

Perspective

Characterization and detection of food allergens using high resolution mass spectrometry: current status and future perspective

Julia Bräcker, and Jens Brockmeyer

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1 Characterization and detection of food allergens using high resolution mass
2 spectrometry: current status and future perspective

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4 Julia Bräcker¹, Jens Brockmeyer¹

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6 ¹Institute of Biochemistry and Technical Biochemistry, Department of Food Chemistry,
7 University of Stuttgart, Stuttgart, Germany

8

9 **Correspondence:** Jens Brockmeyer (jens.brockmeyer@lc.uni-stuttgart.de).

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13

14 **Abstract**

15 Allergic reactions to food are among the major food safety concerns in industrialized countries
16 and it is estimated that approximately 5% of the population suffers from IgE-mediated food
17 allergy. High resolution mass spectrometry has become one of the most important techniques
18 for the molecular characterization of allergens including structural modification, degradation in
19 the gastrointestinal environment or the identification of suitable marker peptides for the
20 development of novel analytical approaches in the last decade. This perspective aims to briefly
21 summarize the current situation and to discuss future developments.

22

23

24 **Food Allergy: some background information**

25 The presence of undeclared allergenic proteins is one of the major food safety concerns in
26 industrialized countries. Current epidemiological data indicate that 5% of the population in
27 westernized countries suffer from food allergy ^{1,2} and the prevalence of allergic reactions has
28 increased significantly over the last decades. Although food allergens are an emerging topic in
29 analytical food chemistry and molecular food sciences, the first description of their adverse
30 effects goes back to the Greco-Roman age, when Hippocrates referred to “hostile humors”
31 responsible for the suffering after the ingestion of cheese and Titus Lucretius Cato coined the
32 sentence: “What is food to one, to another is rank poison” ³.

33 In general, humans develop an active oral immune tolerance against food and allergic reactions
34 are consequently caused by a malfunctional adaptive immune system that generates a specific
35 immune response upon exposure to certain food ⁴. For the vast majority of food allergic
36 reactions, proteins have been identified as the eliciting agents. The basic principles of
37 sensitization and allergic reaction are briefly summarized in Fig.1.

38 The clinical symptoms of allergic reactions range from milder reactions in the oral cavity
39 (itching or sneezing) and bronchial obstruction to severe reactions of the gastrointestinal tract
40 and systemic and life-threatening reactions such as the anaphylactic shock ⁵.

41 About 150-200 foods have allergenic potential as demonstrated in case studies but the vast
42 majority of reactions is elicited by a limited number of allergens that account for about 90% of
43 allergic reactions to food ⁶. The regional differences in the profile of relevant allergens are
44 pronounced and due to insufficient data from developing countries a certain bias in global
45 allergen profile is to be expected ⁷. To enhance product safety and reduce the risk of unintended
46 allergen uptake, different labelling regulations have been introduced worldwide for the most
47 important food allergens and the diversity of these regulations partially also reflect the regional
48 differences in relevant allergen profiles and nutritional habits. An overview of current
49 regulations is exemplified in Table 1. In addition, large clinical research programs aim to more

thoroughly understand the epidemiology of food allergy that allows a risk-based approach based on the identification of threshold levels for different food allergic populations. The EuroPrevall study examined the influence of food intake, immunology, genetic background and socioeconomic factors to identify important risk factors for the development of food allergies in birth cohorts⁸. In addition, comprehensive prevalence data have been established for various allergens in adults and children that also allow to delineate geographic differences⁹ and obtain information of the threshold dose distribution of different allergen in the European population¹⁰. The expert panel of the VITAL (Voluntary Incidental Trace Allergen Labeling) program established reference doses and population threshold levels from clinical challenges of food-allergic individuals. These data provide individual NOAEL and LOAEL¹¹ and reference doses for precautionary labeling¹².

Consequently, the analytical monitoring and quantification of allergens (or analytical surrogates) will be far more important in the future in such risk-based approaches and mass spectrometry-based proteomics has emerged as a very promising approach to address this issue.

