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# vMethod™ Application for Food Testing

## Allergen Screening Solution

# Simultaneous analysis of 12 food allergens in baked and raw food products using the LC-MS/MS QTRAP® 4500 system

***Detection of multiple signature peptides of 12 priority food allergens***

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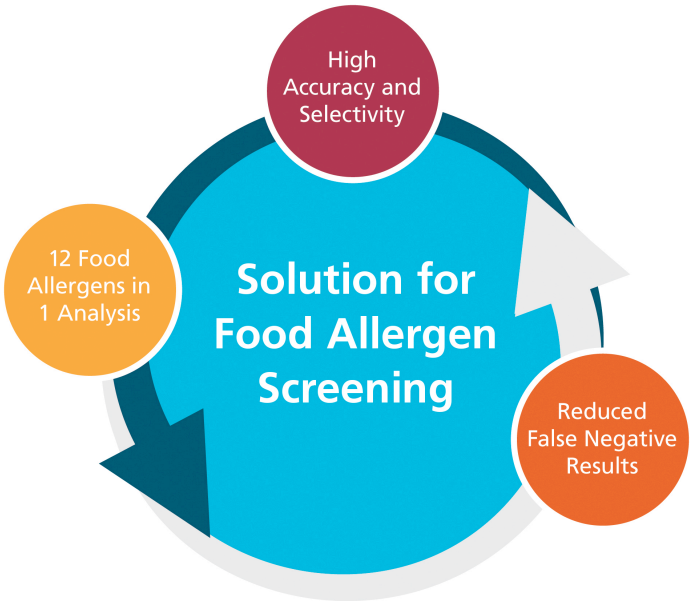
## Introduction

A food allergy is an immune-mediated, adverse reaction to an antigenic protein. Even limited exposure to an antigen can provoke a significant reaction in sensitive individuals, causing rashes, itching and swelling in the mouth, nausea, vomiting, and asthma. Additionally, food allergies are the leading cause of anaphylaxis, an acute, potentially deadly allergic reaction. The prevalence and severity of food allergies are rising, with approximately 150 million people suffering from food allergies worldwide.[1, 2] Presently, there is no cure for food allergies, and sufferers must rely on the correct labeling of foods to avoid consuming allergens. Hence, the development of sensitive and accurate analytical methods to screen for the presence of allergens in food products is necessary for the prevention of potentially life-threatening health problems for allergy sufferers.

Enzyme-linked immunosorbent assays (ELISA) are the most commonly used tests for screening allergens. Although relatively quick and simple to perform, ELISA tests are limited in selectivity and susceptible to cross-reactivity, which can lead to false positive or false negative results. Additionally, most ELISA tests are capable of detecting only one allergen at a time, requiring multiple tests to screen for more than one allergen in a food sample. Therefore, a method that can unambiguously confirm and identify multiple allergens would be invaluable for food screening.

Herein, we present an LC-MS/MS method using the QTRAP® 4500 LC-MS/MS system that detects and screens 12 separate allergenic proteins simultaneously in a single injection. The allergens detected in this method were selected from the guidelines presented in the Codex Alimentarius, a resource developed by the United Nations' Food and Agriculture Organization (FAO) and the World Health Organization (WHO) to harmonize international food standards.[3]




The Codex recommends eight allergenic food groups be declared on the labels of pre-packaged foods: grains, shellfish, eggs, fish, legumes, milk, sulfite, tree nuts.[3] Five of these allergens are detected with this method including eggs, milk, peanuts, soy beans, and tree nuts (almonds, Brazil nuts, cashew nuts, hazelnuts, pecans, pine nuts, pistachios, and walnuts).

To evaluate a range of food products (both raw and bakery goods) for their allergenic content, several unique signature peptides specific to each allergen were identified from tryptic digests of food homogenate extracts. A mixture of 12 allergens was added to bakery product food matrices (either bread or cookie) over a range of known concentrations, and several MRM transitions corresponding to allergenic signature peptides were evaluated simultaneously using the *Scheduled* MRM™ algorithm. Presently, this method can detect allergenic peptides from five of the major classes of allergenic foods at a detection limit of 10 ppm in a variety of food matrices.

