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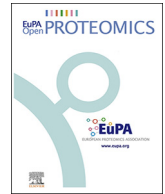
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# Sweet google O' mine—The importance of online search engines for MS-facilitated, database-independent identification of peptide-encoded book prefaces

## A EUPA YPIC challenge entry

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### ABSTRACT

In the recent year, we felt like we were not truly showing our full potential in our PhD projects, and so we were very happy and excited when YPIC announced the ultimate proteomics challenge. This gave us the opportunity of showing off and procrastinating at the same time:) The challenge was to identify the amino acid sequence of 19 synthetic peptides made up from an English text and then find the book that it came from. For this task we chose to run on an Orbitrap Fusion™ Lumos™ Tribrid™ Mass Spectrometer with two different sensitive MS2 resolutions, each with both HCD and CID fragmentation consecutively. This strategy was chosen because we speculated that multiple MS2 scans at high quality would be beneficial over lower resolution, speed and quantity in the relatively sparse sample. The resulting chromatogram did not reveal 19 sharp distinct peaks and it was not clear to us where to start a manual spectra interpretation. We instead used the de novo option in the MaxQuant software and the resulting output gave us two phrases with words that were specific enough to be searched in the magic Google search engine. Google gave us the name of a very famous physicist, namely Sir Joseph John Thomson, and a reference to his book “Rays of positive electricity” from 1913. We then converted the paragraph we believed to be the right one into a FASTA format and used it with MaxQuant to do a database search. This resulted in 16 perfectly FASTA search-identified peptide sequences, one with a missing PTM and one found as a truncated version. The remaining one was identified within the MaxQuant de novo sequencing results. We thus show in this study that our workflow combining de novo spectra analysis algorithms with an online search engine is ideally suited for all applications where users want to decipher peptide-encoded prefaces of 20th century science books.

### 1. Introduction

In this study, we faced the challenge of deciphering the amino acid sequence of 19 PTM-modified peptides. These peptides were synthesized specifically for this challenge and would yield a sentence taken from a book. The goal was to decipher both the peptide sequences and the book they came from.

We received the EuPA YPIC challenge peptides with an attached description stating that one peptide was still carrying a modification of  $m/z$  89.97, probably as a protection group on a Ser, and that three PTMs were used to cipher the letters O (acetylated lysine), B (phosphorylated Ser) and U (methylated Arg). Each peptide should comprise one to five

English words, which with an average English word length of five characters would indicate peptide lengths from five to 25 characters [1].

