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Short communication

Post-chromatographic fixed-charge derivatization for the analysis of hydroxyl-containing compounds by a combination of thin-layer chromatography and matrix-assisted laser desorption/ionization mass spectrometry

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ABSTRACT

A simple and convenient on-spot derivatization has been suggested for the modification of hydroxyl-containing compounds for their analysis by thin layer chromatography/matrix-assisted laser desorption ionization mass spectrometry (TLC/MALDI). The proposed approach was based on post-chromatographic acylation of separated analytes by 3-bromopropionyl chloride with simultaneous quaternization of pyridine. In contrast to the initial alcohols not ionizable in TLC/MALDI conditions, the derivatives, containing permanent positive charge, revealintense peaks of their cationic moieties in MALDI mass spectra recorded directly from TLC plates. The method was tested on a series of mammalian and plant sterols, phenols and terpene alcohols.

1. Introduction

Despite the availability of a number of effective methods for analyzing organic compounds, thin layer chromatography (TLC) remains a key technique in many chemical and analytical laboratories. Its versatility, cheapness and rapidity enable easy and efficient reaction control, qualitative and semi-quantitative analysis of a variety of compounds (natural products, metabolites, pharmaceuticals etc.) [1]. The main drawback of TLC is rather low identification ability of traditionally used chemical and optical approaches and retardation factor (R_F) values. One of the most promising ways to overcome this principal disadvantage is to apply mass spectrometry methods for the detection and identification of analytes resolved by TLC. There are some widely used approaches implying elution of analytes from TLC spots with online transfer to a mass spectrometer [2]. However, mass spectrometric desorption/ionization methods, such as matrix-assisted laser desorption/ionization (MALDI), 'direct analysis in real time' (DART), desorption/electrospray ionization (DESI) seem to be more suitable for this purpose because of a possibility to ionize analytes directly from TLC plates [3–8]. In comparison with MALDI, ambient ionization mass spectrometry methods (DART, DESI) apply more simpler sample preparation procedures, but have problems with TLC spots resolution because of spread ionization zones [9]. One of the problems of TLC/MALDI application, caused by low concentrations of analytes on the adsorbent surface and analytes spots blurring by matrix solutions, can be solved using composite matrices [6] or solvent-free matrices [10]. The other one is due to the nature of MALDI ionization processes occurring in general via protonation, cationization or deprotonation of analytes. This is why, non-polar or low-polarity compounds often do not undergo such processes and, accordingly, cannot be analyzed by MALDI MS. The most convenient approaches allowing the involvement of 'soft' ionization mass spectrometric methods for analysis of such compounds are based on the application of derivatization techniques [11]. Some of them involve the introduction of ionizable group to analytes [12,13]. But the most attractive approaches utilize the formation of salt-like derivatives already having permanent positive or negative charge in their structure. Such fixed-charge derivatization

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methods are widely applied to the analysis of alcohols [14–17], phenols [17,18], amines [19] and thiols [20] by MALDI-MS. However, it is difficult to use these techniques in combination with TLC because of low chromatographic mobility of the salts. So fixed-charge derivatization methods are not suitable for TLC/MALDI as pre-chromatographic procedures. At the same time, they can be used for a post-chromatographic derivatization. This kind of chemical modification methods is well known in traditional TLC analysis where it is mainly used for visualization of TLC spots [21]. But we could not found any examples of its application in the combination TLC/MALDI.

The present paper reports a possible approach for the application of fixed-charge derivatization of preliminary TLC separated alcohols and phenols inside the chromatographic zones followed by recording the MALDI mass spectra directly from TLC plate. Our approach utilizes the straightforward derivatization method recently developed for analysis of alcohols by MALDI-MS [17].

2. Material and methods

2.1. Analytes, solvents and materials

Alcohols, phenols, sterols, steroid alcohols, terpenols and mixture of tocopherols **2-24** (Table 1), 3-bromopropionyl chloride were purchased from Aldrich Chemical Co. (Belgium). Cholesterol **1** was acquired from Dr. Ehrenstorfer GmbH (Germany). For preparation of composite matrices, 1,8,9-anthracenetriol, glycerol and graphite particles (13 μm diameter) were purchased from Sigma (China), Fluka (Austria) and Qingdao Huatai Lubricating and Sealing Science and Technology Co. (China) respectively.

