Gas Liquid Chromatography Analysis of the Fatty Acid Composition of Fats and Oils: A Total System for High Accuracy[1]

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The generally accepted approach to the analysis of fatty acid methyl esters (FAME) by gas liquid chromatography (GLC) is to analyze a standard mixture of known composition and to determine empirical correction factors for individual FAME. These correction factors, which are a composite of the theoretical flame ionization detector (FID) relative response factors and an empirical factor to correct for any system errors that may be present, then are used to correct the raw peak areas of the individual FAME of the sample undergoing analysis. It is proposed that this approach is fundamentally unsound as a means of generating consistently accurate results. Rather, it has been proven that theoretically calculated FID relative response factors are valid, both for the saturated and unsaturated FAME commonly encountered in edible oils, and that these should be used as the only response factors for the correction of raw peak areas. Thus, the proper approach to the generation of highly accurate results is to optimize both equipment and operator technique so that a correct answer is obtained for a primary standard when these theoretical factors are used, rather than to introduce an empirical correction factor other than the theoretical response factor to take account of faulty practice. Eight facets of equipment operation or operator technique have been identified which must be addressed to optimize accuracy.

For the optimum control of oil refinery operation, analytical methods used to monitor the various unit processes should be accurate, reliable and rapid and should deliver meaningful information. GLC of FAME is a technique extensively used to monitor oil composition during the manufacture of edible oil products and has the potential to satisfy all of the above criteria. However, it has been noted (1) that, although GC of FAME has been extensively used as an analytical technique since its introduction in 1952 by James and Martin (2), it is evident there is still "an unacceptable frequency of poor quantitative work and/or state of knowledge amongst analysts who work routinely in this field."

In previous papers (1,3–8), we discussed a number of facets of technique necessary to achieve high accuracy of analysis. However, to date, no paper has been published that deals with the totality of measures necessary to achieve high accuracy. It is the primary aim of this paper to fill this gap. In this regard, high accuracy is defined as a "grade of analysis" in excess of 99.0%.

High accuracy analysis is not just an academic nicety. It is becoming increasingly evident that it is a practical necessity for competent plant control, if the results are to be interpretable in a meaningful way.

DEFINITION OF HIGH ACCURACY

In all of our work aimed at improving accuracy, we have used primary standards of saturated FAME or of saturated triacylglycerols (TAG), and determined the "grade of analysis" as defined by the Smalley Gas Chromatography Check Program for Fatty Acid Analysis (9):

$$\text{Grade} = 100 - \Sigma \left| C_i - C_i^1 \right|$$

where C_i = % content of ester determined

C_i^1 = % content of ester known

MAXIMIZATION OF ACCURACY

We have identified eight facets that need to be addressed to maximize accuracy of analysis:

(a) Use computer or computing integrator.
(b) Use only the theoretical FID relative response factors to correct raw peaks areas.
(c) Use primary standard of saturated FAME to optimize chromatographic parameters.
(d) Use primary standard of saturated TAG to optimize overall procedure.
(e) Optimize ester preparation.
(f) Optimize FID linearity.
(g) Optimize injection technique.
(h) Use "grade of analysis" to identify and correct errors.

Computer or computing integrator. While there is general acceptance that any of the numerous modern computing integrators or computers gives a more accurate and precise assessment of the areas of chromatographic peaks than do any of the previously used techniques, there is a paucity of published objective proof. McNair and Bonelli (10) made a comparison of integration precision using planimeter, triangulation, peak height × half-width, cut and weigh, disc integrator and digital integrator, and showed progressive improvement in coefficient of variation from ±4.06% to ±0.44%. As the computer can more accurately detect beginning and end of peaks than can the digital integrator, and can more logically deal with any baseline variations, it can be predicted that it would be better than the digital integrator. Other evidence of the accuracy and precision of the computer is given in manufacturers' literature; while there is no reason to doubt

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the objectivity of the figures presented, comparison with other techniques is not given.

In spite of the difficulty of proving the case absolutely, it is our contention that the use of a computer or computing integrator is mandatory for high accuracy work. It is obviously advantageous to use a computer for subsequent rapid mathematical treatment of the analytical figures generated.

