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SAMPLING TECHNIQUES IN THE GLASS CAPILLARY GAS CHROMATOGRAPHY OF FATTY ACIDS OF RAPE-SEED

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SUMMARY

The development of erucic acid-free varieties of rape-seed presupposes highly-accurate determinations of fatty acids in rape-seed oil. Comparison of sampling techniques (split, splitless and on-column) indicated that the on-column technique gives the best results. Regular analyses carried out over a long period of time were found to be unreliable, especially if only small samples are taken from biologically very heterogeneous seed material.

INTRODUCTION

Impending legislation concerning the growing of erucic acid-free varieties of rape in Finland has resulted in the need for accurate and reliable fatty acid analyses in the breeding of suitable varieties of rape. As the varieties must be genetically pure as regards the formation of erucic acid, *i.e.* none of the multiple alleles controlling erucic acid synthesis must be present, very low levels of erucic acid may frequently have to be determined during the plant-breeding stage and subsequent monitoring of commercial crops grown from these varieties.

The correlation between the major and minor fatty acid components of rape-seed oil is high. For example, the presence of eicosenic acid in rape-seed oil indicates that the alleles controlling erucic acid synthesis are present. Hence reliable analytical determinations of minor fatty acids, which are of little significance in themselves, are of great importance for plant breeders in characterizing plant genotypes.

Nowadays, gas chromatography is the most common analytical method used in the determination of fatty acids. Very high accuracy and reproducibility can be achieved in gas–liquid chromatographic (GLC) analysis using present-day techniques

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and methods. The relative standard deviation (S_{rel}) for fatty acids, for instance, varies from 1 to 5% in repeated analyses, the precision of course depending on the relative proportions of the different components and their separability. However, such a high reproducibility does not always give a true picture of the reliability of this method because the S_{rel} values frequently presented in the literature have been obtained by repeating the analyses a number of times at short intervals, often within the course of a single day. In plant-breeding studies, on the other hand, one is forced to analyse, almost without exception, large sample lots over very long periods of time. In such cases, the columns almost invariably have to be changed at some time or other during the course of the trial, with the subsequent risk of changes occurring in the overall characteristics of the analytical set-up properties.

In capillary chromatography, the splitting conditions often have to be altered owing to the variation in the concentrations of the components in the samples being analysed. In addition, the instrument itself may have been replaced during the course of the experiment. In order to improve the reliability of the results, the different sources of error inherent in the analytical method have to be identified and an attempt made to estimate the magnitude of these errors for each component in the mixture.

As the greatest problem in GLC, and above all in capillary GLC, is nowadays no longer the choice of a suitable column or its quality, the sampling method and the instrument itself represent the most important sources of error.

The aim of this study was to determine the effect of variations in the splitting ratio and sample size on the reproducibility and accuracy of fatty acid analyses. Furthermore, the split, splitless and on-column methods are compared in connection with fatty acid analyses.

The results obtained by these different methods are compared with each other and with results obtained during the analysis of a large biological sample. In addition, the results of fatty acid analysis on the same selection line, carried out during 3 years, are compared with analyses made on the same line at short intervals.

EXPERIMENTAL

Materials

The cruciferous seed material (Spring turnip rape, *Brassica campestris* L. var. *annua*) used in this work was obtained from the Plant Breeding Institute of Hankkija, Hyrylä, Finland. A selected line (line 43.2) with a low erucic acid content was chosen as the representative sample. Seeds were also taken from a total of 486 plants of the lines (M_1 and M_2) obtained by crossing line P-7622 and line P-7629 for purposes of comparison. The oil from 10–15 seeds was extracted and the methyl esters of the components were prepared by esterification as described earlier[1].

Instrumentation

Gas chromatographic analyses were carried out with a Carlo Erba Fractovap 2300 gas chromatograph using either a split or splitless technique. On-column analyses were performed on a Dani 3200 gas chromatograph. Both instruments were equipped with a flame-ionization detector (FID). In addition, the Dani 3200 was also fitted with an on-column injector and secondary cooling system.

