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TiO_2 nanocrystals - Assisted Laser Desorption and Ionization Time-of-Flight Mass Spectrometric Analysis of Steroid Hormones, Amino Acids and Saccharides. Validation and comparison of methods.

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In the present work, the possibility of application of TiO_2 nanocrystals, of various shape and size, for substrate-assisted laser desorption and ionization time-of-flight mas spectrometric (SALDI TOF MS) quantitative analyses of small molecules (steroid hormones, amino acids and saccharides) was investigated. The parameters, such as homogeneity of the substrate/analyte distribution, reproducibiliy of the measurement, within day, and day-to-day repeatability were determined. The homogeneity on the target plate between different nanocrystals/analyte combinations were compared based on the signal-to-noise values of several analyte signals. Obtained results show that all TiO_2 nanocrystals independently on their shape have a great potential for detection and determination of steroid hormones, amino acids and saccharides with good analytical parameters, and detection limit. On the other hand, reproducibility of the S/N ratio and detectability of the analytes recorded in various modes differ depending on the substrate. All examined molecules were detectable in negative ion mode with TiO_2 NTs, in contrast to all other organic matrices and substrates, and the best reproducibility was obtained with larger nanocrystals, TiO_2 PNSs and TiO_2 NTs, making them a good candidates for for quantitative determination of small molecules.

Introduction

The interference of matrix with analyte signals in low mass range and nonuniform analyte distribution within matrix during their co-crystallization limit the application of conventional MALDI for analysis of low molecular compounds (M < 1500 Da, "small molecules").[1,2] One of the most important features of substrate-assisted laser desorption and ionization mass spectrometry, SALDI MS, on the other hand, is the absence of matrix intrusion in low-mass region since the use of organic matrices is not required. SALDI MS, thus, extends the detectable mass range of small molecules to less than m/z 500.

The term SALDI MS was first introduced by Sunner et al. using graphite as a matrix.[3] Since that time, numerous substrates were tested as matrices and, in general, they enable efficient ionization with minimal analyte fragmentation, and with the possibility of achieving high selectivity, sensitivity and reproducibility of the analysis due to appropriate surface chemistry and morphology.[2] Substrates for SALDI MS techniques often utilize nanoparticles, which absorb the laser energy and efficiently transfer it to analyte.

The performance of SALDI has been improved in terms of a soft LDI process, the detection of both polar and nonpolar compounds. Literature highlights indicate the signal enhancement factors for molecular ions in the SALDI mass spectra.[4] In contrast to MALDI, SALDI process has disadvantage based on the fact that efficiency of generating protonated molecular ions is low.[4]

The use of metal nanoparticles for SALDI MS was originally inspired by Tanaka et al., exploiting a suspension of 30 nm cobalt nanoparticles in glycerol to analyze proteins and synthetic polymers.[5] Besides metals, semiconductors with good UV absorbance are promising candidates for SALDI MS.[6] In the UV region, TiO_2 NPs exhibit strong absorption characteristics (band gap of bulk anatase TiO_2: 3.2 eV).[6] TiO_2-based substrates afford the advantage, being chemically stable in air, chemically modified and readily available.[4,7]

Usual approach for detection molecules in LDI mode which do not have sufficient proton donating activity is to apply higher laser power, which in turn might result in increased onset of side-processes, such as aggregation or fragmentation.[8] However, LDI mass spectrometry, when applicable, has

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limitations related to thermal decomposition of analyte, sensitivity, and variety of compounds classes (mainly, low molecular organic substances), absorbing radiation efficiency of UV lassers.[2,9]

In our previous work, we have demonstrated the advantages of the application of colloidal TiO_2 nanoparticles (5 nm diameter) for the SALDI mass spectrometric analyses of transition metal complexes,[10] and the influence of the presence of inorganic salts on the quality of obtained mass spectra[11]. Those results showed that, although suffering from lower sensitivity of the detection of transition metal complexes compared to organic matrices, TiO_2-assisted LDI spectra were much simpler for interpretation, and their quality was not affected by the presence of inorganic salts. Current study is extended to the investigation of the possibility for quantification of small physiologically-relevant molecules, which are not detectable by the matrix-free approach, by the assistance of three TiO_2 nanocrystals (TiO_2 nanoparticles, TiO_2 NPs, TiO_2 prolate nanospheroids, TiO_2 PNSs and TiO_2 nanotubes, TiO_2 NTs) with different size and shape. The following groups of small molecules were used as the model system:

- **Sex steroid hormones**, which play important roles in maintaining normal reproductive and non-reproductive functions.
- **Aminothiols,** such a glutathione (GSH), cysteine (Cys), and homocysteine (Hcy) and other amino acids are important in the control of vital metabolic processes and in the defense against reactive oxygen species.
- Finally, **carbohydrates** are energy source for all cells in the organism, especially for the brain and the nervous system, and changes in their metabolism might lead to severe disease state.

The usefulness of TiO_2-based substrates towards their sensitivity, reproducibility, rapidity and simplicity through the analyses of substrate and analyzed molecule combination is validated in this work.

Materials and methods

Materials

All tested chemicals, β-cyclodextrin, D-(+)-maltose, glutathione D-(+)-glucose, testosterone, progesterone, estradiol, arabinose, raffinose, DL-methionine, L-alanine, L-cysteine were purchased from Sigma-Aldrich, Taufkirchen, Germany.

Methods

Synthesis of TiO_2 nanoparticles, TiO_2 prolate nanospheroids and TiO_2 nanotubes

A dispersion of TiO_2 NPs was prepared following a procedure described previously.[12] Briefly, $TiCl_4$ solution (cooled to -20°C) was added to cold bidestilated water (at 4°C) in a drop wise manner under vigorous stirring and left at this temperature for 30 min. The solution pH was between 0 and 1. The slow growth of the particles was achieved by dialyze the colloidal dispersion of TiO_2 against the solution (4°C), until its pH reached 3.5. The concentration of TiO_2 in the colloid was determined as described previously.[13]

Titania nanotubes were synthesized according to procedure of Kasuga et al.[14] while the hydrothermal processing (90 min at 250°C, Teflon vessel - Parr acid digestion bomb) of dispersion (pH~5-6) of scrolled titania nanotubes (2.5 mg/mL) was applied for the synthesis of TiO_2 PNSs.

