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Development and Validation of Chromogenic Test for Palm Oil Detection in Milk Fat

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ABSTRACT

Background: In India, the adulteration of low-priced palm oil with ghee is a prevalent issue, prompting the need for effective purity assessment methods. While several techniques exist, they often have inherent limitations. Thus, there's a growing preference for rapid tests in routine quality control.

Methods: Currently, a DPPH-based chromogenic assay is employed to detect palm oil presence in ghee, using DPPH solution.

Result: This assay demonstrates specificity across pure ghee and palm oil samples and exhibits sensitivity to detect palm oil adulteration levels of up to 3%. The developed method has also been validated using a radical scavenging activity assay and triglyceride analysis by GC-FID is considered the most promising and internationally accepted method for testing the authenticity of milk fat. The protocol is efficient, robust and sensitive, suggesting its suitability as a platform test in dairy food testing laboratories for routine quality analysis.

Key words: Adulteration, Ghee, Palm oil, Quality control.

INTRODUCTION

Milk and ghee have long been cherished for their cultural, culinary and nutritional significance (Gupta, 2022; Kauser *et al.,* 2022). Ghee, a form of clarified butter, has been an integral ingredient in various cuisines across different regions of the world. In India, milk production holds a significant position, as the country is not only the largest producer of milk but also a major consumer (NDDB Report, 2023). Additionally, ghee production in India contributes to both domestic consumption and economic growth.

Milk fat, a key component of milk, offers a range of health benefits. It contains essential fatty acids, fat-soluble vitamins (A, D, E and K) and conjugated linoleic acid, which have been associated with positive effects on cardiovascular health, brain function and immune system regulation (Mollica *et al.,* 2021; Ramani *et al.,* 2023). Adulteration involves the addition or substitution of inferior ingredients or the omission of a valued element, which is common in our country. In India, this malpractice has become widespread and it is mostly used to raise quantity and gain high income. Ghee is a costly product and it is 3-4 times more precious than vegetable oils and animal body fat (Gandhi *et al.,* 2023; Hazra *et al.,* 2015). For this reason, it is highly liable for fraudulent activity. In India, economically motivated producer substitutes the expensive clarified butter fat with cheap oils and fats without affecting its identity (Antony *et al.,* 2018; Wasnik *et al.,* 2019; Gimonkar *et al.,* 2021). Palm oil is frequently used for this purpose because of the same fatty acid profile as milk fat, which can have adverse effects on health due to its high saturated fat content and associated risks of cardiovascular diseases (Ismail *et al.,* 2018; Odia *et al.,* 2015). This scenario has tarnished the image of the dairy industry not only in India but also internationally. Numerous press articles have recently been published reporting that ghee adulteration is practiced widely in different parts of the nation. These incidences indicate that the problem of ghee adulteration is becoming more prevalent day by day. In today's world, the challenge of detecting ghee adulteration has taken on a very severe aspect, especially in the era of global competition, in which product quality is not an option, but a requirement.

According to the literature, several methods for determining the authenticity of ghee have been reported in the past, primarily based on physicochemical constants, fatty acid profile, sterol test, colorimetric tests and unsaponifiable constituents, but all of these tests have some drawbacks (Ramani *et al.,* 2025; Aparnathi *et al.,* 2023; Sudhakar *et al.,* 2023; Naik *et al.,* 2023; Sonvanshi *et al.,* 2024; FSSAI, 2011). Due to the unavailability of quick, simple and effective tests for detecting the presence of palm oil. Existing methods for detecting adulteration of foreign fats in ghee are able to help to some extent when the adulterants such as animal body fats or vegetable oils are added individually, but the problem occurs when a mixture of these is added to ghee

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(Ramani *et al.,* 2019). Due to the increase in the cases of fraudulent activities in the dairy sector, a range of novel and more sophisticated technologies to identify the presence of non-dairy substances in dairy products have been developed all over the world (Akshay *et al.,* 2023). Advanced techniques like HPLC and GCMS utilized for adulteration detection are expensive and time taking.

