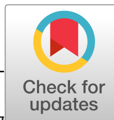

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Proteomic identification of beer brewing products in the ground layer of Danish Golden Age paintings

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The application of mass spectrometry–based proteomics to artworks provides accurate and detailed characterization of protein-based materials used in their production. This is highly valuable to plan conservation strategies and reconstruct the artwork's history. In this work, the proteomic analysis of canvas paintings from the Danish Golden Age led to the confident identification of cereal and yeast proteins in the ground layer. This proteomic profile points to a (by-)product of beer brewing, in agreement with local artists' manuals. The use of this unconventional binder can be connected to the workshops within the Royal Danish Academy of Fine Arts. The mass spectrometric dataset generated from proteomics was also processed with a metabolomics workflow. The spectral matches observed supported the proteomic conclusions, and, in at least one sample, suggested the use of drying oils. These results highlight the value of untargeted proteomics in heritage science, correlating unconventional artistic materials with local culture and practices.

INTRODUCTION

For decades, the application of analytical techniques to cultural heritage objects has been instrumental to guide informed restoration and conservation strategies (*1*). Less intuitively, but maybe even more valuably, this approach enables the treatment of artworks as "historical molecular records" of the culture and the society from which they originate. The materials used for art production directly reflect the accessibility, trade, and customs of the time, as much as the artist's expression and the artistic period.

Although scientific analyses of proteins in artistic objects have been performed for a long time (*2*), mass spectrometry (MS)–based proteomics has been used to investigate historical artistic objects only over the past two decades (*3*). Compared to other analytical techniques, proteomics allows for a detailed and exhaustive characterization of the protein residues in a sample, confidently identifying the type of proteinaceous material present, its taxonomical origin, as well as the extent and type of damage they have accumulated. Because of its high sensitivity, it also requires the same or less amount of starting material needed by other, less informative, approaches routinely used to analyze biomolecules from artistic objects (*4*). All these features make proteomics particularly valuable for the study of cultural heritage materials.

Another highly relevant feature of proteomic analysis is its untargeted nature, which makes it possible to confidently characterize virtually all the proteins contained in a sample, even in complex mixtures, and potentially originating from unconventional sources. On the contrary, other techniques more routinely used for protein analysis in conservation science, such as amino acid analysis by gas chromatography–MS (GC-MS), can only aim at the identification of proteinaceous materials in a limited group of predefined sources. In the investigation of artistic objects, the three most common protein-based materials, egg, animal glue, and milk, are ordinarily used as the sole standards for these analyses. The presence of proteins originating from sources outside this group of arbitrarily preselected standard source materials will not be detected, potentially leading to false-positive results and inaccurate interpretations (*5*).

In this study, protein sequencing by tandem MS (MS/MS) has been used to characterize the protein residues in the ground layers of 10 paintings from the Danish Golden Age, a period of exceptional artistic production in Denmark during the first half of the 19th century CE. In this period, Danish arts flourished despite critical social, political, and economic conditions in the country. The investigated paintings, selected from the collections of the National Gallery of Denmark and the Royal Danish Academy of Fine Arts, were produced by two of the most famous Danish painters and protagonists of the Danish Golden Age: Christoffer Wilhelm Eckersberg, "the father of Danish painting" (*6*), and his student Christen Schiellerup Købke. Eckersberg was a professor at the Royal Danish Academy of Fine Arts for 35 years, a position that made him an important influence on the young Danish artists of that time. Købke was one of these artists, studying at the Academy until 1832. The 10 paintings studied here were carefully selected among those produced by the two artists in the 1820s and 1830s (Table 1 and Fig. 1) to investigate works produced during their time at the Academy and afterward. Since the Academy is known to have provided artistic materials for professors and students, this sample set allowed for a direct comparison of canvases that were most

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Table 1. Description of the analyzed paintings. CWE, C. W. Eckersberg; CSK, C. S. Købke; SMK, Statens Museum for Kunst–The National Gallery of Denmark (Copenhagen, Denmark); Academy, Det Kongelige Akademi for de Skønne Kunster, Akademiraadet–The Royal Danish Academy of Fine Arts (Copenhagen, Denmark).

Artist	Painting short name	Title	Year	Location	Painting code
CWE	A	En russisk flåde til ankers på Helsingørs red—*A Russian Fleet at Anchor near Elsinore*	1826	SMK	KMS1671
	B	To russiske linjeskibe som saluterer—*Two Russian Ships of the Line Saluting*	1827	SMK	KMS1663
	C	En korvet på stabelen. Nyholm med spanteloftbygningen i baggrunden—*A Corvette on the Stocks. Copenhagen*	1828	SMK	KMS6439
	D	Et dansk orlogsskib "Dronning Marie" på 84 kanoner under sejl i Sundet—*The 84-Gun Danish Warship "Dronning Marie" in the Sound*	1834	SMK	KMS255
CSK	E	Udsigt fra loftet på Kornmagasinet ved bageriet i Kastellet—*View from the Loft of the Grain Store at the Bakery in the Citadel of Copenhagen*	1831	SMK	KMS1662
	F	Inger Margrethe Høyen, født Schrøder. Kunsthistorikeren N.L. Høyens moder—*Portrait of Inger Margrethe Høyen, née Schrøder, the Mother of the Art Historian N.L. Høyen*	1832	SMK	KMS1667
	G	Modelfigur. Siddende dreng—*Nude Figure. Sitting Boy*	1833	SMK	KMS6177
	H	Susanne Cecilie Købke, født Købke, kunstnerens hustru—*Portrait of Susanne Cecilie Købke, née Købke, the Artist's Wife*	1836	SMK	KMS3793
	I	Dekorationsmaler C.G. Hilker—*Portrait of the Decorative Artist G.C. Hilker*	1837	SMK	KMS1081
	J	Portræt af billedhuggeren H. E. Freund—*Portrait of the sculptor H.E. Freund*	1838	Academy	KS114

likely prepared by the Academy's craftsmen and those prepared outside the institution. The latter were three paintings produced by Købke in the late 1830s, after he finished his studies (Table 1).

In the 1960s, many of the paintings from the Danish Golden Age located at the National Gallery of Denmark, including those investigated here, were subjected to conservation treatments, such as wax-resin lining, as a preventative measure before being transferred temporarily to other national and international museums (*7*). The lining of paintings is a treatment in which a new canvas is attached onto the back of the original one, in this case, using a mixture of beeswax and dammar resin. As part of this treatment, the edges of the original canvas were formerly often trimmed before applying the lining adhesive and attaching the new canvas. Portions of the trimmings from each painting have been preserved at the National Gallery of Denmark, allowing the sampling of the investigated artworks with no further impact on the original objects.

