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Proteomics in Forensic Sciences: Identification of the Nature of the Last Meal at Autopsy

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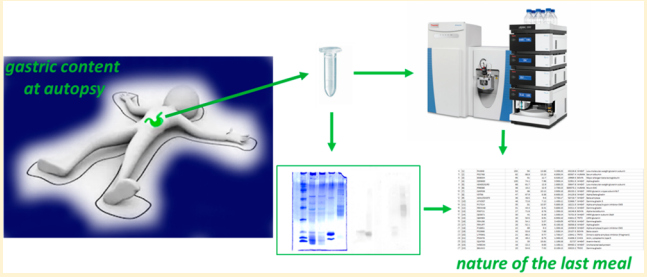
Supporting Information

ABSTRACT: A long-term psychiatric 40 years-old male patient was found dead at 9:00 a.m. in the clinic where he lived. Death was caused by traumatic injuries, which the sanitary staff imputed to a fall. Nurses declared that the patient refused having breakfast, whereas at autopsy the stomach contained 350 g of whitish semifluid material. Using both shotgun and gel-based proteomics, we demonstrated that the chyme contained partly digested milk- and bread-derived proteins, eaten during a recent breakfast. The conflict between evidence and assertions of the attending sanitary staff prompted the Legal Authority to undertake detailed investigations to ascertain facts and possible responsibilities. The herein characterization provides insights in the in vivo mechanisms of gastric breakdown of food proteins in a real meal. β-lactoglobulin was partially resistant to gastric digestion as confirmed by Western blot analysis, in contrast to caseins and wheat gluten proteins, which had been degraded by gastric fluids. In addition to a complex pattern of gastric proteins (e.g., mucin-5AC, pepsin A-3, pepsinogen C, gastric lipase, gastrokine-2, trefoil factors), chyme contained intact proteins and variably sized food-derived polypeptides arising from peptic and nonpeptic proteolytic cleavage as well as heterodimeric disulfide-cross-linked peptides. These findings suggest that the current analytical workflows offer only a partial picture of the real complexity of the human "digestome".



KEYWORDS: *proteomics, forensic sciences, last meal identification, protein gastric digestion, human digestome, cross-linked peptides*

INTRODUCTION

In forensic sciences, proteomics has been used for determining the nature of biological fluids[1] or to reveal organ-specific protein expression patterns.[2,3] In general, the advances of mass spectrometry (MS)-based techniques have overcome many of the drawbacks related to the analysis of scant amounts of samples coming from very complex proteomes[4] and are expected to enlarge the array of tools to solve legal caseworks in the next future.[5] However, despite its great potentiality, proteomics has been confined to a limited number of forensic applications so far and it has not found a way into a routine use yet.[6]

The visual or microscopic inspection of the gastric content of a deceased subject at autopsy is a valuable source of information, as it allows grossly estimating the time since death and time of last meal, based on the appearance of the digesting food.[7] Assessing the molecular nature of the meal before death can provide additional information concealed in the last meal of a deceased, which sometimes could be decisive to frame a crime scene. Food-borne proteins and derived peptides are elective signatures of the ingested foodstuff. However, the precise characterization of the proteins in the ingested meal might be challenging, because chyme contains both intact and variably hydrolyzed proteins, generally coming from a variety of raw and processed matrices, in addition to a number of endogenous gene products.

We applied electrophoresis- and gel-free-based proteomic strategies to characterize the human gastric chyme collected post-mortem at autopsy. In this particular case, we aimed at understanding the nature of the last meal to clarify the circumstances of death and to highlight eventual incongruences between the events and the description of facts reported by defendants. Briefly, a long-time psychiatric male patient aged 40 was living in a private clinic. The man was found dead in prone position on the floor of his room at 9:00 a.m. Causes of death were compatible with multiple traumatic injuries, which the sanitary staff imputed to an accidental fall from bed. Nurses declared the man refused to have breakfast and was fasting since the night before. He assumed the prescribed therapy (delorazepam 2 mg/die, promazine 100 mg/die, valproic acid 500 mg/die and risperidone 3 mg/die) regularly at 8:00 a.m., as

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confirmed by physicians' annotations on medical records. Based on such information, the stomach was expected to be empty,[8] whereas at autopsy it contained a semifluid curd-like whitish matter, whose nature could not be established since the digestion state. Consequently, elucidating the nature of the stomach content became of primary importance to clarify possible incongruences between events and declared circumstances of death, thereby disclosing eventual professional responsibilities.

To the best of our knowledge, this is the first time that proteomics has been applied to determine at a molecular level the composition of the last meal of a human subject deceased under unclear circumstances.

Notably, the herein investigation provides insights in the mechanisms of gastric breakdown of food proteins. In effect, chyme can be considered the result of in human gastric digestion of a real meal, which can be of interest to validate the outcomes of recently devised static or dynamic in vitro models of digestion with physiological relevance.[9]

MATERIALS AND METHODS

A long-time psychiatric male patient aged 40 was found dead in the clinic where he lived. Circumstances of death were unclear and the Legal Authority ordered an autopsy. Body fluids (peripheral blood, urine, bile), stomach content and organ fragments (liver and brain) were sampled during autopsy and stored at −20 °C until analyses. The stomach content (chyme), consisting of 350 gr semifluid whitish material, was sampled entirely. The current study was authorized by the local coroner as "useful for law purposes" in the context of a crime investigation and it did not require further approval by the ethical committees.

