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Headspace Analysis for Residual Hexane in Vegetable Oil

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Abstract To enforce the maximum residue limit for residual hexane (0.005 g/kg) in commercially available Korean vegetable oil, convenient and accurate quantification methods were investigated. Using dual surrogate standards, pentane and heptane were dissolved in ethanol, and then added to hexane-free sunflower oil for setting up the calibration curve. Gas Chromatograph-Flame Ionization Detector with a porous layer open tubular column, indicated good chromatographic separation of hexane from other inhibiting matrix components. The lowest calibration level was 0.5 μg/g, not exceeding a relative standard deviation of 10% (RSD%), and 1.0 μg/g not exceeding a deviation of 22% RSD% using heptane as an internal standard for the Static headspace analysis by using a headspace auto-sampler and manual injection, respectively. The residual hexane was detected in nine of the samples among 87 vegetable oil samples purchased on the local market.

Keywords: hexane, static headspace analysis, porous layer open tubular column, vegetable oil, monitoring

Introduction

There are a couple of methods to make vegetable oil in the industry, depending on the characteristics of the raw material. The solvent extraction method is one of the most common methods used to extract vegetable oil from plant material like soybeans, flaxseed, peanuts, safflower seed, corn-germ, cottonseed, rapeseed, rice bran, sesame, sunflower seed, which contain approximately 20% oil content. Various solvents like hexane, acetone, ethyl acetate and others can also be used for extraction. However, only hexane is allowed for vegetable oil extraction in Korea. The maximum residue level (MRL) for hexane is 0.005 g/kg, which is the legally permissible concentration in vegetable oil in Korea (1).

The extracted unfinished oil goes through various processes like degumming, neutralization, decolorization, and deodorization (2). During this process, the residual hexane is decreased dramatically to the concentration under MRL. A major compound of hexane, *n*-Hexane, has a low acute toxicity level for adult rats by oral administration or inhalation.

Exposure to *n*-hexane concentrations in the air varies from 106 - 8800 mg/m^3 (30 - 2500 mg/L) and has been associated with neuropathy. However, very little information is available on the acute toxicity of *n*-hexane in humans (3). Therefore, it is desirable to reduce the intake amount as low as possible.

The dynamic headspace method is well-known as the best for the environmental sample like water containing trace amount of target materials. However, most dynamic headspace methods require a specially designed injectable apparatus to release adsorbed compounds and inject it directly into a gas chromatograph (GC) system (4, 5). Therefore, it is hardly used in a widespread manner. In order to enforce quality assurance of vegetable oil production by quantifying residual hexane, the methods used must be simple, involving minimal costs, and applying easily available equipment (6). The static headspace - a simple method - is economical because it could be completed even with a GC-flame ionization detector (FID) and gas tight syringe, therefore, fulfilling requirements for the official analysis method. That's why the static headspace method has been widely used for the analysis of volatile compounds like vegetable oils and other matrices (7-10). Even though the static headspace falls behind the dynamic headspace, the MRL 0.005 g/kg is quite high enough to be matched by the static headspace (11). However, no scientific optimization study for a variety of factors have been systematically studied so far in order to accurately and precisely quantify residual hexane in vegetable oils using static headspace.

In this study, many factors that affect the quantification results of residual hexane were optimized for the static headspace analysis. This method was applied in the monitoring of 87 vegetable oil samples collected in Korea.

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Materials and Methods

Reagents The 99.5% purity of hexane and pentane were purchased from Supelco (Bellefonte, PA, USA) while the heptane was purchased from Chem Service (Wester Chester, PA, USA). *N,N*-Dimethylformamide (DMF; 99%) was purchased from YAKURI Pure Chemical Corp. (Japan). The dimethyl sulfoxide was ordered from Junsei Chemical Corp. (Tokyo, Japan). Sunflower oil (Wellga, Yangsan, Korea) as a blank oil was purchased from a local market in Suwon, Korea. Mineral oil as another blank oil, and other solvents were obtained from Sigma-Aldrich (St. Louis, MO, USA)