General aspects for the specific detection of allergens using mass spectrometry

Allergy detection methods currently under development employ the benefits of different mass spectrometry platforms such as triple quadrupole (QQQ), orbitrap or Q-TOF systems. Similar to the detection and quantification of low molecular weight contaminations in food (such as mycotoxins or phytotoxins), triple quadrupole instruments in general still show highest sensitivity and are increasingly used in targeted approaches in food proteomics. One of the first steps in method development in targeted food proteomics in general and in MS-based allergen detection is however the identification of suitable analytical markers. The direct detection of intact food allergens for quantitation purposes from complex food matrices suffers from numerous pitfalls and drawbacks and is currently not suitable for reliable quantitation. The chromatographic separation of intact proteins is often poor, especially in complex matrices such as food and resulting mass spectra of single proteins are highly complex due to the occurrence

of numerous charge states (in ESI-MS) and complex isotopologue distribution. To make it even worse, many allergens show complex proteoform pattern that further complicate data analysis and detection in this so-called top-down approach. Taking all this into account, the specific detection of marker peptides released after enzymatic digest of parent allergens (in general using trypsin) is far more promising and feasible¹³. This bottom-up approach in addition benefits from the efficient chromatographic separation of peptides (that in general lack secondary or tertiary structure), high ionization efficiency of tryptic peptides and predictability and reproducibility of peptide fragments in MS/MS. One of the major aspects for the development of specific targeted bottom-up proteomics analysis is the identification of sensitive and specific marker peptides that allow the quantification of the parent protein/allergen. In principle, the use of bioinformatics resources such as UniProt or NCBI (National Center for Biotechnology Information) can be employed to obtain sequence information of the target protein followed by *in silico* digest resulting in a list of suitable theoretical peptide marker that have to be validated experimentally. The mere use of protein or genome databases for marker identification has however some significant drawbacks. Proteomics and genomics databases for foods are in many cases far from being complete and even for allergens, proteoform data are often lacking or are highly incomplete. In addition, the *in silico* identification of marker does not provide any information concerning their analytical performance with respect to sensitivity, post-translational modifications or the propensity towards processing-induced modifications. An overview on general considerations for the identifications of allergen markers is given in ^{14,15}.

Use of HRMS: marker identification and direct targeted detection

The employment of discovery proteomics for the identification of valid marker peptides has some significant advantages compared to *in silico* analysis. Depending on the analytical task, it allows for the proteome-wide screening of potential marker peptides in food samples and allows the direct experimental validation of key aspects:

- 102 • Does the peptide occur in the given food/matrix?
- 103 • Are modifications of the peptide observed that might interfere with quantitation?
- 104 • How is the signal intensity of the marker peptide in the allergenic food?
- 105 • Is the peptide specific for a given allergen?

106 Though in principle also possible with lower resolution instruments, HRMS strongly increases
107 the number of identified peptides and the statistical confidence of peptide identifications. It has
108 however to be mentioned that instrumentation for HRMS discovery proteomics is in general not
109 available in routine laboratories and that the identification of peptides often still depends on the
110 search of experimental data against databases. In case of highly incomplete databases,
111 discovery-based proteomics can therefore face similar challenges as *in silico* marker
112 identification. Ongoing development in MS instrumentation and bioinformatics software today
113 also allow *de novo* sequencing of peptides without the necessity of databases (or only
114 confirmatory use). Reliable *de novo* sequencing is however strictly dependent on high
115 resolution MS data with very high spectral quality of MS/MS spectra that allow the unequivocal
116 identification of peptides from the precursor ion mass and the MS/MS spectrum. It is strongly
117 advisable to confirm peptides identified by *de novo* sequencing via the subsequent use of
118 synthetic peptide standards. A general overview on identification strategies in bottom-up
119 proteomics is given in Fig. 2.