Ditiothreitol (DTT), iodoacetamide (IAA), guanidine, trifluoroacetic acid (TFA), ammonium bicarbonate (AMBIC), EDTA, and other chemicals were purchased from Sigma-Aldrich (St. Louis, MI). HPLC−MS grade solvents were from Carlo Erba (Milan, Italy). Wheat gluten proteins and whey protein isolate, used as positive controls in Western blot analysis, were from Sigma (code G5400) and from the Fonterra Dairy Co. via the Riddett Institute (New Zealand), respectively.

Toxicological Analyses

Blood sample was first analyzed using immunochemical screening tests from Randox Toxicology (Country Antrim, UK). The subject was found positive to tricyclic antidepressants (TCA), valproic acid (VA) and benzodiazepines (BDZ), which were identified and quantified by gas chromatography (GC−MS), using a Focus gas chromatograph connected to a DSQI single quadrupole mass spectrometer (both from Thermo Fisher, San José, CA). The GC separations were performed using an Rxi-5MS (30 m × 0.25 mm × 0.25 μm) capillary column (Restek, Bellefonte, PA). The analyses were processed using the Xcalibur software (2.0.7 version) from Thermo Fisher. In the case of TCA, 1 mL aliquot of blood sample was hydrolyzed in acid conditions and the digestion carried out at 120 °C for 20 min. Sample was purified by solid phase extraction (SPE); the eluate was dried under nitrogen stream, derivatized with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and analyzed by GC−MS. Results of analysis evidenced the presence of promazine. For BDZ identification, 1 mL blood sample was diluted with 3 mL phosphate buffer (100 mM, pH 6.0). Sample was vortexed and centrifuged, purified by SPE, dried under nitrogen stream, then redissolved in 50 μL ethyl acetate and analyzed in GC−MS. Results of the analysis evidenced the presence of delorazepam. A 1 mL aliquot of blood sample was also analyzed in liquid chromatography/tandem mass spectrometry (LC−MS/MS), using a TripleQuad LC−MS 6400 series from Agilent Technologies (Santa Clara, CA), according to previously described Methods.[10,11] LC−MS/MS analyses highlighted the presence of risperidone and valproic acid. All evidenced analytes were quantified through specific calibration curves. Toxicological analyses were positive with respect to drugs prescribed to the man and all of them were within therapeutic blood ranges (delorazepam, 55.42 ng/mL; promazine, 70.19 ng/μL; risperidone 9.17 ng/mL; valproic acid, 31.73 ng/μL). Results complied with the physicians' annotations on medical records. Therefore, no further toxicological analyses were carried out.

Purification of Proteins/Peptides

Chyme was defrosted on an ice bath and homogenized using an ultraturrax (IKA Co., Germany) for a short time (2 min) on ice to prevent undesired proteolysis. For proteomic analysis, the protein homogenate was prepared according to two different workflows, in order to include either intact proteins or possible protein fragments deriving from pepsinolysis in the identification process.

Workflow No. 1. An aliquot of the homogenized chyme (1 mL) was suspended in 10 mL of a denaturing/reducing buffer (6 M guanidine HCl, 0.3 M Tris, 1 mM EDTA, 10 mM DTT, pH 8.0) and incubated at 56 °C for 1h. After reduction, cysteines were alkylated with IAA (55 mM final concentration) for 40 min at room temperature in the dark. Reduced/alkylated proteins (1 mL) were desalted using size exclusion chromatography (SEC) Econo-pac 10 DG (Bio-Rad, Hercules, CA) prepacked columns, with 6 kDa exclusion limit. The eluting fractions with MW > 6 kDa and <6 kDa were separately collected.

Workflow No. 2. A different 1 mL aliquot containing reduced/alkylated proteins was 10-fold diluted with 25 mM ammonium bicarbonate (AMBIC) pH 7.8 containing DTT in stoichiometric amount (3.5 mg, considering that DTT has two thiol groups) to quench the IAA excess.

Proteins prepared according to the two different procedures were quantified using the Bradford colorimetric protein assay (kit from Bio-Rad) and proteolyzed overnight at 37 °C with modified proteomic grade trypsin (Sigma) at an 1/100 (w/w) enzyme-to-substrate ratio. After proteolysis, tryptic peptides from both the workflows as well as polypeptides <6 kDa obtained by SEC fractionation (workflow no. 1) were purified using prepacked C_{18} Sep-pak cartridges (Waters, Milford, MA), washing with 0.1% aqueous TFA and eluting with 70% acetonitrile (v/v) containing 0.1% TFA. Finally, peptides were concentrated in speed-vac, lyophilized, and redissolved in 0.1% formic acid (v/v) at the approximate concentration of 1 μg/μL for LC−MS/MS analysis.