Headspace sampling, GC-FID and GC-MSD analysis The static headspace analyses were performed by an Agilent 7694 headspace sampler (Agilent, Palo Alto, CA, USA) and manual injection. The headspace was analyzed by an Agilent 6890 GC-FID with a CP-PoraBOND Q porous

layer open tubular (PLOT) column (10 m × 250 μm I.D.) purchased from Varian (Palo Alto, CA, USA). The carrier gas utilized was nitrogen with 1.0 mL/min. The column oven temperature was held constant at 35°C for 3 min, increasing to 200°C with 20°C/min. The split injection port temperature was 220°C and the split ratio was 15:1. For the GC-MSD analysis, an Agilent 6890 GC coupled to Agilent 5973 mass spectrometric detector (MSD) was used. Helium was the gas used as the mobile phase with 1.0 mL/min. The DB-5 capillary column (50 m × 0.25 μm I.D., Agilent J&W Scientific, Folsom, CA, USA) with a film thickness of 0.25 μm, was utilized for GC-MSD analysis. The column oven temperature was held at 40°C for 1 min, increasing to 250°C with 20°C/min. The split injection port temperature was 260°C, whereas the split ratio was 50:1.

Regarding the headspace auto-sampler conditions, the sample oven, valve and transfer line temperatures were set at 100, 110 and 120°C, respectively. The time for sample equilibration, loop fill, loop equilibration, sample injection were 30, 0.5, 0.3 and 0.5 minutes, respectively. The agitation mode did not apply to the sample. For manual static headspace injection, 500 μL of headspace were injected using a 1 mL size gas tight syringe.

The equilibrium of the sample and headspace in the vial was achieved in the sea sand bath located in a heating oven. When the sampling was performed using a gas tight syringe, the same volume (500 μL) of air was injected into the headspace vial. After waiting 10 seconds as the needle placing inside of the headspace vial, the headspace gas were taken for GC injection. All experiments were performed at least three times.

Standard sample preparation for matrix matched quantification analysis To select the blank sample matrix, seven oil matrices - soybean, corn oil, cottonseed oil, sunflower seed oil, salad oil, olive oil and mineral oil - were tested. Among them, the sunflower oil and the mineral oil showed up clean on the chromatogram recording using static headspace, which meant there were no volatile impurities to analyze. Therefore, sunflower oil was used as a blank sample matrix in this study. The diluents for hexane and the surrogates are important for quantifying hexane in oil matrices. The diluents should be well mixed with a sample matrix. Then the target and surrogates could be distributed evenly.

In order to prepare the stock and working standard sample, mineral oil, sunflower oil, 1,2-dichloroethane, 1, 1,1-trichloroethane, isopropyl acetate, *n*-butanal, 1,4-dioxane, 4-methyl-2-pentane, ethanol, DMF and DMSO were tested as the diluent for hexane. It should also not interfere with the target and surrogate peak on the chromatogram recording. Ethanol was selected as the diluent for hexane and surrogates.

Standard mixtures with concentrations in the range of 0.5-5 μg/mL (0.5, 1.0, 2.0 and 5.0 μg/mL) were analyzed to make a calibration curve for quantification. The ethanol-based stock sample containing hexane (100 μg/mL) was added to the sunflower oil to make the working standard mixtures.

Vegetable oil samples for residual hexane monitoring In four major Korea cities (Seoul, Busan, Gwangju and Jeju), 87 vegetable oil samples were collected (Table 1). All samples were in liquid form except for margarine, which was from vegetable sources. The samples were analyzed within one month after being purchased. Pentane and heptane were adopted as surrogate internal standards to reduce the risk of peak overlapping with sample matrix peaks.

Results and Discussion

Capillary column selection for hexane analysis by GC The retention time of hexane on the DB-5 capillary column was approximately 2.2 min. Its peak width was quite large when the analysis was performed by manual static headspace injection. However, the retention time of hexane on the 10 m PLOT column by using manual headspace was approximately 9 min. Also, the retention times of surrogates, pentane and heptane, were 7.7 and 10.2 minutes, respectively. This type of column is well-known for being suitable for light gas analysis. Therefore, it was proved to be able to replace packed and micro-packed columns (12). Thereafter, the headspace analysis was performed with the PLOT column.

Selection of diluent for preparing standard stock sample Among the diluent candidates, sunflower oil and other solvents were tested with spiked hexane. There were no analyzable peaks on the sunflower oil, except for hexane. However, the high viscosity of the sunflower oil made it difficult to prepare a homogenized-stock standard solution. Most of the organic solvents showed the inhibition of hexane and surrogate peaks. While the DMF peak was overlapped with the heptane peak (which is the one of surrogates), ethanol proved the best diluent for hexane and surrogates because the peak did not disturb the target peaks on the PLOT column. Even though ethanol is not supposed to be mixed well with hexane, diluted hexane (100 μg/mL) would distribute evenly with ethanol. The RSD% for hexane (1 μg/g) peaked from five sunflower oil samples was 4.4 and 12.1% by headspace

Table 1. The list of vegetable oil samples for residual hexane monitoring

Variety of sample	Number of samples with different brand names
Sesame oil	23
Olive oil	20
Soybean oil	8
Perilla oil	7
Corn oil	6
Margarine	4
Red pepper seed oil	4
Mixed oil	3
Salad oil	3
Sunflower oil	3
Colza oil (Rape seed oil)	2
Rice-bran oil	2
Chinese pepper oil	1
Safflower oil	1

sampler and manual injection, respectively.

