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Review

Advanced Analytical Strategies for Detecting Non-Milk Fat Adulteration and Species Mixing to Ensure Dairy Authenticity: Current Status and Future Trends

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Abstract

Milk and dairy products are highly vulnerable to Economically Motivated Adulteration (EMA), particularly through the substitution of milk fat with cheaper non-milk fats. This paper presents a comprehensive review of analytical approaches used for detecting vegetable oils (e.g., palm, coconut, sunflower) and animal-origin fats such as pork lard and bovine tallow, as well as the fraudulent mixing of milk from different species. Methods for lipid extraction are examined, including traditional gravimetric procedures such as the Röse–Gottlieb method and high-efficiency alternatives such as Accelerated Solvent Extraction and supercritical fluid extraction. Analytical strategies for fraud detection are evaluated, demonstrating that while fatty acid profiling is widely applied, its sensitivity is limited by natural variability. Greater discriminatory power can often be achieved through triacylglycerol analysis combined with mathematical models such as the Precht formulae (which generate S-values), although performance varies depending on the adulterant matrix, adulteration level, reference population, and analytical protocol. Similarly, sterol profiling, particularly the detection of phytosterols like β-sitosterol, is a valuable marker for vegetable oil adulteration but does not provide an equivalent solution for detecting animal fat adulteration. The potential of rapid, non-destructive screening tools, including Fourier-transform infrared and Raman spectroscopy supported by chemometrics, is also assessed. A central analytical challenge in detecting such fraud lies in the complexity and variability of milk fat composition, which hinders any single analytical method from universally identifying all forms of non-milk fat adulteration; therefore, a tiered strategy combining rapid screening tools with high-resolution confirmatory methods is preferable. Future perspectives highlight the increasing importance of green analytical approaches, artificial intelligence, and portable detection systems for enhancing verification within the global dairy supply chain. However, the effectiveness of AI and chemometric methods depends heavily on the availability of representative training datasets and rigorous external validation to avoid overfitting and ensure reliable application across diverse samples.

Keywords: triacylglycerols; fatty acid profiling; gas chromatography; phytosterols; adulteration detection; Economically Motivated Adulteration (EMA)

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1. Introduction

Food fraud is a significant and escalating global concern, primarily driven by the intentional substitution, addition, or removal of food components for economic gain [1,2], a phenomenon commonly referred to as Economically Motivated Adulteration (EMA) [3,4].

Milk is a nutritionally rich food source that provides essential proteins, fats, carbohydrates, and vitamins necessary for infant development and adult health, and it holds significant economic importance worldwide [1,4–6]. On the other hand, several factors influence milk composition and quality, such as animal physiology, milking technology, management, and hygiene practices [7,8]. Additionally, because milk is widely consumed and forms the basis for numerous high-value products like cheese, butter, and yogurt, it consistently ranks among the most frequently adulterated food items worldwide [3,9]. The dairy industry is a pillar of global food security, yet it is often subject to profit-and-loss pressures that tempt unethical behavior among some traders [10,11].

An additional motivation for dairy fraud is often linked to seasonal fluctuations in milk supply, particularly during lean seasons when the natural production of cows, goats, or sheep falls short of market demand [8,10,12]. To bridge the gap between supply and demand, various fraudulent methods are employed to increase product volume or mask poor quality [5,13]. In many regions, the milk payment system is based on specific chemical parameters such as fat and protein content, creating a strong incentive for traders to manipulate these specific markers [12,14]. Consequently, the authenticity of a dairy product—defined as its adherence to the description provided on the label regarding species origin, provenance, and production method—is frequently compromised [15,16].

Adulteration of milk and dairy products can be carried out through various practices, such as dilution with water, the addition of nitrogen-containing compounds, starches, and other substances, preservation with unauthorized chemicals, and substitution or dilution with milk from other animal species [1,2,9,17,18]. Although these practices represent important aspects of dairy fraud, the substitution of milk fat with non-milk fats is of particular concern because it can be difficult to detect, especially when the added fats have fatty acid profiles that partially overlap with that of milk fat.

The authentication of milk fat is further complicated by its natural compositional variability. Factors such as animal species, breed, diet, season, stage of lactation, and other physiological and environmental factors can influence the fatty acid profile of milk fat. Consequently, changes in individual fatty acids should not be interpreted as definitive evidence of adulteration [3]. To accurately detect such complex manipulations, particularly the intermixing of milk from different species, distinct lipid signatures such as short- and medium-chain fatty acids (C4:0–C12:0) can be used as diagnostic markers in gas chromatography analysis [2,9], effectively complementing molecular tools like Polymerase Chain Reaction (PCR) [19]. These multifaceted fraudulent practices underscore the urgent necessity for robust and standardized analytical methods to verify the authenticity of dairy products and ensure consumer safety [3,20].

Milk fat is a frequent target of high-value fraud, often partially or completely replaced with cheaper vegetable oils, such as palm and coconut, or animal fats, such as lard and tallow [5,21,22]. Due to the high chemical complexity of genuine milk fat, detecting these surreptitious substitutions remains a significant challenge for routine analytical laboratories [3,16,23,24].

This review aims to critically evaluate analytical strategies for detection and quantification of non-milk fat adulteration in milk and dairy products and to identify specific biomarkers, such as fatty acid profiles, triacylglycerol (TAG) compositions, and sterol patterns, integrating them into a practical workflow. A significant focus will be placed on the comparative evaluation of instrumental techniques such as gas chromatography (GC)

and high-performance liquid chromatography (HPLC), together with mathematical and chemometric approaches such as the Precht formulae and Linear Discriminant Analysis (LDA), and rapid, non-destructive screening tools like Fourier-transform infrared (FTIR) and Raman spectroscopy, concluding with the emerging role of artificial intelligence (AI) in data interpretation. By synthesizing these analytical approaches, this review aims to provide a comprehensive framework for analysts and regulatory bodies to effectively monitor and combat dairy fraud by promoting standardized, multi-layered detection strategies. In sum, the overarching objective is to enhance the transparency of the dairy market and ensure that the products reaching consumers are safe, authentic, and ethically labeled.

Literature Search Strategy and Selection Criteria

To ensure a balanced and comprehensive overview of dairy authenticity and non-milk fat adulteration, a structured literature search was conducted across major scientific databases, including Web of Science, Scopus, PubMed, and ScienceDirect. The search covered literature published from 2000 through August 2026, with emphasis placed on standardized reference methods, foundational methodology, and recent analytical developments. To maintain historical accuracy and credit foundational analytical protocols, key seminal studies and original reference methods published prior to 2000 were also included. Primary search terms were combined using Boolean operators and included: ("dairy fraud" OR "milk fat adulteration" OR "foreign fat detection" OR "vegetable fat" OR "vegetable oil" OR "non-milk fat") AND ("triacylglycerol profiling" OR "S-values" OR "ISO 17678" OR "phytosterols" OR "fatty acid profiling" OR "chemometrics" OR "vibrational spectroscopy" OR "FTIR" OR "Raman" OR "lipidomics"OR "Non- milk fat").

Eligibility Criteria:

Inclusion criteria: Peer-reviewed primary research articles, international analytical standards (e.g., ISO, IDF, AOAC), official regulatory frameworks, and authoritative review papers addressing analytical methodologies for detecting foreign fats or species substitution in dairy matrices were included in the review. Only English-language publications were included.

Exclusion criteria: Non-peer-reviewed literature, conference abstracts without full methodology, trade magazine reports, and studies lacking sufficient methodological or diagnostic performance information were excluded.