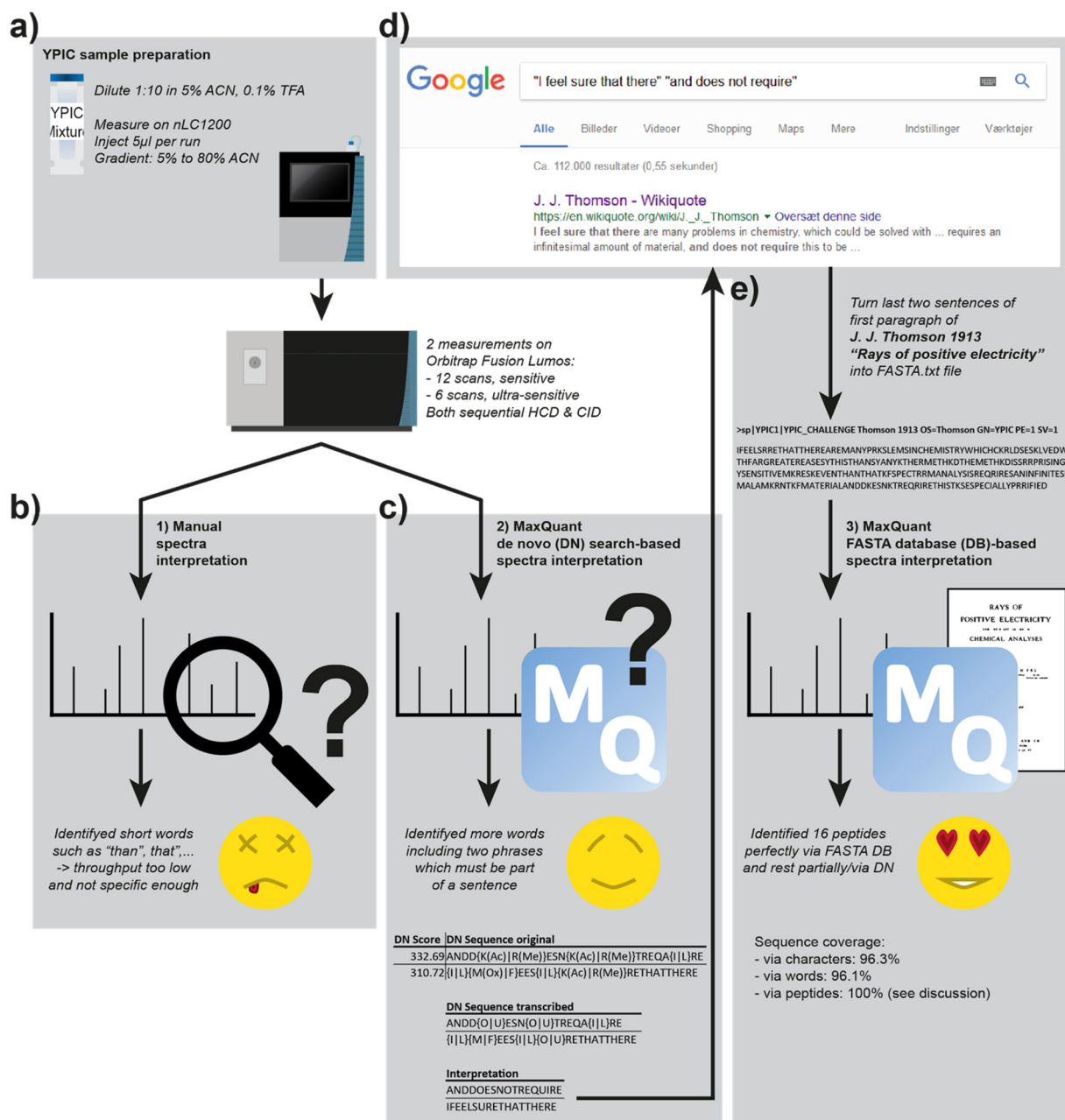
With the information given at hand, we developed an LC–MS/MS based workflow focused on high quality peptide spectra generation using an Orbitrap Fusion Lumos instrument. We reasoned that the physico-chemical properties of the peptides should not be substantially different from tryptic peptides in a standard bottom-up shotgun proteomics experiment. Thus a typical HPLC workflow strategy with standard C18 column and a separating gradient from low to high organic solvent should be applicable.

For MS analysis in a routine proteomics experiment, a

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**Fig. 1. MS-based Workflow to Identify Peptide-encoded Book Prefaces.** The scheme depicts the workflow and results for the identification of the correct text the 19 challenge peptides originated from. a) EuPA YPIC challenge peptides were diluted 1:10 in 5% ACN, 0.1% TFA and 5 µl per injection measured on an nLC1200 + Orbitrap Fusion Lumos system. b) Manual spectra interpretation did not yield enough text-specific peptide sequences. c) MaxQuant de novo spectrum analysis helped in identifying initial peptide phrases. d) Two peptide phrases entered into the search engine Google with quotation marks were enough to identify the correct book from Sir J. J. Thomson. e) Transcribing two sentences from the preface of the book into a FASTA file and searching it with MaxQuant yielded a 100% peptide sequence coverage.