Purification the Large-Sized and Cross-Linked Peptides

An aliquot (1.0 g) of the homogenized chyme was dissolved in the same denaturing buffer as above, without including DTT. The protein/peptide solution was purified with Econo-pac 10 DG columns, eluting with 25 mM AMBIC. The excluded protein fraction (MW > 6 kDa) was collected and filtered with Microcone (Millipore, Badford, MA) cutoff 10 kDa membranes, using a Biofuge Hareaus Centrifuge (3500g, 30 min, 4 °C). The permeate was freeze-dried, redissolved in 0.1% (v/v) formic acid, quantified with the micro-Lowry modified method

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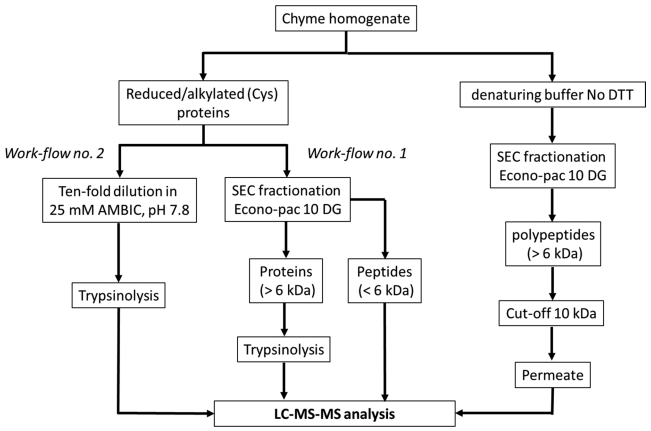
(kit from Sigma) and analyzed by LC−MS/MS. An aliquot of this sample was added with DTT up to 10 mM final concentration (37 °C, 30 min) and subjected to LC−MS/MS analysis as well. The entire workflow of sample preparation is summarized in Scheme 1.

Scheme 1. Schematic of the Workflows of Sample Preparation for Proteomic and Peptidomic Analysis of Chyme Sampled at Autopsy



SDS-PAGE and Western Immunoblotting

The Cys-reduced/alkylated chyme proteins (from both workflows) were separated by 12% SDS-PAGE precast gels (Bio-Rad, Milan, Italy) using a Miniprotean Tetracell device (Bio-Rad, Milan, Italy). In the attempt of detecting intact wheat or bovine milk proteins, especially gliadins or pepsin-resistant β-lactoglobulin (β-Lg), a 10 μg aliquot of the sample was run simultaneously to 8 μg of gluten proteins and 5 μg of bovine whey proteins dissolved in Laemmli buffer. For Western blotting analysis, two additional 10 μg aliquots of the chyme protein fraction (prepared with the workflow no. 1) were loaded onto different lanes of the gel, flanked by gluten and whey proteins (1 μg each), respectively, as the positive controls. After the run, the former part of the gel was stained with G-250 Coomassie Blue Silver, while the latter was electroblotted onto 0.2 μm nitrocellulose membranes using a Trans-Blot Cell (GE healthcare, Milan, Italy) at 400 mA for 1 h at 4 °C. The membrane was blocked for 1 h at room temperature with 5% (w/v) bovine serum albumin (Sigma) in Tris-buffered saline solution containing 0.05% Tween 20 (TBS-T). The membrane was cut and the part containing gluten as the positive control was incubated overnight at 4 °C with antigliadin IgG polyclonal antibody (Sigma) diluted 1/8 000 in TBS-T, while the part containing whey proteins as the positive control was incubated with immunoaffinity purified anti-β-Lg IgG polyclonal antibody developed in rabbit (Abcam Ltd., Cambridge, UK) diluted 1/10 000 in TBS-T. The membrane portions were extensively washed with TBS-T (3 × 10 min) and then incubated for 1 h at room temperature with monoclonal horseradish peroxidase-conjugated rabbit antirabbit IgG antibody (Abcam, Cambridge, UK) diluted in 1/10 000 TBS-T. Afterward, the membrane rinsed with TBS-T (3 × 10 min) and with TBS (1 × 10 min) and finally developed using the enhanced chemiluminescence ECL Prime substrate (GE Healthcare). Immunoreactive bands were visualized using X-ray film (Kodak, Chalons/Saône, France) at various exposure times ranging from 0.5 to 5 min in dark room.

In Gel Protein Digestion

SDS-PAGE protein bands were manually excised, destained through repeated wash in 25 mM AMBIC/acetonitrile (1/1, v/v) and finally in gel-digested with proteomic grade trypsin (~20 μL of a 12.5 ng/μL solution) overnight at 37 °C. Peptides were extracted in 5% formic acid/acetonitrile (1/1, v/v) and dried in speed-vac. Finally, peptide digests were redissolved in 50 μL of 0.1% (v/v) formic acid prior to LC−MS/MS analysis.

LC−MS/MS

LC−MS/MS analyses were performed using an Ultimate 3000 nanoflow ultrahigh performance liquid chromatography (Dionex/Thermo Scientific, San Jose, CA) coupled to a Q-Exactive Orbitrap mass spectrometer (Thermo Scientific). Peptides were loaded through a 5 mm long × 300 μm i.d. precolumn (LC Packings) using a Famos autosampler (Thermo) and separated using an EASY-Spray PepMap C18 column (2 μm, 15 cm × 75 μm) 3 μm particles, 100 Å pore size (Thermo Scientific). Eluent A was 0.1% formic acid (v/v) in LC−MS grade water; eluent B was 0.1% formic acid (v/v) in acetonitrile. For the shotgun proteomics and for the analysis of SEC < 6 kDa fractions, peptides were separated applying a 2−50% gradient of B over 150 min at a 300 nL/min constant flow rate. Nearly 2 μg of peptide mixture were used for the analysis. Peptides resulting from gel-digested proteins purified by SDS-PAGE were separated using the 2−45% B gradient over 35 min, loading 1/10 of the extracts. MS1 precursor spectra were acquired in the positive ionization mode scanning the 1600−300 m/z range, with resolving power of 70 000 full width at half-maximum (fwhm), automatic gain control (AGC) target of 1×10^6 ions and maximum ion injection time (IT) of 256 ms. The spectrometer operated in data-dependent acquisition, selecting up to 10 most intense ions for MS/MS fragmentation, applying a 8 s dynamic exclusion. Fragmentation spectra were obtained at a resolving power of 17 500 fwhm. Ions with one or more than six charges were excluded from MS/MS selection. Spectra were elaborated using the Xcalibur Software 3.1 version (Thermo Scientific).