In the split and splitless trials a free fatty acid phase (FFAP) glass capillary

column (25 m × 0.35 mm I.D.) was used under the following conditions: carrier gas, hydrogen at 0.3 kg/cm^2 (flow-rate 2.9 ml/min), injector temperature 250°C, oven temperature 200°C (isothermal). The splitting ratio was either kept constant at 15:1, or else varied from 500:1, 200:1, 70:1, 15:1, 8:1, 7:1 to 3:1 when testing the effect of the splitting ratio. The sample size was either a constant 0.5 μl, or else varied from 0.1, 0.3, 0.6, 1.2 to 2.4 μl when determining the effect of increasing sample size. The number of successive analyses carried out in the different trials are shown in Tables I–IV.

A longer FFAP column (45 m × 0.35 mm I.D.) was used in the on-column analyses. Nitrogen was used as the carrier gas at 0.9 kg/cm^2 (flow-rate 1.8 ml/min).

Peak areas were measured with Infotronics CRS-208 (for the Carlo Erba gas chromatograph) and Hewlett-Packard 3390 A (for the Dani gas chromatograph) peak integrators.

The standard deviations, coefficients of variation and variances were used as measures of dispersion. The dispersion between the test groups were compared using variances and the *F*-test. Quantitative differences between the groups were tested by Student's *t*-test using the split sampling results as the basis of comparison.

RESULTS AND DISCUSSION

When comparing different methods of sample introduction or their modifications, two basic aspects, namely precision and accuracy, have to be taken into account.

Factors affecting the accuracy of a determination include, in addition to be integrator error, also the changes taking place during the vaporization of the sample, adsorption, decomposition, sample size and the splitting ratio[2–5].

Under constant volume, temperature and pressure, different-sized samples produced momentary changes in pressure in the vaporization chamber, resulting in short-term eddy currents. Such an explosive change in pressure results in the situation in which back pressure in the capillary column prevents the volatilized sample from entering the column[5,6].

If the splitting ratio is large, an appreciable proportion of those incompletely volatilized components of low volatility in the sample will escape through the split before the flow conditions in the system have stabilized[6]. The composition of the vapour entering the column is thus changed. This could be called "discrimination through volatility". Discrimination also takes place when a constant splitting ratio is used as the size of the sample is increased[4,7]. As well as affecting the quantitative results, the sample size also affects the precision of sample series and thus the comparison between different members of the same series. The results of analyses of the methyl esters of fatty acids obtained using the constant splitting ratio method and other methods are compared in Table I.

It can be seen from Table I that there are very significant ($p < 0.01$) and highly significant ($p < 0.001$) quantitative differences between the different methods. In addition, the dispersions of the results of the different sampling methods were either very significantly or highly significantly different in the case of certain components. The mean precision (coefficient of variation, C.V. = 2.3%) for the on-column technique was the best, the mean values for the split and splitless techniques being of

TABLE I

COMPARISON OF SAMPLING TECHNIQUES IN GLC ANALYSIS OF FATTY ACIDS IN RAPE-SEED OIL

Fatty acid	*Split (n = 10)*			*Splitless (n = 6)*			*On-column (n = 6)*		
	Relative peak area (%)	*S.D. (%)*	*C.V. (%)*	*Relative peak area (%)*	*S.D. (%)*	*C.V. (%)*	*Relative peak area (%)*	*S.D. (%)*	*C.V. (%)*
16:0	3.0	0.06	2.1	2.9***,§	0.03	0.9	3.0	0.08	2.7
18:0	1.6	0.04	2.4	1.5	0.15***,§§	10.2	1.4***	0.03	2.3
18:1	58.6	0.19	0.3	58.5	0.19	0.3	57.6***	0.07**	0.1
18:2	20.2	0.10	0.5	20.7***	0.07	0.4	20.7***	0.11	0.5
18:3	12.7	0.07	0.5	12.8**	0.10	0.8	13.0***	0.03	0.3
20:0	0.5	0.04	8.1	0.4**	0.03	7.1	0.4***	0.01**	1.8
20:1	1.7	0.11	6.7	1.5	0.16	10.7	1.8*	0.04**	2.2
20:2	0.2	0.03	21.6	0.1***	0.01***	5.2	0.2	0.01**	5.2
22:0	0.3	0.05	14.1	0.2**	0.04	18.2	0.2***	0.01**	4.7
22:1	1.3	0.10	7.8	1.4	0.09	6.6	1.7***	0.06**	3.5

* $p < 0.05$,
** $p < 0.01$.
*** $p < 0.001$.
§ Student's *t*-test.
§§ *F*-test.

