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IDENTIFICATION OF SUNFLOWER GENOTYPES SUITABLE FOR ORGANIC AGRICULTURE AND DEVELOPMENT OF ANALYTICAL TOOLS FOR THEIR TRACEABILITY

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SUMMARY

1 AGRICOLA GRAINS: COMPANY PRESENTATION

Involved in the agri-food sector since 1930, Agricola Grains S.p.A. stepped up its activity significantly in 1965.

In 1991, the year when organic production was first regulated in Europe under EC Directive 2092/91, the company sensed that the way forward must lie in a renewed care for people and the environment: accordingly, they decided to abandon all interests in conventional chemical agriculture and set up Agricola Grains S.p.A.

Thanks to their enthusiasm and dedication, the company has now become an established international name in the harvesting and marketing of organic cereals, and a technical adviser employing expert staff and using the services of outside consultants.

A reality that operates in compliance with the most scrupulous international certifications.

Our company exports quality Italian organic grain products worldwide, supplying selected raw materials for both animal feed and human consumption.

Closely collaborating with trusted Italian farmers, the company oversees the cultivation of crops from seeding to harvesting, supplying organic seeds directly to growers and ensuring 100% traceability along the agri-food chain. In particular, it produces sunflower oil – both raw and refined - for human consumption. The main services offered are:

- Oil production
- Agronomic Assistance
- Cultivation plans
- Verification of soil characteristics
- Supply of GMO-free seeds certified as organic

- First crop quality analysis
- Crop purchase and marketing with open-ended or closed-ended contracts
- Storage and conservation of goods in certified warehouses
- Drying and roasting with technologically advanced equipment
- Supply of raw materials and feedstuffs for organic livestock production

2 INTRODUCTION

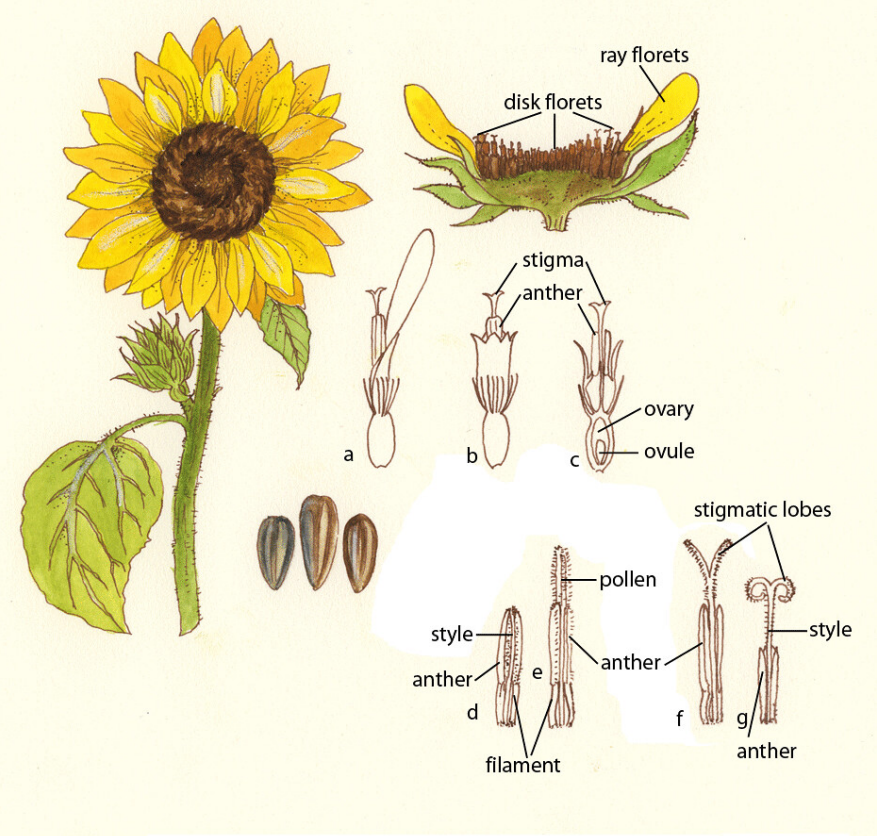
2.1 SUNFLOWER

2.1.1 TAXONOMY, NATURAL HABIT, ORIGIN AND DISTRIBUTION OF THE CULTIVATED SUNFLOWER

Sunflower (*Helianthus annuus* L.) belongs to the Asteraceae family (Asterales order) *Heliantheae* tribe; the genus *Helianthus* includes approximately 50 species with very different morphologies (herbs, shrubs, lianas, etc.) originating in North and Central America, and some species were introduced to the Old World (Heiser et al.,1969). Two important species were cultivated in Central Europe: the common sunflower (*Helianthus annuus*) and the Jerusalem artichoke (*Helianthus tuberosus*).

The basis for the botanical classification of the genus *Helianthus* was proposed by (Heiser et al., 1978) and subsequently refined by other Authors. This approach splits Helianthus into four sections: *Helianthus*, *Agrestes*, *Ciliares* and *Atrorubens*.

The most cultivated species *H. annuus* originally came from North America; it can be found at altitudes between sea level and 3000 metres, in areas with a range of different rainfall characteristics, but essentially in the western two-thirds of the United States, southern Canada and northern part of Mexico. It can be usually found also in open habitats already bothered by human activity.



Flowering shoot, seeds, and median section of capitulum of
Helianthus annuus, sunflower, and details of floral parts:
(a) side view of ray floret (b) side view of disk floret (c) section of tubular floret
(d) - (g) stages in the growth of sytle and stigmas.

2.1.2 MORPHOLOGICAL CHARACTERISTICS

The cultivated sunflower is an erect, hardy, often unbranched, coarse, stout-stemmed annual herb, with a varying height up to 4 m. The stem is robust, circular in section, 3-6 cm in diameter, curved below the head, and woody when mature. It is filled with a white pith that often becomes hollow with age. The root system is a taproot, which can penetrate the soil to a depth of about 3 m, with a large lateral spread of surface roots; however, most of the roots generally remain in the first 50 cm.

Leaves are usually alternate (lower leaves opposite), ovate, cordate, with three main veins, 10-30 cm long, 5-20 cm wide, margin serrate, and carried on long petioles. The colour of the leaves is usually dark green. Lower leaves are larger, broadly ovate or heart-shaped, and attached individually or in pairs. The base of the leaf blade is recurved as it joins a prominent petiole. Teeth

on leaf margins range from inconspicuous to clearly present. Upper leaves are smaller, broadly lance-shaped or ovate, and attached individually to the stem.

The flowering head (capitulum) is heliotropic (rotating to face the sun). Tall, ornamental cultivars usually have more numerous smaller flower heads (although sometimes have giant ones), whereas cultivars for oil extraction are shorter and have a single flower head. Flowering heads may reach up to 35 cm in diameter and have 16-30, yellow to gold, ray flowers surrounding a large central disc containing dark brown to purple flowers. The back of the capitulum is covered with involucral bracts, broadly ovate, with tips drawn out to a fine point. The disc-shaped flowering head is borne terminally on the main stem, 10-50 cm in diameter, sometimes drooping, and containing 800-8000 bisexual florets. Around the margin of the head there are individual ray flowers, which are sterile, brightly coloured, usually yellow, but varying from deep yellow to red. The brown or purplish disc florets are spirally arranged, flowering from the outer to the centre.

The ovary is inferior with a single basal ovule. Fruits are dry, indehiscent achenes, variable in colour (white, brown, black, or often dark with white stripes). Its seed is compressed, flattened oblong, the top truncated and base pointed, 10-25 mm long, 7-15 mm wide. The 1000-kernel weight varies from 50 g to many times this.

Archaeological material of the sunflower was found in several sites in North America. The closest relative of the genus *Helianthus* is *Viguiera*, a large genus whose species range from the southwestern United States to South America.

2.1.3 IMPORTANCE OF THE CROP

The sunflower is native to the western United States where it was an important source of nourishment for local populations. It was introduced in our continent at the beginning of the sixteenth century with ornamental and medicinal purposes. It was considered an oil plant starting from the seventeenth century and only in 1860 did genetic improvement studies begin to increase its oil content.

Sunflower crop reached a major place in European agriculture after the World War I, thanks to the many advantages offered by its cultivation: adaptation to climate change, competitiveness for food and energy, good productivity, extraction of excellent quality oil, both for its nutritional value and for its stability, and good physic-chemical characteristics (Benvenuti and Vannozzi, 2001).

The evolution of sunflower crop at global scale and over a long period is quite remarkable, going from 10 million tons on 9.6 million ha in 1975 to 52 MT on 27 Mha in 2018, with production growing faster than acreage, indicating a very strong technical progress (Pilorgé, 2020).

From the world market point of view, sunflower is the third oilseed produced in the word, with 45 MT per year on the period 2014–2018, representing 9% of the global production, preceded by soybean (60%) and rapeseed (12%), whereas it comes fourth on vegetable oils since the first position is occupied by palm oil.

Sunflower production occurs in a limited number of countries, for two thirds in Europe, mainly Ukraine, Russia, Romania, Bulgaria, Turkey and France. The other major producing countries are Argentina, China, United States, and the South-Eastern part of Africa (South Africa, Tanzania, Uganda, Zambia). The acreage was important in India but dropped drastically from 2.35 in 2006 to 0.5 ten years later and 0.28 Mn ha in 2019 (FAO, 2020).

The top ten countries Ukraine, Federation of Russia, Argentina, China, Romania, Bulgaria, Turkey, Hungary, France and USA represent 84% of the production and 76% of the acreage on the 2014–2018 period. Considered as a single block, the European Union would come in third position after Ukraine and Russia.

Italian acreage increased significantly from the mid-eighties, overcoming 200.000 ha in the mid-nineties, and later stabilizing around 120.000 ha. The main sunflower producing regions in Italy are those in the middle part, Marche, Toscana, and Umbria, with about half of the total acreage,

whereas other regions with increasing interest are Emilia and Piemonte. In the last few years acreage has been shifting more in northern part, also because of higher yields (Canali, 2021).

In the main producing countries yields have shown continuous increase at an important rate: in the last 15 years increases between 25 and 65% were observed, with average annual gains from 1.4% to 3.5%. Nevertheless, variability among years is rather high and tends to hide the overall trends. This variability is probably due to the effect of meteorology, pathogens and pests in single regions. It is for this reason that a large part of the breeding effort is devoted to introducing resistance genes and on improving quantitative traits.

Oil extraction usually occurs in the countries where achenes are produced, which tend to keep the added value of the process. In fact, only about 5% of the seed is exported, compared to 38% of the meals and 55% of the oil obtained. Besides economic reasons, this peculiarity may be explained by the relatively low density due to the large incidence of the hulls, which make sunflower seeds 15–20% bulkier compared to rapeseed and soybean).

Sunflower meals are appreciated as a source of proteins in feed. Non dehulled meals have a rather high fibre content which limits their use only to some livestock, whereas dehulled meals are among the best feed proteins and the utilization of the hull as biofuels or biomaterials makes this option feasible (Ammassari, 2020).

In some regions of northern Italy, like Veneto, the cultivation of arable land in the plains has found in the last decades different uses from traditional food crops, particularly because of the introduction, in the European Union (EU Council, 1994), of the obligation to set aside a part of the object surface request for compensation. These surfaces have become available for innovative agricultural activities tending to non-food productions. Among the productions that found greater interest among operators, oilseed crops to produce biofuels stand out.

The results that emerged seem interesting for a large-scale development of sunflower cultivation, even though there are some constraints, such as the cost of the raw material, the conversion technologies, and some other conditions (stability of supplies in qualitative and quantitative terms, etc.). However, these problems are surmountable, especially if we consider the wide possibilities for improving efficiency deriving from research activity in a highly innovative sector, such as that of bioenergy (Trestini et al., 2019).

2.2 DOMESTICATION AND BREEDING

The evolution of crop plants can be viewed as occurring in distinct phases (Burke et al., 2005). During the initial phase of domestication from its wild progenitor, the proto-domesticate typically experiences changes in a suite of traits collectively known as the "domestication syndrome" (Harlan, 1992). After this initial period of domestication, crop lineages often experience selection for adaptation to diverse environments and then they undergo selection for traits such as yield, quality, and disease resistance. Sunflower was domesticated as a source of edible seeds and then developed into an important oilseed crop (Putt, 1997). Different to what has been observed in other crops, in the case of sunflower domestication appears to have been driven by selection on many loci having small to moderate phenotypic effects (Burke et al., 2002).

Plant breeding in sunflower can be traced back to the early twentieth century in the Soviet Union. The Krasnodar station was funded in 1912, and then became the All-Russia Research Institute of Oil Crops (VNIIMK), still operating. There the funder, Vasily S. Pustovoit, using recurrent selection and the 'half seed' method (testing one half of the seed and keeping the other for planting, was able to increase the oil content from 28-32% up to 54% in the fifties, together also with yields. Among these varieties, Peredovik, VNIIMK 8931, Majak, which became the basis of the subsequent breeding for oil content all over the world (Goryunova, 2019).

A further mayor lip forward in the development of high-yielding varieties was achieved by the exploitation of heterosis. This was made possible on a large scale by the discovery of male sterility

systems; first the one based on a recessive gene (Leclerc, 1966), and then, more importantly, that based on the interaction between mitochondrial genes encoding cytoplasmic sterility factors (PET1-CMS; Leclercq, 1969) and fertility restorers, both from the wild relative *H. petiolaris* (Kinman, 1970). Currently, this is the usual way of the hybrid seed production worldwide.

As in other crops, also in sunflower yield is a complex trait resulting from the expression of various quantitative characters, characterized by low heritability, due to the strong effect of the genotype x environment interaction (Fick, 1978; Fick and Miller, 1997).

The diameter of the head, the thousand seeds weight and the number of seeds per head are commonly used as selection criteria for increasing yield (Ahmad et al., 1991). Furthermore, the yield is also influenced by the length of the flowering cycle, which is a character primarily involved in adaptation to the environment.

The oil content of sunflower seeds is characterised by a relatively high heritability but can be negatively influenced by the lack of water during the reproductive phase (Nel et al., 2002). The oil of commercial varieties generally contains unsaturated fatty acids in a high proportion (about 90%), of which 20% oleic acid (C18: 1) and 70% linoleic (C18: 2). The remaining 10% corresponds to saturated fatty acids, mainly palmitic and stearic. However, the composition in oil is largely influenced by environmental factors, first of all water availability: it seems that the fraction of unsaturated fatty acids grows with increasing water availability and with the precocity of the sowing date (Anastasi et al., 2010). Even if the oil with a high content of linoleic, obtained from the genotypes traditionally cultivated, is still considered to be of good quality for consumption, the development of high oleic cultivars is an important objective considering its higher stability to oxidation, particularly important in the food industry.

Another trait object of the selection for genetic improvement is the resistance to important pathogens of this crop, in particular rust (*Puccinia helianthi*) and downy mildew (*Plasmopara helianthi*). In addition, resistance to broomrape (*Orobanche* spp.) is an important breeding

objective, due to the severe problems posed by this parasite plant to the sunflower crop in many parts of the world, even if it is not relevant in Italy.

A further means for controlling this weed is the use of herbicides; in this respect, the incorporation of herbicide tolerance genes, namely to imidazolinones (IMIs) and sulfonylureas (SUs), which inhibit acetolactate synthase (ALS) activity, is already a fact also in sunflower.

Molecular markers have opened new possibilities for sunflower breeding. Once single genes or chromosomal regions that contain the genetic determinants (quantitative trait loci, QTLs) of a trait of interest are identified, it is possible to use linked markers for their assisted selection (marker-assisted selection, MAS). In sunflower QTLs have been identified that control important agronomic characteristics (thousand seeds weight, number of seeds per head and earliness), oil quantity and quality, resistance to the major pathogens, such as downy mildew (*Plasmopara halstedii*), rust (*Puccinia helianthi*), and white mold (*Sclerotinia sclerotiorum*), and resistance to broomrape (*Orobanche* spp.). Recently, exhaustive reviews have been produced on the status of marker development and utilization for assisted breeding by Dimitrijevic and Horn (2018) and Rauf et al. (2020).

This notwithstanding, breeding for complex polygenic traits is still very challenging. First of all, it requires a precise phenotypic evaluation in order to identify the genomic regions involved in their control; in this direction, platforms for high-throughput phenotyping are being developed for sunflower.

In recent years, high throughput genotyping platforms (e.g., SNP arrays) have been established in sunflower. This will allow GWAS (genome wide association studies) to identify genomic regions involved in complex trait control and on the subsequent selection at the whole genome level. The publication of the sequence of the sunflower genome (Badouin et al., 2017) has provided a further tool for facilitating the breeding efforts.

2.2.1 FERTILITY RESTORER GENES

Sunflower is the second biggest crop after maize cultivated through hybrid seed (Dimitrijevic and Horn, 2017). As explained above, commercial hybrid seed production is dependent on cytoplasmic male sterility (CMS) and male fertility restorer (RF) lines. The female parent (A) is unable to produce functional pollen because CMS is present in its mitochondrial genome, whereas the male parent (C) carries a dominant fertility restorative gene (Rf) in homozygous state in its nucleus. The resulting F1 hybrid is therefore fertile due to the restorative allele of the male parent. A satisfactory restoration of fertility is necessary for high grain filling percentage. The maintenance of the female parental is ensured by pollination with an isogenic line (B) (called maintainer) which differs from the female just for having a normal male-fertile cytoplasm.

More than 70 cytoplasmic male sterility sources are known for sunflower, but only those having with suitable restorer genes can be used in practice. Most of the hybrid sunflower breeding depends on the *PET1* and *Rf1* genes from *H. petiolaris* described by Leclerq (1969) and Kinman (1970), therefore diversification is an urgent breeding goal to reduce genetic vulnerability to diseases and pathogens. Recently a cluster of fertility restorer genes has been identified which are compatible with different CMS sources (Talukder et al., 2019).

2.2.2 GENETIC ARCHITECTURE OF SUNFLOWER GENOME

Genomic data has aided in the elucidation of the evolutionary history of sunflower, and the genetic architecture of at least two important traits (flowering time and the metabolism of oil content) is now better understood (Badouin et al., 2017; Bonnafous et al., 2018). Genome sequences of several of breeding lines showed that the cultivated pan genome is comprised of 61 205 genes, and 27% of these genes vary between breeding lines. A small percentage (1.5%) of the genes are introgressed from wild species, and majority of these genes induce biotic resistance in sunflower. A genetic analysis of male and female lines used in development of sunflower hybrids and compared to open pollinated varieties (OPV) showed male lines had a higher percentage of introgressed genes from

wild species than did the females or OPVs. Genetic analysis of male and female lines also revealed differentiation for biotic resistance, which was complementary to provide better resistance in hybrids.

2.2.3 SSR SUNFLOWER FINGERPRINTING

Among the different marker systems currently available, simple sequence repeat (SSR) are an excellent one for plant variety description and identification because they are simple to use, have usually a prominent level of polymorphism, good coverage of the genome, and quality of information (single locus, co-dominant, reproducible). Recently, SSR markers have also been developed and used for genotyping inbred lines and genetic mapping in the sunflower. Until now, sunflower genotyping studies have only been carried out on a limited number of inbred lines: the highest number analysed by Zhang was 26 (Gentzbittel L., Vear F., Zhang Y.-X., Berville A. and Nicolas P. (1995). Development).

Actually, the method granting better analytical performances in SSR markers' identification is capillary electrophoresis. The main advantages deriving by the use of such experimental technique are listed below:

- Fully automated DNA fragment analysis;
- Ready-to-run gel cartridges;
- Fast processing: 12 samples in 5–12 min;
- Up to 96 samples per run;
- Sample input amounts < 0.1µl;
- Detection limit of 0.1 ng/µl;
- High resolution of 3–5 bp;
- Digital data output.

2.3 FATTY ACIDS

Fatty acids (FAs) are the fundamental building blocks of lipids and form the major component of the body fat. They consist of hydrophilic carboxylic group (head portion) to which lipophilic long chain of alkyl groups (tail portion) is attached. The tail usually contains an even number of carbon atoms. Each fatty acid differs in their chain length, degree of saturation, and position of double bond.

Table below details of the chain length and number and position of double bonds in FAs used as lipid-based excipients.

Fatty Acid Chain Length (Number of Carbon Atoms)	Number and Position (Δ) of Unsaturated Bonds	Common Name	Melting Temperature (°C)
8	0	Caprylic acid	16.5
10	0	Capric acid	31.6
12	0	Lauric acid	44.8
14	0	Myristic acid	54.4
16	0	Palmitic acid	62.9
18	0	Stearic acid	70.1
18	1 Δ 9	Oleic acid	16.0
18	2 Δ 9,12	Linoleic acid	− 5.0
18	3 Δ 6,9,12	γ-Linolenic acid	− 11.0
18	1 Δ 9 (− OH:12)	Ricinoleic acid	6.0
20	0	Arachidic acid	76.1
22	0	Behenic acid	80.0

Based on the number of carbon atoms in the alkyl chain length, FAs can be classified into short-chain (2–4), medium-chain (6–10), and long-chain FAs (12–26).

2.3.1 SATURATION OF FAS

Besides chain length, FAs are distinguished by the number of hydrogen atoms they carry. Those fully loaded, saturated, contain the maximum number of hydrogen atoms they can hold. A fatty acid becomes unsaturated when a pair of hydrogen atoms is removed, thereby creating a double bond between the adjacent carbon atoms where the hydrogens disappeared. Specific enzymes

carry out the desaturation process but only at certain carbon sites. Thus, the location of double bonds is tightly controlled.

The more double bonds a fatty acid has the more unsaturated it is. FAs with one double bond are called monounsaturated. Those with two or more double bonds are polyunsaturated. By convention, FAs can be abbreviated as in this example: 18:2 ω-6, for linoleic acid. Left of the colon is the number of carbon atoms; after the colon is the number of double bonds. The omega and number give the location of the first double bond counted from the methyl end. Often, they are abbreviated without the omega description, e.g., 18:2.

Free rotation around the carbon-carbon bond in the saturated FAs gives the hydrocarbon chain great flexibility; thereby, the steric hindrance is low. These atoms/molecules are packed together by van der Waals forces in crystalline arrays, whereas in unsaturated FAs, the presence of cis double bonds causes bend in the hydrocarbon chain that does not favour tight packing among the atoms/molecules. Thus, their interactions among the molecules are weak, with lower melting points than saturated FAs of similar chain lengths. The melting points of the FAs are also influenced by the length of the hydrocarbon chain. The saturated FAs from 12:0 to 24:0 have a waxy consistency at room temperature, whereas unsaturated FAs with similar carbon chains exist as oily liquids. As animal fats contain a higher number of saturated FAs than vegetable oils, their melting points are higher than the vegetable oils.

Short-chain FAs are water-soluble and absorbed directly from the intestine into the bloodstream. They are usually metabolized for immediate energy needs and are not abundant in most foods, except dairy products. Nutrition and health considerations have emphasized the long-chain FAs with 14 or more carbons. These are stored in membranes as phospholipids and in adipose tissue as triglycerides. In addition to providing energy, long-chain polyunsaturated FAs are constituents of cell membranes where they are available as precursors for several metabolic pathways. They also

have a pronounced effect on membrane protein function (Murphy 1990). It is their behaviour in tissue membranes that markedly influences health.

2.3.2 SUNFLOWER SEED IN HEALTH AND NUTRITION

Sunflower seeds are used to produce oil and can also be consumed directly - sprinkled over cereals, salads, and soups - and mixed with vegetables and snacks, in particular those obtained from varieties of the *confectionary* type. They are a particularly useful dietary supplement, being rich in FAs and used as an ingredient in many pharmaceutical preparations. In fact, they have diuretic and expectorant properties, and are now highly valued for the treatment of bronchial, laryngeal, and pulmonary infections, coughs, and colds. They are also used to reduce the risk of colon cancer, the severity of hot flushes in women going through menopause, and diabetic complications. Sunflower seed oil is used internally to alleviate constipation, as a lubricant, and is used externally as a massage oil, an oil dressing, and in the treatment of skin lesions, psoriasis, and rheumatism. The oilcake is a valuable food for cattle and poultry. The oil is used in cooking, soap, lubricants, and candles, and as biodiesel and biofuel (Adeleke and Babalola, 2020; Pal, 2011)

2.3.2.1 Oleic Acid

Development of high oleic acid lines is an important breeding objective for sunflower. Historically, sunflower contains about 18–25% oleic acid. Because oleic acid is beneficial to human health, the high oleic acid trait was created through mutation breeding and then introgressed into new hybrids. “Pervenet” breeding lines contain a dominant mutation, which increases oleic acid content to more than 89% in the sunflower oil. Commercial varieties with high oleic acid content are available and now account for up to 4% of the total sunflower oil production and enjoy a premium in price. Selection for high oleic acid is expensive and slow due to the laborious gas chromatography and nuclear infrared resonance protocols. The use of molecular markers could facilitate selection in early segregating generations, and only desirable plants would be carried forward into the next generation. Primer sets such as NI-3F/N2-IR have been used successfully to select for the defective

version of the A12- desaturase or FAD2-1D gene which causes the accumulation of important levels of high oleic acid in the sunflower seeds. The primers are perfect markers for this trait because they are completely linked to the causal mutation. This allows genotypes to be selected in all segregating material for which the trait exists and simplifies the use of this trait.

