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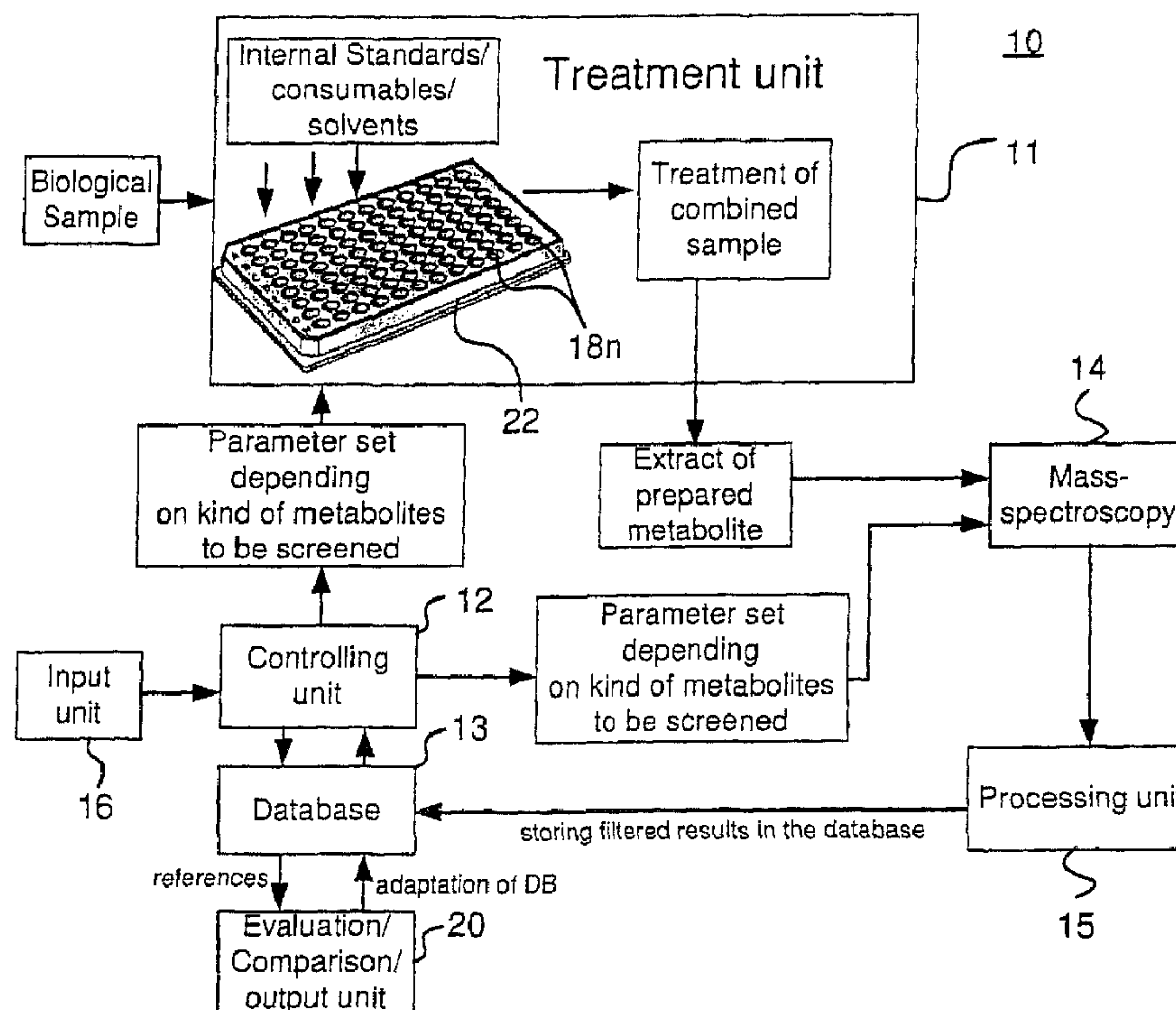
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(72) Inventeurs/Inventors:
RAMSAY, STEVEN LEWIS, AT;
STOEGGL, WOLFGANG MARKUS, AT;
WEINBERGER, KLAUS MICHAEL, AT;
GRABER, ARMIN, AT;
GUGGENBICHLER, WOLFGANG, AT

(73) Propriétaire/Owner:
BIOCRATES LIFE SCIENCES AG, AT

(74) Agent: KIRBY EADES GALE BAKER

(54) Titre : APPAREIL ET PROCEDE D'ANALYSE D'UN PROFIL DE METABOLITES
(54) Title: APPARATUS AND METHOD FOR ANALYZING A METABOLITE PROFILE



(57) Abrégé/Abstract:

The present invention relates to an apparatus for analyzing a metabolic profile in a biological sample. Further, it relates to a method for analysis of a metabolite profile in a biological sample. To provide such a method and an apparatus capable of identifying



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

relevant information in biological samples to provide a metabolite profile of a biological sample in a reliable way within a manageable time an apparatus for analyzing a metabolite profile in a biological sample containing at least one metabolite is proposed comprising: an input unit for inputting the kind of metabolites to be screened; a controlling unit for determining a parameter set for metabolites preparation and for mass spectrometry and analysis depending on the input of the kind of metabolites to be screened; a treatment unit for preparing the metabolites to be screened depending on the determined parameter set; mass spectrometer for performing mass spectrometry analysis on prepared metabolites depending on the parameter set; a database for storing results of analyzing and parameter sets for metabolite preparation and for mass spectrometry analyses; an evaluation unit for evaluating the results of mass spectrometry by use of reference results stored in the database to output an analysis of the metabolites profile.

ABSTRACT

The present invention relates to an apparatus for analyzing a metabolic profile in a biological sample. Further, it relates to a method for analysis
5 of a metabolite profile in a biological sample. To provide such a method and an apparatus capable of identifying relevant information in biological samples to provide a metabolite profile of a biological sample in a reliable way within a manageable time an apparatus for analyzing a metabolite profile in a biological sample containing at least one metabolite is
10 proposed comprising: an input unit for inputting the kind of metabolites to be screened; a controlling unit for determining a parameter set for metabolites preparation and for mass spectrometry and analysis depending on the input of the kind of metabolites to be screened; a treatment unit for preparing the metabolites to be screened depending on
15 the determined parameter set; mass spectrometer for performing mass spectrometry analysis on prepared metabolites depending on the parameter set; a database for storing results of analyzing and parameter sets for metabolite preparation and for mass spectrometry analyses; an evaluation unit for evaluating the results of mass spectrometry by use of
20 reference results stored in the database to output an analysis of the metabolites profile.

1 **Apparatus and Method for Analyzing a Metabolite Profile**

Description

5 The present invention relates to an apparatus for analyzing a drug profile and/or a metabolite profile in a biological sample. Further, it relates to a method for analysis of a drug and/or a metabolite profile in a biological sample.

10 Metabolomics is generally defined as the analysis of a substance or group of substances necessary for or taking part in a particular metabolic process in a human or animal body. It's also known as the metabolome analysis. Metabolomics is an evolving discipline that studies unique chemical fingerprints reflecting metabolic changes related to disease onset and
15 progression. Metabolite profiling, an area within metabolomics, measures small molecules or metabolites, contained in a human cell, tissue or organ, which are involved in primary and intermediary metabolism. The biochemical information resulting from metabolite analysis reveals functional end-points associated with physiological and pathophysiological processes, influenced by
20 both genetic predisposition and environmental factors, such as nutrition, exercise or medication (Harrigan, G.G. & Goodacre, R. (2003) Metabolic profiling: Its role in biomarker discovery and gene function analysis. Kluwer Academic Publishers, Boston/Dordrecht/London; Schmidt, C. (2004), *Journal of the National Cancer Institute*, 96, 732-734; Raudys, S. (2001) *Statistical and neural classifiers*, Springer-Verlag, London; Daviss, B. (2005) *The Scientist*, 19, 25-28).

 Metabolite profiling in combination with data mining approaches have the potential to revolutionize clinical diagnosis and drug development. In
30 particular, big pharma companies are under continuous pressure to discover new targets and novel, more efficacious and safer compounds, and expedite biomarker and drug discovery, and generally lower costs of pharmaceutical development. Therefore they rely increasingly on biotech companies to fill this innovative gap and future pipelines. In this context, innovative
35 bioanalytical and data mining techniques will play a fundamental role in saving costs by reducing time to market and drug attrition rates.

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1 Recently, due to significant advances in high-throughput technologies, a
wider set of the human metabolome - a thus far largely unexplored source of
bioinformation - is now accessible (Beecher, C. (2003). In Harrigan, G.G.,
Goodacre, R. (Ed). *Metabolic profiling: Its role in biomarker discovery and gene*
5 *function analysis* (pp. 311-319). Kluwer Academic Publishers, Boston/
Dordrecht/Londong; Dunn, W.B., Bailey, N.J. & Johnson, H.E. (2005)
Analyst, 130, 606-625). Statistical comparison of metabolite profiles can
expose multivariate patterns that have the potential to revolutionize the
health care system by specifically capturing latent warning signs of up-
10 coming diseases before any disease symptoms show up. Early disease
screening and prevention, opposed to late disease detection and expensive
therapeutic interventions, is probably the primary solution to afford health
care coverage in the future. By definition, these so called biomarkers are
"objectively measured indicators of normal biological processes, pathogenic
15 processes or pharmacological responses to a therapeutic intervention, and
intend to substitute for a clinical endpoint (predict benefit or harm) based on
epidemiologic, therapeutic, pathophysiologic or other scientific evidence"
(Biomarkers Definitions Working Group. (2001) *Clinical Pharmacology and*
Therapeutics, 69, 89-95). Interest in the discovery of novel biomarkers
20 originates from their broad range of potential applications and fundamental
impact on pharmaceutical industry dynamics and current health care sector
principles. Successful implementation of biomarkers in drug discovery can
reduce the time and cost of drug development while the application to
molecular diagnostics will improve patient compliance in clinical settings and
25 reduce unnecessary costs resulting from false diagnosis in addition to late
disease detection (Stoughton, R.B. & Friend, S.H. (2005) *Nature Reviews.*
Drug Discovery, 4, 345-350; Morris, M., & Watkins, S.M. (2005). *Current*
Opinion in Chemical Biology, 9, 407-412; McCandless, S.E. (2004). *Primary*
Care, 31, 583-604).

30

Qualitative and quantitative metabolite profiling technologies comprise a
range of advanced analytical and data processing tools, with the objective of
utilizing potential markers as a result of comparison of small molecule
components of biological systems. Tandem mass spectrometry (MS), for
35 example, detects hundreds of metabolites simultaneously from micro liter
quantities of biological samples, such as whole blood, serum, plasma, urine
or other body fluids from minute amounts, with high precision and

sensitivity (Roschinger, W., Olgemoller, B., Fingerhut, R., Liebl, B. & Roscher, A.A. (2003). *European Journal of Pediatrics*, 162 (Suppl 1), S67-76; Strauss, A.W. (2004). *J Clin Invest* 2004; 113:354-356; Kaltashov, I.A. & Eyles, S.J. (2005) *Mass spectrometry in biophysics: Conformation and dynamics of biomolecules*. Wiley). Quantification is achieved by reference to a wide range of appropriate internal standards. However, the amount of data or results which needs to be interpreted is very voluminous.

For example, WO 03/005628 describes a method for generating, viewing, interpreting and analyzing a quantitative database of metabolites. Further, US 2002/0009740 describes methods for drug discovery, disease treatment and diagnosis using metabolomics. US 6,455,321 describes a method for interpreting tandem mass spectrometry data for clinical diagnosis. US 6,258,605 describes an analytical method to screen the newborn populations' acylcarnitine and amino acids from blood samples.

Therefore, it is an object of the present invention to provide a method and an apparatus capable of identifying relevant information in biological samples to provide a drug and/or metabolite profile of a biological sample in a reliable way within a manageable time.

In one particular embodiment there is provided an apparatus for analyzing a drug and/or metabolite profile in a biological sample containing at least one drug and/or metabolite, comprising: an input unit for inputting the kind of drugs and/or metabolites to be screened; a controlling unit for determining a parameter set for drugs and/or metabolites preparation and for mass spectrometry analysis depending on the input of the kind of drugs and/or metabolites to be screened; a treatment unit for preparing the drugs and/or metabolites to be screened depending on the determined parameter set, the treatment unit comprising an automated liquid handling system, and at least one device for derivatisation of the at least one drug and/or metabolite present in the sample and for subsequent extraction of the derivatives, the

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device comprising: (A) one or more wells, (B) one or more inserts located in the wells, wherein the insert comprises a support comprising a sorbent material for liquids which support is impregnated with at least one organic internal standard; and (C) a retainer holding the insert within the well with a distance between the insert and the walls and the bottom of the well in order to allow a free circulation of fluids around the insert; mass spectrometer for performing mass spectrometry analysis on prepared drugs and/or metabolites depending on the parameter set, for quantification thereof; a database for storing parameter sets for drug and/or metabolite preparation and for mass spectrometry analyses; an evaluation unit for evaluating the results of mass spectrometry by use of reference results stored in the database to output an analysis of the drug and/or metabolite profile.

The object is solved by an apparatus for analyzing a drug and/or metabolite profile in a biological sample containing at least one drug and/or metabolite. The apparatus includes: an input unit for inputting the kind of drugs and/or metabolites to be screened, a controlling unit for determining a parameter set for sample preparation and for mass spectrometry analysis depending on the input of the kind of drugs and/or metabolites to be screened, a treatment unit for preparing the drugs and/or metabolites to be screened depending on the determined parameter set, a mass spectrometer for performing mass spectrometry analysis on prepared drugs and/or metabolites depending on the parameter set, a database for storing results of mass spectrometry analysis and/or parameter sets used for drug and/or metabolite preparation and for mass spectrometry analysis, an evaluation unit for evaluating and comparison of the results derived by mass spectrometry using reference results stored in the database, and for output of the analysis of the drug and/or metabolite profile included in the biological sample under consideration.

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1 The invention is based on the idea, that by providing an apparatus for
preparing a biological sample and for analyzing drugs and/or metabolites in
a biological sample reproducible conditions for analyzing are kept over a wide
range of analyses. Further, by providing an automated process for
5 preparation of the biological sample and for mass spectrometry analysis, the
comprehensive amount of data to be processed could be handled. By
adapting the evaluation of the results of mass spectrometry utilizing
reference results stored in the database, the analyses results derived from
drug and/or metabolite profiles could be improved by use of the knowledge
10 collected during previous analyses. Further, quantification by utilizing
appropriate internal standards is used for quantifying the drugs and/or
metabolites in a biological sample. By use of automated pre-analytical
procedures, the amount of results is reduced. A plurality of standard
operational procedures (SOP) is used for treating and handling a plurality of
15 biological samples in a standardized way depending on the drugs and/or
metabolites to be screened.

In the following, the invention is described with respect to the analysis of
metabolite profiles. However, it should be understood that the invention is
20 not restricted thereto but is also applicable to the analysis of drug profiles
(i.e. for therapeutic drug monitoring, TDM) in a similar way.

In one aspect of the invention a method is provided for analysis of a
metabolite profile in a biological sample comprising the steps: providing the
25 biological sample to an apparatus for analyzing at least one metabolite,
providing information of the metabolites to be screened to the apparatus,
preparing the biological sample depending on the information of metabolites
to be screened, extracting the prepared metabolites, providing the mass
spectrometer with the sample extract, performing one or more mass
30 spectrometry analyses on the prepared metabolites, processing and
evaluating results of mass spectrometry of the metabolites to be screened
and compared with reference values and pre-annotated information of the
targeted metabolites, and generating output data.

35 Thus, the method for analysis of a metabolite profile comprises generally the
steps of sample preparation and separation of the metabolites, analysis by
use of mass spectrometry and data processing.

1 There are two kinds of strategies which may be applied for metabolite
profiling. When using mass spectrometry based metabolite profiling a
qualitative non-targeted and the quantitative targeted approach can be used.
Depending on the selected strategy the extent and type of subsequent data
5 processing is determined.

A targeted profiling scheme is used according to the invention to
quantitatively screen for known small molecule metabolites using multiple
reaction monitoring (MRM), precursor and neutral loss scans. A plurality of
10 different classes of compounds (e.g. amino acids, peptides, acylcarnitines,
monosaccharides, lipids, and phospholipids etc.) is selected for analyzing.
These compounds cover relevant metabolic pathways of a disease being
investigated. The quantification of the metabolites in the compounds of the
biological sample is achieved by reference to appropriate internal standards.

15 Thus, the invention uses a known quantity of an internal standard, which is
combined with the biological sample. By using the apparatus and the method
for metabolite profiling the parts or aliquots of the biological sample to be
screened are combined with a plurality of different internal standards. The
20 internal standards are identified and quantified and, therefore, result in
mass spectrometry data, which correspond to the known concentration.
Further by dividing the biological sample into a plurality of aliquots only
small amounts of biological sample are required. Each aliquot is treated and
prepared in a standardized way, which is controlled by the use of the
25 parameter set derived from the standard operation procedure (SOP). By using
the automated sample preparation (treatment) and separation (e.g.
derivatisation, extraction), depending on the metabolites to be screened, it is
possible to perform a mass spectrometry analysis on each extract of a
respective aliquot, generating a mass spectrometry result having a plurality
30 of peaks of the metabolites included in the prepared and separated aliquot
and the peaks of the internal standards corresponding to known
concentrations.

After mass spectrometry data processing is performed on the results of mass
35 spectrometry analysis. The data processing may use chemometric and
statistical procedures including isotope correction, noise filtering,
normalization and scaling, content based filtering of the results, and

1 quantification and annotation of the targeted detected metabolites utilizing
the known characteristics of the appropriate internal standards and the pre-
annotated information in the database. The processed results are stored and
compared with reference values stored in the database to derive information
5 whether the metabolites in the results belong, for instance, to a healthy or to
diseased patient, or are characteristic for a certain disease stage, or reflect
response to a pharmacological substance or drug. The database may be
adapted based on information derived during comparing the results of the
mass spectrometry with reference values stored in the database. This
10 learning process may be used for providing an adaptation of the parameter
sets and/or standard operational procedure, which are used for treating the
biological samples for analysis of the metabolite profiles. The results of the
method for analyzing metabolite profiles may be so called biomarkers,
characterizing at least one or more very significant metabolites to indicate a
15 disease or change of a normal healthy state of a patient, or response to
pharmacological substance or drug.

Further advantages of the present invention could be derived from the
dependent claims.