TABLE II

COMPARISON OF DIFFERENT MODIFICATIONS OF THE SPLIT-SAMPLING TECHNIQUE

Fatty acid	*Varying split (n = 7)*			*Varying sample size (n = 5)*			*"Hot-needle"-technique (n = 6)*		
	Relative peak area (%)	*S.D. (%)*	*C.V. (%)*	*Relative peak area (%)*	*S.D. (%)*	*C.V. (%)*	*Relative peak area (%)*	*S.D. (%)*	*C.V. (%)*
16:0	3.6**,§	0.48***,§§	13.4	3.4	0.51***,§§	14.9	3.1	0.29***,§§	9.2
18:0	1.5	0.12***	8.3	1.4**	0.07	5.1	1.6	0.01*	0.8
18:1	58.6	0.18	0.3	58.7	0.31	0.5	58.8	0.27	0.5
18:2	20.5**	0.16	0.8	20.3**	0.06	0.3	20.4*	0.16	0.1
18:3	12.7	0.13	1.1	12.7	0.06	0.5	12.8	0.16*	1.2
20:0	0.4*	0.07	16.1	0.4**	0.03	7.7	0.4	0.07	15.6
20:1	1.4**	0.17	12.3	1.7	0.18*	16.6	1.5	0.22	15.0
20:2	0.1***	0.10	19.1	0.1	0.03	18.8	0.1***	0.02	21.1
22:0	0.2**	0.07	33.6	0.2***	0.02	10.4	0.2**	0.06	27.9
22:1	0.9**	0.23	24.4	1.1	0.23*	23.3	1.1	0.33**	30.9

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.
§ Student's *t*-test.
§§ *F*-test.

similar magnitude (C.V. 6.4 and 6.0%). The dispersion of certain compounds varied considerably (*e.g.* $C_{20:2}$ and $C_{22:0}$ in the split mode and $C_{18:0}$, $C_{20:1}$ and $C_{22:0}$ in the splitless technique).

The split-sampling technique is most commonly used because of its ease of operation and, above all, because it avoids overloading and contamination of the column, thus lengthing column life. The split conditions in extended series of experiments may change as a result of changes taking place in the column and flow pressure. In addition, the sample concentrations may vary and so either the splitting ratio or sample size have to be frequently changed. Such changes may have a considerable effect on the results. In order to determine the possible significance of these changes, the effect of varying the splitting ratio and sample size on the results were compared using the split method. In addition, the differences between the so-called hot-needle injection and conventional cold-needle techniques were compared.

The results of the analysis shown in Table II were compared with the results obtained using the constant-split method (Table I). Significant differences were found between both the relative peak area and the dispersions of certain compounds in comparison with the corresponding results for the constant split method.

The considerable variation in the amounts of behenic and erucic acids are of particular interest (*cf.* also Table III). In the case of the varying-split method, the high levels of palmitic acid and low levels of erucic acid can be explained by the fact that larger sample sizes and, on average, smaller splitting ratios were used. The corresponding feature in the results for the varying sample size method were caused by larger sample sizes than those used with the other methods, on average.

The results for methyl palmitate and methyl erucate obtained using different sampling methods and modifications of these methods are presented in Table III.

TABLE III

EFFECT OF SAMPLE SIZE AND SPLITTING RATIO ON RELATIVE PEAK AREA OF METHYL PALMITATE AND METHYL ERUCATE COMPARED WITH RESULTS OBTAINED WITH SPLITLESS AND ON-COLUMN TECHNIQUES

Sampling	*Sample size (μl)*	*Splitting ratio*	*Relative peak area*		*Peak-area ratio* ($C_{16:0}/C_{22:1}$)
			$C_{16:0}$	$C_{22:1}$	
Split	0.5	15:1	3.0	1.3	2.3
Split "hot-needle"	1.0*	15:1	3.1	1.1	2.8
	0.1	15:1	2.9	1.4	2.1
	0.2	15:1	3.4	1.3	2.6
	2.4	15:1	4.3	0.7	6.1
	1.0	200:1	3.2	1.3	2.5
	1.0	70:1	3.3	1.1	3.0
	1.0	8:1	3.6	0.9	4.0
	1.0	3:1	4.2	0.7	6.0
Splitless	0.3**	–	2.9	1.4	2.1
On-column	1.0**	–	3.0	1.7	1.8

* 1.0 μl sample + 1.0 μl *n*-hexane.

