Analysis of Short Chain Fatty Acids from Different Intestinal Samples by Capillary Gas Chromatography

K. Schäfer

Institut für Tierernährung, Fachbereich Veterinärmedizin, Freie Universität Berlin, Brümmerstr. 34, 14195 Berlin, Germany

Key Words

Gas chromatography
Open tubular columns
Short chain fatty acids
Intestinal fluids

Summary

A capillary gas-liquid chromatography method for the analysis of free short chain fatty acids in intestinal samples was developed. Analytical results obtained with intestinal samples are influenced not only by high molecular mass matrix constituents but also by matrix components with a molecular weight smaller than 5000 daltons. To achieve a faithful transfer of SCFA into the chromatographic system it was important to avoid accumulation of involatile deposits on the surface of the inlet liner. Cleaning of the inlet liner lead to a significant weakening of matrix effects. The method described, using an Innowax column, is more effective, particularly with respect to precision and sensitivity, and is clearly superior to packed columns. The capillary is the column of choice for the separation of short fatty acids in samples from the small intestine and stomach.

Introduction

In recent years nutritional research groups have recognized that feedstuffs cannot be fully evaluated solely in terms of their overall digestion in the gastrointestinal tract, but that site and end products of particular components of the digestive process must be separately assessed [1]. Short chain fatty acids (SCFA) are the major endproducts of bacterial fermentation. The main substrates for intestinal fermentation are plant wall polysaccharides (dietary fibres) and carbohydrates (sugar residues) [2, 3]. Non-starch polysaccharides of plant cell walls, e.g. cellulose, hemicellulose and pectins are not hydrolyzed by animal enzymes but are degraded to a greater or lesser extent by bacteria present in the gastointestinal tract, yielding SCFA and gases [4, 5]. A microflora is present in non-ruminants throughout the entire gastrointestinal tract, but its presence and activity become progressively greater towards the large intestine. Thus the major proportion of microbial fermentation occurs in the large intestine (colon and caecum). The total concentration of SCFA in the colon of pigs is around 100 mmol l^{-1}, whereas in the stomach and small intestine SCFA concentrations are comparatively low (1–25 mmol l^{-1}). Of the SCFA in the colon, the small intestine and the stomach, acetic acid is the most abundant, and propionic acid is second. The quantities of butyric and valeric acid that are formed are small [6–8].

In a previous report a method for the gaschromatographic determination of SCFA in intestinal samples has recently been described [9]. The SCFA were separated on a glass column packed with acid modified Carbowax as stationary phase and were detected directly without derivatization. The method is suitable for the routine separation of SCFA in intestinal fluids if the concentration of the acids to be determined amounts to more than 0.2 mmol l^{-1}. Apart from its restricted sensitivity the resolution of the packed column is an additional limitation associated with that particular GC method. Progress in the development of new capillary columns, which are thermally stable and which can withstand repeated injections of water and biological fluids, prompted the use of capillary GC for the separation of SCFA [10–12]. As the concentrations of single acids, particularly of the C4 and C5 acids, in samples from the stomach and small intestine of monogastric animals including the pig, are often lower than 0.2 mmol l^{-1} and at the same time, the matrix of intestinal samples is of great complexity, the potency of the capillary column GC for the direct determination of SCFA in intestinal samples was investigated.

0009-5893/95/05 0550-07 $ 3.00/0

Experimental

Instrumentation

The gas chromatography analysis of SCFA was carried out using the Hewlett Packard 5890 Series II GC equipped with a flame ionisation detector (FID). Data collection and GC control was managed using an HP Vectra and HP 3365 Chem Station. For the automated sample introduction an HP 7673 A automatic sampler was used.

Gas Chromatography

GC separations of SCFA were done on a fused silica large bore capillary column, 30 m × 0.53 mm i.d., bonded with a 1.0 µm cross-linked polyethylene glycol innophase (Hewlett Packard, Innowax). Helium was the carrier gas, at a flow rate of 24 cm^3 min^{-1}. The injector temperature was maintained at 200 °C, the detector at 300 °C. The column temperature, after an initial isothermal period of 2.5 min at 120 °C, was increased to 130 °C at a rate of 8 °C min^{-1}, and was maintained at this temperature for 4 min. The temperature was increased again to 210 °C, and was maintained at the final temperature for 3.3 min. Samples (1 µl) were introduced by splitless injection into a deactivated glass liner, using a packed column injection port.

