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Analysis of genetically modified oils

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Abstract

Genetically modified oils with altered functional or nutritional characteristics are being introduced into the marketplace. A wide array of analytical techniques has been utilized to facilitate developing these oils. This article attempts to review the utilization of these analytical procedures for characterizing both the chemistry and some functionality of these oils. Although techniques to assess oxidative stability in frying and food applications are covered, measurement of nutritional characteristics are not.

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Nomenclature

ACP	Acyl carrier protein
APCI	Atmospheric pressure chemical ionization
cDNA	Complementary deoxynucleic acid
CoA	Coenzyme A
DAG	Diacylglycerol
DGD	Digalactodiacylglycerol
DNPU	Dinitrophenylurethane
ELSD	Evaporative light scattering detection
FID	Flame ionization detection
GC	Gas chromatography
HO	High oleic
HPLC	High performance liquid chromatography
IP	Identity preserved
LIMS	Laboratory information management system
LP	Low palmitic
MAG	Monoacylglycerol
MS	Mass spectrometry
NIR	Near infrared reflectance spectroscopy
NMR	Nuclear magnetic resonance spectroscopy
PC	Phosphotidylcholine
PCR	Polymerase chain reaction
PLS	Palmitoyl-linoleoyl-stearoyl-glycerol
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphisms
TAG	Triacylglycerol
TBA	Thiobarbituric acid
TLC	Thin layer chromatography

1. Genetically modified oils

Genetically modified oils represent some of the most significant new products developed for the oils and fats industry in the past decade. Many of these novel oils possess fatty acid contents that are unique to the crop in which they have been developed. As such, these oils convey improved or different functional and/or nutritional characteristics compared to commodity types.

Many genetically altered oil types are available commercially or are promised to be soon (Table 1). Genetically modified oils fall into two main categories. The primary value of one group is enhanced oxidative stability. These oils are targeted mainly to salad dressing, frying, and spray (food coating) applications and are best represented by the high oleate and low linolenate types. The second general group of genetically modified oils are characterized by altered levels of saturated fatty acids. Elevated levels of palmitate and stearate provide a basestock from a temperate oilseed source for confectionery and margarine applications without hydrogenation. Evidence is accumulating on the nutritional benefit of such a nonhydrogenated product that is essentially free

Table 1
Some genetically modified oils available or in development (in part [180,181])

Oil type	Crops	Developers
High oleate	Sunflower	Dow, DuPont, Instituto de la Grasa
	Canola	Cargill, DuPont
	Soybean	DuPont, Monsanto
	Corn	DuPont
	Peanut	Mycogen, Univ. FL
Low linolenate	Canola	Cargill, DuPont
	Soybean	DuPont
	Linseed	CSIRO
Low saturate	Canola	Cargill, DuPont
	Soybean	DuPont, NC St. Univ.
High palmitate and/or stearate	Sunflower	Instituto de la Grasa
	Canola	Cargill, Monsanto
	Soybean	Monsanto
High laurate	Canola	Monsanto
High medium-chain	Canola	Monsanto
High erucate	Rapeseed	Various

of *trans* fatty acids [1], although the risks associated with consumption at typical levels of dietary intake are not defined completely [2]. Depressed levels of saturated fatty acids, particularly palmitate, can also provide nutritional (and thus marketing) benefits [3], especially if a ''low saturated'' or ''zero saturated fat'' claim can be made on the label. Further on the horizon are genetically modified oils that possess novel (for crude vegetable oils) fatty acyl substitutions, such as hydroxyl or epoxy groups, as well as alkyne or conjugated double bonds. These types represent potential alternatives to industrial oils derived currently from undomesticated or inefficiently domesticated species. Availability of these oils is further in the future mainly due to the very technically demanding requirement of obtaining expression of the desired traits at economically relevant levels.

Genetically modified oils have been developed using either mutation/selection breeding or the tools of biotechnology. In some cases, both techniques have been used to stack traits or enhance trait expression. Regardless of the techniques used to develop any of these new products, commercial success ultimately depends upon market forces. The benefits derived from a given modification must command a price high enough to at least cover the costs of development and, if required, identity preservation and regulatory approval. A comprehensive cost analysis needs to include outlays associated with providing analytical support during development and commercialization of the product.

2. Analysis for gene discovery and gene recombination

2.1. Oil content

Oil is a valuable component of a fully mature seed due to its relatively high caloric value compared to other seed storage constituents. Thus, the economical value of an oilseed is often related directly to oil content. High oil content is a common goal of breeding programs in several

crops, and a minimum oil level must be maintained in order to sell the seed. Since a thorough discussion of the analytical techniques utilized to measure seed oil content is beyond the scope of this review, the topic will be covered only briefly here.

Perhaps the most notable example of elevated oil content is in corn, where decades of selection for high oil types at the University of Illinois has resulted in lines with 21% oil compared to 4–5% in commodity types [4]. High oil corn is currently one of the few specialty genetically modified oil crops planted to significant acres in the United States. Analysis of oil content in seeds has been accomplished traditionally by Soxhlet extraction followed by gravimetric weighing. Supercritical fluid extraction represents an improvement for the former, especially with newer equipment that can process samples serially. Near infrared and pulsed nuclear magnetic resonance spectroscopy have proven essential as secondary methods to support breeding efforts and to evaluate oil quantity at the point of sale (see below).

2.2. Fatty acid composition

Seed oil quality is determined primarily by the fatty acid composition of storage triacylglycerols (TAGs). The number of double bonds, or degree of unsaturation, in the fatty acyl moieties is the prime determinant of melting point, oxidative stability, crystallization properties, and nutritional characteristics. Capillary gas chromatography with flame ionization detection (GC/FID) of fatty acid methyl esters has been the technique of choice for the last 20 years or so for determining the fatty acid composition of seed oils. Peak assignment is almost always based on matching retention times with those of authentic standards, an acceptable protocol when working with altered levels of endogenous fatty acids. Occasionally, additional effort is required to document double bond position using GC combined with mass spectrometry (MS) detection, or double bond orientation using GC combined with infrared detection or argentation thin layer chromatography (TLC). For example, argentation TLC was used to unambiguously identify *cis*-oleate in a high oleic sunflower mutant [5]. Hyphenated chromatographic approaches are also usually required to verify expression of unusual fatty acids (see below).

Success of plant breeding programs to develop modified seed storage oils is dependent upon the ability to rapidly and accurately phenotype large numbers of progeny. Classical mutation/selection plant breeding is a numbers game. The more gene combinations evaluated, the greater chance of identifying desired phenotypes. Even insertion or knockout by biotechnology of genes regulating seed storage lipid composition requires substantial screening to ensure optimal expression, stability across diverse growing environments, and acceptable agronomic performance of derived varieties.

One approach to evaluating the fatty acid phenotype of a large number of samples involves extensive use of process automation integrated closely with streamlined sample tracking and information management [6,7]. The flow of samples and information is outlined in Fig. 1. Personnel at remote locations request analyses that will be performed by a centralized laboratory. Field and laboratory databases are linked dynamically to facilitate association between each sample identification number and its corresponding field plot/row identity information. Samples are submitted to the laboratory in custom 25-well plastic trays that contain barcoded labels that display sample identification. Seeds are crushed, and expelled oil samples are prepared using robotic automation to achieve a very high sample throughput. Fatty acid composition is determined by optimized capillary GC. Custom software facilitates data analysis, which entails

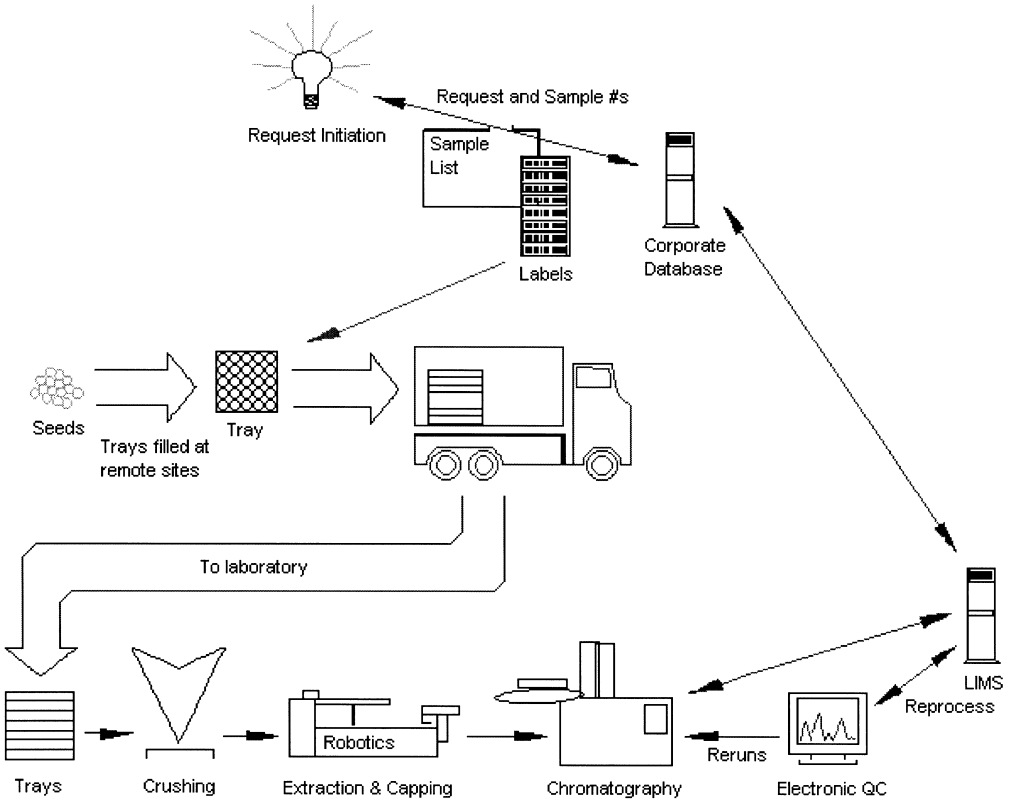

Fig. 1. One approach to high throughput analysis of fatty acid composition in seeds. LIMS, Laboratory Information Management System.

reprocessing acquired chromatograms and rerunning samples. Specialized software, including one program that uses neural net pattern recognition to evaluate the integrity of chromatograms, monitors data quality. Final reports are accessed electronically by the remote laboratory clients.