2.3.3 CURRENT METHODS FOR FATTY ACID DETERMINATION

Gas chromatography (GC) is currently used for FAME quantitative analysis. During the development tests of the method for the determination of methyl esters of fatty acids (FAME) in sunflower oil, a check of the state of the art of the technical-scientific literature was performed. The first tests were carried out according to the official methods described by *Commission Regulation No. 2568/91 (EU Commission 1991)* and *Commission Implementing Regulation No. 2015/1833 (EU Commission 2015)* (described for olive oil matrix) as a reference. In addition, the standardised ISO method 12966:2015 (ISO 2015), which is generic for different kind of lipids, was considered, but was discarded in the early study phase due to the high toxicity of trimethylsulfonium hydroxide, which is used for the trans-esterification.

2.4 ORGANIC AGRICULTURE

Organic farming is an agricultural method aimed at producing food with natural substances and processes. This means that it tends to have a limited environmental impact, as it encourages: use energy and natural resources responsibly, conserve biodiversity, to preserve regional ecological balances, improve soil fertility, maintain water quality.

In addition, organic farming rules promote animal welfare and require farmers to meet the specific behavioural needs of animals.

The EU regulations on organic farming are designed to provide a clear framework for the production of organic products across the EU. The intent is to satisfy consumer demand for reliable organic products, while creating a fair market for producers, distributors and retailers.

For farmers to benefit from organic production methods, consumers need to have confidence in compliance with organic production rules. Therefore, the EU maintains the following strict control and enforcement system to ensure that the rules and regulations regarding organic products are properly adhered to. As organic farming is part of a wider supply chain, which includes the food processing, distribution and retailing sectors, these are also subject to controls.

This regulation governs all sectors of organic production and is based on a series of fundamental principles, such as: ban the use of GMOs, prohibit the use of ionizing radiation, limit the use of artificial fertilizers, herbicides and pesticides, prohibit the use of hormones and limit the use of antibiotics, to be used only if necessary for animal health.

This means that organic producers need to take different approaches to maintain soil fertility and animal and plant health, including: crop rotation, favour the cultivation of nitrogen fixing plants and other green manure crops to restore soil fertility, prohibit the use of mineral nitrogen fertilizers, to reduce the impact of weeds and pests, organic farmers choose resistant varieties and breeds that encourage natural pest control, encourage the natural immune defences of animals, to protect animal health, organic producers must prevent overcrowding.

One of the goals of organic production is to reduce the use of external inputs. All substances used in organic farming to combat harmful organisms or plant diseases must be approved in advance by the European Commission. In addition, specific principles guide the approval of external inputs, such as fertilizers, pesticides and food additives, so that only substances and compounds listed as approved in specific legislation can be used in organic production.

Processed foods are mainly obtained from exclusively agricultural ingredients (no account is taken of the added water and cooking salt). They can also contain preparations based on microorganisms

and enzymes, additives of trace minerals, processing aids and flavourings, vitamins, as well as amino acids and other micronutrients added to food products intended for specific nutritional purposes may be used, but only with prior authorization in accordance with agricultural regulations.

The use of substances and techniques intended to restore the properties lost in processing or storage is not allowed to remedy negligence in processing or that could otherwise mislead the true nature of the products. Non-organic agricultural ingredients can only be used if authorized in the annexes to the legislation or if they have been provisionally authorized by an EU country.

According to Regulation EU 2021/279, traceability of products deriving from organic agriculture is granted by the presence of a national register, where information on the whole chain is entered. This work aims at developing an analytical method able to verify from an experimental – not only documental - point of view the variety and traceability of organic sunflower marketed by Agricola Grains S.p.A. This approach shall include an experimental component related to the identification of genotypes, through molecular research and a second analytical component related to the characterization of the acid facts composition specific for the matrix of organic sunflower.

2.4.1 SUNFLOWER PRODUCTION IN ORGANIC FARMING

The organic sunflower has shown the convenience of this crop in the first and second harvest. The starting point for growing an organic sunflower is the variety choice, considering varieties with high and low oleic content, and making careful considerations on the sowing time, on different agronomic inputs and on the different irrigation availability. The varieties currently available on the market consist of hybrids that differ in the ripening cycle and in the composition of the FAs of the seed. According to the ripening cycle, the hybrids are divided into 3 classes: early, medium and late. The mediums can be further divided into medium-early and medium-late; these two classes are currently the most widespread, since they have the best characteristics both in terms of agronomic characteristics and production levels.

The soil must be worked deeply, to allow a good water retention capacity: normally an autumnal plowing 40-50 cm deep is done; however, a more effective operation is a plowing accompanied by subsoiling. An early preparation of the seedbed is certainly a very useful operation if you want to sow early. Harrowing and grubbing are then carried out during the winter period, so that atmospheric agents have the opportunity to refine the surface layer of soil. This type of processing is not suitable for loamy or sandy soils, which are however not very suitable for the cultivation of this species as they tend to be sterile and not very capable of retaining water.

The ideal sowing period is around the first half of May, and you can also consider the opportunity of a second harvest, sowing until 1 July and threshing in mid-late October. The key factor is to divide the fertilization interventions into three stages: at the bottom, at sowing, in weeding.

The products to be used are digestates and liquid effluents, pelleted and microgranular products, green manure.

Foliar fertilization before the formation of the flower button is recommended to maximize yields. The cost of fertilization, depending on the more or less extreme cultivation path, varies from 150 to 350 euros / ha.

Thanks to the rapidity of development, the sunflower normally has a suffocating effect on weeds; however, it must be protected in the early stages of the cycle. The most frequent weeds are those of corn and chard: *Anagallis arvensis, Polygonum aviculare, Sinapis arvensis, Solanum nigrum, Stachys annua, Chenopodium album, Ammi majus, Setaria viridis, Echinochloa crus-galli*.

The struggle is carried out with weeding, as long as the height of the plants allows it (40 cm).

Harvesting is done when the achenes, whose water content is less than 10%, easily detach from the capitulum; this occurs about 15-20 days after ripening. Complete ripeness is reached when the capitulum and leaves are dry, and the stems are brown in colour. In Italy the sunflower is harvested

from mid-August (in the hottest areas) to mid-September, using adapted wheat combine harvesters or with maize header equipped with a divider for each row.

Yields vary according to the number of plants per surface unit, the number of flowers of the capitulum, the average weight of an achene. The expected yields without irrigation are:

-with sowing at the beginning of May, three fertilizations and harvesting at the beginning of September, the average yield obtained is 3.5 t / ha (Bartolini (2019).

-with sowing in mid-May, fertilization based on digestate only at once and harvested in mid-September, the average yield obtained is 2.9 t / ha.

-with sowing at the beginning of June, ground and foliar fertilization, harvested at the end of September, the average yield obtained is 1.8 t / ha.

3 AIM OF THE STUDY

The experimental activity was concentrated on two objectives:

- the implementation of molecular markers for the genetic traceability of sunflower varieties and the seed obtained thereof, in particular for the routine check of the supplies of raw materials that are delivered for oil extraction, and
- the improvement and validation of a gas-chromatographic method for the determination of FAs composition in sunflower oil.

Regarding variety verification and traceability, the marker systems evaluated were the following:

- SSR markers (12) arranged in two multiplex sets
- SCAR markers for the verification of cytoplasmic male-sterility (*Pet1*) and of fertility restoration factor (*Rf1*)

In addition, in order to allow routine application in an industrial setting, two aims were pursued:

- Development of a suitable protocol for DNA extraction from single seeds
- The implementation of a semi-automated capillary electrophoresis system for fragment analysis of the a.m. markers.

The development and validation of a new GC/FID analytical method for the determination of fatty acids (FAME) in sunflower achenes for improving the quality and efficiency of the analytical workflow in the control of raw and refined materials entering the production chain of Agricola Grains S.p.A. The method will be compared with the currently available reference method based on the evaluation of its performance and suitability to be implemented in routine testing activity.

The analytical performances that are object of validation of the new implemented method are: response linearity, limit of quantification (LOQ), specificity, preciseness, intra-laboratory preciseness, robustness, BIAS. These parameters are used to compare the new developed method and the one considered as reference - Commission Regulation No. 2568/91 (EU Commission 1991) and Commission Implementing Regulation No. 2015/1833 (EU Commission 2015).

4 MATERIALS AND METHODS

4.1.1 MOLECULAR ANALYSES

4.1.2 PLANT MATERIALS

The plant materials used in this study for molecular analyses are shown in Table 1. They include:

- a panel of18 sunflower inbred lines of different origin, all male fertile, including both maintainer and fertility restorer genotypes. They were selected from the collection held at LaRAS to be roughly representative of the diversity of the cultivated germplasm, and they had previously been chosen for some physiological studies in that laboratory (germination in response to sub-optimal temperature conditions);
- a panel of 26 F1 hybrids cultivated in Italy in the last decade, some of which also of current interest for AG.

In the following description, the botanically correct term of “achenes” was replaced, for of simplicity, by the more practical “seeds”. The seeds of all accessions used in this work were stored in paper bags at 15 °C, 50% RH, in the dark.

4.1.3 DNA EXTRACTION

DNA was performed from seedlings, from individual seeds, and from bulked seeds.

For development of the PCR assays high quality DNA of inbred lines was extracted from five seedlings of each genotype. A disk of foliar tissue of about 1 cm^2 was cut, lyophilised, and ground with alumina scales (<2mm) in 1.2 ml collection tubes (Qiagen) tubes held in 96-well plates by means of a Mixer Mill MM 400 (Retsch, Haan, Germany), at a frequency of 30 Hz for 3 min for each side of the plate. The extraction procedure based on CTAB was as described by Saghai Maroof et al.

(1984), with minor modifications. Part of the DNA extracted from the 5 seedlings per line were then mixed to create a composite sample.

DNA of F1 hybrids and their progeny was extracted from individual seeds with the method developed in this research. In order to facilitate grinding, seeds were kept overnight in a freeze drier, then either used directly or after manual dehulling with the help of a scalpel. Embryos were put in the same collection tubes as used for leaves, but containing 2 3-mm stainless steel balls, one of which at the bottom of the tube, the other above the seed to facilitate disruption. One hundred and fifty ml of CTAB extraction buffer were added to the tube, and grinding was performed on the Mixer mill at the same conditions as described above. Two CTAB-extraction protocols were evaluated differing for having one or two purification steps with chloroform.

For extraction from bulk samples at AG laboratory, 20 g of seeds deprived of the pericarp were finely ground for 1 min at max speed using the Retsch GM200 homogenizer. Extraction was performed on an automatic MagCore extractor with a specific Genomic DNA Plant Kit extraction kit, also based on CTAB, following the recommendations of the manufacturer. Sixty mg of the flour obtained by grinding was weighed in a 2ml vial, 400ul of GP1 Buffer and 5ul of RNase were added, the sample was mixed by vortexing and incubated at 65 °C for 20 minutes under stirring. Five ul of Proteinase K were added, followed by re-incubating for 20 min at 65 °C for under stirring. One hundred ul of GP2 Buffer were added, and then incubated on ice for 3 minutes. The filter column was placed inside a 2ml vial without a cap, the entire lysate was transferred over the column, and centrifuged for 3 minutes at 13000rpm. The filter column was discarded and the eluate of about 400ul gently transferred into the specific vial for the MagCore extractor. Extraction was carried out by selecting the 301 MagCore program, specific for plant matrices.

Extracted DNAs were suspended in ultrapure, sterile water and quantified spectrophotometrically (BioSpecNano, Shimadzu). Before use in PCR, aliquots at 40ng /µL were prepared.

4.1.4 SSR ANALYSIS

In this work, two primer sets (Set 2 and Set 3) of those described by Tang et al. (2003) were used (Table 2). Those Authors assembled PCR-multiplex reactions based on primer compatibility, similar melting temperatures, non-overlapping allele size range, map position, and informativeness. The table lists the primer sequences, their map position of the loci and the repeat unit. The 12 markers are all dinucleotide repeats and explore 11 of the 17 sunflower chromosomes (ORS844 and ORS1178 are both located on chromosome 11).

Two platforms were used for fragment analysis:

- LI-COR 4300 DNA Analysis System (from here on LI-COR), an acrylamide gel-based system equipped with a highly sensitive infrared fluorescence detection technology, available at LaRAS;
- QIAxcel Advanced (from here on QIAxcel), an automatic capillary electrophoresis system based on prefilled gel cartridges and ethidium bromide staining, available at the Agricola Grain laboratory.

For both systems, optimal PCR mixture composition was determined by trial and error, mainly by adjusting progressively concentration of the different primer pairs, with the aim of obtaining amplicons of similar intensity among different loci. Starting primer concentration was 0.05 μM for LI-COR, 0.5 μM for the QIAxcel system. More details are given in the RESULTS section.

For analysis on LI-COR, which detects fragments labelled with infrared chromophores, forward primers were modified by adding the 19-bp universal M13-tail to the 5' ends (Oetting et al., 1995; Table 2), and concurrently an M13 primer, end-labelled with either 700- or 800-IRDye®, was present in the PCR mix. According to the final protocol (Table 3), DNA samples are amplified in a 25 μl-PCR reaction mixture containing 1X PCR buffer, 2 mM MgCl2, 0.25 mM each dNTPs, from 0.025 to 0.1 μM each primer, 0.16-0.17 μM fluorescently labelled M13 primer, and 1 unit of AmpliTaq Gold® DNA Polymerase (Life Technologies). "Touchdown" PCR (Don et al., 1991) is used to minimise

spurious amplification, with an initial denaturation step of 15 min at 94°C, followed by 1 cycle of 94°C for 1 min, 65°C for 30 s and 72°C for 1 min. The annealing temperature is then decreased 2°C per cycle in the following 7 cycles until reaching 51°C. Subsequently 27 cycles at 94°C for 1 min, 55-57°C for 30 s, and 72°C for 1 min are performed, with a final extension at 72°C for 10 min.

For analysis on QIAxcel apparatus, markers were first verified in simplex PCRs, and then multiplex reactions were optimised starting from protocol reported in Dean et al. (2013) by evaluating mainly the effects of primer pairs concentration and Taq polymerase on performance. The established amplification mix contains 1X PCR buffer, 2 mM MgCl2, 0.25 mM of each dNTP, from 0.13 to 1 µM of each primer and 1 U of AmpliTaq Gold® DNA polymerase in 20 µl reaction volume (Table 3). The PCR thermal profile used included an initial cycle for Taq activation (94 °C for 15 min), followed by 40 cycles consisting of 60 s at 94 °C, 30 s at 55 °C (for SET2) or 30 s at 57 ° C (for SET3) and 1 min at 72 °C, with final extension at 72 °C for 10 min.

4.1.5 DUPLEX PCR FOR PET1 AND RF1

Regarding the *Pet1* and *Rf1* assays, developed by Rieseberg et al. (1994) and Horn et al. (2004), respectively, an attempt was made to combine them into a single duplex assay that could conveniently be analysed on LI-COR system instead than on agarose gels. For this reason, starting from sequences of the CMS-PET1 fertile (GenBank n. X53537.1) and of CMS-PET1 sterile (GenBank n. X55963.1), a new common forward primer and the sterile cytoplasm specific reverse primers were designed (Table 4).

One hundred twenty ng of template DNA (genomic and cytoplasmic) were used in a PCR mix of 20 µl containing: PCR buffer 1X, $MgCl_2$ 2 mM, 0.2 mM for each dNTP, 10 nM for each of the three CMS *Pet1* primers (CMS1-F-tailed, CMS5-R, and orfH873-R), 300 nM for each of the two Rf1 primers (HRG01-F-tailed e HRG01-R), 100 nM of the universal M13 labelled primer, 0.6 U AmpliTaq Gold® DNA polymerase (Life Technologies).

4.1.6 ELECTROPHORESIS

For preliminary checking of amplification products, electrophoresis was performed in agarose gels 1.8% in 1 X TBE buffer containing 1:10,000 SYBRSAFE fluorescent dye. Products were detected on an UV transilluminator and photographed.

For separation on the LI-COR system PCR products were diluted between 1:10 to 1:20, according to their strength, in loading dye (98% formamide, 0.02 mM EDTA pH 8, 0.08% bromophenol blue). Electrophoresis was performed on 5% polyacrylamide gels, containing 1X TBE and 6 M urea, using 25 cm plates, 0.25 mm thick spacers, and rectangular combs (64 wells). Gels were pre-run for 10 min (1200 V, 40 W, about 47°C), then wells were carefully flushed with a syringe to remove excess of urea. Samples were denatured 8 min at 95°C and then kept on ice until loading. Eight µl of each sample were loaded into the gel using a 8-channel Hamilton syringe. A 50-700 bp molecular weight standard (700/800-IRDye®) was always loaded at least at both ends of the gel and in the middle. Electrophoresis was run at 1300 V for 2.5 h. After each run, usually the gel could be re-loaded with a new set of amplification products, with a maximum number of 3-4 subsequent loadings without appreciable loss of resolution power. Gel images were collected by e-Seq software v3.0 (LI-COR Biosciences), saved as TIFF files (Tagged Image File Format), and subsequently analysed using the software CARESTREAM Molecular Imaging to determine amplicon size.

With the QIAxel system, the QIAxcel DNA (1200) High Resolution Cartridge was chosen as it provides rapid and accurate separation of DNA fragments ranging from 15 bp to 10 kb. For optimal determination of the DNA fragment size using the QIAxcel DNA High Resolution Kit, especially for microsatellite analysis, an alignment marker and a QX Size Marker with a fragment size like the size of the amplicon samples were selected. Given the size of the microsatellite amplicons, it was chosen the alignment marker of 15 bp/1 kb. In some experiments a 15 bp/3 kb was used. The alignment marker is injected from the “Marker 1” position in the buffer tray and co-migrates with the DNA samples and it allows to calibrate variations of migration times across all the channels. Any DNA

size marker can be used if the size of the amplicon falls within the smallest and largest fragments of the reference DNA size marker. We use the QX Size Marker 25bp – 500bp at a final concentration of 10 ng/ µl (as suggested by provider) for size determination. PCR products were prepared with a minimum volume of 10 µl, but only 0.1 µl of the sample are injected by the system into single capillary. The remaining sample was stored at 4°C and re-loaded if necessary. Since the concentration of DNA template used was in the range 10-100 ng/µl and is PCR products amplified from genomic DNA with 30-40 cycles, it was chosen to use the method OM500 but with a modified sample injection time from 10s to 30s. Before each race, a purge of the lines was done.

4.1.7 DATA ANALYSIS

Scoring of gels obtained with LI-COR was performed using the CARESTREAM Molecular Imaging Software ver. 5.0 (New Haven, CT). Bands were automatically detected, artefact were manually eliminated, and their final position was adjusted when obviously altered in their run. Amplicon sizing (in bp with one decimal digit) was performed on the basis of the standard ladder and data were exported to a spreadsheet where genotypes were arranged in columns and markers in rows (allowing 2 rows for each marker in order to accommodate heterozygous loci). For each accession allele calling was performed manually based on their estimated size and supported by eye examination of gel images, also considering the expected repeat unit.

QIAxcel ScreenGel software, provide data and results displayed in both electropherogram and gel image formats. A unique algorithm calculates and generates a tabular display including number of peaks as well as the size, height, width, and area of each peak. For the analysis of the data produced, the profile “Default DNA v.2.0” was chosen. The molecular weight of fragments is given in bp with no decimal digits. The QIAxcel ScreenGel software has the capability to perform automatic allele calling if a suitable “window” for allele identification is provided. However, considering the multiplex format and the dinucleotide nature of the markers used in this work, automatic allele calling was not attempted. The resolution power of the system and the precision of allele size

estimation obtained using QIAxcel were evaluated attributing to each peak the allele identified analysing the same DNA samples with the LI-COR system.

The genetic relationships among the 16 lines as revealed by SSR genotyping were investigated by cluster analysis applying the UPGMA algorithm to Jaccard similarity values calculated between accessions using the statistical package NTSYS-pc (Rohlf, 1997).

4.2 CHEMICAL ANALYSIS

4.2.1 EXTRACTION OF OIL FROM THE SUNFLOWER SEED

In order to improve a new specific method for sunflower samples, different solvents were used for extraction of fatty acids (FAs). As a solubilizer of sunflower oil, n-hexane, was initially tested, followed by petroleum ether (bp 40-60 °C), and tetrahydrofuran (THF). Trans-esterification of FAs was carried out, in all cases, with 2N potassium hydroxide in methanol. The comparison between different analytical techniques was made according to the approach of Niemi et al. (2019), which performed quantification and profile characterization analyses of microalgal FAMEs using GC-FID and GC-mass spectrometry the tests preparation was carried out considering as starting point the official methods described in Commission Regulation No. 2568/91 (EU Commission 1991) and Commission Implementing Regulation No. 2015/1833 (EU Commission 2015). Experiments were conducted on ERM (External Reference Material) made of sunflower seeds obtained in the framework of interlaboratory trial (BIPEA, FR). For each test 10 seed samples were extracted and analysed. For each sample, 50 g of sunflower achenes was grinded with a ZM 200 Retsch at 3000 speed for 1 min. For the extraction of the oil, the Soxhlet'method was used. Approx 5g of the pulverized sample was transferred into an extraction thimble weighing, an equal quantity of anhydrous sodium sulphate was added and mixed with a glass rod. The rod was carefully cleaned with a degreased cotton swab and the sample was covered with it into the extraction thimble. The thimble was placed in a Soxhlet extractor and extract for 6-8 hours with 40-60 ° C light petroleum. The extract was collected in a dried flask containing 3-4 glass balls previously weighed in an

analytical balance to determine the tare. The solvent was removed by distillation with a rotary evaporator and the residue was dried in an oven at 100 °C at atmospheric pressure for 1 hour and 30 min. After this, the extract was kept dry in a silica gel desiccator until the flask was cooled at room temperature and weighted again for tare measurement (flask with only 3-4 glass balls). The operation was repeated until the weight was constant (= the difference between the two successive values of the gross weight was less than 0.1g).

4.2.2 PREPARATION AND PURIFICATION OF THE FATTY ACID METHYL ESTERS FROM OIL

For all samples, the preparation of the fatty acid methyl esters from oils was performed by transesterification with methanolic solution of potassium hydroxide at room temperature. The need of purification by Solid Phase Extraction (SPE), prior to the trans-esterification, depends on the free fatty acids content in the sample (the purification is to be made if oil acidity is > 2.0%), as well as the analytical parameters to be determined. The samples were purified by passing the oil through a silica gel solid-phase extraction cartridge. A silica gel cartridge (1 g in 6 ml tube) was placed in a vacuum elution apparatus and washed with 6 ml of hexane; the washing was performed without vacuum. Then an oil solution (0.12 g approximately) in 0.5 ml of hexane was loaded into the column. The solution was pulled down and then eluted with 10 ml of hexane/diethyl ether (87:13 v/v). The combined eluates were homogenised and divided in two similar volumes. An aliquot was evaporated to dryness in a rotary evaporator under reduced pressure at room temperature. The residue was dissolved in 1 ml of heptane. In this case, the sunflower oils analysed by the laboratory did not need the phase of sample purification before transesterification because their acidity was less than 2%.

4.2.3 TRANSMETHYLATION

Approximately 0.1 g of the oil sample were weighed in a 5 ml screw-top test tube. 2 ml of heptane were added, and the mixture shacked. 0.2 ml of the methanolic potassium hydroxide solution 2N were then added to the tube, a cap fitted with a PTFE joint was put on and tighten, then shaking

vigorously for 30 seconds. The mixture was let to stratify until the upper solution became clear. The upper layer containing the methyl esters was decanted to obtain the heptane solution ready for injection into the GC. These solutions were kept in the refrigerator until analysed by gas chromatography for no more than 12 hours.

4.2.4 GAS-CROMATOGRAPHY

The separation of the constituents and the duration of the analysis depend on the flowrate of the carrier gas in the column, and on the efficiency and permeability of capillary columns, and both are affected by the original matrix. It was thus necessary to identify the best operating conditions for sunflower oils by adjusting the instrument parameters to improve separation and speed up analysis. Some preliminary experiments were performed to determine the best chromatographic conditions for sunflower oil (data not shown). In these tests, changes were made to one parameter at a time, and a set of new conditions that seemed to be suitable for the separation of FAMEs in sunflower oil.

As reported below, the most relevant modifications of chromatographic conditions concerned: temperature of the oven column, carrier gas hydrogen pressure, total flow and split ratio.

