

Reliable Detection of Milk Allergens in Food Using a High-Resolution, Stand-Alone Mass Spectrometer

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Reliable methods are needed for detection of allergenic milk proteins in complex food matrixes. The feasibility of an LC/high-resolution MS method for the analysis of milk proteins in a thermally processed model food (incurred cookies) and in white wine spiked, respectively, with milk powder and caseinate is described. Detection of milk proteins was based on the identification of unique peptides in the tryptic digests of cookie/wine extracts using an RP-HPLC separation coupled to an Exactive™ nonhybrid mass spectrometer using Orbitrap technology. The extremely high mass accuracy and resolution provided by the Orbitrap analyzer allowed a fast preliminary identification of four previously proposed peptide markers of caseins using only accurate values of the m/z of their ions. No interference was observed, despite the complexity of the analyzed matrixes. Moreover, the availability of a high-energy, collisionally activated dissociation cell integrated in the mass spectrometer enabled acquisition of peptide MS/MS-like spectra through post-source fragmentation. Confirmation of peptide marker identity could then be achieved by a comparison between experimental and predicted product ions. The described method shows the great potential of Orbitrap MS as a reliable technique in the field of protein allergen detection once the peptide markers are identified.

Milk contains proteins capable of triggering allergic reactions in sensitive consumers (1). As a consequence, milk is a food allergen (2–4) whose presence in food must be properly labeled. It is generally

acknowledged that even minute amounts of an allergenic food, like milk, can cause severe reactions, although insufficient knowledge is available on clinically relevant threshold levels as established by human oral challenge studies (5). To protect sensitive individuals from the risk of allergic reactions, Directive 2007/68/EC issued in the European Union (6) requires that the 14 most allergenic ingredients added to food products, including milk products, have to be declared on the food label. Because the allergic consumer relies on the information on the label, it is of utmost importance that foods that do not declare milk or milk-derived products as ingredients are truly free of milk proteins. Nevertheless, cross-contact can occur at any stage of food production, thus introducing the hazard of hidden allergens (7).

Milk/dairy products are common ingredients in baked products, such as cookies; thus, there is a high risk that milk allergens are present in these products. As a consequence, there is a need for sensitive methods to ensure compliance with labeling legislation in order to improve consumer protection.

Another important food commodity, wine, can be subject to contamination by milk allergens. Indeed, it is a common practice of wine makers to use fining agents, such as milk proteins (e.g., caseinates), to promote the clarification of wine (8, 9). This practice poses the risk of contaminating wines with trace levels of caseinates, endangering milk-allergic consumers, as pointed out by the opinion issued by the European Food Safety Agency (EFSA; 10). Moreover, EFSA documented the need for reliable methods to confirm analytical results obtained by ELISA of milk allergen in wine (11, 12).

A critical step in allergen detection, whatever method is being used, is the procedure used to extract the allergenic proteins from the food matrix (13). As for milk allergens, the heat applied to the food matrix has been shown to have a negative impact on the final recovery (14).

Immunoassays generally offer a good sensitivity and specificity; however, they may be affected by changes in the

target protein structure or in immunoglobulin binding sites resulting from heating or other food processing (15). As a consequence, the detection of allergens by antibodies may be affected, hampering their final detection and changing the outcome of the test. This is a matter of concern for allergic consumers since hidden milk allergens might remain undetected by immunochemical analysis of food products. It is, therefore, desirable to have a confirmative method for the unambiguous identification of the allergenic compound, and MS-based methods could help in this aim.

The coupling between HPLC and MS/MS has recently gained great attention for the detection of allergens in food (16). For example, LC/quadrupole (Q)-time-of-flight (TOF)-MS methods have been proposed as alternative approach to detect milk allergens in a processed food matrix, exploiting the identification of their peptide markers in the tryptic digest obtained (17, 18). It is well known that mass accuracy is a key parameter for a good peptide attribution in the field of proteomic analysis. Q-TOF-MS systems, traditionally recognized as instruments with high mass accuracy and resolution for peptide analysis, have recently been superseded by some of the latest MS instruments launched on the market, namely those based on Orbitrap technology, often providing mass accuracies in the low ppm range and mass resolutions higher than 10^5 (19). In a proteomic experiment, where a mixture of digested proteins needs to be separated by LC and then directly analyzed by MS, the accuracy achieved by the MS instrument directly translates into the mass tolerances that can be entered in the subsequent database search to identify the digestive peptides, and then the original proteins, with the highest confidence.

Despite these considerations, the potential of LC/Orbitrap MS as an analytical tool for fast screening of milk allergens in food samples has not been investigated so far. This paper explores the capabilities of this technique to identify milk allergens, namely caseins, in complex food matrixes treated with tryptic digestion. This investigation utilized a set of four peptide markers of caseins that have been recently highlighted in one of our papers on the topic (20). In particular, the potential of high-accuracy MS measurements for the recognition of the casein peptide m/z values, even at low concentrations (low ppm range) and in the presence of several potential interfering compounds, was assessed. Subsequently, the high-energy collisionally activated dissociation (HCD) cell in the Orbitrap spectrometer was exploited to perform fragmentation experiments on all the peptides eluted from the chromatographic column and to further confirm peptide sequences through an evaluation of the high resolution MS/MS-like (actually HCD-MS) spectra.