Optimization of headspace volume In a closed vessel, the volatile sample components must be present in the atmosphere of the vial proportional to the concentration of the liquid sample components (13). If the headspace volume is too small or large, it is hard to equilibrate in liquid and atmosphere phases evenly. Therefore, the optimum headspace volume for hexane analysis was tested on the sunflower oil sample.

The headspace sampler vial (20 mL) was filled with various levels (5, 10, 15, 17 and 19 mL) of sunflower oil containing 5 μg/g of hexane. The hexane peak area according to various headspace volume was shown in Fig. 1. The hexane peak area was decreased if the headspace volume was over or less than 15 mL. Therefore, the headspace 15 mL of 20 mL size vial was selected as the optimum headspace volume for analyzing hexane in sunflower oil. For the real analysis, the vegetable oil sample weight was approximately 5 g of the liquid sample, which gave 15 mL of headspace in the 20 mL size vial.

Optimization of equilibrium temperature and time The equilibrium time and temperature are key factors deciding precise and accurate quantification using static headspace analysis. The hexane peak area, according to various heating periods and temperatures, were examined from 10 to 60 min at 100°C and from 40 to 120°C (with 30 min equilibrium time). The hexane peak area reached to the acme and was sustained after 30 min at 100°C (Fig. 2). The equilibrium temperature of 100°C showed up on the top and flat points of the area curve of the hexane peak (with 30 min equilibrium time) (Fig. 3). These results were verified for sunflower oil containing 5 μg/g of hexane using headspace auto-sampler and manual injection.

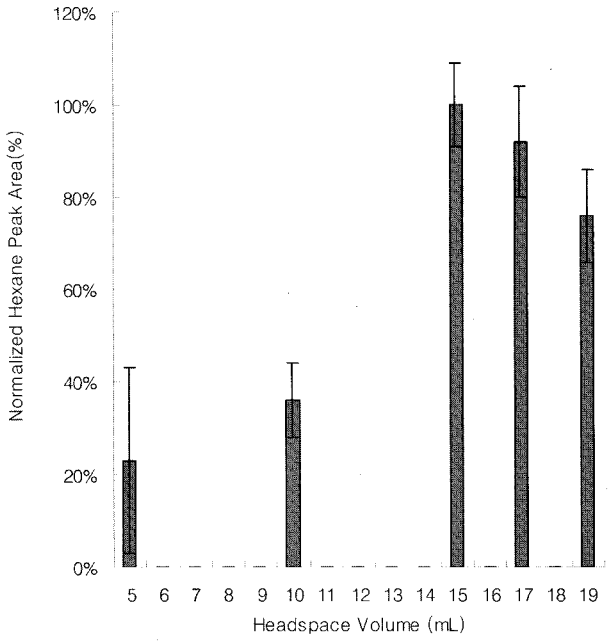

Fig. 1. Normalized hexane peak area according to various headspace volume of 20 mL size vial filled with sunflower oil equilibrated at 100°C for 30 min (5 μg/g of hexane in the sample).

Matrix matched calibration curves by headspace auto-sampler and manual injection The calibration curve was established using internal standard methods with pentane and heptane as the surrogate peaks. The surrogate solution of 100 μL of 500 μg/mL of ethanol, was added to hexane-free sunflower oil (5 g) to make the final 10 μg/g concen-tration. The calibration curve obtained by the headspace auto-sampler and manual injection are shown in Fig. 4 and 5, respectively. Each point of calibration curve was obtained after more than five injections, which were from 121 to 2.9% of RSD%.

The worst reproducibility (at RSD% 121 and 55%), was acquired using 0.5 μg/g of hexane and surrogate heptane and pentane respectively, by manual injection. The erroneous precision of the 0.5 μg/g point of hexane most likely is due to long injection times using a small sample amount. Therefore, when the hexane peak's band broadens, it might summon up inaccurate and variable integration.

