OPTIMISATION OF THE GC METHOD FOR ASSESSING VOLATILE FATTY ACIDS IN RUMEN LIQUID

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Abstract

Accurate determination of the volatile fatty acids (VFA) content of the rumen liquid is indispensable for assessing feedstuffs utilization and effects at ruminal level. Moreover, nowadays feeding strategies require more detailed information such as post-prandial dynamics of VFAs, content of minor VFAs, etc. which leads to the need for precise, rapid and economic determination. In this context, the purpose of this study was to optimize the current GC method by modifying chromatographic conditions and sample preparation in order to reduce the analysis time and the reagents amounts.The optimized method was practically applied by testing VFA from rumen liquid of fistulated wethers challenged with sudden change of the concentrates: forage ratio of the diet from 20:80 to 50:50 (diet 1), 100:0 (diet 2) and 0:100 (diet 3). Rumen content was sampled 4 hours after the morning feeding, prepared and analyzed using the optimized method. Acetic acid content was reduced by diet 1 (72.51 % to 67.46 %) and diet 2 (68.11 % to 64.15 %) whereas diet 3 induced an increase (from 69.95 % to 72.16 %), results that are consistent with the known effects of concentrates: forages ratio. Propionic acid tended to express inverse effects, also consistent with the previous literature reports. These preliminary results showed that the modified GC method enables rapid and sensitive VFA determinations.

Key words: volatile fatty acids, rumen liquid, GC method

INTRODUCTION

Volatile fatty acids (VFA) are substances produced by microbial fermentation in the rumen. Depending of fermented substrate, microbial population, and ruminant forestomach environment different quantities of each type of VFAs are formed [1]. The proportions among individual VFA are of particular interest because each absorbed VFA have different metabolic pathways in the animal organs. The propionic acid/propionate is a substrate for gluconeogenesis and is the main source of glucose in the animal, whereas the acetic acid/acetate and butyric acid/butyrate are nonglucogenic but sources for long-chain fatty acid synthesis [6]. Ruminal branched-chain VFA (isobutyrate and isovalerate) together with valerate and ammonia are an indication of protein ruminal degradation.

Because volatile fatty acids are the main energy source for ruminants [2] and they characterize the metabolism of carbohydrates and proteins in the rumen, their determination is of considerable clinical and diagnostic significance. For ruminants large-scale breeding the VFA analysis is an integral part of the metabolic profile of the rumen liquid and can prevent diseases caused by unbalanced feed rations, organic acid surplus in silage and haylage, and feeding non-wholesome or toxic feeds to animals [4].

The analytical methods for VFA determination can be chromatography, electromigration (capillary isotachophoresis) [4], spectrophotometric method with a commercial kit or five pH point automated titration [5]. The conventional distillation method with subsequent titration is characterized by low selectivity and productivity and is largely no longer in use. Of the chromatographic methods, gas chromatography (GC) is the most widely used, both with filling and capillary columns

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[4]. Gas chromatography is a separation method that separates substances based on their differing affinities to the stationary phase affected by the flowing mobile phase. The mobile phase is gas, the stationary one is a glass sorbent-filled column used with the filling columns, or a thin layer of embedded liquid on the capillary's inner wall used with the capillary columns. The affinity of a substance to the stationary phase is primarily influenced by polarity. The advantage of the capillary columns consists in their higher sensitivity and much more advanced separation of individual acids [4].

The first successful separation of the lower fatty acids (in the ester form) by means of gas chromatography was accomplished more than half a century ago by James and Martin in 1952 [4]. This analytical method is now very easy for applying by regards both to modern technical conditions and to separation media in columns. The GC method is characterized by high accuracy and is more selective than titration method, providing the total acids content and also, detailed information about the concentration of each acid. The GC method does clearly differentiate between *n*- and *iso*- forms of the butyric, valeric and caproic acids and if *iso* forms assessment is needed, the GC method has to be used. This selectivity is lacking in titrimetric and spectrophotometric methods.

In the present paper after optimizing a GC method by modifying chromatographic conditions and sample preparation, the objective was to determine the effects of sudden change of diet on VFAs production in rumen liquid of fistulated wethers.

MATERIALS AND METHODS

Three fistulizated wethers were used and challenged with sudden change of diet (Tabel 1) by modifying the initial concentrates: forage (CF) ratio from 20:80 to 50:50 (diet 1), to 100:0 (diet 2) and to 0:100 (diet 3). Rumen content was sampled 4 hours after challenging. In the end for animals recovering alfalfa hay was administered at discretion. After diet 3 a suppliment of 0.432kg DM concentrate was also administered.

