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High-throughput label-free detection of Ochratoxin A in wine using supported liquid membrane extraction and Ag-capped silicon nanopillar SERS substrates

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Abstract:

Mycotoxins are toxic secondary metabolites produced by fungi, found in a variety of food sources and cause diseases and death in both humans and animals. On-site and easy to use detection would be significant improvement considering the currently used methods. We present an approach, based on surface-enhanced Raman spectroscopy (SERS), where label-free detection of Ochratoxin A (OTA) was achieved in a multiwell SERS detection platform. When quantifying OTA in wine samples the SERS-based detection was used in combination with high throughput supported liquid membrane (SLM) extraction. The obtained detection limit in white wine was 115 ppb and we found that the SERS-based quantification is comparable with LC-MS. However, we also found that the matrix effect from red wine was interfering with both SERS and LC-MS detection. The SLM-SERS method provides a time and cost effective approach for OTA detection, which is a clear advantage over other extraction and direct detection approaches.

Keywords:

Ag SERS substrate, direct label-free detection, Ochratoxin A, parallel artificial liquid membrane extraction, wine

1. Introduction

Ochratoxin A (OTA), a secondary fungal metabolite secreted mainly by *Aspergillus* and *Penicillium* fungi species (Anfossi, Giovannoli, & Baggiani, 2016) is found in a large variety of foods and beverages (Malir, Ostry, Pfohl-Leszkowicz, Malir, & Toman, 2016). This mycotoxin is highly toxic, mutagenic and carcinogenic to most mammalian species (Pfohl-Leszkowicz & Manderville, 2007), therefore the European Commission has established the maximum level of OTA content in foods and foodstuffs (Commission, 2006b). Mycotoxin screening is often limited and mostly done on lot/batch

level, either at the border control or at markets as laid-out in the regulation (EC 401/2006) (Commission, 2006a), which results in the rejection and destruction of the non-compliant lots/batches. However, the irregular fungal growth e.g. throughout a field leads to the production of mycotoxins in ‘hot-spots’ (Shephard, 2008), resulting in uneven distribution of OTA in food samples and increasing inaccuracy of batch-based sampling and analysis.

The main limitation in screening is due to the currently used robust and reliable analytical approaches, which require sophisticated, costly equipment and a well-controlled laboratory environment. In addition, these methods are time-consuming and require trained operators. The used analysis techniques are mostly based on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) (Campone, et al., 2018) or fluorescence detection (Bascarán, de Rojas, Choucino, & Delgado, 2007) and enzyme-linked immunosorbent assays (Liang, et al., 2018; Sun, et al., 2018). Depending on the sample type, most analytic procedures require sample pretreatment methods (Bascarán, et al., 2007; Campone, et al., 2018; Vidal, Bertolín, Ezquerro, Hernández, & Castillo, 2017).

Detection at point-of-production would provide a significant financial and sustainability gain, since it could be used to prevent the mixing of the contaminated raw materials from ‘hot-spot’ in the whole lot/batch, hence greatly reducing the risk of the whole lot being rejected. Unfortunately, current analytical methods are not suitable for being implemented at production sites due to cost, capacity and complexity, therefore significant efforts are focused on the development of reliable sensors and sensing platforms that enable time and cost-effective detection of OTA (Adley, 2014). Various sensors and biosensors have been developed with the potential for detection of OTA in different samples (Bostan, et al., 2017; Ha, 2015). Biosensors are cost-efficient and specific, but they often need several surface modification steps (El-Moghazy, et al., 2016; Liu, et al., 2018; Oliverio, Perotto, Messina, Lovato, & De Angelis, 2017; Song, et al., 2018) and often are used in combination with sample pre-treatment (Ashley, et al., 2017; Bunyakul & Baeumner, 2015; Zhu, et al., 2015). In addition, the sensor stability

is affected by the stability of the recognition element and the reproducibility might be compromised by time-consuming surface modification steps (Bazin, Tria, Hayat, & Marty, 2017; Zhao, et al., 2015).

