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Capillary electrophoresis for short chain organic acids in faeces Reference values in a Mediterranean elderly population

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Abstract

There is increasing evidence that gut microflora and fermentation processes in the large intestine are important for health, and that health-promoting effects are mediated by fermentation products. Usually analytical methods for these compounds are tedious. A simple and rapid procedure of aqueous extraction from the stools has been optimized. After extraction, an aliquot of the aqueous layer was directly injected into the capillary electrophoresis equipment. Oxalic, formic, fumaric, 2-ketoglutaric, succinic, citric, acetic, propionic, 2-ketoisovaleryc, butyric, isovaleric lactic, glyceric 2-hydroxybutyric, and valeric acids were separated and identified. Electrophoretic conditions were: phosphate buffer 234 mM pH 6.10 with 12% (v/v) methanol with a coated capillary at −10 kV of applied potential. The method was validated for a representative group of compounds: acetic, propionic butyric, 2-hydroxybutiric, isovaleric, and oxalic acids, including the comparison of results with ionic chromatography. Finally 136 samples from healthy humans aged 60–80, both male and female living in Spain, were measured. They could be used as reference values for further studies.

Keywords: Carboxylic acids; Short chain fatty acids; Gut microflora; Faecal content; Stools

1. Introduction

Diet is known to modulate immune functions in multiple ways and to affect host resistance to infections. Besides the essential nutrients, non-essential food constituents such as non-digestible carbohydrates may also have an impact on the immune system, especially in the area of the gut-associated lymphoid tissue [1]. Short chain organic acids (SCOA) are carboxylic acids with 1–6 carbon atoms that can include different other functional groups, such a hydroxyl or dicarboxyl, and arise from bacterial fermentation of carbohydrates, protein, peptide and glycoprotein precursors in the colon. SCOA in faeces are biomarkers of physiological processes in the organism as well as of the effect of nutritional interventions. The evaluation of the intestinal microflora activity role related to the immune response in the organism [2]; the study of bacterial fermentation of dietary complex carbohydrates by the large bowel microflora [3,4] or the evaluation of the prebiotic and probiotic properties of different nutritional additives [5,6] are some of the applications. Moreover, possibly, the most important role of short chain carboxylic acids on colonic physiology is their trophic effect on the intestinal epithelium [7]. The main SCOA in faeces are: acetate, propionate and butyrate. These acids are metabolized by the colonic epithelium (butyrate), liver (propionate) and muscle (acetate) [8]. They have been associated with reduced risk of some diseases, including the irritable bowel syndrome, inflammatory bowel disease, cardiovascular disease, and cancer [9–11].

The methods previously employed for SCOA analysis include: gas chromatography (GC) after vacuum distillation [12]; GC or ion chromatography (IC) after ultrafiltration [13] and IC with solid-phase extraction [14]. In all cases the sample pretreatment is laborious and the number of acids measured is low due to the limitations of the techniques. In GC the smaller compounds cannot be separated from the solvent peak and recoveries during derivatization are quite poor.

Recently, a rapid gas chromatographic method for determination of eight SCOAs (acetic acid, propionic acid, *n*-butyric acid, *i*-butyric acid, *n*-valeric acid, *i*-valeric acid, *n*-caproic acid and *n*-heptanoic acid) in colonic and faecal samples from rats and humans has been developed and validated. The methodology involves extraction of the SCOAs in water before a direct injection procedure on a FFAP (designation for a nitroterephthalic acid modified polyethylene glycol polymer) capillary column.

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Nevertheless, there is a general experience of easy contamination of the column and the appearance of ghost peaks with this type of methodology [15].

A headspace solid-phase microextraction procedure in which faecal SCOAs were derivatized on the fibre in-situ with 1-pyrenyldiazomethane has been reported. This method employs a BPX5 fused-silica capillary column and MS detector [16]. All the glassware, headspace vials, magnetic stirrers, must be silanised for 1 h prior to use and the process of derivatization is light-sensitive.