Other strategies have been applied to rapidly extract and transesterify fatty acids from seeds. Simpler logistics result if both processes are accomplished simultaneously on seed tissue in situ. Direct transesterification in situ works well only if the seed coat is disturbed, such as on crushed seeds or excised seed sections (see below). For example, rapid direct methanolysis of oil from crushed canola seed can be accomplished under acid or alkaline conditions with microwave heating [8]. A review of procedures used to produce fatty acid methyl esters has been published fairly recently [9].

Varietal development can be accelerated early in the process by assessing the phenotypic variability of fatty acid composition in individual seeds. This strategy requires nondestructive analysis of the trait in individual seeds. In dicot species with sizeable seeds, such as soybean or sunflower, a portion of the seed not containing the embryonic axis can be excised and subjected to analysis. Transesterification can be accomplished directly on the sections [10], or on homogenates [11] or lipid extracts [12] of the sections. Alternatively, oil can be extracted from seed cores [13]. The remaining portions of the seeds with preferred phenotypes are germinated, usually

in the presence of a contact fungicide. Of course, the fatty acid composition of the tissue sampled must be representative of that of the whole seed. This requirement is met when sampling soybean or sunflower cotyledons [14,15]. Technical difficulties arise when working with monocots such as corn that store oil in the embryo [16], and with crops such as canola that produce very small seeds (see below).

Alterations in TAG fatty acid compositions may be expressed to some degree in other lipid fractions within the seed. This is not surprising given the interrelated biosynthesis of TAGs and membrane lipids [17]. For example, the percentage of palmitic acid in phosphatidylcholine and digalactosyldiacylglycerol, two abundant polar lipids, was significantly lower in developing soybean embryos of a low palmitic mutant compared to a normal type (Table 2). Other soybean TAG fatty acid mutations were observed at the level of total phospholipids (purified by column chromatography), phospholipid class (purified by preparative TLC), and phospholipid stereospecificity (reacted with snake venom phospholipase followed by TLC separation of reaction products [18]. In a different low linolenate mutant, the amount of the predominant linolenate-containing phosphatidylcholine species (16:0/18:3 and 18:3/18:3) extracted from cotyledons and analyzed by high performance liquid chromatography with flame ionization detection (HPLC/FID) was less than in a wild-type control [19]. In a soybean mutant that exhibited elevated oleic acid levels, both the phosphatidylcholine and phosphatidylethanolamine fractions in developing cotyledons had higher oleate contents [20]. Relationships between TAG and phospholipid molecular species compositions (determined by HPLC with evaporative light scattering detection [HPLC/ELSD]) can also be expunged from other soybean [21,22] and canola [23] data.

Alterations in TAG fatty acid compositions are also expressed in the diacylglycerol and total phospholipid fractions purified by TLC from high oleic sunflower seeds [5]. Similar results were found in the phosphatidylinositol, phosphatidylcholine, and phosphatidylethanolamine (all isolated

Table 2
Mean fatty acid composition (mol%) in phosphatidylcholine (PC) and Digalactosyldiacylglycerol (DGD) lipid fractions from embryos of commodity-type (Check) and low palmitic (LP) soybeans at different stages of development[a]

	Stage I		Stage II		Stage III		Stage IV	
	Check	LP	Check	LP	Check	LP	Check	LP
PC								
16:0	12.6	9.9	12.6	9.8	17.6	11.6	17.2	7.7
18:0	11.5	11.2	10.1	10.0	8.2	8.0	6.9	5.9
18:1	15.1	15.2	15.5	13.9	13.0	14.9	12.3	30.0
18:2	30.3	34.3	30.7	36.3	43.2	44.2	53.6	47.5
18:3	30.5	29.4	30.4	30.0	18.0	21.3	9.9	8.9
DGD								
16:0	9.8	7.0	9.0	6.6	10.9	6.7	13.7	5.1
18:0	13.0	10.2	10.1	8.7	5.8	5.0	6.0	4.6
18:1	5.8	4.5	6.3	4.6	5.5	6.6	7.1	7.7
18:2	5.5	7.1	5.1	5.9	18.3	18.1	32.3	23.9
18:3	65.9	71.3	66.1	74.2	59.5	63.6	40.8	58.7

[a] Data obtained in collaboration with E. Lark and J. Browse, Washington State University, Pullman, WA, USA.

by TLC) in three high stearic and one high palmitic sunflower mutants [24]. During germination, the high saturate character of the cotyledons of these mutants reverted to that of a normal fatty acid line except in the CAS-3 mutant, a very high (>25%) stearic acid type [25]. It is unclear if elevated stearate in the cotyledons of these seeds is adverse to germination, as in transgenic high stearic canola [26]. "Spillover" of TAG fatty acid alterations have also been observed in the monoacylglycerol, diacylglycerol, and phosphatidylcholine fractions also isolated by TLC in another high oleic sunflower mutant [27]. In flax, the low linolenic acid character was found in neutral and polar seeds lipids isolated by a combination of TLC and column chromatography [28]. A somewhat different situation was observed to occur in transgenic canola that accumulates seed TAGs with high amounts of laurate, a fatty acid that usually is not found in rapeseed. In this case, a significant amount of lauric acid also accumulated in the diacylglycerols, but little was found in phosphatidycholine of mature seed [29]. Experimental evidence suggested that exclusion of laurate from membrane lipids was due to induction of lauroyl–phosphatidylcholine catabolism, an energetically wasteful process.

One of the limitations to tracking gene expression by oil fatty acid analysis is the time required for the plants to produce seeds. In fast-tracked breeding programs, there is limited time to conduct GC analysis and germplasm selection between harvesting one generation and sowing the next. Although analytical systems, like that described in Fig. 1, assist greatly in reducing data turnaround time to a minimum, the ability to predict seed oil fatty acid composition from that of other tissues would have obvious value. Balanced against the time savings would be working with matrices with lower levels of lipids that are more difficult to extract and transesterify. Harvested tissue must be handled with care since the membrane-associated lipids in these samples are prone to oxidation. Attention must also be paid to the well-documented effect of temperature on fatty acid desaturation [30–32]. Elevated oleic or depressed linolenic acids in leaf lipids could be due to elevated growing temperatures rather than spillover of a fatty acid synthesis mutation. Furthermore, the altered trait would have to be expressed at appreciable levels in the nonseed tissues, which is usually the case only for some mutants, but not most transgenics. In almost all cases, transgenes are inserted behind seed-specific promoters to minimize potential deleterious effects on plant agronomic characteristics.

In spite of these technical limitations, attempts have been made to use the fatty acid composition of lipids extracted from soybean leaves to predict, at least at a gross level, seed oil quality in both low and high palmitate mutants [33]. However, the same was not the case for a high stearate and four low linolenate soybean mutants [18,34], two low linolenate flax mutants [28], or a number of high palmitate, stearate, and oleate sunflower mutants [5,27,35]. In canola lines with very high (i.e. over 80 mole percent) oleate, the oleic acid contents in various phospholipids and galactolipids are also elevated dramatically. For example, the percentage of oleic acid in leaf phosphatidylcholine and digalactosyldiacylglycerol, two abundant lipids in green tissues, was significantly higher in the very high oleic mutant compared to a normal type (Table 3). Similar spillover of the high oleate phenotype has been shown in other very high oleic canola mutants [36,37]. These "ultra" high oleic mutants suffer serious agronomic penalties, presumably due to inhibited expression of a constitutive oleoyl desaturase. In contrast, ultra-high oleic sunflower lines examined in the same study did not exhibit elevated oleate in the same leaf lipid fractions (one shown in Table 4). Restriction of the mutant phenotype to the neutral lipid fraction is probably related to the satisfactory agronomic performance of these sunflower lines.

Table 3
Mean fatty acid composition (mol%) in phosphatidylcholine (PC) and Digalactosyldiacylglycerol (DGD) lipid fractions from leaves of commodity-type (Check) and high oleic (HO) canola[a]

	24-day-old leaves		39-day-old leaves	
	Check	HO	Check	HO
PC				
16:0	19.4	10.9	18.5	13.6
16:3	0.2	0.0	0.3	0.3
18:0	3.9	1.2	5.3	2.5
18:1	10.9	65.4	11.7	48.5
18:2	27.2	2.7	21.9	5.4
18:3	38.4	19.7	42.4	29.7
DGD				
16:0	13.7	11.6	13.3	13.4
16:3	4.2	4.6	3.3	3.2
18:0	2.0	1.5	2.7	1.9
18:1	1.6	5.3	1.3	5.2
18:2	5.0	7.2	7.3	8.4
18:3	73.4	69.7	72.2	67.8

[a] Data obtained in collaboration with E. Lark and J. Browse, Washington State University, Pullman, WA, USA.

Table 4
Mean fatty acid composition (mol%) in phosphatidylcholine (PC) and digalactosyldiacylglycerol (DGD) lipid fractions from leaves of commodity-type (Check) and high oleic (HO) sunflower[a]

	14-day old leaves		65-day old leaves	
	Check	HO	Check	HO
PC				
16:0	23.7	25.2	30.3	29.3
18:0	3.1	4.6	6.7	5.1
18:1	12.7	10.2	8.3	6.6
18:2	34.9	31.9	16.2	15.4
18:3	25.7	28.2	38.5	43.7
DGD				
16:0	13.1	10.7	13.8	13.6
18:0	2.6	2.6	5.1	3.6
18:1	2.0	1.9	1.7	2.1
18:2	3.8	2.9	2.2	2.9
18:3	78.5	81.9	77.2	77.8

[a] Data obtained in collaboration with E. Lark and J. Browse, Washington State University, Pullman, WA, USA.

