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Short communication

Determination of short-chain fatty acids in dietary fiber extracts using ion-exclusion chromatography with suppressed conductivity detection

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ABSTRACT

A new chromatographic method for the sequential determination of short-chain fatty acids is described. Acetic, propionic and butyric acids were determined in dietary fiber extracts using ion-exclusion chromatography equipped with inverse chemical suppression and conductivity detection. The best optimization of the chromatographic conditions were achieved when a 100 mm × 7.8 mm ion-exclusion column with a solution of 0.5 mmol L^{-1} sulfuric acid as eluent in a flow rate of 0.6 mL min^{-1} were employed. The organic acids were sequentially separated in less than 10 min with limits of detection ranging from 1 up to 7.5 μmol L^{-1} and limits of quantification from 5 up to 25 μmol L^{-1}. The linearity of the analytical response was studied in the range of 0.005–10 mmol L^{-1} for acetic acid and 0.025–10 mmol L^{-1} for propionic and butyric acids with coefficients of determination (R^2) ranging between 0.9985 and 0.9999. The method was tested and proved to be selective, precise, accurate, reproducible and highly sensitive. Finally, the method was applied in the analysis of biological samples.

1. Introduction

Dietary fiber (DF) plays an important role as indigestible food components in human nutrition due to their beneficial effects for health. Mainly consisting on non-starch polysaccharides, oligosaccharides and resistant starch, they have water-binding properties thus increasing volume and viscosity of intestinal contents [1–3]. DF are responsible for faecal bulking, enhancing gut motility and lowering transit time. Being indigestible in the small intestine, they finally reach the colon, where they are utilized as fermentation substrates by the gut microflora [4–6]. In the colon, there is a symbiosis through fermentation between the host and intestinal bacteria. The short-chain fatty acids (SCFAs) – acetic, propionic and butyric – released as main microbial fermentation products are rapidly absorbed by the colonic epithelium, which stimulates water and Na^+ absorption [4,6]. Furthermore, especially butyrate is a preferred substrate for the colonocyte. It serves as an energy source and is known to contribute a trophic effect on colonic mucosa [7–9].

The growing interest in determining the formation of short-chain fatty acids is related to the increasing evidence of their positive physiological effects [10]. Thus, several analytical techniques have been reported for the determination of these compounds in different matrices. These include methods such as enzymatic with spectrophotometric detector [11,12], chromatographic [13,14] and electrophoretic [15,16]. Enzymatic methods are well recognized for their high specificity, but require large amounts of reagents and are highly time consuming because only one acid can be determined in each assay [13,16]. However, enzymatic methods are sometimes used as a reference method in order to validate chromatographic and electrophoretic methods [17]. Spectrophotometric methods are tedious and there is no data available on the determination of some acids like succinic, citric and acetic [18]. Capillary electrophoresis also has a very good selectivity, but suffers from lower reproducibility in comparison to enzymatic and chromatographic methods [16]. The speed and selectivity of chromatographic techniques have rendered them more useful in the analysis of organic acids. Gas and liquid chromatography with mass spectrometer detectors (GC–MS, LC–MS) have been considered the best methods so far, owing to their robustness, selectivity, sensitivity and high throughput [19,20]. However, these techniques are greatly limited by high cost and complexity of both instruments and sample preparation [18,21]. Reverse-phase and ion-exchange HPLC with UV spectrophotometric detection [22] have been the most widely used, but it requires complex sample preparation procedures. Anion-exchange chromatography with conductivity detection has been used in the past to determine organic acids in

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juice and wine [23,24]. It is relatively easy, selective and can sequentially determine organic acids and inorganic anions. This becomes a disadvantage in the analysis of organic acids in a matrix containing inorganic anions, due to both interference and the fact that high concentrations of the inorganic anions will in-turn suppress some of the acids [13].

Moreover, in most methods above mentioned large amounts of organic solvents and reagents that are hazardous to the environment and human health are required. Therefore, a simple and environmentally friendly method would be desired to determine SCFAs with minor pretreatment.