The samples were prepared using a proteomic workflow previously used for the characterization of protein residues from archaeological and artistic materials (*8*, *9*). Although this workflow is optimized for the extraction and analysis of peptides and proteins, it is likely that other compounds were coextracted from the samples and measured by the mass spectrometer. Therefore, in the attempt to maximize the recovery of information from the destructive analysis of unreplaceable cultural heritage material, a metabolomics data analysis approach was attempted to investigate the presence of small organic molecules using the measurements acquired with the proteomic workflow. While this approach only provided tentative identifications at this stage, it nevertheless showed a potential to further enrich the information gained by destructive analysis of unique and precious samples through MS.

RESULTS

Protein identification

Various collagen sequences were confidently identified in 8 of the 10 analyzed paintings (table S2). Collagens are structural proteins, abundant in many connective tissues such as the bone, skin, cartilage, and tendons (*10*). Their identification in paintings is associated with the use of animal glue, a material produced by boiling animal tissues, usually leftovers from the skin, leather or parchment production, for a prolonged period of time and often used as an organic binder in the ground layers of paintings and the sizing of painting canvases (*11*). Type I collagens were identified in the samples from eight paintings (A to F, I, and J). These proteins do not allow for tissue discrimination as they are present in most connective tissues. In the samples from five paintings (A, B, F, I, and J), bovine-specific collagen type I alpha-2 (COL1A2) peptides, most probably from cow (*Bos taurus*), were identified. In two of these five samples (B and F), COL1A2 from either sheep (*Ovis aries*) or goat (*Capra hircus*) was also identified. In all the samples in which bovine-specific COL1A2 peptides were identified, nonspecies-specific peptides with sequences matching to bovine and/or ovicaprid collagen were also found and assigned to either of these taxa, following the parsimony principle. In the sample from painting J, bovine type III collagen was also identified. Although collagen type III is present in several connective tissues such as the skin, lungs, liver, intestine, and the vascular system (*12*), the skin is the most likely one to have been used for the production of animal glue to be used in paintings, as no historic recipes for animal glue were found to mention other collagen type III–containing organs. The connection of collagen type III and hide glue in artworks has previously been shown in literature (*13*, *14*). This identification, thus, specifically suggests the use of hide glue in painting J. No collagen traces were observed in the sample from paintings G and H. The protein identifications based on peptide-spectrum match (PSM) against the database of conventional proteinaceous paint binders

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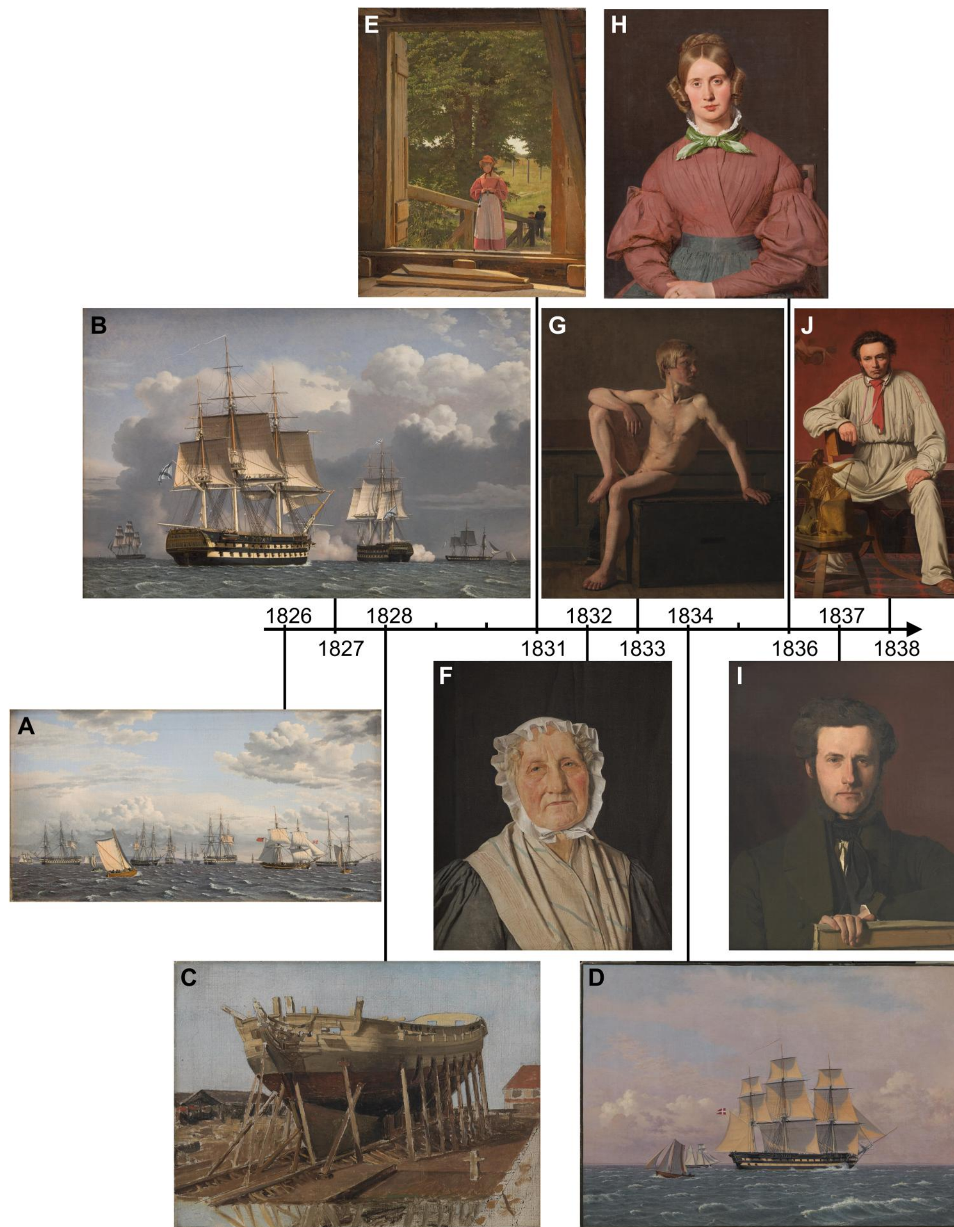

Fig. 1. Analyzed paintings. The 10 artworks by C. W. Eckersberg and C. S. Købke analyzed in this study. The name and the collection number of each artwork (**A** to **J**) are reported in Table 1. For KS114 (J): Copyright Royal Danish Academy–Conservation, M. Scharff.

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are summarized in table S2, whereas the corresponding diagnostic peptides are reported in table S3.

A second MaxQuant search, against the SwissProt database, showed the presence of proteins from cereal species and baker's yeast (*Saccharomyces cerevisiae*) in 7 of the 10 paintings (Table 2, A to G). A database containing all publicly available sequences from several cereal and yeast species (see Materials and Methods) was therefore prepared to perform a third search. As a result, more proteins from baker's yeast and from at least one cereal species were more confidently identified in each of these seven samples. Wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) were the only cereal species identified in C and B, respectively. In the remaining five samples combinations of wheat, barley, buckwheat (*Fagopyrum esculentum*) and rye (*Secale cereale*) were identified (Table 2).

