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ARTICLE

An Ecofriendly Potentiometric Technique to Measure the Iodine Values of Animal Fats

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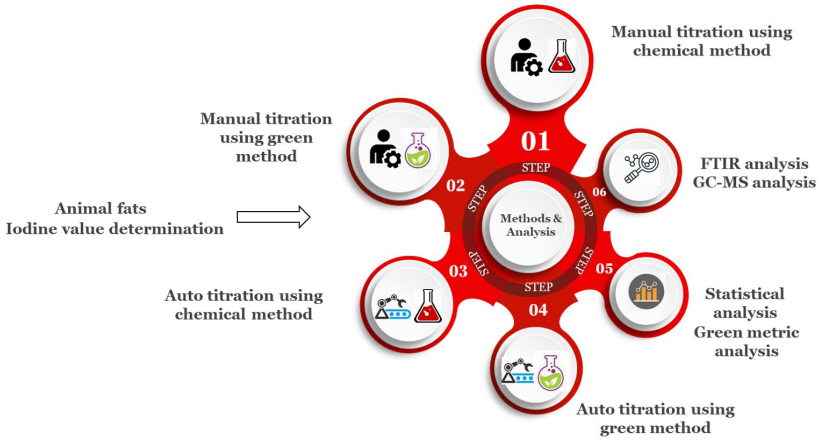

The development of green analytical methods is becoming increasingly important in today's world as we strive to reduce our environmental impact. Wijs' method is one of the analytical experiments used to determine the iodine value in fat/oil that contribute to the environmental impact and are not aligned with the growing demand for eco-friendly practices. The problem that is being addressed is the environmental damage caused by the use of toxic chemicals from traditional chemical methods in analytical experiments. The developed green potentiometric method for determining the iodine value in animal fats yielded notable results. Comparative analysis of the iodine values (g I_2/100 g of oil) of Silkworm pupae oil (SPO), Black soldier larvae oil (BSLO), Milk scum oil (MSO) and Chicken fat oil (CFO) obtained from the traditional Wijs method (Wijs manual WM and Wijs auto WA) and the green method (Green manual GM and Green auto GA) showed close agreement. Students'*t*-test and Snedecor F test were performed and confirmed the accuracy of the four methods at a confidence level of 95% and 99%. The comparable results obtained from the green method validate its accuracy and reliability. Additionally, Fourier-transform infrared spectroscopy (FTIR) and gas chromatography-mass spectrometry (GC-MS) analysis enhanced the understanding of unsaturation levels in animal fats and contributing to a comprehensive assessment of their composition and properties. Moreover, the greenness of analytical procedures was evaluated by Green Analytical Procedure Index (GAPI) and Analytical GREEnness calculator (AGREE). This study advances greener analytical methods and facilitates the analysis of animal fats in various research and industrial applications.

Keywords: potentiometry, animal fats, iodine number, green method, fatty acids, ecofriendly

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INTRODUCTION

Based on Worldometer, India's population is estimated to be around 1.4 billion,[1] with 24% being strictly vegetarian and the rest non-vegetarian. The increasing population drives higher demand in various sectors, especially food production.[2] Mulberry silk production, a major economic contributor, makes India the second-largest silk producer worldwide.[3] The process generates silkworm pupae, containing valuable chemicals like oil, protein, and carbohydrates. Despite their potential, these pupae are mostly discarded or used as fertilizer or animal feed.[4,5] India also leads in milk production, with over 500 large milk processing units.[6] A typical unit processing 200 million L of milk annually requires about 10,000 L of water daily for cleaning, generating significant effluent.[7] This effluent contains suspended solids and organic materials, producing around 90,000 to 100,000 tons of effluent milk scum each year, leading to storage problems and pollution.[8] A study by Aligarh Muslim University highlighted environmental issues around slaughterhouses, with residents reporting water contamination, clogged drains, and unpleasant odors.[9] These animal wastes can be repurposed in industries[10] for products like lubricants,[11] fuel,[12] plasticizer for films,[13] cutting fluid,[14] medical applications,[15] paints, varnishes,[16] and cosmetics.[17] The animal fat oils can be used in different industrial sectors based on the fatty acids present in it. The first step to analyze these oils is to do analytical studies. Analytical studies, particularly the assessment of iodine value in animal fats, are crucial for categorizing industrial products. The iodine value of oil is measured traditionally by using Wijs method (AOCS Official method Cd 1-25)[18] by various sectors. This traditional Wijs method has limitations,[19] which made us to find an eco-friendly alternative, the green potentiometric method, which is environmentally beneficial, cost-effective, accurate, reproducible, and applicable to various materials.[20,21] Benefits of using green potentiometric method for the determination of iodine number of animal fats are listed below.

1. Environmental benefits: Reduce the amount of hazardous waste generated from the analysis but in Wijs's method toxic chemicals are used which are harmful to the environment if not disposed of properly.
2. Cost-effective: Cheaper and readily available reagents are required as compared to traditional methods. Hence reduce the cost of analysis and make it more accessible to researchers and industries.
3. Accurate results: The results obtained with the green potentiometric method are comparable to those obtained with traditional methods such as the Wijs method.
4. Reproducibility: The green potentiometric method can be easily automated and performed with high precision, making it suitable for routine analysis in laboratories and industries.
5. Application to other materials: The green potentiometric method can be applied to other materials besides animal fats and oils, such as for the biodiesel from various oils, which can also benefit from a more environmentally friendly method of analysis.

The comparison of the analytical method carried out in the greener method with the traditional Wijs method based on analysis time, wastage, chemicals used and costs for the analysis are given in Table I.

Table I. Comparative studies with Wijs and green method

Parameter	Traditional Wijs method	Reported potentiometric green method
Time	~1 hour	~10 minutes
Waste	Giving more toxic chemicals at the end of the titration which needed special treatment to discard as well.	Does not have toxic chemicals and also does not need a special treatment to discard it.
Chemicals used	Solvent: Chloroform ($CHCl_3$) Wijs solution (ICl in CH_3COOH) Potassium iodide (KI) Distilled water (H_2O) Sodium thiosulphate ($Na_2S_2O_3$) Starch solution	Solvent: Ethyl alcohol (CH_3CH_2OH) Ethanolic iodine solution (I_2) Distilled water (H_2O) Sodium thiosulphate ($Na_2S_2O_3$) Starch solution
Cost	10X	X

Environmental compatibility of analytical methods is assessed using tools like Green Analytical Procedure Index (GAPI) and Analytical Greenness Metrics (AGREE).[22,23] These metrics evaluate the eco-friendliness of procedures, with GAPI being useful for laboratory practice and AGREE for assessing green analytical chemistry procedures. This paper aims to determine and compare the iodine number (IN) of various animal fats, such as silkworm pupae oil (SPO), black soldier larvae oil (BSLO), milk scum oil (MSO), and chicken fat oil (CFO), using both the Wijs method and a green method. The precision of the obtained iodine values is analyzed statistically using the Student *t*-test and Snedecor F test. Additionally, the level of unsaturation in these animal fats is compared using GC-MS and FTIR analysis. The GAPI and AGREE metrics are employed to evaluate the environmental impact of the iodine number determination methods used.

MATERIALS AND METHODS

Materials and apparatus

Silkworm pupae, black solider larvae, milk scum and chicken fat were procured from local small-scale industries near Kanakapura road, Bengaluru, Karnataka. The oils were extracted in different methods which are discussed later. Totally four oils namely SPO, BSLO, MSO, and CFO were used for further analysis and all the chemicals used in this study are of analytical grade. Chloroform from Isochem, Wijs iodine solution, potassium iodide, starch soluble, sodium thiosulphate (hypo) and iodine resublimed from Nice® Laboratory reagent and ethanol from Changshu Hongsheng Fine Chemical Co., Ltd. were purchased from Bangalore Scientific & Industrial Supplies, Bengaluru. Single distilled water was used for the titration and solution preparation.