Microspore-derived haploid embryos of oilseed rape accumulate TAG in a manner similar to developing seeds [38], and thus they are used to rapidly introduce homozygous mutations. Since haploid cells in culture are readily mutagenized, and microspore-derived embryos are regenerated to double-haploid plants, the technology is utilized to fix recessive traits quickly. Lipids from entire embryos or dissected cotyledons are analyzed [39,40]. Although microspore culturing has been a valuable tool in developing quality traits in canola, screening embryos in culture in lieu of waiting for mature seed has not proven to be very useful in breeding for altered fatty acid profiles [41]. Early phenotype screening in canola could also take advantage of the fact that the linolenate content of pollen storage oil emulates that of seed storage oil [42]. However, this sampling technique is also not used widely if at all. A technique that is used widely in canola is the removal and analysis of one zygotic cotyledon shortly after germination [43]. Seedlings with desired fatty acid profiles are allowed to develop further. However, this procedure is essentially non-destructive sampling of individual seeds, similar to working with soybean or sunflower chips, rather than sampling tissue earlier in a plant's life history to predict seed oil quality.

As already mentioned, since expression of transgenes is almost always restricted to seeds, sampling vegetative tissue of these plants would be useless. However, mature somatic embryos formed from callus during plant regeneration represent early models of the zygotic embryos. Thus, the level of expression of transgenes in mature seeds can be foreseen at this early stage [44], saving time and valuable greenhouse space.

2.3. Triacylglycerol characterization

Reversed-phase HPLC is the current method of choice for determining TAG composition since the technique affords separation of TAG species according to both the number of carbon atoms and the number of double bonds. TAG profiles can also be obtained with high temperature GC, although it is more difficult to achieve separation of TAGs with the same number of carbons but different degrees of unsaturation. Since standards for many TAGs, especially those found in genetically modified oils, are not available commercially, peak identification is best accomplished by MS. Due to the nonpolar nature of TAGs and the mobile phases used to elute them from the HPLC columns, atmospheric pressure chemical ionization (APCI) is the required interface. Lacking HPLC/MS capability, the identity of many TAG peaks can be deduced by knowing the effects of chain length and degree of unsaturation on elution from the column. Some TAGs can also be identified by comparing chromatograms of modified oils and those with normal profiles, since peaks in the former that contain fatty acids with altered levels will similarly be altered. However, quantitation is most often achieved with refractive index, flame ionization, or ELSD. Although reliable data can be achieved from all three detectors, each has drawbacks. Reliance on refractive index detection restricts sensitivity, and gradient elution, required for optimum peak resolution, cannot be performed due to detector instability. Flame ionization detectors are no longer commercially available. ELSD usually requires the use of response factors calculated from the percent of each fatty acid as determined by GC/FID (as does APCI/MS). However, this type of detector calibration is apparently not required for at least one commercial ELSD instrument [45].

As useful as reversed-phase HPLC is for TAG profiling, fatty acid position on the glycerol backbone is not distinguishable without mass spectrometric detection, and then only then with significant limitations.

Altered TAG compositions have been documented in genetically modified sunflower and soybean oils obtained from mutation and selection breeding [46]. TAG profiles were obtained using HPLC/ELSD. Sunflower traits included high oleic and high linoleic, as well as high palmitic or high stearic in a high oleic or linoleic background. Soybean mutants included high palmitic, low palmitic, high stearic, and low linolenic either individually or in combination with others. Alterations in fatty acid composition of the various oils were reflected in their TAG profiles. High saturate hybrids produced seed oils with elevated levels of TAGs containing palmitic, stearic, arachidic, and/or behenic acids. Oils derived from low saturate varieties contained less saturated fatty acid-enriched TAGs. Low saturate oil produced by a high oleic sunflower hybrid was comprised mainly of TAGs with two or more oleic acid moieties. Low linolenic soybean oils were deficient in linolenate-containing TAGs. The distribution of TAGs in oils with two modified fatty acids traits reflected the combined influence of the individual mutations.

As stated above, APCI/MS is a very valuable tool for on-line identification of TAGs eluting from an HPLC analysis. The author's laboratory is among those that rely on this technique for TAG identification of genetically modified oils. The molecular weight of an unknown is determined readily, and data-dependent MS/MS is used to fragment the TAG and measure the molecular masses of the resulting diacylglycerol (DAG) ions (Fig. 2). In the example shown, HPLC/MS analysis of a relatively minor peak gave an $[M+1]^+$ ion at m/z 860. This suggests that the TAG in question could be PLS (16:0-18:2-18:0) with a molecular weight of 859. Tandem mass spectra generated from the fragmentation of the parent ion m/z 860 revealed the three possible DAGs formed, each identified by its molecular weight. It is important to note that the analysis as performed did not elucidate the position of each fatty acid in the TAG molecule. Regardless, the fatty acid composition of an unknown peak could be determined even at very low abundance and in the presence of coeluting peaks, as long as their molecular masses are different. Using this technique, we have been able to identify minor TAGs in some oils that we could not previously. Many of these TAGs elute at the end of reversed-phase HPLC chromatograms and contain very long chain fatty acids. These peaks have sometimes been misidentified in the literature as trisaturated TAGs.

As expected, altered TAG profiles have been found in other genetically modified oils that result from mutations. High saturate sunflower oils have been found by GC to contain higher amounts of saturated fatty acids in some TAG species [47]. TAG profiles of high palmitic, high stearic, and "normal" soybean oils were determined by reversed-phase HPLC [48]. On-line APCI/MS was used to unambiguously determine acyl composition of most of the peaks. As in Fig. 2, identification of individual TAGs was based on the molecular $[M+1]^+$ ion and the DAG fragments. FID was used for quantitation. As found by Reske and coworkers [46], comparison of the TAG profiles of these three oils revealed that the TAGs present were very much a reflection of the fatty acid synthesized. Thus, almost every TAG that contained a palmitic acid moiety was more abundant in the high palmitic oil compared to the other oils. An analogous situation was the case for stearic acid in the high stearic oil. Apparently, both mutations do not affect the acyl selectivity of the acyltransferases. This conclusion is supported by the results of stereospecific analyses of very similar oils conducted by other laboratories (see below), but is testable directly only by metabolic assays.

Using HPLC/ELSD, TAGs were characterized in a low linolenic canola oil [49]. In this oil, every TAG that contained at least one linolenic acid was less abundant compared to two varieties with commodity-type fatty acid compositions. The same was generally true when TAGs characterized by

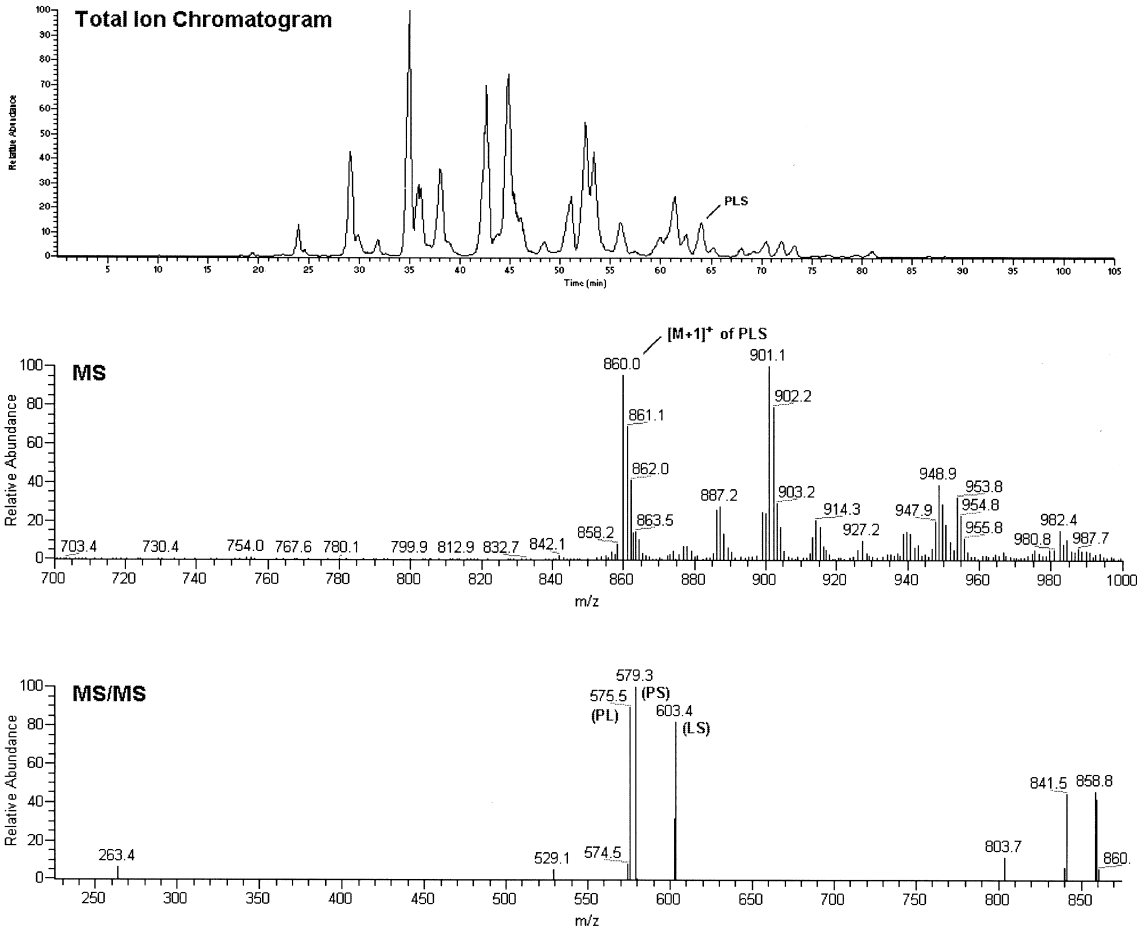

Fig 2. Use of HPLC/APCI/MS/MS for the characterization of 16:0-18:2-18:0 (PLS) in a high saturate soybean oil. PLS, triacylglycerol with palmitate, linoleate and stearate as the esterified fatty acids.