X-ray

The X-ray diffraction (XRD) measurements were carried out on a Bruker D8 ADVANCE diffractometer in theta/theta reflection geometry with parallel beam optics achieved by multilayer Göbel mirror. Diffraction data for a structure analysis were collected in 2θ range from 10° to 80° with steps of 0,05° and 10 s counting time per step.

SALDI TOF MS

A small volume of the sample solutions (0.5 μL) and then the same volume of TiO_2 substrates solutions were applied onto the MALDI target plate. The aqueous suspensions of TiO_2 NPs, PNSs and NTs were used at a concentration of 0.22 mol/L, 0.03 mol/L and 0.12 mol/L, respectively.

Experiments were performed in positive and negative-ion reflector mode on a Voyager DE Pro MALDI TOF mass spectrometer. The samples were irradiated with a 20 Hz nitrogen laser at 337 nm. Spectra were acquired automatically for *m/z* range of 1-1000, except for β-cyclodextrin where *m/z* was in range of 500-2000. Ions produced by laser desorption were energetically stabilized during a delay extraction period of 150 ns. Accelerating voltage was set at +/-20 kV. To obtain a good resolution and signal-to-noise (S/N) ratios, laser fluency was adjusted to slightly higher than threshold (for substrate TiO_2 NPs, TiO_2 PNSs and TiO_2 NTs were 2400, 1950 and 2000 LI, respectively).

Statistical analysis

One sample spot on MALDI plate was visually split on eight sectors while each sector was shot with 120 lasers shots (960 shots per one spot). Based on S/N values obtained in these set of experiments, the homogeneity for each analyte/substrate combination was calculated. For calculation of within day repeatability, we repeat this procedure in triplicate. The same procedure was repeated for three consecutive days for calculation day-to-day precision.

The within-day precision (variance of repeatability) for each combination of substrate/molecule and intermediate precision (a day-to-day repeatability) during three days was analysed using statistic test (ANOVA). In one day measurement all three

sets of eight measurements (each set was sum of 960 spots) were grouped.

Results and discussion

In spite of attempts,[2,6] quantitative MALDI MS analysis has poor shot-to-shot and batch-to-batch reproducibility, caused by inhomogeneous co-crystallization of analytes with matrix, and interference of matrix signals.

Herein, the applicability of TiO_2 nanocrystals of different diameters and shapes, such as colloidal TiO_2 nanoparticles (NPs, average diameter ~ 5 nm), prolate nanospheroids (PNSs, length: 40 - 50 nm, the lateral dimension: 14 - 16 nm) and nanotubes (NTs, length: 100 - 150 nm, average diameter 11 nm), as substrates for SALDI TOF MS quantitative analysis of three groups of low mass molecules (twelve molecules in total) was tested. The analyzed molecules are: *amino acids* (L-cysteine (L-Cys), L-alanine (L-Ala), DL-methionine (DL-Met), and tripepride glutathione (GSH)), sex steroid *hormones* (estradiol - E2, testosterone - T, and progesterone - PRG) and *carbohydrates* (D-(+)-glucose - D-(+)-Glu, D-(+)-maltose - D-(+)-Malt, raffinose - Raff, arabinose - Ara, β-cyclodextrin - β-CD). The identities of signals of tested molecules, in the SALDI spectra are given in Table S1. Substrates, , NPs and NTs have anatase crystalline structures, as we have shown in our previous reports.[15,16] XRD pattern of PNSs revealed also anatase crystal structure of particles (Figure S1).

Selection of small molecule analytes for investigation was driven by their clinical significance and necessity of their fast detection and quantification. Carbohydrates are the exception and they are chosen, due to an absence of reliable and fast methods for their analysis. Concerning the chemical structure, members of one group bear the same functional groups.

T and its metabolite dihydrotestosterone (DHT), PRG and E2 are classified as sex-steroids and they decline with age. Sex steroid hormones are implicated in the cognitive processes of the adult brain.[17] In the last two decades it has been shown that receptors for estrogens, progesterone and androgens are expressed in non reproductive tissue /organs (bone, brain, cardiovascular system) playing a role in their function. Therefore, it is critical to evaluate the impact of sex steroid hormones in the pathophysiology of certain diseases (osteoporosis, Alzheimer', atherosclerosis).[18]

The second group is represented by GSH, L-Cys, L-Ala and DL-Met. The level of GSH and Cys in blood are correlated with several diseases such as hyperhomocysteinemia as a risk factor for atherosclerosis, cardiovascular and chronic kidney disease.[19] Thiol groups are reducing agents, existing at a concentration around 5 mM in animal cells. The normal level of the Cys in the cell is 9.5-11.5 μM.[20] The reduced form of GSH is the most abundant intracellular low molecular weight thiol in the cell and plays essential role in protecting cell from toxic species.[21] Met and Cys are involved in many vital catalytic reactions that keep our body functioning properly. Ala can be easily formed and thus has close links to metabolic pathways such as glycolysis, gluconeogenesis, and the citric acid cycle. It also arises together with lactate and generates glucose from protein via the Ala cycle. Alterations in the Ala cycle that increase the levels of serum alanine aminotransferase (ALT) are linked to the development of type II diabetes.[22]

Carbohydrates are energy sources, but so far, there are no methods for their analyses which is fast and does not require their modification.

In matrix-free approach (LDI mode) analysis, none of analytes yield signals in the mass spectra most likely due to insufficient proton donating activity and absorptivity of analytes in range of laser irradiation wavelength. This indicates that the presence of the assisting agent on the plate is required to obtain corresponding mass spectra.

SALDI TOF mass spectra of steroid hormones, amino acids and carbohydrate

In the first set of experiments, we tested whether the TiO_2 nanocrystals are suitable for the detection of selected molecules. In fact, only the peaks that are clearly not present in the LDI spectra of substrate (Figure S2) showing no overlap with the peaks of substrates were considered for further statistical analysis. It is important to emphasize that the analytes do not yield any detectable signals under LDI conditions (substrate and matrix-free approach); signals appear, on the other hand when nanocrystals were applied.