Solvents (tetrahydrofuran, ethyl acetate, ethyl ether, hexane) and pyridine were of analytical grade (LLC "Himmed", Russia).

TLC plates with fixed silica layer (Macherey-Nagel ALUGRAM Xtra Sil G silica 60) were used for separation of analytes.

2.2. Sample preparation

2.2.1. TLC separation

Solutions of alcohols, phenols, terpenols, sterols, other steroid alcohols or their mixtures in THF (1 mg/ml) were applied onto TLC plates. The plates were developed in a rectangular developing tank (Aldrich Chemical Co., USA) using hexane: ethyl acetate (3:1 v/v) for individual compounds **1-8** and their mixtures, ethyl acetate: acetonitrile (5: 1) for individual compounds **9-11** and their mixtures, hexane: diethyl ether (30:7 v/v) for individual compounds **12-18** and their mixtures, hexane: ethyl acetate (6:1 v/v) for individual compounds **19-24** and their mixtures. The plates were air-dried and placed in a UV cabinet (CAMAG, Switzerland) for spot visualization. Contour of the visualized spots were delineated with a soft graphite pencil. In case of compounds **7**, **17-24** anisaldehyde – sulfuric acid reagent was additionally used for spots visualization as described [17]. TLC plates processed by this reagent were not suitable for further derivatization and were used only for determination of elution zones in parallel experiments.

2.2.2. Derivatization

2 μL of pyridine and 2 μL of 3-bromopropionyl chloride were applied to each spot and then composite matrices, consisting of 1,8,9-anthracenetriol, glycerol and graphite particles, were applied on the plates with a brush, as described [6].

2.3. Equipment and data acquisition

MALDI mass spectra were obtained by desorption/ionization directly from TLC plates using Bruker autoflex speed mass spectrometer equipped with a solid body UV-laser (λ = 355 nm) with 20 μm diameter laser spot on a target. The spectra were recorded in the positive reflectron mode. The maximum energy of the laser was 8 kJ/m^2.

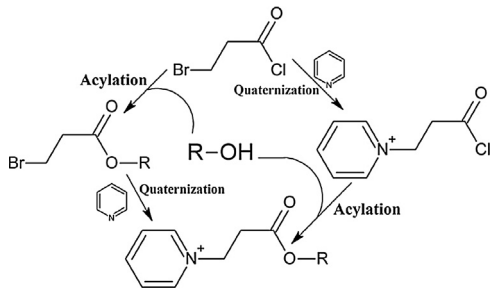
3. Results and discussion

The proposed post-chromatographic derivatization approach is based on the earlier described method of modification of alcohols and phenols, which demonstrate low ionization efficiencies in MALDI conditions and are not detectable by TLC/MALDI. The approach involves their acylation by acyl halides of ω-halogen-substituted fatty acids series with simultaneous quaternization of nitrogenous bases at the expense of terminal halide atom [17]. Most probable reaction mechanisms are given in Scheme 1 for the case of 3-bromopropionyl chloride and pyridine as nitrogeneous base which were used in the present work. Although, this process formally looks as two-steps one, in fact the reaction can be considered as a simply "one-stage" conversion because it occurs without any isolation of by-products and pyridine additionally plays a role of acylation catalyst. Resulting salts have high desorption/ionization efficiencies in MALDI conditions but very low TLC mobility. This fact does not allow using this modification for pre-chromatographic derivatization procedures. At the same time, the process is exother-

Table 1
Tested compounds, their retardation factors (R_F), mass numbers of cationic parts of derivatives, absolute intensities (I, arb.u.) and signal/noise ratios (S/N) of cation peaks in TLC/MALDI mass spectra.

