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Short communication

A novel sampling method for identification of endogenous skin surface compounds by use of DART-MS and MALDI-MS

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ABSTRACT

Identification of endogenous skin surface compounds is an intriguing challenge in comparative skin investigations. Notably, this short communication is focused on the analysis of small molecules, e.g. natural moisturizing factor (NMF) components and lipids, using a novel sampling method with DIP-it samplers for non-invasive examination of the human skin surface. As a result, extraction of analytes directly from the skin surface by use of various solvents can be replaced with the mentioned procedure. Screening of measureable compounds is achieved by direct analysis in real time mass spectrometry (DART-MS) without further sample preparation. Results are supplemented by dissolving analytes from the DIP-it samplers by use of different solvents, and subsequent matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) measurements. An interesting comparison of the mentioned MS techniques for determination of skin surface compounds in the mass range of 50–1000 Da is presented.

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1. Introduction

DART-MS is a powerful technique in measuring analytes without the use of chromatographic separation. The DART ion source provides an open atmospheric pressure interface, which enables direct introduction of solid, liquid, and gaseous samples without time-consuming sample preparation [1]. Analytes are desorbed from surfaces and undergo a soft ionization process based on the formation of ionized water clusters, followed by proton transfer reactions. DART-MS has been widely used for the analysis of biological matrixes [2,3], drugs [4,5], pharmaceuticals [6,7], and explosives [8]. Recently, the characterization of resins by use of DART-MS was introduced by our group [9].

Since introduction of MALDI-MS in the 1980s [10,11], it has become a powerful approach in measuring molecules in their intact form. The important features that make MALDI-MS a practical tool across life sciences are its molecular identification capability and label-free simultaneous detection. MALDI-MS is a useful tool for measuring metabolites [12], lipids [13], peptides, and proteins [14]. More detailed, molecules are softly desorbed from the co-crystallization matrix layer which absorbs the laser beam energy and protects analytes from disruptive energy.

Connected to a time-of-flight (TOF) detector, both techniques – DART-MS and MALDI-MS – are suitable for wide-range screening analyses with good sensitivity and high mass accuracy, while analysis time only takes a few minutes.

This short communication is focused on the analysis of skin surface compounds using a crucial sampling method for non-invasive analysis of the human skin surface. Swabs of the skin were obtained using small glass rods, so-called DIP-it samplers, and analyzed directly by DART-MS or after extraction by MALDI-MS, respectively. According to skin structure morphology, the outermost layer of human skin is the *stratum corneum*, which consists of several layers of dead keratinized cells surrounded by extracellular lipids [15]. Major components in this compartment are cholesterol, free fatty acids (FAs), and ceramides, besides the presence of cholesterol sulfate [16,17]. In addition, the skin surface is covered by a layer of lipids from sebaceous origin, consisting mainly of triacylglycerides (TAGs), wax esters, FAs, squalene, and cholesterol esters [18]. Equally important are the NMF components, a complex mixture of water-soluble compounds with low molecular weights, e.g. amino acids, urea, pyrrolidone carboxylic acid, and lactic acid. The formation of an effective moisture barrier is influenced by the level of *stratum corneum* hydration. Hence, disturbance upon environmental challenge can lead to dry skin [19].

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Published manuscripts focusing on the determination of skin surface compounds in general made use of chromatographic systems prior to mass spectrometric analyses [20]. Our work provides a new insight into the identification of skin surface compounds by use of two powerful and complementary MS techniques without the use of chromatographic separation.

2. Material and methods

2.1. Chemicals and reagents

2,5-Dihydroxybenzoic acid (DHB), 1,8-bis(dimethylamino)-naphthalene (DMAN), 9-aminoacridine-hydrochloride (9-AA), silver trifluoroacetate (AgTFA), and reserpine were purchased from Sigma-Aldrich (Steinheim, Germany). Solvents 2-propanol, methanol (MeOH) and ethanol (EtOH) were obtained from Merck (Darmstadt, Germany). All aqueous solutions were prepared using ultrapure deionized water.

2.2. Sampling and extraction

For the sampling procedure, swabs of the human skin surface were obtained using DIP-it samplers (IonSense, Saugus, USA). Untreated skin of the forearm and forehead of four volunteers (with informed consent) – no application of cosmetic lotions and creams 48 h before sampling – was gently rubbed 10 times with the enclosed end of the glass capillaries. The adherent skin surface material was directly analyzed by DART-MS without further sample preparation.

According to MALDI-MS measurements, adherent skin surface material was dissolved by use of two different solvents. Subsequent to the application of 200 μL 2-propanol and transferring the solution into a new tube, 200 μL deionized water were added to the DIP-it samplers for extraction of water-soluble compounds. Resulting solutions were vacuum-dried and made up to 10 μL with 2-propanol and deionized water, respectively. Applied matrixes and corresponding conditions are depicted in the MALDI-MS part (see below).

A comprehensive overview of the general workflow is shown in Fig. 1.

