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(71) Applicants *(for all designated States except US)*: **KING'S COLLEGE LONDON** [GB/GB]; Strand, London WC2R 2LS (GB). **GUY'S AND ST. THOMAS'NATIONAL HEALTH SERVICE TRUST** [GB/GB]; Guy's Hospital, St Thomas' street, London SE1 9RT (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **DALTON, Raymond, Neil** [GB/GB]; 3 Bickmore Way, Tonbridge, Kent TN9 1ND (GB). **TURNER, Charles** [GB/GB]; 15 Shearman Road, London SE3 9HY (GB).

(74) Agent: **WILLIAMS, Powell**; Morley House, 26-30 Holborn Viaduct, London EC1A 2BP (GB).

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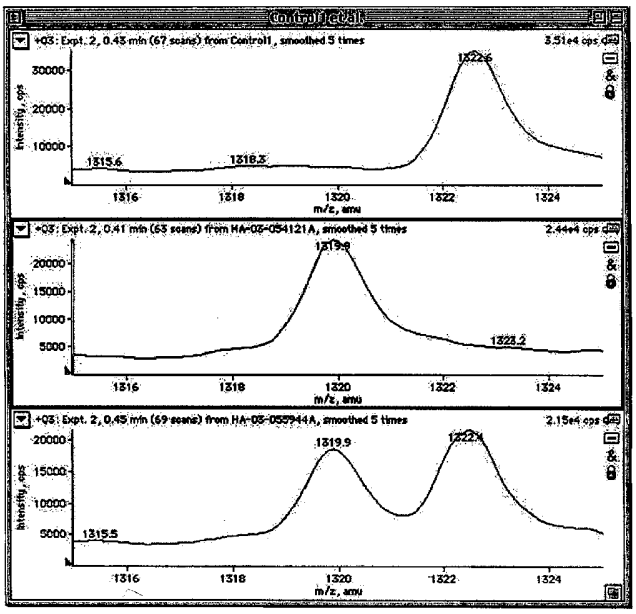
(54) Title: SCREENING METHOD FOR PROTEIN VARIANTS USING MASS SPECTROMETRY



(57) Abstract: A screening method using mass spectrometry, particularly where the ionisation technique produces a multiply-charged spectrum, is used to detect variant peptides, polypeptides and proteins that cause or are indicative of disease. The mass spectrometry is focused on a specific multiply-charged species of the polypeptide of interest, enabling a single targeted ionised species and its corresponding variant (if present) to be measured with greater mass precision. An example of use of this method in a screen for sickle cell disease and sickle cell trait by detecting haemoglobin S and other haemoglobin variants using electrospray mass spectrometry is provided.

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SCREENING METHOD FOR PROTEIN VARIANTS USING MASS SPECTROMETRY

This invention relates to mass spectrometry and more particularly to the use of mass spectrometry to detect variant peptides, polypeptides and proteins that are causes or potential causes of disease in living organisms.

Mass spectrometry has proven to be a most valuable tool for the determination of molecular structures of molecules of many kinds, including biomolecules, and is widely practised today. The technique involves bombardment of the molecular species under examination with electrons or other high energy particles which cause the ionisation and fragmentation of the molecule, resulting in a wide spectrum of ionised particles of varying charge and mass. Soft-ionisation techniques, such as electrospray, result in ionisation but do not primarily cause fragmentation of molecules. The technique is particularly valuable in producing multiply-charged species of proteins and peptides. The advantage is that high molecular weight proteins produce a mass/charge series that can then be analysed within the relatively low mass range of a standard quadrupole. The problem is the complex mass/charge spectrum produced.

As conventionally practised the spectrum is converted into a computer-generated deconvoluted spectrum having a single mass peak for each polypeptide. Current developments of mass spectrometry have centred largely around developing the most effective software necessary for deconvolutional analysis. In spite of continuing improvements in this part of the technique, however, this remains the most demanding and time-consuming component of the procedure for every particular determination. The use of deconvolutional analysis has become indispensable in mass spectrometry aimed at the elucidation of previously unknown or uncertain molecular structures but it has so far proved impossible to simplify the current methodology to significantly reduce the time required for its performance.

Mass spectrometry is also used for the detection of variant proteins and polypeptides implicated in serious diseases. For example, many variant or mutant forms of the polypeptide sub-units of haemoglobin are known to result in various forms of anaemia, and many such mutations are of only one amino acid. The basic molecular structure and amino acid sequences of these proteins, and the corresponding mutations in the DNA encoding them, are already of record in the literature. The complexity of current methods of this kind is well illustrated in the classical mass spectrometry approach described in the paper of B.J. Wild and colleagues (Rapid Identification of Haemoglobin Variants by Electrospray Ionisation Mass Spectrometry, in 'Blood Cells, Molecules, and Diseases (2000) 27(3) May/June: 691-704) to which reference should be made for full details of the procedure, in particular the section on METHODS at page 693 et seq. and Figure 2 at page 697 which shows deconvoluted ESI mass spectra for the normal and one particular variant β haemoglobin chain. Table 1 on page 698 of this quoted paper lists many mass and amino acid changes produced by single base changes in the nucleotide coding triplet and determinable by this method.

It has struck us that when mass spectrometry is used to detect the presence of such variants, for example, as a preliminary screen prior to more definitive methods of diagnosis of disease potential, it is possible to simplify the procedure and to depart radically from the conventional method described above. According to our invention the use of deconvolution analysis is unnecessary and completely avoided. Instead, working from knowledge of the normal (wild-type) polypeptide and its mutants or variants, we focus mass spectrometry on a specific multiply-charged species of the polypeptide of interest and dispense with the recording and analysis of all other data that are at present utilised. This enables us to concentrate data acquisition and, therefore, measure a single targeted ionised species with greater mass precision and to detect with similar mass precision a peak corresponding to the variant if present in the sample tested. Mass accuracy, though desirable, is not essential because knowing the expected mass/charge ratio of the normal polypeptide at any charge value enables simple mass correction to be applied. The correction allows the mass of a variant

polypeptide to be calculated accurately. In important applications, e.g. population screening for variant polypeptides, the actual masses are not required, simply the accurate mass/charge difference of the variant polypeptide from the normal polypeptide.

