Evaluation of the Effect of Tocopherols on the Stability of Biodiesel

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CONTENTS

SUMMARY

A comprehensive study was carried out on the effects of naturally occurring tocopherols and carotenoids on the stability of biodiesel-grade methyl esters. Commercially available tocopherols and carotenoids, α-, γ- and δ-tocopherol, carotene and asthaxanthin, were added to destabilised methyl esters and the solutions were exposed to air at 65^{o}C. The stabilising effect of the added tocopherols and carotenoids was determined from the number of days needed to reach the same increase of viscosity as destabilised methyl ester without tocopherols after 1 day. All three tocopherols stabilised methyl esters; γ- being the most effective and α- the least. The stabilising effect of tocopherols increased with concentration up to an optimum level. Concentrations above this level did not improve stability significantly. The stabilising effect of the tocopherols also depended on the composition of the methyl ester; they were most effective in tallow methyl ester, and had the least effect on sunflower methyl ester. Carotene and asthaxanthin had no effect on the stability of the methyl esters. However an unidentified carotenoid in rape methyl ester changed the oxidation pattern by reducing rates of peroxide and viscosity increase, without affecting overall stability.

INTRODUCTION

Biodiesel, being a mixture of methyl esters of vegetable oil fatty acids, is more susceptible to oxidation than mineral diesel. The lower oxidative stability of biodiesel is caused by the higher level of unsaturation and possibly by the larger amount of dissolved oxygen. A previous study on the storage stability of methyl esters found that there was deterioration during storage, and suggested the addition of antioxidants to ensure storage stability (Thompson *e t al*., 1998). Another study found that there was no measurable deterioration of rapeseed, sunflower and camelina methyl esters stored in closed drums for 18 months (Fröhlich, 1999). The same study however also indicated that methyl esters oxidise when in continuous contact with air, but oxidation can be delayed by the presence of tococopherols and possibly carotenoids. The antioxidant effect of tocopherols in rapeseed and sunflower oils has been reported (Lampi *e t al*., 1999). It was also shown that carotene enhances the stabilising effect of tocopherol (Henry *e t al.*, 1998). The effect of tocopherols and carotenoids on the stability of methyl esters has not been reported previously, but it was shown that both remained in the methyl ester when oils were esterified (Simkowsky, 1997). The objective of the present project is to study the effect of tocopherols and carotenoids on the oxidative stability of biodiesel-grade methyl esters.

METHODOLOGY

Materials

Tocopherols, α-, γ- and δ- and the carotenoids, carotene retinoic acid and astaxanthin were obtained from Sigma-Aldrich (Ireland) Ltd., SME (sunflower methyl ester), RME (rape methyl ester), WCOME (waste cooking oil methyl ester) and TME (tallow methyl ester) were prepared from commercial oils and tallow, CME (camelina methyl ester) was prepared from oil pressed at Oak Park according to a reported method (Fröhlich and Rice, 1995). SME and RME prepared from unrefined oils were obtained from BLT, Wieselburg, Austria and Novaol, Bruck, Austria, respectively. SME was stored in a 2.5 1itre glass container for four years. Tocopherols in the methyl esters were deactivated by gentle stirring at 110°C for about 20 minutes, and the loss of tocopherols was monitored by HPLC. After the deactivation of tocopherols the methyl esters were destabilised by stirring at 100-110°C until peroxide levels increased to 30-35 mg/kg.

Determination of stability

Tocopherols and carotenoids were each weighed directly into approx. 50 ml of destabilised methyl ester and the solution was made up to the required weight. Oxidation of the methyl ester was carried out according to the Schaal accelerated storage test (Jacobs, 1958). Eight 25 g samples were stored for up to 8 days at 65^{o}C in 250 ml beakers of identical geometry, covered with watch glasses. Samples were taken daily.

Free fatty acid levels and viscosities of the collected samples were determined according to ISO 660 and 3104 respectively, and peroxide levels according to the method used for peroxides in oils and fats (AOAC, 1984). AOIT (active oxygen induction time) was determined according to EN 14112 at the University of Graz. Tocopherols were determined by HPLC equipped with a UV detector and an amino-reverse phase column (250 mm). The mobile phase was 80:20 heptane-ethyl acetate and tocopherols were detected at 295 nm (Simkowski, 1997). Coloured compounds (carotenoids) in RME made from unrefined rapeseed oil were separated with the same column, but the detector wavelength was 448 nm, and the mobile phase 50:50 hexane ethyl acetate. The efficiency of antioxidants was determined from the period of stability, defined as the number of days required to reach the same viscosity as destabilised methyl ester (0.5 cSt) after 1 day at 65^{o}C. Correlations between oxidation indicators were determined by linear regression. Methyl esters with low peroxide levels were obtained by heating RME, SME, WCOME and TME (120 g) in a round bottom flask (250 ml) under vacuum (1-2 mm) at 180-200^{o}C until peroxide levels stabilised (15-45 min).

Isolation of carotenoids

The detected carotenoid was isolated by silica gel column chromatography. The column (25 mm od x 300 mm) was made up with silica gel (26 g, 0.063-0.2 mm, Merk 60) in hexane and RME (50 g), prepared from unrefined rapeseed oil, was passed through it. The methyl ester was washed out of the column with hexane until the effluent was colourless (approx. 100 ml), and the orange band absorbed at the top of the column was eluted with ethyl acetate. Collection of the eluent started when the orange band reached the bottom of the column, and it was continued until the eluent became colourless. The solvent was evaporated from the collected fraction, the residual solid (1.7 g) was dissolved in ethyl acetate (2 ml), and added to a silica gel column made up as before. The column was washed with 20% ethyl acetate in hexane and a light yellow band, which eluted from the top of the column, was collected. Evaporation of the solvent yielded a yellow solid (1.7 g), but HPLC indicated that it did not contain the detected carotenoid. The orange band at the top of the column was eluted with 60% ethyl acetate in hexane and collected visually as before. Evaporation of the solvent yielded an orange oil (0.1 g) and HPLC showed a large peak, about 90% of the total peak area, with the retention time of the detected carotenoid, followed by two smaller peaks.

RESULTS

Destabilisation of methyl esters

In order to study the effects of added natural antioxidants on the oxidation stability of methyl esters it was necessary to eliminate all other stabilising factors. Considering that a methyl ester is unstable when its viscosity increases after brief exposure to air, destabilised methyl ester was defined as a methyl ester that increased its viscosity by 0.5 cSt after 1 day at 65^{o}C. Removal of colour and tocopherols from RME was not sufficient for destabilisation; viscosity remained constant for several days (Table 1).

