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# Evaluation of fatty acids in traditional ghee and ashwagandha ghee by gas-chromatography mass spectroscopy

**Vishal Kumar, Tarun Verma and Rohit Sharma**

**Abstract**
This research explores the historical and nutritional aspects of ghee, an anhydrous milk fat deeply rooted in Indian culture. The study delves into its diverse applications, medicinal properties, and regional variations in production methods. A comprehensive analysis of the flavor compounds, including carbonyls, free fatty acids, and lactones, sheds light on the multifaceted nature of ghee flavor. The investigation extends to the isolation and identification of volatile compounds in both traditional and Ashwagandha-incorporated ghee using GC-MS, presenting a thorough discussion on qualitative and quantitative distinctions. Furthermore, the research unveils distinct variations in the fatty acid profiles between control and Ashwagandha ghee, emphasizing the potential implications for quality assessment and detection of adulteration. The study also explores the dietary significance of fatty acids, particularly the rise in linoleic acid consumption and its metabolic connection to arachidonic acid. Contrary to longstanding assumptions, recent findings on anti-inflammatory lipoxins derived from arachidonic acid challenge the notion of its purely pro-inflammatory nature. Additionally, the research provides insights into the Saturated Fatty Acid content of ghee samples from specific regions. Overall, this comprehensive study contributes valuable information to the understanding of ghee, encompassing its historical, chemical, and nutritional dimensions.

**Keywords:** Anti-inflammatory, fatty acids, isolation, nutritional

## Introduction
Ghee, which is anhydrous milk fat, has its origins in India long before any recorded history. The name "ghee" comes from the Sanskrit word "Ghr," which means bright or to make bright. Ayurveda mentions various medicinal properties of ghee, considering it as a coolant that enhances mental and physical well-being (Balakrishnan & Shukla, 2018) [2]. It is believed to have healing effects on ulcers and eye diseases. Ghee also contains conjugated linoleic acid, identified as a recently discovered anti-carcinogenic compound (Ali *et al*., 2016) [1]. Ghee-like products, often referred to as samna and roghan, are widely consumed in Middle Eastern and African nations (Hazra *et al*., 2023) [7]. In these regions, it is common to use not only cow and buffalo milk but also goat, sheep, and camel milk for making these products. In India, people commonly use ghee for cooking, in making sweets, and during religious ceremonies. Ghee has consistently maintained a distinguished position in the hierarchy of Indian diets, characterized by its appealing caramelized flavor and granular texture. (Mehta & Pinto, 2023) [10]. This stands in contrast to butter oil, which exhibits a comparatively bland taste and a smooth, non-granular texture.
In general, there are four ways to manufacture ghee: desi, creamery butter, straight cream, and pre-stratication methods. In India, a significant quantity of ghee is manufactured in rural areas using the traditional desi method, while contemporary dairies employ creamery butter and direct cream methods for ghee production. Desi ghee is more expensive and more favoured by customers since it has a stronger aroma than industrial ghee. The primary driving force behind ongoing research on the nature and role of flavor compounds in ghee is the crucial significance of ghee flavor in its acceptance and marketing.
Current understanding of the ghee flavor indicates that it is chemically a multifaceted attribute. The principal flavor compounds encompass carbonyls, free fatty acids, and lactones.

Among the carbonyls, alkan-2-one or methyl ketones play a significant role in the flavour of ghee (Wadodkar *et al*., 2002) [14]. They are produced by either lipolysis of triglycerides via penicillium moulds during the fermentation of milk or cream, or by hydrolysis of ketonogenic glycerides followed by decarboxylation. Polar carbonyls, including dicarbonyls, alpha-keto-acids, glyoxals, and furfurals, are generated through the fermentation of lactose and citrate, as well as from amino acids and the browning products resulting from lactose caramelization. Unsaturated fatty acids found in milk fat are known to autoxidize and produce aldehydes such as n-alkanals, alka-2-enals, and alka-2-4 dienals (Pattee *et al*., 1983) [12]. Either hydrolysis during processing or lipolysis during fatty acid glyceride ripening and fermentation results in the production of free fatty acids. Lactones are generated during processing through two different processes: lipolysis of glycerides prior to ring closure or hydrolysis of lactogenic glycerides to hydroxy acids followed by ring closure owing to dehydration (lactonization) (Bumbadiya *et al*., 2023) [3].