2. Adulteration of Milk and Dairy Products with Non-Milk Fats and Species Mixing

Beyond general fraudulent practices, the specific substitution of milk fat involves distinct mechanisms depending on the final product. Liquid milk, butter, ghee, cheese, cream, and fermented dairy products should not be considered analytically interchangeable matrices, as differences in matrix composition and fermentation can substantially affect the extraction, detection, and quantification of adulterants. While liquid milk is often adulterated through homogenization with low-cost oils, solid products like butter or ghee are more susceptible to direct blending with solid fats of plant or animal origin. One prevalent form of dairy fraud involves the illegal mixing of milk from different animal species. In many regions, milk from buffaloes or sheep is significantly more expensive than cow milk, leading manufacturers to dilute these premium milks with cheaper cow milk to increase profit margins [2,5]. Identifying the species origin is essential for consumers, not only to prevent economic losses from fraudulent substitution but also due to concerns related to food safety and religion, particularly when milk fat is replaced with other animal fats such as pork lard [16]. This is particularly common in the production of traditional high-value products such as buffalo mozzarella or sheep milk cheeses [25,26]. Detection of

such mixing is complex due to genetic polymorphisms, necessitating advanced analytical techniques like PCR and high-performance liquid chromatography to verify the animal origin declared on labels [1].

2.1. Common Vegetable Fat Adulterants

Among vegetable oils, palm oil and its fractions (palm olein and palm stearin) are the most frequent adulterants used globally. This is due to their extremely low cost, widespread availability, and a fatty acid profile that is misleadingly similar to that of milk fat [27,28]. Research has shown that palm oil can be added to milk, butter, and kajmak without being easily detected by consumers through sensory analysis alone [29,30]. The presence of palm oil in dairy products is often identified by a significant increase in C16:0 (palmitic acid) and C18:1n-9 (oleic acid) levels, as well as the appearance of plant-specific sterols [23]. However, individual fatty acid changes should not be interpreted as definitive evidence of product adulteration because they are naturally present in milk fat, and their concentrations can vary according to genetics, type of farming, animal nutrition, and other physiological or environmental factors [31].

Coconut oil is another highly common vegetable adulterant that is frequently used because it shares some physical properties with butterfat, such as resistance to oxidation. However, coconut oil is uniquely high in lauric acid (C12:0) and capric acid (C10:0), and its addition to milk fat gradually decreases the refractive index and melting point of the final product [2,22].

Other widely utilized vegetable oils for dairy fraud include soybean, sunflower, corn oil, and peanut oil. These are often preferred for their high unsaturated fatty acid content, which can be used to manipulate the nutritional profile of a product for deceptive gains [15,27,32].

2.2. Animal Fat and Rarer Adulterants

Animal-origin fats such as pork lard and bovine tallow are also significant adulterants, particularly in regions where they are readily available as industrial by-products. Pork lard is often used in butter or cheese fraud because its triacylglycerol profile, specifically the higher concentrations of C52 and C54, can mimic certain aspects of milk fat during routine testing [33]. Bovine tallow is used to manipulate the firmness and texture of fat-based dairy products, though it lacks the short-chain fatty acids that are characteristic of genuine ruminant milk fat [9,32]. In some cases, mixtures of animal fats and vegetable oils are used to create “designer oils” that are even harder to detect using traditional chemical constants [24]. On the other hand, fatty acid ratios alone have some limitations, particularly at low levels of milk fat adulteration. Rebechi et al. (2016) reported that fatty acid ratios failed to distinguish milk fat adulterated with less than 15% tallow or lard, whereas Multiple Linear Regression (MLR) models were able to detect adulteration levels above 10% for tallow and 5% for lard [34]. Similarly, TAG profiling combined with Linear Discriminant Analysis (LDA) demonstrated potential for discriminating milk fat from pork lard and bovine tallow, with 94.4% of samples with <10% adulteration correctly classified [32].

Margarine is frequently used as a complex adulterant in products like butter and kajmak because it is already a formulated blend of refined vegetable oils designed to look and feel like dairy fat. Adulteration with margarine may alter the saturated fatty acid content and, depending on the formulation, may increase the presence of trans fatty acids; however, trans fatty acids cannot be considered specific or universal biomarkers of margarine adulteration because their levels vary substantially among margarines and may be low in modern formulations [20,35]. Rarer adulterants documented in research include fish oil, goose fat, and even non-edible mineral oils. Fish oil is sometimes added to mimic

certain nutritional components but can be differentiated from milk fat based on its high levels of long-chain polyunsaturated fatty acids and unique triacylglycerol patterns [3,36].

The use of such a wide variety of substances, from common palm oil to rare animal depot fats, demonstrates the sophisticated nature of modern food fraud and the urgent need for standardized monitoring protocols. To illustrate the extent of this issue, Table 1 provides a comprehensive summary of key investigations concerning non-milk fat adulteration, demonstrating the global diversity of adulterants used and the evolving analytical strategies employed to ensure dairy authenticity.

Table 1. Selected studies on the detection of non-milk fat in dairy products.

Author(s) and Year	Adulterant(s) Detected	Sample Type	Total Samples	Samples Reported as Adulterated	Country
Gutiérrez et al. (2009) [32]	Pork lard, beef tallow, vegetable oils, fish oil	UHT milk fat (market survey)	36	14	Mexico
Uzunov et al. (2018) [29]	Palm oil, coconut oil	Cheese and sour cream	60	23	North Macedonia
Nurseitova et al. (2019) [15]	Rapeseed oil, other veg. oils	Commercial milk	17	8	Kazakhstan
Petronijevic et al. (2019) [9]	Palm fat	Cow and goat cheese	121	35	Serbia
Hajrulai-Musliu et al. (2021) [5]	Palm and coconut oil	Sour cream products	55	4	North Macedonia
Nurseitova et al. (2021) [3]	Vegetable oils (phytosterols)	Butter and sour cream	18	11	Kazakhstan

Note: The reported numbers of adulterated samples are study-specific and should not be interpreted as directly comparable estimates of adulteration prevalence, because the studies differ in sampling design, sample type, analytical methodology, and detection criteria.

The prevalence of dairy fraud through the substitution of milk fat with cheaper non-milk alternatives, such as palm oil, coconut oil, and animal fats like lard and tallow, remains a significant challenge for the global industry [5,22]. Furthermore, the illegal mixing of milk from different animal species, particularly the dilution of high-value buffalo or sheep milk with cheaper cow milk, is a widespread practice aimed at maximizing profit margins [9]. These fraudulent activities not only undermine consumer trust and violate labeling regulations but also pose potential health risks, including undeclared allergens and the introduction of unmonitored substances into the food supply chain [3,20], and can additionally be related to ethical and/or religious concerns.

Uzunov et al. (2018) and other authors underscore the critical importance of continuous market monitoring to ensure transparency and safety [29]. Consequently, the development of robust and standardized methodologies is essential for verifying dairy authenticity in the future. The following section provides a detailed overview of the various analytical methods used for the detection of non-milk fats.

3. Analytical Methodologies for the Detection of Non-Milk Fats in Dairy Products

The verification of dairy product authenticity requires a multi-staged analytical approach, beginning with the extraction of the lipid fraction and progressing through instrumental analysis and, where necessary, quantitative assessment. Because milk fat is a highly complex matrix containing more than 400 fatty acids and numerous triacylglycerol (TAG)

species, a clear distinction between screening, identification, confirmation, and quantification is essential when evaluating analytical methods for authenticity testing [15,16].

Screening refers to the rapid detection of samples that may be adulterated and therefore require further investigation. Screening methods are primarily intended for high-throughput industrial quality control or testing at milk collection points, where rapid and cost-effective analysis is essential [20]. Techniques such as FTIR, NIR, Raman spectroscopy, and rapid chromogenic tests can provide rapid discrimination with limited sample preparation [20,37,38]. However, a positive screening result should be regarded as an indication of a potentially non-authentic sample rather than, by itself, definitive evidence of adulteration.

Identification aims to determine the likely nature and origin of the adulterant on the basis of characteristic chemical markers [3]. For example, the detection of phytosterols such as β-sitosterol can indicate the presence of vegetable-derived fats, while characteristic fatty acid or TAG profiles may provide evidence for the presence of specific vegetable or animal fats [3,28,33]. Importantly, the ability of an analytical method to discriminate experimentally adulterated samples or indicate the likely identity of an adulterant does not, by itself, establish regulatory confirmation.