The reason why nanocrystals made of titania enable mass spectrometric detection of selected compounds and the exact processes, which occur on the substrate surface under laser irradiation, are not fully clarified yet. Numerous authors assume that thermal effects play important role,[4,23] but there are also those, who emphasize the electric/charge transfer and/or photocatalytic processes on the surface of titania-based nanocrystals.[24,25] However, further and carefully planned experiments are required to clarify these fundamental processes in the vacuum/MALDI chamber.

Analyte concentration was in the range from 1 to 11 mM, depending on its mass. In Table 1 is presented the detectability of individual compounds with the peak list and assignment, specifically, analytes that are obtained only with the increased concentrations are presented herein. Representative SALDI TOF mass spectra in positive and negative ion mode are given in Figure 1a and 1b, respectively. We have chosen the most representative mass spectra among spectra acquired with all TiO_2 nanocrystals with almost all detectable signals. The signals taken for statistical evaluation are indicated according to their *m/z* ratio (Table S1 shows identities of detected signals).

Investigated hormones were detectable with TiO_2 PNSs and TiO_2 NTs in positive ion mode while only E2 were unidentified with TiO_2 NPs, at least at tested concentrations. Similarly, T and PRG had low S/N ratios with TiO_2 NPs. In contrast, TiO_2 NTs as substrate gave good quality spectra with hormones in negative ion mode (Figure 1b). With all three TiO_2 substrates we detected only adducts of E2 or T molecules, their corresponding fragments, and alkali (Na^+ or K^+) adducts whereas in the case of PRG only the proton and sodium adducts were detectable.

The spectra of amino acids did not show regularities like hormones, for instance L-Cys and L-Ala were not detected with TiO_2 PNSs, whereas the molecular ion of L-Cys was measurable with TiO_2 NPs. Generally, when TiO_2 NTs were exploited, adducts of all amino acids with alkali ions were detectable in positive ion mode, while in negative ion mode only deprotonated molecules were obtained (Table 1).

In the case of the spectra of L-Ala measured in negative ion mode, all three substrates were demonstrated to be applicable. When S/N intensity of the signals arising from L-Ala is compared, the following order could be obtained: TiO_2 NPs < TiO_2 PNSs < TiO_2 NTs.

To this end, Na^+ adduct of reduced GSH was easily detected with TiO_2 PNSs and TiO_2 NTs, and showed no signal with NPs.

Literature highlights that cationization by sodium and potassium is predominant ionization mechanism for amino acids in SALDI MS analysis, whereas the negative ions could be obtained by the abstraction of a proton.[26] This represents the advantage of used method, due to the fact that spectra are much simpler for interpretation, since no adducts with matrix could be formed.

Furthermore, ions arising from all five carbohydrates were detected with TiO_2 NTs in both ion modes, and Ara was observed only with this substrate, whereas no signals are detectable with others. TiO_2 NPs appeared to be good only for detection of Raff in positive ion mode. Briefly, in positive ion mode with the assistance of all three substrates we detected only alkali adducts for all analyzed molecules, i.e. $[M + Na]^+$ and $[M + K]^+$ whereas negative ion mode spectra of carbohydrates were easier for interpretation and TiO_2 NTs demonstrated to be good substrate. Only the signals arising from β-CD were rather low.

As compared to MALDI MS of tested compounds (data not shown), SALDI MS with TiO_2 substrates gave better sensitivity and reproducibility in negative-ion mode, at least in case of investigated biological molecules. The background generated from TiO_2 NTs is very low, making TiO_2 NTs substrate suitable for the analysis of small molecules in positive ion mode (Figure 1a) while in negative ion mode it exhibited a slightly higher background (Figure 1b).

As obtained data suggested some molecules (T, PRG, L-Ala, L-Cys, and Raff) could be analyzed with TiO_2 nanocrystals with satisfying detectability.

It is possible that when substrate concentration increases, for instance a higher concentration of TiO_2 NPs, the number of nanoparticles around analyzed molecules augments also, resulting in its better detectability.[27] This occurs due to sufficient energy from the laser and, thus, high ionization efficiency. As a side effect of increased concentration of substrate, a high background noise might be generated and poor sensitivity is usually obtained. In accordance to obtained results, we have decided to limit the concentration of NPs to gain satisfactory response with the low background signal.

Homogeneity of analyte/substrate distribution on sample plate

The homogeneity of the sample/matrix co-crystals in MALDI and the distribution of analyte over substrate surface in SALDI approach define reproducibility and accuracy of the measurement.[1,2] Therefore, in the next set of experiments, the homogeneity of our systems was monitored and as illustrated in Figure S3, one sample spot was divided into eight sectors. For calculation and presentation of homogeneity, we used the mean value of coefficient of variation of eight S/N values with nine repetition within three days for each analyte/substrate combination in positive and negative ion mode (Table 2 and 3). These values were used as homogeneity measures for different analyte/substrate combination.

The best homogeneity was achieved with TiO_2 PNSs; the values of relative standard deviation (RSD) were around 20%, except of GSH where TiO_2 NTs gave better results and very satisfactory values of RSD (6.2 - 17.6%). TiO_2 NPs showed worse homogeneity with analyzed molecules, although L-Ala (*m/z* 112.1) and L-Cys (*m/z* 144.2) were exceptions with achieved RSD 9.5-17.1% and RSD 5.7 - 27.3%, respectively. In the last combination, the best mixing was achieved among all studied samples with TiO_2 NPs.

In negative ion mode (Table 3) the molecules were detectable with great majority with TiO_2 NTs, as discussed in the previous chapter. But, three of them, which are detectable with TiO_2 PNSs, gave better homogeneity compared to other analytes: E2 (*m/z* 328.9, RSD 5.3 - 27.3%), Raff (*m/z* 222.0, RSD 6.3 - 14.6%, *m/z* 503.4, RSD 12.6 - 33.2%), and DL-Met (*m/z* 148.2, RSD 8.9 - 17.2%). The only one detectable with TiO_2 NPs was L-Ala (*m/z* 88.1) along with much better reproducibility (3.8 - 13.5%). Negative ion mode in MALDI TOF MS was far less sensitive than one in SALDI TOF MS. The matrix by itself yields a number of peaks, especially in negative ion mode while detection of small molecules is very difficult.[28]

Day-to-day repeatability

Next, to compare the accuracy of measurements with respect to signal intensity and potential use of tested systems for quantitative analysis, within day precision and day-to-day repeatability were calculated.

