performance liquid chromatography–UV detection (HPLC-UV) [6,18,19], HPLC–fluorescence detection [19,20] and liquid chromatography–tandem mass spectrometry (LC-MS/MS) [19] have been used for the determination of capsaicin in several matrices. GC–MS systems provide higher peak resolution and capacity compared to LC-MS systems and is a powerful choice for the determination of volatile and semi-volatile compounds [21].

Although sophisticated analytical instruments have been developed for the determination of several analytes in various matrices, sample preparation methods play important role to obtain suitable sample form to the related instruments [22]. In literature, magnetic/non-magnetic nanoparticles/nanocomposites have gained popularity in different fields as well as analytical chemistry due to its large surface area and unique properties [23]. In dispersive solid phase extraction (DSPE) method, magnetic/non-magnetic nanomaterials have frequently been used to extract and preconcentrate analyte(s) prior to the related instrumentation method [23–25]. Furthermore, the DSPE methods (containing different types of nanoparticles/nanocomposites) have been combined with several instrumentation techniques to extract/preconcentrate various inorganic [26–29] and organic [27,28,30,31] analytes.

Isotope dilution (ID) method is one of the primary analytical methods [32,33] to obtain analytical results with high accuracy and precision by reducing the matrix effects and eliminating analyte-losses [34]. In ID strategies, there are mainly four different experimental designs as single isotope dilution (ID), double isotope dilution (ID^2), triple isotope dilution (ID^3) and quadruple isotope dilution (ID^4) [35]. An isotopically enriched/labelled material belonging to the analyte(s) and a MS system are required to perform ID strategies [36–38]. The ID^4 strategy comprises one sample blend and three calibration blends (containing three different isotope compositions) [35,39] to reduce uncertainties arising from isotopic composition of the sample (r_A), and isotopic composition (r_B) and mass fraction (w_B) of the isotopically enriched/labelled material [35]. In addition, ID strategies have been used as reference analytical approaches to characterize the content of both inorganic [39–41] and organic/organometallic [41–43] analyte(s) in the candidate or available certified reference materials (CRM) or standard reference materials (SRM). For this reason, the ID^4 strategy was employed to obtain accurate and precise analytical results for the capsaicin content in the unspiked/spiked samples during this study.

The aim of this study was to develop rGO/Fe_3O_4 nanocomposites based DSPE method for the preconcentration of capsaicin at trace levels from DW, PP and HS samples before the GC–MS analysis. All influential parameters belonging to the DSPE method were optimized to attain low detection limit for capsaicin. Additionally, the combination of DSPE-GC–MS method and ID^4 strategy was successfully achieved to record highly accurate and precise analytical results. Furthermore, the matrix-matching calibration and the ID^4 strategies were compared to each other in terms of accuracy and precision.

2. Materials and methods

2.1. Chemicals, standards and reagents

Capsaicin (purity ≥ 95 %) and capsaicin-d_3 (purity ≥ 99 %) were bought from Sigma Aldrich (Darmstadt, Germany) and Cayman Chemical (Ann Arbor, USA), respectively. Methanol (purity ≥ 99.8 %), ethanol (purity ≥ 99.9 %) and acetone (purity ≥ 99.5 %) were obtained from IsoLab Laborgeräte GmbH Chemicals (Eschau, Germany) while isopropyl alcohol (purity ≥ 99.8 %), acetonitrile (purity ≥ 99.8 %), hydrogen peroxide (35 %), hydrazine (98 %), ammonium hydroxide (25 %), potassium permanganate (≥99.0 %) and sulfuric acid (95.0 – 97.0 %) were supplied from Merck (Darmstadt, Germany). Ultrapure water (resistivity of 18.2 MΩ·cm) used in all experimental steps and cleaning procedures was produced using Elga PureLab Flex 3 (High Wycombe, England) purification system.

2.2. Instrumentation

During the optimization studies, the combination of a Thermo mass spectrometry (ISQ QD model) and a Thermo gas chromatograph (Trace 1300 model) consisting of a HP-5MS column (60 m length x 250 μm internal diameter x 250 nm film thickness) was employed. During the system analytical performance, recovery studies and the application of ID^4 strategy, an Agilent gas chromatograph (6890 N) integrated with a HP-5MS analytical column (30 m length x 250 μm internal diameter x 250 nm film thickness) was used to achieve the chromatographic separation of the analyte. The gas chromatograph was hyphenated with an Agilent mass spectrometer (5973 N) to qualify and quantify the analyte and its isotopically labelled material. High purity helium, which was set to 2.0 mL/min, was employed as a carrier gas. A single ramp temperature program was applied follow; 1) the initial temperature was set to 100 °C, 2) the temperature was increased from 100 °C to 300 °C at 40 °C/min and 3) the temperature was hold for 2.0 min at 300 °C (holding time was adjusted to 4.0 min during the optimization studies due to the column length). Inlet temperature, injection volume/mode, ionization source temperature, transfer line temperature and quadrupole temperature were adjusted to 290 °C, 1.0 μL/spitless, 230 °C, 280 °C and 150 °C, respectively. Qualifier/quantifier ions were 151/137 for capsaicin while qualifier/quantifier ions were 154/140 for capsaicin-d_3.

