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Chapter

Identification of Peptides and Proteins in Illegally Distributed Products by MALDI-TOF-MS

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Abstract

An analytical strategy based on matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) for identification of peptides and proteins in illegally distributed products is presented. The identified compounds include human growth hormone (hGH), human somatoliberin, anti-obesity drug (AOD), growth hormone releasing peptides (GHRP-2 and GHRP-6), Glycine-GHRP-2 and Glycine-GHRP-6, ipamorelin, insulin aspart and porcine, delta sleep-inducing peptide (DSIP), thymosin β4, insulin like growth factor (IGF), mechano growth factor (MGF), human chorionic gonadotropin (hCG), melanotan II, bremelanotide, dermorphin and body protecting compound (BPC 157). The identification of proteins was mainly based on peptide mass fingerprinting, i.e., *bottom up* approach, while the smaller peptides were identified through *de-novo* sequencing. In cases when a reference standard was available, complementary identification was performed by capillary electrophoresis in double-injection mode (DICE), where a suspicious product was compared with the reference standard through two consecutive injections within the same electrophoretic run.

Keywords: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, double-injection capillary electrophoresis, illegally distributed proteins and peptides

1. Introduction

A broad range of proteins and peptides, for various purposes of enhancement, such as human growth hormone (hGH), i.e., somatropin, can be obtained from the illicit market. These products are mainly marketed as lyophilized formulations in small glass containers often without labelling. The customers are exposed to a range of potential harms, besides from the active components, including bacterial and fungal or viral infections which may arise from the fact that they are administered parenterally.

Figure 1A illustrates the total number of injection vials containing white lyophilized product cake being seized by the Swedish Customs during nine years in the past, i.e., 2010–2018. A large proportion of these samples, i.e., 64%, contained

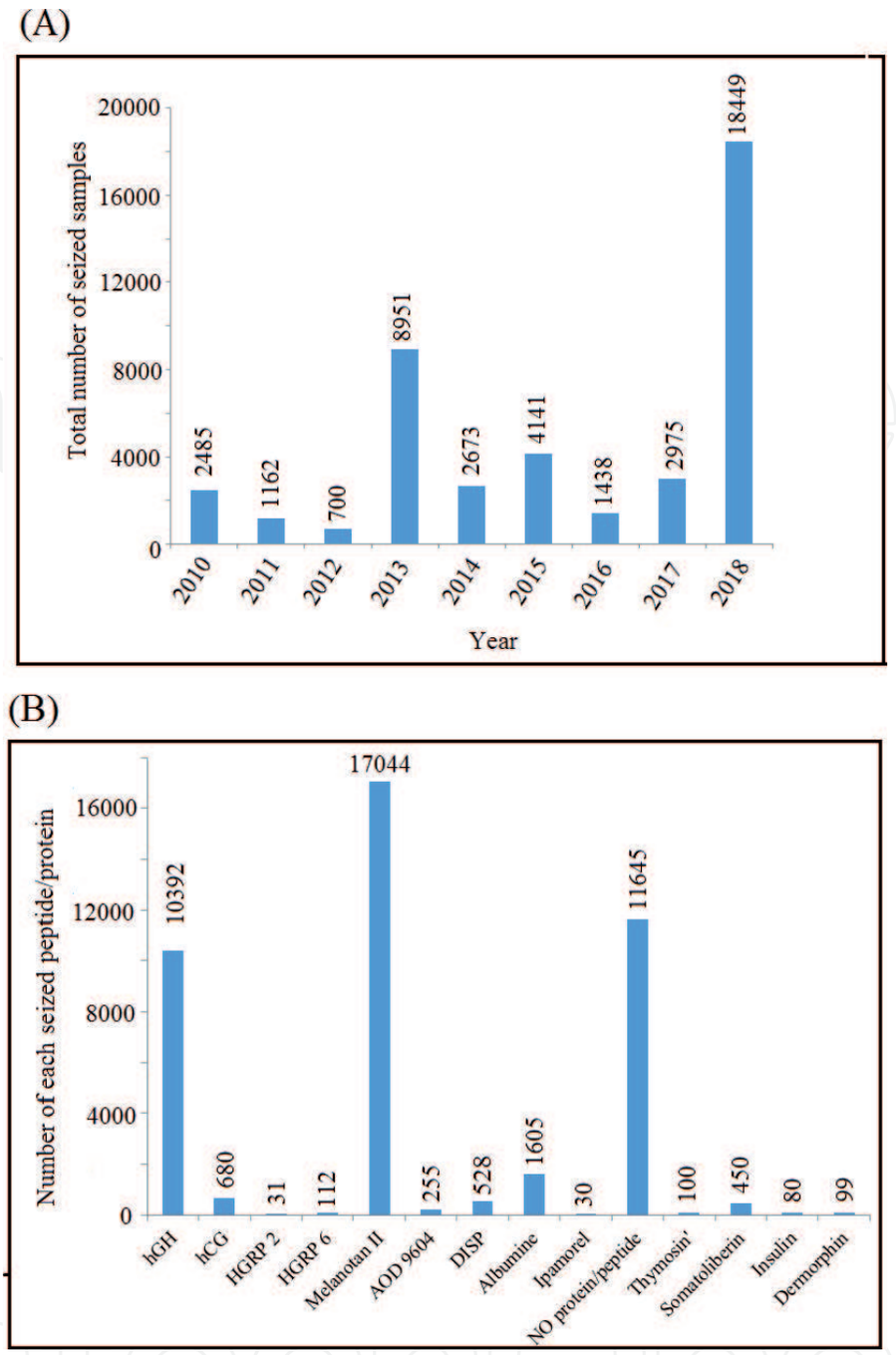


Figure 1.
Schematic illustration of the number of seized illicit products during 2010–2018 in Sweden (A), as well as the active peptides/proteins that have been identified in theses samples (B).

human growth hormone or melanotan II. About a third of the seized vials, i.e., 27%, did not contain any active peptide or protein, while the remaining 9% of the vials contained other compounds (**Figure 1B**).

The concept of a proteolytic peptide pattern, i.e., protein peptide mapping (PPM), being characteristic of a protein was first demonstrated by SDS-PAGE [1]. In 1989, peptide sequencing by automated Edman degradation had a cycle-time of nearly one hour per amino acid residue. Samples of interest often contained complex mixtures of proteins, which usually required separation by SDS-PAGE followed by electroblotting onto a polyvinylidene fluoride (PVDF) membrane [2]. However, a more rapid approach to peptide sequencing is "peptide mass fingerprinting" (PMF). By PMF, proteins are enzymatically cleaved in a predictable manner and the sizes of the generated peptide fragments are specific for different proteins.

Subsequent analysis of the obtained peptides by mass spectrometry (MS) generates mass-to-charge ratio (*m/z*) values in the mass spectrum which in turn give rise to a characteristic "peptide mass fingerprint" of the protein [3, 4]. The fingerprint serves to identify the protein by comparison with *in silico* digests, i.e., search engines attempt to match peptides from *in silico* digested proteins to those measured by the mass spectrometer [5–9]. Peptide mass fingerprinting with MS, which was first demonstrated with fast atom bombardment ionization in 1981, provides the possibility of identifying a protein at nanogram-level [5, 10–12]. Trypsin is a commonly used proteolytic enzyme for PMF, since it is relatively cheap, highly selective, and generates peptides with an average size of about 8–10 amino acids which are ideally suited for analysis by MS. It cleaves principally on the C-terminal side of arginine and lysine with the exception of Arg-Pro and Lys-Pro [2]. Limitations to protein identification by PMF include; I) The protein sequence must be present in a database for a successful protein identification. II) Proteins with extensive post-translational modifications may fail to yield good matches [13]. III) Different isoforms of a protein or alternatively spliced proteins may not be distinguished if the unique sequence regions are not observed in the peptide map. IV) Incomplete proteolytic digestion and differences in peptide ionization provide an incomplete mass fingerprint of the protein. Therefore, a complementary approach to PMF for protein identification is the use of tandem mass spectrometry (MS/MS), whereby tryptic peptide ions from the first stage of MS are dissociated along the backbone and then separated and detected in a second stage of MS to identify primary amino acid sequences [14–16]. Tandem mass spectrometry in conjunction with PMF provides even more specificity, thereby facilitating the identification [17, 18].

Since the innovation of sensitive commercial instrumentation based on MALDI-TOF MS in 1992, the technique has been widely used for protein identification due to its high sensitivity and mass accuracy, speed, extremely low material consumption, absence of multiple charge mass signals and relatively high tolerance toward additives and contaminants such as salts, matrix components and excipients [19–26]. Furthermore, MALDI is a micro-destructive analytical technique and the remaining material on the MALDI target plate can be archived for later analysis. The high sensitivity of MALDI implies that only a small aliquot of the digested protein is required for mass analysis, and the remainder can be used for alternative measurements. MALDI provides additional information regarding the primary structure of the protein by sequencing of selected tryptic peptide ions in post source decay (PSD) mode [27–34]. MALDI in-source decay (ISD) is another attractive method which generates partial sequence information of intact proteins with up to 20–50 amino acid residues [35] (**Figure 2**).

The sequence information from MALDI-PSD or MALDI-ISD analyses can be used to validate protein identification. The singly charged ions generated by MALDI-TOF-MS are a mixture of b-, y- and a-ions accompanied by ions resulting from neutral loss of ammonia or water [36–39].

