Syntax Literate: Jurnal Ilmiah Indonesia p–ISSN: 2541-0849 e-ISSN: 2548-1398
Vol. 9, No. 12, Desember 2024

IDENTIFICATION OF VOLATILE COMPOUNDS IN SOY MILK DRINKS USING SOLID PHASE MICROEXTRACTION-GAS CHROMATOGRAPHY-MASS SPECTROMETRY (SPME-GC-MS)

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Abstrack

The distinctive aroma produced by soy milk is thought to come from volatile compounds. The development and use of soy milk derivative products require a deep understanding and characterization of the flavor profile. The aim of this study was to identify volatile flavoring compounds found in soy milk made from imported and local soybeans. Milk samples extracted using the headspace-SPME method at a temperature of 60°C for 20 minutes, and analyzed using GC-MS. The results obtained, detected as many as 23 major volatile compounds (relative levels> 1%) in both samples. The difference in total volatiles produced between the two samples showed that there was a significant difference (P <0.05), while the difference in relative levels between compounds that often appear such as pentanal in both samples showed insignificant results (P> 0.05). It can be concluded that the origin of soybeans between imported and local soy milk influences the total amount of volatile compounds identified, however, it does not significantly influence the flavour and aroma characteristics caused by the pentanal compound.

Keywords: Volatile Compounds; Soy Milk; SPME; GC-MS

Introduction

Soybeans are the most important seeds in the world because they are widely marketed in the form of original seeds and in the form of by-products whose processing is widely used in various industries. Soybeans are famous for their high protein content of 40% to 41%. Apart from its high protein content, soybeans also contain 35% carbohydrates, 8% to 24% oil, and 5% ash (Balasubramanian & Panigrahi, 2011)(Mendoza-Avendaño et al., 2022). Many people process soybeans into useful soybean derivative products, one of the products that is often processed is soy milk. Soy milk has almost the same amino acid composition as cow's milk, so soy milk can be used as an alternative drink to replace cow's milk for some people who are allergic to lactose and animal protein. In addition, milk from animals also contains animal fat that can increase cholesterol so it is not good to consume it excessively, especially for people who have certain diseases (Mawarni et al., 2018). The development of plant-based milk alternatives such as soy milk has also attracted great interest in diets with a focus on environmentally friendly or balanced nutrition to support health (Pointke et al., 2022). So that pure soy milk products without a mixture of other ingredients (preservatives) are really needed for the community.

Breaking the soybean shells during processing into soy milk products will activate the presence of the lipoxygenase enzyme. This enzyme reacts with fat and produces

How to cite: Sari, A. K. N. W., & Hanwar, D. (2024). Identification of Volatile Compounds in Soy Milk Drinks Using Solid Phase Microextraction-Gas Chromatography-Mass Spectrometry (SPME-GC-MS). *Syntax Literate. (9)12.* http://dx.doi.org/10.36418/syntax-literate.v9i12
E-ISSN: 2548-1398

volatile compounds such as glucosides (causing a chalky taste), saponins (causing bitterness), and the main compound in soy milk, hexanal, which can cause a beany flavor. The process of making soy milk will activate these volatile compounds (Yulifianti et al., 2020). Volatile compounds are not easily removed, this is proven by research conducted by Fuchsmann, *et al*, (2020), which shows that volatile compounds are still detected in human urine after consuming soy-based drinks. On the other hand, volatile compounds such as hexanal can also be used to extend the shelf life of vegetable products because they have good antimicrobial and antifungal properties against pathogenic microorganisms (Fadida et al., 2015). This is reinforced by research that states that only 5% to 10% of volatile compounds contribute to the overall aroma. In addition, the development and use of soybean derivative products requires an in-depth understanding and characterization of the flavor profile as a result of different processing procedures, information is still very lacking. found in previous literature (Achouri et al., 2006). The method commonly used to detect volatile compounds is Solid Phase Microextraction-Gas Chromatography -Mass Spectrometry (SPME-GC-MS) (Al-Rubaye et al., 2017)