2.3. Samples

2.3.1. Samples used in matrix matching calibration strategy

Domestic wastewater (DW) samples were supplied from the Environmental Engineering Department (Yıldız Technical University). A filtration process was implemented to these samples using filter paper and syringe type filter (0.45 μm of pore size) before the use in experiments. After the filtration process, 1.25 g of DW sample was weighed to a clean centrifuge tube (50 mL) and an appropriate amount of standard solution was added onto the sample before the completion to 25.0 g with ultrapure water. The percentage of ethanol was set to 1.0 % (*w/w*) for each spiked sample solution.

Two volunteers found in our research laboratory gave human saliva (HS) samples. Yıldız Technical University Social Sciences Research Ethics Committee (No: 2022.03) approved this study. HS sample (0.50 g) was taken into a clean centrifuge tube (15 mL) and a suitable amount of standard solution was pipetted onto the sample. After, 0.80 g of acetonitrile was added to the solution to acquire protein precipitation, and the solution was filled to 1.60 g with ultrapure water. After the application of vortex mixing (30 s), a centrifugation process was implemented for 10.0 min at 3461 *g* to provide protein precipitation efficiently. After the protein precipitation process, 1.0 g of supernatant was taken into a clean centrifuge tube and then filled up to 40 g with ultrapure water. The contents of ethanol and acetonitrile in each sample solution were 0.50 % (*w/w*) and 1.2 % (*w/w*), respectively.

Four different PP (Banana (B), Kıl (K), Sivri (S) and Samandağ (SA)) were bought from local markets located in İstanbul, Türkiye. In the first step, all PPs were put in an oven at 65 °C for 2 weeks to perform sample drying process. After, all PP samples were grinded to enhance extraction efficiency by increasing surface area. Then, 0.40 g of powdered sample (0.60 g was weighed for the SA-PP sample except other powdered PP samples) was added into a clean centrifuge tube and then completed to 10 g with ethanol to carry out the extraction of capsaicin content. The mixture was mixed using orbital shaker for 30 min and a centrifugation process was applied for 10 min at 3461 *g*. After, the sample solutions were filtered with the help of 0.45 μm syringe filter to remove possible suspended solid particles. Additionally, the S-PP and K-PP sample

solutions were diluted 31.7 and 101.6 folds with ethanol to be compatible with dynamic range of the developed method. Next, 0.20 g of the prepared PPs in ethanol was weighed in a clean centrifuge tube (50 mL) and a proper amount of standard solution was pipetted to the sample solution. Then, the total amount of the spiked solution was completed to 45.0 g with ultrapure water. The ethanol content of PP samples was set to 1.0 % (*w/w*).

2.3.2. Samples employed in ID^4 strategy

DW samples (2.0 g) were spiked to two different concentrations (53.33 μg kg $^{-1}$ and 105.69 μg kg $^{-1}$) and then filled up to 40.0 g with ultrapure water. The ethanol content of DW samples was adjusted to 0.50 % (*w/w*).

The extracted B-PP samples (0.20 g) were added into separate centrifuge tubes and then spiking procedures were applied to two different concentrations (52.25 μg kg $^{-1}$ and 94.67 μg kg $^{-1}$) before the completion to 45.0 g with ultrapure water. In addition, an un-spiked B-PP sample was prepared to perform blank correction. The PP extracts (0.20 g) of Sivri (diluted 31.7 folds), Kıl (diluted 101.2 folds) and Samandağ (diluted 16.5 folds) were taken in separate centrifuge tubes and diluted to 45.0 g with ultrapure water. The reason for the application of different dilutions to each PP sample was to match their capsaicin content to the ID^4 calibration range in order to reach accurate analytical results. The ethanol content for all PP sample solutions was fixed to 1.0 % (*w/w*).

HS samples (0.50 g) were weighed into separate centrifuge tubes and proper amounts of capsaicin standard solution were added onto the samples. After the spiking procedure, the total amount of sample solutions was completed to 1.50 g with acetonitrile to acquire the protein precipitation. Then, 30 s of vortex mixing was applied to obtain homogenous solutions, and the sample solutions were centrifuged for 2.0 min at 3461 *g* to accelerate phase separation. After the centrifugation process, 1.0 g of supernatants was added into separate centrifuge tubes and the total amount of sample solutions was completed to 40.0 g with ultrapure water. The ethanol and acetonitrile content of sample solutions were fixed to 0.50 % (*w/w*) and 1.2 % (*w/w*), respectively.

