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An analysis method for determining residual hexane in health functional food products using static headspace gas chromatography

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Abstract A method for analyzing the contents of residual hexane in health functional food products was developed. The dissolving solvents in the health functional food products and the internal standard selected were *N*,*N*-dimethylacetamide and heptane, respectively. The analysis conditions for headspace-gas chromatography/flame ionization detection (HS-GC/FID) and headspace-gas chromatography/mass spectrometry (HS-GC/MS) were determined as 18 mL of headspace volume, 100°C of headspace oven temperature, and 30 min of equilibration time; a Durabond (DB)-624 column was selected for this analysis. To validate this method, which applies *N*,*N*-dimethylacetamide as a dissolving solvent, the limit of detection and limit of quantification (LOQ) values based on the HS-GC/FID and HS-GC/MS analyses results were found to be 0.10, 0.29 and 0.16, 0.47 mg/L, respectively. The recoveries and coefficient of variation (CV) obtained by HS-GC/MS were 96.39–119.86% and 0.04–1.25%, respectively, better than those obtained by HS-GC/FID. By applying the HS-GC/MS method, it was possible to analyze the content of the residual hexane in 60 different types of health functional food products.

Keywords: hexane, residual solvent, HS-GC/MS, HS-GC/FID, health functional foods products

Introduction

There has been a drastic increase recently in the consumption of health functional food products owing to the economic development and increasing health concerns. In Korea, hexanes have been used as a food additive to extract oil and fat components during the production of edible oils and fats and effectively extract or separate functional raw materials in the production of health functional foods. There is a regulation that the hexane used in these processes must be removed to ensure the safety of products and that the allowable residual hexane in products is limited to less than 0.005 g/kg (1). As the available daily intake (ADI) of hexane is limited by Good Manufacturing Practice (GMP) (2), it is desirable to limit this value as much as possible.

A number of studies have been performed previously on the analysis of hexane in food products. A method to analyze the residual hexane in vegetable oil (3), beeswax (4), annatto extracts (5), and drug substance (6) was developed using headspace gas chromatography (GC). A method to analyze the residual hexane in olive oil (7) was developed using headspace-solid phase microextraction (SPME). In addition, a method to analyze the residual hexane in medical supplies (8) was developed using direct injection GC.

A dynamic headspace GC system is usually used for environmental samples (9,10). Although the SPME method shows a fast analysis time and does not use a solvent, it presents a challenge in terms of collecting the highly concentrated hexane (11). The static headspace GC method is simple and effective because the analysis is conducted using a GC-flame ionization detector (FID) (12,13). Thus, the headspace GC method is generally used to analyze the residual hexane in food products (3,12,13).

In Korea, no real method exists with high reproducibility and fast processing ability to analyze the residual hexane in health functional food products. Therefore, in this study, a method to analyze the residual hexane in health functional food products was established using headspace GC and was verified through a validation process. In addition, content analysis was performed for 60 different health functional products distributed in Korea.

Materials and Methods

Reagents Materials used in this study, including hexane, heptane, benzene, ethanol, toluene, benzyl alcohol, dioxane, dimethylforamide, dimethylsulfoxide, and *N*,*N*-dimethylacetamide were purchased from Sigma-Aldrich (St. Louis, MO, USA) and other analytical reagents were also selected.

Instrumentation Equipment used in this study were GC/FID (6890N; Agilent Technologies, Santa Clara, CA, USA), GC/FID (GC-2010; Shimadzu, Tokyo, Japan), GC/MS (QP-2010; Shimadzu), and the headspace-sampler (HS-20; Shimadzu), which was used as a preprocessing device. The gas flow for the analysis using GC/FID was 1.0 mL/min, and the detector and injection temperatures were 260 and 200°C, respectively. In addition, the analysis was implemented in a 10:1 split mode. The GC/MS analysis was implemented at a flow rate of 1.0 mL/min by applying helium as the carrier gas. The analysis was performed at an ion source temperature of 200°C, interface temperature of 200°C, and ionization energy of 70 eV. The analysis mode used in this process was scanning and selected ion monitoring (SIM), in which the selected ions of hexane and heptane were 57, 41, and 43 *m/z* and 43, 71, and 57 *m/z,* respectively. The analyzed peaks were the identified using NIST 27, NITS 147, and WILEY 7 mass spectrometry libraries (National Institute of Standards and Technology, Gaithersburg, MD, USA).