HPLC/FID from a number of other low linolenic canola oils were compared with those from commodity types [50]. In the latter study, the high oleate character of two high oleate oils was expressed almost exclusively as elevated triolein. TAG profiles were also obtained in canola oils that contain elevated laurate or stearate levels resulting from insertion of a medium-chain thioesterase gene or antisense knockout of stearoyl-ACP desaturase gene, respectively [51]. In this case, HPLC/APCI/MS was used to identify TAG peaks that were quantified by HPLC/FID. Once again, TAG composition present in these canola oils reflected their fatty acid compositions. Again, no selectivity at the level of the acyltransferases was evident.

The introduction of a totally unique TAG in a genetically modified oil provides a very sensitive screening tool for expression of the transgene involved. For example, seed oil from transgenic rapeseed containing the lysophosphatidic acid acyltransferase gene from *Limnanthes douglasii* L. contains trierucoylglycerol due to the insertion of 22:1 at the *sn*-2 position [52]. Since erucic acid is essentially excluded from the *sn*-2 position in conventional high erucic acid rapeseed [53], the

presence of trierucin is tightly coupled to expression of the transgene. Thus, screening for trierucin by high temperature GC is a very sensitive and selective analytical method for this particular transgenic trait [54]. This analytical approach would also eliminate the need for performing complicated positional analysis as described below. A diminutive oil sample from a single cotyledon of a microspore-derived embryo or germinating seed can be used for both TAG and fatty acid analyses, an advantage when dealing with heterogeneous transgene expression and copy number.

Knowing the fatty acid composition at the *sn*-2 position of a genetically modified oil is occasionally desired. Most commonly, oils or purified TAG fractions are treated with an *sn*-1,3-specific lipase, and the resultant *sn*-2 monoacylglycerols (MAGs) are isolated by either TLC or solid phase extraction and then subjected to fatty acid analysis. As a case in point, the high proportion of linoleic acid and the effective exclusion of saturated fatty acids from the *sn*-2 position in both high saturate and commodity-type soybean oils suggest that the same biosynthetic pathway operates in both oil types [46,55]. The same can be said for high saturate sunflower oils [56,57].

Certain functional and nutritional attributes of an oil are governed, in part, by the acyl distribution in the TAGs. Furthermore, screening germplasm for an enhanced ability to incorporate particular fatty acids at the *sn*-2 position is a powerful selection tool for developing oils with increased levels of these fatty acids. For example, development of high erucic rapeseed oils has been restricted by the exclusion of 22:1 from the *sn*-2 position [53]. In an attempt to get around this biochemical limitation, seed oils produced by a collection of high erucic *Brassica napus* L. and *Brassica oleracea* L. lines were subjected to lipolysis using an *sn*-1,3-specific lipase [58]. As expected, erucic acid was essentially not detected at the *sn*-2 position in all oils from the *B. napus* germplasm. However, three lines of *B. oleracea* were found to produce oils with 23% erucate at the *sn*-2 position. Full stereospecific analysis was carried out on two of the three *B. oleracea* oils by chiral HPLC separation of monoacylglycerol di-dinitrophenylurethane (DNPU) derivatives following Grignard-based lipolysis [59]. The analyses revealed only a slight preference for 22:1 at the *sn*-1 or *sn*-3 versus. *sn*-2 positions. No trierucin was found by direct-probe MS. Regardless, it is clear that *B. oleracea* could be used as a source of a gene encoding a 1-acyl-*sn*-glycerol-3-phosphate (lysophosphatidyl) acyltransferase with affinity for erucoyl-CoA. Introduction of this gene using biotechnology or interspecific hybridization could lead to a rapeseed oil with erucic acid levels greater than 66 mole percent.

Measuring the fatty acid content at the *sn*-2 position is particularly relevant when a specific attempt is made to alter the acyl specificity of the lysophosphatidyl acyltransferases. Such a molecular biological approach is required to increase the level of saturated or very long-chain fatty acids that are, for the most part, normally excluded from the *sn*-2 position of TAG [60]. In the economically important example given above, the proportion of erucoyl acid is limited to approximately 66% of the total fatty acids in rapeseed oil, presumably due to the selectivity of its lysophosphatidyl acyltransferase against erucoyl-CoA [61]. To address this, a cDNA encoding a less selective analog of the enzyme from *Limnanthes alba alba* was inserted into *B. napus* [62]. Oil samples were subjected to lipolysis using a lipase from *Rhizopus arrhizus*, and the resulting *sn*-2 MAGs were purified by TLC, transmethylated, and analyzed by GC. In oil from the best expressing transgenic plant, 22% erucate was measured at the *sn*-2 position. Argentation HPLC/ELSD revealed the presence of trierucin (trienoic C_{69}) only in the transgenic plants. The trienoic C_{67} TAG fraction (TAGs that contain 22 carbon atoms and three double bonds) was also more

abundant in transgenic plants due to increased levels of 22:1/22:1/20:1 and 20:1/22:1/22:1. However, total erucate content did not increase. A cDNA encoding a lysophosphatidyl acyltransferase from *Limnathes douglasii* was also introduced into *B. napus* [63]. Oil from the best expressing plant had 28% erucate at the *sn*-2 position, and up to 3% trierucin was confirmed by reversed-phase HPLC. Again, total erucate content did not increase, suggesting that regulation of biosynthesis is not straightforward. Elevated amounts of very long-chain fatty acids, including 20:1 and 22:1, at the *sn*-2 position was also achieved in seed oil of *B. napus* expressing a *sn*-2 acyltransferase gene from yeast [64]. High temperature GC revealed increases in C_{62} to C_{66} TAGs (TAGs that contain at least one very long-chain fatty acid, based of the number of carbon atoms) in the transgenic lines. Positional analysis using pancreatic lipolysis showed small amounts (up to 4.1%) of 22:1 at the *sn*-2 position in the transgenic-derived oils compared to negligible amounts in the non-transformed control. Alterations in fatty acid contents were not observed in the leaves, even though a constitutive promoter was used. In contrast to the two cases where a plant gene was introduced, the proportion of very long-chain fatty acids, such as erucic acid, increased in seed oils from the transgenic plants. Total oil content was also increased. These results suggest that the enhanced flow of acyl groups, including those longer than C_{18}, through the Kennedy pathway was the result of the normally tight regulation of oil biosynthesis being circumvented by the expression of the yeast gene.

The specificity of the lysophosphatidate acyltransferases has also been altered in high laurate canola via expression of a lysophosphatidyl acyltransferase gene from coconut. Resulting transgenic plants produced oils with elevated lauric acid contents at the *sn*-2 position [65]. As in the case with erucate, TAGs were treated with a *R. arrhizus* lipase, but in this case the resulting *sn*-2 MAGs were purified by normal-phase HPLC/ELSD instead of TLC. Collected MAGs were transmethylated, and their fatty acid compositions were determined by GC as before. Argentation HPLC/ELSD was used to quantify trilaurin in the engineered oils. The enrichment of up to 30 mole percent lauric acid at the *sn*-2 position was achieved when the coconut lysophosphatidate acyltransferase gene was stacked with the California bay lauroyl-acyl-carrier protein thioesterase gene. This resulted in the synthesis of more trilaurin (up to 40%) and more total lauric acid (up to 60%).

Reliable methods to perform stereospecific analysis were available by the time genetically modified oils were beginning to be developed. Three methods have been used for this very demanding analysis. All three require chemical or enzymatic hydrolysis of TAGs. One procedure relies on the stereospecific phosphorylation of *sn*-1,2-DAGs to phosphatidic acid, isolation by TLC, and measurement of fatty acid composition by GC [66]. The other two methods utilize either chiral HPLC separation of *sn*-1,2(2,3)-DAGs or MAGs as DNPU derivatives [59] or normal-phase HPLC separation of diasteromeric *sn*-1,2(2,3)-DAGs as (S)-(+)-1-(1-naphthyl)ethyl urethane derivatives [67]. Even with reliable methods available, full stereospecific analysis has been performed on relatively few genetically modified oils. Collectively, the data in the literature to date generally support the hypothesis that the native acyltranferases present in developing oilseeds accommodate the altered levels of acyl groups produced by the introduction or knockout of genes in the fatty acid biosynthetic pathway. Among the TAGs synthesized, the substrate specificity of each acyltransferase in the Kennedy pathway determines fatty acid placement on the glycerol molecule. However, this is apparently not a limitation to overall fatty acid esterification, except in cases such as high erucic acid rapeseed in which the level of 22:1 is capped at approximately 66%

of the fatty acids in the oil [53]. As already described in depth, introduction of a novel acyltransferase with altered substrate specificity is required to surmount this biochemical limitation.