SCFA Calibration Standards

For peak identification and quantification gravimetric standards of the C2–C6 acids (Merck, Larodan) were prepared in a water medium (0.1–2000 mg mL^{-1}). The standards were acidified with 0.5 M oxalic acid.

Gastrointestinal Samples

Stomach-, colon-, and small intestine samples from piglets were prepared as recently described [9]. The samples, which had been stored at – 20 °C, were slowly thawed out and the vials were well shaken before they were chromatographed. In addition to the applied membrane filtration (Sartorius, Minisart, pore size 0.8 µm [9]) some stomach and small intestine samples were submitted to ultrafiltration or ultracentrifugation. The ultrafiltration was done by pouring 2 ml of intestinal sample in a Centrisart I centrifuge tube (Sartorius). After inserting the interior tube carrying the semipermeable membrane (cellulose triacetate; cut off M = 5000), the tube was centrifuged with a fixed rotor for fifteen min (2000 g, room temperature) and the ultrafiltrate was chromatographed. For ultracentrifugation 2 ml of intestinal sample were placed into polycarbonate centrifuge tubes (Beckman) and centrifuged in a Beckman TEL 100 ultracentrifuge at 100 000 g for 2 h at 4 °C.

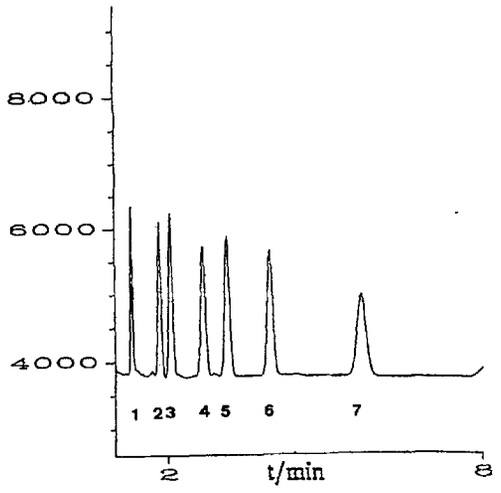

Figure 1
Chromatogram of SCFA from a standard mixture on an Innowax column. Peaks: **1** = acetic acid; **2** = propionic acid; **3** = iso-butyric acid; **4** = butyric acid; **5** = iso-valeric acid; **6** = n-valeric acid; **7** = caproic acid. All standard acids 5 mg l^{-1}. Injection volume 1 µl. Column temperature = 120–210 °C temp. programmed.

Results and Discussion

The inherent characteristics of capillary columns may be a limitation in the capillary GC analysis of crude biological fluids. However, recently new widebore columns have become commercially available [10, 12] which seem very suitable for the determination of SCFA, even if aqueous biological fluids are analyzed. In this investigation a cross-linked polyethylene glycol innophase column (Innowax, Hewlett Packard) was used for the analysis of SCFA in intestinal fluids (colon, small intestine and stomach) of piglets. Sanders [12] directed attention to the qualities of these Innowax columns and he obtained good results when using them for the analysis of SCFA in water and organic solvents.

Retention Time and Precision of SCFA Determinations in Aqueous Media

The peak shape for all seven SCFA chromatogrammed from aqueous solution was sharp, symmetric and well resolved. Figure 1 shows the separation of a C2–C6 aqueous standard mixture using a 30 m large bore Innowax column. The precision of retention times and peak areas was excellent as shown in Table I. There was no effect on the quantitative or qualitative performance when the C2–C6 acids were injected in water, aqueous oxalic acid or aqueous mineral acids. The analysis was completed within 8 minutes.

Intestinal Samples

Although the results for all seven fatty acids in aqueous solution were excellent and demonstrated the good

Table I. Precision of retention time and peak area using an Innowax capillary column. All results were calculated from quintuplicate injections of a SCFA standard mixture C2–C6 (40 mg l^{-1}).