Condition	Official method (olive oil)	Laboratory method (Sunflower oil)
Injector temperature (°C)	250	250
Detector temperature (°C)	250	250
Oven temperature (°C)	165°C (8min) to 210°C at 2°C/min	50°C (7min) to 200°C at 25°C/min and 200°C (8 min) to 230°C at 3°C/min
Carrier gas hydrogen pressure (kPa)	179	101
Total flow (ml/min)	154,0 ml/min	32,5 ml/min
Split ratio	1:100	1:25

Determination of the resolution, R, of two neighbouring peaks I and II, using the formula:

$$R = 2 \times \left(\frac{d_{r(II)} - d_{r(I)}}{w_{(I)} + w_{(II)}} \right) or$$

$$R = 2 \times \left(\frac{t_{r(II)} - t_{r(I)}}{w_{(I)} + w_{(II)}} \right) (USP)(United\ States\ Pharmacopeia)$$

or

$$R = 1{,}18 \times \left(\frac{t_{r(II)} - t_{r(I)}}{w_{0{,}5(I)} + w_{0{,}5(II)}} \right) (EP, BP, JP)(\ European,$$

$$British, and\ \ Japanese\ Pharmacopeia$$

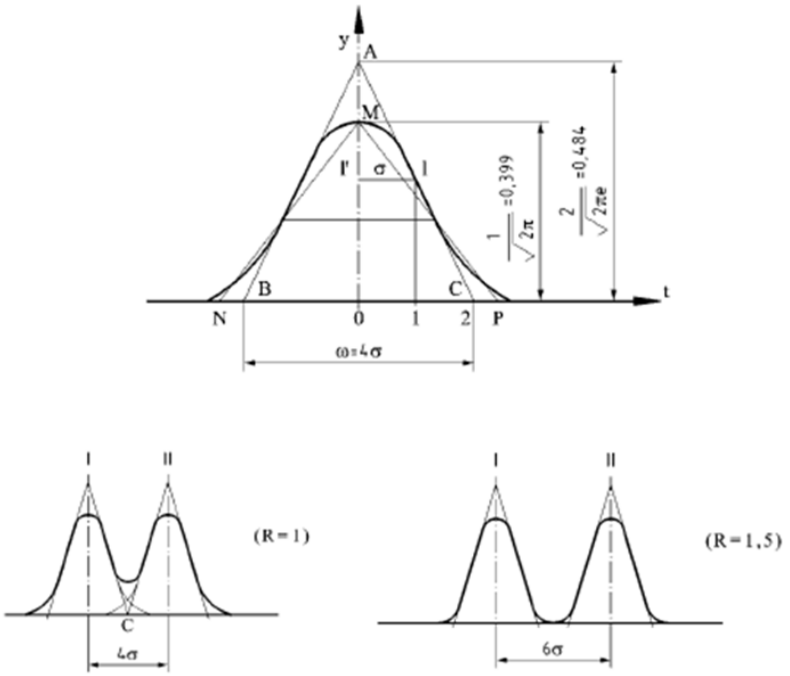
where:

dr(I) is the retention distance of peak I; dr(II) is the retention distance of peak II; tr(I) is the retention time of peak I; tr (II) is the retention time of peak II; ω(I) is the width of the base of peak I; ω(II) is the width of the base of peak II; ω0,5 is the peak width of the specified compound, at mid-height of the peak;

If ω(I) ≈ ω(II), calculate R using the following formulas: R = (dr(II) – dr(I))/ω = (dr(II) – dr(I))/4σ where: σ is the standard deviation.

If the distance dr between the two peaks dr(II) - dr(I) is equal to 4σ, the resolution factor R = 1. If two peaks are not completely separated, the tangents to the inflection points of the two peaks intersect at point C. In order to completely separate the two peaks, the distance between them must be equal to: dr (II) - dr(I) = 6 σ from where R = 1,5.

The laboratory method, specific for sunflower oils, improved the chromatography resolution (as shown below) in order to achieve - for all the detected FAMEs - R>1,5.



4.2.5 STATISTICAL ANALYSIS FOR METHOD VERIFICATION

The analytes considered for the method verification in sunflower oil from ground seeds were the following FAs: palmitic, palmitoleic, heptadecanoic and heptadecenoic, stearic, oleic, linoleic, linolenic, arachidic, eicosenoic, heneicosenoic, behenic, lignoceric, nervonic. The instrument used was GC-FID with 10 µl of sample injection. Initial validation experimental protocol with representative matrix sample (sub-sample from a homogenized sample); Reagent blank; 2 different representative samples of the sunflower matrix.

The instrumental sequence of chemical calibration (initial verification) was represented by: calibration standard in solvent with Certified Reference Material (CRM) or Reference Material (RM) of fatty acid methyl esters (3 or more levels), blank reagent (solvent), 5 extractions of 2 representative sunflower samples (oil and ground seed) for trueness, 10 extractions of 3 representative sunflower samples (oil and ground seed) for precision, 2 representative sunflower samples (oil and ground seed) for reproducibility carried out with different times, operators,

solvents, equipment, calibration standard in solvent with CRM / RM of fatty acid methyl esters (3 or more levels), double-trial for operator qualification.

From the data obtained, the parameters of the following table were determined and verified according to the corresponding criteria, respecting moreover the absence of heteroskedasticity, i.e. verifying that the errors variance was not constant across observations.

PARAMETER	HOW/WHAT	CRITERIA
LINEARITY	Through the calibration curve	Residues < 20%
LOQ	Determine the lowest level of area at which it is demonstrated that the criteria of trueness and precision are met	≤ 1 count
SPECIFICITY	Response of reagent blank and quality control samples	Retention times compared to those of the reference material during method verification ΔRT< 1%
PRECISION RSD_R	Determine the repeatability for the levels considered	≤ CV,r % from method
INTRA-LABORATORY PRECISION RSD_{WR}	Determine intra-laboratory reproducibility from tests under intra-laboratory reproducibility conditions	≤ CV,R % from method
ROBUSTNESS	It can be derived from the validation of the method and verification (during routine analyzes) through the definition of the mean of bias and RSD_{wR}	Look at definitions of RSD_{wR} and BIAS
BIAS	Determine the average of the values considered for accuracy for the levels considered	½ $\sigma_R \leq s_R \leq$ 2/3 σ_R (HORRAT) + PT results

5 RESULTS

5.1 MOLECULAR MARKERS FOR GENETIC TRACEABILITY

5.1.1 DEVELOPMENT OF SSR MULTIPLEX SETS FOR FINGERPRINTING

5.1.1.1 Multiplex analysis on LI-COR system

The optimization of multiplex sets on LI-COR was rather straightforward, due to the previous experience with this system at LaRAS on several crops, as well as to its high sensitivity in detecting the infrared dyes labelling PCR products, therefore the optimization steps are not shown here.

Figures 1 and 2 show the result of the analysis with multiplex set 2 and 3 on the 16 sunflower lines analysed with LI-COR. Both sets produced very clear banding patterns, with bands occupying the range 150-450 bp without overlapping between alleles of different loci. Background noise was absent for set 2 and rather low for set 3, indicating a very good optimization of the amplification protocol preventing the formation of non-specific amplicons. No stuttering was observed at any of the loci under study (even if all of them contain dinucleotide repeats), thus facilitating band sizing and allele calling. This was further facilitated for markers of set 2 since they showed sharp bands of moderate intensity.

Figure 3 reports the amplicon sizes determined with the CARESTREAM Molecular Imaging Software and the allele attribution performed manually. For each genotype two rows are shown to allow the reporting of two alleles due to heterozygosity/heterogeneity (DNA was extracted from a bulk of five individuals per line). Lines turned out rather fixed, with only three of them CRA16UPR, INRA107, and RHA 426) with 1 or 2 heterozygous loci.

All marker loci proved polymorphic among the tested materials. The number of alleles ranged from 2 to 6, with an average of 3.8 and 4.3 (not shown) alleles per locus detected for set 2 and set 3,

respectively; in set 3, null alleles were detected at two loci. Overall, this is a fairly high level of polymorphism, considering the small number of genotypes (sixteen inbred lines) under test.

Alleles likely deriving from insertion/deletion events, revealed by the presence of gaps (>10 bp difference between neighbouring alleles in the size distribution of the alleles within a locus) were observed at four loci.

5.1.1.2 Multiplex analysis on QIAxcel system

Different from the optimization of multiplex assays on LI-COR, the one carried out on QIAxcel was quite difficult, in part due to the novelty of this equipment in our laboratory at Agricola Grains, and on the other hand for to the lower sensitivity of the latter system compared to LI-COR. After the first fruitless attempts to use directly the QIAxcel (no amplicons detected in runs), we decided to go through a preliminary phase of amplification in simplex format with regular verification of expected products in agarose electrophoresis.

The profiles obtained by multiplex amplification of Set 2 and Set 3 before optimization are shown in Figure 4. The concentration is the same for all primer pairs (0.5 μM), but due to different amplification efficiency strong differences in band intensity are present, making difficult analysis on QIAxcel due to the presence of very high peaks for some markers, while for other markers peak would not reach the detection threshold. Therefore, protocol optimization was aimed mainly at improving uniformity in among the amplicons of the different markers in the set. Figure 5 illustrates the optimization steps carried out for multiplex Set 2. It can be noticed that it was necessary to drastically reduce the concentration of primers of the markers producing amplicons of the lowest molecular weights (ORS621, ORS687, and particularly, ORS656), and increase that of the primers for the markers of higher molecular weight (ORS844, ORS674, and ORS810). This is somehow expected, since longer amplicons are less competitive compared to shorter ones in the mix. At the end of the process (step 7) an acceptable balance between amplicons was reached. In Figure 6 the multiplex profile of Set 2 markers for one genotype (INRA293) run on QIAxcel is shown, together

with the runs of the PCR of the single loci. All the expected markers are detectable in the multiplex run (as recognised by their expected size) although some of them still at very low intensity. In addition, spurious bands are visible, in particular at the lowest weights, which were not detected in simplex amplifications, likely attributable to interaction from primers of different pairs.

Amplicon intensity was in general higher for Set 3, for which also a lower background was observed. Figure 7 shows the profiles obtained from multiplex amplification of four different DNA samples. It is worth noting the high degree of correspondence between the amplicons of HA89 and CRA89, two different accessions of the same line (obtained from USDA and CRA-CIN, respectively).

5.1.1.3 Comparison of SSR data obtained with LI-COR and QIAxcel

Table 5 reports the data obtained with Set 2 and 3 in the analysis of the 16 sunflower inbred lines- In order to decrease the sources of variation to a minimum, the same DNA samples were analysed with the two systems. For each sample, the data obtained with Li-COR are based on two replicate lanes, whereas with QIAxcel only one capillary was loaded with each amplification. For each marker, all the alleles are reported, and for each one the size (as average of its occurrences in the materials) and rage of variation is reported. In addition, also reports the estimates of the size and range of variation of the repeat units at each marker locus. It is important to clarify that allele calling based on LI-COR data was applied also to QIAxcel data. The reason for this is that we realised the difficulty, at least at this stage, to perform allele calling based uniquely on size data from QIAxcel, whereas with LI-COR it was possible to rely also on gel images. This is particularly true considering that all markers contain di-nucleotide repeat units which would require a very high-resolution power for correct sizing.

The first thing to be noted is that in many cases the difference size for the same allele in the two system is rather high, reaching 21,5 bp for OSR1178, and in several other cases above 10 bp. In any case, for all markers fragment length estimated with QIAxcel was higher than the one obtained with

LI-COR, but since the true value of fragment size is unknown, it is not possible to determine the bias of the two systems.

In general, the range of allele size was narrower for LI-COR than for QIAxcel. For Set 2 the average ranges were 0.65 and 1.26 for the two systems, respectively, and similar values were observed for Set 3 0.58 vs 1.19. The higher precision observed for LI-COR could in part be attributed to the fact that each sample was replicated and also because the gel imaging system used for band sizing allows to obtain estimates in bp with one decimal digit.

The average size of the repeat unit was calculated for pair of neighbouring alleles, based on their difference in size divided by their difference in terms of repeat units estimated based on the LI-COR gels. On average, the estimates obtained are very close to the expected value of 2 (considering the di-nucleotide repeats) for the two systems (2.07 vs 2.27 for Set 2, and 1.98 vs 2.08 for Set 3, for LI-COR and QIAxcel, respectively). However, a wider variability in repeat unit estimates was observed for QIAxcel than for LI-COR (0.65 vs 0.90 for Set 2, and 0.35 vs 0.76 for Set3).

5.1.1.4 Relationships among lines based on multiplex sets

The relationships among the 16 lines as assessed by cluster analysis based on Jaccard coefficient of similarity calculated on genotype data obtained with the two marker sets are illustrated in Figure 10. The potential for discrimination is evident. It is worth noting that CRA89, an accession of HA89 maintained over the years at CREA, showed no difference at any marker locus compared to the original line. However, the dendrogram did not group accessions based on their belonging to their breeding group (maintainers or restorers), as instead observed by other authors, but this is not unexpected considering the low number of markers tested.

5.1.1.5 DNA extraction procedure from single sunflower seeds

Freeze-drying the material overnight facilitate grinding. Figure 8 shows the setting for the preparation of the achene and the grinding of single de-hulled seeds. The presence of one steel ball below the seed is essential, preventing the formation of a compact pellet of almost intact material

at the bottom of the tube. Grinding in the presence of CYAB extraction buffer facilitates the dispersion of the particles.

Figure 9 shows the profiles obtained with Set 3 in the amplification of DNA samples extracted from leaves, achenes and de-hulled seeds. The quality of the profiles obtained from de-hulled seeds using both the 'complete' and the 'short' protocols is not lower than that of profiles obtained from leaf-extracted DNAs. On the other hand, no usable DNA was extracted from whole achenes subjected to the same grinding procedure.

5.1.1.6 Use of multiplex sets for purity testing

The two marker sets seem to be adequate for testing purity of F1 hybrid seed lots in sunflower. On three different hybrids they revealed 8, 7, and 3 heterozygous loci out of 11 tested (one failed), allowing the detection of progenies from selfing, if present in the lot (Figure 11). Even analysing a small number of individuals (20), it was possible to detect segregation of some markers (ORS 810 in Hybrid 2, or ORS 656 in Hybrid 3) presumably due to parent lines not completely fixed. In addition, individuals with unexpected alleles (Hybrid 2, lane 7) or homozygous at otherwise heterozygous loci (Hybrid 2, lane 20, Hybrid 3 lane 11) may indicate pollen flow from neighbouring fields or defective systems for preventing selfing.

The marker sets proved to be also efficient for assessing product traceability, for example when applied to achenes. Figure 12 represents part of a population of achenes obtained from the cultivation of a given F1 hybrid (n. 4), analysed side-by-side with the latter. Unexpected alleles at some loci may indicate either pollen flow from other sources or impurities present in parental lines. On the other hand, in the population of achenes, the segregation of the markers that were heterozygous in the hybrid is a strong indication of the authentic origin of these materials. In addition, since some product qualities, such as level of oleic acid in the achene, can be expressed at a higher level in the presence of selfing, these markers can provide a tool for the assessment of its level.

5.1.2 MARKERS FOR MALE-STERILITY AND FERTILITY RESTORATION

The aim of the work was to develop a duplex system for the determination of cytoplasmic male sterility *PET1* and its restorative gene *Rf1*, based on assays already described in the bibliography (Rieseberg et al., 1994; Horn et al., 2003) and the sequence information available in databases.

The first step involved reducing the size of the amplifiers, in order to reduce the electrophoretic run times on the DNA Analyzer LI-COR, thus increasing the analysis efficiency. This modification was only possible for the amplification of the cytoplasmic target, of which the two sequence variants of the male fertile and male sterile genotype are known. Compared to the original assay, PCR product for male fertile and male sterile cytoplasm were reduced, respectively, from 870 to 241 bp, and from 1450 to 325 (Figure 13). Interestingly, the band that on the basis of the sequence information was expected to be specific for male fertile genotypes (241 bp), appears instead in both fertile and sterile individuals, and represents a useful positive control of successful amplification, even in the absence of the 325 bp band (Figure 14).

Since the sequences of the SCAR marker associated with the *Rf1* gene were not available, it was not possible to design a new assay for the restoration of male sterility, which therefore remained unchanged, except for the addition of the M13 "tail" to the forward primer. The amplification of this marker therefore produces a specific band of 445 bp in individuals carrying the fertility restoration gene and no band in individuals lacking the gene (Figure 14).

The results of the molecular analysis by duplex PCR of the mitochondrial locus CMS-*PET1* and of the marker associated with the genomic locus *Rf1* conducted on the 18 inbred lines and on 18 commercial F1 hybrids are presented in Table 6. In the majority of cases the outcomes were those expected, with some exceptions.

As regards the maintainer lines (B), it should be remembered that since they are used for the reproduction of male-sterile isolines, they must have fertile cytoplasm and not possess nuclear alleles for the restoration of fertility. The 7 lines analysed for marker CMS PET1 all showed male

fertile cytoplasm, but in one, CRA-16UPR, was found the presence of the marker associated with the *Rf1* allele for the restoration of fertility (this would potentially cause serious problems in production of male-sterile lines). Among the C (restorer) lines, marker allele associated with the fertility restorer *Rf1 was always present*, with the exception of UNIPI-GB2112. Since this material was known to be genetically pure, other hypotheses could be made, such as the presence of a different restorer of CMS-PET1 (e.g. *Rf3*) or of a recombination event between the marker and the gene. Furthermore, with regard to the CMS-PET1 marker, it should be noted that the majority of the C lines considered showed male-sterile cytoplasm. This condition, although not strictly essential for restorative pollinating lines, is useful in maintaining their genetic purity. In fact, the presence of cytoplasmic male sterility allows to highlight a possible heterozygosity of the Rf1 gene, which would manifest itself with the appearance of sterile individuals in the multiplication field. The elimination of these individuals with normal purification operations would tend to limit the frequency of the Rf1 allele (non-restorative) whose presence in the hybrid production field would generate male-sterile offspring.

The analysis of commercial hybrids also produced the expected results: all genotypes revealed the presence of the cytoplasmic determinant of male sterility and the presence of the restorative gene. The only exception is the Heliagol SF 1341, which was characterized by the absence of the *Rf1* marker.

5.2 IMPROVED METHOD FOR FATTY ACID DETERMINATION

5.2.1 COMPARISON OF METHODS

The Annexes below report the elementary data and results obtained for all the FAs tested in the oil from the ground seed samples analysed with the official olive oil method and the new sunflower oil specific one.

In chromatographic separation, the major impact change with respect to the official method was the GC-FID column oven temperature ramp. By modifying this parameter, a better chromatographic

separation between the different FAMEs was obtained, halving the acquisition time of each sample from 50 to 25 minutes. Furthermore, by introducing a smaller flow of matter into the chromatographic column compared to the olive oil method (almost 5 times less), a better chromatographic resolution was noted between the different peaks of FAMEs. The introducing a smaller quantity of carrier gas into the column, allowed some savings of raw materials (H2, N2) for the determination of FAMEs, contributing to lowering the cost of this determination for the laboratory.

Annex 1 shows the elementary data for calculating the average repeatability, the average intra-laboratory reproducibility (the raw data are expressed in %, calculated from the area of each FAME with respect to the sum and the average specificity (indicating the chromatographic retention times found for each FAME) both with the official method and with the new one developed by the laboratory. The calculated values are summarized in Table 7.

Linearity response is calculated by the residue of each calibration point through the difference in relative % compared to the theoretical concentration considered for each calibration point specified for each FAMEs. Table 8 summarizes only the highest percentage of calibration residuals on 6 calibration points, the highest calculated residual percentage value was entered in order to bring out the worst data detected. In this way all the remaining data will be compliant if even the worst is acceptable.

LOQ (Limit of Quantification) were obtained by carrying out 10 analyses at the lowest available level equal to the first calibration point (5 ppm) of the measuring range, each interspersed with a white solvent (petrol ether 40-60). This test determined an average white, which corrects the areas previously obtained. For the calculation of the LoD and LoQ the following formulas were used: LoD = (2x(Blank Correction type deviation)/Mean blank correction) x C MR); LoQ = (10x(Blank Correction deviation)/Mean blank correction) x C MR). Table 8 summarizes only the limits of quantification (LoQ) for both methods.

BIAS was calculated by means of two levels of ERM (external reference materials) received by the proficiency test provider (BIPEA France). The standard deviation of reproducibility σR declared by the method was verified by comparing it with the HORRAT ratio. The Horwitz ratio (HorRat) is a useful index of method performance with respect to precision and is calculated as the ratio between the RSDR as obtained during the study and the RSDR as predicted by the modified Horwitz equation. Method reproducibility is considered as normal when the HorRat value is between 0.5 and 0,66 when the HorRat ratio is calculated as described into the paragraph 4.2.5.

In Annex 2, the elementary data for calculating the residuals of each calibration point for each validated FAME can be found. Table 8 summarizes only the residues with the highest % value.
Annex 3 shows the elementary data used for the calculation of the limit of quantification (LoQ), which was determined through 10 tests at a concentration equal to the first calibration point (5 ppm) interspersed with a matrix blank test. This test determined an average white, which corrects the areas previously obtained. The calculated values are summarized in Table 8.

6 DISCUSSION

The two SSR multiplex sets developed by Tang et al. (2003), for their high level of polymorphism, reproducibility, and clarity of interpretation proved very efficient in detecting genetic variation for the objective of variety purity assessment and traceability in the seed sector and in the downstream production chain. Besides, the availability of efficient assays for monitoring critical steps in seed production, such as the control of pollination (male-sterility and fertility restoration) can contribute to increase the quality and therefore the value of the seed produced in our industrial sector. However, in order to become really applicable in routine testing in the industry laboratory, automation and increase throughput are necessary.

A suitable DNA extraction procedure from single seeds, allowing the handling of a representative sample (at least 50-100 individuals) is a prerequisite for the application of these tools for determining genetic purity in lots of seeds as well as of grains. For example, in order to be able to detect a true level of impurity (e.g., undesired genotypes) equal to 5%, at least 60 individuals need to be tested, ensuring a confidence of 95% to include at least one “contaminant” individual in the sample. Of course, the testing method must be reliable and with an acceptable error rate.

One objective of our study was to evaluate alternatives to the gel-based fragment analysis conducted with LI-COR. Even though we have shown here that the system is fit for purpose, and relatively cheap as far as consumables are concerned, it is instead rather cumbersome and time consuming in particular for gel preparation and loading. Results obtained with the LI-COR system represents a benchmark to which one should compare those obtained with the new candidate method. The investment on the QIAxcel apparatus was intended in fact for the purpose of developing an entire procedure, from DNA extraction to fragment analysis, more easily applicable

in routine testing within the company for genotyping SSR loci to assess the traceability of incoming and outgoing goods from our premises.

We were able to analyse two 6-plex PCR amplification on a QIAxcel; according to our information previous published work report ed at maximum 4-plex reactions. The results are encouraging, even if dinucleotide SSR are not the easiest to use when using a system not having the resolution capability of high-level capillary electrophoresis systems. A possible way around this obstacle would be the use of SSR with repeat units of higher order, such as tetra- or penta-nucleotide microsatellite, for which the resolution power of QIAxcel could be adequate. A search of such markers to be multiplexed has been initiated.

Several difficulties were experienced, particularly establishing conditions for eliminating 'background noises', and for improving sensitivity, resolution power and repeatability. A more careful primer design, and the use of newly developed bioinformatic tools could be used for a more precise multiplex assembly.

As regards the organic sector, the availability of such analytical tools could allow the verification of the varietal identity of the product batches delivered to the industry or trade. It is clear that such an approach would make it possible to bring out situations of illicit use of seeds of varieties recognized as suitable for organic farming for which seed companies were making the effort to produce organic seeds. The effect of this type of controls would be to help create a "virtuous circle" between the various players in the supply chain (transformer, stocker, farmer and seed company) by matching supply and demand for seeds of varietal identity and organic quality certified, with an adequate remuneration of the breeder and the seed producer, and for greater protection of those producers who use organic seeds.

By taking the 'Commission Implementing Regulation (EU) 2015/1833' as a reference method, it was possible to develop a new analytical method for the determination of FAs for sunflower matrix.

In particular, specific new conditions for: oven temperature, split ratio, carrier gas hydrogen pressure, total flow resulted, allowed to obtain a better repeatability and reproducibility, with lower standard deviations when compared to those obtained with official method listed in COI/T.20/Doc. No 33/Rev.1 2017 for all the considered FAMEs.

Tables 7 and 8 summarize the comparison of performance data (% results) between the official method for olive oil (data taken from document COI/T.20/Doc. No 33/Rev.1 2017 quoted in the methods *Commission Regulation No. 2568/91 (EU Commission 1991)* and *Commission Implementing Regulation No. 2015/1833 (EU Commission 2015)* and the new method specifically implemented by the laboratory for sunflower oil.

Table 7 reports for each FA the average values among the replicates. Since the test for heteroskedasticity of variance was not significant, for each parameter the average among the levels is reported.

Table 7 shows the average repeatability, the average intra-laboratory reproducibility and specificity, both obtained with the official method and the new one developed by the laboratory. As can be seen from the results contained in it, better data were found with the method developed by the laboratory compared to the official method for all the verified FAMEs.

