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Analytical Methods

Evaluation of a colorimetric method for measuring the content of FFA in marine and vegetable oils

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ABSTRACT

Free fatty acids (FFA) are produced from triacylglycerides (TAG) through chemical or enzymatic hydrolysis. They are usually associated with undesirable flavour and textural changes when they are present in fats and oils. In the oil processing industry, FFA's are determined to give an indication of the amount of alkali required to remove them as soaps during the refining stage. This is a titration method requiring considerable time and large amounts of sample for oils containing low levels of FFA. Oils containing low levels of FFA, especially marine oils, showed poor repeatability. When the amount of oil required for the analysis (56 g) was used, an emulsion was formed making difficult to obtain a stable change in colour. FASafe is a rapid colorimetric method requiring less than 1 g of oil. Good agreement between the two methods was obtained at lower levels of FFA (<1.0%). For fish oil samples higher results were obtained by FASafe than the AOCS titration method and results for samples with high levels of FFA tended to be lower by FASafe than by the AOCS method.

1. Introduction

Free fatty acids (FFA's) are products of triacylglycerides (TAG) formed either through chemical or enzyme mediated hydrolysis. FFA's are usually associated with undesirable flavour and textural changes when they are present in fats and oils. In the oil processing industry, FFA's are determined to give an indication of the amount of alkali to be used to remove them as soaps during the refining stage. Maximum acceptable levels of FFA are specified in some oils. For example, the EC Regulation 2598/91, amended 2007 classifies olive oils according to the FFA content, also known as acidity degree (AD), expressed as g of oleic acid (OA) for 100 g of oil or %. Extra virgin olive oil must have an AV lower than 0.8 (%, OA), virgin olive oil lower than 2.0 (%, OA) and lampante virgin olive oil higher than 3.3 (%, OA) (Codex Alimentarius Commission, Stan 33, 1981, revised 2-2003). For canola oil, the Canadian General Standards Board. (1987) specifies maximum levels of 1.0% FFA for crude canola oils and 0.05% for refined, bleached and deodorized canola oil. FFAs could also be reported as the percent of lauric acid for coconut and palm kernel oils and lauric acid for palm oil. FFA are often expressed as acid value (AV), defined as the amount of KOH (mg) required to neutralise one gram of oil or fat. An AV of two is equivalent to approximately 1% FFA when the AV is expressed as a % of oleic acid (%, OA). The Codex Alimentarius Commission (Codex-Stan 210, 2003–2005) expresses maximum AV for refined oils (0.6 mg KOH/g oil), cold pressed and virgin oils (4.0 mg KOH/g oil), and virgin palm oils (10.0 mg KOH/g oil).

Reference methods to have been developed by AOCS (Ca 5a-40, 1997), AOAC International (940.28, 2003) and ISO 660: (1996) to measure FFA in oils. These methods use titration with KOH, usually dissolved in alcohol, with phenolphthalein as the colour indicator to mark the neutralisation point. Masking of the colour change by the yellowish/brownish colour of the oils as well as turbidity in many of the matrices, especially marine oils make detection of the end point difficult. The use of a ternary solvent mixture and Alkali blue 6B as an indicator resolves some of these difficulties (Ke & Woyewoda, 1978) but the use of chlorinated hydrocarbons has meant that this modification has not been adopted in the reference methods. The 1996 edition of the ISO method attempts to alleviate these problems by allowing the use of alkali blue 6B or thymolphthalein rather than phenolphthalein, a mixture of diethyl ether (or toluene) and ethanol, titration to a potentiometric endpoint and titration in hot ethanol to help to dissolve the oils and to establish the end-point of the reaction.

