Determination of the Volatile Fatty Acid Content in the Rumen Liquid: Comparison of Gas Chromatography and Capillary Isotachophoresis

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Abstract

Two analytical procedures for determining volatile fatty acids in the rumen liquid were compared: capillary isotachophoresis (ITP) and gas chromatography. Acetic acid, propionic acid, butyric and valeric acids were determined. No significant differences ($n = 53$, $P \geq 0.05$) have been identified in the amounts of acetic and propionic acids and the results are highly consistent. For butyric acid, the method of gas chromatography yielded higher results with a constant error of approximately 0.5 mmol/l in the whole range of concentration. In terms of diagnostics, however, this size of error is non-significant. In the case of valeric acid a proportional error was found. Gas chromatography yielded higher values, the difference being greater for the low values while for the higher values the results are almost identical. However, valeric acid is a minor acid and its determination is of secondary significance to rumen fermentation assessment. The gas chromatography method is able to clearly differentiate between *n* and *iso* forms in the case of butyric and valeric acids. It can be stated that the two analytical methods yield comparable results and both can be used for the needs of practical diagnostics.

The analysis of volatile fatty acids (VFA) - acetic, propionic, butyric and valeric acids - in the rumen is of considerable clinical and diagnostic significance. To a large degree, it characterizes the metabolism of saccharides and nitrogen substances in the ruminant forestomach environment. The VFA determination can detect different disorders already at the subclinical stage and in this way enable timely and effective action. This is especially important with regard to large-scale breeding where prevention is much more efficient and economical than subsequent treatment. Numerous production diseases are caused mainly by flaws in dietetics and feeding technology, unbalanced feed rations, organic acid surplus in silage and haylage, and feeding non-wholesome or toxic feeds to animals. It is mostly in such cases that the VFA analysis as an integral part of the metabolic profile of the rumen liquid proves irreplaceable and provides valuable evidence (Dvořák et al. 1997; Jagoš et al. 1977; Jagoš and Dvořák 1990).

The VFA determination is rather a demanding analytical procedure and typically is not part of routine diagnostics. It requires a special and relatively expensive set of devices. With regard to chemical structure of the relevant substances, there are two groups of appropriate analytical methods to consider – chromatography and electromigration. The conventional distillation methods with subsequent titration are characterized by low selectivity as well as productivity and are largely no longer in use. Of the chromatographic methods, gas chromatography (GC) is the most widely used, both with filling and capillary columns (Baše and Bartoš 1970; Ceccon 1990; Diamantis et al. 2005; Kmošťák and Kolouch 1988; Ewaschuk et al. 2002), whereas liquid chromatography (HPLC) is used less frequently (Wei et al. 2001; Mathew et al. 1997). Of the electromigration methods, capillary isotachophoresis (CITP) (Boček et al. 1978; Dušek et al. 2004) and capillary zone electrophoresis (CZI) (Buchberger et al. 1997) are generally used. Every individual procedure has its benefits and drawbacks. In the agricultural and veterinary practice,

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whether for VFA determination in the rumen liquid or silage, the GC and CITP methods are most widely used. Of them, the GC methods have been used longer, for more than half a century. Since the 1970s, due to their elegant simplicity, also the electromigration methods have become increasingly used in this area.

Given the fact that most laboratories are restricted to using only one of the above described methods, we were interested in the degree of comparability of their results. Therefore, we compared the results of VFA determination obtained using the two most widespread methods – gas chromatography (with a capillary column) and capillary isotachophoresis. We have not found any such comparison in the available literature, although we consider this very important: should any significant differences be found, the measured results would then have to be evaluated in relation to the analytical method used.

Materials and Methods

Analyzed samples

The rumen liquid (n = 53) was sampled by a stomach tube and filtered through an ordinary sieve in the standard way. Approximately 10 ml of the sample was preserved with 2-3 drops of toluene to prevent fermentation. The samples preserved in this way were immediately analyzed or stored at -20 °C temperature pending analysis.

For the ITP analysis, the samples were thoroughly centrifuged (or filtered) and × 200 diluted in distilled water. Prepared in this way, the samples were used for the analysis without any further treatment.