	Retention time (s)		Peak area (counts)	
Acid	Mean	RSD (%)	Mean area	RSD (%)
C2	74.34	0.05	45 771	0.71
C3	103.44	0.06	72 894	0.92
i-C4	115.98	0.05	92 213	1.31
n-C4	151.32	0.08	91 944	0.87
i-C5	178.32	0.04	104 177	1.13
n-C5	227.10	0.04	101 937	1.10
C6	325.20	0.08	104 349	1.37

performance of the column, the performance of the system was lessened when intestinal samples were analyzed. The difficulties observed were unsymmetrical peaks and tailing (stomach and small intestine) and poor reproducibility of peak areas (colon, stomach and small intestine). The reduction is attributed to the adsorption effect of involatile intestinal matrix components. The fact that involatile sample by-products ("dirt") hinder sample transfer is well known as a source of errors [13]. The intestinal samples were introduced by splitless injection, using a packed column injection port. For this, the sample was injected into a heated (200 °C) glass liner with a vaporizing chamber of about 2 ml internal volume. Adsorption of involatile deposits occurred on the surface of the heated and deactivated glass liner. As noted by Grob et al. [14], with "dirty" samples a vaporization step within the injector is desirable to separate volatile solutes from involatile by-products. In this respect, the splitless injection is superior to on-column injection. But "dirt" is also a well-known difficulty when SCFA in biological samples are analyzed as witnessed by others who have encountered similar problems [9, 15].

Acidification with Oxalic Acid

Various procedures for acidification of the analytical system and the injected samples themselves have been employed in order to maintain the performance of the analytical system and prevent the adsorption of SCFA on involatile deposits [15–19]. The addition of oxalic acid (0.5 mmol l^{-1}) to a colon sample was successful and the procedure became more precise. The relative standard deviations of 2–9 % for the C2–C6 acids without the use of oxalic acid improved to less than 3 % after acidification (Table IV). However, the two other intestinal fluids (stomach and small intestine) still presented problems. Even though the quantitative precision for such samples could be improved by use of oxalic acid, the improvement was not substantial. The standard deviation values still approached 10 % for the C2–C4 acids of a stomach sample and the matrix problems still remained.

Ultrafiltration and Ultracentrifugation

Prior to quantification all intestinal samples were subjected to a simple membrane filtration as previously described [9]. Obviously, the concentration of nonvolatile matrix components is usefully diminished by this pretreatment step. As an alternative to membrane filtration, the use of ultrafiltration and ultracentrifugation was then evaluated, in the determination of intestinal SCFA, as a means of producing "clean" intestinal samples without interfering matrix effects. The ultrafiltration technique is easily practicable with the Centrisart system from Sartorius with a standard laboratory centrifuge. In spite of the fact that high molecular mass intestinal matrix components were effectively removed by ultrafiltration and ultracentrifugation, as shown by molecular weight cut-offs, the matrix effects did not disappear and disappointingly, the precision of the SCFA determination was not improved by the application of this separation step. Nevertheless, this result may at least provide some information about the nature of interfering intestinal matrix components. It provides evidence that in intestinal samples, not only are high molecular mass components a source of involatile sample by-products which accumulate on the surface of the glass liner, but also that matrix components with a molecular mass smaller than 5000 daltons, the cut-off of the cellulose triacetate ultrafilter type used, play a part.

Matrix Effects due to Lactose

A SCFA standard mixture containing equal concentrations of C2–C6 acids (50 mg l^{-1}) was spiked with lactose (40 mg l^{-1}) to investigate the potential of a low molecular mass sugar, as encountered in milk, to build involatile sample deposits and thus be responsible for a matrix effect. In Table II relative peak areas of the standard mixture, with and without addition of lactose, are compared after their normalization with the C6 internal standard peak. Each result was calculated from quintuplicate injections of 1 µl volumes at an injection temperature of 200 °C. Relative standard deviations for absolute peak areas were within 1 % for all six fatty acids. With the exception of acetic acid there was no significant difference between the peak areas of the pure SCFA standard mixture and the spiked sample. However, in the case of acetic acid the peak area of the sample containing 4 % lactose, was smaller by 12 %. It was also found that the results obtained with the clean standard sample were identical with those obtained with the standard mixture spiked with lactose, if the clean standard mixture sample was injected after the quintuplicate injections of the "dirty" sample. This demonstrates that, where involatile deposits have already been accumulated on the surface of the glass liner, the matrix effects are not primarily dependent on the sample injected.

