

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
15 February 2007 (15.02.2007)

PCT

(10) International Publication Number  
**WO 2007/016971 A1**

(51) International Patent Classification:

G01N 30/08 (2006.01) G01N 30/14 (2006.01)  
G01N 30/46 (2006.01) G01N 30/88 (2006.01)  
C07K 1/22 (2006.01)

(21) International Application Number:

PCT/EP2005/053608

(22) International Filing Date: 25 July 2005 (25.07.2005)

(25) Filing Language:

English

(26) Publication Language:

English

(71) Applicant (for all designated States except US): **AGILENT TECHNOLOGIES, INC.** [US/US]; 395 Page Mill Road, Palo Alto, CA 94306 (US).

(72) Inventor; and

(75) Inventor/Applicant (for US only): **GRIMM, Rudolf** [DE/DE]; Untertaxetweg 105, 82131 Gauting (DE).

(74) Agent: **BARTH, Daniel**; Herrenberger Str. 130, 71034 Böblingen (DE).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

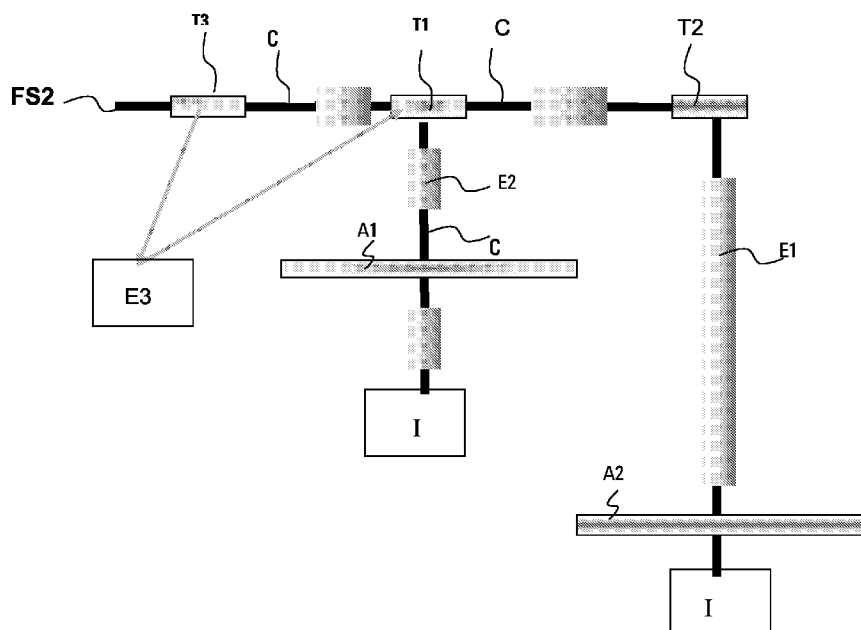
(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: FLUIDIC ANALYSIS WITH HYDROPHOBIC AND HYDROPHILIC COMPOUNDS TRAPPING



(57) Abstract: An analysis system is provided, suitable to analyze fluidic samples (FS1, FS2). Said analysis system comprises at least two trapping columns (T1, T2), wherein the first one is adapted for trapping hydrophobic compounds of the fluidic sample (FS1, FS2) and the second one is adapted for trapping hydrophilic compounds of the fluidic sample (FS1, FS2). The at least two trapping columns (T1, T2) are in fluidic communication and the second trapping column (T2) is arranged downstream to the first trapping column (T1).

WO 2007/016971 A1

## DESCRIPTION

**FLUIDIC ANALYSIS WITH HYDROPHOBIC AND HYDROPHILIC  
COMPOUNDS TRAPPING**

[0001] The present invention relates to analysis of fluidic samples.

5

## BACKGROUND ART

[0002] Analyzing samples consisting of complex mixtures resulting e.g. from biological materials such as cells, organisms or organic tissues became increasingly important during the last decade. Scientists performing research in the domain of biology, biochemistry and in further related domains were focusing on the development of technologies permitting them to precisely, rapidly and efficiently analyze a sample qualitatively and quantitatively.

[0003] Said mixtures may be protein mixtures, polysaccharides, regulatory substances, carbohydrates, nucleic acids, amino acids, nucleotides, nucleosides, fats, fatty acids, and organic acids or metabolic mixtures. The last are usually composed of peptides, sugars, fatty acids, steroids, nucleotides, carbon acids, vitamins, hydrates, nucleic acids, amino acids, nucleotides, nucleosides, fats and a plurality of other additional low molecular compounds (having a molecular weight up to 2 kDa); so metabolites are hydrophobic and hydrophilic metabolic products. Their qualitative and quantitative analysis is named metabolomics.

