UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

CHAPTER 3

MATERIALS AND METHODS

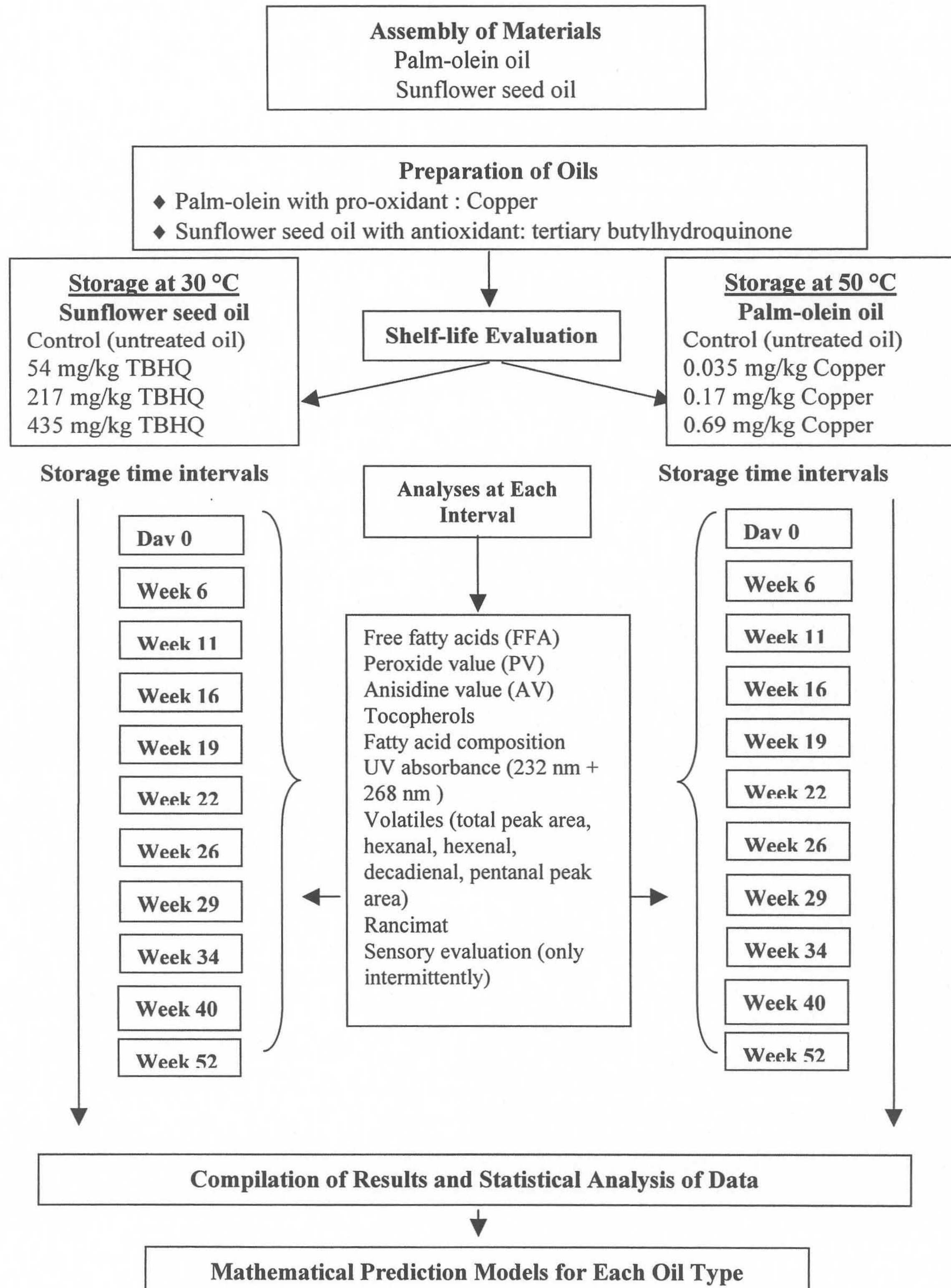

Figure 2: Summary of research methodology for development of prediction models to predict shelf-life of mono- (palm–olein oil) and polyunsaturated oil (sunflower seed oil).