We therefore refer to this new method as one of targeting specific selected ionised species. This method may be applied with advantage to detect not only variants containing amino acids which differ from the norm, as in the many possible known haemoglobinopathies described hereinafter, but also to detect variants in glycosylation patterns of polypeptides having the normal expected amino acid sequences e.g. as found in the molecule transferrin. Furthermore, the method may be utilised to detect deletions or additions of one or more amino acid residues from the expected polypeptide sequence.

The present invention therefore comprises a method of testing a sample by mass spectrometry, where the ionisation technique produces a multiply-charged spectrum, to detect (a) the presence or absence of a known polypeptide or derivative of a polypeptide, or (b) the presence or absence of a variant of a known polypeptide or derivative of a polypeptide, in which scanning of the sample is targeted to selected ionised species of known mass/charge ratio, the absence of the expected value of mass/charge ratio being indicative of the absence of the known polypeptide or derivative thereof, or the presence of the variant polypeptide or derivative thereof being indicated by a shift in mass/charge ratio from the expected value.

The method of the present invention therefore concentrates data acquisition on a restricted mass/charge range (mass window) to include the normal polypeptide and the variant polypeptide or polypeptides of interest. Targeting in this manner is more reproducible and reveals peaks corresponding to the normal mass/charge ratio and any shifts from the norm due to variants present in samples taken from patients who are either homozygous or heterozygous in this respect.

Thus the method may frequently require only one restricted mass window to be targeted. The use of separate windows for the normal and for the variant polypeptide is also possible. Additional mass windows may be used to target other variants or other polypeptides.

The choice of the targeted species is not critical to the invention, although one or more may be preferred for various reasons and at the discretion of the analyst, especially with regard to the need, on occasion, to compromise between sensitivity and specificity. In practice, it may be desirable also to target an ionised species additional to the primary targeted species as a confirmatory measure. For example, this may be done in order to exclude potential interference from singly charged species of another compound extraneous to the investigation which might be falsely interpreted as a positive indication.

The present invention is widely applicable to any of the standard methods of mass spectrometry in use or under development. It will be described hereinafter by way of example for electrospray ionisation quadrupole mass spectrometry, but it will be appreciated by those skilled in this general field of technology that other ionisation methods producing multiply-charged spectra and other mass analysis systems, e.g. time-of-flight (TOF) and magnetic sector, are also possible.

Similarly, the invention is applicable to the detection of a wide range of abnormal forms of the polypeptide or polypeptides of interest. Preferably the normal form is used for mass calibration of the instrument for each group of samples analysed. As a modification of this concept, and for use in large scale screening, e.g. population screening, the mass found in the great majority of 'normal' samples will act as an internal control from which deviations are visually apparent and readily calculated.

Variant haemoglobin proteins that cause various forms of anaemia can be found described in standard textbooks, including 'Clinical Genetics' by Golder N. Wilson, Wiley Liss (2000) at pages 114-119. The above-mentioned B.J. Wild paper also lists

many such amino acid changes at page 698 thereof. All such variations are amenable to detection in accordance with the present invention.

One example of the use of the new method to detect sickle cell disease in accordance with the present invention will now be described in detail with reference to the accompanying drawings of which :-

FIGURE 1 illustrates a typical spectrum obtained by scanning human blood,
FIGURE 2 illustrates the flow injection profile of blood from a normal patient,
FIGURE 3 illustrates scans for normal, SS, and AS patients, and
FIGURE 4 illustrates the overlay of scans of Figure 3.

EXAMPLE: Screening method for the detection of haemoglobin S and other haemoglobin variants using electrospray mass spectrometry

Background

Haemoglobin is the main molecule transporting oxygen in the blood. It is formed of 4 globin polypeptide chains and 4 haem molecules. Haemoglobinopathies are inherited disorders of globin structure or synthesis. Genetic mutations result in amino acid substitutions in globin chains or failure to synthesise globin. Sickle cell disease is associated with the most common pathological mutation, which is found in the β-globin polypeptide chain, where valine is substituted for glutamic acid at position 6. Haemoglobinopathy variants are classically differentiated using methods based on charge separation, essentially isoelectric focussing and cation exchange high performance liquid chromatography. Confirmation has often necessitated genotyping, although, more recently, electrospray quadrupole mass spectrometry has been used.

Sickle cell disease occurs in patients who are homozygous for the sickle mutation and in a limited number of compound heterozygotes containing a copy of the sickle mutation. It is a significant public health problem that has resulted in the

establishment of population screening programmes to identify patients at risk of sickle cell disease. Although the classical methods are relatively simple technologies and there is a considerable knowledge base in their use, both isoelectric focussing and cation-exchange chromatography have drawbacks when applied to population screening. Isoelectric focussing is regarded as unwieldy and difficult to automate, while cation-exchange chromatography, although readily automated carries a significant reagent cost overhead.

Mass spectrometry to identify haemoglobin variants has concentrated on specific confirmation requiring sequential sample clean-up, quadrupole mass spectrometry, triple quadrupole mass spectrometry-mass spectrometry, analysis of tryptic digests, and significant computer time overhead for deconvolutional analysis of the multiply-charged spectrum to re-construct the primary molecular weights. It also requires significant technical expertise. The value of mass spectrometry in haemoglobinopathy screening has also been questioned because, in routine operation, the mass calibration is not sufficiently accurate to be confident of the calculated re-constructed mass. In addition, the mass resolution of bench-top mass spectrometry is inadequate to provide unambiguous identification/diagnosis.