PMF-based protein identification is accomplished by searching a protein sequence database using different search engines such as ProFound [40], Mascot [41], or SEQUEST [15]. A value-based scoring system has been developed that facilitates the identification without accompanying amino acid data [42, 43]. Parameters which are considered to be important for the identification include; molecular mass, protein sequence coverage and the number of matching peptides [42]. However, presence of a signature peptide, being unique for a protein, facilitates the PMF-based identifications [44]. Prior reports suggest that a minimum of four matching peptides and a sequence coverage of at least 20% is necessary for positive PMF-based protein identification [45, 46]. The other alternative strategy

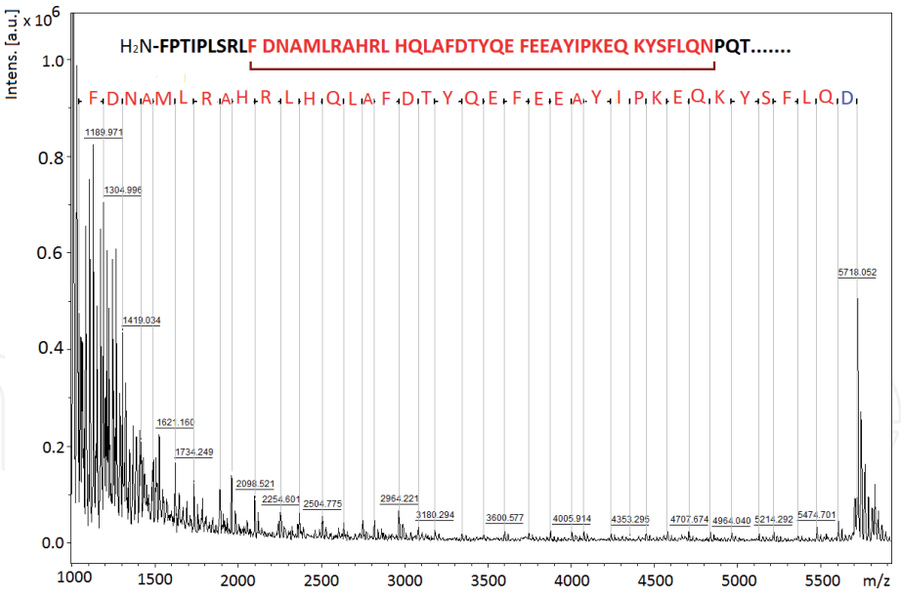


Figure 2.
MALDI in source decay analysis of a suspected illegal somatropin sample. The blue marked amino acid asp (D) is the deamidated form of Asn (N).

for protein identification is the top down approach, where intact molecule ions are subjected to gas-phase fragmentation [47].

Proteins with posttranslational modifications, such as glycosylation, present additional challenges since the masses of the modified peptides are different and thus do not contribute to the identification. In such cases, the protein can be analyzed by capillary electrophoresis (CE), in order to explore the heterogeneity of the protein followed by comparison of its electropherogram with that of the corresponding reference standard [13, 48].

2. Experimental

2.1 Sample preparation

MALDI-TOF-MS is very tolerant to salts and sample matrices, hence it is seldom necessary to desalt the sample. However, sometimes it is necessary to use a C_{18} micro-column in order to fractionate a complex sample or enhance the target analyte concentration.

The sample to be analyzed is mixed with a matrix solution (1:1, v:v), e.g. sinapinic acid (SA) or alpha-cyano-4-hydroxycinnamic acid (ACHCA). One μl of the mixture is deposited on the MALDI target plate and allowed to air-dry (i.e., the dried-droplet method) before being placed in the mass spectrometer [19, 49].

2.2 Proteolysis

The analyte to be digested is dissolved in ammonium bicarbonate (50 mM, pH 7.9). The intact sample is directly analyzed by MALDI in order to determine the molecular mass of the analyte. Then, 200 μl of the solution is digested by addition of 2–10 μl trypsin (200 μg/ml in 10 mM HCl). The reaction is carried out at room temperature or at 37°C for 30 minutes up to 24 hours, depending on peptide or protein in question. It has been found that 30 minutes digestion of somatropin at room temperature generated enough tryptic fragments for the MALDI analyses [50]. For more complex proteins, such as human chorionic gonadotropin, the required time

period for proteolysis is found to be 24 hours at 37°C. Insulin porcine is digested at 37°C for 12 hours, while other peptides are digested at 37°C for 4 hours. In order to enable alkylation of the cysteine residues in a protein or peptide, it is reduced by using DTT or 2-mercaptoethanol (ME) followed by labelling of the free thiol groups with 2-iodoacetamide. The alkylation is carried out through the following procedure:

1. 2.5 μl 100 mM ME is added to 10 μl of the protein solution.

2. The protein is then incubated at 50°C for 15 minutes to reduce the S-S linkages.

3. 2.5 μl 2-iodoacetamide (100 mM) is added into the mixture to interact with free sulfide groups of the cysteine residues at +4°C for 15 to 60 minutes in darkness.

4. 2.5 μl (10 μg/mL) trypsin is added to the mixture for the digestion. The reaction is performed at room temperature or at 37°C [13, 50].

2.3 Apparatus and operating conditions

MALDI-TOF analyses are performed using either an Autoflex or an Autoflex Max (Bruker Daltonics, Bremen, Germany) reflector type time-of-flight mass spectrometer, equipped with a pulsed nitrogen laser working at 337 nm and a smartbeam II laser working at 355 nm, respectively. The Autoflex instrument is operated in the positive ion mode with delayed extraction at an accelerating voltage of 20 kV and a variable voltage reflectron. The parameter settings are optimized to analyze peptides in reflectron mode. Before analysis, the instrument is externally calibrated with Bruker Daltonics standard peptide or protein mixtures. Peptide mass peaks occurring due to autolysis of trypsin (porcine) such as 842.51 and 2211.10 Da are also used for internal calibration. Mass spectra are obtained by averaging 250 laser shots (5× 50 shots) at different positions on the sample surface. All samples being used for post source decay (PSD) analysis are analyzed in the reflectron mode. The autoflex Max instrument TOF/TOF (2 kHz MS and 200 Hz MS/MS) operates in the positive ion mode. Metastable fragmentation is induced by laser (355 nm) without the further use of collision gas. The lyophilized samples are dissolved in 300 μL ammonium bicarbonate buffer (50 mM, pH 7.5). The liquid samples are diluted with same buffer. The wells of MALDI plate are spotted with 1 μl sample/matrix solution (1:1, v:v) and allowed to air dry before being placed in the mass spectrometer. ACHCA is used for analysis of peptides. About 20 mg of ACHCA is mixed in 1 ml of ethanol: acetonitrile (ACN) (1: 1 v/v) and 0.1% trifluoroacetic acid (TFA). SA is used for protein analysis. Two different solutions of SA in water and ethanol are made as follows: 1 - Saturated solution of SA in ethanol and 0.1% TFA; 2 - Saturated solution of SA in 50% acetonitrile (ACN) and 0.1% TFA. Solution 1 is first applied on the MALDI plate on which the sample mixed with SA in 50% ACN and 0.1% TFA (1: 1) is then applied.

3. Results and discussion

Illegally distributed lyophilized or liquid products being suspected to contain pharmacologically active peptides were seized by the Swedish customs. The analyte to be identified is analyzed in both reflectron and linear modes in order to determine its molecular mass (**Figure 3**). Large peptides and proteins are then exposed to trypsin digestion in order to obtain peptide-mass map upon MALDI analysis in

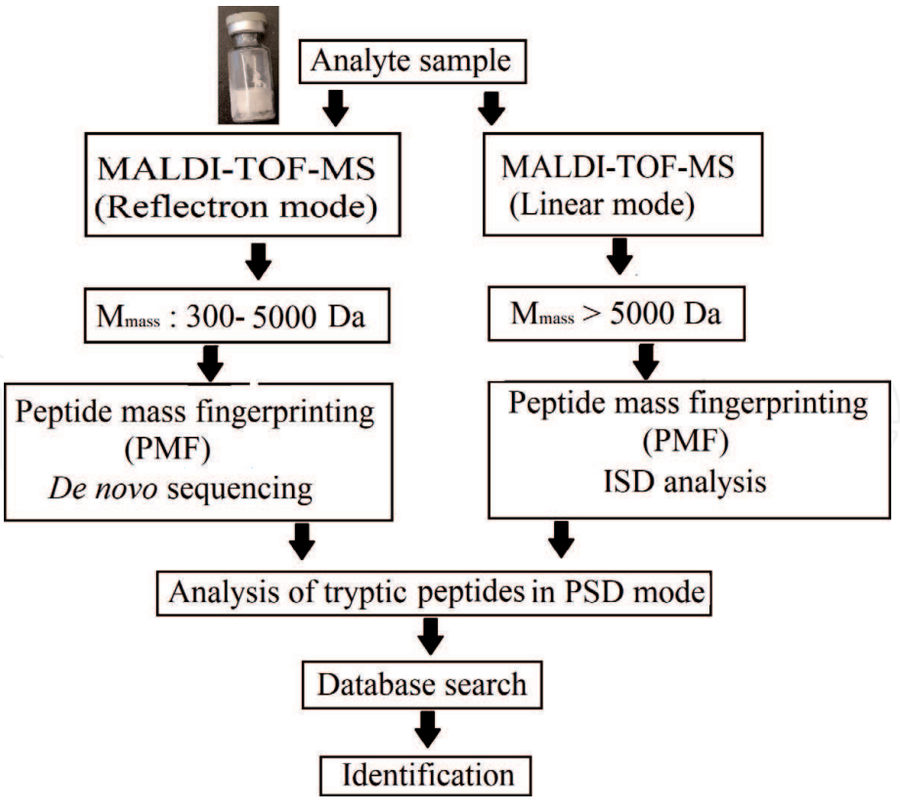


Figure 3.
The sample to be identified is analyzed in both reflectron and linear modes in order to determine the molecular mass of the analyte. Depending on the size of the molecule it will be exposed to enzymatic digestion in order to be identified through PMF. Small peptides used to be identified by de novo *sequencing in PSD mode.*

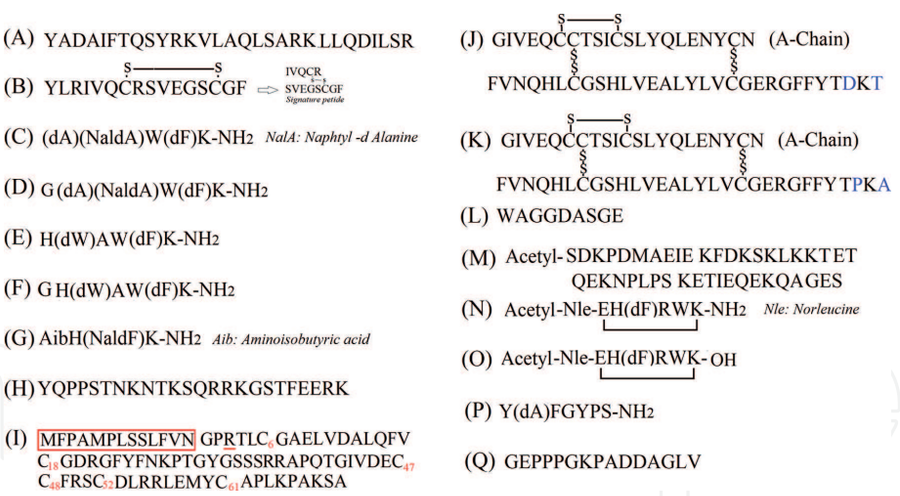


Figure 4.
The primary structure of the analyzed peptides. (A) Somatoliberin, (B) AOD, (C) GHRP-2, (D) glycine-GHRP-2, (E) GHRP-6, (F) glycine-GHRP-2, (G) Ipamorelin, (H) MGF, (I) long-R3-IGF (disulfide bridges: C_6-C_{48}; C_{47}-C_{52} and C_{18}-C_{61}; asp at position 3 is replaced by Arg), (J) insulin Aspart, (K) insulin porcine, (L) DSIP, (M) Thymosine β_4, (N) Melanotan II, (O) Bremelanotide, (P) Dermorphin and (Q) BPC 157. For molecular structures of somatropin and hCG see references [13, 50].

reflectron mode. Small peptides are, on the other hand, analyzed in reflectron mode and/or PSD mode directly. This strategy was applied to the identification of the following peptides and proteins (**Figure 4** and **Table 1**).

