

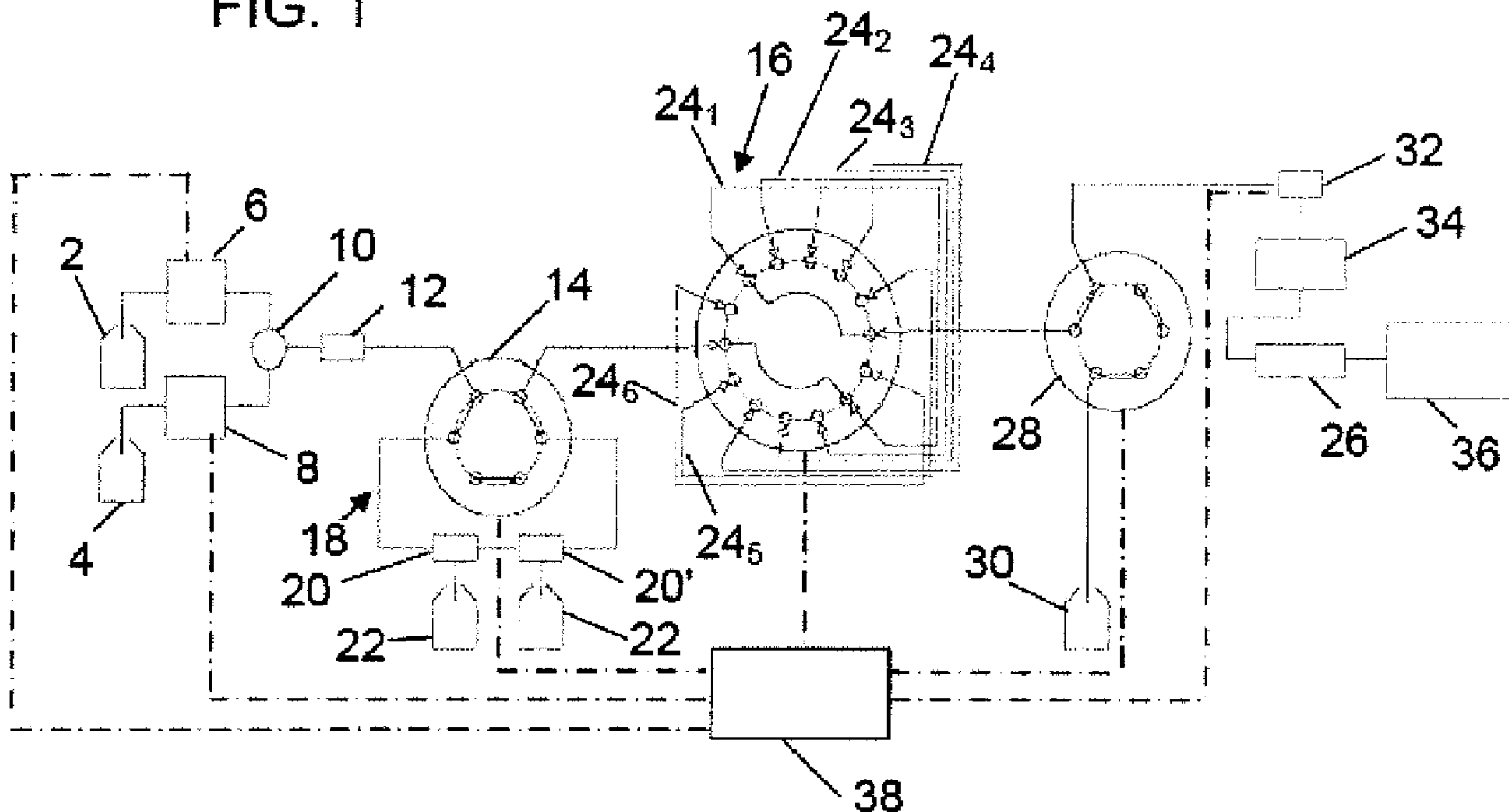


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(54) Titre : DISPOSITIF POUR GENERER DES GRADIENTS DE PHASE MOBILE A MICRO- ET NANODEBIT POUR LA CHROMATOGRAPHIE LIQUIDE A HAUTE PERFORMANCE
 (54) Title: DEVICE FOR GENERATING MICRO- AND NANOFLOW MOBILE PHASE GRADIENTS FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY.

FIG. 1



(57) **Abrégé/Abstract:**

A device for generating micro- and nanoflow mobile phase gradients for high-performance liquid chromatography, characterised by comprising: a generator (2, 4, 6, 8, 10, 12, 14) for micro- and nanoflows of at least two mobile phases in different percentage compositions, an n-position gradient distributor (16) with an inlet port, an outlet port and (n-2)/2 pairs of ports for a like number of capillary loops (24_i), each selectively connectable at one end to the inlet port and at its other end to the outlet port, a flow deviator (28) having its inlet connected to the outlet of the n-position gradient distributor (16), one outlet connected to discharge (30) and

(57) **Abrégé(suite)/Abstract(continued):**

another outlet connected to a micro- and nanoflow meter (32), itself connected to the analytical circuit (26, 36), a control system (38) provided with means for commanding the micro- and nanoflow generator (2, 4, 6, 8, 40, 42, 10) to create the different mobile phase mixture compositions, gradient generating means acting on the implementation and residence times of the gradient distributor (16), means for controlling the throughput of the micro- and nanoflow generator on the basis of the data received from the micro- and nanoflow meter (32), and means for controlling the position of the flow deviator (28).