Confirmation requires the use of an appropriate validated or internationally recognized reference method, together with suitable quality assurance procedures, to establish whether the suspected adulteration can be reliably confirmed for regulatory purposes [39]. For milk fat authenticity, TAG analysis by gas chromatography and the application of the Precht/S-value approach are particularly important because they are incorporated into the international reference framework ISO 17678:2019 [16,39,40]. Thus, a method may demonstrate excellent discrimination between pure and experimentally adulterated samples without necessarily being sufficiently validated or standardized for regulatory confirmation. The reliability of confirmatory analysis also depends on appropriate method validation, quality control, and, where applicable, proficiency testing.

Quantification represents a separate analytical objective, namely the estimation of the level or proportion of adulterants present. Quantitative determination requires an analytical response that has been appropriately calibrated and validated for the intended matrix and concentration range. In dairy fat authenticity testing, TAG profiles combined with mathematical approaches such as the Precht/S-value equations can be used to estimate foreign fat incorporation while accounting for the natural variability of milk fat [9,39,41]. In contrast, a method that successfully classifies samples as authentic or adulterated is not necessarily suitable for quantitative determination. Therefore, quantitative models should be evaluated using appropriate validation procedures, including their performance with independent and representative samples, before being considered suitable for routine or regulatory applications.

Accordingly, the analytical workflow considered in this review follows a sequential framework of screening, identification, confirmation, and quantification, recognizing that individual methods may be appropriate for one or more of these purposes but are not necessarily interchangeable. Within this framework, the extraction of the lipid fraction represents the essential first step, as the quality and integrity of the recovered lipids directly affect the reliability of subsequent chromatographic and spectroscopic analyses. The following sections therefore first examine the principal methodologies for lipid extraction, followed by a comparative evaluation of the instrumental techniques used for the identification, confirmation, and quantification of non-milk fat adulteration.

3.1. Methodologies for Lipid Extraction

The first step in any fat authenticity study is the isolation of the lipid fraction from the dairy matrix. However, it is essential to distinguish between methods designed primarily

for total fat determination and extraction procedures optimized for subsequent compositional analysis [42]. Conventional gravimetric methods, such as the Röse–Gottlieb and Mojonnier methods, are established reference methods for the determination of total milk fat content [43]. These methods rely on alkaline hydrolysis using ammonia to dissolve proteins, followed by repeated liquid–liquid extraction (LLE) using a mixture of diethyl ether and petroleum ether [44,45]. This distinction is critical because extraction recovery alone does not guarantee the preservation of a representative lipid composition, which is a different analytical objective from extraction workflows used before GC-MS lipid profiling or LC-MS lipidomics [16].

While these methods are highly accurate and are used to calibrate routine instruments, they are significantly limited by low sample throughput and high solvent consumption [12,42,46,47]. For detailed chemical characterization, such as fatty acid profiling or sterol analysis, solvent-based extraction methods like the Folch method and the Bligh and Dyer method are more commonly employed. The Folch method involves a two-phase partition of the lipid fraction into an organic phase consisting of a chloroform and methanol mixture, typically in a 2:1 ratio [42,47,48]. After homogenization and filtration, a salt solution (e.g., 2% NaCl) is added to facilitate the separation of the lipid-rich chloroform layer, which is then recovered and evaporated to dryness [5,49]. Importantly, extraction procedures may selectively affect the recovery of different lipid classes and minor authenticity markers. Therefore, the suitability of an extraction procedure depends on the analytical objective: a method optimized for gravimetric total fat recovery may not provide the same lipid class representation as a workflow designed for GC-MS lipid profiling or LC-MS lipidomics [16,42]. While ASE and SFE are emerging as efficient and potentially greener alternatives for specific extraction and analytical applications, they should not be considered replacements for the Röse–Gottlieb method, which remains a reference procedure for total fat determination and regulatory purposes [16,42]. The Bligh and Dyer method follows a similar principle but was originally optimized for leaner tissues, making it more efficient for low-fat dairy products like dried milk powder, because it requires lower solvent volumes and minimizes matrix interference [50,51]. Recent modifications to the Bligh and Dyer protocol have advanced the development of more environmentally friendly fat extraction methods for goat, sheep, and cow cheese by replacing methanol with ethanol and dichloromethane with petroleum ether [52]. One of the developed protocols demonstrated a significant improvement in method greenness without substantially affecting the efficiency of fatty acid extraction.

In conclusion, traditional extraction methods involve unique procedures and, consequently, employ different chemicals that affect the amount of extracted fat and fatty acids, leading to variations in their content [51,52]. For instance, a comparison of the Bligh and Dyer and Mojonnier methods for extracting fatty acids from bovine milk indicated no difference in the total extracted fat and the content of certain saturated fatty acids (SFAs) and unsaturated fatty acids (USFAs) using both methods, including C11:0, C16:0, C18:0, C14:1, and C16:1 [51]. However, most SFAs showed significant differences when the Mojonnier method was compared to the Bligh and Dyer method, with the opposite trend observed for USFAs. Specifically, the Bligh and Dyer procedure is particularly suitable for low-fat and high-moisture dairy matrices because it utilizes reduced solvent volumes and adjustable solvent ratios, thereby effectively avoiding matrix interferences from non-lipid polar components [42]. Short- and medium-chain fatty acids measured by GC were more abundant in Mojonnier fat than in Bligh and Dyer fat, whereas long-chain fatty acids were more prevalent in the latter. Modern laboratories are increasingly shifting toward Accelerated Solvent Extraction (ASE), also known as Pressurized Liquid Extraction (PLE), to streamline the preparation process. ASE utilizes high temperatures (up to 125 °C) and high pressures

(up to 10.3 MPa) to force organic solvents into the sample matrix, significantly increasing extraction efficiency and reducing the volume of solvent required. This automated technique allows for the rapid processing of large batches of cheese or butter samples, providing a clean lipid extract that is immediately ready for chromatographic analysis [9,35,53]. SFE, in particular, maintains high selectivity but primarily removes nonpolar lipid material, potentially excluding polar markers relevant for comprehensive lipidomics [42]. In addition to these methods, emerging green techniques such as ultrasound-assisted and microwave-assisted extraction have shown promise as alternative approaches for extracting dairy fat. The benefits of employing greener extraction techniques include achieving higher-quality products in a more time-efficient manner, lowering energy usage, utilizing environmentally friendly technology, and maintaining a cost-effective balance [54].

For rapid industrial testing where high-precision profiling is not required, the butyrometric (Gerber) method remains the standard routine procedure [55]. This method involves the dissolution of milk proteins, particularly the phospholipid envelopes of milk fat globules, using concentrated sulfuric acid, after which the addition of amyl alcohol helps release the fat [42]. The fat content is then quantitatively separated by centrifugation and read directly from a calibrated butyrometer scale [45,55].

Although the Gerber method is fast and cost-effective, it does not provide an extract suitable for determining the presence of foreign fats, as the harsh chemical treatment causes sulfonation and hydrolysis of unsaturated fatty acids, thereby altering the lipid's fine chemical structure and rendering the extract unsuitable for fatty acid profiling [42,56]. A structured comparison of the extraction methods discussed above is provided in Table 2, which summarizes their fundamental chemical principles, primary applications, and applicability to different dairy matrices.

Table 2. Comparison of fat extraction methods for dairy analysis.