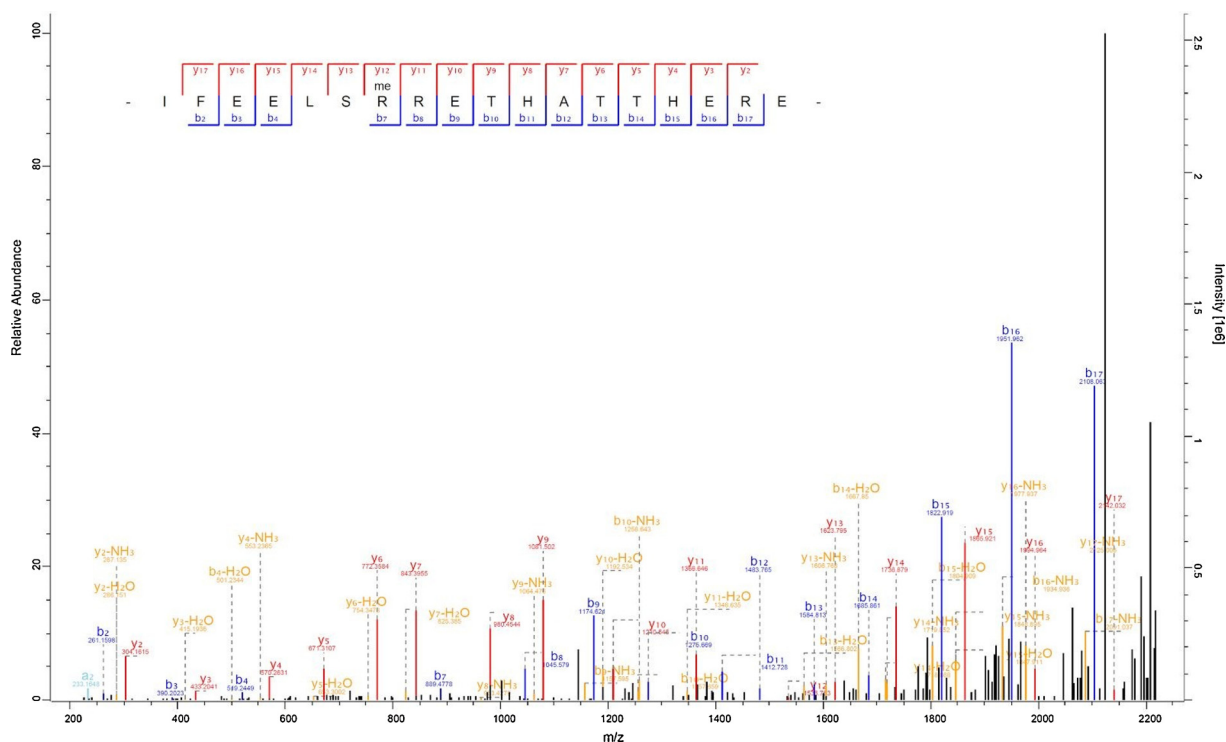
I FEEL SURE THAT THERE ARE MANY PROBLEMS IN CHEMISTRY WHICH COULD BE SOLVED WITH FAR  
 GREATER EASE BY THIS THAN BY ANY OTHER METHOD. THE METHOD IS SURPRISINGLY SENSITIVE —  
 MORE SO EVEN THAN THAT OF SPECTRUM ANALYSIS, REQUIRES AN INFINITESIMAL AMOUNT OF  
 MATERIAL AND DOES NOT REQUIRE THIS TO BE SPECIALLY PURIFIED

**Fig. 2. Peptide Sequence Coverage.** Peptide sequences marked with green boxes were identified without spelling mistakes via the MaxQuant FASTA DB search. Peptides within yellow boxes showed slight spelling mistakes, and the one in blue was only identified by MaxQuant DN search. The phrase “an infinitesimal” was not found, but as indicated in the challenge by “two words still missing”, which would “explain its own absence if combined with ‘small amount’”, this could be the one/two words missing.

**Table 1**

**Peptide Sequence Coverage.** The table lists peptide sequences identified by MaxQuant FASTA DB (highlighted in white) or DN (highlighted in grey). The complete list of peptide sequences found by these two methods is provided in the supplementary. Peptide four was only identified with S instead of S(ph). Peptides 12.1 and 12.2 in FASTA DB and DN search, respectively, were only identified as partial constructs of the target sequences.

Peptide number	Modified sequence	Translated sequence	Score	Length	m/z	Mass	Charge	Fragmentation
1	I FEELSR(me)RETHATTHHERE	I FEELSU RETHATTHHERE	332.96	18	752.377	2254.11	3	CID
2	AREM(ox)ANYPRK(ac)S(ph)LEM(ox)SIN	AREMANYPROBLEMSIN	148.75	17	721.990	2162.95	3	CID
3	HE[MF]I(L)STRYWH(I)LCH *	HEMISTRYWHICH	253.27	13	641604	1927.79	3	HCD
4	CK(ac)R(me)LDSESK(ac)LVED	COULDESOLVED	167.71	13	838.911	1675.81	2	CID
5	WITHFAR	WITHFAR	158.38	7	465.751	929.487	2	CID
6	GREATEREASES(ph)Y	GREATEREASEBY	230.50	13	782.818	NaN	0	CID
7	THISTHANS(ph)Y	TH ISTHAN BY	250.13	10	605.748	1209.48	2	HCD
8	ANYK(ac)THERM(ox)ETHK(ac)D	ANYOTH ERIMETHOD	255.25	14	930.421	1858.83	2	HCD
9	THEM ETHK(ac)DIS	THEMETHDDIS	181.53	11	685.306	NaN	0	HCD
10	SR(me)RPRISINGLY	SURPRISINGLY	85.73	12	723.420	1444.83	2	CID
11	SENSITIVEM(ox)K(ac)RE	SENSITIVEMORE	319.81	13	797.393	1592.77	2	CID
12.1	SK(ac)EVENTHA	SOEVENTHA	191.17	9	528.751	1055.49	2	CID
12.2	S[K(Ac)R(Me)]EVENTHAT *	SOEVENTHAT	169.81	10	579.276	1156.54	2	CID
13	SPECTRR(me)M	SPECTRUM	129.68	8	525.744	1049.47	2	HCD
14	ANALYSISRECRIR(me)RES	ANALYSISREQUIRES	228.73	16	636.341	NaN	0	HCD
15	AM(ox)K(ac)R(me)NTK(ac)FM(ox)ATERIAL	AMOUNTOFMATERIAL	231.71	16	671.020	2010.04	3	CID
16	AN DDK(ac)ESNKA(ac)TRECIR(me)RE	ANDDOESNOTREQUIRE	204.43	17	547.524	2186.07	4	HCD
17	THISTK(ac)S(ph)E	THLSTOBE	213.03	8	512.721	NaN	0	HCD
18.1	SPECIALLY	SPECIALLY	158.53	9	533.268	1064.52	2	CID
18.2	ESPECIALLY	ESPECIALLY	174.21	10	597.789	1193.56	2	CID
19	PR(me)RIFI ED	PURIFIED	89.05	8	530.301	1058.59	2	CID