20

In the following, exemplary embodiments will be described by the
accompanied drawings, in which

Fig. 1 shows a block diagram of an apparatus according to the present
25 invention;

Fig. 2 shows a schematic diagram illustrating components of the treatment
unit according to the present invention;

30 Fig. 3 shows a simplified flowchart according to the method of the present
invention;

Fig. 4 shows a schematic overview for a pre-clinical trial which uses the
inventive method and the apparatus;

35

Fig. 5 shows a schematic illustration for comparing non-targeted and
targeted metabolite profiling according to the present invention;

1 Fig. 6 illustrates results of a mass spectrometry for metabolites and internal standards according to the present invention using targeted metabolite profiling;

5 Fig. 7 describes a cross-sectional view of a singular device preferably used according to the invention containing wells or vials and its assemblage from individual components. Reference number (1) shows a well/vial. Reference number (2) shows an insert comprising an immobilising stationary phase of glass, celluloses or other suitable material (i.e. a porous support) optionally
10 containing internal standards with optional (micro)encapsulation; reference number (3) shows a retainer to hold the porous support in the well or vial, which is chemically inert to derivatives and solvent; reference number (4) shows a filter; reference number (5) shows an outlet, which opens under pressure of centrifugal or gravitational force or vacuum;

15 Fig. 8 describes a neutral loss scan of 135 in negative mode using ion tandem mass spectrometry of phenylthiorurea amino acid derivatives (PTU), showing amino acids from a red blood cell sample and their corresponding stable isotope internal standards prepared with the multi-device described in
20 Example 2;

Fig. 9A describes a precursor scan 184 in positive ion mode (A), showing the multi-devices ability to extract phospholipids from a red blood cell sample of Example 2. For example sphingomyelins and phosphatidylcholines are
25 observable in the m/z range 700 - 840, and lyso phosphatidylcholines in the m/z range 400 - 650;

Fig. 9B describes an MRM scan (multiple reaction monitoring) in positive ion mode of Example 2;

30 Fig. 10 describes an example of how immunotherapy drugs Sirolimus, Everolimus, Cyclosporin A, Tacrolimus, and internal standards Ascomycin and Cyclosporin D from a quality control blood sample are analysed with LCMS to generate quantitative data. The area under the integrated peaks of
35 the internal standard Cyclosporin D and Ascomycin, of known concentrations, are used for comparison against the area under the peak of the immunosuppressants in the five quality control samples containing

1 known concentration amounts. This provides a measure of accuracy for all
four drugs;

Fig. 11 describes a calibrator curve from calibrators for Sirolimus obtained
5 from multi-device with cellulose insert (Example 3);

Fig. 12 describes a calibrator curve from calibrators for Everolimus obtained
from multi-device with cellulose insert (Example 3);

10 Fig. 13 describes a calibrator curve from calibrators for Cyclosporin A
obtained from multi-device with cellulose insert (Example 3);

Fig. 14 describes a calibrator curve from calibrators for Tacrolimus obtained
from multi-device with cellulose insert (Example 3).

15

Before explaining embodiments based on the figures some definitions or
explanations are given.

Internal standard

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An internal standard as used in the invention should be understood to be
any reference materials of known absolute amounts that are used for
comparisons to similar or identical compounds in order to quantify unknown
amounts of compounds present in a given sample. Preferably the internal
25 standard is an organic internal standard. Internal standards as used in the
present invention may belong to the same group or family of compounds to
be analyzed in the biological sample. However, they are preferably labelled
with isotopes in order to properly allow a distinction between the metabolites
of the sample and the internal standard. Any other way of distinguishing the
30 metabolites of the sample from the internal standards, however, may also be
used. For example, non-naturally occurring compounds may also be used as
internal standards.

Specific examples for the internal standard as used in the present invention
35 are indicated in Table 1.

1 **Table 1****Lipids:**

5	abbreviation	Full name	Comments
			Fatty acid chain length
	SM(d18:1/6:0)	N-Hexanoyl-sphing-4-enine-1-phosphocholine	6
	GPCho(9:0/0:0)	1-Nonanoyl-sn-glycero-3-phosphocholine	9
	GPCho(14:0/14:0)	1,2-Ditetradecanoyl-sn-glycero-3-phosphocholine	28
10	GPIns(16:0/16:0)	1,2-Dihexadecanoyl-sn-glycero-3-phospho-(1'-myo-inositol)	32
	GPCho(20:0/20:0)	1,2-Di-(3,7,11,15 tetramethyl hexadecanoyl)-sn-glycero-3-phosphocholine	40
	GPSer(20:0/20:0)	1,2-Di-(3,7,11,15 tetramethyl hexadecanoyl)-sn-glycero-3-phosphoserine	40
	GPSer(6:0/6:0)	1,2-Dihexanoyl-sn-glycero-3-phosphoserine	12

15

Amino acids

20	Abbreviation	Full name	Comments
	13C2-15N-Gly	13C2-15N-Glycine	
	D4-DL-Ala	D4-DL-Alanine	
	15N2-L-Argl	15N2-L-Arginine HCl	
	D3-DL-Asp	D3-DL-Aspartic Acid	
	15N2-L-Asn	15N2-L-Asparagine H2O	
	D3-L-Glu	D3-L-Glutamic Acid	
	D5-L-Gln	D5-L-Glutamine	
25	13C6-L-His	13C6-L-Histidine H2O	
	13C6-L-Ile	13C6-L-Isoleucine	
	13C-L-Lys	13C-L-Lysine 2HCl	
	D3-L-Met	D3-L-Methionine	
	D6-L-Orn	D6-L-Ornithine HCl	
	D5-L-Phe	D5-L-Phenylalanine (ring 5-phe)	
30	D7-L-Pro	D7-L-Proline	
	D3-DL-Se	D3-DL-Serine	
	13C4-L-Thr	13C4-L-Threonine	
	15N2-L-Trp	15N2-L-Tryptophan	
	D4-L-Tyr	D4-L-Tyrosine (ring 4-tyr)	
35	D8-DL-Val	D8-DL-Valine	

1 **Acylcarnitines**

Abbreviation	Full name	side chain length
D3-C0	[d3-methyl]-Carnitine.HCl	C=0
D9-C0	[d9-trimethyl]-Carnitine.HCl	C=0
5 D3-C2	[d3]-Acetyl-L-carnitine.HCl	C=2
D3-C3	[3,3,3-d3]-Propionyl-L-carnitine.HCl	C=3
D3-C4	[4,4,4-d3]-Butyryl-L-carnitine.HCl	C=3
D7-C4	[d7]-Isobutyryl-L-carnitine.HCl	C=4
D3-C5	[5,5,5,-d3]-Valeryl-L-carnitine.HCl	C=4
D9-C5	[d9]-Isovaleryl-L-carnitine.HCl	C=5
10 D3-C6	[6,6,6-d3]-Hexanoyl-L-carnitine.HCl	C=6
D3-C8	[8,8,8-d3]-Octanoyl-L-carnitine.HCl	C=8
D3-C10	[10,10,10-d3]-Decanoyl-L-carnitine.HCl	C=10
D3-C12	[12,12,12-d3]-Dodecanoyl-L-carnitine.HCl	C=12
D3-C14	[14,14,14-d3]-Tetradecanoyl-L-carnitine.HCl	C=14
15 D3-C16	[16,16,16-d3]-Hexadecanoyl-L-carnitine.HCl	C=16
D3-C18	[18,18,18-d3]-Octadecanoyl-L-carnitine.HCl	C=18

20

Reducing monosaccharides

Abbreviation	Full name	Comments
13C6-Glc	13C6-Glucose	

25 **Pyruvate/Lactate**

Abbreviation	Full name	Comments
13C3-Pyr	13C3-Pyruvate	

Creatinine

30

Abbreviation	Full name	Comments
	[d3-methyl]-Creatinine	

35

1 Immunosuppressants

	Abbreviation	Full name(s)	Comments
		Ascomycin	
		Cyclosporin D	
5		32-Desmethoxyrapamycin	

Biological Sample

10 A biological sample as used in the invention should be understood to be any sample of, relating to, caused by, or affecting life or living organisms, biological processes, such as growth and digestion.

15 Examples of a biological sample may include, but are not limited to blood, cell culture supernatant, saliva, tears, urine, blood, serum, plasma, sweat, vaginal fluids, semen, feces, mucous, breast milk, ascites, lymph, pleural effusion, synovial fluid, bone marrow, cerebro-spinal fluid, and washings from bodily cavities (e.g., bronchial lavage), hair, tissue, bones, or teeth.

20 Preferably, the biological sample is a liquid sample. More preferable, the biological sample is blood, and most preferable human blood. Liquid means a state of matter with definite volume but no definite shape at 25°C, like water.

Consumable

25 A consumable should be understood to be any compound suitable for being used in derivatisation and extraction of the metabolites.

Metabolite profile

30 A metabolite profile as used in the invention should be understood to be any defined set of values of quantitative results for metabolites that can be used for comparison to reference values or profiles derived from another sample or a group of samples. For instance, a metabolite profile of a sample from a diseased patient might be significantly different from a metabolite profile of a
35 sample from a similarly matched healthy patient.

1 Metabolites, such as, but not limited to amino acids, peptides, acylcarnitines, monosaccharides, lipids and phospholipids, prostaglandins, hydroxyeicosatetraenoic acids, hydroxyoctadecadienoic acids, steroids, bile acids and glyco- and phospholipids can be detected and/or quantified.

5

Examples for metabolites, which can be detected and/or quantified, are listed in Table 2. In particular, lipid species from C4:X to C46:X (where X, the degree of saturation, ranges from 0 to 8) in any given fatty acid residue are shown. The lipids include also sphingolipids and glycosphingolipids.

10

Amino acids, which can be detected and quantified, are proteogenic or non-proteogenic amino acids. The proteogenic amino acids and the non-proteogenic amino acids, as indicated in Table 2, are preferred.

15 Acylcarnitines from C4:X to C18:X (wherein X is the degree of saturation and ranges from 0 to 8 in any given acid residue) can be detected and/or analyzed. Examples for acylcarnitines which are preferred are also listed in Table 2.

20 Monosaccharides are preferably reducing or non-reducing carbohydrates. Examples of monosaccharides are also listed in Table 2.

Table 2 – Metabolites that are amenable to mass spectrometric analyses according to the invention

25

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35

1 **Lipids:**

	Abbreviation	Full name of lipid subtype	Comments
5		Glycerophospholipids, sphingolipids and glycosphingolipids	Fatty acid chain length
	Sph	Sphingosine	none
	Cer	Ceramide	C6:X – C36:X
10	SM	sphingomyelin	C6:X – C36:X
	Sph pchol	sphingosylphosphorylcholin	none
	Sph dh	dihydrosphingosine	none
15	PC	phoshatidylcholine	C4:X – C46:X
	PI	phosphatidylinositol	C4:X – C46:X
	PS	phosphatidylserine	C4:X – C46:X
20	PC (a)	lysophoshatidylcholine	C4:X – C32:X
	PI (a)	lysophosphatidylinositol	C4:X – C32:X
	PS (a)	lysophosphatidylserine	C4:X – C32:X
25	PC (e)	plasmenylphoshatidylcholine	C4:1 – C32:X
	PC (e)	plasmanyphoshatidylcholine	C4:0 – C32:0

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1 **Amino acids****Proteinogenic amino acids**

		abbreviation	Full name	Comments
5	A	Ala	Alanine	
	D	Asp	Aspartic acid	
	E	Glu	Glutamic acid	
10	F	Phe	Phenylalanine	
	G	Gly	Glycine	
	H	His	Histidine	
15		Xle	Leucine/Isoleucine	
	K	Lys	Lysine	
	M	Met	Methionine	
20	P	Pro	Proline	
	R	Arg	Arginine	
	S	Ser	Serine	
25	T	Thr	Threonine	
	V	Val	Valine	
	W	Trp	Tryptophan	
30	Y	Tyr	Tyrosine	
		ADMA	Asymmetrical dimethyl arginine	LC MS method
		SDMA	Symmetrical dimethyl arginine	LC MS method
35	Q	Gln	Glutamine	
	N	Asn	Asparagine	

1 **Non-proteinogenic amino acids**

5

	abbreviation	Full name	Comments
	O Orn	Ornithine	
	Cit	Citrulline	

Acylcarnitines

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	abbreviation	Full name	Comments
	C0	Carnitine (free carnitine)	C0
	C2:X to C18:X	Acylcarnitine	C0:X to C26:X
	C3:X-OH to C18:2-OH	Hydroxylacylcarnitine	C3-OH to C18:2-OH
	C3:0-DC to C18:2-DC	Dicarboxylacylcarnitines	C3:0-DC to C12:0-DC

Reducing monosaccharides

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Others

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	abbreviation	Full name(s)	Comments
	H	Hexose	
	P	Pentose	
	dH	Deoxyhexose	

	abbreviation	Full name	Comments
	Cr	Creatinine	
		Spermidine	LC MS method
		Spermine	LC MS method

1	Putrescine	LC MS method
	Dopamine	LC MS method
5	Serotonin	LC MS method
	Prostaglandins	LC MS method
	Hydroxyeicosatetraeneoic (HETEs)	LC MS method
10	Hydroxyoctadecadienoic (HODEs)	LC MS method
	Leukatrienes	LC MS method
	Thromboxanes	LC MS method
15	Bile acids	LC MS method
	Sterols	LC MS method
	Cholesterols	LC MS method
20	Vitamins and cofactors	
	Drugs and drug metabolites	LC MS method

25 ***Drug profile***

A drug profile as used in the invention should be understood to be any defined set of values of quantitative results for one or more drugs or drug metabolites in a specified sample. Moreover, immunosuppressants as specific
 30 examples can also be detected and quantified. For example, a drug profile of a transplant patient would give the physician the immediate circulating amounts of one or more drug therapies in use, and future dosages could therefore be increased or decreased according to the quantities measured to achieve best therapeutic range. Such an analysis is designated as
 35 therapeutic drug monitoring (TDM). Immunosuppressants in accordance with the present invention are to be understood as drugs that may be used in immunosuppressive therapy to inhibit or prevent activity of the immune

1 system. Clinically they are used to prevent the rejection of transplanted
organs and tissues and in treatment of autoimmune diseases such as
rheumatoid arthritis, myasthenia gravis, systemic lupus erythematosus,
Crohn's disease, and ulcerative colitis. Immunosuppressants as defined
5 herein basically can be classified into four groups: glucocorticoids,
cytostatics, antibodies, and drugs acting on immunophilins. Preferred
examples of immunosuppressants as used in the present invention are
Cyclosporin A, Sirolimus, Everolimus and Tacrolimus.

10 **Multiple Device**

A multi-device as used in the invention should be understood to be any
multiple devices joined together to form a multi-device such as a microtitre
plate standard format.

15

A microtitre plate as used in the invention should be understood to be any
plastic sample holder used in biology or chemistry research facilities. The
microtitre plate standard was formalized by the Society for Biomolecular
Screening (SBS) in 1996. It typically has 6, 24, 96, 384 or 1536 sample wells
20 arranged in a 2:3 rectangular matrix. The standard governs well dimensions
(e.g. diameter, spacing and depth) as well as plate properties (e.g. dimensions
and rigidity).

The device in multiple format herein named a multi-device may also have a
25 different format. Pre-embedding several vials, as an example 6 wells, give a 6
point calibration with multiple calibrating compounds. Quality control
samples containing known metabolites and/or multiple drug concentrations
are also pre-embedded.

30 The device according to a preferred embodiment of the present invention
additionally comprises a porous support such as cellulose or glass fibre as
examples, preferably retained in at least one well by a chemically inert
retaining structure. The porous support preferably has embedded into it
internal standards in a dry state; optionally microencapsulated (coated) with
35 a protective or covering material or mixture of chemicals, for example
polyethylene glycol 1000, Phosphatidylcholine, glycerol or sorbitol.

1 **Insert**

5 The term 'insert' as used in accordance with a particular preferred embodiment of the invention should be understood to be a porous support containing the internal standards with an optional chemical protectant as mentioned above. The insert may have any geometrical form as long as the insert fits into the well or vial of the device. In a preferred embodiment the insert is arranged within the well or vial of the device by using a retainer. Said retainer is indicated as reference number (3) in Figure 7. In a particular
10 preferable embodiment the retainer (3) allows the insert to be arranged within the well without any direct contact between the insert and the well. Thus, the insert is located above the bottom of the well preferably within a distance of 2 to 10 mm, more preferably of 3 to 5 mm by using the retainer. In other words, in a preferred embodiment there is a so-called "gap" or
15 "distance" between the bottom of the well and the insert and/or the between the walls of the well and the insert. As the retainer in the preferred embodiment any retainer is suitable as long as it allows the formation of the gap between the bottom of the well and the insert. Such an arrangement allows for maximum surface area of the support to precipitate the samples
20 onto. The design further ensures the insert is fully accessed by flow of air or other drying gas around the insert to enable rapid drying of sample after application. This design principal also ensures the insert is fully accessed by flow of solvent from all sides enabling metabolite or drug extraction from sample with minimized protein or salt contaminants. Thus, the pores of the
25 support allow a reaction (derivatisation) to proceed within the support itself, minimizing solvent usage and also subsequent removal as evaporation of excess derivative and solvents is provided by maximum surface area to circulating drying gases (air or nitrogen) around the sample. The increased surface area and solvent mobility around the entire support also ensures
30 high extraction efficiencies using appropriate solvents. In other words, the above-mentioned gap allows an almost free arrangement of the insert within the well and an improved circulation of fluids flowing through the well.

35 Moreover, according to a preferred embodiment of the invention the device may comprise more than one insert arranged in stacks, wherein the respective inserts are more preferably arranged with the above-mentioned gap between each other in order to allow the circulation of fluids.

1 However, it should be understood that the present invention does not necessarily comprise the insert as described above. The insert, however, is used in accordance with a preferred embodiment.

5 **Support**

The support as used in the invention may be any support preferably with at least medium degree, preferably a high degree of porosity. Such a support in principle is known in the prior art and also commercially available.

10

The porosity " Φ " of a medium (i.e. the support) is defined to be the proportion of the non-solid volume to the total volume of material, and is defined by the ratio:

15

$$\Phi = V_p / V_m$$

where V_p is the non-solid volume (pores and liquid) and V_m is the total volume of material, including the solid and non-solid parts.

20 Thus, porosity is a value between 0 and 1, typically ranging from less than 0.01 for solid granite to more than 0.5 for peat and clay, although it may also be represented in percent terms by multiplying the fraction by 100 %. The porous support of the invention has a porosity of at least 30 %, more preferably at least 50 %, even more preferably at least 70 %, and most preferably at
25 least 90 %.

The porous support as used in the insert may be of any suitable material, but it is preferably a solid support. More preferably the porous support is comprised of a sorbent material for liquids (also named liquid sorbent
30 material). Still more preferably the support is consisting of the liquid sorbent material. The sorbent material may be an adsorbent or an absorbent material.

A liquid sorbent material as used in the invention should be understood to be
35 any material that allows solutions of internal standards and subsequent samples for analysis to be adsorbed or absorbed uniformly throughout the pores additionally allowing carrier solvent removal by evaporation.

1 The liquid being ad- or absorbed by the support material can be any kind of liquid, but it is preferably a volatile liquid at atmospheric pressure, for example a liquid having a boiling point less than about 250 degrees Centigrade (C) at atmospheric pressure.