Method Name	Extraction Principle	Primary Use/Application	Author(s) and Year
Röse–Gottlieb	Gravimetric method involving alkaline hydrolysis by ammonia and repeated liquid–liquid extraction (LLE) using diethyl ether and petroleum ether	Reference method for ultimate precision in raw and drinking milk fat content	(Kala et al., 2018; COSMT, 2011) [42,45]
Mojonnier	Gravimetric method essentially same as Röse–Gottlieb; separates the lipophilic ether phase from the rest of the milk sample	Common reference method in North America for milk and dairy products	(Kleyn et al., 1988) [44]
Folch Method	Two-phase partition using a chloroform-methanol-water solvent system (typically 2:1 ratio)	Reference method for detailed fatty acid and sterol profiling; used for marine products and milk	(Hajrulai-Musliu et al., 2021; Folch et al., 1957) [5,47]
Bligh and Dyer	Two-phase partition using chloroform and methanol; optimized for complete separation and clarification	Optimized for lipid extraction from lean tissues and dried dairy products	(Bligh and Dyer, 1959) [48]
Accelerated Solvent Extraction (ASE/PLE)	Uses elevated temperature (up to 125 °C) and pressure (e.g., 10.3 MPa) to increase extraction efficiency and speed	Rapid extraction for TGs and FAs analysis from cheese and fat-based products like Kajmak	(Petronijevic et al., 2019; Radovanovic et al., 2025) [9,35]

Table 2. *Cont.*

Method Name	Extraction Principle	Primary Use/Application	Author(s) and Year
Butyrometric (Gerber)	Dissolution of proteins and fat globule membranes by concentrated sulfuric acid and amyl alcohol	Standard routine method for quick fat content reading in milk, cream, and cheese	(COSMT, 2011; ISO 1966:2018) [45,55]
Supercritical Fluid Extraction (SFE)	Uses supercritical carbon dioxide (sc-CO2) as a solvent under high pressure	"Green" extraction technology for infant formula, buttermilk, and soy-based powders	(Wolf et al., 2003; Astaire et al., 2003) [57,58]
Dry Column Method	Sample is dried with anhydrous sodium sulfate/diatomaceous earth, then lipids are eluted via dichloromethane and methanol	Simultaneous class separation of lipids from milk and buttermilk	(Marmer and Maxwell, 1981; Maxwell et al., 1986) [59,60]
Weibull–Berntrop	Gravimetric method involving hydrolysis by hydrochloric acid followed by extraction with n-hexane	Reference method for infant foods, ice cream, and specialized dairy products	(Kala et al., 2018, ISO, 8262 (1,2,3) -2005;) [42,61–63]
Solid-Phase Microextraction (SPME)	Uses a fiber coated with a silica-based sorbent to extract lipids directly from the sample	Rapid characterization of milk lipidomes (DAG, TAG) without large solvent volumes	(Garwolińska et al., 2017) [64]

Choosing the appropriate extraction technique is crucial, as the purity and structural integrity of the recovered lipid fraction directly influence the accuracy of subsequent chromatographic analysis.

3.2. Instrumental Methods for Fat Analysis

Once the lipid fraction has been isolated, it must be analyzed to identify the chemical "fingerprints" that differentiate milk fat from foreign adulterants. GC is the most widely utilized and internationally recognized reference technique for this purpose [2,9]. The most common application of GC in dairy fraud is the analysis of Fatty Acid Methyl Esters. In this procedure, the extracted fat is transesterified—often using reagents like boron trifluoride (BF_3) in methanol or methanolic potassium hydroxide—to convert complex triacylglycerols into volatile methyl esters [2,65]. These esters are then separated on high-polarity capillary columns (e.g., HP-88 or CP-Sil 88) and detected using a flame ionization detector (FID), allowing for the quantification of individual fatty acids from C4:0 to C24:0 [5,29].

The presence of foreign fats is typically detected through significant deviations in the fatty acid profile. For example, the addition of palm oil is characterized by a sharp increase in palmitic (C16:0) and oleic (C18:1n-9) acids, whereas coconut oil significantly boosts the levels of lauric (C12:0) and capric (C10:0) acids [29].

However, the analytical reliability of fatty acid profiling is heavily influenced by the inherent biological variability of milk fat, which is significantly shaped by animal breed, geographic origin, production system, and stage of lactation [3,66]. Environmental factors such as climate and seasonal changes (e.g., winter vs. summer supply) lead to highly variable and sometimes atypical fatty acid profiles, making interpretation difficult [7,23]. For instance, feeding practices, specifically the transition between fresh pasture grazing and indoor silage-based feeding, can drastically shift the proportions of saturated and unsaturated fatty acids, thereby altering the authentic reference distribution [41,66]. This biological variability can increase the risk of false-positive classification when authentic samples fall outside narrowly defined reference ranges [7]. Conversely, low-level substi-

tutions may be more difficult to distinguish from natural biological variability, as minor additions can be masked by seasonal or herd-level fluctuations [7,41]. To overcome the limitations of fatty acid profiling, researchers utilize intact triacylglycerol (TAG) analysis by GC, which separates TAG molecules based on their total carbon number, typically ranging from C24 to C54 [16,39].

To evaluate potential adulteration, TAG data is processed using the Precht formulae (S-values), an approach refined by Molkentin (2007) and incorporated into the international reference standard ISO 17678:2019 [39,40,67]. In this context, it is crucial to distinguish between the primary analytical objective of detecting a deviation from authentic TAG relationships and the subsequent objective of quantitatively estimating the exact proportion of an adulterant [39]. S-value calculations are primarily used to assess whether the TAG composition deviates from the expected relationships of authentic milk fat; however, the estimation of incorporated levels should be approached with caution. These models provide an approximation of the adulterant proportion based on regression equations, which must account for the inherent natural fluctuations in the milk fat matrix rather than representing a strictly precise analytical quantification [32,39].

While the TAG method is more robust against common feeding variations, the S-value assessment is based on a defined statistical confidence interval (99%), meaning false-positive classifications cannot be entirely excluded for extreme statistical outliers. Furthermore, method limitations apply to technologically processed fats (such as cholesterol-removed or fractionated milk fats) or matrices with high phospholipid content (such as buttermilk), where co-elution can shift S-values outside admissible limits [39].

If the calculated S-value exceeds the admissible limits, the sample is identified as adulterated. Individual studies applying this methodology have reported successful discrimination at approximate adulteration levels in the range of 2–5% for various vegetable oils and animal tallows [32,37]. It is important to clarify that these values represent practical detection ranges achieved within specific experimental frameworks rather than universally validated limits of detection (LOD) [32,39]. The actual capability to detect fraud depends heavily on the specific nature of the adulterant and the degree to which biological variability in the authentic reference samples, driven by factors like season and geographic origin, shifts the baseline distribution [3,32].

An alternative to the official Precht method has been developed, employing short capillary columns with apolar stationary phases that yield results equivalent to those of the packed columns [68]. Additionally, Linear Discriminant Analysis (LDA) and other multivariate statistical techniques are increasingly used to group samples and identify outliers based on their overall TAG 'fingerprint' [69]. These techniques serve as the basis for advanced machine learning and statistical classification models and will be discussed more comprehensively in Section 3.5. In addition to fatty acid profile, lipidomic analysis offers a powerful approach for detecting milk fat adulteration by providing detailed profiling of milk lipid species. Advanced techniques, such as LC–MS and tandem MS, enable the identification and quantification of triglycerides and phospholipids, revealing unique lipid signatures specific to milk origin [70].

While these high-dimensional signatures are promising, they must be evaluated critically regarding their suitability for routine regulatory testing. Current challenges include the high cost of sophisticated mass spectrometry instrumentation (e.g., Q-TOF or Orbitrap MS), the requirement for highly skilled personnel, and significant instrument dependence that limits the transferability of methods between different laboratories [16,71]. Furthermore, the field currently lacks universally standardized protocols and comprehensive, globally interoperable databases for lipid species annotation and isotopic correction, which are essential for ensuring reproducibility and confidence in regulatory enforcement [7,16,71].

Consequently, although lipidomics provides a deeper analytical view than traditional profiling, it is not yet suitable for routine high-throughput screening in standard quality control environments [16,71].

