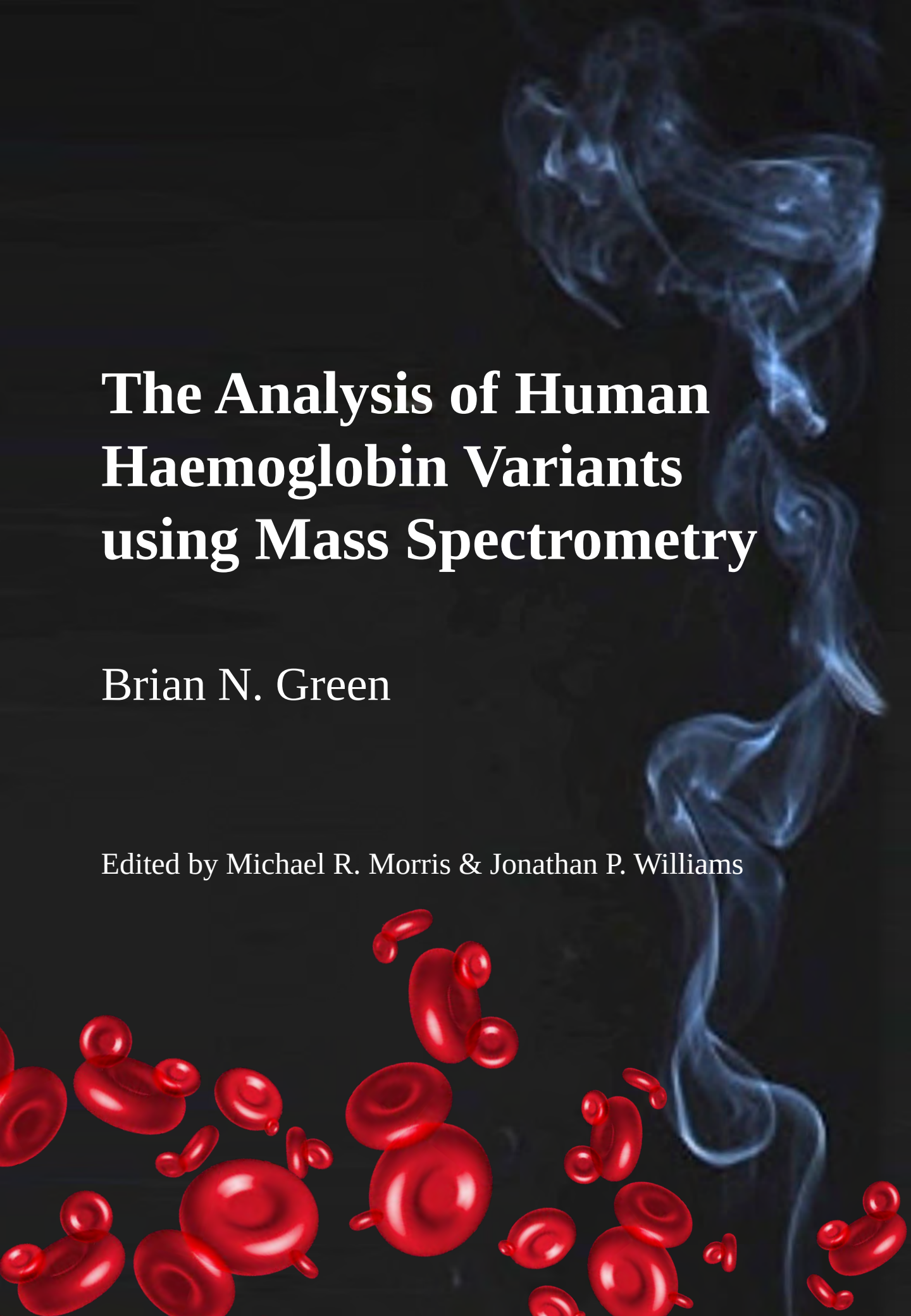

The Analysis of Human Haemoglobin Variants using Mass Spectrometry

Brian N. Green

Edited by Michael R. Morris & Jonathan P. Williams

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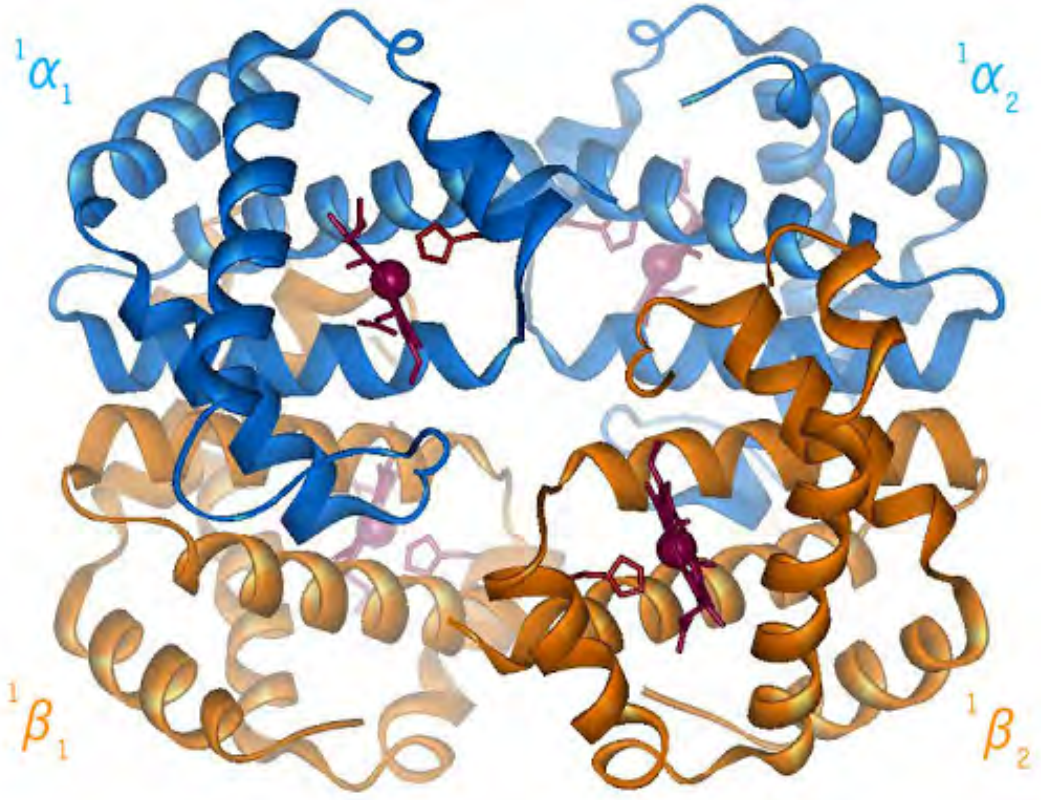
$^1\alpha_1$
$^1\alpha_2$
$^1\beta_1$
$^1\beta_2$

Foreword

This book represents a collation of the haemoglobin studies of Brian Green following the introduction of the Electrospray Ionisation (ESI) technique in VG Biotech in 1988. The data presented were typically acquired on a VG/Micromass triple quadrupole instrument, and the data processed using a MassLynx data system.

One of the challenges of developing analytical approaches for the analysis of specimens of human origin is the availability of 'interesting' samples, and this work would not have been possible without the collaboration of a wide number of individuals who worked with Brian from the early days until his retirement from laboratory work in 2018.

Thanks and acknowledgements are due to Barbara Wild, Adrian Stephens. Ron Oliver, David Roper, David Rees, Cedric Shackleton, Ewa Witkowska, Lisa Farrar, Debbie Mantio, Norman Roberts, Tim Reynolds and Dilip Rai, among many, many others.

Thanks, also, to Mark McDowall for his artistic input and support with the covers.

Mike Morris
Jon Williams

Summer 2021

DISCLAIMER

This work has been published to highlight Brian Green's studies of haemoglobin by mass spectrometry over a thirty-year period from 1988 to 2018.

The book is intended as a reference work to support researchers in the field and to promote the use of mass spectrometry for the identification of haemoglobin variants in the biomedical field, and has been made available by Waters Corporation to support such studies.

The approach is not promoted as a diagnostic device but is intended to support the provision of confirmatory information.

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Preface

A brief history.

When identifying human haemoglobin (Hb) variants by mass spectrometry, the most important parameter associated with the variant chain is an accurate value of its molecular weight (mass) from which the mass change of the variant from normal is derived. Then, based on this mass change and using Genetic Code Tables, a limited number of possible amino acid mutations may be proposed. The first indication that it might be possible to accurately determine the masses of the globin chains in human haemoglobin by mass spectrometry came in 1988, when Covey *et al*[1] published a ground-breaking paper that showed an ion evaporation spectrum of myoglobin (sequence mass 16,951.5 Da) containing a series of ten multiply protonated molecules from which the mass was determined as 16,949.5 ±0.9 Da SD. Other workers soon realised the potential of this new technique as part of the procedure for identifying Hb variants. As early as 1990, Green *et al*[2] had measured the masses of several normal and variant globin chains using a triple quadrupole instrument of modest mass range (*m/z* 2,000). They called the technique Electrospray MS. Subsequently, the technique came to be known as Electrospray Ionization Mass Spectrometry, ESI-MS, which is the name used throughout this book.

Another early indication of the potential of the technique in human haemoglobin analysis was the identification in 1991 of the clinically significant and electrophoretically silent variant, Hb Quebec-Chori, β87Thr→Ile[3]. ESI-MS played a major role in identifying this variant, which had not been previously reported in the literature.

In Covey *et al*[1], the mass of myoglobin was determined by averaging the values calculated from the ions in a series of multiply protonated molecules. Unfortunately, when this method was applied to human haemoglobin analysis, minor components such as the δ-chain, glycated and glutathionylated chains were difficult to assess. In late 1992, however, the maximum entropy based software (MaxEnt)[4] developed at the University of Cambridge by John Skilling, was introduced to disentangle the multiply charged series, and present the results on a molecular weight (mass) scale. This development revolutionised the analysis of the globin chains in diluted blood. MaxEnt is automatic and measures minor components down to ~1% relative abundance. Furthermore, by internally calibrating* the mass scale using one of the normal major chains, the precision in measuring the masses of the other major chains is unrivalled. (Internal calibration: A procedure whereby the mass scale of the multiply charged data from each sample is calibrated on itself before disentangling by MaxEnt.) For example, the precision in determining the mass of the β-chain when using the α-chain for internal calibration has generally been found to be better than ±0.05 Da SD, a hitherto undreamed of precision for measuring the mass of a globin chain in 1992. With these developments, the change in the mass of the variant globin chain from normal could then be used to reliably derive possible amino acid changes from the Genetic Code Tables.

In late 1996, the author of this book and Barbara Wild, then at King's College Hospital, London, began a programme of experiments to identify haemoglobinopathies that had been detected by traditional methods such as cation exchange-HPLC or isoelectric focusing. At this time, blood samples were simply diluted 500-fold in denaturing solvent and introduced directly into the mass spectrometer. Consequently, sodium and potassium adducts of the globin chains were produced from the salts present, which

interfered with measurement of the δ-chain and also sometimes with that of the variant-chain. They were eliminated by briefly shaking the 500-fold diluted blood solution with cation exchange resin beads before introduction into the mass spectrometer. By 2001, this desalting step had been incorporated into a routine procedure for analysing the intact globin chains in whole blood[5]. A rapid (30-minute) method for routinely digesting the globin chains in diluted blood with trypsin was also developed, since tryptic digests are generally needed for identifying the site of the mutation. By 2001[5], 250 samples, which were suspected by traditional methods to contain an unidentified abnormality, had been analysed. Variants were positively identified in 95% of the samples. Hb S and common variants such as Hb C and Hb E were generally not submitted for analysis by mass spectrometry, provided they had been reliably identified by traditional means. Ninety-nine different abnormalities including 36α- and 59β-chain variants were identified and subsequently confirmed by tandem mass spectrometry (see Figure 4 in reference 5). Fifteen of these variants had not been previously reported in the literature, i.e. were novel when first encountered by the author of this book. This programme was discontinued in 2001 with the publication of reference 5.

However, there was a strong interest from several UK hospitals for a service to be provided for identifying haemoglobinopathies, and over the period from 2001-2012, ~4,300 further samples were analysed by the author in a programme supported by Waters Corporation. The abnormalities were identified in more than 98% of these cases. By December 2012, when this service was discontinued, 329 different variants had been identified, namely; 155α (46 novel), 166β (31 novel), 1δ, $2^{G}\gamma$ and 5 hybrids. 77* were novel when first encountered by the author. With few exceptions (<2%), all the samples originated from patients resident in the UK.

*Includes novel variants that were first identified by the author of this book but were subsequently described and named elsewhere.

Brian N Green
Wilmslow, June 2017

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[1] T.R. Covey, R.F. Bonner, B.I. Shushan and J. Henion, The determination of protein, oligonucleotide and peptide molecular weights by ion-spray mass spectrometry, Rapid Commun.Mass Spectrom. **2**, 249-256 (1988).

[2] B.N. Green, R.W.A. Oliver, A.M. Falick, C.H.L. Shackleton, E. Roitman and H.E. Witkowska, Electrospray MS, LSIMS and MS/MS for the rapid detection and characterization of variant hemoglobins, in A.L. Burlingame and J.A. McCloskey (eds), Biological Mass Spectrometry, Elsevier, Amsterdam, 1990, pp129-146.

[3] H.E. Witkowska, B.H. Lubin, Y. Beuzard, S. Baruchel, *et al*., Sickle Cell Disease in a Patient with Sickle Cell Trait and Compound Heterozygosity for Hemoglobin S and Hemoglobin Quebec-Chori, N.Engl.J.Med., **325**, 1150-1154 (1991).

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[5] B.J. Wild, B.N. Green, E.K. Cooper, M.R.A. Lalloz, S. Erten, A.D. Stephens and D.M. Layton, Rapid identification of hemoglobin variants by electrospray ionization mass spectrometry, Blood Cells, Molecules and Diseases, **27**, 691-704 (2001).

CONTENTS

SECTION 1

1.1. The five steps in detecting and identifying human haemoglobin variants by charge sensitive chromatographic means and electrospray ionization mass spectrometry

Introduction

The methods described in this book were developed to routinely detect and positively identify human haemoglobin (Hb) variants by Electrospray Ionization Mass Spectrometry (ESI-MS). They are based on previously known techniques, which were time consuming because derivatisation of the cysteines and various chromatographic separations were undertaken before analysis by ESI-MS. These earlier techniques were drastically simplified to reduce sample preparation and analysis times.

To reduce sample preparation time, solutions for ESI-MS analysis were prepared directly from diluted blood, i.e. without purification, without isolating the variant Hb and without derivatising the cysteines prior to enzymatic digestion. The digest time was reduced from several hours to 30 minutes by first denaturing the Hb in the blood samples; a procedure that takes a couple of minutes. Analysis times were minimised by eliminating chromatographic separation of the mixture of peptides produced by enzymatic digestion prior to analysis by ESI-MS. By these means, the average time to identify a variant has been reduced to less than 2 hours, which includes preparing a report. ESI-MS has been found to positively identify the variant in over 98% of the samples found to contain a variant by traditional methods. It also detects and identifies the majority of variants that are silent by these methods. The five analytical steps in detecting and identifying variants are as follows.

Step 1. Most variants are detected during routine screening by traditional charge sensitive techniques, e.g. cation exchange-HPLC (ce-HPLC) or isoelectric focusing (IEF). Some are detected during diabetic monitoring. Variants that are silent by ce-HPLC or IEF are sometimes detected by ESI-MS when they occur together with a variant that is detected by these charge sensitive techniques.

Step 2. Analysis of 500-fold diluted, denatured and desalted blood by ESI-MS gives the molecular weights (masses) of the globin chains present in the sample. The masses of the major components are determined very accurately, so that the mass difference of a variant from normal can be established to the nearest integer. Then, using the genetic code data given in Tables 1.6.1 and 1.6.2, a limited number of possible single amino acid mutations governed by a single base change in the nucleotide codon can be found to fit this mass difference. The masses of variants that result from amino acid deletions, insertions and extensions are also determined at this stage.

Step 3. This section describes the detection and identification of the hybrid haemoglobins, several of which can be identified directly from their mass spectra. Tandem mass spectrometry of the intact hybrid chains is used for confirmation. A table listing the masses and principle product ions of ten hybrid chains is provided. Also shown are the tandem mass spectra of the normal β-, δ- and $^{G}\gamma$-chains

Step 4. ESI-MS analysis of the peptide mixture produced by digestion of 50-fold diluted and denatured blood with trypsin allows the peptide containing the mutation to be identified. Approximately 50% of variants can be identified at this stage. These include variants in which the tryptic peptide contains only one amino acid that can mutate to give the mass change determined in Step 2 by a single base change in the codon. They also include mutations to or from arginine and lysine. The remaining 50% require sequencing of the variant peptide by tandem MS for identification.

Step 5. Sequencing the tryptic peptide containing the mutation by tandem MS allows the variant to be positively identified. The mutation identified in Step 4 can be confirmed by tandem MS, if deemed necessary.

Table 1.1.1. The atomic weights of the elements associated with haemoglobin analysis[6]

Element	Symbol	Atomic Mass (Da)	Atomic Weight (Da)
Hydrogen	H	1.00782503	1.00794
Carbon	C	12 (exactly, by definition)	12.0107
Nitrogen	N	14.0030740	14.0067
Oxygen	O	15.9949146	15.9994
Sodium	Na	22.9897697	22.989770
Phosphorous	P	30.9737615	30.973761
Sulphur	S	31.9720707	32.065
Potassium	K	38.9637069	39.0983
Iron	Fe	55.9349418	55.845

The atomic masses are the masses of the most abundant isotope of a given element and are used to calculate the monoisotopic masses of small molecules up to ~3,000 Da molecular mass. In all cases except iron, the most abundant isotope also has the lowest mass.

The atomic weights are used to calculate the average masses of compounds, mainly proteins, such as the chains in human haemoglobin.

Reference

(6) J.R. de Laeter, J.K. Bohlke, P. De Bievre, H. Hidaka, H.S. Peiser, K.J.R. Rosman and P.D.P. Taylor, Atomic weights of the elements: Review 2000. Pure Appl. Chem., **75**, 683-800 (2003).

1.2. Haemoglobin.

Human haemoglobin occurs in the red blood cells as a non-covalently assembled tetramer of two dissimilar polypeptide chains (α- and β-globin chains), in which each chain is associated non-covalently with a haem group. Its primary functions are to transport oxygen from the lungs to the organs and tissues of the body and carry carbon dioxide from the tissues to the lungs. It performs the former function via the iron atom in each of the four haem groups, which are non-covalently attached to each chain. Each haem can combine reversibly with one dioxygen molecule. The carbon dioxide released by the respiring tissues is too insoluble to be efficiently conveyed to the lungs but is rendered more soluble by combining with water to form a bicarbonate ion and a proton. The deoxyhaemoglobin acts as a buffer, mopping up the protons and tipping the balance towards the formation of soluble bicarbonate. In the lungs the process is reversed. There, as oxygen binds to the haemoglobin, protons are cast off, driving carbon dioxide out of solution so that it can be exhaled. The reaction between carbon dioxide and water is catalysed by the enzyme carbonic anhydrase.

Normal adult haemoglobin (Hb A) comprises two α-chains of 141 amino acids and two β-chains of 146 amino acids non-covalently assembled into a tetramer ($\alpha_2\beta_2h_4$). The average molecular weights (masses) of the individual chains without heme are α: 15,126.38 Da and β: 15,867.24 Da. The mass of the haem group is 616.50 Da.

In normal adults, the principal component is Hb A, which occurs together with a minor (~3%) component, Hb A_2 ($\alpha_2\delta_2h_4$). In babies at birth, the principal component is fetal Hb, Hb F (80-90%), ($\alpha_2\gamma_2h_4$), comprising two or three γ-chains, $^{G}\gamma$, $^{A}\gamma^{T}$, $^{A}\gamma$, with 10-20% of Hb A. Over 6 months of neonatal life, the Hb F gradually decreases to <1%, and Hb A becomes the major component. The α-chains are encoded by two gene copies on chromosome 16, whilst the non-α-chains are encoded by single gene copies on chromosome 11. One gene of each type is inherited from each parent. In embryonic Hb, which occurs up to approximately 10 weeks from conception, the chains are ζ (zeta, α-like) and ε (epsilon, β-like).

It has been estimated that abnormal haemoglobins (haemoglobinopathies) are carried by approximately 7% of the world population, and that 300-400,000 babies are born each year with severe forms of these diseases[7]. The haemoglobinopathies belong to a group of inherited disorders that are characterised by either reduced synthesis of one or more of the normal globin chains (the thalassaemias) or by the synthesis of one or more structurally abnormal globin chains (the haemoglobin variants).

Variants of any of the major chains can seriously interfere with the function of the assembled tetramer. Currently, the number of variants listed in the Globin Gene Server is in excess of 1,500, and many more are possible. Whilst some variants are clinically significant, most function normally. Nevertheless, once a variant has been detected, it is prudent to identify it, particularly if it occurs in a potential parent, so that appropriate counselling can be given or, if it occurs in a patient with an unexplained abnormal Hb, in order to avoid inappropriate medical treatment due to misdiagnosis.

Reference

[7] D.J. Weatherall and J.B. Clegg. Inherited haemoglobin disorders: an increasing global health problem. Bulletin of the World Health Organisation, **79**, 704-712 (2001).

1.3. The tandem mass spectrometer

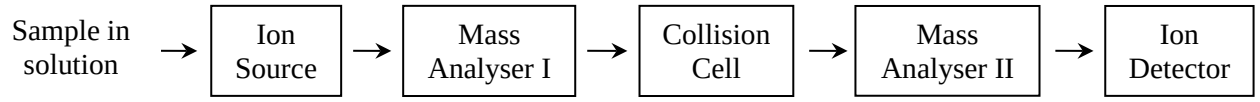


Figure 1.3.1. Schematic diagram of a tandem mass spectrometer

The tandem mass spectrometer can be operated in two modes.

Mode 1. As a simple mass spectrometer (ESI-MS)
The sample in solution is ionized by electrospray ionization in the Ion Source and the resulting ions are separated according to their mass (actually mass-to-charge ratio) by Mass Analyser I, which is scanned to give a mass spectrum. These ions then pass directly to the Ion Detector, where they are converted into electrical signals, which are recorded as a mass spectrum in the associated data system. Mass spectra are produced from either intact globin chains or from the peptides produced by digesting the globin chains with trypsin (tryptic peptides). These spectra are then used to determine the masses and relative abundance of the various components in the sample.

Mode 2. As a tandem mass spectrometer (ESI-MS-MS)
As in Mode 1, ions are generated from the sample in solution, but in this case, ions of a particular mass (the precursor mass) are selected by Mass Analyser I and fragmented by collisions with argon gas in the collision cell. The fragment ions (product ions) are then analysed by Mass Analyser II to give a product ion spectrum. In one application, the precursor ions are selected from a tryptic peptide and the resulting product ion spectrum is used to sequence the peptide. In a second application, the precursor is from an intact globin chain and the resulting spectrum used to confirm the identity of the chain.

1.4. Basic information on the various chains in human haemoglobin

The following tables show the amino acid sequences, terminal groups and masses of the chains in adult, foetal and embryonic haemoglobin.

Table 1.4.1. The masses and sequences of the chains in adult haemoglobin

The Human α-chain (alpha-chain).
N-Terminus = H, C-Terminus = OH.
Average mass = 15,126.3807 Da. Monoisotopic mass = 15,116.8851 Da.

1	6	11	16	21	26	31	36
VLSPA	DKTNV	KAAWG	KVGAH	AGEYG	AEALE	RMFLS	FPTTK
41	46	51	56	61	66	71	76
TYFPH	FDLSH	GSAQV	KGHGK	KVADA	LTNAV	AHVDD	MPNAL
81	86	91	96	101	106	111	116
SALSD	LHAHK	LRVDP	VNFKL	LSHCL	LVTLA	AHLPA	EFTPA
121	126	131	136	141			
VHASL	DKFLA	SVSTV	LTSKY	R			

The Human β-chain (beta-chain).
N-Terminus = H, C-Terminus = OH.
Average mass = 15,867.2406 Da. Monoisotopic mass = 15,857.2497 Da.

1	6	11	16	21	26	31	36
VHLTP	EEKSA	VTALW	GKVNV	DEVGG	EALGR	LLVVY	PWTQR
41	46	51	56	61	66	71	76
FFESF	GDLST	PDAVM	GNPKV	KAHGK	KVLGA	FSDGL	AHLDN
81	86	91	96	101	106	111	116
LKGTF	ATLSE	LHCDK	LHVDP	ENFRL	LGNVL	VCVLA	HHFGK
121	126	131	136	141	146		
EFTPP	VQAAY	QKVVA	GVANA	LAHKY	H		

The Human δ-chain (delta-chain).
N-Terminus = H, C-Terminus = OH.
Average mass = 15,924.3170 Da. Monoisotopic mass = 15,914.2494 Da.

1	6	11	16	21	26	31	36
VHLTP	EEKTA	VNALW	GKVNV	DAVGG	EALGR	LLVVY	PWTQR
41	46	51	56	61	66	71	76
FFESF	GDLSS	PDAVM	GNPKV	KAHGK	KVLGA	FSDGL	AHLDN
81	86	91	96	101	106	111	116
LKGTF	SQLSE	LHCDK	LHVDP	ENFRL	LGNVL	VCVLA	RNFGK
121	126	131	136	141	146		
EFTPQ	MQAAY	QKVVA	GVANA	LAHKY	H		

The abundance of the δ-chain is approximately 3% of the β-chain in normal adults. It doubles with β^0-thalassemia trait in otherwise normal adults.

Table 1.4.2. The masses and sequences of the chains in foetal haemoglobin

The Human $^{G}\gamma$-chain (G-gamma-chain).
N-Terminus = H, C-Terminus = OH.
Average mass = 15,995.2735 Da. Monoisotopic mass = 15,985.2552 Da.

1	6	11	16	21	26	31	36
GHFTE	EDKAT	ITSLW	GKVNV	EDAGG	ETLGR	LLVVY	PWTQR
41	46	51	56	61	66	71	76
FFDSF	GNLSS	ASAIM	GNPKV	KAHGK	KVLTS	LGDA<u>I</u>	KHLDD
81	86	91	96	101	106	111	116
LKGTF	AQLSE	LHCDK	LHVDP	ENFKL	LGNVL	VTVLA	IHFGK
121	126	131	136	141	146		
EFTPE	VQASW	QKMVT	<u>G</u>VASA	LSSRY	H		

The Human $^{A}\gamma^{T}$-chain (A-gamma-T-chain).
N-Terminus = H, C-Terminus = OH.
Average mass = 15,997.2460 Da. Monoisotopic mass = 15,987.2345 Da.

1	6	11	16	21	26	31	36
GHFTE	EDKAT	ITSLW	GKVNV	EDAGG	ETLGR	LLVVY	PWTQR
41	46	51	56	61	66	71	76
FFDSF	GNLSS	ASAIM	GNPKV	KAHGK	KVLTS	LGDA<u>T</u>	KHLDD
81	86	91	96	101	106	111	116
LKGTF	AQLSE	LHCDK	LHVDP	ENFKL	LGNVL	VTVLA	IHFGK
121	126	131	136	141	146		
EFTPE	VQASW	QKMVT	<u>A</u>VASA	LSSRY	H		

The Human $^{A}\gamma$-chain (A-gamma-chain).
N-Terminus = H, C-Terminus = OH.
Average mass = 16,009.3004 Da. Monoisotopic mass = 15,999.2709 Da.

1	6	11	16	21	26	31	36
GHFTE	EDKAT	ITSLW	GKVNV	EDAGG	ETLGR	LLVVY	PWTQR
41	46	51	56	61	66	71	76
FFDSF	GNLSS	ASAIM	GNPKV	KAHGK	KVLTS	LGDA<u>I</u>	KHLDD
81	86	91	96	101	106	111	116
LKGTF	AQLSE	LHCDK	LHVDP	ENFKL	LGNVL	VTVLA	IHFGK
121	126	131	136	141	146		
EFTPE	VQASW	QKMVT	<u>A</u>VASA	LSSRY	H		

The amino acid residues (75 and 136) that differ between these sequences are underlined. Approximately 10% of each chain is N-acetylated in newborns. These chains also occur in adults with hereditary persistence of foetal haemoglobin (HPFH), and other abnormal conditions. Typical ESI mass spectra of these chains in newborns are given in Davison *et al*[8]

Reference

[8] A.S Davison, B.N Green, and N.B. Roberts, Fetal haemoglobin: assessment of glycation and acetylation status by electrospray ionization mass spectrometry, Clin. Chem. Lab. Med., **46**, 1230-38 (2008).

Table 1.4.3 The masses and sequences of the chains in embryonic haemoglobin

The Human ζ-chain (zeta-chain).
N-Terminus = CH_3CO (fully N-acetylated), C-Terminus = OH.
Average mass = 15,547.8885 Da. Monoisotopic mass = 15,538.2068 Da.

1	6	11	16	21	26	31	36
SLTKT	ERTII	VSMWA	KISTQ	ADTIG	TETLE	RLFLS	HPQTK
41	46	51	56	61	66	71	76
TYFPH	FDLHP	GSAQL	RAHGS	KVVAA	VGDAV	KSIDD	IGGAL
81	86	91	96	101	106	111	116
SKLSE	LHAYI	LRVDP	VNFKL	LSHCL	LVTLA	ARFPA	DFTAE
121	126	131	136	141			
AHAAW	DKFLS	VVSSV	LTEKY	R			

The Human ε-chain (epsilon-chain).
N-Terminus = H, C-Terminus = OH.
Average mass = 16,071.6578 Da. Monoisotopic mass = 16,061.4296 Da.

1	6	11	16	21	26	31	36
VHFTA	EEKAA	VTSLW	SKMNV	EEAGG	EALGR	LLVVY	PWTQR
41	46	51	56	61	66	71	76
FFDSF	GNLSS	PSAIL	GNPKV	KAHGK	KVLTS	FGDAI	KNMDN
81	86	91	96	101	106	111	116
LKPAF	AKLSE	LHCDK	LHVDP	ENFKL	LGNVM	VIILA	THFGK
121	126	131	136	141	146		
EFTPE	VQAAW	QKLVS	AVAIA	LAHKY	H		

These chains occur in the first few weeks of gestation after which time the haemoglobin becomes foetal Hb, i.e. mainly α- and γ-chains (Hb F).

Fully acetylated ζ-chain has been observed by ESI-MS at significant levels in *Hydrops Fetalis* babies, when the α-chain was below the detection level. The dominant components were γ-chains with a lower level of β-chain[(9)].

Reference

[(9)] M. Bowers, M.F. McMullin, B.N. Green and F. Jones, Hydrops Fetalis secondary to homozygous alpha thalassemia in a very low incidence area, CME Bulletin Haematology, **2**, 54-56 (1999).

Table 1.4.4. The amino acid composition of the globin chains in adult, foetal and embryonic haemoglobin

															Total Residues
Human α-chain															
A	(Ala)	21	F	(Phe)	7	K	(Lys)	11	P	(Pro)	7	T	(Thr)	9	
C	(Cys)	1	G	(Gly)	7	L	(Leu)	18	Q	(Gln)	1	V	(Val)	13	
D	(Asp)	8	H	(His)	10	M	(Met)	2	R	(Arg)	3	W	(Trp)	1	
E	(Glu)	4	I	(Ile)	0	N	(Asn)	4	S	(Ser)	11	Y	(Tyr)	3	**141**
Human β-chain															
A	(Ala)	15	F	(Phe)	8	K	(Lys)	11	P	(Pro)	7	T	(Thr)	7	
C	(Cys)	2	G	(Gly)	13	L	(Leu)	18	Q	(Gln)	3	V	(Val)	18	
D	(Asp)	7	H	(His)	9	M	(Met)	1	R	(Arg)	3	W	(Trp)	2	
E	(Glu)	8	I	(Ile)	0	N	(Asn)	6	S	(Ser)	5	Y	(Tyr)	3	**146**
Human δ-chain															
A	(Ala)	15	F	(Phe)	8	K	(Lys)	11	P	(Pro)	6	T	(Thr)	5	
C	(Cys)	2	G	(Gly)	13	L	(Leu)	18	Q	(Gln)	5	V	(Val)	17	
D	(Asp)	7	H	(His)	7	M	(Met)	2	R	(Arg)	4	W	(Trp)	2	
E	(Glu)	7	I	(Ile)	0	N	(Asn)	8	S	(Ser)	6	Y	(Tyr)	3	**146**
Human ^{G}γ-chain															
A	(Ala)	11	F	(Phe)	8	K	(Lys)	12	P	(Pro)	4	T	(Thr)	10	
C	(Cys)	1	G	(Gly)	13	L	(Leu)	17	Q	(Gln)	4	V	(Val)	13	
D	(Asp)	8	H	(His)	7	M	(Met)	2	R	(Arg)	3	W	(Trp)	3	
E	(Glu)	8	I	(Ile)	4	N	(Asn)	5	S	(Ser)	11	Y	(Tyr)	2	**146**
Human ^{A}γ^{T}-chain															
A	(Ala)	12	F	(Phe)	8	K	(Lys)	12	P	(Pro)	4	T	(Thr)	11	
C	(Cys)	1	G	(Gly)	12	L	(Leu)	17	Q	(Gln)	4	V	(Val)	13	
D	(Asp)	8	H	(His)	7	M	(Met)	2	R	(Arg)	3	W	(Trp)	3	
E	(Glu)	8	I	(Ile)	3	N	(Asn)	5	S	(Ser)	11	Y	(Tyr)	2	**146**
Human ^{A}γ-chain															
A	(Ala)	12	F	(Phe)	8	K	(Lys)	12	P	(Pro)	4	T	(Thr)	10	
C	(Cys)	1	G	(Gly)	12	L	(Leu)	17	Q	(Gln)	4	V	(Val)	13	
D	(Asp)	8	H	(His)	7	M	(Met)	2	R	(Arg)	3	W	(Trp)	3	
E	(Glu)	8	I	(Ile)	4	N	(Asn)	5	S	(Ser)	11	Y	(Tyr)	2	**146**
Human ζ-chain															
A	(Ala)	16	F	(Phe)	7	K	(Lys)	9	P	(Pro)	5	T	(Thr)	12	
C	(Cys)	1	G	(Gly)	6	L	(Leu)	17	Q	(Gln)	3	V	(Val)	11	
D	(Asp)	8	H	(His)	7	M	(Met)	1	R	(Arg)	6	W	(Trp)	2	
E	(Glu)	6	I	(Ile)	7	N	(Asn)	1	S	(Ser)	13	Y	(Tyr)	3	**141**
Human ε-chain															
A	(Ala)	17	F	(Phe)	9	K	(Lys)	14	P	(Pro)	6	T	(Thr)	6	
C	(Cys)	1	G	(Gly)	9	L	(Leu)	16	Q	(Gln)	3	V	(Val)	13	
D	(Asp)	5	H	(His)	7	M	(Met)	3	R	(Arg)	2	W	(Trp)	3	
E	(Glu)	9	I	(Ile)	5	N	(Asn)	7	S	(Ser)	9	Y	(Tyr)	2	**146**

1.5. Data for the calculation of the molecular masses of peptides and proteins for use in mass spectrometry

The molecular mass of a normally terminated and unmodified peptide or protein (globin chain) may be calculated by summing the masses of the appropriate amino acid residues from Table 1.5.3, and adding the masses of H and OH for the N- and C-termini, respectively (Table 1.5.1). The masses of some alternative terminal groups are also listed in Table 1.5.1. In cases where cysteines are linked by disulphide bonds, the mass of two hydrogen atoms should be subtracted for each disulphide bond in the molecule. The mass changes due to some post-translational modifications of haemoglobin chains are given in Table 1.5.2.

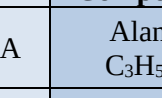
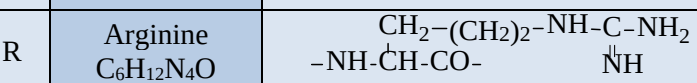
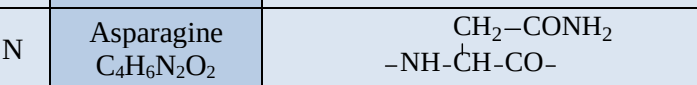
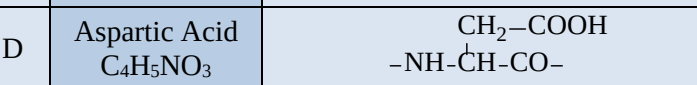
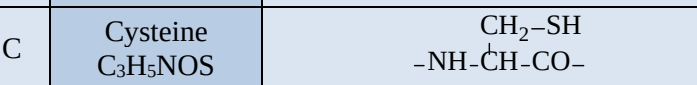
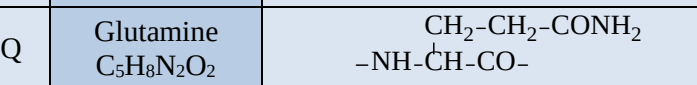
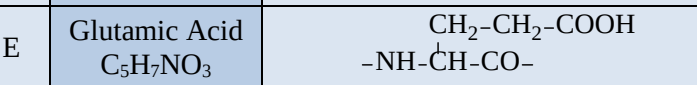
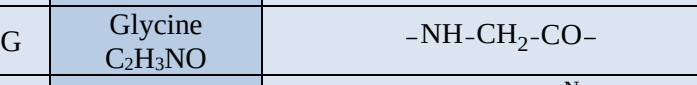
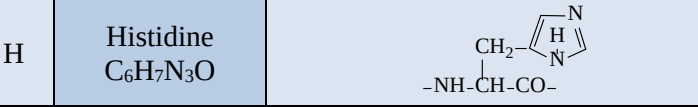
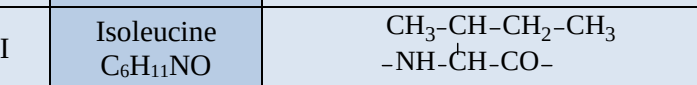
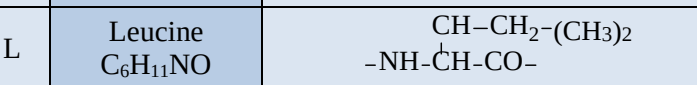
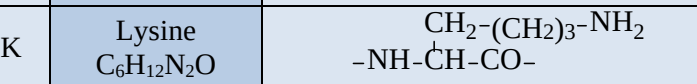
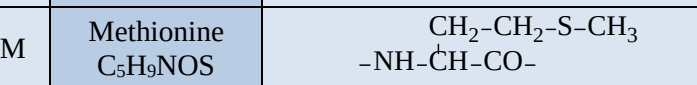
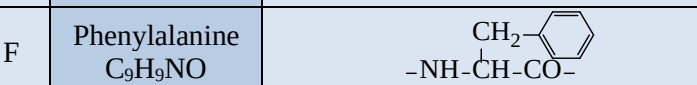
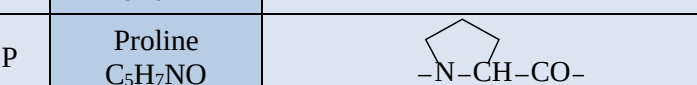
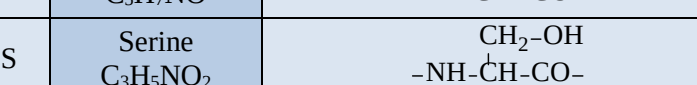
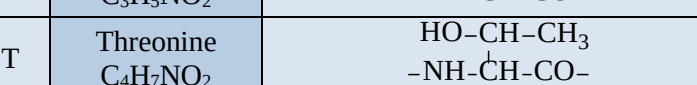
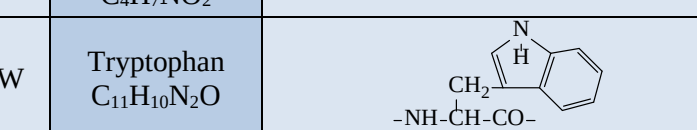
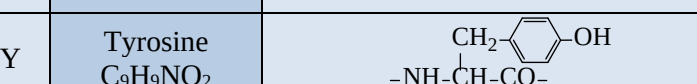
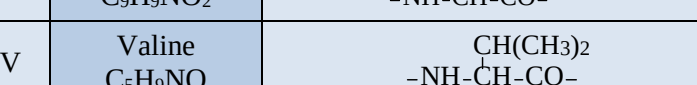
Table 1.5.1. The masses of some terminal groups

	Composition	Monosiotopic Mass (Da)	Average Mass (Da)
N-Terminal Groups			
Hydrogen	H	1.00782	1.0079
N-Formyl	HCO	29.00274	29.0183
N-Acetyl	CH_3CO	43.01839	43.0452
C-Terminal Groups			
Free acid	OH	17.00274	17.0073
Amide	NH_2	16.01872	16.0226

Table 1.5.2. The mass changes due to some post-translational modifications of haemoglobin chains.

Modification	Monoisotopic Mass Change (Da)	Average Mass Change (Da)
Disulphide bond formation	- 2.01565	-2.0159
Deamidation	0.98402	0.9847
Oxidation of Met	15.99491	15.9994
Acetylation	42.01056	42.0373
Carbamoylation	43.00581	43.0251
Pyruvylation	70.00548	70.0477
Sulphation	79.95682	80.0642
Cysteinylation	119.00410	119.1442
Glycation	162.05282	162.1424
Glutathionylation	305.06816	305.3117

Table 1.5.3. The masses and compositions of the twenty commonly occurring amino acid residues

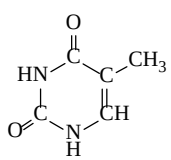
Symbols	Name and Composition	Residue Structure	Monoisotopic Mass (Da)	Average Mass (Da)
Ala, A	Alanine C_3H_5NO		71.03711	71.0788
Arg, R	Arginine $C_6H_{12}N_4O$		156.10111	156.1876
Asn, N	Asparagine $C_4H_6N_2O_2$		114.04293	114.1039
Asp, D	Aspartic Acid $C_4H_5NO_3$		115.02694	115.0886
Cys, C	Cysteine C_3H_5NOS		103.00919	103.1448
Gln, Q	Glutamine $C_5H_8N_2O_2$		128.05858	128.1308
Glu, E	Glutamic Acid $C_5H_7NO_3$		129.04259	129.1155
Gly, G	Glycine C_2H_3NO		57.02146	57.0520
His, H	Histidine $C_6H_7N_3O$		137.05891	137.1412
Ile, I	Isoleucine $C_6H_{11}NO$		113.08406	113.1595
Leu, L	Leucine $C_6H_{11}NO$		113.08406	113.1595
Lys, K	Lysine $C_6H_{12}N_2O$		128.09496	128.1742
Met, M	Methionine C_5H_9NOS		131.04049	131.1986
Phe, F	Phenylalanine C_9H_9NO		147.06841	147.1766
Pro, P	Proline C_5H_7NO		97.05276	97.1167
Ser, S	Serine $C_3H_5NO_2$		87.03203	87.0782
Thr, T	Threonine $C_4H_7NO_2$		101.04768	101.1051
Trp, W	Tryptophan $C_{11}H_{10}N_2O$		186.07931	186.2133
Tyr, Y	Tyrosine $C_9H_9NO_2$		163.06333	163.1760
Val, V	Valine C_5H_9NO		99.06841	99.1326

1.6. DNA Information for haemoglobin

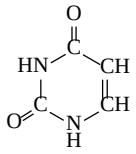
Table 1.6.1. The DNA Codons for the twenty commonly occurring amino acids

Amino Acid	Codon	Amino Acid	Codon	Amino Acid	Codon
Alanine Ala, A	GCT GCC GCA GCG	Glycine Gly, G	GGT GGC GGA GGG	Proline Pro, P	CCT CCC CCA CCG
Arginine Arg, R	CGT CGC CGA CGG AGA AGG	Histidine His, H	CAT CAC	Serine Ser, S	TCT TCC TCA TCG AGT AGC
Asparagine Asn, N	AAT AAC	Isoleucine Ile, I	ATT ATC ATA	Threonine Thr, T	ACT ACC ACA ACG
Aspartic Acid Asp, D	GAT GAC	Leucine Leu, L	TTA TTG CTT CTC CTA CTG	Tryptophan Trp, W	TGG
Cysteine Cys, C	TGT TGC	Lysine Lys, K	AAA AAG	Tyrosine Tyr, Y	TAT TAC
Glutamine Gln, Q	CAA CAG	Methionine Met, M	ATG	Valine Val, V	GTT GTC GTA GTG
Glutamic Acid Glu, E	GAA GAG	Phenylalanine Phe, F	TTT TTC	Chain termination	TAA TAG TGA
ATG also serves as a chain initiation codon. For RNA, replace T by U.					

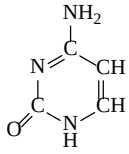
The five major DNA/RNA bases:



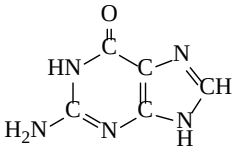
Thymine (T)



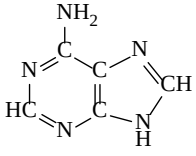
Uracil (U)



Cytosine (C)



Guanine (G)



Adenine (A)

Table 1.6.2. Nominal mass and amino acid changes genetically governed by single base changes in the nucleotide codon.
Amino acid changes from left to right give a mass increase, and changes from right to left give a mass decrease, except for Gln↔Lys and Ile↔Leu, which give no mass change.

Mass Change (Da)	Amino Acid Change	Mass Change (Da)	Amino Acid Change	Mass Change (Da)	Amino Acid Change
0	Gln ↔ Lys	18	Ile ↔ Met	40	Pro ↔ His
	Ile ↔ Leu		Leu ↔ Met	42	Gly ↔ Val
1	Asn ↔ Asp	19	His ↔ Arg	43	Ile ↔ Arg
	Gln ↔ Glu	22	Asp ↔ His		Leu ↔ Arg
	Ile ↔ Asn	23	Asn ↔ His	44	Ala ↔ Asp
	Lys ↔ Glu	24	Leu ↔ His		Cys ↔ Phe
3	Lys ↔ Met	25	Met ↔ Arg	46	Gly ↔ Cys
4	Pro ↔ Thr	26	Ala ↔ Pro	48	Asp ↔ Tyr
9	Gln ↔ His		His ↔ Tyr		Val ↔ Phe
10	Ser ↔ Pro		Ser ↔ Ile	49	Asn ↔ Tyr
12	Thr ↔ Ile		Ser ↔ Leu	53	Cys ↔ Arg
13	Thr ↔ Asn	27	Ser ↔ Asn	55	Thr ↔ Arg
14	Asn ↔ Lys		Thr ↔ Lys	58	Ala ↔ Glu
	Asp ↔ Glu	28	Ala ↔ Val		Gly ↔ Asp
	Gly ↔ Ala		Gln ↔ Arg	59	Pro ↔ Arg
	Ser ↔ Thr		Lys ↔ Arg	60	Cys ↔ Tyr
	Val ↔ Ile	30	Ala ↔ Thr		Ser ↔ Phe
	Val ↔ Leu		Arg ↔ Trp	69	Ser ↔ Arg
15	Ile ↔ Lys		Gly ↔ Ser	72	Gly ↔ Glu
	Leu ↔ Gln		Thr ↔ Met	73	Leu ↔ Trp
16	Ala ↔ Ser		Val ↔ Glu	76	Ser ↔ Tyr
	Phe ↔ Tyr	31	Pro ↔ Gln	83	Cys ↔ Trp
	Pro ↔ Leu	32	Val ↔ Met	99	Gly ↔ Arg
	Ser ↔ Cys	34	Ile ↔ Phe		Ser ↔ Trp
	Val ↔ Asp		Leu ↔ Phe	129	Gly ↔ Trp

The amino acid changes given above can occur by at least one single base change in a codon. However, not necessarily all the codons from a given amino acid can mutate in this way. For example, the codons for Ala (GCT, GCC, GCA and GCG) can all mutate to Val (GTT, GTC, GTA and GTG) by a single base change, but only the last two can mutate in this way to Glu (GAA and GAG).

Example. A blood sample was analysed by ESI-MS to reveal a variant α-chain that was 58 Da heavier than normal, implying the mutation is either Ala→Glu or Gly→Asp. A tryptic digest showed that the mutation occurred in the αT(12-13) peptide, which has the following sequence:

Since there is no Gly in this peptide, the mutation Gly→Asp can be eliminated. Of the six Ala in this peptide, only the one at α120, i.e. J-Meerut, can occur by a single base change in the codon to give Glu (GAG). All the others require two changes, and are therefore extremely unlikely.

Table 1.6.3. The codon sequence of the haemoglobin α_1- and α_2-chains

1	2	3	4	5	6	7	8	9	10	11	12
Val	**Leu**	**Ser**	**Pro**	**Ala**	**Asp**	**Lys**	**Thr**	**Asn**	**Val**	**Lys**	**Ala**
GTG	CTG	TCT	CCT	GCC	GAC	AAG	ACC	AAC	GTC	AAG	GCC

13	14	15	16	17	18	19	20	21	22	23	24
Ala	**Trp**	**Gly**	**Lys**	**Val**	**Gly**	**Ala**	**His**	**Ala**	**Gly**	**Glu**	**Tyr**
GCC	TGG	GGT	AAG	GTC	GGC	GCG	CAC	GCT	GGC	GAG	TAT

25	26	27	28	29	30	31	32	33	34	35	36
Gly	**Ala**	**Glu**	**Ala**	**Leu**	**Glu**	**Arg**	**Met**	**Phe**	**Leu**	**Ser**	**Phe**
GGT	GCG	GAG	GCC	CTG	GAG	AGG	ATG	TTC	CTG	TCC	TTC

37	38	39	40	41	42	43	44	45	46	47	48
Pro	**Thr**	**Thr**	**Lys**	**Thr**	**Tyr**	**Phe**	**Pro**	**His**	**Phe**	**Asp**	**Leu**
CCC	ACC	ACC	AAG	ACC	TAC	TTC	CCG	CAC	TTC	GAC	CTG

49	50	51	52	53	54	55	56	57	58	59	60
Ser	**His**	**Gly**	**Ser**	**Ala**	**Gln**	**Val**	**Lys**	**Gly**	**His**	**Gly**	**Lys**
AGC	CAC	GGC	TCT	GCC	CAG	GTT	AAG	GGC	CAC	GGC	AAG

61	62	63	64	65	66	67	68	69	70	71	72
Lys	**Val**	**Ala**	**Asp**	**Ala**	**Leu**	**Thr**	**Asn**	**Ala**	**Val**	**Ala**	**His**
AAG	GTG	GCC	GAC	GCG	CTG	ACC	AAC	GCC	GTG	GCG	CAC

73	74	75	76	77	78	79	80	81	82	83	84
Val	**Asp**	**Asp**	**Met**	**Pro**	**Asn**	**Ala**	**Leu**	**Ser**	**Ala**	**Leu**	**Ser**
GTG	GAC	GAC	ATG	CCC	AAC	GCG	CTG	TCC	GCC	CTG	AGC

85	86	87	88	89	90	91	92	93	94	95	96
Asp	**Leu**	**His**	**Ala**	**His**	**Lys**	**Leu**	**Arg**	**Val**	**Asp**	**Pro**	**Val**
GAC	CTG	CAC	GCG	CAC	AAG	CTT	CGG	GTG	GAC	CCG	GTC

97	98	99	100	101	102	103	104	105	106	107	108
Asn	**Phe**	**Lys**	**Leu**	**Leu**	**Ser**	**His**	**Cys**	**Leu**	**Leu**	**Val**	**Thr**
AAC	TTC	AAG	CTC	CTA	AGC	CAC	TGC	CTG	CTG	GTG	ACC

109	110	111	112	113	114	115	116	117	118	119	120
Leu	**Ala**	**Ala**	**His**	**Leu**	**Pro**	**Ala**	**Glu**	**Phe**	**Thr**	**Pro**	**Ala**
CTG	GCC	GCC	CAC	CTC	CCC	GCC	GAG	TTC	ACC	CCT	GCG

121	122	123	124	125	126	127	128	129	130	131	132
Val	**His**	**Ala**	**Ser**	**Leu**	**Asp**	**Lys**	**Phe**	**Leu**	**Ala**	**Ser**	**Val**
GTG	CAC	GCC	TCC	CTG	GAC	AAG	TTC	CTG	GCT	TCT	GTG

133	134	135	136	137	138	139	140	141	142
Ser	**Thr**	**Val**	**Leu**	**Thr**	**Ser**	**Lys**	**Tyr**	**Arg**	**STOP**
AGC	ACC	GTG	CTG	ACC	TCC	AAA	TAC	CGT	TAA

Table 1.6.4. The codon sequence of the human β-chain

1	2	3	4	5	6	7	8	9	10	11	12
Val	**His**	**Leu**	**Thr**	**Pro**	**Glu**	**Glu**	**Lys**	**Ser**	**Ala**	**Val**	**Thr**
GTG	CAT	CTG	ACT	CCT	GAG	GAG	AAG	TCT	GCC	GTT	ACT

13	14	15	16	17	18	19	20	21	22	23	24
Ala	**Leu**	**Trp**	**Gly**	**Lys**	**Val**	**Asn**	**Val**	**Asp**	**Glu**	**Val**	**Gly**
GCC	CTG	TGG	GGC	AAG	GTG	AAC	GTG	GAT	GAA	GTT	GGT

25	26	27	28	29	30	31	32	33	34	35	36
Gly	**Glu**	**Ala**	**Leu**	**Gly**	**Arg**	**Leu**	**Leu**	**Val**	**Val**	**Tyr**	**Pro**
GGT	GAG	GCC	CTG	GGC	AGG	CTG	CTG	GTG	GTC	TAC	CCT

37	38	39	40	41	42	43	44	45	46	47	48
Trp	**Thr**	**Gln**	**Arg**	**Phe**	**Phe**	**Glu**	**Ser**	**Phe**	**Gly**	**Asp**	**Leu**
TGG	ACC	CAG	AGG	TTC	TTT	GAG	TCC	TTT	GGG	GAT	CTG

49	50	51	52	53	54	55	56	57	58	59	60
Ser	**Thr**	**Pro**	**Asp**	**Ala**	**Val**	**Met**	**Gly**	**Asn**	**Pro**	**Lys**	**Val**
TCC	ACT	CCT	GAT	GCT	GTT	ATG	GGC	AAC	CCT	AAG	GTG

61	62	63	64	65	66	67	68	69	70	71	72
Lys	**Ala**	**His**	**Gly**	**Lys**	**Lys**	**Val**	**Leu**	**Gly**	**Ala**	**Phe**	**Ser**
AAG	GCT	CAT	GGC	AAG	AAA	GTG	CTC	GGT	GCC	TTT	AGT

73	74	75	76	77	78	79	80	81	82	83	84
Asp	**Gly**	**Leu**	**Ala**	**His**	**Leu**	**Asp**	**Asn**	**Leu**	**Lys**	**Gly**	**Thr**
GAT	GGC	CTG	GCT	CAC	CTG	GAC	AAC	CTC	AAG	GGC	ACC

85	86	87	88	89	90	91	92	93	94	95	96
Phe	**Ala**	**Thr**	**Leu**	**Ser**	**Glu**	**Leu**	**His**	**Cys**	**Asp**	**Lys**	**Leu**
TTT	GCC	ACA	CTG	AGT	GAG	CTG	CAC	TGT	GAC	AAG	CTG

97	98	99	100	101	102	103	104	105	106	107	108
His	**Val**	**Asp**	**Pro**	**Glu**	**Asn**	**Phe**	**Arg**	**Leu**	**Leu**	**Gly**	**Asn**
CAC	GTG	GAT	CCT	GAG	AAC	TTC	AGG	CTC	CTG	GGC	AAC

109	110	111	112	113	114	115	116	117	118	119	120
Val	**Leu**	**Val**	**Cys**	**Val**	**Leu**	**Ala**	**His**	**His**	**Phe**	**Gly**	**Lys**
GTG	CTG	GTC	TGT	GTG	CTG	GCC	CAT	CAC	TTT	GGC	AAA

121	122	123	124	125	126	127	128	129	130	131	132
Glu	**Phe**	**Thr**	**Pro**	**Pro**	**Val**	**Gln**	**Ala**	**Ala**	**Tyr**	**Gln**	**Lys**
GAA	TTC	ACC	CCA	CCA	GTG	CAG	GCT	GCC	TAT	CAG	AAA

133	134	135	136	137	138	139	140	141	142	143	144
Val	**Val**	**Ala**	**Gly**	**Val**	**Ala**	**Asn**	**Ala**	**Leu**	**Ala**	**His**	**Lys**
GTG	GTG	GCT	GGT	GTG	GCT	AAT	GCC	CTG	GCC	CAC	AAG

145	146	147
Tyr	**His**	**STOP**
TAT	CAC	TAA

1.7. Reagents and hardware required for identifying variants by ESI-MS

Solvents.

1. Acetonitrile (HPLC grade). Sigma-Aldrich 34888 or equivalent (2.5 L).

2. Water (HPLC grade). Sigma-Aldrich 27073-3 or equivalent (2.5 L).

3. Concentrated formic acid (Sigma F-4636). Minimum quantity.

Solutions.

Store in glass bottles with glass stoppers. Convenient sizes are 50 mL for every day use and 150 mL for stock solutions. Store at room temperature.

1. 1% aqueous formic acid (by volume). 150 mL bottle.

2. Solution A for 10-fold diluting aqueous solutions of blood and digests ready for introduction into the mass spectrometer source. Prepare by mixing 10 mL of acetonitrile, 4 mL of water and 4 mL of 1% aqueous formic acid or *pro rata* in a 50 mL bottle. Usage is ~1 mL/sample.

3. Wash solution. 50% aqueous acetonitrile containing 1% (by volume) formic acid. For washing syringe between samples. Store in 150 mL and 50 mL bottles.

4. It is convenient to keep some water and some acetonitrile in 50 mL bottles for preparing Solution A. The water is also used for 50-fold diluting blood samples and should be stored in a brown bottle.

Reagents. Preparation is more detailed in Section 2.2. Prepare and store in 1.5 mL microcentrifuge tubes.

1. Denaturing solution for adding to 50-fold diluted blood to denature the Hb prior to digestion with trypsin. Prepare by mixing 500 μL of 1% formic acid with 500 μL acetonitrile. Usage is 20 μL/sample. Store at 5 ^{O}C. Renew after one month.

2. 1 molar aqueous ammonium bicarbonate solution (Sigma A-6141). Usage is 6 μL/sample. Store at 5 ^{O}C.

3. 5 mg/mL aqueous TPCK treated trypsin (Sigma T-1426, 50 mg). Usage is 5 μL/sample. It will remain viable for over 3 months when stored below -20 ^{O}C.

4. 100 mM aqueous dithiothreitol. (Sigma D-0632, 250 mg). Store at 5 ^{O}C. It is occasionally used for reducing disulphide bonds in digests or old blood samples.

Miscellaneous.

1. 20 μL, 200 μL and 1000 μL pipettes. Gilson or equivalent. Usage is 3 or 4 x 200 μL tips /sample.

2. Cation exchange resin beads, 100-200 mesh, hydrogen form. AG 50W-X8, 100g, Cat. No. 143-5441, Bio-Rad Labs. For desalting 500-fold diluted blood.

3. 1.5 mL microcentrifuge tubes. 3 or 4 required/sample.

4. Block heater. To accommodate 20 or 40 x 1.5 mL microcentrifuge tubes. For incubating digest solutions at 37 ^{O}C.

5. Syringe pump, Harvard Apparatus, Type 11 or Type 22. For introducing samples into mass spectrometer source generally at 5 μL/min. Only needed when the mass spectrometer does not have a built-in syringe pump.

6. 100 μL gas-tight syringe plus spares. Hamilton type 1710 with removable needle (22s gauge with blunt tip). For introducing samples using a syringe pump.

7. 4 x 10 μL syringes plus spares. Hamilton type 701. For preparing digest solutions.

8. Mixer.

1.8. Files for installation into MassLynx software

Table 1.8.1. Reference files for mass scale calibration

File Name	Application
Hba.ref	Calibration of denatured Hb spectra using the α-chain
Hbb.ref	Calibration of denatured Hb spectra using the β-chain
DigHbA.ref	Calibration of tryptic digest spectra from adult human Hb
DigHbACT.ref	Calibration of α-chymotrypsin digest spectra from adult human Hb
DigHbF.ref	Calibration of tryptic digest spectra from human foetal Hb
MSMSBeta16.ref	Calibration of tandem mass spectra of the β-chain ion with 16 charges

Table 1.8.2. Sequence of some globin chains

File Name	Chain or Variant Name
HBA_HUMA.emb	Hb α (alpha)-chain
HBB_HUMA.emb	Hb β (beta)-chain
HBD_HUMA.emb	Hb δ (delta)-chain
HBE_HUMA.emb	Hb ε (epsilon)-chain
HBAZ_HUM.emb	Hb ζ (zeta)-chain
HBAgHUMA.pep	Hb $^{A}\gamma$ (A-gamma)-chain
HBAgTHUMA.pep	Hb $^{A}\gamma^{T}$ (A-gamma-T)-chain
HBGgHUMA.pep	Hb $^{G}\gamma$ (G-gamma)-chain
HbLepBal.pep	Hb Lepore-Baltimore. δ-β hybrid chain
HbLepHol.pep	Hb Lepore-Hollandia. δ-β hybrid chain
HbLepWas.pep	Hb Lepore-Boston-Washington. δ-β hybrid chain
HbLincPk.pep	Hb Lincoln Park. β-δ hybrid chain
HbMiyada.pep	Hb Miyada. β-δ hybrid chain
HbNilotic.pep	Hb P-Nilotic. β-δ hybrid chain
HbParchman.pep	Hb Parchman. δ-β-δ hybrid chain
HbPIndia.pep	Hb P-India. β-δ hybrid chain
P-Congo.pep	Hb P-Congo. β-δ hybrid chain
HbKenya.pep	Hb Kenya. $^{A}\gamma$-β hybrid chain. Also called HPFH-7;Kenya
ConstSpring.pep	Hb Constant Spring. α-chain extension
HbTak.pep	Hb Tak. β-chain extension

1.9. Step 1. Cation exchange-HPLC data and its use in identifying human haemoglobin variants by electrospray ionization mass spectrometry

Although information from cation exchange-HPLC (ce-HPLC) data is not essential when identifying variants by electrospray ionization mass spectrometry (ESI-MS), it can sometimes save time by eliminating unlikely mutations. Most variants are detected by ce-HPLC in Step 1. In Step 2, ESI-MS determines the mass change due to the mutation and assigns the mutation to either the α- or β-chain by analysing the intact haemoglobin (Hb) chains present in denatured blood. The denatured blood is then digested with trypsin (Step 4) to produce ~15 tryptic peptides from each chain, thereby narrowing down the position of the mutation to a particular peptide from one of the chains. It is at this stage that ce-HPLC data can be useful for selecting the appropriate table to use when searching for the variant peptide in the mass spectrum of the digest. In some cases, the mass change leads to a unique mutation governed by a single base change in the nucleotide codon. For example, as shown in Table 1.6.2, a 22 Da mass increase over normal would almost certainly be due to the mutation Asp→His, in which case identifying the variant peptide is straightforward. However, in other cases there are several possibilities, e.g., a 14 Da mass increase can be due to one of six mutations. In such cases information from the HPLC data can assist in choosing the appropriate table to use when searching for the variant peptide in the spectrum from the digest.

As well as providing the abundance of the variant, ce-HPLC data can be used to indicate the charge change from normal due to the mutation and hence suggest the most likely mutations. If it is assumed that Arg, Lys and His carry a positive charge, Asp and Glu carry a negative charge and all the other amino acid residues are neutral, then the charge change from normal can be roughly related to the elution time in the cation exchange-HPLC data. If the variant elutes significantly sooner than Hb A_0, then the mutation is assumed to cause the Hb to gain one negative charge (J-like) or two charges (I-like). Conversely, if the variant elutes significantly later than Hb A_0, the mutation is assumed to cause the Hb to gain one positive charge (D-like) or two charges (C-like). If the variant elutes very close to or is coincident with Hb A_0, then the mutation is assumed to be between amino acids carrying a similar charge or carrying no charge, i.e. is silent or nearly so. The Bio-Rad ce-HPLC traces of some variants together with the charge changes predicted by these rules are shown in Figures 1.9.1 and 1.9.2.

Of course, the above rules are over simplified and there are some anomalies. For example, D-Iran (β22Glu→Gln), Figure 1.9.1c, with a predicted increase of one positive charge elutes close to Hb E (β26Glu→Lys), Figure 1.9.1d, with a predicted increase of two positive charges. Most mutations with a predicted charge change of +2 elute much later than Hb E, closer to Hb C (β6Glu→Lys), Figure 1.9.2d or O-Arab (β121Glu→Lys). Despite such anomalies, the above rules appear to work sufficiently well in distinguishing J-/I-like, D-/C-like and silent variants. The following examples illustrate how the rules can be applied in practice.

Suppose a D-like variant was detected by HPLC, and ESI-MS of the intact chains (Step 2) showed that the mutation was associated with the β-chain and increased the mass by 14 Da over normal. Table 1.6.2 shows there are six mutations that can give 14 Da mass increase by a single base change in the nucleotide codon, namely: Asn→Lys, Asp→Glu, Gly→Ala, Ser→Thr, Val→Ile and Val→Leu. Of these mutations, all except Asn→Lys can be discounted, because they would be silent or almost silent by HPLC. Thus, the most likely mutation is Asn→Lys and Table 4.5.6, listing the 'new' peptides would be referred to first when searching for the variant peptide in the digest spectrum.

If a D-like peptide had given 1 Da mass decrease from normal in the β-chain, then different sets of tables would be used (Table 4.4.1 and possibly Table 4.4.2).

If a variant that gave 14 Da mass increase above normal had been essentially silent by HPLC, then the appropriate 'Auto digest simulation' table (Section 4.5) would be used. In this case all the mutations except Asn→Lys would be considered.

Summary
Relating ce-HPLC data to amino acid and charge change.

Assume:

- Arg, Lys and His carry one positive charge
- Asp and Glu carry one negative charge
- All the other amino acid residues carry no charge, i.e. are neutral.

Then, on the whole:

- When the mutation causes a net increase in negative charge, the variant elutes 'well before' Hb A_0 (travels significantly faster than Hb A_0) on ce-HPLC. Examples are: Gly→Asp, Ala→Glu, Ala→Asp, Gln→Glu, Lys→Glu, His→Gln, and Arg or Lys to a neutral residue.

- When the mutation causes no change in charge, the variant elutes 'close to' Hb A_0 on ce-HPLC.

  Examples are: Arg↔Lys, Asp↔Glu and any mutation between neutral residues, e.g. Ser→Pro, Val→Met.

- When the mutation causes a net increase in positive charge, the variant elutes 'well after' Hb A_0 (travels significantly slower than Hb A_0) on ce-HPLC.

  Examples are:
  Asp→Gly
  Glu→Ala
  Glu→Gln
  Asp→Asn
  Glu→Val
  Gln→Arg
  Asn→Lys
  Gln→Lys
  Asp→His
  Glu→Lys.

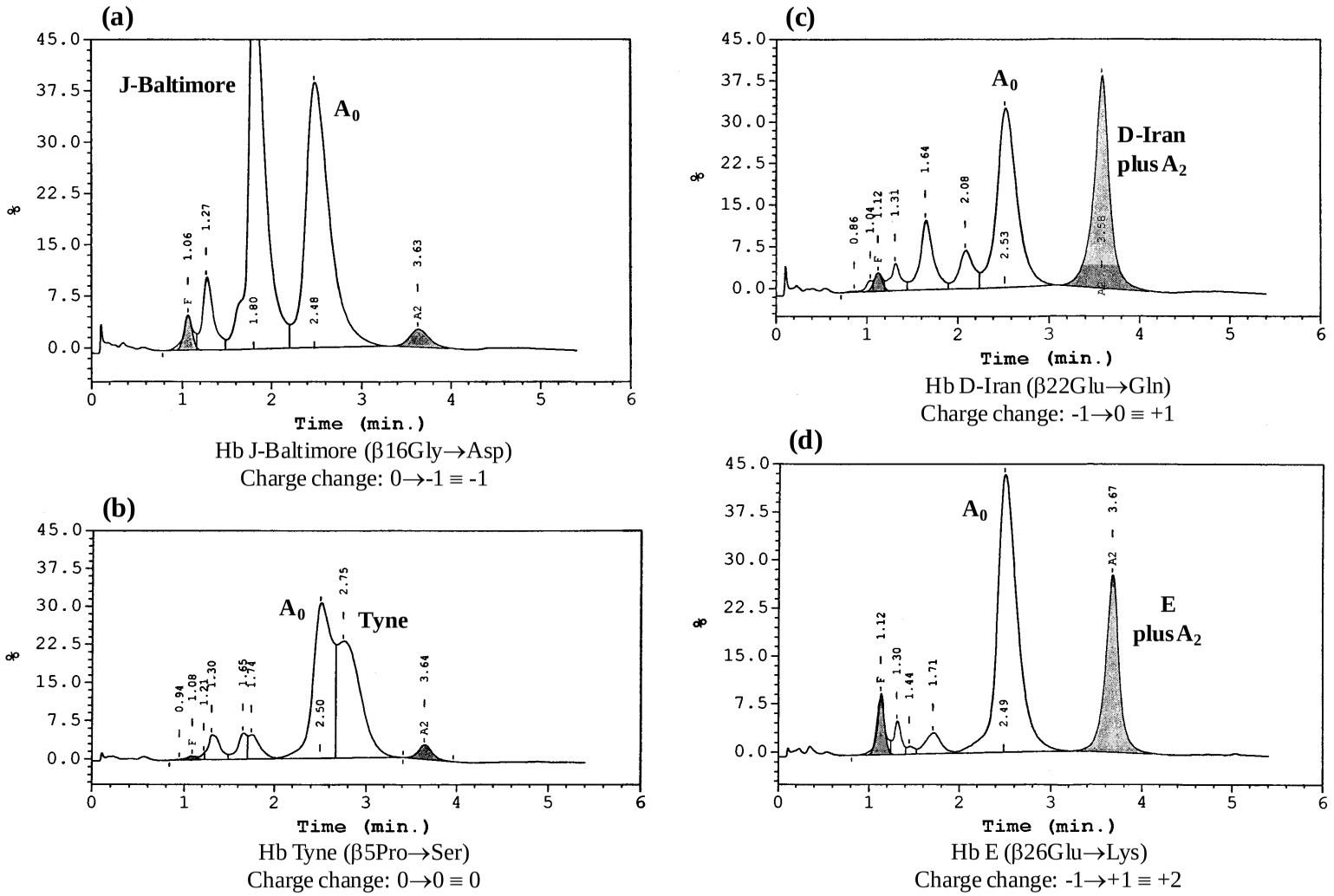


Figure 1.9.1. Cation exchange-HPLC traces showing how the elution time roughly correlates with charge change. Hb E moves anomalously fast but its elution time nevertheless indicates a significant positive charge change.

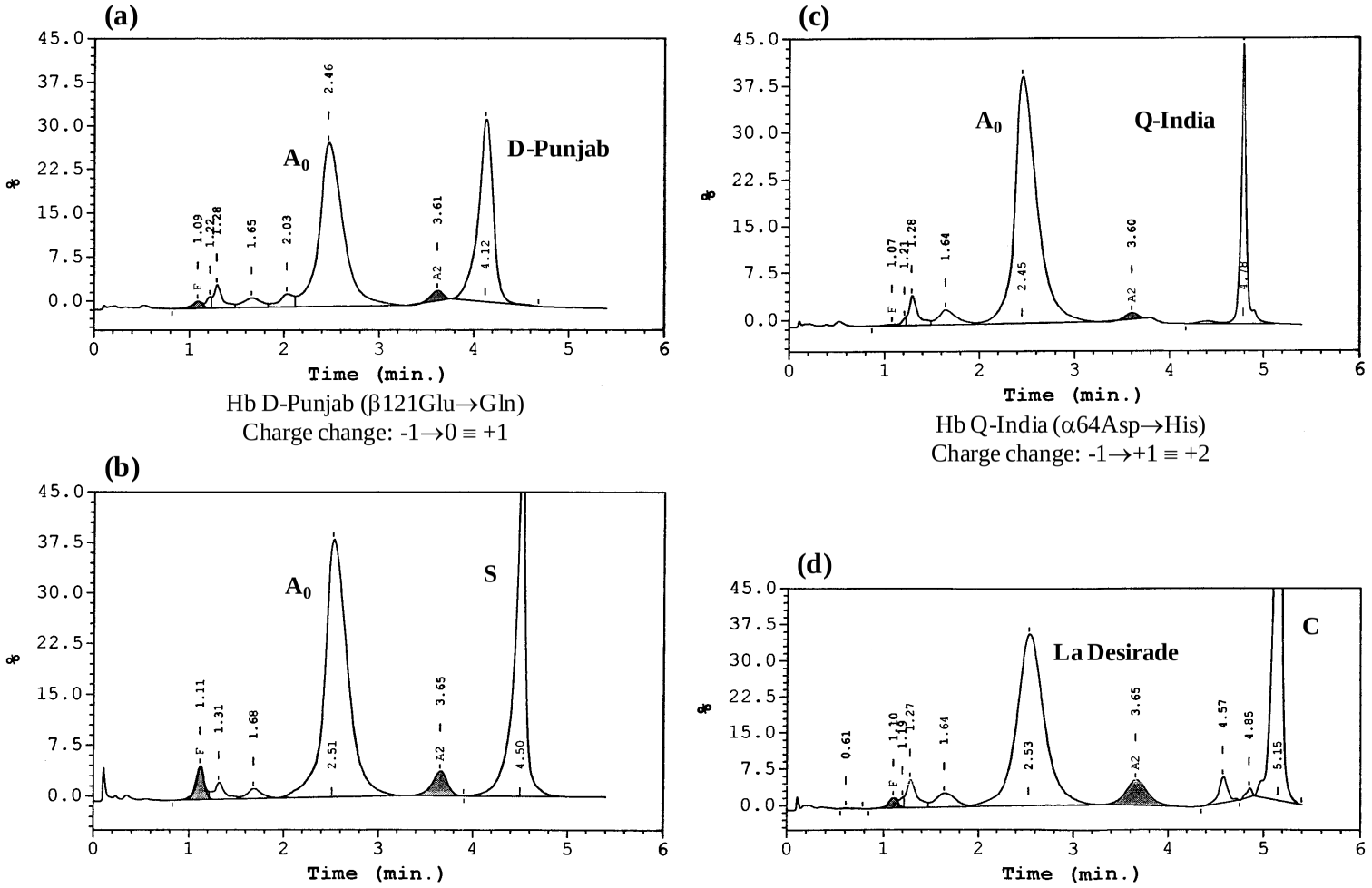


Figure 1.9.2. Cation exchange-HPLC traces of heterozygotes in which the mutation changes the polarity by +1 and +2 charges.

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SECTION 2

2.0. Step 2: The analysis of the globin chains in human haemoglobin by electrospray ionization mass spectrometry (ESI-MS)

2.1. Information obtained from the spectrum of the globin chains.

In contrast to the routine methods used in haematology laboratories, e.g. cation exchange-HPLC (ce-HPLC), which analyse the haemoglobin (Hb) in blood as non-covalent assemblies of globin chains, ESI-MS analyses the Hb in denaturing solution in order to observe the individual globin chains and their derivatives. Blood is diluted 500-fold in 50% aqueous acetonitrile containing 0.2% formic acid. After desalting, this solution is analysed by ESI-MS to give a spectrum in two or three minutes which, after deconvolution by the maximum entropy-based software (MaxEnt), provides the following information.

Figure 2.1.1 shows the electrospray mass spectrum of diluted whole blood over the mass range *m/z* 600-1400 and shows the two distinct multiply protonated molecule series for the α- and β-chains. Also seen is the haem (*m/z* 616.2) that is released from the Hb complex under the denaturing solvent conditions.

To reliably achieve the mass measurement precision required for this work, the acquisition range is limited to *m/z* 930-1210 with a minimum of 32 points per *m/z* unit (Figure 2.1.2a). From the acquired data, the mass-calibrated *m/z* range deconvoluted with MaxEnt is restricted further to *m/z* 980-1180 (Figure 2.1.2b), to give the Hb spectrum on a true mass scale (Figure 2.1.2c).

1. The molecular weights (masses) of the major chains determined with a precision of better than ±0.05 Da (±3.2 ppm) standard deviation, provided that one of the chains (usually the α-chain) is used to internally calibrate the mass scale. The masses of the major chains are usually determined to within 0.2 Da. This accuracy allows the mass change due to the mutation to be derived, and, when a variant is due to a single amino acid change, a limited number of single amino acid mutations can be proposed. This mass change is an important characteristic of the variant that is used in all subsequent identification work. Variant chains that differ in mass by as little as ±1 Da from normal can be detected in heterozygotes, provided their abundance is >10% of the normal chain[1,2].

2. Assignment of a variant to either the α- or the β-chain. With variants in heterozygotes that give <±6 Da mass change from normal, ce-HPLC data are also required.

3. The proportion of the variant chain relative to the normal chain in heterozygotes.

4. The proportion of the δ-chain (equivalent to Hb A_2) relative to total non-α-chains.

5. The levels of glycated α- and β-chains. The Hb A_{1c} level can be derived from the level of glycation on either the α- or β-chain by calibration.

6. The proportions of $^{G}\gamma$, $^{A}\gamma$ and $^{A}\gamma^{T}$ in Hb F (detection limit ~0.5%). The ratio $^{G}\gamma$/(total γ) can be obtained directly from the spectrum.

7. Some variants can be identified from their characteristic spectra or masses, e.g. Hbs St Josef, J-Biskra, Wayne, South Florida, Marseille, P-Nilotic, Lepore-Baltimore, Kenya and Tak (see Section 2.4.6).

8. Detection of variants which are silent by ce-HPLC. Most variants that are silent by ce-HPLC are readily detected by ESI-MS.

9. Detection of carbonic anhydrase 1 (CA1, 28,781.08 Da).

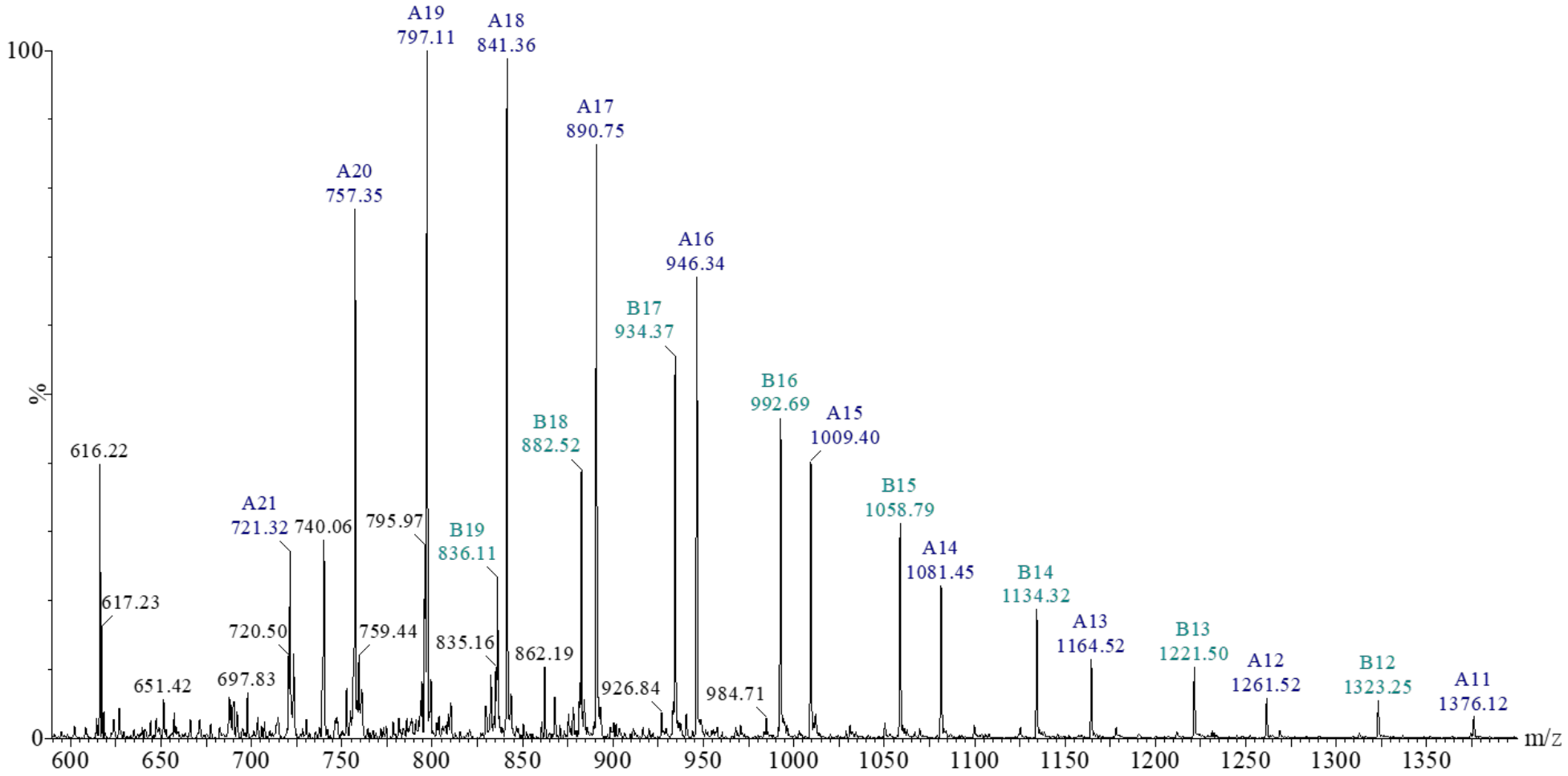

Figure 2.1.1. Full mass spectrum of a diluted sample of whole blood showing the presence of the haem (*m/z* 616.27), and the two multiply protonated series that are formed from the α-chain (A11-A21 protons attached) and β-chain (B12-B19 protons attached).

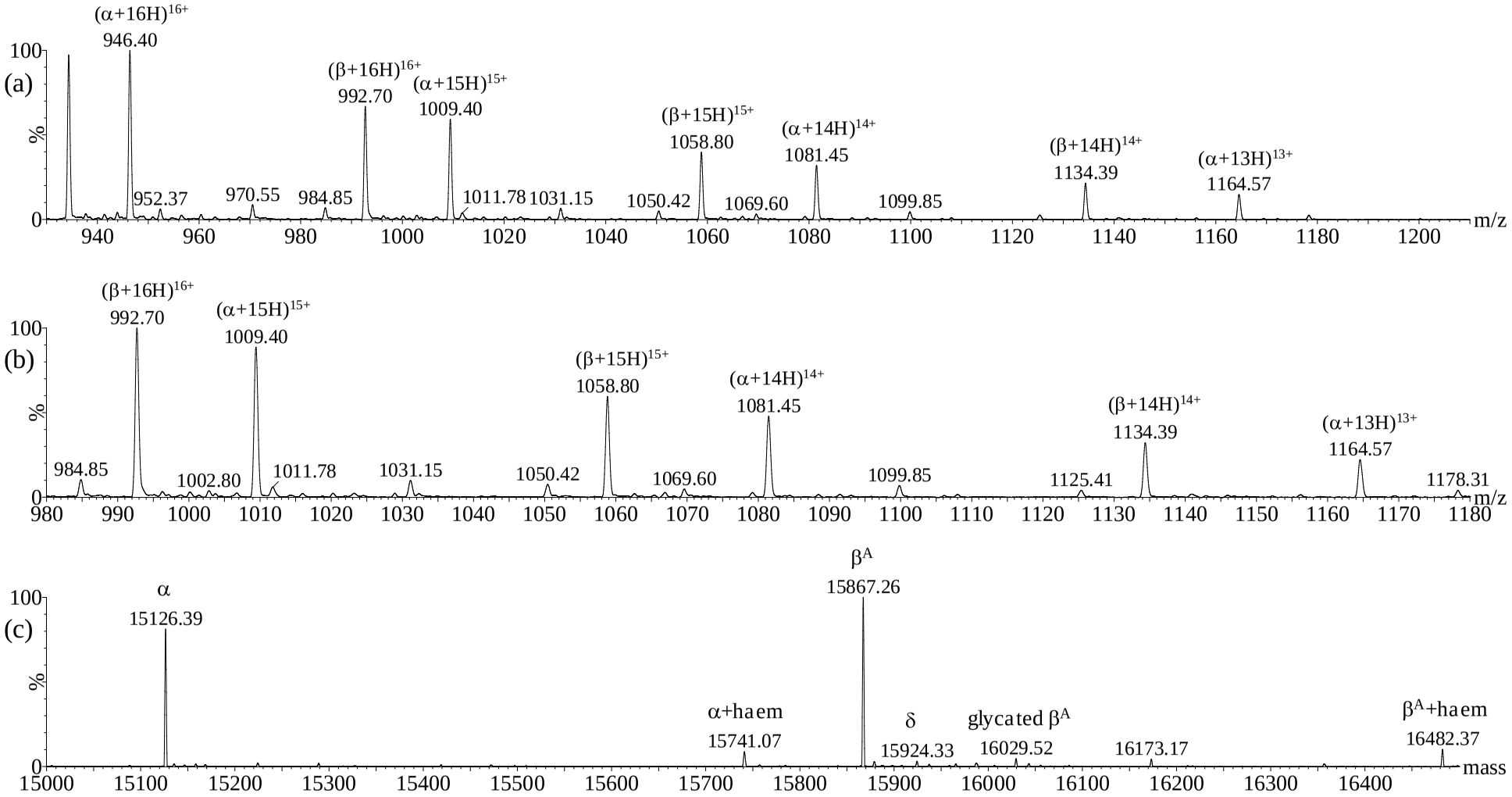

Figure 2.1.2. Figure showing (a) the raw mass spectrum, (b) the mass range required for MaxEnt processing, and (b) the MaxEnt processed spectrum.

2.2. Sample preparation.

2.2.1. Preparing stock solutions from whole blood.

Dilute 10 μL of blood with 490 μL of HPLC grade water to make a stock solution of blood diluted 50-fold. This solution should be stored at -20˚C and should remain viable for several months. It is also used for producing enzymatic digests with trypsin.

2.2.2. Preparing stock solutions from Guthrie cards.

Place four 3 mm diameter spots punched from a Guthrie card in a 1.5 mL microcentrifuge tube and occasionally agitate them with 300 μL of water until most of the haemoglobin has dissolved. Check that the concentration of haemoglobin in the solution is roughly the same as that prepared above (2.2.1.) from a whole blood sample by comparing the colour levels by eye. If necessary, add water or more spots to make the colour intensities similar. If a spot punch is not available, cut out a ~3 x 10 mm strip with scissors. This is roughly equivalent to four 3mm diameter spots. One 3 mm diameter spot contains approximately 2 μL of blood.

2.2.3. Preparing working solutions.

A working solution suitable for ESI-MS analysis may be made by simply diluting 20 μL of the stock solution 10-fold with 180 μL of 0.8:1.0 water:acetonitrile solution containing 0.22% formic acid (solution A) to give an overall dilution from blood of 500. Solution A may be prepared by mixing 5 mL acetonitrile, 2 mL water and 2 mL 1% aqueous formic acid or *pro rata*. The concentration of each major chain in the working solution is ~9 pM/μL (9 μM), based on 15 g of Hb per 100 mL of blood. Figure 2.1.3a shows a typical MaxEnt spectrum from a solution prepared in this way from the blood of a heterozygote for the variant Hb Fontainebleau, α21Ala→Pro, in which the sequence mass of the variant α-chain is 26.04 Da higher than normal.

2.2.4. Preparing desalted working solutions.

Often with heterozygotes, alkali metal adducts of the normal chains (principally Na and K) may interfere with the detection and measurement of variant chains that occur up to ~70 Da higher in mass than the normal chains. These adducts tend to be more serious with α-chain variants, where the variant chain abundance may be 25% or less of the total α-chain abundance. Furthermore, alkali metal adducts interfere with reliable detection and measurement of the δ-chain (Hb A_2). Other cases occur when β-chain variants are at low levels.

Therefore, it is strongly recommended that all diluted blood samples are desalted before analysis. A simple and quick desalting procedure using cation exchange resin beads is as follows. First, wash some beads by placing ~500 mg of cation exchange resin beads (AG 50W-X8, hydrogen form, 100-200 mesh, Cat. No. 143-5441, Bio-Rad Labs) in a fresh 1.5 mL microcentrifuge tube. Add ~1 mL of HPLC grade water and manually shake the mixture for ~30 sec. After allowing the beads to settle (~30 sec), remove as much of the water as can be easily removed with a pipette. Repeat the washing procedure at least once. Store the washed beads at room temperature.

Then, manually agitate 200 μL of the 500-fold diluted working solution as prepared in Section 2.2.3. above with ~20 mg of the washed ion exchange beads for 15-30 seconds. After allowing the beads to settle (~20 seconds), immediately draw 50-100 μL of the supernatant liquid into the syringe that is to be used to introduce the sample into the ESI source. Be careful to avoid drawing beads into the syringe tip. The desalting procedure may introduce non-covalently bound adducts due to impurities from the beads that are 98 and 202 Da higher than the masses of the Hb chains. The 98 Da adducts are probably due to the presence of H_2SO_4 from the beads. Washing the beads prior to use generally reduces such adducts to negligible levels, provided the working solution is not left too long in contact with the beads. Washed beads that have been left standing for more than a few hours, e.g. overnight, should be washed once again before use.

Figure 2.1.3 illustrates the effect of desalting a sample containing the α-chain variant Hb Fontainebleau. Before desalting (Figure 2.1.3a), Na and K adducts associated with the β-chain can be seen at ~10% relative intensity. Since each adduct carries a positive charge, their masses are calculated to occur 21.98 Da (Na-H) and 38.09 Da (K-H) higher than the masses of the α- and β-chains. A third adduct (Na+K-2H) is predicted to occur 60.07 Da higher than the β-chain at 15,927.31 Da, close

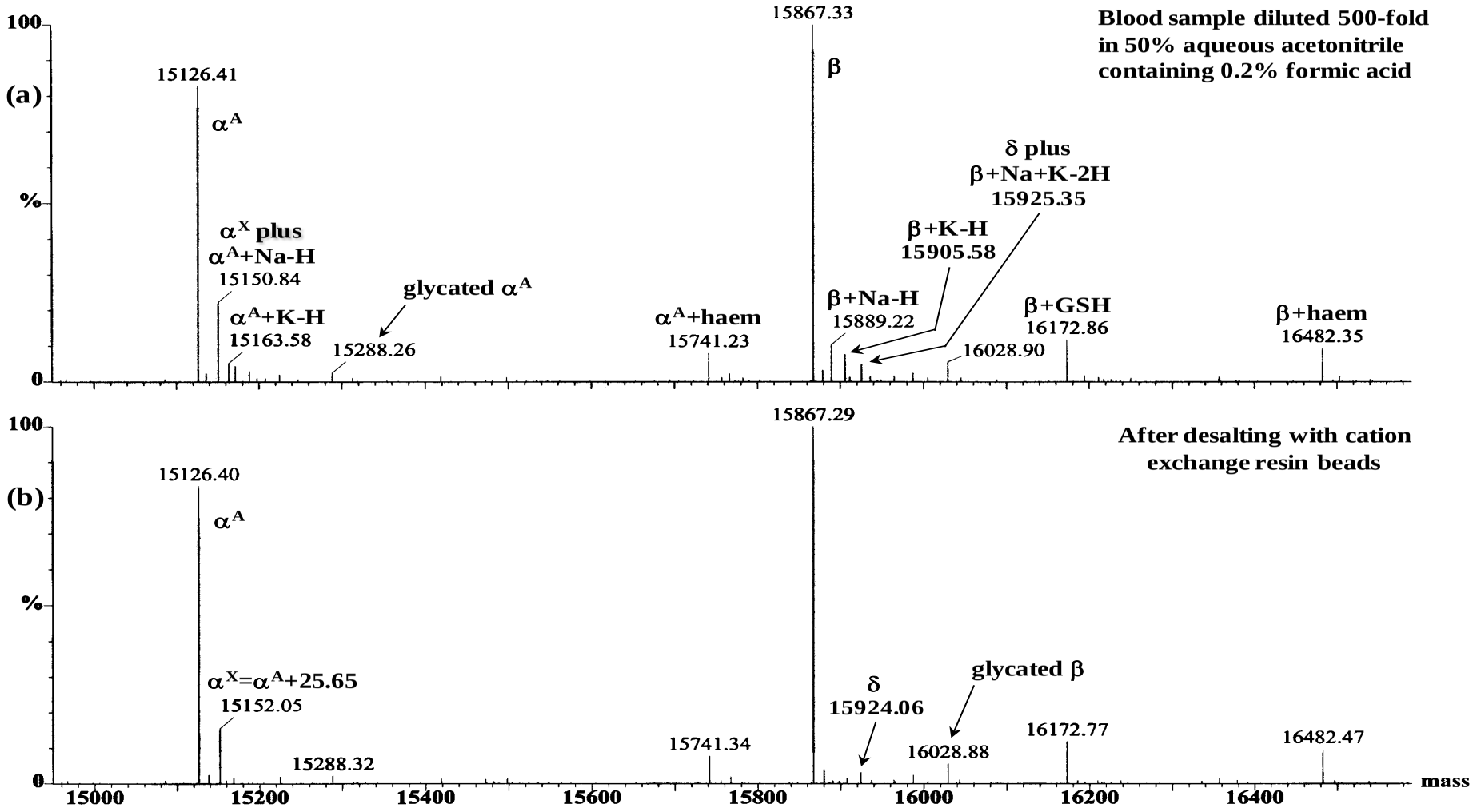

Figure 2.1.3. MaxEnt processed and centred spectra of a Hb Fontainebleau (α21Ala→Pro) heterozygote showing how the masses and intensities of the variant chain and δ-chain improve after desalting. Masses are experimental. GSH: glutathione

to the mass of δ-chain (15,924.32 Da). This erroneously raises the level and mass of the δ-chain to 4.6% and 15,925.35 Da respectively, since Hb components that occur <6 Da apart are not resolved by ESI-MS/MaxEnt. After desalting, (Figure 2.1.3b), the δ-chain level decreased to 3.0% and its mass to 15,924.06 Da.

The sodium adduct associated with the normal α-chain is predicted to occur at 15,148.36 Da. However, it is unresolved from the Fontainebleau variant (sequence mass 15,152.42 Da) and erroneously raises the level of the latter.

Moreover, it lowers the apparent mass of the variant to be 24.43 Da higher than the normal α-chain instead of the predicted 26.04 Da. After desalting, this mass difference increased to 25.65 Da, which, although still slightly lower than predicted, nevertheless rounds to the nominal mass difference of 26 Da, allowing the correct mass difference to be used in predicting potential amino acid changes from the genetic code tables. After desalting, the level of the variant decreased from 21.2 to 15.6%.

2.2.5. Improving the quality of the data from old blood samples by reduction with dithiothreitol.

Adducts often occur in old blood samples that originate from the glutathione present in blood and are covalently bound to β93Cys. They occur 119.14 Da (cysteinylation), 176.20 Da (Cys-Gly addition) and 305.31 Da (glutathionylation) Da higher than the mass(es) of the β-chain(s). For the normal β-chain, their masses are 15,986.38 Da, 16043.44 Da and 16172.55 Da respectively. They may be reduced to the underivatised β-chain(s) by treating an aliquot of the stock solution with dithiothreitol (DTT) as follows. Do not use mercaptoethanol because it produces disulphide bound adducts with accessible cysteines.

Place 20 μL of the stock blood solution (50-fold diluted blood) in a fresh microcentrifuge tube and add 0.5 μL of a 1 M/L solution of ammonium bicarbonate and 2 μL of a 100 mM/L solution of DTT. Mix, pulse centrifuge and incubate at 37 ^{O}C for ~15 minutes. Then add 180 μL of solution A. The resulting solution may be desalted using the procedure described above (2.2.4) and introduced directly into the ESI source of the mass spectrometer.

This procedure can also be useful in cases where the mutation produces a 'new' cysteine with consequently high levels of disulphide bound adducts, e.g. Hb Leeds (β56Gly→Cys). Hb Ta-Li (β83Gly→Cys) forms disulphide linked dimers on standing, which can be reduced to the monomers using DTT in this way.

2.2.6. Overall summary of the procedures for preparing desalted working solutions from whole blood samples.

Prepare a stock solution in a 1.5 mL microcentrifuge tube by diluting 10 μL of whole blood with 490 μL of water (50-fold dilution).

Transfer 20 μL of the stock solution to a fresh 1.5 mL microcentrifuge tube and add 180 μL of solution A (overall 500-fold dilution).

Add ~20 mg of previously washed cation exchange beads. Manually shake the mixture for 15-30 seconds. Allow the beads to settle (~20 seconds) and draw 50-100 μL of the desalted blood solution into the syringe that is to be used for introducing the sample into the ESI source of the mass spectrometer. Be careful to avoid drawing beads into the syringe tip.

Solution A may be prepared by mixing 5 mL of acetonitrile, 2 mL of HPLC grade water and 2 mL of aqueous 1% formic acid or *pro rata*.

2.3. Analysis by ESI-MS.

2.3.1. Sample introduction.

Introduce the working solution of the sample into the ESI source at 5 μL/min. One method is to introduce the sample solution using a syringe pump (e.g. Type 11 or 22, Harvard Apparatus Inc, South Natick, MA) and a gas-tight syringe (e.g. Type 1710 (100 μL) with 22s gauge removable blunt-tip needle, Hamilton Inc). If available, a syringe pump mounted on the mass spectrometer should be used. Two rinses of the syringe with wash solution between samples are generally sufficient to prevent carry-over. A suitable wash solution is composed of 50% aqueous acetonitrile containing 1% formic acid.

2.3.2. Scan and data acquisition parameters.
All the results in this book were produced by a triple quadrupole instrument, which was set to give a peak width at half height of 0.7 *m/z* unit on the *m/z* 1,081.5 ion from the haemoglobin α-chain $(M+14H)^{14+}$, where M is the molecular weight of the α-chain (15,126.38 Da).

Acquire data in the MCA mode for 3 minutes, whilst scanning from *m/z* 930-1210 at 8 seconds/scan. The number of channels per *m/z* unit should be no less than 32.

2.3.3. Critical instrumental parameters
The following four items describe the critical requirements for acquiring and processing globin chain data from blood samples:

1. Data must be acquired with a minimum of 32 data points per *m/z* unit.

2. Acquire the data over the *m/z* range 930-1210. Acquiring over a wider *m/z* range is of no advantage, and doing so may compromise the calibration or deconvolution procedures. The recommended *m/z* range will accommodate most of the variants likely to be encountered in practice including the Constant Springs, the γ-chains and various derivatives of the major chains, e.g. glycated species, haem adducts etc. However, the ratio of the intensity of the α-chain to that of the β-chain is not quantitatively correct.

3. Before deconvoluting the raw data with MaxEnt, the *m/z* scale of the raw data must be internally calibrated, i.e. calibrated on itself, using the multiply protonated normal α-chain peaks. In cases where the normal α-chain is compromised, e.g. by a partly resolved variant, the β-chain should be used.

4. Deconvolution of the baseline subtracted raw data by MaxEnt should normally be undertaken over an input *m/z* range 980-1180, with an output mass range of 14800-16800 Da and an output resolution of 0.2 Da per/channel (per data point). Note that MaxEnt only processes that part of the *m/z* range on display.

The use of instrumentation that isotopically resolves the components is believed to offer no advantage. This is because the lowest isotopic species (the monoisotopic species) from a globin chain has a predicted abundance that is only ~0.1% of the most abundant species which is too small be used. Furthermore, each component consists of nearly 20 isotopic species approximately 1 Da apart and above 10% of the most abundant species. Therefore, two globin chains differing in mass by 1 Da will produce two overlapping patterns each of roughly 20 isotopic species and displaced by 1 Da, which will still require some form of deconvolution in order to establish their masses and relative abundance. It appears that resolving the isotopic species does not improve the ability to resolve closely spaced variants.

2.3.4. Mass scale calibration.
It is extremely rare to encounter a blood sample that does not produce multiply charged normal α-chain peaks as major components. Consequently, these peaks should be used to calibrate the *m/z* scale of each data file on itself, i.e. internally, with considerable benefit to the accuracy of mass determination. The whole calibration procedure may be undertaken automatically in a few seconds from the **raw spectrum** as follows. Note that the calibration procedure uses the acquired *m/z* range irrespective of the range shown on the display.

From the **raw spectrum**, select **Tools,** then select **Make calibration,** and in the **Make new calibration** window select an appropriate **Reference file**, Hba.ref for the α-chain or Hbb.ref for the β-chain (Figure 2.2.1).

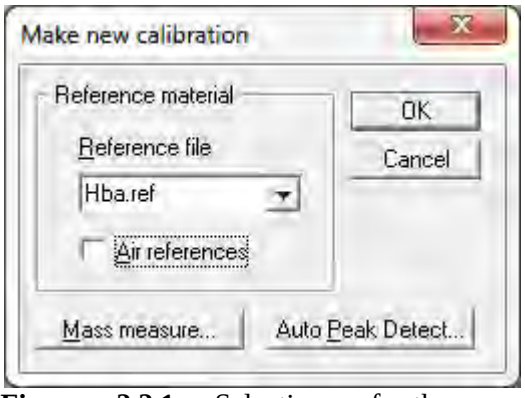

Figure 2.2.1. Selection of the mass calibration file for the α-chain

Select the **Mass Measure** window (Figure 2.2.2).

Select **Background subtract.** Set Polynomial order to 25. Set Below curve (%) to 5.

Select **Smooth.** Set Peak width (Da) to 0.6. Set Number of smooths to 2. Select **Savitsky Golay.** Set Min peak width at half height (channels) to 4.

Select **Centroid top (%).** Set to 50. Press **OK** from the **Mass Measure** window.

Press **OK** from the **Make new calibration** window.

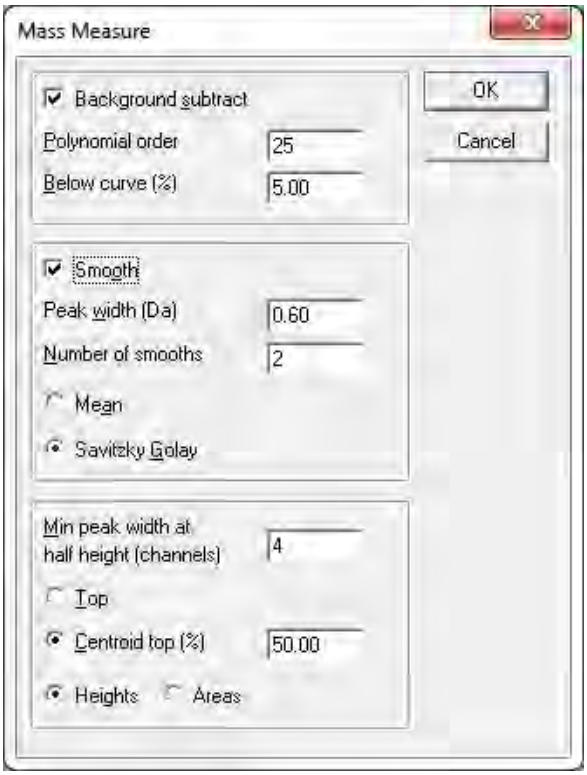

Figure 2.2.2. Typical parameters for automatically calibrating the mass scale of the globin chain *m/z* spectra using the α-chain.

This should calibrate the spectrum in a few seconds and the **Calibrate** report (Figure 2.2.3) should appear to show how well the experimental data fit the reference data.

From the **Calibrate** report, select **Edit** to show the **Calibration Parameters** (Figure 2.2.4).

Typically, these should be as follows:

Select **Perform auto peak matching**. Set Peak window to 0.4. Set Initial error to 0.5. Set Intensity threshold to 0.2. Set Polynomial order to 2. Turn off Intensity weighting.

On some instruments, it may be necessary to increase Initial error. This depends upon how well the mass scale of the mass spectrometer was adjusted before calibrating the mass scale.

Press **OK** to accept the **Calibration Parameters**.

Press **OK** (from the **Calibrate** report) to accept the calibration provided it is satisfactory as indicated by the deviations from the calibration line headed **Residuals** in the **Calibrate** window.

Typically, the deviations from the line should be <±0.01 amu (*sic*, actually *m/z*). The data file is now internally calibrated and will remain with this calibration until calibrated again. The *m/z* spectrum may be recalibrated at any time, and new MaxEnt processed data produced from the recalibrated raw data file. Any earlier MaxEnt processed data will not be affected by recalibrating the raw data.

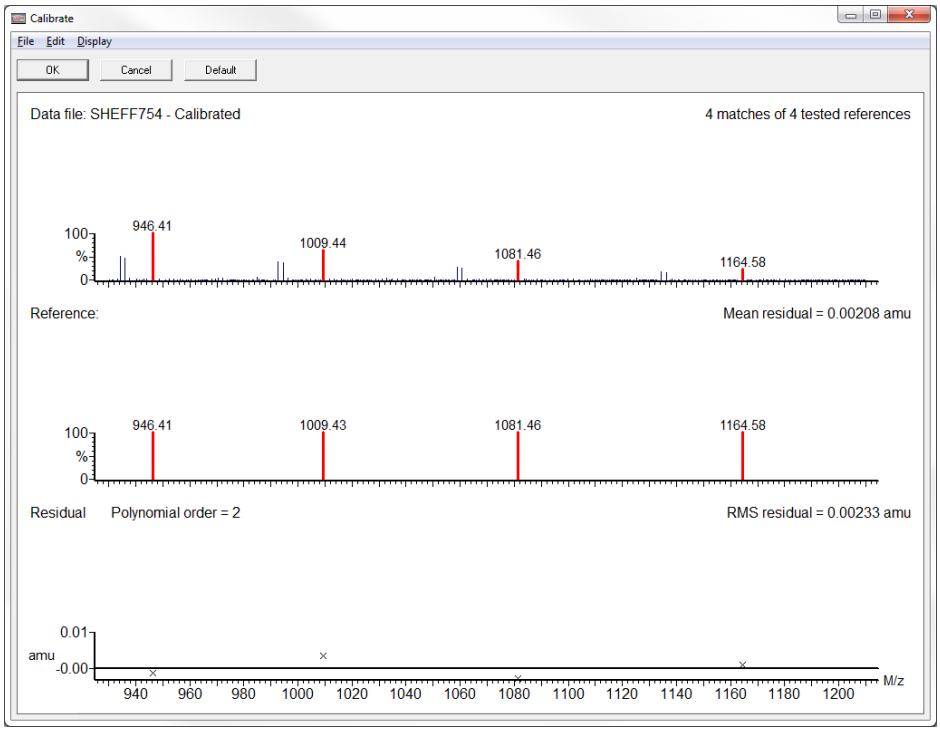

Figure 2.2.3. Typical calibration report from a 500-fold diluted and desalted blood sample using the α-chain for internal calibration.

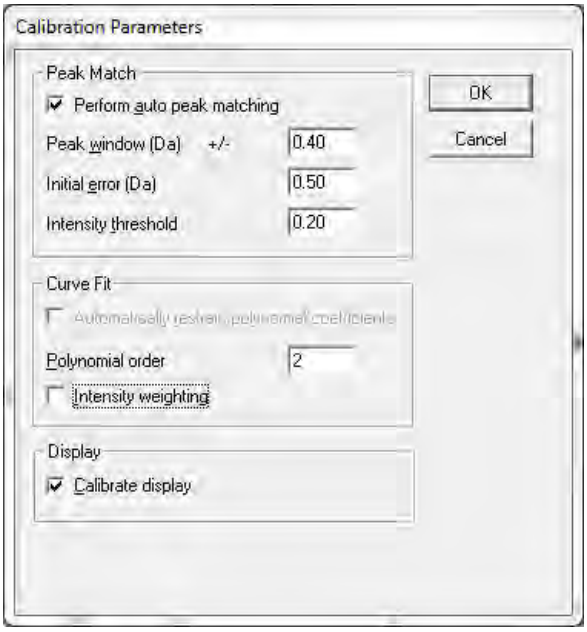

Figure 2.2.4. The parameters for automatically calibrating the mass scale of globin chain *m/z* spectra.

Once the above procedure has been undertaken, subsequent spectra may be automatically calibrated from the raw spectrum by displaying the spectrum, selecting **Tools**, **Make New Calibration**, and pressing **OK** in the **Make New Calibration** window. Finally, press **OK** in the **Calibrate** window to accept the calibration. As stated above, the calibration procedure always calibrates the whole spectrum irrespective of the *m/z* range shown on the display.

In some cases of heterozygotes where an α-chain variant is incompletely resolved from the normal α-chain, it is better to calibrate using the β-chain (Hbb.ref). These cases occur when the mass difference between normal and variant α-chains (ΔM) lies between 9 and ~15 Da. Examples include Le Lamentin (ΔM = -9 Da) and G-Philadelphia (ΔM = +14 Da). This does not apply to cases where the normal and variant α-chains are completely unresolved, i.e. are not apparently present in the *m/z* spectrum. In these cases, the only evidence for suspecting the presence of a variant would be from the ce-HPLC trace. The mass difference would probably lie between 1 and 4 Da.

2.4. Deconvoluting the raw data

The maximum entropy (MaxEnt) software is by far the best method available for condensing the original multiply charged *m/z* data so that each component in the original mixture is presented as a single peak on a true mass scale. MaxEnt automatically processes the multiply charged data and improves the resolution, so that two Hb chains separated by only 6 Da can just be resolved in favourable cases. MaxEnt is also quantitative in so far as the area under a peak in the MaxEnt profile spectrum is a measure of the sum of the intensities of the

multiply charged species from which that peak was derived.

2.4.1. Deconvoluting the raw data using MaxEnt 1

To deconvolute a spectrum by MaxEnt, first **Background Subtract** the raw data (from **Process**, **Subtract**) with polynomial order set to 25, Below curve (%) set to 5 and Tolerance set to 0.01% (Figure 2.2.5). Then display *m/z* 980-1180 (from **Display**, **Range**) and display 2 decimal places (from **Display**, **Peak Annotation**). MaxEnt processes only that part of the spectrum shown on the display. Always background subtract the raw data before processing by MaxEnt and never smooth it.

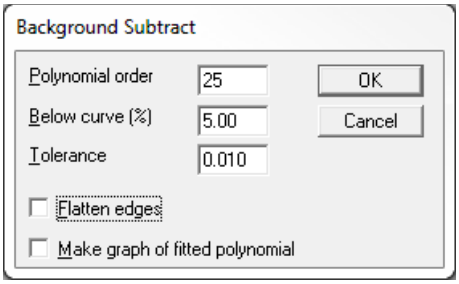

Figure 2.2.5. Typical parameters for background subtracting mass spectra

From **Process**, select **MaxEnt 1**. For routine processing of Hb data, in the **MaxEnt** window (Figure 2.2.6), set Output Mass Range to 14,800:16,800 Da and Output Resolution to 0.2 Da/channel.

Select **Simulated Isotope Pattern** and initially set Spectrometer Blur Width to 0.4 Da (*sic*, actually *m/z*).

Set the Minimum intensity ratios, Left and Right, to 40% and select **Iterate to convergence**. Press **OK** to start MaxEnt processing and allow it to converge. This should take about 15 seconds on a modern computer (2014). Press **OK**.

Select the MaxEnt profile spectrum and **Smooth** it using Peak width (Da) set to 4, number of smooths set to 2. Select **Savitsky Golay**. Press **OK**. Then, **Centre** the smoothed profile spectrum with Create centred spectrum turned off, in order to accurately determine the masses of the components. Set Minimum peak width at half height (channels) to 1 and Centroid top (%) to 90. Press **OK**.

Figure 2.2.6. Typical parameters for MaxEnt processing the background-subtracted raw *m/z* data from 500-fold diluted blood samples. The Spectrometer Blur Width may need adjusting (0.3-0.5) to make the α-chain mass within ±0.1 Da of its sequence mass.

If necessary, adjust the Spectrometer Blur Width and reprocess the original background subtracted data by **MaxEnt 1** in order to make the mass of the α-chain fall within ±0.10 Da of its sequence mass (15,126.38 Da). Increasing the Spectrometer Blur Width increases the mass and *vice versa*.

Finally, produce a bar spectrum in which the intensity of each component is a measure of the sum of the intensities of that component in the multiply charged spectrum by repeating **Centre** with the Create centred spectrum box turned on, select **Areas** and **Add**. Display mass to 2 decimal places. Print the profile and bar spectra on a single page as shown in Figure 2.5. Select the bar spectrum (Figure 2.5b), and produce a mass/intensity list from **Display**, **List Spectrum**. Print the mass/intensity list with Data Threshold set to ~0.5% Full Scale (from **Display**, **View**) as shown in Figure 2.6.

2.4.2. Two special cases requiring atypical processing by MaxEnt.

These are: observation of (a) the Constant Springs and (b) Carbonic anhydrase 1 (CA1, sequence mass 28,781.08 Da) and dimers. Parameters that are different to those given for routine processing are as follows:

The Constant Springs. Display the *m/z* range 955-1210. In the **MaxEnt** window (Figure 2.2.6), set Output Mass Range to 14800:19000 Da and Output Resolution to 0.2 Da/channel.

Carbonic anhydrase (CA1) and dimers. In the **MaxEnt** window (Figure 2.2.6), set Output Mass Range to 14800:32500 Da and Output Resolution to 0.5 Da/channel.

2.4.3. Correcting the mass scale in MaxEnt processed spectra.

Despite using the α-chain for calibration, the measured masses may require correction in order to obtain full accuracy and precision. Although repeated reprocessing of a given data set by MaxEnt can be undertaken to make the α-chain mass equal its sequence mass by adjusting the peak width parameter, a series of measurements made during routine variant analysis indicated a simpler expedient that was quicker to apply in practice. It was found that the mass scale of the MaxEnt output spectrum could be simply adjusted on a linear basis to make the α-chain mass equal its sequence mass, with a worthwhile improvement in the accuracy and precision of the β-chain mass. For example, if the mass of the α-chain is high by 0.05 Da, then 0.05 Da is subtracted from all the components of interest in the MaxEnt output spectrum. The sequence masses of the normal α- and β-chains are 15,126.38 and 15,867.24 Da respectively. The example shown in Figure 2.5 shows how a correction of -0.03 Da applied to the normal β-chain reduced the error from 0.04 to 0.01 Da.

Using this method of correcting the mass and provided 32 channels per *m/z* unit are used to acquire the data, the precision in measuring the mass of the β-chain should be better than ±0.05 Da SD. This precision is particularly useful for detecting variants in heterozygotes, in which the mass of the variant differs from normal by <6 Da. An ability to determine the mass of the β-chain to a precision of better than ±0.05 Da means that ±1 Da α- or β-chain variants in heterozygotes can be detected provided they are present at >10% of total α- or β-chains[(1)] . Moreover, such variants can be assigned to either the α- or β-chain by combining the apparent change in the mass of the β-chain with the polarity change of the variant from normal inferred from ce-HPLC data. See Section 2.4.4.

Figure 2.6 shows how the proportion of the variant was calculated from the %BPI (% Base Peak Intensity) values as 100 x $\alpha^X/(\alpha^X + \alpha^A)$ = 18.6%. This can be related to ce-HPLC data by calculating 100 x $X/(X + A_0)$, where X and A_0 are the percentages of the variant and A_0 respectively. In this example, 100 x $X/(X + A_0)$ was 19.5%.

2.4.4. Assigning variants that give <±6 Da mass change from normal to either the α- or the β-chain with the aid of ce-HPLC data[(†)]

2.4.4.1. Introduction.

A basic limitation of electrospray ionization mass spectrometry (ESI-MS) when used to analyse the globin chains in human haemoglobin (Hb) is that chains differing by <±6 Da from one another are not observed as separate entities. There are about 77 α- and 93 β-chain variants that can occur by single base changes in the nucleotide codon and differ from normal by <±6 Da. When these variants occur in heterozygotes, the mass determined by ESI-MS is the abundance weighted mean of the variant and normal chain masses. Several common β-chain variants, e.g. C, D-Punjab, E and O-Arab, are in this category, since their masses differ from normal by -1 Da. In heterozygotes containing 50 or 25% of these variants, the apparent mass of the β-chain determined by ESI-MS will be lower than normal by 0.50 or 0.25 Da, respectively.

The procedure for establishing the masses of the intact chains by ESI-MS uses one of the major chains for internal calibration of the mass scale, usually the α-chain. In normal Hb, the mass of the β-chain determined in this way is generally within ±0.05 Da SD (±3.2 ppm) of its sequence mass. This degree of accuracy can only be achieved by using one of the major chains for internal calibration. Using the α-chain for calibration, if the measured mass of the β-chain differs from normal by more than say ±0.10 Da, i.e. ±2 SD, the presence of a variant that differs from normal by <±6 Da is indicated. However, since the mass scale has been internally calibrated using the α-chain, the α-chain mass is forced to be correct and the β-chain shows the mass change from normal irrespective of whether the variant is in the α- or the β-chain.

2.4.4.2. Procedure

In order to establish which chain is associated with the variant, the mass change of the variant from normal determined by ESI-MS is combined with the charge change of the variant from normal implied by ce-HPLC data. Variants that travel faster than Hb A_0, i.e. elute well before Hb A_0, are deemed to gain negative charge, e.g. J (-1), I (-2). Variants that travel significantly slower than Hb A_0, i.e. at Hb A_2 and after, are deemed to gain positive charge, e.g. D, S (+1), C, E (+2). Variants that elute very close to Hb A_0, often as shoulders on the side of Hb A_0, are assumed to involve no charge change. Although these rules give a perhaps oversimplified view of charge, they are, nevertheless, useful, with very few exceptions.

Mutations that give zero mass change cannot be detected by ESI-MS analysis of the globin chains, and rely on being detected by ce-HPLC in the first instance. They are readily identified directly from tryptic digests, because they either produce two 'new' peptides (Gln→Lys) or combine two adjacent peptides into one larger peptide (Lys→Gln).

Table 2.1 shows the charge and mass changes produced by all the mutations that give <±6 Da mass-change together with some examples. The amino acid residues Asn, Gln, Ile, Leu, Met, Pro and Thr are assumed to have zero charge, Asp and Glu one negative charge and Lys one positive charge. Hence, a mutation from Glu to Lys (C- or E-like) involves a charge change from -1 to +1, i.e. a net increase of two positive charges. A mutation of Lys to Gln involves a change of +1 to 0, i.e. a net increase of one negative charge.

Table 2.2 shows the four ways in which <±6 Da variants in heterozygotes can be assigned to either the α- or the β-chain by combining the apparent mass change of the β-chain with the charge change implied by the ce-HPLC data. For these rules to apply, the mass scale must be calibrated using the α-chain (Section 2.3.4.). If the β-chain shows a negative (or positive) mass change from normal and the ce-HPLC data indicate that the variant causes respectively an increase in positive (or negative) charge change, then the variant is in the β-chain. However, if the ce-HPLC data indicate a negative (or positive) charge change, still with respectively a negative (or positive) apparent mass change of the β-chain, then the variant is in the α-chain[(1)]. For mutations that give ±1 Da mass change from normal, the proportion of the variant can be estimated approximately as (100x measured mass change from normal)%.

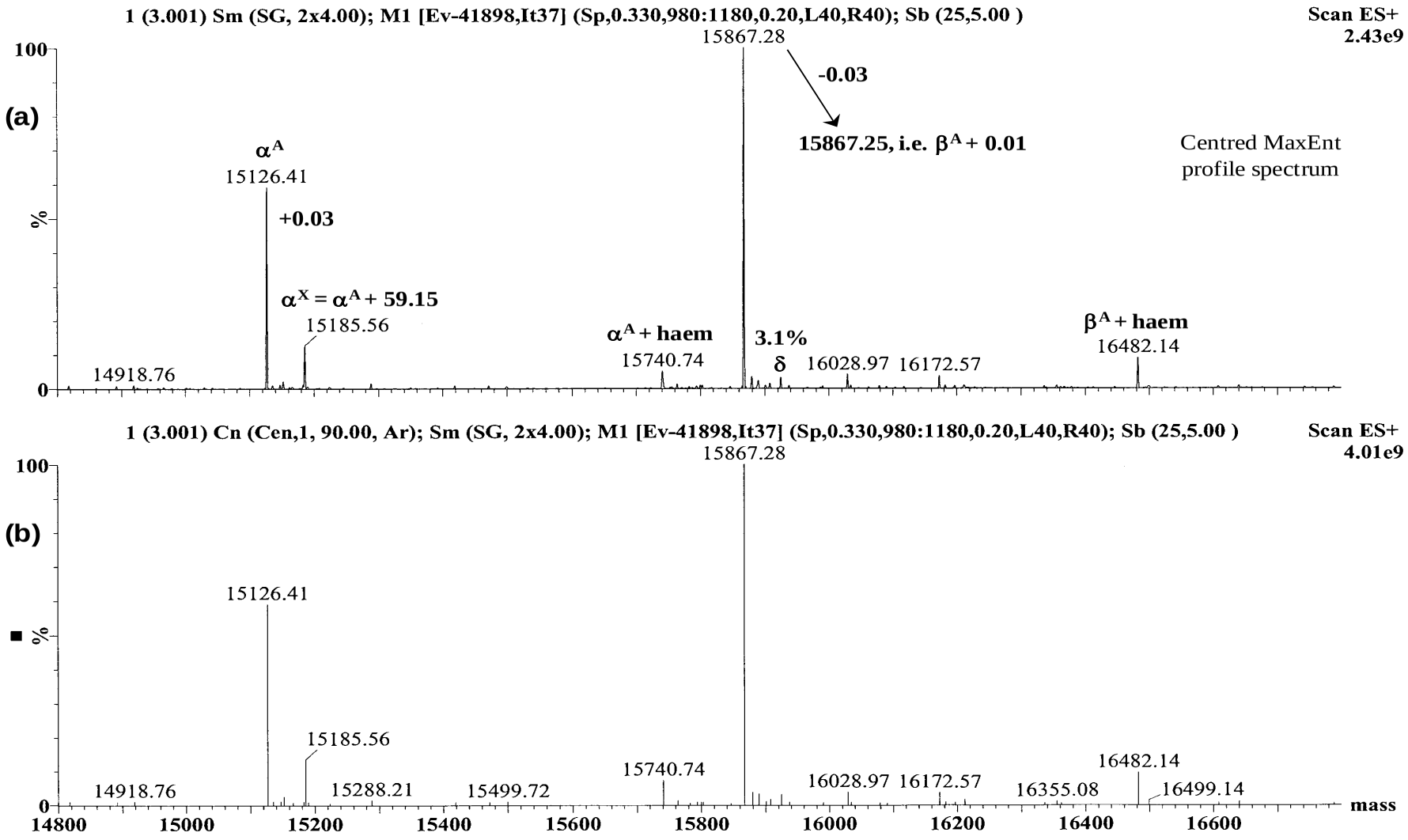

Figure 2.5. MaxEnt processed spectra from a heterozygote for the variant Hb Chiapas, α114Pro→Arg, showing how the β-chain mass is corrected by subtracting the error in the α-chain (0.03 Da). The processing steps are shown in the headers reading from right to left. Masses are experimental.

1 (3.001) Cn (Cen,1, 90.00, Ar); Sm (SG, 2x4.00); M1 [Ev-41898,It37] (Sp,0.330,980:1180,0.20,L40,R40); Sb (25,5.00) Scan ES+

No	Mass	Inten	%BPI	%TIC		No	Mass	Inten	%BPI	%TIC	No	Mass	Inten	%BPI	%TIC	No	Mass	Inten	%BPI	%TIC	No
1:	14817.25	4.15e7	1.04	0.39																	
2:	14891.75	3.38e7	0.84	0.31																	
3:	14918.76	4.32e7	1.08	0.40																	
4:	15126.41	2.37e9	59.10	22.01	α^A																
5:	15135.25	4.60e7	1.15	0.43																	
6:	15146.97	4.53e7	1.13	0.42																	
7:	15151.85	1.02e8	2.54	0.95																	
8:	15166.14	3.28e7	0.82	0.30																	
9:	15182.69	4.35e7	1.08	0.40																	
10:	15185.56	5.43e8	13.54	5.04	α^X																
11:	15190.00	3.40e7	0.85	0.32																	
12:	15223.66	2.15e7	0.54	0.20																	
13:	15288.21	6.11e7	1.52	0.57																	
14:	15418.67	3.55e7	0.88	0.33																	
15:	15471.65	3.42e7	0.85	0.32																	
16:	15499.72	4.14e7	1.03	0.38																	
17:	15740.74	2.96e8	7.39	2.75																	
18:	15763.54	5.22e7	1.30	0.49																	
19:	15782.36	2.23e7	0.56	0.21																	
20:	15793.84	3.70e7	0.92	0.34																	
21:	15799.67	3.68e7	0.92	0.34																	
22:	15802.71	3.73e7	0.93	0.35																	
23:	15845.85	2.14e7	0.53	0.20																	
24:	15867.28	4.01e9	100.00	37.24	β^A																
25:	15879.47	1.54e8	3.83	1.43																	
26:	15889.21	1.36e8	3.38	1.26																	
27:	15900.67	4.72e7	1.18	0.44																	
28:	15907.75	6.93e7	1.73	0.64																	
29:	15924.56	1.28e8	3.20	1.19	δ																
30:	15937.34	3.74e7	0.93	0.35																	
31:	15990.03	2.70e7	0.67	0.25																	
32:	16028.97	1.55e8	3.87	1.44																	
33:	16034.24	3.68e7	0.92	0.34																	
34:	16078.79	2.73e7	0.68	0.25																	
35:	16090.07	2.14e7	0.53	0.20																	
36:	16172.57	1.48e8	3.69	1.37																	
37:	16181.82	3.56e7	0.89	0.33																	
38:	16196.89	3.05e7	0.76	0.28																	
39:	16212.01	6.76e7	1.69	0.63																	
40:	16335.79	2.44e7	0.61	0.23																	
41:	16355.08	4.62e7	1.15	0.43																	
42:	16361.75	2.03e7	0.51	0.19																	
43:	16482.14	3.88e8	9.68	3.61																	
44:	16499.14	5.43e7	1.35	0.50																	
45:	16608.12	3.29e7	0.82	0.31																	
46:	16639.91	3.96e7	0.99	0.37																	
47:	16788.10	2.05e7	0.51	0.19																	

$$100 \times \alpha^X/(\alpha^A + \alpha^X) = 18.6\%$$

$$100 \times \delta/(\beta^A + \delta) = 3.1\%$$

Figure 2.6. Mass and relative intensity list from the spectrum shown in Fig. 2.5b. The proportions of α^X and δ are calculated from the values in the %BPI (%Base Peak Intensity) column.

Table 2.1. Charge and mass changes due to mutations giving <±6 Da mass change.

Mutation	Charge change	True mass change (Da)	Some examples	
			α-chain	β-chain
Glu→Lys	+2	-1	O-Indonesia	C, E, O-Arab,
			Shuangfeng	G-Siriraj, Agenogi
Gln→Lys	+1	0		Alabama
Asp→Asn		-1	Dunn, Titusville	Korle-Bu, Yaizu
			G-Pest, G-Norfolk	Osu Christiansborg
Glu→Gln		-1	Memphis, Oleander	D-Iran, D-Punjab
Met→Lys		-3		Matera
Leu→Ile	0	0	(Cannot be determined by MS)	
Asn→Ile		-1		Schlierbach
Thr→Pro		-4		Valletta
Pro→Thr		4		Linkoping
Lys→Gln	-1	0	J-Wenchang-Wuming	K-Woolwich
Gln→Glu		1	Mexico	Camden
Asn→Asp		1		Alamo, Yoshizuka
Lys→Met		3		Helsinki, Barbizon
Lys→Glu	-2	1	Sudbury	N-Baltimore
Hybrid	(+1)	-2	Lepore-Boston-Washington (with HbA_2)	

Table 2.2. Assignment of the variant to either the α- or the β-chain assuming the mass scale is internally calibrated using the α-chain.

Apparent β–chain mass change[a]	Charge change[b]	Assignment of variant	Assignment of ±1 Da variants
Negative	Positive	β-chain	β-chain - 1 Da
Positive	Negative	β-chain	β-chain + 1 Da
Negative	Negative	α-chain	α-chain + 1 Da
Positive	Positive	α-chain	α-chain - 1 Da

[a] Sign of the apparent mass change of the β-chain from normal.
[b] Polarity change from normal inferred from ce-HPLC data.

2.4.5. Determination of minor components from the spectrum of the globin chains

There are several minor components that can be quantified from the MaxEnt deconvoluted spectrum of the globin chains. They are detailed below.

2.4.5.1. δ-chain (sequence mass 15,924.32 Da)

This is the non-α-chain present in Hb A_2 and normally occurs at about 3% of the β-chain abundance. It is significantly elevated in many cases of β-thalassaemia trait and is particularly useful for distinguishing between a homozygous variant or a variant plus β-thalassaemia when the variant occurs at the same ce-HPLC retention time as Hb A_2, e.g. D-Iran. It appears to be reliable with heterozygotes in which the variant has a similar abundance to the normal β-chain, e.g. ~40% in Hb D-Punjab and Hb S, but not when the variant occurs at low abundance, e.g. ~20%

in Hb E. The level of the δ-chain is usually slightly higher than Hb A_2, because it is defined as δ/(β + δ + total γ), whereas Hb A_2 is determined as a proportion of total Hb. When estimating the abundance of the δ-chain, it is imperative that the diluted blood sample has been carefully desalted before analysis, otherwise the δ-chain level will be erroneously elevated by interference from alkali metal adducts of the normal β-chain (Figure 2.1).

Figure 2.7 shows the centred MaxEnt spectra from (a) a normal sample (δ = 2.9%, Hb A_2 = 2.8% and (b) a sample from a patient with β-thalassaemia trait (δ = 5.8%, Hb A_2 = 6.1%).

The way in which the δ-chain levels are calculated from the %BPI (% base peak intensity) values are illustrated in Figures 2.8 and 2.9. The δ-chain values correlate well with the Hb A_2 values.

Figure 2.10 compares the δ-chain levels in (a) heterozygous D-Punjab (3.5%), (b) homozygous D-Punjab (3.7%) and (c) D-Punjab/β-thalassaemia (5.8%).

Table 2.3 summarises the δ-chain levels observed in 152 D-Punjab samples analysed over several years. The calculated mass of $\beta^{D\text{-}Punjab}$ is 15,866.25 Da.

Table 2.3. The δ-chain levels and mean masses observed for various D-Punjab samples.

	n	δ% ±SD	Mean Mass ±SD (Da)
Heterozygous D-Punjab	116	3.49 ±0.34	15,866.828 ±0.036 (±2.2ppm)
Homozygous D-Punjab	19	3.64 ±0.30	15,866.283 ±0.043 SD (Error = 0.027, 1.7ppm)
D-Punjab/β-thalassaemia	17	6.12 ±0.81	15,866.269 ±0.037 (Error = 0.013, 0.82ppm)

It can be seen from the Table 2.3 that homozygous D-Punjab should be distinguishable from D-Punjab/β-thalassaemia with 98% confidence (±2 SD) by the level of the δ-chain. Actually, the lowest δ-chain level was 5.2% in the D-Punjab/β-thalassaemia samples, which is clearly well above the highest level in the homozygous D-Punjab samples (4.2%).

2.4.5.2. Glycated Hb chains and Hb A_{1c}

Glycation (addition of glucose) occurs on both the α- and β-chains to give covalently bound adducts at calculated masses of 15,288.52 and 16,029.38 Da, respectively, e.g. Figure 2.7. The glycation level (%) on each chain is calculated from the centred MaxEnt spectrum BPI values as:

$$\alpha\text{-chain glycation} = 100\alpha_g/(\alpha_g + \alpha)\%$$

and

$$\beta\text{-chain glycation} = 100\beta_g/(\beta_g + \beta)\%$$

in which α and α_g represent the intensities of the normal and glycated α-chain, and β and β_g represent the intensities of the normal and glycated β-chain (Figure 2.7.).

Either or both of these glycation values may be expressed in terms of HbA_{1c} by calibration with standards. For example, plots of α- and β-glycation against HbA_{1c} for data from a DCCT aligned ce-HPLC instrument gave the following linear regression equations[3]:

α-chain glycation (%) = 0.678 HbA_{1c} -1.479, r = 0.990
β-chain glycation (%) = 1.096 HbA_{1c} -1.694, r = 0.993

Note. These equations are given as an illustration only and may not apply in general.

2.4.5.3. Carbonic anhydrase (CA1, sequence mass 28,781.08 Da)

Although CA1 is believed to have little or no diagnostic significance, it may be measured by processing the background subtracted raw data (*m/z* 980-1,180) with the MaxEnt Output Mass Range and Resolution set to 14800-32500 Da and 0.5 Da per channel respectively. The MaxEnt profile spectrum is then smoothed (2 x 4 Da, SG) and centred (1, top 90%, areas) in the usual way. CA1/(CA1 + α) is ~5% in normal samples. These MaxEnt processing parameters may also be used to detect the presence of dimers of the major chains.

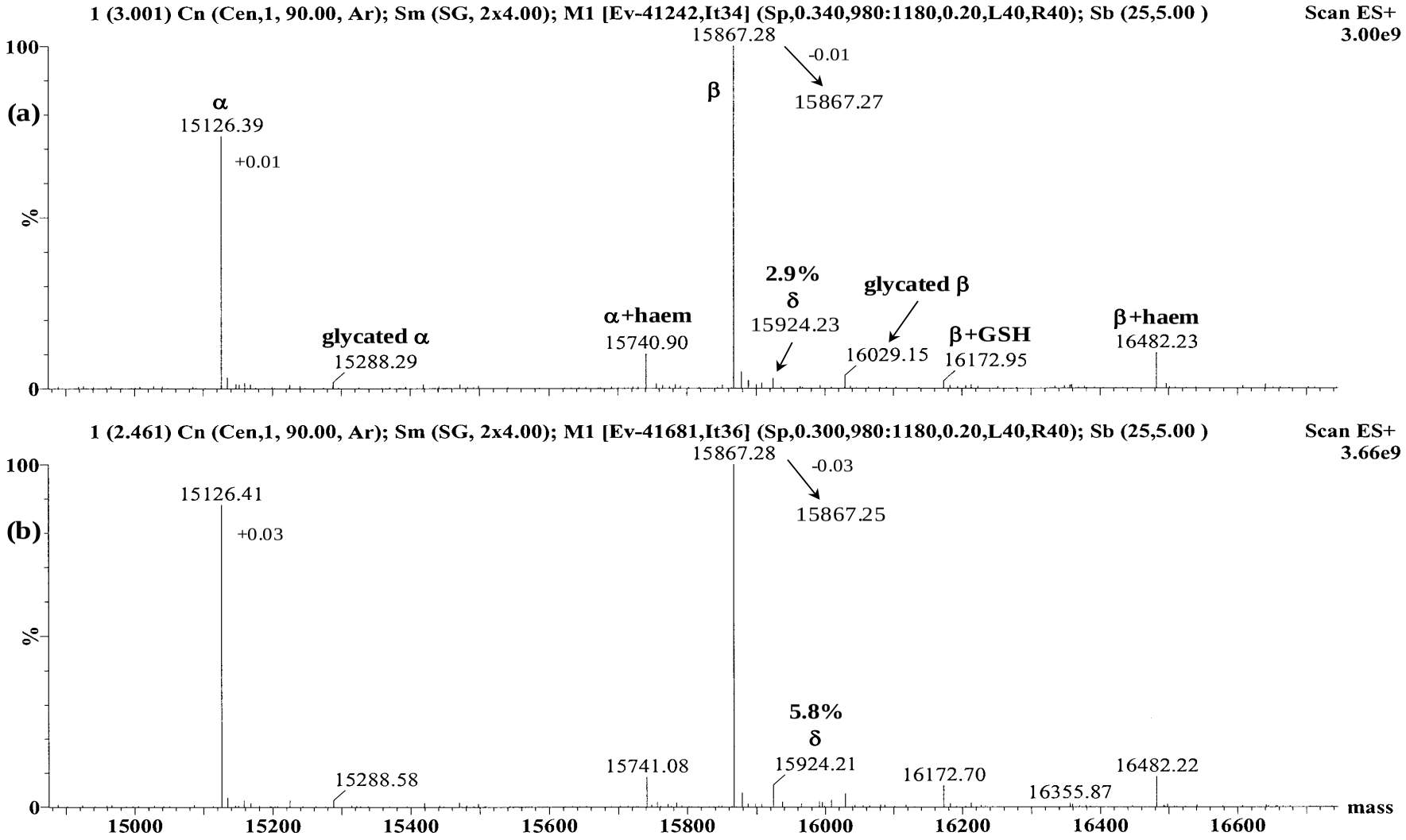

Figure 2.7. Centred MaxEnt spectra from the blood of (a) a normal patient and (b) a patient with β-thalassaemia trait showing the minor components normally present. Note the higher δ-chain level in (b). The glutathione (GSH) adduct tends to increase with sample age.

1 (3.001) Cn (Cen,1, 90.00, Ar); Sm (SG, 2x4.00); M1 [Ev-41242,It34] (Sp,0.340,980:1180,0.20,L40,R40); Sb (25,5.00) Scan ES+

No	Mass	Inten	%BPI	%TIC	No	Mass	Inten	%BPI	%TIC	No	Mass	Inten	%BPI	%TIC	No	Mass	Inten	%BPI	%TIC	No
1:	14918.13	1.61e7	0.54	0.20																
2:	14925.48	2.12e7	0.71	0.26																
3:	14966.05	1.80e7	0.60	0.22																
4:	15028.47	1.90e7	0.63	0.24																
5:	15126.39	2.21e9	73.69	27.41																
6:	15134.82	9.06e7	3.02	1.12																
7:	15147.33	3.88e7	1.29	0.48																
8:	15152.08	3.34e7	1.11	0.41																
9:	15160.06	4.77e7	1.59	0.59																
10:	15167.88	3.44e7	1.15	0.43																
11:	15224.98	3.03e7	1.01	0.38																
12:	15240.11	1.93e7	0.64	0.24																
13:	15288.29	4.91e7	1.64	0.61																
14:	15418.71	3.24e7	1.08	0.40																
15:	15441.36	1.54e7	0.51	0.19																
16:	15471.77	3.36e7	1.12	0.42																
17:	15498.88	2.02e7	0.68	0.25																
18:	15740.90	3.03e8	10.10	3.76																
19:	15755.81	4.06e7	1.36	0.50																
20:	15764.69	2.68e7	0.89	0.33																
21:	15775.08	1.54e7	0.51	0.19																
22:	15783.22	3.20e7	1.07	0.40																
23:	15790.71	2.08e7	0.69	0.26																
24:	15850.87	2.99e7	1.00	0.37																
25:	15867.28	3.00e9	100.00	37.20	β															
26:	15878.58	1.42e8	4.74	1.76																
27:	15888.78	6.98e7	2.33	0.87																
28:	15900.12	3.34e7	1.12	0.41																
29:	15907.86	4.47e7	1.49	0.56																
30:	15924.23	8.82e7	2.94	1.09	δ															
31:	15992.68	2.28e7	0.76	0.28																
32:	16029.15	1.05e8	3.51	1.31																
33:	16172.95	5.90e7	1.97	0.73																
34:	16182.53	2.19e7	0.73	0.27																
35:	16205.06	2.06e7	0.69	0.26																
36:	16212.87	2.93e7	0.98	0.36																
37:	16348.10	2.06e7	0.69	0.26																
38:	16356.19	2.58e7	0.86	0.32																
39:	16358.33	2.90e7	0.97	0.36																
40:	16377.41	1.60e7	0.53	0.20																
41:	16482.23	3.08e8	10.28	3.83																
42:	16496.64	3.48e7	1.16	0.43																
43:	16607.78	1.82e7	0.61	0.23																
44:	16640.29	2.41e7	0.80	0.30																

$100 \times \delta/(\delta + \beta) = 2.9\%$

← No γ-chains

Figure 2.8. Mass/intensity list from the MaxEnt spectrum shown in Figure 2.7a illustrating how the δ-chain level is calculated from the %BPI (%Base Peak Intensity) values.

1 (2.461) Cn (Cen,1, 90.00, Ar); Sm (SG, 2x4.00); M1 [Ev-41681,It36] (Sp,0.300,980:1180,0.20,L40,R40); Sb (25,5.00) Scan ES+

No	Mass	Inten	%BPI	%TIC	No	Mass	Inten	%BPI	%TIC	No	Mass	Inten	%BPI	%TIC	No	Mass	Inten	%BPI	%TIC	No
1:	14888.08	2.49e7	0.68	0.23																
2:	14966.23	1.87e7	0.51	0.18																
3:	15086.45	1.97e7	0.54	0.19																
4:	15126.41	3.23e9	88.17	30.45																
5:	15134.88	1.04e8	2.84	0.98																
6:	15158.31	7.00e7	1.91	0.66																
7:	15167.83	4.08e7	1.11	0.38																
8:	15224.66	7.16e7	1.95	0.67																
9:	15288.58	7.05e7	1.92	0.66																
10:	15419.54	4.16e7	1.14	0.39																
11:	15470.63	4.35e7	1.19	0.41																
12:	15497.79	3.19e7	0.87	0.30																
13:	15741.08	3.23e8	8.80	3.04																
14:	15748.01	2.05e7	0.56	0.19																
15:	15756.39	5.57e7	1.52	0.52																
16:	15771.79	2.96e7	0.81	0.28																
17:	15784.48	4.35e7	1.19	0.41																
18:	15867.28	3.66e9	100.00	34.53	β															
19:	15878.88	1.52e8	4.14	1.43																
20:	15887.46	3.27e7	0.89	0.31																
21:	15898.11	3.24e7	0.88	0.31																
22:	15907.40	2.89e7	0.79	0.27																
23:	15924.21	2.34e8	6.40	2.21	δ															
24:	15937.00	5.53e7	1.51	0.52																
25:	15964.56	3.47e7	0.95	0.33																
26:	15991.04	6.45e7	1.76	0.61																
27:	15995.38	5.05e7	1.38	0.48	$^{G}\gamma$															
28:	16008.73	7.57e7	2.07	0.71	$^{A}\gamma$	$^{G}\gamma + {}^{A}\gamma = 3.45$														
29:	16029.11	1.45e8	3.96	1.37																
30:	16043.30	1.95e7	0.53	0.18																
31:	16080.72	2.43e7	0.66	0.23																
32:	16086.87	2.16e7	0.59	0.20																
33:	16117.63	2.09e7	0.57	0.20																
34:	16172.70	2.29e8	6.25	2.16																
35:	16182.24	3.55e7	0.97	0.33																
36:	16212.51	4.20e7	1.15	0.40																
37:	16227.16	1.80e7	0.49	0.17																
38:	16355.87	3.80e7	1.04	0.36																
39:	16359.48	2.57e7	0.70	0.24																
40:	16482.22	3.27e8	8.91	3.08																
41:	16497.68	2.80e7	0.77	0.26																
42:	16540.58	2.13e7	0.58	0.20																
43:	16641.15	1.99e7	0.54	0.19																

Total non-α = β + δ + $^{G}\gamma$ + $^{A}\gamma$ = 109.85

β/total non-α = 0.9103
δ/total non-α = 0.0583 or 5.8%
Total γ/total non-α = 0.0314 or 3.1%
1.0000

Figure 2.9. Mass/intensity list from the MaxEnt spectrum shown in Figure 2.7b illustrating how the δ- and γ-chain levels are calculated from the values in the %BPI (%Base Peak Intensity) column.

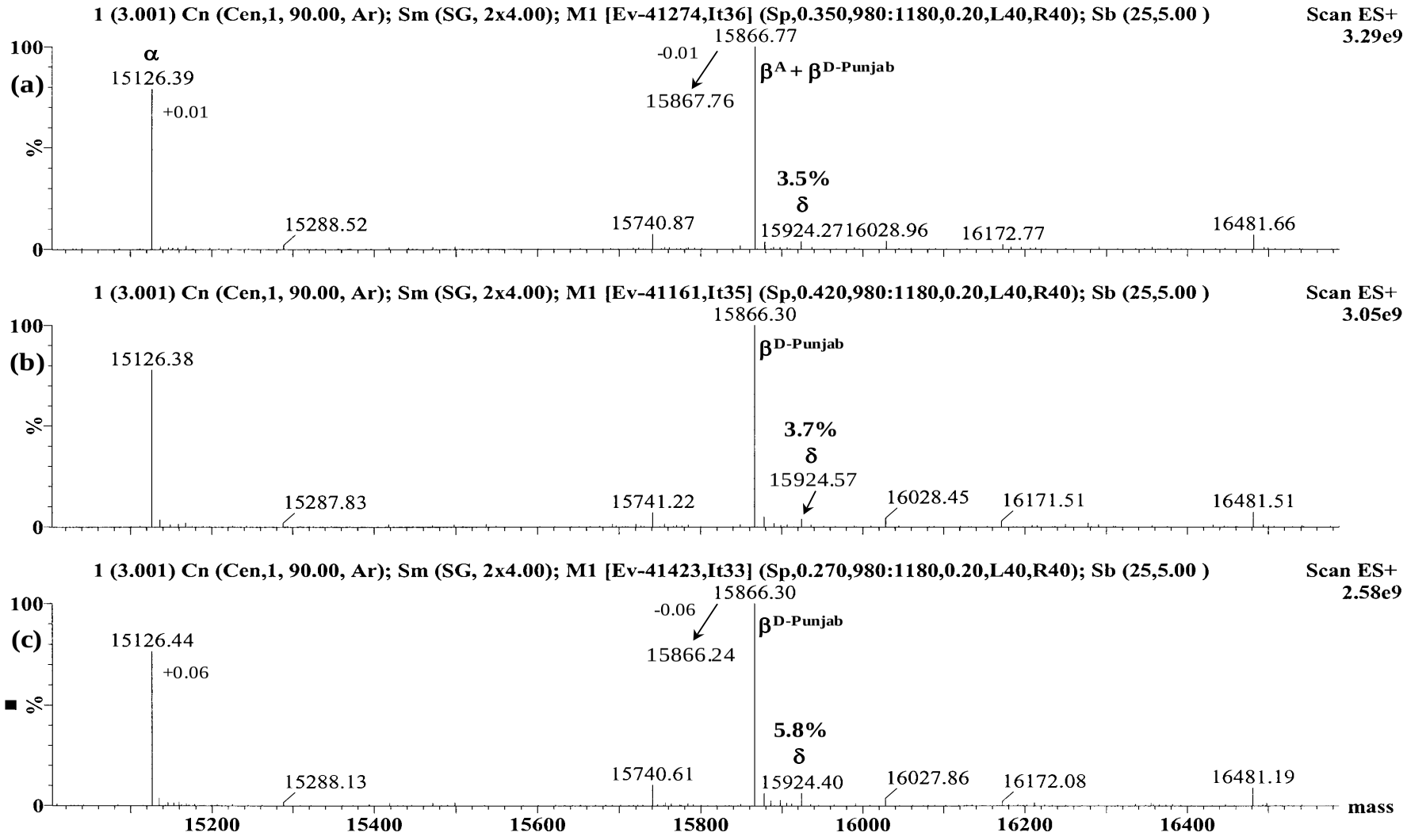

Figure 2.10. Centred MaxEnt spectra from the blood of (a) a D-Punjab heterozygote, (b) a D-Punjab homozygote and (c) a heterozygote for D-Punjab/β-thalassaemia. Note the significant increase in the δ-chain level in (c). Masses are experimental.

2.4.6. Some variants that can be identified from the spectra of their globin chains

Several variants produce MaxEnt processed spectra of the globin chains that are characteristic of the mutation, which allows them to be identified without the aid of ce-HPLC data. Their molecular weights (masses) are probably unique (except Lepore-Hollandia). Some of these mutations occur close to the N-terminus of the α- or β-chain causing retention of the initiator methionine. Others extend the C-terminus or are hybrids, and are summarised in Table 2.4.

Table 2.4. Some variants that can be identified from the mass spectra of the globin chains.

Name	Mutation	Variant[(a)] (%)	Mass (Da)	ΔM[(b)] (Da)
St Jozef	α1Val→Leu			
	α1Val→ (-1)Met-(+1)Leu- - -	18	15,271.61	145.23
J-Biskra	α51-58 or α52-59→0	21	14,361.54	-764.84
Wayne[(c)]	α139-141→α139NTVKLEPR	12	~15,617.5	~491
Natal	α140Tyr-Arg→0	24	14,807.02	-319.36
South Florida	β1Val→Met- - -	9	15,899.31	32.07
	β1Val→ (-1)Met-(1)Met- - -	32	16,030.50	163.26
	β1Val→N-Ac(-1)Met-(+1)Met- - -	9	16,072.54	205.30
Marseille	β2His→Pro			
	β2His→ (-1)Met-(+1)Val-(+2)Pro-	55	15,958.41	91.17
Lepore- Hollandia[(d)]	Hybrid, δ22 - - - β50	10-15	15,836.23	-31.01
	Check that variant elutes in Hb A_2 window to eliminate Gln→Pro.			
Lepore-Baltimore[(d)]	Hybrid, δ50 - - - β86	10-20	15,822.20	-45.04
P-Nilotic	Hybrid, β22 - - - δ50	~20	15,955.33	88.09
	The variant elutes with Hb A_0 by ce-HPLC (Bio-Rad).			
Kenya[(d)]	^{A}γ81 - - - β86	10-20	15,922.23	54.99
HPFH-7	^{G}γ only, no ^{A}γ	7-12	15,994.27	127.03
Tak	β146Stop→147TKLAFLLSNFY	42	17,165.79	1298.55
	The variant elutes in the S-window by ce-HPLC (Bio-Rad).			

Notes

[(a)]. Percentage of the variant in heterozygotes.

[(b)]. Mass difference of the variant chain relative to the normal chain.

[(c)]. Approximately 50% of the variant is de-amidated at α139Asn. Hence the measured mass is ~0.5 Da higher than the sequence mass (15,616.94 Da).

[(d)]. The variant elutes in the Hb A_2 window by ce-HPLC (Bio-Rad).

2.5 References

[(1)] D.K. Rai, W.J. Griffiths, B. Landin, B.J. Wild, G. Alvelius and B.N. Green. Accurate mass measurement by electrospray ionization quadrupole mass spectrometry: detection of variants differing by <6 Da from normal in human hemoglobin heterozygotes. Anal. Chem. **75**, 1978-82 (2003).

[(2)] R.H. Bateman, B.N. Green and M. Morris. Electrospray ionization mass spectrometric analysis of the globin chains in hemoglobin heterozygotes can detect the variants Hb C, D, and E. Clin. Chem. **54**, 1256-7 (2008).

[(3)] J.P. Williams, H. Jackson and B.N. Green. Hb Belleville [β10(A7)Ala→Thr] affects the determination of Hb A_{1c} by routine cation exchange high performance liquid chromatography. Hemoglobin **33**, 45-50 (2009).

2.4.7. The average molecular masses of the normal human haemoglobin chains and some of their derivatives

Table 2.5. The average molecular masses of some of the haemoglobin chains.

	Mass (Da)
α-chain (major component of Hb excl. embryonic)	15,126.38
Carbamoylated α-chain (+CHNO, 43.025)	15,169.41 (in uraemia)
S-Carboxyamidomethylated α-chain (+Cam, 57.05)	15,183.43
Glycated α-chain (+glucose, 162.142)	15,288.52
α-chain + haem – H (non-covalent adduct)	15,741.87
β-chain (major component of adult Hb, Hb A)	15,867.24
Carbamoylated β-chain (+CHNO, 43.025)	15,910.27 (in uraemia)
Pyruvylated β-chain, Hb A_{1b} (+$C_3H_2O_2$, 70.048)	15,937.29 (in old samples)
S-Carboxyamidomethylated β-chain (+2 Cam)	15,981.34
Cysteinylated β-chain (+Cys via -S-S- bond)	15,986.38 (in old samples)
Glycated β-chain (+glucose, 162.142)	16,029.38
β-chain +(Cys-Gly) via -S-S- bond	16,043.44 (in old samples)
Glutathionylated β-chain (+305.31 via -S-S- bond)	16,172.55 (in old samples)
β-chain + haem – H (non-covalent adduct)	16,482.73
δ-chain, in Hb A_2 (~3% in adult Hb)	15,924.32
δ-chain + haem – H (non-covalent adduct)	16,539.81
^{G}γ-chain (major component of foetal Hb, Hb F)	15,995.27
Acetylated ^{G}γ-chain (~10-17% in foetal Hb)	16,037.31
Glutathionylated ^{G}γ-chain	16,300.59 (in old samples)
^{G}γ-chain + haem – H (non-covalent adduct)	16,610.77
^{A}γ^{T}-chain (sometimes in foetal Hb, Hb F)	15,997.25
Acetylated ^{A}γ^{T}-chain (~10-17% in foetal Hb)	16,039.28
Glutathionylated ^{A}γ^{T}-chain	16,302.56 (in old samples)
^{A}γ^{T}-chain + haem - H (non-covalent adduct)	16,612.74
^{A}γ-chain (major component of foetal Hb, Hb F)	16,009.30
Acetylated ^{A}γ-chain (~10-17% in foetal Hb)	16,051.34
Glutathionylated ^{A}γ-chain	16,314.61 (in old samples)
^{A}γ-chain + haem - H (non-covalent adduct)	16,624.79
ζ-chain (zeta-chain, in embryonic Hb)	(15,505.85)
Acetylated ζ-chain (100% in embryonic Hb)	15,547.89
ζ-chain + haem – H (non-covalent adduct)	16,163.38
ε-chain (epsilon-chain, in embryonic Hb)	16,071.66
ε-chain + haem – H (non-covalent adduct)	16,687.15

Based on: C = 12.011 Da, H = 1.00794 Da, N = 14.00674 Da, O = 15.9994 Da, S = 32.066 Da. The average and monoisotopic masses of the haem are 616.50 Da and 616.1773 Da, respectively.

SECTION 3

3.0 Step 3: The detection and identification of the hybrid haemoglobins by electrospray ionization mass spectrometry

3.1. Summary.

In this section it is shown how electrospray ionization mass spectrometry (ESI-MS) can be used to detect and identify the hybrid (fusion) haemoglobins. Some of them can be identified directly from their mass spectra (Lepore-Baltimore, Kenya and P-Nilotic), or from their mass spectra together with ce-HPLC data (Lepore-Hollandia). If necessary, identification may be confirmed by tandem mass spectrometry of the intact hybrid chains, and tandem mass spectra of Lepore-Hollandia, Lepore-Baltimore, Kenya and P-Nilotic are shown. In addition, tandem mass spectra from a Lepore-Boston-Washington (LBW) heterozygote (90% β-chain, 10% LBW chain) are shown. In this case, the LBW-chain and the normal β-chain only differ in mass by 2 Da, and are therefore unresolved in the mass spectra. Tandem mass spectra of the normal β-, δ- and $^{G}\gamma$-chains are also given. The molecular weights and principle product ions from most of the hybrid chains are listed in Table 3.1.

3.2. The hybrid (fusion) haemoglobins.

Four types of hybrid haemoglobins have been described[(1)]. They have the same number of amino acids as the β-chain (146) except for Lincoln Park, which has 145. The Lepores have an N-terminal section of the δ-chain fused to a C-terminal section of the β-chain. The anti-Lepores have an N-terminal section of the β-chain fused to a C-terminal section of the δ-chain. Kenya (also known as HPFH-7; Kenya) has an N-terminal section of the $^{A}\gamma$-chain fused to a C-terminal section of the β-chain. Hb Parchman has a section of the β-chain sandwiched between N- and C-terminal sections of the δ-chain. Their structures, sequence molecular weights (masses) and principal product ions are summarised in Table 3.1. The normal β-, δ- and $^{G}\gamma$-chains are also included in this table.

3.3. Tandem mass spectrometry of the β-, δ-, γ- and hybrid-chains.

Except for the γ-chains, the non-α-chains cleave mainly at the N-terminal side of 36Pro, 51Pro and 124Pro, giving rise to distinctive y''_{111}, y''_{96} and y''_{23} fragments, respectively. Prominent b_{32}-b_{35} and b_{42}-b_{47} fragments associated with cleavage at 36Pro and 51Pro are also produced. The γ-chains differ from the other chains in that 51Pro is replaced by 51Ala, which results in y''_{98} becoming dominant instead of y''_{96}. Nevertheless, they still produce y''_{111}, y''_{23}, b_{32}-b_{35} and b_{42}-b_{47} fragments. Each chain has a characteristic product ion spectrum, which, together with its molecular weight, allows the chain to be positively identified.

3.3.1. Sample preparation

Samples are prepared by simply diluting whole blood 500-fold in 50% aqueous acetonitrile containing 0.2% formic acid as described in Section 2.2.3. Introduce the sample solution as described in Section 2.3.1. The desalting step may be omitted when undertaking tandem MS.

3.3.2. Acquisition of tandem mass spectra from the Tune Page.

On the Tune Page, display the precursor ion with 16 charges. The calculated *m/z* values of the precursor ions are given at the foot of Table 3.1. On triple quadrupole instruments, increase the intensity of the precursor ion by reducing the resolution (HM-Res 1) and increasing the ion energy (Ion Energy 1) until a point is reached beyond which the intensity stops increasing. From **Acquire**, select **Daughter Scan**, select **MCA**. Enter the *m/z* value at the top of the precursor ion as observed on the tune page into **Set Mass** (this may be slightly different to its calculated value). Acquire data over the *m/z* range 500-1,500 with a collision energy of 26V using argon as the collision gas at 2.5 mbar pressure. Ensure the Entrance and Exit apertures are set to a low value, e.g. 2V. If this is an option, set the number of channels per *m/z* unit to 16 as follows. From the **Tune Page**, select **Options**, and from **Set Instrument Threshold**, set, in profile data, Baseline level to 1 and Points per Dalton (*sic m/z* unit) to 16. Acceptable data can usually be obtained by acquiring for 5 minutes.

Reference

[(1)] R.C. Hardison, D.H.K. Chui, B. Giardine, C. Riemer, G.P. Patrinos, N. Aganou, *et al*. HbVar: A relational database of human hemoglobin variants and thalassemia mutations at the globin gene server. Hum.Mutat., **19**, 225-33 (2002) \\globin.bx.psu.edu

Table 3.1. The principal product ions of some normal and hybrid globin chains

Name	β	δ	^{G}γ	Lepore-Hollandia	Lepore-Baltimore	Lepore-Boston-W	Kenya	Miyada	P-Nilotic	Lincoln Park	P-India	P-Congo	Parchman
	β1–146	δ1–146	^{G}γ1–146	δ22–β50	δ50–β86	δ87–β116	^{A}γ80-β87	β12–δ22	β22–δ50	β22–δ50	β87–δ116	β22–δ116	δ-β-δ
Mr	15,867.24	15,924.32	15,995.27	15,836.23	15,822.20	15,865.23	15,922.23	15,897.29	15,955.33	15,856.20	15,953.36	15,926.33	15,980.38
% in het				15% ESI	11% ESI	7-13% Lit	~15%inc δ	17% Lit	24% ESI	14% Lit	23% Lit	NR	1.6% Lit
y_{23}^{4+}	609.2	625.0	634.2	609.2	609.2	609.2	609.2	625.0	625.0	y22 600.2	625.0	625.0	625.0
y_{23}^{3+}	811.9	833.0	845.3	811.9	811.9	811.9	811.9	833.0	833.0	y22 799.9	833.0	833.0	833.0
y_{96}^{11+}	940.6	949.8	949.7	940.6	940.6	944.5	943.1	949.8	949.8	y95 940.8	948.4	945.9	948.4
y_{97}^{11+}	949.7	957.8	957.7	949.7	948.5	952.4	951.0	957.8	957.8	y96 948.7	957.6	955.1	957.6
y_{98}^{11+}	957.7	965.7	965.6	957.7	956.4	960.3	958.9	965.7	965.7	y97 956.7	965.5	963.0	965.5
y_{93}^{10+}		1,016.4sm	1,021.7				1,014.4						
y_{94}^{10+}		1,023.5sm	1,028.8				1,021.5						
y_{95}^{10+}		1,035.0sm	1,037.5				1,030.2						
y_{96}^{10+}	1,034.5	1,044.7	1,044.6	1,034.5	1,034.5	1,038.8	1,037.3	1,044.7	1,044.7	y_{95} 1,034.8	1,043.1	1,040.4	1,043.1
y_{97}^{10+}	1,044.6	1,053.4	1,053.3	1,044.6	1,043.2	1,047.5	1,046.0	1,053.4	1,053.4	y_{96} 1,043.5	1,053.2	1,050.5	1,053.2
y_{98}^{10+}	1,053.3	1,062.1	1,062.0	1,053.3	1,051.9	1,056.2	1,054.7	1,062.1	1,062.1	y_{97} 1,052.2	1,061.9	1,059.2	1,061.9
y_{96}^{9+}	1,149.3	1,160.7	1,160.6	1,149.3	1,149.3	1,154.1	1,152.5	1,160.7	1,160.7	y_{95} 1,149.7	1,158.9	1,155.9	1,158.9
y_{97}^{9+}	1,160.6	1,170.4	1,170.3	1,160.6	1,159.0	1,163.8	1,162.1	1,170.4	1,170.4	y_{96} 1,159.3	1,170.1	1,167.1	1,170.1
y_{98}^{9+}	1,170.2	1,180.0	1,179.9	1,170.2	1,168.7	1,173.5	1,171.8	1,180.0	1,180.0	y_{97} 1,169.0	1,179.8	1,176.9	1,179.8
y_{111}^{11+}	1,104.2	1,112.2	1,110.7	1,104.2	1,102.9	1,106.8	1,104.1	1,112.2	1,112.2	y_{110} 1,103.2	1,112.0	1,109.6	1,112.0
b_{42}^{4+}	1,174.9	1,167.1	1,188.8	1,167.1	1,167.1	1,167.1	1,188.8	1,160.3	1,174.9	1,174.9	1,174.9		
b_{43}^{4+}	1,207.1	1,199.4	1,217.6	1,199.4	1,199.4	1,199.4	1,217.6	1,192.6	1,207.1	1,207.1	1,207.1	1,207.1	1,319.9
b_{44}^{4+}	1,228.9	1,221.2	1,239.4	1,221.2	1,221.2	1,221.2	1,239.4	1,214.4	1,228.9	1,228.9	1,228.9	1,228.9	1,235.7
b_{45}^{4+}	1,265.7	1,257.9	1,276.2	1,257.9	1,257.9	1,257.9	1,276.2	1,251.2	1,265.7	1,265.7	1,265.7	1,265.7	1,272.5
b_{46}^{4+}	1,280.0	1,272.2	1,290.4	1,272.2	1,272.2	1,272.2	1,290.4	1,265.5	1,280.0	1,280.0	1,280.0	1,280.0	1,286.7
b_{47}^{4+}	1,308.7	1,301.0	1,319.0	1,301.0	1,301.0	1,301.0	1,319.0	1,294.2	1,308.7	1,308.7	1,308.7	1,308.7	1,315.5
b_{32}^{3+}	1,124.6	1,114.3	1,143.3	1,114.3	1,114.3	1,114.3	1,143.3	1,105.3	1,124.6	1,124.6	1,124.6		
b_{33}^{3+}	1,157.7	1,147.3	1,176.3	1,147.3	1,147.3	1,147.3	1176.3	1,138.3	1,157.7	1,157.7	1,157.7	1,157.7	1,166.7
b_{34}^{3+}	1,190.7	1,180.4	1,209.4	1,180.4	1,180.4	1,180.4	1209.4	1,171.4	1,190.7	1,190.7	1,190.7	1,190.7	1,199.7
b_{35}^{3+}	1,245.1	1,234.8	1,263.8	1,234.8	1,234.8	1,234.8	1263.8	1,225.8	1,245.1	1,245.1	1,245.1	1,245.1	1,254.1
Precursor	992.7	996.3	1,000.7	990.8	989.9	992.6	996.1	994.6	998.2	992.0	998.1	996.4	999.8

Notes: het: heterozygote, NR: not reported, Lit: Literature, Mr of ^{A}γ is 16,009.32 Da. Precursor has 16 charges. Lincoln Park has same sequence as P-Nilotic except 137Val deleted.

3.3.3. Mass scale calibration.

External mass scale calibration of sample spectra may be undertaken by acquiring a tandem mass spectrum from a normal β-chain obtained from a separate introduction of an appropriate blood sample as follows:

From **Tools**, select **Make calibration**, and in the **Make new calibration** window select MSMSBeta16.ref from **Reference file**.

Select **Background subtract.** Set Polynomial order to 25, Below curve (%) to 5. Tolerance to 0.010.
Select **Smooth.** Set Peak width (Da) to 1.5. Set number of smooths to 2.
Select **Savitzky Golay**. Set Min peak width at half height (channels) to 4.
Select **Centroid top (%)**. Set to 50.
Select **Heights** with the **Create centred spectrum** box turned on.
Press **OK** from the **Make new calibration** window.

This should calibrate the product ion spectrum and a **Calibrate** report should appear to show how well the experimental data fit the reference data. Press **OK** to accept the calibration.

The resulting calibration may then be applied to other tandem mass spectra acquired under the same conditions as the calibration spectrum. These spectra may be acquired either before or after acquiring the calibration spectrum.

Display the spectrum requiring calibration.

From **Tools**, select **Apply Calibration**. This should show the parameters of the current calibration.

Press **OK** to calibrate the spectrum on display.

The calibrated sample spectra are then background subtracted and smoothed using the same parameters as those used to make the calibration. Finally, the spectra are centred with the Create centred box turned off in order to optimise the mass accuracy.

3.4 Examples

All the experimental data were acquired on a Micromass Quattro Ultima. On the following pages are shown the tandem mass spectra from the β-, δ-, and $^{G}\gamma$-chains. Also shown are MaxEnt deconvoluted spectra from Kenya and P-Nilotic heterozygotes. Tandem mass spectra from the latter two Hbs are also given and confirmed their identity.

3.4.1. Hereditary Persistence of Foetal Haemoglobin (HPFH).

Part of the *m/z* spectrum obtained from the analysis of a 500-fold diluted and desalted HPFH sample is shown in Figure 3.1a. The MaxEnt deconvoluted spectrum from these data is shown in Figure 3.1b. It can be seen that the masses of the major non-α components are within experimental error of the sequence masses of the β-, δ-, $^{G}\gamma$- and $^{A}\gamma$-chains (Table 3.1). The putative β^{16+}, δ^{16+} and $^{G}\gamma^{16+}$ ions, at *m/z* 992.7, 996.3 and 1,000.7 respectively, were subjected to tandem MS. The resulting product ion spectra are shown in Figures 3.2. and 3.3. (a) from β^{16+}, (b) from δ^{16+} and (c) from $^{G}\gamma^{16+}$ precursor ions. It can be seen that the three chains produce quite distinct tandem mass spectra and that the product ions listed in Table 3.1, when present, occur within experimental error of their predicted *m/z* values. These results confirm that the sample is from a heterozygote for hereditary persistence of foetal haemoglobin.

3.4.2. Hb Kenya ($^{A}\gamma$80 - - β87), also known as HPFH-7; Kenya.

The literature[1] describes Kenya as a hybrid of $^{A}\gamma$ through 80 with β from 87. Like the β-chain, Kenya has 146 amino acid residues, and occurs together with $^{G}\gamma$ ($^{G}\gamma$/total γ = 96%, i.e. negligible $^{A}\gamma$). In heterozygotes, the fractions of Hbs A, Kenya, F and A_2 are given as 71.9, 14.9, 11.1 and 2.1% respectively (n=7). ESI-MS measurements made on ten Kenya heterozygotes gave mean values for the β^{A}-, (Kenya + δ)- and $^{G}\gamma$-chains of 73.8±4.2, 16.1±4.7 and 10.1±2.0% SD respectively. The Kenya- and δ-chains are too close together (2 Da) to be measured separately. Although the mean proportions agree fairly well with the literature, there was a wide spread of values between individual samples for (Kenya + δ), which varied from 8.6 to 21.7%. The ce-HPLC trace from an Hb Kenya heterozygote is shown in Figure 3.4.

Figure 3.5a shows the MaxEnt deconvoluted spectrum from 500-fold diluted and desalted blood of an Hb Kenya heterozygote. Present are two non-α components whose masses, 15,922.14 Da and 15,995.26 Da, are consistent with the sequence masses of Kenya- and $^{G}\gamma$-chains respectively (Table 3.1). The presence of

both these components strongly suggests Kenya, which was confirmed by tandem MS of the variant ion with 16 charges (*m/z* 996.1). The upper and lower parts of the resulting product ion spectrum are shown in Figures 3.6b and 3.7b respectively. The *m/z* values of the major ions in this spectrum are within experimental error of the theoretical values given in Table 3.1, thus confirming Kenya. For comparison, the corresponding product ion spectra from the normal β-chain are shown in Figures 3.6a. and 3.7a.

3.4.3. Hb P-Nilotic (β22 - - δ50).

This is described in the literature[1] as a hybrid of β through 22 with δ from 50 and is present at 16-21% of total Hb in heterozygotes. It appears to be silent by ce-HPLC. Its sequence mass (15,955.33 Da) is 88.09 Da higher than β^A, a mass change that cannot occur by a single base change in the β-chain nucleotide codon. Thus, the presence of a chain with a mass within experimental error of 15,955.33 Da would strongly suggest P-Nilotic. Figure 3.5b shows the MaxEnt deconvoluted spectrum from a heterozygote (variant abundance 24.3% of total non-α), in which the variant has a mass of 15,955.27 Da, close to the sequence mass of P-Nilotic. This was confirmed by tandem MS of the variant ion with 16 charges (*m/z* 998.2). The upper and lower parts of the resulting product ion spectrum are shown in Figures 3.6c and 3.7c respectively. The *m/z* values of the major product ions are consistent with the calculated values shown in Table 3.1, thus confirming the variant as P-Nilotic.

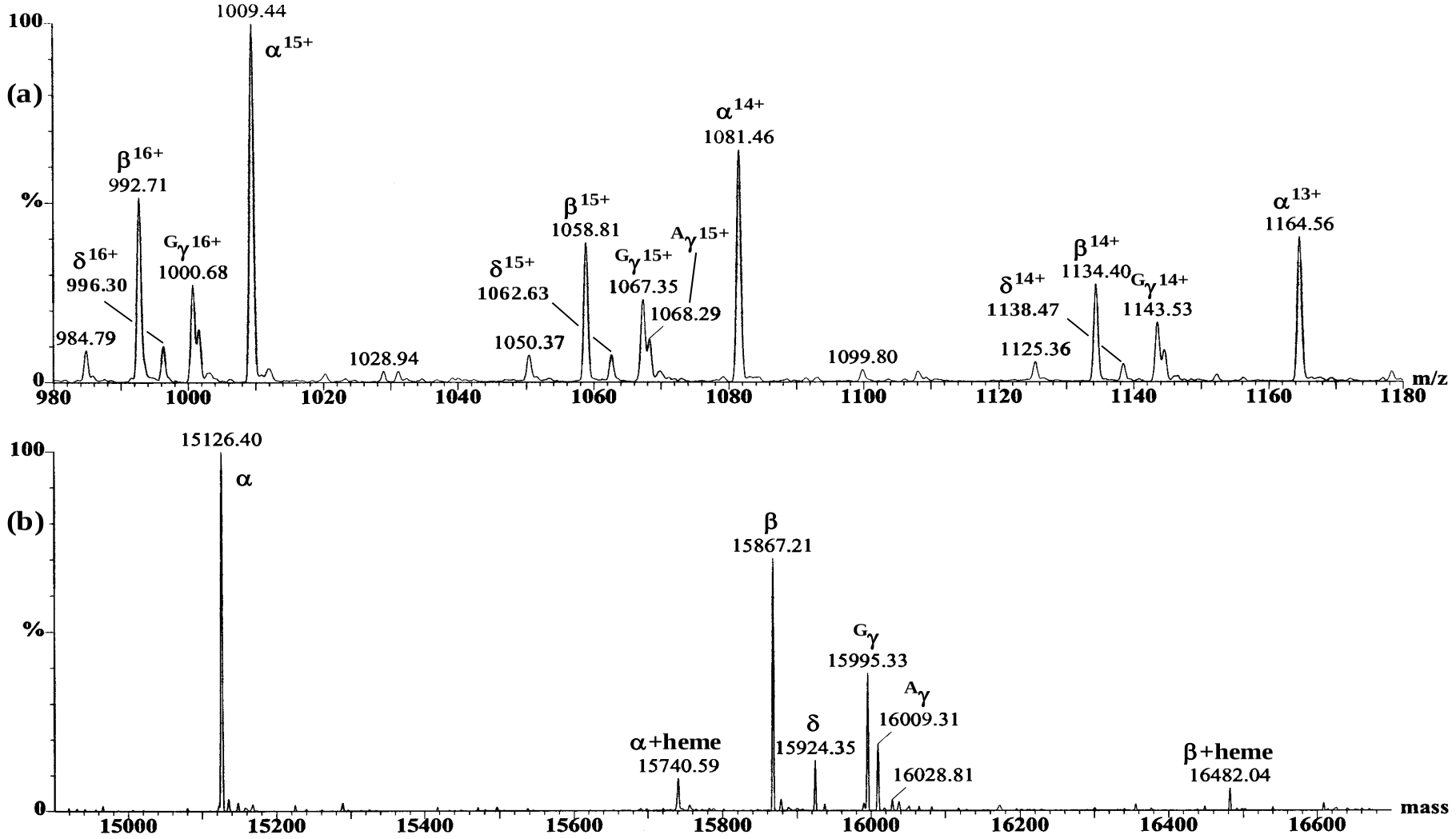

Figure 3.1. Spectra from 500-fold diluted and desalted blood of an adult with HPFH: (a) part of the original *m/z* spectrum and (b) the data in (a) after deconvolution by MaxEnt. Masses and *m/z* values are experimental.

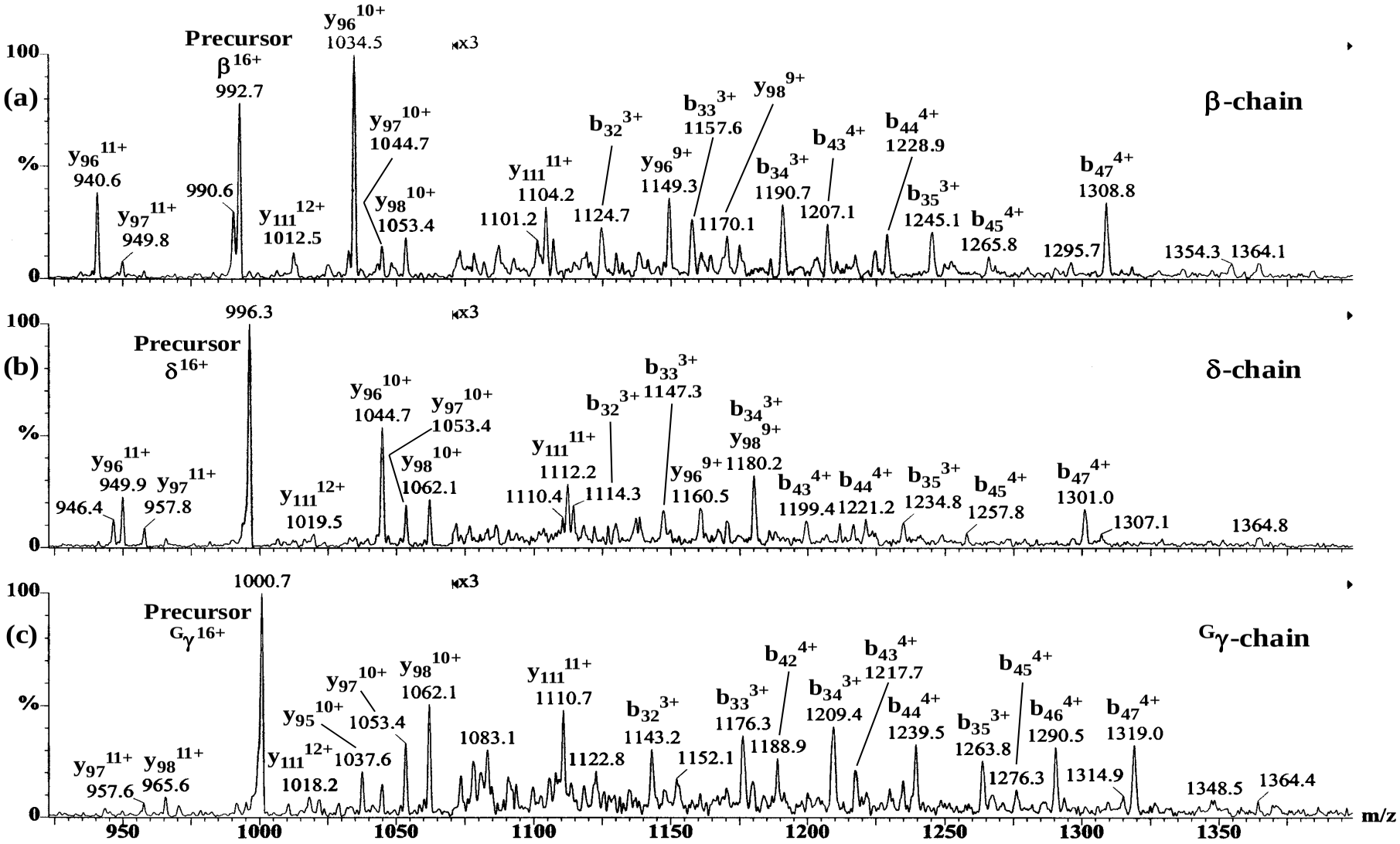

Figure 3.2. Part tandem mass spectra of (a) β^{16+}, (b) δ^{16+} and (c) $^{G}\gamma^{16+}$ precursor ions showing the principal product ions above the precursor. The *m/z* values are experimental. Collision gas was argon at 2.5×10^{-3} mbar pressure. Collision energy was 26V.

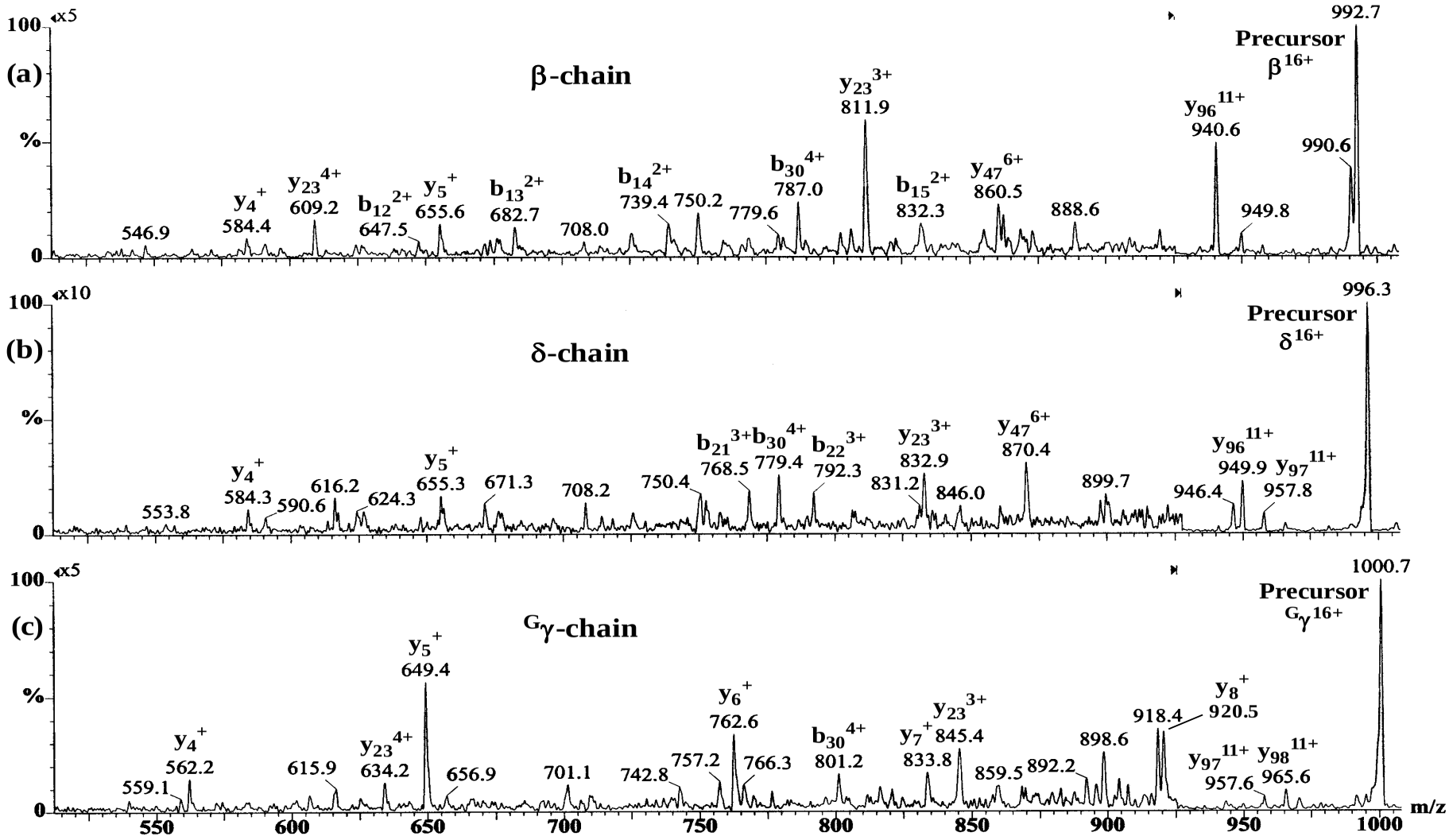

Figure 3.3. Part tandem mass spectra of (a) β^{16+}, (b) δ^{16+} and (c) $^{G}\gamma^{16+}$ precursor ions showing product ions below the precursor. The *m/z* values are experimental.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.1	0.92	1303
F	8.4*	---	1.16	164295
P2	---	4.0	1.33	79324
P3	---	4.1	1.73	80757
Ao	---	66.6	2.52	1306150
A2	16.6*	---	3.55	330136

Total Area: 1961964

F Concentration = 8.4* %
A2 Concentration = 16.6* %

*Values outside of expected ranges

Analysis comments:

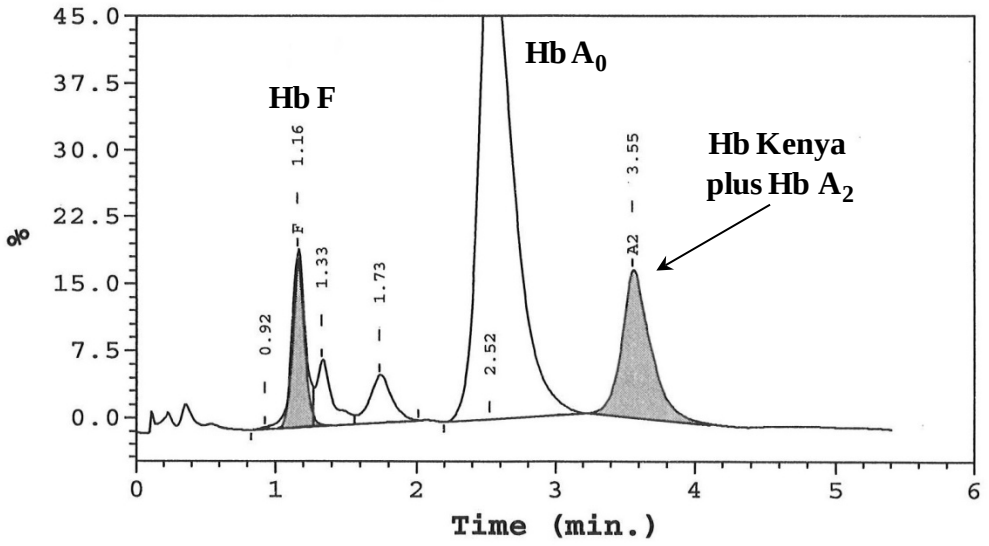

Figure 3.4. Bio-Rad ce-HPLC trace from a Hb Kenya heterozygote.

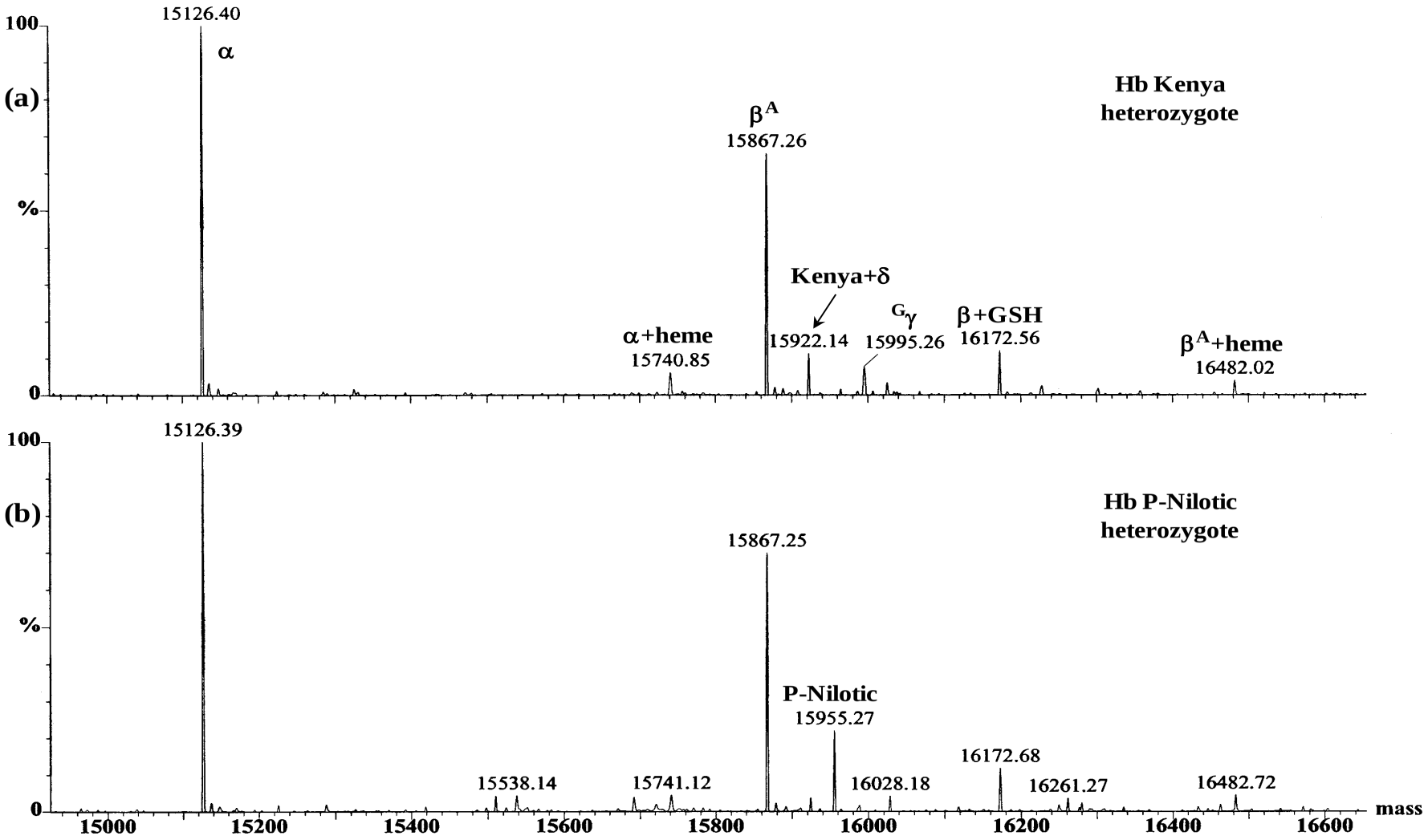

Figure 3.5. Spectra from 500-fold diluted and desalted blood of (a) a Hb Kenya heterozygote and (b) a Hb P-Nilotic heterozygote. Masses are experimental. GSH: glutathione.

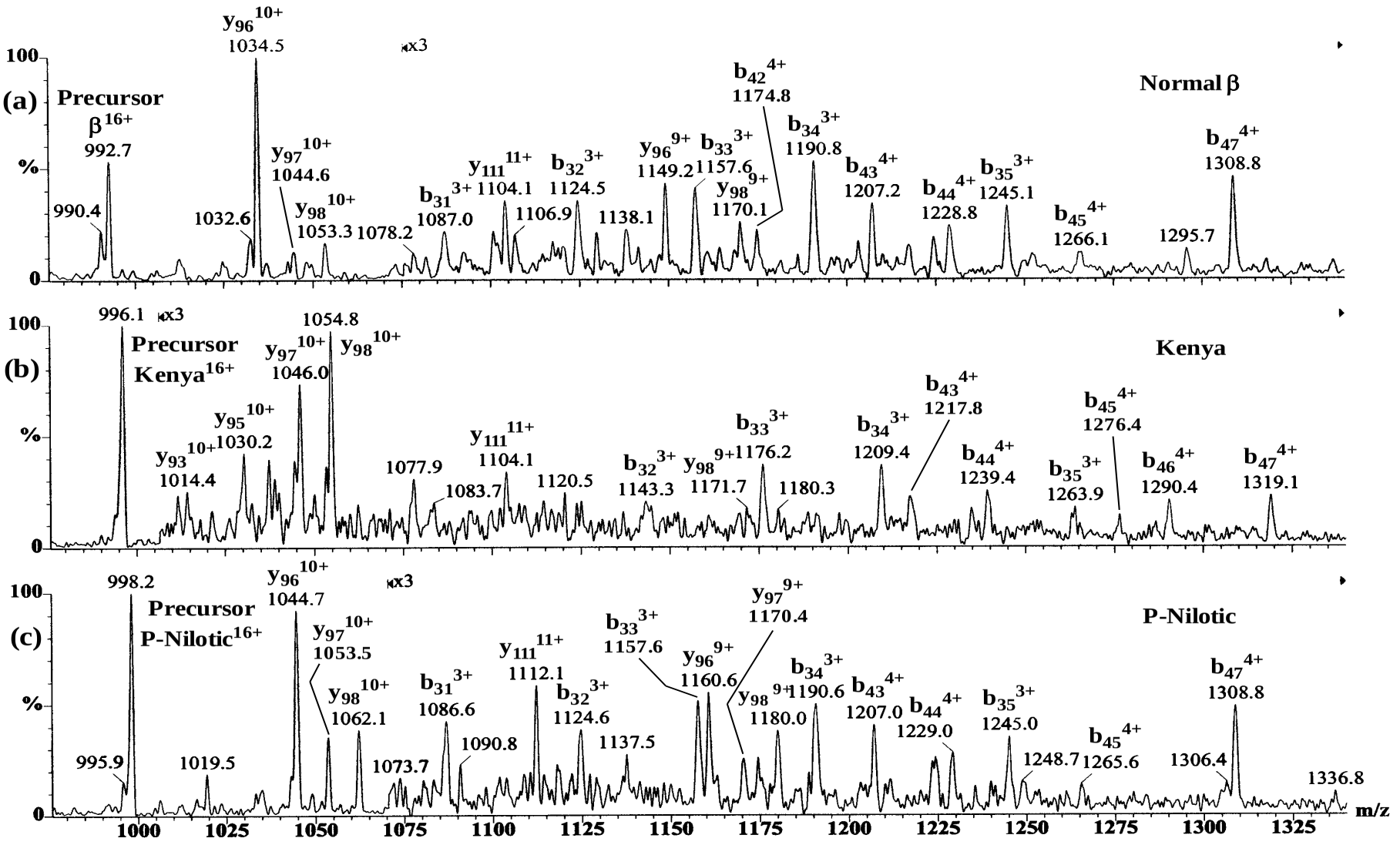

Figure 3.6. Part tandem mass spectra from (a) β^{16+}, (b) Kenya^{16+} and (c) P-Nilotic^{16+} ions showing the region above the precursor. The *m/z* values are experimental. Collision gas was argon at 2.5×10^{-3} mbar pressure. Collision energy was 26V.

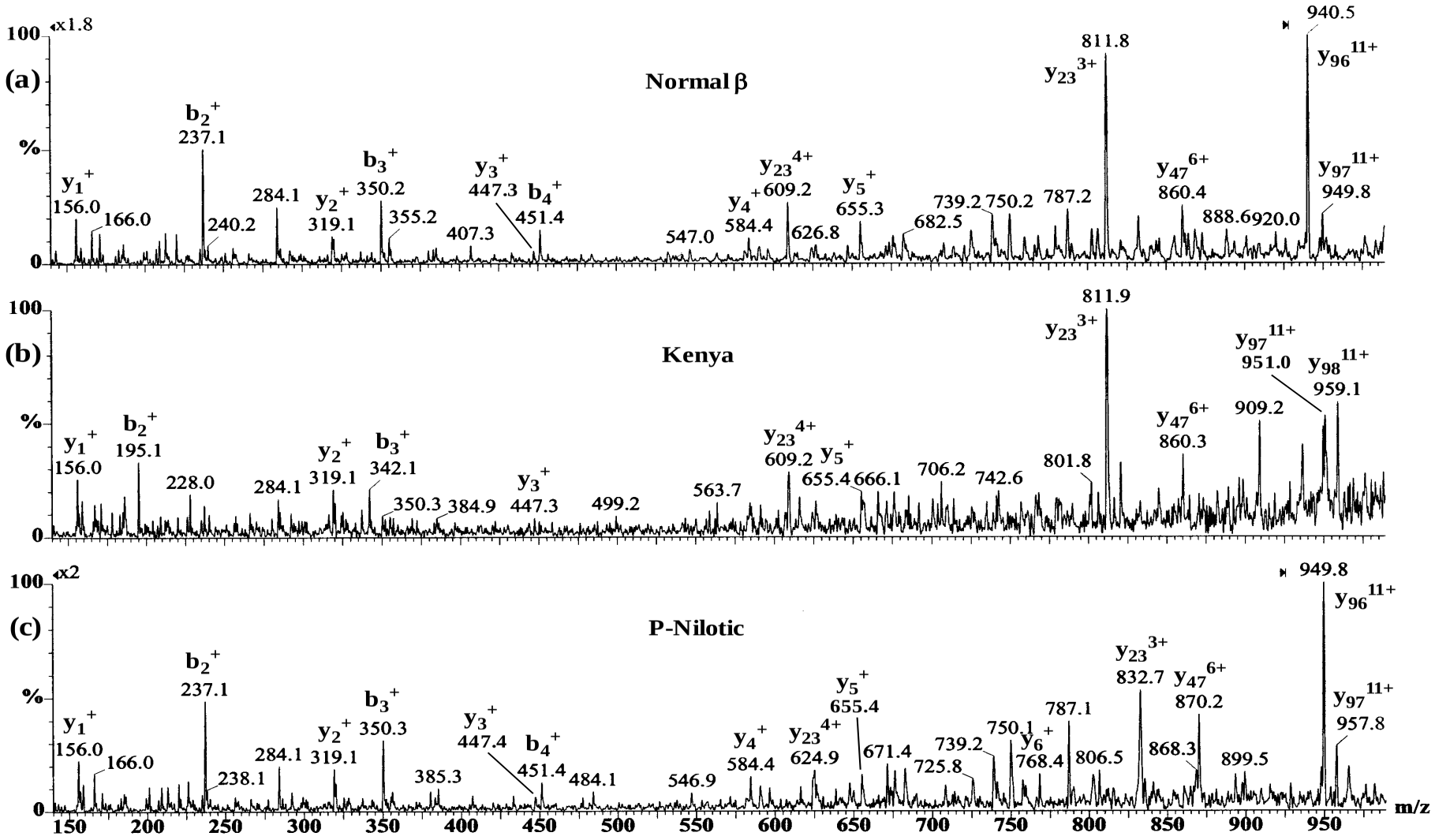

Figure 3.7. Part of tandem mass spectra from (a) β^{16+}, (b) Kenya^{16+} and (c) P-Nilotic^{16+} ions showing the region below the precursor. The *m/z* values are experimental. Collision gas was argon at 2.5×10^{-3} mbar pressure. Collision energy was 26V

3.5. Guidelines for the detection and identification of the Lepore haemoglobins by electrospray ionization mass spectrometry

3.5.1. Introduction.

The Lepore hybrid (fusion) haemoglobins (Hbs) are composed of normal α-chains (141 amino acids) and non-α-chains (146 amino acids). The non-α-chains comprise an N-terminal section of the δ-chain fused to a C-terminal section of the β-chain. Three Lepore Hbs are described in the literature[1] and have been analysed by mass spectrometry[2,3]. Their abundance in heterozygotes ranges approximately from 7-15%[1], and they elute close to Hb A_2 by ce-HPLC (Figure 3.5.1.). Their names, sequences and molecular weights (masses) are given in Table 3.5.1. Lepore-Boston-Washington (LBW) is the most common Lepore.