However, there is an increasing need for the development and validation of easy, rapid and cost-effective methods for the detection of palm oil adulteration in ghee (Hazra *et al.,* 2018). Such methods would enable regular monitoring of ghee quality, ensure consumer safety and support regulatory measures against adulteration practices. A rapid detection method would allow for quick on-site analysis, reducing the dependence on complex laboratory procedures. Moreover, cost-effective techniques would be accessible to a broader range of stakeholders, facilitating widespread adoption and implementation in the dairy industry. This article aims to validate a rapid method for the analysis of palm oil adulteration in ghee, considering the historical and economic significance of milk and ghee, the health benefits of milk fat, the adverse effects of palm oil consumption and the importance of developing practical detection methods. The use of triglyceride analysis by GC is considered the most promising and internationally accepted method for testing the authenticity of milk fat and validating modified tests.

MATERIALS AND METHODS

Chemicals and materials

Analytical grade (AR) methanol, chloroform, hexane, diethyl ether and ethyl acetate were purchased from Thermo Fisher Scientific, Waltham, Massachusetts, USA. DPPH (2,2-Diphenyl-1-picrylhydrazyl) was purchased from Sigma Aldrich, St. Louis, Missouri, USA.

Collection of samples

Cow and buffalo milk were collected from local dairy farmers of Amreli district Gujarat. Palm oil from a reputable brand was purchased from a local store, Amreli.

Samples preparation

For ghee sample preparation

The direct cream method was used to prepare cow and buffalo ghee (Sathiya *et al.,* 2024). To prepare ghee, the cream was heated directly on a flame in a stainless-steel container while being continuously stirred at 120°C. The resulting ghee was then filtered through muslin cloth that was folded six to eight times and finally through Whatman No. 4 filter paper to purify (filter) it further. This process ensured the production of good quality ghee that was free from any impurities. For mixed ghee samples cow and buffalo ghee, samples were mixed in a 1:1 ratio. After completely melting milk fat (ghee) and palm oil at a temperature of 40°C. The adulterated ghee samples were prepared by the addition of Palm oil in four different proportions (1%, 3%, 5%, 7% and10% v/v)into pure ghee. Adulterated ghee samples were stored at 40°C room temperature. During optimization study initially 10%(v/v) adulterated sample were used.

DPPH-based rapid test

To prepare 1.267 mM DPPH dye, accurately 50 mg weighed in a 100 ml. volumetric flask and volume makeup of 100 ml. using ethanol) The solution was covered and kept at a refrigerated temperature. The DPPH chromomeric test was optimized with following conditions.

Two ml of milk ghee was taken in a clean dry test tube and 0.5 ml chloroform was used to dissolve the ghee. Thereafter, 0.75 ml of DPPH solution (1.267 mM) was added to that test tube and mixed for 30 seconds; thereafter observe the colour change. For pure ghee the colour was observed purple but for adulterated ghee samples were changed to violet yellow colour.

Radical scavenging activity (RSA)

The DPPH radical assay was conducted following the procedure outlined by Joshi (2015).

To prepare a stock solution of DPPH radical, 0.45 g of 2,2-diphenyl-1-picrylhydrazyl (with a molecular weight of 394.32) was measured and placed into a 50 ml glass beaker. 25 ml of ethyl acetate was then added to the beaker and the mixture was stirred thoroughly. The beaker was covered and the solution was refrigerated overnight while being stirred slowly and continuously. The next day, the volume of the solution was brought up to 100 ml by adding more ethyl acetate. For the working solution, 1 ml of the stock solution was diluted with ethyl acetate to a final volume of 100 ml, resulting in a concentration of 11.41×10^{-5} mol/liter (Joshi, 2015). For the assay, 0.2 ml of melted ghee was mixed with 3.8 ml of ethyl acetate, followed by the addition of 1 ml of the working stock solution. The absorbance was measured at 520 nm after 10 minutes. For the reference sample, 1 ml of DPPH solution was combined with 4 ml of ethyl acetate. The radical-scavenging activity was determined by calculating the percentage of inhibition.

Triglyceride analysis using GC-FID

The adulteration of ghee was assessed using GC-FID following the ISO 17678 standard method. The analysis utilized an Agilent 7890B GC-FID instrument. For sample preparation, 50 g of the melted ghee sample was filtered through 1 g of sodium sulfate in an Erlenmeyer flask. According to ISO 17678:2010 guidelines, a 1% solution of filtered milk fat in n-hexane was prepared and 1 µl was injected into the GC-FID instrument. The analysis identified 16 peaks using certified reference standards and the S-value was calculated using the formula specified in Table S1 and comparison of the calculated S-values with their respective limits specified in Table S2 (ISO 17678/IDF 202:2010 (E) 2010).

