Proteomics in quality control: Whey protein-based supplements

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A B S T R A C T

The growing consumption of nutritional supplements might represent a problem, given the concern about the quality of these supplements. One of the most used supplements is whey protein (WP); because of its popularity, it has been a target of adulteration with substitute products, such as cheaper proteins with lower biological value. To investigate this type of adulteration, this study used shotgun proteomics analyses by MS 5 (multiplexed, low- and high-collision energy, data-independent acquisition) of WP-based supplements. Seventeen WP-based supplement samples were evaluated. Chicken, maize, rice, potato, soybean, and wheat proteins were considered as probable sources of bovine whey adulteration. Collectively, 523 proteins were identified across all 16 samples and replicates, with 94% of peptides inside a normal distribution within 10 ppm of maximum error. In 10 of the 16 samples analyzed, only proteins from bovine whey could be detected, while in the other samples several other protein sources were detected in high concentrations, especially soybean, wheat, and rice. These results point out a probable adulteration and/or sample contamination during manufacturing that could only be detected using this proteomic approach.

Significance: The present work shows how shotgun proteomics can be used to provide reliable answers in quality control matters, especially focusing on Whey Protein nutritional supplements which are a very popular subject in food and nutrition. In order to achieve an appropriate methodology, careful evaluation was performed applying extremely rigorous quality criteria, established for the proteomic analysis. These criteria and the methodological approach used in this work might serve as a guide for other authors seeking to use proteomics in quality control.

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1. Introduction

The consumption of supplements by athletes and fitness enthusiasts to improve exercise performance has been increasing over the last few years. In some cases, users expect the supplements to fulfill nutritional needs created by an incomplete diet, while in other cases they expect the supplements to enhance their sports performance. However, there is not a consensus among physicians and trainers regarding the benefits of nutritional supplements, because of an enormous quantity of contaminated, faked, or ineffective supplements that might pose serious risks to an athlete’s health or lead to evidence of doping in adverse analytical findings [1–10].

Whey protein (WP), which is the soluble protein fraction in milk serum that is obtained during cheese and casein production, has been studied since the 1970s as a source of high biological-value proteins, as well as bioactive peptides, which might act as antimicrobial, antihypertensive, and immune-response modulator agents [11–13]. A controlled study of 874 athletes in the UK showed that approximately 60% (520) of the athletes use some type of nutritional supplement. The most popular performance-related supplements were creatine, which was used by 36.1% of the supplement-using athletes, followed by WP-based supplements, which were adopted by 30.6% of the users. However, this study has shown that many athletes do not know the reasons why they use a given supplement or, worse, they might use a supplement for a reason that is not compatible with the proven effects of that substance [10].

Unfortunately, the adulteration is extremely common in products derived from powdered milk, the most common cases being the addition of compounds with high nitrogen content that mask the protein content measured by the commonly used Kjeldahl method. This allows the dilution of the milk, which might be advantageous economically [14]. A series of different adulteration methods in these products have been reported, as well as different methods for the detection of these adulterants [15–20].

Another reported method for the adulteration of WP-based supplements is the addition of free amino acids such as glycine, which are cheaper than the whey protein concentrate (WPC) [21]. This might be a little more difficult to determine by the cited methods because, in general, these methods search for other nitrogen-containing compounds...
and not amino acids, which, however, do not have the same nutritional properties of the high biological-value proteins expected to be present in the WP. With this increasing complexity in adulteration procedures, a question is raised as to whether manufacturers could use a cheaper protein source, such as soybean or rice, in order to produce an “undetectable” adulteration in WP-based supplements. Some authors have explored proteomic approaches in the analysis of proteins from whey, and there is some information available on the composition of this product [12,19,22–26]. Also, some studies have explored the potential of proteomics in the quality control of foods, using these techniques for the determination of food safety, microbiological contamination, and authentication [27–33].

Attempts to investigate the protein composition in complex samples through modern proteomics approaches have enabled the identification of low-abundance proteins, which are often lost when two-dimensional electrophoresis gels are used [34]. Therefore, proteomics has evolved to focus on the functionality of the huge datasets acquired through a vast number of analytical technologies. The quality of procedures, orthogonally provided by MS (multiplexed, low-, and high-collision energy, data-independent acquisition (DIA)) [35,36] with increased selectivity and specificity, has recently received great attention. Modern techniques such as a multiplex, high-resolution format MS are valuable for the acquisition required by shotgun proteomics and complex samples [37,38]. This approach is capable to perform serial acquisitions in which the cycle starts by acquiring an MS survey scan with the precursor ion intensity-based selections for MS/MS. With this DIA available on the instrument, major problems such as reproducibility in LC-MS/MS-based protein identification schemes are overcome.