The Solid Phase Microextraction (SPME) technique is a solid extraction method for gas chromatography analysis using the principle of polymer-coated fibers used as an extraction tool. The extracted analytes can be directly analyzed using gas chromatography without the use of solvents that can contaminate the sample (Badulla et al., 2021). This SPME technique involves the adsorption of volatile compounds present in a system (for example a food matrix) onto adsorbent fibers (Achouri et al., 2006). This method has the advantage that its working principle is fast, simple, solvent free and sensitive for analyte extraction. The extraction method using solid phase microextraction (SPME) in gas chromatography is influenced by the extraction time and temperature to obtain optimal extract results (Hanwar et al., 2023). Analysis using gas chromatography (GC) coupled with mass spectrometry (GC-MS) will yield additional information about each separated compound such as molecular mass, elemental composition (when high resolution mass spectrometry is used), functional groups, and in certain cases, molecular geometry and spatial isomerism are also shown. This includes the factors that most determine and influence biogenic volatile compounds in GC–MS analysis (Salih & Celikbicak, 2012). GC-MS combined with SPME has proven to be a useful tool in identifying volatile compounds of various types of food such as fruit, tea, plants and meat products. It is also used to compare the relative amounts of volatile compounds between samples when using the same analytical procedure (Zhang et al., 2021)

The composition profile of volatile compounds in soy milk can vary. This is due to several factors including differences in soybean origin, soybean varieties, soil conditions, climate and cultivation methods which will influence the color, chemical composition, flavor and sensory attributes of the soy milk produced (Adawiyah et al., 2018). Therefore, this study focuses on the comparison of volatile compounds in soy milk derived from imported and local soybeans that are processed traditionally to ensure the purity of soy milk products without a mixture of other ingredients. This is used to determine the effect of differences in the origin of soybeans on the number of volatile compounds in soy milk, which will later be useful for knowing qualities such as aroma profiles, industry standardization parameters, development and improvement of soy milk products.

Research Methods

Materials

The research was carried out at the Analytical Chemistry Laboratory, Faculty of Pharmacy, Universitas Muhammadiyah Surakarta. The tools used are laboratory glassware, SPME (Solid Phase Microextraction Supelco, USA), SPME fiber PDMS/DVB (Polydimethylsiloxane/Divinylbenzene) 65 μm, GC - MS (Gas Chromatography-Mass Spectrometry Detector) Shimadzu - GC 2010 equipped with Shimadzu - GC 2010S, RxiTM-1MS chromatography column (30 m x 0.25 mm, layer thickness 0.25 μm), macro pipette 1000 to 5,000 μL, stative, burette clamp, magnetic stirrer, pan, stove, basin, filter cloth, blender and stirrer. The ingredients used are imported soybeans originating from the USA (United State of America) with the brand (Soybeans, USA BOLA) and local soybeans originating from the Grobogan area, Central Java, Water for injection (pro analysis - IKA Pharmindo, Indonesia), mobile phase helium gas.

Soy Milk Making Process

The soybeans are washed, soaked for 8 hours with a soybean and water ratio of 1:2 (w/v), squeezed to separate the soybean skin and boiled for 5 minutes. Next, crush the soybeans using a blender by adding water in a ratio of 1:5 (w/v). The soybean sprout pulp is wrapped in a filter cloth, then squeezed and the filtrate is collected. The filtrate is boiled until it boils for 5 minutes over medium heat while stirring gently.

SPME Fiber Cleaning

SPME fiber was injected into the Gas Chromatography injection port, left in the injection port for 30 minutes. Such as desorption of samples, and the injector temperature used is 280°C.

Optimization of GC-MS

Optimization of gas chromatography with an MS (Mass Spectrometry) detector requires adjustments to several important parameters to achieve optimal results. This process requires settings such as a programmed increase in column temperature and adjustment of the injection split ratio. This parameter setting is carried out to increase sensitivity, ensure more optimal separation, control the number of samples entering the column, and detect with high accuracy the identification of analyte components present in the sample. Optimization of GC-MS in this study involved the following parameters in **Table 1**.

Table 1. Parameter Optimization of GC-MS

Parameter	Optimization
Inject port temp.	280ºC
Ion Source temp.	200ºC
Interface temp.	250ºC
Gas flow rate	3,0 mL/min
Carrier gas	Helium
Initial Column Pressure	84.6 kPa
Column temp	70°C (5 min) to 270°C (15 min) with a regulated temperature increase 10°C/min
Mode of injection	Split 10 : 1
Total program time	40 min

The initial column temperature of 70ºC was held for 5 minutes; rise 10ºC/minute to 270ºC held for 15 minutes. Split ratio 10 : 1. Analysis was carried out by looking at the chromatogram results, the relative concentration (% Area) obtained and the mass spectra of the samples which had been compared with the internal Willey Library.