Bioinformatics

Raw files or mgf files generated from the LC−MS runs were used to identifying the proteins using the Proteome Discoverer 2.1 software (Thermo Scientific) with SEQUEST search engine or the Protein Prospector Batch-Tag Web tool (http://prospector2.ucsf.edu), respectively. Outputs reported herein are those from the Protein Prospector Batch-Tag Web. Searches in the Uniprot (updated on November 2017) were no taxonomically restricted. Database searching parameters were the following: carbamidomethylcisteine as a static modification; Met oxidation, pyroglutamic acid at N-terminus Gln and Ser/Thr phosphorylation as variable modifications; mass tolerance value of 5 ppm for precursor ion and 10 ppm for MS/MS fragments; trypsin as the proteolytic enzyme; missed cleavage up to 2. The analysis of the <6 kDa SEC fraction was carried out in the same conditions, except selecting "no enzyme" as the cleavage specificity. An additional search relevant to the unfractionated protein extract (workflow no. 2) was performed excluding any proteolytic specificity (no enzyme function), in order to include in the matching possible proteolytic peptide fragments generated by pepsin or proteases with specificity other than trypsin. Searches arising from three technical replicates were merged together using the Protein Prospector Batch-Tag Web options.

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Attempts for identifying disulfide cross-linked peptides were carried out using the specific search engine tool. In this case, modification of cysteines was excluded. Protein identification scores were calculated by Target Decoy Peptide Spectrum Matches (PSMs) filtering, working at 0.01 peptide-level false discovery rate (FDR) that were matched to proteins identified at 1% protein-level FDR.

The comparison and intersection of protein groups for constructing the Venn diagram were performed with the Microsoft Excel 2016 software.

RESULTS AND DISCUSSION

Protein Identification

Along with typical gastric proteins, chyme is expected to contain intact food proteins as well as small-/medium-sized polypeptides produced by pepsinolysis. In order to identify the food-derived proteins in the chyme, previously Cys-reduced and alkylated proteins and their large proteolytic fragments were purified by SEC and digested with trypsin. Peptides were analyzed by LC−MS/MS (Scheme 1, workflow no. 1). Excluding the entries assigned by homology, 359 gene products were confidently identified (FDR 1%), among which 214 with at least two sequenced unique peptides (Supporting Information (SI) Table S1). Top-score protein entries were the expected endogenous proteins, such as mucin-5AC and human serum albumin (hSA). The expected pepsin A-3, pepsinogen C (gastricsin), gastric lipase, gastrokine-2 and trefoil factors (TFF1 and TFF2), these later being structural components of the gastric mucus contributing to maintain the integrity of the mucosa against injurious chemical and bacterial agents,[12] were detected as well.

The occurrence of all the main bovine milk-derived proteins, including the four casein families and whey proteins, along with a number of wheat protein components clearly confirmed that the deceased man had a meal compatible with a typical milk and bread-based Italian breakfast, shortly before the finding of his dead body. In principle, proteomics is able to provide detailed molecular information about the composition of last meal. However, there were no molecular elements to distinguish simple from toasted bread or rusks, in this instance. The occurrence of chicken tubulin β-3, identified through 7 matching peptides, was most likely the result of homology with the human counterpart rather than suggestive of the presence of biscuits or foodstuff containing hen eggs or chicken meat, because the main egg or chicken meat proteins were missing.

Interestingly, β-Lg was identified with 12 unique sequenced peptides, largely dominant over those from caseins, confirming its partial stability of β-Lg to gastric digestion.[13−15] In contrast, caseins were readily hydrolyzed into small peptides, which for the most eluted in the <6 kDa SEC fraction.[15]

It was not possible to infer the exact precise post-mortem interval through the digestion status, because digestion rate and gastric emptying depend on meal composition, viscosity, volume, kind and structure of the foodstuff,[7] in addition to a series of undefined individual factors. Circumstances of death (e.g., mood, eventual anxiety and/or pain, trauma etc.), sex, age as well as antacid drugs can also affect the rate of digestion. Furthermore, although digestion is mainly an active *ante-mortem* process, the gastric digestive fluids can continue their action after decease for an undetermined time.[16] Thus, the estimation of time since death currently should rely on the study of other biochemical parameters, such as electrolytes in vitreous humor,[17] cholesterol levels in peripheral nerve tissue[18−20] and degeneration processes of the soft tissues.[21,22]

The alternative workflow of sample preparation (Scheme 1, workflow no. 2) allowed including the record of proteolytic fragments in the LC−MS/MS. In this case, the whole peptide/protein gastric digests were Cys-reduced and alkylated, subjected to trypsin hydrolysis and analyzed without any prefractionation.

