Extended Problem 1

As a chemist working on 'food standards', you have been supplied with a large and representative sample of 'Soya beans' for the determination of their 'lipid' fatty acid (FA) content. Soya beans contain many types of both saturated and unsaturated fatty acids (mainly present as their esters) and some of these are considered 'essential' to the human diet. As the supplied soya beans are a new source from abroad, the objectives of this analysis are to, i) prepare the beans for analysis, ii) introduce the use of a suitable internal standard (IS) to the sample to determine the "recovery factor" for the FAs through the various processes undertaken, iii) extract and isolate the range of FAs (e.g. stearic, palmitic, oleic, linoleic acids etc.) from the prepared bean sample, iv) derivatise the FAs into a volatile form for measurement (e.g. FAMEs*) and v) quantify the range of FAs, using a suitable instrumental measurement technique, taking advantage of the volatile FAMEs produced.

* FAME: This abbreviation stands for "Fatty Acid Methyl Ester" and is one route used to derivatise the FAs into a more volatile form, for their later separation and measurement.

i) <u>Preparation of sample</u>
You have been provided with 100 g of raw, mature 'Soya Bean' seeds and this is stated to be a representative sample of the bulk.
Q: What sample preparation step(s) could you undertake to bring the fresh sample into a form ready for extraction of the lipid FAs contained therein.
[Hint: See chapter 3 for guidance as to the initial preparation options and don't forget to first, identify the sample and analyte types from the analytical flowchart shown in Figure 3.1. It would also be helpful to investigate, on-line, a little more about the range of fatty acid types you are likely to encounter and the form in which they are 'bound'].

A: In terms of the sample and analyte types identified from the analytical flowchart (Figure 3.1 in Chapter 3), we have a solid, organic-based sample (foodstuff; soya beans) requiring a qualitative and quantitative organic chemistry measurement of the range of molecular compounds (fatty acids) present in the lipid fraction of the foodstuff.
Raw Soya beans are not suitable for human consumption until processed. This usually involves a soaking stage and then using what is often referred to as "wet heat" to cook. Soya beans have a coating known as a "hull" which comes away from the bean with 'processing'. The debate as to whether the analysis should be of either the 'raw-green' or the 'cooked' soya beans, is one for the initial 'defining the problem' step of "why are we measuring this sample" (and hence, "what do we wish to learn from this measurement")?
It was stated in the introduction that there are a range of fatty acid residues (types) associated with the lipid fraction in this sample, some of which are

considered 'essential'; the implication being that the various individual fatty acids require quantification. It is further noted that those 'fatty acid' residues associated with the lipid fraction in many foods are actually present as the esters of these FAs; i.e. the glycerides and sterol esters of free fatty acids. See the example shown below of the former where R refers to a specific FA residue; R_1 refers to the same FA residue while different FAs present are shown as R_1, R_2, R_3 etc.:

Simple Triglyceride

Mixed Triglyceride
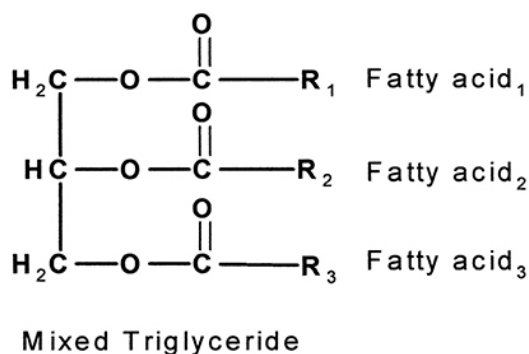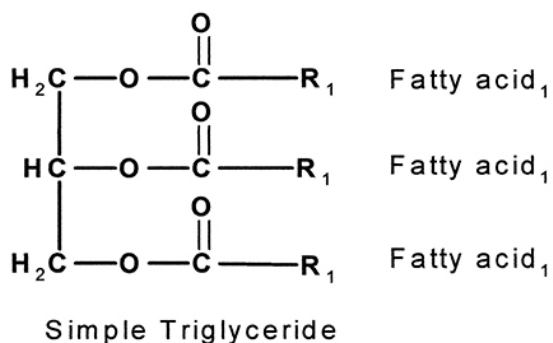
As you are provided with a fresh sample of Soya Beans and this is also a new source material, then you may wish to consider whether the water content is important. As fresh Soya beans (like most vegetable matter) can vary in their water content (raw green Soya beans; ~65 – 70%, while raw air-matured seeds ~ 8 – 10% etc.), it is sometimes helpful to prepare a fully dried sample under controlled conditions. One can then grind and, if required, undertake a sieving process, prior to an extraction step.