Sample Extraction

Extraction of Soy Milk drink samples was carried out using the SPME technique. The material was taken (5 mL) and recorded, then put into a 10 mL measuring flask and added to 10 mL with Water For Injection then vortexed. 5 ml of the dissolved sample was taken and then put into a 20 mL flacon, closed tightly using silicone. Insert the SPME needle into the flacon, then heat it at the SPME optimization temperature and time. When heating the flacon, the SPME fiber is removed from the SPME into the flacon to absorb the analyte. After heating, the analyte is injected into the GC - MS Injection Port, the injection results from SPME are analyzed by looking at the chromatogram results, the relative concentration (% Area) obtained and the mass spectrum of the sample which has been compared with the internal Willey Library.

Optimization of SPME

The sample (5 mL) was added with water for injection in a 10 mL measuring flask and vortexed. A 5 ml sample was taken and placed in a 20 mL flacon. Closed tightly with silicone. Insert the SPME needle into the flacon, then heat it at the SPME optimization temperature and time as in **Table 2.**

Table 2. Extraction Temperature and Time Optimization of SPME

Temperature (ºC)	Time (min)
40ºC	10 min
	20 min
	30 min
50ºC	10 min
	20 min
	30 min
60ºC	10 min
	20 min
	30 min

After heating using the SPME optimization temperature and time, the analyte is injected into the GC-MS injection port, the SPME injection results are analyzed by looking at the chromatogram results, the relative levels (% Area) obtained and the total volatile compounds detected. The mobile phase used in this research is helium gas (Hidayatullah et al., 2022), while the stationary phase used is the column in chromatography (RxiTM-1MS).

Statistical Analysis

Statistical significance of the difference between total volatiles and the difference between the relative levels of certain volatile compounds from soy milk samples, namely, imported soy milk and local soy milk. Each value represents the average of triple SPME analysis, namely one orientation analysis and two replication analyzes. Error bars indicate SD (standard deviation) (Suhendi et al., 2023). The differences in total volatiles in soy

milk and relative levels of certain compounds in soy milk were evaluated using the Independent Samples t-Test, with a 5% probability level.

Result and Discussion

Analysis of the taste and odor profiles of food and drinks is very useful for their quality. Apart from that, taste and odor profiles are also used to determine the right way to manage derivative products from these foods and drinks. One way to find out the taste and odor profile of food or drink is by analyzing the volatile compounds contained. There is even research that states that volatile compounds can also be good antimicrobials. This proves that the identification of volatile compounds in vegetable products, both food and beverages, is very important.

1. Optimization of Solid Phase Microextraction
   a. Effect of Incubation Time

      The effect of extraction time is very important for maximum absorption results. The aim of optimization by increasing the incubation time from 10 minutes, 20 minutes to 30 minutes is to find out at what minute SPME can maximally encourage greater adsorption of volatile compounds into the fiber by increasing the analyte partition balance between the SPME fiber and the sample. The definition of adsorption is the ability of standard volatiles/analytes to adhere to SPME fibers under the conditions studied and measured using an MS detector. In **Figure 1**, you can see the change in total volatiles produced at each time.

