

OrbitrapTM monostage MS versus hybrid linear ion trap MS: application to multi-allergen screening in wine

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Food allergen research has made giant steps in the last years thanks to the features offered by the latest technology of mass analyzers placed on the market allowing multiplex sensitive detection of proteins. Potentials and features of two mass analyzers namely a linear ion trap capable of performing a data dependent or selected reaction monitoring analysis and an OrbitrapTM stand-alone MS enabling a broadband fragmentation without mass selection at highest mass resolving power are herein described and applied to the multiplex screening of allergens in a type of wine chosen as a reference matrix. Quantitative and confirmative capabilities of both platforms were assessed on the specific case study, the multiple detection of egg and milk-related proteins, typically employed in white wines as fining agents. Commercial bioinformatic tools used for a quick allergen identification will be also discussed. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: multi-allergen screening; mass spectrometry; linear ion trap; OrbitrapTM; high-resolution MS; wine

Introduction

Food allergen research has made giant steps in the last decade thanks to the last generation mass spectrometers placed on the market. Mass spectrometry (MS)-based proteomics is commonly performed in a 'shotgun' format where protein allergens are digested to peptides, which are separated and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).^[1] Features offered by the latest technology of mass analyzers have pushed in the direction of developing multi-target and sensitive MS-based methods provided that some requirements are met for the final protein identification. Food matrixes provide additional challenges to the MS analysis, both in terms of experimental design and methodology. A feasibility study of the application of MS-based reference measurement procedures for quantification of protein allergens in wine-like matrix has been recently described by Cryar *et al.*^[2] In general, essential pre-requisites should be taken into account when designing reliable methods for monitoring food allergen contamination such as (1) choice of the more representative target analyte/analytes in case of multiplexing, (2) selection of suitable peptide markers which should be stable to processing and should provide both qualitative and quantitative information about the relevant target protein, (3) optimization of protease digestion protocols to ensure reproducible and robust method development and (4) effective validation and harmonization of results through the use of naturally incurred reference materials. Some guidelines have been recently issued in this regard, to help analysts in facing issues related to the MS-based monitoring of food allergens.^[3]

In a typical shotgun workflow, the key role is played by the capabilities and performances offered by the instrumental setup in use. Due to its excellent selectivity and sensitivity, selected reaction monitoring (SRM) analysis mode using triple quadrupole mass spectrometers (TQ-MS) has been for years the primary technology

for analyte quantification in complex matrices.^[4] By contrast, high-resolution MS (HR-MS) has for long time been considered a more sophisticated tool for in thorough investigations. In general, hyphenated techniques based on the coupling between HPLC and high resolution MS instruments like time-of-flight MS (ToF-MS) or Fourier transform ion cyclotron resonance (FT-ICR) MS have demonstrated over the years their applicability both for analyte identification and quantification, although these systems presented several limitations like the high costs of the instrumentation and the trained staff needed to assure the correct functioning of such sophisticated systems. In addition, the advantages of such machine proved to be not comparable with the SRM-based methods in terms of dynamic range, selectivity and sensitivity.

However, thanks to the recent launch on the market of user-friendly single stage MS platforms also integrated with an external dissociation chamber, the application fields resulted to be considerably expanded also encompassing its use for screening purposes and quantitative analysis. In turn, the latest generations of HR instruments (ToF- or OrbitrapTM-MS) with improved resolution and stability of accurate mass measurements showed great quantitative potential, and various investigations have assessed their effectiveness in quantitative and qualitative analyses of clinical relevance.^[5–7] This can be achieved by performing full MS or full MS/MS acquisition analysis in a selected mass range and by

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applying post-acquisition mass filtering on the total ion current on the basis of the accurate masses expected for the ions of interest.

In particular, the full-scan MS approach using a single-stage OrbitrapTM mass spectrometer grounded its strength on the specificity and sensitivity obtained by stable high-resolution (up to 100 000 at m/z 200) and accurate mass (<5 ppm with external calibration) performance on a routine basis. Furthermore, ion fragmentation is also achieved thanks to high-energy collision-induced dissociation (HCD) chamber generating 'All-Ion-Fragmentation' (AIF) data when further confirmation is required. In a single chromatographic run, both HR-MS full scan and HCD fragmentation can be performed yielding a huge amount of qualitative information on the sample composition, as well as retrospective data mining. A few reports have already proposed a comparison of quantitative performances in the detection of small molecules of LC-TQ-MS with LC/HR-MS, including ToF, Q-ToF-MS^[8–11] and OrbitrapTM-MS.^[4,12–15] For OrbitrapTM technology, previous reports have concluded that equivalent quantitative performance in comparison with the TQ-MS can be obtained.

Here, a further comparison was provided between OrbitrapTM technology and a linear ion trap (LIT) applied to the multiplex detection of proteins in food matrix. Different instrumental features can be exploited depending on the specific configuration of the MS system: either full-scan acquisition at high resolution and mass accuracy, taking advantage from the information retrieved by the HR-MS and HCD spectra, or, multiple options provided by a double stage instrument like a linear ion trap which can be operated under different operating schemes giving valuable contribution to the structural elucidation studies, untargeted screening or multi-targeted experiments.

Typically, the pool of peptides generated after protein enzymatic digestion undergo MS analysis upon chromatographic separation. Hence, marker peptides can be either (1) selected for fragmentation on the basis of their abundance in the 'data-dependent acquisition' mode, or (2) as an alternative, simultaneously activated throughout the entire mass range of co-eluting analytes in 'data-independent acquisition' mode. In the first case, the information of the precursor ion mass, together with a relevant list of fragment ions, are combined to identify the peptide in a sequence database. In case of simultaneous activation, either in the source region or in the collision cell, the direct correlation between precursor and fragments is lost.^[16] However, the correspondence of elution times among precursor and fragments ions can be used to provide reliable peptide identification. In a recent comparison of data-dependent and -independent analysis mode, the authors concluded that fragmentation information was highly comparable.^[17,18]

In this investigation, a comparison of the two analytical approaches applied to the multiplex detection of residual fining agents in white wines will be presented. Potentials and features offered by the two mass analyzers, namely LIT and OrbitrapTM, allowing highly selective data-dependent acquisition mode with low mass accuracy and untargeted data-independent acquisition modes, respectively, will be thoroughly discussed. In particular, lysozyme, ovalbumin and caseinate were selected as target proteins to be monitored in order to trace the presence of fining agents in artificially fortified wine matrices obtained from different grape varieties. Previously optimized sample purification procedure, based on the combination of wine ultrafiltration with tryptic digestion of the dialyzed extracts, was employed.^[19] The final digest was divided into aliquots and subjected to different MS analysis basing on specific features provided by each mass spectrometer. Full-scan high resolution MS (HR-MS) and SRM approaches were compared in

terms of dynamic range, linearity, sensitivity, limits of detection and quantification.