Most of the cereal proteins identified were seed storage proteins, such as hordeins from barley and globulins from wheat (table S4) (*15*). These proteins are expressed only in the plant seeds, indicating that the material used in the ground layers originated from cereal grains. At the same time, many of the yeast enzymes identified, including glyceraldehyde-3-phosphate dehydrogenase, enolase, and pyruvate kinase, have key roles in glycolysis, the metabolic pathway that converts glucose into pyruvic acid. One of the enzymes identified, alcohol dehydrogenase, is part of the alcoholic fermentation pathway. Although this enzyme was only identified in the sample from painting G, no enzymes connected to the Krebs

Table 2. Species confidently identified in each of the analyzed paintings.

Painting	Artist	Species	Total identified proteins	Total identified peptides	Total identified MS/MS spectra
A	CWE	Yeast (*S. cerevisiae*)	13	109	454
		Barley (*H. vulgare*)	3	8	19
		Wheat (*T. aestivum*)	1	2	2
		Cow (*B. taurus*)	2	6	6
B	CWE	Yeast (*S. cerevisiae*)	21	134	454
		Barley (*H. vulgare*)	7	23	46
		Cow (*B. taurus*)	2	18	26
		Sheep/goat (*O. aries/C. hircus*)	1	2	9
C	CWE	Yeast (*S. cerevisiae*)	1	10	20
		Wheat (*T. aestivum*)	6	18	47
		Mammalia	2	12	23
D	CSK	Yeast (*S. cerevisiae*)	17	89	328
		Barley (*H. vulgare*)	3	8	12
		Buckwheat (*F. esculentum*)	2	7	11
		Mammalia	1	2	2
E	CSK	Yeast (*S. cerevisiae*)	11	238	670
		Wheat (*T. aestivum*)	37	201	628
		Barley (*H. vulgare*)	6	20	56
		Buckwheat (*F. esculentum*)	3	16	43
		Mammalia	2	13	17
F	CSK	Yeast (*S. cerevisiae*)	4	47	128
		Buckwheat (*F. esculentum*)	10	77	464
		Barley (*H. vulgare*)	6	16	31
		Wheat (*T. aestivum*)	3	8	15
		Rye (*S. cereale*)	1	2	9
		Cow (*B. taurus*)	2	30	51
		Sheep/goat (*O. aries/C. hircus*)	1	2	16
G	CWE	Yeast (*S. cerevisiae*)	7	74	220
		Buckwheat (*F. esculentum*)	4	23	70
		Barley (*H. vulgare*)	1	2	6
H	CSK	—	—	—	—
I	CSK	Cow (*B. taurus*)	2	7	11
J	CSK	Cow (*B. taurus*)	3	29	67

cycle, the respiratory metabolic pathway alternative to fermentation for energy production, were identified in any of the samples. It is known that yeast will perform alcoholic fermentation rather than the Krebs cycle also under aerobic conditions (*16*). Therefore, this association of enzymes and substrates indicates that the material in the ground layer was subjected to fermentation, during which the baker′s yeast converted the starch most probably associated with the cereal seed storage proteins into ethanol. Such a process may have occurred either before or after the application of the ground layer on the canvas. If the fermentation had occurred after the application of the ground layer to the canvas, then the resulting damage would have been observed, particularly if this had been ongoing after completion of the painting. The production of gaseous carbon dioxide associated with this metabolic pathway would have probably caused the formation of clearly identifiable pinholes within the stratigraphy of the painting. This alteration was not observed for any of the specimens.

The presence of proteins from baker′s yeast could also be due to environmental contamination. However, laboratory contamination was excluded via the comparison of the proteomic profile of the canvas samples with the protocol blanks that were processed alongside. In addition, although protein quantification was not performed, the identification of traces of yeast proteins due to environmental contamination is highly unlikely based on the elevated number of proteins and peptides identified for both cereal species and yeast, supported by a high number of matched MS/

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MS spectra. Last, the identification of cereal and yeast proteins, and in particular of different cereal species, in only a subset of the analyzed samples rules out the contamination of the canvas trimming during storage. The trimmings from paintings A to I were stored in the same space since removal from the artworks, and, therefore, it would be expected that the same storage contamination would be present in all samples. Table S4 reports the details about the identified cereal and yeast proteins, and the corresponding diagnostic peptides are reported in table S5. For yeast, only proteins with species-specific peptides for *S. cerevisiae* are reported, as proteins identified on the basis of nonspecies-specific amino acid sequences cannot be distinguished from contaminating environmental microorganisms. In the sample from painting H, none of the PSM-based searches led to protein identifications, suggesting that no protein was present in a detectable amount.

Protein damage: Deamidation

Deamidation of asparagine and glutamine is one of the most common degradation reactions of proteins and can therefore inform about the preservation state of proteinaceous materials in cultural heritage objects. The level of deamidation of the identified proteins was calculated separately for the two main protein sources identified: animal glue and the cereal- and yeast-containing material. However, as the low (<20) number of collagen peptides identified in each sample is not sufficient to guarantee a reliable calculation of deamidation for animal glue (*17*), deamidation was calculated for noncollagenous proteins only (Fig. 2).

In all specimens but E, the deamidation level of glutamine (Gln or Q) is higher than that of asparagine (Asn or N). The vice versa would be expected on the basis of the kinetics of the deamidation reaction, as deamidation can occur up to 10 times faster on Asn than on Gln (*18*). This unusual result has been previously observed in samples from artworks and paint mock-ups (*4*), but inconsistently throughout the sample sets. The expected proportions, with Asn more deamidated than Gln, have been observed on samples from a wall painting (*9*). The occurrence of this result can therefore not be explained on the basis of these data and should be the object of further research. Furthermore, although some variation in the deamidation levels of Asn and Gln can be observed throughout the sample set analyzed here, no pattern can be found in correlation to the production date of the paintings, the artist, or the identification of different cereal species. To investigate the influence of protein composition, aging, and preparation processes on the level of damage, the obtained results should be compared with a standard of similar composition. However, because of the variable taxonomic composition of the samples and due to the challenge of exactly pinpointing the material used in the ground layers, a comparison with a standard was not performed in this study.

The deamidation level has also been calculated separately for the cereal and yeast proteins (fig. S2). For the samples in which the number of peptides is sufficient to ensure confidence in the calculation, the damage levels of the two protein sources are comparable. The average deamidation level of asparagine is 12% for yeast and 9% for cereal proteins, while for glutamine, the average deamidation values are 21 and 18% for yeast and cereal proteins, respectively.