5

More preferably the liquid sorbent material according to the invention comprises at least one of a carbohydrate material, such as cellulose material, glass fibres, glass beads, polyacrylamide gel, porous plastic inert polymer and porous graphite. The said porous sorbent material may more preferably
10 be comprised of a carbohydrate material or derivative thereof, such as agarose, agar, cellulose, dextran, chitosan, or konjac, carrageenan, gellan, or alginate. The liquid sorbent material is, however, most preferably made of cellulose or glass fibres. The shape of the support or liquid sorbent material is not particularly limited but preferably is of a circular, square or scroll or nautilus dimension. According to the invention the shape of the support or
15 sorbent material is adapted to the shape of the well or vial of the device. As mentioned, the porous support or sorbent material may be fixed or secured in its position in the well or vial by a fixing structure such as a retainer (indicated as (3) in Figure 7).

20

The porous support comprising the liquid sorbent material mainly has two functions. The first is to embed the internal standards (reference material) as described below at predefined concentration ready for addition of the biological sample. The second is the immobilizing of the contents of each
25 sample. This immobilizing step induces cell lysis, protein immobilisation/precipitation and salt and many other drug or metabolite retention from each of the samples. The porosity of the support is then essential for maximal exposure to both the derivatising agents and also the extraction solvent to be added for the analysis.

30

Encapsulation of the Standards

The internal standard in accordance with a preferred embodiment of the invention is encapsulated with a covering material or protecting material
35 protecting the internal standard from degradation and chemical reactivity prior to use. The protection of the internal standard from degradation and chemical reactivity can prevent many forms of breakdown or chemical

1 modification of the internal standard, such as prevention of the action of
sunlight, temperature, and microorganisms, in particular prevention from
any process that transforms the internal standard into breakdown or
degradation products, thereby influencing the outcome of a quantitative
5 analysis.

A protecting/covering material as used in the invention should be understood
to be any material for shielding or designed to shield the internal standard(s)
against degradation.

10 The protecting/covering material according to the invention can be any
material suitable for protecting the internal standard from an environmental
influence as mentioned above. The covering material according to the
invention preferably comprises at least one of a polymer, a micelle-forming
15 compound, a liposome-forming compound and a polyhydroxy compound, or
any mixtures thereof.

If the covering material is a polymer, said polymer as used in the invention is
not particularly limited and understood to be a high molecular weight organic
20 compound, such as having a weight average molecular weight of at least 500
g/mol or at least 1,000 g/mol or at least 5,000 g/mol or at least 10,000 g/
mol, which is either natural or synthetic, whose structure can be represented
by a repeated small unit of a monomer. A synthetic polymer is formed in a
manner known in the art such as by addition or condensation polymerization
25 reaction of monomers. The polymer of the invention may also be a co-
polymer, when two or more different monomers are involved. A homopolymer
is a polymer which is formed from only one type of monomer.

The polymer according to the invention is preferably a polyalkylene glycol
30 homopolymer or copolymer or a mixture thereof. The weight average
molecular weight is preferably about 1000 daltons (Da). More preferably the
polymer according to the invention is a polyethylene glycol (PEG) or
polypropylene glycol (PPG), preferably PEG 1000 having a weight average
molecular weight of about 1000 Da, as it is soluble or miscible with highly
35 polar and less polar to unpolar solvents.

1 If the covering material is a micelle-forming compound said compound as
used in the invention should be understood to be any compound which can
induce submicroscopic aggregation of molecules, as droplets in a colloidal
system. The micelle-forming compound according to the invention is
5 preferably a surfactant.

A surfactant as used in the invention is understood to be any chemical
compound that reduces the surface tension between two liquids; or any
surface-active agent which increases the emulsifying, foaming, dispersing,
10 spreading and wetting properties of a product, in particular any organic
compound whose molecules contain a hydrophilic group at one end and a
lipophilic group at the other end. Suitable surfactants comprise cationic,
anionic, nonionic, and amphoteric surfactants. Preferably the surfactant is
phosphatidyl (C17:0)₂.

15

If the covering material is a liposome-forming compound said compound as
used in the invention should be understood to be any compound which can
build artificial microscopic vesicles consisting of an aqueous core enclosed in
one or more phospholipid layers, used to convey vaccines, drugs, enzymes, or
20 other substances to target cells or organs.

A phospholipid as used in the invention is understood in the general way in
the art and should comprise phosphorous containing lipid, such as lecithin
and cephalin, made of glycerol and fatty acids, with a phosphate group
25 attached. More preferably the liposome forming compound is a phospholipid,
such as a phosphatidyl choline or a phosphatidyl ethanolamine or derivatives
thereof.

If the covering material is a polyhydroxy compound said compound as used
30 in the invention should be understood to comprise at least two hydroxy
groups. Most preferably the polyhydroxy compound is sorbitol and/or
glycerol.

Preferably the encapsulation according to the invention is a
35 microencapsulation. A microencapsulation as used in the invention should
be understood to be any encapsulation of microcapsules, which are small,
preferably microscopic capsules designed to release their contents when

1 broken by pressure, dissolved or melted. In particular, the capsules of the
invention preferably have a diameter of less than 100 micrometer, more
preferably less than 10 micrometer and most preferable less than 1
micrometer.

5

Microencapsulated internal standards are robust in terms of storage and
shipping and are stable regarding oxidation and degradation processes, and
they have a relatively long shelf-life. The microencapsulation is preferably
standardized to prepare synthetic quality control material based on
10 microencapsulated components. This is typically achieved by drying internal
standards and other protected samples down with the covering material in a
solvent that is a suitable solvent for these compounds like a chloroform/
methanol mixture for phospholipids. Typically addition of water to these
samples induces micelle and/or liposome formations to occur, and
15 embedding of these internal or external standard lipophilic protected
compounds is then made possible in water.

For example, the device is prepared as follows: The internal standard,
dissolved in a suitable solvent, is pipetted in a known amount onto a porous
20 support and dried. This procedure is repeated for every internal standard or
class of internal standards to be employed in the device. If an encapsulation
is provided, as the final step, the encapsulating/covering material, preferably
in a suitable solvent, is put onto the support including the internal
standards (i.e. the insert) and dried. The insert is then inserted into the well,
25 preferably by using a securing means or fixing structure such as a retainer.
As an alternative, the support may be inserted into the well before pipetting
the internal standards and the optional covering material onto the support.

Well

30

A well as used in the invention should be understood to be any vial or tube
consisting of a material, which is preferably solvent and derivative resistant,
wherein an extraction or chemical reaction can take place. The one or more
wells (indicated as (18) in Figure 1 and (1) in Figure 7) of the device
35 preferably comprise at least one filter for separating micron size solids, more
preferably exactly one filter for separating micron size solids (indicated as (4)
in Figure 7). The one or more wells of the device preferably comprise at least

1 one outlet (indicated as (5) in Figure 7) for discharging the filtrate. A filter
contained in the well as used in the invention should be understood to be
any porous material a liquid or gas is passed through in order to separate
the fluid from suspended particulate matter. The filter has preferably a pore
5 size of 50 to 0.01 micrometer, more preferably 5 to 0.1 micrometer, and even
more preferable 1 to 0.3 micrometer. Most preferable, the filter has a pore
size of 0.45 micrometer. The filter is located in the well. Moreover, the outlet
according to the invention preferably opens under applied centrifugal force or
reduced pressure, preferably below 500 mbar. The reduced pressure is
10 preferably applied on the side of the outlet of the well. Alternatively, an
increased pressure on the upper side of the well can be applied in order to
ensure a flow from the well to the outlet.

Apparatus

15 Furthermore, the microtitre plate as described above may also be used in an
apparatus for the quantitative analysis of a metabolite profile in a biological
sample. Said apparatus comprises a treatment unit for preparing the
metabolite to be screened comprising an automated liquid handling system,
20 typically combined with devices for derivatisation of the metabolites present
in the sample and for subsequent extraction of the derivatives; a mass
spectrometer for the quantitative targeted mass spectrometry-based analysis,
and a database for storing results of the analysis.

25 The apparatus, also called platform, as used in the invention should be
understood to be any apparatus that enables the complete preparation of a
biological sample ready for analysis by mass spectrometry. This encompasses
processes of derivatization, desalting, concentrating and extraction. This also
includes all possible combinations of some or all of these processes in a fully
30 automated method, preferably incorporating a liquid handling system in
combination with a sample centrifugal device, a sample heating and cooling
device, a sample shaking device, a sample drying device, a sample pipetting
device and a sample homogenization device. A liquid handling system as used
in the invention should be understood to be any mechanical device that
35 enables accurate aspiration and dispensing of many types of solvents in and
out of vials and microtitre plates. A liquid handling system may be controlled

1 by a computer and controlling software used in such a liquid handling
system.

A database as used in the invention should be understood to be any
5 collection of data arranged for ease and speed of search and retrieval.

A targeted mass spectrometry analysis as used in the invention should be
understood to be mass spectrometry analysis, wherein one or more preset ion
pairs are used, specifically defining and representing a known metabolite by
10 a known fragmentation pattern that is characteristic for the corresponding
analyte, for identification of the targeted metabolite. The obtained ion
intensities are used together with the appropriate internal standard to
calculate the concentration of the targeted metabolite. The internal standard
is identified by using a characteristic ion pair (or several), their obtained ion
15 intensities are related to the known concentration of the internal standard
allowing the quantification of a corresponding targeted metabolite. The set of
targeted metabolites is known in advance and can be pre-annotated.
Therefore, detected and quantified metabolites are already annotated
allowing a fast and direct interpretation. A tandem mass spectrometer is
20 particularly preferred as a mass spectrometer capable of MSMS analyses to
distinguish more specifically ion species. Preferably, the apparatus allows for
automated standardized sample preparation and high-resolution tandem
mass analytics procedures. In particular, the automated sample preparation
procedure increases day to day reproducibility of reliable results and lower
25 coefficients of variance (CVs). When, for example, analyzing a derivatized
sugar with a precursor ion scan, the derivative itself can be detected by the
mass spectrometer. In positive ion mode this is preferably the formation of
the phenylmethylpyrazolone (PMP) (MH)⁺ ion at m/z 175. The composition of
the carbohydrate itself or discrete isomers are detectable.

30

Standard operation procedure (SOP)

SOP's are detailed, written instructions with the objective to guarantee high
quality and uniformity of the performance of metabolite analysis. In this
35 context, all the working and processing steps with corresponding parameters,
such as for sample processing, mass spectrometry analysis and data
processing are defined facilitating a standardized metabolite sample

1 collection, handling, preparation, analysis and data processing, which leads
to reproducible results with low intra and inter day variability. Typically, a
specific SOP relates to the analysis of one or more metabolite classes for one
or more sample material types.

5

Parameter set

All the steps and corresponding parameters of the SOP are stored and
managed in a data base. After one or several SOPs are assigned to a sample
10 or sample collection (i.e. samples on a micro titre plate), all parameters for
sample preparation are sent to the control unit to manage and monitor the
sample preparation process in the treatment unit. All steps, including
pipetting, derivatisation, incubation and extraction, are controlled by the
corresponding parameter set. The mass spectrometry (MS) analysis is
15 controlled in a similar way by the control unit applying the corresponding
parameters of the parameter set. The parameters define the MS - method,
such as scan times and positive or negative ionisation for multiple reaction
monitoring (MRM), precursor and neutral loss scans. Furthermore, all the
parameters for identification of targeted metabolites and related internal
20 standards, such as mass pairs and mass tolerances, and for quantification,
such as concentration of internal standards, response factors, detection limit
and linear range are included in the parameter set.

Filtering

25

Data processing involves typically the data reduction step called filtering.
Noise filters reduce the data based on a calculated noise threshold. In this
respect, data below a certain signal to noise ratio is filtered. Content based
filtering of the results leverages, for example, disease specific knowledge to
30 concentrate on relevant metabolic aspects of the disease under investigation.

Comparing and evaluation

After pre-processed data derived from mass spectrometry analysis has been
35 technical validated, statistical analysis can proceed. Depending on the design
of a metabolite profiling study a sample or several samples derived from
healthy controls and patients are compared to reveal differences, i.e.

1 biomarkers that can be utilized to characterize a disease at the molecular
level. In another embodiment, samples are derived from patients
participating in a clinical trial, where a novel drug compound is under
investigation and compared to an approved drug. Evaluation of metabolite
5 profiling shows the pharmacodynamic profiles and difference of the
investigates compounds or dosages. These pharmacodynamic profiles point to
the efficacy and side effects caused by the individual compounds.

In the following, an exemplary configuration of an apparatus according to the
10 present invention is described. Referring to Fig. 1, the apparatus 10 includes
a treatment unit 11 and the mass spectrometer 14. Further, there is a
controlling unit 12 coupled to an input unit 16. The controlling unit 12 is
connected to a database 13. The database 13 is coupled to an evaluation/
comparison and output unit 20. The database 13 receives results of a
15 processing unit 15. The processing unit 15 receives the results from the
mass spectrometer 14. The processing unit 15 may be combined with the
controlling unit 12 or may be realized as separate unit.

In the following, the components of the treatment unit 11, as shown in Fig. 2,
20 are described in more detail. The treatment unit 11 comprises a robotic,
which is handling the micro-titre plate 22. Further, it is handling the
introduction of internal standards, consumables or solvents into the wells of
the micro-titre plate 22. The treatment unit 11 includes a liquid handling
system. The treatment unit 11 further includes an auto sampler, which
25 divides the biological sample into a plurality of aliquots, which are provided
in the wells of the micro-titre plates 22.

In one embodiment of the present invention the micro-titre plate 22 includes
aliquots, which are derived from one biological sample only. However, it is
30 also possible to have different biological samples, which are distributed in
different wells of the micro-titre plate 22.

The treatment unit 11 further includes containers including different
internal standards, consumables or solvents. The robotic is accessing the
35 different containers for adding the internal standards, consumables or
solvents to the respective aliquots in the wells.

1 Further components are required for preparing the plurality of combined
aliquots. It may be required to put the aliquots under vacuum or under
pressure. Additionally, a shaker and/or a centrifuge are arranged for
providing a reliable mixing of the internal standards, consumable and
5 solvents with the aliquots. Additional components of the treatment unit are,
for instance, the fume hood and the pipette washing unit.

The micro-titre plate 22 includes an upper and a lower plate, which may be
separated from each other after the treatment of the combined samples in the
10 treatment unit 11. The upper plate has a holder or retainer for holding a
support, which maybe impregnated with one or more internal standards,
consumables or solvents.

Instead of supplying the internal standards, consumables or solvents in
15 liquid form they may be provided before on the porous support. The porous
support may be inserted in the well in the upper plate. Thus the aliquots of
the biological sample may solubilize the internal standards, consumables or
solvents during treatment.

20 In the following the method for analyzing the metabolite profile in a biological
sample is explained based on Fig. 3.

In step S1 the biological sample to be investigated, e.g. blood of healthy
person, is supplied to the treatment unit 11, where it is divided into a
25 plurality of aliquots by use of an autosampler. The aliquots are supplied
to the wells of the micro-titre plate 22 having 96 wells. The micro-titre plate
22 is then moved by the robotic to a place for inserting internal standards,
consumables or solvents. The determination, which internal standards,
consumables or solvents need to be combined with a certain aliquot, is
30 performed based on the information, which metabolites have to be screened.
In particular by assigning the SOP and the corresponding parameter set to a
sample or sample collection, -the way how to handle the sample(s) is defined
and standardized.

35 In step S2 information, which metabolites have to be screened, is inputted
via the input unit 16, which is supplied to the controlling unit 12. Based on
this information the controlling unit 12 determines the parameter set/SOP,

1 which is used for the sample preparation, separation and for mass
spectrometry analysis of the respective combined and separated biological
samples. The parameter set reflects the processing steps and parameters of
the corresponding standard operational procedure (SOP). The controlling unit
5 12 accesses the database 13 containing data of the parameter sets/SOPs
required for treating a biological sample and for performing the mass
spectrometry analysis. By deriving a parameter set/SOP out of the database
13 the controlling unit 12 determines how to perform the sample
preparation, derivatization and extraction of metabolites. This parameter set
10 may include time values for treating, temperature values for heating and the
kind of consumables/solvents, which should be combined with certain
samples etc.

The user may be asked to input project and sample related information into a
15 laboratory information management system (LIMS), define work list with the
unique sample identifiers, barcodes and assign the standard operation
procedures (SOP). The LIMS can be utilized for tracking the biological
samples during the preparation, the treatment and mass spectrometry
analysis and for quality control and management. In one embodiment, the
20 LIMS can comprise the input, control, processing and evaluation unit and
database, but can also be realized as separate unit with interfaces to the
other units.

In step S5 the aliquots of the biological sample are combined with at
25 least one of an internal standard, consumable and solvents. In particular, it
is possible to combine different consumables/solvents to each aliquot.

After being combined the plurality of combined samples or aliquots are
treated in step S6 depending on the metabolites to be screened. The
30 information which kind of metabolite has to be screened is inputted in the
input unit 16, for instance, by a user. It is further possible to input the
information which metabolites have to be screened by an automated reading
device which reads, for instance, a barcode on the device containing the
biological sample before or during supplying to the treatment unit 11. The
35 barcode indicates, which metabolites have to be screened. The treatment is
performed in the liquid handling system, which may include extraction
according to polarity of solvent mixtures, adding of sample, drying of sample

- 30 -

1 on porous support, derivatisation, drying and extraction by centrifugation (or
vaccum). After the treatment the upper plate of the micro-titre plate 22 is
removed. The lower plate contains the extracted metabolites, which are
provided to the mass spectrometry analysis.

5

In step S7 the extract of each aliquot is provided separately to the mass
spectrometer 14 by use of an auto sampler.

10 In step S8 the mass spectrometry is performed. The mass spectrometry
analysis is controlled in a similar way by the control unit applying the
corresponding parameters of the parameter set 14. The parameters define the
MS - method, such as scan times and positive or negative ionisation for
multiple reaction monitoring (MRM), precursor and neutral loss scans.
Furthermore, all the parameters for identification of targeted metabolites and
15 related internal standards, such as mass pairs and mass tolerances, and for
quantification, such as concentration of internal standards, response factors,
detection limit and linear range are included in the parameter set. In step S9
the result of mass spectrometry analysis are received.

20 As explained above, each extract of an aliquot sample is separately provided
to the mass spectrometry analysis. Thus, the steps S7 to S9 are performed
96 times since there are 96 wells in a micro-titre plate 22.