3.1 Identification of somatropin (hGH)

Recombinant hGH or somatropin consists of 191 amino acids with two disulfide bridges (Cys53-Cys165 and Cys182-Cys189) and promotes proteinogenesis as well as

Peptide	M_{mass}	PMF[a]	PSD[b]	ISD[c]	DICZE[d]	NMR	LC/MS
Somatropin	22115.07 22128.68[e]	X	—	X	X	X	X
Human Somatoliberin	3366.866	X	X	—	—	—	—
AOD (Anti Obesity Drug) HGH fragment 177–191	1813.850	X	X	—	—	—	—
GHRP-2	817.397	—	X	—	—	—	—
Gly-GHRP-2	874.419	—	X	—	—	—	—
GHRP-6	872.433	—	X	—	—	—	—
Gly-GHRP-6	929.455	—	X	—	—	—	—
Ipamorelin	711.385	—	X	—	—	—	—
MGF	2866.469	X	X	—	—	—	—
Long-R3-IGF	9105.385 9111.576[e]	X	X	—	—	—	—
Insulin Porcine	5772.766	X	X	—	X	X	X
Insulin Aspart	5821.611	X	X	—	X	X	X
DSIP	848.318	—	X	—	—	—	—
Thymosin-β_4	4960.474	X	X	—	—	—	—
hCG α - Chain β - Chain α + β	13,431[e] 23,114[e] 36,341[e]	X	X	—	X	—	—
Melanotan-II	1023.502	—	X	—	—	X	—
Bremelanotide	1024.510	—	X	—	—	X	—
Dermorphin	802.337	—	X	—	—	X	X
BPC-157	1418.692	—	X	—	—	X	X
Albumin bovine[f]	> 66,000[e]	X	—	—	—	—	—

[a] *Identification by peptide mass fingerprinting using enzymatic degradation as well as other modifications.*
[b] De novo *sequencing by MALDI- post source decay.*
[c] *Protein sequencing by MALDI- in source decay.*
[d] *Identification and/or impurity profiling by double injection capillary zone electrophoresis.*
[e] *Average molecular mass.*
[f] *Bovin albumin was detected in some of the samples.*

Table 1.
Illegally distributed peptides and proteins that have been analyzed by MALDI-ToF-MS and DICZE. The monoisotopic mass (M_{mass}) of the analytes and the employed analytical methodology is indicated.

fat mobilization and oxidation [51–53]. Recombinant hGH is used as a prescription drug to treat children's growth disorders and adult growth hormone deficiency. In the belief that the beneficial impact of somatropin on the growth can be extrapolated to healthy individuals, it is abused by bodybuilders and athletes [54]. However, many users are unaware of the correct dosage and how to prepare the solution for giving an injection. It has been demonstrated that supra-physiological dosages can have fatal consequences [55]. Apart from the undesired consequences following the abuse of somatropin, our investigations have shown that the illegally marketed products contained high levels of impurities such as endotoxins [50]. Endotoxins are associated with Gram-negative bacteria which can cause severe immune response and diseases in humans [56, 57]. Somatropin was identified through PMF and MALDI-ISD (see **Figure 2**) [48, 58, 59]. The availability of a compendial reference

standard has made it possible to apply double injection capillary zone electrophoresis (DICZE) for both identification and impurity determination of somatropin products [50, 58, 59]. The DICZE-method provided complementary information on the native protein, providing a side by side comparison between the electrophoretic patterns of the reference standard and the analyte to be identified [50].

3.2 Identification of human somatoliberin

Human somatoliberin, growth hormone-releasing hormone (GHRH), constitutes of 44 amino acids without any post-translational modification or disulfide bridge. Somatoliberin was first isolated from two pancreatic islet cell tumors, and subsequently from normal human hypothalamus [60–62]. The MALDI results from determination of the molecular mass, PMF and amino acid sequence revealed that the Asn_8 (N), Gly_{15} (G) and Met_{27} (M) residues have, respectively, been replaced by Gln_8 (Q), Ala_{15} (A) and Leu_{27} (L) during the synthesis (see **Figures 4** and **5**). The peptide was successfully identified by PMF and *de-novo* sequencing of three of the tryptic peptides.

3.3 Identification of an anti-obesity drug (AOD)

The AOD peptide is a fragment of the C-terminus of human growth hormone (fragment 177–191) where a tyrosine is added at the N-terminus. It is a cyclic peptide consisting of 16 amino acids with a disulfide bridge between cysteine residues at positions 7 and 14 in the peptide chain [63] (**Figure 4** and **Table 1**). The fragment is the minimum length of the hGH sequence that retains the lipolytic and antilipogenic properties of hGH [63–65]. The molecular peptide masses of its tryptic peptides complied with the peptide map of hGH fragment 177–191. The existence of the disulfide bridge between C_7 and C_{14} was confirmed upon analysis of the non-reduced tryptic sample (**Figure 6**). This peptide has also been employed as a signature peptide for the identification of hGH [48, 50]. The amino acid sequences of three selected tryptic peptides were also confirmed.

3.4 Identification of growth hormone releasing peptides (GHRP)

GHRP, including GHRP-2, GHRP-6, Gly-GHRP-2, Gly-GHRP-6 and ipamorelin, as an agonist of the gut peptide ghrelin is an endogenous ligand for the growth

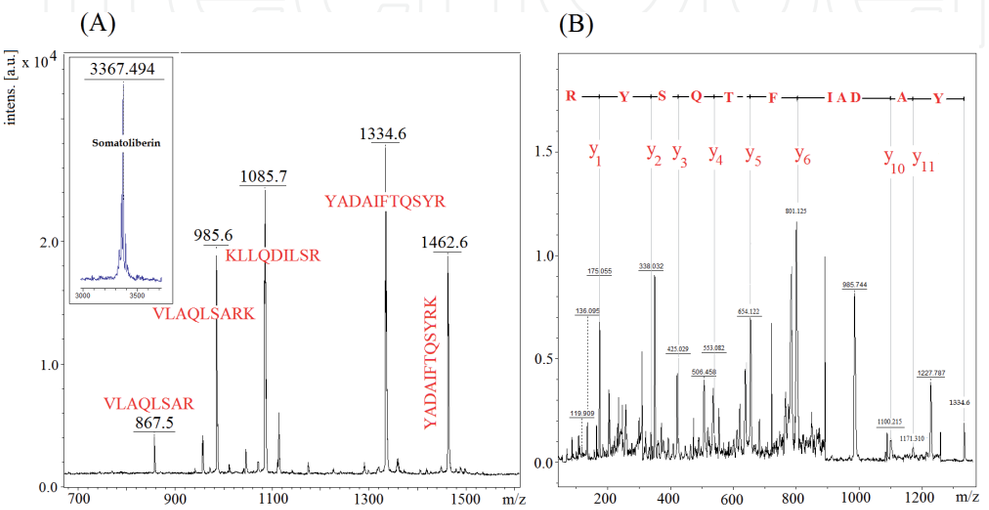


Figure 5.
MALDI-PMF (A) and MALDI-PSD (B) analysis of somatoliberin.

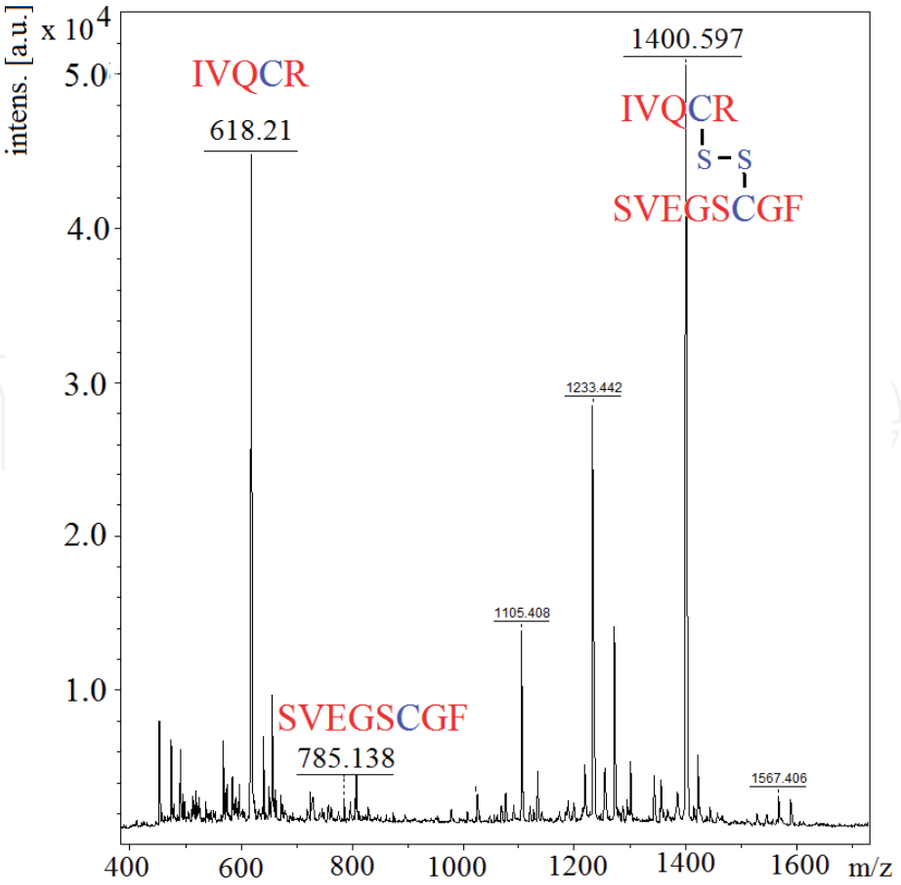


Figure 6.
MALDI-PSD analysis of AOD.

hormone secretagogue receptor [66, 67]. Ghrelin strongly stimulates food intake and GH release in humans [68–70]. These peptides were identified through *de-novo* sequencing. The amino acid sequence of GHRP-6 differs slightly from that of GHRP-2, i.e., the amino acid residues dA and Naphthyl alanine (NalA) in GHRP2 are replaced by H and dW in GHRP-6 (**Figure 4** and **Table 1**) [70].