Theoretical FID relative response factors. Frequently, analysts use no individual response factors when analyzing FAME (i.e., response factor for all FAME = 1). An alternative approach, commonly adopted, is to analyze a primary standard of FAME of known composition and to determine an empirical correction factor for individual FAME to produce the known answer. Neither technique can be accepted as valid for high accuracy work. Ackman and Sipos (11) proposed that a theoretical relative response factor be calculated based on the fact that the FID responds to ions generated by the combustion of the C-H components of the molecule. The detector does not respond to the C=O component. If methyl stearate is given an arbitrary response factor of unity, FAME of shorter chain length will require a relative response factor progressively greater than unity to take account of the progressively lesser content of C-H in the molecule. While the thesis is logical, they did not give proof that could be considered adequate for high accuracy analysis.

It has been shown by Albertyn et al. (1) and by Bannon et al. (3,4) that these theoretical relative response factors are reliable for even-numbered saturated FAME through the range 8:0 to 18:0 inclusive, provided equipment and technique are standardized properly. In a subsequent paper (5), it was shown that the relative theoretical factors apply also to 4:0 and 6:0. In a later paper (6), a technique was developed to determine the relative response factors for a number of unsaturated FAME (18:1, 18:2, 18:3, 20:4 and 22:6); in each case, it was shown that the factor determined in practice was that predicted on theoretical grounds. In the course of work on unsaturated FAME, it was shown that the three further saturated FAME, 17:0, 20:0 and 22:0, also conform to theory.

As a consquence of these publications, it can be concluded that theoretical FID factors are a fundamental constant for all FAME commonly encountered in edible oil manufacture, and must not be modified to take account of other errors of technique. If, on analyzing a primary standard, it is found that a factor other than the theoretical is required to obtain the correct answer, it is an indication that the equipment is not properly optimized, or that some element of technique is faulty, or both. This may be represented as:

$$Fp = Ft \times Fe$$

where Fp = Empirical correction factor determined in practice
F_t = Theoretical FID response factor
F_e = Error factor.

The object of optimization is to ensure that the "error factor" becomes equal to unity. The extent that the "error factor" differs from unity may be taken as a measure of the failure of the analyst to optimize equipment and/or technique. The only acceptable way to resolve this problem is to locate and correct the fault or faults. When equipment and technique are optimized to conform to the theoretical relative response factor using primary standards, it can be stated that any sample of edible oil, when analyzed under the standardized conditions, will yield an accurate result.

No work has been carried out by us on epoxy, hydroxy or other funtionalized FAME, and thus we are unable to predict to what extent this concept might extend to such compounds. Scanlon and Willis (12), however, recently have reexamined the feasibility of calculating effective carbon numbers on a more general basis and have concluded that accurate responses can be predicted in many cases. Thus, it is likely that theoretical response factors can be used for a wider range of fatty acids than those that we have examined.

The most likely reasons for failure to conform to the relative theoretical response factors are faulty ester preparation, detector not operated at optimum linearity and faulty injection technique. These problems are discussed later.

Primary standards. While it is common practice to analyze a primary standard of FAME of known composition and to calculate empirical correction factors from the results obtained, it has been noted that this is considered to be incorrect practice. However, when using the system now described, both FAME and TAG standards are requisite to optimize equipment and technique.

It is inadequate to use only FAME as a standard, because the complete analysis of a sample comprises chemical conversion of TAG into FAME, extraction of FAME into an analyte solution and chromatographic analysis of the resultant solution of FAME. In theory, it might appear to be necessary to demonstrate independently that each of the three facets of the analytical procedure is optimized. In practice, it has been found adequate to divide the process into two components, namely, optimization of sample preparation and optimization of chromatograph performance. Two standards are required so that these two facets can be studied and optimized independently. By using a standard comprised of FAME, one can eliminate all errors that might be introduced as a consequence of faulty methylation or extraction technique. It thereby is possible to concentrate upon optimization of chromatographic technique, paying particular attention to detector linearity and injection technique. Once chromatographic performance is assured, the whole procedure can be optimized by employing a TAG standard. In addition, a TAG standard must be used whenever it is necessary to study and optimize methylation and extraction technique.

