Application of Convective Interaction Media (CIM®) Disk Monolithic Columns for Fast Separation and Monitoring of Organic Acids

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Abstract

The separation of organic acids on the anion-exchange monolithic support, commercially available as Convective Interaction Media (CIM), is presented in this study. It is demonstrated that citric, isocitric, pyruvic, fumaric, malic, and α-ketoglutaric acid can be successfully separated using a CIM monolithic column of suitable user-adjustable length. The effect of the mobile phase composition on the separation is investigated. CIM monolithic columns of adjustable length from 3 to 18 mm are compared regarding the resolution and the back pressure. It is shown that the CIM monolithic column of 12 mm in length enables a good separation of all six organic acids within 3 min and exhibits a linear dependence of back pressure versus flow rate. The resolution and the dynamic binding capacity are found to be flow-unaffected. A filtrated sample of bioprocess supernatant is analyzed without previous pretreatment, which indicates the possibility of online monitoring of small molecules during the bioprocess using CIM monolithic columns.

Introduction

Organic acids are the important metabolites of several biochemical pathways in microorganisms and as a result they are frequently the main product or byproducts in different bioprocesses. One of organic acid's microbial producers is yeast *(Yarrowia lipolytica)*, whose bioprocess represents an alternative to *Aspergillus niger* cultivation for citric acid production on an industrial scale (1).

The production of citric acid involving *Y. lipolytica* is accompanied by the secretion of smaller amounts of several other organic acids. From the point of view of a high production of citric acid, the formation of these byproducts should be minimized. However, to understand the regulatory mechanisms governing the production of organic acids and consequently to optimize organic acid production as described above as well as in all similar bioprocesses, a rapid and simple method for monitoring these compounds is required.

One of the most commonly used methods for organic acid determination is high-performance liquid chromatography (HPLC). By this method, carboxylic acids are separated usually

Table I. Average Resolution Factors of Organic Acids ($\Sigma R_{n-n+1}/5$) and Retention Times of the Last Eluted Peak (t_r) Obtained at Different Phosphate Buffer (c_{buffer}) and NaCl (c_{NaCl}) Concentrations in 20mM Phosphate Buffer on a CIM Monolithic Column of 12-mm length at 5-mL/min Flow Rate

c_{buffer} (mM)	$\Sigma R_{n-n+1}/5$	t_r (min)	c_{NaCl} (mM)	$\Sigma R_{n-n+1}/5$	t_r (min)
500	0.53 (4*)	6.98	400	0.09 (3)	0.51
400	0.65 (4)	7.01	300	0.23 (4)	0.73
300	0.68 (4)	7.96	200	0.89 (5)	1.91
200	0.79 (4)	10.28	150	1.71 (6)	4.29
150	1.06 (5)	13.11	130	2.09 (6)	6.18
100	1.47 (5)	24.15	50	3.62 (6)	47.78

* Number of separated peaks.

Table II. Z Values and R^2 Obtained from Linear Regression Analysis of Organic Acid Retention Data, HETP Values for Organic Acids, and Values of Dynamic Binding Capacity for Organic Acids at Two Different Mobile Phase Flow Rates

Organic acid	Z factor (R^2)	HETP (μm)	Capacity (mg of organic acid per millileter) 5 mL/min	10 mL/min
Pyruvic acid (y = 1)*	1.01 (0.99)	48	1.0	1.0
Malic acid (y = 2)	1.88 (0.99)	12	2.9	3.0
Fumaric acid (y = 2)	1.96 (0.99)	19	6.5	6.8
α-Ketoglutaric acid (y = 2)	1.94 (0.99)	26	7.5	7.6
Citric acid (y = 3)	3.04 (0.99)	26	10.2	10.4
Isocitric acid (y = 3)	3.07 (0.99)	31	12.7	13.2

* Valency of the acidic anion.

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by three different separation mechanisms such as anion-exchange, ion-exclusion, or reversed-phase chromatography in which the separations are generally performed under isocratic flow conditions. In order to achieve satisfactory separation of different organic acid molecules, HPLC columns in length of 10 cm or more are typically used.

