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Direct Quantitation on Paper Chromatograms of Radioactivity From ^{14}C Labelled Fatty Acids

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The present investigation is based on experiments using reversed phase chromatography with horizontal development. It is shown by varying spot size and type of fatty acid that the marginal distribution throughout the thickness of the paper of the molecules to be counted renders no bias to their quantitative evaluation. The said marginal distribution, however, influences the scanning error and it can be recommended to scan low energy labelled paper chromatograms from both sides of the paper and average the results.

The advantages of direct quantitation of radioactive substances separated by paper chromatography need hardly be stressed.

As long as the energy of the radiation from the isotopes used to label the substances in question remains above such a value that the absorption by the paper itself can be reasonably ignored, a simple scanning of the chromatogram with some sort of counting equipment should not offer any difficulties. However, when measuring low energy ^{14}C β-radiation and particularly when counting β-particles from ^{3}H, certain problems do need to be considered.

In the present investigation ^{14}C labelled fatty acids were separated by reverse-phase paper chromatography with horizontal development according to Kaufmann-Mohr [1]. The chromatographic paper used (Schleicher & Schüll 2040bM) had an average weight of 13 mg/cm^2. If one accepts a halving thickness for ^{14}C β-radiation of about 2.7 mg/cm^2 it is seen that in this case only about 4 % of the β-particles emitted from the side of the paper turning away from the counter window and only about 50 % of the β-particles emitted from the center of the paper will have a chance of being registered by the counter. This makes it clear that the geometrical distribution throughout the thickness of the paper of the molecules to be counted and thus the geometrical shape and density of the chromatographic spot to be evaluated, will be of major importance for the final result, while on the other hand self-absorption by the fatty acids will need no special consideration.

In their review article Giddings and Keller [2] point out that in case of an ideal model based partly on random diffusion (in the directions perpendicular

to the direction of flow of the mobile phase) and partly on both random and kinetic diffusion (in the direction of flow of the mobile phase) the shape of a chromatographic spot will be elliptic, with a Gauss-distribution of the molecules along any of the elliptic axes. In the same article reference is made to the experimental work of Åkerfeldt [3] who showed that while the pictured ideal case is often met with in practice, it also can happen that the molecular distribution along the elliptic axes resembles more a rectangular than a Gauss distribution, the latter case perhaps being due to "overloading" of the original spot of application. These considerations apply to the shape and density of the spot along the two coordinate axes (x, y) parallel to the surface of the paper. The marginal distribution of the molecules along the third dimension (z) perpendicular to the surface plane, which is of interest in the present investigation, is not discussed. However, one should not expect the latter distribution to vary from the random component of the x,y distributions except for the fact that the thickness of the paper being small in comparison to the x,y dimensions of the spot, the most probable marginal distribution function along the z axis in case of ideal diffusion will only be a segment of a Gauss curve placed symmetrically about the mean. In case of rectangular x,y distributions the z distribution should of course also be expected to be rectangular.

Based on the above considerations the problem in question can be put as follows:

A quantitative evaluation of the relative distribution of ^{14}C radioactivity between various spots on a paper chromatogram will only be free of bias if each of the spots shows the same geometrical marginal distribution throughout the thickness of the paper. If one accepts a model for the formation of the spots based on diffusion, this means in the present case that the diffusion coefficients should be independent of fatty acid concentrations.

On top of these considerations which refer to ^{14}C quantitation in general there is yet another question which might render a bias to the results in the present particular case, where use is made of reversed phase chromatography with horizontal development. It is the question whether the two liquid phases will have a tendency to separate according to their specific gravity, so that rising R_F values will mean an increasing probability of the mean of the spot to be found in the lower half of the paper, since in the present case the mobile phase is the heaviest one.

EXPERIMENTAL

All scanning was done with equipment constructed by the Health Physics Department at Risö.

The experiments were based on samples containing ^{14}C labelled palmitic and stearic acids. The ratio of measured palmitic activity (P) to measured stearic activity (S) expressed as % of the expected value was used as the dependent variable. Variation between the samples of the stearic and palmitic acid concentration ratios was achieved partly by varying the amount of each of the radioactive fatty acids added, partly by varying their specific activity; in total the concentration ratios of palmitic to stearic acid thus varied from $0.112 \times 1 = 0.112$ to $3.33 \times 8 = 26.7$ in accordance to a variation from 0.112 to 3.33 of the ratio

$$\frac{\text{added } ^{14}\text{C palmitic acid}}{\text{added } ^{14}\text{C stearic acid}} = \text{expected } P/S \text{ and a variation from 1.0 to}$$

Table 1.

Specific activity	Expected P/S 3.33 found P/S	3.33 % of expected	1.00 found P/S	1.00 % of expected	0.112 found P/S	0.112 % of expected	total average
Palmitic = 1.0 μC/mg	2.97	89.3	0.842	84.2	0.1066	95.3	89.4
Stearic = 1.0 μC/mg	3.15	94.6	0.847	84.7	0.0990	88.3	
Palmitic = 0.5 μC/mg	2.90	87.2	1.001	100.1	0.1113	99.5	87.4
Stearic = 2.0 μC/mg	2.53	76.0	0.761	76.1	0.0957	85.4	
Palmitic = 0.25 μC/mg	2.48	74.5	0.970	97.0	0.1083	96.8	95.4
Stearic = 2.0 μC/mg	3.21	96.5	1.064	106.4	0.1131	101.0	
Total average		86.4		91.4		94.4	90.7

8.0 of the ratio $\frac{\text{specific activity of added stearic acid}}{\text{specific activity of added palmitic acid}}$. Each sample was chromatographed twice. Results are given in Table 1.

Based on the above results one gets the following analysis of variance [4] (Table 2).

The question whether a bias owing to a separation of the two liquid phases could be expected was investigated by scanning both sides of each of 7 chromatograms. Results are given in Table 3.

Based on these results it can also be tested [4] whether the new estimate of s^2 derived from the mean of $\frac{1}{2}d^2$ differs from the result 87.2 found in the above analysis of variance:

$$t = \frac{-2.0}{(2 \times 87.2/7)^{\frac{1}{2}}} = -0.4; \; f = 17$$

$$v^2 = \frac{244.9}{87.2} = 2.81; \; f_1 = 7, \; f_2 = 17$$

Table 2.

Variation	SSD	f	s^2	test
Between columns	194.4	2	97.2	$v^2 = \frac{87.3}{87.1} = 1.00; f_1 = 8, f_2 = 9$
Between rows	208.2	2	104.1	
Residual	295.6	4	73.9	
Within sets	783.8	9	87.1	
Total	1 482.0	17	87.2	

Table 3.

% of expected P/S			
Scanning		d = deviation	$\frac{1}{2} d^2$
from top	from bottom	top − bottom	
94.6	106.1	−11.5	66.1
93.0	92.3	0.7	0.3
109.9	88.6	21.3	226.8
76.0	104.1	−28.1	394.8
76.1	111.3	−35.2	619.5
96.5	83.3	13.2	87.1
106.4	81.1	25.3	320.0
	mean	− 2.0	244.9

DISCUSSION

Tests in connection with the analysis of variance show that no bias is to be found from differences in fatty acid concentration, so that all spots will be scanned with the same efficiency independent of their size. Referring to the diffusion model of spot formation, this can be interpreted as proving the equality of the diffusion coefficients.

Also, there is no bias to be found from the location of the mean of the spot depending on the R_F value of the corresponding fatty acid. This is shown by the lack of significance of the t-test for $d \neq 0$ which makes it probable that the average location of the mean of any chromatographic spot will be in the same plane parallel to the surface of the paper. That the found total average of 90.7 % of expected P/S differs from 100 % must thus be due to errors in dilution of the two stock ^{14}C labelled fatty acid solutions used for sample preparations.

The influence of the geometrical marginal distribution of a chromatographic spot along a dimension perpendicular to the surface of the paper is first met with in connection with the scanning error or scanning variance. The mean of $\frac{1}{2}d^2$, where d refers to the deviation between the results of two opposite scannings of the same chromatogram, is significantly larger at the 95 % level than the corresponding value found as the final result of the analysis of variance. This would be in agreement with a theory claiming that the main source of scanning variance was a random difference between the geometrical distributions and with them the geometrical means of the palmitic and stearic spots scanned. The said deviation d would thus be a deviation between one value chosen at random within the general probability distribution of % of expected P/S and the corresponding value with the opposite probability. In this case the mean of $\frac{1}{2}d^2$ should be equal to $2s^2$. As a practical consequence of this explanation it can be recommended to scan low energy labelled paper chromatograms from both sides of the paper and average the results.

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