Experimental

Chemicals

Water and acetonitrile (LC/MS grade), formic acid (HCOOH; MS grade), hydrochloridric iodoacetamide, dithiothreitol (DTT), trypsin (proteomic grade), [Glu]-fibrinopeptide (GFP), and sodium caseinate were

purchased from Sigma-Aldrich (Milan, Italy). RapiGest was from Waters-Micromass (Manchester, UK).

LC/MS Apparatus and Operating Conditions

An HPLC/Ultra (U)-HPLC pump equipped with an autosampler (Accela, Thermo Fisher Scientific, Waltham, MA) was used for the chromatographic separation. The separation was carried out by injecting 10 μ L trypsin digest into a fused-core column (Ascentis Express C18, 100 mm length, 2.1 mm id, 2.7 μ m particle size; Supelco, Milan, Italy) and using a binary elution gradient operated at a flow rate of 150 μ L/min. The solvents used were A) H₂O and B) acetonitrile, both containing 0.1% HCOOH (v/v). The linear gradient adopted for peptide elution was the following: linear from 93 to 50% solvent A in 40 min; from 50 to 30% A in 1 min and then 30% A for 2 min; back to 93% A in 1 min; and re-equilibration for 10 min.

MS and HCD-MS analyses were performed using an Exactive™ nonhybrid, single-stage Orbitrap mass spectrometer (Thermo Fisher Scientific), equipped with an electrospray ionization interface. The spectrometer was operated at a resolution as high as 50,000 and a mass accuracy of 3 ppm.

A 5 μ g/mL standard solution of GFP in acetonitrile–water (50 + 50, v/v) containing 0.1% HCOOH was infused into the instrument to optimize the electrospray parameters for peptide detection. Infusion experiments into the source were carried out by means of an automatic pump delivering the standard solution at a flow rate of 5 μ L/min. The mass accuracy of the instrument was assessed by comparing the experimental m/z with that expected for the peptide ion and was found to be always within 3 ppm.

The optimized MS instrumental parameters were the following: scan range, 200–2000; resolution, high, microscans, 1 Hz; Automatic Gain Control: balanced 1×10^6 ; maximum injection time, 100 ms; sheath gas, 15 arbitrary units; auxiliary gas, 5 arbitrary units; spray kV, 4; capillary T, 250°C; capillary V, 32.50; tube lens V, 130 V; skimmer V, 30; heater T, 30°C.

For HCD-MS acquisitions, the collisional energy was set at 32 eV, and the scan range for HCD-MS spectra was 200–2000 Th. All the other parameters were the same as those used for MS acquisitions.

Search of Proteomic Databases

After the MS and HCD-MS analyses of each peptide marker under study, a list containing the m/z values of the precursor and possible product ions was created and searched against the SwissProt database using ProteinProspector MS-Tag software (<http://prospector.ucsf.edu/prospector/mshome.htm>) in order to elucidate peptide sequence. The following constraints were adopted for database searching: (i) fixed modifications, cysteine carbamidomethylation; (ii) variable modifications, methionine oxidation; (iii) parent (precursor) and fragment (product) ions tolerance, 5 ppm; and (iv) number of allowed missed cleavages during trypsin digestion, 1. For taxonomy, a comparison was made between