3.1 MATERIALS

3.1.1 Palm-olein oil

Two different palm-olein oils were combined for the storage trial. Both oils were fully refined, bleached and deodorised (RBD) oils from Malaysia. One of the oils had been in storage in an amber glass container at – 20°C for approximately one year before the storage trial. The oil was obtained from Hudson and Knight, Durban, South Africa and the other oil (5 litre tin) had been received from Hudson and Knight, Durban, South Africa 6 weeks before the start of the storage trial. The addition of older oil was done to have oil that was comparable to oil that has been transported over a long distance and stored in bulk tankers before distribution. The oils were free of the antioxidant TBHQ. This was confirmed by testing for TBHQ as in the method from Anderson and Van Niekerk (1987) with the deviations as discussed under section 3.3. The oils were mixed in an approximate ratio of two thirds of the fresh oil and one third of the oil from storage. The combined oil was heated slightly to 50°C and stirred well to ensure proper mixing. A total of 7.0 litres were prepared. The fatty acid composition as in Official Methods and Recommended Practices of the AOCS, Method Ce 2-66 with modifications as described in section 3.3 was done on the combined oil and the profile confirmed that the oil was palm-olein oil.

3.1.1.1 Preparation of oil samples containing copper

A 0.3142 mg/ml solution of copper acetate was prepared in methanol. Copper acetate was chosen as it is soluble in methanol and is in organic form similar to fatty acids. Molecular weight of copper acetate is 199.65 g/mol and of copper (Cu^{2+}) 63.546 g/mol. The weighed copper acetate (0.1571 g) was made up to 500 ml in methanol in a volumetric flask. The concentration of copper in the solution was 0.1 mg/ml. Three concentrations of copper in oil were prepared. The first concentration contained 0.035 mg/kg copper. A solution of 0.0319 mg/L copper was prepared by adding 0.51 ml of the copper acetate solution to 1 600 ml oil. The second concentration was 0.17 mg/kg copper in oil. A solution of 1.59 mg/L copper was prepared by adding 2.55 ml of the copper acetate solution to 1 600 ml oil. The third concentration contained 0.69 mg/kg copper in oil. A solution of 6.36 mg/L copper was prepared by adding 10.18 ml copper acetate solution to 1 600 ml oil. Each of the three concentrations were mixed in a two litre glass beaker and left in the oven at 60°C for 3 h with occasional stirring to evaporate the methanol. Aliquots (100 ml) of each concentration, as

well a as control containing no copper, were measured and poured into 100 ml non-transparent (opaque) plastic containers with screw lids ready for storage. A set of four samples (0.035 mg/kg copper, 0.17 mg/kg copper, 0.69 mg/kg copper and the Control) were analysed on Day 0 (zero).

3.1.2 Sunflower seed oil

Deodorised sunflower oil was obtained from SA Oil, Randfontein, South Africa the day after production. No TBHQ was added. This was verified by testing for the presence of TBHQ as in the method of Anderson and Van Niekerk (1987) with the deviations as discussed under section 3.3. To ensure that the oil received was pure sunflower oil, a fatty acid composition determination as in Official Methods and Recommended Practices of the AOCS, Method Ce 2-66 with modifications as described in Analyses section 3.3, was done on the oil. The fatty acid composition resembled that of normal sunflower oil.

