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LC-tandem mass spectrometry as a screening tool for multiple detection of allergenic ingredients in complex foods

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ABSTRACT
In the present investigation, an LC-MS method for sensitive multiplex detection of five allergenic ingredients in a processed food matrix is presented. Cookie was chosen as complex food model and was incurred with egg, milk, soy, hazelnuts and peanuts before baking. Extraction, purification and pre-concentration protocols were applied to ground cookie basing on protocols described elsewhere. Specific instrumental features of a dual cell linear ion trap MS instrument were exploited to identify suitable peptide markers for each allergen and to deliver a sensitive multiplex SRM-based method for the simultaneous detection of common allergenic ingredients which might contaminate such a commodity.

Section: RESEARCH PAPER

Keywords: linear ion trap; mass spectrometry; food allergens; multi-allergen analysis; incurred cookies

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1. INTRODUCTION

Food allergies are reported to be on the rise especially in the last decade [1]. Apart from the intentional incorporation of allergenic ingredients into foods for manufacturing purposes and regulated by the current legislation enforced in Europe (Directive 2007/68) [2], contamination of food by hidden allergens at the moment represents a major health problem for allergic patients. Allergens are defined hidden when they are not declared on the product label as required by the legislation in place in Europe and might unexpectedly reach the end products through several routes of accidental contamination [3].

Different analytical methods have been developed for monitoring food allergen contamination along the food chain. Methods address either the allergen itself or a marker contained in the allergenic food. To support allergen control within HACCP (hazard analysis and critical control points) programs, laboratory immunoassays have been proposed as screening tool due to its ease in use, the relatively high throughputs and the low detection limits reached [4], [5]. On the other hand, an analysis with a higher level of specificity might be required for confirmation of the results obtained. In the last few years mass spectrometry based methods[6], [7] have been considered a promising analytical strategy for food allergens monitoring thanks to the advances made in this technology that enables the reduction of the risk of false positives compared to ELISA methods.

MS based methods in general enable to overcome the several restrictions issued by antibody-based methods, such as enzyme linked immunosorbent assay. It is renowned that antibody based kits can produce false positives, especially when applied to complex or processed food matrices as a consequence of epitope modification or masking effect. In addition the use of such antibody based kits presents limitations in running multiplex analysis. Although a number of papers have been published in this field using Mass Spectrometry as a screening tool for allergens detection in foods [7]-[15], only a few of them are directed to the simultaneous determination of several classes of food allergens in food commodities[12], [16-

19]. Thanks to the innovative configuration and the versatility shown by the dual cell linear ion trap MS used, we have recently developed a method based on micro high performance liquid chromatography (HPLC) and ESI-MS/MS detection for the screening of egg, milk and soy in cookies by monitoring selected peptide markers for each allergenic ingredient [17]. As a follow up of that work we present herein a step forward of such method that includes other allergenic ingredients to be monitored to deliver a multiple selected reaction monitoring (SRM) method for the multi target detection of the allergenic foods like milk, egg, soy, peanut and hazelnut in cookie chosen as complex food matrix. A subset of peptides tracing for five allergenic foods were used to design a multi-target SRM method to detect the presence of each allergenic contaminant in food and the two most sensitive peptide markers/protein were selected in order to retrieve quantitative information.

2. MATERIALS AND METHODS

2.1. Reagents

Acetonitrile (LC–MS grade), formic acid, acetic acid, ammonium bicarbonate, trizma base, Tween 20, hydrochloric acid, iodoacetamide (IAA), dithiothreitol (DTT), egg powder (EP) and skimmed milk powder (MP) were obtained from Sigma–Aldrich (Milan, Italy). Trypsin (proteomic grade) was purchased by Promega (Milan, Italy); Rapigest™ surfactant was purchased by Waters (Milford, MA, USA). Cellulose acetate syringe filters, 1.2 μm (size 25 mm) were purchased by Labochem Science S.r.l. (Sant'Agata di Battiati, CT, Italy), and polytetrafluoroethylene syringe filters, 0.2 μm (size 4 mm) were purchased by Sartorius Italy S.r.l. (Muggiò, MB, Italy). Disposable cartridges PD-10 were purchased from GE Healthcare Life Sciences (Milan, Italy), while ultrafiltration (UF) tubes with 30 kDa cut-off membranes were purchased from Millipore (Billerica, MA, USA). Pre-cooked soy flour (SF) was purchased from a local retailer. Roasted peanuts and hazelnuts were provided by Besana s.p.a. (San Gennaro Vesuviano, NA, Italy).