RESULTS AND DISCUSSION

Optimization of the DPPH test

To enhance the efficiency of the DPPH test, various parameters were investigated and optimized as below:

Optimization of dissolving (ghee) solvent to facilitate the DPPH reaction

Four different solvents: methanol, chloroform, hexane and diethyl ether were used as solvent dissolving solvent. In each solvent, the control sample (pure ghee) and the adulterated samples (10% palm oil added to pure ghee) were dissolved. The colour changes were then observed after 1 mL of DPPH dye was added to each solution. This process was replicated three times and the outcomes are depicted in Fig 1A. Methanol, chloroform and hexane clearly distinguished between the control and adulterated samples. The control samples were purple, while the adulterated samples were light yellow. Diethyl ether, on the other hand, failed to distinguish between the colours of the control and treated samples, which were both a similar yellow shade. Furthermore, in methanol as solvent, a two-layer separation between the solvent and the sample was observed. Applications of chloroform and hexane were given promising colour differences but chloroform was observed most stable for a long time. So, Chloroform was considered the best solvent and was used for the optimization of the solvent quantity.

Optimization of solvent (dissolving ghee) quantity to facilitate the DPPH reaction

In this study, we conducted a comprehensive evaluation of distinct volumes of chloroform, specifically 0.5, 1, 1.5 and 2 ml. The purpose of this evaluation was to ensure consistency in test results, which required the repetition of the procedure three times. The findings of this study are depicted in Fig 1B. It was observed that a discernible differentiation between control and adulterated samples emerged when the ghee samples were dissolved in 0.5 mL of chloroform. In particular, the adulterated samples develop distinct yellow colour, in contrast to the control samples' crimson-purple colour remains constant.

However, a corresponding decrease in colour intensity within the control samples was observed when the ghee samples were dissolved in volumes of 1, 1.5 and 2 ml of chloroform. This reduction in colour intensity made distinguishing between control and adulterated samples difficult, especially at lower volumes. 0.5 mL was considered the optimal volume of chloroform to use in the test and was used in further analyses.

Optimization quantity of sample to facilitate the DPPH reaction

In order to optimise the amount of ghee, samples of 1, 2, 3 and 4 ml were used. The results of the three repetitions of the experiment are given in Fig 1C. With 2 ml samples, a clear colour difference was observed; in control samples a deep purple colour was observed, whilst treated samples showed a light-yellow tint. However, colour difference was not visible in 1 ml samples, 3- and 4-ml samples, control samples displayed a mild purple colour. Therefore, 2 ml of ghee was determined to be the optimal amount for further analysis.

Optimization of quantity of DPPH dye to facilitate the DPPH reaction

Quantities of DPPH dye (0.25, 0.50, 0.75 and 1 ml) were systematically varied to determine the optimal quantity that yielded the most pronounced color differentiation (Fig 1D).

The procedure was replicated three times and the resultant color differentiation was evaluated through graphical representation.0.75 ml of DPPH dye produced the most pronounced colour distinction; control samples exhibited a dark purple hue, whereas adulterated samples appeared yellow. In contrast, with 0.25 mL DPPH dye, no differentiation was observed, whereas 0.50 ml produced slight colour differences. As a result, 0.75 mL of DPPH reagent was determined to be the optimal amount for subsequent analyses.