3.1.2.1 Preparation of sunflower seed oil samples containing TBHQ

A 50 mg/ml stock solution of TBHQ in ethanol was prepared by dissolving 1000 mg TBHQ in 20 ml ethanol and made up in a volumetric flask. The TBHQ solution had to be heated and shaken well until it was dissolved. Three concentrations of TBHQ in oil were prepared. The lowest concentration was 54 mg/kg TBHQ in oil. Stock solution (1.2 ml) was added to 1217 ml sunflower oil. The density of oil of 0.92 was taken into account when the TBHQ content was calculated as mg/kg oil. The second concentration of TBHQ was 217 mg/kg in oil. To prepare the solution 4.8 ml of the stock solution was added to 1217 ml sunflower oil. The third concentration of TBHQ prepared was 435 mg/kg in oil. Stock solution (480 ml) was added to 1217 ml oil. Each prepared oil sample was mixed in a two litre beaker and left in a oven at 50°C for 3 h with occasional stirring to evaporate the ethanol. Aliquots (100 ml) of each concentration, as well as a control containing no TBHQ, were measured into 100 ml size non-transparent (opaque) plastic containers with screw lids. A set of four samples (54 mg/kg TBHQ, 217 mg/kg TBHQ, 435 mg/kg TBHQ and a Control sample) was analysed on Day 0 (zero).

3.2 SHELF-LIFE METHODOLOGY

The palm-olein samples were stored in a Humidity Cabinet that was equipped with a fan where the cabinet was set at 50°C and was monitored by a thermometer. The relative humidity option was not used. A set of samples was taken out at each time interval for analyses. Samples remaining after analyses, or awaiting analyses at a later stage, were stored at – 20°C. The sunflower seed oil samples were stored in an incubator set at 30°C (the incubator was not equipped with a fan). The temperature was monitored by means of a thermometer. A set was taken out of storage at intermittent intervals for analyses. The remaining oil of the samples was stored at – 20°C until completion of analyses. The temperatures of both ovens were monitored weekly. The shelf-life test lasted for a period of 52 weeks (one year) for both oils. The sampling intervals were irregular. The intervals are set out in Figure 2.

3.3 ANALYSES

3.3.1 Chemical methods

All analyses were performed at least in duplicate except where there was insufficient equipment capacity, in which case appropriate repeat analyses were performed.

3.3.1.1 Free fatty acid value

The method determines the amount of free fatty acids present in the oil by dissolving the oil sample in a solvent and neutralising it by titration with sodium hydroxide, using phenolphthalein as indicator. The results are expressed as g/100 g oleic acid, as oleic acid is the main fatty acid present in most oils. The method used was AOCS Method Ca 5a-40, (AOCS, 1997) with the modification that 80 ml solvent (50% toluene/50% isopropanol) was used to dissolve the oil.

3.3.1.2 Peroxide value

Primary oxidation products are measured in terms of milli equivalents of peroxide per 1000 g sample. This is achieved by the addition of potassium iodide which is oxidised to iodine by the peroxides present in the oil. The iodine is measured by titration with "Titrisol"

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

standardised sodium thiosulphate (Merck, Darmstadt, Germany). The method used was the AOCS Method Cd 8-53, (AOCS, 1997).

3.3.1.3 Anisidine value

Secondary oxidation products were measured by determining the *p*-anisidine value. Aldehydic compounds in fats and oils react with *p*-anisidine, in the presence of acetic acid, to form yellowish reaction products. According to the method the intensity of the yellowish compounds is not related only to the amount of aldehydic compounds present, but also to their structure. A double bond in the carbon chain conjugated with the carbonyl double bond increases the molar absorbance four to five times. This is why 2-alkenals and dienals will contribute substantially to the value. This determines the quantity of aldehydes (principally 2-alkenals and 2,4-dienals) present in fats and oils. The AOCS Method Cd 18-90 (AOCS, 1997) was used. *p*-Anisidine was recrystallised according to the method and used for two consecutive days only, after which fresh *p*-anisidine was prepared.

The Totox value was calculated from the peroxide value and the *p*-anisidine value with the formula 2PV +AV.