Regarding IC, efficiency is not good enough to obtain an adequate resolution for some of the acids.

SCOA measurement is an important challenge for several reasons: (i) they do not have a chromophore, (ii) most of them are highly hydrophilic and, therefore, difficult to extract to organic solvents to perform the necessary derivatization to be measured in GC, (iii) the derivatizated compounds are very volatile and part of them are lost during the sample treatment or are difficult to separate from the solvent peak, (iv) a huge number of very similar compounds exist and ion chromatography and HPLC do not show enough efficiency for their resolution in most cases.

Capillary electrophoresis is a green technique and a powerful tool for diagnostic applications suitable for detecting important changes in the metabolic profiles of body fluids. CE represents a high resolution separation method specially suited for aqueous samples, and it has the capability of enabling highly efficient separations of diverse components present in minute sample volumes, with minimum or none sample preparation, the main feature of the technique for the present purpose. Therefore, it is easy to include in routine analytical systems and it is clearly cost-effective. CE has been applied to the measurement of several organic acids in biological fluids [17–21].

CE has only been applied for the determination of seven SCOA in faecal cultures with indirect UV detection [22]. Often wavy baseline disturbances, as well as disturbances resembling real peaks are observed with this type of detection [23].

It is well known that most SCOA have as only chromophore the carboxylic group which shows its absorptium maxima at 200 nm, a wavelength that can be easily employed for detection in CE, working with aqueous buffers. The comparison of sensitivities for this measurement with direct and indirect detection has been a matter of discussion, but our work group has found that for complex samples, such as biological matrices, selectivity and sensitivity are higher with direct detection [18,24].

The objective of this work was to explore the capabilities of CE with direct UV detection to develop a simple, rapid and validated method for SCOA in faeces, useful for measuring large sets of samples, as well as its application in real sample analysis aiming to have reference values for further studies.

2. Materials and methods

2.1. CE apparatus and conditions

CZE was performed on a Beckman System 5500 (P/ACE, Beckman Instrument, Paloalto, USA) equipped with a UV detector set at 200 nm, a column cartridge (Beckman Coulter, Madrid, Spain) coated with polyacrylamide (PAG) 37 cm in total length, with an internal diameter (i.d.) of 50 μm. At the beginning of its use the capillary was conditioned by a pressure flush of 0.1 M HCl (1 min), BGE (10 min) and an electrokinetic flush of electrolyte with 0.5 kV/cm (10 min). In between runs, the capillary was flushed by pressure with BGE (4 min).

All the experiments were carried out at 25 °C. Sample injections were made by pressure at 0.5 psi (33 mbar) for 20 s, as indicated. The separation potential was −10 kV. Injection was done at the cathode and detection at the anode.

The electrophoretic buffer pH 6.10 was prepared with 0.234 M phosphoric acid adjusted to pH with NaOH and 12% (v/v) methanol was added. The current observed under these conditions was 70 μA.

2.2. IC apparatus and conditions

The equipment for ionic chromatography was IC Metrohm Compact-IC 761 system with conductivity detector. The ion exclusion column for determining organic acids was Metrosep Organic Acids (Metrohm, Gomensoro, Madrid, Spain) 7.8 mm × 250 mm, 10 μm particle size and was kept at room temperature. The mobile phase was previously optimized and contained 0.25 mM H_2SO_4/acetone 85:15 (v/v). The flow rate was 0.5 mL/min and the injection volume was 20 μL. The run time was 23 min.

2.3. Reagents

Formic acid sodium salt 100%, lactic acid 97%, succinic acid 99%, fumaric acid 100%, butyric acid 99%, 2-ketoglutaric acid 98%, propionic acid 99.6%, citric acid 100%, glyceric acid 99%, 2-ketoglutaric 98%, valeric acid 99% and isovaleric acid 99% were from Sigma (Alcobendas, Spain), 2-hydroxybutyric acid 95% was from Fluka (Aldrich–Sigma, Alcobendas, Spain), oxalic acid sodium salt 98% from Panreac (Madrid, Spain), acetic acid P.A. was from SDS (Peypin, France), phosphoric acid 85% was from Merck (Darmstadt, Germany), sodium hydroxide from Panreac and methanol from Scharlau (Barcelona, Spain). Buffer solutions and all dilutions were prepared with purified water by a Milli-Q-System (Millipore, Bedford, MA, USA).