In the last decade, several new chromatographic supports have been developed. One group (so called monoliths) exhibits several advantages over conventional chromatographic columns that are filled with porous particles. The main difference is in the exchanging process between the mobile and stationary phase. In the case of the monolithic macroporous matrix, this process is not limited by pore diffusion described thoroughly for conventional chromatographic material. The absence of the pore diffusion enables very short times for the separation process (2–4); flow-unaffected resolution and dynamic binding capacity are advantages, also (5,6). Among the various monoliths, glycidyl metha- crylate-ethylene dimethacrylate (GMA-EDMA) monolithic macroporous media exhibit high mechanical and chemical stability (7) as well as low shrinking and swelling in different solvents or in the buffers of different ionic strengths (8).

Besides the GMA-EDMA monolithic rods that are described extensively in literature (3,9), small-volume disk-shaped and large-volume tube-shaped GMA-EDMA monoliths are commercially available under the trademark Convective Interaction Media (CIM). Bearing different active groups, CIM monolithic columns are used for separation mechanisms such as ion-exchange, affinity, or hydrophobic interaction and are successfully applied for several separations of proteins (2,10–13). Actually, because their typical separation layer thickness is in a range of a few millimeters, the use of CIM monolithic columns were until the present time considered to be limited to the gradient separations of protein macromolecules.

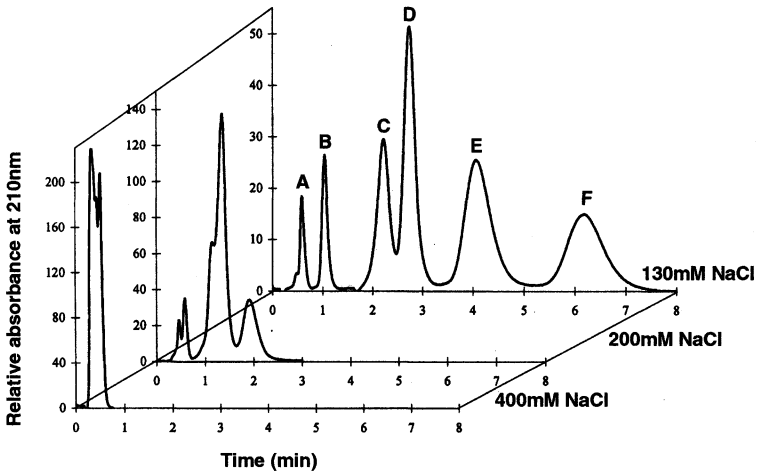

Figure 1. The effect of the mobile phase composition (different NaCl concentrations in 20mM phosphate buffer) on the resolution of the organic acid isocratic separation on a QA CIM column of 12-mm length: pyruvic acid (0.03-g/L sample), A; malic acid (0.5-g/L sample), B; α-ketoglutaric acid (0.2-g/L sample), C; fumaric acid (0.007-g/L sample), D; citric acid (2-g/L sample), E; and isocitric acid (2-g/L sample), F. Conditions: 400mM, 200mM, and 130mM NaCl in 20mM phosphate buffer mobile phases; 5-mL/min flow rate.

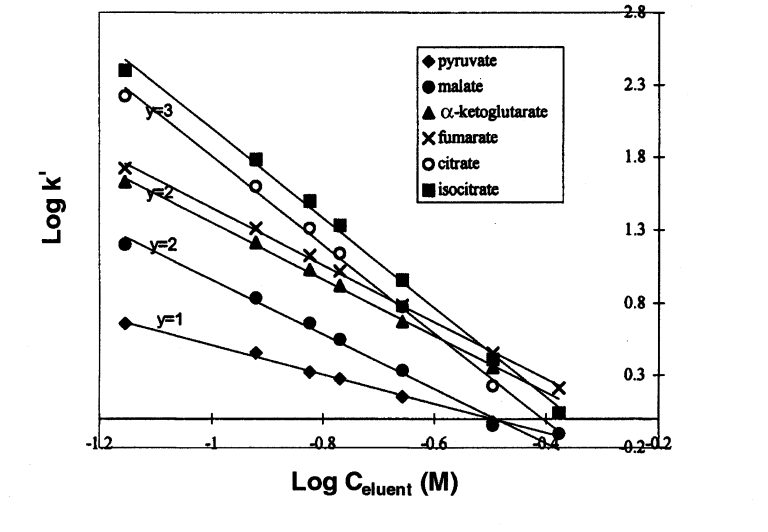

Figure 2. The effect of eluent concentration on the retention behavior of monovalent (y = 1), divalent (y = 2), and trivalent (y = 3) acidic ions.