To enhance the acceptability of commercial ghee, there was a recognized need to comprehend the flavor distinctions between ghee prepared through traditional desi methods and industrial methodologies. This paper presents the isolation of volatile compounds from control ghee and Ashwagandha ghee using the direct injection technique, followed by their separation and identification through GC-MS. The qualitative and quantitative distinctions among the identified flavor compounds in control ghee and ashwagandha ghee are thoroughly discussed and documented.

## Materials and Methods

### Collection and preparation of plant materials

To prepare herbal ghee, Ashwagandha root was acquired from the local Varanasi market, and an aqueous extract was prepared. The herb's authenticity was confirmed by the Center of Advanced Study in Botany. Cow cream with 40% fat, standardized using cow skim milk, was used in the experiment. This cream was refrigerated at 4 °C for 12 hours and manually churned into butter. Glass bottles with a 200 g capacity, essential for the experiment, were purchased from the Varanasi market.

Cow milk was obtained from the local market in Varanasi. After preheating to 45 °C, cream was separated, standardized to 40% fat using cow skim milk, and then cooled to 4 °C, aging overnight. The aged cream was churned into butter with 80.00% fat content. The resulting butter was melted, clarified at 113-115 °C, filtered through muslin cloth, and a cotton pad. Raw Ashwagandha was sun-dried for a day, pounded into coarse powder using a mortar and pestle, and separated as coarse powder (Kashayachurna). The remaining Ashwagandha was further pounded into fine powder using a mixer. 200 grams of coarse Ashwagandha powder were soaked in 4 liters of water overnight. The next day, the container was heated on a gas burner until the decoction was reduced by half (6 hrs). After filtering, the decoction was set aside.

Milk was boiled, and 200 g of ghee was added along with 50 grams of fine Ashwagandha powder (Kalka Churna). The prepared 1-liter decoction (Kashaya) was added and boiled for 2 hours, left to cool overnight, and heating resumed the next morning. During heating, a frothy layer formed on the ghee's surface, and the milk started curdling into a solid consistency after about 6 hours of continuous boiling.

By the 10th hour, a cohesive mud-like paste formed at the container's bottom. Continuous stirring prevented charring of the paste. Around the 12th hour, the frothy layer disappeared, and heating continued until all water evaporated, leaving clear, transparent ghee devoid of froth. A small quantity of the paste was burned to confirm complete water evaporation, indicated by a crackling sound in the fire.

### Concentration and isolation of volatile substances

Prior to use, cleaned splitless glass liners, tweezers, and chromatographic-grade glass wool underwent heat treatment at 300 °C for 36 hours to eliminate any potential volatile contaminants. Sterilized glass liners were prepared with a 4 cm high column of glass wool, each designated for holding individual samples of ghee in the gas chromatograph's injection port. 50 microliters of each ghee sample were introduced onto the glass wool column and purged with helium gas at 150 °C for 30 minutes. To cryofocus the volatiles, the front part of the capillary column was cooled using liquid nitrogen. The glass liners containing the sample-holding glass wool column were swapped with freshly cleaned glass liners before initiating the gas chromatography run.

### Gas chromatography mass spectrometry

The separation of volatile compounds was carried out on a fused silica capillary column (30 m × 0.32 mm ID) coated with DB-WAX (J & W, Fullerton) using a Shimadzu GC, model-17A (Shimadzu, Kyoto, Japan). The initial temperature of the column oven was set at 30 °C for 2 minutes, then programmed to increase to 150 °C at a rate of 3 °C per minute. This temperature was maintained for 2 minutes before further programming to 210 °C at a rate of 4 °C per minute. The final temperature was held for 10 minutes. Helium served as the carrier gas with a flow rate of 1.7 ml per minute. The injector and interface temperatures were kept at 220 °C and 250 °C, respectively. Detection of the separated volatile compounds was performed using a mass spectrometric detector, model QP5000 (Shimadzu).

