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Laser desorption/ionization mass spectrometry of lipids using etched silver substrates

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ABSTRACT

Silver-assisted laser desorption/ionization mass spectrometry can be used for the analysis of small molecules. For example, adduct formation with silver cations enables the molecular analysis of long-chain hydrocarbons, which are difficult to ionize via conventional matrix-assisted laser desorption ionization (MALDI). Here we used highly porous silver foils, produced by etching with nitric acid, as sample substrates for LDI mass spectrometry. As model system for the analysis of complex lipid mixtures, cuticular extracts of fruit flies (*Drosophila melanogaster*) and worker bees (*Apis mellifera*) were investigated. The mass spectra obtained by spotting extract onto the etched silver substrates demonstrate the sensitive detection of numerous lipid classes such as long-chain saturated and unsaturated hydrocarbons, fatty acyl alcohols, wax esters, and triacylglycerols. MS imaging of cuticular surfaces with a lateral resolution of a few tens of micrometers became possible after blotting, i.e., after transferring lipids by physical contact with the substrate. The examples of pheromone-producing male hindwings of the squinting bush brown butterfly (*Bicyclus anynana*) and a fingermark are shown. Because the substrates are also easy to produce, they provide a viable alternative to colloidal silver nanoparticles and other so far described silver substrates.

1. Introduction

Arguably, the most widely used laser-based technique for the analysis of non-volatile biomolecules by mass spectrometry (MS) is matrix-assisted laser desorption/ionization (MALDI) [1]. However, two drawbacks of conventional MALDI are a typically high matrix-derived ion background in the low *m/z* range and the fact that some important lipid classes such as *n*-alkanes or olefins and sterols, which are lacking functional groups, are difficult to ionize by protonation/deprotonation or alkali metal (Na/K) cationization, i.e., via the typical MALDI ionization pathways. Probably for similar reasons, our recently introduced orthogonal time-of-flight (o-TOF) LDI-MS approach also failed to generate molecular ions of *n*-alkanes [2]. In this method, hydrophobic and the laser energy strongly absorbing substrates, namely insect cuticles, served for deposition of the laser energy and thermal desorption of numerous endogenous lipid classes, or of compounds exogenously applied to a similar substrate (e.g., to a fly wing). However, the inability of the approach to ionize alkanes is a substantial shortcoming for chemical ecology applications, because in many species of insects saturated hydrocarbons constitute a major class of the cuticular lipid bouquet. Numerous insect species also use specific, straight-chain as well as methyl-branched alkanes as pheromones [3].

The standard method for the analysis of cuticular extracts is gas chromatography (GC) and in particular if coupled with MS analysis following electron ionization (EI) of separated compounds [4]. An advantage of the EI-based GC/MS method is that, by comparison with databases, a single GC/MS analysis can in many cases enable unambiguous identification of compounds (e.g., with regard to branching positions in methyl-branched hydrocarbons or double bond positions in mono- and polyenes [5]). However, because of decreasing volatilities and typically also decreasing yields of molecular ions with molecular weight (MW), very long-chain hydrocarbons and more polar lipids (e.g., alcohols and acetates) may not be amenable to conventional GC/MS analysis. Numerous papers have recently shown that important classes of cuticular insect pheromones (e.g., very long-chain fatty acyl alcohols [6]

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and triacylglycerols [7]) can therefore be missed by this standard method [8].

One way to enable MALDI MS analysis of even very long-chain alkanes is the use of lithium 2,5-dihydroxybenzoate [9], a lithium salt derived from the classical MALDI matrix 2,5-dihydroxybenzoic acid (DHB). This matrix was, for example, used to characterize the cuticular extract of numerous insects [10] and also to image the distribution of sex pheromones on the cuticle of male *Drosophila melanogaster* fruit flies [11]. Recently, lithiated isoforms of further classical MALDI matrices were introduced that partly provided a further improved analytical sensitivity [12]. Alkanes were detected with limits of detection of ~5 pmol, wax esters with ~100 fmol. An even more common method for laser desorption/ionization (LDI) of *n*-alkanes and further small molecules for MS analysis is the use of transition metals with electronic configurations that enable both an efficient deposition of the laser energy and an efficient coordination of molecular orbitals. Because of its high coordination affinities, especially silver is used for the generation of molecular $[M + Ag]^+$ ions of various lipid classes, including long-chain *n*-alkanes [13,14], olefins and sterols [15], and technical waxes [16]. Silver salts are also frequently added to conventional MALDI matrices for enhancing the ion yields of technical polymers [17], as well as to the stationary phase of thin-layer chromatography (TLC) plates to improve chromatographic separation of alkanes and alkenes [18]. Other transition metals such as gold were also considered but have generally been found less suited than silver (e.g., [17]).

A comprehensive review on UV-LDI MS with silver substrates has recently been provided by Sekuła et al. [19]. On the basis of the literature, the currently most typical way of preparing silver substrates for LDI MS appears to be the use of colloidal, nanometer to ten nanometer-sized silver particles. Colloidal silver has also been used for coating of tissue and subsequent MS imaging (MSI) experiments. For example, in this way the lateral distribution of wax esters on the cuticle of *Arabidopsis thaliana* leaves and petals was visualized [20]. The nanoparticles serve for efficient absorption of the nanosecond-pulsed ultraviolet (UV)-laser light at the laser wavelength (commonly 337 or 355 nm as produced by N_2 and frequency-tripled Nd:YAG lasers, respectively). Presumably, this produces a thermal spike that is in turn inducing desorption of analyte molecules attached to the surface [21]. At the same time, abundant gaseous silver ions are produced by laser ablation and interact with the neutral analyte molecules in the ejected material cloud. Whereas colloidal silver particles are commercially readily available, a certain drawback is that the suspensions cannot be prepared in higher concentrations. To avoid agglomeration, typically stabilizing ligands are therefore added, which can lead to unwanted background signals. Moreover, a uniform coating of hydrophobic surfaces may be more difficult to achieve with the highly polar aqueous solutions.

By adapting sample preparation protocols from secondary ionization mass spectrometry (SIMS) [22], the use of a thin silver layer that was produced by sputtering was also described and, for example, used for LDI MSI of cholesterol in brain tissue sections [23]. The sputter approach provided a particular uniform coating, which in turn enabled MSI with high lateral resolution. However, the requirement of precise control of the optimal, tissue type-dependent film thickness renders this approach technically challenging [23]. As another variant, the use of thin porous silver substrates, produced by galvanic (electrochemical) deposition was recently investigated and, for example, used for the analysis of saturated and unsaturated free fatty acids (FFA) [24]. In yet another approach, nanostructure-initiator mass spectrometry (NIMS) substrates were coated with $AgNO_3$ and with sputtered silver [25,26]. Notably, coarse images of the lateral distribution of cholesterol in mouse brain tissue sections were achieved after blotting the tissue onto the NIMS substrate [25]. Furthermore, the method was also used to image fingerprints [27].

Another parameter of relevance in the analysis of small molecules is the volatility of the compounds. Many compounds exhibit vapor pressures that result to a rapid evaporation in the ion source, in particular at the high vacuum of $\sim 10^{-7}$ mbar of axial-TOF instruments. This could be one reason why most silver-assisted LDI MS results appear to have been collected with instruments that rather adopt a fine-vacuum ion source, where pressures of ~0.1–1 mbar are most typically realized (e.g., [13,20,26]), and to a lower extent using atmospheric pressure (AP-)LDI conditions (e.g., [24]).

Here we describe the use of etched silver foils as sample substrates for UV-LDI MS. Although etched silver foils are widely used as substrates for SIMS [28], their utilization for LDI MS applications has to our knowledge not been reported. We demonstrate the high analytical sensitivity as obtained for UV-LDI-o-TOF MS with these substrates by the example of complex lipid mixtures as extracted from insect cuticle, and in order to characterize LODs, by studying selected synthetic compounds. Second, we show that the highly porous substrates can also be used for blotting lipids from other surfaces for successive MSI experiments. In this way, a lateral resolution in the low ten-micrometer range could be achieved. The examples of a butterfly wing and a fingermark are presented.

2. Experimental design

2.1. Etching of silver substrates

To start the experiments with a material of well-defined purity, silver substrates of 0.5 mm thickness were initially purchased from Goodfellow (art. no. 300652587, Bad Nauheim, Germany; purity > 99.95%) or from Sigma–Aldrich (art. no. 345075, Steinheim, Germany; purity > 99.9%). However, a drawback of these materials is their relatively high price. Later on, all experiments were, therefore, carried out with substrates obtained from a goldsmith supplier (art. No. 98059959, zu Jeddeloh, Winsen, Germany, purity > 99.9%), which can be used equally well. In particular, a similar degree of chemical background (see below) is obtained. Using this material, the costs (calculated for the silver plates only) for producing a 5×5 mm^2 substrate is roughly about 0.1 €.

The initial foils were cut to smaller pieces of about 4×4 mm^2. To obtain an even surface, foils were typically flattened with a hydraulic press; thereby the overall area increased to about 25–30 mm^2. Substrates were then washed with pentane, acetone, ethanol, and deionized water, and etched using 20–30% nitric acid (HNO_3) at 50 °C, until they appeared grayish due to the onset of extensive light scattering by the rough surface (Fig. 1A). Etched surfaces were washed 10 times with water and stored in water before further use. Secondary electron microscopy (SEM) using a field-emission Hitachi S800 instrument (Tokyo, Japan) revealed that a highly porous homogenous surface was produced by the HNO_3 etching, with characteristic structures in the micrometer range (Fig. 1B, C).

2.2. Sample preparation

All standards were purchased from Sigma–Aldrich: 1-tricosanol (C23:0-OH; prod.-no. T9524), *n*-tricosane (C23:0; 263850), *cis*-9-tricosene (C23:1; 859885), *n*-hexacosane (C26:0; 241687), dodecyl arachidate ($C_{32}H_{64}O_2$; C32:0 wax ester; A8671), glyceryl trioleate (triolein; T7140), and a synthetic mix of even-chain *n*-alkanes (C20–C40; 94234). They were diluted to desired concentration in chromatography grade heptane. Aliquots of 0.1 µL were spotted onto the silver substrates. Typically, this volume spread out over an area of approximately 4 mm in diameter, thus covering the central to full area of the prepared silver foils.

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