Table 8 shows the maximum residues (absolute value of the difference between the measured concentration and the theoretical concentration expressed in %) found in the calibration curves, the BIAS calculated and compared through the HORRAT and LOQ ratio, both with the official method and the new one developed by the laboratory. As can be seen from the results contained in it, better data were found with the method developed by the laboratory compared to the official method for all the verified FAMEs.

As a result of this internal validation study, Agricola Grains S.p.A. Laboratory got this test recently accredited according to ISO/IEC 17025 by ACCREDIA. The following FAME is now accredited for the determination of the FAs: Alpha-linolenic acid (omega-3), Arachidic acid, Behenic acid, Eicosenoic

acid, Heneicosanoic acid, Heptadecanoic acid, Heptadecenoic acid, Lignoceric acid, Linoleic acid (omega- 6), Oleic acid, Palmitic acid, Palmitoleic acid, Stearic acid, Tetracosenoic acid.

From a chemical point of view, i.e., the development of a new method for detecting the methyl esters of fatty acids in sunflower seeds, improvements can be noted in all the validation parameters considered with respect to the official method (Commission Regulation No. 2568/91 (EU Commission 1991) and Commission Implementing Regulation No. 2015/1833 (EU Commission 2015)), developed for oils and olive pomace.

A different extraction solvent and different chromatographic conditions were adopted for the method adjustment, to optimize the separation of the various analytes with retention times suitable for the purpose. This was made by adopting the same runs (e.g.: same acceptability criterion for the chromatographic resolution between the two peaks of stearic acid and oleic acid).

If considering oleic acid as an example, improvements can be observed between the application of the official method and the new one developed by the laboratory for sunflower seed samples.

As described in detail in Table 7, for: mean repeatability parameter from 0.08 to 0.03 RSDr %, mean reproducibility parameter from 0.4 to 0.09 RSDR %, mean specificity and mean retention times, values were in line between the two methods, with greater repeatability of the times with the new method developed by the laboratory (Cfr. Annex 1).

Furthermore, as regards the validation parameters presented in Table 8, an improvement between the data produced with the official method compared to those obtained with the new one developed by the laboratory can be observed, too.

In particular, taking oleic fatty acid as an example, % residues can be detected from the theoretical calibration curve from 15.12 for the official method to 11.12% with the new method.

These values are the maximum values found throughout the measuring range for oleic acid (detailed in Annex 2).

In the case of the Bias parameter, as regards all the fatty acids considered, a repeatability standard deviation value lower than the limits dictated by the HORRAT ratio was determined by applying the new method developed by the laboratory.

These results are the fruit of/due to the high repeatability of the method developed by the laboratory, unlike the official method which - after its application to the sunflower matrix - obtained values included in the HORRAT ratio for all the fatty acids considered in the present study.

Finally, as regards the LOQ parameter, values lower than 1 ppm were obtained in both methods but with lower concentrations found in the new method developed by the laboratory. As an example, 0.78 ppm was the result for oleic acid LOQ with the official method, while 0.64 ppm was the one obtained with the new method developed by the laboratory.

A next step in this work could be the application of the presented analytical method on an oil matrix. In the course of our routine experience (data not shown) in the laboratory, we were able to verify the amplifiability of DNA directly on oil samples, making possible to compare the results obtained before pressing (seed) and after pressing on the finished product (oil). In this way, the traceability of the finished product would be guaranteed with a comparative double test between raw material and oil produced.

7 CONCLUSIONS

Thanks to the development and combination of the two analytical methods presented in the previous chapters, it was possible to implement an experimental method for the verification of both traceability and variety of organic sunflower marketed by Agricola Grains S.p.A. A process for a routine quality monitoring was activated: this is based on the joint use of the two methods presented in this work. In particular, in relation to the new FA's determination method, the most significant advantage that Agricola Grains S.p.A. was able to recognize is the greater analytical reliability in the characterization of marketed sunflower, thanks to the greater analytical specificity achieved.

On the basis of all the data obtained by the experimental activities carried out by Agricola Grains S.p.A., we can conclude that the expected goals were successfully obtained.

8 REFERENCES

Ahmad O., Rana M.A., and Siddiqui, S.U.H. (1991) Sunflower seed yield as influenced by some agronomic and seed characters. Euphytica, 56, 137–142. https://doi.org/10.1007/BF00042056

Ammassari P., (2018). Il manuale di conversione al biologico. Schede tecniche

Ammassari P., (2020) Bioreport. L'agricoltura biologica in Italia. Pubblicazione realizzata nell'ambito del Programma Rete Rurale Nazionale 2014-2020 Piano di azione biennale 2021-2023. Scheda progetto CREA 5.2 Azioni per l'agricoltura biologica.

Badouin H. et al., (2017) The sunflower genome provides insights into oil metabolism, flowering and Asterid evolution. Nature, 546, 148–152. https://doi.org/10.1038/nature22380

Bartolini R., (2019) Girasole biologico a contratto, una soluzione agronomica con buone rese e prezzi elevati. Il nuovo agricoltore 25.04.2019.

Battistini E., (2011) Genetic variability and seed vigour in a sunflower germplasm collection, Alma Mater Studiorum Università di Bologna. Dottorato di ricerca in Colture erbacee, genetica agraria, sistemi agroterritoriali, 23 Ciclo.

Benvenuti A., Vannozzi G.P., (2001) Girasole (*Helianthus annuus* L.). In Baldoni R., Giardini L., (eds) "Coltivazioni erbacee. Piante oleifere, da zucchero, da fibra, orticole e aromatiche". Patron, Bologna, pp 59-95.

Bonnafous F., Fievet G., Blanchet N., Boniface M.-C. C., Carrère S., Gouzy J., et al. (2018). Comparison of GWAS models to identify non-additive genetic control of flowering time in sunflower hybrids. Theor. Appl. Genet. 131, 319–332. doi: 10.1007/s00122-017-3003-4

Bruneton J., Wiart C., AHA. Hadi, Richomme P., (1999) 6-Acylcoumarins from Mesua racemosa. Phytochemistry

Burke J.M., Knapp S.J., Rieseberg L.H., (2005) Genetic consequences of selection during the evolution of cultivated sunflower. Genetics, 171, 1933–1940

Burke J.M., Tang S., Knapp S.J., & Rieseberg L.H., (2002) Genetic analysis of sunflower domestication. Genetics 161, 1257–1267

Canali G., (2021) Il girasole in Italia: un mercato in crescita. In Il futuro del girasole in Italia: le prospettive della coltura tra nuova PAC, mercato e ricerca. Webinar di Assosementi, 31 marzo 2021.

Čerpnjak K., Zvonar A., Gašperlin M., Vrečer F., (2013) Lipid-based systems as promising approach for enhancing the bioavailability of poorly water-soluble drugs. Acta pharmaceutica

Chen Q., HJ. Yan, CJ. Yan, GB. Pan, LJ. Wan, GY. Wen (2008) STM investigation of the dependence of alkane and alkane (C18H38, C19H40) derivatives self-assembly on molecular chemical structure on HOPG surface. Surface Science

Coburn J. R., Temnykh S. V., Paul E. M., McCouch S. R., (2002) Design and Application of Microsatellite Marker Panels for Semiautomated Genotyping of Rice (Oryza sativa L.). Cell Biology & Molecular Genetic

Dimitrijevic A., Horn R., (2017) Sunflower hybrid breeding: from markers to genomic selection. Frontiers in Plant Science 8, 2238.c

Enríquez-Fernández B., Álvarez de la Cadena L., Yañez, ME. Sosa-Morale, (2011) Comparison of the stability of palm olein and a palm olein/canola oil blend during deep-fat frying of chicken nuggets and French fries. Journal of food.

EU Council. (1994) COUNCIL REGULATION (EC) No 231/94 of 24 January 1994 amending Regulation (EEC) No 1765 / 92 establishing a support system for producers of certain arable crops. Official Journal of the European Communities, 1994, 2–6.

FAO (2020) FAOSTAT: Food and agriculture data (https://www.fao.org/faostat/en/#home)

Gentzbittel L., Vear F., Zhang Y.-X., Berville A., Nicolas P. (1995). Development of a consensus linkage RFLP map for cultivated sunflower. Theor. Appl. Genet. 90: 1079–1086.

Goryunova S. V., Goryunov D. V., Chernova A.I., Martynova E.U., Dmitriev A.E., Boldyrev S. V., Ayupova A.F., Mazin P. V., Gurchenko E.A., Pavlova A.S., Petrova D.A., Chebanova Y. V., Gorlova L.A., Garkusha S. V., Mukhina Z.M., Savenko E.G., Demurin Y.N. (2019) Genetic and Phenotypic Diversity of the Sunflower Collection of the Pustovoit All-Russia Research Institute of Oil Crops (VNIIMK). Helia 42:45–60. https://doi.org/10.1515/helia-2018-0021

Harlan J. R. (1992). Origins and processes of domestication. In Chapman, G. P. (ed.), Grass Evolution and Domestication. Cambridge University Press, Cambridge, pp. 159–175.

Heiser C.B. (1978) Taxonomy of Helianthus and Origin of Domesticated Sunflower. In Sunflower Science and Technology. Agron. 31-53. Carter J. Ed.

Heiser C.B., Smith D.M., Clevenger S.B., Marin W.C. (1969) The North American sunflowers (*Helianthus*). Memoirs of the Torrey Botanical Club 22:1-218

Hubner S. (2019) Sunflower pan-genome analysis shows that hybridization altered gene content and disease resistance. Nat. Plants 5, 54–62.

Jannin V., Musakhanian J., Marchauda D. (2008) Approaches for the development of solid and semi-solid lipid-based formulations. Advanced Drug Delivery Reviews 60(6):734-46. doi: 10.1016/j.addr.2007.09.006.

Jones C.J., Edwards K.J., Castaglione S., Winfield M.O., Sala F., Van de Wiel C., Bredemeijer G., Vosman B., Matthes M., Daly A., Brettschneider R., Bettini P., Buiatti M., Maestri E., Malcevschi A., Marmiroli N., Aert R., Volckaert G., Rueda J., Linacero R., Vazquez A. & Karp A. (1997) Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. Molecular Breeding volume 3, pages381–390

Karthik Siram., Habibur Rahman S.M., Balakumar K., Duganath N., Chandrasekar R., R.Hariprasad; 2019, Pages 91-115, Biomedical Applications of Nanoparticles)

Katrine Knutsen H., Alexander J., Barreg ard L., Bignami M., B. Br, Ceccatelli S., Dinovi M., Edler L., Grasl-Kraupp B., Hogstrand C., Hoogenboom L., Stefano Nebbia C., Oswald I., Petersen A., Rose M., Roudot A.-C., Schwerdtle T., Vollmer G., Wallace H., Cottrill B., Dogliotti E., Laakso J., Metzler M., Velasco L., Baert K., Angel Gomez Ruiz J.G., Varga E., Vleminckx C. Erucic acid in feed and food. EFSA, 14 (2016), pp. 1-173

Kinman M.L. (1970) New developments in the USDA and state experiment station sunflower breeding programs. Proc. Fourth Int. sunflower conference, Memphis, Tenn., p. 181–183.

Lagow RJ., Felling KW., Youngstrom CR. (2004) Synthesis of perfluorinated functionalized, branched ethers. Journal of fluorine chemistry, 2004

Leclercq P., (1966) Une sterilite male utilisable pour la production de hybrides simples chez le tournesol. Annales de l'Amélioration des Plantes, 16, 135–144.

Leclercq P., (1969) Une sterilite male cytoplasmique chez le tournesol. Annales de l'Amélioration des Plantes, 99–106.

Lijuan S., Zheng L. (2012). Aggregation Behaviour of SurfaceActive Imidazolium Ionic Liquids in Ethylammonium Nitrate: Effect of Alkyl Chain Length, Cations, and Counterions. J. Phys. Chem. B

Liu J. X., Srivastava R., Che P., Howell S. H. (2007). An endoplasmic reticulum stress response in Arabidopsis is mediated by proteolytic processing and nuclear relocation of a membrane-associated transcription factor, bZIP28. Plant Cell 19, 4111–4119. doi: 10.1105/tpc.106.050021

M.L. Senior., Murphy J.P., Goodman M.M., C.W. Utility of SSRs for determining genetic similarities a relationship in maize using an agarose gel system. Stuber Crop Sci 38, 1088–1098 (1998).

Nel A.A., Loubser H.L., Hammes P.S. (2002) Development and validation of relationships between sunflower seed quality and easily measurable seed characteristics for grading purposes. S. Afr. J. Plant Soil, 19, 201–205.

Nettleton J.A. (1995) Introduction to Fatty Acids. In: Omega-3 Fatty Acids and Health. Springer, Boston, MA. Aalen R.B. (1999). Peroxiredoxin antioxidants in seed physiology. Seed Sci. Res. 9: 285–295.

Trevaskis NL., Charman WN., Porter CJH. (2008) Lipid-based delivery systems and intestinal lymphatic drug transport: a mechanistic update. Advanced drug delivery reviews

Owens G. L., Baute G. J., Hubner A., Rieseberg L. H. (2018). Genomic sequences and copy number evolution during hybrid crop development in sunflowers. Evol. Appl. 11, 1–12. doi: 10.111/eva.12603

Paniego N., Echaide M., Munoz M., Fernandez L., Torales S. (2002). Microsatellite isolation and characterization in sunflower (Helianthus annuus L.). Genome 45: 34-43.

Pilorgé E. (2020) Sunflower in the global vegetable oil system: situation, specificities and perspectives. OCL 27:34

Putt E.D. (1997) Early history of sunflower, pp. 1–19 in Sunflower Technology and Production, edited by Schneiter A. American Society of Agronomy, Madison, WI.

Rauf A., Hadda T. Ben., Uddin G., Ramadan M. F. (2017) Fatty Acid Composition and Biological Activities of Oily Fractions from Pistacia integerrima Roots. Chemistry of Natural Compounds

Rauf,S., Warburton M., Naeem A., Kainat W. (2020) Validated markers for sunflower (Helianthus annuus L.) breeding. Ocl, 27, 47. https://doi.org/10.1051/ocl/2020042

Röder M. S., Wendehake K., Korzun V., Bredemeijer G., Laborie D. (2002) Construction and analysis of a microsatellite-based database of European wheat varieties. Theor. Appl. Genet. 106: 67–73.

Scacchi S. (2012) Isolamento, variabilità ed espressione del gene PIMT in girasole. Alma Mater Studiorum Università di Bologna. Dottorato di ricerca in Colture erbacee, genetica agraria, sistemi agroterritoriali.

Shete H., Patravale V. (2013) Long chain lipid-based tamoxifen NLC. Part I: Preformulation studies, formulation development and physicochemical characterization. International Journal of Pharmaceutics

Siram K., Rahman SMH., Balakumar K. (2019) Pharmaceutical nanotechnology: Brief perspective on lipid drug delivery and its current scenario. Biomedical Applications

Caliph SM., Charman WN., Porter CJH. (2000) Effect of Short-, Medium-, and Long-Chain Fatty Acid-Based Vehicles on the Absolute Oral Bioavailability and Intestinal Lymphatic Transport of Halofantrine and Assessment of Mass Balance in Lymph-Cannulated and Non-cannulated Rats. Journal of Pharmaceutical Sciences

Talukder ZI., Ma GJ., Hulke BS., Jan CC, Qi LL (2019) Linkage mapping and genome-wide association studies of the Rf gene cluster in sunflower (Helianthus annuus L.) and their distribution in world sunflower collections. Front Genet 10:216

Tang S., Yu J.-K., Slabaugh M.B., Shintani D.K., Knapp S.J. (2002). Simple sequence repeat map of the sunflower genome. Theor. Appl. Genet. 105: 1124-1136.

TaqMan PCR Reagent Kit Protocol: Part Number 402823 Rev. G, July 2010.

The elimination of primer-dimer accumulation in PCR. Jannine Brownie, Susan Shawcross1, Jane Theaker, David Whitcombe*, Richard Ferrie, Clive Newton and Stephen Little Zeneca Diagnostics, Gadbrook Park, Northwich, Cheshire CW9 7RA, UK and 1Department of Biological Sciences, Manchester Metropolitan University, Chester Street, Manchester M1 5GD, UK

Trestini S., Giampietri E., Boatto V., Povellato A. (2019) A participatory process to design regional policy for rural development: the case of the Veneto Region. Council for Agricultural Research and Economics

Xiong, J., Parsons, L. M., Gao, J. H. & Fox, P. T. (1999) Interregional connectivity to primary motor cortex revealed using MRI resting state images. Hum. Brain Mapp. 8, 151–156.

Yu J.K., Mangor J., Thompson L., Edwards K.J., Slabaugh M.B., and Knapp S.J. (2002). Allelic diversity of simple sequence repeats markers among elite inbred lines in cultivated sunflower. Genome 45: 652-660.

Yu J.K., Tang S., Slabaugh M.B., Heesacker A., Cole G., Herring M., Soper J., Han F., Chu W.C., Webb D.M., Thompson L., Edwards K.J., Berry S., Leon A.J., Olungu C., Maes N. and Knapp S.J. (2003). Towards a saturated molecular genetic linkage map for cultivated sunflower. Crop Sci. 43: 367-387.

9 TABLES

Table 1. List of genotypes utilised in this study.

Material	Name	Source	Pedigree	Caracteristics	Type	Use in this work
Lines						
	CRA181/5-12	CREA	-	-	B	Pet1/Rf1
	CRA-16 UPR	CREA	-	-	B	SSR; Pet1/Rf1
	CRA-89	CREA	VNIIMK 8931 selection	High-oil seed; Single-headed; Resist. to rust	B	SSR; Pet1/Rf1
	CRA-216/3-4	CREA	-	-	B	SSR; Pet1/Rf1
	HA89	USDA	VNIIMK 8931 selection	High-oil seed; Single-headed; Resist. to rust	B	SSR; Pet1/Rf1
	INRA-107	INRA	Cross with H. argophyllus	n.a.	B	SSR; Pet1/Rf1
	INRA-109	INRA	Moroccan	n.a.	B	SSR; Pet1/Rf1
	INRA-293	INRA	Cross with H.tuberosus	Apical branching	C	SSR; Pet1/Rf1
	INRA-295	INRA	Cross with H.resinusus	Apical branching	C	SSR; Pet1/Rf1
	INRA-306	INRA	Cross with H.petiolaris	Apical branching	C	SSR; Pet1/Rf1
	RHA265	USDA	Peredovik/953-102-1-1-41"T66006-2-1-3-1	High-oil-seed. Single-headed; Resist. to rust	C	SSR; Pet1/Rf1
	RHA271	USDA	CMS PI343765/HA119//HA62-4-5/2/T66006-2-1-31-1"T70020	Oil-seed; Recessive branching	C	Pet1/Rf1
	RHA294	USDA	CMS PI343863/BONITA GIANT-MANCHURIAN/HA61/MENNONITE RR	Non-oil seed; Recessive branching; Resist. to downy mildew	C	SSR; Pet1/Rf1
	RHA374	USDA	ARG-R43, Argentinian	Oil-seed; Apical branching	C	SSR; Pet1/Rf1
	RHA419	USDA	RHA373/ARG1575-2	Oil-seed; Apical branching; Resist. to downy mildew	C	SSR; Pet1/Rf1
	RHA426	USDA	RHA409//RHA376*2/PUR (wild H.annuus)	Oil-seed; Full branching; Resist. to imidazolinone	C	SSR; Pet1/Rf1
	RHA464	USDA	RHA418/RHA419/3/RHA 801//RHA 365/PI 413047	High-oil seed; Apical branching; Resist. to rust and downy mildew	C	SSR; Pet1/Rf1
	UNIPI-GB2112	UNIPI	Russian	Apical branching	C	SSR; Pet1/Rf1

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USDA: United States Department of Agriculture
n.a.: not available
B: maintainer line
C: restorer line

continues

Table 1 - continued

Material	Name	Source	Pedigree	Caracteristics	Type	Use in this work
F1 Hybrids						
	Eugenio	Agroservice	-	-	F1	Pet1/Rf1
	Vincenzo	Agroservice	-	-	F1	Pet1/Rf1
	13CSTOL9	Caussade	-	High-oleic	F1	Pet1/Rf1
	13CSTOL10	Caussade	-	High-oleic	F1	Pet1/Rf1
	13CSTLIN15	Caussade	-	-	F1	Pet1/Rf1
	13CSTLIN16	Caussade	-	-	F1	Pet1/Rf1
	13CSTCL1	Caussade	-	-	F1	Pet1/Rf1
	13CSTCL2	Caussade	-	-	F1	Pet1/Rf1
	Solaris	SIS	-	-	F1	Pet1/Rf1
	Montijo	SIS	-	High-oleic	F1	Pet1/Rf1
	Pacific RMO	SIS	-	High-oleic	F1	Pet1/Rf1
	Best	SIS	-	High-oleic	F1	Pet1/Rf1
	Alliance RM	SIS	-	High-oleic	F1	Pet1/Rf1
	Barolo 1338	KWS	-	-	F1	Pet1/Rf1
	Doriana 1340	KWS	-	-	F1	Pet1/Rf1
	Heliagol SF 1341	KWS	-	High-oleic	F1	Pet1/Rf1
	Heliador 1345	KWS	-	High-oleic	F1	Pet1/Rf1
	Helia 2730	KWS	-	-	F1	Pet1/Rf1
	Inotop	Apsovsementi	-	High-oleic	F1	SSR
	Michel	Agroservice	-	High-oleic	F1	SSR
	Ancilla	Agroservice	-	High-oleic	F1	SSR
	LG56.87HO	Limagrain	-	High-oleic	F1	SSR
	SY Excellio	Syngenta	-	High-oleic; Resist. to imidazolinone	F1	SSR
	SY Experto	Syngenta	-	High-oleic; Resist. to imidazolinone	F1	SSR
	MAS 808.OL	MAS SEEDS	-	High-oleic	F1	SSR
	P64HE133	Corteva	-	High-oleic	F1	SSR

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B: maintainer line
C: restorer line

Table 2. Primers used in this study for SSR analysis.

Multiplex set	Marker	Forward[(1)]	Reverse	Chrom.	Repeat
SET2	ORS810	TTCAACGTGCGTGATTAAGG	GCGATTTCTTTGGAGACGAA	15	CT
	ORS674	ACATGAGGGCAAAACAGACA	GCACAAAGACAACCACACCA	16	CT
	ORS844	ACGATGCAAAGAATATACTGCAC	CATGTTTAATAGGTTTTAATTCTAGGG	11	CT
	ORS621	CGCCTTATGCTGAGAGGAAA	CCTGAAGCGAAGAAGAATCG	9	AC
	ORS656	ACGGACGTAGAGTGGTGGAG	TCGTGGTAAGGGAAGACAACA	4	CT
	ORS687	ACCGTTACACTTATTGGTTATTTCATT	GGGGTTTGTTGTTCTGTTTTG	12	CT
SET3	ORS1178	AGCTGACCATGTAAGGTTTGGTT	ATTGTTGAGAGACGAGATGGATG	10	AG
	ORS1248	TGTCCGATCTACCATCTGAAATC	TTAGAGCGAAATCTAGTTACATGAGTG	11	AC/CT
	ORS630	GCACGACCCGGATATGTAAC	TGTGCTGAGGATGATATGCAG	6	GT
	ORS483	CCGAACAACAATCTCCACAA	GGTTTAGGTGTCGCATCACA	13	CT
	ORS457	TGCATACCCAATCTACCAGCTA	AAGACGAAGGTGCAACCAGT	14	AG
	ORS595	TGATGGTAATGCATCGGGTA	CACACCATCCCTTGTAAAATCA	7	CT

(1) For separation on LI-COR, an M13 tail was added to each forward primer at its 5' end. M13-tail sequence: CACGACGTTGTAAAACGAC.

Table 3. PCR mix composition for multiplex Set 2 and 3 for LI-COR and QIAxcel electrophoresis systems.

Set 2				Set 3			
Component	Unit	LI-COR	QIAxcel	Component	Unit	LI-COR	QIAxcel
Buffer	X	1	1	Buffer	X	1	1
MgCl2	mM	2,50	2,00	MgCl2	mM	2,00	2,00
dNTP	mM	0,30	0,25	dNTP	mM	0,25	0,25
ORS810 f+r	µM	0,10	1,00	ORS1178 f+r	µM	0,09	1,00
ORS674 f+r	µM	0,10	1,00	ORS1248 f+r	µM	0,08	1,00
ORS844 f+r	µM	0,09	1,00	ORS630 f+r	µM	0,05	0,50
ORS621 f+r	µM	0,06	0,25	ORS483 f+r	µM	0,07	1,00
ORS656 f+r	µM	0,03	0,13	ORS457 f+r	µM	0,07	0,13
ORS687 f+r	µM	0,08	0,25	ORS595 f+r	µM	0,05	1,00
PrimerM13 800-IRDye	µM	0,17	-	PrimerM13 700-IRDye	µM	0,16	-
AmpliTaq gold	U/reac.	1,0	1,0	AmpliTaq gold	U/reac.	1,0	1,0
DNA	ng/reac.	100,0	80,0	DNA	ng/reac.	60,0	80,0

Table 4. Primers used in this study for analysis of the *Pet1* and *Rf1* determinants.

