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) Preparation of sample

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’].

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

Q_1: In order to ‘validate’ the methodology used (i.e. to assess the overall efficiency of the methodology in terms of being “fit for purpose” for the objective of the analysis), you will need to identify at least one suitable certified reference material (CRM) / reference material (RM), to run alongside the replicate soya bean samples themselves. Can you identify from suitable sources, including those on-line,

at least one suitable CRM or RM to run alongside the replicate samples of Soya Bean?
[Hint: See chapter 6 in the book on 'Reference Materials' for guidance, together with an on-line search using suitable key words including for example CRM, RM – in full or abbreviated, and Soy or Soya bean, Fatty Acids etc.].

Q_2: A known quantity of a suitable internal standard (IS) needs to be added to a known quantity of the processed sample, prior to the extraction step, allowing the efficiency of all the following processing steps (combined) to be monitored and quantified. At this stage we therefore need to consider first 'what would be a suitable internal standard' in preparation for the later question 'how much IS should be added'?

[Hint: See the on-line addition to chapter 5 on internal standardisation for guidance.
Also see the following link which is best copied directly into the search engine in order to work, rather than use any active links available - https://www.sis.se/api/document/preview/918926/
See the emphasis in section 4.2 within this link, covering internal standards.]

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

Q_1: Using the information from section i) above, i.e. identifying the sample and analyte types from the analytical flow chart and using the guidance given in Chapter 3, what procedures could you use to extract and isolate the range of fatty acids present in the bean sample, including the IS added? What would therefore be a suitable quantity of IS to add to your individual replicate samples? and what would be a suitable quantity of sample to take as a replicate?
[Hint: See the introduction section to the on-line work, "Current lipid extraction methods….." in the link below for guidance:
https://microbialcellfactories.biomedcentral.com/track/pdf/10.1186/s12934-017-0633-9]
See also the sections on "solvent extraction" in Chapter 3 of the book and the "on-line additions" to chapter 3.

Q_2: What other procedure must be carried out alongside the sample, from when the extraction process is being undertaken?

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

Q: Given that you have now liberated the free fatty acids fraction from the lipids extract, how might you derivatise this fraction to a more volatile form that can be used to separate the individual components (FAs) for their later measurement?
[Hint: see the links provided from the previous sections, for some guidance]

v) Quantify the range of FAs, using a suitable instrumental measurement technique,
Q: What instrumental measurement technique(s) would you use to quantify the FAs present?
[Hint: see the guidance presented in the tables and sections within Chapter 4, together with the links to the scientific papers from the previous sections]

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

Q: You have processed a 100 g lot of raw, mature, fresh Soya Beans to produce a fine paste-like material **for the quantitative measurement of the range of saturated fatty acids (FAs) present**. A small quantity of this ground 'lot' was separately freeze-dried to determine the water content (identified as 11.0 %) while three 5.00 g sub-samples were taken for the FA extraction process, using a dichloromethane: methanol mixture. To each 5.00 g sub-sample prior to extraction was added 1.00 mL of an equivalent 50 mg mL^{-1} *n*-nonadecanoic acid, present as its FAME, using a micro-pipette.
Extraction, saponification and methylation processes were undertaken prior to a 'clean-up with separation step' using solid phase extraction (SPE). For the latter, conditioning and equilibration of the SPE cartridge (C18-type) was brought about by the sequential addition of acetone and then hexane prior to drawing a 1.0 mL aliquot of the sample's FAMEs solution through it. The retained FAs on the SPE cartridge were then eluted in fractions; the first (Fraction 1) used 6 mL of a 96:4 v/v hexane: acetone mixture to target and release the saturated FAs together with any trans mono-enes, and any cis/cis and trans/trans conjugated linoleic acids (CLAs) that may be present on the cartridge. Other fractions were also eluted separately from the SPE cartridge using selected solvent mixtures. These other fractions were all retained for

possible later investigative and confirmatory work on the unsaturated FA residues and checks for any saturated FAs being present.

The saturated fatty acid fraction (Fraction 1 from the SPE), collected in a 10 mL glass vial, was then blown-down using a gentle nitrogen flow with warming (not above 40^{o}C) to remove the solvent. The solid residue left was then dissolved in **2.0 mL** of hexane, capped-to-seal and 'identity-labelled' for later measurement.