Metrohm autotitrator (Model: 877 Titrino plus) was used with electrode (Model: Metrohm 6.0451.100 Pt/ -5 to 80 °C combined platinum ring electrode) associated with automatic stirrer (Model: 801 Stirrer) and exchange unit where the analyte and titrant is placed respectively to perform potentiometric titrations.

Oil extraction methods

Silkworm pupae and black solider larvae oils were extracted by solvent extraction process. The chicken fat was collected by rendering process. The milk scum oil was obtained by melting and phase separation process. The oil extraction process was detailed in Figure 1.

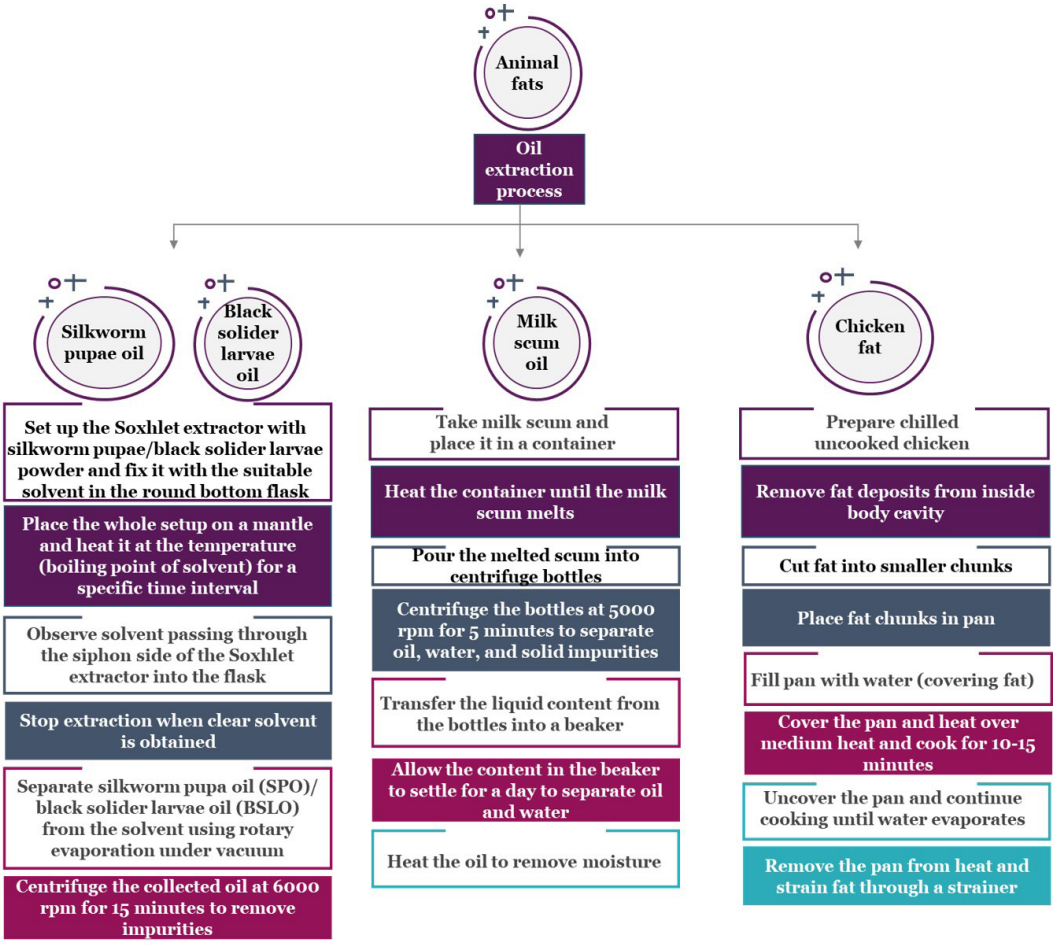


Figure 1. Stepwise procedure for the extraction of SPO, BSLO, MSO, and CFO.

Titration procedure

The following methods were used to determine the IN of SPO, BSLO, MSO, and CFO.

Wijs manual method (WM)

The iodine number of the animal fats and oils were determined by the titration method by using Wijs solution. The oil sample was weighed in an iodine flask according to the expected iodine number. The weighed oil/fat was dissolved in 15 mL of chloroform ($CHCl_3$) followed by adding 25 mL Wijs solution (Iodine monochloride in glacial acetic acid). They were kept closed with glass stopper in dark for 30 minutes along with blank prepared in the same manner except the oil. After 30 minutes, 20 mL of 10% potassium iodide (KI) and 100 mL distilled water were added. They were allowed to stir for 30 seconds. Then titration was started with standardized 0.1 mol L^{-1} $Na_2S_2O_3$ and after reaching light yellow colour, added 3 mL of 1% starch which turns into dark blue in colour. The end point was the disappearance of the blue colour.

Green manual method (GM)

The green method, free from toxic chemicals, involved dissolving the weighed oil/fat in 15 mL ethyl alcohol, stirring vigorously for 2 minutes, heating for 2 minutes at 50 °C, adding 20 mL of 0.1 mol L^{-1} ethanolic iodine solution, and stirring moderately for 5 minutes. Then, 200 mL of cold distilled water (5 °C or less) was added and the mixture was stirred slowly for 5 minutes. The solution was titrated with $Na_2S_2O_3$ until a yellow color appeared. Then, 3 mL of 1% starch solution was added, turning the mixture deep blue. The endpoint was the disappearance of the blue color. For WM and GM methods, the iodine value of the animal fats was calculated using Equation 1.

$$Iodine\ number = \frac{(B - A) \times C \times 12.69}{m} \qquad \text{Equation 1}$$

where: *B* is the volume of the $Na_2S_2O_3$ used for the blank in mL, *A* is the volume of the $Na_2S_2O_3$ used for the sample (oil/fat) in mL, *C* is the concentration of $Na_2S_2O_3$ in mol L^{-1}, *m* is the mass of the sample in g.

Wijs auto method (WA)

The procedure was similar to the WM method, but after adding 150 mL of distilled water, the mixture was stirred for 30 seconds and then titrated using an autotitrator (Model 877 Titrino plus with Metrohm 6.0451.100 Pt electrode) according to Metrohm Application Bulletin 141/4e.[24] The autotitration graphs were analyzed for the different animal fats and are shown in Figures 2 and 3. The autotitration graphs were obtained by creating a method in the autotitrator with Equations 2, 3 and 4 to give the iodine value.

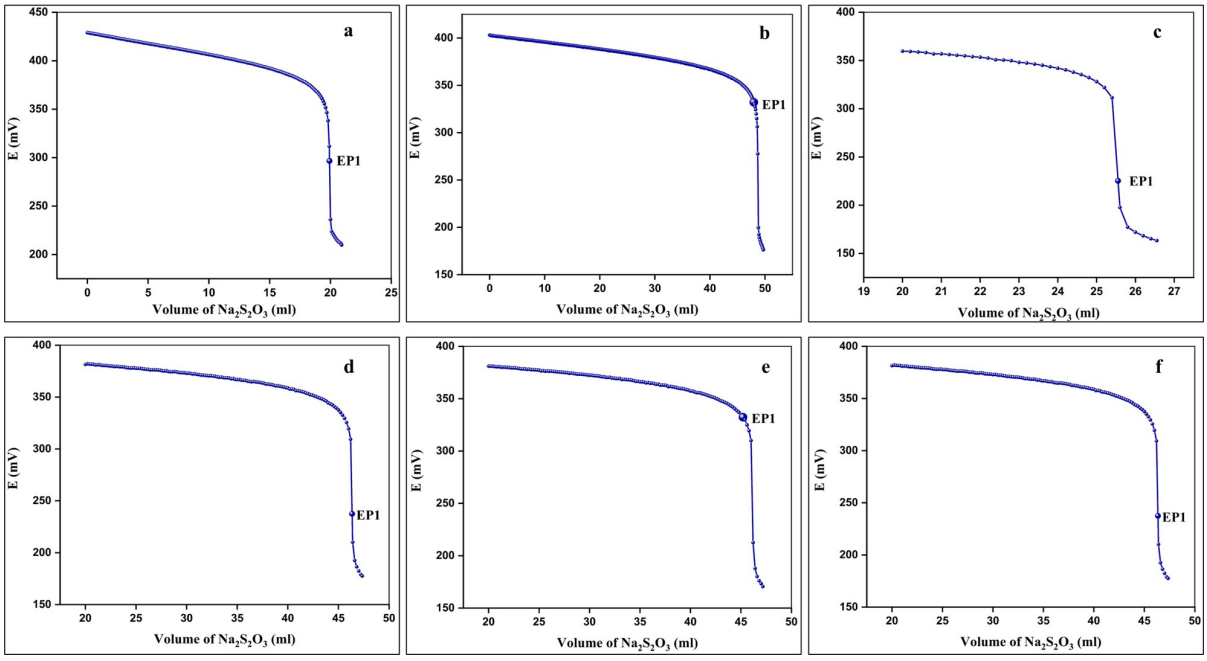


Figure 2. Experimental potentiometric curves for the determination of iodine value by WA. (a) Standardization of $Na_2S_2O_3$ (b) Blank (c) SPO (d) MSO (e) BSLO and (f) CFO.