	Compound	R_F	Cation, *m/z*	I	S/N
1	Cholesterol	0.56[a]	520	27,706	91
2	Ergosterol	0.48[a]	530	51,112	230
3	Estrone	0.42[a]	404	47,842	140
4	β-Sitosterol	0.54[a]	548	15,491	72
5	Stigmastanol	0.56[a]	550	41,058	299
6	Stigmasterol	0.57[a]	546	21,350	168
7	β-Estradiol	0.27[a]	540[e]	23,934	84
8	Testosterone	0.15[a]	422	13,995	56
9	trans-Androsterone	0.80[b]	424	26,759	113
10	Estriol	0.54[b]	422	2098	8
			556[e]	12,474	82
			690[f]	35,695	285
11	Prednisolone	0.63[b]	494	27,815	126
			628[e]	26,147	135
			762[f]	5383	26
12	Bisphenol A	0.12[c]	496[e]	331,778	581
13	Triclosan	0.74[c]	422	21,665	103
14	2-Propylphenol	0.56[c]	270	21,438	79
15	4-Nonylphenol	0.56[c]	354	225,240	1221
16	Thymol	0.58[c]	284	66,022	940
17	3,5-Dimethylcyclohexanol	0.76[c]	262	15,570	98
18	α-Tocopherol	0.32[c]	564	12,592	37
	β- and γ-Tocopherols	0.32[c]	550	233,020	683
	δ-Tocopherol	0.32[c]	536	302,456	856
19	Retinol	0.24[c]	420	56,216	186
20	3-Phenylprop-2-en-1-ol	0.32[d]	268	247,203	454
21	(2E)-3,7-Dimethylocta-2,6-dien-1-ol	0.51[d]	288	66,820	301
22	(2Z)-3,7-Dimethylocta-2,6-dien-1-ol	0.54[d]	288	467,86	308
23	3,7-Dimethylocta-1,6-dien-3-ol	0.48[d]	288	3764	30
24	2-Ethylhexan-1-ol	0.66[d]	264	480,274	967

Solvent systems:
[a] Hexane: ethyl acetate (3:1).
[b] Ethyl acetate: acetonitrile (5: 1).
[c] Hexane: diethyl ether (30: 7).
[d] Hexane: ethyl acetate (6:1).
[e] Products of diacylation and monoquaterization.
[f] Products of triacylation and monoquaterization.



Scheme 1. Mechanisms of the reaction for alcohols derivatization on TLC plate.

mal and does not require any heating or other treatment of the reaction mixture. These qualities make the approach suitable for post-chromatographic derivatization in conjunction with TLC analysis.

Preliminary experiments have shown that the derivatization technique implying reaction of analytes with rather large excess of derivatization agents (7–10 fold), which were applied by a syringe on the spot, leads to TLC spots blurring. Further optimization of application of agents on TLC plates allowed us to determine the best amounts and ratios of them: successive application of 2 μL of pyridine and 2 μL of 3-bromopropionyl chloride on a spot.

The proposed approach was tested on mixtures of mammalian- and phytosterols **1-8**, developed in hexane: ethyl acetate solutions. Separated spots, visualized in UV-cabinet, were delineated with a soft graphite pencil and covered with the composite matrix (1,8,9-anthracenetriol, glycerine and graphite particles) described earlier [6]. MALDI mass spectra registered from TLC plate did not contain any peaks corresponding to the analytes (data not shown). Further the analytes were derivatized with pyridine/3-bromopropionyl chloride by their successive application to the spots. Then the zones of derivatization were covered with composite matrix and