[0004] Accordingly samples being subjected to metabolomics may contain the ensemble of low molecular compounds being produced in an organism due to metabolism, or at least a subfraction of it, thus being of high complexity. So, a human metabolom sample e.g. requires the performance of multiple fractioning- or separating steps due to its composition of up to 10 000 compounds. Said complex mixture needs to be pre-fractionated into its classes prior to performance of further quantitative and/or qualitative analysis.

[0005] US 6849396 B2 to Schneider, Luke V. discloses methods for conducting metabolomics. Purifying of metabolites and conducting metabolic analyses generally involves determining metabolic flux values for a plurality of target analytes by

monitoring the relative isotope abundance of a stable isotope in a substrate labeled with the stable isotope and/or one or more target metabolites formed through metabolism of the labeled substrate. Furthermore, the methods are based on electrophoretic methods to separate the target analytes from other components within the sample being analyzed. Further methods involve administering a substrate labeled with a stable isotope to a subject.

[0006] Referring now to US 20040147040 A1 to Bluggel, M. and Schurenberg, M. describing methods and instrumentation for two or multidimensional separations of biosubstance mixtures, especially protein mixtures, for mass spectrometric analysis.

[0007] The US 20030168392 A1 to Masuda, J. et al. publishes a multi-dimensional liquid chromatography separation system, based on the difference of two or more kinds of separation modes, (e.g., chemical or physical properties of analytes) which may improve separations when samples contain complex mixtures. Here, the analytes separated on the 1<sup>st</sup> analysis system (consisting of the 1<sup>st</sup> column and the 1<sup>st</sup> mobile phase) will be trapped onto individual trapping columns. Then the trapped analytes will be loaded onto the 2<sup>nd</sup> analysis system consisting of the 2<sup>nd</sup> column and the 2<sup>nd</sup> mobile phase. The trapping and loading mechanism consists of a combination of switching valves necessary to produce the serial separations.

[0008] Once the pre-fractionation is done and thus the desired class of metabolites can be handled separately, the appropriate technology can be chosen to carry out the succeeding analytical steps such as proteomics, peptidomics and others. For peptidomics, e.g. one may follow the methods described in US 20040180449 A1 to Hamstra A.J. et al. It refers to methods for separating or purifying samples, for example separating peptides or peptide groups from a protein sample.

## DISCLOSURE

[0009] It is an object of the invention to provide an improved fluidic sample analysis. The object is solved by the independent claims. Preferred embodiments are shown by the dependent claims.

[0010] According to embodiments of the present invention, an analysis system to

analyze fluidic samples is provided. Said analysis system generally comprises two or more trapping columns being connected in series, thereby allowing to perform separation of hydrophobic and hydrophilic compounds.

[0011] In an other embodiment the further separation and characterization is obtained due to combination of trapping columns with analytical columns: Once separated by passing the trapping columns, the hydrophobic and hydrophilic compounds become fractionated directly by use of analytical columns that are connected to the trapping columns, whereby the subsequent identification of the fractionated compounds is facilitated. Accordingly, a poly-dimensional analytical device is provided which allows to carry out analyses without waste of time, since the sample may be analyzed completely in one apparatus.

[0012] Further aspects refer to analyzing said biological fluidic samples comprising hydrophobic and hydrophilic compounds, taking into consideration that it is most desirable to analyze even smallest molecules such as small peptides. Additional advantages result when the system is miniaturized to chip size, accordingly permitting the separation and analysis of very small sample volumes, which means that the number of analyses per time can be increased.

#### BRIEF DESCRIPTION OF DRAWINGS

[0013] Other objects and many of the attendant advantages of embodiments of the present invention will be readily appreciated and become better understood by reference to the following more detailed description of embodiments in connection with the accompanied drawings.

[0014] **Fig. 1** shows schematically the analysis system, comprising a C-18 pre-column connected to a graphite pre-column, and the corresponding analytical columns,

[0015] **Fig. 2** shows schematically the analysis system, comprising a strong cation exchanging (SCX) column and a C-18 pre-column connected to a graphite pre-column, and the corresponding analytical columns, thus it shows a setting being suitable to perform the complex analytical procedure of a metabolomics and proteomics sample,

[0016] **Fig. 3** shows schematically the analysis system of **Fig. 2** with an additional

C-18 pre-column and corresponding analytical column, being coupled via a valve to the channel interconnecting the SCX column with the C-18 pre-column.

[0017] The embodiments of the present invention generally refer to the complete analysis of biological fluidic samples such as metabolites, comprising hydrophobic and hydrophilic compounds, by performance of one single procedure in one single analysis system.