Another established marker for vegetable fat adulteration of dairy products is the analysis of the sterol fraction via GC or high-performance liquid chromatography (HPLC). Authentic milk fat contains cholesterol as its primary sterol (typically over 95%), while vegetable oils contain characteristic phytosterols like β-sitosterol, campesterol, and stigmasterol. The detection of even trace amounts of β-sitosterol is a highly specific marker of the presence of plant-based oils, even when the fatty acid profile remains within seemingly normal ranges [3,15,23]. However, analytical interpretation remains dependent on method performance, matrix effects, and the precise definition of detection thresholds; for instance, the risk of false-positive outcomes must be managed, such as the potential misidentification of *β*-sitosterol with naturally occurring compounds like lanosterol or traces originating from specific animal diets [3,37]. Importantly, while phytosterol analysis is highly effective for detecting plant-derived material, this approach does not address animal-fat substitution (e.g., lard or tallow), which necessitates the use of alternative indicators such as squalene or specific cholestadiene isomers [7,28]. HPLC coupled with a photodiode array detector (PDA) or mass spectrometry (MS) is particularly effective for this analysis, as it can separate and quantify different sterol isomers with high sensitivity [28,41].

3.3. Spectroscopic and Emerging Rapid Methods

In response to the need for faster, non-destructive testing, vibrational spectroscopy has become a vital tool in food fraud detection, offering rapid, specific, and environmentally friendly alternatives to conventional wet-chemical and chromatographic procedures [66,72]. While conventional chromatography and wet-chemical protocols provide high analytical accuracy, their widespread deployment in routine, high-throughput surveillance is frequently constrained by sample destruction, high operational costs, lengthy extraction steps, and the requirement for technical-grade hazardous solvents [72]. Fourier-transform infrared FTIR spectroscopy, specifically in the mid-infrared (MIR) and near-infrared (NIR) ranges, identifies molecular structures by measuring the absorption of light at specific wavelengths corresponding to chemical bonds like C-H, C=O, and P=O [42,73]. Adulterated samples exhibit shifts in these "vibrational fingerprints," which can be identified using chemometric models such as Partial Least Squares (PLS) regression [14,38]. Spectroscopic approaches offer the major advantage of minimal sample preparation and the complete absence of toxic solvents, though their analytical reliability depends heavily on the robustness, breadth, and representativeness of their underlying reference databases [20,41,72].

Raman spectroscopy is another powerful optical technique that measures the inelastic scattering of light to characterize the rotational–vibrational states of lipids. It is particularly effective for the rapid discrimination of vegetable oils and margarine in products like cream and yogurt, often achieving high classification accuracy in classification models [49]. Surface-Enhanced Raman Spectroscopy (SERS) further amplifies these spectral signals, enabling the identification of subtle chemical adulterants, though issues regarding substrate reproducibility and operational cost remain considerations for routine use [72].

Similarly, Fluorescence spectroscopy utilizes the natural luminescent properties of dairy components like riboflavin, vitamin A, and tryptophan to detect structural changes caused by foreign oils [74,75]. These spectroscopic methods are often combined with electronic noses (E-noses) or electronic tongues, which use sensor arrays to mimic human senses and detect volatile organic compounds or taste markers associated with adulteration [76,77]. Combining e-nose and e-tongue systems with contemporary analytical methods, including

gas chromatography–mass spectrometry (GC-MS and GC-MS/MS) and pattern recognition driven by artificial intelligence, significantly improves their accuracy and usefulness [77].

The scientific rigor of dairy authenticity studies relying on vibrational spectroscopy depends heavily on the robust validation of chemometric models. It is essential to distinguish between unsupervised exploratory techniques, such as principal component analysis (PCA), used for initial pattern visualization, and supervised classification methods used to build predictive models [78,79]. Finally, DNA-based techniques and biosensors represent the forefront of high-specificity detection. PCR and duplex-PCR are used primarily to verify the animal species of dairy products, such as detecting cow milk in buffalo mozzarella or sheep milk cheese [21,80]. On the other hand, biosensors use biological receptors like enzymes or antibodies to produce an electrical or optical signal in the presence of specific contaminants or adulterants [6,81]. These emerging technologies offer the potential for portable, on-site testing at village milk collection centers, providing a critical line of defense against the initial stages of food fraud [6,27]. A comprehensive summary of the analytical performance, key chemical markers, and practical applications of these instrumental methodologies is presented in Table 3.

Table 3. Synthesis of research on adulteration types and analytical detection methods.

Author(s) and Year	Adulterant(s)	Sample Matrix	Analytical Method(s)	Key Findings/Results
Gutiérrez et al. (2009) [32]	Pork lard, beef tallow, vegetable oils, fish oil	UHT Milk fat	GC (TAG analysis) + LDA statistical model	Detected inclusion <5%; 14 of 36 market samples were positive for fraud.
Rani et al. (2015) [24]	Vegetable oils	Ghee	RP-TLC (β-sitosterol tracer)	Protocol based on tracers; no sophisticated equipment required for detection.
Uzunov et al. (2018) [29]	Palm oil, Coconut oil	Cheese, Sour cream	GC-FID (Fatty acid profiling)	Palm oil found in 21 samples, coconut in 2; 23 out of 60 total samples were positive.
Salem et al. (2019) [25]	Coconut oil	Milk fat	MP, RI, Sap/Iodine values, GC (FAs)	Adulteration increased RI/Sap values and decreased IV and Melting Point.
Petronijevic et al. (2019) [9]	Palm fat	Cow and Goat Cheese	HPLC-RI (TAG via ECN groups) + PCA	35 of 121 samples were positive; method works with low-cost instrumentation.
Nurseitova et al. (2019) [15]	Rapeseed and other vegetable oils	Commercial Milk	GC-MS (Fatty acids and Sterols)	Phytosterols provided clear evidence of fraud; eight of 17 packages were positive.
Karacaglar et al. (2019) [49]	Vegetable fat blends, Margarine	Cream, Yogurt	Raman Spectroscopy + PCA	Successfully discriminated pure milk fat from vegetable fats and binary mixtures.
Khorsandmanesh et al. (2020) [28]	Palm oil and fractions	Milk fat	GC (Sterols) and HPLC (Squalene)	Sterol analysis combined with squalene detection confirmed fraud at low levels.
Nurseitova et al. (2021) [3]	Various vegetable oils	Butter, Sour cream	GC-MS (Fatty acids and Sterols)	Sterols were more reliable for slight substitutions; 11 of 18 samples were adulterated.

Table 3. *Cont.*

Author(s) and Year	Adulterant(s)	Sample Matrix	Analytical Method(s)	Key Findings/Results
Hajrulai-Musliu et al. (2021) [5]	Palm and Coconut oil	Sour cream	GC-FID (Fatty acid profiling)	Detected fraud in four of 55 samples; identified via C12:0 and C16:0 markers.
Hazra et al. (2021) [27]	Sunflower, Palm, Cotton oils	Milk fat	Chromogenic (ABTS solution)	Visual color change used for rapid on-site detection at collection centers.
Radovanovic et al. (2023) [30]	Palm oil, Margarine, Pig fat	Kajmak (Experimental)	Iodine/Acid values and Sensory evaluation	Iodine value was effective for screening; palm oil and margarine were sensorially undetectable.
Chaikov et al. (2023) [74]	Palm fat	Butter	DLS and Luminescence Spectroscopy	New, inexpensive physical methods for identifying vegetable oil/fats in a matrix.
El-Nabawy et al. (2023) [41]	Palm oil	Milk fat	GC (FAs), RP-HPLC (Sterols), FTIR	Sterol markers (β-sitosterol) were more sensitive than the iodine value.
Salim and Abd El-Aziz (2025) [2]	Palm oil	Buffalo Milk fat	Chromogenic test and Physico-chemical constants	Rapid screening test <1 min; 48.75% of the 400 market samples were positive.
Ramani et al. (2025) [37]	Palm oil	Ghee (Clarified fat)	S-values (GC-TAG), HPLC (Sterols), Chromogenic	S-values and sterols were both detected at 5% inclusion; the Chromogenic test used DPPH dye.