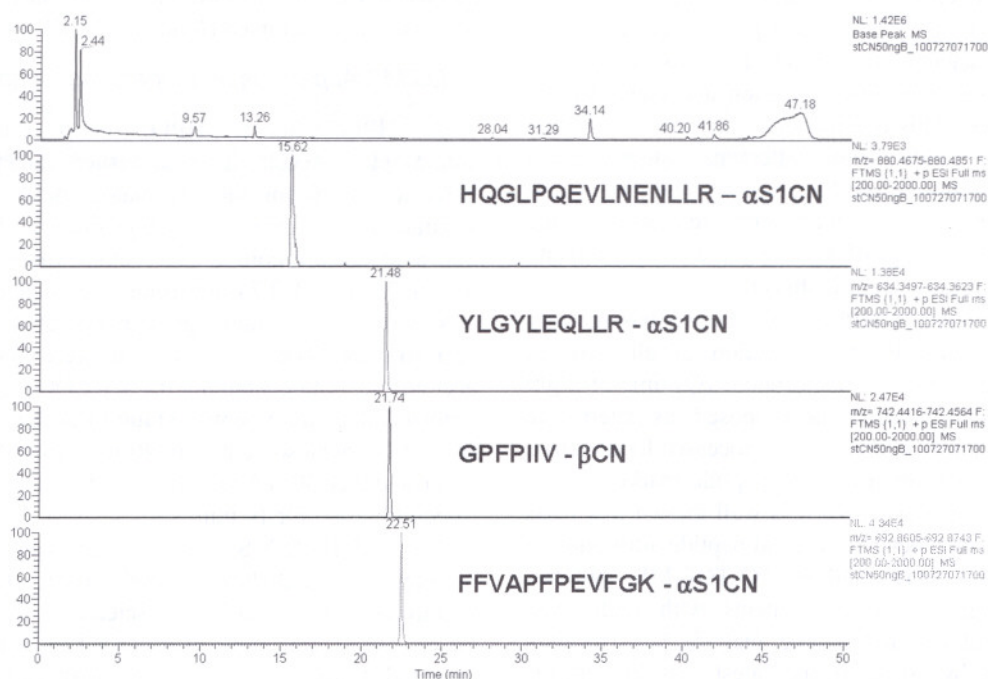


Figure 1. Comparison between the base peak chromatogram and the extracted XICs, referred to the m/z values of four tryptic peptides of caseins obtained after LC/high resolution MS analysis of the tryptic digest of a 0.5 $\mu\text{g/mL}$ caseinate standard solution.

searches performed by setting it as *Bos Taurus*, *Mammals*, or *All* in order to check the ability of the available HCD-MS data to lead to a reliable peptide identification even when large subsets of the proteomic database were searched.

Sample Preparation

The analytical method described was applied to two food matrixes: cookies and wine. In the former case, blind, incurred cookie reference materials spiked with skim milk powder (National Institute of Standards and Technology reference material 1549), produced within the EU MoniQA project, were obtained as a ground powder. These samples were distributed to all members within the MoniQA network to undertake validation studies with ELISA kits. Aliquots of cookie samples, previously analyzed by ELISA and found positive for milk allergens at different levels (estimated concentration between 1.6 and 22 ppm), were subjected to the extraction procedure as described by the ELISA kit manufacturer. Briefly, 2 g ground cookies was extracted with 20 mL 60°C extraction buffer (0.05 M sodium carbonate/bicarbonate, pH 6.9) by vortexing for 10 min. Solids in the samples were allowed to settle; then the supernatant was collected and centrifuged. Supernatant (2 mL) was then placed into Amicon centrifugation filter devices YM-3000 MW (Millipore, Billerica, MA) and dialyzed against the same volume of water. The retentate was lyophilized and redissolved in 0.5 mL RapiGest, 0.1%. A 100 μL volume of this solution was subjected to digestion before injection into the LC/MS apparatus. For wine samples, a total of 12 white

wine samples, either milk allergen-free or fined with caseinate at different levels (1.6, 3, and 6 ppm), were provided by Euroclone S.p.A. (Milan, Italy) and screened for the presence of milk allergen with an ELISA kit (provided by Euroclone) before LC/MS analysis. The sample pretreatment was the same as that described in our previous paper (20). Briefly, 4 mL aliquots were withdrawn from each wine batch and centrifuged for 30 min in ultrafiltration tubes with 5 KDa cut-off membranes (Millipore, Billerica, MA). An additional washing step of 1 mL MilliQ-water (Millipore) was applied to wash off the retentate and obtain a cleaner extract. A final volume of nearly 200 μL was collected, evaporated in a rotational-vacuum-concentrator RVC 2-18 CD (Martin Christ GmbH, Osterode, Germany), and successively resuspended by vortexing 1 min with 100 μL 0.1% RapiGest solution (1 mg RapidGest powder in 1000 μL 50 mM ammonium bicarbonate).

Preparation of Tryptic Digests

Tryptic digestion of cookie and wine extracts was performed as follows: Protein reduction was first performed by adding 5 μL 100 mM DTT solution and incubating the mixture in a thermoshaker at 60°C for 30 min. After cooling the sample to room temperature, 10 μL 200 mM iodoacetamide solution was added, and the mixture was agitated in a thermoshaker for 30 min in the dark. In the final step, 20 μL trypsin was added (final concentration 0.1 $\mu\text{g}/\mu\text{L}$) to the extract, and the tube was gently flicked to mix. Tubes were incubated at 37°C with shaking for 15 h in order to