Nevertheless, the experiment with spiked samples indicates that small sugar molecules, like lactose or

Table II. Relative peak areas of SCFA standard mixtures (50 mg l^{-1}) from four different injections (a–d). Peak areas are normalized by the area of the internal standard C6. a) "clean" standard mixture; b) standard mixture spiked with lactose (40 mg l^{-1}); c) between each injection of standard mixture (spiked with lactose) dichlormethane as cleaning solvent was injected; d) "clean" standard mixture was injected after quintuplicate injections of stomach and small intestine samples. Between the intestinal samples dichlormethane was injected. Relative standard deviations for absolute peak areas for the single acids in all samples were within 2 %.

	SFCFA standard mixture			
	a) "clean"	b) spiked with lactose (4 ppm)	c) spiked with lactose + cleaning vial (dichlormethane)	d) "clean" after intestinal samples + cleaning vial
C2/C6	0.421	0.368	0.430	0.419
C3/C6	0.635	0.625	0.638	0.642
i-C4/C6	0.815	0.792	0.807	0.813
n-C4/C6	0.817	0.795	0.791	0.837
i-C5/C6	0.935	0.945	0.920	0.954
n-C5/C6	0.918	0.897	0.895	0.948

glucose, or other nutrients which escape digestion by enzymes have the potential to act as involatile sample by-products and thereby influence results when SCFA-peak areas are quantified. The real identity of the matrix components is unknown, they could be e.g. breakdown components which are formed during the digestion of feeds or they could even be nutrients themselves, which cause the interfering effects. It seems possible that particles of the macromolecular dietary fibre complex become a disturbing matrix component. The fibre particles, which during their travel in the gastrointestinal tract are gradually degraded by microbial fermentation, travel with the flow of digesta prior to their arrival in the colon [20]. In this context it may be significant that the microbial population differs in terms of numbers and types between the stomach, small intestine and colon of pigs [20].

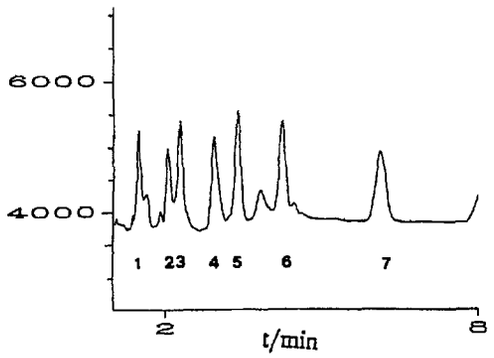

Figure 2

Chromatogram of SCFA from a standard mixture affected by matrix effects. The clean standard mixture (5 mg l^{-1}) was injected after six consecutive injections of a small intestine sample. Peaks and GC-conditions as Figure 1.

Suppression of Matrix Effects

After attempts to eliminate disturbing matrix particles from stomach and small intestine samples by ultrafiltration or ultracentrifugation had failed, attempts to suppress the disturbing effects were investigated by cleaning the deposits from the inlet liner. The involatile components in the intestinal samples, and also in the lactose experiment described above, accumulated in the glass liner forming a brown coloured layer on its surface. However, the effect of such "dirt" on quantitative results could possibly be negligible if accumulation of that "dirt" on the glass liner were prevented. In order to clean the inlet liner after each sample and to dissolve deposits from the surface, to prevent the build-up of a "dirt"-layer, a cleaning vial containing dichlormethane as a purging solvent preceded each intestinal sample vial.

As an experiment a clean SCFA standard mixture (5 mg l^{-1}) was injected after six consecutive injections of a small intestine sample. As shown in Figure 2 the involatile deposits of the preceding intestinal samples affected the peak shape of the SCFA peaks of the clean standard sample injected afterwards. However, the peak shape of the SCFA standard peaks was symmetric when the six injections of the small intestine sample were started with a clean glass liner and a cleaning vial containing dichlormethane followed each small intestine sample injection. As expected, the observed losses of acetic acid, obtained with the SCFA standard mixture spiked with lactose, also disappeared (Table II). The SCFA standard sample experiment was expanded. A clean standard mixture sample (50 mg l^{-1}) was injected after quintuplicate injections of small intestine and stomach samples. Again, each intestinal sample injection was followed by a cleaning vial injection. The relative peak area ratios obtained for this SCFA standard mixture quantification did not significantly differ from expected values (Table II). Furthermore, the relative standard deviations for the stomach sample, which were high (> 10 %) without the use of a cleaning vial, were less than 2 % for the C2 and C3 acids and less than 10 % for the C4 acids when using a cleaning vial. To maintain the performance of the