Table 3.5.1. The sequences and masses of the Lepores and the normal β-chain.

Name	Sequence	Sequence Mass (Da)
Lepore-Hollandia (δ22 - - β50)	δ(1-49) - β(50-146)	15,836.23
Lepore-Baltimore (δ50 - - β86)	δ(1-85) - β(86-146)	15,822.20
Lepore-Boston-Washington (δ87 - - β116)	δ(1-115) - β(116-146)	15,865.23
Normal β-chain	β(1-146)	15,867.24

3.5.2. Intact chain analysis by electrospray ionization mass spectrometry (ESI-MS).

Lepores Hollandia and Baltimore differ significantly in mass from normal β. Thus, in heterozygotes, these two Lepores are readily resolved from normal β when 500-fold diluted blood is analysed by ESI-MS[4,5]. Their masses, determined in this way, can be used to indicate their presence. The mass difference of Hollandia from β^A (-31.01 Da) can also be produced by the β-chain mutation Gln→Pro, but the latter would be almost silent by ce-HPLC, whereas the Lepores co-elute with Hb A_2.

The mass of Lepore-Baltimore (15,822.20 Da) differs from normal β by -45.04 Da, which cannot occur by a single base change in the β-chain codon. Hence, a low-abundance chain with a mass within experimental error of 15,822.20 Da would strongly suggest Lepore-Baltimore.

Unfortunately, LBW is only 2.01 Da lower in mass than β^A, and is not resolved from the latter in heterozygotes. The measured mass is the abundance weighted mean of the two chains, i.e. 0.14 to 0.30 Da lower than β^A assuming 7-15% abundance of LBW. Of the three Lepores, LBW is the most difficult to detect and identify in heterozygotes by ESI-MS, and tandem MS of the intact β^A plus LBW-chains is necessary for confirmation. ESI-mass spectra of (a) Hollandia/β^E, (b) Baltimore/β^A and (c) LBW/β^A heterozygotes are shown in Figure 3.5.2.

3.5.3. Tryptic peptide analysis by ESI-MS.

The tryptic peptides from the Lepores have the same sequence as those from either the β- or δ-chains. There are no peptides specific to a Lepore without also originating from the β- or δ-chains. However, in heterozygotes, provided the δ-chain occurs at a much lower level than the Lepore, elevated levels of Lepore peptides that are the same as the δ-chain yet differ from the β-chain suggest the presence of a Lepore. These peptides are shown in Table 3.5.2. Part spectra of 30-min digests show elevated levels of $\delta T2^{2+}$ (*m/z* 480.29, Figure 3.5.3.), $\delta T3^{2+}$ (*m/z* 628.85, Figure 3.5.4.) and $\delta T5^{2+}$ (*m/z* 1,023.48 (2^{nd} isotope), Figure 3.5.5.) in Baltimore (b) and LBW (c) heterozygotes compared with the control (a). $\delta T2^{+}$ and $\delta T3^{+}$ are also significantly increased over their normal levels (spectra not shown), and $\delta T2^{+}$ (*m/z* 959.531) stands out particularly clearly in the 30-minute digest spectrum.

Table 3.5.2. δ-chain tryptic peptides produced by the Lepores

Peptide	$(M+H)^{2+}$	$(M+H)^{+}$	Produced by
δT2	480.270	959.531	All Lepores and δ-chain
δT3	628.834	1,256.660	All Lepores and δ-chain
δT5	1,022.970	2,044.933	Lepore-Baltimore, LBW and δ-chain
δT10[(a)]	732.844	1,464.679	LBW and δ-chain (peptide contains Cys)

[(a)] Normally occurs in 30 min-digest as $\delta T(10\text{-}11)^{3+}$ at *m/z* 858.08 and $\delta T(10\text{-}11)^{2+}$ at *m/z* 1,286.61 since δ94Asp N-terminal to δ95Lys retards cleavage between δT10 and δT11. The *m/z* values are monoisotopic.

3.5.4. Tandem ESI-MS of the intact chains.
The Lepores give product ion spectra that differ from the β-chain in many respects. The *m/z* values of the b_{42}^{4+} to b_{47}^{4+} and b_{32}^{3+} to b_{35}^{3+} product ions all differ from those of the corresponding β-chain ions, but are the same from all the Lepores and cannot be used to distinguish the latter (Table 3.5.3). Nevertheless, they can be used to establish that a variant is indeed a Lepore and not a β-chain variant. Some of the y''_{96}, y''_{97}, y''_{98} and y''_{111} ions from Baltimore and LBW differ significantly in mass from one another and from their corresponding β- and Hollandia-chain ions. The y''_{96} and y''_{98} ions are particularly useful for positively identifying LBW in heterozygotes.

Table 3.5.3. Calculated *m/z* values of potentially diagnostic product ions in tandem mass spectra from intact β- and Lepore-chain ions with 16 charges.

Ion	β-chain	Hollandia	Baltimore	LBW
b_{32}^{3+}	1,124.6	1,114.3		
b_{33}^{3+}	1,157.7	1,147.3		
b_{34}^{3+}	1,190.7	1,180.4		
b_{35}^{3+}	1,245.1	1,234.8		
b_{42}^{4+}	1,174.9	1,167.1		
b_{43}^{4+}	1,207.1	1,199.4		
b_{44}^{4+}	1,228.9	1,221.2		
b_{45}^{4+}	1,265.7	1,257.9		
b_{46}^{4+}	1,280.0	1,272.2		
b_{47}^{4+}	1,308.7	1,301.0		
y_{96}^{11+}	940.6			944.5
y_{97}^{11+}	949.7		948.5	952.4
y_{98}^{11+}	957.7		956.4	960.3
y_{96}^{10+}	1034.5			1,038.8
y_{97}^{10+}	1,044.6		1,043.2	1,047.5
y_{98}^{10+}	1,053.3		1,051.9	1,056.2
y_{111}^{11+}	1,104.2		1,102.9	1,106.8
Precursor	992.71	990.77	989.90	992.58

Figure 3.5.6. compares tandem mass spectra from (a) Hollandia, (b) Baltimore, (c) heterozygous LBW (i.e. normal β plus ~10% LBW) and (d) the normal β-chain. Figures

3.5.7. and 3.5.8. show sections of these spectra on expanded scales.

With heterozygous Hollandia, the normal β- and Hollandia-chains are resolved. Hence the Hollandia precursor ion (*m/z* 990.8) can be selected without interference from normal β. In the resulting product ion spectrum, the presence of b ions corresponding to Lepores (Figure 3.5.7.) together with y" ions corresponding to β/Hollandia confirms the identity of Hollandia, provided the mass of the intact chain corresponds to that of Hollandia. Similar arguments apply to confirming the presence of Baltimore. In this case, several y" ions with *m/z* values specific to Baltimore are also present, namely $y"_{97}$, $y"_{98}$ with 10 and 11 charges (Figure 3.5.8b) and $y"_{111}$ with 11 charges (Figure 3.5.7b). In the rare occurrence of homozygous LBW or LBW/β-thalassaemia, $y"_{96}$, $y"_{97}$ and $y"_{98}$ with 10 and 11 charges and $y"_{111}$ with 11 charges have *m/z* values specific to LBW.

The situation is different in the case of heterozygous LBW, since the LBW-chain is not resolved from the β-chain. Here, the precursor ion (*m/z* 992.6) contains both the β-chain (~90%) and the LBW-chain (~10%). When the resulting product ion spectrum (Figure 3.5.7c) is compared with the normal β-chain spectrum (Figure 3.5.7d), the presence of minor b_{34}^{3+}, b_{35}^{3+} and b_{47}^{4+} ions (marked * in Figure 3.5.7c) indicate the presence of LBW. Although these ions have *m/z* values common to all the Lepores, they can only originate from LBW in this case, since the precursor ion contains only LBW and normal β. Conclusive further evidence for the presence of LBW are $y"_{96}^{11+}$ and $y"_{96}^{10+}$ ions (*m/z* 944.5 and 1,038.7) in Figure 3.5.8c whose *m/z* values are unique to LBW and positively identify it in the presence of the dominant β-chain. Although less abundant, the *m/z* values of $y"_{98}^{11+}$ and $y"_{98}^{10+}$ are also specific to LBW.

A further complication would arise in detecting and identifying a Hollandia/Sickle (β^S) heterozygote, because the mass of β^S (15,837.26 Da) is only 1 Da higher than that of Hollandia. These species would not be resolved and would have a net mass 0.1-0.2 Da lower than β^S, which would be difficult to detect. Tandem mass spectrometry does not offer an easy solution because the Sickle mutation (β6Glu→Val) occurs close to the N-terminus of the β-chain. Hence all the b ions in Figure 3.5.7a would be 1 Da doublets, again difficult to detect since they would be unresolved, with net masses 0.1-0.2 Da lower than the masses for sickle. A tryptic digest would allow easy detection of the sickle mutation by giving a peptide 30 Da lower than the mass of normal βT1. Since there would be no normal β-chain in the sample and the sequence of the T1 peptide from Hollandia is the same as normal βT1, the presence of a tryptic peptide with the mass of normal βT1 would suggest the presence of Lepore-Hollandia. This suggestion would be reinforced if the abundance of the $\delta T2^{2+}$ and $\delta T3^{2+}$ peptide ions were enhanced relative to a normal control provided the δ-chain abundance was low in the Hollandia/(β^S) heterozygote.

Tandem mass spectra were generated on a Quattro Ultima using argon as the collision gas ($2.5x10^{-3}$ mbar) with 26 volts collision energy. Mass scale calibration employed product ions present in the tandem mass spectrum of the normal β-chain.

3.5.5. Summary of strategy for identifying Lepores.

1. An apparently high level of Hb A_2 (10-15%) may indicate the presence of a Lepore (Figure 3.5.1.).

2. Analyse 500-fold diluted and desalted blood. Intact chains within experimental error of 15,836.23 Da or 15,822.20 Da suggest the presence of Lepore-Hollandia (Figure 3.5.2a) or Lepore-Baltimore (Figure 3.5.2b) respectively. A single non-α-chain between ~15,867.1 Da and ~15,866.9 Da (normal β minus 0.14 to 0.30 Da) is consistent with a normal LBW heterozygote (Figure 3.5.2c), but many other possibilities can give this mass. Estimate the proportion of the δ-chain (15,924.32 Da).

3. Analyse a 30-minute digest of the sample plus a control with a normal level of δ-chain (3-4%). Elevated levels of $\delta T2^{2+}$ (*m/z* 480.27), $\delta T3^{2+}$ (*m/z* 628.83), $\delta T2^{+}$ (*m/z* 959.53), $\delta T3^{+}$ (*m/z* 1,256.66), and $\delta T5^{2+}$ (*m/z* 1,022.97) indicate the Lepores according to Table 3.5.2 and Figs 3.5.3-3.5.5, provided the level of the δ-chain in the sample is low. These results may indicate the presence of a previously unsuspected LBW heterozygote.

4. The identification of the Lepores may be confirmed, if deemed necessary, by tandem MS of the intact variant chains as follows. Analyse the appropriate $(M+16H)^{16+}$ precursor ion from the intact chain by tandem MS to confirm Hollandia (*m/z* 990.8) or Baltimore (*m/z* 989.9). The presence of Hollandia is confirmed provided its mass is within experimental error of 15,836.23 Da and b-ions indicative of a Lepore are present (Table 3.5.4, bold font and Figure 3.5.7a). The presence of Baltimore is confirmed provided its mass is within experimental error of 15,822.20 Da and the ions shown in bold font in Table 3.5.4 are present (Figure 3.5.7b). For LBW in heterozygotes, select the precursor ion at *m/z* ~992.6 (normal β-chain plus LBW-chain). The presence of ions shown bold in Table 3.5.4 and indicated with an asterisk (*) in Figures 3.5.7c and particularly 3.5.8c confirms the presence of LBW.

Conditions for tandem MS.

On triple quadrupole instruments, scan MS2 from *m/z* 500-1500. The collision energy is typically 26V using argon as the collision gas at 2.5 x 10^{-3} mbar collision cell pressure.

Table 3.5.4. Masses of intact chains and *m/z* values of product ions necessary for identifying the Lepores.

Chain name	Mass (Da)[6]	b_{47}^{4+}	$y''_{96}{}^{11+}$	$y''_{98}{}^{11+}$	$y''_{96}{}^{10+}$	$y''_{98}{}^{10+}$	$y''_{111}{}^{11+}$
Lepore-Hollandia[a]	**15,836.23**	**1,301.0**	940.6	957.7	1,034.5	1,053.3	1,104.2
Lepore-Baltimore[a]	**15,822.20**	**1,301.0**	940.6	**956.4**	1,034.5	**1,051.9**	**1,102.9**
Lepore-BW[b]	**15,865.23**	**1,301.0**	**944.5**	**960.3**	**1,038.8**	**1,056.2**	**1,106.8**
Lepore-BW/β^A	15,866.9[c]	**1,301.0**	**944.5**	**960.3**	**1,038.8**	**1,056.2**	1,106.8
Normal β-chain	15,867.24	1,308.7	940.6	957.7	1,034.5	1,053.3	1,104.2

Masses and *m/z* values in bold font are the minimum recommended for confirmation.

[a] Applies to normal heterozygotes, homozygotes and Lepore/β-thalassaemia.

[b] Applies to LBW homozygotes and LBW/β-thalassaemia.

[c] Approximate mass assuming 15% Lepore.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	2.7*	---	1.08	56189
P2	---	5.1	1.27	115349
P3	---	4.1	1.64	93325
A0	---	76.8	2.37	1740930
A2	13.1*	---	3.51	262299

F Concentration = 2.7* %
A2 Concentration = 13.1* %

*Values outside of expected ranges

Analysis comments:

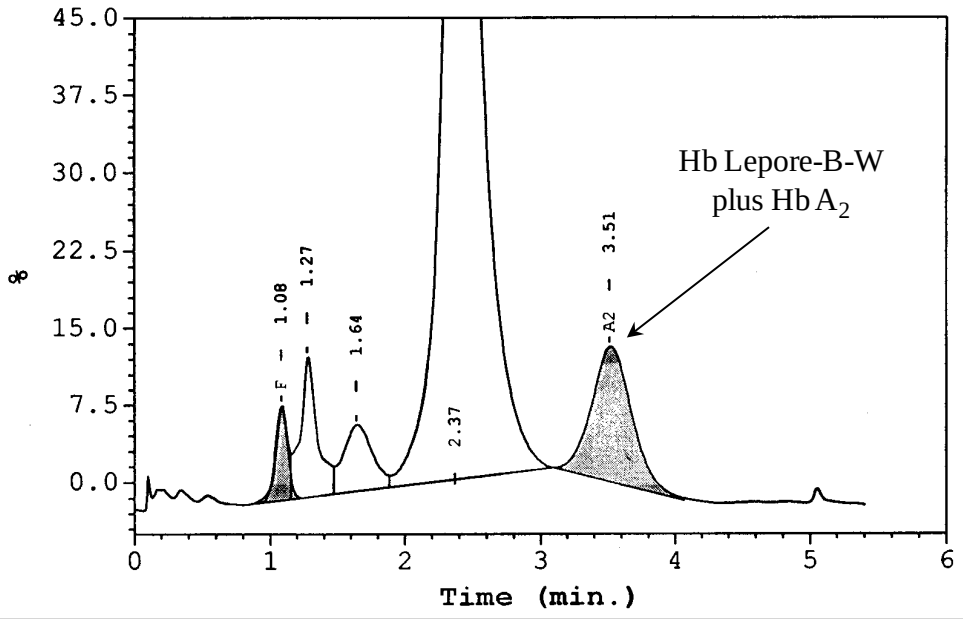

Figure 3.5.1. Bio-Rad ce-HPLC trace from a Lepore-Boston-Washington heterozygote

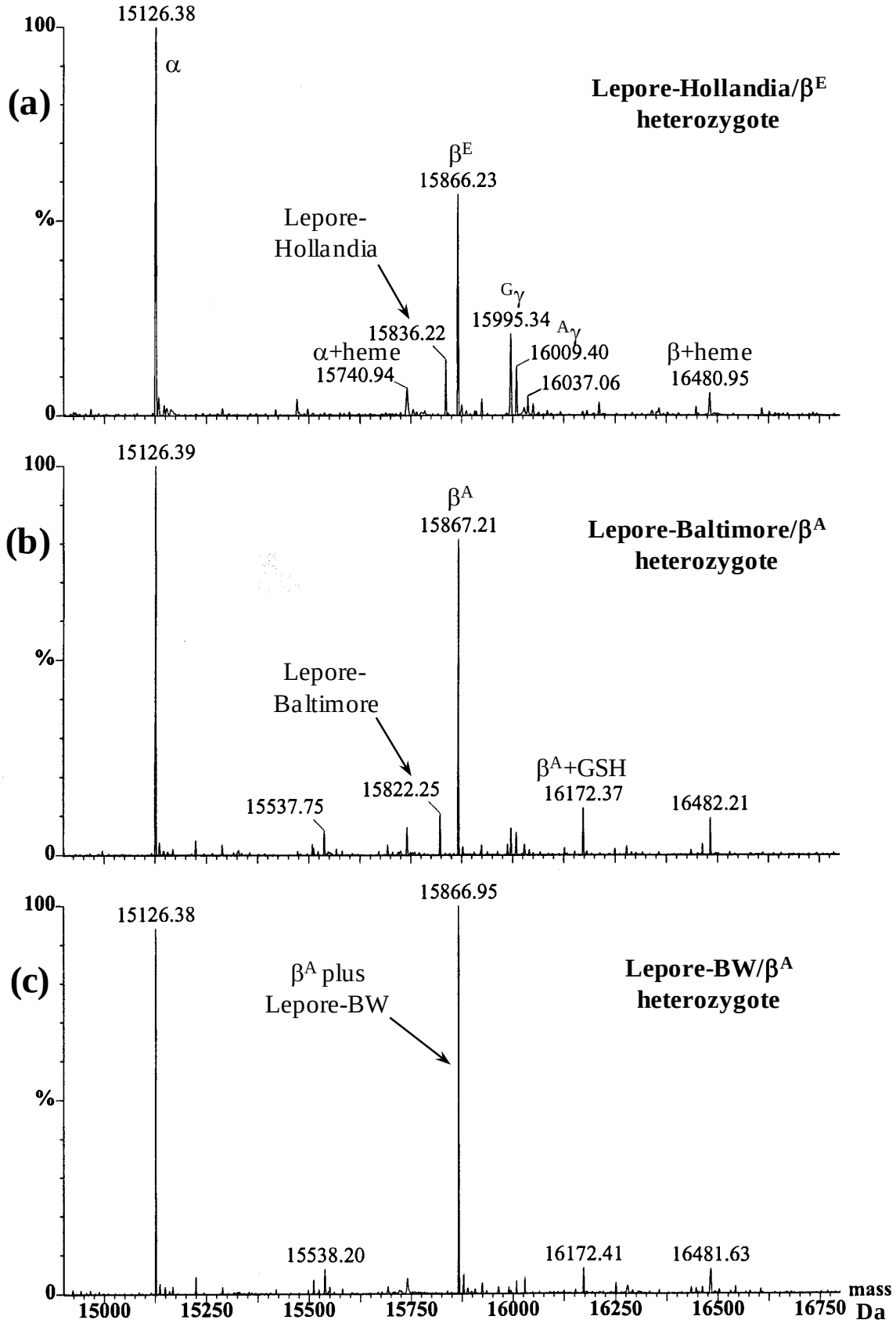

Figure 3.5.2. MaxEnt deconvoluted ESI-mass spectra from three Lepore heterozygotes

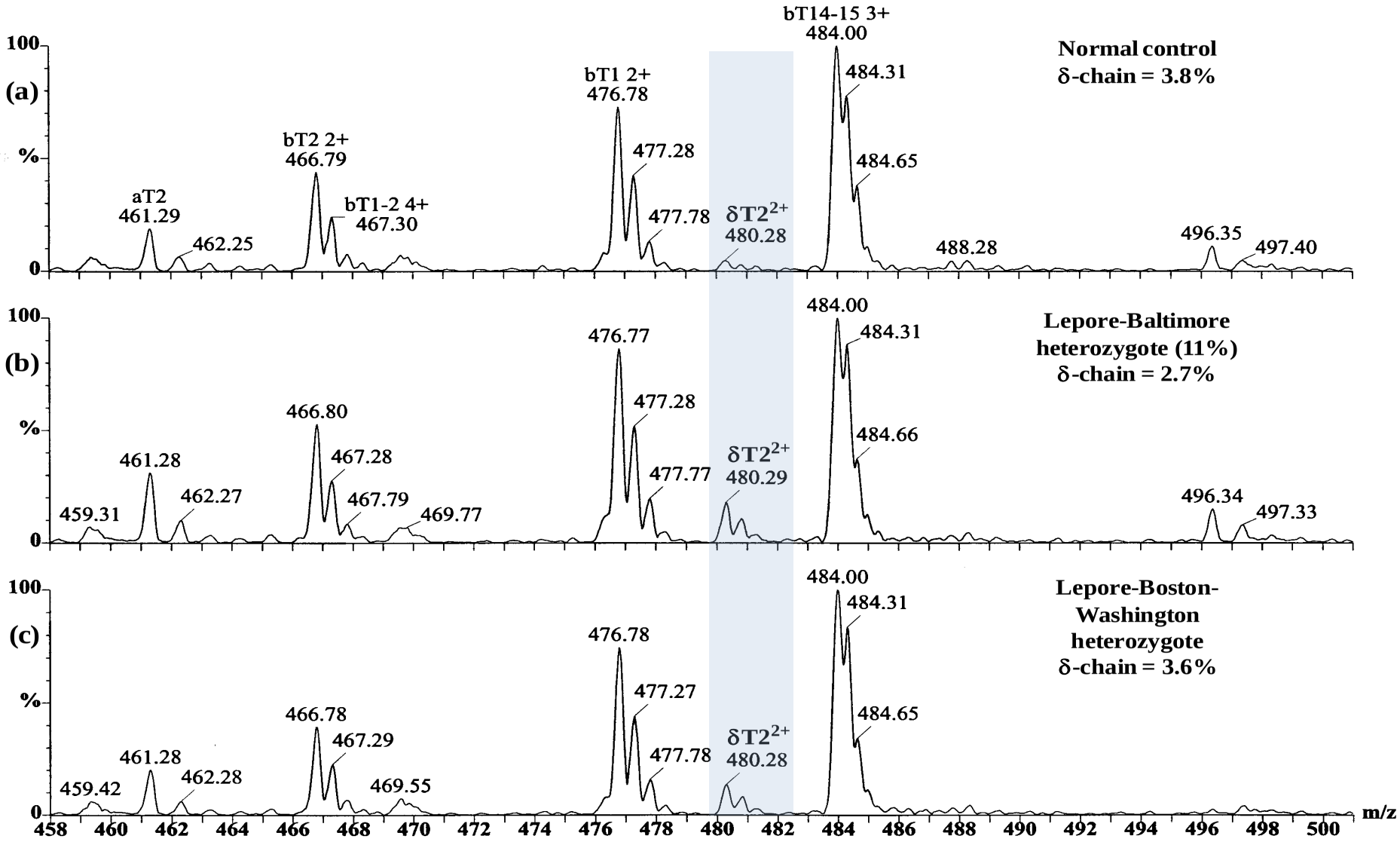

Figure 3.5.3. Part spectra of 30-minute digests showing increased levels of $\delta T2^{2+}$ ions from two Lepores.

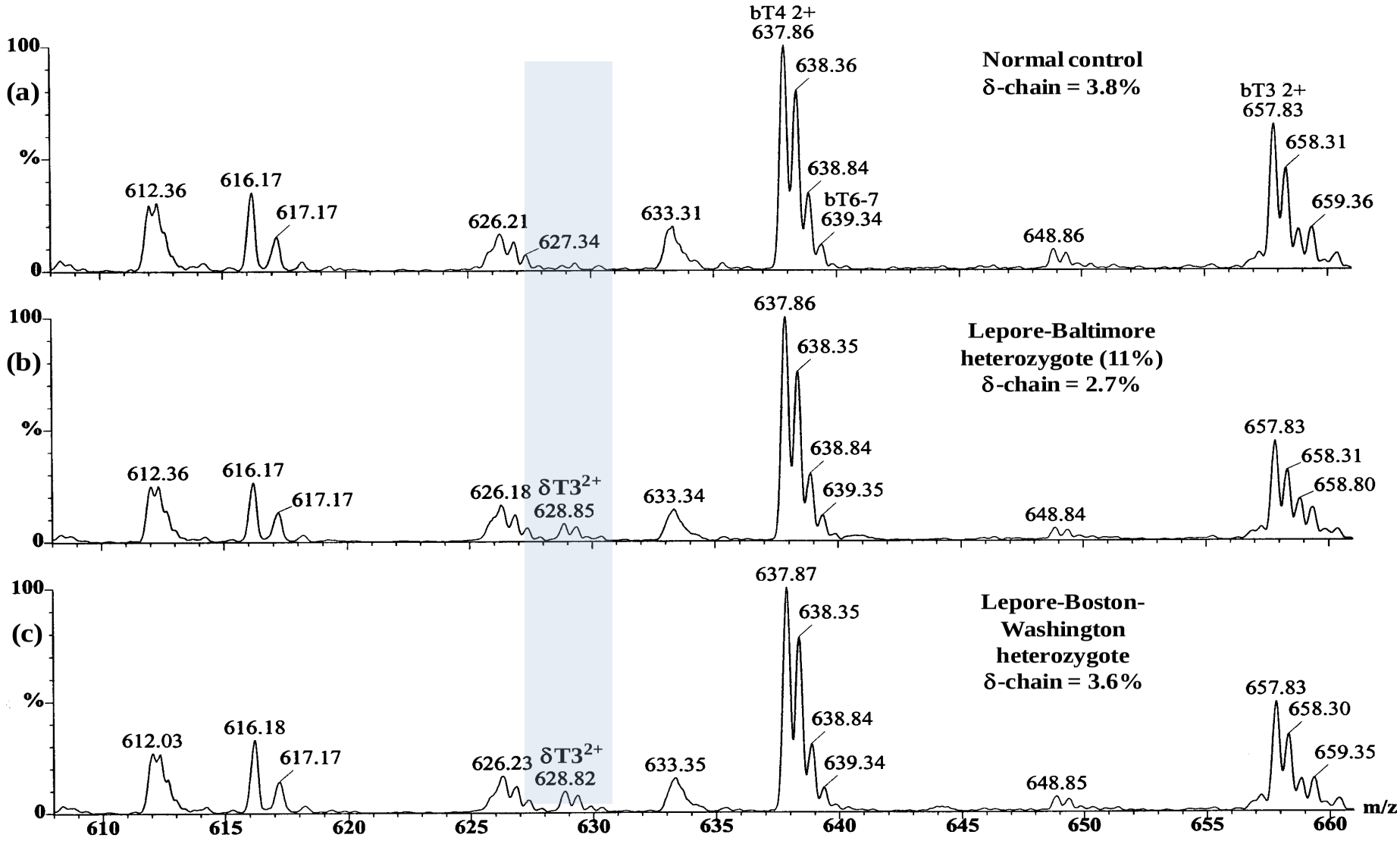

Figure 3.5.4. Part spectra of 30-minute digests showing increased levels of $\delta T3^{2+}$ ions from two Lepores.

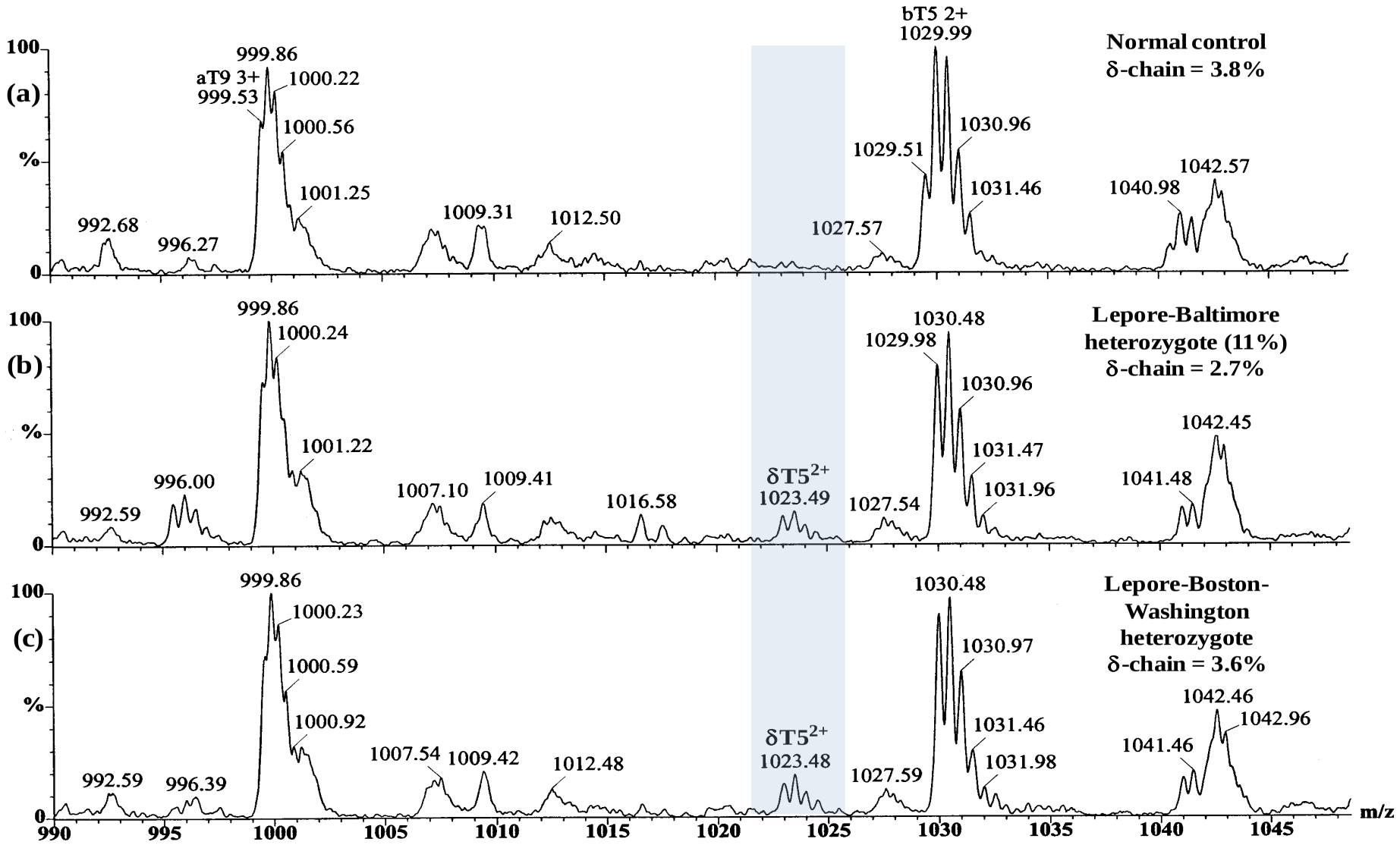

Figure 3.5.5. Part spectra of 30-minute digests showing increased levels of $\delta T5^{2+}$ ions from two Lepores.

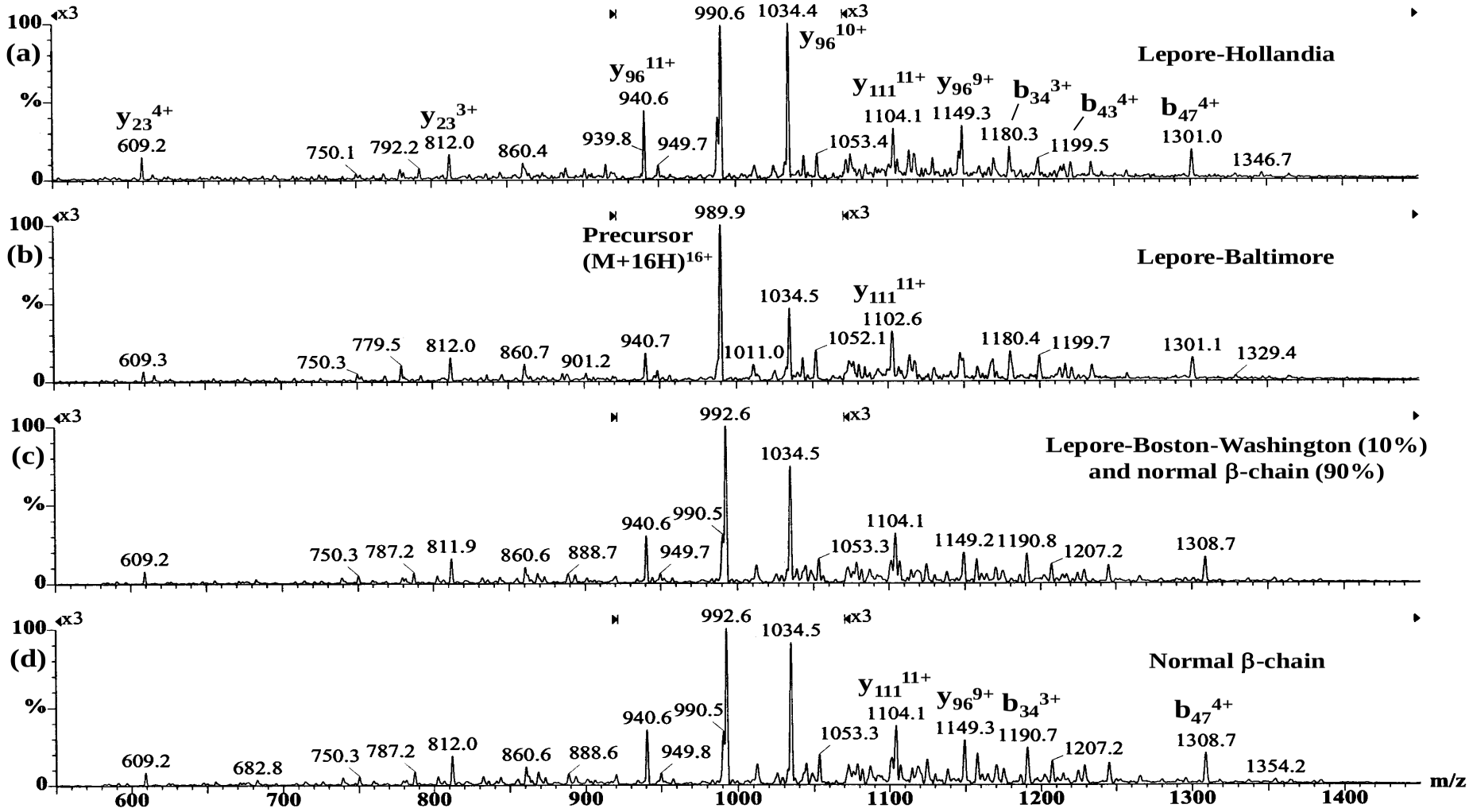

Figure 3.5.6. Part tandem mass spectra from (a) Lepore-Hollandia, (b) Lepore-Baltimore, (c) Lepore-BW heterozygotes and (d) normal β-chain.

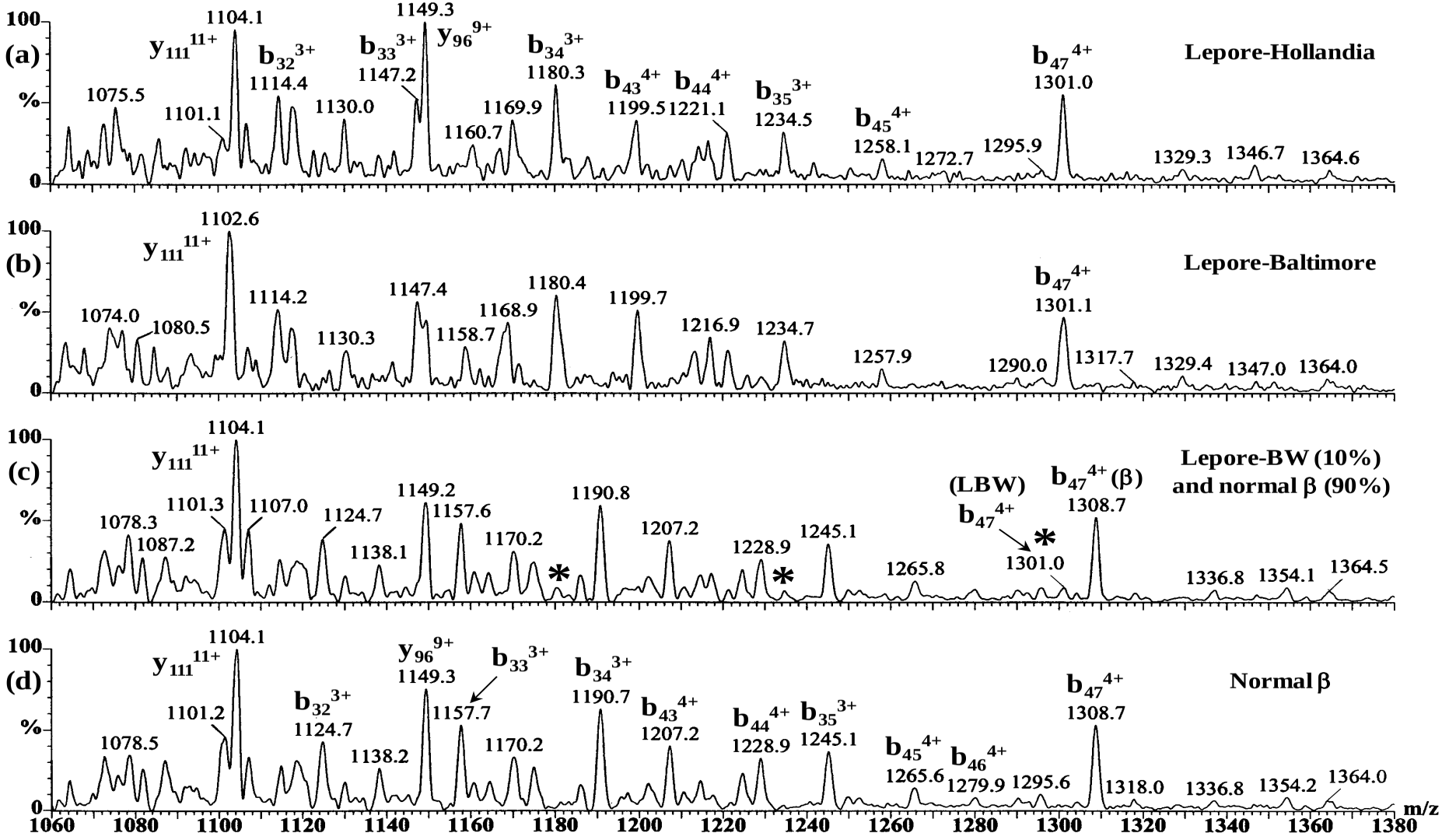

Figure 3.5.7. Part tandem mass spectra from (a) Lepore-Hollandia, (b) Lepore-Baltimore, (c) Lepore-BW heterozygotes and (d) normal β-chain.

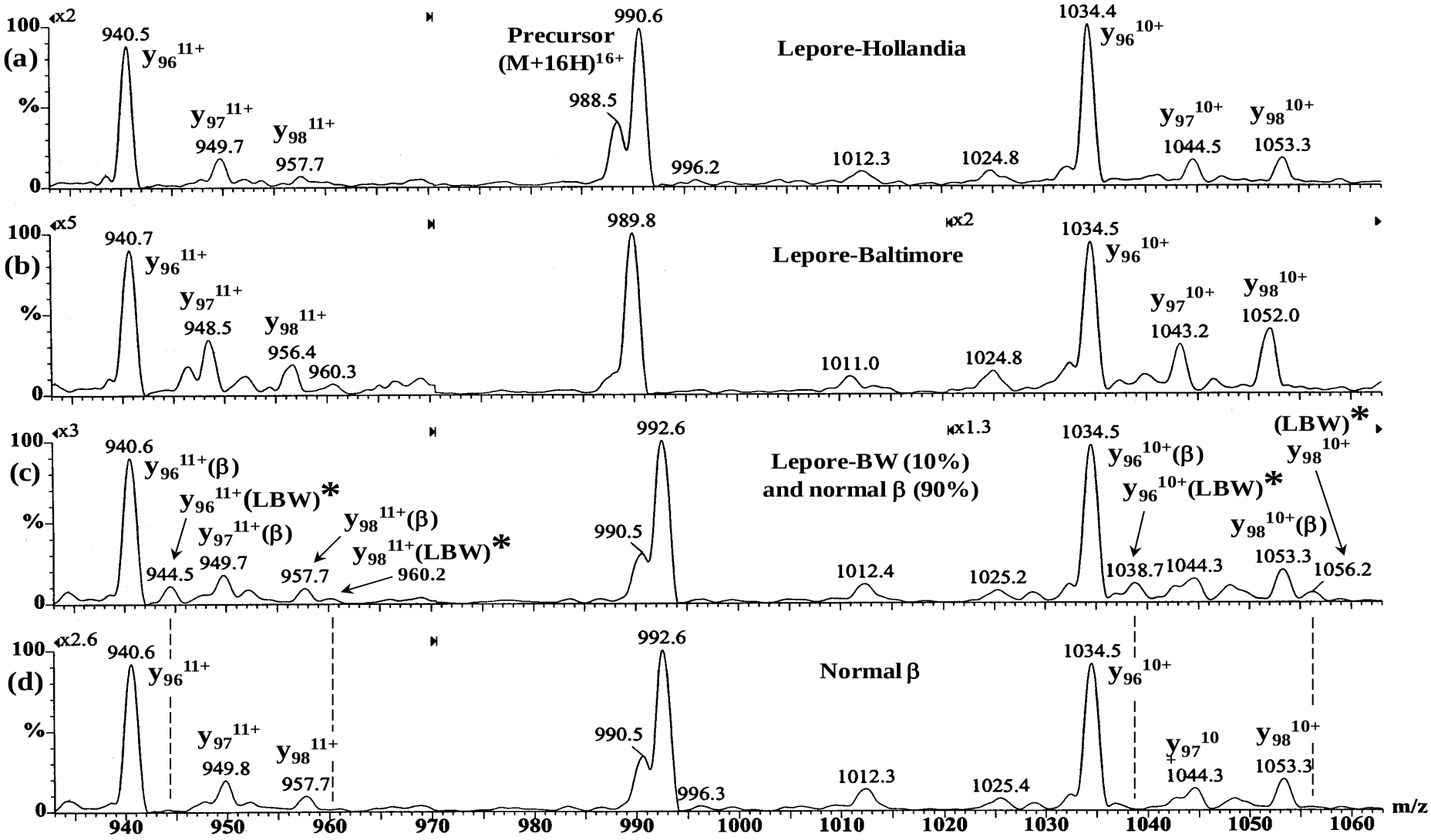

Figure 3.5.8. Part tandem mass spectra from (a) Lepore-Hollandia, (b) Lepore-Baltimore, (c) Lepore-BW heterozygotes, and (d) normal β-chain, showing y_{96} and y_{98} ions characteristic of Lepore-Boston-Washington in (c). Collision energy was 26V using argon as the collision gas.

3.6 References

(1) R.C. Hardison, D.H.K. Chui, B. Giardine, C. Riemer, G.P. Patrinos, N. Aganou, *et al*. HbVar: A relational database of human hemoglobin variants and thalassemia mutations at the globin gene server. Hum.Mutat. **19**, 225-33 (2002). (http://globin.bx.psu.edu).

(2) B.N. Green, R.W.A. Oliver, A.M. Falick, C.H.L. Shackleton, E. Roitman and H.E.Witkowska (1990) Electrospray MS, LSIMS and MS/MS for the rapid detection and characterization of variant hemoglobins, in Biological Mass Spectrometry, A.L. Burlingame and J.A. McCloskey (Eds), pp. 129-146. Elsevier, Amsterdam.

(3) M. De Caterina, P. Esposito, E. Grimaldi, G. Di Maro *et al.*, Characterization of hemoglobin Lepore variants by advanced mass spectrometric methods. Clin. Chem. **38**, 1444-48 (1992).

(4) B.J. Wild, B.N. Green, E.K. Cooper, M.R.A. Lalloz, S. Erten, A.D. Stephens and D.M. Layton. Rapid identification of hemoglobin variants by electrospray ionization mass spectrometry. Blood Cells Mol. Dis. **27**, 671-704 (2001).

(5) D.K. Rai, W.J. Griffiths, B. Landin, B.J. Wild, G. Alvelius and B.N. Green. Accurate mass measurement by electrospray ionization quadrupole mass spectrometry: detection of variants differing by <6 Da from normal in human hemoglobin heterozygotes. Anal. Chem. **75**, 1978-82 (2003).

SECTION 4

4.0. Step 4. Digestion of the haemoglobin in blood with trypsin, nomenclature and the peptides produced in practice

It was shown in Section 2 how analysis of the intact globin chains in abnormal blood samples by ESI-MS gave the mass of the variant chain and hence the mass change of the variant from normal. The variant was also assigned to one of the globin chains by such data. Moreover, with the aid of the genetic code tables, the mass change implied a small number of possible amino acid mutations, but not the position of the mutation in the globin chain. In this section, the haemoglobin (Hb) in diluted blood is denatured and the resulting globin chains digested with trypsin for 30 minutes in order to cut each chain into a number of smaller pieces (tryptic peptides). This mixture of peptides is then analysed by ESI-MS and the resulting spectrum compared with that from a normal sample in order to identify the peptide containing the mutation. In this way, the number of possible positions of the mutation in the globin chain is considerably reduced. It has been found that in roughly half of the samples analysed, only one mutation in the peptide can occur by a single base change in the nucleotide codon, which identifies the variant. In order to identify the variant in peptides containing more than one possible mutation, the variant peptide would need to be sequenced by tandem ESI-MS.

Note that the mixtures of peptides are analysed directly by ESI-MS, i.e. with no prior chromatographic separation. The ESI-mass spectrum from a typical 30-minute tryptic digest of diluted and denatured adult human blood is shown in Figure 4.0.1.

Nomenclature

Trypsin normally cleaves proteins (globin chains in the case of Hb) at the carboxyl or C-terminal (right-hand) side of the amino acid residues arginine (Arg, R) and lysine (Lys, K) except when proline (Pro, P) occurs on the C-terminal side of, and adjacent to, these residues. Tryptic peptides are numbered from the amino or N-terminal (left-hand) end of the globin chain (Figure 4.0.2)**.** For example, αT3 means the third peptide from the N-terminus of the α-chain, and βT5 means the fifth peptide from the N-terminus of the β-chain. βT(10-11) means the tenth and eleventh β-chain peptides joined together.

Tryptic peptides produced in practice: the effect of acidic amino acids

Cleavage is slower when the residues glutamic acid (Glu, E) or aspartic acid (Asp, D) occur close to Arg or Lys. In the context of the normal human Hb α- and β-chains, the rate of cleavage at Lys is drastically reduced when Asp occurs on the N-terminal side of the Lys resulting in the formation of the double peptides αT(1-2), αT(12-13) and βT(10-11) as major components. Tables 4.1.1 and 4.1.2 show respectively the tryptic peptides produced from the normal α- and β-chains.

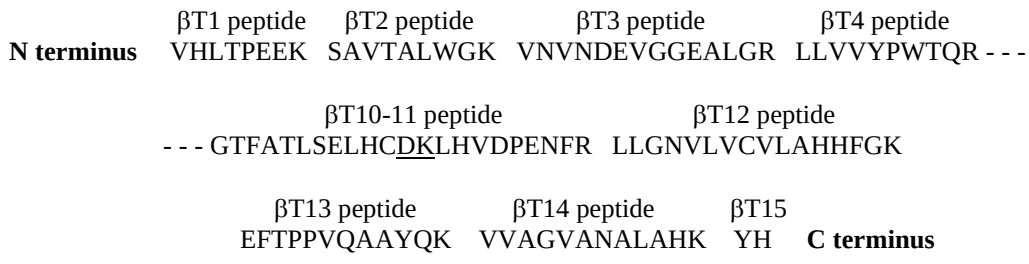

Figure 4.0.2. Parts of the β-chain sequence illustrating various aspects of cleavage by trypsin. The presence of Asp (D) adjacent to Lys (K), (DK underlined in the above sequence), essentially abolishes cleavage at the Lys to give the βT(10-11) peptide. The βT10 and βT11 peptides are not present at useful levels in 30-minute digests.

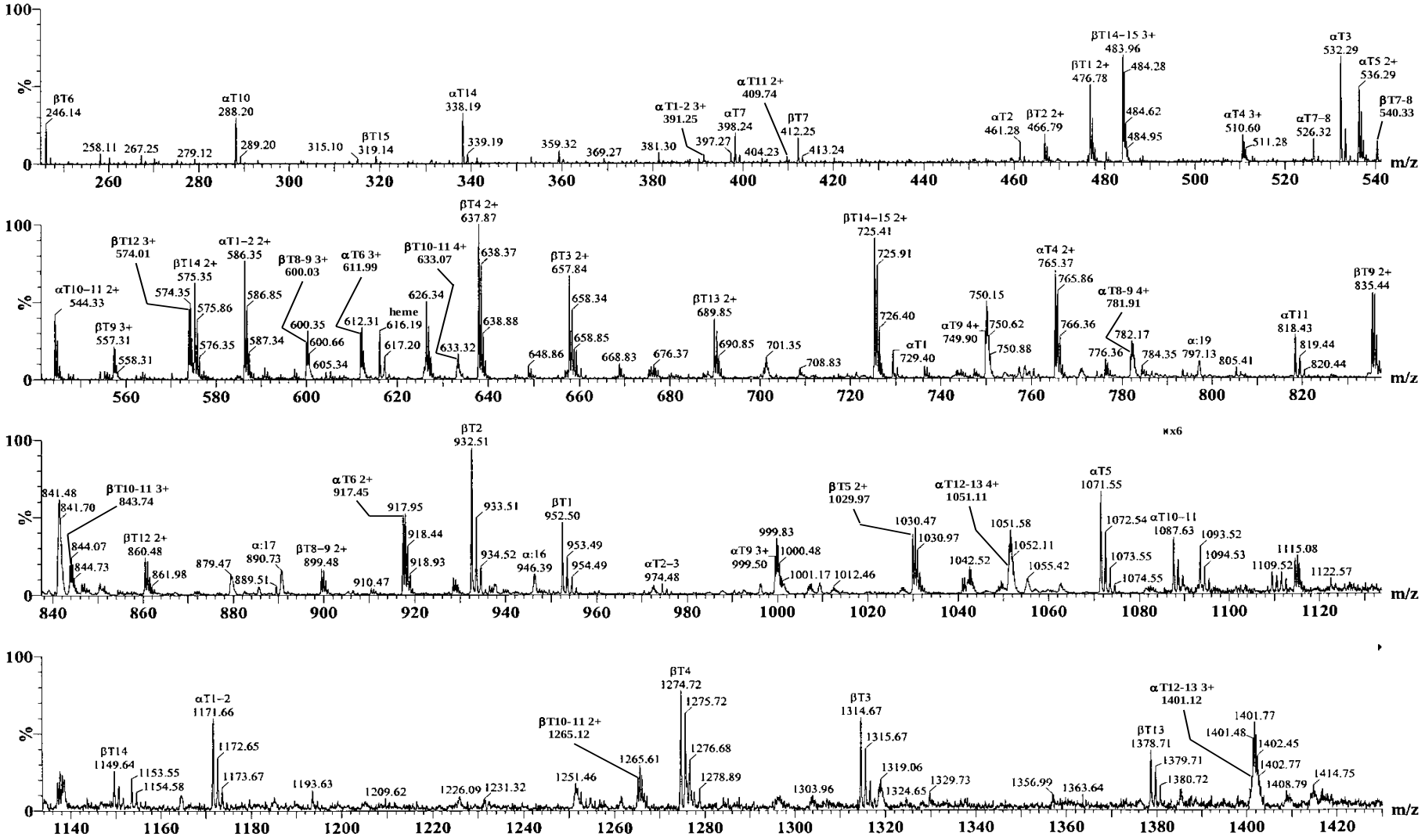

Figure 4.0.1. Part of the electrospray ionization mass spectrum from a typical 30-minute tryptic digest of diluted and denatured adult human blood. *m/z* values are experimental. Residual intact α-chain ions are shown as e.g. α:16 at *m/z* 946.39 (α-chain with 16 protons).

4.1. Procedure for digesting the haemoglobin in blood samples with trypsin in order to produce mixtures of peptides suitable for variant identification by electrospray ionization mass spectrometry (ESI-MS)

Objective. To prepare mixtures of tryptic peptides suitable for identifying variants directly from whole blood samples, i.e. without clean-up, dehaeming, derivatisation or separation.

Preparation of the 50-fold diluted blood stock solution.
Pipette 10 μL of whole blood (generally in EDTA anti-coagulant) into a 1.5 mL microcentrifuge tube and dilute it 50-fold by adding 490 μL of HPLC grade water.

Preparation of the digest solution.
Transfer 100 μL of the stock solution to a fresh 1.5 mL microcentrifuge tube.

Denature the haemoglobin (Hb) by adding 20 μL of denaturing solution. Mix and allow the resulting solution to stand for at least 3 minutes at room temperature (20-25^{O}C).

Then add and mix 6 μL of 1 molar aqueous ammonium bicarbonate (NH_4HCO_3) solution. The solution should become cloudy, indicating that the haemoglobin has been effectively denatured.

Then add and mix 5 μL of 5 mg/mL aqueous TPCK treated trypsin solution (Sigma T-1426). Allow the resulting solution to stand at room temperature, when the precipitate should disappear within approximately 2 minutes of adding the trypsin, as indicated by the solution becoming clear.

Incubate the resulting solution at 37^{O}C.

After 30 minutes, dilute the digest solution 10-fold by adding 900 μL of Solution A. The resulting solution may then be introduced into the mass spectrometer for analysis by ESI-MS. It should remain analytically viable for several weeks when refrigerated at ~4^{O}C.

NB. In order to ensure reproducible introduction of the ammonium bicarbonate and trypsin solutions above, it is recommended that they are dispensed from 10 μL syringes, e.g. Hamilton type 701 with bevel tip. Whilst dispensing the solutions, touch the inner surface of the sample tube about half-way down with the tip of the syringe.

Over 98% of α and β-chain variants can be identified from 30-min tryptic digests as prepared above. Generally, there is little to be gained from digesting over a longer time for two reasons. First, peptides representing the whole of these chains are already present after 30-minutes. Second, the two large 'double' peptides αT(12-13) and βT(10-11) are not significantly cleaved further at α127Lys and β95Lys, respectively, by prolonged digestion. Furthermore, the Cys containing peptides gradually disappear, presumably because they form disulphide-bonded dimers with one another, and which would then require them to be released by reduction with dithiothreitol. However, there are two types of variant that require a special approach:

First, there are a few variants in which a Cys is produced by the mutation and the 'new' Cys containing peptide is not observed in the digest solution. For example, the variant αT9 peptide was not observed in the digest from Hb Nigeria, α81Ser→Cys, until the digest solution was reduced. A similar situation was observed with Hb Porto Alegre, β9Ser→Cys. To observe the variant, reduce the digest solution with dithiothreitol before it is acidified with Solution A, i.e. while the pH is still approximately 8. To do this, place 50 μL of the 30-min digest in a fresh 1.5 mL microcentrifuge tube and add 5 μL of 100 mMolar dithiothreitol (DTT) solution. Incubate at 37^{O}C for 15 minutes. Then add 450 μL of Solution A to give the working solution for analysis by ESI-MS.

Second, there are four α/(α-1 Da) heterozygotes in the αT9 peptide that are very difficult to confirm by tandem MS. A fifth one occurs in the αT(12-13) peptide. These may be identified from α-chymotrypsin digests prepared in exactly the same way as the tryptic digests by simply replacing trypsin with α-chymotrypsin (Sigma C-3142**)**.

4.1.1. Preparation of the reagent solutions used in digesting 50-fold diluted blood with trypsin.

The denaturing solution may be prepared in a 1.5 mL microcentrifuge tube by mixing 500 µL of 1% formic acid with 500 µL of acetonitrile. Store at 4^{O}C. Replace after 6 weeks.

The 1M ammonium bicarbonate (NH_4HCO_3) solution may be prepared as follows. Accurately weigh out *X* mg (50-100 mg) of ammonium bicarbonate (Sigma A-6141) in a 1.5 mL microcentrifuge tube and add 12.6 *X* µL of HPLC grade water. This solution should remain viable for several months when stored at 4^{O}C.

The trypsin solution (5 mg/mL, 0.21 mM) may be prepared as follows. Accurately weigh out *Y* mg (2-3 mg) of TPCK treated trypsin (Sigma T-1426) in a 1.5 mL microcentrifuge tube and dissolve in 200 *Y* µL of HPLC grade water. This solution should remain viable for at least 3 months when stored at -20^{O}C. Such a solution that had been stored for 5 years at -20^{O}C was found to be fully active.

Solution A, for 10-fold diluting aliquots of the digest solutions, may be prepared by mixing 5 mL of acetonitrile, 2 mL of water and 2 mL of 1% HCOOH or *pro rata*. Store at room temperature.

The 100 mM solution of dithiothreitol (DTT) may be prepared as follows. Accurately weigh out *Z* mg (5-10 mg) of dithiothreitol (Sigma D-9760) in a 1.5 mL microcentrifuge tube and add 64.8 *Z* µL of water. This solution should remain viable for at least 1 month when stored at 4^{O}C.

ESI-MS analysis.

Many of the parameters for acquiring data from tryptic digests are instrument type dependent. Therefore, it will be necessary to set them when initially setting up the instrument. The following are typical for a Quattro Ultima tandem quadrupole instrument: Select positive ion mode, capillary = 3.0 kV, 32 channels per *m/z* unit (if available), otherwise use 16 channels. The remaining source and interface parameters are chosen to optimise sensitivity and resolution. For setting up purposes, introduce a 30-min tryptic digest at 5 µL/min flow rate, and optimise the intensity and resolution of the $\beta T14^{2+}$ and $\beta T5^{2+}$ ions at *m/z* 575.3 and 1,030.0 respectively on the tune page. It is often beneficial to use non-standard LM and HM parameters, e.g. 22 and 14 respectively, in order to obtain a more balanced performance across the *m/z* range. Collision cell parameters should be set to give maximum sensitivity. Typical settings are: Entrance = 2V, collision cell = 2-5V, Exit = 40V. Scan from *m/z* 200 to 1,650 is usually adequate. Scan time = 8 sec. Acquisition time = 3 minutes in MCA mode. Internally calibrate i.e. calibrate the *m/z* scale of each digest spectrum on itself, using reference file DigHbA.ref.

4.1.2. Mass scale calibration of the tryptic digest spectrum.

Open the raw data file.
From **Tools**, open **Make Calibration**.
In the **Make new calibration window**, select **Reference file** DigHbA.ref.

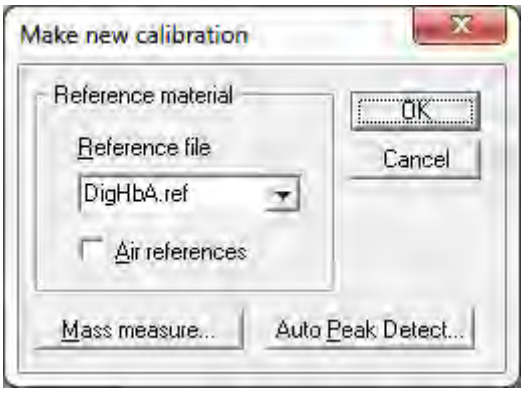

Figure 4.1.2.1. Selection of the appropriate calibration file

Press **Mass measure** to open the **Mass Measure** window.
Select and set the parameters in the **Mass Measure** window as follows.
Select **Background subtract** and set **Polynomial order** to 25 and **Below curve (%)** to 5.00.
Select **Smooth** and set **Peak width (Da)** (sic, actually *m/z*) to 0.60 and set **Number of smooths** to 2.
Select **Savitsky Golay**.
Set Min peak width at half height (channels) to 4.
Select **Centroid top (%)** and set it to 50.00.
Select **heights**.
Press **OK**.

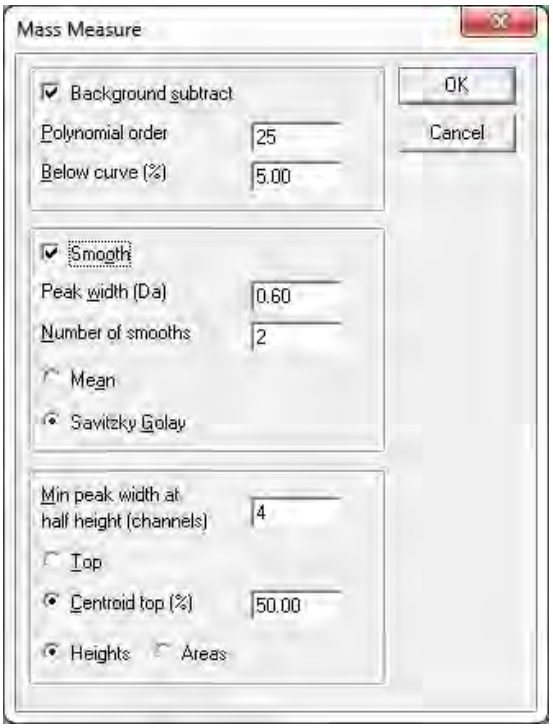

Figure 4.1.2.2. Typical parameters for mass measuring digest mass spectra acquired in MCA mode.

The spectrum should then be calibrated in a few seconds and a Calibration Report displayed, which shows how well the mass spectral peaks fit the reference masses. The errors should generally be less than approximately ±0.02 amu. To remove a peak from the calibration, e.g. a peak that shows excessive error, press the right click the peak with the mouse.

To calibrate subsequent digest spectra, simply open the raw data file, select **Make Calibration** from **Tools** and Press OK from Make new calibration.

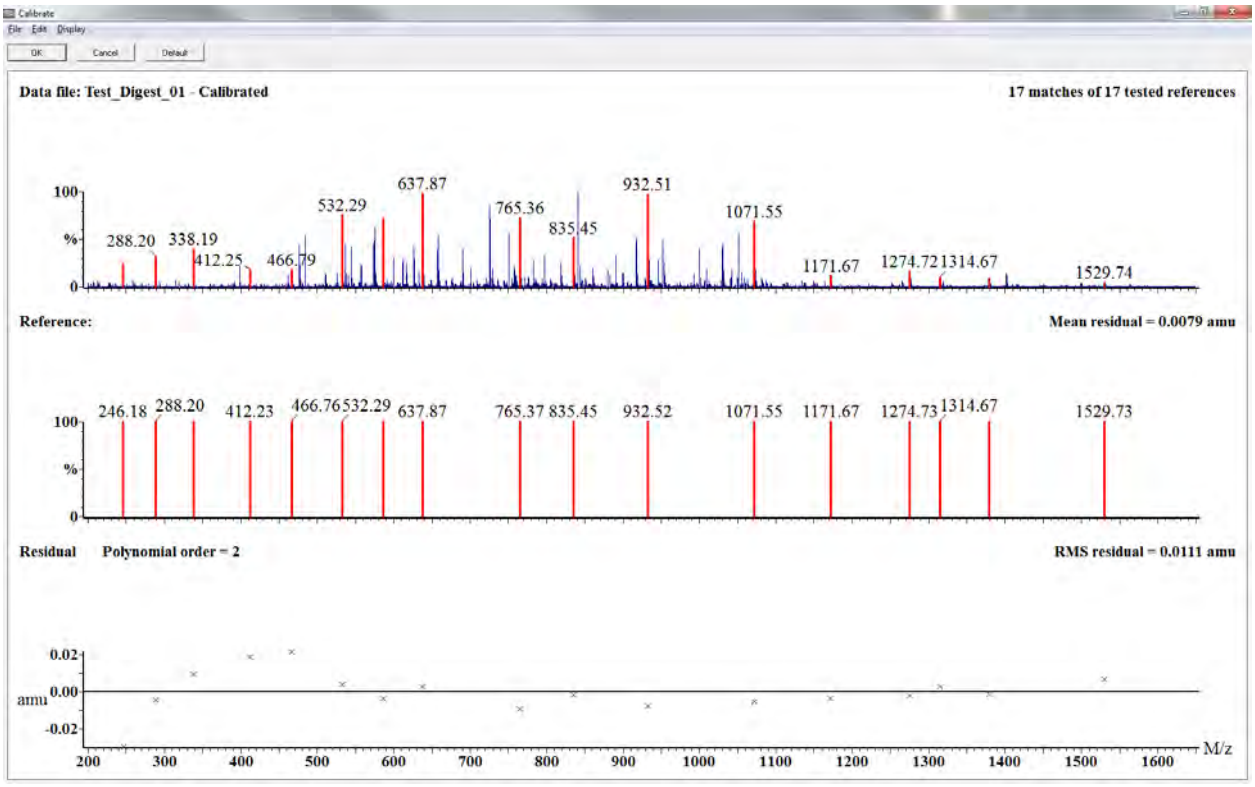

Figure 4.1.2.3. Report following the application of the calibration

The concentration of (α+β) chains in 50-fold diluted blood is approximately $1.9x10^{-4}$ M, based on a haemoglobin concentration in blood of 15 g/100 mL. The concentration of trypsin in the digest solution is 8.2 $x10^{-6}$ M, giving a trypsin to (α+β) chain ratio of 5.8% molar (8.3% by weight).

The following tables show the tryptic peptides together with their masses and *m/z* values predicted to occur in tryptic digests of various globin chains using the AutoDigest Simulation software. The principal *m/z* values observed in practice from 30-minute digests are shown underlined:

Table 4.1.1. α-chain peptides, all values are monoisotopic.

Table 4.1.2. β-chain peptides, all values are monoisotopic.

Table 4.1.3, α-chain peptides, values are monoisotopic up to mass 2,200 Da and average above 2,200 Da.

Table 4.1.4. β-chain peptides, values are monoisotopic up to mass 2,200 Da and average above 2,200 Da.

Table 4,.1.5. δ-chain peptides, all values are monoisotopic. See header to table for further information.

Table 4.1.6. γ-chain peptides, all values are monoisotopic. See header to table for further information.

Table 4.1.1. The masses and *m/z* values of peptides predicted to occur in 30-minute tryptic digests of the **α-chain**. All values are **monoisotopic**. The principal *m/z* values observed in practice are underlined. αT12 and αT13 are not observed at useful levels in 30-minute digests.

```
Data file:    HBA_HUMA.emb,    Mon May 29 12:15:18 2006
Description: HEMOGLOBIN ALPHA CHAIN.   HOMO SAPIENS (HUMAN), PAN TROGLODYTES (CHIMPANZEE), AND PAN PANISCUS
Source:      EMBL P01922, 21-JUL-1986 (REL. 01, LAST SEQUENCE UPDATE)

Average Mass = 15126.3807, Monoisotopic Mass = 15116.8851
N-Terminus = H, C-Terminus = OH
Digest: Trypsin:/K-\P /R-\P
```

Frag#	Res#	Sequence	Mass (Da) Mono→Ave at4500	[M+H]	[M+2H]	[M+3H]	[M+4H]
T1	1-7	(-)VLSPADK(T)	728.41	729.41	365.21	243.81	183.11
T2	8-11	(K)TNVK(A)	460.26	461.27	231.14	154.43	116.07
T3	12-16	(K)AAWGK(V)	531.28	532.29	266.65	178.10	133.83
T4	17-31	(K)VGAHAGEYGAEALER(M)	1528.73	1529.73	765.37	510.58	383.19
T5	32-40	(R)MFLSFPTTK(T)	1070.55	1071.55	536.28	357.86	268.64
T6	41-56	(K)TYFPHFDLSHGSAQVK(G)	1832.88	1833.89	917.45	611.97	459.23
T7	57-60	(K)GHGK(K)	397.21	398.22	199.61	133.41	100.31
T8	61-61	(K)K(V)	146.11	147.11	74.06	49.71	37.53
T9	62-90	(K)VADALTNAVAHVDDMPNALSALSDLHAHK(L)	2995.48	2996.49	1498.75	999.50	749.88
T10	91-92	(K)LR(V)	287.20	288.20	144.61	96.74	72.81
T11	93-99	(R)VDPVNFK(L)	817.43	818.44	409.72	273.49	205.37
T12	100-127	(K)LLSHCLLVTLAAHLPAEFTPAVHASLDK(F)	2966.61	2967.61	1484.31	989.88	742.66
T13	128-139	(K)FLASVSTVLTSK(Y)	1251.71	1252.72	626.86	418.24	313.93
T14	140-141	(K)YR(-)	337.18	338.18	169.60	113.40	85.30
T1-2	1-11	(-)VLSPADKTNVK(A)	1170.66	1171.67	586.34	391.23	293.67
T2-3	8-16	(K)TNVKAAWGK(V)	973.53	974.54	487.78	325.52	244.39
T3-4	12-31	(K)AAWGKVGAHAGEYGAEALER(M)	2042.00	2043.00	1022.01	681.67	511.51
T4-5	17-40	(K)VGAHAGEYGAEALERMFLSFPTTK(T)	2581.26	2582.27	1291.64	861.43	646.32
T5-6	32-56	(R)MFLSFPTTKTYFPHFDLSHGSAQVK(G)	2885.42	2886.43	1443.72	962.81	722.36
T6-7	41-60	(K)TYFPHFDLSHGSAQVKGHGK(K)	2212.08	2213.09	1107.05	738.37	554.03
T7-8	57-61	(K)GHGKK(V)	525.30	526.31	263.66	176.11	132.33
T8-9	61-90	(K)KVADALTNAVAHVDDMPNALSALSDLHAHK(L)	3123.58	3124.58	1562.80	1042.20	781.90
T9-10	62-92	(K)VADALTNAVAHVDDMPNALSALSDLHAHKLR(V)	3264.67	3265.68	1633.34	1089.23	817.17
T10-11	91-99	(K)LRVDPVNFK(L)	1086.62	1087.63	544.32	363.21	272.66
T11-12	93-127	(R)VDPVNFKLLSHCLLVTLAAHLPAEFTPAVHAS LDK(F)	3766.03	3767.04	1884.02	1256.35	942.51
T12-13	100-139	(K)LLSHCLLVTLAAHLPAEFTPAVHASLDKFLAS VSTVLTSK(Y)	4200.30	4201.31	2101.16	1401.11	1051.08
						T12-13 5+ = 841.60	
T13-14	128-141	(K)FLASVSTVLTSKYR(-)	1570.87	1571.88	786.44	524.63	393.73

Table 4.1.2. The masses and *m/z* values of peptides predicted to occur in 30-minute trypic digests of the **β-chain**. All values are **monoisotopic**. The principal *m/z* values observed in practice are underlined. βT10 and βT11 are not observed at useful levels in 30-minute digests.

```
Data file:   HBB_HUMA.emb,   Mon May 29 12:02:11 2006
Description: HEMOGLOBIN BETA CHAIN.   HOMO SAPIENS (HUMAN), PAN TROGLODYTES (CHIMPANZEE), AND PAN PANISCUS
Source:      EMBL P02023, 21-JUL-1986 (REL. 01, LAST SEQUENCE UPDATE)

Average Mass = 15867.2406, Monoisotopic Mass = 15857.2497
N-Terminus = H, C-Terminus = OH
Digest: Trypsin:/K-\P /R-\P
```

Frag#	Res#	Sequence	Mass (Da) Mono→Ave at 4500	[M+H]	[M+2H]	[M+3H]	[M+4H]
T1	1-8	(-)VHLTPEEK(S)	951.50	952.51	476.76	318.18	238.88
T2	9-17	(K)SAVTALWGK(V)	931.51	932.52	466.76	311.51	233.89
T3	18-30	(K)VNVDEVGGEALGR(L)	1313.66	1314.67	657.84	438.89	329.42
T4	31-40	(R)LLVVYPWTQR(F)	1273.72	1274.73	637.87	425.58	319.44
T5	41-59	(R)FFESFGDLSTPDAVMGNPK(V)	2057.94	2058.95	1029.98	686.99	515.49
T6	60-61	(K)VK(A)	245.17	246.18	123.59	82.73	62.30
T7	62-65	(K)AHGK(K)	411.22	412.23	206.62	138.08	103.81
T8	66-66	(K)K(V)	146.11	147.11	74.06	49.71	37.53
T9	67-82	(K)VLGAFSDGLAHLDNLK(G)	1668.88	1669.89	835.45	557.30	418.23
T10	83-95	(K)GTFATLSELHCDK(L)	1420.67	1421.67	711.34	474.56	356.17
T11	96-104	(K)LHVDPENFR(L)	1125.56	1126.56	563.79	376.19	282.40
T12	105-120	(R)LLGNVLVCVLAHHFGK(E)	1718.97	1719.97	860.49	574.00	430.75
T13	121-132	(K)EFTPPVQAAYQK(V)	1377.69	1378.70	689.85	460.24	345.43
T14	133-144	(K)VVAGVANALAHK(Y)	1148.67	1149.67	575.34	383.90	288.17
T15	145-146	(K)YH(-)	318.13	319.14	160.07	107.05	80.54
T1-2	1-17	(-)VHLTPEEKSAVTALWGK(V)	1865.00	1866.01	933.51	622.68	467.26
T2-3	9-30	(K)SAVTALWGKVNVDEVGGEALGR(L)	2227.16	2228.17	1114.59	743.39	557.80
T3-4	18-40	(K)VNVDEVGGEALGRLLVVYPWTQR(F)	2569.37	2570.37	1285.69	857.46	643.35
T4-5	31-59	(R)LLVVYPWTQRFFESFGDLSTPDAVMGNPK(V)	3313.65	3314.66	1657.83	1105.56	829.42
T5-6	41-61	(R)FFESFGDLSTPDAVMGNPKVK(A)	2285.10	2286.11	1143.56	762.71	572.28
T6-7	60-65	(K)VKAHGK(K)	638.39	639.39	320.20	213.80	160.60
T7-8	62-66	(K)AHGKK(V)	539.32	540.33	270.67	180.78	135.84
T8-9	66-82	(K)KVLGAFSDGLAHLDNLK(G)	1796.98	1797.99	899.50	600.00	450.25
T9-10	67-95	(K)VLGAFSDGLAHLDNLKGTFATLSELHCDK(L)	3071.54	3072.55	1536.78	1024.85	768.89
T10-11	83-104	(K)GTFATLSELHCDKLHVDPENFR(L)	2528.21	2529.22	1265.11	843.75	633.06
T11-12	96-120	(K)LHVDPENFRLLGNVLVCVLAHHFGK(E)	2826.51	2827.52	1414.26	943.18	707.64
T12-13	105-132	(R)LLGNVLVCVLAHHFGKEFTPPVQAAYQK(V)	3078.65	3079.66	1540.33	1027.22	770.67
T13-14	121-144	(K)EFTPPVQAAYQKVVAGVANALAHK(Y)	2508.35	2509.36	1255.18	837.12	628.10
T14-15	133-146	(K)VVAGVANALAHKYH(-)	1448.79	1449.80	725.40	483.94	363.21

Table 4.1.3. The masses and *m/z* values of peptides predicted to occur in 30-minute tryptic digests of the **α-chain**. Values are **monoisotopic up to mass 2,200 Da and average above 2,200 Da**. The principal *m/z* values observed in practice are underlined. αT12 and αT13 are not observed at useful levels in 30-min digests.

```
Data file:   HBA_HUMA.emb,   Mon May 29 12:11:56 2006
Description: HEMOGLOBIN ALPHA CHAIN.   HOMO SAPIENS (HUMAN), PAN TROGLODYTES (CHIMPANZEE), AND PAN PANISCUS
Source:      EMBL P01922, 21-JUL-1986 (REL. 01, LAST SEQUENCE UPDATE)

Average Mass = 15126.3807, Monoisotopic Mass = 15116.8851
N-Terminus = H, C-Terminus = OH
Digest: Trypsin:/K-\P /R-\P
```

Frag#	Res#	Sequence	Mass (Da) Mono→Ave at2200	[M+H]	[M+2H]	[M+3H]	[M+4H]
T1	1-7	(-)VLSPADK(T)	728.41	729.41	365.21	243.81	183.11
T2	8-11	(K)TNVK(A)	460.26	461.27	231.14	154.43	116.07
T3	12-16	(K)AAWGK(V)	531.28	532.29	266.65	178.10	133.83
T4	17-31	(K)VGAHAGEYGAEALER(M)	1528.73	1529.73	765.37	510.58	383.19
T5	32-40	(R)MFLSFPTTK(T)	1070.55	1071.55	536.28	357.86	268.64
T6	41-56	(K)TYFPHFDLSHGSAQVK(G)	1832.88	1833.89	917.45	611.97	459.23
T7	57-60	(K)GHGK(K)	397.21	398.22	199.61	133.41	100.31
T8	61-61	(K)K(V)	146.11	147.11	74.06	49.71	37.53
T9	62-90	(K)VADALTNAVAHVDDMPNALSALSDLHAHK(L)	2997.34	2998.35	1499.68	1000.12	750.34
T10	91-92	(K)LR(V)	287.20	288.20	144.61	96.74	72.81
T11	93-99	(R)VDPVNFK(L)	817.43	818.44	409.72	273.49	205.37
T12	100-127	(K)LLSHCLLVTLAAHLPAEFTPAVHASLDK(F)	2968.51	2969.52	1485.27	990.51	743.14
T13	128-139	(K)FLASVSTVLTSK(Y)	1251.71	1252.72	626.86	418.24	313.93
T14	140-141	(K)YR(-)	337.18	338.18	169.60	113.40	85.30
T1-2	1-11	(-)VLSPADKTNVK(A)	1170.66	1171.67	586.34	391.23	293.67
T2-3	8-16	(K)TNVKAAWGK(V)	973.53	974.54	487.78	325.52	244.39
T3-4	12-31	(K)AAWGKVGAHAGEYGAEALER(M)	2042.00	2043.00	1022.01	681.67	511.51
T4-5	17-40	(K)VGAHAGEYGAEALERMFLSFPTTK(T)	2582.92	2583.93	1292.47	861.98	646.74
T5-6	32-56	(R)MFLSFPTTKTYFPHFDLSHGSAQVK(G)	2887.31	2888.32	1444.66	963.45	722.84
T6-7	41-60	(K)TYFPHFDLSHGSAQVKGHGK(K)	2213.44	2214.45	1107.73	738.82	554.37
T7-8	57-61	(K)GHGKK(V)	525.30	526.31	263.66	176.11	132.33
T8-9	61-90	(K)KVADALTNAVAHVDDMPNALSALSDLHAHK(L)	3125.51	3126.52	1563.76	1042.85	782.39
T9-10	62-92	(K)VADALTNAVAHVDDMPNALSALSDLHAHKLR(V)	3266.69	3267.70	1634.35	1089.90	817.68
T10-11	91-99	(K)LRVDPVNFK(L)	1086.62	1087.63	544.32	363.21	272.66
T11-12	93-127	(R)VDPVNFKLLSHCLLVTLAAHLPAEFTPAVHASLDK(F)	3768.44	3769.45	1885.23	1257.15	943.12
T12-13	100-139	(K)LLSHCLLVTLAAHLPAEFTPAVHASLDKFLASVSTVLTSK(Y)	4202.97	4203.98	2102.49	1402.00	1051.75
T13-14	128-141	(K)FLASVSTVLTSKYR(-)	1570.87	1571.88	786.44	524.63	393.73

Table 4.1.4. The masses and *m/z* values of peptides predicted to occur in 30-minute tryptic digests of the **β-chain**. Values **are monoisotopic up to mass 2,200 Da and average above 2,200 Da**. The principal *m/z* values observed in practice are underlined. βT10 and βT11 are not observed at useful levels in 30-min digests.

```
Data file:   HBB_HUMA.emb,   Mon May 29 12:06:36 2006
Description: HEMOGLOBIN BETA CHAIN.   HOMO SAPIENS (HUMAN), PAN TROGLODYTES (CHIMPANZEE), AND PAN PANISCUS
Source:      EMBL P02023, 21-JUL-1986 (REL. 01, LAST SEQUENCE UPDATE)

Average Mass = 15867.2406, Monoisotopic Mass = 15857.2497
N-Terminus = H, C-Terminus = OH
Digest: Trypsin:/K-\P /R-\P
```

Frag#	Res#	Sequence	Mass (Da) Mono→Ave at2200	[M+H]	[M+2H]	[M+3H]	[M+4H]
T1	1-8	(-)VHLTPEEK(S)	951.50	952.51	476.76	318.18	238.88
T2	9-17	(K)SAVTALWGK(V)	931.51	932.52	466.76	311.51	233.89
T3	18-30	(K)VNVDEVGGEALGR(L)	1313.66	1314.67	657.84	438.89	329.42
T4	31-40	(R)LLVVYPWTQR(F)	1273.72	1274.73	637.87	425.58	319.44
T5	41-59	(R)FFESFGDLSTPDAVMGNPK(V)	2057.94	2058.95	1029.98	686.99	515.49
T6	60-61	(K)VK(A)	245.17	246.18	123.59	82.73	62.30
T7	62-65	(K)AHGK(K)	411.22	412.23	206.62	138.08	103.81
T8	66-66	(K)K(V)	146.11	147.11	74.06	49.71	37.53
T9	67-82	(K)VLGAFSDGLAHLDNLK(G)	1668.88	1669.89	835.45	557.30	418.23
T10	83-95	(K)GTFATLSELHCDK(L)	1420.67	1421.67	711.34	474.56	356.17
T11	96-104	(K)LHVDPENFR(L)	1125.56	1126.56	563.79	376.19	282.40
T12	105-120	(R)LLGNVLVCVLAHHFGK(E)	1718.97	1719.97	860.49	574.00	430.75
T13	121-132	(K)EFTPPVQAAYQK(V)	1377.69	1378.70	689.85	460.24	345.43
T14	133-144	(K)VVAGVANALAHK(Y)	1148.67	1149.67	575.34	383.90	288.17
T15	145-146	(K)YH(-)	318.13	319.14	160.07	107.05	80.54
T1-2	1-17	(-)VHLTPEEKSAVTALWGK(V)	1865.00	1866.01	933.51	622.68	467.26
T2-3	9-30	(K)SAVTALWGKVNVDEVGGEALGR(L)	2228.49	2229.50	1115.25	743.84	558.13
T3-4	18-40	(K)VNVDEVGGEALGRLLVVYPWTQR(F)	2570.93	2571.94	1286.47	857.99	643.74
T4-5	31-59	(R)LLVVYPWTQRFFESFGDLSTPDAVMGNPK(V)	3315.80	3316.81	1658.91	1106.27	829.96
T5-6	41-61	(R)FFESFGDLSTPDAVMGNPKVK(A)	2286.59	2287.60	1144.30	763.21	572.66
T6-7	60-65	(K)VKAHGK(K)	638.39	639.39	320.20	213.80	160.60
T7-8	62-66	(K)AHGKK(V)	539.32	540.33	270.67	180.78	135.84
T8-9	66-82	(K)KVLGAFSDGLAHLDNLK(G)	1796.98	1797.99	899.50	600.00	450.25
T9-10	67-95	(K)VLGAFSDGLAHLDNLKGTFATLSELHCDK(L)	3073.48	3074.49	1537.75	1025.50	769.38
T10-11	83-104	(K)GTFATLSELHCDKLHVDPENFR(L)	2529.82	2530.82	1265.92	844.28	633.46
T11-12	96-120	(K)LHVDPENFRLLGNVLVCVLAHHFGK(E)	2828.34	2829.35	1415.18	943.79	708.09
T12-13	105-132	(R)LLGNVLVCVLAHHFGKEFTPPVQAAYQK(V)	3080.65	3081.66	1541.33	1027.89	771.17
T13-14	121-144	(K)EFTPPVQAAYQKVVAGVANALAHK(Y)	2509.89	2510.90	1255.95	837.64	628.48
T14-15	133-146	(K)VVAGVANALAHKYH(-)	1448.79	1449.80	725.40	483.94	363.21

Table 4.1.5. The masses and *m/z* values of peptides predicted to occur in 30-minute tryptic digests of the **δ-chain**. All values are **monoisotopic**. Solid underlines denote observable species differing from β-chain species. Broken underlines denote δ-chain species likely present but masked by β-chain species.

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Data file:    HBD_HUMA.emb
Description: HEMOGLOBIN DELTA CHAIN.   HOMO SAPIENS (HUMAN).
Source:       EMBL P02042, 21-JUL-1986 (REL. 01, LAST SEQUENCE UPDATE), 01-JUN-1994 (REL. 29, LAST ANNOTATION UPDATE)
Printed:      Mon Sep 08 12:59:24 2008

Average Mass = 15924.3170, Monoisotopic Mass = 15914.2494
N-Terminus = H, C-Terminus = OH
Digest: Trypsin:/K-\P /R-\P
```

Frag#	Res#		Sequence	Mass (Da) Mono→Ave at4500	[M+H]	[M+2H]	[M+3H]	[M+4H]	[M+5H]
T1	1-8	*	(-)VHLTPEEK(T)	951.50	952.51	476.76	318.18	238.88	191.31
T2	9-17		(K)TAVNALWGK(V)	958.52	959.53	480.27	320.52	240.64	192.71
T3	18-30		(K)VNVDAVGGEALGR(L)	1255.65	1256.66	628.83	419.56	314.92	252.14
T4	31-40	*	(R)LLVVYPWTQR(F)	1273.72	1274.73	637.87	425.58	319.44	255.75
T5	41-59		(R)FFESFGDLSSPDAVMGNPK(V)	2043.92	2044.93	1022.97	682.32	511.99	409.79
T6	60-61	*	(K)VK(A)	245.17	246.18	123.59	82.73	62.30	50.04
T7	62-65	*	(K)AHGK(K)	411.22	412.23	206.62	138.08	103.81	83.25
T8	66-66	*	(K)K(V)	146.11	147.11	74.06	49.71	37.53	30.23
T9	67-82	*	(K)VLGAFSDGLAHLDNLK(G)	1668.88	1669.89	835.45	557.30	418.23	334.78
T10	83-95		(K)GTFSQLSELHCDK(L)	1463.67	1464.68	732.84	488.90	366.93	293.74
T11	96-104	*	(K)LHVDPENFR(L)	1125.56	1126.56	563.79	376.19	282.40	226.12
T12	105-116		(R)LLGNVLVCVLAR(N)	1268.76	1269.77	635.39	423.93	318.20	254.76
T13	117-120		(R)NFGK(E)	464.24	465.25	233.13	155.75	117.07	93.86
T14	121-132		(K)EFTPQMQAAYQK(V)	1440.67	1441.68	721.34	481.23	361.18	289.14
T15	133-144	*	(K)VVAGVANALAHK(Y)	1148.67	1149.67	575.34	383.90	288.17	230.74
T16	145-146	*	(K)YH(-)	318.13	319.14	160.07	107.05	80.54	64.63
T1-2	1-17		(-)VHLTPEEKTAVNALWGK(V)	1892.02	1893.02	947.02	631.68	474.01	379.41
T2-3	9-30		(K)TAVNALWGKVNVDAVGGEALGR(L)	2196.17	2197.17	1099.09	733.06	550.05	440.24
T3-4	18-40		(K)VNVDAVGGEALGRLLVVYPWTQR(F)	2511.36	2512.37	1256.69	838.13	628.85	503.28
T4-5	31-59		(R)LLVVYPWTQRFFESFGDLSSPDAVMGNPK(V)	3299.63	3300.64	1650.82	1100.89	825.92	660.93
T5-6	41-61		(R)FFESFGDLSSPDAVMGNPKVK(A)	2271.09	2272.10	1136.55	758.04	568.78	455.23
T6-7	60-65	*	(K)VKAHGK(K)	638.39	639.39	320.20	213.80	160.60	128.69
T7-8	62-66	*	(K)AHGKK(V)	539.32	540.33	270.67	180.78	135.84	108.87
T8-9	66-82	*	(K)KVLGAFSDGLAHLDNLK(G)	1796.98	1797.99	899.50	600.00	450.25	360.40
T9-10	67-95		(K)VLGAFSDGLAHLDNLKGTFSQLSELHCDK(L)	3114.54	3115.55	1558.28	1039.19	779.64	623.92
T10-11	83-104		(K)GTFSQLSELHCDKLHVDPENFR(L)	2571.22	2572.23	1286.62	858.08	643.81	515.25
T11-12	96-116		(K)LHVDPENFRLLGNVLVCVLAR(N)	2376.31	2377.32	1189.16	793.11	595.09	476.27
T12-13	105-120		(R)LLGNVLVCVLARNFGK(E)	1714.99	1716.00	858.50	572.67	429.76	344.01
T13-14	117-132		(R)NFGKEFTPQMQAAYQK(V)	1886.90	1887.91	944.46	629.97	472.73	378.39
T14-15	121-144		(K)EFTPQMQAAYQKVVAGVANALAHK(Y)	2571.33	2572.33	1286.67	858.12	643.84	515.27
T15-16	133-146	*	(K)VVAGVANALAHKYH(-)	1448.79	1449.80	725.40	483.94	363.21	290.77

* δ-chain sequence is same as β-chain sequence

Note: Although δT(10-11) and δT(14-15) have essentially the same mass, the latter is unlikely to be present.

Table 4.1.6. The masses and *m/z* values of peptides predicted to occur in 30-minute tryptic digests of the **γ–chains**. All values are **monoisotopic**. Solid underlines denote observed species. Broken underlines denote species not observed at useful levels in 30-minute digests.

BIOLYNX - PROTEIN REPORT Average masses of $^{A}\gamma$- and $^{A}\gamma^{T}$-chains are 16009.3004 and 15997.2460 Da respectively.

Data file: HBGgHUMA.pep
Description: Haemoglobin G-gamma chain. HOMO SAPIENS (HUMAN), AND PAN TROGLODYTES (CHIMPANZEE).
Source: EMBL P02096, 21-JUL-1986 (REL. 01, LAST SEQUENCE UPDATE), 01-FEB-1996 (REL. 33, LAST ANNOTATION UPDATE)
Printed: Wed Mar 25 15:43:47 2009

Average Mass = 15995.2735, Monoisotopic Mass = 15985.2552
N-Terminus = H, C-Terminus = OH
Digest: Trypsin:/K-\P /R-\P

75γ is Thr in $^{A}\gamma^{T}$ & 136γ is Ala in $^{A}\gamma$ and $^{A}\gamma^{T}$

Frag#	Res#		Sequence	Mass (Da) Mono→Ave At 4500	[M+H]	[M+2H]	[M+3H]	[M+4H]	
T1	1-8		(-)GHFTEEDK(A)	961.41	962.42	481.71 }	321.48 }	241.36	
T2	9-17		(K)ATITSLWGK(V)	975.54	976.55	488.78 }	326.19 }	244.89	
T3	18-30		(K)VNVEDAGGETLGR(L)	1315.64	1316.64	658.83	439.55	329.92	
T4	31-40	*	(R)LLVVYPWTQR(F)	1273.72	1274.73	637.87	425.58	319.44	Same as βT4
T5	41-59		(R)FFDSFGNLSSASAIMGNPK(V)	1988.93	1989.94	995.47	663.98	498.24	
T6	60-61	*	(K)VK(A)	245.17	246.18	123.59	82.73	62.30	Same as βT6
T7	62-65	*	(K)AHGK(K)	411.22	412.23	206.62	138.08	103.81	Same as βT7
T8	66-66	*	(K)K(V)	146.11	147.11	74.06	49.71	37.53	Same as βT8
T9	67-76		(K)VLTSLGDAIK(H)	1015.59	1016.60	508.80	339.54	254.91	
T10	77-82		(K)HLDDLK(G)	739.39	740.39	370.70	247.47	185.85	
T11	83-95		(K)GTFAQLSELHCDK(L)	1447.68	1448.68	724.85 }	483.57 }	362.93	
T12	96-104		(K)LHVDPENFK(L)	1097.55	1098.56	549.78 }	366.86 }	275.40	
T13	105-120		(K)LLGNVLVTVLAIHFGK(E)	1693.03	1694.04	847.52	565.35	424.27	
T14	121-132		(K)EFTPEVQASWQK(M)	1448.69	1449.70	725.35	483.91	363.18	Similar to βT(14-15)
T15	133-144		(K)MVTGVASALSSR(Y)	1177.61	1178.62	589.81	393.55	295.41	
T16	145-146	*	(R)YH(-)	318.13	319.14	160.07	107.05	80.54	Same as βT15
T1-2	1-17		(-)GHFTEEDKATITSLWGK(V)	1918.94	1919.95	960.48	640.66	480.74	
T2-3	9-30		(K)ATITSLWGKVNVEDAGGETLGR(L)	2273.17	2274.17	1137.59	758.73	569.30	
T3-4	18-40		(K)VNVEDAGGETLGRLLVVYPWTQR(F)	2571.34	2572.35	1286.68	858.12	643.84	
T4-5	31-59		(R)LLVVYPWTQRFFDSFGNLSSASAIMGNPK(V)	3244.64	3245.65	1623.33	1082.55	812.17	
T5-6	41-61		(R)FFDSFGNLSSASAIMGNPKVK(A)	2216.09	2217.10	1109.05	739.71	555.03	
T6-7	60-65	*	(K)VKAHGK(K)	638.39	639.39	320.20	213.80	160.60	Same as βT(6-7)
T7-8	62-66	*	(K)AHGKK(V)	539.32	540.33	270.67	180.78	135.84	Same as βT(7-8)
T8-9	66-76		(K)KVLTSLGDAIK(H)	1143.69	1144.69	572.85	382.24	286.93	
T9-10	67-82		(K)VLTSLGDAIKHLDDLK(G)	1736.97	1737.98	869.49	580.00	435.25	
T10-11	77-95		(K)HLDDLKGTFAQLSELHCDK(L)	2169.05	2170.06	1085.53	724.03	543.27	
T11-12	83-104		(K)GTFAQLSELHCDKLHVDPENFK(L)	2527.22	2528.22	1264.62	843.41	632.81	1Da lower than βT(10-11)
T12-13	96-120		(K)LHVDPENFKLLGNVLVTVLAIHFGK(E)	2772.57	2773.58	1387.29	925.20	694.15	
T13-14	105-132		(K)LLGNVLVTVLAIHFGKEFTPEVQASWQK(M)	3123.71	3124.72	1562.86	1042.25	781.94	
T14-15	121-144		(K)EFTPEVQASWQKMVTGVASALSSR(Y)	2608.30	2609.30	1305.16	870.44	653.08	
T15-16	133-146		(K)MVTGVASALSSRYH(-)	1477.73	1478.74	739.88	493.59	370.44	
T9	67-76		(K)VLTSLGDATK(H)	1003.55	1004.56	502.79	335.53	251.90	In $^{A}\gamma^{T}$
T15	133-144		(K)MVTAVASALSSR(Y)	1191.63	1192.64	596.82	398.22	298.91	In $^{A}\gamma$ and $^{A}\gamma^{T}$

* γ-chain sequence is same as β-chain sequence

4.1.3. Procedure for labelling (annotating) the peaks in adult haemoglobin digest spectra with symbols representing the α- and β-chain tryptic peptides

Introduction.
As part of the procedure for identifying variants, diluted haemoglobin samples are digested with trypsin to produce mixtures of tryptic peptides, which are analysed by ESI-MS. In each of the resulting spectra, normally acquired over the *m/z* range 200-1,650, there are 15 α- and 15 β-chain tryptic peptides, as shown in Tables 4.1.1. and 4.1.2., respectively. The ultimate objective is to identify the peptide containing the variant and hence identify the variant.

The following describes a procedure that assigns the tryptic peptides to peaks in a given digest spectrum, thus acting as an aid towards identifying the variant.

Procedure.
First, make a **centered** spectrum of the digest file to which the tryptic peptides are to be assigned, as follows. This spectrum is assumed to be raw MCA data that has not already been centered as shown in Figure 4.1.2.3. Open the digest spectrum file and display the default *m/z* range.

Select **Process**, **Subtract**. Set **Polynomial order** to 25, **Below curve (%)** to 5 and **Tolerance** to 0.01.

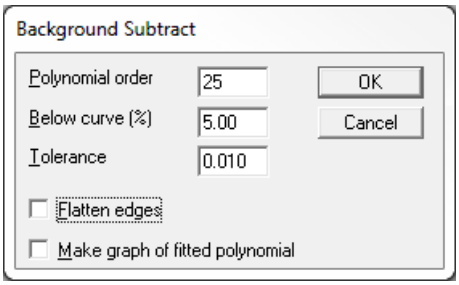

Figure 4.1.3.1. Typical parameters for background subtracting mass spectra

Select **Process**, **Smooth**. Set **Peak width (Da)** to 0.5 and **Number of smooths** to 1. Select **Savitzky Golay**.

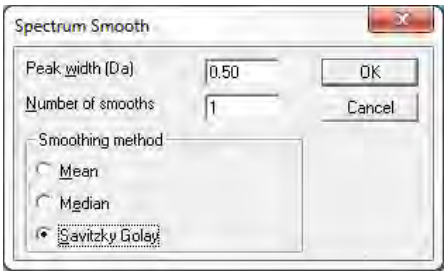

Figure 4.1.3.2. Typical parameters for smoothing digest mass spectra acquired in MCA mode.

Select **Process**, **Spectrum Center**. Set **Min peak width at half height (channels)** to 1. Set **Centroid top (%)** to 50. Check the **Create centered spectrum** box. Select **Heights** and **Replace**.

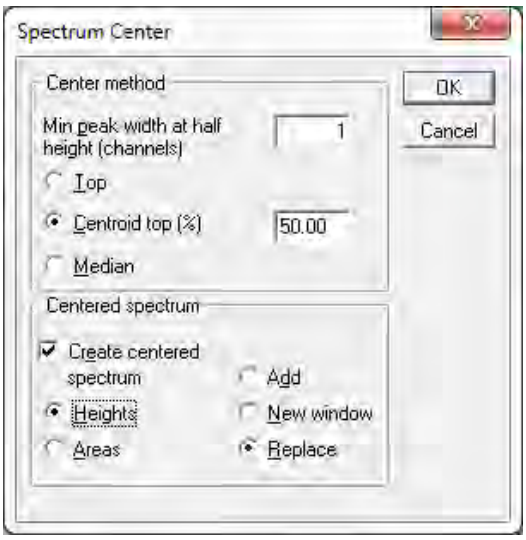

Figure 4.1.3.3. Typical parameters for peak centering mass spectra

This is the centered spectrum to which the tryptic peptides will be assigned.

4.1.3.1. Annotating the α-chain tryptic peptides.

From **BioLynx** and the **Protein/Peptide Editor**: import the EMBL α-chain (HBA_HUMA.emb).

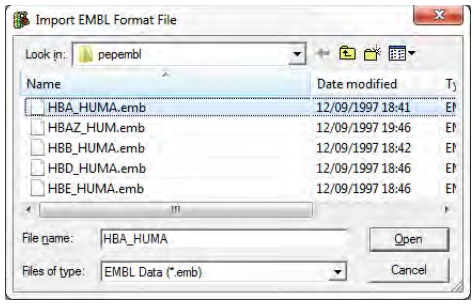

Figure 4.1.3.4. Select the α-chain from Import.

This will show the single letter amino acid sequence of the human α-chain:

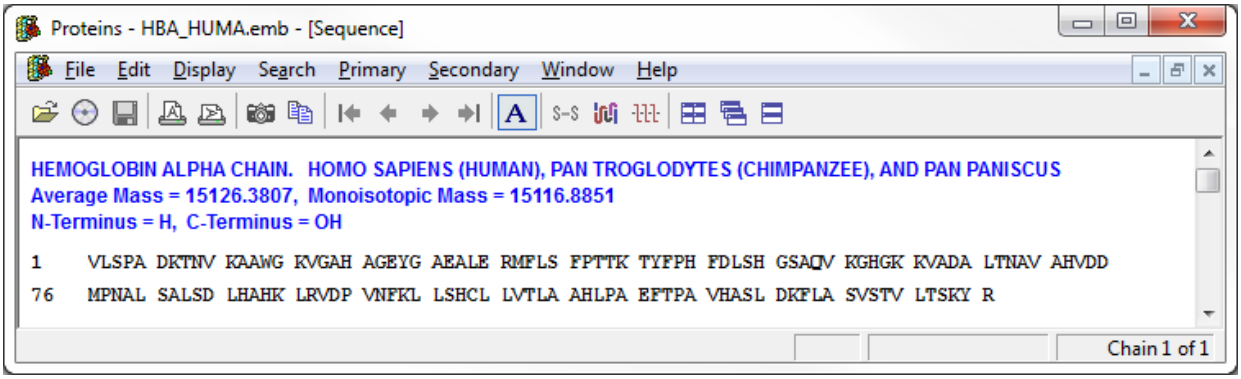

Figure 4.1.3.5. The sequence of the α-chain.

Select **Primary**, **Auto Digest Simulation**.

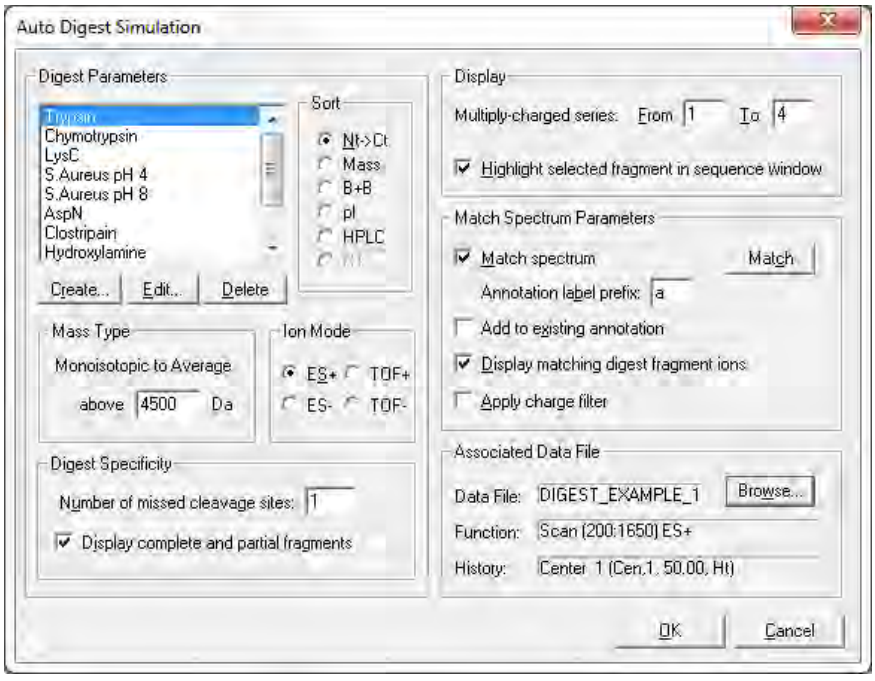

Figure 4.1.3.6. Parameters required for annotating the α-chain tryptic peptides.

Set **Digest Parameters** to **Trypsin**, **Sort** Nt→Ct. Set **Monoisotopic to Average above** to 4,500 Da.

Set **Number of missed cleavage sites:** to 1, and check **Display complete and partial fragments**.

Set **Multiply-charged series From** 1 **To** 4 and check **Highlight selected fragment in sequence window**.

Check **Match Spectrum**.

Set **Annotation label prefix** to a (for α).

Check **Display matching digest fragment ions**.

Press **Match**. Set **Mass window (amu)** to ±0.20 and **Spectrum Threshold** to 1% Full Scale.

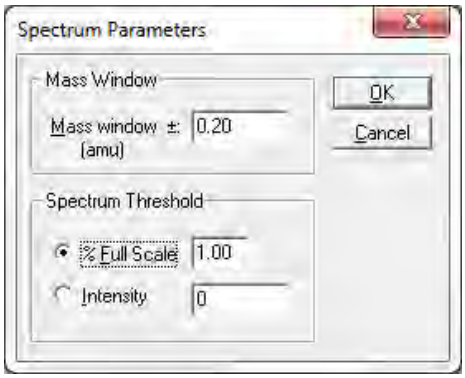

Figure 4.1.3.7. Recommended parameters for peak matching.

Press **Browse** to confirm the currently active spectrum.

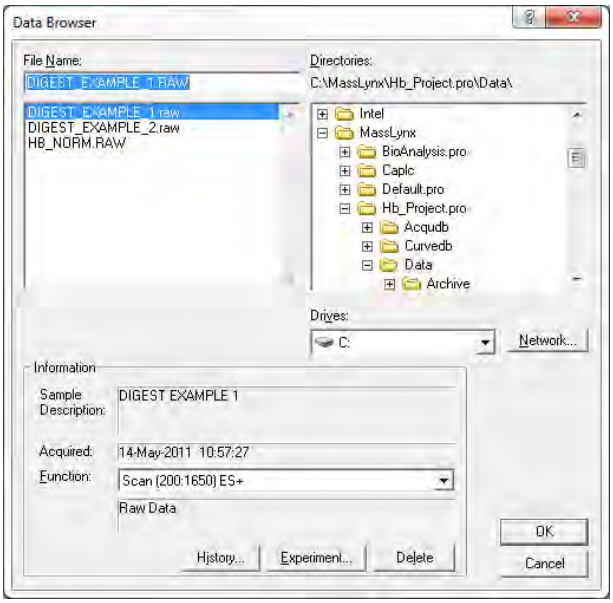

Figure 4.1.3.8. Ensures that the spectrum to be annotated is selected.

Select **History** and select **Center** Spectrum.

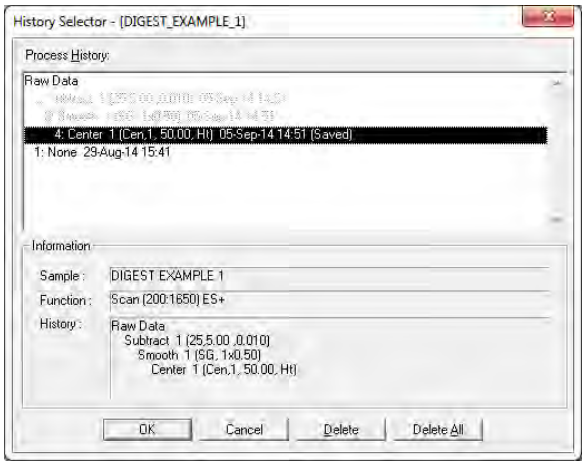

Figure 4.1.3.9. Select the Centered spectrum from History.

Press **OK** three times to annotate the spectrum and return a page of α-chain tryptic peptides.

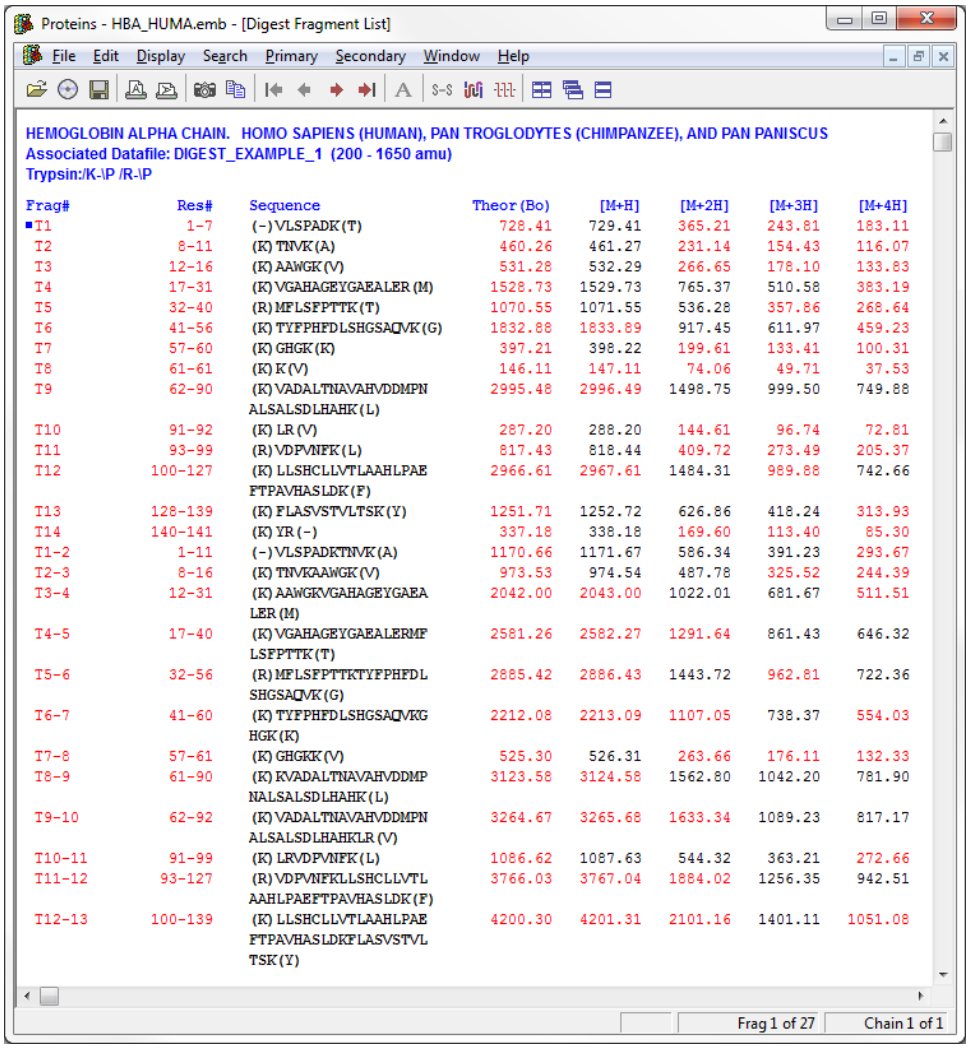

Figure 4.1.3.10. The full list of α-chain tryptic peptides.

All the α-chain tryptic peptides should now be annotated, as shown in Figure 4.1.3.11.

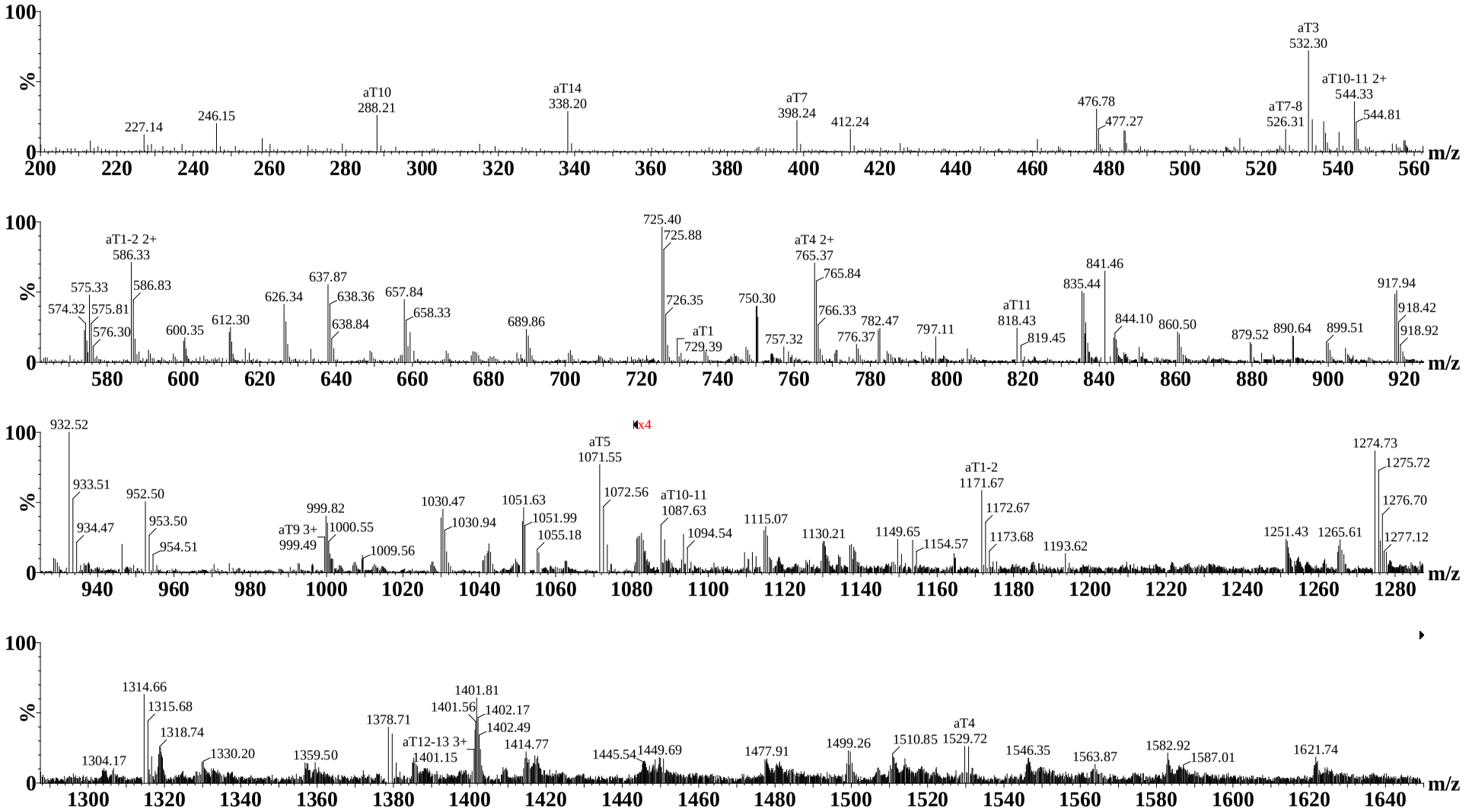

Figure 4.1.3.11. The centered spectrum annotated with the α-chain tryptic peptides. A 4x magnification range >*m/z* 1,080 has been applied for clarity.

4.1.3.2. Annotating the β-chain tryptic peptides.

The procedure is the same as that described for the α-chain, with the following exceptions:
From **BioLynx** and the **Protein/Peptide Editor**: import the EMBL β-chain (HBB_HUMA.emb).

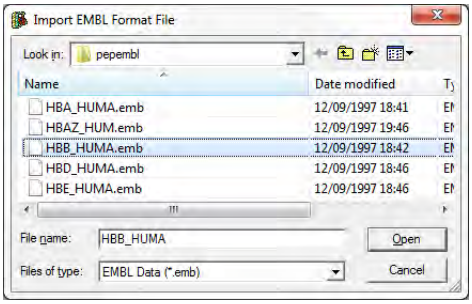

Figure 4.1.3.12. Select the β-chain from Import.

This will show the single letter amino acid sequence of the human β-chain:

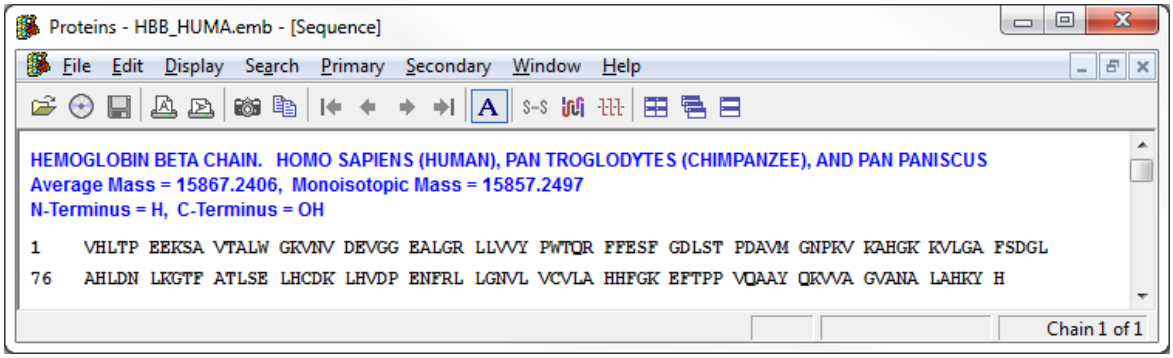

Figure 4.1.3.13. The sequence of the β-chain.

Select **Primary**, **Auto Digest Simulation**.

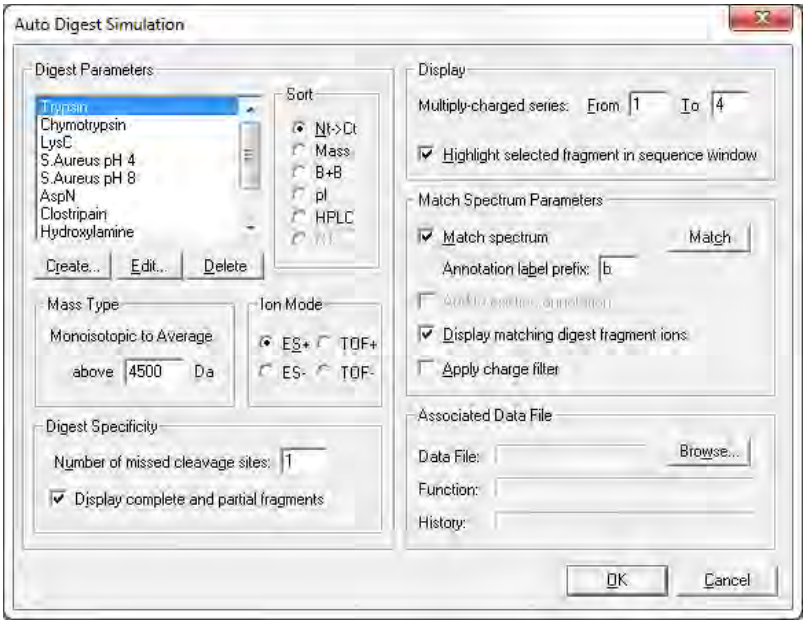

Figure 4.1.3.14. Parameters required for annotating the β-chain tryptic peptides.

Set **Annotation label prefix** to **'b' (for β).**

Press **Browse**, when the currently active spectrum should be highlighted.

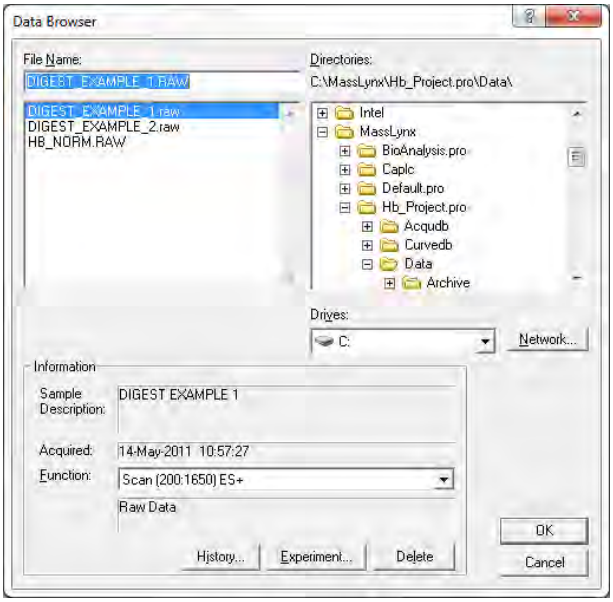

Figure 4.1.3.15. Ensures that the spectrum to be annotated is selected.

Select **History** and select **Center Spectrum**.

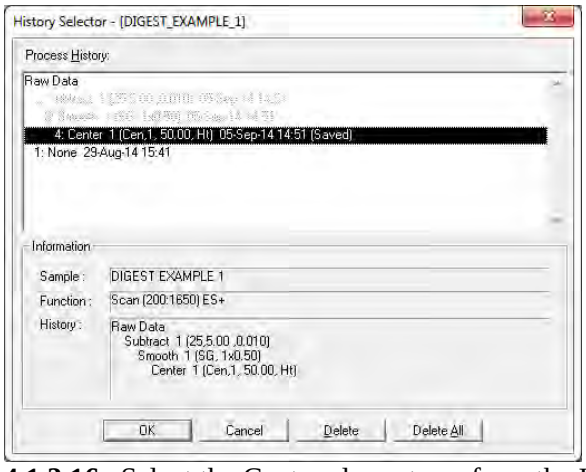

Figure 4.1.3.16. Select the Centered spectrum from the History.

Press **OK** twice to return to the **Auto Digest Simulation** page.

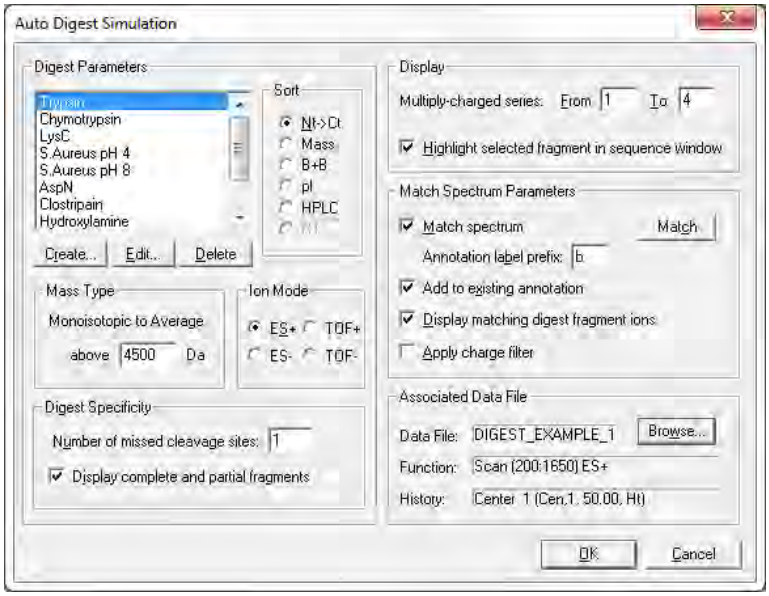

Figure 4.1.3.17. Parameters required for annotating the β-chain tryptic peptides.

Select **Add to existing annotation**. This option should only be available when the α-chain peptides have been annotated. If it is not selected, the β-chain peptides will not be annotated and the α-chain peptides will remain annotated.

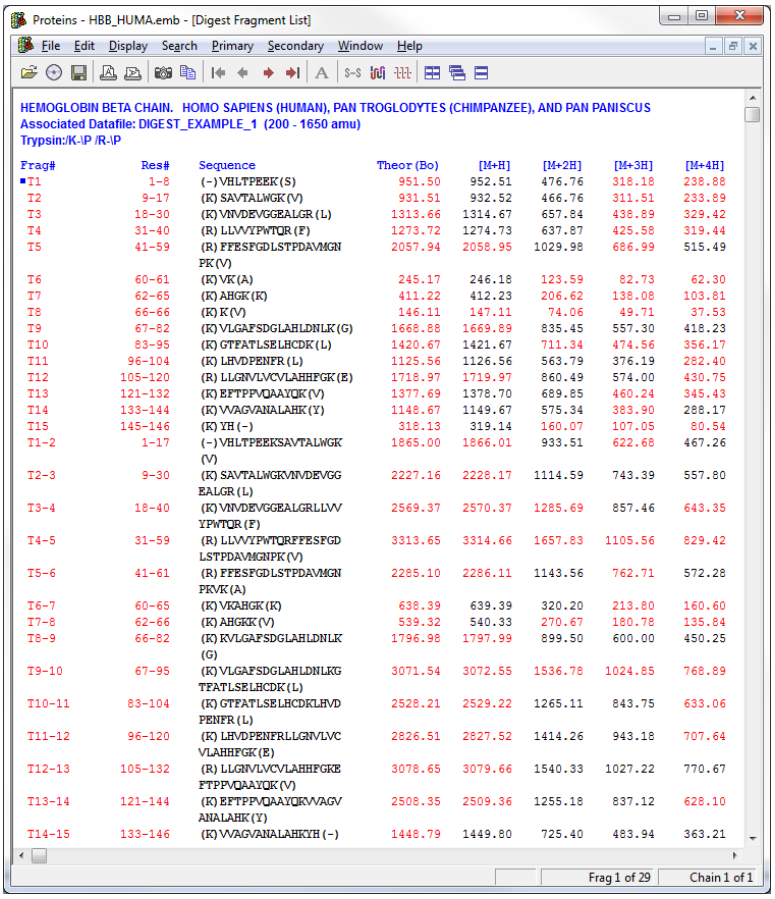

Figure 4.1.3.18. The full list of β-chain tryptic peptides.

All the α- and β-chain tryptic peptides should now be annotated, as shown in Figure 4.1.3.19.

The peaks associated with this spectrum will remain annotated as shown in Figure 4.1.3.19. Hence, when the original MCA data file is opened, the spectrum may be displayed as a background-subtracted and smoothed MCA spectrum, as shown in Figure 4.1.3.20.

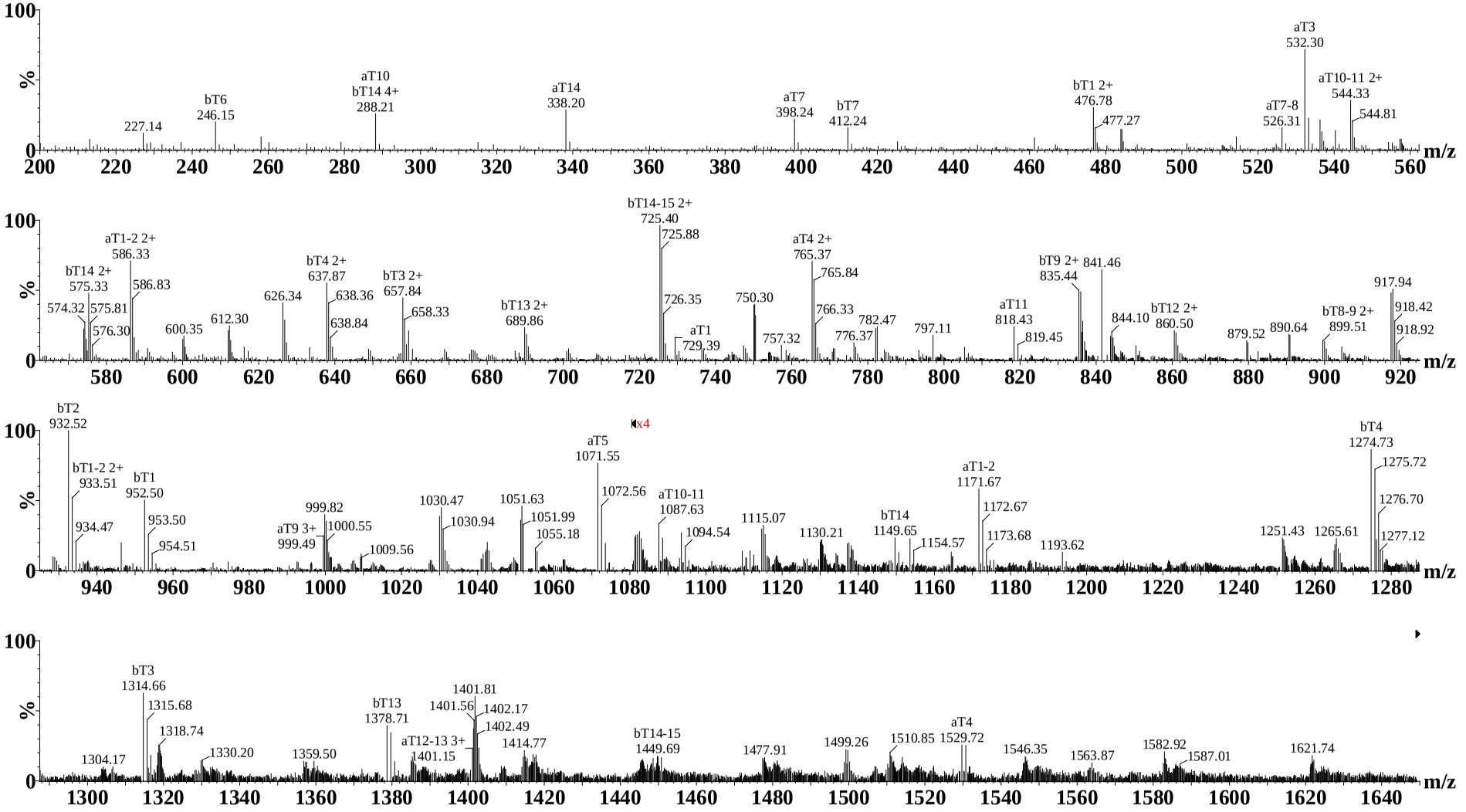

Figure 4.1.3.19. The centered spectrum annotated with both the α- and β-chain tryptic peptides. A 4x magnification range >*m/z* 1,080 has been applied for clarity.

The peaks in the spectra from other haemoglobins may be similarly annotated by selecting appropriate chain sequences. For example, with foetal haemoglobin, substitute the $^{G}\gamma$-chain for the β-chain in Figure 4.1.3.12. The α-chain selected in Figure 4.1.3.4 will, of course, remain unchanged.

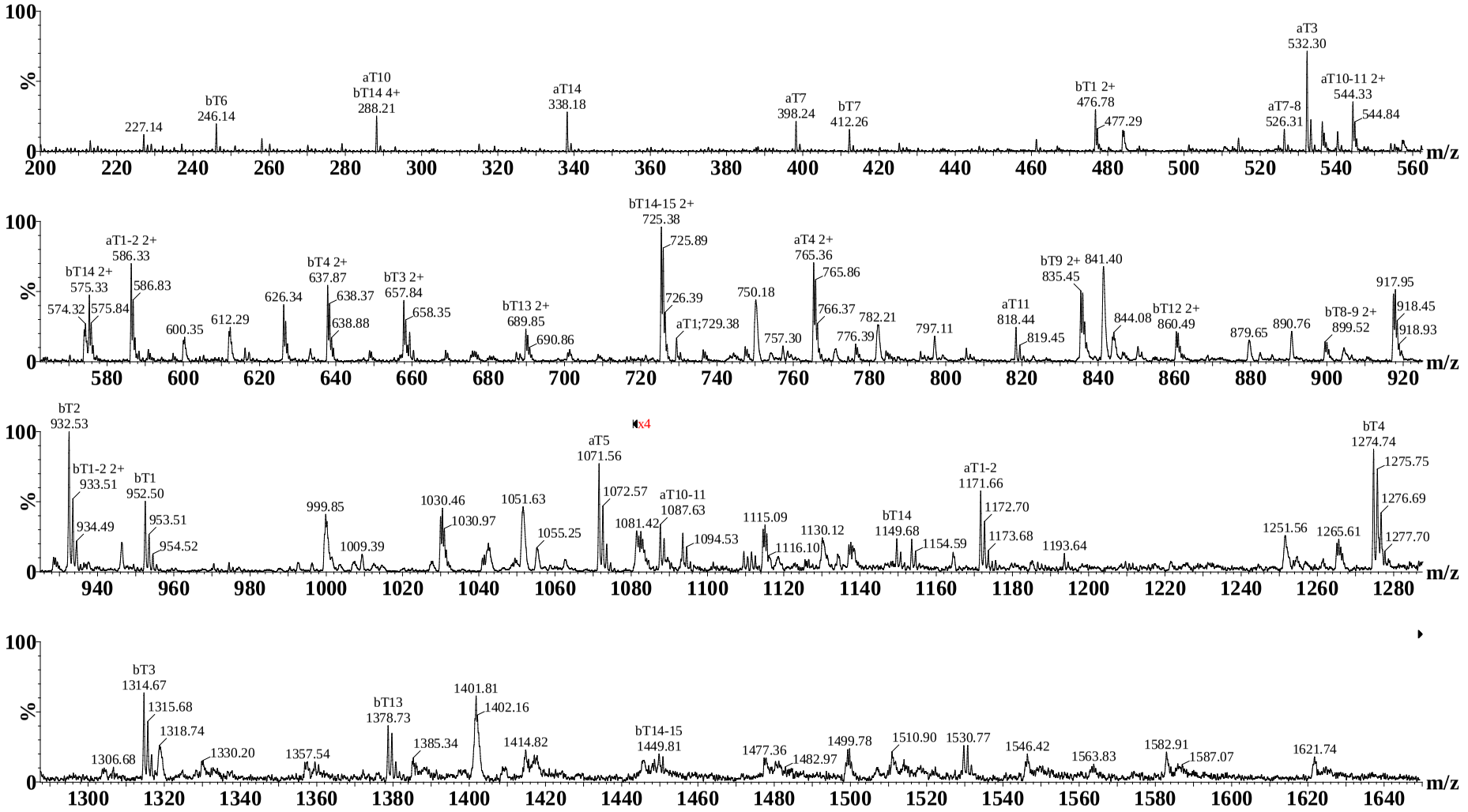

Figure 4.1.3.20. MCA spectrum annotated with both the α- and β-chain tryptic peptides. A 4x magnification range >*m/z* 1,080 has been applied for clarity.

4.1.4. Summary of the tables devised to assist in searching for variant tryptic peptides.

4.2. Tables to assist in searching for tryptic peptides produced from **α-chain** variants that differ from normal by **0, ±1, ±3 and ±4 Da**.

Table 4.2.1. The 37 mutations and *m/z* values of tryptic peptides that are produced as a result of single amino acid changes giving **<±6 Da** change in the mass of the **α-chain** and **do not involve Lys**.

Table 4.2.2. The pairs of tryptic peptides resulting from the seven single amino acid changes that give **<±6 Da** in the mass of the **α-chain** and involve a mutation **to Lys**.

Table 4.2.3. The 32 tryptic peptides and their *m/z* values that result from a single amino acid change giving **<±6 Da** change in the mass of the **α-chain** and involve a mutation **from Lys**.

4.3. Tables to assist in searching for tryptic peptides from **α-chain** variants that **involve Arg or Lys** and give a mass change **>±6 Da** from normal (excluding Arg↔Lys).

4.4. Tables to assist in searching for tryptic peptides produced from **β-chain** variants that differ from normal by **0, ±1, ±3 and ±4 Da**, and do not involve Lys.

Table 4.4.1. The 44 mutations and *m/z* values of tryptic peptides that are produced as a result of single amino acid changes that give **<±6 Da** change in the mass of the **β-chain** and **do not involve Lys**.

Table 4.4.2. The pairs of new tryptic peptides resulting from the twelve single amino acid changes that give **<±6 Da** in the mass of the **β-chain** and involve a mutation **to Lys**.

Table 4.4.3. The 30 tryptic peptides and their *m/z* values that result from a single amino acid change giving **<6 Da** change in the mass of the **β-chain** and involve a mutation **from Lys**.

4.5. Tables to assist in searching for tryptic peptides produced from **β-chain** variants that involve **Arg** or **Lys** and give a mass change **>±6 Da** from normal (excluding Arg to Lys).

4.6. Tables to assist in searching for some tryptic peptides produced from **α- and β-chain** variants that involve the mutations **Xxx↔Asp** when adjacent to Lys.

Table 4.6.1. Some **α6Asp→Xxx** mutations that can occur by a single base change in the nucleotide codon.

Table 4.6.2. Some **α126Asp→Xxx** mutations that can occur by a single base change in the nucleotide codon.

Table 4.6.3. Some **β94Asp→Xxx** mutations that can occur by a single base change in the nucleotide codon.

Table 4.6.4. This table was devised to assist in searching for some of the tryptic peptides resulting from the mutation **Xxx→Asp** when **Xxx is adjacent to Lys**.

4.2. Tables to assist in searching for tryptic peptides produced from α-chain variants that differ from normal by 0, ±1, ±3 and ±4 Da

These tables were devised to assist in searching for tryptic peptides from α-chain variants that cannot be resolved from the normal α-chain when analysing the globin chains in heterozygotes. This situation arises when the normal and variant masses differ by <±6 Da. Within these limits, there are seven mass changes (0, ±1, ±3 and ±4 Da) that are produced by a single amino acid change governed by a single base change in the nucleotide codon. There are 80 such variants in the α-chain including four Leu→Ile mutations. Three types of mutation are considered:

- First, there are mutations that do not involve Lys. These simply change the mass of the tryptic peptide and are listed in Table 4.2.1.
- Second, there are mutations that involve an amino acid change to Lys, which creates a new cleavage site, and hence two new peptides in the tryptic digest (Table 4.2.2.).
- Third, there are mutations that involve an amino acid change from Lys, which removes a tryptic cleavage site (Table 4.2.3.).
- The mutation Leu→Ile produces neither a mass change nor a new cleavage site and thus cannot be detected by mass spectrometry. It is not included in the Tables. Of the 18 Leu in the α-chain, only four at α91, α100, α101 and α113 can mutate to Ile by a single base change in the codon.

The *m/z* values of the variant peptide ions are given in the Tables. For peptides below mass 2,300 Da, only monoisotopic *m/z* values are given (normal font). For peptides above mass 2,300 Da, average *m/z* values are given in italics beneath the monoisotopic values.
Monoisotopic masses are based on: C: 12.0 Da, H: 1.0078250 Da, N: 14.0030740 Da, O: 15.9949146 Da and S: 31.9720718 Da. Average masses are based on: C: 12.011 Da, H: 1.00794 Da, N: 14.00674 Da, O:15.9994 Da and S: 32.066 Da.
NL: Not listed in http://globin.bx.psu.edu
I: interference.
ΔM is the nominal mass change of the variant α-chain from normal (Da).
Variants that have been identified and *m/z* values that have been observed are shown in bold font.
All the mutations shown in the tables can occur by a single base change in the DNA codon.
Unless otherwise stated, the variants are in the α1 or α2 gene. αCT: α-chymotrypsin
Cleavage by trypsin at α7Lys and α127Lys is hindered by α6Asp and α126Asp, respectively, giving mainly αT1-2 and αT12-13 peptides. These peptides are included in the tables.

Table 4.2.1. The 37 mutations and *m/z* values of tryptic peptides that are produced as a result of single amino acid changes giving <±6 Da change in the mass of the α-chain and do not involve Lys.

Peptide Mutation	ΔM	Name	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
αT1						
α6Asp→Asn	**-1**	**Dunn**		**243.482**	**364.719**	**728.431**
α4Pro→Thr	4	NL		245.142	367.209	733.410
αT2						
α9Asn→Ile	-1	NL		154.110	230.661	460.314 (I)
α9Asn→Asp	1	NL		154.757	231.632	462.256 (I)
α8Thr→Pro	-4	NL		153.098	229.143	457.277 (I)
αT1-2						
α6Asp→Asn	-1	Dunn		390.900	585.846	1,170.685
α9Asn→Ile	-1	NL		390.908	585.859	1,170.710
α9Asn→Asp	1	NL		391.556	586.830	1,172.653
α8Thr→Pro	-4	NL		389.896	584.341	1,167.674
α4Pro→Thr	4	NL		392.560	588.336	1,175.664

Peptide Mutation	ΔM	Name	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
αT4						
α23Glu→Gln	**-1**	**Memphis**	382.944	**510.255**	**764.879**	**1,528.751**
α27Glu→Gln	-1	NL				
α30Glu→Gln	**-1**	**G-Honolulu**		**510.255**	**764.879**	1,528.751
Beware confusion with O-Padova. Distinguish by ce-HPLC. G-Honolulu elutes with Hb A_2						
αT5						
α38Thr→Pro	-4	NL		356.525	534.284	1,067.560
α39Thr→Pro	-4	NL				
α37Pro→Thr	4	NL		359.188 (I)	538.279	1,075.550
αT6						
α47Asp→Asn	**-1**	**Arya**	458.983	**611.641**	**916.958**	**1,832.908**
α54Gln→Glu	**1**	**Mexico**	459.475	**612.297**	**917.942**	1,834.876
α41Thr→Pro	-4	NL	721.364	961.483	1,441.721	2,882.434
α44Pro→Thr	4	NL	460.228	613.301	919.448	1,837.887
αT9 Overlay normal & suspect variant $\alpha T9^{3+}$ or $\alpha T9^{4+}$ to detect -1 Da variants. Identify using α-CT						
α64Asp→Asn	-1	**G-Waimanalo**	749.632	**999.172**	**1,498.257**	2,995.506
			750.097	*999.793*	*1,499.186*	*2,997.363*
α68Asn→Ile	-1	NL	749.637	999.182	1,498.269	2,995.531
			750.107	*999.806*	*1,499.206*	*2,997.403*
α74Asp→Asn	**-1**	**G-Pest**	749.632	**999.172**	**1,498.257**	2,995.506
			750.097	***999.793***	***1,499.186***	*2,997.363*
α75Asp→Asn	-1	Matsue-Oki				
α78Asn→Ile	-1	NL				
α85Asp→Asn	**-1**	**G-Norfolk**	749.632	**999.172**	**1,498.257**	2,995.506
α68Asn→Asp	1	Ube-2	750.124	999.830	1,499.241	2,997.474
			750.589	*1,000.450*	*1,500.170*	*2,999.333*
α78Asn→Asp	1	J-Singa				
α67Thr→Pro	-4	NL	748.880	998.170	1,496.751	2,992.495
			749.346	*998.792*	*1,497.684*	*2,994.360*
α77Pro→Thr	4	NL	750.877	1,000.834	1,500.746	3,000.485
			751.340	*1,001.451*	*1,501.672*	*3,002.336*
αT11						
α94Asp→Asn	**-1**	**Titusville**		273.158	**409.233**	**817.457**
α97Asn→Ile	-1	NL		273.166	409.245	817.482
α97Asn→Asp	1	Cheektowaga, α2		273.814	410.217	819.425
α95Pro→Thr	4	Godavari		274.817	411.722	822.436
αT12						
α116Glu→Gln	-1	Oleander	742.413	989.548	1,483.818	2,966.629
			742.890	*990.185*	*1,484.773*	*2,968.538*
α126Asp→Asn	-1	Tarrant	742.413	989.548	1,483.818	2,966.629
(special case, see Table 4.6.2)			*742.890*	*990.185*	*1,484.773*	*2,968.538*
α108Thr→Pro	-4	NL	741.660	988.545	1,482.313	2,963.618
			742.139	*989.183*	*1,483.271*	*2,965.534*
α118Thr→Pro	-4	NL				
α114Pro→Thr	4	Jura, α2	743.658	991.208	1,486.308	2,971.608
			744.134	*991.842*	*1,487.259*	*2,983.511*
α119Pro→Thr	4	NL				

Peptide Mutation	**ΔM**	**Name**	**$(M+4H)^{4+}$**	**$(M+3H)^{3+}$**	**$(M+2H)^{2+}$**	**$(M+H)^{+}$**
αT12						
α116Glu→Gln	-1	Oleander	742.413	989.548	1,483.818	2,966.629
			742.890	*990.185*	*1,484.773*	*2,968.538*
α126Asp→Asn	-1	Tarrant	742.413	989.548	1,483.818	2,966.629
(special case, see Table 4.6.2)			*742.890*	*990.185*	*1,484.773*	*2,968.538*
α108Thr→Pro	-4	NL	741.660	988.545	1,482.313	2,963.618
			742.139	*989.183*	*1,483.271*	*2,965.534*
α118Thr→Pro	-4	NL				
α114Pro→Thr	4	Jura, α2	743.658	991.208	1,486.308	2,971.608
			744.134	*991.842*	*1,487.259*	*2,983.511*
α119Pro→Thr	4	NL				

Peptide Mutation	**ΔM**	**Name**	**$(M+5H)^{5+}$**	**$(M+4H)^{4+}$**	**$(M+3H)^{3+}$**	**$(M+2H)^{2+}$**
αT12-13 Overlay normal and suspect $\alpha T(12\text{-}13)^{3+}$ or $\alpha T(12\text{-}13)^{4+}$ ions to detect α/(α-1 Da) variants in heterozygotes. Identify using α-CT digests.						
α116Glu→Gln	**-1**	**Oleander**	840.871	1,050.837	1,400.781	2,100.667
			841.406	*1,051.505*	*1,401.671*	*2,102.002*
α126Asp→Asn	-1	Tarrant	Expect cleavage at 127Lys to give variant αT12 and normal αT13 peptides. See also Table 4.6.2			
α108Thr→Pro	-4	NL	840.269	1,050.085	1,399.777	2,099.161
			840.805	*1,050.754*	*1,400.670*	*2,100.500*
α118Thr→Pro	-4	NL				
α134Thr→Pro	-4	NL				
α137Thr→Pro	-4	NL				
α114Pro→Thr	4	Jura, α2	841.867	1,052.082	1,402.440	2,103.156
			842.400	*1,052.748*	*1,403.329*	*2,104.489*
α119Pro→Thr	4	NL				

Peptide Mutation	**ΔM**	**Name**	**$(M+4H)^{4+}$**	**$(M+3H)^{3+}$**	**$(M+2H)^{2+}$**	**$(M+H)^{+}$**
αT13						
α134Thr→Pro	-4	NL	312.936	416.912	624.864	1,248.720
α137Thr→Pro	-4	NL				

Table 4.2.2. The pairs of tryptic peptides resulting from the seven single amino acid changes that give less than ±6 Da change in the mass of the α-chain and involve a mutation to Lys. Thus, a new cleavage site is produced, creating two new tryptic peptides. These mutations give a significant increase in positive charge and are readily detected by ce-HPLC and IEF.

The presence of these new peptide ions at their predicted *m/z* values identifies the mutation.

Mutation	**ΔM**	**Name**	**$(M+3H)^{3+}$**	**$(M+2H)^{2+}$**	**$(M+H)^{+}$**
Peptides					
α23Glu→Lys	-1	Chad			
αT4a, 17VGAHAGK			213.791	320.183	639.358
αT4b, 24YGAEALER			303.488	454.728	908.448
α27Glu→Lys	**-1**	**Shuangfeng**			
αT4a, 17VGAHAGEYGAK			**353.846**	**530.265**	1,059.522
αT4b, 28ALER			163.433	244.646	**488.283**
α30Glu→Lys	**-1**	**O-Padova**			
αT4a, 17VGAHAGEYGAEALK			458.234	686.847	1,372.686
αT4b, 31R					175.120
αT4, 17VGAHAGEYGAEALKR			**510.268**	**764.898**	1,528.787

The αT4a peptide from O-Padova does not occur in 30-minute digests leading to possible confusion with G-Honolulu. Hence, variant αT4 is observed. Use ce-HPLC data to distinguish. On the ce-HPLC, G-Honolulu elutes with Hb A_2. O-Padova elutes just before Hb C.

α32Met→Lys	-3	NL			
αT5a, 32K					147.113
αT5b, 33FLSFPTTK			314.177	470.761	940.514
α54Gln→Lys	0	NL			
αT6a, 41TYFPHFDLSHGSAK			536.260 (I)	803.887	1,606.765
αT6b, 55VK				123.595	246.182(I)
α76Met→Lys	-3	Noko			
αT9a, 62VADALTNAVAHVDDK			513.599	769.895	1,538.781
αT9b, 77PNALSALSDLHAHK			491.932 (I)	737.395	1,473.781
α116Glu→Lys	**-1**	**O-Indonesia**			
αT12a, 100LLSHCLLVTLAAHLPAK			**600.691** (I)	**900.532 (I)**	**1,800.06(I)**
αT12b, 117FTPAVHASLDK			395.881	593.317	1,185.627
αT12b-13, 117FTPAVHASLDKFLASVSTVLTSK					
			807.113	**1,210.166**	2,419.324
			807.609	***1,210.910***	2,420.812

Expect little or no cleavage at -KP- in Noko above, i.e. look for -3 Da mass change in αT9.
There is little or no cleavage between αT12b and αT13 in O-Indonesia due to α126Asp adjacent to α127Lys. Hence αT(12b-13) is observed.

Table 4.2.3. The 32 tryptic peptides and their *m/z* values that result from a single amino acid change giving <±6 Da change in the α-chain mass and involve a mutation from Lys. Thus, a tryptic cleavage site is abolished and two adjacent tryptic peptides are combined into one larger new peptide. These mutations give a significant increase in negative charge and are readily detected by ce-HPLC and IEF. The presence of the new peptide at its predicted *m/z* values identifies the mutation. αLys139 (AAA) cannot mutate to Met (ATG) by a single base change in the codon.

Peptide Mutation	**ΔM**	**Name**	**$(M+4H)^{4+}$**	**$(M+3H)^{3+}$**	**$(M+2H)^{2+}$**	**$(M+H)^{+}$**
αT1-2						
α7Lys→Gln	0	J-Brainerd, α2	293.664	391.216	586.320	1,171.632
α7Lys→Glu	1	Kurosaki	293.910	391.544	586.812	1,172.616
α7Lys→Met	3	NL	294.409	392.210	587.811	1,174.614
αT2-3						
α11Lys→Gln	0	J-Wenchang-Wuming		325.507	487.757	974.506
α11Lys→Glu	1	NL	244.628	325.835	488.249	975.490
α11Lys→Met	3	NL	245.128	326.501	489.248	977.488
αT1-2-3						
α11Lys→Gln	**0**	**J-W-Wuming**	421.981	**562.306**	**842.955**	1,684.902
α11Lys→Glu	1	NL	422.227	562.634	843.447	1,685.886
α11Lys→Met	3	NL	422.727	563.300	844.446	1,687.884
αT3-4						
α16Lys→Gln	0	NL	511.498	681.661	1,021.988	2,042.968
α16Lys→Glu	**1**	**I, I-Philad'ia**	511.744	**681.989**	**1,022.480**	**2,043.952**
α16Lys→Met	3	Harbin	512.243	682.655	1,023.479	2,045.950
αT5-6						
α40Lys→Gln	**0**	**Linwood, α2**	**722.354**	**962.803**	**1,443.700**	2,886.393
			722.825	*963.431*	*1,444.642*	*2,888.277*
α40Lys→Glu	1	Kariya	722.600	963.131	1,444.192	2,887.377
			723.071	*963.759*	*1,445.135*	*2,889.262*
α40Lys→Met	3	Kanagawa	723.099	963.797	1,445.191	2,889.374
			723.592	*964.454*	*1,446.176*	*2,891.345*
αT6-7						
α56Lys→Gln	0	NL	554.019	738.356	1,107.030	2,213.053
α56Lys→Glu	**1**	**Shaare Zedek**	554.265	**738.684**	**1,107.522**	2,214.037
α56Lys→Met	3	NL	554.765	739.350	1,108.521	2,216.035
αT7-8						
α60Lys→Gln	0	NL	176.096	263.641	526.274	
α60Lys→Glu	1	Dagestan		176.425	264.133	527.258
α60Lys→Met	3	NL		177.090	265.132	529.256
αT8-9						
α61Lys→Gln	0	NL	625.716	781.893	1,042.188	1,562.778
			626.102	*782.376*	*1,042.832*	*1,563.743*
α61Lys→Glu	1	Miyagi	625.913	782.139	1,042.516	1,563.270
			626.299	*782.622*	*1,043.160*	*1,564.236*
α61Lys→Met	3	NL	626.312	782.639	1,043.182	1,564.269
			626.716	*783.143*	*1,043.854*	*1,565.277*

Peptide Mutation	ΔM	Name	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
αT9-10						
α90Lys→Gln	**0**	**Bergerac**	653.934	**817.166**	**1,089.218**	1,633.323
			654.337	*817.669*	*1,089.889*	*1,634.330*
α90Lys→Glu	**1**	**Sudbury**	**654.131**	**817.412**	**1,089.546**	1,633.815
			654.534	*817.915*	*1,090.217*	*1,634.822*
α90Lys→Met	3	Handa,	654.530	817.911	1,090.212	1,634.814
		Munakata	*654.950*	*818.436*	*1,090.912*	*1,635.864*
αT11-12						
α99Lys→Gln	0	NL	754.206	942.506	1,256.338	1,884.004
			754.687	*943.107*	*1,257.140*	*1,885.206*
α99Lys→Glu	1	Turriff	754.403	942.752	1,256.666	1,884.496
			754.884	*943.353*	*1,257.468*	*1,885.699*
α99Lys→Met	3	NL	754.803	943.251	1,257.332	1,885.495
			755.301	*943.874*	*1,258.163*	*1,886.740*
αT11-12-13						
α99Lys→Gln	0	NL	1000.946	1,250.930	1,667.571	2,500.852
			1001.579	*1,251.722*	*1,668.626*	*2,502.436*
α99Lys→Glu	1	Turriff	1001.142	1,251.176	1,667.899	2,501.344
			1001.776	*1,251.968*	*1,668.955*	*2,502.928*
α99Lys→Met	3	NL	1001.542	1,251.675	1,668.565	2,502.343
			1002.193	*1,252.489*	*1,669.649*	*2,503.969*
αT12-13						
α127Lys→Gln	0	NL	841.061	1,051.074	1,401.096	2,101.141
			841.594	*1,051.740*	*1,401.985*	*2,102.473*
α127Lys→Glu	1	No name, α2	841.258	1,051.320	1,401.424	2,101.633
			841.791	*1,051.987*	*1,402.313*	*2,102.965*
α127Lys→Met	3	NL	841.657	1,051.820	1,402.090	2,102.632
			842.207	*1,052.507*	*1,403.007*	*2,104.007*
αT12-13-14						
α139Lys→Gln	0	NL	904.894	1,130.865	1,507.485	2,260.723
			905.467	*1,131.581*	*1,508.439*	*2,262.155*
α139Lys→Glu	1	Hanamaki-1,α1	905.091	1,131.111	1,507.813	2,261.215
		Hanamaki-2,α2	*905.664*	*1,131.828*	*1,508.767*	*2,262.647*

Peptide Mutation	ΔM	Name	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
αT13-14						
α139Lys→Gln	0	NL	393.717	524.620	786.426	1,571.843
α139Lys→Glu	1	Hanamaki-1,α1	393.963	524.948	786.918	1,572.827
		Hanamaki-2,α2				

4.3. Tables to assist in searching for tryptic peptides from α-chain variants that involve Arg or Lys and give a mass change >±6 Da from normal (excluding Arg↔Lys).

These tables were devised to assist in systematically searching tryptic digest spectra for variant α-chain peptides from mutations that involve Arg or Lys (excluding Arg↔Lys) and the mass change of the variant is >±6 Da from normal. These mutations either create a new tryptic cleavage site and hence cut the peptide containing the mutation into two new smaller peptides or abolish one of the cleavage sites causing two adjacent peptides to be combined into one new larger peptide. Since the masses of such peptides are not simply related to the masses of the corresponding normal peptides, it is helpful to have tables available that give the *m/z* values of these new peptides. The following 23 tables list the *m/z* ratios of all the α-chain tryptic peptide ions that involve mutations to or from Arg and Lys, give a mass change greater than ±6 Da and are governed by a single base change in the DNA codon.

For peptides below mass 2,300 Da, only monoisotopic values are given (normal font).

For peptides above mass 2,300 Da, average values are given beneath the monoisotopic values.

Unless otherwise stated, the variants can be in either the α1 or the α2 gene.

Mutation	Nominal Mass Change (Da)	Number of Mutations	Table
Arg→Cys	-53	1	4.3.1
Arg→Gln	-28	1	4.3.2
Arg→Gly	-99	3	4.3.3
Arg→His	-19	1	4.3.4
Arg→Leu	-43	2	4.3.5
Arg→Met	-25	1	4.3.6
Arg→Pro	-59	2	4.3.7
Arg→Ser	-69	2	4.3.8
Arg→Thr	-55	1	4.3.9
Arg→Trp	30	2	4.3.10
Asn→Lys	14	4	4.3.11
Cys→Arg	53	1	4.3.12
Gln→Arg	28	1	4.3.13
Gly→Arg	99	7	4.3.14
His→Arg	19	10	4.3.15
Leu→Arg	43	18	4.3.16
Lys→Asn	-14	11	4.3.17
Lys→Ile	-15	1	4.3.18
Lys→Thr	-27	11	4.3.19
Met→Arg	25	2	4.3.20
Pro→Arg	59	7	4.3.21
Ser→Arg	69	4	4.3.22
Trp→Arg	-30	1	4.3.23

Table 4.3.1. The new tryptic peptides produced by the three Arg→Cys mutations in the α-chain
Mass change: -53.092 Da (monoisotopic), -53.043 Da (average)

Mutation Name	Peptide	Mass (M)	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
α31Arg→Cys	αT4-5	2,528.172	633.051	843.732	1,265.094	2,529.179
NL		*2,529.878*	*633.477*	*844.301*	*1,265.947*	*2,530.886*
α92Arg→Cys	αT10-11	1,033.527	259.390	345.517	517.771	1,034.535
NL						
α141Arg→Cys	αT14	284.083			143.049	285.091
Nunobiki						

Notes.
Monoisotopic mass and *m/z* values are in normal font; average values are in italics.
NL: Not listed in http://globin.bx.psu.edu.
This mutation causes a negative polarity change in the α-chain.
Variants identified by MS and ions observed in digest spectra are shown in bold font.
Of the mutations shown, only Nunobiki is possible by a single base change in the DNA codon (CGT→TGT).

Table 4.3.2. The new tryptic peptides produced by the three Arg→Gln mutations in the α-chain
Mass change: -28.043 Da (monoisotopic), -28.057 Da (average)

Mutation Name	Peptide	Mass (M)	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
α31Arg→Gln	αT4-5	2,553.221	639.313	852.081	1,277.618	2,554.229
NL		*2,554.864*	*639.724*	*852.629*	*1,278.440*	*2,555.872*
α92Arg→Gln	**αT10-11**	**1,058.576**	265.652	353.866	**530.296**	**1,059.584**
J-Cape Town						
α141Arg→Gln	αT14	309.133			155.574	310.140
NL						

Notes.
Monoisotopic mass and *m/z* values are in normal font; average values are in italics.
NL: Not listed in http://globin.bx.psu.edu.
This mutation causes a negative polarity change in the α-chain.
Variants identified by MS and ions observed in digest spectra are shown in bold font.
Of the mutations shown, only J-Cape Town is possible by a single base change in the DNA codon (CGG→CAG).

Table 4.3.3. The new tryptic peptides produced by the three Arg→Gly mutations in the α-chain
Mass change: -99.080 Da (monoisotopic), -99.136 Da (average)

Mutation Name	Peptide	Mass (M)	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
α31Arg→Gly	αT4-5	2,482.184	621.554	828.402	1,242.100	2,483.192
NL		*2,483.785*	*621.954*	*828.936*	*1,242.900*	*2,484.793*
α92Arg→Gly	αT10-11	987.539	247.893	330.187	494.777	988.547
NL						
α141Arg→Gly	αT14	238.095			120.056	239.103
J-Camaguey						

Notes.
Monoisotopic mass and *m/z* values are in normal font; average values are in italics.
NL: Not listed in http://globin.bx.psu.edu.
This mutation causes a negative polarity change in the α-chain.
Variants identified by MS and ions observed in digest spectra are shown in bold font.
All these mutations are possible by a single base change in the DNA codon.

Table 4.3.4. The new tryptic peptides produced by the three Arg→His mutations in the α-chain
Mass change: -19.042 Da (monoisotopic), -19.046 Da (average)

Mutation Name	Peptide	Mass (M)	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
α31Arg→His	αT4-5	2,562.221	641.563	855.082	1,282.188	2,563.229
NL		*2,563.874*	*641.976*	*855.633*	*1,282.945*	*2,564.882*
α92Arg→His	αT10-11	1,067.576	267.902	356.867	534.796	1,068.584
NL						
α141Arg→His	**αT14**	**318.133**			160.074	**319.141 (I)**
Suresnes						

Notes.
Monoisotopic mass and *m/z* values are in normal font; average values are in italics.
NL: Not listed in http://globin.bx.psu.edu.
This mutation causes a negative polarity change in the α-chain.
Variants identified by MS and ions observed in digest spectra are shown in bold font.
I: interference from $\beta T15^{+}$ ion.
This mutation was confirmed by tandem MS of the intact variant $(M+16H)^{16+}$ ion.
Of the mutations shown, only α141Arg can mutate to His by a single base change in the DNA codon (CGT→CAT).

Table 4.3.5. The new tryptic peptides produced by the three Arg→Leu mutations in the α-chain Mass change: -43.017 Da (monoisotopic), -43.028 Da (average)

Mutation Name	Peptide	Mass (M)	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
α31Arg→Leu	αT4-5	2,538.247	635.569	847.090	1,270.131	2,539.254
NL		*2,539.893*	*635.981*	*847.639*	*1,270.954*	*2,540.900*
α92Arg→Leu Chesapeake	**αT10-11**	**1,043.602**	261.908	348.875	**522.809**	**1,044.609**
α141Arg→Leu Legnano	αT14	294.158			148.087	295.166

Notes.
Monoisotopic mass and *m/z* values are in normal font; average values are in italics.
NL: Not listed in http://globin.bx.psu.edu.
This mutation causes a negative polarity change in the α-chain.
Variants identified by MS and ions observed in digest spectra are shown in bold font.
Of the mutations shown, only Chesapeake and Legnano are possible by a single base change in the DNA codon.

Table 4.3.6. The new tryptic peptides produced by the three Arg→Met mutations in the α-chain Mass change: -25.061 Da (monoisotopic), -24.989 Da (average)

Mutation Name	Peptide	Mass (M)	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
α31Arg→Met	αT4-5	2,556.203	640.059	853.075	1,279.109	2,557.211
NL		*2,557.932*	*640.491*	*853.652*	*1,279.974*	*2,558.940*
α92Arg→Met NL	αT10-11	1,061.558	266.397	354.860	531.787	1,062.566
α141Arg→Met NL	αT14	312.114			157.065	313.122

Notes.
Monoisotopic mass and *m/z* values are in normal font; average values are in italics.
NL: Not listed in http://globin.bx.psu.edu.
This mutation causes a negative polarity change in the α-chain.
Variants identified by MS and ions observed in digest spectra are shown in bold font.
Of the mutations shown, only α31Arg→Met is possible by a single base change in the DNA codon (AGG→ATG).

Table 4.3.7. The new tryptic peptides produced by the three Arg→Pro mutations in the α-chain Mass change: -59.048 Da (monoisotopic), -59.071 Da (average)

Mutation Name	Peptide	Mass (M)	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
α31Arg→Pro	αT4-5	2,522.215	631.562	841.746	1,262.115	2,523.223
NL		*2,523.850*	*631.970*	*842.291*	*1,262.933*	*2,524.858*
α92Arg→Pro	αT10-11	1,027.570	257.900	343.531	514.793	1,028.578
Monou						
α141Arg→Pro	αT14	278.127			140.071	279.135
Singapore						

Notes.
Monoisotopic mass and *m/z* values are in normal font; average values are in italics.
NL: Not listed in http://globin.bx.psu.edu.
This mutation causes a negative polarity change in the α-chain.
Variants identified by MS and ions observed in digest spectra are shown in bold font.
Of the mutations shown, only Monou and Singapore are possible by a single base change in the DNA codon.

Table 4.3.8. The new tryptic peptides produced by the three Arg→Ser mutations in the α-chain Mass change: -69.069 Da (monoisotopic), -69.109 Da (average)

Mutation Name	Peptide	Mass (M)	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
α31Arg→Ser	**αT4-5**	**2,512.194**	629.056	**838.406**	**1,257.105**	2,513.202
Prato		***2,513.811***	*629.461*	***838.945***	***1,257.914***	*2,514.819*
α92Arg→Ser	αT10-11	1,017.550	255.395	340.191	509.783	1,018.557
NL						
α141Arg→Ser	αT14	268.106			135.061	269.114
J-Cubujuqui						

Notes.
Monoisotopic mass and *m/z* values are in normal font; average values are in italics.
NL: Not listed in http://globin.bx.psu.edu.
This mutation causes a negative polarity change in the α-chain.
Variants identified by MS and ions observed digest spectra are shown in bold font.
Of the mutations shown, only Prato and J-Cubujuqui are possible by a single base change in the DNA codon.

Table 4.3.9. The new tryptic peptides produced by the three Arg→Thr mutations in the α-chain Mass change: -55.053 Da (monoisotopic), -55.083 Da (average)

Mutation Name	Peptide	Mass (M)	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
α31Arg→Thr	αT4-5	2,526.210	632.560	843.078	1,264.113	2,527.218
NL		*2,527.838*	*632.967*	*843.621*	*1,264.927*	*2,528.846*
α92Arg→Thr	αT10-11	1,031.565	258.899	344.863	516.790	1,032.573
NL						
α141Arg→Thr	αT14	282.122			142.069	283.129
NL						

Notes.
Monoisotopic mass and *m/z* values are in normal font; average values are in italics.
NL: Not listed in http://globin.bx.psu.edu.
This mutation causes a negative polarity change in the α-chain.
Variants identified by MS and ions observed in digest spectra are shown in bold font.
Of the mutations shown, only α31Arg→Thr is possible by a single base change in the DNA codon (AGG→ACG).

Table 4.3.10. The tryptic peptides produced by the three Arg→Trp mutations in the α-chain Mass change: 29.978 Da (monoisotopic), 30.026 Da (average)

Mutation Name	Peptide	Mass (M)	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
α31Arg→Trp	αT4-5	2,611.242	653.818	871.422	1,306.629	2,612.250
NL		*2,612.946*	*654.245*	*871.990*	*1,307.481*	*2,613.954*
α92Arg→Trp	αT10-11	1,116.597	280.157	373.207	559.306	1,117.605
Cemenelum						
α141Arg→Trp	αT14	367.153			184.584	368.161
NL						

Notes.
Monoisotopic mass and *m/z* values are in normal font; average values are in italics.
NL: Not listed in http://globin.bx.psu.edu.
This mutation causes a negative polarity change in the α-chain.
Variants identified by MS and ions observed in digest spectra are shown in bold font.
Of the mutations shown, only α31Arg→Trp and α92Arg→Trp are possible by a single base change in the DNA codon.

Table 4.3.11. The new tryptic peptides produced by the four Asn→Lys mutations in the α-chain
Mass change: 14.052 Da (monoisotopic), 14.070 Da (average)

Mutation Name	Peptide	Mass (M)	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
α9Asn→Lys	αT2a	247.153				248.161
Park Ridge	αT2b	245.174				246.182
	αT1-2a	957.550		320.191	479.783	958.557
α68Asn→Lys	**αT9a**	**716.407**		239.810	**359.211**	**717.415**
G-Philadelphia	**αT9b**	**2,311.138**	**578.792**	**771.387**	**1156.577**	2,312.146
		2,312.593	***579.156***	***771.872***	***1,157.304***	*2,313.600*
	αT8-9a	**844.502**		282.508	**423.259**	**845.510**
α78Asn→Lys	**αT9a**	**1,765.867**	442.475	**589.630**	**883.941**	1,766.875
Stanleyville II	**αT9b**	**1,261.678**	316.427	**421.567**	**631.847**	**1,262.686**
	αT8-9a	**1,893.962**	474.498	**632.328**	**947.989**	1,894.970
α97Asn→Lys	αT11a	556.322			279.169	557.330
Dallas	αT11b	293.174			147.598	294.182

Notes.
Monoisotopic mass and *m/z* values are in normal font; average values are in italics.
This mutation causes a positive polarity change in the α-chain.
Variants identified by MS and ions observed in digest spectra are shown in bold font.
All these mutations are possible by a single base change in the DNA codon (AAC→AAA or AAG).

Table 4.3.12. The new tryptic peptides produced by the single Cys→Arg mutation in the α-chain
Mass change: 53.092 Da (monoisotopic), 53.043 Da (average)

Mutation Name	Peptide	Mass (M)	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
α104Cys→Arg	αT12a	624.371		209.131	313.193	625.379
NL	αT12b	2,413.337	604.342	805.453	1,207.676	2,414.345
		2,414.831	*604.716*	*805.952*	*1,208.424*	*2,415.839*
	αT12b-13	3,647.034	912.766	1,216.686	1,824.525	3,648.042
		3,649.290	*913.330*	*1,217.438*	*1,825.653*	*3,650.298*

Notes.
Monoisotopic mass and *m/z* values are in normal font; average values are in italics.
NL: Not listed in http://globin.bx.psu.edu.
This mutation causes a positive polarity change in the α-chain.
Variants identified by MS and ions observed in digest spectra are shown in bold font.
This mutation is possible by a single base change in the DNA codon (TGC→CGC).
αT(12b-13) is more likely to be observed than αT12b because α126Asp hinders cleavage by trypsin at α127Lys.

Table 4.3.13. The new tryptic peptides produced by the single Gln→Arg mutation in the α-chain Mass change: 28.043 Da (monoisotopic), 28.057 Da (average)

Mutation Name	Peptide	Mass (M)	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
α54Gln→Arg	**αT6a**	**1,633.764**	409.449	**545.596**	**817.890**	1,634.772
Shimonoseki	αT6b	245.174				246.182

Notes.
All mass and *m/z* values are monoisotopic.
This mutation causes a positive polarity change in the α-chain.
Ions observed in the digest spectrum are shown in bold font.
This mutation is possible by a single base change in the DNA codon (CAG→CGG).

Table 4.3.14. The tryptic peptides produced by the seven Gly→Arg mutations in the α-chain Mass change: 99.080 Da (monoisotopic), 99.136 Da (average)

Mutation Name	Peptide	Mass (M)	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
α15Gly→Arg	**αT3a**	**502.265**				**503.273**
Ottawa, Siam	αT3b	146.106				147.113
α18Gly→Arg	**αT4a**	**273.180**				**274.188**
Handsworth	**αT4b**	**1,372.637**	344.167	458.554	**687.326**	**1,373.645**
α22Gly→Arg	αT4a	609.335		204.119	305.675	610.343
NL	αT4b	1,036.483	260.128	346.502	519.249	1,037.490
α25Gly→Arg	αT4a	958.462	240.623	320.495	480.239	959.470
NL	αT4b	687.355		230.126	344.685	688.363
α51Gly→Arg	**αT6a**	**1,418.673**	355.676	**473.899**	**710.344**	**1,419.681**
Russ	αT6b	531.302			266.659	532.310
α57Gly→Arg	αT7a	174.112				175.120
L-Persian Gulf	**αT7b**	**340.186**			171.101	**341.194**
α59Gly→Arg	αT7a	368.192			185.104	369.200
Zurich-Albisrieden	αT7b	146.106				147.113

Notes.
All mass and *m/z* values are monoisotopic.
NL: Not listed in http://globin.bx.psu.edu.
This mutation causes a positive polarity change in the α-chain.
Variants identified by MS and ions observed in digest spectra are shown in bold font.
All these mutations are possible by a single base change in the DNA codon.
In Russ, interference from αT3 prevents observation of αT6b.

Table 4.3.15. The new tryptic peptides produced by the ten His→Arg mutations in the α-chain Mass change: 19.042 Da (monoisotopic), 19.046 Da (average)

Mutation Name	Peptide	Mass (M)	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
α20His→Arg	αT4a	401.239			201.627	402.247
Hobart	αT4b	1,164.541	292.143	389.188	583.278	1,165.549
α45His→Arg	αT6a	682.344		228.456	342.180	683.352
Fort de France	αT6b	1,187.594	297.906	396.872	594.805	1,188.601
α50 His→Arg	αT6a	1,281.614	321.411	428.213	641.815	1,282.622
Aichi	αT6b	588.323		197.116	295.169	589.331
α58His→Arg	αT7a	231.133				232.141
NL	αT7b	203.127				204.135
α72His→Arg	αT9a	1,099.599	275.907	367.541	550.807	1,100.606
Daneshgah-Tehran	αT9b	1,932.936	484.242	645.320	967.476	1,933.944
α87His→Arg	αT9a	2,678.333	670.591	893.786	1,340.175	2,679.341
Iwata		*2,679.992*	*671.006*	*894.339*	*1,341.004*	*2,681.000*
	αT9b	354.202			178.109	355.209
α89His→Arg	αT9a	2,886.429	722.615	963.151	1,444.223	2,887.437
Tamano		*2,888.212*	*723.061*	*963.745*	*1,445.114*	*2,889.220*
	αT9b	146.106				147.113
α103His→Arg	αT12a	487.312			244.664	488.320
Contaldo	αT12b	2,516.346	630.094	839.790	1,259.181	2,517.354
		2,517.976	*630.502*	*840.333*	*1,259.996*	*2,518.984*
	αT12b-13	3,750.043	938.519	1,251.022	1,876.029	3,751.051
		3,752.435	*939.117*	*1,251.820*	*1,877.225*	*3,753.443*
α112His→Arg	αT12a	1,408.822	353.213	470.615	705.419	1,409.830
Strumica	αT12b	1,594.836	399.717	532.620	798.426	1,595.843
	αT12b-13	2,828.532	708.141	943.852	1,415.274	2,829.540
		2,830.275	*708.577*	*944.433*	*1,416.145*	*2,831.283*
α122His→Arg	αT12a	2,471.372	618.851	824.799	1,236.694	2,472.380
NL		*2,472.982*	*619.253*	*825.335*	*1,237.499*	*2,473.990*
	αT12b	532.286			267.151	533.294
	αT12b-13	1,765.983	442.503	589.669	883.999	1,766.990

Notes.

Monoisotopic mass and *m/z* values are in normal font; average values are in italics.

NL: Not listed in http://globin.bx.psu.edu.

This mutation causes a positive polarity change in the α-chain.

Variants identified by MS and ions observed in digest spectra are shown in bold font.

In α103, α112 and α122His→Arg, αT(12b-13) is more likely to occur than αT12b because α126Asp hinders cleavage at α127Lys.

All these mutations are possible a single base change in the DNA codon (CAC→CGC).

Table 4.3.16. The tryptic peptides produced by the eighteen Leu→Arg mutations in the α-chain Mass change: 43.017 Da (monoisotopic), 43.028 Da (average)

Mutation Name	Peptide	$(M+5H)^{5+}$	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
α2Leu→Arg	αT1a					274.188
Chongqing	αT1b				259.135	517.262
	αT(1b-2)			320.511	480.262	959.516
α29Leu→Arg	αT4a			429.875	644.308	1,287.608
NL	αT4b					304.162
α34Leu→Arg	αT5a				227.118	453.228
Queens, Ogi	αT5b			227.459	340.685	680.362
α48Leu→Arg	**αT6a**			361.507	**541.757**	**1,082.506**
Montgomery	**αT6b**			271.813	**407.215**	**813.422**
α66Leu→Arg	αT9a				266.148	531.289
NL	αT9b		632.565	843.084	1,264.122	2,527.236
			632.958	*843.608*	*1,264.909*	*2,528.810*
α80Leu→Arg	αT9a		495.746	660.659	990.484	1,979.961
Ann Arbor	αT9b			360.193	539.786	1,078.565
α83Leu→Arg	αT9a		563.534	751.043	1,126.061	2,251.114
NL	αT9b			269.809	404.210	807.411
α86Leu→Arg	αT9a		642.320	856.091	1,283.632	2,566.257
Moabit			*642.716*	*856.619*	*1284.424*	*2,567.841*
	αT9b				246.638	492.268
α91Leu→Arg	αT10a					175.120
NL	αT10b					175.120
α100Leu→Arg	αT12a					175.120
NL	αT12b		714.388	952.182	1,427.768	2,854.529
			714.847	*952.793*	*1,428.685*	*2,856.363*
	αT12b-13	818.451	1,022.812	1,363.414	2,044.617	4,088.226
		818.971	*1,023.461*	*1,364.279*	*2,045.915*	*4,090.822*
α101Leu→Arg	αT12a					288.204
NL	αT12b		686.117	914.487	1,371.226	2,741.445
			686.557	*915.073*	*1,372.106*	*2,743.204*
	αT12b-13	795.835	994.541	1,325.719	1,988.075	3,975.142
		796.339	*995.172*	*1,326.559*	*1,989.335*	*3,977.662*
α105Leu→Arg	αT12a			243.468	364.698	728.388
NL	αT12b		576.071	767.759	1,151.134	2,301.261
	αT(12b-13)	707.798	884.495	1,178.991	1,767.983	3,534.958
		708.234	*885.041*	*1,179.718*	*1,769.073*	*3,537.139*

Mutation Name	**Peptide**	**$(M+5H)^{5+}$**	**$(M+4H)^{4+}$**	**$(M+3H)^{3+}$**	**$(M+2H)^{2+}$**	**$(M+H)^{+}$**
α106Leu→Arg	αT12a			281.162	421.240	841.472
NL	αT12b		547.800	730.064	1,094.592	2,188.177
	αT12b-13	685.181	856.224	1,141.296	1,711.441	3,421.874
		685.602	*856.751*	*1,141.998*	*1,712.493*	*3,423.979*
α109Leu→Arg	αT12a			385.563	577.840	1,154.672
Suan-Dok, α2	αT12b		469.500	625.664	937.992	1,874.976
	αT12b-13	622.541	777.924	1,036.896	1,554.841	3,108.673
		622.923	*778.401*	*1,037.533*	*1,555.795*	*3,110.582*
α113Leu→Arg	αT12	602.932	753.413	1,004.215	1,505.819	3,010.630
San Antonio, α2		*603.316*	*753.894*	*1,004.856*	*1,506.779*	*3,012.551*
	αT12-13	**849.672**	**1061.838**	**1,415.448**	2,122.667	4,244.327
		850.208	***1062.508***	***1,416.342***	*2,124.009*	*4,247.009*
α125Leu→Arg	αT12a		692.633	923.175	1,384.258	2,767.508
Plasencia			*693.078*	*923.768*	*1,385.148*	*2,769.288*
	αT12b					262.140
	αT12b-13		374.715	499.284	748.423	1,495.837
α129Leu→Arg	αT12-13a	654.963	818.452	1,090.933	1,635.895	3,270.783
NL		*655.384*	*818.978*	*1,091.634*	*1,636.947*	*3,272.887*
	αT13a					322.188
	αT13b			331.526	496.785	992.563
α136Leu→Arg	αT12-13a	786.437	982.794	1,310.056	1,964.580	3,928.152
Toyama		*786.937*	*983.419*	*1,310.889*	*1,965.830*	*3,930.652*
	αT13a			327.191	490.283	979.558
	αT13b					335.193

Notes.
Monoisotopic *m/z* values are in normal font; average values are in italics.
NL: Not listed in http://globin.bx.psu.edu.
This mutation causes a positive polarity change in the α-chain.
Variants identified by MS and ions observed in digest spectra are shown in bold font.
All these mutations are possible by a single base change in the DNA codon.
With α100 and α101Leu→Arg, αT12b and αT(12b-13) contain Cys. With α105, α106, α109 and α125Leu→Arg, αT12a contains Cys. With all these mutations, αT(12b-13) is more likely to occur than αT12b because α126Asp hinders cleavage at α127Lys.
With α113Leu→Arg, αT12 and αT(12-13) contain Cys. Here, α114Pro inhibits cleavage at α113Arg. Also, αT(12-13) is more likely to occur than αT12 because α126Asp hinders cleavage at α127Lys.
With α129 and α136Leu→Arg, αT(12-13)a contains Cys. Also, αT(12-13a) is more likely to occur than αT13a because α126Asp hinders cleavage at α127Lys.

Table 4.3.17. The tryptic peptides produced by the eleven Lys→Asn mutations in the α-chain
Mass change: -14.052 Da (monoisotopic), -14.070 Da (average)

Mutation Name	Peptide	$(M+5H)^{5+}$	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
α7Lys→Asn Tatras	**αT1-2**		290.160	386.544	**579.312**	**1157.617**
α11Lys→Asn	αT2-3		240.878	320.835	480.749	960.490
Albany-Suma	αT1-3		418.478	557.634	835.947	1,670.887
α16Lys→Asn Beijing	**αT3-4**	406.597	507.994	**676.989**	**1,014.980**	2,028.953
α40Lys→Asn	**αT5-6**	575.282	718.850	**958.131**	**1,436.692**	2,872.377
Saratoga Springs, α1		575.656	719.318	958.755	1,437.629	2,874.250
Villiers le Bel, α2						
α56Lys→Asn	αT6-7	440.614	550.515	733.684	1,100.023	2,199.037
Belliard		*440.882*	*550.851*	*734.132*	*1,100.693*	*2,200.379*
α60Lys→Asn Zambia	**αT7-8**				256.633	**512.258**
α61Lys→Asn	αT8-9	622.913	778.389	1,037.516	1,555.770	3,110.533
J-Buda		*623.297*	*778.869*	*1,038.156*	*1,556.730*	*3,112.452*
α90Lys→Asn	**αT9-10**	**651.131**	**813.662**	**1,084.546**	**1,626.315**	3,251.623
J-Broussais		***651.531***	***814.162***	***1,085.214***	***1,627.316***	*3,253.625*
α99Lys→Asn	αT11-12	751.403	939.002	1,251.666	1,876.996	3,752.984
Beziers, α1		*751.882*	*939.600*	*1,252.464*	*1,878.193*	*3,755.378*
	αT11-13	**998.142**	**1,247.426**	1,662.899	2,493.844	4,986.681
		998.774	***1,248.215***	*1,663.951*	*2,495.422*	*4,989.836*
α127Lys→Asn	αT12-13	838.258	1,047.570	1,396.424	2,094.133	4,187.258
Jackson		*838.789*	*1,048.234*	*1,397.309*	*2,095.459*	*4,189.911*
α139Lys→Asn	αT13-14		390.213	519.948	779.418	1,557.828
Fukui, α1	αT12-14	*902.661*	*1,128.075*	*1,503.764*	*2,255.141*	*4,509.275*

Notes.
Monoisotopic mass and *m/z* values are in normal font; average values are in italics.
This mutation causes a negative polarity change in the α-chain.
Variants identified by MS and ions observed in digest spectra are shown in bold font.
All these mutations are possible by a single base change in the DNA codon.

Table 4.3.18. The tryptic peptide produced by a Lys→Ile mutation in the α-chain
Mass change: -15.011 Da (monoisotopic), -15.015 Da (average)

Mutation Name	Peptide	$(M+5H)^{5+}$	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
α139Lys→Ile Novel	**αT12-14**	**901.899**	**1,127.122**	1,502.493	2,253.236	4,505.463
		902.472	***1,127.838***	*1,503.449*	*2,254.669*	*4,508.330*
	αT13-14		389.973	519.628	778.938	1,556.869

Notes.
Monoisotopic mass and *m/z* values are in normal font; average values are in italics.
This mutation causes a negative polarity change in the α-chain.
Ions observed in the digest spectrum are shown in bold font.
Although there are eleven Lys in the α-chain, only α139Lys→Ile can occur by a single base change in the DNA codon (AAA→ATA).

Table 4.3.19. The tryptic peptides produced by the eleven Lys→Thr mutations in the α-chain
Mass change: -27.047 Da (monoisotopic), -27.069 Da (average)

Mutation Name	Peptide	$(M+5H)^{5+}$	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
α7Lys→Thr Nayarit, α2	αT1-2		286.911	382.212	572.815	1144.621
α11Lys→Thr	αT2-3		237.630	316.504	474.251	947.495
NL	αT1-3	332.385	415.229	553.302	829.450	1,657.891
α16Lys→Thr Boa Esperanca, α2	αT3-4	403.998	504.745	672.658	1,008.483	2,015.958
α40Lys→Thr	αT5-6	572.683	715.601	953.799	1,430.195	2,859.382
Pisa, α1		573.057	716.069	954.422	1,431.130	2,861.251
α56Lys→Thr	αT6-7	438.015	547.266	729.353	1,093.525	2,186.042
Thailand		*438.282*	*547.601*	*729.799*	*1,094.194*	*2,187.380*
α60Lys→Thr NL	αT7-8				250.135	499.263
α61Lys→Thr	αT8-9	620.314	775.140	1,033.184	1,549.273	3,097.538
J-Anatolia		*620.697*	*775.619*	*1,033.823*	*1,550.230*	*3,099.453*
α90Lys→Thr	**αT9-10**	648.532	**810.413**	**1,080.215**	1,619.818	3,238.628
J-Rajappen, α1		*648.932*	***810.912***	***1,080.881***	*1,620.817*	*3,240.626*

Mutation Name	Peptide	$(M+5H)^{5+}$	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
α99Lys→Thr	αT11-12	748.804	935.753	1,247.335	1,870.498	*3,739.989*
NL		*749.282*	*936.351*	*1,248.132*	*1,871.693*	*3,742.379*
	αT11-13	995.543	1,244.177	1,658.567	2,487.347	4,973.685
		996.174	*1,244.965*	*1,659.618*	*2,488.923*	*4,976.837*
α127Lys→Thr	αT12-13	835.659	1,044.322	1,392.093	2,087.635	4,174.263
St. Claude		*836.189*	*1,044.984*	*1,392.976*	*2,088.960*	*4,176.912*
α139Lys→Thr	αT13-14		386.964	515.616	772.920	1,544.832
Tokoname	αT12-14	899.492	1,124.113	1,498.481	2,247.217	4,493.427
		900.062	*1,124.825*	*1,499.431*	*2,248.642*	*4,496.276*

Notes.
Monoisotopic *m/z* values are in normal font; average values are in italics.
NL: Not listed in http://globin.bx.psu.edu.
This mutation causes a negative polarity change in the α-chain.
Variants identified by MS and ions observed in digest spectra are shown in bold font.
All these mutations are possible by a single base change in the DNA codon.

Table 4.3.20. The new tryptic peptides produced by the two Met→Arg mutations in the α-chain Mass change: 25.061 Da (monoisotopic), 24.987 Da (average)

Mutation Name	Peptide	Mass (M)	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
α32Met→Arg	αT5a	174.112				175.120
NL	αT5b	939.507		314.177	470.761	940.514
α76Met→Arg	**αT9**	**3,020.543**	**756.144**	**1,007.855**	1,511.279	3,021.551
Walpole		***3,022.329***	***756.590***	***1,008.451***	*1,512.172*	*3,023.337*

Notes.
Monoisotopic mass and *m/z* values are in normal font; average values are in italics.
NL: Not listed in http://globin.bx.psu.edu.
This mutation causes a positive polarity change in the α-chain.
Variants identified by MS and ions observed in digest spectra are shown in bold font.
Both mutations are possible by a single base change in the DNA codon (ATG→AGG).
Cleavage by trypsin at α76Arg is inhibited by α77Pro, hence variant αT9 is observed.

Table 4.3.21. The new tryptic peptides produced by the seven Pro→Arg mutations in the α-chain Mass change: 59.048 Da (monoisotopic), 59.071 Da (average)

Mutation Name	Peptide	Mass (M)	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
α4Pro→Arg	αT1a	473.296			237.656	474.304
Goree	αT1b	332.170				333.177
	αT1b-2	774.424		259.149	338.220	775.431
α37Pro→Arg	αT5a	799.405		267.476	400.710	800.413
Boumerdes	αT5b	348.201				349.209
α44Pro→Arg	**αT6a**	585.291			293.653	**586.299 (I)**
Kawachi	**αT6b**	1,324.652		442.559	**663.334**	**1,325.660**
α77Pro→Arg	αT9a	1,696.820	425.213	566.615	849.418	1,697.828
GuiZhou	αT8-9a	1,824.915	457.237	609.313	913.465	1,825.923
	αT9b	1,375.721	344.938	459.581	688.868	1,376.729
α95Pro→Arg	αT11a	388.207				389.215
St Luke's, α1	**αT10-11a**	657.392		220.139	**329.704**	**658.400 (I)**
	αT11b	506.285			254.150	**507.293**
α114Pro→Arg	**αT12a**	1,658.965	415.749	**553.996**	**830.491**	1,659.973
Chiapas	αT12b	1,384.699	347.183	462.574	693.357	1,385.707
	αT12b-13	2,618.396	655.607	**873.806**	1,310.206	2,619.404
α119Pro→Arg	αT12a	2,204.214	552.061	735.746	1,103.115	2,205.222
NL	αT12b	839.450	210.870	280.825	420.733	840.458
	αT12b-13	2,073.147	519.295	692.057	1,037.581	2,074.155

Notes.
All mass and *m/z* values are monoisotopic.
NL: Not listed in http://globin.bx.psu.edu.
This mutation causes a positive polarity change in the α-chain.
Variants identified by MS and ions observed in digest spectra are shown in bold font.
I: Interference.
All these mutations are possible by a single base change in the DNA codon.
Peptide αT12a contains Cys.
Peptide αT(12b-13) is more likely to occur than αT12b because 126Asp hinders cleavage at 127Lys.

Table 4.3.22. The tryptic peptides produced by the eleven Ser→Arg mutations in the α-chain
Mass change: 69.069 Da (monoisotopic), 69.109 Da (average)

Mutation Name	Peptide	Mass (M)	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
α49Ser→Arg	**αT6a**	1,194.582	299.653	399.202	**598.299**	**1,195.590**
Savaria, α2	**αT6b**	725.382		242.802	**363.699**	**726.390**
α84Ser→Arg	**αT9a**	2,363.190	591.805	**788.738**	**1,182.603**	2,364.198
Etobicoke		*2,364.666*	*592.174*	***789.230***	***1,183.341***	*2,365.674*
	αT9b	719.372		240.798	**360.694**	**720.379**
α102Ser→Arg	**αT12a**	400.280			201.148	**401.288**
Manitoba I, α2	**αT12b**	2,653.405	**664.359**	**885.476**	1,327.710	2,654.413
Manitoba II, α1		*2,655.117*	***664.787***	***886.047***	*1,328.567*	*2,656.125*
Manitoba III, α2	**αT12b-13**	3,887.102	**972.783**	**1,296.708**	1,944.559	3,888.110
		3,889.576	***973.402***	***1,297.533***	*1,945.796*	*3,890.584*
α133Ser→Arg	αT13a	691.402		231.475	346.709	692.410
Val de Marne	αT12-13a	3,939.996	911.007	1,214.340	1,821.006	3,641.004
		3,642.328	*911.590*	*1,215.117*	*1,822.172*	*3,643.336*
	αT13b	647.385		216.803	324.701	648.393

Notes.
Monoisotopic mass and *m/z* values are in normal font; average values are in italics.
NL: Not listed in http://globin.bx.psu.edu.
This mutation causes a positive polarity change in the α-chain.
Variants identified by MS and ions observed in digest spectra are shown in bold font.
Although there are eleven Ser in the α-chain, only Savaria, Etobicoke, Manitoba and Val de Marne can occur by a single base change in the DNA codon (AGC→CGC or AGA or AGG).

Table 4.3.23. The new tryptic peptides produced by the single Trp→Arg mutation in the α-chain
Mass change: -29.978 Da (monoisotopic), -30.026 Da (average)

Mutation Name	Peptide	Mass (M)	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
α14Trp→Arg	αT3a	316.361				317.368
Evanston	αT3b	203.241				204.249

Notes.
All mass and *m/z* values are monoisotopic.
This mutation causes a positive polarity change in the alpha chain.
This mutation is possible by a single base change in the DNA codon (TGG→CGG or AGG).

4.4. Tables to assist in searching for tryptic peptides produced from β-chain variants that differ in mass from normal by 0, ±1, ±3 and ±4 Da.

These tables were devised to assist in searching for tryptic peptides from β-chain variants that cannot be resolved from the normal β-chain when analysing the globin chains in heterozygotes. This situation arises when the normal and variant masses differ by <±6 Da. Within these limits, there are seven mass changes (0, ±1, ±3 and ±4 Da) that are produced by a single amino acid change governed by a single base change in the codon. There are 89 such variants in the β-chain. Three types of mutation are given. First, there are mutations that do not involve Lys. These simply change the mass of the tryptic peptide and are listed in Table 4.4.1. Second, there are mutations that involve an amino acid change to Lys, which creates a new cleavage site, and hence two new peptides in the digest (Table 4.4.2). Third, there are mutations that involve an amino acid change from Lys, which removes a cleavage site (Table 4.4.3). The mutation Leu→Ile produces neither a mass change nor a new cleavage site and thus cannot be detected by mass spectrometry. It is not included in the Tables. Of the 18 Leu in the β-chain, only 3 at β68, 81 and 105 (CTC) can mutate to Ile (ATC) by a single base change in the codon.

The *m/z* values of the variant peptide ions are given in the Tables. For peptides below mass 2,300 Da only monoisotopic *m/z* values are given (normal font). For peptides above mass 2,300 Da average *m/z* values are given in italics beneath the monoisotopic values.
Monoisotopic masses are based on: C: 12.0 Da, H: 1.0078250 Da, N: 14.0030740 Da, O: 15.9949146 Da and S: 31.9720718 Da. Average masses are based on: C: 12.011 Da, H: 1.00794 Da, N: 14.00674 Da, O: 15.9994 Da and S: 32.066 Da.
NL: Not listed in http://globin.bx.psu.edu. I: interference.
ΔM is the nominal mass change of the variant β-chain from normal (Da).
Variants that have been identified and *m/z* values that have been observed are shown in bold font.
All the mutations shown can occur by a single base change in the nucleotide codon.
Cleavage by trypsin at 95Lys is hindered by 94Asp giving mainly βT(10-11). Cleavage at 144Lys is also slow giving both βT(14-15), βT14 & βT15. These cases are included in the tables.
The special cases of Alamo, β19Asn→Asp and Bunbury, β94Asp→Asn are also included in the tables.

Table 4.4.1. The 44 mutations and *m/z* values of tryptic peptides that are produced as a result of single amino acid changes giving <±6 Da change in the mass of the β-chain and do not involve Lys.

Peptide Mutation	**ΔM**	**Name**	**$(M+3H)^{3+}$**	**$(M+2H)^{2+}$**	**$(M+H)^{+}$**
βT1					
β6Glu→Gln	-1	Machida	317.847	476.267	951.526
β7Glu→Gln)	-1	Bellevue III			
β4Thr→Pro	-4	Benin City	316.844	474.762	948.515
β5Pro→Thr	4	NL	319.507	478.757	956.505
βT2					
β12Thr→Pro	-4	NL	310.180	464.767	928.526
βT3					
β19Asn→Ile	-1	NL	438.574	657.357	1,313.707
β21Asp→Asn	-1	Cocody	438.566	657.345	1,313.681
β22Glu→Gln	**-1**	**D-Iran**		**657.345**	**1,313.681**
β26Glu→Gln	**-1**	**Novel, King's Mill**	438.566	**657.345**	**1,313.681**
β19Asn→Asp	**1**	**Alamo**, special case	**743.722**	**1,115.080**	2,229.152

β19Asp in Alamo hinders cleavage at β17Lys, causing the variant βT(2-3) peptide to occur in a similar way to the αT(10-11) peptide from the α-chain. Beware confusion with Nagasaki, β17Lys→Glu.

Peptide Mutation	ΔM	Name	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
βT4					
β39Gln→Glu	1	Vaasa	425.909	638.359	1,275.710
β38Thr→Pro	-4	Hazebrouck	424.249	635.870	1,270.731
β36Pro→Thr	4	Linköping	426.912	639.864	1,278.721
βT5					
β43Glu→Gln	-1	Hoshida	686.660	1,029.486	2,057.964
β47Asp→Asn	-1	G-Copenhagen			
β52Asp→Asn	**-1**	**Osu Christiansborg**		**1,029.486**	**2,057.964**
β57Asn→Ile	-1	NL	686.668	1,029.499	2,057.989
β57Asn→Asp	1	J-Daloa	687.316	1,030.470	2,059.932
β50Thr→Pro	-4	NL	685.656	1,027.981	2,054.953
β51Pro→Thr	4	NL	688.320	1,031.976	2,062.943
β58Pro→Thr	4	NL			
βT9					
β73Asp→Asn	**-1**	**G-Accra, Korle-Bu**	**556.974**	**834.958**	1,668.907
β79Asp→Asn	**-1**	**Yaizu**			
β80Asn→Ile -1	NL		556.983	834.970	1,668.933
β80Asn→Asp	1	Valley Park	557.630	835.942	1,670.875
βT(8-9)					
β73Asp→Asn	-1	G-Accra, Korle-Bu	599.673	899.005	1,797.002
β79Asp→Asn	-1	Yaizu			
β80Asn→Ile	-1	NL	599.681	899.018	1,797.027
β80Asn→Asp	1	Valley Park	600.329	899.989	1,798.970
βT10					
β90Glu→Gln	-1	NL	474.235	710.849	1,420.690
β94Asp→Asn	**-1**	**Bunbury,** special case	474.235	**710.849**	**1,420.690**
Normal βT11			376.193	**563.786**	**1,126.565**
The Bunbury mutation allows cleavage at 95Lys to give the variant βT10 and normal βT11 peptides.					
β84Thr→Pro	-4	NL	473.231	709.343	1,417.679
β87Thr→Pro	-4	Valletta	473.231	709.343	1,417.679
βT11					
β99Asp→Asn	-1	Kempsey	375.865	563.294	1,125.581
β101Glu→Gln	-1	Rush	375.865	563.294	1,125.581
β102Asn→Ile	-1	NL	375.874	563.307	1,125.606
β102Asn→Asp	1	NL	376.521	564.278	1,127.549
β100Pro→Thr	4	Bellevue II	377.525	565.784	1,130.559

Mutation Peptides	ΔM	Name	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
βT(10-11)						
β90Glu→Gln	-1	NL	632.815	843.417	1,264.622	2,528.236
			633.216	*843.952*	*1,265.424*	*2,529.840*
β99Asp→Asn	**-1**	**Kempsey**	632.815	**843.417**	**1,264.622**	2,528.236
β101Glu→Gln	-1	Rush				
β102Asn→Ile	-1	NL	632.821	843.425	1,264.634	2,528.261
			633.226	*843.965*	*1,265.444*	*2,529.880*
β102Asn→Asp	1	NL	633.307	844.073	1,265.606	2,530.204
			633.708	*844.608*	*1,266.409*	*2,531.809*
β84Thr→Pro	-4	NL	632.062	842.413	1,263.116	2,525.225
			632.465	*842.951*	*1,263.922*	*2,526.836*
β87Thr→Pro	**-4**	**Valletta**	**632.062**	**842.413**	**1,263.116**	2,525.225
			632.465	*842.951*	*1,263.922*	*2,526.836*
β100Pro→Thr	4	Bellevue II	634.060	845.077	1,267.111	2,533.215
			634.459	*845.610*	*1,267.911*	*2,534.813*
βT12						
β108Asn→Ile	-1	Schlierbach		573.677	860.011	1,719.014
β108Asn→Asp	**1**	**Yoshizuka**		**574.324**	**860.983**	1,720.957
βT13						
β121Glu→Gln	**-1**	**D-Punjab, D-Los Angeles**		459.911	**689.362**	**1,377.717**
β127Gln→Glu	1	Complutense		460.567	690.346	1,379.685
β131Gln→Glu	**1**	**Camden**			**690.346**	**1,379.685**
β123Thr→Pro	-4	NL		458.907	687.857	1,374.706
β124Pro→Thr	4	NL		461.570	691.852	1,382.696
β125Pro→Thr	4	NL				
βT14						
β139Asn→Ile	-1	NL		383.577	574.862	1,148.716
β139Asn→Asp	1	Geelong		384.225	575.833	1,150.658
βT(14-15)						
β139Asn→Ile	-1	NL	362.965	483.618	724.923	1,448.838
β139Asn→Asp	1	Geelong	363.451	484.265	725.894	1,450.781

Table 4.4.2. The pairs of new peptides resulting from the twelve single amino acid changes that give <±6 Da change in the β-chain mass and involve mutation to Lys, thus creating a new cleavage site. These mutations give a significant increase in positive charge and are readily detected by ce-HPLC and IEF. The presence of these new peptide ions at their predicted *m/z* values identifies the mutation.

Mutation Peptides	**ΔM**	**Name**	**$(M+3H)^{3+}$**	**$(M+2H)^{2+}$**	**$(M+H)^{+}$**
β6Glu→Lys	**-1**	**C**			
βT1a, 1VHLTPK				**347.717**	**694.425**
βT1b, 7EK				138.582	**276.156**
β7Glu→Lys	**-1**	**G-Siriraj**			
βT1a, 1VHLTPEK			275.161	412.238 (I)	**823.468**
βT1b, 8K					147.113
βT(a+b), 1VHLTPEKK			317.859	476.285 (I)	951.563
β22Glu→Lys	**-1**	**E-Saskatoon**			
βT3a, 18VNVDK				287.664	**574.320 (I)**
βT3b, 23VGGEALGR			253.477	**379.712**	**758.416**
βT3(a+b), 18VNVDKVGGEALGR			438.578	**657.363**	**1,313.718**
β26Glu→Lys	**-1**	**E**			
βT3a, 18VNVDEVGGK			306.163	**458.741**	**916.474**
βT3b, 27ALGR				**208.635**	**416.262**
β39Gln→Lys	**0**	**Alabama**			
βT4a, 31LLVVYPWTK			373.559	**559.835**	**1,118.661**
βT4b, 40R					175.120
β43Glu→Lys	**-1**	**Novel, Hornchurch**			
βT5a, 41FFK				221.129	**441.250**
βT5b, 44SFGDLSTPDAVMGNPK			545.928	**818.388 (I)**	**1,635.769**
β55Met→Lys	-3	Matera			
βT5a, 41FFESFGDLSTPDAVK			553.935	830.399	1,659.791
βT5b, 56GNPK			139.082	208.119 (I)	415.231
β90Glu→Lys	**-1**	**Agenogi**			
βT10a, 83GTFATLSK			275.489	**412.730**	**824.452**
βT10b, 91LHCDK			205.769	308.150	**615.292**
(βT(10b-11), 91LHCDKLHVDPENFR			574.951	**861.923**	1,722.839
β101Glu→Lys	-1	British Columbia			
βT(10-11a), 83GTFATLSELHCDKLHVDPK			704.358	1,056.034	2,111.060
βT11a, 96LHVDPK			236.807	354.706	708.404
βT11b, 102NFR				218.619	436.231
β121Glu→Lys	**-1**	**O-Arab**			
βT13a, 121K					147.113
βT13b, 122FTPPVQAAYQK			417.225	**625.333**	**1,249.658**
βT13(a+b), 121KFTPPVQAAYQK			459.922 (I)	**689.380**	**1,377.753**

Mutation Peptides	ΔM	Name	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
β127Gln→Lys	0	Brest			
βT13a, 121EFTPPVK			273.154	409.227	817.446
βT13b, 128AAYQK			194.108	290.659	580.310
β131Gln→Lys	0	Shelby			
βT13a, 121EFTPPVQAAYK			417.553	625.825	1,250.642
βT13b, 132K					147.113
βT13(a+b), 121EFTPPVQAAYKK			460.251	689.872 (I)	1,378.737 (I)

Table 4.4.3. The 30 tryptic peptides and their *m/z* values that result from a single amino acid change giving <±6 Da change in the mass of the β-chain and involve a mutation from Lys. Thus, a tryptic cleavage site is eliminated and two adjacent tryptic peptides are combined. These mutations give a significant increase in negative charge and are readily detected by ce-HPLC and IEF. The presence of the new peptide ions at their predicted *m/z* values identifies the mutation. Three of the Lys at β66, 120 and 132 (AAA) cannot mutate to Met (ATG) by a single base change in the codon.

Peptide Mutation	ΔM	Name	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
βT(1-2)						
β8Lys→Gln	0	J-Luhe	467.250	622.664	933.492	1,865.976
β8Lys→Glu	1	N-Timone	467.496	622.992	933.984	1,866.960
β8Lys→Met	3	Nakano	467.995	623.658	934.983	1,868.958
βT(2-3)						
β17Lys→Gln	**0**	**Nikosia**	557.787	**743.382**	**1,114.569**	2,228.131
β17Lys→Glu	1	Nagasaki	558.035	743.710	1,115.061	2,229.115
β17Lys→Met	3	NL	558.534	744.376	1,116.060	2,231.113
βT(5-6)						
β59Lys→Gln	**0**	**NL**	572.275	762.697	**1,143.542**	2,286.075
β59Lys→Glu	**1**	**I-High Wycombe**	572.521	763.025	**1,144.034**	2,287.059
β59Lys→Met	3	NL	573.020	763.691	1,145.032	2,289.057
βT(6-7)						
β61Lys→Gln	0	Pocos de Caldas	160.595	213.791	320.183	639.358
β61Lys→Glu	1	N-Seattle	160.841	214.119	320.675	640.342
β61Lys→Met	3	Bologna	161.341	214.785	321.674	642.340
βT(7-8)						
β65Lys→Gln	0	J-Cairo		180.768	270.649	540.289
β65Lys→Glu	1	NL		181.096	271.141	541.273
β65Lys→Met	3	J-Antakya		181.762	272.140	543.271

Peptide Mutation	**ΔM**	**Name**	**$(M+4H)^{4+}$**	**$(M+3H)^{3+}$**	**$(M+2H)^{2+}$**	**$(M+H)^{+}$**
βT(8-9)						
β66Lys→Gln	0	NL	450.24	599.989	899.479	1,797.950
β66Lys→Glu	1	I-Toulouse	450.489	600.317	899.971	1,798.934
βT(9-10)						
β82Lys→Gln	0	Tsurumai	768.883	1,024.842	1,536.759	3,072.510
			769.367	*1,025.486*	*1,537.725*	*3,074.443*
			783.629	*1,044.503*	*1,566.251*	*3,131.495)*
β82Lys→Glu	1	Gambara	769.129	1,025.170	1,537.251	3,073.494
			769.613	*1,025.814*	*1,538.218*	*3,075.427*
β82Lys→Met	**3**	**Helsinki**	769.629	**1,025.836**	**1,538.250**	3,075.492
			770.134	***1,026.509***	***1,539.259***	*3,077.510*
βT(9-10-11)						
β82Lys→Gln	0	Tsurumai	1,045.770	1,394.024	2,090.532	4,180.056
			1,046.422	*1,394.894*	*2,091.836*	*4,182.665*
β82Lys→Glu	1	Gambara	1,046.016	1,394.352	2,091.024	4,181.040
			1,046.668	*1,395.222*	*2,092.329*	*4,183.650*
β82Lys→Met	3	Helsinki	1,046.515	1,395.018	2,092.023	4,183.038
			1,047.189	*1,395.916*	*2,093.370*	*4,185.733*
βT(10-11)						
β95Lys→Gln	0	NL	633.052	843.733	1,265.096	2,529.183
			633.451	*844.266*	*1,265.895*	*2,530.781*
β95Lys→Glu	**1**	**N-Baltimore**	633.298	844.061	1,265.588	2,530.167
			633.697	***844.594***	***1,266.387***	*2,531.766*
β95Lys→Met	3	J-Cordoba	633.797	844.727	1,266.586	2,532.165
			634.218	*845.288*	*1,267.429*	*2,533.849*
βT(12-13)						
β120Lys→Gln	0	Takamatsu	770.661	1,027.212	1,540.313	3,079.619
			771.159	*1,027.876*	*1,541.310*	*3,081.613*
β120Lys→Glu	1	Hijiyama	770.907	1,027.540	1,540.806	3,080.603
			771.405	*1,028.205*	*1,541.803*	*3,082.598*
βT(13-14)						
β132Lys→Gln	**0**	**K-Woolwich**	628.086	**837.112 (I)**	**1,255.164**	2,509.320
			628.470	***837.624***	***1,255.932***	*2,510.856*
β132Lys→Glu	1	Takasago	628.332	837.440	1,255.656	2,510.304
			628.716	*837.952*	*1,256.425*	*2,511.841*
βT(13-14-15)						
β132Lys→Gln	**0**	**K-Woolwich**	**703.117**	**937.153**	**1,405.225**	2,809.443
			703.549	***937.730***	***1,406.091***	*2,811.174*
β132Lys→Glu	1	Takasago	703.363	937.481	1,405.717	2,810.427
			703.796	*938.058*	*1,406.583*	*2,812.158*
βT(14-15)						
β144Lys→Gln	0	NL	363.196	483.925	725.384	1,449.760
β144Lys→Glu	1	Mito	363.442	484.253	725.876	1,450.744
β144Lys→Met	**3**	**Barbizon**	363.941	**484.919**	**726.875**	1,452.742

4.5. Tables to assist in searching for tryptic peptides from β-chain variants that involve Arg or Lys and give a mass change >±6 Da from normal (excluding Arg↔Lys).

These tables were devised to assist in systematically searching tryptic digest spectra for variant β-chain peptides from mutations that involve Arg or Lys (excluding Arg↔Lys) and the mass change of the variant is >±6 Da from normal. These mutations either create a new tryptic cleavage site and hence cut the peptide containing the mutation into two new smaller peptides or abolish one of the cleavage sites causing two adjacent peptides to be combined into one new larger peptide. Since the masses of such peptides are not simply related to the masses of the corresponding normal peptides, it is helpful to have tables available that give the *m/z* values of these new peptides. The following 20 tables list the *m/z* ratios of all the β-chain tryptic peptides that involve mutations to or from Arg and Lys, give a mass change greater than ±6 Da and which are governed by a single base change in the DNA codon.

Mutation	Nominal Mass Change (Da)	Number of Mutations	Table
Arg→Gly	-99	3	4.5.1
Arg→Met	-25	3	4.5.2
Arg→Ser	-69	3	4.5.3
Arg→Thr	-55	3	4.5.4
Arg→Trp	30	3	4.5.5
Asn→Lys	14	6	4.5.6
Cys→Arg	53	2	4.5.7
Gln→Arg	28	3	4.5.8
Gly→Arg	99	13	4.5.9
His→Arg	19	9	4.5.10
Leu→Arg	43	18	4.5.11
Lys→Asn	-14	11	4.5.12
Lys→Ile	-15	3	4.5.13
Lys→Thr	-27	11	4.5.14
Met→Arg	25	1	4.5.15
Pro→Arg	59	7	4.5.16
Ser→Arg	69	2	4.5.17
Thr→Arg	55	1	4.5.18
Thr→Lys	27	1 or 2	4.5.19
Trp→Arg	-30	2	4.5.20

Table 4.5.1. The new tryptic peptides produced by the three Arg→Gly mutations in the β-chain Mass change: -99.080 Da (monoisotopic), -99.136 Da (average)

Mutation Name	Peptide	$(M+5H)^{5+}$	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
β30Arg→Gly	βT(3-4)	495.065	618.579	824.436	1236.151	2,471.293
NL		*495.367*	*618.957*	*824.940*	*1,236.906*	*2,472.805*
β40Arg→Gly	βT(4-5)	643.922	804.650	1,072.531	1,608.292	3,215.576
NL		***644.340***	***805.174***	***1,073.229***	***1,609.339***	***3,217.670***
β104Arg→Gly	βT(11-12)	546.494	682.866	9,10.152	1,364.724	2,728.440
Nimes		*546.848*	*683.309*	*9,10.742*	*1,365.609*	*2,730.210*
	βT(10-12)	827.025	1,033.530	1,377.703	2,066.051	4,131.095
		827.564	*1,034.203*	*1,378.602*	*2,067.399*	*4,133.789*

Notes.
Monoisotopic *m/z* values are in normal font; average values are in italics.
NL: Not listed in http://globin.bx.psu.edu.
This mutation causes a negative polarity change in the β-chain.
All these mutations can occur by a single base change in the DNA codon (AGG→GGG).
With β30Arg→Gly, AGG→GGG probably abolishes splicing at the normal 5' splice. Variant β-chain was not detected, i.e. β^0-thal.
With β104Arg→Gly, peptides βT(11-12) and βT(10-12) contain Cys. βT(10-12) is more likely to be observed than βT(11-12) because β94Asp hinders cleavage at β95Lys

Table 4.5.2. The new tryptic peptides produced by the three Arg→Met mutations in the β-chain Mass change: -25.061 Da (monoisotopic), -24.989 Da (average)

Mutation Name	Peptide	$(M+5H)^{5+}$	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
β30Arg→Met	βT(3-4)	509.869	637.084	849.109	1,273.160	2,545.312
NL		*510.197*	*637.494*	*849.656*	*1,273.980*	*2,546.951*
β40Arg→Met	βT(4-5)	658.725	823.155	1,097.204	1,645.302	3,289.595
Taipei-Tien		*659.170*	*823.710*	*1,097.944*	*1,646.412*	*3,291.817*
β104Arg→Met	βT(11-12)	561.298	701.371	934.825	1,401.733	2,802.459
Bad Salzuflen		*561.678*	*701.845*	*935.458*	*1,402.682*	*2,804.357*
	βT(10-12)	841.829	1,052.034	1,402.376	2,103.061	4,205.114
		842.394	*1,052.740*	*1,403.317*	*2,104.472*	*4,207.936*

Notes.
Monoisotopic *m/z* values are in normal font; average values are in italics.
NL: Not listed in http://globin.bx.psu.edu.
This mutation causes a negative polarity change in the β-chain.
Variants identified by MS and ions observed in digest spectra are shown in bold font.
All these mutations can occcur by a single base change in the DNA codon (AGG→ATG).
With β104 (Arg→Met), peptides βT(11-12) and βT(10-12) contain Cys. βT(10-12) is more likely to be observed than βT(11-12) because β94Asp hinders cleavage by trypsin at β95Lys.

Table 4.5.3. The new tryptic peptides produced by the three Arg→Ser mutations in the β-chain Mass change: -69.069 Da (monoisotopic), -69.109 Da (average)

Mutation Name	Peptide	$(M+5H)^{5+}$	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
β30Arg→Ser Tacoma	βT(3-4)	501.067	626.082	834.440	**1,251.156**	2,501.304
		501.373	*626.464*	*834.949*	***1,251.919***	*2,502.831*
β40Arg→Ser Austin	βT(4-5)	649.924	812.153	1,082.534	1,623.297	3,245.587
		650.346	*812.680*	*1,083.238*	*1,624.352*	*3,247.697*
β104Arg→Ser Camperdown	βT(11-12)	552.496	690.368	920.155	1,379.729	2,758.450
		552.854	*690.815*	*920.751*	*1,380.622*	*2,760.237*
	βT(10-12)	833.027	1041.032	**1,387.707(I)**	2,081.057	4,161.105
		833.570	*1041.710*	***1,388.611***	*2,082.412*	*4,163.816*

Notes.
Monoisotopic *m/z* values are in normal font; average values are in italics.
This mutation causes a negative polarity change in the β-chain.
Variants identified by MS and ions observed in digest spectra are shown in bold font.
All these mutations can occur by a single base change in the DNA codon (AGG→AGT or AGC).
With Tacoma heterozygotes, variant $\beta T(3\text{-}4)^{2+}$ (*m/z* 1,251.156) should be observed together with normal $\beta T3^{+}$ (1,314.675) and $\beta T4^{+}$ (*m/z* 1,274.73) at reduced intensity relative to $\alpha T5^{+}$ *m/z* 1071.60.
With Camperdown, βT(11-12) and βT(10-12) contain Cys. βT(10-12) is more likely to be observed than βT(11-12) because β94Asp hinders cleavage at β95Lys. See also notes on Sherwood Forest below.

Table 4.5.4. The new tryptic peptides produced by the three Arg→Thr mutations in the β-chain Mass change: -55.053 Da (monoisotopic), -55.083 Da (average)

Mutation Name	Peptide	$(M+5H)^{5+}$	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
β30Arg→Thr Munroe	βT(3-4)	503.870	629.586	839.112	1,258.164	2,515.320
		504.178	*629.970*	*839.625*	*1,258.933*	*2,516.858*
β40Arg→Thr NL	βT(4-5)	652.727	815.657	1,087.206	1,630.305	3,259.603
		653.151	*816.187*	*1,087.913*	*1,631.366*	*3,261.724*
β104Arg→Thr Sherwood Forest	βT(11-12)	555.299	693.872	924.827	1,386.737	2,772.466
		555.659	*694.322*	*925.426*	*1,387.636*	*2,774.26*
Reduced	βT(10-12)	835.830	**1,044.536**	**1,392.379**	2,088.064	4,175.121
		836.375	***1,045.217***	***1,393.286***	*2,089.425*	*4,177.843*
With -S-S- bond.	βT(10-12)	835.427	**1,044.032**	**1,391.707**	2,087.057	4,173.105
		835.972	***1,044.713***	***1,392.614***	*2,088.417*	*4,175.827*

Notes.
Monoisotopic *m/z* values are in normal font; average values are in italics.
NL: Not listed in http://globin.bx.psu.edu.
This mutation causes a negative polarity change in the β-chain.
Variants identified by MS and ions observed in digest spectra are shown in bold font.
All these mutations can occur by a single base change in the DNA codon (AGG→ACG).
With Sherwood Forest, βT(10-12) is observed because β94Asp hinders cleavage at β95Lys. This peptide contains both β-chain Cys, which form an intra-chain disulphide bond during incubation of the digest.

Table 4.5.5. The tryptic peptides produced by the three Arg→Trp mutations in the β-chain
Mass change: 29.978 Da (monoisotopic), 30.025 Da (average)

Mutation Name	Peptide	$(M+5H)^{5+}$	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
β30Arg→Trp	βT(3-4)	520.877	650.844	867.456	1,300.680	2,600.351
NL		*521.200*	*651.247*	*867.994*	*1,301.487*	*2,601.966*
β40Arg→Trp	βT(4-5)	669.733	836.914	1,115.550	1,672.821	3,344.634
NL		*670.173*	*837.464*	*1,116.283*	*1,673.920*	*3,346.832*
β104Arg→Trp	βT(11-12)	572.306	715.130	953.171	1,429.253	2,857.498
Sainte Eugenie		*572.681*	*715.599*	*953.796*	*1,430.190*	*2,859.372*
	βT(10-12)	852.837	1,065.794	1,420.723	2,130.580	4,260.153
		853.397	*1,066.494*	*1,421.656*	*2,131.979*	*4,262.951*

Notes.
Monoisotopic *m/z* values are in normal font; average values are in italics.
NL: Not listed in http://globin.bx.psu.edu.
This mutation causes a negative polarity change in the β-chain.
All these mutations can occur by a single base change in the DNA codon (AGG→TGG).
With Sainte Eugenie, peptides βT(11-12) and βT(10-12) contain Cys. βT(10-12) is more likely to be observed than βT(11-12) because β94Asp hinders cleavage at β95Lys.

Table 4.5.6. The tryptic peptides produced by the six Asn→Lys mutations in the β-chain
Mass change: 14.052 Da (monoisotopic), 14.070 Da (average)

Mutation Name	Peptide	Mass (M)	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
β19Asn→Lys	βT3a	245.174				246.182
D-Ouled Rabah	**βT3b**	1,100.546	276.144	367.857	**551.281**	**1,101.554**
	βT3	1,327.710		**443.578**	**664.863**	**1,328.717**
β57Asn→Lys	βT5a	1,846.845	462.719	616.623	924.430	1,847.853
G-Ferrara	βT5b	243.158				244.166
	βT5	**2,071.993**	519.006	691.672	**1037.004**	2,073.000
β80Asn→Lys	βT9a	1,441.757	361.447	481.593	721.886	1,442.764
G-Szuhu, Gigu	βT9b	259.190				260.197
	βT9	1,682.936	421.742	561.986	842.476	1,683.943
β102Asn→Lys	βT11a	836.439		279.821	419.227	837.447
Richmond	βT(10-11a)	2,239.094	560.781	747.373	1120.555	2,240.102
	βT11b	321.180				322.188
β108Asn→Lys	**βT12a**	**429.295**			215.655	**430.303**
Presbyterian	**βT12b**	**1,321.733**	331.441	**441.585**	**661.874**	1,322.741
β139Asn→Lys	**βT14a**	**642.407**		215.143	322.211	**643.414**
Hinsdale	**βT14b**	**538.323**			270.169	**539.331**
	βT(14b-15)	**838.445**		280.489	**420.230**	**839.453**

Notes.
All masses and *m/z* values are monoisotopic.
This mutation causes a positive polarity change in the β-chain.
Variants identified by MS and ions observed in digest spectra are shown in bold font.
All these mutations can occur by a single base change in the DNA codon.
With D-Ouled Rabah, cleavage is slow at 19Lys. Hence abundance of βT3b peptide is low. Mechanism appears to be similar to slow cleavage by trypsin at 92Arg in the normal α-chain (β-17KVKVDEV- is similar to α-90KLRVDPV-). If necessary, prove the mutation occurs at 19Lys by tandem MS on $βT3^{2+}$ ions. The variant $βT3^{2+}$ ion needs higher collision energy (25V) than the normal $βT3^{2+}$ ion (19V) using argon collision gas.
With G-Ferrara, β58Pro inhibits cleavage at β57Lys. Hence variant βT5 is observed.
With G-Szuhu, β79Asp is likely to hinder cleavage at β80Lys. Hence variant βT9 is likely to be observed.
In Richmond and Presbyterian, peptides βT(10-11)a and βT12b respectively contain Cys.
With Richmond, variant βT(10-11)a is more likely to be observed than βT11a because β94Asp hinders cleavage at β95Lys.

Table 4.5.7. The new tryptic peptides produced by the two Cys→Arg mutations in the β-chain
Mass change: 53.092 Da (monoisotopic), 53.043 Da (average)

Mutation Name	Peptide	Mass (M)	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
β93Cys→Arg	**βT10a**	**1,230.636**	**411.220**	**616.326**	**1,231.644**
Okazaki	βT10b	261.133			262.140
	βT(10b-11)	**1,368.679**	457.234	**685.347**	**1,369.686**
β112Cys→Arg	βT12a	882.565	295.196	442.290	883.573
Indianapolis	βT12b	907.503	303.509	454.759	908.511

Notes.
All mass and *m/z* values are monoisotopic.
This mutation causes a positive polarity change in the β-chain.
Variants identified by MS and ions observed in digest spectra are shown in bold font.
Both mutations can occur by a single base change in the DNA codon (TGT→CGT).
With Okazaki, the βT(10b-11) peptide occurs in 30-min digest because cleavage at β95Lys is hindered by β94Asp.

Table 4.5.8. The tryptic peptides produced by the three Gln→Arg mutations in theβ-chain
Mass change: 28.043 Da (monoisotopic), 28.057 Da (average)

Mutation Name	Peptide	Mass (M)	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
β39Gln→Arg	βT4a	1,145.660	382.894	573.838	1,146.668
Tianshui	βT4b	174.112			175.120
β127Gln→Arg	βT13a	844.444	282.489	423.230	845.452
Dieppe	βT13b	579.302		290.659	580.310
β131Gln→Arg	βT13a	1,277.640	426.888	639.828	1,278.648
Sarrebourg	βT13b	146.106			147.113

Notes.
All mass and *m/z* values are monoisotopic.
This mutation causes a positive polarity change in the β-chain.
All these mutations can occur by a single base change in the DNA codon (CAG→CGG).

Table 4.5.9. The tryptic peptides produced by the thirteen Gly→Arg mutations in the β-chain
Mass change: 99.080 Da (monoisotopic), 99.136 Da (average)

Mutation Name	Peptide	Mass (M)	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
β16Gly→Arg	**βT2a**	**902.497**		301.840	**452.257**	**903.505**
D-Bushman	βT2b	146.106				147.113
β24Gly→Arg	βT3a	829.429		277.484	415.723	830.437
Riverdale-Bronx	βT3b	601.318		201.447	301.667	602.326
β25Gly→Arg	βT3a	886.451		296.491	444.233	887.459
G-Taiwan-Ami	βT3b	544.297			273.156	545.305
β29Gly→Arg	βT3a	1256.636	315.167	419.887	629.326	1,257.644
NL	βT3b	174.112				175.120
β46Gly→Arg	**βT5a**	**831.392**		278.138	**416.704**	**832.399**
Gainesville-GA	**βT5b**	**1,343.639**	336.918	448.888	**672.827**	**1,344.647**
β56Gly→Arg	**βT5a**	**1,817.829**	455.465	606.951	**909.923**	1,818.837
Hamadan	**βT5b**	**357.201**			179.608	**358.209**
β64Gly→Arg	βT7a	382.208			192.112	383.216
NL	βT7b	146.106				147.113
β69Gly→Arg	βT9a	386.264			194.140	387.272
Kenitra	βT9b	1,399.710	350.935	467.578	700.863	1,400.717
β74Gly→Arg	βT9a	863.450		288.825	432.733	864.458
Aalborg	βT9b	922.524		308.516	462.270	923.531
β83Gly→Arg	βT10a	174.112				175.120
Muskegon	βT10b	1,363.644	341.919	455.556	682.830	1,364.652
	βT(10b-11)	2,471.190	618.805	824.738	1,236.603	2,472.198
β107Gly→Arg	βT12a	400.280			201.148	401.288
Burke	βT12b	1,435.776	359.952	479.600	718.896	1,436.784
β119Gly→Arg	βT12a	1,689.950	423.495	564.325	845.983	1,690.958
Angoulime	βT12b	146.106				147.113
β136Gly→Arg	βT14a	443.286			222.651	444.293
‘tlangeland	βT14b	822.471		275.165	412.243	823.479
	βT(14b-15)	1,122.593	281.656	375.206	562.305	1,123.601

Notes.
All mass and *m/z* values are monoisotopic. NL: Not listed in http://globin.bx.psu.edu.
This mutation causes a positive polarity change in the β-chain.
Variants identified by MS and ions observed in digest spectra are shown in bold font.
All these mutations can occur by a single base change in the DNA codon.
Peptides βT10b, βT(10b-11), βT12b (Burke) and βT12a (NL) contain Cys.

Table 4.5.10. The new tryptic peptides produced by the nine His→Arg mutations in the β-chain
Mass change: 19.042 Da (monoisotopic), 19.046 Da (average)

Mutation Name	Peptide	Mass (M)	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
β2His→Arg	**βT1a**	**273.180**				**274.188**
Deer Lodge	**βT1b**	**715.375**		239.466	**358.695**	**716.383**
β63His→Arg	βT7a	245.149				246.157
Zurich	βT7b	203.127				204.135
	βT(7b-8)	331.222				332.230
β77His→Arg	βT9a	1,104.593	277.156	369.205	553.304	1,105.601
Costa Rica	βT9b	601.344		201.456	301.680	602.351
β92His→Arg	βT10a	1,093.577	274.402	365.533	547.796	1,094.585
Mozhaisk	βT10b	364.142				365.150
	βT(10b-11)	1,471.688	368.930	491.570	736.852	1,472.696
β97His→Arg	βT11a	287.196				288.204
NL	βT(10-11a)	1,689.851	423.471	564.291	845.933	1,690.859
	βT11b	875.414	---------	292.812	438.715	876.422
β116His→Arg	**βT12a**	**1,268.764**	318.199	423.929	**635.390**	**1,269.772**
Sfax	**βT12b**	**487.254**			244.635	**488.262**
β117His→Arg	βT12a	1,405.823	352.464	469.615	703.919	1,406.831
P-Galveston	βT12b	350.195				351.203
β143His→Arg	**βT14a**	**1,039.614**	260.911	347.546	**520.815**	**1,040.622**
Abruzzo	βT14b	146.106				147.113
	βT(14b-15)	446.228			224.122	447.236
β146His→Arg	βT15	337.175				338.183
Cochin-Port Royal	βT(14-15)	1,467.831	367.966	490.285	734.923	1,468.839

Notes.
All mass and *m/z* values are monoisotopic.
NL: Not listed in http://globin.bx.psu.edu.
This mutation causes a positive polarity change in the β-chain.
Variants identified by MS and ions observed in digest spectra are shown in bold font.
All these mutations can occur by a single base change in the DNA codon.
Peptides βT10b and βT(10b-11) in Mozhaisk; βT(10-11a) in β97His→Arg; βT12a in Sfax and P-Galveston contain Cys.

Table 4.5.11. The tryptic peptides produced by the eighteen Leu→Arg mutations in the β-chain
Mass change: 43.017 Da (monoisotopic), 43.028 Da (average)

Mutation Name	Peptide	Mass (M)	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
β3Leu→Arg	βT1a	410.239			206.127	411.247
NL	βT1b	602.291		201.772	302.153	603.299
β14Leu→Arg	**βT2a**	**603.334**		202.119	302.675	**604.342**
Sogn	**βT2b**	**389.206**			195.611	**390.214**
β28Leu→Arg	βT3a	1,143.552	286.896	382.192	572.784	1,144.560
Chesterfield	βT3b	231.133				232.141
β31Leu→Arg	βT4a	174.112				175.120
Hakkari	βT4b	1,160.634	291.166	387.886	581.325	1,161.642
β32Leu→Arg	βT4a	287.196				288.204
Castilla	βT4b	1,047.550	262.895	350.191	524.783	1,048.558
β48Leu→Arg	βT5a	1,003.440	251.868	335.488	502.728	1,004.448
Okaloosa	βT5b	1,115.528	279.890	372.851	558.772	1,116.536
β68Leu→Arg	βT9a	273.180				274.188
NL	βT9b	1,456.731	365.191	486.585	729.373	1,457.739
β75Leu→Arg	βT9a	920.472	231.126	307.832	461.244	921.479
Pasadena	βT9b	809.440	203.368	270.821	405.728	810.447
β78Leu→Arg	βT9a	1,241.652	311.421	414.892	621.834	1,242.660
Quin-Hai	βT9b	488.260			245.138	489.267
β81Leu→Arg	βT9a	1,583.806	396.959	528.943	792.911	1,584.813
Baylor	βT9b	146.106				147.113
β88Leu→Arg	βT10a	651.334		218.119	326.675	652.342
Boras	βT10b	830.359		277.794	416.187	831.367
	βT(10b-11)	1,937.905	485.484	646.976	969.961	1,938.913
β91Leu→Arg	**βT10a**	**980.493**		327.839	**491.254**	**981.501**
Caribbean	βT10b	501.201			251.608	502.208
	βT(10b-11)	**1,608.747**	403.195	**537.257 (I)**	**805.381**	16,09.755
β96Leu→Arg	βT11a	174.112				175.120
NL	βT(10-11a)	1,576.767	395.200	526.597	789.391	1,577.775
	βT11b	1,012.473		338.499	507.244	1,013.480
β105Leu→Arg	βT12a	174.112				175.120
NL	βT12b	1,605.881	402.478	536.302	803.949	1,606.889

Mutation Name	Peptide	Mass (M)	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
β106Leu→Arg	βT12a	287.196				288.204
Terre Haute	βT12b	1,492.797	374.207	498.607	747.406	1,493.805
β110Leu→Arg	βT12a	670.413		224.479	336.214	671.420
NL	βT12b	1,109.580		370.868	555.798	1,110.588
β114Leu→Arg	βT12a	1,084.643		362.555	543.329	1,085.651
NL	βT12b	695.350		232.791	348.683	696.358
β141Leu→Arg	βT14a	855.493		286.172	428.754	856.501
Olmsted	βT14b	354.202				355.209
	βT(14b-15)	654.324		219.116	328.170	655.332

Notes.
All mass and *m/z* values are monoisotopic.
NL: Not listed in http://globin.bx.psu.edu.
This mutation causes a positive polarity change in the β-chain.
Variants identified by MS and ions observed in digest spectra are shown in bold font.
I: interference.
All these mutations can occur by a single base change in the DNA codon.
In Boras and Caribbean, βT10b and βT10b-11 contain Cys. Also, βT10b-11 is more likely to occur than βT10b because β94Asp hinders cleavage at β95Lys.
In β96Leu→Arg, βT(10-11a) contains Cys. Also, βT(10-11a) is more likely to occur than βT11a because β94Asp hinders cleavage at β95Lys.
In β105Leu→Arg, β106Leu→Arg and β110Leu→Arg, βT12b contains Cys.
In β114Leu→Arg, βT12a contains Cys.

Table 4.5.12. The tryptic peptides produced by the eleven Lys→Asn mutations in the β-chain
Mass change: -14.052 Da (monoisotopic), -14.070 Da (average)

Mutation Name	Peptide	$(M+5H)^{5+}$	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
β8Lys→Asn	βT(1-2)		463.746	617.992	926.484	1,851.961
Limassol			*464.027*	*618.367*	*927.047*	*1,853.086*
β17Lys→Asn	βT(2-3)		554.285	738.710	1,107.562	2,214.116
J-Amiens			*554.613*	*739.148*	*1,108.218*	*2,215.429*
β59Lys→Asn	**βT(5-6)**		568.771	758.025	**1,136.534**	2,272.060
J-Lome			*569.138*	*758.515*	***1,137.268***	*2,273.529*
β61Lys→Asn	βT(6-7)			209.119	313.175	625.342
Hikari						
β65Lys→Asn	βT(7-8)				263.641	526.274
J-Sicilia						
β66Lys→Asn	βT(8-9)		446.739	595.317	892.471	1,783.934
Ulm			*447.009*	*595.676*	*893.009*	*1,785.011*
β82Lys→Asn	βT(9-10)		765.379	1,020.170	1,529.751	3,058.494
Providence			*765.860*	*1,020.811*	*1,530.712*	*3,060.416*
	βT(9-11)	834.014	1,042.266	1,389.352	2,083.524	4,166.041
		834.534	*1,042.915*	*1,390.218*	*2,084.823*	*4,168.638*
β95Lys→Asn	**βT(10-11)**	503.840	**629.548**	**839.061**	**1,258.088**	2,515.168
Detroit		*504.157*	***629.945***	***839.590***	***1,258.881***	*2,516.755*
β120Lys→Asn	βT(12-13)	613.927	767.157	1,022.540	1,533.306	3,065.604
Riyadh		**614.324**	**767.652**	**1,023.201**	**1,534.297**	**3,067.586**
β132Lys→Asn	βT(13-14)		624.582	832.440	1,248.156	2,495.305
Yamagata			*624.963*	*832.948*	*1,248.919*	*2,496.830*
	βT(13-15)	559.892	699.613	932.481	1,398.217	2,795.427
		560.236	*700.043*	*933.054*	*1,399.077*	*2,797.147*
β144Lys→Asn	**βT(14-15)**		359.692	**479.253**	**718.376**	1,435.745
Andrew-Minneapolis			*359.909*	***479.543***	***718.811***	*1,436.614*

Notes.
Monoisotopic *m/z* values are in normal font, average values are in italics.
This mutation causes a negative polarity change in the β-chain.
Variants identified by MS and ions observed in digest spectra are shown in bold font.
Hb Providence partly deamidates to give β82Lys→Asn and β82Lys→Asp. Above values assume β82Lys→Asn. βT(9-11) will probably occur because β94Asp hinders cleavage at β95Lys.
All these mutations are possible by a single base change in the DNA codon.
Peptides βT(9-10), βT(9-11), βT(10-11), βT(12-13) contain Cys.

Table 4.5.13. The tryptic peptides produced by three Lys→Ile mutations in the β-chain
Mass change: -15.011 Da (monoisotopic), -15.015 Da (average)

Mutation Name	Peptide	$(M+5H)^{5+}$	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
β66Lys→Ile NL	βT(8-9)		446.500	594.997	891.992	1,782.976
β120Lys→Ile Jianghua	βT(12-13)	613.735 *614.135*	766.917 *767.416*	1,022.220 *1,022.886*	1,532.826 *1,533.825*	3,064.645 *3,066.642*
β132Lys→Ile NL	βT(13-14)	499.675 *499.983*	624.342 *624.727*	832.121 *832.634*	1,247.677 *1,248.447*	2,494.346 *2,495.885*

Notes.
Monoisotopic *m/z* values are in normal font; average values are in italics.
NL: Not listed in http://globin.bx.psu.edu.
This mutation causes a negative polarity change in the β-chain.
Although there are eleven Lys in the β-chain, only those mutations shown can occur by a single base change in the DNA codon (AAA→ATA).
Peptide βT(12-13) contains Cys.

Table 4.5.14. The tryptic peptides produced by the eleven Lys→Thr mutations in the β-chain
Mass change: -27.047 Da (monoisotopic), -27.069 Da (average)

Mutation Name	Peptide	$(M+5H)^{5+}$	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
β8Lys→Thr	βT(1-2)		460.497	613.660	919.987	1,838.965
Rio Grande			*460.778*	*614.034*	*920.547*	*1,840.087*
β17Lys→Thr	βT(2-3)		551.036	734.379	1,101.064	2,201.120
NL			*551.363*	*734.815*	*1,101.719*	*2,202.430*
β59Lys→Thr	**βT(5-6)**		565.522	753.693	**1,130.036**	2,259.064
J-Kaohsiung			*565.888*	*754.182*	***1,130.769***	*2,260.530*
β61Lys→Thr	**βT(6-7)**			204.788	**306.677**	**612.347 (I)**
Novel						
β65Lys→Thr	βT(7-8)				257.143	513.279
NL						
β66Lys→Thr	βT(8-9)		443.491	590.985	885.973	1,770.939
Chico			*443.759*	*591.343*	*886.510*	*1,772.012*
β82Lys→Thr	βT(9-10)		762.131	1,015.838	1,523.253	3,045.499
Rahere			*762.610*	*1,016.478*	*1,524.212*	*3,047.417*
	βT(9-11)	831.415	1,039.017	1,385.020	2,077.027	4,153.045
		831.934	*1,039.666*	*1,385.885*	*2,078.324*	*4,155.639*
β95Lys→Thr	βT(10-11)		626.299	834.729	1,251.590	2,502.172
NL			*626.695*	*835.257*	*1,252.382*	*2,503.756*
β120Lys→Thr	βT(12-13)	611.328	763.908	1,018.208	1,526.808	3,052.608
NL		***611.724***	***764.403***	***1,018.868***	***1,527.798***	***3,054.587***
β132Lys→Thr	βT(13-14)		621.333	828.108	1,241.659	2,482.309
Cook			*621.714*	*828.616*	*1,242.419*	*2,483.831*
	βT(13-15)	557.293	696.364	928.149	1,391.720	2,782.432
		557.636	*696.793*	*928.721*	*1,392.578*	*2,784.148*
β144Lys→Thr	βT(14-15)		356.443	474.922	711.879	1,422.749
NL			*356.660*	*475.210*	*712.312*	*1,423.615*

Notes.
Monoisotopic *m/z* values are in normal font; average values are in italics.
NL: Not listed in http://globin.bx.psu.edu.
This mutation causes a negative polarity change in the β-chain.
Variants identified by MS and ions observed in digest spectra are shown in bold font.
All these mutations can occur by a single base change in the DNA codon.
Peptides βT(9-10), βT(9-11), βT(10-11), βT(12-13) contain Cys.
With Rahere, βT(9-11) will probably occur because β94Asp hinders cleavage at β95Lys.

Table 4.5.15. The new tryptic peptides produced by the single Met→Arg mutation in the β-chain
Mass change: 25.061 Da (monoisotopic), 24.989 Da (average)

Mutation Name	Peptide	Mass (M)	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
β55Met→Arg	βT5a	1,686.789	563.271	844.402	1,687.797
NL	βT5b	414.223		208.119	415.231

Notes.
All mass and *m/z* values are monoisotopic.
NL: Not listed in http://globin.bx.psu.edu.
This mutation causes a positive polarity change.
This mutation can occur by a single base change in the DNA codon (ATG→AGG).

Table 4.5.16. The tryptic peptides produced by the seven Pro→Arg mutations in the β-chain
Mass change: 59.048 Da (monoisotopic), 59.071 Da (average)

Mutation Name	Peptide	Mass (M)	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
β5Pro→Arg	βT1a	624.371	209.131	313.193	625.379
Warwickshire	βT1b	404.191		203.103	405.199
β36Pro→Arg	βT4a	761.480	254.834	381.748	762.488
Sunnybrook	βT4b	589.297		295.656	590.305
β51Pro→Arg	**βT5a**	**1,304.604**	435.876	**653.310**	**1,305.612**
Willamette	**βT5b**	**830.396**	277.806	416.206	**831.403**
β58Pro→Arg	**βT5a**	**1,988.894**	663.972	**995.455**	**1,989.902**
Dhofar,	**βT5b**	**373.269**			**374.277**
β100Pro→Arg	βT11a	638.350	213.791	320.183	639.358
New Mexico	βT(10-11a)	2,041.005	681.343	1,021.510	2,042.013
	βT11b	564.266		283.141	565.273
β124Pro→Arg	βT13a	551.270		276.643	552.278
Khartoum	βT13b	903.481	302.168	452.749	904.489
	βT13	**1,436.741**	479.922	**719.378**	**1,437.749**
β125Pro→Arg	βT13a	648.323	217.116	325.169	649.331
NL	βT13b	806.429	269.817	404.222	807.437

Notes.
All mass and *m/z* values are monoisotopic.
NL: Not listed in http://globin.bx.psu.edu.
This mutation causes a positive polarity change in the β-chain.
Variants identified by MS and ions observed in digest spectra are shown in bold font.
All these mutations can occur by a single base change in the DNA codon.
In Dhofar, βT5b is 59KVK, where VK is βT6.
In New Mexico, βT(10-11a) is more likely to occur than βT11a because β94Asp hinders cleavage at β95Lys. Also, βT(10-11a) contains Cys.
In Khartoum, β125Pro inhibits cleavage at β124Arg. Hence, βT13 is observed instead of the two new peptides shown above.

Table 4.5.17. The tryptic peptides produced by two of the Ser→Arg mutations in the β-chain
Mass change: 69.069 Da (monoisotopic), 69.109 Da (average)

Mutation Name	Peptide	Mass (M)	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
β72Ser→Arg	βT9a	661.391	221.472	331.703	662.399
Headington	βT9b	1,094.572	365.865	548.294	1,095.580
β89Ser→Arg	βT10a	764.418	255.814	383.217	765.426
Vanderbilt	βT10b	743.327	248.784	372.671	744.335
	βT(10b-11)	1,850.873	617.966	926.445	1,851.881

Notes.
All mass and *m/z* values are monoisotopic.
NL: Not listed in http://globin.bx.psu.edu.
This mutation causes a positive polarity change in the β-chain.
Variants identified by MS and ions observed in digest spectra are shown in bold font.
Although there are 5 Ser in the β-chain, only Headington and Vanderbilt can occur by a single base change in the DNA codon (AGT→AGA or CGT).
In Vanderbilt, βT(10b-11) is more likely to be observed than βT10b because β94Asp hinders cleavage by trypsin at β95Lys. Also βT(10b-11) contains Cys.

Table 4.5.18. The tryptic peptides produced by one of the Thr→Arg mutations in the β-chain
Mass change: 55.053 Da (monoisotopic), 55.083 Da (average)

Mutation Name	Peptide	Mass (M)	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
β87Thr→Arg	βT10a	550.286			276.151	551.294
NL	βT10b	943.443		315.489	472.729	944.451
	βT(10b-11)	2,050.990	513.755	684.671	1,026.503	2,051.997

Notes.
All masses and *m/z* values are monoisotopic.
NL: Not listed in http://globin.bx.psu.edu.
This mutation causes a positive polarity change in the β-chain.
Peptides βT10b and βT(10b-11) contain Cys.
Although there are 7 Thr in the β-chain, only the one shown (β87Thr) can mutate to Arg by a single base change in the DNA codon (ACA→AGA).

Table 4.5.19. The tryptic peptides produced by two Thr→Lys mutations in the β-chain
Mass change: 27.047 Da (monoisotopic), 27.069 Da (average)

Mutation Name	Peptide	Mass (M)	$(M+4H)^{4+}$	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
β50Thr→Lys	βT5a	1,175.550		392.858	588.783	1,176.558
Edmonton	βT5b	927.448		310.157	464.732	928.456
	βT5	2,084.988	522.255	696.004	1,043.502	2,085.996
β87Thr→Lys	**βT10a**	**522.280**			262.148	**523.288**
D-Ibadan	βT10b	943.443		315.489	472.729	944.451
	βT(10b-11)	**2,050.990**	**513.755**	**684.671**	**1,026.503**	2,051.997

Notes.
All mass and *m/z* values are monoisotopic.
NL: Not listed in http://globin.bx.psu.edu.
This mutation causes a positive polarity change in the β-chain.
Variants identified by MS and ions observed in digest spectra are shown in bold font.
Peptides βT10b and βT(10b-11) contain Cys.
Although there are 7 Thr in the β-chain only β87Thr→Lys can occur by a single base change in the codon (ACA→AAA).
The mutation in Edmonton requires two base changes in the DNA codon (ACT→AAG or AAA), or the codon at β50 was ACA. This conflict is mentioned in the Web summary for Edmonton.

Table 4.5.20. The new tryptic peptides produced by the two Trp→Arg mutations in the β-chain
Mass change: -29.978 Da (monoisotopic), -30.026 Da (average)

Mutation Name	Peptide	Mass (M)	$(M+3H)^{3+}$	$(M+2H)^{2+}$	$(M+H)^{+}$
β15Trp→Arg	**βT2a**	**716.418**	239.814	**359.217**	**717.426**
Belfast	βT2b	203.127			204.135
β37Trp→Arg	βT4a	858.533	287.185	430.274	859.541
Rothschild	βT4b	403.218		202.617	404.226

Notes.
All mass and *m/z* values are monoisotopic.
This mutation causes a positive polarity change in the β-chain.
Variants identified by MS and ions observed in digest spectra are shown in bold font.
Both mutations can occur by a single base change in the DNA codon.

4.6. Tables to assist in searching for some tryptic peptides produced from α- and β-chain variants that involve the mutations Xxx↔Asp when adjacent to Lys

When proteins are digested with trypsin, cleavage normally occurs at the C-terminal side of the amino acid residues Arg (R) and Lys (K), except when Pro (P) occurs C-terminally and adjacent to these residues. This latter situation does not arise with the α-, β- and γ-chains in human haemoglobin. However, when Asp (D) occurs adjacent to Lys cleavage is severely inhibited at 6DK and 126DK in the normal α-chain and at 94DK in the normal β-chain causing the peptides shown below to occur as major components in 30-minute digests.