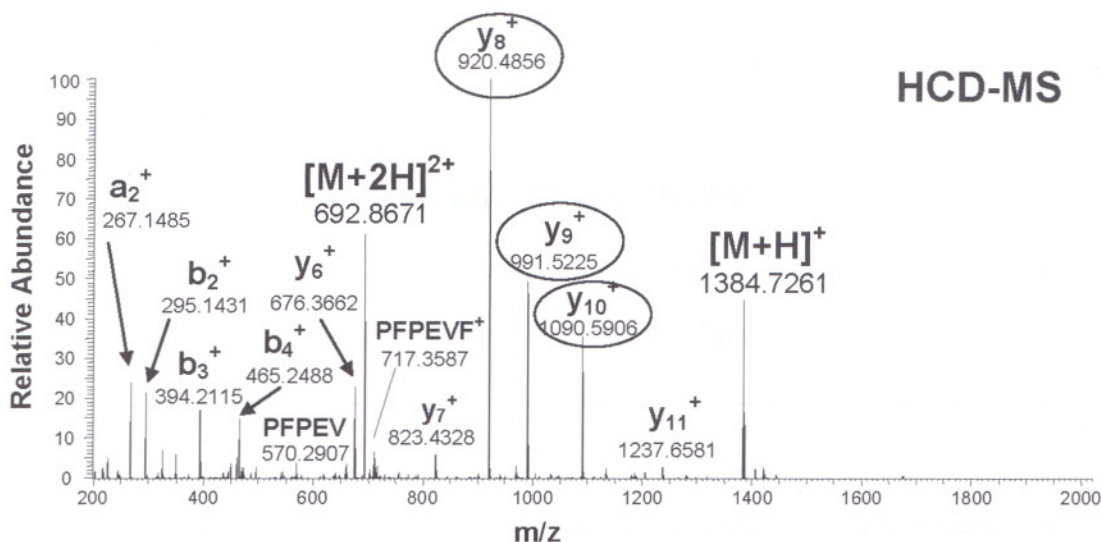


Figure 2. HCD-MS spectrum averaged under the chromatographic peak ($t_r = 22.51$ min) corresponding to the m/z 692.867 doubly charged ion shown in Figure 1. The product ion assignments are referred to the α -S1 casein peptide FFVAPFPEVFGK, assigned to that m/z after LC/MS measurements.

ensure complete digestion. Then, 10 μ L 1 M HCl solution was added to the digest, incubated for 30 min at 37°C, and centrifuged at 16 000 $\times g$ for 10 min. The pellet was discarded, and the supernatant was carefully transferred into microvials prior to LC/MS analysis.

Results and Discussion

Identification of Peptide Markers from LC/MS and LC/HCD-MS Data

The capabilities of the Orbitrap technology in identifying peptides arising from the tryptic digestion of potentially allergenic caseins to be used as markers, even in complex matrixes, was evaluated in the first stage of the present investigation. In particular, the combination of MS and HCD-MS data with the high accuracy and resolution provided by the Orbitrap instrumentation was exploited to confirm the identity of peptide markers.

Attention was focused on a set of casein tryptic peptides included among those recently identified by the same authors (although at higher concentration levels) in a Q-TOF-MS study (20): GPFPIIV from β -casein (singly charged ion, exact m/z 742.450); and FFVAPFPEVFGK, YLGYLEQLLR, and HQGLPQEVLENLLR from α S1 casein (all doubly charged ions, exact m/z 692.869, 634.356, and 880.476, respectively).

A 0.5 μ g/mL caseinate standard solution was considered first; the LC/MS base peak chromatogram obtained for its tryptic digest is shown in the upper panel of Figure 1. None of the visible peaks was found to correspond to one of the cited peptides, yet a tentative ion current extraction, performed on the exact m/z of their main ions, suggested their presence. The experimental m/z values were then determined from high-resolution mass spectra averaged under the corresponding peaks, and were found to be the following (values rounded to

the third decimal place): 742.449, 692.867, 634.356, and 880.476. Since the highest discrepancy between these values and the corresponding exact ones is -2.9 ppm, and the available mass accuracy is 3 ppm, the MS data provided first, reasonable evidence for the detection of the cited casein peptides in the sample.

The extracted ion chromatograms (XICs) were then obtained for the four experimental m/z values and are shown in Figure 1. Note that, due to the high mass resolution available, very narrow extraction windows (maximum half width of 0.009 Th) could be adopted. As shown in Figure 1, the peaks tentatively related to the four peptides exhibited excellent S/N values, although no evidence of their presence had been obtained from the base peak chromatogram, possibly due to the low concentration of digested caseinate.

High-resolution HCD-MS acquisitions were performed on the same sample by a systematic fragmentation in the HCD cell of the mass spectrometer. The ions related to all the species eluting from the chromatographic column were fragmented before being transferred into the Orbitrap. Very useful structural information could be retrieved for the four casein peptides based on these measurements.

For example, the HCD-MS spectrum averaged under the chromatographic peak [retention time (t_r) = 22.51 min] relevant to the m/z 692.867 ion is reported in Figure 2. As apparent, all of the peaks with a relative abundance higher than 7% could be assigned to product ions related to the casein peptide sequence compatible with that m/z , FFVAPFPEVFGK. Moreover, two unique proline-initiated internal ions and the y_{11}^+ ion could be assigned to minor peaks. The figure demonstrates that, provided HPLC achieves perfect separation between the peptide of interest and other matrix components, an MS/MS-like spectrum can be obtained by using a single-stage mass spectrometer and subjecting the corresponding ion to the HCD process before entering the