The goal of this work was to develop a methodological approach using shotgun mass spectrometry-based proteomics in order to identify, quantify, and determine the protein sources of WP-based supplements, and to apply this method to commercial supplement samples to show whether or not some type of adulteration and/or contamination may be occurring in these products.

2. Materials and methods

2.1. Samples

In total, sixteen samples of WPC or WP isolate (WPI)-based supplements, as described in Table 1, were acquired in the Brazilian local market and analyzed in this work. Samples of previously hydrolyzed WP-based supplements were not evaluated.

2.2. Protein extraction

The samples were homogenized in their own bottles before sampling. Then, 1 g of supplement powder was weighed precisely and dissolved in 30 mL of a solution containing urea (6 mol·L$^{-1}$), ammonium bicarbonate (50 mM) and sodium dodecyl sulfate (SDS) (1% v/v). The solutions were vortexed for 30 s and then centrifuged at 14,000 rpm for 40 min. A 500 μL aliquot of the supernatant was washed and concentrated using a 3 kDa filter (Amicon Ultra®, Millipore). Five washes were carried out using a 50 mM ammonium bicarbonate buffer at pH 8.0, and centrifugation was resumed at 12,000 rpm for 10 min at 4 °C. To collect the filtered and concentrated proteins, the filter was inverted and centrifuged at 500g for 2 min at 4 °C. The sample was transferred to a new tube and stored at −20 °C until the next step.

2.3. Protein quantification

The samples were quantified by using the 2D Quant Kit (GE Healthcare) and following the manufacturer’s instructions: An analytical curve of bovine serum albumin (BSA) was obtained using 0, 10, 20, 30, 40, and 50 μg of protein, with 5 μL of each sample used for quantification. The absorbance was measured at 480 nm in a Spectramax 190 spectrophotometer using Softmax Pro v.5.4.1 software (Molecular Devices), and the total protein concentration was obtained by interpolation.

2.4. Protein digestion for nanoUPLC-MS$^6$ analysis

After quantification, 50 μg of protein were diluted with 50 mM ammonium bicarbonate buffer to achieve a final volume of 60 μL. Then, 25 μL of 0.2% RapiGest SF (Waters, Manchester, UK) were added per sample [39], followed by incubation at 80 °C for 15 min. After this period, 2.5 μL of 100 mM diithiothreitol (DTT) (Little Chalfont, UK) were added to each sample; the samples were then homogenized and incubated at 60 °C for 30 min. Afterward, 2.5 μL of 300 mM 2-iodoacetamide (IAA) (Sigma Aldrich, St. Louis, US) were added to each tube, the samples were homogenized again, and then were incubated in the absence of light for 30 min. Trypsin (Promega, Madison, USA) was added at a 1:100 enzyme:protein ratio and the samples were digested overnight at 37 °C.

After digestion, 10 μL of 5% v/v trifluoroacetic acid were added to each sample, followed by homogenization and incubation at 37 °C for 90 min. The samples were centrifuged at 16,000g for 30 min at 6 °C, and the supernatant was transferred to the final sample vials. Prior to injection, 5 μL of a solution consisting of 1 pmol·μL$^{-1}$ of MPDS (MassPREP digestion standard, yeast alcohol dehydrogenase (ADH), UNIPROT Entry P00330, Waters, USA) were added, leading to a final concentration of 25 fmol·μL$^{-1}$, and 85 μL of 3% acetonitrile (ACN) with 0.1% formic acid were then added, so that a final protein concentration of 250 ng·μL$^{-1}$ was reached in each vial. The samples were then placed in the auto-sampler and kept at 4 °C for the nanoUPLC-MS$^6$ analysis.

