Validation of a time saving method for saponification value estimation using microwaves technologies

Andrea Milani[1]✉
Liliana Folegatti[1]

Innovhub Stazioni Sperimentali per l'Industria S.r.l.
Food Chemistry, Technology and Safety Team

The use of microwave technology is increasing in routine laboratories especially for synthesis reaction and sample preparation procedure in the last decades. This technology strongly affects the reaction rate reducing analysis time and side reactions and increasing percentage yield and reproducibility. Nevertheless, microwaves are mainly used in sample preparation for chromatographic analysis while only few works have been published regarding bromatological determinations.
The aim of this work is to improve the determination of the saponification number avoiding the use of laboratory heaters, reducing the space required in the lab and taking advantage of microwave technologies to reduce sample preparation times improving reaction rate. For this purpose, the method developed with the use of microwaves was compared with the official ISO 3657:2020 method for animal and vegetable fats and oils and European Pharmacopoeia ones for cosmetic raw materials.
Keywords: Saponification, Microwaves Technologies, Validation, Vegetable Oils, Cosmetic Raw Materials

INTRODUCTION

One of the most common indices to evaluate oils and fats quality is the saponification value, that is the measurement of free and esterified acids in lipid-based products. As described in the ISO 3657:2020 [1] method, the analysis is based on saponification of a known amount of sample with excess of KOH ethanolic solution; the remaining alkali solution is then back titrated with HCl acid solution in presence of phenolphtalein as an indicator. Furthermore, the number of moles of fatty acids in the sample, reacting stoichiometrically one to one with KOH, are strictly related to the difference between the total KOH number of moles in the early solution and the titrant ones needed to reach the indicator colour turning from purple to colourless/white. Thus, saponification value shows changes inversely proportional to the length of fatty acyl chains constituting triacylglycerols.
However, despite the historicity of the analysis, only few works are available in literature on this topic. Some authors demonstrated how this parameter can be used to highlight the adulteration of cow and buffalo milk with coconut oil [2, 3]. In fact, the typical saponification value of coconut oil ranges from 243 to 262 mg KOH/g, due to its amount of lauric and myristic fatty acids [4, 5], that is significantly higher than milk value, usually varying from 213 to 227 mg KOH/g fat due to the abundance of short (C4–C6) and medium chain (C8–C12) fatty acids [6, 3]. However, except for producer countries, coconut oil can result to be an expensive product for the adulteration of dairy products so, on the other side the saponification value allows to detect adulterations with cheaper vegetable oils or fats rich in long chain fatty acids (C16 and 18), characterised by a saponification value of 168-196 mg KOH/g oil and for

✉ CORRESPONDING AUTHOR:
Via Giuseppe Colombo, 79,
20133, Milano
e-mail address:
andrea.milani@mi.camcom.it
Telephone number: 02 85153556

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this reason bringing to an overall saponification value reduction of the hypothetical mixture.
Although the ISO 3657:2020 reference method is easy and accurate, it is time consuming (the saponification must be complete before titration and this step takes approximately one hour) and adequate laboratory spaces are needed especially if a large number of samples has to be processed.
Therefore, like many other authors in recent years [7,8,9], in order to optimise the analysis, reduce time and costs, a new reliable and time-saving method for determining the saponification value would be preferable.
To our knowledge only one attempt to improve this method has been made by Umarani et al. [10] using a domestic microwave oven and few ice pieces introduced on the saponification solution to avoid excessive solvent evaporation.
In the last years, the use of microwave technology has become widely diffused through the scientific community and laboratory microwave ovens able to process several samples together (up to 24 per run) are now easily available. Furthermore, microwave appliances can resist really high pressure and temperature conditions improving yields and speeding up reactions.
Finally, this technology can be termed as 'e-chemistry' because it is easy, effective, economic, and ecofriendly [11], improves reproducibility and reduces both side reactions and operation times [12,13,14] also helping in minimising environmental pollution.
In this work, we have developed a simple, accurate and faster procedure for the determination of the saponification value, reducing the saponification time and space needed to perform the analyses using microwaves technology.
The ISO 3657:2020 method and the microwave-assisted one were compared firstly using some reference material vegetable oils and then with the most diffused vegetable oils on the market. Furthermore, the microwave method (MW) is also applied to some widely diffused cosmetic raw materials obtaining comparable results to the official method.