Excluding possible homologies, the number of proteins harvested with the workflow no. 2 (129, of which 78 with at least two sequenced peptides, SI Table S2) was lower if compared to the workflow no. 1, perhaps due to the lack of a protein enriching step. For instance, some minor milk proteins escaped identification (e.g., lactoferrin) or were identified through a lower number of matching peptides (e.g., lactadherin, 2 vs 14 sequenced peptides). On the other hand, many food-derived proteins were identified with increased sequence coverage, because of a higher number of matching peptides, compared to the workflow no. 1. For example, β-Lg and α-lactalbumin (α-La) were identified through 23 and 13 unique peptides, respectively, compared to 12 and 8 peptides of the SEC-based workflow no. 1. Notably, both high frequency genetic variants of β-Lg, namely β-Lg A and β-Lg B, were almost entirely sequenced, accounting for a recorded sequence coverage of 94.4% referred to the mature proteins. The β-Lg coverage (86.5%) appears lower in the SI Table S2 since it takes into account the signal peptides. This finding confirmed that food proteins, also including whey proteins, were extensively digested and a relatively abundant amount of polypeptide fragments eluted in the <6 kDa fraction when purified by SEC.

Considering the possible presence of peptides generated by pepsin hydrolysis or by cleavages other than the tryptic ones, the LC−MS/MS run of peptides obtained from the workflow no. 2 was repeated with "no enzyme" specificity. In this case, milk- and wheat-derived components, became largely dominant over the endogenous gene products (SI Table S3). Overall, 213 proteins were identified, among which 138 with at least two sequenced peptides. The number of sequenced peptides strikingly increased, including a plethora of proteolytic fragments generated by nonspecific hydrolysis. In fact, gliadin and glutenins produced hundreds of peptide fragments, while 95 and 27 peptides matched the β-Lg and α-La sequences, respectively. Fragments from both a low molecular weight glutenin subunit (Uniprot accession R4JB48) and β-Lg covered the entire sequence of the mature proteins. The coverage of all the casein families remarkably increased as well, although their phosphorylated regions (caseinphosphopeptides) escaped detection, probably owing to their intrinsic low detectability further hindered by ion suppression effects in very complex mixtures.

Taking into account the detection of small-sized peptides, the coverage of proteins described as digestion stable, such as wheat serpins, α-amylase/trypsin inhibitors and nonspecific lipid transfer proteins, increased, suggesting that they were at least partly degraded by gastric digestion. On the other hand, the partial stability to gastric digestion of these proteins or derived large domains could underlie their food allergenic potential.[23]

Surprisingly, several milk- and wheat-derived peptides had varying C-terminal amino acids, including unexpected D, G, N, Q, A, and S residues among the others (SI Table S4). In other terms, the sequence of many peptides exhibited cleavage

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specificity other than those of trypsin and pepsin, suggesting that additional proteases and/or peptidases could contribute to gastric degradation.

The Venn diagram of the intersections among the protein groups identified according to the three different proteomic procedures, that is, workflow no. 1, workflow no. 2 tryptic digests, workflow no. 2 nonspecific cleavage, is shown in Figure 1. A limited number of proteins (53) was shared by the three procedures, demonstrating that the outcomes of a "digestomic" characterization could be strongly dependent on the analytical and postanalysis strategy adopted. This finding was in part expected, since the targets of the analytical processes were different. Therefore, in general, the workflows for establishing the nature of the last meal should be case-by-case driven by the experimental end point.

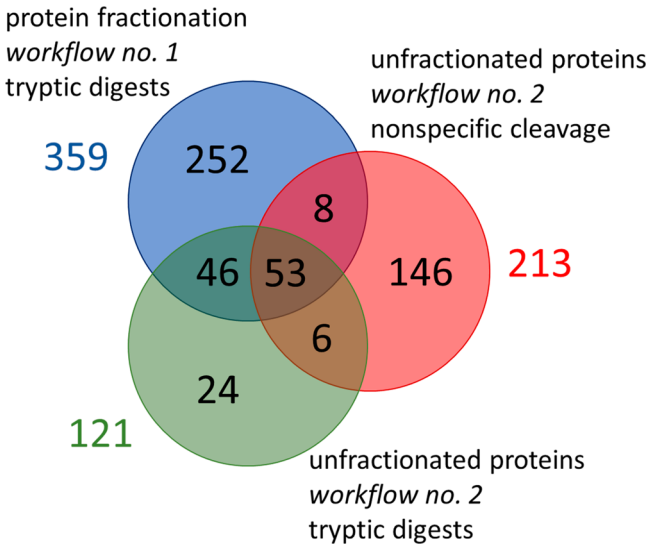

Figure 1. Venn diagram reporting the intersection among protein entries identified with three different analytical and postanalytical proteomic strategies. The number of total protein entries include those identified with a single peptide at 1% FDR.

Peptide Identification

Over 250 different peptides, belonging to 30 different gene products, were sequenced by LC−MS/MS of the <6 kDa fraction of SEC (SI Table S5). The identification confirmed that food-derived proteins were for the most wheat storage components, including the main subfamilies of gliadins and glutenins, and bovine milk proteins. β-casein-derived fragments were dominant over those from β-Lg, supporting the much higher susceptibility of caseins to gastric digestion if compared to β-Lg. Consistently with the above findings, many peptides did not arise from canonical pepsin cleavages. Notably, gastric digests contained α-gliadin 33-mer homologue peptides (e.g., the peptide with $[M + H]^+$=3747.89, SI Tables S4 and S5) exhibiting punctual substitutions compared to the canonical 33-mer, since it derived from α-gliadin genetic variants.[24]

The inventory of identified peptides and the coverage at the protein level, were further increased when the <6 kDa peptide fraction was subjected to tryptic digestion prior to LC−MS/MS analysis (data not shown).