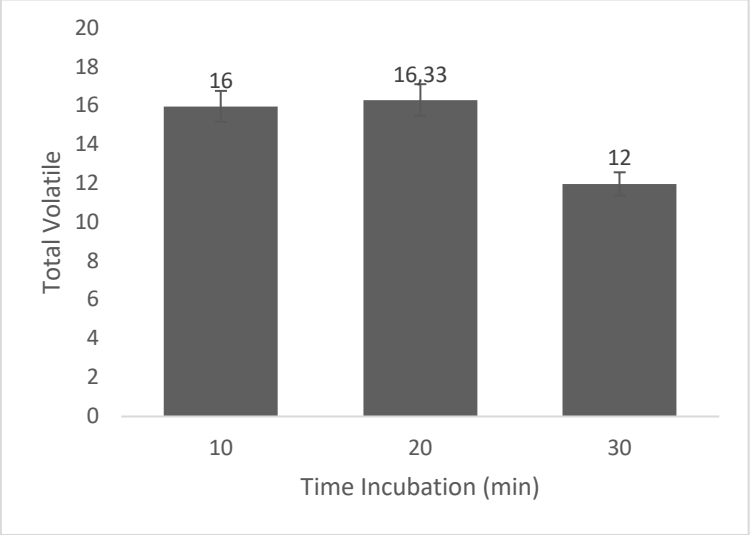

Figure 1. Effect of incubation time on total volatiles

The graph shows that there was an increase in the total volatiles detected in the 20th minute, namely 16.33 volatile compounds. After the 20th minute, there was a decrease in the total volatiles detected, namely at the 30th minute there were 12 volatile compounds. The decrease in total volatiles at 30 minutes probably occurred due to reverse diffusion, resulting in analytes that had been absorbed into the SPME fiber diffusing back into the sample because they had reached the maximum equilibrium point. Therefore, an incubation time of 20 minutes is suitable for use in sample analysis (optimal volatile recovery).

b. Effect of Incubation Temperature

In analysis using the SPME method, apart from setting the incubation time during sample preparation, it is also necessary to set the appropriate temperature so that later sample analysis produces maximum results. Here we use 3 temperatures for optimization in the SPME method, namely at 40; 50; and 60ºC. The aim of optimizing by adjusting the incubation temperature is to accelerate the release of analytes from the sample matrix and increase the analyte diffusion coefficient so that the analytes can reach the fiber quickly and an equilibrium partition occurs between the analyte and the fiber. A graph of the differences in volatile compounds detected at each temperature can be seen in **Figure 2.**

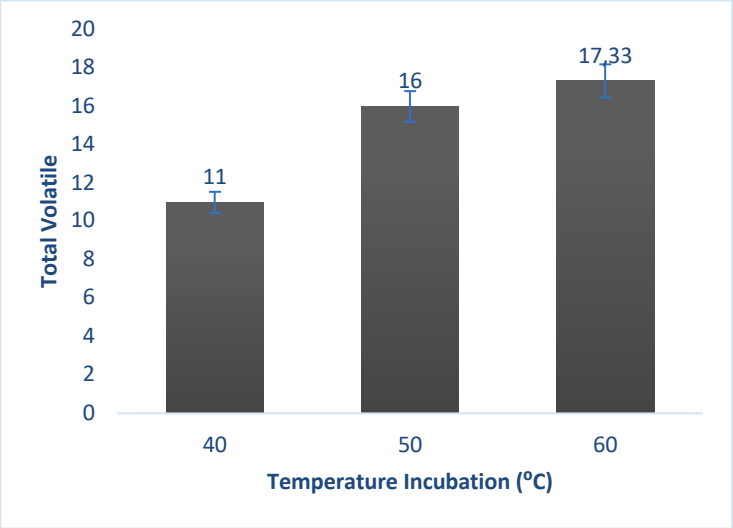

Figure 2. Effect of incubation temperature on total volatiles

From the results obtained, it is known that there was an increase in total volatiles from the lowest temperature, namely 40ºC, namely 11 volatile compounds, increasing to a temperature of 50ºC, namely 16 volatile compounds and reaching a peak at a temperature of 60ºC, namely 17.33 volatile compounds. This shows that the higher the sample extraction temperature, the more analytes are absorbed into the SPME fiber, so that this can increase the number of volatile compounds identified. In this way, the incubation temperature of 60ºC is suitable for use in sample analysis (recovery of total volatiles).

2. Analysis of Volatile Compounds of Soy Milk Samples

a. Sample Extraction

In the analysis of volatile compounds in imported and local soy milk samples, the first thing that must be done is to extract the sample. In this research, sample extraction was carried out using the headspace technique, which is a technique with the principle that the analyte must not come into direct contact with the SPME fiber. The SPME fiber used in this research is PDMS/DVB (Polydimethylsiloxane/Divinylbenzene) 65 µm fiber. These fibers are more efficient in sampling highly volatile organic compounds. The advantage of using this coating is that the shorter extraction time speeds up the analysis process and

also ensures linear extraction of compounds over time (Balasubramanian & Panigrahi, 2011).