Metabolomics library and in silico spectra matching

To identify potential small organic molecules coextracted and analyzed together with the peptide mixtures by liquid chromatography (LC)–MS/MS, a metabolomics data analysis approach for the putative identification of small molecules using library matching through the Global Natural Products Molecular Networking Platform (GNPS) (*19*) and in silico spectral matching through DEREPLICATOR (*20*–*22*) was attempted. A total of four mass spectral features from the molecular networking approach could be matched to known molecules within the GNPS spectral libraries with a spectral similarity (cosine) score above 0.8: α-cyclodextrin (cluster ID 39836; parent ion mass, 1945.65 Da), cyclic adenosine diphosphate ribose (cluster ID 15324; parent ion mass, 1083.14 Da), maltopentaose (cluster ID 33898; parent ion mass, 1657.56 Da), and maltotetraose (cluster ID 23259; parent ion mass, 1297.43 Da) (fig. S3). α-Cyclodextrin, maltopentaose, and maltotetraose were found within the same molecular family, i.e., were connected components in the obtained network, containing several

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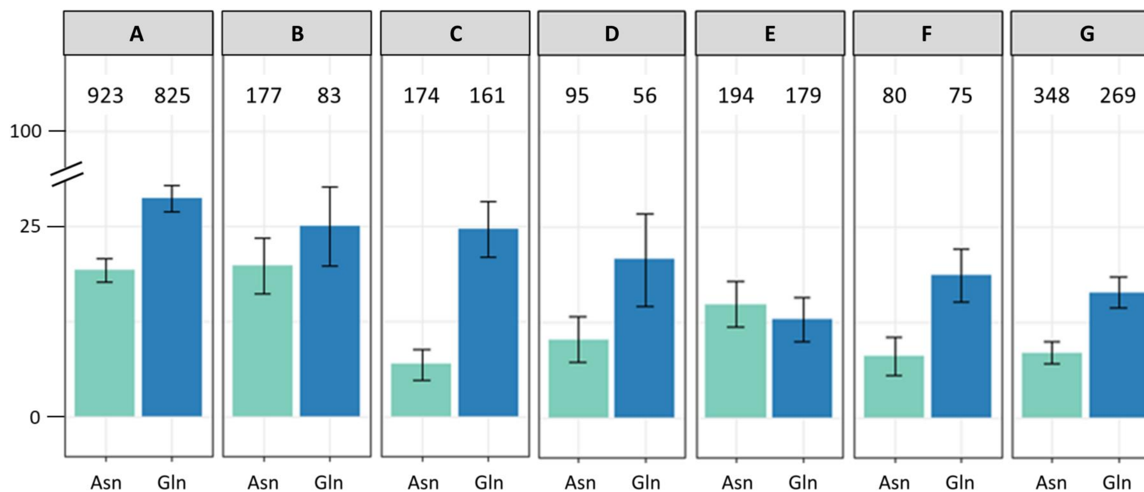

Fig. 2. Deamidation level of cereal and yeast proteins. (**A** to **G**) Percentage of deamidation of asparagine and glutamine residues for the species-specific cereal and yeast proteins identified in seven of the 10 analyzed samples. Error bars indicate SD around 1000 bootstrap replicates. Sample identifiers are shown at the very top, while the number of peptides used for the calculation is indicated for each bar.

polysaccharide structural analogs found in the samples from paintings B, F, H, and J. MS/MS spectra matching that of α-cyclodextrin have also been identified in some of the blanks. Spectral matches for this compound are therefore likely due to environmental contamination. Cyclic adenosine diphosphate ribose, found in the samples from paintings E, F, and A, did not show chemical structural similarity to any other mass spectral feature. However, this compound is a universal metabolite present in many different species, from plants to animals (*23*). It cannot therefore be excluded that this spectral match represents environmental contamination, and, accordingly, it cannot be confidently assigned to the ground layer of the painting.

The search for spectral analogs resulted in 197 spectral matches in total, many of which correspond to compounds also found as matches in the GNPS search. Azelaic acid was one of the matching mass spectral features in the sample from painting H. This acid is one of the main products of the oxidation of unsaturated glycerides and is formed during the curing of drying oils (*24*). Two reliable matches were found, one with a parent mass of 752.52 Da (cluster ID 2857) and one with a parent mass of 754.34 Da (cluster 2911) (fig. S4). This tentative identification suggests the presence of a lipid-based material in the samples. In this case, the parent ion could be a triglyceride containing azelaic acid as one of the fatty acid moieties, based on the comparison with the masses of compounds typically detected in drying oils (*25*). However, the mass of neither spectral match corresponds to the known triglycerides contained in drying oils, and the data acquired in this study are not sufficient to determine the identity of the parent ion(s).

Using DEREPLICATOR (*20*, *22*) and DEREPLICATOR VarQuest (*21*), a total of 14 and 28 mass spectral features with $P < 1 \times 10^{-8}$ and a false discovery rate (FDR) < 3% were found to match to in silico predicted spectra, respectively. However, a manual check of the spectra matching quality led to reducing the number of reliable matches to 3 and 9, respectively. The presence of many of the compounds found as matches is not plausible, given the nature of the samples, and these matches are probably due to the presence of an unidentified homolog or to contamination.

Nonetheless, some of the spectral matches observed can be connected to the use of lipidic and cereal- and yeast-containing materials as ground layer binders. The DEREPLICATOR algorithm found matches for cyclolinopeptide B in the samples from paintings B and J and cyclolinopeptide E in the samples from paintings A and I (fig. S5). Cyclolinopeptides are produced by linseed (*Linum usitatissimum*) (*26*), and their presence is possibly related to the use of linseed oil as one of the organic binders in the ground layers of the paintings. Among the matches found by the VarQuest algorithm, two might be connected with the identifications of the proteomic analysis: esperin in the samples from paintings A and D and acidocin in the samples from paintings E and F (fig. S6). These two compounds are produced by various micro-organisms in the *Bacillus* and *Lactobacillus* genera respectively, both of which can attack and cause spoilage in food products such as dough, bread, and beer (*27*–*29*). Matches to these two compounds were only found in samples in which proteomics identified cereal and yeast proteins. For each spectral match considered reliable, the comparisons of the experimental and library standard or predicted MS/MS spectra are shown in figs. S3 to S6.

DISCUSSION

In the early decades of the 19th century, the Royal Danish Academy of Fine Arts would commonly supply professors and students with artistic materials, in particular, canvases already primed. The 10 paintings investigated in this study were specifically chosen to allow characterization of the ground layers of canvases prepared by craftsmen working within and outside of the Academy workshop. Four paintings produced by Eckersberg (A to D) while he was a professor and three paintings produced by Købke (E to G) during his studies at the Academy (1822–1832) were investigated, together with three paintings by Købke (H to J) dating from the second half of the 1830s, after he had left the Academy. Eckersberg's diaries report that he often used the services of a succession of helpers at the Academy for the preparation of canvases with white or off-white grounds (*30*). He continued to use these canvases until the late 1830s, when commercially primed painting canvases, mainly imported from Dresden (Germany), became available in Copenhagen (*31*). Differences in the stratigraphy make it easy to distinguish between the canvases prepared at the Academy and the imported ones. The former consist of a single white layer, whereas the latter have double grounds with a lower reddish-brown or pale brown layer and an upper white or pale gray layer (*31*). None of the 10 paintings investigated in this study present a stratigraphy to suggest that these were commercial canvases imported from Dresden. Moreover, there is no record of conservation treatments performed on the selected paintings before lining in the 1960s (*7*), when the samples analyzed here were detached from the rest of the artwork. Therefore, the current study looked at the protein composition of the original ground layers of the canvases as prepared at the Academy.