2.4. The preparation of calibration and sample blends used in the application of ID^4 strategy and the calculation of capsaicin concentration in the spiked and un-spiked samples

The prepared calibration and sample blends were detailly summarized in Table S1 and S2, respectively. The concentration of capsaicin in the spiked B-PP, DW, HS samples and K-PP, S-PP and SA-PP samples were calculated using the formula of ID^4 strategy below [35].

$$w_X = -\frac{\begin{array}{l} w_{X^*-2}\bullet\frac{m_{X^*(X^*Y-2),\ aq}}{m_{Y(X^*Y-2),\ aq}}\bullet w_{X^*-3}\bullet\frac{m_{X^*(X^*Y-3),\ aq}}{m_{Y(X^*Y-3),\ aq}}\bullet(r_{XY}-r_{X^*Y-1})\bullet(r_{X^*Y-2}-r_{X^*Y-3})+ \\ w_{X^*-1}\bullet\frac{m_{X^*(X^*Y-1),\ aq}}{m_{Y(X^*Y-1),\ aq}}\bullet w_{X^*-3}\bullet\frac{m_{X^*(X^*Y-3),\ aq}}{m_{Y(X^*Y-3),\ aq}}\bullet(r_{XY}-r_{X^*Y-2})\bullet(r_{X^*Y-3}-r_{X^*Y-1})+ \\ w_{X^*-1}\bullet\frac{m_{X^*(X^*Y-1),\ aq}}{m_{Y(X^*Y-1),\ aq}}\bullet w_{X^*-2}\bullet\frac{m_{X^*(X^*Y-2),\ aq}}{m_{Y(X^*Y-2),\ aq}}\bullet(r_{XY}-r_{X^*Y-3})\bullet(r_{X^*Y-1}-r_{X^*Y-2}) \end{array}}{\begin{array}{l} w_{X^*-1}\bullet\frac{m_{X^*(X^*Y-1),\ aq}}{m_{Y(X^*Y-1),\ aq}}\bullet(r_{XY}-r_{X^*Y-1})\bullet(r_{X^*Y-2}-r_{X^*Y-3})+ \\ w_{X^*-2}\bullet\frac{m_{X^*(X^*Y-2),\ aq}}{m_{Y(X^*Y-2),\ aq}}\bullet(r_{XY}-r_{X^*Y-2})\bullet(r_{X^*Y-3}-r_{X^*Y-1})+ \\ w_{X^*-3}\bullet\frac{m_{X^*(X^*Y-3),\ aq}}{m_{Y(X^*Y-3),\ aq}}\bullet(r_{XY}-r_{X^*Y-3})\bullet(r_{X^*Y-1}-r_{X^*Y-2}) \end{array}}\frac{m_Y(XY),\ aq}{m_X(XY),\ aq} \quad (1)$$

w_X: The concentration of capsaicin in the sample solution.

w_{X^*-a}: The concentration of capsaicin standard solution (a: 1, 2 or 3).

$m_{X^*(X^*Y-a),aq}$: The mass of capsaicin standard solution in a calibration blend (a: 1, 2 or 3).

$m_{Y(X^*Y-a),aq}$: The mass of capsaicin-d_3 standard solution in a calibration blend (a: 1, 2 or 3).

$m_{X(XY),aq}$: The mass of a sample solution.

$m_{Y(XY),aq}$: The mass of capsaicin-d_3 standard solution in a sample solution.

r_{X^*Y-a}: The isotope ratio obtained from a calibration blend via the peak area of capsaicin dividing by the peak area of capsaicin-d_3. (a: 1, 2 or 3).

r_{XY}: The isotope ratio obtained from a sample blend via the peak area of capsaicin dividing by the peak area of capsaicin-d_3.

2.5. DSPE procedure

A standard/sample solution (8.0 mL) was added into a clean centrifuge tube (containing 30 mg of rGO/Fe_3O_4). Then, pH 7.0 of buffer solution (1.50 mL) was pipetted onto this solution. After the addition of buffer solution, vortex mixing (45 s) was implemented to increase the interactions between the adsorbent and the analyte molecules. After, the magnetic adsorbent was collected to the bottom of tube using an external magnetic field (neodymium magnet) to apply decantation process. After the application of decantation process, a centrifugation process (1.0 min, 3461 *g*) was conducted to remove possible liquid parts adsorbed by the adsorbent in order to increase the DSPE method repeatability. Then, the excess liquid part arising from the standard/sample solution was transferred to the waste container with the help of a micro-pipette. After the removal of excess liquid part, 75 μL of ethanol was added onto the analyte-rich adsorbent and then this mixture was vortexed for 15 s for the desorption process. In the final step, the adsorbent was collected to the bottom of tube and the analyte-rich organic phase (approximately 60 μL) was pipetted to a clean insert vial to send the GC–MS system.

2.6. The synthesis of rGO/Fe_3O_4 nanocomposites

In the first step, Hummer method was used to obtain graphene oxide (GO) from graphite powder. During the synthesis of GO, 2.0 g of graphite powder was dissolved in 46 mL of concentrated sulfuric acid (95 – 97 %) and mixed with the help of a magnetic stirrer for 60 min. After, this solution was taken into an ice bath and then 6.0 g of potassium permanganate was slowly added onto the solution. After the addition of potassium permanganate, 92 mL of ultrapure water was pipetted onto this mixture and then stirred using magnetic field for 60 min. After the mixing process, 280 mL of ultrapure water was added

onto the mixture and 10 mL of peroxide (35 %) was pipetted to finalize the reaction after the addition of ultrapure water. The obtained heterogeneous solution was held in dark medium for 1 day. Next, the decantation process was applied to separate the synthesized GO from the liquid part. After the decantation process, the synthesized GO was washed with HCl (5.0 %), acetone and ultrapure water to remove possible impurities. In the final step, the obtained GO powder was dried in a laboratory oven at 50 °C through a day [44,45].