Other methods for determining free fatty acids in vegetable oils have included gas chromatography (AOCS Ca 5d-1, 2001) and FTIR spectroscopy (Al Alawi, Van de Voort, & Sedman, 2004) but the most common alternative method has involved spectrophotometry. One successful method involves the determination of copper soaps complexes in aromatic solvents (Baker, 1964; Lowry & Tinsley, 1976) that was also used for lipase determinations, (Kwon &

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Table 1
Comparison of results for FFA determination by the AOCS titration method and the FASafe spectrophotometric method for marine and vegetable oils for data from two laboratories

Oil[b]	GRL						Saftest						Within labs between methods				Within methods between labs			
													Variance comparison[a] AOCS/FASafe		Means comparison[a] (AOCS–FASafe)		Variance comparison[a] GRL/Saftest		Means comparison[a] (GRL-Saftest)	
	AOCS			FaSafe			AOCS			Fasafe			GRL	Saftest	Saftest	GRL	FASafe	AOCS	FASafe	AOCS
	Mean (%)	Std. dev.	N^c	Mean	Std. dev.	N^c	Mean (%)	Std. dev.	N^c	Mean	Std. dev.	N^c	*F*	*F*	Diff. (%)	Diff. (%)	*F*	*F*	Diff. (%)	Diff. (%)
Algal H	1.663	0.040	10	1.539	0.031	10	1.522	0.042	10	1.466	0.070	10	1.640	0.370	**0.124**	**0.056**	0.890	**0.200**	**0.141**	**0.073**
Algal M1	0.239	0.012	10	0.310	0.017	10	0.244	0.005	10	0.291	0.018	9	0.498	**0.081**	**−0.071**	**−0.047**	**6.080**	0.970	−0.005	**0.019**
Algal M2	0.610	0.011	10	0.659	0.0166	10	0.608	0.014	10	0.658	0.016	7	0.470	0.800	**−0.049**	**−0.050**	0.640	1.090	0.002	0.001
Fish H	1.310	0.038	10	1.317	0.027	10	1.255	0.038	10	1.360	0.064	10	1.981	0.360	−0.007	**−0.105**	0.990	**0.180**	**0.055**	−0.043
Fish L	0.178	0.017	10	0.291	0.008	10	0.282	0.090	8	0.285	0.018	10	**4.260**	**25.280**	**−0.113**	−0.003	**0.040**	**0.210**	**−0.104**	0.006
Fish M	0.532	0.050	10	0.712	0.009	10	0.631	0.027	10	0.736	0.048	10	**28.450**	0.330	**−0.180**	**−0.105**	3.400	**0.040**	**−0.099**	−0.024
Herring H	1.329	0.033	10	1.076	0.0303	9	1.437	0.193	10	1.028	0.075	9	1.220	**6.600**	**0.253**	**0.409**	**0.030**	**0.160**	**−0.108**	0.048
Herring M1	0.443	0.023	9	0.516	0.012	10	0.493	0.019	10	0.508	0.028	10	3.520	0.470	**−0.073**	−0.015	1.360	**0.180**	**−0.050**	0.008
Herring M2	0.624	0.032	9	0.689	0.013	10	0.693	0.017	10	0.684	0.039	9	**5.920**	**0.180**	**−0.065**	0.009	3.770	**0.110**	**−0.069**	0.005
Menhaden H	1.234	0.035	10	1.252	0.025	10	1.186	0.025	8	1.336	0.033	9	1.860	0.540	−0.018	−0.150	1.950	0.560	**0.048**	**−0.084**
Menhaden M1	0.651	0.055	10	0.761	0.018	10	0.647	0.047	9	0.728	0.050	10	**8.770**	0.884	**−0.110**	**−0.081**	1.330	**0.130**	0.004	0.033
Menhaden M2	0.320	0.016	10	0.464	0.011	10	0.392	0.010	10	0.456	0.040	9	2.100	**0.063**	**−0.144**	**−0.064**	2.570	**0.080**	**−0.072**	0.008
Canola H	1.264	0.025	9	0.889	0.0526	10	1.203	0.030	10	0.982	0.033	9	0.230	0.840	**−0.375**	**−0.221**	0.720	2.610	**0.061**	**−0.093**
Canola M2	0.789	0.023	10	0.680	0.0261	10	0.727	0.018	10	0.735	0.017	8	0.800	1.140	**−0.109**	0.008	1.760	2.510	**0.062**	**0.055**
Canola M1	0.366	0.014	10	0.391	0.0112	9	0.365	0.012	10	0.452	0.018	9	1.670	0.450	**0.026**	**0.087**	1.400	0.380	0.000	**0.061**
Flax L	0.137	0.005	8	0.227	0.0048	10	0.136	0.003	8	0.234	0.008	9	1.270	**0.120**	**0.089**	**0.098**	3.600	0.340	0.001	**0.007**
Flax M2	0.660	0.013	10	0.652	0.0379	10	0.641	0.019	10	0.682	0.034	9	**0.110**	0.320	−0.008	**0.041**	0.440	1.250	**0.019**	0.030
Flax M1	0.493	0.009	9	0.484	0.0269	10	0.486	0.037	10	0.534	0.031	9	**0.120**	1.420	−0.010	**0.048**	**0.060**	0.770	0.007	**0.051**
Olive H	1.411	0.032	10	1.214	0.0511	10	1.364	0.026	10	1.391	0.034	9	0.400	0.620	**−0.197**	0.027	1.500	2.310	**0.047**	**0.177**
Olive L	0.158	0.010	10	0.229	0.0075	10	0.17	0.004	10	0.244	0.006	9	1.780	0.400	**0.071**	**0.074**	**6.430**	1.440	**−0.012**	**0.016**
Olive M	0.568	0.015	10	0.547	0.0436	10	0.545	0.010	10	0.605	0.033	9	**0.120**	**0.100**	−0.021	**0.060**	2.100	1.770	**0.023**	**0.058**
Safflower H	1.362	0.024	10	1.179	0.0539	10	1.345	0.021	10	1.244	0.027	10	**0.190**	0.610	**−0.182**	**−0.101**	1.240	3.950	0.017	**0.064**
Safflower M1	0.278	0.010	10	0.302	0.0143	10	0.271	0.005	10	0.319	0.021	9	0.510	**0.050**	**0.024**	**0.048**	**4.630**	0.470	0.007	0.016
Safflower M2	0.605	0.014	10	0.541	0.0313	10	0.591	0.012	10	0.603	0.021	8	0.200	0.300	**−0.064**	0.012	1.450	2.170	**0.015**	**0.062**