Ipamorelin is a penta-peptide, being derived from GHRP-1 [71]. Ipamorelin like the other GHR-peptides, stimulates production of growth hormone [72]. Incorporation of aminoisobutyric acid (Aib) in the peptide chain increases the stability of the peptide (**Figure 4**) [73].

3.5 Identification of mechano growth factor (MGF) and long-R3 insulin-like growth factor (IGF-1)

MGF is a unique, spliced variant of IGF-1. MGF induces muscle cell proliferation in response to muscle stress and injury [74]. MGF and Long-R3-IGF1 were identified in several confiscated samples. Long-R3-IGF-1, an analogue of IGF-1, has 13 additional amino acids at its N-terminus (**Figure 4** and **Table 1**). IGF-1 mediates the anabolic and mitogenic activity of GH [75–77]. MGF and Long-R3-IGF1 were identified by sequence coverages of 100% and 43%, respectively (**Table 2** and **Figure 7**).

3.6 Identification of insulin porcine and insulin aspart

Insulin regulates the cellular uptake, utilization, and storage of glucose, amino acids, and fatty acids and inhibits the breakdown of glycogen, protein, and fat. Since more than one decade ago the illegal use of insulin has been noticed

Peptide fragments	Theoretical m/z $[M + H]^+$	Determined m/z $[M + H]^+$
YQPPSTNKNTKSQRRKGSTFEERK	2869.169	2869.422
Glu-C digestion of the peptide[a]:		
YQPPSTNKNTKSQRRKGSTFEE	2584.809	2584.816
Trypsin digestion of the peptide:		
GSTFEERK[b]	953.458	953.574
YQPPSTNK[b]	934.453	934.525
GSTFEER[b]	825.363	825.469
SQR[b]	390.199	390.186
NTK[b]	362.193	362.302

[a]*Glu-C cleaves at the C-terminus of either aspartic or glutamic acid residues.*
[b]*The amino acid sequence of the peptide was determined.*

Table 2.
MALDI peptide mass fingerprinting-data from analysis of mechano growth factor.

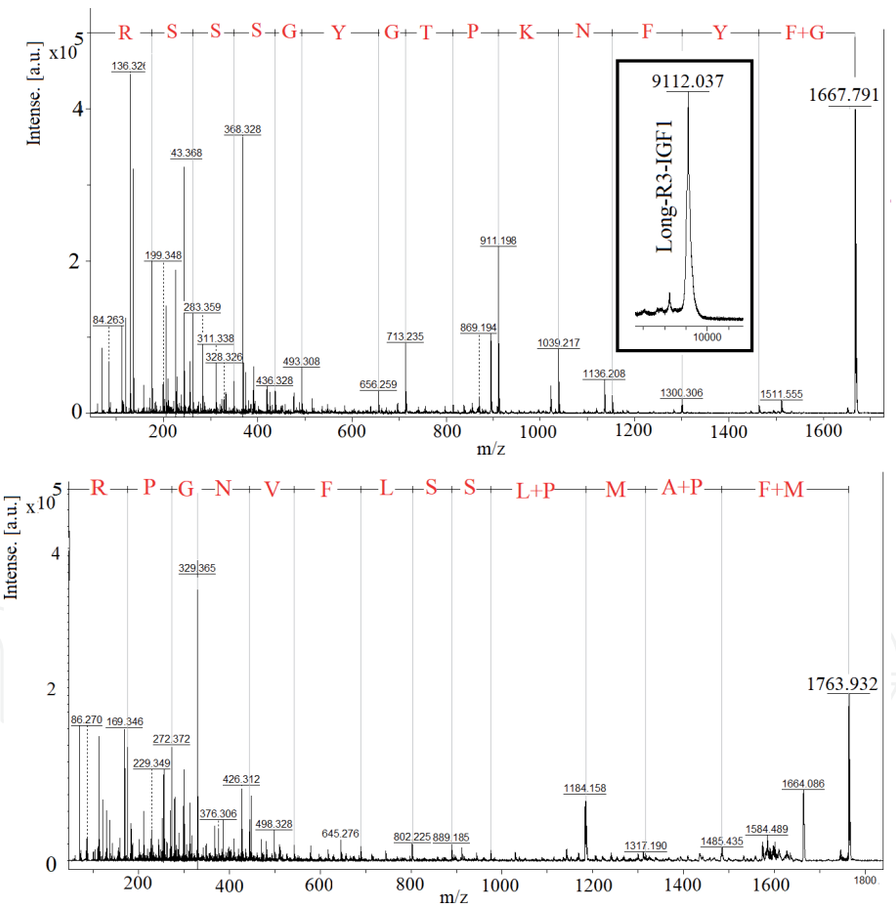


Figure 7.
MALDI analysis of intact long-R3-IGF and MALDI-PSD analysis of two tryptic peptides, i.e., m/z 1667.771 and m/z 1763.887.

[78]. However, the misuse and wrong administration of insulin could cause the, so called, dead in the bed syndrome [79]. In bodybuilding, insulin works such as testosterone or hGH to consolidate muscle tissue. Insulin also prevents breakdown of muscles and vanishes rapidly from the body, since it has a very short half-time ($t_{1/2}$) [80].

Several illegal products containing insulin porcine or aspart have been analyzed. Insulin is composed of two peptide chains, i.e., A and B, which are joined by two inter-chain disulfide bonds. The A chain also contains an intra-chain disulfide bond (**Figure 4**). The results summarized in **Table 3**, demonstrate the applied strategy for the identification of porcine and insulin aspart. The insulin molecules were reduced using a potent reducing agent, i.e., 2-mercaptoethanol (ME). MS-analysis of the reduced samples resulted in a mass spectrum consisting of several signals from both reduced A and B chains. The A and B chains generated three and four signals, respectively, corresponding to the ME-modified peptide as described in **Table 3**. It is to be noted that the amino acid residues P and A at positions 28 and 30 in the B-chain, respectively, have been replaced by D and T in insulin aspart. Therefore, these insulin molecules are distinguished upon these differences. The tryptic digestion of the B chain yielded three peptide fragments of different sizes (**Figure 8** and **Table 3**). The molecular masses of these peptides were determined accurately, and the amino acid sequence of the tryptic peptides were determined in PSD-mode.

Insulin	**Theoretical m/z $[M + H]^+$**	**Determined m/z $[M + H]^+$**
Porcine (intact)	5774.635	5774.632
Aspart (intact)	5822.612	5822.618
Peptide chains from Insulin porcine:		
$[A\text{-chain} + Na]^+$	2404.990	2404.758
$[A\text{-chain} +1ME + Na]^{+a}$	2480.988	2480.769
$[B\text{-chain} + H]^+$	3398.682	3398.460
$[B\text{-chain} +1ME + H]^{+a}$	3474.680	3474.486
Peptide chain from Insulin aspart:[b]		
$[B\text{-chain} + H]^+$	3446.667	3446.434
$[B\text{-chain} + Na]+$	3468.648	3468.487
$[B\text{-chain} +1ME + H]^{+a}$	3522.665	3522.422
$[B\text{-chain} - (GFFYTDKT) + H]^{+c}$	2487.228	2487.030
$[B\text{-chain} - (GFFYTDKT) + 1ME + H]^{+a, c}$	2563.226	2563.302
Tryptic peptides from Insulin aspart:		
$[GFFYTDK + H]^+$	877.399	877.317
$[GFFYTDKT + H]^+$	978.457	978.446
$[B\text{-chain} - (GFFYTDKT) + H]^{+c, d}$	2487.217	2487.030
Tryptic peptides from Insulin porcine:		
$[GFFYTPK + H]^+$	859.425	859.345
$[GFFYTPKA + H]^+$	930.462	930.337
$[B\text{-chain} - (GFFYTPKA) + H]^{+c}$	2487.228	2487.234
$[B\text{-chain}-(GFFYTPKA) + 1ME + H]^{+a, c}$	2563.226	2563.129

[a]*Beta mercaptoethanol (ME) was used as reducing agent.*
[b]*The A-chains of insulin aspart and Insulin porcine are identical.*
[c]*Trypsinated B-chain.*
[d]*These peptides originate from insulin aspart, see* ***Figure 8.***

Table 3.
MALDI-TOF-MS analysis of insulin porcine and aspart.