2.2. Preparation of incurred cookie samples.

Cookies incurred with egg, milk, soy, hazelnut and peanut were prepared according to the following recipe: 418 g of wheat flour, 180 g of sugar, 1 g of salt, 2 g of sodium bicarbonate, 90 g of extra virgin olive oil, 160 g of water, and 6.4 g of each allergenic ingredient (skimmed milk powder, egg powder, pre-cooked soy flour, ground roasted peanut and ground roasted hazelnut). Allergenic ingredients were first homogenized and then added to the dry mixture (wheat flour, sugar, salt and sodium bicarbonate) at a final concentration of 10000 μg/g. Subsequently, olive oil and water were added and the final total weight was estimated to be approximately 900 g. Incurred dough was left mixing 40 min before being further portioning in 10 g aliquots which were spread in open cookie tins of about 7 cm in diameter and approximately 1 cm in height and baked at 200°C for 12 min. After cooling down, cookies were weighted in order to re-scale the actual allergen concentration to the final baked matrix. The highest incurred cookies corresponded to 8756 μg/g (μg of allergenic ingredients per g of matrix). For the production of blank cookie samples the amount of allergenic foods was replaced by wheat flour (a total of 32 g).

Both blank and incurred cookies were finely milled in a blender at 17,000 rpm (Steril mixer 12 model 6805-50, PBI International) by iteration of four cycles of blending (30 s) and rest (10 s) in order to prevent material overheating. Ground blank and incurred cookies were passed through a 1 mm sieve, spread on a large tray (50cm × 50cm) and manually mixed for homogeneity purpose. A total of 5 subsamples (10 g each), were taken respectively from each stock powder produced (blank and incurred) and subsequently combined to form a single representative sample (about 50 g each). Two serial dry dilutions of the incurred sample were prepared by mixing ground blank and incurred samples in the ratio 1:3, up to reach the final theoretical concentration of 973 μg/g. Such concentration level was used as reference sample for all further experiments.

2.3. Preparation of calibration curves

Four points calibration curves were prepared for incurred cookies to cover the range 20– 243 μg/g. Calibration curves were prepared by serial dilutions of incurred cookie extracts at 973 μg/g concentration level, with appropriate volumes of a blank cookie extract. All the sample at each concentration levels were purified and pre-concentrated by ultrafiltration with dimensional cut-off membranes basing on protocols detailed elsewhere [17], [19].

2.4. Enzymatic digestion

The finalextract was denatured by heating for 15 min at 95°C and subsequently diluted in the surfactant/denaturing agent Rapigest™ (dilution 1:2) to reach a final volume of 100 μL. Protein enzymatic digestion was carried out by addition of suitable amount of trypsin as specific cleavage enzyme, to reach the approximately ratio 1/50, enzyme/protein. Reaction was stopped after overnight incubation by acidifying the sample with HCl 1 M and the final extract was filtered through 0.2 μm PTFE filters before chromatographic injection.

2.5. HPLC–MS/MS analysis and database search.

The HPLC–MS system consisted of a UHPLC pump provided with an autosampler and an ESI interface connected to a Linear Ion Trap Mass Spectrometer Velos Pro™ (Thermo Fisher Scientific, San Josè, USA). Peptides separation was accomplished on an Acclaim™ PepMap100 analytical column (Thermo Fisher, San Josè, US), 1 mm × 15 cm × 3 μm, 100 Å porosity at a flow rate of 60 μL/min and the following elution gradient was used: 0–40 min solvent A reduced from 85 % to 45 %, 40–42 min further reduction from 45 to 10 %, constant for 10 min, 52–54 min back to 85 % and constant at this composition to allow a 15 min column conditioning before next injection (solvent A = H_2O + 0.1 % formic acid; solvent B = CH_3CN/H_2O, 80/20 v/v + 0.1 % formic acid). For SRM acquisition mode, a six segments acquisition scheme was set up, screening a total of 21 peptides. Each segment counted up to four scan events, in which each selected peptide was isolated and activated by CID with a normalized collision energy of 35 %, and ion current related to the three most intense transitions was recorded within a 3 m/z window.

3. RESULTS AND DISCUSSIONS

3.1. Selection of peptide markers.

One of the most crucial steps in designing a multi-target screening MS-based method for food allergen detection in complex food matrices, is the appropriate selection of target peptides that should fulfil specific requirements to be considered reliable allergens markers.

