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METHODS FOR DETERMINATION OF FATTY ACIDS ADAPTED TO A BREEDING PROGRAM

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The aim of quality breeding is to produce new varieties with improved fatty acid composition and improved quality of the meal. Such varieties which are called double-zero varieties also bring about an increased content of linoleic acid and a diminished content of linolenic acid. In the breeding work all common methods for improved fatty acid composition, including the half-seed technique, are used which implies that oil from a lot of sources should be analysed. The methods for fatty acid determination must be applicable to samples from a seed lot, from a single plant as well as from one cotyledon. The samples from seed lots come from variety tests or they may also arise from the seed production. The usual quantity is 5 grams. The samplings from single plants may arise from single plant selection or from single plants or a new variety. The normal size of the samples is about 100 seeds but when the plants originate from the greenhouse we sometimes have to use 30 mg (about 10 seeds). The half-seed technique may be a part of a crossing program or a purification of a contaminated new variety. In addition to these samples originating from the breeding program we also determine the erucic acid content of farmers' samples which is a part of the quality analysis that serves as base for the compensation of the farmers' deliveries. In a breeding program it is necessary to carry out a large number of analyses during a short period of time which means that this must be done with a minimum of work contribution. To effect this we have simplified the different methods of determination of the fatty acid pattern as much as possible and tried to adapt the treatment of the different samplings to a standard method.

For the analysis of the fatty acids we earlier used both paper and gas chromatography but now all analyses are made with the later technique. There are several reasons for this. We now have 8 automatic injectors with a daily capacity of about 1200 analyses, if the duration of each chromatogram does not exceed 7 minutes. Also, the interest in the other fatty acids beside erucic acid has increased; even in samples where the main interest is erucic acid we may control other fatty acids. Finally, the results of gas chromatography are much safer than those obtained from paper chromatography.

The triglycerides must be extracted and transesterified before gas chromatography is used. While most laboratories use methylate in methanol for this process we are now as a rule using ethylate in ethanol. Previously, the seed samples were extracted in steel tubes sealed with rubber stoppers. When we tried to extract and transesterify with a mixture of petroleum ether and methylate in methanol in one step the rubber stoppers appeared not to be resistent to methanol. They were, however, resistent to ethanol so we changed to ethylate in ethanol as transesterification reagent. For samples which are to be compared with official comparative trials we still use methylate in methanol.

As a rule a 0.5 M solution of sodium methylate in methanol, prepared by the addition of sodium to methanol, is used as transesterification reagent. In order to simplify the preparation of the reagent we have changed to a 0.02 M solution of both our reagents prepared by the addition of sodium hydroxide to methanol and ethanol (99.5 %) respectively. With these reagents the transesterification is complete within 15 minutes and if there occur a formation of free fatty acids the quantity will be too small to be detectable. We have found that 0.02 M or there about will be the optimal concentration of the reagents. With a lower concentration the transesterification rate is too slow and with a higher concentration the formation of free fatty acids will cause some problems.

In the half-seed technique the seeds are sown in peat pots or in plant growth pouches. The outer cotyledons are removed as soon as the seeds have germinated. The cotyledons are then placed in small test tubes and crushed with a steel rod. 100 µl of petroleum ether/0.02 M sodium hydroxide in 99.5 % ethanol (2/3) are added and the tubes are left overnight at room temperature. The solvent is allowed to evaporate and 50 µl of petroleum ether is added. Through this operation, evaporation and addition of solvent, the resulting solution will be more concentrated and free from glycerol present in the original solution. A small volume (2-5 µl) is then transferred by a capillary tube to aluminium capsules fitting Perkin-Elmer's automatic injector. This autosampler is normally loaded with 100 capsules but in our version the magazine holder is elongated and it is possible to load it with 210 samples (Fig. 1).

As mentioned previously, the samples from single plants are divided into two groups. When the harvest of the plants is small 30 mg of seeds are weighed to get a more uniform size of the sample. Normally, however, 0.6 ml of seeds (about 100 seeds) are used. The small samples are simultaneously extracted and transesterified at the same time in steel tubes containing one steel ball and 5 ml petroleum ether/0.02 M sodium hydroxide in ethanol (2/3) in a shaking machine. After one hour the solutions are filtered into test tubes containing 5 ml of a salt solution. As soon as the two layers have separated, the benzine phases are transferred to small test tubes and are then ready for gas chromatography. The larger samples are treated in nearly the same way except that the seeds are crushed in small paper bags by means of a vice and transferred to test tubes for extraction and transesterification. 8 ml of the ethylate reagent is added and after a short shaking the tubes are left over night. In this case 10 ml of salt solution is added. The application of the vice has greatly simplified the analysis as the seeds can be crushed in the paper bags in which they are delivered from the oil crop division. We intend to change the vice to a power-driven mangle (Fig. 2).