Column For the determining columns, HP-1ms UI (30 m×0.25 mm I.D., 0.5 μm), DB-624 (30 m×0.25 mm I.D., 1.4 μm), HP-PLOT/Q (30 m×0.32 mm I.D., 20 μm), and HP-voc (30 m×0.2 mm I.D., 1.12 μm) were purchased from Agilent Technologies. In the oven conditions for each column, the HP-1ms UI column was maintained at a temperature of 35°C for 10 min and was heated up to 250°C at a rate of 50°C/min. The DB-624 column was maintained at a temperature of 35°C for 10 min and was heated to 40°C at the rate of 15°C/min. It was maintained at 40°C for 10 min and was heated to 235°C at a rate of 50°C/min; it was then maintained at 235°C for 8 min. The HP-PLOT/Q column was heated from 200 to 220°C at a rate of 2°C/min; it was then maintained at 220°C for 3 min. The HP-voc column was maintained at a temperature of 40°C for 10 min and was then heated to 50°C at a rate of 15°C/min; after this, it was heated to 240°C at a rate of 50°C/min.

Headspace sampler A preprocessing condition for the samples was established using the Headspace-sampler (HS-20; Shimadzu). The sample line temperature conditions of 110°C, transfer line temperature of 120°C, pressurizing time, pressure equilibration time, load time, and injection time were configured with an interval of 1 min. For selecting a headspace volume, sunflower seed oil was added to 20 mL vials in aliquots of 1, 2, 5, 10, and 15 mL, and 50 μL of each hexane (100 μg/mL) and heptane (500 μg/mL) sample. In the analysis of these samples, the hexane areas were verified. For determining the oven temperature of the headspace sampler, 2 mL of the sunflower seed oil without hexane was added to a 20-mL vial, and 50 μL of both hexane (100 μg/mL) and heptane (500 μg/mL) was added. The sample was then heated at different oven temperatures of 80, 90, 100, 110, 130, and 150°C for 30 min. In the analysis of this sample, the hexane areas were verified. For configuring the heating time of the headspace sampler, 2 mL of the sunflower seed oil was added to a 20-mL vial, and 50 μL of both hexane (100 μg/mL) and heptane (500 μg/mL) were also added to the vial. Then, the sample was heated at an oven temperature of 100°C for 10, 30, 40, 50, and 60 min. In the analysis of this sample, the hexane areas were verified.

Method validation For obtaining the limit of detection (LOD) and limit of quantification (LOQ), the calibration curves were produced using low concentration samples. Then, the values of $3.3\times\sigma/S$ (gradients of the calibration curves) and $10\times\sigma/S$ were determined to be the LOD and LOQ, respectively, using the average of the standard deviations (SD, σ) of the seven analyses for the medium concentration.

Precision and accuracy were expressed in intra-day of data analyzed three times by spiked concentration of three levels, and expressed in inter-day of data analyzed for 3 day-to-day.

Preparation of standard solutions For the hexane standard solution, a hexane standard product of 1,000 mg was added to a volumetric flask using an analytical balance, and the sample was then stirred in a vortex to dissolve the hexane in *N*,*N*-dimethylacetamide. The final sample was measured to be 10,000 mg/L. For the internal standard solution, a hexane standard product of 40 mg was added to a volumetric flask. Then, the sample was stirred in a vortex to dissolve it in *N*,*N*-dimethylacetamide to obtain 400 mg/L of the sample. The standard solution was stored at room temperature and was used to produce the standard and test solutions for determining the calibration curves. The standard solution for the calibration curves was fabricated as follows: The standard substance solution was produced by diluting the hexane concentration to 40–2,000 mg/L, and 50 μL of the produced standard solution was added into vials to produce a final concentration of 1–50 mg/L. For the internal standard solution, 50 μL of the produced undiluted standard solution (400 mg/L) was added to a vial to produce a final concentration of 10 mg/L.

Sample preparation The samples identified for the analysis of their residual hexane contents were selected from those produced through an extraction process of health functional food products based on the Food Additives Code (1). Sixty different products were purchased to implement the experiments, including various health functional food products such as omega-3 fatty acid containing oils, γ-linolenic acid containing oils, conjugated linoleic acids, lecithin, octacosanol containing oils, lutein, extracts of *Oenothera biennis*, coenzyme Q-10, milk thistle supplements, and phosphatidylserine.