MATERIALS AND METHODS

The reference materials used in this study were vegetable oils bought at B.I.P.E.A. (Proficiency testing programs Paris – FRANCE) while vegetable oils analysed as commercial samples were taken from the market or supplied directly by Associazione Granaria - Milan (Italy); some laboratory samples with certified value were used for cosmetic raw materials.
Potassium hydroxide (KOH) 0.5 mol/l solution in ethanol and hydrochloric acid (HCl) 0.5 mol/l standard volumetric solutions were used for saponification and titration respectively with phenolphthalein solution (0.1 g/100 ml of 96% ethanol) as indicator. All chemicals and solvents used with analytical purity were purchased from Sigma-Aldrich (Milan, Italy).
The reference method used was "Determination of saponification value EN ISO 3657:2020 applied to animal and vegetable fats and oils" [1], applicable to crude and refined vegetable fats and Pharmacopeia 01/2008:20506 [15] for cosmetic raw materials.

MICROWAVE-ASSISTED SAPONIFICATION

The saponification of vegetable oils was done with an ETHOS X microwave system (MW) equipped with FastEX rotor of 12 vessels in PTFE with disposable glass vial from Milestone Srl (Milan, Italy).

SAMPLE PREPARATION

The sample to be saponified was added to a 100 ml microwave vessel in a different amount depending on the expected saponification value suggested respectively by the ISO 3657:2020 method for vegetable oils reported on Table I and by Pharmacopeia 01/2008:20506 for cosmetic raw materials reported on Table II. Then, the stir bar and 25.0 ml of ethanolic KOH 0.5 M solution were added to the glass vial with a two-mark bulb 25 ml pipette. The glass vial is then transferred inside the PTFE vessel for subsequent microwave saponification carried out in an ETHOS X microwave system equipped with the FastEX rotor from Milestone Srl (Milan, Italy). The treatment temperature (120°C) was reached within 5 min at 800 W and maintained for 15 min, under constant magnetic stirring. After cooling, the exceeding amount of KOH solution is directly titrated with HCl 0.5 M solution into the glass vial, using from 0.5 to 1 ml of the colour indicator solution (Phenolphthalein) until the colour of the indicator changes at the equivalence point (from pink/purple to white or colourless depending on the analysed sample). While most of the vegetable oils can be titrated at room temperature, coconut oil, palm oil and the cosmetic raw material should be titrated

Table I - Oil sample amount based on expected saponification value

Expected Saponification Value	Sample Amount (g)
150 to 200	2.2 to 1.8
200 to 250	1.7 to 1.4
250 to 300	1.3 to 1.2
> 300	1.1 to 1.0

Table II - Cosmetic sample amount based on expected saponification value

Expected Saponification Value	Sample Amount (g)
< 3	20
3 to 10	15 to 12
10 to 40	12 to 8
40 to 60	8 to 5
60 to 100	5 to 3
100 to 200	3 to 2.5
200 to 300	2 to 1
300 to 400	1 to 0.5

Table III - Results, mean, Standard Deviation, % Standard Deviation reference value and repeatability limit (r) for eight different reference material oils using the ISO 3657:2013 standard procedure

	ISO Method							
Sample	A	B	C	Mean mg KOH/g	SD mg KOH/g	% RSD	Reference Value mg KOH/g	r*
Conventional Sunflower Oil	189.7	190.2	189.9	190.0	0.3	0.1	190.5	1.5
Sesame Oil	188.9	188.7	189.2	188.9	0.2	0.1	189.2	1.5
Mix Refined Oils	189.2	189.8	190.0	189.7	0.4	0.2	189.2	2.5
Fish Oil	189.2	189.1	188.0	188,8	0.6	0.3	188.6	4.0
Grapeseed Oil	192.3	190.9	190.3	191.2	1.0	0.5	191.3	6.2
Crude Rapeseed Oil	190.7	190.3	190.1	190.4	0.3	0.2	190.7	1.9
Palm Oil	200.1	200.6	200.8	200.5	0.4	0.2	197.6	2.2
Coconut Oil	257.1	256.9	257.4	257.1	0.3	0.1	255.3	1.5