Surface-enhanced Raman spectroscopy (SERS) is an advanced analytical technique widely used in different areas including food safety and quality control (Durucan, Rindzevicius, Schmidt, Matteucci, & Boisen, 2017; Gillibert, Huang, Zhang, Fu, & de La Chapelle, 2018; Zheng & He, 2014), biochemistry and biology (Hakonen, et al., 2016; Morelli, Zór, et al., 2017), disease diagnosis (Lauridsen, et al., 2017), forensic detection (Hakonen, et al., 2018) as well as in environmental studies (Hakonen, et al., 2017; Patze, et al., 2017). This well-established vibrational spectroscopic technique has also been used for OTA detection (Ganbold, et al., 2014; Gillibert, Triba, & de la Chapelle, 2018; Zhao, et al., 2015). Recently, this technique has also been presented as a suitable platform for direct detection of several mycotoxins (Li, Yan, Tan, Lu, & Han, 2019).

Generally, in sensor development, direct, label-free detection of a trace amount of target molecules in a complex sample matrix is challenging due to the presence of interfering compounds (Andreasen, et al., 2018; Liu, et al., 2018; Morelli, Zór, et al., 2017). To improve the selectivity of the detection method, the assays often include either sample pretreatment steps (Ashley, et al., 2017; Bunyakul & Baeumner, 2015), surface functionalization (Oliverio, et al., 2017), and/or labeling methods (Cao, et al., 2017) which add to the cost and complexity of the detection method.

In recent years, we have been developing SERS-based detection methods, where we combine SERS with simple sample pretreatment steps for direct detection of analytes in complex sample matrices (Durucan, et al., 2017; Morelli, Andreasen, et al., 2017; Morelli, et al., 2018; Morelli, Zór, et al., 2017). There are a wide range of sample preparation, clean-up and extraction methods successfully used in combination with various sensing approaches, such as liquid-liquid extraction (El-Moghazy, et al., 2016; Zhu, et al., 2015), solid phase extraction (Huang, et al., 2018; Park & Seo, 2019), immunoaffinity column (Abd-Elghany & Sallam, 2015) and supported liquid membrane (SLM) extraction

(Andreasen, et al., 2018; Morelli, Andreasen, et al., 2017). Although the choice of sample pretreatment method depends on both the sample matrix and the detection method, immunoaffinity column is widely used for the extraction/pre-concentration of OTA (Abd-Elghany & Sallam, 2015; Bascarán, et al., 2007) due to its high selectivity, despite its high cost, limited lifetime, and usage of large amounts of solvents. SLM extraction is based on a single mass-transfer step, driven by the pH difference between two aqueous phases, separated by a hydrophobic organic solvent impregnated in a porous membrane (Jönsson & Mathiasson, 1992). SLM extraction, although is not highly specific, is an attractive sample preparation method, which has been applied for extraction of numerous compounds from various complex media (Morelli, Andreasen, et al., 2017; Palmarsdottir, Thordarson, Edholm, Jönsson, & Mathiasson, 1997; Vårdal, et al., 2017). Additionally, membrane extraction has been implemented for extraction of OTA from wine (Almeda, Arce, & Valcárcel, 2008; Romero-González, Frenich, Vidal, & Aguilera-Luiz, 2010).

In this study, we present the application of a high throughput SLM extraction method used in combination with a multiwell SERS detection unit for the first time. For SERS, a highly uniform (Schmidt, Hübner, & Boisen, 2012) silver (Ag)-capped silicon nanopillars SERS substrate was immobilized in the bottom of a multiwell platform for direct label-free detection of OTA. As a case study, commercial red and white wine samples spiked with OTA were used to evaluate the compatibility and usability of the proposed extraction and detection method with real samples.

2. Experimental

2.1. Chemicals

Ochratoxin A (OTA), ammonium hydroxide (25%), acetic acid (AA), acetonitrile (ACN), ethanol and dodecyl acetate (DDA) all were supplied by Sigma-Aldrich (St. Louis, MO, USA). The 2 mg/ml stock solution of OTA was prepared in ethanol and then OTA working standard solutions were ob-

tained by diluting the stock solution using ammonium water (AW) (25 mM, pH 10), AA (0.2 M) or acidified ammonium hydroxide (AW-AA) to the desired concentration. Solutions were prepared in ultrapure water (Millipore Corporation, Billerica, MA, USA). The high throughput SLM platform, parallel artificial liquid membrane extraction (PALME) developed by Gjelstad et al (Gjelstad, Rasmussen, Parmer, & Pedersen-Bjergaard, 2013), was utilized for SLM extraction, included commercially available 96-well polypropylene microplates with a volume of 0.5 mL per well. The 0.5 mL well plate was used as a donor plate (Sigma-Aldrich), while a MAIPN4550 96-well MultiScreen-IP Filter Plate with 0.45 μm pore size polyvinylidene fluoride membrane (Merck Millipore, Burlington, MA, USA) in the bottom that was used as an acceptor phase. A polyolefin polymeric sealing tape for multiwell plates (Nalgene Nunc, Rochester, NY, USA) was used to cover the acceptor wells to avoid solvents evaporation and spilling during the agitation step. The white and red wine samples were bought from a local supermarket and were stored in the refrigerator before analysis.