Limit of detection (LOD) of the DPPH test optimized

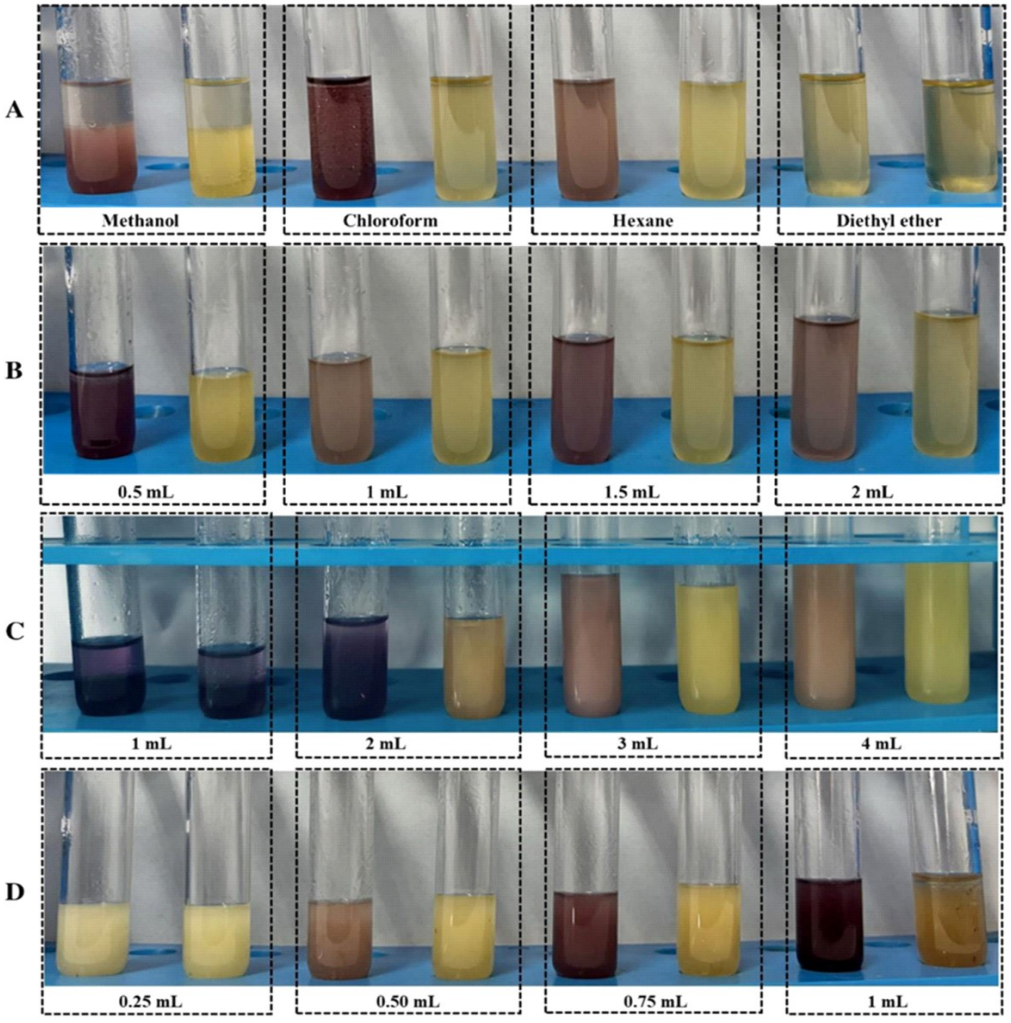
Optimized test condition was used to find the LOD value of this DPPH based test. The result is depicted in Fig 2. It was observed (Fig 2) that ghee adulterated with palm oil @ 3% or more able to differentiate from pure ghee easily. Earlier Ramani *et al.,* (2018) reported to palm oil adulteration in ghee at 5% or more; but few modifications described in this study increased the efficiency of this chromomeric test.

Radical scavenging activity (RSA) of ghee

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, which measures the ability to neutralize DPPH radicals, was used to assess the radical scavenging activity (RSA) of ghee. Fig 3 depicts the experimental results, which show that the RSA of the control ghee sample was significantly lower than that of the palm oil or spike ghee sample. This difference is most likely due to the increased antioxidant compounds by the addition of palm oil. When compared to the control ghee sample, ghee spiked with palm oil had the greatest potential for quenching DPPH radicals. Ghee contained approximately 22.92% of its radical scavenging activity due to the presence of antioxidant compounds, whereas palm oil contained a significantly higher percentage of 86.62%. The addition of a small amount of palm oil (1%), to the ghee sample, did not affect its radical scavenging activity. However, adding more than 3% palm oil resulted in a significant increase in radical scavenging activity, as given in Fig 3. This result was well correlated with rapid chromomeric test.