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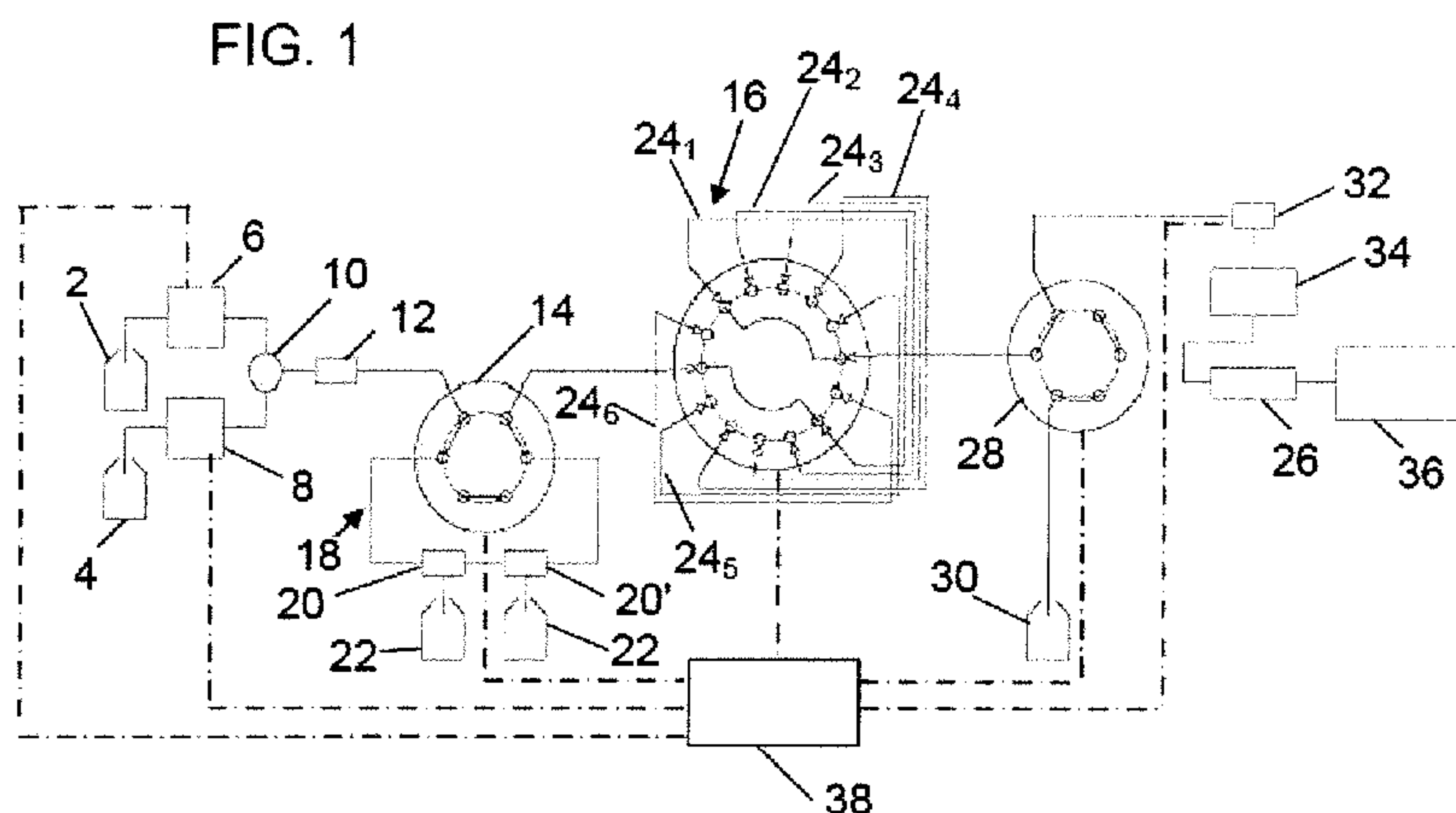
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(54) Title: DEVICE FOR GENERATING MICRO- AND NANOFLOW MOBILE PHASE GRADIENTS FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY.



(57) Abstract: A device for generating micro- and nanoflow mobile phase gradients for high-performance liquid chromatography, characterised by comprising: a generator (2, 4, 6, 8, 40, 42, 10) for micro- and nanoflows of at least two mobile phases in different percentage compositions, an n-position gradient distributor (16) with an inlet port, an outlet port and $(n-2)/2$ pairs of ports for a like number of capillary loops (24_i), each selectively connectable at one end to the inlet port and at its other end to the outlet port, a flow deviator (28) having its inlet connected to the outlet of the n-position gradient distributor (16), one outlet connected to discharge (30) and another outlet

connected to a micro- and nanoflow meter (32), itself connected to the analytical circuit (26, 36), a control system (38) provided with means for commanding the micro- and nanoflow generator (2, 4, 6, 8, 40, 42, 10) to create the different mobile phase mixture compositions, gradient generating means acting on the implementation and residence times of the gradient distributor (16), means for controlling the throughput of the micro- and nanoflow generator on the basis of the data received from the micro- and nanoflow meter (32), and means for controlling the position of the flow deviator (28).

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DEVICE FOR GENERATING MICRO- AND NANOFLOW MOBILE
PHASE GRADIENTS FOR HIGH-PERFORMANCE LIQUID
CHROMATOGRAPHY

The present invention relates to a device for generating micro- and
5 nanoflow mobile phase gradients for high-performance liquid
chromatography.

Recent decades have seen a progressive miniaturization of high-
performance liquid chromatography (HPLC) systems, in particular with
regard to the mobile phase flows utilized. This is because flow reduction
10 particularly enables the consumption of costly toxic solvents to be limited;
it also results in a reduction in injection volumes, and hence enables very
small sample quantities to be processed. In addition, if concentration-
sensitive sensors are used, a sensitivity increase results because of the
smaller dilution of solvent in the column and a better signal-noise ratio.

15 Finally, the new liquid chromatography-mass spectrometry (LC-
MS) coupling techniques enable working at flows less than 1 $\mu\text{L}/\text{min}$, to
obtain higher sensitivity and limit possible contamination risks.

However one of the problems encountered when working at very
low flows consists of the lack of instrumentation able to generate reliable
20 mobile phase gradients, with only a small delay and homogeneous mixing
of solvents. The generation of a gradient at flows less than 1 $\mu\text{L}/\text{min}$
certainly requires an adequate pumping system, able not only to carefully
and homogeneously mix the mobile phases, but also to transfer them into
the column in a reproducible manner and with minimum delay. However,
25 under these conditions the commercially available instrumentation is
compelled to operate at its limit, and hence the performance reliability is
often questionable. Moreover the effective gradient profile presents an
unsatisfactory pattern compared with that set.

One of the most common ways of generating micro- and nanoflow mobile phase gradients is the split-flow technique using a splitter inserted between the HPLC pumps and the injector. This solution is limited by poor control of the gradient actually transferred into the column, and consequently of poor reproducibility. One of the initial attempts to achieve nano-scale gradients was to store a specific gradient in a capillary and to then transfer it into the column with a syringe pump. Drawbacks of this system are poor practicality, a limited choice of gradients to be formed, and poor control of the shape of the gradient.

Another known system, known as the exponential dilution method, consists of rapidly passing the mobile phase flow from the weak eluent, contained in a mixing chamber, to the strong eluent, with generation of a gradient of concave exponential profile.

The drawback of this system is the limited number of gradient types which can be generated.

To overcome this drawback, it has already been proposed to fill the mixing chamber with the weak eluent and to cause the strong eluent to flow in a programmed manner into a dynamic mixer, in order to be able to control the gradient variation with time.