Time-of-flight mass spectrometry has been applied to neonatal sickle cell disease screening in the paper by U.A. Kiernan and colleagues (High-Throughput Analaysis of Hemoglobin from Neonates Using Matrix-assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry, in Clinical Chemistry (2002) 48(6);947-949. The technique generates singly-charged polypeptide ions obviating the need for deconvolutional analysis. Unequivocal diagnosis, in a limited number of samples, was demonstrated using peptide mapping of tryptic digests. This provides an attractive solution to the problem of neonatal sickle cell disease screening. However, the sample preparation is relatively complex when compared to current screening methods and the technology is not readily available

in neonatal screening laboratories where metabolite analysis using electrospray mass spectrometry-mass spectrometry is now routine.

Our objective was to develop a simplified mass spectrometric approach that could be applied in a routine screening programme (general, ante-natal, and neonatal) to identify patients at risk of sickle cell disease or other haemoglobinopathies.

The approach is generally applicable to haemoglobinopathies but the identification of patients at risk of sickle cell disease is described. The sickle protein is a mutation of the normal β-globin chain. The molecular weight of the normal human β-chain is 15867.2 daltons and the molecular weight of the sickle protein is 15837.2 daltons. Mass scanning (500-1500 m/z) of an inject of diluted human blood produces a series of multiply charged ions (see attached Figure 1). This mass/charge series provides the raw data for the deconvolutional analysis that generates the original masses of the haemoglobin chains and their adducts.

The first concept that we consider original is to realise that in any deconvolutional analysis the precision and accuracy of the initial masses in the mass/charge series will determine the outcome of the deconvolutional analysis. If, however, using knowledge of the mass/charge series for normal human β-globin we target a particular mass/charge species, e.g. 12, and measure that precisely and accurately then the rest of the mass/charge series becomes irrelevant and the original mass is very easy to calculate and requires no deconvolutional analysis. The precision with which the mass/charge ratio can be measured depends on the mass range used and the acquisition time. Targeting a particular normal human β-globin mass/charge species, e.g. 12, using a narrow mass charge window (1315-1325 a.m.u.) that includes both normal and sickle variants means that the mass precision is significantly enhanced for a given acquisition time. The latter being an important consideration for a high-throughput screening method.

The second concept that we consider original is the use of the normal human β-globin chain, whose is mass is known, to accurately calibrate the mass spectrometer for each group of samples analysed. This can be done physically before analysis by calibrating the machine using a control normal haemoglobin blood sample or by applying a simple correction to the data post-analysis. A simpler approach, conceptually and practically, particularly when it comes to population screening, is to use the knowledge that the majority of samples are normal and to identify abnormal β-chains by looking for deviation from the normal mass. In the case of sickle protein the mass difference is 30 daltons less than in the normal β-chain. That implies, at a mass/charge ratio of 12, the difference will be 2.5 plus or minus experimental error.

Sample preparation

3mm blood spots are punched into a deep-well polypropylene 96 well plate. 0.5 ml of deionised water is added to each well, the plate is capped, and mixed gently for 10 min. If liquid anti-coagulated blood samples are used then the blood sample is mixed and diluted 1:200 (5 μl/ml) with deionised water and the diluted sample transferred to a deep-well polypropylene 96 well plate and capped.

Sample delivery

We used Shimadzu pumps and a Gilson 215 autosampler. Sample (5 μl) is injected into the solvent (acetonitrile:water 50:50 with 0.025% formic acid) stream flowing at 50 μl/min.

Mass spectrometry

We used an Applied Biosystems API2000. This is a triple quadrupole mass spectrometer but simple Q3 scans are performed. The standard polypropylene glycols (PPGs) positive ion state-file is used. Two scan experiments, m/z 1315-

1325 and m/z 1215-1225; the latter scan is looking at the 13-charged β-chain and acts as an internal confirmation of the primary 1315-1325 data. 116 scans are performed and the total inject to inject time is 75-90 seconds.

Results

An example of the flow-injection profile and the 2 scans, m/z 1215 - 1225 and m/z 1315 -1325, in a blood sample from a normal subject is shown in Figure 2. The normal β-globin chain can be seen at m/z 1322.6 and 1220.9, the 12 and 13 charged species, respectively.

In Figure 3, examples of the m/z 1315 - 1325 scans for the 12 charged β-globin chains are shown for a normal subject (control), a sickle cell disease patient (SS) with two copies of the sickle protein and no normal β-globin chain, and a heterozygous subject (AS) with equivalent amounts of the sickle protein and normal β-globin chain. Note in the heterozygous subject that the m/z difference between the normal and abnormal protein is the predicted -2.5. As the charge is 12 then actual mass difference of the proteins is the expected 30 daltons. Figure 4 shows the same scans overlayed to emphasise the difference observed.

An initial experiment to look at the system in routine operation was undertaken. The first objective was to check how robust the system is with a significant number of samples (96) analysed sequentially: to check for mass drift and effects on the mass spectrometer source. Secondly to determine the precision with which the m/z ratios can be measured and specificity in differentiating normal, AS, and SS.