It has to be underlined that a remarkable fraction of MS/MS spectra remained unassigned, as a consequence of several concomitant factors, such as incompleteness of annotation (e.g., database of wheat proteins), unsatisfactory quality of the

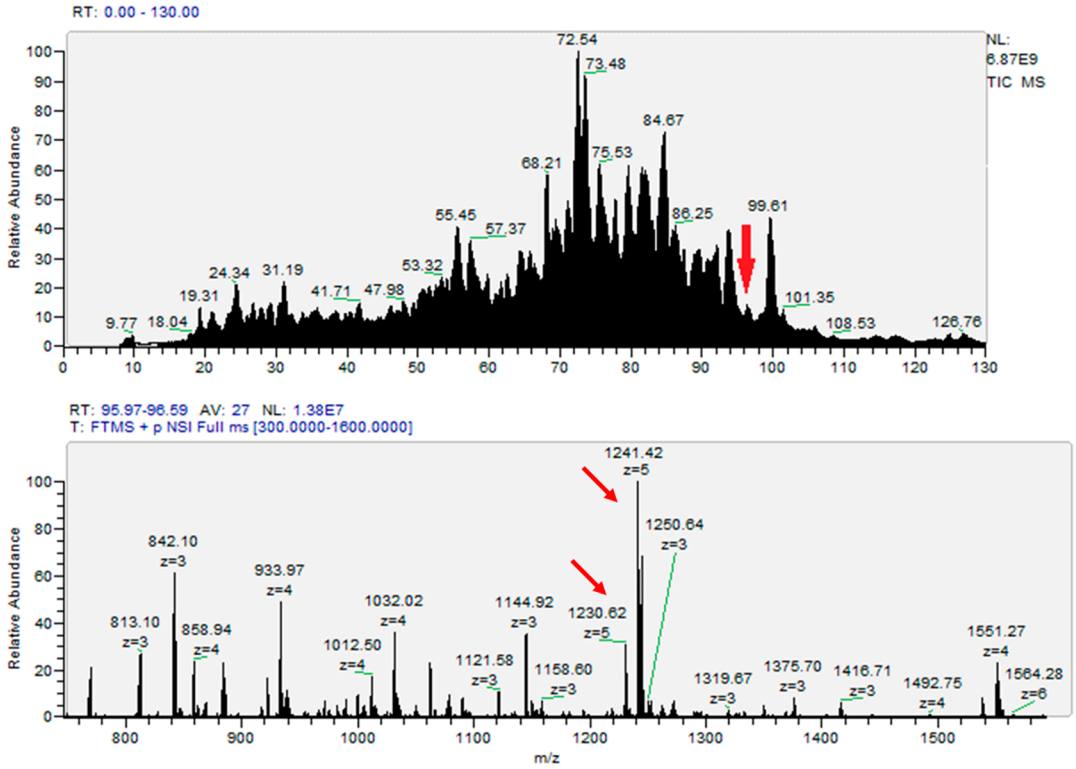

Figure 2. LC−MS/MS analysis of unreduced polypeptides (>6 kDa) purified by SEC and filtered with a 10 kDa cutoff: TIC chromatogram (upper panel) and exemplificative spectrum at retention time 95.97−96.58 min (lower panel), including signals of S−S cross-linked peptide f(41−69)−f(139−162) of β-Lg A and B genetic variants ($[M+5H]^{5+}$ signals indicated by arrows).

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fragmentation spectra, dimension of the peptide fragments exceeding 4.0−4.5 kDa.

One of the factors limiting the comprehensive and accurate characterization of a peptide digestome is the probable release of S−S cross-linked peptides, which are difficult to be identified using the ordinary pipeline of peptidomic exploration. A few of these components have been previously identified using MALDI-TOF MS analysis.[15] The release of cross-linked peptides that might remain undetected has important implications when proteins have to be classified according to their digestibility, especially in the perspective of the allergenicity risk assessment.[23,25] To increase the peptide coverage we reduced and alkylated cysteines prior to LC−MS/MS analysis. However, the information about expected disulfide cross-linked peptides can be preserved. To this purpose, the unreduced protein extract from chyme was fractionated by SEC and the >6 kDa components were further subjected to filtration with cutoff 10 kDa membranes. The LC−MS/MS analysis of the permeate disclosed the presence of a high number of large polypeptides with complex fragmentation patterns compatible with S−S cross-linked heterodimers or even S−S concatenated heteropolymers (e.g., fragments from α-La). The TIC chromatogram (Figure 2, upper panel) and the exemplificative extracted spectrum at retention time 95.97−96.58 min (Figure 2, lower panel) exhibited a very complex pattern of signals with MW ranging between 2.4 and 6.4 kDa. The signal at m/z 1230.62 relevant to a peptide with five positive charges (monoisotopic MH^+ = 6145.11) matched the S−S cross-linked peptide f(41−69)−f(139−162) of β-Lg variant B (theoretical MH^+ = 6145.12). A signal peptide at 1242.63 was detected at a partly overlapping retention time and most likely was the S−S cross-linked peptide f(41−69)−f(139−162) of the β-Lg variant A (monoisotopic MH^+ = 6203.12, theoretical MH^+ = 6203.13), further substantiating the assignment. Interestingly, these unexpected tryptic-like peptides were much less intense than many others, probably generated by pepsin or nonspecific hydrolysis, which remained unassigned. The analysis of the same peptide fraction after reduction of disulfide bonds demonstrated the disappearance of the supposed cross-linked peptides and the release of the monomeric fragments of β-Lg (not shown).