Like TAG composition, altered stereospecific positioning of fatty acids on the glycerol backbone has been found in the few genetically modified oils subjected to this analysis to date. Stereospecific analysis using the diasteromeric urethane DAG-based method has been performed on a number of genetically modified sunflower and soybean oils derived from mutagenesis [46]. As expected, in all oils, saturated fatty acids were restricted to the *sn*-1 and *sn*-3 positions of the glycerol. However, acyl groups were not arranged in a *sn*-1,3-random, *sn*-2-random manner. In the sunflower oils, both palmitic and stearic acids showed preferences for the *sn*-3 over *sn*-1 position, especially in those with elevated palmitate or stearate. Oleic and linoleic acids were distributed over all three positions, their compositions affected by the abundance of palmitic and stearic acids at *sn*-1 and *sn*-3. In the soybean oils, neither palmitic nor stearic acids had consistent preferences for either the *sn*-1 or *sn*-3 positions. Oleic and linoleic acids were distributed over all positions, with *sn*-2 preferred. Linolenic acid was largely absent from the *sn*-2 position, except in oils with high levels of saturates occupying the external positions. However, the distribution of a particular fatty acid across the three positions can be affected by the amount of the fatty acid in the oil. For example, stearic acid was found preferentially at the *sn*-1 position, except in high saturate oils in which it accumulated to a greater extent at the *sn*-3 position.

Oils derived from two high oleic mutant and two "normal" sunflower varieties were subjected to stereospecific analysis using both the phosphatidic acid- and urethane DAG-based methods [68]. The authors indicated that incomplete resolution of the DAG urethane derivatives limited the reliability of the data generated using this method. Performing the HPLC analyses at very low temperatures [46] would probably diminish this problem. Regardless, their results were similar to those of Reske and coworkers [46]. Furthermore, a regression model was constructed that showed that the content of each fatty acid at each stereospecific position changed at the same rate as its content in the oil. This phenomenon was not observed in the sunflower oils analyzed by Reske and co-workers [46], perhaps due to their analysis of oils derived from more than one mutation.

Stereospecific analysis has also been performed on high palmitic, low palmitic, high stearic, and low linolenic (and combinations thereof) soybean oils derived from mutagenesis [69]. These authors used the DNPU-DAG-based method. Again, the results agreed well with those of Reske and coworkers [46], which is not surprising given the very similar genetic backgrounds of the germplasms used in the two studies. The results of both studies demonstrated that the distribution of many of the fatty acids across the three positions on the glycerol was affected by the amount of the fatty acid in the oil.

2.4. Unusual fatty acids

Most genetically modified oils offered commercially or under development (Table 1) are designed for edible applications. However, introduction of an unusual fatty acid into a domesticated oilseed could potentially yield a reliable, economic source of high-value feedstocks for the oleochemical industry. Indeed, fatty acids with altered positions of unsaturation [70], or with hydroxy [70,71] or epoxy [44,71] groups on the acyl moieties can convey rather useful properties. Most of the same methods used to characterize "normal" fatty acids in an oil are used to identify and quantify these unusual moieties. However, structural information obtained by MS has been invaluable for identification of novel fatty acids.

Only a few domesticated edible oilseed crops produce unusual fatty acids with potential utility for industrial processes. Wild species that produce novel fatty acids have been targeted for domestication, although these efforts have been hindered by the time and effort required producing high-yielding cultivars, as well as the lack of agricultural and marketing infrastructures. Another approach is to use genetic engineering to introduce these traits into existing widely domesticated oilseed species. Although GC/FID with authentic retention time standards has been used successfully to identify and quantify unusual fatty acids in transgenic oils, combined GC/MS coupled with various derivatization techniques is clearly more powerful for this application.

GC/MS analysis of dimethyldisulfide adducts of fatty acid methyl esters has been used to document the presence of petroselinic acid ($18{:}1^{\Delta 6}$) and its biosynthetic precursor $16{:}1^{\Delta 4}$ in transgenic tobacco callus transformed with a specific acyl–acyl carrier protein desaturase from coriander, a plant in which petroselinic acid is the predominant seed oil fatty acid [72]. Using argentation TLC and phosphorimaging, radiolabeled versions of both fatty acids (as their methyl esters) were found in transgenic tobacco suspension cell cultures that were fed [1-^{14}C]-acetate [73], thus providing direct evidence for insertion of the new biosynthetic pathway in tobacco. In a separate transformation effort using the same cDNA insert, petroselinate was found to accumulate, albeit at very low (0.4%) amount, in oil from mature seeds produced by transgenic sunflower plants [74]. The selectivity afforded by GC/MS was essential for quantifying the small amount of dimethyldisulfide methyl petroselinate in the presence of abundant and closely eluting dimethyldisulfide methyl oleate. These authors also found an extremely low level of petroselinate in the phosphatidylcholine fraction isolated by TLC from the transgenic sunflower seeds, suggesting that the low level of expression in the TAG is perhaps not due to incorporation of this usual fatty acid in the polar lipids but rather due to a deficiency elsewhere in the biosynthetic pathway, such as the lack of a specific Δ6 thioesterase [75].

GC/MS analysis of trimethylsilyl derivatives of fatty acid methyl esters has been used to document expression of oleate Δ12-hydroxylase from castor bean in transgenic *Arabidopsis thaliana* plants [76]. Ricinoleic (12-OH, $18{:}1^{\Delta 9}$), densipoleic (12-OH, $18{:}2^{\Delta 9,15}$), lesqueroleate (14-OH, $20{:}1^{\Delta 11}$), and auricoleic (14-OH, $20{:}2^{\Delta 11,17}$) acids were confirmed in the *A. thaliana* plants. The different lipid classes were separated by TLC and subjected to GC/MS. From this analysis, it was demonstrated that the hydroxylated fatty acids were excluded from the polar lipids, save for only small amounts of densipoleic acid. TAGs that contained 0, 1, or 2 hydroxy fatty acids were also separated by TLC and subjected to *sn*-2 positional analysis using *R. arrhizus* lipase and TLC purification of the reaction products. Hydroxy fatty acids were found esterified at all three positions, with the bulk being at *sn*-1 and *sn*-3. Trihydroxyacylglycerol was not detected, presumably due to limited expression of hydroxylated fatty acids in transgenic *Arabidopsis* oils (17% of total in the best case in this paper). A Δ12-hydroxylase from *Lesquerella fendleri* has been expressed recently in both *A. thaliana* and *B. napus*, resulting in accumulation of hydroxy fatty acids in their seed oils [77]. Ricinoleic acid has also been detected in offspring of somatic hybrids between *B. napus* and *Lesquerella fendleri* [78]. A castor bean oleate Δ12-hydroxylase gene was also expressed in tobacco, where up to 13.2% ricinoleate accumulated in the seed oil [15]. No expression was observed in transgenic sunflowers, however.

GC/MS has also been used to demonstrate expression of two conjugated fatty acids (as their methyl esters) in transgenic soybean tissue [79]. Expressed sequence tags generated from cDNA libraries prepared from developing seeds *of Momordica charantia* and *Impatiens balsamina*, plants

that produce significant amounts of conjugated fatty acids, were used to identify cDNAs that encode a diverged form of the $18{:}1^{\Delta 12}$ desaturase. Insertion of full-length cDNAs into soybean resulted in α-eleostearic ($18{:}3^{\Delta 9cis,11trans,13trans}$) and α-parinaric ($18{:}4^{\Delta 9cis,11trans,13trans,15cis}$) acids together accumulating to 17% of the weight of somatic embryos. Although the position and configuration of the conjugated fatty acid methyl esters were not determined, their identification was based on retention times and mass spectra that were identical to those from *M. charantia* and *I. balsamina* extracts. The expression level in seeds, and the presence or absence of any deleterious effects on agronomic characteristics of the plants have yet to be reported.

The same experimental strategy employed to clone the desaturases involved in the synthesis of conjugated double bonds was used to isolate a gene from *Vernonia galamensis* that is responsible for the formation of Δ12-epoxy fatty acids [80]. GC and GC/MS of methyl esters have been used to analyze this unusual fatty acid in plant extracts. In this case, the lipid fraction from transgenic soybean somatic embryos contained up to 8% Δ12-epoxy $18{:}1^{\Delta 9cis}$.

It is anticipated that combined HPLC/MS will prove to be as useful in identifying TAGs in industrial oils as it has been in the characterization of TAGs in other genetically modified oils.

2.5. Non-acylglycerol lipid constituents

Triacylglycerols constitute the vast bulk of lipids in oilseeds, and as such represent a major portion of the value of the lipid fraction in commodity or genetically modified seeds. However, other non-acylglycerol lipid components also have economic merit. Two such "minor" constituents are tocopherols and phytosterols, both recovered from the deodorization step during processing of crude vegetable oils [81]. Tocopherols and phytosterols in vegetable oils are analyzed commonly by either HPLC or GC. GC analysis is usually performed on trimethylsilyl derivatives.

Tocopherols are natural antioxidants that inhibit lipid oxidation in foods and biological systems by stabilizing hydroperoxy and other free radicals [82]. *Alpha*-tocopherol extracted from oilseeds is sold as a nutritional supplement with vitamin E activity. Therefore, increasing the amount of tocopherols in an oilseed crop has potential economic benefits.

Tocopherol content varies considerably across and within the major oilseed crops [83]. However, little effort has been expended to date to specifically modify tocopherol levels. Mutations have been introduced in sunflower that resulted in the accumulation of β-, γ-, and δ-tocopherols in an oil that usually contains only the α homolog [84]. The mutations affected the activity of methyl transferases leading to the synthesis of α-tocopherol. In a reverse process, δ-tocopherol methyltransferase has been expressed in transgenic *A. thaliana*, resulting in the near-total conversion of δ-tocopherol to α-tocopherol [85]. The amount of total tocopherols in the *Arabidopsis* seed oils was unchanged.