Finally, to check the stability of the samples:
A 96 well plate was prepared containing 8 blood spot samples from each of 10 control subjects (total 79 because an extra mis-punched SS included), 8 blood spot

samples from 1 heterozygote sickle trait (AS) subject, and 8 blood spot samples (total 9 including 1 mis-punched sample) from 1 homozygote sickle cell (SS) patient. The plate was analysed on 4 consecutive days. The basic data confirms that the system is sufficiently robust for screening purposes. Only 1 control sample, on day 1, failed to inject. There was no evidence of any mass drift or cumulative effect of 96 consecutive injections on the mass spectrometer. On all 4 days all the normal, AS, and SS could be correctly identified by visual inspection. The daily precision of the m/z ratio for the normal β-globin chain and the sickle protein are shown in Tables 1 and 2, respectively. Daily primary mass calculations, i.e. (m/z * 12)-12, for the normal β-globin chain are shown in Table 3. Note they are not the same as the theoretical values. Calibrating the mass spectrometer using a normal blood sample could have been done but, in fact, the mass of the normal β-globin chain is known and can be used to re-calculate the mass of the sickle protein, Table 4. The check data using the m/z 1215 -1225 scan of the 13 charged protein provides confirmation in every analysis. The complete set of data for each day is not shown herein.

The area of each signal was calculated using Turboquant, the basic data processing package provided with the API2000. The data was exported to Excel and the ratio of the areas of the normal β-globin chain and the sickle protein calculated and simple rules applied that enabled electronic identification of normal, sickle trait, and sickle cell disease profiles. At this stage the rules are crude but would be refined during analysis of a large population screen. The data are not shown.

Liquid blood samples from adult patients with AS or SS have been analysed and successfully identified in over 200 cases (data not included).

Note that this test is not diagnostic. The mass resolution on the API2000 is insufficient to measure the re-constructed mass to an accuracy of 1 dalton. The precision data defines the potential inaccuracy, in the region of +/- 3 daltons. In

addition, the mass of the sickle protein is not unique. The value of the test is that it identifies those samples from patients who are at risk of sickle cell disease; these samples can then go forward for confirmatory testing. In terms of population screening the importance of the test is that the vast majority of samples can be identified as normal and reported as a negative risk for sickle cell disease.

Benefits

The method described enables the application of electrospray mass spectrometry, a robust screening technology routinely available in many neonatal screening laboratories, to the identification of patients at risk of sickle cell disease. The method is simpler and faster than either isoelectric focussing or cation-exchange high performance liquid chromatography. The method requires no costly reagents or consumables. The whole process from sample preparation through to identification and reporting of samples from patients at risk of sickle cell disease can be readily automated. The drawbacks associated with mass spectrometry have specifically been addressed. Sample preparation has been minimised. Using specific mass/charge ratio scans has significantly reduced data acquisition time but still increased the number and, therefore, the quality of the scans. Using the obvious knowledge of the molecular weights of the normal human β-globin chain and the sickle protein means that the mass system can be very accurately calibrated and the sickle protein identified by a simple mass/charge shift. The actual molecular weight of the sickle protein can be calculated without the need for complex deconvolutional analysis that normally requires a significant computer time overhead unsuitable as a population screening application.

The system described also includes an internal check by using the 13 charged species as an internal validation of the 12 charged data. After the analytical run normal and sickle profiles can be simply identified by visual inspection or the data analysis can be automated using the peak areas or heights to apply simple rules for the electronic identification of normal β-globin, heterozygote sickle protein, and

homozygote sickle protein. Finally, the mass spectrometer and running solvent used is the same as that already used for screening for amino acid and fat oxidation disorders: implies that screening for patients at risk of cell disease can be run immediately before or after the present mass spectrometry-mass spectrometry screening and maximising resource utilisation.

Alternative Examples of the Invention

The method presented describes Q3 scanning using a triple quadrupole mass spectrometer. Although Q1 scanning is possible the scans, in our experience, were less consistent. The particular mass/charge ratio demonstrated, the 12 charged β-chain, is not unique as is clear from the use of the 13 charged β-chain as an internal check. The 12 charged β-chain was used for mathematical convenience and it is a relatively high mass, so less likely to suffer from interference, but well within the mass range of the instrument. On an instrument with a greater mass range it would be possible to use, for instance, the 10 charged β-chain and the mass difference with sickle protein would be 3 daltons enabling a more specific differentiation.

The same approach can be used for other β-globin chain haemoglobinopathies, specifically on a targeted mass/charge ratio or by increasing the mass/charge scan range to include all β-globin chain variants.

The same approach can be used for α-, δ–, or γ-globin chain haemoglobinopathies, again specifically on a targeted mass/charge ratio or by using a mass/charge scan range to include all relevant globin chain variants. For example, the molecular weight of the normal human α-globin chain is 15126.4 daltons so the 12 charged protein has a mass/charge ratio 1261.5.

In more general terms, the same approach can be applied to identifying any abnormal human protein. Because the majority of subjects are normal a known

target mass/masses can be proposed, used as a mass calibration, and abnormal modifications, genetic or post-translational, identified. For most other human proteins a significant initial sample clean-up will be required to separate the protein from the other major components like haemoglobin and albumin.

The specificity of the approach can be increased using alternative mass spectrometry techniques with greater mass resolution, e.g. time-of-flight mass spectrometry. With greater mass resolution it will be possible to investigate globin variants to within a unit mass. It will still not allow a specific diagnosis in the majority of instances because the mass is rarely unique. However, the potential possible variants would be significantly reduced and confirmation follow-up be more targeted.