Ion-exclusion chromatographic separation has been commonly used for the determinations of aliphatic organic acids in a variety of matrices as well as non-ionic analytes of significant pharmaceutical interest including alcohols and carbohydrates [18,21,25,26]. In ion-exclusion chromatography, separation is accomplished using dilute mineral acids as mobile phase, to maintain organic acids in their undissociated forms, and separated ions are detected using suppressed or non-suppressed conductometric or UV detection [25].

The current mechanism of ion-exclusion chromatography proposes that the sulfonic groups are fixed mostly on the surface of the polystyrene-divinylbenzene (PS-DVB) resin and form a negatively charged shield on the polymeric surface, often referred to as the "Donnan membrane" [27,28]. This "membrane" separates the moving fraction of the eluent (i.e. the mobile phase) from the static, occluded component of the eluent (i.e. the stationary phase). Once the analytes enter into the column, they interact with the sulfonated PS-DVB copolymer in such way that the dissociated fraction of the analyte is repelled from the vicinity of the "Donnan membrane" into the bulk of the interstitial eluent, while the protonated fraction penetrates the membrane and enters into the occluded fraction of the eluent, where it may experience additional retention by surface adsorption onto the unfunctionalized parts of the resin [27–30]. As higher is the pK_a of an individual acid, the higher is the protonated fraction and consequently the longer is its retention time. Anomalies for analyte acids showing significantly different retention times but having almost identical pK_a values have been explained by the increased hydrophobic character of some acids, which leads to increased hydrophobic adsorption [27–30].

This paper describes an ion-exclusion chromatographic method with inverse chemical suppression for the determination of three organic acids in dietary fiber extracts. The main task of the inverse chemical suppressor is to reduce the self-conductivity of the eluent. This is achieved by using ion-exchange to replace the highly conductive protons by cations with a relatively low conductivity. The conditions that resulted in the best isocratic separation of the organic acids were checked for linearity, precision and accuracy.

2. Experimental

2.1. Reagents

High-purity reagents and deionized water (Millipore, Milli-Q System) were used for all preparations. Standard solutions of the organic acids (acetic, propionic and butyric) were prepared from their sodium salts (analytical-reagent grade; Acros Organics). A mixed 100 mmol L^{-1} stock standard solution was prepared for each organic anion. Calibration standards were prepared from 1 mmol L^{-1} mixed standard solution using a series of dilutions.

2.2. Instrumentation

The determination of the organic acids was performed using the "MIC-2 Advanced" modular IC system (Metrohm AG, Herisau, Switzerland). It is composed of a serial double-piston high-pressure pumping unit, a two-channel peristaltic pump for use with the Metrohm Suppressor Module, separation center, conductivity detector and an interface to connect with PC. Chromatograms were recorded using the Metrohm IC Net 2.3 software. The system was run in the isocratic mode with the column in the room temperature. Ion-exclusion chromatography with inverse suppression and conductivity detection was used to separate all the organic acids.

For ion-exclusion, samples were injected via a 10 μL loop and eluted at a flow rate of 0.6 mL min^{-1} and pressure of 1.1 MPa through a Metrosep 6.1005.210 organic acids analytical column (100 mm × 7.8 mm, particle size of 10 μm, with polystyrene-divinylbenzene copolymer packing material functionalized with sulfonic acid groups). Solutions of sulfuric acid and sulfuric acid/acetone were used as eluent at different concentrations. The eluents were filtered through a 0.22 μm Millipore paper filter and then degassed by vacuum and ultrasonification. The choice of eluent was mainly guided by their abilities to totally ionize in solution at low concentrations (strong acids or acids which behave as strong acids). It was also guided by their compatibility with the detection mode and polarity. The suppressor system was regenerated by a solution of 50 mmol L^{-1} LiCl solution pumped through a suppressor unit simultaneously with deionized water. The conductivity detector was operated in the positive mode at a full scale of 10.0 μS cm^{-1}. The peak areas and retention times were recorded and used to calculate chromatographic parameters.