2.3. DART-MS – data acquisition and analysis

DART analyses were performed by use of a DART ion source (IonSense, Saugus, USA) coupled to a JEOL AccuTOF orthogonal acceleration single-reflectron TOF mass spectrometer (JEOL, Eching, Germany). The ion source was operated in positive ion mode at 250 °C. Helium was used as the ionizing gas at a flow rate of ~3 L min^{-1}. Electrodes were set as follows: needle voltage 3000 V, grid electrode voltage 250 V, and discharge electrode voltage 150 V. Mass spectra were recorded using a TOF detector operating at a resolving power greater than 6000 (FWHM definition, full width at half maximum) at *m/z* 609.28 from protonated reserpine. Thus, a mass accuracy of 2 ppm and less was achieved for all signals. Analyte ions were identified by elemental composition calculation (monoisotopic masses, software *Elemental Composition Estimation,* MassCenter Main, version 1.3.0.1000, Jeol, Eching, Germany) and verified by analysis of reference substances. Settings of the mass spectrometer were as follows: peaks voltage 600 V, ring lens voltage 5 V, orifice 1 voltage 20 V, orifice 2 voltage 5 V. All spectra were acquired in a mass range of *m/z* 50–1000 and with a spectrum recording interval of 0.2 s.

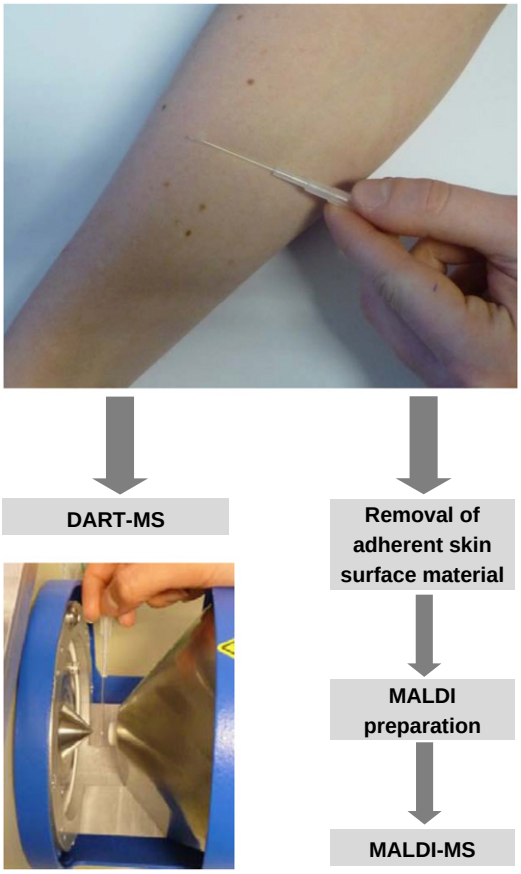

Fig. 1. Comprehensive overview of the sampling method and subsequent steps until analysis. After gently swabbing the skin surface, the DIP-it sampler was measured directly by DART-MS. According to the MALDI-MS procedure, another DIP-it sampler was applied to an in-tube analyte removal. MALDI-MS measurements were carried out subsequent to matrix application.

2.4. MALDI-MS – data acquisition and analysis

MALDI measurements were performed by use of an Autoflex III TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) controlled by the flexControl 3.3 software package (Bruker Daltonics).

2-propanol solutions (1 μL) were mixed with 20 μL DHB matrix (20 mg mL^{-1} in 2-propanol) and 20 μL DHB–AgTFA matrix (20 mg mL^{-1} DHB and 1 mg mL^{-1} AgTFA in 2-propanol), respectively, and mass spectra were recorded in positive reflector mode, setting the low-mass deflector cutoff to 400 Da. For measurements of FAs, DMAN (20 mg mL^{-1} in EtOH) was used as the matrix, acquiring in negative reflector mode with the low-mass deflector cutoff set to 200 Da. To determine further compounds in negative ion mode, 1 μL of the aqueous solution was mixed with 20 μL 9-AA (20 mg mL^{-1} in 1:1 MeOH/deionized water), measuring in a mass range of 200–600 Da. Additionally, aqueous and 2-propanol solutions were analyzed on a nano-assisted laser desorption/ionization (NALDI) target (Bruker Daltonics) in reflector positive ion mode choosing a mass range of *m/z* 50–1000.

3. Results and discussion

3.1. DIP-it sampling

DIP-it sampling was used as a non-invasive method for the examination of the human skin surface. Swabbing the skin surface with the enclosed end of the DIP-it samplers approximately 10 times is advisable to obtain a sufficient quantity of the

Table 1
Schedular summary of detectable skin surface compounds in the mass range of 50–1000 Da by use of DART-MS and MALDI-MS, respectively.