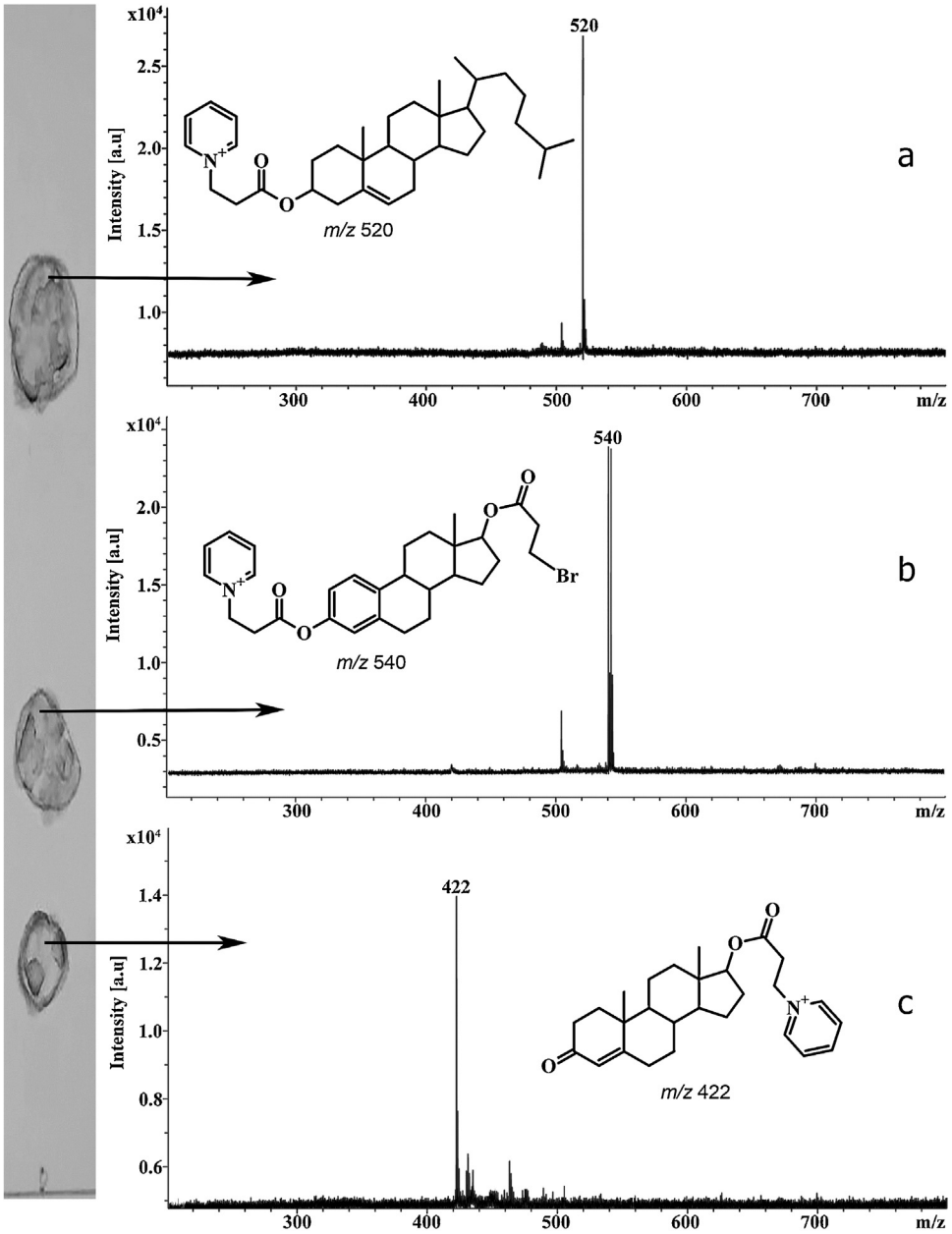


Fig. 1. MALDI mass spectra of steroid alcohols derivatized on TLC plate: cholesterol (a), β-estradiol (b, positions of bromopropionyl and pyridiniumpropionyl groups may vary) and testosterone (c).