However, soya beans are noted to contain a high percentage of oil (lipid fraction) which can make the 'grinding' process and post handling problematic. The simplest approach, (but noted not always the most appropriate), may be to take the sample, **as received**, and then blend or grind a large quantity of it. This would result in a 'homogeneous' paste-like material which can then be sub-sampled for the various analytical requirements. Reducing the particle size and increasing the surface area in this way will allow for a more efficient "extraction" stage

afterwards, while separate sub-samples of the paste-like material may be taken for measurement of their water content using freeze-drying.

ii) Use of a suitable i) reference material for validation purposes and ii) internal standard for the sample to determine the "recovery Factor"

A_1: A number of sources can be found on-line, usually those that are identified with certain recognised or official publications (e.g. peer-assessed papers in journals, validated standardised methods by government bodies etc. Examples include AOAC, EA, EPA, ISO etc.) together with recognised suppliers of reference / certified reference materials, such as NIST, LGC, BAS, NRCC etc.

As stated in the book, it is preferable to use a CRM / RM, that is as close as possible to the sample under identification, in both type and form in order to validate the methodology. Unfortunately not every sample type / form is readily available. Compromises therefore have to be made and these deficiencies identified in any subsequent report. In this case, one of the closest materials available where the validation of a Fatty Acid content has been performed, is on the "oil" that has been extracted from a solid soya bean source. It is noted that fatty acid validation processes have also been performed on other solid vegetable materials and on fish tissues, which contain similar FA components. For the record, to date, the following CRMs/RMs have been identified as possible validation reference materials:

BCR-162R Soya Bean Oil

NIST-3250 (and NIST-3251) Saw Palmetto

NIST-3274 Botanical Oils

NIST-2387 Peanut Butter

NIST-3275 Fish Oils

NIST-1946 Fish Tissue

Remember that the chosen reference material (CRM, RM, in-house RM etc.) should be taken through the same steps / processes as the sample(s) itself and at the same time, in order to fully validate the methodology. It is of course noted that an authentic solid 'reference material' will have been pre-prepared to demonstrate homogeneity and stability and hence part of the sample preparation step has been completed for

you. Therefore, it may well be that the reference material is introduced at the "extraction stage" of the method, alongside the replicate samples, and would include addition of any internal standard employed (see the next section (ii) on choice and use of an internal standard).

A_2: If at this stage we have a large quantity of a 'homogeneous' paste-like material prepared in order to sub-sample, then we can weigh out suitable quantities (as replicates) for analysis. This 'suitable quantity' will be considered later in section iii). It is noted that in natural products that include compounds containing fatty acid residues, the carbon number of the fatty acids (FAs) is usually 'even' – i.e. C14; C16, C18; C18:1; C18:2; C20… etc. The first number refers to the carbon atoms in the main chain including the acid group (i.e. COO⁻). For example, the stearic acid residue, C18 can be shown as:

stearic acid
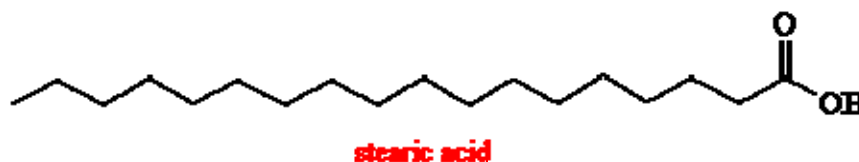
Where there is a second number, e.g. C18:1, this refers to the number of unsaturated bonds; in the above example C18:1, this means there is one unsaturated bond. This unsaturated bond is often situated at its natural (C_{10}=C_9) central position, to give what is known as an omega-9 fatty acid, i.e. cis-9-octadecenoic acid, or $CH_3(CH_2)_7CHCH(CH_2)_7COOH$; 'trivial' name Oleic Acid. Note that the <u>natural</u> 'cis' arrangement about the double bond is expected (compared with the 'trans' arrangement found in some processed FAs). For information, the naturally occurring C18:2 and C18:3 refer to linoleic and linolenic acids which are the natural cis poly-unsaturated FAs having two and three double bonds, respectively.

A suitable internal standard (IS) would therefore be one which closely mimics the physical and chemical properties of the analyte(s) under investigation but is also <u>not present in the original sample</u> in any significant concentration. It is noted from a simple on-line investigation / study, of the fatty acid residues expected from lipids including those from the triglycerides, that the range of long chain FAs we are interested in could cover carbon lengths from C10 up to C24. However, the saturated, mono-unsaturated and polyunsaturated FAs of greater significance are those from around the C16 - C18 and C20 – C22 chain lengths. Hence, a fatty acid residue based upon a chain length such as C19, would serve as a suitable internal standard (IS); i.e. the *n*-nonadecanoic acid would be a good starting point. Knowing that, if it was present (which it is not!), the glyceryl ester of this