For analysis of the majority of oil types, FAME and TAG primary standards containing the even-numbered saturated fatty acids from 8:0 to 18:0 inclusive are recommended. As discussed later, the composition should simulate fully hydrogenated coconut oil. When it is necessary to analyze fats that contain fatty acids of very low molecular weight, additional FAME and TAG standards may be required (5), including methyl butyrate or tributyrin, respectively, the composition of each standard simulating the fat to be analyzed.

The technique for preliminary analysis of the components of standard mixtures and precautions necessary to ensure the accuracy of the calculation have been detailed by Albertyn et al. (1) and by Bannon et al. (5). Primary standards are comprised only of saturated FAME or TAG, respectively, because purity of the standard and nature of impurities in saturated components can be determined according to techniques already published (1,5). Unsaturated FAME and TAG are not used in any primary standard because it has not been found possible to determine their purity to the accuracy required for this work by direct techniques, or to maintain the composition of any standard that contains unsaturated ingredients subject to autoxidation. As it has been shown (6) that the relative response factors of unsaturated FAME determined in practice conform to those predicted by theory, there is no necessity to incorporate unsaturated lipids in any standard to be used for optimization of equipment or technique.

Optimization of ester preparation. Bannon et al. (3,4) investigated the stoichiometry of conversion of TAG into FAME and the efficiency of extraction of FAME into the analyte solution. They showed that two generally accepted official methods (AOCS and ISO) are not acceptable for high accuracy work when the sample contains low molecular weight FAME. Of these two, the AOCS method was found to be particularly poor and gave a grade of analysis for the coconut standard of only 94.21%±1.05%. The ISO method was found to be only marginally better, giving a grade of 95.65±0.55. The reason for the poor grade in each of these methods is that, because the recommended extraction procedure is very gentle, the low molecular weight FAME are not quantitatively extracted into the analyte phase. Thus the AOCS method failed to extract approximately 50% of 6:0, 25% of 8:0 and 5% of 10:0 FAME. In the case of the ISO method, extraction was slightly improved because of a slightly more vigorous extraction technique.

Two methods were developed (3,4) and shown to be superior to both the AOCS and ISO methods. By using a transesterification/boron trifluoride method to convert TAG into FAME, and by optimizing the extraction of FAME into the analyte solution (3), grade was increased to 99.10%±0.05%. Small losses of 6:0 and 8:0 were still evident, but were much less than those found when AOCS and ISO methods were used. When sodium methoxide was used for the methanolysis reaction and the polarity of the extractant increased by the addition of a small amount of diethyl ether (4), grade was increased further to 99.32%±0.13% and 8:0 was quantitatively extracted. Small losses of 6:0 were still evident and it thus is recommended that these methods not be used if it is important to quantitate accurately any FAME of chain length shorter than 8:0.

Although both of these methods are equally suitable for high accuracy work within the applicable range of chain lengths, the methoxide technique is preferred for edible oil factory control. It is quicker (two minutes instead of six), extends quantitative extraction down to 8:0 and reacts only with TAG. Free fatty acid is not methylated, so the result is theoretically better for factory control, free fatty acids being removed during the course of refining.

If it is necessary to analyze butterfat, the method of Bannon et al. (5) is recommended. This method has been shown to give quantitative recovery of FAME down to and including 4:0; it is an improvement over the method of Christopherson and Glass (13) in that it yields an analyte solution that is stable for a long time. The analyte solution produced by the Christopherson and Glass method is not stable with time as saponification proceeds as a secondary reaction after conversion of TAG into FAME. As methyl butyrate saponifies significantly faster than do longer chain esters, the composition of the analyte solution changes noticeably in as little as 15 minutes.

The improved method (5), however, is designed for a specific application and should not be considered as a general method for the preparation of FAME. Craske et al. (8) confirmed the more rapid saponification of low molecular weight FAME and also showed that the rate of conversion of TAG to the corresponding FAME decreased with increasing chain length. As a consequence, the recommendation was made that the Bannon et al. method (5) should be used only for its specific application and, when used, the procedure should be strictly followed and carefully standardized. By contrast, the accuracy and the wider applicability of the methoxide method (4) were confirmed.