**Fig. 3. Example Peptide Sequence “I feel sure that there”.** The spectrum shows annotated a-, b- and y-ions for the peptide sequence IFEELSR(me)RETHATTHHERE, with R(me) translating to U. The spectrum was recorded following CID fragmentation.

fragmentation strategy optimized to the sample and instrument at hand with lower MS2 scan resolutions and shorter injection times would be preferable to facilitate a higher scan speed [2]. However, we speculated that the challenge sample, even though lacking post-synthesis peptide purification, should contain less peptide variants than e.g. a standard tryptic HeLa proteome. Thus, a high spectrum quality for confident amino acid sequence determination would be desirable over a high peptide throughput with an as fast as possible MS scan speed.

We therefore decided to measure the sample twice with two different high resolution MS2 settings including both CID and HCD fragmentation on the same precursor. Using two different fragmentation approaches should further increase the quality for peptides which have a preference for better fragmentation with either CID or HCD.

Taken all these consideration into account we ended with the workflow described in Fig. 1a.

## 2. Results and discussion

When first inspecting the resulting chromatogram (see Supp. Fig. 1), we saw highly abundant peaks and began manual interpretation of the spectra in the two raw files we recorded (see Fig. 1b). We were able to identify individual sequences forming words such as “than” and “that” (data not shown). While these findings indicated that we had indeed generated usable data, the throughput and specificity of this approach were both far too low for a meaningful analysis and identification of the book we were looking for. Instead, we needed an automated, high-throughput data analysis strategy.

Since the peptides were not tryptic and the phrases were not derived from any organism’s genome, we could not start with a classic FASTA database search (DB) approach, which is why we tried out the de novo (DN) search feature of MaxQuant v1.5.8.4i (see Fig. 1c) [3]. This

feature tries to de novo annotate amino acid sequences for all spectra from a raw file without resorting to a predefined FASTA database. It can also include PTMs in this process and even assigns a DN quality score to each annotation. After using this feature with our two raw files, we did indeed acquire lots of annotations, which we sorted by DN score in a highest to lowest order. After transcribing PTM-modified residues to their respective letters, the highest scoring peptide with a DN score of 332.69 yielded the sequence “ANDD{O|U}ESN{O|U}TREQA{I|L}RE”. Although wrongly spelled with an A instead of U, this had to indicate the phrase “and does not require” being part of the text which we were looking for. Since the synthetic peptides were not purified, there should be a high chance of finding wrongly or partly synthesized versions of the target sequences. Thus it could very well be possible for misspelled versions to score higher than the actual target sequences. The five next highest DN scoring peptides were variations of this sequence, but the seventh transcribed into a perfectly spelled “I feel sure that there”.

After identifying these two phrases, we next wanted to see if we could already now pinpoint which text they originated from. Since manually reading through literally decades of scientific research publications would be neither high-throughput nor specific, we instead relied on the power of a second automated database search tool - Google (see Fig. 1d). Since the identified sequences seemed to be part of sentences, we entered them with quotation marks into the search query. This would ensure that Google does not search for the words individually, but preserves their appearance as phrases. Indeed, the first hit of the more than 112,000 search pointed to the wikiquote entry of Sir Joseph John Thomson. To avoid a false-positive hit, we also tested the alternative search engines Yahoo and Bing. While the former only identified two search hits, with the first correctly being the J. J. Thomson text, Bing only identified it as its seventh hit. Other articles with unhelpful, but at least for a PhD student relatable topics such as “Why do we sleep, anyway?” scored higher instead.

