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ANALYSIS OF ART OBJECTS USING MASS SPECTROMETRY

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ABSTRACT

This presentation reviews the latest progress in the identification of the proteinaceous materials and organic dyes presented in art and in historical building materials using mass spectrometry as a dominant method.

The method of the peptide mass mapping for identification of proteinaceous binders in the colour layers of easel paintings and in the historical mortars was developed in our laboratory. It is based on specific enzymatic cleavage and subsequent analysis of the peptide mixture by MALDI-TOF MS (matrix-assisted laser desorption/ionisation time of flight mass spectrometry). The obtained mass spectrum creates a "mass fingerprint", which enables reliable identification by comparison of the spectrum of the analysed sample with those of reference samples from our library. The developed method was tested on the model and real colour layers as well as on the fresh and naturally aged model mortar samples and on the several hundred years old building materials.

The direct analysis of organic dyes by mass spectrometry is based on identification of their molecular ions. No preliminary treatment of the sample is needed. The method was successfully applied on the microscopic amounts of pure dyes and organic pigments, on the fragments of colour layers in the weight of a few micrograms, and on the individual textile fibres.

INTRODUCTION

Identification of Proteins in Colour Layers of Easel Paintings and Historical Building Materials

Identification of a small amount of a protein (in order of tens of picomoles) is nearly impossible by traditional methods of chemical analysis – e.g. gas chromatography [1-3], pyrolysis-gas chromatography [4,5], and high performance chromatography [4]. The situation is further complicated when a protein mixture of variable composition should be identified in complex matrix containing dyes, oils, inorganic pigments, lime, etc.; moreover, the analysed materials come often from the Middle Ages or even ancient times and the proteins in them could have undergone various modifications (e.g. oxidation, photodecomposition, microbial digestion) over the centuries.

Luckily, during the last two decades, the methodology for mastering this challenging task has been developed by biochemists. The branch of biochemistry that seeks to identify all or at least the great majority of proteins occurring in cell, tissue or even entire organism under the given physiological or pathological conditions is called proteomics. Its experimental procedure is usually based on three steps:

1. Protein (as pure as possible) is specifically cleaved with certain protease under defined conditions. Bovine trypsin, which cleaves peptide chains after the positively charged amino acids of lysine and arginine, is used most frequently. Thus, the mixture of peptides, specific for the given protein, is obtained.

2. Then the mass spectrum of the mentioned peptide mixture is measured. The set of molecular mass values ("peak list") corresponding to individual peptides is characteristic for the analysed protein and can be considered as its fingerprint.
3. The obtained peak list together with other data (biological species, possible posttranslational modifications of amino acids etc.) is then submitted to a software tool (usually publicly available) and searched against a certain protein database, which (hopefully) leads to protein identification. The software tools usually offer information on the statistical plausibility of protein identification as well.

This procedure is called Peptide Mass Mapping (PMM), which is defined as a means of identifying proteins by comparing observed masses (*m/z* values) with predicted masses of digested proteins contained in a database.

Recently, we have used the technique described above for the identification of proteinaceous binders [6-8]. We anticipated that peptide mass fingerprint, which can be obtained from a relatively well-defined protein mixture (animal glue, egg, milk, etc.), would be more characteristic for the mixture than the mutual ratios of individual amino acids (the parameter used by traditional chromatographic techniques mentioned above). By this method it could be possible to identify not only a single proteinaceous binder, but even combinations of their different types, which is totally out of the question for any other analytical method. Experience from biochemical experiments allowed us to expect that the method can be very sensitive; in our experiments the detection limit for e.g. β-casein (the characteristic milk protein) was about 18 femtomoles [9], which means that sufficient amount of sample can be less than 0.5 μg in the case of the colour layer of paintings. Such a low detection limit is essential for the analysis of historical mortars in which the protein concentration can be very low and the amount of sample must be proportionally larger. The detection limits of traditional analytical methods are higher by several orders of magnitude and consumption of sample is much higher as well [10].

Different types of mass spectrometers can be used for determination of molecular weights of peptides resulting from specific enzyme cleavage. Nowadays, matrix-assisted laser desorption/ionisation – time of flight (MALDI-TOF) mass spectrometry is probably the most popular method because it does not require preliminary chromatographic separation of peptides and thus is relatively fast with high throughput potential. All our data, which will be reported further on, were measured by this type of mass spectrometer.

In the last decade, also other modern biochemical methods have been used for analysis of protein binders [6,11]. E.g. the group led by A. Heginbotham identified egg proteins in a 17th century painting using immunofluorescent microscopy and ELISA method [11].