The drawback of this system is a poor versatility of achievable gradient profiles.

Another known system consists of generating an exponential nanoflow gradient by introducing into two successive mixing chambers firstly the weak eluent and then the strong eluent, and then transporting them into the column using one pump at a time.

The drawback of this known solution consists of a limited gradient and profile selection, in addition to a consistent gradient delay.

US 7,135,111 describes a nanoflow gradient elution device. It comprises a first pump for mixing different solvents and transporting them, a second pump for transporting a transfer solution, an injector, a column and a detection system. Two loops enable different solvent mixtures to be temporarily stored, these being transferred by the first microflow pump and then transported into the nanoflow column by the second pump, by a modification in the internal system connections and hence in the flow direction.

The difficulty of this known solution is the difficulty of delivering the mobile phase at constant flow when working with nanolitres/min.

US 7,141,161 describes a gradient pump arrangement able to transfer eluents to a chromatograph continuously, at specific time intervals and at a constant flow of the order of nanolitres/minute while the composition of two or more eluents is modified. The gradient pump arrangement includes a ten-port multi-position valve, a first loop connected to an isocratic pump and a second loop, through which the eluents are transferred.

The drawback of this known solution consists of a considerable complexity of the pump and valve system, making it particularly difficult to automate the instrumentation.

An object of the invention is to eliminate the aforesaid drawbacks by providing a new arrangement for generating micro- and nanoflow mobile phase gradients for high-performance liquid chromatography, as described in claim 1.

Two preferred embodiments of the present invention are further clarified hereinafter with reference to the accompanying drawings, in which:

Figure 1 is a schematic view of a first embodiment of the device of the invention, with two conventional HPLC pumps connected to a splitter device,

5 Figure 2 shows five replicas of the chromatographic separation of a mixture of four components at a predetermined concentration,

Figure 3 shows the profile of a stepless trimmed gradient,

10 Figure 4 shows three different profiles of a gradient from 0% to 100% of CH₃CN in H₂O under particular chromatographic conditions,

Figure 5 shows the expected theoretical pattern of the three profiles of Figure 4,

15 Figure 6 is a schematic view of a second embodiment of the device of the invention with two HPLC syringe pumps for micro- and nanoflows.

With reference to Figure 1, the first embodiment of the device of the invention is intended for generating micro- and nanogradients.

20 It comprises two bottles 2, 4 of two solvents A and B; these are connected via two traditional reciprocating HPLC pumps 6, 8 to a mixer 10 connected to a sintered filter 12 with 0.5 μm pores. This filter is connected to the inlet of a multi-position valve 16 via a splitter circuit indicated overall by 18.

25 This splitter circuit comprises a valve 14 and two identical PEEKsil capillaries (25 or 50 μm I.D.) connected to the valve via a circuit of small dead volume, and also connected to discharge 22. The splitter circuit 18 enables the desired flow to be obtained from the high flow generated by the HPLC pumps 6, 8 by virtue of the impedance offered by the two

capillaries 20, 20' and by the chromatography column 26, fed by the device of the invention.

The multi-position valve 16 is of traditional type, for example model EMTCS6UW of the Valco Instruments Co. Inc., Huston, Tx, and is provided with fourteen ports, two of which form the inlet and outlet, while the other twelve represent the connection points for six loops 24_i, formed with PEEK tubing of internal diameter 250 μm , to store the mobile phase.

The first five loops 24₁-24₅ are of equal volume of 50 μL , whereas the sixth loop has a volume of 150 μL . It contains the final gradient composition, its greater volume being useful to complete the chromatographic analysis and to clean the column 26.

The outlet port of the multi-position valve 16 is connected to the inlet of a two-position valve 28, having one outlet connected to discharge 30 and the other outlet connected to a micro- and nanoflow meter 32 connected to an injector 34 for the chromatography column 26, with which a detector 36 is associated.

The operation of each device component, and in particular of the two two-position valves 14 and 28 and the multi-position valve 16, is coordinated by a control system 38, providing organized automatic operation of the device.

In operation, the first scheduled operation consists of loading the six loops 24_i associated with the multi-position valve 16. To achieve this loading, the two two-position valves 14 and 28 are set with the valve 14 by-passing the splitter device 16 and the valve 28 connecting the outlet of the multi-position valve 16 to discharge 30.

In this configuration the pumps 6 and 8 can provide a high flow, of about 0.8-1.0 mL/min to accelerate the procedure. The control system

activates a control program for the pumps 6 and 8, correlated with a program of implementation times for the multi-position valve 16, to distribute the different mobile phase compositions of increasing eluting power within the six loops 24_i.

5 For example, to generate a gradient from 100% of solvent A contained in the bottle 2, to 100% of solvent B contained in the bottle 4, the first loop 24₁ is selected and simultaneously the pump 6 is programmed to alone pump 100% of solvent A; for a flow of 0.8-1.0 mL/min, the time for filling the loop would in theory be just a few seconds,
10 however in practice it is advisable to wait a couple of minutes for conditioning. Thus after a few minutes the loop 24₁ is completely loaded with its corresponding mobile phase composition, and the control system activates the multi-position valve 16 to connect the second loop 24₂ to the valve 14; at the same time the operation of the pumps 6 and 8 is modified
15 such that the pump 6 pumps 80% of solvent A and the pump 8 pumps 20% of solvent B.

After another couple of minutes the third loop 24₃ is selected and the pumps are further modified, such as to pump 60% of solvent A and 40% of solvent B.

20 The process is then repeated until the sixth loop 24₆ is selected, with the pump 8 being programmed to alone deliver 100% of solvent B.

Having completed loading of the loops 24_i, the first loop 24₁ is again selected, with 100% solvent A passage; the flow is then reduced and the two two-position valves 14 and 28 are switched over to insert the
25 splitter circuit and connect the multi-position valve 16 to the analytical circuit. The system processes the data arriving from the nanoflow meter

32 and regulates the flow of the pump 6 to obtain the desired flow in the column 26.

In this configuration, a micro- or nanoflow of solvent A, originating from the splitter circuit 16, continuously recharges the first loop 24₁ with the weaker eluent (generally water) to condition the column 26.

Following introduction of the sample and commencement of chromatographic analysis, the multi-position valve 16 is activated to transfer into the column 26 the contents of the next loop 24₂, loaded with a mobile phase composition of greater eluting power. This process is repeated sequentially for all loops. The pattern of the gradient which is to be generated is obtained by varying the implementation and residence times. The solvent A, which is used without variation to push the contents of each loop until termination of the analysis, enables a constant mobile phase flow to be delivered.

