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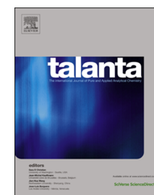

On plate graphite supported sample processing for simultaneous lipid and protein identification by matrix assisted laser desorption ionization mass spectrometry

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ABSTRACT

The simultaneous identification of lipids and proteins by matrix assisted laser desorption ionization-mass spectrometry (MALDI-MS) after direct on-plate processing of micro-samples supported on colloidal graphite is demonstrated. Taking advantages of large surface area and thermal conductivity, graphite provided an ideal substrate for on-plate proteolysis and lipid extraction. Indeed proteins could be efficiently digested on-plate within 15 min, providing sequence coverages comparable to those obtained by conventional in-solution overnight digestion. Interestingly, detection of hydrophilic phosphorylated peptides could be easily achieved without any further enrichment step. Furthermore, lipids could be simultaneously extracted/identified without any additional treatment/processing step as demonstrated for model complex samples such as milk and egg. The present approach is simple, efficient, of large applicability and offers great promise for protein and lipid identification in very small samples.

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1. Introduction

The simultaneous identification (and quantification) of lipids and proteins as efficiently and rapidly as possible is gaining increasing importance in the—omics science [1,2]. Indeed, the integration of lipidomics and proteomics data could be of major importance for recognizing biological pathways [3,4], identifying micro-organisms [5,6], evaluating food quality/safety [7–9], or for the diagnosis, prognosis and understanding of the molecular mechanisms involved in disease development [10–13]. Typically this is pursued using independent approaches aimed at obtaining separate proteomic and lipidomic data that are finally combined and interpreted.

Although, mass spectrometry (MS) with soft ionization methods, *e.g.*, electrospray (ES) and matrix assisted laser desorption (MALD) ionization (I), has become the common key technique in current analytical platforms for proteomic and lipidomic investigations, the sample pre-treatments are quite different. For protein sequencing, sample digestion with proteolytic enzymes (e.g., trypsin) is strictly required in the so called bottom-up approach. Digestion is generally performed in aqueous solution and presents a number of drawbacks, being time-consuming, tedious, and inconvenient for automation [14]. On the other hand, lipids are usually extracted by organic solvents, as chloroform and methanol, according to different protocols the most popular being those proposed by Folch [15] and Bligh–Dyer [16].

Recently, a protocol based on the combination of extraction solvents for sequential MALDI-MS analysis of both lipids and proteins contained in a single small paint sample was described [17–19]. In order to permit a simultaneous determination of lipids and proteins in a single MALDI MS experiment, a step forward is, however, required by devising a suitable pretreatment step compatible with both analyte classes possibly performed in situ, *i.e.* directly on the MALDI plate.

Various recent studies have reported the use of graphite for assisted laser desorption ionization (GALDI), in the form of solid

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plates [20], layers on a metal target [21], suspension in glycerol [22] or 2-propanol [23], or as a powder [24]. Pre-coating of MALDI-MS targets with silicone/graphite has been proposed for large-scale proteomic experiments allowing for increased sample loading and more efficient desalting by on-target washing [25]. Alternatively, graphite has been used as a target coating to improve matrix crystallization, leading to a considerable enhancement of signal intensities of peptides and proteins [26]. Moreover, GALDI has been used for imaging mass spectrometry (IMS) analysis of cerebrosides by forming a thin film of colloidal graphite on rat brain tissue [27].

Here, we propose a simple approach for simultaneous MALDI-MS analysis of proteins (peptides) and lipids where all the wet chemistry steps for micro-samples processing (e.g., protein digestion, desalting, matrix addition and concomitant lipid extraction) are performed in situ using a target plate modified by colloidal graphite.

2. Experimental section

2.1. Chemicals and reagents

α-Cyano-4-hydroxycinnamic acid (CHCA), ACTH 18-39 fragment, angiotensin I, renin, bovine serum albumin (BSA), α-caseins, lactoglobulin, ovalbumin, egg yolk powder, trypsin proteomic grade, ammonium bicarbonate, trilaurin (LLL), trimyristin (MMM), tripalmitin (PPP), and tristearin (SSS) were obtained from Sigma-Aldrich (Sigma Aldrich, St. Louis, MO, USA). HPLC grade water, acetonitrile (ACN), trifluoroacetic acid (TFA), and methanol (MeOH) (Sigma Aldrich) were used without further purification. α-Cyano-4-chlorocinnamic acid (CClCA) was synthesized according to a previously reported procedure [8]. Whole bovine milk samples were purchased at local supermarkets. Conducting graphite paint (code no. AGG3418) (in isopropanol) containing colloidal graphite and a thermoplastic resin as binder was obtained from Agar Scientific (Stansted, UK).

2.2. Analytical protocol

Protein standards and egg yolk sample were prepared in water at 0.5 mg/mL concentration. Bovine milk was simply diluted in water (1:10) and processed as described in the following.

Sample solutions (usually 1 μL) were applied on the target well previously covered by a small drop of conducting graphite paint.

For on spot protein digestion, 1 μL of trypsin solution (0.04 μg/μL in 50 mM NH_4HCO_3) was deposited, followed by 1 μL of 50 mM NH_4HCO_3. The target plate was placed in a Petri dish containing a wet piece of paper (to ensure a moisty environment), and kept in the oven at 40 °C for 10 min. Then, the spots were allowed to air dry and 1 μL of CClCA (10 mg/mL in ACN:MeOH 1:2 with 0.1% TFA) was added and the MALDI MS analysis (positive ion mode) was run simultaneously for peptides and lipids fractions.

2.3. Instrumentation

A Micromass M@LDI™-LR time-of-flight mass spectrometer (Waters MS Technologies, Manchester, UK) equipped with a nitrogen laser (337 nm wavelength), reflectron optics and a fast dual micro-channel plate (MCP) detector was used.

Applied potentials for positive ion acquisition mode (mass range 400–4000 *m*/*z*) were: pulse, 2610 V; source, 15,000 V; reflectron, 2000 V; MCP 1900 V. A time lag focusing (TLF) delay of 500 ns was used. The resulting spectra were averaged, background subtracted, and smoothed by a Savitzky–Golay algorithm.

For the sake of clarity, spectra are shown in the ranges 400–900 *m*/*z* for lipids and 900–4000 *m*/*z* for peptides. Mass calibration was performed using a peptide mixture composed of ACTH, renin and angiotensin for peptide analysis and a triacylglycerols mixture composed of trilaurin, trimyristin, tripalmitin, and tristearin for lipid analysis.

2.4. Database search

The raw MS files relevant to tryptic digests were searched on the Swiss-Prot non-redundant database utilizing the Mascot (or MS-Fit) server. The search was performed against the *Chordata* (or *Gallus gallus*) database for egg samples, *Mammalia* (or *Bos taurus*) for milk samples, setting methionines oxidation as a variable modification. A mass tolerance of 0.15 Da was set and a maximum of three missed cleavages were accepted. Alternatively, the search was performed using the FindPept database creating a list of all possible proteins contained in egg and milk and searching raw MS files relevant to tryptic digests against one protein at a time. Lipids were assigned by using the LIPID MAPS database [http://www.lipidmaps.org/] and literature information.

3. Results and discussion

In order to set up the best conditions for in situ proteolysis, model samples of single standard proteins such as BSA, β-lactoglobulin (β-lg), and α-casein were used. The protocol described in the experimental section was assessed after testing different incubation times, trypsin/protein ratios, amount of conducting graphite paint, MALDI matrices, and buffer concentrations. Fig. 1 reports the MALDI mass spectra acquired on BSA (A), β-lactoglobulin (B), and α-casein (C) after on spot digestion of the graphite supported samples by using CClCA as a matrix. BSA is a globular protein containing 607 amino acids (aa), 85 tryptic cleavage sites, and a large number of disulfide bridges which reduce trypsin accessibility; β-lactoglobulin is a globular protein of bovine whey that contains 162 aa and two disulfide bonds (Cys66–Cys160 and Cys106–Cys119); α-s1-casein is a mammary gland specific protein (containing 214 aa) rather simple to digest due to the absence of disulfide bridges. The *m*/*z* values reported in the spectra of Fig. 1 were confidently identified after database search as tryptic peptides of BSA, β-lg and α-s1-casein with sequence

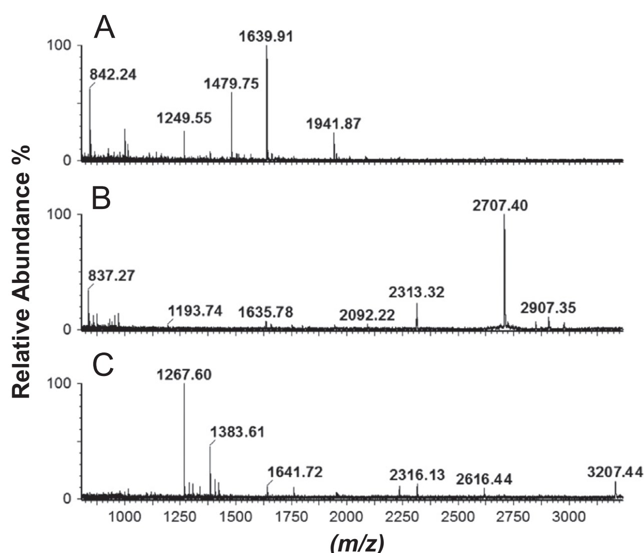

Fig. 1. MALDI-TOF mass spectra of the tryptic digest obtained from direct on spot digestion of BSA (A), β-lactoglobulin (B), and α-casein (C) model proteins.

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