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GC-FID Method Development and Validation Parameters for Analysis of Palm Oil (*Elaeis guineensis* Jacq.) Fatty Acids Composition

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Abstract The variety of available techniques and analytical methods applied in laboratories calls for the need of defining the scope, range of application and quality of information obtained using each of them with reference to internationally agreed standards. Of these techniques, Gas Chromatography with Flame Ionization Detector (GC-FID) is most used in fatty acids analysis. Method development and validation is useful for GC and other analytical procedures. This paper outlines the importance of method validation and elaborates on typical validation parameters with special reference to their application in the analysis of fatty acids of palm oil. The first section presents background information on stages in analytical method processing, quantitative and qualitative analysis, GC and its use in the analysis of lipid extracts. Critical components for a method are highlighted such as sampling, sample preparation (FAME derivatization), instrument analysis conditions and method standardization. Validation characteristics like Specificity, Linearity and range, Accuracy, Precision, LOD, LOQ, Robustness, Ruggedness and System suitability are presented in the last section with a brief view on some statistical methods used in method validation. It is hoped that this review will facilitate the work of analysts and the scientific community by enhancing efficient method development and validation which matches international standards.

Keywords: *GC-FID, FAME preparation, method development, validation characteristics, fatty acids, Elaeis guineensis Jacq., Gas Chromatography*

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1. Introduction

The numerous sources of hydrocarbons combined with the variety of available techniques and analytical methods have created confusion among the end users of data in regards to the appropriate methodologies. Users, government laboratories and private laboratories disagree on the scope, range of application and quality of information obtained from using different methods [1]. Due to recognized differences and complexity of biopharmaceuticals and other products [2], qualitative and quantitative analysis is fundamental to pharmaceutical product development [3] and food manufacturers for quality control, purity determination, and for the detection of adulterants [4].

Determining the degree of fatty acid unsaturation of a product is difficult because foods can contain a complex mixture of saturated, monounsaturated, and polyunsaturated fatty acids with a variety of carbon chain lengths [4].

This situation can be circumvented by analytical chemistry which deals with methods for determining the chemical composition of samples. It is thus necessary to calibrate each instrument or equipment and devise a specific method for the analysis of each sample type. Analytic method development and validation are key elements of any product development program [3]. Method validation has a long history in the pharmaceutical and biopharmaceutical industries [2]. Method development and validation of a process is needed to confirm that the analytical procedure employed for a specific test is suitable for its intended use. It helps to improve the reliability, consistency and accuracy of analytical data [5].

The major stages of a typical analytical method processing are [5]:

- Sample collection, sampling and labeling;
- Sample transportation from field to laboratory and storage in appropriate conditions;
- Sample preparation: extraction, drying, FAME preparation i.e. esterification, transesterification etc;
- Instrument configuration and method development: e.g. GC injector, oven and detector temperature settings, gas pressure and flow rate, retention time definition etc.
- Sample analysis: introduction into vials, injection into GC, acquisition process;
- Data handling: software processing, detector signal integration parameters, results format development (method and instrument information, graphs and tables of values etc.);
- Report generation: data analysis (statistics...), validation parameters, printing of results tables, chromatograms, regression curves etc;
- Archiving, release of information to end users.

Some analytical methods include thin layer chromatography (TLC), high-performance liquid chromatography (HPLC) coupled with detectors such as refractive index (RI) detector, flame ionization detector (FID), density detector, evaporative light scattering detector (ELSD), and mass spectrometer detector. These techniques have been employed to identify and quantify glycerides and FAME. All these techniques require long analytical procedures and in some cases co-elution of components is verified, such as in mixtures containing low-molecular weight triglycerides (TG) and high molecular weight diglycerides (DG) [6]. The discovery of gas phase chromatography is credited to Archer John Porter Martin and Richard Laurence Millington Synge who published the theory of separation chromatography in 1941 [7,8]. The GC technique revolutionized the study of lipids by making it possible to determine the complete fatty acid composition of a lipid in a very short time [9,10]. Gas Chromatography seems to be the most appropriate method for qualitative and quantitative analysis of isolated lipid extracts.

Analytical processes for oils like palm oil often use Gas chromatography (GC) with flame ionization detector (GC–FID) method with derivatization. Palm oil from *Elaeis guineensis* Jacq. is made up of mainly triglycerides with some mono and diglycerides. Crude palm oil (CPO) contains much saturated fatty acid (palmitic acid) considered to be detrimental to health. Nutritionally, saturated fats are of particular concern, because an excess in the diet leads to their accumulation in the cardiovascular system, resulting in several health related problems. CPO analysis by GC is required for the appreciation of variability of its fatty acids composition among parents and progeny in view of creating improved oil palm breeding materials that will produce oil of better quality [4].

Capillary GC-FID is widely used for the quantitative analysis of hydrocarbon compounds and oil analyses [11]. For precise quantitative analysis, it is essential to calibrate the GC–FID system using calibration standards for each analyte. The response factor method (determined by the analysis of standard materials or other methods prior to measurement of the analytes) is also used for calibration of the GC–FID. The FID response for hydrocarbons is generally proportional to the carbon number of the analyte [12]. Quantitative analysis of test compounds is sometimes carried out using one of the compounds in the samples as an internal calibration standard, and the analytical results compared with the reference values. Such calibration method is known as primary ratio method. In this system, only one standard material is required and the cost for analysis is greatly reduced compared to the conventional calibration method using calibration standards for each analyte [12].

The four critical components for a method are: sampling, sample preparation, instrument analysis conditions and standardization. During the preliminary method development stage, all individual components should be investigated before the final method optimization. This gives the scientist a chance to critically evaluate the method performance in each component and streamline the final method optimization [3]. Early in a project, the method development should be kept at a minimum and for a small number of samples, a tedious work up procedure can be afforded. Further on in the project, it may be worthwhile to make efforts towards automation and convenience in the method [13]. A compound can often be measured by several methods. The choice of analytical methodology is based on many considerations, such as: chemical properties of the analyte and its concentration, sample matrix, the speed and cost of the analysis, type of measurements i.e. quantitative or qualitative and the number of samples [13].

A qualitative method yields information on the chemical identity of the species in the sample. The simplest qualitative analysis involves a comparison of the retention times between a chromatographic peak containing an unknown compound and peaks obtained for reference samples using more than one stationary phase. [13]. On the other hand, a quantitative method provides numerical information regarding the relative amounts of one or more of the species in the sample. In quantitative analysis, the goal is to determine the exact amount of analyte molecules in a sample. Most often two different analytes of equal concentration give different detector responses in chromatography, therefore the detector responses must be measured for known concentrations of each analyte, expressed as a standard curve [13]. In a GC–FID measurement, standard materials (e.g. SIGMA 189-1) for each target compound are essential for quantitative analysis [12]. Retention time specificity for the sample and the standard methyl esters are determined as well as the calibration curve and the correlation coefficient. The accuracy and precision of the method are evaluated by analyzing standards and samples and by determining their recovery. Mean values of recovery ratios for standards and samples should be closest to 100% for a given concentration. The limit of detection (LOD) and limit of quantification (LOQ) for the GC column and detector are also determined. The method validated in such process can be used for the quantification of fatty acids composition. It could equally be adapted for quality control in food analysis laboratories for studying variability of fatty acids composition of fats and oils [14].

