# Rapid Method for the Gas-Chromatographic Determination of Volatile Fatty Acids in Rumen Fluid

Bernard G. Cottyn and Charles V. Boucque

A rapid method for the gas-chromatographic determination of volatile fatty acids (VFA, $C_2$ to $C_5$) in rumen fluid with the aid of flame ionization detection is based on the direct analysis of the rumen fluid without previous extraction. After filtration of the rumen fluid, metaphosphoric acid is added to precipitate proteins, and formic acid (5%) is used to eliminate the disturbing "ghosting" effect. The clear supernatant obtained after centrifugation is injected directly into the column. The analysis of one rumen sample requires about 8 minutes. For the quantitative determination, a nonautomatic integrator is used. The individual VFA are calculated by comparing with a standard. The recovery and precision of the gas-chromatographic techniques are discussed.

The energy requirements of ruminants are mainly covered by absorption through the rumen wall of the short-chain fatty acids. The molecular proportion of the different VFA is closely related to energy utilization and the animal's production. For nutritionists, a rapid and accurate method for fatty acid analysis in rumen fluid is therefore of great importance. In recent years, gas chromatography has appeared to be the most suitable technique.

James and Martin (1952) perfected the first gas-chromatographic separation of both straight- and branched-chain lower fatty acids. Because of their volatility, the lower fatty acids were analyzed as esters, and later the acids themselves were determined directly.

The direct determination of the free VFA was of great importance, as the earlier esterification procedures generally used, such as those with boron trifluoride and diazomethane, were very tedious and inelegant. The method of Gehrke and Lamkin (1961), later specially adapted by Fenner and Elliot (1963) for rumen fluid analysis, made use of the formerly generally employed thermal conductivity detection. Apart from the lower sensitivity, the disadvantage of this was the fact that aqueous solutions could not be used. Mainly, water does not damage the detector, but causes excessive tailing of the peaks. This makes a quantitative study of the individual acids impossible. Thus it was necessary to separate the volatile fatty acids quantitatively from the original aqueous sample and dissolve them in a suitable solvent (ethyl ether, hexane).

The perfection of the very sensitive flame ionization detector, which is insensitive to water, has obviated the above difficulties. Rumen liquor can now be easily analyzed without extraction and solution in an organic solvent.

The former classical pretreatment of the samples consisted of collecting the acids in water through steam distillation of the rumen liquor with $H_2SO_4$. The distillate was titrated and made alkaline with NaOH and evaporated to dryness, after which, depending on the method used, Na salts were dissolved after acidification either in an organic solvent (Fenner and Elliot, 1963) or in water (Kaufmann and Rohr, 1967; Kromann *et al.*, 1967). This classical preparation of the samples was also used by Shelley *et al* (1963). The method used here is based on the principle of direct injection of the centrifuged rumen fluid without extensive sample preparation.

National Institute for Animal Nutrition, Gontrode (Ghent), Belgium.

## EXPERIMENTAL PROCEDURE

The first gas-chromatographic analysis of very dilute aqueous fatty acid solutions was reported by Emery and Koerner (1961) and was further developed for blood and rumen fluid analysis by Erwin *et al.* (1961).

**Ghosting Effect.** The difficulty of using aqueous solutions was the "ghosting" or "repeater" effect. This phenomenon was cited by Emery and Koerner (1961) and Erwin *et al.* (1961). The term refers to the adsorption of small quantities of fatty acids when aqueous solutions of these acids are injected into the column. These components, which are adsorbed on the solid support, are expelled during the following analysis and exaggerate the peaks of the following sample. Injection of a pure water sample produces a chromatogram of small ghost peaks with the same retention time as the detected fatty acids; this difficulty prevented accurate quantitative analysis.

Ackman and Burgher (1963) added formic acid in the carrier gas stream with the aid of a cold-finger trap to prevent this effect. A variation of this technique for the elimination of the ghost pattern consisted of the direct addition of formic acid to the rumen fluid to be analyzed. This procedure was first reported by Decker (1962) and later applied by Carlstrom *et al.* (1965), Van Eenaeme *et al.* (1965), and Cottyn (1966).

Experience indicates that a concentration of 5% formic acid in the liquids to be analyzed (unknown and standard solutions) is sufficient to reduce the ghosting effect to an insignificant level. According to Carlstrom *et al.* (1965), the formic acid which passes the column first saturates the solid support and makes the adsorption of the VFA impossible. The extent of the ghosting effect would also depend on the nature of the stationary phase of the column. Van Eenaeme *et al.* (1965) found less ghosting with polar than with nonpolar stationary phases.