The comparative data in the table highlight that while traditional physicochemical constants (such as iodine and saponification values) and chromogenic tests are excellent for rapid, low-cost screening, they often lack the sensitivity to detect low-level (<10%) adulteration. GC remains the most prevalent tool, with TAG analysis generally outperforming simple fatty acid (FA) profiling because it provides a unique molecular fingerprint that is less affected by the animal's diet.

Techniques such as Raman, FTIR, and dynamic light scattering (DLS) have emerged as powerful tools for rapid screening, with several studies reporting exceptionally high classification accuracy for detecting adulterants such as palm oil or foreign milk species under controlled laboratory conditions [71,74]. However, it is critical to distinguish proof-of-concept discrimination achieved under controlled experimental conditions from the diagnostic accuracy expected in authentic market surveillance [82]. The high accuracy values reported in the literature are often linked to specific, homogeneous datasets and validation designs that may not fully encapsulate the inherent complexity of routine applications [71,78]. In particular, predictive performance may decline significantly when models are challenged with independent samples containing biological, geographical, and seasonal variability, as well as instrumental drift and differences in processing technologies [3,82]. For example, it has been observed that machine learning models can experience a 15–30% drop in classification accuracy when tested against data distributions that differ from their training environment, such as variations in raw materials across different climates or supply chains [71]. Consequently, a high numerical performance in a laboratory setting does not guarantee generalizability; robust diagnostic reliability can only be

ensured through rigorous external validation using truly independent test sets that reflect real-world variability across production seasons and instruments [71,82].

3.4. Comparative Evaluation of Methodologies

The selection of an analytical method depends on the specific requirements of the study, balanced between accuracy, cost, speed, and environmental impact. Regulatory status is strictly linked to standardized and validated procedures rather than to instrumental platforms in general. For example, ISO 17678:2019 specifies a gas chromatographic method for determining milk fat purity based on the analysis of triacylglycerols (TAGs), while GC-based sterol analysis is widely used for the specific detection of plant-derived sterols [3,39,83].

However, these techniques are inherently destructive, require highly trained personnel, and involve expensive, technical-grade chemicals for sample preparation [3,20]. In contrast, FTIR and Raman spectroscopy offer the major advantage of being non-destructive and extremely rapid, making them ideal for screening high volumes of samples in industrial settings [14,49]. Furthermore, emerging optical techniques such as Hyperspectral Imaging (HSI) and multi-sensor data fusion approaches are increasingly enhancing the scope of non-destructive quality control by offering spatial-spectral information and higher resistance to misclassification in complex matrices [72]. When defining the analytical scope of these methodologies, they should be prioritized according to their intended use:

Fatty acid (FA) profiling provides an economical and well-established first-line screening tool, though it is often limited by high biological variability that can mask low-level adulteration [7,66].

Sterol analysis serves as a highly specific marker for vegetable fat substitution, as phytosterols provide strong and specific evidence of the presence of plant-derived fat [7,37].

The TAG approach (Precht/ISO) offers broader applicability, as it is validated for detecting both vegetable oils and animal tallows [16,39].

Advanced lipidomics via LC-MS provides high-dimensional information on intact molecular species but currently faces significant barriers regarding global standardization and high operational costs [16,71].

To optimize market surveillance, a two-tier decision framework is proposed:

Level 1: Rapid Screening. Utilizing vibrational spectroscopy (FTIR/Raman), this level aims for high-throughput industrial monitoring. It offers a rapid turnaround (minutes) and low per-sample operational costs but depends strongly on validated chemometric models and representative reference databases to account for natural sources of milk compositional variability, including season, environmental conditions, individual animal variation, and lactation stage [20,66,72].

Level 2: Confirmatory Testing. Samples flagged as potentially non-compliant at Level 1 are subjected to standardized chromatographic methods (ISO 17678:2019 or sterol analysis by GC-MS). This level requires longer analysis times (hours to days) and higher per-sample costs due to specialized personnel and solvent requirements but relies on standardized chromatographic procedures and provides substantially stronger analytical and regulatory evidence [7,39,42].

The environmental and economic aspects also play a significant role in method comparison. Traditional reference methods like Röse–Gottlieb are being phased out of routine use in favor of ASE and scCO2 extraction, which align with "green chemistry" principles by minimizing solvent waste [9,53]. Furthermore, while the initial acquisition cost of spectroscopic equipment is high, the low operational cost and lack of consumables make them more economical in the long run compared to chromatography [20,73]. Ultimately, a combination of rapid spectroscopic screening for initial detection and high-resolution

GC/HPLC verification for legal confirmation provides the most robust framework for ensuring dairy authenticity [1,22].

The technical and operational trade-offs between these analytical strategies are summarized in Table 4, which highlights their respective detection limits and practical utility. The current research emphasizes that while fatty acid profiling is convenient for detecting massive substitutions, only sterol and TAG analyses are sufficiently sensitive to detect "discrete" or slight adulterations (below 10%) without being misled by natural variations in milk composition.

Table 4. Comparative performance of analytical methods for fat adulteration.

Method Category	Primary Adulterant Type Detected	Primary Biomarkers	Reported Adulteration Levels Detectable in Selected Studies	Main Advantages	Limitations	Author(s) and Year
Sterol Analysis (GC-MS/HPLC)	Vegetable fat/oils	Phytosterols (β-sitosterol, Campesterol, Stigmasterol)	Trace levels <10% [3]; 5% β-sitosterol [28,41]	Highly specific marker for vegetable oils; independent of seasonal cow diet or breed.	Not effective for detecting animal fat (lard/tallow) which lacks phytosterols.	(Nurseitova et al., 2021; Khorsandmanesh et al., 2020; El-Nabawy et al., 2023) [3,28,41]
TAG Analysis (GC-FID + S-values)	Vegetable fat/oils and animal fats	Triacylglycerol patterns (C24–C54)	≤5% [32]; 2–8% [39]; 2–5% [16]	International reference method (ISO 17678); detects both animal and vegetable fats; highly reliable.	Requires complex mathematical models (Precht formulae) and high equipment costs.	(Cossignani et al., 2019; Gutiérrez et al., 2009; Molkentin, 2007) [16,32,39]
Fatty Acid Profiling (GC-FID)	Vegetable fat/oils	Individual FAs (C4:0, C12:0, C16:0, C18:1n-9)	~10% for general FA-profile detection [5,29]; 5% when specific FA markers are used [35]	Standard equipment in most labs; identifies massive fraud and specific oil types.	Highly affected by seasonal variation and animal feed; high risk of false negatives.	(Hajrulai-Musliu et al., 2021; Uzunov et al., 2018; Radovanovic et al., 2025) [5,29,35]
Vibrational Spectroscopy (FTIR/Raman)	Vegetable fats/oils and selected chemical adulterants	Molecular "Fingerprints"	1% for selected vegetable-fat mixtures under controlled conditions [49]; 1–5% for selected adulterants [20]; study-specific performance reported for other adulterants [14]	Rapid results (<2 min); non-destructive; requires no toxic solvents or chemicals.	Accuracy depends heavily on the robustness of chemometric databases (PCA/LDA).	(Ceniti et al., 2023; Vlasiou, 2023; Karacaglar et al., 2019) [14,20,49]
Physical/ Chemical Constants	Vegetable fat/oils and animal fats	Iodine Value, Saponification Value, RI	25% [2,22]; 18% [30]	Very low cost; can be performed without sophisticated instrumentation.	Low sensitivity; cannot determine the specific type of adulterant used.	(Salim and Abd El-Aziz, 2025; Salem et al., 2019; Radovanovic et al., 2023) [2,22,30]

Table 4. *Cont.*

Method Category	Primary Adulterant Type Detected	Primary Biomarkers	Reported Adulteration Levels Detectable in Selected Studies	Main Advantages	Limitations	Author(s) and Year
Chromogenic Tests (DPPH/ABTS)	Vegetable fat/oils	Visual color change	10% [2]; 1% [21]; 5% [10]	Designed for on-site screening at collection centers; results in <1 min.	Semi-quantitative; primarily optimized for detecting palm oil.	(Salim and Abd El-Aziz, 2025; Hazra et al., 2021; Ramani et al., 2018) [2,10,27]

Regarding the trade-off between speed and precision, spectroscopic methods (FTIR/Raman) are preferred for their speed and high classification accuracy when using proper statistical models. However, GC-FID remains an important reference technique for confirmatory and regulatory analysis. In this context, detecting low levels of adulteration remains challenging because of the natural variability in milk fat composition, which makes a multiparametric analytical approach essential.