Table IV - Results, mean and reference value for eight different reference material oils using the microwaves saponification

	MW Saponification							
Sample	A	B	C	D	E	F	Mean mg KOH/g	Reference Value mg KOH/g
Conventional Sunflower Oil	189.9	190.6	190.9	190.9	190.0	190.6	190.5	190.5
Sesame Oil	189.0	188.8	190.2	188.5	189.5	189.2	189.2	189.2
Mix Refined Oils	189.6	189.8	190.1	189.5	189.1	189.7	189.6	189.2
Fish Oil	188.6	189.0	189.4	189.6	188.5	189.9	189.2	188.6
Grapeseed Oil	192.4	192.1	192.7	192.4	191.5	192.8	192.2	191.3
Crude Rapeseed Oil	191.0	191.4	191.1	191.4	190.3	191.4	191.1	190.7
Palm Oil	201.5	201.6	201.0	202.9	201.7	202.5	201.9	197.6
Coconut Oil	258.2	259.5	259.1	260.0	259.5	259.9	259.4	255.3

while still warm to avoid sample solidification that can affect the final result of the analysis.

STATISTICS

The experiments were made at least in triplicate for both the MW and ISO 3657:2020 method. The blank tests were carried out following the procedure specified using 25.0 ml of ethanolic potassium hydroxide solution but omitting the test portion. The results were expressed in mg KOH/g fat as the mean values, standard deviation (SD) and relative standard deviation (% RSD). Furthermore, saponification number values were subjected to analysis of variance (ANOVA) to calculate the precision under conditions of repeatability, intermediate reproducibility, and accuracy [16].

RESULT AND DISCUSSION

The ISO 3657:2020 method was first applied in triplicate to eight different reference materials: seven vegetable oils (conventional sunflower oil, sesame oil, mix of refined oils, grapeseed oil, crude rapeseed oil, palm oil and coconut oil) and a fish oil. The results obtained were comparable with the assigned value for all samples, with a % RSD reaching a maximum value of 0.5 as reported on Table III. The repeatability limit generally showed good results except for fish oil and grapeseed oil that have a quite high value.

Then the saponification with MW was applied on the same eight matrixes, six times for each sample and the results are reported on Table IV. Also, in this case results were comparable with the assigned value and showed a great repeatability of the analyses with % RSD values really close to those obtained with the ISO 3657:2020 official method and even better in the case of fish oil and grapeseed oil (Table V). Furthermore, compared to the ISO method, lower values of repeatability limit were observed.

For the sample processed by microwave saponification then the accuracy was also calculated.

The accuracy was evaluated comparing the average

Table V - Standard Deviation, % Standard deviation, repeatability limit and trueness for eight different reference material oils using the microwaves saponification

Sample	SD mg KOH/g	% RSD	r*	Trueness
Conventional Sunflower Oil	0.4	0.2	1.6	0.014
Sesame Oil	0.6	0.3	2.2	0.001
Mix Refined Oils	0.3	0.2	1.1	0.143
Fish Oil	0.6	0.3	2.0	0.186
Grapeseed Oil	0.7	0.4	1.7	0.297
Crude Rapeseed Oil	0.5	0.2	1.6	0.141
Palm Oil	0.7	0.3	2.5	1.421
Coconut Oil	0.7	0.3	2.4	0.813

*Repeatability limit

Table VI - Mean, Standard Deviation and % Standard Deviation of intermediate reproducibility for three different reference material oils using the microwaves saponification repeated in six different days

	MW Saponification									
Sample	A	B	C	D	E	F	Mean mg KOH/g	SD mg KOH/g	% RSD	Reference Value mg KOH/g
Crude Rapeseed Oil	191.4	191.2	191.1	191.5	190.4	191.4	191.2	0.4	0.2	190.7
Palm Oil	199.4	200.8	200.3	201.1	200.0	200.7	200.4	0.6	0.3	200.4
Coconut Oil	259.7	259.5	259.5	259.3	259.7	259.8	259.6	0.2	0.1	259.6