Experimental

Reagents and instruments

Acetonitrile and water (both LC-MS grade), formic acid, hydrochloric acid, iodoacetamide, dithiothreitol (DTT), lysozyme (LYS), ovalbumin (OVA) and sodium caseinate (CNate) were provided from Sigma-Aldrich (Milan, Italy). Trypsin (proteomic grade) was purchased from Promega (Milan, Italy); the RapigestTM surfactant was purchased from Waters (Milford, MA, USA). Ultrafiltration (UF) tubes with 10-kDa cut-off membranes were purchased from Millipore.

Stock solutions of LYS, OVA and CNate were prepared in ammonium bicarbonate 50 mM at the concentration of 1000 µg/ml and were used for wine fortification. Four different white wines, produced from (A) Falanghina, (B) Pinot Grigio delle Venezie, (C) Greco di Tufo and (D) mixed Bombino Bianco and Trebbiano grape varieties, were purchased from a local retailer. For calibration purposes each type of wine was fortified with LYS, OVA and CNate to reach the following concentration levels: 0.2, 0.5, 1, 2 µg/ml. Wine aliquots were successively subjected to purification procedure and enzymatic digestion described elsewhere.^[6] The final acidified trypsin-treated digest was centrifuged and the supernatant split in two aliquots further analyzed respectively by Thermo ScientificTM ExactiveTM MS and Velos ProTM MS.

The chromatographic separation of peptides pool was carried out by injecting 10 µl of sample in a C18 Kinetex column (100 mm × 2.1 mm × 2.6 µm, 100 Å), purchased from Phenomenex (Castel Maggiore, Bo, Italy), and using a binary elution gradient operating at a flow rate of 200 µl/min. The eluents were: A) H₂O + 0.1% HCOOH and B) CH₃CN + 0.1% HCOOH. Peptide elution was accomplished by using the following linear gradient: 0–23 min, linear from 90 to 65% solvent A; 23–26 min, isocratic; 26–28 min, from 65 to 50%; 28–33 min, isocratic; 33–40 min, linear from 50 to 5% solvent A; 40–55 min, isocratic. In the following 5 min solvent A was raised to 90% and kept for 10 min to allow equilibration.

Velos ProTM MS acquisitions were performed in two different acquisition modes. First, Nth order double play Data-DependentTM Acquisition (DDA) mode was applied in order to activate Collisional-Induced Dissociation (CID) of the top 20 most intense peaks of the full MS spectrum. Recorded data was searched against customized databases for allergenic proteins identification by Proteome DiscovererTM (PD) software (version 1.3). Afterwards, selected transitions of candidate peptide markers were identified and employed for setting up a single segment/six events acquisition scheme. The segment corresponded to the entire duration of the LC run; the six events were represented by SRM acquisition in which each selected peptide marker was isolated and activated by CID with a normalized collision energy of 35%, and ion current related to the two most intense transitions were recorded within a 3 m/z window.

ExactiveTM MS acquisitions were based on a single segment/two events acquisition scheme. The segment corresponded to the entire duration of the LC run; the two events were represented, respectively, by a MS full scan and a HCD-MS all ion fragmentation (AIF) acquisition (collision energy 40 eV), both accomplished in a 200–2000 m/z range.

The ESI settings for both interfaces were the following: sheath gas flow (arbitrary units), 25, auxiliary gas flow (arbitrary units), 5, spray voltage, 4 kV, capillary temperature, 320 °C, heater temperature, 30 °C. The ExactiveTM mass spectrometer was set up at

resolving power 50 000 (full width at half a maximum at m/z 200) with a maximum injection time of 100 ms and 1-ms-long microscans. The Velos ProTM mass spectrometer was set up at 'Normal' scan rate corresponding to a resolving power 5400 $M/\Delta M$ (scan rate 33 kDa/s) with a maximum injection time of 50 ms/1 microscan, for full MS, and 100 ms/4microscans for MS/MS acquisitions. Mass spectral data were acquired and processed by using Xcalibur software (Thermo Electron).

Results and discussion

Features provided by the latest technology of mass analyzers have pushed in the direction of developing multi-target, sensitive MS-based analytical methods for food allergen detection. Still, foods pose important challenges for the development of MS-based analytical methods, mostly related to the choice of the target analyte and the effects of food processing and food matrices on the protein extraction efficiency and subsequent MS analysis.

The 'shotgun' proteomic approach, typically preferred in the specific research field, is based on the enzymatic digestion of allergenic proteins to peptides, which are separated by liquid chromatography (LC) and detected by single MS for precursor ion identification, followed by tandem mass spectrometry (MS/MS) for amino acid sequence confirmation. Enzymatic peptides are then selected and analyzed as qualitative and quantitative markers of the proteins under investigation. Such peptide selection and its further use as marker can be performed in different ways basing on the specific instrumental features available.

Tandem mass spectrometry has played a pivotal role in the multi-residue analysis and screening of contaminants in foods thanks to the high sensitivity offered by the selected transitions to be monitored in the SRM method designed. This has been in general achieved by triple Q instruments that offered the unique advantage of a very challenging detection limits thanks to the filtering fragmenting and scanning features occurring in three spatially distinguished compartments. Thus, the sensitivity and specificity in the SRM approach are achieved by removal of background ions in the first quadrupole and transmission of a unique fragment in the third quadrupole; however, the selective nature of SRM acquisition can mask valuable information such as co-eluting matrix ions, degradation products or adducts of the analytes of interest.