120 HRMS is not only used for allergen marker identification but also for detection and
121 quantification. Though QQQ instruments in general provide excellent sensitivity, this can be at
122 the cost of specificity especially in targeted proteomics. Complex foods consist of
123 metaproteomes from different species resulting in incredible complex peptide composition after
124 enzymatic digest. Highest sensitivity of targeted proteomics approaches on QQQ instruments is
125 achieved by recording several SRM/MRM transitions and the isolation window for analytes in
126 the first quadrupole is in general between $m/z = 0.2-0.5$. Peptides with near isobaric precursor
127 ion masses and overlapping 2 or 3 SRM transitions can occur in such complex samples and

might lead to false positive results in case of similar retention times. This matrix interference has been exemplified in a recent study using an MRM approach for the detection of nut allergens also demonstrating that increase of specificity can be obtained by including MS³ experiments in MRM mode¹⁶. HRMS strongly increases statistical confidence in peptide identification and a straightforward approach is the rapid HRMS screening of precursor ion masses with subsequent triggering of MS/MS spectra to confirm the peptide sequence. This strategy can be used for a semi-untargeted monitoring of samples and allows in principle also for the retrospective analysis of samples. One example is the analysis of foods material used for oral challenges. HRMS coupled to LC and ion mobility separation was employed for the semiquantitative and qualitative profiling of peanut allergens in raw and roasted peanuts¹⁷ and allowed to identify for which allergens roasting affected extractability¹⁷. This approach was exemplified for the detection of the allergenic fining agents caseinate, lysozyme and ovalbumin in white wine on an Orbitrap instrument. The precursor ion mass was determined in high resolution for the detection of specific marker peptides. The limits of detection were in the range of 1.0 µg/ml¹⁸. For the detection of peanut in tree nut samples a similar approach using HRMS screening was successfully employed¹⁹. The multiplexed tree nut and peanut allergen detection from five different species was performed using HRMS in full scan mode at maximal resolution on an orbitrap instrument. The exact mass of 44 marker peptides together with the expected isotopologue pattern was used as readout from extracted ion chromatograms²⁰ for identification and quantification. The limit of detection of the most sensitive peptides were between of 1.0-5.7 µg/g for the different allergenic foods.

Structural characterization of allergens using HRMS

A further area of growing interest is the multifaceted structural characterization of allergens using the potential of high resolution mass spectrometry. This includes the:

- detection of processing-induced modifications

- 154 • structural polymorphisms and proteoforms *in planta*
- 155 • modification and degradation kinetics during gastrointestinal metabolism
- 156 • processing after uptake by immune cells e.g. to elucidate T-cell and B-cell epitopes
- 157 • investigation of the topology of antibody allergen complexes after stabilization via
- 158 cross-linking and H/D exchange

159 Some of the above mentioned topics of interest are exemplified by the following studies:

160 To investigate the extractability of specific peanut allergens after processing (roasting) of
161 peanut material used for food challenges HRMS (Q-TOF combined with ion mobility) was
162 applied²¹. Extractability of allergens from the cupin superfamily (Ara h 1 and 3) were
163 significantly reduced in contrast to prolamins (Ara h2 and 6) which were not affected. Defatting
164 of peanut material strongly reduced the content of the oleosins Ara h a10 and Ara h 11 which
165 are associated with oil bodies in the peanut. In a similar study, the effect of roasting on walnut
166 allergens was investigated²².

167 For the characterization of structural heterogeneity (polymorphisms and post-translational
168 modifications) of 2S albumins from mustard and hazelnut *in planta*, a combination of
169 proteomics approaches on intact protein level (top-down), subunit level (middle-down) and
170 peptide level (bottom-up) was applied^{23,24}.

171 Up to 41 proteoforms of the hazelnut 2S allergen Cor a 14 were identified that in addition also
172 differed significantly between hazelnut varieties. For the mustard 2S albumins eight consensus
173 isoforms were identified that were present in nine different mustard samples while other
174 isoforms previously described on DNA level were not detectable on protein level. This
175 pronounced degree of heterogeneity might influence allergenic potential of single isoforms and
176 has to be taken into account for the development of allergen detection methods.

177 A pronounced sequence heterogeneity has also been reported for the major birch pollen allergen
178 Bet v 1 with at least 18 isoforms and it has to be noted that this heterogeneity is currently not
179 reflected in the available diagnostics products²⁵.

Mass spectrometry was also employed in several studies for the more detailed characterization of the degradation of food allergens in gastrointestinal model systems. The degradation kinetics of different hazelnut allergens was analyzed *in vitro* in a model system comprising of the oral, gastric and intestinal phase and demonstrated pronounced differences in stability of allergens belonging to different protein superfamilies²⁶. These studies also allow to identify structures responsible for sensitization via the gastrointestinal tract. In a similar study, digestion-resistant peptides from peanut conglutinin were identified using HRMS and immobilized trypsin on magnetic beads and the conformational stability of these peptides was modeled²⁸. The release of digestion-resistant peptides from peanut in a static GI-model and their IgE-binding capacity was characterized in another study²⁹.