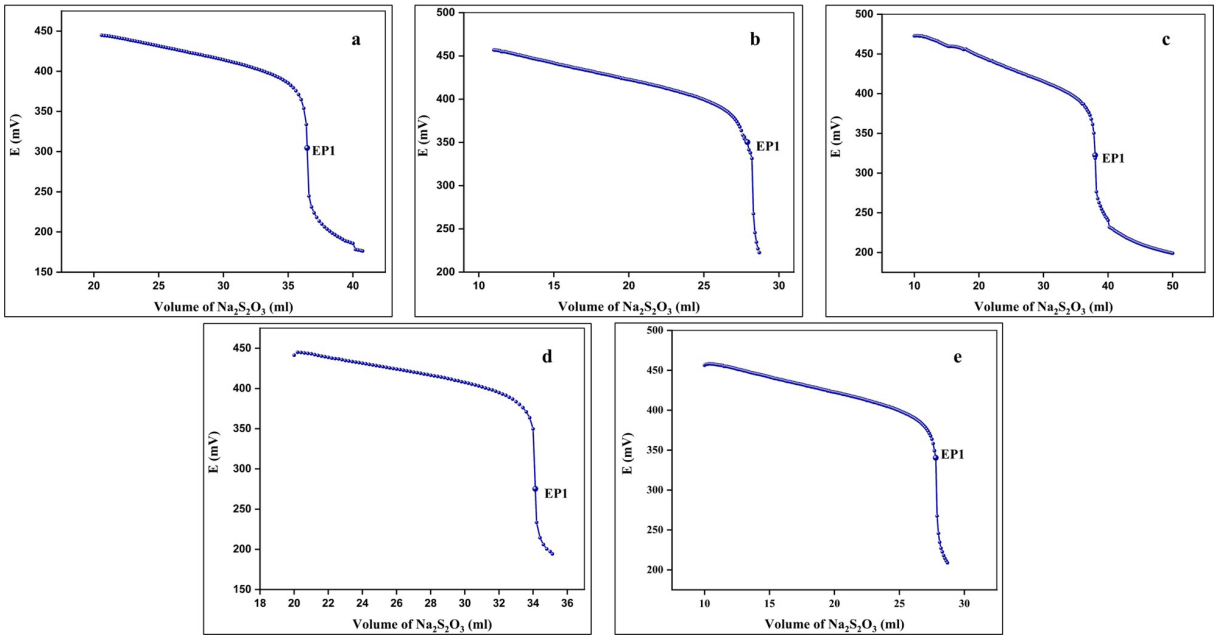


Figure 3. Experimental potentiometric curves for the determination of iodine value by GA. Blank (b) SPO (c) MSO (d) BSLO and (e) CFO.

Green auto method (GA)

The procedure followed the GM method until the addition of 200 mL cold distilled water. After 5 minutes of slow stirring, the mixture was autotitrated using the Model 877 Titrino plus with Metrohm 6.0451.100 Pt electrode. The iodine values were calculated using the autotitrator and reported.

Titer

Standardization of $Na_2S_2O_3$ was done in the autotitrator by dissolving 70 mg of potassium iodate with 80 mL of distilled water followed by adding 10 mL of 10% of potassium iodide and 25 mL of 0.5 M H_2SO_4. The solution becomes dark brown and the iodine liberated is calculated using Equation 2.

$$Titer = \frac{m_s \times 6}{V_{EP1} \times c(\mathrm{Na_2S_2O_3}) \times M_A} \qquad \text{Equation 2}$$

where:

$Titer$: Titer of the selected titrant

m_s : Mass of standard in mg

6: Stoichiometric factor

V_{EP1} : Titrant consumption until the first equivalence point in mL

$c(\mathrm{Na_2S_2O_3})$: Concentration of the selected titrant in mol L^{-1}; here c($Na_2S_2O_3$) = 0.1 mol L^{-1}

M_A: Molecular weight of the analyte; here 214.00 g/mol

Blank

The blank solution is prepared as in the WA and GA methods without the fat/oil and is titrated against $Na_2S_2O_3$ titrant and obtained the end point EP1 which is mentioned in the Figure 2(b) using Equation 3.

$$Blank = V(mL)$$ Equation 3

where:

$V(mL)$: Blank value consumption for the used quantity of solvent in mL

Sample

The sample solution is prepared as in the WA and GA methods, is titrated against $Na_2S_2O_3$ titrant and obtained the end point EP1 which is mentioned in the Figure 2(c–f) and calculated using the Equation 4.

$$IV = \frac{V_{EP1} \times f \times c(\mathrm{Na_2S_2O_3}) \times M_A}{10 \times m_s}$$ Equation 4

where:

IV : Iodine value of the sample in g iodine/100 g

V_{EP1} : Titrant consumption until the first equivalence point in mL

V_{Blank} : Blank value consumption for the used quantity of solvent in mL

$c(\mathrm{Na_2S_2O_3})$: Concentration of the selected titrant in mol L^{-1}; here c($Na_2S_2O_3$) = 0.1 mol L^{-1}

F : Correction factor without unit

M_A : Molecular weight of the analyte; here 126.90 g mol^{-1}

m_s : Sample size in g

10 : Conversion factor

Oil characterization

The FTIR analysis for all oil samples was conducted by placing the liquid samples onto the diamond crystal surface of an attenuated total reflectance (ATR) spectrophotometer. Each spectrum was collected within the wavenumber range of 4000 to 400 cm^{-1} with a resolution of 4 cm^{-1}, performing 64 scans using a Shimadzu ATR spectrophotometer. The fatty acid methyl ester content of SPO and MSO was analyzed by gas chromatography using an Agilent 7890B GC-MS system with a 30 m × 320 µm × 0.25 µm capillary column. Samples were diluted in hexane, and 1 µL was injected into the system. The injector temperature was set at 290 °C, and the detector temperature was maintained at 150 °C with a gas flow rate of 1 mL/min. The fatty acid profiles of BSLO and CFO were reviewed from the literature and reported.

Statistical analysis

Precision

Precision refers to the repeatability or reproducibility of measurements. It measures how close repeated measurements are to each other. In the context of iodine value determination, precision assesses the consistency of results when the same sample is tested multiple times. Calculation of standard deviation or relative standard deviation from replicate measurements. Low values indicate high precision. The titration is replicated for five times in every method to achieve its repeatably and accuracy.