In this paper we investigated for the first time the feasibility of a linear ion trap MS for the multiplex screening of food allergens in wine with pros and cons of this MS setup. Compared to the classical ion trap instruments placed on the market, this linear ion trap MS instrument based on a system of dual pressure cell enabled to work at different scan speed according to the need. The system can be operated at the highest scan speed in case of a data-dependent acquisition reaching approximately 33 kDa per sec (according to what reported in the specifications), with the advantage of screening a wide range of MW in a very short time; on the other hand, more resolved MS spectra could be acquired in case of peptide in thorough investigation also involving mass peak deconvolution as requested in case of polypeptides or mid-size proteins. In addition, the ion trap allows performing sequential MS experiments that can be helpful in structural elucidation studies. On the other hand, a classical SRM scheme based on the transitions of the most intense peptide ions could be also designed and implemented on this MS configuration. High-resolution MS is a more recent approach and as such has been only in the most recent years object of investigation and observation. In particular, such a system based on a single stage based

system provided with Orbitrap technology has represented a very competitive tool for its easy in execution and rapidity for the multi-target screening of contaminants in food commodities. The benefits provided by this last generation technology over the classical MS/MS methods are considerable. Among the advantages of collecting HR-MS spectra that represent huge data packages containing tons of information that can be temporarily stored and re-utilized for any kind of thorough investigation and might provide more insights in the type of sample analyzed in a second time.

In this frame, a comparative study of two mass analyzers and relevant potentials featured in the development of analytical approaches for multiplex detection of residual fining agents in wines was herein reported. In particular, performances of a dual-pressure linear ion trap (Velos ProTM, Thermo ScientificTM) and an OrbitrapTM benchtop single stage mass spectrometer (ExactiveTM, Thermo ScientificTM) were compared.

Given the aim of the present work, allergen-free Falanghina white wine, purchased from a local retailer, was taken as reference wine matrix. Wine fortification was carried out by direct addition of purified proteins stock solutions of lysozyme (LYS), ovalbumin (OVA) and caseinate (CNate), previously highlighted to be reliable markers for the presence of egg and milk-derived residual fining agents employed in winemaking procedure.^[19] The wine artificially fortified at the final concentrations of 0.2, 0.5, 1 and 2 $\mu\text{g/ml}$, for each protein was further submitted to the optimized sample purification procedure, based on the combination of ultrafiltration and tryptic digestion of the dialyzed wine extracts.^[19]

Selection of peptide markers

As already mentioned, the first step in the development of a MS-based method for food allergen detection is the proper selection of peptide markers which have to fulfill specific requirements to be considered reliable marker tracing for the relevant target protein. Thus, a preliminary untargeted survey MS analysis of tryptic digests was required to identify most abundant peptides which could following enter the list of potential candidate markers to be employed for designing the targeted SRM method.

Velos ProTM mass spectrometer provides DDA mode to fit this purpose. In particular, DDA N^{th} order double play (top 20) was selected in which the analyzer performs two dependent scan events during the whole chromatographic run. First, the full MS scan event, second the full MS/MS scan event whose activation was enabled on the 20 most intense ions of the spectrum, provided that a minimum threshold level is reached. This approach potentially results in a huge number of MS/MS spectra, once the constraints set up to trigger the second dependent scan event have been fulfilled. In order to keep only useful information related to potential candidate peptide markers, ion charge screening was enabled and only double and triple charged ions were allowed to trigger CID activation. Injection of digests on the whole concentration range was performed searching for peptides which were still detectable also at low spike levels. Raw data were processed via commercial software PD (version 1.3), and identification was achieved by Sequest search against a customized food allergen database containing a total of about 150 entries. The rationale behind the choice of a restricted database relies on the will of reducing the searching time and improving the protein coverage. As previously demonstrated by our group, such data mining resulted in a higher number of specific peptides identified compared to Sequest searches against general UniProt database.^[20] The software output was validated by visual inspection of the analyst and

only MS/MS spectra that met the following criteria were taken into account: (1) all fragments with an abundance higher than 10% relative to the most abundant fragment in each MS/MS spectra recorded should be correctly attributed, (2) at least three consecutive peptide fragments of either y- or b- ions should be clearly detected and (3) a significant total ion current (≥ 500) of the MS/MS spectra recorded should be displayed. Spectra considered validated by the analyst and showing a good reproducibility (15% variance) in the same charge state had therefore to fulfill the mentioned requirements. In Table 1 is reported the resulting filtered list of peptides, for each spike level of wine matrix.

Final selection of two candidate peptide markers for each protein was carried out, preferring only the most abundant peptides from the list, identified with the highest score which were reproducibly detected in the same charge state (possibly doubly charged), in the whole concentration range. The following peptides, NTDGSTDYGLQINSR and FESNFNTQATNR for lysozyme, GGLEPINFQTAADQAR and LTEWTSSNVMEER for ovalbumin and YLGYLEQLLR and FFVAPFPEVFGK (belonging to α S1-casein) for caseinate were selected as meeting all the requirements. For final

confirmation as peptide markers, a BLAST search against general UniProt database was performed for these six peptides, by setting an expectation value threshold of 10 in order to avoid false positives and assessing their uniqueness towards the proteins of interest.

With an ExactiveTM mass spectrometer (Thermo Fisher Scientific) the selection of candidate marker peptides should be performed with a total different approach, because stand-alone OrbitrapTM is unable to perform the isolation of the precursor ion preliminary to the fragmentation (*A/F*); thus, the direct correlation between precursor and fragments is lost. All the ions arriving from the ESI source are passed through the C-trap into a multipole collision cell where they are fragmented using high-energy collision dissociation (HCD).