All data for the six days (A, B, C, D, E, F) are reported as mean of three analyses

Table VII - Mean, Standard Deviation, % Standard Deviation, % Horwitz Standard Deviation and HORRAT value for three different reference material oils considered

Parameter	Rapeseed Oil	Palm Oil	Coconut Oil	Rapeseed Oil (ISO)
Mean (means of six different days)	191.2	200.4	259.6	190.2
SD	0.4	0.6	0.2	1.8
RSD	0.2	0.3	0.1	0.9
RSD % Horwitz	0.49	0.51	0.64	-
HORRAT Value	0.38	0.55	0.10	-

of six measurements with the declared value of a certified reference material with a composition very similar to the matrixes under examination.
To verify the result reliability, a Student t-test was performed (with a significance value of 95%). Based on the positive results the test gave, it was possible to declare that the method provides accurate results at the chosen significance level.
All results obtained showed values lower than 1 exception made for the palm oil with an accuracy of 1.420, nevertheless, all samples' trueness agreed with the difference between the calculated and theorical t-Student (Table V).
The microwave method described in this study was in-house validated by assessing the precision, expressed in terms of standard deviation for repeatability and intermediate reproducibility calculated by Horwitz equation; correctness was then calculated with the reference value. Finally, our values were compared with those of the ISO 3657:2020 method for rapeseed oil.

PRECISION

The precision of the method was determined by carrying out six analyses under repeatability conditions on reference materials, in which the tests were performed on the same day and by the same technician. The value below the absolute difference between two single test results, is expected to be found with a 95% of probability. In the intermediate reproducibility conditions, the experiments were carried out over six different days in triplicate and results are reported on table VI.
Experimental intermediate reproducibility values (RSD_R%) were used to calculate an acceptable predictive value obtained by applying the Horwitz equation, an empirical relationship between the acceptable precision and analyte concentration.
The results of the precision study are illustrated in Table VII for the three reference materials used.
The ratio between the relative standard deviation % (RSD%) under intermediate precision and the RSD% calculated by Horwitz equation is an indicator of the precision of the analysis and it is known as HORRAT value (Table VII).
Usually, HORRAT is used to indicate the presence of analytical problems that compromise the precision of the analysis: values lower than 1 indicate a good analytical precision, values between 1 and 1.5 are acceptable results while values above 2 highlight analytical issues.
Once the new method showed to be effective, it was applied, together with the ISO one, to several samples representing the main vegetable oils available on the market and used by industries in food production. For each sample the analyses were performed in triplicate for both methods and the results are reported on Table VIII.
Both methods showed results with a good repeatability, but a general lower RSD was obtained with the MW one. The better result of MW is due to the more homogeneous saponification process compared to the traditional heating processes and the constant agitation thanks to the magnetic stir bar obtaining a complete and constant homogenization of the sample during heating process.
The method using microwave saponification was also applied in triplicate on cosmetic products. The values are reported in Table IX with reference value.
As can be seen, both saponification techniques provide excellent analytical results for determining the saponification number. Microwave extraction with

Table VIII - Results, mean, Standard Deviation and % Standard Deviation for the main categories of vegetable oils available in the market