[0018] According to a preferred aspect, the analysis system is composed of two different trapping columns or pre-columns T1, T2, respectively; one of which being adapted to trap hydrophobic compounds, the other one being adapted to trap hydrophilic compounds. These two trapping columns are in fluidic communication, the second trapping column T2 being arranged downstream to the first trapping column T1. Of course, one could arrange a plurality of trapping columns in series, but the ones trapping hydrophobic compounds generally need to be upstream the ones trapping hydrophilic compounds. So, the trapping columns T1, T2 provide the device for a first analytical dimension.

[0019] Then, each of the trapping columns T1, T2 is connected to an analytical column A1, A2; said analytical columns A1, A2 providing a second analytical dimension due to the further fractionations of the components according to an organic gradient.

[0020] In a preferred embodiment, a C-18 pre-column is selected as the first trapping column T1, and a graphite pre-column as second trapping column T2. Downstream to the C-18 pre-column T1 an analytical C-18 column A1 is located and downstream to the second pre-column T2, which is a graphite pre-column, an analytical graphite column A2 is arranged.

[0021] If it is desired to refine the resolution of the analytical system one can place a third type of column in the arrangement: The third column T3 is arranged upstream to the first trapping column T1, and said third column T3 is standing in fluidic communication with the first trapping column T1. One might choose a strong ion exchanging column (SCX) in order to have the option of separating compounds comprised of metabolom and proteome containing sample.

[0022] Generally, the fluidic communication between two of the trapping columns T1, T2, T3 that are connected in series as well as between a trapping- and an analytical column A1, A2 could be provided by use of a tube or a channel C; the one or the other channel C could be equipped with a valve or a fluid flow controller in order to determine the fluid, eluent or sample fluid flow SF. If one single column of the type "third column" T3 or "first column" T1 is merging into more than one downstream column, the interconnecting tube or channel C could be provided with a valve, said valve being coupled to one or more bypassing systems. The bypassing system could comprise another trapping and analyzing column T1', A1', as shown in **Fig. 3**.

[0023] The embodiments of the analysis system described above could be advantageously miniaturized to micro size, thus leading to a further embodiment: A microfluidic device which comprises the analysis system of the above embodiments. The microfluidic device could be designed as single- or as a multilayer microfluidic device, permitting to perform an economic analytical procedure with a high resolution.

[0024] **Fig. 1** shows schematically how the method according to one aspect of the present invention could be realized: First, the fluidic sample is injected into the first trapping column T1 of the analysis device, which is a C-18 pre-column herein. Said column serves as trap for the hydrophobic components such as hydrophobic metabolites of the sample. Then, the fluid is directed immediately onto a second trapping column T2 that is a graphite column. Said second column T2 is the suitable means in order to trap the hydrophilic metabolites or compounds, respectively. The second trapping column T2 is located downstream to the first one, accordingly the fluidic sample flows in the C-18 pre-column T1 firstly, and then in the graphite column T2.

[0025] If desired, one may connect a plurality of first and second trapping columns T1, T2, wherein firstly hydrophobic metabolites trapping columns and secondly the hydrophilic metabolites trapping columns are passed by the sample.

[0026] Once the hydrophobic and hydrophilic metabolites are trapped in a first step onto different columns T1, T2 and thus separated successfully, in a second step elution might be performed. As known so those skilled in the art, elution requires the

rinsing of the selected column with a fluid or eluent, respectively.

[0027] In a first elution step E2, elution of the components trapped on the second column T2 is done, which is the graphite pre-column T2. Said components were directed onto an analytical graphite column A2 and fractionated according to an organic gradient. Then, identification I and quantification of the separated components is done by means of mass spectroscopy (MS) and/or MS/MS- spectroscopy.

[0028] In a second elution step E1, elution of the components trapped on the first column – the C-18 pre-column – T1 is done, said components are flushed using an eluent onto an analytical C-18 column A1 and they become separated according to an organic gradient. As described in the first elution step E2, identification and quantification of the separated components is done by means of mass spectroscopy (MS) and/or MS/MS- spectroscopy. The MS-systems coming into question are TOF (time of flight spectroscopy), Q-TOF (Quadrupole-Time-Of-Flight spectroscopy) or FT-MS (Fourier transformed mass spectroscopy), an other option is the use of ion-trap mass spectrometer.

[0029] The analytical performance is known to those skilled in the art.

[0030] So, the above method enables the scientist to perform a time-saving analysis of highly complex samples, combining the steps of fractionation or separation, respectively, and identification efficiently.