```
                1     6
αT1-2           VLSPADKTNVK

                  100                           126
αT12-13         LLSHCLLVTLAAHLPAEFTPAVHASLDKFLASVSTVLTSK

                83            94
βT10-11         GTFATLSELHCDKLHVDPENFR
```

The following three tables were devised to assist in searching for variants that involve mutations from the three Asp shown above, i.e. α6Asp→Xxx (Table 4.6.1), α126Asp→Xxx (Table 4.6.2) and β94Asp→Xxx (Table 4.6.3).

Table 4.6.1. Some α6Asp→Xxx mutations that can occur by a single base change in the nucleotide codon. Masses and *m/z* values are monoisotopic. Variants identified by ESI-MS are shown in bold font. ΔM: Mass change. NL: Not listed in http://globin.bx.psu.edu. Unless otherwise stated, the mutation can be in either the α1 or the α2 gene.

Mutation Name	**Nominal ΔM (Da)**	**Mass (Da)**	**αT1^{2+}**	**αT1^{+}**
α6Asp→Ala Sawara	-44	684.417	343.216	685.425
α6Asp→Asn Dunn	**-1**	**727.423**	**364.719**	**728.431**
α6Asp→Glu NL	14	742.423	372.219	743.430
α6Asp→Gly Swan River	-58	670.401	336.209	671.409
$\alpha_2$6Asp→His Galliera II	22	750.439	376.227	751.447
$\alpha_1$6Asp→His Galliera I	22	750.439	376.227	751.447
α6Asp→Tyr Woodville	**48**	**776.443**	**389.229**	**777.451**

Mutation Name	**Nominal ΔM (Da)**	**Mass (Da)**	**αT1^{2+}**	**αT1^{+}**
α6Asp→Val Ferndown	-16	712.448	357.232	713.456
α6Asp→0 Boyle Heights	**-115**	**613.380**	**307.698**	**614.388**
			αT2^{2+}	**αT2^{+}**
Normal αT2 peptide		460.265	231.140	461.272

Table 4.6.2. Some α126Asp→Xxx mutations that can occur by a single base change in the nucleotide codon. For peptides below mass 2,300 Da, only monoisotopic *m/z* values are given in normal font. For peptides above mass 2,300 Da, average *m/z* values are given beneath the monoisotopic values. Variants identified by ESI-MS are shown in bold font. ΔM: Mass change. NL: Not listed in http://globin.bx.psu.edu. Unless otherwise stated, the mutation can be in either the α1 or the α2 gene.

Mutation Name	**Nominal ΔM (Da)**	**αT12^{4+}**	**αT12^{3+}**	**αT12^{2+}**
$\alpha_1$126Asp→Ala Verdun	-44	731.662 *732.134*	975.213 *975.843*	1,462.316 *1,463.260*
α126Asp→Asn Tarrant	-1	742.413 *742.890*	989.548 *990.185*	1,483.818 *1,484.773*
$\alpha_1$126Asp→Glu Burlington	14	746.163 *746.643*	994.548 *995.188*	1,491.318 *1,492.279*
α126Asp→Gly West One	**-58**	**728.158** ***728.627***	**970.541** ***971.167***	1,455.308 *1,456.247*
$\alpha_1$126Asp→His Sassari	22	748.167 *748.650*	997.220 *997.864*	1,495.326 *1,496.291*
α126Asp→Tyr Montefiore	48	754.668 *755.158*	1,005.888 *1,006.542*	1,508.329 *1,509.309*
α126Asp→Val Fukutomi	-16	738.670 *739.148*	984.557 *985.194*	1,476.331 *1,477.287*
α126Asp→0 NL	-115	713.902 *714.364*	951.534 *952.150*	1,426.797 *1,427.721*
		αT13^{3+}	**αT13^{2+}**	**αT13^{+}**
Normal αT13 peptide		418.244	**626.862**	**1,252.715**
		αT13-14^{3+}	**αT13-14^{2+}**	**αT13-14^{+}**
Normal αT(13-14) peptide		524.632	786.444	1,571.880

Table 4.6.3. Some β94Asp→Xxx mutations that can occur by a single base change in the nucleotide codon. The *m/z* values are monoisotopic. Variants identified by ESI-MS are shown in bold font. ΔM: Mass change. NL: Not listed in http://globin.bx.psu.edu.

Mutation Name	**Nominal ΔM (Da)**	**βT10^{3+}**	**βT10^{2+}**	**βT10^{+}**
β94Asp→Ala NL	-44	459.900	689.346	1,377.684
β94Asp→Asn Bunbury	**-1**	**474.235**	**710.849**	**1,420.690**
β94Asp→Glu NL	14	479.235	718.348	1,435.689
β94Asp→Gly Chandigarh	-58	455.228	682.338	1,363.668
β94Asp→His Barcelona	22	481.907	722.357	1,443.705
β94Asp→Tyr Geldrop St Anna	48	490.575	735.359	1,469.710
β94Asp→Val NL	-16	469.244	703.361	1,405.715
β94Asp→0 NL	-115	436.221	653.827	1,306.647
		βT11^{3+}	**βT11^{2+}**	**βT11^{+}**
Normal βT11 peptide		376.193	**563.786**	**1,126.565**

Table 4.6.4. Some variants that result from the mutation Xxx→Asp when Xxx is adjacent to Lys and that can occur by a single base change in the nucleotide codon . For peptides below mass 2300, monoisotopic *m/z* values are given in normal font. For peptides above mass 2300, average *m/z* values are given in italics below the monoisotopic values. Variants identified and the *m/z* values of the peptides observed by ESI-MS are shown bold. ΔM: Nominal mass change. NL: Not listed in http://globin.bx.psu.edu.

Mutation Name	**Peptide ΔM (Da)**	**$(M+4H)^{4+}$**	**$(M+3H)^{3+}$**	**$(M+2H)^{2+}$**	**$(M+H)^{+}$**
α10Val→Asp NL	αT(1-3) 16	425.980	567.638	850.952	1,700.897
α15Gly→Asp I-Interlaken	αT(3-4) **58**	526.008	701.009	1,051.009	2,101.010
	αT3			295.651	**590.294[a]**
α30Glu→Asp NL	αT(4-5) -14	642.820 *643.231*	856.757 *857.306*	1,284.632 *1,285.455*	
α55Val→Asp NL	αT(6-7) 16	558.018	743.688	1,115.028	2,229.048
α59Gly→Asp Adana	αT(7-8) 58		195.444	292.662	584.316
α89His→Asp NL	α(9-10) -22	811.667 *812.167*	1,081.886 *1,082.553*	1,622.326 *1,623.325*	
β7Glu→Asp Stockholm	βT(1-2) -14	463.755	618.004	926.502	1,851.997
β16Gly→Asp J-Baltimore	**βT(2-3) 58**	572.299 *572.640*	**762.730** ***763.184***	**1,143.590** ***1,144.272***	2,286.173 *2,287.536*
β29Gly→Asp[b] Lufkin	βT(3-4) 58	657.851 *658.250*	876.798 *877.331*	1,314.693 *1,315.492*	2,628.379 *2,629.977*
β64Gly→Asp J-Calabria	βT(7-8) **58**		200.116	299.670	**598.331**
		$(M+5H)^{5+}$	**$(M+4H)^{4+}$**	**$(M+3H)^{3+}$**	**$(M+2H)^{2+}$**
β83Gly→Asp[c] Pyrgos	**βT(9-11) 58**	**848.426 *848.955***	**1,060.280 *1,060.942***	1,413.371 *1,414.254*	2,119.553 *2,120.876*
	βT(8-11)	**874.045 *874.590***	**1,092.304 *1,092.986***	1,456.070 *1,456.978*	2,183.600 *2,184.964*
β119Gly→Asp Fannin-Lubbock I	**βT(12-13) 58**	628.338 *628.745*	**785.171 *785.679***	**1,046.559 *1,047.236***	1,569.334 *1,570.350*
β119Gly→Asp /β111Val→Leu Fannin-Lubbock II	**βT(12-13) 72**	631.142 *631.550*	**788.675 *789.186***	1,051.231(I) *1,051.912(I)*	1,576.342 *1,577.364*
β143His→Asp Rancho Mirage	βT(14-15) -22				

Notes.

a. In I-Interlaken, there is interference at *m/z* 701.01 and 1,051.01, which prevents observation of the variant αT(3-4) peptide. However, the variant $\alpha T3^{+}$ ion was observed with sufficient intensity to allow identification.

b. Although the mutant Asp is adjacent to Arg, it has potential to hinder cleavage at the Arg.

c. In this mutation, the new Asp occurs on the C-Terminal side of the Lys.

4.7. Examples of variants in which cleavage by trypsin at Lys is severely hindered by an adjacent Asp.

4.7.1. Identification of a Hb Bunbury heterozygote.

Analysis of a blood sample gave the mass of the β-chain as 15,866.81 Da, i.e. (β^{A}-0.43) Da, suggesting a β^{A}/(β^{A}-1 Da) heterozygote. Figures 4.7.1 and 4.7.2 show part spectra from 30-min tryptic digests of (a) a normal control and (b) the blood sample. The presence of the variant $\beta T10^{2+}$ ion (Figure 4.7.1) at its calculated value together with the normal $\beta T11^{2+}$ ion (Figure 4.7.2) identifies the mutation as β94Asp→Asn, Hb Bunbury. Note that although there are three additional mutations in the βT10-11 peptide that would lower the mass by 1 Da, only the Bunbury mutation can produce the variant $\beta T10^{2+}$ and the normal $\beta T11^{2+}$ ions at the *m/z* values shown.

4.7.2. Identification of a Hb J-Baltimore heterozygote.

The mass of the β-chain in a blood sample was 58 Da higher than normal, suggesting two possible mutations, either Gly→Asp or Ala→Glu. Part spectra of the tryptic digests from the blood sample and a control are shown in Figure 4.7.3. The spectrum from the variant sample (Figure 4.7.3b) shows an intense doubly charged ion at *m/z* 1,143.58, which corresponds to the $\beta T(2\text{-}3)^{2+}$ ion (calculated *m/z* 1,143.59) characteristic of the mutation β16Gly→Asp, J-Baltimore.

4.7.3. Identification of a Hb Pyrgos heterozygote.

This blood sample also gave a β-chain mass that was 58 Da higher than normal, suggesting either Gly→Asp or Ala→Glu. However, in this case the part spectra from 30-min digests of control and sample (Figure 4.7.4) show how the formation of the βT(9-10-11) and βT(8-9-10-11) tryptic peptides result from the mutation β83Gly→Asp, Hb Pyrgos.

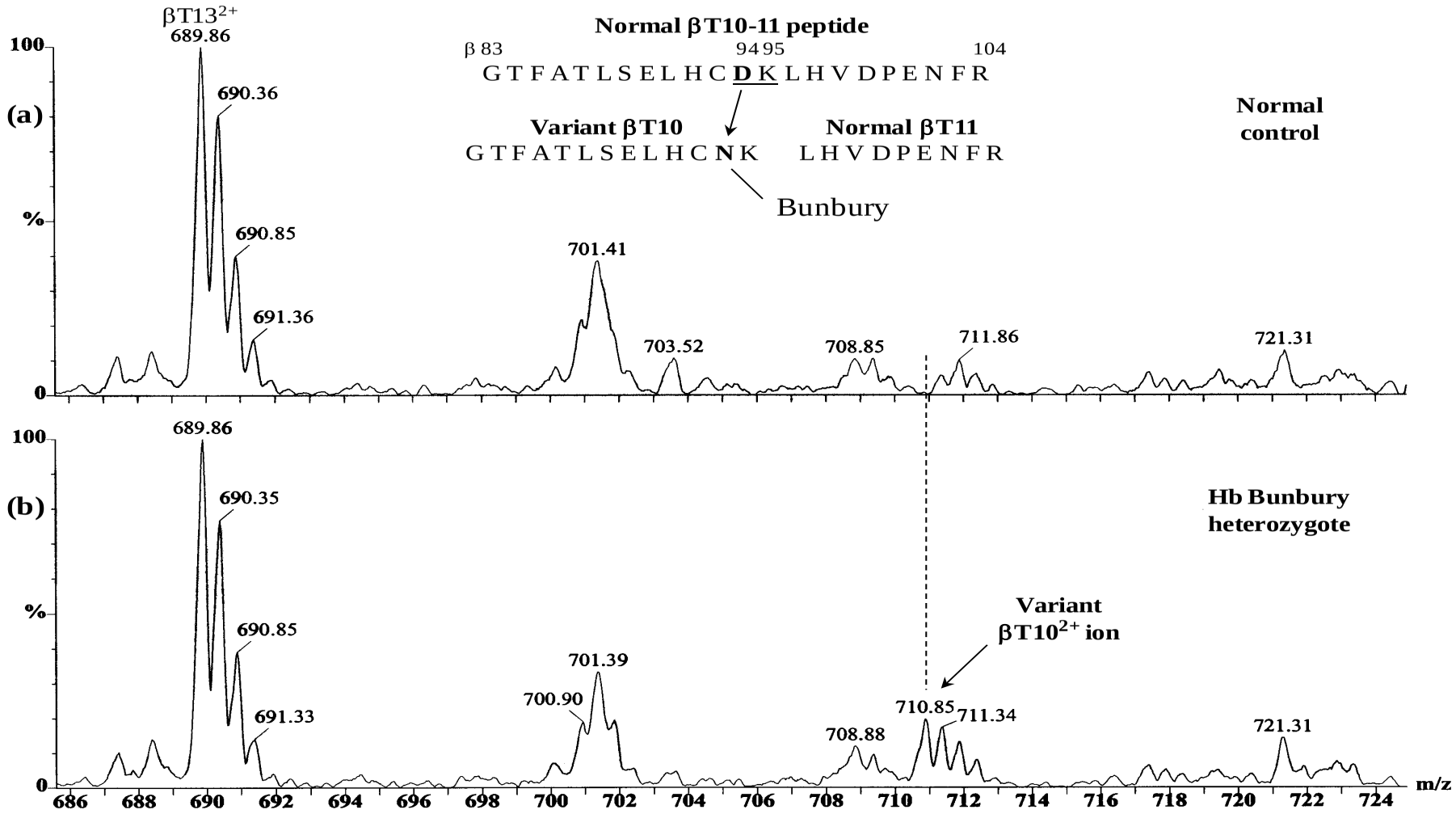

Figure 4.7.1. Part spectra from 30-min digests of (a) a normal control and (b) a Hb Bunbury heterozygote showing the variant $\beta T10^{2+}$ ion. The presence of this ion together with the appearance of the normal $\beta T11^{2+}$ ion in Figure 4.7.2b identifies the mutation as β94Asp→Asn.

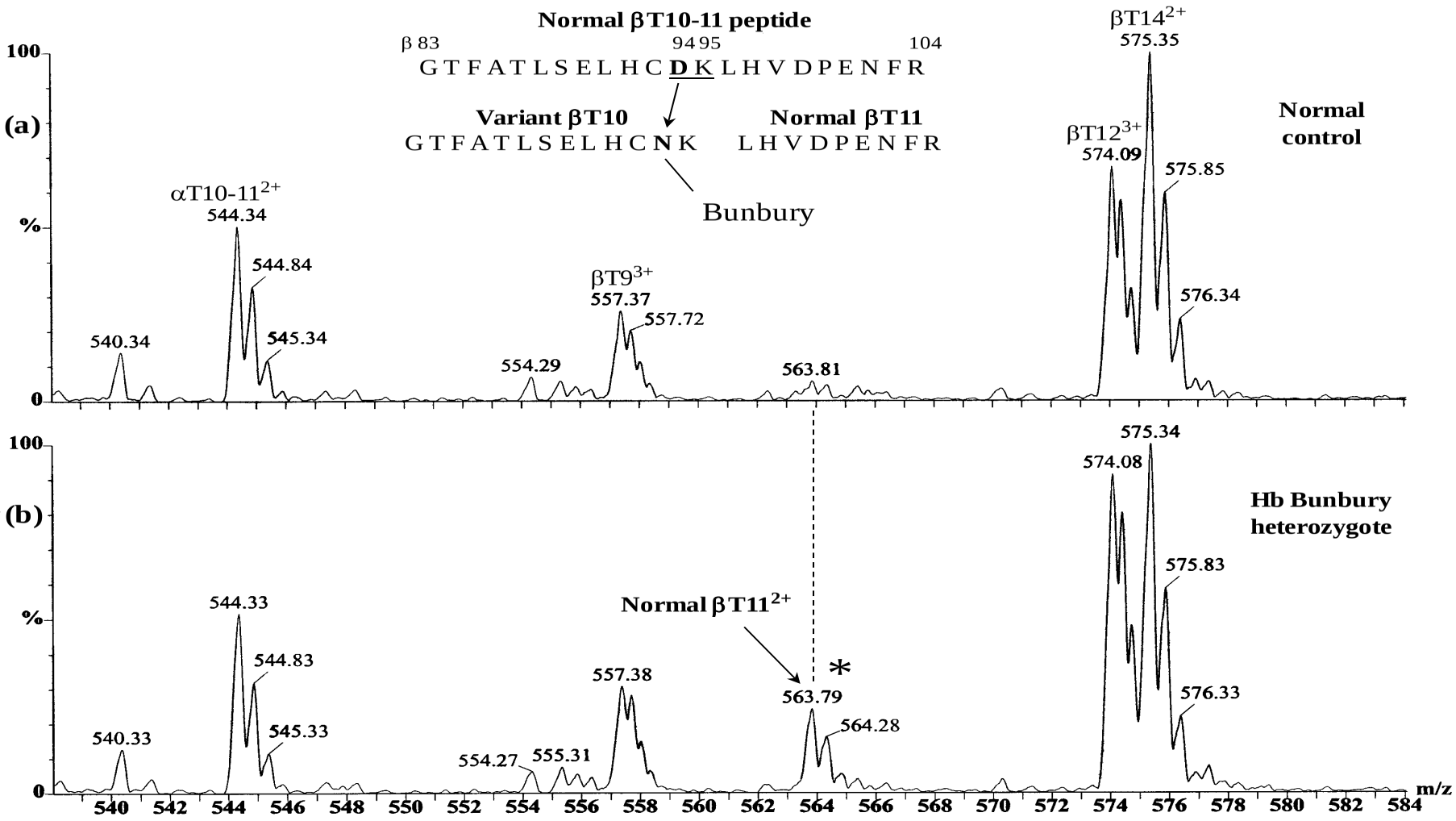

Figure 4.7.2. Part spectra from 30-minute digests of (a) a normal control and (b) a Hb Bunbury heterozygote showing the normal $\beta T11^{2+}$ ion significantly enhanced in (b) as a result of the mutation.

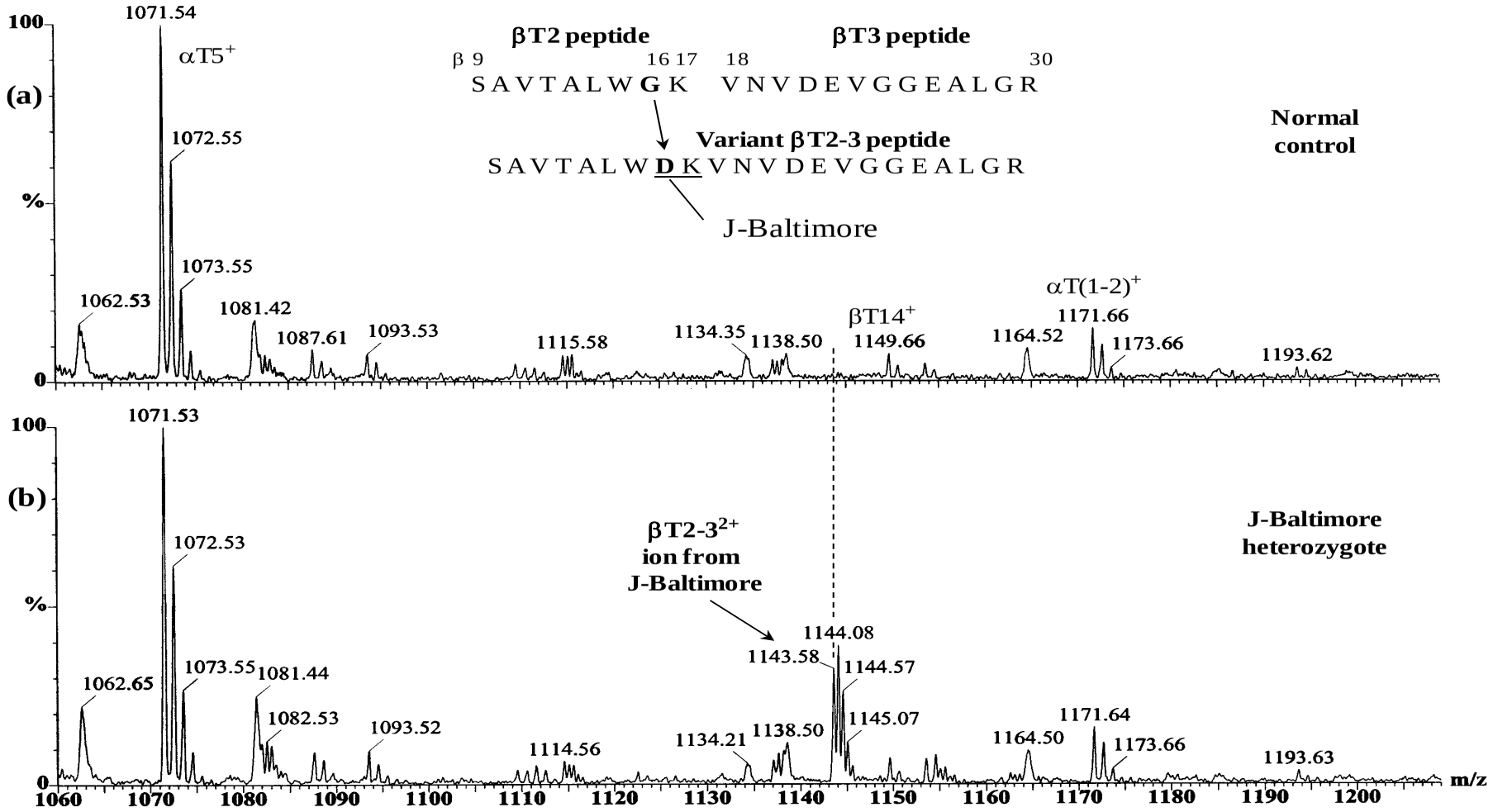

Figure 4.7.3. Part spectra of 30-minute digests from (a) a normal control, and (b) a J-Baltimore heterozygote showing the characteristic βT2-3^{2+} ion caused by the mutation to 16Asp hindering cleavage at 17Lys. Calculated monoisotopic m/z of βT2-3^{2+} = 1,143.59.

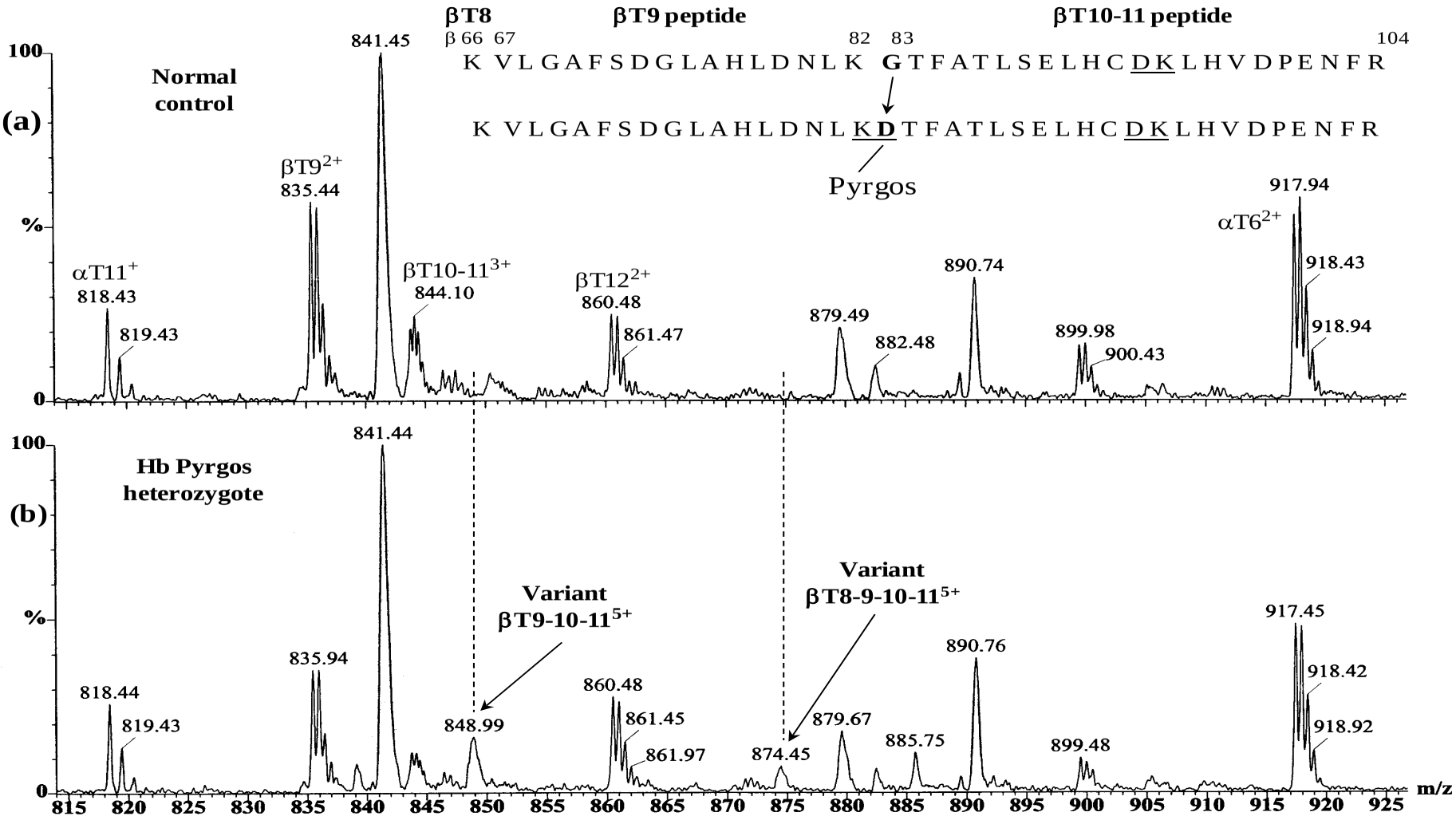

Figure 4.7.4. Part spectra of 30-minute digests from (a) a normal control and (b) a Hb Pyrgos heterozygote showing the characteristic ions caused by the mutant 83Asp hindering cleavage at 82Lys. Note the reduced relative intensities of the $\beta T9^{2+}$ and $\beta T(10\text{-}11)^{3+}$ ions.

SECTION 5

5.0. Tandem Mass Spectrometry

The principles of mass spectrometry and tandem mass spectrometry were outlined in Section 1.3. In this section the processes for defining mutations in a number of examples from both the α- and β-chains are presented.

5.1. Fragmentation of peptides

Peptide fragmentation is generally referenced to the fragmentation of the peptide bond, according to the Roepstorff-Fohlman nomenclature[1]. The convention describes the retention of the charge on either the N-terminus (a, b, c fragments) or the C-terminus (x, y, z fragments) of a peptide and also references the number of amino acids retained in the charged fragment, relative to the N- or C-termini.

For a peptide of four amino acid residues (R_1, R_2, R_3 and R_4) shown in Figure 5.1.1.

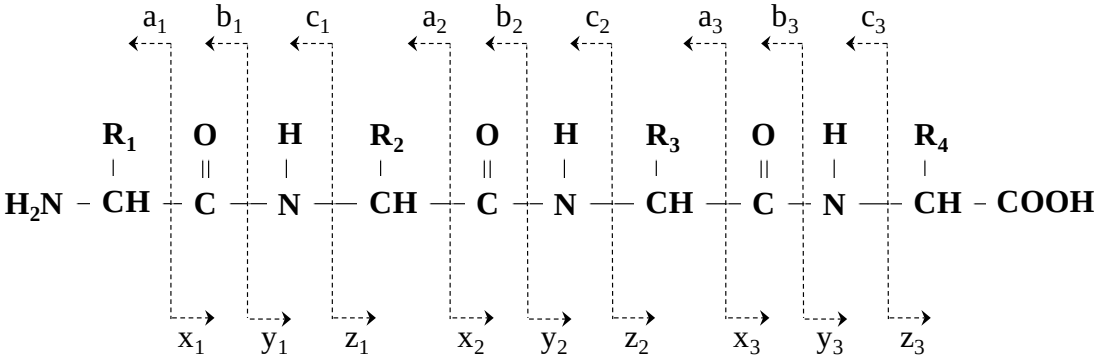

Figure 5.1.1. Tetrapeptide fragmentation.

Under low-energy collision-induced dissociation, peptides typically fragment across C -- N linkage to yield b and y fragment ions as illustrated in Figure 5.1.2. During the formation of the **y** ions, there is typically a rearrangement of two hydrogen atoms in order for the y-fragment to retain the charge. This is typically denoted **y**".

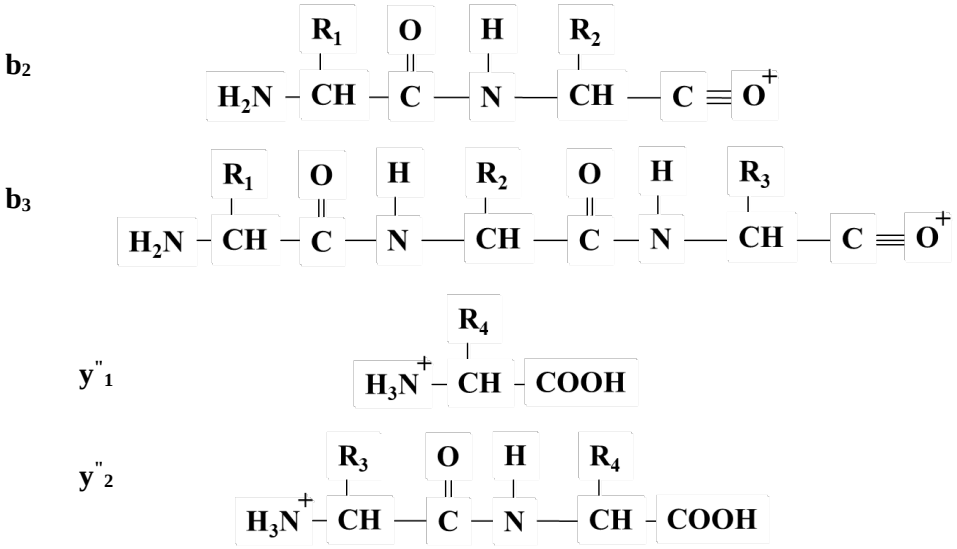

Figure 5.1.2. Typical low-energy fragment ions from the tetrapeptide shown in Figure 5.1.1.

Reference

[1] P. Roepstorff and J. Fohlman, Proposal for a Common Nomenclature for Sequence Ions in Mass Spectra of Peptides, Biomed.Mass Spectrom., 11 (1984) 601.

5.2. βT1 Comprehensive Identification of Hb S (β6Glu→Val)

Generally, the Sickle variant, β6Glu (E) →Val (V), is identified in hospital haemoglobinopathy screening laboratories by various routine methods, which include ce-HPLC or IEF. Occasionally, however, confirmation of its identity is requested, when, for example, the fraction of the Sickle variant in a heterozygote deviates significantly from its normal value (~40%). The following describes the results of analysing a Sickle heterozygote.

βT1 Peptide

β	1	2	3	4	5	6	7	8
b		2	3	4	5	6	7	8
	V	H	L	T	P	E	E	K
y"	8	7	6	5	4	3	2	1

↓

V

Sickle

Figure 5.2.1. Sequence of the βT1 tryptic peptide showing the numbering of the b- and y"-series.

The ce-HPLC trace in Figure 5.2.2. shows a significant signal in the S-window at 4.49 min and indicates that the mutation exhibits a positive charge change from the normal Hb.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	5.8	---	1.13	74859
P2	---	1.1	1.33	14865
P3	---	0.6	1.71	7730
Unknown	---	2.8	2.32	38206
A0	---	16.4	2.59	221626
A2	6.8	---	3.69	92424
S-window	---	66.7	4.49	899410

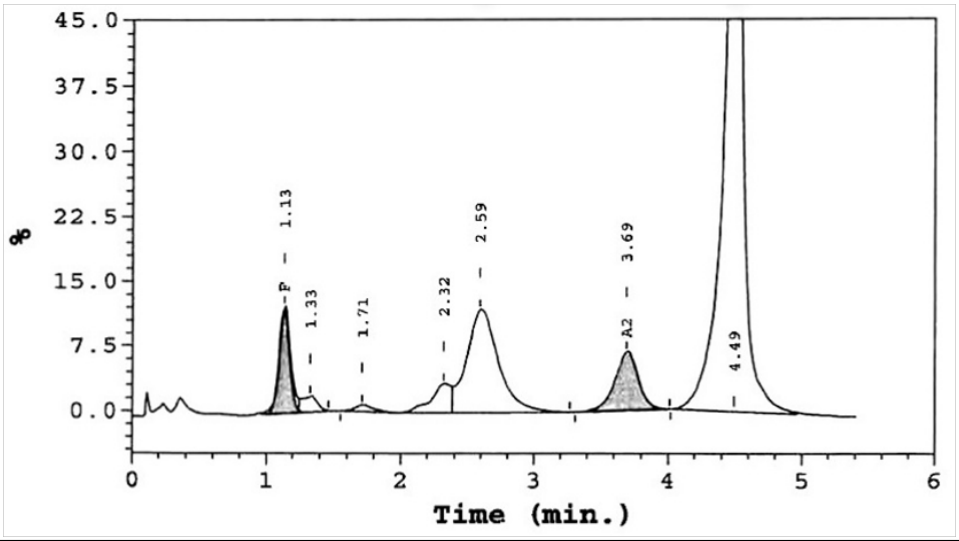

Figure 5.2.2. ce-HPLC trace of the Sickle variant

Figure 5.2.3. shows the spectrum from a typical Sickle heterozygote. The presence of a variant β-chain at 15,837.34 Da, 29.98 Da lower than normal, is consistent with Hb S (Sickle), but could be due to four other mutations Thr→Ala (7 possibilities), Trp→Arg (2 possibilities), Ser→Gly (5 possibilities), Met→Thr (1 possibility) or Glu→Val (8 possibilities).

In Figure 5.2.4. are part spectra from 30-minute tryptic digests of (a) normal control and (b) a Sickle heterozygote showing the $\beta T1^{2+}$ ions at *m/z* 476.76 (normal) and *m/z* 461.80 (variant). The region containing the $\beta T1^{+}$ ions - *m/z* 952.50 (normal) and *m/z* 922.54 (variant) is shown in Figure 5.2.5. Both figures show that the Sickle mutation occurs in the βT1 peptide. However, either β6Glu (GAG) or β7Glu (GAG) could mutate to Val (GTG) by a single base change in the codon. Hence, tandem MS is needed to distinguish β6Glu→Val (Hb Sickle) from β7Glu→Val (Hb Haaglanden, non-sickling). A third possibility, β4Thr (ACT) →Ala (GCT), can usually be discounted because it is likely to be silent by ce-HPLC. Thus, if an S-like variant is detected by ce-HPLC, then β4Thr→Ala can be eliminated.

Figures 5.2.6.-5.2.8. show diagnostic parts of tandem mass spectra from (a) the normal, and (b) the Sickle $\beta T1^{2+}$ ions. Complete tandem mass spectra are compared in Figure 5.2.9. The collision energy was 18V with argon as the collision gas at 2.5×10^{-3} mbar pressure.

The Hb S mutation, β6Glu→Val, may be positively identified and distinguished from β7Glu→Val and β4Thr→Ala when the criteria in items 1 and 2 below are met. Items 3-5 give strong supporting evidence.

1. The y''_1 ion is present at *m/z* 147.1 in the variant spectrum (Figure 5.2.9).
2. The y''_2 ion remains at its normal value (*m/z* 276.1) in the variant spectrum, whilst y''_3 decreases from *m/z* 405.2 in the normal spectrum to 375.2 in the variant spectrum (Figure 5.2.6). If the mutation were β7Glu→Val, then y''_2 would appear at *m/z* 246.1 in the variant spectrum instead of at *m/z* 276.1.
3. The internal fragment PV occurs at *m/z* 197.1 in the variant spectrum instead of PE at *m/z* 227.1 in the normal spectrum (Figure 5.2.7).
4. The b_4 ion occurs at its normal value (*m/z* 451.3) in the variant spectrum, whilst b_6 occurs at *m/z* 647.2 instead of its normal value at *m/z* 677.2 (Figure 5.2.8). The b_4 ion at *m/z* 451.3 in the variant spectrum eliminates β4Thr→Ala.
5. Provided β4Thr→Ala has been eliminated, supporting evidence for Sickle would be given by TPV (*m/z* 298.2) replacing normal TPE (*m/z* 328.2) (Figure 5.2.9.) and by HLTPV (*m/z* 548.3) replacing normal HLTPE (*m/z* 578.2) (Figure 5.2.8.). Both of these internal fragments would occur at their normal values were β7Glu→Val present. The *m/z* 548.3 ion (b_5) was detected at a very low level in the control (due to β5Pro?). Hence, it is probably mainly HLTPV in the variant spectrum.

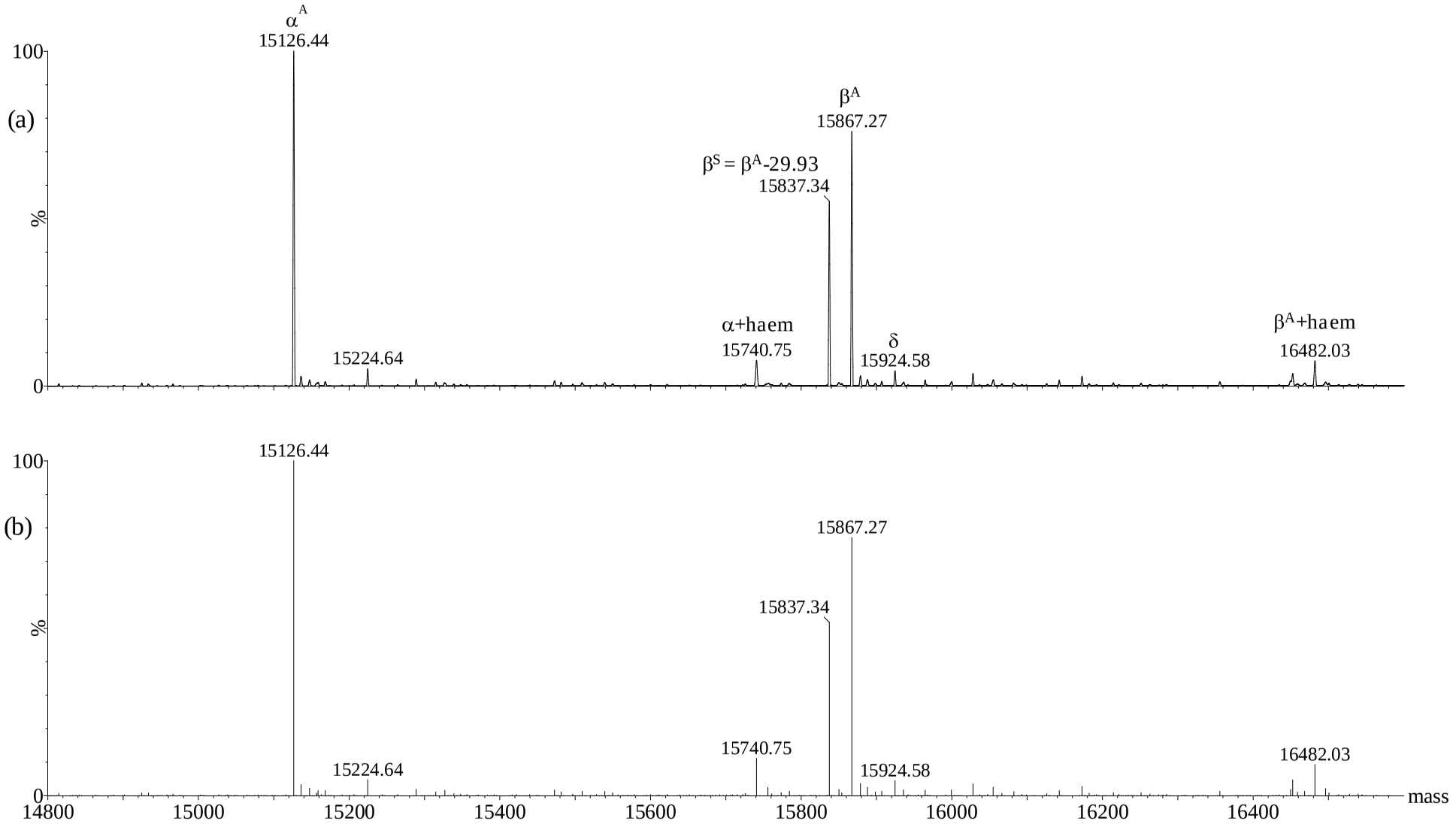

Figure 5.2.3. MaxEnt deconvoluted ESI-mass spectrum of a Hb Sickle heterozygote (a) profile, and (b) centred.

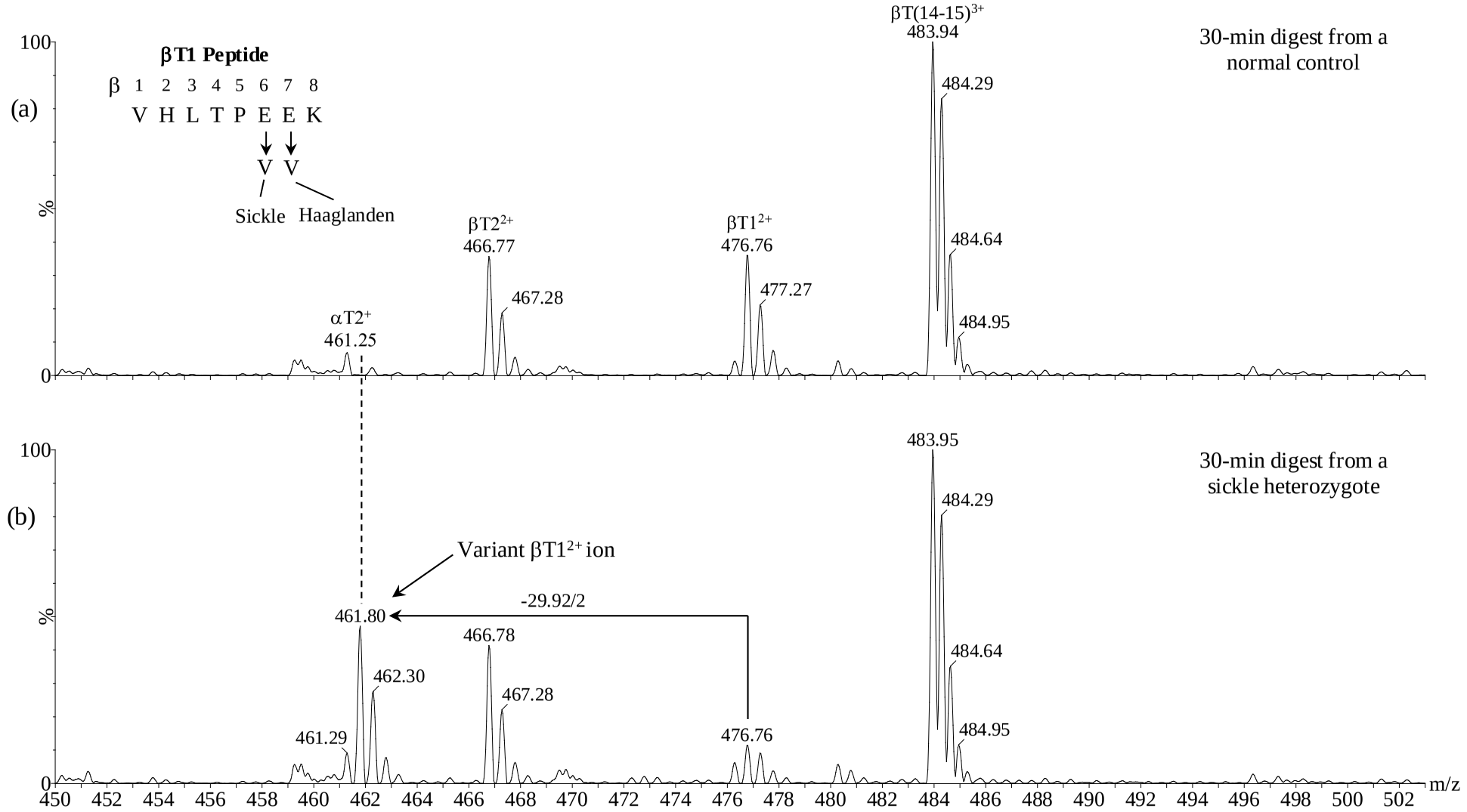

Figure 5.2.4. Part spectra from 30-min digests of (a) normal Hb, and (b) a Sickle heterozygote showing the $\beta T1^{2+}$ ions.

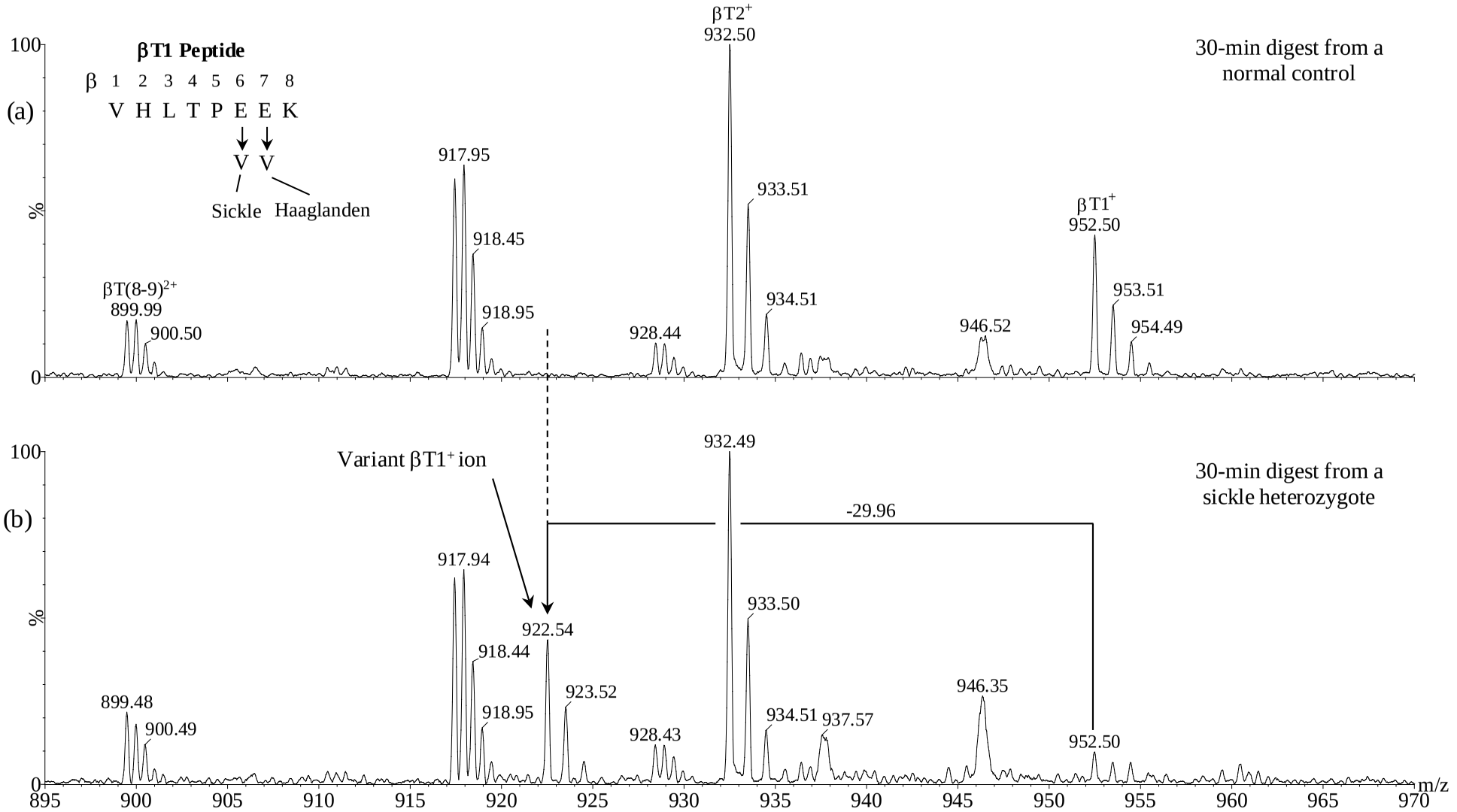

Figure 5.2.5. Part spectra from 30-min digests of (a) normal Hb and (b) a Sickle heterozygote showing the $\beta T1^{+}$ ions.

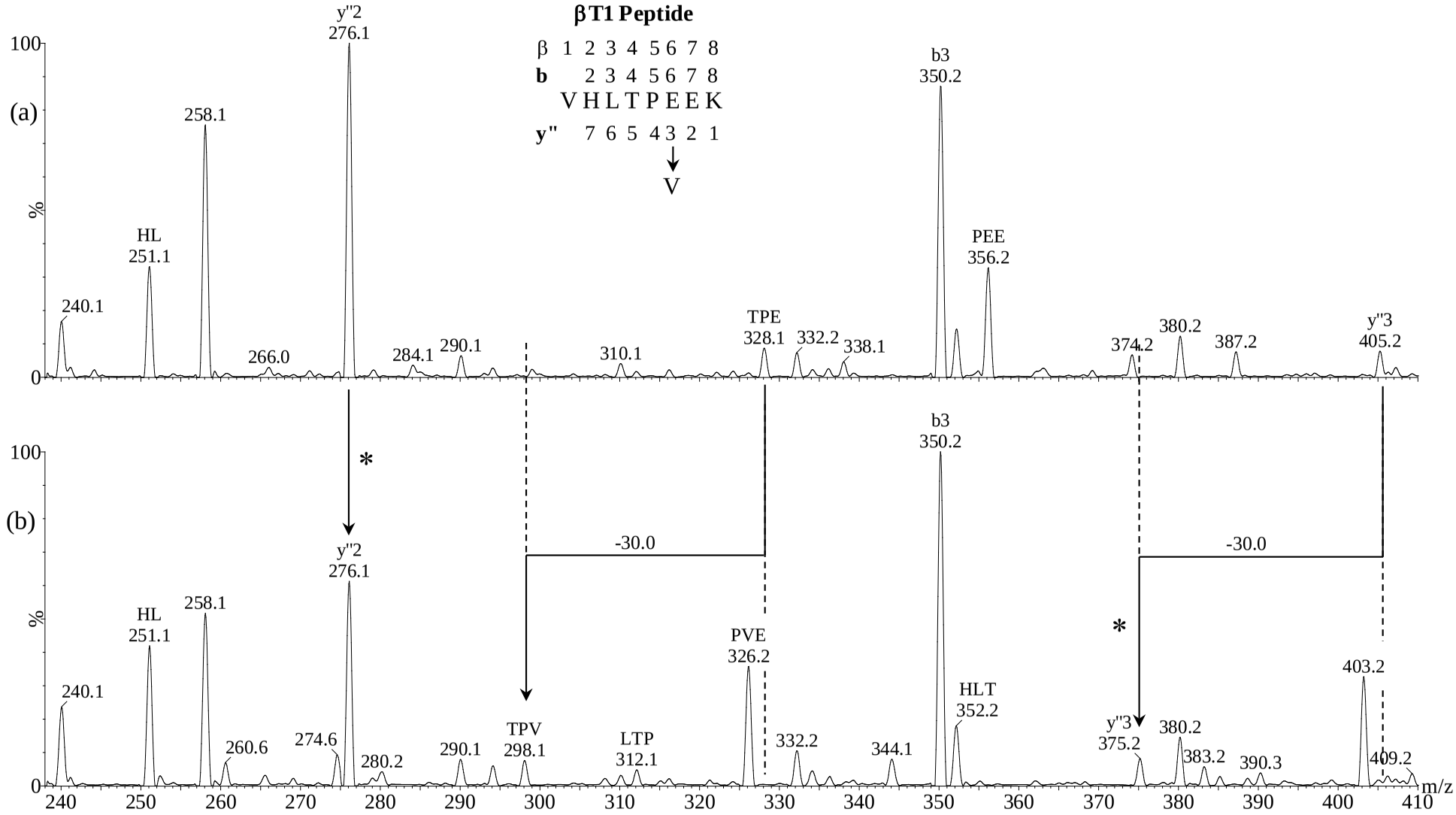

Figure 5.2.6. Part tandem mass spectra from (a) normal Hb, and (b) Sickle $\beta T1^{2+}$ ions showing diagnostic y″2 and y″3 ions.

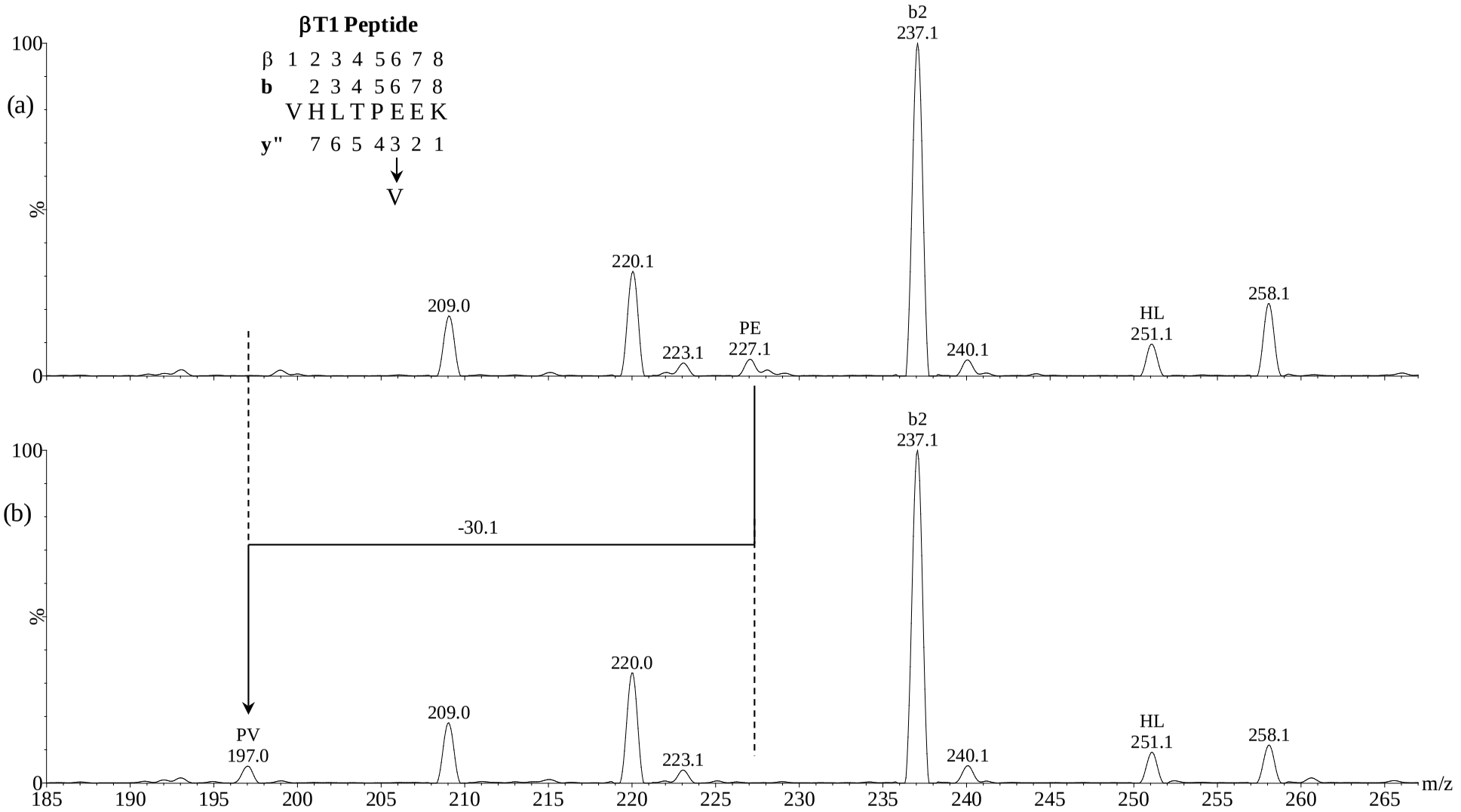

Figure 5.2.7. Part tandem mass spectra from (a) normal Hb, and (b) Sickle $\beta T1^{2+}$ ions showing diagnostic PE and PV ions.

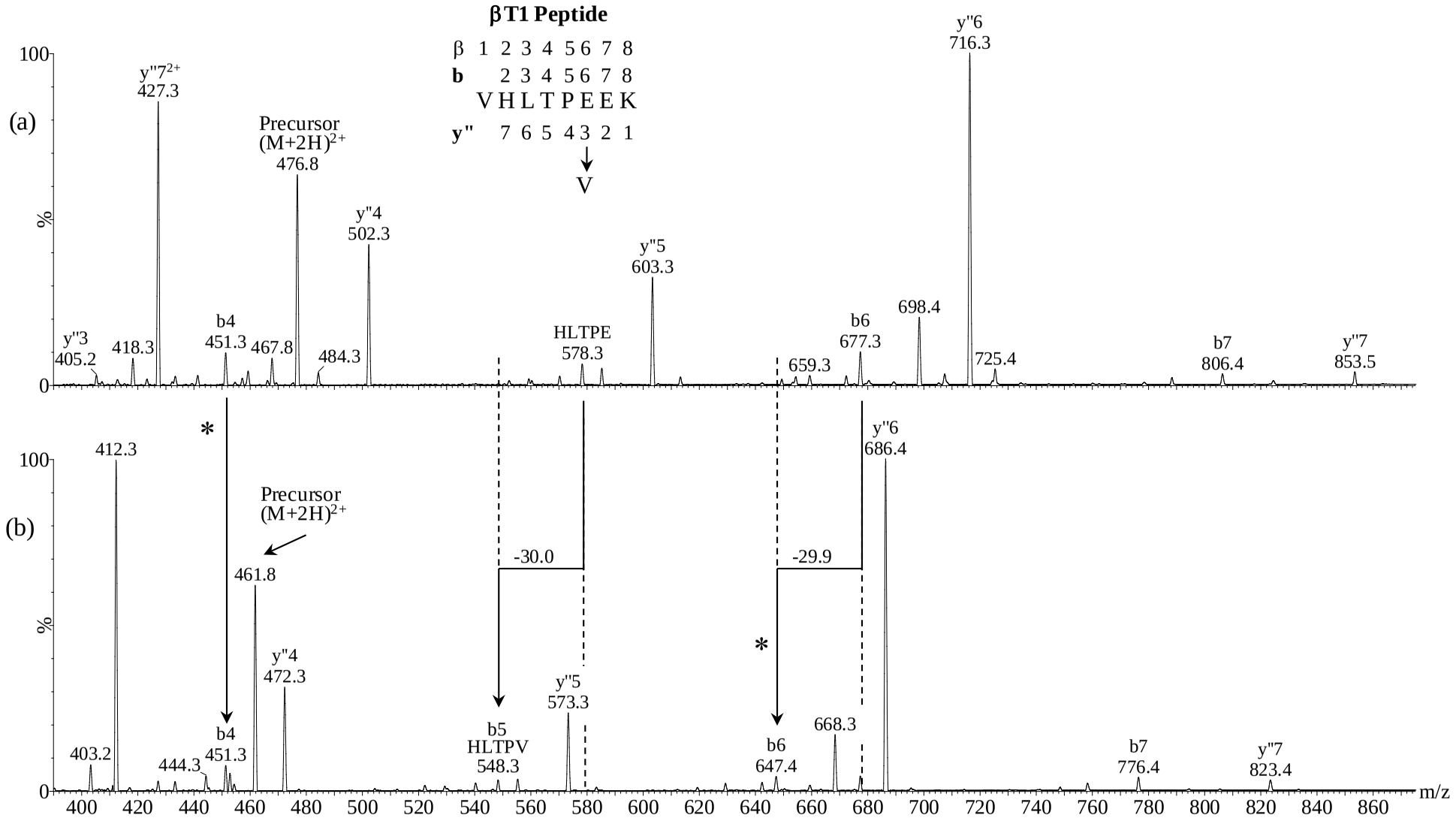

Figure 5.2.8. Partial product ion spectra from normal and Sickle βT1^{2+} ions showing diagnostic b_4, b_6 and HLTPV ions.

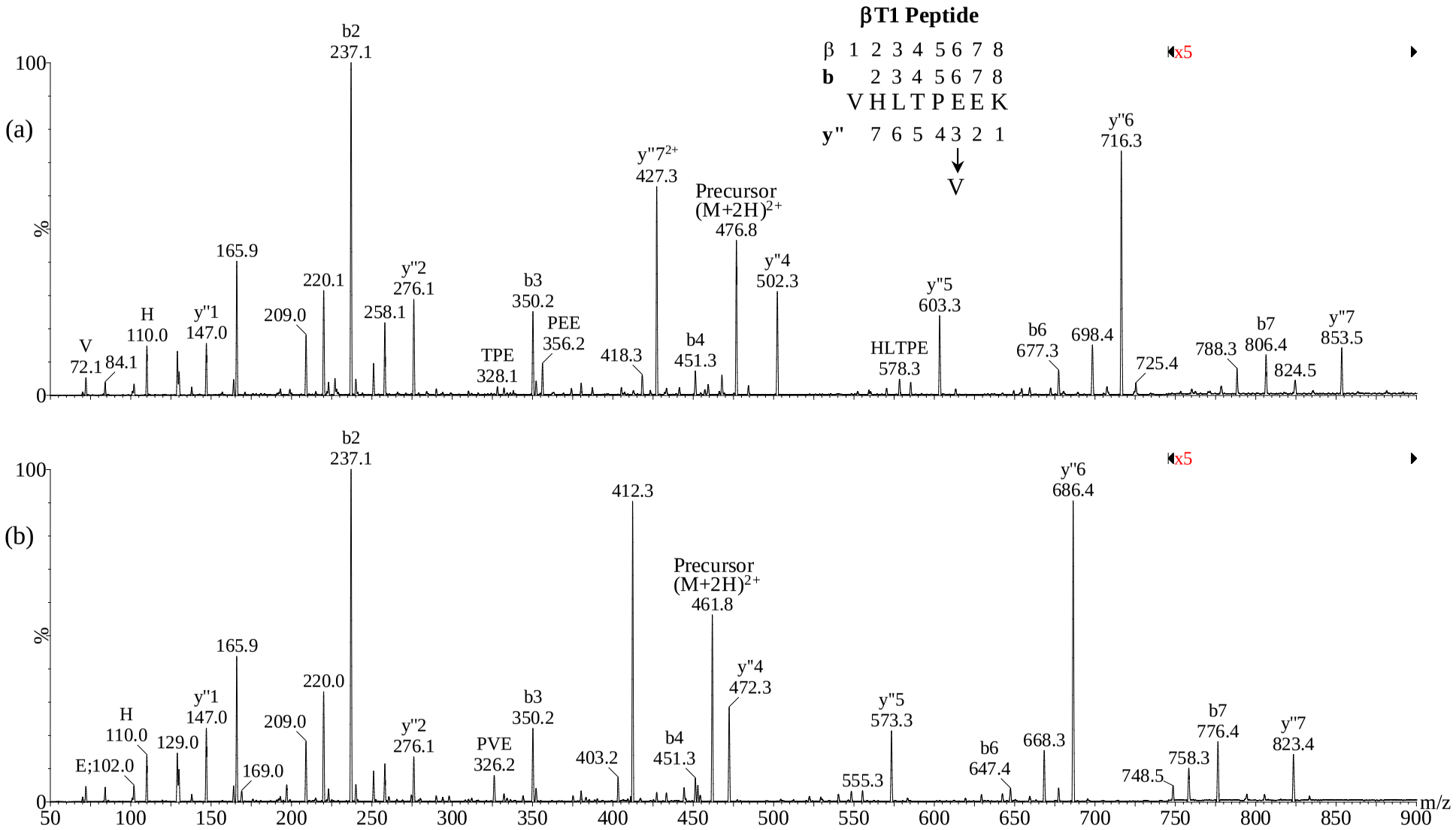

Figure 5.2.9. Complete product ion spectra of (a) normal Hb and (b) Sickle $\beta T1^{2+}$ ions.

5.3. α-chain mutation examples

This section contains examples of the determination of amino acid mutations in the α-chain and have been selected to illustrate certain characteristics that can be investigated using the approaches described in this book. The entries denoted (Novel) were novel when first analysed by mass spectrometry, the name indicates the current recorded name in the literature.

Section	Tryptic Peptide	Mutation	Name
5.3.1.	αT1	α1Val→Leu, Initiator Met retained	St.Jozef
5.3.2.	αT1	α3Ser→Pro	Central Middlesex (Novel)
5.3.3.	αT2	α11Lys→Gln	J-Wenchang-Wuming
5.3.4.	αT3	α12Ala→Asp	J-Paris-I
5.3.5.	αT4	α20His→Gln	LeLamentin
5.3.6.	αT4	α21Ala→Pro	Fontainebleau
5.3.7.	αT1	α31Arg→Ser	Prato
5.3.8.	αT5	α38Thr→Ile	Chelsea (Novel)
5.3.9.	αT6	α51Gly→Ser	Riccarton
5.3.10.	αT6	α54Gln→Arg	Shimonoseki
5.3.11.	αT6	α56Lys→Glu	Shaare Zedek
5.3.12.	αT7	α57Gly→Arg	L-Persian Gulf
5.3.13.	αT9	α64Asp→His	Q-India
5.3.14.	αT9	α68Asn→Lys	G-Philadelphia
5.3.15.	αT9	α74Asp→His	Q-Thailand
5.3.16.	αT9	α78Asn→Lys	Stanleyville-II
5.3.17.	αT9	α87His→Tyr	M-Iwate
5.3.18.	αT9	α90Lys→Asn	J-Broussais
5.3.19.	αT11	α94Asp→Tyr	Setif
5.3.20.	αT12	α112His→Asp	Hopkins-II
5.3.21.	αT12	α120Ala→Glu	J-Meerut
5.3.22.	αT13	α139-141 → α139NTVKLEPR	Wayne

Table 5.3.1. List of the mutation illustrations for α-chain.

5.3.1. αT1 - Hb St. Jozef (α1Val→Leu and initiator Met retained)

Hb St. Jozef is the result of a mutation in which the α1 amino acid is changed from Val to Leu through a single base change in the codon GTG→CTG, and the initiator Met is retained.

αT1 Peptide

	1	2	3	4	5	6	7
α	V	L	S	P	A	D	K
	↓						
	M	L	L	S	P	A	D	K

Figure 5.3.1.1. Sequence of the Hb St. Jozef αT1 tryptic peptide.

A blood sample from an anaemic patient showed no obvious abnormality by ce-HPLC (Figure 5.3.1.2.).

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	0.3	---	1.11	6408
Unknown	---	0.7	1.24	13480
P2	---	3.7	1.32	71635
P3	---	7.6	1.80	146887
A0	---	84.8	2.50	1636202
A2	2.6	---	3.66	55894

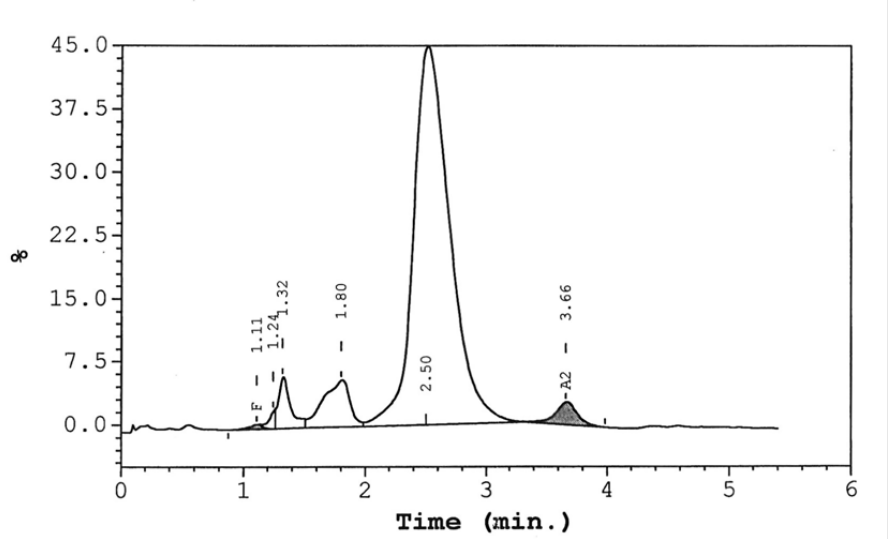

Figure 5.3.1.2. ce-HPLC trace for Hb St. Jozef.

Analysis by ESI-MS of the 500-fold diluted blood from this patient however, revealed an α-chain heterozygote in which the variant chain was 15,271.62 Da, 145.26 Da higher in mass than normal and was 14.8% the intensity of the total α-chains (Figure 5.3.1.3.). Furthermore, 30-minute tryptic digests of the heterozygote and a normal Hb showed that the mutation is at the N-terminus of the α-chain and confirmed the mass excess of the variant over normal as 145.26 Da. This mass excess strongly suggests the variant is Hb St. Jozef, as shown in Table 2.4. headed: "Some variants that can be identified from the mass spectra of the globin chain". The mutation in St. Jozef is α1Val→Leu with retention of the initiator Met.

Figure 5.3.1.4 shows the diagnostic region of the tryptic digest mass spectra for (a) normal Hb and (b) the variant Hb. A new signal is observed in the lower panel at *m/z* 437.77 corresponding to the $\alpha T1^{2+}$ ion from the 30-minute digest of the St. Jozef heterozygote. This is further supported by the data in the lower panel of Figure 5.3.1.5 and the singly charged $\alpha T1^{+}$ signal at *m/z* 874.46.

Product ion spectra from the $\alpha T1^{2+}$ tryptic fragment ions from St Jozef at two collision energies are shown in Figures 5.3.1.6(a) and (b) showing the diagnostic b_2 (*m/z* 245.1) and immonium (M, *m/z* 104.1) ions. The spectra are consistent with the proposed αT1 peptide from the St. Jozef mutation.

Note that if only the mass change is considered, the mutation could be either Val→Leu or Val→Ile. However, Val→Leu requires only one base change in the codon (GTG→CTG), whereas Val→Ile requires two base changes (GTG→ATT or ATC or ATA) and is therefore much less likely. In other words, the sample analysed in this work is much more likely to be St Jozef than α1Val→Ile.

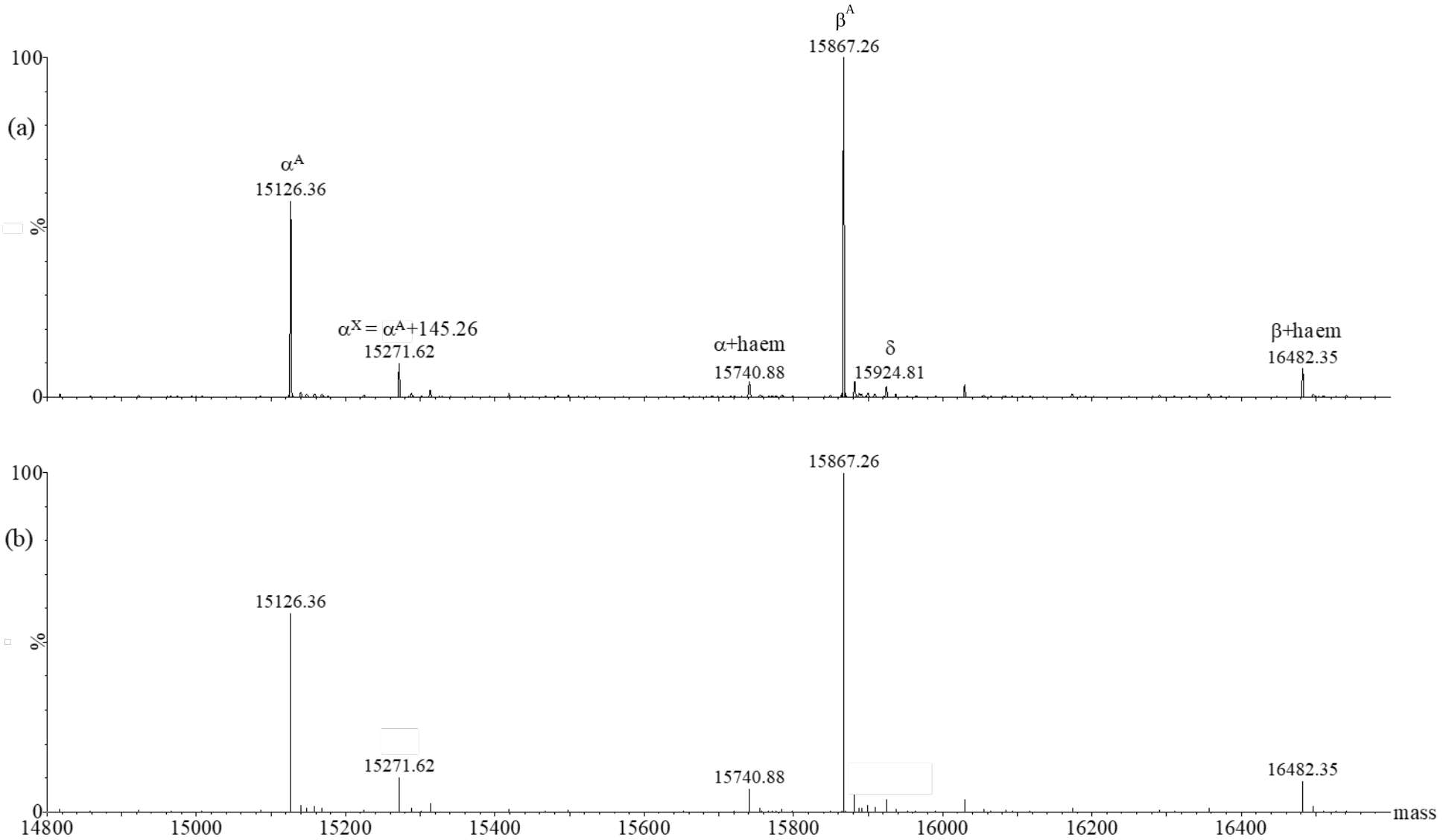

Figure 5.3.1.3. Deconvoluted mass spectrum of a Hb St. Jozef heterozygote (α1Val→Leu and initiator Met retained) (a) profile, and (b) centred. A variant was observed at 15,271.62 Da which was 14.8% of the total α-chains.

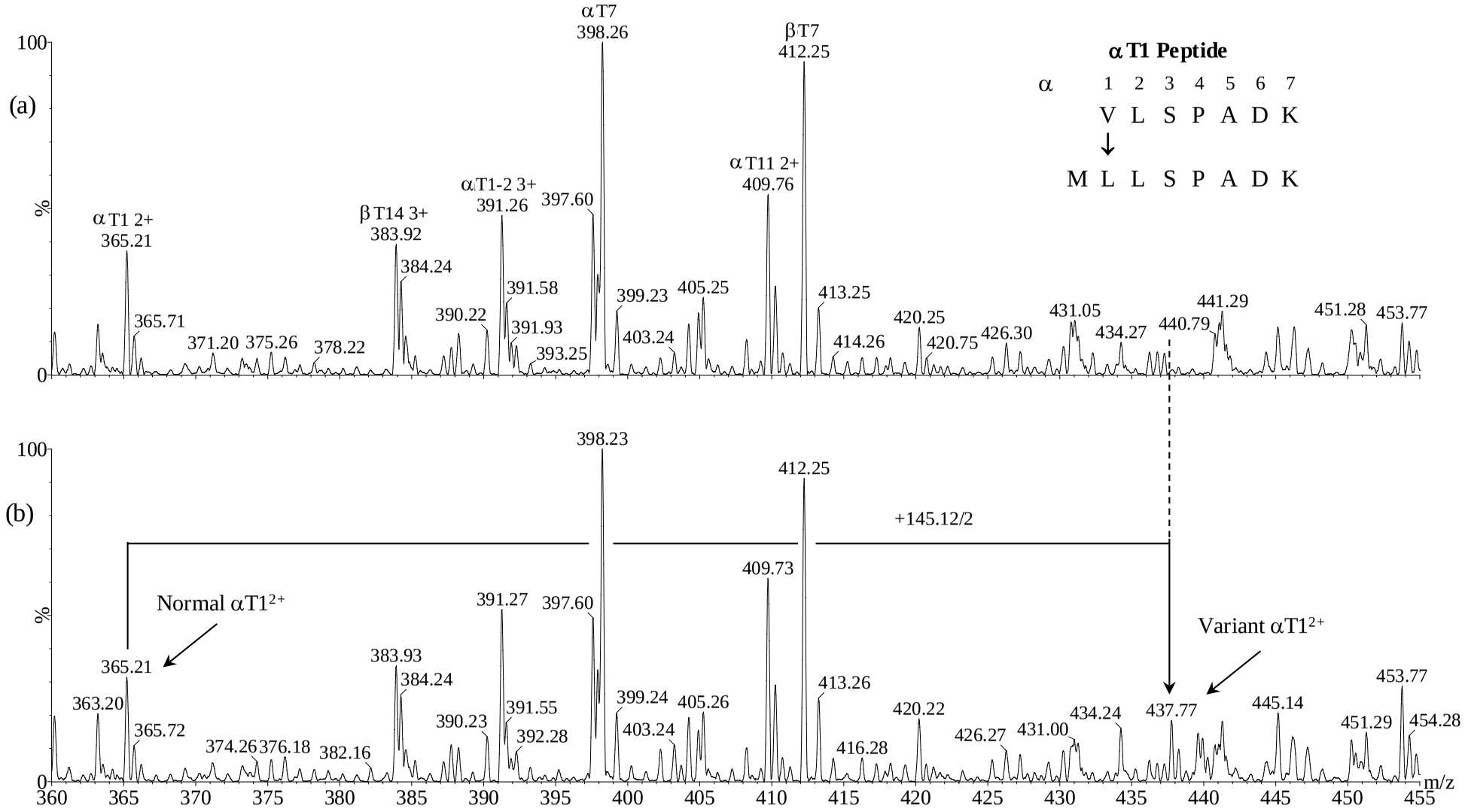

Figure 5.3.1.4. Part spectra from 30-min digests of (a) normal Hb, and (b) the Hb St. Jozef heterozygote showing the $\alpha T1^{2+}$ ions.

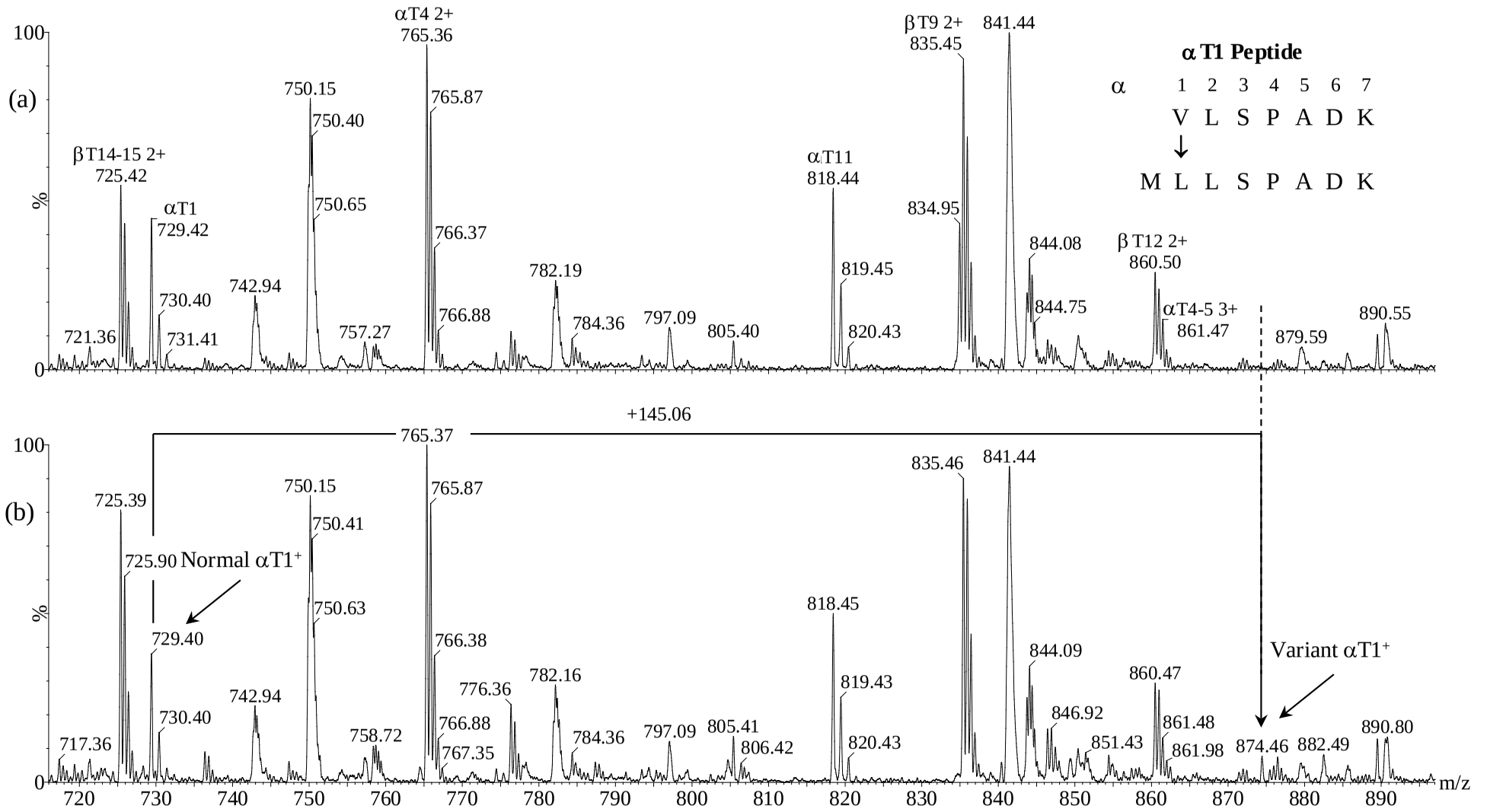

Figure 5.3.1.5. Part spectra from 30-min digests of (a) normal Hb, and (b) the Hb St. Jozef heterozygote showing the $\alpha T1^{+}$ ions.

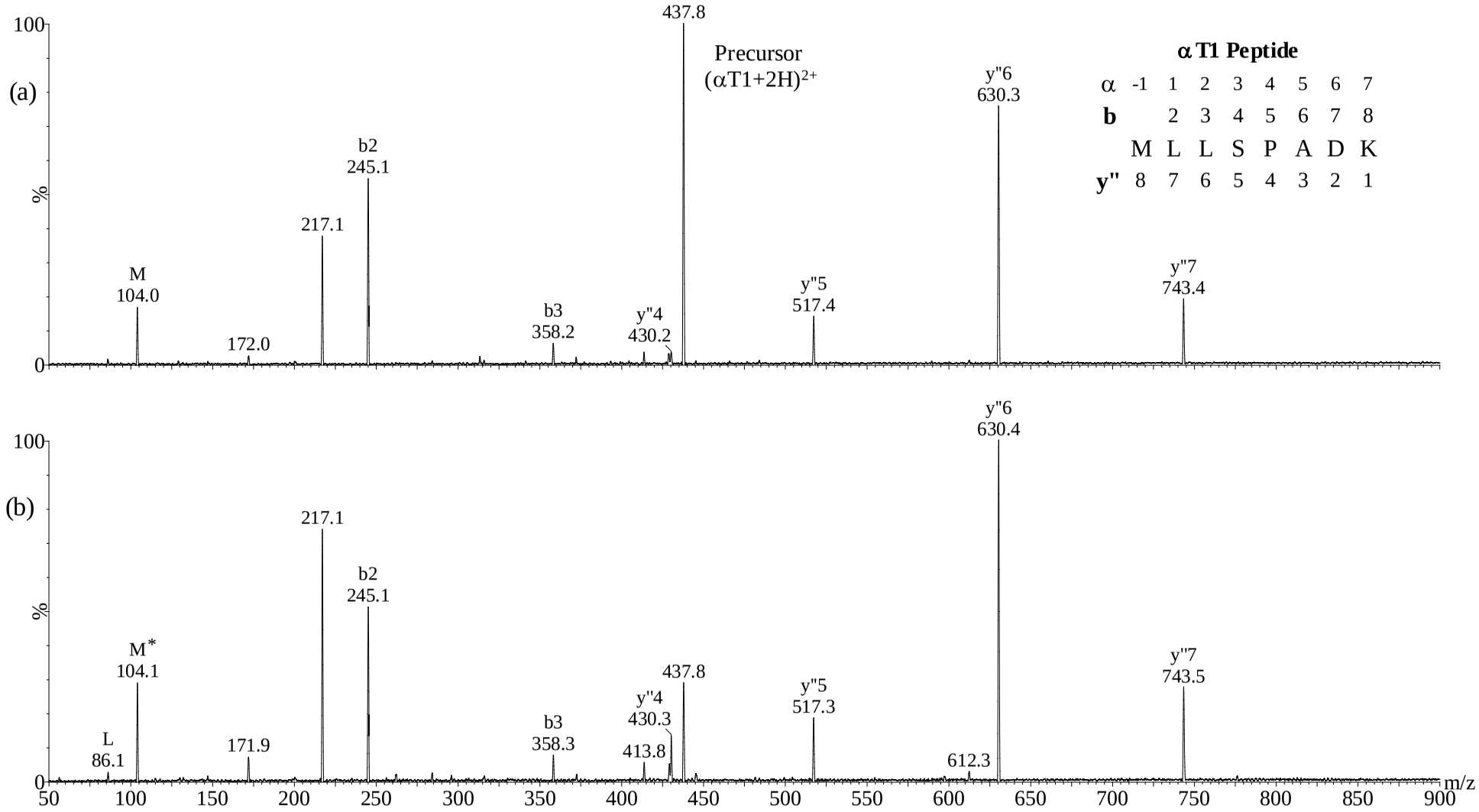

Figure 5.3.1.6. Product ion spectra from the $\alpha T1^{2+}$ tryptic fragment of Hb St. Jozef (α1Val→Leu and initiator Met retained) at (a) 12V collision voltage and (b) 14V collision voltage, showing diagnostic immonium (M*) and b_2 ions.

5.3.2. αT(1-2) - Hb Central Middlesex (α3Ser→Pro)

Hb Central Middlesex is the result of a mutation in which the α3 amino acid residue is changed from Ser to Pro through a single base change in the codon, TCT→CCT.

Tryptic cleavage at 7Lys (K) is severely restricted by 6Asp (D), resulting mainly in the production of αT(1-2).

αT(1-2) Peptide

α	1	2	3	4	5	6	7	8	9	10	11
	V	L	S	P	A	D	K	T	N	V	K
			↓								
			P								

Figure 5.3.2.1. Sequence of the Hb Central Middlesex αT(1-2) tryptic peptide.

A blood sample was submitted for analysis by mass spectrometry even though the ce-HPLC trace (Figure 5.3.2.2.) appeared normal.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	0.3	---	1.12	8081
P2	---	5.2	1.31	144251
P3	---	8.4	1.66	235834
A0	---	83.2	2.34	2326377
A2	2.8	---	3.64	82556

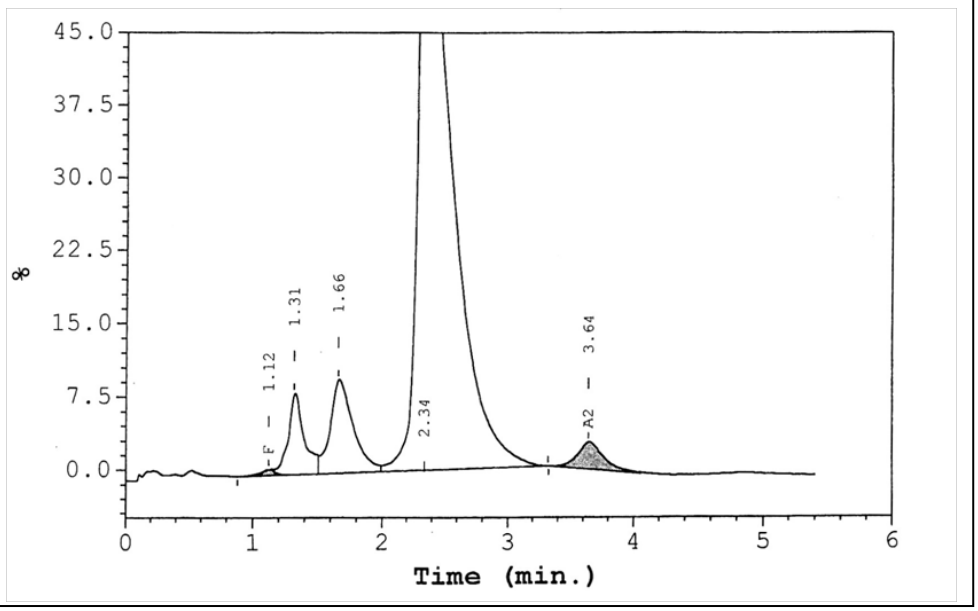

Figure 5.3.2.2. ce-HPLC trace for Hb Cental Middlesex

ESI-MS analysis of the blood sample diluted 500-fold, however, revealed an α-chain heterozygote in which the intensity of the variant chain was 22% of total α-chains at a mass of 15,136.37 Da, 10 Da higher in mass than the normal α-chain (Figure 5.3.2.3). There is only one single amino acid change that can give 10 Da increase in mass by a single base change in the codon, namely Ser→Pro (TCT→CCT)

Figure 5.3.2.4. shows the diagnostic part of a 30-minute tryptic digest of (a) normal Hb and (b) the α-chain heterozygote. The increase in mass of the $\alpha T(1\text{-}2)^{2+}$ tryptic fragment at *m/z* 591.37 in the lower panel places the mutation in the αT(1-2) peptide, and thus identifies the mutation as α3Ser→Pro.

Figures 5.3.2.5. shows a comparison of the product ion spectra for the $\alpha T(1\text{-}2)^{2+}$ tryptic fragments for (a) normal Hb and (b) the variant Hb. The 10 Da mass increase at b_3^+ (*m/z* 310.0) fragment and $y''_9{}^{2+}$ fragment (*m/z* 485.3) in the lower panel confirms the mutation as α3Ser→Pro, Hb Central Middlesex.

This variant was novel when first analysed by mass spectrometry.

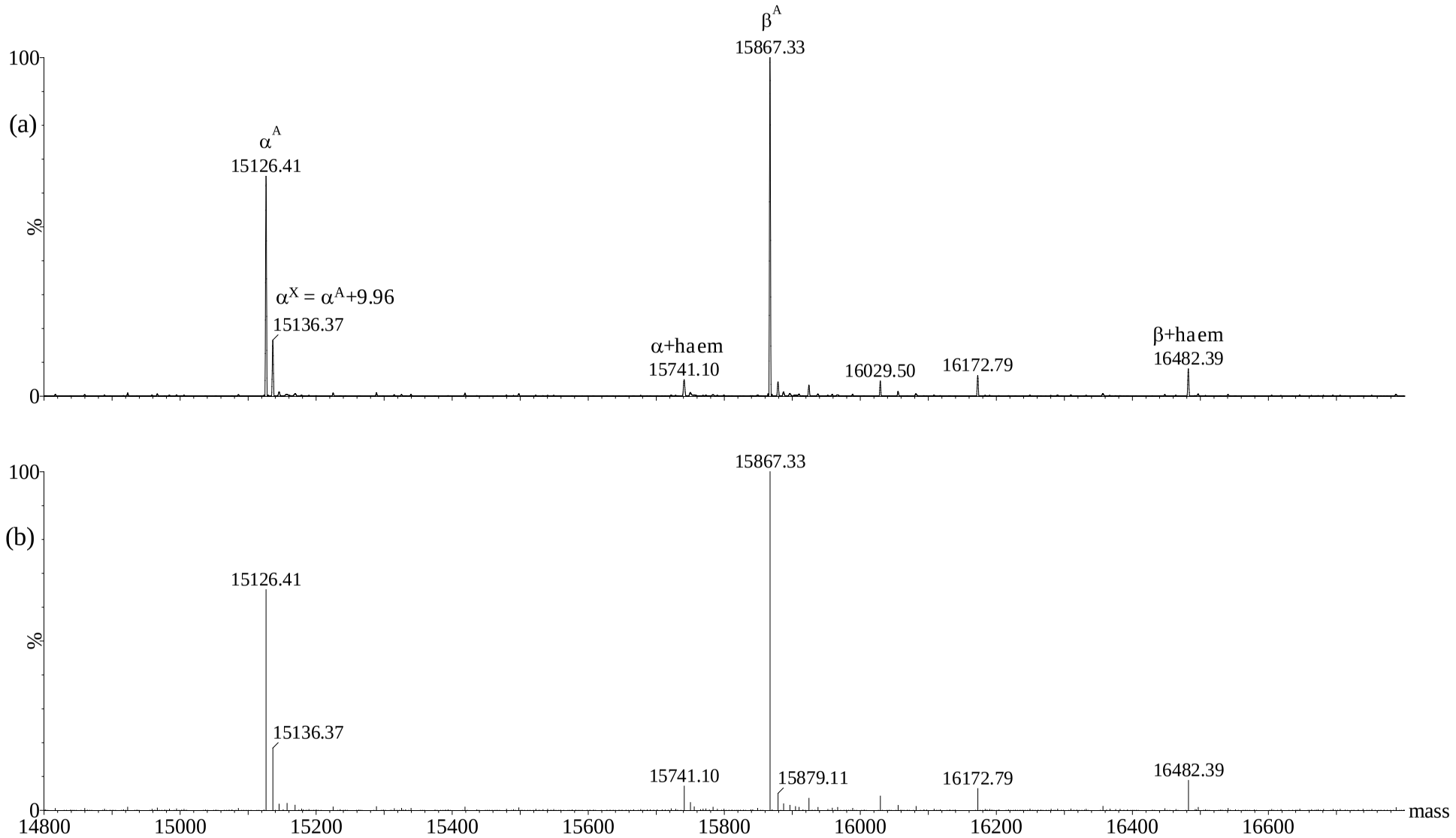

Figure 5.3.2.3. Deconvoluted mass spectrum of Hb Central Middlesex (α3Ser→Pro) showing the presence of a signal at 15,136.37 Da at approximately 22% of the total α-chains.

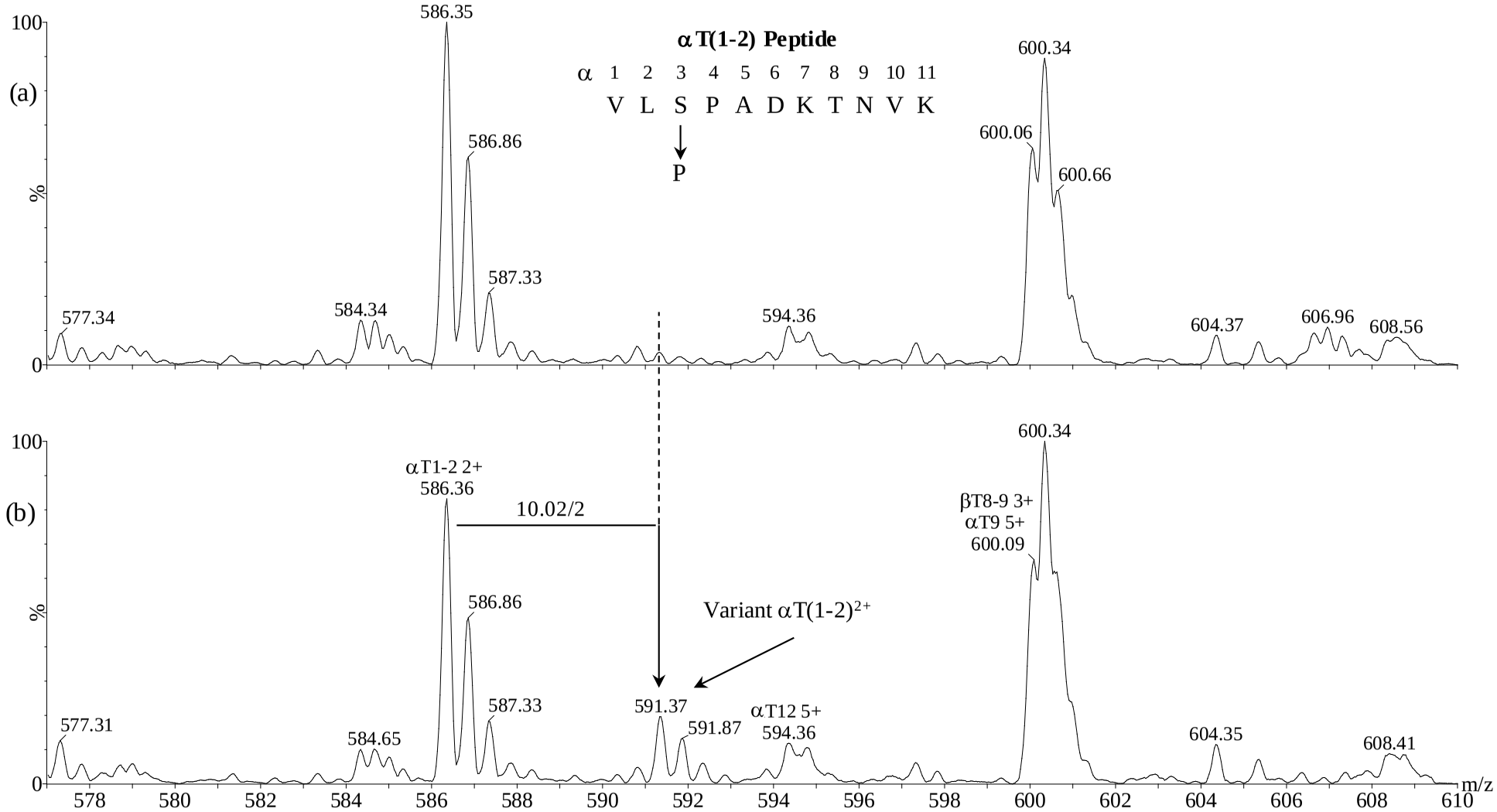

Figure 5.3.2.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Central Middlesex heterozygote.

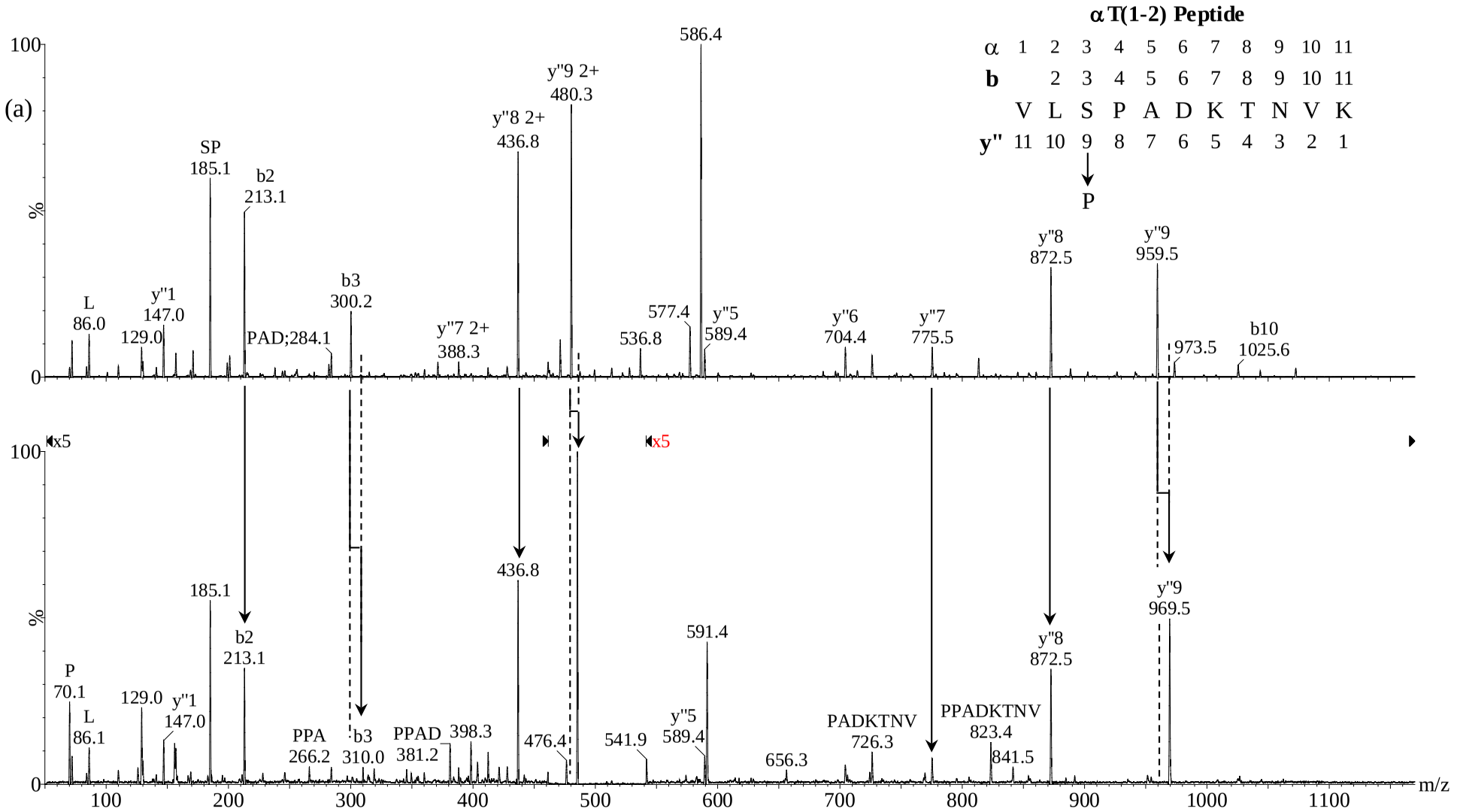

Figure 5.3.2.5. Product ion spectra of the $\alpha T(1\text{-}2)^{2+}$ tryptic fragments from (a) normal Hb and (b) the Hb Central Middlesex heterozygote. The 10 Da mass increase at b_3 and y''_9 confirms the mutation α3Ser→Pro.

5.3.3. αT2 - Hb J-Wenchang-Wuming (α11Lys→Gln)

Hb J-Wenchang-Wuming is the result of a mutation in which the α11 amino acid residue is changed from Lys to Gln through a single base change in the codon, AAG→CAG.

αT(1-2-3) Peptide

α	1	2	3	4	5	6	7	8	9	10	11		12	13	14	15	16
	V	L	S	P	A	D	K	T	N	V	K		A	A	W	G	K
											↓						
	V	L	S	P	A	D	K	T	N	V	Q	A	A	W	G	K	

Figure 5.3.3.1. Sequence of the Hb J-Wenchang-Wuming αT(1-2-3) tryptic peptide.

Note that tryptic cleavage at α7Lys is severely restricted by α6Asp, resulting mainly in the production of αT(1-2). The replacement of Lys with Gln at α11 prevents tryptic cleavage, and results in an extended peptide covering the first three tryptic fragments of the α-chain, αT(1-2-3).

A blood sample was submitted for analysis by ESI-MS, because the ce-HPLC trace (Figure 5.3.3.2.) showed an abnormally high P3 response. The high P3 (mainly due to the variant) implies the variant causes a negative polarity change.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
P1	---	0.5	0.70	6806
F	1.3	---	1.09	16873
Unknown	---	0.5	1.24	7072
P2	---	2.5	1.34	33638
P3	---	27.3	1.70	366401
A0	---	65.5	2.50	879444
A2	2.1	---	3.62	32439

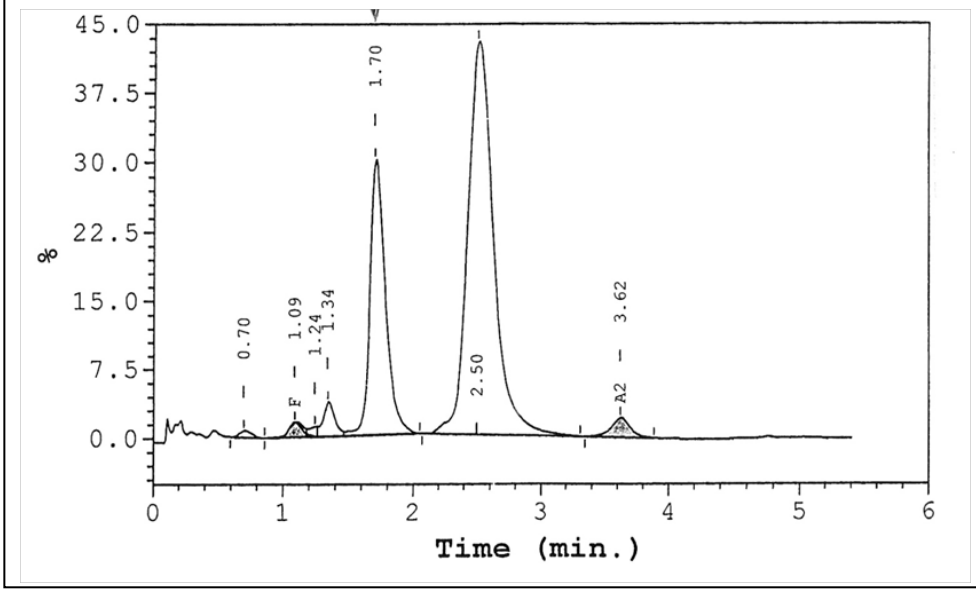

Figure 5.3.3.2. ce-HPLC trace for Hb Wenchang-Wuming.

ESI-MS of the 500-fold diluted blood sample (Figure 5.3.3.3.) showed peaks that correspond in mass to normal α- and β-chains, implying the mutation produces no mass change. From a single codon change with no change in mass infers either Gln↔Lys or Leu↔Ile, with the former indicated owing to the negative charge change.

Low HbA_2 suggests the variant is in the α-chain. Moreover, there is only one amino acid change that is governed by a single base change in the nucleotide codon and gives zero mass change, namely Gln↔Lys. Since this change gives a negative change in polarity as shown by the ce-HPLC response, the mutation is Lys→Gln. There is only one other mutation that could give zero mass change, i.e. Leu→Ile, but this can be discounted because it would give no polarity change.

Reference is made to Table 4.2.3. The tryptic peptides and their *m/z* values that result from a single amino acid change giving <±6 Da change in the α-chain mass and involve a mutation from Lys, and a tryptic cleavage site is abolished with two adjacent tryptic peptides being combined into one larger new peptide. These mutations give a significant increase in negative charge and are readily detected by ce-HPLC and IEF. The presence of the new peptide at its predicted *m/z* values identifies the mutation.

Figure 5.3.3.4. shows the appearance of a peak at *m/z* 562.31, attributable to a new $\alpha T(1\text{-}2\text{-}3)^{3+}$ tryptic peptide ion, which supports the mutation α11Lys→Gln, Hb J-Wenchang-Wuming. There is further evidence for this mutation in Figure 5.3.3.5. with the presence of a peak at *m/z* 842.95, attributable to the new $\alpha T(1\text{-}2\text{-}3)^{2+}$ tryptic peptide signal.

Figure 5.3.3.6. shows a marked decrease in the relative intensity of the $\alpha T3^{+}$ signal at *m/z* 532.29, which would be expected if one of the α-chain genes had mutated to allow the formation of a new αT(1-2-3) tryptic peptide.

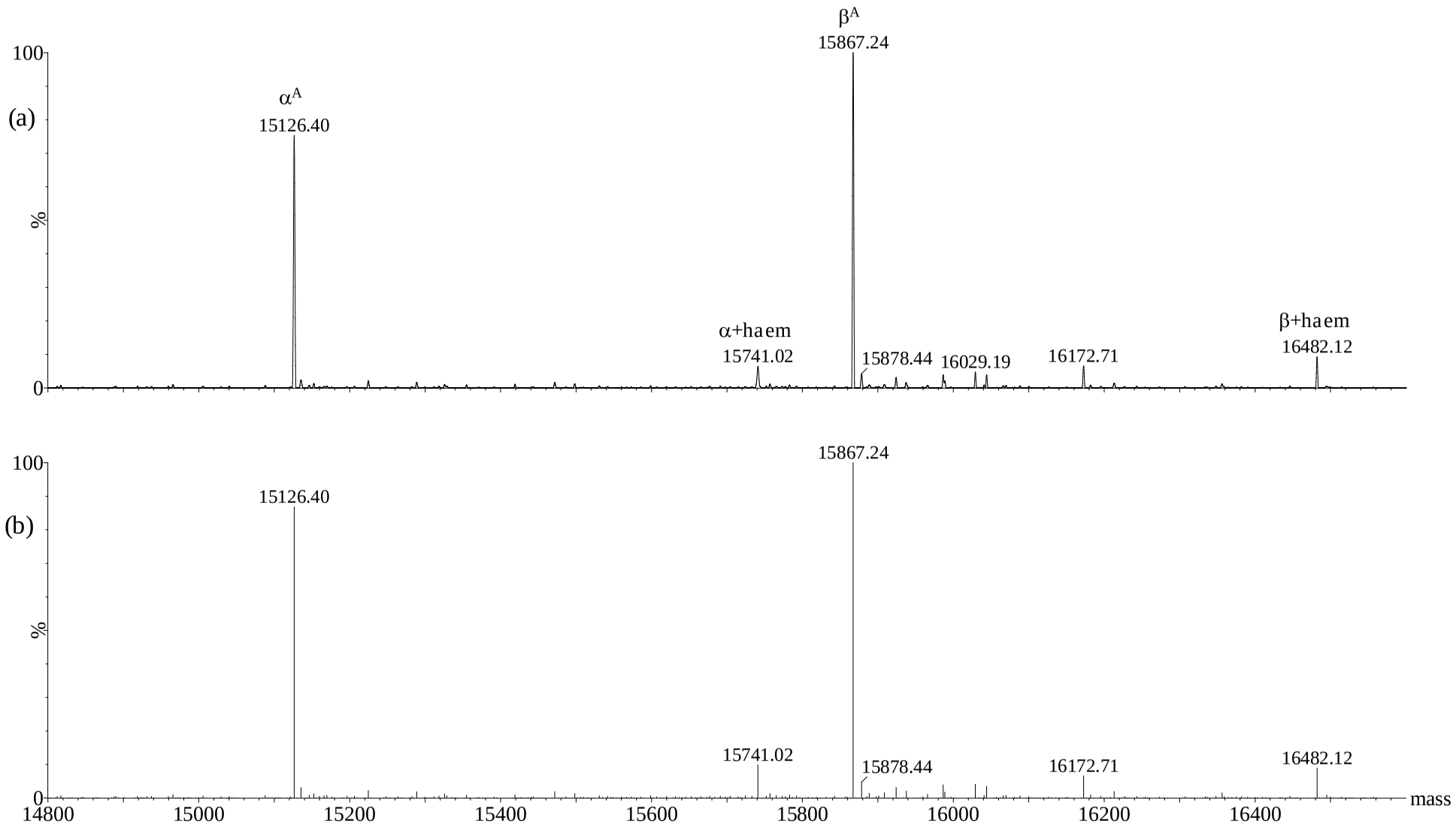

Figure 5.3.3.3. Deconvoluted mass spectrum of Hb J-Wenchang-Wuming (α11Lys→Gln). No mass change is apparent in the α-chain, as would be expected for the 11Lys→Gln amino acid change. Since HPLC shows 'J-like' variant, probably Lys→Gln.

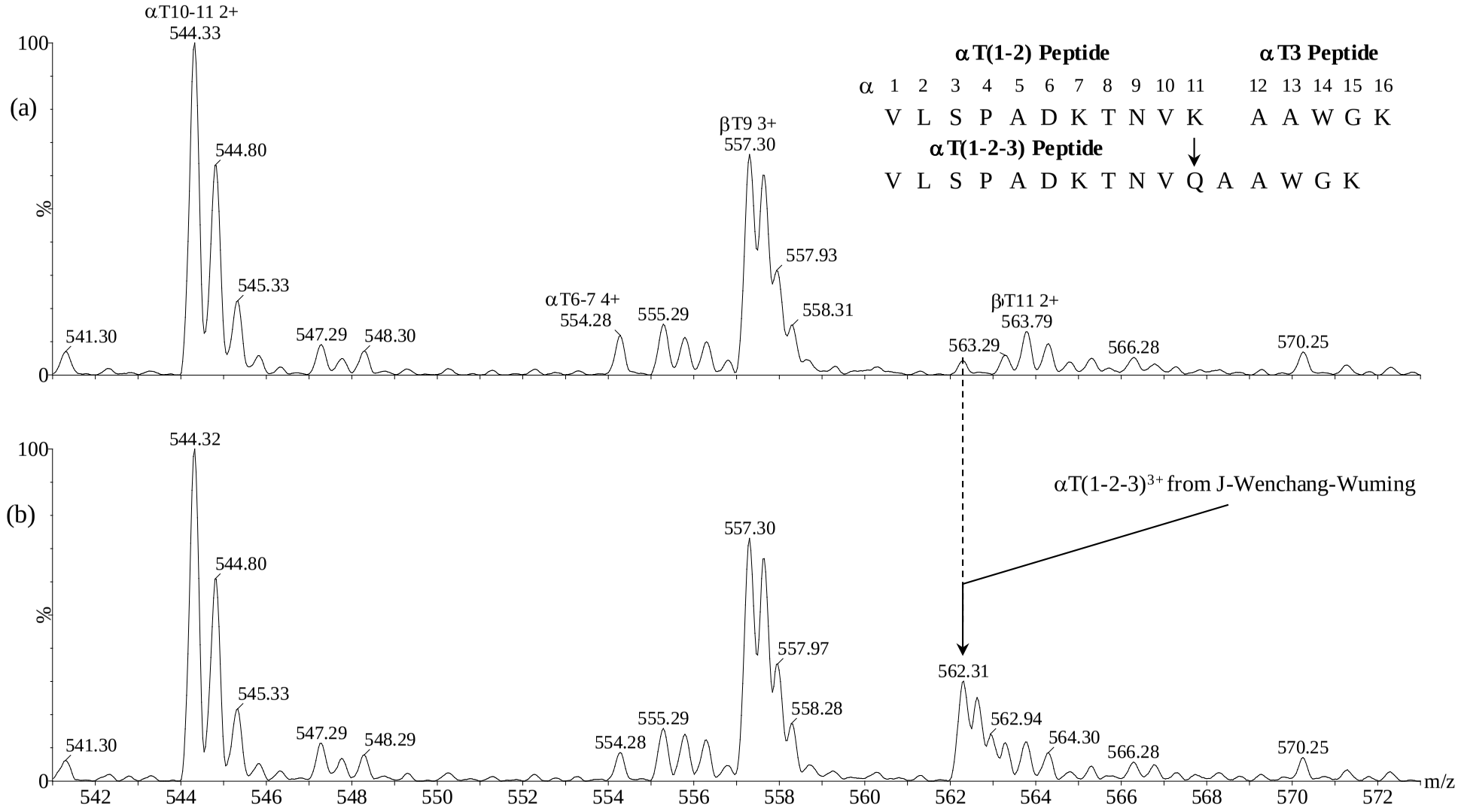

Figure 5.3.3.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb J-Wenchang-Wuming (α11Lys→Gln). Note the appearance of $\alpha T(1\text{-}2\text{-}3)^{3+}$ at *m/z* 562.31.

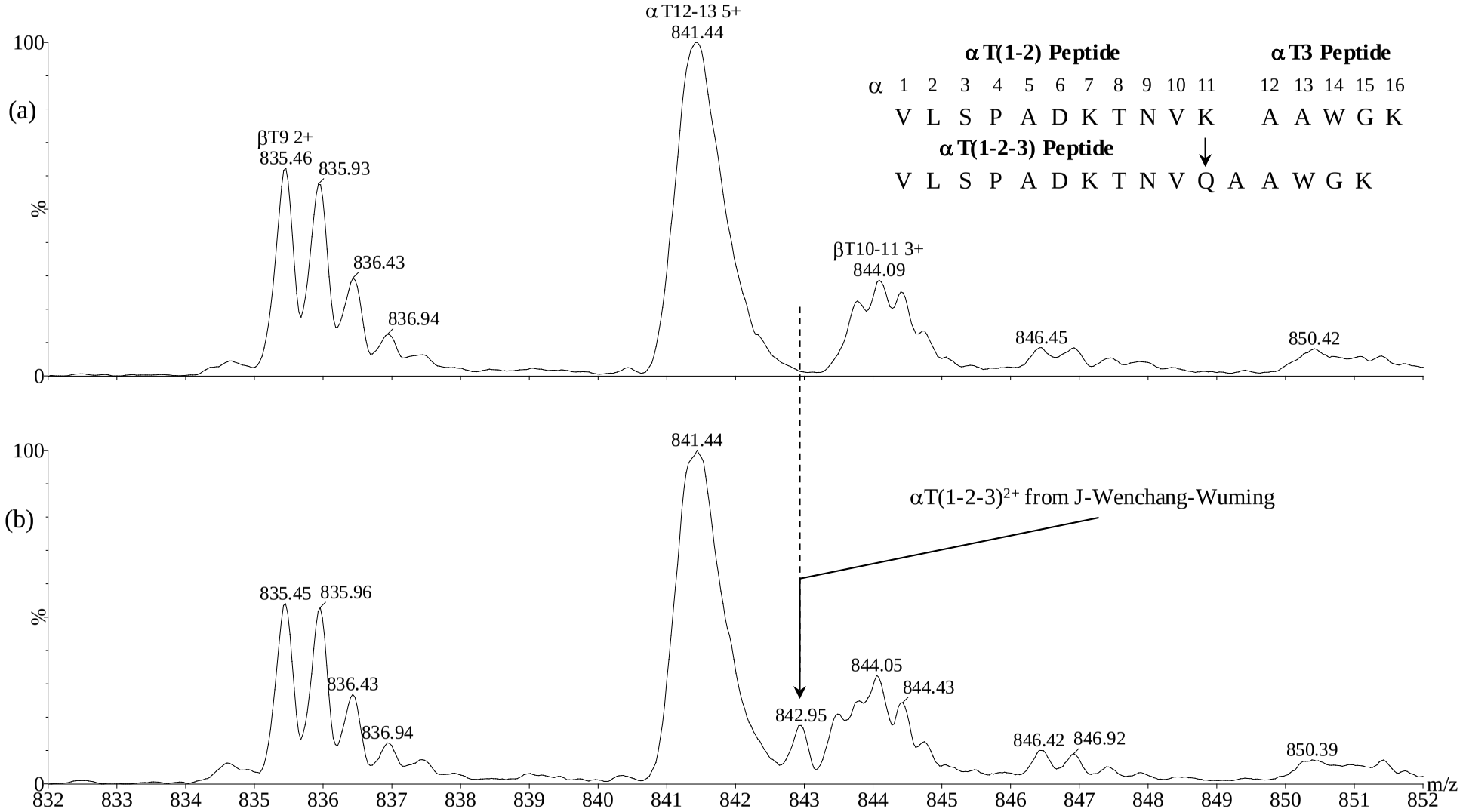

Figure 5.3.3.5. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb J-Wenchang-Wuming (α11Lys→Gln). Note the appearance of $\alpha T(1\text{-}2\text{-}3)^{2+}$ at *m/z* 842.95.

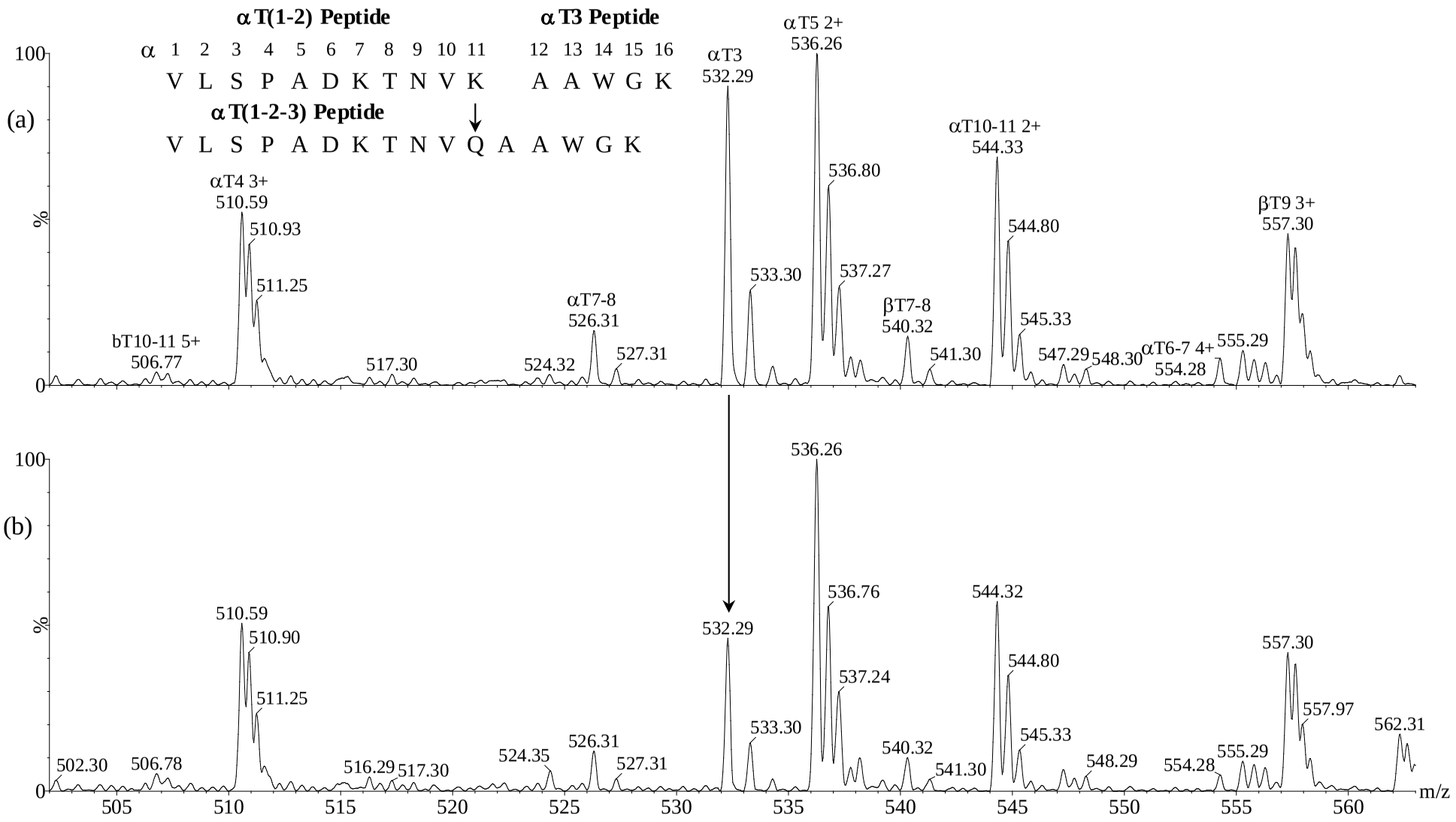

Figure 5.3.3.6. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb J-Wenchang-Wuming (α11Lys→Gln). Note the marked reduction in relative intensity of $\alpha T3^{+}$ ion at *m/z* 532.29.

5.3.4. αT3 - Hb J-Paris-I (α12Ala→Asp)

Hb J-Paris-I is the result of a mutation in which the α12 amino acid residue is changed from Ala to Asp through a single base change in the codon, GCC→GAC.

αT3 Peptide

α	12	13	14	15	16
	A	A	W	G	K
	↓				
	D				

Figure 5.3.4.1. Sequence of the Hb J-Paris-I αT2 tryptic peptide.

A blood sample was submitted for analysis by ESI-MS because the ce-HPLC trace (Figure 5.3.4.2.) showed an abnormally high P3. The high P3 (mainly due to the variant) implies the variant causes a negative polarity change.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.2	0.99	2767
F	2.0	---	1.14	29690
P2	---	4.7	1.29	79890
P3	---	24.8	1.80	420792
A0	---	64.9	2.49	1099335
A2	3.4	---	3.67	61934

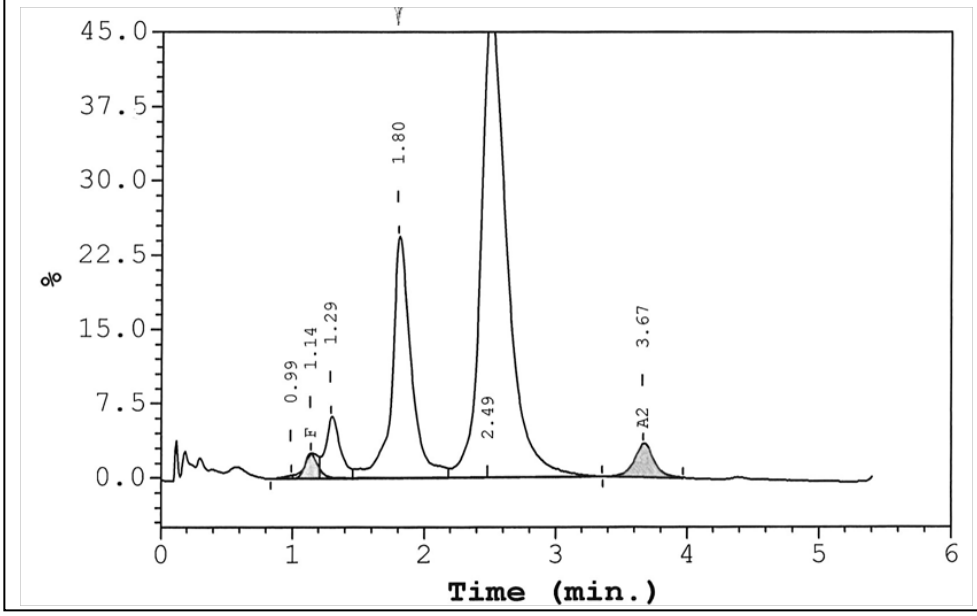

Figure 5.3.4.2. ce-HPLC trace for Hb J-Paris-I

The spectrum obtained by ESI-MS analysis of the blood sample diluted 500-fold is shown in Figure 5.3.4.3. and reveals an α-chain heterozygote at mass 15,170.18 Da, 44 Da heavier and+ 26.5% of the intensity of the total α-chains. The mass increase of 43.80 Da heavier than the normal α-chain, implies the mutation is Ala→Asp, as this mutation is the only possibility that gives a significant negative polarity change. Cys→Phe (TGC→TTC) would give the same mass change, but would not result in a charge change and can be discounted.

Figure 5.3.4.4. shows the diagnostic part of the spectra from (a) normal Hb and (b) the variant heterozygote. The increase in intensity of the *m/z* 576.38 peak from 23% to 38% shows that the mutation occurs in the αT3 peptide. There are two possible sites of mutation in the αT3 tryptic peptide; α12Ala→Asp (Hb J-Paris-I) and α13Ala→Asp (Hb Little Waltham).

Figure 5.3.4.5. shows product ion spectra of the $\alpha T3^{+}$ tryptic fragments of (a) normal Hb and (b) the variant under investigation. The occurrence of all the y" ions up to and including $y"_4$ at the same mass in both the normal and variant precursor ion spectra, and the mass increase of the b_1 ion at *m/z* 187.0 in the lower panel, identifies the mutation as α12Ala→Asp, Hb J-Paris-I.

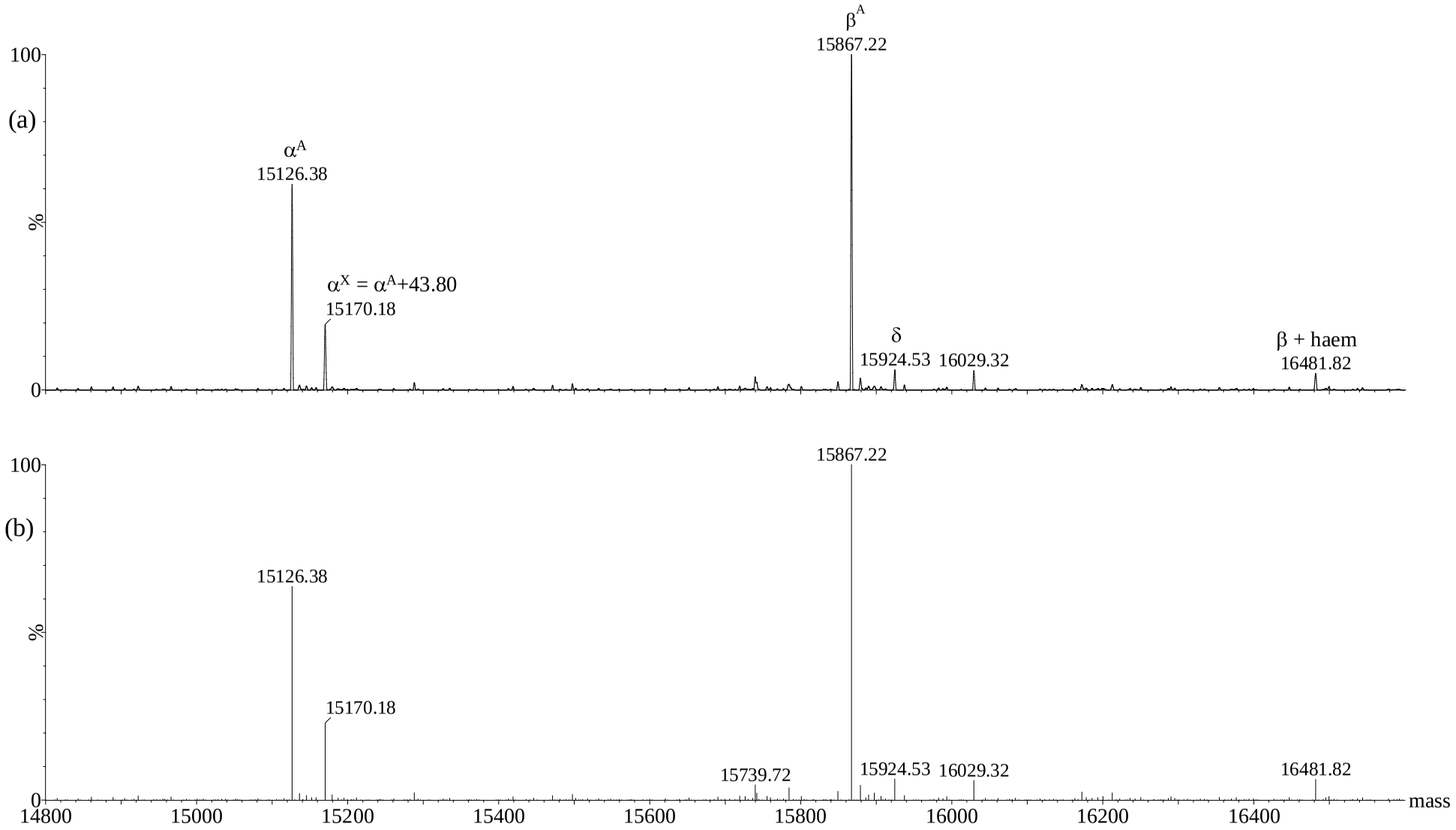

Figure 5.3.4.3. Deconvoluted mass spectrum of Hb J-Paris-I (α12Ala→Asp) showing the presence of a signal at 15,170.18 Da at approximately 26.5% of the total α-chains. A 44 Da mass increase and 'J-like' suggests Ala→Asp.

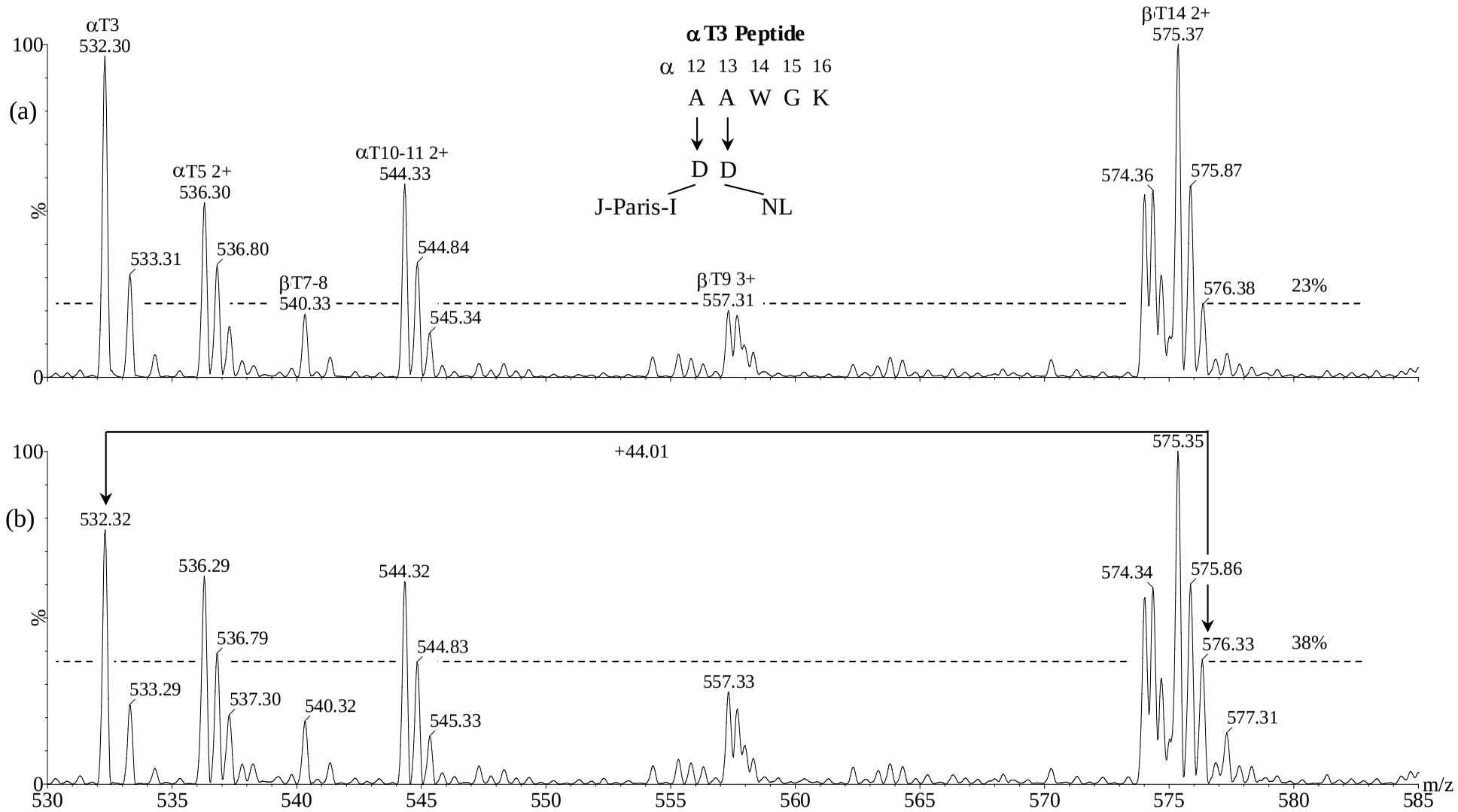

Figure 5.3.4.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) the Hb J-Paris-I heterozygote.

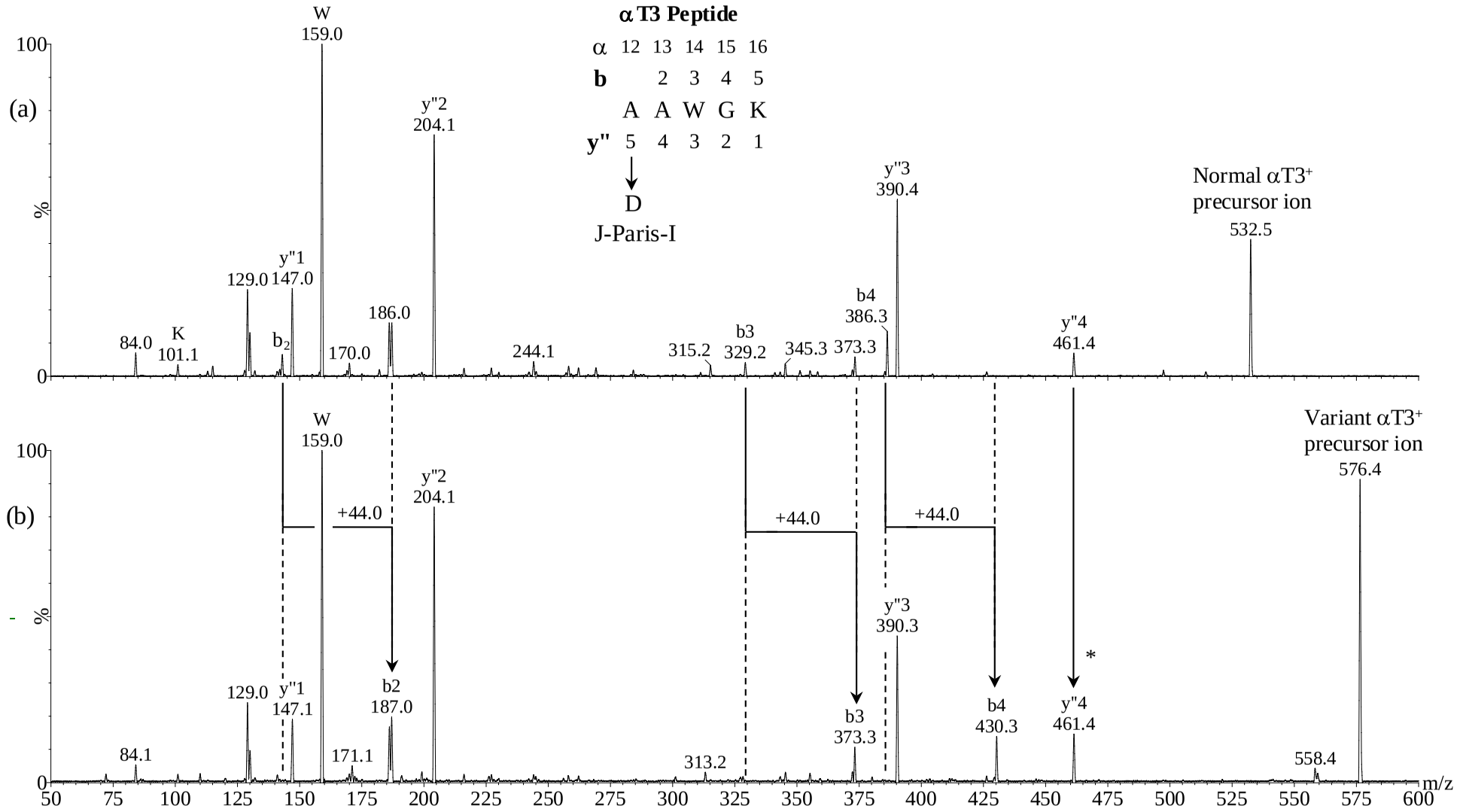

Figure 5.3.4.5. Product ion spectra of the αT3^{+} tryptic fragment of (a) normal Hb and (b) Hb J-Paris-I. The presence if the y"-ions, particularly y"$_4$, at the same mass from both normal and variant precursor ions, identifies the mutation as α12Ala→Asp, Hb J-Paris-I.

5.3.5. αT4 - Hb LeLamentin (α20His→Gln)

Hb LeLamentin is the result of a mutation in which the α20 amino acid residue is changed from His to Gln through a single base change in the codon, CAC→CAA.

αT4 Peptide

α	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
	V	G	A	H	A	G	E	Y	G	A	E	A	L	E	R
				↓											
				Q											

Figure 5.3.5.1. Sequence of the Hb LeLamentin αT4 tryptic peptide.

A blood sample was submitted for analysis by mass spectrometry because the ce-HPLC trace (Figure 5.3.5.2.) showed an abnormally high P3 response (23.4%). The high P3 (mainly due to the variant) implies the variant causes a negative polarity change.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.4	1.02	6681
F	0.5	---	1.08	6417
Unknown	---	0.6	1.26	9386
P2	---	3.5	1.36	52571
P3	---	23.4	1.62	355086
A0	---	69.4	2.46	1054910
A2	2.2	---	3.64	34921

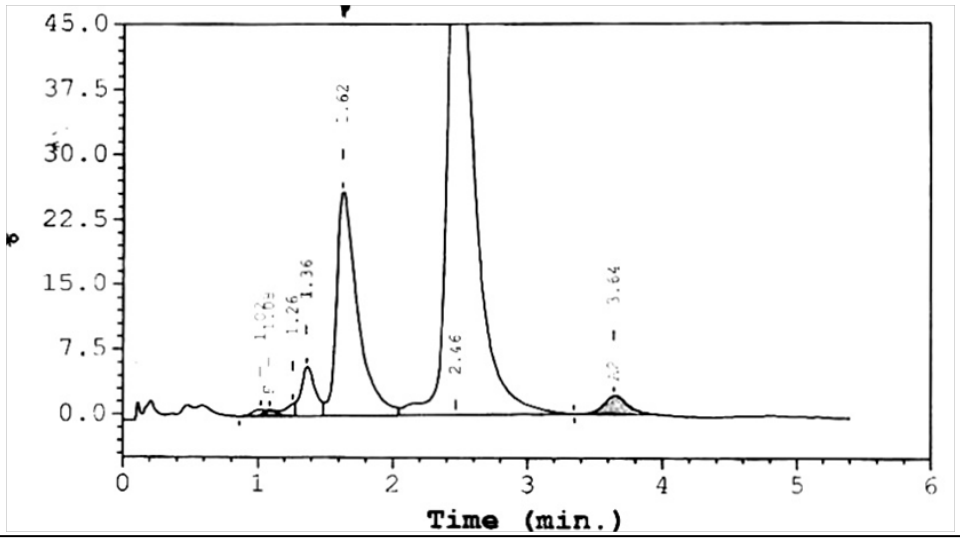

Figure 5.3.5.2. ce-HPLC trace for Hb LeLamentin

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.3.5.3.) revealed an α-chain heterozygote in which the mass of the variant chain was 8.96 Da lower than normal at 15,117.38 Da, and the intensity of the variant chain was 23.6% of total α-chains.

There is only one single amino acid change that can give 9 Da decrease in mass by a single base change in the codon, namely His→Gln (10 possibilities).

Figure 5.3.5.4. shows the diagnostic part of the tryptic digest spectrum for (a) normal Hb and (b) the variant Hb. The appearance of a new $\alpha T4^{2+}$ tryptic fragment at *m/z* 760.86 indicates that the mutation is

in the αT4 tryptic peptide and identifies the mutation as α20His→Gln, since it is the only possibility in this peptide.

Figures 5.3.5.5. shows part of the product ion mass spectra for the $\alpha T4^{2+}$ precursors of (a) normal Hb and (b) the variant Hb. The -9 Da mass change in the y''_{12} ion at *m/z* 1293.5 and no change in the y''_{11} ion at *m/z* 1,165.2 confirms the mutation as α20His→Gln, Hb LeLamentin.

Further evidence for the mutation is shown in the lower panel of Figure 5.3.5.6. with the appearance of a Gln immonium ion at *m/z* 101.1 and no His immonium ion at *m/z* 110.0, as well as the mass decrease of the b_4 fragment at *m/z* 356.2.

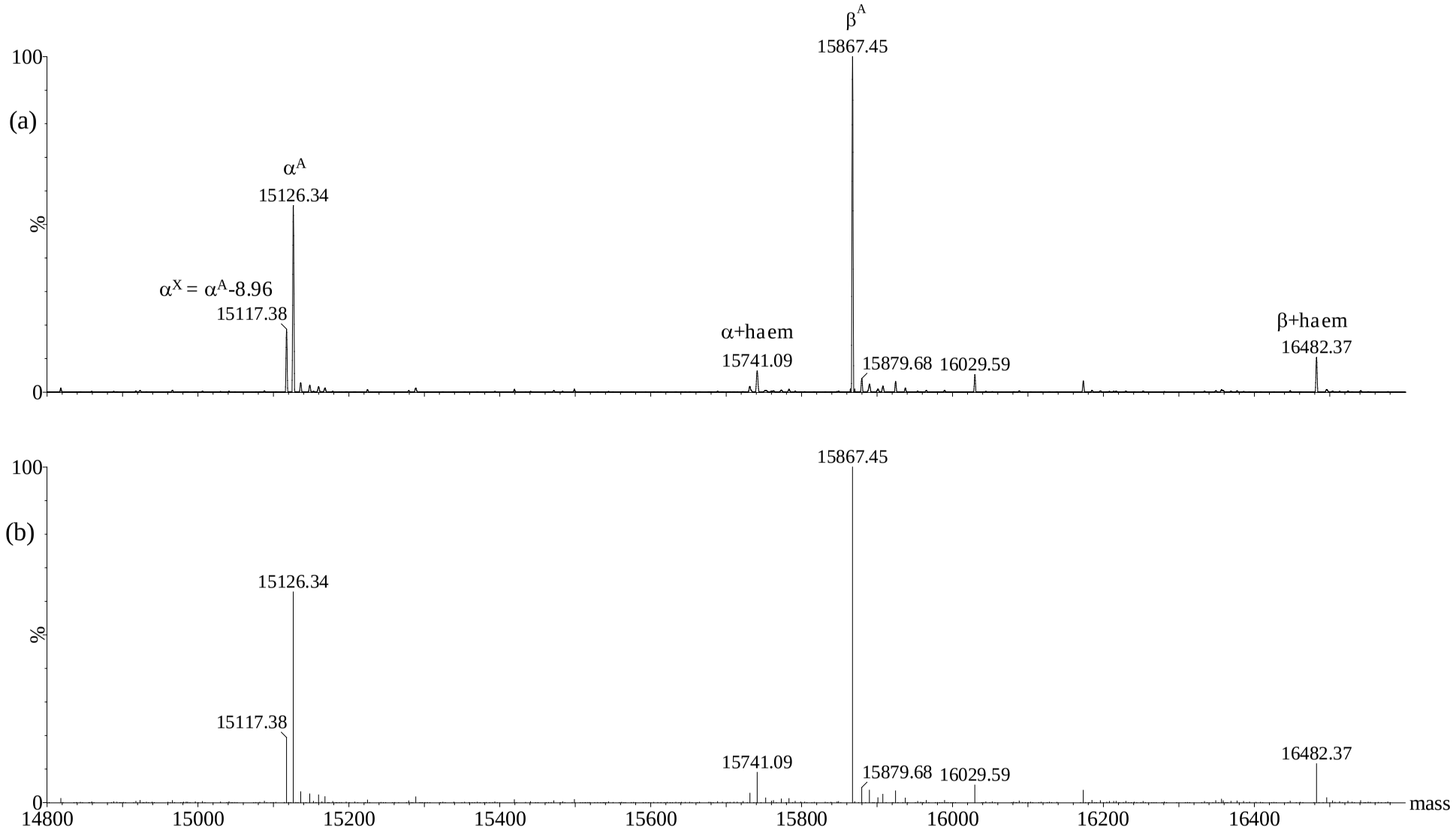

Figure 5.3.5.3. Deconvoluted mass spectrum of the blood sample showing the presence of an α-chain variant at 15,117.38 Da, which is 23.6% of the total α-chains.

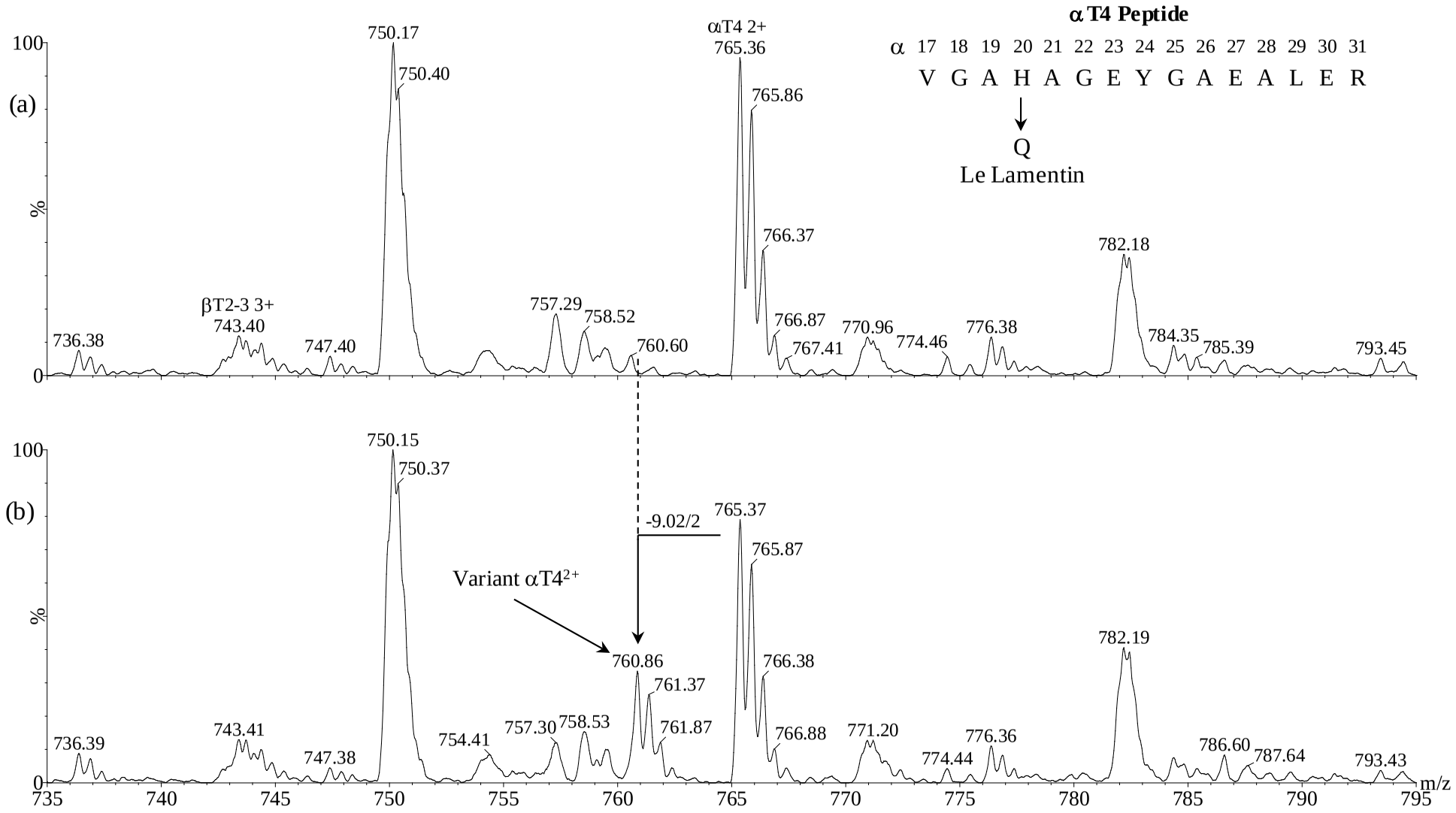

Figure 5.3.5.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb LeLamentin heterozygote. The presence of the mutation in the αT4 peptide identifies the mutation as α20His→Gln, since it is the only possibility in this peptide.

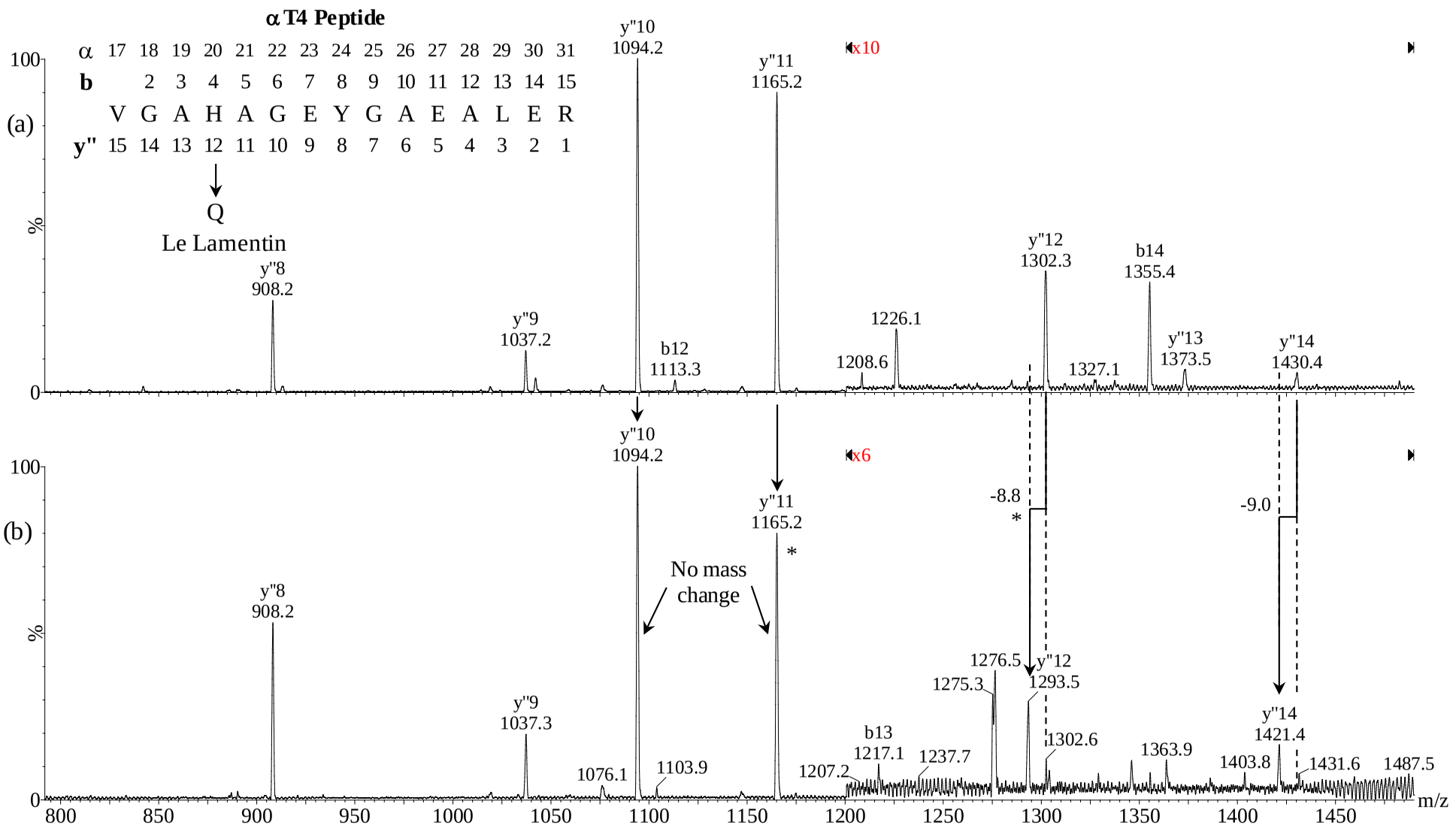

Figure 5.3.5.5. Partial Product ion spectra of the $\alpha T4^{2+}$ tryptic fragment of (a) normal Hb and (b) Hb LeLamentin (α20His→Gln). The 9 Da mass decrease at $y"_{12}$ and b_4 (Figure 5.3.5.6.) confirms the mutation α20His→Gln.

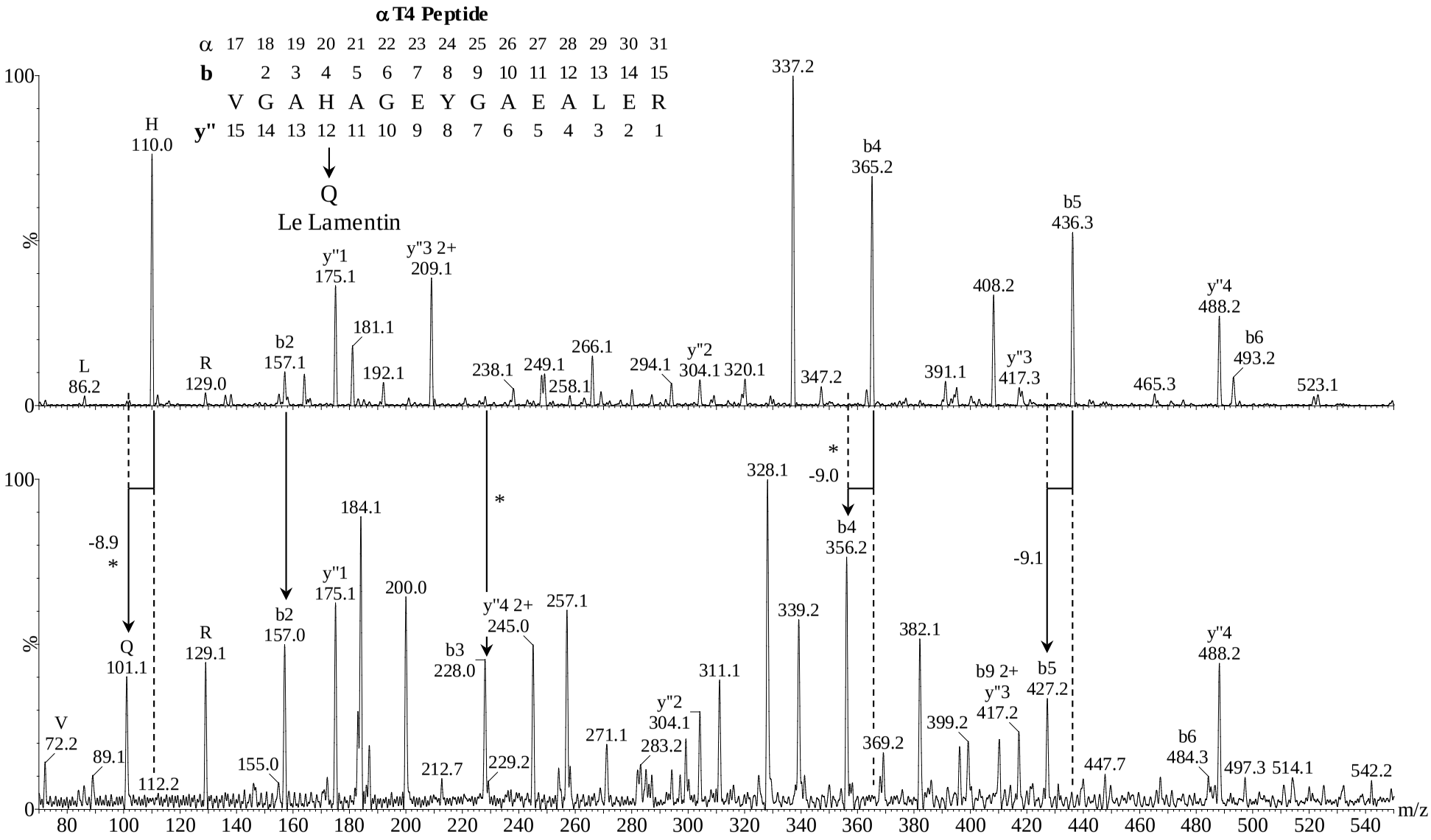

Figure 5.3.5.6. Partial Product ion spectra of the $\alpha T4^{2+}$ tryptic fragment of (a) normal Hb and (b) Hb LeLamentin. The 9 Da mass decrease at $y"_{12}$ (Figure 5.3.5.5.) and b_4 confirms the mutation α20His→Gln.

5.3.6. αT4 - Hb Fontainebleau (α21Ala→Pro)

Hb Fontainebleau is the result of a mutation in which the α21 amino acid residue is changed from Ala to Pro through a single base change in the codon, GCT→CCT.

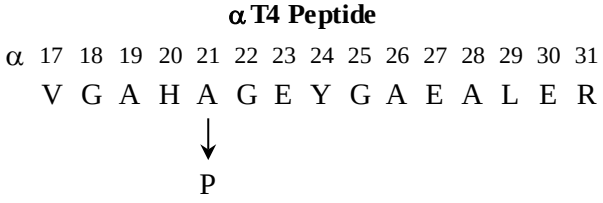

Figure 5.3.6.1. Sequence of the Hb Fontainebleau αT4 tryptic peptide.

A blood sample was submitted for analysis by mass spectrometry because the ce-HPLC trace (Figure 5.3.6.2.) showed an abnormal peak that eluted at 2.77 min, just after A_0, suggesting no charge change in the variant.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
P1	---	0.1	0.66	1133
F	0.3	---	1.04	3843
Unknown	---	0.9	1.21	11211
P2	---	3.8	1.30	49167
Unknown	---	1.1	1.48	14406
P3	---	7.0	1.65	91288
A0	---	72.1	2.41	944920
Unknown	---	13.6	2.77	178179
A2	1.2	---	3.51	16776

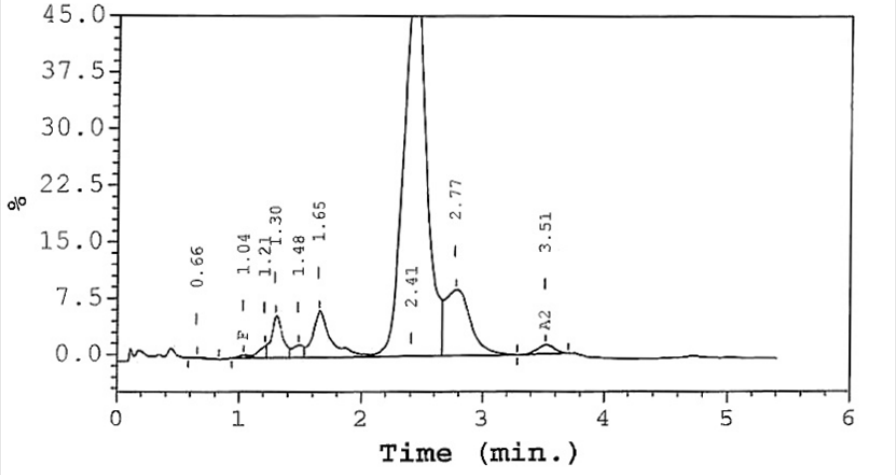

Figure 5.3.6.2. ce-HPLC trace for Hb Fontainebleau

The spectrum obtained by ESI-MS analysis of the blood sample diluted 500-fold (Figure 5.3.6.3.) revealed an α-chain variant at 15,151.65 Da, 25.23 Da higher in mass than the normal α-chain. The signal is of low intensity, and may be affected by the Na adduct of the α-chain, so consideration is given to potential mutations of +25 Da and +26 Da: Met→Arg (discounted as no significant charge change by ce-HPLC), Ala→Pro (21 possibilities), His→Tyr (10 possibilities), Ser→Ile (11 possibilities) or Ser→Leu (11 possibilities).