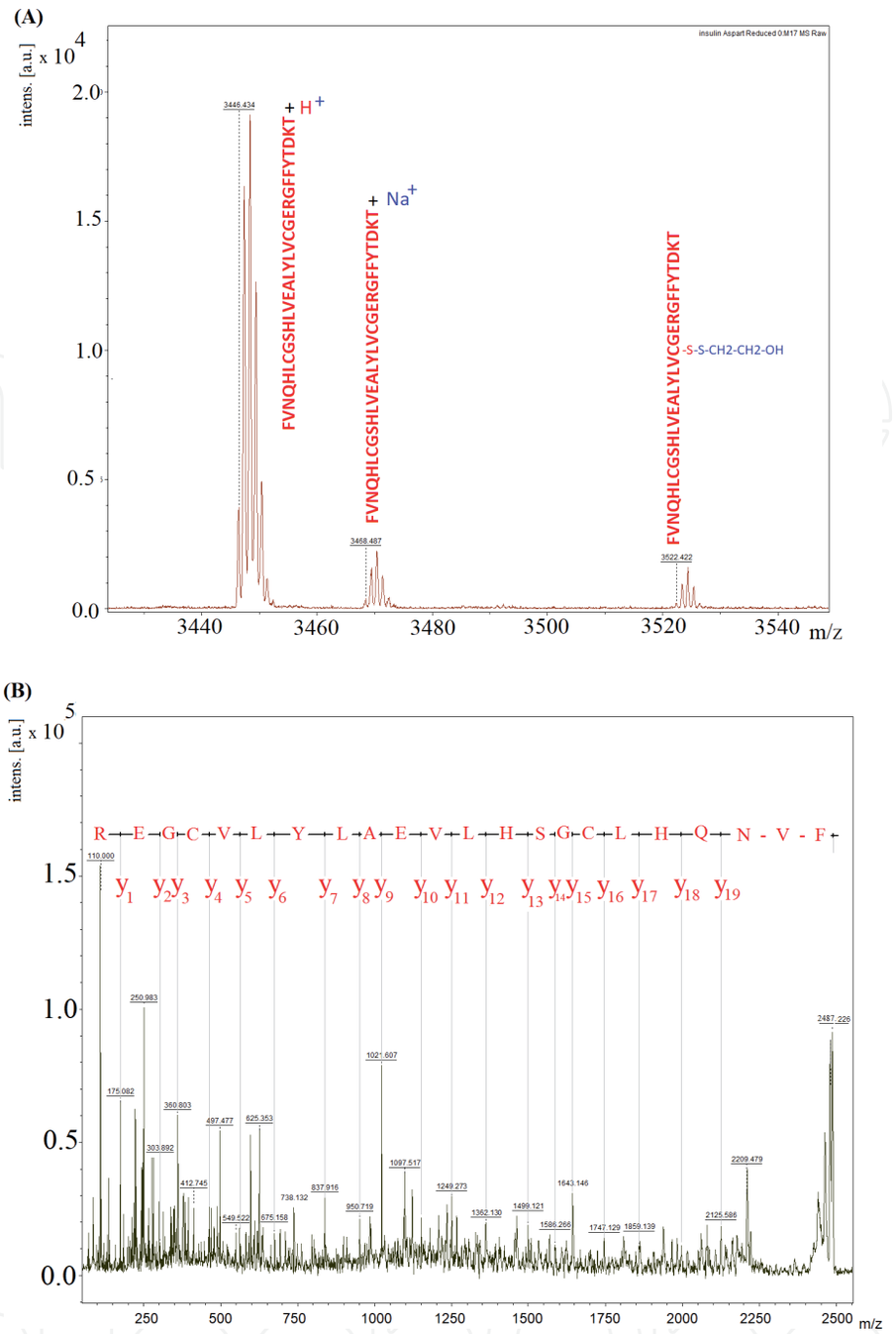


Figure 8.
MALDI analysis of insulin aspart; analysis of reduced B-chain (A), MALDI-PSD analysis of tryptic B-chain (B), see ***Table 3****.*

Double-injection capillary electrophoresis has also been applied for the identification of insulin molecules [81].

3.7 Identification of delta sleep-inducing peptide (DSIP)

The nonapeptide delta DSIP was first isolated from the cerebral venous blood of rabbits in an induced state of sleep during the mid-70s [82]. It was primarily believed to be involved in sleep regulation due to its apparent ability to induce slow-wave sleep in rabbits. However, it has been demonstrated that short-term treatment of chronic insomnia with DSIP is not likely to be of major therapeutic benefit [83]. The peptide is marketed illegally presumably for the treatment of insomnia. The peptide was directly exposed to the PSD analysis in order to confirm its molecular mass and amino acid sequence (**Figure 4** and **Table 1**).

3.8 Identification of thymosin β_4

Synthetic thymosin is a peptide consisting of 43 amino acids with artificial acetylation of the N-terminus (see **Figure 4** and **Table 1**). Thymosin has the potential of playing a significant role in tissue development, maintenance, repair, pathology and other important biological activities [84]. Some important biological activities of thymosin are related to the peptide sequence $L_{17}KKTET_{22}$ [85]. Illegally distributed thymosin products are claimed to promote a variety of beneficial biological functions, such as muscle building. The peptide was identified through PMF and de-novo sequencing of the tryptic peptides (**Table 4**).

3.9 Identification of human chorionic gonadotropin (hCG)

Human chorionic gonadotropin (hCG) is a glycoprotein hormone consisting of α (92 amino acids) and β-subunits (145 amino acids) being noncovalently associated [86]. These subunits are, however, highly cross-linked internally through disulfide bridges, i.e., the α-subunit has five disulfide bridges [87], while the β-subunit has six [87, 88]. The protein is heavily glycosylated where oligosaccharides are attached to the protein backbone through asparagine and serine residues and constitute approximately 30% of the molecular mass [89]. The protein has been identified using MALDI-TOF-MS and DICZE [13, 50]. Approximately 40% of the amino acid sequence of hCG was confirmed upon PMF (**Table 5**) [13].

Peptide fragments	**Theoretical m/z $[M + H]^+$**	**Determined m/z $[M + H]^+$**
Ac-SDKPDMAEIEKFDKSKLKKTETQEKNPLPSK ETI EQE-KQAGES	4961.484	4960.987
KTETQEKNPLPSKETIEQEKQAGES	2829.401	2829.219
Ac-SDKPDMAEIEKFDKSKLK	2151.090	2151.124
Ac-SDKPDMAEIEKFDKSK	1909.911	1909.698
Ac-SDKPDMAEIEKFDK[a]	1694.784	1694.765
NPLPSKETIEQEK	1512.780	1512.768
SKLKKTETQEK	1319.743	1319.729
Ac-SDKPDMAEIEK	1304.594	1304.498
ETIEQEK	876.421	876.356
TETQEK[a]	735.342	735.356
NPLPSK[a]	655.367	655.354
FDKSK	624.325	N.D.[b]
QAGES	491.199	N.D.[b]
SKLK	475.314	N.D.[b]
FDK[a]	409.198	409.196
LKK[a]	388.282	388.286
LK[a]	260.187	260.168
SK[a]	234.135	234.151

[a] *The amino acid sequence of the peptide was determined in the PSD mode.*
[b] *N.D. = Not detected.*

Table 4.
MALDI peptide mass fingerprinting data from analysis of thymosin β_4.

Peptide fragments	Peptide position in the peptide chain	Theoretical m/z $[M + H]^+$	Determined m/z $[M + H]^+$
AYPTPLR	α-hCG; 36–42	817.446	817.482
TMLVQK	α-hCG; 46–51	719.402	719.414
STNR	α-hCG; 64–67	477.231	477.165
VTVMGGFK	α-hCG; 68–75	838.439	838.471
SK	β-hCG; 1–2	234.135	243.142
PR	β-hCG; 7–8	272.161	271.996
EPLR	β-hCG; 3–6	514.288	514.291
EPLRPR	β-hCG; 3–8	767.442	767.469
DVR	β-hCG; 61–63	389.204	389.228
FESIR	β-hCG; 64–68	651.336	651.359

Table 5.
MALDI-PMF and MALDI-PSD analysis of human chorionic gonadotropin. The identified peptides from the α and β subunits are presented in the table below.

The identification was confirmed by DICZE analysis of illegal samples together with the corresponding reference standard [13, 50].

3.10 Identification of melanotan II (MII) and bremelanotide

Melanotan, a melanocortin receptor agonist, is a cyclic-lactam bridge heptapeptide which induces melanogenesis (i.e., tanning of the skin), by activation of the MC_1 receptor, being an analogue to alpha melanocyte hormone (α-MSH) [90]. The cyclic, lactam bridged structure of MII induces increased lipophilicity (**Figure 4**) [91].

Skin-tanning products that claim to contain MII are being advertised and sold on the illicit drug market. Injection of MII can result in systemic toxicity and rhabdomyolysis [90]. Bremelanotide (formerly PT-141) is an active metabolite of MII (**Table 1**).

These peptides were identified through the *top-down* approach by MALDI in PSD mode as illustrated in **Figure 9**.

3.11 Identification of dermorphin

Dermorphin is a μ-opioid receptor-binding peptide that causes both central and peripheral effects [92] (**Figure 4** and **Table 1**). This peptide, being originally isolated from the skin of the south American tree frog *Phyllomedusa sauvagii,* is classified as one of the strongest mammalian endogenous analgesic opioids [93, 94]. Dried frog skin containing dermorphin, has been used as a therapeutic agent by the Matses tribes of the upper Amazonian basin, to treat cuts during hunting expeditions [95]. The analgetic effects of dermorphin has been demonstrated in rat, horse, dog and white sea cod [92, 94]. It has been used illegally in horse racing as a pain killing agent, allowing horses to run even if injured.

This peptide, which was detected in several samples, was identified by MALDI in the PSD mode (**Figure 10**). The molecular structure was confirmed by NMR spectroscopy.

3.12 Identification of body protecting compound 157 (BPC 157)

BPC 157 being a partial sequence of body protecting compound (BPC) (M_{mass} = 40 kDa) is a synthetic peptide, which is composed of fifteen amino acids

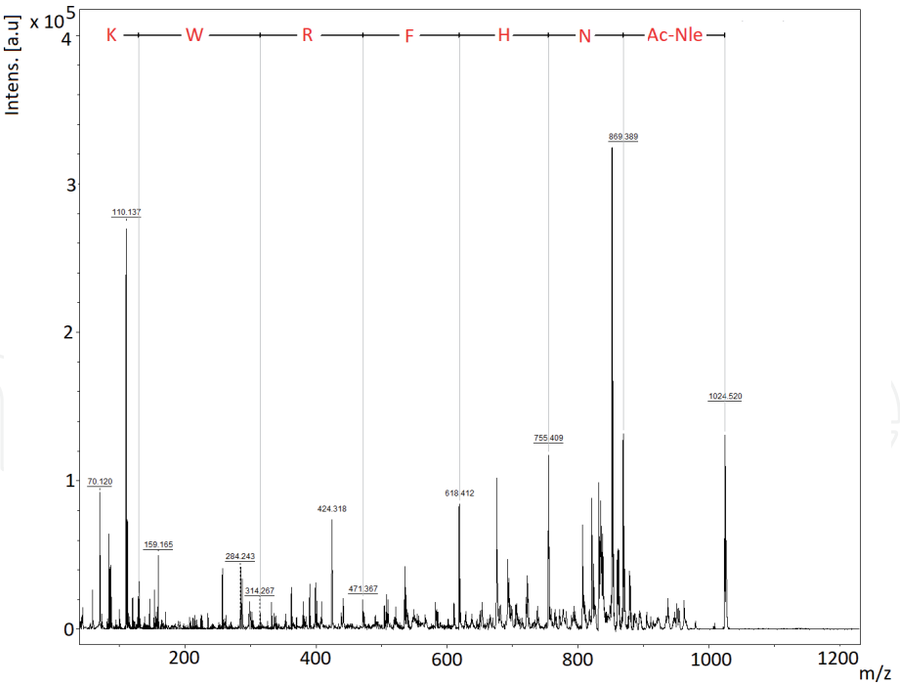


Figure 9.
MALDI-PSD analysis of melanotan II.