In the first step of the synthesis of rGO/Fe_3O_4, the GO (250 mg) was added into 250 mL of ultrapure water and stirred for 60 min to attain a homogeneous solution. After, a solution (50 mL) containing iron salts (12.12 g of $Fe(NO_3)_3{\cdot}9H_2O$ and 4.17 g of $FeSO_4{\cdot}7H_2O$) was added onto the GO solution. After, the temperature of this mixture was increased to 80 °C and 30 mL of ammonia solution (25 %) was pipetted onto the mixture. After the addition of ammonia solution, the reaction temperature was increased to 90 °C and then 250 μL of hydrazine was added onto the mixture in order to reduce the GO. The reaction was performed under the nitrogen atmosphere by mixing with the help of a magnetic stirrer for 4.0 h. The synthesized rGO/Fe_3O_4 nanocomposites were dried in a laboratory oven at 50 °C through 1 day after washing processes with ethanol and ultrapure water to remove possible impurities. The synthesized rGO/Fe_3O_4 was characterized in our previous studies [44,46]. The synthesized rGO/Fe_3O_4 nanocomposites were used as an adsorbent for the preconcentration of capsaicin from the DW, HS and PP samples.

3. Results and discussion

All influential parameters belonging to the developed DSPE method were optimized to enhance the signal to noise ratio of analyte in addition to obtaining repeatable results. Three replicates for each variable in an optimization study were performed to calculate mean and standard deviation values.

3.1. The effect of adsorbent type and amount

Five different nanoparticles/nanocomposites (cobalt, nickel, zirconium, Fe_3O_4 nanoparticles and rGO/Fe_3O_4 nanocomposites) were investigated the effect of tested nanoparticles/nanocomposites on the preconcentration yield for the analyte. In the result of this optimization study, Fe_3O_4 nanoparticles and rGO/Fe_3O_4 nanocomposites provided analytical signals while an analytical signal was not obtained from cobalt, nickel and zirconium nanoparticles. As can be seen in Fig. S1, rGO/Fe_3O_4 nanocomposites, which were selected as optimum adsorbent type, given higher analytical signals than Fe_3O_4 nanoparticles. Additionally, rGO in rGO/Fe_3O_4 nanocomposites might enhance the extraction efficiency due to π–π interactions [47] between rGO and aromatic ring in capsaicin molecule [48]. For this reason, rGO/Fe_3O_4 nanocomposites could show higher preconcentration efficiency than Fe_3O_4 nanoparticles.

In addition, the optimization of adsorbent amount was performed in the range of 10 – 50 mg to augment the signal to noise ratio belonging to the analyte since high volume of desorption solvent is required to efficiently desorb the analyte if high amount of adsorbent is used [49]. As can be seen in Fig. 1, a linear increment was observed from 10 mg to 30 mg and a slight decrement in the analytical signals was recorded at 50 mg of nanocomposites. For this reason, 30 mg of rGO/Fe_3O_4 nanocomposites were determined as optimum amount to avoid high amount of adsorbent.

3.2. The effect of buffer pH and volume

Ionic strength of a solution affects the adsorption of an analyte on a adsorbent positively or negatively [50]. For this purpose, various buffer solutions having seven different pH values were examined on the preconcentration efficiency. In addition, an experiment was performed with non-buffered solutions to compare the buffered standard solutions. The solutions buffered with pH 7 provided the highest analytical signals in terms of mean value of peak areas (Fig. S2). Hence, pH 7 of buffer solution was used in further experiments.

Furthermore, volume of buffer solution was optimized to obtain repeatable analytical signals. As can be seen in Fig. S3, the lowest analytical signals were recorded for 0.50 mL of buffer solution (pH 7) while 1.0 mL, 1.5 mL and 2.0 mL of buffer solutions (pH 7) gave almost the same analytical signals. Higher standard deviation value was observed for 1.0 mL of buffer solution than 1.5 mL and 2.0 mL of buffer solutions, therefore; optimum buffer solution volume was selected as 1.5 mL.

3.3. The effect of eluent type and volume

In a DSPE method, eluent type is an important parameter in terms of selectivity, extraction capability and compatibility [51] to reach low detection limits. Hereby, five different organic solvents (methanol, ethanol, isopropyl alcohol, acetonitrile and acetone) were tested to efficiently desorb the analyte from the adsorbent surface. In the result of this optimization study, ethanol was determined as optimum eluent type because of the highest analytical signals (Fig. S4). In addition, ethanol volume was optimized to enhance the signal to noise ratio belonging to the analyte. For this purpose, 75, 100, 150, 200 and 250 μL of eluent volumes were tried and a linear decrement in peak areas was observed while increasing the eluent volume (Fig. 2). Therefore, 75 μL of ethanol, which was enough to reach low detection/quantification limit for the analyte, was chosen as optimum volume.