OXFORD
UNIVERSITY PRESS

hypothetical C19 FA would release the C19 residue. Adding a simple ester of this FA would react and mimic in many ways the actual FAs from the lipids in the food itself. Therefore, a known amount of the simple ***n*-nonadecanoic acid methyl ester** could be added to each of the weighed out, homogenised sample(s) to act as the IS. The quantity to add is considered in section iii).

iii) <u>Extract and isolate the range of FAs (e.g. Oleic, linoleic, stearic, palmitic acids etc.)</u>
<u>from the prepared bean sample</u>

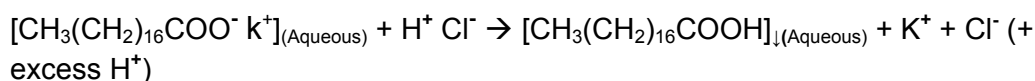A_1: Extraction of lipids and quantity of IS to add:
Considering the organic nature of both the sample and the analytes of interest together with the knowledge that these FAs are present as, for example, the esters in the 'oily' lipid fraction, then their extraction (through solubility) would best be served using particular organic solvents. Using the principle that 'like dissolves like' and knowing that the sample and analytes cover a range of polarity (from relatively 'non-polar to polar organic' – see on-line additions to chapter 3 on "solvent extraction") then it is understandable that the guidance given in the on-line work, "Current lipid extraction methods….." identifies a range of suitable solvents. Particular emphasis was on the original work of Folch et al. (1957; see reference at the end of this extended problem). Either a mixture of chloroform:methanol (2:1, v/v) or its updated, more safety conscious derivative, i.e. the mixture of dichloromethane:methanol (2:1v/v) was used. You may have already prepared a large quantity of the fresh "soya bean", now homogenised to a paste. Hence, extraction could well be based upon shaking and mixing a relatively large, say 5.00 g sample of this 'homogenate' with up to 100 mL of either of the mixed solvent systems shown above (e.g. as 2 x 50.0 mL lots). Of course, <u>before this extraction</u>, you need to consider 'how much of the chosen IS should be added' to the weighed sample. One 'rule of thumb' would be to add <u>at least the</u> <u>same quantity of IS</u> to the original sample prior to its extraction, <u>as the expected or predicted quantity (or covering the range) of the 'analyte(s)'</u> present in the weighed sample. As stated previously, the IS will undergo the same processes as those of the sample's analytes, therefore it must be present at a suitable level for measurement alongside the analytes at the measurement step. This will be dependent to some extent upon the methodology adopted (losses incurred, fractions taken, efficiency factors etc.). As an example, from various sources (see on-line links etc.) the stearic (C18:0) acid content of raw soya beans can cover the range 0.8 to 1.2% m/m (while, for example, Palmitic (C16:0) acid is slightly higher, around 2% m/m). Therefore, in our 5.00 g freshly ground soya bean sample we might predict around 50 mg of a Stearic acid residue. The **equivalent mass** of an IS can be achieved by adding a 1.00 mL micropipette volume of a 50.0 mg mL^{-1} solution of the ***n*-nonadecanoic acid** (present as its methyl ester; or something similar to this 50

mg, but <u>the exact quantity added – volume and concentration- of this IS must be known</u>) to each of the weighed out, homogenised replicate samples. The resulting extraction solution, after shaking and mixing with the sample, would require filtering to remove the protein fraction. The sample solids left behind and all filtering apparatus would be washed with 3 x 10 mL lots of the solvent mixture adopted (i.e. chloroform or dichloromethane : methanol), to help with the quantitative transfer. All extracts and washings should be collected into a suitable vessel that allows any later, separation of layers containing the analytes of interest.
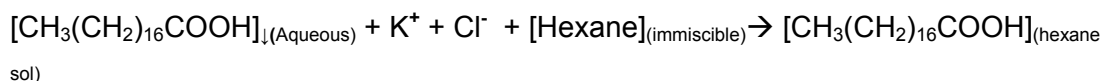
This will help to provide you with the "total lipid extract" from each extracted homogenised sample, which should now contain both the bound and free FAs together with the IS.

Saponification and isolation of total FAs from the total lipid extract

In order to "isolate" the FAs (originally present as, for example their esters, in the extraction solution), the extract can first be saponified. This process hydrolyses the cholesterol esters, the tri-glycerides and the phospholipids (with, for example, potassium or sodium hydroxide) and converts all the bound fatty acids into their water-soluble soaps (for example, their potassium or sodium salts). These can then be liberated, from their aqueous phase as their **actual** free fatty acids by adding a suitable quantity in excess, of a strong acid (e.g. Conc. HCl). For example for Stearic acid; C18:O :

$$[CH_3(CH_2)_{16}COO^- k^+]_{(Aqueous)} + H^+ Cl^- \rightarrow [CH_3(CH_2)_{16}COOH]_{\downarrow(Aqueous)} + K^+ + Cl^- (+ \text{ excess } H^+)$$

The liberated free fatty acid, of essentially non-polar character will have little to no solubility in the aqueous phase, which is of a relatively high polarity and contains ionic salts. It is now possible to use the process of 'liquid-liquid' extraction, where two immiscible liquids are in contact and the target analyte(s) is much more soluble in one of these compared with the other. For these FAs to dissolve, a suitable non-polar immiscible solvent would be hexane. A small volume of this can be shaken with the total aqueous phase and then isolated using a "separating funnel"; this would be repeated two more times to ensure complete transfer and the fractions combined.