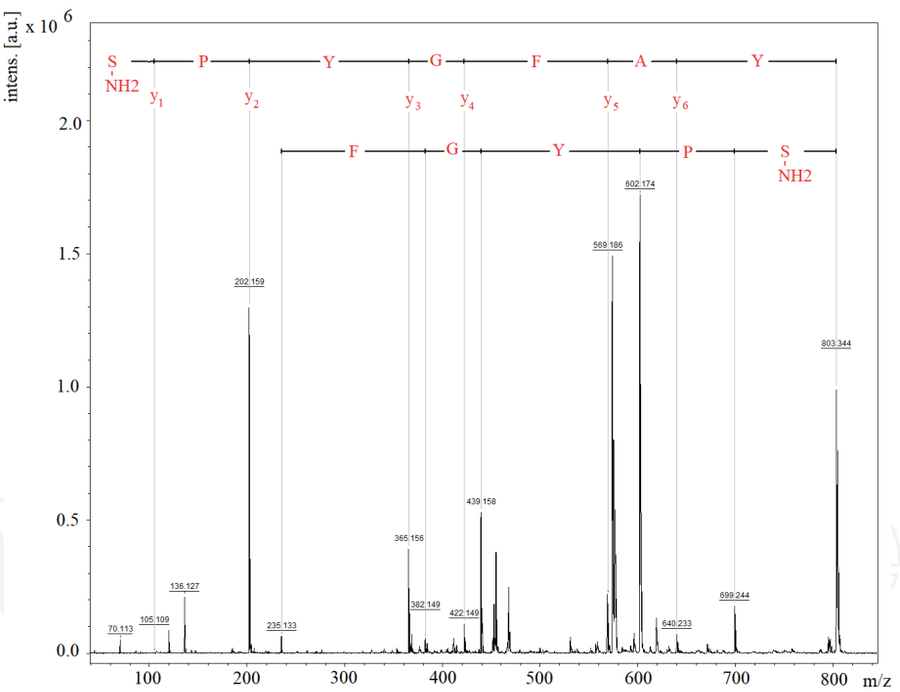


Figure 10.
MALDI-PSD analysis of dermorphin.

(**Figure 4** and **Table 1**). BPC was discovered and isolated from mouse gastric juice in response to stress stimuli in the gut mucosa [96]. BPC 157 is also known as Bepcin and PL. 14,736 or PL 10 [97]. This peptide fragment was speculated to be responsible for the BPC's physiological and protective effects [96]. However, it is unclear whether this peptide is endogenous to humans. BPC 157 is suggested to aid in tendon, ligament and muscle healing, and therefore its use as a quick injury healing in the sporting world is appealing. However, no proper clinical trials in human subjects have yet been performed to investigate the healing capability and the harmful effects of this compound [97].

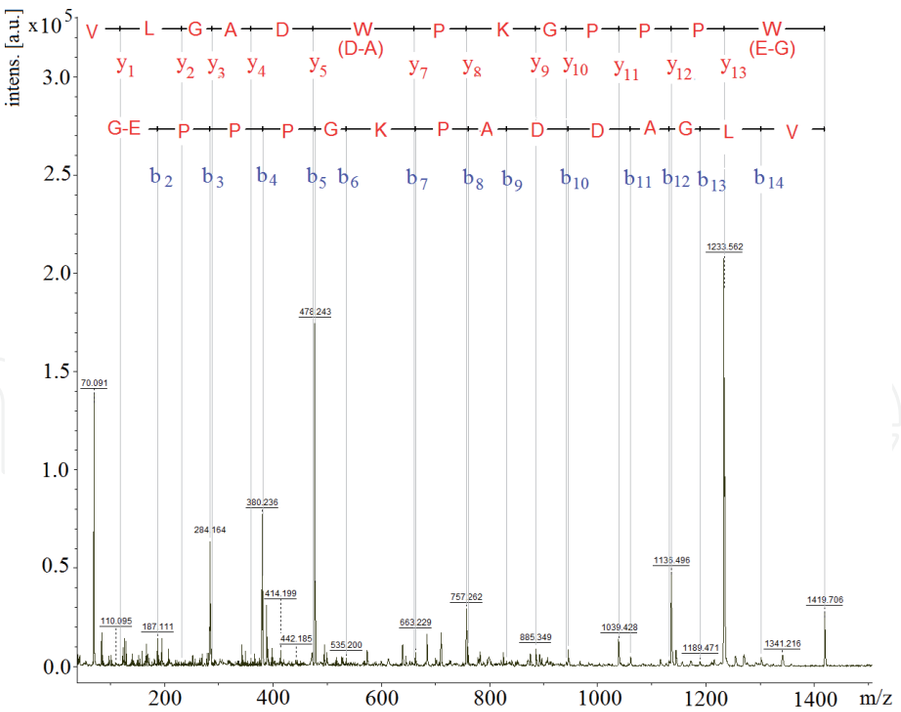


Figure 11.
MALDI-PSD analysis of BPC 157.

BPC 157 was recently identified in several confiscated vials for injection. The identification was carried out by MALDI in both PSD and reflectron modes (**Figure 11**). The amino acid sequence of the peptide was confirmed by NMR spectroscopy and LC-QTOF-MS.

4. Conclusions

The proposed methods, based on PMF by MALDI-TOF-MS as well as analysis with DICZE, provided an efficient procedure for the identification of peptides and proteins in illegally distributed samples. The use of trypsin as a proteolytic enzyme generated peptide fragments which covered 40 to 80% of the amino acid sequences of the analyzed proteins. The presence of a signature peptide in the peptide map facilitated the analyte identification considerably. MALDI-TOF-MS was also applied in the PSD mode for the amino acid sequencing of selected tryptic peptides as well as small peptides, such as ipamorelin.

The double-injection CE method provided complementary information on the native protein in the presence of a reference standard. This provided the possibility of performing a comparison between the electrophoretic patterns of the reference standard and the analyte to be identified. In addition, the double-injection based identifications were carried out by comparing the corrected migration time of the analyte and the observed migration time of the reference standard.

Abbreviations

ACHCA	α-cyano-4-hydroxycinnamic acid
ACN	Acetonitrile
Aib	Aminobutyric acid
BPC	Body protecting compound

DICZE	Double-injection capillary electrophoresis
DSIP	Delta sleep-inducing peptide
GH	Growth hormone
GHRP	Growth hormone releasing peptide
GHRH	Growth hormone releasing hormone (somatoliberin)
hCG	Human chorionic gonadotropin
hGH	Human growth hormone
IGF-1	Insulin like growth factor 1
ISD	In source decay
Nle	Norleucine
PMF	Protein mass fingerprinting
PSD	Post source decay
SA	Sinapinic acid
TFA	Trifluoroacetic acid

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References

[1] Cleveland DW, Fischer SG, Kirschner MW, Laemmli UK. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. Journal of Biological Chemistry. 1977; 252: 1102-1106.

[2] Matsudaira P. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. Journal of Biological Chemistry. 1987;262:10035-10038.

[3] Domon B, Aebersold R. Mass Spectrometry and protein analysis. Science. 2006;312:212-217. DOI: 10.1126/science.1124619.

[4] Han X, Aslanian A, Yates III JR. Mass spectrometry for proteomics. Current Opinion in Chemical Biology. 2008;12:483-490. DOI: 10.1016/j.cbpa.2008.07.024.

[5] Yates III JR, Speicher S, Griffin PR, Hunkapiller T. Peptide mass maps: a highly informative approach to protein identification. Analytical Biochemistry. 1993, 214, 397-408. DOI: 10.1006/abio.1993.1514.

[6] Aebersold R, Goodlett DR. Mass spectrometry in proteomics. Chemical Reviews. 2001;101:269-295. DOI:org/10.1021/cr990076h.

[7] Pappin DJC, Hojrup P, Bleasby AJ. Rapid identification of proteins by peptide-mass fingerprinting. Current Biology.1993;3:327-332. DOI:10.1016/0960-9822(93)90195-t.

[8] Pandey A, Mann M. Proteomics to study genes and genomes. Nature. 2000;405:837-846. DOI:10.1038/35015709.

[9] Aebersold R, Mann M. Mass spectrometry-based proteomics. Nature. 2003;422: 198-207. DOI:10.1038/nature01511.

[10] Barber M, Bordoli RS, Sedgewick RD, Tyler AN. Fast Atom Bombardment of Solids (F.A.B.): A New ion source for mass spectrometry. Journal of the Chemical Society, Chemical Communications.1981;11:325-327. DOI: org/10.1039/C39810000325.

[11] Henzel WJ, Watanabe C. Protein identification: the origins of peptide mass fingerprinting. Journal of American Society of Mass Spectrometry. 2003;14:931-942. DOI:10.1016/S1044-0305(03)00214-9.

[12] Jensen ON, Podtelejnikov AV, Mann M. Identification of the components of simple protein mixtures by high-accuracy peptide mass mapping and database searching. Analytical Chemistry. 1997;69:4741-4750. DOI: org/10.1021/ac970896z.

[13] Durgaryan A, Rundlöf T, Lavén M, Amini A. Identification of human chorionic gonadotropin hormone in illegally distributed products by MALDI-TOF mass spectrometry and double-injection capillary zone electrophoresis. Analytical Methods. 2016;8:4188-4196. DOI:org/10.1039/C6AY01078D.

[14] Shevchenko A, Sunyaev S, Loboda A, Shevchenko A, Bork P, Ens W, Standing K G. Charting the proteomes of organisms with unsequenced genomes by MALDI-Quadrupole Time-of-Flight Mass Spectrometry and BLAST homology searching. Analytical Chemistry. 2001;72:1917-1923. DOI:org/10.1021/ac0013709.

[15] Eng JK, McCormack AL, Yates JR. An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database.

Journal of American Society of Mass Spectrometry. 1994;5:976-989. DOI. org/10.1021/jasms.8b00502.

[16] Zubarev RA, Kelleher NL, McLafferty FW. Electron Capture Dissociation of Multiply Charged Protein Cations. A nonergodic process. Journal of American Chemical Society. 1998;120:3265-3266. DOI:org/10.1021/ja973478k.

[17] Shevchenko A, Jensen ON, Podtelejnikov AV, Sagliocco F, Wilm M, Vorm O, Mortensen P, Shevchenko A, Boucherie H, Mann M. Linking genome and proteome by mass spectrometry: Large-scale identification of yeast proteins from two dimensional gels. Proceedings of the National Academy of Sciences. 1996;93:14440-14445. DOI:org/10.1073/pnas.93.25.14440.

[18] Damodaran S, Wood TD, Nagarajan P, Rabin RA. Evaluating peptide mass fingerprinting-based protein identification. Genomics proteomics bioinformatics. 2007;5:152-157. DOI:org/10.1016/S1672-0229(08)60002-9.

[19] Karas M, Hillenkamp F. Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. Analytical Chemistry. 1988;60:2299-3201. DOI:org/10.1021/ac00171a028.

[20] Pan C, Xu S, Zhou H, Fu Y, Ye M, Zou H. Recent developments in methods and technology for analysis of biological samples by MALDI-TOF-MS. Analytical and Bioanalytical Chemistry Research. 2007;387:193-204. DOI: 10.1007/s00216-006-0905-4.