A sample of 0.1 g (powder phase) or 1 mL (oil base) was added to a 20 mL headspace vial to which 50 μL of the internal standard (IS) was added. Then, *N*,*N*-dimethylacetamide was applied to the sample to produce a final volume of 2 mL. The sample was sealed and stirred for 2 min and then used as a test solution.

Statistical analysis All statistical analysis was conducted using the Statistical Analysis System software (SAS User Guide, ver. 6., SAS Institute, Inc., Cary, NC, USA). Mean values and standard deviations were calculated, and Duncan's multiple range tests were implemented. All experiments were performed in triplicate. A probability (*p*) level of 0.05 was considered significant.

Results and Discussion

Optimization of the dissolving solvent and internal standard (IS) As the residual hexane included in health functional food products has low solubility in water (14), organic solvents were used as dilution solvents in these experiments, i.e., dissolution experiments were implemented for ethanol, toluene, benzyl alcohol, dioxane, dimethylforamide, dimethylsulfoxide, and *N*,*N*-dimethylacetamide to obtain the hexane areas, LOD, and LOQ.

For dimethylsulfoxide, ethanol, toluene, benzyl alcohol, and *N*,*N*-dimethylacetamide, the blanks showed no peaks during the analysis. The hexane standard and IS were clearly separated and detected. In the case of dimethylforamide, the heptane used as the IS and the solvent peak were detected in an overlapped manner. In the case of dioxane, a solvent peak was detected between the hexane standard and IS. In addition, the peak areas for hexane were large in ethanol and toluene. Furthermore, benzyl alcohol and *N*,*N*-dimethylacetamide produced large peaks after applying a calibration process using the IS. Rocheleau *et al*. (8) reported the existence of different impurities that presented interferences in an organic solvent analysis process. Urakami *et al*. (14) reported a high peak intensity ratio of hexane to benzyl alcohol and *N*,*N*-dimethylacetamide, similar to the results presented in this study.

In the first stage, the LOD and LOQ values of hexane were produced using ethanol, toluene, benzyl alcohol, and *N*,*N*-dimethylacetamide on the basis of the peak areas and resolutions for each solvent (Table 1). The LOD and LOQ values for benzyl alcohol were low, of the order of 0.09 and 0.28 mg/L, respectively, followed by *N*,*N*-dimethylacetamide with LOD and LOQ values at 0.10 and 0.30 mg/L, respectively. Toluene and ethanol represented the next lowest levels. Oh *et al.* (3) showed that the most proper diluent of residual hexane is ethanol because there are no interferences of the peaks in the sunflower seed oil. This agrees with the results of the present study. However, the LOD and LOQ levels in ethanol were higher than those in the other diluents. Thus, in this study, benzyl alcohol and *N*,*N*-dimethylacetamide were selected as the dissolving solvents for the analysis of residual hexane.

Table 1. Limit of detection (LOD) and limit of quantification (LOQ) of hexane in matrix-medium (unit: mg/L)

Matrix media	Ethanol	Toluene	Benzyl alcohol	Dimethylacetamide
LOD	0.79	0.22	0.09	0.10
LOQ	2.38	0.67	0.28	0.30

In the results of analyzing the linearity of hexane using heptane as an IS, the R^2 value in the calibration curve of hexane using an external standard substance analysis method was 0.9991. Furthermore, the R^2 value in the calibration curve of heptane using an internal standard substance analysis method was 0.9995. Therefore, it was showed that heptane can be used as an internal standard substance for analyzing hexane.

In addition, there were no peaks that disturbed the retention time of hexane in benzyl alcohol or *N*,*N*-dimethylacetamide, which were thus selected as solvents. Moreover, the ratio between the target and reference ions in the hexane and IS was verified under the SIM mode. It was verified that the retention time values in hexane and IS were in agreement (data not shown).