Table 1 of 4
Mass precision for the 12 charged normal β-chain

	Day 1	Day 2	Day 3	Day 4
Mean	1322.79	1322.81	1322.67	1322.79
SD	0.07	0.07	0.09	0.07
CV (%)	0.005	0.005	0.007	0.005
Min	1322.6	1322.7	1322.5	1322.6
Max	1322.9	1322.9	1322.8	1322.9
n	78	79	79	79

NB 78 samples on day 1 because 1 sample failed to inject

Table 2 of 4
Mass precision for the 12 charged sickle β-chain

	Day 1	Day 2	Day 3	Day 4
Mean	1320.31	1320.38	1320.20	1320.33
SD	0.10	0.08	0.14	0.11
CV (%)	0.007	0.006	0.010	0.008
Min	1320.2	1320.3	1319.9	1320.1
Max	1320.5	1320.5	1320.4	1320.5
n	17	17	17	17

Table 3 of 4

Primary mass reconstruction

normal β-chain (should be 15867.2)

(Multiply * 12)-12

	Day 1		Day 2	
Mean	1322.79	15861.5	1322.81	15861.7
Min	1322.6	15859.2	1322.7	15860.4
Max	1322.9	15862.8	1322.9	15862.8

	Day 3		Day 4	
Mean	1322.67	15860.0	1322.79	15861.5
Min	1322.5	15858.0	1322.6	15859.2
Max	1322.8	15861.6	1322.9	15862.8

Table 4 of 4

Correction of sickle β-chain molecular weight using normal β -chain to calibrate.
Could calibrate machine daily using normal β –chain but simple correction applied:

Sickle β-chain – actual MW 15837.2

Average calculated MW

Day 1	15837.4
Day 2	15838.1
Day 3	15837.5
Day 4	15837.7

CLAIMS

1. A method of testing a sample by mass spectrometry, where the ionisation technique produces a multiply-charged spectrum, to detect (a) the presence or absence of a known polypeptide or derivative of a polypeptide, or (b) the presence or absence of a variant of a known polypeptide or derivative of a polypeptide, in which scanning of the sample is targeted to selected ionised species of known mass/charge ratio, the absence of the expected value of mass/charge ratio being indicative of the absence of the known polypeptide or derivative thereof, or the presence of the variant polypeptide or derivative thereof being indicated by a shift in mass/charge ratio from the expected value.

2. A method according to claim 1, in which the ionisation technique is one which, for the polypeptide or variant under test, produces multiply charged ionised species thereof.

3. A method according to claim 1 or 2, in which one ionised species is targeted.

4. A method according to claim 1, 2, or 3, in which the scan is targeted to a species having the mass/charge ratio of the expected normal value and also to one or more species having the mass/charge ratio of an expected variant or variants.

5. A method according to claim 4, in which scanning is carried out with a restricted mass window (a range of atomic mass units) which includes the normal polypeptide and the variant being tested for, or with separate mass windows for each substance being tested for.

6. A method according to any of claims 1 to 5, in which an additional charged species is targeted for confirmatory purposes.

7. A method according to any of the preceding claims, in which, when used for large scale screening, the mass of the normal samples provides a control from which any deviations are apparent.

8. A method according to any of the preceding claims, in which electrospray ionisation mass spectrometry is used.

9. A method according to any of the preceding claims, in which a variant tested for is a variant of a blood protein.

10. A method according to claim 9, in which the blood protein is haemoglobin.

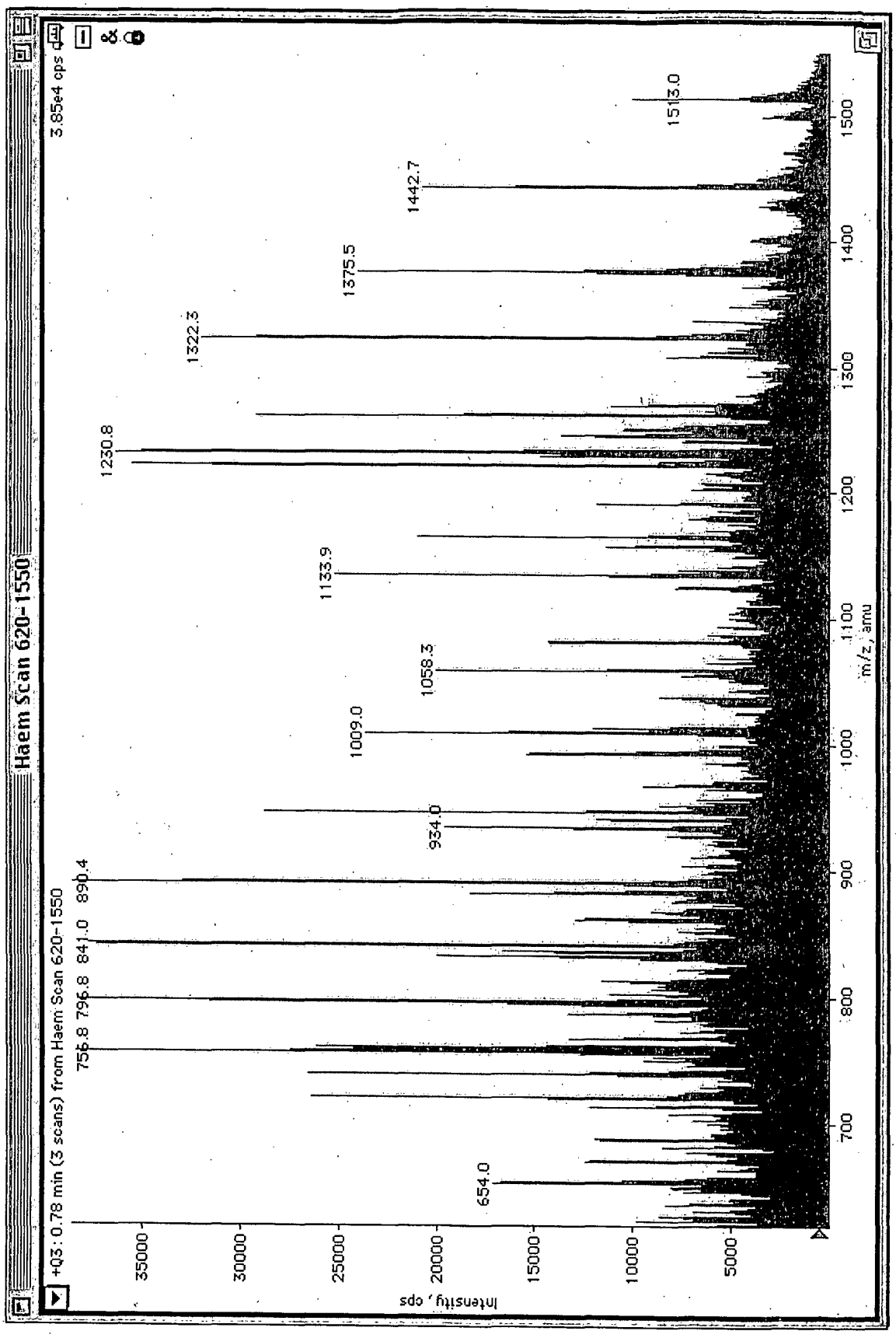
11. A method according to claim 10, in which the variant tested for is a variant β sub-unit of haemoglobin.