There has been some uncertainty about the nature of the relationship between tocopherol content and fatty acid composition in genetically modified seed oils. Is the generally positive correlation between tocopherol content and the level of TAG unsaturation, associated with oil instability [86], amplified in oils with altered fatty acid profiles? Most experimental evidence does not support this concept. Altering TAG fatty acid composition did not affect tocopherol quantity or quality in high palmitic, stearic, oleic, or linoleic acid sunflowers [87–89] or canola [23,89]. Furthermore, the genes governing oleic acid and tocopherol compositions are not linked [90]. However, positive correlations between tocopherol and linolenic acid contents have been reported

in low linolenic acid soybean oils [89,91]. It is likely that this association is a consequence of the very narrow gene pool of this phenotype, especially with the lack of a direct biochemical link between the synthesis of tocopherols and storage lipids [92]. Increasing tocopherol levels with decreasing percentages of linolenic acid has also been found in genetically modified soybean oils [21], although the seeds used in this study were obtained from diverse sources, and thus is not possible to account for the potential influence of growing conditions on expression of the two traits.

Plant-derived sterols or phytosterols are an important basestock for the health and nutrition industry. Phytosterols are used by the cosmetic industry as emulsifiers and provide more than 75% of the world's precursors for hormonal steroid production [81]. Two margarines that contain substantially elevated phytosterol contents are currently on the market in a number of countries. Although the source and composition of the sterols incorporated into the two products differ, use of either has been shown to significantly lower blood serum cholesterol [93,94]. As with tocopherols, increasing the amount of phytosterols in an oilseed crop has potential economic benefits.

Like tocopherols, phytosterol content varies considerably across and within oilseed species [83]. Also like tocopherols, little work has gone into modifying phytosterol contents in oilseeds. In oil palm, introgression of genes from *Elaeis oleifera*, a species normally found growing wild, resulted in a hybrid that produced seed oil with a substantially greater amount of phytosterols (and carotenes) [95]. Phytosterols have also be examined in some temperate oils with altered fatty acid profiles. It is clear from these reports that modification of TAG fatty acid composition did not affect phytosterol quantity or composition in high palmitic, stearic, oleic, and linoleic acid sunflower oils [87,88,96], nor in high palmitic, high stearic, low saturate, and low linolenic acid soybean oils [96]. Although the amounts of a few phytosterols in two high oleic safflower oil have been shown to be slightly different compared to those in four high linoleic (normal) safflower oils [97], the limited number of oils evaluated and the lack of replication prohibits the extrapolation to a general phenomenon from these results. Genetic independence of phytosterols and fatty acids would indeed be predicted due to their unrelated biosynthetic pathways. Tocopherol and phytosterol contents are also generally not correlated in the sunflower and soybean oils.

2.6. Lipoxygenase

Lipoxygenases are enzymes that catalyze the oxidative breakdown of polyunsaturated fatty acids to catabolites that can impart undesirable flavors to seed oils [98]. Soybean mutants have been produced that lack one or more of the lipoxygenase isomers in their seeds [99]. Although the economic value of the trait is uncertain (see below), some effort has gone into developing methods to rapidly screen lipoxygenase activity in soybean seeds. Electrophoresis, a conventional method for detecting lipoxygenases, is costly and time-consuming. Measuring enzyme activity by following either formation of conjugated double bonds with a spectrophotometer or consumption of oxygen with a recording electrode are faster procedures, but both are hampered by the requirement for homogenized seed extracts.

An alternative protocol more suited for large-scale breeding support is a colorimetric assay that measures the lipoxygenase-catalyzed conversion of exogenous linoleate or linolenate to hydroperoxides [100]. The test for lipoxygenase-1 is conducted directly on thin cotyledon slices in test

tubes. Hydroperoxides produced are detected by the oxidation of iodide ion to iodine, which in turn reacts with starch. In the assay for lipoxygenase-2 and-3, crushed seeds and refined soybean oil are transferred to filter paper sheets. Hydroperoxides formed are visualized by the oxidation of ferrous thiocyanate to ferric thiocyanate. Another set of colorimetric assays for lipoxygenase activity is based on measuring the reactivity of lipoxygenase-catalyzed products of linoleate [101]. Lipoxygenase-1 and-2 activities are measured by the bleaching of methylene blue, and lipoxygenase-3 activity is determined by the bleaching of β-carotene. Since each test is specific of an isozyme, the triple null and individual double nulls can be readily distinguished.

Enzyme-linked immunoassay (ELISA) is another rapid assay format developed for lipoxygenases [102–105]. Use of monoclonal antibodies renders this assay specific for each isozyme. Very high sample throughput is facilitated by a combination of 96-well microtiter plates and robotic liquid handling, although crude extracts are required. ELISA-based testing for lipoxygenase in soybean seeds is also available commercially [106].

2.7. Non-chromatographic methods for fatty acid composition

Although GC is a well-proven tool for accurate determination of fatty acid composition in seed oils, the technique requires trained personnel to operate and maintain expensive instrumentation. Furthermore, sample preparation requires toxic chemicals and is destructive, necessitating population sampling and/or seed chipping. These attributes are less than ideal for supporting breeding efforts to develop modified seed oils. Often just an estimate of fatty acid composition is required in order to select for advancement seeds that have a reasonable probability of possessing a desired phenotype. This is especially true early in a breeding program when prodigy seed is limited, or when a modified oil trait is being introduced by backcrossing into germplasm with favorable agronomics. In these cases, a less accurate, preferably nondestructive assay is desired to cull samples prior to GC analysis. Three non-chromatographic approaches have been used to address this need: colorimetric spot tests, spectroscopic analysis, and use of molecular markers.

The chemistry underlying the thiobarbituric acid (TBA) test for the presence of peroxides in oxidized vegetable oils is the basis of a spot test somewhat specific for linolenic acid content. The assay has been used to select low linolenic rapeseed [107] and soybean [108] seeds. Seeds were crushed against a sheet of filter paper on which 2-TBA was applied. Oxidation of fatty acids was induced by exposure to ultraviolet light. The filters were heated to expedite the reaction of trienoic fatty acids (essentially linolenic acid) to form a cyclic peroxide that in turn forms malonaldehyde, resulting is a red or dark brown spot. Linolenate content of samples was estimated by carefully comparing the color of spots with those formed from low linolenate and control varieties. Although the procedure does not require expensive instrumentation (i.e. a gas chromatograph), working with soybeans the author has found this protocol, as well as a modification of it, somewhat cumbersome to perform. Furthermore, reliability and robustness of the response (and thus quality of the calls) depends heavily on both providing uniform ultraviolet irradiation and on operator experience. Thus, for soybeans at least, the assay is usable only to screen out genotypes with excessively high linolenic acid contents [108].

Spectroscopy is used routinely to measure levels of major seed constituents such as protein, oil, and starch. Near infrared reflectance (NIR) and nuclear magnetic resonance (NMR) spectrometers are commonplace in grain quality laboratories and grain elevators. These instruments are

very simple to operate, and rapidly generate fairly accurate data on whole seeds without destroying samples. As such, they are ideal for point of sale analysis, for which they have found wide application (see below). Near infrared transmission spectroscopy can be use to segregate individual maize kernels for oil content, although it is not as accurate as NMR for this purpose [109]. However, the technique is useful for breeding selection since percent protein and starch can be estimated concurrently. Many other papers have been published describing the use of spectroscopy for analysis of oil quality. Most involve measuring total oil content, constituents that arise from processing, or classifying different vegetable oils with a goal of detecting adulteration. These applications of spectroscopy do not specifically address distinguishing genetically modified oil types, and thus are beyond the scope of this review. Clearly, it would be highly advantageous to use nondestructive spectroscopy for both constituent analysis and fatty acid composition. However, to date this has generally not been the case, although some spectrometers have been evaluated for this purpose.

NIR spectroscopy is undoubtedly the most widely used spectroscopic method in agriculture today. Several groups have demonstrated the ability of NIR spectroscopy to predict fatty acid composition in several different oilseed crops. Using multivariate statistics, a calibration was developed using 408 soybean lines with commodity-type seed oil fatty acid compositions grown at two locations in Minnesota [110]. A rather small validation set of 26 samples was applied against the calibration. Reasonable (at least for use as a breeding screen) correlation coefficients (0.66–0.71) were obtained for stearic, oleic, linoleic, and linolenic acids when using ground seed. The prediction degenerated (R^2s between 0.38 and 0.56) when whole seeds were used, an obvious handicap for high throughput screening of breeding material. The calibration predicted poorly the percent of palmitic acid, probably due to little variability of the trait in the calibration sample set. Inclusion of modified oil types may improve performance of this calibration.

Initial attempts to use of NIR spectroscopy to estimate fatty acid content in rapeseed met with mixed success. In one study, NIR calibrations were correlated poorly with fatty acid content even though the 215 samples used for the calibration were obtained over five growing seasons and from many different locations in western Canada [111]. Performance for linoleic, linolenic, and saturated fatty acids improved when the reference data were expressed as the actual concentration presented to the instrument, thus correcting for differences in oil content. However, even in this case, prediction statistics were inadequate for routine breeding support. Use of a sample set with a wider range of fatty acids yielded calibrations that provided quantitative information on some of the components, but not at the desired level of accuracy [112,113]. More recent work with large sample sets of intact seeds of *Brassica* spp. obtained from plants grown in different environments over several years yielded very good prediction statistics [114–117]. The quality of the calibration was related directly to the variability in fatty acid content. Thus, oleic, linoleic, linolenic, and erucic acids were predicted best when mutants with altered levels of these fatty acids were represented in the calibration sets. Again, results for saturated fatty acids were not as good, presumably due to less variability in the seeds used for calibration. Another NIR spectroscopy calibration was made covering 495 seed samples from 26 genera and 128 species of the family Brassicaceae [118]. The resulting equations could be used to estimate oil content and concentrations of oleate, linolenate, and erucate for potential breeding purposes. The results for palmitate, stearate, and linoleate were less promising. The technique was expanded to estimate oil content and oleic and erucic acid compositions in individual *B. napus* seeds [119]. The NIR data

predicted percent linoleic and linolenic acids poorly. Recently, a NIR spectroscopy calibration was constructed from 30 high and low erucic acid rapeseed samples grown at two locations [120]. Intact and single seeds, as well as oil extracted in diethyl ether were scanned. Spectral regions were found that correlated with percent linoleic acid in the oil ($R^2 = -0.93$), seeds ($R^2 = -0.87$), and single kernels ($R^2 = -0.86$). The latter correlation might have improved if the gas chromatographic reference data were obtained from individual kernels rather than from samples comprised of several seeds. Furthermore, high and low erucic acid (and thus oleic acid) contents were readily distinguishable, although the degree of resolution of these traits was not determined due to the lack of samples with intermediate levels. Regardless, results from individual seeds have obvious utility for breeding efforts.