Figure 5.3.6.4. shows the diagnostic region of the 30-minute digest spectrum for (a) normal Hb and (b) the variant Hb. A signal is observed in the lower panel at *m/z* 778.40, corresponding to a 26.02 Da mass

increase in the $\alpha T4^{2+}$ ion. A corresponding mass increase is also observed for the variant $\alpha T4^{3+}$ at *m/z* 519.29 in the lower panel of Figure 5.3.6.5. There are five potential sites of mutation in the αT4 tryptic fragment include: α20His→Tyr (Hb Necker Enfants-Malades), α21Ala→Pro (Hb Fontainebleau) and three, previously unreported, Ala→Pro mutations at α19, α26 and α28.

Figure 5.3.6.6. shows the product ion spectrum of the $\alpha T4^{2+}$ tryptic peptide for (a) normal Hb and (b) the variant Hb. No mass change is observed in the lower panel for the b_4 fragment at *m/z* 365.3, nor the y" series to $y"_{10}$ at *m/z* 1,094.6. The 26 Da mass increase is observed for the $y"_{11}$ fragment at *m/z* 1,191.7 confirms the mutation as α21Ala→Pro, Hb Fontainebleau.

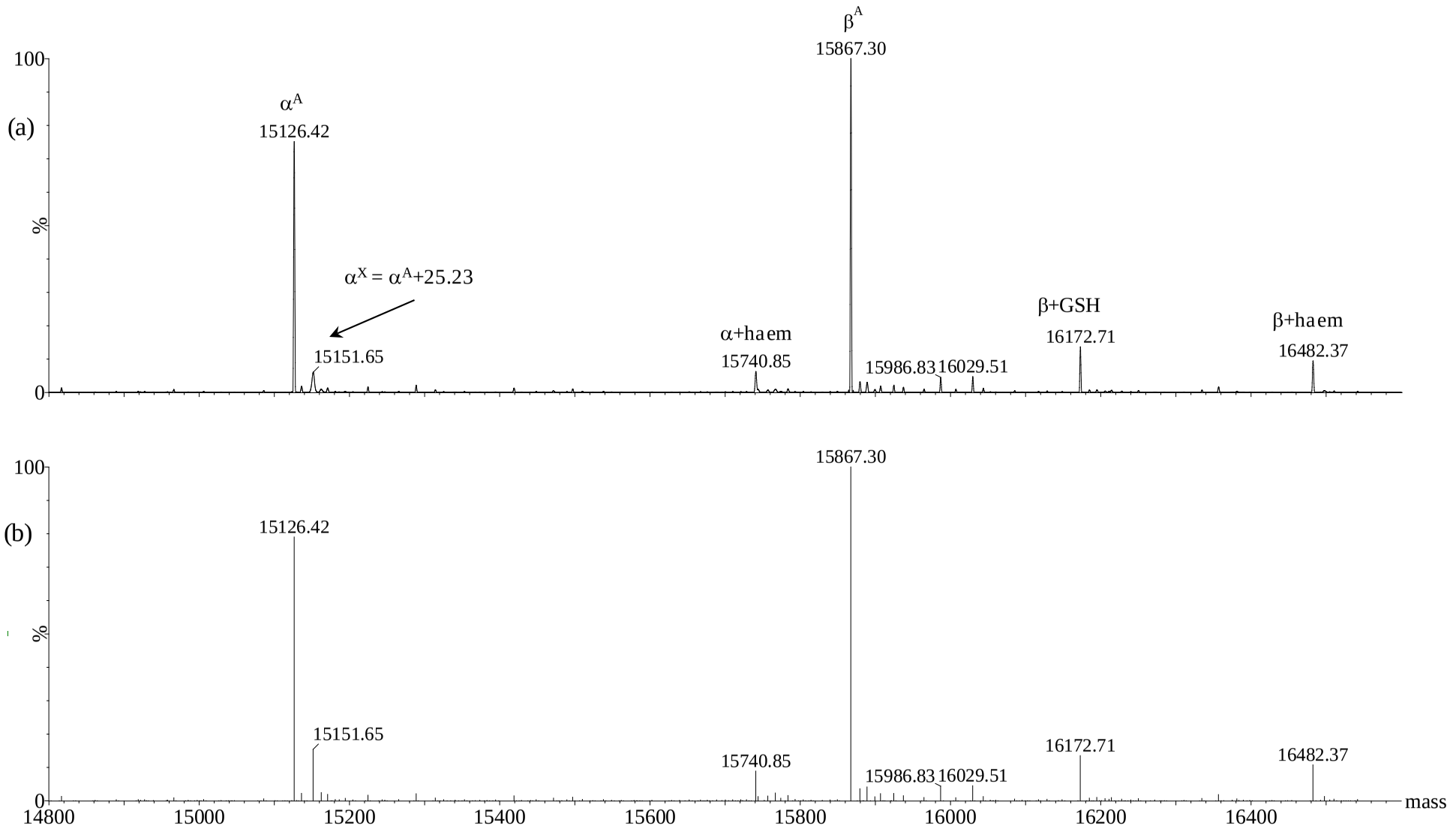

Figure 5.3.6.3. Deconvoluted mass spectrum of the blood sample showing the presence of an α-chain variant at 15,151.65 Da which is 16.3% of the total α-chains.

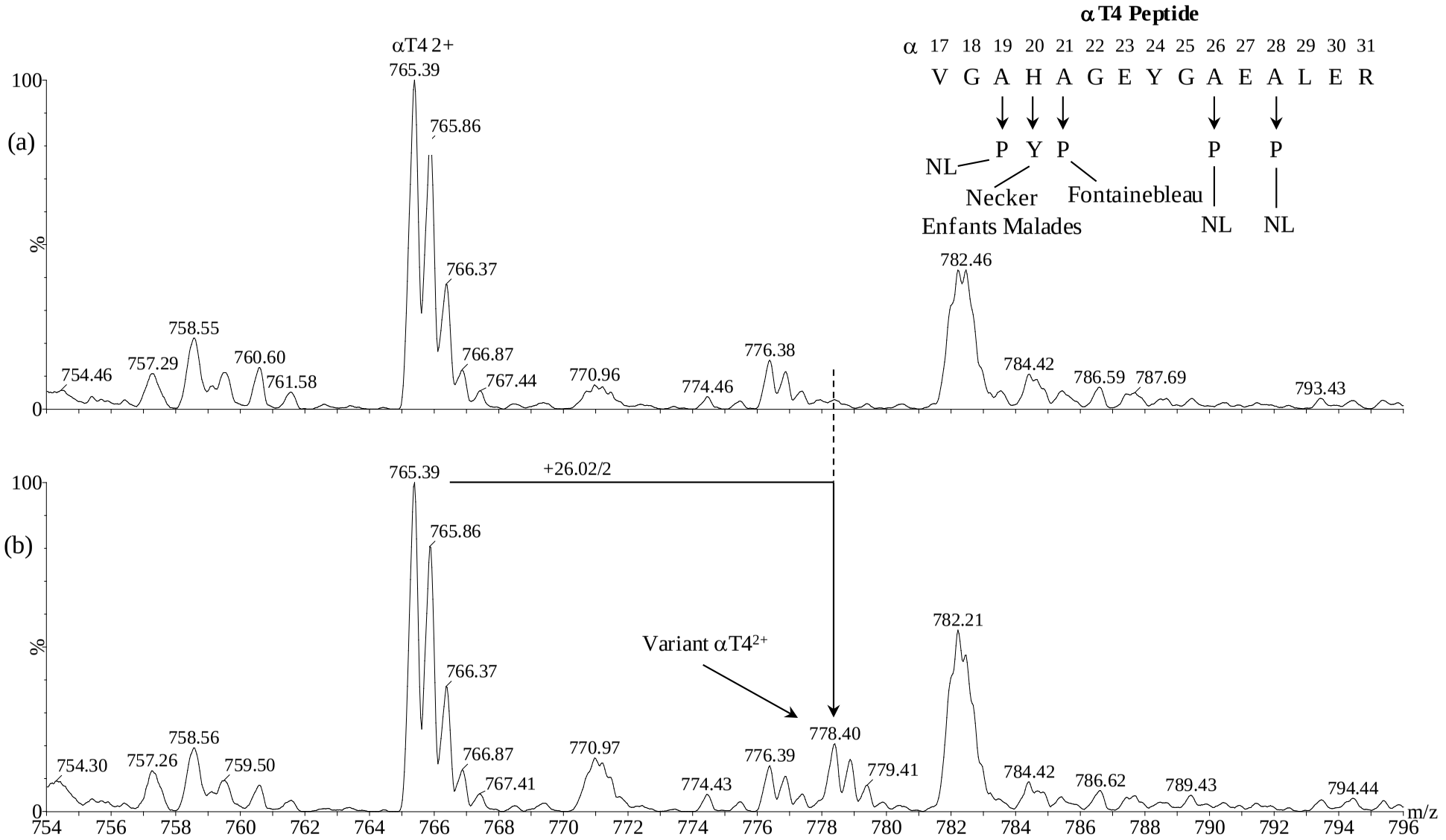

Figure 5.3.6.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) the blood sample.

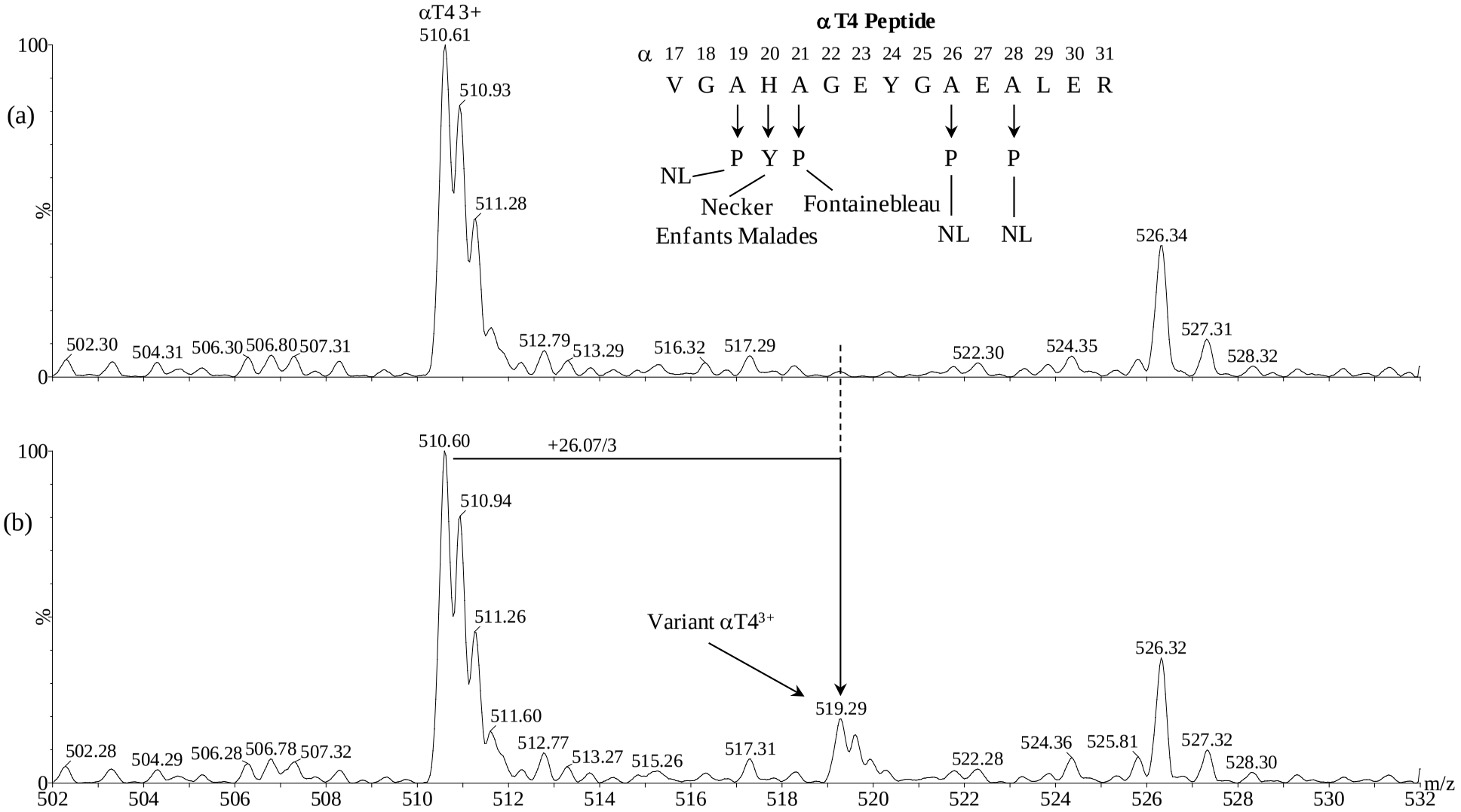

Figure 5.3.6.5. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) the blood sample.

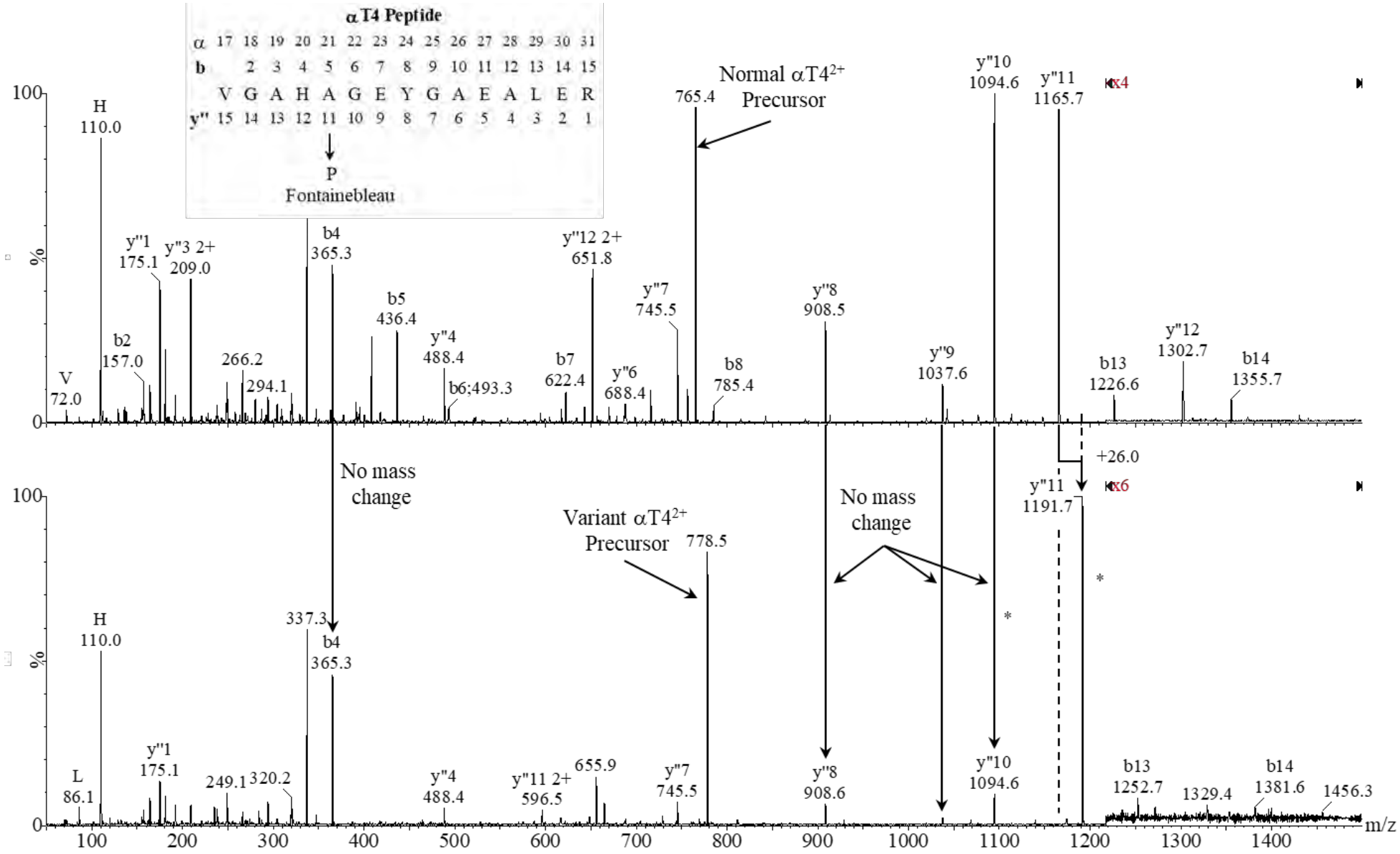

Figure 5.3.6.6. Product ion spectra of the $\alpha T4^{2+}$ tryptic fragments of (a) normal Hb and (b) the blood sample. The 26 Da mass increase at y''_{11} identifies the mutation as α21Ala→Pro.

5.3.7. αT4 - Hb Prato (α31Arg→Ser)

Hb Prato is the result of an α-chain mutation in which the α31 amino acid residue is changed from Arg to Ser through a single base change in the codon AGG→AGC or AGT. The mutation of the Arg residue results in the loss of a tryptic cleavage site, together with the formation of an αT(4-5) tryptic peptide.

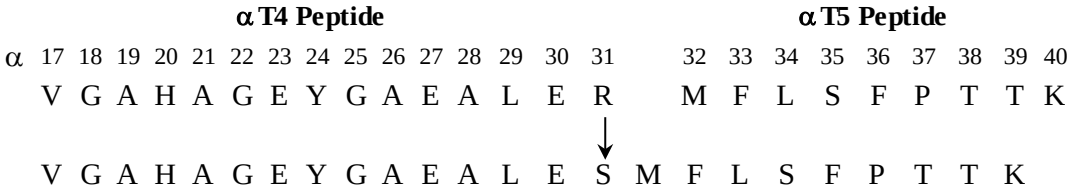

Figure 5.3.7.1. Sequence of the Hb Prato αT(4-5) tryptic peptide.

A blood sample was submitted for analysis by mass spectrometry because the ce-HPLC trace (Figure 5.3.7.2) showed an abnormally high P3 value (14.3%). The high P3 (mainly due to the variant) implies the variant causes a negative polarity change.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.9	1.03	14186
F	0.9	---	1.14	15899
Unknown	---	2.3	1.22	38257
P2	---	5.6	1.30	92114
P3	---	14.3	1.63	235774
A0	---	74.6	2.38	1227471
A2	1.6	---	3.60	22525

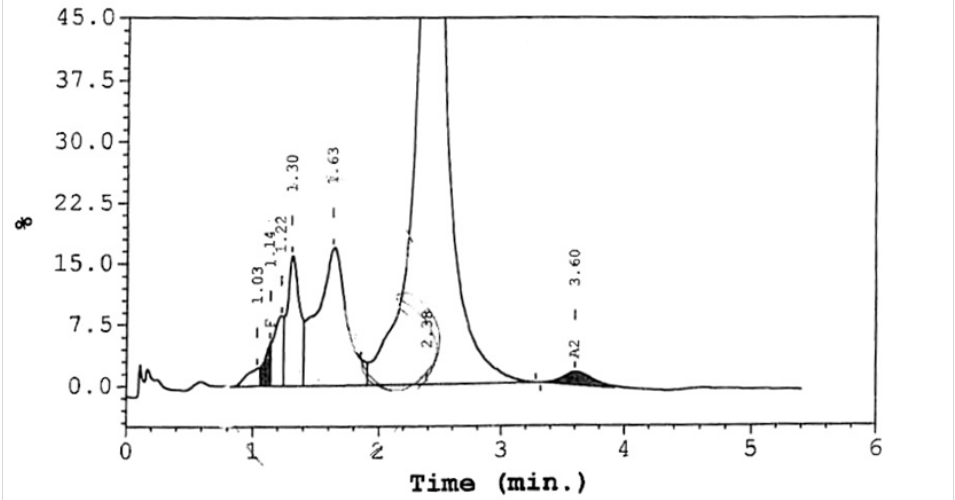

Figure 5.3.7.2. ce-HPLC trace for Hb Prato

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.3.7.3.) revealed an α-chain heterozygote in which the mass of the variant chain at 15,057.23 Da, 69.14 Da lighter than normal. The intensity of the variant chain was 21.0% of total α-chains. A mass difference of -69 Da can only be achieved with an Arg→Ser nutation (3 possibilities). As indicated above, loss of an Arg residue would result in the formation of a 'double' tryptic peptide. Possible sites of mutation are α31, α92 or α141, though α92 is unlikely as it would require two base changes in the codon.

Figure 5.3.7.4. shows the diagnostic part of the tryptic digest spectrum for (a) normal Hb and (b) the variant Hb. A signal is observed in the lower panel at *m/z* 838.43 that is consistent with the formation of

an $\alpha T(4\text{-}5)^{3+}$ tryptic fragment, and this is further supported by the signal in the lower panel of Figure 5.3.7.5. at *m/z* 1257.06 ($\alpha T(4\text{-}5)^{2+}$).

Figure 5.2.6.6. shows the product ion spectrum of the $\alpha T(4\text{-}5)^{3+}$ precursor, and the spectrum is fully consistent with the expected sequence of the $\alpha T(4\text{-}5)$ tryptic peptide, confirming the mutation as α31Arg$\rightarrow$Ser, Hb Prato.

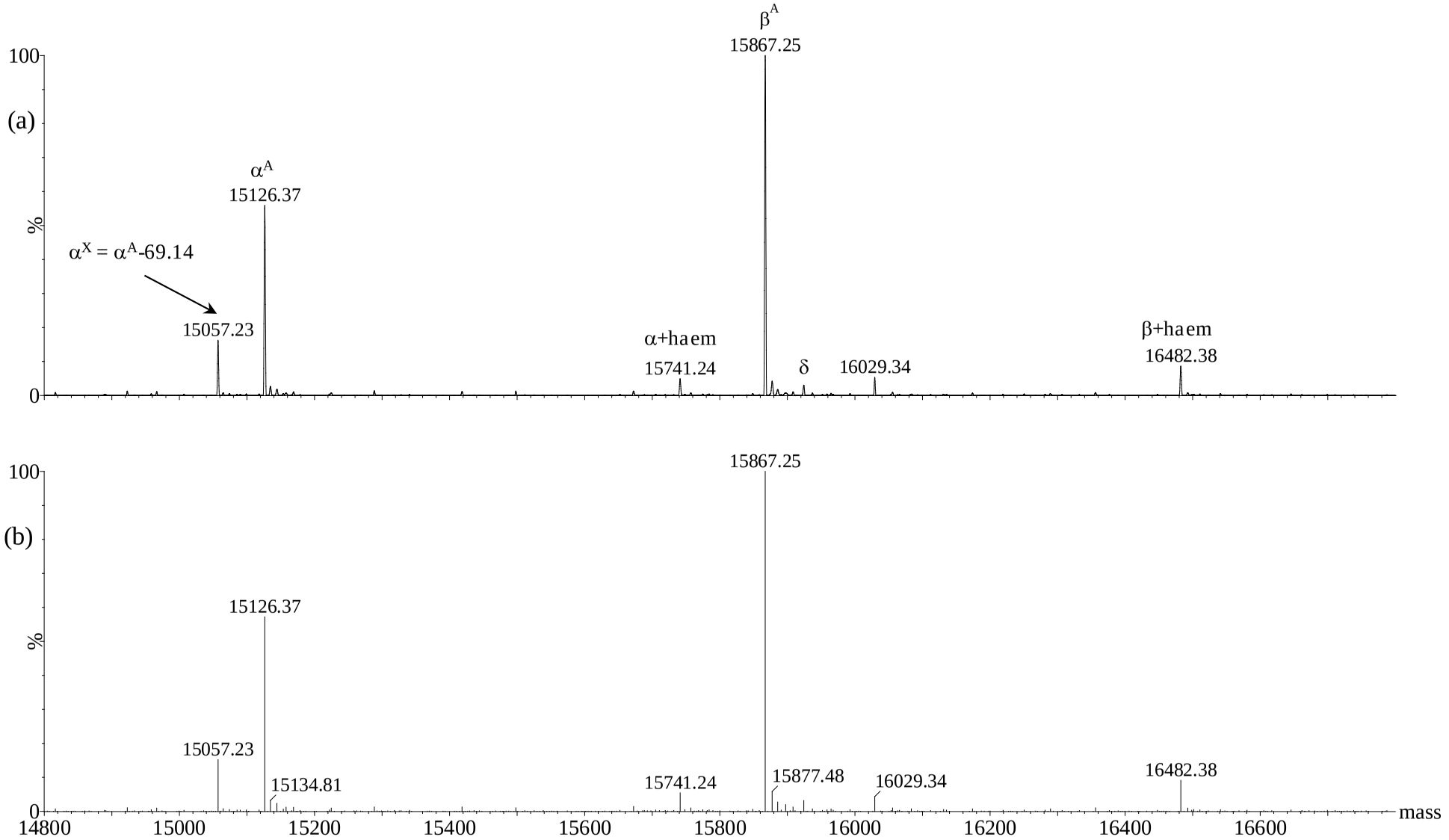

Figure 5.3.7.3. Deconvoluted mass spectrum of Hb Prato (α31Arg→Ser) showing the presence of a signal at 15,057.23 Da at approximately 21% of the total α-chains. The 69 Da mass decrease can only arise from an Arg→Ser mutation.

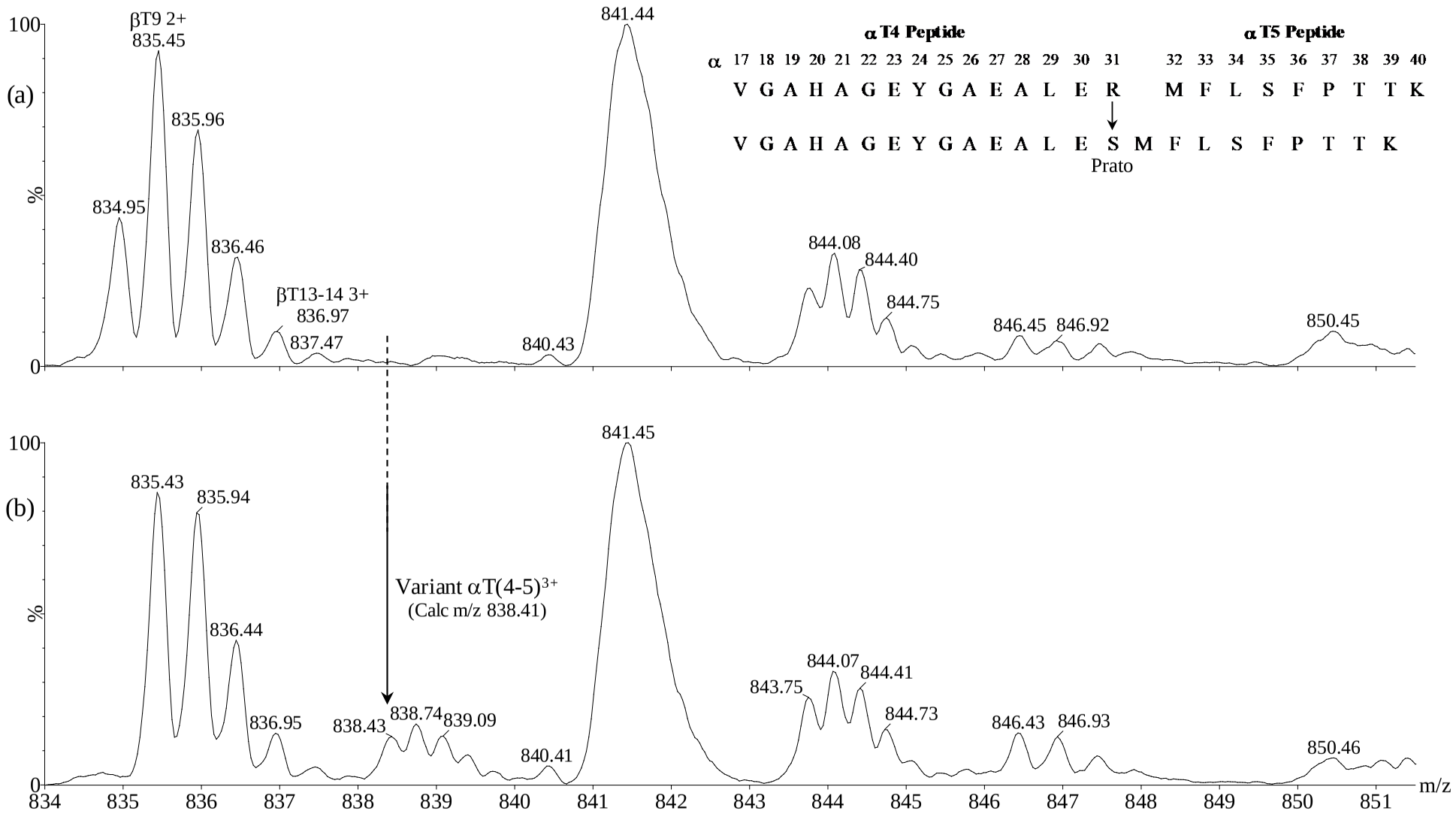

Figure 5.3.7.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) the blood sample. The presence of the $\alpha T(4\text{-}5)^{3+}$ ion at *m/z* 838.43 identifies the mutation as α31Arg→Ser, Hb Prato.

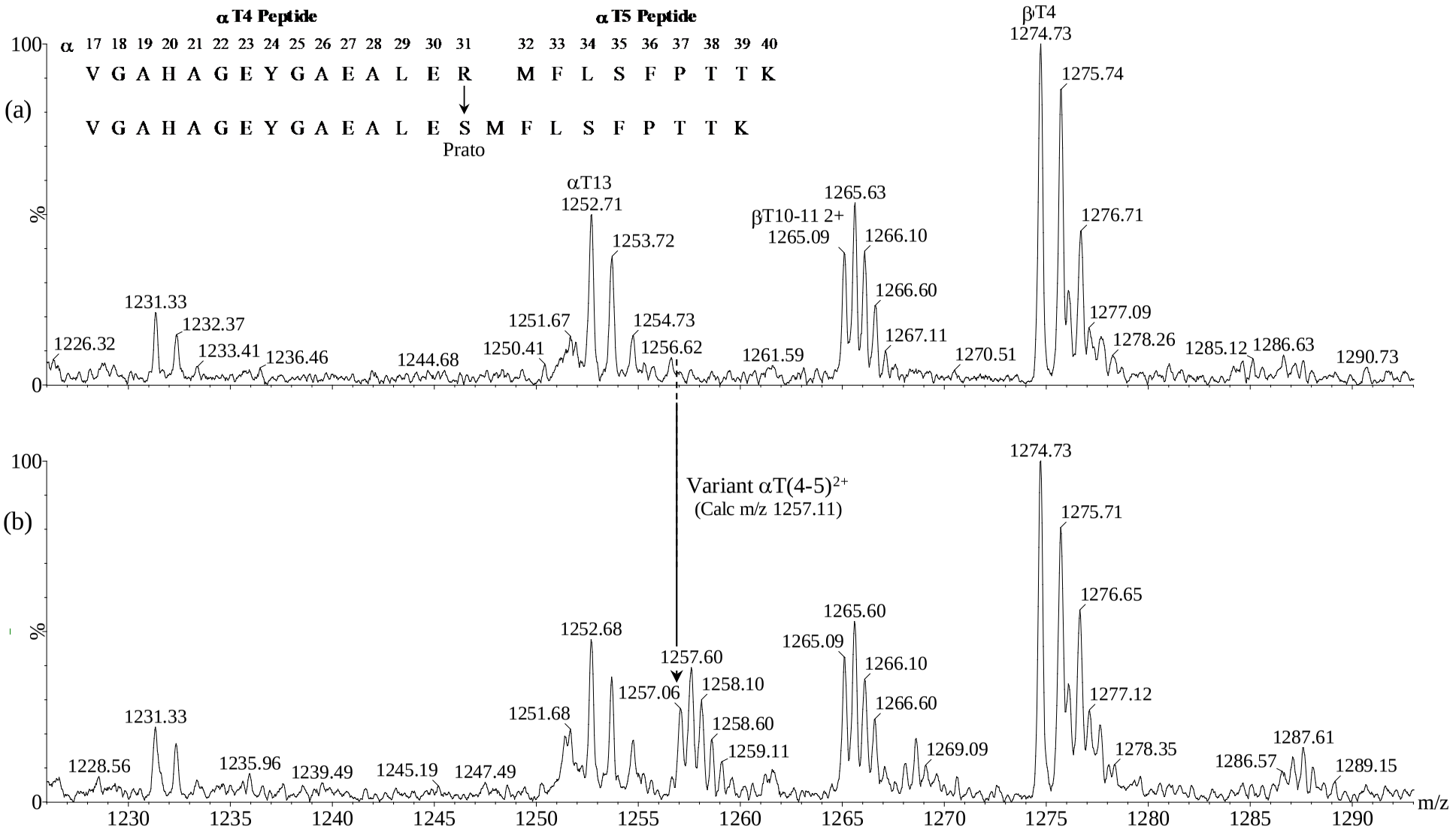

Figure 5.3.7.5. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Prato heterozygote.

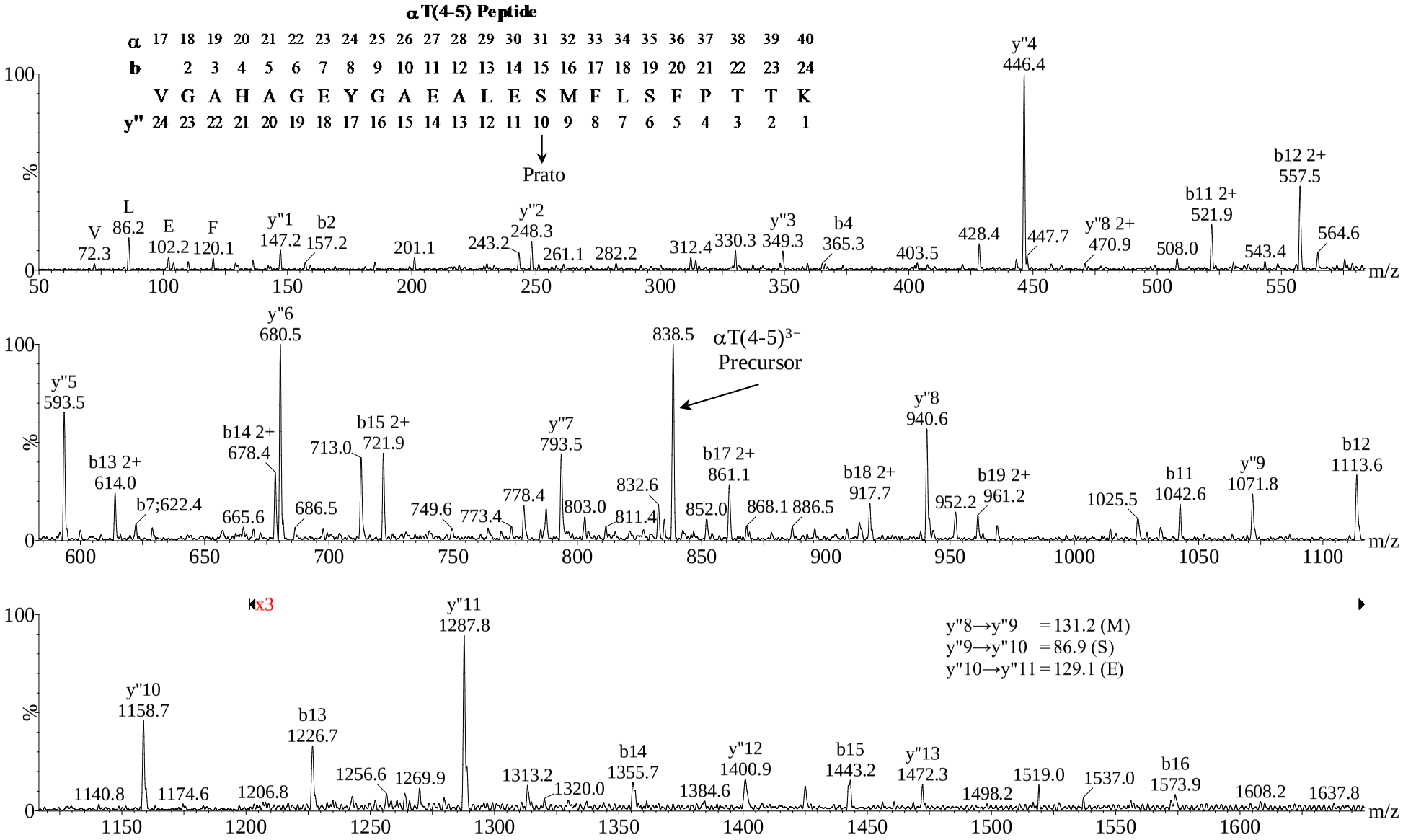

Figure 5.3.7.6. Product ion spectra of the αT(4-5)$^{3+}$ tryptic fragment of Hb Prato. This spectrum is fully consistent with the expected sequence of the αT(4-5) peptide, and confirms the mutation α31Arg→Ser.

5.3.8. αT5 - Hb Chelsea (α38Thr→Ala)

Hb Chelsea (novel) is the result of an α-chain mutation in which the α38 amino acid residue is changed from Thr→Ile through a single base change in the codon ACC→ATC.

αT5 Peptide

α	32	33	34	35	36	37	38	39	40
	M	F	L	S	F	P	T	T	K
							↓		
							I		

Figure 5.3.8.1. Sequence of the Hb Chelsea αT5 tryptic peptide.

A blood sample was submitted for analysis by mass spectrometry because the ce-HPLC trace (Figure 5.3.8.2.) presented a shoulder that appeared slightly later than A_0, suggesting no charge change in the variant.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	0.2	---	1.06	3368
Unknown	---	0.2	1.24	5588
P2	---	4.5	1.35	100212
P3	---	3.1	1.72	68601
A0	---	88.5	2.45	1987654
A2	3.0	---	3.64	79548

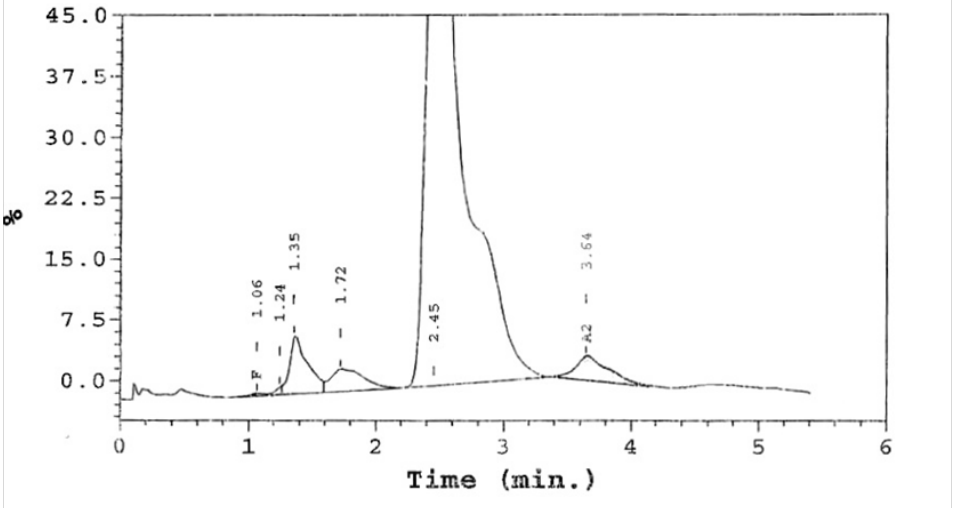

Figure 5.3.8.2. ce-HPLC trace for Hb Chelsea

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.3.8.3.) revealed an α-chain heterozygote in which the α-chain was 23.5% of total α-chains and the mass of the variant chain was 15,138.36 Da, 11.90 Da heavier than normal. The only mutation that gives a 12 Da mass increase and fits the table of nominal mass and amino acid changes genetically governed by a single base change in the codon is Thr→Ile (9 possibilities).

Figures 5.3.8.4. shows the diagnostic parts of the tryptic digest spectra from (a) normal Hb and (b) the variant Hb. The appearance of a peak at *m/z* 542.30 is consistent with the variant occurring in the $\alpha T5^{2+}$ tryptic fragment. This is further supported by the appearance of an $\alpha T5^{+}$ signal at *m/z* 1,083.58 in the lower panel of Figure 5.3.8.5. These results place the mutation in the αT5 peptide, with the possible sites of mutation as α38Thr→Ile (Hb Chelsea) or α39Thr→Ile (not previously reported), both of which can be achieved through a single base change in the codon ACC→ATC.

Figure 5.3.8.6 shows the partial product ion spectra of the $\alpha T5^{+}$ ion from (a) normal Hb and (b) the variant Hb. A 12 Da increase is observed for the y''_3 fragment at *m/z* 361.3 and an internal fragment, PI, at *m/z* 211.2 in the lower panel. These data identify the mutation as mutation as α38Thr→Ile, Hb Chelsea.

This variant was first reported following analysis by mass spectrometry.

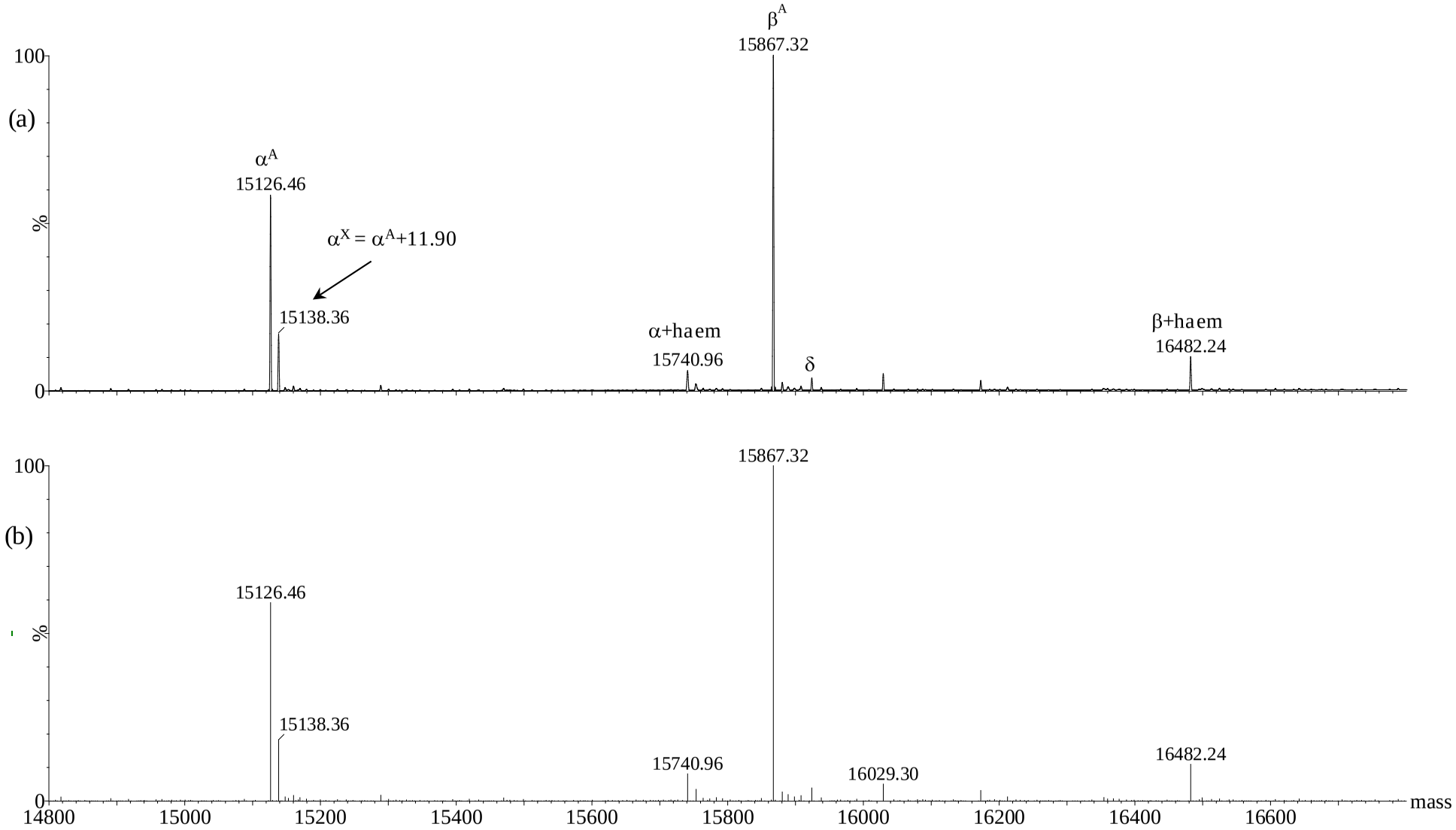

Figure 5.3.8.3. Deconvoluted mass spectrum of Hb Chelsea (Novel) (α38Thr→Ile) showing the presence of a signal at 15,138.36 Da at 23.5% of the total α-chains. For 12 Da mass increase Thr→Ile is the only possibility for a single base change in the codon.

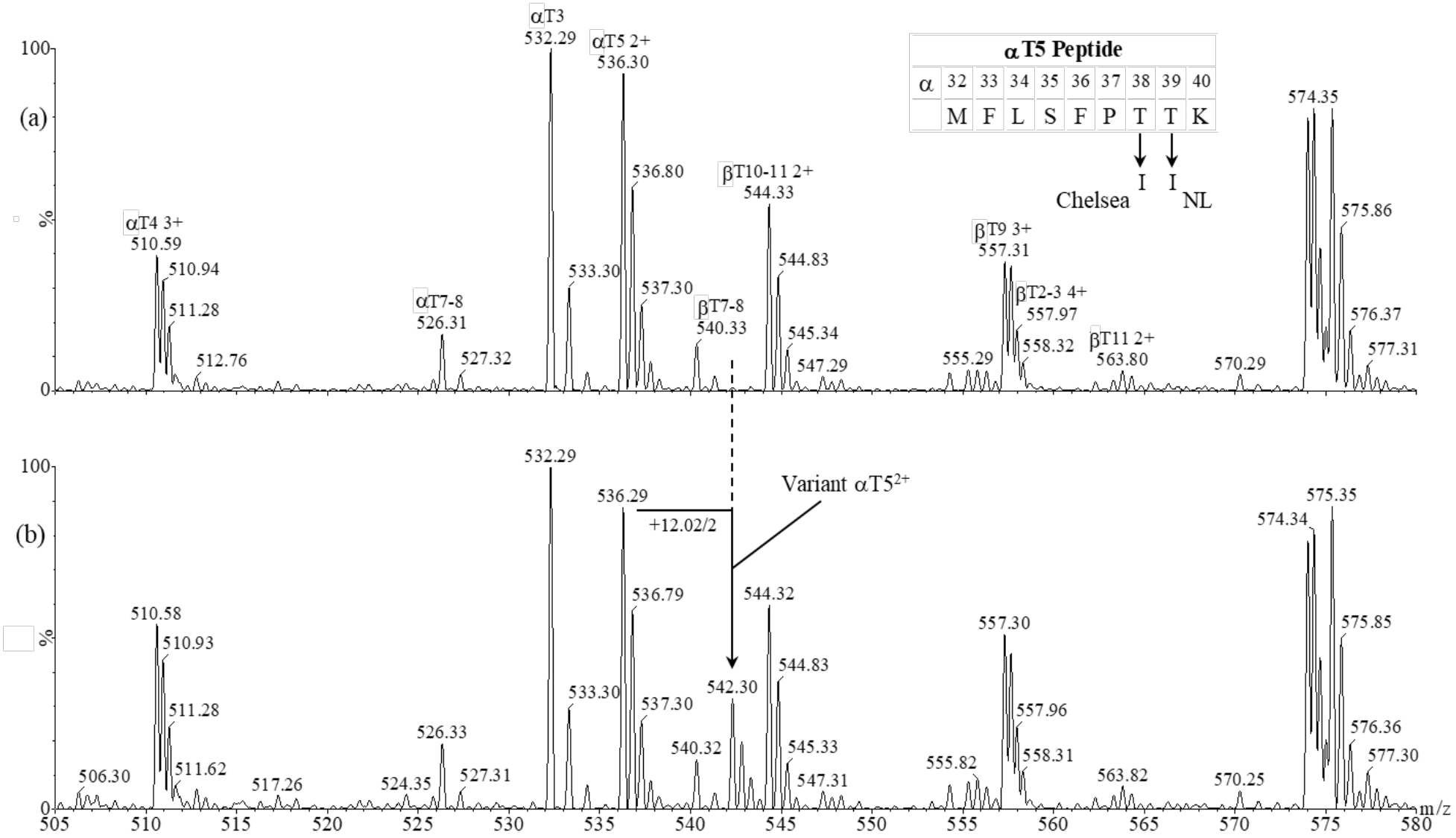

Figure 5.3.8.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Chelsea heterozygote. Both of the indicated mutations can occur by a single base change in the codon (ACC→ATC).

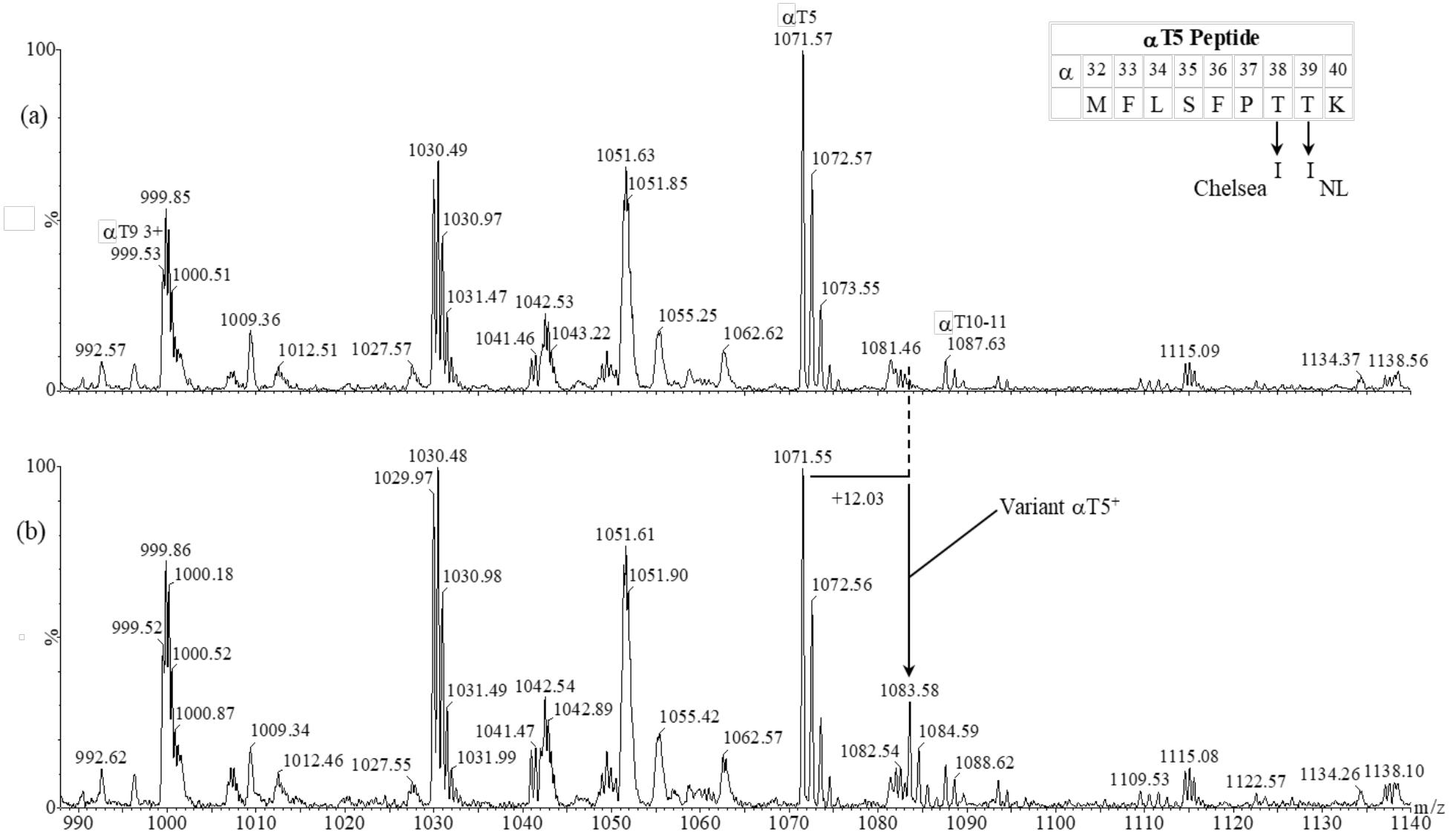

Figure 5.3.8.5. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Chelsea heterozygote.

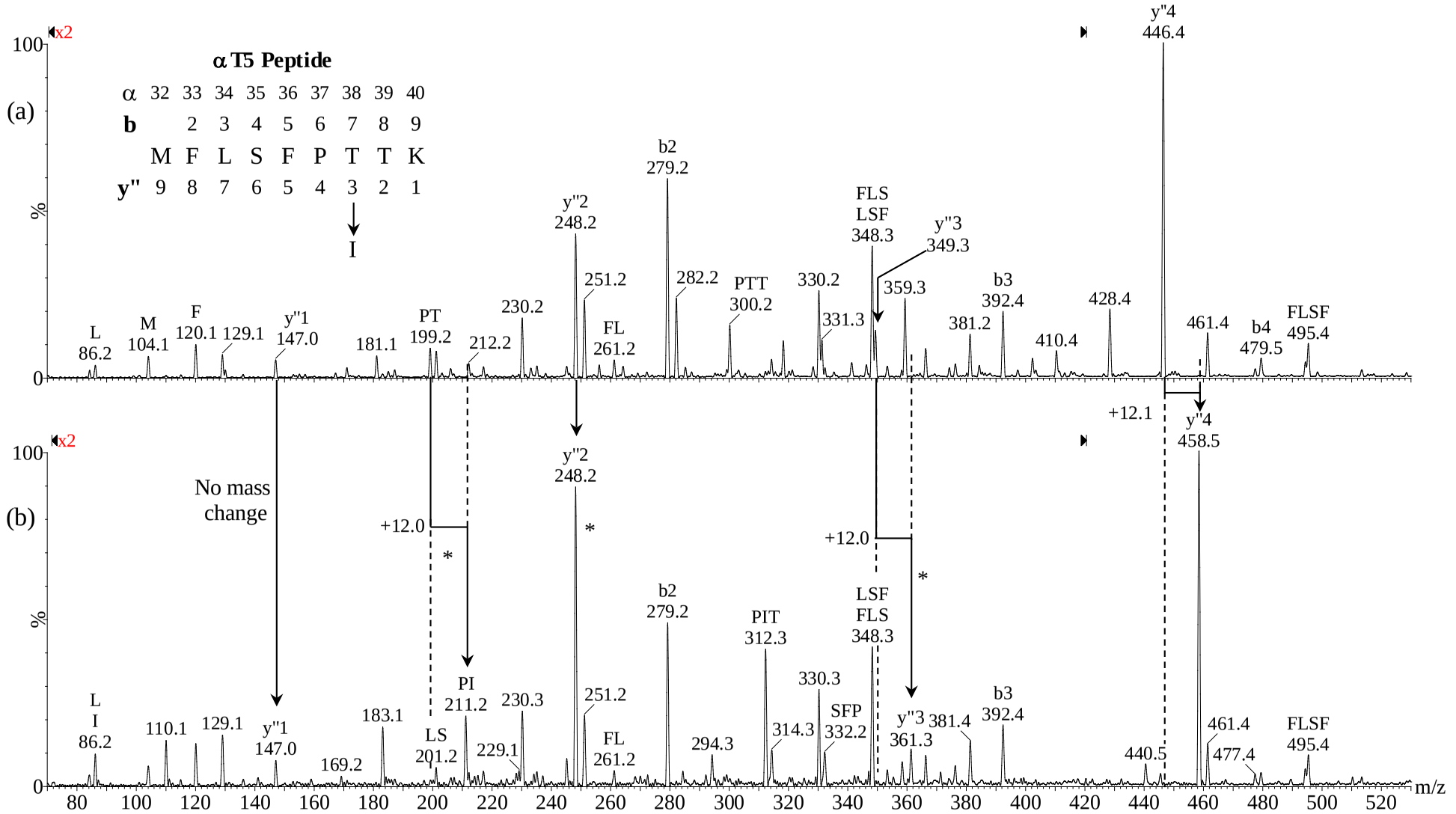

Figure 5.3.8.6. Partial Product ion spectra of the αT5^{+} tryptic fragment of (a) normal Hb and (b) Hb Chelsea (α38Thr→Ile). The 12 Da mass increase at y"$_3$ at *m/z* 361.3 identifies the mutation as α38Thr→Ile and is strongly supported by the 12 Da mass increase in the internal fragment PT→PI.

5.3.9. αT6 - Hb Riccarton (α51Gly→Ser)

Hb Riccarton is the result of an α-chain mutation in which the α51 amino acid residue is changed from Gly to Ser through a single base change in the codon GGC→AGC.

αT6 Peptide

α	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56
	T	Y	P	F	H	F	D	L	S	H	G	S	A	Q	V	K
											↓					
											S					

Figure 5.3.9.1. Sequence of the Hb Riccarton αT6 tryptic peptide.

A blood sample was submitted for analysis by mass spectrometry because of a signal observed on the HPLC trace (Figure 5.3.9.2.) with an unresolved component apparent tailing the A_0 peak at 3.12 min.

Parameter	Value %	Time min.	Area
P00	1.3	0.57	15.4
F	1.1	0.78	13.5
P01	0.3	1.19	3.5
P02	6.6	1.89	79.9
P03	4	2.36	48.1
A0	82.4	3.12	995.8
P04	1.3	3.96	16.2
A2	0.2	4.31	2
P05	1.3	5.16	15.4
P06	0.7	6.14	8.4
P07	0.9	6.73	10.6

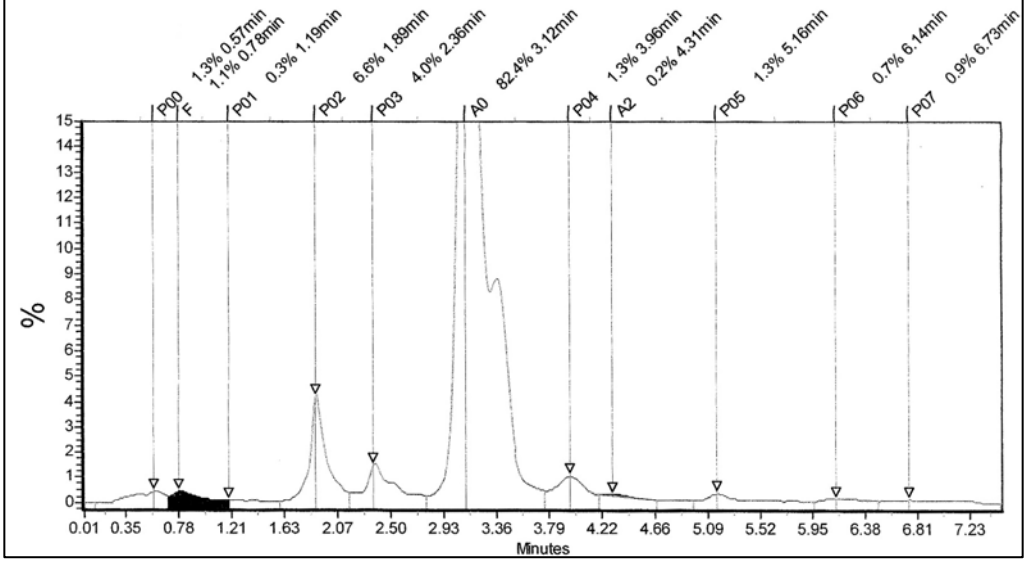

Figure 5.3.9.2. Tosoh HPLC trace for Hb Riccarton

The variant was silent on the ce-HPLC trace (Figure 5.3.9.3.) indicating no charge change as the result of the mutation.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.1	1.02	1082
F	0.2	---	1.12	4158
Unknown	---	0.6	1.26	12135
P2	---	4.6	1.34	89193
P3	---	3.6	1.73	70235
A0	---	88.3	2.58	1727574
A2	2.7	---	3.69	53122

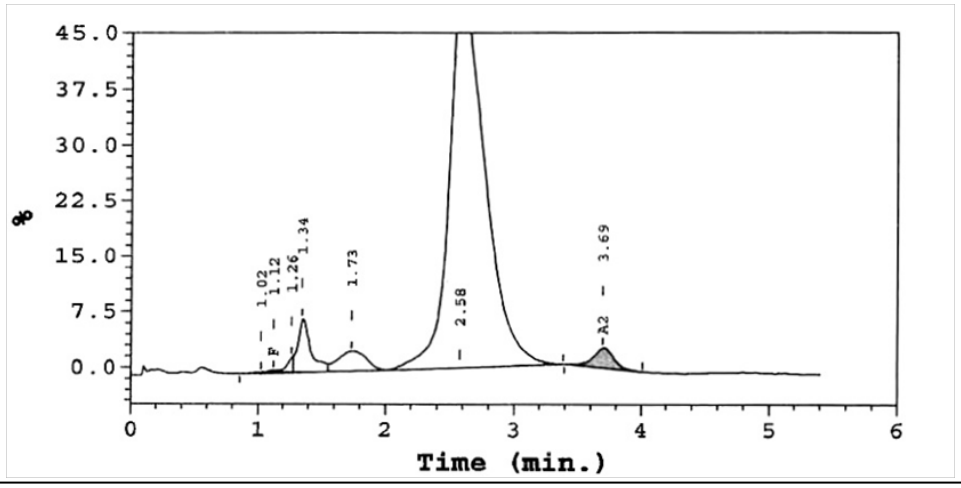

Figure 5.3.9.3. ce-HPLC trace for Hb Riccarton

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.3.9.4.) revealed an α-chain heterozygote in which the α-chain was 20.6% of total α-chains and the mass of the variant chain was 15,156.39, 30.01 Da heavier than normal. A single codon change giving a mass increase of +30 Da and no charge change infers Ala→Thr (21 possibilities), Gly→Ser (7 possibilities) or Thr→Met (9 possibilities).

Figure 5.3.9.5. shows the diagnostic part of the tryptic digest spectra from (a) normal Hb and (b) the variant Hb. The appearance of a peak at *m/z* 622.33 in the lower panel is indicative of a +30 Da mass change in the $\alpha T6^{3+}$ tryptic fragment. There are two possible sites of mutation in this peptide at α51Gly→Ser (Hb Riccarton) or α53Ala→Thr (not previously reported).

Figure 5.3.9.6. shows the partial product ion mass spectrum $\alpha T6^{3+}$ ion from (a) normal Hb and (b) the variant Hb. The mass change of the y''_6 fragment at *m/z* 619.5, and subsequent y'' fragments, identifies the mutation as α51Gly→Ser, Hb Riccarton.

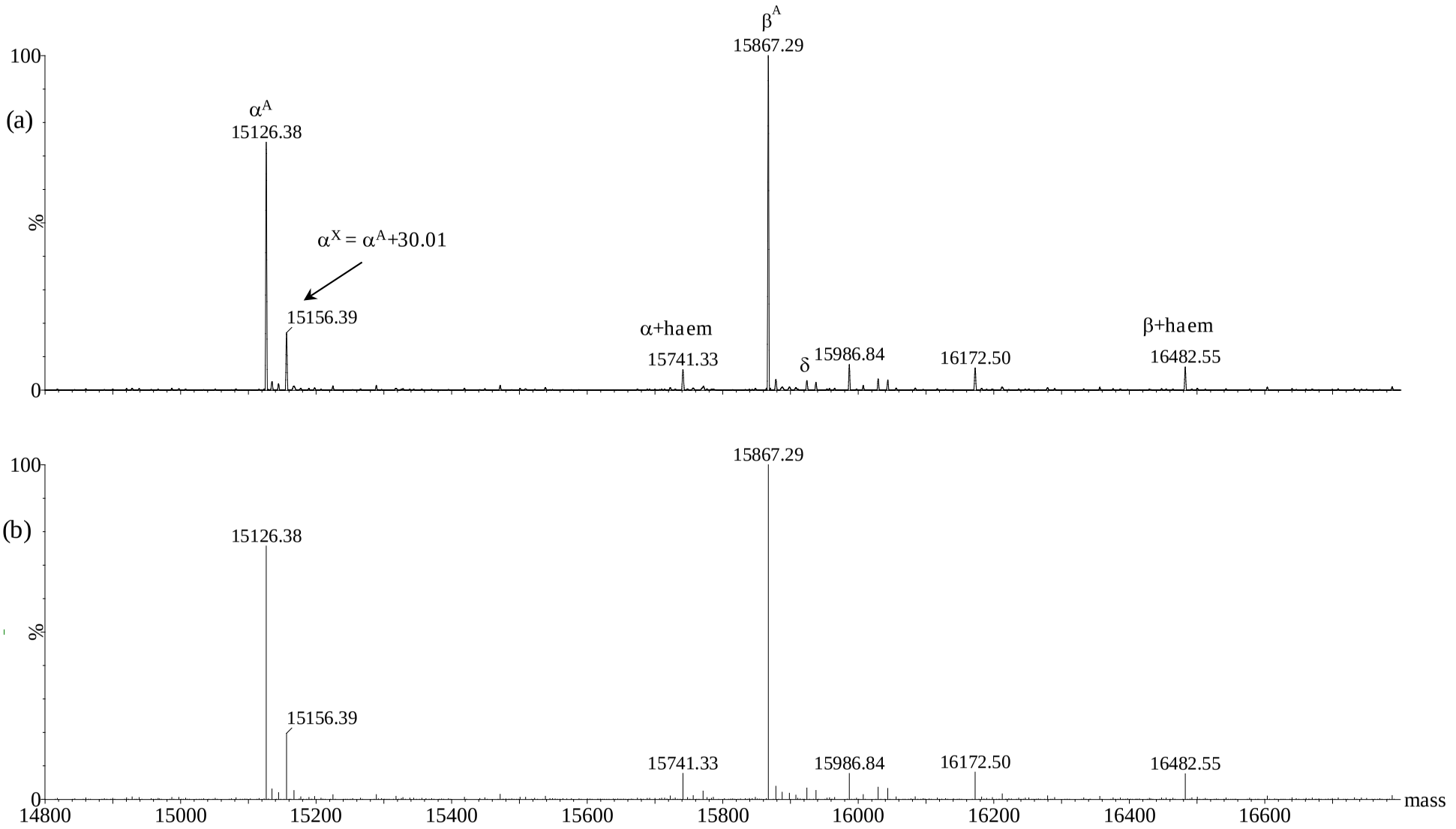

Figure 5.3.9.4. Deconvoluted mass spectrum of Hb Riccarton (α51Gly→Ser) showing the presence of a signal at 15,156.39 Da at approximately 20% of the total α-chains. For a 30 Da mass increase and no charge change; Ala→Thr, Gly→Ser or Thr→Met.

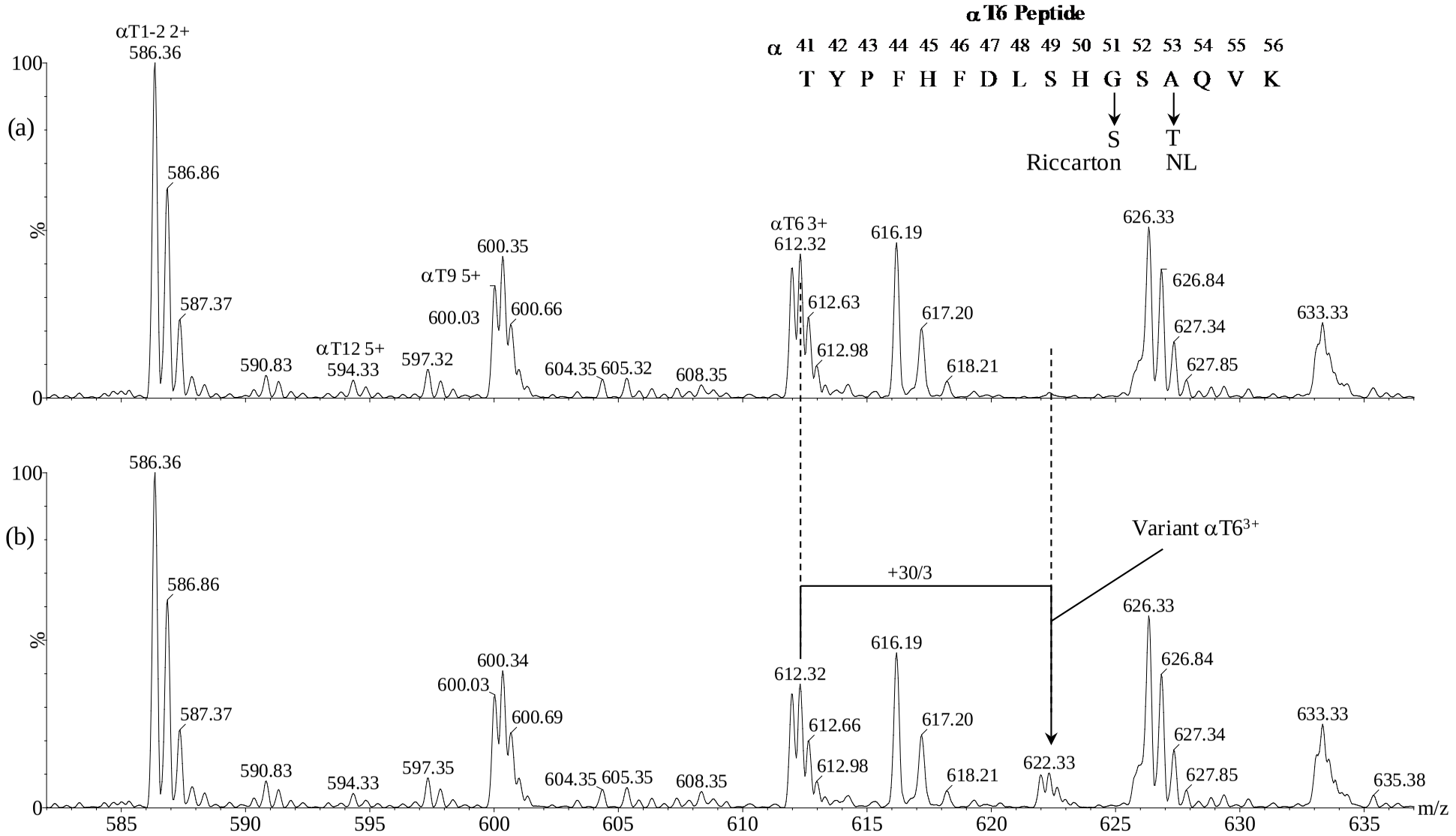

Figure 5.3.9.5. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Riccarton heterozygote. Mutation is identified in αT6 peptide as either α51Gly→Ser or α53Ala→Thr.

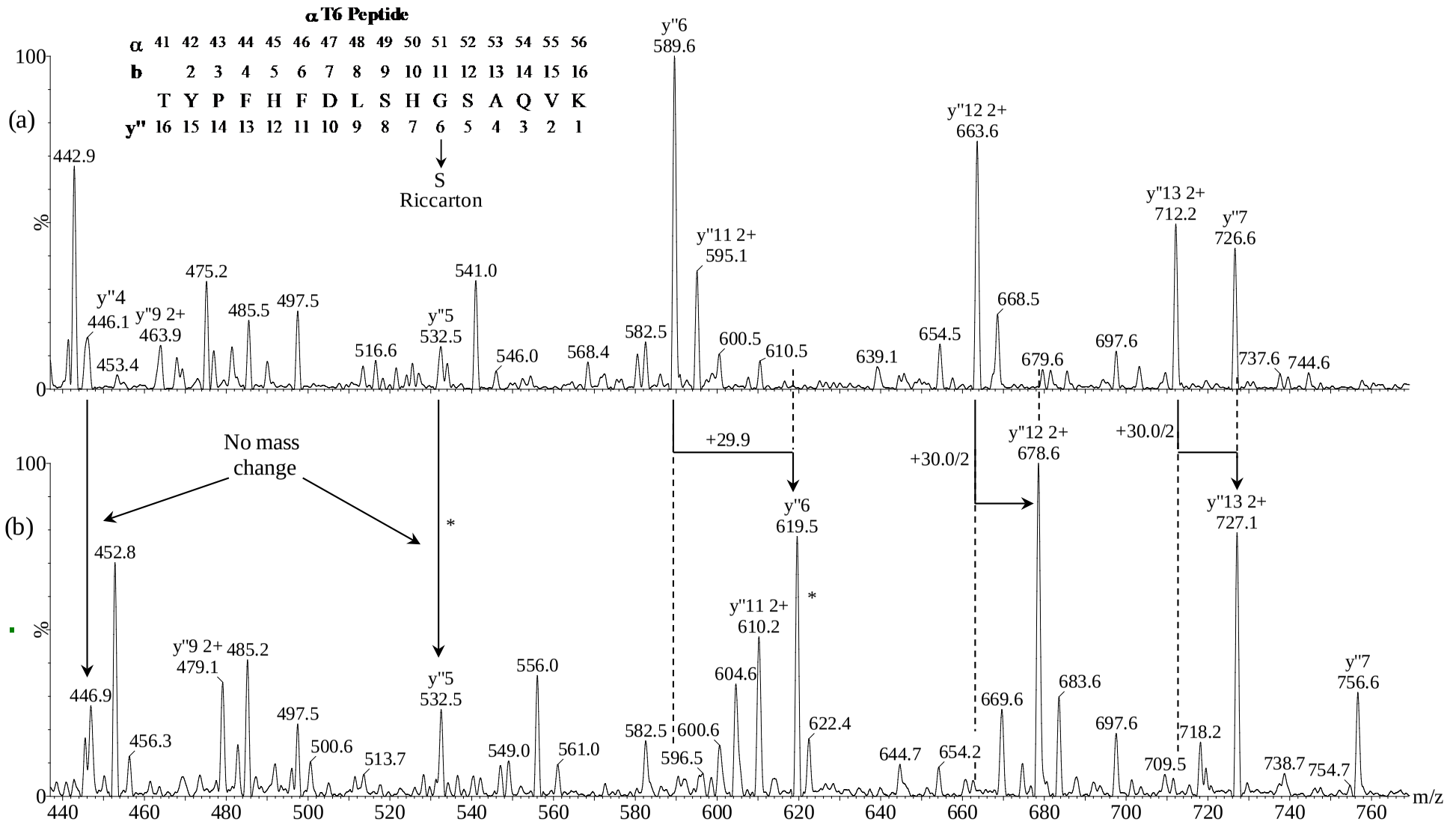

Figure 5.3.9.6. Partial product ion spectra of the $\alpha T6^{2+}$ tryptic fragment of (a) normal Hb and (b) Hb Riccarton. The 29.9 Da mass increase at $y"_6$ identifies the mutation as α51Gly→Ser.

5.3.10. αT6 - Hb Shimonoseki (α54Gln→Arg)

Hb Shimonoseki is the result of an α-chain mutation in which the α54 amino acid residue is changed from Gln to Arg through a single base change in the codon CAG→CGG.

The mutation to the Arg residue results in an additional tryptic cleavage product, as shown in Figure 5.3.10.1.

αT6 Peptide

α	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56
	T	Y	P	F	H	F	D	L	S	H	G	S	A	Q	V	K
							αT6a							↓		**αT6b**
	T	Y	P	F	H	F	D	L	S	H	G	S	A	R		V K

Figure 5.3.10.1. Sequence of the Hb Shimonoseki αT6 tryptic peptides.

A blood sample was submitted for analysis by mass spectrometry because the ce-HPLC trace (Figure 5.3.10.2.) showed an abnormally high response in the S-window (20.9%) at 4.39 min, indicating a positive charge change.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	0.2	---	1.09	2869
Unknown	---	0.8	1.24	12316
P2	---	2.7	1.38	42604
Unknown	---	0.4	1.57	6659
P3	---	2.4	1.74	37515
A0	---	69.4	2.50	1094911
A2	2.9	---	3.68	50329
S-window	---	20.9	4.39	329613

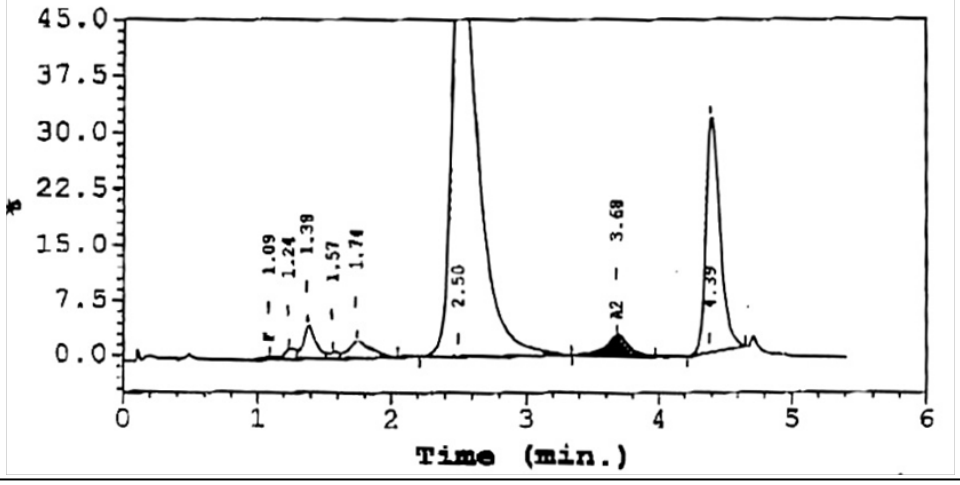

Figure 5.3.10.2. ce-HPLC trace for Hb Shimonoseki.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.3.10.3.) revealed an α-chain heterozygote in which the α-chain was 19.7% of total α-chains and the mass of the variant chain was 15,154.42 Da, 27.95 Da heavier than normal. A single codon change giving a mass increase of +28 Da could be Ala→Val (neutral), Gln→Arg (positive, 1 possibility, Hb Shimonoseki) or Lys→Arg (neutral).

In the human α-chain, there is only one Gln residue that could mutate by a single base change in the codon, resulting in an additional peptide cleavage with trypsin at α54Gln→Arg.

Figures 5.3.10.4. shows the diagnostic region of the tryptic digest spectrum for (a) normal Hb and (b) the variant Hb. The appearance of a peak at *m/z* 817.88 is consistent with the formation of a new αT6a^{2+} tryptic fragment from a mutation at α54. The new tryptic fragment is also observed in the lower panel of Figure 5.3.10.5. at *m/z* 545.48, confirming the mutation as α54Gln$\rightarrow$Arg, Hb Shimonoseki.

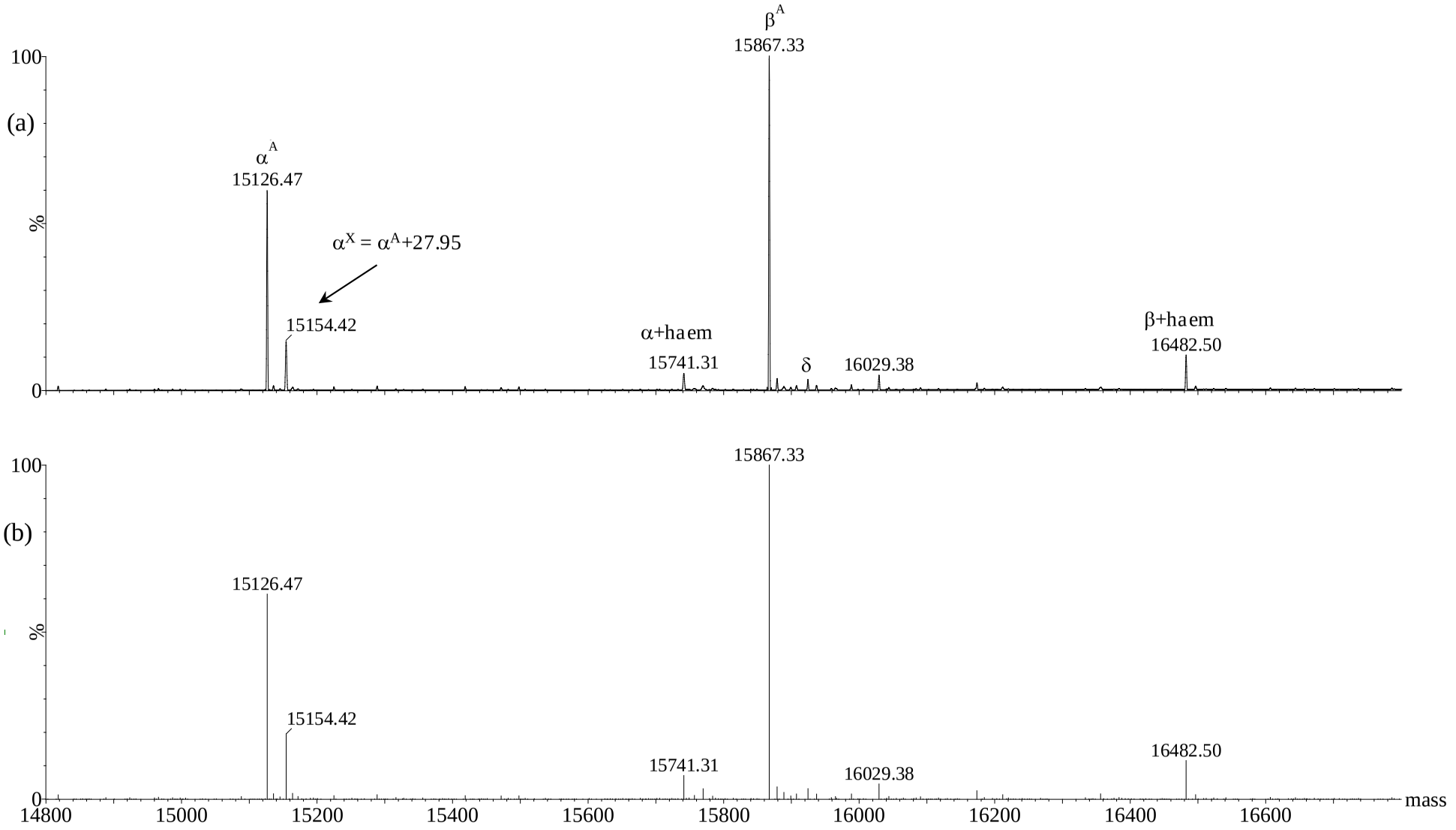

Figure 5.3.10.3. Deconvoluted mass spectrum of Hb Shimonoseki (α54Gln→Arg) showing the presence of a signal at 15,154.42 Da at 24.1% of the total α-chains. For a 28 Da mass increase, the possibilities are: Ala→Val (neutral), Gln→Arg (positive) and Lys→Arg (neutral).

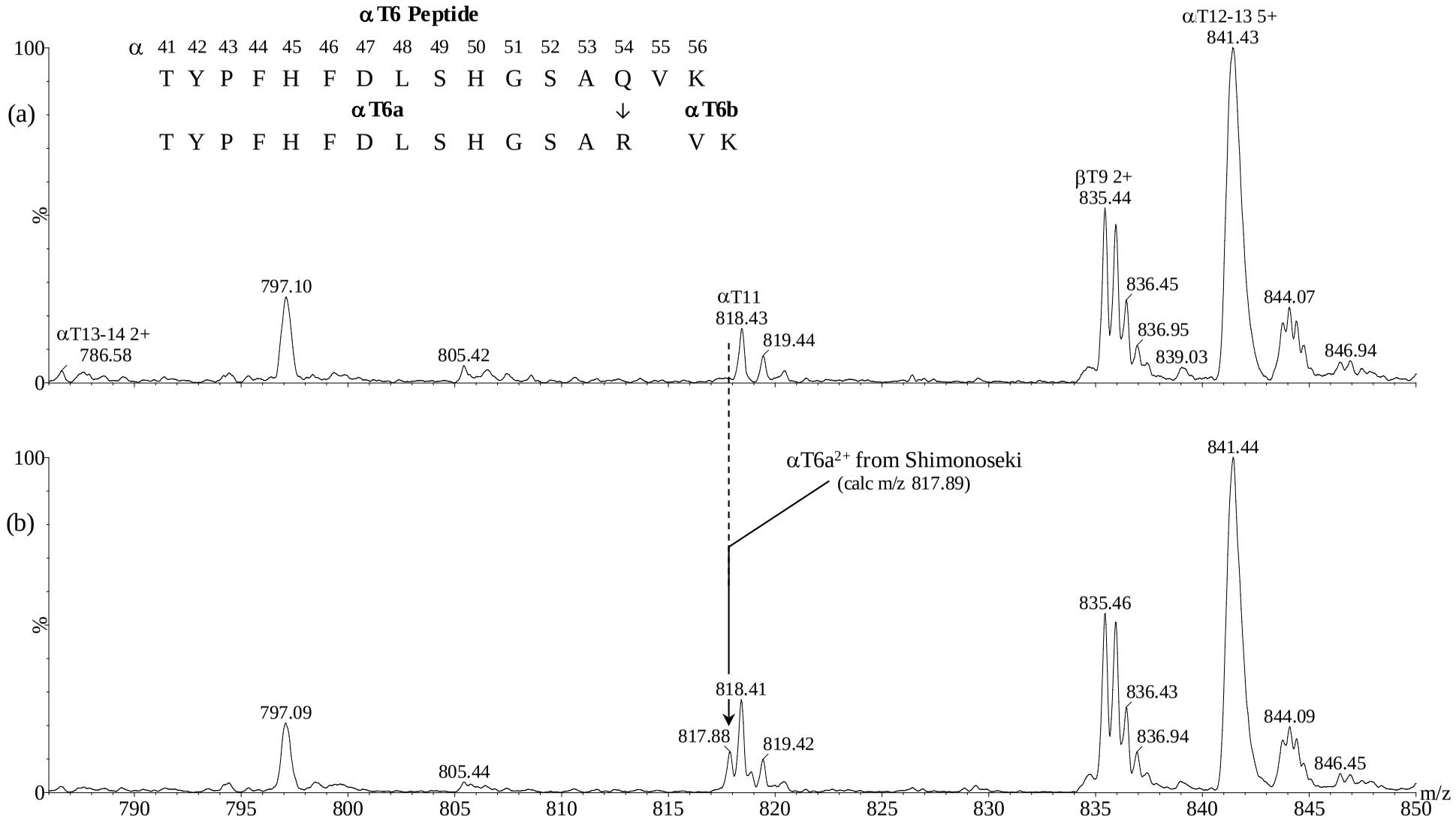

Figure 5.3.10.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Shimonoseki heterozygote. The appearance of the peak at *m/z* 817.88 is consistent with α54Gln→Arg, Hb Shimonoseki.

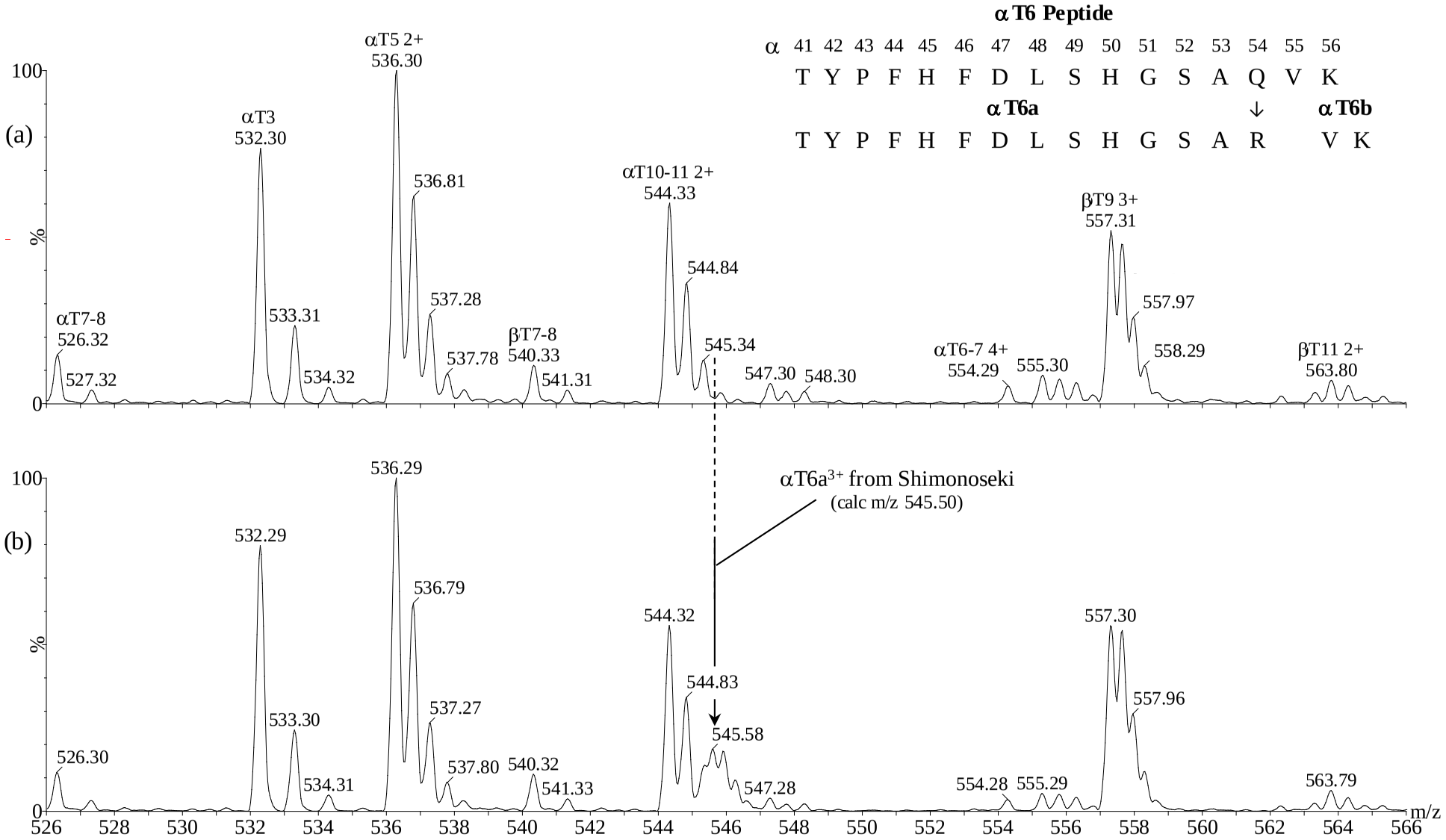

Figure 5.3.10.5. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Shimonoseki heterozygote. The appearance of the peak at *m/z* 545.58 is consistent with α54Gln→Arg, Hb Shimonoseki.

5.3.11. αT6 - Hb Shaare-Zedek (α56Lys→Glu)

Hb Shaare-Zedek is the result of an α-chain mutation in which the α56 amino acid residue is changed from Lys to Glu through a single base change in the codon AAG→GAG.

The mutation of the Lys residue results in the loss of a tryptic cleavage product, and the formation of a combined αT(6-7) tryptic peptide.

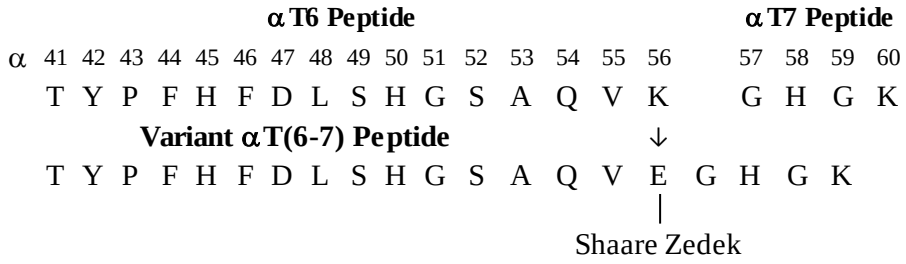

Figure 5.3.11.1. Sequence of the Hb Shaare-Zedek αT(6-7) tryptic peptide.

A blood sample was submitted for analysis by mass spectrometry because the ce-HPLC trace (Figure 5.3.11.2.) showed an abnormally high F response (22.7%), suggesting a negative charge change in the variant.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	22.7	---	1.09	561206
P2	---	3.2	1.32	82111
P3	---	6.0	1.71	151290
A0	---	66.3	2.40	1675782
A2	2.0	---	3.66	56273

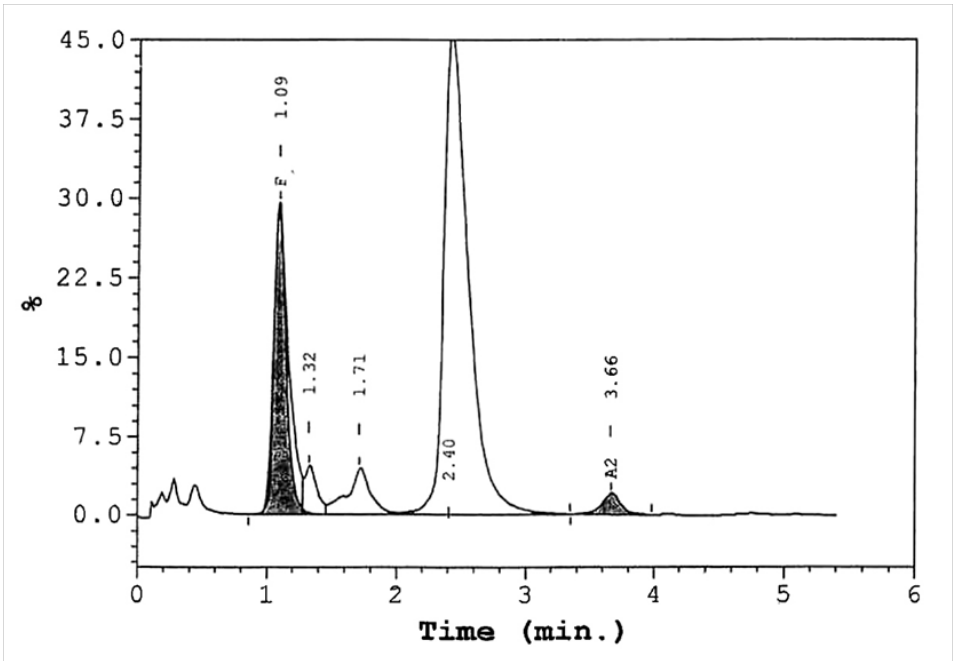

Figure 5.3.11.2. ce-HPLC trace for Hb Shaare-Zedek

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.3.11.3.) revealed a β-chain with a mass lower than expected (15,867.01 Da), following calibration of the mass scale from the α-chain. According to Table 2.1 (Section 2.4.4.), a negative change in the apparent mass of the β-chain coupled with a

negative charge change inferred from the ce-HPLC Data, assigns the variant as (α-chain + 1 Da) heterozygote.

Mutation from a single codon change offers four possibilities for a single amino acid change giving rise to a 1 Da increase in mass: Asn→Asp (4 possibilities), Gln→Glu (1 possibility) or Lys→Glu (11 possibilities). Coupled with the negative charge change observed on the ce-HPLC trace, the most likely change is Lys→Glu which would all lead to the loss of a tryptic fragment on digestion when compared to normal Hb.

Figure 5.3.11.4. shows the diagnostic part of the tryptic digest spectrum for (a) normal Hb and (b) the variant Hb. The appearance of a signal at *m/z* 738.67 in the lower panel is consistent with the formation of an $\alpha T(6\text{-}7)^{3+}$ tryptic fragment, and implies the loss of a tryptic cleavage site at α56 Lys→Glu. This is further supported by the data in the lower panel of Figure 5.3.11.5. with the appearance of a signal at *m/z* 1107.57 in the lower panel attributable to the $\alpha T(6\text{-}7)^{2+}$ tryptic fragment.

Figure 5.3.11.6. shows the partial product ion spectrum of the $\alpha T(6\text{-}7)^{2+}$ tryptic fragment of the variant Hb, and the data are consistent with the proposed sequence of the αT(6-7) tryptic fragment of α56Lys→Glu, Hb Shaare-Zedek.

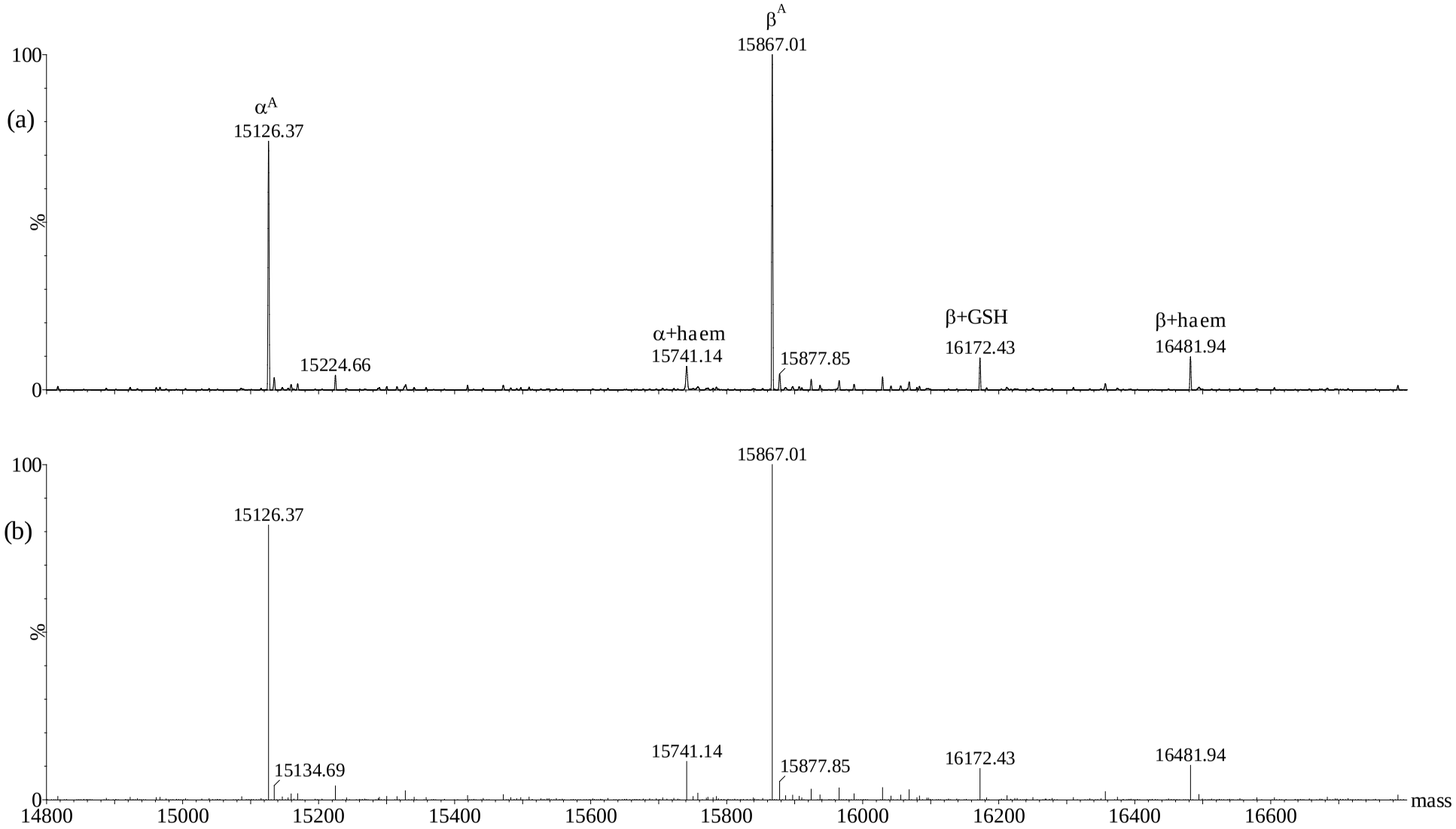

Figure 5.3.11.3. Deconvoluted mass spectrum of Hb Shaare-Zedek (α56Lys→Glu) showing the presence of a single signal at 15,126.37 Da. A possible (α-chain + 1 Da) heterozygote, with a polarity change from normal, is inferred from ce-HPLC Data.

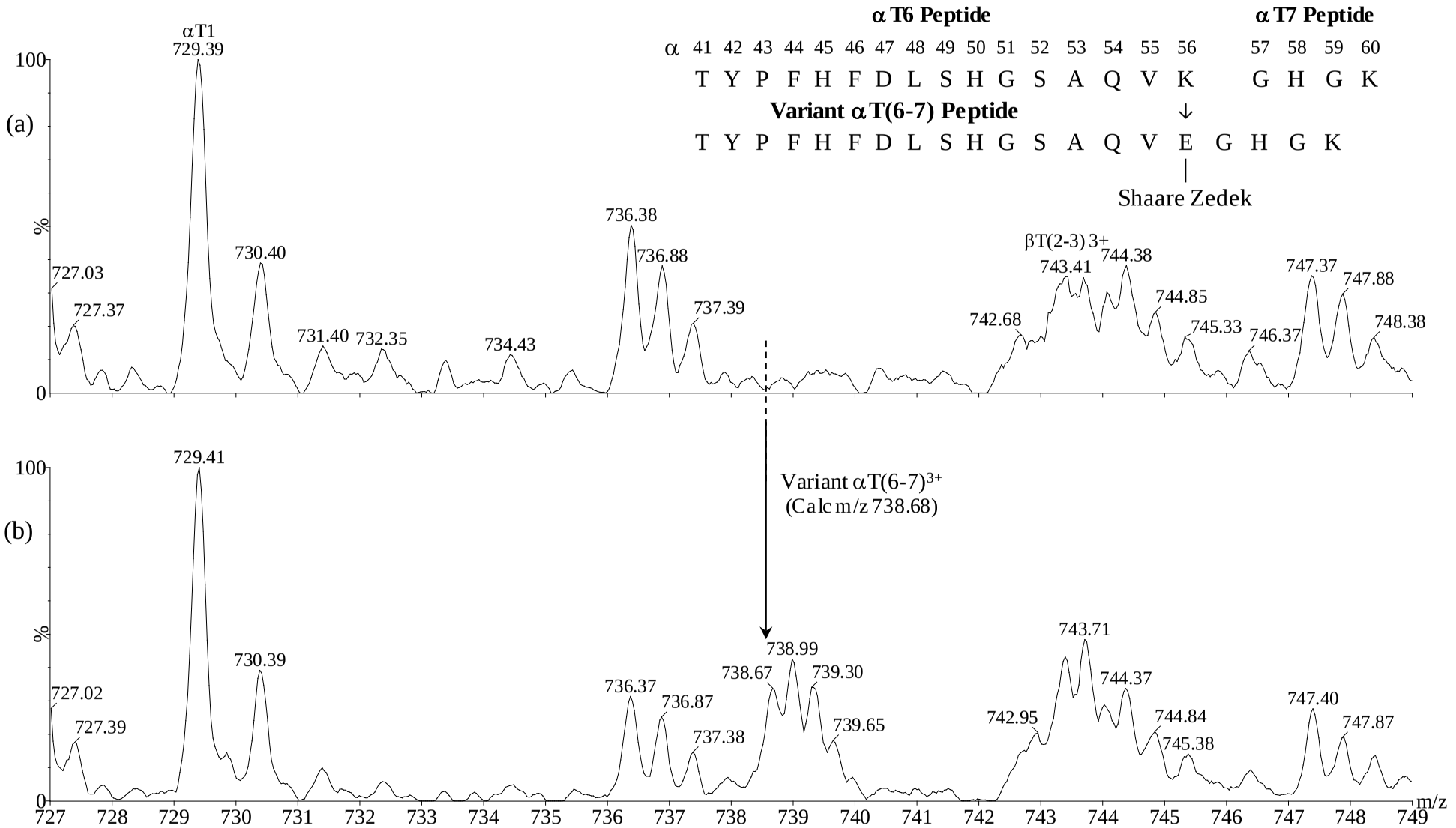

Figure 5.3.11.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Shaare-Zedek heterozygote. The presence of the $\alpha T(6\text{-}7)^{3+}$ ion at *m/z* 738.67 identifies the mutation as α56Lys→Glu, Hb Shaare-Zedek.

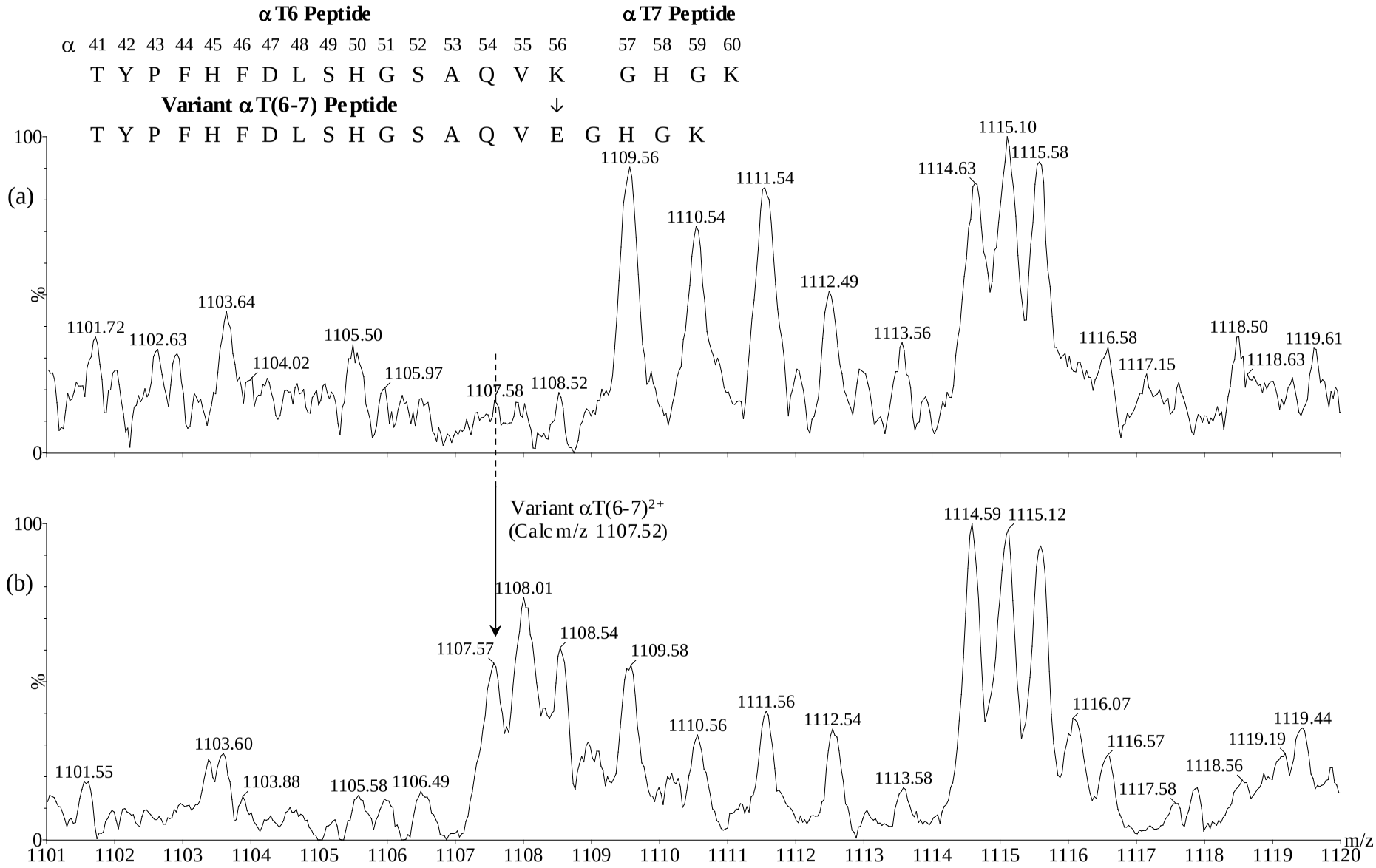

Figure 5.3.11.5. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Shaare-Zedek heterozygote. The presence of the $\alpha T(6\text{-}7)^{2+}$ ion at *m/z* 1107.57 identifies the mutation as α56Lys→Glu, Hb Shaare-Zedek.

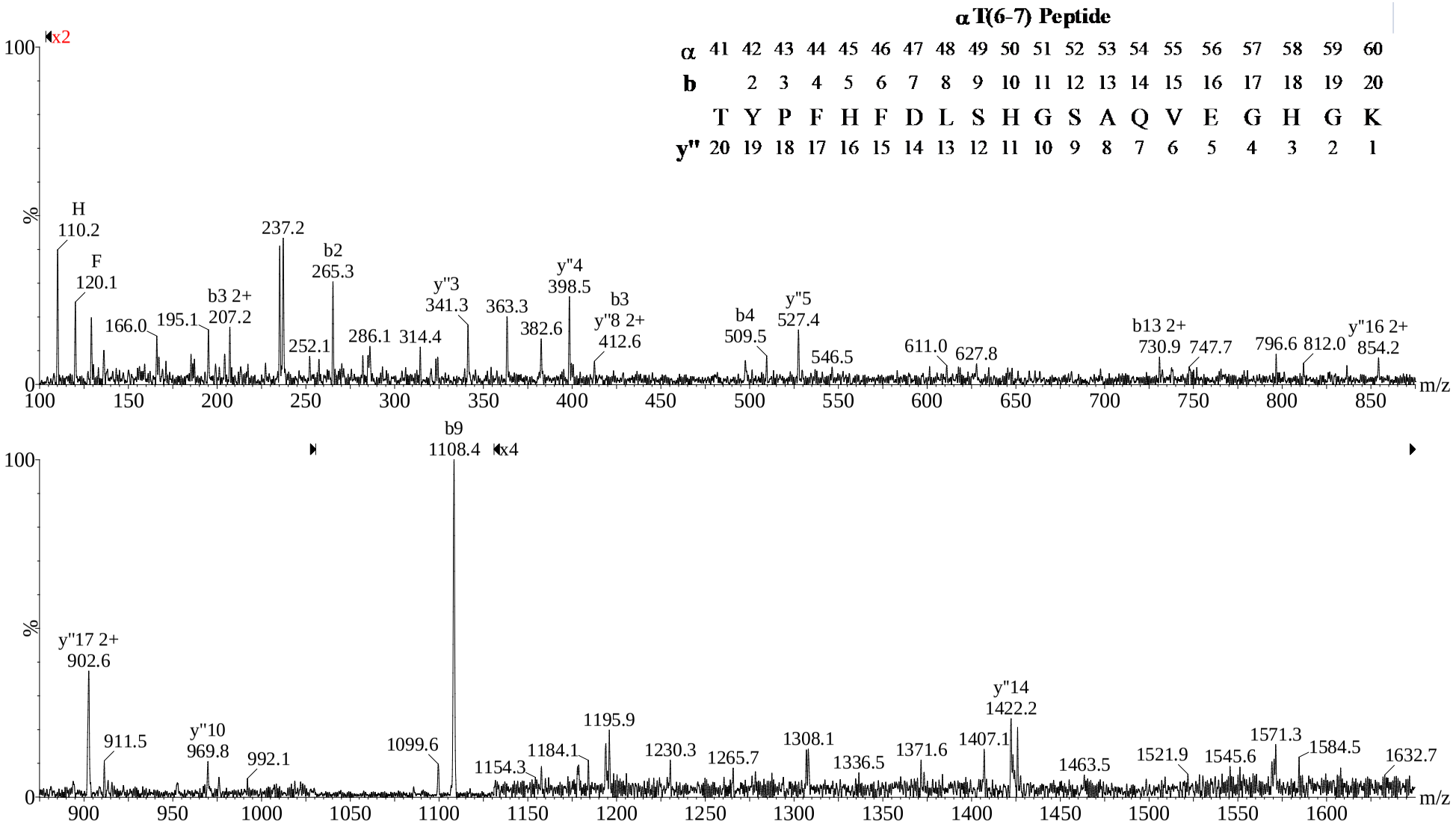

Figure 5.3.11.6. Partial product ion spectrum of the $\alpha T(6\text{-}7)^{2+}$ tryptic fragment of Hb Shaare-Zedek (α56Lys→Glu). The fragmentation observed is consistent with the sequence of the $\alpha T(6\text{-}7)$ peptide.

5.3.12. αT7 - Hb L-Persian Gulf (α57Gly→Arg)

Hb L-Persian Gulf is the result of an α-chain mutation in which the α57 amino acid residue is changed from Gly to Arg through a single base change in the codon GGC→CGC.

The mutation to the Arg residue results in an additional tryptic cleavage product, as shown in Figure 5.3.12.1.

αT7 Peptide

α	57	58	59	60
	G	H	G	K
	↓		**αT7b**	
	R		H	G	K

Figure 5.3.12.1. Sequence of the Hb L-Persian Gulf αT7 tryptic peptides.

A blood sample was submitted for analysis by mass spectrometry because the ce-HPLC trace (Figure 5.3.12.2.) showed an abnormally high response in the S-window (18.6%) at 4.50 min, indicating a positive charge change as a result of the mutation.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.0	0.96	833
F	0.3	---	1.05	4964
Unknown	---	0.8	1.25	14304
P2	---	4.1	1.33	76409
P3	---	5.4	1.69	100805
A0	---	66.9	2.49	1258952
A2	3.2	---	3.62	65793
S-window	---	18.6	4.50	349925
Unknown	---	0.5	4.72	9844

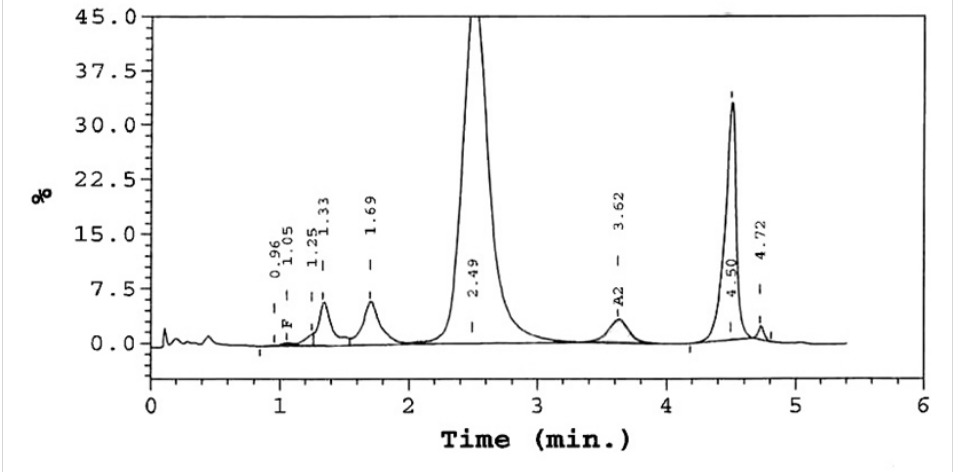

Figure 5.3.12.2. ce-HPLC trace for Hb L-Persian Gulf.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.3.12.3.) revealed an α-chain heterozygote with a variant observed at 15,225.47 Da, 99.11 Da heavier than normal and 21.5% of total α-chains. A single codon change giving a mass increase of +99 Da could be Gly→Arg (positive) or Ser→Trp (neutral). The positive shift in the ce-HPLC trace indicates a positive charge change and infers the Gly→Arg mutation (7 possibilities).

Figure 5.3.12.4. shows the diagnostic part of the tryptic digest spectra from (a) normal Hb and (b) the variant Hb. The appearance of a peak at *m/z* 341.21 is consistent with the mutation α57Gly→Arg, and thus the formation of a new αT7b peptide, HGK.

Figure 5.3.12.5 shows a comparison of the product ion spectra from *m/z* 341.4 from (a) normal Hb and (b) the variant Hb. The data in the lower panel are consistent with the expected sequence of the new αT7b tryptic peptide as HGK, and the purported mutation α57Gly→Arg, Hb L-Persian Gulf. However, the variant is **not** positively identified.

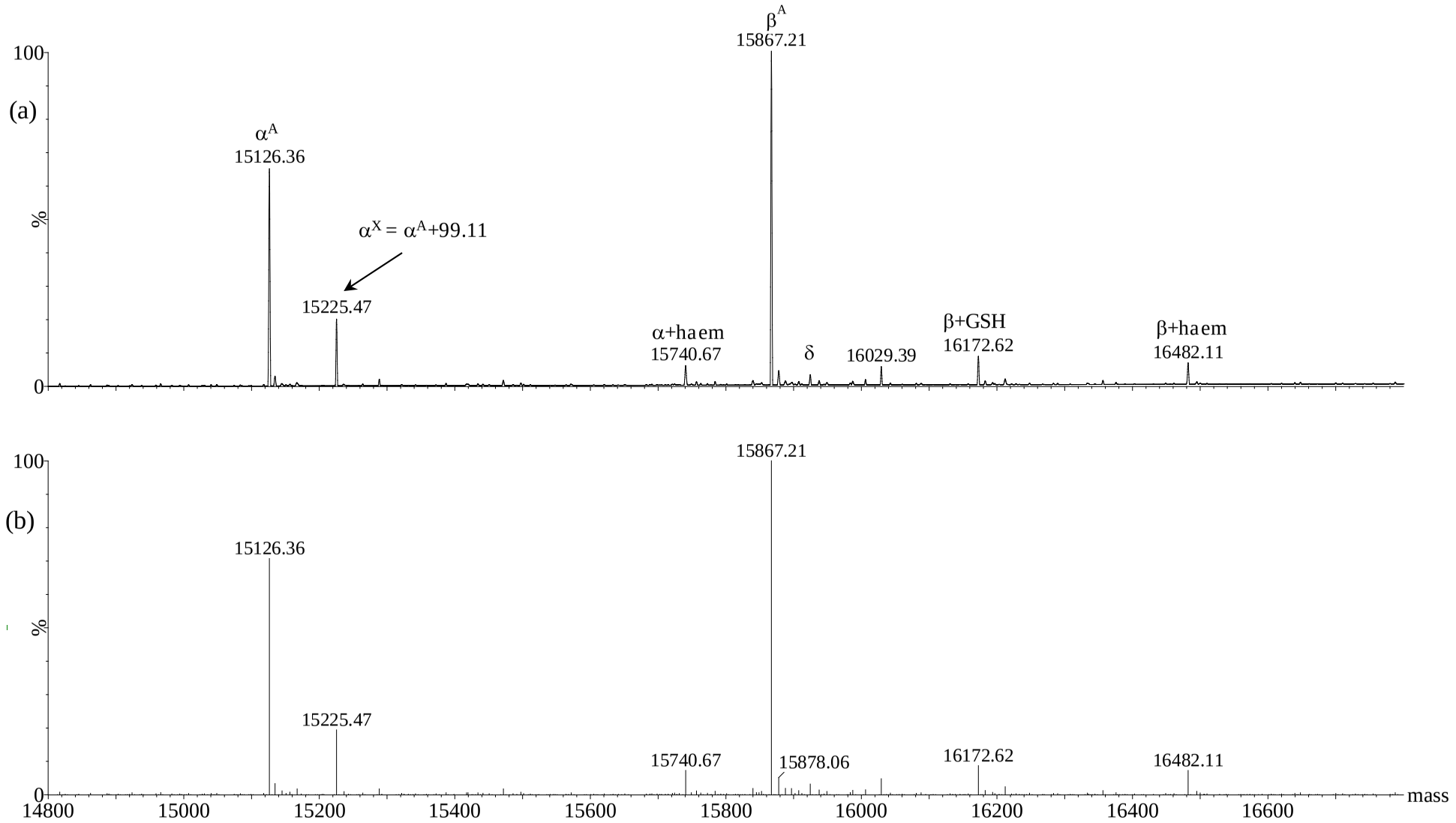

Figure 5.3.12.3. Deconvoluted mass spectrum of Hb L-Persian Gulf (α57Gly→Arg) showing the presence of a signal at 15,225.47 Da at approximately 21.5% total α-chains. For a mass increase of 99.11 Da, coupled with a positive charge change, Gly→Arg only.

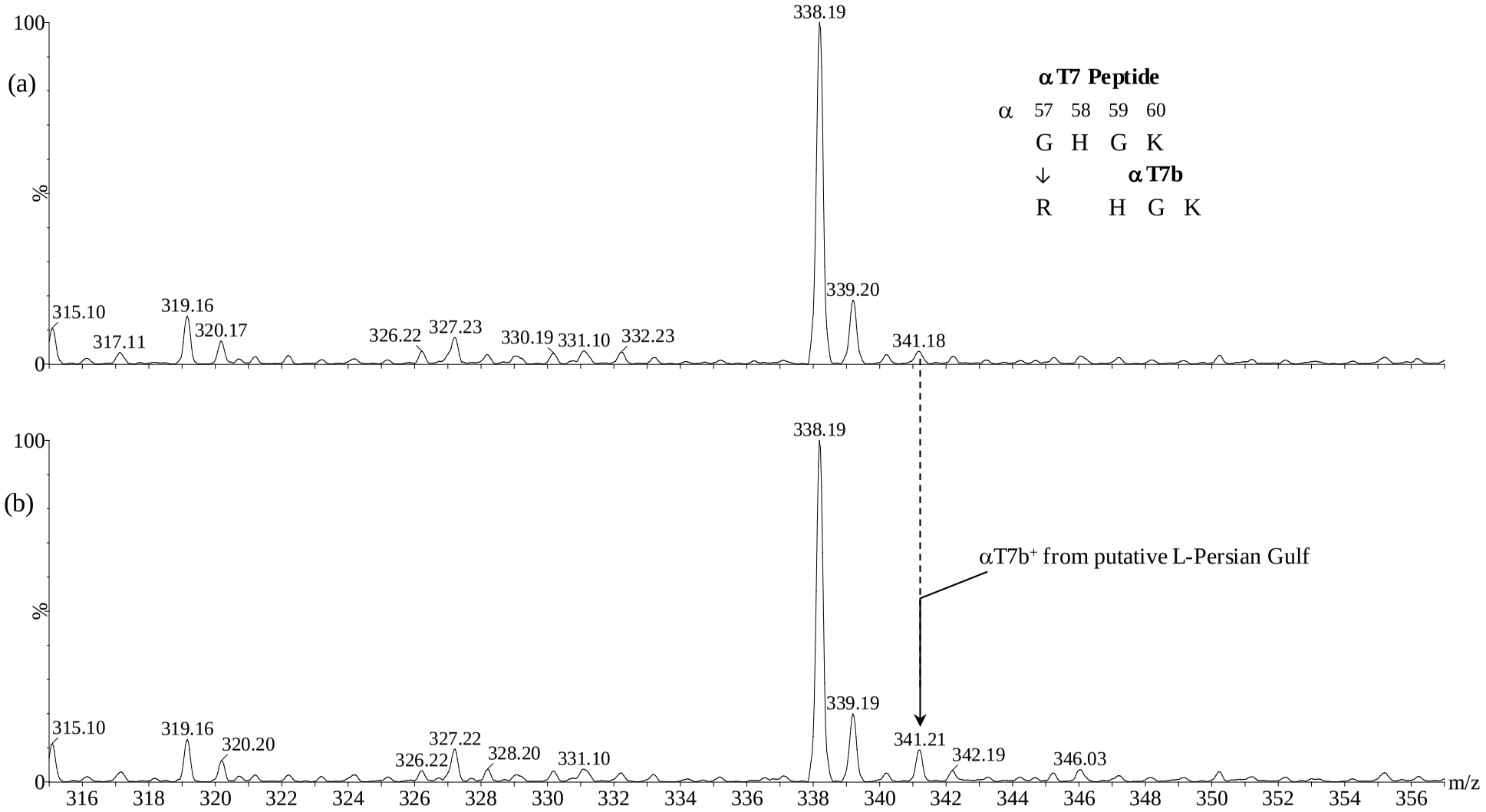

Figure 5.3.12.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) the variant Hb L-Persian Gulf heterozygote.

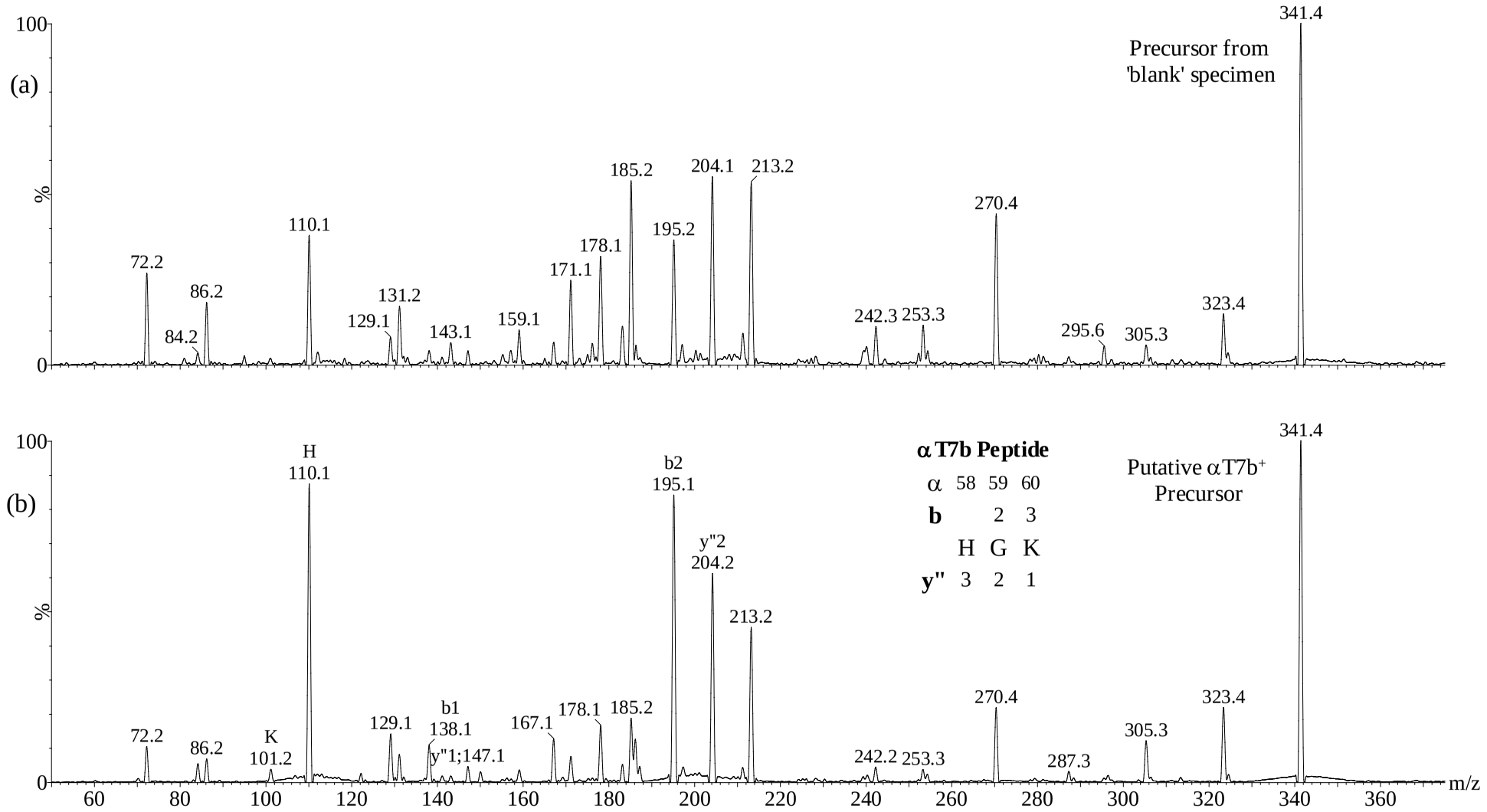

Figure 5.3.12.5. Product ion spectra of the putative αT7b$^+$ tryptic fragment of (a) normal Hb and (b) Hb L-Persian Gulf. The data are consistent with α57Gly→Arg, but **not** positively identified.

5.3.13. αT9 - Hb Q-India (α64Asp→His)

Hb Q-India is the result of an α-chain mutation in which the α64 amino acid residue is changed from Asp to His through a single base change in the codon GAC→CAC.

αT9 Peptide

α	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
	V	A	D	A	L	T	N	A	V	A	H	V	D	D	M	P	N	A	L	S	A	L	S	D	L	H	A	H	K
			↓																										
			H																										

Figure 5.3.13.1. Sequence of the Hb Q-India αT9 tryptic peptide.

A blood sample was submitted for analysis by mass spectrometry because the ce-HPLC trace (Figure 5.3.13.2.) showed an abnormally high unknown response (18.0%) at 4.80 min. The high unknown response indicates that the variant causes a significant positive charge change.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	0.3	---	1.10	7286
Unknown	---	0.6	1.23	14078
P2	---	3.8	1.31	96216
P3	---	3.1	1.70	78104
A0	---	71.1	2.41	1804038
A2	2.8	---	3.63	73649
S-window	---	0.3	4.46	8331
Unknown	---	18.0	4.80	456344

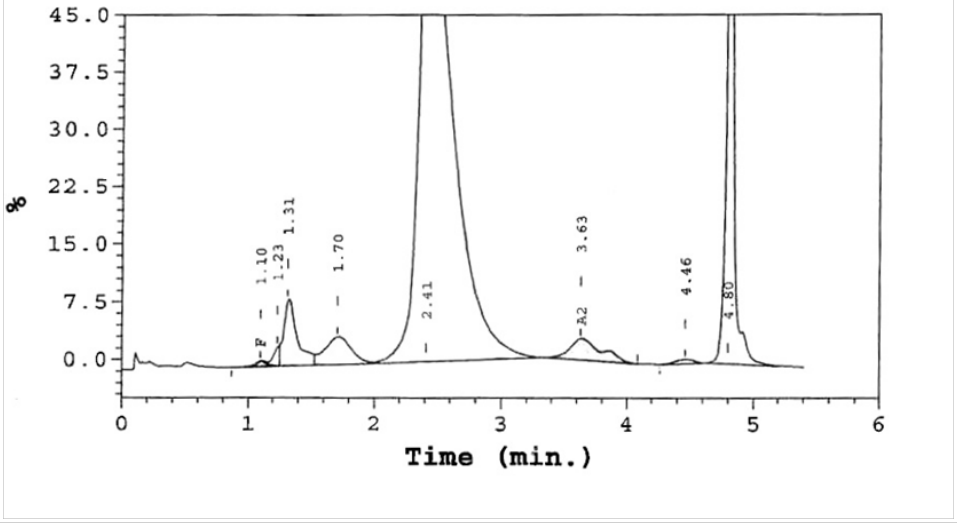

Figure 5.3.13.2. ce-HPLC trace for Hb Q-India.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.3.13.3.) revealed an α-chain heterozygote at 15,148.14 Da, 21.75 Da heavier than normal with the variant α-chain being 20.2% of total α-chains. A single codon change giving a mass increase of +22 Da could only be Asp→His (8 possibilities) and is consistent with the positive charge change observed in the ce-HPLC trace.

Figure 5.3.13.4. shows the diagnostic part of the tryptic digest spectra from (a) normal Hb and (b) the variant Hb. The appearance of a peak at *m/z* 755.67 in the variant spectrum is consistent with the Asp→His mutation in the $\alpha T9^{4+}$ tryptic fragment. There are four possible sites of mutation in this peptide from a single base change in the codon; α64Asp→His (Hb Q-India), α74Asp→His (Hb Q-Thailand), α75Asp→His (Hb Q-Iran) or α85Asp→His (Hb Canuts).

Figure 5.3.13.5. shows product ion spectra from the $\alpha T9^{4+}$ tryptic fragment of (a) normal Hb and (b) the variant Hb. No mass change in the b_2 fragment at *m/z* 171.1, and the +22 Da mass change in the b_3 fragment at *m/z* 308.2 in the lower panel identifies the mutation as α64Asp$\rightarrow$His, Hb Q-India.

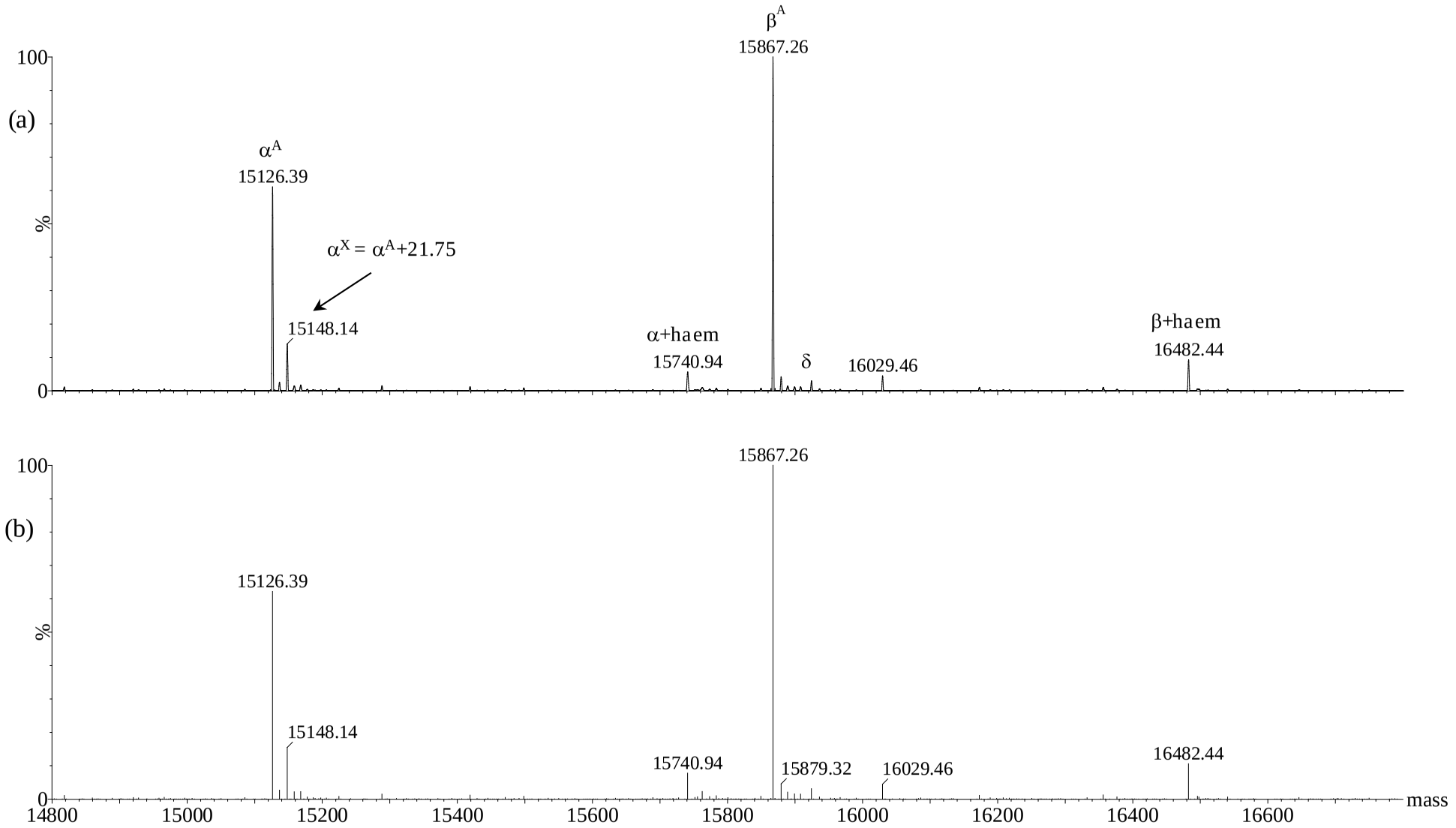

Figure 5.3.13.3. Deconvoluted mass spectrum of Hb Q-India (α64Asp→His) showing the presence of a signal at 15,148.14 Da at approximately 20% total α-chains. A mass increase of 22 Da can only be achieved with the mutation Asp→His.

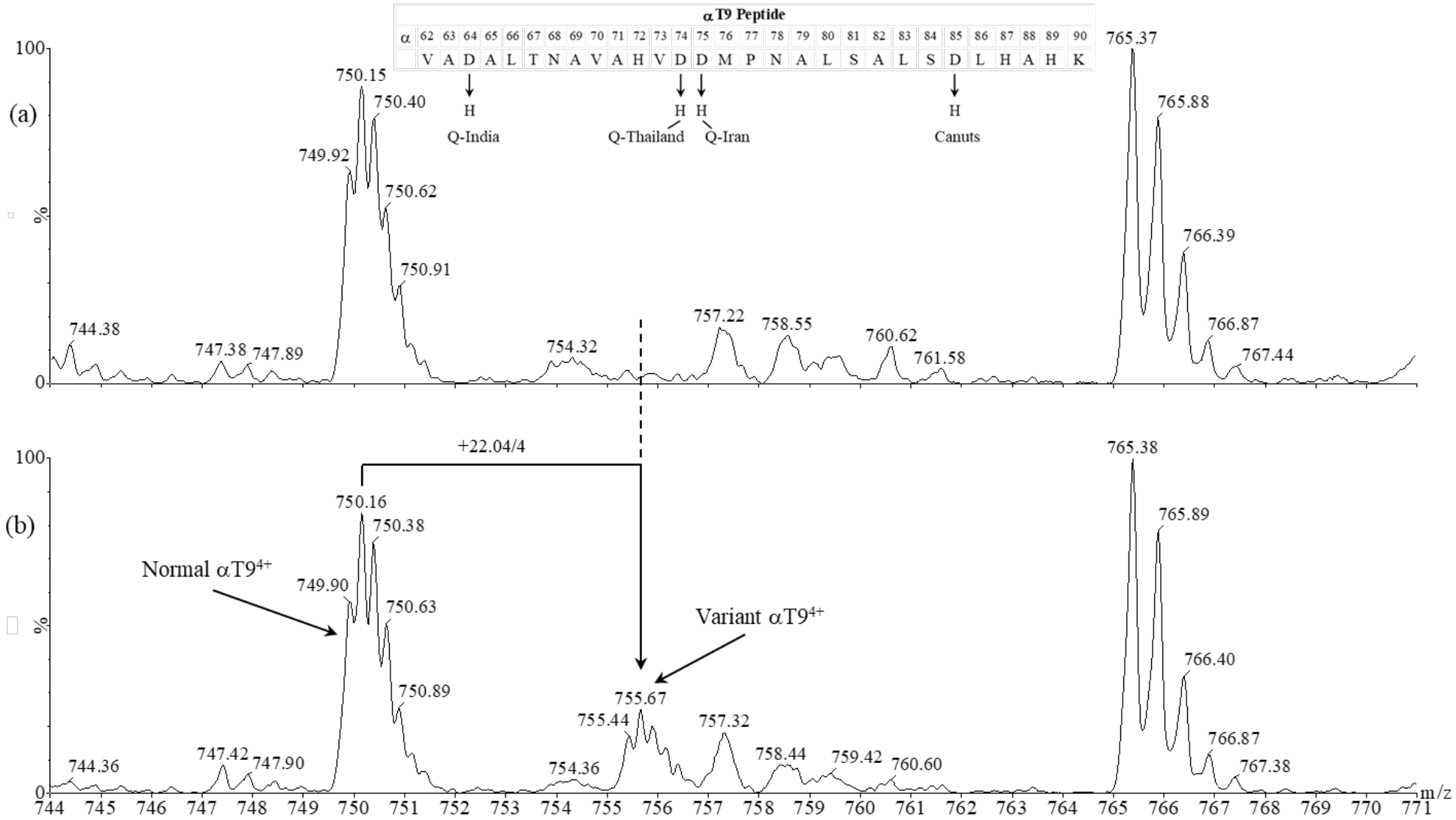

Figure 5.3.13.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Q-India heterozygote.

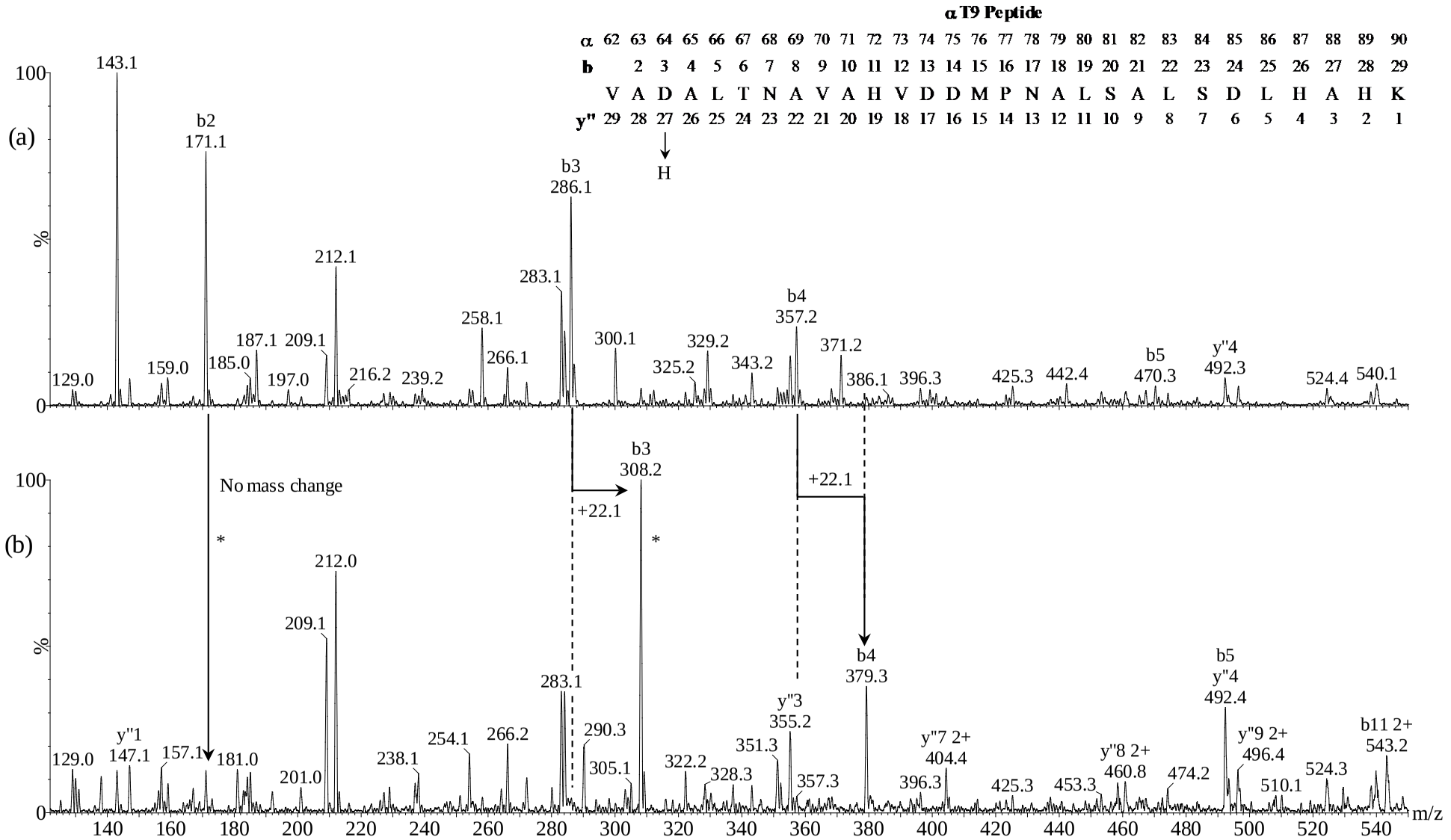

Figure 5.3.13.3. Product ion spectra of the $\alpha T9^{4+}$ tryptic fragment of (a) normal Hb and (b) Hb Q-India. The 22 Da mass increase at b_3 identifies the mutation as α64Asp→His.

5.3.14. αT9 - Hb G-Philadelphia (α68Asn→Lys)

Hb G-Philadelphia is the result of an α-chain mutation in which the α68 amino acid residue is changed from Asn to Lys through a single base change in the codon AAC→AAG or AAA.

The mutation to the Lys residue results in an additional tryptic cleavage product, as shown in Figure 5.3.14.1.

αT9 Peptide

α	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
	V	A	D	A	L	T	N	A	V	A	H	V	D	D	M	P	N	A	L	S	A	L	S	D	L	H	A	H	K

αT9a Peptide ↓ **αT9b Peptide**

V A D A L T K A V A H V D D M P N A L S A L S D L H A H K

Figure 5.3.14.1. Sequence of the Hb G-Philadelphia αT9 tryptic peptides.

A blood sample was submitted for analysis by mass spectrometry because the ce-HPLC trace (Figure 5.3.14.2.) showed an abnormally high response in the D-window (31.2%) at 4.11 min, indicating a positive charge change.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.0	0.99	701
F	0.1	---	1.08	2858
Unknown	---	0.5	1.20	10194
P2	---	3.1	1.27	68884
P3	---	2.6	1.63	58277
Unknown	---	1.8	2.09	40009
A0	---	58.7	2.42	1312692
A2	1.2	---	3.54	27177
D-window	---	31.2	4.11	696873
S-window	---	0.8	4.57	18442

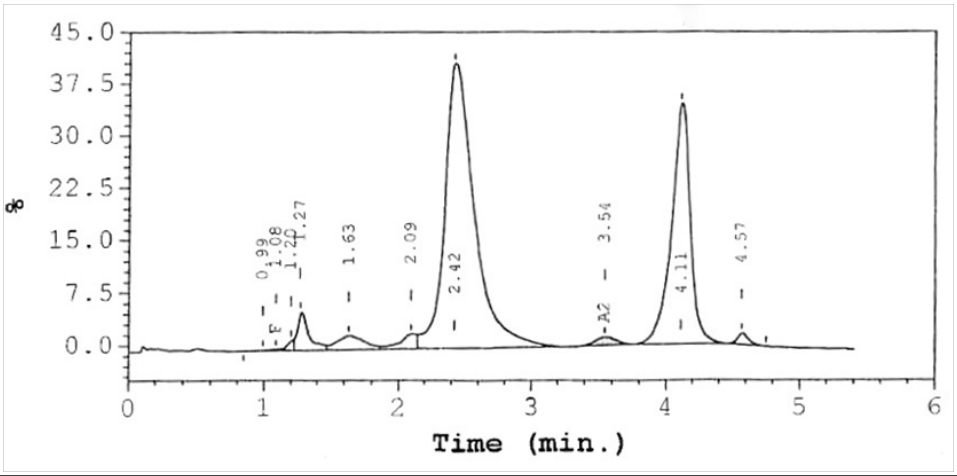

Figure 5.3.14.2. ce-HPLC trace for Hb G-Philadelphia.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.3.14.3.) revealed an α-chain heterozygote at 15,140.37 Da in which the α-chain was approximately 36% of total α-chains and the mass of the variant chain was 13.91 Da heavier than normal. A single codon change giving a mass increase of +14 Da could be Asn→Lys (4 possibilities), Asp→Glu (8 possibilities), Gly→Ala (7 possibilities), Ser→Thr (11 possibilities), Val→Ile (13 possibilities) or Val→Leu (13 possibilities).

However, the shift in the ce-HPLC trace shows a positive charge change and indicates the Asn→Lys mutation is most likely. Mutation to Lys will likely result in an additional fragment on tryptic digestion.

Figure 5.3.14.4. shows the diagnostic part of the tryptic digest spectra from (a) normal Hb and (b) the variant Hb. The appearance of peaks at *m/z* 717.49 (αT9a^{+}) and *m/z* 771.74 (αT9b^{3+}) are consistent with the formation of two new tryptic peptides from the αT9 peptide with the mutation α68Asn→Lys, and the formation of an additional tryptic cleavage site at α68. Further evidence is shown in the lower panel of Figure 5.3.14.5. for the tryptic digest of the variant Hb and the appearance of the αT9b^{2+} signal at *m/z* 1,157.08. These data confirm the mutation as α68Asn→Lys, Hb G-Philadelphia.

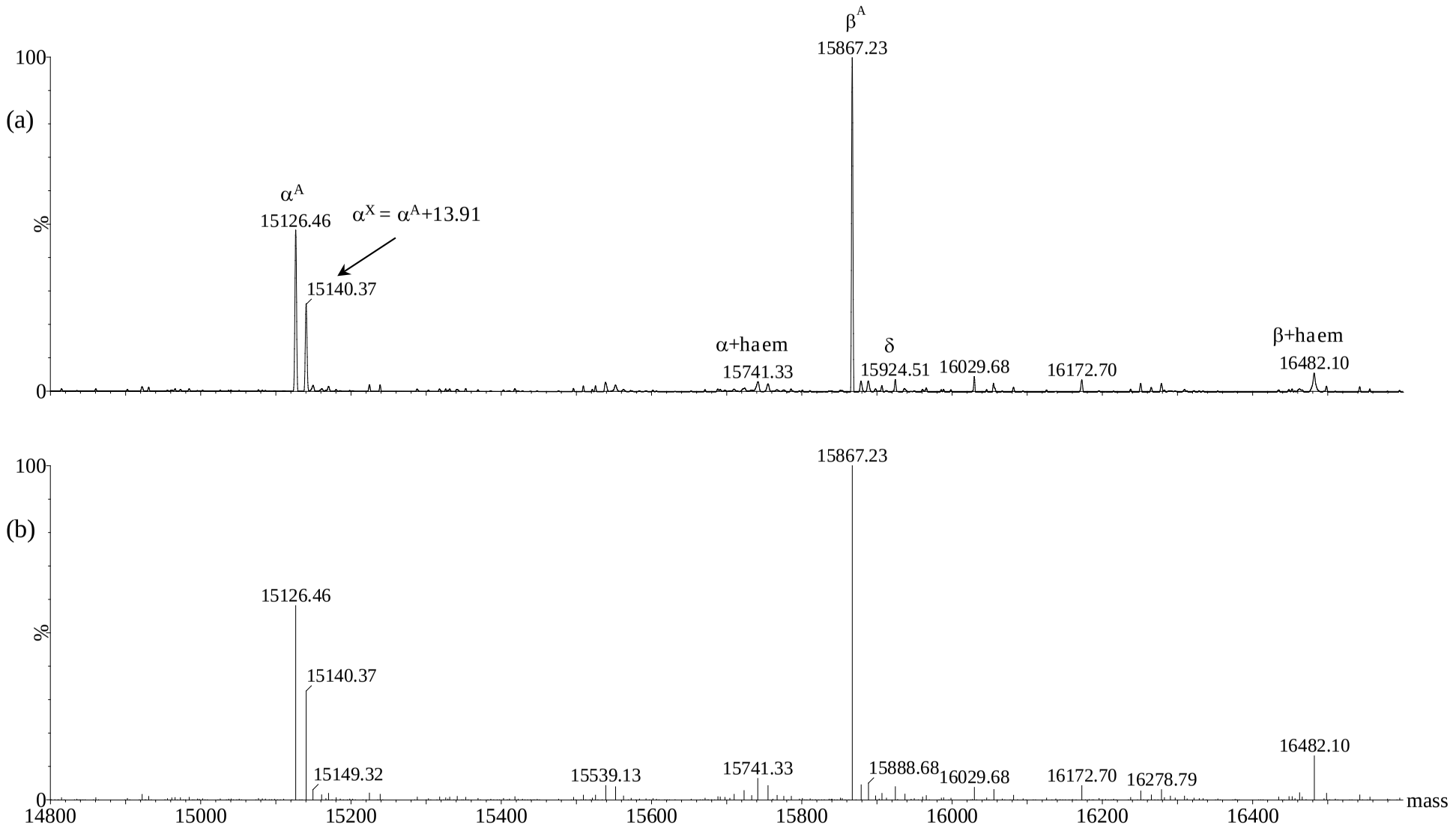

Figure 5.3.14.3. Deconvoluted mass spectrum of Hb G-Philadelphia (α68Asn→Lys) showing the presence of a signal at 15,140.37 Da at approximately 36% of the total α-chains.

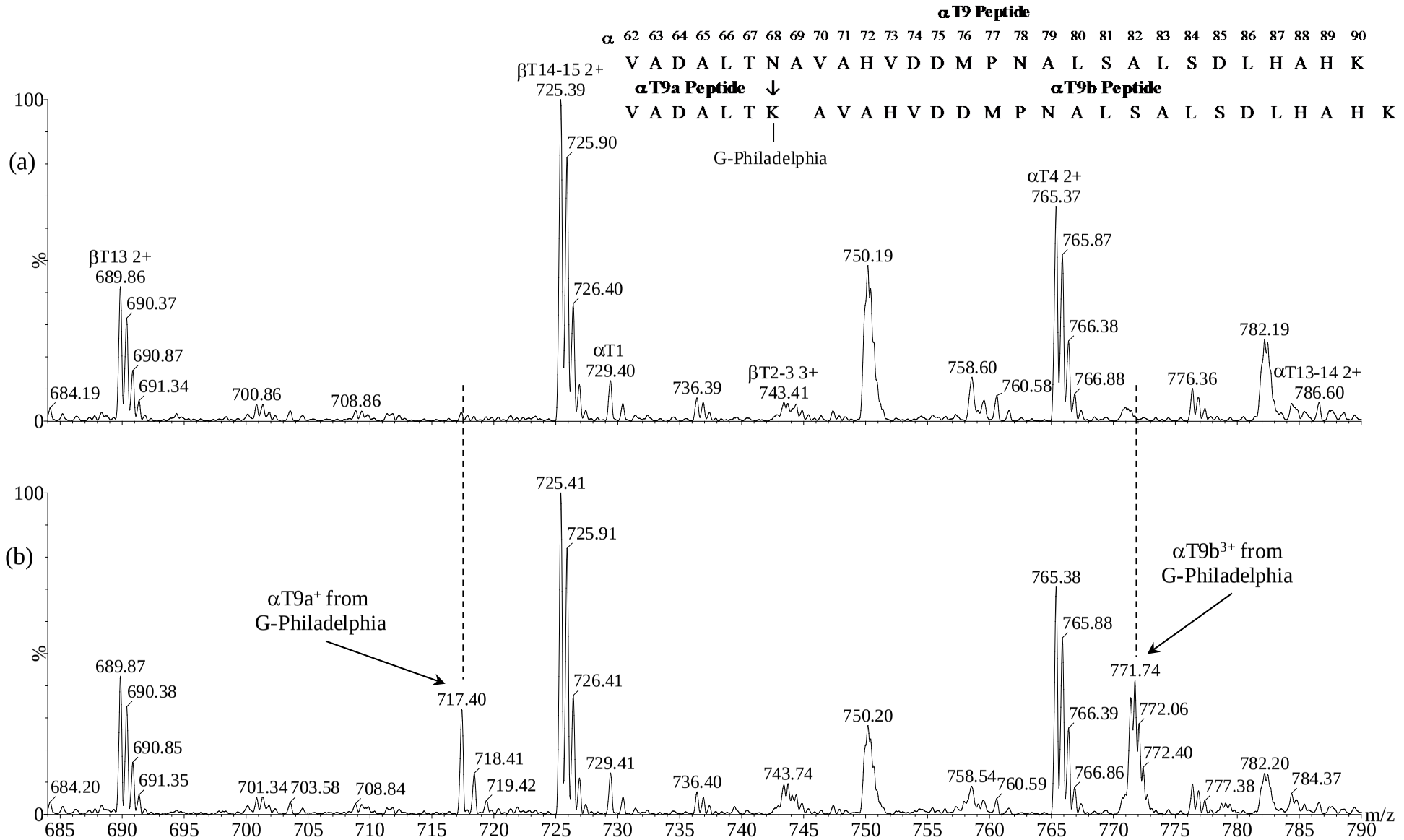

Figure 5.3.14.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb G-Philadelphia heterozygote. The variant spectrum is consistent with the formation of two new tryptic peptides in Hb G-Philadelphia (α68Asn→Lys).

239

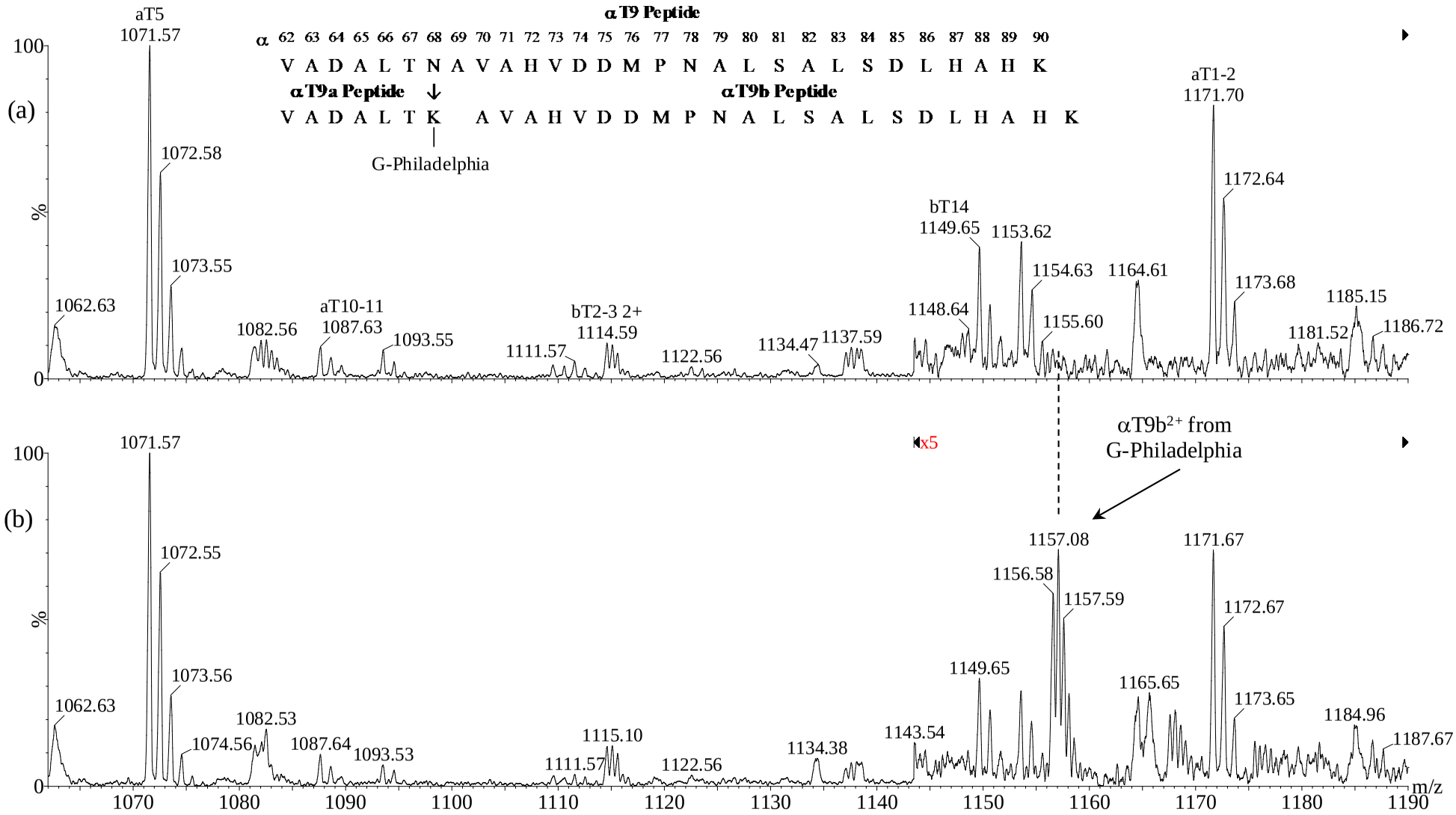

Figure 5.3.14.5. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb G-Philadelphia heterozygote. The variant spectrum is consistent with the formation of two new tryptic peptides in Hb G-Philadelphia (α68Asn→Lys).

5.3.15. αT9 - Hb Q-Thailand (α74Asp→His)

Hb Q-Thailand is the result of an α-chain mutation in which the α74 amino acid residue is changed from Asp to His through a single base change in the codon GAC→CAC.

αT9 Peptide

α	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
	V	A	D	A	L	T	N	A	V	A	H	V	D	D	M	P	N	A	L	S	A	L	S	D	L	H	A	H	K
													↓																
													H																

Figure 5.3.15.1. Sequence of the Hb Q-Thailand αT9 tryptic peptide.

A blood sample was submitted for analysis by mass spectrometry because the ce-HPLC trace (Figure 5.3.15.2.) showed an abnormally high response in the S-window (31.4%) at 4.66 min, indicating a positive charge change.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	0.1	---	1.11	1363
Unknown	---	0.5	1.25	9565
P2	---	2.5	1.33	52887
P3	---	2.1	1.72	43148
A0	---	60.6	2.49	1266772
A2	2.8	---	3.66	60545
S-window	---	31.4	4.66	656662

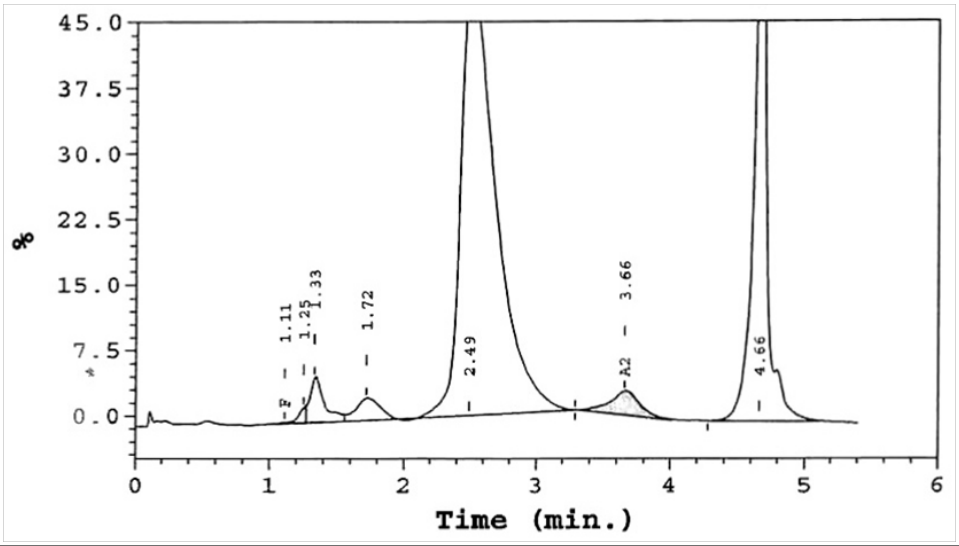

Figure 5.3.15.2. ce-HPLC trace for Hb Q-Thailand.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.3.15.3.) revealed an α-chain heterozygote at 15,148.40 Da, 22.00 Da heavier than normal, and 31.9% of total α-chains. A single codon change giving a mass increase of 22 Da could only be Asp→His (8 possibilities, positive charge change).

Figure 5.3.14.4. shows the diagnostic parts of the tryptic digest spectra from (a) normal Hb and (b) the variant Hb. A new peak is observed in the lower panel at *m/z* 755.44 corresponding to a mass increase of 22 Da in the $\alpha T9^{4+}$ tryptic fragment. This is further supported by the change in intensity ratio of the $\alpha T9^{3+}$ tryptic fragments at *m/z*/ 999.54 and *m/z* 1,006.96 in the lower trace (variant) of Figure 5.3.14.5. compared to the same peaks in the upper panel. There are four possible sites of mutation in the αT9

peptide that could give rise to a +22 Da mass change; α64Asp→His (Hb Q-India), α74Asp→His (Hb Q-Thailand), α75Asp→His (Hb Q-Iran) or α85Asp→His (Hb Canuts).

Figures 5.3.14.6. shows the partial product ion spectra for the $\alpha T9^{3+}$ precursor for (a) normal Hb and (b) the variant Hb. No mass change is observed for the y''-series ions up to y''_{16}, whereas the y''_{17} ion shows an increase of 22 Da at *m/z* 1,857.6. No mass change is observed for the b_{12} fragment at *m/z* 1,163.1, but a new signal is observed in the lower panel for the b_{13} fragment at *m/z* 1,324.4, confirming the mutation as α74 Asp→His, Hb Q-Thailand.

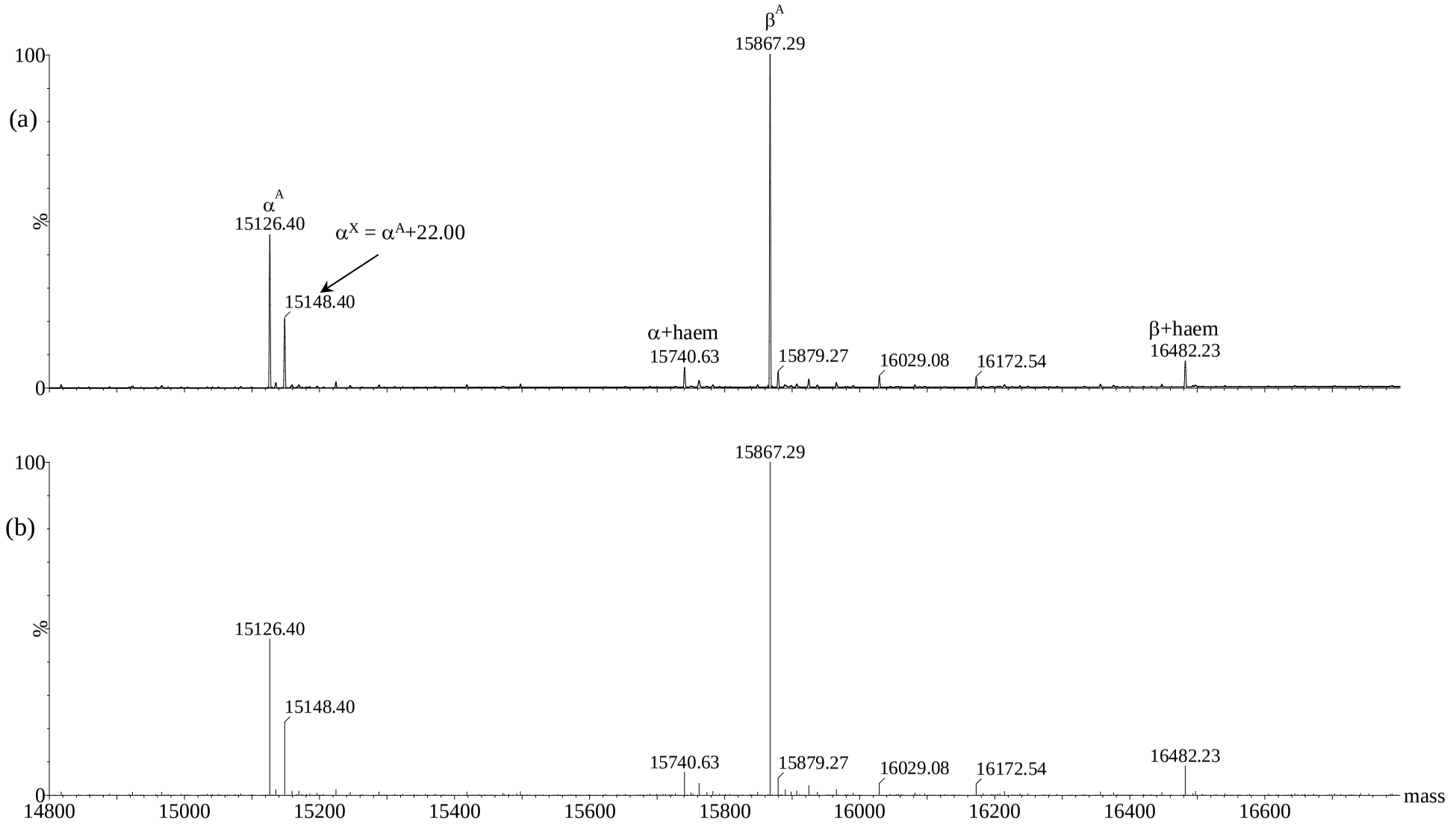

Figure 5.3.15.3. Deconvoluted mass spectrum of Hb Q-Thailand (α74Asp→His) showing the presence of a signal at 15,148.40 Da at approximately 32% of the total α-chains. The 22 Da increase in mass can only be attributed to the Asp→His mutation.

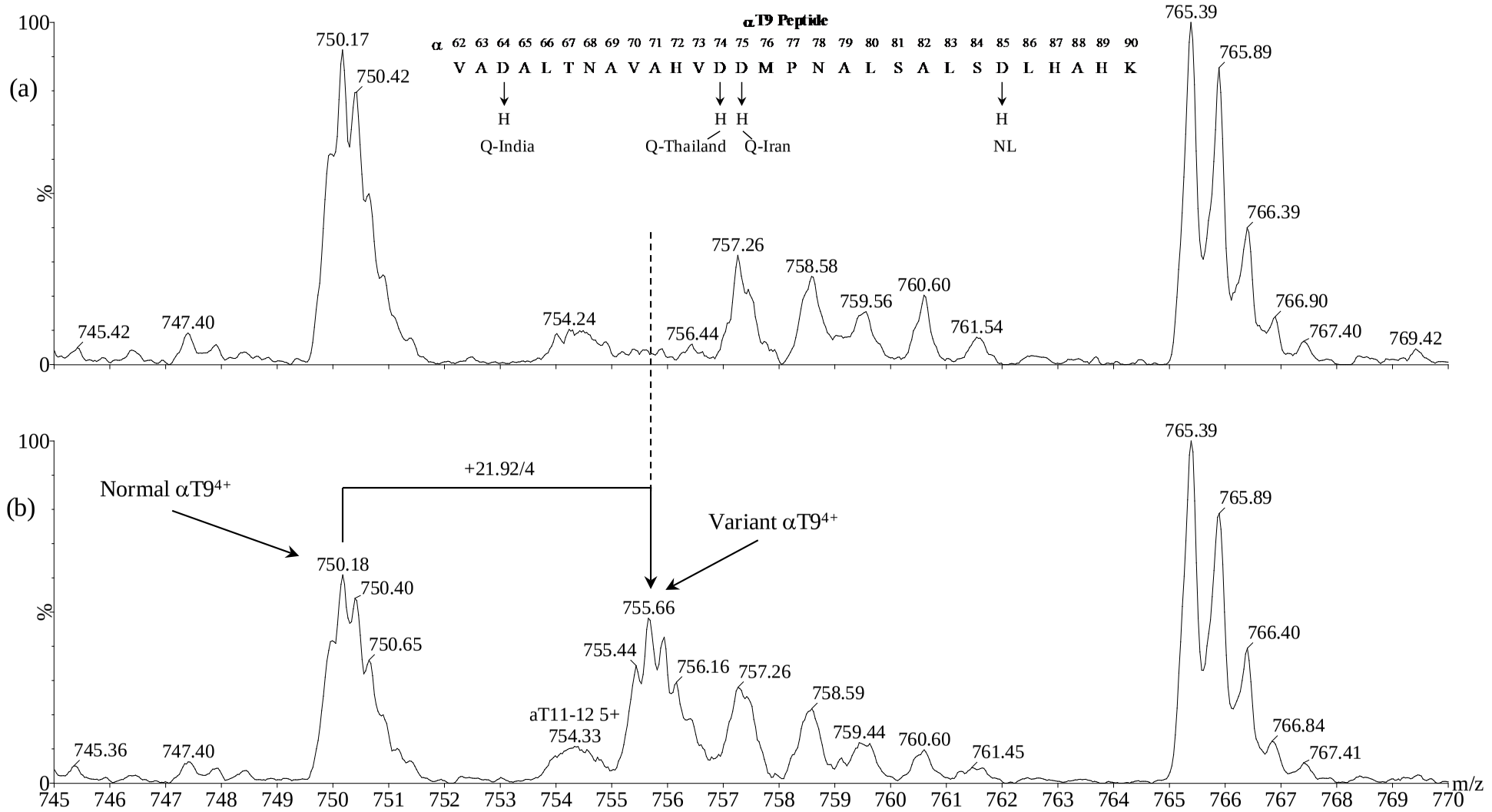

Figure 5.3.15.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Q-Thailand heterozygote. The 21.92 Da mass increase in the $\alpha T9^{4+}$ peak is consistent with the Asp→His in the αT9 tryptic peptide.

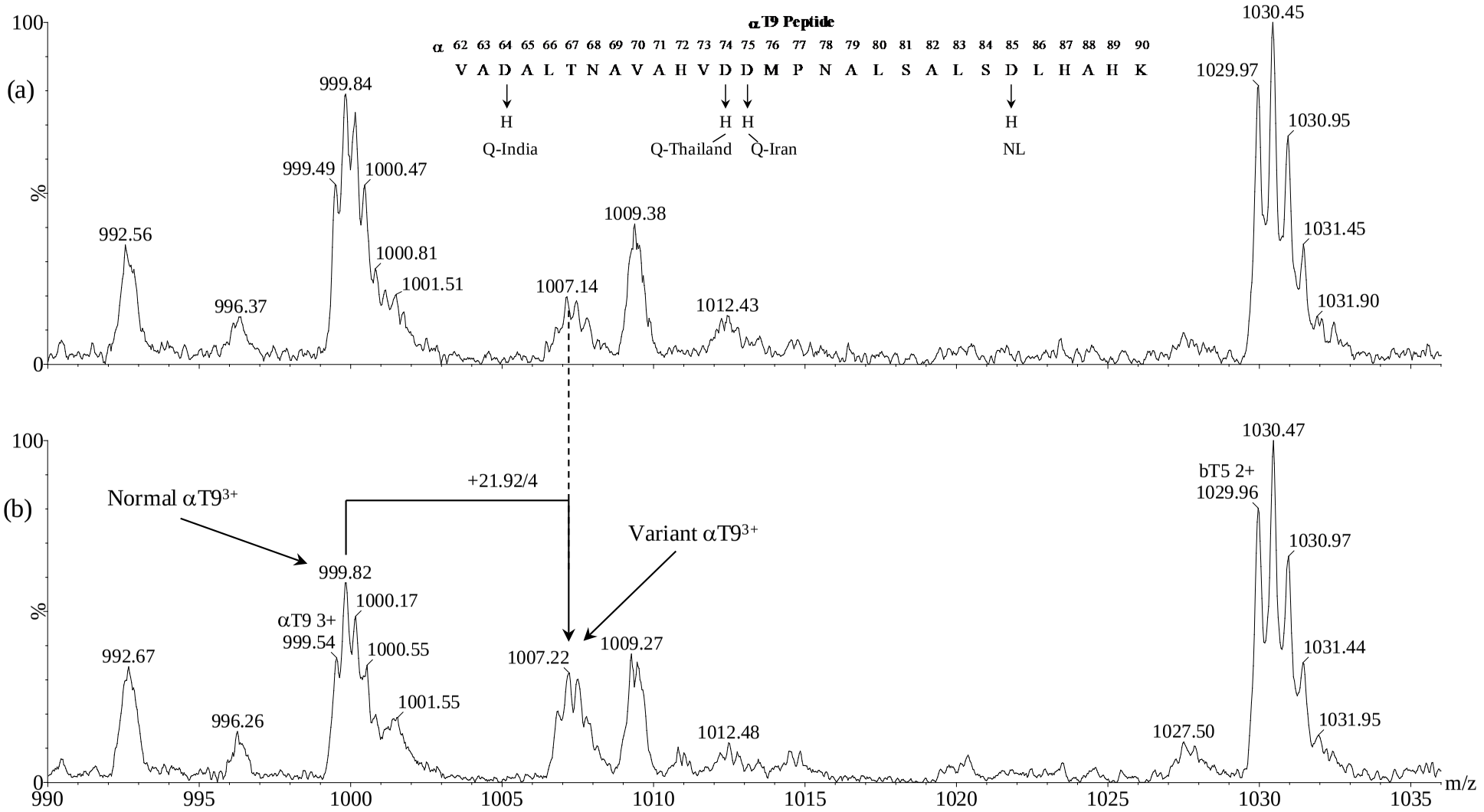

Figure 5.3.15.5. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Q-Thailand heterozygote. The 21.92 Da mass increase in the $\alpha T9^{3+}$ peak is consistent with the Asp→His mutation in the αT9 tryptic peptide.

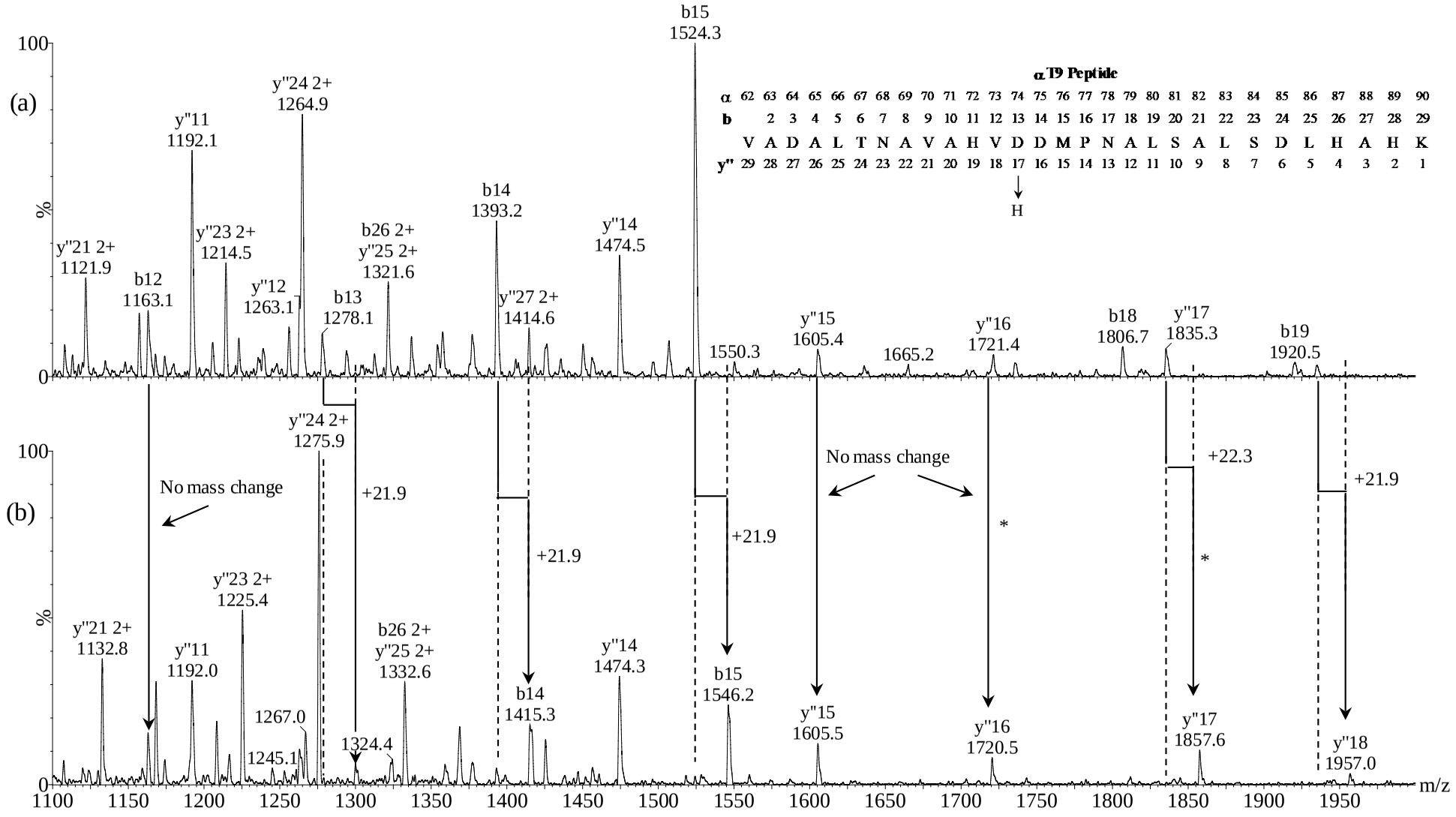

Figure 5.3.15.6. Partial Product ion spectra of the $\alpha T9^{3+}$ tryptic fragment of (a) normal Hb and (b) Hb Q-Thailand. The 22 Da mass increase at b_{13} and y''_{17} identifies the mutation as α74Asp→His, Hb Q-Thailand.

5.3.16. αT9 - Hb Stanleyville-II (α78Asn→Lys)

Hb Stanleyville-II is the result of an α-chain mutation in which the α78 amino acid residue is changed from Asn to Lys through a single base change in the codon AAC→AAG.

The mutation to the Lys residue results in an additional tryptic cleavage product, as shown in Figure 5.3.16.1.

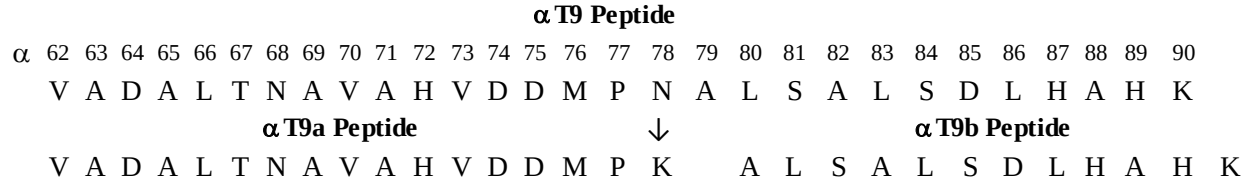

Figure 5.3.16.1. Sequence of the Hb Stanleyville-II αT9 tryptic peptides.

A blood sample was submitted for analysis by mass spectrometry because the ce-HPLC trace (Figure 5.3.16.2.) showed an abnormally high response in the S-window (20.4%) at 4.29 min, suggesting a positive charge change.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.2	1.05	3463
F	0.3	---	1.13	5726
P2	---	7.6	1.31	170307
P3	---	3.5	1.69	77798
Unknown	---	2.7	2.19	61002
A0	---	63.3	2.48	1422275
A2	1.8	---	3.62	38573
S-window	---	20.4	4.29	458864
Unknown	---	0.5	4.61	10634

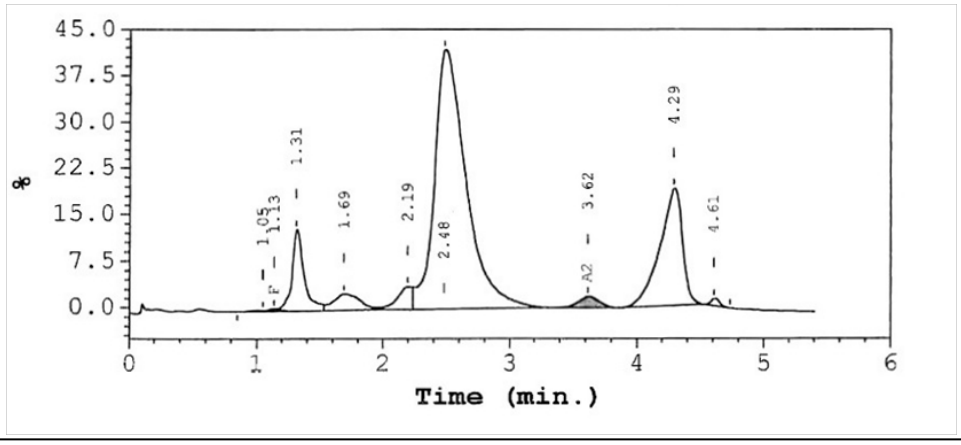

Figure 5.3.16.2. ce-HPLC trace for Hb Stanleyville-II.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.3.16.3.) revealed an α-chain heterozygote at 15,140.18 Da, 13.84 Da heavier than normal and approximately 28% of total α-chains. A single codon change giving a mass increase of +14 Da could be Asn→Lys (4 possibilities), Asp→Glu (8 possibilities), Gly→Ala (7 possibilities), Ser→Thr (11 possibilities), Val→Ile (13 possibilities) or Val→Leu (13 possibilities). However, the shift in the ce-HPLC trace shows a positive charge change and indicates the Asn→Lys mutation. Mutation to Lys will likely result in an additional fragment on tryptic digestion.

Figure 5.3.16.4. shows the diagnostic part of the tryptic digest spectra from (a) normal Hb and (b) the variant Hb. The appearance of peaks at *m/z* 589.96 (αT9a^{3+}) and *m/z* 632.37 (αT9b^{2+}) is consistent with the formation of two new tryptic peptides from the αT9 peptide with the mutation α78Asn$\rightarrow$Lys, and the additional tryptic cleavage site at α78. Further evidence is show in the lower panel of Figure 5.3.16.5. for the tryptic digest of the variant Hb and the appearance of the αT9a^{2+} signal at *m/z* 883.94. These data confirm the mutation as α78Asn$\rightarrow$Lys, Hb Stanleyville-II.

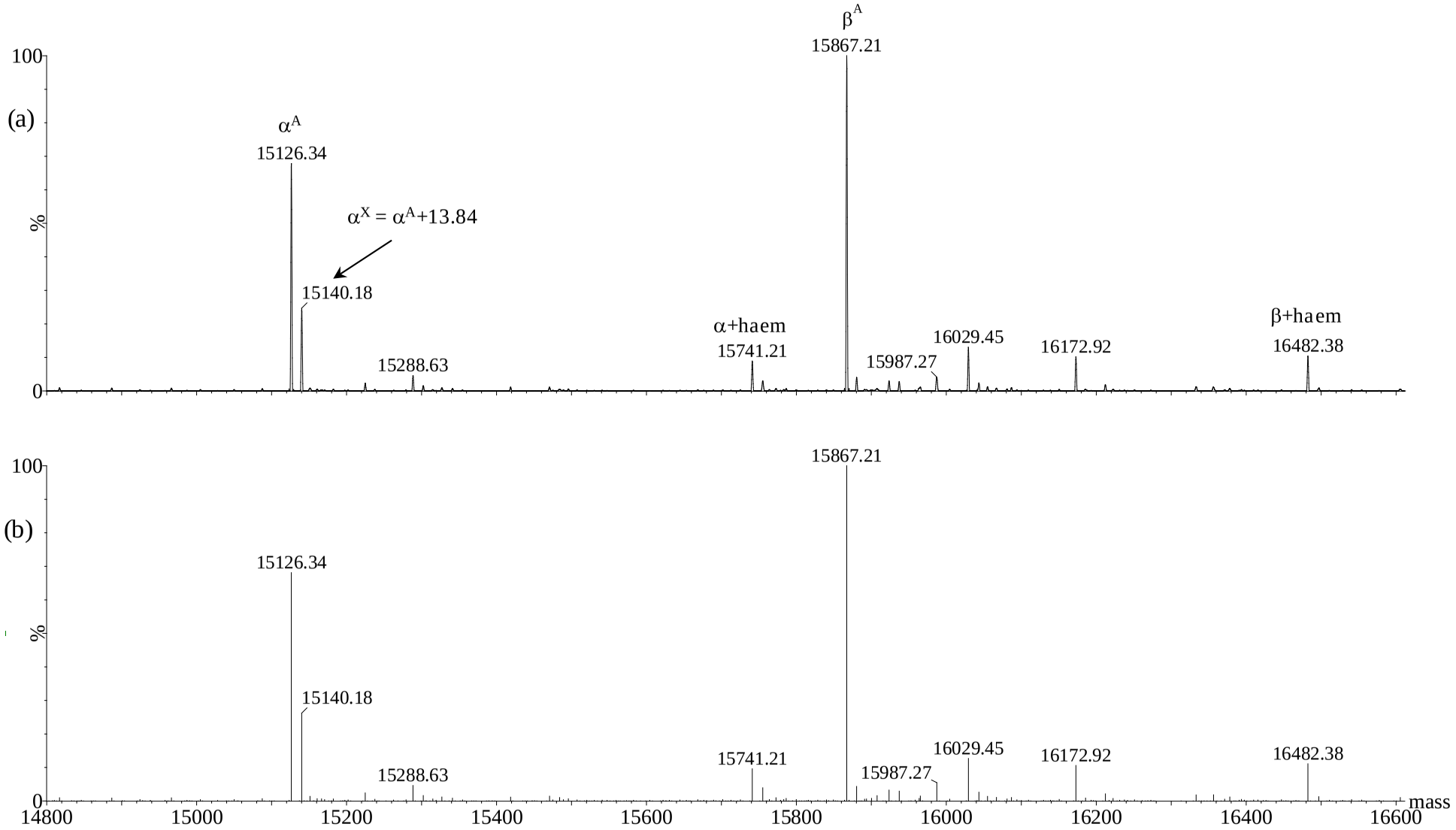

Figure 5.3.16.3. Deconvoluted mass spectrum of Hb Stanleyville-II (α78Asn→Lys) showing the presence of a signal at 15,140.18 Da at approximately 28% of the total α-chains.

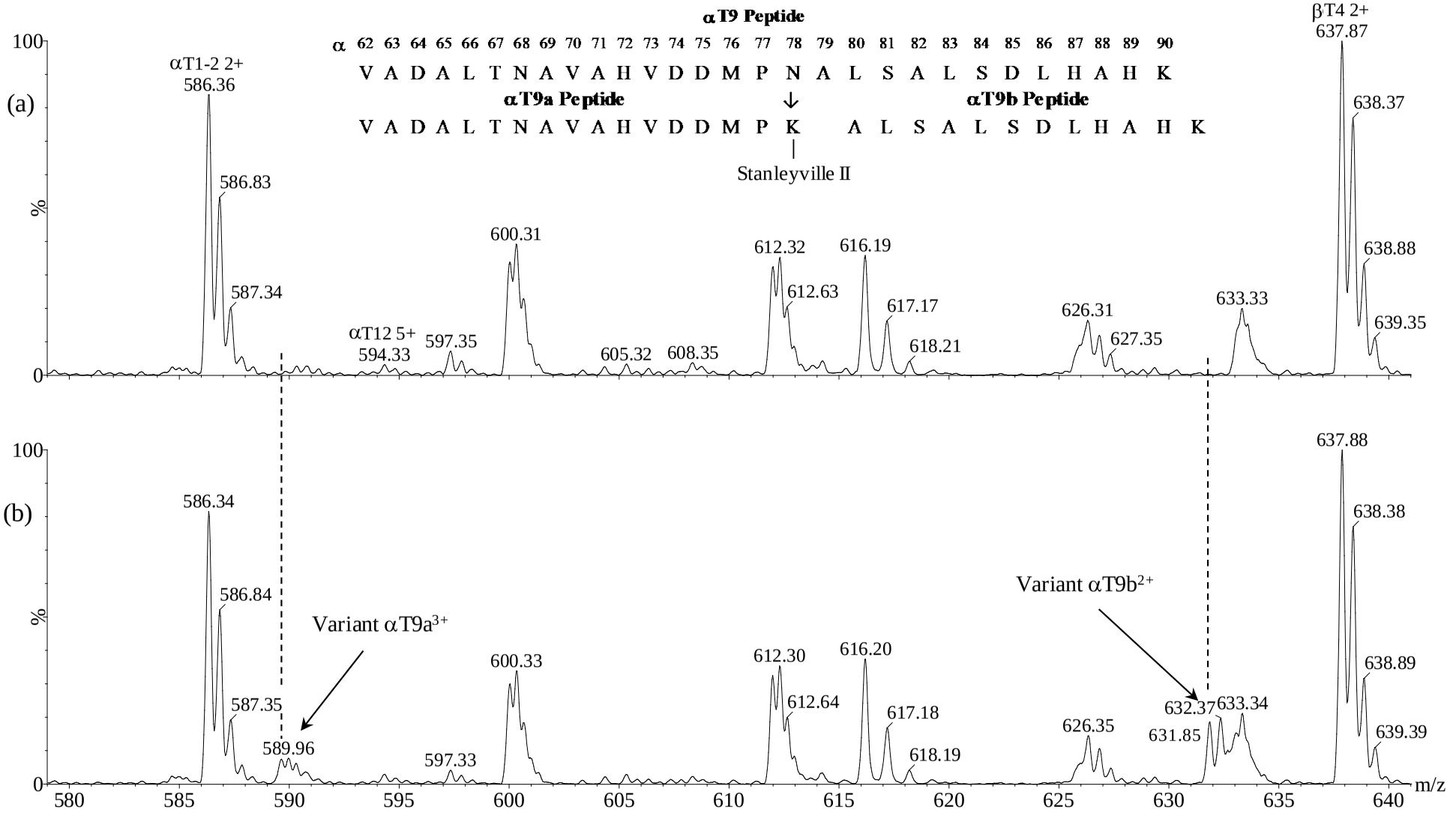

Figure 5.3.16.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Stanleyville-II heterozygote. The variant spectrum is consistent with the formation of two new tryptic peptides in Hb Stanleyville-II (α78Asn→Lys).

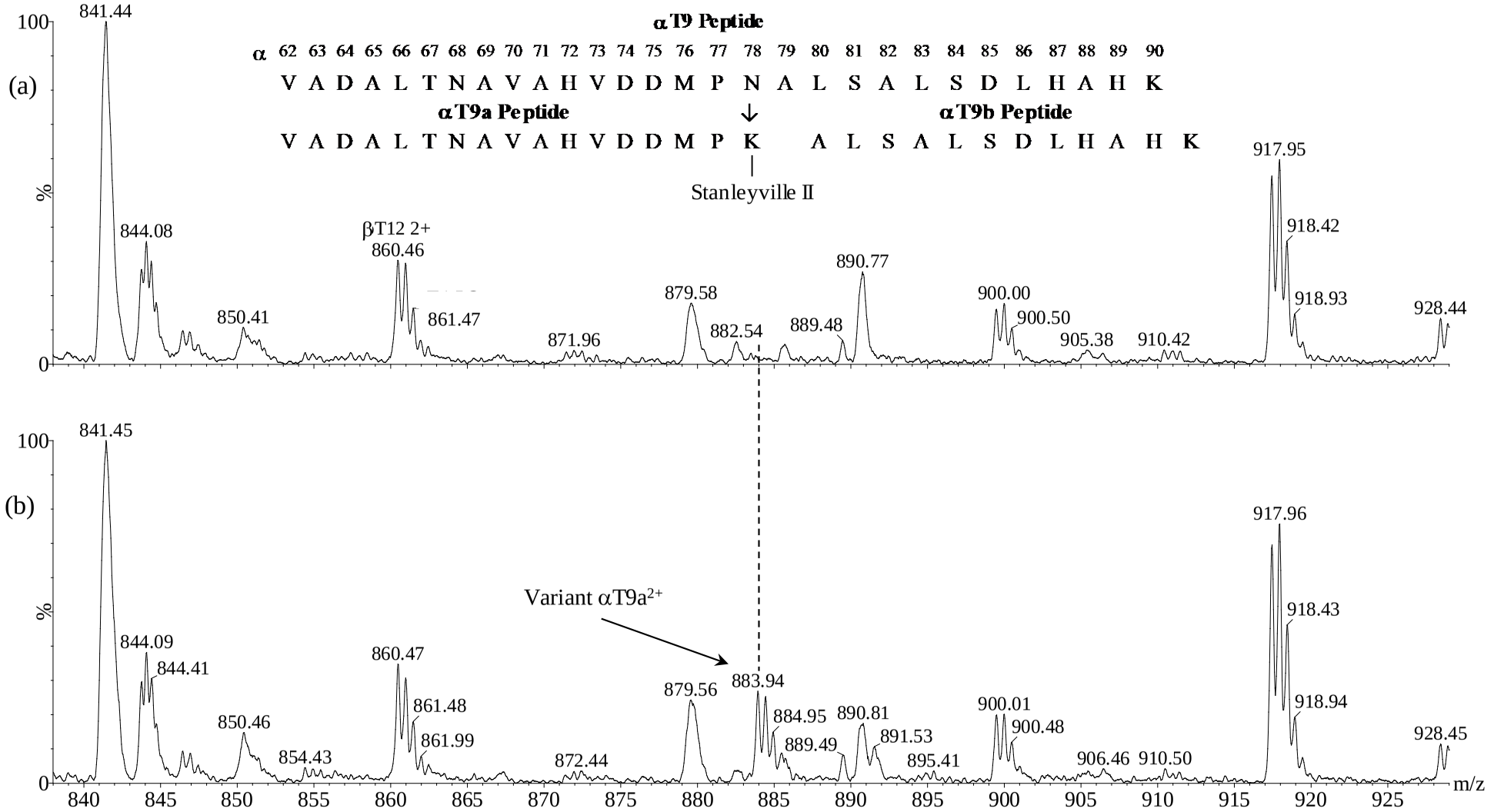

Figure 5.3.16.5. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Stanleyville-II heterozygote. The variant spectrum is consistent with the formation of two new tryptic peptides in Hb Stanleyville-II (α78Asn→Lys).

5.3.17. αT9 - Hb M-Iwate (α87His→Tyr)

Hb M-Iwate is the result of an α-chain mutation in which the α87 amino acid residue is changed from His to Tyr through a single base change in the codon CAC→TAC.

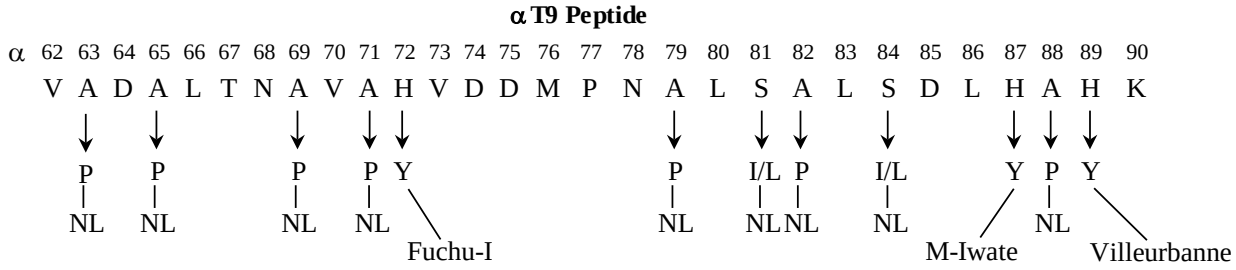

Figure 5.3.17.1. Sequence of the Hb M-Iwate αT9 tryptic peptide.

No ce-HPLC trace was available for this variant.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.3.17.2.) revealed an α-chain heterozygote at 15,152.08 Da, 25.67 Da heavier than normal and 30.2% of total α-chains. A single codon change giving a mass increase of +26 Da could be Ala→Pro (21 possibilities), His→Tyr (10 possibilities), Ser→Ile (11 possibilities) or Ser→Leu (11 possibilities).

Figure 7.3.17.3. shows part of the tryptic digest spectrum for (a) normal Hb and (b) the variant Hb. The appearance of a peak at *m/z* 1,008.54 in the lower panel is consistent with the formation of a new $\alpha T9^{3+}$ tryptic fragment. Within the αT9 peptide, there are twelve possible sites of mutation that could arise from a single base change in the codon, except: α81Ser(TGC)→Leu/Ile and α84Ser(AGC)→Leu. Of the remaining possibilities, seven have not previously been reported. The mutation in the αT9 peptide is further supported by the appearance of the $\alpha T9^{4+}$ ion at *m/z* 756.66 in the lower panel of Figure 5.3.17.4. and the $\alpha T9^{2+}$ ion at *m/z* 1,512.25 in Figure 5.3.17.5.

Figure 5.3.17.6. shows the partial product ion spectra of the $\alpha T9^{3+}$ tryptic fragment of (a) normal Hb and (b) the unknown variant. The 26 Da mass increase at y''_4 at *m/z* 518.4 in the lower panel identifies the mutation as α87His→Tyr, Hb M-Iwate.