2.5. Mass spectrometry and protein identification

The peptides from digestion of the WP-based supplements were then submitted to a shotgun analysis. The instrument used was a Synapt high definition mass spectrometer (HDMS) (Waters, Manchester, UK), as described elsewhere [40], equipped with a nanoACQUITY ultra-performance liquid chromatography (nanoUPLC) system (Waters, Milford, USA) and coupled to a hybrid quadrupole/ion mobility mass spectrometry/orthogonal acceleration time-of-flight (Q-IMMS-orTOF) MS geometry. Qualitative and quantitative experiments were conducted with the following gradient (in which A is deionized water and B acetonitrile, both with 0.1% (v/v) of formic acid): 7 to 40% of B in 86 min; followed by a cleaning column gradient of 40 to 85% of B for 4 min; then from 85% to 7% of B in 2 min. The flow rate was kept at 600 nL·min$^{-1}$ during a total run time of 100 min. A nanoACQUITY UPLC HSS T3 1.8 μm, 100 μm × 100 mm column (pH 3) was utilized in conjunction with a Symmetry C$^{18}$, 5 μm trap column. Typical on-column sample loads were 500 ng of total protein digests for each sample injected. For all measurements, the mass spectrometer was operated in the resolution mode and all analyses were performed using nano-electrospray ionization in the positive ion mode (nanoESI+) and a NanoLockSpray (Waters, Manchester, UK) ionization source. The lock mass channel was sampled every 30 s. The mass spectrometer was calibrated with a MS/MS spectrum of [Glu$^1$]-Fibrinopeptide B human (Glu-Fib) solution (320 fmol·μL$^{-1}$) delivered through the reference sprayer of the NanoLockSpray source. The signal [M + 2H]$^+$ = 785.8426 was used for an initial single-point calibration and MS/MS fragment ions of Glu-Fib were used to obtain the final instrument calibration. Multiplexed DIA scanning (MS$^6$) MS analyses were performed with a Synapt HDMS mass spectrometer (Waters, Manchester, UK) [41], which was automatically set to switch between standard MS (6 eV) and elevated collision energies (MS$^6$ 15–55 eV) applied to the trap ‘T’-wave CID (collision-induced dissociation) cell with argon gas. The trap collision cell was adjusted for 6 eV, using a millisecond scan time, and previously was adjusted based on the linear velocity of the chromatography peak delivered through nanoACQUITY UPLC to
get a minimum of 20 scan points for each extracted ion chromatogram, both in low-energy and at high-energy transmission at the orthogonal acceleration time-of-flight (oa-TOF) for a mass range from m/z 50 to 2000. The radio frequency (RF) offset (MS profile) was adjusted such that the nanoUPLC-MS² data were effectively acquired from m/z 400 to 2000, which ensured that any masses less than m/z 400 that were observed in the high-energy spectra arose from dissociations in the collision cell.

2.6. Database searching and quantification

Protein identifications and the quantitative data package were generated by the use of dedicated algorithms [42] that searched against a database using ion accounting and the Hi3 (Top3) based quantitation method, as described in the literature [43,44]. The utilized databases were reversed “on-the-fly” during the database queries, and appended to the original database to access the false positive rate of identification. For proper spectra-processing and database-searching conditions, a ProteinLynx Global Server software package with Apex 3D, Peptide 3D, and Ion Accounting informatics v3.0.2 (Waters, UK) was used. The UNIPROT protein databank with the specific annotations for bovine, chicken, maize, rice, potato, soybean, and wheat was utilized. Search conditions were based on taxonomies (Bos taurus, Gallus gallus, Zea mays, Oryza sativa subsp. Japonica, Solanum tuberosum, Glycine max and Triticum aestivum), with the maximum number of cleavages missed by trypsin allowed up to 1, and variable modifications by carbamidomethyl (C), acetyl N-terminal, and oxidation (M) set at a default maximum of 4% of the false discovery rate (FDR). Data filtering and quality parameters applied to the identified and quantified proteins for the described samples and replicates were analyzed using data filtering through the following criteria. At the peptide level, the maximum allowed peptide error tolerances were 10 ppm for precursor ions, a maximum of 20 ppm for fragment ions prior to database searching, and an in-source fragment percentage of 10% (maximum), as described elsewhere [43,44]. At protein level, the following quality-control parameters were applied across all replicates and samples: quantified