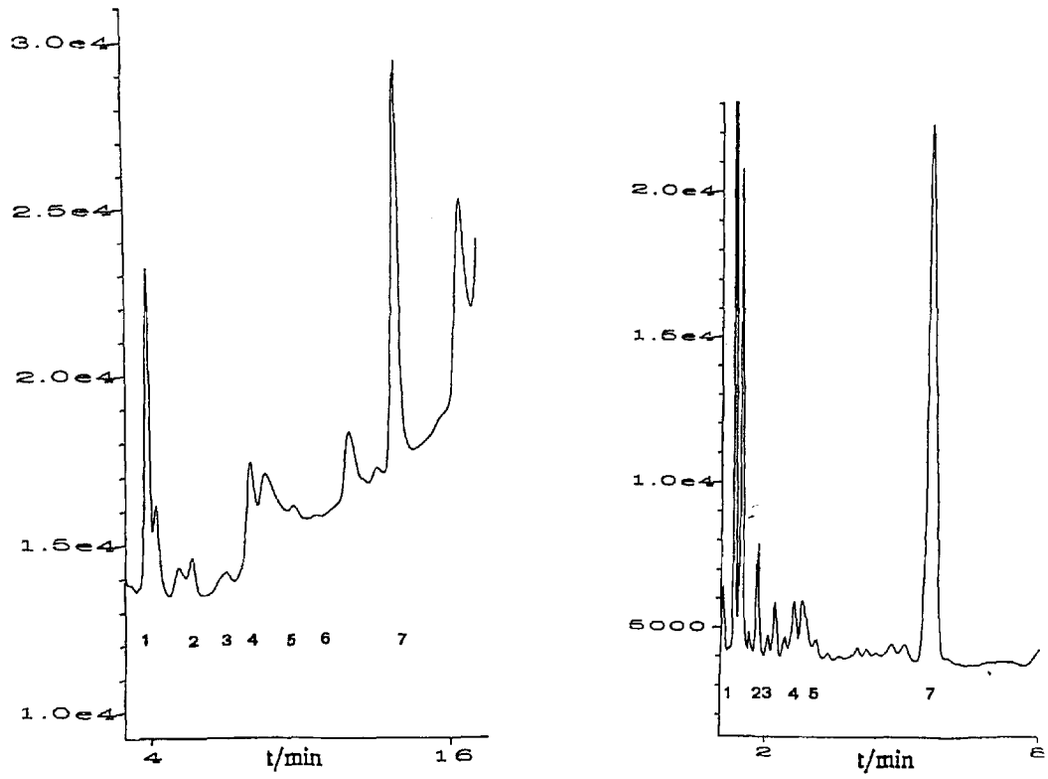

Figure 3

GC separation of SCFA on a packed column (chromatogram on the left) and on an Innowax capillary column (chromatogram on the right) from a small intestine sample. The quantified SCFA concentration values for the sample are given in Table IV. Peaks and GC-conditions as Figure 1.

Table III. Recovery of SCFA. A stomach sample was spiked with a SCFA standard mixture (50 mg l^{-1}). Per cent values are the means from triplicate injections.

	C2	C3	i-C4	n-C4	i-C5	n-C5	C6
Recovery %	96.1	95.6	97.8	96.0	100.3	99.2	95.0
$\pm s_x$	3.2	1.6	1.6	2.6	12.2	8.2	2.4

analytical system the injection of dichlormethane was performed after every intestinal sample analysis. The precision of the quantification was controlled regularly with a SCFA standard mixture. When reproducibilities of results became worse, indicating the building of deposits on the surface of the glass liner – in spite of the cleaning – the glass liner was replaced by a new one. Replacement was necessary after about 30–40 injections of intestinal samples.

The quantification of SCFA-peaks in intestinal samples by comparing peak areas of SCFA calibration mixtures and peak areas of intestinal samples is accurate as long as matrix effects are negligible. The validity of using standard mixtures for the calibration of intestinal peak areas was checked by spiking a stomach sample with a 50 ppm SCFA standard mixture (a cleaning vial was included). Poor recovery results would be an indication that matrix effects must be taken into account. But as shown in Table III, good recoveries were obtained in the range from 95 to 100 %. The quantification of intestinal samples could thus be done without involving standard addition methods, using only calibration standards. The linearity of the calibration response was checked with SCFA standard acids C2–C6 at four concentrations. The linearity of the measured standards C2, C3, n-C4, n-C5, C6 can be described by the regression lines (C2–C6 respectively):

$$y = 0.0239 + 0.2466x \pm 0.00115 \quad (r = 1.00),$$
$$y = -0.0034 + 0.4628x \pm 0.00449 \quad (r = 0.999),$$
$$y = 0.0259 + 0.7193x \pm 0.00407 \quad (r = 1.00),$$
$$y = -0.0456 + 0.9440x \pm 0.01082 \quad (r = 0.999),$$
$$y = -0.1455 + 1.1171x \pm 0.02549 \quad (r = 0.999).$$

The calibration curve was linear up to concentrations of at least 2000 mg l^{-1} (i.e. 33.3 mmol l^{-1} for acetic acid). Higher concentrations were not evaluated because those levels of concentration are far above those normally found in diluted intestinal samples.

Table IV. SCFA-concentrations of intestinal samples after quantification using packed column GC and capillary column GC. All results were calculated from quintuplicate injections of the intestinal samples (colon, stomach, and small intestine); concentrations are expressed in mmol l^{-1}.

Sample / Column	C2	C3	i-C4	n-C4	i-C5	n-C5	
Colon							
packed	62.63	14.43	0.60	11.24	1.86	1.61	×
	0.74	0.19	0.01	0.15	0.03	0.02	$\pm s_x$
	1.19	1.32	2.28	1.30	1.42	1.35	RSD %
capillary	60.94	18.17	0.42	10.57	0.79	1.94	
	0.54	0.20	0.01	0.24	0.02	0.01	
	0.89	1.10	2.57	2.32	2.42	0.52	
Stomach							
packed	14.61	1.88	0.17	0.46	0.31	0.11	
	0.69	0.08	0.06	0.03	0.04	0.06	
	4.70	4.12	34.21	6.50	12.91	56.50	
capillary	15.45	1.28	0.04	0.42	0.12	–	
	0.22	0.04	0.05	0.04	0.02	–	
	1.45	1.83	12.50	9.50	16.67	–	
Small intestine							
packed	10.33	0.60	0.33	1.20	0.79	–	
	0.10	0.09	0.03	0.09	0.03	–	
	0.97	16.04	7.68	8.40	0.38	–	
capillary	10.79	0.84	0.08	0.33	0.005	–	
	0.17	0.03	0.01	0.03	0.001	–	
	1.61	3.82	14.75	9.09	2.00	–	

Comparison of Packed Column – Capillary Column Results

After adding a cleaning vial prior to the stomach or small intestine vial, the separation of SCFA in intestinal fluids was possible with good quantitative precision. The Innowax capillary column was used for about three hundred injections of intestinal samples. The quantitative performance of the capillary did not change. It appeared worthwhile to compare some results obtained with a packed column method described earlier [9] with the present capillary GC procedure. After a delay of about three months the same intestinal samples were subjected to SCFA-analysis by capillary GC. In the meantime the intestinal samples were kept deep frozen. The comparative results for three different intestinal samples are presented in Table IV. The concentration values of a colon sample obtained with the Innowax capillary column showed good agreement with results obtained with the packed column. The agreement of the concentrations for a stomach and a small intestine sample were good for acetic and propionic acid. The higher butyric acid and valeric acid concentrations obtained with the packed column GC may be explained by the better performance of the capillary GC to separate SCFA from coeluting peaks in the presence of a complex intestinal matrix. Figure 3 shows the chromatograms obtained with both columns from a small intestine sample. The differences between the two chromatograms illustrate the superior performance of the capillary column. Due to the higher efficiency and selectivity of the Innowax column, significant improvements in resolution, peak shape, and symmetry were obtained for all the fatty acids, particularly the C4 and C5 acids. Analysis by the capillary method took only half the time. The capillary GC procedure was more precise than the packed column procedure. Table IV shows the relative standard deviations of the SCFA-concentrations using the Innowax column. The precision of the capillary method was good for high and medium acid concentrations and was also acceptable at low concentrations (< 0.1 mmol l^{-1}).