The results are received in the processing unit 15. In the processing unit 15
25 step S10 is performed including the processing of data, which includes noise
filtering, content based filtering of the results, quantification, and annotation
of the detected metabolites. The processed and filtered results are stored in a
database 13 in step S11. The processing unit 15 detects the targeted
metabolites and corresponding internal standards and calculates the
30 concentrations of the metabolites, as the concentrations of the internal
standard of a respective sample are known. After calculation of the
concentrations the filtering is performed. Since there is a huge amount of
mass spectrometry data, the derived results need to be filtered, wherein
different filtering methods may be applied to reduce the amounts of data.
35 Then the filtered data are stored. After having stored the prepared or
processed results of the mass spectrometry analysis the results can be
compared to reference values, which are stored in the database 13 in step S12

1 to derive information, which indicate a normal or pathogenic disease state or
the pharmacological response to therapeutic intervention.

The results are outputted in step S13 by use of a monitor or a printout.
5 Based on the information, which may be derived during comparing the
results of the mass spectrometry with reference values stored in a database
13, the database may be adapted. This learning process may be used for
providing an adaptation of the parameter sets, which are used for treating
the biological samples for analyses of the metabolites profile.

10

With reference to Figure 4 an example will be given for deriving a plurality of
results for creating a metabolite profile of a biological sample.

A pre-clinical trial on diabetes mellitus type II exploiting a qualitative and
15 quantitative metabolite profiling approach was conducted for metabolically
characterization of a disease mouse model and detailed pharmacodynamic
description of a novel candidate drug class. Six groups were studied
consisting of healthy and diseased mice either treated, untreated or treated
with an approved drug.

20

The six groups are in the first row including a diseased group (T2D), healthy
group, a drug candidate treated diseased group, a drug candidate treated
healthy group, a diseased group treated with an approved drug and a healthy
group treated an approved drug. For each group a predetermined number of
25 animals are tested. For each animal four materials are used as biological
samples. In this embodiment, an urine sample, a plasma sample, a red blood
sample and a liver sample are taken from each animal. Each sample is
provided in the inventive apparatus and is tested on four different classes for
metabolites. Each class of metabolites (amines, carnitines, sugars, lipids) is
30 characterized by a specific standard operational procedure (SOP). It is noted
that for analyzing red blood a different procedure is required than for testing
urine. Further, the internal standards, consumables and solvents to be
combined are different. Finally, the treatment and separation of the
combined aliquots is different. However to perform a substantiated test each
35 sample of each group is screened by use of four different SOPs.

1 After performing the whole test procedure more than 120000 quantitative
features of these tests are derived. These quantitative features need to be
analyzed by use of statistical procedures. Additionally, using a knowledge
database data mining can be performed.

5

For deriving information for qualitative LC-MS metabolite profiling, mouse
urine and plasma samples were separated by reversed phase
chromatography, and analyzed with a quadrupole time of flight (qTOF) mass
spectrometer equipped with an electrospray source. Raw spectra were
10 filtered, aligned, and scaled, followed by statistical analysis of the data. As to
quantitative targeted MS/MS analysis, mouse urine, plasma, erythrocytes
and liver samples were derivatized (amino acids, acylcarnitines, sugars) and
extracted by solid-phase or in Folch solution (glyco- and phospholipids) with
a liquid-handling-system, and analyzed by flow injection in combination with
15 multiple reaction monitoring (MRM), precursor and neutral loss scans with a
triple quadrupole (QqQ) MS equipped with an electrospray source.
Concentrations were calculated from the raw MS spectra, filtered, normalized
(to creatinine, total protein), and scaled, succeeded by statistical analysis
and biochemical interpretation of the pre-annotated metabolites. The
20 procedure is illustrated in Fig. 5.

The quantitative targeted metabolite profiling concentrates on analytes
(internal standards) that are pre-defined, pre-annotated, and detected by
MRM, precursor and neutral loss scans. Flow injection analysis (FIA) allows
25 signal averaging over a steady stream of ion (TIC) leading to robust signals.
Characteristic mass transitions are used for identification of metabolites and
associated internal standards (ISs). De-isotoping is used for protein analysis.
However, isotope correction is also recommended for certain small molecule
compounds and classes. These algorithms utilize calculated isotope
30 percentages of targeted analytes to correct the measured peak intensities for
their isotopic overlap. Finally, metabolite concentrations are calculated by
relating the known concentrations of internal standards with the measured
ion counts per second (cps).

35 The inventive targeted metabolite profiling methodology utilized in a liquid-
handling-system for fully automated and parallel sample preparation in
micro-titre format guaranteeing high reproducibility and low coefficients of

1 variation (CVs). Furthermore, analytes and corresponding metabolites were annotated in advance so as to enable fast and direct biochemical and biological interpretation.

5 Up to 825 metabolites were obtained from each compartment and comparison of the groups enabled identification of the animal disease model and facilitated the immediate biochemical characterization of drug effects.

Fig. 6 illustrated the result of mass spectrometry, wherein different amino
10 acids are shown in comparison with peaks of known internal standards (indicated with *).

Targeted metabolite concentration profiling facilitates higher throughput and the versatility for standardized analysis of various biofluids and tissues,
15 which is especially important for comprehensive disease characterizations, and efficacy and toxicity assessments in animal model experiments. Direct or surrogate, uni or multivariate markers are revealed by data mining techniques with the objective to describe diseases at the molecular level, which are subsequently often used to study metabolic and pharmacodynamic
20 changes in various compartments and organs.

In general, the identical technology can be applied in various stages of pharmaceutical development, ranging from cell-based systems, and animal models to clinical studies. For example, putative biomarkers discovered and
25 verified in the preclinical phase, such as for the characterization of normal biological and pathogenic processes or pharmacological responses to a therapeutic intervention can be clinically validated with the same analytical technology in human studies. In an intended diagnostic application, clinical studies will have to assess the predictive performance and generalization
30 power of candidate biomarkers in clinical routine, where typically high specificity is required to rule out other diseases.

Each successful metabolite profiling biomarker discovery study relies on a carefully planned experimental design with clear defined objectives, a
35 detailed plan and quality control procedures in advance, as is common practice in controlled clinical trials. Well thought-through experimental

1 designs maximize the information obtained for a given experimental effort,
yielding to valid and objective conclusions.

Experimental flaws and bias jeopardizes the predictive performance and
5 generalization power of statistically determined biomarkers. In this context,
metabolite profiling has to learn from the past, where insufficient
experimental design and deficient reproducibility in early clinical validation
studies have restrained the widespread use of serum protein profiling
technologies.

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Of course, beyond statistical significance lies the problem of biological
significance. Just because an expression or concentration change is
statistically significant does not always imply that the change has any affect
on the underlying biology. Some genes, protein or metabolites are tightly
15 regulated so that small changes in abundance are biologically relevant, while
others are loosely regulated and can vary considerably with no biological
effect.

In summary, when carrying out the analysis of a metabolite profile according
20 to the invention a quantity of hundreds of metabolites can be analysed
simultaneously from microtitre quantities of biological material with high
speed, precision and sensitivity using pre-analytical steps. Quality assured
(QA) data is generated from individual samples in the matter of minutes and
interpreted employing cutting edge statistical software tools. This method
25 also overcomes hitherto to existing analytical bottlenecks through pre-
analytical standardization and automation, and user-friendly statistical and
biochemical data interpretation. This integration of all components in the
method of the invention into a new technology platform will make
"biochemical fingerprinting" accessible for widespread application and will
30 expedite the spread of metabolomics.

Examples

The present invention will be further illustrated by the following non-limiting
35 examples.

1 *Preparation and conditions of the multi-device*

One multi-device was prepared using 7 mm cellulose spots (cut from generic
card - 10 539 859, Schleicher Schuell Biosciences GmbH, Dassel, Germany)
5 as the porous support in each of the 96 wells of a Solvinert microtitre plate
(MSRP N04, Millipore Corp. MA, USA). These were fixed into place with
manufactured retainers made from polypropylene (Biocrates, Tirol, Austria).

To analyse a selected subset of metabolites, in this case, amino acids,
10 acylcarnitines and phospholipids from a sample, a selection of suitable
internal standards of amino acids, acylcarnitines and lipids labelled with
stable isotopes to represent all the twenty proteogenic phosphatidylcholines,
sphingomyelins, and lyso species of each were used. These were pre-
embedded into the porous support of the multi-device by pipetting known
15 amounts of each internal standard class, allowing each to dry within the
porous support before adding the next mixture of internal standards,
allowing to dry and so forth. In this example there were added acylcarnitines
followed by amino acids and last, a mixture of phospholipid internal
standards in a water solution containing 0.1 % w/w polyethyleneglycol 1000
20 (PEG 1000), a compound which served dual purposes. As a surfactant, PEG
1000 resides in the pores of the porous support coating all internal
standards offering a protective barrier to otherwise degradative actions of
exposure to oxygen and water.

25 When completely dry the multi-device technical validation samples were then
added to the first five wells of the multi-device.

Well 1: a blank,

30 Wells 2 and 3: control mixtures of unlabelled metabolites,

Well 4: a quality control with low concentration metabolites (normal
levels or 1 times), and

35 Well 5: a quality control with high concentration metabolites (levels 10
times normal).

1 The multi-device containing pre-imbedded internal standards with additional control samples in wells 1 to 5 is then stored ready to use at 4 °C.

Method of Using the Multi-Device

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[Example 1]

For example purposes only, the following is a description of how the device as specified above is used to process samples for analysis of a selection of
10 metabolites.

To analyse a subset of metabolites, amino acids, acylcarnitines and phospholipids from a sample, a selection of suitable internal standards of amino acids, acylcarnitines and lipids, stable isotope labelled to represent all
15 the twenty proteogenic amino acids, the most abundant acylcarnitines and phospholipids including phosphatidylcholines, sphingomyelins, and lyso species of each were used. Upon addition of a predefined amount of sample, typically 10 µl of plasma, the internal standards and amino acids of the sample are mixed within the confines of the pores of the insert. Any
20 subsequent treatment that causes loss or degradation of metabolites will therefore be correlated for by the internal standard. Derivatisation of the amino acids can then take place within the confines of the pores of the insert. The derivatising reagent in this example consists of 15 µl of a 5% phenylisothiocyanate in a 1:1:1 solution of pyridine, water, ethanol. This
25 derivatisation process occurs at room temperature in less than 20 minutes. As the derivatising solution is completely volatile it can be simply removed under a gentle stream of nitrogen or vacuum at room temperature. The addition of a methanol solution containing 10mM ammonium acetate extracts the derivatised amino acids, acylcarnitines and the phospholipids
30 simultaneously from the porous device into the methanol solvent. The microtitre plate of choice for this purpose has additional properties. It has a 0.45 micron filter and a liquid outlet, that only opens under centrifugal force or vacuum, built into the bottom of each well. The methanol extract from the sample is then simply collected via centrifugation into a capture-microtitre
35 plate, placed under the microtitre plate containing device. Mass spectrometry analysis of the solution from each well can then take place, typically using an autosampling instrument to deliver the sample to the mass spectrometer.

1 [Example 2]

The following will demonstrate that the device can be used to process samples for analysis of a selection of metabolites.

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The multi-device upon accurate addition of 10 µl of blood samples from one patient to each well is mixed with the internal standards within the confines of the pores of the porous support (insert). Any subsequent treatment that causes loss or degradation of metabolites will therefore be correlated to the internal standard. Derivatization was carried out as in Example 1 and the resulting solutions from each well are then analyzed by mass spectrometry methods, typically using an autosampling instrument to deliver the sample to the spectrometer.

15 Results from the mass spectrometric measurements of the metabolites derivatized and extracted with the multi-device are graphically depicted in Figure 8 and Figure 9 showing the amino acids, the phospholipids and the acylcarnitines, respectively.

20 The quantities of the amino acids and acylcarnitine metabolites are shown in Table 3. also showing the accuracy and the variance of the values obtained using the multi-device.

Table 3 - The quantities accuracy and reproducibility of the amino acids, lipids, lactate, creatinine and glucose from a single sample measured 10 times are shown in Table 3 and were obtained using the multi-device.

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1 **Amino Acids**

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Sample Name	QAlow C2	QAlow C3	QAlow C4	QAlow C5	QAlow C6	QAlow C7	QAlow C8	QAlow C9	QAlow C10	QAlow C11	QAlow C12	mean [μmol/l]	std	CV [%]
Arginine-PTC	64.8	71.0	67.5	68.7	62.3	63.1	67.9	65.6	67.4	66.5	63.7	66.2	2.6	4.0
Phenylalanine-PTC	74.0	71.6	72.1	81.5	70.1	69.2	70.4	73.6	72.5	72.9	76.2	73.1	3.4	4.7
Proline-PTC	114.4	117.1	124.5	120.4	120.2	120.6	137.2	118.2	127.5	126.3	120.6	122.5	6.2	5.1
Lysine-PTC	102.2	94.4	108.4	110.5	93.0	102.1	102.1	98.1	98.9	101.8	102.1	101.2	5.2	5.1
Histidine-PTC	90.0	97.0	95.4	89.4	93.0	89.2	97.7	82.5	88.5	95.3	100.6	92.6	5.2	5.6
Tryptophane-PTC	35.8	39.0	42.7	38.2	36.6	36.6	37.4	38.9	34.8	34.8	35.7	37.3	2.3	6.2
Tyrosine-PTC	93.4	99.9	95.7	92.7	94.8	103.7	95.2	97.2	86.1	87.4	83.6	93.6	6.0	6.4
α-Leucine-PTC	174.5	181.2	158.1	191.9	164.1	153.8	167.5	157.2	150.2	158.6	157.1	164.9	12.8	7.8
Valine-PTC	117.0	96.9	108.7	121.7	116.3	114.6	115.7	134.2	111.7	116.9	121.0	115.9	9.1	7.9
Ornithine-PTC	78.7	81.7	58.8	67.9	69.3	68.9	71.6	68.9	74.8	74.9	74.4	71.8	6.2	8.6
Methionine-PTC	44.4	38.2	34.9	41.5	44.7	38.8	37.8	43.2	36.2	36.0	36.3	39.3	3.6	9.1
Citrulline-PTC	28.3	24.8	28.0	23.5	20.6	25.2	26.1	25.0	28.0	29.4	25.8	25.9	2.5	9.7
Glutamine-PTC	455.8	455.6	445.7	364.4	352.0	372.8	369.0	374.2	322.0	355.7	369.1	385.1	45.6	11.8
Serine-PTC	182.1	153.7	176.7	177.1	191.9	188.5	173.4	137.8	132.6	134.0	154.9	163.9	22.1	13.5
Threonine-PTC	21.3	26.0	31.0	34.4	23.5	29.5	25.7	29.4	25.0	24.2	29.0	27.2	3.8	14.0
Alanine-PTC	219.6	316.7	227.8	282.3	298.7	267.5	205.8	177.8	203.0	245.4	210.9	241.4	44.4	18.4
Asparagine-PTC	236.4	250.9	204.5	179.4	168.4	136.5	170.1	192.4	230.5	206.7	157.0	193.9	35.7	18.4
Glycine-PTC	320.9	282.0	272.0	297.9	412.6	422.9	252.8	268.5	225.1	226.8	278.0	296.3	66.2	22.4
Glutamic Acid-PTC	2115.4	1508.6	1694.6	1220.8	2118.8	1560.0	2137.9	2770.8	2137.9	1563.6	1984.8	1892.1	431.0	22.8

1 **Acylcarnitines**

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Sample Name	QAlow C2	QAlow C3	QAlow C4	QAlow C5	QAlow C6	QAlow C7	QAlow C8	QAlow C9	QAlow C10	QAlow C11	QAlow C12	mean [μmol/l]	std	CV [%]
C2	8.487	9.674	9.529	9.007	8.845	9.105	9.847	9.477	9.928	8.552	9.137	9.24	0.49	5.4
C18:1	0.161	0.233	0.218	0.216	0.193	0.193	0.207	0.225	0.205	0.191	0.180	0.20	0.02	10.4
C8:1	0.161	0.233	0.218	0.216	0.193	0.193	0.207	0.225	0.205	0.191	0.180	0.20	0.02	10.4
C0	38.351	48.949	51.179	53.867	53.869	52.218	61.745	60.195	56.018	52.762	56.524	53.24	6.21	11.7
C12-DC	0.021	0.015	0.017	0.013	0.015	0.017	0.015	0.017	0.016	0.017	0.017	0.02	0.00	12.4
C14:2	0.041	0.050	0.044	0.035	0.047	0.052	0.041	0.050	0.035	0.047	0.045	0.04	0.01	13.1
C8	0.232	0.306	0.352	0.274	0.200	0.212	0.264	0.250	0.258	0.276	0.299	0.27	0.04	16.4
C12	0.043	0.051	0.071	0.047	0.043	0.043	0.044	0.048	0.046	0.045	0.055	0.05	0.01	16.7
C12:1	0.028	0.025	0.030	0.031	0.027	0.035	0.035	0.030	0.040	0.034	0.019	0.03	0.01	18.6
C16:1	0.038	0.016	0.036	0.037	0.030	0.035	0.030	0.033	0.024	0.030	0.035	0.03	0.01	20.2
C3	0.293	0.401	0.524	0.376	0.274	0.324	0.309	0.435	0.423	0.473	0.423	0.39	0.08	20.5
C14:1	0.110	0.106	0.103	0.113	0.113	0.141	0.174	0.118	0.172	0.108	0.117	0.13	0.03	20.6
C4:1	0.110	0.106	0.103	0.113	0.113	0.141	0.174	0.118	0.172	0.108	0.117	0.13	0.03	20.6
C7-DC	0.050	0.036	0.044	0.052	0.040	0.039	0.066	0.055	0.036	0.064	0.041	0.05	0.01	22.5
C5-M-DC	0.192	0.204	0.184	0.202	0.191	0.209	0.210	0.316	0.177	0.136	0.160	0.20	0.05	22.7
C4-OH	0.106	0.080	0.100	0.167	0.138	0.129	0.170	0.151	0.175	0.115	0.121	0.13	0.03	23.7
C11	0.006	0.008	0.010	0.010	0.013	0.008	0.010	0.007	0.012	0.008	0.006	0.01	0.00	23.9
C16	0.136	0.204	0.188	0.169	0.127	0.096	0.132	0.137	0.100	0.124	0.178	0.14	0.04	24.6
C4:1-DC	0.148	0.182	0.241	0.167	0.111	0.097	0.165	0.146	0.226	0.127	0.218	0.17	0.05	28.3