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Flavor</th>
<th>Labeled ingredients</th>
<th>Labeled manufacturer observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Strawberry</td>
<td>Whey protein concentrate, hydrolyzed wheat protein, mix of vitamins and minerals (magnesium, zinc and vitamin B6), xanthan gum thickener, silicon dioxide, flavor identical to natural strawberry, sucralose sweetener and red dye.</td>
<td>Contains milk and soy ingredients; manufactured in a facility that processes egg and fish ingredients.</td>
</tr>
<tr>
<td>2</td>
<td>Chocolate</td>
<td>Whey protein concentrate (whey protein concentrate, soy lecithin), corn maltodextrin, cocoa powder (processed with alkali), natural and artificial flavor, salt, whey protein isolate, carrageenan, xanthan gum, acetaldehyde potassium and sucralose.</td>
<td>Gluten free. Contains milk and soy ingredients.</td>
</tr>
<tr>
<td>3</td>
<td>Cookies</td>
<td>Concentrate whey protein, cookies (natural identical aroma) and sucralose.</td>
<td>Gluten free.</td>
</tr>
<tr>
<td>4</td>
<td>Strawberry</td>
<td>Protein blend (whey protein concentrate, whey protein isolate), natural and artificial flavors, beet powder (color), cellulose gum, soy lecithin, xanthan gum, calcium phosphate, calcium carbonate, acetaldehyde potassium, malic acid, citric acid, sucralose.</td>
<td>Made in a GMP facility on equipment that process milk, soy, egg, peanuts, tree nuts, fish, shellfish and wheat.</td>
</tr>
<tr>
<td>5</td>
<td>Strawberry</td>
<td>Just-WHEY Protein blend [whey protein concentrate (milk), whey protein isolate], natural and artificial flavors, lecithin (soy), cellulose gum, red beet powder, citric acid, acetaldehyde potassium, and sucralose.</td>
<td>Gluten free. Sugar free.</td>
</tr>
<tr>
<td>6</td>
<td>Chocolate</td>
<td>Whey protein concentrate, cocoa powder, flavor identical to natural chocolate, guar gum thickener and sweetener sucralose.</td>
<td>Gluten free. Contains lactose. May contains traces of milk and egg.</td>
</tr>
<tr>
<td>7</td>
<td>Chocolate</td>
<td>Whey protein concentrate, cocoa powder, calcium arginine chelate, flavoring, caramel coloring INS 150d, sucralose.</td>
<td>Sugar free. Gluten free.</td>
</tr>
<tr>
<td>8</td>
<td>Strawberry</td>
<td>Whey protein concentrate, hydrolyzed wheat protein, maltodextrin, artificial strawberry flavor, artificial banana flavor, dyes amaranth (INS 123), tartrazine (INS 102) and sunset yellow (INS 110).</td>
<td>Made in a GMP facility on equipment that process milk, soy, egg, peanuts, tree nuts, fish, shellfish, and wheat.</td>
</tr>
<tr>
<td>9</td>
<td>Strawberry</td>
<td>Whey protein concentrate, aroma identical to natural strawberry, thickener xanthan gum, antiwetting agent silicon dioxide, sweetener sucralose, and red dye Bordeaux.</td>
<td>Gluten free.</td>
</tr>
<tr>
<td>10</td>
<td>Cookies and cream</td>
<td>Pure whey (ultrafiltered whey concentrate [ comprised of -45% β-lactoglobulin, -20% a-lactalbumin, -15% glycomacropeptides, -10% immunoglobulins, -6% bovine serum albumin, -1% lactoperoxidase, protease-peptone, lysosome], whey isolates, partially hydrolysed whey peptides][milk], natural and artificial flavors, cookie bits [sugar, wheat flour, cocoa (processed with alkali), partially hydrogenated vegetable oil, (soybean and/or cottonseed), salt, natural and artificial flavors], lecithin (soy), acetaldehyde potassium, sucralose.</td>
<td>Gluten free. Sugar free.</td>
</tr>
<tr>
<td>11</td>
<td>Strawberry</td>
<td>Whey protein concentrate, whey protein isolate, artificial strawberry flavor, artificial sweetener sucralose, soy lecithin stabilizer.</td>
<td>Gluten free.</td>
</tr>
<tr>
<td>12</td>
<td>Chocolate</td>
<td>Whey protein concentrate, cocoa powder, vitamin C, vitamin E, vitamins B1, B2 and B12, minerals: phosphorus, calcium, magnesium, iron, zinc, chromium, selenium, aroma identical to natural vanilla, thickeners: xanthan gum and guar gum, artificial sweeteners: sodium cyclamate, sodium saccharin and sucralose.</td>
<td>Gluten free.</td>
</tr>
<tr>
<td>13</td>
<td>Chocolate</td>
<td>Whey protein concentrate (WPI), artificial sweetener sucralose and chocolate flavor identical to natural.</td>
<td>Gluten free. Sugar free.</td>
</tr>
<tr>
<td>14</td>
<td>Strawberry and banana</td>
<td>Whey protein isolate, whey protein concentrate, hydrolyzed wheat protein, maltodextrin, artificial strawberry flavor, artificial banana flavor, dyes red amaranth (INS 123), tartrazine (INS 102) and sunset yellow (INS 110).</td>
<td>Gluten free.</td>
</tr>
<tr>
<td>15</td>
<td>Vanilla</td>
<td>Protein matrix (whey protein concentrate, whey protein isolate and hydrolyzed whey protein), glucose, thickener CMC, guar gum and xanthan gum, linsed oil, sea salt, artificial sucralose and acetaldehyde potassium, flavoring natural and artificial lactase enzyme.</td>
<td>Gluten free.</td>
</tr>
<tr>
<td>16</td>
<td>Chocolate</td>
<td>Mixed whey protein (whey protein concentrate, whey protein isolate and hydrolyzed whey protein), modified waxy maize starch (waxy maize), cocoa powder, magnesium pyruvate, zinc L-aspartate, chromium picolinate, pyridoxine hydrochloride (B6 vitamin), xanthan gum thickener and sucralose.</td>
<td>Gluten free.</td>
</tr>
</tbody>
</table>