No modification to the conventional chromatograph is required to install the device of the invention. This aspect is particularly important, as it enables any commercially available conventional HPL chromatograph to work effectively with micro- and nanoflows.

Because of the low flow used, the small diameter of the loops 24_i, and the connection capillaries, no turbulence phenomenon or mixing occurs; consequently once completely filled, the six loops 24_i are able to provide eluent sufficient for different analyses, depending on the flow.

The control system stores in its memory the quantities of solvent used by each loop 24_i during each analysis, according to the type of gradient and the flow used: this means that before the loops 24_i have been completely emptied of the corresponding mobile phase composition, and

hence before they are incapable of generate the correct gradient, a new loading procedure is automatically started.

To evaluate the characteristics and performance of the device of the invention, the intraday repeatability was verified by carrying out five analyses of a mixture of four compounds monuron, difenoxuron, linuron and azinphos ethyl, each at a concentration of 5 mg/L.

The separations were obtained with an Agilent Zorbax 150 mm x 75 μm column packed with 3.5 μm particles of phase C 18, using a linear gradient from 0% to 100% of CH_3CN in H_2O in 16 min; injection volume 50 nL and detecting with UV at $\lambda = 230$ nm.

Figure 2 shows the high retention time repeatability in the five analyses, the relative standard deviation being less than 0.19%.

Table 1 shows the retention times relative to the chromatograms of Figure 2, for a more detailed evaluation of the intraday repeatability.

15

TABLE 1

Compound	Inj 1	Inj 2	Inj 3	Inj 4	Inj 5	\bar{X}	SD	RSD (%)
Monuron	19.497	19.473	19.553	19.457	19.507	19.4974	0.03678	0.18864
Difenoxuron	21.847	21.780	21.833	21.847	21.830	21.8274	0.027628	0.12657
Linuron	22.877	22.780	22.847	22.837	22.843	22.8368	0.035302	0.15458
Azinphos Ethyl	24.657	24.557	24.640	24.630	24.640	24.6248	0.039124	0.15888

In order to also evaluate the interday repeatability, ten chromatographic separations per day were carried out for five consecutive

working days. The compounds analysed were: methomyl, monuron, difenoxuron, linuron, each at a concentration of 197 mg/L. The gradient used was from 100% H₂O to 100% CH₃CN in 16 min. Flow 360 nL/min; column Agilent Zorbax C18 3.5 μm, 150 mm x 75 μm; injection volume 60 nL; UV detection at λ = 230 nm.

The following Table 2 shows the averages of the retention times of each compound for each working day and the standard deviation relative to the daily averages. Excellent interday repeatability can be observed for each compound.

10

TABLE 2

Compound	\bar{X} Day 1	\bar{X} Day 2	\bar{X} Day 3	\bar{X} Day 4	\bar{X} Day 5	RSD %
Methomyl	10.59	10.55	10.68	10.81	10.44	1.31
Monuron	14.87	14.86	14.71	14.69	14.88	0.63
Difenoxuron	17.21	17.13	16.98	16.94	17.10	0.65
Linuron	19.15	19.04	18.89	18.91	18.99	0.55

In general it is advisable to use a gradient without steps, in order to obtain a constant variation in the mobile phase composition with time. In this respect, sudden mobile phase variations, typical of a stepped pattern, correspond to rapid polarity changes, which can interfere unexpectedly with the chromatographic separation in terms both of resolution and of reproducibility.

With nanoflows, the different mobile phases contained in continuous loops alternate to mix together within the spaces of the multi-position valve 16 and the connections, to avoid sudden variations in solvent composition.

5 Figure 3 shows a stepless trimmed gradient.

Figure 4 shows the profiles of three 12 minute gradients obtained at a flow of 400 nL/min, using different valve operating programs.

The loops 24_i were filled with the following solvent compositions:
loop 24_1 : 100% H₂O - 0% CH₃CN; loop 24_2 : 80% H₂O - 20% CH₃CN; loop
10 24_3 : 60% H₂O - 40% CH₃CN; loop 24_4 : 40% H₂O - 60% CH₃CN; loop 24_5 :
20% H₂O - 80% CH₃CN; loop 24_6 : 0% H₂O - 100% CH₃CN.

The operating programs were the following: loop 24_2 for 0.30 minutes, loop 24_3 for 1 minute, loop 24_4 for 1.30 minutes, loop 24_5 for 9.00 minutes for curve a, which represents the concave gradient; loop 24_2 for 3
15 minutes, loop 24_3 for 3 minutes, loop 24_4 for 3 minutes, loop 24_5 for 3 minutes for curve b, which represents the linear gradient; loop 24_2 for 9.00 minutes, loop 24_3 for 1.30 minutes, loop 24_4 for 1 minute, loop 24_5 for 0.30 minutes for curve c, which represents the convex gradient.

The expected theoretical patterns are shown in Figure 5. The
20 traces relative to absorbance are very well differentiated, as shown by the implementation times selected. This is particularly important, considering the extremely small operative flow.

The high correspondence between the expected and real gradient pattern under different conditions enables the form of the gradient to be
25 set both via a table of implementation times, and by a more intuitive graphical approach. In this respect, the control system makes it possible to display the gradient profile on the computer screen as a function of the

implementation times: each change in the set times is immediately reflected as a corresponding change in the displayed profile.

In the same manner, each gradient profile modification, obtained by dragging the graph with the computer pointer, determines a
5 corresponding modification in tabled implementation times. Consequently each gradient form can be easily programmed by establishing the tabled time parameters or the graphic profile.

When a linear gradient is required the command "Linear Gradient" can be used which, based on the selected duration for the gradient,
10 automatically calculates the implementation times and displays the gradient profile.

In the embodiment shown in Figure 6, the device of the invention differs from that shown in Figure 1 by replacing the reciprocating pumps 6 and 8 by micro- and nanoflow syringe pumps 40 and 42. This enables
15 nanoflows to be obtained without using the splitter circuit 18.

For the rest, the device is similar, with identical components carrying the same reference numerals.

From the foregoing it is apparent that the device of the invention is particularly advantageous, as it enables micro- and nanoflows to be
20 generated automatically, with high flexibility in the choice of the gradient pattern, in accordance with analytical requirements, and always with very high accuracy and reproducibility.