**Apparatus.** The apparatus used was an F&M Model 810 gas chromatograph, equipped with a double flame ionization detector and a Minneapolis Honeywell 1-mv. recorder fitted with a nonautomatic Kipp integrator (Fa Kipp & Zonen, The Netherlands). The integrator is provided with three counters. Two are used alternately

to read the individual areas of the successive peaks. After the first peak, the readout is manually switched to the second counter. The first counter may then be read and reset to zero while the second evaluates the next peak. The third counter gives the value of the accumulated area under all peaks. The integrator is equipped with a built-in zero control. By this means, the zero line of integration—i.e., the base line from which the areas of the recorded trace are evaluated—may be set at an arbitrary point on the recorder scale. A stainless steel column (4 foot × 1/8 inch) packed with 20% Tween 80 on Diatoport W.A.W. 40- to 60-mesh was used.

A temperature-programmed cycle from 90° to 130° C., rising by 4° C. per minute, was used. The injection block was maintained at 265° C. to provide rapid vaporization of the injected fluid. Nitrogen was used as a carrier gas with a flow rate of 60 ml. per minute. The hydrogen flow rate to the detector was 50 ml. per minute, and the air flow was 380 ml. per minute. Under these conditions, the analysis of a rumen fluid sample requires about 8 minutes from acetic to valeric acid.

**Sample Preparation.** Samples were taken by suction from fistulated sheep. After immediate pH determination and filtration through muslin, a few drops of toluene were added to inhibit fermentation. The samples may be analyzed immediately or stored in a freezer at −15° C. They are analyzed for VFA, $NH_3$, and total volatile fatty acids. For the analysis, 5 ml. of the strained rumen fluid is pipetted into a centrifuge tube, after which 1 ml. of a 3 to 1 v./v., solution of metaphosphoric acid (25%) and formic acid is added. The metaphosphoric acid precipitates the proteins which contaminate the column. After 30 minutes, the contents are centrifuged at 4000 r.p.m. for 20 minutes. The clear supernatant is ready for direct injection.

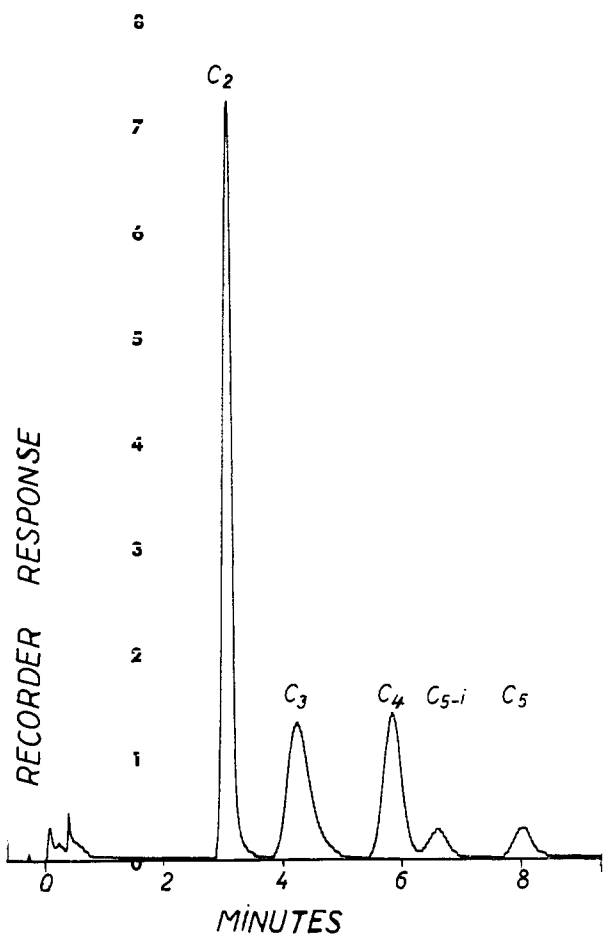


**Figure 1. Chromatogram of a typical rumen fluid**

**Quantitative Analysis.** For the quantitative evaluation, the method of the internal standard was used previously (Cottyn, 1966). This method was best suited to an accurate analysis. With it, it is possible to obviate the delicate question of keeping all variables constant during a day's work. The injected sample volume should be specially mentioned here. As an internal standard, Carlstrom *et al.* (1965) employed isovaleric acid and Cottyn (1966) used caproic acid.

The disadvantage of both is, however, the presence of minute amounts of isovaleric acid and occasional traces of caproic acid in the rumen fluid to be analyzed; as these quantities remain more or less constant, a correction can be applied for this. Because of the difficulty of choosing a suitable internal standard, this method was not used.