** Diluted: 1:100.

It is evident that the proportion of methyl palmitate increased, and that of methyl erucate decreased as the sample size was increased or the splitting ratio decreased. These relative quantitative changes can be attributed to the effect of discrimination through volatility (see above). It is apparent that sample sizes of greater than 1 μl, or splitting ratios of less than 15:1, give very unreliable results for these components. This is further borne out by the results for peak ratios. However, a splitting ratio of 70:1 resulted in a relative decrease in the peak-area ratio for erucic acid. This is without doubt a result of interaction between the splitting ratio and sample size. The peak-area ratio of 1.8 obtained with the on-column technique proved to be the correct one after a prepared mixture of these two compounds had been analysed.

As the same sample (line 43.2) was used in all these runs, the sum of variances of all ten components in the different methods can be used as a measure of the differences between the different methods. Sources of variation, number of observations, variances and their share out of the total variance, as well as coefficients of variation for comparison purposes, are presented in Table IV.

The corresponding values for the material consisting of six samples, taken from the same selection line (line 43.2) over a period of 3 years, are shown in Table IV. The analyses were carried out by the split method under conditions which correspond to the constant-split method used elsewhere in this study. Despite the fact that a number of different columns were used during this period, the same liquid stationary phase (FFAP) was used. The source of variance shown in Table IV includes the variation caused by the fact that a small sample (10–15 seeds) only was selected from the seed material, which had a large degree of biological variation. In addition, the selection variance includes the variation arising from the isolation, transesterification and of course GLC analysis of the fatty acids. As well as the earlier mentioned sources of variance, the 3-year term variance also includes the variance arising from replacement of the column and slight adjustments to the carrier gas flow-rate. The total variance presented in Table IV has been estimated from the total variance of the two selection line crosses (M_1 and M_2).

It is evident from Table IV that the selection and the 3-year term variances are the only sources of variance as far as the sum of the variances of the ten fatty acids is

TABLE IV

VARIANCES AND COEFFICIENTS OF VARIATION IN FATTY ACID ANALYSIS

Source of variance	*n*	*Variance*	*Percentage of total variation*	*C.V. (%)*
Split:				
Constant	10	0.082	0.6	6.4
Varying split	5	0.420	2.8	12.9
Varying sample size	7	0.507	3.4	9.8
"Hot needle"	7	0.373	2.5	12.3
3-year term	6	4.512	30.3	11.3
Splitless	6	0.111	0.7	6.0
On-column	6	0.029	0.2	2.8
Selection	20	4.364	29.3	13.6
Total variation	486	14.902	100.0	–

concerned. On the other hand, the proportion of the sum of the variances arising from the GLC method (0.2–3.4%) are negligible and appear to be of no practical importance in plant breeding studies if the size of the sample is kept sufficiently large. However, methodological errors are important in the determination of certain components. The variances for palmitic acid in the case of the varying split in the varying sample size methods are 0.23 and 0.26, the corresponding values for the constant-split method varying in the biological materials (M_1 and M_2) from 0.10 to 0.23 (*cf.* also Table III).

CONCLUSIONS

It was found in the study that the analysis of fatty acids by different sampling methods gave results which differed significantly from each other as regards both precision and accuracy. The on-column technique (C.V. 2.8%) gave the most reliable results, the sum of variances of all ten different components being in this case only 0.2% of the total variation. The largest source of error proved to be selection combined with analyses carried out over a long period of time (*ca.* 30% of the total variation). This can be mainly attributed to the fact that only 10–15 seeds were analysed from the biologically very heterogeneous material. However, it should be remembered that the variance also includes the variation due to transesterification.

The sample size and splitting ratio proved, in the case of palmitic and erucic acids, to be highly critical. The most reliable results in the case of the split technique were obtained with small sample sizes (0.1–0.5 μl) and a splitting ratio of 15:1.

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