12. A method according to claim 10, in which the variant tested for is a variant α, δ, or γ sub-unit of haemoglobin.

13. A method according to claim 11, in which the variant sub-unit is indicative of sickle cell disease.

14. A method according to claim 11, 12, or 13, in which the targeted species has mass/charge of 12 or 13.

15. A method according to any of the preceding claims, used as a preliminary screening of patients at risk of developing a disease associated with the variant polypeptide or polypeptide derivative tested for.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1009.0
1058.3
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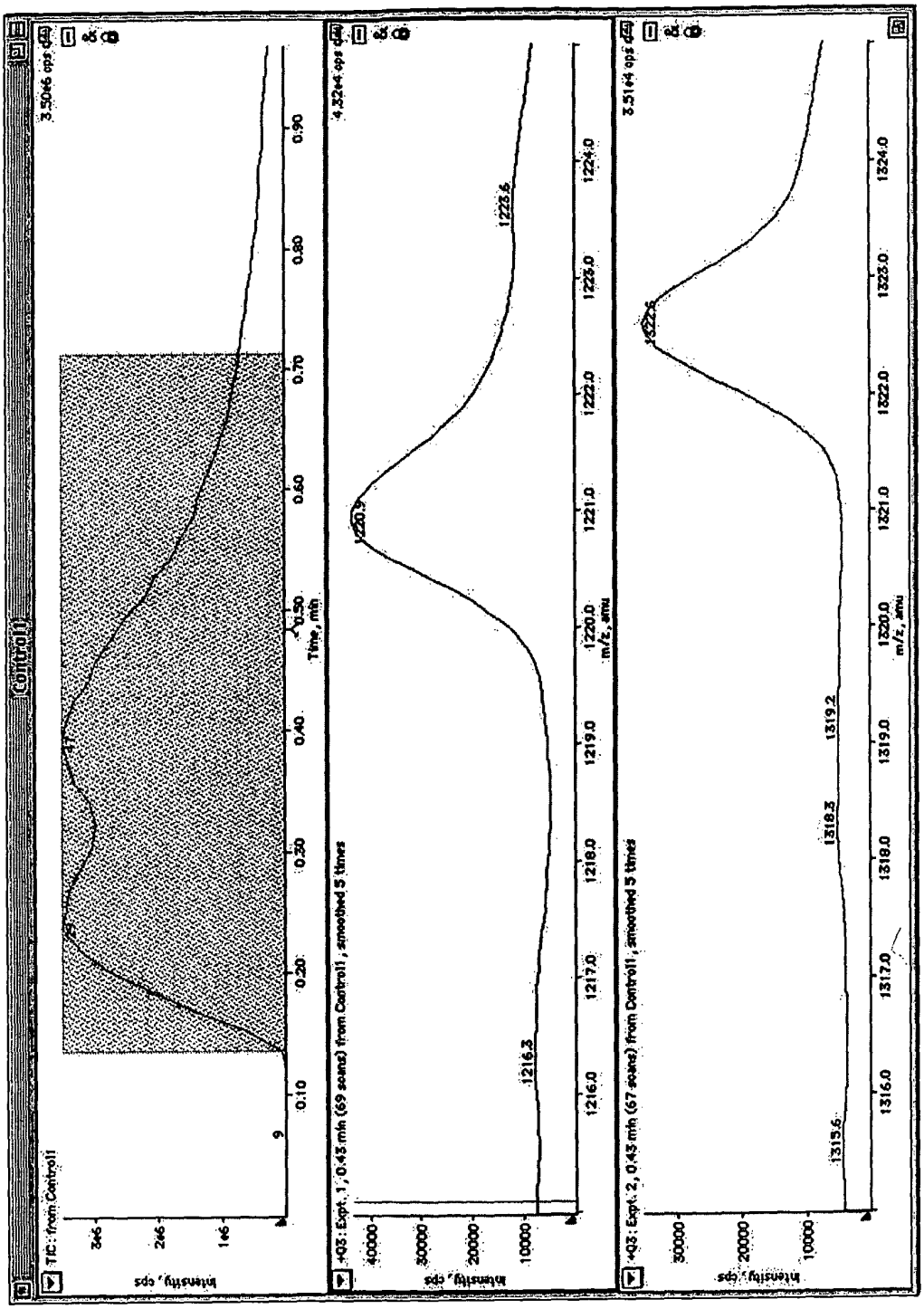
Intensity, cps
m/z, amu