Sample extraction is carried out using samples of the same type but from different origins. The first sample uses soy milk originating from imported soybeans from the USA (United State of America) with the brand (Soybeans, USA BOLA) while the second sample uses soy milk from soybeans originating from the Grobogan area, Central Java. Each sample was extracted with the time and temperature obtained during optimization, namely at 60ºC for 20 minutes. Apart from that, the sample volume used must be properly regulated so that it is not touched by the SPME fiber during extraction. The volume used is 5 mL for each sample analyzed.

b. Identification of Volatile Compound

The results of qualitative analysis using GC-MS show that total volatile compounds have been identified in imported and local soy milk. In this study, compounds were grouped that frequently appeared in the analysis of samples of imported soy milk and local soy milk, each in three copies (orientation, replication 1 and replication 2), with relatively high levels. Relative concentration is defined as the proportion of the peak area of each component relative to the total area of all peaks in the chromatogram. It is often used to determine the relative composition of each component in a sample.

The grouping of compounds from all samples can be seen from the cluster analysis results in Table 3. Cluster data analysis is used to classify the GC-MS analysis results based on the presence of compounds that frequently appear in each analysis. Compounds that appear frequently and have relatively high levels (>1%) are major compounds, while compounds with relatively small levels (<1%) are minor compounds (Novi et al., 2024). It can be seen in **Table 3**, a list of major volatile compounds which have relative levels (>1%), contained in imported and local soy milk samples with 2 replications. It is shown that there are around 23 compounds identified.

Table 3. Volatile compounds in local and imported soy milk with 2 replications

No	Name Compound	Relative levels (% area) Import			Relative levels (% area) Local		
		Ori	Rep 1	Rep 2	Ori	Rep 1	Rep 2
1	**Pentanal (**Pentanal, n-pentanal valeraldehyde)	2,42	7,02	1,93	2,47	9,57	2,05
2	**Ester** (O-(2-Methylethyl) ester of carbamothioic acid and 4-hexenoic acid,2,2,5-trimethyl, ethylester)	1,84	3,64	4,10	-	-	8,75
3	**Siloksan** (Decamethylcyclopentasiloxane and octamethylcyclotetrasiloxane)	2,09	8,10	1,58	1,26	-	2,51
4	Benzaldehyde	-	6,73		-	5,73	-
5	Benzenemethanol, benzyl alcohol	-	-	61,74	-	-	11,70
6	Acetophenon semicarbazone	1,97	-	-	-	-	-
7	1,2-Benzenediol, 3,5bis(1,1-dimethylethyl)	1,05	-	-	-	-	2,51
8	1-undecen-3-ol. 1-phenyl	3,22	-	-	-	-	-
9	Methoxy, phenyl, oxime	1,54	-	-	-	-	-

No	Name Compound	Relative levels (% area) Import			Relative levels (% area) Local		
		Ori	Rep 1	Rep 2	Ori	Rep 1	Rep 2
10	Cyclohexane, 1-(1,1-dimethylethyl)	2,00	2,76	-	2,60	-	-
11	Endo-isofenchol, bicyclo(2,2,1)heptane-2-ol	-	1,70	-	-	-	-
12	1,3-oxazetidin-2-one, 3-phenyl	-	-	3,24	-	-	-
13	Propane, 1,1-dicloro, 1,1-dichloropropane	-	-	3,38	-	-	-
14	3-butenoic acid, vinylacetic acid	-	-	1,51	-	-	-
15	EZ-3-methyl-2,4-hexadiene, 2,4-hexadiena,3-methyl	-	-	1,93	-	-	-
16	Benzenemethanol, alpha-(1-aminoethyl, norephedrine	-	-	1,93	-	-	-
17	2,2-diphenyl-1,3,2-benzo-dioxa-4H-tellune	-	-	1,13	-	-	-
18	Acetamide, 2-fluoro	27,51	-	-	-	-	2,52
29	D-alanine	-	-	7,06	-	-	-
20	Cyclopentene, 3 heptyl	-	-	-	5,00	3,90	8,75
21	Cyclohexanol,2-(1-methylethyl)	-	-	-	-	3,90	-
22	1-Napthalenecarboxaldehyde	-	1,14	-	-	-	-
23	1-octen-3-ol	1,29	-	-	-	-	-

Note : (-) Relative Levels (<1%) not counted

The major compounds identified in the two soy milk samples with high similarity were pentanal, alcohol, siloxane, ester, benzaldehyde, while for cyclopentene, it only appeared in the local soy milk samples. Many other compounds were detected but in relatively small levels (<1%) or below the instrument threshold so they were not counted in the total volatile compounds. The results of statistical analysis show that the type of soybean between imported and local has a significant effect on the total number of volatiles identified. ($P < 0.05$) Figure 3. This significant difference probably occurs due to differences in the origin of the soybeans used in making soy milk, including age, origin, time and method of harvesting, drying and soybean production processes.