Unidentified antioxidants in tocopherol-free RME were deactivated by passing the methyl ester through a silica gel column or heating at 110^{o}C for 4 hours. Passing tocopherol-free RME through silica gel to remove possible unidentified antioxidants increased its stability, but prolonged heating destabilised it (Table 1). The difference between the three RMEs was the peroxide level; seemingly peroxides must be increased to a certain level to destabilise the methyl ester. Oxidation of SME with a range of peroxide levels indicated that the peroxide level at which the methyl ester is destabilised is about 30 mmole/kg (Table 2). Other methyl esters, namely RME, WCOME and TME with peroxide levels of 30-35 mmole/kg also increased their viscosities after 1 day at 65^{o}C (Table 3). Consequently, the peroxide levels of the methyl esters used for the evaluation of tocopherols were adjusted to 30-35 mmoles/kg.

Table 1: Viscosity increase of RME stored at 65°C with tocopherols and colour removed

Treatment	Control	RME passed through silica gel	RME heated at 110°C for 4 hr
Day	Viscosity increase (cSt)		
1	0.00	0.00	0.70
2	0.00	0.00	1.03
3	0.00	0.00	1.51
4	0.18	0.00	2.13
5	0.30	0.05	2.72
6	0.59	0.10	3.38
7	1.38	0.37	3.96

Table 2: Stability of antioxidant-free SME

	Stability (days)	Peroxide level
SME-1	2	7.9
SME-2	2	9.3
SME-3	1	18.2
SME-4	0	32.0
SME-5	0	35.0

Table 3: Viscosity increase and peroxide level of destabilised RME, WCOME and TME

Day	RME		WCOME		TME	
	Viscosity increase [cSt]	Peroxide levels [mmole/kg]	Viscosity increase [cSt]	Peroxide levels [mmole/kg]	Viscosity increase [cSt]	Peroxide levels [mmole/kg]
0		32		36		33
1	0.36	80	0.39	78	0.18	55
2	0.79	122	1.03	122	0.30	70

Tocopherols

Effect of added tocopherols on the stability of SME

The effect of tocopherols on the oxidation rate of SME is shown in Tables 4-6. SME was used because it is regarded as the least stable of the available biodiesel grade methyl esters. Considering that atmospheric oxidation of fatty acid methyl esters leads to the formation of oligomers and polymers which increase viscosity, differences in viscosity between initial and oxidised samples were used as the indicator of oxidation. Monitoring of viscosities during exposure to air at 65°C shows that all three tocopherols delay oxidation (Tables 4-6). The period of stability, the period when viscosity is less than 0.5 cSt higher than the initial value (viscosity increase of destabilised SME after 1 day at 65°C), seems to depend on the type and amount of antioxidant added. At natural antioxidant levels, 250-500 mg/kg of α-tocopherol in SME (Table 4), stability is about the same as before destabilisation, and viscosities start to increase after 1 day in accelerated storage. At higher tocopherol levels however, viscosities can remain stable (<0.5 cSt increase) for a much longer period.

Of the three antioxidants, γ-tocopherol proved to be the most effective. A concentration of 250 mg/kg γ-tocopherol stabilised SME for 6 days (Table 6), whereas the same amounts of α- and δ- tocopherols had stabilising effects of only 1 day (Table 4) and 3 days (Table 5), respectively. The stabilising effect also increased with the amount of antioxidant, but little additional stability was gained above 1000 mg/kg of α- and 500 mg/kg of γ- and δ-tocopherols. At all levels of α-tocopherol, viscosity started to increase very slowly after Day 1, whereas with δ- and γ-tocopherols, particularly at higher levels, it remained stable for several days.

In all samples tocopherol levels decreased during accelerated storage, but not at a uniform rate (Tables 4-6). Generally 50% of the antioxidant was lost after the first day, but losses were more gradual thereafter. Tocopherols did not prevent viscosity increases, but the rate of increase was much slower (0.01-0.1 cSt/day) while they were present. This is particularly noticeable in the case of α-tocopherol, where the daily increase of viscosity was below 0.1 cSt while the antioxidant remained above 100 mg/kg (Table 4). Once the tocopherols were deactivated, viscosity increased at about the same rate as that of destabilised SME. The period of stability more or less corresponded to the time when effective

amounts of antioxidants were present in the methyl ester.

Table 4: Effect of α-tocopherol levels on viscosity increase of stored SME; increases below 0.5 cSt underlined

α-tocopherol added (mg/kg)									
	0	250		500		1000		2000	
Day	Viscosity increase [cSt]	Viscosity increase [cSt]	Tocoph. level [mg/kg]	Viscosity increase [cSt]	Tocoph. level [mg/kg]	Viscosity increase [cSt]	Tocoph. level [mg/kg]	Viscosity increase [cSt]	Tocoph. level [mg/kg]
1	0.48	**0.04**	36	**0.06**	47	**0.02**	485	**0.05**	1074
2	0.90	1.12	11	**0.40**	11	**0.06**	383	**0.12**	869
3	1.29	1.83	n.d.[a]	1.50	n.d.	**0.12**	346	**0.22**	728
4	2.75	3.58	n.d.	1.62	n.d.	**0.22**	287	**0.32**	418
5	3.81	5.03	n.d.	3.33	n.d.	**0.24**	160	**0.40**	418
6	4.88	7.37	n.d.	4.67	n.d.	**0.26**	78	**0.46**	300
7	6.83	8.28	n.d.	5.90	n.d.	0.77	n.d.	**0.49**	261
8	8.41	13.77	n.d.	9.60	n.d.	1.75	n.d.	0.80	15

- [a]n.d. = not detected

Table 5: Effect of δ-tocopherol levels on viscosity increase of stored SME; increases below 0.5 cSt underlined