[21] Tanaka M, Waki H, Ido Y, Akita S, Yoshida T. Protein and polymer analysis up to *m/z* 100,000 by laser ionization time-of-flight mass spectrometry. Rapid Communication in Mass Spectrometry. 1988;2:151-153. DOI:org/10.1002/rcm.1290020802.

[22] Hrabák J, Chudáčková E, Walková R. Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry for detection of antibiotic resistance mechanisms: from Research to Routine Diagnosis. Clinical Microbiology Reviews. 2013;26:103-114. DOI: 10.1128/CMR.00058-12.

[23] Henzel WJ, Billeci TM, Stults JT, Wong SC, Grimley C, Watanabe C. Identifying proteins from two-dimensional gels by molecular mass searching of peptide fragments in protein sequence databases. Proceedings of the National Academy of Sciences USA.1993;90:5011-5015. DOI:10.1073/pnas.90.11.5011.

[24] James P, Quadroni M, Carafoli E, Gonnet G. Protein identification by mass profile fingerprinting. Biochemical and Biophysical Research Communications. 1993;195:58-64. DOI: 10.1006/bbrc.1993.2009.

[25] Amini A, Dormady SJ, Riggs L, Regnier FE, The impact of buffers and surfactants from micellar electrokinetic chromatography on matrix-assisted laser desorption ionization (MALDI) mass spectrometry of peptides. Effect of buffer type and concentration on mass determination by MALDI-time-of-flight mass spectrometry. Journal of Chromatography A. 2000;894:345-355. DOI: 10.1016/s0021-9673(00)00798-6.

[26] Padliya ND, Wood TD. Improved peptide mass fingerprinting matches via optimized sample preparation in MALDI mass spectrometry. Analytica Chimica Acta. 2008;627:162-168. DOI:org/10.1016/j.aca.2008.05.059.

[27] Nilsson E, Amini A, Wretlind B, Larsson A. Pseudomonas aeruginosa infections are prevented in cystic fibrosis patients by avian antibodies binding *Pseudomonas aeruginosa* flagellin. Journal of Chromatography B.

2007;856:75-80. DOI: 10.1016/j.jchromb.2007.05.029.

[28] Spengler B, Kirsch D, Kaufmann R, Jaeger E. Peptide sequencing by matrix-assisted laser-desorption mass spectrometry. Rapid Communications in Mass Spectrometry. 1992;16:105-108. DOI: 10.1002/rcm.1290060207.

[29] Doucette A, Craft D, Li L. Protein concentration and enzyme digestion on microbeads for MALDI-TOF peptide mass mapping of proteins from dilute solutions. Analytical Chemistry. 2000;27:3355-3362. DOI: 10.1021/ac000176j.

[30] Kaufmann R, Spengler B, and Luetzenkirchen F. Mass Spectrometric Sequencing of Linear Peptides by Product-ion Analysis in a reflectron time-of-flight mass spectrometer using matrix-assisted laser desorption ionization. Rapid Communications in Mass Spectrometry. 1993;7:902-910. DOI: 10.1002/rcm.1290071010.

[31] Spengler B. Post-source decay analysis in matrix-assisted laser desorption/ionization mass spectrometry of biomolecules. Journal of Mass Spectrometry. 1997, 32, 1019-1036. DOI: 10.1002/(SICI)1096-9888(199711)32:10<1019::AID-JMS595>3.0.CO;2-G.

[32] Purcell AW, Gorman JJ. The use of post source decay in matrix assisted laser desorption-ionisation mass spectrometry to delineate T cell determinants. Journal of Immunological Methods. 2001;249:17-31. DOI: 10.1016/s0022-1759(00)00361-6.

[33] Talbo GH, Suckau D, Malkoski M, Reynolds EC. MALDI-PSD-MS analysis of the phosphorylation sites of caseinomacropeptide. Peptides. 2001;22:1093-1098. DOI: 10.1016/s0196-9781(01)00426-0.

[34] Hoteling AJ, Owens KG. Improved PSD and CID on a MALDI TOFMS. Journal of the American Society of Mass Spectrometry. 2004;15:523-535. DOI: 10.1016/j.jasms.2003.12.004.

[35] Demeure K, Quinton L, Gabelica V, De Pauw E. Rational selection of the optimum MALDI matrix for top-down proteomics by in-source decay. Analytical Chemistry. 2007;*79*:8678-8685. DOI: org/10.1021/ac070849z.

[36] Wattenberg A, Organ AJ, Schneider K, Tyldesley R, Bordoli R, Bateman RH. Sequence dependent fragmentation of peptides generated by MALDI quadrupole time-of-flight (MALDI Q-TOF) mass spectrometry and its implications for protein identification. Journal of the American Society for Mass Spectrometry. 2002;13:772-783. DOI: 10.1016/S1044-0305(02)00414-2.

[37] McCormack AL, Somogyi A, Dongré AR, Wysocki VH. Fragmentation of protonated peptides: surface-induced dissociation in conjunction with a quantum mechanical approach. Analytical Chemistry. 1993;65:2859-2872. DOI: 10.1021/ac00068a024.

[38] Dongré AR, Somogyi A, Wysocki VH. Surface-induced Dissociation: An effective tool to probe structure, energetics and fragmentation mechanisms of protonated peptides. Journal of Mass Spectrometry. *1*996;**31:**339-350. DOI: 10.1002/(SICI)1096-9888(199604)31:4<339::AID-JMS322>3.0.CO;2-L.

[39] Tsaprailis G, Nair H, Somogyi A, Wysocki VH, Zhong W, Futrell JH, Summerfield SG, Gaskell SJ. Influence of secondary structure on the fragmentation of protonated peptides. Journal of the American Chemical Society. 1999;**121:**5142-5154. DOI: 10.1021/ja982980h.

[40] Zhang WZ, Chait BT. ProFound: an expert system for protein identification

using mass spectrometric peptide mapping information. Analytical Chemistry. 2000;72:2482-2489. DOI: 10.1021/ac991363o.

[41] Perkins DN, Pappin DJC, Creasy JM, Cottrell JS. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis. 1999;20:3551-3567. DOI: 10.1002/(SICI)1522-2683(19991201)20:18<3551::AID-ELPS3551>3.0.CO;2-2.

[42] Damodaran S, Wood TD, Nagarajan P, Rabin RA. Evaluating peptide mass fingerprinting-based protein identification. Genomics, Proteomics & Bioinformatics. 2007;5:152-157. DOI: 10.1016/S1672-0229(08)60002-9.

[43] Holcapek M, Jirásko R, Lísa M. Recent developments in liquid chromatography-mass spectrometry and related techniques. Journal of Chromatography A. 2012;1259:3-15. DOI: 10.1016/j.chroma.2012.08.072.

[44] Ji J, Chakraborty A, Geng M, Zhang X, Amini A, Bina M, Regnier FE. Strategy for qualitative and quantitative analysis in proteomics based on signature peptides. Journal of Chromatography B. 2000;745:197-210. DOI: 10.1016/S0378-4347(00)00192-4.

[45] Biron DG, Brun C, Lefevre T, Lebarbenchon C, Loxdale HD, Chevenet F, Brizard JF, Thomas F. The pitfalls of proteomics experiments without the correct use of bioinformatics tools. Proteomics. 2006;6:5577-5596. DOI: 10.1002/pmic.200600223.

[46] Barrett J, Brophy PM, Hamilton JV. Analysing proteomic data. International Journal for Parasitology. 2005;35:543-553. DOI: 10.1016/j.ijpara.2005.01.013.

[47] Sze SK, Ge Y, Oh H, McLafferty FW. Top-down mass spectrometry of a 29-kDa protein for characterization of any posttranslational modification to within one residue. Proceedings of the National Academy of Sciences. 2002;99:1774-1779. DOI: 10.1073/pnas.251691898.

[48] Amini A. Separation of somatropin charge variants by multiple-injection CZE with Polybrene/chondroitin sulfate A double-coated capillaries. Journal of Separation Science. 2013;36:2686-2690. DOI: 10.1002/jssc.201300327.

[49] Avinash AP, Cheng-Kang C, Chen-Hao W, Wen-Ping P. Forced dried droplet method for MALDI sample preparation. Analytica Chimica Acta. 2018;1031:128-133. DOI:org/10.1016/j.aca.2018.05.056.

[50] Amini A, Rundlöf T, Rönnquist K, Tydén M, Turunen T, Korhola P and Perolari A. A protocol for the quality assessment of illegally distributed human growth hormones with respect to identity, purity, endotoxin level and microorganism content. Analytical Methods. 2015;7: 8857-8864. DOI: org/10.1039/C5AY01390A.

[51] Hober FF and Haymond MW. Human growth hormone prevents the protein catabolic side effects of prednisone in humans. Journal of Clinical Investigation. 1990;86: 265-272. Doi: 10.1172/JCI114694.

[52] Dupin P, Galinou F and Bayol A. Analysis of recombinant human growth hormone and its related impurities by capillary electrophoresis. Journal of Chromatography A. 1995;707: 396-400. Doi.org/10.1016/0021-9673(95)00324-G.

[53] Somatropin for injection. European Pharmacopoeia (Ph. Eur.). 0951 (01/2008).

[54] Crist DM, Peake GT, Egan PA and Waters DL. Body composition response to exogenous GH during training in highly conditioned adults Journal of

applied Physiology. 1988;65: 579-584. DOI: 10.1152/jappl.1988.65.2.579.

[55] Takala J, Ruokonen E, Webster NR, Nielsen MS, Zandstra DF, Vundelinckx G and Hinds CJ. Increased mortality associated with growth hormone treatment in critically ill adults. The New England Journal of Medicine. 1999;341: 785-792. DOI: 10.1056/NEJM199909093411102.

[56] McCuskey R, Urbaschek R and Urbaschek B. The microcirculation during endotoxemia. Cardiovascular Research. 1996;32: 752-763. Doi. org/10.1016/S0008-6363(96)00113-7.

[57] Carcillo JA and Cunnion RE. Septic shock. Critical Care Clinics. 1997;13:553-574. DOI: 10.1016/s0749-0704(05)70328-6.

[58] Amini A. Rapid identification of somatropin by peptide-mass fingerprinting, using MALDI-TOF mass spectrometry. Pharmeuropa Bio & Scientific Notes, 2009;1: 11-6.

[59] Webster J and Oxley D. Peptide mass fingerprinting: protein identification using MALDI-TOF mass spectrometry. Methods in Molecular Biology. 2005;310: 227-240. DOI: 10.1007/978-1-59259-948-6_16.