[0031] One may wish now to carry out proteomics and metabolomics in one procedure: This can be done following another preferred method, using the analytical system being equipped with three types of columns, which are connected in series; see **Fig. 2**:

[0032] First, the complex fluidic sample FS2 is acidified by adding of acids such as acetic acid or formic acid or another suitable weak acid, charging peptide- and amine-compounds positively. Then, the fluidic sample FS2 is injected into the analytical system, being directed immediately onto a third column T3, which is an SCX column (strong cation exchange column) herein.

[0033] The positively charged compounds are retained in said third column T3,

whereas the fluidic sample FS2 with the neutral or negatively charged compounds passes said column and flows through the C-18 pre-column T1. The C-18 pre-column T1 retains hydrophobic metabolites and the uncharged peptides.

[0034] The second column T2 – which is the last one in the series and which is a graphite pre-column–, retains the hydrophilic metabolite compounds and the hydrophilic and small, non-charged peptide compounds.

[0035] In the following, the compounds retained in the graphite pre-column T2 are eluted when the elution step E1 is performed, thus they are flushed from the graphite-pre-column T2 onto an analytical graphite column A2. Herein, further fractionation is performed according to an organic gradient. Then, an identification step I is done by use of mass spectroscopy and/or MS/MS analyses as described above.

[0036] Afterwards, the compounds, which have been retained on the C-18 pre-column T1 become eluted in a second elution step E2 and are directed onto an analytical C-18 column A1, becoming fractionated according to an organic gradient, and being subjected to an identification step I, too.

[0037] Now, after the first and the second trapping- and analytical columns T1, T2, A1, A2 were unloaded, the components that have been retained on the SCX column become eluted, too, accordingly a third elution step is performed. This may be done according to a salt step gradient elution. Therefore, the components can be flushed with saline solution from the third column T3; the gradient is obtained by increasing the salt concentration step-wise. One may take 20 mM, 40 mM, 60 mM, 80 mM, 100 mM, 250 mM, 500 mM ammonium acetate, e.g.

[0038] Another option is to flush them by use of the back flush method on the analytical C-18 column T2 in order to perform fractionation according to an organic gradient. Subsequently an identification step I is performed.

[0039] **Fig. 3** depicts a further advantageous aspect: If it is desired to perform the eluting of the third column T3 in parallel to the eluting of the pre-columns T1, T2, one could choose an analysis system of **Fig. 3**, being provided with another first trapping column. Said additional pre- and analytical columns T1', A1' are coupled via a valve V

to the channel C1, which provides the fluidic communication between the SCX column and the C-18 pre-column. So, first the fluidic sample FS2 is inject into the analysis system, then an eluent such as a saline solution elutes the trapped positively charged molecules from the SCX column and brings them via the bypass comprised of valve V  
5 and additional channel C' to the additional trapping- and analyzing column T1', A1'.

[0040] Said method may be performed by use of conventional trapping columns having lab-size, or it can be advantageously be performed by use of a microfluidic arrangement: One could design micro sized C-18 columns as well as micro sized graphite columns. A chip could be designed on which chip the micro pre-columns  
10 (SCX, C-18 column and graphite-column) T1, T1', T2, T3 and the analytical columns (analytical C-18 column and analytical graphite-column) A1, A1', A2 are arranged.

[0041] It is most advantageous to retain components of samples of this complex composition, comprised of proteomic and metabolomic compounds, by direct combination of strong cationic exchange column, C-18 pre column and graphite pre-  
15 column, providing a complete retaining of that components, performing elution until the corresponding analytical column and performing analyses afterwards.

## CLAIMS

1. An analysis system to analyze fluidic samples (FS1,FS2),  
said analysis system comprising at least two trapping columns, wherein:
  - said at least two trapping columns are in fluidic communication,
  - 5 - a first trapping column (T1) is adapted for trapping hydrophobic compounds of the fluidic samples (FS1, FS2), and a second trapping column (T2) of said at least two trapping columns is adapted for trapping hydrophilic compounds of the fluidic samples(FS1, FS2),  
wherein the second trapping column (T2) is arranged downstream of the first
  - 10 trapping column (T1).
2. The analysis system of claim 1, comprising at least one analytical column per each of said trapping columns (T1,T2), wherein:
  - a first analytical column (A1) is connected downstream to the trapping column (T1) and
  - 15 - a second analytical column (A2) is connected downstream to the trapping column (T2).
3. The analysis system of claim 2 or any one of the above claims, comprising at least one of the features:
  - the first trapping column (T1) is a C – 18 pre-column,
  - 20 - the analytical column (A1) which is connected downstream to the C – 18 pre-column is an analytical C – 18 column,
  - the second trapping column (T2) is a graphite pre-column,
  - the analytical column (A2) which is connected downstream to the graphite pre-column is an analytical graphite column.
- 25 4. The analysis system of claim 1 or any one of the above claims, wherein at least one third trapping column (T3) is arranged upstream to the first trapping column (T1), and said third trapping column (T3) is in fluidic communication with the first trapping column (T1).
5. The analysis system of claim 4 or any one of the above claims, wherein said third
- 30 trapping column (T3) is a strong ion exchange column.