Data herein are only preliminary and the precise identification of the cross-linked peptides in gastric or gastrointestinal digests would require dedicate investigations. The attempts to characterize these peptides using a number of common bioinformatics tools were unsuccessful, probably also due to the overall size of the peptides and to an insufficient MS/MS fragmentation pattern. However, a great multitude of signals occurred all along the TIC chromatogram of Figure 2, supporting the evidence that the protein digests from real food matrices could be much more complex than actually believed and the current ordinary workflows of "digestome" analysis might be far from comprehensive.[26]

SDS-PAGE and Immunoblotting

Investigating the in vivo susceptibility to hydrolysis of milk proteins and gliadins was particularly interesting, in consideration of their role as food allergens (both milk proteins and gliadins) or celiacogenic proteins (gliadins). The SDS-PAGE of chyme proteins and parallel Western blot analysis are shown in Figure 3. Bovine whey proteins and gluten proteins were used as the positive controls for detecting β-Lg and gliadins, respectively. The chyme protein bands resulting from SDS-PAGE separation were identified by LC−MS/MS of the in-gel tryptic digests (Table 1). The electrophoretic pattern (Figure 3, left panel) was dominated by hSA, both as the intact protein and derived lower molecular weight fragments. Intact hSA, occurring at significant amount in the gastric chyme, migrated at apparent molecular weight slightly higher than those expected, due to the previous carbamidomethylation of cysteins (overall 35 Cys residues). Simultaneous occurrence of intact hSA and its proteolytic fragments could be distinctive of the gastric material, suggesting possible forensic applications, for instance to distinguish vomit from other body fluids. Mucin-5AC fragments were also relatively abundant, whereas the corresponding full-length polypeptide probably was the high molecular weight band that did not enter the SDS gel. Among the endogenous proteins detected by SDS-PAGE, chyme contained several gene products already described in the gastric environment, contributing to the formation of mucus or deriving from sloughing off of epithelial cells. Surprisingly, pepsin/pepsinogen was not detected among the main bands of the SDS-PAGE. Interestingly, β-Lg was detected as an intact protein in SDS-PAGE, as confirmed by Western blot (Figure 3, middle panel), thus supporting its partial stability to human gastric juices which has been amply demonstrated in vitro,[13−15] ex vivo[27] and, recently, in vivo in piglets.[28] Thus, β-Lg is a candidate protein marker to assess the consumption of milk, which can be targeted with protein-oriented analytical methods, such as electrophoresis and Western blotting. Differently, α-La was for the most digested by pepsin and only faint amounts were detected by LC−MS/MS of the SDS-PAGE band no. 7. Similar to hSA, β-Lg migrated at molecular weight slightly higher than the corresponding standard due to the Cys-alkylation (overall 5 Cys residues). Intact caseins were missing in the gastric chyme, confirming that they are readily digested by pepsin. Gliadins were completely degraded by pepsin into smaller peptides too, thus confirming the previous findings.[29] Gliadins only produced two faint antigliadin immunoreactive bands at ca. 25 and 21 kDa (Figure 3, right panel). For this

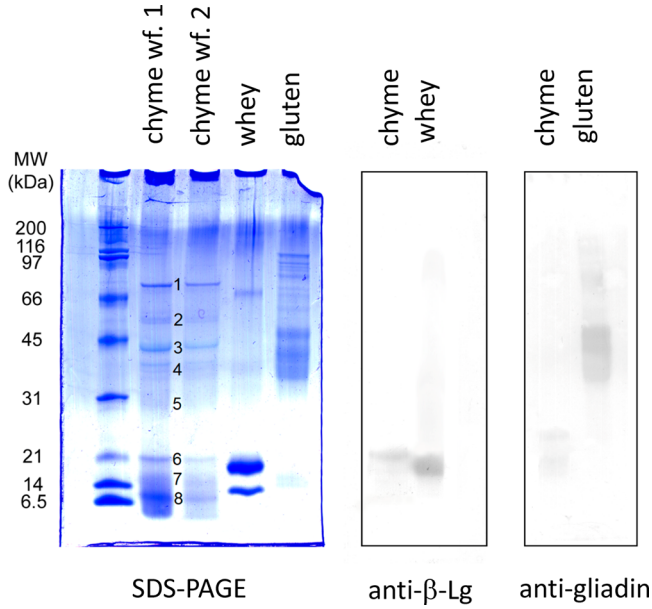

Figure 3. SDS-PAGE electrophoresis (left panel), anti-β-Lg (middle panel) and antigliadin (right panel) Western blot analyses of the chyme. For the Western blot analyses, the chyme prepared with the workflow no. 1 (wf. 1) was used.