Student t test

The obtained iodine values from various methods were compared by two statistical methods namely Student's test and Snedecor F-test using Equations 5 and 6.[25]

$$t = \frac{x_a - x_b}{\sqrt{\frac{(n_a + n_b)(n_a S_a^2 + n_b S_b^2)}{n_a n_b (n_a + n_b - 2)}}}$$ Equation 5

where:

t : calculated Student value, derived from comparing the arithmetic means obtained from different methods for the same set of samples

x_a and x_b : arithmetic means of the results obtained from the two methods

n_a and n_b : number of parallel determinations carried out in each method to calculate the mean

S_a and S_b : standard deviations of methods A and B

$(n_a + n_b - 2)$: degree of freedom

Snedecor F-test (the variance ratio test)

$$F = \frac{S_A^2}{S_B^2}$$ Equation 6

where:

S_A^2 and S_B^2 : variances of methods A and B

Greenness evaluation in the titration methods

GAPI and AGREE are the metric tools used to assess the greenness of an iodine value determination by WM, WA, GM and GA. The GAPI was analyzed from the software ComplexGAPI v.0.2 beta and the AGREE was done from the AGREE which can be found from the online free website https://agreeprep.anvil.app/. Sample preparation analysis and pre-analysis processes are the two sessions in GAPI. The criteria and the conditions of these two sessions were given in Figure 4. AGREE has 10 green analytical chemistry (GAC) criteria to decide the percentage greenness of the process and the principles were mentioned in Figure 5. The procedures for assigning scores based on the principles of the GAPI and AGREE metrics are explained in Tables S1 and S2 in the supplementary material.

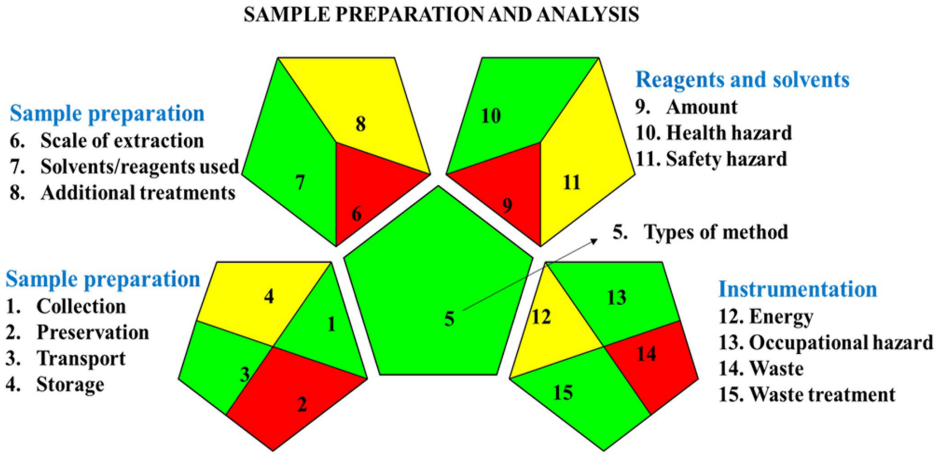


Figure 4. Green Analytical Procedure Index principles.

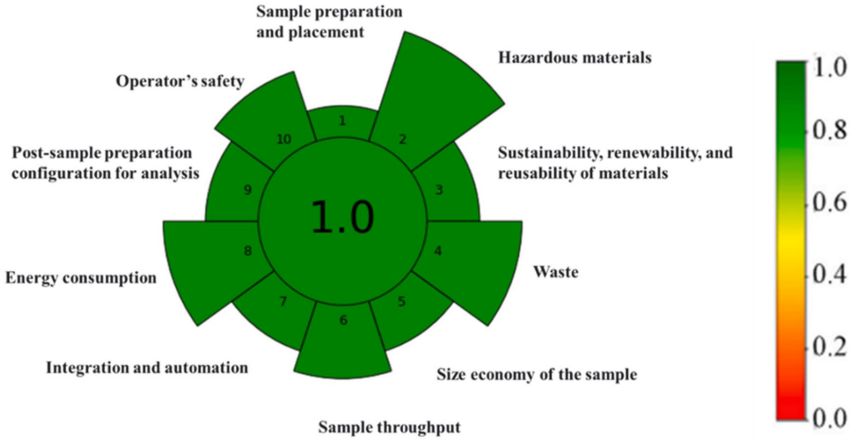


Figure 5. AGREE principles.

RESULTS AND DISCUSSION

Iodine values of animal fat oils obtained by titration methods

Four oils namely SPO, BSLO, MSO and CFO were extracted from the raw materials and collected in a glass vial for iodine number determination. Visually, it was observed that SPO, BSLO and CFO were in yellow color whereas MSO was in brown color as shown in Figure 6. The Wijs method (manual and auto)[26] involves treating oil samples with iodine monochloride, resulting in halogenation and evolution of excess iodine, which is then titrated with sodium thiosulfate. The green method uses ethanolic iodine solution, and the iodine value (IN) is calculated from titration results using specific formulas for GM and GA methods. Scheme I illustrates the titration process in GM and GA method. Table II shows that SPO and CFO have similar iodine values, followed by BSLO and MSO, indicating their degree of unsaturation as in the order of SPO ≈ CFO > BSLO > MSO.

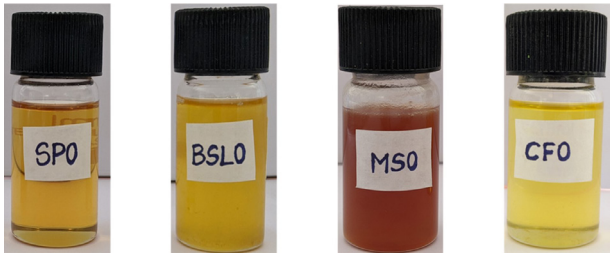
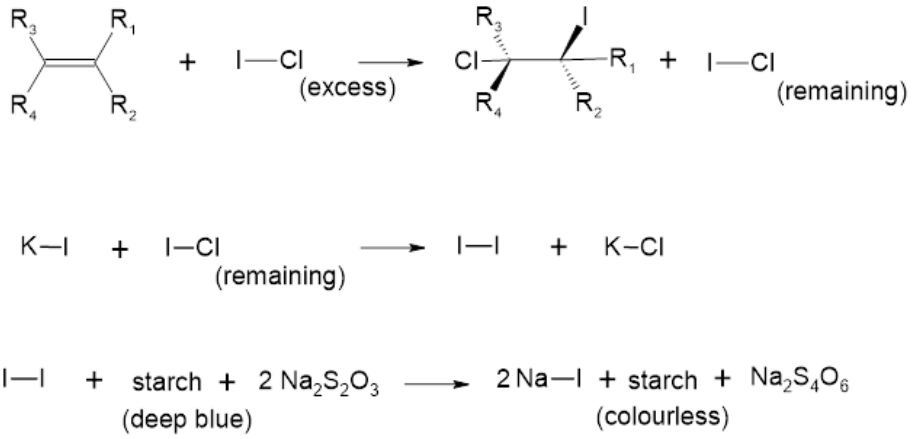


Figure 6. Visual representation of animal fat oils.



Scheme I. Chemical reaction involved in Green method.

Table II. Iodine number of the animal fats from manual and autotitrations using Wijs and green methods. Numbers of determination (n) taken were 5/oil sample.

Oil sample	Wijs manual method (WM) (g I_2/100g oil)	RSD %	Wijs auto method (WA) (g I_2/100g oil)	RSD %	Green manual method (GM) (g I_2/100 g oil)	RSD %	Green auto method (GA) (g I_2/100g oil)	RSD %
SPO	121.202 ± 0.72	0.33	123.17 ± 0.54	0.24	120.082 ± 0.57	0.26	119.52 ± 0.94	0.44
BSLO	41.902 ± 0.56	2.30	35.308 ± 0.55	0.86	38.398 ± 0.77	1.13	35.036 ± 0.60	0.96
MSO	22.378 ± 1.04	2.61	23.732 ± 1.21	2.86	22.642 ± 0.98	2.42	24.274 ± 0.63	1.46
CFO	122.218 ± 1.81	0.83	122.264 ± 1.14	0.52	120.812 ± 0.84	0.38	120.316 ± 1.08	0.50