Locus	Primer	Name	Sequence (5'-3')
Pet1	Forward[(1)]	new	AATCCCTTTCTTTCCGTTGGTC
	Reverse (fertile)	orfH873-R[(2)]	TCTAGGAACGGGCTGCCCCGGGATTTCCAC
	Reverse (sterile)	new	AGGGGGAGAATGCTTTGAG
Rf1	Forward[(1)]	HRG01-F[(3)]	TATGCATAATTAGTTATACCC
	Reverse (fertile)	HRG01-R[(3)]	ACATAAGGATTATGTACGGG

[(1)] For separation on LiCOR 4300, an M13 tail was added at each forward primer at its 5' end. M13-tail sequence: CACGACGTTGTAAAACGAC.

[(2)] Primer as in Rieseberg et al. (1994)

[(3)] Primers as in Horn et al. (2003)

Table 5. Average and range sizes of alleles, differences in repeat units and in size between consecutive alleles (letters), average size of RU obtained for the 16 sunflower lines with multiplex sets 2 and 3 with LiCOR and Qiaxcel systems.

SET	Marker	Allele	Diff. in RU[1]	LiCOR				Qiaxcel				LiCOR vs Qiaxcel
				Size	Range	Diff. in size[2]	Avg. size of RU	Size	Range	Diff. in size[2]	Avg. size of RU	Diff. in size
			(n.)	(bp)	(bp)	(bp)	(bp)	(bp)	(bp)	(bp)	(bp)	(bp)
SET 1	ORS 810	F		409,0	1,1			413,6	2,0			-4,6
		I	3	401,8	1,3	7,2	2,40	405,3	3,0	8,3	2,78	-3,5
	ORS 674	F		356,3	1,1			367,7	2,0			-11,4
		H	2	352,7	0,5	3,6	1,80	361,5	1,0	6,2	3,11	-8,8
		I	1	351,6	0,5	1,1	1,13	360,0	0,0	1,5	1,50	-8,4
		K	2	345,6	0,3	5,9	2,96	355,0	0,0	5,0	2,50	-9,4
	ORS 844	F		308,7	0,8			319,2	1,0			-10,5
		G	1	306,5	1,8	2,2	2,22	316,4	1,0	2,8	2,77	-9,9
		Q	10	287,4	0,5	19,2	1,92	292,0	2,0	24,4	2,44	-4,6
		R	1	284,5	0,3	2,9	2,88	288,5	3,0	3,5	3,50	-4,0
		S	1	282,5	0,0	2,0	1,98	286,0	0,0	2,5	2,50	-3,5
	ORS 621	F		250,3	1,0			256,3	1,0			-6,0
		K	5	240,7	0,3	9,6	1,92	246,7	1,0	9,7	1,93	-6,0
		L	1	239,4	0,0	1,3	1,33	245,0	0,0	1,7	1,67	-5,6
		M	1	236,1	0,8	3,3	3,25	242,6	1,0	2,4	2,40	-6,5
		N	1	233,2	0,5	2,9	2,95	239,0	0,0	3,6	3,60	-5,8
		O	1	232,2	0,0	1,0	1,00	238,0	0,0	1,0	1,00	-5,8
	ORS 656	F		207,7	0,0			217,0	2,0			-9,3
		G	1	206,1	0,5	1,6	1,60	215,3	1,0	1,7	1,67	-9,2
		I	2	202,2	1,3	3,9	1,97	210,3	2,0	5,1	2,54	-8,1
		J	1	199,9	0,8	2,2	2,22	207,7	1,0	2,6	2,58	-7,7
	ORS 687	F		165,5	1,4			172,6	2,0			-7,2
		G	1	163,8	0,5	1,7	1,73	172,6	3,0	0,04	0,04	-8,8
	Average				0,65		2,07		1,26		2,27	
	St. Dev.						0,65				0,90	

(1) Difference in number of Repeat Units between two neighbouring alleles at a locus , based on LiCOR electrophoregrams.

(2) Difference in base pairs between two neighbouring alleles at a locus , based either on LiCOR or Qiaxcel electrophoregrams.

Table 5. Continued

SET	Marker	Allele	Diff. in RU[1]	LiCOR				Qiaxcel				LiCOR vs Qiaxcel
				Size	Range	Diff. in size[2]	Avg. size of RU	Size	Range	Diff. in size[2]	Avg. size of RU	Diff. in size
			(n.)	(bp)	(bp)	(bp)	(bp)	(bp)	(bp)	(bp)	(bp)	(bp)
SET 2	ORS 1178	F		429,1	0,0			450,0	0,0			-21,0
		H	2	426,1	1,8	2,9	1,46	447,3	2,0	2,7	1,33	-21,2
		I	1	423,6	0,3	2,6	2,58	445,0	0,0	2,3	2,33	-21,5
		J	1	422,0	0,6	1,5	1,53	442,0	0,0	3,0	3,00	-20,0
		null		n.a.	n.a.			n.a.	n.a.			n.a.
	ORS 1248	F		374,9	1,5			377,2	2,0			-2,3
		G	1	373,0	1,5	1,9	1,89	376,0	2,0	1,2	1,17	-3,0
		H	1	371,0	0,0	2,0	2,00	373,5	1,0	2,5	2,50	-2,5
	ORS 630	F		352,1	0,0			361,0	0,0			-8,9
		G	1	349,7	1,5	2,4	2,40	359,7	5,0	1,3	1,33	-10,0
		H	1	347,4	1,2	2,3	2,30	358,0	2,0	1,7	1,67	-10,6
		J	2	343,0	0,3	4,4	2,19	352,5	1,0	5,5	2,75	-9,5
		L	2	339,1	0,0	3,9	1,96	349,0	0,0	3,5	1,75	-9,9
		null		n.a.	n.a.			n.a.	n.a.			n.a.
	ORS 483	F		269,8	0,8			273,6	1,0			-3,8
		G	1	268,0	1,4	1,8	1,78	272,1	2,0	1,5	1,48	-4,1
		H	1	265,7	0,6	2,3	2,28	271,5	1,0	0,6	0,63	-5,8
		N	6	254,2	0,0	11,5	1,92	259,0	0,0	12,5	2,08	-4,8
	ORS 457	F		229,4	1,2			238,3	2,0			-8,9
		G	1	227,4	0,6	2,0	1,97	236,3	1,0	1,9	1,92	-8,9
		H	1	225,4	0,3	2,0	2,00	234,2	1,0	2,2	2,17	-8,8
		I	1	224,2	0,6	1,2	1,20	232,7	2,0	1,5	1,50	-8,5
	ORS 595	F		142,0	0,0			155,0	0,0			-13,0
		I	3	137,0	0,3	5,0	1,67	148,8	2,0	6,2	2,06	-11,8
		J	1	135,2	0,0	1,8	1,80	148,0	0,0	0,8	0,83	-12,8
		K	1	133,2	0,3	2,0	2,00	144,7	2,0	3,3	3,33	-11,5
		M	2	128,2	0,0	5,1	2,53	138,0	0,0	6,7	3,33	-9,8
		Z	13	103,0	0,4	25,1	1,93	109,0	2,0	29,0	2,23	-6,0
	Average				0,58		1,97		1,19		1,97	
	St. Dev.						0,35				0,76	
SET 1 & 2	Average				0,61		1,98		1,18		2,08	
	St. Dev.						0,54				0,83	

(1) Difference in number of Repeat Units between two neighbouring alleles at a locus , based on LiCOR electrophoregrams.

(2) Difference in base pairs between two neighbouring alleles at a locus , based either on LiCOR or Qiaxcel electrophoregrams.

Table 6. Results of the duplex assay for the detection of the *Pet1* male sterile cytoplasm and of the *Rf1* fertility restorer gene in lines and F1 hybrids.

Material	Name	Source	Type	CMS PET1[3]	Rf1 HRG01[4]
Lines					
	CRA181/5-12	CREA	B	F	-
	CRA-16 UPR	CREA	B	F	+
	CRA-89	CREA	B	F	-
	CRA-216/3-4	CREA	B	F	-
	HA89	USDA	B	F	-
	INRA-107	INRA	B	F	-
	INRA-109	INRA	B	F	-
	INRA-293	INRA	C	S	-
	INRA-295	INRA	C	S	+
	INRA-306	INRA	C	S	+
	RHA265	USDA	C	F	+
	RHA271	USDA	C	S	+
	RHA294	USDA	C	S	+
	RHA374	USDA	C	S	+
	RHA419	USDA	C	S	+
	RHA426	USDA	C	S	+
	RHA464	USDA	C	F	+
	UNIPI-GB2112	UNIPI	C	F	-
F1 Hybrids					
	Eugenio	Agroservice	F1	S	R
	Vincenzo	Agroservice	F1	S	R
	13CSTOL9	Caussade	F1	S	R
	13CSTOL10	Caussade	F1	S	R
	13CSTLIN15	Caussade	F1	S	R
	13CSTLIN16	Caussade	F1	S	R
	13CSTCL1	Caussade	F1	S	R
	13CSTCL2	Caussade	F1	S	R
	Solaris	SIS	F1	S	R
	Montijo	SIS	F1	S	n.d.
	Pacific RMO	SIS	F1	S	R
	Best	SIS	F1	S	R
	Alliance RM	SIS	F1	S	R
	Barolo 1338	KWS	F1	S	R
	Doriana 1340	KWS	F1	S	R
	Heliagol SF 1341	KWS	F1	S	-
	Heliador 1345	KWS	F1	S	R
	Helia 2730	KWS	F1	S	R

[2] B = maintainer line; C = restorer line; F_1 = hybrid

[3] F = fertile cytoplasm; S = sterile cytoplasm

[4] R = presence of the marker allele associated with fertility restorer Rf1; - = absence of Rf1

Table 7. Mean performance data obtained by the application of the official method and the new laboratory method for determination of FAME. Repeatability, reproducibility, and specificity based on elementary data from Annex 1.

FAME	Mean Repeatability (Precision RSD_R%)		Mean Reproducibility (Intra-lab precision RSD_{WR}%)		Mean Specificity (Retention time, min.)	
	Official method	New lab. method	Official method	New lab. method	Official method	New lab. method
Palmitic acid C16:0	0,9	0,7	4,1	3,4	8,29	8,30
Palmitoleic acid C16:1	1,1	0,9	4,5	3,9	8,43	8,42
Heptadecanoic acid C17:0	2,1	1,9	5	4,1	8,76	8,78
Heptadecenoic acid C17:1	2	1,4	15	5,9	8,92	8,92
Stearic acid C18:0	0,8	0,3	3,3	2,1	9,41	9,38
Oleic acid C18:1	0,08	0,03	0,4	0,09	9,56	9,56
Linoleic acid (omega-6) C18:2	0,4	0,08	1,8	1,1	9,88	9,89
Alpha-Linolenic acid (omega-3) C18:3	1,5	0,8	3,3	1,9	10,20	10,23
Arachidic acid C20:0	2,8	1,8	9,3	4,1	10,75	10,75
Eicosenoic acid C20:1	5,3	3,7	8,7	5,8	10,95	10,96
Heneicosanoic acid C20:2	5,3	4,2	8,8	4,9	11,61	11,51
Behenic acid C22:0	13,5	9,8	15	12,9	12,59	12,60
Lignoceric acid C24:0	22,8	19,7	35	27,9	14,87	14,88
Tetracoseoic acid C24:1	21,3	17,7	34,9	22,6	15,20	15,22

Table 8. Performance data obtained by the application of the official method and by the validation of the new laboratory method for determination of FAME. Linearity and bias based on elementary data from Annex 2, LOQ based on data from Annex 3.

FAME	Linearity		Bias		LOQ (ppm)	
	Official method	New lab. method	Official method	New lab. method	Official method	New lab. method
Palmitic acid C16:0	18,21%	13,65%	0,0774≤0,0982≤0,1032	0,0774≤0,0061≤0,1032*	0,9	0,56
Palmitoleic acid C16:1	17,20%	11,19%	0,01394≤0,0154≤0,0186	0,01394≤0,0059≤0,0186*	0,8	0,61
Heptadecanoic acid C17:0	15,82%	13,64%	0,0037≤0,00434≤0,00492	0,0037≤0,0002≤0,00492*	0,82	0,6
Heptadecenoic acid C17:1	19,98%	17,02%	0,0377≤0,0488≤0,0503	0,0377≤0,0005≤0,0503*	0,99	0,93
Stearic acid C18:0	18,44%	8,64%	0,0302≤0,0379≤0,0403	0,0302≤0,0007≤0,0403*	0,82	0,72
Oleic acid C18:1	15,12%	11,12%	0,2284≤0,2989≤0,3045	0,2284≤0,0025≤0,3045*	0,78	0,64
Linoleic acid (omega-6) C18:2	18,22%	11,33%	0,0623≤0,0783≤0,0831	0,0623≤0,0031≤0,0831*	0,78	0,52
Alpha-linolenic acid (omega-3) C18:3	19,73%	11,22%	0,0141≤0,0179≤0,0187	0,0141≤0,0011≤0,0187*	0,89	0,66
Arachidic acid C20:0	18,30%	10,31%	0,0154≤0,0188≤0,0205	0,0154≤0,0015≤0,0205*	0,66	0,47
Eicosenoic acid C20:1	19,90%	18,88%	0,0115≤0,0130≤0,0153	0,0115≤0,0013≤0,0153*	0,87	0,63
Heneicosanoic acid C20:2	19,40%	18,67%	0,0115≤0,0144≤0,0153(#)	0,0115≤0,0002≤0,0153*(#)	0,76	0,59
Behenic acid C22:0	15,94%	11,54%	0,0079≤0,0099≤0,0105	0,0079≤0,0010≤0,0105*	0,9	0,7
Lignoceric acid C24:0	18,98%	16,46%	0,0076≤0,0084≤0,0101	0,0076≤0,0016≤0,0101*	0,99	0,81
Tetracosanoic acid C24:1	19,24%	17,27%	0,0076≤0,0092≤0,0101(°)	0,0076≤0,0010≤0,0101*(°)	0,87	0,75

(#): Precision value referred to Eicosenoic Acid C20:1 in COI/T.20/Doc. No 33/Rev.1 2017

(°): Precision value referred to Lignoceric Acid C24:0 in COI/T.20/Doc. No 33/Rev.1 2017

*: sR values lower for high repeatability

10 FIGURES

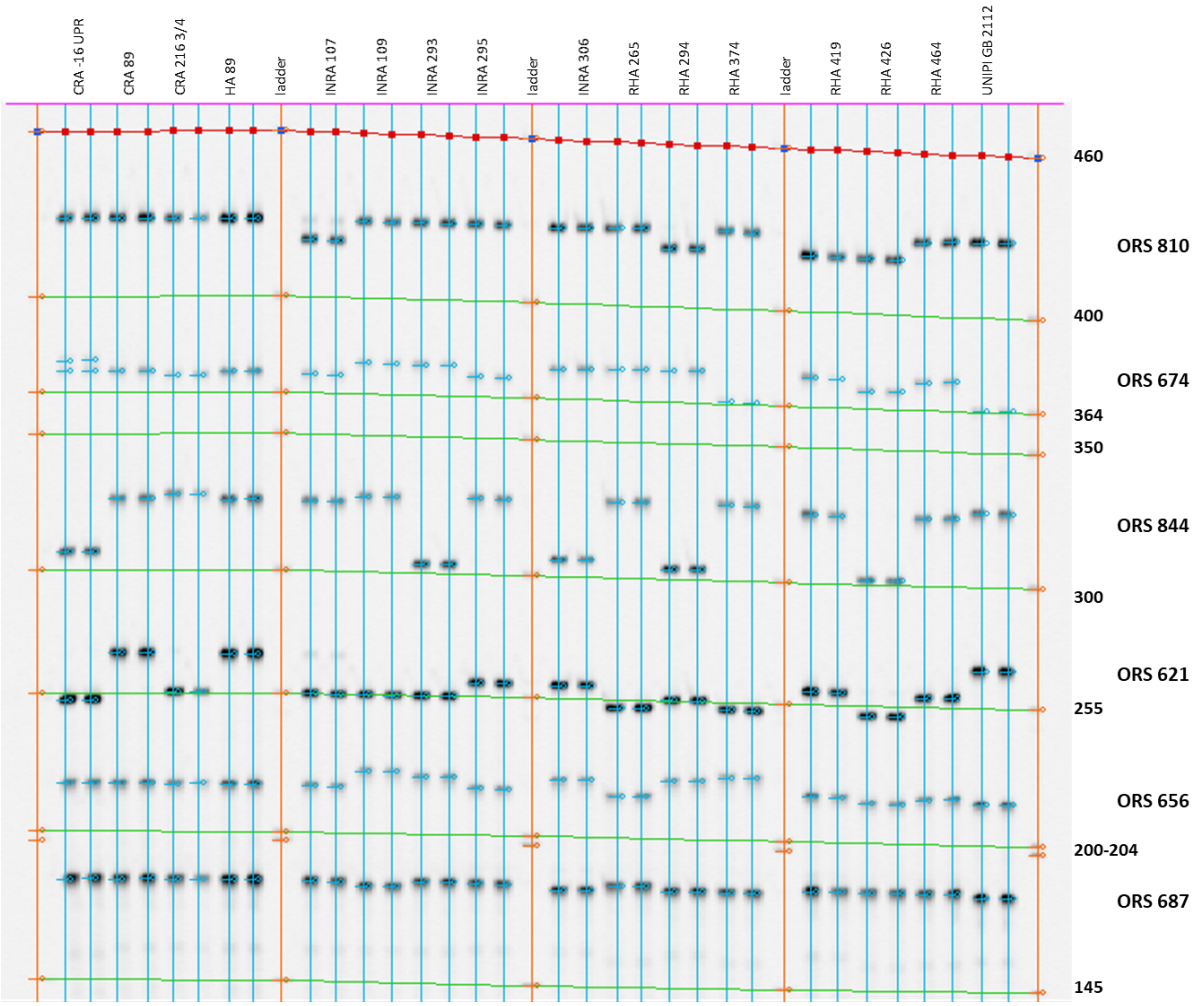


Figure 1. Amplification products obtained for the 16 sunflower lines with multiplex Set 2 separated on a LI-COR 4300 equipment. Each genotype is analysed in duplicate. On the right the name of the loci and the values of the molecular weight standard in bp. On the gel is superimposed the grid generated by the CARESTREAM Molecular Imaging Software used for amplicon size determination.

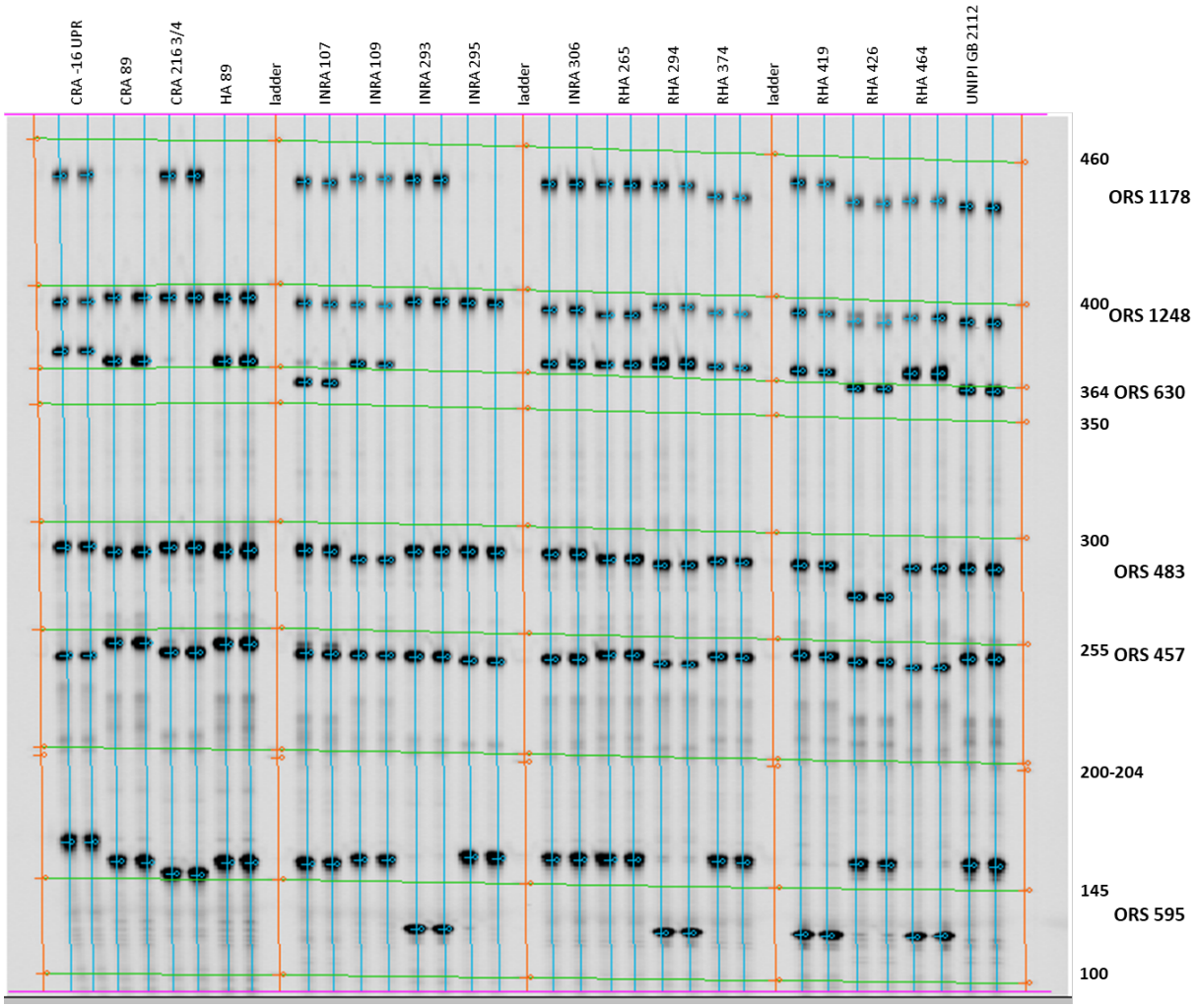


Figure 2. Amplification products obtained for the 16 sunflower lines with multiplex Set 3 separated on a LI-COR 4300 equipment. Each genotype is analysed in duplicate. On the right the name of the loci and the values of the molecular weight standard in bp. On the gel is superimposed the grid generated by the CARESTREAM Molecular Imaging Software used for amplicon size determination.