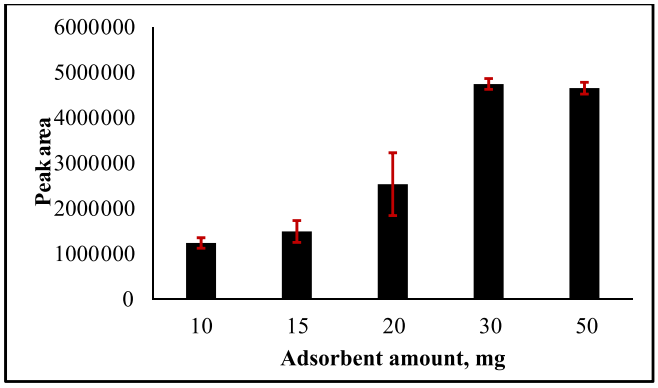

Fig. 1. The effect of adsorbent amount. Conditions: 5.0 mg/kg of capsaicin standard solution, 1.5 mL of pH 7 buffer solution, rGO/Fe_3O_4 used as adsorbent, 45 s of vortex mixing and 100 μL of ethanol as eluent.

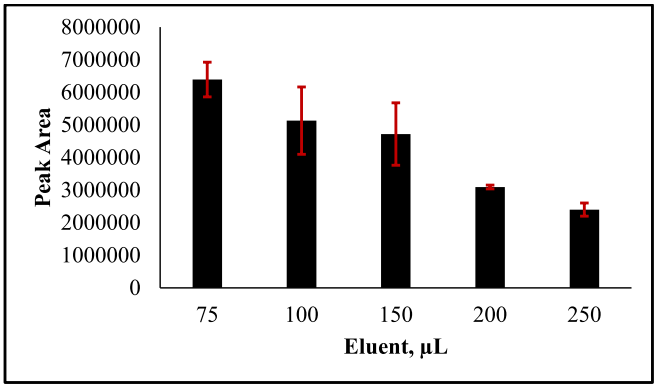

Fig. 2. The effect of eluent volume. Conditions: 5.0 mg/kg of capsaicin standard solution, 30 mg of rGO/Fe_3O_4, 1.5 mL of pH 7 buffer solution, 45 s of vortex mixing and ethanol as eluent.

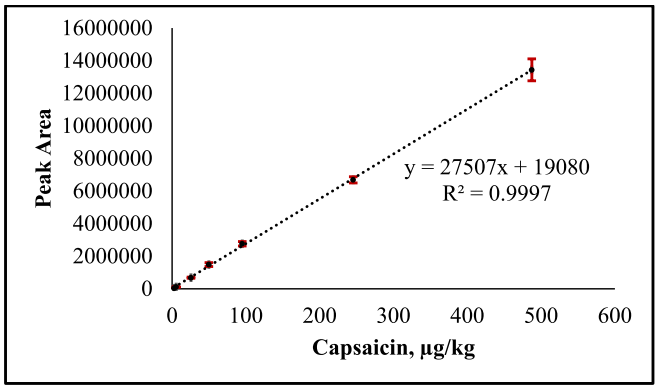

Fig. 3. The calibration plot obtained from the DSPE-GC–MS.

3.4. The effect of vortex mixing period

The adsorption efficiency of an analyte on a nanoparticle/nanocomposite depends on the contact period between the analyte and the adsorbent [52]. Therefore, three different vortex periods between 15 – 45 s were tested and also an experiment was conducted with a non-mixed solution. The peak areas belonging to the analyte linearly increased from 0 s to 45 s (Fig. S5). During this optimization experiment, vortex periods longer than 45 s were not tested to keep short of sample preparation period. In the result of this optimization study, 45 s of vortex period, which was enough to obtain low detection limits, was chosen as optimum one.

3.5. Analytical figures of merit for the developed DSPE-GC–MS method and literature comparison

Aqueous standard solutions of capsaicin prepared between 1.14 µg/kg – 487.35 µg/kg (containing nine different concentrations) were treated with the developed DSPE method and then the analyte-rich phases were sent to the GC–MS system. The calibration plot given in Fig. 3 was constructed between 2.66 µg/kg – 487.35 µg/kg (containing eight different concentrations) with high linearity (coefficient of determination (R^2 = 0.9997)). Limit of detection/quantitation (LOD/LOQ) values were calculated as 0.54/1.80 µg/kg via the formulas below. Additionally, percent relative standard deviations (%RSD) for the lowest concentration in calibration plot (2.66 µg/kg), 25.59 µg/kg and 245.44 µg/kg were recorded as 12.6 % (n = 6), 5.1 % (n = 3) and 2.8 % (n = 3), respectively. System analytical performance values for the developed analytical method are summarized and made comparison with literature findings in Table 1.

$$LOD = 3 \bullet \frac{SD_L}{m}$$

$$LOQ = 10 \bullet \frac{SD_L}{m}$$

SD_L: Standard deviation (n = 6) for the lowest concentration in calibration plot.

m: Calibration plot slope.