GC-MS analysis

The fatty acid composition of a fat directly influences its unsaturation level. Fats with a higher proportion of unsaturated fatty acids will have a higher degree of unsaturation i.e., increase in iodine number. Conversely, fats with a higher proportion of saturated fatty acids will have a lower degree of unsaturation. The fatty acid composition was determined by gas chromatography for the identification and quantification of individual fatty acids in a fat sample. From Table III, the total unsaturation of animal fat oils was estimated and the order of unsaturation was found as follows: SPO ≈ CFO > BSLO > MSO. SPO is having the highest total unsaturation of 69.36% (comprises of 32.62% of α-Linolenic acid and 30.25% of oleic acid) among all the four animal fat oils estimated. Followed by CFO which has similar range of unsaturation as SPO i.e., 67.52% of unsaturation (43.94% of oleic acid and 18.26% of linoleic acid), BSLO (39.07%) and MSO having 24.46% of unsaturation. From the degree of unsaturation of oils, the iodine value can be determined. In the fatty acids of the oil, the unsaturated bonds react with the halogens (addition reaction

takes place) during the titration process. The obtained iodine values from different titration methods (WM, WA, GM and GA) goes align with the GC-MS analysis. SPO and CFO were shown the highest iodine values as compare to other animal fats. This proves the fact that the higher the degree of unsaturation in the oil, the higher will be the iodine value, and their comparison is provided in Table IV.[5,27,28]

Table III. Fatty acid composition (% of total fatty acid methyl esters) of various animal fat oils

Fatty acids	SPO	BSLO	MSO	CFO
C10:0 Capric acid	-	1.76	2	-
C12:0 Lauric acid	0.07	35.72	3.13	0.64
C14:0 Myristic acid	0.23	5.03	12.34	1.62
C15:0 Pentadecanoic acid	-	-	1.4	-
C16:0 Palmitic acid	22.86	13.78	38.97	25.39
C17:0 Heptadecanoic acid	-	0.20	0.91	-
C18:0 Stearic acid	6.97	2.81	14.08	4.84
C16:1 Palmitoleic acid	0.75	2.12	1.65	5.32
C17:1 Heptadecanoic acid	-	0.18	0.32	-
C18:1 n-9c Oleic acid	30.25	18.28	21.08	43.94
C18:2 n-6c Linoleic acid	5.74	15.02	1.22	18.26
C18:3 n-3 α-Linolenic acid	32.62	1.95	0.19	-
C18:4 n-3 Stearidonic acid	-	0.54	-	-
C20:5 n-3 Eicosapentaenoic acid	-	0.98	-	-
Total unsaturation	**69.36**	**39.07**	**24.46**	**67.52**

Table IV. Comparison between the average iodine values determined from WM, WA, GM, and GA methods and the unsaturation content in GC-MS data

Oil sample	Iodine value in average	GC-MS data
SPO	121.99	69.36
BSLO	37.66	39.07
MSO	23.25	24.46
CFO	121.4	67.52

FTIR analysis

The relationship between FTIR results and iodine value lies in the fact that FTIR can help identify and quantify specific functional groups associated with unsaturated bonds, such as C=C bonds. Characteristic absorption peaks related to the unsaturation in the oil can provide indirect information about the iodine value or the degree of unsaturation in the oil. The relationship between FTIR results and iodine value is not a direct quantitative correlation. Other factors such as the presence of other functional groups, impurities,

or variations in oil composition can also influence the FTIR spectra and iodine value. Nevertheless, the combination of FTIR analysis and iodine value determination can provide complementary information about the chemical characteristics and unsaturation level of oils.

FTIR analysis reveals functional groups responsible for unsaturation in animal fats (Figure 7), with specific peaks for unsaturated bonds (Figure 8). Peaks at 3003-3007 cm^{-1} indicate CH stretching of double bonds, while other bands confirm C=C stretching and vibrations of methylene and methyl groups.[29,30] Higher monounsaturated fatty acids (MUFA) in oils result in higher peak intensities, with CFO having the highest MUFA content and corresponding peak intensities in the range of 3003-3007 cm^{-1}. Unsaturation is also indicated by FTIR bands at 1651, 966–974, and 719–721 cm^{-1}, corresponding to C=C stretching and –HC=CH– bending and rocking vibrations. The 2954 cm^{-1} peak is due to $–CH_3$ vibrations, and methylene ($–CH_2$) vibrations appear at 2924 and 2852 cm^{-1}. A strong band at 1743 cm^{-1} indicates carbonyl group stretching (C=O) in triglycerides. Similar peaks were reported by Irnawati et al. for lard and chicken fat.[31] Additional peaks at 1463, 1238, 1161, and 1377 cm^{-1} are associated with methylene and methyl bending vibrations,[32] while a band at 1103 cm^{-1} confirms ester groups.[33] Table III shows monounsaturated fatty acids (MUFA) content as 31% (SPO), 20.58% (BSLO), 23.05% (MSO), and 49.26% (CFO), with higher MUFA content resulting in higher peak intensities. The peak intensity order at 3003 to 3007 cm^{-1} is CFO > SPO > BSLO > MSO. A weak band at 1651 cm^{-1}, due to C=C stretching of disubstituted cis C=C in linoleic and oleic acids, is less pronounced in MSO due to its lower content of these acids, as shown in Figure 8.

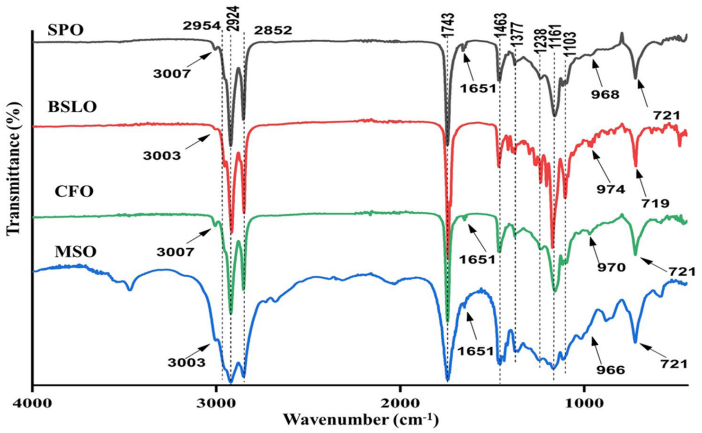


Figure 7. FTIR spectra for SPO, BSLO, CFO, and MSO.

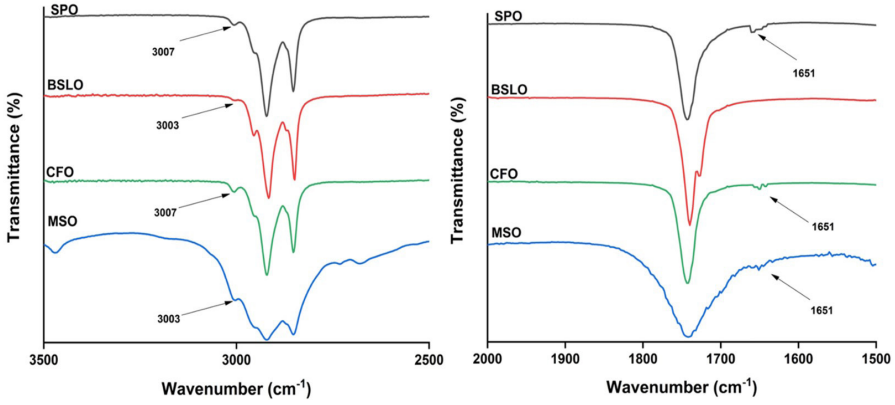


Figure 8. Enlarged view of FTIR in the functional group region.

Statistical analysis

Precision

The obtained iodine values of the animal fats by four different methods were analyzed and their mean and standard deviation of the five titrations per oil/fat sample and their percentages of relative standard deviation (RSD) were reported in Table II. Their average is calculated and their standard deviation (error bars) are mentioned in Figure 9.

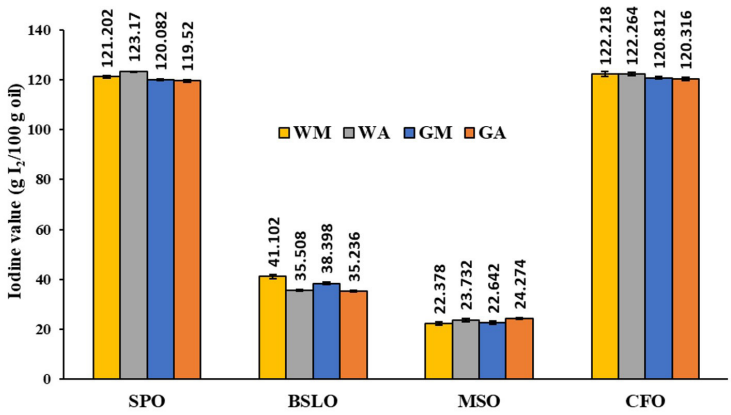


Figure 9. Bar graph represents different animal fats vs their iodine values.