In order to draw down a list of potential candidate markers for the selected allergens, a preliminary untargeted MS analysis in data dependent acquisition (DDA) mode was carried out on the cookies protein extract (sample incurred at 973 μg/g), followed by purification on size-exclusion columns. Raw data were processed via commercial software Proteome Discoverer™ (version 1.4) and protein assignment was accomplished via Sequest HT scoring algorithm by searching MS/MS spectra against a customized database (DB), restricted to food allergens and other contaminants. A list of peptides was generated and the MS/MS spectra providing the highest matching with the predicted fragmentation patterns were further validated by analyst inspection, as already detailed in a previous paper [17] and only MS/MS peptide spectra containing at least three consecutive peptide fragments of either y- or b-ions were selected as good candidate. Finally, three most intense transitions were chosen for each internally validated peptide and these were further used to build up an SRM acquisition scheme aiming at developing a sensitive and selective method for the detection of target proteins in a complex matrix like cookies.

In order to design a proper MS acquisition method, parameters such as number of segments along the LC run, number of scan events per segment, acquisition scan rate (and consequently resolution) and acquisition range, were optimized. Basing on the expected elution time for each candidate peptide marker, the acquisition run in SRM mode was split into six segments, and a maximum of four scan events per segment were recorded for each peptide marker monitored. Notably, after peptide isolation and fragmentation, the sum of ion current related to the three most intense transitions was recorded.

MS analyses were carried out both in allergen-free and incurred cookie extracts in order to confirm the absence of any interfering peak at the expected retention times and to evaluate the resolution obtained by chromatographic separation; this comparison allowed to highlight some co-elution problems encountered with two candidate peptides, namely peptide ESYFVDAQPK, 592.3 m/z belonging to the α-chain of β-conglycinin (soy protein), and peptide DQSSYLQGFSR, 644.3 m/z belonging to a fragment of conarachin (peanut protein) as depicted in Figure 1. As a consequence, these peptides were further excluded from the list of candidate markers since not considered a reliable marker in incurred baked cookies. A part from the two mentioned peptides that were excluded from the list, due to the absence of any matrix interferences with the other candidate markers selected, these chromatographic conditions were considered suitable for our purposes and a total of 19 peptides deemed suitable markers for allergen detection in cookies. A typical representation of the multi-target analysis achieved in the SRM mode is shown in Figure 2 where an overlay of nineteen chromatographic traces is reported.

3.2. Assessment of quantitative performances.

In order to select the best quantitative markers within the list of nineteen peptide markers included into the SRM-MS/MS instrument method, matrix-matched curves were obtained in incurred cookies and the relevant SRM traces were recorded. The two most sensitive peptides for each allergenic food category were then selected as quantifier peptides (Table 1).

Response linearity was assessed on the whole concentration range under investigation (20–243 μg/g) with Fisher-Snedecor F-test. The ratio between regression and residual variance was compared with the critical F value with the proper freedom degrees at 99% of confidence, proving that the linear correlation was significant. Limits of detection (LOD) and quantification (LOQ) were estimated as the minimum concentration of an added allergenic ingredient that can be detected, at S/N equals 3 and 10, respectively (the standard deviation of the calibration line intercept was used in this case as an estimate of noise). Table 1 reports an overview of method performances for a total of ten peptides selected for the 5 allergenic ingredients. As appearing from the table, some limit values calculated for each allergenic ingredient resulted very similar irrespective of the specific marker monitored, thus confirming the reliability of both peptides selected for quantitative analysis. Besides, LODs of each allergenic ingredient under consideration in incurred cookies were found to be ranging from 10 μg/g for egg to 8 μg/g for milk and peanut, while the highest sensitivity reached for soy and hazelnut was down to 5 μg/g (allergen/food matrix).