Table 1
List of the sixteen samples analyzed in this work and their labeled descriptions by the manufactures.
proteins with a minimum average peptide per protein of 3 across all samples, and an expected dynamic range of 3 logs based on multi-channel plate (MCP) detection. Briefly, this detector plate corresponds to 2 chevron (V-shaped) aligned plates and behaves as a fast-electron multiplier, where a single ion event can result in $1 \times 10^7$ electrons being produced over a 4 to 5 ns period. This event is followed by a time-to-digital converter (TDC) that is a PCI-based acquisition device. The high-impact ion flux caused by these multi-charged ions, such as peptides in the complex samples, strike the front MCP, causing an electron shower within the plate that is then multiplied across a pair of plates. The voltage spike created on the anode is recorded by the TDC as an ion arrival event — together with its characteristic time-of-flight data, which are buffered and passed to the host, embedded PC acquisition system (EPCAS) technology, for processing and data-event recording [45]. This ion flux momentum over a single MS spectra-detection amplitude range corresponds to at least 3 logs of dynamic range. With the unbiased DIA acquisition, the detection dynamic range over the sample was calculated.

3. Results

Evaluating the quality of the acquired proteomic data, we observed at the peptide level that the percentage of peptides identified as missed cleavages, variable modifications, and in-source fragments were less than 10%, 7%, and 3%, respectively, as described in Fig. S1 (see support information). These low numbers for peptides formed by these processes indicates good quality data.

At the protein level, collectively in all samples, 523 proteins were obtained along with 22 non-replicated decoys. After concatenation and replication filtering, 63% of replicated proteins were identified and quantified across all 16 samples and replicates along with 8 MPDS proteins with 94% of peptides assigned to all reported proteins inside a normal distribution of 10 ppm (see support information Fig. S2) with an average of 8 peptides per protein. After the protein quality criteria was applied (minimum of 3 peptides per protein and a dynamic range of 3 logs, Fig. S3), 211 proteins were considered for all further analyses and interpretations that follow in this work.

Among this 211 proteins, 162 were assigned to *Bos taurus*, 19 to *Triticum aestivum*, 25 to *Glycine max*, 1 to *Oryza sativa*, 1 assigned to *Solanum tuberosum*, 1 protein to *Zea mays*, and 2 to *Gallus gallus*. The non-bovine proteins were identified in 6 of the 16 samples and were distributed as shown in Fig. 1.

All information about the proteins and peptides is available as supplemental materials, i.e., a complete peptide and protein list. Also the information on the dynamic range of both identified and quantified proteins with at least with 3 orders of magnitude (log10 [counts]) is presented (Figure S3).