A two-event acquisition scheme was set up, referred to, respectively, *full-scan HR-MS* and *HCD-HR-MS* scan. In the first event, ions arriving from the source are directly diverted into the OrbitrapTM analyzer using a C-trap and detected with high mass accuracy; in the second, ions are first driven into the HCD cell, and then their eventual product ions are transferred into the OrbitrapTM analyzer. Peptide identification was achieved by combining the accurate value of the peptide *m/z* ratio retrieved from *full MS scan*

Table 1. List of peptides identified in four samples of white wine produced by Falanghina grape variety, fortified with variable concentrations of LYS, OVA and CNate stock solutions. Identification was achieved by commercially available software Proteome DiscovererTM, v.1.3 comparing experimental full MS/MS spectra recorded in Data-DependentTM acquisition mode using Velos ProTM mass spectrometer with a customized protein database

Protein	Peptide sequence	Observed <i>m/z</i> , charge			
		2 μ g/ml	1 μ g/ml	0.5 μ g/ml	0.2 μ g/ml
OVA	NVLQPSSVDSQTAMVLVNAIVFK	821.1, +3	821.2, +3	1231.1, +2	–
	NVLQPSSVDSQTAmVLVNAIVFK	826.5, +3	1239.2, +2	1239.1, +2	–
	ILELPFASGTmSMLVLLPDEVSGLEQLESINFEK	–	1294.1, +3	–	–
	VTEQESKPVQMMYQIGLFR	1142.9, +2	1142.6, +2	1142.0, +2	–
	ELINSWVESQTNGIIR	930.0, +2	620.4, +3	930.0, +2	930.0, +2
	EVVGSAAEGVDAASVSEEFK	–	1005.2, +2	1005.5, +2	–
	ISQAVHAAHAEINEAGR	887.7, +2	887.7, +2	592.0, +3	887.5, +2
	GGLEPINFQTAADQAR	844.4, +2	844.4, +2	844.7, +2	844.4, +2
	LTEWTSSNVMEER	791.5, +2	791.5, +2	791.4, +2	791.4, +2
	LTEWTSSNVmEER	799.4, +2	–	799.3, +2	–
	AFKDEDTQAMPFR	778.4, +2	778.3, +2	–	–
	AFKDEDTQAmPFR	524.6, +3	–	–	–
	HIATNAVLFFGR	–	673.4, +2	–	–
	DILNQITKPNDDVYSFSLASR	1141.6, +2	–	–	–
	NVLQPSSVDSQTAMVLVNAIVFKGLWEK	1025.2, +3	–	–	–
LYS	NTDGSTDYGLQINSR	877.7, +2	877.8, +2	877.6, +2	877.7, +2
	FESNFNTQATNR	714.9, +2	715.0, +2	715.0, +2	714.8, +2
	IVSDGNGMNAWVAWR	838.9, +2	838.4, +2	837.9, +2	838.3, +2
	IVSDGNGmNAWVAWR	–	564.9, +3	846.9, +2	–
	KIVSDGNGMNAWVAWR	902.7, +2	–	–	–
	GTDVQAWIR	523.3, +2	523.5, +2	523.3, +2	523.3, +2
	GYSLGNWVcAAK	–	663.4, +2	–	–
	HGLDNYR	–	–	437.8, +2	–
α S1-CN	EPmIGVNQELAYFYPELFR	1159.2, +2	1159.1, +2	–	–
	EPmIGVNQELAYFYPELFR	778.4, +3	1167.1, +2	–	–
	HQGLPQEVNLNLLR	587.6, +3	880.5, +2	–	–
	HIQKEDVPSEK	–	446.7, +3	–	–
	YLGYLEQLLR	634.6, +2	634.7, +2	–	–
α S2-CN	FFVAPFPEVFGK	693.0, +2	692.9, +2	692.9, +2	–
	ALNEINQFYQKFPQYLYQGPIVLPWDQVK	–	1353.8, +3	–	–
β -CN	DMPIQAFLLYQEPVLPVGR	1094.2, +2	729.8, +3	–	–
	DmPIQAFLLYQEPVLPVGR	1101.6, +2	1101.7, +2	–	–
κ -CN	SPAQLQWQVLSNTVPAK	–	990.6, +2	–	–