The structural characterization of the complex formation of allergens with antibodies is also analyzed with mass spectrometry via H/D footprinting. To this end, exchange of H with D is analyzed for the free complex partners (allergen and antibody) and for the binary complex. Binding epitopes of the complex show reduced or no H/D exchange and can be identified via this technique²⁷.

Future outlook

Mass spectrometry and especially high-resolution mass spectrometry is of hugely growing importance for the molecular characterization and analytical detection of allergens. Taking also into consideration the dynamic technological advancement of mass spectrometers with constantly emerging novel hybrid instruments, the addition of further separation dimensions such as ion mobility, or the renaissance of capillary electrophoresis as a separation technique, it is estimated that HRMS will be of growing importance in the future. Proteomics in food and allergy analysis is however still in its infancy and a variety of issues need to be addressed. We do have sequence information for a large proportion of allergens but the structural variety and reactivity in food is still far from being fully understood. The development of MS-based analytical approaches for the quantification of allergens in food is a highly dynamic field with

206 constant and rapid improvement but the absolute quantitation of allergens, especially from
207 processed food, is still beset with pitfalls. HRMS has just started to show its potential in the
208 field of molecular allergy sciences and as methodologies and technology continue to develop,
209 we will gain e.g. a deeper understanding of mechanisms influencing allergenicity in food by
210 matrix interferences and identify in more detail the structural elements relevant in sensitization
211 and allergic reaction. This might also contribute to pave the way for precision diagnostics and a
212 more personalized risk assessment and, one day, novel and efficient therapeutic approaches.

213

214 **Conflict of interest**

215 *The authors have declared no conflicts of interest.*

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321

322 **Table1**

323 **Table 1** Examples for mandatory allergen labelling worldwide (according to FARRP International regulatory chart, March
324 8, 2018, <https://farrp.unl.edu/IRChart>). Major allergens (“big 8”) are underlined.^a Countries with mandatory
325 allergen labelling are: Argentina, Bolivia, Brazil, Chile, Colombia, Costa Rica, Cuba, Mexico, Nicaragua,
326 Venezuela. ^b labelling of crustacean mandatory for crab, shrimp and prawn. ^c labelling of fish mandatory for
327 mackerel. ^d labelling mandatory for milk from mammary gland of farmed animals. ^e labelling of milk mandatory
328 for lacteal secretion from cows. ^f labelling of tree nuts mandatory for walnuts. ^g labelling of wheat not mandatory
329 in Argentina, Chile and Mexico. ^h labelling of sulfites mandatory for concentrations ≥10 mg/kg. ⁱ labelling
330 mandatory for sulfites directly added or ≥10 mg/kg. ^k labelling of sulfites mandatory for concentrations of
331 ≥10 mg/kg in all countries except of Brazil (labelling mandatory in any case). ^l labelling of latex (natural rubber)
332 mandatory in Brazil.

Allergenic Food	Codex Alimentarius	Australia	Canada	“Central and South America” ^a	China	European Union	Japan	Korea	United States
<u>Crustacea</u>	X	X	X	X	X	X	(X) ^b	(X) ^b	X
<u>Egg</u>	X	X	X	X	X	X	X	X	X
<u>Fish</u>	X	X	X	X	X	X		(X) ^c	X
<u>Milk</u>	X	X	X	X	X	(X) ^d	X	X	(X) ^e
<u>Soy</u>	X	X	X	X	X	X		X	X
<u>Peanut</u>	X	X	X	X	X	X	X	X	X
<u>Tree nuts</u>	X	X	X	X	X	X		(X) ^f	X
<u>Wheat</u>	X	X	X	(X) ^g	X	X	X	X	X
Cereals with gluten	X	X	X	X	X	X			
Celery						X			
Lupin		X				X			
Mustard			X			X			
Mollusks		X	X			X			
Sesame		X	X			X			
Sulfites	(X) ^h	(X) ^h	(X) ⁱ	(X) ^k		X			X
Buckwheat							X	X	
Peach								X	
Pork								X	
Tomato								X	
Bee pollen / Propolis		X							
Royal jelly		X							