Figure 5.3.17.7. shows the diagnostic part of the chymotryptic digest spectrum of (a) normal Hb and (b) the variant Hb. The presence of the variant $\alpha(81\text{-}87)^+$ ion at *m/z* 768.38 is fully consistent with the mutation α87His→Tyr, Hb M-Iwate. Figure 5.3.17.8. shows the product ion spectrum of the new chymotryptic fragment, $\alpha CT9^+$, of Hb M-Iwate (α87His→Tyr), and this spectrum is fully consistent with the sequence of the variant chymotryptic peptide

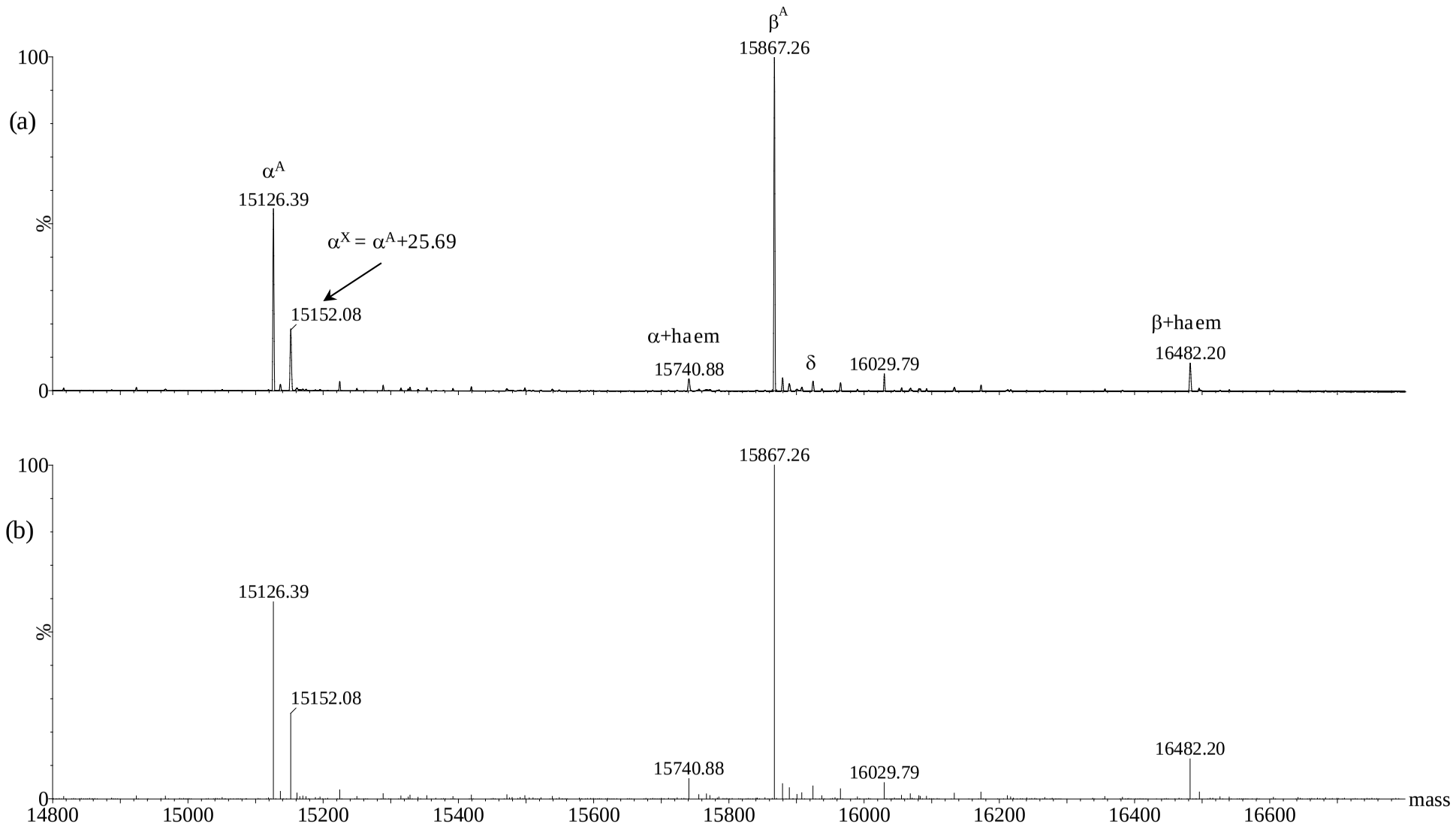

Figure 5.3.17.2. Deconvoluted mass spectrum of Hb M-Iwate (α87His→Tyr) showing the presence of a signal at 15,152.08 Da at approximately 30% of the total α-chains.

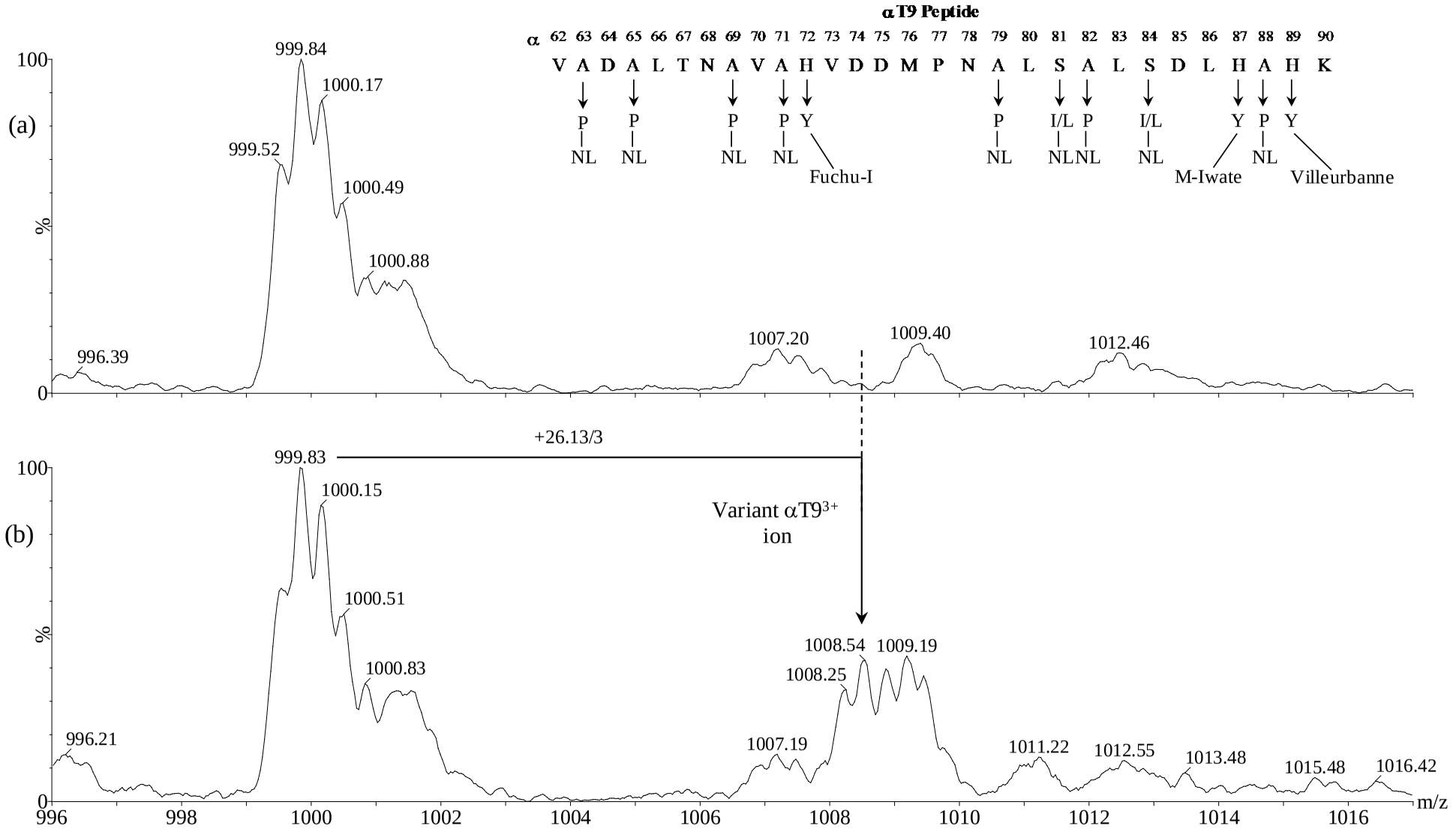

Figure 5.3.17.3. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb M-Iwate heterozygote. All the mutations shown can occur by a single base change in the codon except: 81Ser(TGC)→Leu/Ile and 84Ser(AGC)→Leu.

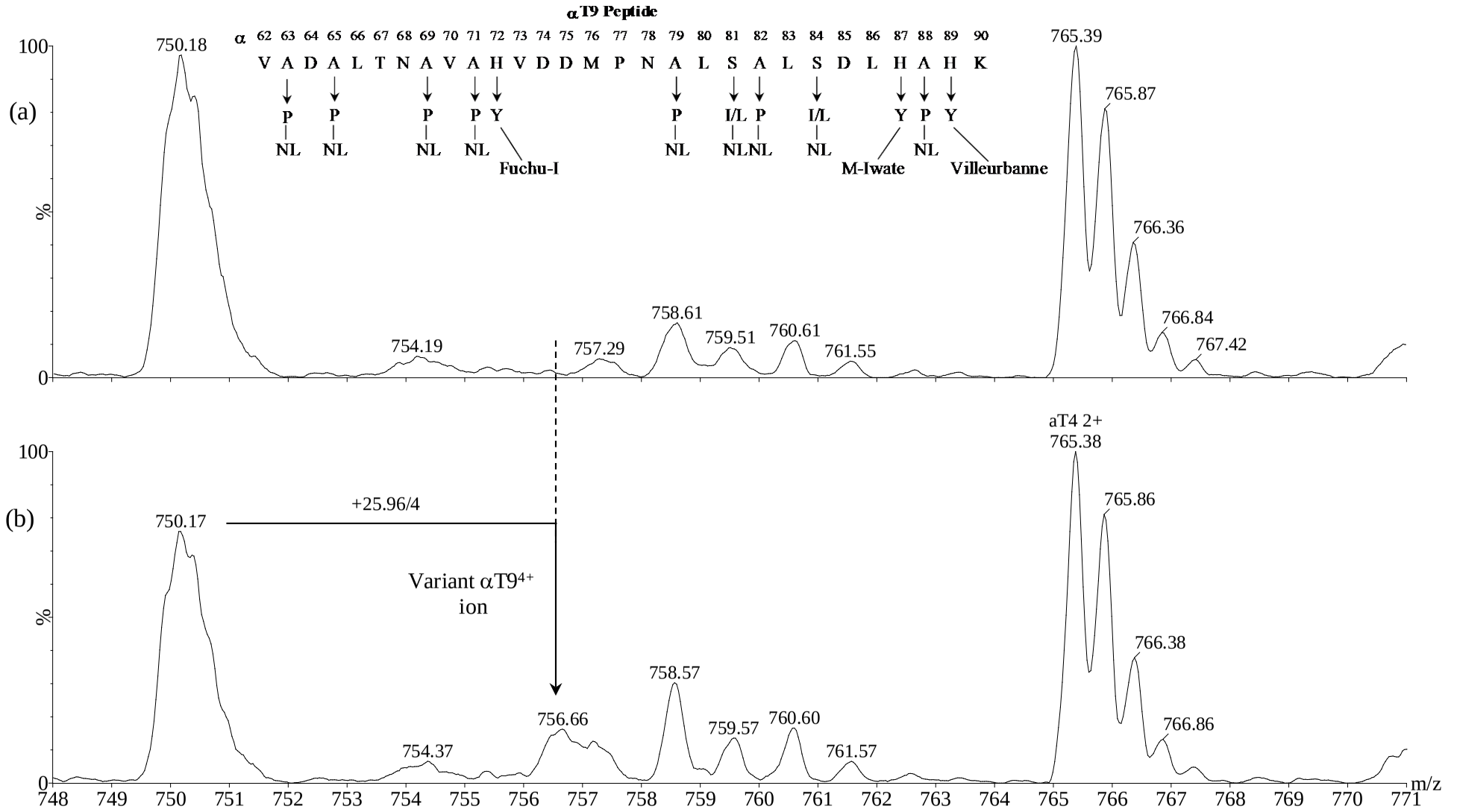

Figure 5.3.17.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb M-Iwate heterozygote.

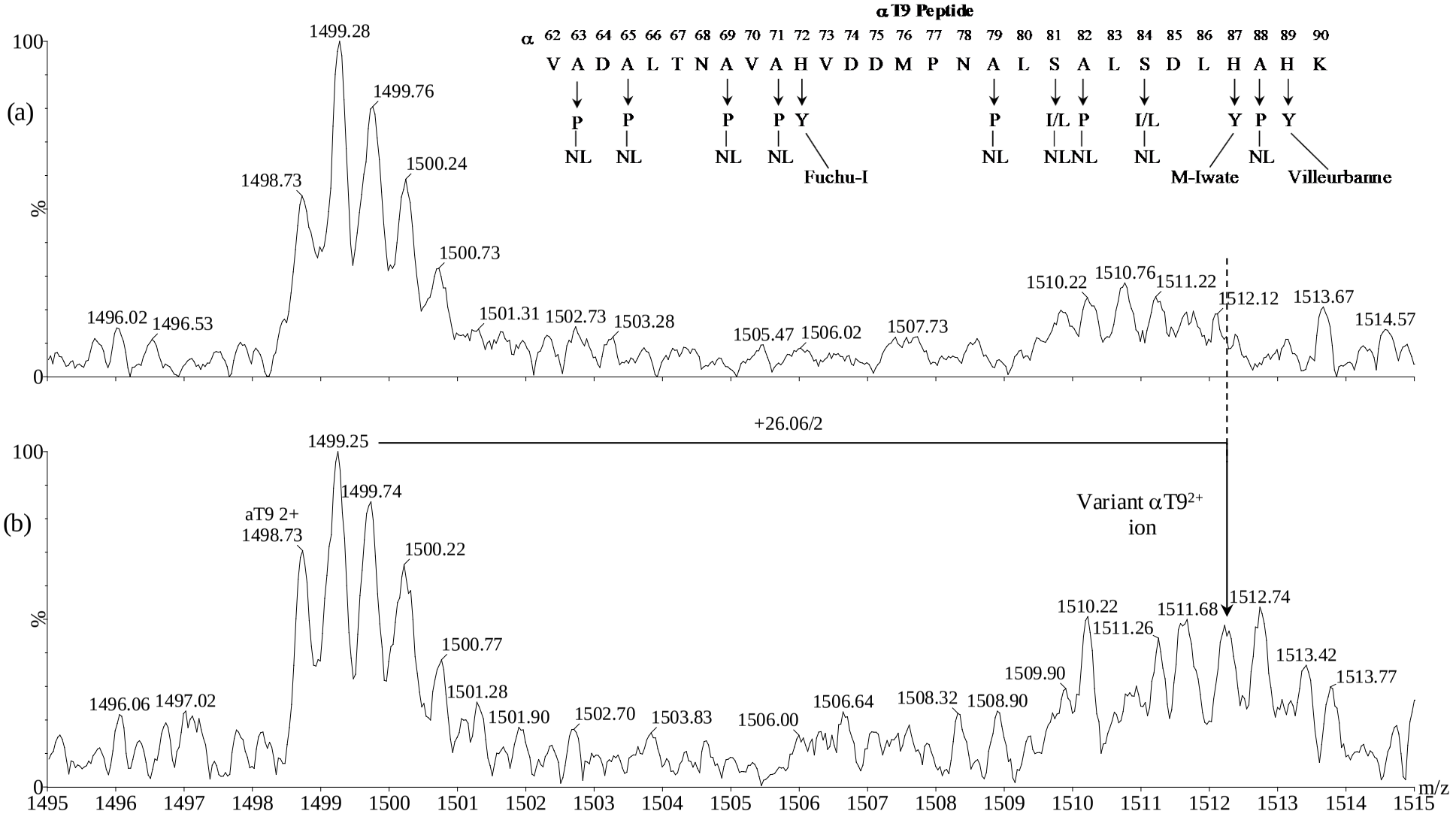

Figure 5.3.17.5. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb M-Iwate heterozygote.

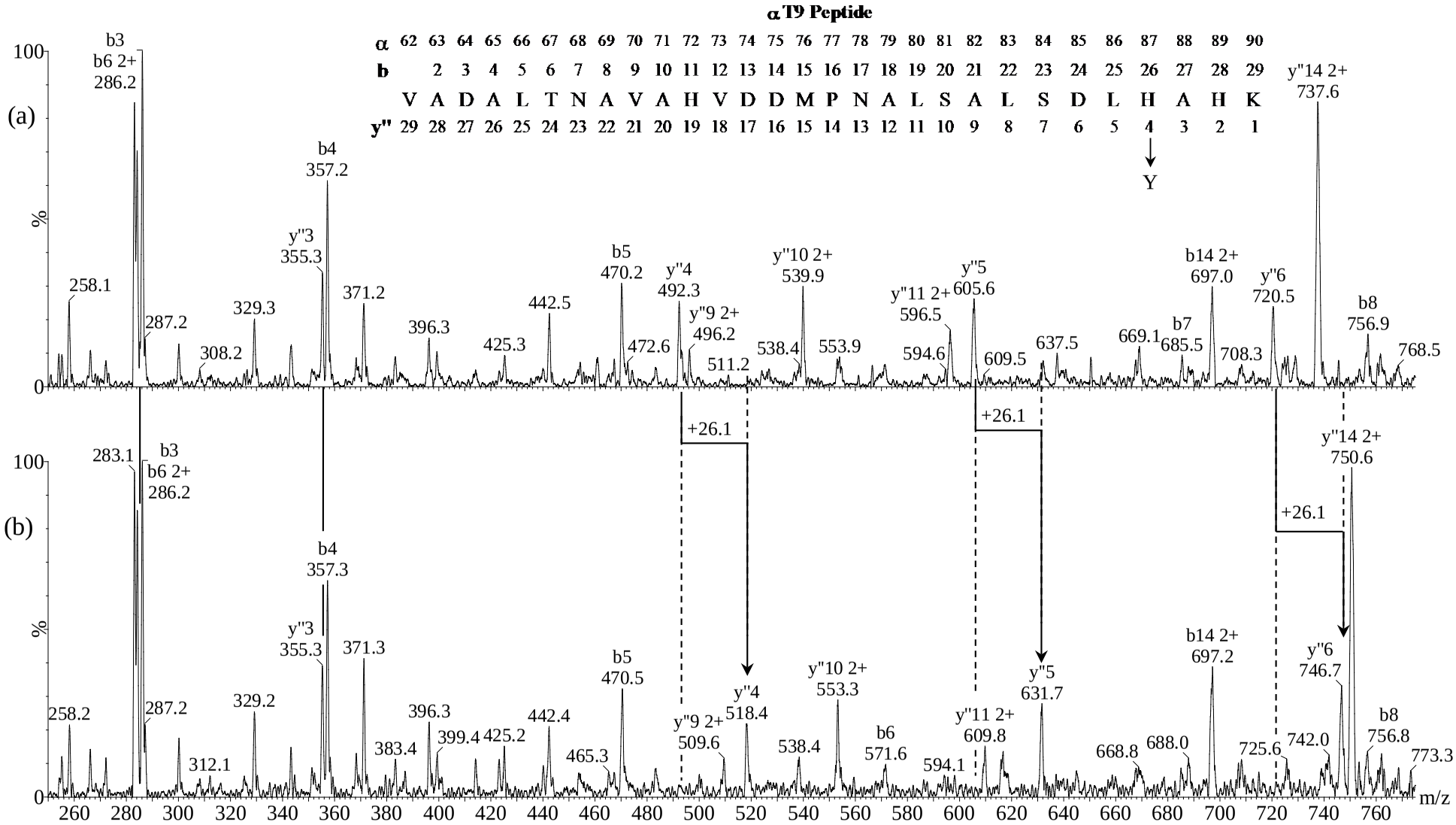

Figure 5.3.17.6. Partial product ion spectra of the $\alpha T9^{3+}$ tryptic fragment of (a) normal Hb and (b) Hb M-Iwate. The 26 Da mass increase at $y"_4$ identifies the mutation as α87His→Tyr.

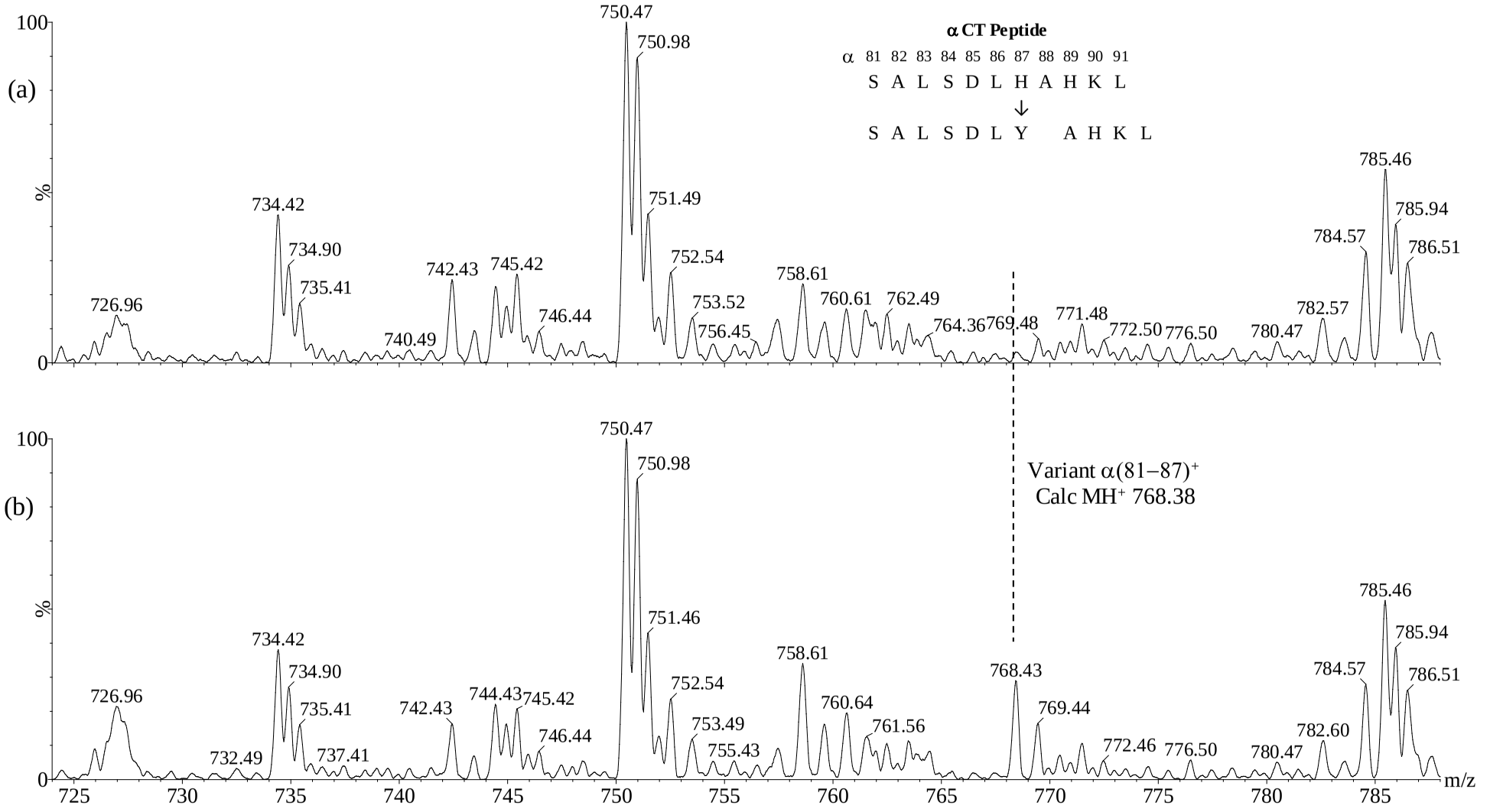

Figure 5.3.17.7. Diagnostic part of the chymotryptic digest spectrum of (a) normal Hb and (b) Hb M-Iwate. The presence of the variant $\alpha(81\text{-}87)^+$ ion is fully consistent with the mutation α87His→Tyr, M-Iwate.

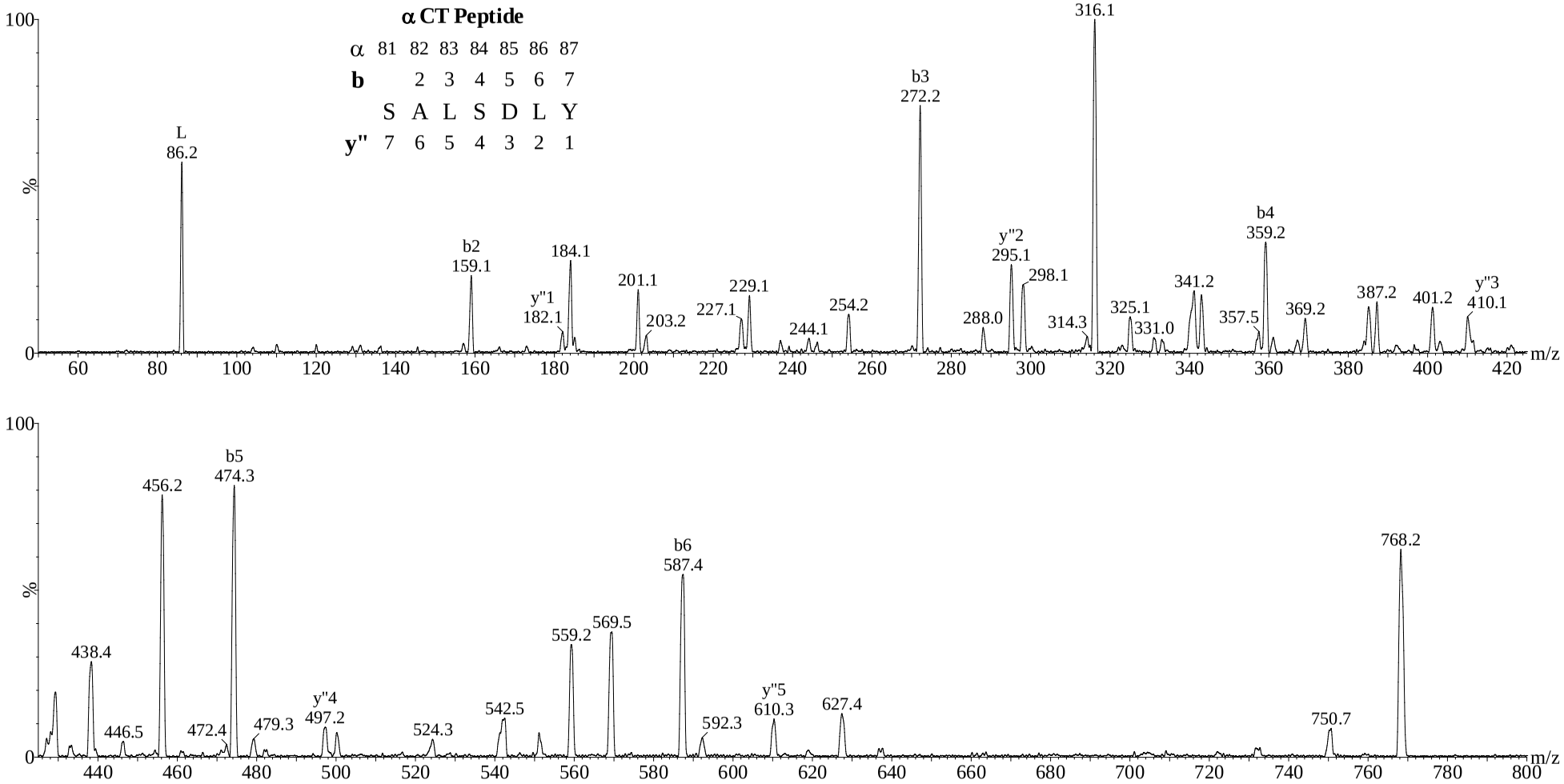

Figure 5.3.17.8. Product ion spectrum of the new αCT9^{+} (*m/z* 768.2) chymotryptic fragment of Hb M-Iwate (α87His→Tyr). This spectrum is fully consistent with the sequence of the variant αCT9 peptide.

5.3.18. αT9 - Hb J-Broussais (α90Lys→Asn)

Hb J-Broussais is the result of an α-chain mutation in which the α90 amino acid residue is changed from Lys to Asn through a single base change in the codon AAG→AAC or AAT.

The mutation of the 90Lys residue results in the loss of a tryptic cleavage product, and the formation of a combined αT(9-10) tryptic peptide.

		αT9 Peptide																										αT10 Peptide	
α	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92
	V	A	D	A	L	T	N	A	V	A	H	V	D	D	M	P	N	A	L	S	A	L	S	D	L	H	A	H	K	L	R
																													↓		
	V	A	D	A	L	T	N	A	V	A	H	V	D	D	M	P	N	A	L	S	A	L	S	D	L	H	A	H	N	L	R

Figure 5.3.18.1. Sequence of the Hb J-Broussais αT(9-10) tryptic peptide.

A blood sample was submitted for analysis by ESI-MS because the ce-HPLC trace (Figure 5.3.18.2.) showed an abnormally high P3 response (24.5%), indicating that there is a negative polarity change in the variant.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
P1	---	0.1	0.73	1360
Unknown	---	0.1	0.94	2428
F	2.4	---	1.09	46426
P2	---	3.7	1.28	78328
P3	---	24.5	1.66	523514
A0	---	67.4	2.44	1439759
A2	2.2	---	3.61	44018

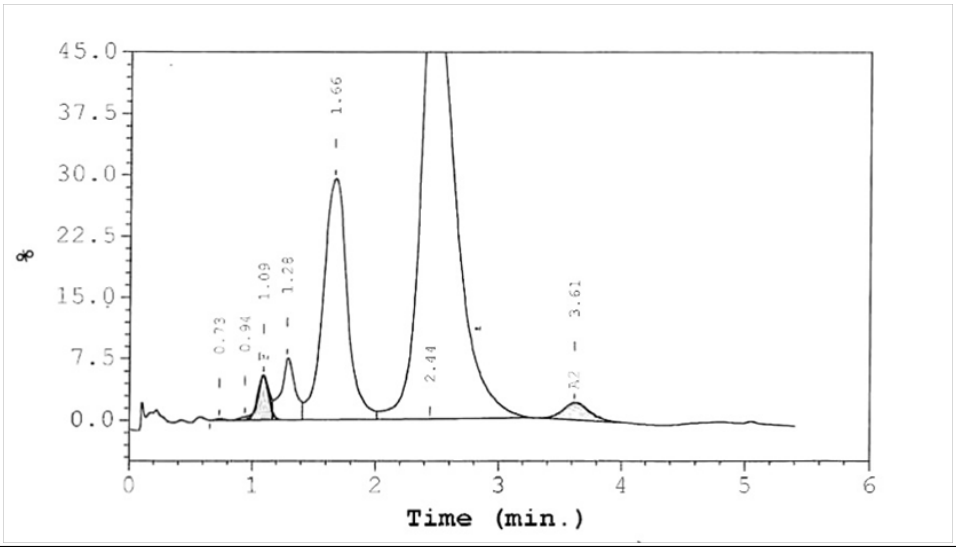

Figure 5.3.18.2. ce-HPLC trace for Hb J-Broussais.

The spectrum obtained by ESI-MS analysis of the blood sample diluted 500-fold is shown in Figure 5.3.18.3. It reveals an α-chain heterozygote in which the variant-chain is 20.3% of the total α-chains and has a mass of 15,112.36 Da, 14.05 Da lower than the normal α-chain. This suggests, coupled with the negative polarity change observed in the ce-HPLC trace that, from a single base change in the codon, the most likely mutation is Lys→Asn (11 possibilities).

Figure 5.3.18.4. shows a diagnostic part of the tryptic digest spectra from (a) normal Hb and (b) the variant Hb. The appearance of a triply charged ion at *m/z* 1,084.55 in the lower panel is consistent with the formation of a combined αT(9-10) peptide with the mutation α90 Lys→Asn, and the loss of the αT10 fragment that is observed for the normal Hb sample. This is further supported by the appearance of a quadruply charged ion at *m/z* 813.94 (calculated *m/z* 813.91) for the αT(9-10) peptide in Figure 5.3.18.5. Note: The *m/z* value in this case refers to the second isotope of the isotopic envelope.

Additionally, in normal Hb samples, the $\alpha T11^{2+}$ tryptic peptide is not normally observed following tryptic digestion. However, Figure 5.3.18.6. shows presence of the $\alpha T11^{2+}$ at *m/z* 409.74 for the variant sample. Figure 5.3.18.7 shows the product ion spectrum of the $\alpha T11^{2+}$ tryptic fragment of Hb J-Broussais (α90Lys→Asn), and these data are fully consistent with the sequence of the αT11 peptide.

Figure 5.3.18.8. shows the product ion spectrum for the $\alpha T(9\text{-}10)^{3+}$precursor. The data, in particular the y''_2 (*m/z* 288.2) and y''_3 (*m/z* 402.3) fragments, confirm the mutation as α90 Lys→Asn, Hb Broussais.

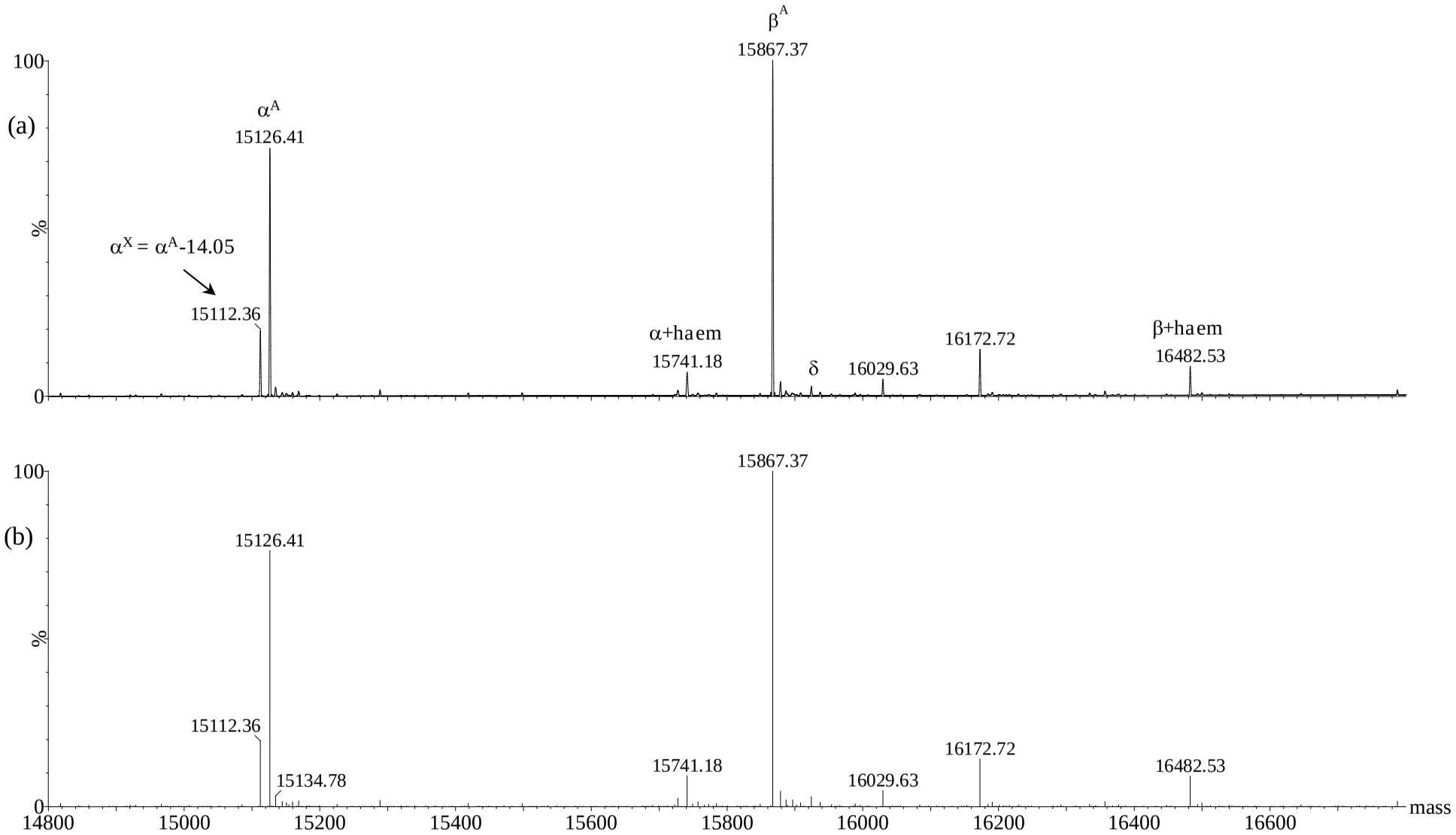

Figure 5.3.18.3. Deconvoluted mass spectrum of Hb J-Broussais (α90Lys→Asn) showing the presence of a signal at 15,112.36 Da at approximately 20% of the total α-chains. For a mass loss of 14 Da, and 'J-like', probably Lys→Asn.

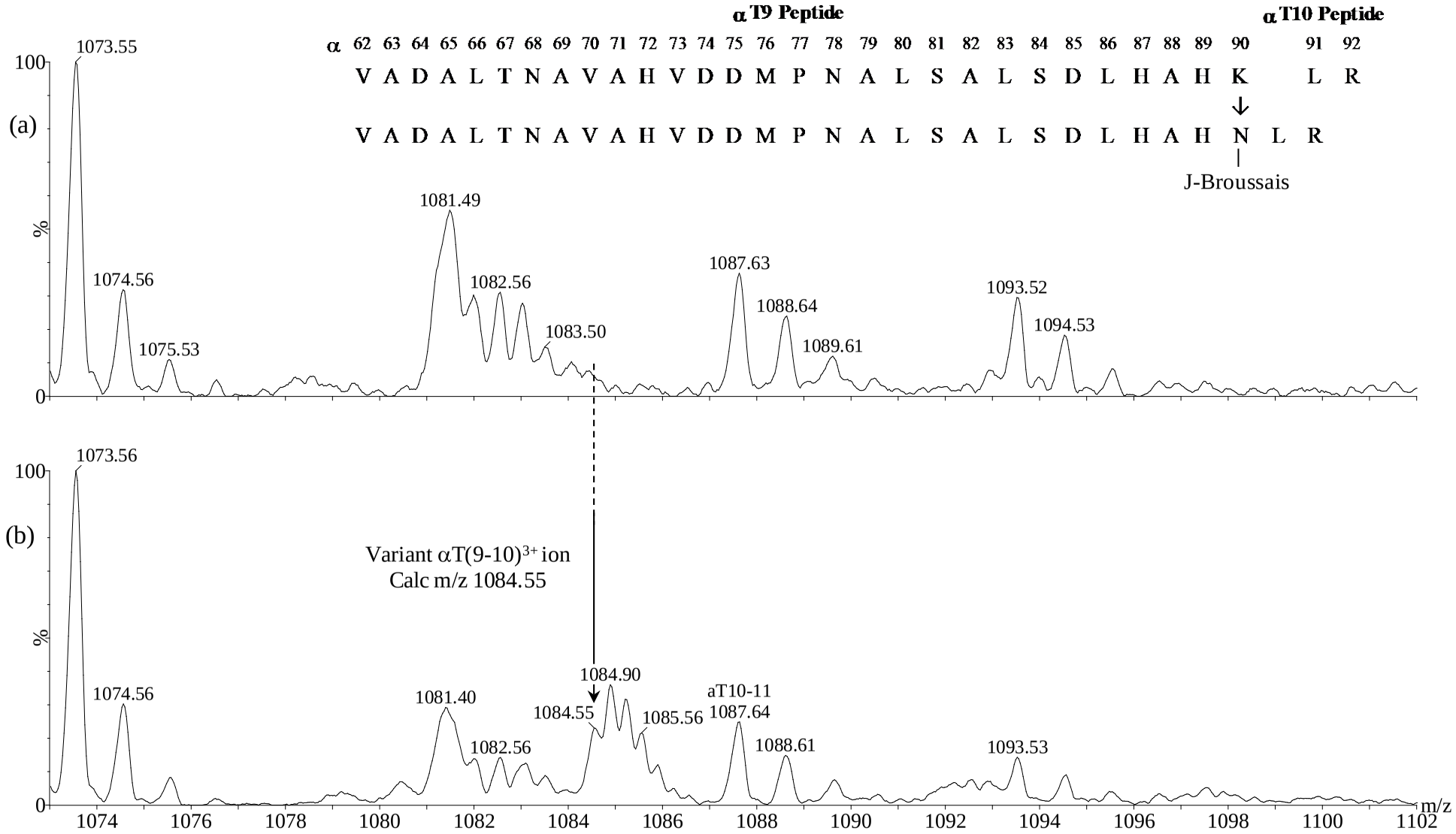

Figure 5.3.18.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb J-Broussais heterozygote.

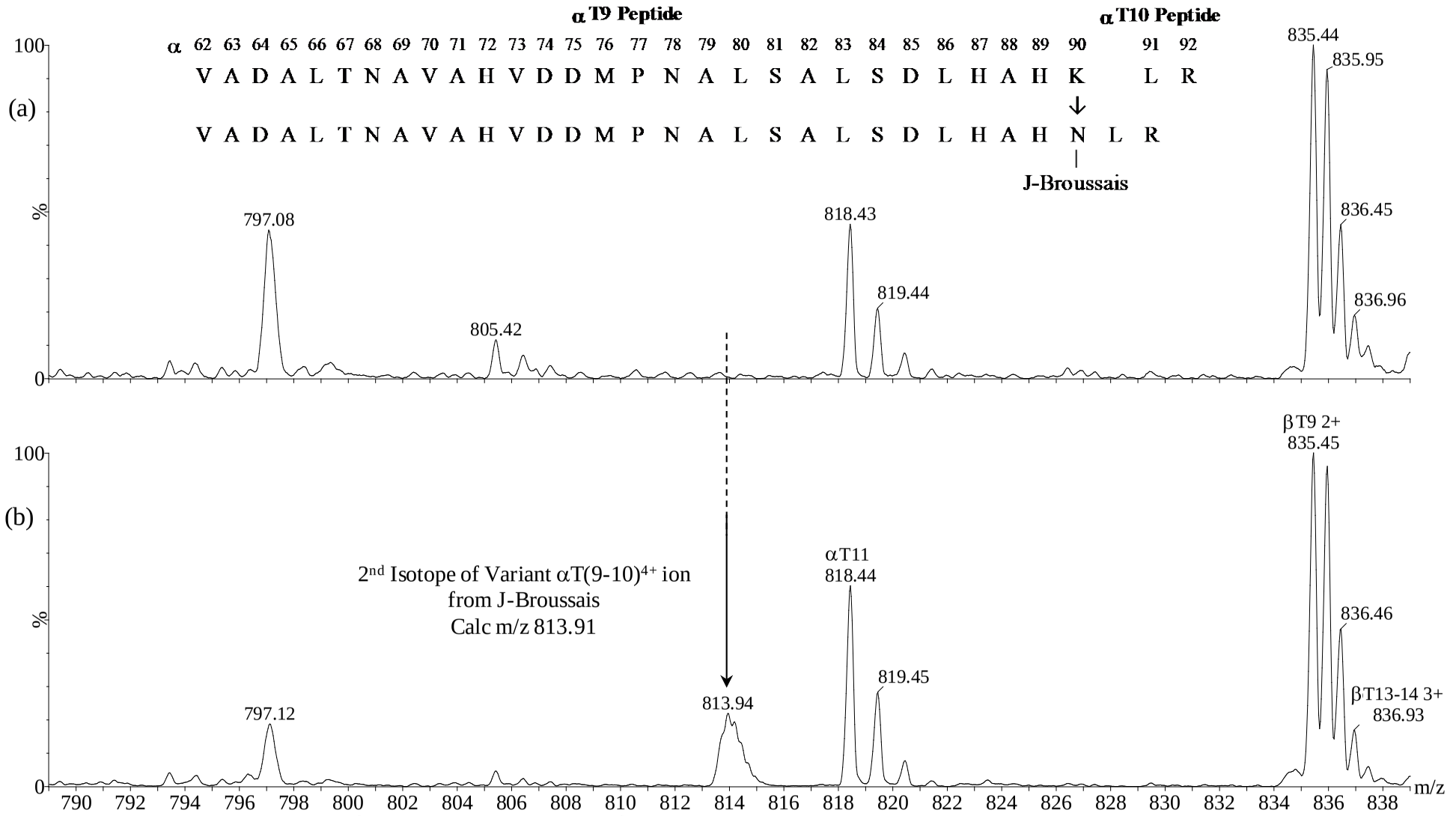

Figure 5.3.18.5. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb J-Broussais heterozygote.

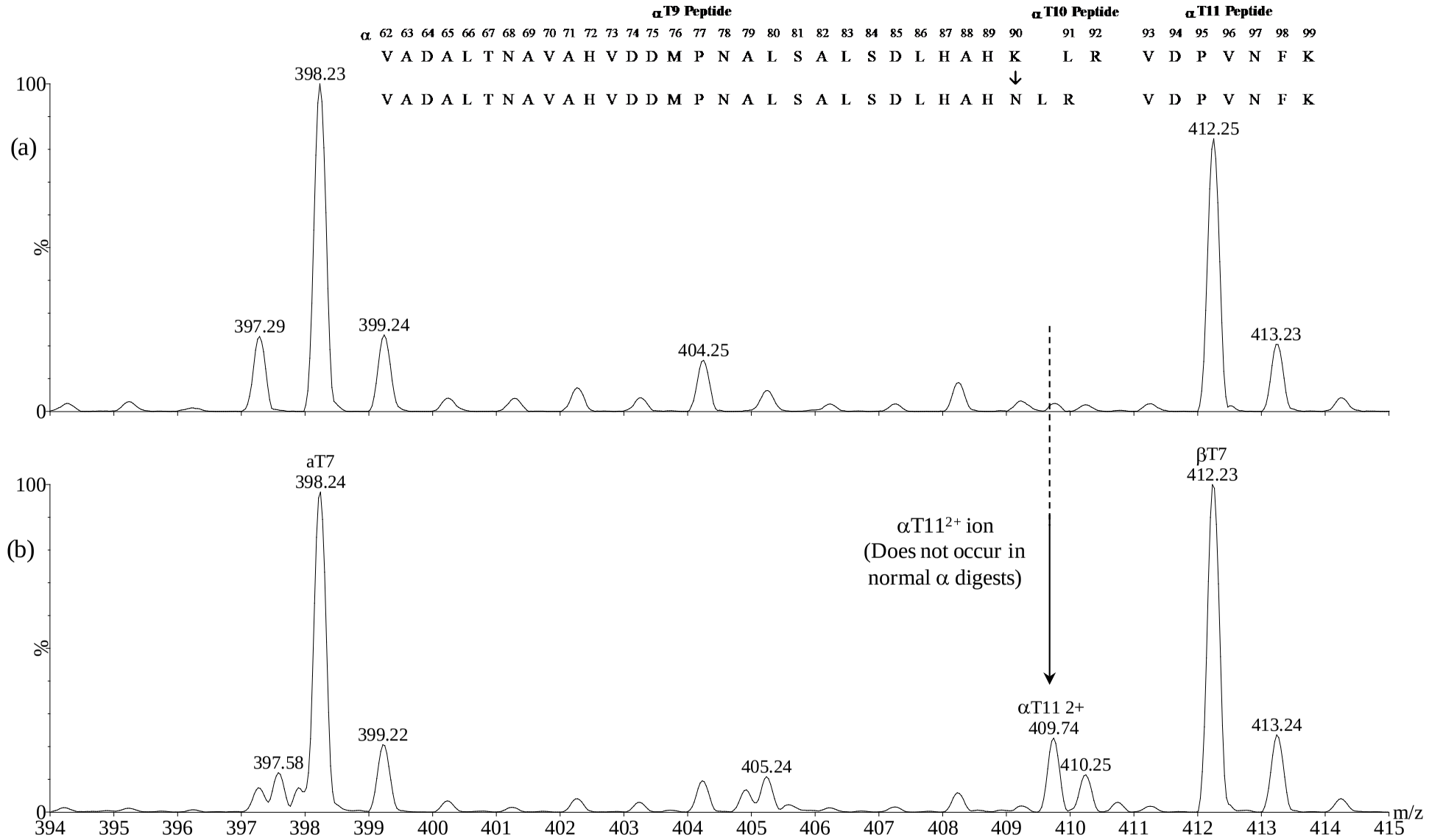

Figure 5.3.18.6. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb J-Broussais heterozygote. The αT11 does not usually appear but is possibly present in Hb J-Broussais owing to the loss of charge at α90. Identity of αT11 is confirmed in Figure 5.3.18.7.

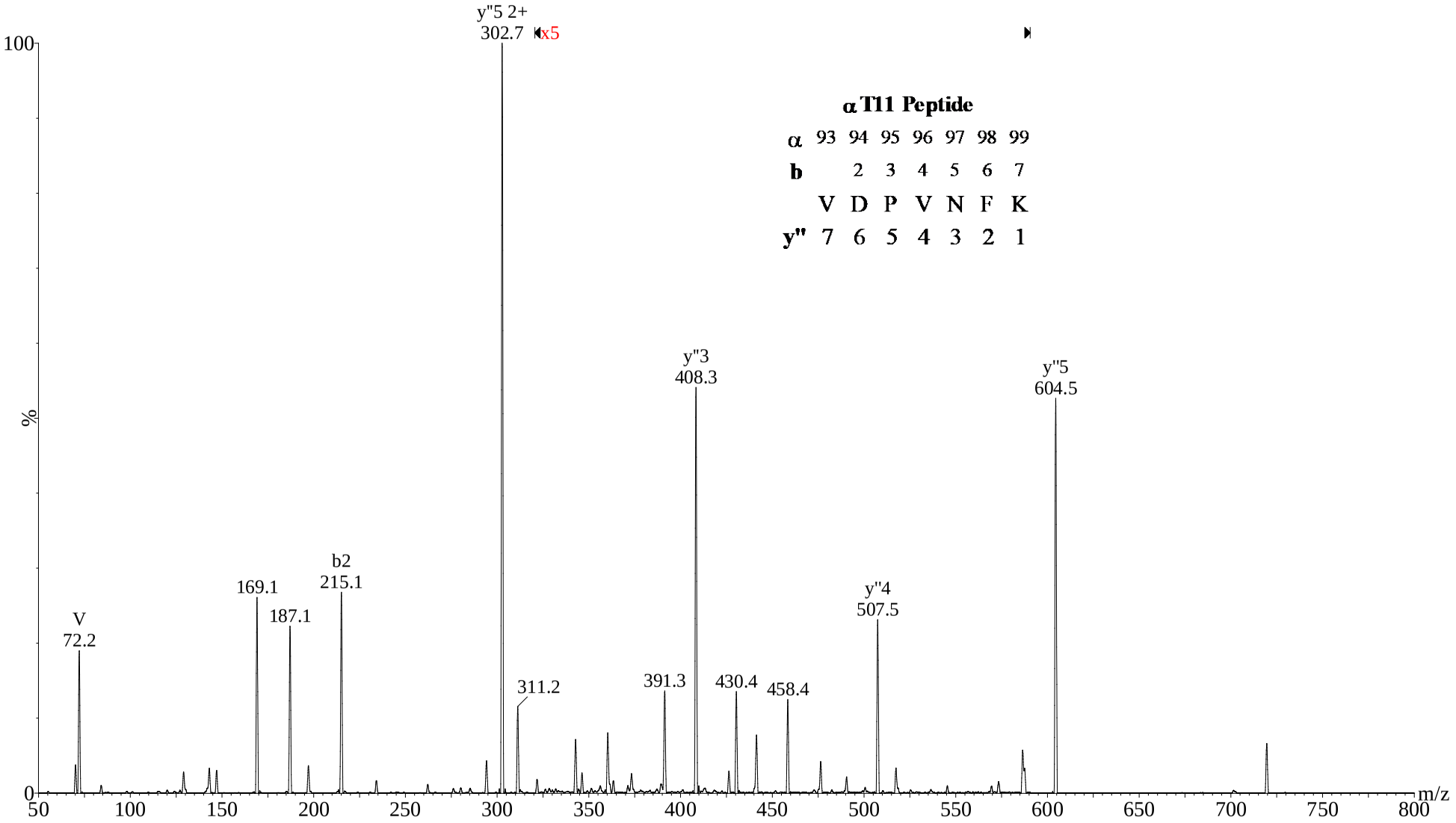

Figure 5.3.18.7. Product ion spectrum of the $\alpha T11^{2+}$ tryptic fragment of Hb J-Broussais (α90Lys→Asn). This spectrum is fully consistent with the sequence of the αT11 peptide that is not normally observed in Normal Hb tryptic digests owing to hindrance of the α90Lys.

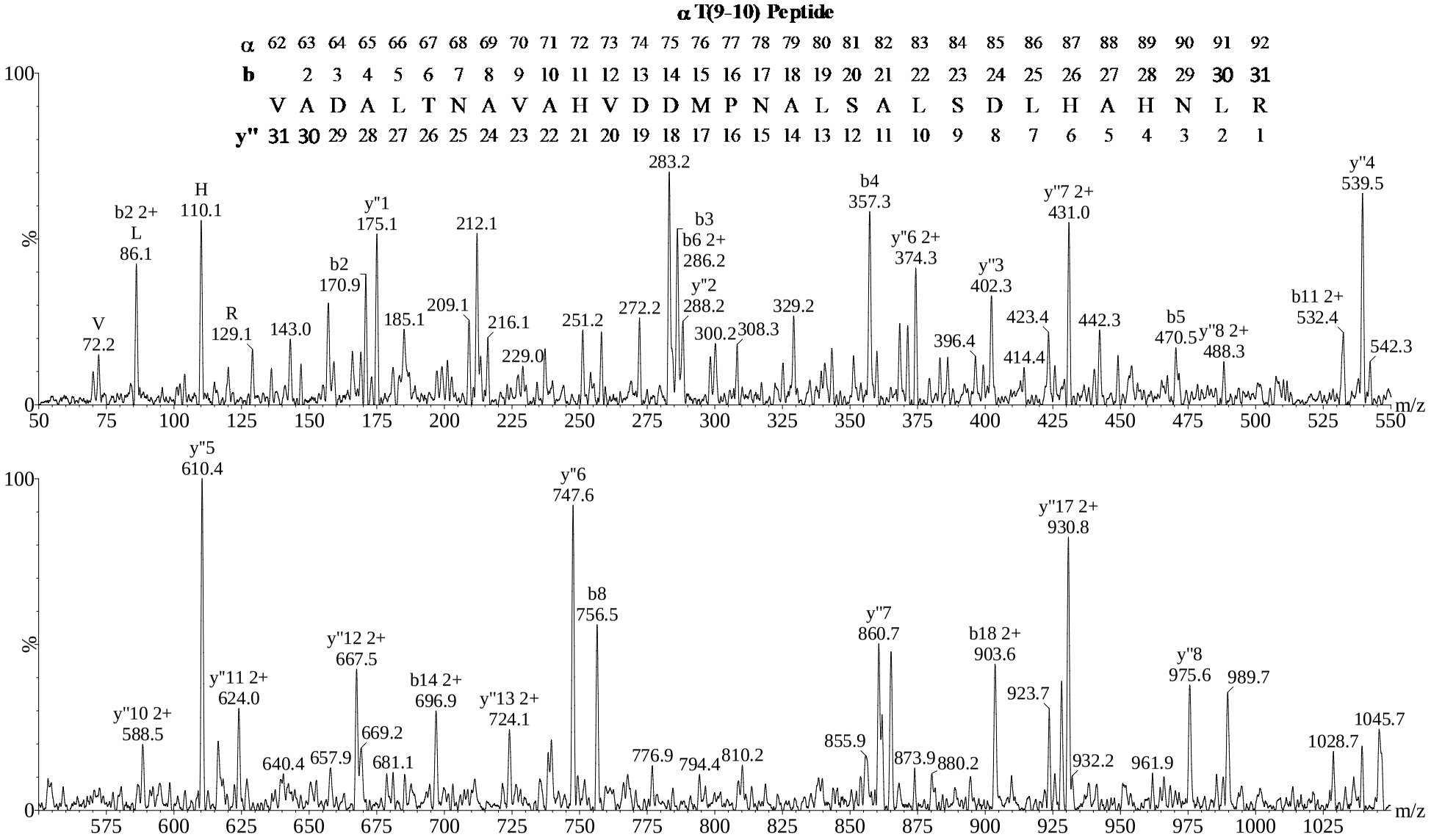

Figure 5.3.18.8. Partial product ion spectra of the $\alpha T(9\text{-}10)^{3+}$ tryptic fragment of Hb J-Broussais. This spectrum is fully consistent with the sequence of the variant αT(9-10) peptide with α90Lys→Asn.

5.3.19. αT11 - Hb Setif (α94Asp→Tyr)

Hb Setif is the result of an α-chain mutation in which the α94 amino acid residue is changed from Asp to Tyr through a single base change in the codon GAC→TAC.

αT11 Peptide

α	93	94	95	96	97	98	99
	V	D	P	V	N	F	K
		↓					
		Y					

Figure 5.3.19.1. Sequence of the Hb Setif αT11 tryptic peptide.

A blood sample was submitted for analysis by mass spectrometry because the ce-HPLC trace (Figure 5.3.13.2.) showed an abnormally high unknown response (16.7%) at 4.72 min, indicating a positive charge change arising from the mutation.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	0.4	---	1.05	8256
Unknown	---	0.7	1.25	18161
P2	---	3.4	1.34	83210
Unknown	---	0.6	1.52	15928
P3	---	3.2	1.72	78987
A0	---	72.1	2.38	1771346
A2	2.6	---	3.65	68278
Unknown	---	16.7	4.72	410991

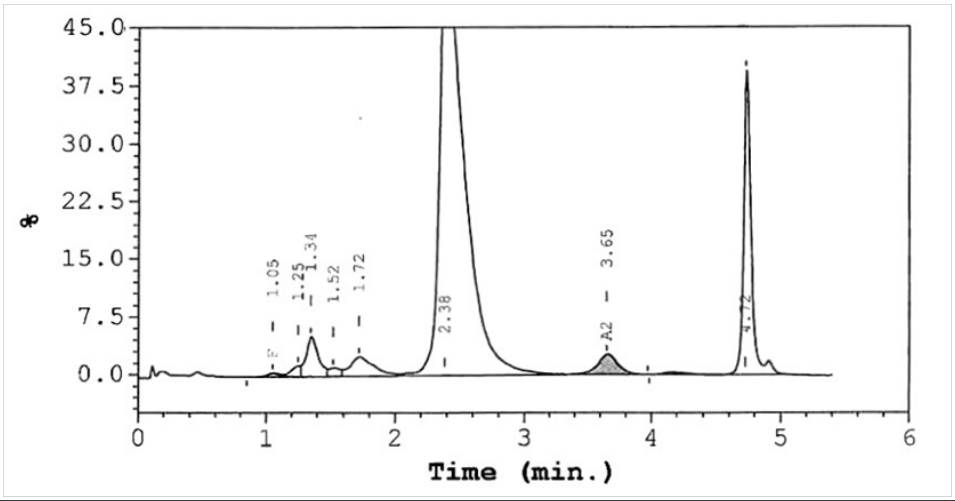

Figure 5.3.19.2. ce-HPLC trace for Hb Setif.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.3.19.3.) revealed an α-chain heterozygote in which the α-chain was 19.1% of total α-chains at a mass of 15,173.98 Da, 47.64 Da heavier than normal. A single codon change giving a mass increase of +48 Da could be Val→Phe (neutral) or Asp→Tyr (positive). The shift in the ce-HPLC trace indicates a positive charge change and infers the Asp→Tyr mutation (8 possibilities).

Figure 5.3.19.4. shows a diagnostic part of the tryptic digest spectra from (a) normal Hb and (b) the variant Hb. The appearance of a peak at *m/z* 866.48 for the variant $\alpha T11^{+}$ tryptic fragment in the lower panel is consistent with the mutation occurring in the αT11 peptide, and could only be α94Asp→Tyr,

Hb Setif. This is further supported by the presence of the doubly charged $\alpha T11^{2+}$ tryptic fragment in the lower panel of Figure 5.3.19.5. at *m/z* 433.76.

In Figure 5.3.19.6. the very low abundance of the $\alpha T(10\text{-}11)^{2+}$ ion, together with the variant αT11 peptide in Hb Setif, strongly supports the view that the cleavage at α92Arg is normally hindered by α94Asp.

Figure 5.3.4.7. shows product ion spectra from (a) normal $\alpha T11^{2+}$ precursor ion and (b) the variant $\alpha T11^{2+}$ precursor ion. The immonium ion at *m/z* 136.0 confirms the presence of Tyr in the variant. The occurrence of all the y" ions up to and including $y"_6$ at the same *m/z* in both the normal and variant precursor ion spectra, and the mass increase of 48 Da in the $y"_6{}^{2+}$ fragment in the lower panel confirms the mutation as α94Asp→Tyr, Hb Setif.

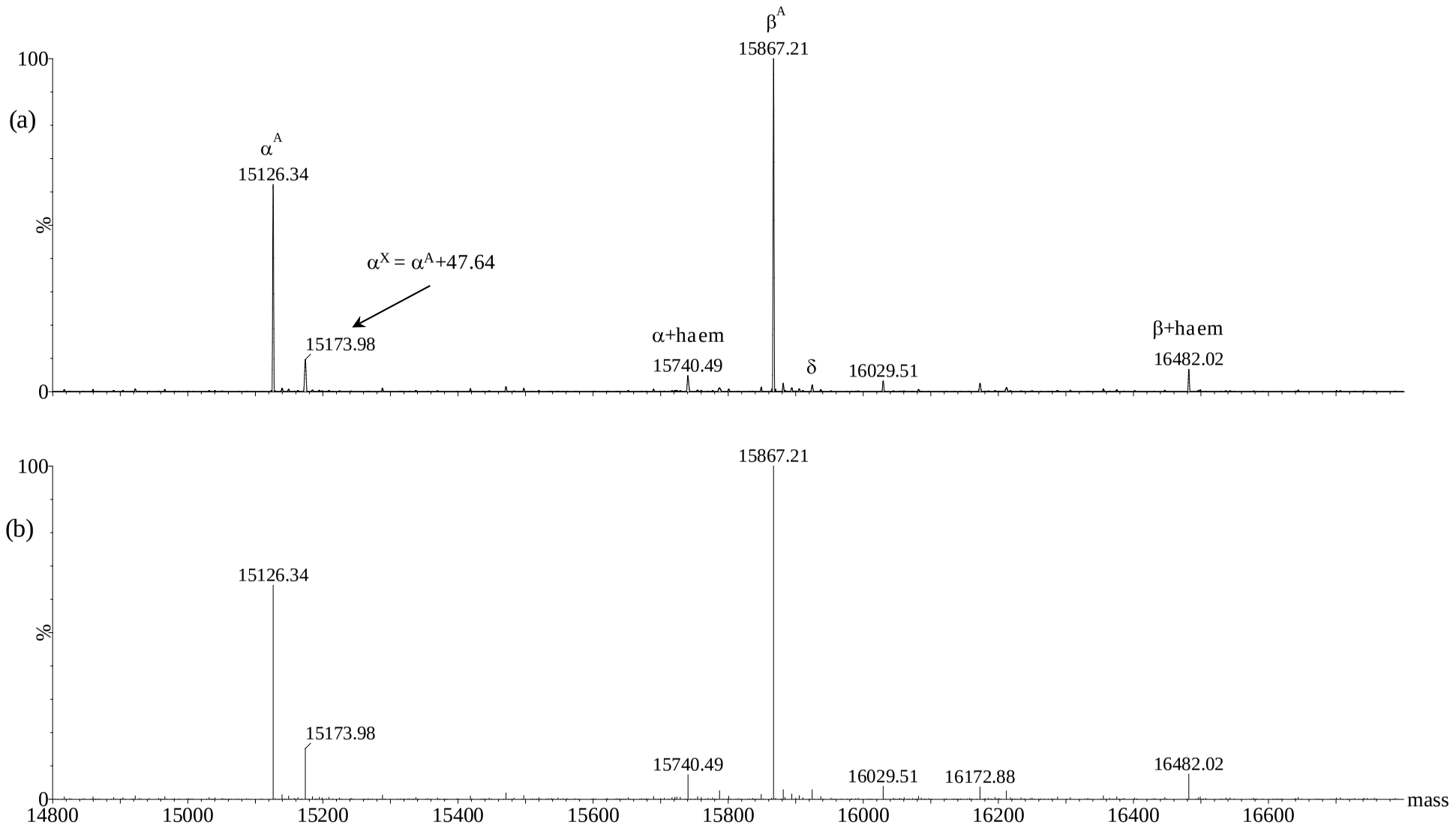

Figure 5.3.19.3. Deconvoluted mass spectrum of Hb Setif (α94Asp→Tyr) showing the presence of a signal at 15,173.98 Da and approximately 19% of the total α-chains. The +48 Da mass difference, coupled with a positive charge change, can only relate to an Asp→Tyr change.

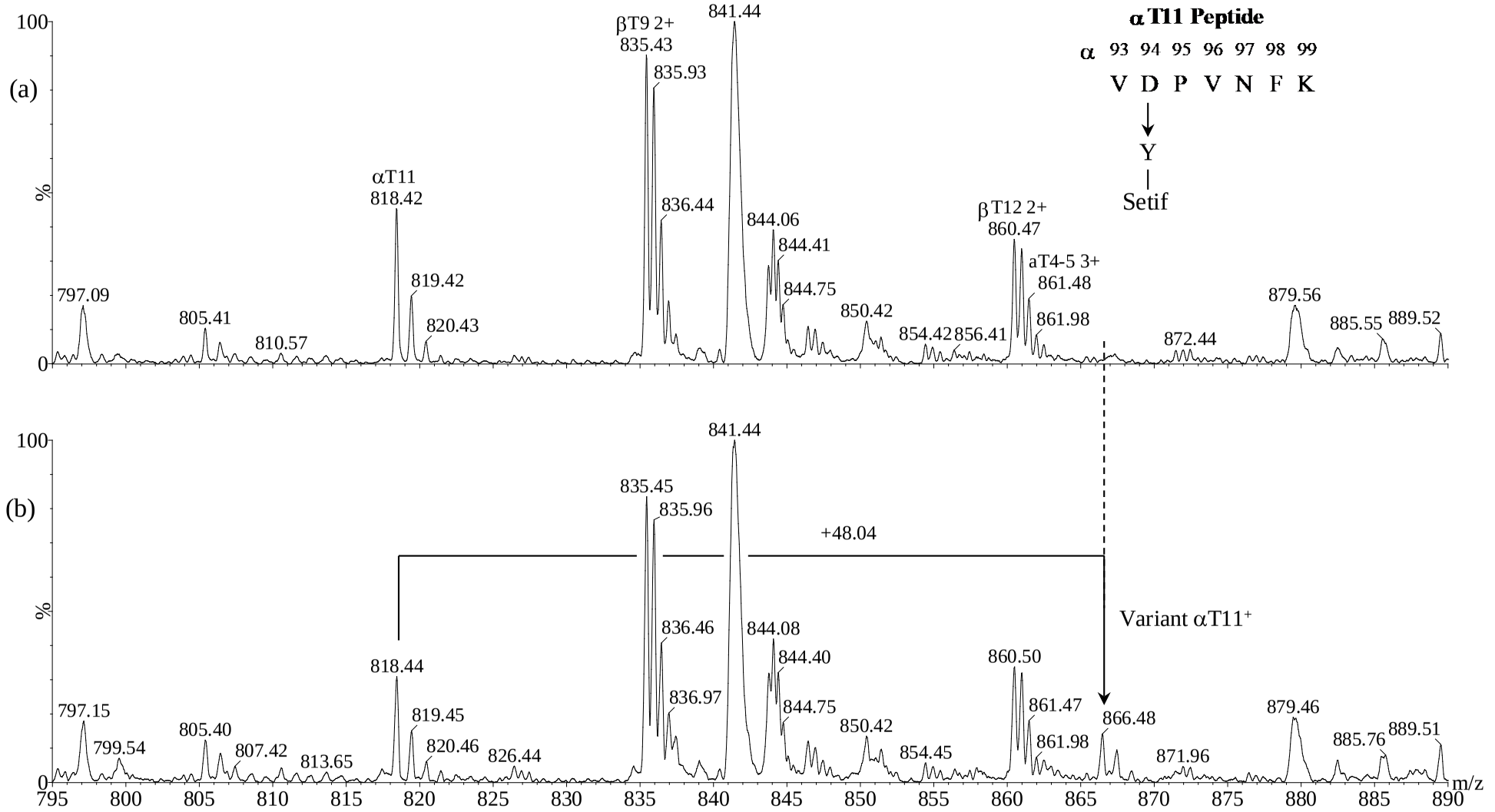

Figure 5.3.19.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Setif heterozygote.

271

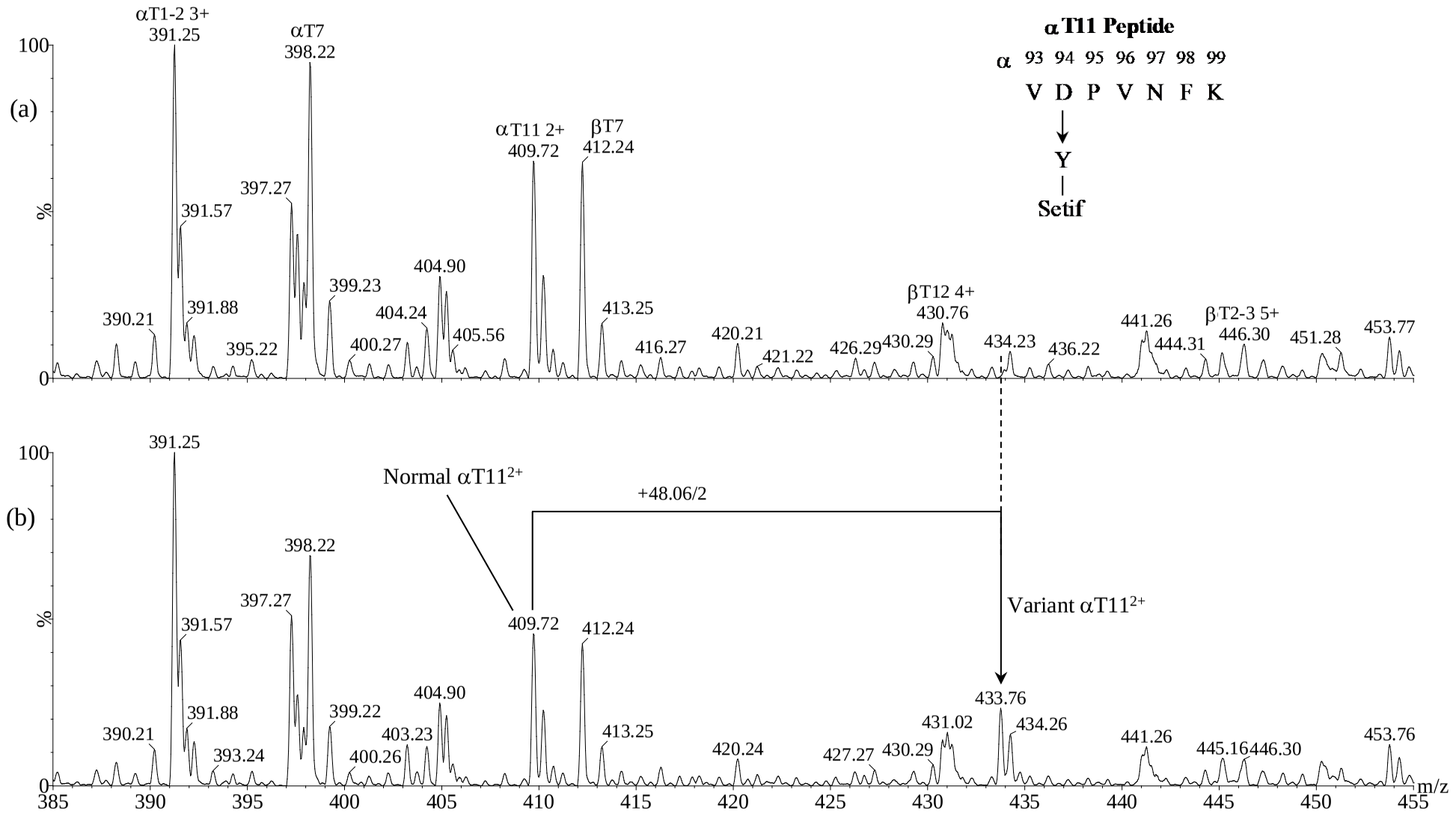

Figure 5.3.19.5. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Setif heterozygote. The variant $\alpha T11^{2+}$ ion was only observed after reducing the cone voltage from its normal 80/45 to 80/35.

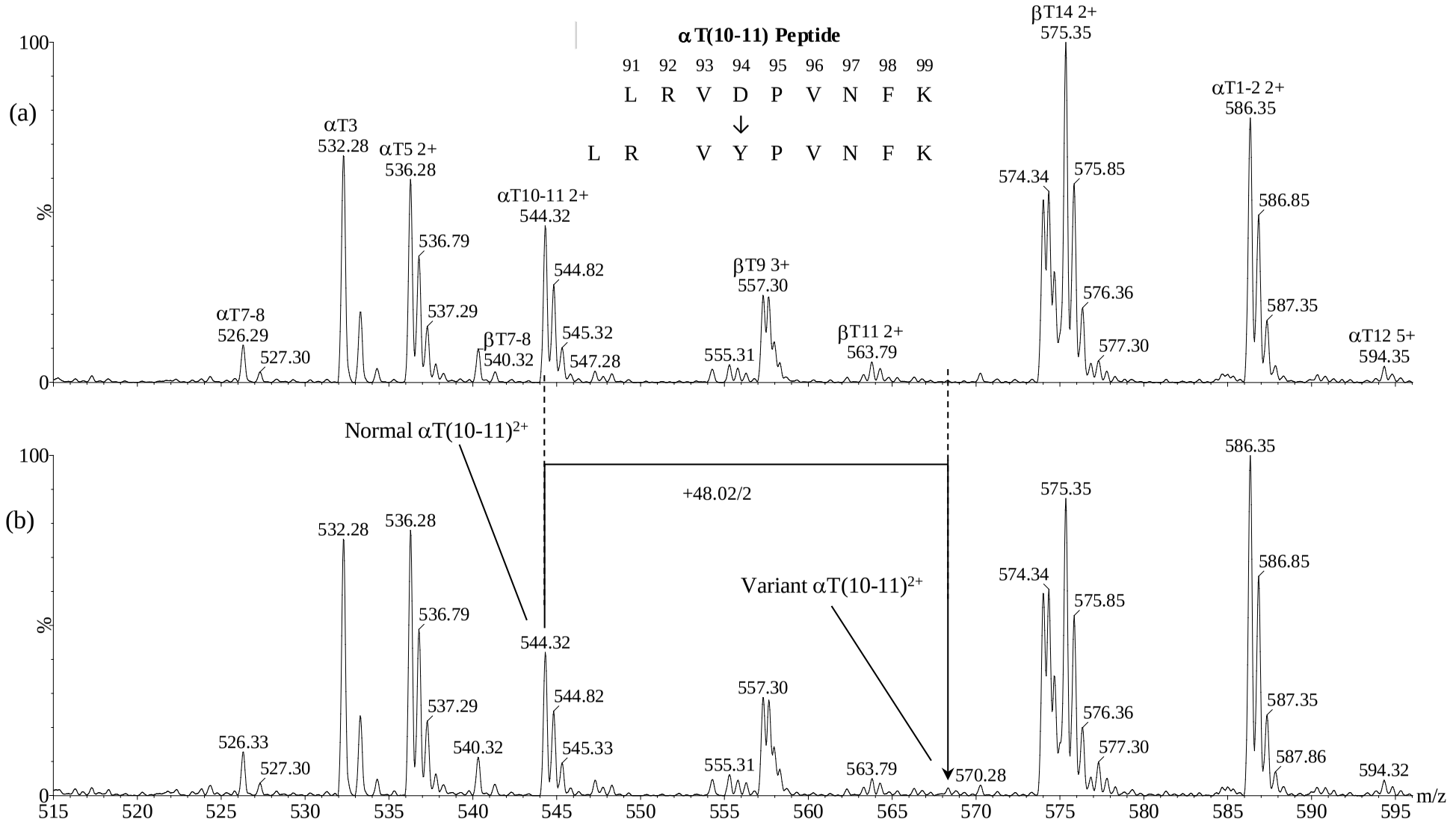

Figure 5.3.19.6. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Setif heterozygote. The very low abundance of the $\alpha T(10\text{-}11)^{2+}$ ion in the variant spectrum, together with the variant αT11 peptide in Figures 5.3.19.4. and 5.3.19.5., strongly supports the view that the cleavage at α92Arg is normally hindered by α94Asp.

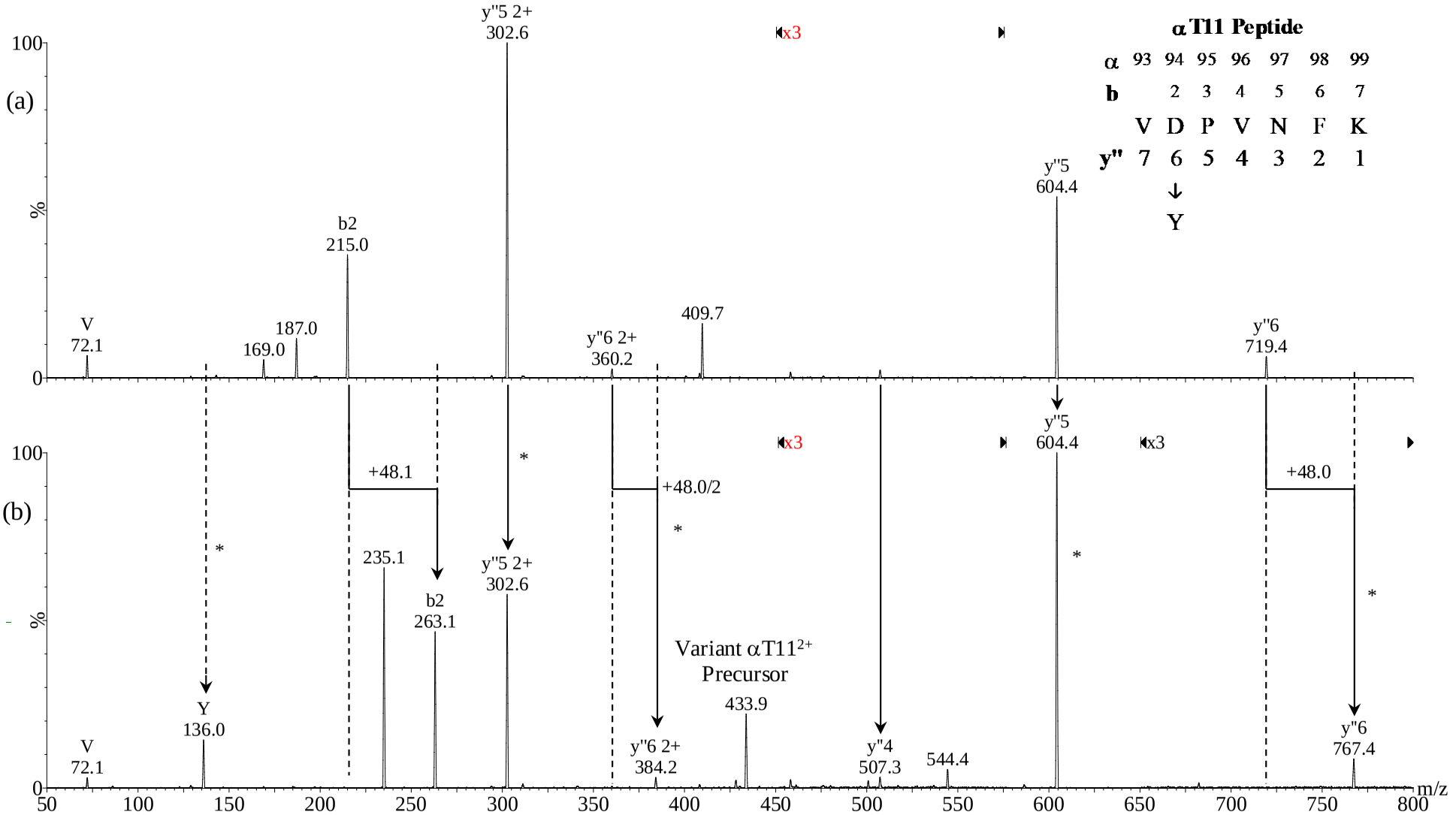

Figure 5.3.19.7. Product ion spectra of the $\alpha T11^{2+}$ tryptic fragment of (a) normal Hb and (b) Hb Setif. The 48 Da mass increase of $y''_6{}^{+}$ and $y''_6{}^{2+}$ confirms the mutation α94Asp→Tyr and is also supported by the presence of the Y immonium ion in the variant spectrum.

5.3.20. αT12 - Hb Hopkins-II (α112His→Asp)

Hb Hopkins-II is the result of an α-chain mutation in which the α112 amino acid residue is changed from His to Asp through a single base change in the codon CAC→GAC.

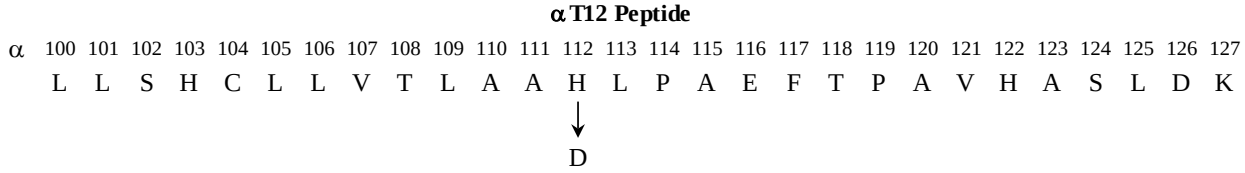

Figure 5.3.20.1. Sequence of the Hb Hopkins-II αT12 tryptic peptide.

A blood sample was submitted for analysis by mass spectrometry because the ce-HPLC trace (Figure 5.3.5.2.) showed an abnormally high P3 response (15.8%) at 1.54 min and implies a negative charge change.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.1	0.92	1855
F	1.0	---	1.04	14328
Unknown	---	0.6	1.24	9579
P2	---	3.8	1.34	61276
P3	---	15.8	1.54	254180
A0	---	76.1	2.47	1223244
A2	2.4	---	3.63	42492

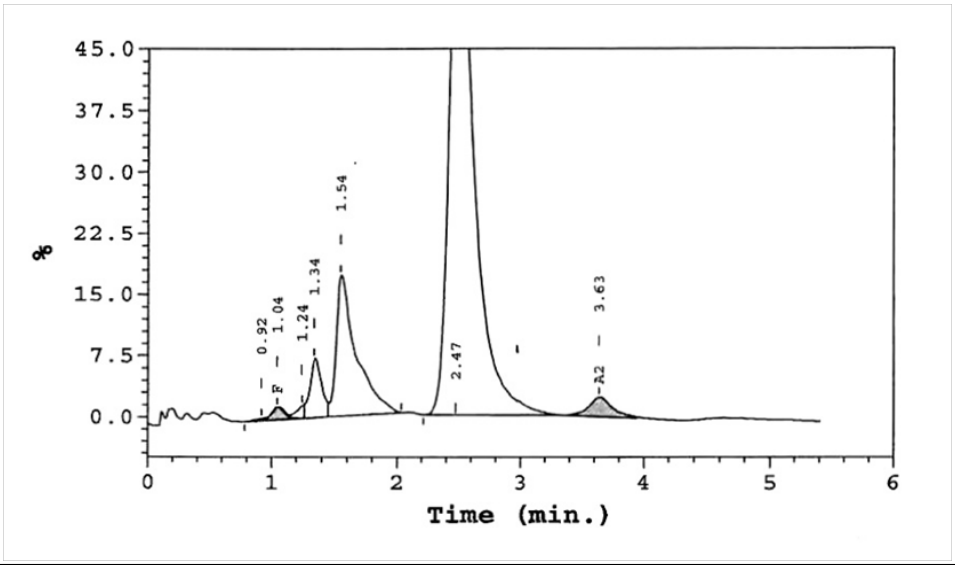

Figure 5.3.20.2. ce-HPLC trace for Hb Hopkins-II.

The spectrum obtained by ESI-MS analysis of the blood sample diluted 500-fold is shown in Figure 5.3.20.3. and reveals an α-chain heterozygote in which the variant-chain is 14.6% of the total α-chains at 15,104.48 Da, 21.92 Da lighter than the normal α-chain. The mass difference indicates that the mutation can only be His→Asp (10 possibilities) through a single base change in the codon, and this is also supported by the negative polarity change in the ce-HPLC trace.

Figure 5.3.20.4. shows the diagnostic part of the spectra from (a) normal Hb and (b) the variant Hb heterozygote. The appearance of a peak at *m/z* 1,046.01 in the variant spectrum supports the mutation His→Asp occurring in the αT(12-13) peptide, in which there are three possible sites of mutation through

a single base change in the codon - α103His→Asp (not previously reported), α103His→Asp (Hb Hopkins II) and α122His→Asp (not previously reported).

Figure 5.3.20.5. shows partial product ion spectra from (a) normal $\alpha T(12\text{-}13)^{4+}$ precursor ion and (b) the variant $\alpha T(12\text{-}13)^{4+}$ precursor ion. The occurrence of all the b ions up to and including b_{12}^{2+} at the same mass in both the normal and variant precursor ion spectra, coupled with the 22 Da mass decrease in the b_{13}^{2+} (*m/*z 676.3) in the variant spectrum, identifies the mutation as α112His→Asp, Hb Hopkins II. The identification is further supported in Figure 5.3.20.6. with the 22 Da mass change in the $y''_{28}{}^{2+}$ ion at *m/z* 1,474.0 in the variant spectrum.

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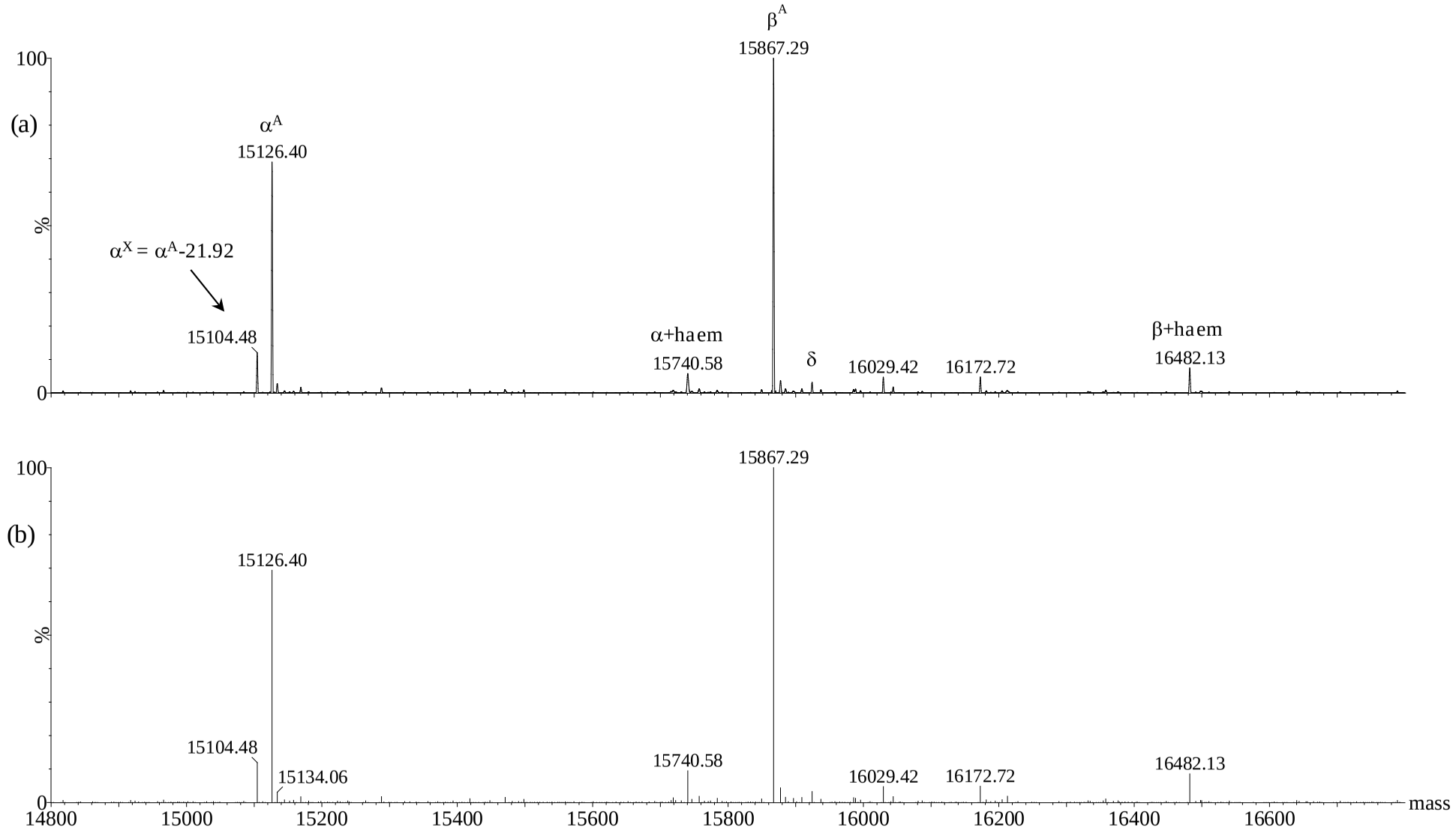

Figure 5.3.20.3. Deconvoluted mass spectrum of Hb Hopkins-II (α112His→Asp) showing the presence of a signal at 15,104.48 Da, at approximately 15% of the total α-chains. The -22 Da mass difference can only relate to a His→Asp change.

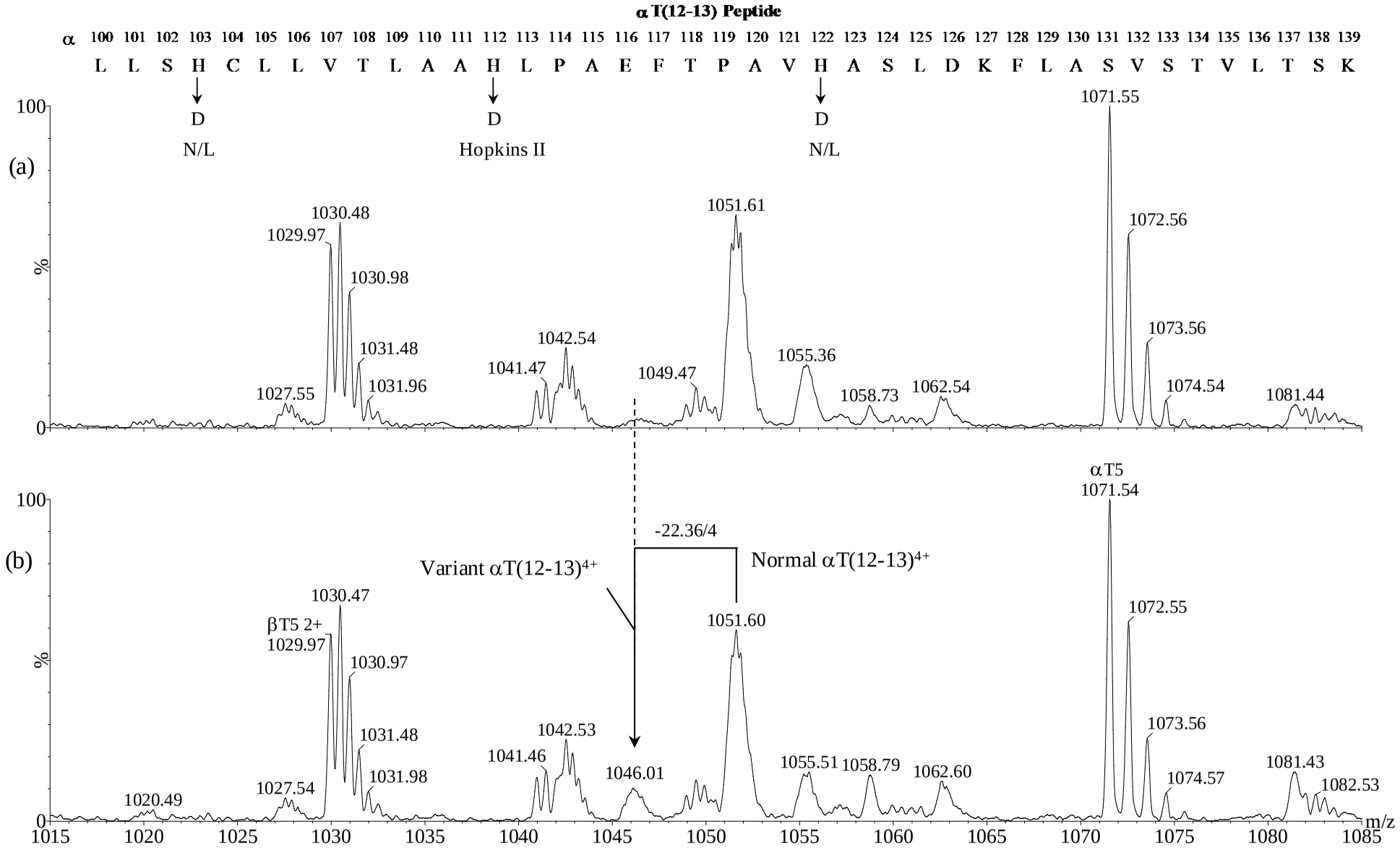

Figure 5.3.20.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Hopkins-II heterozygote.

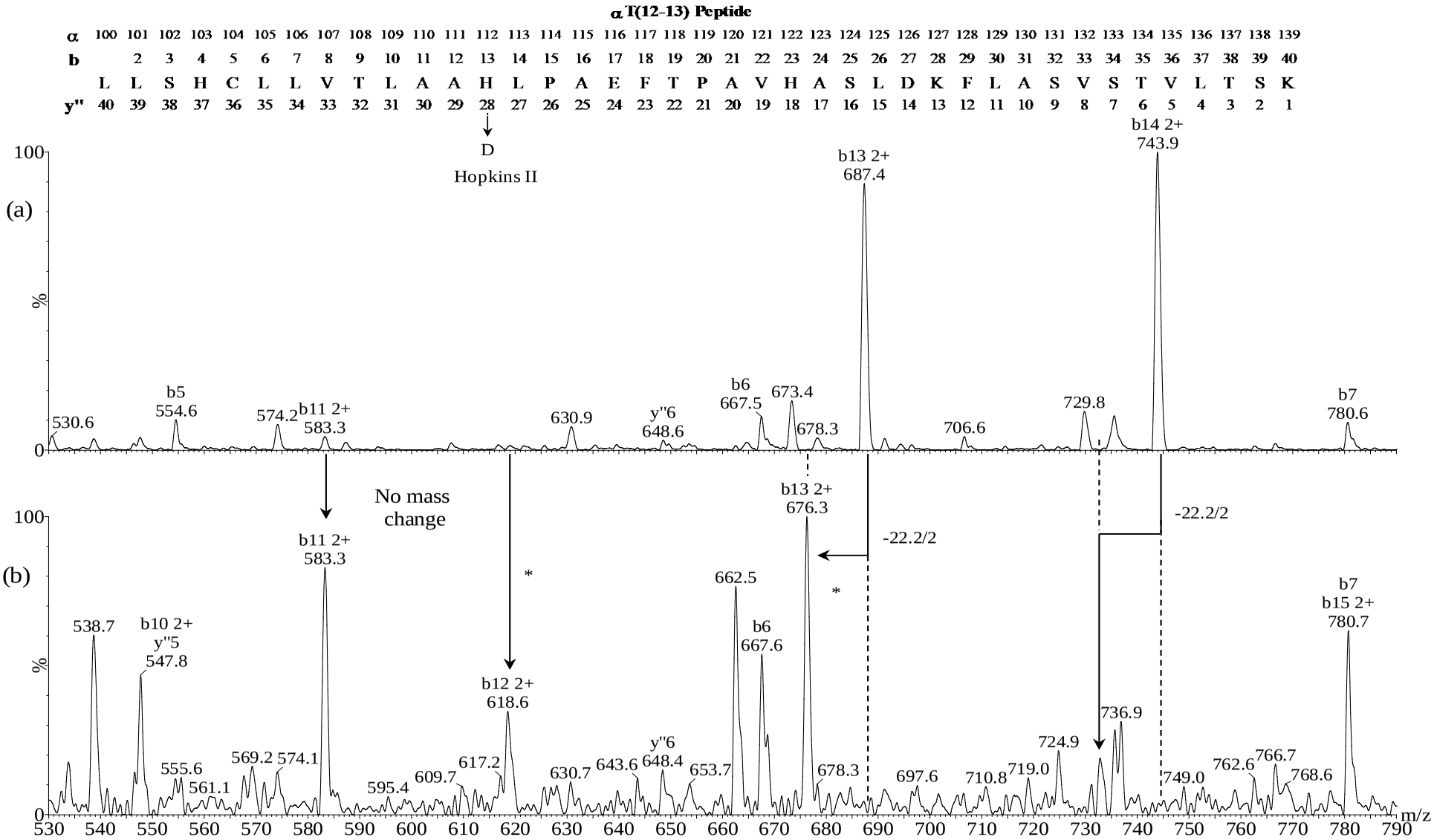

Figure 5.3.20.5. Partial product ion spectra of the $\alpha T(12\text{-}13)^{4+}$ tryptic fragment of (a) normal Hb and (b) Hb Hopkins-II . The 22.2 Da mass decrease at b_{13}^{2+} identifies the mutation as α112His→Asp.

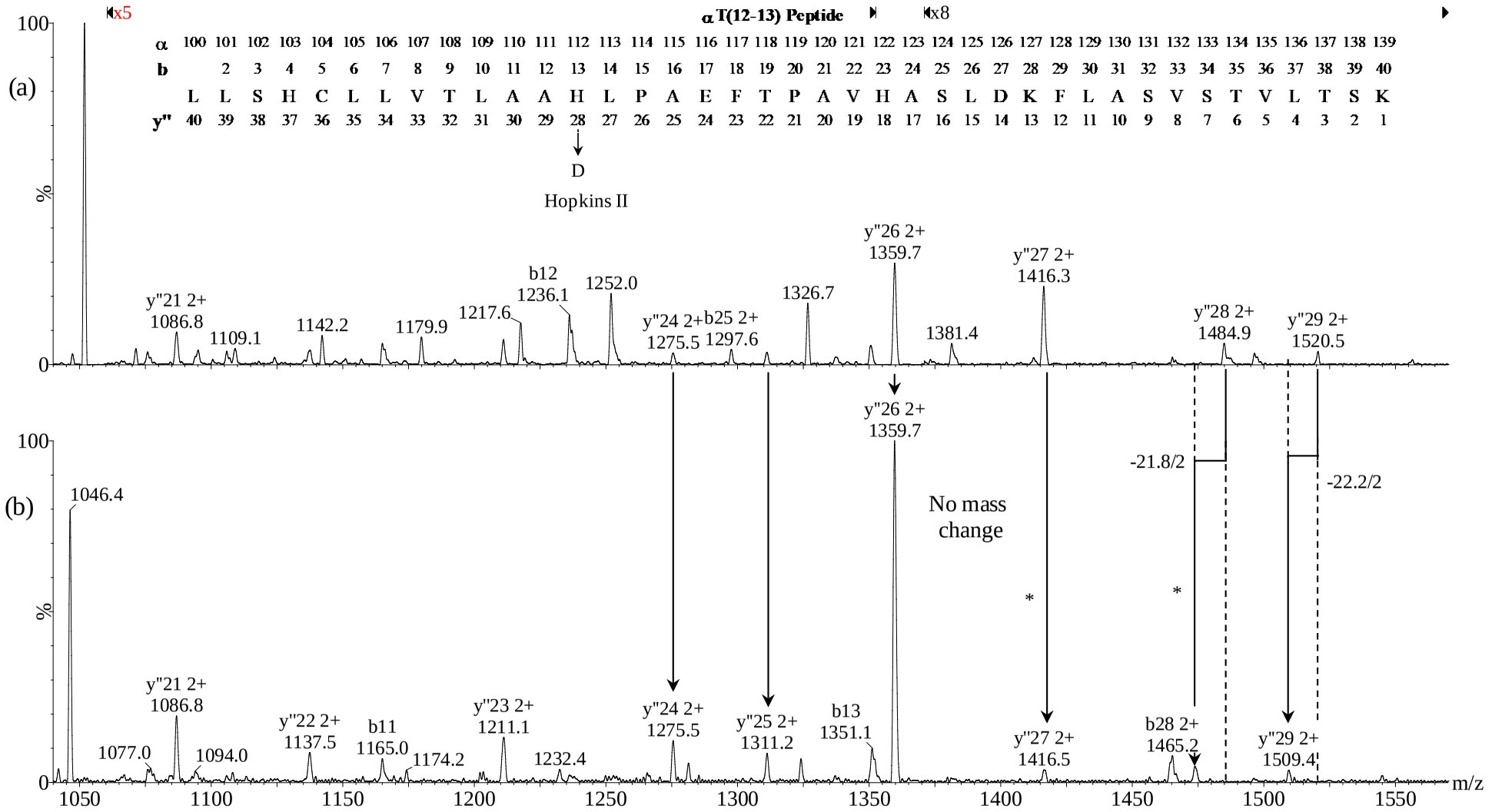

Figure 5.3.20.6. Product ion spectra of the $\alpha T(12\text{-}13)^{4+}$ tryptic fragment of (a) normal Hb and (b) Hb Hopkins-II. The 21.8 Da mass decrease at $y"_{28}{}^{2+}$ identifies the mutation as $\alpha 112 His \rightarrow Asp$.

5.3.21. αT12 - Hb J-Meerut (α120Ala→Glu)

Hb J-Meerut is the result of an α-chain mutation in which the α120 amino acid residue is changed from Ala to Glu through a single base change in the codon GCG→GAG. The codons for the six Ala amino acid residues in the αT(12-13) peptide are:

NL	NL	NL	J-Meerut	NL	NL
α110	α111	α115	α120	α123	α130
GCC	GCC	GCC	GCG	GCC	GCT

The Glu codons are GAA and GAG, thus only α120Ala (GCG) can mutate to Glu (GAG) by a single base change in the codon.

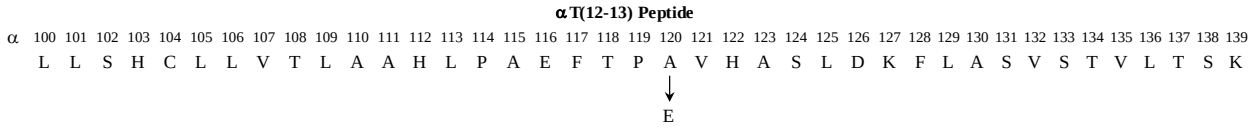

Figure 5.3.21.1. Sequence of the Hb J-Meerut αT(12-13) tryptic peptide.

A blood sample was submitted for analysis by mass spectrometry because the ce-HPLC trace (Figure 5.3.21.2.) showed an abnormally high unknown response (19.4%) at 1.95 min, indicating a negative charge change.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.2	1.00	3017
F	1.3	---	1.09	19987
Unknown	---	0.8	1.22	12670
P2	---	5.1	1.30	86047
P3	---	3.5	1.67	58340
Unknown	---	19.4	1.95	324591
A0	---	67.7	2.52	1132829
A2	2.2	---	3.62	35629

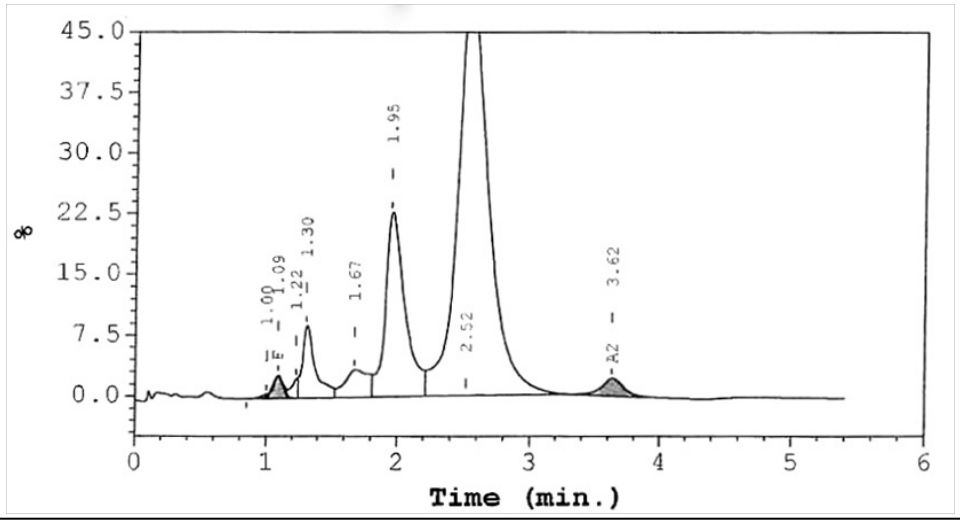

Figure 5.3.21.2. ce-HPLC trace for Hb J-Meerut.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.3.21.3.) revealed an α-chain heterozygote in which the α-chain was 21.5% of total α-chains and the mass of the variant chain was 15,184.52, 58.14 Da heavier than normal. A single codon change giving a mass increase of +58 Da could

be Ala→Glu (negative, 21 possibilities) or Gly→Asp (neutral, 7 possibilities). The shift in the ce-HPLC trace indicates a negative charge change and infers the Ala→Glu mutation.

Figure 5.3.21.4. shows the diagnostic part of the spectra from (a) normal Hb and (b) the variant Hb. The appearance of a peak at *m/z* 852.97 (αT(12-13)$^{5+}$) in the lower panel shows that the mutation occurs in the αT(12-13) peptide. There are six possible sites for Ala→Glu mutation, but only α120Ala (GCG) can mutate to Glu (GAG) through a single base change in the codon. Further evidence supporting the mutation in the αT(12-13) is shown in Figure 5.3.21.5. with the appearance of the αT(12-13)$^{4+}$ at *m/z* 1,066.13 in the variant spectrum.

Figures 5.3.21.6. and 5.3.21.7. show diagnostic mass ranges from the product ion spectra of the αT(12-13)$^{4+}$ from (a) normal Hb and (b) the variant Hb. The 57.8 Da increase in mass for the y"$_{21}$ (*m/z* 2,229.9) ion eliminates α130Ala→Glu and α123Ala→Glu as possible sites of mutation in Figure 5.3.21.6. The lack of mass change in the b_{14}^{2+} (*m/z* 744.0) precludes α110Ala→Glu and α111Ala→Glu in Figure 5.3.21.7.

The only remaining possibilities are α115 and α120, and the 57.8 Da mass increase for the y"$_{21}$ at *m/z* 2,229.9 (Figure 5.3.21.6.) confirms the mutation α120Ala→Glu, Hb J-Meerut.

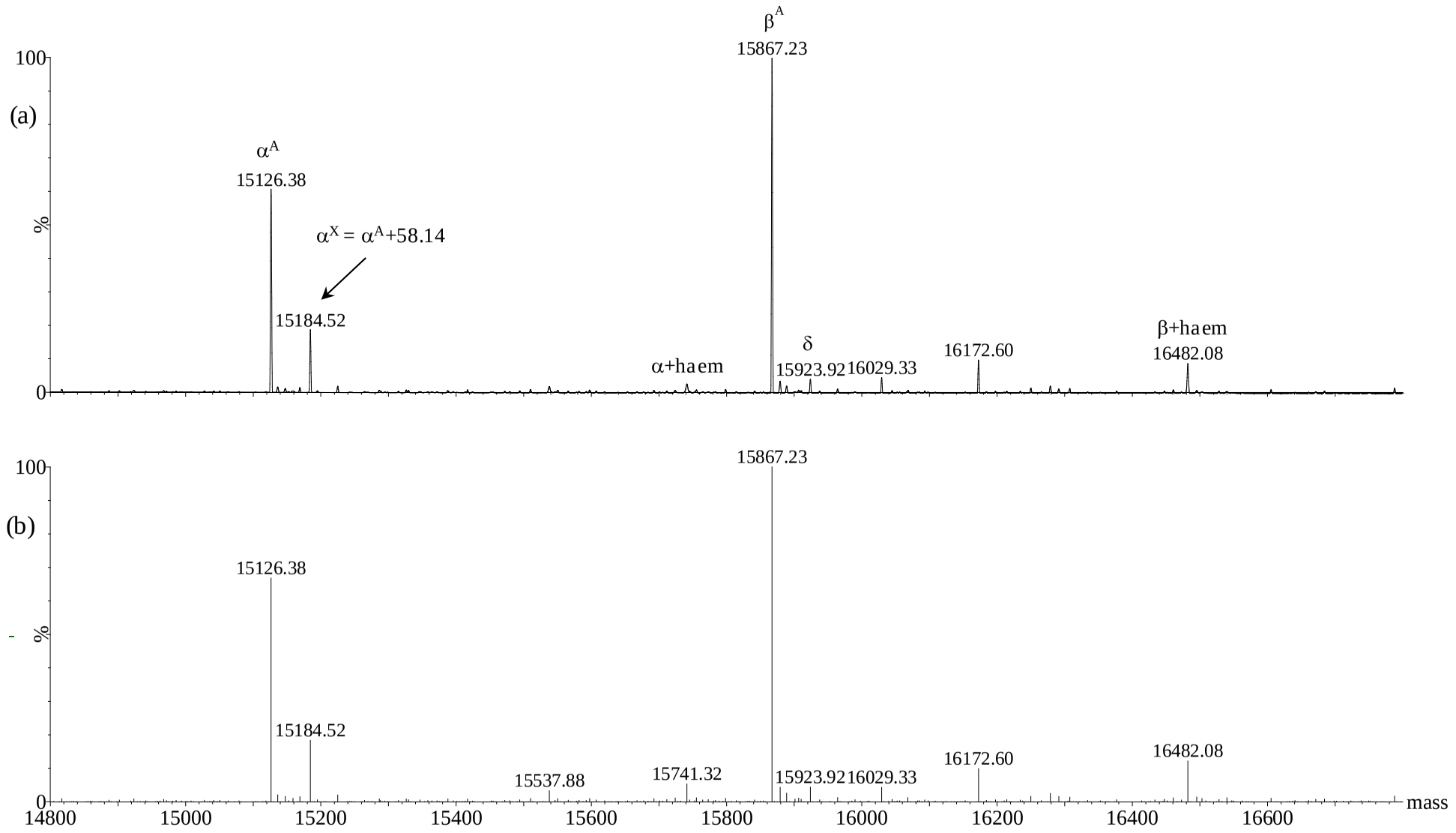

Figure 5.3.21.3. Deconvoluted mass spectrum of Hb J-Meerut (α120Ala→Glu) showing the presence of a signal at 15,184.52 Da at approximately 21.5% of the total α-chains. A +58 Da mass change could arise from Ala→Glu (21 possibilities) or Gly→Asp (7 possibilities).

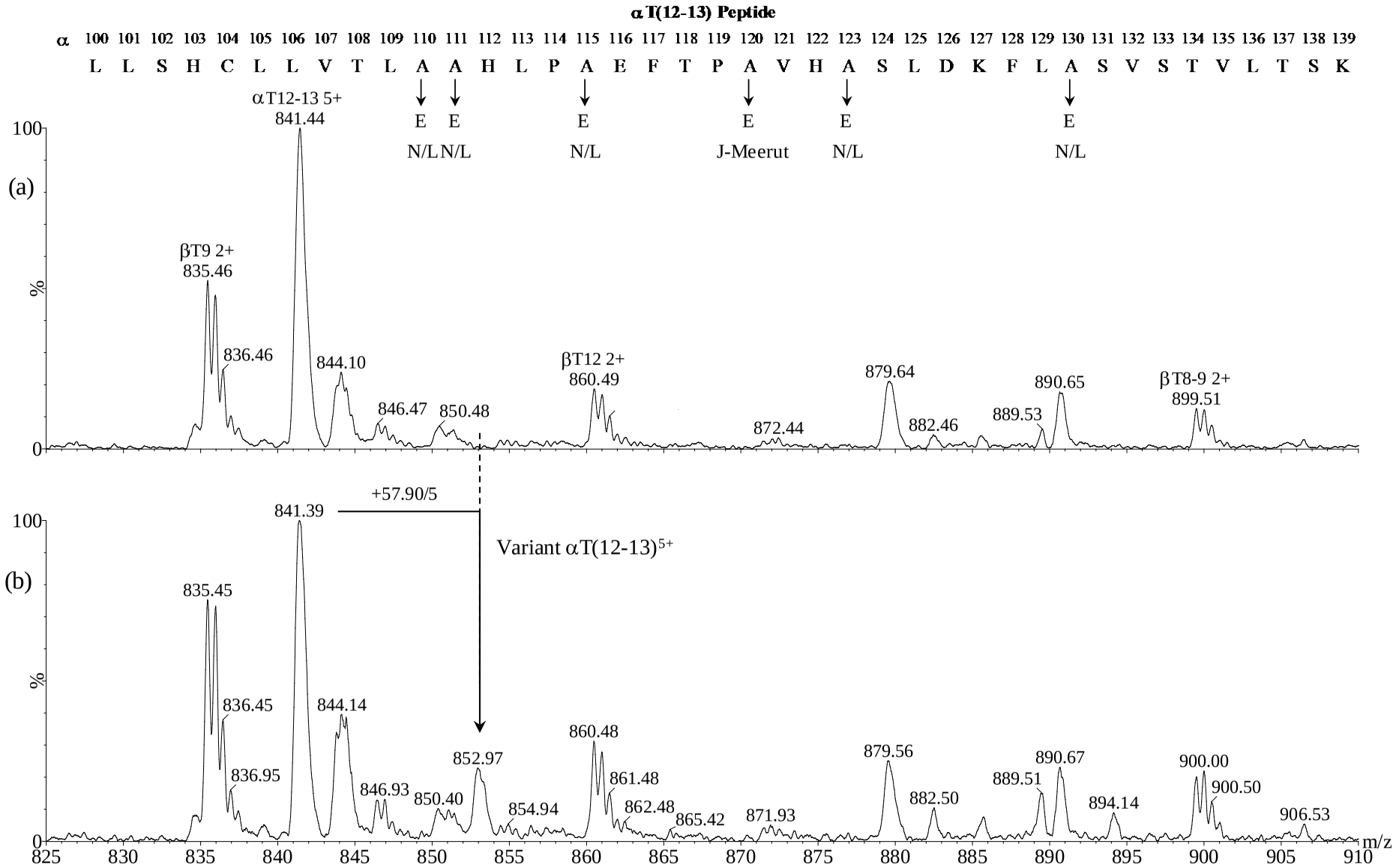

Figure 5.3.21.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb J-Meerut heterozygote. Of the six possible mutations in this peptide, only α120Ala can mutate to Glu by a single base change in the codon.

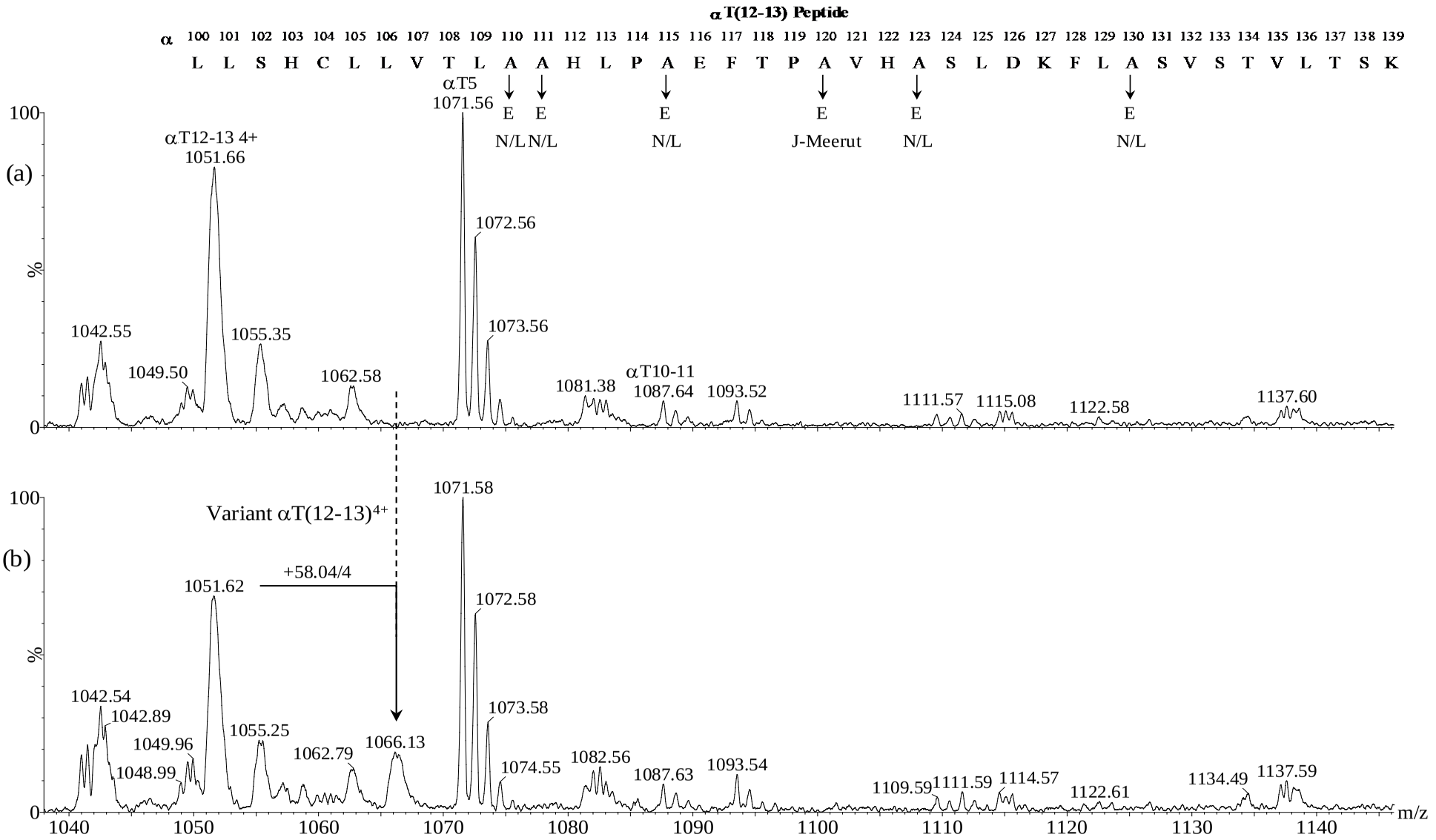

Figure 5.3.21.5. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb J-Meerut heterozygote.

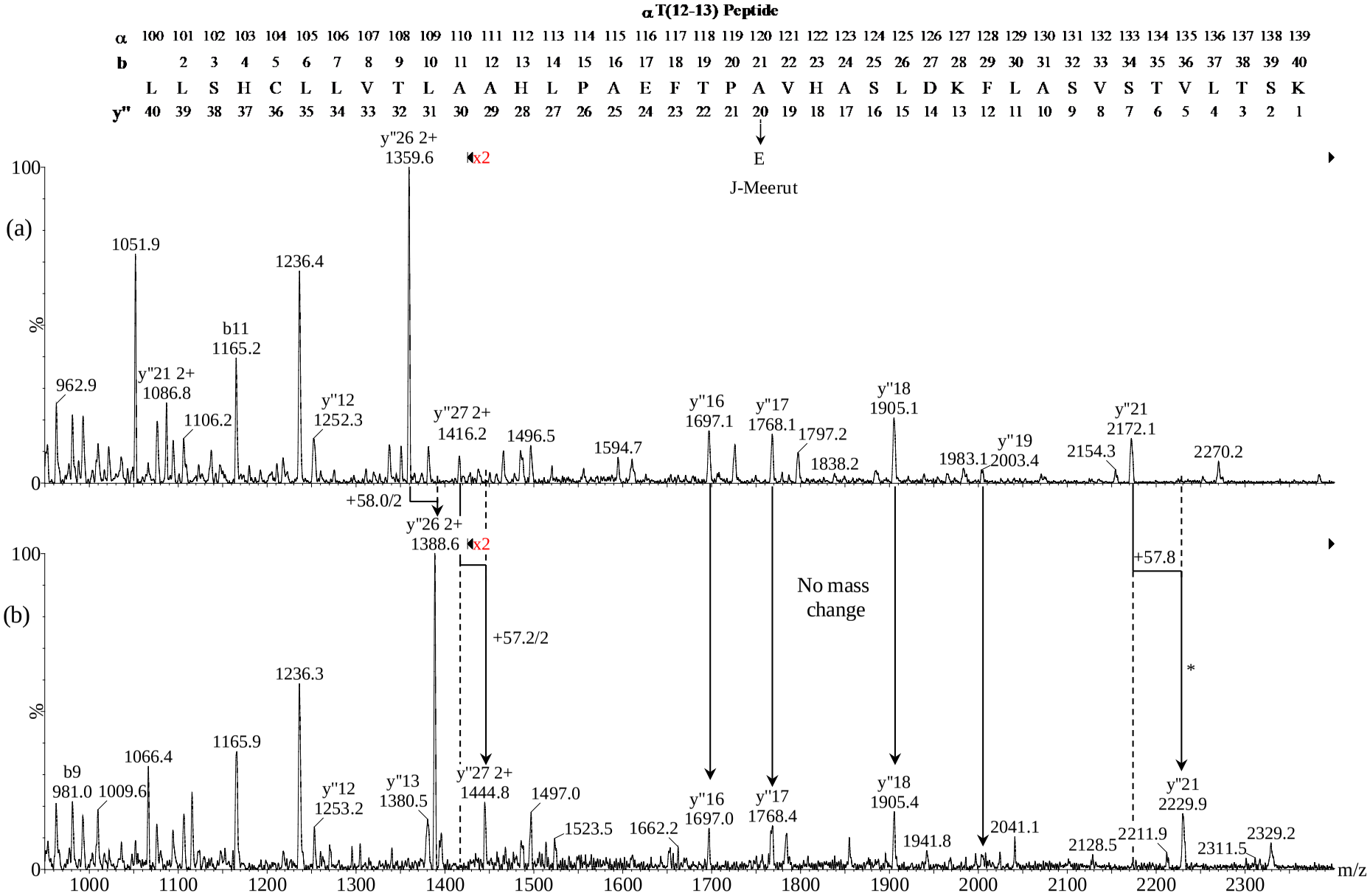

Figure 5.3.21.6. Product ion spectra of the αT(12-13)$^{4+}$ tryptic fragment of (a) normal Hb and (b) Hb J-Meerut. The 57.8 Da mass increase at y"$_{21}$, and no mass change at y"$_{19}$ supports the mutation at α120Ala→Glu by precluding the mutation of Ala→Glu at α123 and α130.

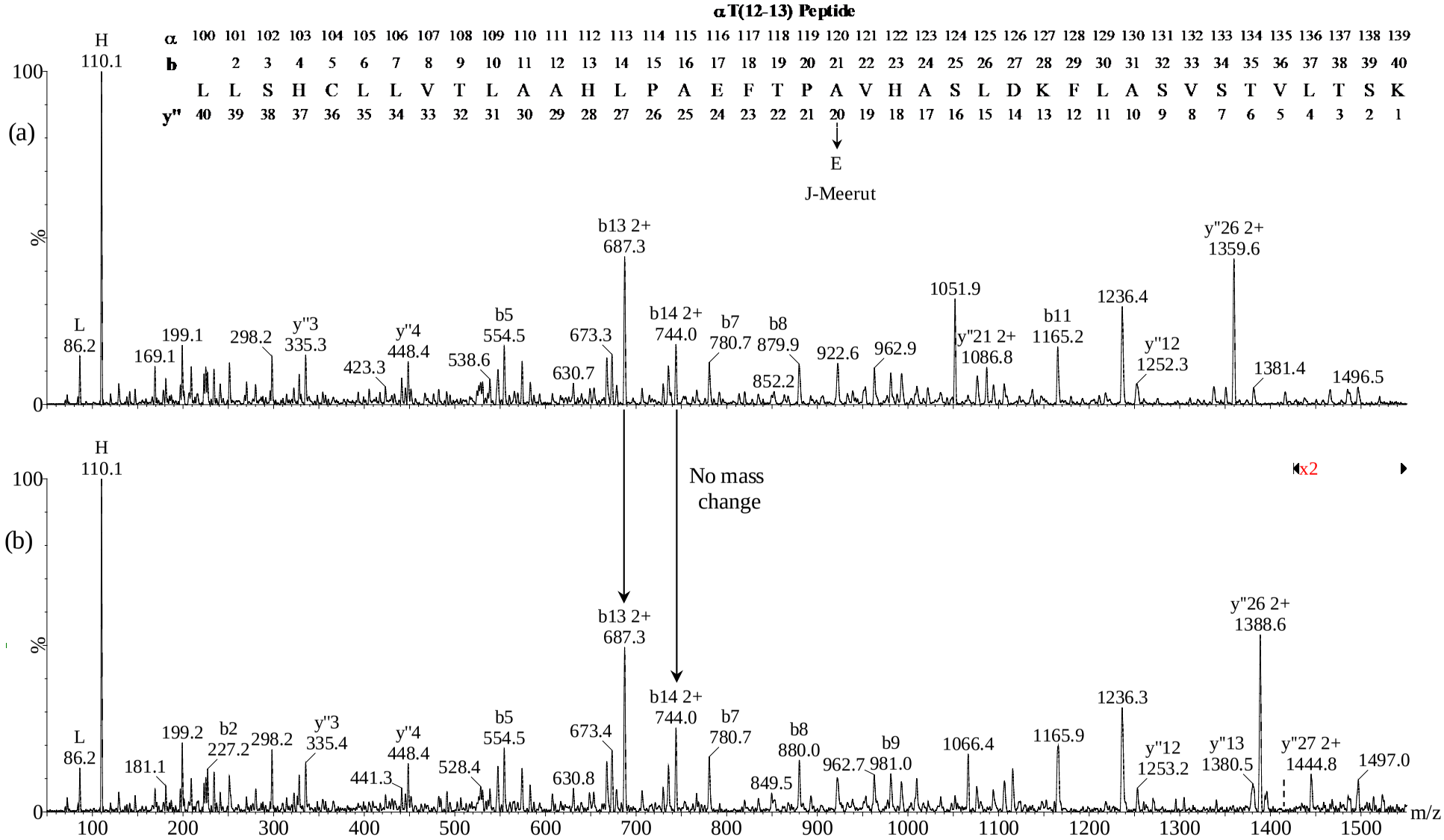

Figure 5.3.21.7. Partial Product ion spectra of the αT(12-13)$^{4+}$ tryptic fragment of (a) normal Hb and (b) Hb J-Meerut (α120Ala→Glu). No mass change at b_{13}^{2+} precludes the mutation of Ala→Glu at α110 and α111.

5.3.22. αT13 - Hb Wayne (α139–141 →139 NTVKLEPR)

Hb Wayne is the result of an α-chain mutation in which the α139–141 amino acid residues are changed from -Lys-Tyr-Arg to -Asn-Thr-Val-Lys-Leu-Glu-Pro-Arg. This occurs in the α2 gene through the deletion of a single codon giving rise to a frameshift.

The inclusion of a Lys residue results in an additional tryptic cleavage product, as shown in Figure 5.3.22.1. (α143-146).

αT(12-13) Peptide (Wayne)

α	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142
	L	L	S	H	C	L	L	V	T	L	A	A	H	L	P	A	E	F	T	P	A	V	H	A	S	L	D	K	F	L	A	S	V	S	T	V	L	T	S	N	T	V	K

Wayne		αT(12-13) Peptide																										αT14 Peptide			
α	--	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146
	--	P	A	V	H	A	S	L	D	K	F	L	A	S	V	S	T	V	L	T	S	N	T	V	K	L	E	P	R

Figure 5.3.22.1. Sequences of the Hb Wayne αT(12-13) tryptic peptide, and the αT(12-13) and αT14 peptides.

A blood sample was submitted for analysis by mass spectrometry because the Tosoh HPLC trace (Figure 5.3.22.2.) showed an abnormally high response at 1.80 min.

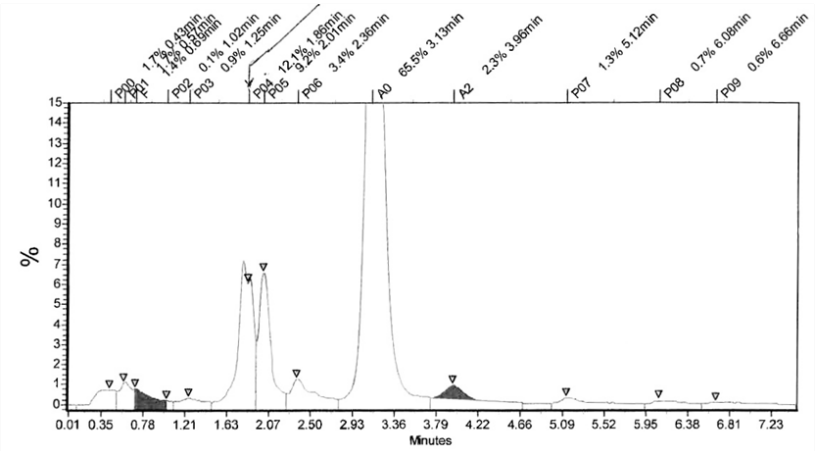

Figure 5.3.22.2. HPLC trace for Hb Wayne.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.3.22.3.) revealed an α-chain heterozygote in which the α-chain was 19.6% of total α-chains and the mass of the variant chain was 15,617.55 Da, 491.14 Da heavier than normal. The mass of the variant is characteristic of the mutation α(139-141) →α139NTVKLEPR, Hb Wayne.

Figure 5.3.22.4. shows part the diagnostic part of the tryptic digest spectra from (a) normal Hb and (b) the variant Hb. The appearance of a variant $\alpha T(12\text{-}13)^{4+}$ peak at *m/z* 1,130.41 is consistent with the mutation and extension α(139-141)→α139NTVKLEPR, Hb Wayne. This is further supported in Figure 5.3.22.5. and the appearance of a peak consistent with the variant $\alpha T(12\text{-}13)^{5+}$ at *m/z* 904.50 and Figure 5.3.22.6. with the appearance of the variant $\alpha T14^{+}$ at *m/z* 514.30.

Figure 5.3.22.7. shows the product ion spectrum from the variant $\alpha T(12\text{-}13)^{4+}$ precursor ion. The data are consistent with the αT(12-13) peptide from Hb Wayne.

Figure 5.3.22.8. shows the product ion spectrum from the variant $\alpha T14^{+}$ precursor ion. The data are consistent with the αT14 peptide from Hb Wayne.

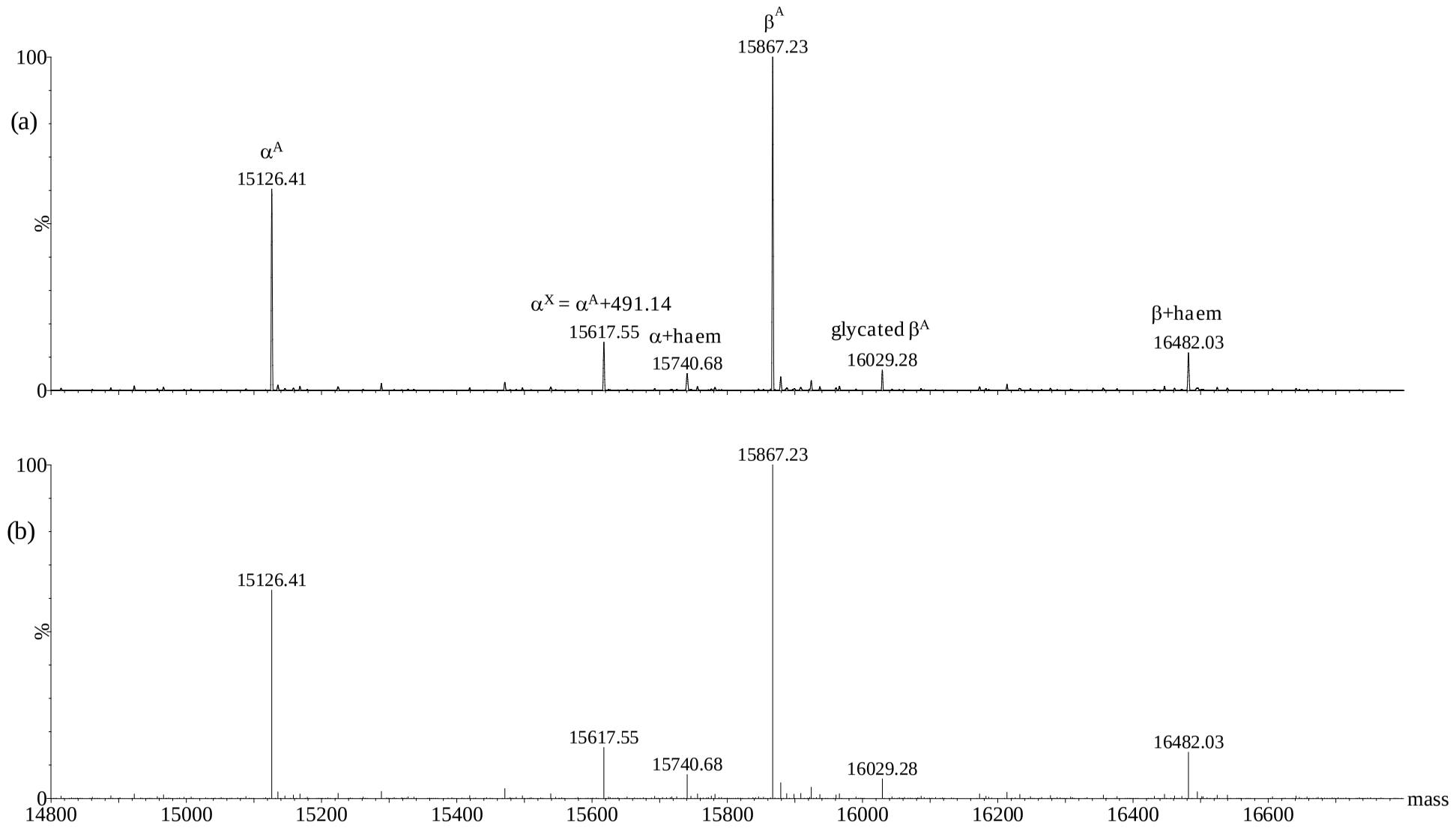

Figure 5.3.22.3. Deconvoluted mass spectrum of Hb Wayne (α139–141 →139 NYVKLEPR) showing the presence of a signal at 15,617.55 Da and the normal α-chain peak (15,126.41 Da). The variant α-chain represents approximately 20% of the total α-chains.

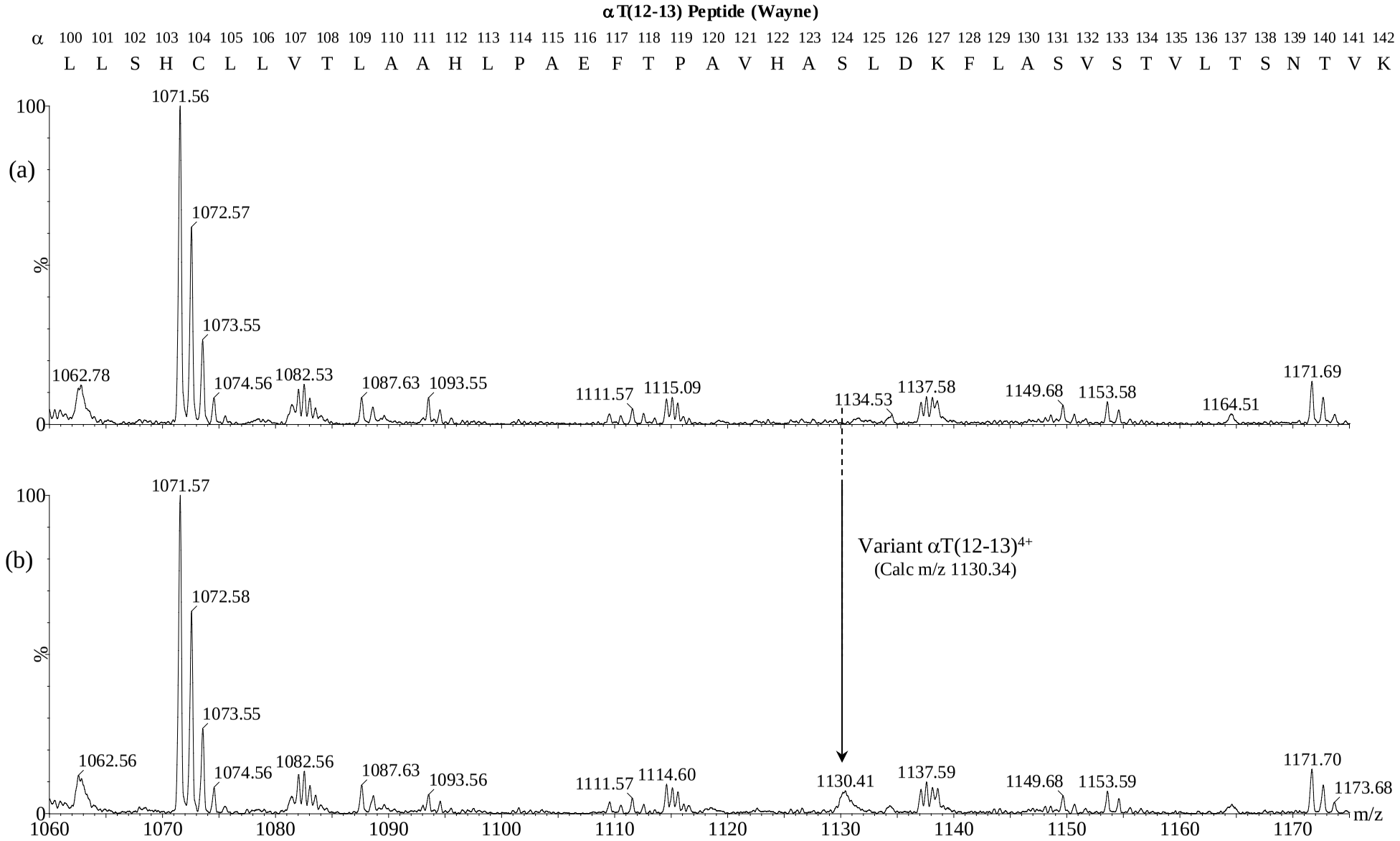

Figure 5.3.22.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Wayne heterozygote.

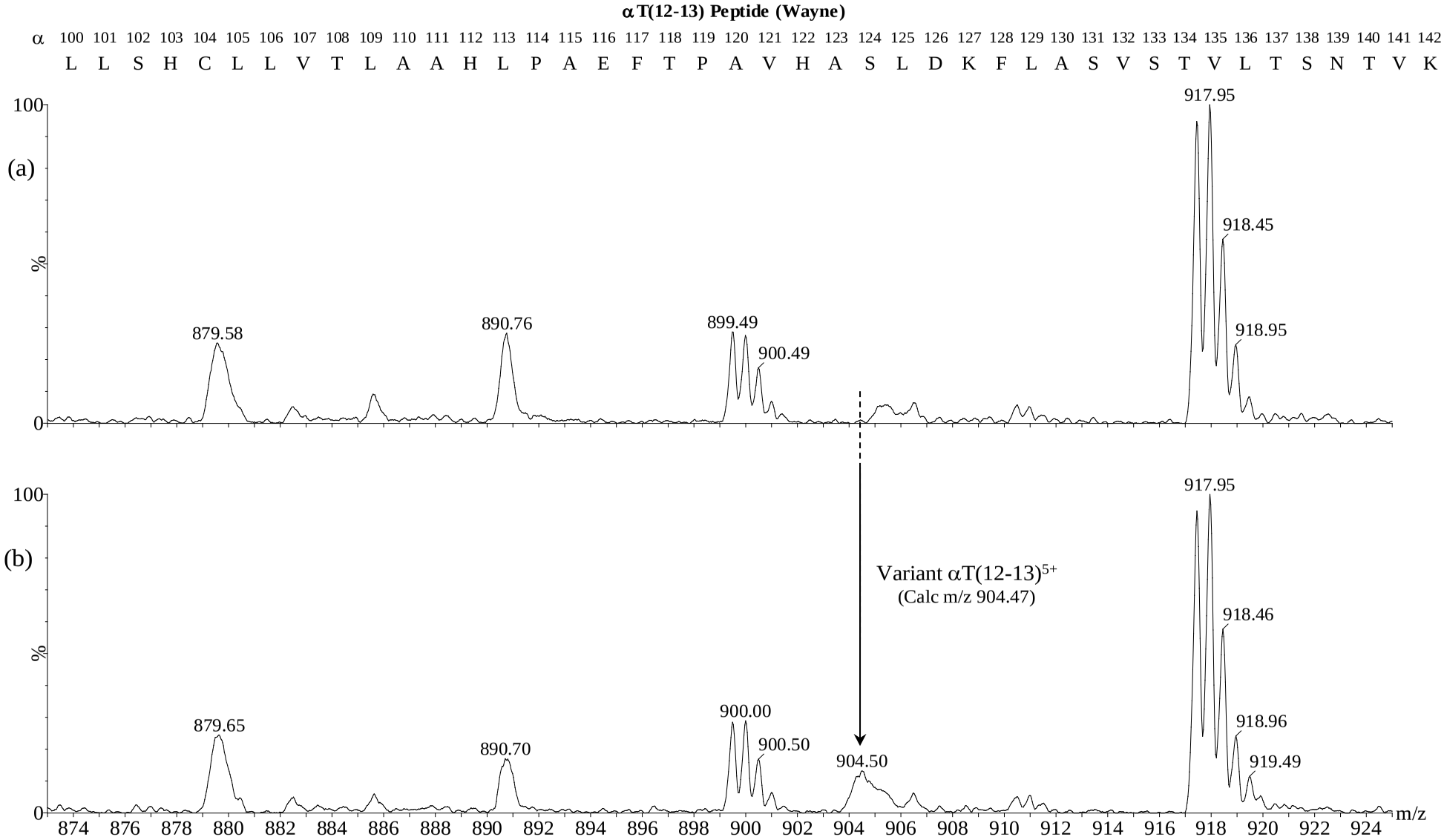

Figure 5.3.22.5. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Wayne heterozygote.

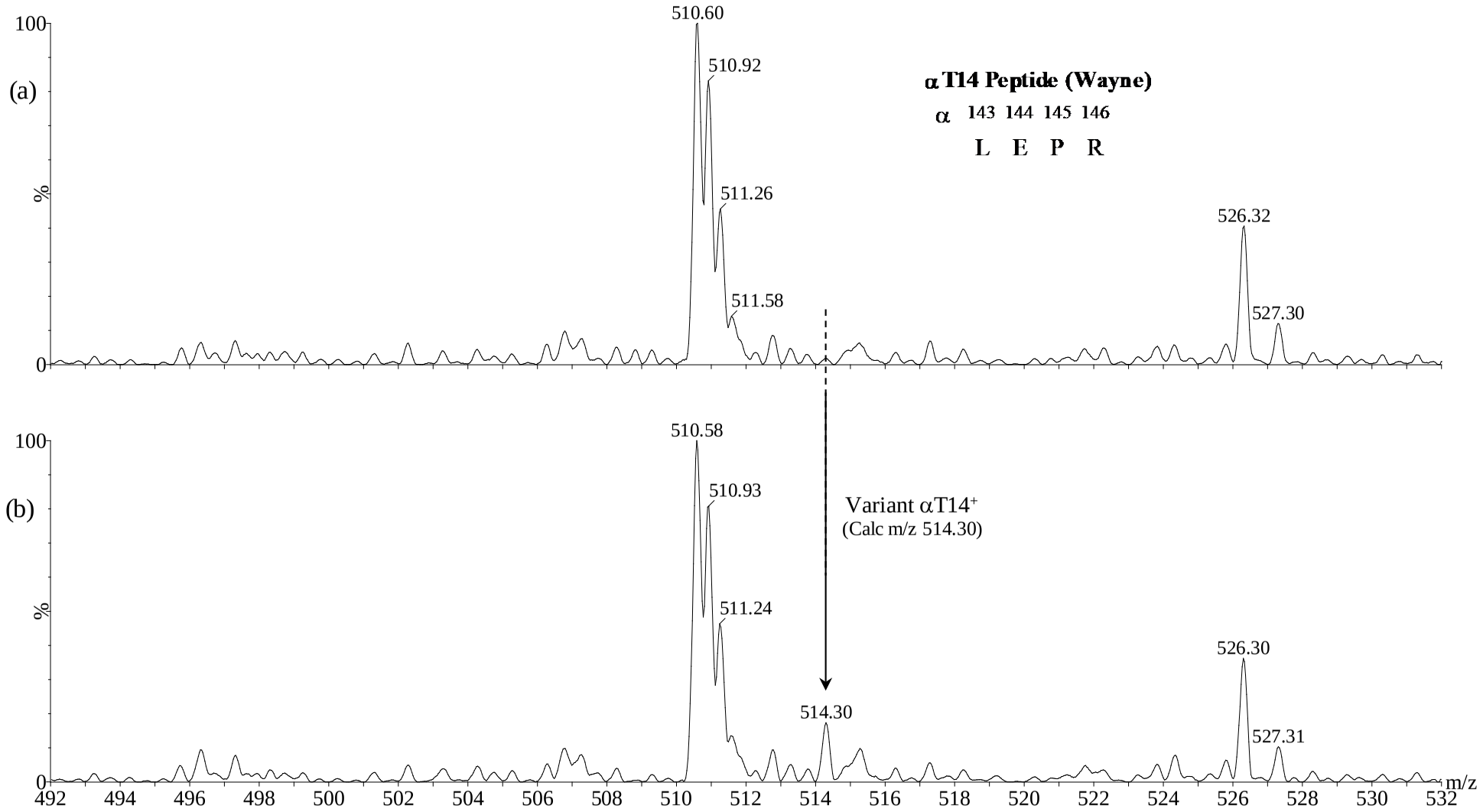

Figure 5.3.22.6. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Wayne heterozygote.

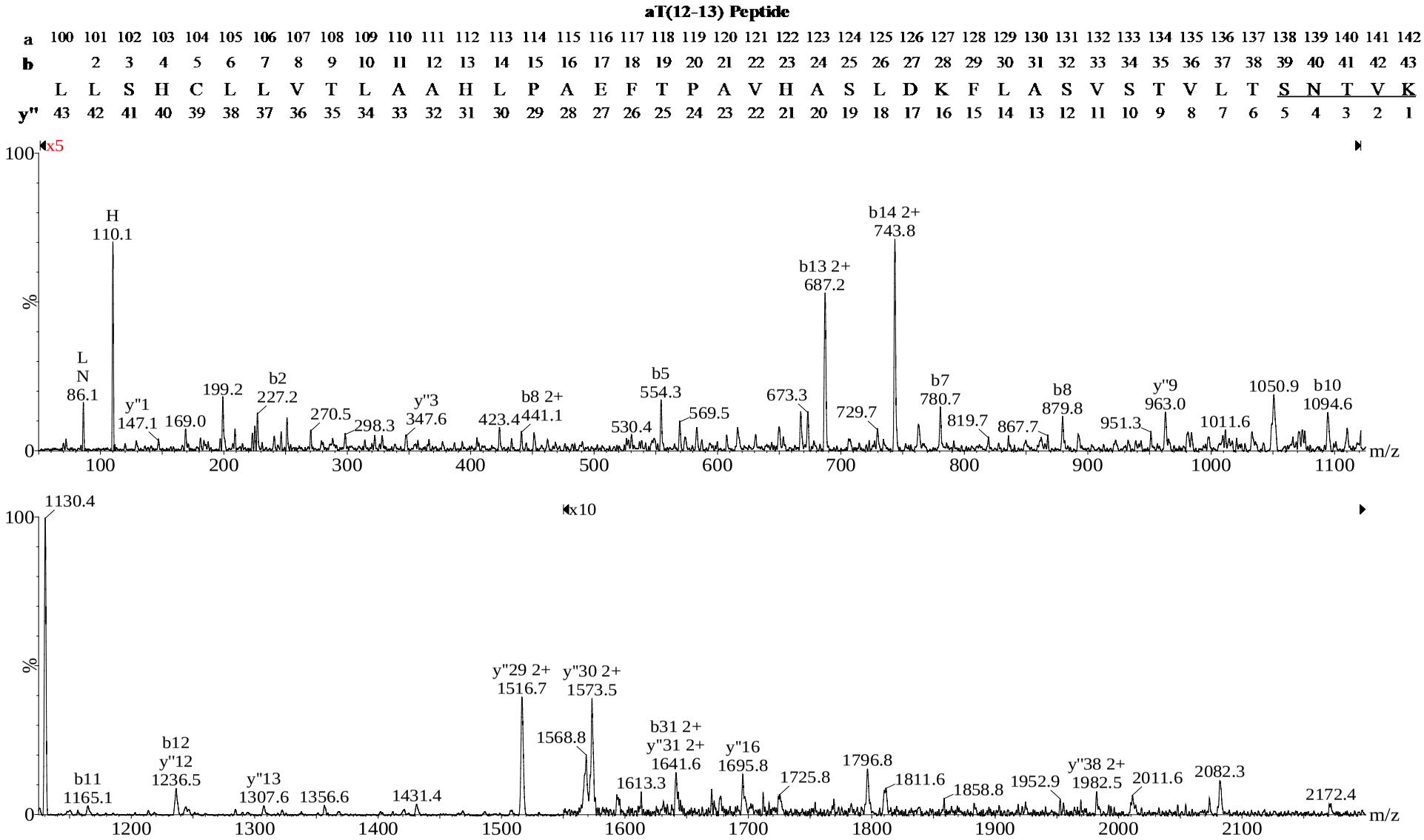

Figure 5.3.22.7. Partial product ion spectrum of the αT(12-13)$^{4+}$ tryptic fragment of Hb Wayne (α139–141 →139 NTVKLEPR). The spectrum is consistent with the expected sequence for Hb Wayne (α(139–141) →139 NTVKLEPR).

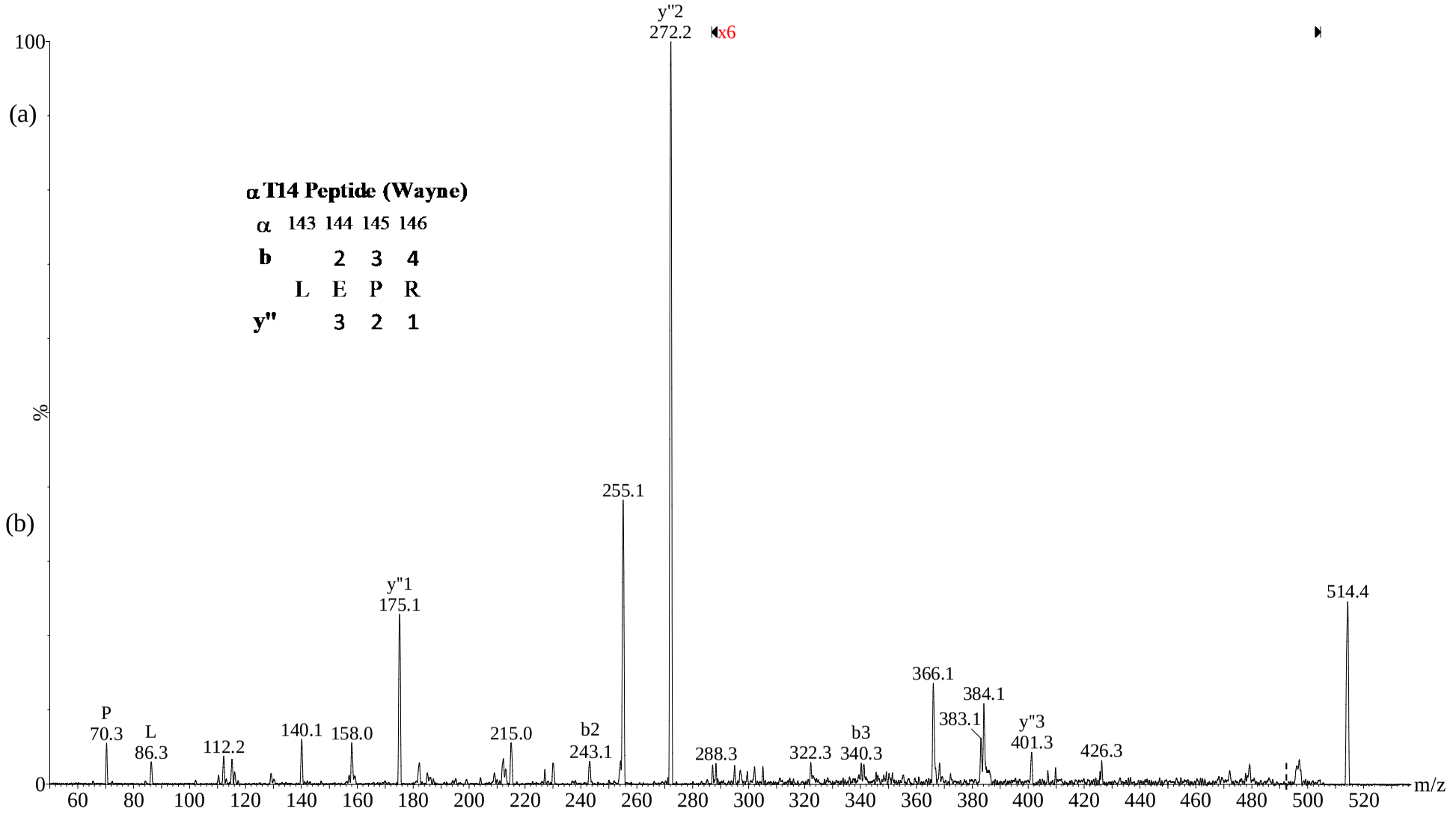

Figure 5.3.22.6. Product ion spectrum of the αT14^{+} (LEPR) tryptic fragment of Hb Wayne (α(139–141) →139 NTVKLEPR), consistent with the expected sequence for Hb Wayne.

5.4. β-chain mutation examples

This section contains examples of the determination of amino acid mutations in the β-chain and have been selected to illustrate certain characteristics that can be investigated using the approaches described in this book. The entries denoted (Novel) were novel when first analysed by mass spectrometry, the name indicates the current recorded name in the literature.

Section	Tryptic Peptide	Mutation	Name
5.4.1.	βT1	β1Val→NAc-Ala	Raleigh
5.4.2.	βT1	β1Val→Met, Initiator Met retained	South Florida
5.4.3.	βT1	β2His→Pro, Initiator Met retained	Marseille
5.4.4.	βT1	β5Pro→Ser	Tyne
5.4.5.	βT1	β6Glu→Lys	C
5.4.6.	βT1	β6Glu→Val/ β58Pro→Arg	C-Ziguinchor, S/Dhofar in same chain
5.4.7.	βT2	β9Ser→Cys	Pôrto Alegre
5.4.8.	βT2	β16Gly→Asp	J-Baltimore
5.4.9.	βT3	β19Asn→Lys	D-Ouled Rabah
5.4.10.	βT3	β22Glu→Gln	D-Iran
5.4.11.	βT3	β26Glu→Lys	E
5.4.12.	βT4	β36Pro→Ser	North Chicago
5.4.13.	βT5	β42Phe→Ser	Hammersmith
5.4.14.	βT5	β52Asp→Asn	Osu Christiansborg
5.4.15.	βT5	β58Pro→His	Sheffield (Novel)
5.4.16.	βT6	β61Lys→Thr	Novel
5.4.17.	βT7	β64Gly→Asp	J-Calabria
5.4.18.	βT9	β82Lys→Arg	Taradale (Novel)
5.4.19.	βT10	β87Thr→Pro	Valletta
5.4.20.	βT11	β98Val→Met	Köln
5.4.21.	βT11	β104Arg→Lys	Alzette
5.4.22.	βT12	β106Leu→Pro	Southampton
5.4.23.	βT12	β109Val→Leu	Johnstown
5.4.24.	βT13	β121Glu→Gln	D-Los Angeles D-Punjab
5.4.25.	βT13	β121Glu→Lys	O-Arab
5.4.26.	βT13	β24Pro→Gln	Ty Gard
5.4.27.	βT14	β135Ala→Val	Alperton (Novel)

Table 5.4.1. List of the mutation illustrations for β-chain.

5.4.1. βT1 - Hb Raleigh (β1Val→NAc-Ala)

Hb Raleigh is the result of a β-chain mutation in which the β1 amino acid residue is changed from Val to Ala through a single base change in the codon GTG→GCG, followed by acetylation of the N-terminal Ala.

βT1 Peptide

β 1 2 3 4 5 6 7 8

V H L T P E E K

↓

NAc-A

Figure 5.4.1.1. Sequence of the Hb Raleigh βT1 tryptic peptide.

A blood sample was submitted for analysis by mass spectrometry because the ce-HPLC trace (Figure 5.4.1.2.) showed an abnormally high P2 response (46.3%) at 1.30 min, indicating a negative charge change.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	0.9	---	1.07	15038
P2	---	46.3	1.30	831657
P3	---	2.9	1.68	52846
A0	---	46.9	2.44	842673
A2	2.7	---	3.61	52954

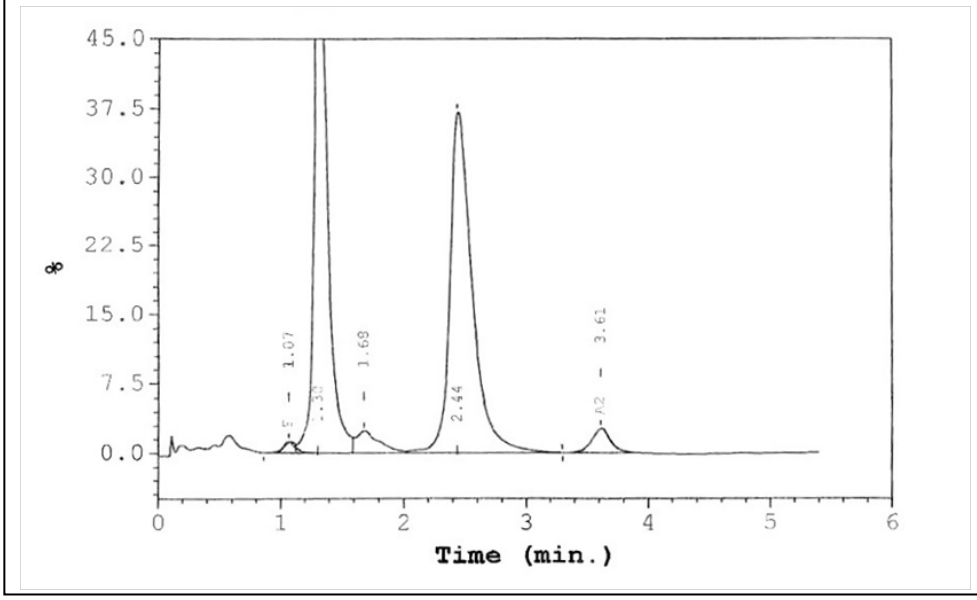

Figure 5.4.1.2. ce-HPLC trace for Hb Raleigh

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.4.1.3.) revealed a β-chain heterozygote in which the variant β-chain was 53.8% of total β-chains and the mass of the variant chain was 15,881.15, 13.83 Da heavier than normal. A single codon change giving a mass increase of +14 Da could be Asn→Lys (positive), Asp→Glu (positive), Gly→Ala (neutral), Ser→Thr (neutral), Val→Ile (neutral), or Val→Leu (neutral). The shift in the ce-HPLC trace indicates a strongly negative charge change, and indicates that none of the single base change codon mutations would be likely. Mutation Val→Ala is possible through a single base change in the codon (Δm -28 Da) followed by acetylation (Δm +42 Da), giving an overall mass increase of +14 Da.

Figure 5.4.1.4. shows the diagnostic part of the tryptic digest spectra from (a) normal Hb and (b) the variant heterozygote. The appearance of a variant $\beta T1^+$ peak at *m/z* 966.48, 13.97 Da heavier than the normal $\beta T1^+$ peak, indicates that the mutation has occurred in the βT1 tryptic peptide.

Figure 5.4.1.5. shows product ion spectra from (a) normal $\beta T1^+$ precursor ion and (b) the variant $\beta T1^+$ precursor ion. The variant b_2 fragment at *m/z* 966.49 is 13.9 Da heavier than the normal b_2 ion, indicating that the mutation must occur in the first two amino acid residues. All the y" ions up to and including $y"_7$ are present at the same mass in both the normal and variant product ion spectra, which places the mutation at the N-terminus, and is consistent with the proposed mutation β1Val→Ac-Ala, Hb Raleigh.

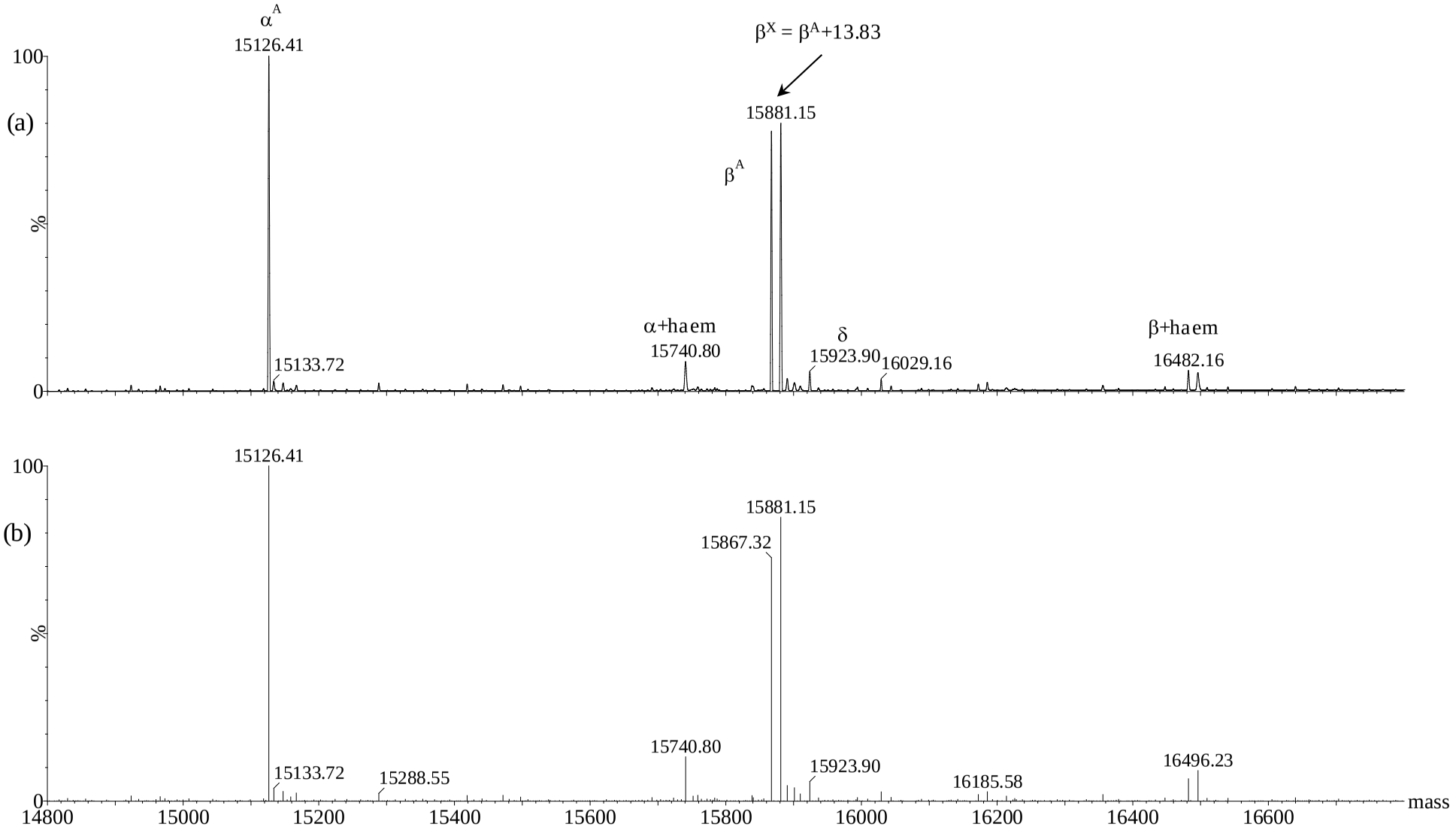

Figure 5.4.1.3. Deconvoluted mass spectrum of Hb Raleigh (β1Val→NAc-Ala) showing the presence of a signal at 15,881.15 Da at approximately equal intensity of the normal β-chain peak (15,867.32 Da).

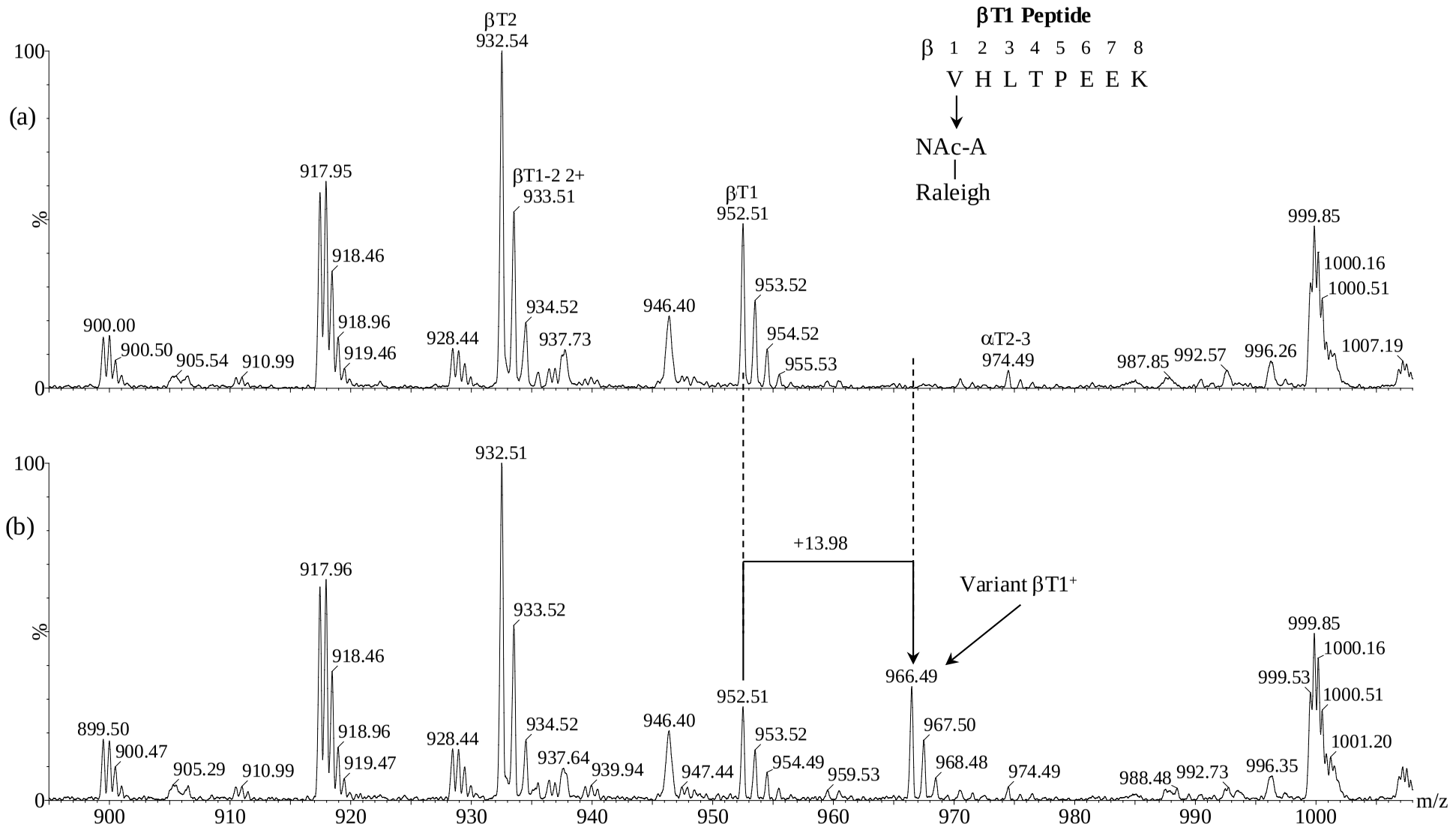

Figure 5.4.1.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Raleigh heterozygote.

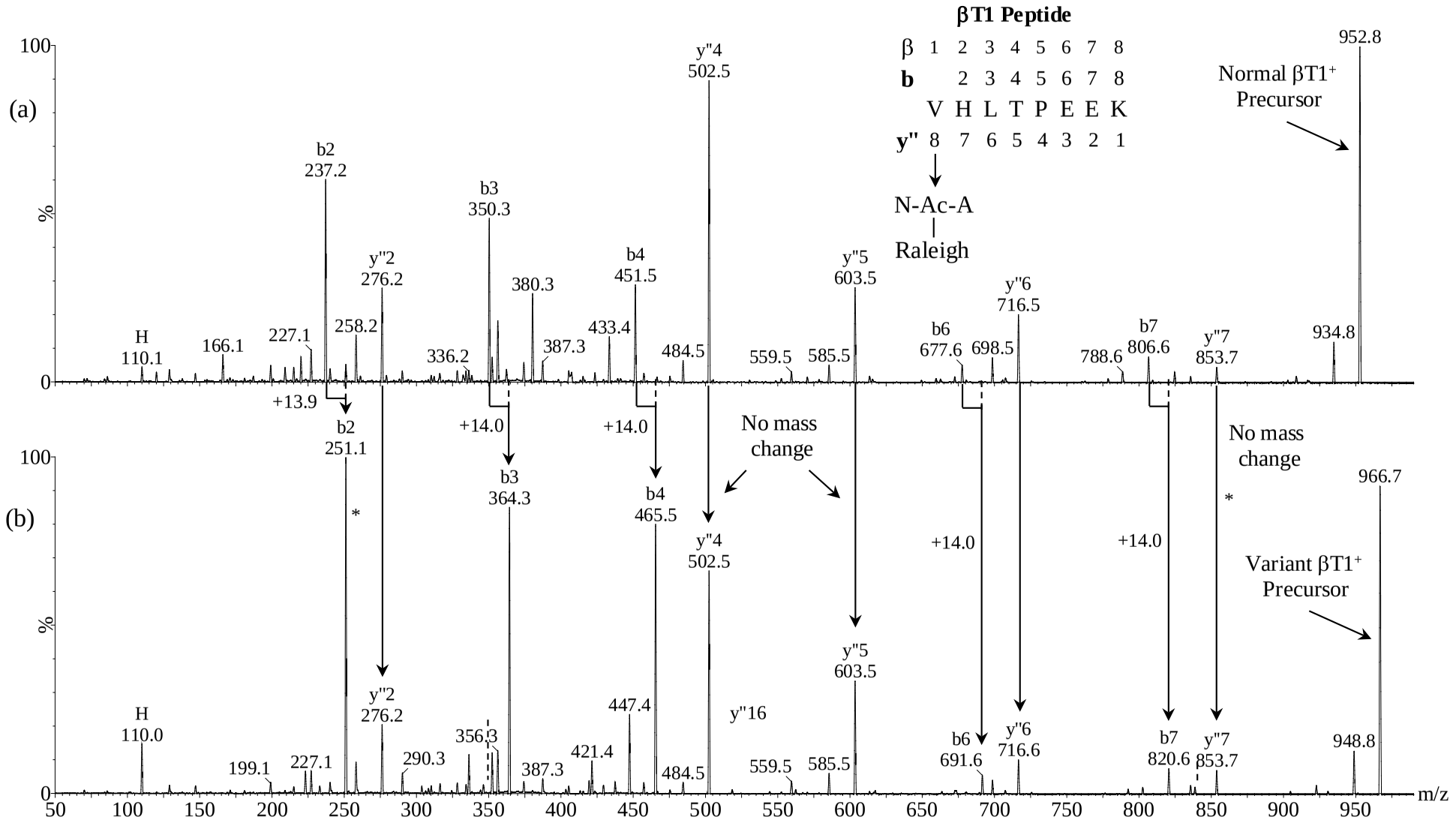

Figure 5.4.1.5. Product ion spectra of the βT1^{+} tryptic fragment of (a) normal Hb and (b) Hb Raleigh. All the ions up to, and including, y"$_{7}$ show no mass change. This places the mutation at the N-terminus and is consistent with β1Val→NAc-Ala.

5.4.2. βT1 - Hb South Florida (β1Val→Met initiator Met retained)

Hb South Florida is the result of a β-chain mutation in which the β1 amino acid residue is changed from Val to Met through a single base change in the codon GTG→ATG, and the initiator Met is retained.

βT1 Peptide

β	-1	1	2	3	4	5	6	7	8
		V	H	L	T	P	E	E	K
		↓							
	M	M	H	L	T	P	E	E	K

Figure 5.4.2.1. Sequence of the Hb South Florida βT1 tryptic peptide.

A blood sample was submitted for analysis by mass spectrometry because the ce-HPLC trace (Figure 5.4.2.2.) showed an abnormally high unknown response (10.5%) at 0.636 min, indicating a negative charge change.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.2	0.103	5606
Unknown	---	0.5	0.160	14329
A1a	---	0.9	0.198	25482
A1b	---	1.4	0.271	36971
F	0.3	---	0.422	11130
Unknown	---	0.3	0.528	8408
Unknown	---	10.5	0.636	283711
A1c	5.8	---	0.882	98497
P3	---	4.2	1.503	114009
A0	---	75.3	1.720	2038513
A2	2.7	---	2.883	71749

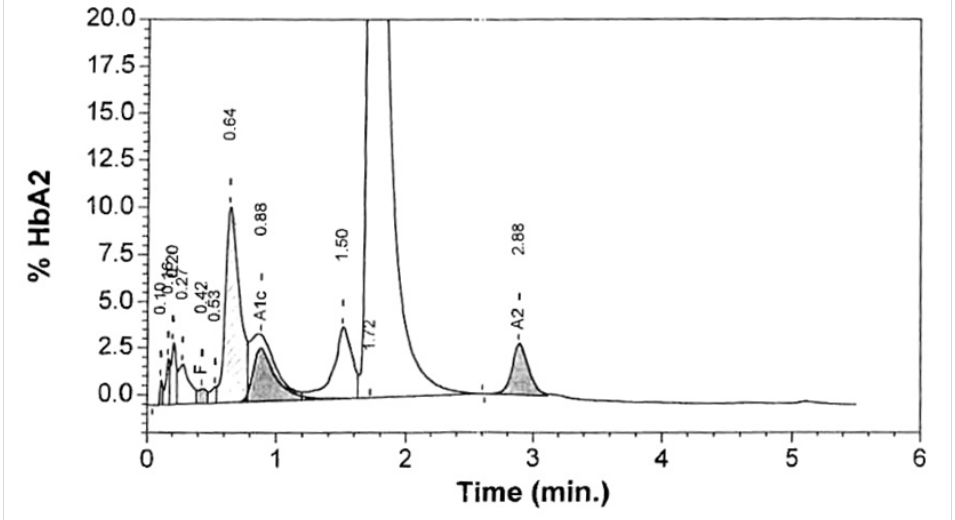

Figure 5.4.2.2. ce-HPLC trace for Hb South Florida

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.4.2.3.) revealed a β-chain heterozygote in which the variant β-chain had a mass of 16,030.36 Da, 163.08 Da heavier than normal. The variant peak is unresolved from the normal glycated Hb at 16,029.38 Da. Peaks are also observed at 15,889.15 Da (loss of Met from the variant) and 16,072.48 Da (N-acetylation of the variant).This spectrum is characteristic of the variant Hb South Florida, β1Val→Met, plus the retention of the initiator Met.

Figure 5.4.2.4. shows the diagnostic part of the spectra from (a) normal Hb and (b) the variant heterozygote. The appearance of the $\beta T1^{+}$ tryptic fragment at *m/z* 1,115.50 in the variant spectrum (+162.99 Da) indicates that the mutation has taken place near the N-terminus of the chain. The product ion spectra (Figure 5.4.2.5.) shows peaks in the variant spectrum (b_3 (*m/z* 400.2), b_4 (*m/z* 513.2) and b_5 (*m/z* 614.4) that are consistent with the expected peaks from Hb South Florida.

Figure 5.4.2.6. shows the variant $\beta T1^{2+}$ (*m/z* 558.2) product ion spectrum that is consistent with the amino acid sequence of the variant βT1 tryptic peptide of Hb South Florida.

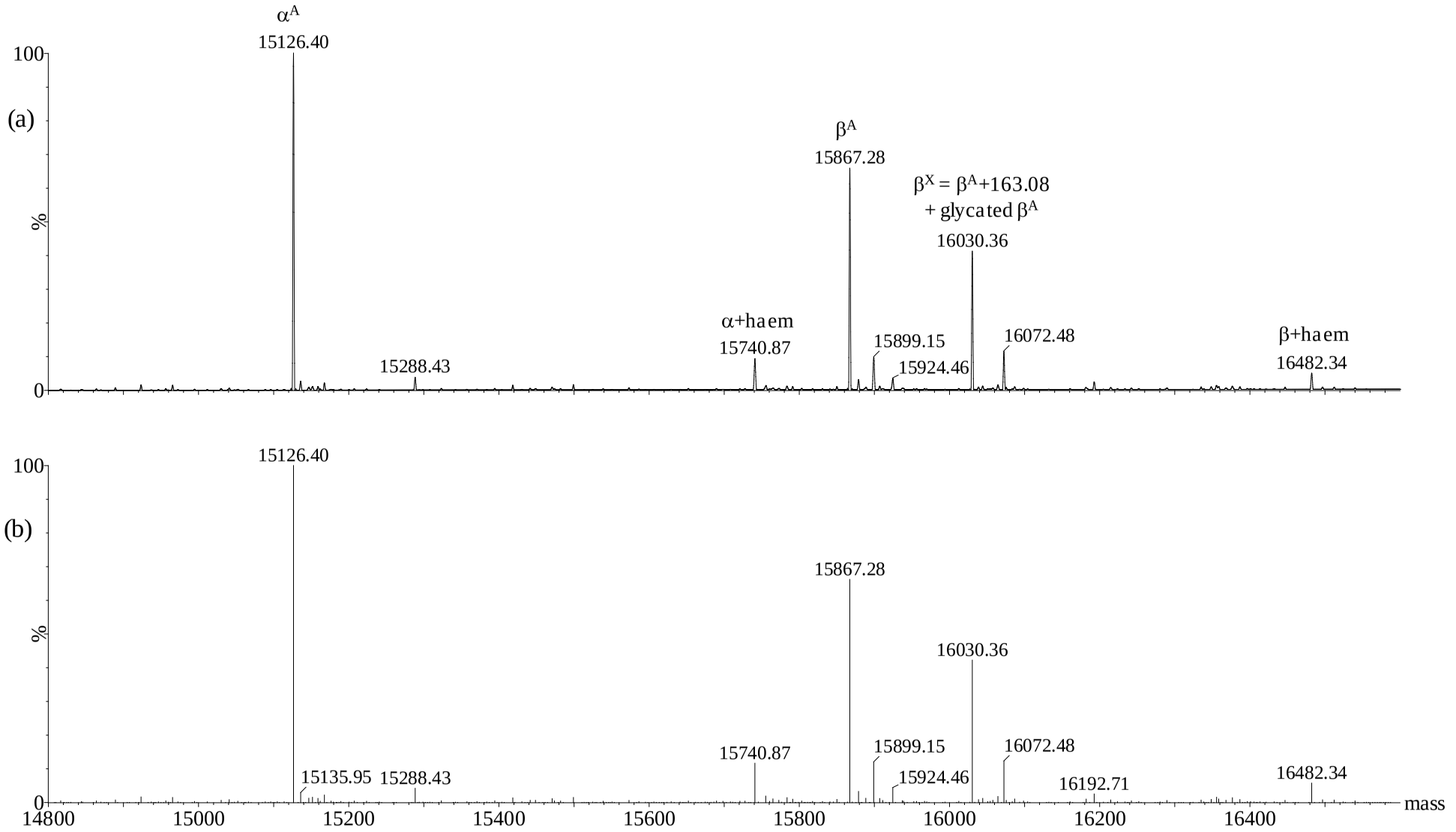

Figure 5.4.2.3. Deconvoluted mass spectrum of Hb South Florida (β1Val→Met and initiator Met retained) showing the presence of a signal at 16,030.36 Da at approximately 75% intensity of the normal β-chain peak (15,867.28 Da). A peak is also observed at 15,899.15 Da (loss of Met), and 16,072.49 Da (N-acetylation). The spectrum is characteristic of Hb South Florida, β1Val→Met and retention of the initiator Met.

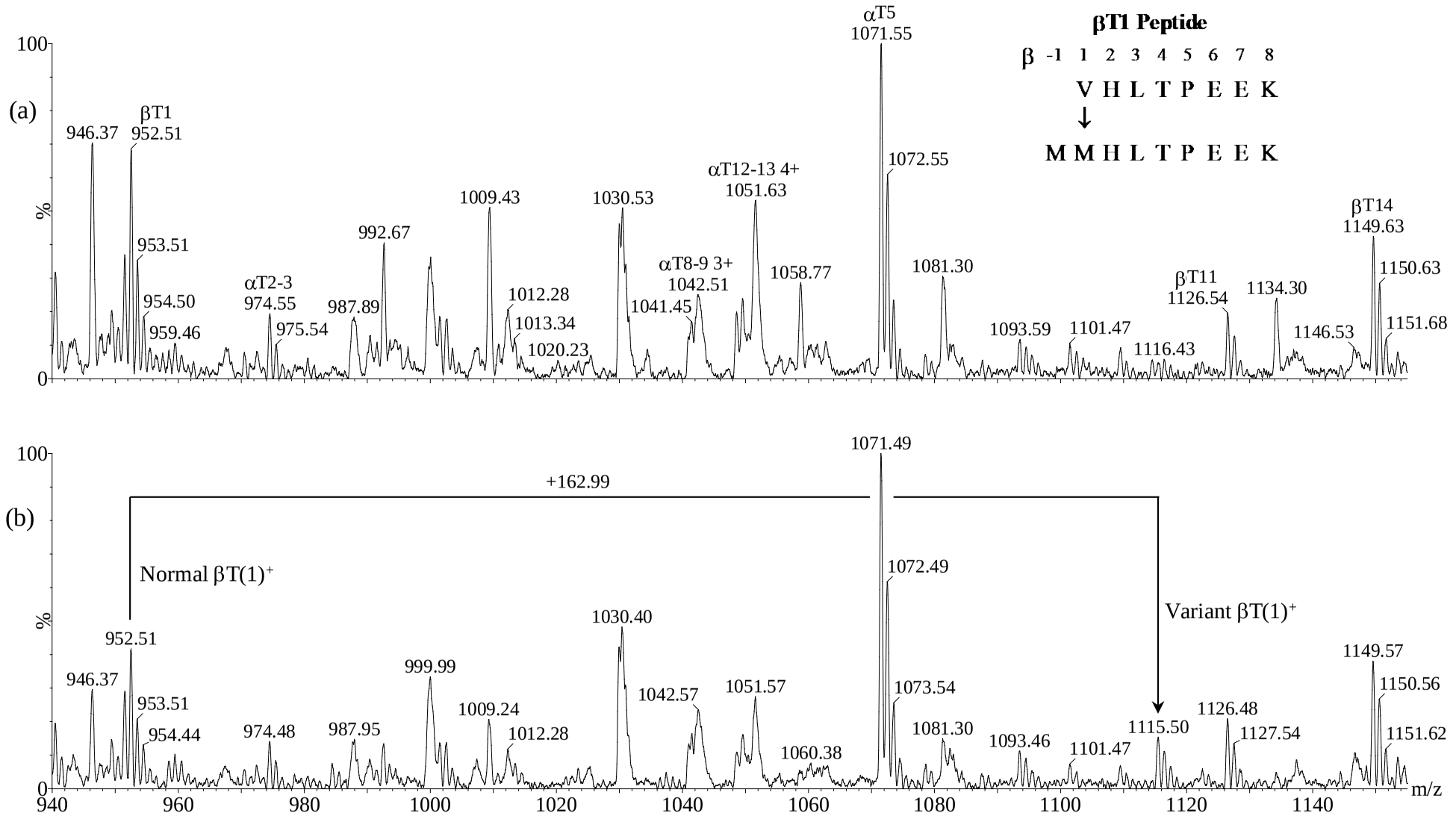

Figure 5.4.2.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb South Florida heterozygote.

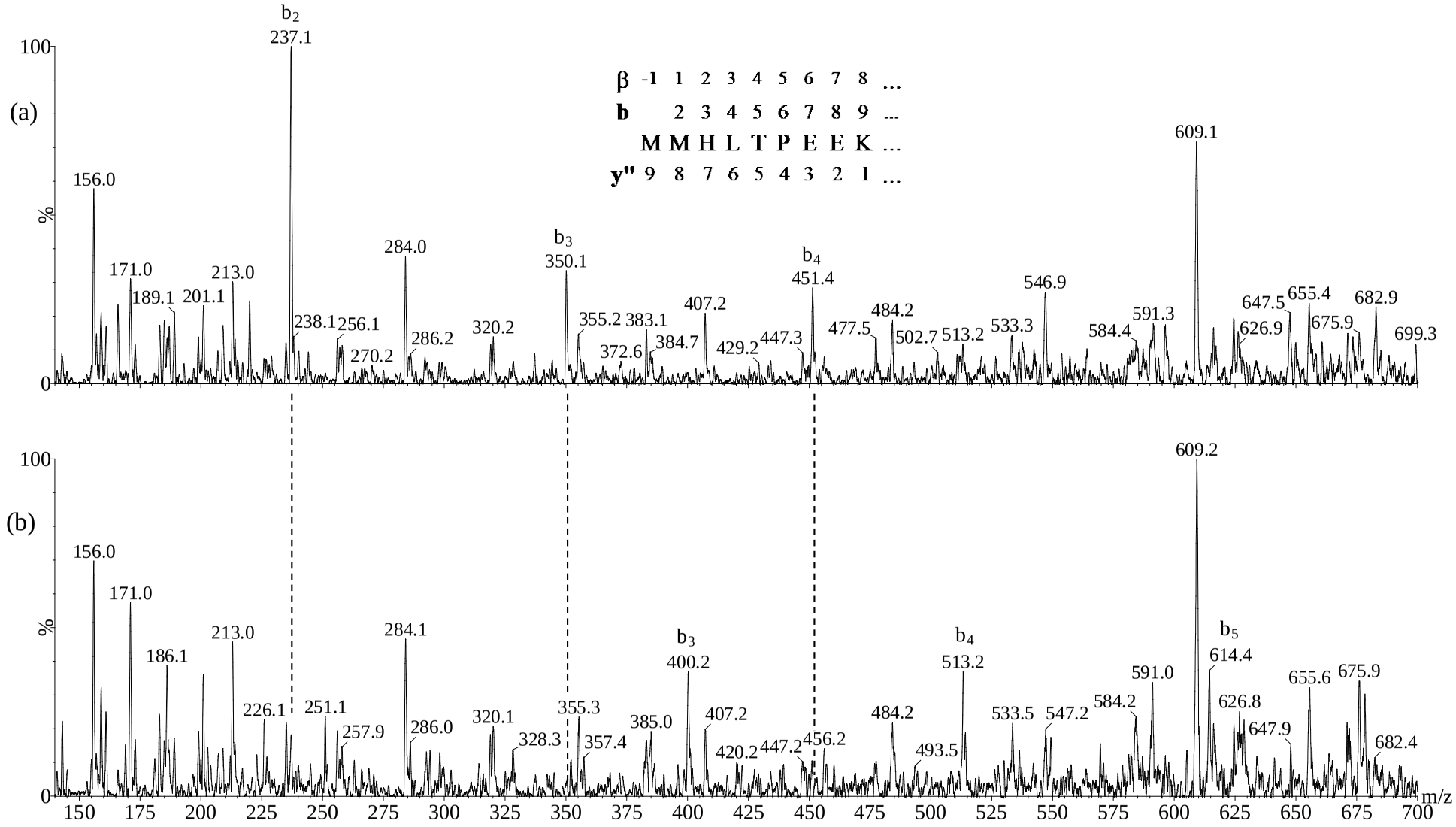

Figure 5.4.2.5. Partial product ion spectra of the βT1^{+} tryptic fragment of (a) normal Hb and (b) Hb South Florida (β1Val→Met and initiator Met retained). This part of the spectrum infers that the mutation occurs at the N-terminus of the β-chain. In the lower spectrum, b_3, b_4 and b_5 are consistent with the expected peaks from Hb South Florida.

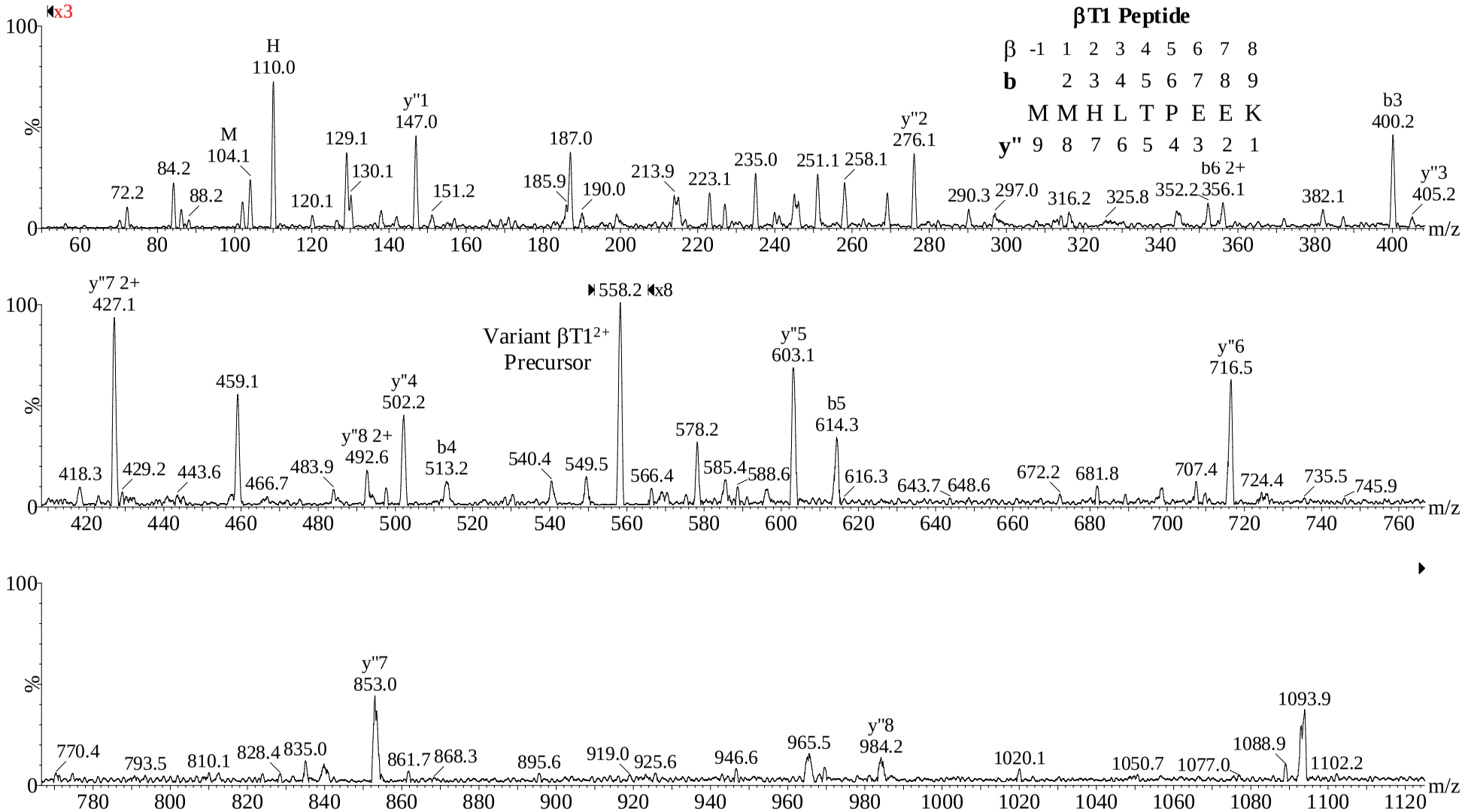

Figure 5.4.2.6. Product ion spectrum of the βT1^{2+} tryptic fragment of Hb South Florida (β1Val→Met and initiator Met retained). Apart from a couple of major peaks that are unassigned, the spectrum is totally consistent with the sequence shown, and confirms the variant as Hb South Florida.

5.4.3. βT1 - Hb Marseille (β2His→Pro initiator Met retained)

Hb Marseille is the result of a β-chain mutation in which the β2 amino acid residue is changed from His to Pro through a single base change in the codon CAT→CCT, and the initiator Met is retained.

βT1 Peptide

β -1 1 2 3 4 5 6 7 8

V H L T P E E K

↓

M V P L T P E E K

Figure 5.4.3.1. Sequence of the Hb Marseille βT1 tryptic peptide.

A blood sample was submitted for analysis by mass spectrometry because the ce-HPLC trace (Figure 5.4.5.2.) showed an abnormally high P2 response (49.9%). The high P2 (mainly due to the variant) implies the variant causes a significant negative polarity change.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	0.3	---	1.03	5342
P2	---	49.9	1.26	989791
P3	---	6.3	1.70	125816
A0	---	41.4	2.52	820426
A2	1.8	---	3.61	40375

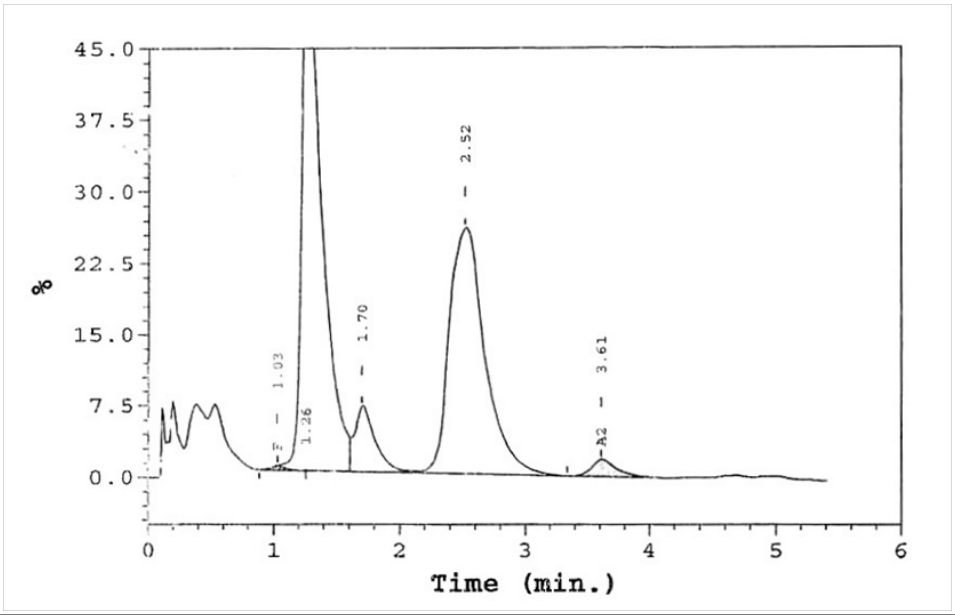

Figure 5.4.3.2. ce-HPLC trace for Hb Marseille.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.4.3.3.) revealed a β-chain heterozygote in which the variant β-chain was 56.9% of total β-chains, and the mass of the variant chain was 15,958.39 Da, 91.14 Da heavier than normal. No single amino acid mutation can give rise to a mass increase of 91 Da.

Figure 5.4.3.4. shows a diagnostic part of the tryptic digest spectra from (a) normal Hb and (b) the variant Hb. The appearance of a signal at *m/z* 522.30 in the lower panel shows an increase in mass for the $\beta T1^{2+}$ tryptic fragment of 90.98 Da, indicating that the mutation has taken place in the first tryptic fragment. This is further supported by the appearance of a peak in the lower panel of Figure 5.4.3.5. at *m/z* 1,043.56,

at 91.06 Da heavier than the normal $\beta T1^{+}$ tryptic fragment. As the mutation occurs in the βT1 tryptic peptide, there is suspicion that the initiator Met (131 Da) may have been retained thus a mass balance of 40 Da would suggest a His→Pro mutation from a single base change in the codon. There is only one His residue in the βT1 tryptic peptide.

This information, coupled to that from the ce-HPLC trace, identifies the mutation as β2His→Pro (-40 Da), with the retention of the initiator Met (+131 Da), Hb Marseille. MS/MS of the βT1 tryptic peptide was not required for a positive identification.

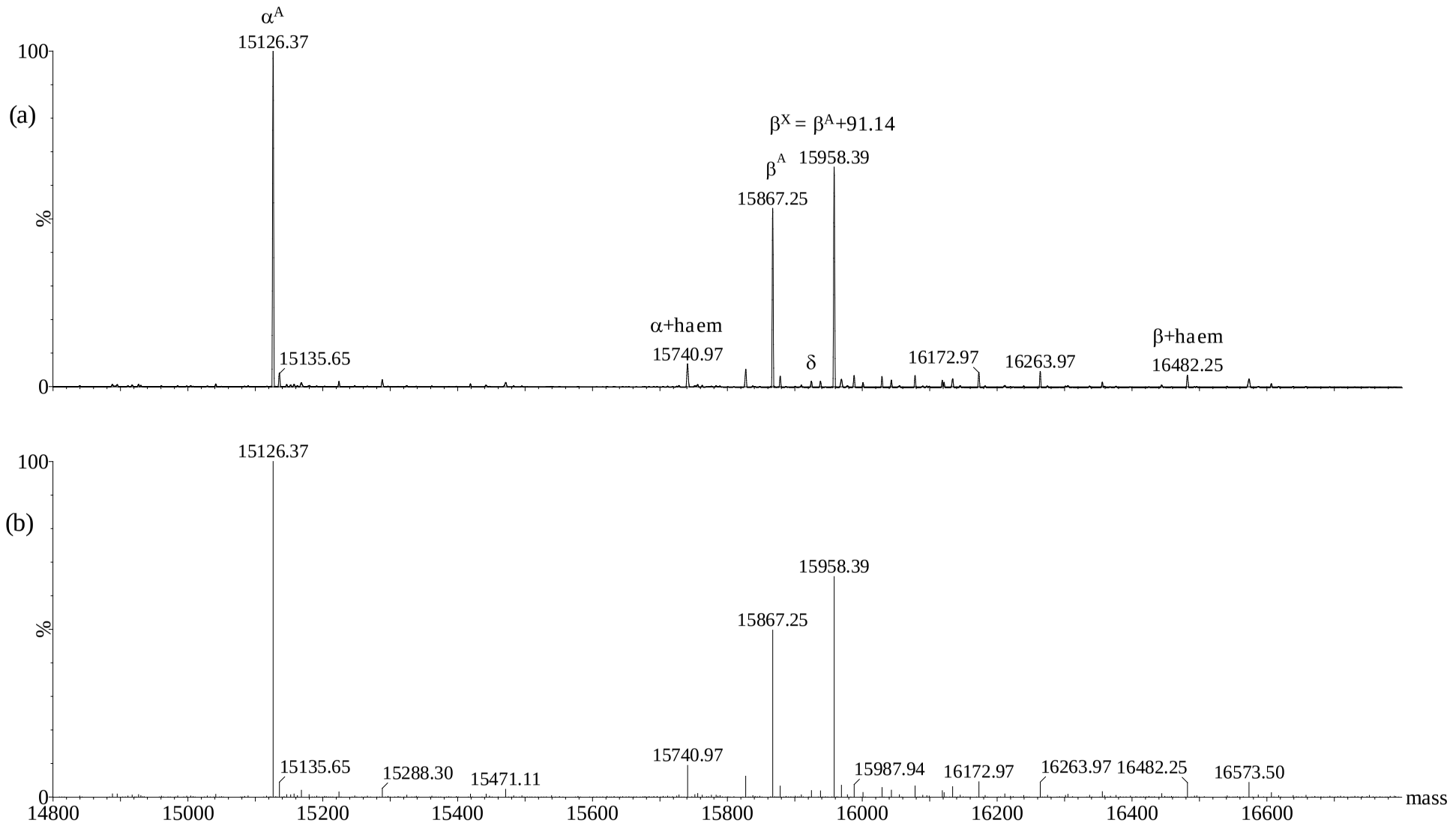

Figure 5.4.3.3. Deconvoluted mass spectrum of Hb Marseille (β2His→Pro and initiator Met retained) showing the presence of a signal at 15,958.39 Da at approximately 115% intensity of the normal β-chain peak (15,867.25 Da). This identifies the variant as Hb Marseille, β(-1)Met-(+1)Val-(+2)Pro-Leu.

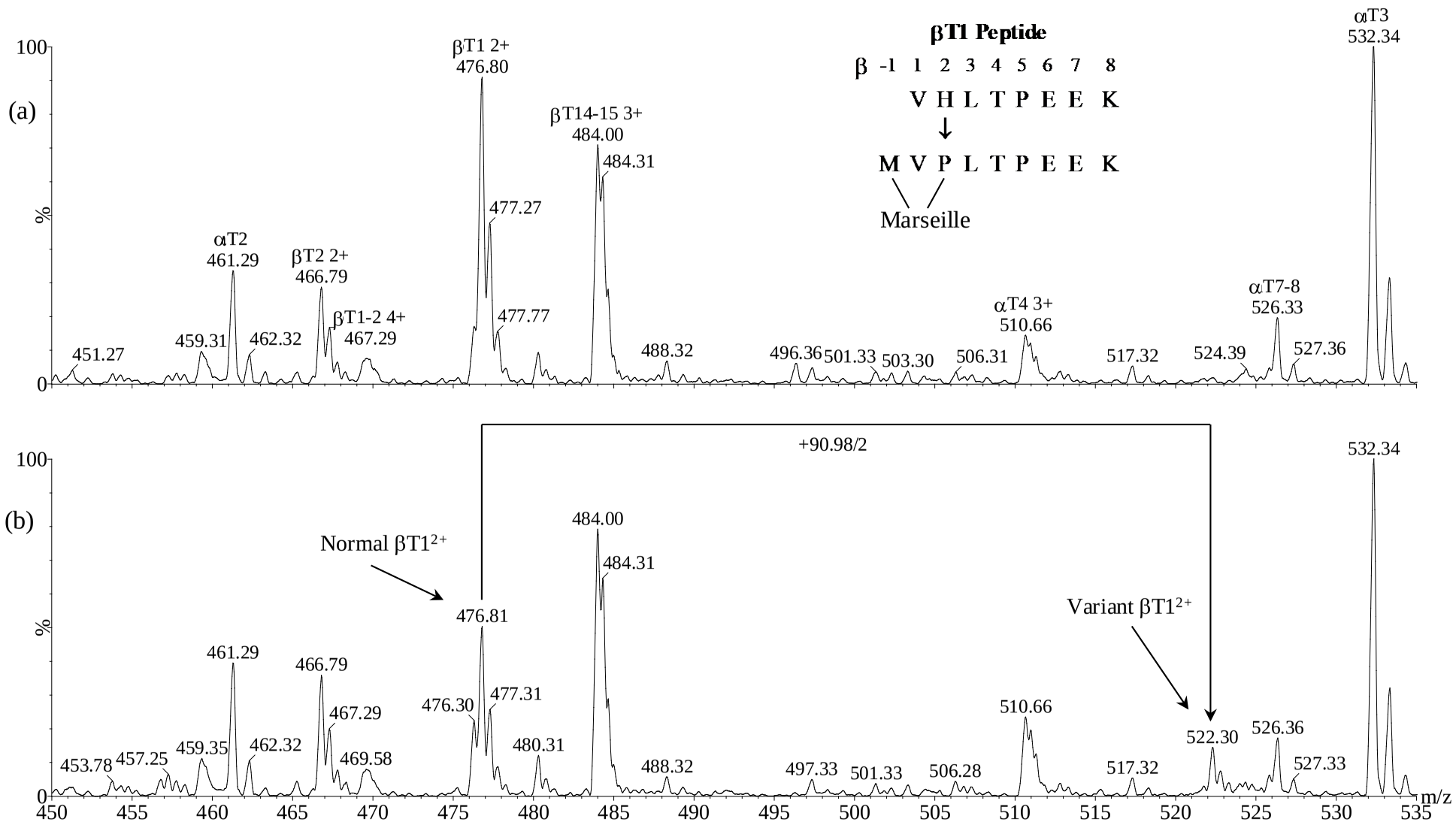

Figure 5.4.3.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Marseille heterozygote.

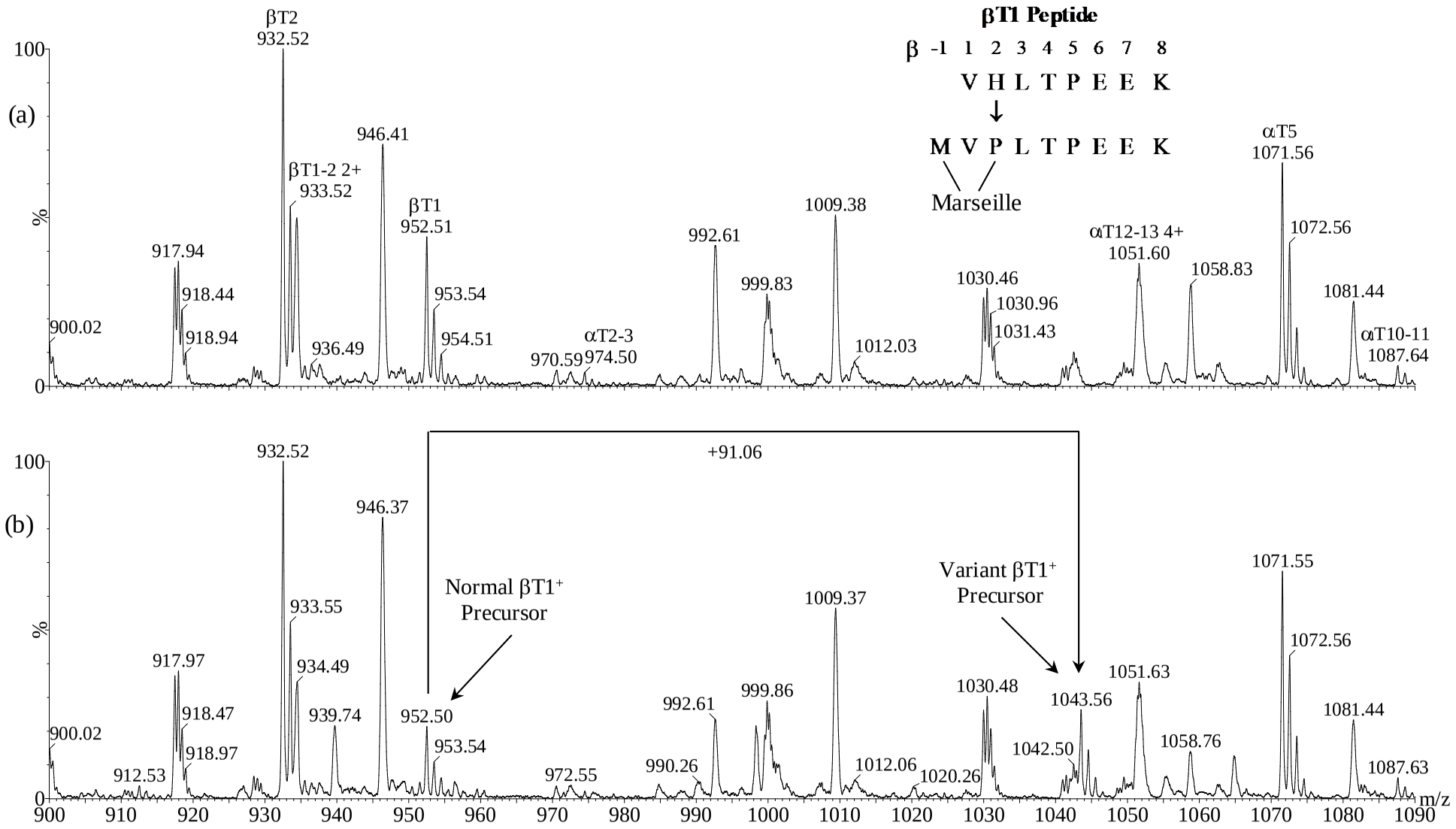

Figure 5.4.3.5. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Marseille heterozygote.

5.4.4. βT1 - Hb Tyne (β5Pro→Ser)

Hb Tyne is the result of a β-chain mutation in which the β5 amino acid residue is changed from Pro to Ser through a single base change in the codon CCT→TCT.

βT1 Peptide

β 1 2 3 4 5 6 7 8

V H L T P E E K

↓

S

Figure 5.4.4.1. Sequence of the Hb Tyne βT1 tryptic peptide.

A blood sample was submitted for analysis by mass spectrometry because the ce-HPLC trace (Figure 5.4.4.2.) showed an abnormal response (42.0%) at 2.68 min, slightly later than A_0, indicating, most likely, no charge change.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.0	0.97	700
F	0.1	---	1.06	2220
Unknown	---	0.7	1.23	11351
Unknown	---	1.6	1.32	26667
P2	---	3.4	1.36	56743
P3	---	4.0	1.77	68172
A0	---	44.5	2.45	751393
Unknown	---	42.0	2.68	709955
A2	3.3	---	3.61	61522

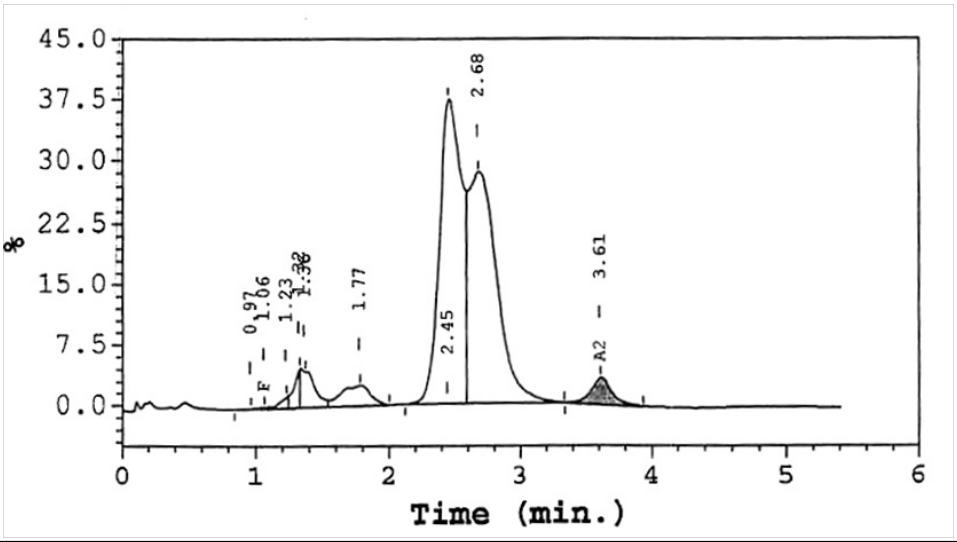

Figure 5.4.4.2. ce-HPLC trace for Hb Tyne

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.4.4.3.) revealed a β-chain heterozygote in which the variant β-chain was approximately equal intensity to the normal β-chain, and the mass of the variant chain was 15,857.21 Da, 10.08 Da lighter than normal. A single codon change giving a mass decrease of 10 Da is Pro→Ser (7 possibilities).

Figure 5.4.4.4. shows the diagnostic part of the tryptic digest spectra from (a) normal Hb and (b) the variant heterozygote. The appearance of a signal at *m/z* 471.77 in the lower panel indicates that the mutation occurs in the βT1 peptide. Based on the mass difference of the whole chain, there is only one

mutation that could give rise to a mass difference of -10 Da from a single base codon change, β4 Pro→Ser. This is further supported by the data in Figure 5.4.4.5. showing the appearance of a variant $\beta T1^{+}$ ion at *m/z* 942.48.

Figure 5.4.4.6. shows the product ion spectra from the $\beta T1^{2+}$ precursor for (a) normal Hb and (b) the variant Hb. The occurrence of all the y" ions up to and including $y"_3$ at the same mass in both the normal and variant precursor ion spectra, and the mass shift for the $y"_4$ at *m/z* 492.4 by -10.0 Da in the lower panel, confirms the mutation as β4 Pro→Ser, Hb Tyne.

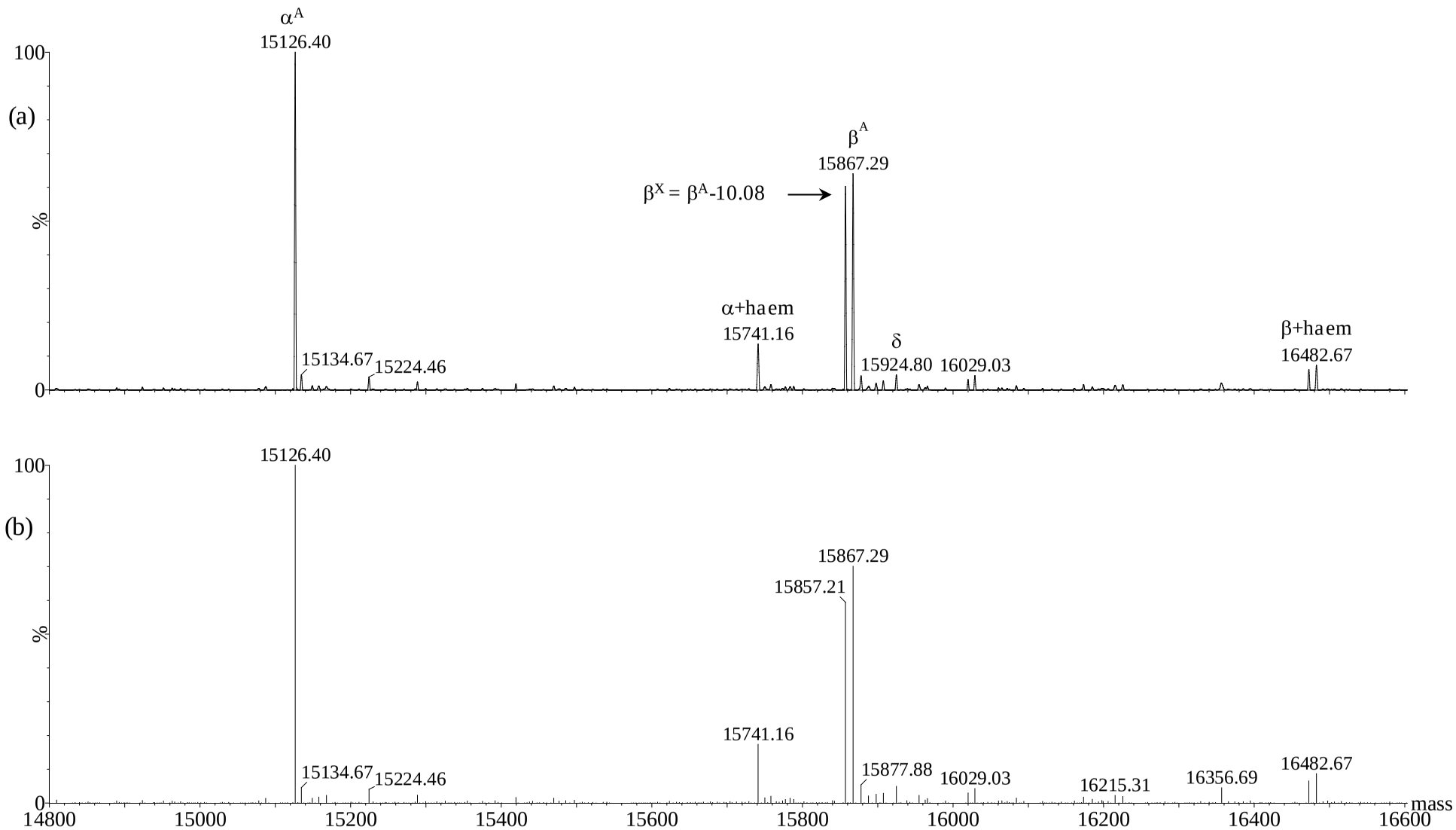

Figure 5.4.4.3. Deconvoluted mass spectrum of Hb Tyne (β3Pro→Ser) showing the presence of a signal at 15,857.21 Da at approximately equal intensity of the normal β-chain peak (15,867.29 Da). A mass decrease of 10 Da can only be Pro→Ser for a single base change in the codon.

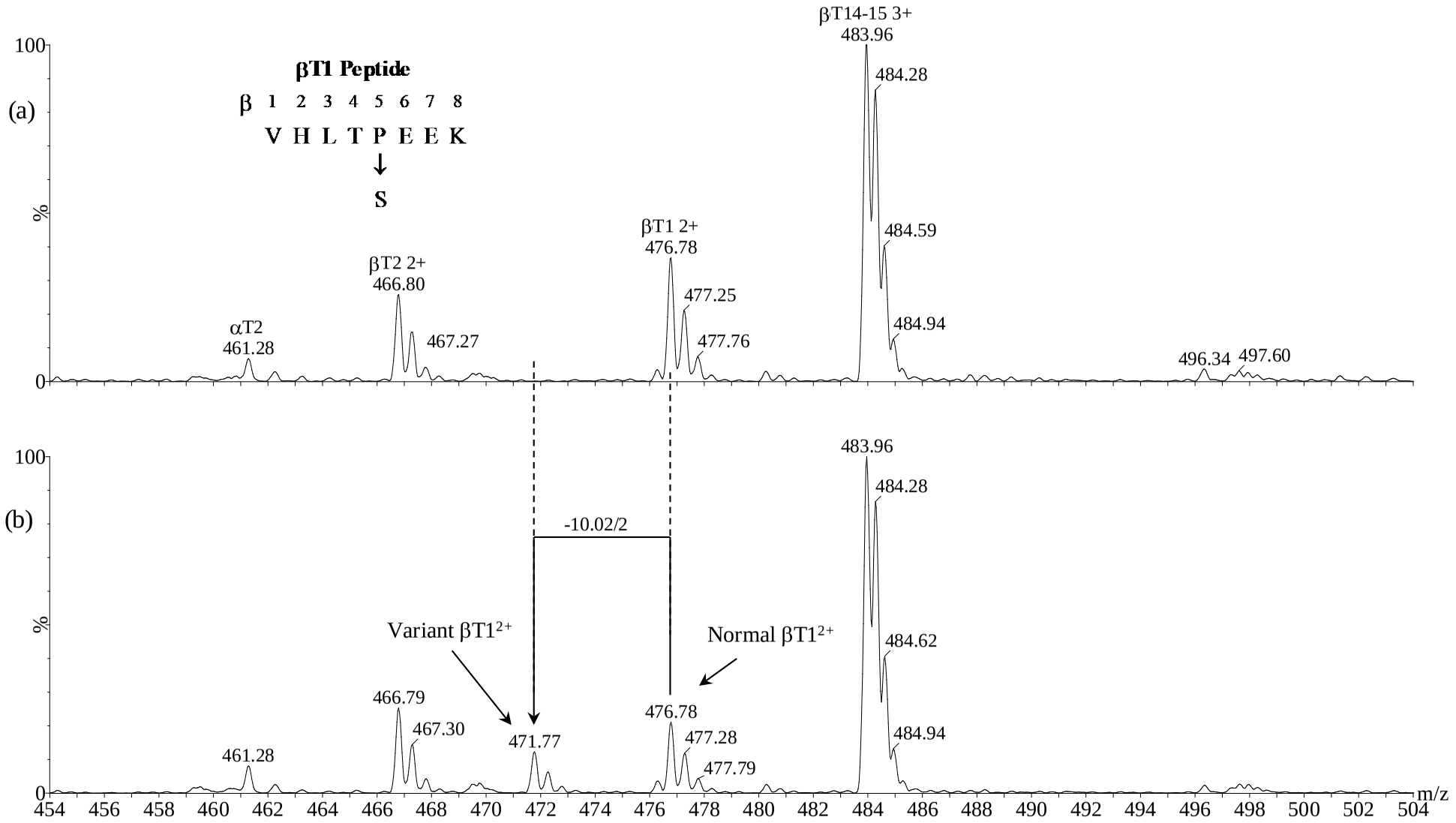

Figure 5.4.4.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Tyne heterozygote.

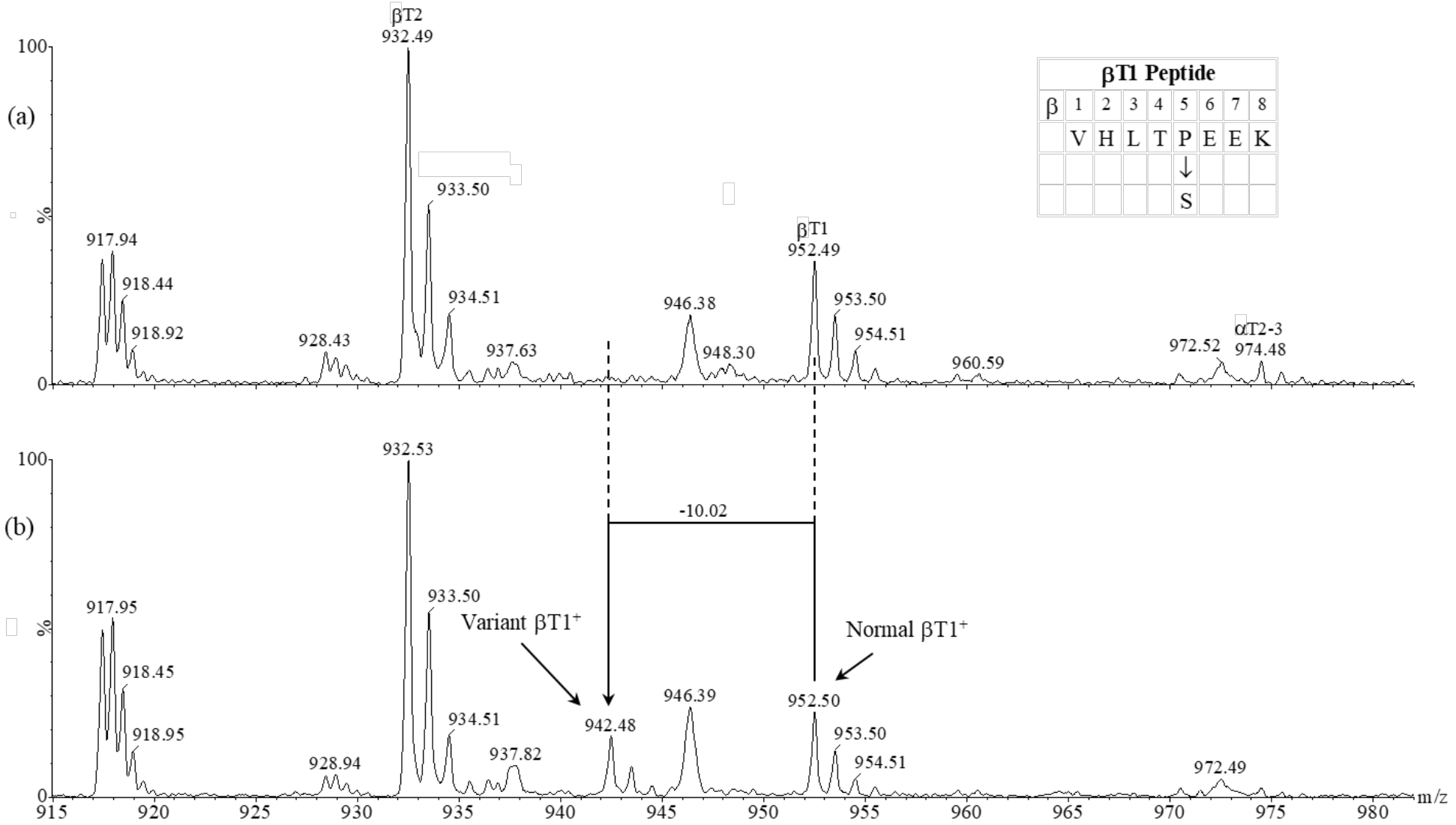

Figure 5.4.4.5. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Tyne heterozygote.

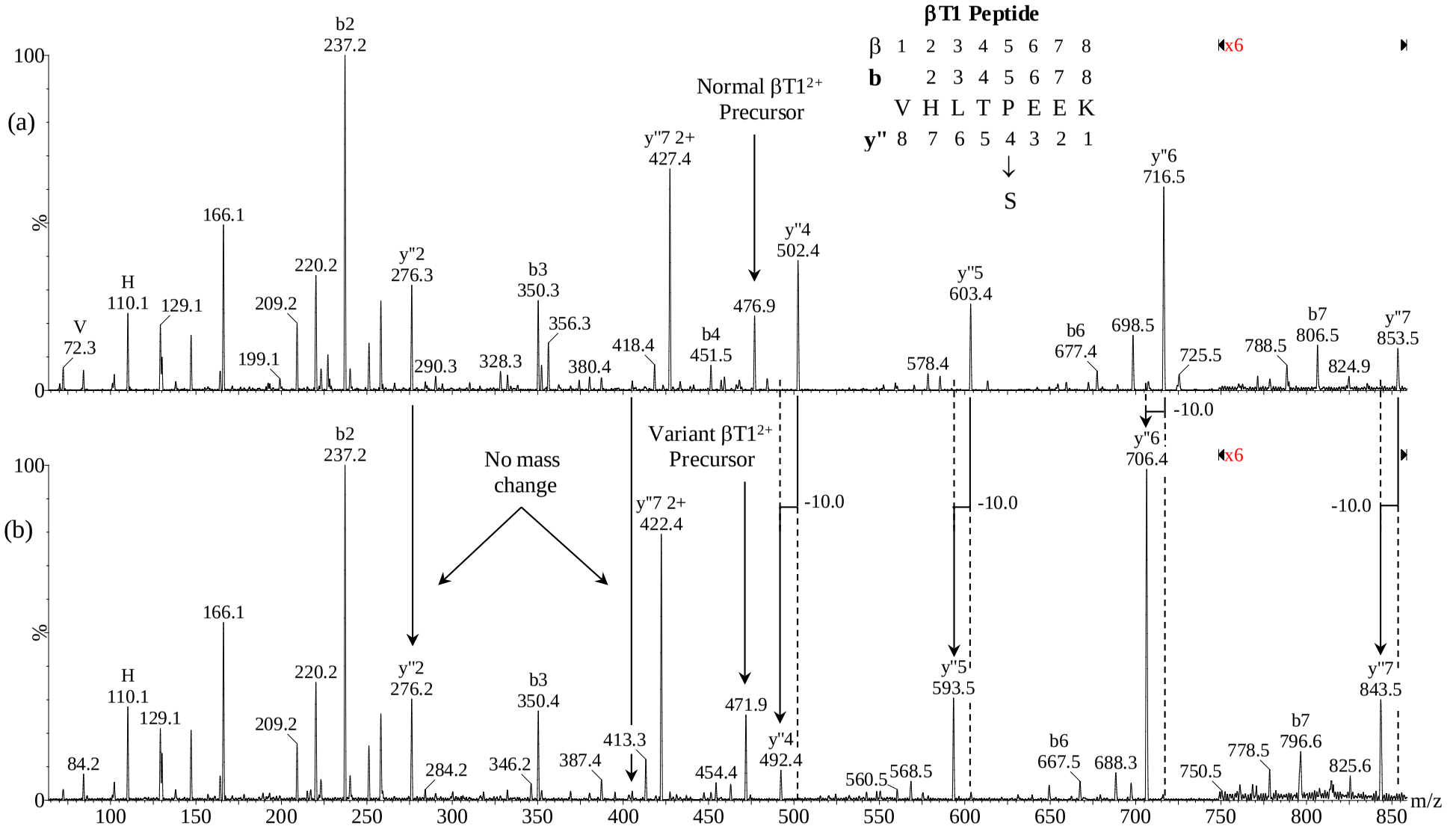

Figure 5.4.4.6. Product ion spectra of the βT1^{2+} tryptic fragment of (a) normal Hb and (b) Hb Tyne. The 10 Da mass decrease at y"$_4$ confirms the mutation β5Pro→Ser.