2.2. Instruments

All SERS measurements were performed with a DXRxi Raman Imaging Microscope (Thermo Fisher Scientific Inc., Waltham, MA, USA). The optical microscope was coupled to a spectrometer 5 cm^{-1} FWHM and $\pm 2 \text{ cm}^{-1}$ wavenumber accuracy. SERS spectra were collected at 780 nm with a laser power of 10 mW, with a 10x objective lens, 50 μm slit and an estimated laser spot of 3.6 μm diameter. All the spectra were collected three times for 0.05 s in each spot. SERS maps were collected on the whole surface of each chip (three maps/three times scanning on each chip), with a 100 μm collection step and an overall collection time of 3 min. Also, triplicate measurements were acquired for all experiments.

Liquid chromatography was carried out on an Ultimate 3000 uHPLC (Thermo Fisher, Waltham, MA, USA) instrument. The separation was performed on a Gemini, C18 (3 μm , 2 mm $\times$ 100mm) col-

umn from Phenomenex (Torrance, CA, USA). ACN/water (70:30 v/v) was used as a mobile phase. Mass spectra were acquired by a Bruker EVOQ triple quadrupole mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an ESI interface in the positive-ion mode. A scanning electron microscope (SEM) (Zeiss Supra 40VP field emission scanning electron microscope) was used for the characterization of the Ag-capped silicon nanopillars.

2.3. Fabrication of SERS chip and the multiwell measurement chamber

The Ag-capped nanopillars substrates were fabricated based on the previously described method (Schmidt, et al., 2012; Wu, et al., 2015). The fabrication was carried out using reactive ion etching over a 4-inch Si wafer with an Advanced Silicon Etcher (Surface Technology Systems MESC Multiplex ICP) at an SF₆: O₂ flow ratio of 1.12, platen power of 120 W and a chamber pressure of 36 mTorr followed by 1 min oxygen plasma cleaning. Lastly, Ag metal (225 nm) was deposited over the whole wafer using e-beam evaporation with a rate of 10 Å/s. Subsequently, the wafer was diced into single 4 x 4 mm chips using a Laser Micromachining tool (3D-Micromac AG, D-09126 Chemnitz, Germany).

SERS spectra were obtained directly from liquid samples utilizing a custom made multi-well system. The 24-well system consisted of chip-sized wells on a microscope slide (75 x 26 mm). Wells with an inner volume of 38.5 µL were created by laser ablating (Epilog Mini 18 30 W, Epilog, USA) 24, 4.5 x 4.5 mm size openings in a 1.5 mm thick poly(methyl methacrylate) (PMMA) (Axxicon Moulds, Eindhoven, The Netherlands) sheet. In addition, a 0.15 mm thick pressure sensitive adhesive (ARcare 90106, Adhesive Research, Limerick, Ireland) cut with a Silhouette Cameo Plotter (Silhouette America, Inc., Utah, US) was used to assemble the PMMA piece with well opening with a flat glass microscope slide (t = 1 mm), serving as the bottom of the wells. The individual SERS chips were placed in the wells, prior to measurements.

2.4. SLM procedure

The SLM extraction was set up on the PALME platform according to the reported procedure (Vårdal, et al., 2017). Briefly, 250 μ L of OTA solution in AA (0.2 M, pH 3) or wine samples (pH 3-3.5) was pipetted into the donor well. Also, 2.5 μ L of DDA was dropped and dispersed on the surface of the membrane at the bottom of the acceptor plate. Then the acceptor plate was placed on top of the donor plate and they were fitted tightly. Subsequently, 50 μ L of AW 25 mM, pH 10 as an acceptor phase was pipetted into the acceptor well to give a theoretical OTA concentration factor of 5. Finally, a polymer sealing tap was applied to cover the acceptor plate in order to avoid the spilling of the acceptor phase during agitation step. This set up was fixed on a vibrational shaker (VWR[®] Mini Shakers, 230V, Radnor, PA, USA) and agitated vigorously at a rate of 900 rpm for 30 min. For SERS-based detection 30 μ L of the acceptor phase was transferred into a vial and was acidified to pH 3 with 30 μ L of AA 0.2 M (AW-AA 1:1 v/v). Finally, 30 μ L of the acidified extract was pipetted into a measuring chamber, which contained the Ag SERS substrate. For LC-MS analysis, after SLM extraction and acidification as described above, the samples were mixed with ACN (1:1 v/v) and transferred to HPLC vials.