[a] Figures in bold are significant at $p > 0.05$.
[b] Letters H, M, L refer to general levels of high medium and low, respectively.
[c] *N* = number of analyses. Each laboratory was given 10 samples for analyses but results more than ±2 standard deviations (Std. dev.) from the mean were dropped as outliers.

Rhee, 1986; Sahahsrabudhe, 1982) and in a flow injection system for routine assays in foodstuffs (Puchades, Suescun and Maquiera, 1994). Enzyme methods are also used. Recently, FASafe™, a colorimetric test kit developed by Safety Associated Inc. for determining free fatty acids in vegetable and marine oils, was granted Performance Tested Method status by AOAC International (Gordon, 2004).

The goal of this study was to compare the FASafe™ method and the AOCS Official Method Ca-5a-40 to measure FFA contents in marine and vegetable oils. This study also reports some problems encountered with the AOCS method.

2. Materials and methods

2.1. Materials

Vegetable (linseed, canola, olive and safflower) and marine (menhaden, fish, herring and algal) oils with different amounts of FFA (designated low, medium and high) were donated by several processing companies. Each of the 24 oil type/FFA level samples was subdivided into 2 randomly numbered sets of 20 × 20 mL aliquots, one set for analysis by AOCS official method and the other for the FASafe™ analysis for a total of 960 samples. Each of the two laboratories participating in the study (Canadian Grain Commission (CGC), Grain Research Laboratory, Winnipeg, Canada and Saftest Inc., Tempe, USA) received a complete set of 480 samples, enough for 10 repetitions by each method for the 24 oil type/FFA levels.

At the CGC, a series of control samples were also prepared from a refined, bleached and deodorized canola commercial canola oil by adding oleic acid (>99% pure from Nu-Chek Prep Inc., Elisian MN, USA). The base level of FFA in the commercial canola oil was estimated by both AOCS Official method and FASafe™ methods (Table 1) but the AOCS Official method results only were used to calculate the FFA value of the base oil. Two sets of three samples (FFA content A, B and C) were prepared to cover the expected levels of FFA in the test samples. Due to the large amount of check sample required, sufficient check sample was prepared to allow 7.05 g of check sample per test. This amount is smaller than the amounts recommended by the AOCS method (1997) but corresponds to the amount in the AOAC International method (2003).