C L A I M S

1. A device for generating micro- and nanoflow mobile phase gradients for high-performance liquid chromatography, characterised by comprising:

- 5 - a generator (2, 4, 6, 8, 40, 42, 10) for micro- and nanoflows of at least two mobile phases in different percentage compositions,
- an n-position gradient distributor (16) with an inlet port, an outlet port and $(n-2)/2$ pairs of ports for a like number of capillary loops (24_i), each selectively connectable at one end to the inlet port and at its other end
10 to the outlet port,
- a flow deviator (28) having its inlet connected to the outlet of the n-position gradient distributor (16), one outlet connected to discharge (30) and another outlet connected to a micro- and nanoflow meter (32), itself connected to the analytical circuit (26, 36),
- 15 - a control system (38) provided with means for commanding the micro- and nanoflow generator (2, 4, 6, 8, 40, 42, 10) to create the different mobile phase mixture compositions, gradient generating means acting on the implementation and residence times of the gradient distributor (16), means for controlling the throughput of the micro- and nanoflow
20 generator on the basis of the data received from the micro- and nanoflow meter (32), and means for controlling the position of the flow deviator (28).

2. A device as claimed in claim 1, characterised in that the micro- and nanoflow generator comprises at least two bottles (2, 4) containing
25 different solvents, a reciprocating pump (6, 8) associated with each bottle, a mixer (10) for the flows generated by said pumps, and a splitter circuit (18).

3. A device as claimed in claim 2, characterised in that the splitter circuit (18) comprises a valve (14) controlled by said control unit (38) to switch between a position of connection to said splitter circuit (18) and a position by-passing this latter.

5 4. A device as claimed in claim 3, characterised in that said splitter circuit comprises at least one capillary (20, 20') connected to said valve (14) and to discharge (22).

5. A device as claimed in claim 1, characterised in that the micro- and nanoflow generator comprises at least two bottles (2, 4) containing
10 different solvents, a syringe pump (40, 42) associated with each bottle, and a mixer for the micro- and nanoflows generated by said syringe pump.

6. A device as claimed in claims 2 or 5, characterised in that a filter (12) is interposed between said mixer (10) and said gradient distributor (16).

15 7. A device as claimed in claim 6, characterised in that said filter (12) is made of sintered steel with pores of the order of 0.5 μm .

8. A device as claimed in claim 1, characterised in that the n-1 loops (24₁-24₅) of the gradient distributor (16) have the same volume, whereas the nth loop (24₆) has a greater volume.

20 9. A device as claimed in claim 8, characterised in that the n-1 loops (24₁-24₅) of the gradient distributor (16) have a volume of the order of 50 μL , whereas the nth loop (24₆) has a volume of the order of 150 μL .