3.5. Chemometrics and Statistical Validation

Statistical and chemometric approaches used to interpret analytical data should be distinguished as unsupervised or supervised, as they address fundamentally different questions. Principal component analysis (PCA) is an unsupervised method used for dimensionality reduction, exploratory data analysis, visualization of sample relationships, trend detection, and identification of potential outliers [84]. Because PCA does not incorporate predefined class information, the separation of sample groups in a PCA score plot should be interpreted as evidence of a multivariate structure rather than a validated classification result. In contrast, Linear Discriminant Analysis (LDA) is a supervised classification method that uses predefined class membership to derive discriminant functions and maximize separation between groups [85]. Consequently, the apparent discrimination obtained by LDA cannot be evaluated solely from the graphical separation of samples; the classification performance must be assessed using appropriate validation procedures and independent test data [78].

Machine learning approaches, including supervised classification algorithms, represent a further methodological category. Unlike PCA, supervised machine learning models learn relationships between analytical variables and predefined response classes and can accommodate complex, including nonlinear, relationships [79,86]. Their analytical validity therefore depends not only on classification accuracy but also on appropriate training/test partitioning or cross-validation, prevention of data leakage, class balance, selection of relevant features, and evaluation using independent samples. Reported accuracy alone can be misleading, particularly when datasets are small, imbalanced, or insufficiently representative of the variability encountered in routine analysis. Appropriate performance measures include sensitivity, specificity, precision, F1-score, balanced accuracy, and, where relevant, receiver operating characteristic (ROC) analysis [78,82,86,87].

Artificial intelligence (AI) should not be considered a single statistical method interchangeable with PCA or LDA. Rather, AI encompasses a broad range of computational approaches, including machine learning and deep learning algorithms, which may be used for classification, prediction, feature extraction, or pattern recognition. Their applicability to analytical authentication and adulteration studies should therefore be judged according to the specific algorithm, data structure, training strategy, validation design, and interpretability of the resulting model [78,79,82]. For review purposes, PCA is most

appropriately considered an exploratory unsupervised tool, whereas LDA and supervised machine learning approaches should be evaluated as predictive classification methods. This distinction is important because exploratory separation observed in unsupervised models does not necessarily translate into reliable predictive performance in independent samples [78,79,82].

For supervised models such as LDA, PLS-DA, and machine learning methods, particular attention should be paid to overfitting, especially in analytical datasets with many measured variables relative to the number of samples [78,85]. Preprocessing, feature/variable selection, dimensionality reduction, and hyperparameter optimization should be performed exclusively within the training data during cross-validation to avoid information leakage [82]. Nested cross-validation is particularly appropriate when model parameters or variables are optimized, as it separates model selection from the estimation of predictive performance [88]. Class imbalance should also be considered because unequal numbers of authentic and adulterated samples can result in misleadingly high overall accuracy; therefore, stratified sampling, class weighting, or resampling strategies should be applied within the training data, with sensitivity, specificity, and balanced accuracy reported alongside conventional accuracy [89].

For supervised classification, confusion matrices provide additional information on class-specific errors and should be considered alongside sensitivity, specificity, and balanced accuracy. Where feasible, confidence intervals should also be reported to indicate the uncertainty associated with the estimated classification performance, particularly when datasets are relatively small or class sizes are unequal. Therefore, a high overall accuracy should not be interpreted as sufficient evidence of model robustness without considering the class-specific performance and uncertainty of the estimates [71,82].

Cross-validation and model evaluation should be performed at the level of independent samples rather than individual analytical replicates when multiple measurements originate from the same specimen. Replicate measurements or aliquots derived from the same sample should remain within the same training, validation, or test partition. If measurements from the same specimen are distributed across different partitions, the resulting information leakage can substantially inflate apparent classification performance and produce overoptimistic estimates that do not reflect true inter-sample variability [71,78,82]. This consideration is particularly relevant for dairy authenticity studies, where multiple spectra or analytical measurements may be obtained from a single milk-fat sample. For quantitative prediction of adulterant concentration, model performance should preferably be assessed using complementary metrics such as RMSECV, RMSEP, R^2, and bias, with RMSEP obtained from genuinely independent samples where possible.

Finally, external validation using samples independent of the calibration/model-development dataset is essential for evaluating generalizability. This is particularly relevant for instrumental analysis because differences between instruments, laboratories, acquisition conditions, and sample preparation procedures may affect model transferability [82]. For dairy authenticity applications, external validation should, where feasible, include samples representing biological and production variability beyond that present in the model-development dataset, such as different production seasons, geographical regions, and animal breeds, in addition to different analytical instruments or laboratories. Such independent validation provides a more realistic assessment of whether a model can be transferred to samples outside the original experimental setting [3,82].

Taken together, these considerations highlight that no single analytical approach is optimal for all authenticity testing scenarios. Method selection should therefore be guided by the intended purpose of analysis, balancing speed, analytical specificity, standardization, operational requirements, and the need for confirmatory evidence. Based on these

principles, the principal analytical approaches can be broadly organized into three application levels: rapid screening, confirmatory analysis, and advanced/research applications (Table 5).

Table 5. Decision-oriented framework for selecting analytical approaches for dairy authenticity assessment.

Application Level	Representative Methods	Target Adulterants/ Applications	Major Confounders	Approximate Time	Personnel	Character	Degree of Standardization
Rapid Screening	FTIR, Raman, NIR, chromogenic tests [14,20,49]	Vegetable fats/oils; selected chemical adulterants	Matrix variability; chemometric robustness	Seconds/ minutes	Low–moderate	Mainly non-destructive	Low–moderate; method-dependent
Confirmatory Analysis	GC-FID, GC-MS, HPLC-MS [7,16,32,39,40]	Vegetable and animal fats; specific lipid biomarkers	Sample preparation; instrument requirements	Hours/days	High	Mainly destructive	High for standardized procedures
Advanced/ Research Applications	LC-MS lipidomics; AI/ML; multimodal data fusion [71,79,82,86]	Complex adulteration; molecular profiling; emerging applications	Small datasets; overfitting; domain shift; lack of reference datasets	Hours/days	High/ specialized	Mainly destructive for MS-based methods	Low–moderate; ongoing standardization

This framework emphasizes that rapid screening is primarily intended for high-throughput surveillance, whereas standardized chromatographic methods provide more robust confirmatory evidence. Advanced lipidomic and AI-based approaches currently have greater value in research and emerging authenticity applications.

4. Future Trends: Green Chemistry and Artificial Intelligence in Dairy Authenticity

The future of dairy authenticity testing is rapidly evolving toward high-efficiency systems that prioritize sustainability, real-time monitoring, and automated data processing. A primary driver in this evolution is the adoption of green chemistry principles, which aim to reduce the environmental and health risks associated with traditional laboratory practices [49,90]. Traditional reference methods, such as Röse–Gottlieb or Folch extraction, are increasingly scrutinized for their heavy reliance on hazardous organic solvents like chloroform and methanol [16,17]. Consequently, there is a global push to replace these with "greener" alternatives, such as supercritical fluid extraction (SFE) using sc-CO2, which provides a selective, non-toxic isolation of the lipid fraction [9,53].

The most significant trend in sustainable analysis is the shift toward non-destructive spectroscopic techniques, which require no chemical reagents and produce zero waste [14,20]. Methods such as FTIR, Raman, and near-infrared (NIR) spectroscopy allow for the rapid "fingerprinting" of milk fat, preserving the sample's integrity for further testing if necessary [21,91]. Furthermore, research is moving toward the miniaturization and simplification of these tools, leading to the development of handheld devices that can be used directly at milk collection centers [14,92]. This shift not only aligns with environmental goals but also significantly lowers the cost per analysis, making high-level quality control accessible to smaller producers [20,50].