SET/MARKER		CRA 16UPR	CRA 89	CRA 216 3/4	HA 89	INRA 107	INRA 109	INRA 293	INRA 295	INRA 306	RHA 265	RHA 294	RHA 374	RHA 419	RHA 426	RHA 464	UNIPI GB 2112	Allele n.
SET 2	ORS 810	409,5	409,5	409,0	409,0	401,6	408,8	408,8	408,8	408,8	409,3	402,7	409,3	401,6	401,3	408,5	408,8	2
		409,5	409,5	409,0	409,0	401,6	408,8	408,8	408,8	408,8	409,3	402,7	409,3	401,6	401,3	408,5	408,8	
		F	F	F	F	I	F	F	F	F	F	I	F	I	I	F	F	
	ORS 674	356,5	352,8	351,3	352,8	351,8	356,5	356,3	352,3	356,0	356,8	356,8	345,8	355,7	351,6	356,0	345,5	4
		352,8	352,8	351,3	352,8	351,8	356,5	356,3	352,3	356,0	356,8	356,8	345,8	355,7	351,6	356,0	345,5	
		F/H	H	I	H	I	F	F	H	F	F	F	K	F	I	F	K	
	ORS 844	287,6	307,5	308,6	306,8	306,5	308,6	284,4	308,9	287,1	309,1	284,6	308,9	306,0	282,5	305,8	308,3	5
		287,6	307,5	308,6	306,8	306,5	308,6	284,4	308,9	287,1	309,1	284,6	308,9	306,0	282,5	305,8	308,3	
		Q	G	F	G	G	F	R	F	Q	F	R	F	G	S	G	F	
	ORS 621	233,4	250,8	236,5	250,3	236,0	235,8	236,0	240,9	240,6	232,9	236,3	233,2	240,6	232,2	239,4	249,8	6
		233,4	250,8	236,5	250,3	236,0	235,8	236,0	240,9	240,6	232,9	236,3	233,2	240,6	232,2	239,4	249,8	
		N	F	M	F	M	M	M	K	K	N	M	N	K	O	L	F	
	ORS 656	202,5	202,5	202,8	202,5	202,0	207,7	206,1	202,0	205,9	200,2	206,4	207,7	201,5	199,4	201,5	200,2	4
		202,5	202,5	202,8	202,5	202,0	207,7	206,1	202,0	205,9	200,2	206,4	207,7	201,5	199,4	201,5	200,2	
		I	I	I	I	I	F	G	I	G	J	G	F	I	J	I	J	
	ORS 687	165,7	165,7	166,0	166,0	165,4	163,8	166,0	165,4	163,6	165,7	164,1	163,8	164,9	164,9	164,6	163,6	2
		165,7	165,7	166,0	166,0	165,4	163,8	166,0	165,4	163,6	165,7	164,1	163,8	164,9	164,9	164,6	163,6	
		F	F	F	F	F	G	F	F	G	F	G	G	F	F	F	G	
SET 3	ORS 1178	426,2	null	425,9	null	423,7	425,7	425,7	null	425,4	425,9	426,2	422,3	429,1	422,0	423,4	421,8	4 + NULL
		427,2	null	426,9	null	423,7	425,7	425,7	null	425,4	425,9	426,2	422,3	429,1	422,0	423,4	421,8	
		H		H		I	H	H		H	H	H	J	F	J	I	J	
	ORS 1248	373,9	375,7	375,1	374,8	372,7	372,7	374,8	374,5	372,7	371,0	375,1	373,0	374,2	374,2	373,6	372,4	3
		373,9	375,7	375,1	374,8	372,7	372,7	374,8	374,5	372,7	371,0	375,1	373,0	374,2	371,0	373,6	372,4	
		G	F	F	F	G	G	F	F	G	H	F	G	F	F/H	G	G	
	ORS 630	352,1	348,0	null	347,4	346,8	346,8	null	null	349,1	349,4	350,6	350,0	349,4	342,9	349,7	343,2	5+NULL
		352,1	348,0	null	347,4	339,1	346,8	null	null	349,1	349,4	350,6	350,0	349,4	342,9	349,7	343,2	
		F	H		H	H/L	H			G	G	G	G	G	J	G	J	
	ORS 483	270,3	268,3	270,0	268,6	268,9	265,4	269,5	269,5	269,5	267,7	266,0	268,0	267,5	254,2	267,5	267,5	4
		270,3	268,3	270,0	268,6	268,9	265,4	269,5	269,5	269,5	267,7	266,0	268,0	267,5	254,2	267,5	267,5	
		F	G	F	G	G	H	F	F	F	G	H	G	G	N	G	G	
	ORS 457	224,4	230,2	225,6	229,0	229,0	225,3	225,3	223,8	225,3	227,6	224,4	227,6	229,0	227,0	225,3	229,3	4
		224,4	230,2	225,6	229,0	225,6	225,3	225,3	223,8	225,3	227,6	224,4	227,6	229,0	227,0	225,3	229,3	
		I	F	H	F	F/H	H	H	I	H	G	I	G	F	G	H	F	
	ORS 595	142,0	133,1	128,2	133,4	133,1	135,2	103,2	137,1	136,8	137,1	103,2	137,1	102,9	136,8	102,9	137,1	6
		142,0	133,1	128,2	133,4	133,1	135,2	103,2	137,1	136,8	137,1	103,2	137,1	102,9	136,8	102,9	137,1	
		F	K	M	K	K	J	Z	I	I	I	Z	I	Z	I	Z	I	

Figure 3. Amplicon sizes (bp) and alleles (letters) obtained for the 16 sunflower lines with multiplex sets 2 and 3 with LI-COR 4300. Amplicon sizes were determined with the CARESTREAM Molecular Imaging Software. Allele calling was done manually. For each locus letters were attributed starting from the "longest" allele that was given an "F" (blue cells).

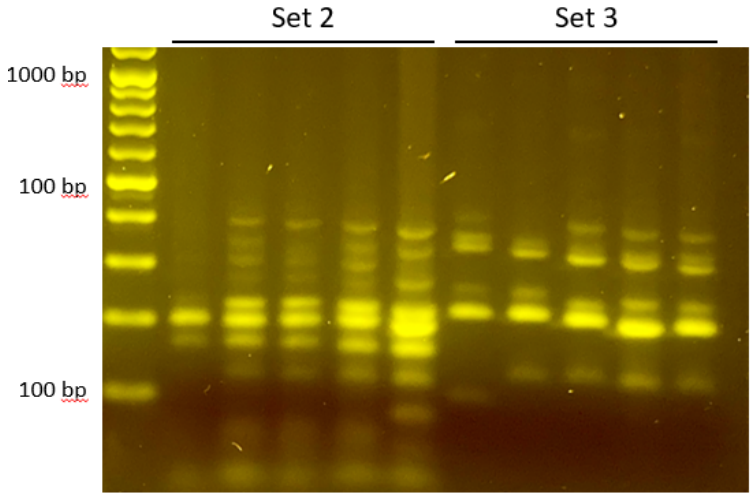


Figure 4. Agarose gel electrophoresis (1.8%, 1 X TBE) showing amplification pattern of multiplex Set 2 and 3 before optimization.

Step		0	1	3	2	5	4	6	7	final
Buffer	X	1	1	1	1	1	1	1	1	1
MgCl2	mM	2	2	2	2	2	2	2	2	2
dNTP	mM	0,25	0,25	0,25	0,25	0,25	0,25	0,25	0,25	0,25
ORS810	µM	0,5	**0,65**	**0,8**	**1**	**1**	**2**	1	1	1
ORS674	µM	0,5	**0,65**	**0,8**	**1**	**1**	**2**	1	1	1
ORS844	µM	0,5	**0,65**	**0,8**	**1**	**1**	**2**	1	1	1
ORS621	µM	0,5	0,5	**0,3**	**0,25**	**0,25**	**0,5**	0,25	0,25	0,25
ORS656	µM	0,5	0,5	**0,3**	**0,25**	**0,25**	**0,25**	**0,125**	0,125	0,125
ORS687	µM	0,5	0,5	**0,3**	**0,25**	**0,25**	**0,5**	0,25	0,25	0,25
Taq	Units	0,5	0,5	0,5	0,5	0,5	0,5	0,5	**0,75**	**1,0**
DNA	ng	80	80	80	80	80	80	80	80	80

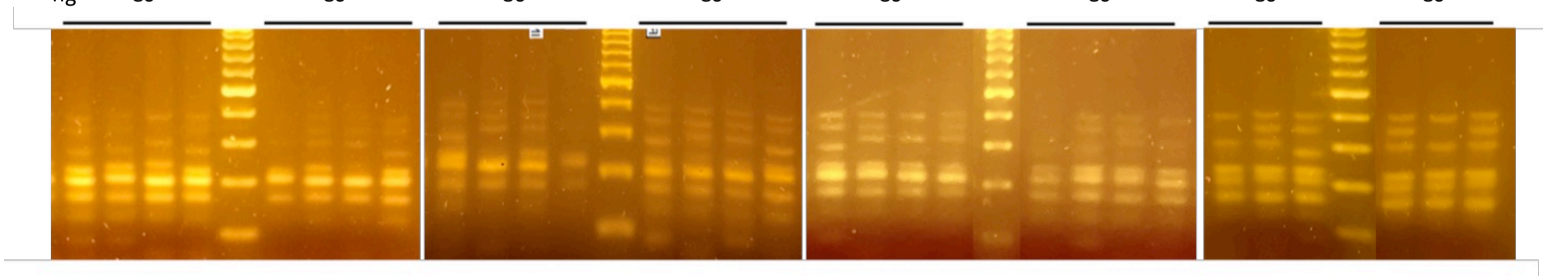


Figure 5. Agarose gel electrophoresis (1.8%, 1 X TBE) illustrating the optimization steps of reaction composition for multiplex PCR for Set 2 markers for separation on the QIAxcel system. At each stem the conditions modified are highlighted in bold. A similar process was followed for multiplex Set 3. Final results of the optimization process are shown in Figures 6 and 7.

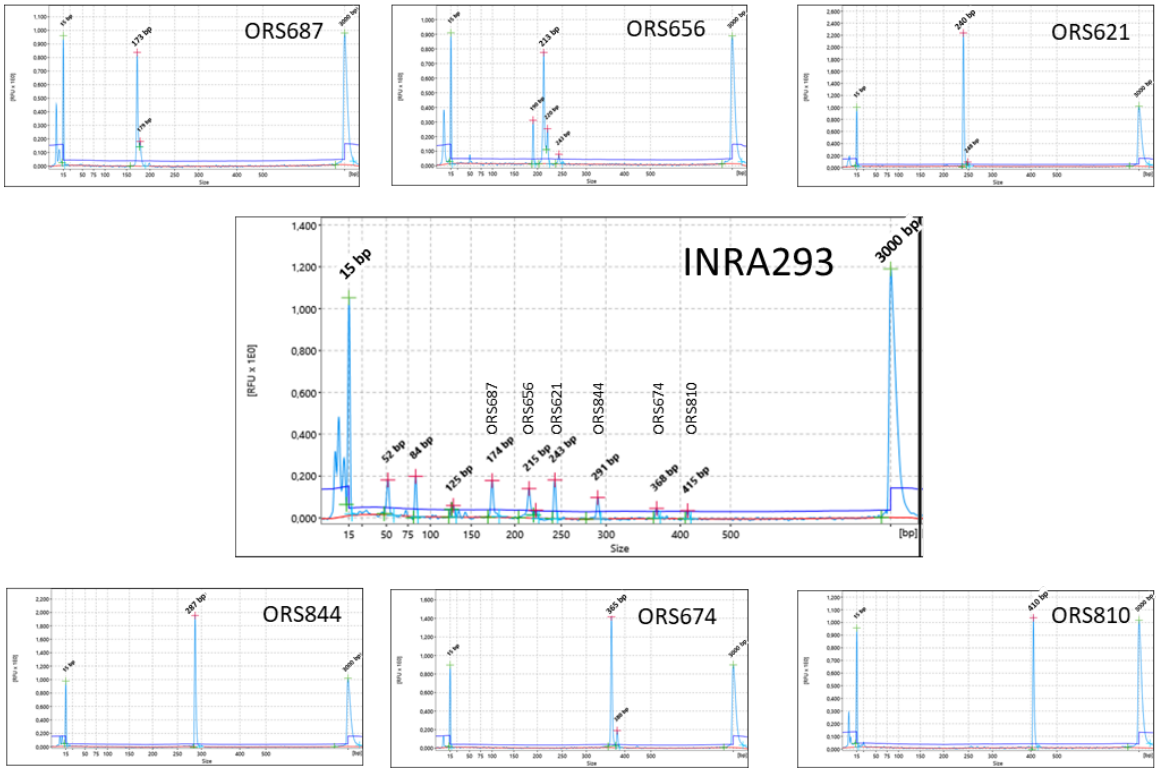


Figure 6. SSR profiles on QIAxcel system of Set 2 markers on an inbred line (RHA293) obtained by multiplex PCR (middle) or simplex PCR of single loci (top and bottom).

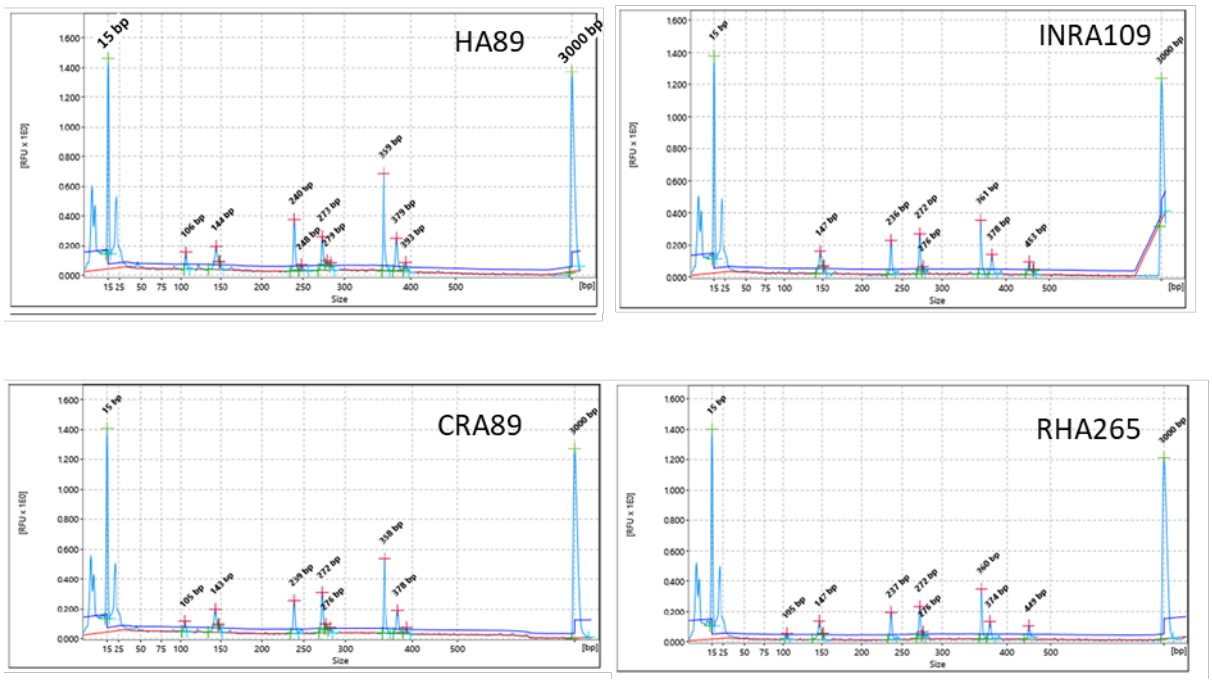


Figure 7. SSR profiles obtained by multiplex PCR of Set 3 on four of the sixteen inbred lines tested, followed by electrophoretic separation with QIAxcel. Please note that HA89 and CRA89 are two accessions of the same line.

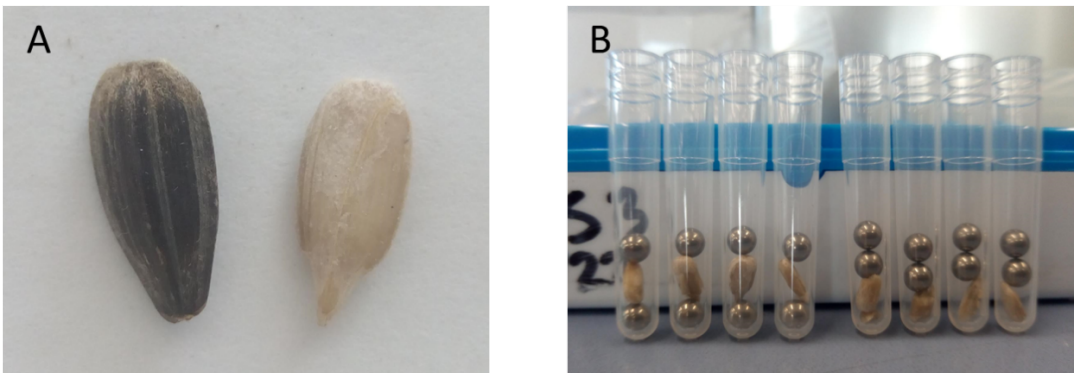


Figure 8. A: sunflower whole achene (with pericarp) (left); de-hulled achene (naked seed) (right). The seed coat is visible as a thin membrane covering the embryo. B: tubes containing naked seeds and stainless-steel balls for grinding. The setting on the left proved more efficient for grinding.

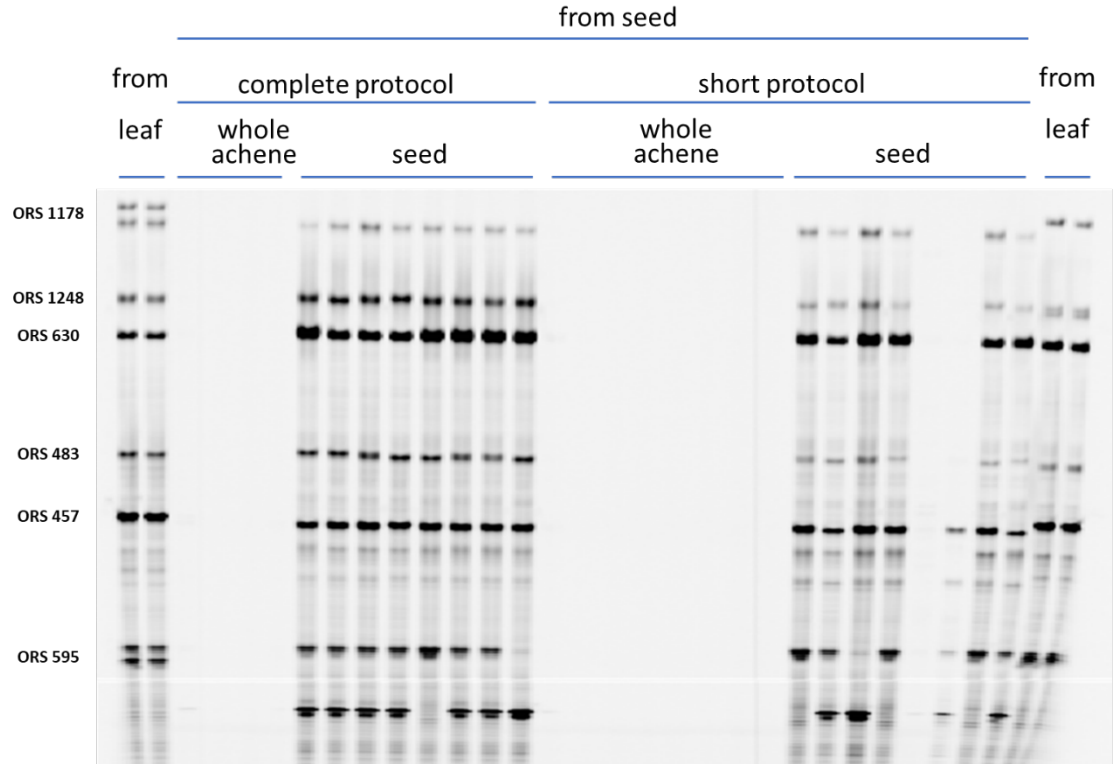


Figura 9. SSR profiles obtained with multiplex Set 3 on DNA extracted from seeds compared to DNA extracted from leaves, either by a complete or a short protocol, either from whole achenes or embryos. Leaf and seeds belonged to different genotypes.

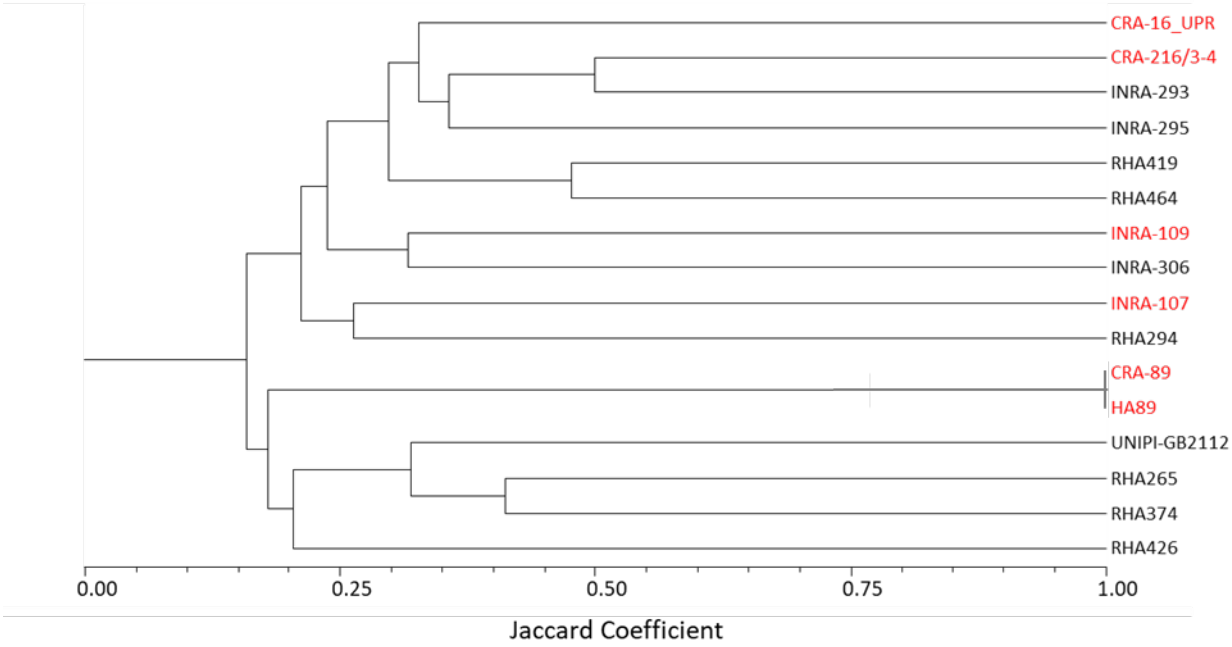
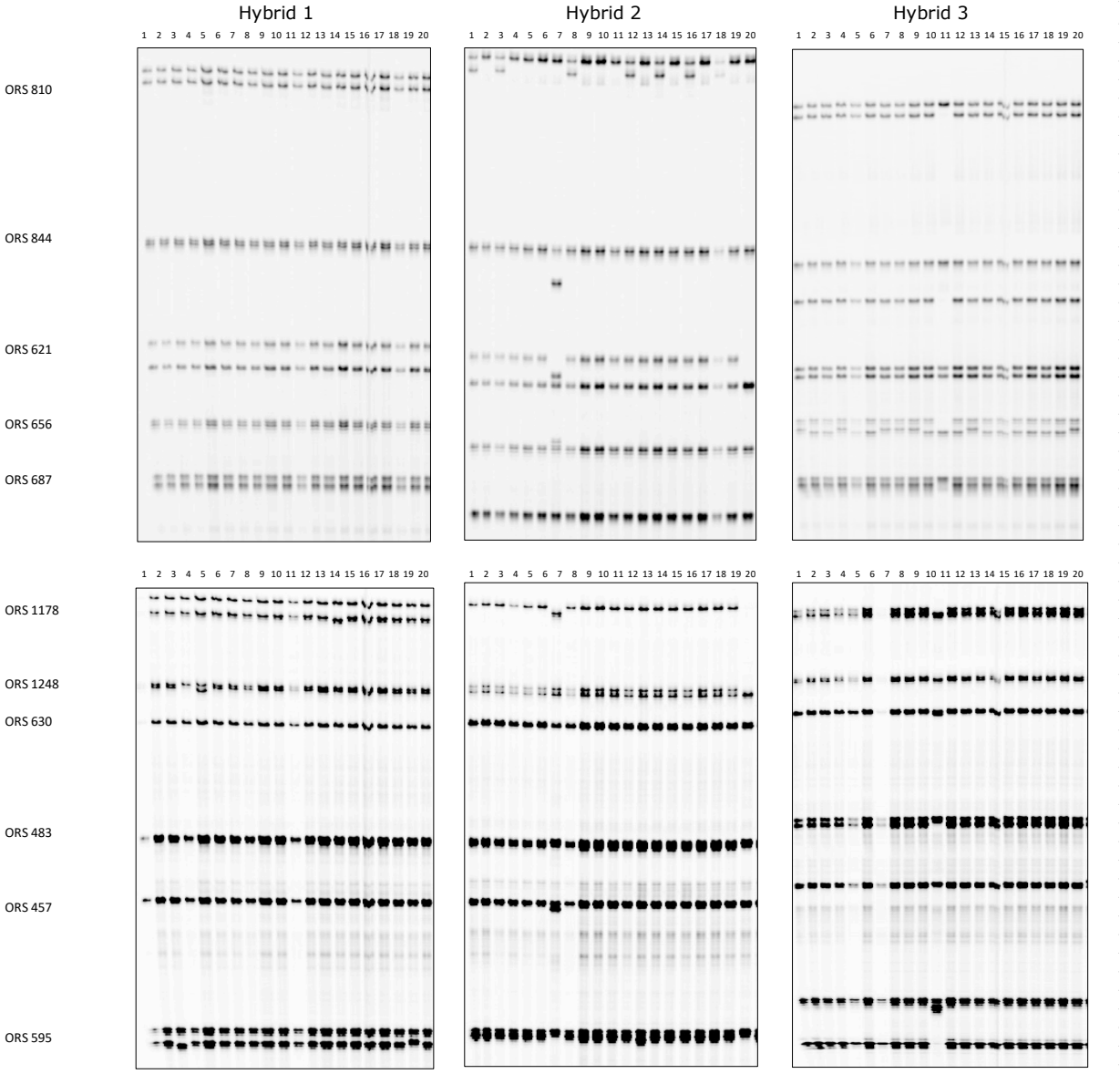


Figure 10. UPGMA dendrogram obtained from the analysis of the 12 SSR loci on the 16 sunflower lines. In red maintainer lines, in black restorer lines.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
ORS 810	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m
ORS 844	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
ORS 621	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
ORS 656	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
ORS 687	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
ORS 1178	-	H	H	H	H	H	H	H	H	H	H	H	F	H	H	H	H	H	H	H
ORS 1248	-	H	H	O	F	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
ORS 630	-	O	O	O	F	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
ORS 483	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
ORS 457	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
ORS 595	H	H	F	H	H	H	H	H	H	H	H	H	H	H	H	H	H	F	H	H
Polymorphic loci	8																			

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
ORS 810	H	O	H	O	O	O	O	H	O	O	O	H	O	H	O	H	O	H	O	O
	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m
ORS 844	O	O	O	O	O	O	H	O	O	O	O	O	O	O	O	O	O	O	O	O
ORS 621	H	H	H	H	H	H	F	H	H	H	H	H	H	H	H	H	H	H	H	O
ORS 656	O	O	O	O	O	O	F	O	O	O	O	O	O	O	O	O	O	O	O	O
ORS 687	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
ORS 1178	O	O	O	O	O	O	F	O	O	O	O	O	O	O	O	O	O	O	O	O
ORS 1248	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	O
ORS 630	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
ORS 483	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
ORS 457	O	O	O	O	O	O	F	O	O	O	O	O	O	O	O	O	O	O	O	O
ORS 595	H	H	H	H	H	H	H	O	H	H	H	H	H	H	H	H	H	H	H	O
Polymorphic loci	3																			

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
ORS 810	H	H	H	H	H	H	H	H	H	H	O	H	H	H	H	H	H	H	H	H
	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m
ORS 844	H	H	H	H	H	H	H	H	H	O	H	H	H	H	H	H	H	H	H	H
ORS 621	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
ORS 656	F	H	H	F	H	H	F	F	F	H	O	H	F	H	H	H	H	H	H	F
ORS 687	H	H	H	H	H	H	H	H	H	O	H	H	H	H	H	H	H	H	H	H
ORS 1178	H	H	H	H	H	H	-	H	H	H	O	H	H	H	H	H	H	H	H	H
ORS 1248	O	O	O	O	O	O	-	O	O	O	O	O	O	O	O	O	O	O	O	O
ORS 630	O	O	O	O	O	O	-	O	O	O	F	O	O	O	O	O	O	O	O	O
ORS 483	H	H	H	H	H	H	-	H	H	H	O	H	H	H	H	H	H	H	H	H
ORS 457	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
ORS 595	H	H	H	H	H	H	H	H	H	H	F	H	H	H	H	H	H	H	H	H
Polymorphic loci	7																			

Figure 11. Analysis of F1 seed lots with the two multiplex SSR sets. For each lot, 20 individuals were tested. Genotypes were classified as follows: H=heterozygous; O=homozygous; F=with foreign (unexpected) allele (either homo- or heterozygous); m=missing data; nd=not determined. Since parent lines were not available for genotyping, unexpected alleles could only be presumed based on their lower frequency.