The developed analytical method has superiority in terms of LOD values when the developed method is compared to literature findings (except LC-MS/MS and SFDF-LPME-GC–MS). When the developed DSPE-GC–MS method was compared to our previous published study (SFDF-LPME-GC–MS) [7], the developed method has less chemical consumption, less extraction steps (without derivatization step) and chlorinated solvent-free. When the evaluation was made in terms of the dynamic range values, the proposed method provided a similar or lower dynamic range than several literature findings. Furthermore, it is possible that the synthesized nanocomposites can be functionalized to obtain a wider dynamic range by increasing the surface area of the synthesized nanocomposites and the physical/chemical interactions between the analyte and the adsorbent. Additionally, the developed DSPE method can be coupled with the sensitive analytical instrumentation methods such as LC-MS/MS and GC–MS/MS to reach low detection/quantification limits for capsaicin.

Table 1
System analytical performance of DSPE-GC–MS method for the determination of capsaicin and literature comparison.

Method	Dynamic range	LOD[a]	LOQ[b]	Reference
DSPE-GC-MS[c]	2.66 – 487.35 µg/kg	0.54 µg/kg	1.80 µg/kg	Current study
HPLC-UV[d]	–	0.09 mg/kg	0.30 mg/kg	[6]
HPLC-DAD[e]	1 – 200 mg/L	0.018 mg/L	0.062 mg/L	[18]
rGO-SPCE based voltammetry[f]	1.1 – 25.0 µmol/L (~336 – 7635 µg/L)	0.3 µmol/L (~ 92 µg/L)	–	[14]
UAE-GC-MS[g]	0.01 – 1.0 mg/L	0.014 mg/L	0.069 mg/L	[15]
HPLC-UV[d]	250 – 10000 µg/L	73 µg/L	243 µg/L	[19]
HPLC-FD[h]	15 – 1000 µg/L	3 µg/L	11 µg/L	
LC-MS/MS[i]	0.5 – 250 µg/L	0.08 µg/L	0.28 µg/L	
Bare GC based ECL[j]	0.1 – 100 µmol/L (~30.5 – 30541 µg/L)	0.094 µmol/L (~ 28.7 µg/L	–	[53]
GC–MS after HMDS derivatization[k]	25 – 810 µg/L	7 µg/L	25 µg/L	[16]
DLLME-UHPLC-HRMS[l]	–	1.5 µg/L	5.0 µg/L	[54]
SFDF-LPME-GC-MS[m]	1.03 – 507.0 µg/kg	0.33 µg/kg	1.10 µg/kg	[7]
SPME-GC-MS[n]	109 – 1323 µg/L	14 µg/L	69 µg/L	[55]

[a] *LOD: Limit of detection.*
[b] *LOQ: Limit of quantification.*
[c] *DSPE-GC–MS: Dispersive solid phase extraction – gas chromatography–mass spectrometry.*
[d] *HPLC-UV: High performance liquid chromatography–UV detection.*
[e] *HPLC-DAD: High performance liquid chromatography–diode array detector.*
[f] *rGO-SPCE based voltammetry: Reduced graphene oxide screen-printed carbon electrode based voltammetry.*
[g] *UAE-GC–MS: Ultrasound assisted extraction – gas chromatography–mass spectrometry.*
[h] *HPLC-FD: High performance liquid chromatography–fluorescence detector.*
[i] *LC-MS/MS: Liquid chromatography–tandem mass spectrometry.*
[j] *Bare GC based ECL: Bare glassy carbon electrode based electrogenerated chemiluminescence.*
[k] *GC-MS after HMDS: Gas chromatography–mass spectrometry after 1,1,1,3,3,3-hexamethyldisilazane derivatization.*
[l] *DLLME-UHPLC-HRMS: Dispersive liquid–liquid microextraction – ultra–high performance liquid chromatography–high resolution mass spectrometry.*
[m] *SFDF-LPME-GC–MS: Spraying-based fine droplet formation-liquid phase microextraction – gas chromatography–mass spectrometry.*
[n] *SPME-GC–MS: Solid phase microextraction – gas chromatography–mass spectrometry.*

3.6. Matrix-matching recovery experiments

The preparation of all sample solutions used in the matrix-matching recovery experiments was detailly explained in Section 2.3.1. In the calculation of DW recovery results, the calibration plot constructed from the spiked DW II was used to calculate the concentration of spiked DW I and vice versa. The same approach applied to the DW samples was implemented to the HS samples in order to determine recovery results. In the calculation of recovery results for PP samples, the calibration plot built using the spiked B-PP samples was employed to calculate the concentration of capsaicin in the spiked S-PP, K-PP and SA-PP samples.

For PP samples, blank corrections were applied to calculate the recovery results. During the calculation of recovery results, a formula found below was used.

$$\%Recovery = \frac{C_E}{C_T} \bullet 100$$

C_E : Experimental concentration calculated via matrix-matching calibration plot equation using peak area belonging to the analyte obtained from the GC–MS system after the application of DSPE method.

C_T : Spiked concentration.

The obtained recovery results via the matrix-matching calibration method showed that there are different matrix effects on the analyte arising from the HS sample matrices while acceptable recovery results for the DW and PP samples were obtained. In the result of this study, the matrix-matching calibration method can be employed to attain relatively accurate analytical results for the DW and PP samples. On the other hand, ID^4 strategy was combined with the developed DSPE-GC–MS method to obtain more accurate and precise analytical results by eliminating the possible matrix effects and instrumental/experimental errors. The combination of ID^4 strategy and DSPE-GC–MS method and the obtained analytical results by DSPE-GC-ID^4-MS were explained in *Section 3.8 and 3.9* in detail.