The mass spectrometer operated in an electron impact mode with an electron energy of 70 eV. Identification of volatile compounds involved comparing their retention times (RT) with authentic compounds and utilizing computerized comparisons of their mass spectra with known compounds from standard NIST and Wiley libraries. When authentic compounds were not available, tentative identification was performed by comparing the mass spectra with known compounds from standard libraries. Quantification of volatile compounds utilized n-undecane (Sigma, USA) as an internal standard, considering the response factor as 1.0. Each sample of control and ashwagandha ghee underwent GC-MS analysis three times, maintaining identical experimental conditions. The repeated GC-MS data for each sample exhibited consistent similarities in terms of both the numbers and identifications of the detected flavor compounds. The reported results represent the average values obtained from the three replications.

The identification of GC-MS fatty acid peaks in all samples was reaffirmed through mass spectrometric data base analysis of standard compounds generated by a computer. Peak amplitudes were employed in the analysis to indicate the abundance of specific fatty acids.

## Results and Discussion

The fatty acids compositions of samples are presented in Table 1. The fatty acid profile of control ghee and Ashwagandha ghee reveals notable differences:

**Table 1:** Fatty acid Composition of Control ghee and Ashwagandha ghee

| S. No. | Parameter | Control ghee | Ashwagandha ghee |
|---|---|---|---|
| | **Test results** | | |
| 1. | **Saturated Fatty Acids** | | |
| | A. Mysistic Acid (C14/0) | 7.26±0.20 | 3.95±0.20 |
| | B. Stearic acid (C18/0) | 7.68±0.20 | 7.65±0.20 |
| 2. | Monounsaturated Fatty Acid | | |
| | A. Palmitic Acid (C16/1) | 9.53±0.20 | 13.96±0.20 |
| | B. Oleic Acid (C18/1) | 10.19±0.20 | 16.95±0.20 |
| 3. | **Polyunsaturated Fatty Acid** | | |
| | A. Linolic Acid (C18/3) | 13.41±0.20 | 21.04±0.20 |
| | B. Linolenic Acid (C18/3) | 15.37±0.20 | 21.36±0.20 |
| | C. Arachidonic Acid (C20/4) | 16.03±0.20 | 22.40±0.20 |