10. A device as claimed in claim 1, characterised in that the gradient distributor (16) consists of an n-position valve.

FIG. 1

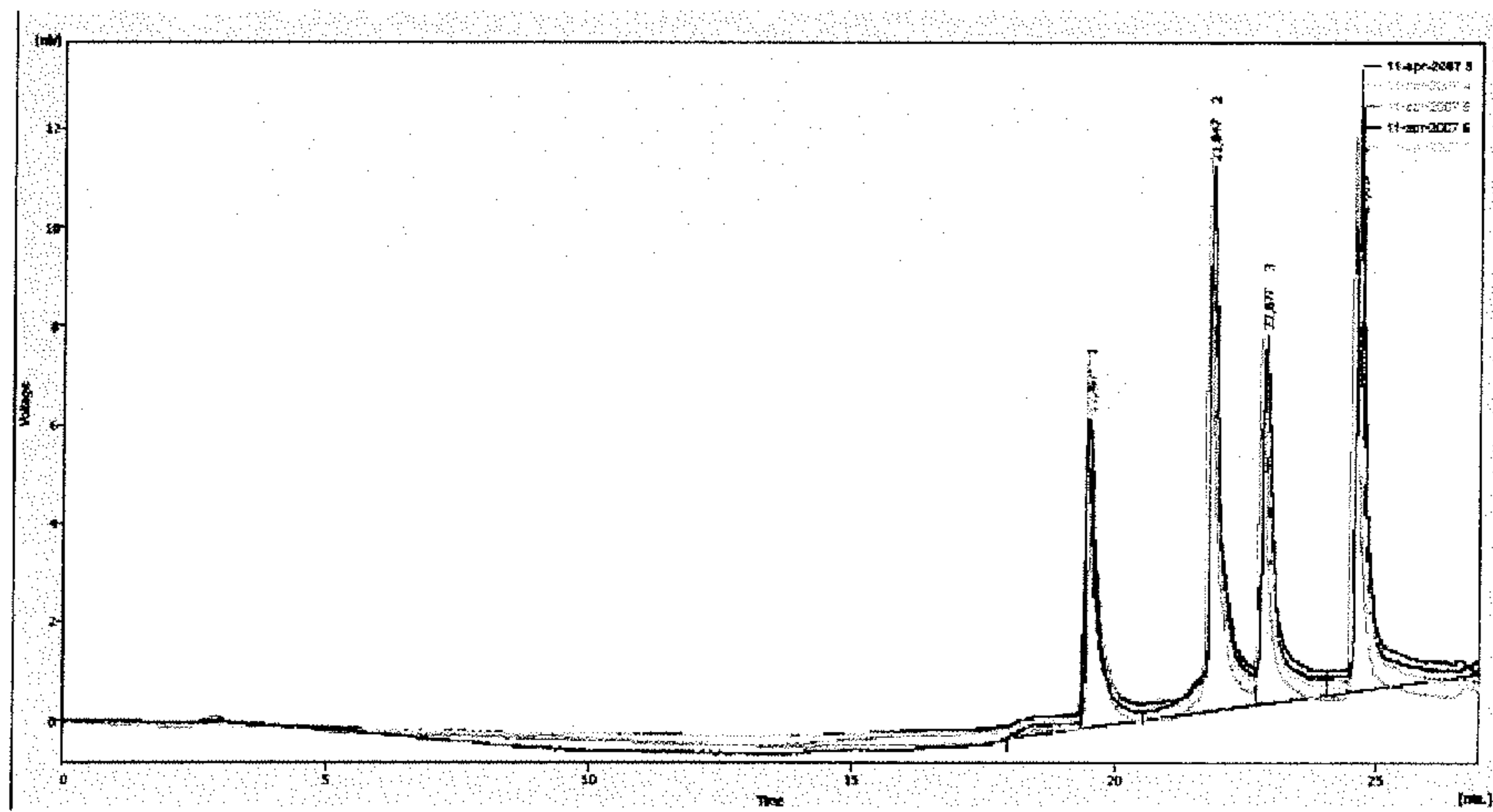
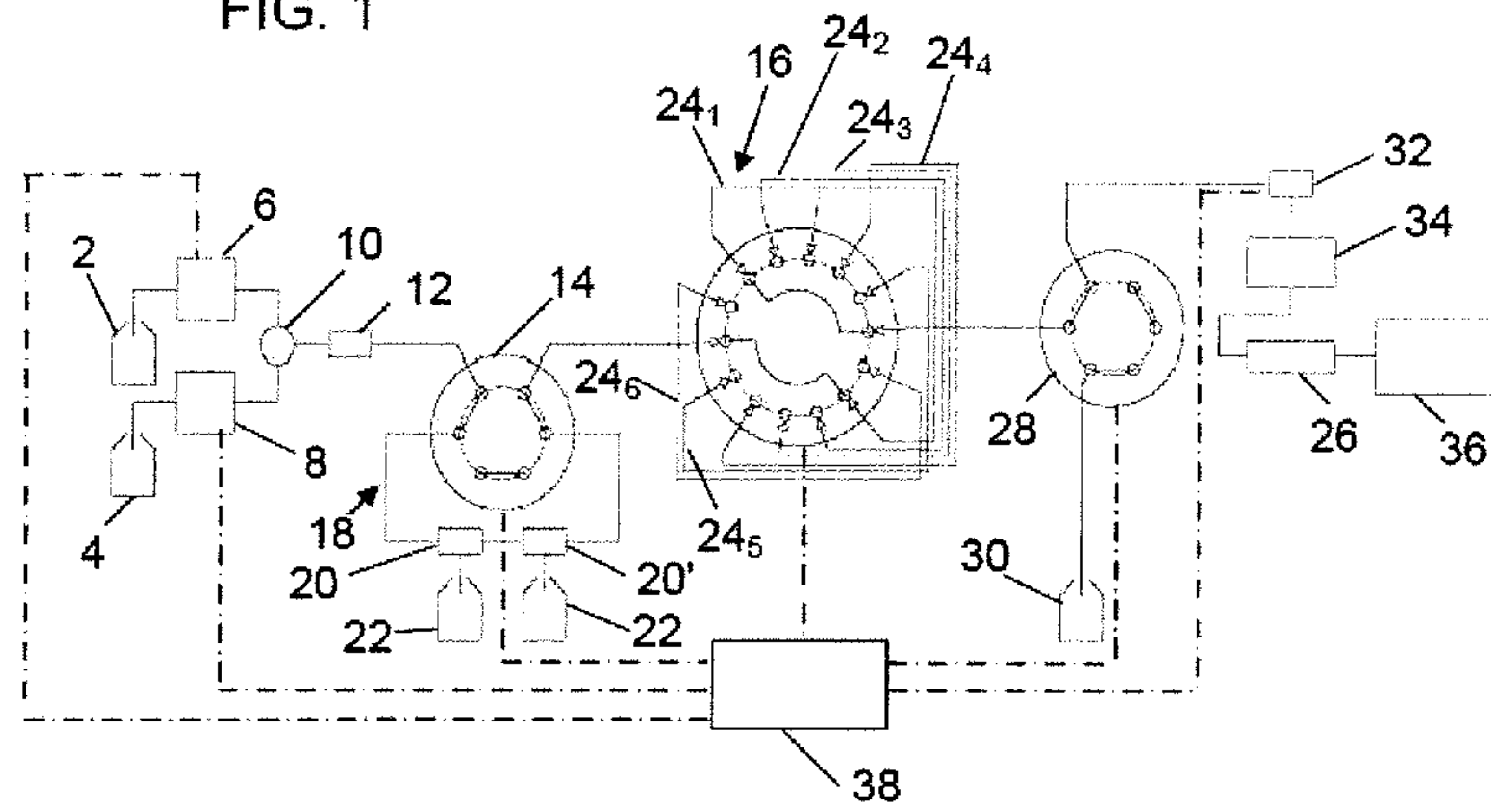