Be it qualitative or quantitative, each analytical method contains a number of steps that can be simplified in a flow chart as a combination of information and operations with the typical structure: input-process-output [13]. Input

includes the initial information such as the purpose of the analysis. Processing involves sampling, sample preparation, analytical chromatography, detection and data processing, while output is the analytical results [13]. The main focus of this write-up is to present the basic procedures for analytical method development and validation with particularly emphasis on gas chromatography (GC) and its application in the analysis of fatty acids composition of palm oil.

2. Sampling, Choice of Materials and Sample Preparation

2.1. Sampling

In method development and validation, it is important to write a sampling plan with the aim of utilizing the information to check the sampling protocols [15]. A sampling plan defines the objectives and provides specific description of the data to be collected, the interval of data collection, and the subjects from whom the data will be collected. Outlines of the various decisions that must be introduced into a sampling plan should include: choosing the sampling location; Selecting the size and number of samples; selecting the frequency of sampling and type of sample; choice of sampling devices, equipment, containers and cleansing and preservation requirements [15].

2.2. Choice of Materials, Reagents and Analytes

For GC analysis, it is important to use only high quality derivatization reagents to ensure that no artifacts are present during analysis [4]. The choice of solvent to be used for extraction of analytes is very important. The U.S. EPA has removed methods that use Freon-113 as an extraction solvent and, as an alternative, recommended the use of the method EPA-1664: n-Hexane Extractable Material and silica gel treated with n-hexane extractable material by extraction and gravimetry [1]. Only derivatization reagents with low moisture are recommended, as the esterification reaction can be hindered by the presence of water. The storage conditions of derivatization reagents should be strictly adhered to, as some are susceptible to degradation during long-term storage [4]. Some derivatization reagents include: BCl3-Methanol (12% w/w); BF3-Methanol (10% w/w); BF3-Butanol (10% w/w); Methanolic Base (0.5 N); Methanolic HCl (0.5 N); Methanolic HCl (3 N); and Methanolic H2SO4 (10% v/v) [4].

One of the first considerations when deciding which procedure to adopt for preparing ester derivatives of an analyte for GC is the lipid composition of the samples to be analyzed [10]. If these are free fatty acids alone, mixtures containing significant amounts of free acids or mixtures of unknown composition that might contain free acids, then acid-catalyzed procedures are generally preferred [10]. However, alkaline transesterification procedures are so rapid and convenient that they should be considered for mixed lipid samples that contain no unesterified fatty acids or for single lipid classes containing ester-bound fatty acids [10]. If a single method is required for use with all routine lipid samples, hydrogen chloride (5%) or sulfuric acid (2%) in methanol, despite the comparatively long reaction times needed, is probably the best general-purpose reagent available [10]. It is not inconvenient to have more than one method in routine use in the laboratory, i.e. sulfuric acid in methanol for those samples containing free fatty acids, and sodium methoxide in methanol for all other lipid classes with the exception of sphingolipids for which special procedures are necessary [10].

A further consideration when deciding on a reagent is the fatty acid composition of the samples to be esterified. If short-chain fatty acids or others of unusual structure are present, appropriate methods must be adopted. Polyunsaturated fatty acids must always be handled with care and should not be subjected to more vigorous conditions than are necessary. Reagents that are perfectly satisfactory when used under optimized conditions can be destructive to fatty acids if used carelessly [10]. However, the analysis of fatty acids in the free form instead of as fatty acid methyl esters results in easier and quicker sample preparation. Additionally, artifact formation that may result from a derivatization procedure is eliminated [4].

The chemical standards to be used are also an important aspect. Standards for the determination of free fatty acids should be purchased from a chemical manufacturer with knowledge in the preparation, handling, storage, and shipment of volatile analytes e.g. Sigma-Aldrich [4]. Characterized Reference Oils are offered that can be used as controls or check samples, providing an excellent means of standardizing applications and comparing results to others. Several convenient kits of either derivatized FAMEs or underivatized fatty acids are also offered, so analysts can formulate their own mixes. [4]. AOCS Animal and Vegetable Reference Mixes are also available. Each quantitative mix is similar to the fatty acid distribution of certain oils and conforms to the requirements of AOCS Method Ce 1-62 [4,16].

Another important aspect of GC method development and validation is the choice of column to be used. For the GC analysis of free fatty acids, a specialized column that will not allow the adsorption of active carboxyl groups is required. The Nukol, with its acidic characteristic, is well-suited for this application, allowing chromatography with excellent peak shapes. To confirm identification, very efficient capillary GC columns with the ability to resolve a large number of peaks are required. Omegawax columns provide highly reproducible analyses, being specially tested for reproducibility of FAME equivalent chain length (ECL) values and resolution of key components [4].

Moreover, all glassware must be scrupulously cleaned as soon as possible after use by rinsing with the last solvent used. This should be followed by detergent washing with hot water, and rinses with tap water and organic-free reagent water. Drain the glassware and dry in an oven at 130 ℃ for several hours or rinse with methanol and drain. Store dry glassware in a clean environment [17].

2.3. Sample Preparation for Method Development and Analysis

Before beginning the GC method development, it is important to review what is known about the sample. The goal of the analysis should be defined at this point and considerations must be given to the number of samples to be analyzed, available equipment, etc. The nature of the sample (e.g. whether it is hydrophilic or hydrophorbic,

whether it contains protolytic functions, etc) determines the best approach to method development [13,18]. Generally, a chemical analysis is performed on only a small fraction of the material whose composition is being sought. The composition of this sample must reflect as closely as possible the average composition of the bulk of the material [13].

The process by which a representative fraction is being acquired is termed sampling. Some samples require a pre-treatment prior to analysis because of the need to remove interferences or to concentrate sample analytes. The sample pre-treatment development can at times be more complex than the separation itself. The goals of the separation should be specified at the beginning of the method development [13]. Moreover, before the sample is injected during the GC method development, the detector should be selected to be sensitive to all sample components of interest. The final procedure should meet all the goals that have been defined at the beginning of the method development and when the method for quantitative use is finalized it should be validated [13].

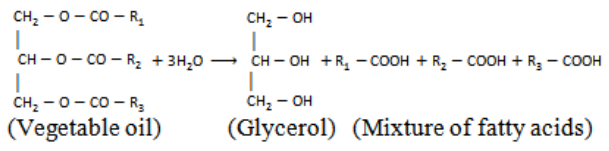
2.3.1. Fatty Acids Methyl Ester Preparation

GC can be used to analyze fatty acids either as free fatty acids or as fatty acid methyl esters. Methyl esters are the favourite derivatives for GC analysis of fatty acids [10].

After lipid extraction, it is necessary to transform the different constituents into specific derivatives in order to render them more volatile. Fatty acids are not quite volatile and thus need preparation or derivatization which is possible through two approaches: (1) either by a two-steps reaction involving saponification followed by esterification [19]; (2) or by a single step reaction known as transesterification. The following are two primary reasons to analyze fatty acids as fatty acid methyl esters [4]: (1) In their free, underivatized form, fatty acids may be difficult to analyze because these highly polar compounds tend to form hydrogen bonds, leading to adsorption issues. Reducing their polarity may make them more amenable for analysis. (2) To distinguish between the very slight differences exhibited by unsaturated fatty acids, the polar carboxyl functional groups must first be neutralized, and also by degree of unsaturation, position of unsaturation, and even the cis vs. trans configuration of unsaturation [4]. The two main chemical reactions that occur during methylation are hydrolysis and esterification.