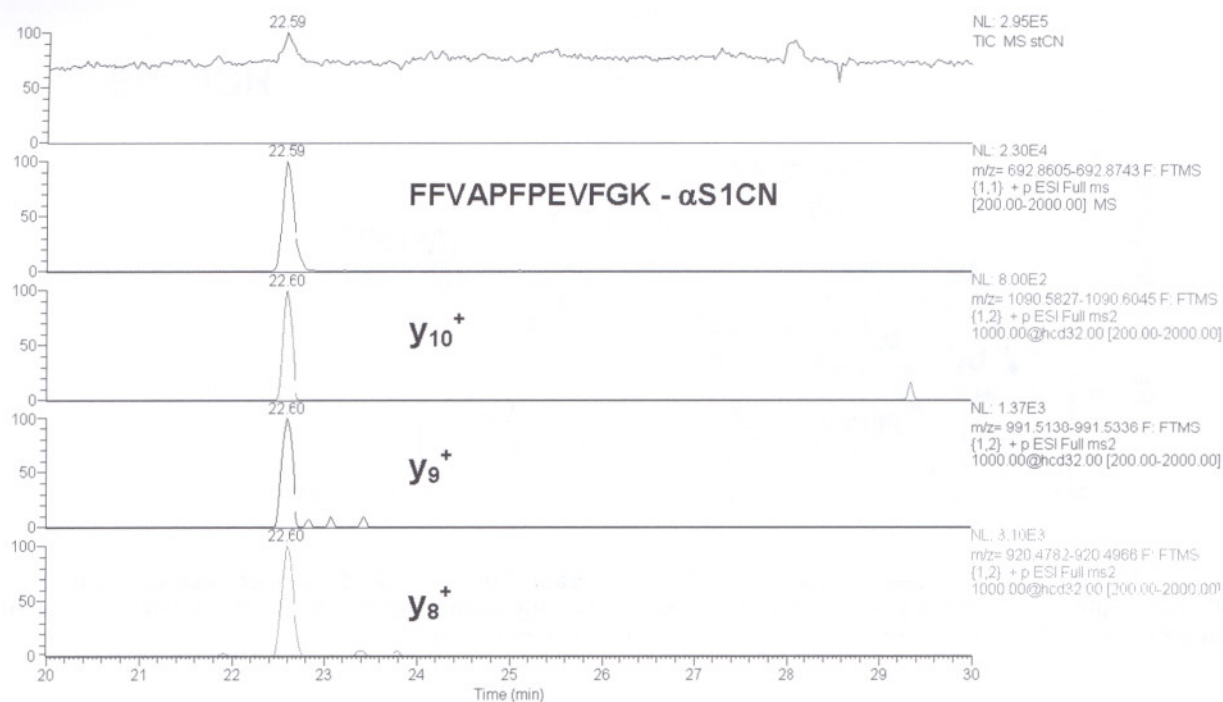


Figure 3. Detailed view of the TIC-MS chromatogram, the XIC obtained by extracting the m/z 692.867 ion current from it, and the XICs obtained from the HCD-MS chromatogram of the m/z values 1090.591, 991.523, and 920.486, corresponding to the main product ions of the FFVAPFPEVFGK peptide.

Orbitrap analyzer. Additionally, high accuracy/resolution MS/MS-like data can be obtained, thus facilitating the discrimination between concurrent candidate sequences during a subsequent database search.

A database search was performed by the MS-Tag software inputting the list of m/z values for product ions with relative abundance higher than 5% among those observed in the HCD-MS spectrum. It is worth noting that a, c, x, z, and internal ions were included among the possibilities, due to the high energy involved in HCD fragmentation. Even when the entire SwissProt database was searched (taxonomy setting: *all*), the casein peptide FFVAPFPEVFGK was found to be the best candidate, with 10 of 12 input product ions found to match with predicted ones. The worst discrepancy observed between experimental and exact m/z was -3.5 ppm.

The m/z values of the first three product ions in order of abundance, highlighted in Figure 2 (m/z 1090.591, 991.523, and 920.486) and all belonging to the $-y$ series, were used subsequently to obtain XICs from the HCD-MS total ion current (TIC) traces. By comparison, on a selected t_r interval of the MS TIC chromatogram, the XIC obtained by extracting the m/z 692.867 ion current from it and the three XICs obtained from the HCD-MS chromatogram are shown in Figure 3. The alignment between the major peaks in the five traces under comparison is apparent, suggesting that the XICs obtained from the HCD-MS chromatogram for a specific peptide product ion can be considered as selected reaction monitoring (SRM)-like chromatograms.

Among the casein markers under study, a potentially more critical case was represented by peptides YLGYLEQLLR and GPFPIIV, partly coeluting from the chromatographic column (Figure 1). In this case the HCD-MS spectra were first averaged under the entire peak of each species, using the XIC for their quasimolecular ions (m/z 634.355 and 742.448, respectively) as a guide. A spectral background correction was then performed by subtracting the HCD-MS spectra averaged in a time interval corresponding to the peak overlapping region. The resulting HCD-MS spectra are reported in Figure 4, together with product ion assignments based on the hypothesized peptide sequence in each case. It is apparent that, due to the background correction, no cross-interference (i.e., related to peaks arising from the fragmentation of the other peptide) is observed. In the case of the m/z 742.448 ion, four peaks not assignable to the expected peptide sequence were observed among those having relative abundance higher than 5%. Nonetheless, they did not prevent GPFPIIV from being the best scored sequence when a database search was performed on the entire SwissProt database using all the HCD-MS peaks overcoming that abundance threshold.