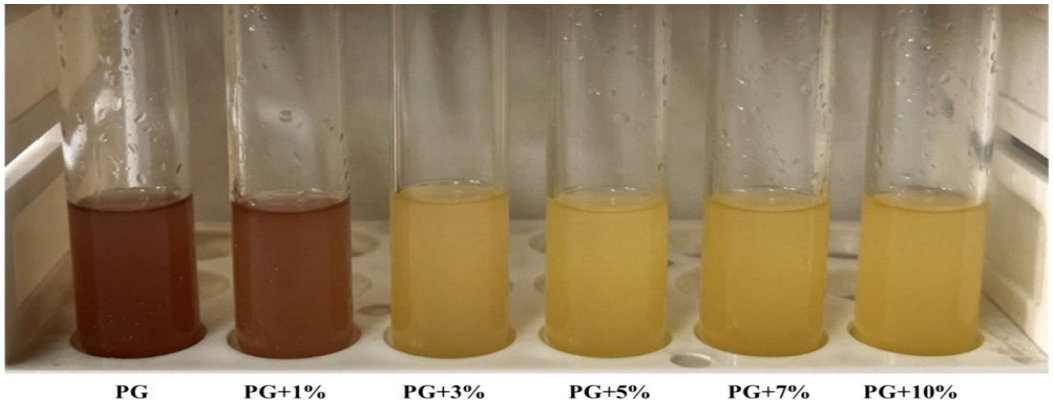
Triglyceride analysis using GC-FID followed by S-value analysis

The ISO 17678:2010 method is utilized for triglyceride analysis, providing an effective means to assess the purity of milk fat. This method defines a specific triglyceride profile characteristic of pure milk fat, consisting of 16 peaks with even carbon numbers ranging from C24 to C54. The variations in triglyceride composition between milk fat and foreign fats are utilized to verify the authenticity of milk fat through GC analysis of its triglyceride profile. The inclusion



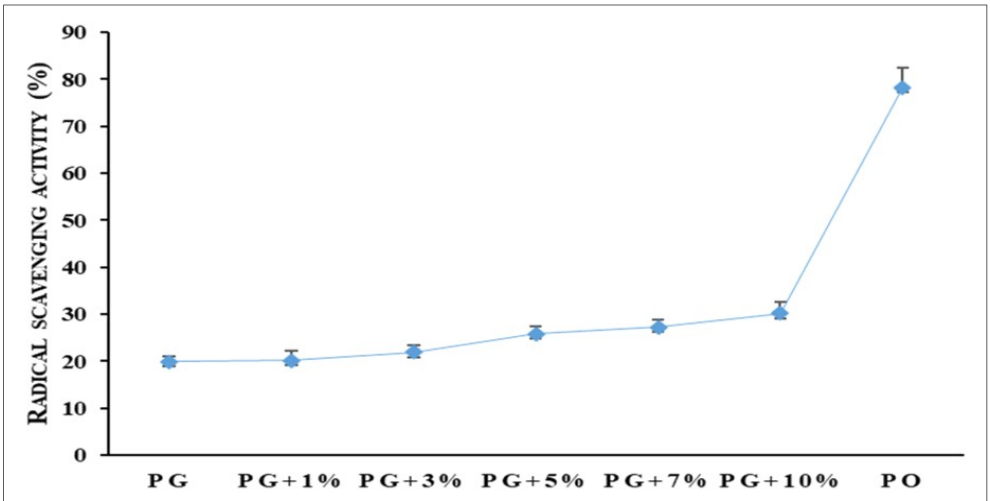
A-Optimization of different solvents to facilitate the DPPH reaction; B-Optimization of solvent quantity to facilitate the DPPH reaction; C-Optimization quantity of sample to facilitate the DPPH reaction; D-Optimization of quantity of DPPH dye to facilitate the DPPH reaction.

Fig 1: Optimization of the DPPH test.



PG- pure ghee; PO- palm oil;

Fig 2: Detection Limit of the Optimized DPPH Test.



All the experiments were conducted in six replicates (n = 6). Significant ($p<0.05$) differences from each other. PG- Pure ghee; PO- Palm oil.

Fig 3: Radical scavenging activity of pure ghee and adulterated samples.

Table 1: Standardized (S)-limits of pure ghee and adulterated ghee samples.