[60] Guillemin R, Brazeau P, Bohlen PE, Esch R, Ling N and Wehrenberg W. Growth hormone-releasing factor from a human pancreatic tumor that caused acromegaly. Science. 1982;218: 585-587. DOI: 10.1126/science.6812220.

[61] Rivier J, Spiess JL, Thorner MO and Vale W. Characterization of a growth hormone-releasing factor from a human pancreatic islet tumour. Nature. 1982;300: 276-278. DOI: 10.1038/300276a0.

[62] Ling N, Esch R, Böhlen P, Brazeau P, Wehrenberg W and Guillemin R. Isolation, primary structure, and synthesis of human hypothalamic somatocrinin: growth hormone-releasing factor. Proceedings of the National Academy of Sciences of the USA. 1984;81: 4302-4306. DOI: 10.1073/pnas.81.14.4302.

[63] Ogru E, Wilson JC, Heffernan M, Jiang WJ, Chalmers DK, Libinaki R and Ng FM. The conformational and biological analysis of a cyclic anti-obesity peptide from the C-terminal domain of human growth hormone. Journal of Peptide Science. 2000;56: 388-397. DOI: 10.1034/j.1399-3011.2000.00771.x.

[64] Wijaya E and Ng FM. Effect of an antilipogenic fragment of human growth hormone on glucose transport in rat adipocytes. Biochemistry and molecular biology international. 1993;31: 543-552.

[65] Ho KK, O'Sullivan AJ and Hoffman DM. Metabolic Actions of Growth Hormone in Man. Endocrine Journal. 1996;43: S57-S63. DOI: 10.1507/endocrj.43.suppl_s5.

[66] Bowers CY, Momany FA, Reynolds GA and Hong A. On the in vitro and in vivo activity of a new synthetic hexapeptide that acts on the pituitary to specifically release growth hormone. Endocrinology. 1984;114: 1537-1545. DOI: 10.1210/endo-114-5-1537.

[67] Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H and Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature. 1999;402: 656-660. DOI: 10.1038/45230.

[68] Takaya K, Ariyasu H, Kanamoto N, Iwakura H, Yoshimoto A, Harada M, Mori K, Komatsu Y, Usui T, Shimatsu A, Ogawa Y, Hosoda K, Akamizu T, Kojima M, Kangawa K. and Nakao K. Ghrelin strongly stimulates growth hormone release in humans. The Journal of Clinical Endocrinology and Metabolism.

2000;85: 4908-2911. DOI:10.1210/jcem.85.12.7167.

[69] Wren A M, Small CJ, Ward HL, Murphy KG, Dakin CL, Taheri S, Kennedy AR, Roberts GH, Morgan, DGA, Ghateli MA, Bloom SR. The Novel Hypothalamic Peptide Ghrelin Stimulates Food Intake and Growth Hormone Secretion. Endocrinology. 2000;141: 4325-4328. DOI: org/10.1210/endo.141.11.7873.

[70] Popovic V, Damjanovic S, Micic D, Djurovic M, Dieguez C and Casanueva FF. Blocked growth hormone-releasing peptide (GHRP-6)-induced GH secretion and absence of the synergic action of GHRP-6 plus GH-releasing hormone in patients with hypothalamopituitary disconnection: evidence that GHRP-6 main action is exerted at the hypothalamic level. The Journal of Clinical Endocrinology and Metabolism. 1995;80: 942-947. DOI: 10.1210/jcem.80.3.7883854.

[71] Semenistaya E, Zvereva I, Thomas A, Thevis M, Krotov G and Rodchenkov G. Determination of growth hormone releasing peptides metabolites in human urine after nasal administration of GHRP-1, GHRP-2, GHRP-6, Hexarelin, and Ipamorelin. Drug Testing and Analysis. 2015;7: 919-925. DOI: 10.1002/dta.1787.

[72] Isidro *M and* Cordido F. Growth Hormone Secretagogues. Combinatorial Chemistry & High Throughput Screening, 2006;9:175-180. DOI:10.2174/138620706776055458.

[73] Banerjee A, Pramanik A, Bhattacharjya S and Balaram P. Omega amino acids in peptide design: incorporation into helices. Biopolymers. 1996;39: 769-777. DOI: 10.1002/(SICI)1097-0282(199612)39:6%3C769::AID-BIP4%3E3.0.CO;2-T.

[74] Kandalla PK, Goldspink G, Butler-Browne G, Mouly V. Mechano Growth Factor E peptide (MGF-E), derived from an isoform of IGF-1, activates human muscle progenitor cells and induces an increase in their fusion potential at different ages. Mechanisms of Aging and Development. 2011;132:154-162. DOI:10.1016/j.mad.2011.02.007.

[75] Laron Z. Biodrugs. Somatomedin-1 (recombinant insulin-like growth factor-1): clinical pharmacology and potential treatment of endocrine and metabolic disorders. 1999;11:55-70. DOI: 10.2165/00063030-199911010-00006.

[76] Green H, Morikawa M, Nixon T. A dual effector theory of growth-hormone action. Differentiation, 1985;29:195-198. DOI:org/10.1111/j.1432-0436.1985.tb00316.x.

[77] Dubaquié Y, Mortensen DL, Intintoli A, Hogue DA, Nakamura G, Rancatore P, Lester P, Sadick MD, Filvaroff E, Fielder PJ, Lowman HB. Selective Insulin-Like Growth Factor I Variants: Engineering, Biodistributions, and Clearance. Endocrinology. 2001;142: 165-173. DOI: org/10.1210/endo.142.1.7864.

[78] Sonksen PH. Insulin, growth hormone and sport. Journal of Endocrinology. 2001;170:13-25. DOI: 10-1677/joe.0.1700013.

[79] Weston PJ. The dead in bed syndrome revisited: a review of the evidence. Diabetes Management. 2012;2:233-241.

[80] Duckworth WC, Bennett RG, Hamel FG. Insulin Degradation: Progress and Potential. Endocrine Reviews. 1998;19:608-624. DOI:org/10.1210/edrv.19.5.0349.

[81] Amini A. Double-injection capillary electrophoresis for the identification of analytes. Electrophoresis. 2014;35:2915-2921. DOI:org/10.1002/elps.201400229.

[82] Schoenenberger GA, Maier PF, Tobler HJ, Monnier M. A naturally occurring delta-EEG-enhancing nonapeptide in rabbits. Pflügers Archiv -European Journal of Physics. 1977;369;99-109.

[83] Bes F, Hofman W, Schuur J, Van Boxtel C. Effects of Delta Sleep-Inducing Peptide on Sleep of Chronic Insomniac Patients. Neuropsychobiology. 1992;26:193-197. DOI:10.1159/000118919.

[84] Sosne G, QiuP, Goldstein AL, Wheater M. The biological activities of thymosin β4 defined by active sites in short peptide sequences. The FASEB Journal. 2010;24: 2144-2151. DOI:org/10.1096/fj.09-142307.

[85] Mannherz HG, Hannappel E. The beta-thymosins: intracellular and extracellular activities of a versatile actin binding protein family. Cell Motility and the Cytoskeleton. 2009;66:839-851. DOI: 10.1002/cm.20371.

[86] Stenman UH, Tiitinen A, Alfthan H, Valmu L. The classification, functions and clinical use of different isoforms of HCG. Human Reproduction Update 2006;12:769-784. DOI:org/10.1093/humupd/dml029.

[87] Mise T, Bahl OP. Assignment of disulfide bonds in the alpha subunit of human chorionic gonadotropin. Journal of biological Chemistry.1980;255:8516-8522.

[88] Mise T, Bahl OP. Assignment of disulfide bonds in b sub-unite of human chorionic gonadotropin. Journal of Biological Chemistry. 1981;256:6587-6592.

[89] Elliott MM, Kardana A, Lustbader JW, Cole LA. Carbohydrate and peptide structure of the alpha- and beta-subunits of human chorionic gonadotropin from normal and aberrant pregnancy and choriocarcinoma. Endocrine. 1997;7:15-32. DOI: 10.1007/BF02778058.

[90] Nelson ME, Bryant SM, Aks SE. Melanotan II injection resulting in systemic toxicity and rhabdomyolysis. Clinical Toxicology. 2012, **50**,1169-1173. DOI: 10.3109/15563650.2012.740637**.**

[91] Hadley ME. Discovery that a melanocortin regulates sexual functions in male and female humans. Peptides, 2005;26:1687-1689. DOI:org/10.1016/j.peptides.2005.01.023.

[92] Robinson MA, Guan F, McDonnell S, Uboh CE, Soma LR. Pharmacokinetics and pharmacodynamics of dermorphin in the horse. Journal of Veterinary Pharmacology and Therapeutics, 2014;38:321-329. DOI:org/10.1111/jvp.12179.

[93] Broccardo M, Usenko AB, Uranova MG, Guzevatykh LS, Kamensky AA, Andreeva LA, Alfeeva LY, Myasoedov NF, Giannini E, Improta G, Emel'yanova TG. In vitro and in vivo opioid activity of [DPro^6]dermorphin, a new dermorphin analogue. Peptides, 2003;24:419-428. DOI:org/10.1016/S0196-9781(03)00057-3.

[94] Chervova LS, Lapshin DN, Kamenskii AA. Pain Sensitivity of Trout and Analgesia Induced by Intranasal Administration of Dermorphin. Doklady Biological Sciences.1994;338:424-425.

[95] Negri L, Melchiorri P, Lattanzi R. Pharmacology of amphibian opiate peptides. Peptides, 2000;21:1639-1647. DOI: 10.1016/s0196-9781(00)00295-3.

[96] Sikirić, P Petek M, Ručman R, Seiwerth S, Grabarević Z, Rotkvić, I, Turković B, Jagić V, Mildner B, Duvnjak M, Lang N, Danilović Z, Cviko A, Kolega M, Sallmani A, Djačić S, Bura M, Brkić T, Banić M, Dodig M, Corić V,

Šimičević V, Veljaca M, Erceg, D, Ježek D, Simunić-Banek L, Skroza N, Bulić K, Buljat G, Hanževački M, Orihovać V, Lučinger D, Culig J, Separović J, Marović, A, Miše S, Suchanek E, Matoz W, Perović D, Gjurašin M, Mikulandra S, Derniković K, Cuk V, Karakas I. A new gastric juice peptide, BPC. An overview of the stomach-stress-organoprotection hypothesis and beneficial effects of BPC. The Journal of Physiology. 1993;87: 313-327. DOI: org/10.1016/0928-4257(93)90038-U.

[97] Cox HD, Millera GD, Eichnera D. Detection and in vitro metabolism of the confiscated peptides BPC 157 and MGF R23H. Drug Testing and Analysis. 2017;9:1490-1498. DOI: 10.1002/ dta.2152.