Application of the Method to Food Samples

The first application of the method to food matrixes was made on blind incurred cookie reference materials spiked with skim milk powder. The same casein peptides investigated in the caseinate standard solutions, arising from its most

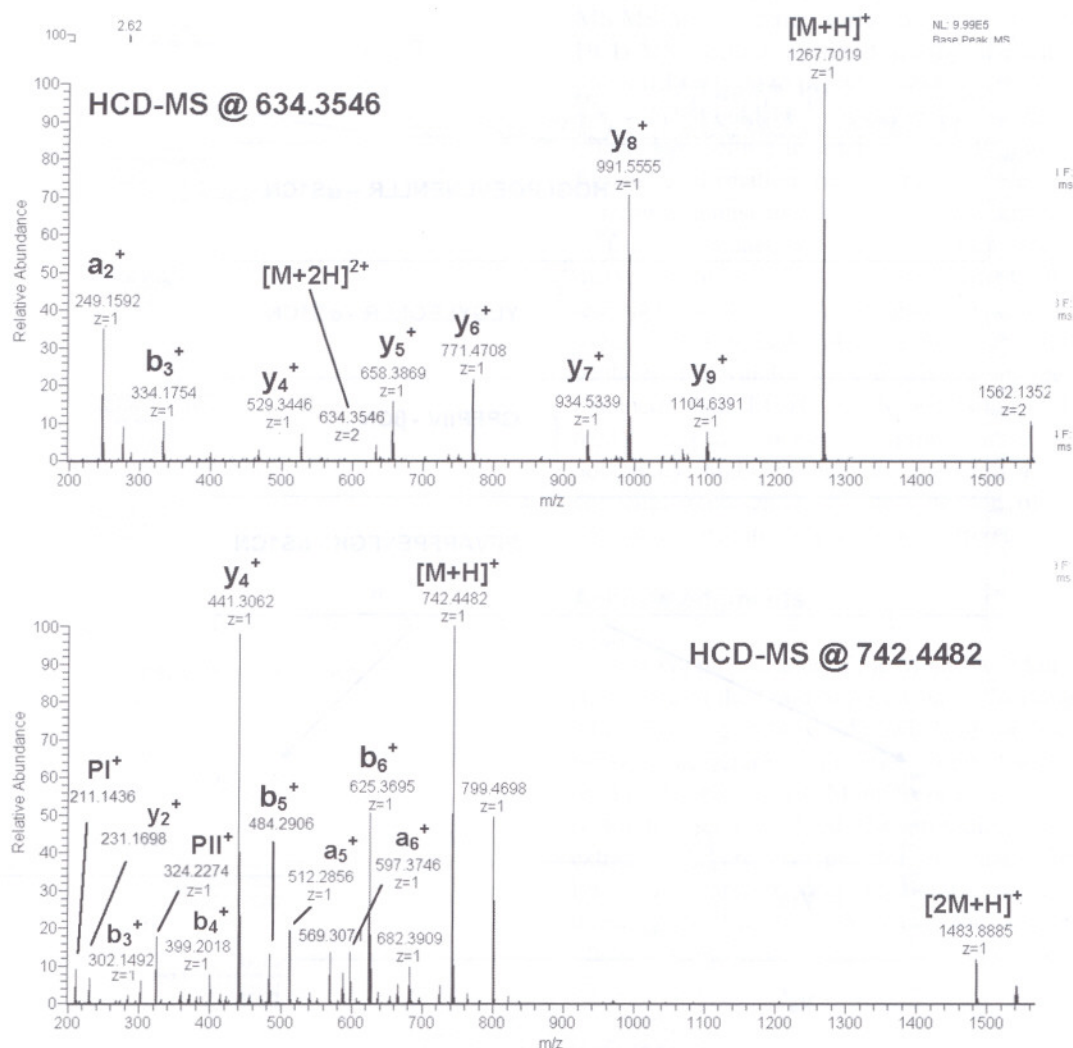


Figure 4. HCD mass spectra obtained for the m/z 634.356 (doubly charged) and 742.449 (singly charged) ions, related to peaks partly coeluting in the LC/MS chromatogram of a caseinate tryptic digest (Figure 1). The product ion assignments reported are based on peptide sequences YLGYLEQLLR and GPFPIIV, previously proposed for the two ions using high resolution MS data. See text for details about spectral averaging and spectral background subtraction.

abundant proteins, α_1 CN and β CN, were chosen as potential markers of milk powder.

The LC/MS base peak and the XICs for the cited peptides, relative to the tryptic digest of an incurred cookie extract found to be positive to caseins after ELISA tests (concentration ≤ 22 ppm), are reported in Figure 5. As expected, the base peak trace appears to be considerably more complex than that obtained for the caseinate standard, due to the complexity of the cookie extract. Nonetheless, the peaks for caseinate peptide markers can still be detected at a high S/N with negligible interference in the XIC traces due to the selectivity provided by the mass accuracy of the Orbitrap and by the ion current extraction window.