6. The analysis system of claim 4 or any one of the above claims, wherein a tube or a channel (C) provides a fluidic communication between the trapping columns (T1, T2, T3) and/or analytical columns (A1, A2) connected in series.
7. The analysis system of claim 6 or any one of the above claims, wherein said tube or the channel (C) comprises at least one of the following features:
  - a valve (V),
  - a fluid flow controller.
8. The analysis system of claim 6 or any one of the above claims, wherein:
  - the tube or the channel (C) comprises a valve (V),
  - a bypassing tube or channel (C') is provided and being coupled to the valve (V) which is comprised in the channel (C) or tube which provides the fluidic communication between the third trapping column (T3) and the first trapping column (T1), thus, the valve (V) permits the bypassing of a fluid which is not meant to be directed onto the trapping columns (T1, T2).
9. The analysis system of claim 8 or any one of the above claims, comprising: an additional trapping column (T1') and an additional downstream analyzing column (A1'), both of which being in fluidic communication, wherein the trapping column (T1') is coupled to the valve (V).
10. A microfluidic device comprising the analysis system of claim 1 or any one of the above claims.
11. The microfluidic device of the preceding claim, wherein the microfluidic device is a multilayer microfluidic device.
12. A method of analyzing fluidic samples (FS1, FS2), comprising:
  - injecting said fluidic sample (FS1, FS2) into an analytical system,
  - directing the fluidic sample (FS1, FS2) onto at least two trapping columns (T1, T2) standing in fluidic communication and being connected in series,
  - trapping hydrophobic compounds comprised in said fluidic sample (FS1, FS2) in at least one first trapping column (T1),
  - trapping hydrophilic compounds comprised in said fluidic sample (FS1, FS2) in

at least one second trapping column (T2),  
with the second trapping column (T2) being arranged downstream to the first  
trapping column (T1).

13. The method of claim 12 or any one of the above claims, comprising at least one  
5 of the features:

- eluting the hydrophilic compounds of said second trapping column (T2),
- directing the eluted hydrophilic compounds onto an analytical column (A2)  
being connected downstream to said second trapping (T2) column, whereby  
fractionation of the hydrophilic compounds is performed according to an organic  
10 gradient,

- identifying the hydrophobic compounds subsequent to fractionation,
- eluting the hydrophobic compounds of said first trapping column (T1),
- directing the eluted hydrophobic compounds onto an analytical column (A1)  
being connected downstream to said first trapping column (T1), whereby  
15 fractionation of the hydrophobic compounds is performed according to an organic  
gradient,

- identifying the hydrophobic compounds subsequent to fractionation.

14. The method of claim 12 or any one of the above claims, comprising at least one  
of the features:

- 20 - acidifying said fluidic sample (FS2), whereby peptides and amines comprised  
by the fluidic sample (FS2) become - at least partially - positively charged, prior  
to injecting said fluidic sample (FS2) into the analytical system,

- directing said fluidic sample onto a third trapping column (T3) being arranged  
upstream to the first trapping column (T1) prior to directing the fluidic sample  
25 (FS2) onto said first and second trapping column (T1,T2), whereby the positively  
charged peptides and amines become trapped in said third trapping column (T3).

15. The method of the preceding claim, comprising at least one of the features:

- subjecting the first trapping column (T1) and the analytical column (A1) to  
elution so that they are free from hydrophobic and hydrophilic compounds,

- 30 - eluting of said peptides and amines stepwise from said third trapping column  
(T3),

- directing the eluted peptides and amines onto the first trapping column (T1),
  - trapping said peptides and amines on said first trapping column (T1),
  - eluting said peptides and amines,
  - directing the eluted peptides and amines onto the analytical column (A1),
- 5       thereby obtaining fractionation according to an organic gradient,
- identifying the fractionated peptides and amines subsequent to fractionation.
16.   The method of claim 14 or any one of the above claims, comprising at least one of the features:
- eluting of said peptides and amines stepwise from said third trapping column
- 10       (T3),
- bypassing the eluted peptides and amines via a valve (V) and via a channel (C') onto an additional trapping column (T1'),
  - eluting said peptides and amines,
  - directing the eluted peptides and amines onto the analytical column (A1'),
- 15       thereby obtaining fractionation according to an organic gradient,
- identifying the fractionated peptides and amines subsequent to fractionation.
17.   The method of claims 15 or any one of the above claims, comprising:  
          performing the stepwise elution by use of a saline solution.