2.2. AOCS titration method

The FFA content of each oil was determined using the AOCS Official method Ca 5a-40 (1997). Some modifications to the method (sample size) were applied as described above and below.

2.3. FASafe™ method

Reagents and samples were equilibrated to room temperature 20° ± 3 °C. An aliquot of 100 μL was treated with proprietary test reagents and the developed colour was measured on a colorimeter using a 570/690 nm filter after incubation at 37–44 °C for 10 min.

The test is based on a pH indicator reaction that takes place in a stabilized reagent (isopropanol). FFAs interact with the pH indicator and the decrease in absorbance at 570 nm is measured. The resulting absorbance values are logarithmically related to FFA concentrations and decrease with FFA concentration. The results were compared to a standard curve made from samples of oleic acid supplied with the kit. Adjustments to volumes of sample were made to ensure that the colorimeter reading was optimal for the amount of FFA in the sample.

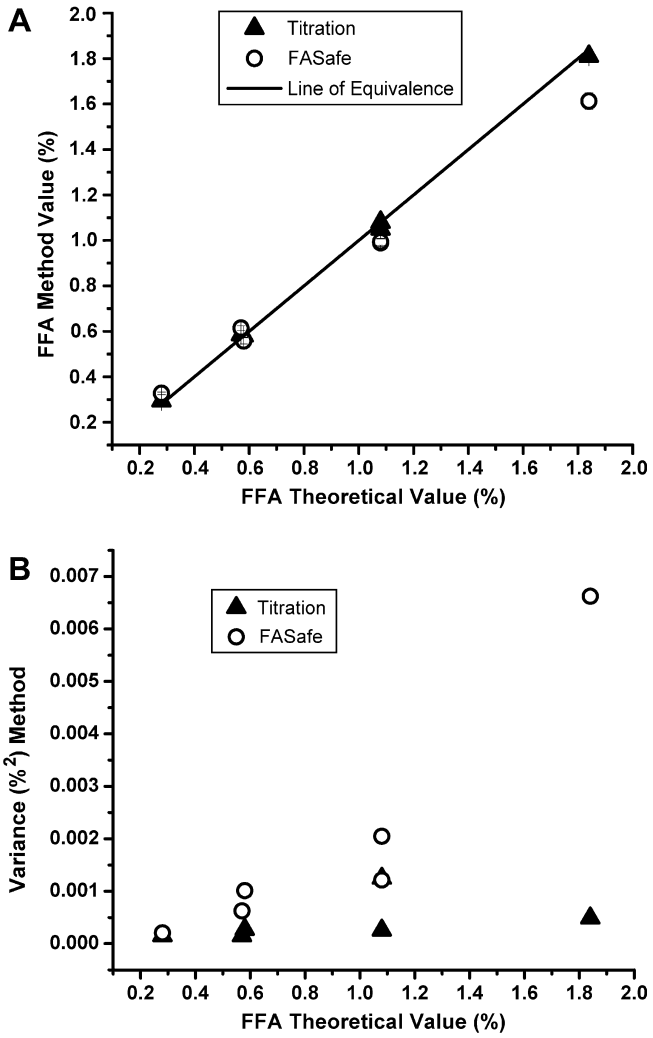

Fig. 1. Comparison of mean values and variances for the AOCS titration method and the FASafe spectrophotometric method for determination of FFA levels in prepared control samples of pure canola oil.