The characteristic t_r alignment between the main peaks observed in the XIC traces obtained for one of the peptides precursor ions (m/z 692.867) and for three of its main product

ions is shown in the Figure 5 inset as an example of the potential of HCD-Orbitrap MS in confirming the identity of allergen peptide markers, even when complex food matrixes are considered.

In order to check the method on a different complex matrix, nine white wine samples fined with caseinate at three different concentration levels below 10 ppm, labeled as low (1.6 ppm), medium (3 ppm), and high (6 ppm), three samples for each level, were analyzed by LC/high resolution MS. For the sake of comparison, three caseinate-free wine samples were also analyzed (Figure 6, panel A). LC/MS and LC/HCD-MS data relevant to one of the blank samples and one of those fined at low caseinate levels are compared in Figure 6, focusing on the peptide marker FFVAPFPEVFGK. Its presence is apparent in the case of the fined wine sample (panel B), with peaks in the XICs relevant to three of its

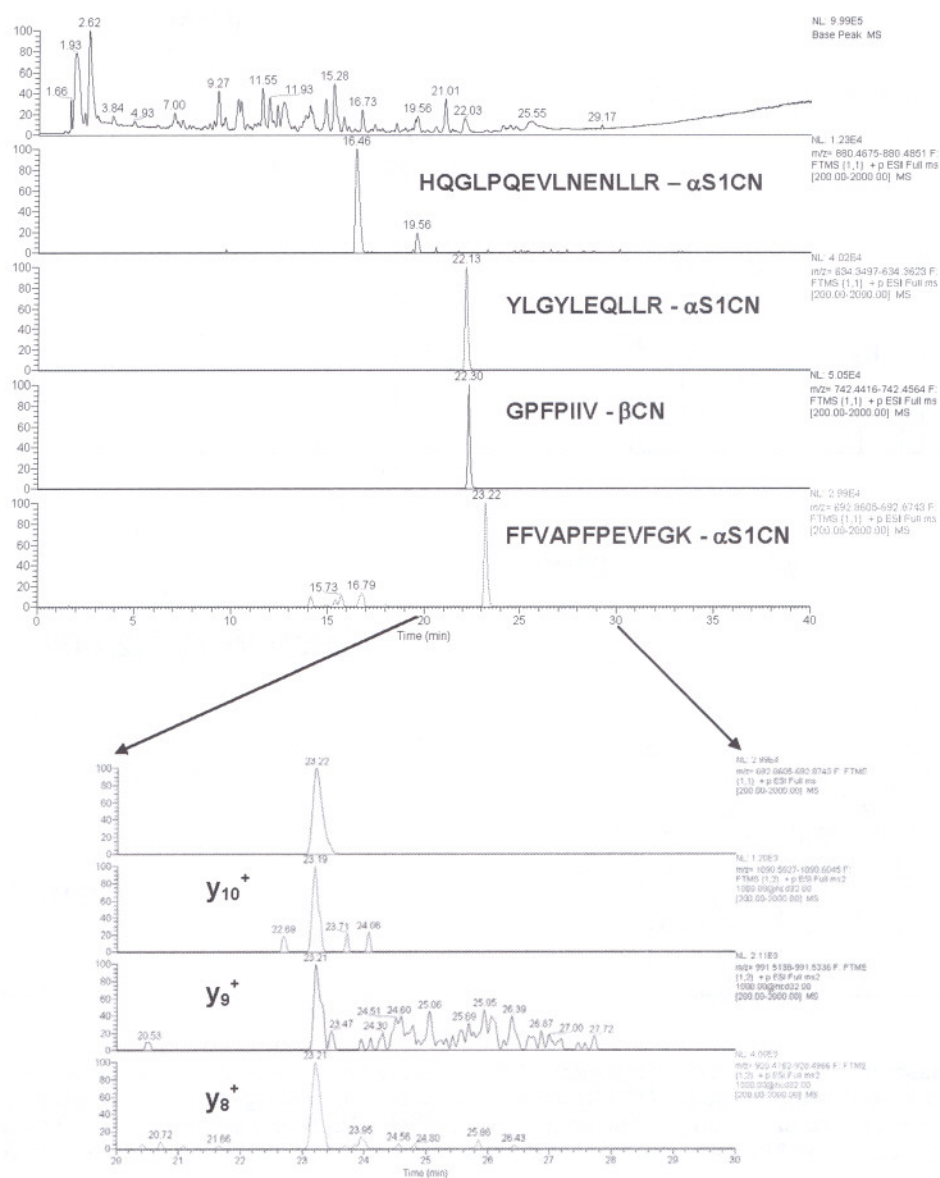


Figure 5. LC/MS base peak and XICs for the indicated casein tryptic peptides obtained by analyzing the tryptic digest of an incurred cookie extract found to be positive to caseins after ELISA. The XICs obtained from the HCD-MS chromatogram for m/z 1090.591, 991.523, and 920.486, corresponding to the main product ions of the FFVAPFPEVFGK peptide, are compared with the XIC of the precursor in the inset.

product ion easily detectable and aligned with that related to the singly charged quasimolecular ion.