Optimization of the HS condition In the analysis using a headspace sampler, it is difficult to obtain equilibrium between a sample solution and the atmosphere if the headspace volume is either too large or small (3). Thus, in this study, the optimal headspace volume was selected to precisely analyze the residual hexane contents in health functional food products. Sunflower seed oil that does not include hexane was added to 20-mL vials in aliquots of 1, 2, 5, 10, and 15 mL, respectively. Then, 100 μL each of hexane (100 ppm stock) and heptane (500 ppm stock) were added to the vials and equalized at 100°C for 30 min. The smaller was the sample volume used, the larger was the hexane area presented. For example, a sample volume of 1 mL showed the largest hexane area. However, it caused an over-pressurized condition that exceeded the configured pressure of 100 kPa. Thus, the sample volume was selected as 2 mL (headspace volume of 18 mL), which demonstrated excellent reproducibility regardless of the pressure. Oh *et al*. (3) showed that the best liquid sample volume is 5 mL (headspace volume of 15 mL), when 5 μg/g of hexane is applied to the sunflower seed oil. This is different from the results of this study, which showed the optimum hexane concentration to be 100 ppm.

The equilibration time and temperature are very important factors in the analysis based on static headspace GC to obtain accurate and precise quantitative analysis (3). To verify the hexane area according to the oven temperatures of the headspace, 2 mL of the sunflower seed oil was added to a 20 mL vial, and 100 μL each of hexane (100 μg/L) and heptane (500 μg/L) were added to the vial. Then, the experiment was conducted at oven temperatures of 80, 90, 100, 110, 130, and 150°C with an equilibration time of 30 min. Although the hexane peak area increased according to the increase in the oven temperature, the headspace oven temperature was set at 100°C to

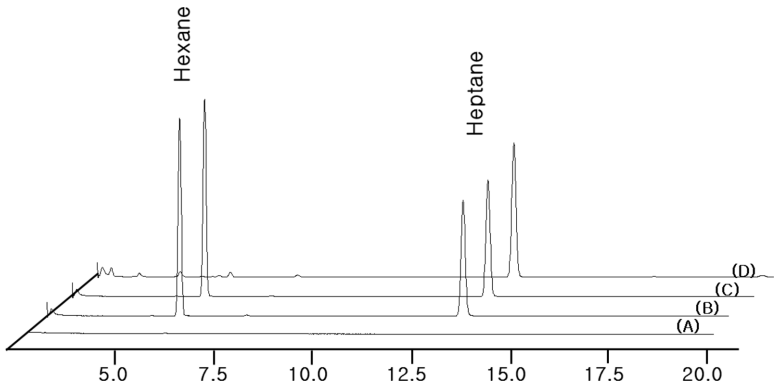

Fig. 1. Specificity of each analyte using HS-GC/MS method with *N,N*-dimethylacetamide solvent. (A) Chromatogram of *N,N*-dimethylacetamide blank. (B) Chromatogram of standard hexane and internal standard (heptane). (C) Chromatogram of standard hexane and internal standard (heptane) spiked sample (sunflower oil). (D) Chromatogram of sample.

Table 2. Detection of limit (LOD) and quantification of limit (LOQ) of *n*-hexane analysis in *N*,*N*-dimethylacetamide (DMA) and benzyl alcohol (BA) using HS-GC/FID and HS-GC/MS

Detector	Dissolving solvent	Slope	Intercept	R^2	LOD (mg/L)	LOQ (mg/L)
HS-GC/FID	DMA	0.14	-0.0280	0.9998	0.10	0.29
	BA	0.12	0.0100	0.9936	0.09	0.28
HS-GC/MS	DMA	0.14	0.0000	0.9996	0.16	0.47
	BA	0.11	-0.0100	0.9862	0.23	0.71

limit the configured pressure to 100 kPa.

For configuring the equilibration time of the headspace sampler, 2 mL of the sunflower seed oil and 100 μL of both hexane (100 ppm stock) and heptane (500 ppm stock) were added to a 20 mL vial, and the sample was maintained at 100°C for 10, 30, 40, 50, and 60 min. No significant hexane peak was produced at any of these equilibration times; therefore, the headspace heating time was configured to 30 min. Although Cheng *et al*. (6) reported the equilibration temperature and time for the concurrent analysis of different solvents as 140°C and 15 min, respectively, it is possible to analyze it at a lower temperature because the boiling point of hexane is 69.0°C (6). Oh *et al*. (3) showed that the best equilibration temperature and time were 100°C and 30 min, respectively, when 5 μg/g of hexane was added to sunflower seed oil, which agrees well with the results of the present study.