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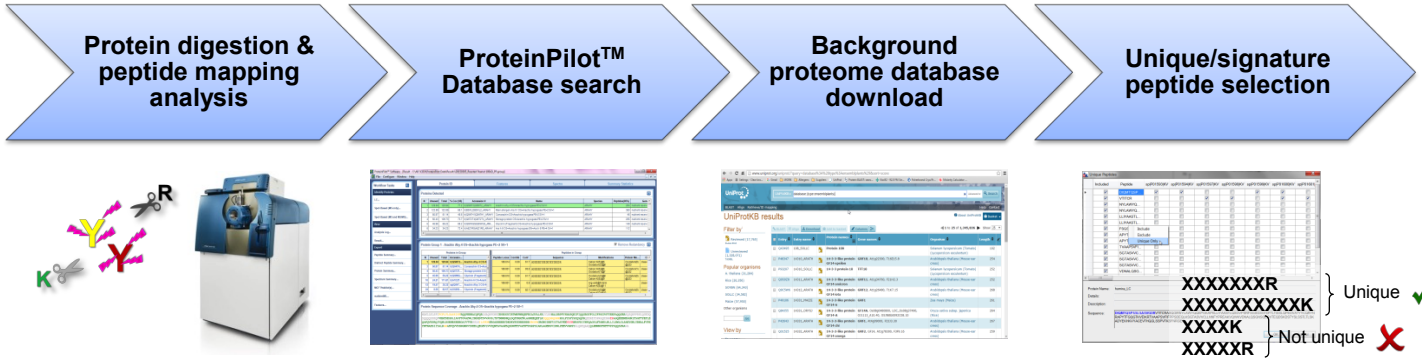


**Figure 1.** Signature peptide selection workflow using the SCIEX TripleTOF® 6600 system and ProteinPilot™ software

## Experimental

### Sample Preparation

To prepare bread and cookie homogenates, unbaked gluten-free bread or cookie mixes (100 g) were supplemented with 10 to 500 ppm (by weight) of each of the following 12 allergenic foods: eggs, milk, peanuts, soy, almonds, Brazil nuts, cashew nuts, hazelnuts, pecans, pine nuts, pistachios, and walnuts. The fortified foods were then cooked according to manufacturer's specifications. The food samples (raw nuts, baked goods) were finely homogenized using a coffee grinder. Each homogenate (1 g) was defatted by extracting twice with hexane and dried by evaporation in the fume hood. Extraction buffer (4 mL) was added to the defatted homogenates, which were then centrifuged prior to the removal of supernatants (500 µL). Reducing reagent (50 µL) was added to supernatants at 60°C for 1 hr. After cooling (25°C), samples were alkylated using a cysteine blocking reagent (25 µL). Trypsin (20 µg) was added to modified proteins (3 to 12 h) in calcium chloride/ammonium bicarbonate buffer to obtain tryptic peptides for signature peptide analysis prior to neutralization with formic acid (30 µL). Digested samples