2.4. Samples

Samples were kindly provided by Grupo Leche Pascual (Aranda de Duero, Spain) and corresponded to both males and females around 70 years age (range 60–80 years). The specimens were stored at −80 °C until the day of the assay. Prior to the analysis, defrosted samples (1 g) were mechanically shaken for 30 min with purified water (10 mL) and then, they were left to settle for 15 min, finally they were centrifuged at 2000 × *g* for 15 min and the clear supernatant was directly injected.

A pool was prepared with 10 randomly selected samples for method development and validation.

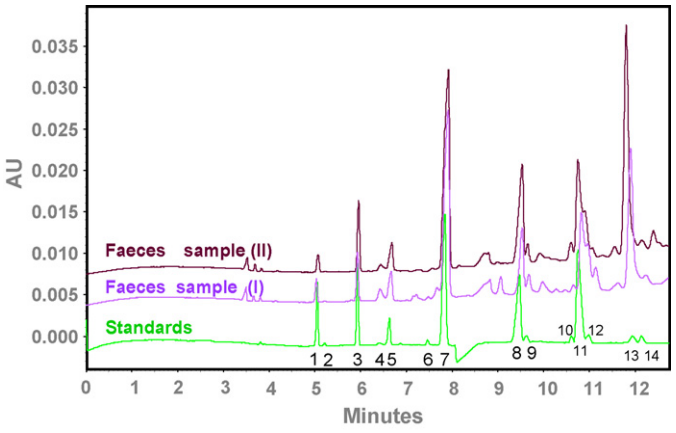

Fig. 1. Electropherograms from a mixture of standards of short chain organic acids and two different faecal extracts. Standards: 1, oxalic (200 μM); 2, formic (125 μM); 3, fumaric (40 μM); 4, 2-ketoglutaric (39 μM); 5, succinic (175 μM); 6, citric (25 μM); 7, acetic (3.25 mM); 8, propionic (1.50 mM); 9, lactic (62 μM); 10, glyceric (246 μM); 11, butyric (1.87 mM); 12, 2-hydroxybutyric (400 mM); 13, valeric (100 μM); 14, isovaleric acids (90 μM). Electrophoretic conditions: phosphate buffer 234 mM pH 6.10 plus 12% (v/v) methanol. Coated capillary. Voltage: −10 kV.

2.5. Validation study

Optimization of the separation was developed simultaneously with all the standards and in parallel with samples, and, although calibration was performed for all the tested analytes, a complete validation was performed only with a representative group of them: acetic, propionic, butyric, 2-hydroxybutyric, isovaleric and oxalic acids, due to the amount of work. Individual 20 mM stock solutions of each organic acid were prepared in purified water and stored at −20 °C; at this concentration samples can be spiked for identification without appreciable dilution. On the day of the analysis they are adequately diluted.

Selectivity was also checked by comparing the results obtained in 10 individual random samples by two orthogonal techniques: CE and IC, for those acids that could be determined with IC.

Linearity of response for standards was tested for the whole set (15 SCOAS) by assaying in triplicate five levels of concentrations, covering all the expected values ranging from 25 to 200% of medium values found in a preliminary assay. Individual ranges are described in Tables 1 and 2. Recovery was estimated by comparing the values obtained in spiked samples prepared into the linearity range, taking into account the endogenous concentrations, which had been previously measured in the samples.

Within-day precision was tested to check the constancy of both extraction and instrumental response to a given analyte. For this purpose, the assay was performed with ten calibrators and ten samples, in the mid-range of the calibration curve. Intermediate precision was tested in the same way, but on a different day, with the buffer and all the reagents freshly prepared.