1 **Lipids**

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Sample Name	QAlow C2	QAlow C3	QAlow C4	QAlow C5	QAlow C6	QAlow C7	QAlow C8	QAlow C9	QAlow C10	QAlow C11	QAlow C12	mean [μmol/l]	std	CV [%]
GPCho 36:3a	190.12	219.29	195.68	224.00	250.66	236.77	231.16	199.46	207.93	210.60	198.76	214.9	19.2	8.9
SM d18:1/16:0	311.11	387.86	330.25	339.33	346.71	381.94	419.57	307.61	337.20	348.34	380.74	353.7	34.8	9.8
GPCho 36:2e	22.84	25.00	20.99	22.67	21.71	21.29	23.19	23.91	27.44	19.20	21.12	22.7	2.2	9.9
GPCho 32:1a	55.79	50.54	58.33	55.17	58.33	67.42	64.89	62.50	67.03	56.25	48.00	58.6	6.4	10.9
GPCho 32:0a	7.69	7.37	7.73	5.20	7.87	6.71	7.82	6.63	7.74	9.23	7.07	7.4	1.0	13.6
LGPCCho 18:2a	6.17	6.43	6.17	6.00	8.55	6.45	6.52	4.35	7.93	7.28	6.21	6.6	1.1	16.7
GPCho 34:1p	76.84	76.34	87.50	93.10	73.96	82.02	80.85	127.50	82.42	83.33	82.00	86.0	14.7	17.2
GPCho 36:1p	243.16	268.82	354.16	327.59	300.00	277.53	288.30	425.00	290.11	269.79	259.00	300.3	51.8	17.3
LGPCCho 18:1p	160.00	174.19	215.28	168.97	165.62	180.90	156.38	257.50	180.22	138.54	164.00	178.3	32.4	18.2
LGPCCho 18:0e	40.24	47.37	51.38	43.35	47.19	65.10	53.63	59.64	50.97	67.69	37.88	51.3	9.7	18.8
GPCho 38:1a	18.95	21.51	29.17	17.24	25.00	22.47	28.72	25.00	26.37	25.00	34.00	24.9	4.8	19.3
LGPCCho 18:0p	17.28	28.57	21.61	16.67	24.34	21.94	27.54	19.02	17.68	26.49	18.01	21.7	4.4	20.2
GPCho 30:0a	11.73	21.43	12.35	12.67	14.47	14.19	15.22	9.78	13.41	15.89	16.15	14.3	3.0	21.2
GPCho 38:2a	41.86	56.25	63.42	80.00	65.85	87.50	78.12	82.35	70.27	79.31	50.00	68.6	14.6	21.3
GPCho 34:0e	6.79	8.57	6.79	9.33	11.84	9.03	11.59	13.04	12.20	10.60	9.32	9.9	2.1	21.3
GPCho 32:1p	25.26	15.05	30.56	19.54	19.79	22.47	22.34	23.75	16.48	17.71	16.00	20.8	4.6	22.3
GPIns 38:4	13.52	20.27	15.11	14.77	10.75	17.97	16.27	15.24	15.62	13.51	11.61	15.0	2.7	18.0
GPIns 36:2	7.32	5.48	4.00	8.00	3.58	5.22	6.78	6.35	6.88	4.32	3.23	5.6	1.6	29.2

1 **Lactate, Glucose and Creatinine**

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Sample Name	QAlow C2	QAlow C3	QAlow C4	QAlow C5	QAlow C6	QAlow C7	QAlow C8	QAlow C9	QAlow C10	QAlow C11	QAlow C12	mean [μmol/l]	std	CV [%]
Lactate	21513	24282	25798	25443	22912	22519	23778	21673	22400	24968	23625	23537	1473	6.3
Glucose	3826	4250	4383	4104	4235	4288	4486	4156	3949	3983	3981	4149	202	4.9
Creatinine	213.62	270.39	247.02	243.48	242.64	233.51	280.19	263.16	262.74	238.50	244.91	249.1	18.7	7.5

1 [Example 3]

Therapeutic drug monitoring

5 Immunosuppressants are required to inhibit organ rejection after transplantation. The immunosuppressants used are Everolimus, Cyclosporin A, Tacrolimus, Sirolimus, and Mycophenolic acid. Therapeutic drug monitoring results prepared from a suitably prepared multi-device similarly as described above is shown here to further illustrate the use of the multi-
10 device and support the claims of the invention.

Preparation and conditions of multi-device

This multi-device was prepared with exactly the same method as described
15 above, but instead using a single 8 mm cellulose spot (cut from generic card - 10 539 859, Schlicher Schuell, Biosciences GmbH, Dassel, Germany) as the porous support.

Into the two multi-devices wells were placed a methanol solution (20 µl)
20 containing Everolimus (200 ng/mL) (Sigma, Vienna, Austria), an internal standard for Sirolimus and Tacrolimus, and Cyclosporin D (400 ng/mL) (Sigma, Vienna, Austria), an internal standard for Cyclosporin A, was pipetted (Gilson 20 µl pipette) onto the porous supports of the multi-device and allowed to dry at room temperature for 30 minutes. Calibrator mixture
25 and quality control levels I-V (whole blood calibrator set (level 0-6) for immunosuppressants, ClinChek R whole blood control for immunosuppressants, Recipe Chemicals and Instruments GmbH, Munich, Germany) were reconstituted according to manufactures instruction and both stored at -20 °C. Prior to use, six calibrator solutions with increasing
30 concentrations of Cyclosporin D and Everolimus and five quality control solution with various concentrations of Cyclosporin A, Tacrolimus, Sirolimus and Everolimus were thawed and allowed to reach room temperature around 23 °C. Into six wells 20 µl of each six calibrators were pipetted (20 µl Gilson pipette) onto porous supports of multi-device. The five quality controls were
35 pipetted into five separate wells porous supports of multi-device. To the multi-device was added acetonitrile (HPLC grade) immediately (Gilson 200 ml pipette) onto the multi-devices porous supports and instantly shaken with an

1 orbital shaker at less than 600 rpm for 30 minutes. The eluant was collected
by placing a 300 µl capacity microtitre capture plate under the device and
then centrifugation of the two at 500 g for 6 minutes. The eluant was then
analyzed by mass spectrometric technique based on a published method (T.
5 Koal, M. Deters, B. Casetta, V. Kaefer, Simultaneous determination of four
immunosuppressants by means of high speed and robust on-line solid phase
extraction-high performance liquid chromatography-tandem mass
spectrometry, J. Chromator. B, Analyt. Technol. Biomed. Life Sci. 2004 Jun
15, 805(2); 215-222). A representative example of how the results are
10 obtained and calculated are presented in Figure 10 for Cyclosporin A
analysis with LCMS to generate quantitative data. The areas under the
integrated peaks of the internal standard Cyclosporin D were used for
comparison against the area under the peak of the immunosuppressant
Cyclosporin A in the five quality control samples containing known amounts.

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Figures 11 to 14 show linear standard curves for all four
immunosuppressants Cyclosporin A, Tacrolimus, Everolimus and Sirolimus
using cellulose supports as inserts within the multi-device. Table 4 shows
the calculated concentrations and the actual for accuracy comparisons of the
20 five quality assurance materials analysed.

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			Analyte Concentration (ng/mL)	Calculated Concentration (ng/mL)	Accuracy (%)				Analyte Concentration (ng/mL)	Calculated Concentration (ng/mL)	Accuracy (%)
Cyc A		Calibrator 0	0.2	0.201	101	Sirolimus	Calibrator 0		0	No Peak	N/A
		Calibrator 1	46.7	43.8	93.7		Calibrator 1		2.4	2.81	117
		Calibrator 2	115	116	101		Calibrator 2		6.6	6.38	96.7
		Calibrator 3	304	315	103		Calibrator 3		12.7	12.8	101
		Calibrator 4	483	472	97.7		Calibrator 4		19.6	19.6	99.8
		Calibrator 5	777	820	106		Calibrator 5		29	31.5	109
		Calibrator 6	1940	1900	97.9		Calibrator 6		49.4	46.7	94.4
		QA1	61	45.3	74.2		QA1		3.04	2.46	80.8
		QA2	116	95.5	82.3		QA2		8.65	10.1	117
		QA3	254	220	86.7		QA3		15.3	12.6	82.4
	QA4	474	391	82.6		QA4		0	No Peak	N/A	
	QA5	1340	1310	98.1		QA5		0	No Peak	N/A	
Tacrolimus		Calibrator 0	0.1	No Peak	N/A	Everolimus	Calibrator 0		0	< 0	N/A
		Calibrator 1	2.1	2.17	103		Calibrator 1		2.1	2.46	117
		Calibrator 2	5.6	5.38	96		Calibrator 2		6	5.71	95.1
		Calibrator 3	10.9	10.5	95.9		Calibrator 3		12.3	12.5	101
		Calibrator 4	15.8	16.1	102		Calibrator 4		18.2	18.3	100
		Calibrator 5	21.9	22	101		Calibrator 5		25.3	27.1	107
		Calibrator 6	38.8	38.9	100		Calibrator 6		46.5	44.4	95.5
		QA1	3.23	3.69	114		QA1		3.48	3.18	91.3
		QA2	6.6	7.35	111		QA2		11.1	10.8	97.3
		QA3	13.2	14.8	112		QA3		18.2	18.5	102
	QA4	0	0.246	N/A		QA4		0	No Peak	N/A	
	QA5	0	0.355	N/A		QA5		0	No Peak	N/A	

1 **Industrial Applicability**

The invention makes possible a versatile and standardized analysis of various biofluids and tissues. For example, current in-house capacities can
5 demonstrate simultaneous and fully automated sample preparation and analysis, generating more than 1000 quantitative and annotated data points from 10 µl of dried blood within 6 minutes of MS machine time covering various classes of metabolites within more than 100 annotated pathways. Thereby the invention for the first time overcomes most of the bottlenecks in
10 (pre)analytics, automatization and data processing and interpretation that have prohibited so far wide-spread quantitative metabolomics mining.

Compared to prior art analytical methods and devices, the quantitative analysis of the invention is extremely rugged and the results are highly
15 reproducible. In particular, the metabolite data is much superior to comparable proteome or transcriptome data. Only 10 µl blood or serum or 20 µl urine or less than 100,000 cultured cells are needed.

The performance features of the analytical method and the device can meet
20 both research (discovery) application and subsequently clinical diagnostic standards. This ensures or makes possible quality assured data, standardized data, which is comparable from laboratory to laboratory, rapid turn-around time, "ready to go" implementation (hits), easily received data interpretation and visualization and a very high degree in automatization and
25 standardization (SOPs). The overall costs/data point makes the metabolome information orders of magnitudes less expensive than proteome information.

The quantitative information obtained by the method or the device of the invention covers pathways and metabolites in a systemic (system biology)
30 context and scalable fashion. Thereby, a representative functional picture or screen shot or metabolic fingerprint of intermediary metabolism can be finally derived from arrays of marker metabolites.

Moreover, functional end-point information that is annotated and can
35 conveniently be linked to information sources of the proteome, transcriptome and genome, recruiting metabolome information for system biology needs.

1 The device and the method can be used in an integrated tool (software and
analytical) suitable to establish a new "standard" for simultaneous generation
of large scale quantitative identified and annotated metabolite profiles and
the study of complex and dynamic multiple biomarker patterns. Moreover,
5 commercially available hardware components, consisting of a liquid handling
system for automated and standardized sample preparation and a mass
spectrometer for MS-MS analytics, can be integrated by proprietary and
protected designed consumable-based products and application software,
comprising (pre-) analytical procedures and innovative modules for quality
10 controlled data processing, technical validation and documentation,
statistical analysis and biochemical interpretation.

The sample preparation time in the present invention (hit-based in batch of
90 samples/microtitre tray) is only roughly 2 h, and will be further reduced
15 by means of parallelization through the scheduling software. A wide range of
specific internal standards for quantification is pre-formulated in proprietary
chemistry as integral part of usually one or two step reaction preparation
and application hits, as is contained all necessary material for QC and QA in
combination with software and SOPs.

20

Industrial applications include biomarker discovery and commercialization
with the objective to utilize validated biomarkers for disease diagnosis,
treatment efficacy or toxicity. The main applications in pharmaceutical
development include the areas drug metabolism and pharmacokinetics,
25 toxicology and safety, drug efficacy and pharmacodynamics. Other fields
comprise clinical diagnostics and theranostics, where, for example, early,
sensitive and specific diagnosis and accurate staging facilitates disease
prevention instead of costly interventions and allows personalized treatment,
and where therapeutic effects can be specifically monitored supporting
30 personalized treatment. Further application areas include, but are not
limited to, nutrition industry, wellness, homeland security, and basic
biology.

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Claims

1. An apparatus for analyzing a drug and/or metabolite profile in a biological sample containing at least one drug and/or metabolite, comprising:

5 an input unit for inputting the kind of drugs and/or metabolites to be screened;

a controlling unit for determining a parameter set for drugs and/or metabolites preparation and for mass spectrometry analysis depending on the input of the kind of drugs and/or metabolites to be screened;

10 a treatment unit for preparing the drugs and/or metabolites to be screened depending on the determined parameter set, the treatment unit comprising an automated liquid handling system, and at least one device for derivatisation of the at least one drug and/or metabolite present in the sample and for subsequent extraction of the derivatives, the device
15 comprising:

(A) one or more wells,

(B) one or more inserts located in the wells, wherein the insert comprises a support comprising a sorbent material for liquids which support is impregnated with at least one organic internal standard; and

20 (C) a retainer holding the insert within the well with a distance between the insert and the walls and the bottom of the well in order to allow a free circulation of fluids around the insert;

a mass spectrometer for performing mass spectrometry analysis on prepared drugs and/or metabolites depending on the parameter set,
25 for quantification thereof;

a database for storing parameter sets for drug and/or metabolite preparation and for mass spectrometry analyses; and

an evaluation unit for evaluating the results of mass spectrometry by use of reference results stored in the database to output an analysis
30 of the drug and/or metabolite profile.

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2. The apparatus as claimed in claim 1, wherein the treatment unit is further provided for combining the sample with at least one of internal standards (ISTD), consumables and solvents or a combination thereof, wherein the combining is performed depending on the input of the kinds
5 of drugs and/or metabolites to be screened.

3. The apparatus as claimed in claim 2, wherein the combined sample(s) is/are treated according to the supplied parameter set in the treatment unit to provide a prepared drug and/or metabolite.

4. The apparatus as claimed in any one of claims 1 to 3, wherein the prepared drugs and/or metabolites are supplied to the mass spectrometer after being extracted.

5. The apparatus as claimed in any one of claims 1 to 4, including a processing unit for at least one of the steps of data filtration, concentration calculation, normalization, verification and annotation of mass spectrometry results.

6. The apparatus as claimed in any one of claims 1 to 5, wherein a plurality of samples are provided as multiple combinations to the treatment unit for being prepared by various methods/parameter sets with different internal standard ISTD, consumables or solvents to give multiple prepared extracts.

7. A method for analysis of a drug and/or metabolite profile in a biological sample comprising the steps of:

providing the sample to an apparatus as claimed in any one of claims 1 to 6 for analyzing drugs and/or metabolites;

providing information of the drugs and/or metabolites to be screened to the apparatus;

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determining a parameter set for screening for drugs and/or metabolites based on that parameter set;

preparing the sample depending on the determined parameter set;

derivatising and extracting the prepared drugs and/or metabolites;

5 providing the mass spectrometer with the sample extract;

performing one or more mass spectrometry analyses on the prepared drugs and/or metabolites for quantification thereof;

evaluating and processing results of mass spectrometry of the drugs and/or metabolites to be screened and comparing with reference
10 values; and

generating output data from the step of evaluating and processing indicative of the drug and/or metabolite profile.

8. The method as claimed in claim 7, wherein the step of providing
15 information of the drugs and/or metabolites to be screened comprises further:

prompting for a checklist of at least one of a sample number, internal standard (ISTD), consumable, solvent, standard operating procedure (SOP) type, or laboratory information management system
20 (LIMS) style of tracking.

9. The method as claimed in claim 7 or 8, further comprising the steps of:

combining the sample with at least one ISTD, consumable or
25 solvent or a combination thereof; and

treating the combined sample(s) depending on the parameter set determined by the apparatus; wherein the parameter set is supplied depending on the information of the drugs and/or metabolites to be screened.

30

10. The method as claimed in any one of claims 7 to 9, wherein the step of preparing the sample comprises further: derivatising and

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extracting the prepared drugs and metabolites from aliquoted samples from the sample.

11. The method as claimed in any one of claims 7 to 10 further

5 comprising the steps of:

dividing the sample into aliquots of small samples;

combining each of the aliquoted samples with different ISTD, consumables or solvents;

10 treating each of the aliquoted samples according to the parameter set;

providing the aliquoted samples combined with different ISTD, consumables or solvents to the mass spectrometer and analyzing drugs and/or metabolites based on the parameter set;

15 processing the mass spectrometer results comprising at least one of the steps: data filtration, concentration calculation, normalization, verification, linkage to pre-annotated biochemical and functional properties and storage of results and generated drug and/or metabolite concentration profile to a database; and

20 after storing mass spectrometry results in the database evaluating and comparing the results to reference results stored in the database.

12. The method as claimed in claim 11, wherein the step of evaluating and comparing the results to reference results stored in the database comprises further:

25 statistically and chemometrically analysing the drugs and/or metabolites;

visualizing the drugs and/or metabolites onto predefined and new biochemical pathways; and

30 identifying drug and/or metabolite biomarker candidates for further evaluation and validation.