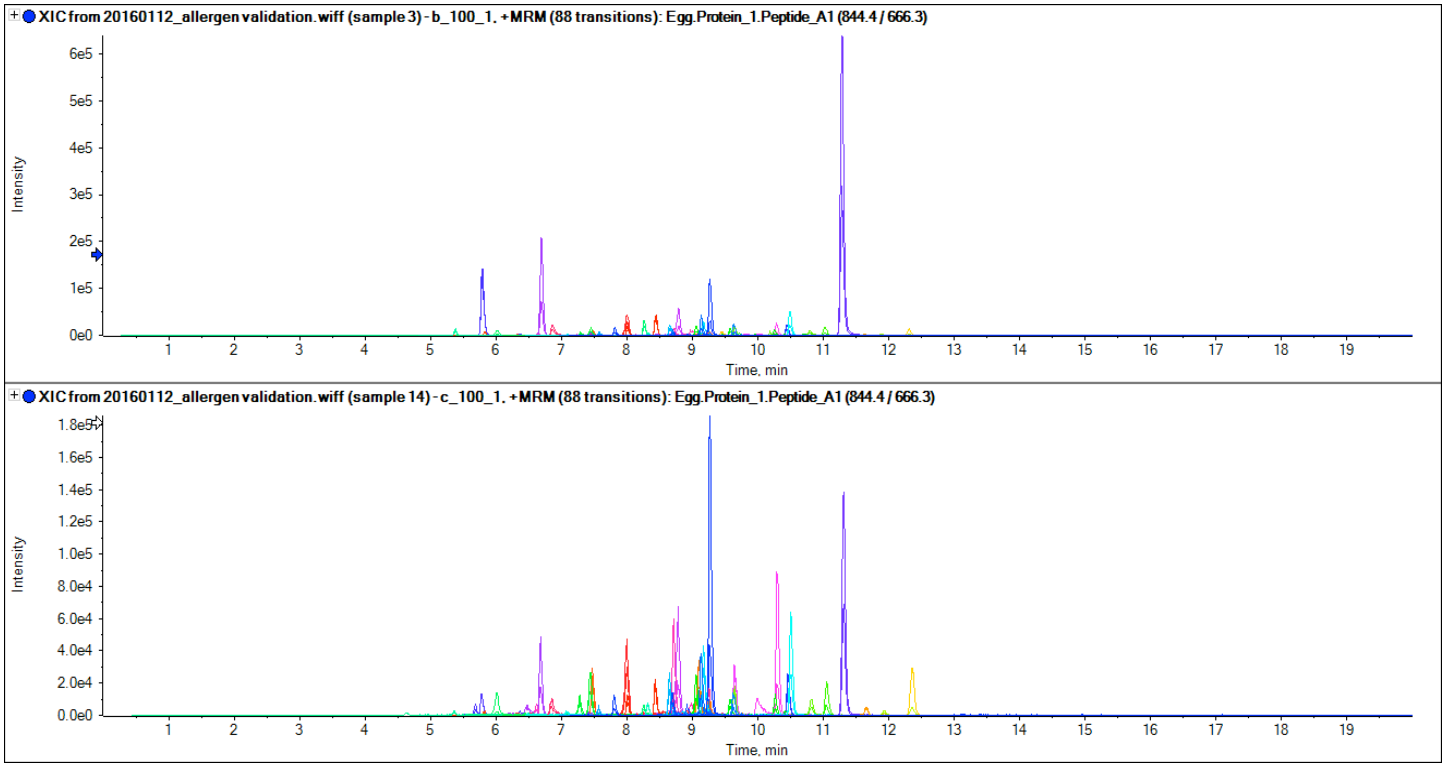


**Figure 2.** Extracted ion chromatograms (XIC) from LC-MS/MS analysis of bread (top) and cookie (bottom) homogenates fortified with egg, milk, peanut, soy, and nut proteins at100 ppm. Multiple peaks corresponding to allergenic tryptic peptides are displayed.

(500 µL) were centrifuge-filtered using a 10 kDa MWCO filter prior to LC-MS/MS analysis.

**LC Separation**

Tryptic peptides (30 µL injection volume) were chromatographically separated using a Shimadzu Prominence UFLC$_{XR}$ system equipped with a Phenomenex Kinetex C18 column (2.6 µm, 100 x 3 mm). A linear gradient was employed over 12 min at a flow rate of 300 µL/min using 0.1% formic acid in water and 0.1% formic acid in acetonitrile.

**MS/MS Detection**

To identify signature peptides for allergen screening, peptide maps of various allergenic foods (eggs, milk, peanuts, soy beans, and tree nuts) were acquired using a TripleTOF® 6600 LC-MS/MS System (Figure 1). The strategy for the selection of signature peptides can be found in more detail in the Results and Discussion.

To screen foods for allergens, a SCIEX QTRAP® 4500 system with Turbo V™ source in positive ESI mode was employed using an ion source temperature of 500°C. The *Scheduled* MRM™ algorithm was used to analyze food samples for 12 allergens in a single injection by multiplexing the detection of multiple MRM transitions for allergenic signature peptides.

## Results and Discussion

Signature peptides were chosen for each allergen based on: 1) their uniqueness compared to background proteins; and 2) their sensitivity of detection. Further details on peptide sequences, their relative abundance, and possible post-translational modifications were generated using the ProteinPilot™ software's protein database search features after LC-MS/MS analysis of peptides on a TripleTOF® 6600 System (Figure 1). The list of selected peptides was refined by removing peptide sequences susceptible to further reaction (e.g., post translational modification, Maillard reaction) during food processing or baking.