δ-tocopherol added [mg/kg]									
	0	250		500		1000		2000	
Day	Viscosity increase [cSt]	Viscosity increase [cSt]	Tocoph. level [mg/kg]	Viscosity increase [cSt]	Tocoph. level [mg/kg]	Viscosity increase [cSt]	Tocoph. level [mg/kg]	Viscosity increase [cSt]	Tocoph. level [mg/kg]
1	0.48	**0.12**	66	**0.01**	364	**0.04**	535	**0.06**	1268
2	0.90	**0.28**	47	**0.10**	229	**012**	446	**0.09**	1027
3	1.29	**0.34**	39	**0.11**	217	**0.08**	422	**0.09**	951
4	2.75	0.89	11	**0.21**	93	**0.12**	345	**0.13**	772
5	3.81	0.96	n.d.[a]	**0.22**	89	**0.21**	254	**0.19**	583
6	4.88	1.72	n.d.	**0.36**	52	**0.30**	149	**0.25**	562
7	6.83	2.39	n.d.	**0.43**	43	**0.37**	128	**0.29**	407
8	8.41	3.71	n.d.	0.77	23	**0.49**	68	**0.39**	140

- [a]n.d.= not detected

Table 6: Effect of γ-tocopherol levels on viscosity increase of stored SME; increases below 0.5 cSt underlined

γ-tocopherol added [mg/kg]							
	0	250		500		1000	
Day	Viscosity increase [cSt]	Viscosity increase [cSt]	Tocoph. level [mg/kg]	Viscosity increase [cSt]	Tocoph. level [mg/kg]	Viscosity increase [cSt]	Tocoph. level [mg/kg]
1	0.48	**0.00**	128	**0.00**	227	**0.05**	532
2	0.90	**0.00**	80	**0.04**	202	**0.06**	426
3	1.29	**0.01**	*57*	**0.07**	120	**0.07**	323
4	2.75	**0.07**	*57*	**0.02**	*88*	**0.05**	226
5	3.81	**0.15**	*63*	**0.07**	*88*	**0.15**	178
6	4.88	**0.23**	*63*	**0.31**	*67*	**0.15**	*98*
7	6.83	0.50	*63*	**0.23**	*67*	**0.30**	*67*
8	8.41	1.00	*25*	**0.23**	*67*	**0.14**	143

- Values in *italic*:tocopherol determinations not accurate because HPLC peaks changed shape

Stabilisation of methyl esters with added δ-tocopherol

Stabilisation of methyl esters other than SME (i.e. RME, WCOME and TME) with tocopherol was also investigated. δ-Tocopherol was used, because it is more effective than α-tocopherol and it could be determined more accurately than γ-tocopherol. δ-Tocopherol (1000 mg/kg) was added to the methyl esters and they were oxidised for 8 days as per SME. It was not possible to obtain exact stabilisation times as before because RME, WCOME and TME had significant amounts of tocopherol left after 8 days of oxidation (Table 7). However, oxidation indicators such as peroxide levels and antioxidant losses after 8 days could be used to estimate comparative stabilising effects.

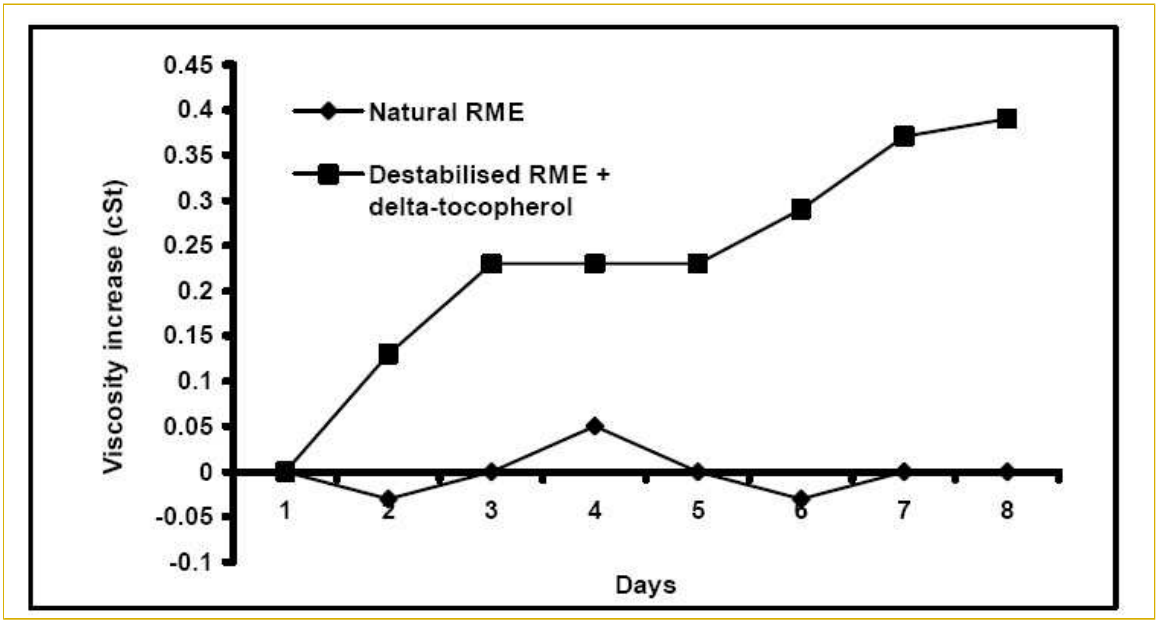
The stabilising effect of δ-tocopherol, and possibly of other tocopherols, in addition to concentration, seems to depend also on the composition of the methyl ester. The antioxidant was considerably more effective in stabilising TME, WCOME and RME than SME. About 93% of the tocopherol in SME was lost and peroxide level increased to 115 mmole/kg after 8 days of oxidation. On the other hand, 83% of the tocopherol remained in TME, and there was no increase in peroxide level during the same period, indicating that all dissolved oxygen was taken up by the tocopherol. Peroxide increase and tocopherol losses in RME and WCOME were between the two extremes. Hence, according to the obtained data, the stabilising effect of δ-tocopherol in the four methyl esters is in the order TME>WCOME>RME>SME.

Table 7: Methyl esters with 1000 mg/kg δ-tocopherol added; oxidation indicators after 8 days

Methyl ester	δ-tocopherol level (mg/kg)	Viscosity increase after 8 days storage (cSt)	Peroxide increase (mmole/kg)
SME	68	0.49	115
RME	188	0.34	24
WCOME	428	0.39	19
TME	834	n.d.	n.d.