FIG. 2

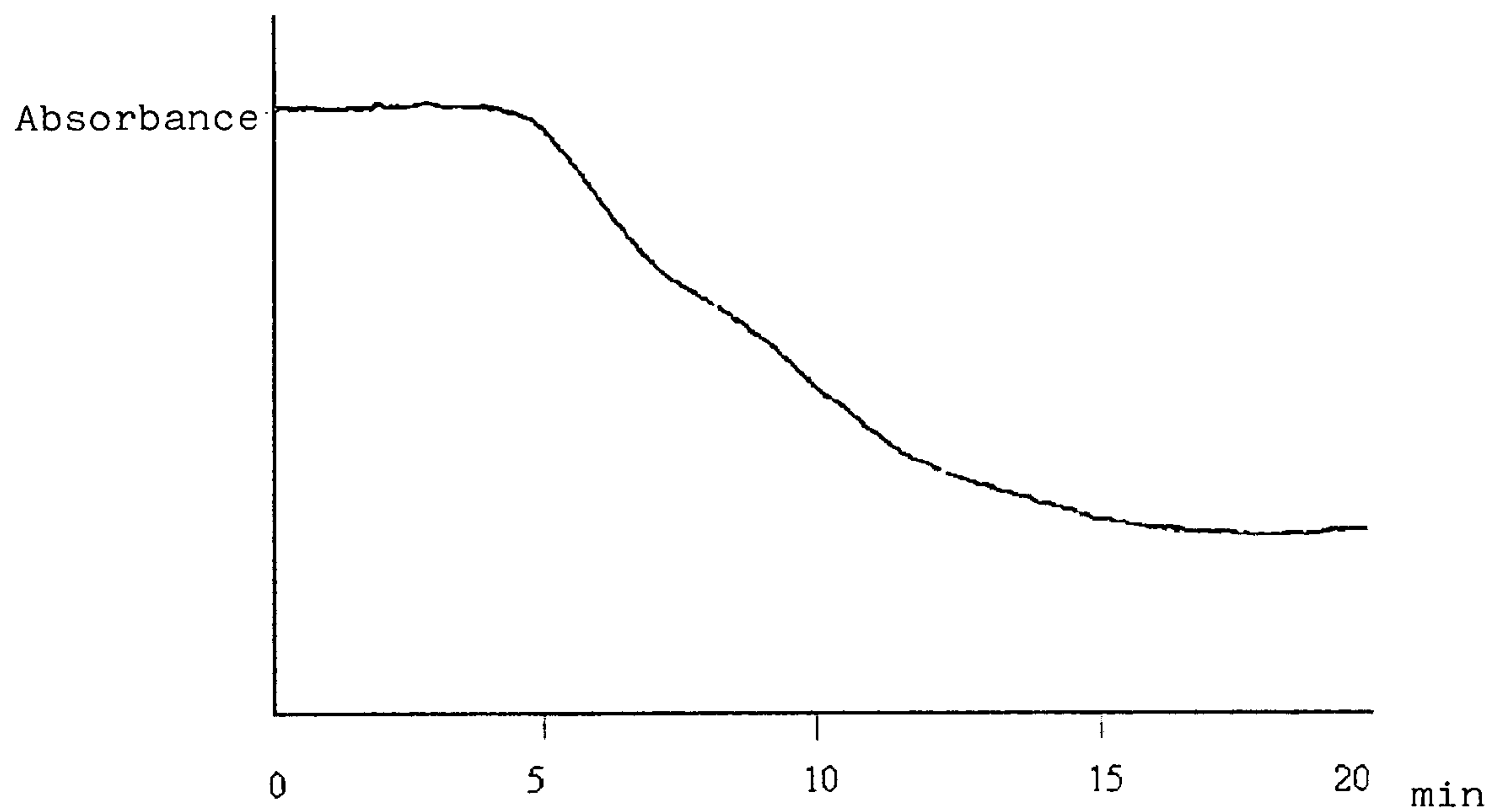


FIG. 3

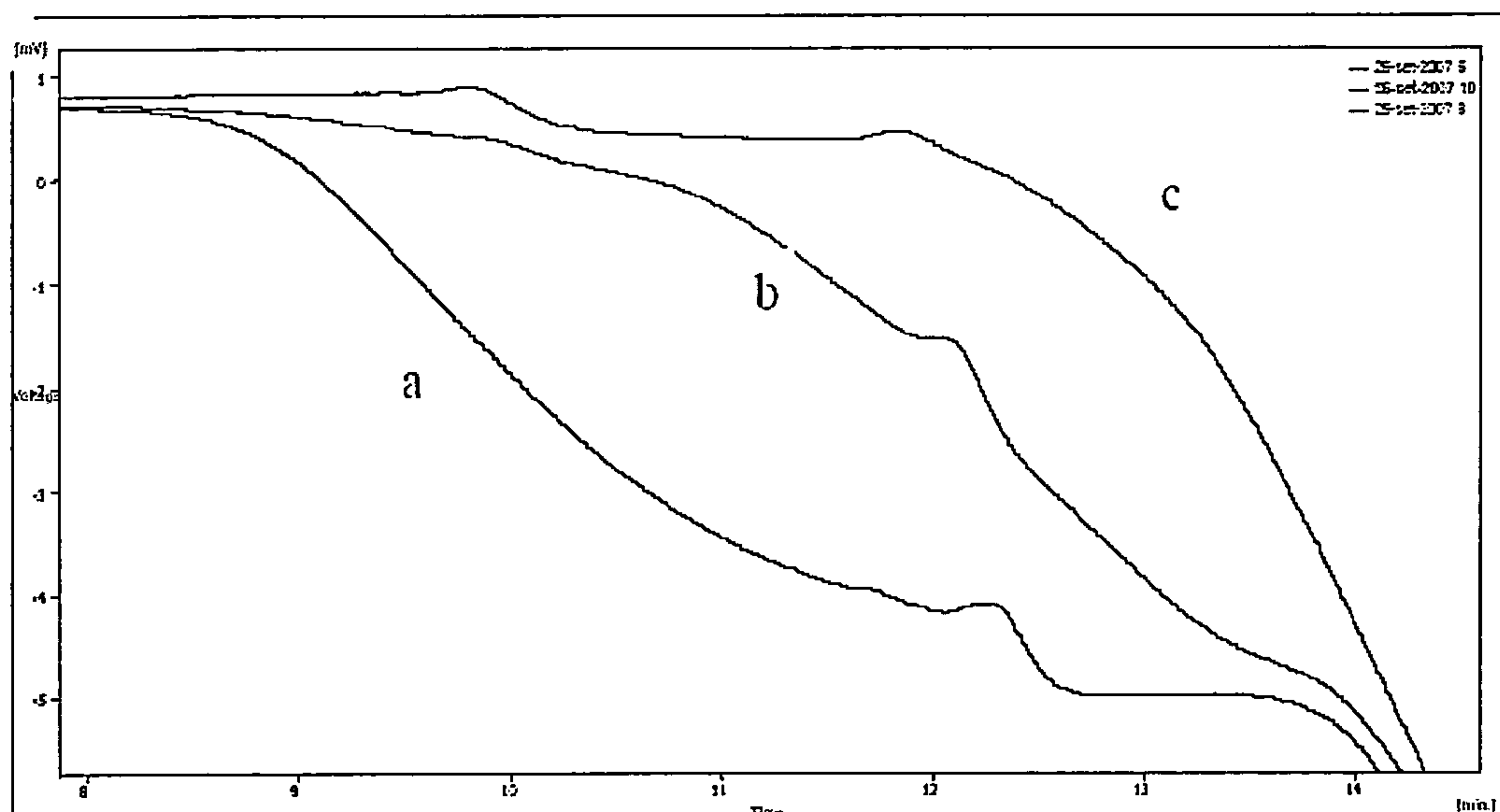


FIG. 4