Proteins from baker's yeast and several species of cereals were confidently and exclusively identified in the seven paintings (A to G) produced, or at least started, when both artists were at the Academy: between 1826 and 1833. To the best of the authors' knowledge, these proteins have never been identified in an artwork before, and the identification of the source material is not straightforward. Some of these proteins, particularly those from baker's yeast, could also be present because of environmental contamination. However, the amount of evidence for yeast and cereal proteins is overwhelmingly high in those paintings where it is observed, too high to be simply due to contamination. At the same time, if the identification of yeast was due to contamination during storage, then all the samples should show more or less the same amount of contamination, considering that they have been conserved and stored in the same way. However, in the samples from paintings H, I, and J, yeast proteins are completely absent. The specific yeast proteome identified also suggests that its presence is not due to contamination, as it was not a random yeast proteome, but proteins were closely associated with glycolysis and alcoholic fermentation. Yeast contamination in the laboratory space can be excluded on the basis of the absence of yeast proteins in the protocol blanks. Last, contamination from modern baker's yeast can be excluded on the basis of the damage level of the identified yeast proteins, which is comparable to that of the cereal proteins and compatible with nonmodern proteins (fig. S2).

Many different types of materials, either individually or mixed, might explain this protein profile, as discussed in detail in Supplementary Text. Among these, on the basis of (i) the literature on the

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use of such materials in artwork production, (ii) their protein composition when known, and (iii) their mechanical properties, two sources can be considered the most likely: fermented flour glue and alcoholic brewing products. Flour glue is a paste made with flour and water, usually by adding the flour to boiling water and cooking until a thick consistency is achieved (*32*). Starch glues, consisting of wheat starch obtained after separation from the gluten proteins, have also been widely used in painting production. However, since proteins are removed during their production, the proteome profile of starch glues would not be compatible with the cereal proteins identified in the samples analyzed here. To prevent it from rotting, the raw flour paste can be fermented, cooked, dried, and then pulverized (*33*). Flour glue is reported as a component of several ground layer recipes (*11*, *34*–*36*). To the best of the authors' knowledge, the protein composition of flour glue has never been characterized. Nonetheless, it is known that wheat flour and dough contain the main cereal proteins: albumins, globulins, gliadins, and glutenins (*37*–*40*). These types of proteins would probably be present also in fermented flour glue, together with proteins from yeast, although the fermentation and cooking processes might have substantially affected their amount and integrity. Several proteins from these families were also identified in the investigated samples (table S4).

The material used might have also been a product, or by-product, of a brewing process, such as in the production of beer or aquavit. Beer was the most common drink in 19th-century Denmark, since water needed to be treated in some way, often by brewing, before it was safe to drink (*41*). Beer was also used in the arts, in the cleaning and restoration of paintings (*42*), and Danish literature specifically reports its use as adhesive, paint binder, and for painting lining (*43*–*45*). Note that the paintings studied in this work were all subjected to lining in the 1960s by applying the wax-resin technique (*7*), which involves a mixture of beeswax and a resin used as the lining adhesive. This adhesive does not ordinarily contain any material originating from cereal or yeast. Even in the unlikely case that the one used to line the studied paintings exceptionally did, its identification as the possible contaminating source of the cereal and yeast proteins can be confidently excluded. This is because the tacking edges were removed before the adhesive was applied to the already trimmed canvas, as standard lining practice at the National Gallery of Denmark in the 1960s, and as described in the conservation reports. The high content of sugar in beer might be responsible for its fast drying and strength as a binder (*44*). The protein composition of barley and lager beers has previously been studied (*46*–*53*), and these results have been compared with the proteomes of the samples analyzed here. Many cereal and yeast proteins confidently identified in the samples analyzed in this study overlap with those previously found in beer (table S6).

Aquavit, another traditional brewing product in Scandinavia, is a distilled spirit obtained from the fermentation of grains and/or potatoes. During distillation, the most volatile components of the "wort," including alcohol, are separated and collected, to be then diluted to form the final product. Proteins are macromolecules and, hence, have low volatility. Accordingly, the amount of proteins contained in the final product, i.e., aquavit, is probably too low to justify the number of proteins identified in the paintings analyzed here. Therefore, if derived from the production of aquavit, then the detected material is probably a by-product of the initial fermentation process, rather than the final distillation product. Although aquavit was initially prepared purely using grains, the easier cultivation of potatoes in Scandinavia has made the tuber a worthy substitute since the 18th century (*54*). While no potato proteins were identified in any of the investigated samples, the use of a by-product of a pure grain aquavit brewing cannot be excluded.

In the samples from five paintings (A and D to G), multiple sources of seed storage proteins were identified among barley, buckwheat, and wheat. Multiple cereals can be used for the production of both flour glue and brewing products. Recipes for flour glue most commonly report the use of wheat and/or rye flours (*55*), although literature sources also report a mixture with other cereals, such as barley and buckwheat (*33*). In beer brewing, barley is the most traditional cereal ingredient (*56*) and was identified in six of the seven samples containing cereal proteins (Table 2). Other types of cereals, including wheat and buckwheat, can be used, and barley mixed with wheat was used for brewing beer in preindustrial Denmark (*41*). After fermentation of the beer, the final product is separated from residual yeast, one of the main by-products of beer brewing, which can be collected and reused in other productions. In preindustrial Copenhagen in particular, brewers would reuse the highest-quality yeast for their own brewing but were obliged by law to give part of the by-product to the city bakers (*41*). Any yeast that was left after this could be sold, often to be used as animal feed, and might therefore have been purchased by the Academy to be used as artistic material.