2.3.1.1. Hydrolysis of Fatty Acids

Hydrolysis results in a mixture of fatty acids and glycerol from triglycerides as indicated by the following reaction (reaction 1) in which R1, R2 and R3 are linear carbon chains:



2.3.1.2. Esterification of Fatty Acids

The esterification of fatty acids to fatty acid methyl esters is performed using an alkylation derivatization reagent. The esterification reaction involves the condensation of the carboxyl group of an acid and the hydroxyl group of an alcohol in the presence of a catalyst. The catalyst protonates an oxygen atom of the carboxyl group making the acid much more reactive. An alcohol then combines with the protonated acid to yield an ester with the loss of water. The catalyst is removed with the water. Esterification is illustrated in reactions (2) and (3). The alcohol that is used determines the alkyl chain length of the resulting esters; the use of methanol will result in the formation of methyl esters whereas the use of ethanol will result in ethyl esters [4].

$$\underbrace{R-COOH}_{\text{Fatty acid}} + \underbrace{R'-OH}_{\text{Alcohol}} \overset{H_2SO_4}{\Longleftrightarrow} \underbrace{R-COO-R'}_{\text{FAME}} + H_2O \quad (2)$$

$$C-COOH + CH_3OH \xrightarrow{BCl_3} R-COO-CH_3 + H_20 \quad (3)$$

Where R and R' are linear carbon chains.

2.3.1.2.1. Acid-catalyzed esterification

Carboxylic acids can be esterified by alcohols in the presence of a suitable acidic catalyst [9]. The initial step is protonation of the acid to give an oxonium ion, which undergoes an exchange reaction with an alcohol to give the intermediate, and this in turn can lose a proton to become an ester. Each step in the process is reversible but in the presence of a large excess of the alcohol, the equilibrium point of the reaction is displaced so that esterification proceeds to completion. However, in the presence of water, which is a stronger electron donor than are aliphatic alcohols, formation of the intermediate is not favoured and esterification will not proceed fully [9]. The following typical esterification procedure (using BCl3-methanol) can be used as a guideline and could be altered to meet the needs of a specific application [4]:

- Samples can be derivatized neat or after dissolving in solvent. If appropriate, dissolve sample in a nonpolar solvent (such as hexane, heptane, or toluene). If the sample is in an aqueous solvent, first evaporate to dryness then use neat or dissolved in an organic, non-polar solvent.
- Weigh 1-25 mg of sample into a 5-10 mL micro reaction vessel.
- Add 2 mL BCl_3-methanol, 12% w/w. A water scavenger (such as 2,2-dimethoxypropane) can be added at this point.
- Heat at 60 ℃ for 5-10 minutes. Derivatization times may vary depending on the specific compound(s) being derivatized.
- Cool, then add 1 mL water and 1 mL hexane.
- Shake the reaction vessel (it is critical to get the esters into the non-polar solvent).
- After allowing the layers to settle, carefully transfer the upper (organic) layer to a clean vial. Dry the organic layer by either:
  a) passing through a bed of anhydrous sodium sulfate during the transfer step to the clean vial;
  b) adding anhydrous sodium sulfate to the clean vial then shaking.
- To determine the proper derivatization time, analyze aliquots of a representative sample using different derivatization times. Plot peak area (y-axis) vs derivatization time (x-axis). The minimum time to

use is when no further increase in peak area is observed with increasing derivatization time (where the curve becomes flat).

- If it is suspected that complete derivatization is never achieved, use additional reagent or re-evaluate temperature.
- It is important to prepare a reagent blank, along with the samples, to identify any issues that may arise.

For GC analysis, the fatty acid components of lipids are converted to the simplest convenient volatile derivative, usually methyl esters, though other esters may be preferred for specific purposes. Although fatty acids can occur in nature in the free (unesterified) state, they are most often found as esters, linked to glycerol, cholesterol or long-chain aliphatic alcohols, and as amides in sphingolipids [9]. The physical state of the lipids can vary. For example, they can be isolated as pure lipid classes or remain as a mixed lipid extract. Various procedures are described with reference to the preparation of methyl esters from the more common carbon 14 (C14) to carbon 22 (C22) fatty acids, mainly in the free state or bound to lipids by ester or amide bonds [9]. In fact, methyl esters offer excellent stability, and provide quick and quantitative samples for GC analysis [4].

2.3.1.2.2. Transesterification

Ester exchange or transesterification begins with initial protonation of the ester followed by addition of the exchanging alcohol to give the intermediate, which can be dissociated via the transitions state to give the ester. Each step is reversible and in the presence of a large excess of the alcohol, the equilibrium point of the reaction is displaced so that the product is almost entirely the required ester. Water must be excluded, as it would produce some hydrolysis by dissociation of an intermediate analogous to a free acid [9]. The preferred conditions for acid-catalyzed esterification of carboxylic acids or transesterification of existing esters are therefore a large excess of the appropriate alcohol and the absence of water [4,9]. It may be possible to obtain water-free conditions by adding anhydrous sodium sulfate to the reaction medium [9,26]; a better practice in general is to operate with dry reagents and glassware. A critical practical point is the choice of acid as catalyst. This must facilitate the reaction but should not cause unwanted side effects. In principle, the methodology can be used with any alcohol component, but in practice it is limited to those alcohols that can be eliminated from the reaction medium by selective evaporation, i.e. methanol to perhaps pentanol [9].

2.3.1.2.3. Artefacts of trans/esterification procedures

During esterification, the operator must exercised continuous vigilance to detect contamination of samples by impurities in the reagents or from any other source. Middleditch cit. [10] has compiled information on compounds that can be troublesome in chromatographic analyses. The samples themselves may be a source of contaminants or artefacts, e.g. from the fatty acids, endogenous cholesterol, other sterols or their esters, and other lipids [10]. All solvents (including water) and reagents should be of the highest grade and may have to be distilled before use to remove non-volatile impurities, especially when preparing very small quantities of esters. Extraneous substances can be introduced into samples from a variety of sources, for example filter papers, soaps, hair preparations, tobacco smoke and laboratory grease; care must be taken to recognize and avoid such contaminants [10].

Phthalate esters used as plasticizers are probably the most common contaminants and are encountered whenever plastic ware of any kind is in contact with solvents, lipid samples, reagents and even distilled water. They can enter preparations through the brief contact between disposable pipette tips and reagents. Phthalate esters can interact with transesterification reagents to give mono- and sometimes di-methyl esters. Basic catalysts react more rapidly than acids [10].

During GC analysis, the precise point of elution relative to fatty acid derivatives is dependent on the nature of the phthalate ester and the stationary phase, but typically is in the same range as the C18 to C22 fatty acids [10]. Samples containing polyunsaturated fatty acids should be handled under nitrogen whenever possible, and antioxidants such as 2,6-di-tert-butyl-4-methylphenol (BHT) may be added to solvents and reagents to minimize autoxidation [10]. In GC analyses, BHT emerges as a sharp peak, which can interfere with the analysis of methyl myristate with packed columns but not usually when fused silica capillaries are used; in HPLC, it often emerges close to the solvent front where it can be a nuisance with UV detection. Boron trifluoride is known to interact with BHT to produce methoxy derivatives as artefacts that co-elute with methyl pentadecanoate or hexadecenoate on GC analysis. This does not appear to be a problem with other acidic reagents [10].