In Table 4 and 5, calculated F values for all combinations were showed (F ratio is the ratio of two mean square values. If the null hypothesis is true, calculated F value does not exceed F_{crit}. For eight measurements, F_{crit} is 5.1. A large F ratio (higher than F_{crit}) means that variation among group average values is more than one expects to see by chance). Day-to-day variation exceeded variation within days in those cases in which the calculated F value exceeds F critical value (F_{crit} = 5.1). Under applied experimental conditions, variation within days for the most analyte/substrate pairs was very good (less than 10%) (Table 4 and 5) when compared to literature data.[6,27,29] The best results were, however, obtained for molecule/substrate pair in positive ion mode: T (*m/z* 423.6)/PNSs - RSD 2.1%, Raff (*m/z* 365.3)/PNSs RSD - 2.7%. In negative ion mode, the best results were obtained for L-Ala (*m/z* 88.1)/NPs - RSD 2.9%.

Based on above mention results, TiO_2 PNSs and TiO_2 NTs are potential matrices for quantitative determination of molecules.

In general, the best agreement of values day-to-day variations of the same molecule/substrate pair was achieved with TiO_2 PNSs with RSD values lower than 25%. This repeatability was excellent and also in accordance with data for combination of catechin-modified TiO_2 NPs and steroid hormones where the batch-to-batch variations were less than 15%, n = 7.[29]

TiO_2 NTs as potential substrates for quantitative analysis of molecules in positive ion mode gave minimal values of day-to-day variation for particular molecules. Variation for the most analyzed molecules with TiO_2 NTs was less than 10% (for example: Raff (*m/z* 365.3) – RSD 5.8%, L-Ala (*m/z* 112.1) - RSD 2.4%) while for other molecules RSD was around 20%. Similar results were presented in the study of Lee and al.[6] where the within day variation was less than 10% over 50 spots in the same sample, and day-to-day variation was less than 15% for three different batches (each for 50 spots). These results also confirm that TiO_2 NTs are very good substrates for the detection and determination of amino acids.

In negative ion mode, the smallest values for day-to-day variation with TiO_2 NTs were achieved for GSH (*m/z* 306.3) - RSD 5.7%), Ara (*m/z* 149.1) - RSD 5.9%) and Raff (*m/z* 222.0) - RSD 6.0%. The only one detected in negative mode with TiO_2 NPs was L-Ala that had very low values of day-to-day variation and within day variation, RSD 3.7% and 2.9%, respectively. When TiO_2 PNSs was used as substrate RSD values for day-to-day variations were from 3.7% (Raff) to 15.9% (DL-Met), and for within day were from 5.2%(Raff) to 9.1% (DL-Met).

Limit of the detection

Finally, detection limit (LODs) of combinations analyte/substrate was investigated. Under the optimal conditions, we obtained a calibration curve of S/N values for all twelve analysed molecules against their concentration in range 0.02-112 mM. LODs and linear range of analytes are summarized in Table 6 for positive ion mod and Table 7 for negative ion mode. The correlation coefficients (R^2) in linear range were from 0.637 to 0.999. The majority of obtained correlation coefficients were around 0.9, but for some peaks this value was even lower. TiO_2 PNSs gave the best results for the correlation coefficients.

In positive ion mode (Table 6) the minimal values of LODs for L-Ala, Ara, β-CD, L-Cys, E2, GSH, DL-met, PRG, and Raff were achieved with TiO_2 NTs in the range of 0.02 to 0.69 mM. TiO_2 PNSs gave low values of LODs with D-(+)-Glu, GSH, DL-Met, PRG, and Raff in the range of 0.02 to 0.64 mM. Sensitivity of detection of these compounds, however, might be increased with modified nanoparticles, such catechine modified TiO_2 nanoparticles,[29] but this is beyond the scope of the present work.

Because of the poorer ionization efficiencies of Cys, relative to that GSH, and greater noise in low-mass region (*m/z*<200), their LODs were higher than ones for GSH.[27]

In negative ion mode (Table 7) both TiO_2 PNSs and TiO_2 NTs gave satisfactory LODs values for the most analyzed molecules, with the respect that TiO_2 NTs gave the smaller values in range of 0.02 for GSH to 7.54 mM for L-Cys. Similar to one observed in positive ion mode, LOD for L-Cys is higher than for GSH for all substrates. TiO_2 NPs among the analyzed molecules were the most suitable for detection of PRG and L-Ala, with LODs of 0.82 and 0.55 mM, respectively.

Conclusions

We have investigated the possibility to use TiO_2 nanocrystals of different shapes and size as substrates for SALDI TOF mass spectrometric analysis of selected physiologically - relevant small molecules such as: steroid hormones, amino acids and carbohydrates. According to obtained data, all TiO_2 nanocrystals could be used as substrates independently of their size and shape, however, giving various spectra quality with respect to the homogeneity, repeatability, sensitivity and detection limit and, having various potential for quantitative MS analyses.

In conclusion our results show that:

- Larger titania based nanocrystals (PNSs and NTs) gave the spectra with much higher reproducibility and easier for interpretation.
- Steroid hormones were detected with best performances when TiO_2 PNSs were used as substrates.
- Amino acids, GSH and carbohydrates were detectable both as positive and negative ions with TiO_2 NTs.
- The best homogeneity was achieved with TiO_2 PNSs for the most of analyses molecules (exception was GSH where TiO_2 NTs gave better results).
- In general TiO_2 NTs have the highest potential for quantification of most molecules in positive ion mode.

