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AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD WITH COLUMN SWITCHING FOR THE DETERMINATION OF NEUROTRANSMITTERS AND RELATED COMPOUNDS, ASCORBIC ACID AND URIC ACID IN TISSUE EXTRACTS

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SUMMARY

An automated high-performance liquid chromatographic method with electrochemical and fluorimetric detection and on-line data evaluation is described for the simultaneous measurement of indoleaminergic and catecholaminergic neurotransmitters, some of their metabolites and precursors and ascorbic and uric acids. Deproteinized tissue extracts from the central nervous system or peripheral organs are injected without prior purification (recovery > 90%). A switching system enables the compounds to be passed as necessary through one, two or three reversed-phase columns, which are then eluted simultaneously (analysis time 25 min). Fifty samples per day can be analysed with a precision of 95% for neurotransmitters and about 90% for ascorbic and uric acids.

INTRODUCTION

In a previous paper [1] we described a method for the determination of catecholamine and indoleamine neurotransmitters and some of their precursors and metabolites using two reversed-phase high-performance liquid chromatographic (HPLC) systems. Whereas the indoleamines could be quantified by direct injection of a tissue extract, the catecholamines had first to be isolated by the alumina adsorption method. This meant that at least 10 mg of tissue were necessary for a complete analysis.

In the course of our work, we needed to be able to investigate neurotransmitter systems in smaller discrete areas of the central nervous system (CNS) (e.g., micropunches) in a single chromatographic run with direct injection of

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extracts. It is difficult to develop a method capable of doing this, because the various compounds have widely differing retentions on reversed-phase columns. In addition, with electrochemical detection (ED), which is at present the most specific and sensitive method for certain biological amines, most tissue extracts produce a very large initial peak, which interferes greatly with quantification of the more polar compounds, unless the analysis time is excessively long. Thus, with most of the simpler systems only a limited number of selected metabolites or precursors can be determined. Literature up to 1983 can be found in our previous paper, and has been summarized by Lasley et al. [2]. More recent publications have offered the following solutions to this problem:

(1) Gradient elution [3, 4]. This method not only requires a gradient mixer but also is not fully compatible with ED. The use of dual electrodes does not seem to eliminate completely the effect of the gradient on the baseline [4]. A recent paper [5] describes a gradient method using alkyl boronates as catechol-specific mobile phase pairing agents and ED. This seems to give good results, as the concentration of organic modifier (methanol) does not rise above 20%.

(2) More efficient separation using columns with 3-μm particles [6] or microbore columns (1 mm I.D.) [7]. The use of these columns needs experience and attention to technical details, and a great disadvantage is that the large initial peak is not eliminated and more or less overlaps with the peaks of compounds with short retention times.

(3) Use of column switching. This method has been applied by a number of workers [8—11] to systems of varying complexity. It seems that column switching can be of advantage in the HPLC of biological materials.

In our earlier method for indoleamines we already used a switching device to prevent overloading of the electrochemical detector caused by the initial peak. We therefore decided to modify this method, using column switching, in order to achieve the separations which we required.

Preliminary experiments showed that problems arise when attempts are made to combine column switching with ED, particularly in stabilization of the baseline. Certain conditions must be fulfilled if a workable system is to be obtained (the ionic strength and pH of the buffers running through each electrochemical detector must be constant; pressure changes must be minimized). Taking these factors into account, we developed a method based on the following "cascade" principle: the first cut from any column (containing slightly retained compounds) is loaded by a switching device on to another column. Subsequently, the long-retained compounds are resolved on the original column and the second column is eluted simultaneously with the same or a different buffer (within the limits mentioned above) to resolve the compounds with low retention times. Norepinephrine (NE), epinephrine (E), dopamine (DA), serotonin (5-HT), tyrosine (Tyr), tryptophan (Trp), 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA) and 3-methoxy-4-hydroxyphenylethylene glycol (MHPG) could be determined in a two-column system. At a later stage we also wanted to determine ascorbic acid (Asc), and uric acid (Ur) as a blood—brain barrier marker, so we added a third unit (pump, column and detector) to the system. Thus it is possible to add or delete separation units according to the analytical problem that has to be solved.

EXPERIMENTAL

Materials

The materials were as described in our previous paper [1]. In addition, MHPG piperazine salt was obtained from Calbiochem (La Jolla, CA, U.S.A.) and α-methyltryptophan (α-meTrp) from Sigma (St. Louis, MO, U.S.A.).