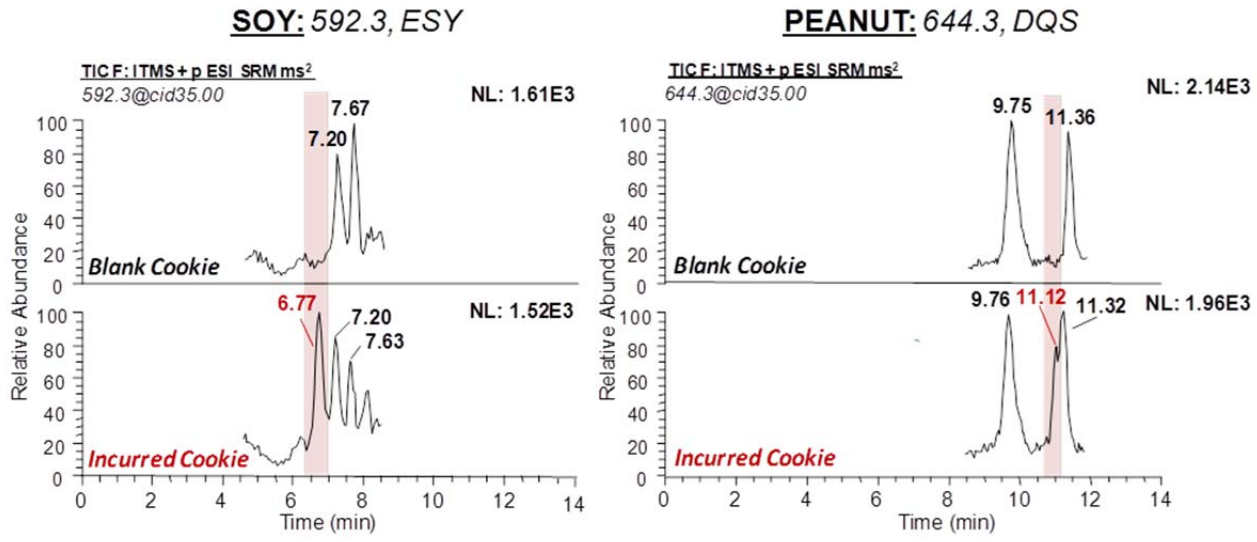

Figure 1. Comparison of SRM chromatographic traces recorded for blank and incurred cookie (243 μg/g), for two candidate peptide markers 592.3 (ESYFVDAQPK) and 644.3 (DQSSYLQGFSR), belonging to soy and peanut, respectively.