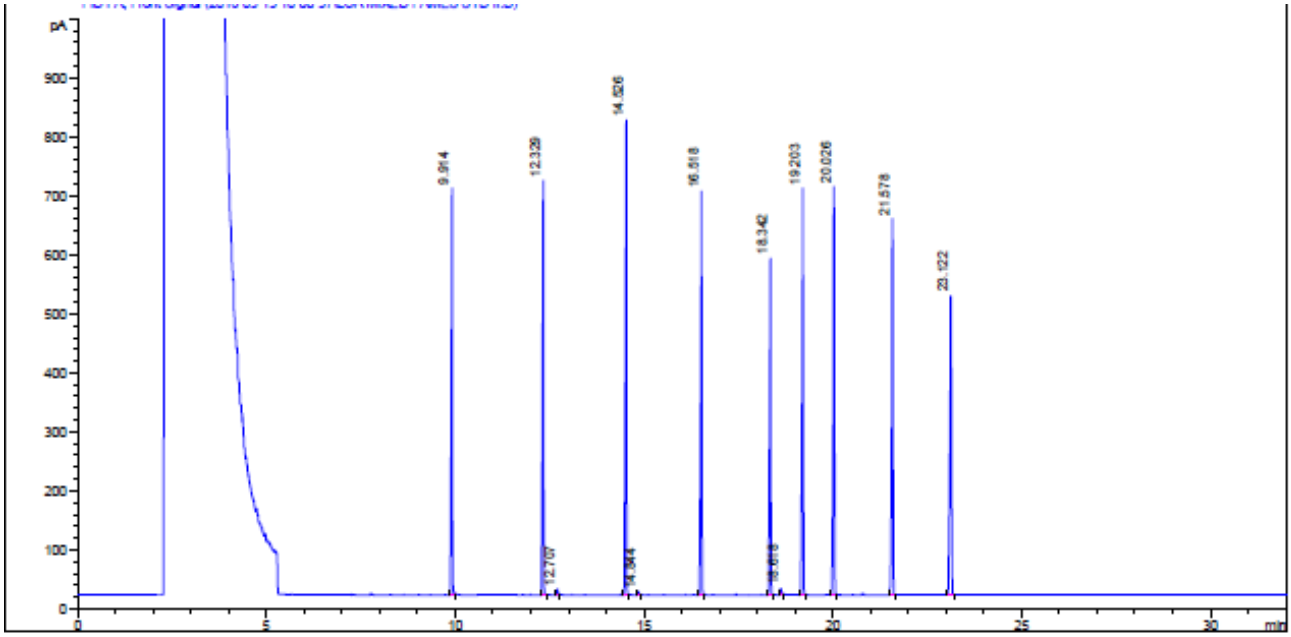
Gas chromatography with a flame ionisation detector (GC-FID) was used (**see Chapter 4 of book**) to identify and quantify the saturated fatty acids present in the collected fraction 1 and to produce a calibration graph from known quantities of the IS. The instrumental conditions used were:
Column: 30 metre by 0.32 mm id with 0.25 µm film thickness – (5% phenyl:95% dimethylsiloxane)
Carrier gas: Nitrogen (1 mL / min)
Injection: Splitless mode; 1µL with injector port held at 250^{o}C
Programme: Column oven; initial T = 60^{o}C, then 10^{o}C rise / min up to 280^{o}C; Hold at 280^{o}C for 10 min (run time / sample = 32 mins.)
Flame ionisation detector: Temp. = 300^{o}C; Hydrogen flow = 40 mL / min,
Air flow = 400 mL / min, Nitrogen make-up flow = 15 mL / min.

The following measurement results were obtained:

The calibration values for the C19 (FAME of the *n*-nonadecanoic acid) internal standard (IS) Concentration in mM vs peak area (counts; AU) are shown below

C19 Calibration	Peak Area (AU)
mM quantity	
0.000	n/d
0.025	105.5
0.025	105.7
0.025	105.1
0.100	434.1
0.100	436.4
0.100	435.2
0.500	2278.6
0.500	2264.9
0.500	2247.2
1.500	6673.9
1.500	6620.9
1.500	6566.3
2.500	11004.0
2.500	10792.7
2.500	10902.8

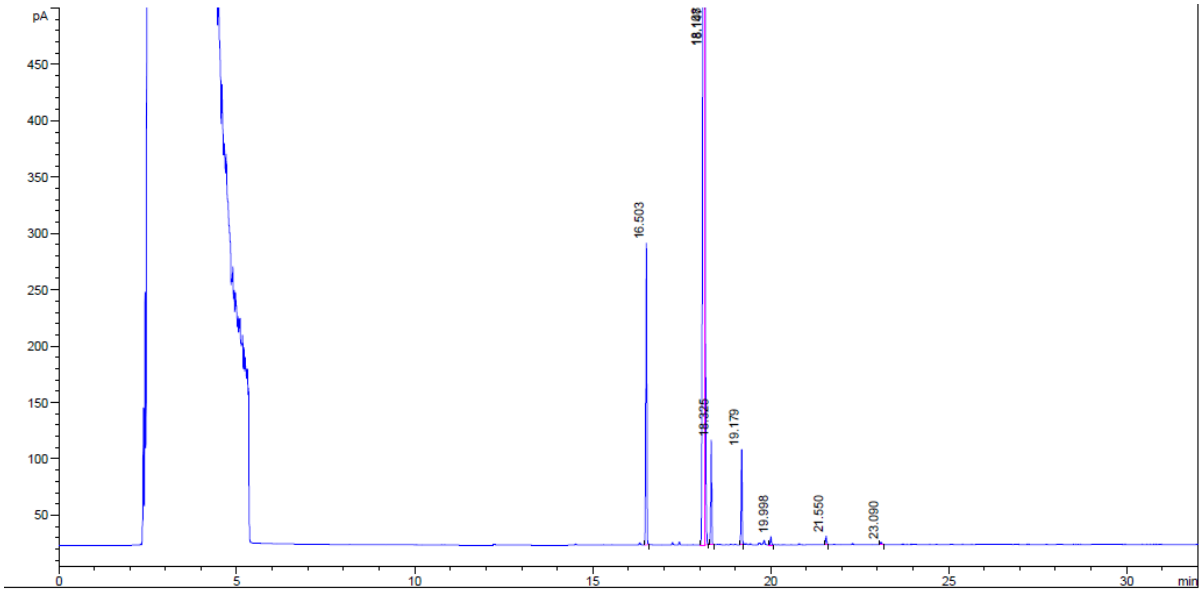
Below is shown the chromatogram (from GC-FID) from the injection of a standard set of FAMEs at the same concentration (50 mg/L in terms of the FA) with “even” carbon numbers ranging from C10 to C24 (in order) and including the C19 IS, together with all their integrated area counts.