In this context it should be noted that the precision achieved when quantifying SCFA depended on their respective concentration level. The relative standard deviation of an aqueous standard containing all acids in equal concentration of 40 mg l^{-1} was 1–1.5 %. However, the standard deviation values increased up to 8 % for the fatty acids when the concentration of the internal standard was in the range of 3–5 mg l^{-1}. Concentration values of the C4 and C5 acids which were quantified in stomach and small intestine samples dropped down to 40 µmol^{-1} (3.5–4 mg l^{-1}) in some samples. The concentrations mentioned above are not measured, but calculated ones, as the dilution of the samples (dilution factor of 5 [9]) must be taken into account. The lowest C4 and C5 values obtained from intestinal samples came close to the limit of quantification. The estimation of the limit of detection for SCFA in water, based on the mean value plus three times the standard deviation of standard acids containing 100 µg l^{-1} of the appropriate acid, indicate values of 50–100 µg l^{-1}. The quantification of 200–500 µg l^{-1}, i.e. approximately 5 µmol^{-1} for each single acid in an intestinal sample, is reliable.

Conclusions

Even after ultrafiltration intestinal samples still contain low-molecular by-products which have an effect on quantitative results. By cleaning the inlet linear, matrix effects became much weaker and the reproducibilities of results improved. The described capillary method has a low detection limit, it allows the quantification of low SCFA-concentration levels from different intestinal samples.

A comparison of the two columns shows that the capillary one is the column of choice for the separation of SCFA in small intestine and stomach samples. The higher efficiency and selectivity of the Innowax gives more reliability to the results. However, colon and rumen samples are "easier" with respect to SCFA-concentration levels and also with respect to disturbing matrix by-products. For those samples accurate quantitative results can also be obtained by packed column methods. As with packed columns the performance of the columns can be restored, packed column GC can be an alternative to capillary GC when SCFA in colon and rumen samples have to be quantified.

References

[1] *M. F. Fuller,* In Vitro Digestition for Pigs and Poultry. The Rowett Research Institute Greenburn Road Bucksburn Aberdeen, XII. Wallingford C.A.B. International (1991).

[2] *V. C. Mason,* Proceedings of the Nutritional Society **43,** 45 (1984).

[3] *J. H. Cummings,* Lancet **i,** 1206 (1983).

[4] *A. C. Longland,* In Vitro Digestion for Pigs and Poultry, 3. Wallingford C.A.B. International (1991).

[5] *H. Graham,* In Vitro Digestion for Pigs and Poultry, 35. Wallingford C.A.B. International (1991).

[6] *K. E. Bach Knudsen, B. Borg Jensen,* Proc. Vth International Symposium on Digestive Physiology in Pigs, Pudoc Wageningen, 389 (1991).

[7] *F. X. Roth, B. Eckel, M. Kirchgessner, U. Eidelsburger,* J. Anim. Physiol. a. Anim. Nutr. **67,** 148 (1992).

[8] *J. v. d. Meulen, J. G. M. Bakker,* Proc. Vth International Symposium on Digestive Physiology in Pigs 440 (1991).

[9] *K. Schäfer,* Chromatographia **39,** 706 (1994).

[10] *D. V. McCalley,* J. High Res. Chromatogr. **12,** 465 (1989).

[11] *L. Ceccon,* J. Chromatogr. **519,** 369 (1990).

[12] *W. Sanders,* Application Note 228 – 225, Hewlett Packard Co (1993).

[13] *K. Grob, Jr.,* J. Chroamtogr. **287,** 1 (1984).

[14] *K. Grob, Th. Läubli, B. Brechbühler,* J. High Res. Chromatogr. & Chromatogr. C. **11,** 462 (1988).

[15] *E. M. Stansbridge, G. A. Mills, V. Walker,* J. Chromatogr. **621,** 7 (1993).

[16] *R. F. Fussell, D. V. McCalley,* Analyst **112,** 1213 (1987).

[17] *M. F. Mehran, N. Golhar, M. Mehran, W. J. Cooper,* J. High Res. Chromatogr. & Chromatogr. C. **11,** 610 (1988).

[18] *G. C. Cochrane,* J. Chromatogr. Sci. **13,** 440 (1975).

[19] *M. H. Henderson, T. A. Steedman,* J. Chromatogr. **244,** 337 (1982).

[20] *B. Ratcliffe,* In vitro Digestion for Pigs and Poultry, 19. Wallingford C.A.B. International (1991).

Received: Nov 11, 1994
Revised manuscript received: Jan 19, 1995
Accepted: Feb 14, 1995