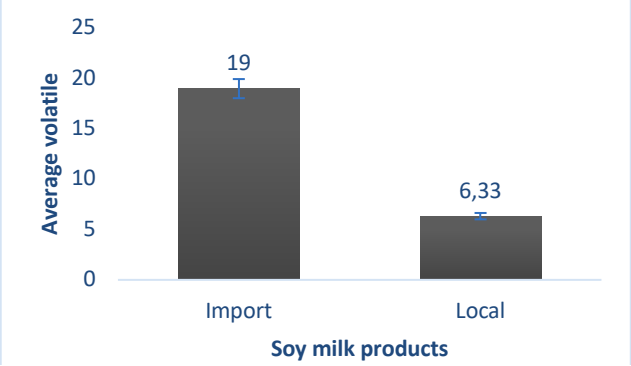

Figure 3. Total volatile compounds detected in imported and local soy milk

The major compounds identified in the two soy milk samples greatly influenced the taste and aroma characteristics of soy milk. The taste resulting from major compounds such as carbonyl compounds such as pentanal in soy milk has a green, woody pleasant odor, nutty aroma, and gives a slightly sweet taste like honey. Meanwhile, the carbonyl compound benzaldehyde reportedly does not really contribute to the taste of soy milk, however, benzaldehyde has a masking effect because its aroma resembles cherry or almond. The alcohol compounds contained here such as 1-octen-3-ol describe an earthy, mushroom-like, and slightly vegetable aroma and have a taste like green, oily, and faintly fatty (creamy feel). Siloxane compounds generally have no taste, but these compounds usually have benefits such as natural preservatives in food to slow the spoilage process and maintain the texture and taste of the product. The ester compound here is defined as having a honey-like aroma and a floral taste. The distribution of GC-MS chromatogram peaks for the major compounds can be seen in **Figure 4 & 5.**