Several proteins are found in bovine whey, being β-lactoglobulin, α-lactalbumin, bovine serum albumin (BSA), and immunoglobulins the most abundant proteins of the whey fraction [46]. In fact, evaluating all the detected proteins, the prevalent contributions for *Bos taurus* proteins were from α-lactalbumin (UNIPROT entry name – LALBA_BOVIN) and β-lactoglobulin (LACB_BOVIN), followed by an “uncharacterized protein” G5ESH7_BOVIN, which is a variant of β-lactoglobulin. These results were in agreement with previously reported proteomic data for bovine whey [46]. The protein distributions for *Bos taurus* are reported in Fig. 2.

For *Triticum aestivum* the most abundant proteins were glutenin GLT0, GLT2, GLT4, and GLT5, followed by gamma-gliadin (GDBB_WHEAT & GDB2_WHEAT) (Fig. 3), and for *Glycine max*, glycamin G1, G2, and G4 were prevalent, followed by beta-conglycinin alpha and beta (GLCA_SOYBN & F7077_SOYBN) (Fig. 4).

The distribution of the quantified proteins was evaluated by comparing the percentage of contribution of β-lactoglobulin and its variants, other bovine proteins and non-bovine proteins to the total protein content quantified within each sample, as presented in Fig. 5.

4. Discussions

Whole milk is a complex fluid that presents a wide range of proteins, and therefore a very large dynamic range is expected [47,48]. Caseins and proteins from whey represent 80% and 20% of the total proteins in milk, respectively [49], while among whey proteins, the most abundant are lactoglobulins and lactalbumins which was indeed the results found in the proteomics evaluation reported in this work (Fig. 2).

There are several types of WP-based supplements on the market. WPC-based supplements, in which the whey is submitted to ultrafiltration to recover the proteins in their native form, are among the most common [46,50]. WPC terminology is being used for the spray-dried whey, having more than 25% and up to 90% of the WPC protein composition [51]. Whey protein isolate (WPI) is also a very common type of WP-based supplement, the main difference being that WPIs are more pure (more fractionated) than WPCs, as non-protein components (e.g., lactose and salts) are partially removed, thus “isolating” only the proteins. So, WPI generally presents higher amounts of protein compared to WPC. In both WPC and WPI, a mixture of native intact proteins is available but whey can also be submitted to a further step of hydrolysis, forming whey protein hydrolysate (WPH). In WPH, the native proteins are then submitted to acidic or enzymatic hydrolysis, forming more bioavailable peptides and amino acids, which are more rapidly absorbed when ingested than are intact proteins [46], and due to this reason, WPH supplements were not analyzed in this work.

Commonly, commercial whey supplements are a blend of these three types of WP (WPC + WPI + WPH) so as to reduce the final cost and provide an intermediate benefit for the consumer (for instance, because WPH is far more expensive than WPC). In the production processes, the addition of other sources of proteins, such as soybean proteins, is also common. The problem arises when this addition is not declared, and the consumer acquires a product as 100% proteins obtained from bovine whey, but in fact it is not. The detection of adulteration with proteins of lower biological values is, therefore, more challenging from an analytical point of view. This work demonstrated, along with previously reported results, that shotgun proteomics is a very suitable method for detecting this type of adulteration, as it allows the direct analysis of these complex protein mixtures, such as WP blends with other species. By rapidly providing a global profile of the proteins within the mixture, using pre-selected protein datasets, probable adulteration sources can be unambiguously detected [52–56].

From the labeled ingredients declared by the producers (Table 1), we can observe that, in some WP-based supplements, the addition of other protein sources is declared. For example, samples 1, 8, and 14 have declared hydrolyzed wheat protein in their composition, while sample 16 has waxy maize declared on the label. So, for these samples, proteins other than whey are indeed expected to be detected. In fact, samples 1, 8, and 14 contained other sources of proteins, not only from bovine whey and wheat, but from rice, chicken and soybean.
which indicates adulteration, contamination during processing or the use of impure ingredients (Fig. 1). In sample 16, waxy maize proteins were not detected as expected.