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Molecule	Substrate TiO_2	Detectability		*m/z* values		Detectable at higher concentration	
		Positive	*Negative*	*Positive*	*Negative*	*Positive*	*Negative*
		Ion mode		*Ion mode*		*Ion mode*	
E2	**NPs**	-	-	**/**	**/**	**407.6, 423.6, 439.7**	/
	PNSs	+	+	**407.6,** 423.6*	**328.9**	439.7*	
	NTs	+	-	**407.6, 423.6, 439.7**	**328.9**		
T	**NPs**	+	-	**401.6, 423.6**	**/**		**399.6**
	PNSs	+	-	**423.6**	**/**		**399.6**
	NTs	+	+	**401.6, 423.6**	**399.6**	401.6*	
PRG	**NPs**	+	-	**315.5, 337.5**	**/**		**313.5**
	PNSs	+	-	**337.5**	**/**		**313.5**
	NTs	+	+	**337.5**	**313.5**	315.5*	
L-Ala	**NPs**	+	+	**90.1, 112.1, 128.2**	**88.1**		**88.1**
	PNSs	-	+	**/**	88.1*	**112.1**	
	NTs	+	+	**112.1, 128.2**	**88.1**		
L-Cys	**NPs**	+	-	**121.2,** 144.2*	**/**	**144.2**	**120.2**
	PNSs	-	-	**/**	**/**	**144.2**	**120.2**
	NTs	+	+	**144.2, 167.1**	**120.2**		
DL-Met	**NPs**	+	-	172.2*	**/**	**150.2, 172.2, 188.2**	**148.2**
	PNSs	+	+	**172.2, 195.2**	**148.2**		
	NTs	+	+	**172.2, 188.3, 195.2**	**148.2**		
GSH	**NPs**	+	-	330.3*	**/**	**308.3, 330.3, 346.4**	**306.3**
	PNSs	+	-	**308.3, 330.3,** 346.4*	**/**		**306.3**
	NTs	+	+	**330.3, 346.4**	**306.3**		
D-(+)-Glu	**NPs**	-	-	**/**	**/**	**203.2, 219.3**	179.2*
	PNSs	+	-	**203.2**	**/**		179.2*
	NTs	+	+	**203.2, 219.3**	**179.2**		
D-(+)-Malt	**NPs**	-	-	**/**	**/**	**365.3**	**222.0**
	PNSs	+	-	**365.3**	**/**		**222.0**
	NTs	+	+	**365.3**	**222.0**		
β-CD	**NPs**	-	-	**/**	**/**	**1158.0, 1174.1**	
	PNSs	+	-	**1158.0**	**/**		
	NTs	+	+	**1158.0, 1174.1**	**1134.0**		
Raff	**NPs**	+	-	**203.2, 365.3, 527.4, 543.5**	**/**		**222.0**
	PNSs	+	+	**185.1, 203.2, 365.3, 384.3, 527.4**	**222.0, 503.4**	185.1*, 203.2***, 543.5**	
	NTs	+	+	**185.1, 203.2, 365.3, 527.4, 543.5**	**222.0, 503.4**	185.1*, 203.2*	
Ara	**NPs**	-	+	**/**	149.1*	**173.1, 189.2**	**149.1**
	PNSs	-	+	**/**	149.1*	**173.1,** 189.2*	**149.1**
	NTs	+	+	**173.1, 189.2**	**149.1**		

It is detectable, but S/N valuse are lower than 50, that is our empirical value.

Table 1: Detectability of molecules with TiO_2 substrates. The table comprises all signals (*m/z* ratio) arising from analyte detectable with particular substrate.

Molecule	*m/z*	RSD, %		
		NPs	PNSs	NTs
E2	407.6	/	9.1-21.3	10.4-18.7
T	401.6	20.3-61.3	/	27.3- 45.8
	423.6	22.1-56.5	6.0- 16.3	13.2-27.8
PRG	315.5	59.6-107.0	/	/
	337.5	65.0-94.0	7.9- 21.9	11.3-24.2
L-Ala	90.1	10.6-24.2	/	/
	112.1	9.5-17.1	/	5.5- 17.5
	128.2	10.9-17.1	/	14.0- 25.2
L-Cys	121.2	9.7-38.8	/	/
	144.2	5.7-27.3	/	31.8- 21.7
	167.1	/	/	11.3- 34.0
DL-Met	172.2	/	6.6- 14.7	5.9- 38.9
	188.3	/	/	5.4- 27.9
	195.2	/	6.4-18.6	7.1- 48.5
GSH	308.3	/	7.2-38.8	/
	330.3	/	11.5-78.4	6.2-17.6
	346.4	/	/	8.2-31.01
D-(+)-Glu	203.2	/	3.3-20.9	14.4-45.3
	219.3	/	/	11.1-56.7
D-(+)-Malt	365.3	/	6.0-17.4	9.8-28.1
β-CD	1158.0	/	9.1- 17.8	17.8-48.1
	1174.1	/	/	11.3-54.5
Raff	185.1	/	5.4- 23.1	16.1-65.5
	203.2	21.3-57.2	6.4- 25.1	23.9-65.2
	365.3	32.3-74.8	5.4- 12.6	5.3-29.4
	384.3	/	4.4- 18.2	/
	527.4	32.3-62.8	7.7- 14.8	9.8-30.7
	543.5	29.6-58.2	/	18.8-49.9
Ara	173.1	/	/	8.2- 26.4
	189.2	/	/	18.4- 39.3

Table 2: Homogeneity of sample onto the SALDI plate in positive ion mode.

Molecule	*m/z*	RSD, %		
		NPs	PNSs	NTs
E2	328.9	/	5.3-27.3	11.0-30.8
T	399.6	/	/	16.5-34.0
PRG	313.5	/	/	18.6- 36.1
L-Ala	88.1	3.8-13.5	/	5.0-27.3
L-Cys	120.2	/	/	6.5-19.8
DL-Met	148.2	/	8.9-17.2	11.0-29.3
GSH	306.3	/	/	16.5-27.8
D-(+)-Glu	179.2	/	/	14.2-25.1
D-(+)-Malt	222.0	/	/	6.3-37.1
Raff	222.0	/	6.3-14.6	14.4-32.7
	503.4	/	12.6-33.2	26.8-63.0
Ara	149.1	/	/	16.8-41.8

Table 3: Homogeneity of sample onto the SALDI plate in negative ion mode.