5.4.5. βT1 - Hb C (β6Glu→Lys)

Hb C is the result of a β-chain mutation in which the β6 amino acid residue is changed from Glu to Lys through a single base change in the codon GAG→AAG.

The mutation to the Lys residue results in an additional tryptic cleavage product, as shown in Figure 5.4.5.1.

βT1 Peptide

β 1 2 3 4 5 6 7 8

V H L T P E E K

↓

V H L T P K E K

Figure 5.4.5.1. Sequence of the Hb C βT1 tryptic peptide.

EXAMPLE 1

A blood sample was submitted for analysis by mass spectrometry, with the ce-HPLC trace (Figure 5.4.5.2) showing the presence of a peak at 5.10 minutes in the C-window that is of approximately equal intensity to the A_0 signal. There is also a significant presence of Hb F at 1.17 minutes (72.4%).

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	2.1	0.12	68604
Unknown	---	3.0	0.19	98887
Unknown	---	12.9	0.37	427488
P1	---	1.4	0.77	44800
F	72.4	---	1.17	2151744
A0	---	7.7	2.51	254105
C-window	---	7.9	5.10	261092

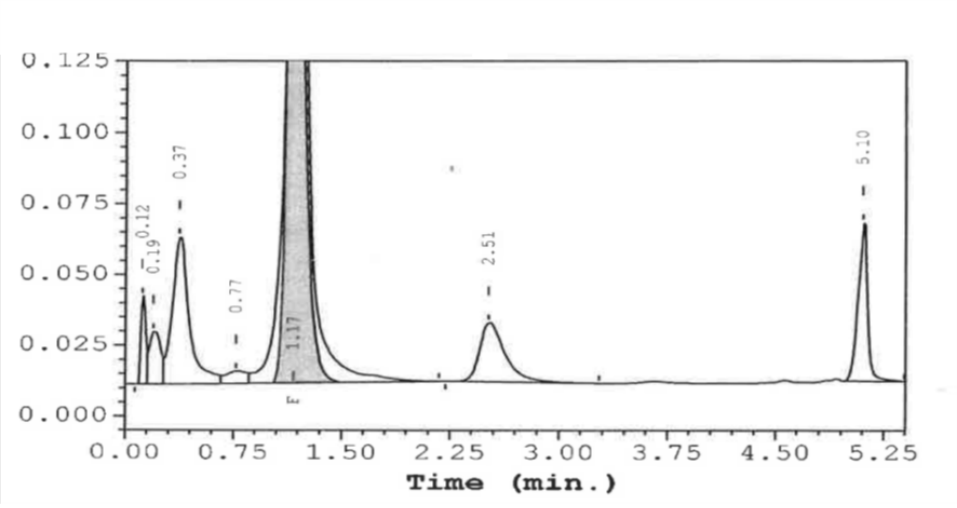

Figure 5.4.5.2. ce-HPLC trace for Hb C in a newborn specimen.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.4.5.4.) revealed a signal at 15,866.84 Da, which suggests a $\beta^A/(\beta^A\text{-}1)$ heterozygote. The γ-chain doublet at 15,995.33 Da / 16,009.11 Da indicates that this sample is from an infant.

Figures 5.4.5.5. and 5.4.5.6. show comparisons of the tryptic digest of (a) normal Hb and (b) the submitted sample in the diagnostic regions. New signals are observed in the lower panels corresponding to the appearance of the $\beta T1a^+$ (*m/z* 694.44 in Figure 5.4.5.5.) and $\beta T1a^{2+}$ (*m/z* 347.72 in Figure 5.4.5.6.).

The formation of a pair of new tryptic T1 peptides suggests that the mutation involves the inclusion of a tryptic-cleavable residue (K or R). The mass of the βT1a peptide confirms β6Glu→Lys, Hb C.

EXAMPLE 2

A sample was received for analysis in which the ce-HPLC trace (Figure 5.4.5.3) is dominated by the peak at 5.91 minutes, assigned as Hb C (91.1%). The shift in retention time indicates a positive charge change in the mutation.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	1.0	---	1.06	14747
P2	---	0.1	1.39	1022
P3	---	0.1	1.82	1972
A0	---	0.6	2.65	10410
A2	3.2	---	3.66	56398
S-window	---	3.1	4.55	51775
Unknown	---	0.7	4.93	11454
C-window	---	91.1	5.12	1519476

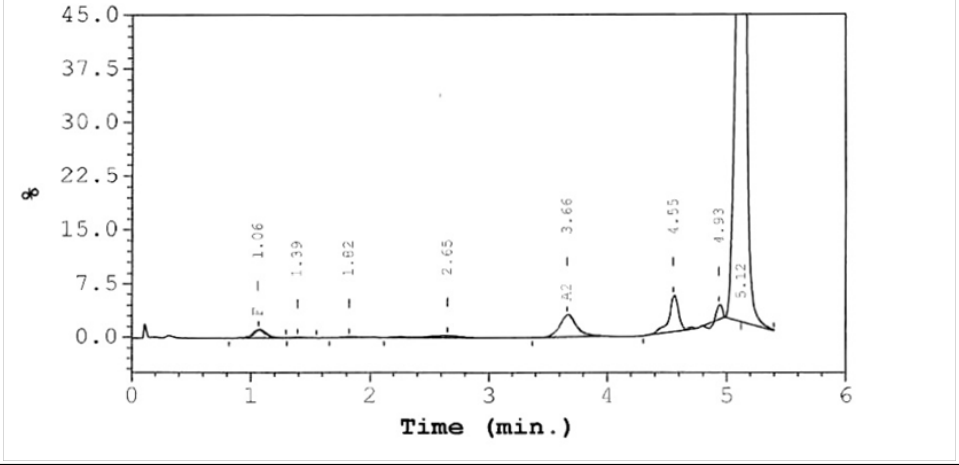

Figure 5.4.5.3. ce-HPLC trace for Hb C in a newborn specimen.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.4.5.7.) shows the presence of a single signal at 15,866.33 Da, which suggests either a (β^A-1)/(β^A-1) homozygote or (β^A-1)/ β-thalassaemia.

Figure 5.4.5.8. shows a comparison of the tryptic digest of (a) normal Hb and (b) the submitted sample in the diagnostic region. New signals are observed in the lower trace corresponding to the appearance of the $\beta T1b^+$ (*m/z* 276.16) and $\beta T1a^{2+}$ (*m/z* 347.72).

Figure 5.4.5.9. highlights the presence of the newly formed $\beta T1a^+$ (*m/z* 694.43) and the absence of the normal $\beta T1^{2+}$ (*m/z* 476.76). The absence of the normal $\beta T1^+$ is also indicated in Figure 5.4.5.10. These data support the hypothesis that the specimen is homozygous for the mutation β6Glu→Lys, Hb C.

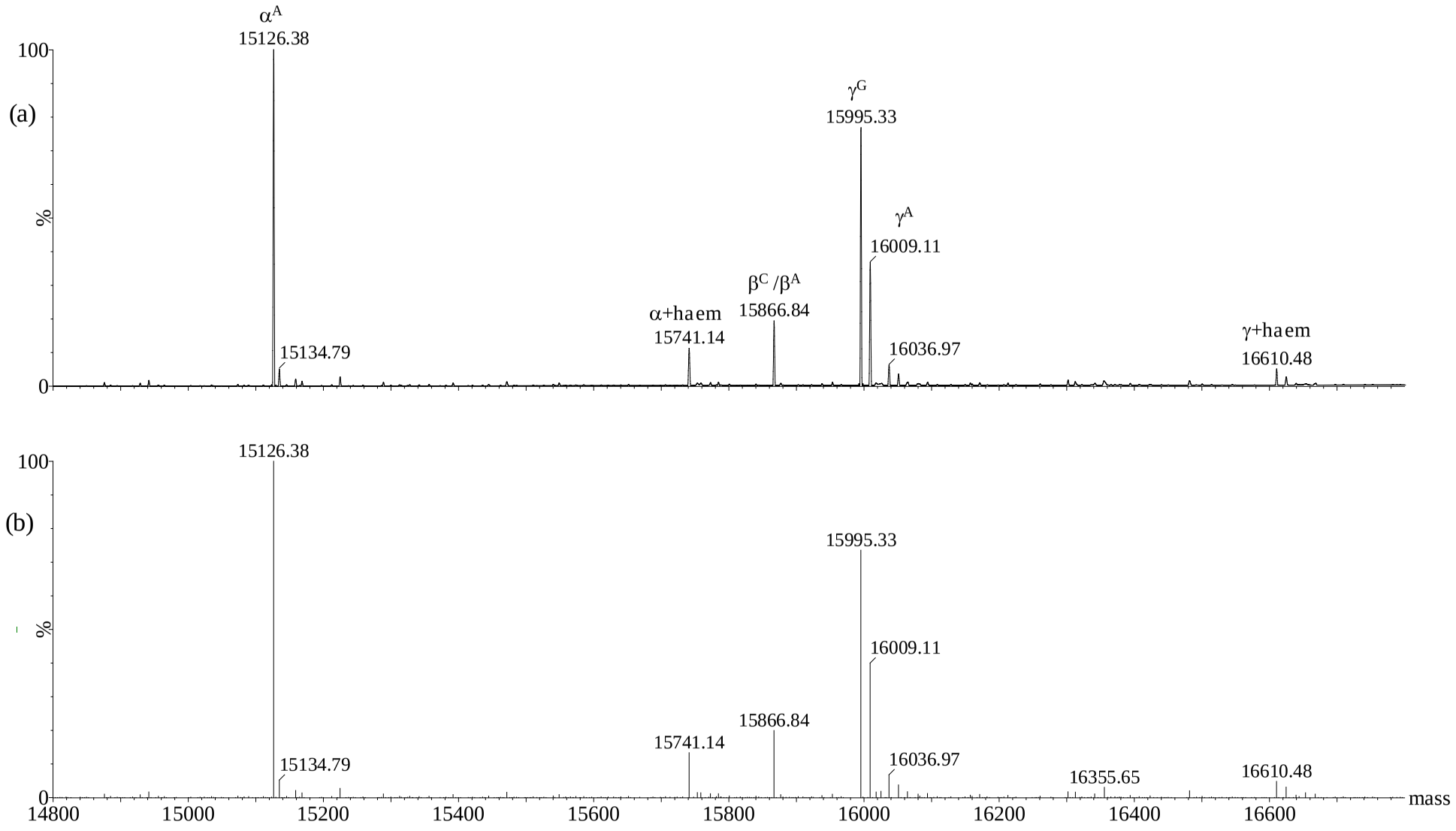

Figure 5.4.5.4. Deconvoluted mass spectrum of Hb C (β6Glu→Lys) showing the presence of a signal at 15,866.84 Da in the presence of Hb F (γ^G, 15,995.33 Da and γ^A,16,009.11 Da). The measured mass is indicative of a $\beta^A/(\beta^A\text{-}1)$ heterozygote.

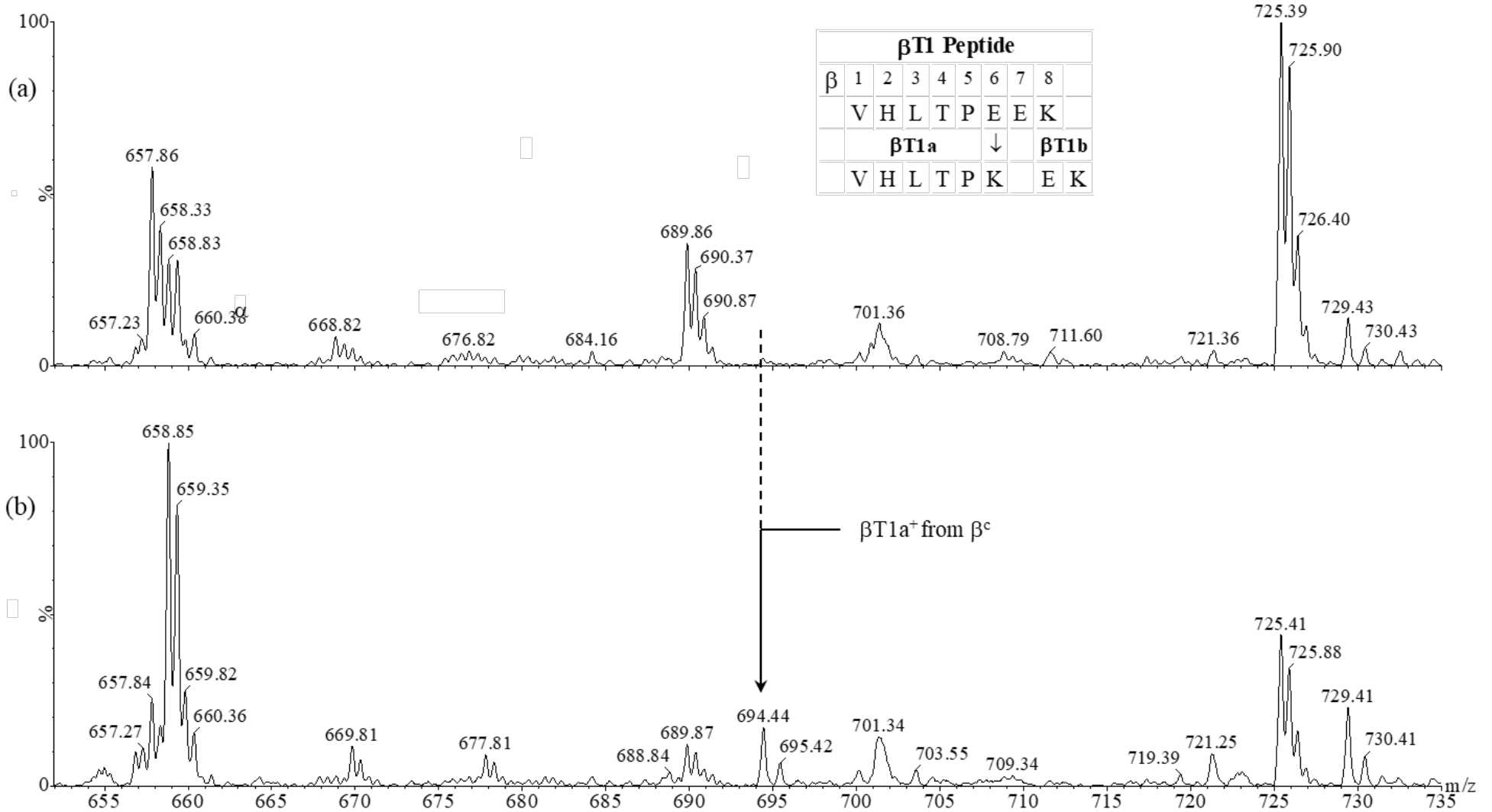

Figure 5.4.5.5. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb C heterozygote.

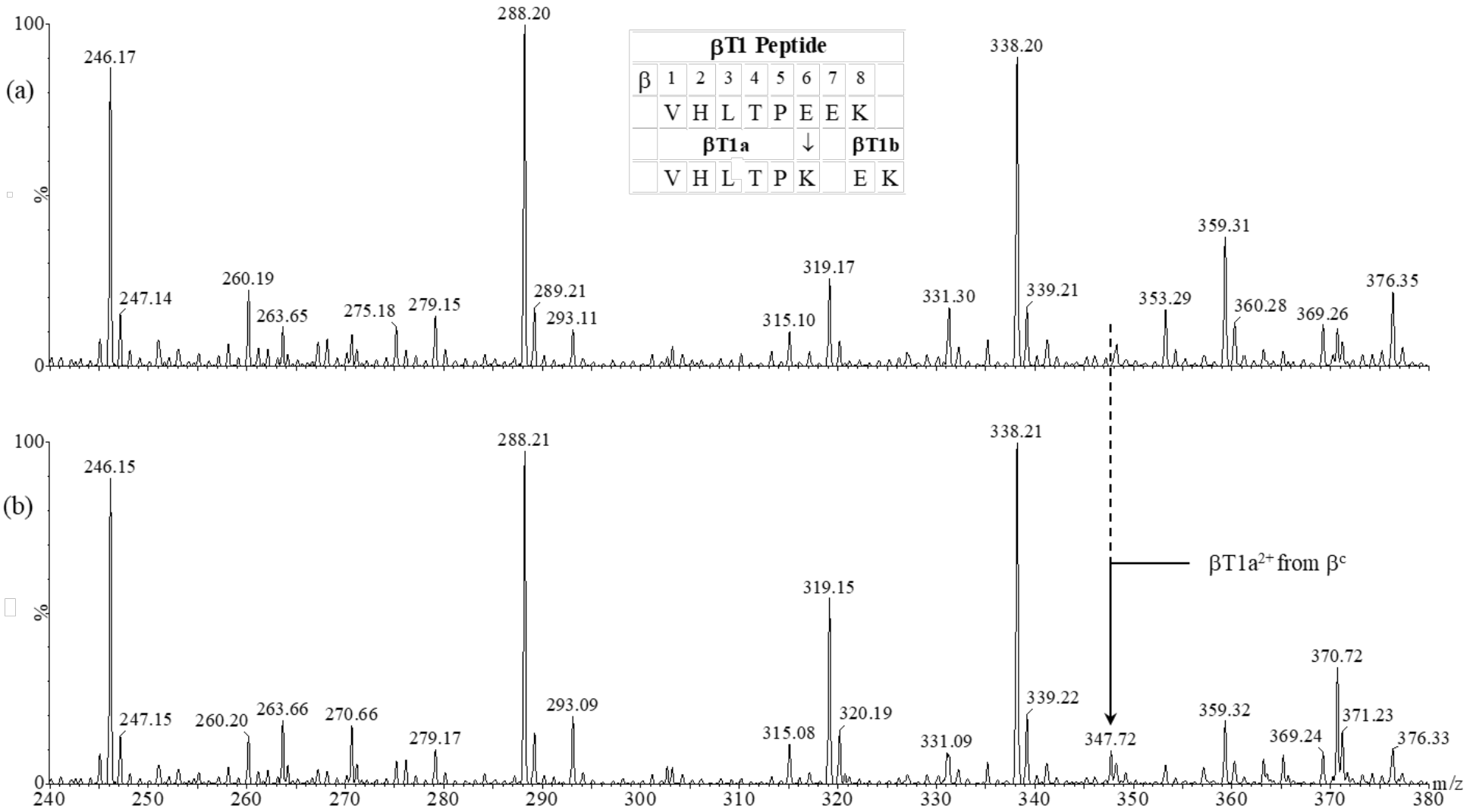

Figure 5.4.5.6. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb C heterozygote.

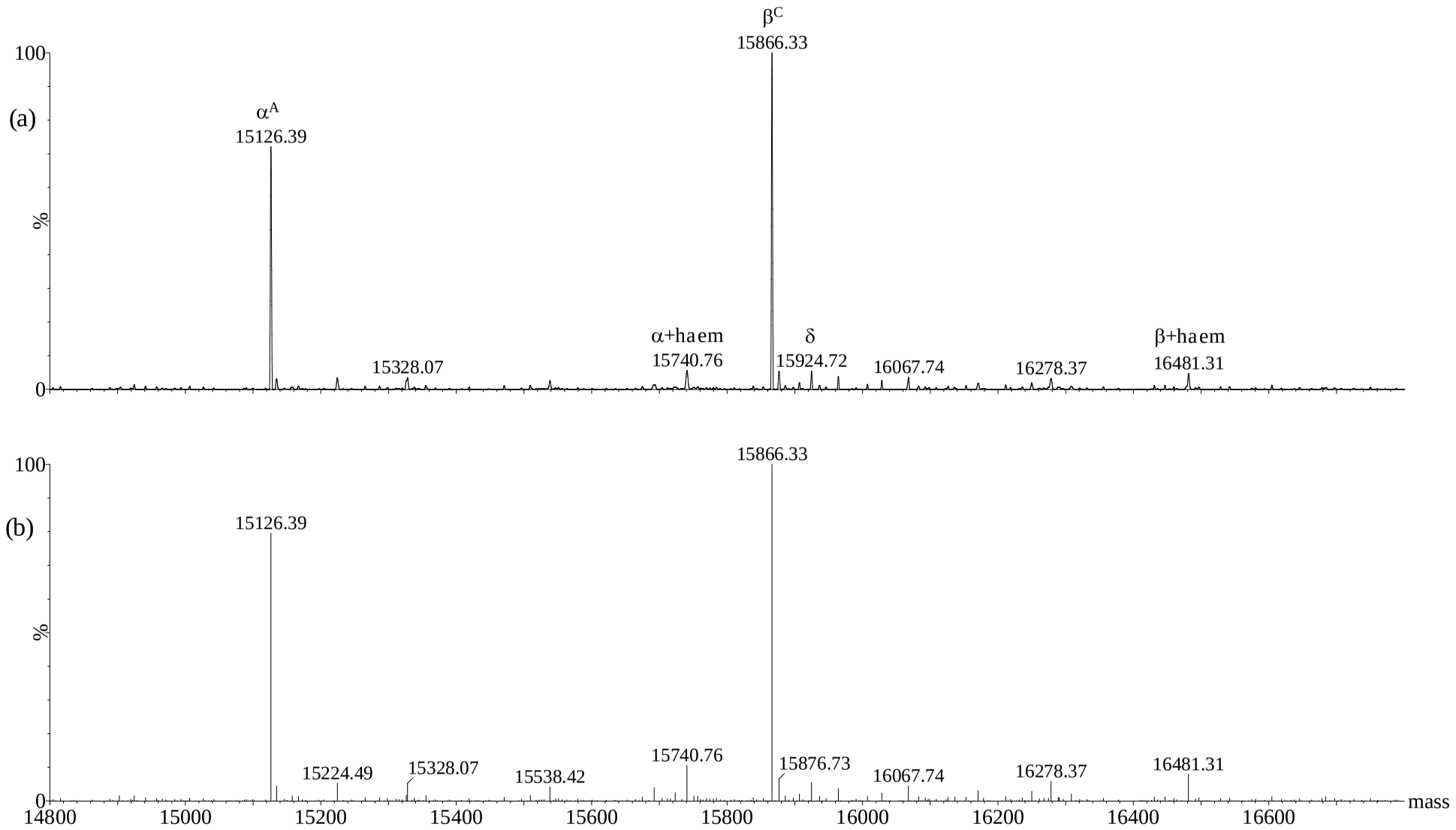

Figure 5.4.5.7. Deconvoluted mass spectrum of Hb C (β6Glu→Lys) showing the presence of a signal at 15,866.33 Da. The measured mass is indicative of a (β^A-1) homozygote, (β^A-1)/(β^A-1) or (β^A-1)/β-thal.

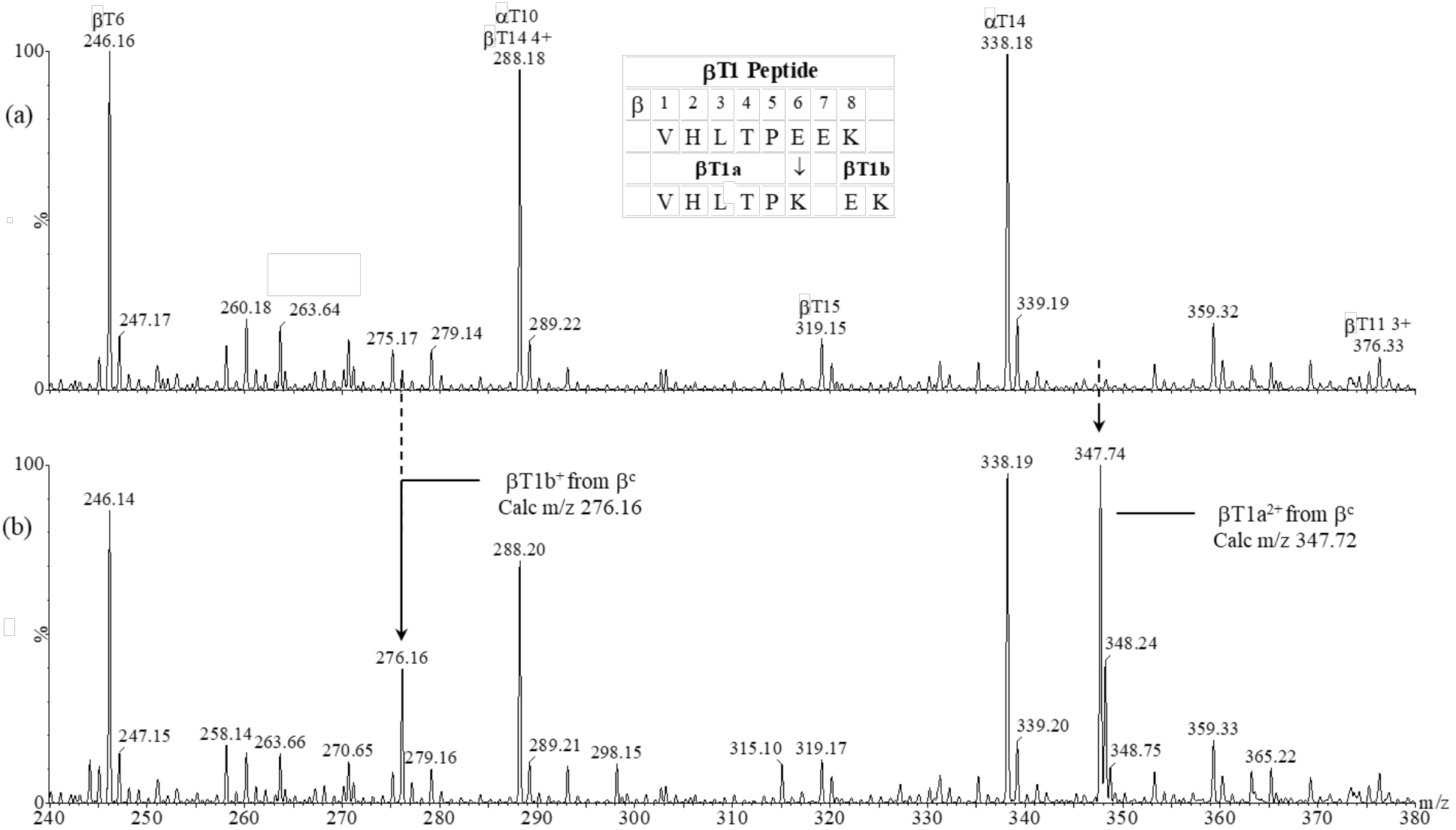

Figure 5.4.5.8. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb C homozygote.

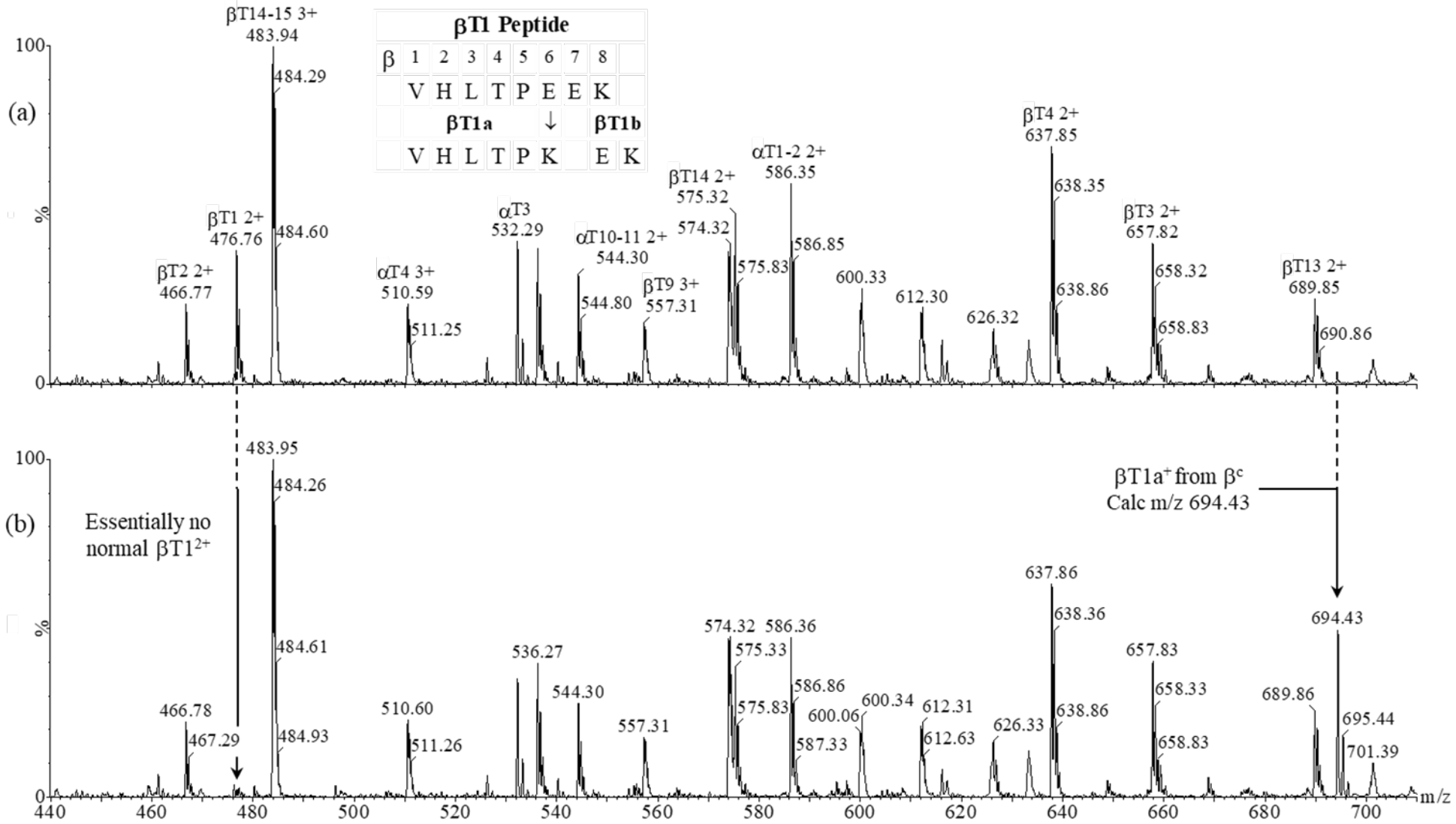

Figure 5.4.5.9. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb C homozygote.

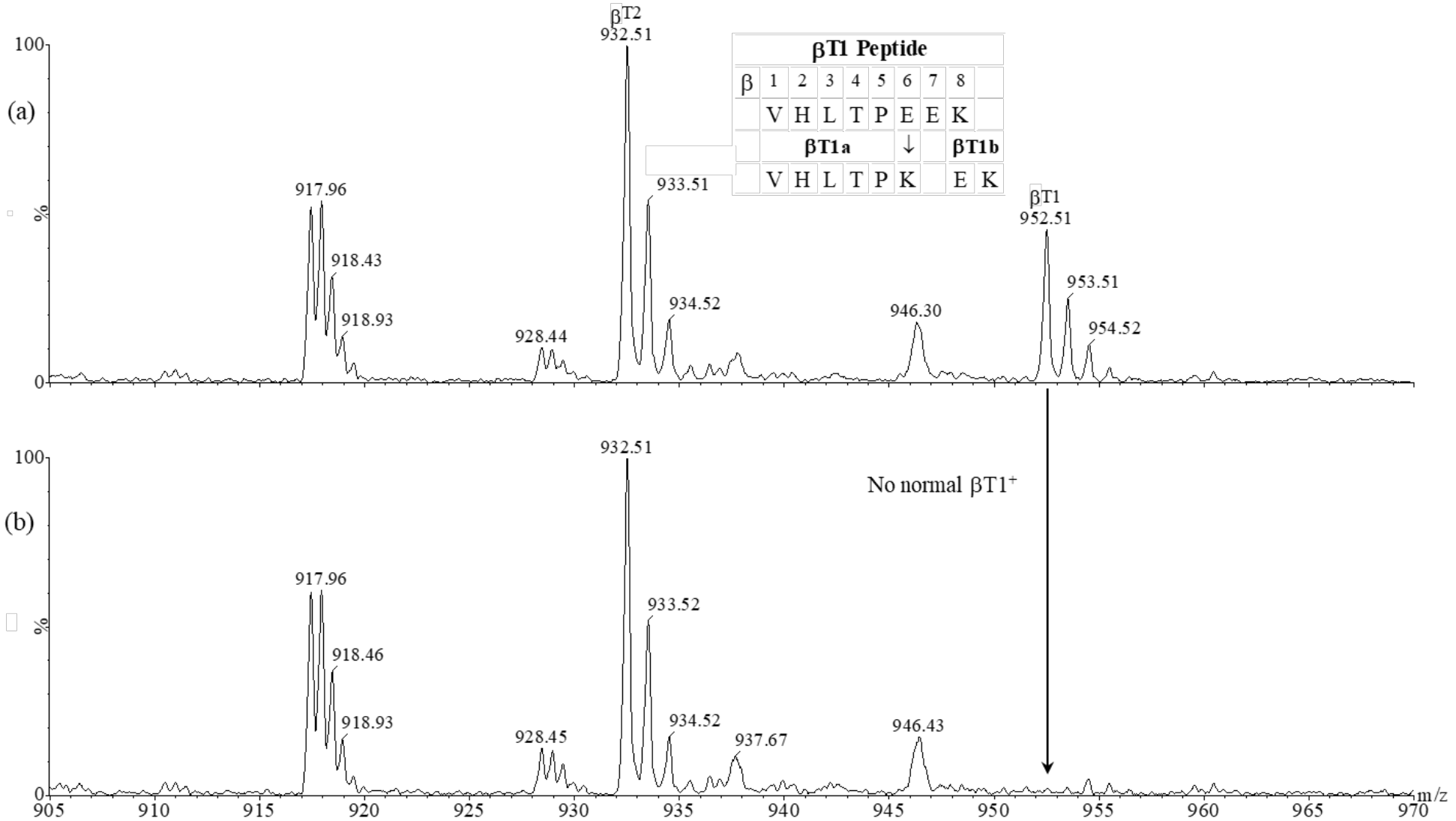

Figure 5.4.5.10. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb C homozygote.

5.4.6. βT1 - Hb Ziguinchor (β6Glu→Val and β58Pro→Arg)

Hb Ziguinchor is the result of a double mutation in the β-chain in which the β6 amino acid residue is changed from Glu to Val through a single base change in codon 6 CAG→CTG (Hb Sickle), and the β58 amino acid residue is changed from Pro to Arg through a single base change in codon 58 CCT→CGT (Hb Dhofar).

The mutation to the Arg residue results in an additional tryptic cleavage product, as shown in Figure 5.4.6.1.

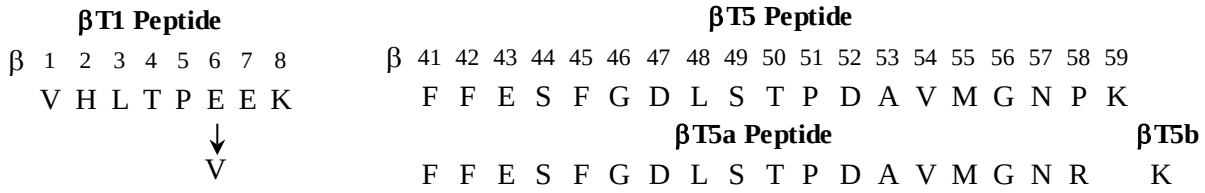

Figure 5.4.6.1. Sequences of the Hb Ziguinchor βT1 and βT5 tryptic peptides.

There is no ce-HPLC trace available for Hb Ziguinchor.

A blood sample was received for analysis that showed Hb C-like characteristics on HPLC. ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.4.6.2.) revealed a β-chain heterozygote in which the variant β-chain was 38.9% of total β-chains, and the mass of the variant chain was 15,896.22 Da, 28.93 Da heavier than normal. There is no single base change in the codon that can give rise to such a mass increase, implying that the sample contains a double mutation, or a mutation/modification, in a single beta chain.

Figure 5.4.6.3. shows one of the diagnostic regions of the tryptic digest spectrum of (a) normal Hb and (b) the variant Hb. A signal is observed at *m/z* 461.81 and is doubly charged, corresponding to the $\beta T1^{2+}$ tryptic peptide showing a decrease in mass of 30 Da from normal. This is confirmed in Figure 5.4.6.4. with the appearance of a signal at *m/z* 922.52, corresponding to the singly charged mutant βT1 peptide.

Figure 5.4.6.5. shows a region of the tryptic digest mass spectrum in which a new doubly charged signal is observed at *m/z* 995.46. The mass difference of -59 Da observed is consistent with the Hb Dhofar mutation, β58Pro→Arg. The β58Arg will give rise to an additional tryptic peptide.

Figure 5.4.6.6. shows the partial product ion spectrum of the $\beta T1^{2+}$ tryptic fragment of the specimen, in which the y''_3 ion (*m/z* 375.3) and the internal TPV fragment (*m/z* 298.3) confirm the mutation β6Glu→Val, Hb S.

Figure 5.4.6.7. shows the product ion spectrum of the $\beta T5a^{2+}$ tryptic fragment, and the observed fragments from the *m/z* 995.6 precursor are consistent with the mutation β58Pro→Arg.

This combination of the two mutations in a single β-chain - β6Glu→Val (Hb S) and β58Pro→Arg (Hb Dhofar) - is recorded in the literature as Hb Ziguinchor.

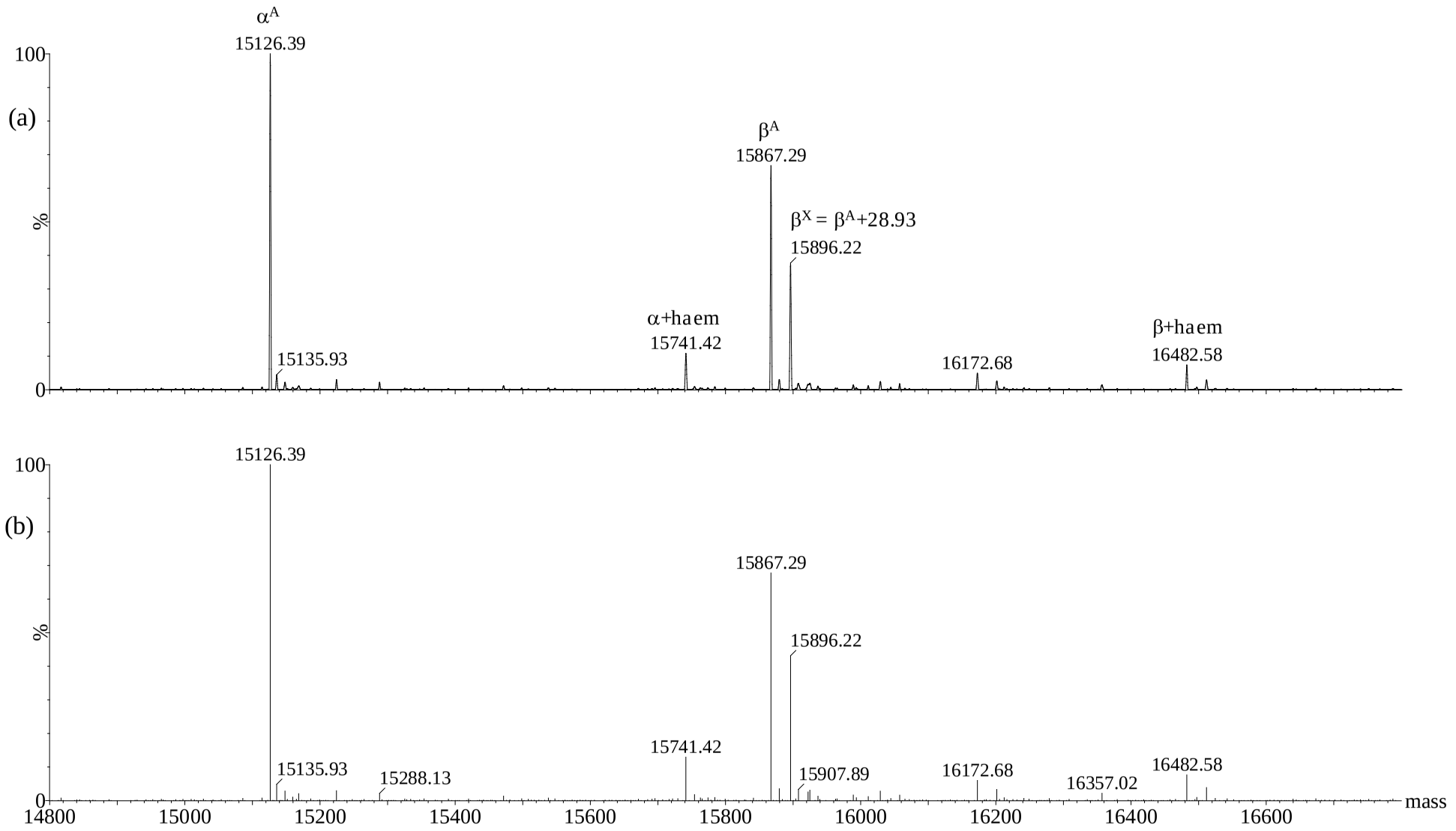

Figure 5.4.6.2. Deconvoluted mass spectrum of Hb Ziguinchor (β6Glu→Val and β58Pro→Arg) showing the presence of a signal at 15,896.22 Da at approximately 55% intensity of the normal β-chain peak (15,867.29 Da). The 29 Da mass increase suggests the presence of two mutations.

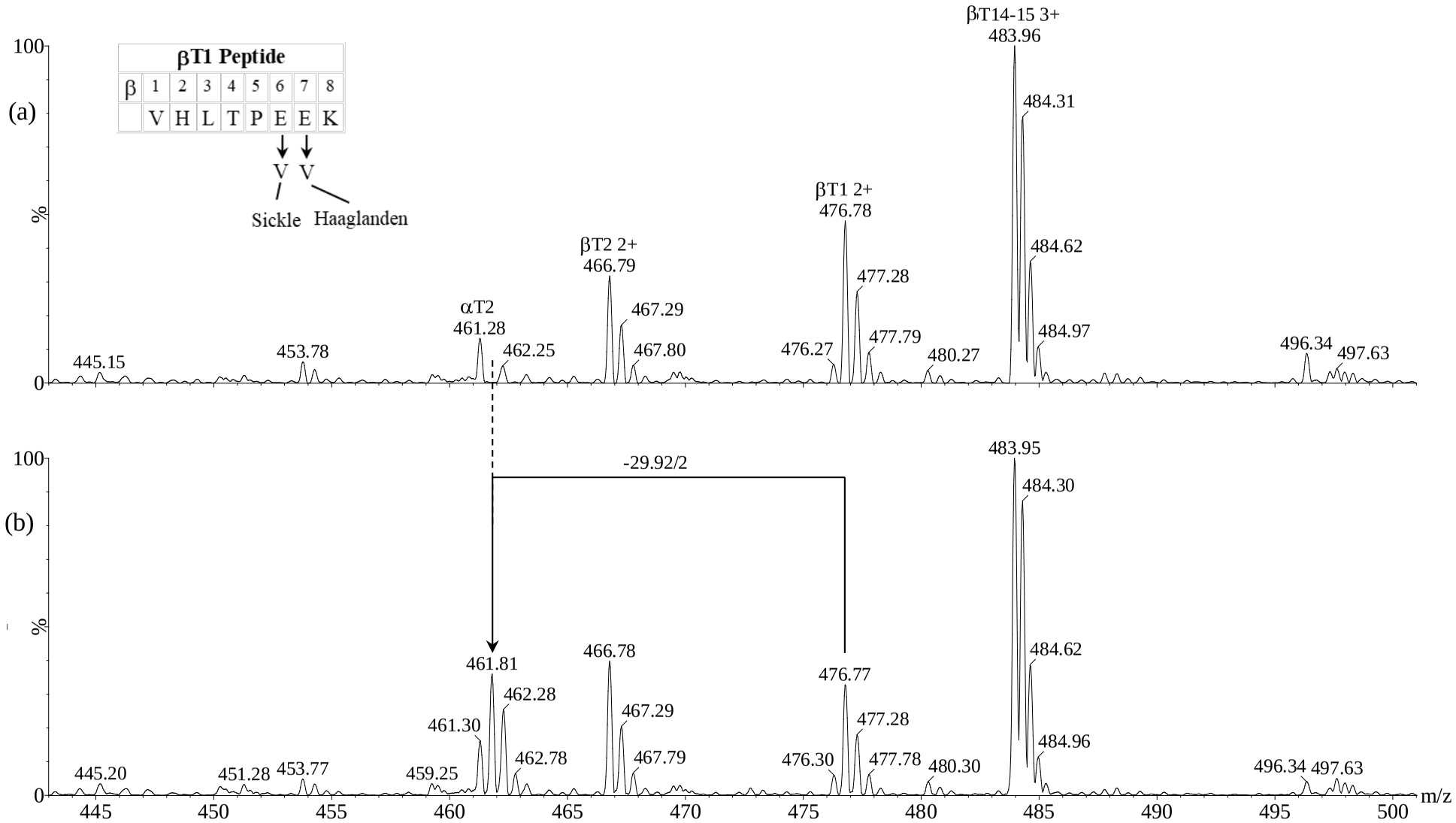

Figure 5.4.6.3. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Ziguinchor heterozygote. These data indicate that a mass decrease of 30 Da is present in the βT1 tryptic peptide.

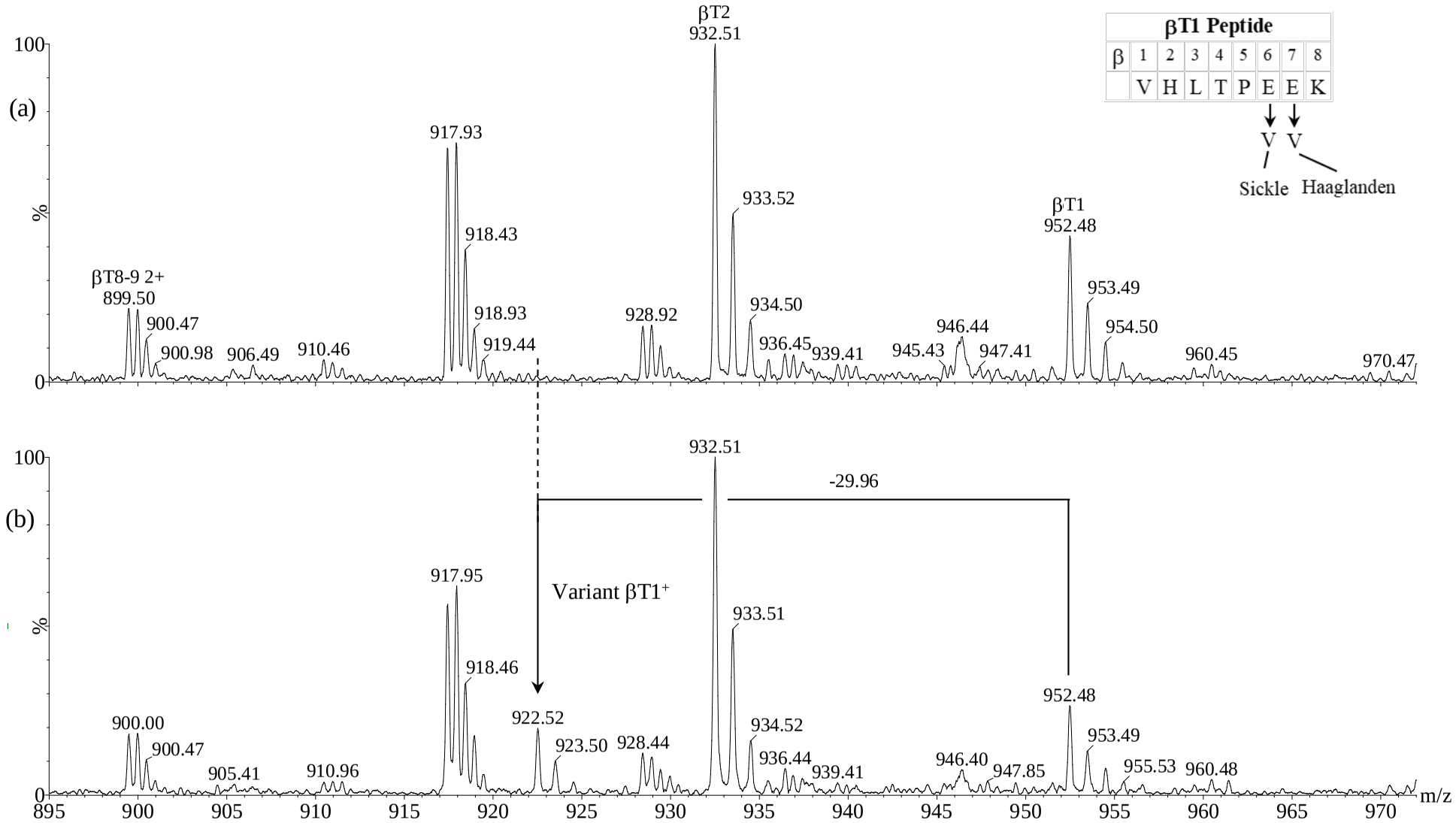

Figure 5.4.6.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Ziguinchor heterozygote. These data indicate that a mass decrease of 30 Da is present in the βT1 tryptic peptide.

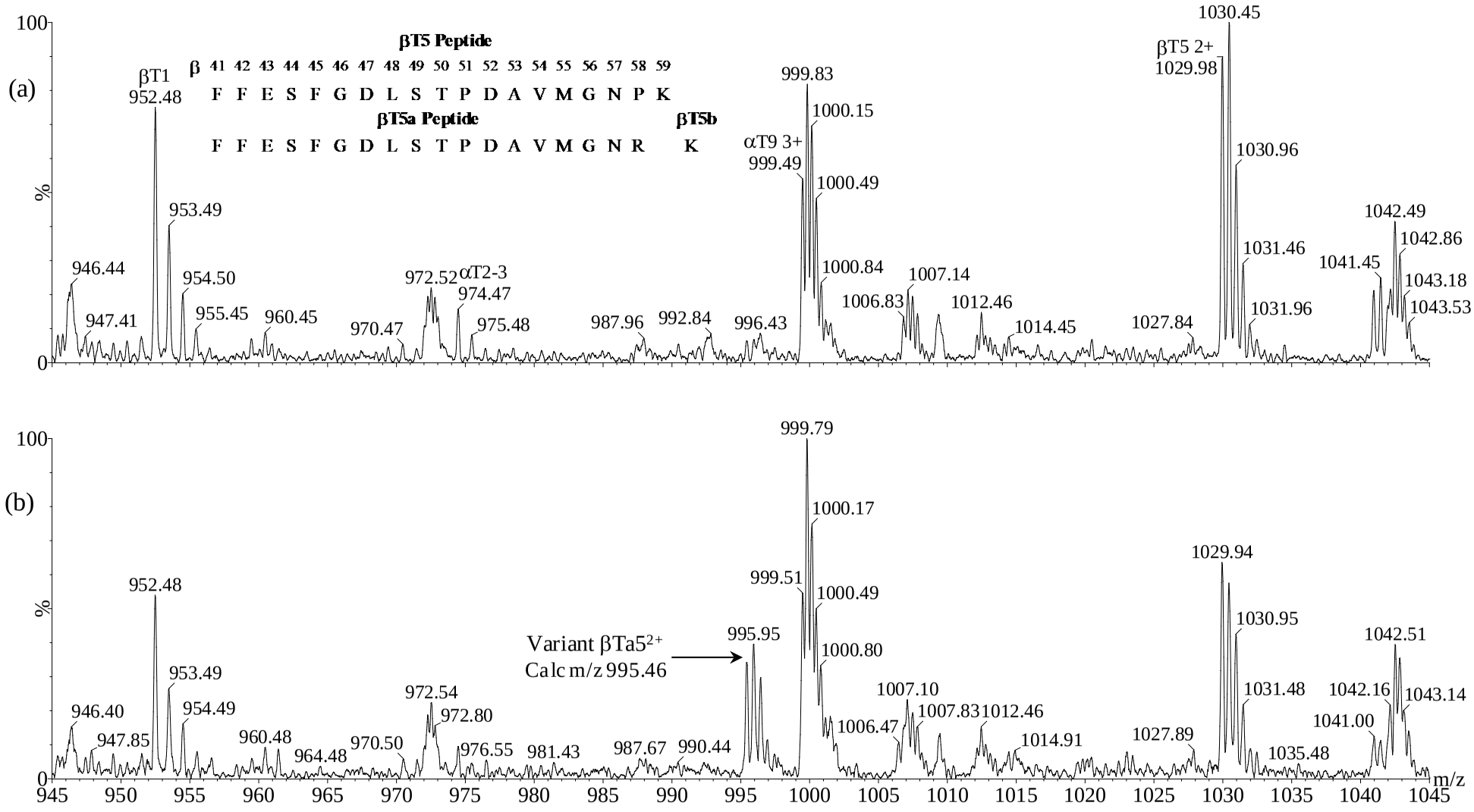

Figure 5.4.6.5. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Ziguinchor heterozygote. A new signal has appeared at *m/z* 995.46 and is consistent with the Hb Dhofar mutation (β58Pro→Arg).

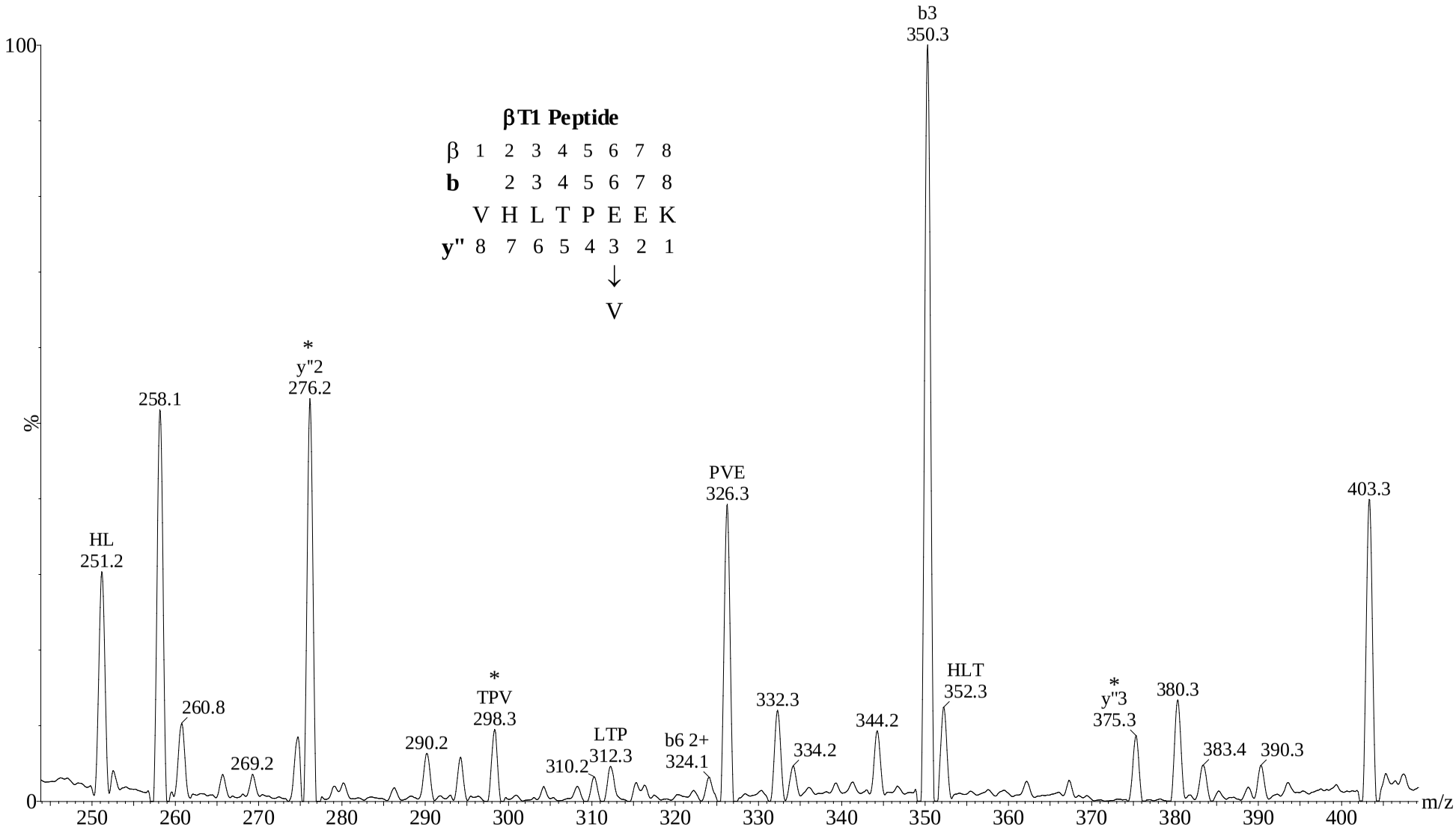

Figure 5.4.6.6. Partial Product ion spectra of the $\beta T1^{2+}$ tryptic fragment of Hb Ziguinchor. The $y"_3$ ion and the internal TPV fragment confirm the mutation β6Glu→Val, Hb Sickle.

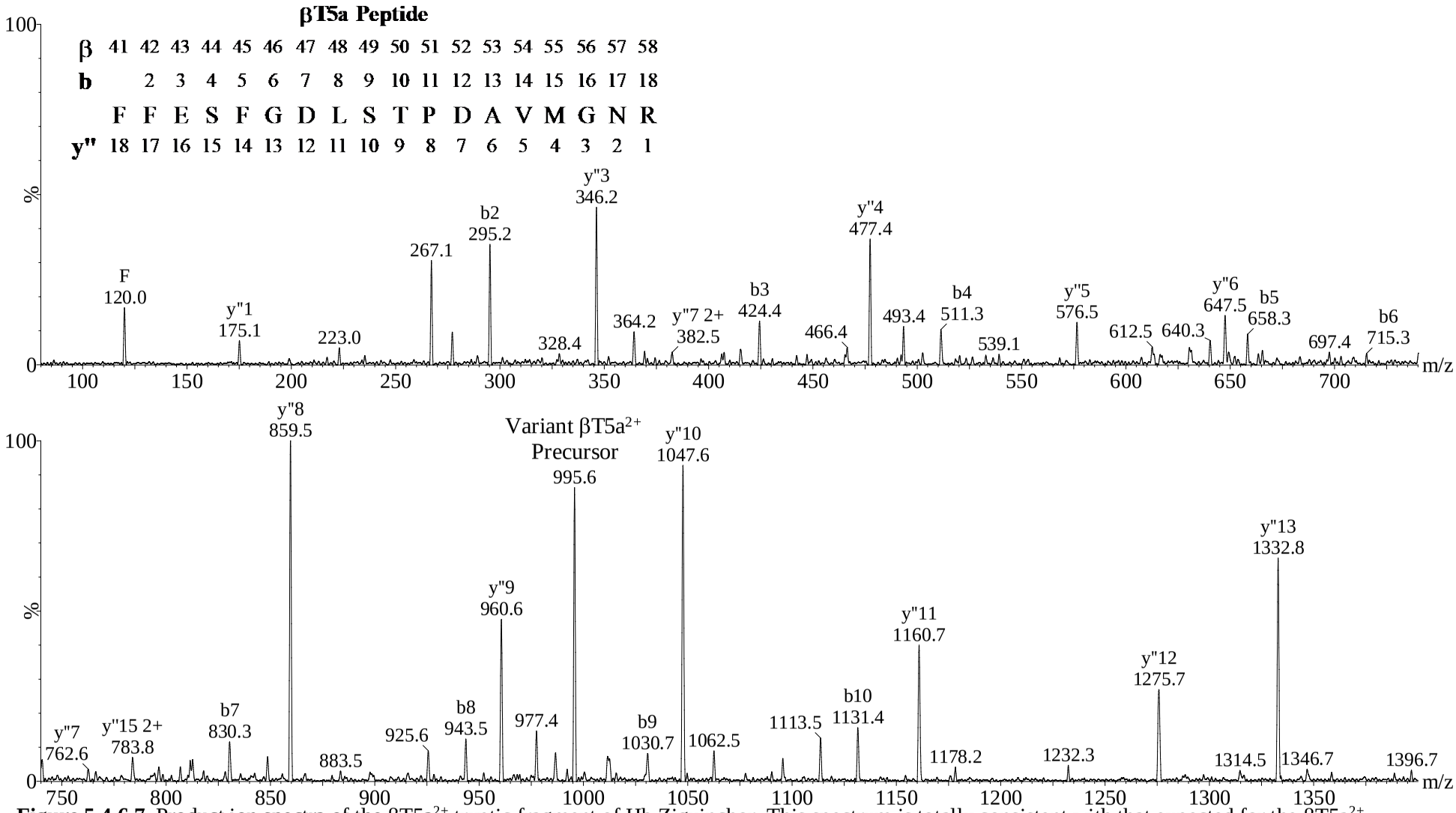

Figure 5.4.6.7. Product ion spectra of the $\beta T5a^{2+}$ tryptic fragment of Hb Ziguinchor. This spectrum is totally consistent with that expected for the $\beta T5a^{2+}$ owing to the mutation β58Pro→Arg.

5.4.7. βT2 - Hb Porto Alegre (β9Ser→Cys)

Hb Porto Alegre is the result of a β-chain mutation in which the β9 amino acid residue is changed from Ser to Cys through a single base change in the codon TCT→TGT.

βT2 Peptide

β	9	10	11	12	13	14	15	16	17
	S	A	V	T	A	L	W	G	K
	↓								
	C								

Figure 5.4.7.1. Sequence of the Hb Porto Alegre βT2 tryptic peptide.

There is no ce-HPLC trace available for Hb Porto Alegre. The sample was received as a dried blood spot with reports of sickle and a fast band on electrophoresis and IEF.

This is an example of a specimen containing two β-chain mutations, in separate chains.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.4.7.2.) revealed the presence of signals at 15,837.41 Da and 15,883.33 Da, indicating the possibility of a mutation in each of the β-coding genes. The (β^A+16) Da signal would be expected to be of similar intensity to the other chain, and the intensity of the mutation is increased after reduction with DTT (lower panel). Figure 5.4.7.3. shows the presence of a dimer at 31,764.51 Da, that disappears after reduction with DTT, and suggests the presence of Cys residue in the (β^A+16) Da species.

Figure 5.4.7.4. shows a comparison of a diagnostic region of the tryptic digest spectra of the baby, father and mother, showing that the mutation giving rise to the mass decrease of 30 Da in the βT1 tryptic fragment is also carried by the father, and indicates a mutation at either β6Glu→Val (Hb S) or β7Glu→Val (Hb Haaglanden).

Figure 5.4.7.5. shows the product ion spectra of the $\beta T1^{2+}$ tryptic fragment of (a) normal and (b) variant #1 in which this mutation is identified by the mass difference in the y''_3 fragment (*m/z* 298.3 in the lower panel) as β6Glu→Val, Hb S.

Figure 5.4.7.6. shows a comparison of the deconvoluted mass spectra for the mother's specimen (a) after the sample had been in solution for 15hr at 25°C and (b) after reduction with DTT. This samples shows similar characteristics to the baby specimen discussed in Figure 5.4.7.2., with the increased signal from the variant following DTT reduction. Figure 5.4.7.7. shows the presence of a dimer at 31,764.51 Da, that disappears after reduction with DTT. This suggests the presence of Cys residue in the (β^A+16) Da species. The carbonic anhydrase is a contaminant.

Figure 5.4.7.8. shows a comparison of the tryptic digest mass spectra from (a) baby, (b) father, (c) mother and (d) mother after reduction with DTT. The normal $\beta T2^{2+}$ tryptic peptide at *m/z* 466.78 is apparent in each specimen, with the proposed variant at *m/z* 474.77 in the baby's and mother's specimens, but absent in the specimen from the father. The single point mutation from a single base change in the codon giving rise to a +16 Da mass change could occur at β9Ser→Cys (Hb Porto Alegre), β10Ala→Ser (not previously reported), β11Val→Asp (Hb Windsor) or β13Ala→Ser (not previously reported). This is further supported by the singly charged $\beta T2^{+}$ tryptic peptides shown for the same specimens in Figure 5.4.7.9.

Figure 5.4.7.10 shows a comparison of the product ion spectra from the $\beta T2^{2+}$ precursor for (a) normal Hb and (b) the submitted sample. The 16 Da increase in the mass for the b_2 fragment (*m/z* 175.0) , and no mass change in y''_7 (*m/z* 675.3) and y''_8 (*m/z* 774.4) confirms the mutation as β9Ser→Cys, Hb Porto Alegre.

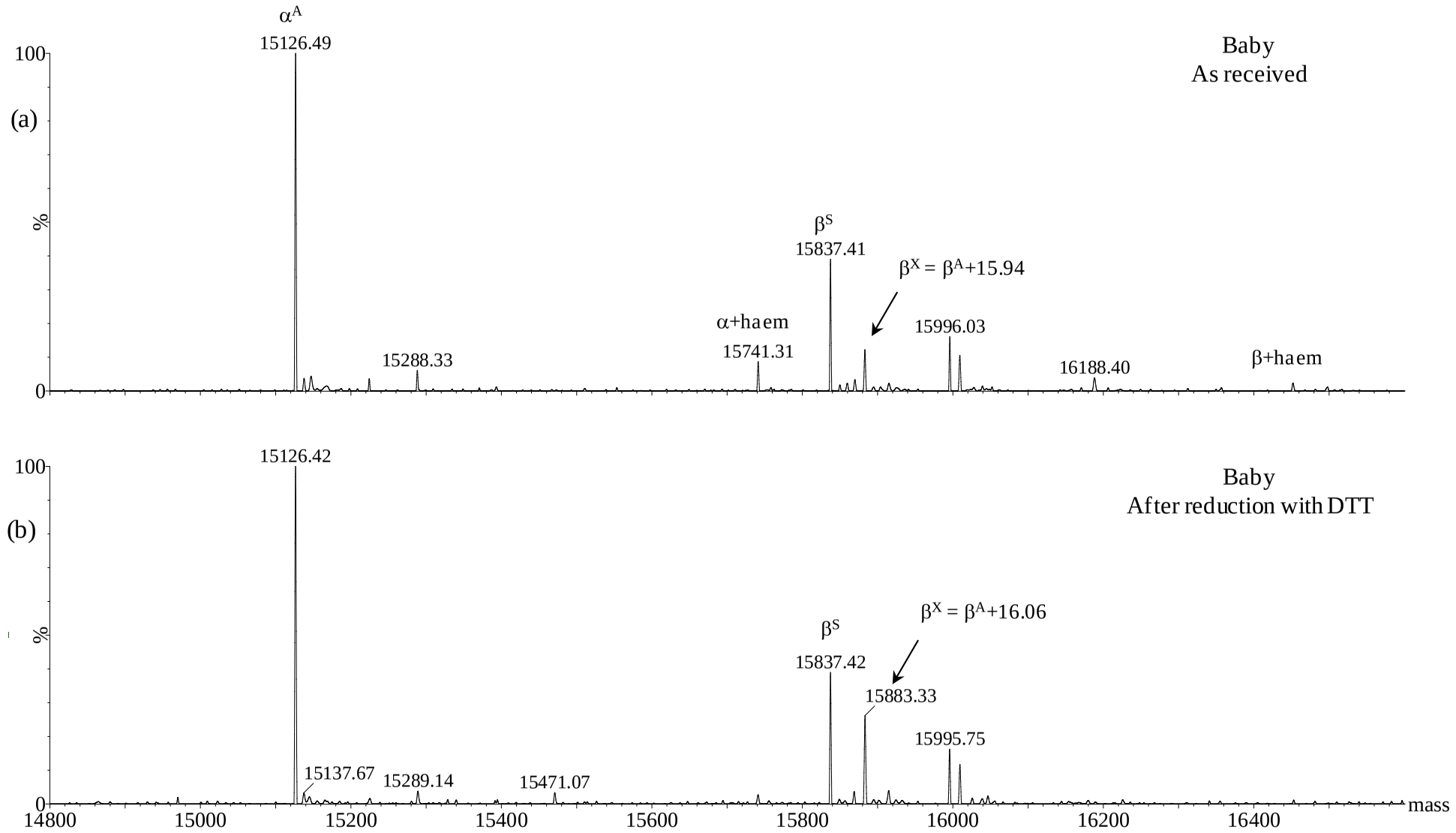

Figure 5.4.7.2. Deconvoluted mass spectrum of Hb Porto Alegre (β9Ser→Cys, 15,883.33 Da), heterozygous with a variant consistent with Hb S (β6Glu→Val, 15,837.42 Da).

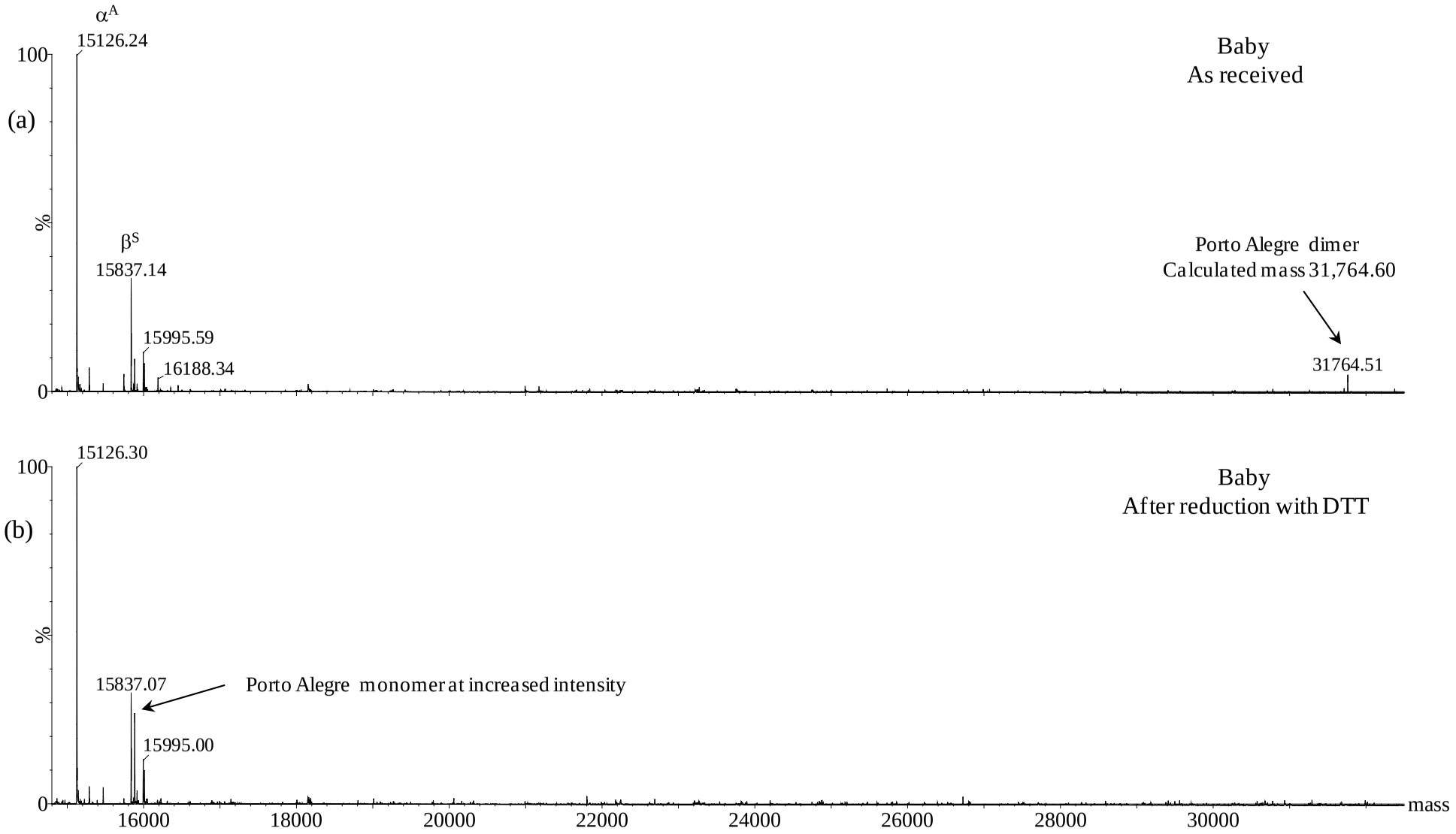

Figure 5.4.7.3. Deconvoluted mass spectrum of Hb Porto Alegre (β9Ser→Cys, 15,883.33 Da), heterozygous with a variant consistent with Hb S (β6Glu→Val, 15,837.42 Da). In the non-reduced spectrum (a) a dimer is observed for the Porto Alegre variant.

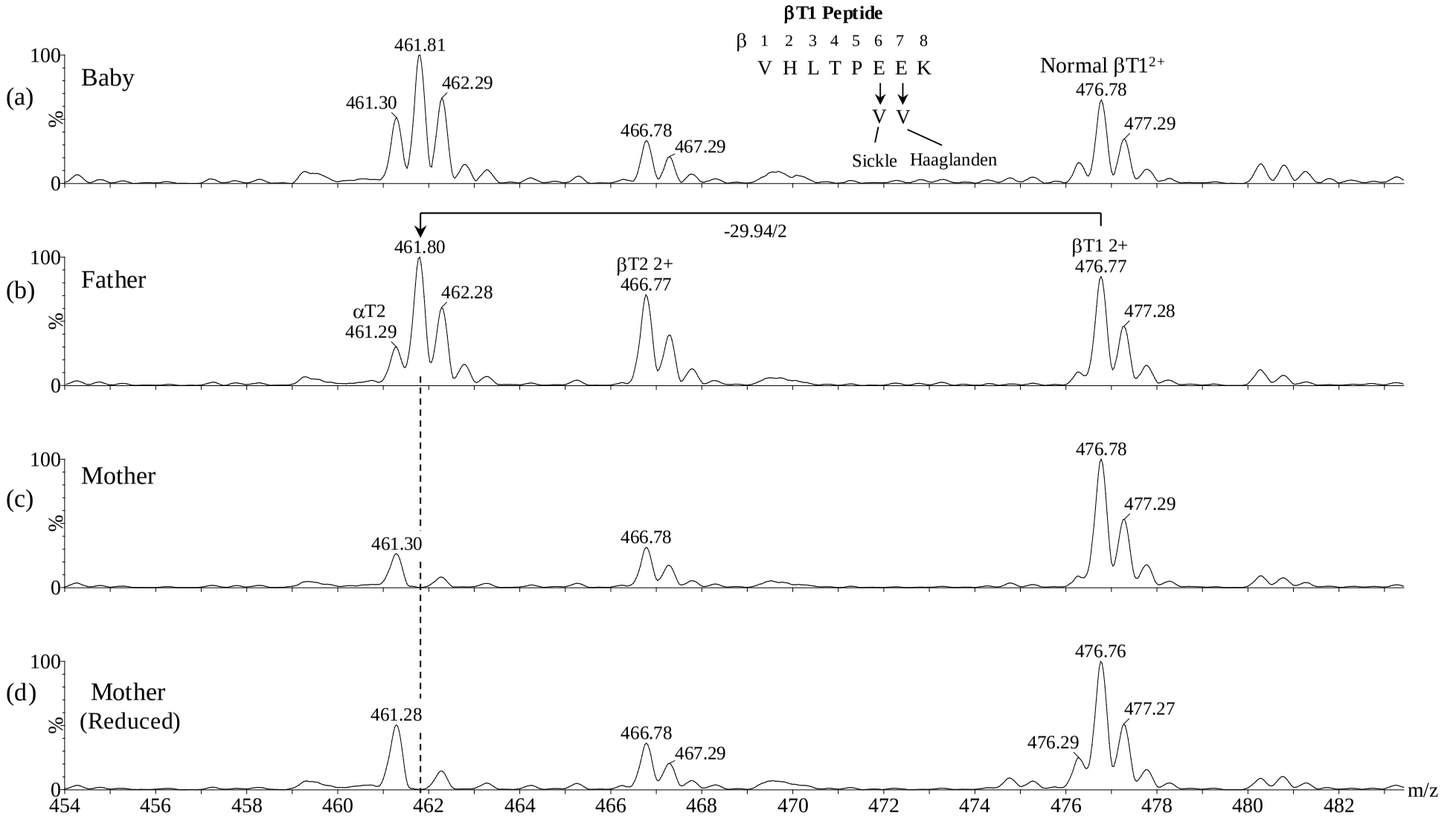

Figure 5.4.7.4. Tryptic digest mass spectra for (a) Baby, (b) Father, (c) Mother and (d) Mother after treatment with DTT. These data show that the first variant (mass decrease 30 Da) occurs at either β6 or β7 in Baby and Father, and is consistent with β^S.

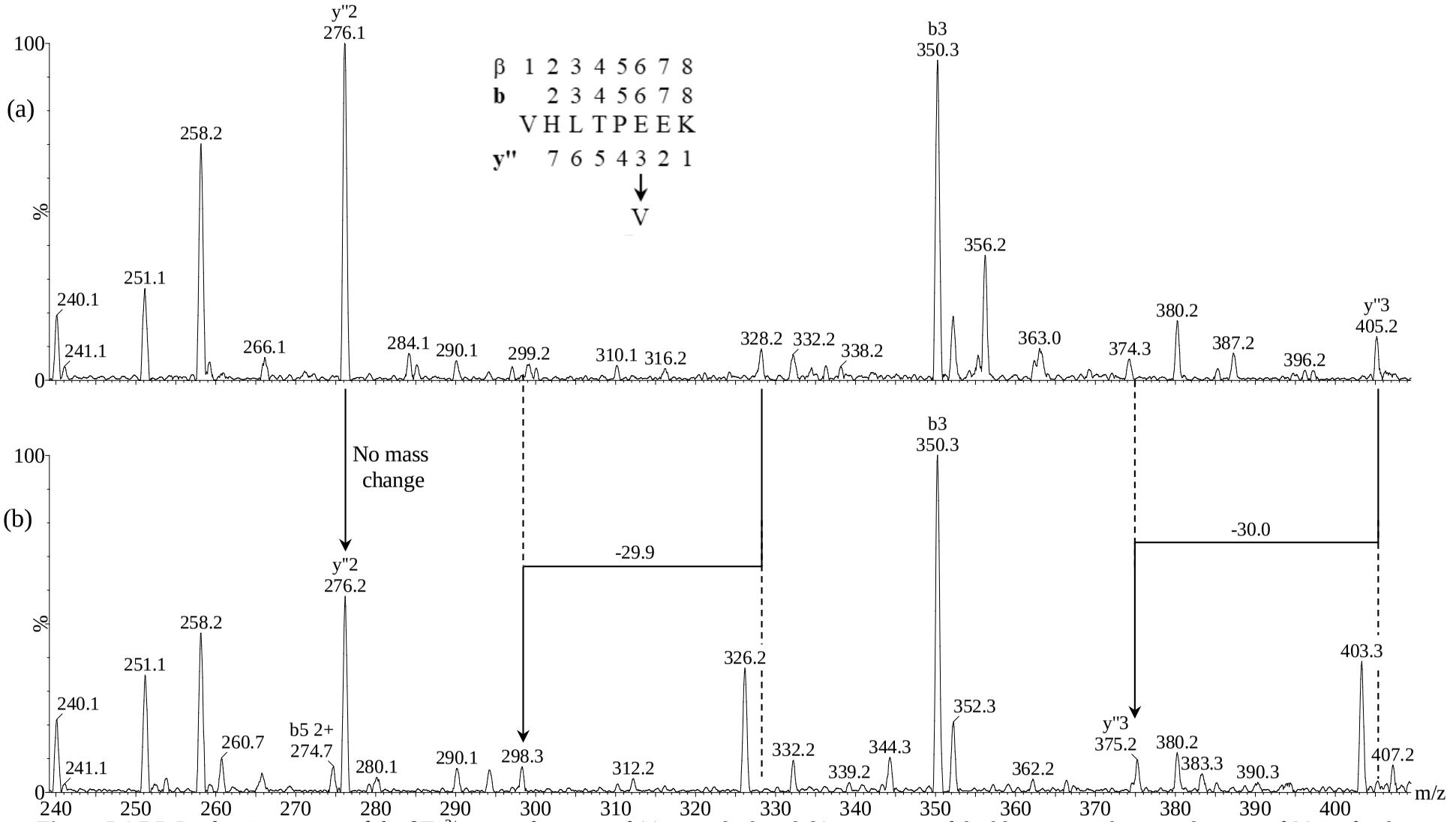

Figure 5.4.7.5. Product ion spectra of the $\beta T1^{2+}$ tryptic fragment of (a) normal Hb and (b) Variant #1 of double variant. The mass decrease of 30 Da for the $y"_3$ peak identifies this mutation as β6Glu→Val, Hb S.

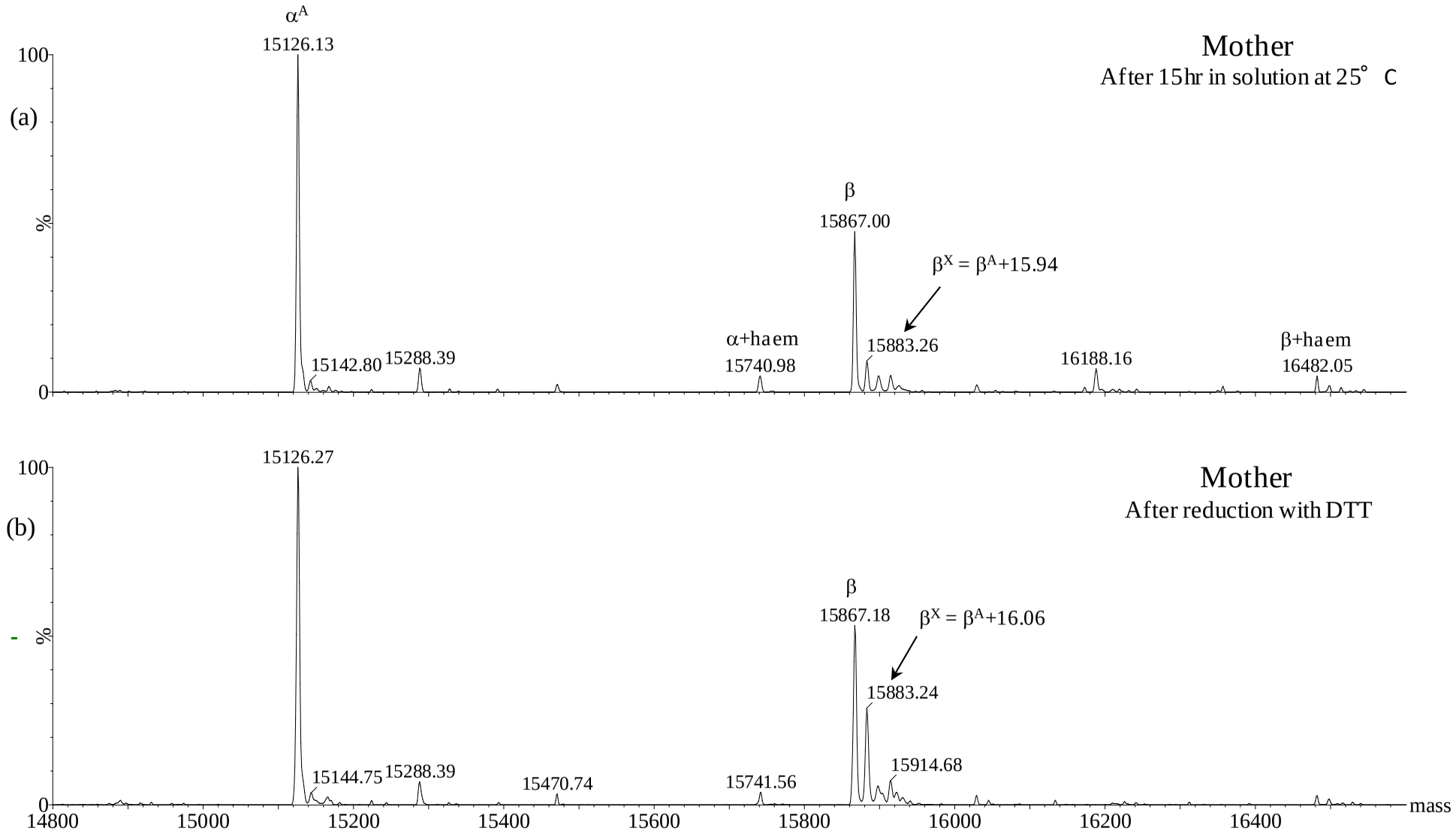

Figure 5.4.7.6. Deconvoluted mass spectrum of Hb Porto Alegre (β9Ser→Cys) showing the presence of a signal at 15,883.26 Da at approximately 50% intensity of the normal β-chain peak (15,867.18 Da).

339

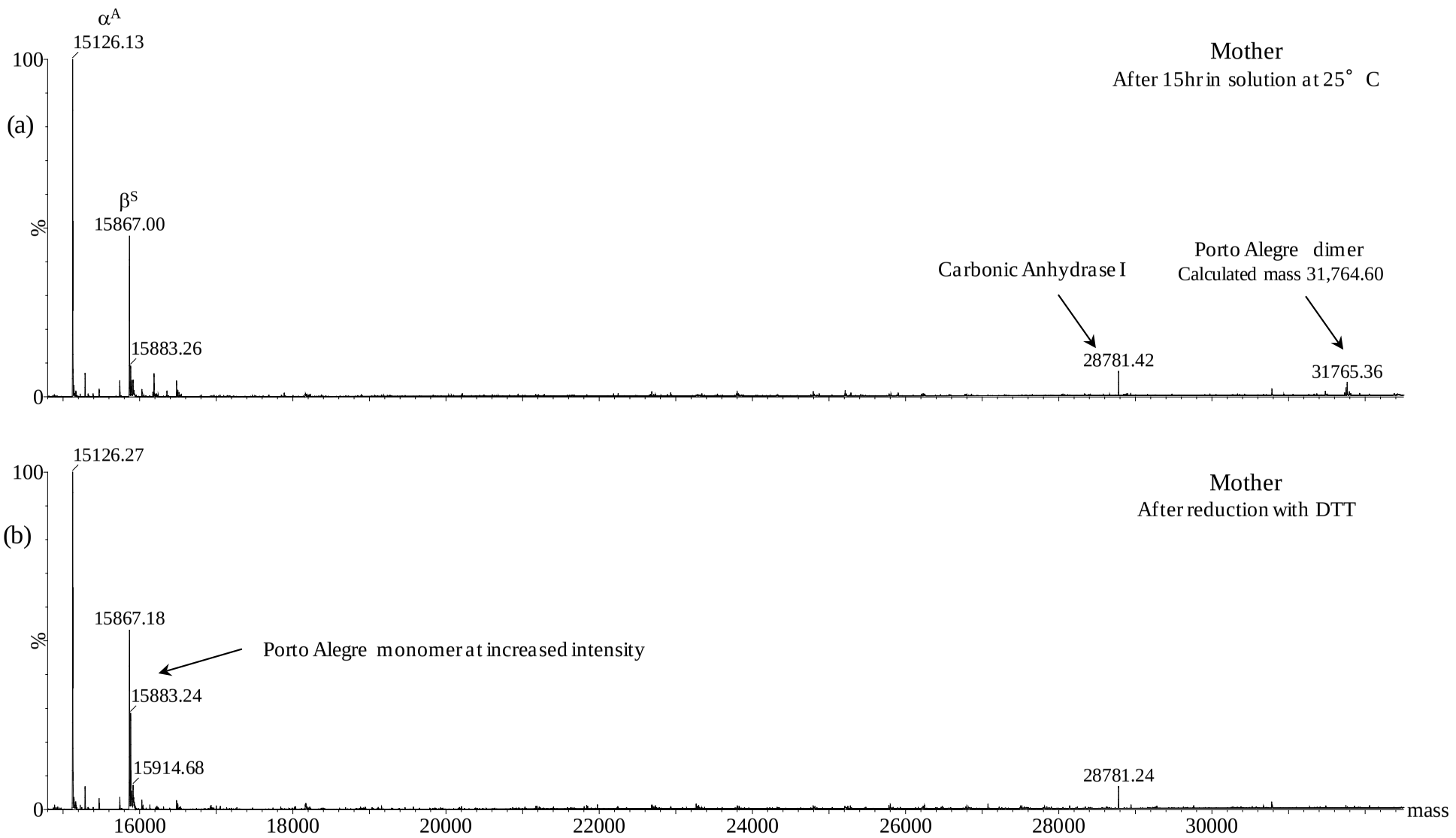

Figure 5.4.7.7. These spectra show the Porto Alegre β-chain dimer close to its calculated mass in the 'as received' (a) sample. The dimer disappears upon reduction with DTT with a marked increase in the intensity of the monomer of the β-chain variant.

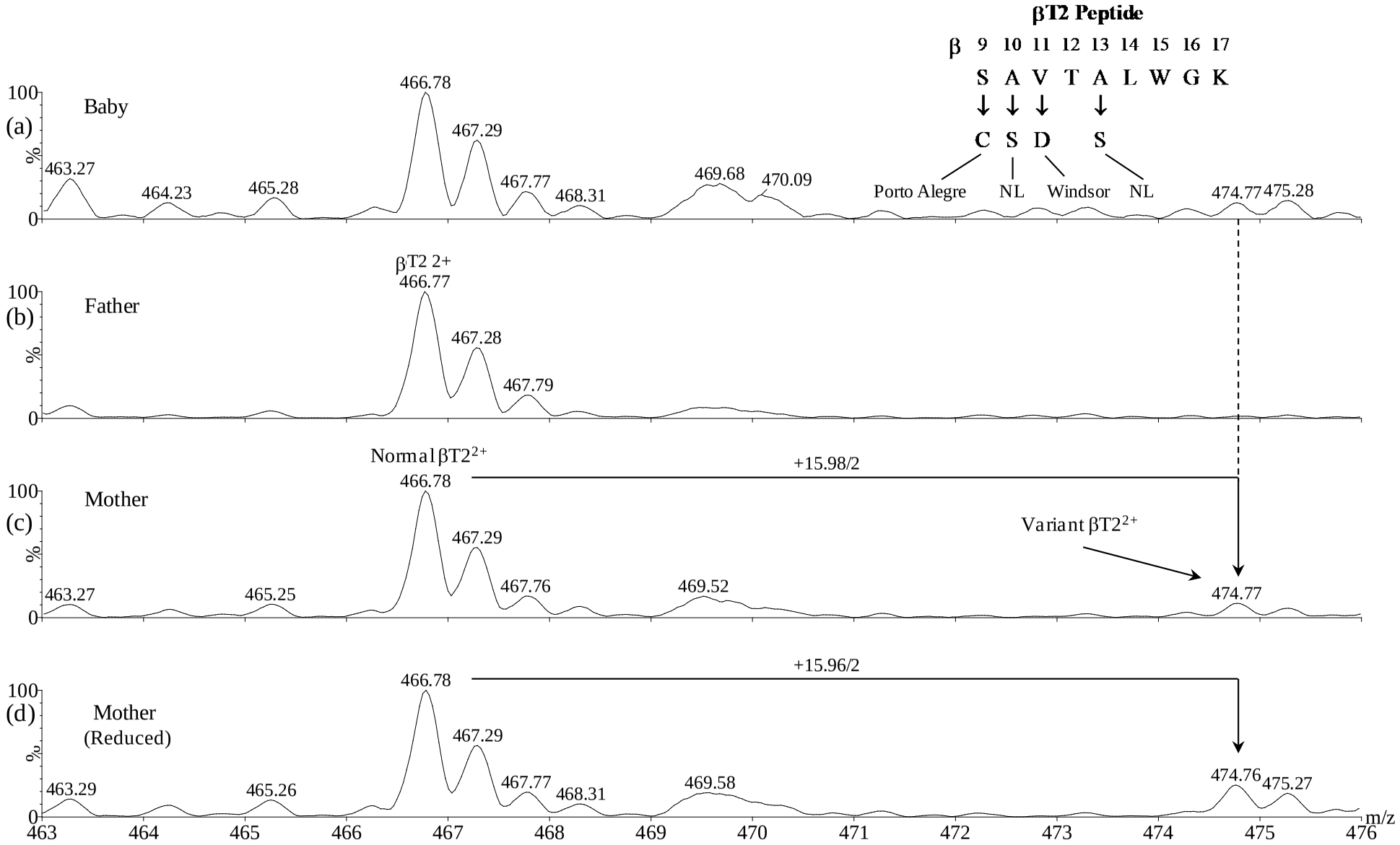

Figure 5.4.7.8. These tryptic digest spectra show that the mutation occurs in the βT2 peptide.

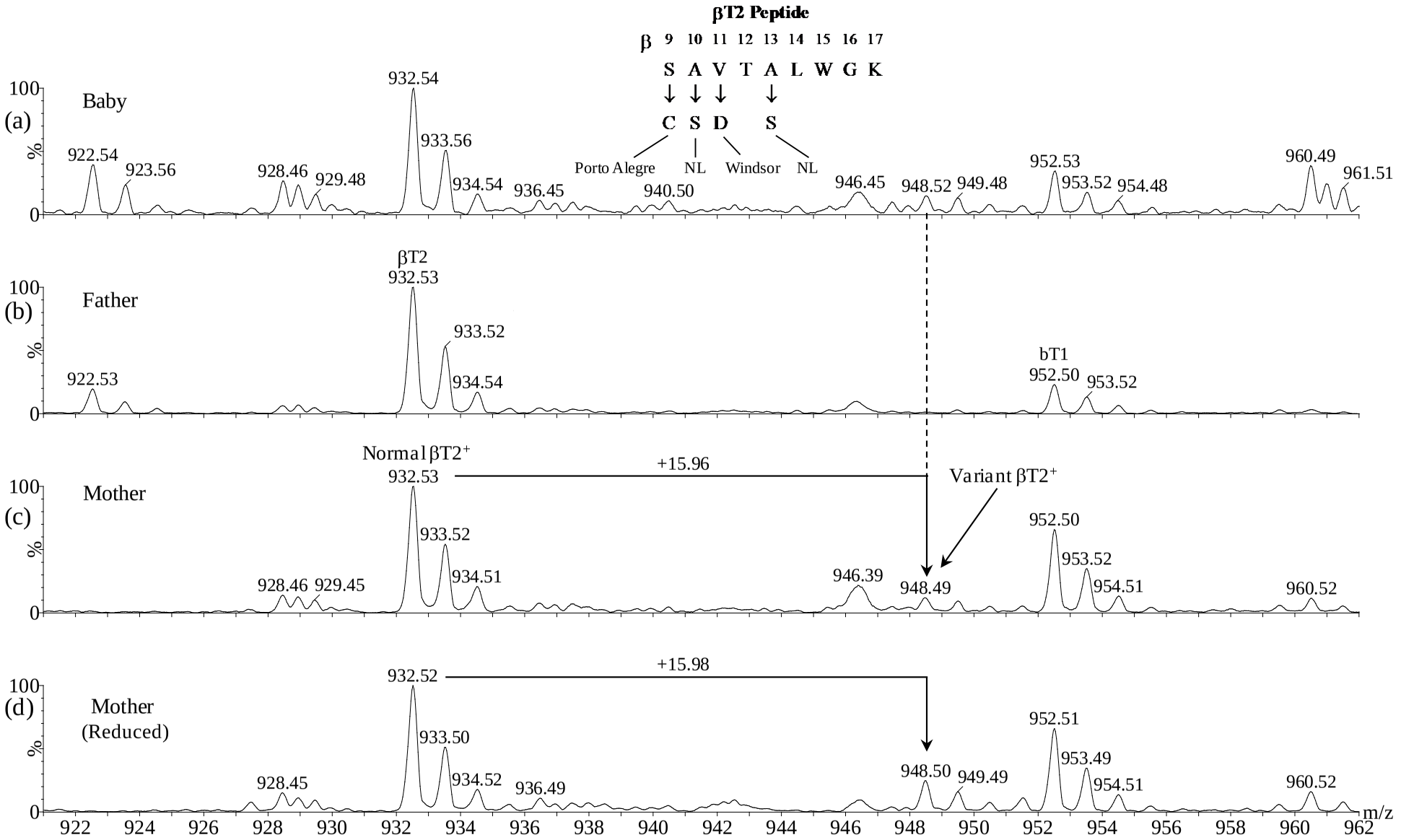

Figure 5.4.7.9. These tryptic digest spectra show that the mutation occurs in the βT2 peptide.

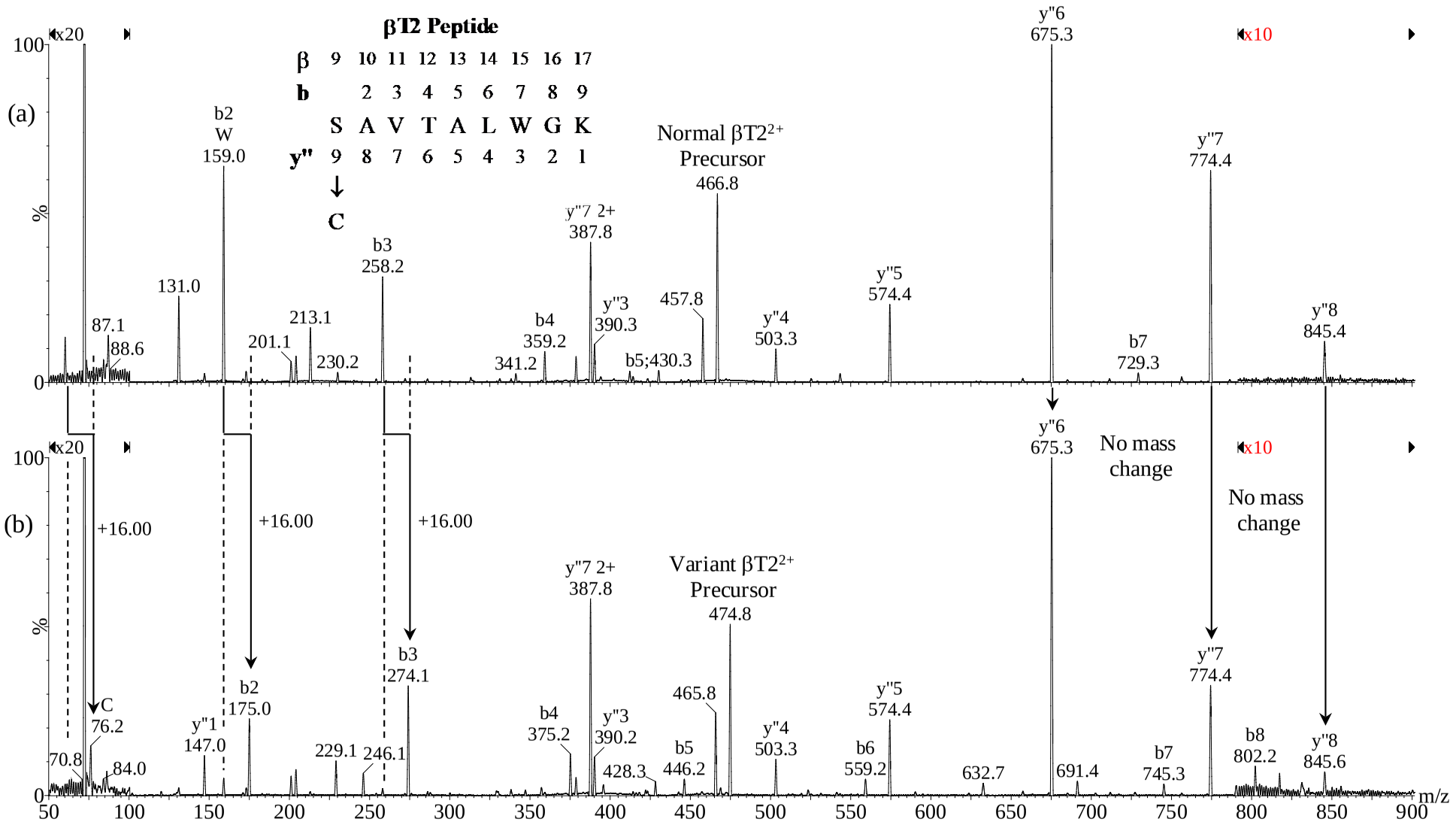

Figure 5.4.7.10. Product ion spectra of the $\beta T2^{2+}$ tryptic fragment of (a) normal Hb and (b) Hb Porto Alegre. The 16 Da increase in mass for the b_2 ion, and no mass change in $y"_7$ and $y"_8$ confirm that the mutation is β9Ser-Cys, Hb Porto Alegre.

5.4.8. βT2 - Hb Belfast (β15Trp→Arg)

Hb Belfast is the result of a β-chain mutation in which the β15 amino acid residue is changed from Trp to Arg through a single base change in the codon TGG→AGG or CGG.

The mutation to the Arg residue results in an additional tryptic cleavage product, as shown in Figure 5.4.8.1.

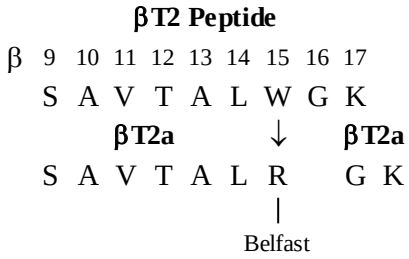

Figure 5.4.8.1. Sequence of the Hb Belfast βT2 tryptic peptides.

A blood sample was submitted for analysis by mass spectrometry because the ce-HPLC trace (Figure 5.4.8.2.) showed a response (31.9%) in the S-window and indicates a positive charge change.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	1.8	---	1.08	30084
Unknown	---	0.5	1.24	7708
P2	---	2.0	1.34	34621
Unknown	---	0.6	1.51	10670
P3	---	2.3	1.70	39297
A0	---	57.6	2.42	979079
A2	3.0	---	3.62	55790
S-window	---	31.9	4.31	542242

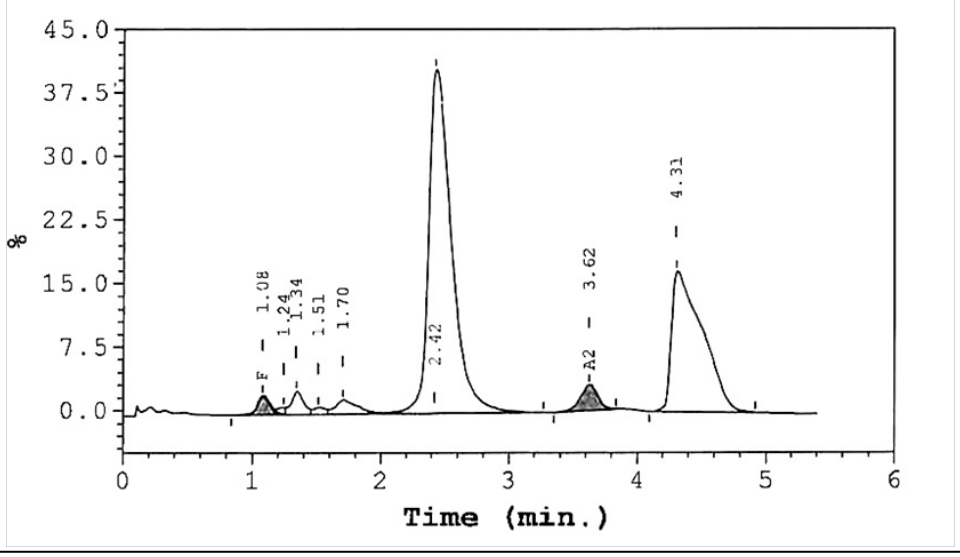

Figure 5.4.8.2. ce-HPLC trace for Hb Belfast.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.4.8.3.) revealed a β-chain heterozygote in which the variant β-chain was approximately 50% the intensity of the normal β-chain, and the mass of the variant chain was 15,837.08 Da, 30.03 Da lighter than normal. A single codon change giving a mass decrease of 30 Da coupled with the appearance in the HPLC S-window suggests either Trp→Arg (2 possibilities) or Glu→Val (4 possibilities).

Figure 5.4.8.4. shows the diagnostic part of the spectrum for the tryptic digest of (a) normal Hb and (b) the variant Hb which shows the appearance of a new peak at *m/z* 717.44, which is consistent with a new tryptic peptide, $\beta T2a^{+}$, derived from the βT2 fragment in which the Trp residue is changed to Arg, identifying the mutation as β15Trp→Arg, Hb Belfast. This is further supported by data in Figure 5.4.8.5. with the appearance of a new signal at *m/z* 359.22, $\beta T2a^{2+}$ in the lower panel.

Figure 5.4.8.6. shows the product ion spectrum of the $\beta T2a^{2+}$ tryptic fragment, which is consistent with the mutation β15Trp→Arg, Hb Belfast.

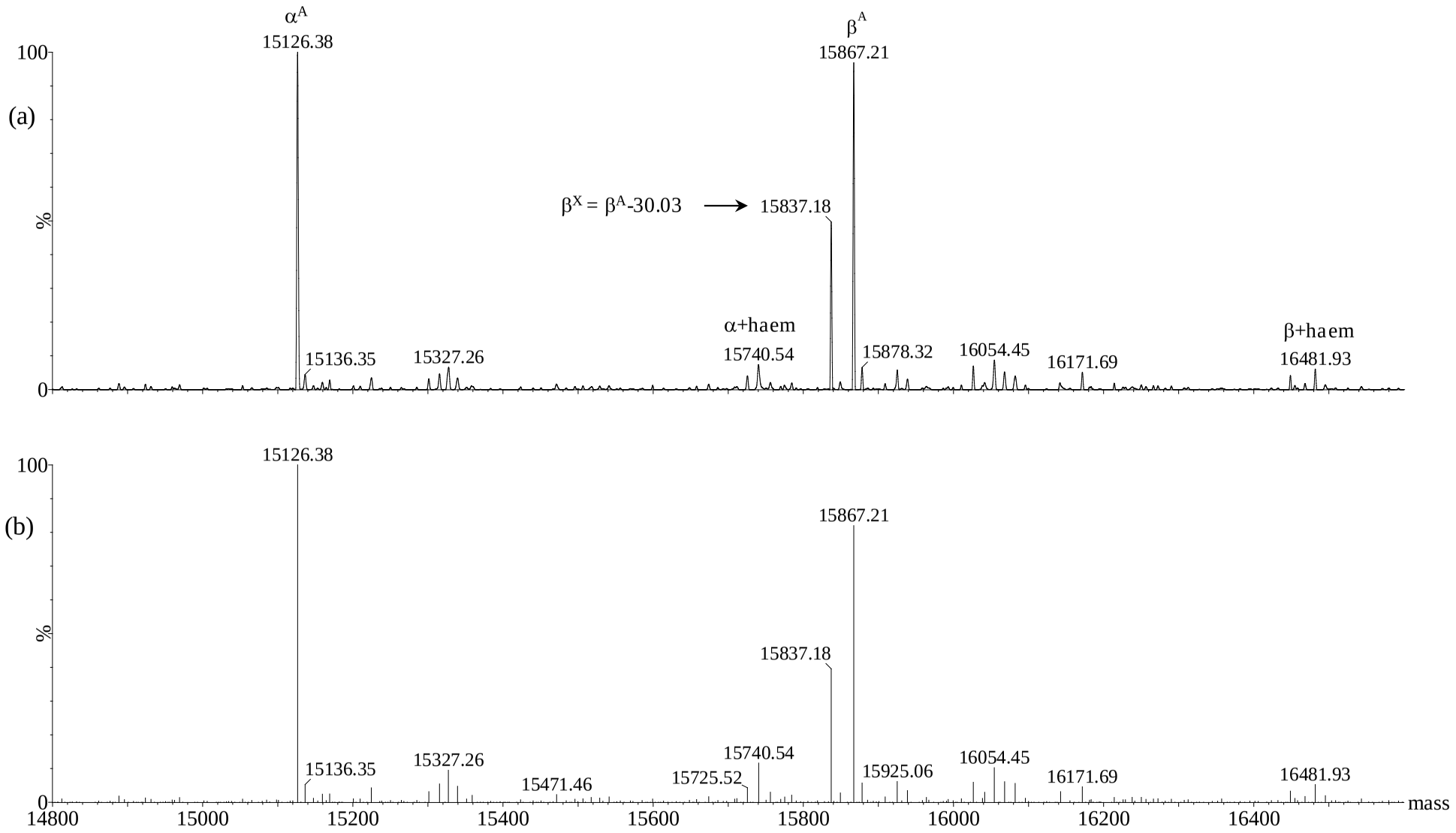

Figure 5.4.8.3. Deconvoluted mass spectrum of Hb Belfast (β15Trp→Arg) showing the presence of a signal at 15,837.18 Da at approximately 50% intensity of the normal β-chain peak (15,867.21 Da). The mass decrease of 30 Da and S-window by HPLC suggests Trp→Arg or Glu→Val.

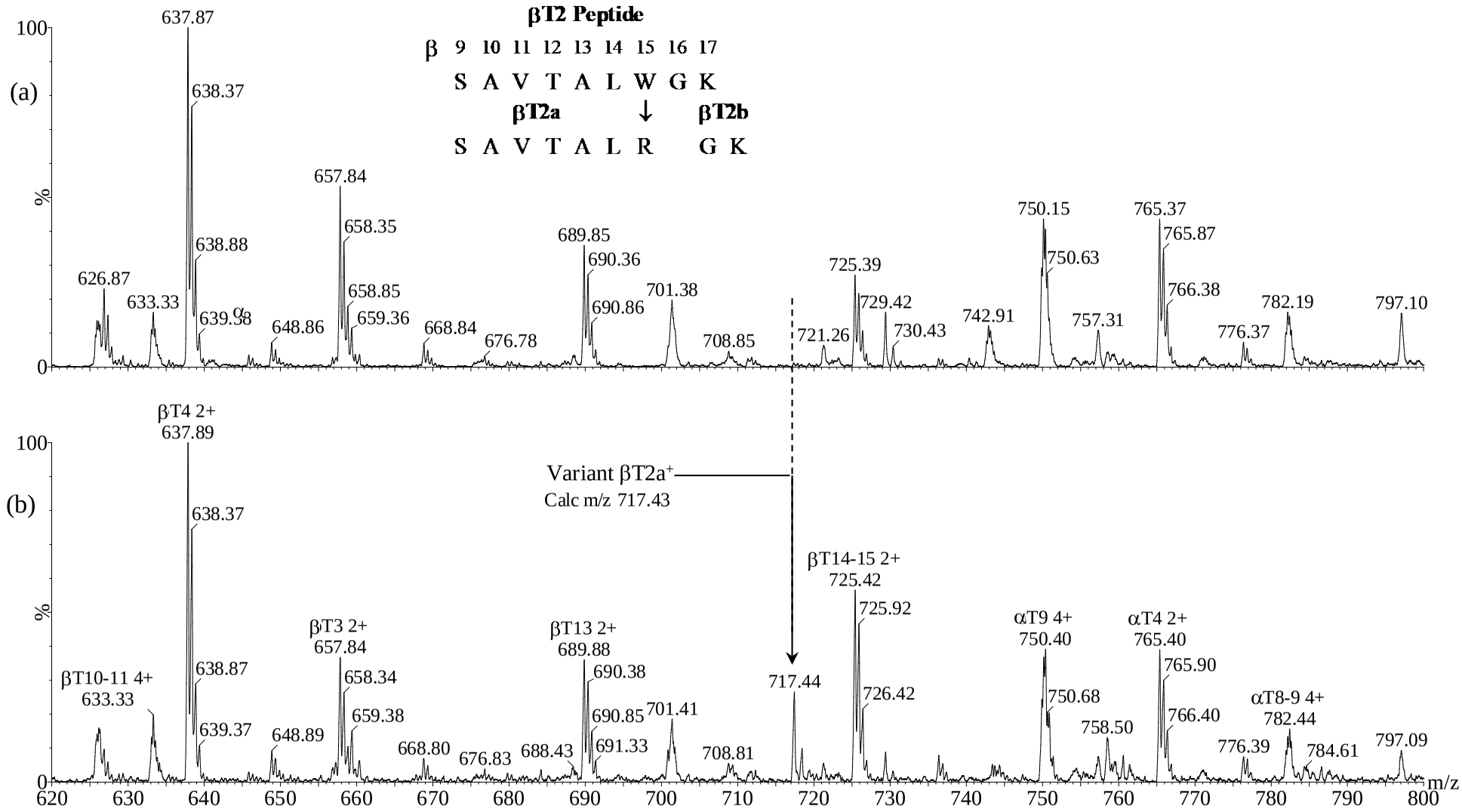

Figure 5.4.8.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Belfast heterozygote. The appearance of the $\beta T2a^{+}$ ion at *m/z* 717.44 identifies the mutation as β15 Trp→Arg, Hb Belfast.

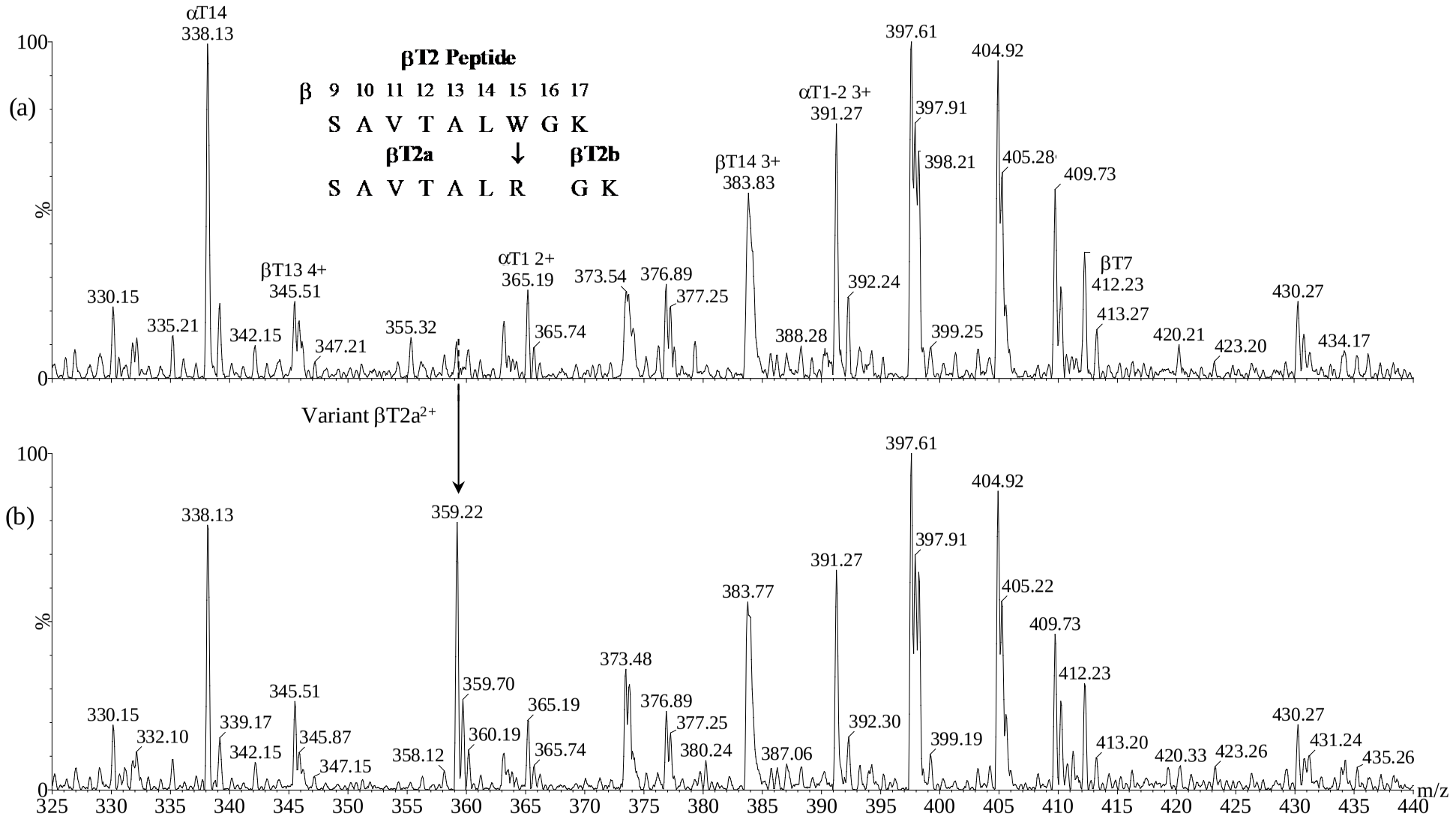

Figure 5.4.8.5. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Belfast heterozygote. The appearance of the $\beta T2a^{2+}$ ion at *m/z* 359.22 identifies the mutation as β15Trp→Arg, Hb Belfast.

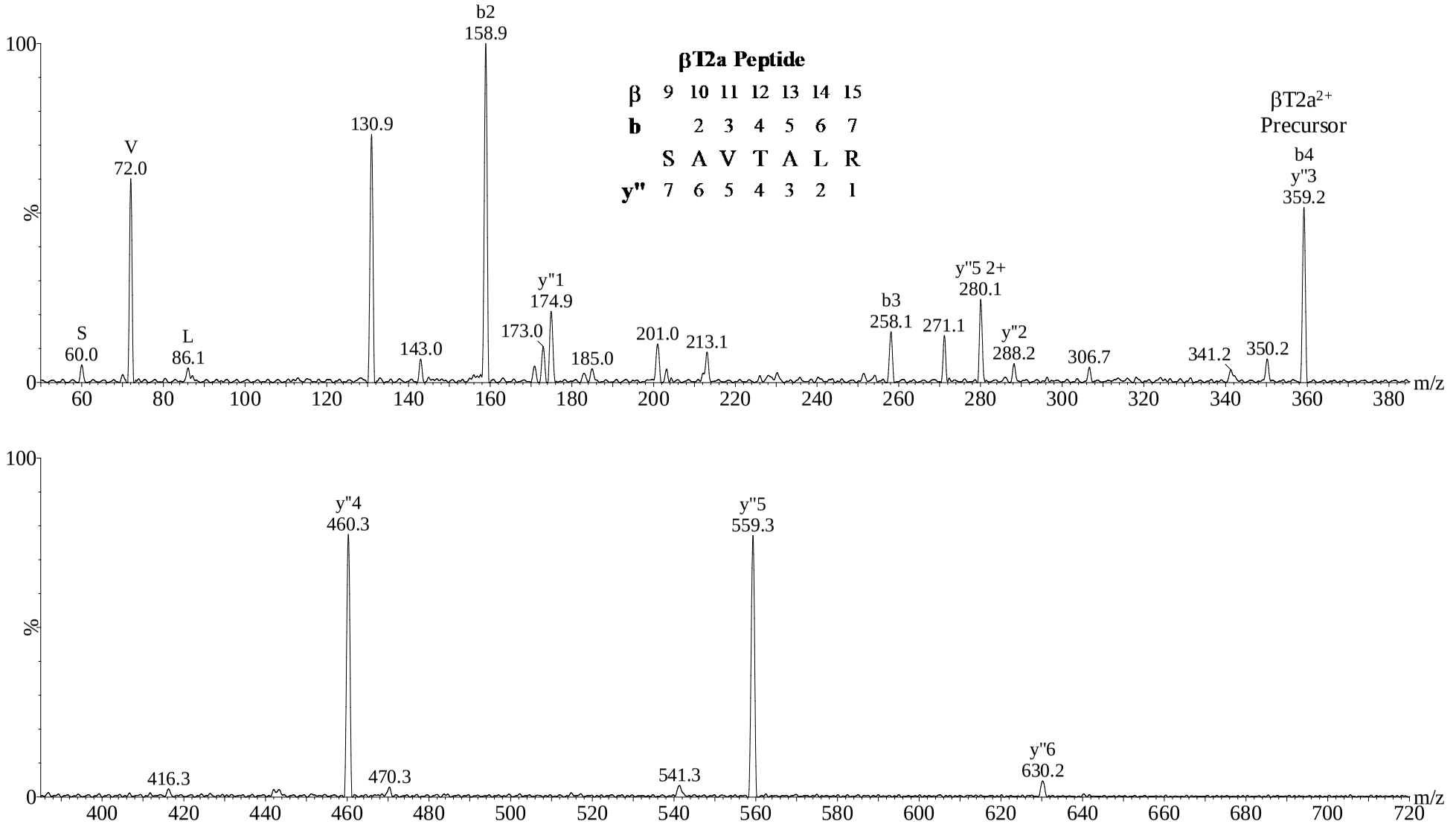

Figure 5.4.8.6. Product ion spectra of the βT2^{2+} tryptic fragment of (a) normal Hb and (b) Hb Belfast. This spectrum is consistent with the mutation β15Trp→Arg, Hb Belfast.

5.4.9. βT2 - Hb J-Baltimore (β16Gly→Asp)

Hb J-Baltimore is the result of a β-chain mutation in which the β16 amino acid residue is changed from Gly to Asp through a single base change in the codon GGC→GAC.

The mutation to Asp next to a tryptic digestion Lys inhibits the cleavage of βT2 and βT3 fragments, and results in a combined βT(2-3) peptide.

	βT2 Peptide									βT3 Peptide														
β	9	10	11	12	13	14	15	16	17		18	19	20	21	22	23	24	25	26	27	28	29	30
	S	A	V	T	A	L	W	G	K		V	N	V	D	E	V	G	G	E	A	L	G	R
								↓ βT(2-3) Peptide															
β	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	
	S	A	V	T	A	L	W	D	K	V	N	V	D	E	V	G	G	E	A	L	G	R	

Figure 5.4.9.1. Sequence of the Hb J-Baltimore βT(2-3) tryptic peptide.

A blood sample was submitted for analysis by mass spectrometry because the ce-HPLC trace (Figure 5.4.9.2.) showed an abnormal response (45.4%) in the P3 window at 1.87 min, indicating a negative charge change.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
P1	- - -	0.2	0.81	2928
Unknown	- - -	1.5	1.00	27473
F	1.3	- - -	1.08	22124
Unknown	- - -	1.3	1.24	24319
P2	- - -	2.2	1.36	39398
P3	- - -	45.4	1.87	827371
A0	- - -	45.1	2.46	821143
A2	3.0	- - -	3.67	57444

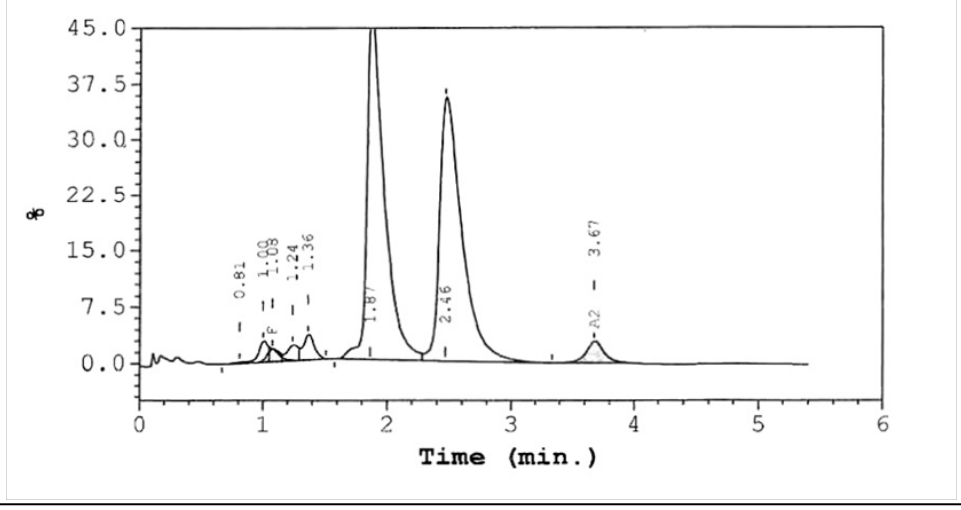

Figure 5.4.9.2. ce-HPLC trace for Hb J-Baltimore.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.4.9.3.) revealed a β-chain heterozygote in which the variant β-chain was approximately equal intensity to the normal β-chain, and the mass of the variant chain was 15,925.24 Da, 57.97 Da heavier than normal. A single base change in the codon giving a mass increase of 58 Da could be Ala→Glu (15 possibilities) or Gly→Asp (13 possibilities), and these mutations are supported by the charge change in the ce-HPLC.

Figure 5.4.9.4. shows the diagnostic part of the tryptic digest spectrum for (a) normal Hb and (b) the variant Hb. A peak at *m/z* 1,143.63 is apparent in the lower panel and not observed in the normal spectrum, and is consistent with the formation of the combined $\beta T(2\text{-}3)^{2+}$ tryptic fragment. This occurs as the proposed mutation β16Gly→Asp inhibits the tryptic cleave at β17Lys.

Further evidence of the missed cleavage is shown in Figure 5.4.9.5. lower panel with the appearance of the peak at *m/z* 762.73, which is consistent with the $\beta T(2\text{-}3)^{3+}$ peptide, and further supports the hypothesis that the specimen was heterozygous β16Gly→Asp, Hb J-Baltimore.

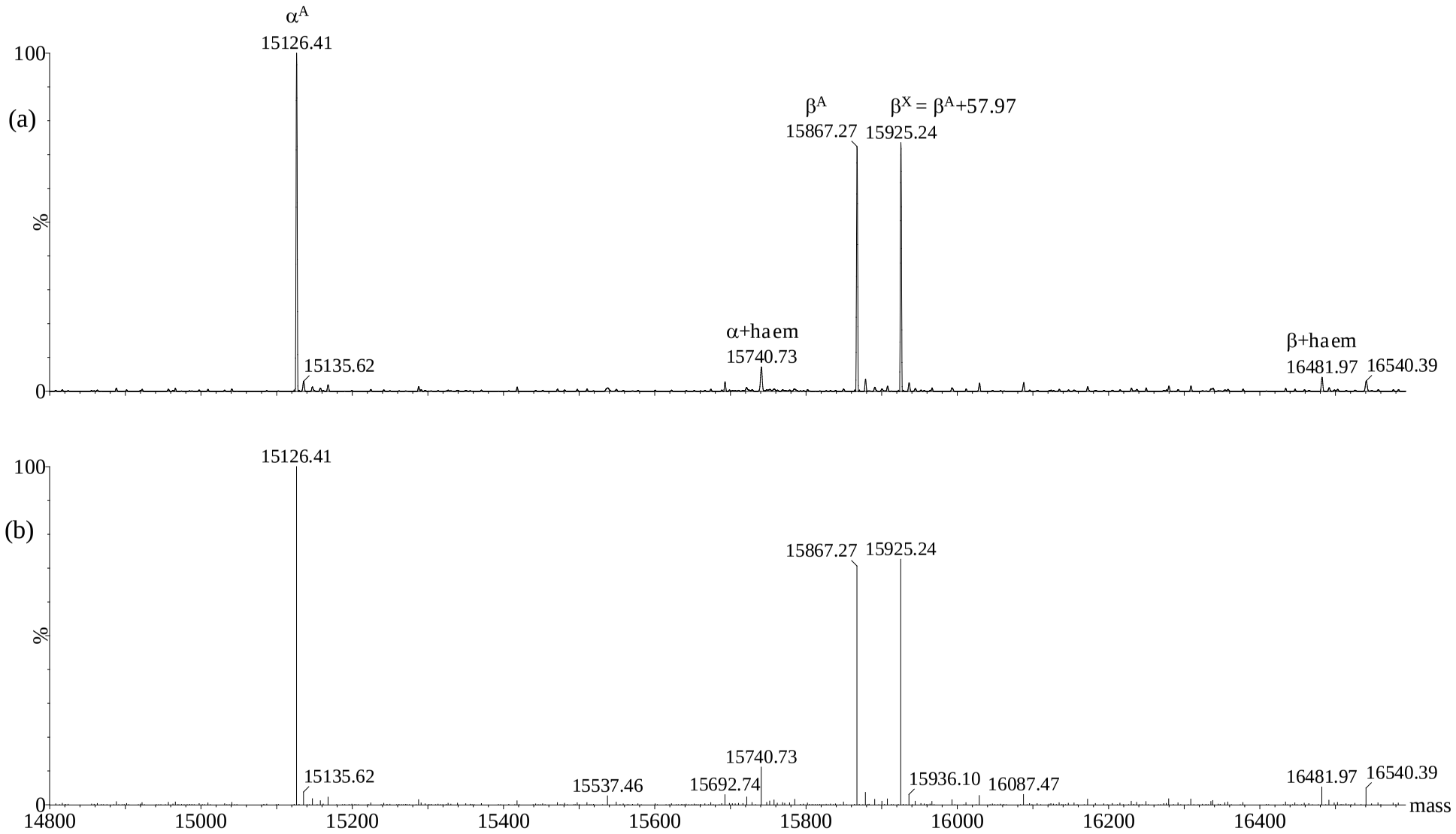

Figure 5.4.9.3. Deconvoluted mass spectrum of Hb J-Baltimore (β16Gly→Asp) showing the presence of a signal at 15,925.24 Da at approximately equal intensity of the normal β-chain peak (15,867.27 Da). The mass increase of 58 Da can be attributed to Gly→Asp or Ala→Glu.

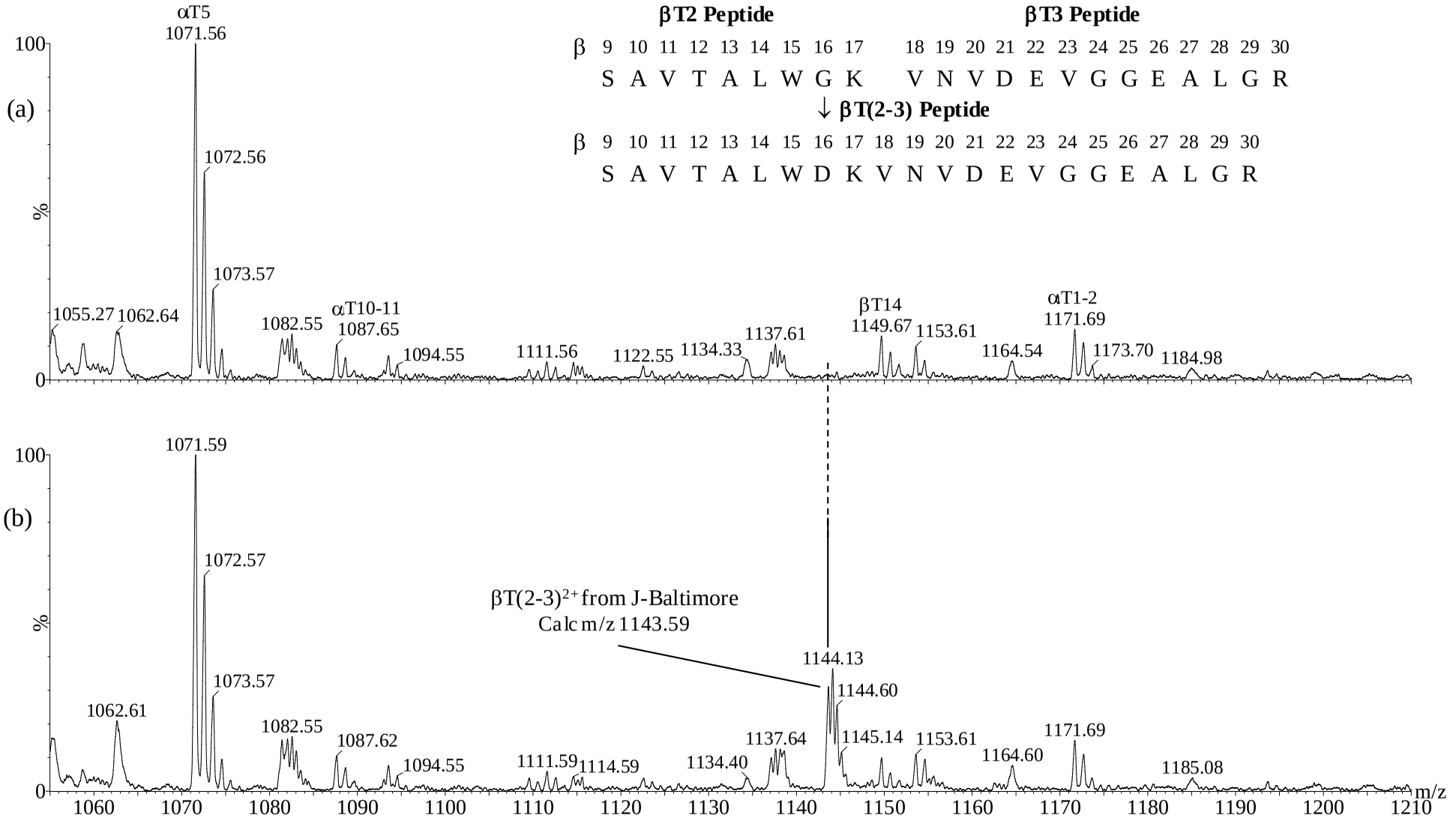

Figure 5.4.9.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb J-Baltimore heterozygote. The doubly charged peak at *m/z* 1,143.63 is consistent with the presence of the βT(2-3) tryptic peptide.

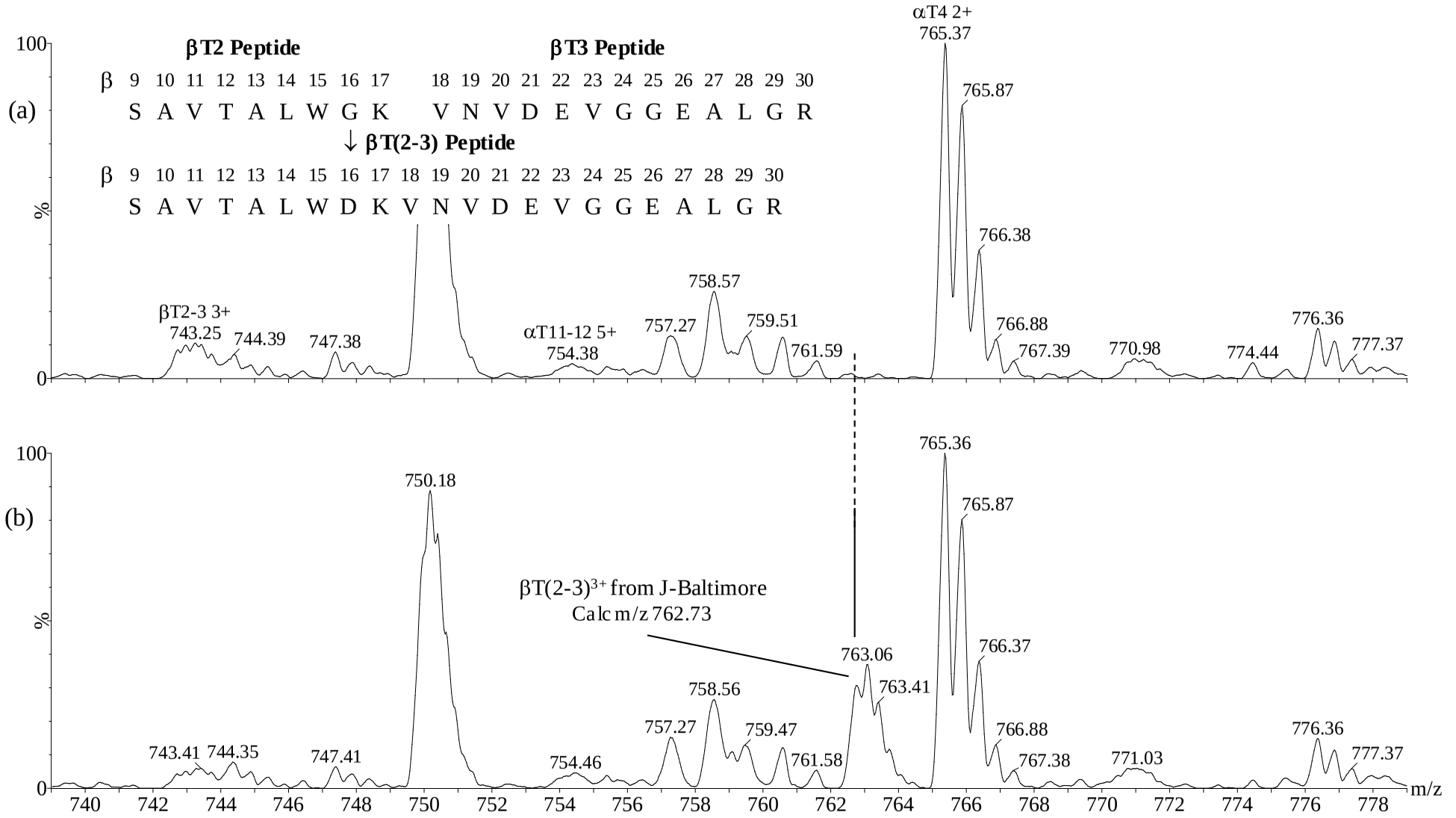

Figure 5.4.9.5. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb J-Baltimore heterozygote. The triply charged peak at *m/z* 762.75 is consistent with the presence of the βT(2-3) tryptic peptide.

5.4.10. βT3 - Hb D-Ouled Rabah (β19Asn→Lys)

Hb D-Ouled Rabah is the result of a β-chain mutation in which the β19 amino acid residue is changed from Asn to Lys through a single base change in the codon AAC→AAA or AAG.

The mutation to the Lys residue results in an additional tryptic cleavage product, as shown in Figure 5.4.10.1.

βT3 Peptide

β 18 19 20 21 22 23 24 25 26 27 28 29 30

V N V D E V G G E A L G R

↓ **βT3b Peptide**

V K V D E V G G E A L G R

Figure 5.4.10.1. Sequence of the Hb D-Ouled Rabah βT3 tryptic peptides.

A blood sample was submitted for analysis by mass spectrometry because the ce-HPLC trace (Figure 5.4.10.2.) showed a significant response (43.7%) in the A_2 region at 3.66 min and suggests a positive charge change.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	2.5	---	1.13	49609
Unknown	---	0.8	1.23	16000
P2	---	2.3	1.31	47244
P3	---	5.2	1.69	106597
A0	---	47.5	2.51	966297
A2	43.7	---	3.66	847132

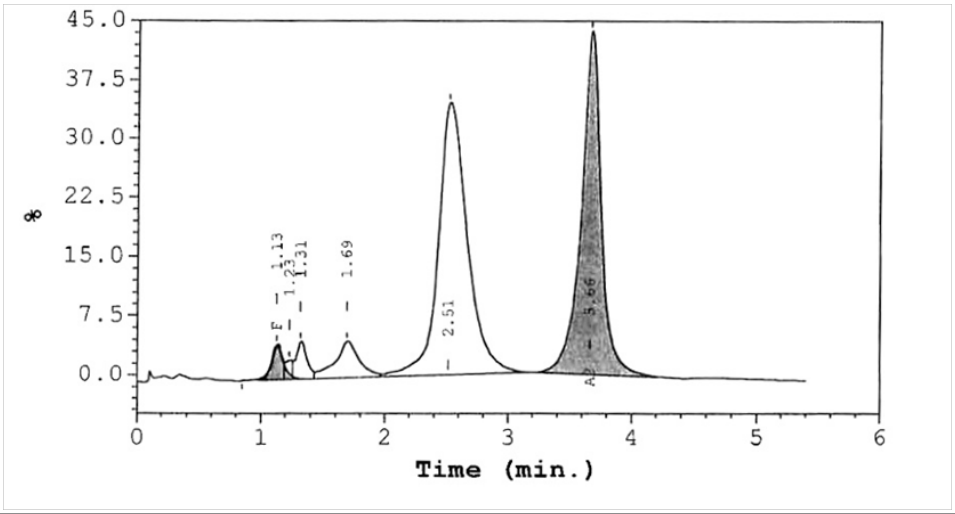

Figure 5.4.10.2. ce-HPLC trace for Hb D-Ouled Rabah.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.4.10.3.) revealed a β-chain heterozygote in which the variant β-chain was approximately equal intensity to the normal β-chain, and the mass of the variant chain was 15,881.24 Da, 13.99 Da heavier than normal. A single codon change giving a mass increase of 14 Da coupled with a positive charge change is probably Asn→Lys (6 possibilities).

Figure 5.4.10.4. shows the diagnostic part of the tryptic digest spectrum for (a) normal Hb and (b) the variant Hb. In the lower panel, a new signal is observed at *m/z* 664.87, attributable to a mass increase of 14 Da in the $\beta T3^{2+}$ fragment. In the βT3 tryptic peptide, this suggests that the mutation occurs at

β19Asn→Lys. The mutation Asn→Lys also gives rise to the possibility of an additional tryptic cleavage product, and this is supported in Figure 5.4.10.5 by the appearance of a new signal at *m/z* 551.29, which is consistent with the formation of the new $\beta T3b^{2+}$ tryptic fragment.

Figure 5.4.10.6. shows the product ion spectra of the $\beta T3^{2+}$ tryptic fragment for (a) normal Hb and (b) the variant Hb. The +14 Da change in mass of the b_2 and y''_{12} fragments confirms the mutation as β19Asn→Lys, Hb D-Ouled Rabah.

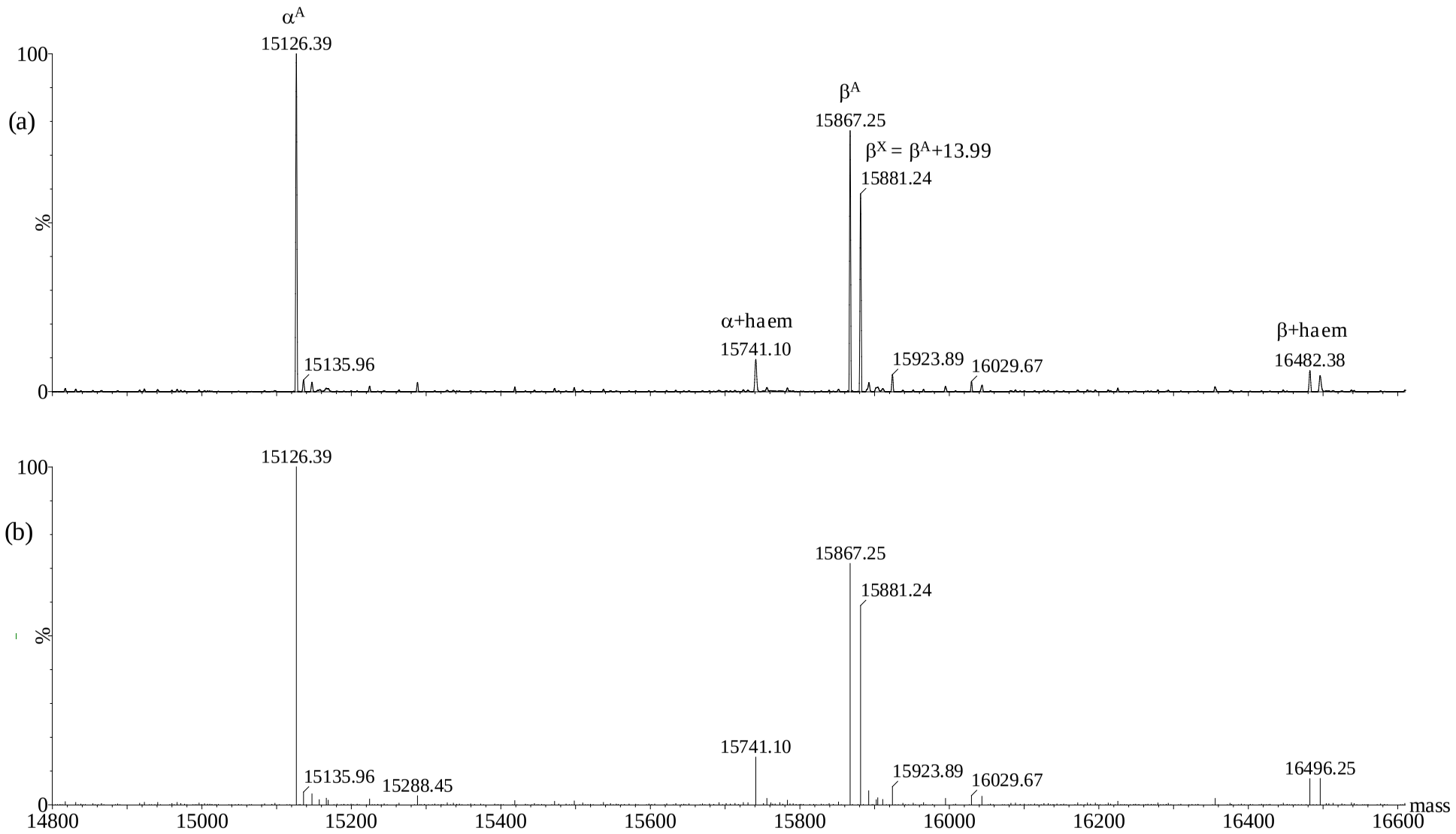

Figure 5.4.10.3. Deconvoluted mass spectrum of Hb D-Ouled Rabah (β19Asn→Lys) showing the presence of a signal at 15,881.24 Da at approximately 80% intensity of the normal β-chain peak (15,867.25 Da). The 14 Da mass increase, coupled with a positive charge change suggests Asn→Lys.

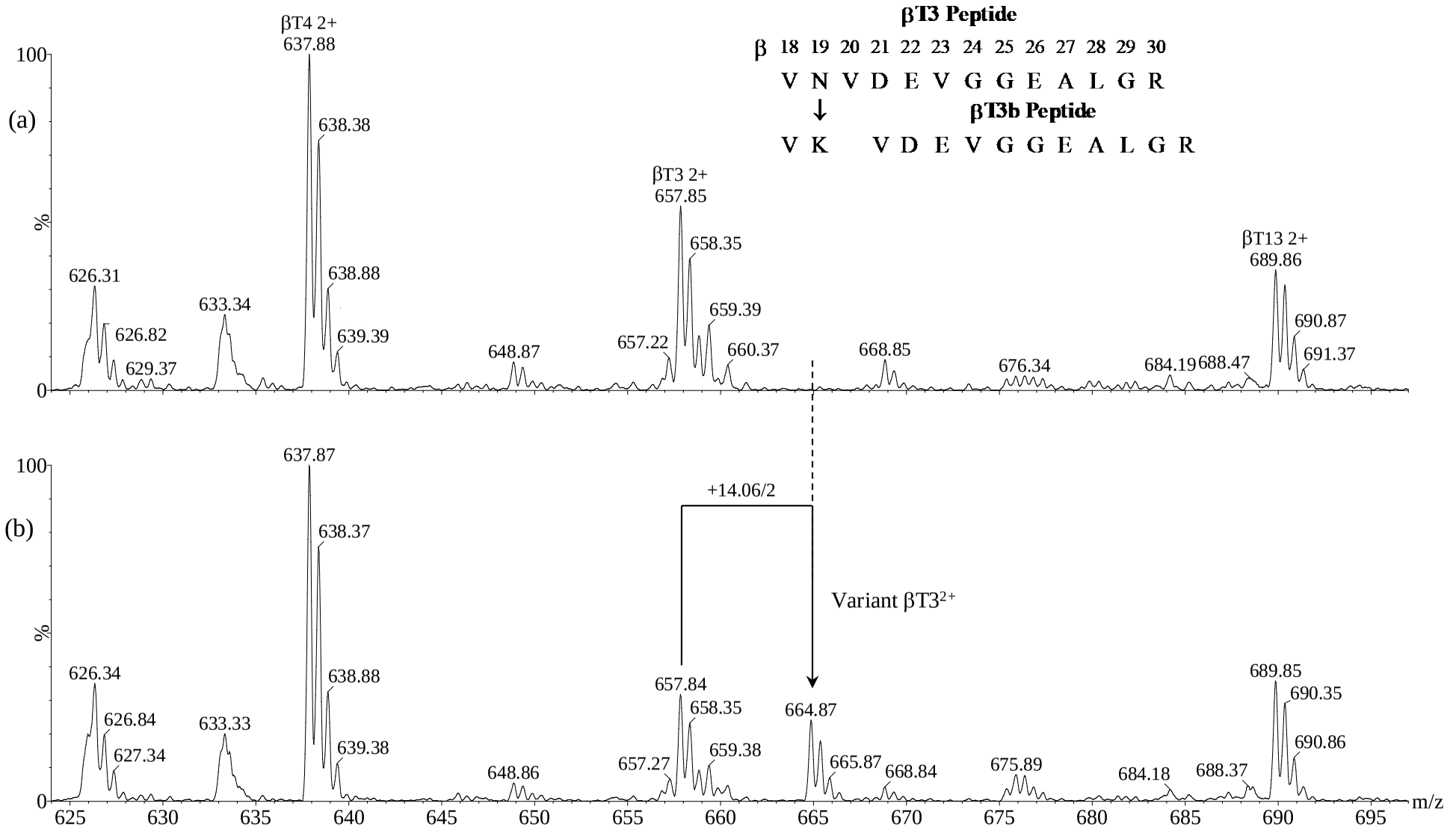

Figure 5.4.10.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb D-Ouled Rabah heterozygote.

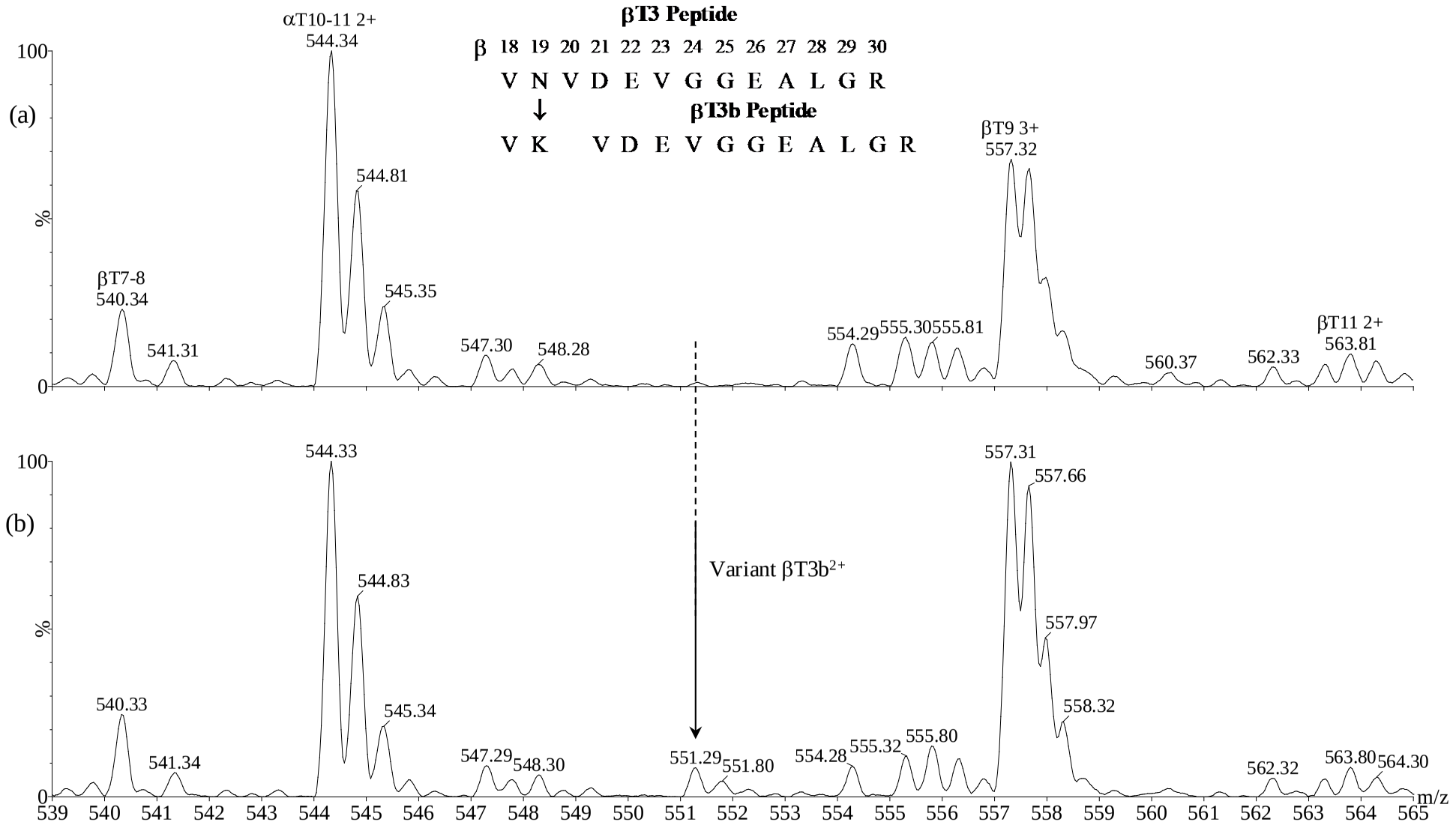

Figure 5.4.10.5. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb D-Ouled Rabah heterozygote.

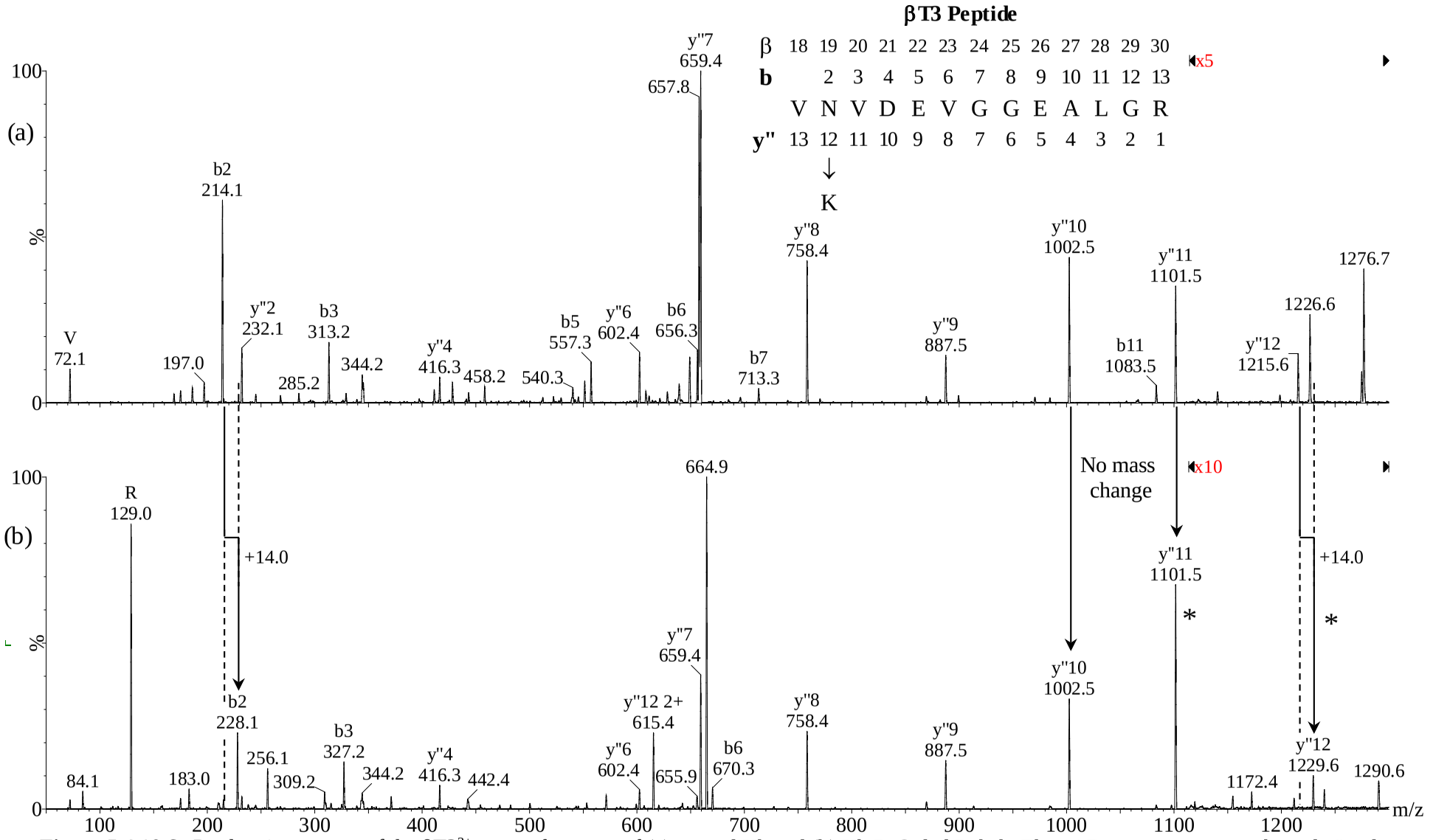

Figure 5.4.10.6. Product ion spectra of the $\beta T3^{2+}$ tryptic fragment of (a) normal Hb and (b) Hb D-Ouled Rabah. The 14 Da mass increase at b_2 and $y"_{12}$ places the mutation at β19 and confirms the mutation β19Asn→Lys.

5.4.11. βT3 - Hb D-Iran (β22Glu→Gln)

Hb Iran is the result of a β-chain mutation in which the β22 amino acid residue is changed from Glu to Gln through a single base change in the codon GAA→CAA.

βT3 Peptide

β	18	19	20	21	22	23	24	25	26	27	28	29	30
	V	N	V	D	E	V	G	G	E	A	L	G	R
					↓								
					Q								

Figure 5.4.11.1. Sequence of the Hb D-Iran βT3 tryptic peptide.

A blood sample was submitted for analysis by mass spectrometry because the ce-HPLC trace (Figure 5.4.11.2.) showed a significant response (40.1%) in the A_2 region at 3.57 min, indicating a positive charge change.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
P1	---	0.0	0.71	655
Unknown	---	0.4	0.94	8971
F	0.2	---	1.08	4344
Unknown	---	0.3	1.26	6677
P2	---	2.3	1.36	50196
P3	---	4.5	1.75	98579
Unknown	---	1.0	2.15	21588
A0	---	46.3	2.48	1009249
A2	40.1	---	3.57	978429

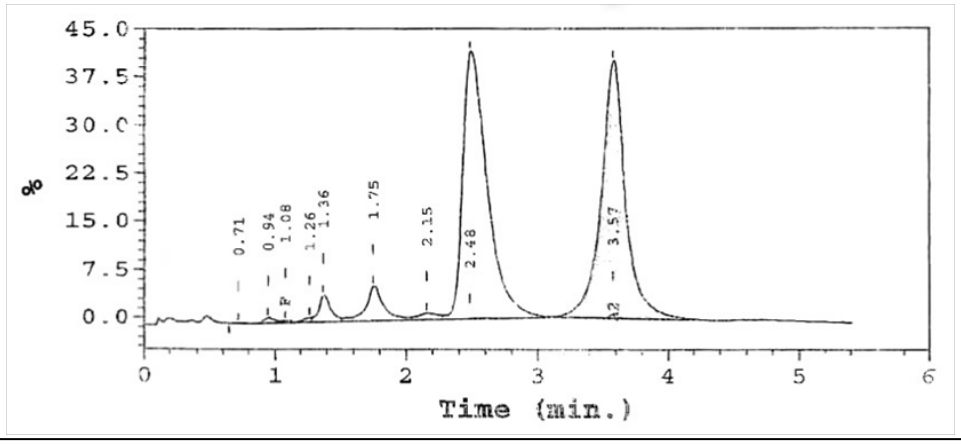

Figure 5.4.11.2. ce-HPLC trace for Hb D-Iran.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.4.11.3.) revealed a signal at 15,866.78 Da, which suggests a $\beta^A/(\beta^A\text{-}1)$ heterozygote.

Figure 5.4.11.4 shows the diagnostic part of the tryptic digest spectrum for (a) normal Hb and (b) the variant Hb. The appearance of a $\beta T3^{2+}$ signal at *m/z* 657.34 in the lower trace is consistent with a mutation giving rise to a -1 Da mass change in the βT3 tryptic fragment. There are three sites in the βT3 tryptic peptide that could support this mass change from a single base change in the codon, β21Asp→Asn (Hb Cocody), β22Glu→Gln (Hb D-Iran) and β26Glu→Gln (Hb King's Mill). This is further supported by the appearance of the $\beta T3^{3+}$ signal at *m/z* 1,313.68 in the lower panel of Figure 5.4.11.5. There was no evidence of the formation of an additional tryptic fragment, which precludes the Glu→Lys mutation.

Figure 5.4.11.6. shows a comparison of the product ion mass spectra of the $\beta T3^{2+}$ ions for (a) normal Hb and (b) the variant Hb. The 1 Da mass decrease at b_5 (*m/z* 556.5) and y''_9 (*m/z* 1,100.7) confirms the mutation as β22Glu→Gln, Hb D-Iran.

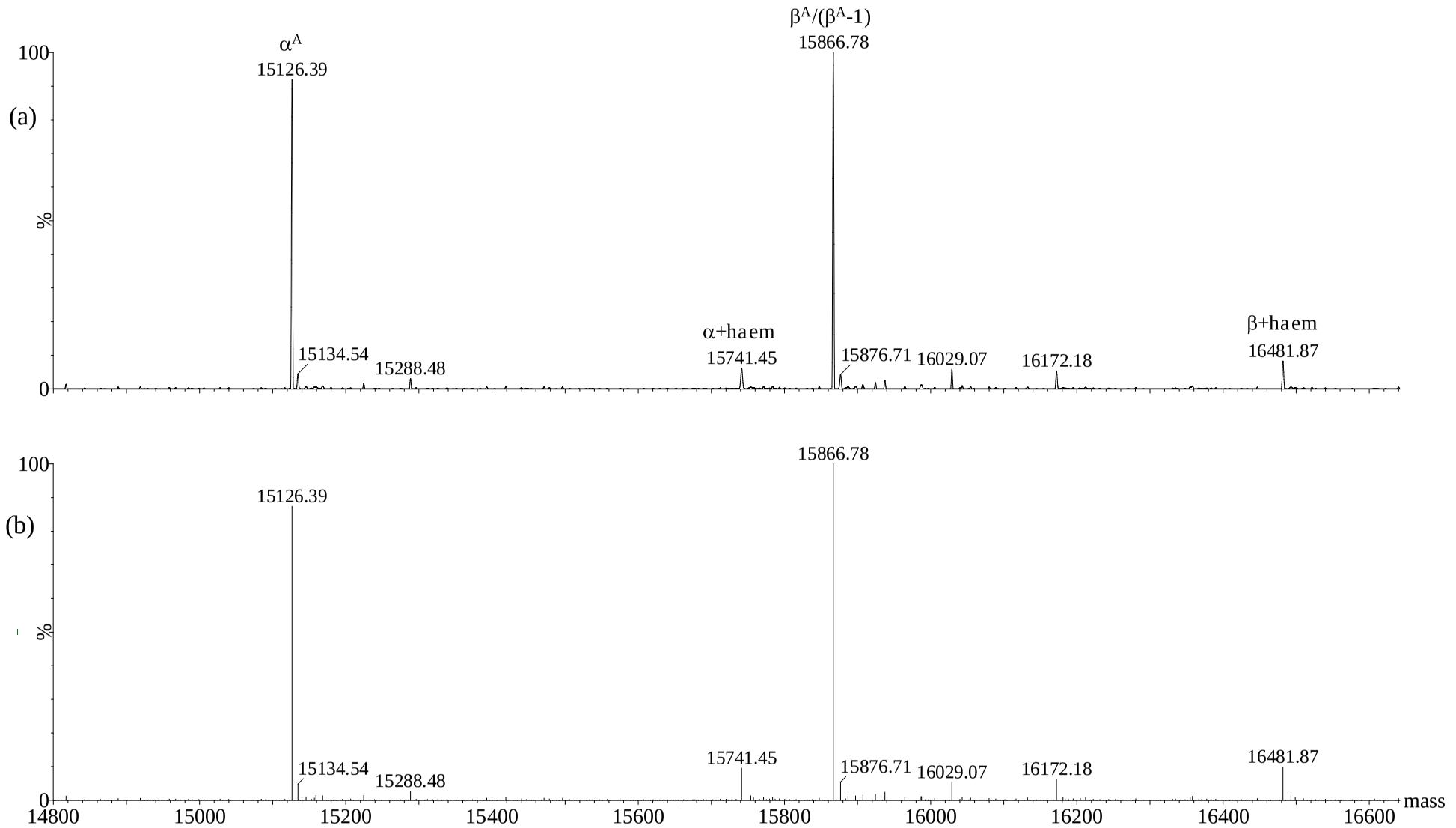

Figure 5.4.11.3. Deconvoluted mass spectrum of Hb D-Iran (β22Glu→Gln) showing the presence of a signal at 15,866.78 Da. The mass suggests a $\beta^A/(\beta^A\text{-}1)$ heterozygote.

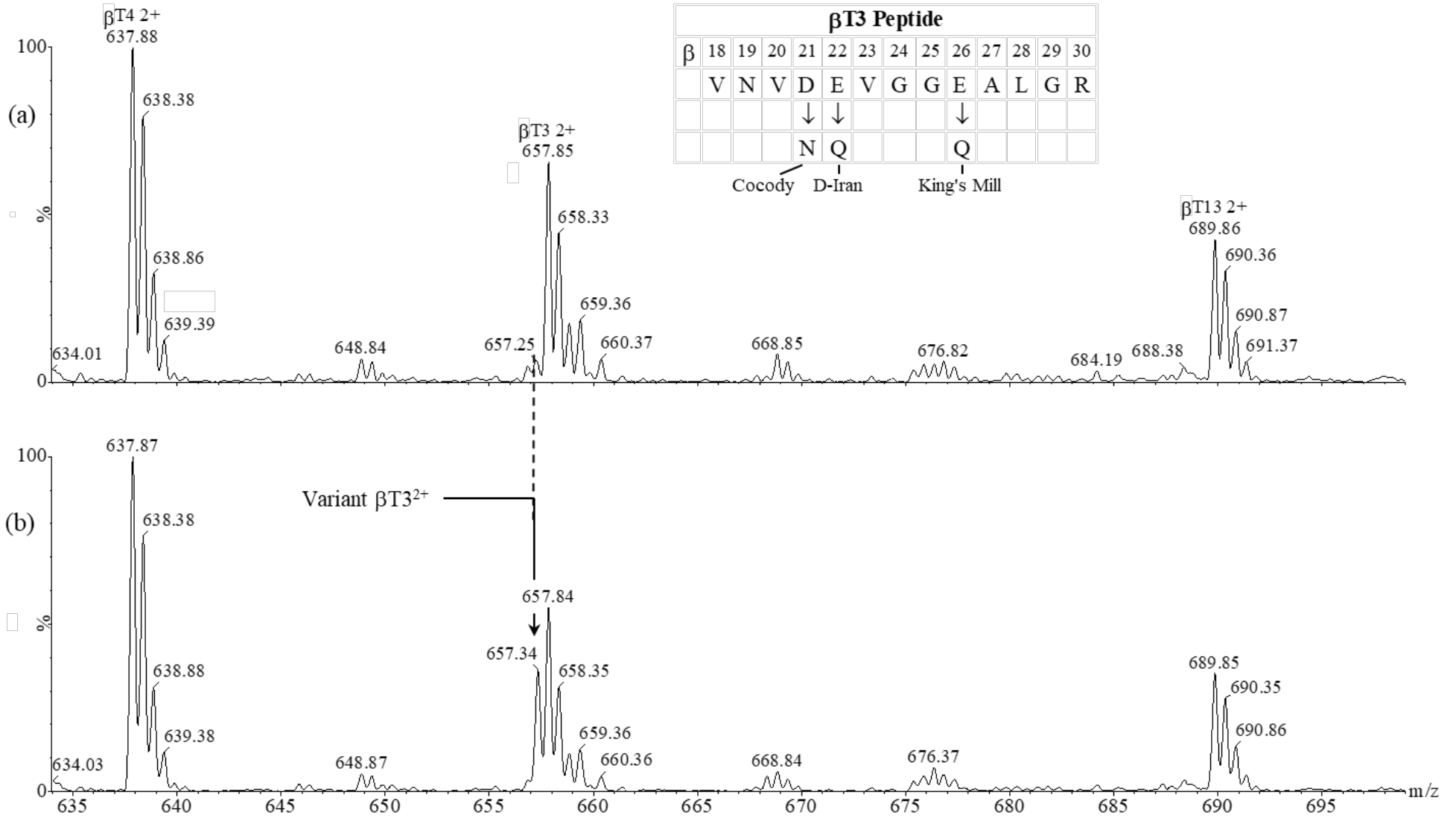

Figure 5.4.11.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb D-Iran heterozygote.

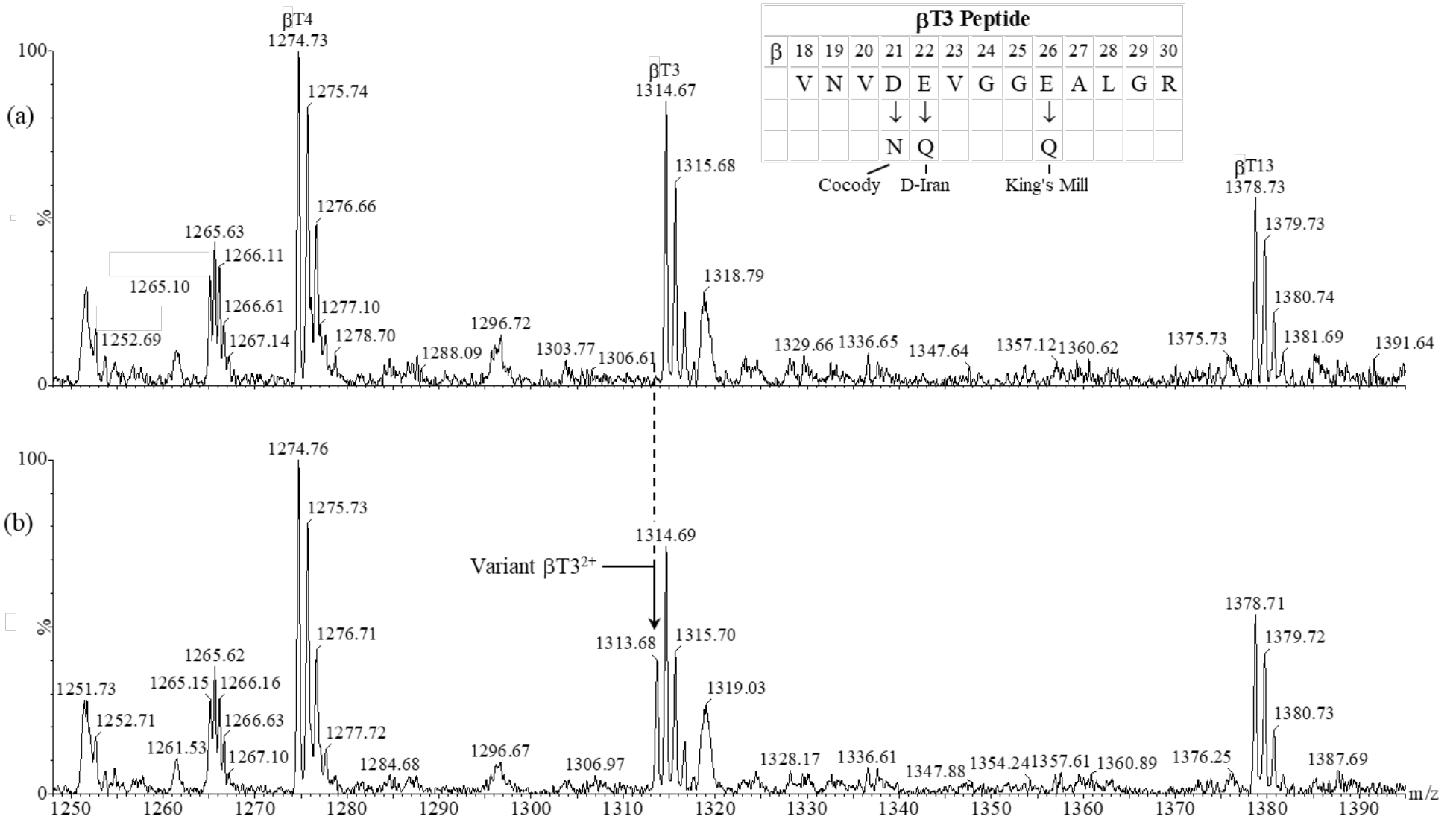

Figure 5.4.11.5. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb D-Iran heterozygote.

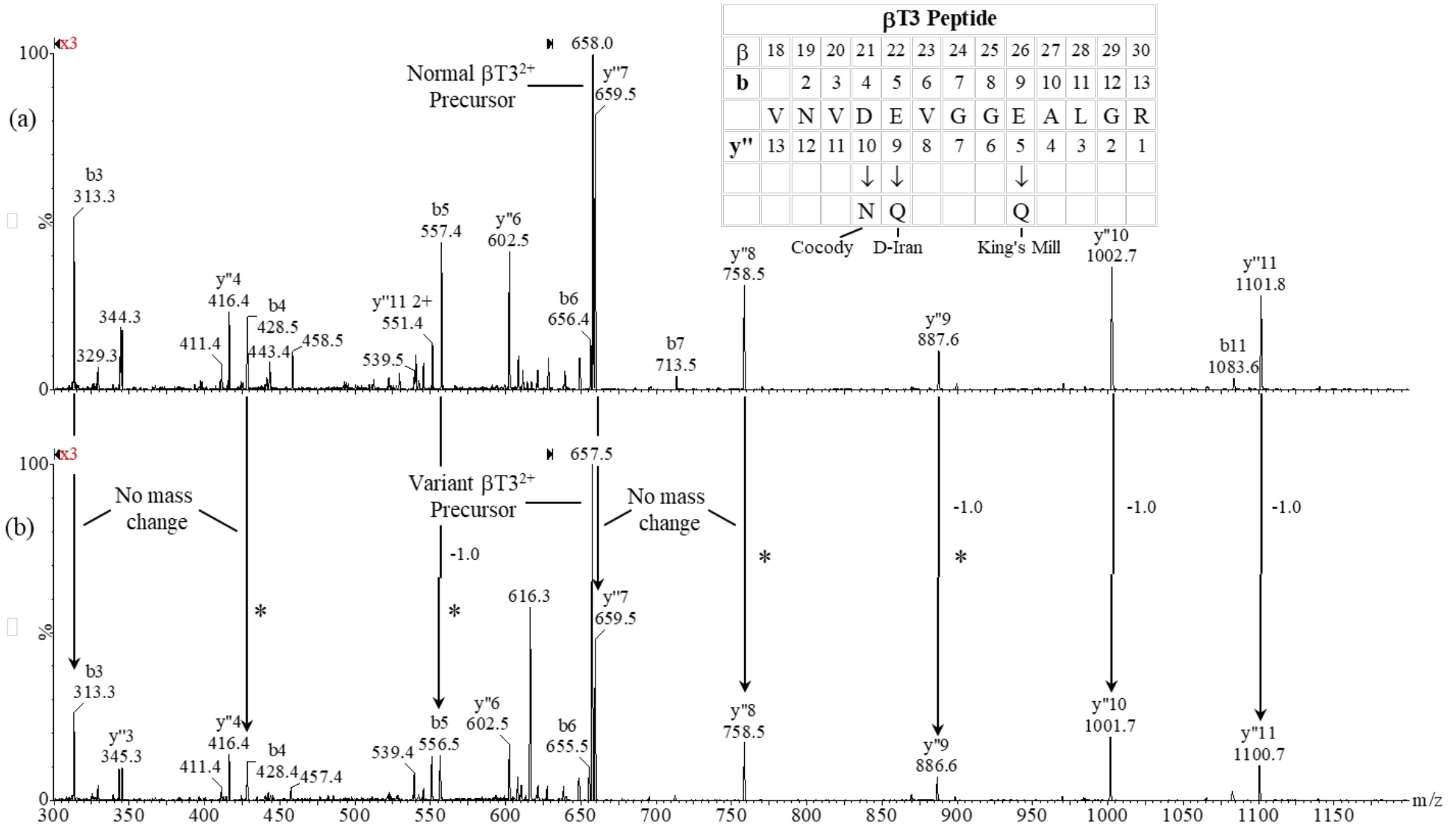

Figure 5.4.11.6. Product ion spectra of the $\beta T3^{2+}$ tryptic fragment of (a) normal Hb and (b) Hb D-Iran. The 1 Da mass decrease at b_5 and y''_9 identifies the mutation as β22 Glu→Gln.

5.4.12. βT3 - Hb E (β26Glu→Lys)

Hb E is the result of a β-chain mutation in which the β26 amino acid residue is changed from Glu to Lys through a single base change in the codon GAG→AAG.

The mutation to the Lys residue results in an additional tryptic cleavage product, as shown in Figure 5.4.12.1.

βT3 Peptide

β 18 19 20 21 22 23 24 25 26 27 28 29 30

V N V D E V G G E A L G R

βT3a Peptide ↓ **βT3b Peptide**

V N V D E V G G K A L G R

Figure 5.4.12.1. Sequence of the Hb E βT3 tryptic peptides.

A blood sample was submitted for analysis by mass spectrometry because the ce-HPLC trace (Figure 5.4.12.2.) showed a significant response (25.4%) in the A_2 region at 3.67 min, indicating a positive charge change.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	4.7	---	1.11	81862
P2	---	2.7	1.29	49131
Unknown	---	0.6	1.43	10844
P3	---	3.4	1.70	61281
A0	---	63.1	2.49	1141073
A2	25.9	---	3.67	465033

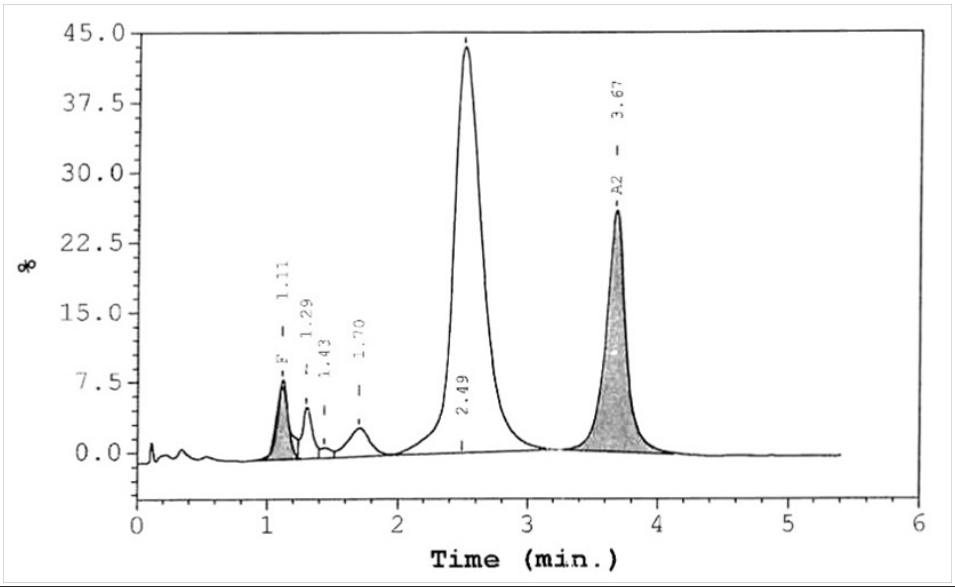

Figure 5.4.12.2. ce-HPLC trace for Hb E.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.4.12.3.) revealed a signal at 15,867.06 Da, which suggests a $\beta^A/(\beta^A\text{-}1)$ heterozygote with the $(\beta^A\text{-}1)$ component being at the lower percentage as indicated in the ce-HPLC trace.

Figure 5.4.12.4 shows the diagnostic part of the tryptic digest spectrum for (a) normal Hb and (b) the variant Hb. The appearance of peaks at *m/z* 416.27 and *m/z* 458.76 indicate that new tryptic peptides have been formed, and these are attributable to a possible mutation in the βT3 peptide in which a Lys

has been inserted, therefore either β22Glu→Lys (Hb E-Saskatoon) or β26Glu→Lys (Hb E), both of which would present as a positive charge change in the ce-HPLC trace.

Figure 5.4.12.5. shows another region of the tryptic digest spectrum for (a) normal Hb and (b) the variant Hb. The additional peak observed at *m/z* 916.47 in the lower panel is consistent with the ^{12}C isotope of the newly formed $\beta T3a^{+}$ tryptic fragment.

The masses observed, and the formation of the additional tryptic peptide(s), are consistent with the mutation β26Glu→Lys, Hb E.

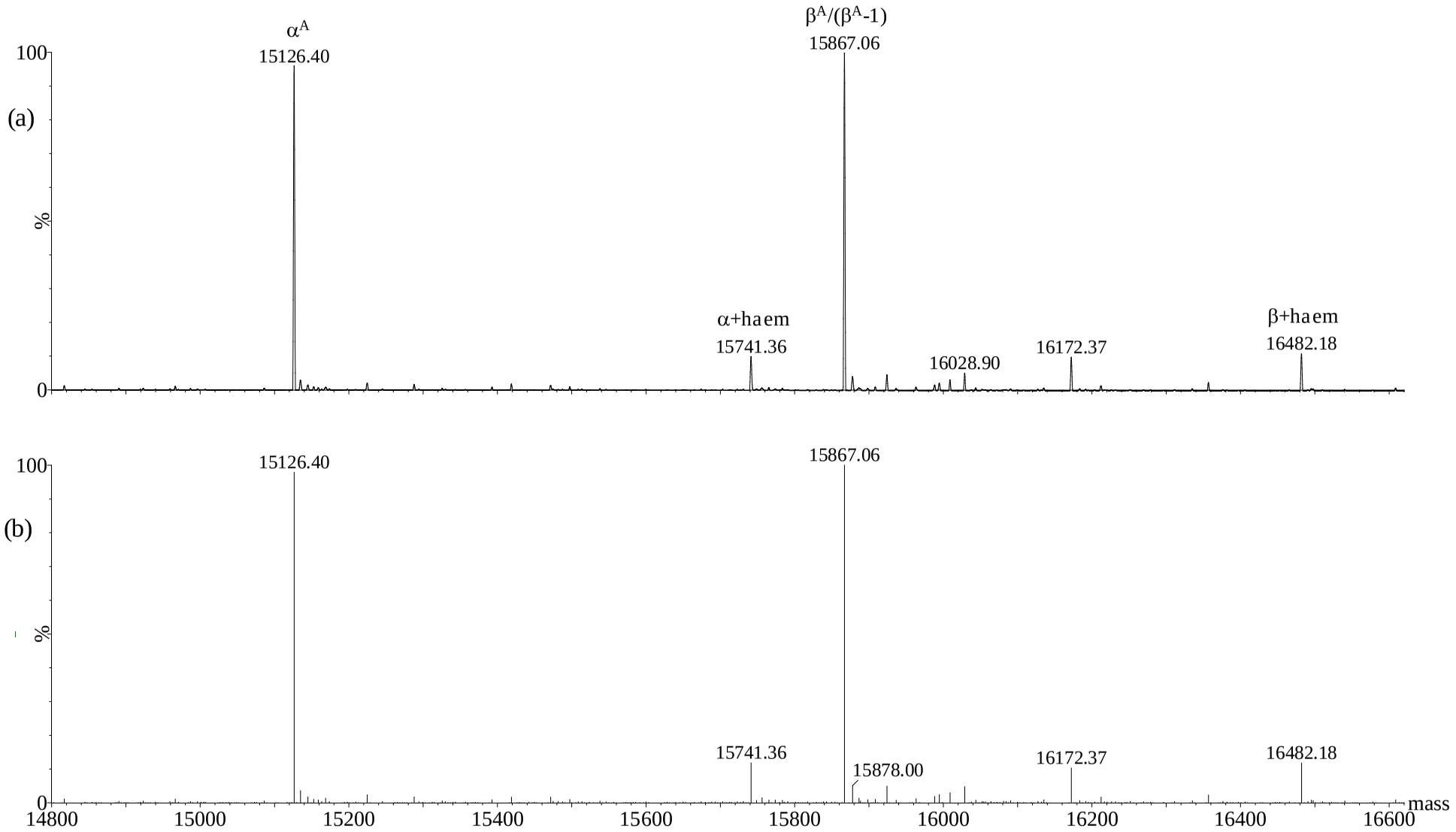

Figure 5.4.12.3. Deconvoluted mass spectrum of Hb E (β26Glu→Lys) showing the presence of a signal at 15,866.78 Da. The mass suggests a $\beta^A/(\beta^A\text{-}1)$ heterozygote.

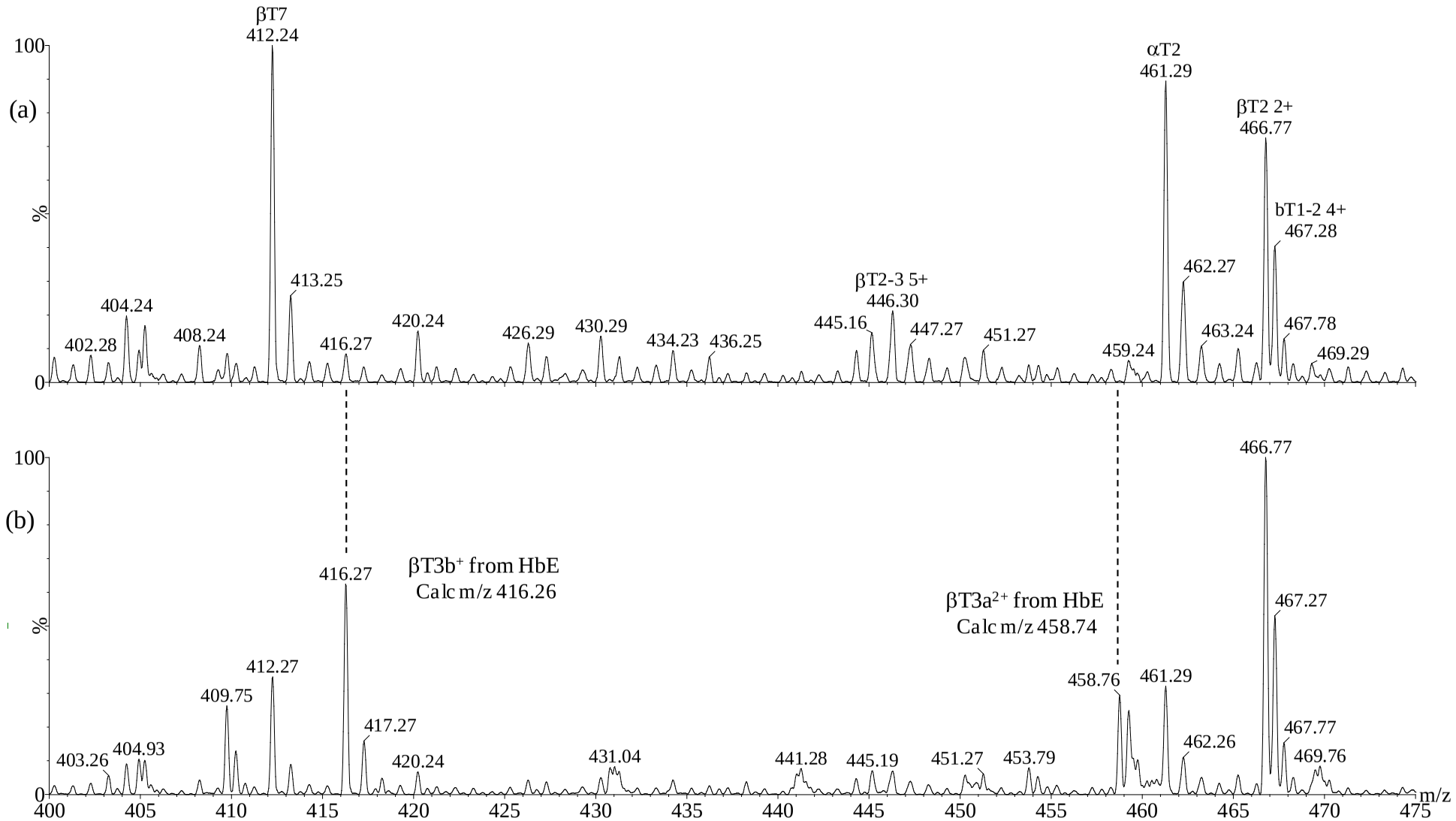

Figure 5.4.12.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb E heterozygote.

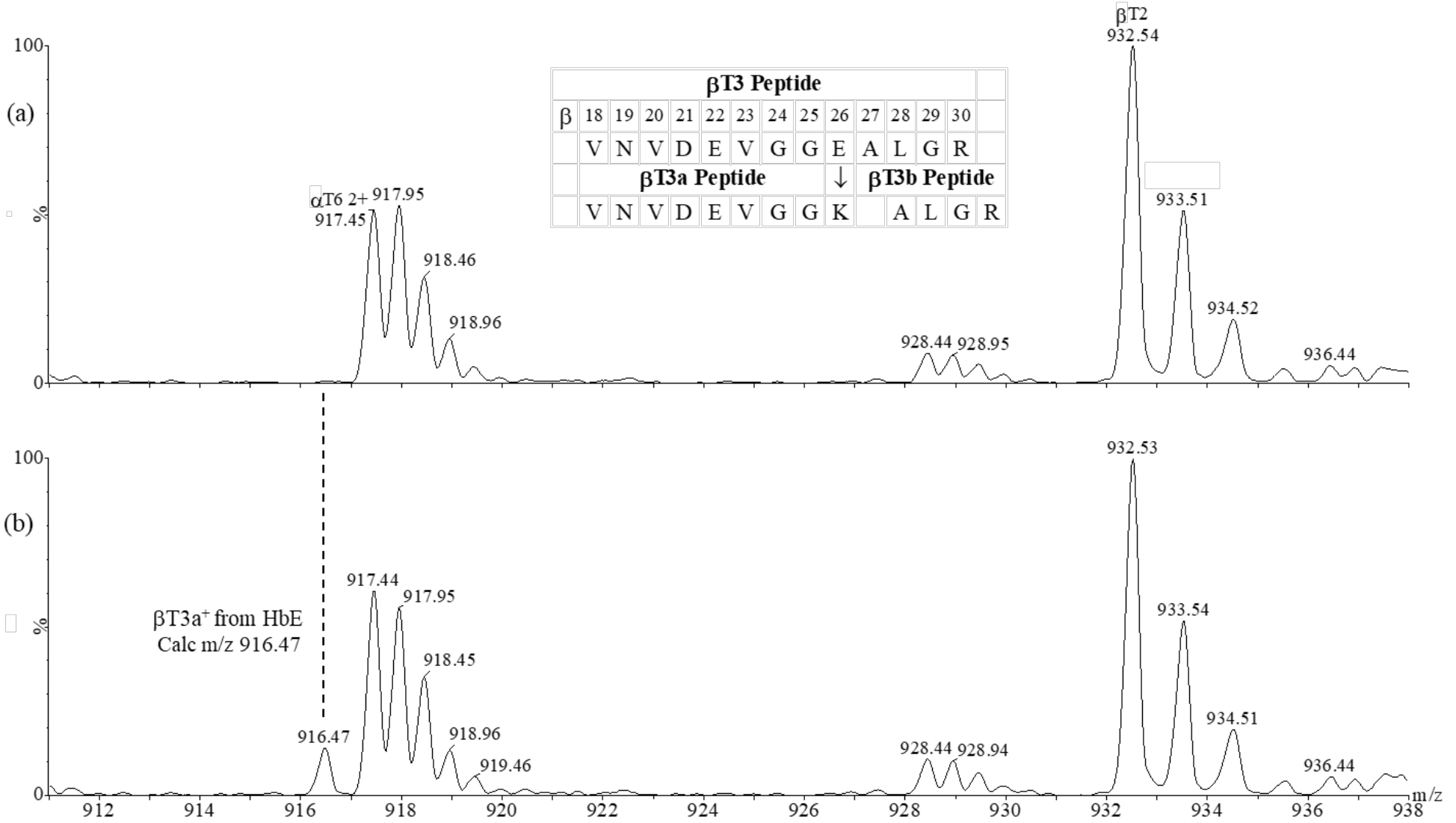

Figure 5.4.12.5. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb E heterozygote.

5.4.13. βT4 - Hb North Chicago (β36Pro→Ser)

Hb North Chicago is the result of a β-chain mutation in which the β36 amino acid residue is changed from Pro to Ser through a single base change in the codon CCT→TCT.

βT4 Peptide

β	31	32	33	34	35	36	37	38	39	40
	L	L	V	V	Y	P	W	T	Q	R
						↓				
						S				

Figure 5.4.13.1. Sequence of the Hb North Chicago βT4 tryptic peptide.

There is no ce-HPLC trace available for Hb North Chicago.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.4.13.2.) revealed a signal at 15,857.20 Da, 10.01 Da lighter than a normal β-chain, and infers a mutation Pro→Ser (7 possibilities). The normal and variant signals are approximately equal intensity.

Figure 5.4.13.3. shows the diagnostic part of the tryptic digest spectrum for (a) normal Hb and (b) the variant Hb. A new signal is observed in the lower panel at *m/z* 632.37 that is attributable to the mutated $\beta T4^{2+}$ fragment and is consistent with the -10 Da mass change. This is further supported by the data in Figure 5.4.13.4. in the comparison of the digest spectra for (a) normal Hb and (b) the variant Hb, in which an additional signal is observed in the lower panel at *m/z* 1,264.71 and is consistent with the mutated $\beta T4^{+}$ tryptic fragment.

Figure 5.4.13.5. shows a comparison of the product ion spectra from the $\beta T4^{2+}$ precursors for (a) normal Hb and (b) the mutated β-chain. The 10 Da shift in mass of the y''_5 fragment ion at *m/z* 677.1 in the lower panel identifies the mutation as β36Pro→Ser, Hb North Chicago.

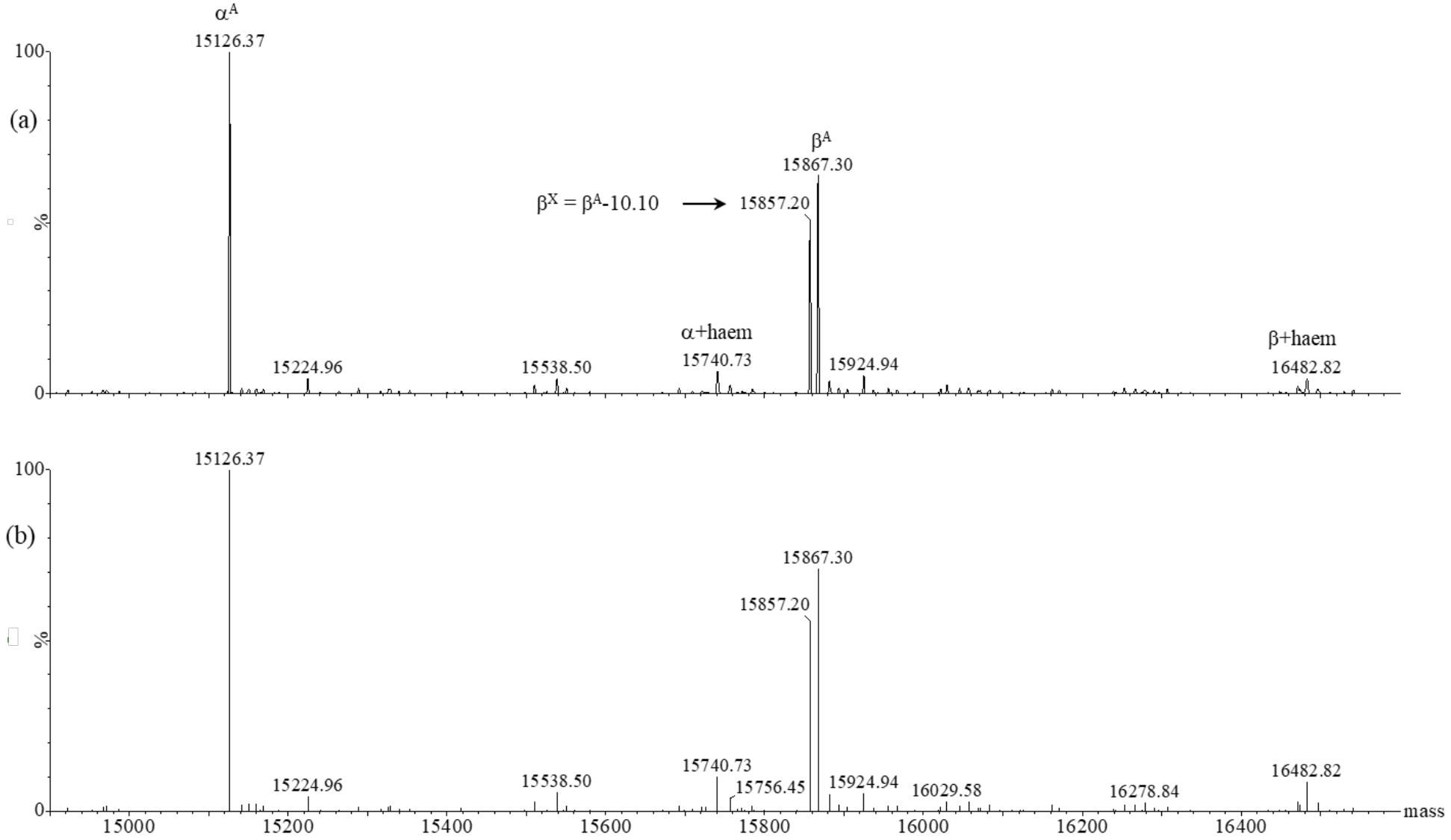

Figure 5.4.13.2. Deconvoluted mass spectrum of Hb North Chicago (β36Pro→Ser) showing the presence of a signal at 15,857.20 Da at approximately 80% intensity of the normal β-chain peak (15,867.30 Da). The mass decrease of 10 Da can only arise from a Pro→Ser mutation.

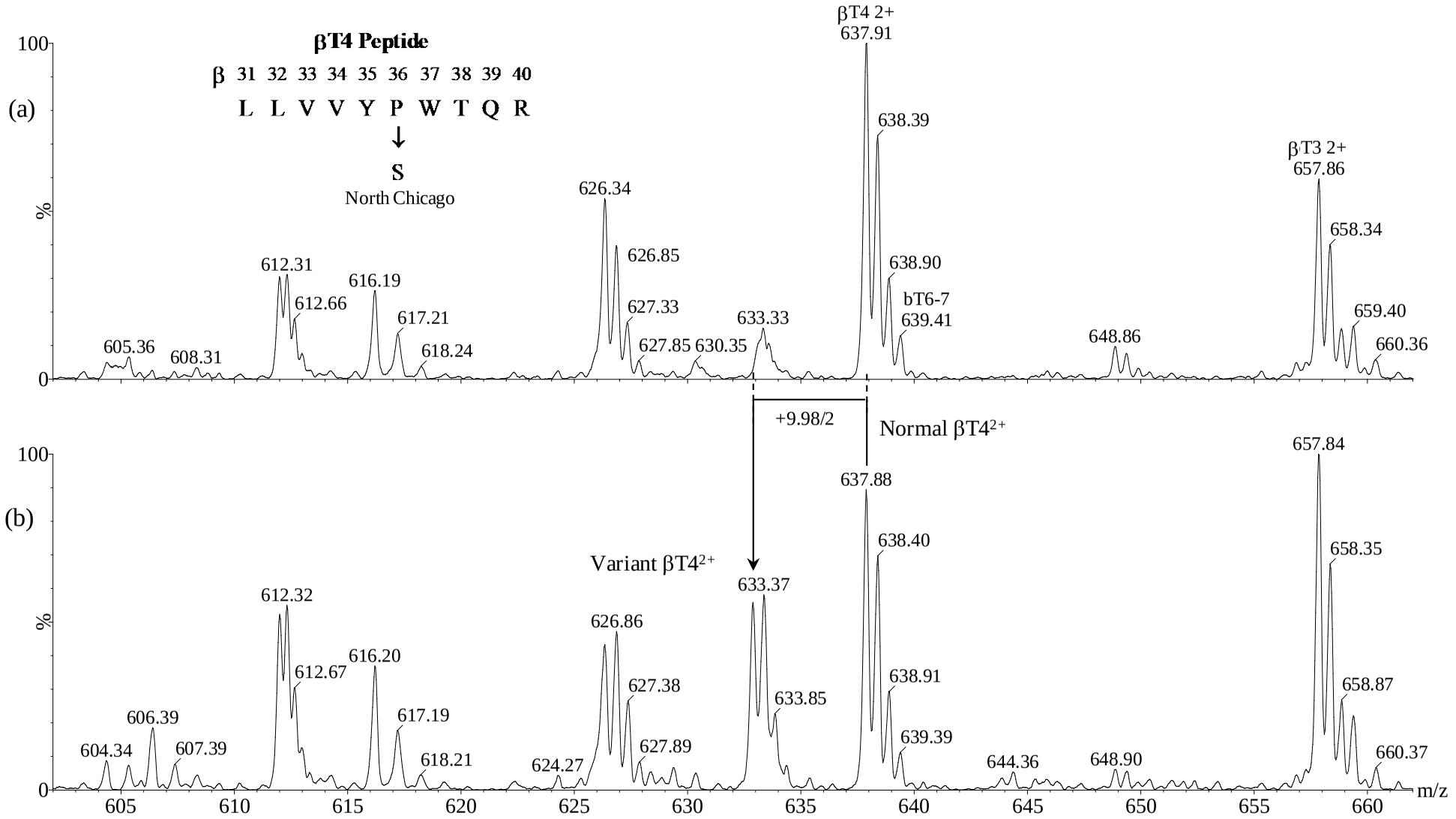

Figure 5.4.13.3. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb North Chicago heterozygote.

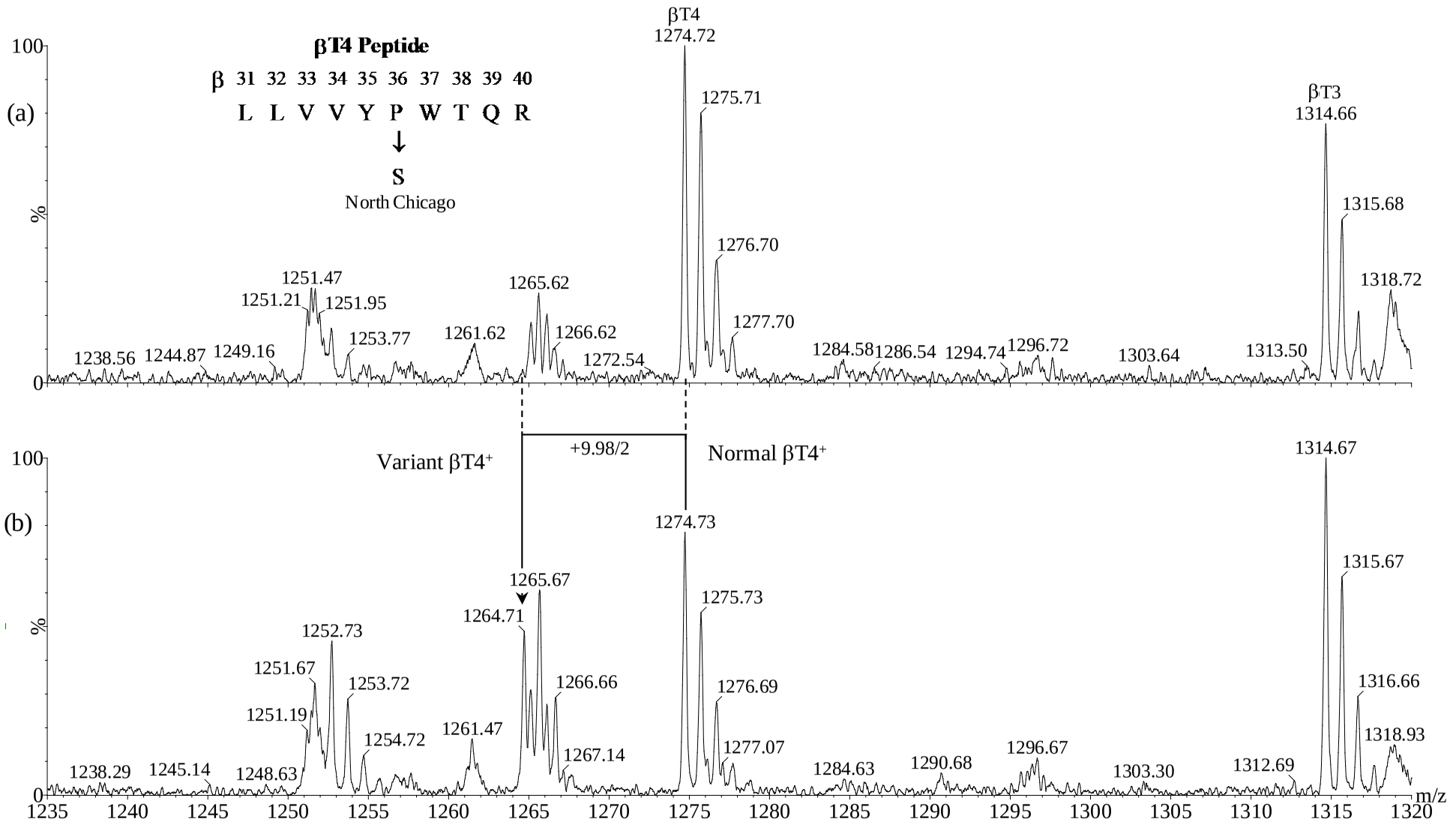

Figure 5.4.13.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb North Chicago heterozygote.

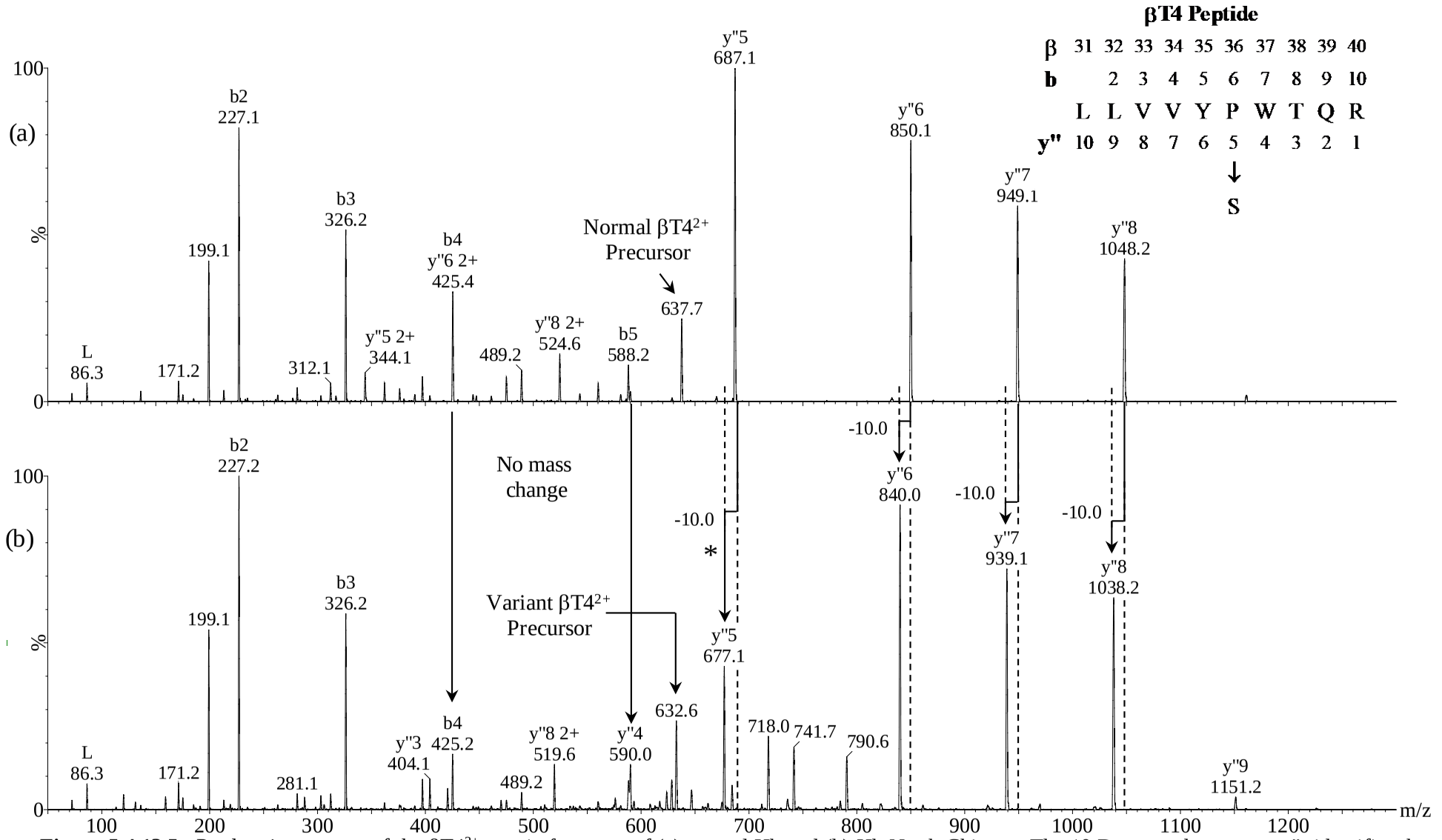

Figure 5.4.13.5. Product ion spectra of the $\beta T4^{2+}$ tryptic fragment of (a) normal Hb and (b) Hb North Chicago. The 10 Da mass decrease at $y"_5$ identifies the mutation as β36Pro→Ser.

5.4.14. βT5 - Hb Hammersmith (β42Phe→Ser)

Hb Hammersmith is the result of a β-chain mutation in which the β42 amino acid residue is changed from Phe to Ser through a single base change in the codon TTT→TCT.

βT5 Peptide

β	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59
	F	F	E	S	F	G	D	L	S	T	P	D	A	V	M	G	N	P	K
					↓														
					S														

Figure 5.4.14.1. Sequence of the Hb Hammersmith βT5 tryptic peptide

A sample was received for analysis that exhibited a normal ce-HPLC trace (Figure 5.4.14.2.) with 70.8% A_0, 17.3% F and 3.2% A_2.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	17.3	---	1.12	205884
P2	---	2.2	1.28	25608
P3	---	1.7	1.69	19606
A0	---	70.8	2.45	822502
A2	3.2	---	3.60	32335
D-window	---	2.9	4.17	34184
C-window	---	1.9	5.09	22312

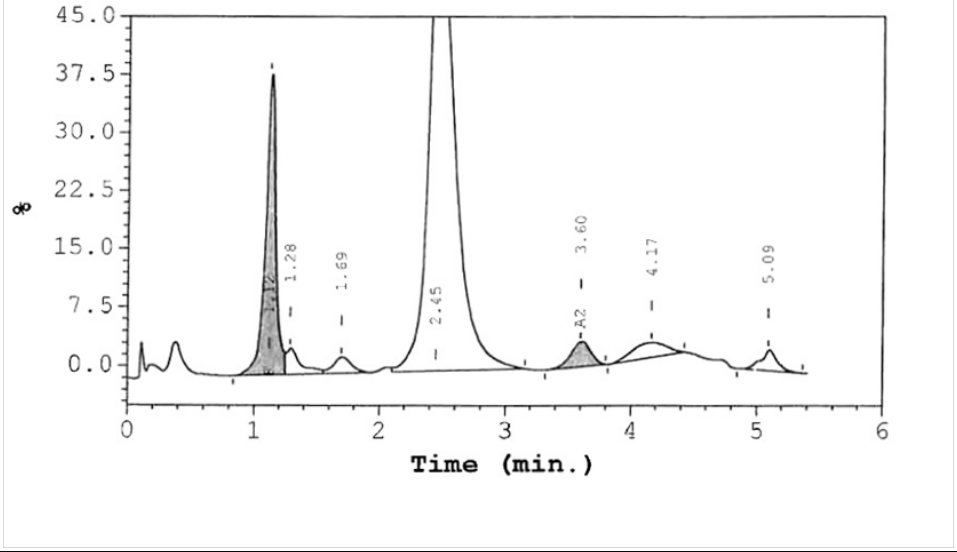

Figure 5.4.14.2. ce-HPLC trace for Hb Hammersmith.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.4.14.3.) revealed a signal at 15,807.12 Da, 60.29 Da lighter than, and approximately 35% intensity of, the normal β-chain. A mass difference of -60 Da implies either Tyr→Cys (3 possibilities) or Phe→Ser (8 possibilities) from a single base change in the codon.

Figure 5.4.14.4. shows a diagnostic part of the mass spectrum of the tryptic digest of (a) normal Hb and (b) the variant Hb. A significantly increased signal is observed at *m/z* 999.66 that is consistent with the mutated $\beta T5^{2+}$ fragment, with some underlying $\alpha T9^{3+}$, and this reduces the mutation possibilities to

Phe→Ser; β41Phe→Ser (Hb Denver), β42Phe→Ser (Hb Hammersmith) or β45Phe→Ser (Hb Cheverly).

Figure 5.4.14.5. shows the *m/z* 1,050 - 1,900 region of the product ion spectrum of the variant $\beta T5^{2+}$ tryptic fragment. The digest conditions were extended to 12 hours to remove interference from the underlying $\alpha T9^{3+}$. The masses of all the y" ions are unchanged up to $y"_{17}$, limiting the mutation possibilities to β41 or β42.

Figure 5.4.14.6. shows the *m/z* 1,050 - 1,900 region of the product ion spectrum of the variant $\beta T5^{2+}$ tryptic fragment. The absence of b_2, b_3 and b_4 at normal masses confirms β41Phe→Ser (Hb Denver) or β42Phe→Ser (Hb Hammersmith).

Further examination of the high mass (*m/z* 1,380 - 2,100) portion of the $\beta T5^{2+}$ product ion spectrum in Figure 5.4.14.7 shows the appearance of a mutated $y"_{18}$ ion at *m/z* 1,851.8, confirming the mutation as β42Phe→Ser, Hb Hammersmith.

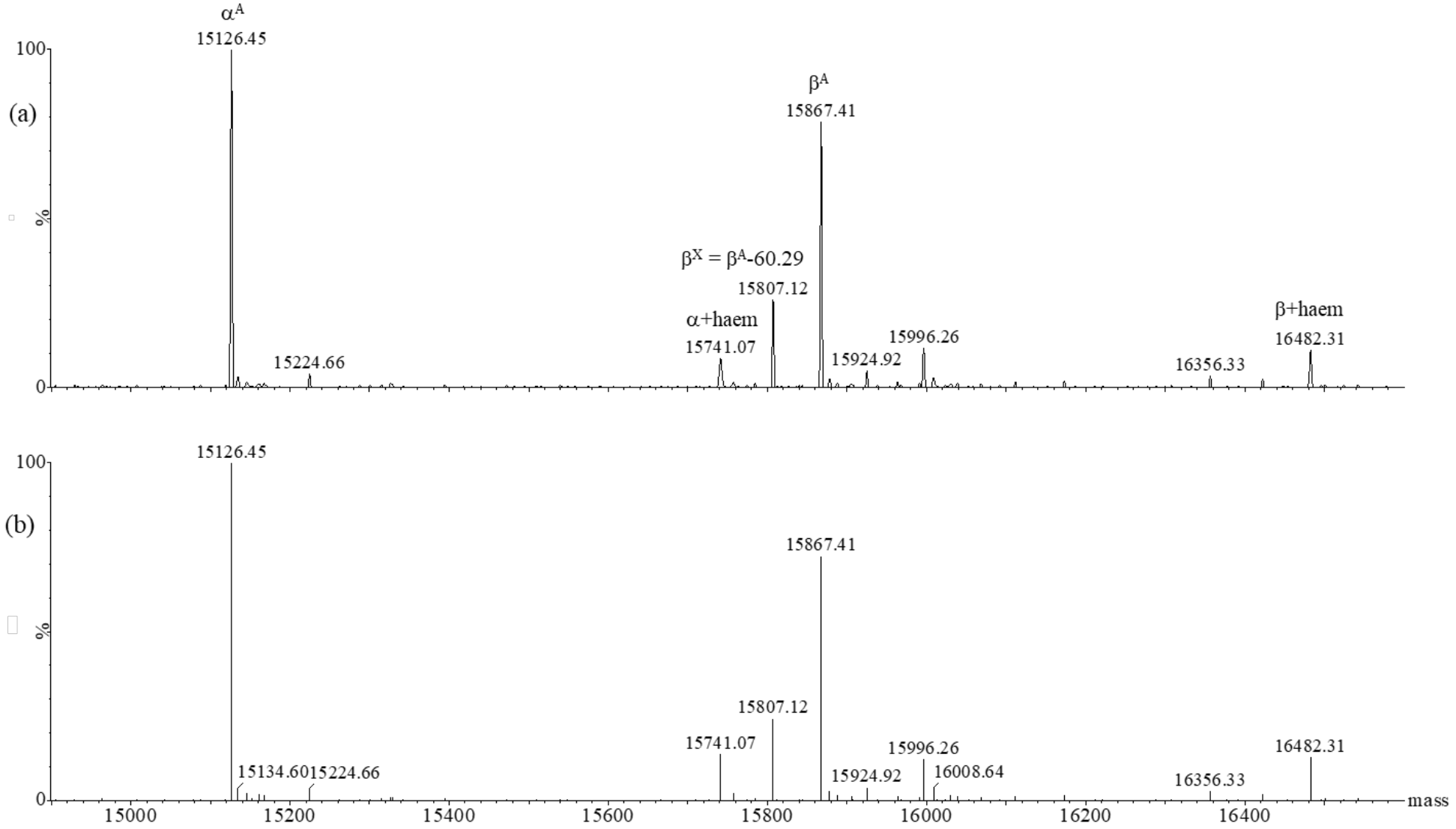

Figure 5.4.14.3. Deconvoluted mass spectrum of Hb Hammersmith (β42Phe→Ser) showing the presence of a signal at 15,807.12 Da at approximately 35% intensity of the normal β-chain peak (15,867.41 Da). The 60 Da mass decrease can be assigned to Tyr→Cys or Phe→Ser.

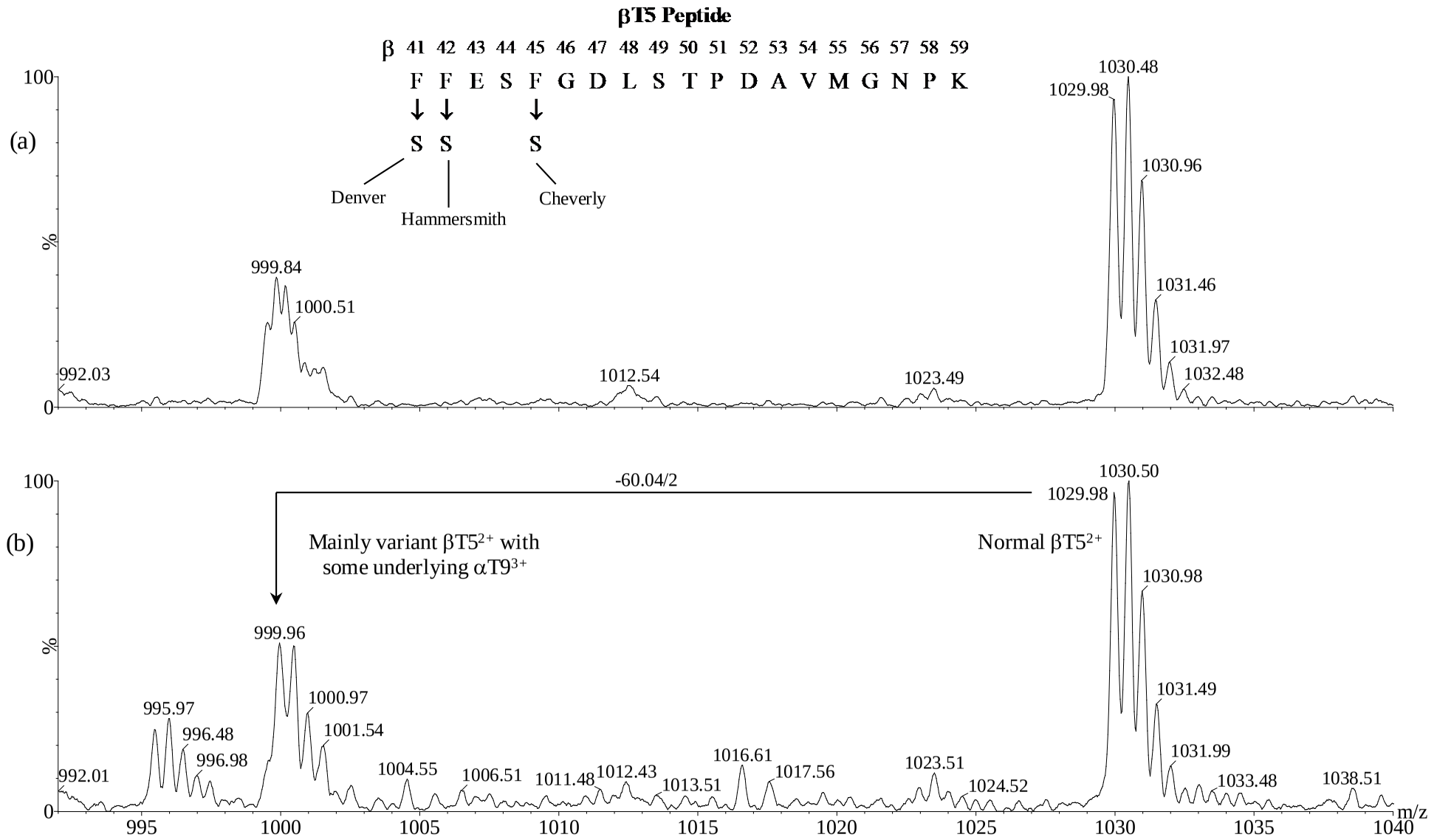

Figure 5.4.14.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Hammersmith heterozygote.

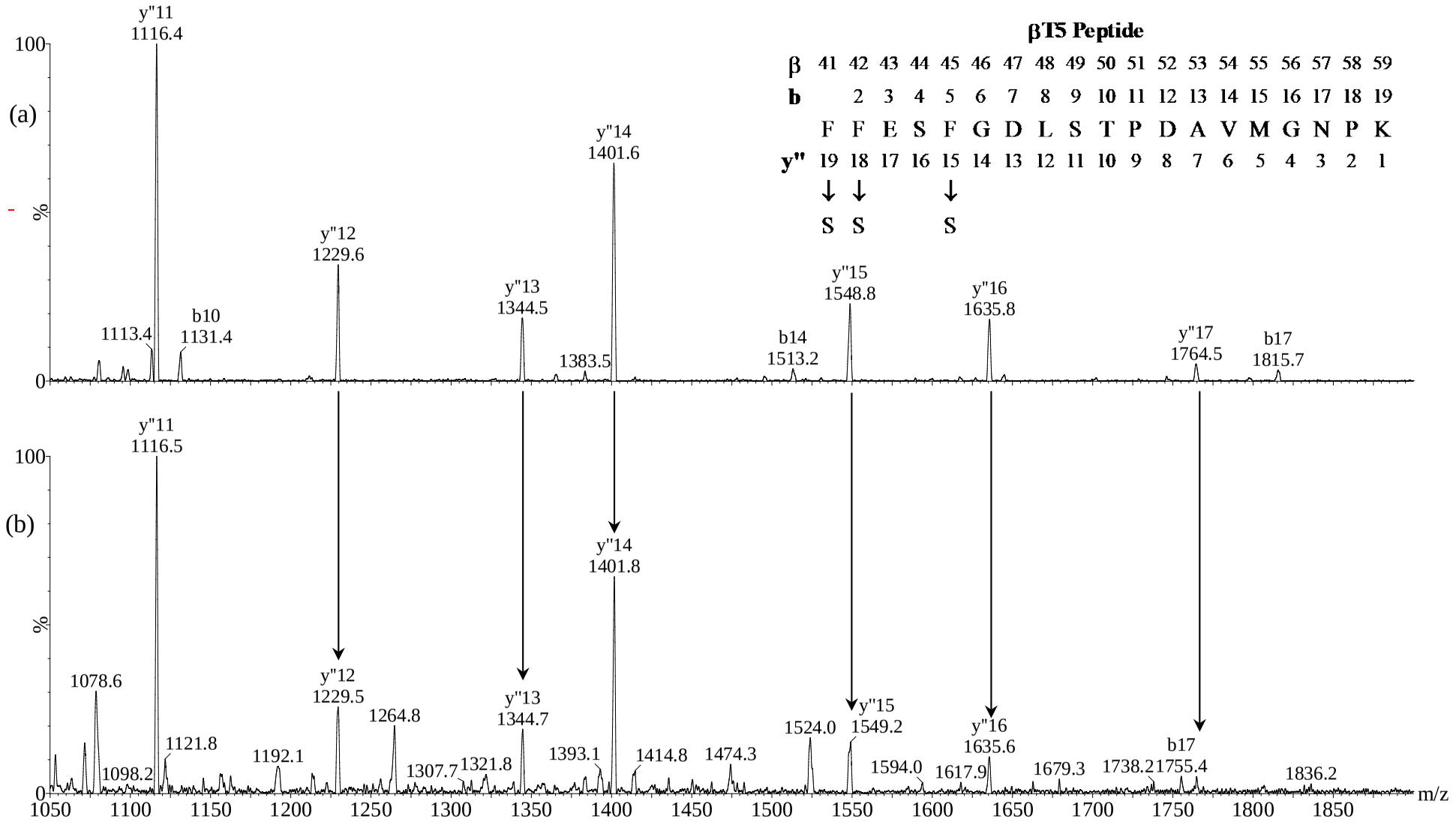

Figure 5.4.14.5. Partial product ion spectra of the $\beta T5^{2+}$ tryptic fragment of (a) normal Hb and (b) Hb Hammersmith (β42Phe→Ser). The masses of all y" ions up to $y"_{17}$ are unchanged, implying the mutation is either β41Phe→Ser (Hb Denver) or β42Phe→Ser (Hb Hammersmith). β45Phe→Ser (Hb Cheverly) is excluded.

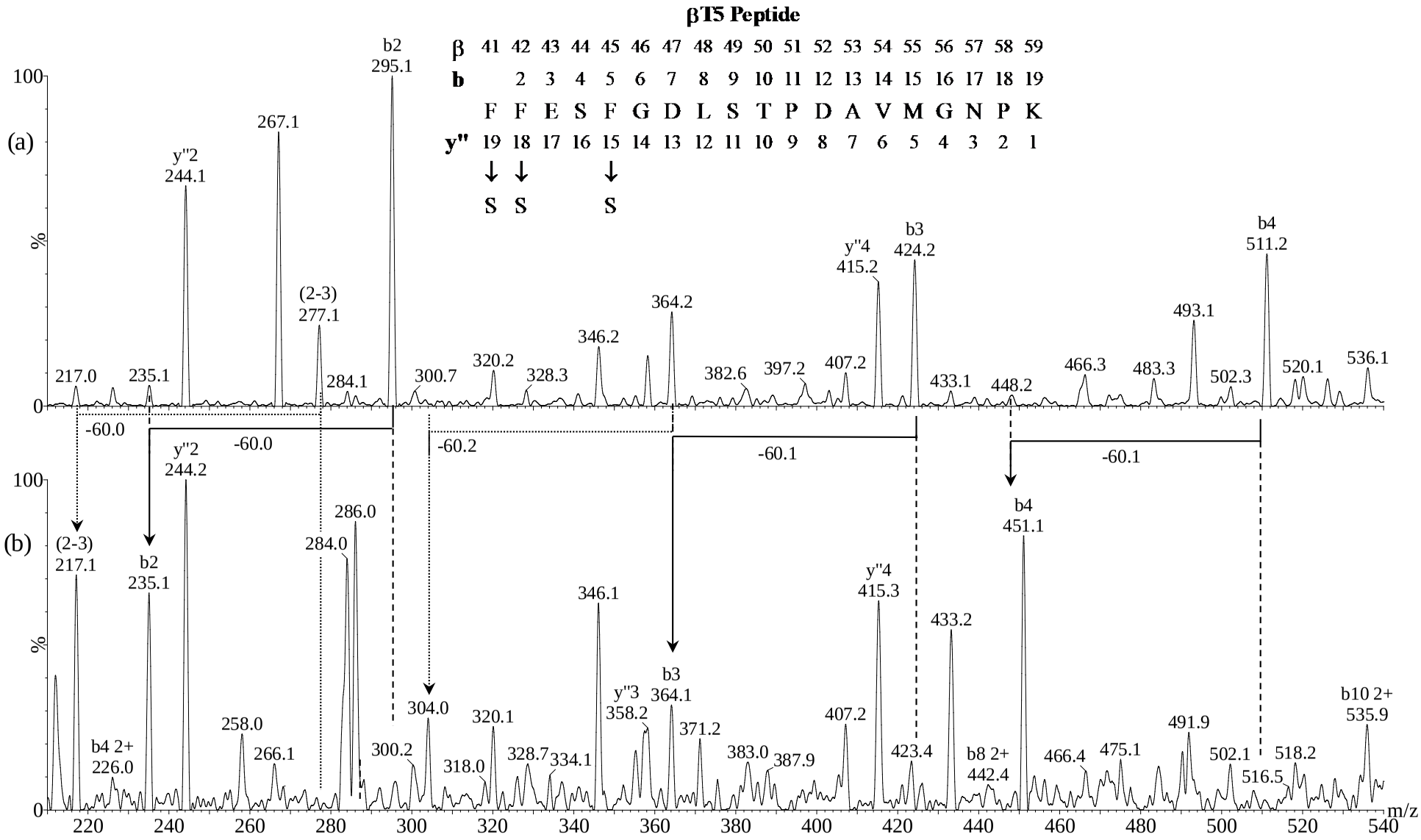

Figure 5.4.14.6. Partial Product ion spectra of the $\beta T5^{2+}$ tryptic fragment of (a) normal Hb and (b) Hb Hammersmith (β42Phe→Ser). The absence of b_2, b_3 and b_4 at normal masses confirms β41Phe→Ser (Hb Denver) or β42Phe→Ser (Hb Hammersmith). The 60 Da mass decrease of internal fragments 2-3 (*m/z* 217.1) and 2-4 (*m/z* 304.0) favour β42Phe→Ser.

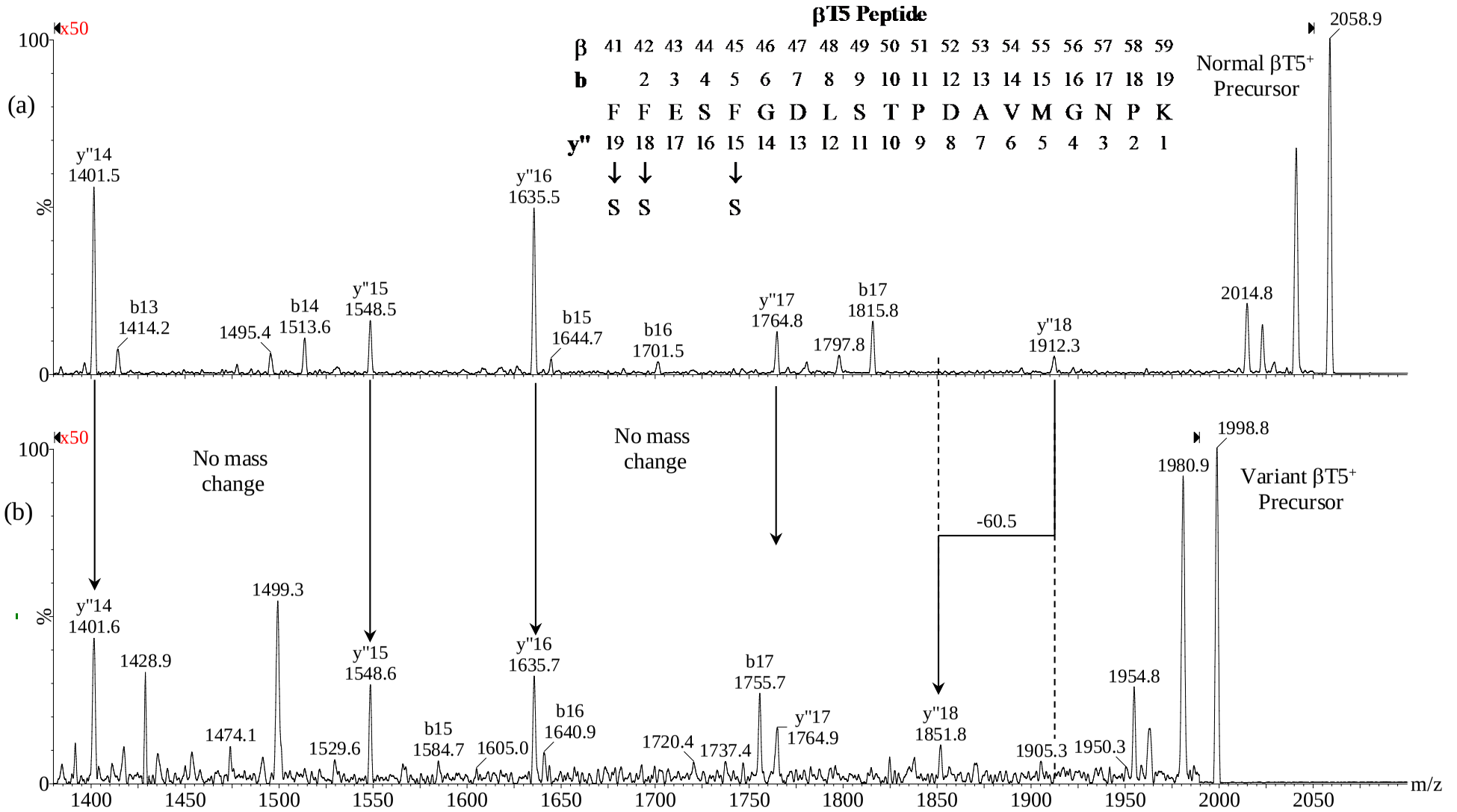