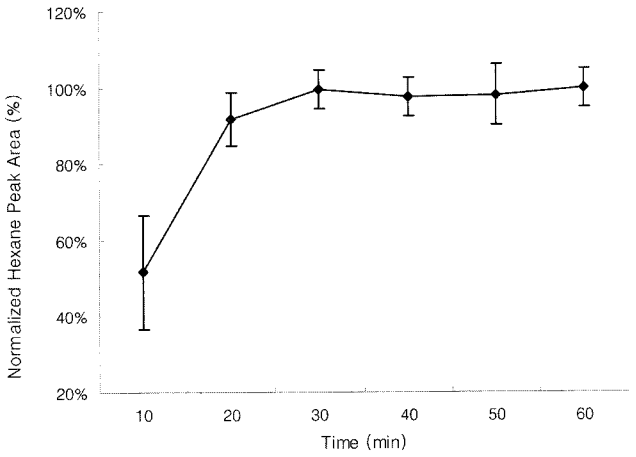

Fig. 2. Normalized hexane peak area according to various headspace equilibrium time at 100°C with 15 mL headspace volume of 20 mL size vial filled with sunflower oil (2 μg/g of hexane in the sample).

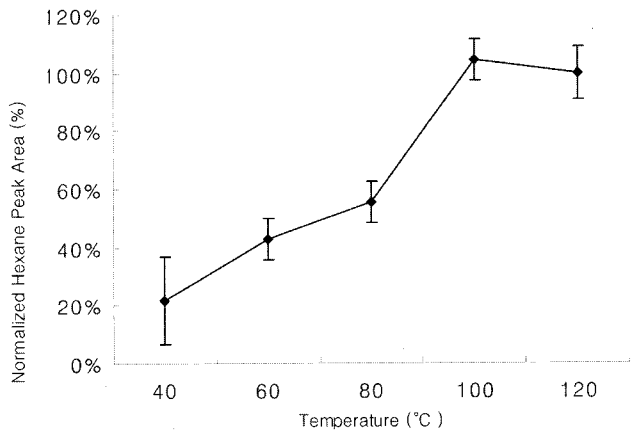

Fig. 3. Normalized hexane peak area according to various headspace equilibrium temperature for 30 min equilibrium time with 15 mL headspace volume of 20 mL size vial filled with sunflower oil (2 μg/g of hexane in the sample).

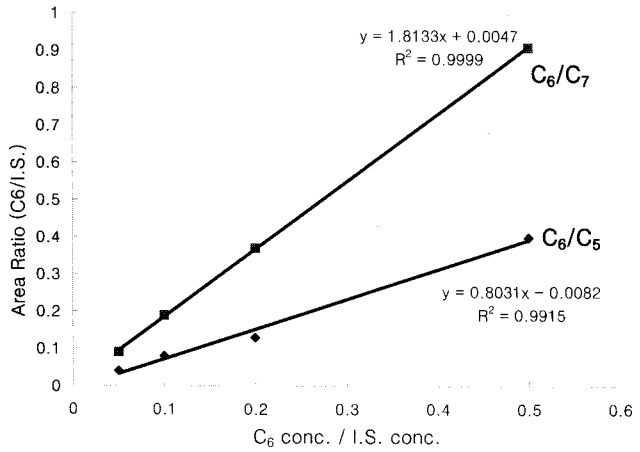

Fig. 4. The calibration curve for hexane analysis by headspace sampler (The concentration of surrogate, pentane and heptane in sunflower oil was 10 μg/g each.).

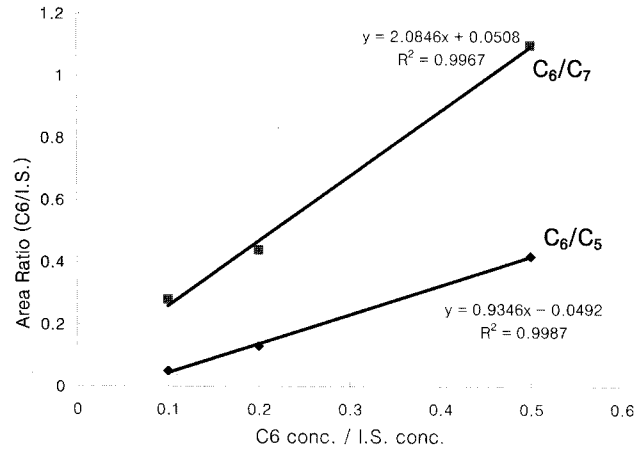

Fig. 5. The calibration curve for hexane analysis by manual headspace injection (The concentration of surrogate, pentane and heptane in sunflower oil was 10 μg/g each.)