A

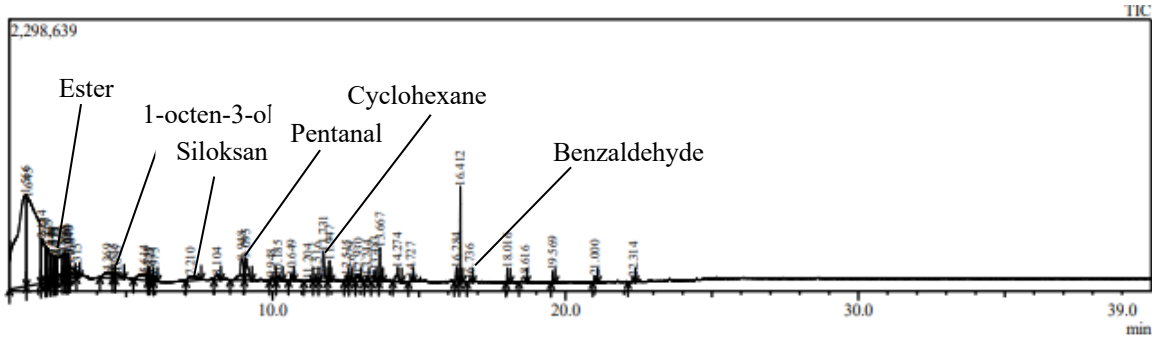

Figure 4.(A) Chromatogram of volatile compounds in imported soy milk

B

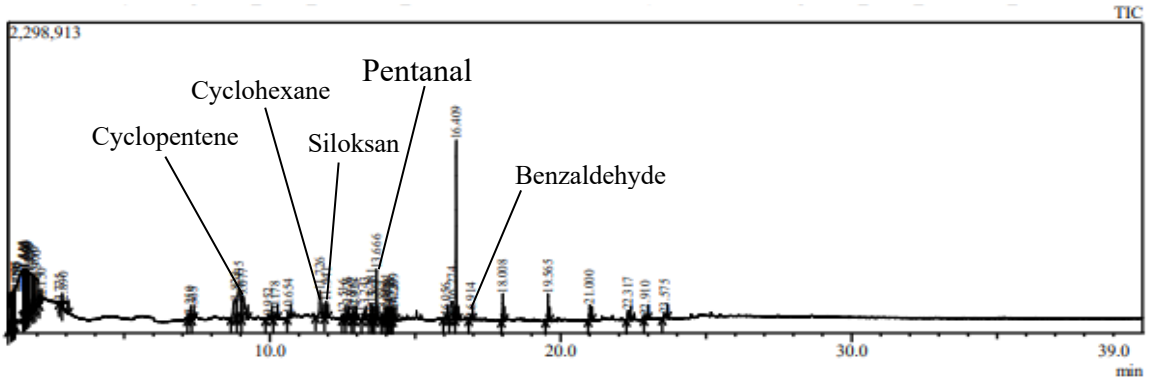

Figure 5 (B) Chromatogram of volatile compounds in local soy milk

The differences in volatile compound components in soy milk are not only based on the total volatiles produced. This data is not enough to show the taste and odor profile of soy milk. Therefore, more specific research is needed, by comparing the relative levels of major compounds (>1%) and compounds that frequently appear in both samples with the resulting triplicate analysis. The compound that appeared in the six analyzes was. The imported samples of pentanal compounds in orientation, replication 1, and replication 2 each had a relative level of 2.42%; 7.02%; 1.93%, while local samples of pentanal compounds in orientation, replication 1, and replication 2 each had relative levels of 2.47%; 9.57%; 2.05%, the relative content graph can be seen in **Figure 6**.

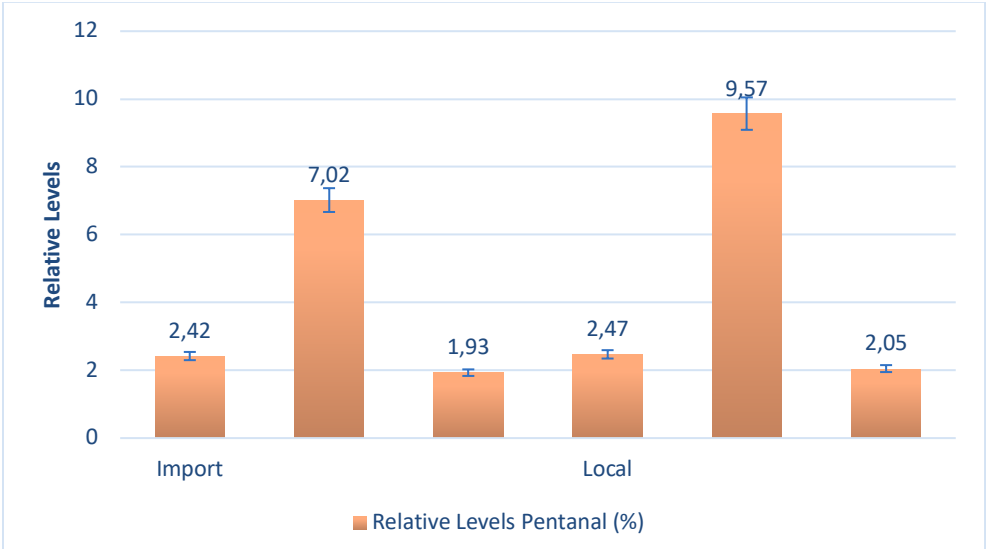

Figure 6. Comparison of relative levels of major compounds from imported and local soy milk

The relative levels of each sample obtained were subjected to statistical analysis. The results showed that the pentanal compound in imported and local soy milk had no significant effect on the relative levels produced ($P > 0.05$). This shows that the taste and odor characteristics produced by the pentanal compound do not significantly influence the differences in the origin of the samples used.

Conclusion

The Solid Phase Microextraction - Gas Chromatography (SPME - GC) method is an appropriate method to use to identify the volatile compound content in soy milk products. This research shows that good optimization for analyzing imported and local soy milk samples is at a temperature of 60ºC for 20 minutes. The maximum volume used is 5 mL to prevent direct attachment of the sample to the SPME fiber. The difference in total volatiles produced between the two soy milk samples shows that there is a significant difference ($P < 0.05$), so it can be concluded that the origin of imported and local soybeans used to make soy milk influences the total number of volatile compounds identified. Furthermore, the differences in relative levels between the pentanal compounds in the two soy milk samples showed insignificant results ($P > 0.05$), so it can be concluded that the taste and aroma characteristics produced by the pentanal compounds did not significantly influence the differences in the origin of the samples used.

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