S-limits for pure cow milk fats as per ISO standard							
Equations	Limits	PG	PG + 1% PO	PG + 3% PO	PG + 5% PO	PG + 7% PO	PG + 10% PO
S1	98.05-101.95	99.55±0.14	99.46±0.30	99.31±0.19	99.17±0.23	89.94±0.34	98.67±0.29
S2	99.42-100.58	99.75±0.35	99.84±0.23	99.95±0.21	100.04±0.31	100.42±0.38	101.05±0.28
S3	95.90-104.10	100.17±0.23	99.12±0.29	97.08±0.27	95.27±0.15	93.54±0.24	90.82±0.37
S4	97.96-102.04	100.24±0.19	101.41±0.16	100.96±0.36	101.80±0.35	102.40±0.21	103.12±0.26
S total	95.68-104.32	99.06±0.25	98.21±0.21	96.30±0.31	94.74±0.24	94.74±0.33	88.64±0.30

The experiments were done in triplicate (n=3). All outcomes were mean of replicates.PG- Pure ghee; PO- Palm oil.

Table S1: Equation for calculate the S-value of pure ghee and adulterated ghee samples.

S-value	Equation employed
S1	= (2.0983C30) + (0.728C34) + (0.6927C36) + (0.6353C38) + (3.7452C40) (1.2929C42) + 1.3544C44) + (1.7013C46) + (2.5283C50)
S2	= (3.7453C32) + (1.1134C36) + (1.3648C38) + (2.1544C42) + (0.4273C44) +(0.5809C46) + (1.2926C48) + (1.0306C50) +(0.9953C52) + (1.2396C54)
S3	= (3.6644C28) + (5.2297C30) (12.5073C32) + (4.4285C34) (0.2010C36) + (1.2791C38) + (6.7433C40 – (4.2714C42 + (6.3739C46)
S4	= (6.5125C26) + (1.2052C32) + (1.7336C34) + (1.7557C36) + (2.2325C42) + (2.8006C46) +(2.5432C52)+ (0.9892C54)
S total	= (2.7575C26) + (6.4077C28) + (5.5437C30) (15.3247C32) + (6.2600C34) + (8.0108C40) (5.336C42) + (0.6356C44) + (6.0171C46)

C26 to C54 represents percentage of each major triglycerides fraction containing respective number of carbon atoms in total triglycerides.

Table S2: Specification on limits of S-values for pure ghee and adulterated ghee samples.

S-value	Equations	Limits
S1	Sunflower; Soya bean; Rape seed; olive; Linseed; maize germ; Wheat germ; Cotton seed and fish oil	98.05-101.95
S2	Coconut and palm kernel fat	99.42-100.58
S3	Palm oil and beef tallow detection	95.90-104.10
S4	Lard	97.96-102.04
S total	Total general formula for any foreign fat	95.68-104.32

of any foreign fat or combination of foreign fats in milk fat significantly alters its triglyceride composition, resulting in a noticeable shift in its S-value(s) beyond the established standard limits (Table S2). To address this variability, ISO 17678 establishes S limits based on five regression equations derived from the triglyceride profiles of pure milk fat, encompassing 14 foreign fats, including 11 vegetable oils and 3 animal body fats (Table S1). Triglyceride analysis followed by S-value calculation was conducted on samples of pure ghee and adulterated ghee, as presented in Table 1. This analysis method effectively identifies the presence of palm oil in ghee or milk fat using equation S3, enabling detection of palm oil adulteration at or above 5% (Table 1). Therefore, the modified method holds promise to ensure the quality of ghee at 3% and above (Fig 2).

Conclusion

Detection of foreign oil or fat, in milk fat is similar to detecting the presence of tap water in river water. A straightforward and efficient modification was made to a simple and speedy DPPH-based chromogenic technique to detect the presence of palm oil in ghee. Remarkably, this modified protocol successfully identified even a 3% adulteration of palm oil in ghee, consistently yielding the same results across 50 trials. The method was validated using a radical scavenging activity assay and triglyceride analysis by GC-FID for milk fat authenticity. Consequently, this simplified test holds promise for implementation in routine quality control laboratories, offering an efficient solution for palm oil detection in ghee.

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Conflict of interest

The authors declare no conflict of interest in the presented research work.

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