Optimization of the GC analysis It is very important to select proper GC columns and detectors to effectively separate hexane from foods. For determining the columns when analyzing hexane, a non-polarity column of HP-1ms UI (30 m×0.25 mm I.D., 0.5 μm), a medium polarity column of DB-624 (30 m×0.25 mm I.D., 1.4 μm), and a column of HP-PLOT/Q (30 m×0.32 mm I.D., 20 μm) coated with solid particles were used to analyze the gas and an HP-voc (30 m×0.2 mm I.D., 1.12 μm) was used to analyze the volatile substances. When comparing these columns, there were differences in the RT values between the hexane and IS. The DB-624 column showed a higher peak than the other columns and so was used to analyze the hexane (data not shown). In addition, Cheng *et al*. (6) showed that the DB-624 column, which has a moderate polarity, has been generally used to determine the residual solvents.

Method validation for hexane analysis To validate the method in this study, the detector used for analyzing hexane was GC/FID and GC/MS and the dissolving solvents were *N*,*N*-dimethylacetamide and benzyl alcohol. The results of these conditions were then compared.

The specificity of the optimized HS-GC/MS method was validated by comparing the blank solvent (*N*,*N*-dimethylacetamide), standard hexane solution, and IS. Figure 1 shows their chromatograms. There were no interference peaks in the blank solvent, and it showed an excellent resolution between the standard hexane solution and IS. Moreover, *m/z* 57 was employed as the quantification ions for hexane and *m/z* 41 and 43 were employed as its respective identification ions; these were evaluated in the spiked samples (sunflower seed oil). All these ions were detected at the same respective retention time in the standard solution and were within 20% of the relative ion intensities specified between the standard and sample spectra.

The hexane standard solution for producing the calibration curves was applied to the experiment at concentrations of 1, 2, 5, 10, 15, and 50 mg/L (Table 2). In terms of the solvent usage for *N*,*N*-dimethylacetamide and benzyl alcohol, the correlation coefficient (R^2) values of the calibration curves of GC/FID were 0.9983 and 0.9936, respectively, and showed excellent linearity. In the case of GC/MS, the values were 0.9996 and 0.9862, respectively, and represented excellent linearity. In addition, on the basis of the inter-day experiment over three days, it was verified that the changes in the slope values of the calibration curves were consistent.

For obtaining the LOD and LOQ values, the calibration curves were

Table 3. Accuracy and precision of *n*-hexane at three different concentration in *N*,*N*-dimethylacetamide (DMA) and benzyl alcohol (BA) using HS-GC/FID and HS-GC/MS

Detector	Dissolving solvent	Concentration	Intra-day		Inter-day	
		(mg/L)	Accuracy (%)	CV[1)] (%)	Accuracy (%)	CV (%)
GC/FID	DMA	2	142.21	9.82	126.56	0.96
		10	94.36	2.29	96.31	1.39
		50	101.42	0.68	100.62	0.93
	BA	5	133.67	6.72	127.89	0.81
		10	85.38	16.00	95.04	1.11
		50	100.86	0.08	100.81	0.74
GC/MS	DMA	2	119.86	0.73	118.90	0.81
		10	96.39	0.43	96.86	1.25
		50	100.50	0.04	100.46	0.11
	BA	2	109.46	11.59	115.63	1.10
		10	96.91	1.32	95.96	0.56
		50	99.91	0.30	100.14	0.19

[1)]CV, coefficient of variation.

produced using low concentration samples. Then, the values of 3.3×σ/S (gradients of the calibration curves) and 10×σ/S were determined to be the LOD and LOQ, respectively, using the average of the standard deviations (SD, σ) of the seven times analyses for the medium concentration. In the results of the analysis using GC/FID (Table 2), the values of the LOD and LOQ of the *N*,*N*-dimethylacetamide solvent were 0.10 and 0.29 mg/L, respectively, and the benzyl alcohol solvent showed similar values as 0.09 and 0.28 mg/L, respectively. In the results of the analysis using GC/MS (Table 2), the values of LOD and LOQ for the *N*,*N*-dimethylacetamide solvent were 0.16 and 0.47 mg/L, respectively, and these values for the benzyl alcohol solvent were 0.23 and 0.71 mg/L, respectively. The benzyl alcohol solvent showed higher values than the *N*,*N*-dimethylacetamide solvent. The residual hexane contents for all the detectors and dissolving solvents used in the experiment showed lower values than the Korean residue limits in foods (1). Ramos (15) showed that for the residual hexane contents in five drug substances analyzed using HS-GC/FID, the values of LOD and LOQ were 3.85 and 8.00 ppm, respectively. These are higher values than the results obtained in this study.