A calibration was developed using just 30 seed samples obtained from 15 sunflower varieties grown in a single field but over three successive growing seasons [121]. Not every variety was harvested each year. Although most of the samples had commodity-type fatty acid profiles, several mid-oleic types were included. Bulked machine-husked seed, individual hand-husked kernels, and diethyl ether-extracted oils were scanned. As in the work with rapeseed, spectral regions were found that were highly correlated ($R^2 > -0.97$) with percent linoleic acid in each sample type. Although developed on a very small number of samples, the results with hand-husked kernels that retain viability are encouraging for breeding purposes. Recently, a calibration was also developed using 387 intact seed samples from sunflower lines grown in the greenhouse [122]. Again, fatty acid mutants were represented in the sampling. A subset of 120 samples was also scanned as husked seed, meal, and oil. Excellent predictions were obtained for all the major fatty acids in the husked seed, meal, or oil, with R^2 values above 0.90. The calibration did less well with intact seed (R^2 values between 0.76 and 0.85). Robustness of this calibration waits testing with samples from different locations and years. Diethyl ether-extracted seed oils from the various mutants could be classified as high saturate, high oleic, high saturate/high oleic, or commodity-type using just two wavelengths (1800 and 2110 nm) [122,123]. This suggests that a less expensive discrete filter spectrometer could be used for this purpose, a significant advantage at the point of sale. Since no wavelength discriminated palmitic from stearic acids, distinguishing these two traits still required whole spectrum analysis [122]. Using a special adapter for a monochromatic instrument, the authors recently developed calibration equations for oleic and linoleic acids in single intact achenes using several hundred samples with a range of fatty acid compositions [122,124]. Coefficients of determination were 0.88 for both fatty acids in an external validation set of 100 samples, and 0.83 and 0.80 for oleic and linoleic acids, respectively, in two greenhouse-grown F_2 populations segregating for these fatty acids. The lower coefficients of determination and standard errors of prediction for the F_2 populations are expected since these plants were grown in different environments than those used for the calibration and validation. It is for this reason that robust calibrations are constructed from samples obtained from many different growing locations and years. Regardless, the calibration was useful at identifying high oleic types (i.e. above 70%), but could not readily distinguish relatively minor differences in oleic acid content among high oleic individuals. Again, such a nondestructive assay performed on single seeds has obvious utility for germplasm development.

Pulsed NMR is used widely to measure seed oil content in seed quality laboratories. Over two decades ago, ^{13}C-NMR was proposed for determining fatty acid content in intact seeds [125]. Magic-angle spinning ^{13}C-NMR was applied to several genetically modified canola lines [126].

Spectra acquired over just 10 min yielded reasonable results for single seeds. However, the technique as presented did not distinguish individual saturated or monounsaturated fatty acids due to resonance overlap. Application of NMR techniques to the analysis of oils and fats has been reviewed recently [127]. For a number of reasons, NMR has not been used widely for determining fatty acid composition in genetically modified seeds. This is due mostly to the high cost and limited automation associated with the fairly high-resolution instruments required for the analysis, as well as the high accuracy of results generated with GC. Still, NMR and other spectroscopy techniques hold promise for the measurement of fatty acid content in intact oilseeds.

Association of a modified oil phenotype with molecular markers represents an indirect technique to follow gene recombination. Use of molecular markers is especially useful in tracking the gene(s) of interest through a backcrossing program, when the altered oil trait(s) are combined with genes required for successful commercialization of the plant variety, such as those governing yield, disease resistance, and other essential agronomic properties. There are several different molecular marker techniques in use, but their descriptions are beyond the scope of this review. However, most share the fundamental advantages of actual or potential high throughput and the ability to sample vegetative tissue, thus allowing selections to be made well in advance of reproductive development. Since it is desirable to maximize the genetic linkage between the marker(s) and the gene of interest, setting up a molecular marker assay can require significant resources. Perhaps the biggest disadvantage in applying marker technology for this application is inherent in the indirect nature of the technique. As many molecular biologists have come to find out, presence of a gene does not necessarily determine level of its expression or stability of its expression across many different environments.

Regardless, some effort has been expended to develop marker-assisted selection programs for modified oil traits, although from the author's perspective implementation has been minimal. This is perhaps due to the high cost of setting up the assays, possible lack of legal rights to use the gene itself as a (perfect) marker, and the high quality and reasonable cost of GC-based fatty acid composition data. Molecular markers associated with the low linolenate phenotype in canola have been identified in a *B. napus* double-haploid population derived from crossing the public low linolenate line 'Apollo' with a high linolenate line. Bulked segregant analysis was used to develop these random amplified polymorphic DNA (RAPD) marker [128]. Sixteen markers were distributed over three loci that explained 51% of the variation in 18:3 in the population used. Interestingly, the rapeseed *fad*3 gene encoding the microsomal Δ15-desaturase was one of the markers used, and this gene mapped near a locus that controlled only 14% of the variation in 18:3 content. Other groups have also developed molecular markers for the altered oil phenotypes in *Brassica* spp., including restriction fragment length polymorphisms (RFLP) for linolenate and erucate [129], RAPDs for linolenate [130–134] and palmitate [135], PCR amplified specific alleles for oleate [136], and codominant sequence characterized amplified regions [137]. A RFLP map has also been constructed for the altered fatty acid traits in soybean [138]. DNA fragment length polymorphism, single-strand conformational polymorphism, and simple sequence repeat markers were developed recently for Δ9-stearoyl-acyl carrier protein desaturases for potential use in developing a high stearate sunflower oil [139].

Colorimetric spot tests have not been utilized widely to screen genetically modified oils. The lack of popularity of these tests is due mainly due to the availability of easier methods providing required analytical instrumentation is available, which is most often the case. Overall, spectroscopy

represents a rapid, nondestructive tool for screening less subtle differences in seed oil fatty acid content. Better predictions are obtained with better instrumentation, but the associated capital costs can be prohibitive. Molecular markers for oil quality traits are applied best in conjunction with those for agronomic characteristics. This coupled approach spreads out analytical costs between multiple traits. Thus, it is the author's opinion that the combination of high accuracy and high sample throughput afforded by GC when automated and streamlined [6], represents the optimum analytical approach to fatty acid analysis of genetically modified oils.

3. Determining improved functionality

Regardless of the fatty acid profile of an oil, the arrangement of fatty acids on the glycerol backbone, or the levels of minor constituents, the ultimate value of a genetically modified oil is determined by the degree of improved functionality over commodity types. A specific trait will fail economically if it is not of sufficient value to an end user to recoup the costs associated with its development and supply. This consideration is especially relevant with edible oils, an industry long driven by substituting, blending, low cost supplying, and tight operating margins. Therefore, testing performance and product suitability is essential for a thorough analysis of any genetically modified oil.

Practical considerations dictate that functional testing of a new oil be done on a small scale, often with just of few kilograms of product. Inferences are made from the results of these tests on the anticipated performance of the oil at a production-level scale. Several university, government, and industry laboratories have set up to do this type of testing.

Functional assays of oils can be divided into two main categories. The first group of assays assess oxidative stability, either during storage, "accelerated" storage (i.e. heated moderately), or frying. These tests attempt to address the needs of the bottled and frying oil segments. A second line of testing involves using the oil in an actual product or for an established process other than frying. This could entail fabricating foods or lubricating machinery with the oil being tested. These assays provide a measure of substitutability. The two testing categories are complementary, for oxidative stability is an essential criterion for an oil in a food product or its use in an industrial process.

Oxidative stability tests are carried out on oils maintained for different durations at various temperatures. Since light can induce oxidation [140], test oils are often stored both in the light and in darkness. Samples are periodically tested for quality using standard parameters such as free fatty acids, total polar compounds, peroxide value, anisidine value, TBA value, active oxygen method value, volatile compounds, conjugated dienoic acids, oxidized TAG (hydroperoxides), and flavor/odor as judged by a trained sensory panel. Stability of an oil is compared most often with that of commodity types with or without hydrogenation, other genetically modified oils, and blends. High oleic sunflower [56,87,141,142], safflower [141], corn [143], soybean [144], and peanut [145] oils have proven very stable in these tests. The same was observed with TAGs from high saturate sunflower oils [56], as well as with low linolenic [146,147] and high stearic [147] soybean oils. Canola oils with lower amounts of polyunsaturated fatty acids or higher amounts of oleic, stearic, or lauric acids also exhibited enhanced oxidative stability [148–150]. Compared to a single control oil, four experimental corn oils with slightly elevated saturate levels exhibited a slower

increase in peroxide values upon storage at 60C in the dark [151]. Substitution of oleic acid for linoleic acid at the *sn*-2 position of TAG molecules also conveys improved stability in both soybean [152,153] and canola oils [50,150]. Oxidative stability was also better when a saturated fatty acid was substituted for an unsaturated fatty acid at the *sn*-2 position of soybean TAGs [154].