Figure 1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1220.9
1223.6
+Q3: Expt. 2, 0.43 min (67 scans) from Control1, smoothed 5 times
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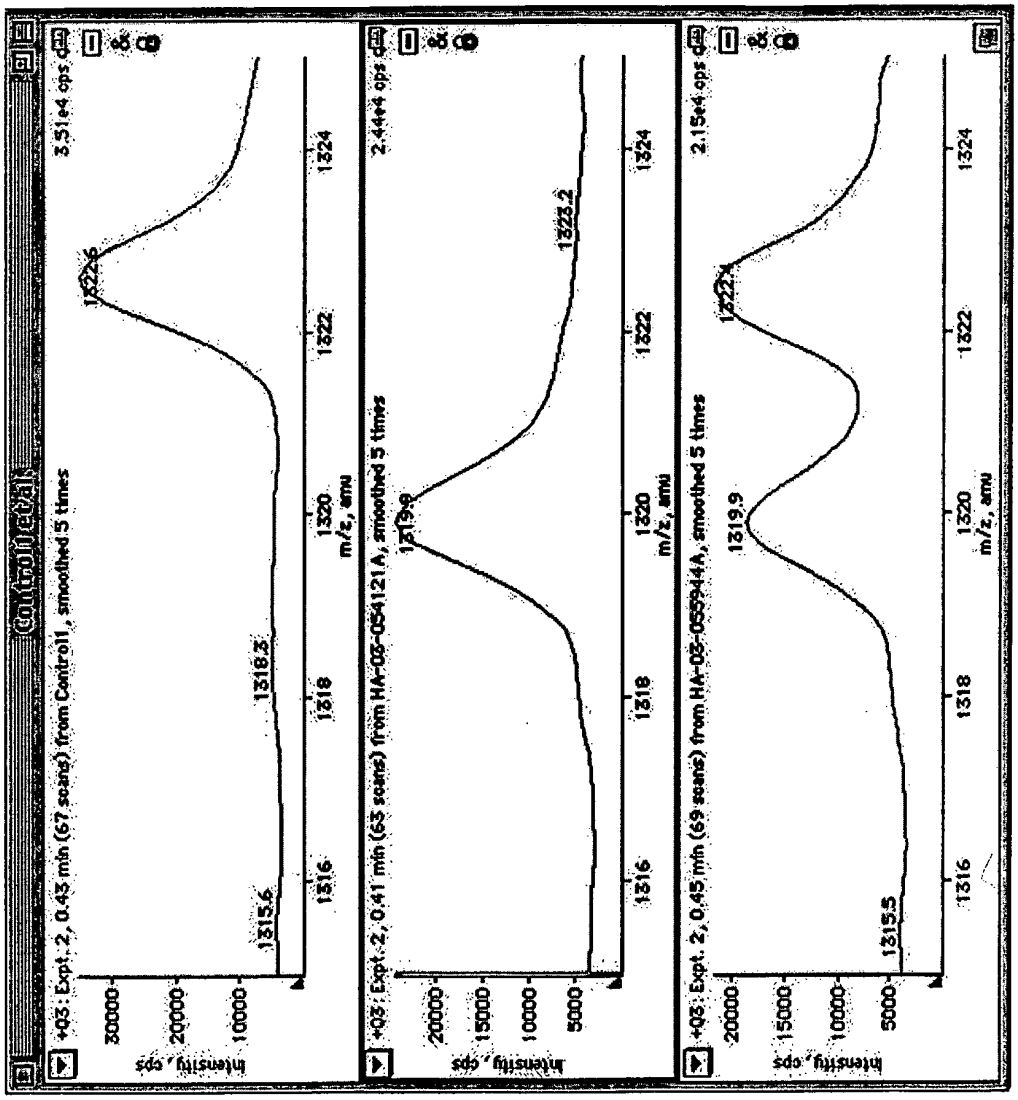
1319.2
1322.5
m/z, amu

Figure 2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10000
5000
1319.9
1323.2
1316
1318
1320
1322
1324
m/z, amu
+03: Expt. 2, 0.45 min (69 scans) from HA-03-055944A, smoothed 5 times
2.15e4 cps
Intensity, cps
20000
15000
10000
5000
1315.5
1319.9
1322.4
1316
1318
1320
1322
1324
m/z, amu