As noted, this partition effect is based upon solubility, as "like dissolves like" (polarity etc.; see both the book and the on-line addition to sections in and to, chapter 3 on "solvent extraction")

$$[CH_3(CH_2)_{16}COOH]_{\downarrow(Aqueous)} + K^+ + Cl^- + [Hexane]_{(immiscible)} \rightarrow [CH_3(CH_2)_{16}COOH]_{(hexane\ sol)}$$
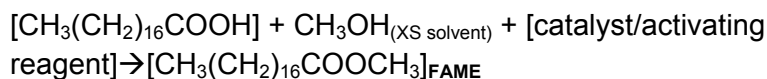
Rotary evaporation of the isolated FAs in the combined hexane fractions will remove the hexane solvent and provide you with a measure of your "total free fatty acid" fraction.

A$_2$: The other procedure that must be carried out alongside the sample, from when the extraction process is being undertaken, is the preparation of "procedural blanks". A number of solvent blanks can be taken through the exact same procedures as (and alongside) the samples from this stage and throughout, so as to analyse alongside the analytes at the measurement step. This will allow you to correct for associated contamination and background effects from the practical methodology. You should undertake replicate 'procedural blanks' and also consider some blanks having the IS added, for later evaluation.

iv) Derivatise the free FAs into a volatile form for measurement (e.g. FAMEs*)

A: All the free fatty acids present at this stage can be derivatised into their more volatile methyl esters (FAMEs) using methanol along with a suitable "activating reagent". One efficient route is to employ a 'boron trifluoride' activating reagent in a 'methanol' solvent. One other route is to use methanol with a sulfuric acid catalyst (98:2, v/v).

$$[CH_3(CH_2)_{16}COOH] + CH_3OH_{(XS\ solvent)} + [catalyst/activating\ reagent] \rightarrow [CH_3(CH_2)_{16}COOCH_3]_{\textbf{FAME}}$$

Isolation of the prepared FAMEs using a suitable non-polar solvent, such as hexane (again, see "solvent extraction" in the on-line addition section to Chapter 3) will provide you with your total FAME (and sterol) fraction ready for the measurement step.

v) Quantify the range of FAs, using a suitable instrumental measurement technique,

A: We have deliberately prepared the FAMEs of the FAs isolated from the lipid fraction within the soya beans, in order to take advantage of particular techniques.

The superior separation capabilities of Gas Chromatography (GC) should allow us to separate the individual FAs when present as their stable, volatile methyl esters (FAMEs). Using a suitable column and temperature programme, a measure of what different types of FA are present in the original extract, i.e. qualitative information,

should be possible when a suitable detector is used to identify each FA's retention time and / or mass fragmentation pattern.

When this separation technique is combined with a suitable detector and calibrated against known FAME standards, then a measure of how much of each FA is present in the extract can be obtained, i.e. quantitative information. Detectors can be simple but sensitive such as a flame ionisation detector (FID; to give GC-FID) or more complex and sensitive but more informative such as a mass spectrometer (MS; to give GC-MS). However, some potential 'interference' problems can arise because of the complex range of FAs present; a result of the limitations to separation of certain FA residues present with close to near identical retention times.

Additional information:

<u>Preparation steps – 'clean up' using a separation stage to remove possible 'interferences'</u>

It is noted that extraction of lipids from organic samples (i.e. animal and plant-based sources) using organic solvents can produce complex mixtures which may require 'clean-up' or separation steps at a suitable point in the process in order to avoid possible interferences in the later measurement step. This 'clean-up' process may be achieved by using, for example i) particular solvent types (variable solubility effects), ii) certain liquid column techniques (LC; normal or reversed phase systems; e.g. silica columns to help separate neutral and polar lipid fractions, etc.), iii) targeted solid phase extraction techniques (modified SPE to either retain the target analytes or retain possible interferents as a pre-separation step), iv) thin-layer chromatography (TLC) of the FAMEs etc., to isolate particular fractions.

However, it is important to ensure an internal standard (IS) is chosen that follows each required (separated) fraction during the 'clean-up' or simplification process, so that it is present at the measurement step together with the analytes of interest.