Frying tests usually involve continuous or discontinuous (batch) deep fat frying of bread cubes, potatoes, or various frozen foods with or without replenishing the oil. Oil samples are withdrawn periodically, and quality of the oil and/or food is measured by changes in some or all of the following: Lovibond color, fatty acid composition, non-esterified (free) fatty acids, total polar compounds, volatile compounds, DAGs, TAG polymers, hydroperoxides, conjugated dienoic acids, peroxide value, anisidine value, soap value, foam height, and room odor and oil and food flavor as judged by sensory panels. Comparisons are made against commodity and/or high-stability, usually hydrogenated, commercial frying oils, fats, and blends. In these tests, high stearic soybean [155], low linolenic soybean [155–161], low linolenic canola [49,156,162,163], high oleic canola [162–164], high oleic corn [143], high oleic sunflower [157,165–170], and high oleic safflower oils [171] overall performed better than commodity types, and performed at or near as well as hydrogenated products. Although fatty acid composition was a main determinant of frying stability, minor components such as tocopherols also played a role. Interestingly, a basal level of polyunsaturated fatty acids appeared to be required for maximum flavor in freshly fried foods [164,169]. Foods fried in ultrahigh oleic oils did not exhibit optimal initial flavor, but did tend to maintain flavor stability over time.

Spray tests are designed to evaluate the ability of applied oils to improve the appearance and shelf life of a cracker or other snack. Samples are evaluated for flavor by sensory panels at lengthy intervals, usually in the order of months. As in frying tests, oils with decreased linolenate or elevated oleate performed well compared to high-stability commercial spray oils [157]. The same trend should be the case for high saturate oils.

Genetically modified oils have been evaluated for use in several food applications. Non-dairy coffee creamers also require high-stability oils due to the need for extended shelf life. Many of the same oxidative stability assays employed in frying tests are used to evaluate oils for this application. It has been shown that high oleic sunflower oil is suitable for nondairy creamers [157], and high laurate canola oil should be as well. Margarine has been made using high saturate oils or palmitate- and stearate-enriched fractions thereof. TAG composition, solid fat index, and dropping point are standard indicators of margarine functionality. In addition, differential scanning calorimetry, texture analysis, and sensory evaluation can be performed on finished products. Six different high stearic soybean oil samples were evaluated for suitability for the manufacturing of a margarine with little or no *trans* fatty acids [55]. The TAG profile of each oil was determined by HPLC/FID. Those oils with a greater proportion of disaturated TAG molecules performed the best. However, it is important to properly sample seeds from lots of the same genetic background. If the 25% stearic soybean oil reported to contain 27.7% disaturated TAGs [46] was used to represent line A-90 instead of the 17% stearic oil that contained just 17.2% disaturated TAGs [55], a more favorable solid fat index profile would probably have been obtained. Regardless, all six high stearic oils lacked the solids content required for the manufacture of even a soft tub margarine. However, addition of hardstock [9] or random interesterification [172,173] yielded oils with physical properties compatible to a soft tub margarine. It remains to be seen if the cost associated with the extra processing can be justified.

One potential product with interesting properties is a soybean oil extracted from seeds that lack lipoxygenases. Mutants possessing only lipoxygenase-1,-2, or-3 exhibited reduced production of both hydroperoxide (estimated by the 1,3-diethyl-2-TBA value) and hexanal [99]. A mutant lacking all three seed lipoxygenase isozymes produced the least amount of both catabolites. The authors suggested that the triple mutant could be used to produce soy-based foods with enhanced oxidative stability. This conclusion was not tested directly, but rather was based on the reduced production of hydroperoxides and hexanal in the presence of exogenous polyunsaturated fatty acids, which are abundant in many foods. Overall, the value to oil quality of mutants deficient in lipoxygenase activity is uncertain. Thermal inactivation of lipoxygenases improves the stability of partially processed commodity soybean oils, and the treatment resulted in the same benefit for oils derived from a line lacking lipoxygenase-1 [174]. In a more recent study, oils that lack one or two of the lipoxgenase isozymes were used to fry bread cubes [175]. Fatty acid composition, formation of conjugated dienoic acid and TAG polymers, peroxide value, and bread flavor were measured. The authors did not find improved stability in oils from the lipoxygenase-null mutants. Oils from regular soybeans and from those deficient in one, two, or three of the lipoxygenase isozymes were stored at 35 and 60°C in the light and dark [160,176]. The amount of linolenic acid, tocopherols, hexanal, and other volatiles, as well as peroxide values and flavor were measured to assess the influence of lipoxygenase on oxidative stability. The authors concluded again that the absence of lipoxygenase did not improve flavor or stability. This result is perhaps expected since lipoxygenases are essentially removed from refined soybean oil during processing. As long as seeds are not stressed post-harvest, lipoxygenase and lipase activities should not significantly degrade oil quality.

Some of the most important functional testing has been performed by substituting an altered oil for the one(s) used ordinarily in an established product, and then conducting a battery of performance assays. This large-scale testing can represent a substantial commitment by the end user, since production equipment is dedicated for the project. Flavor of a well-established, familiar product can be critical to the success of a modified oil in a food application. Higher oxidative stability is not always better. For example, potato chips fried in a 78% oleic canola oil exhibited excellent oxidative stability, but had a less intense (and presumably desirable) flavor than those prepared in a 68% oleic canola oil [164]. It was suggested that the level of linolenic acid was also important to the flavor of the potato chips, since the higher oleic oil also had a higher amount of 18:3 (4.2% compared to 3.1% in the 68% oleic oil).

Genetically modified vegetable oils, like commodity types, are most often associated with food applications. However, chemically modified vegetable oils have been used, although in relatively small volumes, for various industrial purposes. Such oils have utility as biodegradable lubricants, chemical precursors, or diesel oil substitutes. Certainly, creating the required chemical modifications in planta by genetic engineering represents a potentially economic substitute for chemical processing. Exclusive use of the resulting oils in nonedible applications should also circumvent concerns about food safety and genetically modified organisms.

One approach to engineer an oil with enhanced fuel properties is to increase the level of short-chain fatty acids. Such an oil could be used as a diesel fuel substitute without the processing costs associated with forming fatty acid methyl esters. To this end, physical properties relevant to diesel oil functionality were measured in an oil derived from a *Cuphea viscosissima* mutant with increased amounts of caproic and caprylic acids [177]. Boiling point, vapor pressure, and heat of

vaporization were determined. Due to limited oil availability, a synthetic mixture containing tricaproin, tricaprylin, trimyristin, tripalmitin, and triolein formulated to the same fatty acid composition as the *Cuphea* oil was used for viscosity tests. The *Cuphea* oil outperformed other vegetable oils, but was inferior to rapeseed methyl esters. There was also a problem with crystallization at 25°C. It is also unknown if similar functionality would be obtained with the *Cuphea* oil that undoubtedly contains many more TAGs than the simulated mixture. Clearly, additional genetic modifications are required for a TAG-based diesel fuel. An engine "torque test" that measures in an accelerated manner the tendency of diesel fuels to deposit coke in fuel injectors was also performed [178]. Synthetic analogs that contained 60–80% short-chain fatty acids deposited statistically the same amount of cake as number 2 diesel fuel. The results could be used to set specifications for vegetable oil-based diesel fuel substitutes.

Another approach to develop an industrially useful vegetable oil is to engineer a fatty acid molecule that contains additional chemically active sites, such as double bonds with unusual position and/or configuration, triple bonds, hydroxy groups, or epoxy groups. These oils would be candidates for industrial feedstocks. Analysis of these unusual fatty acids has already been discussed. Much less reactive oils would also be useful as industrial lubricants with good oxidative stabilities. For example, high oleic soybean oil was evaluated using rotating bomb (a measure of oxidative stability), hydraulic pump wear, viscosity change, and total acid number change (a measure of TAG hydrolysis to free fatty acids) tests [179]. Augmented with anti-wear and anti-oxidation additives, the high oleic soybean oil performed as well or better than other vegetable oil-based and medium-grade petroleum-based lubricants [80,179].

4. Identity preservation of genetically modified oils

The economic value of genetically modified oils can only be captured if they are kept separate from commodity seed from seed production through use in finished products. Therefore, a system to preserve the unique identity of these oils is required, especially when there is a reasonable likelihood of co-mingling with commodity types. One such situation where this could occur is at county elevators where both identity preserved (IP) and commodity seeds are delivered. It is at this point of sale that efforts to establish effective IP protocols have been focused. Constraints on the analytical technologies used to distinguish IP from commodity deliveries are imposed by the nature of the elevator environment. The analytical process employed must be rapid, inexpensive, and relatively simple to perform. Use of elaborate sample preparation, complex instrumentation, and hazardous chemicals would be problematic. It is no surprise perhaps that spectrophotometric methods are envisioned to fulfill the requirements of point of sale confirmation of genetically modified oils. Alternatives to gas chromatographic determination of fatty acid composition are emerging with the need for rapid, on-site analysis. At the present time, however, there is little in place at the point of sale to meet this need.

One example of using a fairly simple spectrophotometer for IP determination in an elevator environment is the employment of a refractometer to estimate percent oleate in high oleic acid sunflower seeds. Crude oil, expelled by crushing seeds in a simple press, is placed on the measuring prism. Refractive index is read and the percent oleate is determined from a standard curve. The technique is simple, very quick, relatively inexpensive, and fairly accurate. It is vital, though,

to ensure that the oil read is representative of oil from all the seeds in the sample. An additional sampling challenge arises when large truck or barge shipments need to be evaluated.

Clearly, a procedure that uses whole or ground seed is preferred over one that necessitates preparation of an extract. Standard NIR instruments already in many elevators for the measurement of percent protein, oil, and moisture probably are not capable of executing IP fatty acid determinations at the accuracy required. Developing and implementing alternative technologies is consistent with commercialization of IP oils.

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