The lowest calibration level was fixed as 1 μg/g of hexane in the sunflower oil by manual headspace injection. The correlation coefficient (R^2) was 0.997 and the RSD% of each point did not exceed 22% with the surrogate heptane. However, when pentane was used as a surrogate for the manual headspace injection, the correlation coefficient was 0.999 and the RSD% was 61% for hexane 1 μg/g level (Table 2). The reason for the failing precision with surrogate pentane was due to the large solvent peak tailing caused the inaccurate integration of the hexane peak. For the calibration curve (obtained by the headspace auto-sampler), all points in the calibration curve showed good precision with low RSD% (not exceeding 10%). This was due to the precise valve injection system could make the very narrow sample band on the GC capillary column. Therefore, the lowest calibration level of hexane was set as 0.5 μg/g by headspace auto-sampler.

Determination of residual hexane in various vegetable oil products A total of 87 samples were analyzed using a static headspace auto-sampler and manual injection system for residual hexane monitoring. The detected hexane peak was confirmed by GC-MSD. The hexane was detected in the nine samples with concentrations ranging from trace to 2.8 and from trace to 11.2 μg/g by headspace auto-sampler and manual injection method, respectively (Table 3). The sample in which the hexane concentration was under 1.0 μg/g, was marked using the "trace" by manual injection method because the lowest calibration level of manual injection method was 1.0 μg/g.

The detected amounts of hexane in liquid samples over 1.0 μg/g were identical within 20% of RSD% between the headspace auto-sampler and manual injection. However the results for margarine, which is a unique solid sample in this study, were substantially different depending on the methods, headspace auto-sampler and manual injection method used.

One of the major reasons for the large differences between the auto- and manual-analysis results for the

Table 3. The amount of residual hexane in the samples among the 87 vegetable oil samples analyzed using static headspace auto-sampler and manual-injection method

Sample Name (First letter of the company name)	Residual amount (μg/g) by Headspace auto-sampler	Residual amount (μg/g) by Manual headspace injection
Soybean oil (J)	1.4	1.7
Soybean oil (D)	0.7	Trace*
Sesame oil (C)	0.9	Trace*
Chinese pepper oil (M)	1.2	1.2
Rice-bran oil (S)	0.7	Trace*
Corn oil (S)	0.6	Trace*
Sesame oil (S)	Trace*	Trace*
Margarine No. 1 (O)	2.8	6.0
Margarine No. 2 (O)	2.2	11.2

*Trace means the concentration level is in between lowest calibration level and method detection limit.

Table 2. Relative standard deviation(%) of relative peak area for hexane with internal standards pentane and heptane in each concentration point of standard curve

Concentration of hexane in vegetable oil (μg/g)*	The samples injected by headspace auto-sampler		The samples injected by gas tight syringe manually	
	C_6/C_5	C_6/C_7	C_6/C_5	C_6/C_7
0.5	10	10	55	120
1.0	6	4	61	17
0.2	2	6	19	12
0.5	8	5	25	22

*The amount of internal standard was 10 μg/g.

margarine samples, might be due to the poor homogeneity of surrogate standards in the solid matrix. Moreover, there was the large possibility of allowing the volatile pentane and heptane to be lost during the melting process of margarine too. The external calibration without surrogate internal standards showed reduced discrepancy from 73 to 26% and from 134 to 100% between auto- and manual-analysis for two margarine samples.

The result indicates that the surrogate standards made the result more inaccurate for solid vegetable oils like margarine. For the solid matrix samples, other analysis methods should be considered.

In conclusion, the static auto- and manual-headspace analysis methods may be a suitable tools for the quantitative analysis method of residual hexane in vegetable liquid oil samples for the purpose of enforcing Korean MRL 0.005 g/kg. However, the manual static headspace method could not precisely measure the amount of residual hexane under 1.0 μg/g of hexane content due to higher calibration levels.

The PLOT column should be adapted for the manual-static headspace method to compensate for the broadened peak width as a result of long injection times using a high volume gas tight syringe. Among the 87 vegetable oil samples collected in four major cities in Korea, nine samples were found containing residual hexane.

The residual hexane amounts of the seven liquid oil samples were under 1.7 μg/g. This is the highest amount quantified using the manual headspace injection method. The quantified amount of residual hexane from the two margarine samples should be reanalyzed using other suitable methods optimized for solid sampling.

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