The 5 g sample from a seed lot is extracted in a steel tube containing 3 stell balls and 40 ml petroleum ether by milling for 15 minutes in a shaking machine. After sedimentation, 2 ml of the solution is transferred to a test tube and 6 ml of 0.02 M sodium hydroxide in methanol is added. After shaking the tube is left for 30 minutes at 50°C and 10 ml of the salt solution is added. The tube is shaken and as soon as the two layers have separated the benzine phase is transferred to a test tube fitting Varian autosamplers (Fig. 3).

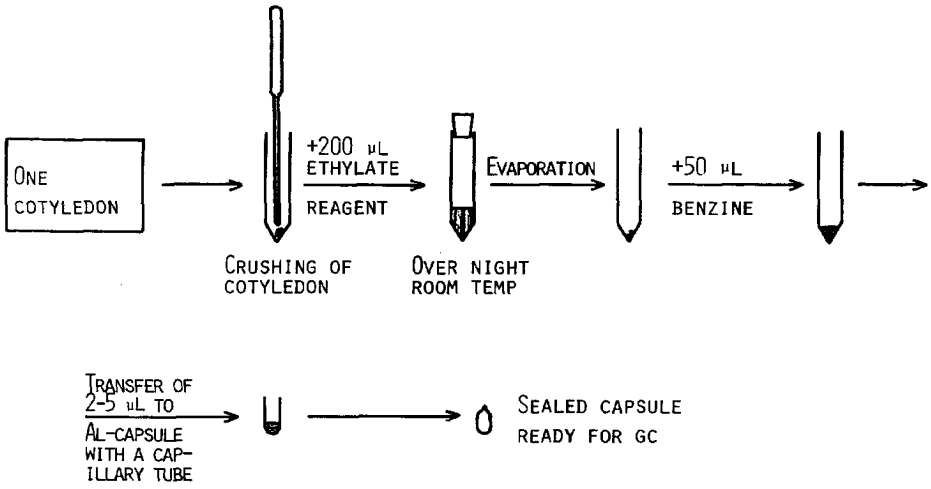

FIG. 1. PREPARATION OF COTYLEDONS FOR AUTOMATIC GAS CHROMATOGRAPHIC DETERMINATION OF FATTY ACIDS

ETHYLATE REAGENT: PETROLEUM ETHER/0.02 M SODIUM HYDROXIDE IN ETHANOL (2/3)

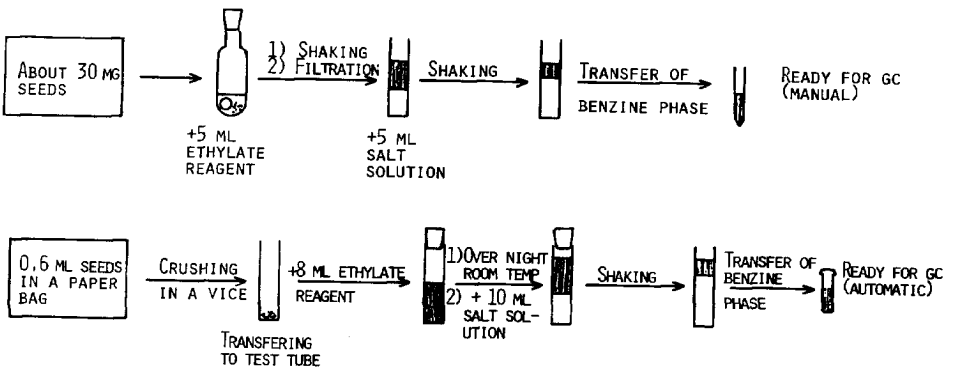

FIG. 2. PREPARATION OF PLANT SAMPLES FOR GC

ETHYLATE REAGENT: SEE FIGURE 1

SALT SOLUTION: SODIUM CHLORIDE (80 G) AND SODIUM HYDROGEN SULPHATE (3 G) IN WATER (1 LITRE)

In addition to the breeding program we also determine the erucic acid content of samples from farmers´ seed lots. This is part of the oil content determination according to the Svalöv method which is used as an officially accepted method for price regulation. From this analysis a solution of oil in petroleum ether / ethanol (3/1) is obtained. 1 ml of this solution is transferred to a test tube and 0.5 ml of petroleum ether / 0.05 M sodium hydroxide in 99.5 % ethanol (1/1) is added. The tube is shaken and is then left for one hour at room temperature. The solution is then ready for gas chromatography. In this case glycerol is present in the solution and this has to be considered at the final calculation (Fig. 4).