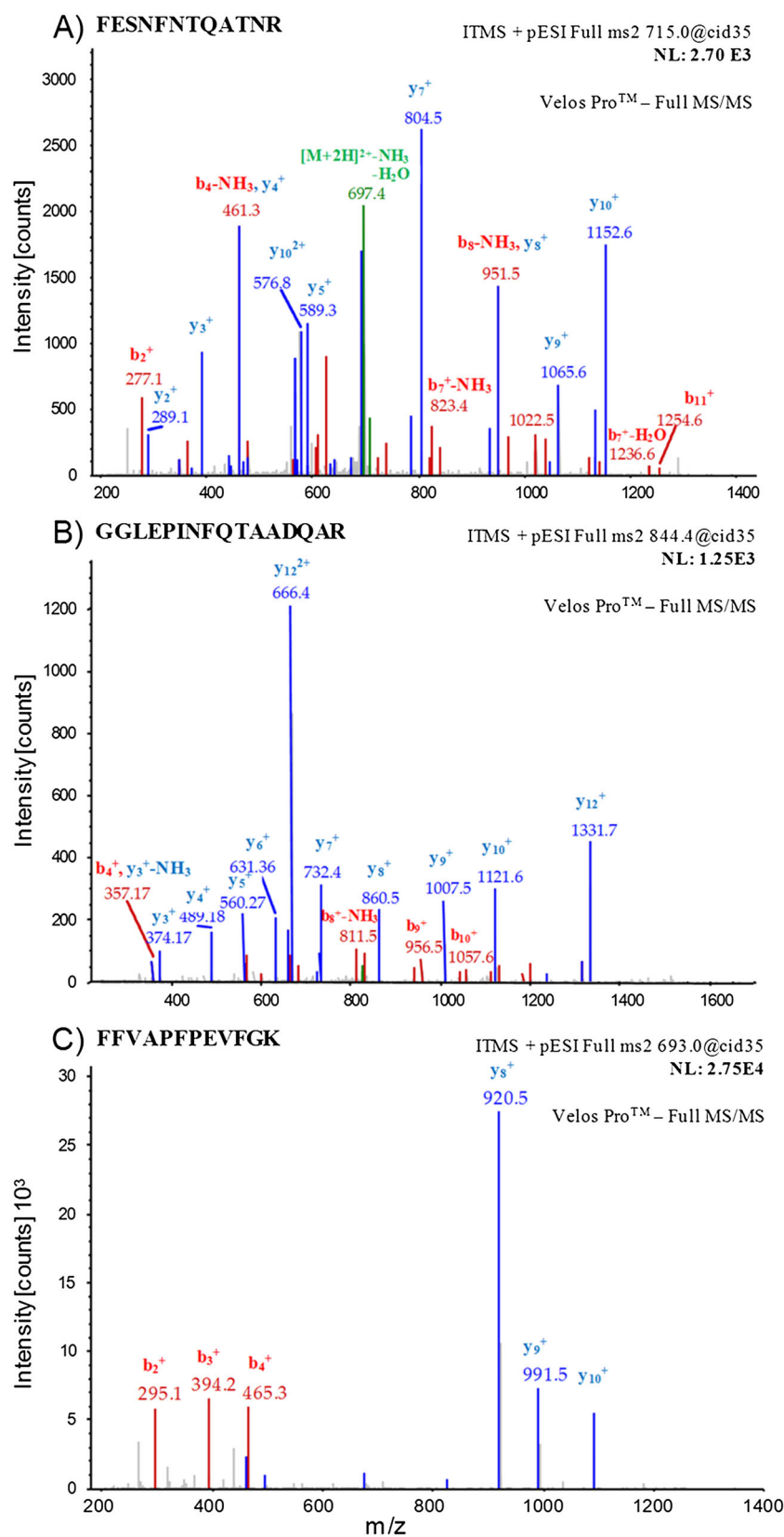


Figure 1. Overlay of typical Full MS/MS spectrum matches acquired with Velos Pro™ in DDA acquisition mode, referred to (A) marker 714.8 (LYS, FESNFNTQATNR), (B) 844.4 (OVA, GGLEPINFQTAADQAR) and (C) 692.9 (αS1-CN, FFVAPFPEVFGK), respectively. Transition assignments were achieved via software.

acquisitions and the relevant fragment ions extracted from *HCD-HR-MS* traces. *FindPept* software, available on the *ExPASy* portal,^[21] was used to find correlations between experimental *m/z* ratios and predicted ones in protein tryptic digests. A confirmation of the amino acid sequence for each peptide was achieved through the analysis of the *HCD-HR-MS* eXtracted Ion Chromatograms (XIC) obtained by extraction of ion current related to specific fragment ions belonging mainly to the *y*-series as predicted by MS-Product software (included into the Protein Prospector proteomic portal^[22]). The alignment in terms of retention time between peaks in the *full-scan HR-MS* and *HCD HR-MS* XICs allowed retrieving MS/MS-like information. In the present investigation the occurrence of at least three consecutive product ions belonging to the *y*-series (the prevailing type of ions in *HCD-MS* spectra) was considered as the preferred criterion to confirm the amino acid sequence of the peptide. It is worth noting that the described approach has been already exploited successfully in our laboratories to confirm the sequences of egg-white and caseinate-related tryptic peptides.^[19,23,24] The list of candidate peptide markers obtained with DDA acquisition were confirmed with the OrbitrapTM-based MS/MS-like approach.

Beyond the quite comparable final results obtained, the two approaches showed differences in the selection of suitable peptide markers. The main advantage of DDA by Velos ProTM combined with data mining via software was the fast information processing which become crucial when a high number of target analytes must be considered simultaneously (in the present case we tested the method on a limited number of proteins). As a result, the complex data elaboration required by the second approach would not be effective once multiple information about tens of proteins are searched. Still, DDA acquisition required a careful choice of several acquisition parameters which have to limit the number of precursor ions triggering the dependent MS/MS experiment, only to cases which are more likely to be peptides of interest. These parameters should usually be compromise values among number of spectra, amount of information recorded, possible interferences from matrix components and acquisition speed. All information about precursor ions not fulfilling these parameters are lost.

On the other hand, the two-event method tested with the OrbitrapTM mass analyzer (*full-scan HR-MS* and *HCD HR-MS*) boasts the unique feature of fragmenting, without a preliminary specification, virtually every ion generated inside the ionization source, thus keeping a larger amount of information about the sample under investigation. The high accuracy of mass

assignment, both on the precursor and the fragments ions, provided by OrbitrapTM technology allows achieving the same selectivity achieved in DDA modes. It deserves to be mentioned that in particular situations, not encountered during the present investigation, e.g. if the precursor ion of interest is not abundant and enters the HCD cell almost at the same time as another, more abundant, ion (due to significant co-elution of the corresponding compounds), the attribution of fragment ions to the correct parent peptide can become a critical issue.

Features of OrbitrapTM and LIT-MS platforms for multiallergen detection in wine

After the careful selection of suitable peptide markers, the multiplex detection of target proteins for quantitative purposes can be accomplished on the Velos ProTM by SRM acquisition mode that is a targeted approach where selected product ion masses are monitored according to the chosen transitions, while ignoring the rest of ions. This permits very selective and sensitive detection particularly useful when analysis is performed on complex food matrices, due to removal of background ions and improvement of the signal/noise ratio.

In order to design such instrumental method for LYS, OVA and CNate detection, *full MS/MS* spectrum matches of previously selected peptide markers identified by PD software were carefully analyzed highlighting the two most abundant transitions to be monitored in the SRM acquisition (see Fig. 1 for some examples of typical assigned spectra provided by PD search). The selected transitions for each peptide reported in Table 2 were employed to compile an SRM instrumental method. One segment corresponding to the entire duration of the LC run was set up, with six scan events in which each peptide marker was isolated and activated and the ion current related to the two most intense transitions was recorded within a 3 *m/z* window. The latter method was applied to the analysis of tryptic digests of Falanghina wines fortified at various concentration levels within the range of 0.2–2 µg/ml. An overlay of typical SRM traces of the six peptide markers referred to wine sample fortified at the lowest concentration is reported in Fig. 2.