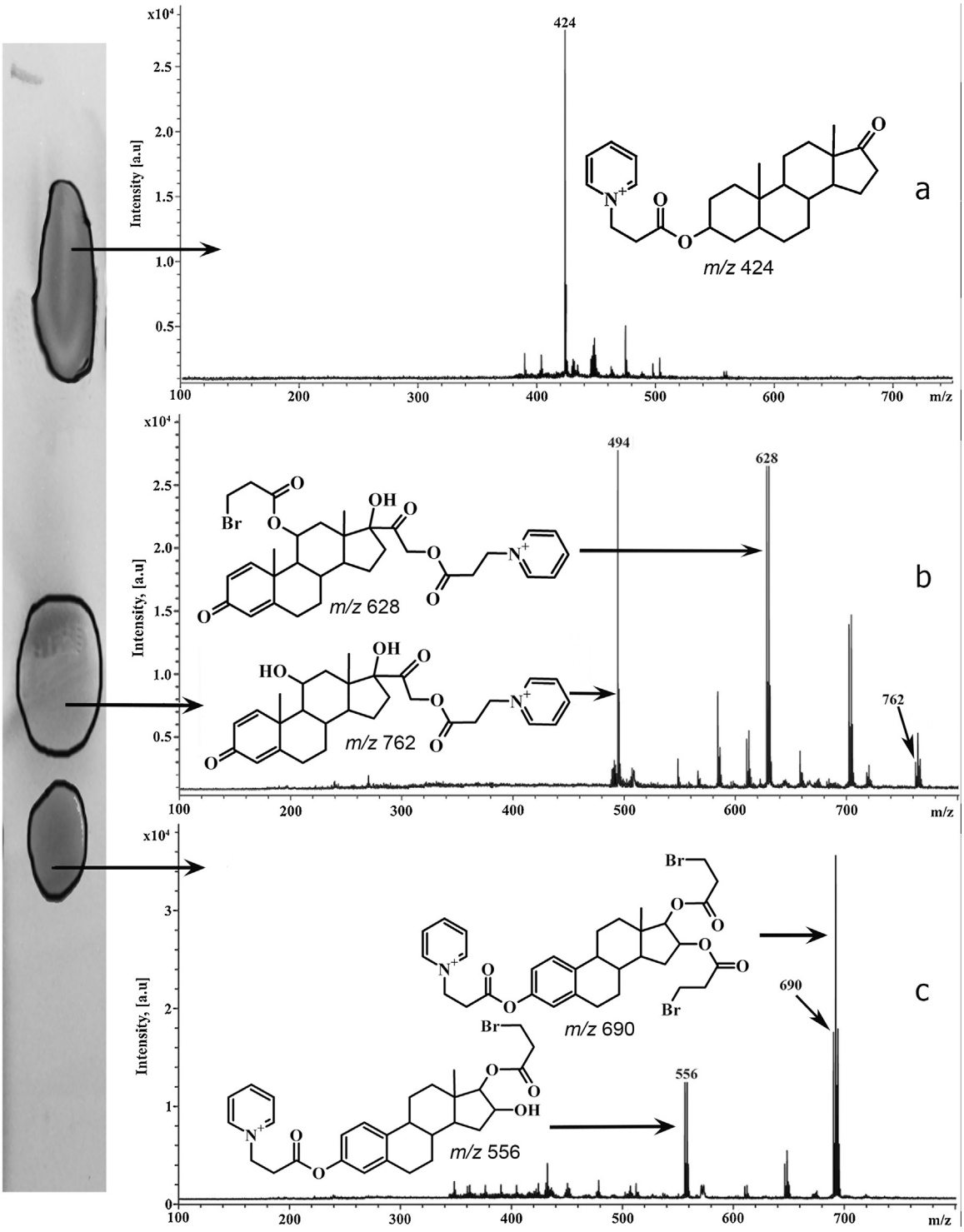


Fig. 2. MALDI mass spectra of steroid alcohols derivatized on TLC plate: *trans*-androsterone (a), prednisolone (b), and estriol (c) (in two latter cases, positions of bromopropionyl and pyridiniumpropionyl groups may vary).

subjected to MALDI-MS analysis. In this case, MALDI mass spectra revealed intense peaks of cationic moieties of corresponding derivatives (Fig. 1). Important to note that the application of the solvent-free combined MALDI matrix (see Experimental) eliminates spots blurring. At the same time, glycerol, which is part of the combined matrix, facilitates the transfer of analyte molecules from the sorbent layer to the surface, thereby facilitating the desorption of the cations.