Student t-test

Statistical analysis comparing iodine numbers across four methods for animal fats was performed using Student's *t*-test,[25,34] with results detailed in Table V. The degree of freedom (df) for this analysis was 8. The critical values of *t* for two tailed tests from the *t* test table were found to be 2.306 for the significance level α = 0.05 and 3.355 for the significance level α = 0.01. The obtained *t* values were less than the critical values of the significance level (α = 0.05 and 0.01). So, we failed to reject the null hypothesis and concluded that there was no significant difference between the means obtained by the two methods. Therefore, we concluded that the alternative method gave similar results to the conventional method and used as a suitable alternative. The proposed methods (Green manual and green auto) were statistically equivalent to the Wijs manual and Wijs auto method at 0.95 confidence level, that the mean iodine values obtained by the four methods were not significantly different. Moreover, complete concordance was observed at 0.99 confidence level.

Table V. Comparison of four methods for determining iodine values in animal fats using paired Student's *t*-test. Numbers of determination (n) taken were 5/oil sample. The degrees of freedom (df) for this analysis were calculated as n1 + n2 − 2 = 8.

Oil sample	Student's *t* test values					
	WM vs WA	WM vs GM	WM vs GA	WA vs GM	WA vs GA	GM vs GA
SPO	1.72	0.96	1.13	3.10	2.68	0.40
BSLO	2.51	1.15	2.60	2.42	0.26	2.56
MSO	0.67	0.14	1.24	0.55	0.31	1.11
CFO	0.01	0.56	0.71	0.81	0.99	0.28

WM = Wijs manual, WA = Wijs auto, GM = green method, GA = green auto.

Snedecor F test

From Table VI, the Snedecor F values were calculated for the six different probabilities and to determine the difference in the variances. The degree of freedom (df1) for the first set of values and (df2) for the second set of values were 4. The critical values of F from the F distribution table were found to be 6.38 and 15.97 for the significance level α = 0.05 and 0.01 respectively. The obtained F values were less than the critical values of F from the F distribution table having the significance level α = 0.05 and 0.01. So, we rejected the alternative hypothesis and accepting the null hypothesis and concluded that there was no significance difference in the variation of iodine values between the two methods. Finally, it could be inferred that all the methods utilized in this study exhibit similar precision at a 95% and 99% confidence level.

Table VI. Comparison of four methods for determining iodine values in animal fats: Statistical analysis using Snedecor F test. The number of determinations (n) taken was 5/oil sample. The degrees of freedom (df1 and df2) for this analysis were calculated as n − 1 = 4.

Oil sample	Snedecor F test values					
	WM vs WA	WM vs GM	WM vs GA	WA vs GM	WA vs GA	GM vs GA
SPO	1.75	0.62	1.67	1.09	2.94	0.37
BSLO	9.37	4.74	7.72	1.97	1.21	1.62
MSO	1.35	1.13	2.71	1.53	3.67	2.39
CFO	2.53	4.67	2.81	1.84	0.89	1.65

WM = Wijs manual, WA = Wijs auto, GM = green method, GA = green auto.

Green metrics analysis

GAPI analysis

The analysis of four methods namely WM, WA, GM, and GA using GAPI was assessed and documented in Table S3 of the supplementary material and corresponding pictograms are exhibited in Figure 10-a to -d respectively. When delving into a comprehensive explanation of pentagrams, the first subset revolves around sample collection, which remains consistent across all four methods. The second facet pertains to preservation, where no specific preservation method was required for any of the four methods. Moving to transportation (3rd aspect), all four methods did not necessitate transportation measures. The 4th feature revealed that the Wijs method did not impose specific storage conditions, while the green method was mandated to store under normal conditions at 10 °C. The fifth aspect elucidated the type of methods employed, noting that simple procedures were requisite for all four methods prior to analysis. The sixth consideration concerned the scale of extraction, with all methods falling under the micro extraction category. The seventh facet involved solvents/reagents, wherein the Wijs method employed non-green solvents (manual and auto), while the green method opted for environmentally friendly solvents. The eighth element underscored additional treatments, revealing that none of the four methods necessitated supplementary procedures.

The second set concentrates on reagents and solvents, encompassing the ninth aspect, which delved into the amount used for sample preparation; consistently, the sample amount ranges from 10 to 100 mL across all methods. The tenth facet pertained to the health hazards of reagents and solvents used. The Wijs method was associated with serious injuries on short-term exposure and was deemed a known or suspected small animal carcinogen (NFPA = 4), whereas the green method posed slight toxicity and slight irritability, with an NFPA health hazard score of 0 or 1 and no distinct hazards. The eleventh consideration examined safety hazards. For the Wijs method, the highest NFPA flammability or instability score was 4, while the green method incurred a score of 0 or 1 and no unique hazards. The twelfth aspect scrutinized

energy consumption by instruments revealing that all four methods consumed ≤0.1 kWh per sample. The thirteenth element delved into occupational hazards, noting that the Wijs method released vapors into the atmosphere, while the green method ensured hermetic sealing of the analytical process. The fourteenth facet, waste, contrasts the waste generated by the Wijs method (>10 mL) and the green method (<1 mL). Lastly, the fifteenth element, waste treatment, highlights that the Wijs method did not entail specific waste treatment, whereas the green method facilitated recycling.