Figure 3

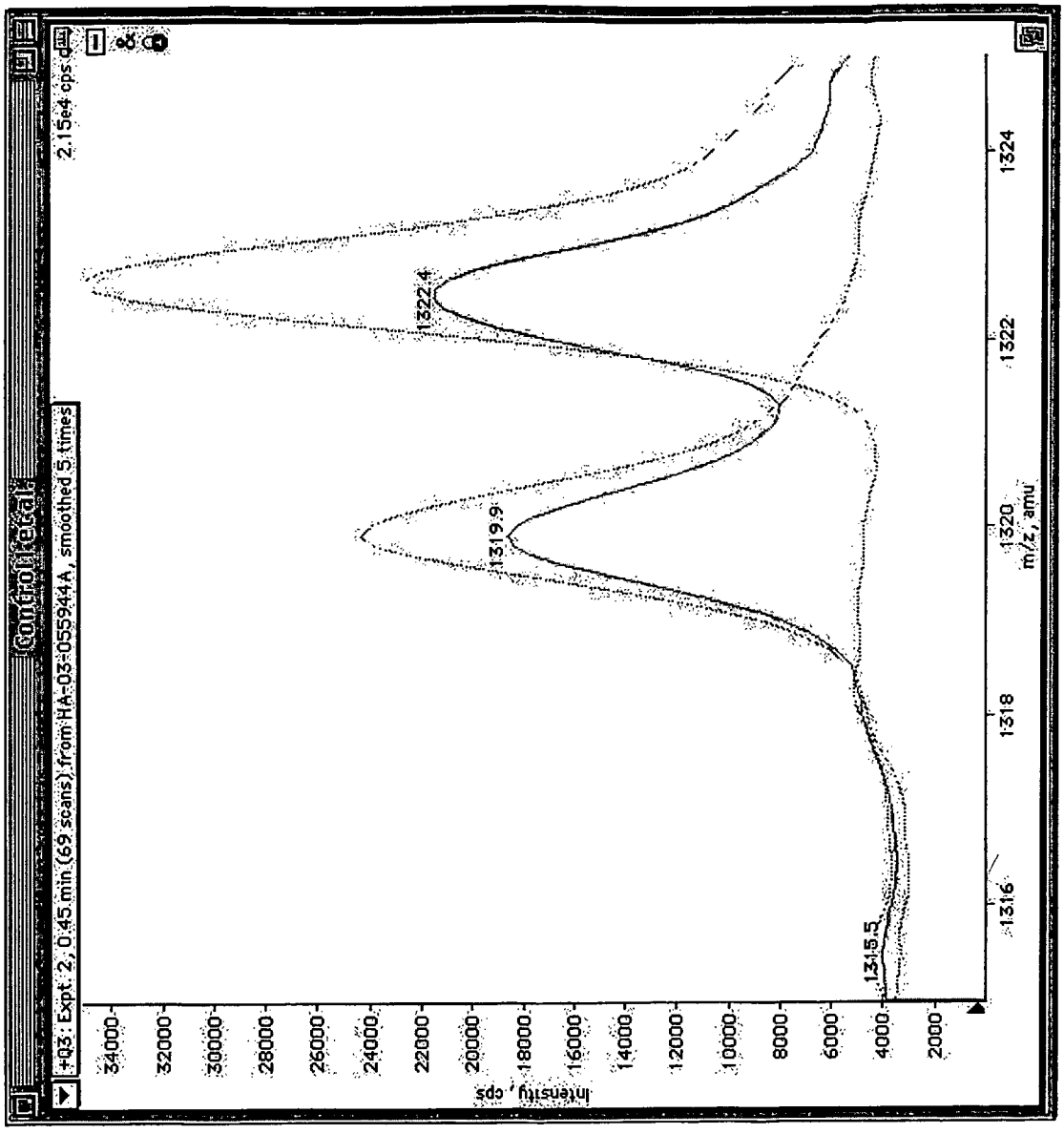
Control fetal
+Q3: Expt: 2, 0.45 min (69 scans) from HA-03-0559444A, smoothed 5 times
2.15e4 cps
1322.4
1319.9
1315.5
Intensity, cps
m/z, amu
34000
32000
30000
28000
26000
24000
22000
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
1316
1318
1320
1322
1324

Figure 4

INTERNATIONAL SEARCH REPORT

International Application No
/GB2004/001528

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/72

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N

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

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

EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SHUSHAN B ET AL: "A rapid accurate flow injection method for the determination of glycated hemoglobin using IonSpray Mass Spectrometry (ISP-MS) and a relatively low-cost single analyzer MS system" CLINICAL CHEMISTRY, vol. 44, no. 6 PART 2, June 1998 (1998-06), page A150, XP001194496 & 50TH ANNUAL MEETING OF THE AMERICAN ASSOCIATION OF CLINICAL CHEMISTRY; CHICAGO, ILLINOIS, USA; AUGUST 2-6, 1998 ISSN: 0009-9147 abstract	1-5,8-15
Y	----- -/--	7

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

° Special categories of cited documents :

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

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

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

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

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

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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 July 2004	30/07/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Weber, P

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WILD B J ET AL: "Rapid identification of hemoglobin variants by electrospray ionization mass spectrometry." BLOOD CELLS, MOLECULES & DISEASES. 2001 MAY-JUN, vol. 27, no. 3, May 2001 (2001-05), pages 691-704, XP002288209 ISSN: 1079-9796 cited in the application page 695, right-hand column, line 19 - line 22	7
A		1-6,8-15
X	LIU TAO ET AL: "Protein isoforms observed by ultrahigh resolution capillary isoelectric focusing-electrospray ionization mass spectrometry" SHENGWU HUAXUE YU SHENGWU WULI XUEBAO, vol. 34, no. 4, July 2002 (2002-07), pages 423-432, XP008032790 ISSN: 0582-9879 page 427, right-hand column, paragraph 2 - page 431, left-hand column, paragraph 1 figures 5,6	1-6,8-14
X	KOBOLD UWE ET AL: "Candidate reference methods for hemoglobin A-1c based on peptide mapping" CLINICAL CHEMISTRY, vol. 43, no. 10, 1997, pages 1944-1951, XP002288207 ISSN: 0009-9147 abstract page 1946, left-hand column, paragraph 2 - right-hand column, paragraph 2 page 1947, left-hand column, last paragraph - page 1948, left-hand column, paragraph 1 figure 3	1-5,8-15

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WAN H Z ET AL: "Rapid method for monitoring galactosylation levels during recombinant antibody production by electrospray mass spectrometry with selective-ion monitoring" JOURNAL OF CHROMATOGRAPHY, ELSEVIER SCIENCE PUBLISHERS B.V. AMSTERDAM, NL, vol. 913, no. 1-2, 13 April 2001 (2001-04-13), pages 437-446, XP004232810 ISSN: 0021-9673 page 439, left-hand column, last paragraph - right-hand column, paragraph 1 page 442, left-hand column, paragraph 2 - page 443, left-hand column, paragraph 1 figures 3,4	1-5,8
X	VAN DORSSELAER A ET AL: "MASS SPECTROMETRY ANALYSES OF RECOMBINANT HIRUDINS 7 KDA" BIOCHEMISTRY, vol. 28, no. 7, 1989, pages 2949-2956, XP002288208 ISSN: 0006-2960 page 2951, left-hand column, paragraph 3 - last paragraph figures 1,6	1-5
A	KIERNAN URBAN A ET AL: "High-throughput analysis of hemoglobin from neonates using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry" CLINICAL CHEMISTRY, vol. 48, no. 6, June 2002 (2002-06), pages 947-949, XP002288210 ISSN: 0009-9147 cited in the application the whole document	1-15