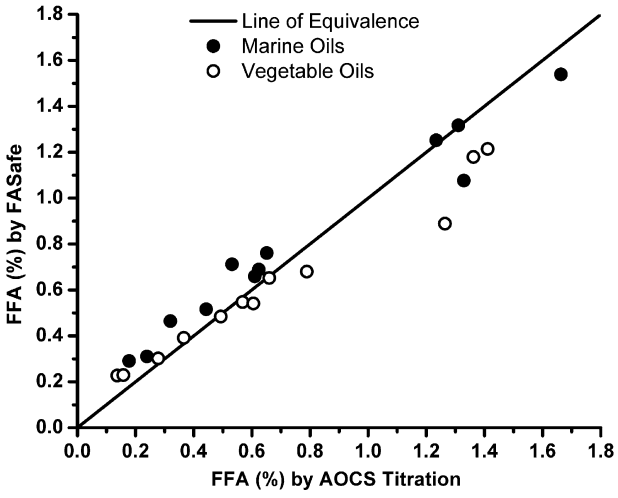

Fig. 2. Comparison of mean values for the AOCS titration method and the FASafe spectrophotometric method for determination of FFA levels in marine and vegetable oils. Results from 8 to 10 sub samples of each oil type tested both methods at each of two laboratories.

2.4. Statistical analysis

Statistical procedures used were from either SAS version 9.1 for windows (SAS Institute Inc., Cary, NC, USA.), Origin version 7.5 (Originlab Corporation, Northhampton MA, USA), Graphpad Instat version 3.05 (GraphPad Software, San Diego CA, USA) or Microsoft Excel 2002 (Microsoft Corporation, Seattle, WA, USA). In addition, repeatability and reproducibility were determined according to AOCS Official Method M 1-92 Determination of Precision of Analytical Methods.

3. Results and discussion

3.1 General observations on the AOCS titration method

It was more difficult to determine the end point of the analyses when 28 g of oil or more were used for the FFA determination. The results of the fish oils tested with 56 g of oil showed poor repeatability as shown by the higher standard deviation. When 56 g of oil was used, an emulsion was formed, when the end point of the reaction was obtained it then disappeared; the definition of the end point was a stable change in colour and this was difficult to achieve following the AOCS method. Our laboratory actually uses as routine, a secondary method which employs a ternary solvent mixture and was developed to combat the problems of highly coloured marine oils (Ke & Woyewoda, 1978). For the current reference methods, it would seem preferable in the case of highly-coloured oils to use 7 g of oil recommended by the AOAC Official method (940.28).

3.2. Comparison of methods using the prepared control samples

The control samples prepared at CGC were tested as check samples throughout the analysis of the fish and vegetable oils at that location using both the titration and the Saftest procedure. There was good agreement between the two methods at lower levels of FFA, (Fig. 1A) but the FASafe method gave lower results than the titration method at more than 1% FFA. The variances of both methods increased with increasing levels of FFA (Fig. 1B) but the increase was more important for the FASafe method.

3.3. Comparison of methods using fish and vegetable oil samples tested at two different laboratories

In the analysis of the data, results received that were more than ±2 standard deviations from the mean value for that sample-laboratory-method combination were excluded from the overall assessment. It was felt that, in a laboratory situation with repeated results, these results would have fallen outside of the control parameters that would be set. Even with these dropped analyses, at least 9 good data points were obtained for 43 of the 48 sample sets and only one set was reduced to 7 repetitions (Table 1). Statistical analysis (Table 1), including a factorial analysis of variance (not shown), showed that there were significant interactions between laboratories, methods and samples for both the mean value and the variability. In particular, for some samples, particularly among the fish oil samples the AOCS titration method had a greater variability, as demonstrated by the *F*-test, than the FASafe method. The reverse situation was true for the vegetable oil samples. A plot of the mean values (Fig. 2) shows that the fish oil samples gave somewhat higher results by FASafe than the AOCS titration method. Results for samples with high levels of FFA tended to be lower by FASafe than by the AOCS method.

While two laboratories are insufficient to develop precision data suitable for a published method (Fiebig, 2006), it is possible, at least, to get an idea of the within and between laboratory precision for the study in question using nested analysis of variance (Youden & Steiner, 1975). The results (Table 2) show that, for the AOCS titration method, several oils gave high relative repeatability and reproducibility values. These oils (Fish L, Fish M, Herring H and possibly Menhaden M2) were highly coloured making the endpoint difficult to see. The relative repeatability and reproducibility

Table 2
Precision data[a] for analysis of fish and vegetable oil samples in two different laboratories using two methods