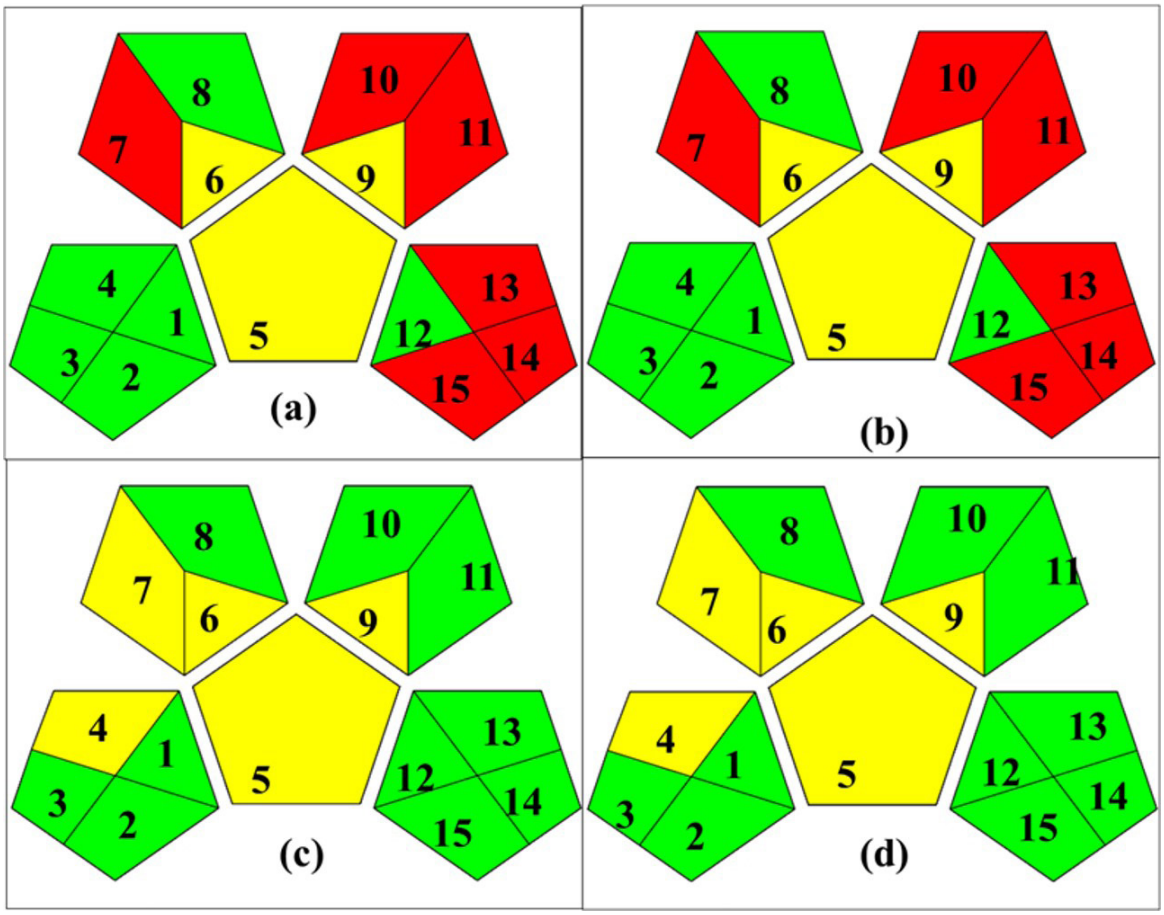


Figure 10. GAPI analysis for (a) WM (b) WA (c) GM (d) GA method.

AGREE analysis

Employing AGREE for four methods namely WM, WA, GM, and GA, the green method's proportion has been assessed through the color-coded segments in the pie chart, vividly depicted in Figure 11. The AGREE values for WM, WA, GM, and GA methods were recorded as 0.54, 0.66, 0.83, and 0.91, respectively. Across all four methods, the initial criterion aligns as "green," given that sample preparation and placement were executed within the designated space. In the subsequent criterion, WM and WA showed an orange hue due to the utilization of hazardous substances like chloroform and Wijs Iodine solution. Conversely, GM and GA methods involved the use of ethanol and iodine in ethanol, resulting in a different color.

The third criterion relating to material sustainability and renewability was inapplicable for all methods. However, GM and GA allowed for material reuse, while WM and WA did not. Turning to the fourth criterion, WM and WA generated hazardous waste, whereas GM and GA did not. Under the fifth criterion, the sample's size economy was notably low, leading to a "green" classification for all samples.

The sixth criterion, sample throughput, pertains to the overall duration of sample preparation, which remained consistent across all methods within one hour. The seventh criterion evaluates the integration of sample preparation steps, with all methods exhibited a limited number of steps. However, WA and GA demonstrated full green automation, while WM and GM remained fully manual. Addressing the eighth criterion, energy consumption remains uniform across all four methods, showing a certain level of "green" alignment. The ninth criterion evaluates the post-sample preparation configuration for analysis, which was deemed favorable across all methods. Operator's safety was assessed in the tenth criterion. Except for the WM method, where operators might inhale hazardous chemicals, all other methods were considered safe for operators. The comprehensive assessment of AGREE principles is presented in a detailed format in Table S4 of the supplementary material.

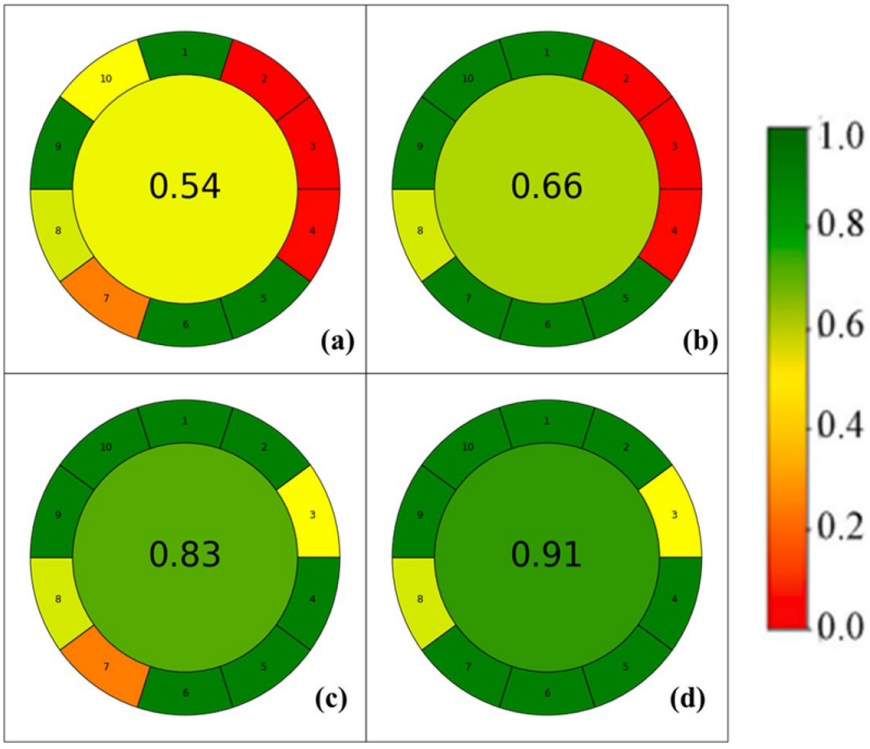


Figure 11. AGREE analysis for (a) WM method (b) WA method (c) GM method (d) GA method.

The comparison of the outcomes of the GAPI and AGREE metrics and the results in the Table VII concludes that it is good to do the iodine value estimation of animal fats by GA method.

Table VII. Comparison of the outcomes of the two metrics

Method	GAPI colors	AGREE score
WM	G = 6, Y = 3, R = 6	0.54
WA	G = 6, Y = 3, R = 6	0.66
GM	G = 10, Y = 5, R = 0	0.83
GA	G = 10, Y = 5, R = 0	0.91

CONCLUSIONS

The green potentiometric analysis method proposed here is greener than the traditional Wijs method in both manual and auto method. As per the cost of the chemicals and safety in the toxic basis, proposed greener method is quite favourable. As comparison experimentally within the four methods mentioned, all the methods statistically give equivalent results. Moreover, Green Auto (GA) method was recommended for determining iodine values due to its accuracy, ease of use, low toxicity, and minimal environmental impact.

Conflict of interest

The authors declare that the search was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

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SUPPLEMENTARY MATERIAL

Procedures to assign scores based on the principles of GAPI and AGREE metrics

Table S1. GAPI procedure to assign scores based on the principles of GAC

Pentagram	GAC criteria	Condition
Sample Preparation and Analysis		
Sample preparation		
1	Collection	1. In-line 2. On-line or at-line 3. Off-line
2	Preservation	1. None 2. Chemical or physical 3. Physico-chemical
3	Transport	1. None 2. Required
4	Storage	1. None 2. Under normal conditions 3. Under special conditions
5	Type of method	1. No sample preparation 2. Simple procedures 3. Extraction required
6	Scale of extraction	1. Nano-extraction 2. Micro-extraction 3. Macro-extraction
7	Solvents/reagents used	1. Solvent-free methods 2. Green solvents/reagents 3. Non-green solvents/reagents
8	Additional treatments	1. None 2. Simple treatments 3. Advanced treatments

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Table S1. GAPI procedure to assign scores based on the principles of GAC (continuation)

Pentagram	GAC criteria	Condition
Reagents and solvents		
9	Amount	1. <10 mL (<10 g) 2. 10–100 mL (10–100 g) 3. >100 mL (>100 g)
10	Health hazard	1. Slightly toxic, slight irritant, NFPA health hazard score of 0 or1. No special hazards 2. Moderately toxic; could cause temporary incapacitation; NFPA = 2 or 3. 3. Serious injury on short-term exposure; known or suspected small animal carcinogen; NFPA = 4.
11	Safety hazard	1. Highest NFPA flammability or instability score of 0 or 1. No special hazards. 2. Highest NFPA flammability or instability score of 2 or 3, or a special hazard is used. 3. Highest NFPA flammability or instability score of 4.
Instrumentation		
12	Energy	1. <= 0.1 kWh per sample 2. >= 1.5 kWh per sample 3. > 1.5 kWh per sample
13	Occupational hazard	1. Hermetic sealing of the analytical process 2. Emission of vapors to the atmosphere
14	Waste	1. <1 mL (<1 g) 2. 1–10 mL (1–10 g) 3. >10 mL (>10 g)
15	Waste treatment	1. Recycling 2. Degradation, passivation 3. No treatment