Optimization of detector linearity. It has been shown by Albertyn et al. (1) that, when an FID is optimized for sensitivity (commonly carrier and hydrogen flow rates about equal), it is not necessarily at its optimum for linearity. For the particular detector examined, linearity was optimum at a considerably higher hydrogen:nitrogen ratio than that required for maximum sensitivity (ca. 1.5 instead of ca. 1.0). When operated in this manner, sensitivity was ca. 5–10% lower than optimum, but this is of no consequence when compared to the advantage of improved linearity and, hence, accuracy. A FAME standard simulating hydrogenated coconut oil methyl esters was used again in this work because the high concentration of early-eluting methyl laurate places a high demand upon the linear response of the detector. If linearity can be demonstrated for this standard, no linearity problem will be experienced in the analysis of any other sample encountered in practice.

The optimum for linearity quoted above applies only to the particular detector investigated and may fall at a different hydrogen:nitrogen ratio for another detector. The important point is that the optimum for linearity should be determined for each detector. It also may be noted that this work was carried out using packed columns; consequently, large sample sizes passed through the detector. When capillary columns and small samples are used, problems of optimizing the detector for linearity are much reduced.

A distinction must be made between detector linearity and amplifier linearity, as the latter also may give rise to errors in some instruments. The several amplifiers at our disposal never have shown evidence of significant problems in this respect, and we suspect that most modern amplifiers have excellent linearity. However, we would predict that any such problems could be detected using the coconut-type FAME standard if the

observed errors could not be corrected by adjusting the hydrogen to nitrogen ratio.

Column selection. In contrast to earlier times, column selection has become an almost insignificant factor in FAME analysis, the only requirements being that the column be inert and of adequate efficiency and polarity for the separations required. With state-of-the-art materials and technology, these requirements are readily satisfied by a large number of options. To this extent, we have never observed significant activity in glass or fused silica columns as far as any of the fatty acids encountered in commercial edible oils are concerned, but this cannot be said of metal columns. While highly polar phases such as polyesters and cyanosilicones remain mandatory for packed columns in most cases, fused silica open tubular (FSOT) columns permit the selection of a larger number of phases. Moderately polar phases such as bonded Carbowax thus may be used successfully, but highly polar phases such as DEGS, SP-2330 and Sil 88, all of which are readily available in FSOT columns, remain advantageous in minimizing the time of analysis as a consequence of the larger α-values for similar chain length saturated and unsaturated fatty acids. Notwithstanding the option of using packed or even megabore (530 μ I.D.) FSOT columns, we see no point in using anything other than FSOT columns of 0.2–0.35 mm I.D., which offer obvious benefits with respect to resolution and speed of analysis.

Optimization of sample introduction. Because of our strong preference for the use of FSOT columns in the split injection mode, we have confined our comments to the problem of sample introduction for this configuration. An excellent broader treatment for other configurations has been given by Jennings and Mehran (14).

There is wide acceptance that the most reliable method of introducing a sample to a capillary column to optimize quantitativity is cold injection of a solution directly into the column. However attractive on-column injection might appear in theory, it is not yet an option for factory control. As first introduced, the technique called for the dexterous use of delicate equipment that could not be recommended as a technique for factory technicians. Automatic injectors now appearing on the market may eliminate this objection, but a far more intractable problem still remaining is the fact that commercial samples contain small amounts of relatively non-volatile material, e.g., sterols, tocopherols, traces of unconverted TAG and polymeric materials, and regular injection of this type of material leads to rapid degradation of column performance. As a consequence, it is requisite to employ a technique in which the sample is vaporized in a pre-injection zone so that non-volatile impurities can be trapped and only the required volatile components are transferred to the column for analysis. In practice, this implies that a sample-splitting technique must be used. However, publications in which the problems of sample-splitting are discussed are legion. Bayer and Liu (15) listed nine phenomena that have been proposed to account for sample discrimination, namely:

(a) Selective evaporation of molecules of different sizes from the syringe needle (16–22).
(b) Changes in splitting ratio caused by pressure waves (18,23,24).
(c) Changes in splitting ratio caused by variations in gas viscosity and by condensation of solvent at the column inlet (16).
(d) Incomplete evaporation and limited speed of evaporation of the sample (18,25–28).
(e) Insufficient mixing of sample vapor with carrier gas (27–30).
(f) Different rates of diffusion of molecules of different sizes (23,28).
(g) Aerosol formation and droplet splitting (16,27).
(h) Adsorption on liner surface (18,21).
(i) Explosive evaporation and adsorption of less volatile components at cold parts of the carrier gas inlet system (17,31).