Multiply charged ions rarely appear in MALDI conditions, so it was of interest to check applicability of the suggested method to the analysis of steroid polyols. Estriol **10**, used as a pregnancy marker, pharmaceutically active prednisolone **11** and *trans*-androsterone **9**, which is capable of diol formation owing to possible carbonyl group enolyzation, were taken as examples. In all cases MALDI mass spectra revealed peaks (*m*/*z* 424 for **9**, *m*/*z* 436 for **10** and *m*/*z* 494 for **11**) of monoacylated/quaternerised analytes (Fig. 2, the mixture was developed in ethyl acetate: acetonitrile solution). Mass

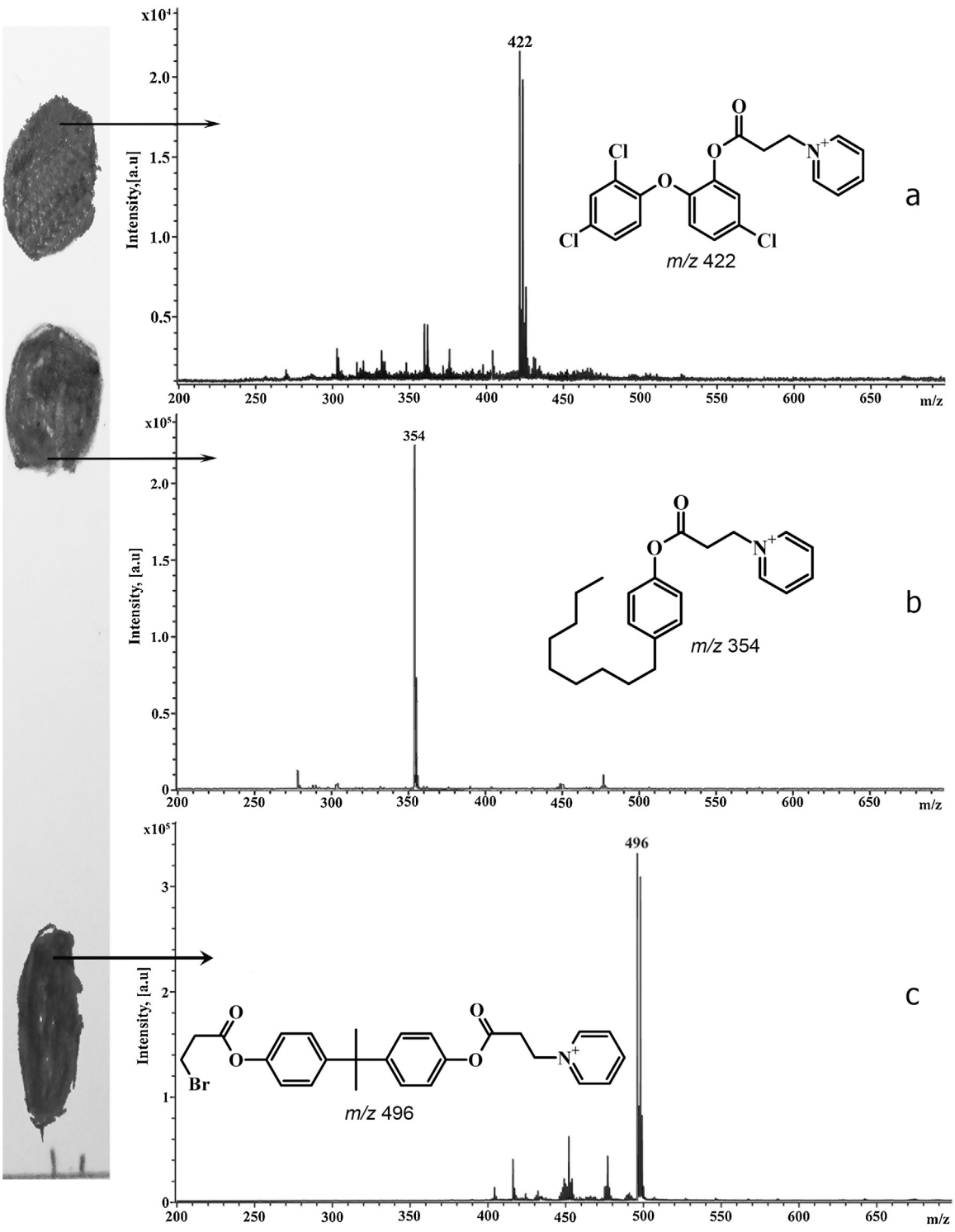


Fig. 3. MALDI mass spectra of triclosan (a), 4-nonylphenol (b) and bisphenol A (c), derivatized on TLC plate.