Thomson was a physicist and Nobel prize laureate from England, and is credited as one of the discoverers of the electron. More importantly with respect to this challenge, he also was the first person to measure the mass-to-charge ratio of positively charged molecules and the slowly dying unit Th ( $m/z$ ) is actually named after him. These findings, which are often regarded as the birth of the field of mass spectrometry, were described in his 1913 publication “Rays of positive electricity and their application to chemical analyses” [4]. The preface of this book, written by Thomson himself, contains the two phrases we identified in our peptide mixture. Since the book in question played such an important role in the development of modern mass spectrometers, we were positive that we had found the correct text sequence. To confirm this, we next transformed the preface into a FASTA file and searched the two raw files again with MaxQuant. We transcribed the letters B, O and U into their PTM-modified amino acid counterparts, and searched the raw files applying unspecific digestion with a minimum amino acid sequence length of six. We did this two times, using the whole preface the first time (data not shown), and the second time only two sentences that due to their high number of peptide sequence hits had to be the correct text (see Fig. 1e). With this approach we were able to identify 16 out of 19 peptides without any spelling mistakes (See Fig. 2). Fun fact: the peptide corresponding to the sequence “specially”, as written in the preface, was also found as “especially”.

The remaining three peptides were still identified, although with spelling mistakes or truncations (see Table 1): 1) The peptide “could be solved” was misspelled with “s” instead of “b”. In the challenge it was mentioned that one peptide carries a possible protection group on a Ser. We argue that the phosphorylated Ser, which would have translated into the letter B, got dephosphorylated, thus leading to the S in our sequence. 2) The peptide “so even than that of” was only identified partially as “so even tha”. In addition, MaxQuant DN search also found the variant “so even that”. The sequence “of” was only found in the correctly identified peptide “amount of material”. Since we do not find

evidence of peptides with a “tha\_tha” motif, it is intriguing for us to speculate that the peptide was synthesized with only “that”, as indicated by the DN results. Since the sequential “of” was missing as well, this peptide could have been particularly difficult to synthesize. 3) The final missing peptide “chemistry which” was only identified without its N-terminal C via MaxQuant DN search. We speculate that the Cys might not have been carbamidomethylated, but oxidized instead, which is why our approach could not find it.

The phrase “an infinitesimal” could not be found. However, the challenge stated that two words were missing in the sample, with one of them being interchangeable with “small amount”. This fits our missing two words perfectly. We thus identified variants of all 19 peptides.

Eleven out of 19 high-scoring peptide identifications resulted from fragmentation with CID, as compared to eight for HCD. This illustrated that for non-tryptic peptides, both fragmentation techniques can have complementary power in producing high-quality MS2 spectra. Interestingly, twelve out of the 21 spectra considered in Table 1 were recorded with the more sensitive top6 method, indicating that the higher MS2 resolution and longer fill times increased the overall spectrum quality as expected. To confirm if the DB search algorithm identified all peptides correctly, we manually checked the annotated spectra (see Supp. Fig. 2). As an example, we present the annotated spectrum of our initially identified peptide “I feel sure that there”, which is the first one in the text, with a nearly perfect b- and y-ion series covering 100% of the amino acid sequence (see Fig. 3).

### 3. Conclusion

We conclude that our combined MaxQuant DN and DB + Google search strategy proved extremely successful for the initial annotation of peptide sequences, identification of the correct book and text paragraph, and the final confirmation of all 19 peptide sequences in the sample. The only limitation we encountered, was that it was not possible for us to identify the peptide reported to carry a protection group on Ser. This is due to the fact that MaxQuant requires precise information on chemical composition for modifications it has to search, as not even an arbitrary protection group on Ser of the same mass yielded any true hits. Importantly, our approach highlights that due to the sheer number of impure peptide variants (even including ambiguities such as specially/especially), putting together the whole preface text without errors just from the peptide sequences would be a very tedious process. The importance of Google for solving comparable tasks can thus not be stressed enough.

This challenge has been a true pleasure to work on and has resulted in many good and quite different talks over the coffee machine. We thank the organizers for preparing the challenge including peptide synthesis and required logistics to ship them out, and are looking forward to use our MS-skills again on the (hopefully coming) next EuPA YPIC challenge.

### Data Availability

Data are available via ProteomeXchange with identifier PXD007693 [5,6].

### Acknowledgments

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Thrones, and of course the Dalai Lama for always believing in us. Tusind tak!

#### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:.

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