<u>Measurement step and the Internal Standard (IS):</u>

It is noted that a known quantity of the chosen IS is added to a known quantity of the processed sample prior to extraction of its FA lipid fraction. At the later measurement step, where a known volume of the FAME fraction is injected into, for example the GC-detector system under a strict temperature-controlled programme, the internal standard and the sample's FAs (all as FAMEs) will each display their individual retention times. This will allow a level of 'identification' by comparing these retention times against those of a standard set of FAs, run as their methyl esters (qualitative information) under the same temperature-controlled programme. If a calibration is also performed using known increasing quantities of the IS, then a calibration graph can be prepared (based upon the integrated area response for each known quantity of the IS's calibrant solutions) against which the IS from the processed sample can be measured (quantitative information on the IS). Therefore **the ratio of** the "amount

of IS recovered" (IS_R) which is measured using, for example the GC-FID (from the final processed sample) and quantified from the IS calibration graph, **to that of** the "amount of IS added" (IS_A) to the sample before extraction, may be considered as the 'recovery factor' (RF):

$$RF = \quad IS_R / IS_A$$

This recovery factor can be used to "scale" each of the FA quantities obtained at the measurement step back to the original sample (see the On-line Chapter 5 topic "Internal Standardisation" and section 3 within this).

To a first approximation, in the quantification of the individual FAs, **if** the detector (e.g. FID) has the same response value (i.e. area counts) for each FA as that of the IS (e.g. on a mass to mass basis or on a molar basis etc.), then the IS calibration may be used to gauge the quantity of the individual FAs present. Here, **you are assuming** the IS calibration graph and each individual FA's calibration graph will have the same gradient (slope).

In reality, this assumption will introduce an error and a correction will be required. To address this, a 'calibration response set' can also be injected and measured under the same conditions as the IS calibrants. This 'set' comprises **standards of all of the FAs** under investigation including the IS and all are **present at the same concentration / quantity** (e.g. all present at say 50.0 mg/L, in the form of their FAME). From the individual FA responses (FA_x ; as area counts) compared with the IS response (as area counts), a ratio; IS / FA_x can be calculated. This can then be used as a correction factor for each FA value calculated against the IS calibration graph.

Of course, the latter approach of using a 'calibration response set' is sometimes taken to avoid producing individual calibration graphs for each of the analyte FAs. If, for example, you were looking for 10 FAs, then by not adopting the above approach, this would mean producing 10 individual FA calibration graphs, each FA having four or five calibrants. Then, together with calibration blanks this would mean over ~50 standard solutions would need to be run, just to calibrate the individual FAs. This might be considered excessive, particularly if you were running only one sample in triplicate!

One other point to remember concerning units is that while 1 mole of a FAME contains the equivalent of 1 mole of the FA (therefore 1mM of a FAME ≡ 1mM of the FA), a correction is required when a mass based unit is employed. For example, stearic acid has a RMM of 284.48 g/mol while its fatty acid methyl ester (FAME), methyl stearate has a RMM of 298.50 g/mol. Hence, it must be clear when stating mass-based units for FAs whether the mass is based upon i) the absolute quantity of

a FAME present or ii) on the equivalent amount of FA contained within the FAME present!

Problem based upon the above 'Fatty Acids in Soya Bean' topic

A_i: Using the supplied information, calculate i) which saturated fatty acids are present in the sample;

Here we can use the retention time and pattern spacing of the standard FAMEs to 'identify' which FA residues are present in the chromatogram from the processed sample extracts. If the GC programme used for both is the same (and it is reproducible) then the following table can be constructed for the sample;

Carbon No. of saturated Fatty Acid	Standard FAMEs Peak Retention time (mins)	Soya Bean Sample 1 Peak Retention time (mins)	Comments FA trivial Name
C10:0	9.91	ND*	C10 Capric acid
C12:0	12.33	ND	C12 Lauric acid
C14:0	14.53	ND	C14 Myristic acid
C16:0	16.52	16.50	C16 Palmitic acid
C18	(see notes; and answer to 'suitable internal standard)	18.12	C18:2 linoleic acid-cis
C18:0	18.34	18.32	C18:0 Stearic acid
C19:0 [Internal Standard]	19.20	19.18	C19:0 *n*-nonadecanoic acid
C20:0	20.03	20.00	C20 Arachidic acid
C22:0	21.58	21.55	C22 Behenic acid
C24:0	23.12	23.09	C24 Lignoceric acid

*ND = not detected

The above fatty acids are therefore identified using their retention time (and relative pattern), under the conditions of the measurement step using GC-FID.

A_{ii}:

Using the above information, calculate ii) how much of each saturated fatty acid is present in the original sample of Soya Beans?