Parallel to these green initiatives, the integration of AI and machine learning (ML) is revolutionizing the interpretation of complex analytical data [93–95], with AI serving as a supportive tool that improves data interpretation rather than replacing established analytical techniques.

Unlike conventional chemometric approaches, which often rely on linear dimensionality reduction and unsupervised pattern recognition (e.g., PCA), AI models provide significant advantages in nonlinear feature extraction and the ability to handle high-dimensional lipidomic and proteomic datasets [79]. These approaches improve data interpretation by enabling automated spectral interpretation and uncovering complex, multi-layered relationships between variables that remain hidden in traditional analysis [71,86].

Because milk fat is naturally variable, traditional static limits for fatty acids often result in false positives; however, ML models can account for seasonal and biological fluctuations [3,38]. Advanced algorithms, including Support Vector Machines (SVM), Random Forest, and Deep Learning, are now used to process high-dimensional spectral data with classification accuracies often exceeding 98% [95–97]. Convolutional neural networks (CNN) have specifically demonstrated success in detecting multiple adulterants simultaneously, such as mixtures of different vegetable oils and chemical fillers, in a single automated run [93,94].

However, the inclusion of AI does not inherently guarantee improved analytical reliability. The quality and representativeness of reference samples remain more critical than algorithmic complexity [78,82]. In authenticity research, a sophisticated classifier trained on a narrow collection of genuine samples may perform worse in practice than a simpler, parsimonious model (following Occam's Razor) trained on a well-designed reference population that captures real-world uncertainty [78,98]. Major obstacles currently limiting AI scalability in dairy forensics include the reliance on small datasets that are prone to overfitting, a widespread lack of external validation on independent test data, and the "black-box" nature of deep learning, which limits interpretability for regulators. Furthermore, domain shift between laboratories and the absence of standardized reference datasets remain significant hurdles for cross-platform deployment [71,78]. Looking forward, the most promising trend is the implementation of multimodal data fusion, which is more robust than simply applying increasingly complex algorithms to a single analytical platform [79,82]. By combining orthogonal evidence from fatty acids, triacylglycerols (TAGs), sterols, vibrational spectroscopy, and possibly species-specific molecular markers (e.g., proteins like α-casein or α-lactalbumin), researchers can create a holistic molecular fingerprint that minimizes the risk of false results [79,99].

Finally, the fusion of portable hardware with AI-driven software (version 3.6.1, 2017) is paving the way for "smart" food forensics, where smartphone-based imaging and biosensors provide instant results [6,17]. Digital image colorimetry, coupled with pattern recognition tools, allows even non-specialized personnel to detect deviations in milk quality through simple mobile applications [81,100]. These emerging technologies represent a shift from centralized laboratory testing toward a decentralized, transparent supply chain where fraud can be detected at every stage from farm to fork [1,101]. Ultimately, the combination of eco-friendly extraction and intelligent data processing will provide the robust framework necessary to safeguard the global dairy market against increasingly sophisticated fraudulent practices [14,20].

The current trajectory of dairy authenticity research suggests that the integration of green chemistry and AI-driven screening is no longer merely a trend, but a fundamental necessity for global supply chain integrity. While high-resolution chromatography remains the indispensable legal reference, the practical prevention of fraud increasingly depends on the deployment of rapid, intelligent tools at the point of milk collection. Moving forward, the synergy between sustainable extraction methods and robust digital forensics will define the next generation of food safety, ensuring that consumer trust is maintained through a transparent 'farm-to-fork' framework.

5. Conclusions

Food fraud, particularly the Economically Motivated Adulteration of dairy products, represents a persistent and evolving threat to the global food supply chain. Milk and its derivatives are uniquely vulnerable due to their high nutritional value, universal demand, and the significant price premium associated with pure milk fat. This research has comprehensively reviewed the diverse landscape of dairy fraud, focusing on the surreptitious substitution of expensive milk fat with cheaper vegetable oils, such as palm and coconut oil, or animal-origin fats like pork lard and bovine tallow. Furthermore, the practice of species mixing, such as diluting premium buffalo or sheep milk with more abundant cow milk, remains a widespread tactic used to deceive consumers and increase profit margins.

The effectiveness of combating dairy fraud depends heavily on the robustness and sensitivity of analytical detection methods. As demonstrated throughout this review, no single analytical method provides a universal solution for dairy authenticity assessment. Rapid spectroscopic and other screening approaches are valuable for high-throughput monitoring, whereas chromatographic TAG and sterol analyses provide more specific confirmatory evidence depending on the suspected adulterant. Traditional physicochemical constants and chromogenic tests can provide useful, low-cost screening options for substantial adulteration, but their sensitivity and specificity are generally insufficient for the reliable detection of low-level or complex adulteration.

In contrast, high-resolution instrumental techniques, such as GC and HPLC, remain key approaches for definitive legal verification. TAG analysis offers broader applicability for detecting both vegetable and animal foreign fats, while sterol analysis is particularly effective for identifying vegetable fat substitution through the presence of phytosterols. However, animal fat adulteration remains more challenging to detect than vegetable fat substitution, as it may lack distinctive plant-specific biomarkers. In addition, natural variation in milk fat composition arising from factors such as season, breed, geographical origin, and animal diet remains an important confounding factor, particularly for fatty acid-based and spectroscopic approaches. In the future, the dairy industry is expected to transition toward more sustainable and technologically advanced monitoring frameworks. The integration of green chemistry principles reduces the environmental footprint of laboratories by phasing out toxic solvents in favor of non-destructive spectroscopic methods and supercritical fluid extraction. Simultaneously, the application of AI and ML is providing new opportunities for data interpretation and multimodal analysis, but increased algorithmic complexity alone does not guarantee improved analytical reliability.

Overall, safeguarding the integrity of the dairy market requires a multilayered analytical approach. By combining rapid portable screening tools for real-time monitoring with high-precision chromatographic verification for regulatory enforcement, the industry can better protect consumer rights and public health. Future progress will depend not only on more sensitive instrumentation or AI but also on representative authentic reference databases, harmonized validation protocols, interlaboratory studies, transparent reporting of diagnostic performance, and robust evaluation of chemometric model transferability across seasons, breeds, geographical regions, matrices, and instruments. These factors are essential for translating promising methods from controlled experimental studies into reliable routine dairy surveillance and regulatory use. This review underscores the necessity for continuous methodological innovation and standardized and representative global reference databases to ensure that dairy products reaching consumers are safe, authentic, and transparently labeled.

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Abbreviations

The following abbreviations are used in this manuscript:

EMA	Economically Motivated Adulteration
PCR	Polymerase Chain Reaction
SNF	Solid-not-fat
TAG	Triacylglycerol
GC	Gac chromatography
HPLC	High-performance liquid chromatography
LDA	Linear Discriminant Analysis
FTIR	Fourier-transform infrared
AI	Artificial intelligence
LLE	Liquid–liquid extraction
SFAs	Saturated fatty acids
USFAs	Unsaturated fatty acids
ASE	Accelerated Solvent Extraction
PLE	Pressurized Liquid Extraction
Sc-CO_2	Supercritical carbon dioxide
FID	Flame ionization detector
PDA	Photodiode array detector
MS	Mass spectrometry
MIR	Mid-infrared
NIR	Near-infrared
FA	Fatty acid
DLS	Dynamic Light Scattering
GC-MS	Gas chromatography–mass spectrometry
SFE	Supercritical fluid extraction
ML	Machine learning
RMSECV	Root mean square error of calibration of cross-validation
RMSEP	Root mean square error of prediction
AOAC	Association of Official Agricultural Chemists
CNN	Convolutional neural network
E-nose	Electronic nose
E-tongue	Electronic tongue

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