2.5. Preparation of the sample

For detection of OTA in wine samples, standard solutions of OTA were spiked into the red and white wine. OTA was extracted from the spiked wine samples using the SLM extraction procedure and was detected as mentioned above both with SERS and LC-MS.

2.6. Data analysis and SEM imaging

Samples were measured on three SERS chips as described in section 2.2 and each chip was scanned three times. Therefore, SERS maps ($\sim 3.5 \times 3.5$ mm each) for a given OTA concentration con-

sisting of ~1200 SERS spectra/maps were obtained from each SERS chip. Next, each SERS map was averaged to yield a single SERS spectrum representing the map. The baseline correction was performed on the averaged spectrum in the spectral region between 980 and 1050 cm^{-1} and the SERS intensity of the characteristic OTA vibration mode (Gillibert, Triba, et al., 2018) at 1003 cm^{-1} was plotted vs. OTA concentration (Figures 2, 4 and 5). The SERS data error bars represent standard deviations between three SERS chips for a given OTA concentration. The data treatment was performed using OMNICxi Raman imaging and MATLAB R2018b software packages. The limit of detection (LOD) was calculated based on $3\sigma/s$ where (σ) is the standard deviation of blank and (s) is the slope of the calibration curve. To characterize the fabricated AgNP SERS substrates, scanning electron microscopy (SEM) images were obtained using a Zeiss Supra 40 VP SEM microscope (Carl-Zeiss, Germany). For HPLC measurements the data files were processed using Quant Analysis (Bruker Daltonics, Bremen, Germany). Ion chromatograms of m/z 404.0895 $\pm$ 5ppm (OTA) were extracted and integrated. Concentrations of OTA were calculated based on the obtained linear calibration curves. All the plotted calibration curves with confidence and prediction intervals were provided (supplementary material) and an F-test was performed for evaluation the correlation between SERS and LC-MS data the correlation between LC-MS and SERS data was determined based on the obtained R^2 values.

3. Results and discussion

3.1. Principle of SLM extraction and optimization of SERS-based detection of OTA

Taking into consideration the various sample matrices where OTA can be present, as well as the rather low maximum levels of OTA content in food and beverages (2 ppb) (Commission, 2006b), a sample clean-up and concentration steps are commonly used in combination with the various detection methods (Almeda, et al., 2008; Andrade & Lanças, 2017; Andreasen, et al., 2018; Giovannoli, Passini, Di Nardo, Anfossi, & Baggiani, 2014; Morelli, Andreasen, et al., 2017). As briefly described

in the Introduction, in SLM extraction a thin layer of an apolar organic solvent is deposited in a porous membrane, which separates two aqueous phases with different pH. The working principle of the extraction is based on the transportation of the target analyte from the sample (aqueous donor phase) where the analyte is not ionized through the organic phase to the aqueous acceptor phase in the ionized/charged form. As it is illustrated in Figure 1a, in the donor phase (AA, pH 3) OTA is in the neutral form (pKa value of 4 (for carboxyl group of phenylalanine) and 7.1 (for hydroxyl group of phenol)(Malir, et al., 2016)). As illustrated in Figure 1a in the PALME setup, the volume of the acceptor (50 μ L) is smaller than the donor (250 μ L), enabling a theoretic 5-fold concentration of the sample. The uncharged molecules will be extracted into the organic phase (DDA) and subsequently they will diffuse to the acceptor phase (AW, pH 10) as deprotonated OTA with a negative charge that prevents OTA to diffuse back through the apolar solvent membrane. In this case, the OTA extracted from buffer or sample matrix (wine) was measured with SERS as depicted in Figure 1b, using the multiwell platform (Figure 1d). The SEM image of the used SERS substrate, with Ag-capped silicon nanopillars presented in Figure 1c.