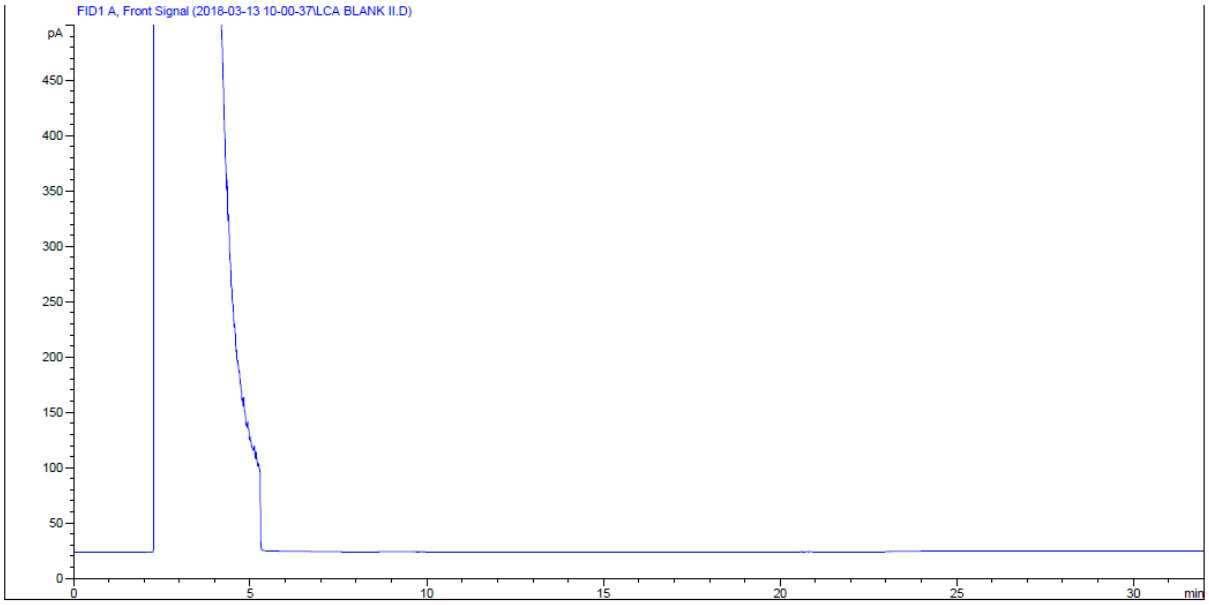
#	Meas. Ret. Time	Area	Height
1	9.914	1382.751	689.506
2	12.329	1432.156	702.220
3	12.707	20.120	10.057
4	14.526	1740.010	804.552
5	14.844	10.772	5.387
6	16.518	1505.978	683.269
7	18.342	1313.020	570.243
8	18.618	25.712	10.744
9	19.203	1581.039	689.542
10	20.026	1623.909	692.820
11	21.578	1517.440	637.749
12	23.122	1521.045	506.651

Figure: The chromatogram (from GC-FID) from the injection of a standard set of FAMEs at the same concentration (50 mg/L in terms of the FA) with “even” carbon numbers ranging from C10 to C24 (in order) and including the C19 IS and their integrated area counts.

Below are chromatograms for i) one of the samples and ii) an example of a ‘blank’ injection from the ‘procedure’. The area counts are shown with these.



#	Meas. Ret. Time	Area	Height
1	16.503	543.403	267.660
2	18.108	2175.178	789.138
3	18.147	1226.630	662.567
4	18.325	189.941	93.004
5	19.179	176.368	84.167
6	19.998	14.772	6.972
7	21.550	17.208	7.657
8	23.090	5.766	2.064



#	Meas. Ret. Time	Area	Height

Q: Using all the above information, calculate i) which <u>saturated</u> fatty acids are present in the sample and ii) how much of each is present in the original sample?