Stability of methyl esters with natural tocopherols

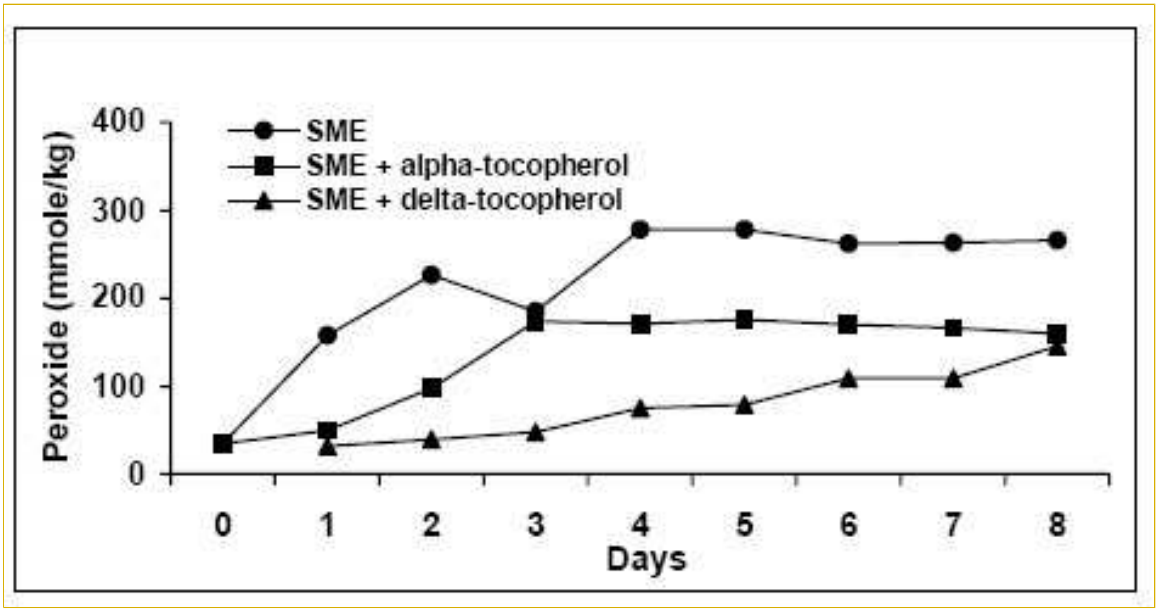
Methyl esters with natural tocopherols (i.e. prepared from unrefined oils) were found to be more stable in terms of viscosity increase and rate of peroxide formation than destabilised methyl esters with similar amounts of added antioxidants. The viscosity of destabilised SME with 500 mg/kg α-tocopherol was constant for only one day (Table 1), and its peroxide level increased by 65 mmoles/kg in the first two days of oxidation. On the other hand, SME prepared from unrefined oil with 300 mg/kg α-tocopherol stored with about 10% headspace for four years, showed constant viscosity for two days and its peroxide levels increased by only 20 mmole/kg during the same period.Similarly, destabilised RME with1000 mg/kg added tocopherol increased its viscosity and peroxide level in eight days by 0.3 cSt and 23 mmole/kg, respectively; whereas, RME prepared from unrefined oil, with 200 and 300 mg/kg α- and γ-tocopherols, increased its peroxide level by only 6 mmole/kg and its viscosity remained constant during the same period (Fig. 1). The greater susceptibility of destabilised methyl esters with added antioxidants to oxidation is probably due to the presence of deactivated tocopherols formed during destabilisation, which are reported to be pro-oxidants (Jung and Ming, 1992).



- **Fig. 1:** Oxidation of natural RME vs destabilised RME with 1000 mg/kg δ-tocopherol added

Effect of tocopherols on peroxide levels

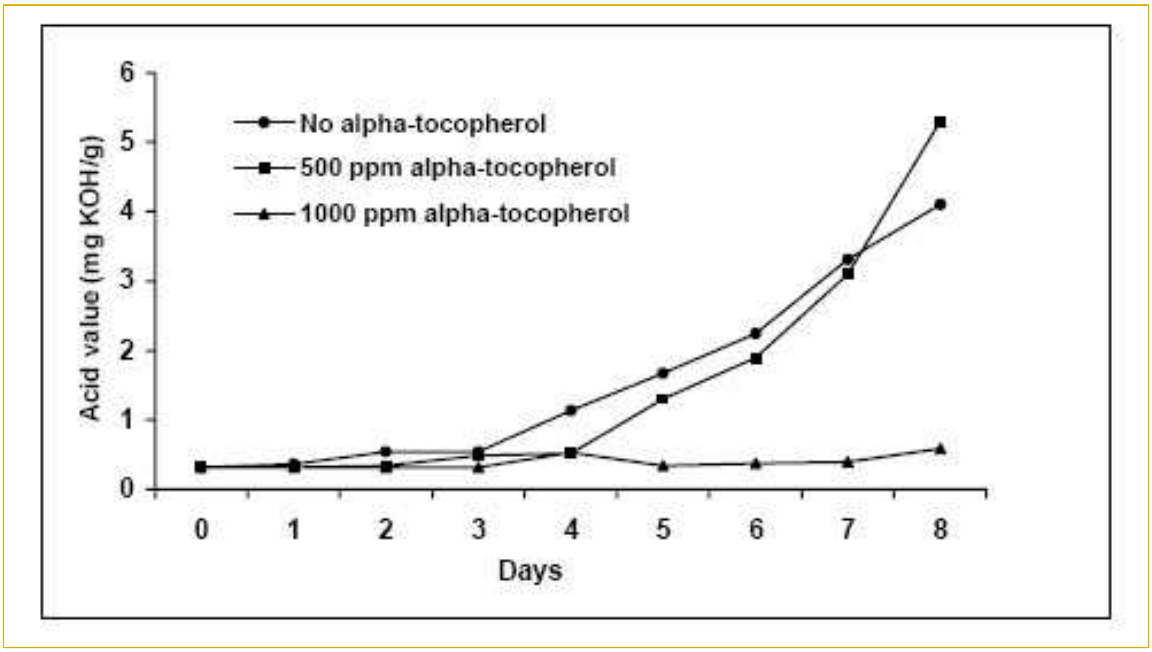
Monitoring of peroxide levels indicated that the effect of tocopherols on oxidation was to slow down the build up of peroxides, which in turn stabilises the viscosity. Seemingly, a certain critical peroxide level must be reached before there is a significant increase of viscosity, possibly over 70-80 mmole/kg in SME, RME and WCOME but lower in TME (Table 3). Tocopherols extend the time needed to reach the critical peroxide level. In general, peroxide levels remain below 80 mmole/kg as long as effective amounts of antioxidants remain in the methyl ester. The delaying effect depends on the type of tocopherol. Destabilised SME reached a peroxide level of 150 mmole/kg after only 1 day of oxidation, whereas with 500 mg/kg α-tocopherol it took 3 and with the same amount of δ-tocopherol 8 days to reach the same level (Fig. 2).