	ISO Method						MW Saponification					
Sample	A*	B*	C*	Mean	SD	% RSD	A*	B*	C*	Mean	SD	% RSD
Cocoa Butter	189.2	192.1	195.6	192.3	2.6	1.4	192.3	192.5	193.0	192.6	0.3	0.2
Extra Virgin Olive Oil	190.4	190.2	193.9	191.5	1.7	0.9	193.4	193.7	193.7	193.6	0.1	0.1
Conventional Soybean Oil	182.8	192.8	193.2	189.6	4.8	2.5	190.4	190.8	191.1	190.8	0.3	0.2
Mais Oil	190.4	190.3	191.5	190.7	0.5	0.3	191.2	191.9	190.7	191.2	0.5	0.3
Conventional Sunflower Oil	190.5	189.2	184.2	188.0	2.7	1.5	189.0	189.9	190.7	189.9	0.7	0.4
Olive Oil	191.1	193.6	190.8	191.8	1.3	0.7	191.6	191.4	190.8	191.3	0.4	0.2
Peanut Oil	183.2	188.0	187.6	186.2	2.2	1.2	188.4	189.3	190.1	189.3	0.7	0.4
Coconut Oil	256.0	257.3	261.4	258.2	2.3	0.9	259.4	259.7	260.1	259.7	0.3	0.1
Palm Oil	190.7	199.4	197.5	195.9	3.8	1.9	197.5	197.3	197.4	197.4	0.1	0.0
HO Sunflower Oil	182.9	188.8	190.4	187.4	3.2	1.7	194.1	193.6	193.8	193.8	0.2	0.1
Avocado Oil	187.1	194.0	190.4	190.5	2.8	1.5	191.0	191.0	190.5	190.8	0.2	0.1
HO Soybean Oil	187.6	191.0	190.2	189.6	1.4	0.8	191.0	191.0	190.5	190.8	0.2	0.1
HO Rapeseed Oil	186.9	184.2	189.8	187.0	2.3	1.2	187.5	188.4	188.6	188.2	0.5	0.3
Safflower Oil	190.7	185.8	190.8	189.1	2.3	1.2	192.1	191.9	191.9	192.0	0.1	0.1
Conventional Rapeseed Oil	188.0	187.9	189.3	188.4	0.6	0.3	191.0	190.5	191.1	190.9	0.2	0.1
Sesame Oil	187.5	188.2	187.3	187.6	0.4	0.2	187.9	187.7	188.6	188.1	0.4	0.2
Linseed Oil	188.6	188.9	190.4	189.3	0.8	0.4	190.6	190.6	191.3	190.8	0.3	0.2

***** mg KOH/g

Table IX - Results, mean, Standard Deviation and % Standard Deviation for some cosmetics ingredients frequently used on cosmetic products formulation

Sample	A	B	C	Mean mg KOH/g	SD mg KOH/g	% RSD	Reference Value mg KOH/g
Trioctyldodecyl citrate	142.4	142.2	142.9	142.5	0.4	0.3	145.5
Hydrogenated castor oil dimer dilinoleate	185.3	188.6	186.2	186.7	1.7	0.9	188.2
Vegetal Stearine	206.3	203.7	208.2	206.0	2.3	1.1	207.3
Blend of Mono-, Di- and Triglycerides	277.5	277.7	277.1	277.4	0.3	0.1	284.0
Isostearyl isostearate	103.8	102.8	102.7	103.1	0.6	0.6	103.0
Dipentaerythrityl tetrabehenate/polyhydroxy stearate	183.8	183.0	183.7	183.5	0.4	0.2	184.0
Glyceryl Undecilenate	209.9	209.5	208.9	209.5	0.5	0.2	207.3
Polyglyceryl-10Pentahydroxystearate	127.3	127.0	126.4	126.9	0.5	0.4	130.0

Milestone ETHOS X is performed in a few minutes and multiple samples can be analysed simultaneously, in safe and constantly monitored conditions.
Microwave-assisted saponification uses closed vessels allowing to reach higher pressures and consequently temperatures above sample solution atmospheric boiling point; the increased solubility of the analytes and lower viscosity of the solvent speed up the reaction with the matrix reducing analysis time.

CONCLUSION

The method developed seems reliable to be used for saponification number evaluation as a possible alternative to the ISO 3657:2020 official one, in terms of repeatability, intermediate reproducibility and accuracy. Advantages are the use of MW as heating source that saponify the samples in less time (20 min compared to 1 h of the official method), the better results of this analysis compared to ISO method is due also to the constant agitation allowed by the magnetic stir bar during both saponification and titration processes. Furthermore, the rotor able to host up to 24 samples reduce the necessary laboratory space and times needed to analyse several samples.
Compared to the conventional saponification techniques for the SV determination (ISO 3657:2020 and European Pharmacopoeia 01/2008:20506), the obtained microwaves-based saponification values suggest the new method as a more sustainable and rapid alternative approach.

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Conflict of interest

Authors declare no conflict of interest.

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