2.3. Source of fibers

Fruits were purchased from organic cultivar. Pumpkins and papaya were cut, dried at 40 °C and powdered. Pumpkin seeds and kale were dried at 40 °C and powdered. Apples, guavas, plums and grapes were blended and lyophilized. The powders were stored at −20 °C.

2.4. Source of faecal bacterial inoculum

Fresh faeces were collected from three rats consuming a normal diet. Faecal samples were diluted 1 g faeces: 6 mL saline phosphate buffered (PBS) pH 7.4.

2.5. Batch-culture fermentation

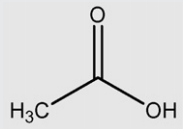
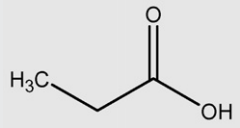
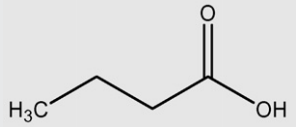
The procedure for *in vitro* fermentation with faecal inoculum was based on Velazquez et al. [5]. Briefly, 0.5 g of fiber was added into a 100 mL sterile bottle. Fibers were hydrated with 40 mL of PBS with the oxygen reducing enzyme supplement (Oxyrase® For Broth, Mansfield, OH, USA) following the manufacturer's instructions. The bottles were tightly closed to maintain anaerobic conditions generated by the enzyme supplement and stored at 4 °C for 12–16 h to allow adequate hydration of the fibers.

Each bottle was inoculated with 10 mL of faecal solution and tightly closed. Samples were placed and gently shaken in a water bath kept at 37 °C for 24 h. Duplicate aliquots (2 mL) from each substrate bottle were mixed with 1 mL of copper sulfate solution (10 g L^{-1}) to inhibit further microbial growth. Aliquots were frozen at −20 °C until preparing for SCFAs analysis. For analysis of SCFAs, samples were centrifuged at 12,000 rpm and 4 °C for 15 min.

3. Results and discussion

In order to separate SCFAs listed in Table 1, several parameters were considered in the optimization process of the chromatographic conditions. First, the choice of the stationary phase, and the next step, the mobile phase like the optimal concentration and the flow rate were investigated.

Table 1
Selected SCFAs with their respective pK_a values.

SCFAs	Chemical structure	pK_a (at 25 °C) [36]
Acetic acid		4.76
Propionic acid		4.87
Butyric acid		4.83

The initial assays were carried out with a Metrosep 6.1006.520 *A Supp 5* analytical column (150 mm × 4.0 mm, particle size of 5 μm, with polyvinyl alcohol packing material functionalized with quaternary ammonium groups) using as mobile phase a mixed solution of sodium carbonate and bicarbonate at 3.2 and 1.0 mmol L^{-1}, respectively, according to the column specification supplied by the manufacturer (Metrohm AG). However, in these conditions, it was not possible to separate sequentially SCFAs because the analytes were eluted together from the column and could not be identified. Then, all other experiments were performed on an ion-exclusion column (Metrosep 6.1005.210 organic acids analytical column, previously described), considering the retention mechanism based on Donnan exclusion effects.

Since the instrument was operated in the isocratic mode, variation in the concentrations of the mobile phases was performed in separate runs. Diverse sulfuric acid solutions at different concentrations, ranging between 0.25 and 10 mmol L^{-1}, were tested as eluent, according to the literature data [13,31,32]. In addition, the influence of different percentages of the organic modifier in the mobile phase was also investigated. The last evaluation concerns the optimization of the flow rate.

The optimum concentration of sulfuric acid and the best flow rate that yielded an acceptable isocratic separation of all organic acids in the mixture were 0.5 mmol L^{-1} and 0.6 mL min^{-1}, respectively.

The influence of an organic modifier on the separation of organic acids has been well documented [24,25,33]. It is thought to contribute to the reduction of the retention of carboxylic acids by the stationary phase due to the lipophilic properties of the alkyl group in the solvents [24,25,33].

In this work, acetone (HPLC grade), with concentrations ranging between 5 and 15%, was tested as an organic modifier in the mobile phase composed by the diluted sulfuric acid solutions. The results indicate that the retention times gradually decreased as the acetone content of the mobile phase was increased, and at an acetone content of 15%, the peak-to-peak separation was incomplete.