For each allergen, two unique proteins, two unique peptides per protein, and two MRM transitions per peptide were chosen to ensure confidence in the identification of an allergen. To monitor many MRM transitions during a single injection, the *Scheduled* MRM™ Algorithm was employed, where individual MRM transitions were monitored for a short period during their expected retention time, decreasing the total number of concurrent MRM experiments during a cycle and allowing cycle time and dwell time to be maintained. This approach maximized the S/N for signature peptide detection and allows the method to be expanded as new allergenic markers are identified.

To identify multiple allergens in the same food sample, a total of 88 MRM transitions corresponding to 44 allergenic peptides, from eggs, milk, peanuts, soy beans, and tree nuts, were characterized (Figure 2). Of these 44 peptides, 40 transitions corresponded to peptides with unique sequences not shared by background proteins. The LC-MS/MS-based screening method deployed here simultaneously detected 12 allergenic proteins from 5 major classes of food allergens (egg, milk, peanut, soy and tree nuts) that had been fortified into bakery products at varying concentrations.

To show that signature peptide signals were linear in response to increasing allergen levels, calibration curves for each peptide and its three transitions were generated over a wide dynamic range

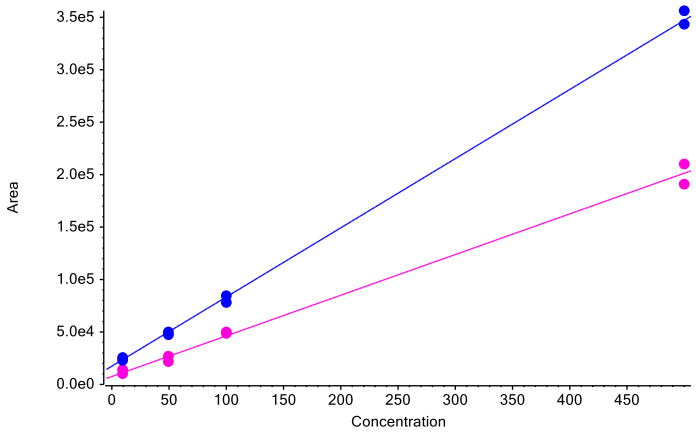


**Figure 3a.** Calibration lines of a hazelnut peptide from 0 to 500 ppm. Two MRM transitions were monitored: fragment 1 (blue) and fragment 2 (pink)

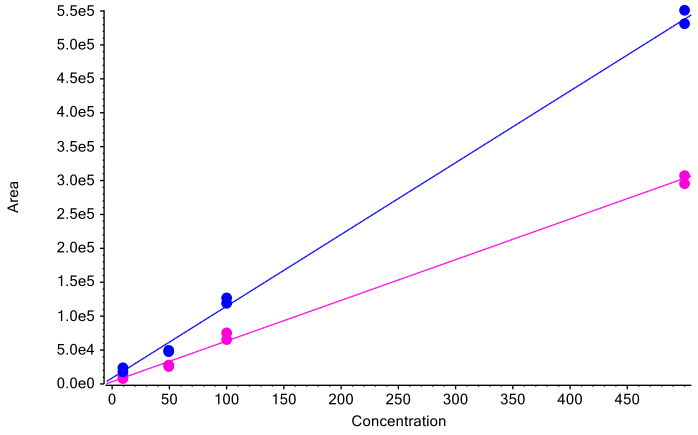


**Figure 3b.** Calibration lines of a peanut peptide form 0 to 500 ppm. Two MRM transitions were monitored: fragment 1 (blue) and fragment 2 (pink)