For the gas chromatography we have at our disposal 17 columns, all directly connected with a central minicomputer (Altema AB) for the collection and calculation of all data. We have three Perkin-Elmer autosamplers with a total capacity of 630 samples per loading working with the capsule technique and five Varian autosamplers with a total capacity of 300 samples per loading. This implies that most of our gas chromatograms might be injected automatically but that we also can use the manual columns if necessary. The type of columns and other parameters are traditional. The columns usually are 6-feet glass columns with 2 mm inner diameter packed with 5 % BDS on Varaport 30 80-100 mesh or 100-120 mesh. Depending on the demands we optimate the conditions and the time per chromatogram varies between 5 and 15 minutes.

In our efforts to improve the separation between the different fatty acids in combination with a reasonably short time per gas chromatogram we have started to investigate the use of capillary columns. The aims are in part complete separation between the fatty acids and rapidity, in part separation of erucic acid from the other fatty acids combined with a very short duration time. To investigate this we started with a 50-meter capillary column coated with the silicone phase SILAR-9CP. The 50-meter column of course created a perfect separation but as we in the last case described above are interested in a short running time and only a good separation of erucic acid from the rest of the fatty acids, we decided to try with a 10-meter capillary column of the same type. With an oven temperature of 215^{o}C and 1 ml/min of nitrogen through the column a 3-minutes chromatogram separated erucic acid from the rest of the fatty acids. This can probably be improved even more. If this short capillary column can be used with the autosampler working with the capsule technique the daily capacity per instrument should be about 400 samples. However, this work is still under investigation.

We have now described a number of simplifications especially as regards the sample preparation step. However, most are very simple ones but in the daily routine work these improvements are very important.

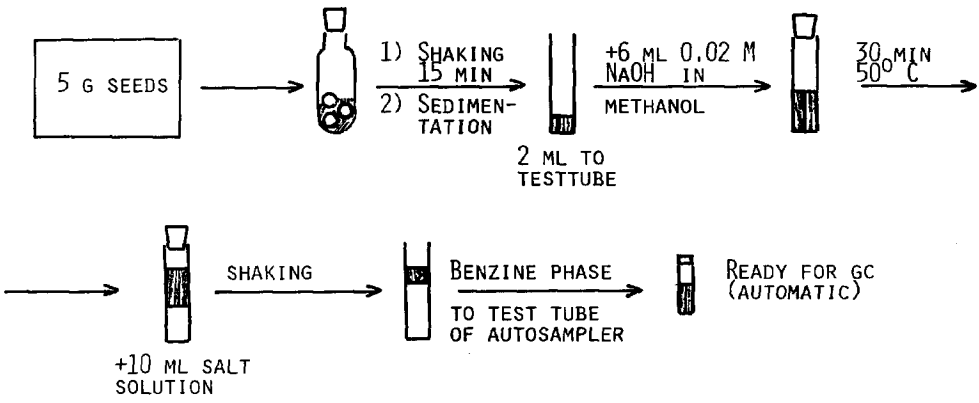

FIG. 3. PREPARATION OF SAMPLES FROM SEED LOTS FOR GC
SALT SOLUTION: SEE FIGURE 2

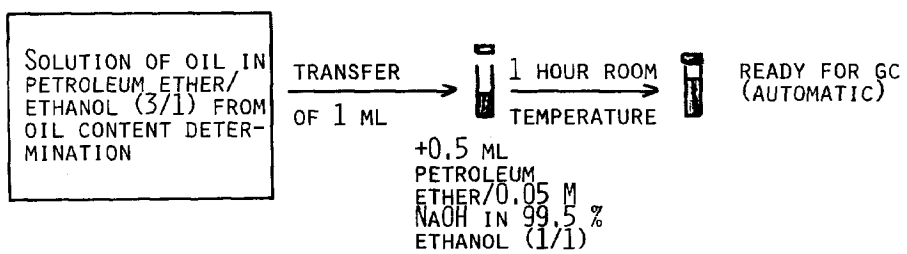

FIG. 4. PREPARATION OF SAMPLES FROM FARMERS SEED LOTS FOR GC