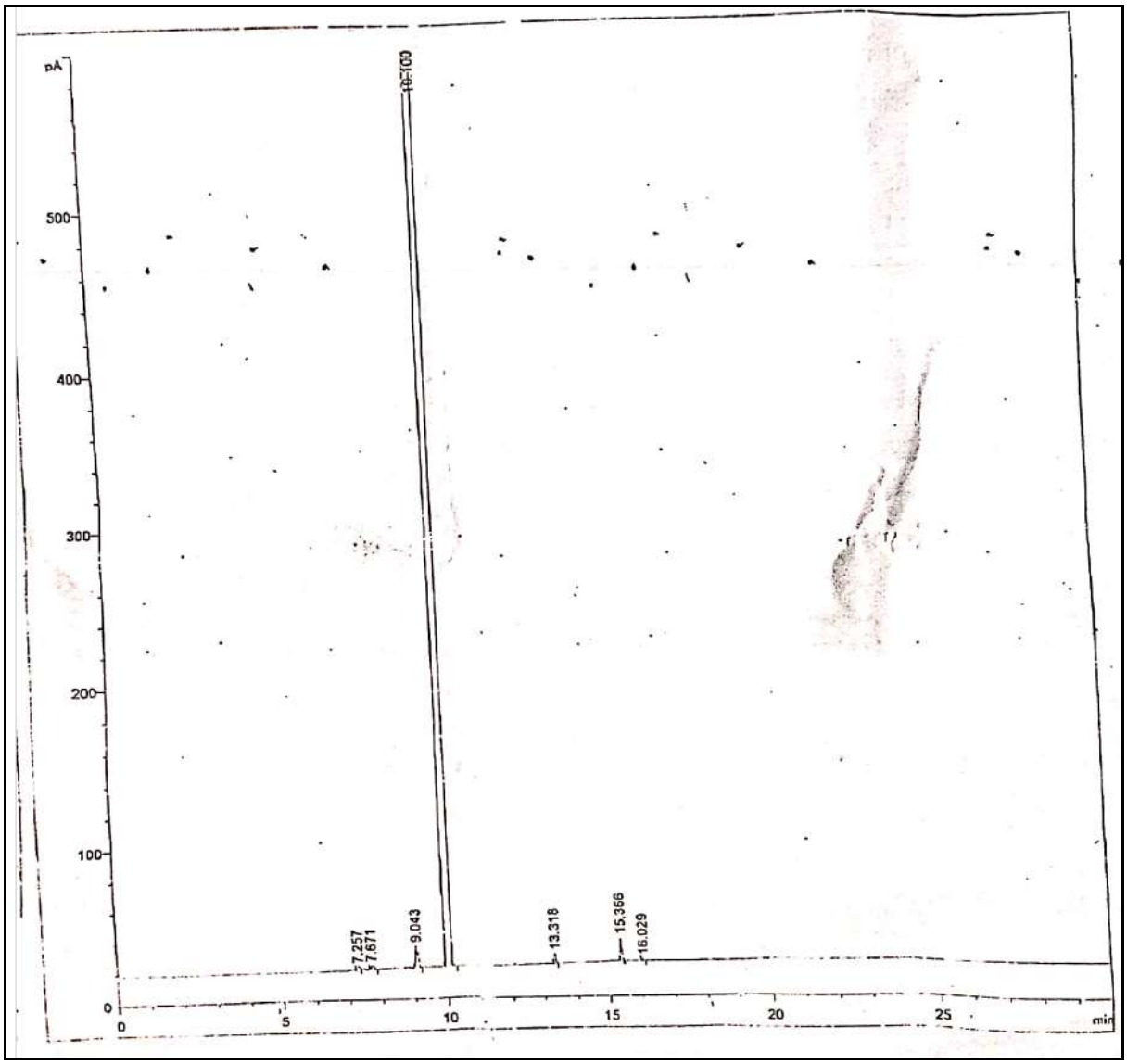


**Fig 1:** Fatty-Acid Profile of Control ghee

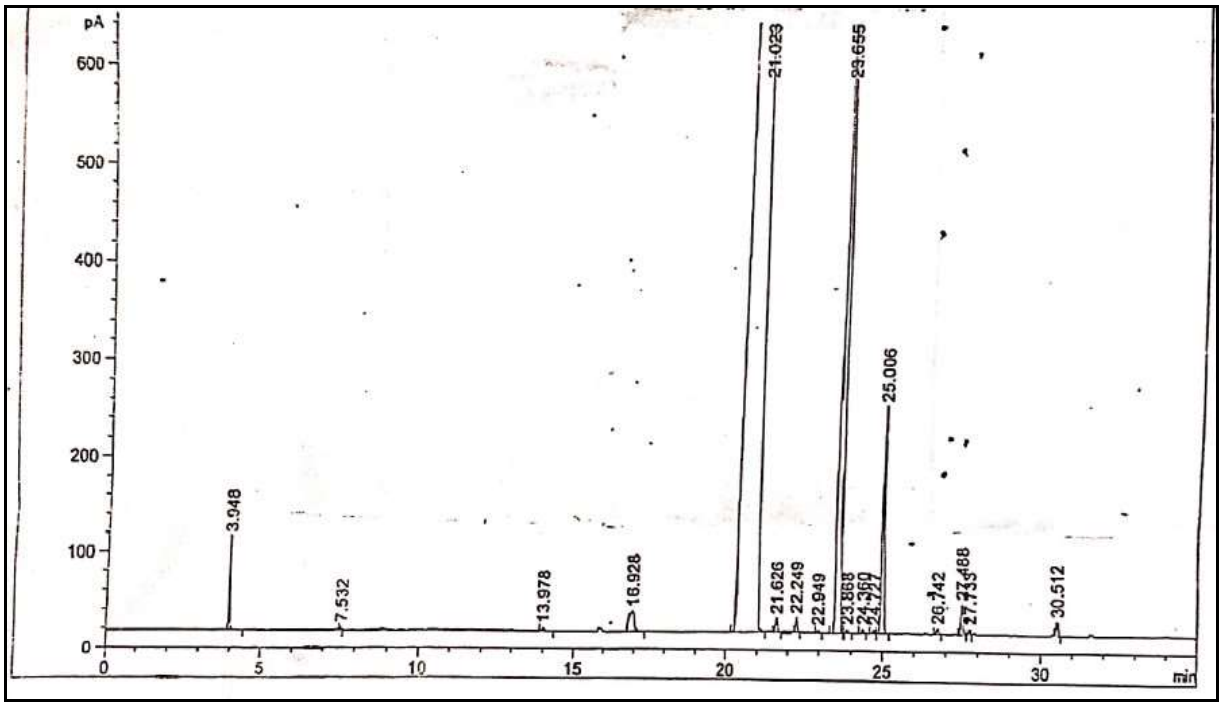


**Fig 2:** Fatty-Acid Profile of Ashwagandha ghee

**Saturated Fatty Acids**

**Mysistic Acid (C14/0):** Control ghee has a higher content (7.26) compared to Ashwagandha ghee (3.95).

**Stearic acid (C18/0):** Control ghee and Ashwagandha ghee exhibit similar values (7.68 and 7.65, respectively).

**Monounsaturated Fatty Acids**

**Palmitc Acid (C16/1):** Ashwagandha ghee shows a higher content (13.96) compared to control ghee (9.53).

Oleic Acid (C18/1): Similar to palmito acid, Ashwagandha ghee has a higher value (16.95) compared to control ghee (10.19).

**Polyunsaturated Fatty Acids**

**Linolic Acid (C18/3):** Ashwagandha ghee displays a significantly higher content (21.04) compared to control ghee (13.41).

**Linolenic Acid (C18/3):** Similar to linolic acid, Ashwagandha ghee has a higher value (21.36) compared to control ghee (15.37).

**Arachidonic Acid (C20/4):** Ashwagandha ghee exhibits a higher content (22.40) compared to control ghee (16.03).

The chemical analysis of fatty acids in ghee offers insights into the quality and flavor characteristics of ghee samples (Yang *et al*., 2024) [15]. These results serve as valuable indicators for assessing, detecting, and investigating potential adulteration in various ghee products. Among the samples analyzed, palmitic acid emerged as the predominant fatty acid. Furthermore, the presence of specific fatty acids or the ratios between certain acids could be pivotal in the identification or detection of adulteration in ghee. Differences in fatty acid composition could be attributed to variations in feeding practices, seasonal and climatic conditions, and other influencing factors (Hanuš *et al*., 2018) [6]. The outcomes of the current study align well with previous research, indicating palmitic acid as the primary fatty acid within the range of 37.14% to 41.58%. Subsequently, oleic acid was identified as the second most prevalent fatty acid, with concentrations ranging from 19.49% to 25.12%. In their study, Johnson and Saikia (2009) [8] similarly identified palmitic and oleic acids as the prevailing fatty acids in Indian ghee.