Figure 5.4.14.7. Partial Product ion spectra of the βT5^{+} tryptic fragment of (a) normal Hb and (b) Hb Hammersmith (β42Phe→Ser). The 60 Da mass decrease at y"$_{18}$ identifies the mutation β42Phe→Ser, Hb Hammersmith.

5.4.15. βT5 - Hb Osu Christiansborg (β52Asp→Asn)

Hb Osu Christiansborg is the result of a β-chain mutation in which the β52 amino acid residue is changed from Asp to Asn through a single base change in the codon GAT→AAT.

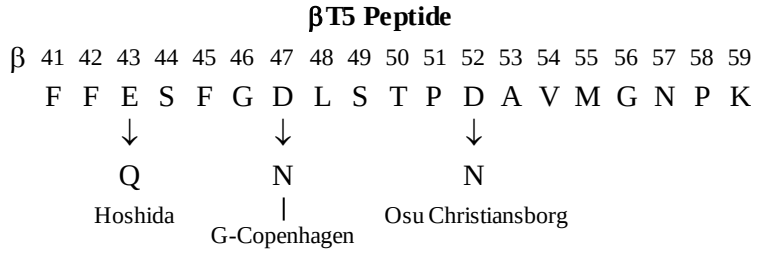

Figure 5.4.15.1. Sequence of the Hb Osu Christiansborg βT5 tryptic peptide.

A blood sample was submitted for analysis by mass spectrometry because the ce-HPLC trace (Figure 5.4.15.2.) showed a significant response (39.3%) in the A_2 window at 3.77 min and infers a positive charge change.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.1	1.01	1097
F	0.7	---	1.12	11399
Unknown	---	0.7	1.24	12530
P2	---	2.9	1.32	53409
P3	---	4.5	1.75	81561
A0	---	53.7	2.50	976648
A2	39.3	---	3.77	681816

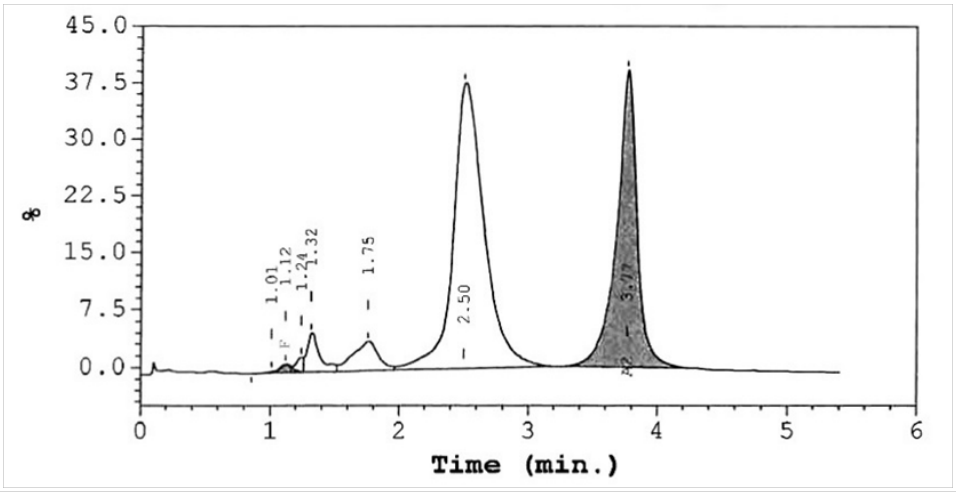

Figure 5.4.15.2. ce-HPLC trace for Hb Osu Christiansborg.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.4.15.3.) shows the presence of a single signal at 15,866.91 Da, which suggests a $\beta^A/(\beta^A\text{-}1)$ heterozygote, with a low (<50%) percentage of the $(\beta^A\text{-}1)$ variant.

Figure 5.4.15.4. shows the diagnostic part of the tryptic digest mass spectrum for (a) normal Hb and (b) the variant Hb. The lower panel shows the appearance of a signal at *m/z* 2,019.48 adjacent to the normal $\beta T5^{2+}$ peak with a mass difference of -1 Da (0.5 *m/z*). This indicates that the mutation is present in the βT5 tryptic peptide and could arise from β43Glu→Gln (Hb Hoshida), β47Asp→Asn (Hb G-

Copenhagen) or β52Asp→Asn (Hb Osu Christiansborg) to be consistent with the observed charge change.

Figure 5.4.15.5. shows the partial product ion spectra of the $\beta T5^{2+}$ tryptic fragment of (a) normal Hb and (b) the variant Hb. A mass decrease of -1Da is observed at y''_9 (*m/z* 927.6) and no mass change is seen at y''_7 (*m/z* 716.5), identifying the mutation as β52Asp→Asn, Hb Osu Christiansborg.

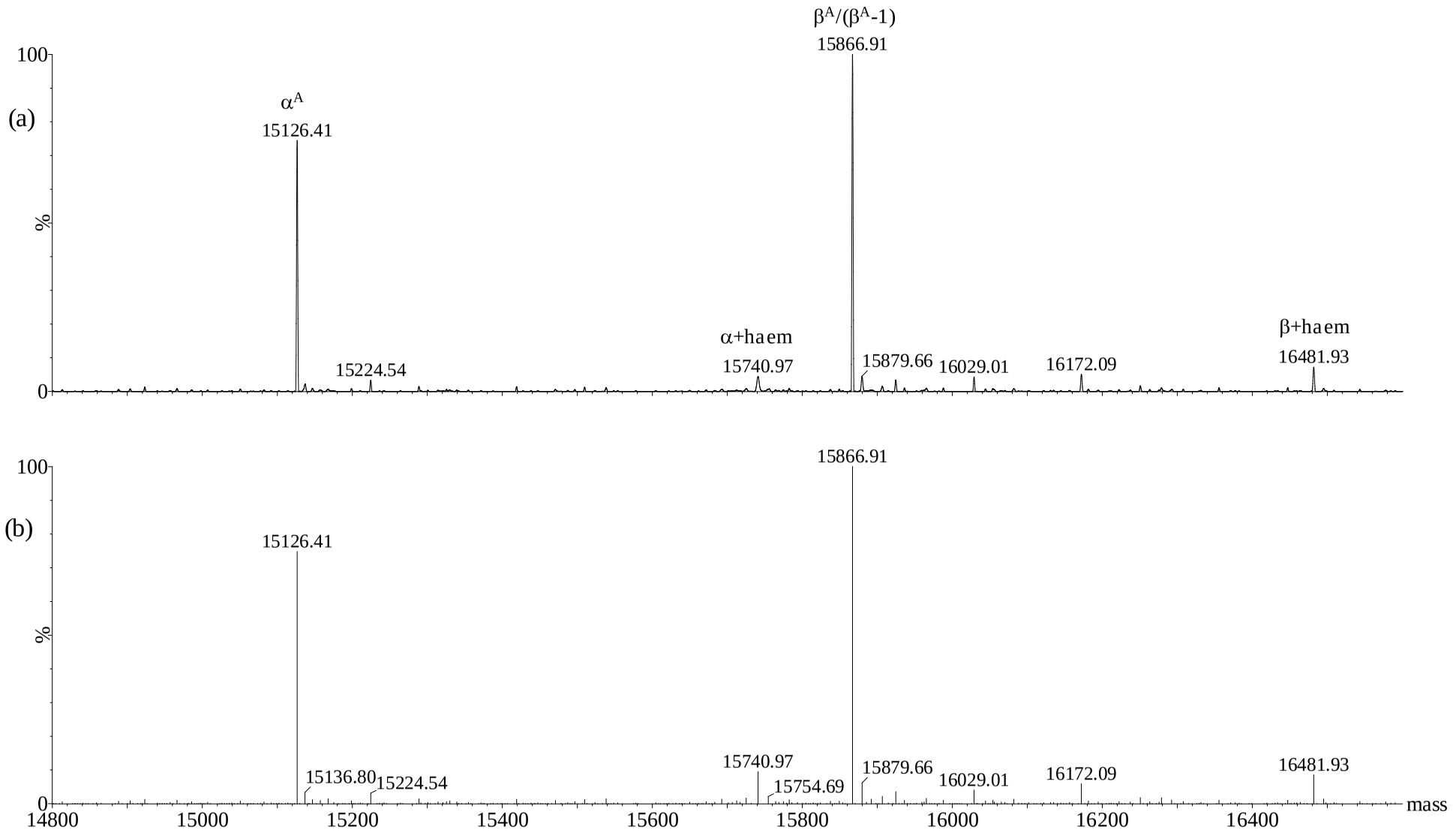

Figure 5.4.15.3. Deconvoluted mass spectrum of Hb Osu Christiansborg (β52Asp→Asn) showing the presence of a signal at 15,866.91 Da The mass suggests a $\beta^A/(\beta^A\text{-}1)$ heterozygote.

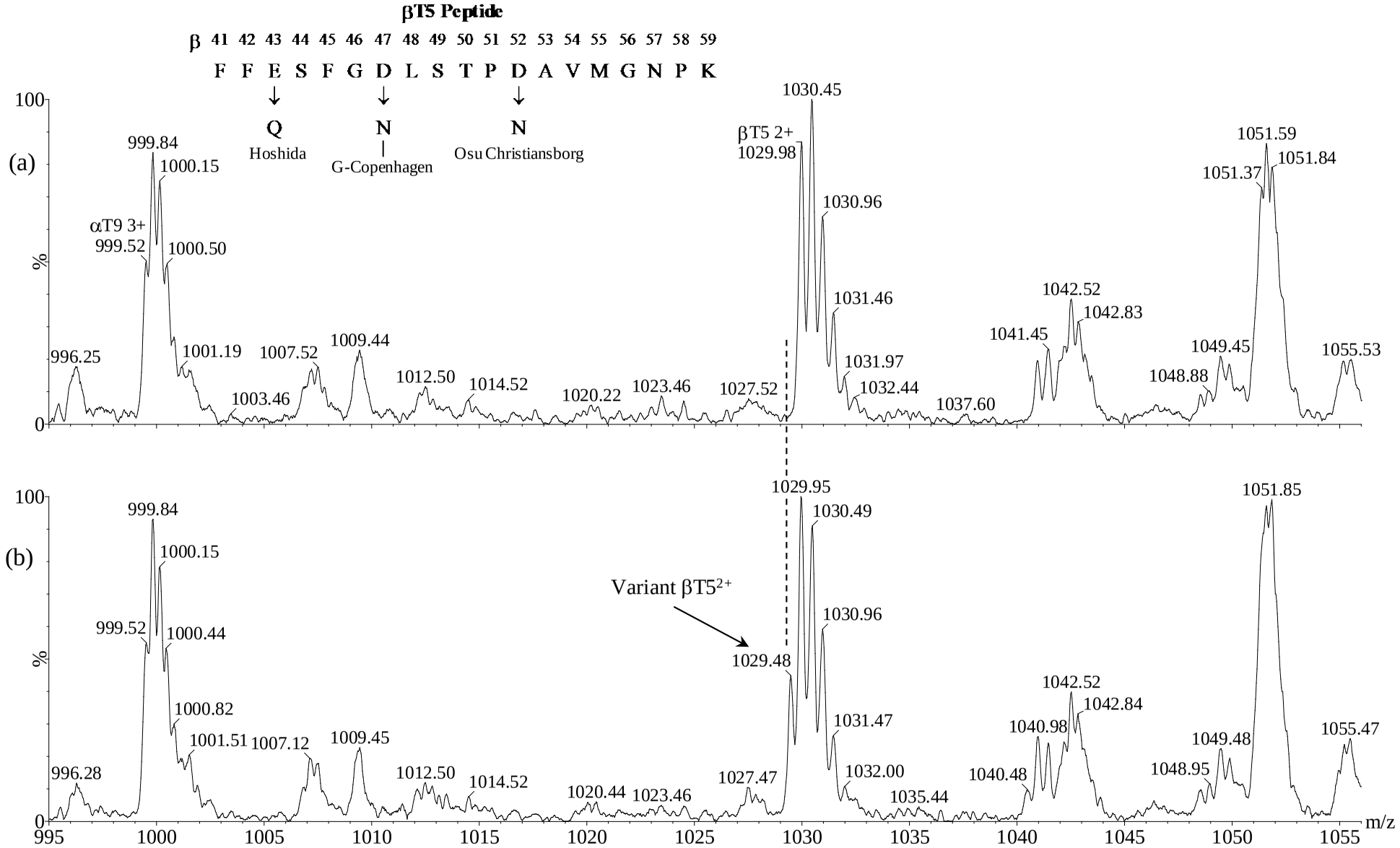

Figure 5.4.15.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Osu Christiansborg heterozygote.

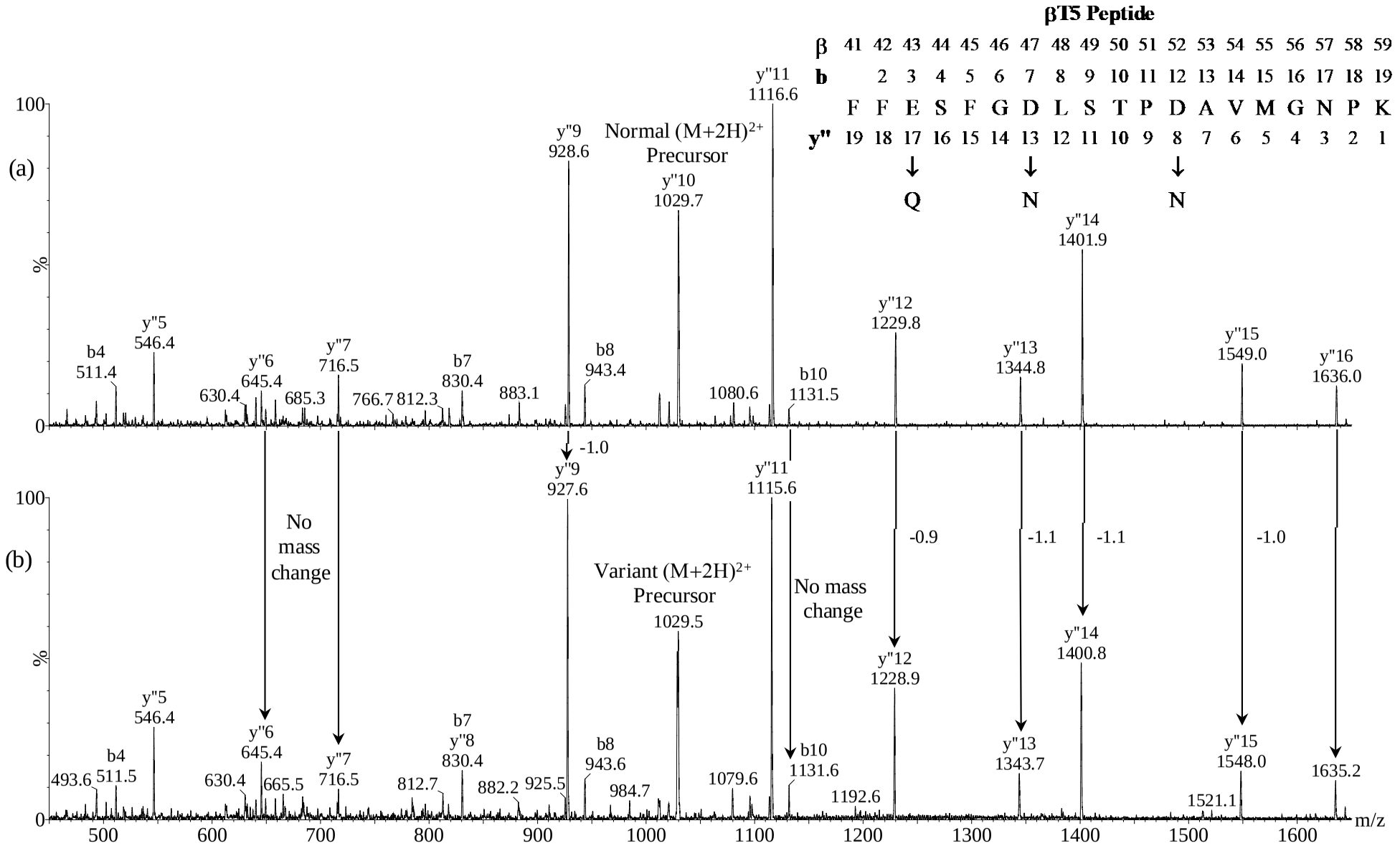

Figure 5.4.15.5. Partial Product ion spectra of the $\beta T5^{2+}$ tryptic fragment of (a) normal Hb and (b) Hb Osu Christiansborg. The 1 Da mass decrease at y''_9 and no mass change at y''_7 identifies the mutation as β52Asp→Asn.

5.4.16. βT5 - Hb Sheffield (β58Pro→His)

Hb Sheffield is the result of a β-chain mutation in which the β58 amino acid residue is changed from Pro to His through a single base change in the codon CCT→CAT.

βT5 Peptide																		
β	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59
	F	F	E	S	F	G	D	L	S	T	P	D	A	V	M	G	N	P	K
											↓							↓	
											H							H	
											North Manchester							Sheffield	

Figure 5.4.16.1. Sequence of the Hb Sheffield βT5 tryptic peptide.

There is no ce-HPLC trace available for Hb Sheffield.

A sample was received for analysis that exhibited an abnormal peak (39.9%) just after A_0 by HPLC, indicating the presence of a possible mutation with a slightly positive charge change. The abnormality was detected during routine diabetic monitoring.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.4.16.2.) revealed a signal at 15,907.22 Da, 39.96 Da heavier, and approximately the same intensity as, the normal β-chain. A mass difference of +40 Da is only attributable to a Pro→His mutation through a single base change in the codon, with seven possible sites of mutation in the β-chain.

Figure 5.4.16.3. shows the diagnostic part of the tryptic digest spectra for (a) normal Hb and (b) the variant Hb. The appearance of a peak at *m/z* 1,050.47 in the lower panel is consistent with a mutation in the βT5 fragment with a +40 Da mass change. In the βT5 tryptic fragment, there are two possible sites of mutation: β51Pro→His (Hb North Manchester) or β58Pro→His (Hb Sheffield).

Figure 5.4.16.4 shows a comparison of part of the product ion spectra of the $\beta T5^{2+}$ tryptic fragments of (a) normal Hb and (b) the variant Hb. A mass change of +40 Da is observed for the y''_2 fragment ion (*m/z* 284.2 in the lower panel) that persists for the increasing y" fragments, and identifies the mutation as β58Pro→His, Hb Sheffield.

This variant was novel when first analysed by mass spectrometry.

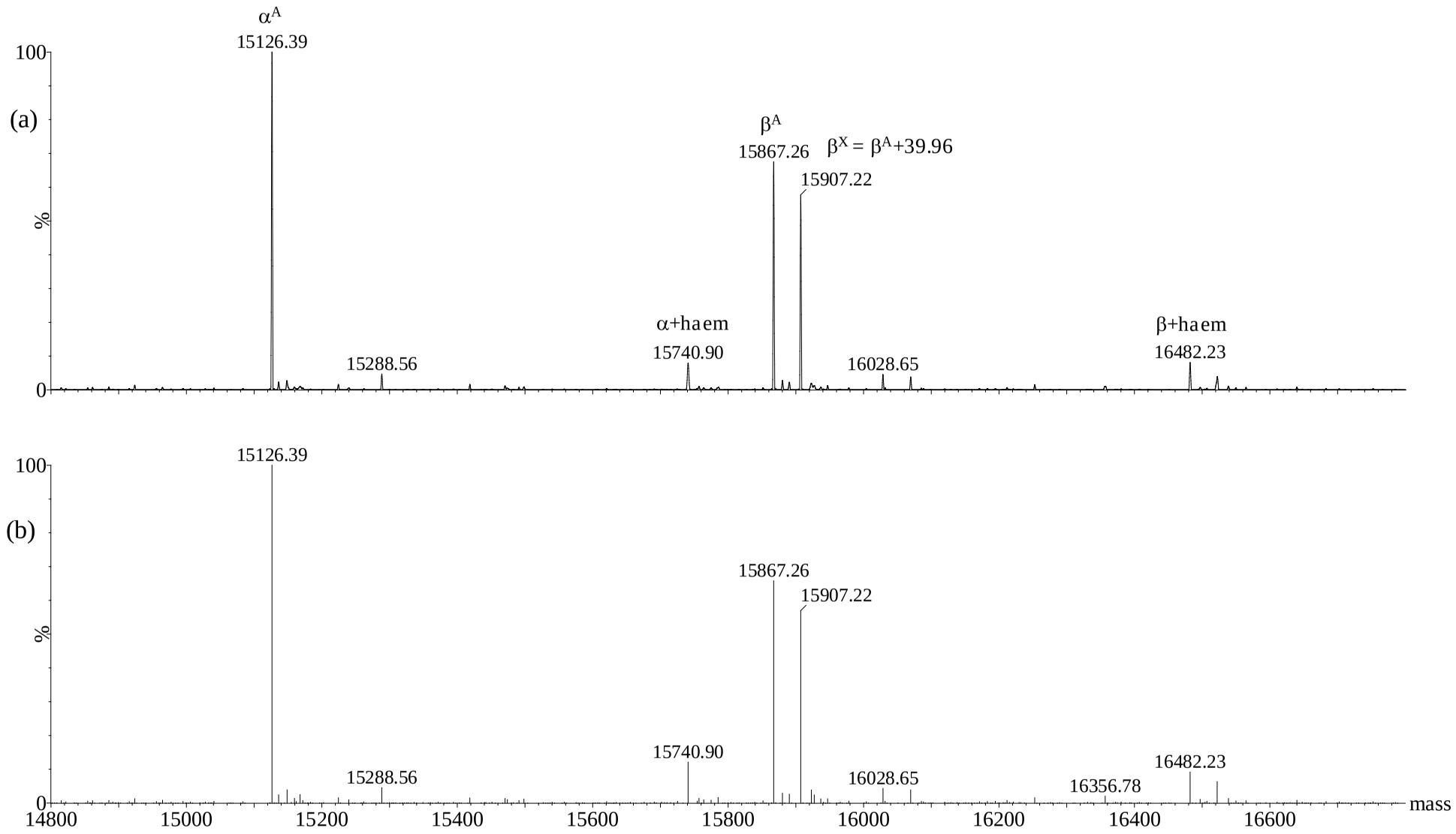

Figure 5.4.16.2. Deconvoluted mass spectrum of Hb Novel, Sheffield (β58Pro→His) showing the presence of a signal at 15,907.22 Da at approximately 90% intensity of the normal β-chain peak (15,867.26 Da). The 40 Da mass increase suggests Pro→His (7 possibilities).

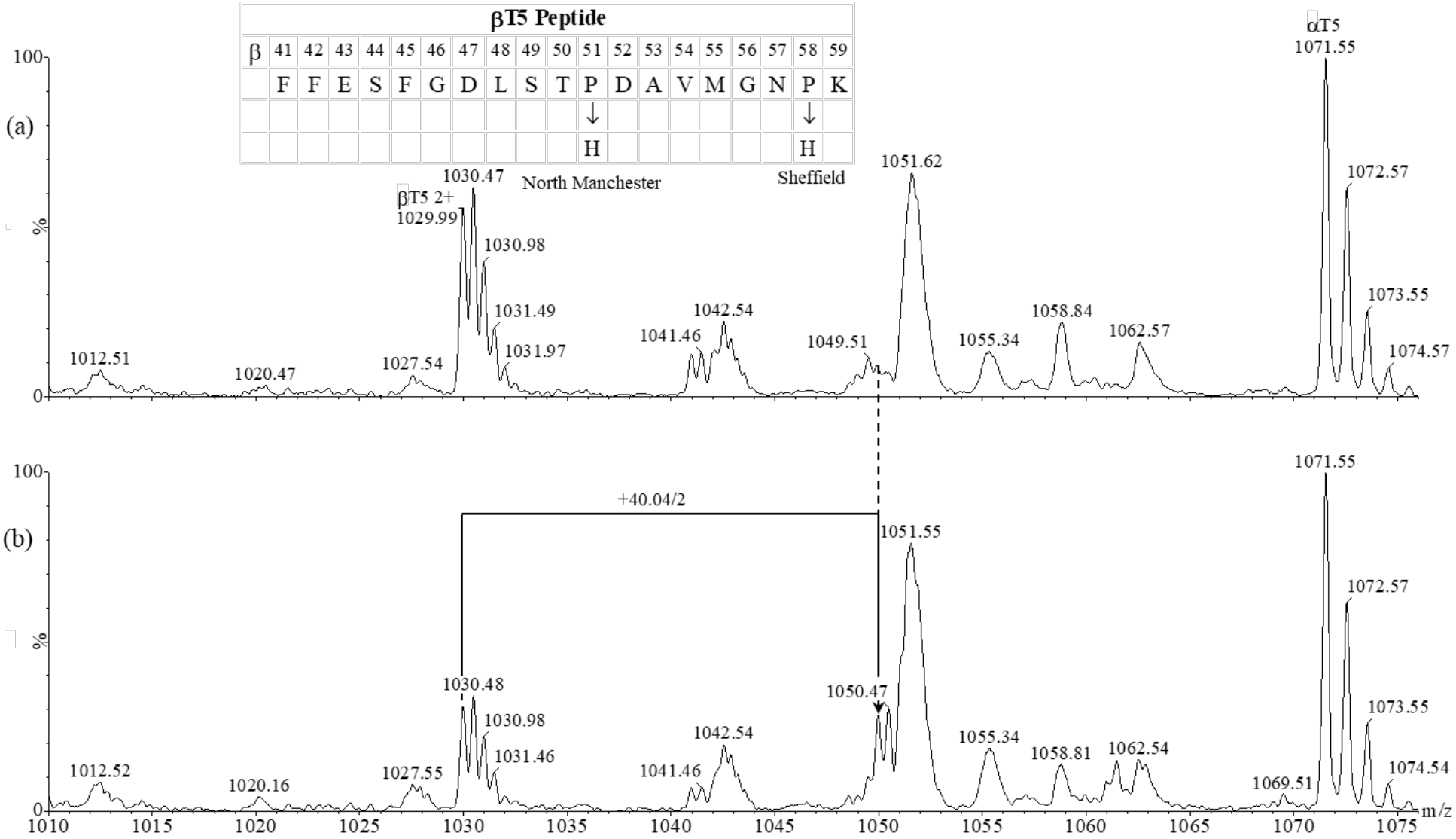

Figure 5.4.16.3. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Novel, Sheffield heterozygote.

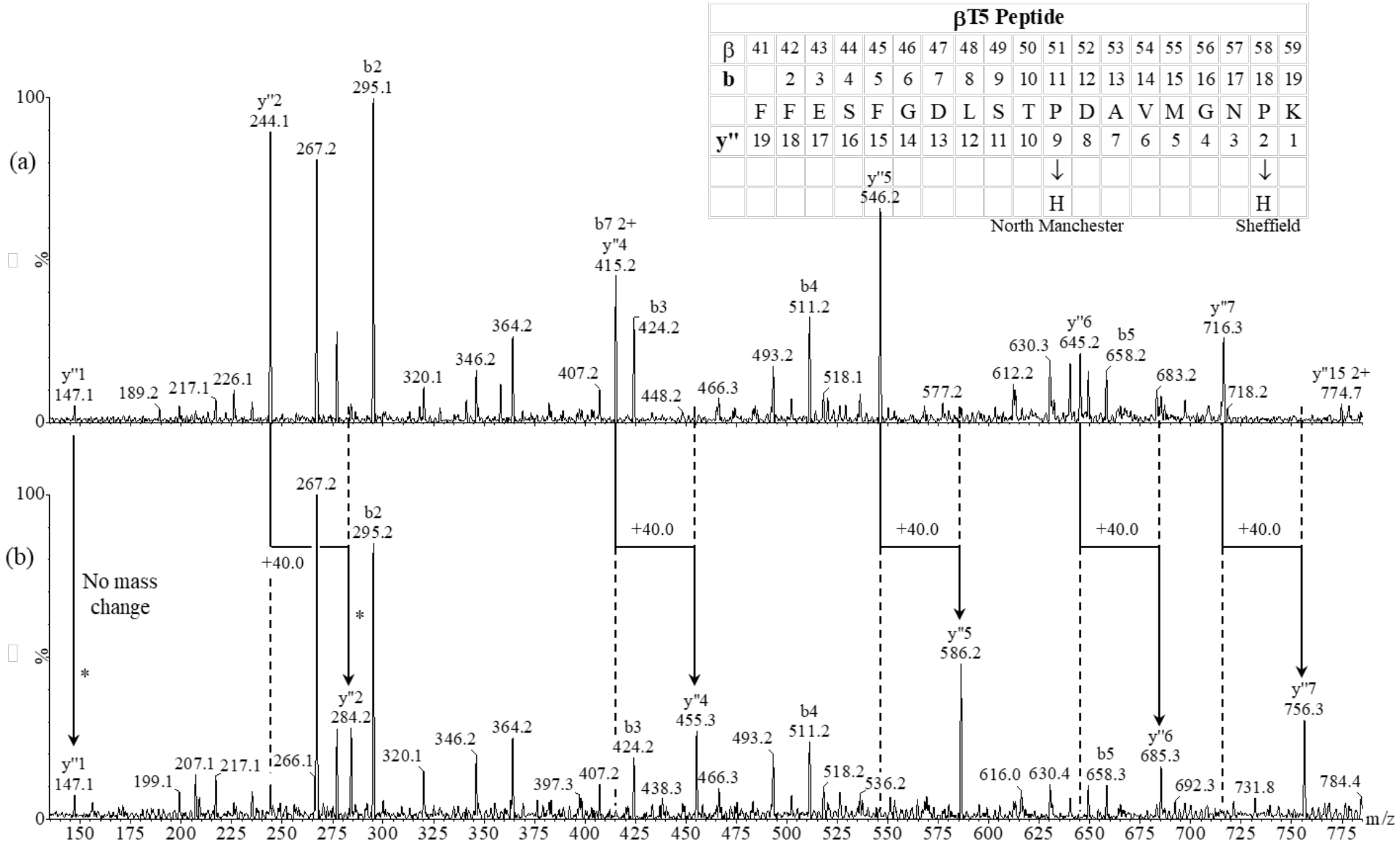

Figure 5.4.16.4. Product ion spectra of the $\beta T5^{2+}$ tryptic fragment of (a) normal Hb and (b) Hb Sheffield. The 40 Da mass increase at y''_2 identifies the mutation as β58Pro→His, Hb Sheffield.

5.4.17. βT6 - Novel (β61Lys→Thr)

Hb Novel is the result of a β-chain mutation in which the β61 amino acid residue is changed from Lys to Thr through a single base change in the codon AAG→ACG.

The mutation of the Lys residue results in the loss of a tryptic cleavage product, and the formation of a combined βT(6-7) tryptic peptide.

βT6 & 7 Peptides

β	60	61		62	63	64	65
	V	K		A	H	G	K
		↓					
	V	T	A	H	G	K	

Figure 5.4.17.1. Sequence of the Hb Novel βT(6-7) tryptic peptide.

There is no ce-HPLC trace available for Hb Novel. A sample was received for analysis in which was reported with a 38% variant showing a negative charge change, and J-like characteristics.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.4.17.2.) revealed a signal at 15,840.20 Da, 27.0 Da lighter than, and approximately equal intensity of, the normal β-chain. A mass difference of -27 Da from a single base change in the codon, together with a negative charge change, suggests a mutation Lys→Thr, of which there are 11 possibilities in the β-chain. Loss of a Lys residue would also lead to the loss of a tryptic fragment and the generation of a new, heavier tryptic fragment.

Figure 5.4.17.3. shows that diagnostic part of the tryptic digest spectra for (a) normal Hb and (b) the variant Hb. A new signal is observed at *m/z* 306.68 in the lower panel that is consistent with the formation $\beta(6\text{-}7)^{2+}$ from a combined β(6-7) tryptic peptide, identifying the mutation as β61Lys→Thr, which has not previously been reported. Further evidence of the formation of the new tryptic peptide is shown in Figure 5.4.17.4. with a comparison of the tryptic digest spectra from (a) normal Hb and (b) the variant Hb, in which the lower panel exhibits a change in the peak at *m/z* 612.31 which is composed of the 2nd isotope of the $\alpha T6^{3+}$ and 1st isotope of the $\beta T(6\text{-}7)^{+}$.

Figure 5.4.17.5 shows the product ion spectrum of the $\beta T(6\text{-}7)^{2+}$ tryptic fragment, and the data are consistent with the mutation β61Lys→Thr, Hb Novel.

This variant was novel when first analysed by mass spectrometry.

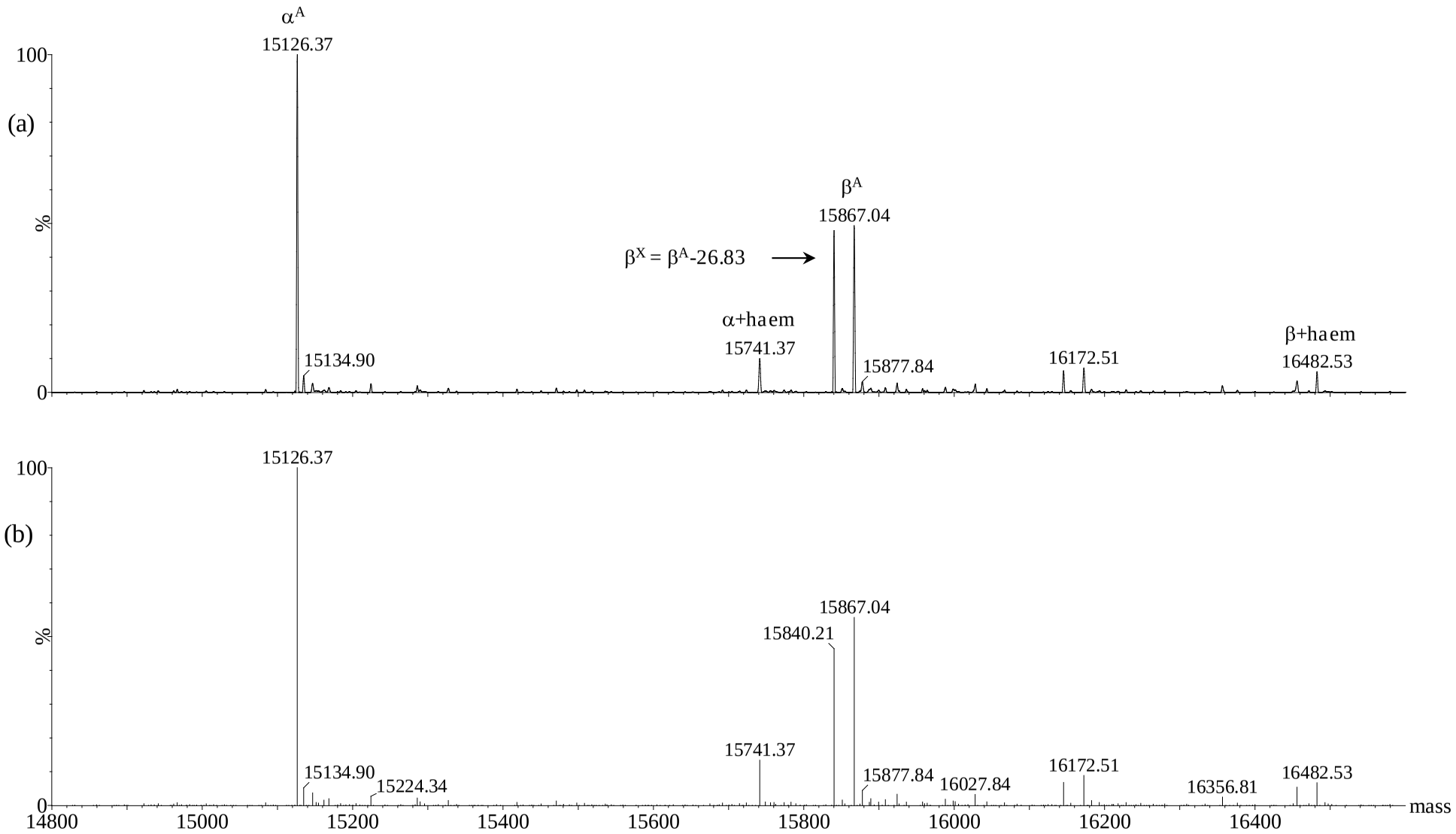

Figure 5.4.17.2. Deconvoluted mass spectrum of Hb Novel (β61Lys→Thr) showing the presence of a signal at 15,840.21 Da at approximately equal intensity of the normal β-chain peak (15,867.04 Da). The 27 Da mass decrease, and J-like characteristics, suggest the mutation Lys→Thr.

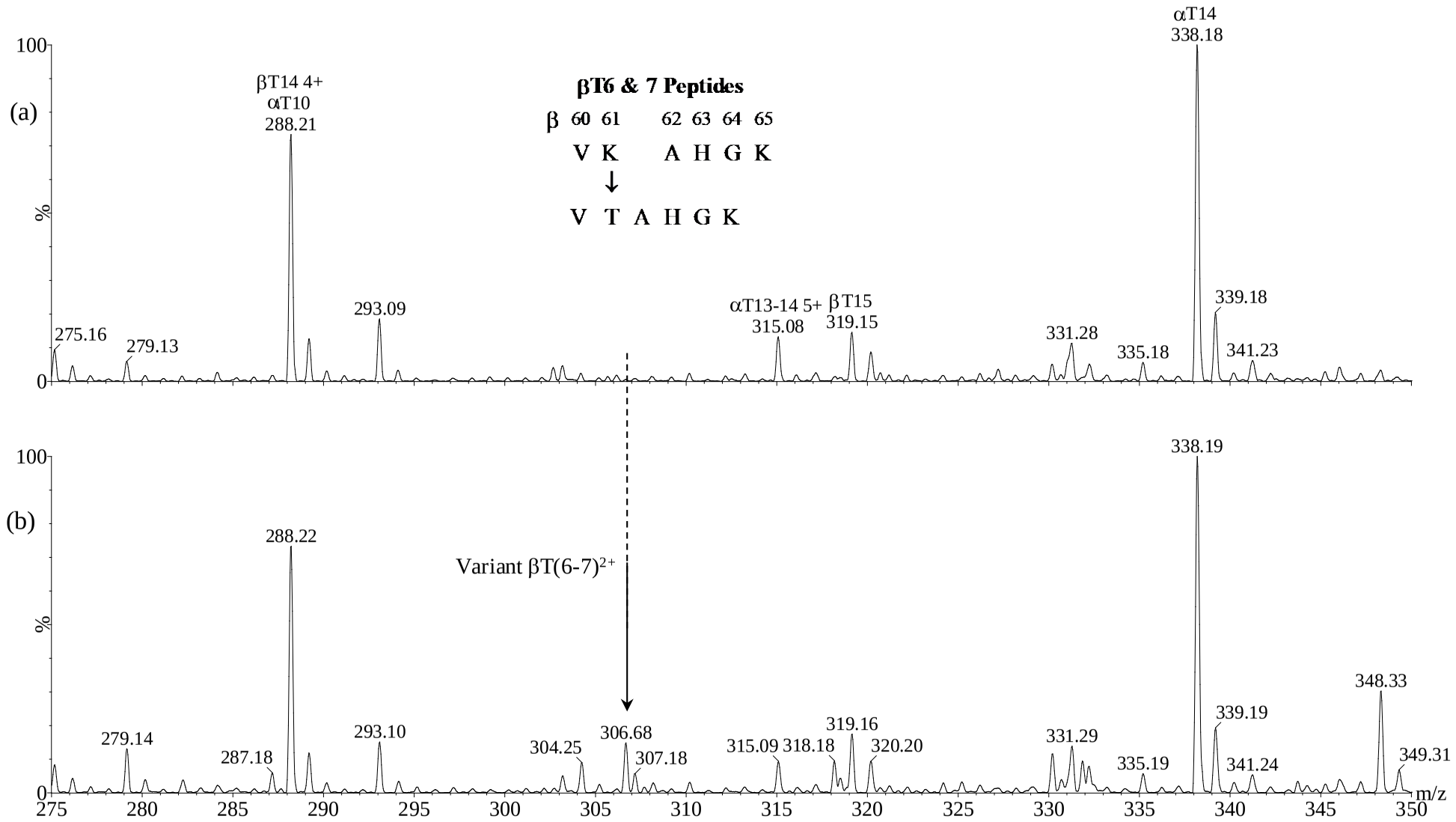

Figure 5.4.17.3. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Novel heterozygote. The presence of the ion at *m/z* 306.68 identifies the mutation as β61Lys→Thr.

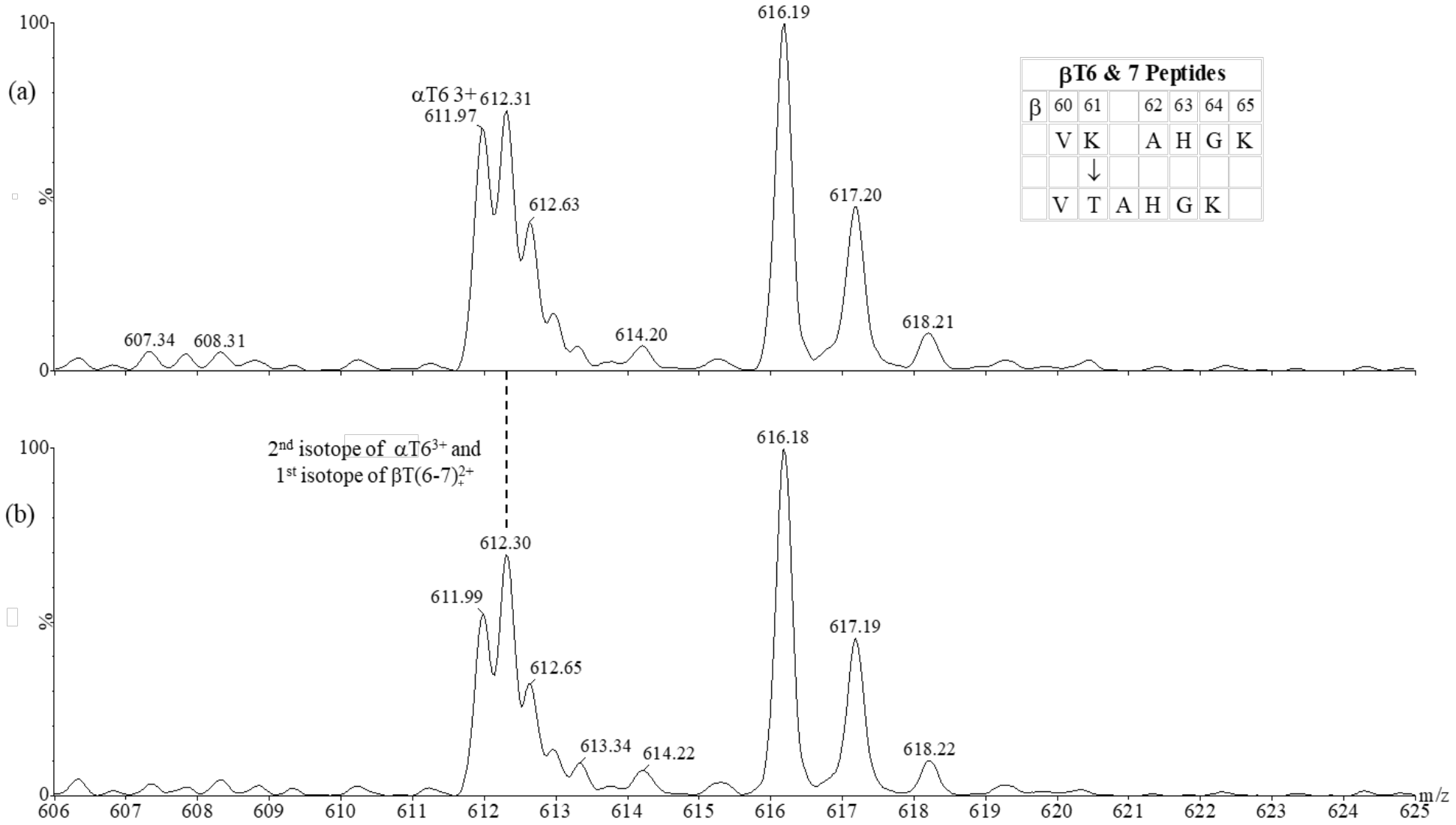

Figure 5.4.17.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Novel heterozygote (β61Lys→Thr).

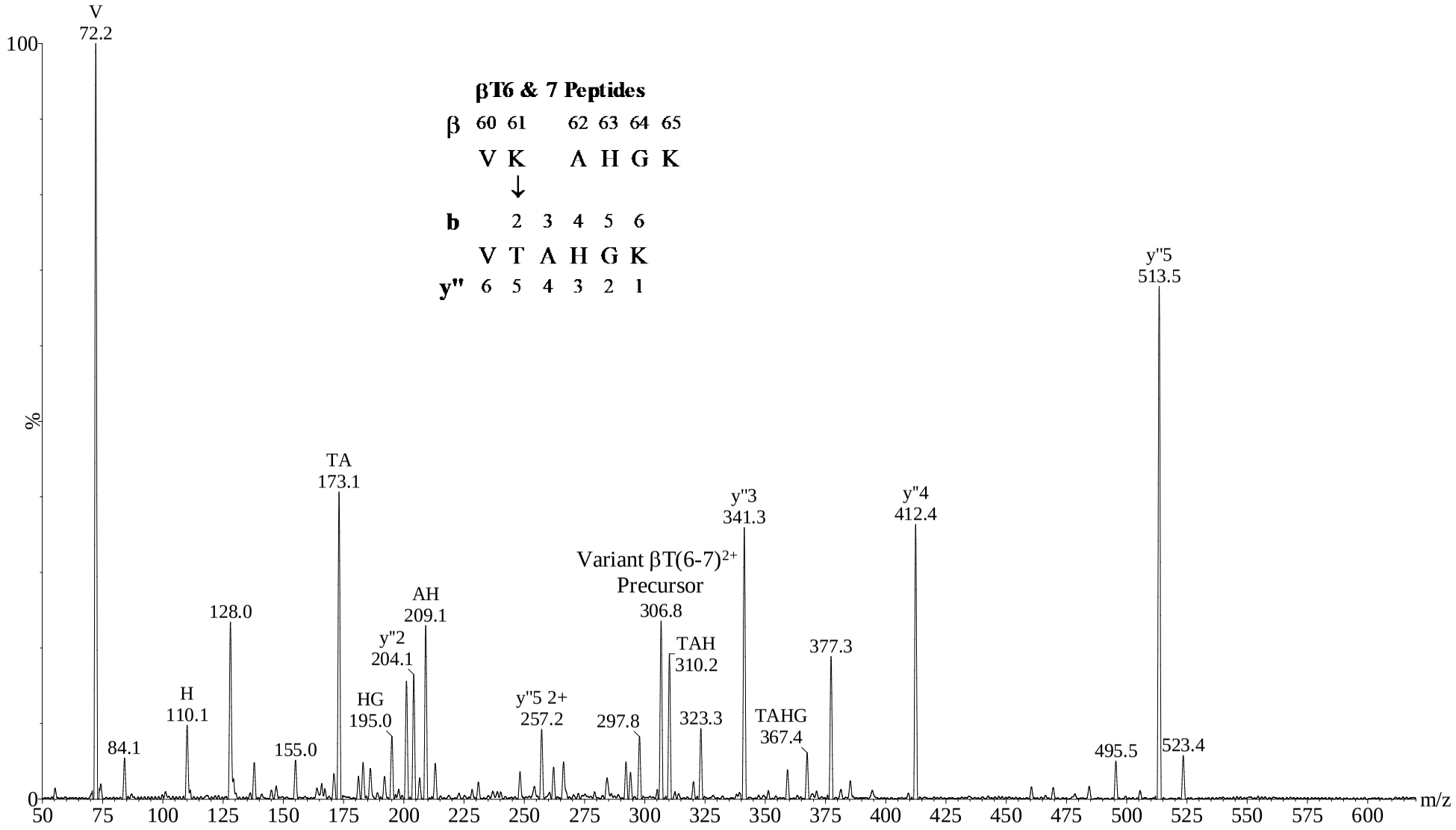

Figure 5.4.17.5. Product ion spectra of the $\beta T(6\text{-}7)^{2+}$ tryptic fragment of Hb Novel. This spectrum is consistent with the sequence of the β(6-7) peptide and confirms the mutation β61Lys→Thr.

5.4.18. βT7 - Hb J-Calabria (β64Gly→Asp)

Hb J-Calabria is the result of a β-chain mutation in which the β64 amino acid residue is changed from Gly to Asp through a single base change in the codon GGC→GAC.

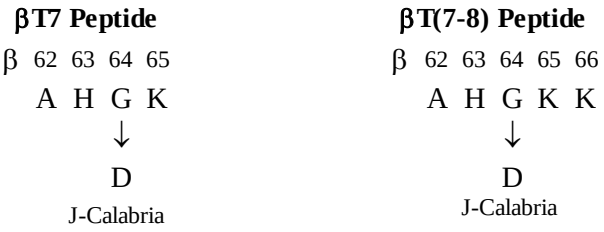

Figure 5.4.18.1. Sequence of the Hb J-Calabria βT7 and βT(7-8) tryptic peptides.

The mutation to Asp inhibits the cleavage at 65Lys, with the possibility that both βT7 and βT(7-8) may be apparent in the tryptic digest spectra.

A sample was received for analysis that exhibited an unknown response in the normal ce-HPLC trace (Figure 5.4.18.2.) with 32.8% at 1.95 min, suggesting a negative charge change.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	2.9	---	1.12	76132
Unknown	---	7.0	1.29	170514
P2	---	3.3	1.66	79171
P3	---	32.8	1.95	797081
A0	---	50.9	2.34	1237992
A2	2.5	---	3.56	70808

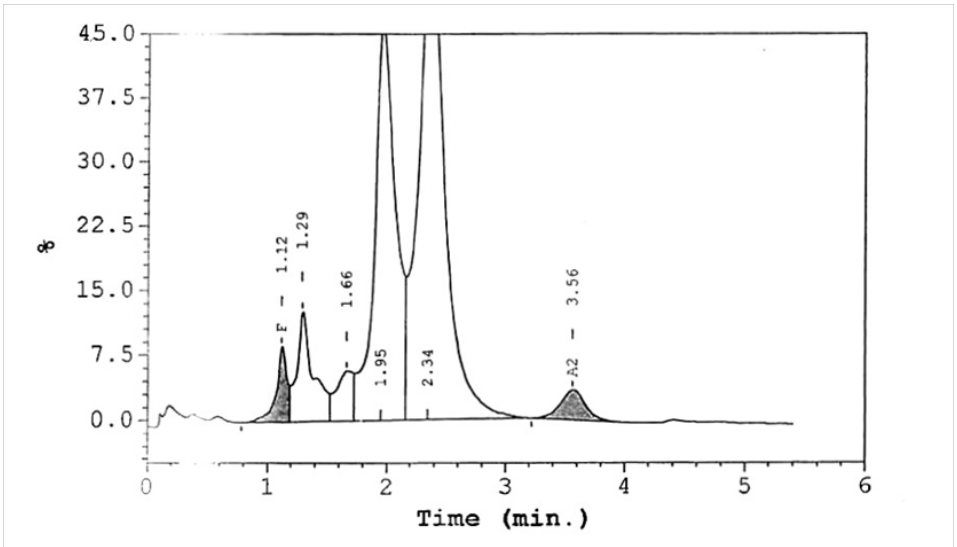

Figure 5.4.18.2. ce-HPLC trace for Hb J-Calabria.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.4.18.3.) revealed a signal at 15,925.18 Da, 57.92 Da lighter than, and approximately 42% intensity of, the normal β-chain. A mass difference of +58 Da implies either Ala→Glu (21 possibilities) or Gly→Asp (13 possibilities) from a single base change in the codon.

Figure 5.4.18.4. shows a diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) the variant Hb. The appearance of a peak at *m/z* 598.33 is indicative of a mutated βT(7-8)$^{+}$, and identifies the mutation as β64Gly→Asp. This is further supported by the spectrum in the lower panel of Figure

5.4.18.5. with the appearance of a minor peak at *m/z* 470.27, $\beta T7^{+}$. The low intensity of this signal is expected as the β64Asp would inhibit the tryptic digestion at β65Lys.

Figure 5.4.18.6. shows a comparison of the product ion spectra of the $\beta T(7\text{-}8)^{+}$ tryptic fragment of (a) normal Hb and (b) the variant Hb. The 58 Da mass increase in the mass of the y''_3 at *m/z* 390.4, and the same increase in the mass of the b_3 ion at *m/z* 324.2, confirms the mutation as β64Gly→Asp, Hb J-Calabria.

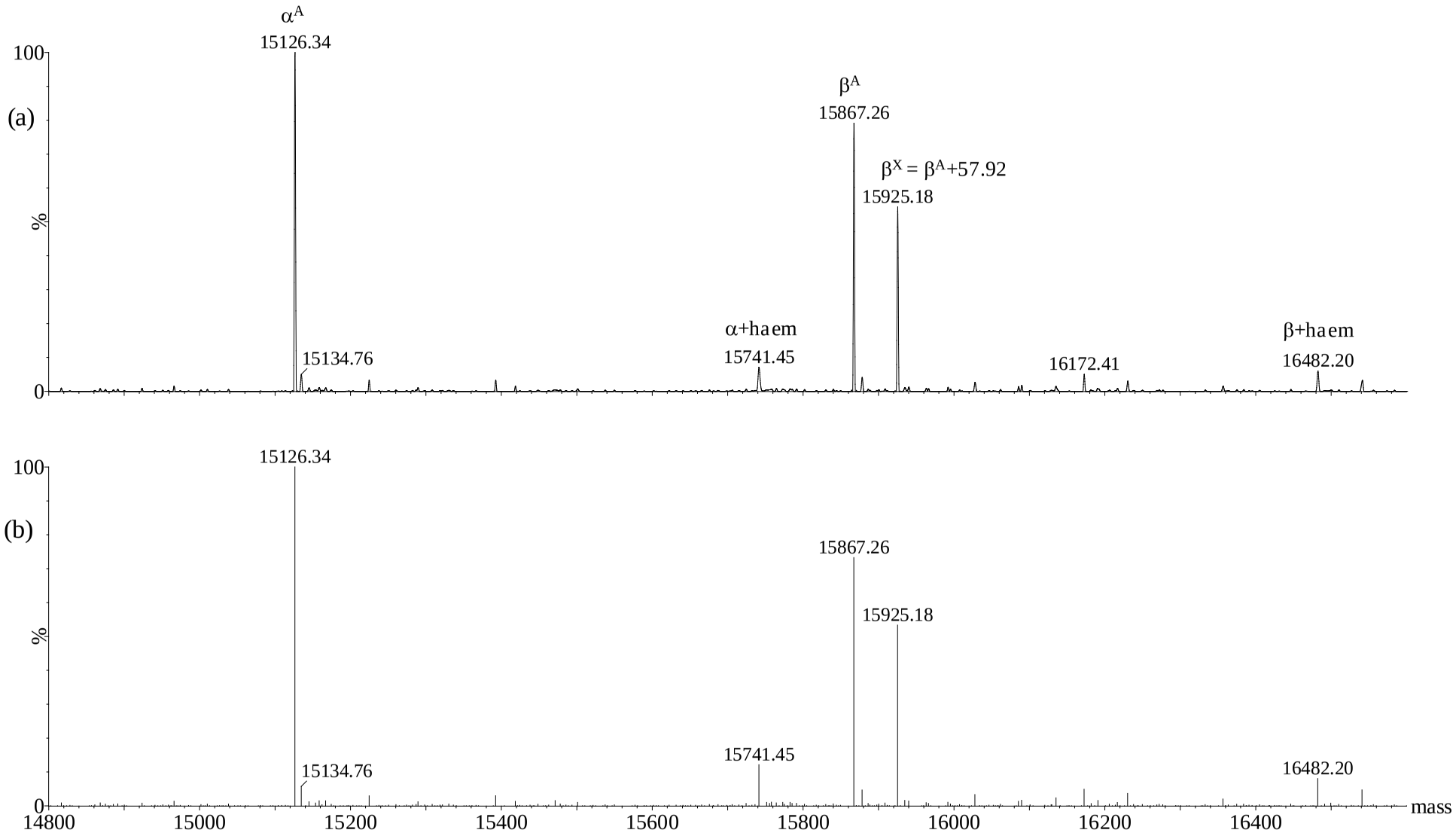

Figure 5.4.18.3. Deconvoluted mass spectrum of Hb J-Calabria (β64Gly→Asp) showing the presence of a signal at 15,925.18 Da at approximately 75% intensity of the normal β-chain peak (15,867.26 Da). The 58 Da mass increase suggests the mutations Ala→Glu or Gly→Asp.

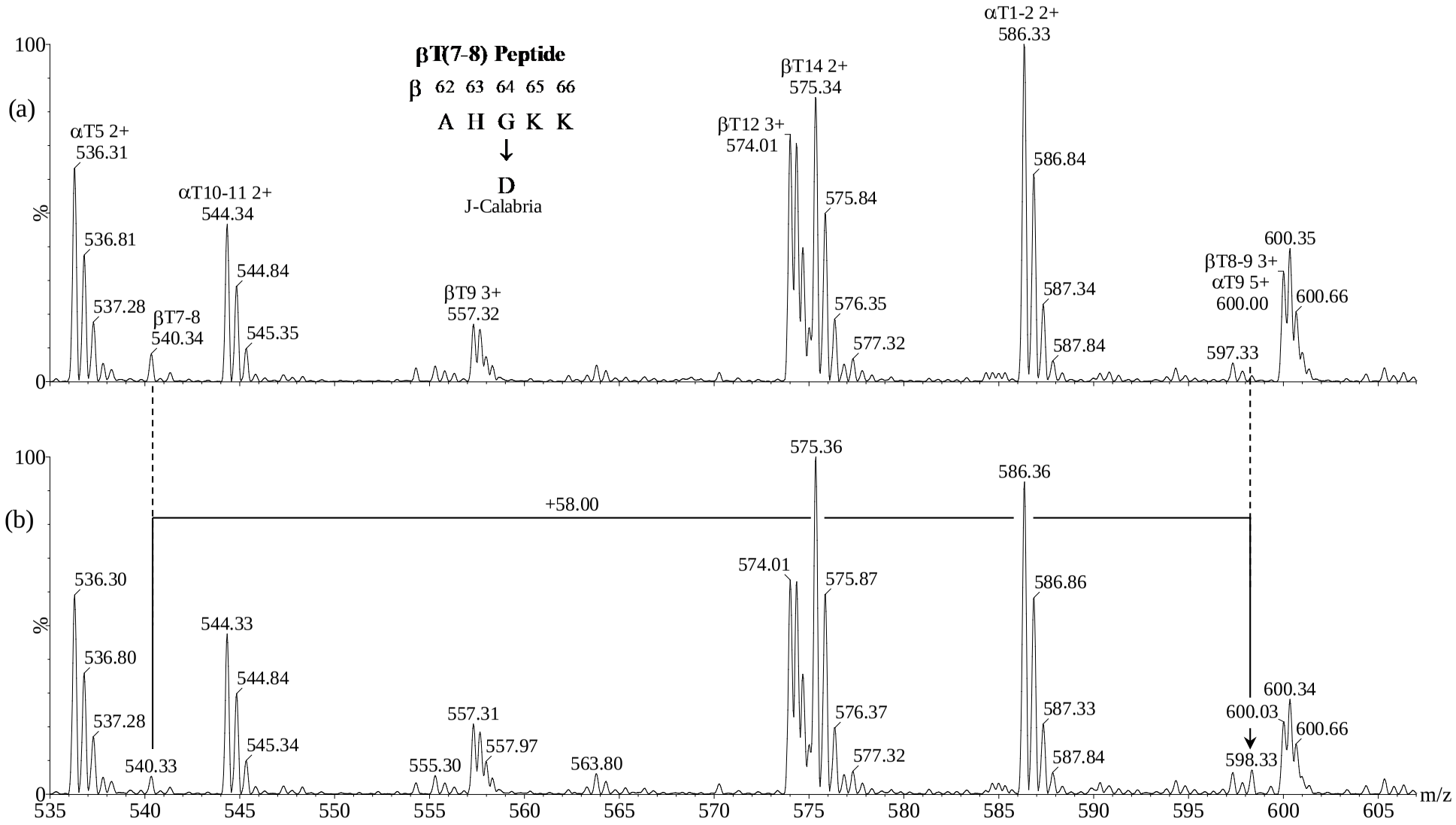

Figure 5.4.18.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb J-Calabria heterozygote. These data identify the mutation as β64Gly→Asp since it is the only mutation that can occur by a single base change in the codon.

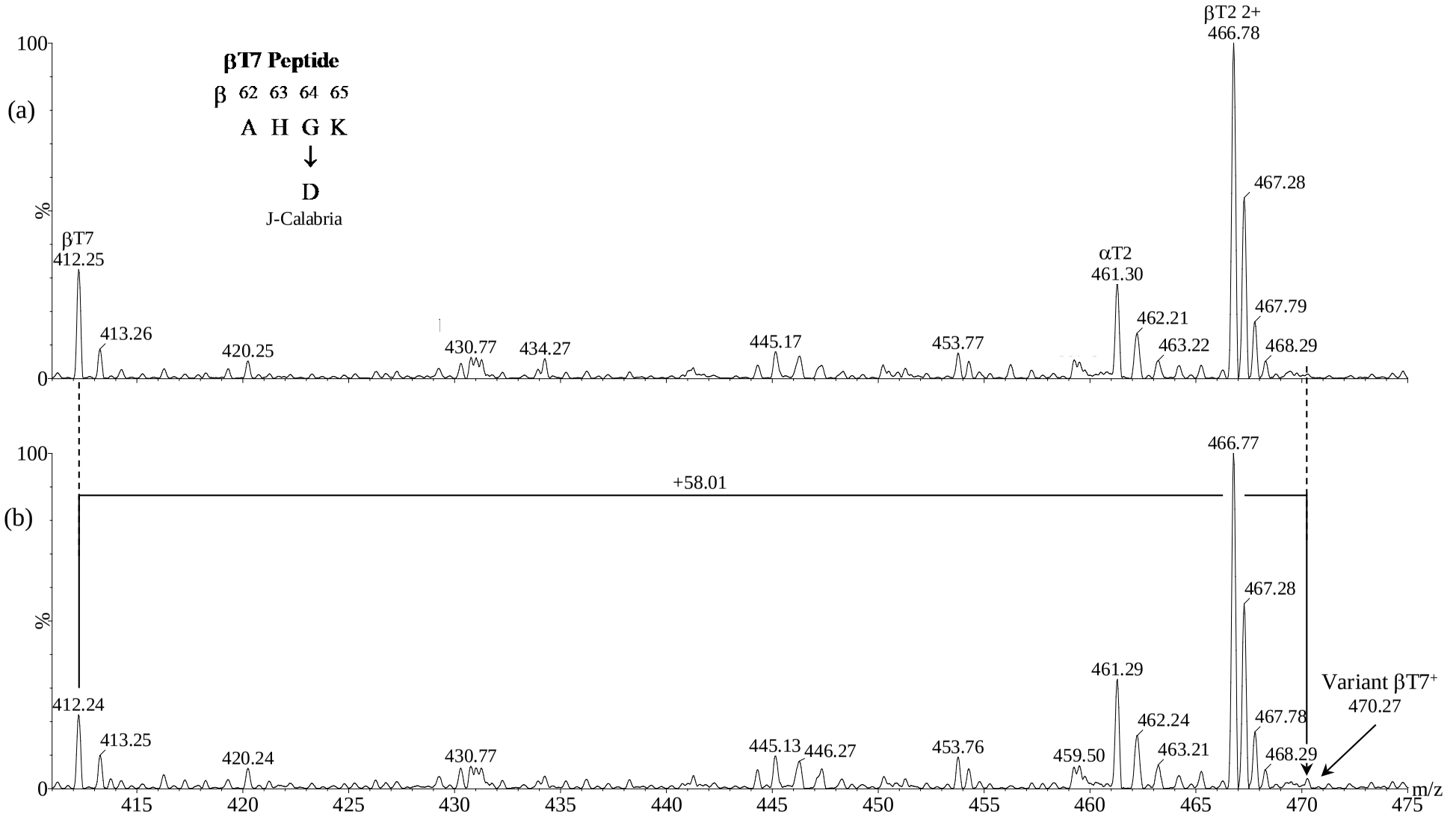

Figure 5.4.18.5. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb J-Calabria heterozygote. These data identify the mutation as β64Gly→Asp since it is the only mutation that can occur by a single base change in the codon.

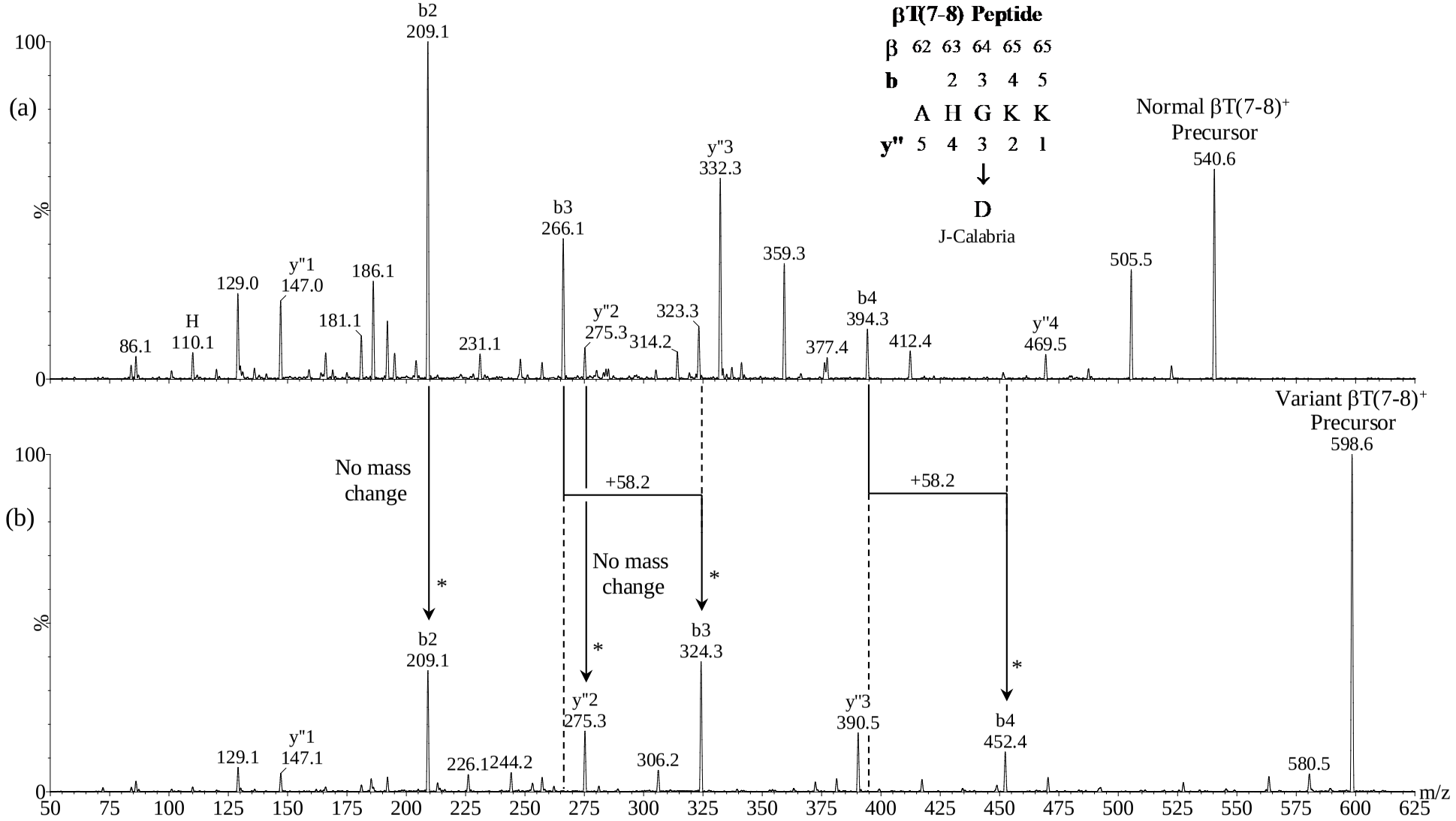

Figure 5.4.18.6. Product ion spectra of the $\beta T(1\text{-}2)^{2+}$ tryptic fragment of (a) normal Hb and (b) Hb J-Calabria. The 58 Da mass increase at b_3 and y''_3 confirms the mutation β64Gly→Asp, Hb J-Calabria.

5.4.19. βT9 - Hb Taradale (β82Lys→Arg)

Hb Taradale is the result of a β-chain mutation in which the β82 amino acid residue is changed from Lys to Arg through a single base change in the codon AAG→AGG.

Although a Lys residue is mutated, the replacement by Arg does not significantly affect the products observed during tryptic digestion.

βT9 Peptide

β	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82
	V	L	G	A	F	S	D	G	L	A	H	L	D	N	L	K
				↓						↓						↓
				V						V						R
				Marineo						Harlequin						Taradale

Figure 5.4.19.1. Sequence of the Hb Taradale βT9 tryptic peptide.

A sample was received of analysis that exhibited an unknown peak in the ce-HPLC trace (Figure 5.4.19.2.) at 2.45 mins and approximately equal intensity to A0. The slight shift in retention time suggests a slight increase in positive charge.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	0.3	---	1.08	4560
Unknown	---	0.3	1.20	5805
Unknown	---	2.2	1.34	37979
P2	---	2.2	1.44	38743
P3	---	3.7	1.79	64381
Unknown	---	42.4	2.45	732157
A0	---	44.7	2.93	772533
A2	1.4	---	3.56	24836

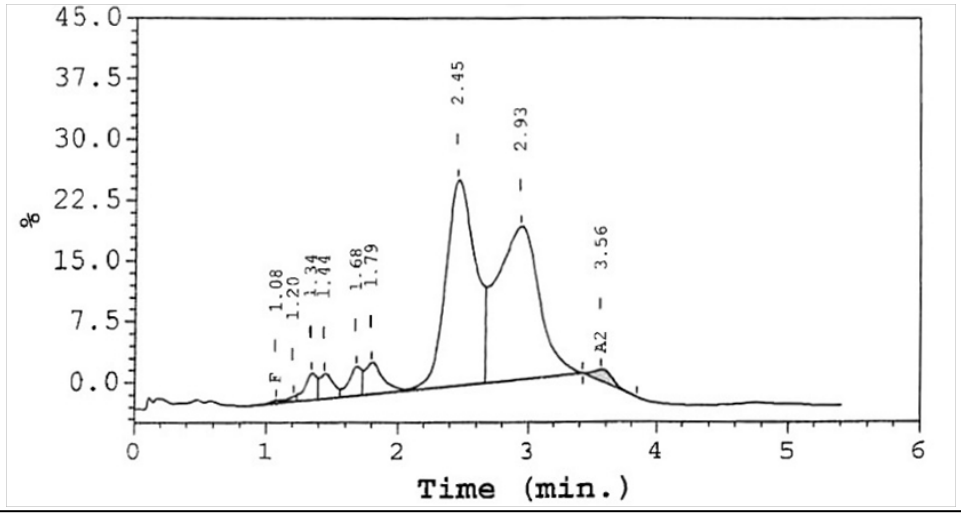

Figure 5.4.19.2. ce-HPLC trace for Hb Taradale.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.4.19.3.) revealed a signal at 15,895.25 Da, 27.96 Da heavier than, and approximately equal intensity to, the normal β-chain. A mass difference of +28 Da implies either Ala→Val (15 possibilities), Gln→Arg (1 possibility) or Lys→Arg (11 possibilities) from a single base change in the codon.

Figure 5.4.19.4. shows a diagnostic part of the mass spectrum of the tryptic digest of (a) normal Hb and (b) the variant Hb. A peak is observed in the lower trace at *m/z* 849.46 (not labelled) for the $\beta T9^{2+}$ variant ion, indicating that the mutation could have taken place at β70Ala→Val (Hb Marineo), β76Ala→Val (Hb Harlequin) or β82Lys→Arg (Hb Taradale). This is further supported by the peak at *m/z* 566.67 for the $\beta T9^{3+}$ tryptic fragment in Figure 5.4.19.5.

Figures 5.4.19.6 and 5.4.19.7. show partial product ion spectra of the $\beta T9^{2+}$ tryptic fragment of (a) normal Hb and (b) the variant Hb. The persistence of the b_1 to b_{14} ions in the variant spectra eliminates the possibility of the mutation being β70Ala→Val or β76Ala→Val. The 28 Da mass increase at y''_1 (*m/z* 175.1, lower panel Figure 5.4.19.6.) and all subsequent y" ions identifies the mutation as β82Lys→Arg, Hb Taradale.

This variant was novel when first analysed by mass spectrometry.

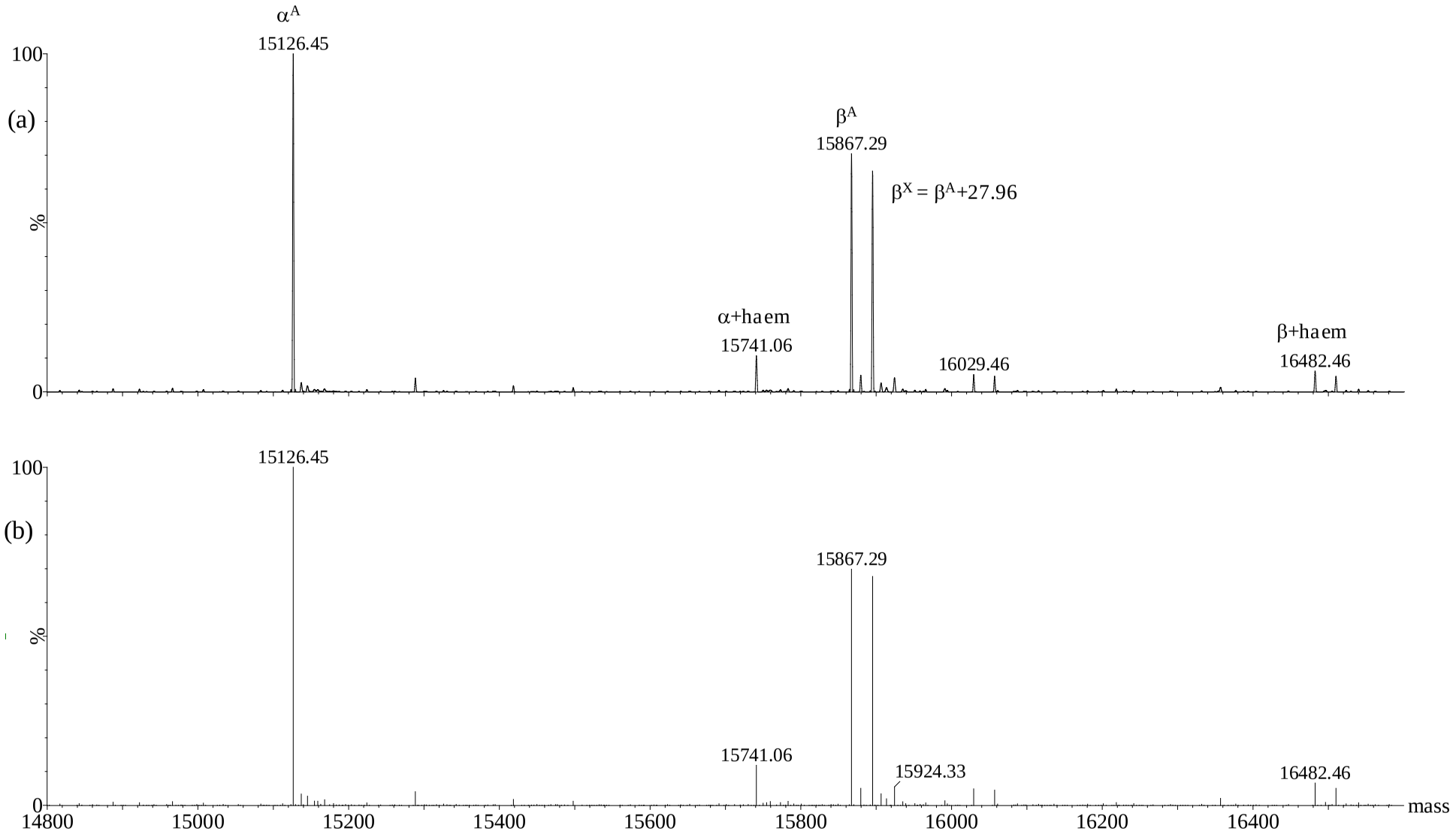

Figure 5.4.19.3. Deconvoluted mass spectrum of Hb Taradale (β82Lys→Arg) showing the presence of a signal at 15,895.26 Da at approximately 95% intensity of the normal β-chain peak (15,867.29 Da).

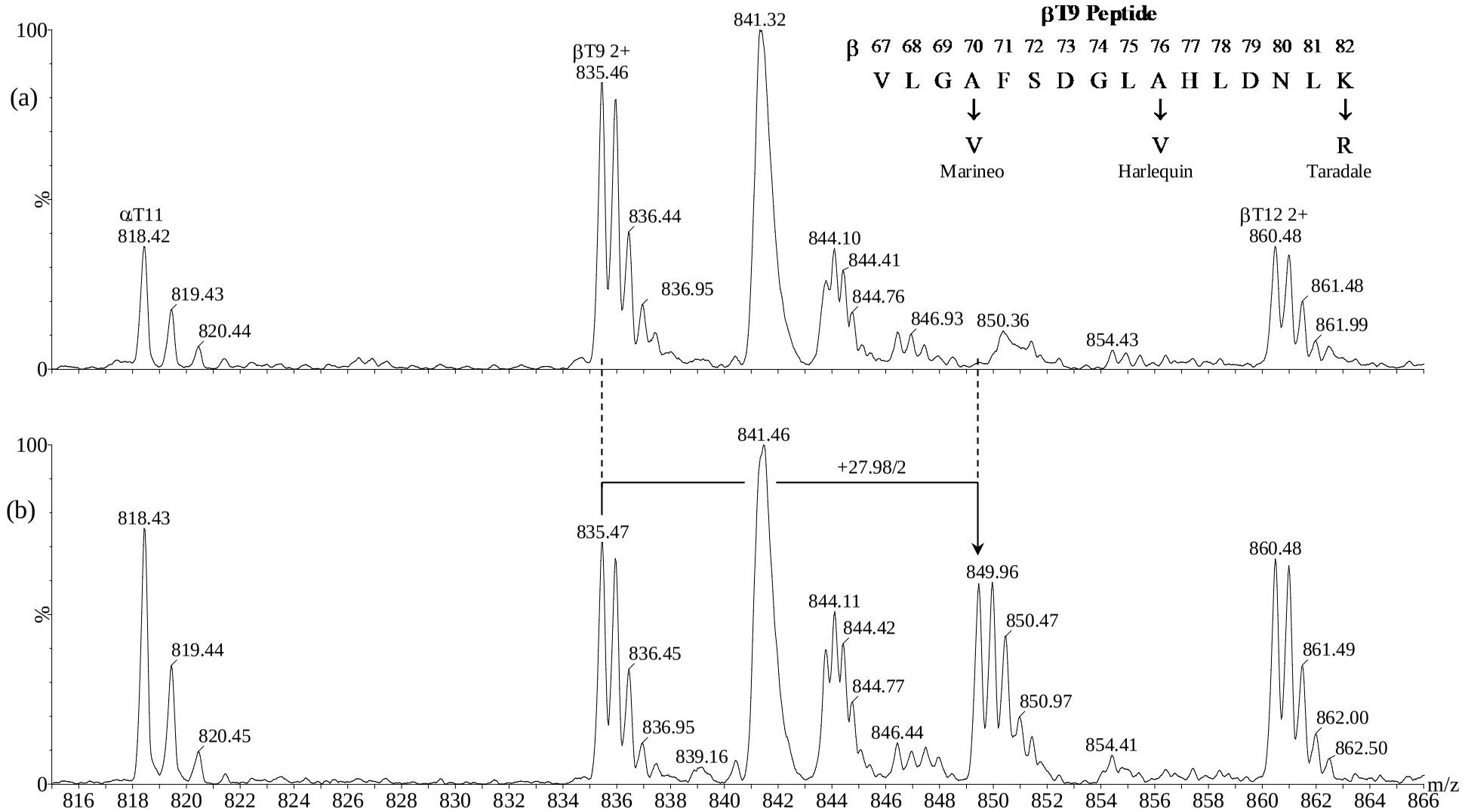

Figure 5.4.19.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Novel, Taradale heterozygote.

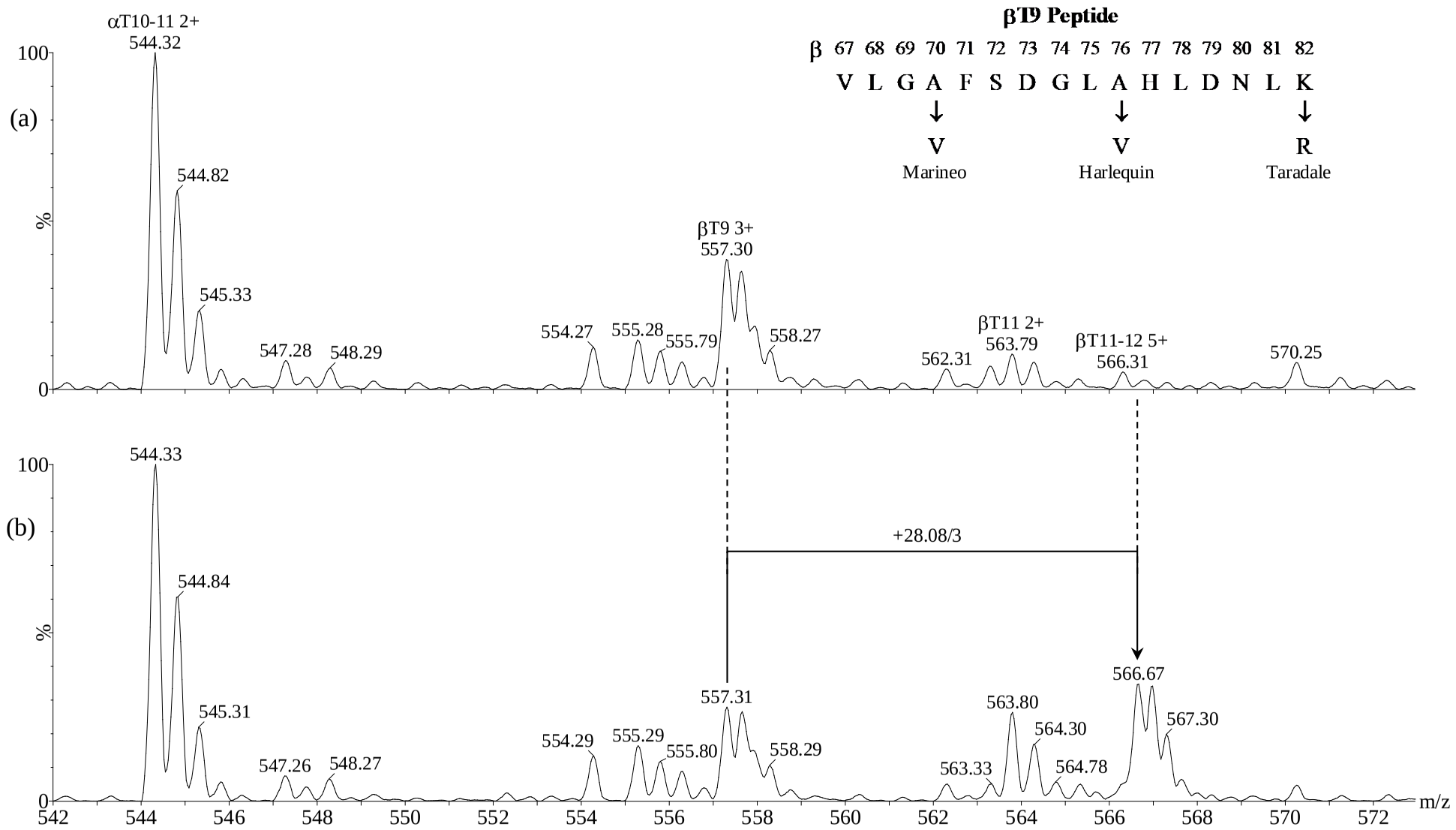

Figure 5.4.19.5. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Novel, Taradale heterozygote.

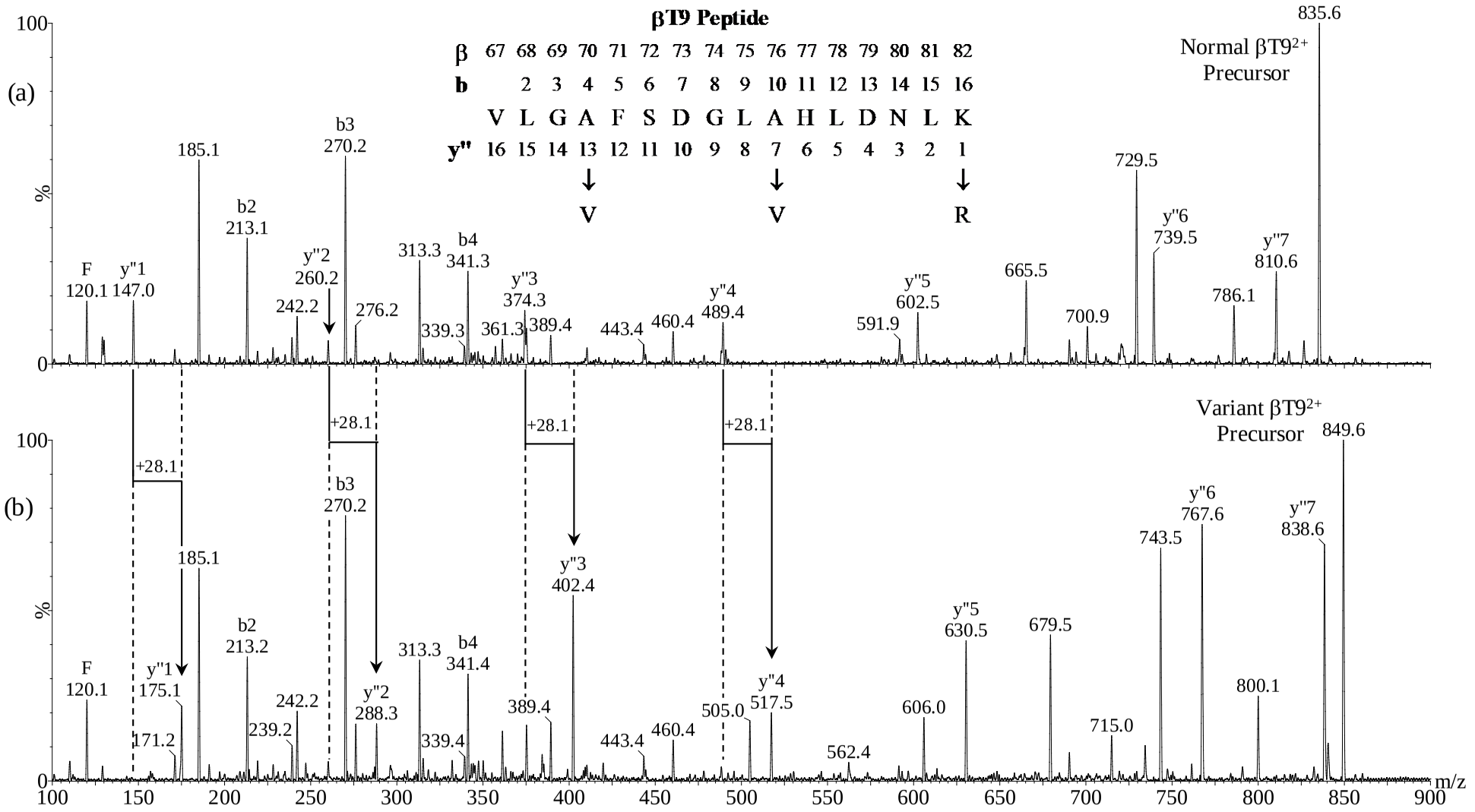

Figure 5.4.19.6. Partial Product ion spectra of the $\beta T9^{2+}$ tryptic fragment of (a) normal Hb and (b) Hb Novel, Taradale. The 28 Da mass increase at y''_1 and all subsequent y" ions identifies the mutation as β82Lys→Arg.

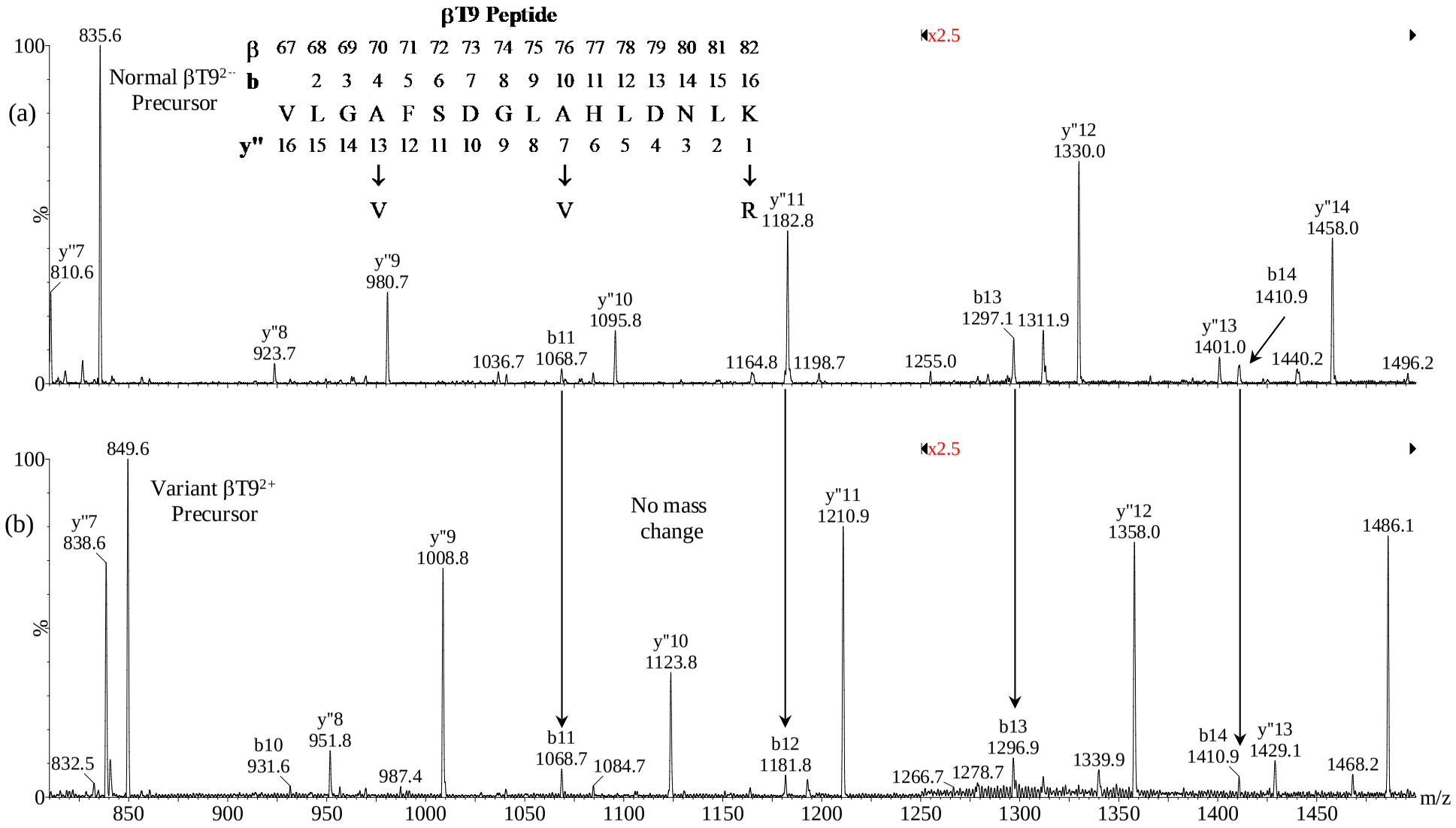

Figure 5.4.19.7. Partial Product ion spectra of the $\beta T9^{2+}$ tryptic fragment of (a) normal Hb and (b) Hb Novel, Taradale (β82Lys→Arg). b_{11} to b_{14} show no mass change, which eliminates β70Ala→Val and β76Ala→Val.

5.4.20. βT20 - Hb Valletta (β87Thr→Pro)

Hb Valletta is the result of a β-chain mutation in which the β87 amino acid residue is changed from Thr to Pro through a single base change in the codon ACA→CCA.

βT(10-11) Peptide

β	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104
	G	T	F	A	T	L	S	E	L	H	C	D	K	L	H	V	D	P	E	N	F	R
		↓			↓																	
		P			P																	
		NL			Valletta																	

Figure 5.4.20.1. Sequence of the Hb Valletta βT(10-11) tryptic peptide.

A sample was received for analysis that showed an essentially normal ce-HPLC trace (Figure 5.4.20.2.).

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	2.6	---	1.12	65408
Unknown	---	1.7	1.24	41532
P2	---	4.2	1.32	104917
P3	---	4.3	1.72	108716
A0	---	84.3	2.49	2119758
A2	2.7	---	3.64	73593

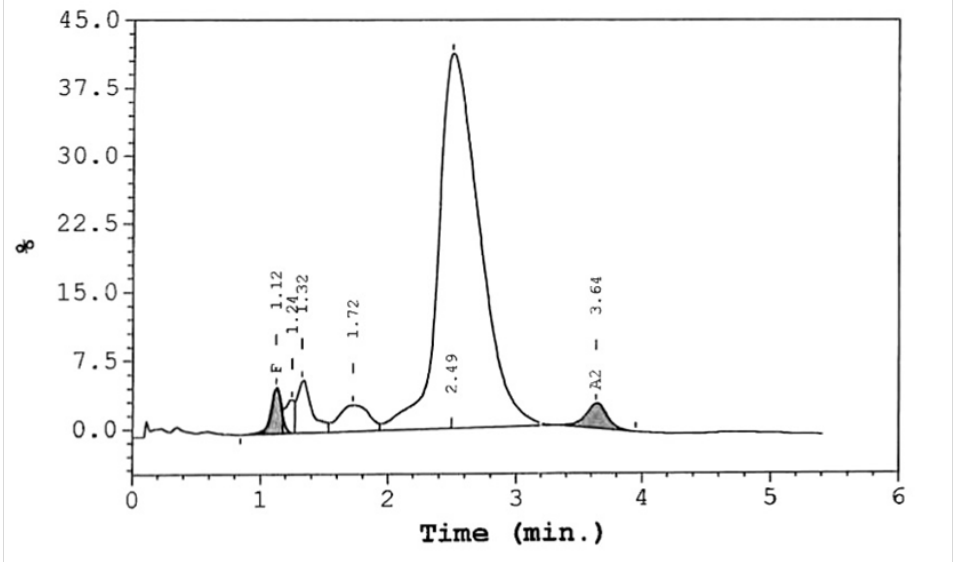

Figure 5.4.20.2. ce-HPLC trace for Hb Valletta.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.4.20.3.) revealed a signal at 15,865.25 Da and implies the presence of a $\beta^A/(\beta^A\text{-}4)$ heterozygote. A mass difference of -4 Da implies Thr→Pro (7 possibilities) mutation from a single base change in the codon.

Figure 5.4.20.4. shows the diagnostic region of the tryptic digest spectrum for (a) normal Hb and (b) the variant Hb. The peak at *m/z* 1,263.06 in the lower panel indicates that the mutation has occurred in the combined $\beta(10\text{-}11)^{2+}$ tryptic fragment, giving the possible sites of mutation as β84Thr→Pro (not previously reported) or β87Thr→Pro (Hb Valletta). There is also supportive evidence of the appearance of the $\beta(10\text{-}11)^{3+}$ at *m/z* 842.45 in the lower panel of Figure 5.4.20.5.

Figure 5.4.20.6. shows the diagnostic part of the product ion spectra of (a) normal Hb and (b) the variant Hb of the $\beta(10\text{-}11)^{2+}$ tryptic fragment with the mass shift of -4Da observed in the lower panel for the $y''_{18}{}^{2+}$ (*m/*z 1,075.4) and $y''_{19}{}^{2+}$ (*m/z* 1,110.9) fragments. The high relative intensity of the $y''_{18}{}^{2+}$ ion is consistent with the facile cleaving at the N-terminal side of the 'new' Proline and identifies the mutation as β87Thr→Pro, Hb Valletta.

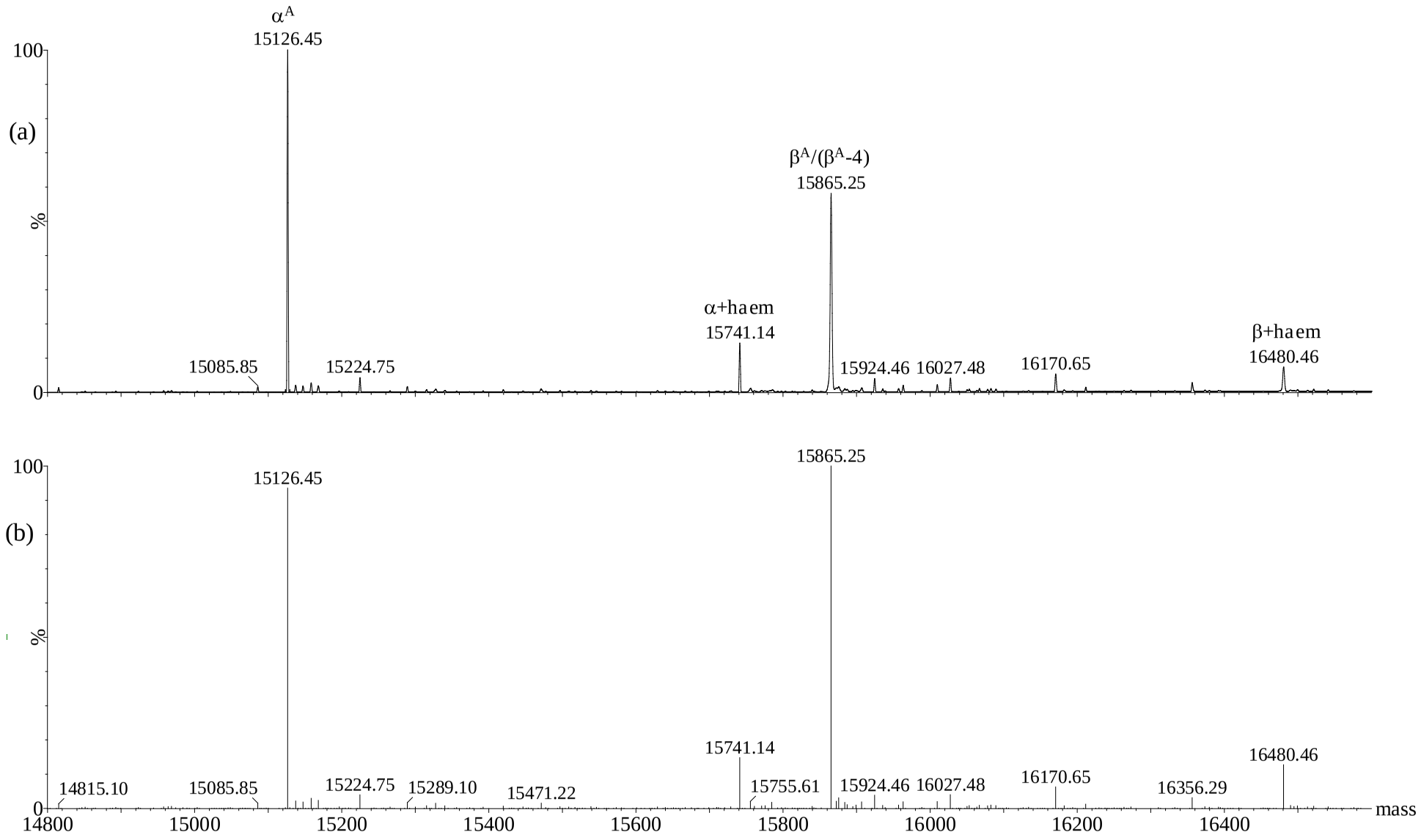

Figure 5.4.20.3. Deconvoluted mass spectrum of Hb Valletta (β87Thr→Pro) showing the presence of a signal at 15,865.25 Da. The (β^A-2.06) is consistent with a (β^A-4)/β^A heterozygote.

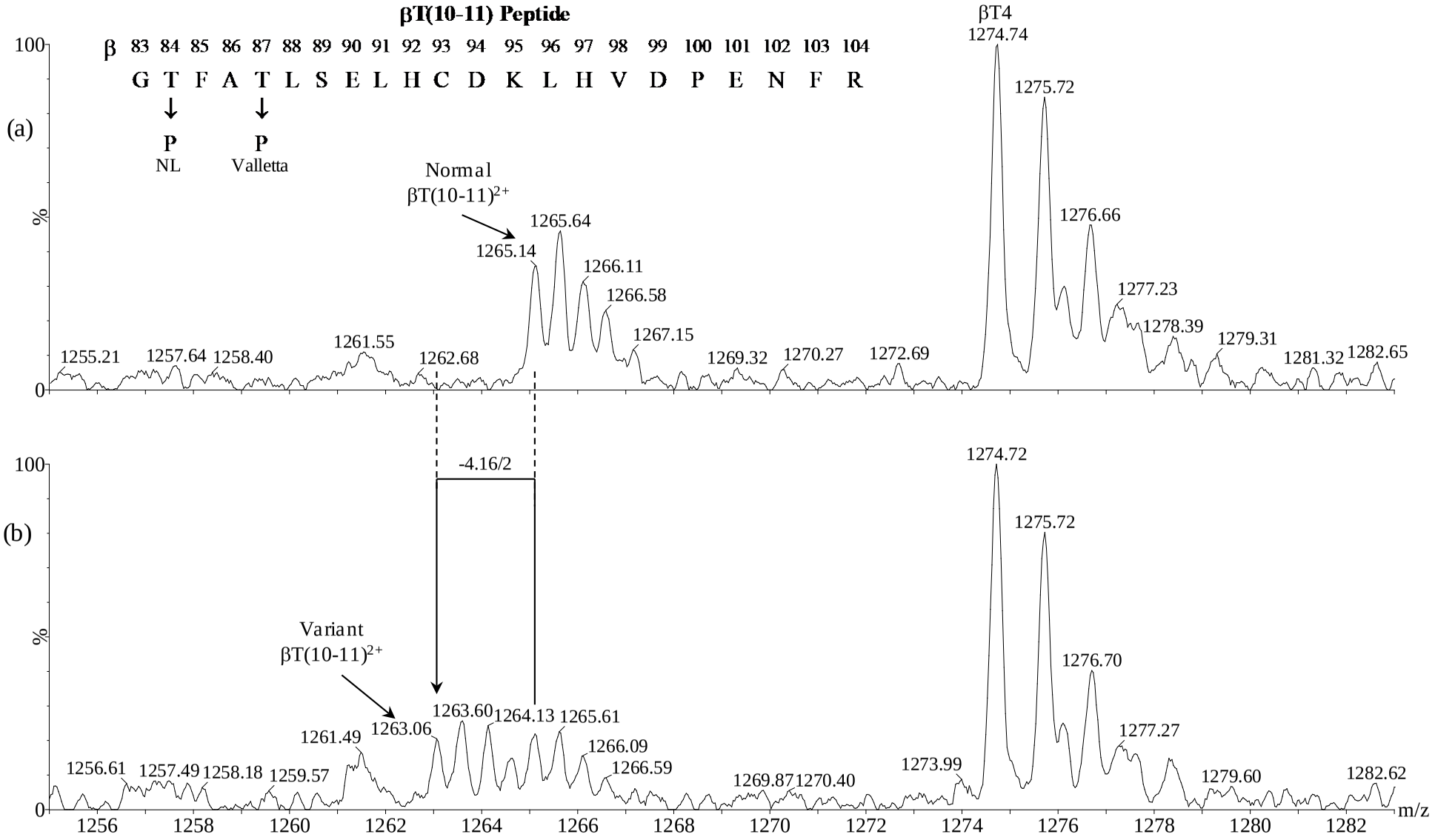

Figure 5.4.20.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Valletta heterozygote. There are two possible 4 Da mass decreases possible in the β(10-11) tryptic peptide, both of which can occur by a single base change in the codon.

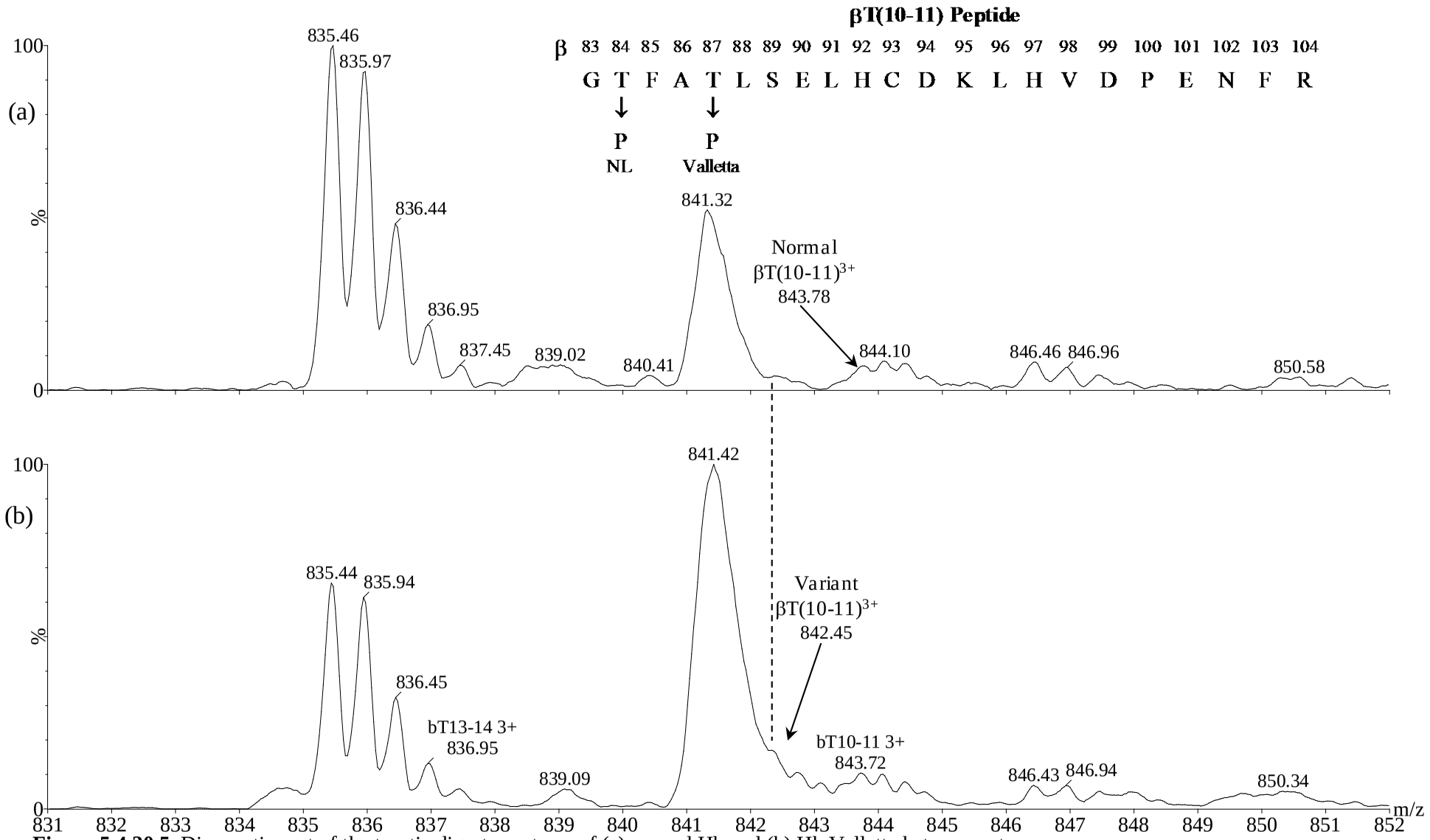

Figure 5.4.20.5. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Valletta heterozygote.

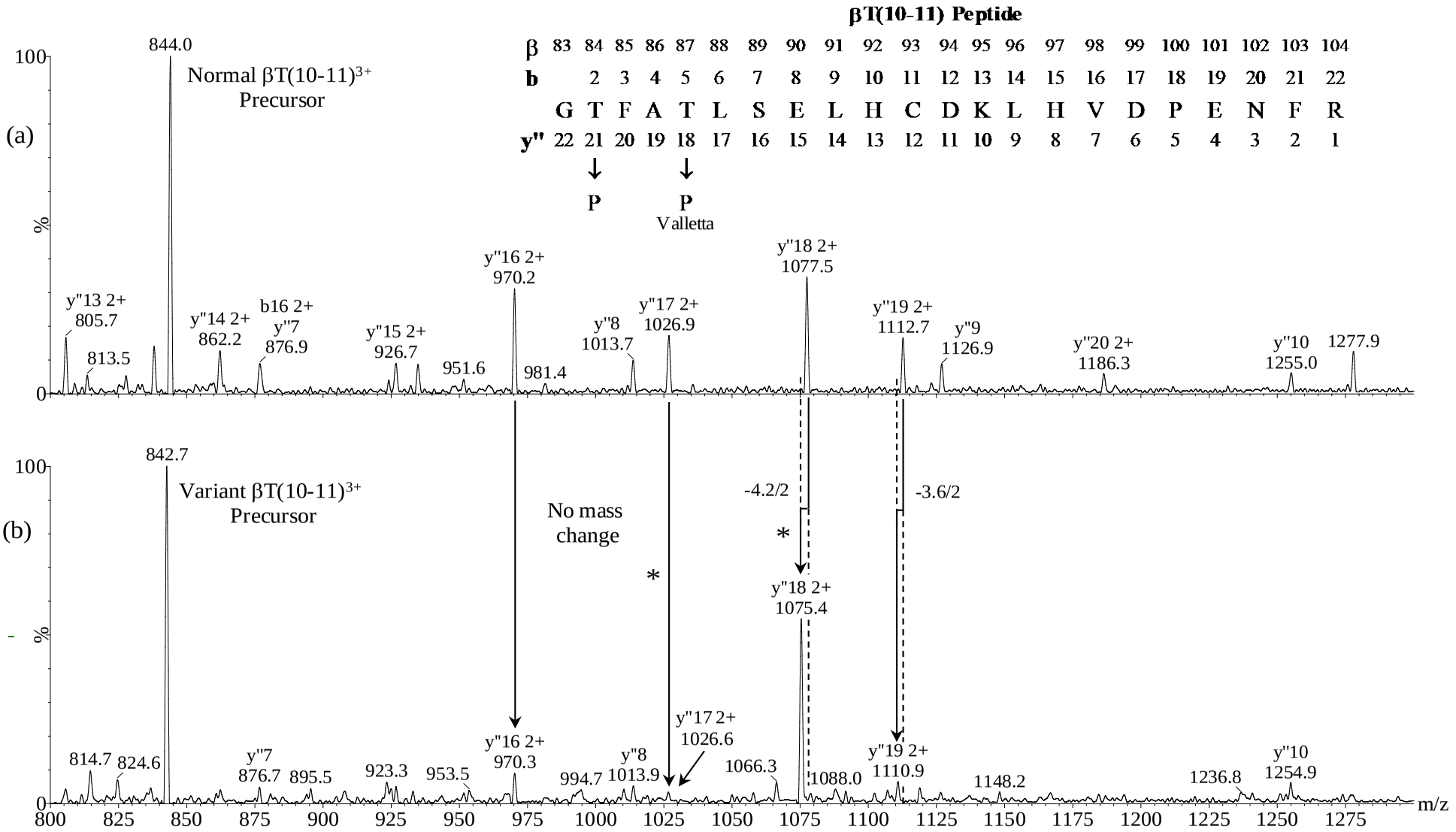

Figure 5.4.20.6. Partial Product ion spectra of the βT(10-11)$^{2+}$ tryptic fragment of (a) normal Hb and (b) Hb Valletta (β87Thr→Pro). The 4 Da mass decrease at y"$_{18}^{2+}$ identifies the mutation as β87Thr→Pro, Hb Valletta. The high relative intensity of the y"$_{18}^{2+}$ ion is consistent with the facile cleaving at the N-terminal sides of the 'new' Proline.

5.4.21. βT21 - Hb Köln (β98Val→Met)

Hb Köln is the result of a β-chain mutation in which the β amino acid residue is changed from Val to Met through a single base change in the codon GTG→ATG.

βT(10-11) Peptide

β	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104
	G	T	F	A	T	L	S	E	L	H	C	D	K	L	H	V	D	P	E	N	F	R
																↓						
																M						

Figure 5.4.21.1. Sequence of the Hb Köln βT(10-11) tryptic peptide.

A blood sample was submitted for analysis by mass spectrometry because the ce-HPLC trace (Figure 5.4.21.2.) showed a response (9.3%) in the C-window at 4.94 min.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	0.5	---	1.10	4327
Unknown	---	0.4	1.24	3431
P2	---	1.0	1.32	9803
P3	---	1.6	1.67	14861
A0	---	83.8	2.55	792613
A2	3.5	---	3.63	33084
C-window	---	9.3	4.94	87990

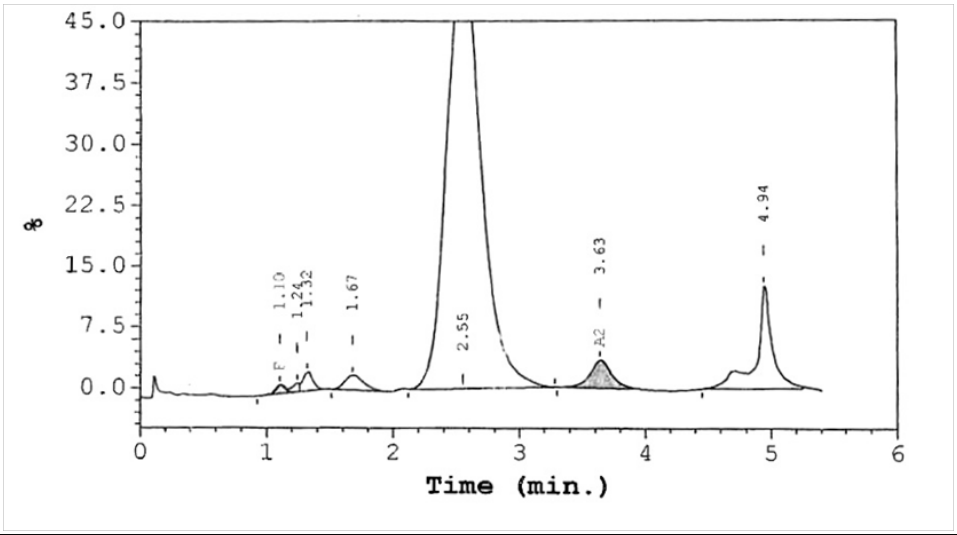

Figure 5.4.21.2. ce-HPLC trace for Hb Köln.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.4.21.3.) revealed a signal at 15,899.25 Da, 31.90 Da heavier than a normal β-chain. This infers a mutation Val→Met from a single base change in the codon, of which there are 13 possibilities. The variant signal is approximately 50% the intensity of the normal β-chain.

Figure 5.4.21.4. shows the diagnostic region of the tryptic digest spectrum for (a) normal Hb and (b) the variant Hb. The peak at *m/z* 1,281.15 (not labelled) in the lower panel indicates that the mutation has occurred in the combined $\beta(10\text{-}11)^{2+}$ tryptic fragment, giving the possible site of mutation as β98Val→Met (Hb Köln). There is also supportive evidence with the appearance of the $\beta(10\text{-}11)^{3+}$ ion at *m/z* 854.43 in the lower panel of Figure 5.4.21.5.

Figures 5.4.21.6. shows the diagnostic part of the product ion spectra of (a) normal Hb and (b) the variant Hb of the $\beta(10\text{-}11)^{3+}$ tryptic fragment with no mass change observed for the y''_5 (*m/z* 662.5) and y''_6 (*m/z* 777.4) ions, but a mass shift of +32 Da for the y''_7 (*m/z* 908.5) and higher fragments. Supporting evidence is also observed in Figure 5.4.21.7. where a 32 Da mass increase in the b_{16} fragment (*m/z* 1,784.4) is observed. These data identify the mutation as β98Val→Met, Hb Köln.

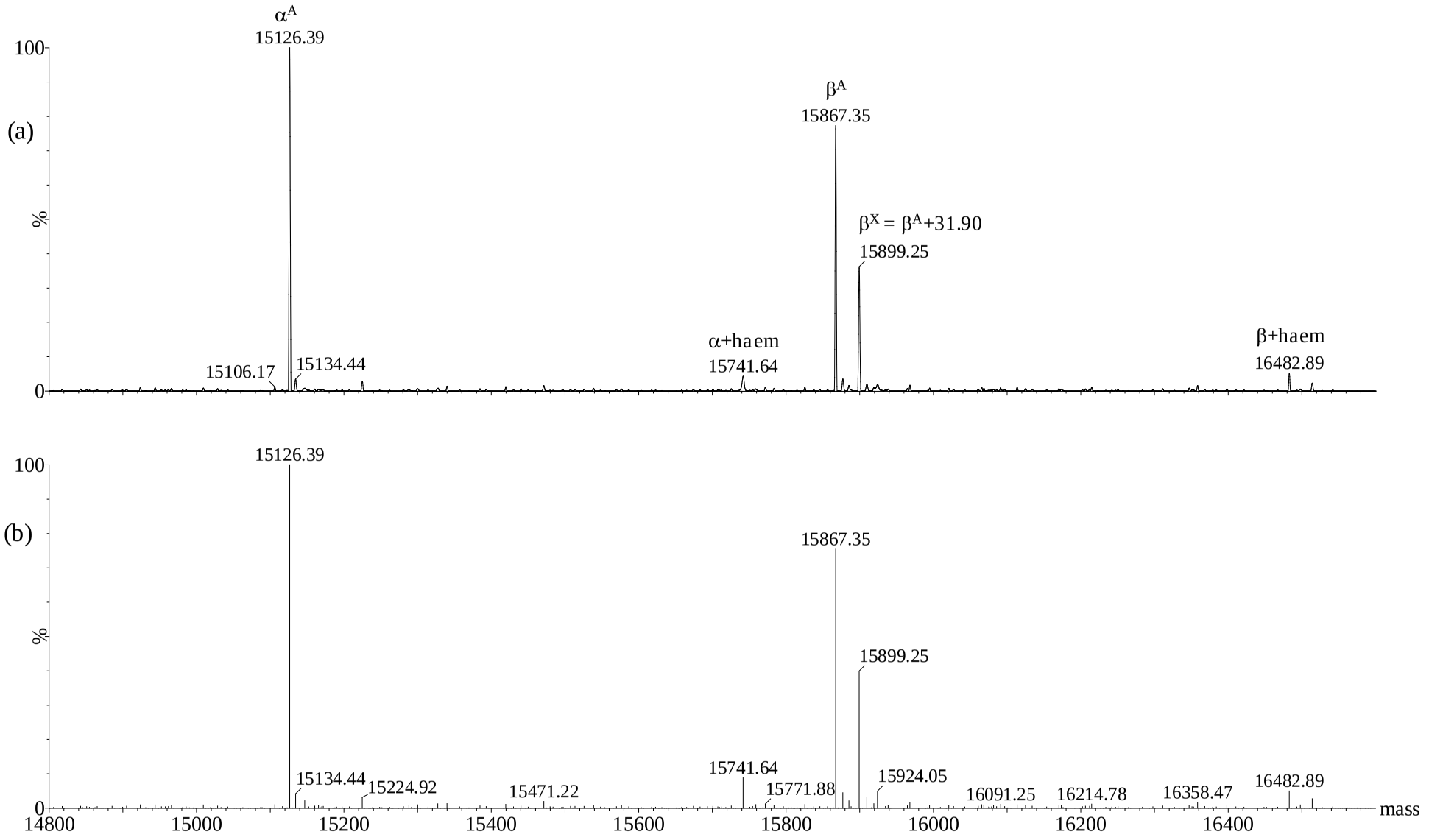

Figure 5.4.21.3. Deconvoluted mass spectrum of Hb Köln (β98Val→Met) showing the presence of a signal at 15,899.25 Da at approximately 55% intensity of the normal β-chain peak (15,867.35 Da). The 32 Da mass increase is only possible for a Val→Met single mutation.

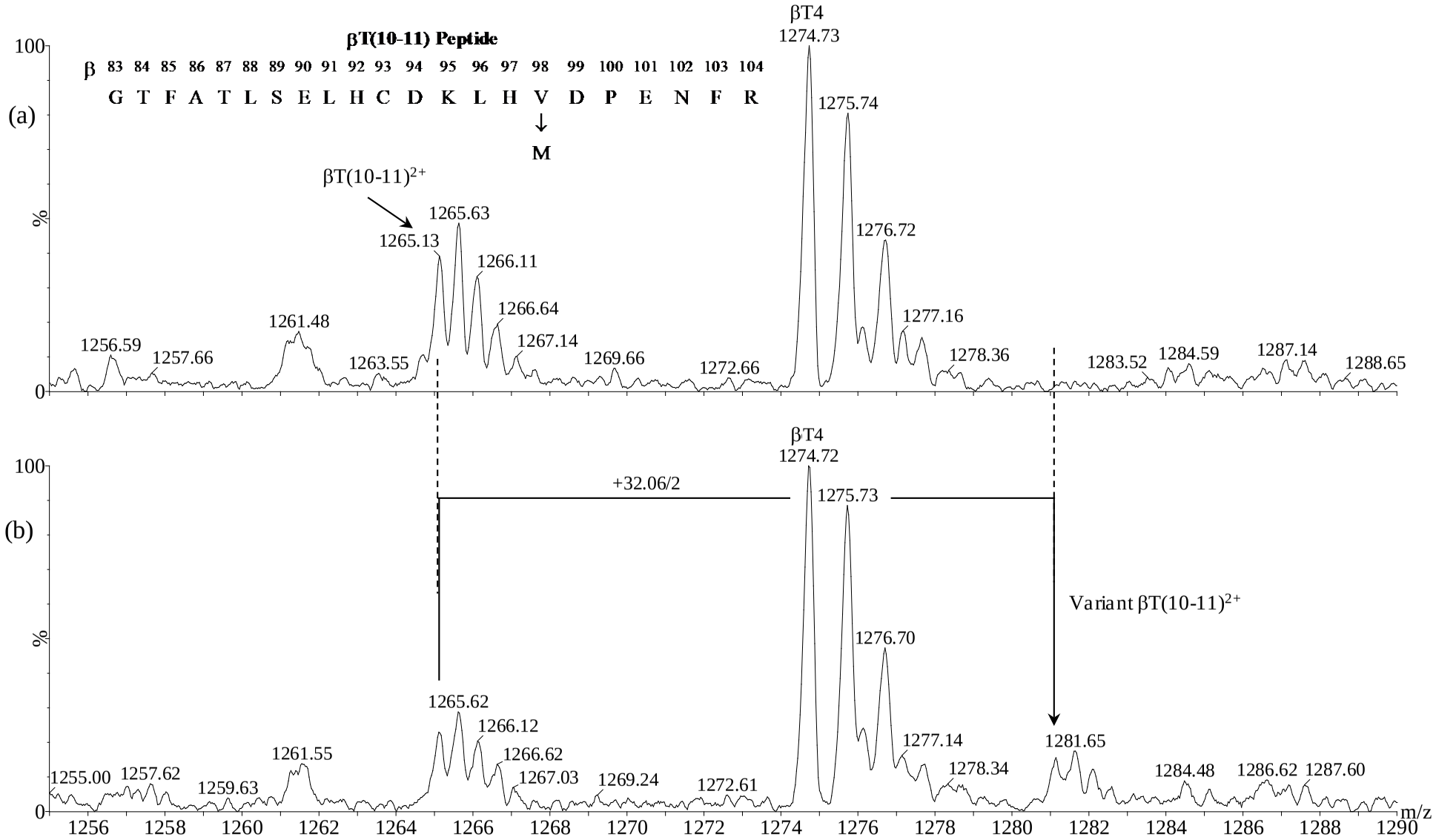

Figure 5.4.21.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Köln heterozygote.

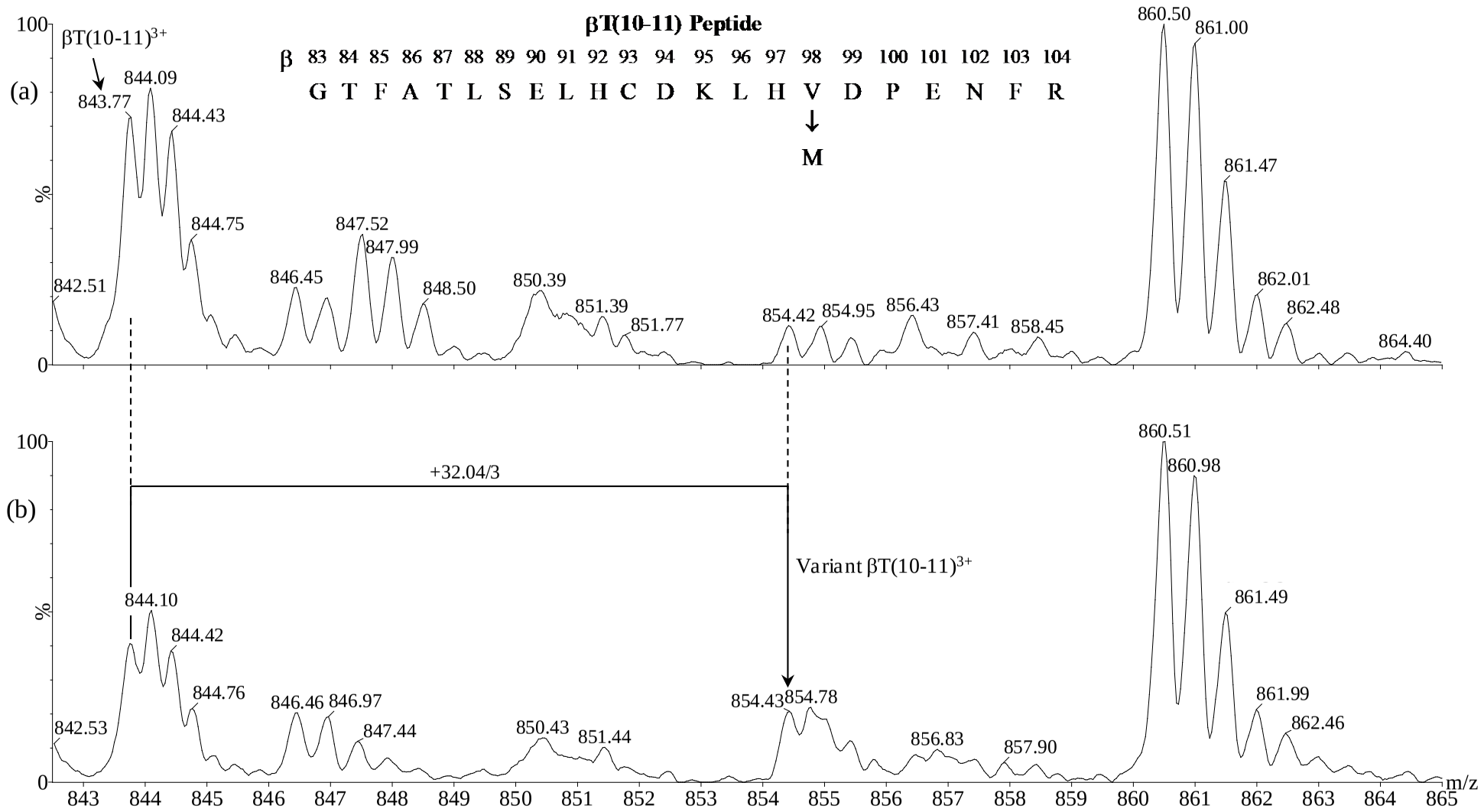

Figure 5.4.21.5. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Köln heterozygote.

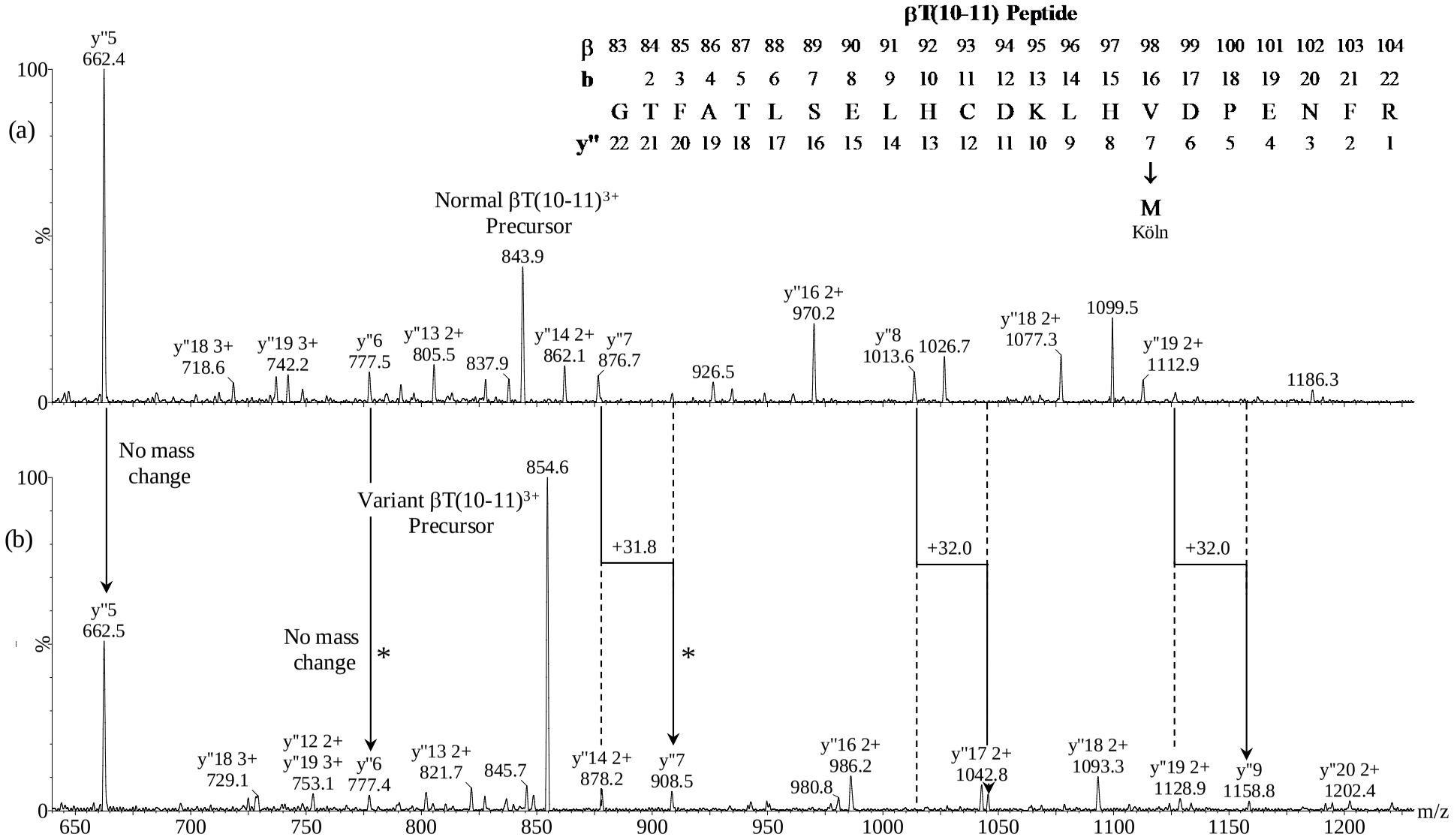

Figure 5.4.21.6. Partial Product ion spectra of the βT(10-11)$^{3+}$ tryptic fragment of (a) normal Hb and (b) Hb Köln. The 32 Da mass increase at y"$_7$ confirms the mutation as β98Val→Met.

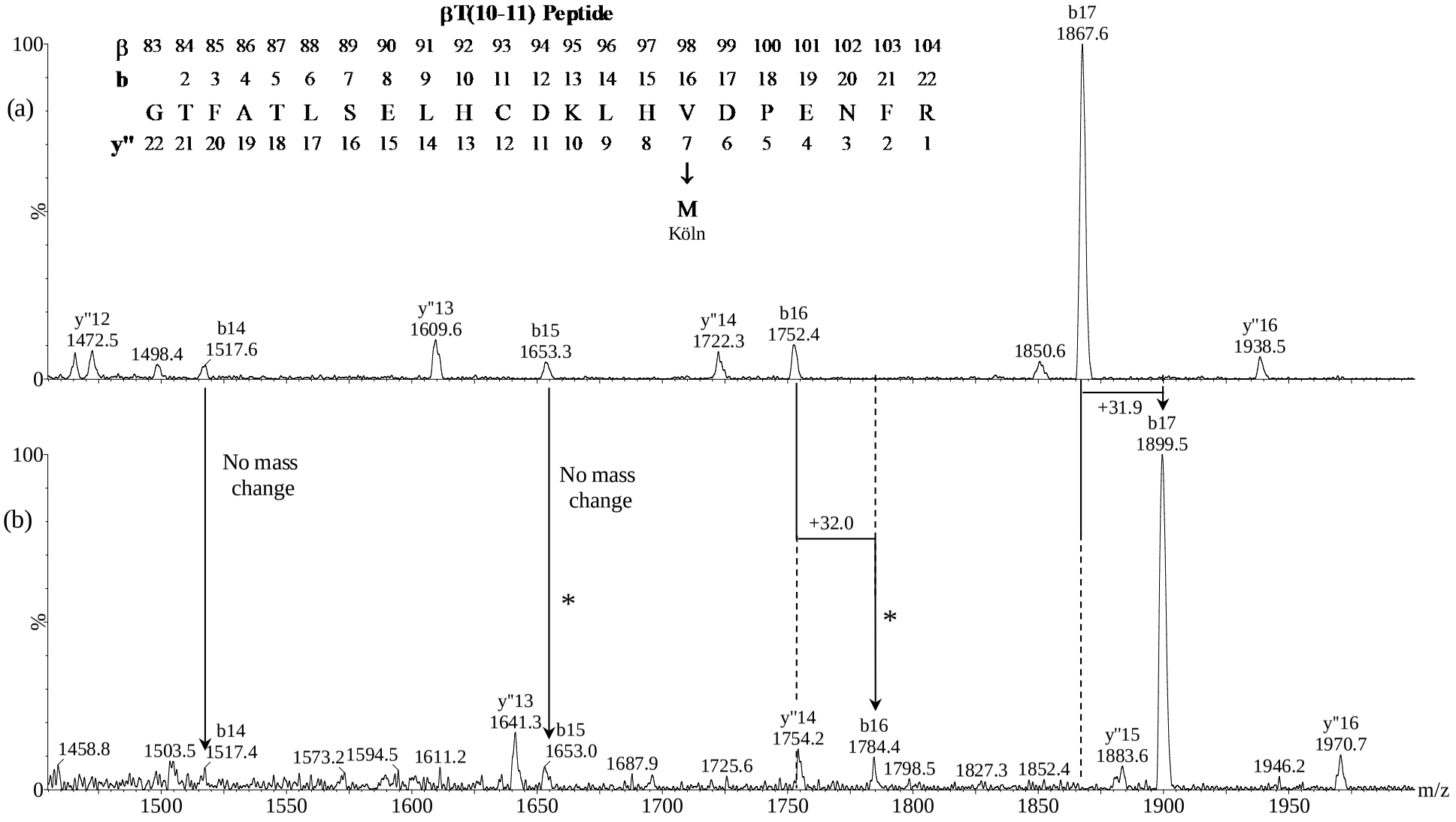

Figure 5.4.21.7. Partial Product ion spectra of the βT(10-11)$^{3+}$ tryptic fragment of (a) normal Hb and (b) Hb Köln. The 32 Da mass increase at b_{16} confirms the mutation as β98Val→Met.

5.4.22. βT21 - Hb Alzette (β104Arg→Lys)

Hb Alzette is the result of a β-chain mutation in which the β104 amino acid residue is changed from Arg to Lys through a single base change in the codon AGG→AAG.

The replacement of Arg by Lys will not affect the formation of tryptic digest fragments.

βT(10-11) Peptide

β	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104
	G	T	F	A	T	L	S	E	L	H	C	D	K	L	H	V	D	P	E	N	F	R
																↓						↓
																A						K
																Djelfa						Alzette

Figure 5.4.22.1. Sequence of the Hb Alzette βT(10-11) tryptic peptide.

A sample was received for analysis in which an abnormal peak was observed in the ce-HPLC trace (Figure 5.4.22.2) slightly before A_0 at 2.30min, suggesting no charge change.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.1	0.97	1264
F	0.9	---	1.10	17686
Unknown	---	2.4	1.23	51849
P2	---	2.1	1.36	46152
P3	---	4.4	1.70	96373
Unknown	---	42.5	2.30	920930
A0	---	44.7	2.47	969916
A2	2.7	---	3.67	63539

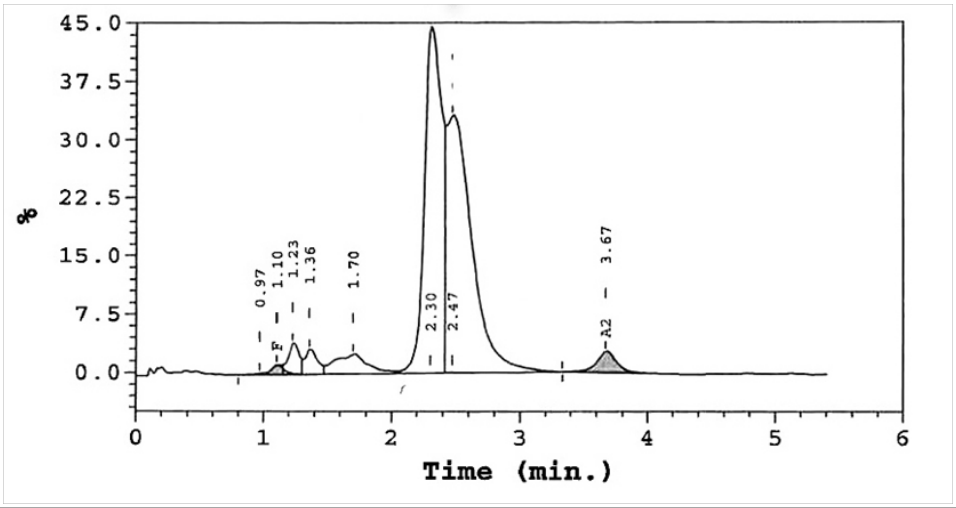

Figure 5.4.22.2. ce-HPLC trace for Hb Alzette.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.4.22.3.) revealed a signal at 15,839.27 Da, 27.96 Da lighter than, and approximately equal intensity to, the normal β-chain. A mass difference of -28 Da implies either Val→Ala (13 possibilities), Arg→Gln (3 possibilities) or Arg→Lys (3 possibilities) from a single base change in the codon.

Figure 5.4.22.4. shows the diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) the variant Hb. The presence of a peak consistent with $βT(10\text{-}11)^{3+}$ tryptic fragment in the lower panel eliminates the possibility of the β104Arg→Gln mutation, leaving β98Arg→Gln (Hb Djelfa) or

β104Arg→Lys (Hb Alzette) as the possibilities. The presence of a peak appearing at *m/z* 1,251.19 (not labelled) as the $\beta T(10\text{-}11)^{2+}$ variant in the lower panel of Figure 5.4.22.5. is also consistent with this hypothesis.

Figure 5.4.22.6. shows the partial product ion spectrum of the $\beta T(10\text{-}11)^{2+}$ for (a) normal Hb and (b) the variant Hb, The 28 Da mass decrease in the y''_1 (*m/z* 147.1) and other y" fragments identifies the mutation as β104Arg→Lys, Hb Alzette.

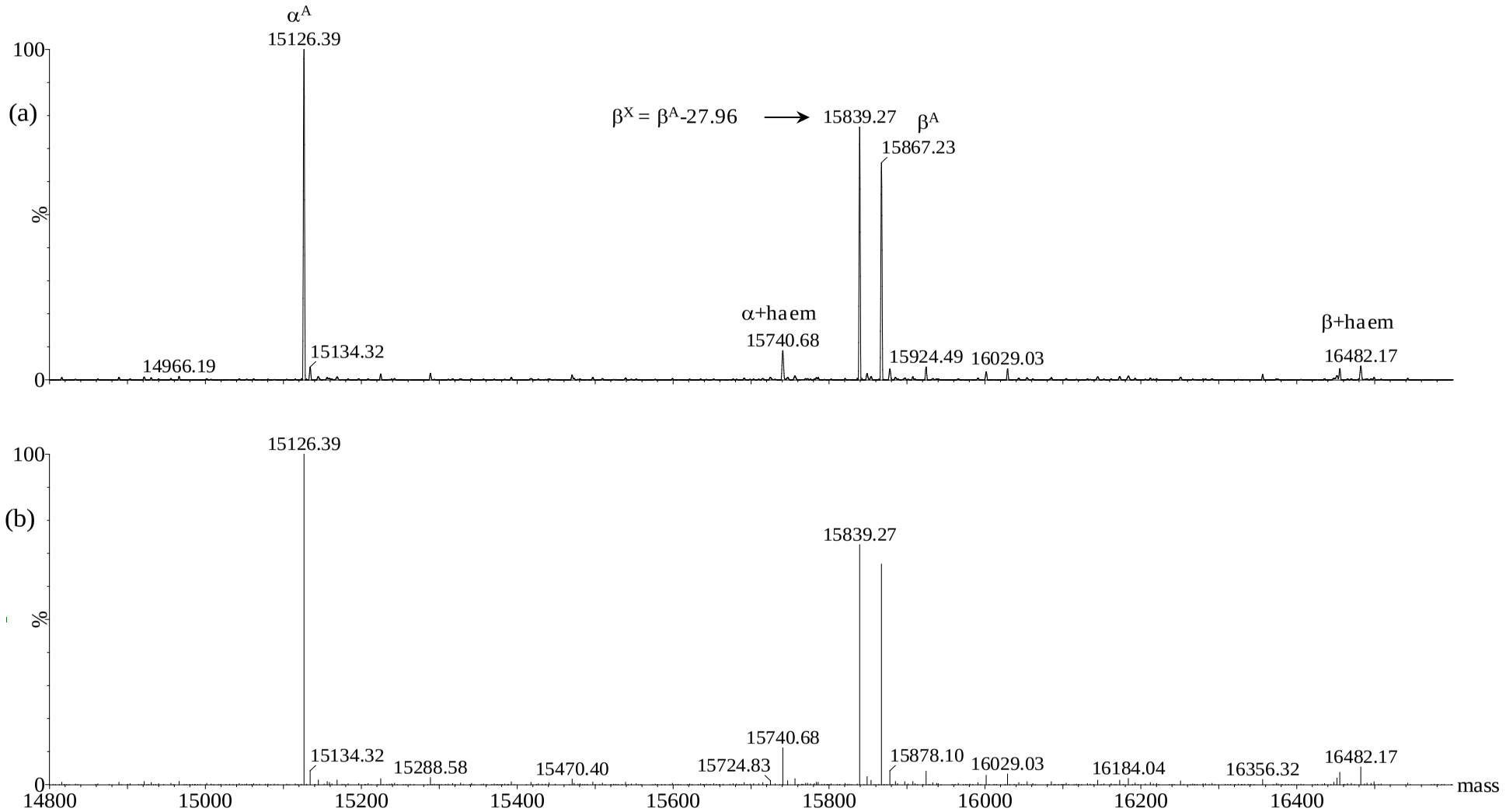

Figure 5.4.22.3. Deconvoluted mass spectrum of Hb Alzette (β104Arg→Lys) showing the presence of a signal at 15,839.27 Da at approximately equal intensity of the normal β-chain peak (15,867.23 Da). For a decrease in mass of 28 Da and neutral in respect to charge, mutation is either Val→Ala or Arg→Lys.

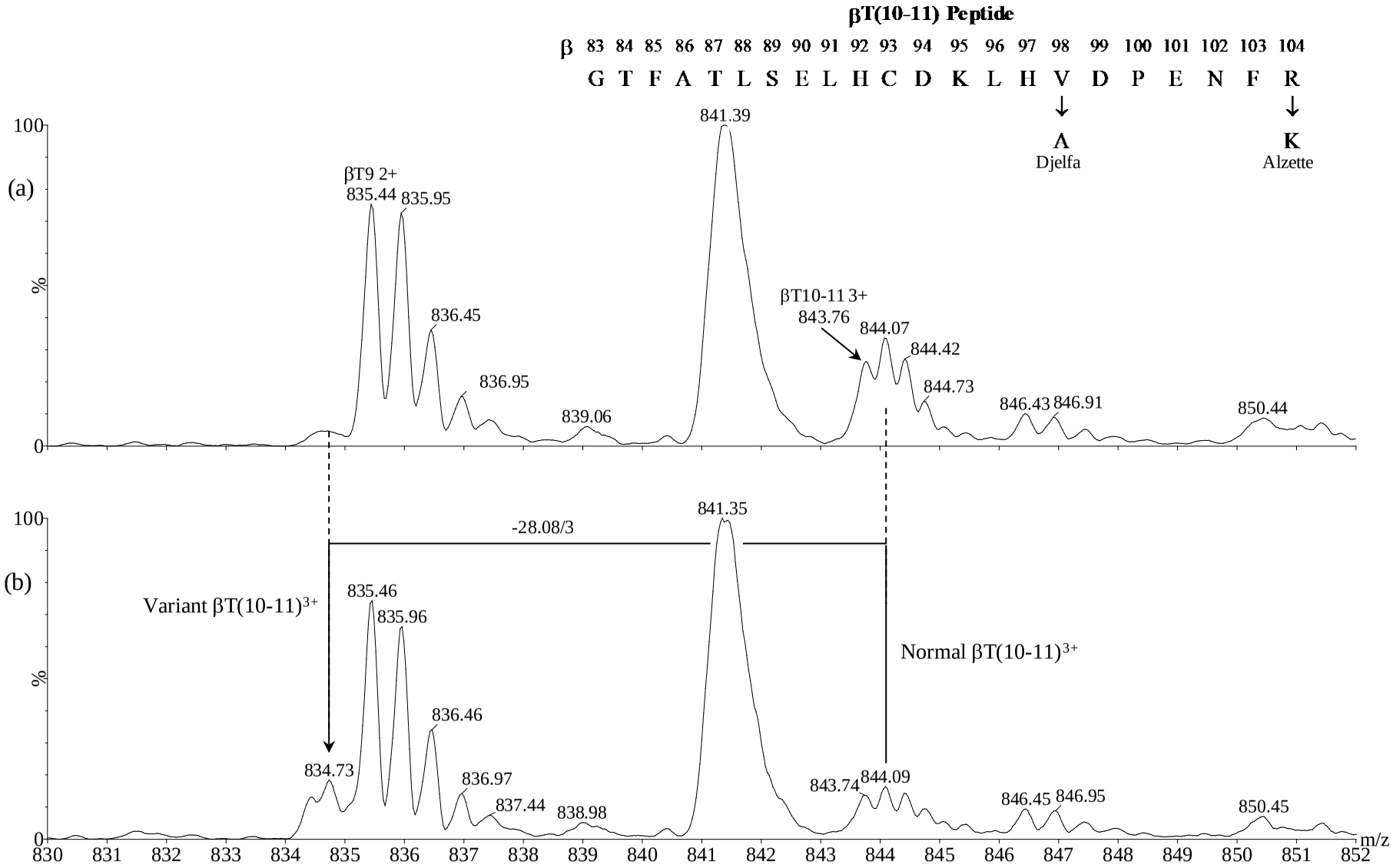

Figure 5.4.22.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Alzette heterozygote.

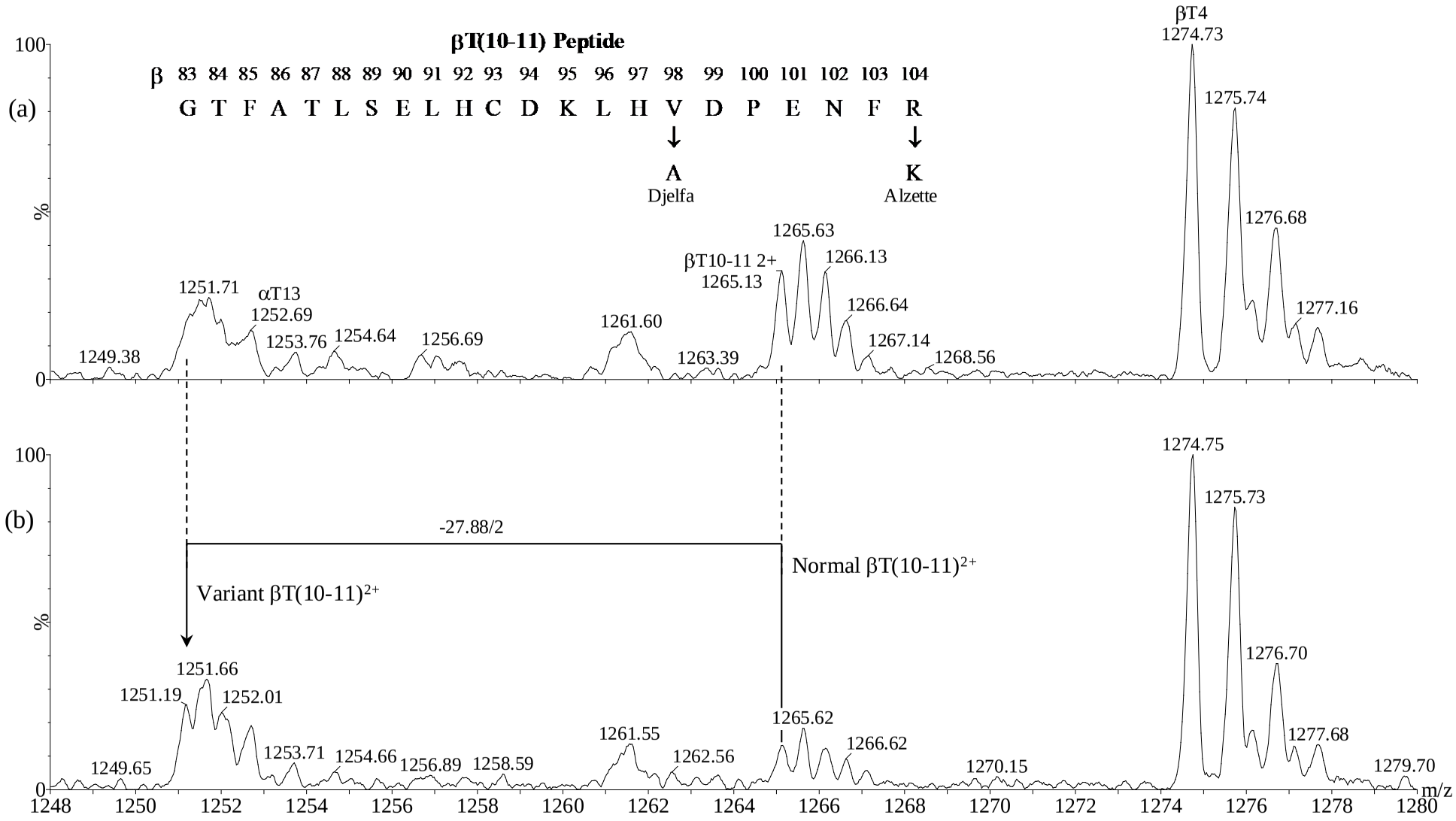

Figure 5.4.22.5. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) Hb Alzette heterozygote.

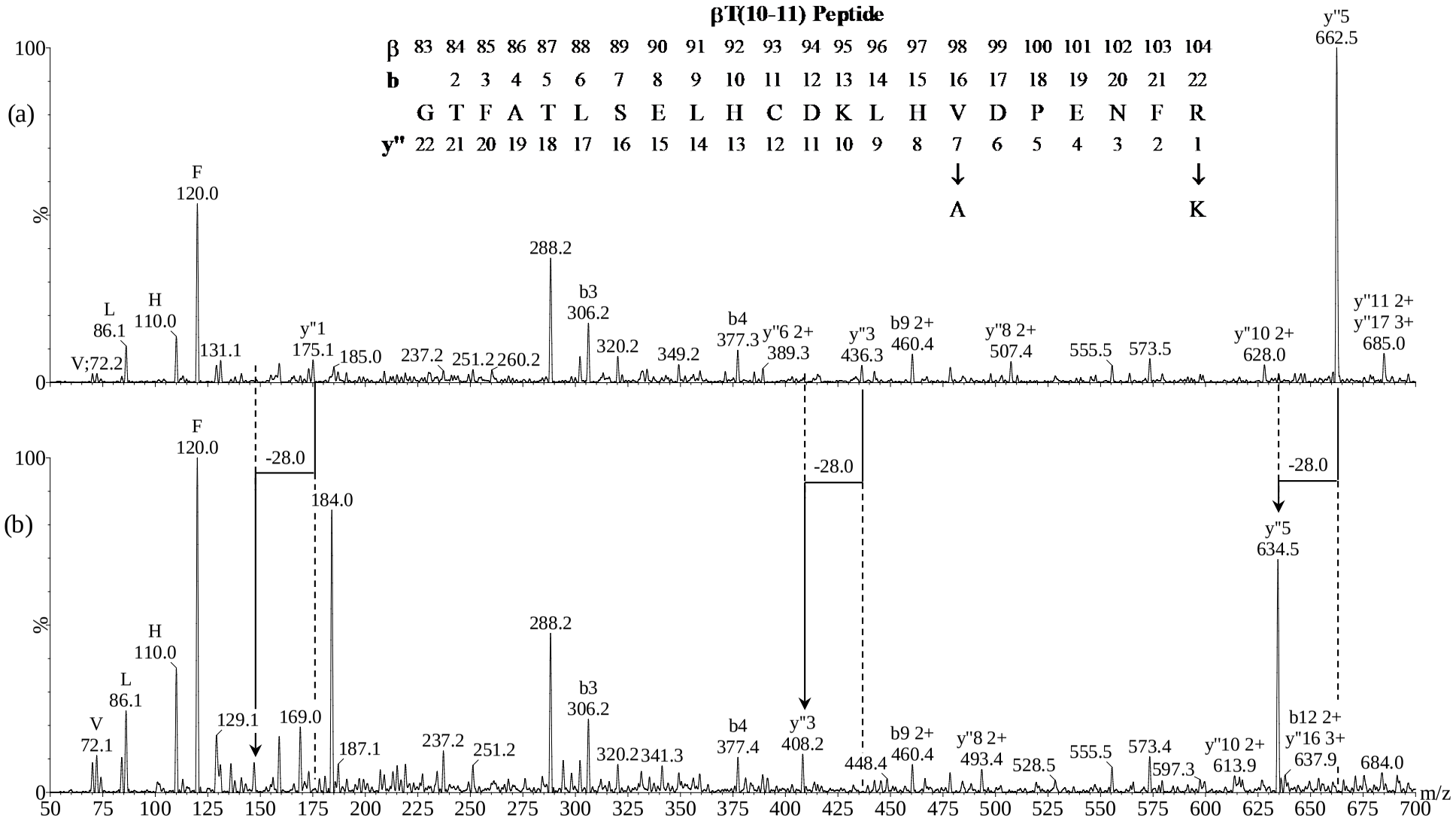

Figure 5.4.22.6. Partial product ion mass spectra of the βT(10-11)$^{2+}$ tryptic fragment of (a) normal Hb and (b) the Hb Alzette heterozygote. The 28 Da mass decrease at y"$_1$ confirms the mutation β104Arg→Lys.

5.4.23. βT22 - Hb Southampton (β106Leu→Pro)

Hb Southampton is the result of a β-chain mutation in which the β106 amino acid residue is changed from Leu to Pro through a single base change in the codon CTG→CCG.

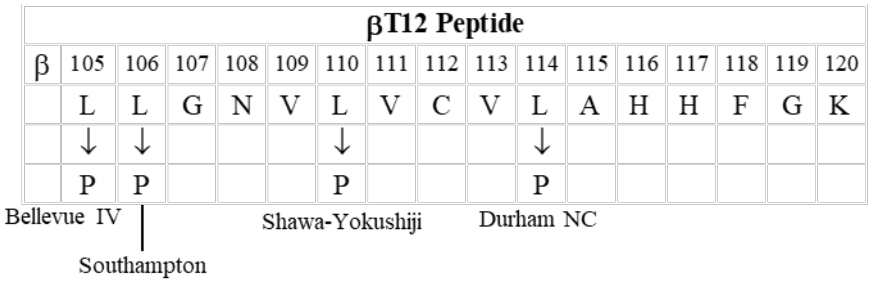

Figure 5.4.23.1. Sequence of the Hb Southampton βT12 tryptic peptide.

There is no ce-HPLC trace available for Hb Southampton. The sample was sent for investigation by mass spectrometry owing to an abnormality observed in the $^{A}\gamma/\,^{G}\gamma$ measurement for possible hereditary persistence of foetal haemoglobin.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.4.23.2.) revealed a signal at 15,850.86 Da, 16.37 Da lighter than, and approximately 20% of the intensity of, the normal β-chain. The low intensity of the variant suggests that the protein is unstable, and a mass difference of -16 Da implies either Asp→Val (7 possibilities), Cys→Ser (2 possibilities), Ser→Ala (5 possibilities), Tyr→Phe (3 possibilities) or Leu→Pro (18 possibilities) from a single base change in the codon.

Figure 5.4.23.3. shows a partial product ion spectrum of the $(M+16H)^{16+}$ precursor of (a) normal Hb and (b) the variant Hb. The b_{47}^{4+} fragment at *m/z* 1,308.3 shows no mass change, and indicates that the mutation does not lie between β1 and β47, and no mass change in the $y''_{23}{}^{4+}$ ion at *m/z* 811.7 indicates that the variant does not lie between β124 and β146. The mass difference of -16 Da in the $y''_{96}{}^{10+}$ ion at *m/z* 1,032.7, indicates that the mutation has occurred between β51 and β123, inclusive.

Figure 5.4.23.4. shows the diagnostic region of the tryptic digest spectrum for (a) normal Hb and (b) the variant Hb. The appearance of a peak in the lower trace at *m/z* 587.79 is consistent with the mutation of the $\beta T12^{3+}$ (β105-120), with all the possible mutations in this fragment Leu→Pro; β105 (Hb Bellevue IV), β106 (Hb Southampton), β110 (Hb Shawa-Yokushiji) and β114 (Hb Durham NC).

Figure 5.4.23.5. shows the partial product ion spectra of the $\beta T12^{3+}$ tryptic fragment for (a) normal Hb and (b) the variant Hb. For the variant, there is no mass change in any of the y'' ions until the y''_{15} fragment with a mass decrease of 16 Da observed at *m/z* 824.7, confirming the mutation as β106Leu→Pro, Hb Southampton.

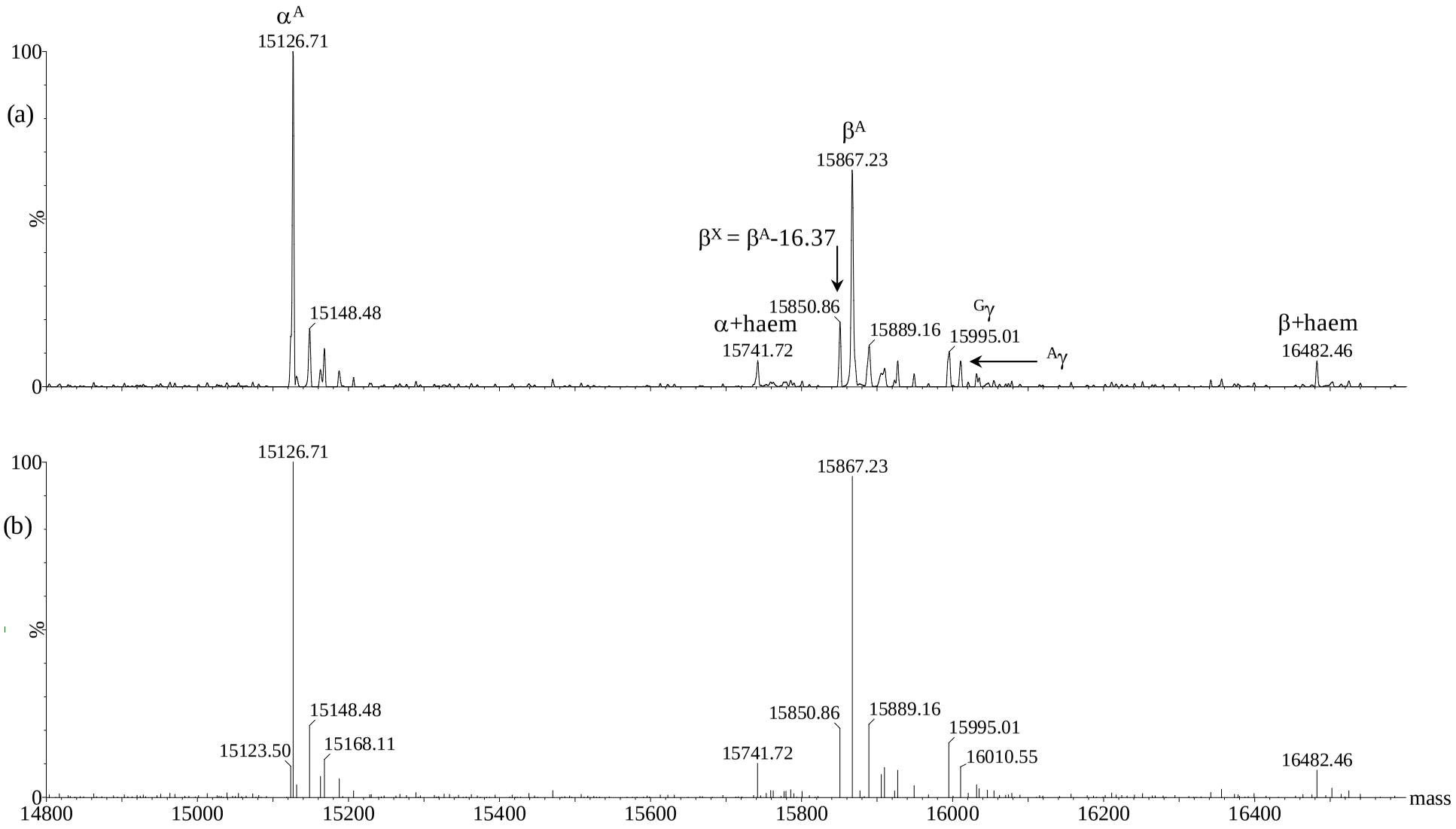

Figure 5.4.23.2. Deconvoluted mass spectrum of Hb Southampton (β106Leu→Pro) showing the presence of a signal at 15,850.86 Da at approximately 20% intensity of the normal β-chain peak (15,867.23 Da).

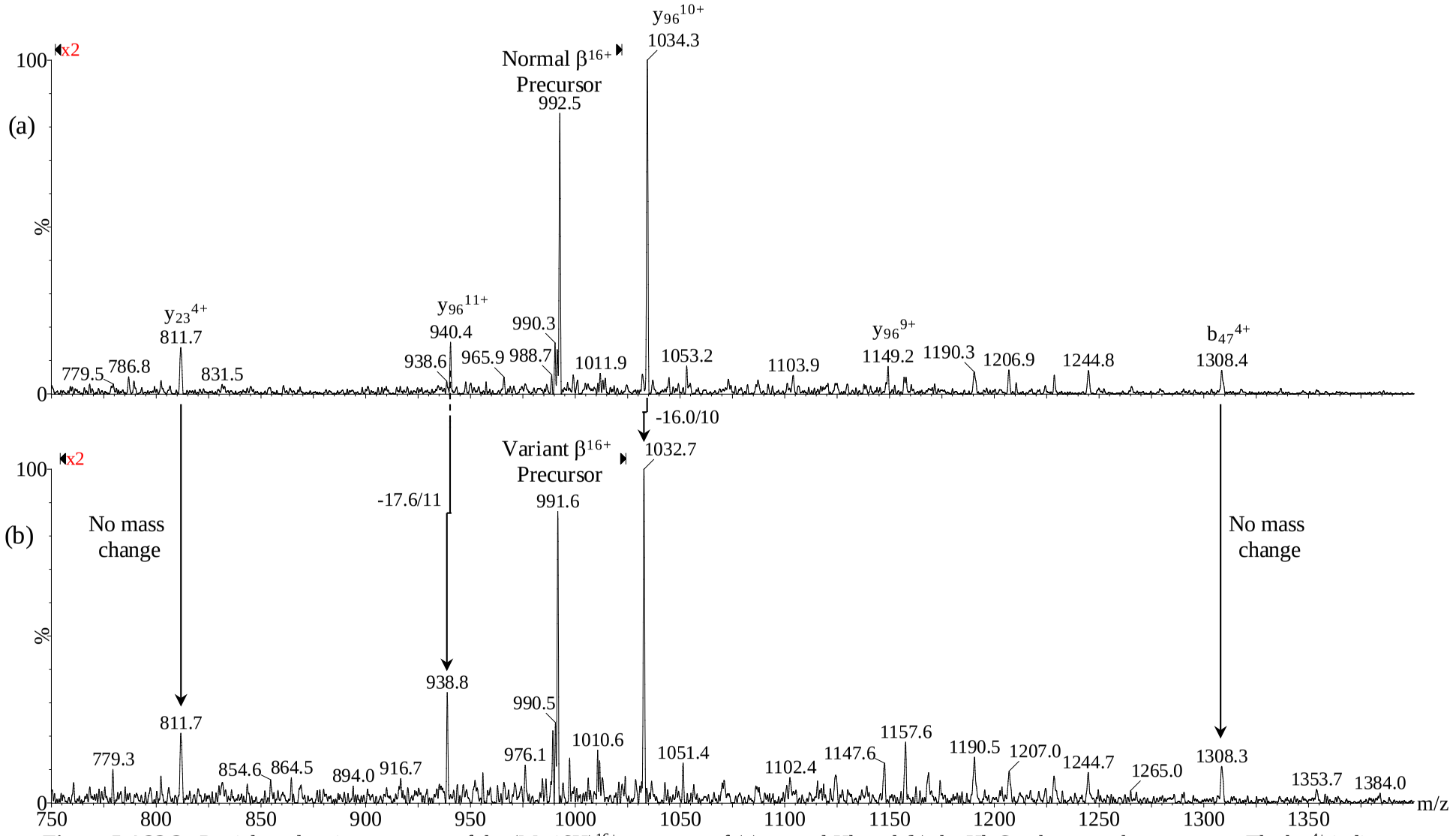

Figure 5.4.23.3. Partial product ion spectrum of the $(M+16H)^{16+}$ precursor of (a) normal Hb and (b) the Hb Southampton heterozygote. The b_{47}^{4+} indicates that the variant does not lie between β1 and β47, and the $y''_{23}{}^{4+}$ indicates that the variant does not lie between β124 and β146.

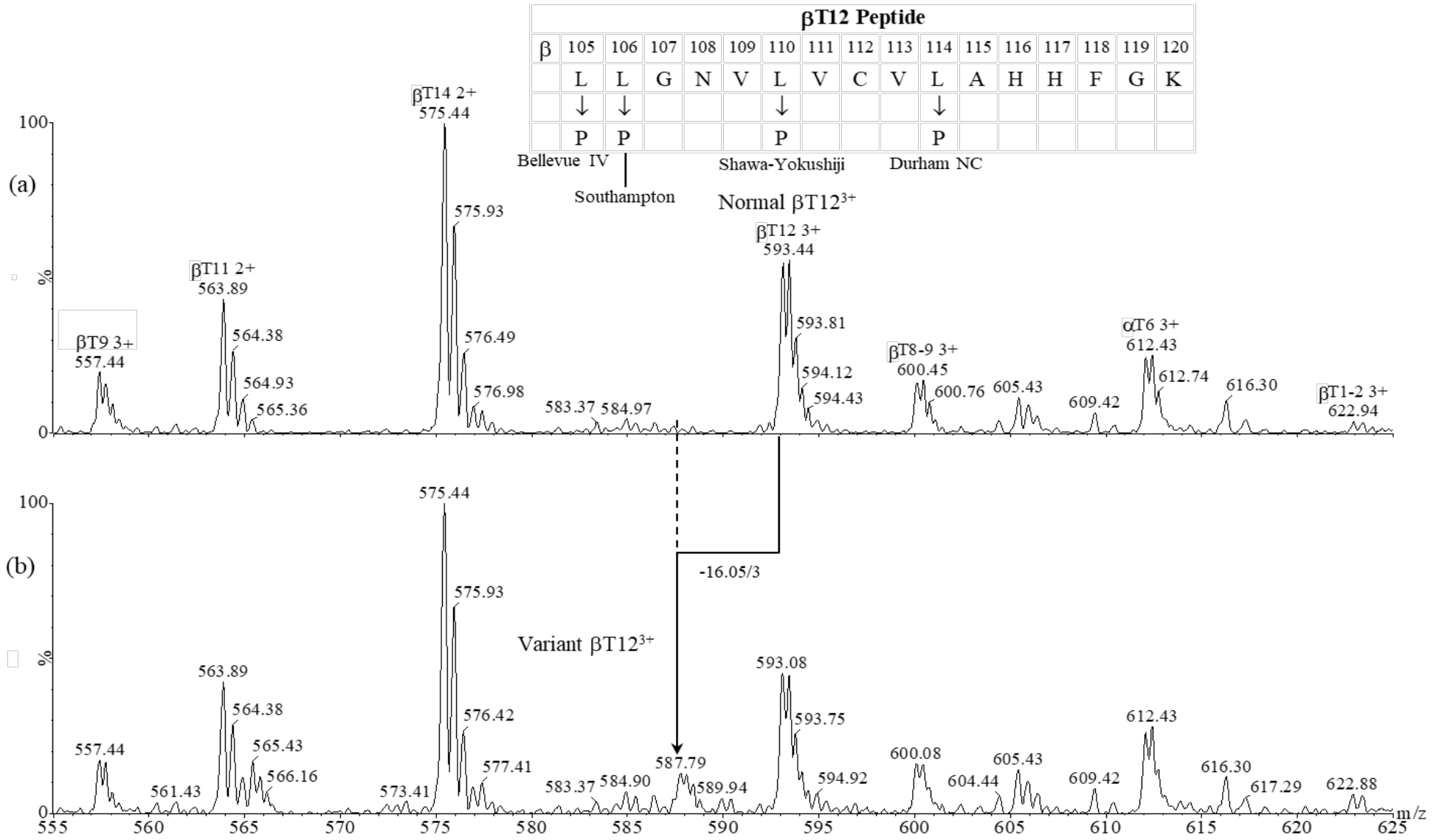

Figure 5.4.23.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) the Hb Southampton heterozygote. βT12 (β105-120) contains four Leu residues which can give rise to a mass decrease of 16 Da.

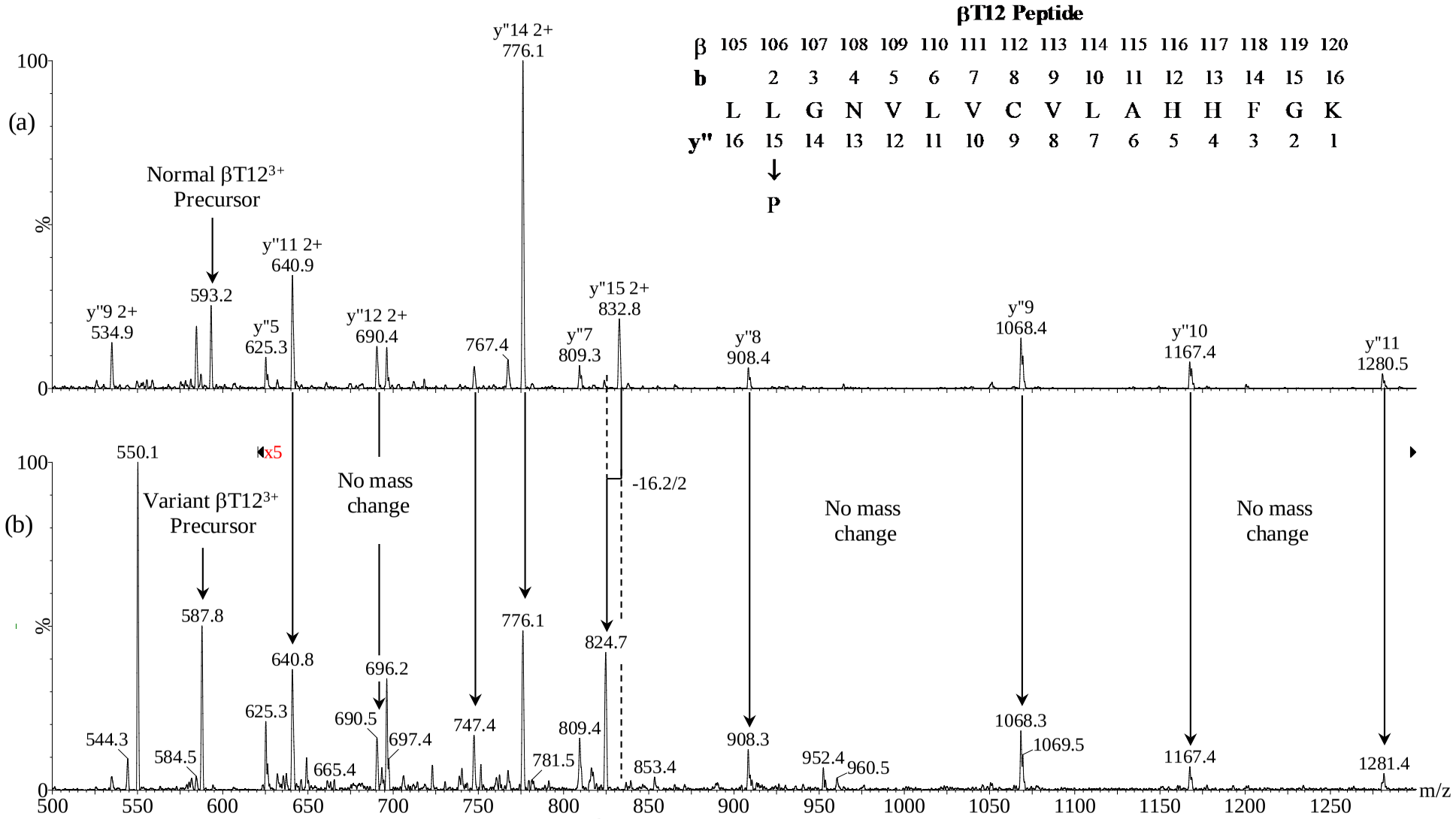

Figure 5.4.23.6. Partial product ion mass spectra of the βT12^{3+} tryptic fragment of (a) normal Hb and (b) the Hb Southampton heterozygote. These spectra exhibit almost complete y" series at 1^+, 2^+ or 3^+ ions. There is no change in mass between the normal and the variant $y"_1 \rightarrow y"_{14}$, and a decrease in mass of 16 Da at $y"_{15}{}^{2+}$, confirming the mutation β106Leu→Pro.

5.4.24. βT12 - Hb Johnstown (β109Val→Leu)

Hb Johnstown is the result of a β-chain mutation in which the β109 amino acid residue is changed from Val to Leu through a single base change in the codon GTG→CTG or TTG.

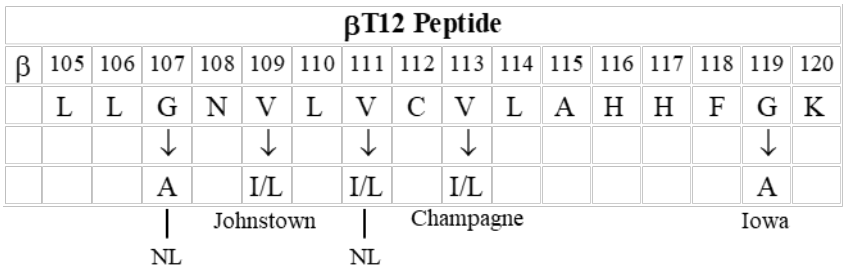

Figure 5.4.24.1. Sequence of the Hb Johnstown βT12 tryptic peptide.

There is no ce-HPLC trace available for Hb Johnstown. The sample was sent for analysis by mass spectrometry for a reported high oxygen affinity.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.4.24.2.) revealed a signal at 15,881.17 Da, 13.91 Da heavier than, and approximately similar intensity to, the normal β-chain. A mass difference of +14 Da implies either Asn→Lys (6 possibilities), Asp→Glu (7 possibilities), Gly→Ala (13 possibilities), Ser→Thr (5 possibilities), Val→Ile (18 possibilities) or Val→Leu (18 possibilities) from a single base change in the codon. Phenotypic results discount Asn→Lys, leaving 61 possible sites of mutation at this stage.

Figure 5.4.24.3. shows the diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) the variant Hb comparing the $\beta T12^{2+}$ tryptic fragments. An additional peak is observed in the lower panel at *m/z* 867.51 consistent with the mutation of +14 Da occurring in the βT12 tryptic fragment. This is further supported by the data in the lower panel of Figure 5.4.24.4. with the appearance of the mutated $\beta T12^{3+}$ tryptic fragment at *m/z* 578.76. These data are consistent with the mutations: β107Gly→Ala (not previously reported), β197Val→Leu/Ile (Hb Johnstown), β111Val→Leu/Ile (not previously reported), β113Val→Leu (Hb Champagne) or β119Gly→Ala (Hb Iowa).

Figure 5.4.24.5. shows the partial product ion mass spectra of the $\beta T12^{2+}$ tryptic fragment of (a) normal Hb and (b) the variant Hb. The 14 Da mass increase of the b_5 fragment at *m/z* 511.3 in the lower panel confirms the mutation as either β109Val→Leu (Hb Johnstown) or β109Val→Ile (not previously reported). Mass spectrometry cannot distinguish between Leu and Ile. However, β109Val→Ile is unlikely as two base changes in the specific codon are required for this mutation.

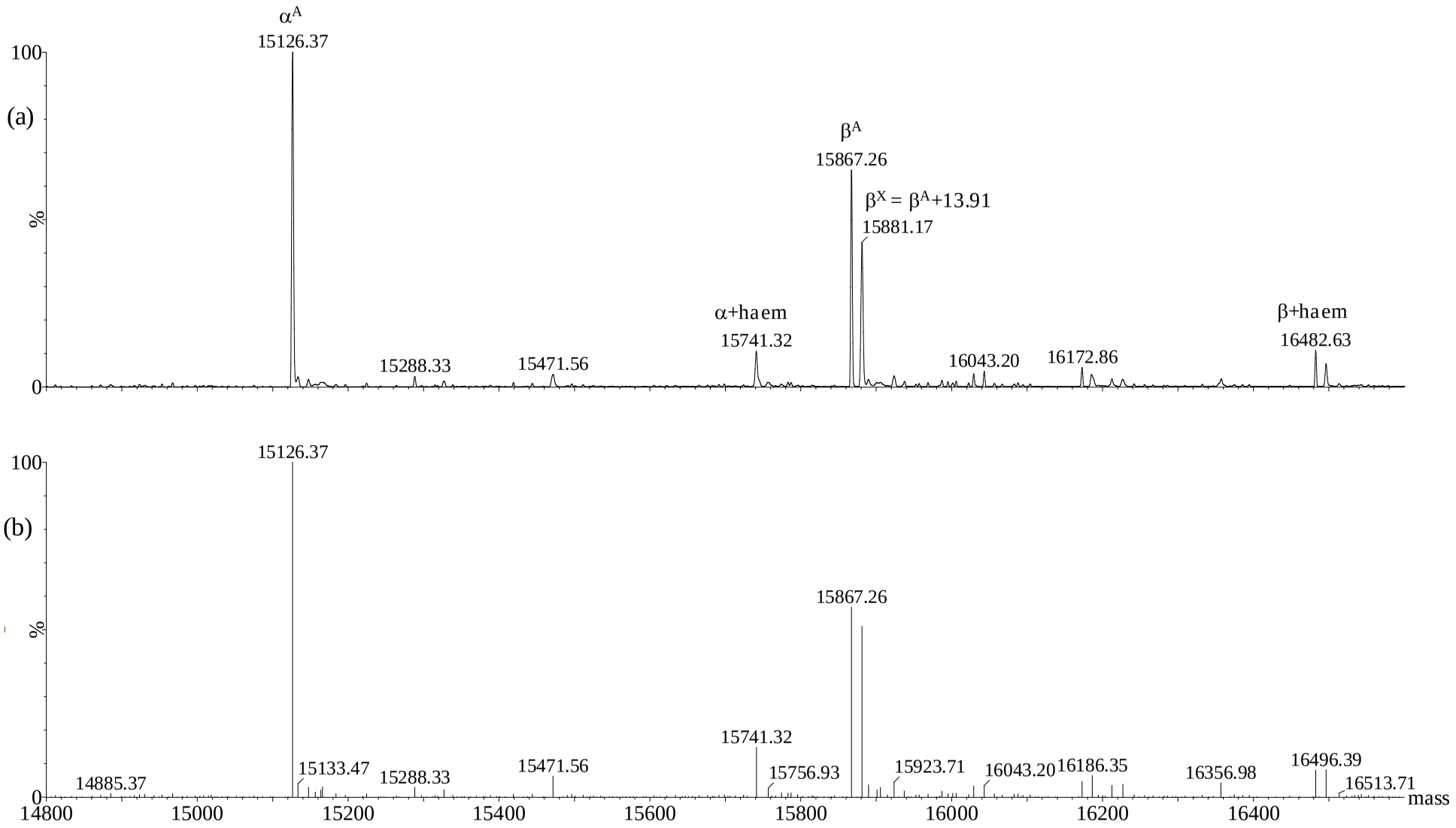

Figure 5.4.24.2. Deconvoluted mass spectrum of Hb Johnstown (β109Val→Leu) showing the presence of a signal at 15,881.17 Da at approximately 90% intensity of the normal β-chain peak (15,867.26 Da).

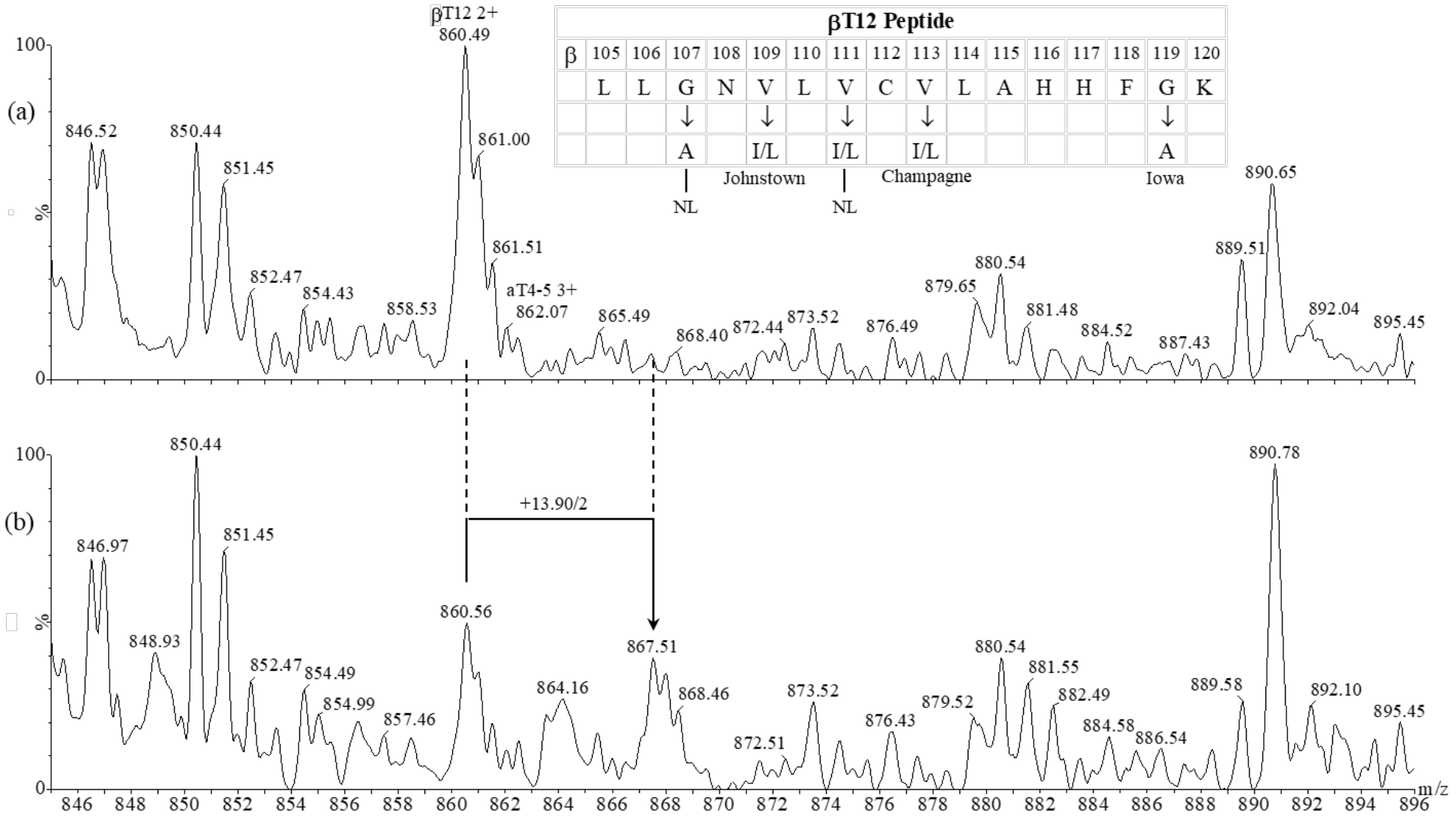

Figure 5.4.24.3. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) the Hb Johnstown heterozygote. The mutation is confirmed as +14 Da by the ion at *m/z* 867.51 ($\beta T12^{2+}$) in the lower panel.

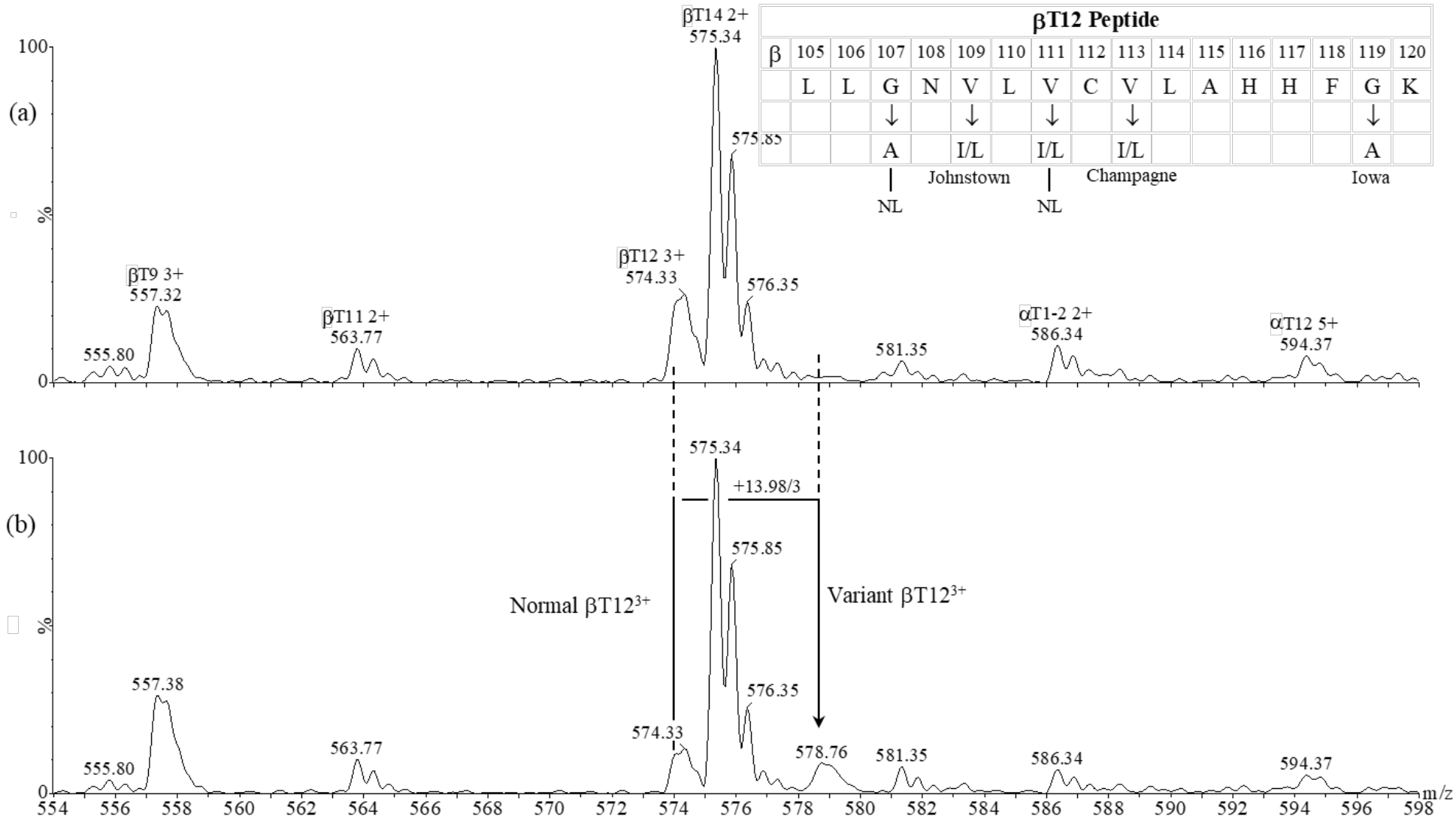

Figure 5.4.24.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) the Hb Johnstown heterozygote. There are eight possible mutations that can give a 14 Da mass increase from a single amino acid mutation.

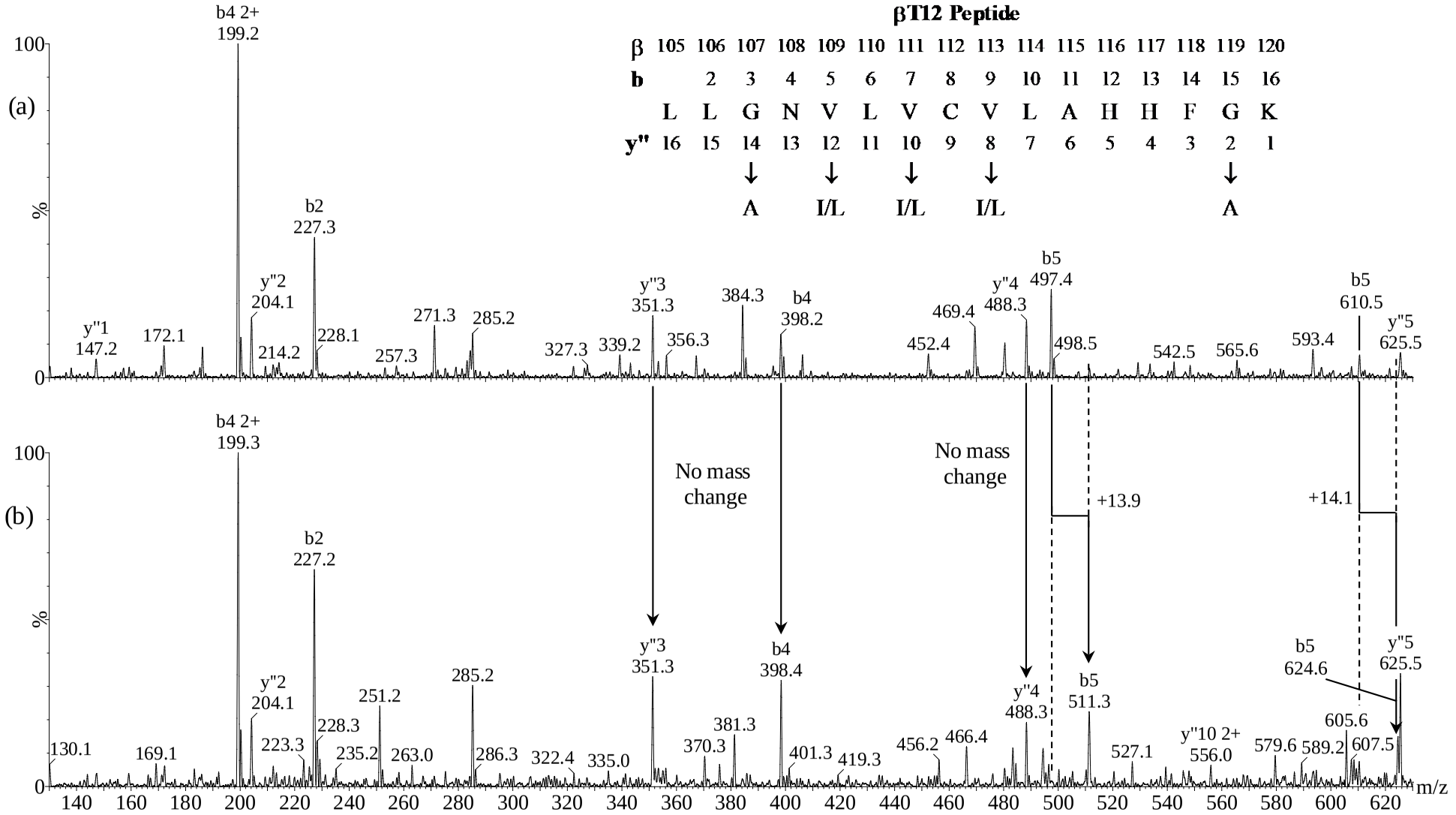

Figure 5.4.24.5. Product ion mass spectra of the $\beta T12^{2+}$ tryptic fragment of (a) normal Hb and (b) the Hb Johnstown heterozygote. The 14 Da mass increase at b_5 confirms the mutation as either β109Val→Leu (Hb Johnstown) or β109Val→Ile (not listed). Mass spectrometry cannot distinguish between Leu and Ile. However, β109Val→Ile is unlikely as two base changes in the codon are required for this mutation.

5.4.25. βT13 - Hb O-Arab (β121Glu→Lys)

Hb O-Arab is the result of a β-chain mutation in which the β121 amino acid residue is changed from Glu to Lys through a single base change in the codon GAA→AAA.

βT13 Peptide

β	121	122	123	124	125	126	127	128	129	130	131	132
	E	F	T	P	P	V	Q	A	A	Y	Q	K
	↓					**βT13b Peptide**						
	121		122	123	124	125	126	127	128	129	130	131	132
	K		F	T	P	P	V	Q	A	A	Y	Q	K
	O-Arab												

Figure 5.4.25.1. Sequence of the Hb O-Arab βT13 tryptic peptide.

A blood sample was submitted for analysis by mass spectrometry because the ce-HPLC trace (Figure 5.4.25.2.) showed a significant response (36.9%) at 4.88 min, indicating a significant positive charge change.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	1.3	---	1.09	19780
Unknown	---	0.4	1.27	6993
P2	---	2.0	1.37	33154
Unknown	---	0.3	1.56	5403
P3	---	2.3	1.73	37522
A0	---	52.5	2.48	871697
A2	2.7	---	3.67	46951
D-window	---	0.9	4.04	15495
S-window	---	0.6	4.64	10518
Unknown	---	36.9	4.88	612925

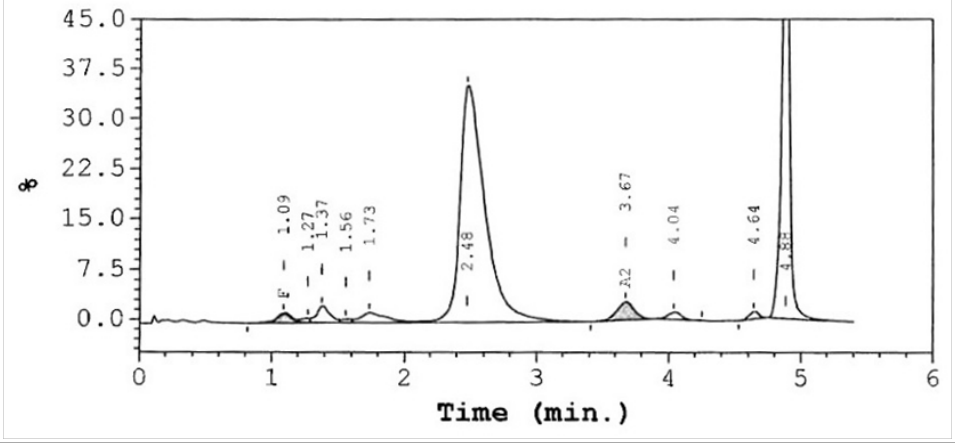

Figure 5.4.25.2. ce-HPLC trace for Hb O-Arab.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.4.25.3.) revealed a signal at 15,866.89 Da, which is consistent with a $\beta^A/(\beta^A\text{-}1)$ heterozygote. A mass difference of -1 Da with a significant positive charge change suggests that the mutation is either Glu→Gln (8 possibilities) or Glu→Lys (8 possibilities) from a single base change in the codon. If the mutation is Glu→Lys, this will likely result in an additional tryptic fragment.

Figure 5.4.25.4. shows the diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) the variant Hb. Peaks are observed at *m/z* 689.38 in the lower panel, attributable to the proposed $\beta T(13a+b)^{2+}$ and at *m/z* 625.34, assigned as the new $\beta T13b^{2+}$. Figure 5.4.25.5. shows a higher mass range than that shown in Figure 5.4.25.4. with a tryptic fragment being seen at *m/z* 1,249.68, attributed to the $\beta T13b^{+}$ tryptic fragment. These data unambiguously identify the mutation as β121Glu→Lys, Hb O-Arab.

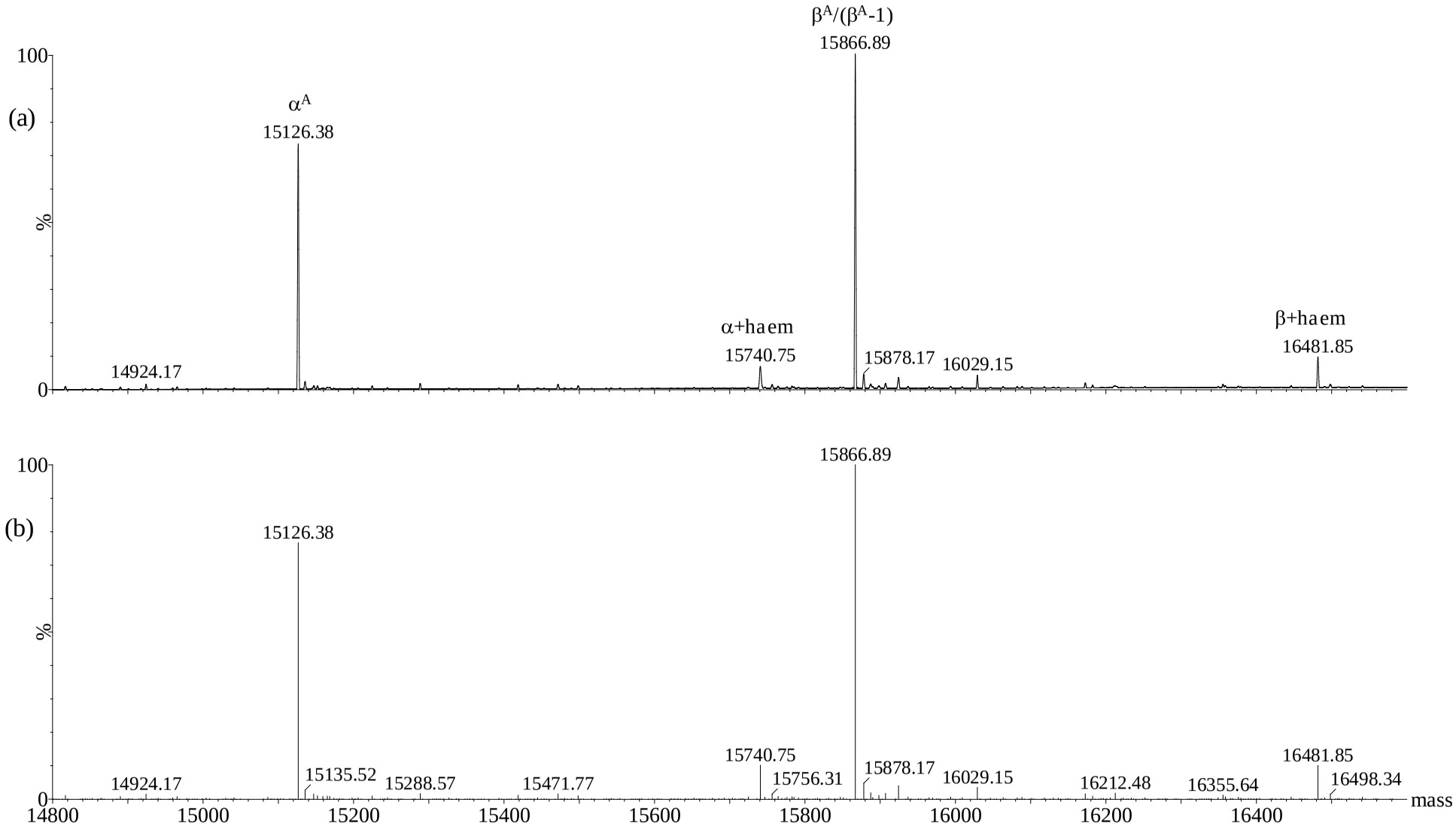

Figure 5.4.25.3. Deconvoluted mass spectrum of Hb O-Arab (β121Glu→Lys) showing the presence of a single signal at 15,866.80 Da. This is suggestive of a $\beta^A/(\beta^A\text{-}1)$ heterozygote.

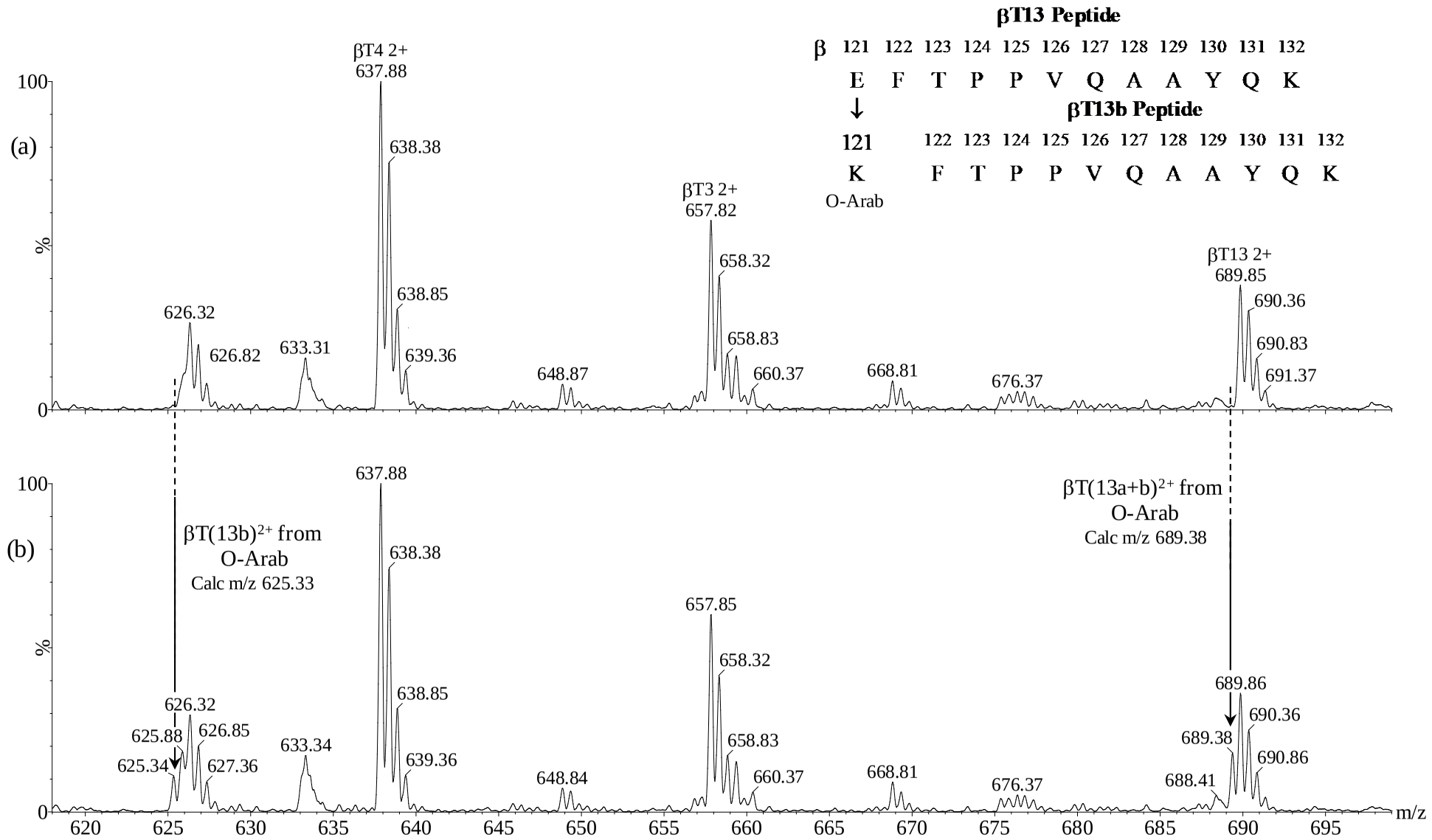

Figure 5.4.25.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) the Hb O-Arab heterozygote. This unambiguously identifies the mutation as β121Glu→Lys.

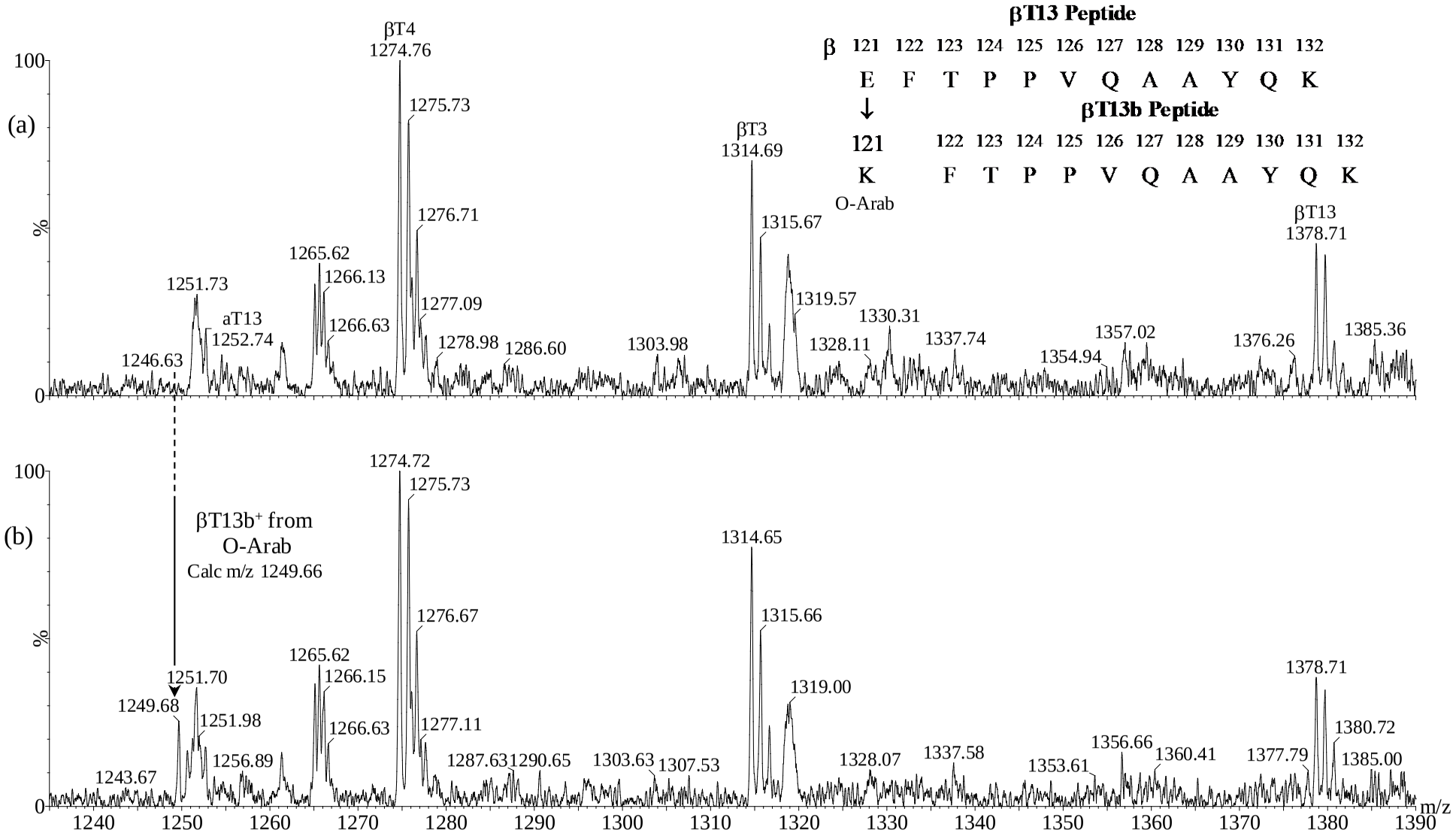

Figure 5.4.25.5. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) the Hb O-Arab heterozygote. This unambiguously identifies the mutation as β121Glu→Lys.

5.4.26. βT13 - Hb D-Punjab (D-Los Angeles) (β121Glu→Gln)

Hb D-Punjab is the result of a β-chain mutation in which the β121 amino acid residue is changed from Glu to Gln through a single base change in the codon GAA→CAA.

βT13 Peptide

β	121	122	123	124	125	126	127	128	129	130	131	132
	E	F	T	P	P	V	Q	A	A	Y	Q	K
	↓											
	Q											

D-Punjab
D-Los Angeles

Figure 5.4.26.1. Sequence of the Hb D-Punjab βT13 tryptic peptide.

A blood sample was submitted for analysis by mass spectrometry because the ce-HPLC trace (Figure 5.4.26.2.) showed a significant response (33.8%) in the D-window at 4.16 min, indicating a significant positive charge change.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	0.3	---	1.11	4696