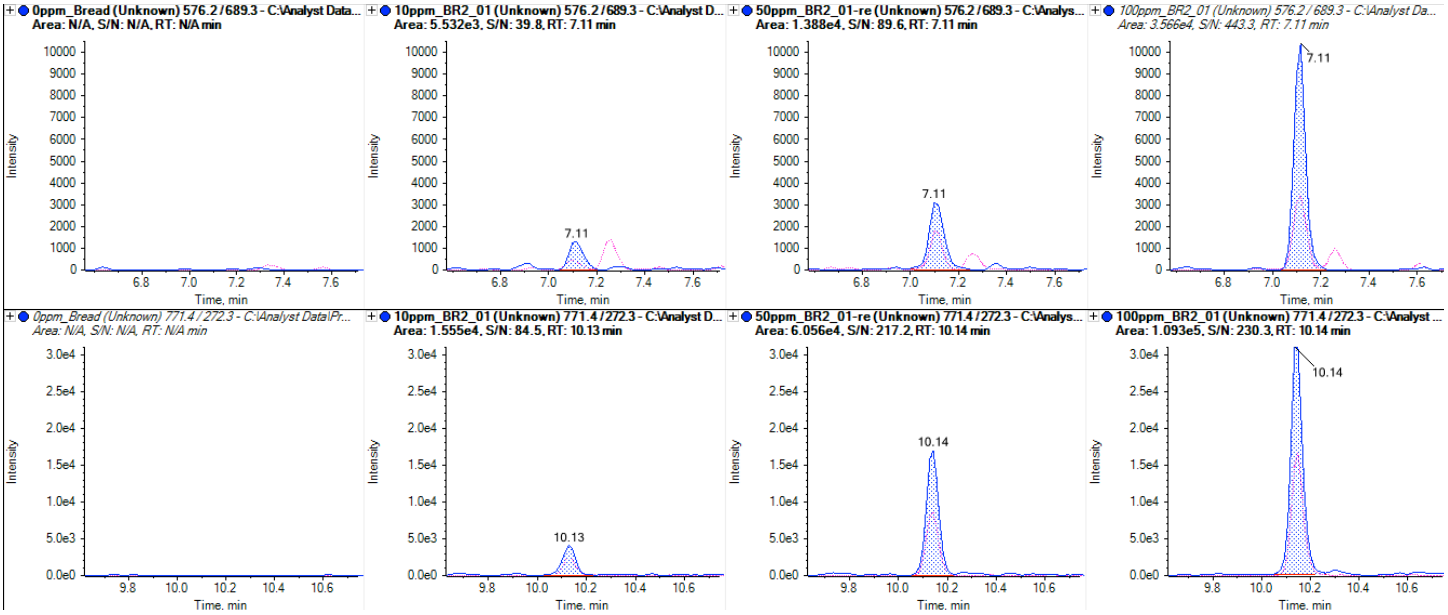


**Figure 4.** Extracted ion chromatograms for the signature peptide, protein 1 peptide 1, from hazelnut (top) and peanut (bottom). Varying concentrations of allergen (0, 10, 50 and 100 ppm) were added to bread samples. Two different MRM transitions for protein 1, peptide 1 are shown (blue, and pink traces).

(0 to 500 ppm) with good reproducibility in matrix (Figures 3a and 3b). MRM transitions were linear over a broad dynamic range and resulted in regression values over 0.95 for all allergens.

All allergenic peptides were detected at concentrations as low as 10 ppm (Figure 4) and generated signals proportional to the quantity of supplemented allergen.

One advantage of the LC-MS/MS method over ELISA-based detection methods is that multiple allergens can be detected in the same sample with one injection. To ensure that a high standard of performance was maintained as throughput increased with the multiplexed LC-MS/MS method, two separate allergen detection methods were directly compared. Signature peptides for select allergens (hazelnut and peanut) were analyzed using two separate ELISA kits and with the LC-MS/MS based method. In general, there was good correlation between the calculated concentrations obtained from ELISA and LC-MS/MS with $r^2 \geq 0.99$ (Figure 5). However, results from the ELISA-based tests underestimated the concentrations of hazelnut and peanut supplements in bread and cookie matrices, especially at higher concentrations.