During GC analysis, artefact peaks are produced when traces of basic transesterification reagents remaining in samples are introduced into GC columns [10]. Non-lipid contaminants or non-ester by-products of esterification reactions like cholesterol can be removed by preparative TLC on plates coated with silica gel G and developed in a solvent system of hexane-diethyl ether (9:1, v/v), though there may be some selective loss of short-chain esters if these are present. Dimethyl acetals formed from plasmalogens have been separated from methyl esters by preparative TLC with toluene or dichloroethane as mobile phase, when esters migrate more rapidly [10].

2.3.1.2.4. FAME preparation with methanolic HCl

The most commonly used reagent for the preparation of methyl esters is 5% anhydrous hydrogen chloride in methanol, prepared by bubbling dry gaseous hydrogen chloride into dry methanol [9]. Hydrogen chloride in methanol (or another alcohol) can be used to esterify free fatty acids or to transesterify fatty acids linked by ester bonds to glycerol or cholesterol. The main disadvantage is the comparatively long reflux time needed for complete reaction to be achieved. As with other acid catalysts, it is not suited for certain fatty acids with sensitive functional groups, such as epoxyl, cyclopropane or cyclopropene rings [9]. Gaseous hydrogen chloride is available commercially or can be prepared when needed by dropping concentrated sulfuric acid onto fused ammonium chloride or into concentrated hydrochloric acid in a Kipp's apparatus [9,20].

Hartman & Lago (1973) cit. [9] suggested that methanolic hydrogen chloride might be prepared by adding ammonium chloride and sulfuric acid to methanol. In a typical esterification procedure using methanolic hydrogen chloride, the lipid sample is dissolved in at least a 100-fold excess of the reagent and the solution is refluxed for about two hours or is held at 50 ℃ overnight (30 minutes at 50 ℃ will suffice for free acids alone) [9]. At the end of this time, water is added and the required esters are extracted thoroughly into an appropriate solvent such as diethyl ether, hexane or light petroleum. The solvent layer is washed with dilute potassium bicarbonate solution to remove excess acid and dried over anhydrous sodium or magnesium sulfate (or anhydrous calcium chloride) and the esters are recovered after removal of the solvent by evaporation under reduced pressure on a rotary film evaporator or in a gentle stream of nitrogen [9]. The reaction may also be performed in a sealed tube so that higher temperatures and shorter reaction times are possible.

Longer reaction times are required as the molecular weight of the alcohol is increased. All fatty acids are esterified at approximately the same rate by methanolic hydrogen chloride, so there are unlikely to be differential losses of specific fatty acids during the esterification step. On the other hand, special precautions are necessary to ensure quantitative recovery of short-chain esters. Certain classes of simple lipids such as cholesterol esters and triacylglycerols are not soluble in methanolic hydrogen chloride alone and an inert solvent must be added to effect solution before the reaction will proceed. It has been claimed that spurious components, which may interfere with GC analyses, are formed in hydrogen chloride-methanol solutions [9]. Such artefacts may be formed, apparently from the methanol, with a variety of acidic catalysts if superheating of the solution is allowed to occur in the presence of oxygen.

2.3.1.2.5. FAME preparation with methanolic H_2SO_4

A solution of 1 to 2% concentrated sulfuric acid in methanol has almost identical properties to 5% methanolic hydrogen chloride, and is very easy to prepare [9]. The same reaction times are usually recommended for H2SO4- and HCl-methanol. It has been shown (Lie Ken Jie & Yan-Kit, 1988 cit. [9]) that free acids are esterified especially rapidly with the former and a microwave oven as an energy source. Inert solvents must be added to effect solution of simple lipids. Free fatty acids were esterified very rapidly by heating in 10% sulfuric acid in methanol until the reflux temperature was reached, but this procedure cannot be recommended for polyunsaturated fatty acids, as sulfuric acid is a strong oxidizing agent [9]. Very long reflux times (up to six hours), excessive sulfuric acid concentrations (20%) or high temperatures (170 ℃) will lead to the formation of coloured by-products and the destruction of polyenoic fatty acids. With the dilute reagent and moderate temperatures, however, there is no evidence for side effects, and under such conditions the reagent was approved by the Instrumental Committee of the American Oil Chemists' Society even though it has the same drawbacks as other acidic catalysts with sensitive fatty acids [9].

2.3.1.2.6. FAME preparation with BF_3-methanol

The Lewis acid (boron trifluoride) in the form of its coordination complex with methanol is a powerful acidic catalyst for the esterification of fatty acids. Esterification of free fatty acids has been completed in two minutes with 12 to 14% boron trifluoride in methanol under reflux [9]. Morrison and Smith cit. [9] showed that the reagent could be used to transesterify most lipid classes (inert solvent must be added to effect solution of simple lipids), although in general longer reaction times are necessary than with free fatty acids. For example, in this reagent cholesterol esters are transesterified in 45 minutes at 100 ℃ in a sealed Teflon TM-lined screw-top tube. Boron trifluoride can be used with other alcohols; ethyl, propyl and butyl esters have been prepared in this way [9].

However, BF3-methanol has serious drawbacks. Lough cit. [9] first reported that methoxy artefacts were produced from unsaturated fatty acids by addition of methanol across the double bond when very high concentrations of boron trifluoride in methanol (50%) were used. It is possible that the side-reactions are exacerbated by the presence of oxidized lipids. In addition, it has been reported that sample size is critical with substantial losses sometimes occurring with samples of less than 200mg. There is some evidence that artefact formation is most likely with aged reagents [9].

Boron trifluoride-methanol suffers from the same disadvantages as other acidic reagents with fatty acids with labile functional groups, although there is a suggestion that it produces by-products quantitatively in some instances and that this may be of analytical value. Solutions of boron trifluoride in methanol obtained commercially should therefore be checked carefully before use and periodically in use. The reagent has a limited shelf life at room temperature and should be kept refrigerated. The reagent has the approval of the American Oil Chemists' Society and of IUPAC amongst others [9]. It is highly popular, but possibly because it is one of the few such reagents that can be purchased from commercial suppliers. However, this reagent has high acid content and many known side reactions compared with other analogous reagents. Nonetheless, BF3 in methanol can be used in a similar manner to prepare methyl esters, although the reaction is slower than when BF3 is the catalyst [9].

FAME can be prepared with other acidic catalysts. Aluminium trichloride appears to be as effective as boron trifluoride as a catalyst for transesterification, but it has not been tested with a wide range of samples. Phenyl esters of fatty acids have been prepared by acid-catalysed esterification with p-toluenesulfonic acid as catalyst. Similarly, a strong cation-exchange resin in a fixed bed has been used as part of an HPLC system for post-column transesterification of lipids [9].

2.4. Fatty Acid Analysis

Analysis of fatty acids from biological or food samples generally involves three steps: extraction of lipids [55] [56], conversion of the extracted lipids to fatty acid methyl esters (FAME) [65] and analysis of the FAME using gas-liquid chromatography (GC) for the fatty acid profile [54]. As mentioned above, FAMEs are usually prepared by transesterification using hydrochloric acid, sulphuric acid, or borontrifluoride in methanol [54]. After FAME preparation as presented in the previous section, the next

step in method development is the analysis of the FAME samples. Analytical methods for oils, like palm oil, often use gas chromatography with flame ionization detection (GC-FID) [27]. Many workers have used gas chromatography (GC) [52,53,60,63] and other techniques [50,51] to analyze fatty acids with several goals such as for taxonomy and classification of microorganisms [43], for nutritional (health) [54,58,59,64], and analytical issues [44-49]. Fatty acid analysis of whole blood or other samples (e.g., adipose, erythrocytes, phospholipids) is often done to assess the fatty acid status in tissues in relation to physiopathological conditions or to monitor the fatty acid status in populations.