For the gas chromatography (GC) analysis, the samples were prepared in the following way: 200 μl of metaphosphoric acid (25%) and formic acid (3 : 1) mixture was added to 1 ml of rumen liquid (Cottyn and Boucque 1968). After 30 min of centrifugation, the clear supernatant was × 10 diluted in water and injected in the chromatograph.

Chemicals used

Metaphosphoric acid, ε-aminocaproic acid (EACA), hydroxyethylcellulose (HEC), caproic acid – Sigma-Aldrich, CR, HCl – refined by isothermic distillation, formic acid (Lachema Brno, CR). Standard solutions of appropriate concentration were prepared from the individual substances of analytical purity (Sigma-Aldrich, CR).

CITP Analysis

The analyses were carried out in a two-capillary isotachophoretic analyser IONOSEP 2002 (RECMAN Laboratory technology, CR). The internal diameter of the pre-separation capillary was 0.60 mm and that of the analytical capillary 0.25 mm. Detection was carried out with contactless high-frequency conduction detectors. The leading electrolyte – 10 mM HCl + 22 mM EACA + 0.05 % HEC (pH 4.5). The terminal electrolyte – 5 mM caproic acid. The driving power in the pre-separation capillary – 120 μA, in the analytical capillary – 40 μA, during the detector passage – 20 μA. Thirty μl of sample were applied per column using an autosampler. The analysis time was approximately 20 min.

GC Analysis

The analyses were conducted on a 6820 GC System gas chromatograph, Agilent Technologies. A FFAP capillary column was used, 30 m × 250 μm × 0.25 μm (Quadrex Corporation). Carrier gas – nitrogen, flow 1.0 ml/per min, detector – FID, temperature programme used: 60–200 °C (20 °C/min, 10 min), injector – 250 °C, detector – 300 °C. The injector was equipped with a glass liner of glass wool to separate particles of dirt from the sample.

The samples were dosed by a HT 300A automatic dosing device at an injection size of 1 μl using the split method and a 30:1 splitting ratio. The analysis time is approximately 15 min.

Statistical analysis

Results were expressed as mean, standard deviation (S.D.), parameters of regression lines and Sudent´s paired t-test was used. Non significant differences were defined at $p \geq 0.05$.

Results

The separation of individual substances was very good in both cases and all the acids are clearly differentiated. When CITP is used, a phosphate zone appears near the lactic acid, yet the separation of the two zones is sufficient. The GC method used for butyric and valeric acids also separates their n and iso forms. No such separation happens within the CITP application. This is one of the GC advantages and if iso forms assessment is needed, the GC method has to be used.

Table 1 summarizes statistical processing of the measured results. The results for acetic

Table 1. Comparison of individual volatile fatty acid concentrations (mmol/l) determined by capillary isotachophoresis (CITP) and gas chromatography (GC) methods

	Acetic acid		Propionic acid		Butyric acid		Valeric acid	
n = 53	CITP	GC	CITP	GC	CITP	GC	CITP	GC
Mean	66.41	66.65	28.54	28.40	13.85	14.43	3.09	3.59
± S.D.	10.28	10.61	6.57	6.83	2.83	3.05	0.75	0.83
P	N.S.		N.S.		0.001		0.001	

Table 2. Coefficients of regression lines of statistical comparison of individual volatile fatty acids determined by capillary isotachophoresis (CITP) and gas chromatography (GC) methods

Analyte	Coefficients of the regression line		
n = 53	a	b	r
Acetic acid	0.873	0.99	0.9591
Propionic acid	0.387	1.011	0.9812
Butyric acid	0.316	1.017	0.9582
Valeric acid	0.876	0.854	0.8362

cGC = a + b c$CITP$

and propionic acids were highly consistent and no significant differences between the two methods were found. This was a very positive finding, since the two majority acids make up the greatest part of the total VFA and most pathological conditions are accompanied by changes in concentrations of just these substances. In addition, the regression straight line indicators and correlation coefficients (see Table 2) indicate a very close relationship between the results of the two measurements. Figs 1 and 2 demonstrate a close correspondence of the results, the regression line and the "identity line" (Y = X → a = 0, b = 1) being almost identical in these instances. In this study, the CITP method was selected as an independent variable and the GC method as a dependent variable. If these methods were switched, the regression lines would certainly have different coefficients, still the results would be interpreted similarly.