- **Fig. 2:** Effect of 500 ppm of α and δ-tocopherols on peroxide levels of stored SME

Effect of tocopherols on FFA levels

Along with peroxides, FFAs (free fatty acids, expressed here as acid values) also increased during oxidation (Fig. 3). FFAs were formed when peroxides were converted into secondary oxidation products, and their increase indicated that secondary oxidation was taking place. While tocopherols were present there was no significant increase of FFAs, and acid values remained below 0.5 mgKOH/g. However, once the tocopherols were lost FFAs began to rise and continued to rise more rapidly as the oxidation progressed.



- **Fig. 3:** Effect of α-tocopherol on FFA levels of stored SME

Effect of peroxide levels on methyl ester stability

It was shown earlier that the stability of the methyl ester increased as peroxide levels were reduced. Hence, the possibility of stabilising methyl esters by minimising peroxides was investigated. Peroxide levels of TME and WCOME could be reduced to below 2 mmole/kg under vacuum at 180-200^{o}C, but the minimum obtainable levels for RME and SME under the same conditions were 2 and 4 mmoles, respectively.

Reduced peroxide levels stabilised both SME and TME, although not as much as tocopherols. The rates of peroxide formation and viscosity increase were almost halved when compared to those with initial peroxide levels normally found in these methyl esters. Stabilisation of WCOME with reduced peroxide level was however comparable to stabilisation with tocopherols. There was no detectable increase of viscosity during the first 10 days of oxidation and the rate of peroxide build-up was lower than that of WCOME with 1000 mg/kg added δ-tocopherol (Table 7).On the other hand, RME was destabilised when peroxide levels were minimised by the present method.

Carotenoids

Effects of carotenoids on the stability of methyl esters

The antioxidant effect of β-carotene in combination with γ-tocopherol has been reported previously (Henry, 1998). This work has shown that RME is considerably more stable than CME during oxidation, although their tocopherol composition, particularly γ-tocopherol levels are almost identical (Fröhlich, 1999). However, RME made from unrefined oil showed a much higher absorbance at 448 nm than CME, and the absorbance decreased along with tocopherols during oxidation. The visible spectrum of RME showed three absorption maxima at 423, 448 and 475 nm, indicating the presence of a carotenoid, which could contribute to the stabilisation of the methyl ester.

Astaxanthin and retinoic acid

Initially the effects of a strong carotenoid antioxidant, astaxanthin, and a carotene derivative, retinoic acid were investigated. The carotenoids were added to destabilised SME at the rate of 500 and 100 mg/kg, and were oxidised for 8 days. Neither retinoic acid nor asthaxanthin had any effect on the stability of the methyl esters; both viscosities and peroxide levels increased at the same rate as in SME before the addition of carotenoids.

β-Carotene

The effect of β-carotene was also examined because it was reported to be present in rapeseed oil (Patterson, 1989), and we could also detect it in RME. Hence, if carotene stabilises RME it should also stabilise CME which has similar tocopherol composition. Sufficient carotene was added to CME to give the same absorbance as RME at 448 nm and some α-tocopherol was also added to obtain the same tocopherol composition. In addition β-carotene was added to CME

to give a carotene level higher and lower than found in RME. Oxidation of the samples with added β-carotene and the corresponding controls indicated that β-carotene did not improve the stability of CME, irrespective of the amounts added, or if α-tocopherol was also added. The CME samples with added β-carotene had approximately the same rate of increase of viscosity as destabilised CME (Table 8).

Identification of other carotenoids

Considering that carotene astaxanthin and retinoic acid did not stabilise SME, attempts were made to identify other carotenoids, by separating the orange coloured compounds in RME by HPLC. Conditions used for the separation of tocopherols at 448 nm showed only carotene, but with a more polar mobile phase, in addition to carotene, four compounds were also separated. Carotene accounted for only about 10-15% of the peak area of the separated compounds, and about 60% corresponded to a single compound with much higher retention time than carotene. The other three compounds were present in smaller amounts.

Table 8: Effects of carotene on the stability of CME: increase of viscosity(cSt) during accelerated storage

Treatment day	CME natural	+α-tocopherol	+α-tocopherol +carotene	CME destabilised
	Viscosity increase of stored CME (cSt)			
2	0.14	0.03	0.26	0.32
4	0.34	0.27	0.62	0.76
6	0.85	0.64	0.86	1.45
8	1.15	1.82	1.50	2.66

Treatment day	CME natural	+Carotene 10 mg/kg	+Carotene 100 mg/kg	CME destabilised
2	0.14	0.31	0.32	0.32
4	0.34	0.64	0.62	0.76
6	0.85	1.18	1.75	1.45
8	1.15	2.68		2.66

The detected compound was present in RME from different sources at about the same level (relative to carotene) as tocopherols (Table 9). A very small amount of the compound was present in CME; it was absent from TME and SME, and only 7% of the compound was left in RME after heating for 3 hours at 110°C. In addition, when RME with high absorbance at 448 nm (prepared from raw oil) was heated at 110^{o}C, 5 hours were required to remove the tocopherols, whereas from RME with low absorbance (prepared from refined oil) tocopherols were removed within 20 minutes. Differences in tocopherol deactivation times could indicate that along with tocopherols, the detected compound could also stabilise RME.

Table 9: Compound detected at 448 nm and β-carotene in different methyl esters

Methyl ester	Absorbance 450 nm [a]	β -carotene [mg/kg]	Detected compound [b]	Peak area of detected compound (%)
RME 1	0.57	47	247	67
RME 2	0.38	31	140	63
RME 3	0.53	83	316	70
SME 1	0.01	n.d.[c]	n.d.	
CME 1	0.08	n.d.	17	100
TME 1	0.01	n.d.	n.d.	
RME 2 (heated at 110°C)	0.11	n.d.	17	

- [a]10% solution in hexane
- [b]mg/kg β-carotene used as reference
- [c]n.d.= not detected

Isolation of the carotenoid

The carotenoid detected by HPLC was isolated from RME by column chromatography as an orange-coloured oil about 0.2% of the methyl ester. HPLC chromatogram indicated that apart from the carotenoid there were two additional compounds with longer retention times. However, the carotenoid corresponded to 82% of the total peak area and the other two compounds were 15% and 3%, respectively. Absorbance spectra of the isolated substance gave three maxima at 423, 448 and 452 nm, indicating a structure similar to carotene.