To start this process, we first need to calculate the "recovery factor", RF, identified and defined previously; i.e. the ratio of the "amount of IS recovered" (IS_R) from the sample using the IS calibration, to that of the "amount of IS added" (IS_A) to the sample in the beginning; this may be considered the recovery factor (RF):

$$RF = IS_R / IS_A$$

We therefore need to construct a calibration graph using the C19 standard data and hence, the calibration values for the C19 (FAME of the *n*-nonadecanoic acid) IS concentration in mM vs peak area (counts; AU) give us:
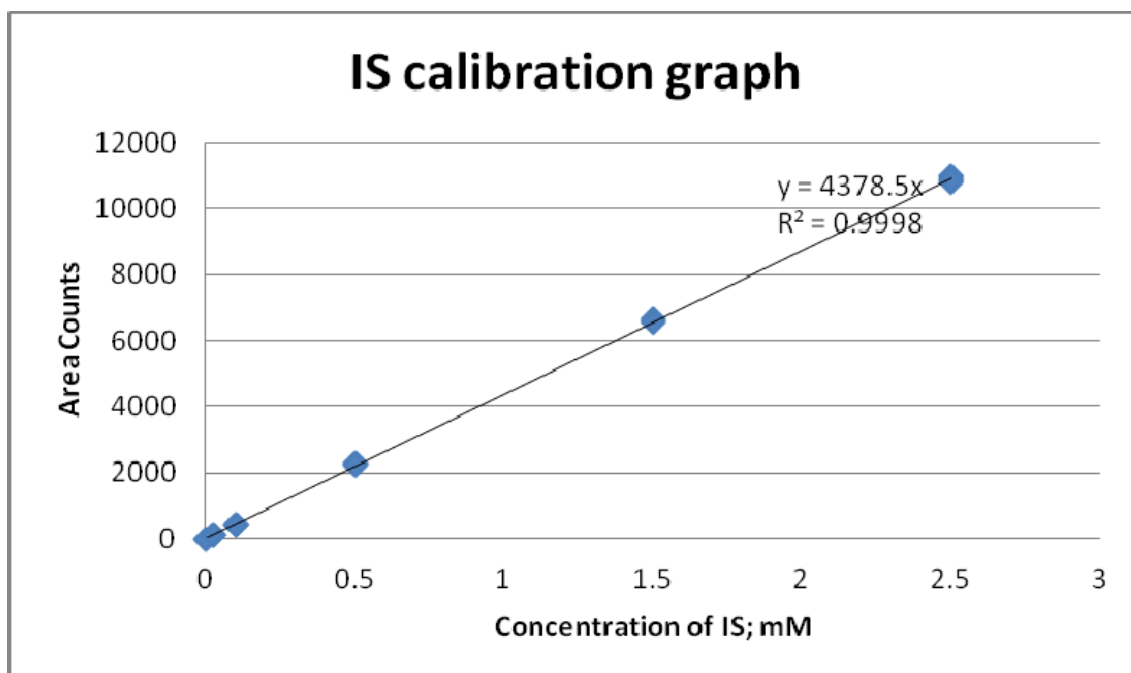
The above is based upon the below values, where each point is plotted and the equation of the trend-line shown:

mM quantity IS	Area Counts
0	0
0.025	105.5

OXFORD
UNIVERSITY PRESS

0.025	105.7
0.025	105.1
0.1	434.1
0.1	436.4
0.1	435.2
0.5	2278.6
0.5	2264.9
0.5	2247.2
1.5	6673.9
1.5	6620.9
1.5	6566.3
2.5	11004
2.5	10792.7
2.5	10902.8

As the sample's FAMEs were run under the same programme conditions, the 'Area Counts' for the IS within the sample can be used to calculate the quantity of IS present in the measured fraction.

It is first noted that the molar mass for the *n*-nonadecanoic acid is: 298.5038 g/mol and secondly, it is noted that the law of constant composition means that the ratio of the mass of a selected FA divided by the mass of its FAME will be a constant and that one mole of a selected FAME contains one mole of its FA. If all mass concentrations are expressed in terms of their FA, then from the IS calibration graph and its equation of the line:

Area Counts (y) = 4378.5x ; where x = concentration in mM

And noting that the Area Counts for the IS in the sample is: 176.4; as shown in the table below:

Carbon No. of saturated Fatty Acid	Standard FAMEs Peak Retention time (mins)	Soya Bean Sample 1 Peak Retention time (mins)	Soya Bean Sample 1 Peak Area Counts	Standard FAMEs Peak Area Counts (50 mg / L)
C16:0 Palmitic acid	16.52	16.50	543.4	1506.0
C18:2 linoleic acid-cis:cis *	(see notes; and answer to 'suitable internal	18.11 + 18.15	[2175] + [1227]	1313.0