Recently, it has been shown that the same CIM disk monolithic columns can also be used for separations under isocratic flow conditions. The isocratic separations of plasmid DNA conformers (14), oligonucleotides (15,16), and peptides (16) in the ion-exchange mode have been demonstrated, and the isocratic reversed-phase separation of steroid mixtures have been obtained (16). These results indicate the possibility of also applying CIM monoliths for the isocratic separation of several other small-charged molecules. Because the average analysis time using CIM disk monolithic columns is approximately 1–3 min, these supports can be a method of choice for the separation of organic acids intended for in-process control.

In this study, a simple method for the fast separation and monitoring of organic acids at room temperature is developed. Strong anion-exchange CIM quaternary amine (QA) disks commonly applied for efficient gradient separation of proteins were used, forming length-adjustable CIM monolithic columns. The effects of the mobile phase composition, flow rate, and column length on the quality of the separation were studied.

Experimental

Equipment

An isocratic HPLC system included one HPLC Pump 64, an injection valve with a 20-µL sample loop, a variable wavelength ultraviolet–visible detector with a 10-µL volume flowcell (0.1-s response time), and a data-processing system. All of these instruments were obtained from Knauer (Berlin, Germany). All separations were carried

out at room temperature. The wavelength in all measurements was set to 210 nm. The flow rate was monitored by a validated digital flowmeter (K-3773, Phase Separation, UK).

Separation unit

Separations were performed on length-adjustable anion-exchange CIM QA monolithic columns comprising of 1 to 6 CIM disks, all from BIA Separations (Ljubljana, Slovenia). The CIM disks were macroporous GMA-EDMA monolithic polymers bearing strong anion-exchange active groups (CIM QA disks). The diameter of a single CIM disk was 12 mm with a thickness of 3 mm. The length of the CIM monolithic column was varied by merging a different number of CIM disks in a single CIM housing.

Mobile phase

Double-deionized water purified with a Milli-Q system (Millipore, Bedford, MA) was used throughout. All chemicals were of analytical reagent grade. K_2HPO_4 and KH_2PO_4 were purchased from Kemika (Zagreb, Croatia) and NaCl from Fluka (Buchs, Switzerland). The mobile phase was prepared by dissolving a required amount of salt in the distilled water and adjusting the pH value to pH 8.0 with HCl.

Sample

Synthetic sample

Citric, isocitric (three-sodium salt), pyruvic (sodium salt), and fumaric acid were supplied by Sigma (St. Louis, MO), and α-ketoglutaric (di-sodium salt) and malic acid were purchased from Fluka. All organic acid standards were of analytical reagent grade. They were dissolved in a mobile phase, and afterwards, pH values were adjusted to pH 8.0.

Bioprocess sample

A sample of *Y. lipolytica* fermentation broth that is described in detail elsewhere (17) was filtered through the 0.45-µm filter before injection.

Results and Discussion

The organic acids that were commonly secreted into the cultivation broth during the *Y. lipolytica* bioprocess were pyruvic acid, citric acid (as the main product), and several other acidic metabolites of the Tricarboxylic acid (TCA)-cycle such as malic, fumaric, α-ketoglutaric, and isocitric acid (18). To obtain a complete picture of the organic acid composition in the cultivation broth, the possibility of separating all of the stated organic acids was studied with the intention of achieving optimal separation conditions regarding the mobile phase composition, thickness of the separation material, and mobile phase flow rate. In regards to the criteria for a quality separation, the average peak resolution (sum of all peak resolutions divided by the number of possible resolution values, which is 5) and analysis time were chosen. A resolution value greater than 2.0 was considered as satisfactory.

Effect of the mobile phase composition

The main difference between CIM and classical chromato-

Table III. Average Resolution Factors of Organic Acids ($\Sigma R_{n-n+1}/5$) and Retention Times of the Last Eluted Peak (t_r) Obtained at Different CIM Column Lengths (L) at a 5-mL/min Flow Rate and at Different Mobile Phase Flow Rates on a CIM Monolithic Column of 12-mm Length

L (mm)	$\Sigma R_{n-n+1}/5$	t_r (min)	Flow rate (mL/min)	$\Sigma R_{n-n+1}/5$	t_r (min)
3	0.57 (5)*	1.1	5.24	2.08	6.2
6	0.83 (6)	2.51	7.58	2.0	4.33
9	1.45 (6)	4.37	10.3	1.87	3.24
12	2.09 (6)	6.18	15.3	1.57	2.19
15	2.15 (6)	8.69			
18	2.26 (6)	10.61			

* Number of separated peaks.