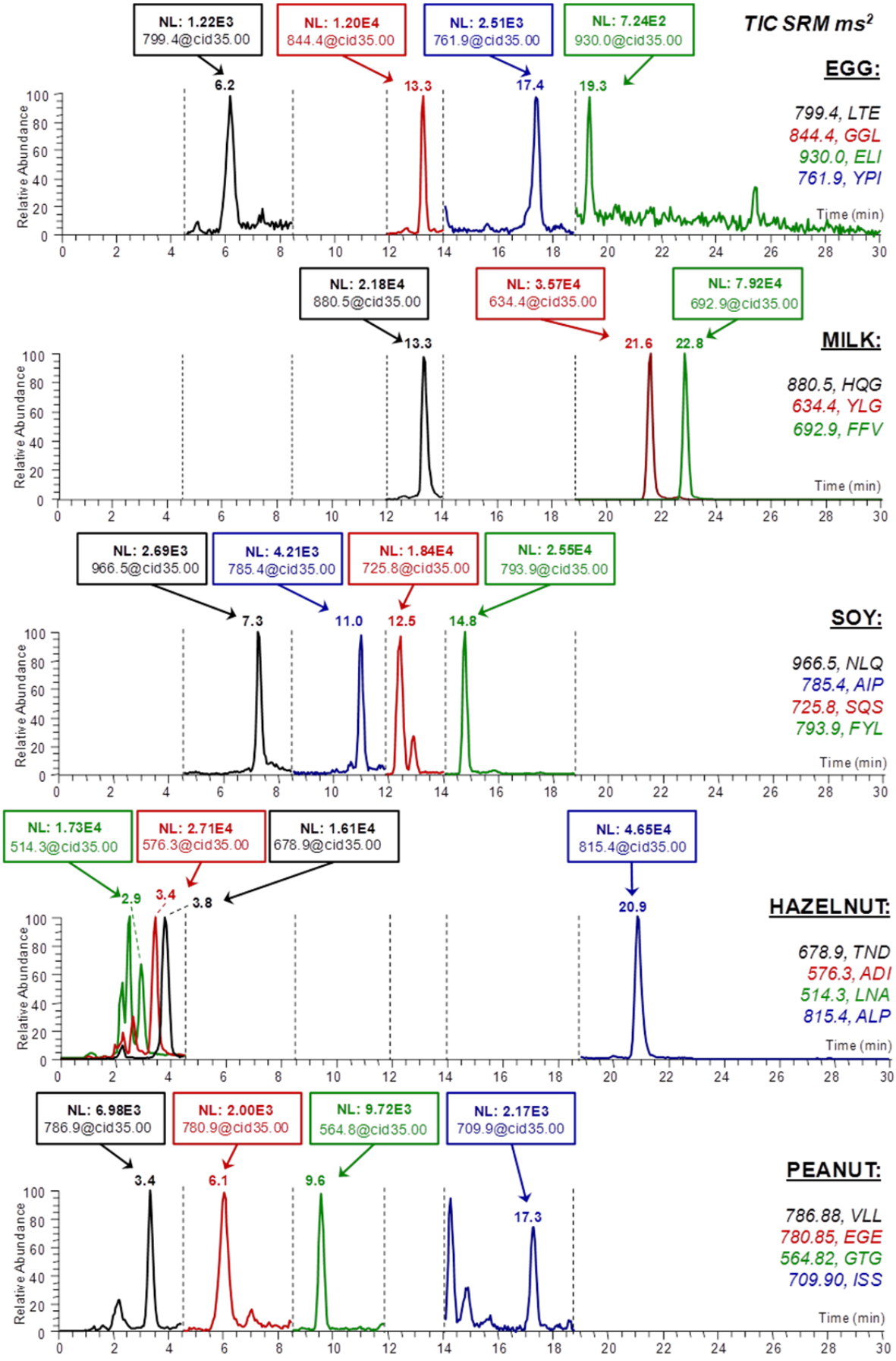

Figure 2. Overlay of typical ion chromatograms recorded in SRM acquisition mode for the selected peptide markers. The entire chromatographic run was divided into six acquisition time segments: (i) 0-4.5 min; (ii) 4.5-8.5 min; (iii) 8.5-12.0 min; (iv) 12.0-14.1 min; (v)14.1-18.8 min; (vi) 18.8-30.0 min.

Table 1. Summary of the quantitative performances provided by the LC-SRM method for selected peptide markers of five allergenic ingredients in cookies. The accession number refers to on-line Uniprot Database.

PEPTIDE m/z	*Allergenic food*	*Protein (Accession number)*	*Peptide sequence*	*Transitions*	*Retention time (min)*	*LOD (µg/g)*	*LOQ (µg/g)*	Slope	R^2
844.4	Egg	Ovalbumin (P01012)	GGLEPINFQTAADQAR	y_{12}^{+2}; y_7^+; y_{10}^+	13.3±0.1	10	30	536±11	0.998
761.9	Egg		YPILPEYLQCVK	b_4^+; y_8^+; y_9^+	17.5±0.1	15	51	192±6	0.995
692.9	Milk	Alpha-S1-casein (P02662)	FFVAPFPEVFGK	y_8^+; y_9^+; y_{10}^{+2}	22.9±0.1	8	26	4120±70	0.998
634.4	Milk		YLGYLEQLLR	y_5^+; y_6^+; y_8^+	21.6±0.1	13	40	2140±60	0.997
725.8	Soy	Glycin G1 (P04776)	SQSDNFEYVSFK	$[M+2H]^{+2}$-H_2O; y_3^+; y_{10}^+	12.5±0.1	5	18	1054±13	0.990
793.9	Soy	11S globulin-like protein (Q8W1C2)	FYLAGNQEQEFLK	y_{11}^{+2}; y_9^+; y_{10}^+	14.8±0.1	8	27	1210±20	0.998
815.4	Hazelnut		ALPDDVLANAFQISR	y_7^+; y_8^+; y_{13}^{+2}	21.0±0.1	5	16	3330±30	0.999
576.3	Hazelnut		ADIYTEQVGR	$[M+2H]^{+2}$-H_2O; y_6^+; y_7^+	3.5±0.1	10	32	1530±30	0.997
786.9	Peanut	Conarachin Fragment(Q6PSU3)	VLLEENAGGEQEER	y_{12}^{+2}; y_7^+; y_8^+	3.4±0.1	8	30	389±7	0.998
564.8	Peanut		GTGNLELVAVR	y_3^+; y_7^+; y_6^+	9.6±0.1	9	30	526±11	0.997

4. CONCLUSIONS

In the present investigation, an LC-MS method for sensitive multiplex detection of egg, milk, soy, hazelnuts and peanuts allergenic ingredients in incurred cookie matrix was presented. Suitable peptide markers for each allergenic ingredient were selected basing on several parameters which proved the reliability of the identification. Specific transitions of MS/MS spectra were identified to build up a highly selective and sensitive LC-multiplex SRM-based method for the simultaneous detection of egg, milk, soy, hazelnuts and peanuts cookie.