The ground layer of 3 of the 10 paintings (A, G, and I) analyzed here were previously studied via amino acid analysis with GC-MS (*57*). In that work, the authors concluded that the canvas of paintings A and G contained egg proteins and a drying oil, whereas the canvas of painting I only contained a drying oil. In the present study, egg proteins were not detected in any of the samples, whereas proteins from yeast and cereal species were confidently identified in canvas of paintings A and G. Collagen was also identified in the canvas of paintings A and I, although with only six and seven peptides, respectively (table S2). This suggests that a very small amount of glue is present, probably too little for hydroxyproline, the amino acid residue used as marker for the presence of animal glue in GC-MS, to be detected at a reliable level. Moreover, since the identification is based on the comparison with three standards: egg, milk, and animal glue, the presence of a different protein source will interfere with the statistical analysis of the results, as mentioned in previous literature (*5*). The amino acid profile obtained for the sample will either be unidentified or falsely assigned to one of the three standards. Therefore, the presence of cereal and yeast proteins in the canvas of paintings A and G might have resulted in a profile more similar to egg than to animal glue and milk, leading to the inaccurate identification of the former. Nonetheless, it is possible that GC-MS would have identified the material detected via proteomics if this had been included among the standards used for comparison. Therefore, future studies should focus on the combination between these two techniques, taking advantage of the untargeted nature of the proteomics protocol for identification of previously undocumented proteinaceous materials present in artworks, to be included in routine analysis of large sample sets using GC-MS.

By combining the results from the present proteomic study and previous analyses on the canvas of paintings A and G (*7*, *57*), a wider idea of the ground layer composition can be achieved. The results of GC-MS, microscopic, and spectroscopic techniques indicate the

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presence of lead white, calcium sulfate, and a drying oil. Besides the cereal- and yeast-containing material, the proteomics analysis identified animal glue from bovine and/or ovicaprine species in eight of the specimens analyzed, including painting A. Artists' manuals report that ground layers containing animal glue have good absorbency and therefore limit the spread of the paint, but they are prone to cracking and not very flexible (*34*). On the contrary, oil-based ground layers are very flexible, but not very absorbent. The identification of both materials in the investigated paintings hints at the will of the craftsmen preparing the canvases to achieve a balance between these two properties. However, animal glue and drying oil do not mix well given the hydrophilic and hydrophobic properties of the two materials, respectively. High-sugar materials, such as treacle and honey, were occasionally used as emulsifying and plasticizing agents in the 18th and 19th centuries (*34*, *58*–*62*). In the ground layers studied, a material such as fermented flour glue or a beer- or aquavit-brewing (by-)product, containing cereal starch, might have, thus, been added as an emulsifying and plasticizing agent to improve the elasticity and stability of the layer. However, in the mock-up canvas ground layers prepared to test the mechanical properties of these materials (see the Supplementary Materials), fermented flour glue showed very poor cohesion and extensive cracking (fig. S1), suggesting against the use of this material in the ground layers analyzed in this study.

Among the most likely options about the identity of such material, the use of beer or brewing residual yeast requires the least number of assumptions. The use of fermented flour glue is documented as mounting adhesive in Japan (*33*), but its use in Europe and in painting production is, to the best of the authors' knowledge, undocumented. Furthermore, the presence of different species of cereals suggests that the detected proteins derived from a mixture of materials from multiple, different productions, rather than a material specifically prepared to be used as a binder in the ground layers, as would have been the case for the fermented flour glue. As for aquavit, the only compatible material would be a by-product of the distillation of pure grain-based aquavit, which was not as widespread as the potato-based one in 19th-century Denmark. On the contrary, both beer and residual yeast seem to fit with the proteomic profile detected in the samples of paintings A to G. Residual yeast, in particular, appears to have high emulsifying power, as shown by mock-up canvas ground layers prepared to test this theory (see the Supplementary Materials). In addition, residual yeast could have been bought by the Academy as waste material from the beer production taking place in Copenhagen, whereas beer itself was a very precious product in 19th-century Denmark, even used as currency to pay salaries (*41*). Thus, it can be speculated that the material identified here is most probably residual yeast, rather than beer, although the collected evidence is not sufficient to confidently exclude any of the materials suggested above.

The MS data acquired via the proteomics protocol were also analyzed by a data analysis workflow used in metabolomics studies. This approach is limited by the application of a small-molecule data analysis workflow to an experimental protocol optimized to extract ancient protein remains, only unintentionally coextracting other compounds. Furthermore, the MS measurement in this case is not optimized for small molecules, as the lower limit of the mass/charge ratio (m/z) range recorded by MS analysis was set to 350 (see Materials and Methods). Because of these limitations, the reported spectral matches can at best be considered tentative identifications, as they can only suggest the presence of structural analogs of the matching compounds. Despite this, this approach was tested to maximize the recovery of molecular information, integrating the proteomic results with those derived from the investigation of small molecules such as flavor compounds and metabolites.

Several spectral matches were obtained. Multiple spectral matches supported the tentative identification of maltotetraose and maltopentaose, compounds commonly present in cereals, starch, and even wort. Although this result is compatible with the identification of a cereal- and yeast-containing material, matching experimental spectra were also detected in the samples of paintings H to J, where proteins from these sources were not identified. Accordingly, the presence of these compounds cannot be strictly associated with cereal seed proteins and could instead originate from other sources. On the other hand, spectral matches for esperin and acidocin were found only in samples containing cereal and yeast proteins, from paintings A and D and paintings E and F, respectively. Esperin is an antibiotic produced by *Bacillus mesentericus* (*63*), a food contaminant also found in bread dough and beer (*27*). Acidocin is a bacteriocin produced by *Lactobacillus acidophilus* (*29*), one of the *Lactobacillus* species infamously known for infecting beer (*64*), but that can also be used to increase the beverage's sourness (*65*) and in sourdough baking products (*66*). *L. acidophilus* is also present in the human body, particularly in the digestive tract and the mouth (*67*), and, thus, acidocin contamination from human handling of the paintings and samples cannot be excluded. As a matter of fact, the detection of potential contaminants rather than only substances connected to the proteomic identifications indicates the capability of the metabolomics workflow to produce credible and reliable results, within the discussed limitations.

Several spectral matches suggest the presence of a drying oil in some of the samples. Matches for cyclolinopeptides, produced in *L. usitatissimum*, i.e., linseed (*26*), were found in the samples of four paintings (A, B, I, and J). Two of these samples, A and I, showed presence of a drying oil, probably from linseed, when studied with GC-MS (*57*). Several spectra matching the fragmentation pattern of azelaic acid were found in the sample of painting H (fig. S4), from which no protein was confidently identified. Azelaic acid is one of the products formed in the oxidative curing of drying oils (*24*), which were often used for the preparation of canvases (*34*). The observation of spectral features matching azelaic acid thus suggests that a drying oil was used as binder in the ground layer of this painting. Although further studies are needed to assess the reliability of this application, this result suggests that metabolomic analysis has great potential to further enrich the results of the proteomic analysis of a painting microsample.