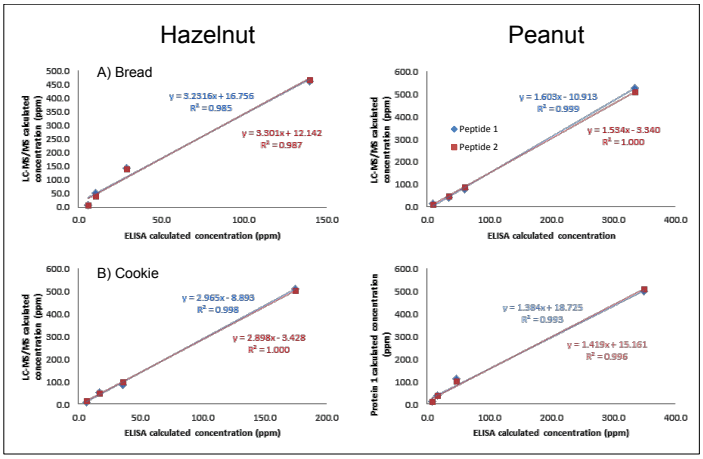


**Figure 5.** Comparison of allergen concentrations detected using ELISA vs. LC-MS/MS methods for two peptides (blue and orange) and two matrices, bread (top) and cookie (bottom)

To verify the effectiveness of the LC-MS/MS method for detecting allergens in commercial food samples, bakery products (cookies) containing a variety of allergens were screened using the signature peptide method (Figure 6). Allergen-related signals were not detected in cookie samples that were egg-, milk- and nut-free. However, cookies and bread products that listed hazelnuts and peanuts as ingredients tested positive using the LC-MS/MS method. Other allergens were identified, including egg and milk.

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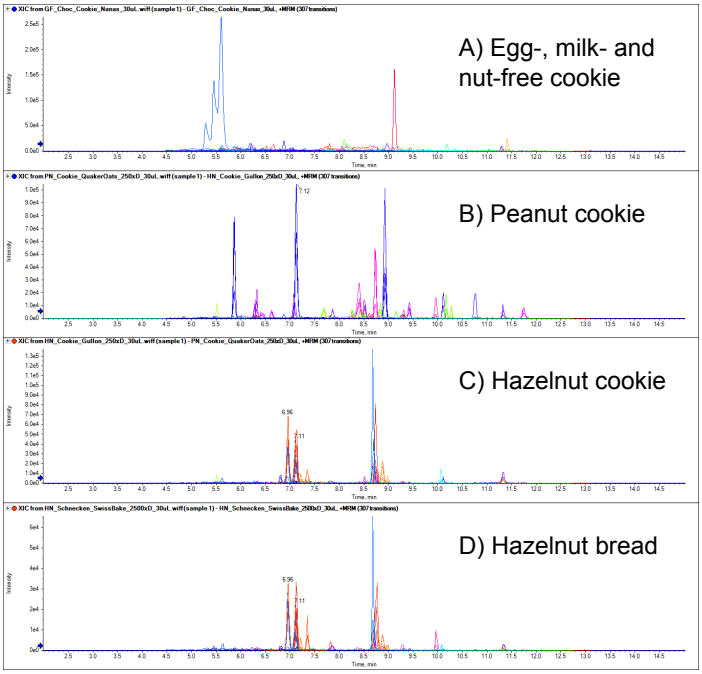


**Figure 6.** Extracted ion chromatograms of (A) Egg-, milk-, and nut-free cookie, (B) peanut cookie, (C) hazelnut cookie, and (D) hazelnut bread.

## Summary

We have developed a multi-allergen screening tool using an LC-MS/MS method that can detect 12 food allergens in commercial products by identifying several MRM transitions corresponding to unique signature peptides for each allergen and multiplexing their detection into a single injection. In total, there are 88 MRM transitions representing peptides from the egg, milk, peanut, soy, and tree nut allergen groups. Unlike ELISA methods, this LC-MS/MS analysis detects multiple peptides from each allergic protein, thus improving method specificity and minimizing the potential for false positive and false negative results. Using only a single sample preparation method and a multiplexed data acquisition, more allergens than previously reported[4] were screened and differentiated from other food ingredients contained in baked food matrix.

## References

1 R. Valenta et al.: 'Food allergies: the basics' Gastroenterology 148 (2015) 1120-1131

2 A. Sage: 'Food Allergen Analysis Using LC-MS-MS' LCGC The Column 10 (2015) 13-29

3 Codex Alimentarius Commission: 'Report of the Twenty-Second Session of the Codex Committee on Food Labelling' (1993) downloaded at http://www.codexalimentarius.org/

4 S. Lock et al.: 'The Detection of Allergens in Bread and Pasta by Liquid Chromatography Tandem Mass Spectrometry' Application Note SCIEX (2010) #1830610-0



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