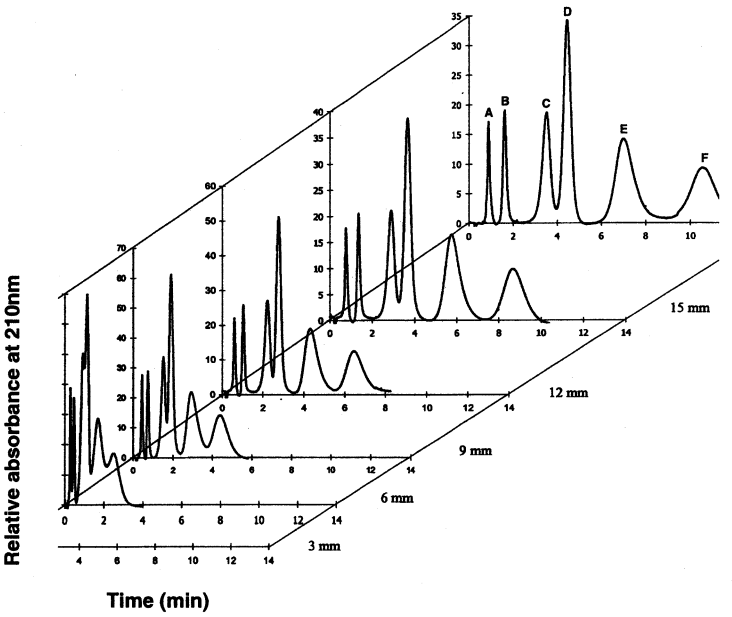

Figure 3. The effect of the monolith thickness (number of CIM disks in the CIM housing) on the resolution of the organic acid isocratic separation: pyruvic acid (0.03-g/L sample), A; malic acid (0.5-g/L sample), B; α-ketoglutaric acid (0.2-g/L sample), C; fumaric acid (0.007-g/L sample), D; citric acid (2-g/L sample), E; and isocitric acid (2-g/L sample), F. Conditions: 130mM NaCl in 20mM phosphate buffer mobile phase, CIM disk monolithic column separation unit comprising of 1–6 QA CIM disks; 5-mL/min flow rate.

graphic support is that of a hydrodynamic nature; the surface chemistry is equal for both. Therefore, a starting method for organic acid separation was taken from literature (19) using phosphate buffer as a mobile phase. In Table I, the equated resolutions and retention times of the final eluted peak at different phosphate buffer compositions are presented. By changing the mobile phase concentration from higher to lower values, resolution improved. However, this prolonged the analysis time. Still, even at the lowest buffer concentration of 100mM, we were not able to separate all six organic acids when having the analysis time close to 24 min at a mobile phase flow rate of 5 mL/min.

To obtain improvement in the resolution, a major part of the phosphate buffer was replaced with NaCl. In Figure 1, chromatograms of organic acid separations at three different NaCl concentrations (400mM, 200mM, and 130mM) in 20mM phosphate buffer are presented. From these chromatograms as well as from Table I, it is evident that lowering the NaCl concentration has the same effect on the resolution and analysis time as described for pure phosphate buffer. However, in this case, the resolution drastically improved reaching the resolution value of 3.62 at the displacer salt concentration of 50mM NaCl. Because the analysis time exceeded 45 min at a flow rate of 5 mL/min, we chose the mobile phase concentration of 130mM NaCl in 20mM phosphate buffer as an optimal mobile phase having the analysis time at approximately 8 min (Figure 1). This mobile phase was used for all further organic acid separations.

To improve the understanding of organic acid retention properties at different NaCl concentrations, we calculated the capacity factors (k') of the carboxylate analytes and correlated the log k' values as the function of log C_{eluent} (Figure 2). As can already be concluded from Figure 1, changes in the eluent concentration had a significant effect on the analyte retention characteristics. Increasing the NaCl concentration led to decreased capacity factors. However, the capacity factors decreased differently for each group of acidic anions with certain valencies resulting in an intersection of some lines. Consequently, as it has already been described in literature (20), by changing the eluent concentration, the elution order of organic acids could be reversed. However, in the eluent concentration range in which the separation of all six organic acids was achieved, the elution order did not change.

For the further understanding of retention behavior, Z factors were determined from the slopes of the plots shown in Figure 2. From Table II, it is evident that Z factors increased with the increasing valency of the acidic anions. Z factors for tricarboxylate citrate and isocitrate were higher than Z factors for dicarboxylate malate, α-ketoglutarate, and fumarate and higher than the Z factor for monocarboxylate pyruvate anions, which had the lowest value. This is sensible because at the described elution conditions, all organic acids were fully dissociated. Thus, with the increase of acidic anion valency, the number of binding sites also increased and as a consequence more eluent was needed to solvate the anions. Moreover, Z factors were practically equal to the valency of the acidic anions for the organic acids of all three valencies that were obtained together with high correlation coefficients (R^2) for the linear regression analysis of data in agreement with a dominant ion-exchange chromatography (IEC) elution mode. From these results, it can be concluded that the retention was mainly the consequence of IEC effects (21).

To compare the efficiency of CIM monolithic columns with the efficiency of other columns used for organic acid separations, high equivalent to the theoretical plate (HETP) values for all six organic acids were estimated (Table II). Thus, the HETP value of malic acid for a CIM disk column was 12 µm, which was approximately twice less than the HETP value of the same acid for Aminex column HPX-87H, estimated to be 25 µm (22). As a result, CIM columns had higher efficiency providing the possibility of performing separations on shorter separation layers.

Table IV. Parameters of Linearity and Limit of Detection for the Separation of Pyruvic, Malic, α-Ketoglutaric, Fumaric, Citric, and Isocitric Acid on CIM Monolithic Columns of 12-mm Lengths

Organic acid	Limit of Detection (g/L)	Upper linear concentration value (g/L)	R^2	Slope	Intercept
Pyruvic acid	0.0005	4	0.9997	49.987	-3.0046
Malic acid	0.0076	6	0.9984	3.6872	0.2037
Fumaric acid	0.00047	0.2	0.9998	558.63	-0.2519
α-Ketoglutaric acid	0.0031	4	0.9999	39.33	0.2112
Citric acid	0.0313	15	0.9997	3.8999	0.3142
Isocitric acid	0.0305	20	0.9998	3.3749	-0.0167

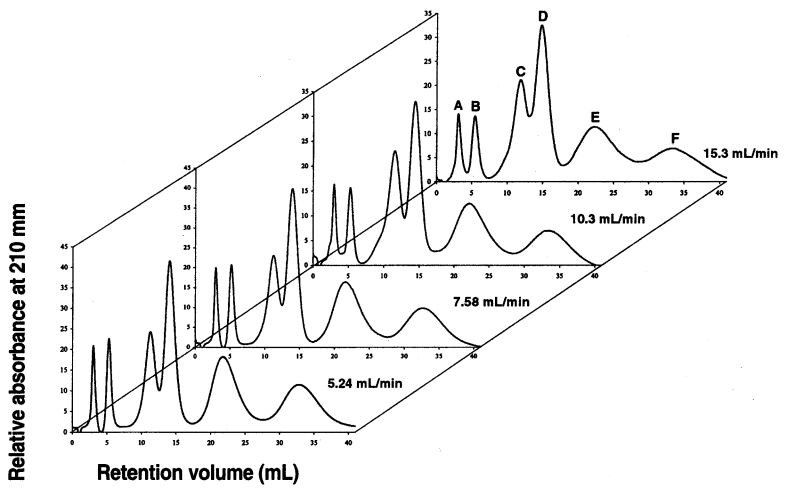

Figure 4. The effect of flow rate on the organic acid isocratic separation on the QA CIM monolithic column of 12-mm length: pyruvic acid (0.03-g/L sample), A; malic acid (0.5-g/L sample), B; α-ketoglutaric acid (0.2-g/L sample), C; fumaric acid (0.007-g/L sample), D; citric acid (2-g/L sample), E; and isocitric acid (2-g/L sample), F. Conditions: 130mM NaCl in 20mM phosphate buffer mobile phase; 5.24-, 7.58-, 10.3-, and 15.3-mL/min flow rates.

Effect of the thickness of the separation layer

The length of the column is another parameter that affects the peak resolution in the isocratic mode, because a longer length of the separation material increases the number of theoretical plates. CIM monolithic columns offer a unique possibility to vary a column length by placing different numbers of the CIM disks into a single CIM housing. This can be done by the user simply by opening a CIM housing and inserting the desired number of CIM disks. In this way, the total column length is a sum of the number of CIM disks placed in the CIM housing multiplied by the thickness of the CIM disk. It has already been shown in the case of isocratic separation of oligonucleotides that separation on the CIM monolithic column is improved significantly by increasing its length, which exhibits no distortion of the peak shape or resolution because of a discontinued bed (15). Chromatograms in Figure 3 show the same tendency also in the case of organic acid separation. The average resolutions equated from these chromatograms are shown in Table III.

The CIM monolithic column of length 3 mm (a single CIM disk) was not able to separate all six acids. Its average resolution was only 0.57. By placing two CIM disks in the CIM housing, a CIM monolithic column of 6 mm length was obtained, and it can be further extended to the length of 18 mm using 6 CIM disks in a single CIM housing. The resolution increased each time (reaching the maximal value of 2.26). Of course, a longer separation layer resulted in a longer analysis time (Table III) and higher back pressure. The relationship between the analysis time and separation layer thickness was linear with a correlation coefficient of 0.9935. Furthermore, it was also found that the back pressure changed linearly with the CIM column length resulting in a correlation coefficient of 0.9913. However, even at the longest CIM monolithic column, the back pressure was still moderate (reaching only 1.4 MPa at the flow rate of 5 mL/min).

Effect of the flow rate

It has been shown that in contrast to the conventional particle chromatographic material, the resolution achieved in CIM monoliths is not affected by the flow rate of the mobile phase neither in a gradient (8) nor isocratic mode (15). In contrast to the previous studies carried out on a single CIM disk, the CIM disk monolithic column of 12 mm (built up of four CIM disks) was used to investigate the effect of the flow rate on the isocratic separation of organic acids. Data in Table III exhibit a decreasing of the average resolution with increasing flow rate; although at the highest mobile phase flow rate of 15.3 mL/min, the average resolution was still above 1.5. However, this tendency in the resolution proved to be in a high degree accountable to the HPLC system itself. Namely, data-recording time intervals of the system were too large, which at high flow rates apparently causes a decrease of peak heights while the peaks in all cases still remain well-separated. This can be seen in Figure 4 in which the comparison of the chromatograms normalized on the elution volume obtained at 5.24, 7.58, 10.3, and 15.3 mL/min is presented.

The main advantage of working at high flow rates is certainly a shorter analysis time, which means great time savings in eventual downstream processes. As it is evident from Table III, the retention time of the last peak was shortened from approximately 6 min to approximately 2 min when the flow rate was increased from 5 to 15 mL/min. However, the applicability of chromatographic material for the purification of molecules on a preparative scale is largely conditioned by a high binding capacity of the material. Because dynamic binding capacities for CIM monolithic columns that are measured for proteins reach relatively high values (25–30 mg of protein per milliliter) (5), we examined them also for organic acids. As evident from Table II, dynamic binding capacities are dependent on the specific organic acid employed and increase with the valency of the organic acid (ranging between 1 and 13 mg of organic acid per milliliter). Furthermore, measuring the dynamic capacities at two different mobile phase flow rates showed that they were unaffected by the flow rate, which was in contrast to the dynamic capacities of conventional chromatographic materials. However, the flow-unaffected binding capacity characteristic of CIM monolithic columns has already been presented in the case of proteins (5,8). This important feature of CIM material enables the performance of organic acid separations at higher flow rates without loosing binding capacity and quality of separation, thus opening the possibility of the application of CIM monoliths for the eventual purification of organic acids from cultivation broths.

However, when working at high flow rates, the limiting factor is the back pressure of the CIM column. Similar to the linear relation between the flow rate and the back pressure found for a single CIM disk monolithic column (8), the same relationship was found for a CIM disk monolithic column containing several CIM disks. Thus, this feature seems to be common for all CIM

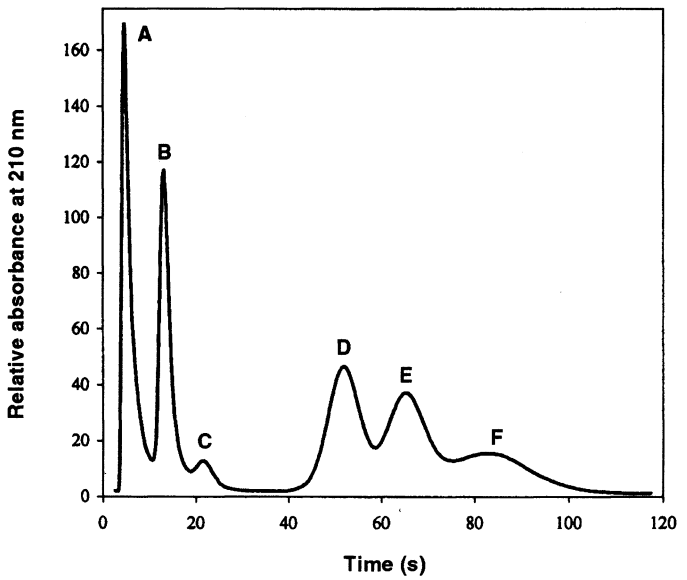

Figure 5. Analysis of organic acids in the *Y. lipolytica* cultivation broth using the QA CIM monolithic column of 12-mm length: unidentified peak, A; pyruvic acid, B; malic acid, C; α-ketoglutaric acid, D; fumaric acid, E; and citric acid, F. Conditions: 130mM NaCl in 20mM phosphate buffer mobile phase; 17-mL/min flow rate.

disk monolithic columns. Therefore, one can easily estimate the characteristics of a particular CIM disk monolithic column that facilitate its application.

Detection of organic acids in cultivation broth

The quantities of organic acids that accumulate in a *Y. lipolytica* cultivation broth are different; besides, they depend on the composition of the cultivation medium as well as cultivation conditions. Citric acid as the main product accumulates in the cultivation broth in the highest concentration (which is approximately 10 g/L at the end of the batch process), whereas concentrations of other organic acids are only a few grams per liter (23). To examine the possibility of the application of the method using the QA CIM column for organic acid detection in the concentration ranges that appear in the real samples, we determined the range of linearity as well as the limit of detection for each acid.

In determining the linearity of the method, different concentrations of organic acids were injected. The parameters of the calibration curves for each acid are summarized in Table IV. The high values for R^2 showed good method linearity. Typically, the linear range is of three orders of magnitude and is of course dependent on the injection loop volume. The detection limit was considered as the concentration at which the signal-to-noise ratio of more than 10 was obtained. Because in this case a loop of 20 µL was used, a detection limit could be further lowered using a larger volume injection loop. However, when samples of high organic acid concentrations are to be analyzed, the samples should be diluted before analysis in order to fall in the linear range of the method.

To verify the method, we investigated the efficiency of the CIM QA support when real samples were applied. The sample of *Y. lipolytica* bioprocess broth was used. It is important to emphasize that no dialysis or any other pretreatment of the sample except a filtration was performed. This approach has already been successfully used for the analysis of lignin peroxidase isoforms (24). The feature is supposedly because of the high group density, which results in a significant capacity in the presence of salt (5). In Figure 5, the separation of the TCA-cycle acids and pyruvic acid in the broth using a QA CIM disk monolithic column of 12 mm length at a flow rate of 17 mL/min is presented. As can be concluded from the chromatogram, no isocitric acid was present in the sample. The first unretained peak was a sum of the broth components that did not bind to the anion-exchange material and did not interfere with the analysis. The whole separation was performed in 2 min. Even at so high of a flow rate, the back pressure of the complete HPLC system was only 10 MPa. Therefore, it can be concluded that CIM chromatography can be a method of choice for the separation of organic acids whenever fast analysis without sample pretreatment is required.

Conclusion

CIM ion-exchange monolithic columns demonstrated to be an efficient medium for the fast separation of large molecules (and recently for smaller molecules such as peptides and oligonucleotides, also). A successful separation of organic acids further enlarges this set to even smaller highly charged molecules. The unique possibility to adopt a CIM disk monolithic column length by a user to match particular system requirements makes these supports attractive for many different applications. This feature (combined with a relatively low back pressure and a practically flow-unaffected resolution and dynamic binding capacity) offers more possibilities for a method optimization in regards to the separation and speed. However, the possibility of analysis directly from fermentation broth without a particular pretreatment indicates the possible application of CIM columns as a multipurpose sensor with a short response time.

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Manuscript accepted June 29, 2000.