C18:0 Stearic acid	standard) 18.34	18.33	189.9	
C19:0 *n*-nonadecanoic acid [Internal Standard]	19.20	19.18	176.4 [slope of graph = 4378.5 area counts / mM]	1581.0
C20:0 Arachidic acid	20.03	20.00	14.77	1623.9
C22:0 Behenic acid	21.58	21.55	17.21	1517.4
C24:0 Lignoceric acid	23.12	23.09	5.77	1521.0

*Polyunsaturated (di): see earlier notes

Then, the concentration, x, of the IS in the processed sample in mM is given by:

176.4 = 4378.5x
Therefore x = 0.0402(9) mM.
This concentration value, the number of millimoles of IS per litre [0.0402(9) mM] can provide us with the absolute number of millimoles present in the final volume of solution made up for measurement; i.e. that solution which provided the 1 μL injection into the GC-FID. This final solution was stated to be made up to **2.0 mL** using hexane and capped.
Therefore, if the concentration is 0.0402(9) mmoles per liter of solution then in **2.0 mL** there are:

0.0402(9) x 2.0 / 1000 = 8.05(8) x 10^{-5} mmoles of IS

Now, 1.0 mL of a 50 mg mL^{-1} IS (C19 FA; *n*-nonadecanoic acid) was added to the 5.00 g sample at the beginning, before the extraction. The number of mmoles IS of this solution can be calculated using the RMM of the IS (298.5038 g / mole);

50.0 mg mL^{-1} IS added = 50.0 mg actually added
50.0/298.5038 = 0.167(5) mmoles IS added
The 'Recovery Factor' ratio RF is therefore:
8.05(8) x 10^{-5} / 0.167(5) = 4.81(1) x 10^{-4}

[The alternative approach to calculating the RF is to convert all units to mg rather than mM]

As the IS (*n*-nonadecanoic acid) has a RMM of 298.5038 g/mol

then,

in terms of mg / L in the processed sample, we see that from the calibration graph we have

0.0402(9) mM x 298.5038 = 12.0(3) mg / L ;

therefore there are 12.0(3) x 2 / 1000 mg of IS in the final **2.0 mL** of solution;

= 0.0240(5) mg

The original concentration of IS added was 50.0 mg mL^{-1} which = 50.0 mg

Therefore the ratio for the RF becomes

0.0240(2) / 50.0 = <u>4.81(1) x 10^{-4}</u> = RF

Now that we have the recovery factor, we can use this later on to "scale" the quantity of each FA measured in the processed sample (from the processed GC-FID sample 1 peak area results) to that in the original 5.00 g sample used at the beginning.

But first, we have to calculate the quantity of each FA, measured in the processed sample by GC-FID.

<u>Approximation assuming equal response of the 'detector' (FID) to all FAs on a molar or mass basis:</u>

If we assume that the slope of the IS calibration (the sensitivity or unit response) is the same for all FAs (as identified before in our notes as a first approximation, to simplify);
then we can use the equation of the IS calibration graph (Area Counts (y) = 4378.5x) to calculate the number of mM of each FA in the processed sample from the area counts shown in the table above. The following table can therefore be produced:

Carbon No. of saturated Fatty Acid	Soya Bean Sample 1 Peak Retention time (mins)	Soya Bean Sample 1 Peak Area Counts (y)	Conc. x in mM based upon IS slope of (y/x) = 4378.5	RMM of FA in g / mole	Quantity of FA (mg) in 5.00 g of raw sample^Δ	Quantity of FA (mg) in 100 g of raw sample

C16:0 Palmitic acid	16.50	543.4	0.124(1)	256.430	**132.(3)**	**2646 ≡ 2.65 % m/m**
C18:2 linoleic acid-cis:cis *	18.11 +	2175 +	0.496(7)	280.452	**579.(1)**	**11,58(2) ≡ 11.6 % m/m**
[C18:1 / C18:3] e.g. Oleic and linolenic acids (?)	[18.15]	[1227]	[0.280(2)]	[~ 282]	**[328.(5)]**	**[657(0)] [≡ ~6.6 % m/m]**
C18:0 Stearic acid	18.32	189.9	0.0433(7)	284.484	**51.2(9)**	**102(6) ≡ 1.0(3) % (m/m)**
C19:0 *n*-nonadecanoic acid [Internal Standard; IS]	19.18	176.4 [slope of graph = 4378.5 area counts / mM]	0.0402(9)	298.504	**50.0**	**IS added (≡ 1000 or 1 % m/m)**
C20:0 Arachidic acid	20.00	14.77	0.00337 ‡	312.54	**4.3(8) ‡**	**87.7 ‡**
C22:0 Behenic acid	21.55	17.21	0.00393 ‡	340.59	**5.5(6) ‡**	**111 ‡**
C24:0 Lignoceric acid	23.09	5.77	0.00132 ‡	368.63	**2.0(2) ‡**	**40.5 ‡**