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REFERENCES

[1] L. Monaci, R. Pilolli, E. De Angelis G. Mamone, Mass Spectrometry in Food Allergen Research, in Comprehensive Analytical Chemistry. Y. Picó Editor. Publisher, Elsevier, Amsterdam (Netherlands), Oxford (UK), Waltham (USA), 2015, ISBN 978-0-444-63340-8, 359-393.

[2] European Commission, Commission Directive 2007/68/EC.Off. J. Eur. Union L130(2007)11–14.

[3] S. Hattersley, R. Ward, A. Baka, R.W.R. Crevel, Advances in the risk management of unintended presence of allergenic foods in manufactured food products - An overview. Food Chem. Toxic. 67(2014) 255-261.

[4] L. Monaci, A. Visconti, Immunochemical and DNA-based methods in food allergen analysis and quality assurance perspectives, Tr. Food Sci. Technol. 21 (2010) 272-283.

[5] R. Pilolli, L. Monaci, A. Visconti, Advances in biosensor development based on integrating nanotechnology and applied to food-allergen management. Tr. Anal. Chem. 47 (2013) 12-26.

[6] L. Monaci, A. Visconti, Mass spectrometry-based proteomics methods for analysis of food allergens. Tr.Anal. Chem. 28 (2009) 581-591.

[7] P.E.Johnson, S. Baumgartner, T. Aldick, C. Bessant, V. Giosafatto, J. Heick, G. Mamone, G. O'Connor, R. Poms, B. Pöpping, A. Reuter, F. Ulberth, A. Watson, L. Monaci, E.N. Mills, Current perspectives and recommendations for the development of mass spectrometry methods for the determination of allergens in foods. J. AOAC Int. 94 (2011) 1026-1033.

[8] A. Cryar, C. Pritchard, W. Burkitt, M. Walker, G. O'Connor, D.T. Burns, M. Quaglia, Towards absolute quantification of allergenic proteins in food-lysozyme in wine as a model system for metrologically traceable mass spectrometric methods and certified reference materials. J. AOAC Int. 96 (2013) 1350-1361.

[9] B. Pöpping, S.B. Godefroy, Allergen detection by mass spectrometry the new way forward. J. AOAC Int. 94 (2011)1005-1005.

[10] G. Picariello, G. Mamone, F. Addeo, P. Ferranti, The frontiers of mass spectrometry-based techniques in food allergenomics. J. Chromatogr. A, 1218 (2011) 7386-7398.

[11] L. Monaci, I. Losito, F. Palmisano, A. Visconti, Reliable detection of milk allergens in food using a high-resolution, stand-alone mass spectrometer. J. AOAC Int. 94 (2011) 1034-1042.

[12] J. Heick, M. Fischer, B. Pöpping, First screening method for the simultaneous detection of seven allergens by liquid chromatography mass spectrometry. J.Chromatogr. A 1218 (2011) 938-943.

[13] P. Lutter, V. Parisod, H. Weymuth, Development and validation of a method for the quantification of milk proteins in food products based on liquid chromatography with mass spectrometric detection. J. AOAC Int. 94 (2011) 1043-1059.

[14] L. Monaci, I. Losito, F. Palmisano, M. Godula, A. Visconti, Towards the quantification of residual milk allergens in caseinate-fined white wines using HPLCcoupled with single-stage Orbitrap™ mass spectrometry. Food Addit. Contam. 28 (2011) 1304-1314.

[15] L. Monaci, E. De Angelis, S.L. Bavaro, R. Pilolli, High Resolution-Orbitrap™ based Mass Spectrometry for a prompt detection of peanuts in nuts products. Food Addit. Contam. A, 32 (2015) 1607-1616.

[16] L. Monaci, I. Losito, E. De Angelis, R. Pilolli, A. Visconti, Multi-allergen quantification of fining-related egg and milk proteins in white wines by high-resolution mass spectrometry. Rapid Commun. Mass Spectrom. 27 (2013) 2009-2018.

[17] L. Monaci, R. Pilolli, E. De Angelis, M. Godula, A. Visconti Multi-allergen detection in food by micro-HPLC coupled to a dualcell linear ion trapmass spectrometer. J ChromatogrA 1358 (2014) 136–144.

[18] R. Pilolli, E. De Angelis, M. Godula, A. Visconti,L. Monaci, Orbitrap™ monostage MS versus hybrid Linear ion trap MS: application to multi-allergen screening in wine.J. Mass Spectrom. 49 (2014) 1254- 1263.

[19] R. Pilolli, E. De Angelis, L. Monaci, Speeding-up sample handling for multiplex MS/MS allergens detection in a processed food, *submitted to Food Chemistry.*