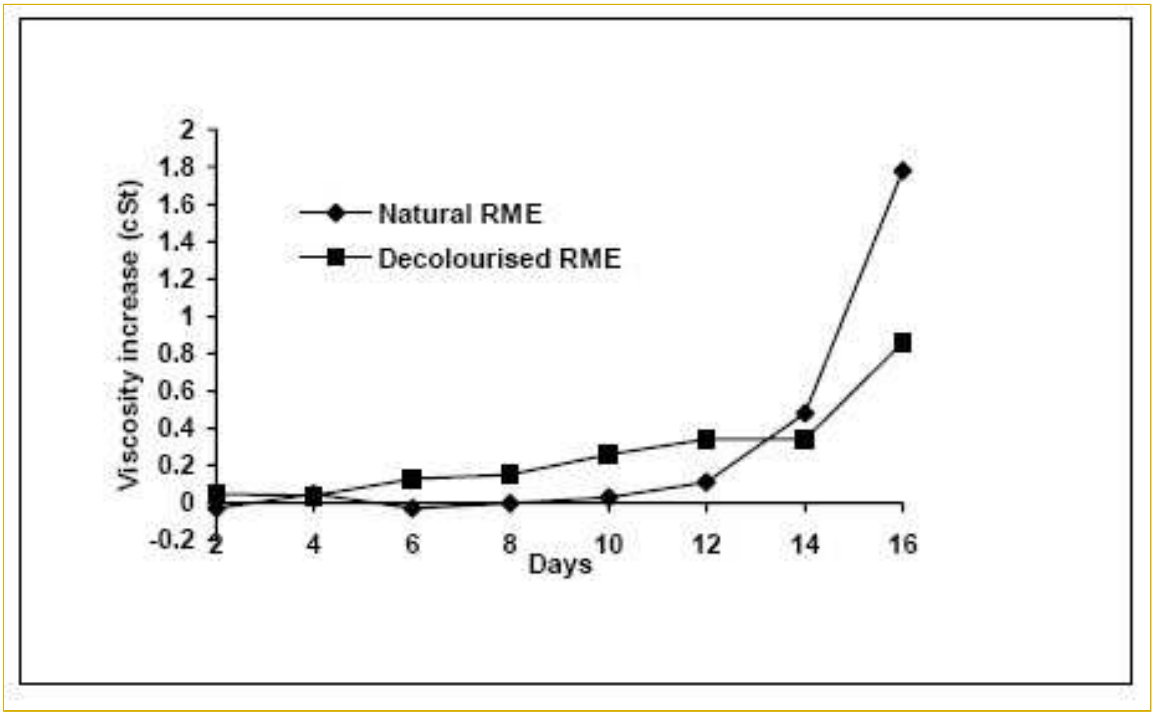
Mass spectrum of the carotenoid peak gave two abundant fragments at 323 and 339 m/z, and the largest fragment at 662 m/z was of relatively low abundance. The sum of the two most abundant fragments was also 662, which indicated that the fragment at 662 could be the molecular ion of a compound with m.w. of 663. It will be necessary however to obtain IR and NMR spectra of the pure carotenoid peak for further structural elucidation.

Effects of the detected carotenoid

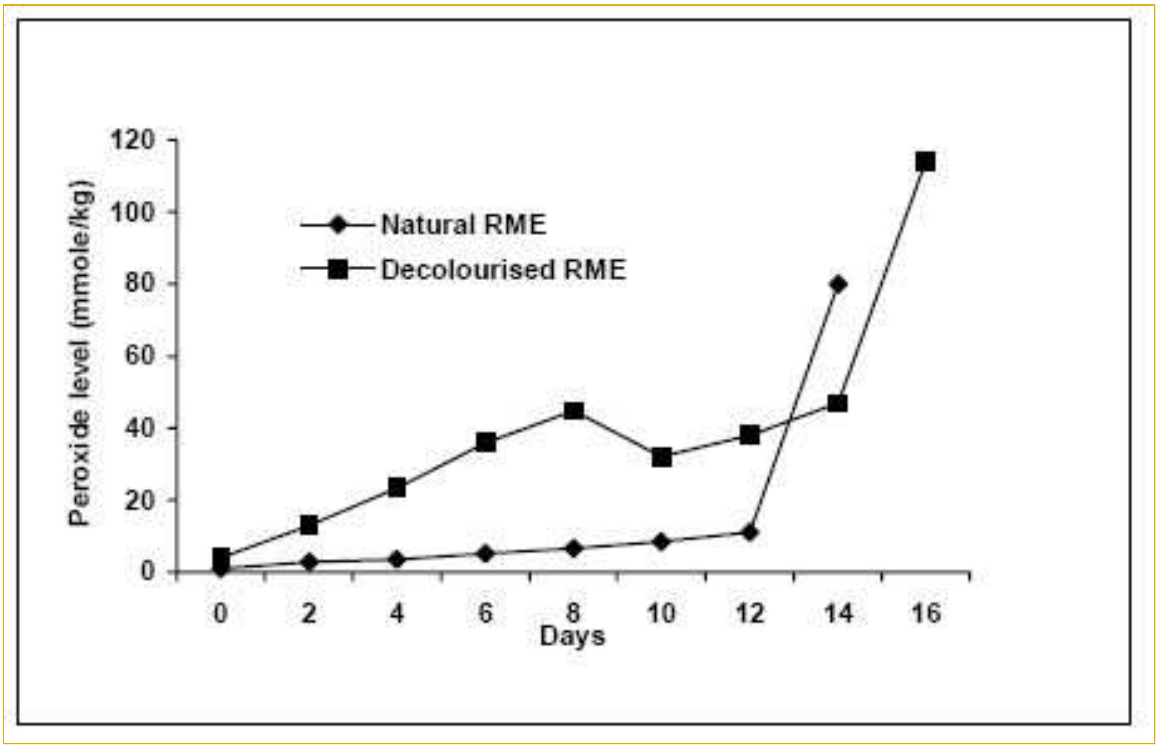
The effect of the detected carotenoid on the stability of RME was determined by comparing oxidative stabilities of normal and carotenoid-free RMEs. Carotenoids could be removed from RME by brief heating at 80^{o}C with Fuller's Earth. The method is used for the decolourisation of RME during refining. Analysis of decolourised RME by HPLC and absorbance at 448 nm indicated that all carotenoids were removed, but there was no significant loss of tocopherols.