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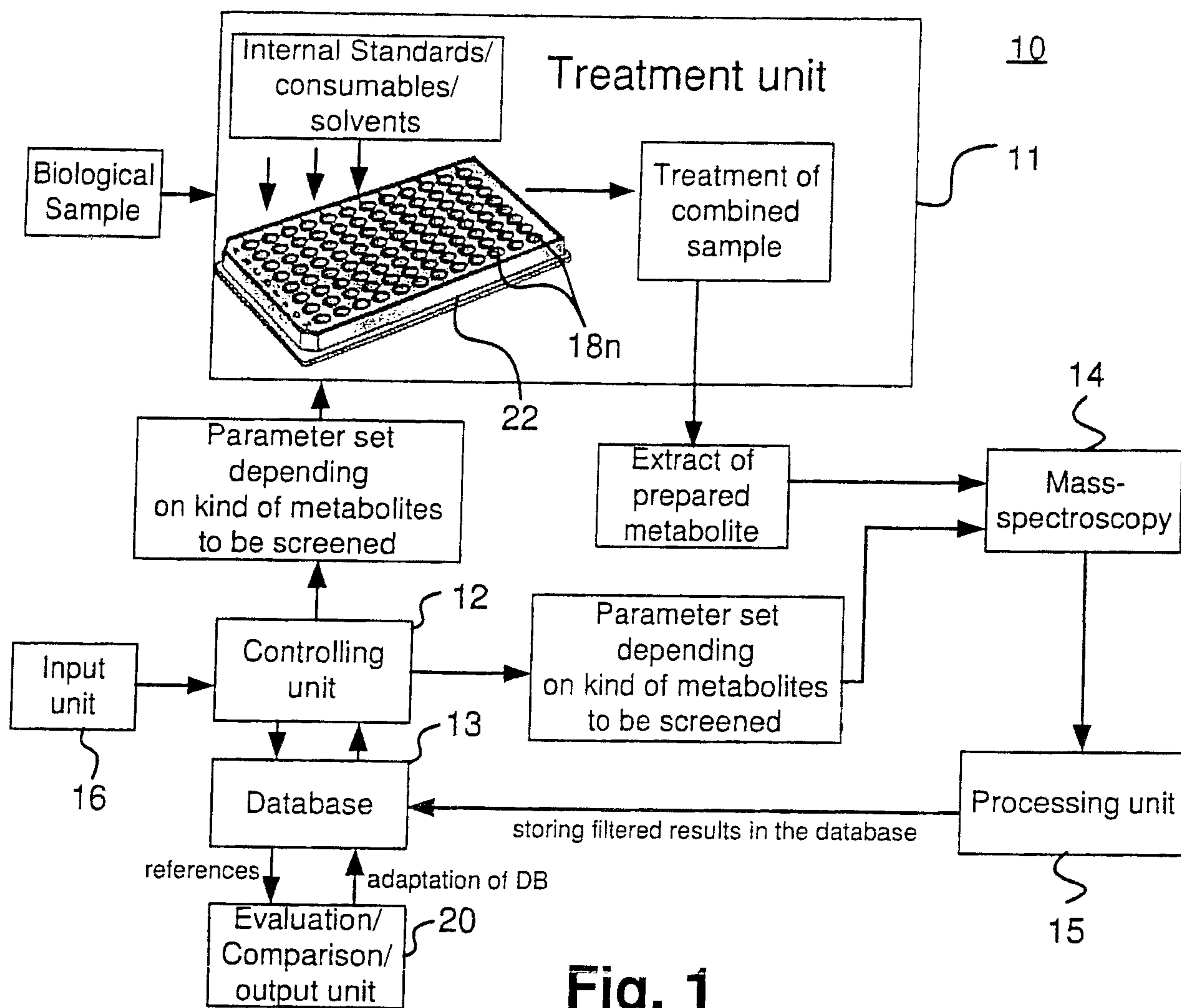


Fig. 1

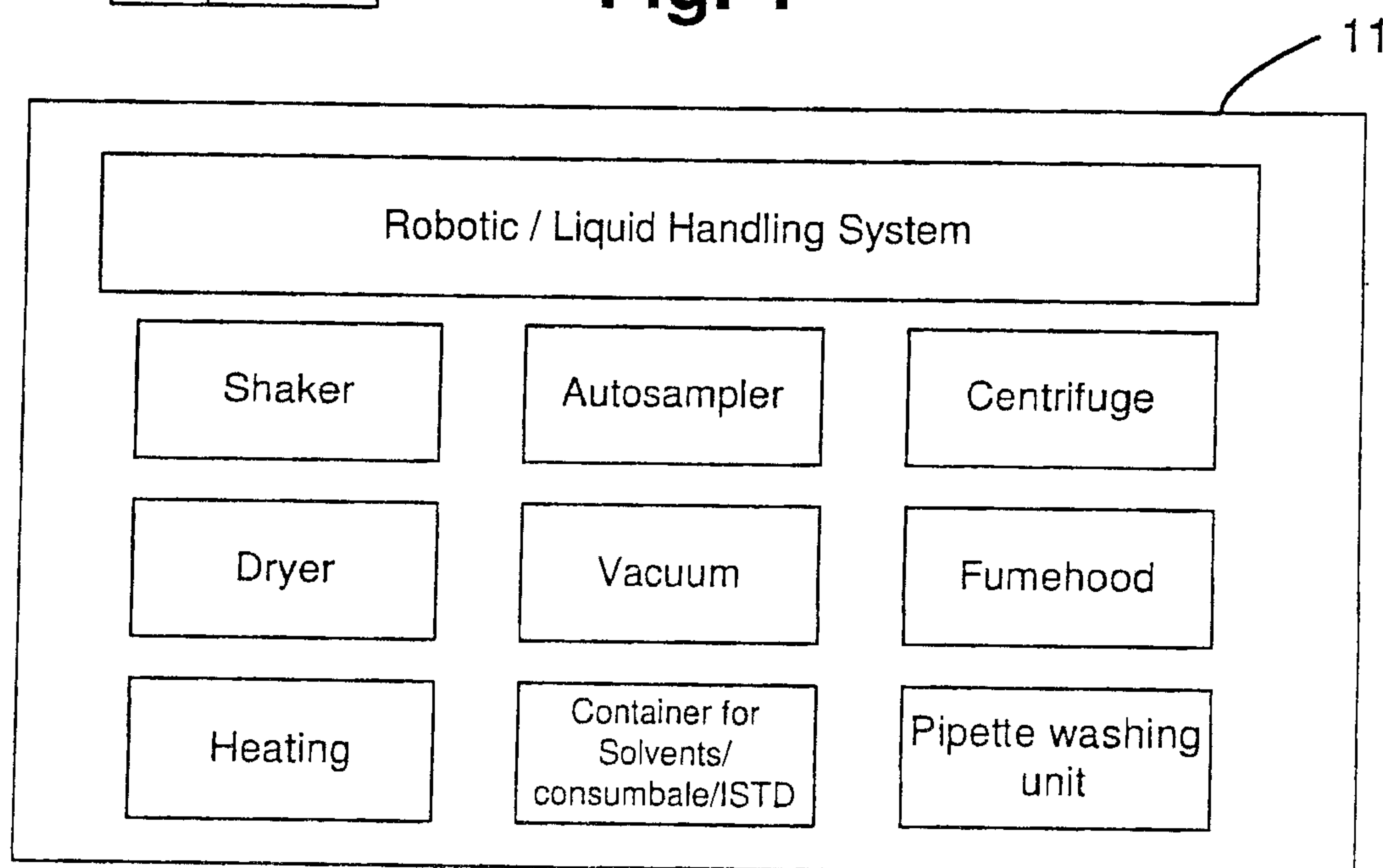


Fig. 2

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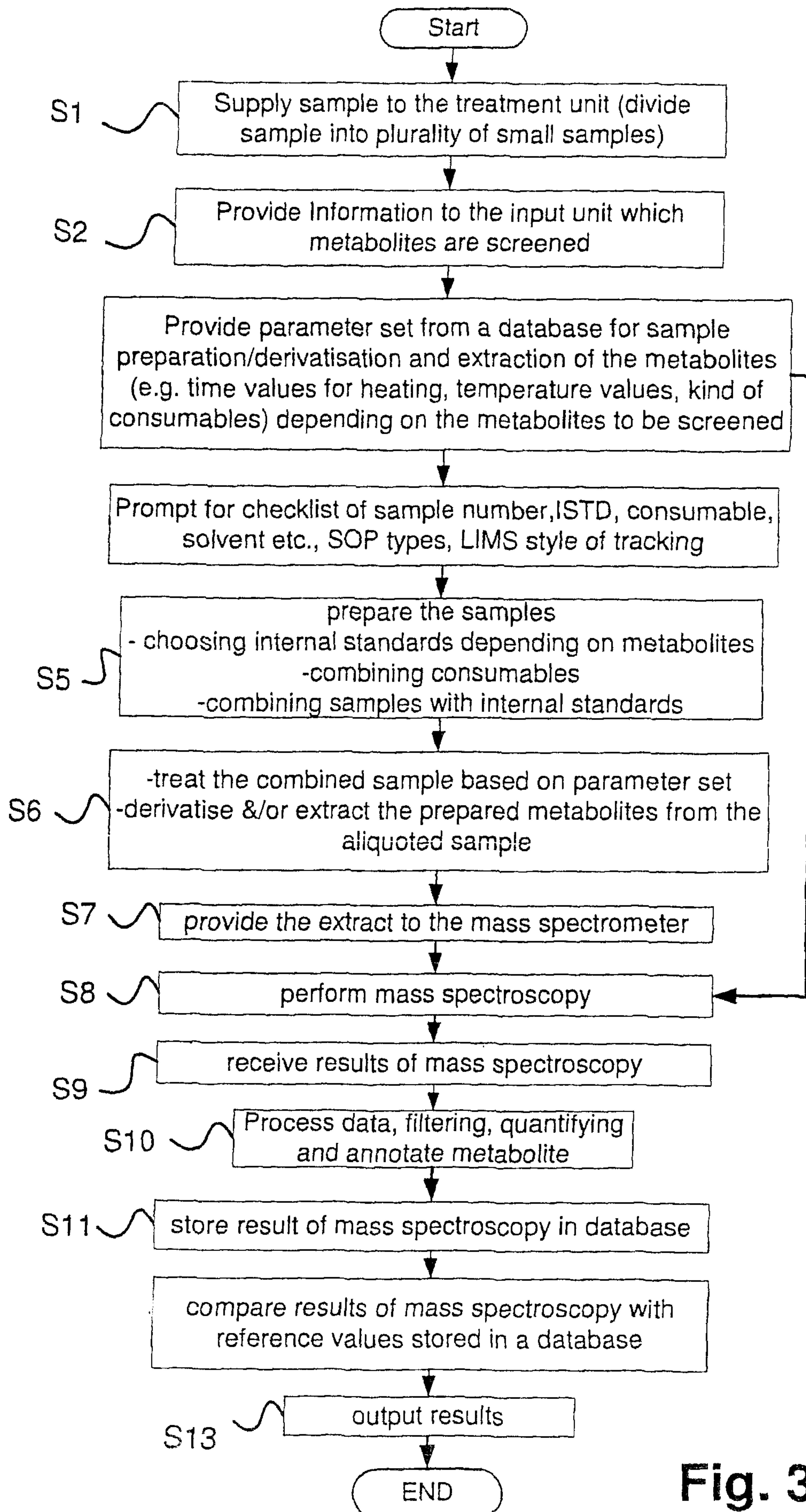


Fig. 3

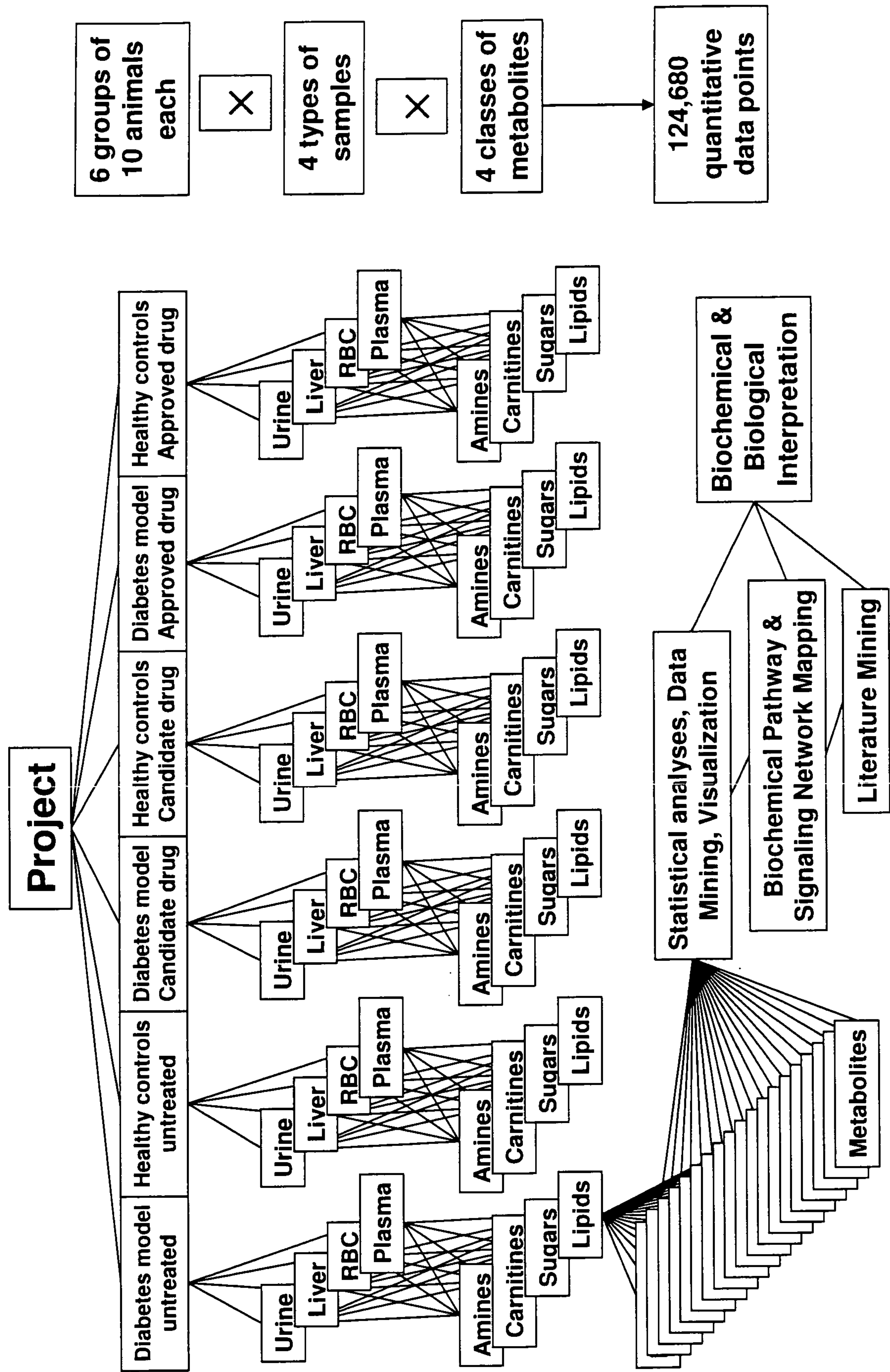


Fig. 4

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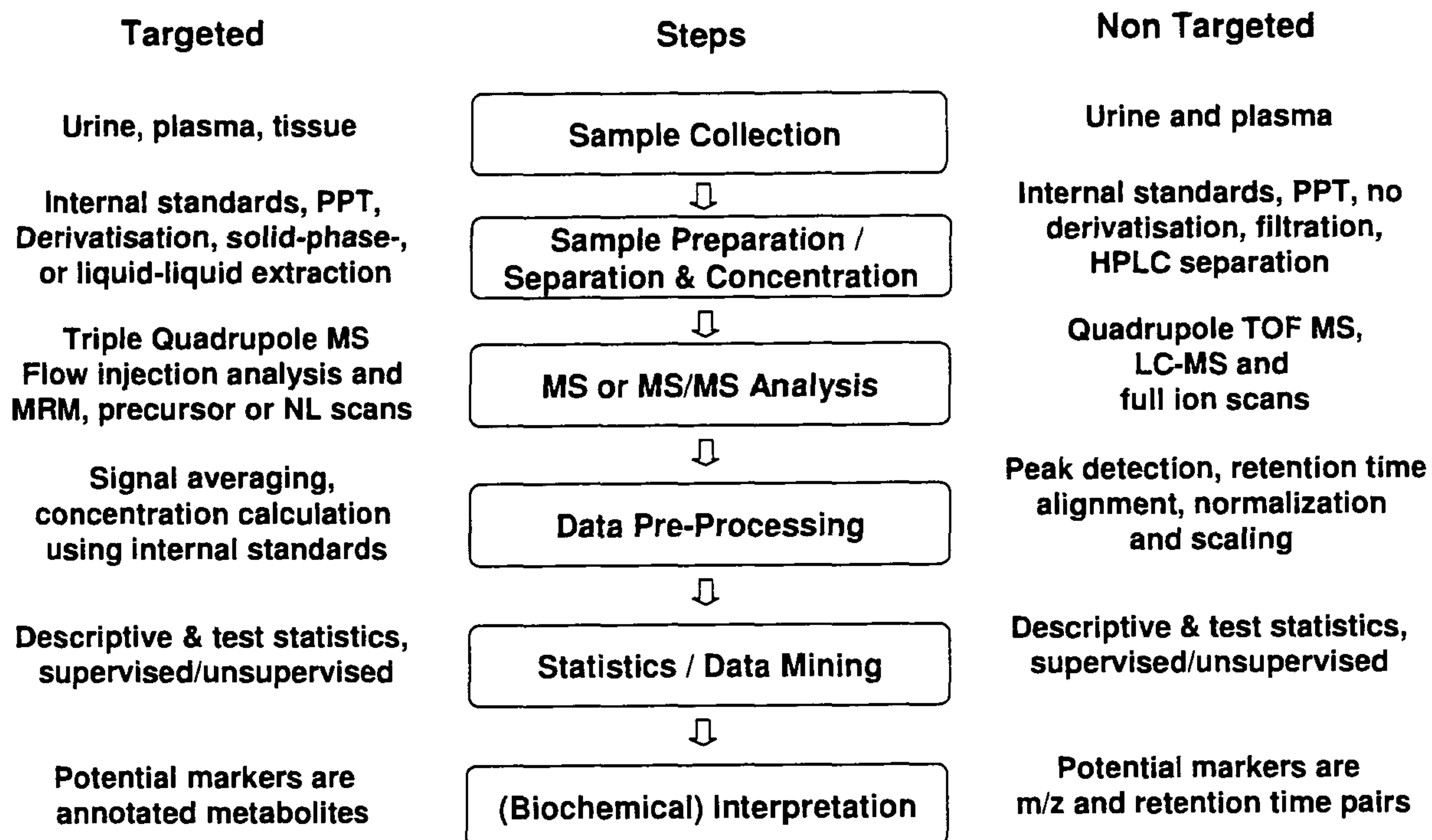