Figure 1. a) Schematic illustration of OTA extraction with SLM using the PALME setup; b) SERS-based detection of OTA after extraction and acidification; c) SEM image of Ag-capped silicon nanopillars used as SERS substrate; d) multiwell system with incorporated SERS chip; and e) SERS spectra of OTA (1 ppm) in AW, AA and AW-AA on Ag SERS substrate; inset: SERS spectra of OTA in AW-AA on Au and Ag SERS substrates.

Initially, we evaluated the direct, label-free SERS-based detection of OTA using both Ag and gold (Au)-capped silicon nanopillars. As it is presented in Figure 1e inset, it was found out that OTA in AW-AA has a good affinity towards Ag-capped nanopillars. Indeed, OTA could anchor on Ag atoms

on the surface of nanopillars through the formation of a coordination bond or through simple electrostatic attraction due to the presence of oxygen-containing functional groups such as carbonyl, carboxylic and phenolic groups on its structure (Dinh, Quy, Huy, & Le, 2015; Ebrahiminezhad, Bagheri, Taghizadeh, Berenjian, & Ghasemi, 2016). Also, the π - π interaction between C in aromatic benzene rings and d orbital of Ag could be responsible for OTA affinity to Ag SERS substrate (Anderson, McDevitt, & Urbach, 1984). Moreover, there is a possibility of interaction between the chlorine group of OTA and the Ag surface. In other words, chlorine atoms could be adsorbed on the Ag surface (Gava, Kokalj, de Gironcoli, & Baroni, 2008).

When further optimizing the detection step, it was observed that in AA (pH 3) when OTA is neutral, the SERS signal obtained from OTA is significantly higher than when the analyte was negatively charged in AW (pH 10). This effect could be explained by a previous report, where it was shown that an alkaline condition leads to the formation of open-ring OTA and consequently lower SERS signals (Bazin, et al., 2013). Therefore, for further measurements, the samples were acidified with AA to reach pH 3 after SLM extraction (Figure 1e) as also illustrated in Figure 1a. The typical spectra of OTA (in the range of 950-1150 cm^{-1}) is presented in Figure 1e. The appeared peak at 1003 cm^{-1} was used for quantification.

3.2. Quantification of OTA using SERS

After optimization of the experimental parameters, OTA was measured on Ag-SERS substrate in different concentrations from 0.02 to 1 ppm in AW-AA. Typical SERS signals for the increasing concentration of OTA and a typical calibration curve are presented in Figure 2a and b, respectively. We found a good linear dependency between Raman intensity of the representative OTA peak at 1003 cm^{-1} and OTA concentration in the range of 0.02-1 ppm with a linear calibration equation of $y=117.08x + 18.601$ and correlation coefficient of 0.9969. Also, LOD was calculated 0.02 ppm according to the

~~obtained calibration curve.~~ In Figure S1a it is shown the calibration curve with the confidence and prediction band.

Figure 2. a) SERS spectra of different concentrations of OTA in AW-AA (0-1 ppm); b) Calibration curve of OTA in AW-AA with a linear range from 0.02 to 1 ppm.

3.3. SLM extraction and OTA detection from real sample

OTA can be present both in several foodstuffs (e.g. cereals, grapes, coffee beans) as well in foods (e.g. flour, bread) and beverages (e.g. coffee, wine). There are varieties of extraction methods, which are suitable for extraction of mycotoxins from a certain type of food matrices. For instance, in the case of cereals, it is common to use solid and liquid phase extraction as nonspecific extraction methods prior to HPLC-based analysis (Zhou, Xu, Huang, Cai, & Ren, 2017). Recently liquid-based extraction has also been used in combination with SERS detection (Li, et al., 2019). SLM extraction, although nonspecific, has been shown to be suitable for extraction of OTA from wine (Almeda, et al., 2008; Romero-González, et al., 2010), therefore was chosen for our case study. To verify the applicability of the method, SLM extraction was implemented to extract OTA from spiked white and red wine samples. It is noteworthy that as the pH of wine samples used for this study was acidic (below pH 4) by nature, it was not necessary to lower the pH before SLM extraction. Due to the complexity of wine samples, especially the presence of anthocyanin (da Silva, Guerra, Klein, & Bergold, 2017) in red wine, we were not able to record the SERS signal from OTA by measuring directly in wine without SLM clean up. Figure 3 shows the measured spectra of white wine and spiked white wine with 0.2 ppm OTA, before and after SLM extraction. It is clear that without SLM extraction OTA cannot be detected directly from white wine. As can be seen, there is no peak at 1003 cm^{-1} , where OTA has been detected previously, neither in white wine (blank) or in OTA spiked white wine before SLM extrac-