In the case of the blank sample, a peak is observed for the latter at a reasonable t_r , but its intensity is four orders of magnitude lower and, more importantly, no signal at all can be seen in the XIC traces arising from LC/HCD-MS chromatograms, i.e., those related to product ions. This finding suggests that the cited peak is likely due to the ionic noise and has no relationship with the presence of the FFVAPFPEVFGK peptide, as expected for a blank sample.

In light of the results obtained from cookie and wine extracts, LC/MS based on high resolution MS and HCD-MS

appears to be very promising for the detection of trace levels of milk allergens in complex food matrixes, even when the pretreatment of the latter is unable to reduce their complexity significantly. A further important step in the method development would be to assess the quantitative capabilities of the proposed approach. Work is in progress in this direction.

Conclusions

The coupling between HPLC separation and MS detection based on a single-stage Orbitrap mass spectrometer equipped

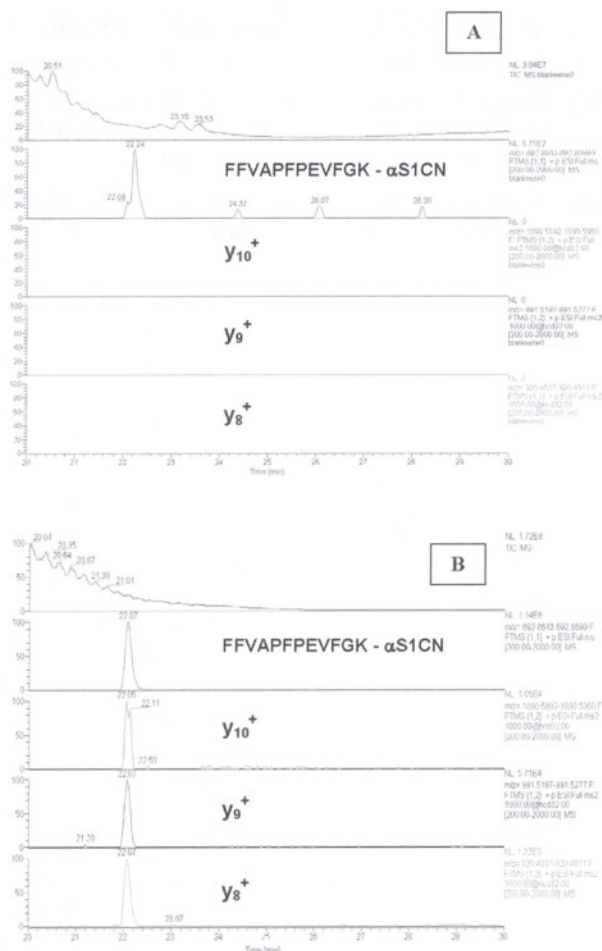


Figure 6. Base peak and XIC chromatograms (mass tolerance = 4 ppm) relative to: (A) a blank wine sample; and (B) a wine sample fined at low caseinate level (= 1.6 ppm). The XICs were obtained for the m/z 692.867 ion (FFVAPFPEVFGK) and its main product ions.

with an HCD cell enabled the identification of four peptide markers of caseins in the tryptic digests of the extracts of two food commodities, cookie, and white wine, spiked with low levels (few ppm) of skim milk powder and caseinate, respectively, to simulate a contamination by milk allergens.

Due to the high mass accuracy and resolution provided by the Orbitrap analyzer, the presence of the four peptides in the tryptic digests could be easily confirmed by extracting, with very narrow windows (maximum half width of 0.009 Th), the ion current relevant to the m/z of their quasimolecular ions. Despite the complexity of the samples under investigation and the lack of a comprehensive purification procedure, no significant interference was observed in the XICs.

The presence of an HCD cell in the Orbitrap spectrometer enabled a further check of the peptide markers' identity, similar to that usually accomplished by MS/MS but with the

advantage of high mass resolution and accuracy. Indeed, MS/MS-like spectra were obtained by averaging the HCD-MS spectra acquired during the elution of peaks previously related to peptide markers. The list of product ion m/z values could be then exploited for database searches, effected by setting tolerances of a few ppm and leading to further confirmation of the peptide identity even when searching against an entire proteomic database.

Currents related to specific product ions of the peptide markers could be also extracted from LC/HCD-MS traces, leading to SRM-like chromatograms. The latter could be very useful for a reliable identification of partially coeluting peptides, for which mixed fragmentation spectra would be obtained from LC/HCD-MS acquisitions. The described method appears to be very promising as a reliable, and potentially high throughput, approach to the analytical challenge represented by the detection of trace levels of protein allergen in complex food matrixes.

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