To make the injection volume (5 μl.) reproducible, a special Hamilton syringe with a Chaney adaptor was used. Twice a day, a standard mixture of aqueous VFA of approximately the same composition as the rumen fluid was injected. The unknown concentrations of the individual VFA in the rumen fluid to be analyzed are calculated by comparing the areas of the peaks to those of the standard. Naturally, the dilution factor resulting from the addition of HCOOH and $HPO_3$ must be taken into account.

## RESULTS AND DISCUSSION

The described method was used for several hundreds of rumen sample analyses (Figure 1) without any apparent deterioration of the column owing to the presence of all sorts of components in the injected rumen fluid. No special cleaning of injection or detector block seemed necessary. Columns with a diameter of 1/8 inch instead of 1/4 inch appeared to be markedly better. The only disadvantage of the columns with Tween 80 as a stationary phase is the imperfect separation of propionic and isobutyric acids. However, only traces of isobutyric acid appeared in the rumen fluid. For the analysis of aqueous solutions of silage extracts and rumen fluid with columns with Carbowax 20 M, De Vuyst *et al.* (1964) experienced the same difficulties, while the time used for analysis was considerably longer.

The most perfect separation of the acids was obtained by the use of sebacic acid (Jackson, 1964; Cottyn, 1966) as stationary phase, but the duration of analysis is also considerably longer. With these columns, there is also a more rapid loss of efficiency.

The nearly perfect horizontal base line (Figure 1) with a sensitivity setting of 10 × 128 allows an undisturbed working of the connected integrator. To determine the accuracy of the gas-chromatographic measurements, five different aqueous standard solutions of fatty acids were analyzed (Table I).

To demonstrate the reproducibility of the method, five replicates of a rumen sample withdrawn from a sheep fed

Table I. Analysis of Synthetic Mixtures of Volatile Fatty Acids

| Sample Number | Acetic Acid, Mg./100 Ml. VFA Present | VFA Found | Recovery, % | Propionic Acid, Mg./100 Ml. VFA Present | VFA Found | Recovery, % | *n*-Butyric Acid, Mg./100 Ml. VFA Present | VFA Found | Recovery, % | *n*-Valeric Acid, Mg./100 Ml. VFA Present | VFA Found | Recovery, % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 210 | 214 | 101.9 | 99.3 | 97.1 | 97.8 | 48 | 46.5 | 96.9 | 23.45 | 22.0 | 93.8 |
| 2 | 262.5 | 273.2 | 104.1 | 99.3 | 97.3 | 98.0 | 24 | 25.1 | 104.6 | 46.9 | 45.2 | 96.4 |
| 3 | 315 | 307.0 | 97.5 | 198.6 | 204.0 | 102.7 | 96 | 93.1 | 97.0 | 93.8 | 94 | 100.2 |
| 4 | 420 | 405.0 | 96.4 | 198.6 | 207.0 | 104.2 | 96 | 94.3 | 98.2 | 46.9 | 48.1 | 102.6 |
| 5 | 262.5 | 265.0 | 101.0 | 49.65 | 49.0 | 98.7 | 48 | 49.6 | 103.3 | 23.45 | 22.4 | 95.5 |

Table II. Precision in the Analysis of a Rumen Sample

| Sample Number | % Acids, Mole % of Total: Acetic acid | Propionic acid | *n*-Butyric acid | Isovaleric acid | *n*-Valeric acid |
|---|---|---|---|---|---|
| 1 | 63.8 | 17.9 | 14.7 | 1.5 | 2.1 |
| 2 | 63.3 | 18.0 | 15.2 | 1.6 | 1.9 |
| 3 | 62.7 | 18.3 | 15.4 | 1.5 | 2.1 |
| 4 | 63.5 | 17.8 | 15.0 | 1.6 | 2.1 |
| 5 | 63.3 | 17.8 | 15.1 | 1.7 | 2.1 |
| Average | 63.3 | 18.0 | 15.1 | 1.6 | 2.1 |
| Std. dev. | ±0.40 | ±0.20 | ±0.26 | ±0.09 | ±0.09 |
| Var. coeff. | 0.63 | 1.11 | 1.72 | 5.63 | 4.29 |

a diet of 50% concentrates and 50% hay pellets were analyzed, after which the standard deviation and the coefficient of variation were determined (Table II).

The described method can be used for the routine analysis of rumen fluid samples. As mentioned above, the time for carrying out a programmed analysis cycle was 8 minutes. Under the described conditions of preparing the rumen fluid samples, one analyst can easily perform 15 to 20 rumen fluid analyses daily. The connected integrator excludes the time-consuming work of measuring the peaks by triangulation, planimetry, cutting and weighing, or other laborious methods. Accurate measurement by the integrator requires a perfect horizontal base line (Figure 1).

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