Colour layers of art-works are a combination of powder dyes or pigments with binders, serving for their conjugation. The addition of organic binders restricts oxidation of the pigments and other constituents, therefore increasing stability of the art-works. Although the binders form the minority of the colour layer, the painting techniques are derived from them; at present the most common techniques are oil and tempera.

According to the predominant component, the binders are usually divided into protein, oil, polysaccharide, and resin binders. In this paper we shall focus on protein binders but it is worth mentioning that in the majority of natural "non-protein" binders a minority protein component is usually present as well. Thus many of the analytical techniques described here

can be (with certain limitations) applied to them as well. Although in colour layers of art-works and particularly in paintings protein binders are relatively abundant (up to 10 %), their identification is often limited by a small amount of sample, usually available for analysis (tens or hundreds micrograms at most [10,12]).

Early on ancient Egypt and Rome, builders added various inorganic and organic additives to the mortar. From the inorganic materials they used, amongst other things, pulverized bricks, potsherds, broken glass, pottery, ash or clay as filling agents to replace locally scarce and expensive lime and sand [13,14].

The organic additives can be divided into two groups. The first group contains the compounds used as the filling or reinforcing materials like straw, sawdust, other plant filamentous materials, and animal piles (mainly from horse and goat). The second group contains the so-called modifiers that, even in small amounts, can adjust certain mortar properties; they may change the water distribution in fresh mortar and the speed of its desiccation during which they initiate crystallization and thus change the properties of hardened mortar, e.g. extending workability time, improving cohesion, increasing firmness, etc. [13,15].

Identification of Red Organic Dyestuffs

The identification of organic dyes and pigments in the heterogeneous mixtures present in the colour layers has not been satisfactorily solved. Almost all of analytical methods needs the liquid sample. The extracts of anthraquiones have been analysed by thin layer chromatography (TLC) [16-18], high performance liquid chromatography (HPLC), high performance chromatography on reverse phase (RPHPLC) [19-32], and electrospray ionisation-mass spectrometric detection (ESI-MSD). The detection limits for the mentioned methods are shown in Table I. Standards of carminic acid, emodin, purpurin, alizarin, and laccaic acid, the typical natural red dyes that vary in substituents on positions 9,10 of anthraquione molecule, have been studied by capillary electrophoresis combined with electrospray [33]. Newly tested MALDI-TOF mass spectrometry and mass spectrometry with electrospray ionisation, both in negative mode, were used for confirmation of carminic acid in the mixture with linseed oil [34]. The liquid samples that are necessary for these methods are obtained by dissolving of samples in acids with the following extraction. The disadvantage of sample dissolving is the breakdown of some red dyes that are not stabile in acidic condition [35,36].

Methods such as infrared and visible spectroscopy that deals with analysis of solid samples can provide complementing information. Both methods indirectly confirmed the presence of anthraquiones dyes in Byzantic manuscript [37]. Direct identification of red dyes due to content of characteristics proteins, which are depending on the dye origin, and due to molecular ions of the all types of organic dyes can be realised using MALDI-TOF mass spectrometry [38-40]. In the last period we have founded that it is possible to measure mass spectra of many organic dyes and pigments in the MALDI-TOF instrument without application of any matrix.

Method	Dyestuff	Amount	Detection limit
TLC-IR	purpurin	-	$20 \cdot 10^6$ ng/ml [41]
HPLC-UV	anthraquinones	-	100 ng/g [20]
RPHPLC-UV	anthraquinones	-	$1\text{-}1.5 \cdot 10^6$ ng/ml [21]

Voltammetry	anthraquinones, flavons, indigo, Prussian blue	< 1 mg	0.1 (%) [35]
ESI-MS with UV/VIS detection	anthraquinones	0.5 mg	100-500 ng/ml [33]
ESI-MSD	anthraquinones	0.6 mg	30-90 ng/ml [42]
MALDI-TOF MS	anthraquinones, flavons, indigo,copper phthalocyanine	< 1 μg [43]	-

Table I. The analytical methods that have been used for identification of red organic dyestuffs used in colour layers. TLC-IR - thin layer chromatography with infrared detection, (RP)HPLC UV- high performance liquid chromatography (on reverse phase) with ultraviolet detection, ESI-MSD - electrospray ionisation-mass spectrometric detection, MALDI-TOF MS - matrix-assisted laser desorption/ionisation time of flight mass spectrometry.

EXPERIMENTALS

Materials and Chemicals

Trypsin (TPCK) from Promega Corporation, trifluoracetic acid and 2,5-dihydroxybenzoic acid both from Sigma, acetonitrile (p.a.) and ammonium hydrogen carbonate from Lachema Brno were used. The organic dyes were supplied from Sigma-Aldrich (alizarin) and Fluka (purpurin). The analysed textile fibres come from the India's shoes from the beginning of 20th century.

Digestion of Proteins by Trypsin

The powdery sample (e.g. a mortar) was mixed with 60-100 μl of trypsin solution (2 μg in 100 μl of 50 mmol/l NH_4HCO_3/$(NH_4)_2CO_3$ buffer pH 7.5) so that the sample was covered. About 100 mg of mortar powder are needed.

Sample Measurement by MALDI-TOF Mass Spectrometry

The 3 μl of the concentrated peptide solution was mixed with 5 μl of 2,5-dihydroxybenzoic acid (~ 7 mg in 500 μl of the mixture of acetonitrile/0.1% trifluoracetic acid, 1/2 (v/v)). The resulting mixture was applied to the MALDI steel plate and left to crystallise. Afterwards, the MALDI-TOF mass spectra were measured by BIFLEX IV instrument (Bruker, Germany) under appropriate conditions (potential 19 kV on the plate and 15,05 kV on the deflector, positive reflector mode – potential 20 kV, laser intensity 50 or 60 %). The range of detected masses was from 600 to 3000 Da. For the external calibration the standard mixture of peptides M-Pep was used.

The red dyestuffs and textile fibres were mixed with distilled water and left to dry on the steel plate. The "stickled" samples were measured in the negative mode by the same instrument under the analogical conditions. The range of detected masses was from 0 to 1000 Da. For the external calibration the standard mixture of peptides M-Pep was used.

RESULTS AND DISCUSSION

Protein Analyses

For many years it had been generally assumed that the cleaved protein must be a component of a diluted water solution or at least well accessible from a hydrophilic phase. At the beginning of 2004, we tried to apply trypsin solution directly to the surface of art-work colour layer. Surprisingly, trypsin was able to cleave the proteins incorporated in totally insoluble matrices. This fact is extremely important for protein identification in binders of historical

materials. A drop of trypsin solution can be just deposited on a sample of colour layer and soluble peptides are released into solution that can be subsequently submitted for further analysis. Similarly, the pulverized (insoluble) building material (e.g. mortar) can be mixed with an appropriate volume of trypsin solution.

The method of Peptide Mass Mapping was firstly used for the identification of proteinaceous binders in colour layers of art works. Thanks to the protein analysis of Munch′s paintings, which was presented on Art05 conference (Lecce Italy) [44], the presumption of casein tempera was overcame. The occurrence of egg tempera and animal glue were also found in the paintings of M. Coxie [4] and J. Zitterer [45].

The presence of protein additives in the mortars, mainly collagens (animal glues), was confirmed in many Czech sacral and secular historical buildings e.g. in Lemberk castle, chateau in Náměšť nad Oslavou, Saint Catherine rotunda in Znojmo [46], and baroque Lobkowicz palace at Prague castle. Collagen materials were added to the mortars, most probably, to improve their cohesion. On the other hand, the egg proteins, found in Svojšín church and Pernštejn castle (Czech Republic), improve the mortar workability and accelerate the firmness of the plasters [4,47]. The relatively common milk proteins guarantee the firmness of the construction; they were identified in the third oldest bridge in the Czech Republic (14th century) situated in Roudnice nad Labem.

The MALDI-TOF MS will most likely remain the method of the "first election" for the peptide mass mapping because of the simple sample preparation and mainly because of the rapid spectra recording (without the chromatographic separation). It can be supposed that in future more sophisticated mass spectrometry methods will be used in this analytical field; they are slower, but they can offer more information about the binder present. The lower number of peaks will then be sufficient for identification because the peptides will be sequenced and compared with single protein database. For instance, the nanoLC/nanoESI/Q-q-TOF MS/MS has recently been applied to model samples that contained ovoalbumin and whole egg; the whole egg was identified in real samples taken from early renaissance paintings [48].

The Organic Dyestuffs Analyses

The red organic pigments, which are contained in art objects and textile fibres, were analysed by mass spectrometry in negative reflector mode. The coloured cotton and silk rags were taken from India shoes from the beginning of 20th century. The separated fibres from the rags were "sticked" on the steel plate by the distilled water and the mass spectrum was obtained (Fig. 1). Two peaks with the m/z ratios of alizarin (240.3 Da) and purpurin (256.3 Da) were safely identified. Both dyestuffs are contained in plant *Rubia tinctoria* that has been used for dyeing of textiles and for preparation of painters' pigments. Presumably the shoes were manufactured using this plant that have been used as a colouring agent in India since the 3rd millennium BC.

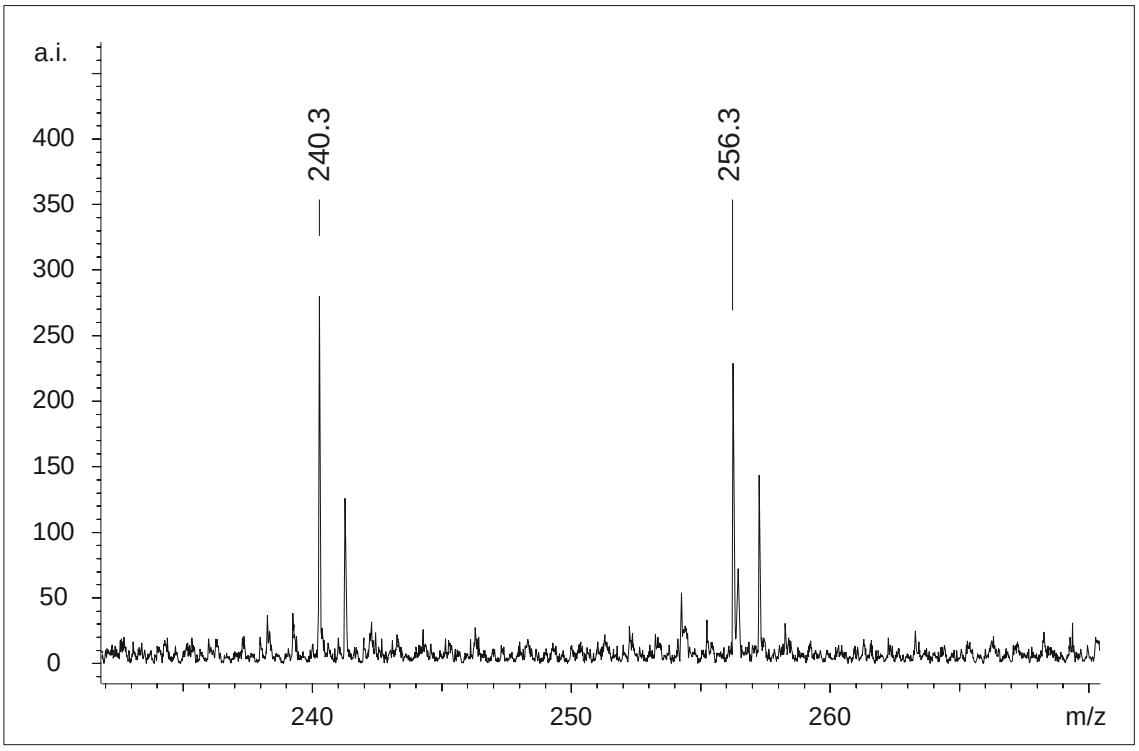

Figure 1. The mass spectrum of red textile fibres from India's shoes from the beginning of 20th century. The mass peaks of alizarin (240.3) and purpurin (256.3) are labelled.

CONCLUSIONS

The method of the peptide mass mapping for identification of proteinaceous binders in the colour layers of easel paintings and in the historical mortars was developed in our laboratory. It is based on specific enzymatic cleavage of proteins and subsequent analysis of the resulting peptide mixture by MALDI-TOF MS. The obtained mass spectrum creates a "mass fingerprint", which enables reliable identification of binders by comparison of the spectrum of the analysed sample with those of reference samples from our library. The developed method was tested on the model and real colour layers as well as on the fresh and naturally aged model mortar samples and on the several hundred years old building materials.

The measurement of the natural dyes without any matrix can be called laser desorption/ionisation (LDI); it is highly promising for identification thanks to its non-destructive character and extreme simplicity of sample preparation and measurement itself. In contrast to other analytical methods the advantages of this method is the no preliminary sample treatment and rapid analysis that takes several minutes. LDI mass spectrometry was successfully used for direct identification of red dyestuffs from cotton and silk fibres. Both anthraquionone dyes from traditionally used plant (*Rubia tinctoria*) were found in the mass spectrum.

In the Czech Republic we are the only laboratory that performs this special kinds of analyses. We will willingly cooperate with all laboratories and ateliers that need protein and organic pigments identification for research and restoration purposes.

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