The principal advantage of the untargeted data-independent analysis provided by ExactiveTM mass analyzer relies on the fact that the same chromatographic runs previously discussed for peptide marker selection can be reconsidered for obtaining additional information, without further waste of sample, solvents and time. XICs generated using a 5-ppm mass tolerance (around the theoretical

Table 2. Comparison of LOD, slope and linear correlation coefficient obtained by Velos-ProTM SRM acquisition and ExactiveTM Full MS acquisition for Falanghina grape wine artificially fortified with lysozyme, ovalbumin and caseinate stock solutions, in the range of 0.2–2 µg/ml

Allergenic protein	Marker peptides	EXACTIVE TM <i>Full-scan HR-MS</i>		VELOS PRO TM <i>SRM (CID)</i>		
		LOD	R ²	Fragments	LOD	R ²
LYS	FESNFNTQATNR <i>z</i> = +2, 714.829 ^a	0.18	0.981	<i>y</i> ₅ ⁺ , <i>y</i> ₆ ⁺	0.19	0.983
	NTDGGSTDYILQINSR <i>z</i> = +2, 877.421 ^a	0.18	0.981	<i>y</i> ₄ ⁺ , <i>y</i> ₅ ⁺	0.2	0.977
OVA	LTEWTSSNVMEER <i>z</i> = +2, 791.364 ^a	0.3	0.962	<i>y</i> ₈ ⁺ , <i>y</i> ₉ ⁺	0.19	0.982
	GGLEPINFQTAADQAR <i>z</i> = +2, 844.424 ^a	0.4	0.948	<i>y</i> ₁₂ ⁺ , <i>y</i> ₁₂ ⁺	0.2	0.987
CN	YLGYLEQLLR <i>z</i> = +2, 634.356 ^a	0.3	0.973	<i>y</i> ₆ ⁺ , <i>y</i> ₈ ⁺	0.2	0.985
	FFVAPFPEVFGK <i>z</i> = +2, 692.869 ^a	0.3	0.971	<i>y</i> ₈ ⁺ , <i>y</i> ₉ ⁺	0.3	0.974

^aThe *m/z* values reported were the expected monoisotopical values calculated for protonated peptides double charged.

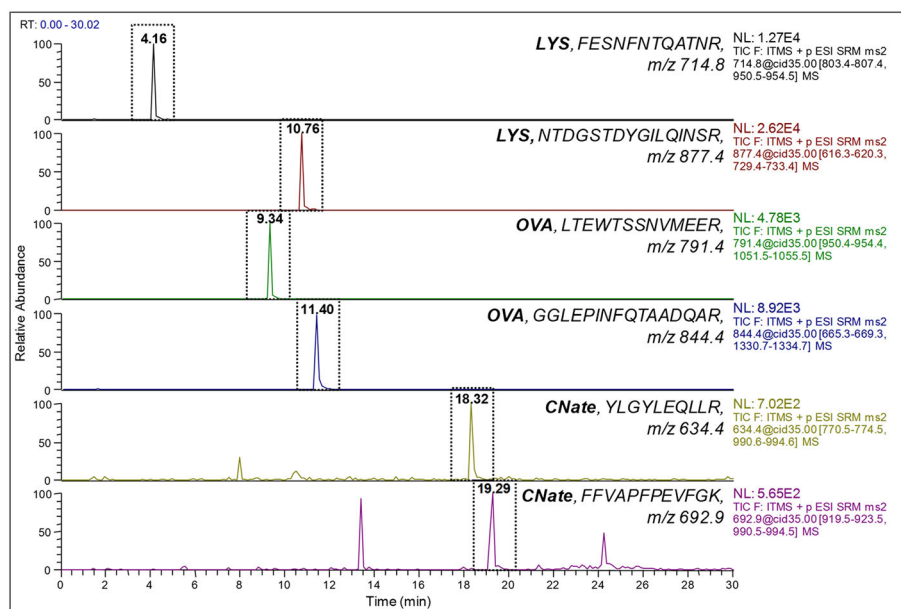


Figure 2. Overlay of the Velos ProTM total ion current SRM chromatograms obtained for selected peptide markers for lysozyme (LYS), ovalbumin (OVA) and caseinate (CNate), and referred to a Falanghina wine sample fortified with standard solutions of purified proteins at 0.2 μ g/ml each.

m/z value) were reconsidered, and no significant visible difference between the SRM chromatograms (Fig. 2) and HR-MS XICs (Fig. 3) was displayed with regard to specificity, background noise and baseline level. In fact, thanks to the high resolution offered by the OrbitrapTM system a very narrow mass extraction window was applied and the six peptide marker peaks can be clearly displayed with a good S/N, after filtration despite the run in *full-scan MS* acquisition mode. Confirming what previously reported,^[13,25] the robustness of the HR full-scan approach depended on the stability of both resolution and mass accuracy and a resolution of 50 000 at m/z 200 in HR full-scan acquisition with an adequate mass window provided selectivity comparable to SRM acquisition.

The accuracy of quantitative information provided by an MS method depends, among others, also on the duty cycle of each experiment resulting in a different sampling rate across the

chromatographic peak. Generally speaking, the time taken for a low-resolution scan on a LIT is shorter than what required for high-resolution scans on an OrbitrapTM. Here, the LIT was operated in a single segment-six SRM events method with the simultaneous monitoring of two transitions (within 3 m/z wide window) per peptides. On the other hand, one-segment-two event acquisition scheme was employed for OrbitrapTM-based analysis, including *full-scan HR-MS* and *HCD-HR-MS* scan both accomplished over the range of 200–2000 m/z . The number of data points acquired by both the analyzers was always >7 for the six peptide markers at all concentration levels. Like expected, LIT acquisition provided a higher sampling rate, which in perspective can be further increased if the six SRM events would be divided into different acquisition segments basing upon each respective chromatographic retention time.