Significant differences were found for butyric and valeric acids. Based on these results it is impossible to clearly define the cause of the differences. As the GC method produces higher values for butyric acid, possible superponation of peaks can be considered where an unknown substance from the sample matrix manifests a chromatographic behaviour identical to the analyte and in this way increases the resulting value. It follows from Fig. 3 that this increase is approximately constant for butyric acid in the whole range of concentration (the regression line is almost parallel to the "identity line"), which supports this idea. Verifying this possibility would involve the use of different separation conditions that is even a different chromatographic column, which goes beyond the scope of this work. Although the differences were statistically relevant, an average difference of about 0.4–0.6 mmol/l was not clinically relevant to the degree that would affect
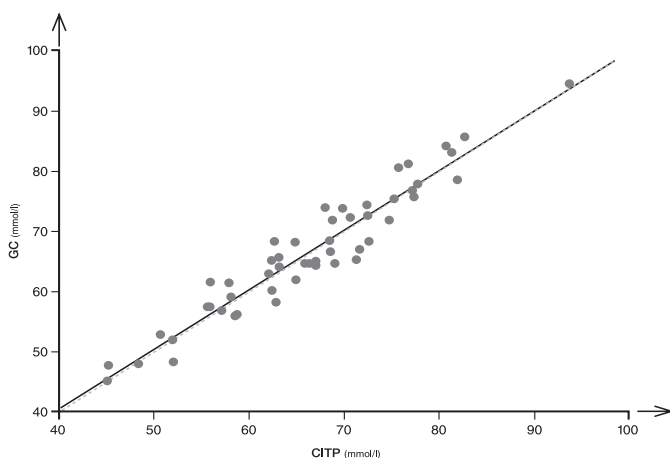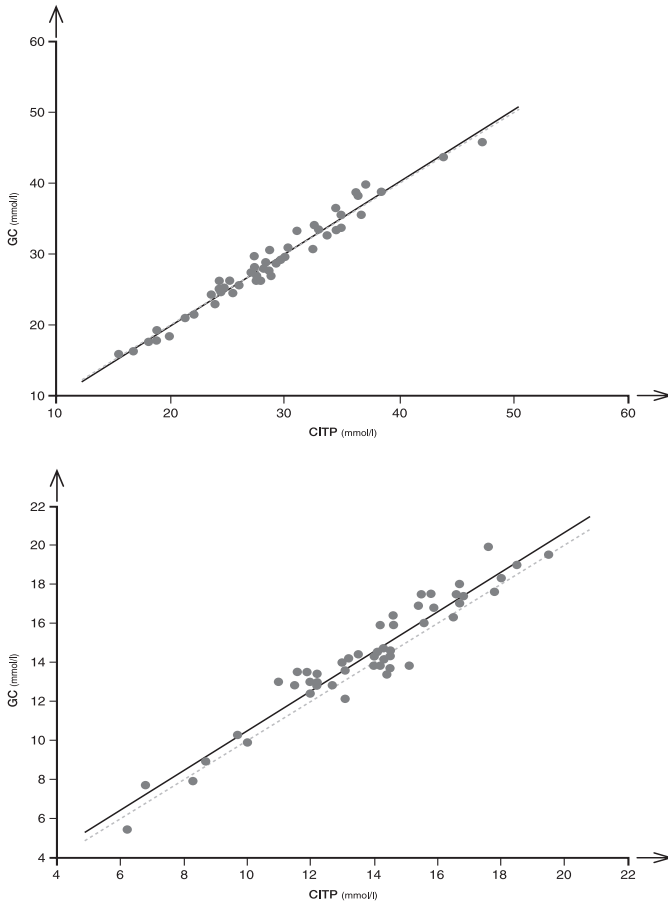