Both carotenoid-free and natural RME were oxidised at 65^{o}C for 16 days. The difference between the oxidative stabilities of the two RMEs was minimal; both methyl esters increased their viscosities by about the same amount after 14 days (0.33 and 0.48). However, the oxidation patterns were different. The viscosity of natural RME remained constant for 10 days, and started to increase on Day 12, whereas the viscosity of decolourised RME began to increase very slowly after Day 6 (Fig. 4). Similarly, the rate of peroxide increase of natural RME was only 0.8 mmoles per day, but it increased suddenly to 35 mmole/day on Day 12. On the other hand, peroxide build up in decolourised RME was 5.8 mmole/day and the first large increase, 30 mmole, occurred on Day 14 (Fig. 5). The deactivation rate of the detected carotenoid was about the same as that of γ-tocopherol, and both were completely deactivated at the same time (Day 14).

The effect of the isolated carotenoid on SME was also examined. The yellow solid (4.5g), isolated from 250 g RME after the first silica gel column chromatography, was added to destabilised SME with 250 mg/kg δ-tocopherol. Absorbance of the SME at 448 nm increased from 0.02 to 0.4 au, which is within the range of observed absorbances of natural RME. SME with the added carotenoids and the control were oxididised by accelerated storage for 8 days. Instead of the expected oxidation rate reducing effect however the added yellow solid increased both peroxide and viscosity rates. Reasons for the increased oxidation rates could be the prooxidant effects (Jung and Ming, 1992) of noncarotenoids in the yellow solid, and the high initial peroxide levels, which increased from 31 to 40 mmole/kg when the solid was dissolved in SME. The experiment will need to be repeated with the orange oil obtained from the isolated solid, which contains far less coextracted materials.



- **Fig. 4:** Increase in viscosities, natural vs decolourised RME



- **Fig. 5:** Peroxide increase, natural vs decolourised RME

DISCUSSION

Tocopherols

The results obtained in the present work indicate that all three commercially available natural antioxidants, α-, γ- and δ-tocopherols, which are by-products of vegetable oil refining, were effective in stabilising SME. γ-Tocopherol was the most effective and α- the least, and stabilisation also depended on tocopherol concentration up to a certain level. Added δ-tocopherol also stabilised RME, WCOME and TME and probably α- and γ-tocopherols would also be effective. Tocopherols occurring naturally in RME (α and γ-) and SME (α-) were found to be more effective than added tocopherols.

Sunflower and rapeseed oils, the main feedstocks for biodiesel-grade methyl ester in the EU, are stabilised by naturally occurring α- and γ- tocopherols, which also stabilise their methyl esters. Attempts must be made therefore to ensure that these tocopherols remain near their original levels in the methyl esters when the oils are esterified. The natural tocopherol levels in rapeseed oil (100-200mg/kg α-T, 250-350mg/kg γ-T) stabilise RME sufficiently to meet EU specifications (European Committee for Standardisation, 2001), provided they are retained during esterification. However, sunflower oil contains only α-tocopherol which at its natural levels (300-400 mg/kg), was found to be the least effective tocopherol (Table 3), and TME and most WCOMEs contain no significant amounts of natural antioxidants. Considering that 500 mg/kg γ-tocopherol gives almost the same stability to destabilised SME as the natural antioxidants to RME (Table 6, Fig. 4), it should be possible to stabilise SME, WCOME and TME sufficiently with γ-tocopherol to meet the EU specification. Tocopherols, α- and γ-, are by-products of rapeseed oil refining and inexpensive mixtures of the two antioxidants are widely available.

Carotenoids

The carotenoids added to SME and CME (β-carotene, astaxanthin and retinoic acid) had no detectable effect on the stability of the methyl esters. However, an unknown carotenoid detected in RME did change the oxidation pattern by reducing the rate of peroxide increase and stabilising viscosity, even if it did not improve its overall stability when compared to decolourised RME. There is a good correlation between the carotenoid peak areas and γ-tocopherol levels during oxidation (r^2 0.95), and both compounds were fully deactivated at the same time.

The detected carotenoid is probably not another antioxidant, because antioxidants are normally deactivated at different rates. It took only 4 days to deactivate α-tocopherol in RME, whereas 12 days were needed for γ-tocopherol. The compound behaves more like a “co-antioxidant”; it improved the antioxidant effect of γ-tocopherol while present, but it did not change its deactivation time. Considering that the compound can be isolated from RME by a relatively simple technique (see Methodology), its properties and effects on the oxidation of methyl esters should be investigated further.

technique (see Methodology), its properties and effects on the oxidation of methyl esters should be investigated further.

Stabilisation with low peroxide levels

Apart from tocopherols, low peroxide levels obtained by heating the methyl ester at 180-200^{o}C under vacuum were also found to delay the oxidation of SME, WCOME and TME, but not of RME. The method is used routinely in vegetable oil refining to deodorise and to reduce the peroxide levels of refined oils. However, methyl esters made from fresh vegetable oils normally have peroxide levels of 2-6 mmole/kg, and the small gain in stability from reduction of peroxide levels could be cancelled out by the loss of some tocopherols, during heating under vacuum. TME does not have any antioxidants, but it would need to be evaluated whether the small improvement in stability, when reducing peroxide level from 5 to 2 mmole/kg, justifies the cost of heating to high temperature under vacuum.

Reduction of peroxide level of WCOME to below 2 mmole/kg raised its stability almost to the level of natural RME (Figs. 4, 5), the most stable commercial methyl ester known at present. Hence the high temperature treatment under vacuum could be considered for WCOMEs with high peroxide levels. Similarly, reduction of peroxides could be very useful in the recovery of old methyl esters with poor stability which usually have high peroxide levels. Heating under vacuum until peroxide levels are minimised, followed by addition of suitable antioxidants, could yield methyl ester of acceptable stability.

Determination of stability

Ideally, a methyl ester is stable when no oxidation takes place, i.e. peroxides are not being formed, and all of the dissolved oxygen is absorbed by the antioxidant. In this case, the beginning of primary oxidation, that is peroxide formation, can be taken as a measure of stability. However, in the present work only TME with 1000mg/kg δ-tocopherol showed ideal stability; in all other methyl esters peroxide levels started to increase while tocopherols were still present. Consequently, the beginning of secondary oxidation, indicated by the increase of viscosity could also be taken as a measure of stability.