In conclusion, MS-based proteomics identified animal glue and a material containing cereal grains and baker's yeast as binders in the ground layers of canvas paintings produced by C. W. Eckersberg and C. S. Købke during the Danish Golden Age while at the Royal Danish Academy of Fine Arts. The use of this recipe can be traced to the Academy workshops, where canvases would have been prepared for both professors and students. To the best of the authors' knowledge, cereal and yeast proteins have never been identified in artworks before. Although the exact identification of this material cannot be indisputably defined exclusively on the basis of the proteomic analysis, the most likely source of cereal grains and baker's yeast proteins is a (by-)product of beer brewing. Local literature

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sources reporting the common use of beer as artistic material and the evaluation of the mechanical properties, the availability, and the cultural and social importance of beer brewing products and by-products in Denmark further support this interpretation. Future studies should focus on the detailed characterization of these materials and their protein composition, starting from standard and reference materials such as the mock-ups produced in this project. This will, in turn, allow for higher accuracy in the identification of the products used in artworks and possibly confirm the interpretation of the results obtained in this work.

The metabolomics characterization of small molecules coextracted with peptides represents a promising approach to increasing the molecular information that can be extracted, without further sampling, from the MS-based analysis of cultural heritage objects. In the future, the development of optimized protocols to maximize simultaneous coextraction of proteins and small molecules from the same sample will allow the reconstruction of a richer molecular profile in multiple classes of artistic and archaeological objects.

Last, this work highlights the importance of investigating the presence of unconventional artistic materials, in connection to their historical and cultural relevance. In 19th-century Denmark, beer brewing was a paramount part of the culture and economy. Therefore, the identification of a beer-brewing (by-)product in a set of Danish Golden Age artworks is highly relevant for unveiling previously ignored applications concerning a productive activity so meaningful in the history of Denmark and Danish society. This identification was only possible because of the untargeted nature of the MS-based proteomic protocol used. Further application of proteomics to the study of artworks will potentially unravel more currently unknown associations between society and art production, enhancing the relevance of this approach beyond its value for conservation and restoration purposes.

MATERIALS AND METHODS

Samples

The ground layers of 10 paintings produced during the Danish Golden Age were investigated. Samples were removed from trimmed portions of the tacking edge of the canvases, which had been cut off before wax-resin lining of the paintings in the 1960s and are now stored at the scientific department of the Statens Museum for Kunst in Copenhagen (paintings A to I) and the Institute of Conservation of Det Kongelige Akademi for de Skønne Kunster also in Copenhagen (painting J). Presently, the paintings themselves are also still located at those respective institutions (Table 1). An example of one of the canvas trimmings and the sample removed for proteomic analysis are shown in fig. S7.

All the canvases consisted of fairly tightly woven, plain weave fabrics with thread counts between 12 and 16 threads/cm in both directions. Judging by their appearances, the yarns are made of linen or, in some cases, possibly hemp, but no fiber identification was carried out. Examination of the paintings by Eckersberg has shown that their canvases were cut from larger, preprimed pieces (*31*).

Using a scalpel, canvas samples were cut from the trimmings removed in the 1960s lining campaign, placed in separate tubes, and weighed. The mass of the collected samples was in the range of 3.8 to 28.6 mg, mostly represented by the plant fibers forming the canvas and thus substantially larger than samples typically used for the study of cultural heritage objects with MS-based protocols [see, for example, (*4*, *68*)]. The ground layer and the underlying canvas were processed together, and no attempt was made to separate them. The amount of proteinaceous material analyzed was, accordingly, substantially smaller than the actual weight of each sample.

Sample preparation

The experimental protocol adopted was previously described by Mackie 2018 (*9*). Briefly, each canvas sample was placed in a 1.5-ml Eppendorf Protein LoBind tube and processed along with blank controls to monitor for laboratory contamination. Proteins were solubilized via incubation for 2 hours at 80°C with 100 μl of an aqueous buffer containing 2 M guanidinium chloride (GuHCl), 10 mM tris(2-carboxyethyl)phosphine, 20 mM chloroacetamide, and 100 mM trisaminomethane (tris). The pH was adjusted to approximately 8.0 using 10% ammonium hydroxide when needed. The solid samples and extracted proteins in solution were then subjected to a double-step enzymatic digestion. First, proteins were digested under agitation for 2 hours at 37°C in-solution with 0.2 μg of rLysC (Promega). The solution was then brought to a final concentration of 0.6 M GuHCl using 25 mM tris in 10% acetonitrile (ACN) in water. The second digestion step occurred overnight under agitation at 37°C with 0.8 μg of trypsin (Promega). Samples were then acidified to pH 2 using 10% trifluoroacetic acid (TFA) to quench the digestion. After centrifugation of the tubes for at least 1 hour at 21,000*g*, to avoid transferring any solid debris into the next step, peptides were collected on in-house made C18 extraction StageTips (*69*). The StageTips were prepared following a protocol based on Rappsilber 2007 (*70*), then conditioned with 150 μl of methanol, followed by 0.1% TFA in 80% ACN, and washed with 0.1% TFA in water. The sample solutions were loaded in 200 μl increments, followed by two washings with 0.1% TFA in water. StageTips were stored at −18°C until MS analysis.

Nano–liquid chromatography–tandem mass spectrometry

The purified peptides were eluted from the StageTips using 30 μl of 40% ACN in water. Extracts were concentrated in a vacuum centrifuge at 40°C until approximately 3 μl of solution were left and then rehydrated with 5 μl of 0.1% TFA and 5% ACN in water. The peptides were then separated on a 15-cm-column (75 μm in inner diameter) in-house laser pulled and packed with 1.9-μm C18 beads (Dr. Maisch, Germany) on an EASY-nLC 1200 (Proxeon, Odense, Denmark). A 77-min gradient was used: Buffer A was 0.1% formic acid in Milli-Q water, and buffer B was 80% ACN and 0.1% formic acid. The peptides were separated with increasing buffer B, going from 5 to 30% in 50 min, 30 to 45% in 10 min, and 45 to 80% in 2 min, held at 80% for 5 min before dropping back down to 5% in 5 min, and held for 5 min. Flow rate was 250 nl/min. The column temperature was maintained at 40°C using an integrated column oven. To hinder cross-contamination, a wash-blank method using 0.1% TFA and 5% ACN was run in between each sample. The chromatographic parameters were the same for all samples.

In a pilot round of measurements, one aliquot of the peptide extracts from paintings A, I, and J were measured using a Q-Exactive HF mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The mass spectrometer was operated in data-dependent top 12 mode. Spray voltage was 2 kV, S-lens radio frequency level

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was at 50, and the heated capillary was kept at 275°C. Full scan mass spectra were recorded at a resolution of 120,000 at *m*/*z* 200 over the *m*/*z* range of 350 to 1400 with a target value of 3×10^6 and a maximum injection time of 25 ms. Higher-energy collisional dissociation (HCD)-generated product ions were recorded in the *m*/*z* range of 200 to 2000 with a maximum ion injection time set to 45 ms. The target value was 2×10^5, and spectra were recorded at a resolution of 30,000. Normalized collision energy was set at 28%, and the isolation window was 1.2 *m*/*z* with the dynamic exclusion set to 20 s. In the main round of measurements, a second aliquot of the peptide extracts from paintings A and I, two aliquots of the peptide extract from painting J, and one aliquot of the peptide extracts from paintings C and G were analyzed using Q-Exactive HF mass spectrometer with improved instrumental parameters, namely, in data-dependent top 10 mode, increasing the MS/MS resolution to 60,000, and the MS/MS maximum injection time to 108 ms. The peptide extracts from paintings B, D, E, F, and H were measured on a Q-Exactive HF-X mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), because of an instrument upgrade during the study. Experimental parameters for the Q-Exactive HF-X were set as on the Q-Exactive HF in the main round of measurements, increasing the MS/MS maximum ion injection time to 118 ms, in accordance with the instrument's optimized acquisition speed. A summary of the MS parameters set differently between the two instruments is reported in table S1. The downstream proteomics data analysis relied exclusively on data generated in the main round of measurements, while the metabolomic analysis relied on data generated in both the pilot and the main round of measurements.