Some manufacturers indicate also that the WP-based supplements are produced in facilities that process other types of foods (a precautionary indication for the remote possibility of presence of food allergens), as in the cases of samples 2, 5, and 10 (Table 1). But the fact that they are WP-based supplements should mean that other protein sources are expected to be found in low concentrations. In samples 2 and 5, basically only WP could be identified, while for sample 10 a very low relative abundance of maize protein was found, which is probably due to some contamination in the processing as this extremely low abundance would not be economically interesting to justify an adulteration. From the data presented in Fig. 5, it is possible to notice that the relative contribution of non-bovine proteins is very low in samples 7 and 10. However, samples 1, 3, 8 and 14 present levels of non-bovine proteins which are not negligible, thus indicating serious contamination or even adulteration in these cases. Of these, samples 1, 8 and 14 declare the addition of hydrolyzed wheat which could partially justify the high levels of non-bovine proteins found even though when comparing these data to those presented in Fig. 1 it is possible to notice that more non-declared soybean proteins were identified in these samples than wheat proteins itself. It could be expected the presence of soybean proteins to be related with the addition of soy lecithin but these samples have not declared this addition in their labeled compositions (Table 1).

β-lactoglobulin has been reported to be the major protein component of the whey fraction with an abundance of about 70–80% of the WP. Thus, observation of the data in Fig. 5 raises another question regarding the quality of the WP-based supplements that did not present any (or presented very low levels – e.g. sample 10) of non-bovine proteins. The relative composition of the bovine proteins in some of these samples (2, 7, 12 and 16) is in agreement with the expected profile for the WP. However, the other samples, i.e. 4, 5, 6, 9, 10, 11, 13 and 15 are not in agreement with this observation, being the relative

**Fig. 2.** Protein distribution sized by protein quantitation over *Bos taurus* taxonomy (UNIPROT entry names). Proteins not labeled means that they are not named individually and are considered as “other” in this plot.

**Fig. 3.** Protein distribution sized by protein quantitation over *Triticum aestivum* taxonomy (UNIPROT entry names). Proteins not labeled means that they are not named individually and are considered as “other” in this plot.
\(\beta\)-lactoglobulin contribution in these samples lower than 20% of the bovine proteins. This could indicate the use of low quality WP or even of the whole milk in the production of these supplements.

This fact is quite noteworthy, and points out that several WP supplements do not contain only proteins from whey, as expected, but indeed consists of several sources of proteins. WP is only being consumed more extensively because of the well-known good nutritional properties of its proteins. Yet, in the presence of other protein sources, these nutritional properties may be compromised. In this work we cannot state for sure that the presence of these indicates deliberate adulteration. They can be added intentionally to decrease the manufacturing costs, or they can be sourced in contaminations during processing or by the addition of 'impure' ingredients to the supplements. However, whether or not the additions of other protein sources are intentional, consumers are not getting what they expect, as these supplements are intended to have their protein sources entirely from whey. All the data presented here suggest that more quality control over WP products must be implemented so as to regulate the protein composition of these supplements. Moreover, the results presented here have practical implications related to the possible occurrence of undeclared proteins, which can act as allergens (e.g., soybean or wheat proteins), and also concerning the requirements of specific categories of consumers (e.g., not declaring the presence of animal proteins for vegetarians/vegans).

5. Conclusions

This work presented a proteomics analysis of 16 samples of WP supplements via shotgun MS\(^6\) (multiplexed DIA) in a Synapt HDMS mass spectrometer. After concatenation, the number of all unique identified proteins was 523, with 22 decoys. The total number of both identified and quantified proteins was, collectively, 211. Of these, 162 were assigned to \textit{Bos taurus}, 19 to \textit{Triticum aestivum}, 25 to \textit{Glycine max}, 1 to \textit{Oryza sativa}, 1 assigned to \textit{Solanum tuberosum}, 1 protein to \textit{Zea mays}, and 2 to \textit{Gallus gallus}.

In most of samples, only proteins derived from bovine were detected, but in 6 of the 16 analyzed samples, proteins from other sources were found with high relative abundances which suggest product defilement or contamination during processing. We have also discussed the low levels of \(\beta\)-lactoglobulin in some samples which might indicate the addition of whole milk instead of WPC or WPI in these supplements.

This work draws attention to the fact that many purchasers are not getting what they pay for: that is, a product with proteins mostly from bovine whey. In the majority of samples, not only the proteins with high biological prices and costs from bovine whey were detected. From this, we conclude that more quality control and by protein identification and quantitation could be implemented to regulate the supplement’s market. Furthermore, some care should be exercised in the use and choice of this type of nutritional supplements, as it has been shown that some of them are mixtures with other undesired proteins.

Transparency document

The Transparency document associated with this article can be found, in online version.

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