333

334 **Figure legends**

335 Figure 1: Overview on sensitization and allergic reaction against food

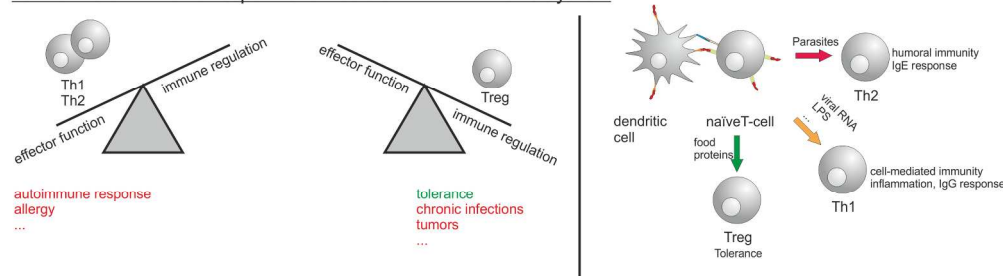
336 Upper lane: oral tolerance is an active process mediated via the immune system. The immune
337 response is mediated via specific T cells (Th 1 or 2) after contact to specific antigenic structures
338 such as virus RNA, or protein structures from parasites. In nonallergic individuals, uptake of
339 food proteins leads to the formation of specific regulatory T cells (Treg) that mediate persistent
340 oral tolerance. Middle lane: The course of sensitization in food allergy. After uptake of
341 allergens and presentation to the immune system a Th2 response is initiated instead of oral
342 tolerance which leads to the generation of plasma cells from B cells that produce specific IgE
343 antibodies against the respective food allergen. Binding of IgE on the surface of effector cells
344 (mast cells) is the final step of the sensitization. Lower lane: Allergic reaction. Following
345 sensitization, the uptake of allergens leads to binding of epitopes to specific IgE antibodies and
346 IgE cross-linking on the surface of effector cells. This induces the release of potent mediators
347 from effector cell granules which are causative for the allergic symptoms.

348

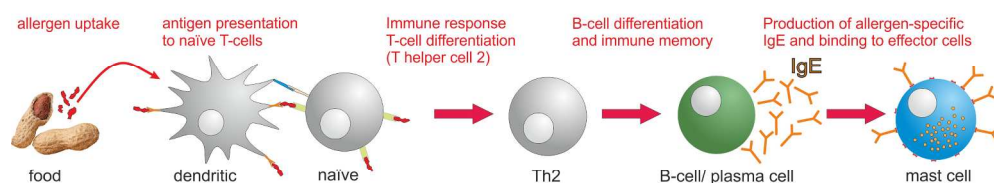
349 Figure 2: Graphical overview on bottom-up proteomics, database- assisted peptide identification
350 and *de novo* sequencing.

351

Tolerance or immune response: a decision of the immune system



Sensitization



Allergic reaction

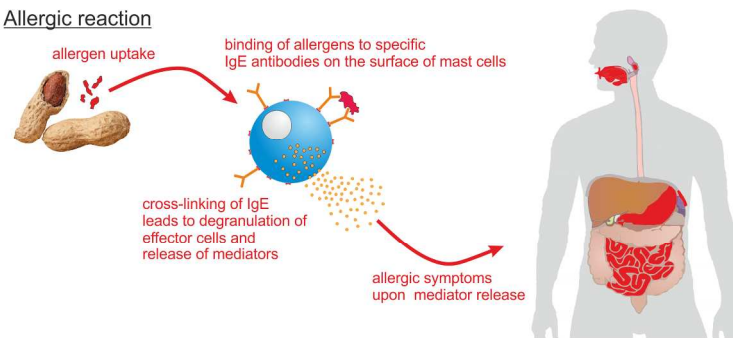
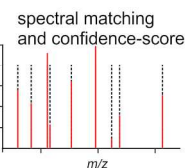
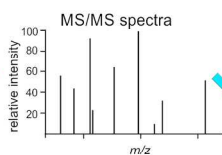
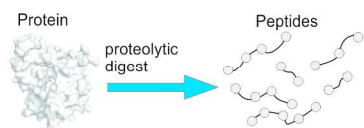
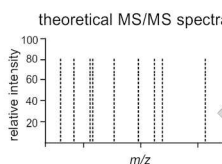
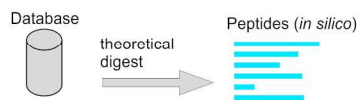
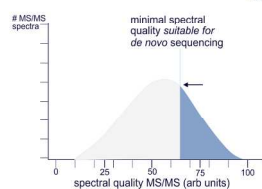