The AOAC Official method 996.06 is recommended for analysis of foods for fatty acid information [54]. A rapid and inexpensive method to analyze fatty acid composition of blood lipids by GC without lipid extraction is now available [57]. The information obtained from fatty acid analysis is useful in relating to dietary fat intakes to health outcomes [54,61]. In oil palm breeding, this can help to identify materials that produce low amounts of saturated fatty acids, which can then be exploited for hybridization [59,62].

3. Standard Method Development and Validation Parameters

3.1. Method Development

Several steps are involved in method development and validation. In the case of analytical procedures like GC and HPLC, these steps might include review of information on samples to be analyzed, definition of separation goals; assurance of special procedure requirements, sample pretreatment if any; detector selection and setting; separation conditions optimization; check for problems or special procedure requirements; recovery of purified material; quantitative calibration and qualitative method development; and method validation for release to laboratories [3].

3.1.1. GC-FID Instrument Configuration and Analytical Conditions

Before using the GC-FID for sample analysis, the instrument has to be configured usually by a field engineer from the manufacturing company. After installation and configuration of the instrument, a method is prepared with the help of the method editor. This requires computer skills and knowledge of the sample to be analyzed. Plate 1 shows screen shots of method preparation stages as developed on the Focus GC-FID in the IRAD La Dibamba Lipids Analysis Laboratory (LAL) for the analysis of fatty acids composition of oil palm (*Elaeis guineensis* Jacq.). The method preparation process which is done on the computer connected to the GC-FID with appropriate data management software involves defining the method title, detection, integration, calculation and report parameters. The results format, operator's identification and analytical information are equally defined in the process (Plate 1 a & b). The sample amount and injection procedure are developed for the autosampler (Plate 1 c) while the data sheet (Plate 1 d) for samples allocation and management by the GC is prepared.

Figure 1. Thermo Scientific Focus GC-FID with computer and printer (Source: author)

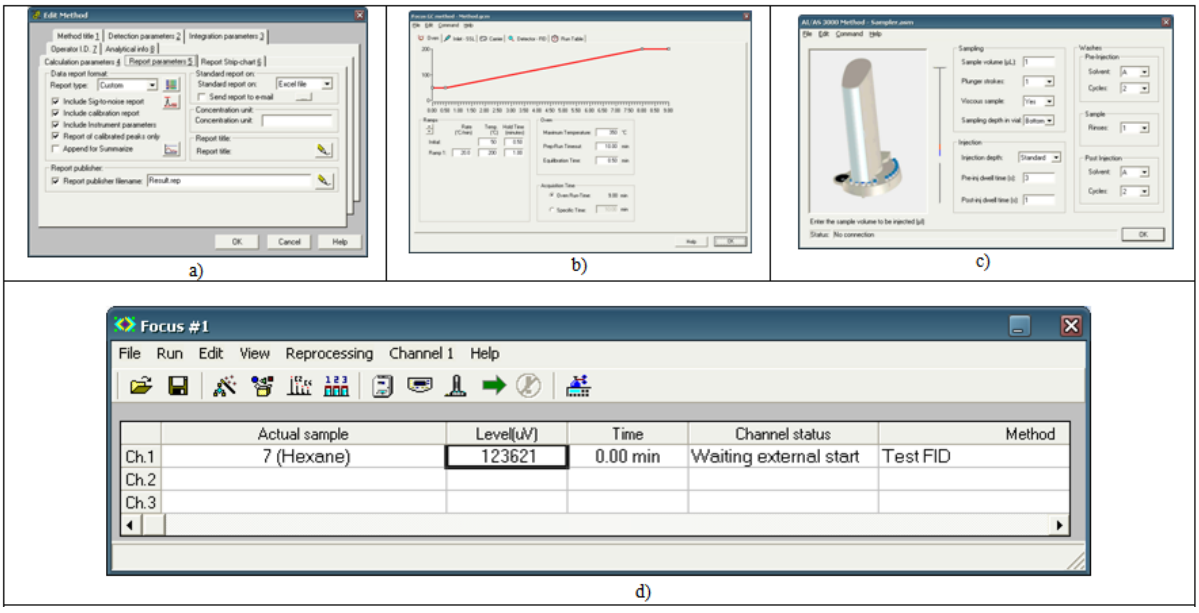


Plate 1. Illustrations of **a)** GC method editor; **b)** Oven temperature program; **c)** AS/IA 3000 Auto-sampler program ; **d)** Samples table ; Programming of GC-FID as in a) to d) was done with reference to [1] and [21]. The GC was installed with a 30m 0.32 mm DB-5 (95 metil-5%-fenil polisiloxane) fused silica capillary column. The computer software used here is the Chrom-Card data system

3.1.2. Focus GC-FID Instrument Calibration and Method Development

Once the GC-FID is configured and the analytical conditions defined, it is appropriate to run a blank sample like n-hexane (Figure 2) to appreciate the defined instrument parameters. Smith et al., 1981 cit. [13] stated that for quantification analysis, three methods of calibration are common: (i) external standard (ii) the internal standard method and (iii) the standard addition methods.

3.1.2.1. External Standard Calibration Method

External standard (E.S.) calibration method is a simple but less precise method and is used when the sample separation is simple and small or when no instrumental variations are observed. To construct a standard curve, standard solutions containing known concentrations of the analyte must be prepared and fixed volumes injected into the column [13]. The resulting areas or heights of the peaks in the chromatogram are measured and plotted versus the amount injected (Plate 2). Unknown samples are then prepared, injected and analyzed in exactly the same manner, and their concentrations are determined from the calibration plot. The term "external standard calibration" implies that the standards are analyzed in chromatographic runs that are separate from those of the unknown sample [13].