Table S2. AGREE procedure to assign scores based on 10 principles of GAC

Principle number	GAC criteria	Condition
1	**Sample preparation placement** Procedures that avoid excessive sample transportation and apply sample preparation that is integrated in analytical procedure are favoured.	1. In-line/In situ – sample preparation is carried out in the investigated object. It usually integrates sampling and sample preparation 2. On-line/In situ – sample preparation is performed in situ, sampling and sample preparation are performed in the same place using permanently installed devices with the overall operation being typically fully automated 3. On-site – sample preparation is performed on site, with the sample preparation device being brought to the sampling site 4. Ex situ – sample preparation is performed in the laboratory after sample collection and transportation.
2	**Hazardous materials** The use of toxic materials, including the use of acids and bases for derivatization and digestion, should be avoided.	Based on the amount (g or mL) of toxic materials used during the analysis, the green analysis was calculated.
3	**Sustainability, renewability, and reusability of materials** The application of materials from sustainable and renewable sources should be prioritized. It is calculated as the ratio of the mass of sustainable and renewable materials to the total mass of materials used.	1. Only sustainable and renewable materials are used several times 2. >75% of reagents and materials are sustainable or renewable 3. 50–75% of reagents and materials are sustainable or renewable, but can only be used once 4. Materials are not sustainable or renewable, but are used several times 5. 25–50% of reagents and materials are sustainable or renewable 6. <25% of reagents and materials are sustainable or renewable, but can only be used once
4	**Waste** In analytical chemistry, all material inputs, including the sample preparation stage, can be treated as waste.	Based on the amount of waste, the score board value changes.
5	**Size economy of the sample** Smaller sample sizes should be favoured, as long as sample representativeness is assured.	Input the sample size in g or mL.
6	**Sample throughput** Sample throughput determines the overall duration of the sample preparation stage.	Input the number of samples that can be prepared in one hour. Based on that, the score changes.

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Table S2. AGREE procedure to assign scores based on 10 principles of GAC (continuation)

Principle number	GAC criteria	Condition
7	**Integration and automation** Sample preparation should be simplified and automated if possible.	1. Select the number of discrete steps in the sample preparation procedure: a. steps or fewer b. 3 steps c. 4 steps d. 5 steps e. 6 steps or more 2. Select the degree of automation: a. Fully automated systems b. Semi-automated systems c. Manual systems
8	**Energy consumption** The power consumption per analysis should be minimized.	Input the energy consumption [Wh] per sample, accounting for the sample throughput.
9	**Post-sample preparation configuration for analysis** The prepared sample matrix should be compatible with the analytical instrument, which in turn affects the overall environmental impact of the procedure.	Select the final determination technique or its closest analogue: 1. Simple, readily available detector: smartphones, desktop scanners, paper strips, etc. 2. Spectrophotometry, surface analysis techniques, voltammetry, potentiometry, etc. 3. GC and HPLC with non-MS detection, atomic absorption spectroscopy, capillary electrophoresis, etc. 4. Liquid chromatography, gas chromatography with quadrupole detection, etc. 5. Advanced MS with high energy and/or noble gas consumption: ICP-OES, ICP-MS, etc.
10	**Operator's safety** Hazards associated with the procedure, including physical ones, should be minimized.	The number of distinct hazards of chemical (threats indicated in pictograms labelling used chemicals) and physical nature: 1. No hazard or exposure 2. 1 hazard 3. 2 hazards 4. 3 hazards 5. 4 or more hazards

Analysis of GAPI and AGREE for WM, WA, GM, and GA to evaluate the greenness of iodine value determination

Table S3. GAPI analysis

Method	Evaluation	Color indication in pictogram
WM	1. In-line	Green
	2. None	Green
	3. None	Green
	4. None	Green
	5. Simple procedures	Yellow
	6. Micro-extraction	Yellow
	7. Non-green solvents/reagents	Red
	8. None	Green
	9. 10–100 mL	Yellow
	10. Serious injury on short-term exposure; known or suspected small animal carcinogen; NFPA = 4.	Red
	11. Highest NFPA flammability or instability score of 4.	Red
	12. <= 0.1 kWh per sample	Green
	13. Emission of vapors to the atmosphere	Red
	14. >10 mL	Red
	15. No treatment	Red
WA	1. In-line	Green
	2. None	Green
	3. None	Green
	4. None	Green
	5. Simple procedures	Yellow
	6. Micro-extraction	Yellow
	7. Non-green solvents/reagents	Red
	8. None	Green
	9. 10–100 mL	Yellow
	10. Serious injury on short-term exposure; known or suspected small animal carcinogen; NFPA = 4.	Red
	11. Highest NFPA flammability or instability score of 4.	Red
	12. <= 0.1 kWh per sample	Green
	13. Emission of vapors to the atmosphere	Red
	14. >10 mL	Red
	15. No treatment	Red

(continues on next page)

Table S3. GAPI analysis (continuation)

Method	Evaluation	Color indication in pictogram
GM	1. In-line	Green
	2. None	Green
	3. None	Green
	4. Under normal conditions	Yellow
	5. Simple procedures	Yellow
	6. Micro-extraction	Yellow
	7. Green solvents/reagents	Yellow
	8. None	Green
	9. 10–100 mL	Yellow
	10. Slightly toxic, slight irritant, NFPA health hazard score of 0 or1. No special hazards	Green
	11. Highest NFPA flammability or instability score of 0 or 1. No special hazards.	Green
	12. <= 0.1 kWh per sample	Green
	13. Hermetic sealing of the analytical process	Green
	14. <1 mL	Green
	15. Recycling	Green
GA	1. In-line	Green
	2. None	Green
	3. None	Green
	4. Under normal conditions	Yellow
	5. Simple procedures	Yellow
	6. Micro-extraction	Yellow
	7. Green solvents/reagents	Yellow
	8. None	Green
	9. 10–100 mL	Yellow
	10. Slightly toxic, slight irritant, NFPA health hazard score of 0 or 1. No special hazards	Green
	11. Highest NFPA flammability or instability score of 0 or 1. No special hazards.	Green
	12. <= 0.1 kWh per sample	Green
	13. Hermetic sealing of the analytical process	Green
	14. <1 mL	Green
	15. Recycling	Green

Table S4. AGREE analysis

Method	Evaluation
WM	P1: In-line/In situ P2: 45 mL P3: Materials are not sustainable or renewable, but are used several times 25–50% of reagents and materials are sustainable or renewable P4: 45 mL P5: 0.1 g P6: 1 P7: (1) 2 steps or fewer (b) Manual systems P8: 50 Wh P9: Simple, readily available detector: smartphones, desktop scanners, paper strips, etc. P10: 2 hazards
WA	P1: In-line/In situ P2: 45 mL P3: Materials are not sustainable or renewable, but are used several times 25–50% of reagents and materials are sustainable or renewable P4: 45 mL P5: 0.1 g P6: 1 P7: (1) 2 steps or fewer (b) Fully automated systems P8: 50 Wh P9: Simple, readily available detector: smartphones, desktop scanners, paper strips, etc. P10: No hazards or exposure
GM	P1: In-line/In situ P2: 0 mL P3: <25% of reagents and materials are sustainable or renewable, but can only be used once P4: 0 mL P5: 0.1 g P6: 1 P7: (1) 2 steps or fewer (b) Manual systems P8: 50 Wh P9: Simple, readily available detector: smartphones, desktop scanners, paper strips, etc. P10: No hazard or exposure
GA	P1: In-line/In situ P2: 0 mL P3: <25% of reagents and materials are sustainable or renewable, but can only be used once P4: 0 mL P5: 0.1 g P6: 1 P7: (1) 2 steps or fewer (b) Fully automated systems P8: 50 Wh P9: Simple, readily available detector: smartphones, desktop scanners, paper strips, etc. P10: No hazard or exposure