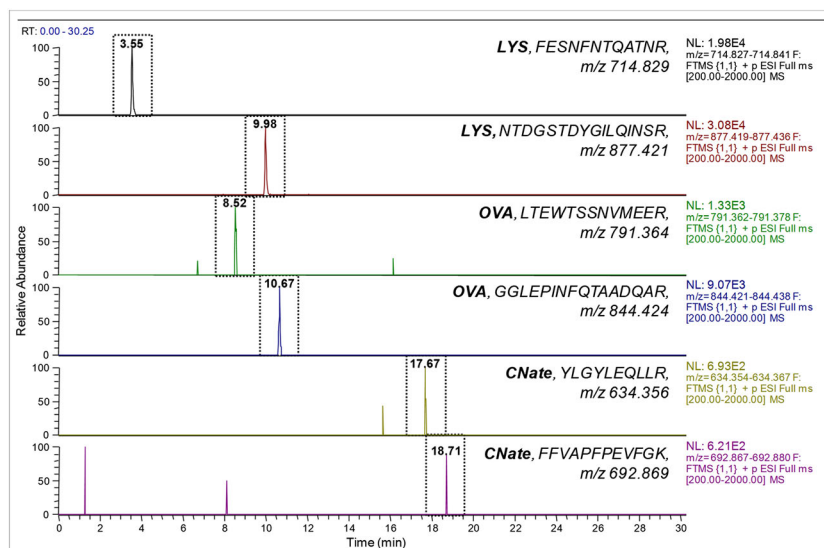


Figure 3. Overlay of the OrbitrapTM extracted ion current (XIC) chromatograms obtained for selected peptide markers for lysozyme (LYS), ovalbumin (OVA) and caseinate (CNate), and referred to a Falanghina wine sample fortified with standard solutions of purified proteins, at 0.2 μ g/ml each.

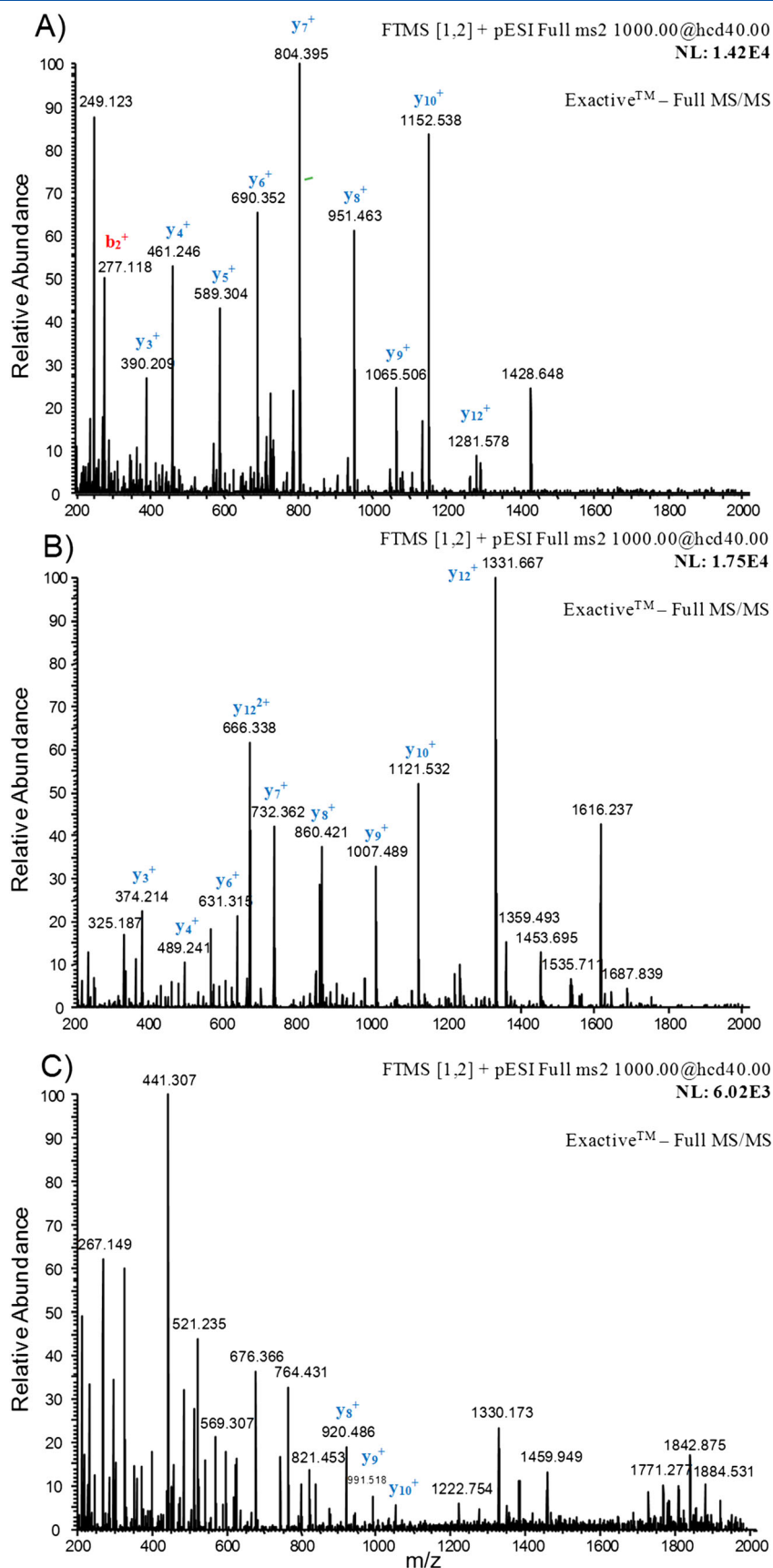


Figure 4. Overlay of typical HCD-HR All ion fragmentation MS spectra obtained with Orbitrap™ of marker (A) 714.829 (LYS, FESNFTQATNR), (B) 844.424 (OVA, GGLEPINFQTAADQAR) and (C) 692.869 (α S1-CN, FFVAPFVFVGK), respectively, recalled at retention times 3.6, 10.7 and 18.7 min, respectively.