*Polyunsaturated (di): see earlier notes

‡ Result to check against limit of quantitation for method (LOQ)

Δ See equation below

To illustrate the calculation, we can use the IS as a guide. We know that from the sample's chromatogram, the IS is present at a concentration of 0.0402(9) mM and that 50.0 mg of IS was added to the original sample at the beginning; therefore:

The Final solution prepared for injection into the GC was made up to **2.0 mL** (from this a 1 µL injection was made). Therefore using the IS value of 0.0402(9) mM

0.0402(9) mM IS = 0.0402(9) x 2.0 / 1000 mmoles = 0.0805(8) µmols of IS present in the final solution of **2.0 mL**. Converting to mass units using the RMM of the IS we have:

0.0805(8) µmoles x 298.5038 g/mole = 24.0(5) µg IS per 2 mL of final solution for injection into the GC

Using the RF to 'scale' back-up to the original 5.00 g of sample:

24.0(5) µg of IS / RF (4.81(1) x 10^{-4}) = 50,000 µg IS added to the 5.00 g original sample

50,000 µg = 50 mg, which is the correct quantity of IS added (notes show we added 1 mL of 50 mg/mL IS solution).

The above full calculation as an equation (**Δ**) can be shown as:

[No. of mM of FA_X x 2.0 / 1000] x [RMM of FA_X] / RF   = mg of FA_X in the 5.00 g original sample (**Δ**)

Values for the quantity of each FA are shown in the table above. Both for those present in 5.00 g of sample and scaled further to show the quantity in 100 g of sample are presented (the latter is used by government and industry sources for foods; per 100 g foodstuff).

Correction when there is an unequal response of the 'detector' (FID) to FAs on a mass vs molar basis:

If each FA has a different response from the detector, based upon unit quantity (molar or mass) then a correction is required. Essentially, the slope of the calibration graph for the IS will not be the same as that for each of the FAs in the series (C16, C18, C18:2, C20 etc.). This correction can be achieved by identifying the difference in response from the FID using a standard set of known-quantity FAs run under the same conditions as previously used with all the FAs of interest (including the IS, with those identified in the sample) on the GC instrument. This will allow a response ratio to be calculated and then used to correct. Ideally, an equi-molar set of FAs would be used on the GC-FID instrument to calculate the scaling required. Alternatively, an equi-mass set of FAs would be run and their responses (as area counts) first corrected to their molar contribution, and then used as a factor to correct the final concentration in the foodstuff. The reader is reminded that all previous calculations were based upon the slope of the IS calibration as mM concentration.

Any values calculated for the concentration of the FAs in the sample have already been corrected for their difference in RMM but not for their difference in response (response factor) to the FID. The product of this response factor and the concentration of the fatty acid in the sample (previously based upon an equi-molar

response) will correct for this difference. Values obtained from this exercise are shown below. To correct any FA values using these 'Response Factors', divide the FA values in the above table by their specific Response Factor shown. For example, the uncorrected value for Stearic Acid is: 1.0(3) %

The corrected Stearic Acid value is: 1.0(3) / 0.791 = 1.3(0) %

Carbon No. of saturated Fatty Acid	Standard FAMEs Peak Area Counts (50 mg / L)	RMM of FA in g / mole	mM of FA in standard FAMEs (mmoles/L)	Area counts per 1 mM of FA	Response Correction Factor to slope (based upon mM) ‡
C16:0 Palmitic acid	1506.0	256.430	0.1950	7723	0.818(2)
C18:2 linoleic acid-cis:cis *		280.452			
C18:0 Stearic acid	1313.0	284.484	0.1758	7469	0.791(3)
C19:0 n-nonadecanoic acid [Internal Standard; IS]	1581.0	298.504	0.1675	9439 (IS)	1.000
C20:0 Arachidic acid	1623.9	312.54	0.1600	10,150	1.075
C22:0 Behenic acid	1517.4	340.59	0.1468	10,337	1.095
C24:0 Lignoceric acid	1521.0	368.63	0.1356	11,217	1.18(8)

*Polyunsaturated (di): see earlier notes

‡ Calculated as the ratio of the responses; FAx / IS.

The links already provided give some guidance and /or further links to these processes and validated techniques.

See also:

Folch, J., Lees, M. and Stanley G.H.S (1957) A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry* **226**, 497-509.

FSA (2010) *The Composition of Foods*,

https://www.gov.uk/government/publications/composition-of-foods-integrated-dataset-cofid

https://www.gov.uk/government/collections/national-diet-and-nutrition-survey

Food Composition USDA - https://www.nal.usda.gov/fnic/food-composition