Chromatographic system

The apparatus and connections used are shown in Fig. 1. The chromatographic system for the separation of indoleamines, consisting of a refrigerated autosampler (WISP 710B; Waters Assoc., Milford, MA, U.S.A.), an HPLC pump (P_1) with flow-rate adjustable in steps of 0.01 ml/min (Spectroflow 400; Kratos, Ramsey, NJ, U.S.A.), two semi-microbore reversed-phase columns in series (C1; RP-18 Spheri-5, 5 μm spherical particle size, 100 + 220 mm, 2.1 mm I.D.), a fluorescence detector (Model FS 970; Schoeffel Instruments, Kratos) set at a wavelength of 225 nm for excitation and with a 7-54 band-

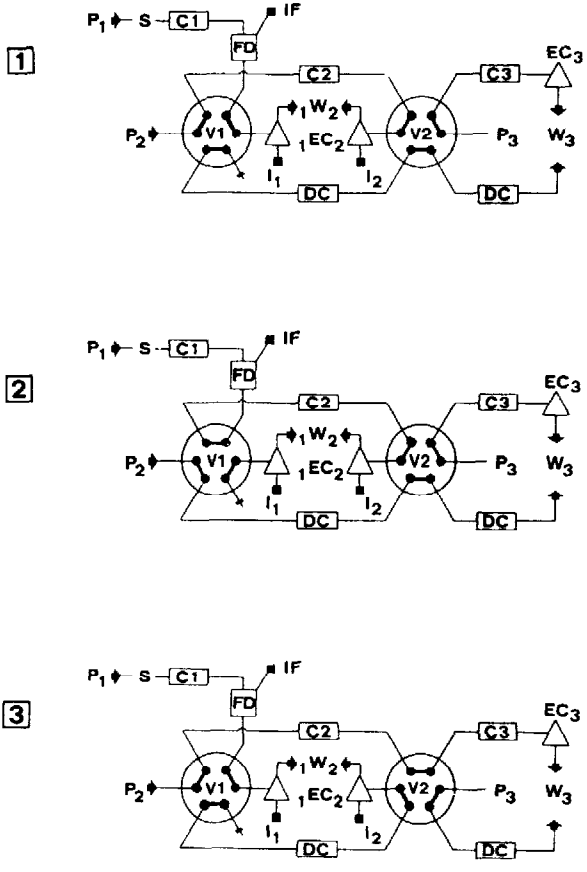

Fig. 1. Configuration of the column-switching apparatus. Status 1: from injection to 2.33 min, after 8.90 min; C1, C2 and C3 all independent. Status 2: 2.33—5.75 min; C1 and C2 in series, C3 independent. Status 3: 5.75—8.90 min; C1 independent, C2 and C3 in series. P = Pump; C = column, S = autosampler; V = switching valve; FD = fluorescence detector; EC = electrochemical detector (△); I = integrator (■); W = waste; DC = discarded column.

pass filter for emission, and an electrochemical detector (EC_1), was as described previously [1], except that two integrators (3390 A; Hewlett-Packard, Avondale, PA, U.S.A.) were used instead of one (IF and I_1). The switching system, which was controlled by integrator IF through a 19400A sample/event control module (Hewlett-Packard), consisted of a column-switching module (V1; Gynkotek, Munich, F.R.G.) and an automatic six-port valve (V2; Rheodyne 70/40). Two additional pumps (P_2 and P_3; LC 410, Kontron Analytic, Zurich, Switzerland) were required, two additional pulse-damping devices (Portmann, Therwil, Switzerland) and two additional electrochemical detectors (EC_2 and EC_3; 656 VA, Metrohm, Herisau, Switzerland) with glassy carbon electrodes set at 0.8 V versus the Ag/AgCl reference electrodes. Each pump was connected to its own manometer, as pressure control is important in a switching system. Only one additional integrator was needed: the outputs from EC_1 and EC_3 were recorded by the same integrator I_1 (3390A, Hewlett-Packard) using a sample/event control module and relay as described previously [1].

Column C2 for the separation of catecholamines was RP-8 Spheri-5, 5 μm spherical particle size, 220 × 4.6 mm I.D. (Brownlee Labs., Santa Clara, CA, U.S.A.) and column C3 for ascorbic and uric acids, RP-8 Spheri-5, 5 μm spherical particle size, 100 × 4.6 mm I.D. (Brownlee Labs.). Two discarded columns (DC) were mounted as shown.

The mobile phases containing sodium dihydrogen phosphate (0.1 *M*) and EDTA (0.08 m*M*) were adjusted to pH 3.5 with phosphoric acid. Mobile phase 1 (for P_1) contained in addition 0.025 m*M* sodium *n*-octyl sulphate and 8.5% acetonitrile; mobile phase 2 (for P_2) contained 0.35 m*M* sodium *n*-octyl sulphate and 4% acetonitrile. Mobile phase 3 (for P_3) was chosen to be the same as mobile phase 2. For optimal separation the amount of sodium *n*-octyl sulphate in mobile phase 2 had to be increased slightly as the column aged (from 0.35 to 0.6 m*M* in six months of constant use). The mobile phases were filtered and degassed as described previously [1]. When the system was not in use, all mobile phases were recycled. During use, mobile phase 1 could be recycled for about two weeks without affecting the baseline, but it was better not to recycle mobile phases 2 and 3. The flow-rates were as follows: P_1, 0.26 ml/min; P_2 0.9 ml/min; P_3 0.3 ml/min. The whole system was at room temperature.

Sample preparation

Tissue from the CNS or peripheral organs was homogenised by sonification in ice and centrifuged as described previously [1], except that 0.4 *M* instead of 0.1 *M* perchloric acid was used. α-MeTrp ($2 \cdot 10^{-6}$ *M*) was added as an internal standard. These processes were carried out as quickly as possible, and tissues and extracts were stored at −80°C if injection could not be performed immediately, in order to prevent the decomposition of ascorbic acid [12].

Automated operation

In the development of a successful switching method, certain disturbing factors must be minimized. There should be very little pressure build-up on switching, as this might damage any detector that is not bypassed by the

switching system, in our case the fluorescence detector. We solved this problem by switching from a semi-microbore column (C1) requiring a very low flow-rate to a normal column (C2), resulting in additional pressure of only 20—50 bar. A fluorimeter in the position shown is advantageous not only for the detection of Tyr, Trp, 5-HT and the internal standard α-meTrp, but also for providing information for timing the switching correctly. This pressure problem did not arise when switching from C2 to C3 because there was no detector involved.

Steps should also be taken to keep the conditions in the electrochemical detectors as constant as possible, otherwise baseline artefacts make integration difficult. This means that all the buffers used should have the same ionic strength and pH, although concentrations within certain limits of ion-pairing reagent and organic modifier do not seem to play a role. In addition, any drastic pressure loss, such as that occurring when a pump is connected to waste through an electrochemical detector, must be avoided. The two DCs were mounted for this purpose, as shown in Fig. 1.

The stability of the detectors is increased if the three mobile phases are mixed as little as possible in each run. This means that the time during which a second mobile phase passes through a column must be reduced to a minimum. The compounds to be analysed can be divided into three groups: group 1, highly retained compounds (5-HT, Trp, α-meTrp, 5-HIAA, HVA) quantified on C1; group 2, slightly retained compounds (NE, E, MHPG, Tyr, DA) quantified on C2, except for Tyr, which has to be detected fluorimetrically; group 3, very slightly retained compounds (Asc and Ur) quantified on C3. Switching was regulated on C1 according to the retention time of MHPG (the slowest in group 2) and on C2 that of Ur (the slowest in group 3). Mobile phase 1, for long-retained compounds, contained only a moderate amount of the ion-pairing reagent sodium *n*-octyl sulphate so that the analysis time was not excessive. Before injection into C1, the semi-microbore column, C1, C2 and C3 and their detectors were equilibrated independently with their respective mobile phases (Fig. 1, status 1). This status continued for 2.33 min, that is, until shortly before the solvent front reached V1. V1 was then switched, resulting in status 2 (C1 and C2 in series, C3 independent). During this status, the compounds of groups 2 and 3 emerging from C1 were loaded on to C2 instead of passing through EC_1, while the pressure on P_2 was maintained by the DCs. When MHPG had been loaded on to C2 (monitored by the fluorescence detector), V1 returned to its original position (5.75 min). At the same time V2 was switched, resulting in status 3 (C1 independent, C2 and C3 in series). During this status, three operations were being performed: (1) C1 was being eluted, permitting quantification of the highly retained compounds (group 1) electrochemically or fluorimetrically; (2) C2 was being eluted with mobile phase 2, which contained a much higher concentration of ion-pairing reagent than mobile phase 1 and less acetonitrile, and was therefore more effective for the separation of catecholamines; (3) the pressure on P_3 was being maintained by a discarded column. The first compounds to emerge from C2 were Asc and Ur. When Ur had been loaded on to C3 (8.9 min), V2 was also switched back to its starting position (the time of emergence of Ur could be monitored with EC_2 in a separate run omitting status 3). The system was again

in status 1, which continued until the end of the analysis (25 min). During this time the three columns were eluted simultaneously. Group 2 compounds were detected by EC_2 and group 3 by EC_3. The output of EC_3 was recorded on I_1 between 9.40 and 12.60 min.

It is an advantage of our method that the solvent peak is switched away and never passes through the first two electrochemical detectors, where it would greatly contribute to detector instability. Therefore, these detectors could be set at high sensitivity (5 or 10 nA) for the quantification of the amines, 5-HIAA and HVA. The third required only low sensitivity (0.5 μA) in any case because Asc and Ur are generally present in high concentrations. At the end of the run no re-equilibration was necessary and the next sample could be injected immediately. The maximum possible throughput was therefore about 50 samples per day.

Generally, 20 μl of the supernatant were injected into the system, requiring a total volume of about 40 μl. The use of a refrigerated sampler was advantageous as some of the compounds, especially 5-HIAA and ascorbic acid, are unstable in standard solutions at room temperature [13], although less so in extracts. The yield was checked for each analysis by controlling the area of the internal standard peak (average yield 93 ± 5%). Calibration solutions were frozen (−80°C) in portions and diluted before use to three concentrations covering those found in the tissue to be analysed. The electrochemical detectors had to be activated regularly if the accuracy of the calibration was to be maintained. EC_3 (Asc and Ur) in particular became insensitive very quickly (after about 150 injections). HVA could also be quantified much more reliably with a newly activated electrode.

Computer evaluation

The method for computer evaluation described in our previous paper [1] was modified so that data could be collected from three HP integrators instead of only one. The following changes were made.

Hardware. Two extra serial interfaces (CCS-7710-A) for the Apple IIe; two extra cables to connect the HP integrators with the two RS-232-C in the Apple.

Software. The interface driver, which is written in Assembler (the machine language), had to be changed so that the incoming data could be selected in a user-defined sequence from the three interfaces. No clock card or external timing is needed because the integrators are all started at the same time by the WISP autosampler but are not stopped simultaneously (IF, 20 min; I_1, 22 min; I_2, 25 min). The integrators send the collected data to the Apple IIe one after the other, and there is enough time to record one storage-file (Chro-file) before the next one comes. There is one Chro-file for each integrator, i.e., three per run. The Chro-files can be either binary or ASCII. All the original options of our Basic Multi program can be used except that for data reduction, which had to be expanded. Data needed for statistical calculations (MICRO-SPSS 4.1 for Apple IIe) are transferred from the Chro-files and collected in one matrix, which is stored in a new, single ASCII data file, together with extra information if required. For use with three integrators, the data array described in our previous paper [1] had to be enlarged horizontally by adding one extra

matrix column for each variable and one for each integrator (a matrix with N Chro-files and $M \times 3$ defined variables). ASCII files can also be easily transferred to another computer, e.g., a MacIntosh plus, for further data reduction and/or statistical computation.

RESULTS AND DISCUSSION

Fig. 2 (left) shows the chromatograms of a synthetic mixture of twelve compounds, and Fig. 2 (right) those of an extract of CNS tissue [rat brain without cerebellum and pons-medulla oblongata; 1:10 (w/v) in 0.4 M perchloric acid]. Trace A shows the output from the fluorimeter with quantification of Tyr, 5-HT, Trp and α-meTrp. There are two unidentified peaks (U) in the brain extract. Trace B shows the output of EC_1 from 5 to 9.40 min

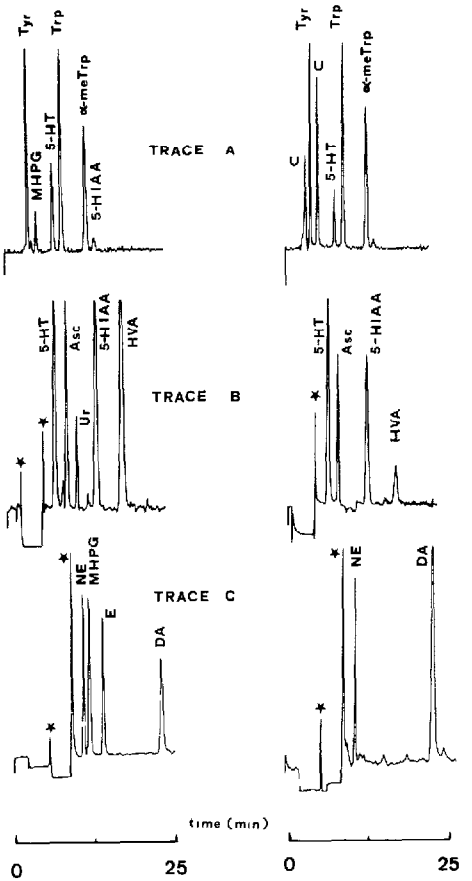

Fig. 2. Typical chromatograms with chromatographic conditions as described in the text. Injection volume, 20 μl. Trace A: fluorescence detection, column 1. Trace B: electrochemical detection, column 1, 0—9.40 min, 12.60—25 min; column 3, 9.40—12.60 min. Trace C: electrochemical detection, column 2. Left: a synthetic mixture of twelve substances. Amounts injected: indoleamines, 10 pmol; catecholamines, HVA and MHPG, 15 pmol; Trp, 40 pmol; Ur, 200 pmol; Tyr, 400 pmol; Asc, 1000 pmol. Right: extract of rat brain (without cerebellum and pons-medulla oblongata), 1:10 (w/v) in 0.4 M perchloric acid.

(5-HT) and from 12.60 to 25 min (5-HIAA and HVA), and also the output of EC_3 from 9.40 to 12.60 min (Asc + Ur). Trace C shows the output from EC_2 (NE, MHPG, E and DA). There is baseline separation of all compounds. The periods of negative baseline and peaks marked with asterisks in the two lower traces are switching artefacts. As can be seen, they do not interfere with integration because, as explained before, care was taken to keep the conditions in the electrochemical detectors as constant as possible.

Table I lists k' values and typical retention times in the system for compounds of neurochemical interest. Compounds with very short retention times such as 3,4-dihydroxymandelic acid (DOMA), 3,4-dihydroxyphenylalanine (DOPA), 3,4-dihydroxyphenylethylene glycol (DOPEG) and vanillylmandelic acid (VMA) cannot be resolved under these conditions and they also interfere slightly with the fluorescence detection of tyrosine. However, they are generally present in very low concentrations and have low fluorescence at the wavelength used, so the error is small. It should be possible to separate them with a suitable choice of column and mobile phase. Unfortunately, 3,4-dihydroxyphenylacetic acid (DOPAC) eluted at approximately the same time as 5-HT. When it was present in large amounts the two compounds caused a double peak in EC_1. In this instance 5-HT was quantified fluorimetrically, as DOPAC has a low fluorescence at the wavelength used. In addition, 3-methoxynorepinephrine (normetanephrine, NM) and 5-hydroxytryptophan (5-HTP) could be determined in EC_2 if required.

TABLE I

k' VALUES AND RETENTION TIMES (t_R) OF SOME COMPOUNDS OF NEUROCHEMICAL INTEREST

Compound	Column 1				Column 2		Column 3	
	FD		EC_1		EC_2		EC_3	
	k'	t_R (min)	k'	t_R (min)	k'	t_R (min)	k'	t_R (min)
Asc							0.05	10.19
Ur							0.21	11.75
DOMA	0.25	3.57						
DOPA	0 27	3.62			0.59	11.69		
Tyr	0.33	3.80						
DOPEG	0.34	3.81						
VMA	0.44	4.11						
NM	0.49	4.24			1.22	16.35		
5-HTP	0.73	4.93			1.04	15.00		
MHPG	0.75	5.00			0.60	11.74		
5-HT	1.90	8.26	2.02	8.60				
Trp	2.39	9.65						
α-MeTrp	3.93	14.06						
DOPAC			2.07	8.74				
5-HIAA			4.39	15.36				
HVA			5.67	19.00				
NE					0.42	10.42		
E					0.73	12.72		
DA					1.47	18.16		

In addition to the classical neurotransmitters and metabolites, we were also interested in their amino acid precursors, Trp and Tyr, and in the compounds Asc and Ur. The determination of Trp and Tyr entails the use of a fluorimeter as these amino acids are not oxidizable at the potentials generally used for ED. For the determination of Asc and Ur, an extra column and detector were required. A knowledge of the concentrations of these compounds in tissues is of importance in attempts to correlate pathological changes with changes in neurotransmitter systems. It is known that increased concentrations of uric acid in cerebrospinal fluid indicate disturbances of the blood—brain barrier [14]. A recent paper [10] described a simple HPLC method for the determination of uric and ascorbic acids together with catecholamines (NE, E, DA) in a variety of extracts. However, no metabolites or precursors were determined.

Our system has been in operation for many months with only slight modifications to the mobile phases. After filling the tray of the refrigerated sampler, the method runs automatically. The within-day variation was less than 5% for NE, E, MHPG, DA, 5-HT, HVA, Tyr and Trp, for ascorbic acid it was 7% and for uric acid about 10%. The day-to-day variation was approximately the same as the within-day variation when the system was regularly recalibrated, i.e., about every 80 injections for EC_1 and EC_2 and 50 injections for EC_3 or twice a week. We have applied our method to extracts of rat brain and spinal cord (normal and pathological), mouse brain and peripheral tissue and human cerebrospinal fluid. The minimum amounts that could be measured in the system were 20—40 pg for NE, E, DA, 5-HT, 5-HIAA, HVA and MHPG. 200 pg for Trp and 700 pg for Tyr. The settings given under Experimental (fluorescence detector 0.5 μA, electrochemical detector 10 nA for amines, 0.5 μA for Asc and Ur) are suitable for the analysis of CNS tissue extracted 1:10 (w/v) with perchloric acid. For the analysis of very small amounts (e.g., micropunches) the tissue can be homogenised 1:20 or even 1:50. There is still a good margin of sensitivity available, as the baselines of the electrochemical detectors are acceptable with the setting of 1 nA. In addition, the injection volume can be increased. However, a disadvantage of our system is that we inject into a semi-microbore column, so that 50 μl is the upper limit for a good separation.

There are three other publications describing the application of column switching and ED to the measurement of biogenic amines, viz., those by Levine and Milstien [9], Niederwieser et al. [8] and Cuisinaud et al. [10]. In the methods of Levine and Milstien and Niederwieser et al., the substances with short retention times are switched from a short column (3 μm particle size) to a longer column, where they are retained with no flow while the longer retained compounds are eluted from the first column. An important factor in the success of this method seems to be the use of a 3-μm packing. In our preliminary experiments we attempted a similar method using a 5-μm column; we experienced great difficulty in timing the switching so that peaks were quantitatively transferred, owing to insufficient resolution of the components. It is of interest that Levine and Milstien [9], who are mainly interested in the metabolites of norepinephrine and dopamine with short retention times, have also recognized the importance of switching away the solvent peak and maintaining the electrochemical detector under stable conditions. The method

of Cuisinaud et al. is similar to ours, the compounds being passed through one, two, three or four columns depending on their retention times (total column length 850 mm, particle size 10 μm). However, in this method also the compounds are retained for a certain time with no flow, and the columns are eluted one after the other, in contrast to the simultaneous elution in our method. The advantage of serial elution is that only one detector and integrator are required, the disadvantage being that the analysis time is much longer. The method of Cuisinaud et al. [10] has been applied by Julien et al. [11] for the measurement of catecholamines and their metabolites in urine, but with a two-step pretreatment (ethyl acetate extraction and alumina adsorption). Hence their results cannot be compared with ours, which are obtained by direct injection of a deproteinized extract.

In conclusion, we have confirmed that column switching can be successfully combined with ED, providing a useful technique for the analysis of tissue extracts. Our system is flexible and could be modified by the addition or subtraction of columns, or by the use of different buffers, to solve other analytical problems. It might also be possible to improve the separation by the use of 3-μm columns, so that fewer columns would be needed. However, the problem of the high pressure caused by these columns would first have to be solved.

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