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Table 1. LC−MS/MS-Based Identification of the in Gel-Trypsinized SDS-PAGE Isolated Protein Bands[a]

band	identification	species	uniprot accession	unique peptides	coverage (%)
1	serum albumin	human	P02768	22	31.4
	mucin-5AC	human	P98088	8	1.5
2	serum albumin	human	P02768	8	12.8
	α-1-antitrypsin	human	P01009	7	11.2
	mucin-5AC	human	P98088	6	1.4
	hornerin	human	Q86YZ3	5	2.8
	semenogelin-2	human	Q02383	4	10.3
	gastric tryacilglycerol lipase	human	P07098	4	11.7
3	filaggrin-2	human	Q5D862	7	5.1
	filaggrin	human	P20930	6	1.2
	junction plakogloblin	human	P14923	5	6.2
	desmoplakin	human	P15925	11	3.4
	desmoglein-1	human	Q02413	4	4.8
	suprabasin	human	Q6UWP8	6	15.6
4	serum albumin	human	P02768	19	29.7
	mucin-5AC	human	P98088	4	0.9
5	serum albumin	human	P02768	17	25.5
	fibrillin-1	human	P35555	4	1.1
6	β-lactoglobulin	bovine	P02754	9	34.3
	gastrokine-2	human	Q86XP6	5	21.2
	β-amylase	barley	P16098	4	6.9
7	fatty acid-binding protein, epidermal	human	Q01469	4	25.9
	α-lactalbumin	bovine	P00711	3	18.1
	α-amylase/trypsin inhibitor CM16	wheat	P16159	4	28.0
8	serum albumin	human	P02768	9	13.1
	β-lactoglobulin	bovine	P02754	6	26.4
	hornerin	human	Q86YZ3	5	2.8
	serotransferrin	human	P02787	4	6.3
	transgelin	human	Q01995	5	21.4
	serpin Z1B	wheat	P93693	3	5.9

[a]Only proteins or polypeptide fragments identified with at least three unique peptides are reported.

reason, peptide-oriented techniques, such as LC−MS/MS or competitive ELISA, could better fit the detection of caseins or gluten proteins in the chyme, especially when digestion might have progressed.

The assessment of other kinds of protein foodstuff should be evaluated case-by-case. Indeed, the pepsin stability/susceptibility of most food proteins, which has been already established in vitro, should be confirmed in vivo, eventually in processed foodstuff and in the presence of other components constituting a real meal.[30] To this purpose, milk contains a multitude of minor proteins, whose individual behavior in the stomach is only partly known.[31] Therefore, it could be evaluated in order to individuate novel milk-specific protein probes. Similarly, some specific wheat-derived protein domains (*e. g.* α2-gliadin 33-mer peptide and homologues) are known to survive even very prolonged exposure to gastrointestinal proteases[32] and could serve as peptide markers of wheat products. Lipid transfer proteins and α-amylase/trypsin inhibitors are other possible candidate protein targets of wheat, because at least in part they survive gastric degradation.

CONCLUSION

The characterization at the molecular level of the gastric content at autopsy confirmed that the patient had a meat prior to death, contrarily to what was declared by the attending sanitary staff. This meal consisted of milk and (toasted) bread, compatible with a typical Italian breakfast. Based on these findings, the Legal Authority undertook further investigations in order to ascertain facts and to disclose possible responsibilities.

The current study offers a proof of principle that MS-based proteomics could be a potent tool enabling the access to a higher informative level if compared to the simple visual or microscopic inspection of the gastric content sampled at autopsy. In this sense, the contribution of proteomics will allegedly increase supporting crime investigations and forensic caseworks in the next future. Conversely, proteomics of the gastric content is unsuited to establish the precise time since death.

The characterization of the chyme was the occasion to provide a shapshot of the human digestome arising from in vivo gastric degradation of food proteins in a real meal. Data about gastric stability of food proteins substantially mirror those obtained in vitro and ex vivo simulating the kinetics of human gastric digestion for isolated food systems. On the other hand, the outcomes of this work support the concept the real digestomes could be much more complex than what estimated with the ordinary peptidomic workflows, since they also include large-/medium-sized polypeptides and S−S-cross-linked peptide oligomers, which generally escape the simple electrophoresis or LC−MS/MS-based analyses. These aspects could have relevant implications related to the exploitation of digestive models to assess the food allergenicity risk. Further insights about the in vivo degradation of foodstuff could be obtained with dedicate characterizations of the digestive material sampled from the small intestine, which could be source of information when the post-mortem interval is significantly longer than in the current case.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.8b00159.

Table S1. Protein entries identified by LC−MS/MS in the SEC-purified chyme extract (>6 kDa), according to the workflow no. 1 (XLSX)

Table S2. Protein entries identified in the chyme extract by LC−MS/MS according to the workflow no. 2, searching tryptic peptides (XLSX)

Table S3. Protein entries identified in the chyme extract by LC−MS/MS according to the workflow no. 2, searching peptides with nonspecific cleavage (XLSX)

Table S4. List of PSM peptides identified in the chyme extract by LC−MS/MS according to the workflow no. 2, searching peptides with nonspecific cleavage (XLSX)

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Table S5. List of peptides purified by SEC (fraction <6 kDa) identified by LC−MS/MS in the chyme extract (XLSX)

The mass spectrometry data sets are available and can be exchanged by the authors upon request (PDF)

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

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