Fig. 5

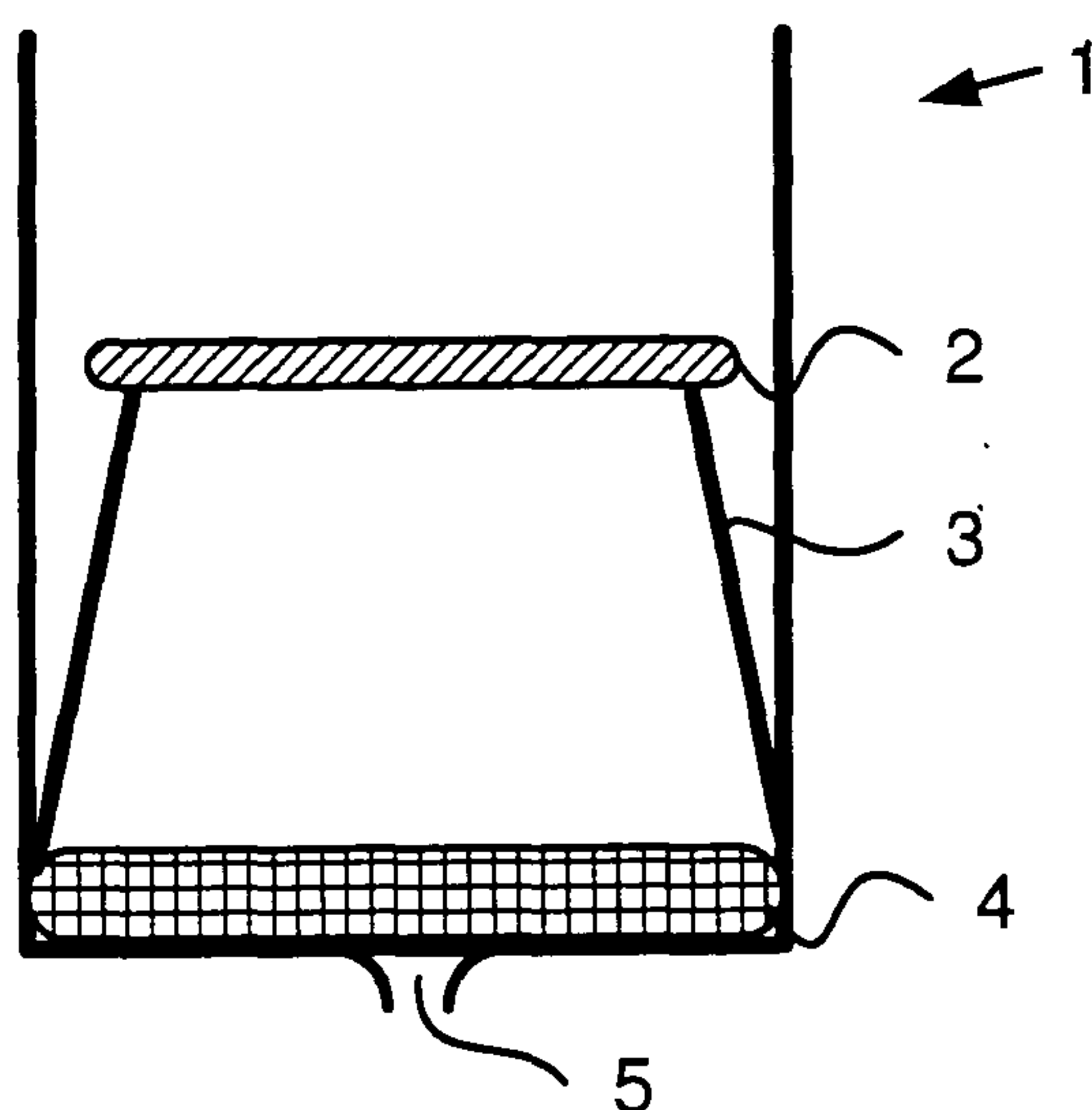


Fig. 7

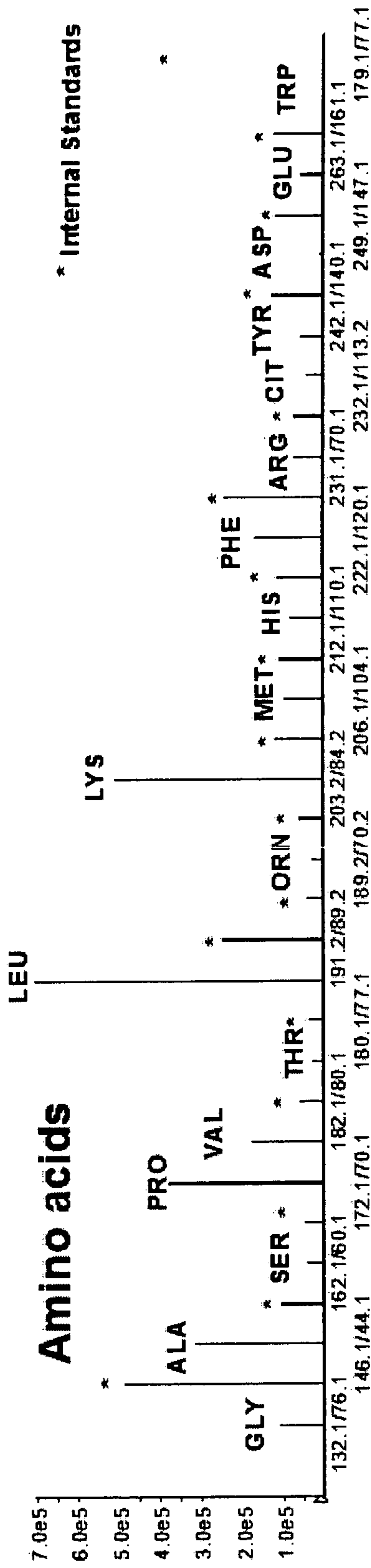


Fig. 6

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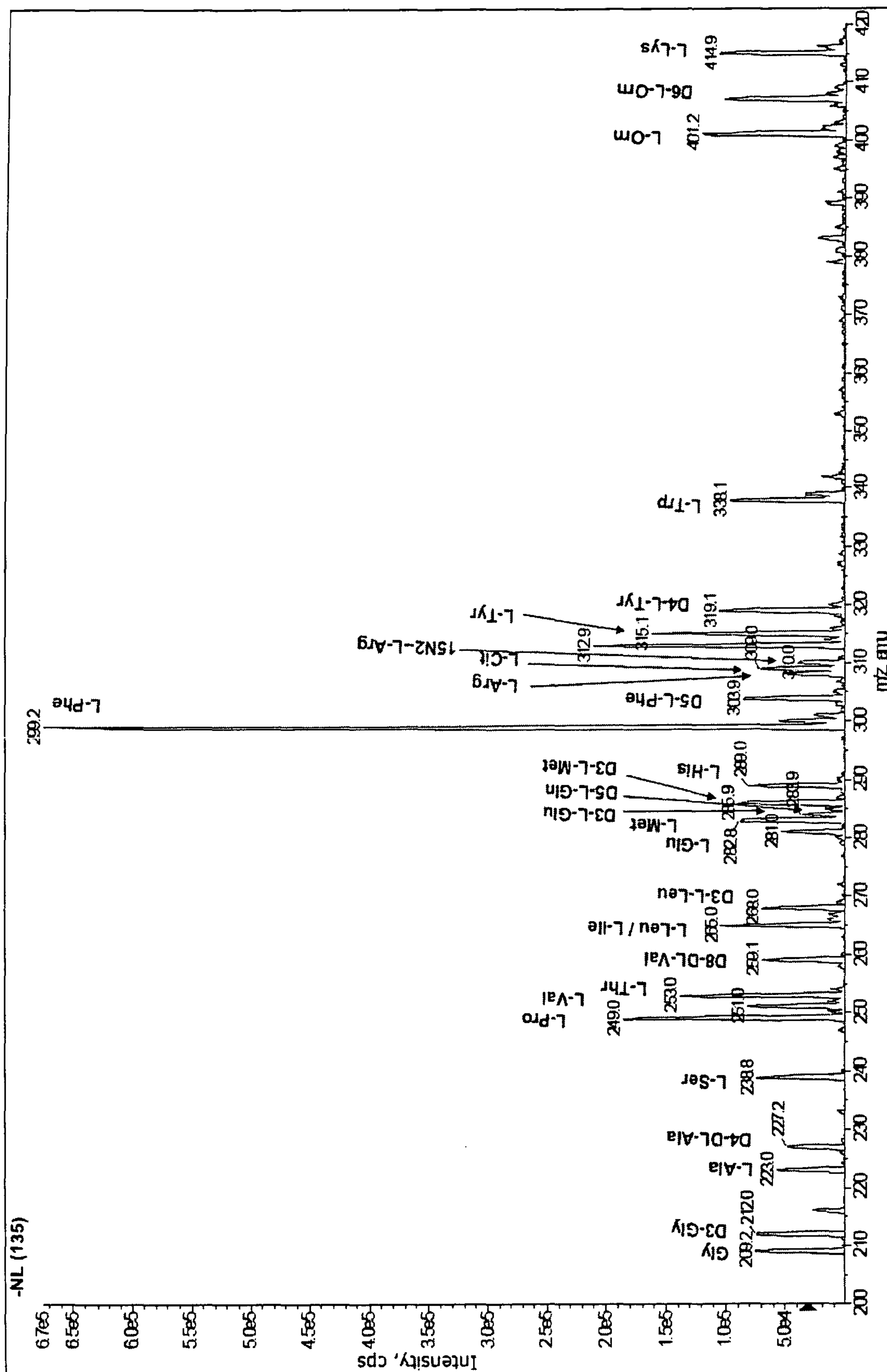


Figure 8. Phenylthiourea derivatives of a selection of amino acid standards and internal standards (stable isotopes)

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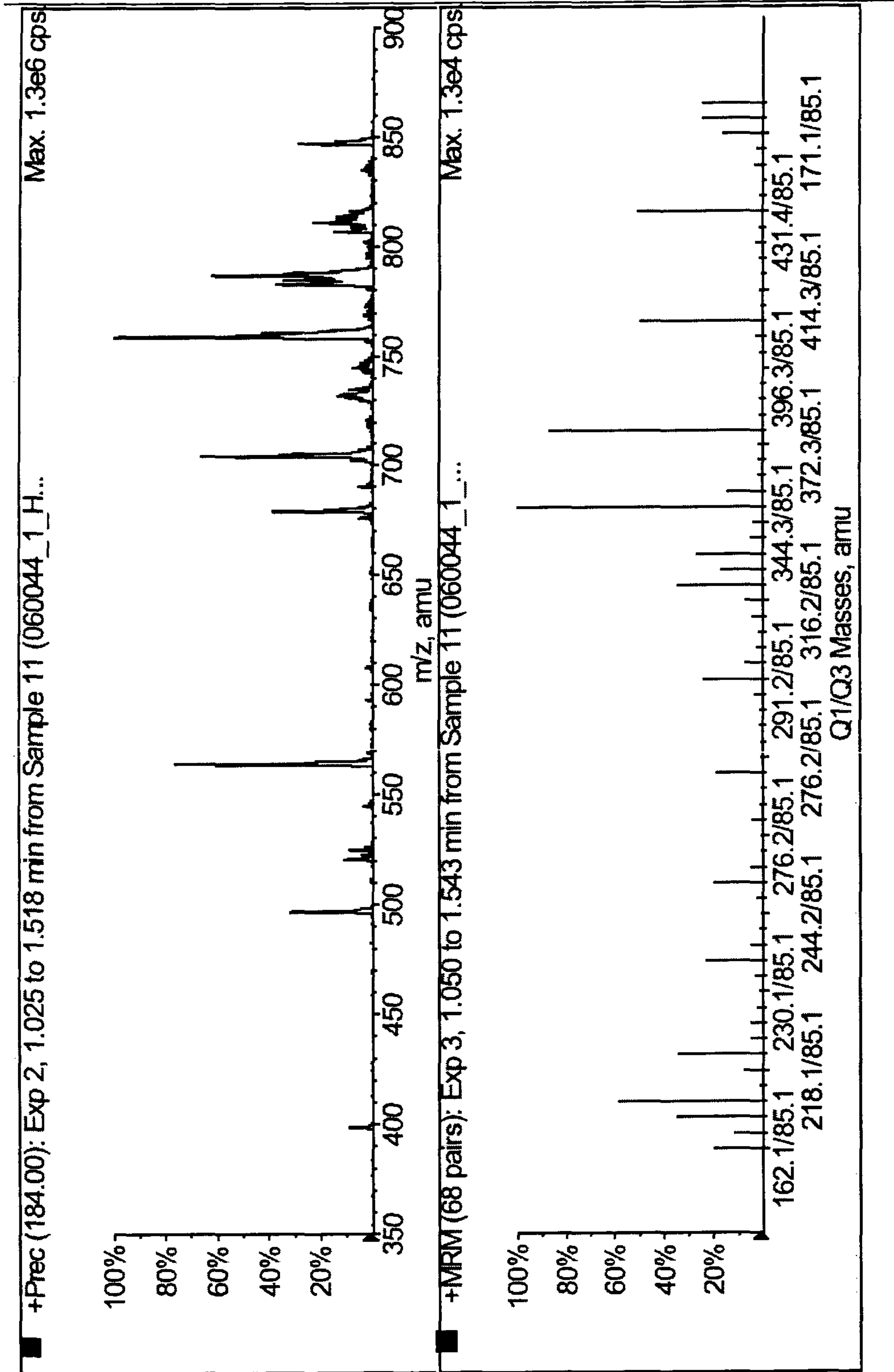


Figure 9. A shows precursor ion of 184 specific for choline-containing lipids like sphingomyelins and phosphatidylcholines and B; multiple reaction monitoring (MRMs) of individual acylcarnitines, all extracted from plasma

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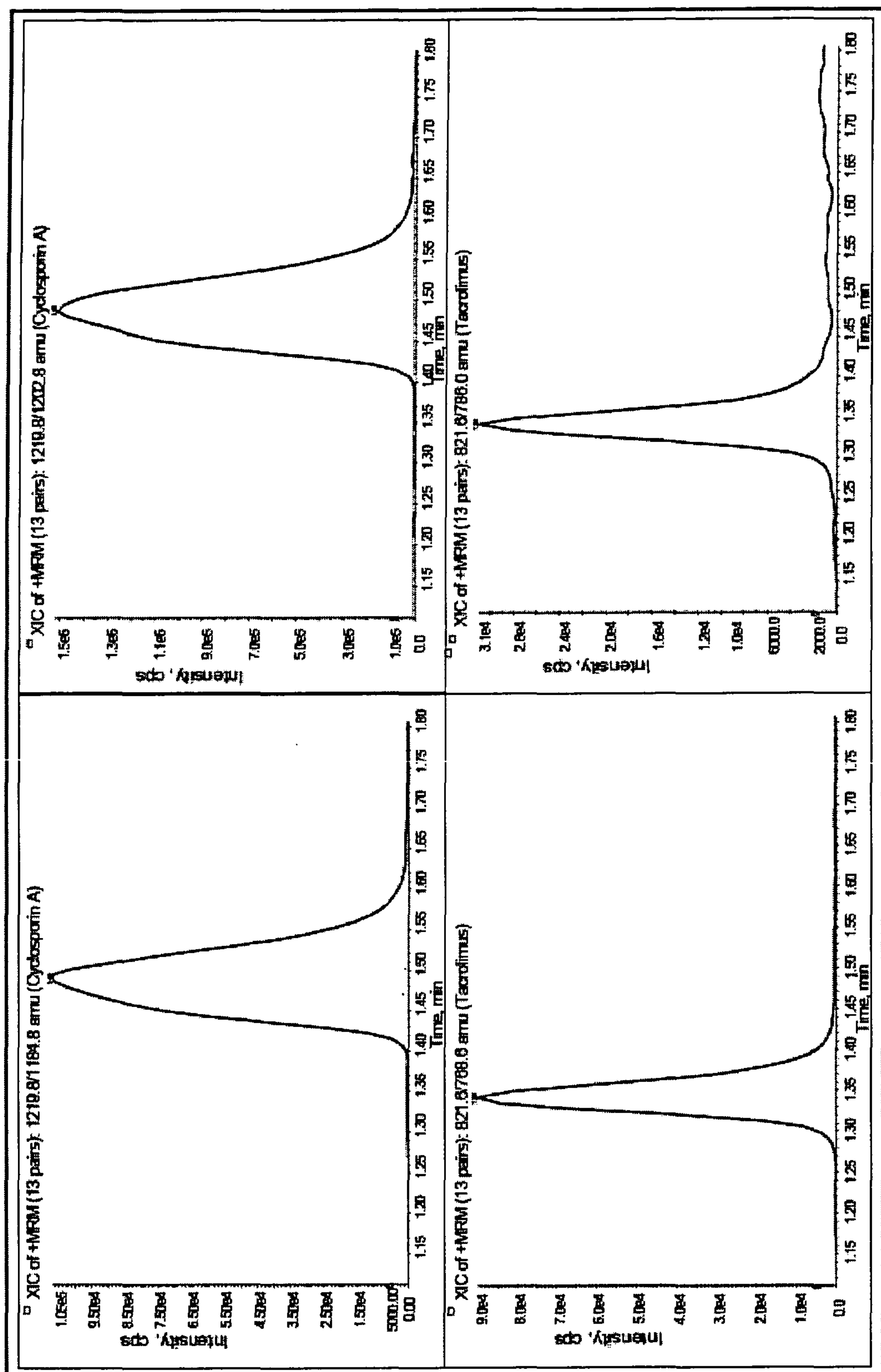


Figure 10a. Multiple MRM transitions of therapeutic drugs Cyclosporin A and Tacrolimus from a quality control blood sample

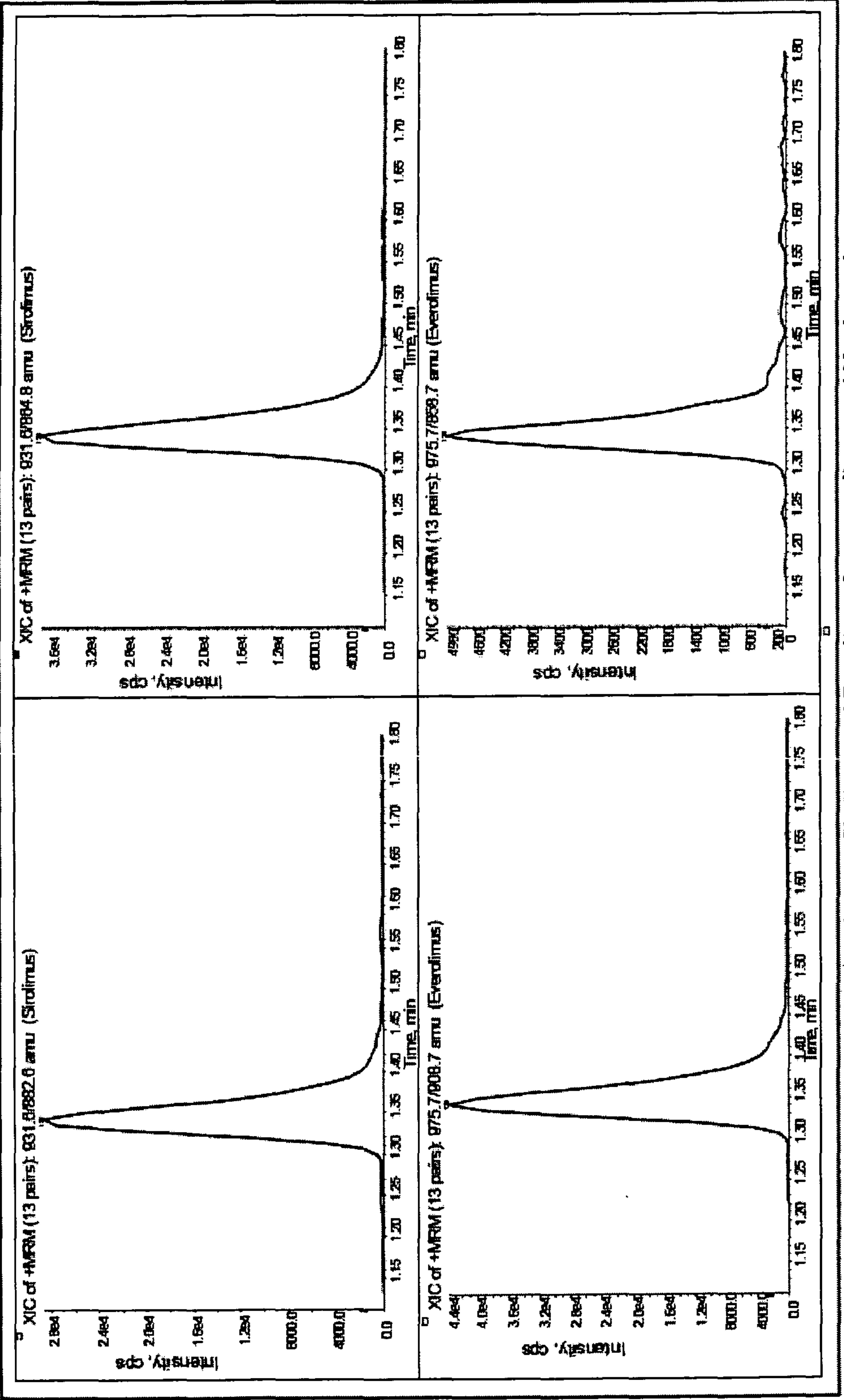


Figure 10b. Multiple MRM transitions of therapeutic drugs Sirolimus and Everolimus from a quality control blood sample