Figure 1: Overview on sensitization and allergic reaction against food

Upper lane: oral tolerance is an active process mediated via the immune system. The immune response is mediated via specific T cells (Th 1 or 2) after contact to specific antigenic structures such as virus RNA, or protein structures from parasites. In nonallergic individuals, uptake of food proteins leads to the formation of specific regulatory T cells (Treg) that mediate persistent oral tolerance. Middle lane: The course of sensitization in food allergy. After uptake of allergens and presentation to the immune system a Th2 response is initiated instead of oral tolerance which leads to the generation of plasma cells from B cells that produce specific IgE antibodies against the respective food allergen. Binding of IgE on the surface of effector cells (mast cells) is the final step of the sensitization. Lower lane: Allergic reaction. Following sensitization, the uptake of allergens leads to binding of epitopes to specific IgE antibodies and IgE cross-linking on the surface of effector cells. This induces the release of potent mediators from effector cell granules which are causative for the allergic symptom

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bottom-up proteomicsdatabase-assisted data evaluationde novo sequencing

apply software-assisted de novo sequencing

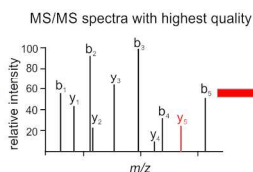
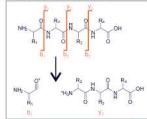
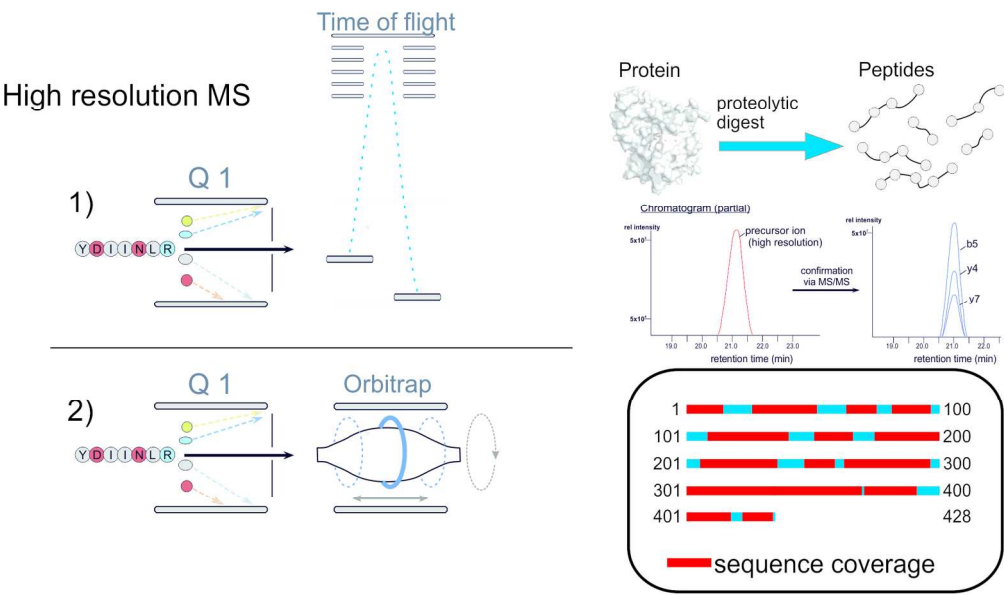
LFQTLR
de novo peptide sequence

Figure 2: Graphical overview on bottom-up proteomics, database- assisted peptide identification and de novo sequencing.

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TOC graphic

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