Unknown	---	0.7	1.25	9925
P2	---	3.1	1.32	43567
P3	---	2.6	1.69	37639
Unknown	---	2.0	2.06	29126
A0	---	55.7	2.54	793646
A2	1.7	---	3.65	24806
D-window	---	33.8	4.16	481469

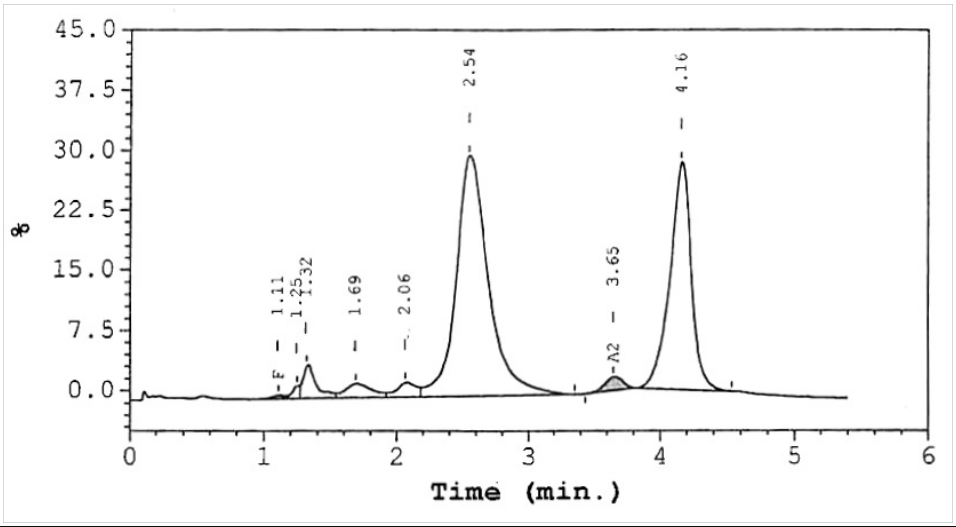

Figure 5.4.26.2. ce-HPLC trace for Hb D-Punjab.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.4.26.3.) revealed a signal at 15,866.80 Da, which is consistent with a $\beta^A/(\beta^A\text{-}1)$ heterozygote. A mass difference of -1 Da with a significant positive charge change suggests that the mutation is either Glu→Gln (8 possibilities) or Glu→Lys (8 possibilities) from a single base change in the codon. If the mutation is Glu→Lys, this will likely result in an additional tryptic fragment.

Figure 5.4.26.4. shows the diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) the variant Hb. A peak is observed at *m/z* 689.37 in the lower panel, attributable to the proposed $\beta T13^{2+}$.

The isotope patten is a combination of the normal and variant $\beta T13^{2+}$ isotope patterns overlapping. Unlike the data for Hb O-Arab (Section 5.4.25.) no new peak is observed at *m/z* 625.3, indicating an additional tryptic fragment has <u>not</u> been formed, and the data are therefore consistent with the mutation β121Glu→Gln. Figure 5.4.26.5. shows a higher mass range than that shown in Figure 5.4.25.4. with a tryptic fragment being seen at *m/z* 1,377.73 attributed to the $\beta T13^{+}$, and no peak being observed at *m/z* 1,249.68. These data unambiguously identify the mutation as β121Glu→Lys, Hb D-Punjab (D-Los Angeles).

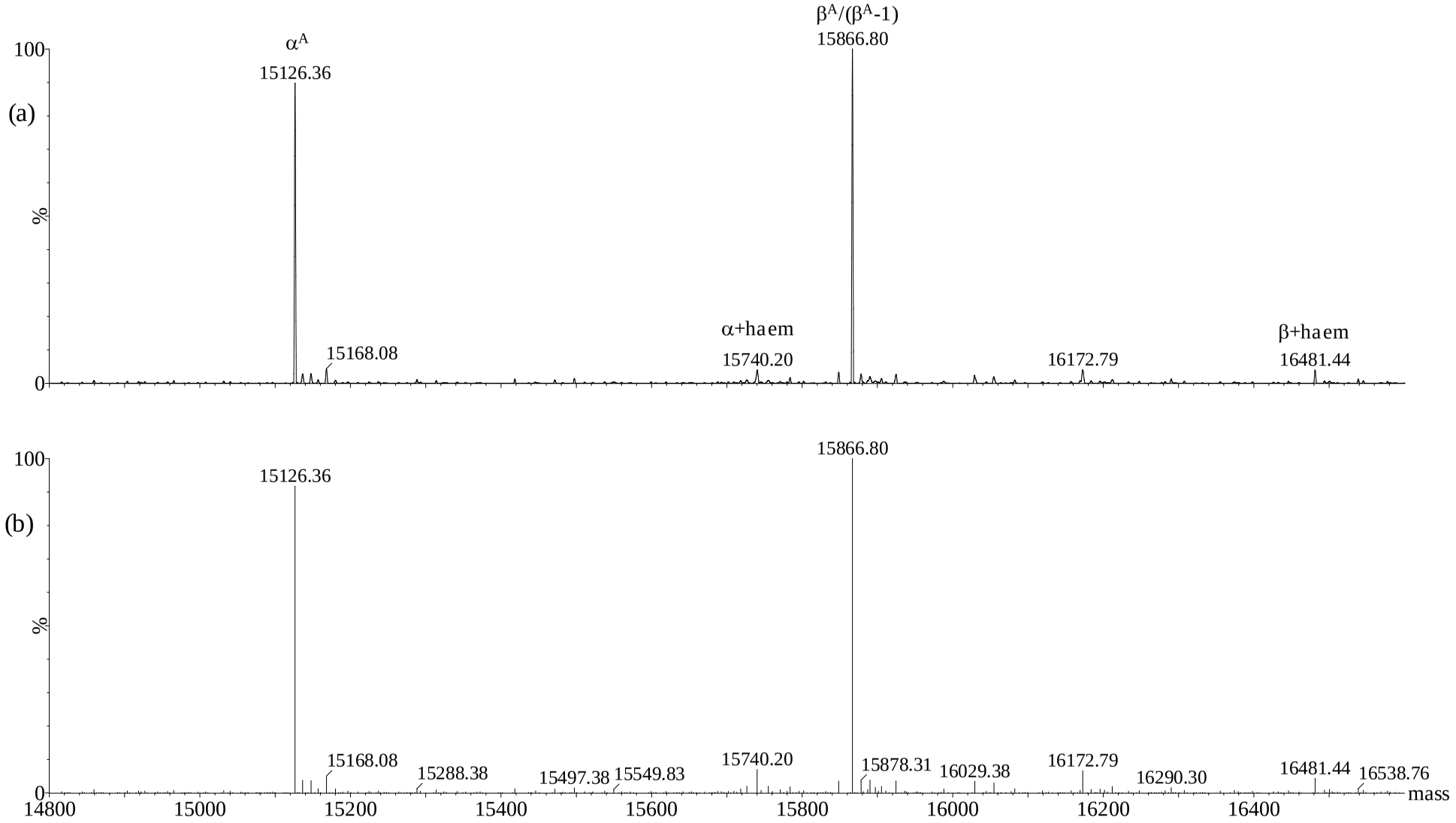

Figure 5.4.26.3. Deconvoluted mass spectrum of Hb D-Punjab (β121Glu→Gln) showing the presence of a single signal at 15,866.80 Da. This is suggestive of a $\beta^A/(\beta^A\text{-}1)$ heterozygote.

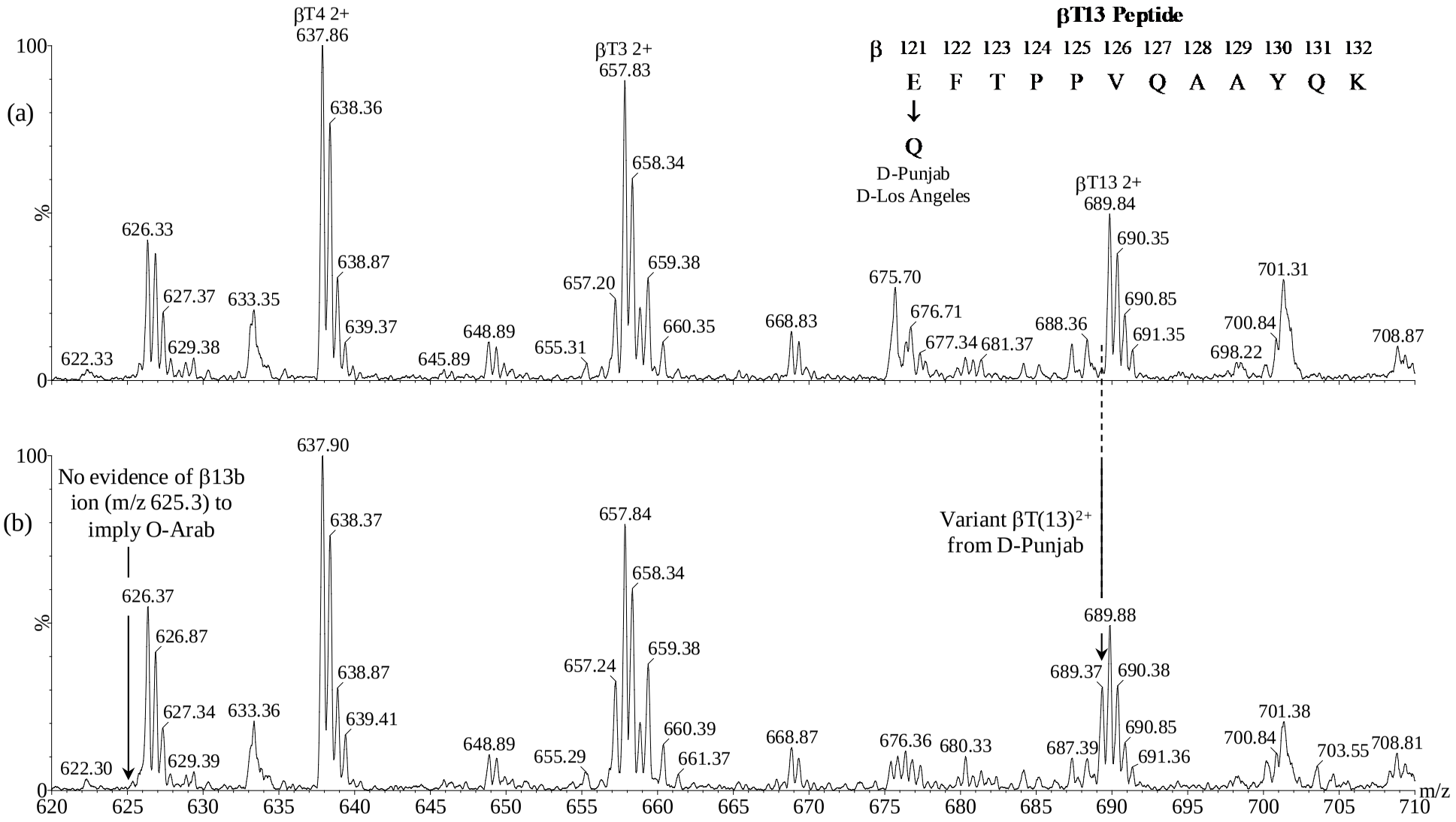

Figure 5.4.26.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) the Hb D-Punjab heterozygote. The only possibilities for a 1 Da mass decrease in this peptide from a single base change in the codon are β121Glu→Gln and β121Glu→Lys (Hb O-Arab).

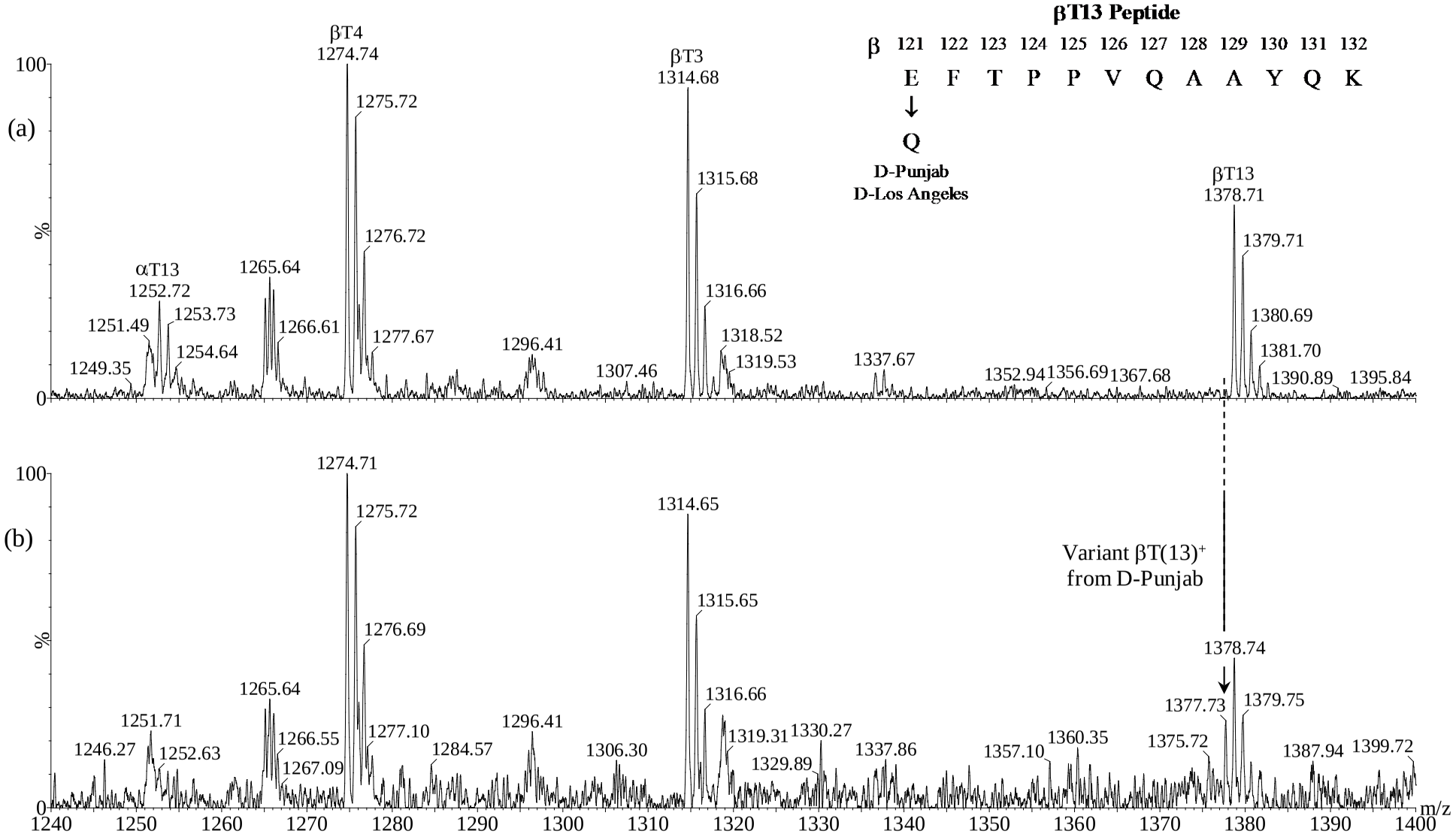

Figure 5.4.26.5. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) the Hb D-Punjab heterozygote. The only possibilities for a 1 Da mass decrease in this peptide from a single base change in the codon are β121Glu→Gln and β121Glu→Lys (Hb O-Arab).

5.4.27. βT13 - Hb Ty Gard (β124Pro→Gln)

Hb Ty Gard is the result of a β-chain mutation in which the β124 amino acid residue is changed from Pro to Gln through a single base change in the codon CCA→CAA.

βT13 Peptide

β	121	122	123	124	125	126	127	128	129	130	131	132
	E	F	T	P	P	V	Q	A	A	Y	Q	K
				↓	↓							
				Q	Q							
				Ty Gard	NL							

Figure 5.4.27.1. Sequence of the Hb Ty Gard βT13 tryptic peptide.

A blood sample was submitted for analysis by mass spectrometry because the ce-HPLC trace (Figure 5.4.27.2.) showed a significant response (36.8%) at 2.29 min, indicating no significant charge change.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	0.2	---	0.98	1902
Unknown	---	2.2	1.27	27459
P2	---	2.2	1.35	27925
P3	---	4.0	1.68	50071
Unknown	---	36.8	2.29	461647
A0	---	51.0	2.52	639312
A2	3.2	---	3.64	44860

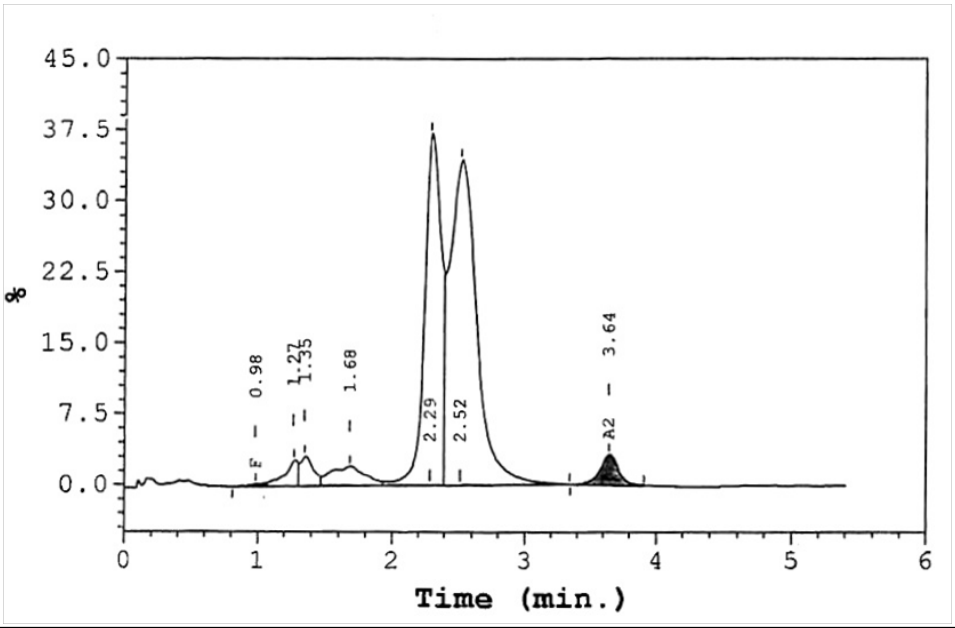

Figure 5.4.27.2. ce-HPLC trace for Hb Ty Gard.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.4.27.3.) revealed a signal at 15,892.29 Da, 31.02 Da heavier than, and approximately 80% of the intensity of the normal β-chain. A mass difference of +31 Da can only arise from Pro→Gln (7 possibilities) from a single base change in the codon.

Figure 5.4.27.4. shows the diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) the variant Hb comparing the $\beta T13^{2+}$ tryptic fragments. An additional peak is observed in the lower panel at *m/z* 705.36, consistent with the mutation of +31 Da occurring in the βT13 tryptic fragment. In the

βT13 tryptic fragment there are two potential sites of mutation: βT124Pro→Gln (Hb Ty Gard) or βT125Pro→Gln (not previously reported).

Figure 5.4.24.5. shows the partial product ion mass spectra of the $\beta T13^{2+}$ tryptic fragment of (a) normal Hb and (b) the variant Hb. A mass change of +31 Da is observed for the b_4 fragment at *m/z* 506.3 in the lower panel, as well as a similar mass change in the y''_9 fragment at *m/z* 1,032.8, identifying the mutation as βT124Pro→Gln, Hb Ty Gard.

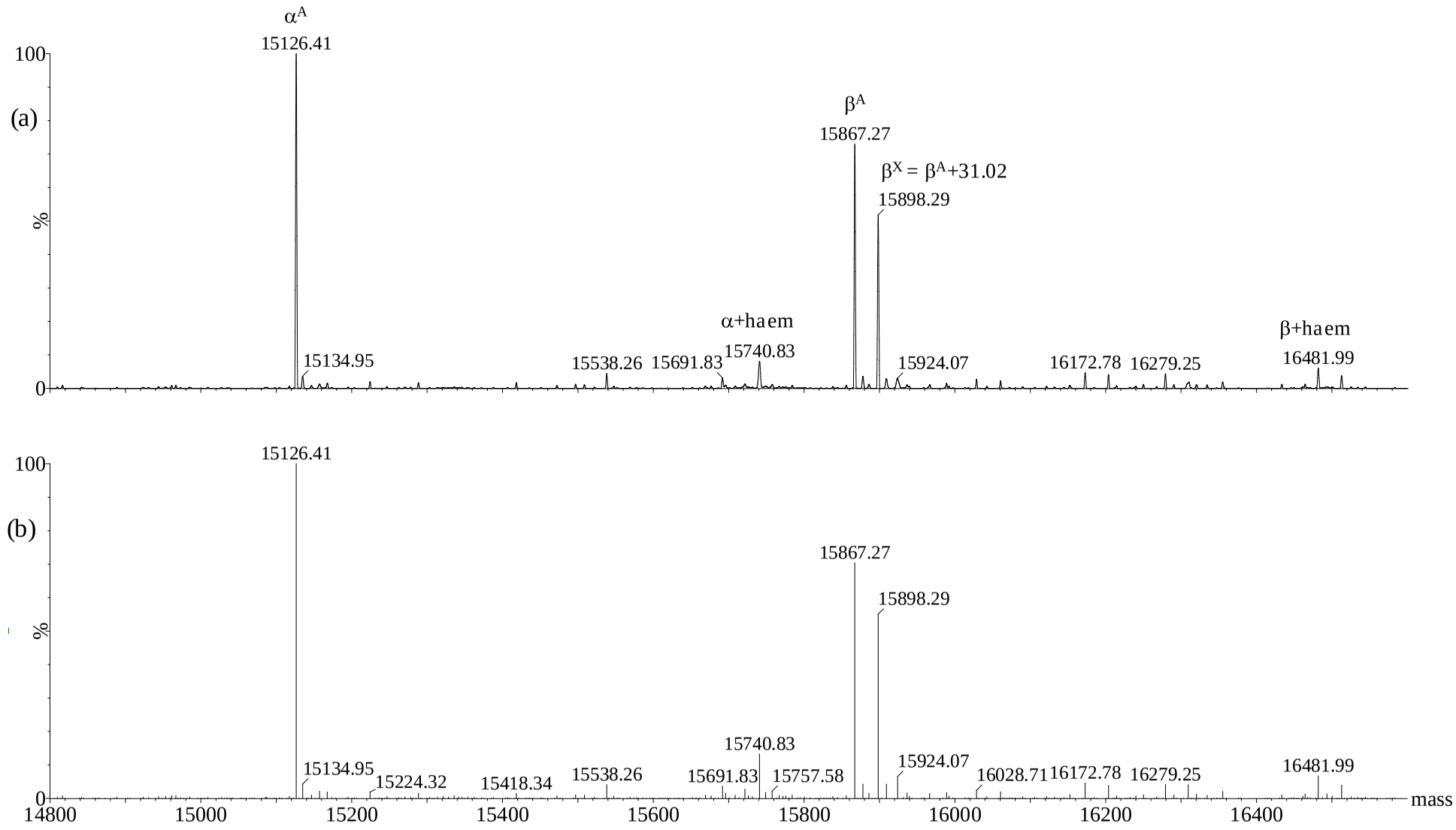

Figure 5.4.27.3. Deconvoluted mass spectrum of Hb Ty Gard (β124Pro→Gln) showing the presence of a signal at 15,898.29 Da at approximately 80% intensity of the normal β-chain peak (15,867.27 Da). The mass increase of 31 Da can only arise from Pro→Gln from a single base change in the codon.

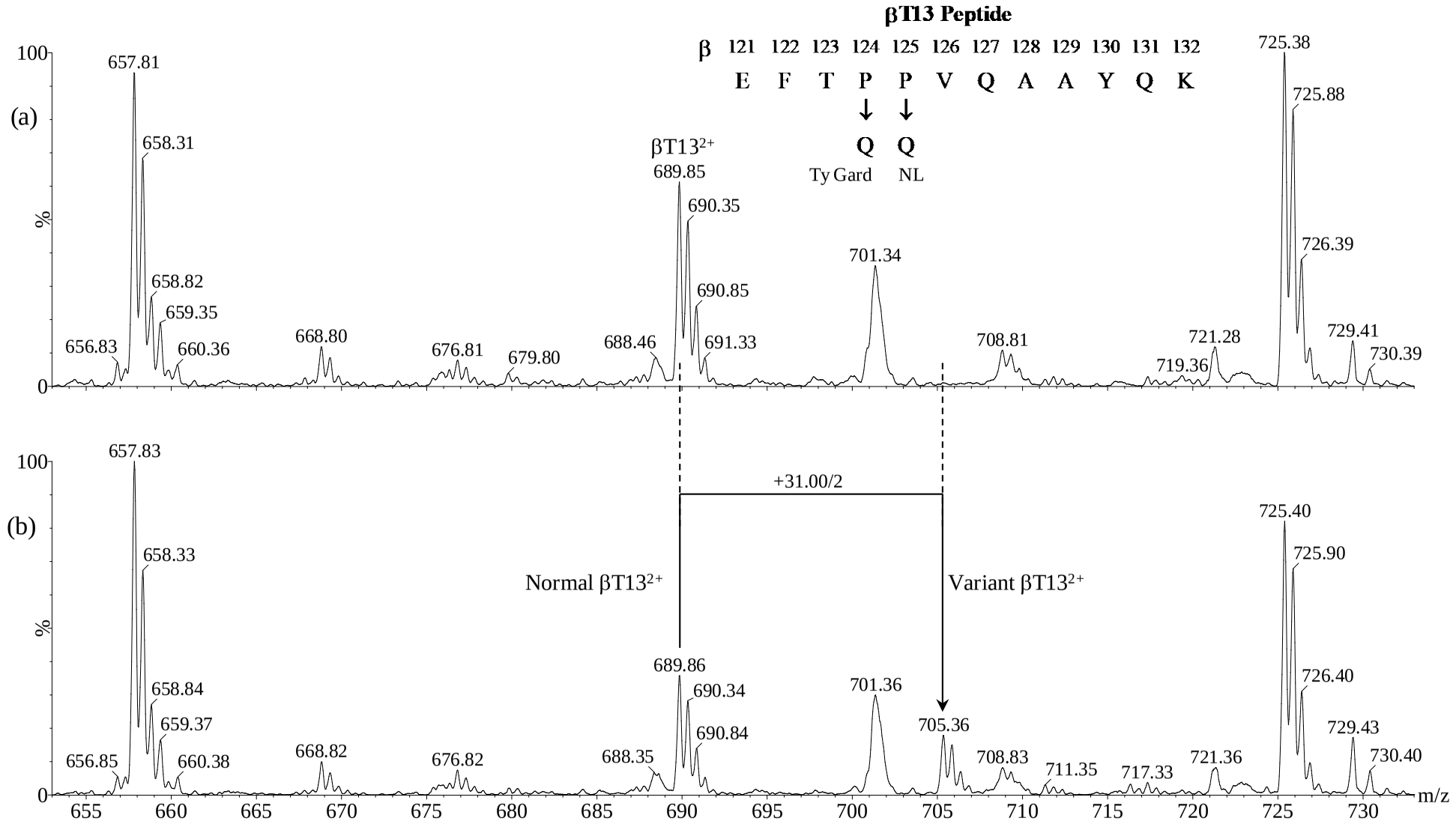

Figure 5.4.27.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) the Hb Ty Gard heterozygote.

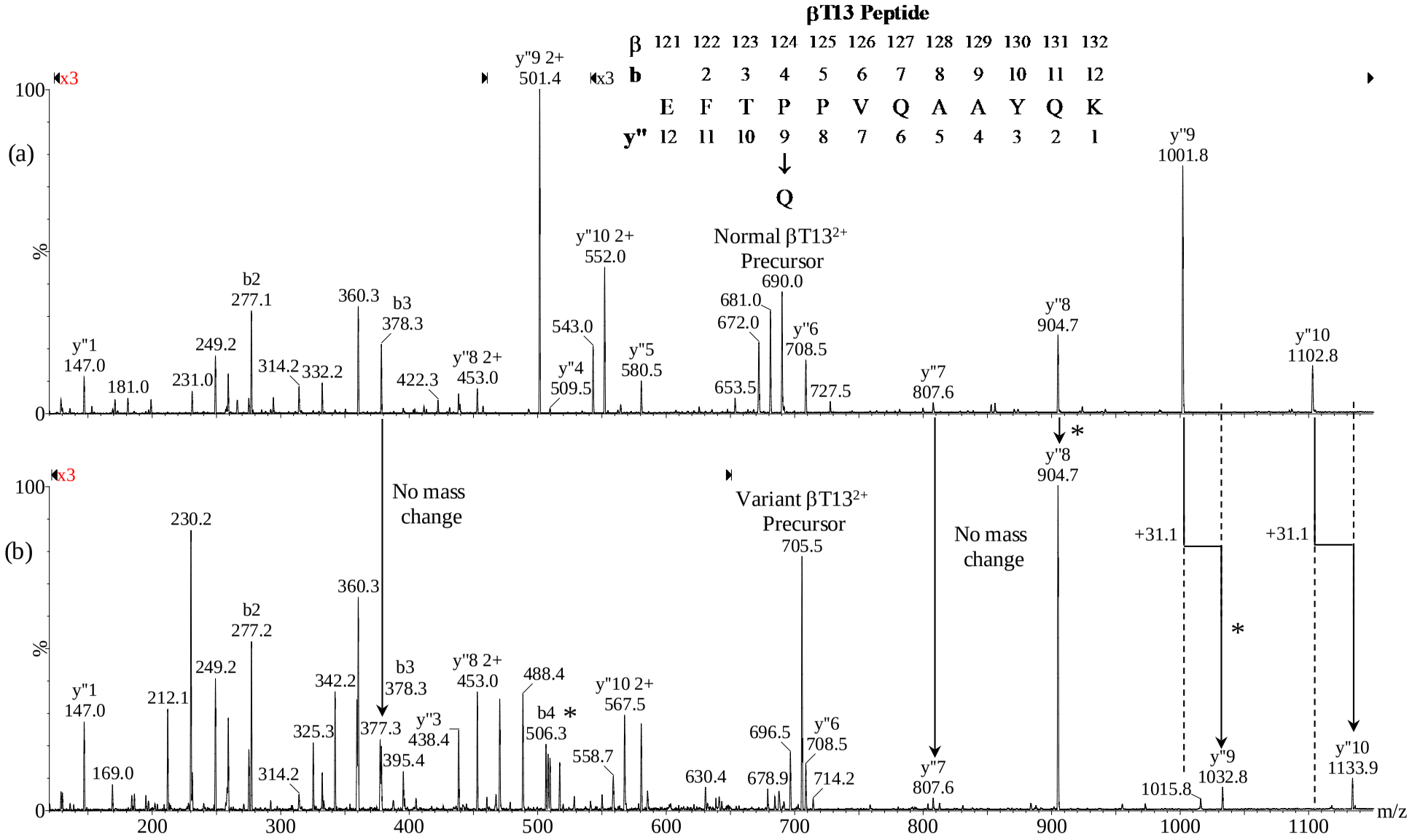

Figure 5.4.27.5. Partial product ion mass spectra of the βT13^{2+} tryptic fragment of (a) normal Hb and (b) the Hb Ty Gard heterozygote. The 31 Da mass increase at b_4 and y''_9 identifies the mutation as β124Pro→Gln.

5.4.28. βT14 - Hb Alperton (β135Ala→Val)

Hb Alperton is the result of a β-chain mutation in which the β135 amino acid residue is changed from Ala to Val through a single base change in the codon GCT→GTT.

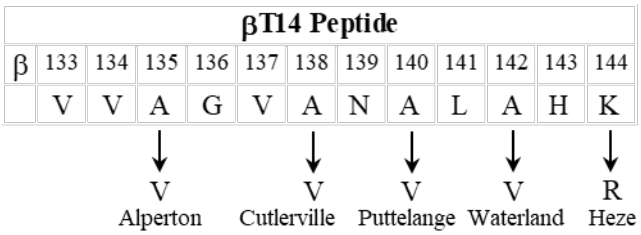

Figure 5.4.28.1. Sequence of the Hb Alperton βT14 tryptic peptide.

A blood sample was submitted for analysis by mass spectrometry because the ce-HPLC trace (Figure 5.4.28.2.) showed a mildly elevated response (5.6%%) in the A_2 region at 3.62 min. The sample was also reported as showing β-thalassaemia trait.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.3	1.04	3693
F	1.1	---	1.12	13889
P2	---	6.4	1.29	94392
P3	---	3.9	1.69	57478
A0	---	82.5	2.71	1211000
A2	5.6	---	3.62	87103

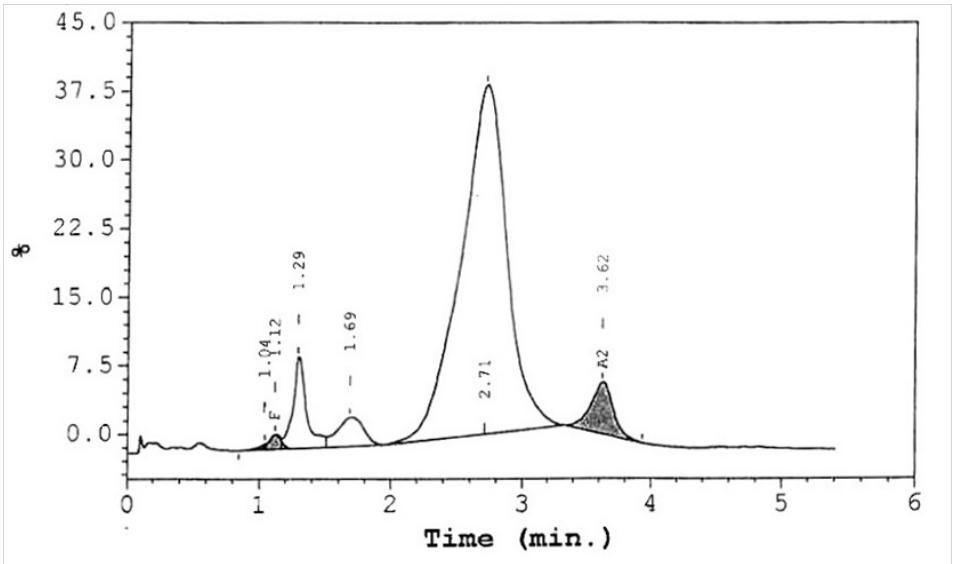

Figure 5.4.28.2. ce-HPLC trace for Hb Alperton.

ESI-MS analysis of the 500-fold diluted blood sample (Figure 5.4.28.3.) revealed a single peak at 15,895.41 Da, 28.14 Da heavier than the normal β-chain. As this was silent by ce-HPLC, no charge change is anticipated, and the likely mutations are: Ala→Val (15 possibilities) or Lys→Arg (11 possibilities) from a single base change in the codon.

Figure 5.4.28.4 shows the diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) the variant Hb. The appearance of a peak in the lower trace at *m/z* 589.36 is consistent with the +28 Da mutation occurring in the $βT14^{2+}$ tryptic fragment ion. The lack of a signal corresponding to the normal $βT14^{2+}$ fragment suggests this is either a homozygous mutation, or a β-thalassaemia trait. Possible sites of mutation in the βT14 tryptic fragment include: β135Ala→Val (Hb Alperton), β138Ala→Val (Hb

Cutlerville), β140Ala→Val (Hb Puttelange), β142Ala→Val (Hb Waterland) or β144Lys→Arg (Hb Heze).

Figure 5.4.28.5. shows the partial product ion mass spectra of the $\beta T14^{2+}$ tryptic fragment of (a) normal Hb and (b) the variant Hb. No mass change is observed in the y'' ion series until the +28 Da mass change in the $y''_{10}{}^{2+}$ (*m/z* 490.4) and $y''_{10}{}^{+}$ (*m/z* 979.6) fragments, confirming the mutation as β135Ala→Val, Hb Alperton.

This variant was novel when first analysed by mass spectrometry.

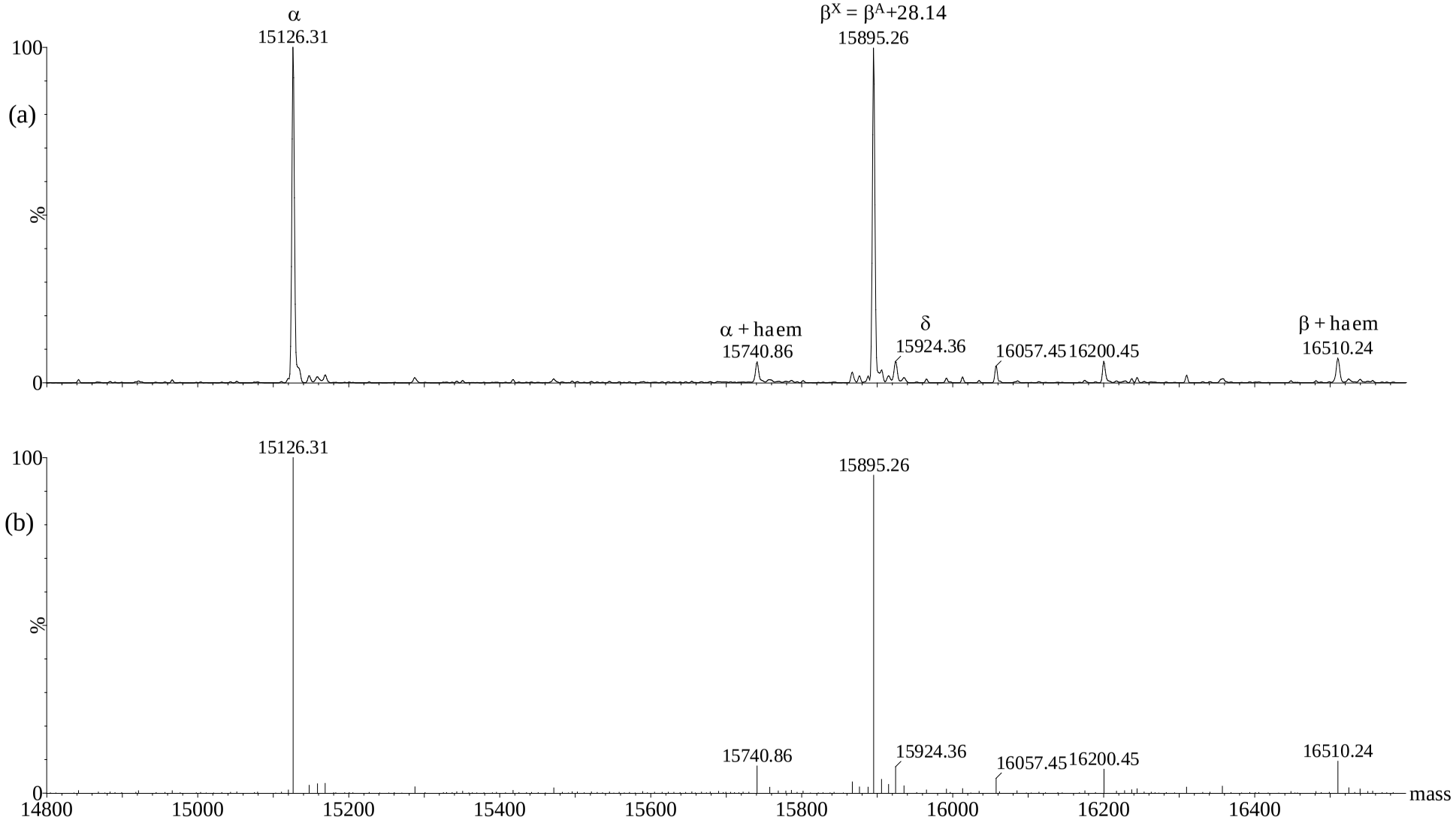

Figure 5.4.28.3. Deconvoluted mass spectrum of Hb Alperton (β135Ala→Val) showing a signal at 15,895.26 Da. For a 28 Da mass increase, and silent by ce-HPLC, possibilities for the mutation are Ala→Val or Lys→Arg.

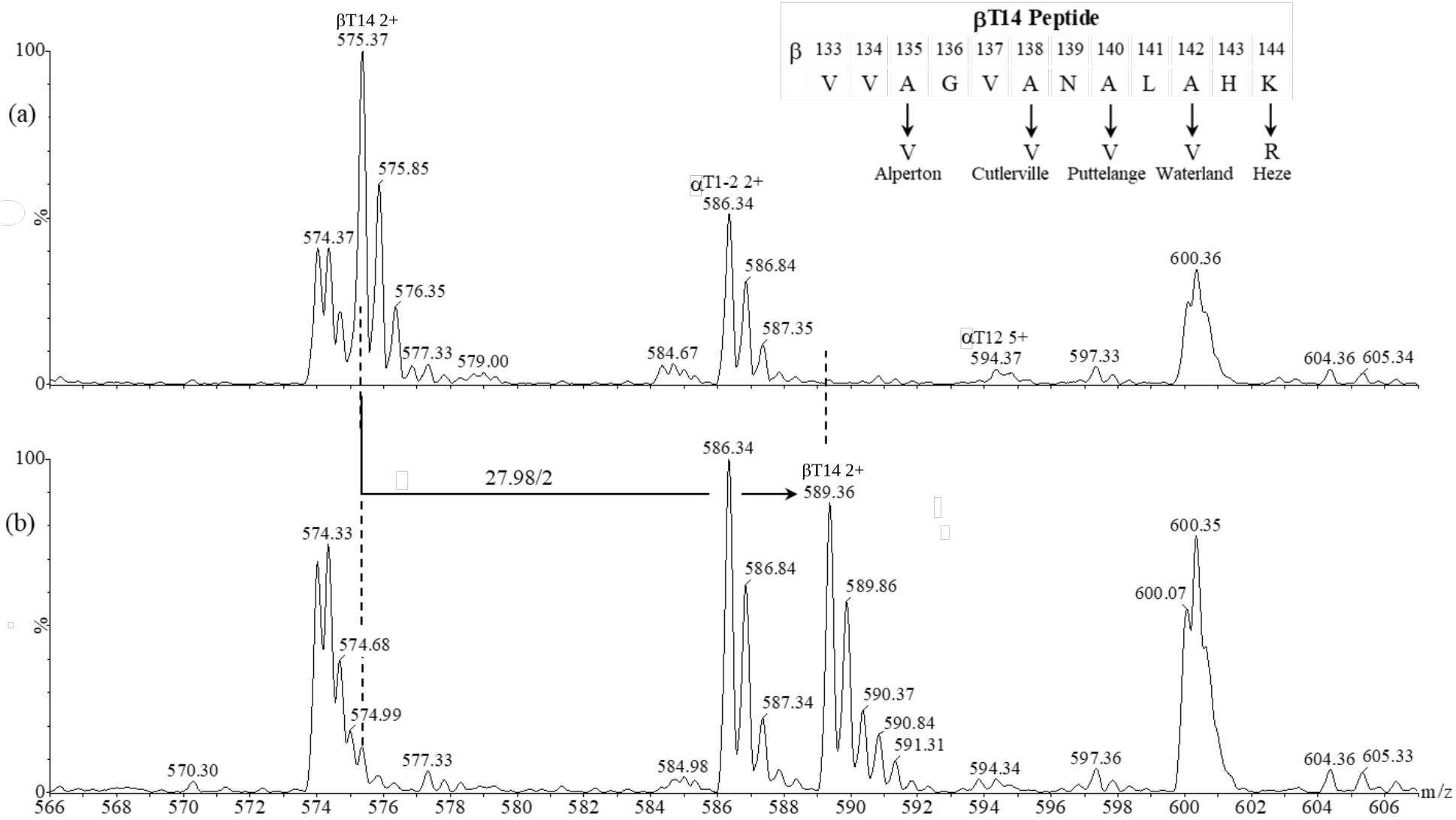

Figure 5.4.28.4. Diagnostic part of the tryptic digest spectrum of (a) normal Hb and (b) the Hb Alperton.

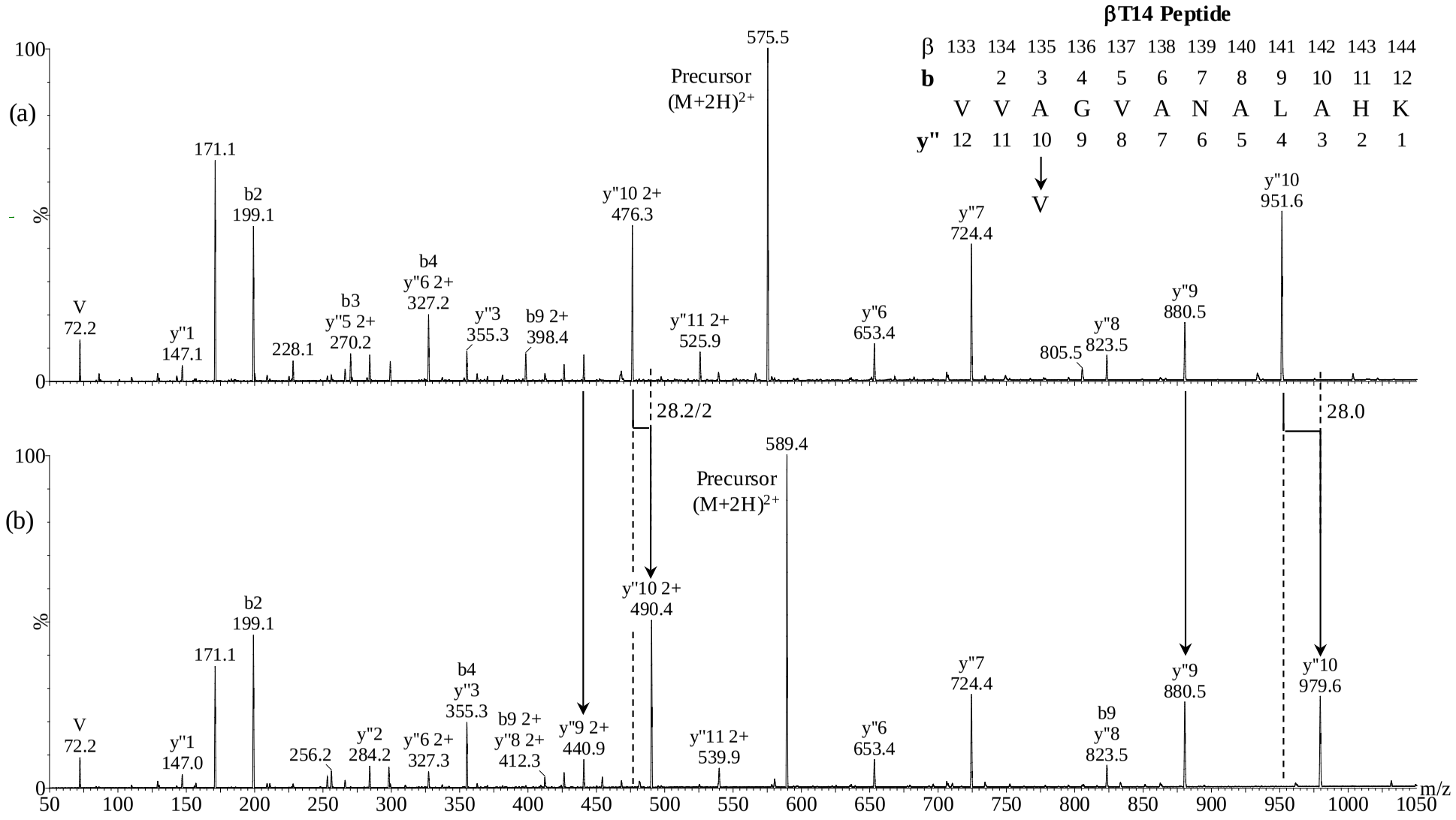

Figure 5.4.28.5. Product ion mass spectra of digest mass spectra of the $\beta T14^{2+}$ species of (a) normal Hb and (b) Hb Alperton. The 28 Da mass increase at b_3 and y''_{10} identifies the mutation as β135 Ala→Val, Hb Alperton.

SECTION 6

6.1. Variants identified by electrospray ionization mass spectrometry

Generally detected by ce-HPLC or IEF.

In the Tables for α-chain and β-chain shown below, the tryptic peptides are highlighted with shading. The examples shown in detail in Section 5 are also highlighted with a heavy border.

α-chain variants

	Mutation	Name	Comments	%	ΔM	TMS
αT1	α1Val→Leu Initiator Met retained	St.Jozef (α2)	Mr 15,271.61	15	145	N
	α3Ser→Pro	Central Middlesex (Novel)		22	10	N
	α5Ala→Asp	J-Toronto		31	44	N
	α6Asp→Asn	Dunn		~13	-1	N
	α6Asp→Tyr	Woodville		9	48	NY
	α6Asp→0	Boyle Heights		7	-115	NY
	α7Lys→Asn	Tatras		25	-14	N
αT2	α9Asn→Ser	Zurich-Hottingen (Novel)		9	-27	Y
	α9Asn→Thr	Broomfield (Novel)		19	-13	N
	α11Lys→Gln	J-Wenchang-Wuming		(27)	0	N
αT3	α12Ala→Asp	J-Paris-I		~30	44	Y
	α13Ala→Pro	Ravenscourt Park (Novel)		10	26	Y
	α14Trp→Cys	Bladensburg		4	-83	N
	α15Gly→Arg	Ottawa		21	99	N
	α15Gly→Asp	I-Interlaken, J-Oxford		24/30	58	NY
	α16Lys→Asn	Beijing		26	-14	N
	α16Lys→Glu	I		~28	1	N
αT4	α18Gly→Arg	Handsworth		14	99	N
	α18Gly→Ser	Novel		21	30	Y
	α19Ala→Pro	Novel		23	26	Y
	α19Ala→Thr & O-Arab	Novel		30	30	Y
	α20His→Gln	LeLamentin		26.0±0.6 (n=13)	-9	N
	α21Ala→Pro	Fontainebleau		16.0±1.1 (n=12)	26	Y
	α22Gly→Asp	J-Medellin		22	58	Y
	α23Glu→Gln	Memphis		~17	-1	Y
	α23Glu→Val	G-Audhali		14	-30	Y
	α26Ala→Val	Campinas		24	28	Y
	α27Glu→Ala	Hackney (Novel)		15	-58	Y
	α27Glu→Asp	Hekinan		17	-14	Y
	α27Glu→Gly	Fort Worth		4 & 9	-72	Y

	Mutation	Name	Comments	%	ΔM	TMS
αT4	α27Glu→Lys	Shuangfeng		~11	-1	N
	α27Glu→Val	Spanish Town		12	-30	Y
	α29Leu→Val	Novel		19	-14	Y
	α30Glu→Gln	G-Honolulu	[Distinguish	~31	-1	Y
	α30Glu→Lys	O-Padova	by ce-HPLC]		-1	Y
	α31Arg→Ser	Prato		21	-69	N
αT5	α32Met→Thr	Novel		18	-30	Y
	α38Thr→Ala	Beaconsfield (Novel)		23	-30	Y
	α38Thr→Ile	Chelsea (Novel)		25	12	Y
	α40Lys→Asn	Saratoga Springs	mE	17	-14	N
	α40Lys→Gln	Linwood	mE	(25)	0	N
αT6	α41Thr→Ser	Miyano	E	27	-14	Y
	α42Tyr→His	Barika		22	-26	Y
	α44Pro→Ala	Milne (Novel)		30	-26	Y
	α44Pro→Arg	Kawachi		25	59	NY
	α46Phe→Val	Hillingdon (Novel)		25	-48	Y
	α47Asp→Asn	Arya		~23	-1	N
	α47Asp→His	Hasharon		19	22	N
	α47Asp→Tyr	Kurdistan		21	48	N
	α48Leu→Arg	Montgomery		22	43	N
	α48Leu→Pro	Reading (Novel)		17	-16	Y
	α49Ser→Arg	Savaria (α2)		22	69	N
	α50His→Asp	J-Sardegna		29	-22	Y
	α50His→Leu	Dublin (Novel)		26	-24	Y
	α50His→Tyr	South Yorkshire (Novel)		31	26	Y
	α51Gly→Arg	Russ		20	99	N
	α51Gly→Ser	Riccarton (α1)	Affects measurement of HbA_{1C} by ce-HPLC	20.7±0.6 (n=70)	30	Y
	α51Gly→Ser	Riccarton-II (α2)		26	30	Y
	α(51-58)→0 or α(52-59)→0	J-Biskra		21	-765	N
	α52Ser→Ala	Cheshire (Cheshire)		24	-16	Y
αT6	α52Ser→Pro & S	Novel		30	10	N
	α54Gln→Arg	Shimonoseki		25	28	N
	α54Gln→Glu	Mexico		~29	1	Y
	α54Gln→His	Novel, Princes Risborough		22	9	N
	α54Gln→Pro	Dhaka (Novel)		18	-31	N

	Mutation	Name	Comments	%	ΔM	TMS
αT6	α55Val→Ile or Leu	Novel or Roubaix		25	14	Y
	α56Lys→Glu	Shaare Zedek		(25)	1	N
αT7	α57Gly→Arg	L-Persian Gulf		22	99	N
	α57Gly→Asp	J-Norfolk		27	58	Y
	α59Gly→Ser	Parma (α1)		15	30	Y
	α60Lys→Asn	Zambia		19	-14	N
αT9	α62Val→Ala	Novel		7	-28	Y
	α63Ala→Asp	J-Pontoise		19	44	Y
	α63Ala→Thr	Novel		25	30	Y
	α64Asp→Asn	G-Waimanalo	α-CT	~24	-1	Y
	α64Asp→His	Q-India		19	22	Y
	α68Asn→His	Jeddah (α1)/St. Truiden (α2)		22/43	23	Y
	α68Asn→Lys	G-Philadelphia		36	14	N
	α68Asn→Tyr	Chelmsford (Novel)		24	49	Y
	α70Val→Gly	Edinburgh (Novel)		7	-42	Y
	α71Ala→Glu	J-Habana		25	58	Y
	α71Ala→Thr & D-Punjab	Hatfield (Novel)		27	30	Y
	α72His→Gln	Gouda		23	-9	Y
	α74Asp→Asn	G-Pest	α-CT	~15	-1	Y
	α74Asp→Gly	Chapel Hill		20	-58	Y
	α74Asp→His	Q-Thailand (α1)	with α-thal	32	22	Y
	α74Asp→Tyr	Uttoxeter (Novel)		17	48	Y
	α74Asp or α75Asp→0	Watts		10	-115	Y
	α75Asp→His	Q-Iran		22	22	Y
	α75Asp→Tyr	Winnipeg		15	48	Y
	α76Met→Arg	Walpole (Novel)		9	25	N
	α76Met→Thr	Aztec		19	-30	Y
	α77Pro→His	Toulon		24	40	N
	α78Asn→Lys	Stanleyville II		25	14	N
	α81Ser→Cys	Nigeria		As received 25 Reduced 35	16	Y
	α84Ser→Arg	Etobicoke		18	69	N
	α85Asp→Asn	G-Norfolk	α-CT	~20	-1	Y
	α85Asp→Glu	Aylesbury (Novel)		24	14	Y

	Mutation	Name	Comments	%	ΔM	TMS
αT9	α85Asp→Tyr	Atago		21	48	Y
	α85Asp→Val	Inkster (α2)		28	-16	Y
	α86Leu→Val	Ridgewood (Novel)		26	-14	Y
	α87His→Tyr	M-Iwate	Cyanosis	25	26	Y
	α88Ala→Glu	Wroclaw (Novel)		22	58	Y
	α89His→Gln	Enfield (Novel)		24	-9	Y
	α89His→Leu	Luton	E with α-thal	39	-24	Y
	α90Lys→Asn	J-Broussais		22	-14	N
	α90Lys→Gln	Bergerac		(31)	0	N
	α90Lys→Glu	Sudbury (Novel)		~22	1	N
	α90Lys→Thr	J-Rajappen		26	-27	N
αT10	α91Leu→Phe	Vientiane		25	34	N
	α92Arg→Gln	J-Cape Town	mE	29/38	-28	N
	α92Arg→Leu	Chesapeake		23	-43	N
αT11	α94Asp→Asn	Titusville		?	-1	N
	α94Asp→Glu	Roanne		22	14	Y
	α94Asp→Gly	Capa (α1)		24	-58	Y
	α94Asp→Tyr	Setif		17	48	N
	α95Pro→Ala	Denmark Hill		29	-26	N
	α95Pro→Arg	St Luke's (α1)		11	59	N
	α95Pro→Leu	G-Georgia		17	16	N
	α98Phe→Tyr & Sickle	Mill Hill (Novel)		19	16	Y
	α99Lys→Asn	Fulton (Novel)		18	-14	N
αT12	α102Ser→Arg	Manitoba		11	69	N
	α103His→Tyr	Charolles (α1), Lombard (α2)		37	26	Y
	α107Val→Met	Novel		26	32	Y
	α108Thr→Asn	Bleuland		14	13	Y
	α110Ala→Val	White Rose (Novel)		16	28	Y
	α112His→Asp	Hopkins-II		15	-22	Y
	α113Leu→Arg	San Antonio		26	43	Y
	α114Pro→Ala	Broomhill (Novel)		19	-26	Y
	α114Pro→Arg	Chiapas		19	59	N
	α115Ala→0	Towson (α2?)		27	-71	Y
	α116Glu→Gln	Oleander	α-CT	~23	-1	N
	α116Glu→Lys	O-Indonesia		~22	-1	N

	Mutation	Name	Comments	%	ΔM	TMS
αT12	α117-118 Leu or Ile inserted	(Phnom Penh, Ile inserted (α1)		18	113	Y
	α119Pro→Leu	Diamant	α-CT	9	16	Y
	α120Ala→Glu	J-Meerut		21.7±0.7 (n=11)	58	N
	α121Val→Met	Owari		22	32	Y
	α122His→Gln	Westmead		17	-9	Y
	α123Ala→Asp	Novel	α-CT	8	44	Y
	α123Ala→Thr	Santa Barnabas		19	30	Y
	α123Ala→Val	Pressath (Novel)		19	28	Y
	α124Ser→Phe	Batley (Novel)		10	60	Y
	α126Asp→Gly	West One		22	-58	N
αT13	α130Ala→Pro	Sun Prairie	α-CT	15	26	Y
	α131Ser→Ala & LeLamentin	Novel		21	-16	Y
	α134Thr→Ser	Kenton (Novel)		21	-14	Y
	α136Leu→Met	Chicago		36	18	Y
	α139Lys→Ile	Novel		24	-15	N
	α139-141 → α139NTVKLEPR	Wayne	Mr ~15,617.5	12	491	N
αT14	α140Tyr-Arg→0	Natal	Mr 1,5807.02	24	-319	N
	α140Tyr→His	Ethiopia		23	-26	N
	α141Arg→His	Suresnes		29	-19	N
	α141Arg→0	Koelliker[(a)]			-156	N
	α142Stop→142Gln	Constant Spring	Mr 18,481.29 Bio-Rad 5.04 min	3	3355	Y
	α142Stop→142Glu	Seal Rock	Mr 18,482.28 Bio-Rad 4.79 min	1.2	3356	Y

β-chain variants

	Mutation	Name	Comments	%	ΔM	TMS
βT1	β1Val→NAc-Ala	Raleigh		54	14	N
	β1Val→Gly	Watford (Novel)		42	-42	N
	β1Val→Met Initiator Met retained	South Florida	Mr 16,030.50	49 incl. glycated β^A	163	N
	β2His→Arg	Deer Lodge		48	19	N
	β2His→Gln	Okayama		53	-9	N
	β2His→Gln/ β26 Glu→Lys	Novel Okayama/E in same chain		27	-10	N
	β2His→Pro, initiator Met retained	Marseille	Mr 15,958.41	55	91	N
	β4Thr→Pro	Benin City (Novel)		~33	-4	N
	β5Pro→Ala	Gorwihl (Novel)		48	-26	N
	β5Pro→Ser	Tyne		43.6±2.1 SD (n=19)	-10	N
	β6Glu→Lys	C			-1	N
	β6Glu→Lys/ β83Gly→Asp	C-New Cross (Novel) C/Pyrgos in same chain		47	57	N
	β6Glu→Lys/ β98Val→Met	Kingsbury (Novel) C/Köln in same chain	SHA Mr 15,898.36	As received 8.7 Reduced 11	31	Y
	β6Glu→Val	Sickle			-30	Y
	β6Glu→Val/ β58Pro→Arg	C-Ziguinchor, S/Dhofar in same chain	Mr 15,896.33	39	29	Y
	β7Glu→Gly	G-San José		40	-72	Y
	β7Glu→Lys	G-Siriraj		~35	-1	NY
βT2	β9Ser→Cys	Pôrto Alegre	Dimerises	Reduced 40	16	Y
	β10Ala→Thr	Belleville (Novel)		46	30	Y
	β10Ala→Val	Iraq-Halabja		47	28	Y
	β13Ala→Val	Novel		52	28	Y
	β14Leu→Arg	Sogn		30	43	N
	β15Trp→Arg	Belfast	HzB	30	-30	N
	β15Trp→Cys	Garston (Novel)		27	-83	N
	β16Gly→Arg	D-Bushman		48	99	N

	Mutation	Name	Comments	%	ΔM	TMS
βT2	β16Gly→Asp	J-Baltimore	Diagnostic βT2-3^{2+} ion at *m/z* 1,143.59	50.7±0.8, incl δ (n=18)	58	N
	β17Lys→Gln & G-Philadelphia	Nikosia	Diagnostic βT2-3^{2+} ion at *m/z* 1,114.59	---	0	N
βT3	β18Val→Gly	Sinai-Baltimore		40	-42	Y
	β19Asn→Asp	Alamo		(49)	1	Y
	β19Asn→Lys	D-Ouled Rabah		45	14	Y
	β20Val→Met	Olympia	ME	50	32	Y
	β21Asp→Val	Rocky Mountain (Novel)		38	-16	N
	β22Glu→Ala	G-Coushatta		46	-58	Y
	β22Glu→Gln	D-Iran		~45	-1	Y
	β22Glu→Gly	G-Taipei		43	-72	Y
	β22Glu→Lys	E-Saskatoon	ce-HPLC re D-Iran	(46)	-1	Y
	β24Gly→Asp	Moscva		38	58	Y
	β26Glu→Asp	Marijampolė (Novel)		46	-14	Y
	β26Glu→Gln	King's Mill (Novel)		~51	-1	Y
	β26Glu→Lys	E	Target cells	~25	-1	N
	β27Ala→Val	Grange-Blanche	mE	50	28	N
	β30Arg→Ser	Tacoma	HzB	43	-69	N
βT4	(β31Leu→Pro, not identified by MS	Yokohama	HzB, SHA	~7	-16	--
	β36Pro→Ser	North Chicago	E	44	-10	N
	β38Thr→Asn	Hinwil	mE	43	13	N
	β39Gln→Lys	Alabama		(40)	0	N
	β40Arg→Lys	Athens-GA		49	-28	N
βT5	β42Phe→Cys	Little Venice (Novel)	*de novo* HzB, SHA	As received 19 Reduced 21	-44	Y
	β42Phe→Leu/(Ile)	Louisville	mHA, HzB, α-CT	34	-34	Y
	β42Phe→Ser	Hammersmith	*de novo*, HzB, SHA	24	-60	Y
	β43Glu→Gly	Haringey (Novel)		47	-72	N
	β43Glu→Lys	Hornchurch (Novel)		~40	-1	N
	β45Phe→Ser	Cheverly	HzB	42	-60	Y
	β46Gly→Arg	Gainesville-GA		37	99	N
	β46Gly→Glu	K-Ibadan		40	72	N

	Mutation	Name	Comments	%	ΔM	TMS
βT5	β47Asp→Tyr	Maputo		44	48	Y
	β47Asp→Val	Muravera		38	-16	Y
	β51Pro→Arg	Willamette	Target cells	35	59	N
	β51Pro→His	North Manchester (Novel)		50	40	Y
	β52Asp→Ala	Ocho Rios		46	-44	Y
	β52Asp→Asn	Osu Christiansborg		40	-1	Y
	β52Asp→Gly & D-Punjab	Hokusetsu		---	-58	Y
	β52Asp→His	Summer Hill		44	22	Y
	β54Val→Ile & S & Stanleyville II	Askew (Novel)		---	14	Y
	β56Gly→Arg	Hamadan		51	99	N
	β56Gly→Asp	J-Bangkok		51	58	N
	β56Gly→Cys	Leeds (Novel)		42	46	N
	β57Asn→His & Stanleyville II	Sidcup (Novel)		40	23	N
	β57Asn→Lys	G-Ferrara		34	14	Y
	β58Pro→Arg	Dhofar		20 & 40	59	N
	β58Pro→His	Sheffield (Novel)		46	40	Y
	β59Lys→Asn	J-Lome		50	-14	N
	β59Lys→Gln	Hillsborough (Novel)		(48)	0	N
	β59Lys→Glu	I-High Wycombe		~43	1	N
	β59Lys→Thr	J-Kaohsiung		47	-27	N
βT6	β61Lys→Thr	Novel		45	-27	N
βT7	β64Gly→Asp	J-Calabria		42	58	NY
βT9	β69Gly→Asp	Rambam, J-Cambridge		51	58	Y
	β69Gly→Ser & Hasharon	City of Hope		51	30	Y
	β70Ala→Asp	Seattle	HzB, mHA	25	44	Y
	β71Phe→Ser	Christchurch	HzB, HA	30	-60	N
	β73Asp→Asn	G-Accra, Korle-Bu		50	-1	Y
	β74Gly→Val	Bushwick	HA	35	42	Y
	β77His→Asp	J-Iran		50	-22	N
	β77His→Tyr	Fukujama		48	26	NY
	β79Asp→Asn	Yaizu		~40	-1	Y
	β79Asp→His	Tigraye		43	22	Y
	β80Asn→Tyr	Hounslow (Novel)		43	49	N
	β82Lys→Arg	Taradale (Novel)		48.5±0.6 (n=25)	28	Y

	Mutation	Name	Comments	%	ΔM	TMS
βT9	β82Lys→Met	Helsinki	E	---	3	N
βT10	β83Gly→Asp	Pyrgos	βT(9-10-11)$^{5+}$	54	58	N
	β83Gly→Cys	Ta-Li	Dimerises	Reduced 41	46	N
	β85Phe→Ser	Buenos Aires		35	-60	Y
	β86Ala→Val	Novel, Izmir		47	28	Y
	β87Thr→Ile	Quebec-Chori (Novel)		~40	12	Y
	β87Thr→Ile & β6Glu→Val	Quebec-Chori with Sickle cell trait	Moderate to severe sickle cell disease	60	12	Y
	β87Thr→Lys	D-Ibadan		48	27	N
	β87Thr→Pro	Valletta		~50	-4	Y
	β90Glu→Asp	Pierre-Bénite	E	48	-14	Y
	β90Glu→Gly	Roseau-Pointe a Pitre		11	-72	Y
	β90Glu→Lys	Agenogi		~44	-1	N
	β91Leu→Arg	Caribbean		32	43	N
	β91Leu→Pro	Sabine	HzB, SHA	As received 7 Reduced 26	-16	Y
	β93Cys→Arg	Okazaki		48	53	N
	β94Asp→Asn	Bunbury	mE	~43	-1	N
	β95Lys→Asn	Detroit		50	-14	N
	β95Lys→Glu	N-Baltimore		~46	1	Y
βT11	β97His→Asn	Santa Clara		52	-23	Y
	β98Val→Met	Köln	HzB, mHA	Reduced 35	32	NY
	β99Asp→Asn	Kempsey	E	~48	-1	Y
	β99Asp→His	Yakima	E	43	22	N
	β101Glu→Ala	Youngstown (Novel)		43	-58	Y?
	β101Glu→Asp	Potomac	E	46	-14	Y
	β103Phe→Ile or Leu	Saint Nazaire or Heathrow	E	41	-34	Y
	β103Phe→Val	Sparta	mE	18	-48	Y
	β104Arg→Lys	Alzette		52	-28	Y
	β104Arg→Ser	Camperdown		51	-69	N
	β104Arg→Thr	Sherwood Forest		54	-55	N
βT12	β105Leu→Val	L'Aquila (Novel)	mHA	42	-14	Y
	β106Leu→Pro	Southampton	*de novo*, SHA	18	-16	Y
	β108Asn→Asp	Yoshizuka		~50	1	N
	β108Asn→Lys	Presbyterian		40	14	N

	Mutation	Name	Comments	%	ΔM	TMS
βT12	β108Asn→Ser	Santa Juana		44	-27	N
	β109Val→Leu	Johnstown	mE	47	14	Y
	β109Val→Met	San Diego	E	54	32	Y
	β111Val→Phe	Peterborough	mHA	34	48	N
	β113Val→Glu	New York	Silent by ce-HPLC	43	30	Y
	β116His→Arg	Sfax		55	19	N
	β116His→Tyr	Rhode Island (Novel)		45	26	Y
	β118Phe→Cys	Harrow		39	-44	N
	β118Phe→Tyr	Minneapolis-Laos		45	16	Y
	β119Gly→Asp & Sickle	Fannin-Lubbock I		---	58	N
	β111Val→Leu/ β119Gly→Asp	Fannin-Lubbock II		40	72	Y
βT13	β121Glu→Gln	D-Los Angeles, D-Punjab		~40	-1	N
	β121Glu→Lys	O-Arab		~34	-1	N
	β121Glu→Val	Beograd		40	-30	N
	β124Pro→Arg	Khartoum		35	59	Y
	β124Pro→Gln	Ty Gard	E	41	31	Y
	β125Pro→Thr & Lepore B-W	Novara (Novel)		---	4	Y
	β126Val→Ala & D-Punjab	Beirut		49	-28	N
	β126Val→Glu	Hofu		37	30	Y
	β126Val→Leu	Molfetta		49	14	N
	β128Ala→Asp	J-Guantanamo	Target cells	45	44	Y
	β128Ala→Val	Sitia		44	28	Y
	β129Ala→Asp	J-Taichung		36	44	Y
	β129Ala→Val	La Desirade		39	28	Y
	β131Gln→Glu	Camden		~46	1	Y
	β131Gln→His	Silver Springs		42	9	Y
	β132Lys→Gln	K-Woolwich		(44)	0	N
βT14	β133Val→Ala	Renert (Novel)		43	-28	Y
	β134Val→Glu	North Shore	mHA	31	30	Y
	β135Ala→Asp	Beckman		51	44	Y
	β135Ala→Val	Alperton (Novel)	also with β-thal	48	28	Y
	β136Gly→Ala	Petit Bourg		52	14	Y
	β136Gly→Asp & S	Hope		50 (incl δ)	58	N
	β139Asn→Lys	Hinsdale		47	14	N
	β140Ala→Asp	Himeji		28	44	Y

	Mutation	Name	Comments	%	ΔM	TMS
βT14	β140Ala→Asp	Himeji		28	44	Y
	β142Ala→Thr	Inglewood		45	30	Y
	β143His→Arg	Abruzzo	E	43	19	N
	β143His→Tyr	Old Dominion/ Burton-upon-Trent		49.3±0.9 (n=6)	26	N
	β144Lys→Asn	Andrew-Minneapolis	E	52	-14	N
	β144Lys→Met	Barbizon		~45	3	N
βT15	β145Tyr→Cys	Rainier	(strong -S-S-), E	45	-60	NY
	β145Tyr→His	Bethesda	E	46	-26	N
	β146His→Leu	Cowtown	E	53	-24	N
	β146His→Tyr	Bologna-St.Orsola	ME	54	26	N
	β147TKLAFLLSNFY	Tak	Mr 17,165.79	42	1299	N

δ-chain variants

Mutation	Name	ΔM
δ16Gly→Arg	Hb A_2' or Hb B_2	99

^{G}γ-chain variants

Mutation	Name	Comment	ΔM
^{G}γ63His→Tyr	F-M-Osaka	Cyanosis	26
^{G}γ65Lys→Asn	F-Clarke		-14

Hybrids

Mutation	Name	ΔM	TMS
δ22 - - - - β50	Lepore-Hollandia (15836.23 Da)	-31	N
δ50 - - - - β86	Lepore-Baltimore (15822.20 Da)	-45	N
δ87 - - - - β116	Lepore-Boston-Washington (15865.23 Da)	-2	Y
^{A}γ80 - - - - β87	Kenya (15922.23 Da)	55	N
β22 - - - - δ50	P-Nilotic (15955.33 Da)	88	N

Notes

ΔM: Nominal mass change from normal due to mutation.

%: 100 x Variant / (variant + normal) in heterozygotes. Determined from %BPI values in MaxEnt processed data, which was centred using areas.

(%): Determined from HPLC data as 100 x Variant / (Variant + A_0).

In all +58 Da variants, δ is included in intensity of variant.

~%: Estimated using mass change from normal. Less accurate than %.

[a] Post translational modification

E: erythrocytosis, mE: mild erythrocytosis, ME: marked erythrocytosis

mHA or SHA: mild or severe haemolytic anaemia (HA)

Cyan: Cyanosis

HzB: Heinz bodies

α-CT: Recommend α-chymotrypsin digest for identification.

AR: From as received sample. Red: reduced with dithiothreitol.

β119Gly→Asp/β111Val→Leu, for example, implies both mutations occur in the same chain.

β126Val→Ala & D-Punjab, for example, implies the variant occurs in a compound heterozygote.

TMS: Indicates whether or not the tryptic peptide containing the variant needs sequencing by tandem MS in order to identify the variant.

N: Tandem MS is not required. Identifying the tryptic peptide containing the variant identifies the variant. This occurs when there is only one mutation in the peptide that can give the mass change from normal (ΔM) by a single base change in the nucleotide codon. This also occurs when the variant involves Arg or Lys (excluding Arg↔Lys) to either create a 'new' tryptic cleavage site and hence two 'new' smaller peptides or abolish an existing cleavage site to combine two adjacent peptides into one 'new' larger peptide.

Y: Tandem MS is required when two or more possibilities occur in the tryptic peptide containing the variant and ce-HPLC data are not available for comparison purposes.

NY: Tandem MS may be required due to poorly defined peptide ions in the digest, or when ce-HPLC data are not available to establish the polarity change due to the mutation.

155 α-chain variants including 46 that were novel when first submitted for analysis by mass spectrometry.

166 β-chain variants including 31 that were novel when first submitted for analysis by mass spectrometry.

1 δ-chain variant.

2 $^{G}\gamma$-chain variants

5 hybrids.

Total: 329 different variants, including 77 that were novel when first submitted for analysis by mass spectrometry.

Yokohama is not included in the total number of β-chain variants.

SECTION 7

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About the Author

Brian Noel Green was born on Christmas Day, 1933 in Urmston Cottage Hospital on the western fringes of Manchester, UK. With two younger brothers, he lived behind an ironmonger's shop kept by his mother. His father was a draughtsman at Metropolitan Vickers Electrical Company (MV) and had converted what was originally meant to be the living room into a workshop where he repaired radios in his spare time. Thus, Brian developed an early interest in electronics and, because one of his brothers was building a 5-inch gauge model steam locomotive, also gained a useful background in metalworking. In 1955, he fulfilled one of his early ambitions to gain an amateur radio licence (call-sign G3KCB).

Brian studied at Manchester Grammar School and gained a scholarship to Manchester University, from where he graduated in 1955 with a B.Sc. in Honours General Science Section II. He then spent two years as a college apprentice at MV in Trafford Park, Manchester working in various departments in the Company. The Research Department appeared to be more interesting than heavy electrical engineering so in 1957, Brian joined the group under John Waldron, which, amongst other scientific instrumentation projects, was involved in the development of mass spectrometers. An early recollection was of John Beynon and the development group obtaining a 'typical' spectrum of the $CO/N_2/C_2H_4$ triplet between flashovers on the MS8 - the forerunner to the MS9. John Waldron was believed to have stated that the guarantee on the MS8 would be null and void if John Beynon introduced any organic chemicals into it.

Working under Robert Craig, Brian's first project was to develop a residual gas analyser based on a 2-inch radius, 180° permanent magnet (MS10). He then spent some time working on the production version of the double-focusing spark-source instrument (MS7), and in 1961 he installed one of these at the RCA labs in Princeton, NJ. This was the first mass spectrometer that Associated Electrical Industries (AEI) exported to the USA (MV became AEI in 1960).

From 1962, Brian joined the team developing AEI's 12-inch radius high-resolution double-focusing instrument, the MS9, and was involved in the installation of several of the first instruments. In those days, the only method of recording spectra was on a photographic paper chart. A major disadvantage was that exact mass measurement was undertaken by peak matching; a very time-consuming procedure. At the Montreal ASMS meeting in 1964, Klaus Biemann (MIT) described a method of recording and automatically mass-measuring whole spectra acquired on a photoplate using a focal plane instrument (CEC 21-110). This announcement created panic at AEI, and a program was started immediately by John Halliday aiming to show that it was feasible to electrically record and mass measure entire 10,000 resolution spectra at 10 seconds/decade in mass with 10 ppm mass accuracy. This work culminated in the installation of an MS9 at Yale University Medical School (Sandy Lipsky) in early 1965, and Brian then spent 26 weeks showing experimentally, with Walt McMurray's help, that digitised data from analogue signals could be accurately mass measured. At that time, the fastest available digitizer operated at 1.5 kHz and the data were tape recorded and played back at a slower speed, a somewhat impracticable procedure. Nevertheless, AEI's three papers given at the ASMS meeting that year set the basis for the later explosion of data system processing of mass spectra as computers and digitisers developed. Brian met his future wife, Nancy, during his stay at Yale, and they married in 1967.

After an excursion into ESCA with Mickey Barber, Brian left AEI and re-joined Robert Craig in 1972 at the recently formed (1969) VG Micromass Company (MM) in Winsford, Cheshire, where he became technical manager with responsibility for the design, development and application of single- and double-focusing mass spectrometers for organic analysis. He was directly involved in the design and

development, in 1973, of the first double-focusing instrument made by VG: the 5-inch radius magnetic sector MM 7070. The first instrument had vertical geometry for Knudsen cell work, but thereafter, all organic instruments manufactured by VG Micromass had horizontal geometry in order to facilitate the high pumping speed needed for chemical ionisation. Over 500 MM 7070s had been manufactured when the instrument was superseded in 1987.

In 1974, Brian moved with the organic analysis part of the business (VG Organic) to Altrincham. In 1976, he was involved with the design and development of the MM ZAB (**Z**ero **A**lpha **B**eta aberrations), a 11.754-inch radius double-focusing magnetic sector instrument. This was designed to a performance specification proposed by John Beynon (University of Swansea) in order to undertake MIKES (mass-analysed ion kinetic energy) experiments. Consequently, the ZAB was a reverse-geometry instrument.

A significant milestone in sector instrument design was the development of fully laminated magnets using grain-oriented 0.3 mm steel strip. Brian did the design work and Peter Burns the mechanical design and manufacture (1980). This innovation considerably increased the scanning speed of magnetic sectors, reduced hysteresis, stray fields and defocusing effects, and gave a 20% increase in the mass range compared with previous solid-core magnets. It was also introduced on the ZAB magnet and led to VG Analytical receiving the Queen’s Award for Technological Achievement in 1987.

Following the 1980 invention of fast-atom bombardment (FAB) ionisation by Mickey Barber and the UMIST group, the mass range of labile organic compounds directly accessible to analysis by mass spectrometry increased dramatically. Brian worked closely with the UMIST team on the early applications of FAB and worked to develop extended mass range instruments that were capable of taking advantage of the new ionisation technique. The analysis of intact insulin (1982) spawned an interest in protein analysis that has remained with Brian until the present.

The application of mass spectrometry in the environmental field, particularly dioxin analysis, stimulated the development of specifically tailored instrumental control and processing software. In this field, sensitivity is paramount. Since the method used GC/MS with helium as the carrier gas, a simple inventive step, not published at the time (1985), was the development of an EI source that operated near to the ionisation potential of helium. This significantly reduced the suppression of sample ions by helium ions, and substantially increased the sensitivity. Derivatives of the MM 7070 developed during this period are still in use today and are considered to be the reference standard for dioxin determination.

Brian's wife, Nancy, died in 1985, the same year he was recognised by the Crown for his contributions to mass spectrometry when he was appointed an Officer of the Order of the British Empire. Whilst taking a subsequent break with his younger daughter, he met a piano dealer from whom he bought a player piano for £50. After substantial renovation, this became one of Brian's main out-of-work interests.

Brian continued in his development role within the organic group of companies and was the driving force behind the development of electrospray ionisation (ESI) within the Company. In 1988, work was started, with Stuart Jarvis, developing an ESI source on the already existing quadrupole instrument. The first instrument was delivered to Robin Aplin (University of Oxford) in 1990. Alone, or in combination with liquid chromatography, this softest of the ionisation techniques created an explosion in the application of mass spectrometry to the analysis of labile molecules and brought mass spectrometry to a much wider audience in the life, pharmaceutical and clinical sciences. ESI also extended the mass range of molecules that could be analysed by mass spectrometry, considerably expanding the areas of interest to at least 500kDa molecular weight. Brian's most cited collaborative paper (278 citations) is the 1992 discussion on the application of Maximum Entropy algorithms (developed by John Skilling) to the disentanglement of electrospray multiply charged mass-to-charge ratio data in order to generate spectra on a true mass scale.

In 1996 Brian was recognised by the British Mass Spectrometry Society and received the Aston Medal, which is awarded to scientists who have worked in the UK for "outstanding contributions to knowledge in the biological, chemical, engineering, mathematical, medical, or physical sciences relating directly to outstanding exploitation, application or development of mass spectrometry".

Since the mid-90's, Brian has focused much of his spare time on the application of mass spectrometry to the analysis of proteins, particularly haemoglobin and related molecules. He has developed protocols for the rapid identification of variants in human haemoglobin by mass spectrometry and has identified more than 320 different variants including 77 that had not been previously reported in the literature at the time they were analysed.

In October 2017, Brian celebrated 60 years of involvement with mass spectrometry. He is a named author on more than 140 scientific publications (110 since 1990), the majority of which have been written in collaboration with academic and industrial partners. Brian has two daughters, Katy and Meg, and two grandchildren.

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This book is a collation of the studies of Brian Green OBE on the analysis of human haemoglobin variants by mass spectrometry over the period from the introduction of Electrospray Ionisation in the late 1980s to his retirement in 2018.

The work describes the five steps that he employed in determining the mutations in variant haemoglobins following the identification of a suspicious result from routine clinical investigations. Over the years he developed a number of tools and procedures that allowed for the unambiguous determination of amino acid mutations in the globin chains, and the underlying mass spectrometry experiments that underpin these conclusions.

Examples are given for mutations in each of the tryptic digest peptide chains for both α- and β-globin, with a step-by-step guide to sample preparation, data collection, data processing and data analysis.

During the course of these studies 329 different human haemoglobin variants were characterised, namely; 155 α-chains, 166 β-chains, 1 δ-chain, 2 $^{G}\gamma$-chains and 5 hybrids. 77 variants (46 α-chain and 31 β-chain) were novel when first encountered by the author. With few exceptions (<2%), all the samples originated from patients resident in the UK.