3.3.1.4 Oxidative stability index (Rancimat)

The time from the start of the accelerated stability test to the point at which rapid oxidation occurs is called the induction period and gives an indication of the relative oxidative stability of the oil or fat. This is measured by heating the sample in a thermostated heater, while bubbling purified air through the sample at a constant rate. The effluent air is passed through deionised water and the conductivity of the water is measured for polar oxidation products (mainly formic acid) by an electrode. The method used was AOCS Method Cd 12b-92 (AOCS, 1997). A Rancimat 679 Metrohm Ltd, Switzerland was used. Sample (2.50 g) was weighed and air was bubbled through at a rate of 20 l/h. The temperature used to conduct the test for the sunflower seed oil samples was 110°C and for the palm-olein oil samples 120°C. This was because monounsaturated oils such as palm-olein are much more stable than polyunsaturated oils such as sunflower seed oil and are therefore normally subjected to higher temperature to ensure an induction period less than 48 h, which is the normal maximum run time for the Rancimat instrument.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

3.3.1.5 Tocopherols

Tocopherols were measured by normal phase HPLC using a fluorescence detector with excitation at 295 nm and emission at 330 nm as described by Van Niekerk (1973) and Van Niekerk (1975) and amended as in AOCS Method Ce 8-89 (AOCS, 1997). The oil was made up to volume in the mobile phase, 1 % isopropanol in hexane. The mobile phase was dried over sodium sulphate, as the water content of the mobile phase is of critical importance and drying with sodium sulphate regulates it. Separation was obtained by using a 25 cm Lichrosorb Diol 5µm column (Merck) and applying a flow rate of 0.8 ml/min. The detection limit for tocopherols is 0.1 mg/100 g.

3.3.1.6 Volatile compounds

Volatile compound analyses were determined using an in-house method of static headspace Gas chromatography (GC)-Flame ionisation (FID) analysis. A Genesys equilibrium headspace sampler and a Varian 3800 GC with a Restek Rtx-5, 30 m, 0.25µm film thickness, 0.32 mm ID (poly-5 % diphenyl / 95 % dimethylsiloxane) (Chromspec, PA, USA) column was used. Two g sample (accurate to four decimal places) was weighed in a 20 ml headspace vial and sealed with a crimp cap. The samples were equilibrated at 80°C for 240 min. after which 120 µl of the headspace was injected splitless to the column. The line temperature was 150°C and the loop temperature 120°C. The temperature program of the oven was 40°C, held for one min, and increased to 240°C at a rate of 10° C/min. The detector was set at 240°C. Three volatiles: hexanal, trans-2-hexenal and trans,trans-2,4-decadienal, were determined and standards were prepared with all three in one sample. The following approximate concentrations were used for the calibration: 0.5, 1.0, 5.0, 10.0, 20.0, 30.0, 40.0 and 50.0 mg/kg. These were made up accurately to three decimal places in fresh oil. Fresh sunflower oil was obtained from Unifoods, Boksburg, South Africa and was collected directly after the deodorisation step. A blank run of the oil was performed to ensure that the three volatile peaks that would be determined were absent or minimally present in the oil. Two palm-olein oils were tested and the cleaner oil was selected as blank and was used to make up the standards.

3.3.1.7 Fatty acid composition

The determination of the fatty acid composition procedure was based on the Official Methods and Recommended Practices of the AOCS Method Ce 2-66 (AOCS, 1997) by preparing

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

methyl esters which are separated and determined by capillary GC with FID. BF_3-methanol reagent was used for the derivitisation after transesterification with 0.5 M NaOH in methanol. Three drops of oil sample were derivatised and taken up in 2 ml. heptane. One µl of the prepared sample was injected onto an Omegawax™ 320 (Supelco, Johannesburg, South Africa), fused silica capillary 30m column, 0.32 mm ID and 0.25 µm film thickness as supplied by Supelco. The oven temperature program was 180°C for 15 min, after which it increased to 210°C at 5 °C/min and held at 210°C for 18 min. The injector was set at 230°C and the detector at 240°C. The fatty acids were expressed as g/100 g fatty acids.