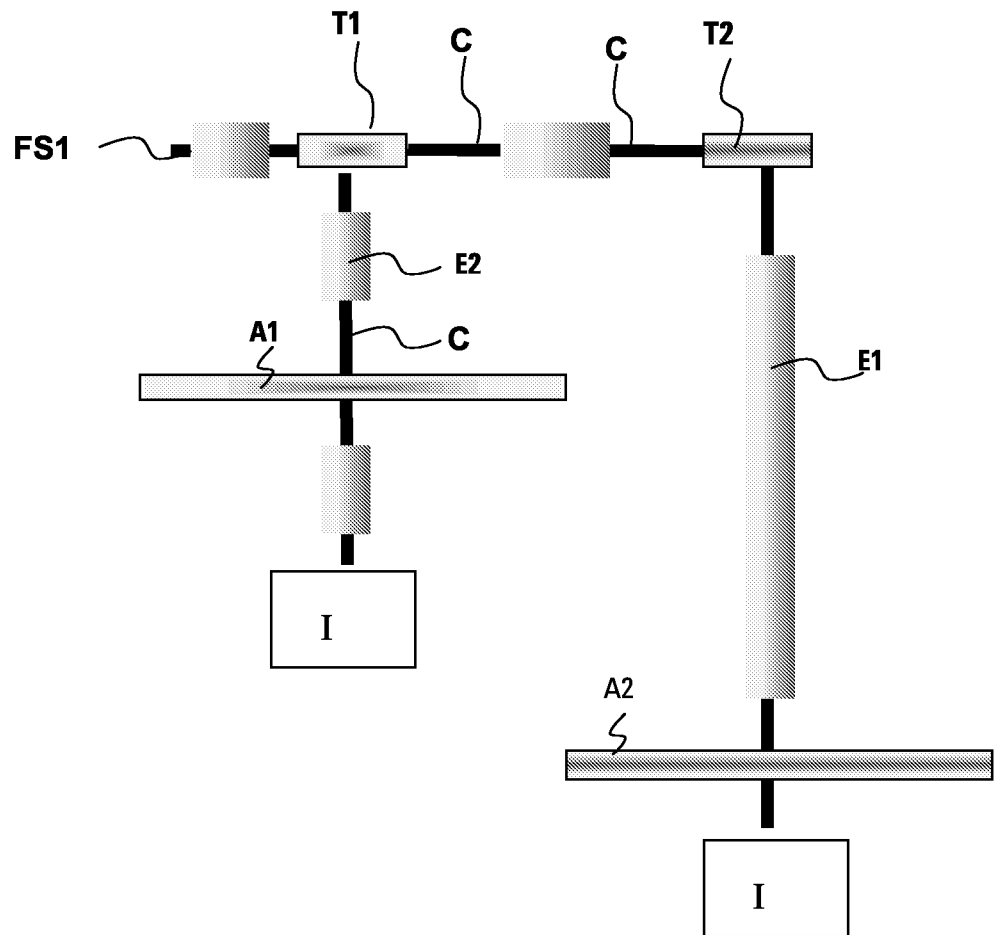


Fig. 1