Molecule	*m/z*	ANOVA Day-to-day, %			ANOVA Within Day, %			F F_{crit} 5.143		
		NPs	PNSs	NTs	NPs	PNSs	NTs	NPs	PNSs	NTs
E2	407.6	/	7.4	8.1	/	13.3	14.5	/	1.9	0.1
T	401.6	26.4	/	33.5	53.5	/	13.9	0.3	/	18.5
	423.6	17.3	12.9	10.1	33.0	2.1	7.0	0.2	115.6	7.3
PRG	315.5	19.6	/	/	36.6	/	/	1.9	/	/
	337.5	58.4	20.5	8.8	101.3	9.7	11.4	0.0	14.5	2.8
L-Ala	90.1	4.1	/	/	28.0	/	/	0.9	/	/
	112.1	9.7	/	2.4	26.2	/	7.5	0.6	/	1.3
	128.2	11.7	/	27.3	27.2	/	7.0	0.4	/	46.5
L-Cys	121.2	14.2	/	/	34.6	/	/	1.5	/	/
	144.2	16.2	/	4.5	32.6	/	20.1	0.3	/	0.9
	167.1	/	/	7.3	/	/	20.8	/	/	0.6
DL-Met	172.2	/	13.4	9.4	/	13.8	12.6	/	3.9	2.7
	188.3	/	/	16.3	/	/	13.5	/	/	5.4
	195.2	/	30.2	21.1	/	5.3	13.2	/	98.1	8.6
GSH	308.3	/	33.1	/	/	27.6	/	/	5.1	/
	330.3	/	45.4	6.4	/	62.0	12.2	/	2.6	0.2
	346.4	/	/	29.0	/	/	20.7	/	/	6.9
D-(+)-Glu	203.2	/	5.7	18.2	/	10.2	25.9	/	0.0	2.5
	219.3	/	/	8.0	/	/	23.9	/	/	1.3
D-(+)-Malt	365.3	/	4.9	18.3	/	9.9	13.0	/	0.3	7.0
β-CD	1158.0	/	12.3	16.5	/	22.0	32.2	/	1.3	0.2
	1174.1	/	/	35.9	/	/	29.6	/	/	5.4
Raff	185.1 +	/	18.9	18.7	/	16.3	38.6	/	5.0	0.3
	203.2	39.0	14.0	19.4	43.9	11.5	34.7	3.4	5.4	0.1
	365.3	22.5	14.1	5.8	53.5	2.7	16.6	1.5	81.3	0.6
	384.3	/	13.5	/	/	9.0	/	/	7.7	/
	527.4	25.5	11.0	6.6	57.8	7.7	16.2	1.6	7.0	0.5
	543.5	24.7	/	24.3	36.6	/	31.3	2.4	/	2.8
Ara	173.1	/	/	16.7	/	/	8.9	/	/	11.5
	189.2	/	/	18.6	/	/	26.3	/	/	2.5

Table 4: The values of variations day-to-day and within day repeatability in the positive ion mode.

Molecule	*m/z*	ANOVA Day-to-day, %			ANOVA Within Days, %			F F_{crit} 5.143		
		NPs	PNSs	NTs	NPs	PNSs	NTs	NPs	PNSs	NTs
E2	328.9	/	14.7	28.0	/	7.2	15.2	/	13.6	11.2
T	399.6	/	/	35.8	/	/	18.3	/	/	12.5
PRG	313.45	/	/	20.0	/	/	16.1	/	/	5.6
L-Ala	88.1	3.7	/	10.0	2.9	/	7.3	6.0	/	6.7
L-Cys	120.2	/	/	7.9	/	/	9.7	/	/	3.0
DL-Met	148.2	/	15.9	9.8	/	9.1	11.3	/	10.2	3.2
GSH	306.3	/	/	5.7	/	/	21.5	/	/	1.2
D-(+)-Glu	179.2	/	/	21.6	/	/	14.1	/	/	8.0
D-(+)-Malt	222.0	/	/	26.3	/	/	25.3	/	/	4.3
Raff	222.0	/	7.8	6.0	/	5.2	10.4	/	7.7	0.0
	503.4	/	3.7	16.7	/	10.5	30.3	/	1.4	0.1
Ara	149.1	/	/	5.9	/	/	30.0	/	/	1.1

Table 5: The values of variations day-to-day and within day repeatability in negative ion mode.

Molecule	*m/z Positive ion mode*	LOD mM			Linear range mM			R^2		
		NPs	PNSs	NTs	NPs	PNSs	NTs	NPs	PNSs	NTs
E2	407.6	3.11	6.12	0.45	*	0.3-41.0	0.3-1.6	*	0.955	0.866
	423.5	2.71	1.15	0.29	*	0.3-4.1	0.3-1.6	*	0.849	0.938
	439.7	3.05	1.48	0.30	*	0.3-8.2	0.3-1.6	*	0.925	0.936
T	401.6	0.47	/	5.11	*	/	0.1-20.0	*	/	0.816
	423.6	5.28	0.31	2.35	0.5-50.0	0.04-2.0	2.0-20.0	0.974	0.927	0.987
PRG	315.5	3.33	0.23	0.46	1.0-10.0	*	0.5-10.0	0.833	*	0.985
	337.5	2.80	2.15	0.46	0.5-10.0	0.5-10.0	0.5-10.0	0.837	0.910	0.985
L-Ala	90.1	2.81	/	/	0.6-56.1	/	/	0.993	/	/
	112.1	8.98	2.28	0.06	2.2-56.1	0.6-11.2	0.6-2.2	0.962	0.918	0.999
	128.2	11.02	/	0.55	0.6-56.1	/	0.6-2.2	0.899	/	0.917
L-Cys	122.2	16.05	/	35.22	4.1-82.5	/	4.1-123.8	0.925	/	0.839
	144.2	4.22	26.62	0.69	4.1-41.3	8.3-123.8	4.1-16.5	0.981	0.908	0.997
	167.1	/	/	5.12	/	/	4.1-16.5	/	/	0.872
DL-Met	150.2	1.39	/	0.10	6.7-33.5	/	0.3-3.4	0.998	/	0.998
	172.2	3.46	1.07	0.96	6.7-33.5	0.3-13.4	0.3-3.4	0.987	0.984	0.869
	188.3	1.61	/	0.78	6.7-33.5	/	0.3-3.4	0.997	/	0.910
	195.2	/	0.64	0.87	/	0.3-3.4	0.3-3.4	/	0.938	0.890
GSH	308.3	3.55	/	3.74	6.5-32.5	/	0.7-16.3	0.984	/	0.896
	330.3	5.43	0.09	0.32	3.3-32.5	3.3-16.3	0.2-1.6	0.952	0.999	0.937
	346.4	2.19	7.94	0.56	6.5-32.5	6.5-32.5	0.3-3.2	0.994	0.925	0.950
D-(+)-Glu	203.1	4.85	0.42	0.57	5.6-55.5	0.3-2.8	0.3-5.6	0.986	0.960	0.978
	219.3	3.73	0.12	1.04	5.6-55.5	0.3-2.8	0.3-5.6	0.992	0.997	0.929
D-(+)-Malt	365.3	7.75	0.08	0.84	5.6-27.8	0.1-2.8	0.1-2.8	0.935	0.968	0.881
β-CD	1158.0	0.83	/	0.17	0.9-8.8	/	*	0.984	/	*
	1174.1	2.29	/	0.03	0.9-8.8	/	*	0.891	/	*
Raff	185.1	3.34	0.04	0.03	1.0-9.9	0.1-0.4	0.1-0.4	0.908	0.988	0.994
	203.1	2.67	0.09	0.02	1.0-9.9	0.1-1.0	0.1-0.4	0.883	0.931	0.997
	365.3	17.73	0.22	0.70	9.9-39.7	0.1-1.0	0.1-0.4	0.637	0.921	0.957
	384.3	/	0.18	0.60	/	0.1-1.0	0.4-2.0	/	0.943	0.999
	527.4	10.97	0.24	0.70	9.9-39.7	0.1-1.0	0.1-0.4	0.821	0.907	0.962
	543.5	1.28	0.02	0.10	*	0.2-1.0	0.1-0.4	*	0.999	0.910
Ara	173.1	4.9	0.70	0.57	0.3-33.3	0.3-6.7	0.3-3.3	0.941	0.977	0.960
	189.2	3.66	1.38	0.5	0.3-33.3	0.3-6.7	0.3-3.3	0.966	0.916	0.949
/ This signal can`t be detected with this TiO_2 substrate.										
**** It can`t be calculated from the graphic.***										