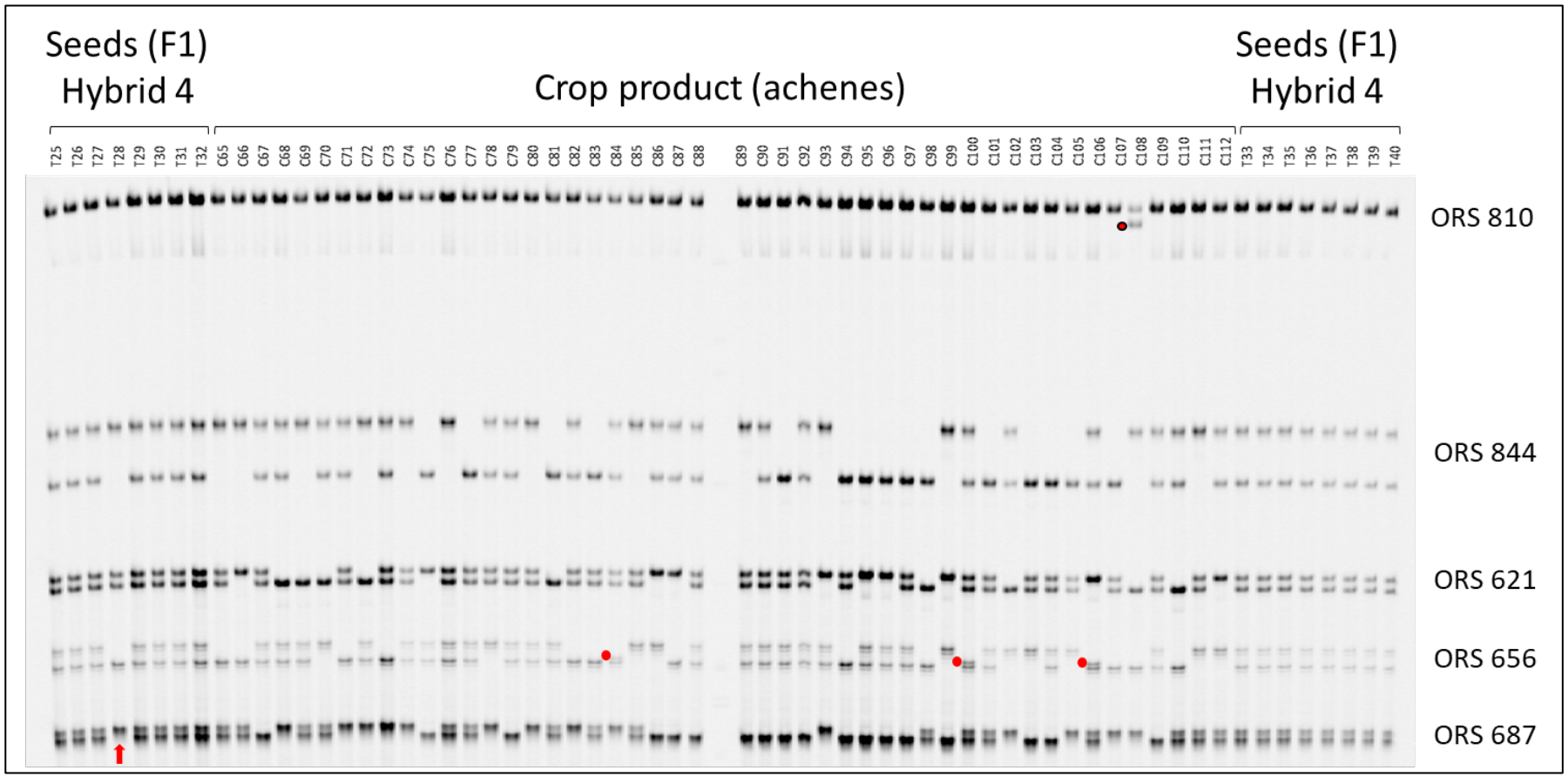


Figure 12. Multiplex SSR profiles of Set 2 with LI-COR 4300 of Hybrid 4 seeds.and their progeny obtained from production field. Red arrow indicates an individual F1 seed with unexpected homozygous loci. Red dots indicate unexpected alleles.

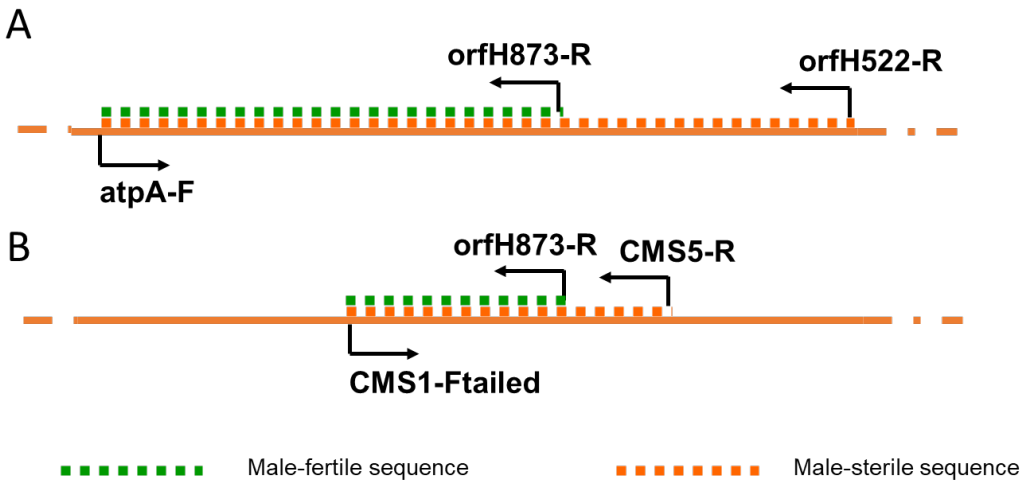


Figure 13. Adaptation of the assay for the detection of CMS *Pet1* described by Rieseberg *et al.* (1994) (A) to the LI-COR apparatus by shortening amplicons lenght (B).

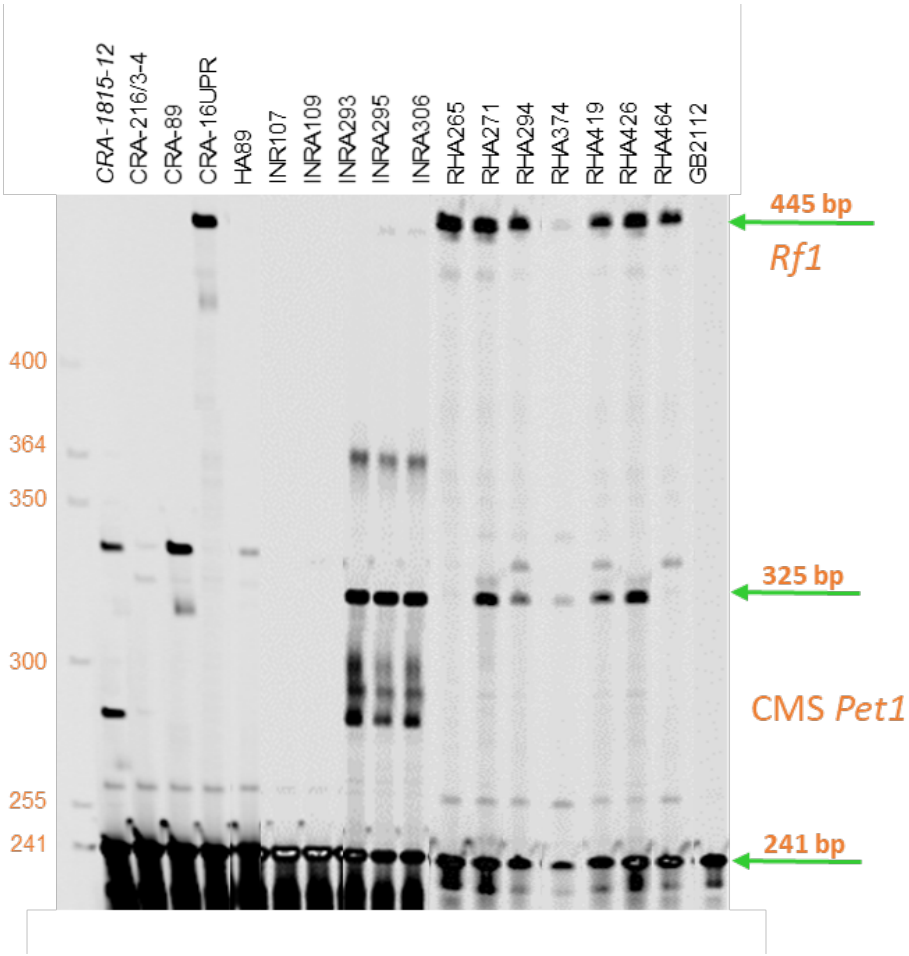


Figure 14. Duplex amplification of cytoplasmic male sterile sequence of *Pet1* and of *Rf1* nuclear marker in a set of maintainer and restorer lines. The 325 bp product is specific of the male sterile cytoplasm, the241 product is common to both male sterile and male fertile. Note the presence of *Rf1* in the maintainer CRA-16UPR, and its absence in the restorer UNIPI-GB2112 (both undesired).

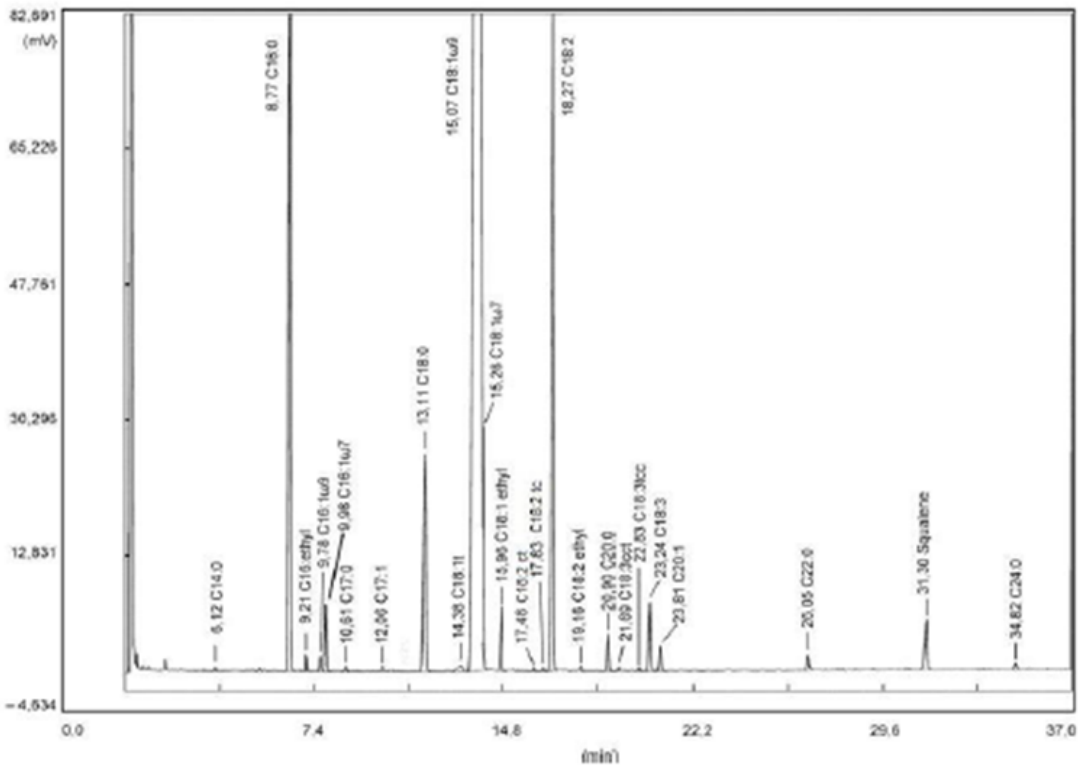


Figure 9. Gas chromatographic profile obtained by the new method specific for sunflower oil.

11 ANNEXES

Annex 1. Elementary data of peak area percentage for repeatability (RSD_R%) and reproducibility (RSD_{WR}%), and of retention time for specificity (expressed by Retention Time, RT) obtained by the application of the official method and the new laboratory method for determination of FAME.

FAME	Repeatability (Precision)		Reproducibility (Intra-lab precision)		Specificity (Retention time, min.)	
	Official method	New laboratory method	Official method	New laboratory method	Official method	New laboratory method
Palmitic acid C16:0	11,21	11,11	10,94	11,04	8,29	8,3
	11,05	11,01	11,68	11,34	8,31	8,3
	11,23	11,21	11,46	11,56	8,28	8,3
	11,01	11,11	10,76	10,87	8,3	8,29
	11,25	11,26	11,46	11,56	8,28	8,3
	11,15	11,1	11,99	11,88	8,3	8,3
	11,23	11,11	10,77	10,98	8,27	8,31
	11	11,03	11,75	11,78	8,31	8,3
	11,04	11,04	10,73	10,86	8,27	8,3
	11,1	11,1	11,05	11,02	8,32	8,32
	RSD_R% = 0,9	***RSD_R% = 0,7***	***RSD_{WR}% = 4,1***	***RSD_{WR}% = 3,4***	***RT_{mean} = 8,3***	***RT_{mean} = 8,3***
Palmitoleic acid C16:1	0,1	0,1	0,1	0,1	8,42	8,41
	0,1	0,1	0,1	0,1	8,43	8,41
	0,1	0,1	0,1	0,1	8,44	8,41
	0,1	0,1	0,1	0,1	8,41	8,41
	0,1	0,1	0,09	0,09	8,45	8,43
	0,1	0,1	0,09	0,09	8,43	8,41
	0,09	0,1	0,1	0,1	8,44	8,44
	0,1	0,1	0,1	0,1	8,41	8,41
	0,09	0,09	0,09	0,1	8,43	8,41
	0,1	0,1	0,1	0,1	8,41	8,42
	RSD_R% = 1.1	***RSD_R% = 0.9***	***RSD_{WR}% = 4.5***	***RSD_{WR}% = 3.9***	***RT_{mean} = 8.4***	***RT_{mean} = 8.4***
Heptadecanoic acid C17:0	0,08	0,08	0,08	0,09	8,77	8,78
	0,08	0,08	0,08	0,09	8,75	8,78
	0,08	0,08	0,08	0,09	8,78	8,78
	0,08	0,08	0,08	0,08	8,79	8,78
	0,08	0,08	0,08	0,09	8,77	8,76
	0,09	0,09	0,09	0,09	8,77	8,78
	0,08	0,08	0,09	0,08	8,74	8,75
	0,08	0,08	0,08	0,09	8,73	8,78
	0,08	0,08	0,1	0,08	8,77	8,78
	0,08	0,09	0,1	0,08	8,77	8,79
	RSD_R% = 2,1	***RSD_R% = 1,9***	***RSD_{WR}% = 5***	***RSD_{WR}% = 4,1***	***RT_{mean} = 8.8***	***RT_{mean} = 8.8***
Heptadecenoic acid C17:1	0,06	0,05	0,06	0,05	8,91	8,92
	0,06	0,05	0,05	0,05	8,91	8,92
	0,05	0,05	0,05	0,05	8,93	8,92
	0,06	0,05	0,06	0,05	8,91	8,92
	0,06	0,05	0,07	0,05	8,93	8,93
	0,06	0,05	0,07	0,05	8,91	8,92
	0,06	0,05	0,05	0,05	8,94	8,94
	0,06	0,05	0,05	0,05	8,91	8,92
	0,06	0,05	0,05	0,05	8,95	8,92
	0,06	0,05	0,05	0,06	8,91	8,93
	RSD_R% = 2	***RSD_R% = 1,4***	***RSD_{WR}% = 15***	***RSD_{WR}% = 5,9***	***RT_{mean} = 8.9***	***RT_{mean} = 8.9***

continues

Annex 1 - continued

FAME	Repeatability (Precision)		Reproducibility (Intra-lab precision)		Specificity (Retention time, min.)	
	Official method	New laboratory method	Official method	New laboratory method	Official method	New laboratory method
Stearic acid C18:0	3,38	3,38	3,31	3,45	9,4	9,38
	3,4	3,38	3,46	3,39	9,4	9,38
	3,39	3,38	3,52	3,38	9,42	9,38
	3,41	3,39	3,45	3,43	9,44	9,37
	3,43	3,37	3,47	3,46	9,4	9,38
	3,4	3,38	3,54	3,55	9,4	9,35
	3,41	3,39	3,48	3,36	9,42	9,38
	3,38	3,39	3,26	3,37	9,4	9,38
	3,45	3,4	3,38	3,52	9,4	9,38
	3,35	3,38	3,22	3,55	9,43	9,39
	RSD R% = 0,8	***RSD R% = 0,3***	***RSD WR% = 3,3***	***RSD WR% = 2,1***	***RT mean = 9.4***	***RT mean = 9.4***
Oleic acid C18:1	34,88	34,9	34,84	34,88	9,55	9,56
	34,91	34,91	35,1	34,91	9,55	9,56
	34,91	34,91	34,88	34,91	9,55	9,56
	34,94	34,91	34,98	34,95	9,58	9,59
	34,86	34,89	34,8	34,86	9,55	9,56
	34,92	34,92	34,78	34,92	9,56	9,57
	34,91	34,91	34,99	34,91	9,55	9,56
	34,91	34,91	34,71	34,91	9,57	9,56
	34,85	34,91	34,74	34,85	9,55	9,55
	34,86	34,92	34,67	34,86	9,58	9,56
	RSD R% = 0,08	***RSD R% = 0,03***	***RSD WR% = 0,40***	***RSD WR% = 0,09***	***RT mean = 9.5***	***RT mean = 9.6***
Linoleic acid (omega-6) C18:2	51,92	51,92	53,54	52,34	9,88	9,88
	52,08	52	52,13	52,01	9,88	9,89
	51,9	51,95	50,93	51,93	9,86	9,89
	51,8	51,9	52,74	51,61	9,88	9,87
	51,88	51,91	52,67	51,03	9,88	9,89
	51,46	51,86	51,11	51,55	9,87	9,89
	52,18	51,97	51,99	51,98	9,88	9,89
	51,99	51,98	51,02	51,03	9,88	9,9
	51,79	51,95	52,98	52,24	9,89	9,89
	52,12	51,91	51,21	52,86	9,88	9,9
	RSD R% = 0,4	***RSD R% = 0,08***	***RSD WR% = 1,8***	***RSD WR% = 1,1***	***RT mean = 9.9***	***RT mean = 9.9***
Alpha-Linolenic acid (omega-3) C18:3	1,29	1,31	1,35	1,3	10,21	10,22
	1,28	1,31	1,29	1,32	10,21	10,22
	1,31	1,31	1,32	1,33	10,21	10,23
	1,33	1,33	1,38	1,34	10,2	10,22
	1,31	1,31	1,37	1,35	10,21	10,25
	1,34	1,34	1,34	1,28	10,19	10,22
	1,31	1,31	1,31	1,29	10,21	10,27
	1,3	1,31	1,28	1,29	10,18	10,22
	1,29	1,32	1,27	1,3	10,21	10,22
	1,28	1,31	1,25	1,34	10,2	10,27
	RSD R% = 1,5	***RSD R% = 0,8***	***RSD WR% = 3,3***	***RSD WR% = 1,9***	***RT mean = 10.2***	***RT mean = 10.2***

continues

Annex 1 – continued

FAME	Repeatability (Precision)		Reproducibility (Intra-lab precision)		Specificity (Retention time, min.)	
	Official method	New laboratory method	Official method	New laboratory method	Official method	New laboratory method
Arachidic acid C20:0	0,37	0,37	0,43	0,38	10,74	10,75
	0,36	0,36	0,44	0,36	10,74	10,75
	0,35	0,36	0,35	0,35	10,77	10,74
	0,34	0,37	0,36	0,34	10,74	10,75
	0,37	0,37	0,37	0,37	10,74	10,75
	0,37	0,37	0,38	0,38	10,74	10,77
	0,37	0,37	0,4	0,37	10,75	10,75
	0,35	0,38	0,44	0,35	10,74	10,76
	0,37	0,37	0,37	0,38	10,78	10,75
	0,37	0,37	0,35	0,35	10,74	10,75
	RSD R% = 2,8	***RSD R% = 1,8***	***RSD WR% = 9,3***	***RSD WR% = 4,1***	***RT mean = 10.7***	***RT mean = 10.7***
Eicosenoic acid C20:1	0,33	0,32	0,38	0,33	10,94	10,95
	0,35	0,35	0,36	0,35	10,94	10,96
	0,36	0,33	0,33	0,32	10,94	10,95
	0,38	0,35	0,35	0,37	10,94	10,95
	0,39	0,35	0,38	0,38	10,96	10,95
	0,34	0,33	0,32	0,34	10,94	10,98
	0,35	0,36	0,36	0,36	10,95	10,95
	0,35	0,35	0,38	0,37	10,94	10,97
	0,35	0,35	0,4	0,33	10,94	10,95
	0,33	0,33	0,43	0,33	10,97	10,94
	RSD R% = 5,3	***RSD R% = 3,7***	***RSD WR% = 8,7***	***RSD WR% = 5,8***	***RT mean = 10.9***	***RT mean = 10.9***
Heneicosanoic acid C20:2	0,03	0,03	0,03	0,02	11,61	11,52
	0,03	0,03	0,03	0,03	11,6	11,5
	0,03	0,03	0,03	0,03	11,6	11,54
	0,02	0,03	0,02	0,03	11,63	11,5
	0,03	0,03	0,03	0,03	11,6	11,5
	0,03	0,03	0,03	0,03	11,6	11,54
	0,03	0,03	0,02	0,03	11,65	11,5
	0,03	0,03	0,03	0,03	11,6	11,5
	0,03	0,03	0,03	0,03	11,64	11,53
	0,03	0,03	0,03	0,03	11,6	11,5
	RSD R% = 5,3	***RSD R% = 4,2***	***RSD WR% = 8,8***	***RSD WR% = 4,9***	***RT mean = 11.6***	***RT mean = 11.5***
Behenic acid C22:0	0,7	0,73	0,8	0,73	12,59	12,6
	0,65	0,75	0,75	0,65	12,59	12,6
	0,63	0,74	0,74	0,79	12,57	12,6
	0,67	0,71	0,63	0,76	12,59	12,59
	0,75	0,65	0,6	0,62	12,59	12,6
	0,81	0,63	0,62	0,65	12,58	12,57
	0,83	0,6	0,59	0,55	12,59	12,6
	0,64	0,73	0,55	0,66	12,59	12,6
	0,59	0,78	0,5	0,84	12,6	12,61
	0,54	0,83	0,59	0,78	12,59	12,6
	RSD R% = 13,5	***RSD R% = 9,8***	***RSD WR% = 15,0***	***RSD WR% = 12,9***	***RT mean = 12.6***	***RT mean = 12.6***

Continues

Annex 1 – continued

FAME	Repeatability (Precision)		Reproducibility (Intra-lab precision)		Specificity (Retention time, min.)	
	Official method	New laboratory method	Official method	New laboratory method	Official method	New laboratory method
Lignoceric acid C24:0	0,24	0,25	0,21	0,23	14,87	14,88
	0,3	0,33	0,31	0,31	14,87	14,88
	0,26	0,35	0,25	0,25	14,86	14,9
	0,31	0,28	0,31	0,32	14,87	14,88
	0,39	0,2	0,43	0,41	14,85	14,88
	0,32	0,24	0,28	0,35	14,87	14,91
	0,27	0,19	0,22	0,25	14,87	14,88
	0,24	0,24	0,2	0,22	14,87	14,88
	0,2	0,24	0,13	0,18	14,88	14,86
	0,41	0,25	0,43	0,42	14,87	14,87
	RSD_R% = 22,8	***RSD_R% = 19,7***	***RSD_{WR}% = 35***	***RSD_{WR}% = 27,9***	***RT_{mean} = 14.9***	***RT_{mean} = 14.9***
Tetracoseoic acid C24:1	0,12	0,11	0,13	0,11	15,19	15,21
	0,1	0,1	0,08	0,1	15,21	15,21
	0,11	0,09	0,1	0,11	15,19	15,23
	0,1	0,08	0,05	0,1	15,19	15,21
	0,09	0,11	0,15	0,09	15,18	15,25
	0,12	0,1	0,13	0,12	15,19	15,21
	0,13	0,1	0,08	0,12	15,23	15,21
	0,15	0,14	0,1	0,16	15,19	15,22
	0,17	0,1	0,05	0,17	15,19	15,21
	0,1	0,07	0,09	0,1	15,21	15,23
	RSD_R% = 21,3	***RSD_R% = 17,7***	***RSD_{WR}% = 34,9***	***RSD_{WR}% = 22,6***	***RT_{mean} = 15.2***	***RT_{mean} = 15.2***

Annex 2. Linearity elementary data obtained by the application of the official method and the new laboratory method for determination of FAME.