Limits of quantification were calculated with Eurachem method [25], by injecting six replicates of each standard at five levels of concentration ranging from 20.0–325.0 μM for acetic acid, 9.3–150.0 μM for propionic, 11.6–187.5 μM for butyric, 7.8–125 μM for 2-hydroxybutiric, 3.9–62.5 μM for isovaleric and 4.9–39.0 μM, for oxalic acids and taking 10% as acceptance limit.

3. Results and discussion

The short chain organic acids under study were selected after a bibliographic review [4,15,16,26,27] and included those acids that in our experience appear in the profile and consisted of oxalic, formic, fumaric, ketoglutaric, succinic, citric, acetic, propionic, lactic, ketoisovaleric, glyceric, butyric, 2-hydroxybutiric, valeric and isovaleric acids. Separation was tested under the same conditions previously developed by our group [18,19,21,24,28] and was slightly modified to optimize the separation and detection of the acids under study in these

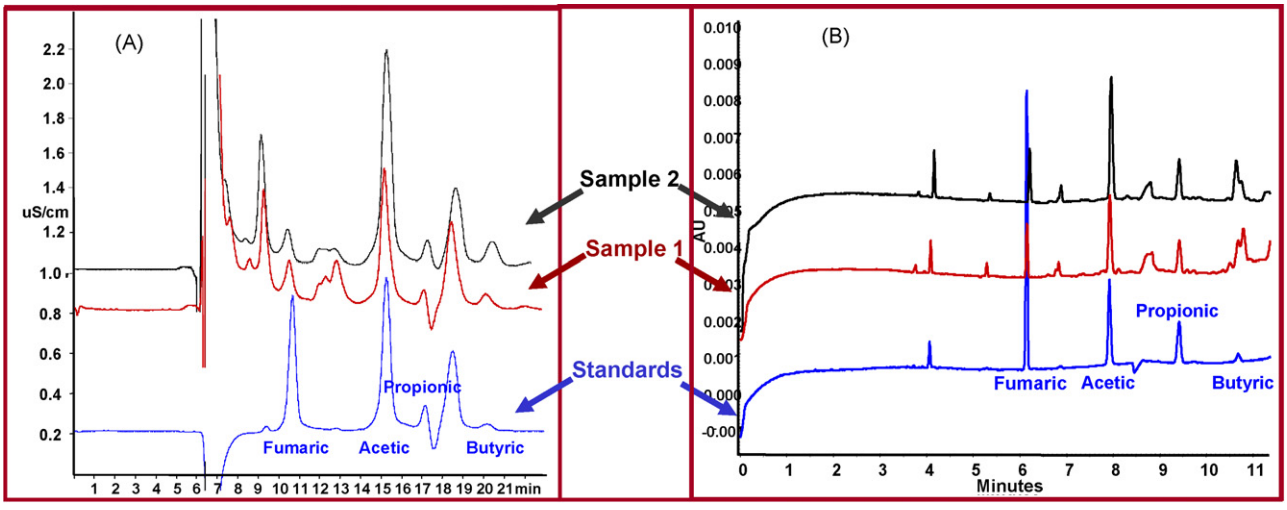

Fig. 2. Comparison of IC and CE efficiencies: (A) chromatograms of standards: fumaric, acetic, propionic and butyric acids and two faecal extract with IC method. Experimental conditions: Metrosep Organic Acids column 7.8 mm × 250 mm, mobile phase: 0.25 mM H_2SO_4/acetone 85:15 (v/v), flow rate 0.5 mL/min. (B) The same standards and samples analysed with CE method. Electrophoretic conditions: phosphate buffer 234 mM pH 6.10 plus 12% (v/v) methanol. Coated capillary. Voltage: −10 kV.

Table 1
Validation parameters obtained for acetic, propionic, butyric, 2-hydroxybutiric, isovaleric and oxalic acids

	Acids					
	Acetic	Propionic	Butyric	2-Hydroxy butyric	Isovaleric	Oxalic
Standards linearity						
Intercept	−250.2 ± 5041.7	−1428.5 ± 4069.2	1760.4 ± 4593.1	−336.5 ± 632.0	−76.95 ± 456.65	−379.6 ± 482.5
Slope	15674.3 ± 487.3	32129.1 ± 1229.3	36736.4 ± 1126.9	17405.3 ± 523.1	48861.5 ± 1302.6	30578.3 ± 1239.9
r	0.999	0.998	0.999	0.999	0.999	0.997
Range (mM)	1.96–15.66	0.78–6.23	0.96–7.67	0.25–2.00	0.07–0.58	0.08–0.64
Recovery	98.3 ± 1.7	94.9 ± 1.4	103.0 ± 3.0	104.8 ± 6.3	106.6 ± 2.3	104.3 ± 1.5
Instrumental precision						
Repeatability ($N = 10$) (%)	1.2	1.3	1.4	1.1	0.5	1.6
Intermediate ($N = 20$) (%)	1.1	1.2	1.1	2.5	1.7	3.5
Method precision for samples						
Repeatability ($N = 7$) (%)	9.4	9.5	8.2	5.5	7.7	5.7
Intermediate ($N = 14$) (%)	10.8	13.0	8.1	9.1	8.8	14.5
LOQ (μM)	35.6	16.1	30.6	49.6	18.2	10.4

Table 2
Linearity equations for: formic, 2-ketoglutaric, fumaric, valeric, succinic, lactic, citric, glyceric acids

Acids	Formic	2-Ketoglutaric	Fumaric	Valeric	Succinic	Lactic	Citric	Glyceric
Intercept	−58.7 ± 60.9	−239.4 ± 135.8	−1146 ± 1608	137.6 ± 327.5	−705.2 ± 698.2	840.5 ± 294.6	−133.6 ± 130.7	−212.7 ± 359.0
Slope	4990 ± 134	82945 ± 2901	623675 ± 18243	49301 ± 1594	47010 ± 1654	29894 ± 1940	101129 ± 2131	40174 ± 584
r	0.999	0.998	0.999	0.999	0.998	0.995	0.999	0.999
Range (mM)	0.09–0.74	0.009–0.076	0.02–0.17	0.05–0.40	0.08–0.70	0.03–0.25	0.01–0.10	0.1–1.0

Table 3
CE analysis of SCOAs in 136 adult people

SCOA mean ± S.D.	CE method (μmol/g) wet faeces ($n = 30$)	GC mmol kg^{-1} wet faeces ($n = 8$) [27]	GC-headspace μmol/g dry faeces (n unknown) [16]
Acetic	49.5 ± 1.6	59.3 ± 6.2	48.96
Butyric	19.9 ± 0.9	14.6 ± 5.2	8.0
Propionic	16.5 ± 0.6	13.1 ± 4.9	17.71
Oxalic	0.56 ± 0.03		
Isovaleric	2.4 ± 0.3	1.1 ± 0.2	3.28
Formic	0.40 ± 0.02		
2-Ketoglutaric	0.33 ± 0.02		
Succinic	1.9 ± 0.1		
Lactic	1.2 ± 0.2		
Fumaric	0.33 ± 0.02		
Citric	0.16 ± 0.02		
Glyceric	0.40 ± 0.02		
2-Hydroxybutiric	15.4 ± 0.7		

Results expressed as the mean with the standard deviation. Comparison with other previously reported results.

specific samples. Buffer concentration was assayed from 0.200 to 0.234 M, MetOH percentage from 10 to 15% and injection time from 5 to 20 s. Final conditions were those described under experimental conditions and were chosen taking into consideration mainly the simultaneous resolution between critical pairs such as lactic and propionic, butyric and 2-hydroxybutiric and citric and acetic.

Identification was performed by migration time as compared with pure standards and by spiking. Moreover, this operation can be done automatically by co-injection in the equipment. Fig. 1 shows the short organic acids identified as present in two different human faecal extracts.

3.1. Validation results

A summary of the validation parameters for the selected acids is shown in Table 1, including both standards and samples and will be briefly discussed.

Selectivity is always a problematic issue in this type of analysis because the analytes lack of a characteristic chromophore and coelutions should be discarded in some way. Firstly the high efficiency of CE separation permits the discarding of closely eluting compounds during the identification step because the spiking produces peak distortion. Secondly, during our assay, IC was employed as orthogonal technique to compare the results obtained for some of the acids, as not all of them could be separated with this technique. Fig. 2 shows the highest efficiency obtained with the CE method as compared to IC during the analysis of the same solution of standards and two different faecal extracts. The mean and standard error obtained with both methods for propionic, acetic and butyric acids after measuring 18 individual samples were compared and statistically tested in Fig. 3. The paired t-test showed no significant differences

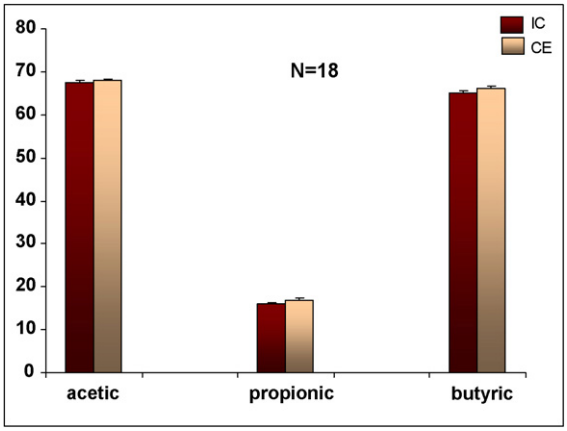

Fig. 3. Accuracy study: analysis of acetic, propionic and butyric acids in 18 different faecal samples with IC and CE methods. (A) Histogram shows calculated concentrations, mean and standard error, for acetic, propionic and butyric acids. (B) Paired t-test ($n = 18$).

	Texp (95% two tails)	p value
Acetic	0.22	0.829
Propionic	1.72	0.103
Butyric	0.48	0.635
	t tab: 2.11	

Paired t-test ($n = 18$).

between the results obtained with the two methods for these acids.

Regarding the validation parameters obtained, the standards fit the linear model ($r > 0.99$) for all of the organic acids and no bias was found, because the confidence limits of the intercept include the zero value. Table 2 includes the results for the corresponding equations in those acids that were not submitted to full validation. Recoveries ranged from 94.9 to 106.6% and taking into account their R.S.Ds., they do not differ statistically from 100% ($p < 0.05$). Intra-assay precision ranged from 0.5 to 1.6% for standards ($n = 10$) and inter-assay precision from 1.1 to 3.5% ($n = 20$), being the lowest values for oxalic acid due to the low level of concentration tested. When seven samples prepared from the same pool were run in the same assay, the daily R.S.Ds. ranged from 5.5 to 9.5% and from 8.1 to 14.5% in different days. Theoretical limits of quantification calculated by the Eurachem method for these acids ranged from 10.4 to 49.6 μM, low enough considering the levels in samples and similar to others previously published [15].

3.2. Results in a Mediterranean population

Finally the method was applied to 136 samples corresponding to people both male and female aged 60–80 and living in Spain. Results are summarised in Table 3 and those that could be compared, agree with previously published values obtained with different methods [16,27]. Moreover, various population survey data have shown that faecal SCFA production is in the order: acetate > propionate ≥ butyrate in a molar ratio of approximately 60:20:20 [26], similar to what has been shown here.

4. Conclusion

A rapid and simple method has been developed and validated for SCOA analysis in faeces that requires only aqueous extraction, and direct injection in CE. Validation parameters are similar to others previously published, but sample treatment is easier than most of them and it also permits the detection of fifteen total acids, including formic acid, which cannot be detected with other methods without gross errors. This development, due to its rapidity and efficiency, can be especially useful to test potential benefits of prebiotics and probiotics on populations, where a large number of samples need to be measured in order to obtain statistical significance.

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