Dietary fatty acids (FAs) significantly influence the onset and advancement of various prevalent diseases. Particularly, polyunsaturated fatty acids (PUFA) are crucial for normal growth, cellular function, signaling, and immune response (Radzikowska *et al*., 2019) [13]. Consequently, disturbances in PUFA levels may contribute to the pathogenesis of numerous chronic diseases. The consumption of linoleic acid (LA) has experienced a significant surge in the last century, primarily attributed to a substantial rise in vegetable oil intake (Mercola & Adamo, 2023) [11]. Concurrently, there has been a notable decline in the overall consumption of n-3 polyunsaturated fatty acids (ALA, EPA, and DHA). Moreover, linoleic acid (LA) has the potential to undergo metabolism into arachidonic acid (AA), a key participant in inflammatory processes (Kikut *et al*., 2020) [9]. AA can act as a substrate for generating certain pro-inflammatory eicosanoids, contributing to the synthesis of inflammatory mediators like tumor necrosis factor alpha (TNFα) and interleukin-1 (IL-1) (Calder, 2009) [4]. With this comprehension, there has been a longstanding assumption that the primary n-6 polyunsaturated fatty acids (PUFA), linoleic acid (LA), and arachidonic acid (AA) elicit a pro-inflammatory response, while n-3 PUFAs are believed to possess anti-inflammatory properties. Consequently, an increased consumption of n-6 polyunsaturated fatty acids (PUFA), combined with a diminished intake of n-3 PUFA, could potentially foster a pro-inflammatory milieu, thereby contributing to chronic inflammation—a recognized risk factor for the onset of numerous chronic diseases.

Despite the metabolic connection between linoleic acid (LA) and arachidonic acid (AA), the presumption that both molecules exhibit pro-inflammatory characteristics may not be entirely accurate. Moreover, recent findings reveal the existence of lipoxins derived from AA, demonstrating anti-inflammatory and pro-resolving properties. The outcomes of the current study align well with previous research by (Erfani *et al*., 2020) [5], The Saturated Fatty Acid (SFA) content in

samples from Ilam, Kermanshah, and Hamedan was documented as 63.3%, 60.4%, and 64.85%, respectively. Meanwhile, the monounsaturated fatty acid content for Ilam, Kermanshah, and Hamedan samples was reported as 30.1%, 31.37%, and 29.7%, respectively. Oleic acid emerged as the predominant monounsaturated fatty acid (MUFAs). Additionally, the total Polyunsaturated Fatty Acids (PUFAs) in the samples from Ilam, Kermanshah, and Hamedan were 3.89%, 4.01%, and 3.06%, respectively, with linoleic acid exhibiting the highest concentration among all PUFAs.

## Conclusion

In conclusion, this research provides a comprehensive exploration of ghee, its historical significance, and diverse applications rooted in Indian culture. The study highlights its esteemed position in Indian diets, distinguished by a unique caramelized flavor and granular texture, setting it apart from butter oil. The investigation into ghee manufacturing methods, including the traditional desi approach and contemporary industrial processes, underscores the importance of flavor in its acceptance and marketing. The chemical complexity of ghee flavor is unveiled, revealing a multifaceted attribute composed of carbonyls, free fatty acids, and lactones. The role of alkan-2-one or methyl ketones, produced through various processes like lipolysis and hydrolysis, in shaping ghee's flavor is emphasized. This understanding is crucial for ongoing research aimed at enhancing the acceptability of commercial ghee. The research extends its focus to the isolation and identification of volatile compounds in both control ghee and Ashwagandha-incorporated ghee. Through GC-MS analysis, the qualitative and quantitative distinctions among flavor compounds are thoroughly discussed and documented. The exploration of fatty acid profiles in both ghee variants offers valuable insights for quality assessment and detection of potential adulteration. The inclusion of Ashwagandha in the preparation of herbal ghee adds a medicinal dimension to the study, showcasing the integration of traditional herbal practices with modern food production methods. The meticulous description of the preparation process, from acquiring Ashwagandha root to the final production of Ashwagandha ghee, provides a practical understanding of herbal ghee production. The research also contributes to the broader discourse on dietary fatty acids, emphasizing the significance of polyunsaturated fatty acids (PUFA) and the potential implications of altered PUFA levels on chronic diseases. The investigation challenges longstanding assumptions about the pro-inflammatory nature of linoleic acid (LA) and arachidonic acid (AA), introducing the concept of anti-inflammatory lipoxins derived from AA.

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