tion. The absence of signal in the spectrum of the spiked sample could be attributed to the complex composition of wine (Campone, et al., 2018; Giovannoli, et al., 2014) and the presence of interfering compounds in its matrix. Surface fouling of the SERS substrate by non-Raman active compounds has been previously reported (Morelli, Zór, et al., 2017). However, we were able to detect OTA in the wine samples spiked with OTA (Figure 4) after SLM extraction.

Figure 3. SERS spectra of white wine and spiked (0.2 ppm) white wine before and after SLM extraction; Inset: SERS spectrum of OTA detected from spiked white wine after SLM extraction.

In order to assess the efficiency of the SLM extraction and to define the detection limit and dynamic range of the sensor, OTA was SLM extracted from AA, white and red wine. Wine samples were spiked with various concentrations of OTA (0.05, 0.1, 0.35, 0.5, 0.75 and 1 ppm) and extracted by SLM extraction, acidified and measured using a SERS-based detection as previously described. The calibration curves and the related SERS spectra are presented in Figure 4 and 5. By comparing the calibration curve of OTA extracted from AA with OTA measured directly in AW-AA, the enrichment factor was calculated by dividing the donor concentration before, with the acceptor concentration after SLM extraction. We found that the enrichment factor obtained with LC-MS (3.2 ± 0.82 , $n=3$) and SERS (2.3 ± 0.34 , $n=3$) were comparable but lower than the theoretical concentration factor of 5. However, extracting from wine samples, we noticed that the enrichment factor obtained with SERS was 0.75 ± 0.04 and 0.48 ± 0.06 for white wine and red wine, respectively, which is lower than the extraction done from AA (2.3 ± 0.34). This decrease is probably due to the interfering compounds, which are not SERS active but may foul the surface of the sensor (Morelli, Zór, et al., 2017) leading to lowered signal in SERS, considering that the enrichment for white wine obtained with LC-MS was comparable (4.3 ± 0.98) with the one obtained when extracting OTA from AA. In addition, we also found that the

enrichment factor calculated based on data obtained by LC-MS from red wine extract was much higher (8 ± 1.5) than when extraction was performed from AA, which is a false positive and overestimation of the extracted OTA amount as a result of the interfering compounds from red wine. As the LC-MS analysis did not include internal standards a too high concentration may have been measured by LC-MS using external standard due to a positive matrix effect. This was not examined further in this study.

Figure 4. a) SERS spectra of OTA extracted from AA (0-1 ppm). Inset: related calibration curve (0.05-1 ppm); SERS spectra of OTA extracted from b) white wine (0-1 ppm), Inset: calibration curve of OTA in white wine and c) red wine (0-1 ppm), inset: calibration curve of OTA in red wine.

Figure 5. Calibration curve of OTA in white (a) and red wine (b) after SLM extraction using SERS and LC-MS. Inset: Correlation between data obtained with SERS and LC-MS

Based on our results we can conclude that a more efficient clean-up method with a larger enrichment factor is needed, especially for red wine samples which will enable reduction of matrix effects by dilution. Nevertheless, when comparing the data obtained from SERS and LC-MS (Figure 5), considering the respective enrichment factors, we found that the obtained data are comparable, ~~Based on a performed F test we found a correlation between SERS and LC MS for red wine.~~ based on the R^2 values (0.88 for white and 0.85 for red wine, respectively) obtained, when correlating the LC-MS result with the obtained SERS data (inset Figure 5a and b). However, it needs to be taken into consideration that the background signal in the case of red wine observed in the SERS data (Figure 4c) indicates the presence of sample matrix, which could have also affected the results obtained with LC-MS. Considering the effect of interferences after SLM, we were able to quantify OTA with SERS using cali-

bration curves plotted with OTA spiked into the sample matrix (red or white wine) and SLM extracted. Figure 4 shows the calibration curves obtained after SLM using AA and real sample matrix and the confidence and prediction bands are represented in Figure S1b, c and d. It was found that, in the concentration range of 0.05–1 ppm, the linear equation of $y = 93.266x - 2.6246$ ($r = 0.9983$) and $y = 54.194x + 3.072$ ($r = 0.967$) were obtained for OTA extracted from white wine and red wine, respectively. Also, in the case of white wine sample the LOD was 155 ppb (0.15 ppm) and 306 ppb (0.3 ppm) for red wine.