Fig. 1. Graphic comparison of volatile fatty acid concentrations determined by capillary isotachophoresis (CITP) and gas chromatography (GC) methods – dispersion charts (n = 53), 1 - acetic acid

Figs 2 and 3. Graphic comparison of volatile fatty acid concentrations determined by capillary isotachophoresis (CITP) and gas chromatography (GC) methods – dispersion charts (n = 53), 2 - propionic acid, 3 - butyric acid

the result interpretation in relation to standard or pathological values. As the standard values for butyric acid are within the range of 10–20 mmol/l, the difference causes an increase of some 5% in a 10 mmol/l value and only a 2.5% increase in a 20 mmol/l value, which nears the limit of reproducibility of the method itself. Identification of significance is in this case determined by a relatively high number of measurements (n = 53), so that even with a small effect achieved the result was considered as significant. The GC method does clearly differentiate between n- and iso- forms in the two acids. In the electrolyte system used, the CITP method will not distinguish the two forms and determine this acid as a sum of both forms. This may also be the cause of ´slight´ differences between the results. Though in the literature some modifications are described of e.g. alcohol addition to electrolytes leading to distinguishing of n- and iso- forms of butyric acid in sugar-making solutions (K v a s n i c k a et al. 1993), this rather complicates the preparation of those solutions and our experience with rumen liquid analyses has not been convincing in this respect. However, this differentiation is not even necessary for practical evaluation of the current status of rumen fermentation, as iso- form of the butyric acid only constitutes several per cent of the total butyric acid and an increase only takes place in the major n- forms in most cases.

Valeric acid is a minor acid forming a maximum of 5% of the total VFA. In this case, the differences between the two methods were the greatest, the GC method yielding significantly higher results. The regression line equation shows that the difference between the results decreases as the values increase. E.g. for a value of 3.0 (CITP) the GC results are higher in value by approximately 20%, for 4.0 they increase by approximately 15% and for a value of 6.0 the results are practically the same. The effect is obvious from Fig. 4 at first sight. As regards a possible explanation of these results, we can consider that, as in the case of butyric acid, with GC used the complete separation
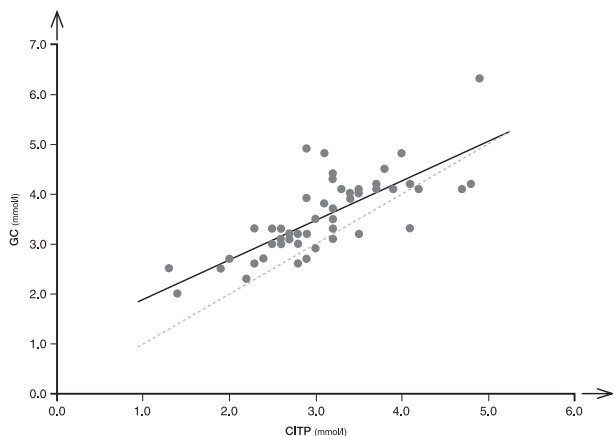
Fig. 4. Graphic comparison of volatile fatty acid concentrations determined by capillary isotachophoresis (CITP) and gas chromatography (GC) methods – dispersion charts (n = 53)
4 – valeric acid

of *n-* and *iso-* forms takes place, whereas when CITP is used such separation does not take place and valeric acid is assessed as a zone created by two forms of the same acid. This effect is more pronounced at low concentrations while it diminishes at higher ones. As valeric acid is a minor VFA and its concentration does not bring any qualitatively new information in most cases (it is frequently not even evaluated within routine diagnostics), we decided not to study the difference any further.

Discussion

Gas chromatography and capillary isotachophoresis are analytical methods based on completely different principles.

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 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 (James and Martin 1952). The following dynamic development of this analytical method as regards both its technical conditions and separation media used advanced to the present stage where we can identify the lower fatty acids through direct injecting without derivatisation required. For this purpose a range of water-resistant sorbents have been developed, some of which are able to also identify butyric acid in a single injection, i.e. a substance with rather different physically-chemical properties, but also valued in relation to assessing the condition of rumen fermentation (Fussell and McCalley 1987; Steverink 1984; Supelco 1996). At present, extensive literature on this subject testifies to an ever growing interest in identifying these substances in a wide range of materials (Yang and Choong 2001; Henderson and Steedman 1982). The advantage of the capillary columns consists in their higher sensitivity and much more advanced separation of individual acids.