Oil	AOCS titration method							FASafe spectrophotometric method						
	Mean	S_r	S_R	r	R	RS_r	RS_R	Mean	S_r	S_R	r	R	RS_r	RS_R
Algal H	1.663	0.041	0.107	0.115	0.300	2.47	6.44	1.539	0.054	0.073	0.152	0.205	3.52	4.75
Algal M1	0.239	0.009	0.010	0.026	0.027	3.89	4.01	0.310	0.017	0.021	0.049	0.060	5.64	6.86
Algal M2	0.610	0.013	0.012	0.036	0.034	2.11	2.01	0.659	0.016	0.015	0.045	0.042	2.46	2.28
Canola H	1.264	0.028	0.050	0.078	0.141	2.20	3.97	0.889	0.044	0.078	0.124	0.219	4.99	8.79
Canola m1	0.366	0.013	0.013	0.037	0.035	3.65	3.47	0.391	0.015	0.045	0.042	0.126	3.84	11.53
Canola M2	0.789	0.021	0.048	0.058	0.135	2.62	6.11	0.680	0.022	0.044	0.063	0.125	3.29	6.55
Fish H	1.310	0.038	0.054	0.107	0.150	2.91	4.08	1.317	0.049	0.056	0.138	0.156	3.73	4.24
Fish L	0.178	0.061	0.094	0.170	0.262	34.16	52.59	0.291	0.014	0.014	0.039	0.039	4.79	4.76
Fish M	0.532	0.040	0.079	0.113	0.222	7.59	14.93	0.712	0.034	0.037	0.096	0.102	4.81	5.13
Flax L	0.137	0.004	0.004	0.012	0.012	3.16	3.02	0.227	0.007	0.008	0.019	0.023	2.95	3.57
Flax m1	0.493	0.027	0.026	0.077	0.074	5.55	5.34	0.484	0.029	0.045	0.080	0.126	5.94	9.30
Flax M2	0.660	0.016	0.020	0.045	0.057	2.44	3.07	0.652	0.036	0.040	0.101	0.113	5.53	6.18
Herring H	1.329	0.139	0.167	0.388	0.467	10.44	12.54	1.076	0.057	0.064	0.161	0.179	5.33	5.94
Herring M1	0.443	0.021	0.040	0.059	0.113	4.73	9.08	0.516	0.022	0.021	0.061	0.060	4.22	4.16
Herring M2	0.624	0.025	0.055	0.071	0.154	4.04	8.80	0.689	0.029	0.027	0.080	0.076	4.15	3.96
Menhaden H	1.234	0.031	0.045	0.086	0.125	2.49	3.61	1.252	0.030	0.066	0.083	0.184	2.37	5.25
Menhaden M1	0.651	0.051	0.049	0.144	0.136	7.89	7.48	0.761	0.038	0.043	0.106	0.119	4.99	5.61
Menhaden M2	0.320	0.014	0.052	0.038	0.147	4.22	16.37	0.464	0.029	0.028	0.080	0.078	6.19	5.99
Olive H	1.411	0.029	0.043	0.082	0.122	2.09	3.08	1.214	0.044	0.132	0.122	0.369	3.60	10.86
Olive L	0.158	0.008	0.011	0.021	0.031	4.82	6.99	0.229	0.007	0.013	0.020	0.035	3.04	5.50
Olive M	0.568	0.013	0.020	0.036	0.057	2.29	3.56	0.547	0.039	0.055	0.109	0.155	7.11	10.12
Safflower H	1.362	0.022	0.024	0.063	0.068	1.65	1.79	1.179	0.043	0.061	0.120	0.171	3.62	5.17
Safflower m1	0.605	0.008	0.009	0.022	0.026	1.33	1.52	0.541	0.018	0.020	0.050	0.057	3.27	3.79
Safflower M2	0.278	0.013	0.016	0.036	0.045	4.60	5.78	0.302	0.027	0.052	0.077	0.147	9.06	17.36

Each laboratory analysed each sample approximately 10 times (Table 1). Data calculated from nested analysis of variance {Youden & Steiner 1975 #23660}.

[a] S_r = standard deviation of repeatability, S_R = standard deviation of reproducibility, r = repeatability, R = reproducibility, RS_r = relative standard deviation of repeatability RSR = relative standard deviation of reproducibility.