New Laboratory method						*Official method*					
X=FAME (ppm)	Experimental-Y (Area)	Expected-Y (Area)	Residue (%)	Calibration curve		X=FAME (ppm)	Experimental-Y (Area)	Expected-Y (Area)	Residue (%)	Calibration curve	
				Y	x					Y	x
Palmitic Acid C16:0											
5,71	216864	187267	13,65	74983	-240513	5,71	228423	193234	18,21	75984	-240635
11,41	626796	615047	1,87			11,41	626919	626343	0,09		
28,53	1895409	1898386	0,16			28,53	1893053	1927191	1,77		
57,05	3901934	4037286	3,47			57,05	3901934	4094258	4,70		
114,1	7864545	8315084	5,73			114,1	7864890	8429151	6,69		
285,25	21346002	21148480	0,93			285,25	21045590	21433829	1,81		
Palmitoleic acid C16:1											
10,95	27084	24053	11,19	72165	-765794	10,95	262103	223635	17,20	71175	-555732
21,89	901996	813899	9,77			21,89	902119	1002291	9,99		
54,73	3252386	3183439	2,12			54,73	3250030	3339681	2,68		
109,45	6596960	7132672	8,12			109,45	7901934	7234383	9,23		
218,9	13629536	15031138	10,28			218,9	13629881	15024497	9,28		
547,25	39350577	38726537	1,59			547,25	39050165	38394841	1,71		
Heptadecanoic Acid C17:0											
7,37	257512	244637	5	72358	-288641	7,37	125247	108137	15,82	52358	-277741
14,74	900836	777914	13,65			14,74	494560	494014	0,11		
36,85	2312481	2377748	2,82			36,85	1784567	1651648	8,05		
73,7	4794008	5044136	5,22			73,7	3845671	3581036	7,39		
147,4	10039947	10376914	3,36			147,4	7045610	7439813	5,30		
368,5	26551810	26375245	0,66			368,5	16340579	19016145	14,07		

New Laboratory method						*Official method*					
X=FAME (ppm)	Experimental-Y (Area)	Expected-Y (Area)	Residue (%)	Calibration curve		X=FAME (ppm)	Experimental-Y (Area)	Expected-Y (Area)	Residue (%)	Calibration curve	
				Y	x					Y	x
Heptadecenoic Acid C17:1											
8,88	398417	330607	17,02	68235	-275319	8,88	249582	311887	19,98	58244	-205319
17,76	1095667	936534	14,52			17,76	900836	829094	8,65		
44,4	2832919	2754313	2,77			44,4	2312481	2380714	2,87		
88,8	5517678	5783946	4,83			88,8	4794008	4966748	3,48		
177,6	11146625	11843212	6,25			177,6	10039947	10138815	0,98		
444	30328314	30021008	1,01			444	26551810	25655017	3,50		
Stearic Acid C18:0											
5,05	332996	306934	7,83	66537	-28744	5,05	334186	282152	18,44	63544	-38744
10,09	672524	642613	4,45			10,09	672524	602413	11,64		
25,23	1518472	1649648	8,64			25,23	1518472	1564466	2,94		
50,45	3347022	3328041	0,57			50,45	3347022	3167040	5,68		
100,9	6765858	6684827	1,2			100,9	6765858	6372825	6,17		
252,25	16730374	16755183	0,15			252,25	16730374	15990179	4,63		
Oleic Acid C18:1											
8,42	638242	660897	3,55	73683	40491	8,42	740345	643121	15,12	71550	40672
16,84	1223225	1281304	4,75			16,84	1356705	1245569	8,92		
42,1	2828037	3142524	11,12			42,1	3305683	3052915	8,28		
84,2	5780990	6244557	8,02			84,2	6732019	6065157	10,99		
168,4	11899056	12448623	4,62			168,4	10738755	12089642	11,17		
421	30966928	31060823	0,3			421	32491038	30163096	7,72		
Linoleic acid C18:2											
9,74	499602	452259	9,48	71956	-248589	9,74	263272	321941	18,22	68956	-349686
19,48	1300489	1153107	11,33			19,48	1100589	993569	10,77		
48,7	3197783	3255650	1,81			48,7	3188783	3008452	5,99		
97,4	6569324	6759889	2,9			97,4	6969324	6366589	9,47		
194,8	13129781	13768366	4,86			194,8	14578345	13082865	11,43		
487	35076089	34793798	0,8			487	35682003	33231691	7,37		

New Laboratory method						*Official method*					
X=FAME (ppm)	Experimental-Y (Area)	Expected-Y (Area)	Residue (%)	Calibration curve		X=FAME (ppm)	Experimental-Y (Area)	Expected-Y (Area)	Residue (%)	Calibration curve	
				Y	x					Y	x
Heptadecenoic Acid C17:1											
5,8	84241	91522	8,64	62441	-270634	5,8	84241	5529	1423,67	60457	-345120
11,6	508717	453679	10,82			11,6	508717	356178	42,83		
29	1384796	1540148	11,22			29	1384796	1408124	1,66		
58	3323230	3350930	0,83			58	3323230	3161369	5,12		
116	6639754	6972494	5,01			116	6639754	6667857	0,42		
290	17981221	17837186	0,8			290	17981221	17187323	4,62		
Arachidic Acid C20:0											
6,18	199129	181089	9,06	70900	-257073	6,18	222126	187772	18,30	70805	-249803
12,36	650127	619250	4,75			12,36	640292	625346	2,39		
30,9	1753023	1933736	10,31			30,9	2247810	1938071	15,98		
61,8	4050554	4124546	1,83			61,8	4390183	4125946	6,40		
123,6	8248354	8506164	3,13			123,6	8839456	8501695	3,97		
309	21774619	21651021	0,57			309	24928204	21628942	15,25		
Eicosenoic Acid C20:1											
7,54	390923	343302	12,18	73199	-208619	7,54	370892	309334	19,90	68923	-210346
15,08	753023	895223	18,88			15,08	763920	829014	7,85		
37,7	2533871	2550985	0,68			37,7	2645736	2388055	10,79		
75,4	5045160	5310590	5,26			75,4	5329281	4986456	6,88		
150,8	9579448	10829799	13,05			150,8	10572821	10183257	3,83		
377	27904898	27387425	1,85			377	28364610	25773663	10,05		
Heneicosenoic Acid C20:2											
5,19	372599	336900	9,58	74015	-46868	5,19	354281	327667	8,12	72019	-46114
10,37	885118	720668	18,58			10,37	836671	700727	19,40		
25,93	1837545	1871972	1,87			25,93	1743011	1821347	4,30		
51,85	3620510	3790812	4,7			51,85	3871203	3688087	4,97		
103,7	7265775	7628492	4,99			103,7	7713671	7422288	3,93		
259,25	19308829	19141531	0,87			259,25	21345722	18624890	14,61		

New Laboratory method						*Official method*					
X=FAME (ppm)	Experimental-Y (Area)	Expected-Y (Area)	Residue (%)	Calibration curve		X=FAME (ppm)	Experimental-Y (Area)	Expected-Y (Area)	Residue (%)	Calibration curve	
				Y	x					Y	x
Behenic Acid C22:0											
5,16	205611	220180	7,09	74252	-162961	5,16	234821	220411	6,54	70272	-142193
10,32	682023	603321	11,54			10,32	675931	583015	15,94		
25,8	1720218	1752744	1,89			25,8	1837210	1670827	9,96		
51,6	3566309	3668449	2,86			51,6	3819371	3483847	9,63		
103,2	7256043	7499859	3,36			103,2	7519201	7109887	5,76		
258	19108440	18994089	0,6			258	20471010	17988008	13,80		
Lignoceric Acid C24:0											
6,84	74334	62094	16,47	64083	-376235	6,84	73650	90906	18,98	62137	-334113
13,68	553540	500422	9,6			13,68	556921	515925	7,95		
34,2	1739572	1815409	4,36			34,2	1937290	1790983	8,17		
68,4	3663449	4007053	9,38			68,4	3318719	3916078	15,25		
136,8	8088086	8390341	3,74			136,8	7831711	8166270	4,10		
342	21721541	21540204	0,83			342	24182012	20916844	15,61		
Tetracosenoic Acid C24:1											
7,08	115161	111959	2,78	78414	-443209	7,08	119312	103437	15,35	75371	-430192
14,16	806412	667128	17,27			14,16	759651	637067	19,24		
35,4	2266976	2332633	2,9			35,4	2381821	2237955	6,43		
70,8	4980901	5108476	2,56			70,8	4716212	4906103	3,87		
141,6	10096329	10660161	5,58			141,6	11283712	10242398	10,17		
354	27559796	27315218	0,89			354	28102811	26251283	7,05		

Annex 3. Elementary data for LOQ determination obtained by the application of the official method and the new laboratory method for determination of FAME.

	New laboratory method			***Official method***		
Palmitic Acid C16:0	L1 (Area)	Mean Blank (Area)	Blank Correction (Area)	L1 (Area)	Mean Blank (Area)	Blank Correction (Area)
	5,02	0	5,02	5,06	0	5,06
	5,02	0	5,02	4,97	0	4,97
	4,98	0	4,98	4,90	0	4,90
	5,07	0	5,07	5,12	0	5,12
	4,94	0	4,94	4,98	0	4,98
	4,97	0	4,97	4,96	0	4,96
	4,97	0	4,97	5,00	0	5,00
	5,04	0	5,04	5,12	0	5,12
	5,1	0	5,1	5,13	0	5,13
	5,09	0	5,09	5,17	0	5,17
		Mean	5,02		Mean	5,04
		Std Dev	0,06		Std Dev	0,09
		CMR (L1 Conc. ppm)	5		CMR (L1 Conc. ppm)	5
		LoD (ppm)	0,11		LoD (ppm)	0,18
		LoQ (ppm)	0,56		LoQ (ppm)	0,90
Palmitoleic acid C16:1	L1 (Area)	Mean Blank (Area)	Blank Correction (Area)	L1 (Area)	Mean Blank (Area)	Blank Correction (Area)
	715188	0	715188	723719	0	723719
	726949	0	726949	723821	0	723821
	728160	0	728160	721921	0	721921
	737074	0	737074	721820	0	721820
	721424	0	721424	723311	0	723311
	727145	0	727145	731291	0	731291
	722975	0	722975	721210	0	721210
	734298	0	734298	742109	0	742109
	744070	0	744070	731210	0	731210
	739661	0	739661	757571	0	757571
		Mean	729694,4		Mean	729798,3
		Std Dev	8956,8		Std Dev	11744,2
		CMR (L1 Conc. ppm)	5		CMR (L1 Conc. ppm)	5,00
		LoD (ppm)	0,12		LoD (ppm)	0,16
		LoQ (ppm)	0,61		LoQ (ppm)	0,80
Heptadecanoic Acid C17:0	L1 (Area)	Mean Blank (Area)	Blank Correction (Area)	L1 (Area)	Mean Blank (Area)	Blank Correction (Area)
	491706	0	491706	501281	0	501281
	501639	0	501639	502912	0	502912
	500832	0	500832	492181	0	492181
	505326	0	505326	502121	0	502121
	494545	0	494545	503121	0	503121
	499601	0	499601	503238	0	503238
	500771	0	500771	502821	0	502821
	505163	0	505163	508219	0	508219
	511568	0	511568	510291	0	510291
	508879	0	508879	524120	0	524120
		Mean	502003		Mean	505030,5
		Std Dev	6057,5		Std Dev	8206,4
		CMR (L1 Conc. ppm)	5,00		CMR (L1 Conc. ppm)	5,00
		LoD (ppm)	0,12		LoD (ppm)	0,16
		LoQ (ppm)	0,60		LoQ (ppm)	0,81

continues

	New laboratory method			***Official method***		
Heptadecenoic Acid C17:1	L1 (Area)	Mean Blank (Area)	Blank Correction (Area)	L1 (Area)	Mean Blank (Area)	Blank Correction (Area)
	686965	0,31	686964,69	686984	0,31	686983,69
	695211	0,31	695210,69	693482	0,31	693481,69
	697507	0,15	697506,85	695701	0,15	695700,85
	702983	0,16	702982,84	702192	0,16	702191,84
	685660	0,33	685659,67	682912	0,33	682911,67
	693793	0,32	693792,68	692812	0,32	692811,68
	698272	0,34	698271,66	697281	0,34	697280,66
	721336	0,36	721335,64	721928	0,36	721927,64
	711347	0,34	711346,66	714929	0,34	714928,66
	723006	0,33	723005,67	721121	0,33	721120,67
		Mean	701607,7		Mean	700933,905
		Std Dev	13091,6		Std Dev	13853,7
		CMR (L1 Conc. ppm)	5,00		CMR (L1 Conc. ppm)	5,00
		LoD (ppm)	0,19		LoD (ppm)	0,20
		LoQ (ppm)	0,93		LoQ (ppm)	0,99
Stearic Acid C18:0	L1 (Area)	Mean Blank (Area)	Blank Correction (Area)	L1 (Area)	Mean Blank (Area)	Blank Correction (Area)
	358297	0	358297	357618	0	357618
	368388	0	368388	361919	0	361919
	366072	0	366072	367191	0	367191
	368209	0	368209	369282	0	369282
	362173	0	362173	358729	0	358729
	369188	0	369188	361719	0	361719
	362167	0	362167	367192	0	367192
	372924	0	372924	371911	0	371911
	372346	0	372346	371913	0	371913
	374749	0	374749	375119	0	375119
		Mean	367451,3		Mean	366259,3
		Std Dev	5303,3		Std Dev	6001,0
		CMR (L1 Conc. ppm)	5,00		CMR (L1 Conc. ppm)	5,00
		LoD (ppm)	0,14		LoD (ppm)	0,16
		LoQ (ppm)	0,72		LoQ (ppm)	0,82
Oleic Acid C18:1	L1 (Area)	Mean Blank (Area)	Blank Correction (Area)	L1 (Area)	Mean Blank (Area)	Blank Correction (Area)
	714676	0,6	714675,4	727381	0,6	727380,4
	717989	1,15	717987,85	732911	1,15	732909,85
	716664	0,79	716663,21	716352	0,79	716351,21
	728982	0,73	728981,27	728391	0,73	728390,27
	702252	0,99	702251,01	729121	0,99	729120,01
	700770	1	700769	712830	1	712829
	701959	0,95	701958,05	739101	0,95	739100,05
	707073	0,95	707072,05	702811	0,95	702810,05
	714542	0,92	714541,08	729121	0,92	729120,08
	705356	0,89	705355,11	712729	0,89	712728,11
		Mean	711025,4		Mean	723073,903
		Std Dev	9070,9		Std Dev	11261,1
		CMR (L1 Conc. ppm)	5,00		CMR (L1 Conc. ppm)	5,00
		LoD (ppm)	0,13		LoD (ppm)	0,16
		LoQ (ppm)	0,64		LoQ (ppm)	0,78
Linoleic acid C18:2	L1 (Area)	Mean Blank (Area)	Blank Correction (Area)	L1 (Area)	Mean Blank (Area)	Blank Correction (Area)
	890569	0,43	890568,57	892011	0,43	892010,57
	898571	0,37	898570,63	875101	0,37	875100,63
	884626	0,15	884625,85	911810	0,15	911809,85
	902488	0	902488	891028	0	891028
	880015	0	880015	893011	0	893011
	879216	0,17	879215,83	865910	0,17	865909,83
	884016	0,17	884015,83	898719	0,17	898718,83
	893587	0	893587	892721	0	892721
	906201	0	906201	872918	0	872918
	887727	0	887727	898182	0	898182
		Mean	890701,47		Mean	889140,971
		Std Dev	9345,3		Std Dev	13852,2
		CMR (L1 Conc. ppm)	5,00		CMR (L1 Conc. ppm)	5,00
		LoD (ppm)	0,10		LoD (ppm)	0,16
		LoQ (ppm)	0,52		LoQ (ppm)	0,78

continues

	New laboratory method			*Official method*		
Alpha-linolenic acid C18:3	L1 (Area)	Mean Blank (Area)	Blank Correction (Area)	L1 (Area)	Mean Blank (Area)	Blank Correction (Area)
	361632	0	361632	356181	0	356181
	368037	0	368037	367191	0	367191
	368166	0	368166	360191	0	360191
	365450	0	365450	365789	0	365789
	356170	0	356170	359876	0	359876
	365674	0	365674	367198	0	367198
	358376	0	358376	371911	0	371911
	356512	0	356512	378191	0	378191
	362273	0	362273	361719	0	361719
	368169	0	368169	369181	0	369181
		Mean	363045,9		Mean	365742,8
		Std Dev	4766,0		Std Dev	6514,1
		CMR (L1 Conc. ppm)	5,00		CMR (L1 Conc. ppm)	5,00
		LoD (ppm)	0,13		LoD (ppm)	0,18
		LoQ (ppm)	0,66		LoQ (ppm)	0,89
Arachidic Acid C20:0	L1 (Area)	Mean Blank (Area)	Blank Correction (Area)	L1 (Area)	Mean Blank (Area)	Blank Correction (Area)
	365536	0	365536	370101	0	370101
	367739	0	367739	373131	0	373131
	374319	0	374319	361719	0	361719
	374080	0	374080	368191	0	368191
	367507	0	367507	371812	0	371812
	373887	0	373887	364910	0	364910
	372640	0	372640	363928	0	363928
	373237	0	373237	378001	0	378001
	375533	0	375533	366292	0	366292
	373322	0	373322	367712	0	367712
		Mean	371780		Mean	368579,7
		Std Dev	3481,3		Std Dev	4836,8
		CMR (L1 Conc. ppm)	5,00		CMR (L1 Conc. ppm)	5,00
		LoD (ppm)	0,09		LoD (ppm)	0,13
		LoQ (ppm)	0,47		LoQ (ppm)	0,66
Eicosenoic Acid C20:1	L1 (Area)	Mean Blank (Area)	Blank Correction (Area)	L1 (Area)	Mean Blank (Area)	Blank Correction (Area)
	536789	0	536789	546191	0	546191
	548868	0	548868	558210	0	558210
	549504	0	549504	558211	0	558211
	553135	0	553135	556271	0	556271
	543003	0	543003	548109	0	548109
	545382	0	545382	554918	0	554918
	547432	0	547432	557019	0	557019
	548795	0	548795	539827	0	539827
	562511	0	562511	542719	0	542719
	554960	0	554960	573241	0	573241
		Mean	549037,9		Mean	553471,6
		Std Dev	6960,9		Std Dev	9671,3
		CMR (L1 Conc. ppm)	5,00		CMR (L1 Conc. ppm)	5,00
		LoD (ppm)	0,13		LoD (ppm)	0,17
		LoQ (ppm)	0,63		LoQ (ppm)	0,87
Henicosenoic Acid C20:2	L1 (Area)	Mean Blank (Area)	Blank Correction (Area)	L1 (Area)	Mean Blank (Area)	Blank Correction (Area)
	404380	0	404380	412928	0	412928
	416820	0	416820	435373	0	435373
	411922	0	411922	423731	0	423731
	411148	0	411148	428472	0	428472
	405803	0	405803	418327	0	418327
	411882	0	411882	428282	0	428282
	406173	0	406173	427384	0	427384
	409906	0	409906	431773	0	431773
	420070	0	420070	427182	0	427182
	412098	0	412098	424011	0	424011
		Mean	411020,2		Mean	425746,3
		Std Dev	4879,9		Std Dev	6446,6
		CMR (L1 Conc. ppm)	5,00		CMR (L1 Conc. ppm)	5,00
		LoD (ppm)	0,12		LoD (ppm)	0,15
		LoQ (ppm)	0,59		LoQ (ppm)	0,76

continues

	New laboratory method			*Official method*		
Behenic Acid C22:0	L1 (Area)	Mean Blank (Area)	Blank Correction (Area)	L1 (Area)	Mean Blank (Area)	Blank Correction (Area)
	429957	0	429957	464991	0	464991
	448749	0	448749	458201	0	458201
	445978	0	445978	447281	0	447281
	446936	0	446936	452919	0	452919
	439105	0	439105	471912	0	471912
	442541	0	442541	452171	0	452171
	445005	0	445005	457219	0	457219
	446297	0	446297	446181	0	446181
	453195	0	453195	448102	0	448102
	445826	0	445826	453431	0	453431
		Mean	444358,9		Mean	455240,8
		Std Dev	6247,3		Std Dev	8166,1
		CMR (L1 Conc. ppm)	5,00		CMR (L1 Conc. ppm)	5,00
		LoD (ppm)	0,14		LoD (ppm)	0,18
		LoQ (ppm)	0,70		LoQ (ppm)	0,90
Lignoceric Acid C24:0	L1 (Area)	Mean Blank (Area)	Blank Correction (Area)	L1 (Area)	Mean Blank (Area)	Blank Correction (Area)
	380366	0	380366	371810	0	371810
	390584	0	390584	381729	0	381729
	385104	0	385104	387261	0	387261
	395523	0	395523	387203	0	387203
	392801	0	392801	390287	0	390287
	395407	0	395407	382921	0	382921
	395523	0	395523	397161	0	397161
	382150	0	382150	387201	0	387201
	398684	0	398684	388273	0	388273
	395045	0	395045	399011	0	399011
		Mean	391118,7		Mean	387285,7
		Std Dev	6369,9		Std Dev	7705,9
		CMR (L1 Conc. ppm)	5,00		CMR (L1 Conc. ppm)	5,00
		LoD (ppm)	0,16		LoD (ppm)	0,20
		LoQ (ppm)	0,81		LoQ (ppm)	0,99
Tetracosenoic Acid C24:1	L1 (Area)	Mean Blank (Area)	Blank Correction (Area)	L1 (Area)	Mean Blank (Area)	Blank Correction (Area)
	531065	0	531065	547191	0	547191
	531595	0	531595	537192	0	537192
	540559	0	540559	548231	0	548231
	544433	0	544433	544627	0	544627
	521597	0	521597	538191	0	538191
	535407	0	535407	556292	0	556292
	526213	0	526213	538219	0	538219
	521375	0	521375	523740	0	523740
	540406	0	540406	548210	0	548210
	528539	0	528539	531491	0	531491
		Mean	532118,9		Mean	541338,4
		Std Dev	8011,0		Std Dev	9468,7
		CMR (L1 Conc. ppm)	5,00		CMR (L1 Conc. ppm)	5,00
		LoD (ppm)	0,15		LoD (ppm)	0,17
		LoQ (ppm)	0,75		LoQ (ppm)	0,87

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