Organic solvents are extensively used in chromatography and for this reason they are a matter that has to concern due to the characteristics such as: high flammability, volatility, and toxicity. This is a motivation to eliminate the need of organic modifier solvents in the mobile phase, generating a "green chemistry" ion-exclusion chromatographic method. Therefore, the mobile phase contained 0% acetone (i.e. only sulfuric acid solution at 0.5 mmol L^{-1}) was selected, considering that, in these conditions, a satisfactory separation was achieved.

Acetic, propionic and butyric acids were determined in fermented extracts of dietary fiber samples using the proposed method. The presence of these compounds was confirmed by comparing their retention times with those of the standards.

The values of pK_a of these acids, shown in Table 1, are similar to each other. However, can be observed from the trend of retention times that an increase in the number of lipophilic carbon atoms for the same functional groups (aliphatic carboxylic acids) increases the hydrophobic interaction between the compounds and the stationary phase, hence an increase in the retention factor, i.e. the retention time of acetic < propionic < butyric [13,26]. Several chromatographic parameters evaluated for the three compounds, including retention time, retention factor, selectivity coefficient, resolution and number of theoretical plates, are shown in Table 2.

The retention factor, k', which is a measure of the migration rate of the analyte on the column, was calculated as the ratio of the difference between the actual retention time and the dead volume. The dead volume was considered as the distance from the beginning of the chromatogram at time $t = 0$ to the solvent front. The selectivity, α, of the method that is a measure of the separability of two compounds eluting adjacent to each other was calculated according to Eq. (1) [25]:

$$\alpha = \frac{k'_2}{k'_1} \quad (1)$$

where k'_2 and k'_1 are the retention factors of two adjacent peaks (compounds).

The quality of separation in each case was evaluated by calculating the resolution, R, of the peaks according to Eq. (2) [25]:

$$R = 2\frac{t_{R_2} - t_{R_1}}{W_{t_1} + W_{t_2}} \quad (2)$$

where t_{R_1} and t_{R_2} are the retention times for the first and second peaks, respectively; W_{t_1} and W_{t_2} are the base widths between the tangents in time unit for the first and second peaks, respectively.

The selectivities and resolutions of the peaks presented in Table 2 are with respect to the previous adjacent peak. For example, the selectivity and resolution of the propionic acid peak is in relation to the acetic acid peak and the butyric acid peak is in relation to the propionic acid peak. All the selectivity coefficients, α, were greater than one, indicating that a mixture of the compounds can be separated. This is acceptable especially in isocratic separations [13,34]. The chromatographic efficiency, representing the ability of

Table 2
The chromatographic parameters evaluated for the selected SCFAs.

Chromatographic parameters	Acetic acid	Propionic acid	Butyric acid
Retention time, t_R (min)	5.21 ± 0.01	6.12 ± 0.01	7.56 ± 0.01
Retention factor, k'	1.13 ± 0.01	1.51 ± 0.01	2.10 ± 0.01
Selectivity coefficient, α	–	1.33 ± 0.01	1.39 ± 0.01
Resolution, R	–	1.98 ± 0.01	1.59 ± 0.01
Theoretical plates, N	3709 ± 15	1765 ± 13	605 ± 2

Table 3
Analytical parameters obtained for the selected SCFAs.

Parameter	Acetic acid	Propionic acid	Butyric acid
Linear range (mmol L^{-1})	0.005–10	0.025–10	0.025–10
Sensitivity (mV s L $mmol^{-1}$)	6374.4	6127.2	4921.2
Coefficient of determination, R^2	0.9985	0.9999	0.9997
Concentration range (mmol L^{-1})	0.1–20	0.05–7	0.04–8
Limit of detection (mmol L^{-1})	0.001	0.0075	0.0075
Limit of quantification (mmol L^{-1})	0.005	0.025	0.025
Precision (%R.S.D.)	1.06	0.84	0.96
Recovery (%)	98.3 ± 0.8	97.2 ± 1.1	97.4 ± 1.4