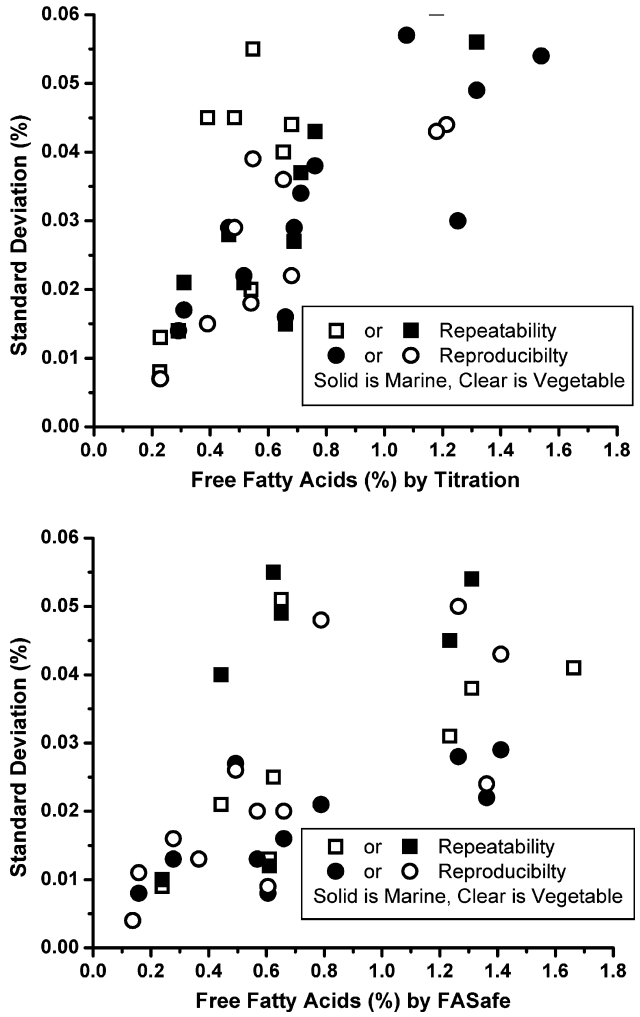

Fig. 3. Comparison of precision values (repeatability and reproducibility standard deviations) for the AOCS titration method and the FASafe spectrophotometric method for determination of FFA levels in marine and vegetable oils. Results from 8 to 10 sub samples of each oil type tested both methods at each of two laboratories.

for these oils using the FASafe method was much less than when using the titration method. The only oil that seemed to give problems to the FASafe method was Safflower M2 which had high relative reproducibility.

Examination of the relationship between the mean value and repeatability or reproducibility standard deviation (Fig. 3), without including the samples with unusually high relative values, show that there is no real difference between the type of oil and the precision value (or even between the two precision values although this latter effect might be a result of a low number of laboratories). The repeatability and reproducibility increased linearly with the level of free fatty acids and the increase seemed to be more rapid for the titration method. This is in contrast to the results from the prepared control samples (Fig. 1) and indicates that the "real world" samples gave a more robust estimate of the precision.

The results suggested that the two methods gave similar results, however a bias was introduced when using the FASafe method. This bias was small since there was no statistical difference between the two sets of data (titration FFA and FASafe FFA). Overall, the FASafe method could be used as an alternative to the AOCS titration method to measure FFA content of a broad variety of oils. There are several advantages in using the FASafe method over the AOCS titration method: (1) the small sample size requirement, less than 1 g of oil whereas AOCS method requires between 7.05 and 56.4 g of oil (This is an important advantage when measuring FFA in the oil from seeds since usually the FFAs are lower than 1%), (2) the FASafe method uses isopropyl alcohol as organic solvent, this means that the test can be run in a setting where no fume-hood is necessary and (3) the FASafe is fast and easy to use making routine analyses easy to perform. The main draw back of the FASafe method is the cost of analysis, $2.86 for one sample in duplicate. This is higher than the cost of an analysis (in duplicate) using the AOCS method, mainly due to the cost of the solvent (ethanol).

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