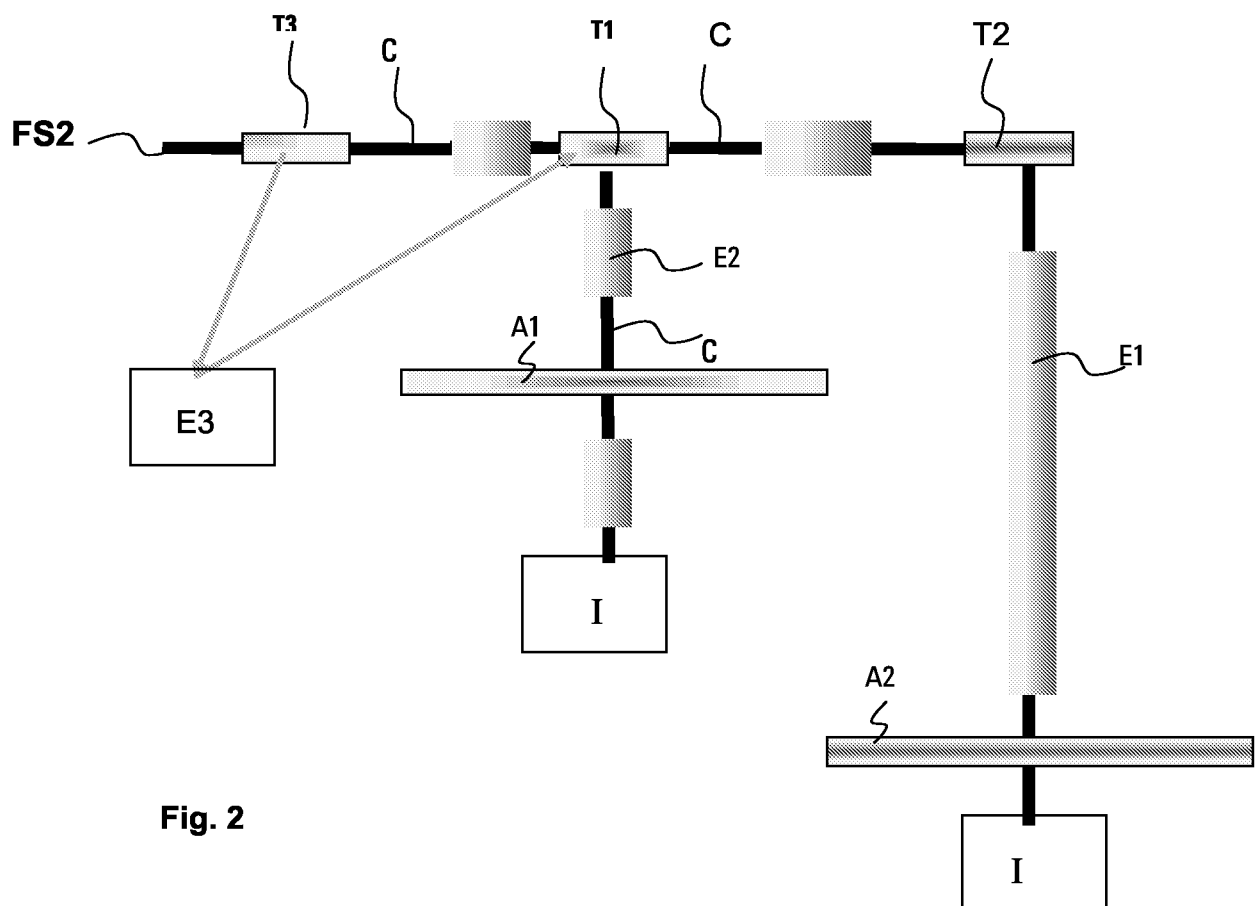
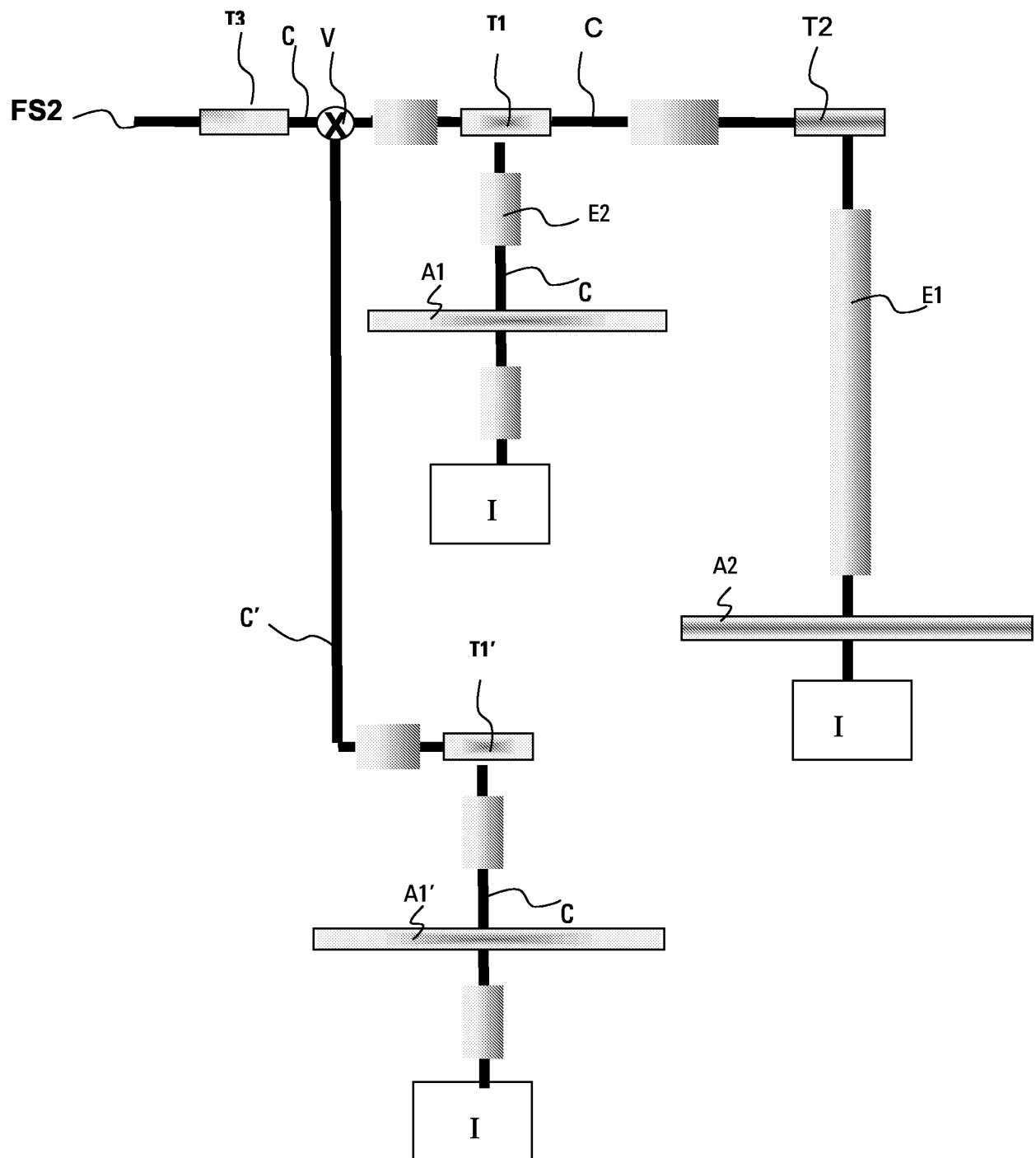