Table 6: Limit of detections, linearities and correlation coefficients for all analyzed molecules with TiO_2 substrates in positive ion mode.

Molecule	*m/z Negative ion mode*	LOD mM			Linear range mM			R^2		
		NPs	PNSs	NTs	NPs	PNSs	NTs	NPs	PNSs	NTs
E2	328.9	/	6.64	0.36	/	0.3-41.0	0.3-1.6	/	0.921	0.909
T	399.6	/	3.24	1.45	/	0.5-10.0	1.0-5.0	/	0.815	0.902
PRG	313.5	0.82	0.77	0.20	1.0-5.0	0.5-20.0	0.5-5.0	0.967	0.987	0.997
L-Ala	88.1	0.55	1.88	0.26	0.6-5.6	0.6-5.6	0.6-2.2	0.983	0.829	0.981
L-Cys	120.1	10.00	16.83	7.54	4.1-123.8	8.3-82.5	4.1-16.5	0.989	0.930	0.759
DL-Met	148.2	3.12	0.36	0.23	6.7-33.5	0.3-3.4	0.3-1.3	0.997	0.979	0.958
GSH	306.3	6.10	1.02	0.02	6.5-48.8	3.3-16.3	0.2-1.6	0.969	0.995	0.999
D-(+)-Glu	179.2	23.00	0.89	0.54	5.6-55.5	0.3-5.6	0.3-5.6	0.763	0.948	0.980
D-(+)-Malt	222.0	12.95	0.45	0.11	2.8-41.6	0.1-2.8	0.1-2.8	0.826	0.962	0.998
Raff	222.0	3.97	0.22	0.21	1.0-9.9	0.4-2.0	0.1-1.0	0.972	0.983	0.927
	503.4	/	0.08	0.73	/	0.4-2.0	0.4-2.0	/	0.998	0.842
Ara	149.1	8.41	1.12	1.61	0.3-66.6	0.3-6.7	0.3-13.3	0.974	0.943	0.965
/ This signal can`t be detected with this TiO_2 substrate.										

Table 7: L Limit of detections, linearities and correlation coefficients for all analyzed molecules with TiO_2 substrates in negative ion mode.