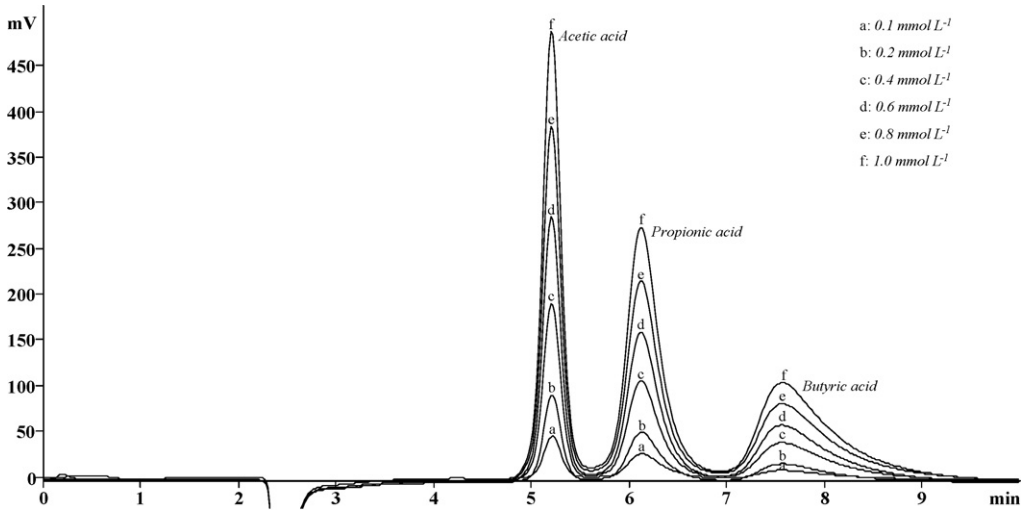


Fig. 1. Merged chromatograms showing the separation of the SCFAs in a gradient of concentration (0.1–1 mmol L^{-1}).

separation with minimal dispersion of the compound, was evaluated through the number of theoretical plates according to Eq. (3) [25]:

$$N = 16\left(\frac{t_R}{W_t}\right)^2 \quad (3)$$

where t_R is the retention time of the peak and W_t is the base width between the tangents drawn to each side of the peak.

A typical chromatogram showing the separation of the three SCFAs in a gradient of concentration (0.1–1 mmol L^{-1}) is shown in Fig. 1.

The limits of detection (LOD) and quantification (LOQ) were obtained by using a signal-to-noise ratio of 3:1 and 10:1, respectively. The obtained values are shown in Table 3. The LOD values vary from 1 to 7.5 μmol L^{-1} and the LOQ values vary from 5 to 25 μmol L^{-1} (without preconcentration). These detection limits are similar or even lower than those observed by other authors

Table 4
Concentrations of the selected SCFAs in a pumpkin sample having seven different compositions.

Sample	SCFAs concentration (mmol L^{-1})		
	Acetic	Propionic	Butyric
Pumpkin: 0.50 g of pulp	2.64 ± 0.01	0.18 ± 0.01	0.26 ± 0.01
Pumpkin: 0.40 g of pulp + 0.10 g of seed	12.15 ± 0.01	1.64 ± 0.01	1.81 ± 0.01
Pumpkin: 0.30 g of pulp + 0.20 g of seed	5.21 ± 0.01	0.80 ± 0.01	1.65 ± 0.01
Pumpkin: 0.25 g of pulp + 0.25 g of seed	5.57 ± 0.01	0.90 ± 0.01	1.78 ± 0.01
Pumpkin: 0.20 g of pulp + 0.30 g of seed	11.63 ± 0.01	3.01 ± 0.01	2.24 ± 0.01
Pumpkin: 0.10 g of pulp + 0.40 g of seed	6.07 ± 0.01	0.96 ± 0.01	1.92 ± 0.01
Pumpkin: 0.50 g of seed	13.00 ± 0.01	5.86 ± 0.01	4.33 ± 0.01