spectrum of *trans*-androsterone derivatization products does not contain any peaks of doubly acylated molecules. At the same time, ions corresponding to products of double (*m*/*z* 556 for **10** and *m*/*z* 628 for **11**) and triple (*m*/*z* 690 for **10** and *m*/*z* 762 for **11**) acylation but with only single quaternization were also present. We suppose, that the last ones are due to dequaternization processes in the course of desorption/ionization. The ratio of their intensities corresponds to steric hindrances preventing acylation of hydroxyl groups. In fact, in the case of **10** (Fig. 2c), the intensity of the ion peak corresponding to the product of triple acylation/single quaternization (*m*/*z* 690) is much higher than that (*m*/*z* 762) for the compound **11** (Fig. 2b), bearing tertiary hydroxyl group.

The described derivatization approach was also tested for the case of phenols, which are the permanent analytical objects owing to their potential health hazards. Although phenols are less reactive in acylation processes, the discussed method of derivatization-TLC/MALDI mass spectrometry turned out to be applicable to them as well. We showed that it is useful for detection of bisphenol A

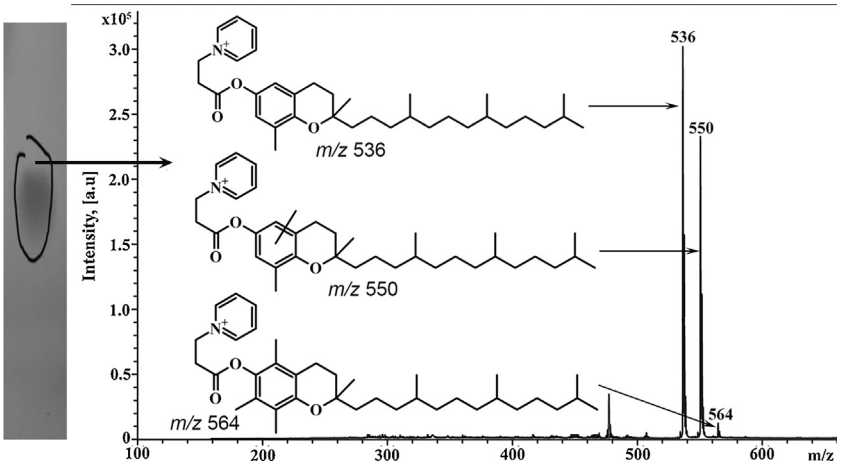


Fig. 4. MALDI mass spectrum of tocopherols mixture, derivatized on TLC plate.

12 (known as used in plastics production), triclosan **13** (widely applied as antibacterial and antifungal agent), as well as industrially important 2-propylphenol **14**, 4-nonylphenol **15** and thymol **16** (3,5-dimethylcyclohexanol **17** was added to some mixtures to compare reactivity of analytes). The mixtures of them were developed in hexane: diethyl ether solutions. All recorded from TLC plates MALDI mass spectra contained intense peaks of cations (for example, see Fig. 3).

The last branch of analytes covered terpenols and phenols (tocopherols **18**, mixtures of retinol **19,** phenylprop-2-en-1-ol **20** and dimethyloctadienols **21-23**); 2-ethylhexanol **24** was added to some of these mixtures to compare reactivity of analytes. In these cases the same MALDI MS results were achieved (Fig. 4). Although we did not succeed in chromatographic resolving of tocopherols mixture, its sum mass spectrum contained all peaks of the derivatives and their ratio corresponded to GC/MS data for this mixture. It should be noted, that despite steric hindrances for acylation of tertiary hydroxyl group of **23**, the derivative formed and was detected although the worst absolute intensity of peak and signal/noise ratio were recorded.

4. Conclusions

We demonstrated that the proposed approach (derivatization-TLC/MALDI mass spectrometry) can be effectively applied to the detection of non-ionizable alcohols and phenols primarily resolved by planar chromatography and subsequent mass spectra recording directly from TLC plate. Recorded mass spectra contain intense peaks of cationic parts of derivatives which are suitable for determination of their molecular weights. Formation of multi-acylated derivatives provides an opportunity to determine the number of free hydroxyl groups in the analytes.

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