Isotachophoresis belongs to the group of electrophoretic procedures. It is based on the mobility of charged particles in the electrical field, which depends on the size of charge of the particle and its mass.

In order to identify carboxyl acids with the CITP method, several electrolyte systems are used in practice. The selection of the leading electrolyte is essentially determined by the dissociation invariables of the substances that we want to separate (pK_a: acetic acid – 4.76, propionic acid 4.85, butyric acid 4.82). A pH value of the leading electrolyte must therefore be near this area. For example β-alanine, EACA or urotropine are quite frequently used. The end electrolyte is chosen based on the acids that we want to identify;

caproic acid or MES (4-morpholineethansulfonic acid) are among the most frequently used. Through the right choice of an electrolyte system we can therefore achieve successful separation of exactly those substances that are of greatest interest to us. The CITP method advantage is that a single analysis enables identifying of a number of other useful substances at the same time, without any requirements for sample modification, e.g. lactic acid, pyruvic acid, formic acid, oxalic, citric or succinic acids and others, some of them useful for the rumen diagnostics. The electrolyte system (EACA, pH 4.5) selected by us is rather widely used and suitable for reliable analyzing of all the important components. The method's sensitivity is satisfactory at usual concentrations whereas at very low concentrations of e.g. lactic acid (values in the real sample below 0.5 mmol/l) the identification can be repeated using a less diluted sample. Still such values are within normal limits and from the diagnostic point of view are basically irrelevant.

Therefore CITP is an appropriate method for analyzing the volatile fatty acids in the rumen. It reliably detects all diagnostically valuable components, does not require any pre-treatment of the sample (except for centrifugation and dilution of the sample with water), and due to considerable dilution of the electrolyte solutions the need for chemicals is low. On the other hand, the CITP method generally requires very exacting levels of purity of the used chemicals (sometimes even PA purity is insufficient and re-purification is necessary), and also the used distilled water must be given careful attention (underestimation of these factors will result in impurity zones that may interfere with the analysis). Some of the widespread electrolyte systems can now be obtained commercially.

It is interesting to learn that such a high level of consistency has been found in the methods based on the separation principles differing to such an extent.

Rimanoczyne and Nagy (1991) compared the identifications of acetic, propionic and butyric acids by the methods of gas and liquid chromatography in different biological materials (including the rumen liquid) without identifying any significant differences.

It can be concluded that identification of the described substances by modern separation procedures yields essentially identical results and no special attention needs to be given to the analytical method used when interpreting the findings.

Stanovení těkavých mastných kyselin v bachorové tekutině: srovnání plynové chromatografie a kapilární isotachoforézy

Byly srovnány dva analytické postupy pro stanovení těkavých mastných kyselin v bachorové tekutině – plynová chromatografie a kapilární izotachoforéza. Stanovovány byly kyselina octová, propionová, máselná a valerová. V případě kyseliny octové a propionové nebyly nalezeny významné rozdíly (n = 53, $P \geq 0.05$) a výsledky byly ve velmi dobré shodě. V případě kyseliny máselné metoda plynové chromatografie dávala výsledky vyšší s konstantní chybou cca 0.5 mmol/l v celém koncentračním rozsahu. Z hlediska diagnostiky jde o chybu zanedbatelnou. V případě kyseliny valerové byla nalezena chyba proporcionální. Plynová chromatografie dávala vyšší výsledky, rozdíl se projevuje výrazněji v nižších hodnotách, ve vyšších hodnotách se výsledky prakticky scházejí. Kyselina valerová je však kyselinou minoritní a její stanovení pro posouzení bachorové fermentace má druhořadý význam. Metoda plynové chromatografie je schopna rozlišit u kyseliny máselné a valerové *n* a *iso* formu. Obecně lze konstatovat, že oba způsoby analýzy dávají srovnatelné výsledky a pro potřeby praktické diagnostiky lze používat obě uvedené metody.

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