When evaluating the performance of the SERS sensor we found that the relative standard deviations in the case of standard calibration curve (Figure 2b) was $17 \pm 6\%$ (6 concentration point, each in triplicate), while this variation was higher ($20 \pm 11\%$) in the case of calibration in wine samples (Figure 4c and d) obtained after SLM extraction (2 times, 6 concentration points in triplicates). The found variation is comparable with values previously obtained with the same SERS nanopillar (Morelli, Zór, et al., 2017). Additionally, the sensitivity of the sensor was similar when comparing the calibration curve in AW-AA (Figure 2b) and AA (Figure 2a) after SLM, with LOD of 65 ppb and 116 ppb, respectively. However, based on the slope of the linear fitting, the sensitivity of the calibration curve obtained from white wine (Figure 4b) was three times lower, compared to AA (Figure 2a), with LOD of 155 ppb. Additionally, the sensitivity of the calibration obtained from red wine (Figure 4c) was two times lower versus the white wine, with LOD of 306 ppb, probably due to the sample matrix, present in red wine samples even after extraction. Moreover, in the case of the calibration curve obtained from red wine the R^2 value was 0.74, while this was found to be 0.99 and 0.98, when the extraction was performed from white wine and AA respectively.

Keeping in mind that simple, cost efficient, direct and label-free detection of OTA from real samples is challenging, even when combined with sample clean-up and concentration methods, the here achieved LOD using SERS with SLM extraction is promising. However, given the LOD achieved

with the SERS sensor (155 and 306 ppb), the enrichment in the SLM extraction process needs to be improved in order to be able to detect levels of OTA according to the current legislation (Commission, 2006b). An increased enrichment with the PALME setup can be achieved by further optimization of the oil phase or by decreasing the volume of the acceptor chamber. In addition, a consecutive SLM extraction step could also be implemented for additional enrichment. With other SLM extraction setups, especially when extraction was performed in flow condition, a 50-500 fold extraction has been shown (Payán, Jensen, Petersen, Hansen, & Pedersen-Bjergaard, 2012; Wang, Saridara, & Mitra, 2005).

Comparing the presented SERS-based detection with other sensing strategies, we realized that the sensors developed for OTA detection are often only used for detection of OTA in standard buffer solution, resulting in a very low, down to ppt level LOD (Liang, et al., 2018; Song, et al., 2018; Zhu, et al., 2015). However, when these sensors were used for the detection of OTA in real samples, such as wine, the LOD was much higher and comparable with the achieved LOD by the here proposed sensing approach (Almeda, et al., 2008; Novo, Moulas, Prazeres, Chu, & Conde, 2013). On the other hand, when comparing the reached LOD in wine with other methods, where commonly used sample extraction /clean-up methods were applied in combination with traditional analytical techniques, it is evident that the presented strategy needs further improvement (Vidal, et al., 2017).

4. Conclusion

The strength of this method is the direct measurement of OTA from wine samples, following a one-step extraction. When used in combination with a more efficient enrichment method, the limit of detection of the sensor can be further improved. The proposed method is an important step towards fast and cost efficient label-free detection of OTA on-site, especially considering the commercially available SERS substrates (SERS Substrates - Low cost and high performing SERS substrate; SERS

Substrates - StellarNet; Surface Enhanced Raman Spectroscopy (SERS) for Food Analysis » SPECTROSCOPY.TV) as well as the access to miniaturized, portable Raman systems (Serstech | Chemical intelligence solutions; TruScan RM Analyzer for Material Testing - DK). The same method could be optimized to a range of other mycotoxins, such as aflatoxins.

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Highlight

- Direct, label-free detection of Ochratoxin A was achieved on Ag nanopillar substrate.
- Supported liquid membrane extraction was used for the extraction of Ochratoxin A.
- High throughput extraction was combined with SERS detection in multiwell platform.
- The SERS data from wine sample was comparable with LC-MS results based on R^2 values.
- The method is an important step towards cost efficient detection of Ochratoxin A.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: