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GAS CHROMATOGRAPHIC DETERMINATION OF VOLATILE FATTY ACIDS IN AQUEOUS MEDIA

ITS USE IN BOVINE RUMEN FLUID AND IN SILAGE

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The importance of the volatile fatty acids (C_2—C_5) produced in the rumen has been increasingly appreciated in recent years. At the same time it has been shown that the volatile fatty acids are of value in the estimation of silage quality. A relatively rapid and exact method for the determination of these acids is therefore of importance. Such a method will be described here, and the short survey of some results from the determination in rumen fluid and silage is given only as an example of the use of the method.

The problem of determination of the volatile fatty acids (V.F.A.) is a classical one. Owing to the very great resemblance of characters in the homologous series (and the iso-acids) the difficulties are great. If the sample is in aqueous solution, the problem is not made easier. Formerly, the most commonly used method was a strictly accomplished water-vapour distillation followed by titration of portion after portion of the distillate. As the acids distil partly as a mixture, the method is difficult and uncertain.

Partition chromatography is, of course, the method of choice for the determination of a homologous series of acids. The column variety has been used by, e.g., *Elsden* (3), *Keeney* (6),

Moyle et al. (9), *Wise* (11), and *Wiseman & Irvin* (12), and the paper variety by *Duncan & Porteous* (2), *Lindqvist & Storgårds* (7), and *Miettinen & Virtanen* (8), and others. Gas chromatography seems, however, to be the variety that best combines rapidity and exactness.

In their development of gas chromatography, *James & Martin* (4, 5) showed that separation and identification of the V.F.A. was possible. The determination of these acids in an aqueous medium involves special problems, however.

The first and most obvious of these difficulties, the large quantity of water — in rumen fluid, for instance, the non-aqueous content is only a few per cent, and the total amount of V.F.A. about 0.5 per cent — is nowadays easily overcome with the aid of the water insensitive flame ionization detector and the great sensitivity of modern gas chromatographs. By far the greatest problem is that a small portion of the V.F.A. seems to become more or less permanently adsorbed onto the solid support. If the samples are non-aqueous, the solid support will soon be saturated and the disturbance thus eliminated. But water dissolves and sweeps away some of the adsorbed V.F.A., and if water is injected a "ghost picture" of small peaks will develop, as if the water had contained a little V.F.A. If two aqueous samples of V.F.A. are injected in succession, the peaks of the second sample will be exaggerated by the "ghost peaks" originating from the first one. Phosphoric acid has been used to saturate permanently the solid support, but this method has not met with complete success.

Ritter & Hänni (10), working with cheese, briskly water-vapour distilled the sample after adding a fairly large amount of sulphuric acid, neutralized the distillate, evaporated it to complete dryness, and treated the dry V.F.A. salts with solid sodium bisulphate in a non-aqueous volatile solvent. In this way they obtained a non-aqueous solution of the acids, which can be chromatographed without difficulty. We used this method for some time in the determination of the V.F.A. in rumen fluid and silage, but it was found that although the proportions between the acids given by the method were correct, the absolute content of the sample could not be determined this way.

Up to now, the simplest and most accurate method for aqueous solutions of V.F.A. seems to be that of *Decker* (1). He adds to the sample as much as 5 % of formic acid. The formic acid passes the column first of the V.F.A. and saturates the solid

Table

	Acetic	Propionic	Iso-Butyric	Butyric	Valeric	Total
Lactating cows, $\bar{x} \pm \sigma$	46.57 ± 9.39	13.45 ± 2.22	1.06 ± 0.25	11.05 ± 1.99	1.02 ± 0.36	73.22 ± 11.79
% of total	63.6	18.4	1.4	15.1	1.4	
Dry cows, $\bar{x} \pm \sigma$	46.98 ± 9.14	13.95 ± 3.62	1.23 ± 0.42	9.23 ± 1.67	0.98 ± 0.36	72.45 ± 13.22
% of total	64.8	19.3	1.7	12.7	1.4	
t-test	0.9>P>0.8	0.5>P>0.4	P<0.02*	P<0.001***	0.6>P>0.5	0.8>P>0.7

Mean values ($\bar{x} \pm \sigma$) of the concentrations (in mEq per litre) of volatile fatty acid in 111 samples of rumen fluid from lactating cows and in 40 samples from dry cows, and statistical comparison between the two groups.

support, so that the succeeding V.F.A. cannot be adsorbed. The flame ionization detector is insensitive to formic acid and therefore this acid does not disturb the registration of the sample V.F.A. The method is not wholly effective, but the remaining "ghost peaks" are insignificant.

Gas chromatography is not by itself a quantitative technique. In spite of the great exactness of the modern apparatus, small variances in temperature and gas pressure (carrier gas, and fuel and air for the flame ionization detector) cannot be avoided during a day's work and, above all, the injection volume cannot be kept very constant. The easiest and most accurate way to make the method quantitative is the use of an internal standard. We have not been able to find a better internal standard than iso-valeric acid (β-methyl butyric acid), which gives a peak about midway between butyric and n-valeric acid. In the sample types with which we have worked (rumen fluid and silage) the disadvantage is that iso-valeric acid is present in the sample, but since the content is small and fairly constant, as we have found at determinations of more than a hundred samples by *Ritter & Hänni*'s method, a correction for it can be included in the factors used to determine the concentration of the V.F.A.

The procedure of preparing a sample for gas chromatography is as follows: The sample (rumen fluid or silage juice) is centrifuged at 3000 r.p.m. for 5 minutes. 2 ml of the liquid portion are taken, and to this sample are added first exactly 0.1 ml of a mixture of 100 ml of formic acid and 13 ml of iso-valeric acid, and then a knife's point of mercury chloride. In this condition the sample can be kept at — 15°C for at least 10 days before the V.F.A. determination.

The mixture of formic and iso-valeric acids is used as long as there is anything left of it. Factors for V.F.A. calculation are frequently determined by gas chromatography of an accurately known aqueous solution of the V.F.A., treated as a sample by adding a portion of the mixture of formic and iso-valeric acids to it.

We used the Perkin-Elmer 116 E gas chromatograph*) and 2-m columns of the Perkin-Elmer type BA, i.e. 20 % of a mixture

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of 90 % di(ethyl hexyl) sebacate and 10 % sebacic acid on Celite 545, 60—100 mesh.*) Helium was used as a carrier gas. Other conditions might be chosen individually. However, at a temperature of 140 °C, and a flow rate of about 200 ml per minute the determination of acetic to valeric acid requires about 10 minutes and that of acetic to caproic acid (present in some poor-quality silages) about 18 minutes.

The method was used in a series of samples of rumen fluid from healthy lactating and dry cows of the Swedish Red and White breed, the latter being fed a lower-energy ration. The samples were withdrawn by suction, in the morning, two to three hours after feeding. The results are shown in the appended table. It will be seen that there is a highly significant difference in butyric acid, the concentration in lactating cows being higher than in dry cows, a probably significant difference in iso-butyric acid, the concentration in lactating cows being lower, but no significant difference in any other acid, nor in the total V.F.A. content.

We also studied a number of silage samples and found:

1. In good-quality silages there are only insignificant amounts of V.F.A. other than acetic and propionic acid;

2. In poor-quality silages the butyric acid content in particular is sometimes highly elevated, but the contents of the other V.F.A. can also be raised. In some cases not insignificant amounts of caproic acid, absent in good-quality silage, are present;

3. In some cases poor-quality silages show no abnormalities in the content of the V.F.A.

In case 2 above it is possible that sometimes the content of iso-valeric acid is also raised, but the error in the determination deriving from this deviation is probably moderate.

Finally, we regret that lactic acid, being a hydroxy acid, cannot be determined by this method. It is all the more unfortunate, as the concentration of lactic acid is of utmost interest in the process of ensiling and, therefore, of importance to the quality of silage.

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*) We have also used columns with Chromosorb W, acid-washed, 60—80 mesh, as the supporting medium, and with equally good result.

In those cases when knowledge of its presence or absence in silage or rumen fluid is required we use a rapid semi-quantitative paper-chromatographical method as a complement to the V.F.A. determination described above.

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SUMMARY

A rapid quantitative method for the gas chromatographic determination of volatile fatty acids in aqueous media is described. It includes the use of formic acid for the elimination of "ghost peaks" and of iso-valeric acid as an internal standard. The method has been used for the examination of bovine rumen fluid and of silage.

Statistical treatment of the result shows that the fluid from lactating cows has a significantly higher content of butyric acid than has the fluid from dry cows; such a difference could not be demonstrated as to acetic, propionic and valeric acid.

Some experience from the determination of the volatile fatty acids in silage is also reported.

ZUSAMMENFASSUNG

Gaschromatographische Bestimmung flüchtigen Fettsäuren in wässriger Lösung.

Ihre Verwendung auf Panseninhalt und Silage.

Eine schnelle gaschromatographische Analysemethode für flüchtigen Fettsäuren in wässriger Lösung wird beschrieben, wo Ameisensäure für die Elimination von „Gespenstgipfeln" und Isovaleriansäure als inneres Standard verwendet wird. Die Methode ist auf Panseninhalt von Kühen und auf Silage benutzt.

Statistische Auswertung der Ergebnisse zeigt, dass der Panseninhalt laktierenden Kühen eine signifikant grössere Menge Buttersäure enhält als der der trockenen Kühen; ein solcher Unterschied war jedoch für Essigsäure bzw. Propionsäure und Valeriansäure nicht nachweisbar.

Erfahrungen bei der Bestimmung flüchtigen Fettsäuren in Silage werden auch erwähnt.

SAMMANFATTNING

Gaskromatografisk bestämning av flyktiga fettsyror i vattenlösning.

Analyser av våminnehåll och ensilage.

En snabb gaskromatografisk metod för bestämning av flyktiga fettsyror i vattenlösning beskrives. I denna metod användes myrsyra för borttagande av „spöktoppar" och isovaleriansyra som inre standard. Metoden har tillämpats på våminnehåll från kor och på ensilage.

Statistisk behandling av resultaten visar att hos mjölkande kor har våminnehållet en signifikant högre halt smörsyra än hos sinkor; någon sådan skillnad har ej kunnat påvisas för ättiksyra eller propionsyra och valeriansyra.

Vissa erfarenheter gjorda vid bestämning av flyktiga fettsyror i ensilage redovisas också.

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