Data analysis: Proteomics

The data analysis with the MaxQuant software (*71*) (version 1.6.1.0) was performed in three consecutive searches. The MS/MS spectra were first matched against a reference, in-house curated database containing all the publicly available sequences for the most common proteinaceous paint binders (collagens, egg proteins, and milk proteins). The following variable modifications were included: oxidation of methionine, deamidation of asparagine and glutamine, conversion of N-terminal glutamine to pyroglutamic acid, conversion of N-terminal glutamic to pyroglutamic acid, and hydroxyproline. To investigate the presence of protein residues originating from additional sources, the spectra were then matched against the SwissProt database (downloaded January 2017) (*72*), which contains a large selection of manually reviewed protein sequences from a wide variety of species. The same variable modifications as in the first search were included.

On the basis of the identifications in the second search, a database containing protein sequences from cereal and yeast species was built, and modifications identified in literature in wort and beer (*47*, *73*, *74*) were selected as variable modifications to try to further increase peptide identifications. The third database contains all protein sequences publicly available on Uniprot from the following species: *F. esculentum*, *H. vulgare*, *Humulus lupulus*, *Oryza sativa* subsp. *japonica*, *S. cereale*, *T. aestivum*, and the genus *Saccharomyces*. The following were included as variable modifications: oxidation of methionine, deamidation of asparagine and glutamine, acetylation of the N terminus, condensation product of 3-deoxyglucosone, hexose addition, double hexose addition, and glycation of the N terminus.

For all searches, the software was set to search for tryptic peptides with up to a maximum of five modifications per peptide, and carbamidomethylation was set as a fixed modification. The minimum peptide length was set to 7, with up to two missed cleavages. The FDR for both peptides and proteins was set to 0.01, and the minimum Andromeda score for unmodified and modified peptides was set to 40. Mass tolerances for precursor and fragment ions were set to 4.5 and 20 parts per million, respectively. Contaminant proteins were assessed using the default "contamination.fasta" file bundled to MaxQuant, containing common laboratory contaminants (*75*), including primate keratins, excess trypsin, and bovine serum albumin. Peptides assigned to contaminant proteins were filtered out and not considered any further.

Proteins were considered confidently identified if at least two unique nonoverlapping peptides were observed, unless otherwise specified. Peptides were considered species-diagnostic when, after a search against the entire National Center for Biotechnology Information nonredundant (nrNCBI) protein database via the BLAST alignment tool (*76*), they were assigned to a single species or if only one of the matching species can be considered plausible on the basis of the historical and geographical origin of the samples. The search against the BLAST database was automated using an R script, Get_SpSpPept_list.R, which is available for download (see Data and Materials Availability). The workflow started with a blastp (v2.2.26) search against the nrNCBI nucleotide database using each of the identified peptides as query and the following parameters: -e 200000 -W 2 -G 9 -E 1 -M PAM30 -f 16 -C 0 -A 15 -m 0 -a 30. To identify peptides that could be assigned to a single species (species-specific peptides), only blast hits with 100% coverage and 100% identity were considered. In this case, a peptide was considered to be species-specific if only one species was present among its hits. Then, from the list of species-specific peptides, all the possible combinations of peptide sequences potentially leading to the same identification were created by substituting three possibly ambiguous amino acid couples (Leu/Ile, which have the same molecular mass; Asn/Asp and Gln/Glu, due to deamidation converting the former residues into the latter ones). A second blast search was then performed against nrNCBI database using the same parameters mentioned above and discarding peptides where one or more of their alternative combinations had one or more hits (100% coverage and 100% identity) with a different species than the original peptide.

To validate the endogenous origin of the proteins identified and exclude laboratory contamination and sample cross-contamination, blanks and instrument washes were also analyzed, following the same protocol as the sample data (the data from the protocol blanks are available for download, see Data and Materials Availability). Last, the deamidation level was calculated using the deamidation tool described in Mackie 2018 (*9*) and publicly available on GitHub (*77*).

Data analysis: Metabolomics

To retrieve chemical structural information from small molecules (above the 350 *m*/*z* lower MS measurement limit; see nLC-MS/MS methods), the proprietary Thermo .raw MS data files were exported to .mzML format using ProteoWizard's MSConvert (version 3.0) (*78*) and submitted to mass spectral molecular networking within the GNPS (*19*). Data were filtered by removing all MS/MS fragment ions within ±17 Da of the precursor *m*/*z*. MS/MS

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spectra were window filtered by choosing only the top 6 fragment ions in the ±50-Da window throughout the spectrum. The precursor ion mass tolerance was set to 0.1 Da, and the MS/MS fragment ion tolerance was set to 0.02 Da. A network was then created where edges were filtered to have a cosine score above 0.6 and more than five matched peaks. Further, edges between two nodes were kept in the network if and only if each of the nodes appeared in each other's respective top 10 most similar nodes. Last, the maximum size of a molecular family was set to 100, and the lowest scoring edges were removed from molecular families until the molecular family size was below this threshold. The spectra in the network were then searched against all GNPS spectral libraries. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.7 and at least five matched peaks. The term "tentative identification" was used in the text to refer to spectral matches respecting the established threshold for similarity scores. An additional library search was performed using the "Search Analogs" option allowing for a maximum of 200-Da shift between the precursor m/z of the query and library spectrum. The molecular network was created using the classical molecular networking workflow (version release 23).

In silico chemical structural information of peptidic natural products was retrieved through DEREPLICATOR (*20*) and DEREPLICATOR VarQuest (*21*). Precursor and fragment ion mass tolerances were set to 0.02 Da, maximum charge and isotopic shift to 2, and minimum numbers of bonds was set to 3. Both Na and K adducts were searched. DEREPLICATOR and DEREPLICATOR VarQuest were run within the GNPS analysis environment.

Supplementary Materials

This PDF file includes:
Supplementary Text
Figs. S1 to S7
Table S1
Legends for tables S2 to S6
References

Other Supplementary Material for this manuscript includes the following:
Tables S2 to S6

View/request a protocol for this paper from *Bio-protocol*.

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