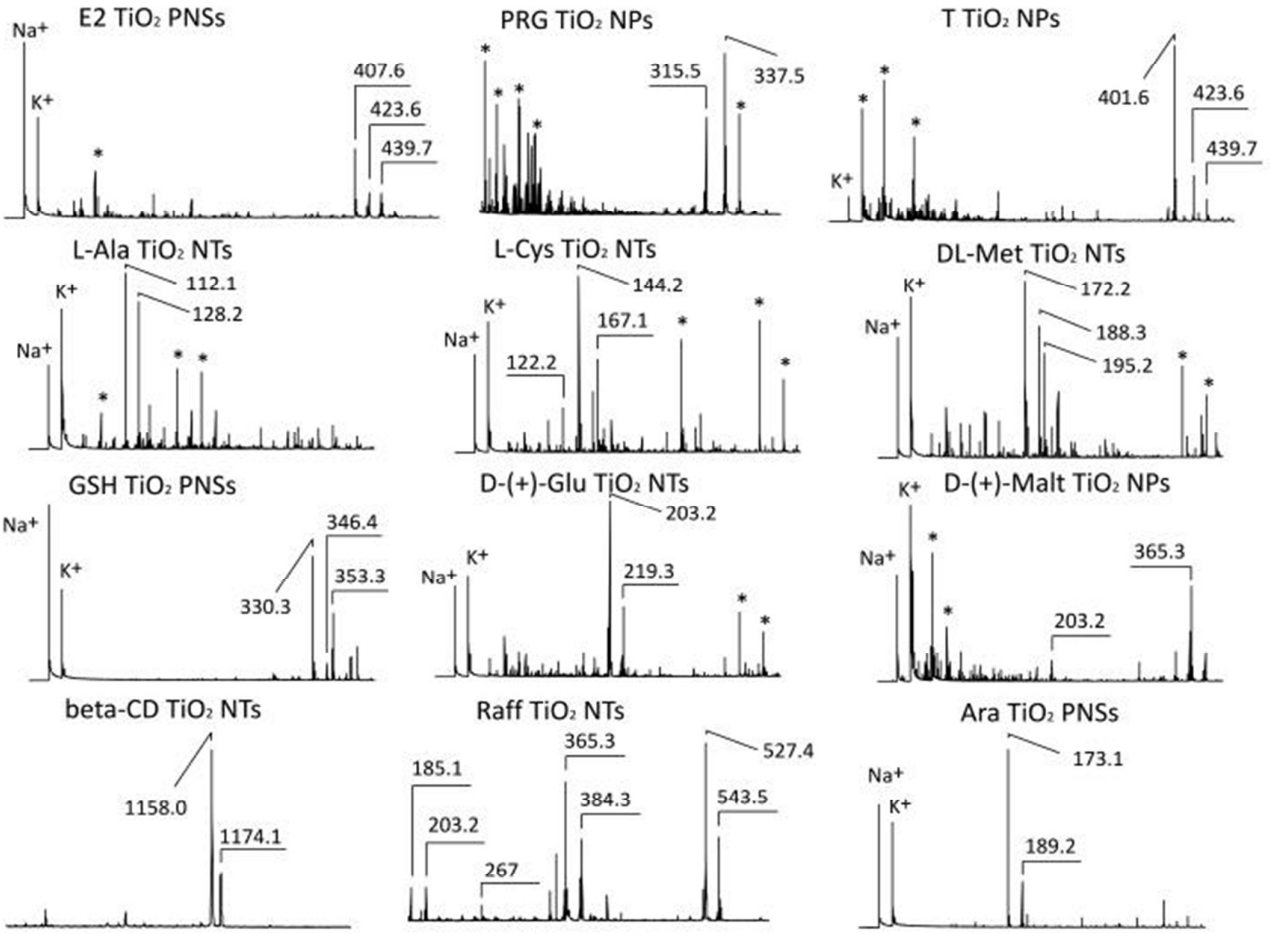

Figure 1a: Positive ion mode SALDI TOF mass spectra of E2, T, PRG, L-Ala, L-Cys, DL-Met, GSH, D-(+)-Glu, D-(+)-Malt, β-CD, Raff, Ara with one of substrates that gave the major number of peaks of analyzed molecules which S/N values were analyzed further.

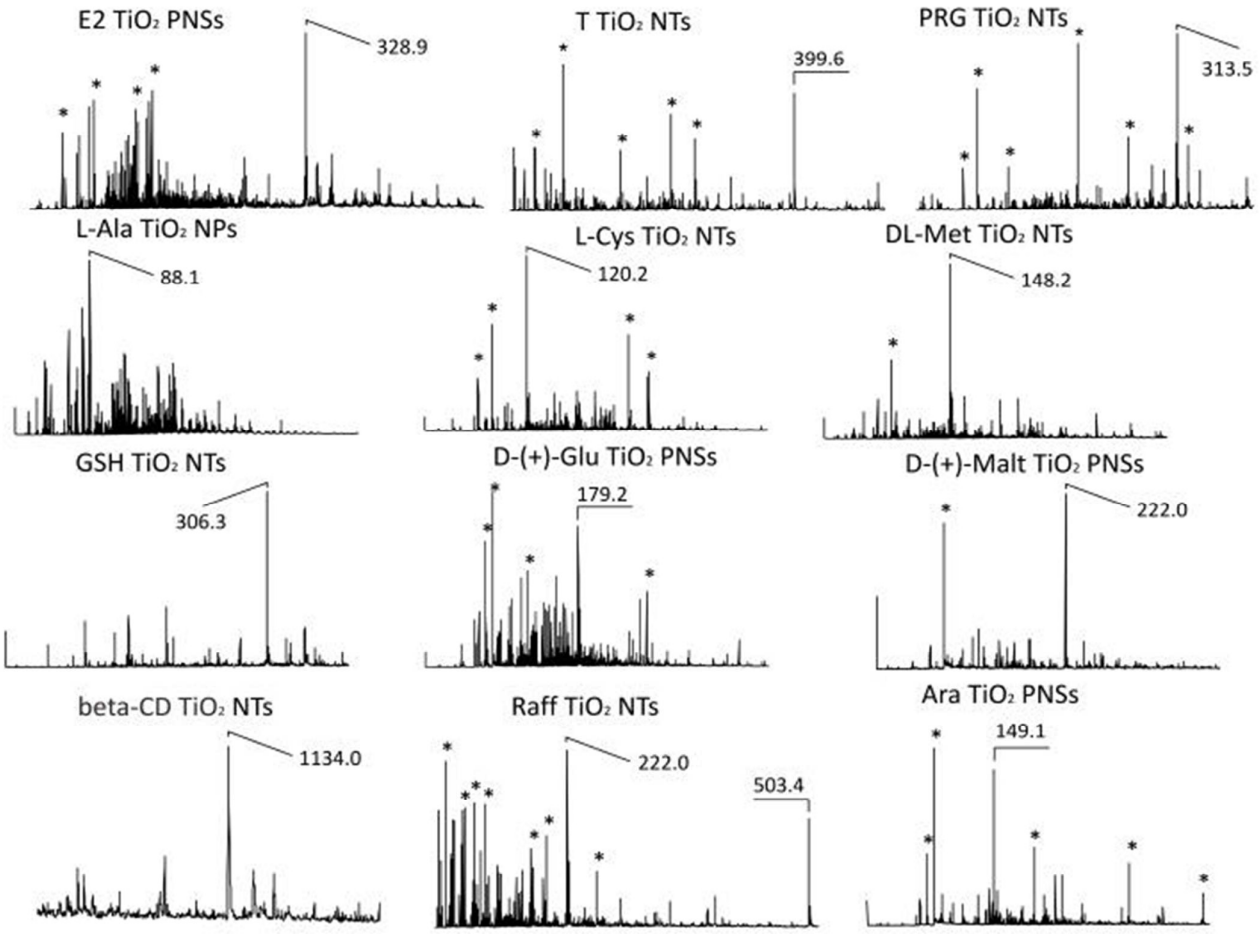

Figure 1b: Negative ion mode SALDI TOF mass spectra of E2, T, PRG, L-Ala, L-Cys, DL-Met, GSH, D-(+)-Glu, D-(+)-Malt, β-CD, Raff, Ara with one of the substrates that gave the major number of peaks of analyzed molecules which S/N values were analyzed further.