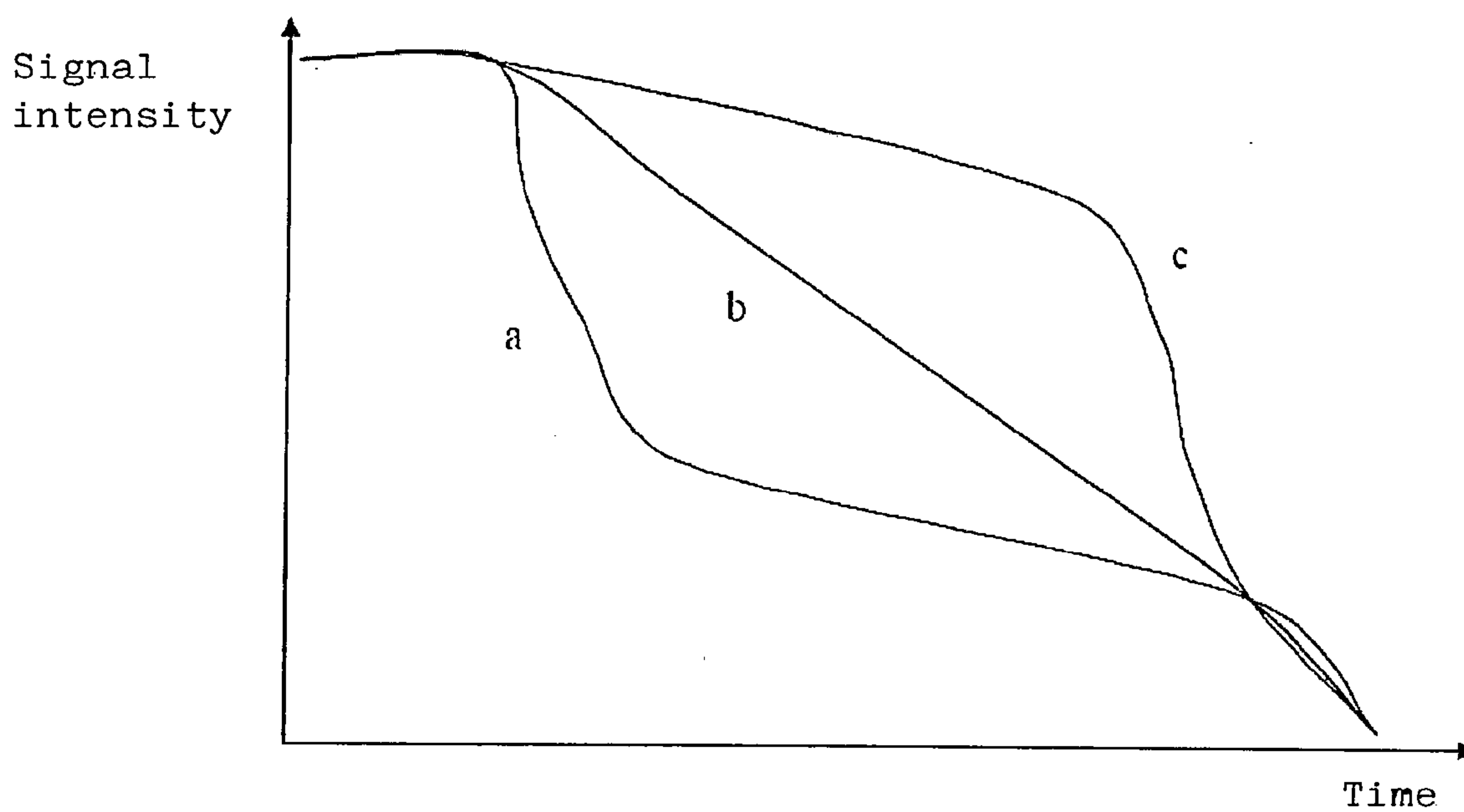


FIG. 5

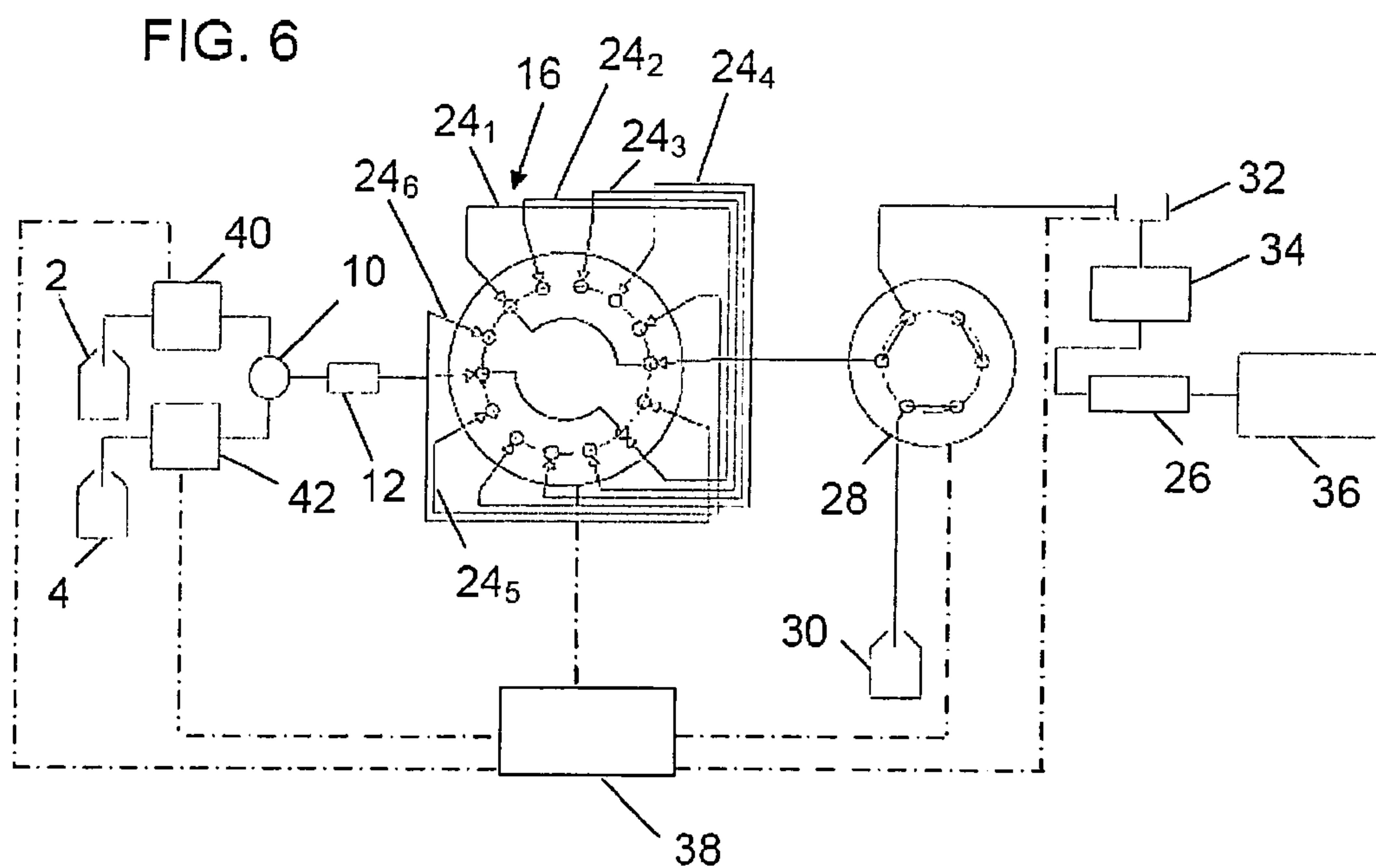


FIG. 6

FIG. 1