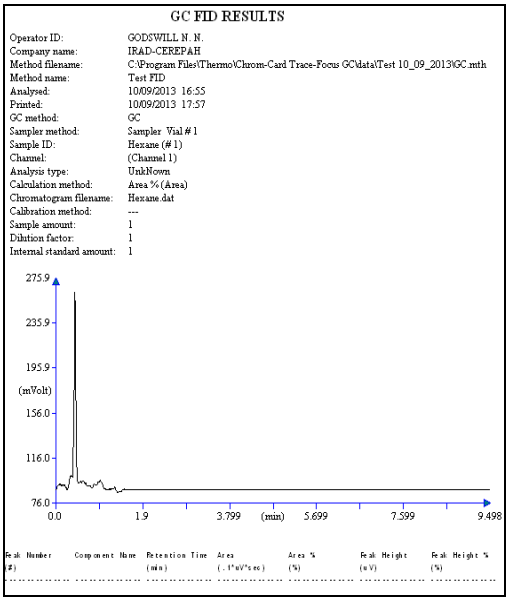


Figure 2. Chromatogram of n-Hexane

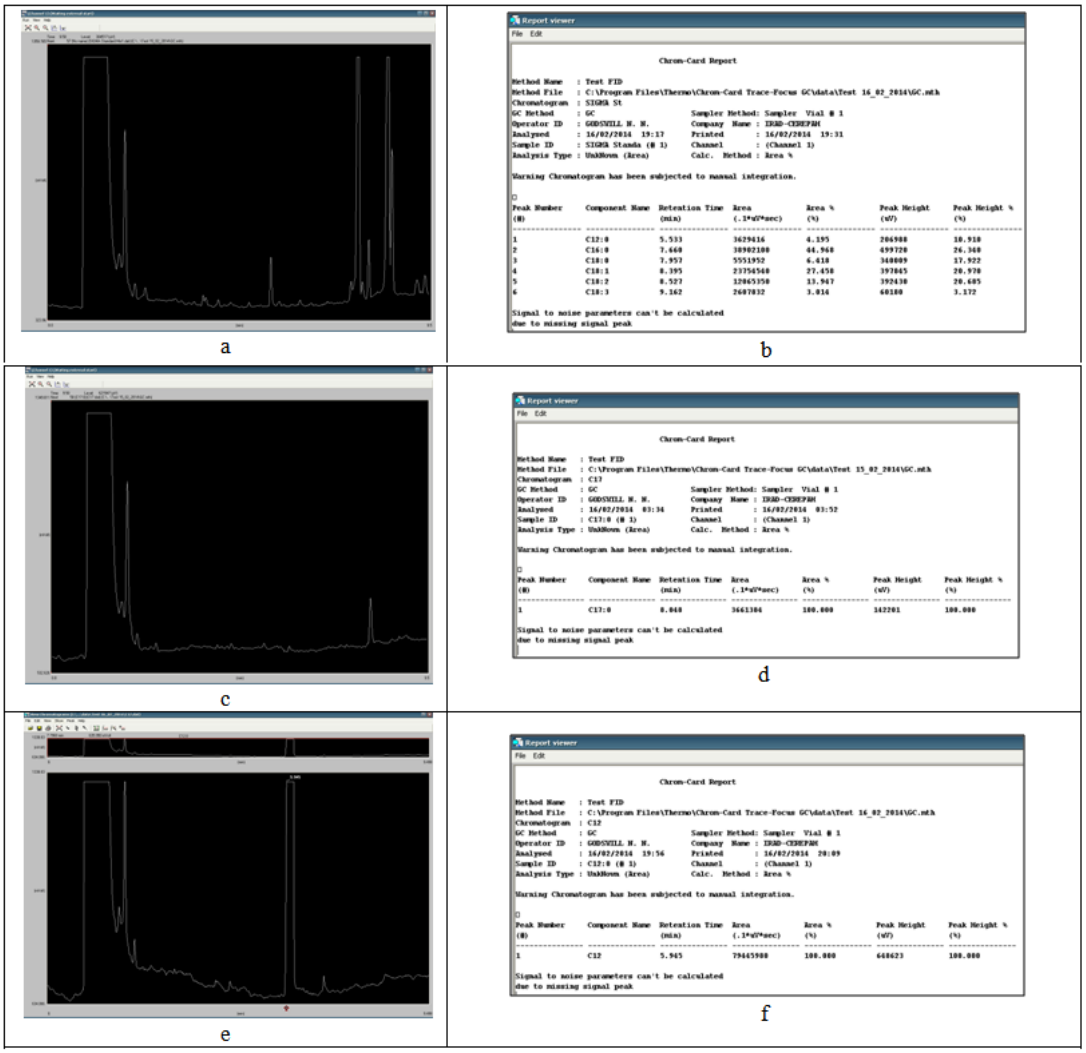


Plate 2. Screen shot with chromatograms of **a)** SIGMA 189-1 standard mix chromatogram **b)** Chrom-Card Result table for SIGMA **c)** C17:0 chromatogram **d)** Chrom-Card result generated one day after GC analysis **e)** C12:0 Chromatogram **f)** Chrom-Card report for C12:0. These chromatograms are an illustration of the use of external standard calibration method. They represent the chromatograms of SIGMA 189-1 standard mix (composition 16:0; 18:0; 18:1; 18:2; 18:3; 20% /sample) and C17: 0 diluted with n-hexane and analyzed with GC-FID in the LAL of La Dibamba in February 2014 with reference to [22]

3.1.2.2. The Internal Standard Calibration Method

Most often a compound with similar structure is selected as a standard. According to Burlingame et al. (1998) cit. [13], the internal standard (I.S.) method which is more accurate has become a very popular technique not only in chromatography, but also in quantitative HPLC-MS methods. Meanwhile, Dudley et al., 1978 cit [13] and [23] observed that the I.S. technique can compensate for both instrumental and sample preparation errors and variations like those from dilution and extraction.

Both the accuracy and precision of quantitative data increase if I.S. is included in the procedure. The I.S. should be similar but not identical to the analyte, and the two should be well resolved in the chromatographic step. The standard curves are obtained from standards of blank samples spiked with different known concentrations of the analyte of interest and addition of an I.S. at constant concentrations. Also, to the unknown sample the same constant concentration of the I.S. is added and the standard samples are processed in parallel with the unknown samples. In the calibration curve, the ratios of analyte to I.S. peak area (or height) are plotted versus the concentration of the analyte [13]. Smith et al. (1981) cit. [13] stated that a proper I.S. in a bioanalytical chromatographic method should fulfill the following requirements:

- be well resolved from the compound of interest and other peaks;
- not be present in the sample;
- be similar in retention to the analyte;
- be available in high purity (not contaminated with the analyte);
- be stable;
- should resemble the analyte in all sample preparation steps;
- be of similar structure as the analyte;
- be of similar concentration as the analyte.

Contrary to bioanalytical (analysis of drugs in biological fluids) approaches, no recommendations exist for the selection of internal standard in biotechnological analysis [13]. Meanwhile, the standard addition method is often used in cases when it is not possible to obtain suitable blank matrices. The approach is to add different weights of analyte to the unknown sample, which initially contains an unknown concentration of the analyte. After the chromatographic analysis, peak areas (or heights) are plotted versus the added concentration. Extrapolation of the calibration plot provides the original unknown concentration of the analyte. A standard addition method that possesses even greater accuracy and precision is obtained if one incorporates an internal standard [13,24].

3.2. Analytical Method Validation

Method validation is an essential component of the measures that a laboratory should implement to allow it to produce reliable analytical data [25]. The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose [3,28,29]. Validation of analytical methods is directed to the four most common types of analytical procedures [28]:

- Identification tests;
- Quantitative tests for impurities' content;
- Limit tests for the control of impurities;
- For pharmaceutical procedures, validation is directed to quantitative tests of the active moiety in samples of a drug substance or drug product or other selected component(s) in the drug product.

Identification tests are intended to ensure the identity of an analyte in a sample. This is normally achieved by comparison of a property of the sample (e.g., spectrum, chromatographic behavior, chemical reactivity, etc) to that of a reference standard [28].

3.2.1. Validation Protocol

Validation protocol is a written plan stating how method validation will be conducted and defining acceptance criteria. For example, the protocol for a manufacturing process identifies processing equipment, critical process parameters/operating ranges, product characteristics, sampling, test data to be collected, number of validation runs, and acceptable test results [30]. A method validation protocol could be simple or exhaustive. An exhaustive validation protocol could include details on how solutions are going to be made, the experimental design, how the calculations are going to be performed as well as any software to be utilized [31]. Some of the items that are necessary to be specified in the validation protocol are [31]:

- the analytical method for a given product or drug substance;
- the test to be validated;
- the parameters for each test, including type and number of solutions and number of tests;
- the acceptance criteria for each parameter based on an internal standard operational procedure (product or method-specific adaptations may be necessary and are acceptable, if justified);
- list of batches of the substance and/or products;
- for a drug product, the grade or quality of the excipients used in the formulation;
- list of reference materials to be used in the validation experiments;
- information on the instruments and apparatus to be used;
- responsibilities [author, chemists, analytical research project leader, quality assurance, etc.].

3.2.2. Validation Characteristics or Analytical Parameters

Typical analytical parameters or validation characteristics used in assay validation are Specificity, Linearity and range, Accuracy, System and Method Precision, Detection Limit, Quantitation Limit, Robustness, Ruggedness and System suitability [5,28,32,33].

All relevant data collected during validation and formulae used for calculating validation characteristics should be submitted and discussed as appropriate [28]. It is the responsibility of the applicant to choose the validation procedure and protocol most suitable for their product. Revalidation may be necessary in the following circumstances [28]: changes in the synthesis of the product (e.g. drug substance); changes in the composition of the finished product; and changes in the analytical procedure. During the validation process, standard stock such as SIGMA and calibration solutions (analytes and

other standards) are prepared and stored for stability. In GC-FID analysis of CPO for instance, the quantities of fatty acids in the SIGMA and CPO samples in the aliquots could be used for linearity determination [34].

3.2.2.1. Focus GC-FID Method Parameters for CPO Analysis

To develop the GC-FID method needed for CPO analysis, the GC column temperature program has to be defined. An appropriate carrier gas such as nitrogen should equally be supplied in a given flow-rate and split/splitless mode [35].

3.2.2.2. Sample Preparation and GC Analysis

Sample preparation mainly from the point of view of time requirements and feasibility [18] for the GC-FID analysis is imperative. Concentrations of stock standard solutions are defined and prepared at different concentrations in the solvent. Calibration curve should be prepared in a given range [35] for all palm oil samples. The fatty acid methyl esters of all standards and samples are prepared and the methylated samples are injected on GC column in repetitions on multiple days to show reproducibility and robustness. For each concentration, several determinations are performed and the averages calculated. The data are processed with an appropriate data system like Chrom-Card for Focus GC-FID and the response versus the column load are plotted [27].

3.3. Method Validation Characteristics

Method validation is an important requirement in the practice of chemical analysis. It is one of the measures required by a laboratory to be recognized and to comply with national and international regulations in all areas of analysis [25]. The objective of the analytical procedure should be clearly understood since this will govern the validation characteristics under evaluation. Typical analytical parameters used in assay validation (Specificity, Linearity and range, Accuracy, System and Method Precision, Detection and Quantitation Limit, Robustness, Ruggedness and System suitability) are evaluated using solutions of calibrated concentrations [5,6,28,36,37].

3.3.1. Repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision [28]. The repeatability and ruggedness study signifies the reproducibility of the method [38]. Chromatographic conditions are determined as repeatability by measuring replicates of standard solution (comparing peak areas and retention times), peak symmetry and number of theoretical plates [39]. For GC-FID method validation, the repeatability and selectivity of the system has to be checked for individual components [11,14,27]. According to [28], repeatability should be assessed using a minimum of 9 determinations covering the specified range for the procedure (e.g. 3 concentrations/3 replicates each); or a minimum of 6 determinations at 100% of the test concentration.

3.3.2. Stability of Compounds

The European Criteria 2002/657 state that the stability of the analyte in solvent during storage, in matrix during storage and/or sample preparation and in extract during storage and/or analysis should be tested. However, if working solutions are renewed before every batch of samples, the stability of the target compounds in solvent is not considered problematic and therefore may not be investigated in the study. Similarly, matrix stability may not be tested when samples are extracted just after sampling. Since the GC-FID apparatus used in a given study is also used for other routine analysis, the stability of extracts and derivates need to be studied after short-term (e.g. 4 weeks) storage. A one-way ANOVA or Kruskal-Wallis ($p>0.05$) test could be applied on the peak area ratios of the target analytes and the internal standards to detect significant effects (like degradation) of the short-term storage [36].

3.3.3. Quantification and Detection Limits

The quantification limit (QL) or limit of quantitation (LOQ) is defined as the lowest analyte concentration that can be determined with accuracy and precision in a sample [6,28]. This parameter is used particularly for the determination of impurities and/or degradation products [28]. Meanwhile, the detection limit (DL) or limit of detection (LOD) is defined as the lowest analyte concentration that can be identified in a sample, but not necessarily quantified, under the stated experimental conditions [6,28].

GC-FID method validation also involves the LOD and LOQ for individual components [11,14,27,37]. Sample quantification is performed through the calibration curves [6]. The LOD and LOQ can be calculated based on the following formulae [14]:

$$LOD = 3*SE/a;$$

$$LOQ = 10*SE/a,$$

Where: SE = Standard Error and a = the slope.

Daily calibration could be checked in at least three points of the standard mix chromatogram. If checked items correspond to know concentrations, then the standard mix (e.g. SIGMA) and analyte (e.g. palm oil) samples can be run for the day; if not, the equipment should be recalibrated before controls and samples are run [14]. The resulting chromatograms of standards and analyte samples can then be compared.

3.3.4. Trueness

For method validation in the case where no certified reference material is available, the trueness of the analytical method could be assessed through the recovery of additions of a standard mixture of the target analytes in ultrapure solvent. According to the European criteria 2002/657, the directive for the control of analytical methods for matrices of animal origin, the trueness of the method has to be in the interval -50 % to +20 % for a mass fraction of $\leq$ 1000 ng.l-1 [36].

3.3.5. Specificity of Method

The specificity of an analytical method is its ability to measure accurately and specifically the analytes in the presence of compounds (impurities, degradants, matrix, etc) that may be expected to be present in the sample matrix [28,40]. The specificity of the analytical method is determined by injecting the placebo solution under the

same experimental conditions as per the assay [5]. The authors of [6] investigated the specificity of their developed GC method by analyzing a typical sample of the catalytic glycerolysis of methyl oleate followed by identification of chromatographic elution peaks using reference solutions of the analytes. The developed method is considered specific if under the mentioned conditions, a well established retention time (RT) is obtained for a sample analyzed. Resolution should be > 3.5 to indicate a very good separation of peaks [14].

3.3.6. Linearity and Range

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample [28]. In method validation, linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear relationship, test results should be evaluated by appropriate statistical methods [5]. The correlation coefficient, y-intercept, slope of the regression line and residual sum of squares should be recorded and a plot of the data included, with an analysis of the deviation of the actual data points from the regression line [28]. A calibration using reference substances is carried out for quantitative determination of sample components [6,41]. For the establishment of linearity, a minimum of 5 concentrations is recommended [28].

In a study by [35], linearity, precision, accuracy, limit of detection (LOD), limit of quantitation (LOQ), and recovery parameters were determined for validation of method with nine levels (0.001-1 g/mL) calibration series and three analyses at each concentration level. According to the study, samples for recovery experiments are spiked with the target oil sample and allowed for 60 min before performing the extraction. The recoveries are calculated as the percentage using extraction process after spiking from 25 to 100 ng/g with three different levels of oil (n=9). Precision and relative standard errors (accuracy) are determined on spiked oil-free samples at three concentrations by calibration curve prepared.

The linearity of an analytical method for assay is done by injecting a series of samples of each solution and recording chromatograms for various standard preparations prepared from stock in a given range (80% to 120%) into the chromatograph covering different concentrations. A plot (calibration curve) is drawn between the concentrations vs peak response of analyte [14,27]. The slope is reported, and the intercept and regression coefficient from the plot obtained [11].

The linearity of the method is verified by calculation of the coefficient of regression of the calibration curves. High correlation coefficient values for all the analytes indicate excellent linearity in a wide compositional range [6]. In this case, validation criterion is the correlation coefficient which should be above 0.99 for all analytes or calibration curves [6,14,27,34]. Thus to validate the method, the curve fitting should not be less than 99.9% and the calibration curve obtained should be linear while the correlation coefficient value obtained should be above 0.999%, indicating a direct proportionality between the concentrations of standard solutions and peak areas [5,14,27].

3.3.7. Range

Range is the interval between the upper and lower levels of analyte (inclusive) that have been demonstrated to be determined with precision, accuracy and linearity using the defined method [25]. The specified range in method validation is normally derived from linearity studies and depends on the intended application of the procedure. It is established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure [28]. The test should be done in a way that provides the same final dilution as the normal procedure produces, and the range of additions should encompass the same range as the procedure-defined calibration validation. Once the calibration is linear, the slopes of the usual calibration function and the analyte additions plot can be compared for significant difference [25].

References related to method validation suggest a minimum of five or six concentration levels equally spaced across the concentration range, at least in duplicate [25,42]. If the study range is selected at a wider scope (e.g. factor of 200 and above), the analytical data would normally tend to become heteroscedastic where the deviation between replicates becomes bigger at higher concentrations tested [25]. Linearity was observed by [38] in the concentration range of 2-20 µg/ml for celecoxib.

3.3.8. Method Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness [28]. Accuracy should be established across the specified range of the analytical procedure. Several methods of determining accuracy are available [28] which include: application of an analytical procedure to an analyte of known purity (e.g. reference material); comparison of the results of the proposed analytical procedure with those of a second well-characterized procedure, the accuracy of which is stated and/or defined (independent procedure); accuracy may be inferred once precision, linearity and specificity have been established.

The method accuracy is evaluated from recovery experiments using reference samples containing known amounts of standard substances. To determine the accuracy of the method, standard solutions are run as samples. Accuracy may often be expressed as percent recovery by assay of known analyte [5]. The accuracy will be determined by calculation of the mean recoveries ± SD of several levels of concentrations [34]. The intra-day and inter-day RSD's are determined for analyzed samples. Accuracy should be assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range (e.g., 3 concentrations /3 replicates each of the total analytical procedure) [28]. The criterion of accuracy is verified by calculation of recoveries and accuracy's interval of ratios of found quantities on the introduced quantities. The recoveries obtained with the proposed method should be closest or better than those obtained by other methods reported

before [34]. The recovery percentage for each analyte is calculated from the following equation [6]:

$$R_j\,(\%) = \frac{\bar{x}_{obs\,j}}{\bar{x}_{ref\,j}} 100$$

Where R*j* is the Recovery of sample *j*, X_{obs} is the average concentration of compound *j* determined from GC analysis and X_{refj} the actual concentration of *j* in standard samples.

If the concentrations achieved are close to the theoretical values of previous day's calibration curve, and the rate of recovery is closest to 100%, then the method is accurate [6,14].

3.3.9. Precision Evaluation

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. If it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution. The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements [28]. The method precision is evaluated by repeated analysis of each sample. The authors of [6] evaluated a GC method precision by consecutively analyzing a sample 6 times.

System precision on its part is determined by measuring the peak response of the sample for a number of replicate injections of the standard solution preparation as per the proposed method. Acceptance criteria: the relative standard deviation for the replicate standard preparations of same batch should not be more than 2 % [5]. The precision of the proposed method could be verified by calculation of the repeatability's RSD and intermediate precision's RSD of six preparations done successively during one day and the following 3 consecutive days [34]. Data of standard deviations (SD) and relative standard deviations (RSD) for analyzed compounds should show a good repeatability of quantitative results [6]. Recovery ratios calculated at check points of the calibration curve should be closest to 100% [14].

3.3.10. Robustness

The evaluation of robustness should be considered during the method development phase. The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage [28]. It depends on the type of procedure under study. Robustness is determined by analysis of aliquots from homogeneous lots by differing physical parameters that may differ but are still within the specified parameters of assay like change in gas flow rate, column temperature and mobile phase ratio of the GC. The relative standard deviation should be less than 2% [5]. Examples of typical variations for robustness are stability of analytical solutions and extraction time. In the case of liquid chromatography, examples of typical variations are: influence of variations of pH in a mobile phase; influence of variations in mobile phase composition; different columns (different lots and/or suppliers); temperature; and flow rate. In the case of gas chromatography, examples of typical variations are: different columns; temperature; and flow rate [28].

3.3.11. Reproducibility or Ruggedness

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology). It is assessed by means of an inter-laboratory trial [28]. The ruggedness of an analytical method is thus defined as the degree of reproducibility of test result obtained by the analysis of sample under a variety of normal test conditions. For instance, a different analyst using a different GC-FID system with a different and similar column on a different day should be carried out for the experiment [5]. In a validation procedure, the method that shows excellent reproducibility is then selected for eventual sample derivatization [6].

3.3.12. System Suitability

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated [28]. For GC-FID method development, this parameter is evaluated to determine suitability of chromatographic system for the method of analysis by establishing parameters like peak tailing factor, number of theoretical plates and % RSD of samples and standard preparations on daily basis [5]. For validation, the column efficiency should not be less than 2000 theoretical plates while the tailing factor for the analyte peak should not be more than 2 and the relative standard deviation for the replicate injections not more than 2 % [5].

4. Statistical Tools for Method Development and Validation

During method development and validation, test results should be evaluated by appropriate statistical methods, for example, by comparing both the averages and the standard deviations [6], and/or by calculation of a regression by the method of least squares [28]. In some cases, to obtain linearity between assays and sample concentrations, the test data may need to be subjected to a mathematical transformation prior to the regression analysis. Data from the regression line itself may be helpful to provide mathematical estimates of the degree of linearity [28]. Statistical tools like One way analysis of variance (ANOVA) and Bonferroni's multiple comparison tests can be used for the determination of significance of the process in method development and validation [38].

5. Conclusion

The information presented in this paper outlines method development and validation parameters and procedures useful for GC-FID and other analytical procedures. It is an effort to facilitate the work of analysts with reference to

the data from scientific papers and ICH guidelines. Typical analytical method development and validation parameters are also presented with possible recommended and/or reference formulae and values. Some preliminary analysis done with the Thermo Scientific Focus GC–FID of the Lipids Analysis Laboratory (LAL) of IRAD La Dibamba are included to demonstrate some information included in this paper. A precise method with the same equipment which is needed for the analysis of fatty acids composition of crude palm oil from *Elaeis guineensis* Jacq. is under development and validation. Since this paper also presents an overview of method development and validation parameters, it is hoped that information from here will serve the scientific community by enhancing efficient method development and validation which matches international standards and which can be applied by product developers and analysts in quality control laboratories and institutes.

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