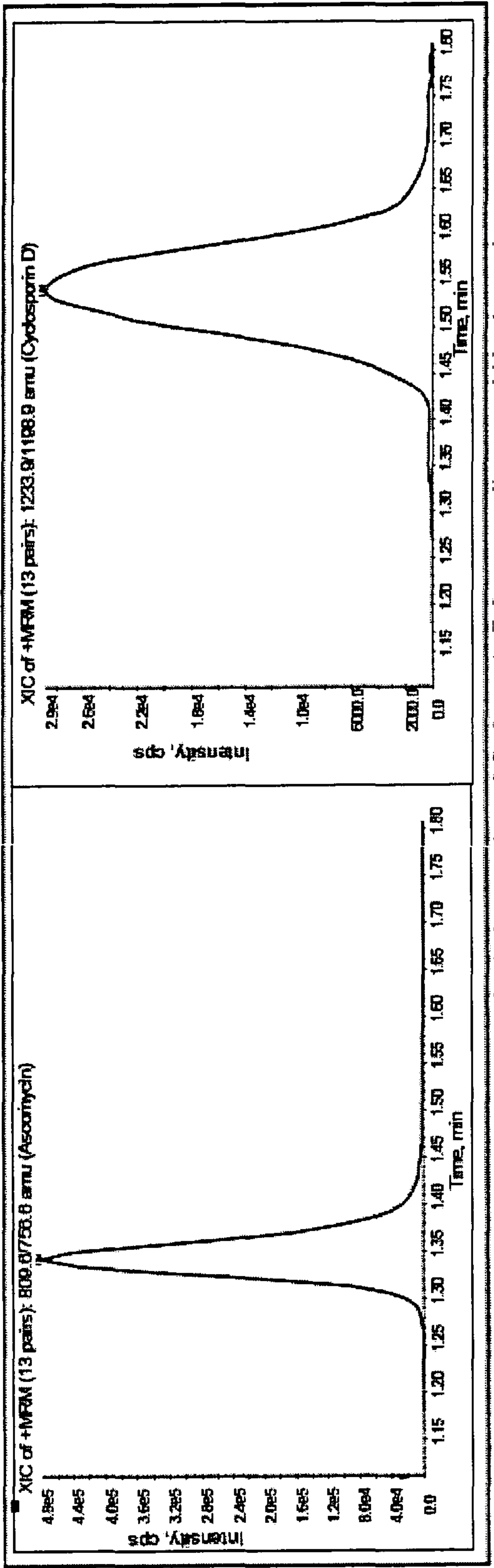
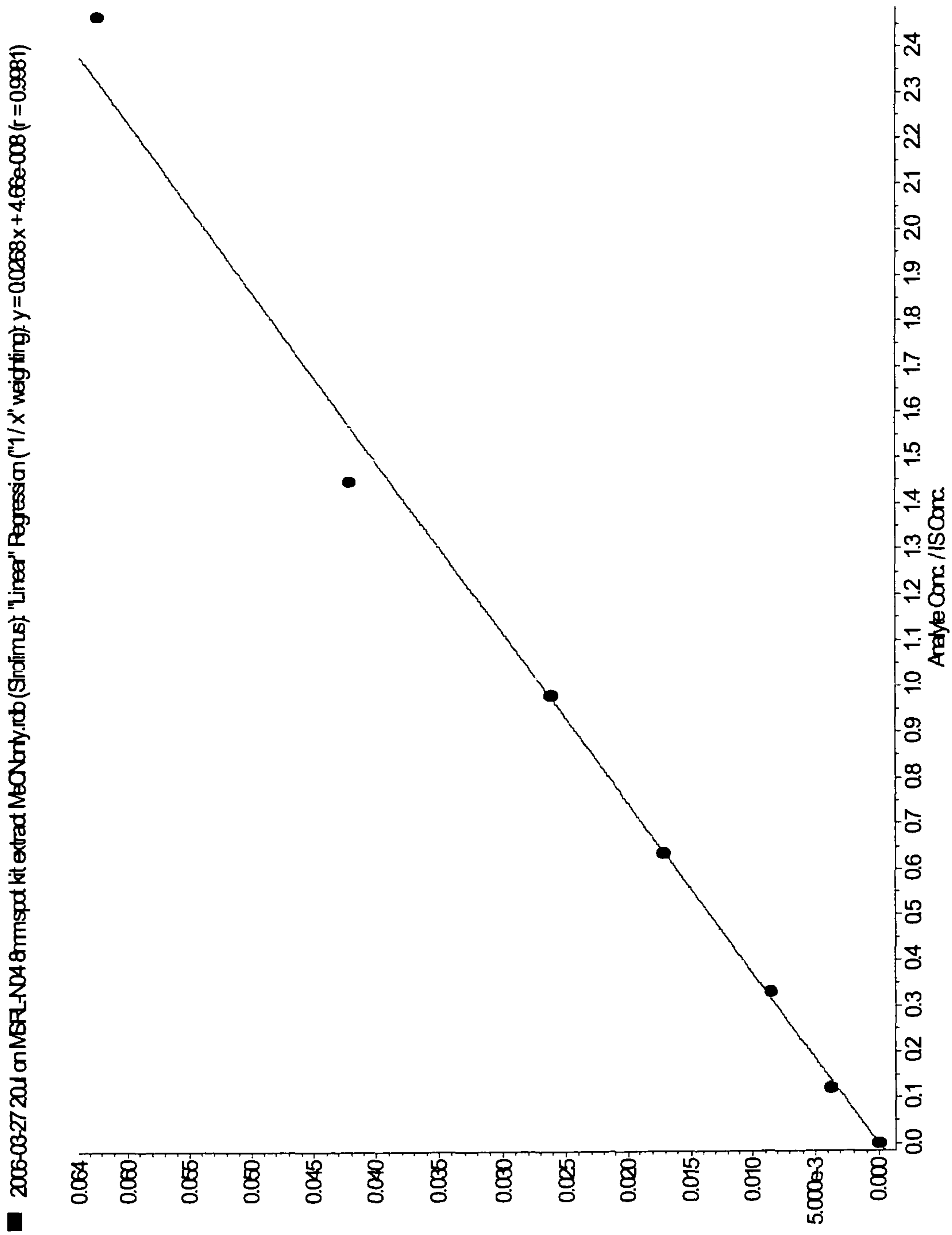


Figure 10c. Multiple MRM transitions of Internal standards Ascomycin and Cyclosporin D from a quality control blood sample

11 / 14Figure 11. Sirolimus calibration curve ($r = 0.9981$)

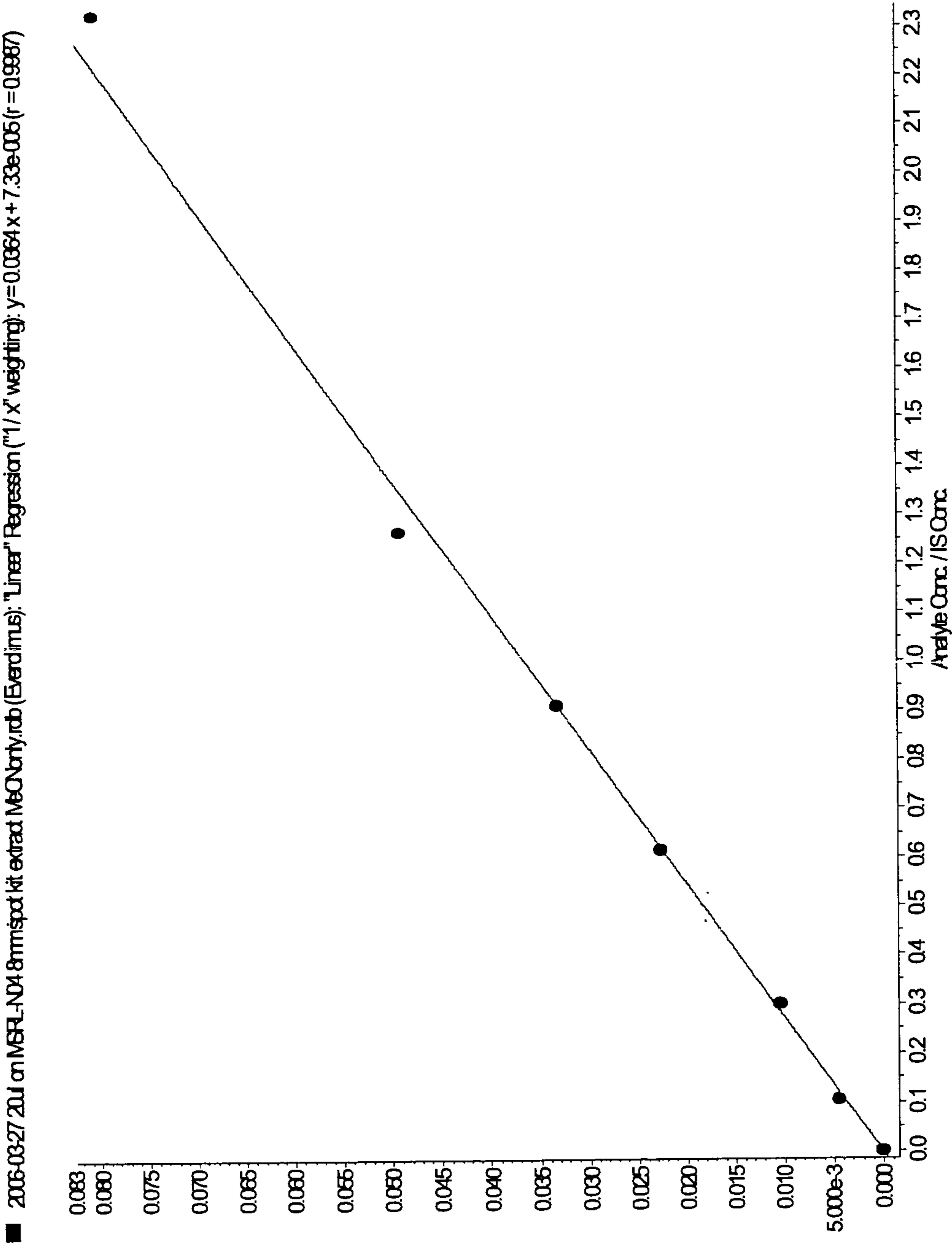
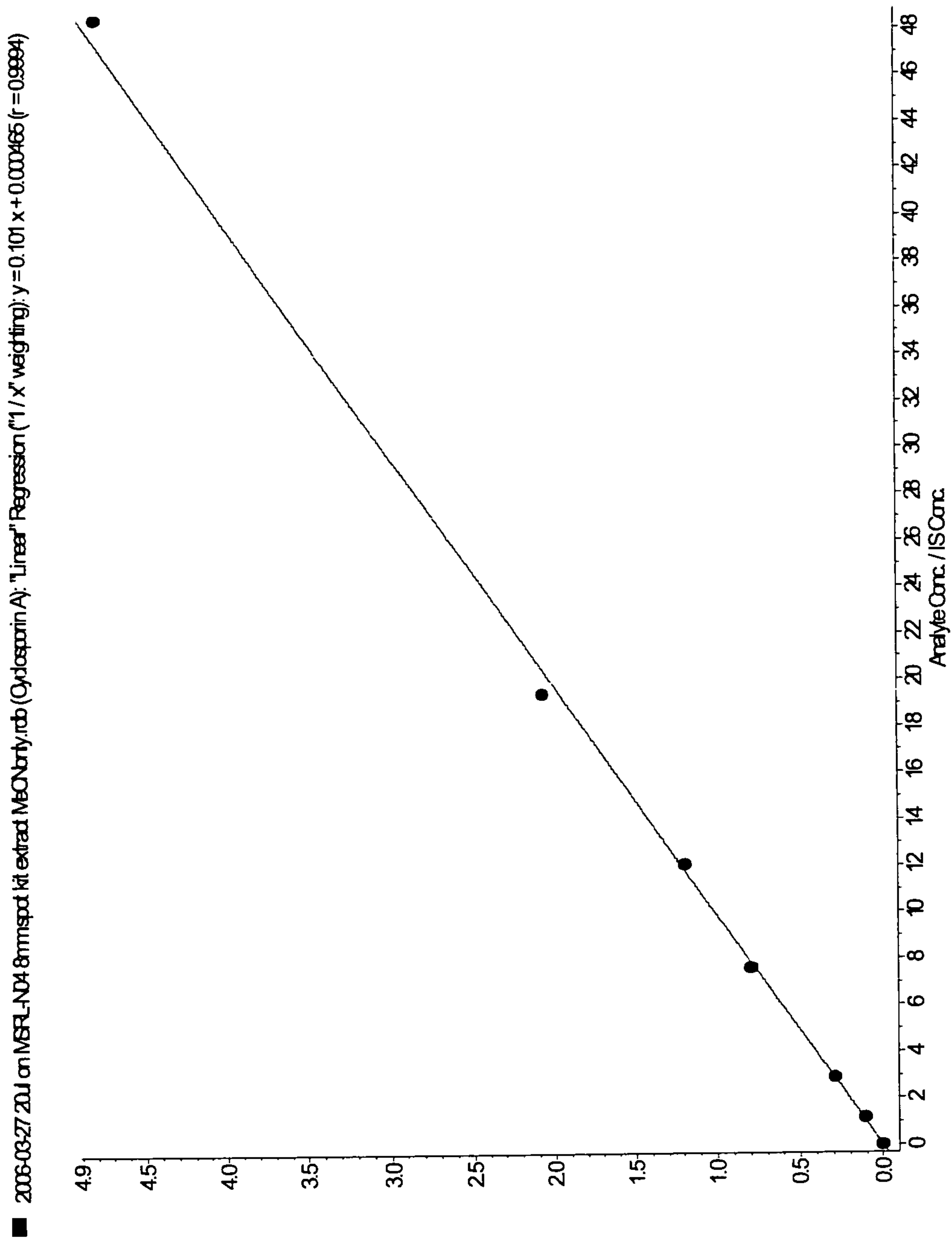
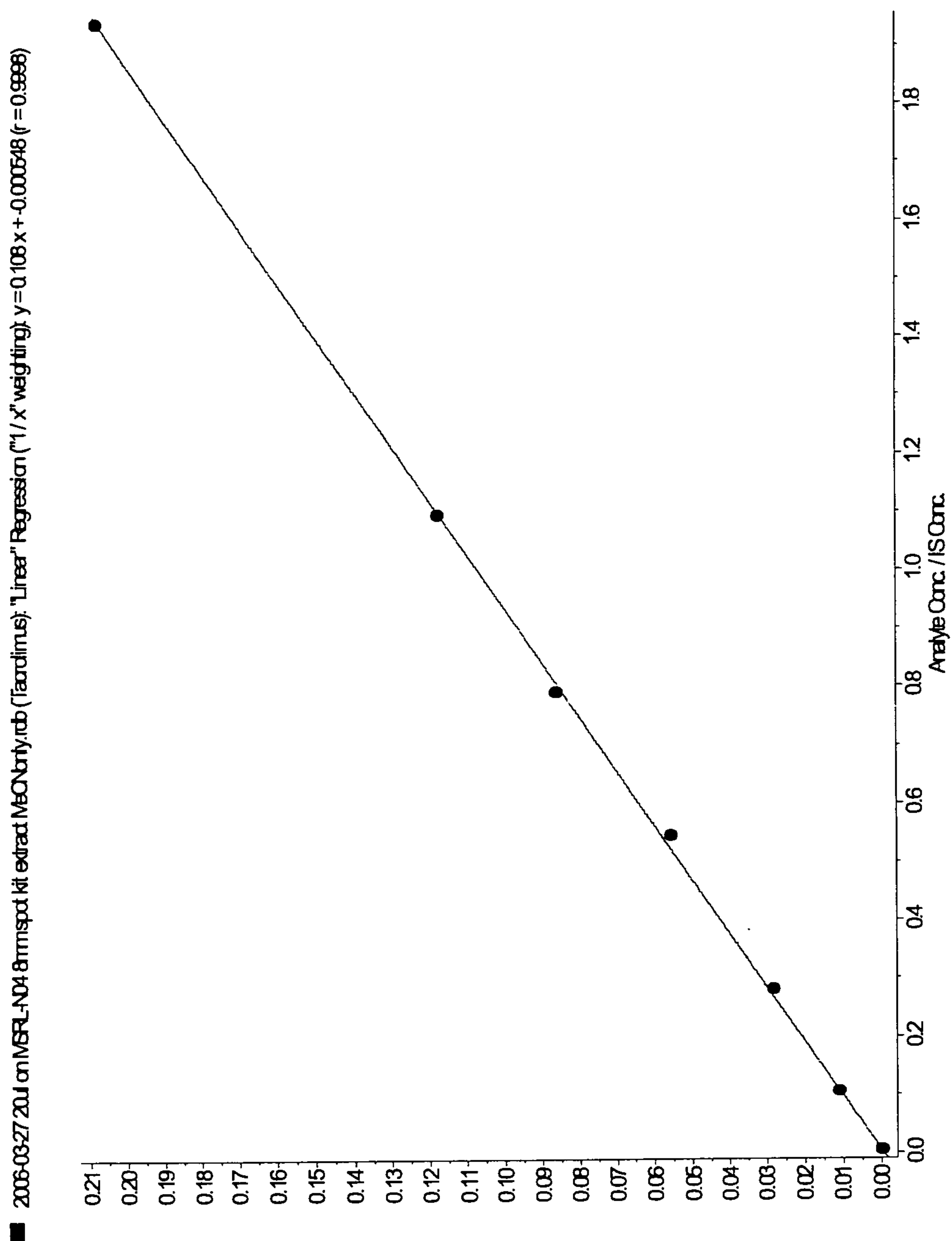


Figure 12. Everolimus calibration curve (r=0.9987)

13 / 14Figure 13. Cyclosporin A (Cyc A) calibration curve ($r = 0.99994$)

14 / 14Figure 14. Tacrolimus calibration curve ($r=0.9998$)