For verifying the precision and accuracy of the experiment, an intra-day assay analysis, which tests the same sample three times a day under the same sample conditions, and an inter-day assay, which tests the sample for three days, were performed (Table 3). In the results of the triplicate analyses for each solvent with a concentration of 2, 10, and 50 mg/L, respectively, using HS-GC/FID, the recoveries and CV of the *N*,*N*-dimethylacetamide solvent were 94.4–142.2 and 0.7–9.8%, respectively, and those of the benzyl alcohol solvent were 85.4–133.7 and 0.1–16.0%, respectively. In the case of the analyses using HS-GC/MS, the recoveries and CV of the *N*,*N*-dimethylacetamide solvent were 96.39–119.86 and 0.04–1.25%, respectively, and those of the benzyl alcohol solvent were 95.96–115.63 and 0.19–11.6%, respectively. Antolín *et al*. (4) reported mean recoveries±SD of 92.3±15.4–108.6±15.8% for the analysis of residual *n*-hexane in the active ingredient of beeswax using HS-GC/FID. Ramos (15) reported that the recoveries for the analysis of the residual hexane in five drug substances using HS-GC equipped with FID were 98.5–103.2% for the range of 463–831 ppm.

In the method validation, HS-GC/FID and HS-GC/MS were suitable for the experiment. In addition, both the tested dissolving solvents were found to be suitable. However, the benzyl alcohol solvent did not readily dissolve the sample because of its viscosity. Thus, *N*,*N*-dimethylacetamide solvent was chosen as the optimal solvent.

Monitoring of health functional food products The optimized and validated HS-GC/MS method was used to analyze the residual hexane contents in 60 different health functional food products by considering the characteristics of various mixtures in these products (Table 4).

On the basis of the analyses of residual hexane, the omega-3-fatty-acid-containing oils in these health functional food products were present up to 0.66±0.02 mg/kg (average 0.45±0.35 mg/kg, *n*=11), the γ-linolenic-acid-containing oils were measured at ND–0.44±0.01 mg/kg (average 0.39±0.21 mg/kg, *n*=8), and the conjugated linoleic acid was measured at ND–1.09±0.00 mg/kg (average 0.74±0.73 mg/kg, *n*=11). The other seven products, including lecithin, produced results of the order of ND–0.85±0.33 mg/kg. Oh *et al*. (3) reported that the analyses of the residual hexane in 87 commercial vegetable oils using HS-GC/FID showed values of the order of trace–2.8 mg/kg. Ito *et al*. (5) reported that the residual hexane in six commercial bixin products using HS-GC/FID was of the order of ND–0.7 ppm. In this study, hexane was measured at less than 5 mg/kg, which complies with the maximum residue limits regulated by the Food Additives Code (1).

Although the residue limit of the residual hexane in health functional food products in Korea are required to be regulated at less than 0.005 g/kg, there are few methods of analyzing hexane with high reproducibility and fast performance. Thus, in this study, a

Table 4. Residual hexane contents detected in commercial functional health food products

Samples	No. of samples	Hexane contents (mg/kg)[1)]	
		Range	Average
Omega-3 fatty acid containing oils	11	ND[2)]–0.66±0.02	0.45±0.35
γ-Linolenic acid containing oils	8	ND–0.44±0.01	0.39±0.21
Conjugated linoleic acid	11	ND–1.09±0.00	0.74±0.73
Lecithin	2	0.27±0.04–0.85±0.33	0.56±0.41
Octacosanol containing oils	4	ND–0.54±0.05	0.31±0.15
Lutein	8	ND–0.50±0.06	0.38±0.28
Extract of *Oenothera biennis*	2	ND–0.26±0.03	0.26±0.17
Coenzyme Q-10	5	ND–0.12±0.04	0.08±0.05
Milk thistle supplement	7	ND	ND
Phosphatidylserine	2	0.72±0.02–0.81±0.51	0.76±0.06
Total	60		

[1)]Data are mean±SD (*n*=3).
[2)]ND, not detected and/or less than LOD.

method of analyzing the residual hexane in health functional food products using HS-GC/FID or HS-GC/MS was established and validated. Using the established HS-GC/MS method, the residual hexane contents in 60 different health functional food products, which represent various matrix characteristics, were successfully analyzed. Therefore, the method developed in this study can contribute to the analysis of the residual hexane in health functional food products.

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Disclosure The authors declare no conflict of interest.

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