Fig. 2

**Fig. 3**



# INTERNATIONAL SEARCH REPORT

International application No  
PCT/EP2005/053608

## A. CLASSIFICATION OF SUBJECT MATTER

INV. G01N30/08 G01N30/46 C07K1/22  
ADD. G01N30/14 G01N30/88

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                     | Relevant to claim No. |
|-----------|------------------------------------------------------------------------------------------------------------------------|-----------------------|
| X         | US 4 577 492 A (HOLBA ET AL)<br>25 March 1986 (1986-03-25)<br>column 3, line 1 - column 4, line 46<br>-----            | 1,4,12,<br>13         |
| Y         | WO 98/46623 A (MARSDEN, JOHN, CHRISTOPHER;<br>AGNER, ERIK) 22 October 1998 (1998-10-22)<br>page 2, lines 5-30<br>----- | 14-17                 |
| A         | -----<br>-/--                                                                                                          | 1-17                  |

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

### \* Special categories of cited documents :

\*A\* document defining the general state of the art which is not considered to be of particular relevance

\*E\* earlier document but published on or after the international filing date

\*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

\*O\* document referring to an oral disclosure, use, exhibition or other means

\*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*G\* document member of the same patent family

Date of the actual completion of the international search

11 April 2006

Date of mailing of the international search report

20/04/2006

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Müller, T

# INTERNATIONAL SEARCH REPORT

International application No  
PCT/EP2005/053608

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                      | Relevant to claim No. |
|-----------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Y         | ISSAQ H J ET AL: "Multidimensional separation of peptides for effective proteomic analysis"<br>JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL SCIENCES & APPLICATIONS, ELSEVIER, AMSTERDAM, NL,<br>vol. 817, no. 1,<br>5 March 2005 (2005-03-05), pages 35-47,<br>XP004743612<br>ISSN: 1570-0232                               | 14-17                 |
| A         | the whole document                                                                                                                                                                                                                                                                                                      | 1-17                  |
| Y         | OPITECK G J ET AL: "COMPREHENSIVE TWO-DIMENSIONAL HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE ISOLATION OF OVEREXPRESSED PROTEINS AND PROTEOME MAPPING"<br>ANALYTICAL BIOCHEMISTRY, ACADEMIC PRESS, SAN DIEGO, CA, US,<br>vol. 258, no. 2, 1 May 1998 (1998-05-01),<br>pages 349-361, XP000960771<br>ISSN: 0003-2697 | 14-17                 |
| A         | page 351; figures 1,2                                                                                                                                                                                                                                                                                                   | 1-17                  |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2005/053608

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s) | Publication<br>date     |
|-------------------------------------------|---------------------|----------------------------|-------------------------|
| US 4577492                                | A                   | 25-03-1986                 | NONE                    |
| W0 9846623                                | A                   | 22-10-1998                 | AU 7062198 A 11-11-1998 |
|                                           |                     | EP 0975658 A2 02-02-2000   |                         |
|                                           |                     | US 6245238 B1 12-06-2001   |                         |