In spite of the fact that some authors consider that it is not possible to construct a non-discriminating split injector (16,24), there are others who have made positive contributions to the design of split injectors of improved quantitative performance.

Bayer and Liu (15) concluded that change in the viscosity of the gas phase and recondensation of the solvent in the column were the main causes of discrimination, and reported a split injection technique which gave negligible discrimination.

Jennings (26) designed a splitter in which injection, vaporization, mixing, expansion and splitting all occur in a glass insert. A novel feature of this injector is the "inversion cup" to promote mixing of the vaporized sample with the carrier gas. Vaporization and mixing were further improved by packing the vaporization area with quartz wool. An injector of this design has been adopted as standard in the Hewlett Packard Model 5880 used in our work.

Purcell (32) stressed the importance of precluding changes in gas viscosity at the split vent during the sample splitting process and also maintained that it was essential to have isokinetic velocity of vent and column streams, i.e., the linear velocity of gas-flow through the column and that of the vent stream should be equal at the point of splitting.

In our work (7) on sample introduction to splitting injectors, we found three factors to be of major importance in achieving highly accurate results: avoidance of needle discrimination when injecting, very rapid vaporization of the sample and rapid, complete homogenization of the sample with the carrier gas stream. High speed of injection was found to be a highly effective means of avoiding needle discrimination. Rapid vaporization of the sample was promoted by using high injector temperatures, relatively dilute solutions of analyte in the solvent and the smallest sample size commensurate with obtaining a chromatogram that could be accurately quantitated. Good mixing of the vaporized sample with the carrier gas was achieved with a number of injector insert designs; with improvement of design, it was found possible to achieve linear splitting over a wider range of operating conditions. Splitting of the vaporized sample under conditions at, or not too far from, those required for isokinetic sampling was found to be important for the less-efficient insert designs investigated, but decreased in

importance markedly as the insert design was improved.

The important points to be recognized are these:

(a) While sample introduction to splitting systems is a common source of error, the highly accurate quantitative analysis of FAME on a basis which uses only the theoretical FID relative response factors to correct peak areas can be carried out by split injection capillary GLC, providing a number of well-defined parameters are addressed.

(b) It is probable that the various phenomena that can contribute to error vary in significance from instrument to instrument. Hence, optimum conditions may vary from system to system.

(c) It is possible to overcome the problems and it is mandatory to do so to obtain consistently reliable quantitative results.

The alternative of altering the response factor to cater for malpractice is not acceptable.

Use of grade of analysis. While it is evident that the grade of analysis is a simple measure of the overall excellence of analytical technique, an examination of the size and the sign of the errors of the individual FAME is an excellent indicator of why any particular analysis is inaccurate and may be used to guide the analyst in the selection of operating parameters that will improve the accuracy of analysis. In our work developing improved methods for preparing FAME (3,4), the phenomenon of increasing negative error with decreasing chain length was interpreted as inefficiency of extraction of low molecular weight FAME. With optimization of extraction technique, 10:0 FAME was extracted quantitatively; by addition of diethyl ether to the solvent, 8:0 was also extracted quantitively. In our work on fats that contain low molecular weight fatty acids (5), the same approach was used to monitor (a) the efficiency of extraction of the esters, (b) the rapid saponification of methyl butyrate and (c) the relatively slow conversion of tristearin to methyl stearate, to devise an optimum method of preparation of esters of this type and to optimize injection technique. In our later investigation of differential rates of transmethylation and subsequent saponification (8), the magnitude, trend and sign of the error of individual FAME were again the techniques used to elucidate the phenomena. Clearly, a detailed examination of the most significant individual errors that give rise to a loss of grade is a most powerful aid to the improvement of accuracy.

CONCLUDING REMARKS

By application of the measures described, it is possible consistently to achieve grades in excess of 99% and, with very careful work, grades in excess of 99.5%. It cannot be emphasized too strongly that accuracy of analysis is not just an academic nicety, but a practical necessity. It is becoming increasingly evident that while much valuable work can be accomplished when analysis grades within the range 99-99.5% are achieved, it is beneficial to achieve a grade in excess of 99.5% to facilitate reliable analytical control of plant operation under all circumstances.

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