The rumen liquid was sampled through a rumen canula and strained through 4 layers of cheesecloth and an approximately 10 mL-portion of each filtered sample was acidified by mixing with 2.5 mL of 25% phosphoric acid (w/v) to reach a pH below two. Samples were stored in a freezer (–18^{O}C) until GC analysis. Before determination, the defrosted samples were centrifuged 10 minutes at 7000 x g, at -3^{O}C and then the supernatants mixed in equal part with distilled water were centrifuged at 14000 x g for 15 minutes, at -3^{O}C. The new supernatants represented the samples for GC analysis.

A sample volume of 1 µL was injected under split mode into a gas chromatograph (Varian, 430-GC) equipped with a capillary column Elite-FFAP with a length of 30 m, an inner diameter of 320 µm, and a film thickness of 0.25 µm (Perkin Elmer, USA). The carrier gas was hydrogen, flow 1.5 ml/per min. The injector was set at 250^{O}C and the split rate was 1:40. The flame ionization detector (FID) was set to 200^{O}C, and the column oven was set to 110^{O}C. The oven temperature was increased to 170^{O}C at a rate of 12^{O}C/min, where it was held for 9.5 min. The analysis time was 10 minutes.

RESULTS AND DISCUSSIONS

The method was validated only by linearity of calibration curve and by reproducibility. For linearity parameter a commercial standard mixture of VFA (Supelco, USA) with concentration of 10 mmol/L of each acid was used in a concentration range of 1–10 mmol/L to create the calibration curves. The correlation factors (R^2) of each of the corresponding regression lines for calibration were > 0.9986 indicating good relationship between the measured response (area of the peak) and the acids concentrations. Reproducibility as a precision parameter was tested by repeated testing of 8 mmol/L standard solution and the relative standard deviation obtained was 6.82%, less than 10%, the limit for the analytic (VFA) level.

Figure 1 illustrates an example of separation for volatile fatty acids obtained by this method for a rumen content sample. The

peaks for all the samples chromatograms have good resolution, no interferent peaks, constant retention time. In Table 2 are presented the quantities results (milimoles, mM/L) of chromatogram from figure 1 and the specific retention time for each VFA. The peaks heights (detector signal, μV) and area are also calculated.

After diet changing only the results for acetic, propionic and butyric acids were retained for discussion of the effects of changing the diet CF ratio, as being the most influenced by this ratio. Others VFAs are in small amounts and important for evaluating impact of others dietary factors (e.g. slight modifications of rumen bacterial flora). The results are presented in Table 3.

The level of acetic acid which is the main product of activity of cellulolytic bacteria is more stable during the day and the results confirm direct correlation with forage content in the diet. A decrease of mean molar proportion of acetate in diet 1 (from 72.51% to 67.46%) was recorded as a consequence of decreasing forage to 50 percents in the diet. And also for diet 2 was a decrease from initial 68.11% to 64.5% in the order of extreme decreasing of forage to 0 percents in the diet. In diet 3 with high level of hay the acetate proportion increased from an initial percent of 69.95% to 72.16% influencing the postruminal fiber digestion.

Propionic acid which is produced by starch fermenting bacteria can reach maximum concentration in one hour after feeding. Ceresnakova[3] reported for cows values of propionic acid proportions of 13% before feeding, 37% at 1 h post-feeding a ration with wheat grain 29% included in the dry matter, and a decrease at 20% after 4.5 h. In our test diet 1 did not induce an important increase for propionate molar proportion (22.73% comparing to initial 20.38%). In diet 3 the propionic proportion was reduced to 17.96% from initial 20.04% but this decrease in propionate molar proportion is a consequence of a rising in acetate concentration contributing to total VFAs rather than an increase in propionate concentration. Diet 2 induced o slight decrease (21.89% vs. 22.81%) even an increase was expected. It is possible that result to be influenced by the sample preparation and this suggests improving of this step of analysis.

Butyrate is quantitatively the least important of the three major VFAs in the rumen fluid, usually contributing only 0.05 M to 0.1 M to the total VFA pool [7]. Butyrate is used as an energy source and for milk fat synthesis. In this test the butyrate molar proportion increased in diet 1 (from 7.1% to 9.8%) and more consistent increased in diet 2 (9.06% to 13.59%). In diet 3 no significant difference was recorded between the initial (10%) and final sampling (9.87%).

The rumen concentrations of total VFAs in the tested wethers was not influenced by the composition of diets even there were extreme variations in the diet structure but for short period of time. These concentrations could not be compared for diets not isocaloric or not isoproteic but the intention of our study was only to observe the influence of diet structure to proportions of individual volatile fatty acids. It was concluded that the influences of pH and temperature are necessary to be tested for more accurate results. Also, moderate modifications of rations are indicated for future testing and of course, sudden diet change must be settled for a minimum of 7 daysto have a real impact on VFAs content.

Table 1Structures of the wethers diets

	Initial common diet 20:80 C:F ratio (kg DM feed intake/animal/day)	Diet 1 50:50 C:F ratio (kg DM as meal for challenging)	Diet 2 100:0 C:F ratio (kg DM as meal for challenging)	Diet 3 0:100 C:F ratio (kg DM as meal for challenging)
Concentrate	0.484	0.432	0.865	0
Alfalfa hay	1.938	0.434	0	0.850
Total	2.422	0.866	0.865	0.850

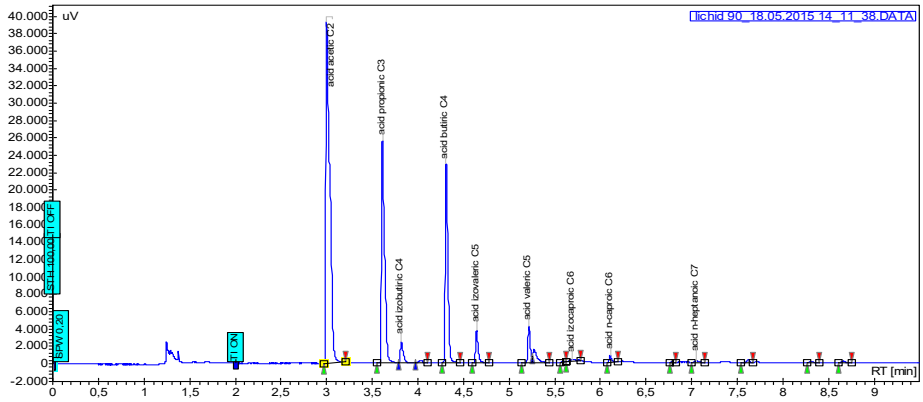

Fig. 1 Example of a GC-chromatogram for VFA in a sample of rumen liquid from fistulizated wether

Table 2 Example for calculation of concentration of each VFA in a sample of rumen liquid

#	Peak Name	Time [min]	Quantity [mM]	Height [uV]	Area [uV.min]	Area % [%]
1	acid acetic C2	3.00	30.93	39205.5	1982.4	46.573
2	acid propionic C3	3.61	7.70	25558.1	1012.1	23.778
3	acid izobutiric C4	3.82	0.54	2461.2	92.8	2.179
5	acid butiric C4	4.31	4.23	22993.9	742.0	17.433
6	acid izovaleric C5	4.64	0.53	3679.3	118.0	2.772
7	acid valeric C5	5.22	0.54	4239.0	124.6	2.926
10	acid izocaproic C6	5.69	0.07	205.6	18.5	0.434
11	acid n-caproic C6	6.11	0.09	846.1	23.2	0.545
13	acid n-heptanoic C7	7.05	0.03	200.4	9.2	0.215
Total			44.65	102142.0	4256.6	100.000

Table 3 Effect of diet on volatile fatty acids concentrations/molar proportions

molar proportion of VFA's (mM/100 mM)	Diet 1 50:50 C:F ratio		Diet 2 100:0 C:F ratio		Diet 3 0:100 C:F ratio	
	initial	after diet changing	initial	after diet changing	initial	after diet changing
Acetic acid C2	72.51%	67.46%	68.11%	64.5%	69.95%	72.16%
Propionic acid C3	20.38%	22.73%	22.81%	21.89%	20.04%	17.96%
Butyric acid C4	7.1%	9.8%	9.06%	13.59%	10.0%	9.87%
Total VFAs	100% (98.5 mM)	100% (103.28 mM)	100% (95.65 mM)	100% (72.85 mM)	100% (77.2 mM)	100% (107.15 mM)

CONCLUSIONS

The presented preliminary results showed that the modified GC method is easy applicable for rumen samples and enables rapid and sensitive determination of VFAs shifts upon dietary changes, such as modification of the concentrates: forages ratio.

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