3.3.1.8 Tertiary butylhydroquinone (TBHQ)

The oils were tested for the presence of the synthetic antioxidant TBHQ before using the oils in the shelf-life tests. The method used was that of Anderson and Van Niekerk (1987) with modifications to the mobile phase. TBHQ was determined in the oil directly using normal-phase HPLC with fluorescence detection. One g (weighed accurately to four decimals) of oil was dissolved in the mobile phase consisting of 6 % isopropanol in hexane and made up to 10 ml. The mobile phase was dried over sodium sulphate, to adjust the moisture below a critical level. The excitation of the fluorescence detector was set at 309 nm and the emission at 340 nm and separation was done by a 25 cm Lichrosorb Diol 5µm column supplied by Merck.

3.3.1.9 Conjugated diene and triene values

The shift in the position of the double bond of polyunsaturated oils, that results as one of the first steps of oxidation (primary oxidation), is measured by the conjugated diene value (CV) (White, 1995). The determination of the CV was done according to ISO/DIS 3656 method (ISO/DIS, 1989). The oil sample was dissolved in 25 ml iso-octane and the absorbance was measured at 232 (diene) and 268 (triene) nm. The CV will also be referred to as UV at 232 and the conjugated trienes as UV at 268 nm.

3.3.1.10 Moisture

The initial moisture contents of the two oils were determined by a vacuum oven method where the moisture loss was measured gravimetrically. The sample was weighed (2 g) accurately to 4 decimal places into a dried, clean, weighed aluminium dish. The dish, covered partially with the lid, was placed in a vacuum oven at 70 °C (± 5 °C) under pressure of *c*. 280

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

mm Hg. Whilst drying, a slow stream of dry air (approx. 2 bubbles/s) was introduced into the oven. The samples were dried overnight (16-18 h) or longer until constant weight. Once the drying period was completed the dish was cooled in a dessicator and weighed.

The moisture content was calculated as follows:

$$\text{g/100 g moisture} = \frac{(\text{dish} + \text{lid} + \text{wet sample}) - (\text{dish} + \text{lid} + \text{dry sample}) \times 100}{\text{mass of wet sample}}$$

3.3.1.11 Iron and copper

The iron and copper contents were determined by atomic absorption spectroscopy (AAS) after dry ashing to convert organic matter into inorganic compounds. Ashing was done by weighing 5-10 g oil sample into clean, preweighed silica dishes. Samples were placed in a cold muffle furnace that was heated very slowly 10 °C/h to 520 °C. On reaching temperature the samples were left overnight. The resulting ash was moistened with 2 ml conc. HNO_3, dried, dissolved by the addition of a ml conc. HCl and made up to volume with deionised water. The mineral determinations were carried out according to the instructions given by the manufacturer of the Perkin-Elmer Model 5000 AAS instrument, Buckinghamshire, England. The operating procedures for each element were as follows:

Element	Flame type	Wavelength	Slit setting	Light source
Copper (Cu)	Air acetylene	324.8 nm	4 (0.7 nm)	HCL*
Iron (Fe)	Air acetylene	248.3 nm	3 (0.2 nm)	HCL

* HCL = Hollow Cathode Lamp

Calculation: $$\text{mg/100 g} = \mu\text{g/ml} \times \frac{\text{final vol}}{\text{mass}} \times \text{dilution} \times \frac{100}{1000}$$

where µg/ml = obtained from standard curve (Y = A + Bx).

3.3.2 Sensory evaluation

The method used was by odour evaluation of the oils. The four samples of one of the oil types, from a specific storage time interval, were evaluated at a time. For example, the four sunflower oil samples, Control, 54 mg/kg TBHQ, 217 mg/kg TBHQ and 435 mg/kg TBHQ, taken out at Week 22, were evaluated at the same time. The panel consisted of people

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

working at CSIR Bio/Chemtek, Pretoria at the time of the evaluation. Half of the panellists selected had previous experience in oil odour evaluation and the rest of the panellists had to be informed of the characteristics of rancid oil and were presented with rancid oil for evaluation in order to identify typical rancidity odour in oil. A panel of 10-12 people was used. Sample (15 ml) was measured into a 50 ml glass beaker and covered with aluminium foil. Fresh oil was used as a standard to remind the panellist of the characteristics of good quality oil. The standard sample, also measured into a 50 ml beaker, was presented to the panellist at the same time as the test samples. The samples were heated to approximately 50°C before being presented to the panellist. The score sheet listed a choice of four categories to guide the panellist in the evaluation of the samples (as compared to the standard sample). The four categories were as follows:

1) Odour bland, weak characteristic odours
2) Weak off-odour or loss of characteristic odour
3) Moderate off-odour, slightly rancid
4) Strong off-odour, rancid, painty

In the case of the odour evaluation of palm-olein oil an additional standard sample was presented to the panellists. The sample contained a small amount of added copper acetate. This was done because some of the samples presented to the panellists contained copper acetate and it was necessary that they could differentiate between the copper acetate odour, if they could detect it, and normal off-odours due to rancidity. This was not done with the sunflower oils, which had TBHQ added, as TBHQ does not have a strong characteristic odour.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

3.4 MODELLING

Mathematical modelling was done by multiple regression analysis on STATISTICA® Kernel release 5.1M 1998 Edition, StatSoft Inc., Tulsa, USA. Multiple regression was performed where the dependant variable was the shelf-life, and the independent variables were the values obtained for the various analyses such as PV, FFA, OSI, etc at the determined shelf-life. The programme selected relevant variables with forward stepwise regression where the independent variables were individually added or deleted from the model at each step until the "best" regression model was obtained. The *F to enter* value, which determines how significant the contribution of a variable in the regression equation has to be in order to be added to the equation, was set at 3.0 where the minimum *F to enter* value is 0.0001. The *F to remove* value, which determines how "insignificant" the contribution of a variable in the regression equation has to be in order to be removed from the equation, was set at 1.5, where the minimum *F to remove* value is 0.0. The *tolerance* of a variable, which is defined as one minus the squared multiple correlation of this variable with all other independent variables in the regression equation, was set at 0.0001.

The storage time at which the oils were considered rancid was chosen by a combination of PV and AV. A PV higher than 20-25 meq/kg (Yousuf Ali Khan et al, 1979; Tian et al, 1999) and an AV higher than 10 mmol/kg (White, 1995) were used as criteria to determine at which storage time the oils were rancid. The different cases used in the modelling were generated by taking the time at which the oil was deemed rancid, e.g. at Week 22 the Control of palm-olein was deemed rancid, and attributing the agreeing shelf-life of 0 weeks to the case. The oil was rancid at Week 22 and thus had no shelf-life. The remainder of the cases were calculated by working backwards to the start of the storage trial so that as in this example, the next case for the Control was at Week 19 with a shelf-life of 3 weeks followed by Week 16 with a shelf-life of 6 weeks and so on. In the same manner all the cases for each treatment is calculated from the time it was deemed rancid with its agreeing shelf-life up till Day 0. The Totox value was excluded from the modelling, as it is a combination of the AV and the PV. Models that based onset of rancidity on the sensory evaluation were also done. The following models had been designed to predict the shelf-life of the oils:

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Palm-olein oil:

Model 1 Normal function values of all the variables

Model 2 Squared values of all the variables

Model 3 Weighted values of normal function values of all the variables (done by the division of all the values by the standard deviation of each analysis)

Model 4 Weighted values of squared values of all the variables

Model 5 Ideal model including all the variables, normal and squared values

Model 6 Practical model using only well-known, easy to use methods, namely FFA, PV, OSI, conjugated diene and triene value

Model 7 OSI and its squared value

Model 8 Ideal model, based on sensory evaluation with all variables, normal and squared values

Model 9 Practical model based on sensory evaluation

Model 10 OSI and its squared value based on sensory evaluation

Sunflower seed oil:

The same models were done for the sunflower seed oil. The Ideal Model 5 was additionally performed with the exclusion of the TBHQ values.

The models selected, based on their correlation coefficients and p-values defined in the Results 4.1.3.1 section, were validated by the jackknife approach (Hair et al, 1998). The method is based on the "leave-one-out" principle, where the modelling was performed with the exception of one case. All the cases used in the models were excluded one by one and the models were then tested with the excluded case.