3.7. The application of standard addition strategy for the determination of capsaicin content in PP samples

Standard addition calibration approach was used for the calculation of capsaicin content in the PP samples. For this purpose, the individual standard addition calibration plot for each PP sample using the unspiked/spiked PP samples (Fig. S6). The capsaicin content for S-PP, K-PP and SA-PP samples were calculated as 114.5 ± 3.6 mg/kg, 518.4 ± 42.6 mg/kg and 174.9 ± 12.9 mg/kg, respectively. All sample dilutions were taken into account when the calculation of capsaicin content in the PPs were made.

3.8. Equilibration period between capsaicin and capsaicin-d_3

Before the application of ID^4 strategy, the equilibration between analyte and its isotopically enriched/labelled material should be optimized to record stable isotope ratio using peak area values [56]. For this reason, the equilibration period between capsaicin and capsaicin-d_3 was investigated in the range of 0 – 300 min to reach accurate and precise results. In the result of this study, significant changes in the isotope ratio were not observed from 0 min to 300 min (Fig. S7). Therefore, the prepared blends can be directly sent to the GC–MS system without any holding period.

3.9. Combination of ID^4 strategy and DSPE-GC–MS method

After the equilibration period, the ID^4 strategy and the DSPE-GC–MS method were combined with each other to record high accurate and precise analytical results by eliminating matrix effects and reducing uncertainties. The used calibration and sample blends were detailly explained in Section 2.4. After the combination of ID^4 strategy and DSPE-GC–MS method, highly accurate and precise analytical results for the spiked DW I, B-PP and HS II samples were obtained as 98.2 %–99.3 %, 99.7 %–100.7 % and 99.4 %–99.8 % (Table 3), respectively. When the comparison of the obtained recovery results via ID^4 strategy were made with the matrix-matching calibration strategy (Table 2), the implementation of ID^4 strategy provided superior analytical results in terms of accuracy and precision. Moreover, the obtained recovery results show that the matrix effects on the analyte and experimental/instrumental errors reduced after the combination of DSPE-GC–MS method and ID^4 strategy. Additionally, chromatograms belonging to the sample blends of, B-PP, DW I and HS II samples are given in Fig. S8 and the selected ion chromatograms obtained from A*B-2-100a calibration blend (Table S1) can be seen in Fig. S9. As a result of this study, the developed DSPE-GC-ID^4-MS method can be used as a reference analytical method to obtain highly accurate and precise analytical results.

Table 2
Recovery results for DW, PP and HS samples obtained from matrix-matching calibration strategy.

Sample	Spiked concentration, μg/kg	Found concentration ± SD*, μg/kg	Recovery, %	±SD* for Recovery (%)
DW I[a]	8.98	6.94 ± 0.29	77.3	3.2
	25.61	24.40 ± 1.50	95.3	5.9
	48.86	47.74 ± 2.09	97.7	4.3
	94.95	98.72 ± 3.92	104.0	4.1
	251.30	248.80 ± 7.84	99.0	3.1
DW II[b]	9.50	7.87 ± 0.29	82.9	3.1
	25.51	26.02 ± 1.44	102.0	5.7
	50.71	49.07 ± 3.07	96.8	6.1
	96.60	103.05 ± 2.21	106.7	2.3
	258.52	257.96 ± 12.64	99.8	4.9
S-PP[c]	29.40	32.62 ± 2.82	111.0	9.6
	61.11	76.97 ± 2.42	126.0	4.0
	85.58	103.89 ± 12.09	121.4	14.1
	142.78	161.38 ± 5.67	113.0	4.0
K-PP[d]	29.35	32.05 ± 0.45	109.2	1.5
	56.94	63.03 ± 3.80	110.7	6.7
	84.50	85.15 ± 5.50	100.8	6.5
	143.18	148.41 ± 8.57	103.7	6.0
SA-PP[e]	27.95	31.88 ± 2.71	114.1	9.7
	59.86	59.73 ± 5.03	99.8	8.4
	91.40	90.59 ± 5.46	99.1	6.0
	142.43	134.67 ± 6.08	94.5	4.3
HS I[f]	30.18	16.43 ± 2.54	54.5	8.4
	53.35	28.05 ± 2.74	52.6	5.1
	103.26	60.82 ± 2.33	58.9	2.3
	181.78	111.38 ± 7.91	61.3	4.3
	233.21	154.58 ± 14.68	66.3	6.3
HS II[g]	30.88	56.74 ± 2.21	183.8	7.2
	52.65	84.78 ± 1.81	161.0	3.4
	103.82	167.70 ± 16.00	161.5	15.4
	177.59	274.36 ± 11.04	154.5	6.2
	240.69	364.99 ± 9.96	151.6	4.1

* *Standard deviation: n = 3 for DW I, DW II, HS I and HS II, and n = 4 for S-PP, K-PP and SA-PP.*

[a] *DW I: Domestic wastewater I.*
[b] *DW II: Domestic wastewater II.*
[c] *S-PP: Sivri pepper.*
[d] *K-PP: Kıl pepper.*
[e] *SA-PP: Samandağ pepper.*
[f] *HS I: Human saliva I.*
[g] *HS II: Human saliva II.*

Table 3
Recovery results for DW I, B-PP and HS II samples obtained from ID^4 strategy.