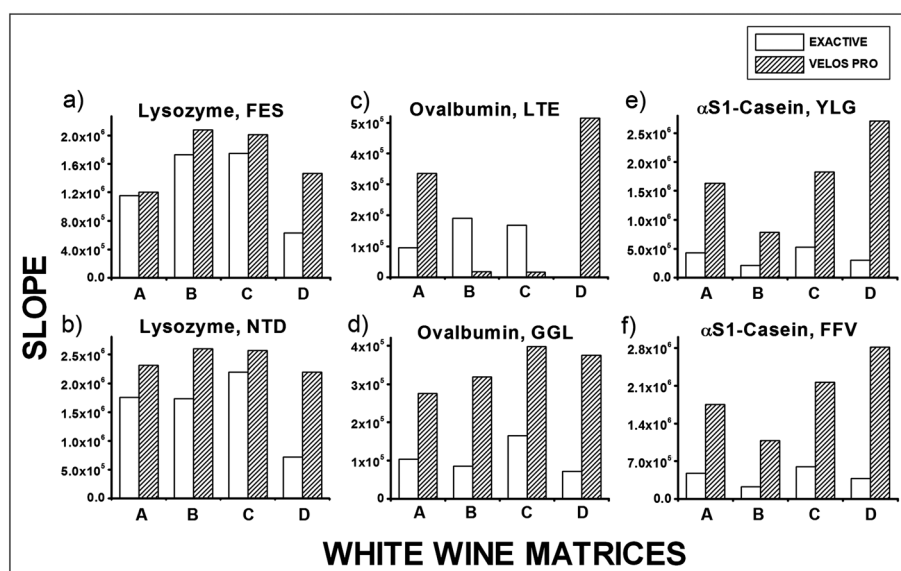


Figure 5. Sensitivity comparison of the two mass analyzers ExactiveTM (solid columns) and Velos ProTM (dashed columns): slopes obtained for the calibration curves of lysozyme (a, b), ovalbumin (c, d) and caseinate (e, f) marker peptides, in different artificially contaminated wine matrices: Falanghina (A), Pinot Grigio (B), Greco di Tufo (C) and mixed Bombino Bianco and Trebbiano (D) grape varieties.

Although the number of data points for HR-MS acquisition is slightly smaller, it did not result in poorer quantitative performance compared to SRM, as discussed in the following.

For low-resolution LIT acquisitions, calibration curves were obtained by plotting SRM peak areas *versus* the concentration of the corresponding protein. Differently, two options are available with OrbitrapTM acquisition for calibration purposes: either integration of HR-MS XICs or integration of HCD HR-MS XICs. Given the complexity of the real food matrices, and the absence of precursor ion selection in the AIF mode available on the ExactiveTM platform, HCD-HR-MS acquisition is usually less sensitive. As a fact, notwithstanding MS/MS like information can be retrieved through ion current extraction of specific transitions and alignment of XIC peaks basing on the retention time, the HCD-HR-MS spectra remain composite spectra populated by ions arising from the contemporaneous fragmentation of other co-eluting species. In order to partially overcome this issue, a narrow time window was applied to recall the MS spectrum under the specific peptide peak along with MS subtraction across the ascending and the descending part of the peak, resulting in a clearer fragmentation pattern which could be easily attributable to the specific precursor peptide.

For reliable quantification of target analytes, HR-MS XIC peaks were exploited whereas HCD-HR-MS traces were considered only for confirmation of peptide identity. The LOD values were calculated for both HR and SRM data set referred to the minimum concentration of the added purified protein that can be detected, at $S/N = 3$ (the standard deviation of the calibration line intercept was used in this case as an estimate of noise) and are reported in Table 2.

From the comparison of SRM and *full-scan* HR-MS curves, it emerged that the two approaches allowed a satisfactory linear correlation in the whole concentration range investigated, with comparable limits of detection achieved by using both MS platforms.

Finally, it has to be pointed out that the LODs achieved by only applying precursor mass filtration were much lower than those calculated when both fragments needed to be detected in the HCD-HR-MS spectrum for confirmative purposes. The minimum

concentration at which both fragments could be clearly detected in the HCD-HR-MS spectra corresponded to 0.2 $\mu\text{g/ml}$ for LYS, 0.2 $\mu\text{g/ml}$ for OVA and 0.5 $\mu\text{g/ml}$ for CN. In Fig. 4 were reported typical HCD-HR-MS spectra of marker peptides undergoing fragmentation with at least two fragments clearly highlighted.

Evaluation of differential contribution of sample matrix onto MS detection

In general, the robustness of the HR full-scan approach strictly depends on the resolving power and mass accuracy of the specific instrument. However, mass accuracy often deteriorates significantly in complex matrix due to unresolved interferences which in turn affects identification and selectivity, as well as quantification. In principal, this makes HR-MS potentially more sensitive than SRM-MS to matrix effects.

In order to check this issue both MS platforms were proved to the analysis of various wine matrices produced by different grape varieties. In particular, four wines were considered produced from Falanghina (A), Pinot Grigio (B), Greco di Tufo (C) and mixed Bombino Bianco and Trebbiano (D) grapes, and relevant sensitivity in terms of slope of the calibration curves were compared. Results are reported in Fig. 5, for the six peptide markers. As a general evidence, both platforms appeared to be subjected to matrix effects, resulting in variable sensitivity as a function of the wine matrix selected. Still, Velos ProTM, generally resulted in higher slopes, probably due to a lower matrix effect compared to the single-stage OrbitrapTM-based mass spectrometers with the exception of OVA marker LTE.

Conclusions

In this communication, potentials and features offered by two mass analyzers namely a linear ion trap and an OrbitrapTM mass spectrometer will be presented and compared each other applied to the food allergen field. Quantitative and confirmative capabilities of both platforms have been assessed and discussed

along with their strengths and weaknesses for the multiallergen screening in wine matrices.

Both approaches proved to be equally suited for quantification and screening purposes. The main advantage of full-scan high resolution MS lies in the possibility to apply post-run data processing for a retrospective analysis. This approach proves a higher flexibility than the classical MS/MS method where a proper decision has to be made in the very beginning during the experimental design, to reach the desired sensitivity. On the other hand, for confirmative purposes, when confirmatory fragment ions are needed, the sensitivity shown by the SRM method proved to be higher than that offered by activating the full ion fragmentation option offered by the monostage HR-MS. Though both approaches still suffer from matrix effects, a more intensive clean-up is required for single stage OrbitrapTM analysis compared to MS/MS, where detection selectivity depended on the accuracy of mass assignments which is mainly affected by the ratio of analyte relative to co-eluting matrix compounds.

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