However, even the beginning of viscosity increase was not an entirely suitable criterion in the present work, for in many destabilised methyl esters with added tocopherols, viscosity started to increase, albeit very slowly, once the methyl ester was exposed to air at 65^{o}C. Hence another criterion for stability was established, which compared the stability of methyl ester with tocopherol to that of destabilised methyl ester. The period of stability was defined as the time in days required to reach the viscosity reached by the destabilised methyl ester after 1 day at 65^{o}C. The obtained times corresponded to the first sharp increase of viscosity and also to the complete deactivation of tocopherols (Tables 4-6).

While the time needed to reach the initial viscosity increaseofdestabilised methyl ester is probably an accurate indicator of stability, the method cannot be routinely used for commercial methyl esters. There is a long waiting period, up to 14 days in the case of stable RMEs, and accurate determination of eight to ten viscosities is very time consuming. Furthermore, there is either no increase of viscosity while the methyl ester is stable, or the slow increase is not necessarily continuous. Consequently, it does not seem to be possible to predict the period of stability accurately from initial viscosity increases.

Prediction of stability from the obtained experimental data

Peroxides are primary oxidation products, and it was shown that they need to reach certain levels before a sharp increase of viscosity indicates irreversible secondary oxidation. Therefore the rate of increase of peroxide levels might be an indicator of stability. Peroxide rates can be determined easily from peroxide levels, and provided they are continuous and linear it should be possible to obtain an indication of oxidation stability from peroxide rates determined after 2-3 days of oxidation.

Peroxide rates were determined from the linear portion of the peroxide curves; correlation coefficients ranged from 0.94-0.99. However, the correlation coefficient (Table 10) between peroxide rates and periods of stability determined from viscosity increase was only 0.80, which is not sufficiently high for the prediction of stability. Possible reasons for the poor correlation could be that peroxide rates were not always linear up to the time when viscosity increase indicated destabilisation. Furthermore, peroxide rates can be misleading. The determined peroxide rate of decolourised RME (not included in the correlation) is about six times as high as that of natural RME (Fig. 5), but in terms of viscosity increase they both have the same stability (12 days).

Viscosity-based periods of stability and peroxide rates were also correlated with AOIT (active oxygen induction time), which is normally used to determine stabilities of edible oils, i.e. time before rancidity. The correlation coefficient between AOITs and the two sets of oxidation parameters indicated that there was no useful correlation between the two sets of values (Table 11). The result is not entirely unexpected considering that AOIT indicates the time for the appearance of aldehydes (rancidity), whereas stabilisation time based on viscosity increase corresponds to the beginning of rapid polymerisation. According to the proposed mechanism of oxidation of linoleic acid, formation of aldehydes occurs in the chain propagation step, but polymerisation in the later termination step (Labuza, 1971). At present there is no available data to indicate how the relative rates of these oxidation reactions are influenced by tocopherols and the

composition of the methyl ester.

Considering that both tocopherol levels and absorbance at 448 nm decrease gradually during oxidation, it should be possible to predict the stability of RME from the initial values of these parameters. Absorbance determinations are probably more convenient, because tocopherols need to be determined by HPLC. Peak heights of the detected carotenoid correlate well with absorbance at 448nm (r^2 0.98), and both show a linear decrease (r^2 0.98) during oxidation. Also there is a good linear correlation between absorbance at 448 nm and tocopherol levels (r^2 0.95). Hence it should be possible to determine the period of stability from three or four initial absorbance values**.** In addition, if absorbance of the RME is known at the time of preparation, a simple ratio of actual and initial and absorbance should give a good indication of the loss of stability. The possibility of using absorbance of RME for the prediction of stability should be investigated further.

Table 10: Correlations between different indicators of methyl ester stability

Correlation	Regression coefficient (r^2)
Viscosity increase vs peroxide rate	0.79
Viscosity increase vs FFA increase	0.58
Viscosity increase vs AOIT[a]	0.48
Peroxide rate vs FFA increase	0.55
Peroxide rate vs AOIT	0.19
FFA increase vs AOIT	0.47

- [a]Active oxygen induction time

Table 11: Indicators of methyl ester stability

Methyl ester	Viscosity increase[a]	Peroxide rate(mmole/day)			AOIT[c]
		No. of days [b]	Regression coefficient (r^2)	Rate	
SME α-T250	1			44	
SME α-T500	2			28	1.04
SME α-T1000	6	6	0.94	9.7	1.08
SME α-T2000	7	7	0.96	13.4	1.48
SME δ-T250	3	4	0.99	18.2	0.14
SME δ-T500	7	5	0.97	9.8	0.84
SME δ-T1000	8	7	0.96	8.3	1.11
SME δ-T2000	9	8	0.99	6.2	2.80
SME g-T250	6	6	0.97	7.8	0.80
SME γ-T500	>8	5	0.99	4.2	1.11
SME γ-T1000	>8	7	0.92	5.4	1.45
RME δ-T1000	>8	8	0.95	2.7	3.27
WME δ-T1000	>8	8	0.93	2.2	4.14
TME δ-T1000	>8			0	26.5
NRME	12	12	0.98	0.8	7.78
NRME decol.	12	8	0.94	5.2	6.71
WME lowpx	10	10	0.96	1.0	2.41

- [a]No. of days of viscosity <0.5 cSt
- [b]No of days of linear peroxide rate
- [c]Active oxygen induction time

CONCLUSIONS

Tocopherols, α-, δ-, and γ- delay the oxidation of SME, RME, WCOME and TME, in some cases by more than a factor of 10 compared to methyl esters without tocopherols. γ-Tocopherol is the most effective of the three, α-tocopherol the least, and their antioxidant effect increases with concentration up to an optimum level. Above the optimum level the increase in antioxidant effect with concentration is relatively small.

The stabilising effect of tocopherols was also found to depend on the composition of the methyl ester. The order of effectiveness is: TME>WCOME>RME>SME.

Oxidation of methyl esters begins with the build-up of peroxides; viscosity starts to increase only after peroxides reach a certain level. Tocopherols stabilise the methyl esters by reducing the rate of peroxide formation, thereby extending the time needed to reach the level where viscosity starts to increase.

The carotenoids astaxanthin and retinoic acid have no detectable effect on the stability of SME. Similarly β-carotene added to CME along with some α-tocopherol, to give the same absorbance at 448 nm as RME, has no stabilising effect on the methyl ester. A carotenoid detected in RME changes its oxidation pattern by reducing the rates of increase of both viscosity and peroxide levels. However, the detected carotenoid does not extend the period of stability of RME.

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