Sample	Spiked concentration, μg/kg	Found concentration ± SD*, μg/kg	Recovery %	±SD* for Recovery (%)
DW I[a]	53.33	52.97 ± 1.60	99.3	3.0
	105.69	103.83 ± 2.04	98.2	2.0
B-PP[b]	52.25	52.10 ± 1.76	99.7	3.4
	94.67	95.30 ± 2.29	100.7	2.4
HS II[c]	52.40	52.28 ± 1.78	99.8	3.4
	105.29	104.66 ± 2.13	99.4	2.0

* *Standard deviation for 81 combinations.*

[a] *DW I: Domestic wastewater I.*
[b] *B-PP: Banana pepper.*
[c] *HS II: Human saliva II.*

3.10. The application of ID^4 strategy for the determination of capsaicin content in PP samples

The ID^4 strategy was also applied to PP samples (S, K and SA) in order to determine their capsaicin contents. The used calibration and sample blends during the calculation of capsaicin content of S-PP, K-PP and SA-PP samples are given in Table S1 and Table S2, respectively. After the application of ID^4 strategy, different capsaicin contents (309.5 ± 11.8 mg/kg for S-PP, 873.7 ± 26.7 mg/kg for K-PP and 165.3 ± 5.1 mg/kg for SA-PP) than matrix-matching calibration strategy was recorded. This difference between the applied two strategies might be due to different matrix effects at different spiked concentrations in the matrix matching calibration strategy. The obtained results from the ID^4 strategy provided more reliability results according to the obtained recovery results (Table 3).

4. Conclusion

The rGO/Fe_3O_4 nanocomposites based DSPE method was elaborately optimized to decrease the detection/quantification limit of GC–MS system for the determination of capsaicin in DW, PP and HS samples. Additionally, the developed DSPE-GC–MS method was combined with the ID^4 strategy to obtain high accurate and precise results. Under the optimum chromatographic and DSPE conditions, dynamic range, LOD and LOQ were recorded as 2.66 – 487.35 μg/kg with high coefficient of determination (0.9997), 0.54 μg/kg and 1.80 μg/kg, respectively. Although a relatively wide dynamic range was obtained from the proposed analytical protocol, the adsorption capacity of the used amount of rGO/Fe_3O_4 could limit the ability to achieve a wider linear range. In further studies, surface modification can be performed for the synthesized nanocomposites to increase the surface area of the adsorbent and the physical/chemical interactions between the analyte molecules and the adsorbent in order to obtain a wider dynamic range in addition to lower LOD and LOQ values. Two different strategies (matrix-matching calibration and ID^4) were used to evaluate the applicability and accuracy of DSPE-GC–MS method. Recovery experiments were conducted with the spiked DW, PP and HS samples. Percent recovery results for the spiked DW, PP and HS samples were calculated via matrix-matching calibration strategy in the range of 77.3 %–106.7 %, 94.5 %–126.0 % and 52.6 %–183.8 %, respectively. The combination of DSPE-GC–MS and ID^4 provided excellent recovery results between 98.2 %–100.7 % for the spiked DW, PP and HS samples. When the comparison between the recovery results obtained from the matrix-matching calibration method and the ID^4 strategy was performed, it can easily be seen that the ID^4 strategy gave more reliable results. Therefore, the use of combination of ID^4 strategy and DSPE-GC–MS method is recommended to accurately and precisely determine capsaicin content in the presented samples instead of matrix-matching calibration method or standard addition method. Furthermore, the content of capsaicin in S-PP, K-PP and SA-PP samples were calculated as 114.5 ± 3.6 mg/kg, 518.4 ± 42.6 mg/kg and 174.9 ± 12.9 mg/kg using the standard addition calibration method, respectively. Different results for capsaicin content in S-PP (309.5 ± 11.8 mg/kg), K-PP (873.7 ± 26.7 mg/kg) and SA-PP (165.3 ± 5.1 mg/kg) were obtained from the ID^4 strategy compared to the standard addition calibration method. It is clear that the capsaicin content in PP samples calculated via the ID^4 strategy are more accurate and precise than the standard addition calibration method to be considered the obtained recovery results. In addition, the developed DSPE-GC-ID^4-MS method can be used as a reference analytical method for the determination of capsaicin in DW, PP and HS samples.

CRediT authorship contribution statement

Süleyman Bodur: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sezin Erarpat Bodur:** Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Selim Gürsoy:** Validation, Methodology, Investigation. **Merve Fırat Ayyıldız:** Validation, Methodology, Investigation. **Bedrihan Kartoğlu:** Validation, Methodology, Investigation. **Hilal Akbıyık:** Validation, Methodology, Investigation. **Ömer Tahir Günkara:** Validation, Investigation. **Sezgin Bakırdere:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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Data availability

Data will be made available on request.

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