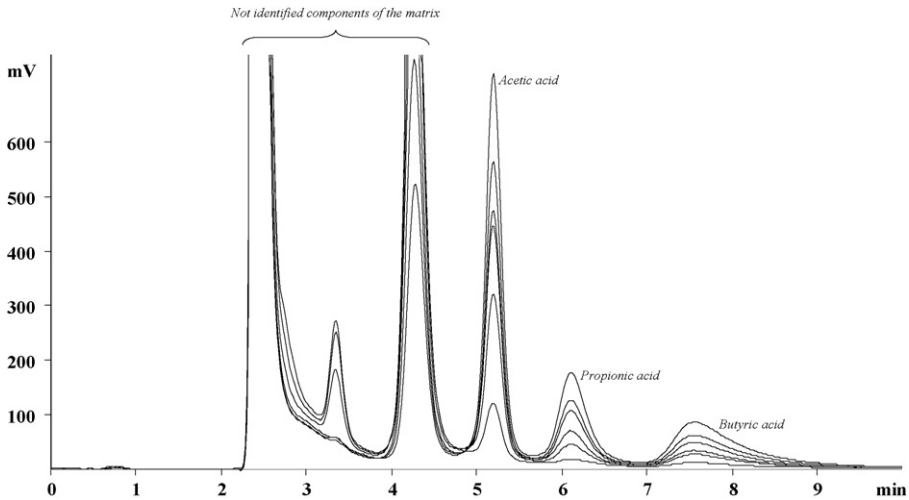


Fig. 2. Merged chromatograms showing the separation of the components (the compounds of the matrix and the SCFAs) of some randomly selected samples.

[10,14,31,32,35]. The linearity of the analytical response was studied for each compound. Table 3 shows the optimum linear response range for each compound. As it can be observed a good linearity was obtained in all cases.

In order to check the precision of the method, seven replicate analysis of a standard solution on different days were performed. The precision expressed in terms of the relative standard deviation (R.S.D.) always remained around 1% for all the studied SCFAs. The accuracy of the method was evaluated from the recovery analysis, preparing spiked samples in quadruplicate at three levels of concentration higher than LOQ. The values obtained in all of the cases, shown in Table 3, were satisfactory from the analytical point of view, although some losses occurred during the experiments have provided results just below of 100%.

The fermented extracts of different dietary fibers, including pumpkin, kale, papaya, apples, guavas, plums and grapes, were analyzed according to the proposed method. In Table 4 are shown the concentrations of the acetic, propionic and butyric acids in a pumpkin sample having seven different compositions. The merged chromatograms, illustrated in Fig. 2, exhibit the separation of the components (the compounds of the matrix and the SCFAs) of some randomly selected samples. As can be seen, practically no impurities or interfering substances were found in the chromatograms, which are similar for all studied samples.

The analytical performance of the proposed method was evaluated by comparison with other methods reported in literature and that has been applied for the analysis of biological samples [10,14,31,32,35]. Thus, the proposed method provides good statistical accuracy and precision, a wide linear range, high sensitivity and satisfactory efficiency. Moreover, this method is simple and rapid with no loss in sensitivity, so it may be recommended for routine analysis, being considered a viable option from the analytical, environmental and economic point of view.

4. Conclusion

An ion-exclusion chromatographic method for the determination of SCFAs has been developed. The described method using the suppressed ion-exclusion chromatography is well suited for the rapid, accurate, precise and sequential determination of acetic, propionic and butyric acids in dietary fiber extracts without any derivatization, giving results in less than 10 min. Moreover, the advantages of this method over the others include its simplicity, low cost of materials and reagents needed for the analysis and the absence of organic modifier solvents in the mobile phase, generating a “green chemistry” ion-exclusion chromatographic method.

The evaluated chromatographic parameters suggested a satisfactory analytical performance of the proposed method, although the number of theoretical plates for butyric acid has been low. However, from the analytical point of view, despite the low chromatographic efficiency obtained for the butyric acid, the sensitivity of this component was not affected significantly by width of its chromatographic peak.

Finally, this method can be easily applied for routine analysis in scientific laboratories of universities and industries that have interest in these analytes. In addition, the information provided by this new method can also be used for other types of aqueous matrices.

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