	Class	Analyte		Ion type	*m/z* calculated
DART	**NMF compounds**	Urea		$[M+H]^+$	61.04
		Glycine		$[M+H]^+$	76.04
		Alanine		$[M+H]^+$	90.06
		Lactic acid		$[M+H]^+$	91.04
		Glycerol		$[M+H]^+$	93.06
		Serine		$[M+H]^+$	106.05
		Proline		$[M+H]^+$	116.07
		Threonine		$[M+H]^+$	120.07
		Pyrrolidone carboxylic acid		$[M+H]^+$	130.05
		Leucine/Isoleucine		$[M+H]^+$	132.10
		Urocanic acid		$[M+H]^+$	139.05
		Histidine		$[M+H]^+$	156.08
	Lipophilic compounds	FA C14:0		$[M+H]^+$	229.22
		FA C16:1		$[M+H]^+$	255.23
		FA C 16:0		$[M+H]^+$	257.25
		FA C18:2		$[M+H]^+$	281.25
		FA C18:1		$[M+H]^+$	283.26
		FA C18:0		$[M+H]^+$	285.28
		Cholesterol and esters		$[M-H_2O+H]^+$	369.35
		Squalene		$[M+H]^+$	411.40
		TAG fragment C24:0		$[M-FA+H]^+$	439.38
		TAG fragment C26:0		$[M-FA+H]^+$	467.41
		Wax ester C32:1		$[M+H]^+$	479.48
		TAG fragment C28:0		$[M-FA+H]^+$	495.44
		Wax ester C34:2		$[M+H]^+$	505.50
		Wax ester C34:1		$[M+H]^+$	507.51
		TAG fragment C30:1		$[M-FA+H]^+$	521.46
		TAG fragment C30:0		$[M-FA+H]^+$	523.47
		Wax ester C36:2		$[M+H]^+$	533.53
		Wax ester C36:1		$[M+H]^+$	535.55
		TAG fragment C32:0		$[M-FA+H]^+$	551.50
		Wax ester C38:2		$[M+H]^+$	561.56
		TAG fragment C34:1		$[M-FA+H]^+$	577.52
		Wax ester C40:2		$[M+H]^+$	589.59
		Wax ester C40:1		$[M+H]^+$	591.61
		Wax ester C40:0		$[M+H]^+$	593.62
		Wax ester C42:2		$[M+H]^+$	617.62
MALDI	**Solution**	**Analyte**	**Matrix**	**Ion type**	***m/z* calculated**
	2-propanol solution	FA C16:1	DMAN	$[M-H]^-$	253.22
		FA C16:0	DMAN	$[M-H]^-$	255.23
		FA C18:2	DMAN	$[M-H]^-$	279.23
		FA C18:1	DMAN	$[M-H]^-$	281.25
		FA C18:0	DMAN	$[M-H]^-$	283.26
		FA C20:1	DMAN	$[M-H]^-$	309.28
		FA C20:0	DMAN	$[M-H]^-$	311.29
		Cholesterol	DHB+AgTFA	$[M+^{107}Ag]^+$	493.26
				$[M+^{109}Ag]^+$	495.26
		Squalene	DHB+AgTFA	$[M+^{107}Ag]^+$	517.30
				$[M+^{109}Ag]^+$	519.30
		Cer(d18:0/23:0)	DHB/NALDI	$[M+H]^+$	638.64
		Cer(d18:0/25:0)	DHB/NALDI	$[M+H]^+$	666.68
		TAG C46:2	DHB/NALDI	$[M+Na]^+$	797.66
		TAG C46:1	DHB/NALDI	$[M+Na]^+$	799.68
		TAG C46:0	DHB/NALDI	$[M+Na]^+$	801.69
		TAG C47:2	DHB/NALDI	$[M+Na]^+$	811.68
		TAG C47:1	DHB/NALDI	$[M+Na]^+$	813.70
		TAG C48:2	DHB/NALDI	$[M+Na]^+$	825.69
		TAG C48:1	DHB/NALDI	$[M+Na]^+$	827.71
		TAG C48:0	DHB/NALDI	$[M+Na]^+$	829.73
		TAG C49:2	DHB/NALDI	$[M+Na]^+$	839.71
		TAG C49:1	DHB/NALDI	$[M+Na]^+$	841.73
		TAG C50:2	DHB/NALDI	$[M+Na]^+$	853.73
		TAG C50:1	DHB/NALDI	$[M+Na]^+$	855.74
		TAG C51:2	DHB/NALDI	$[M+Na]^+$	867.74
		TAG C51:1	DHB/NALDI	$[M+Na]^+$	869.76
	Aqueous solution	Cholesterol sulfate	9-AA	$[M]^-$	465.30

9-AA, 9-aminoacridine; **AgTFA**, silver trifluoroacetate; **Cer**, ceramide; **DHB**, 2,5-dihydroxybenzoic acid; **DMAN**, 1,8-bis(dimethylamino)naphthalene; **FA**, fatty acid; ***m/z***, mass-to-charge ratio; **NALDI**, nano-assisted laser desorption/ionization; **NMF**, natural moisturizing factor; **TAG**, triacylglyceride

endogenous compounds. An accurate yield cannot be specified; this is attributed to only trace amounts being handled. Hence, weighing of the DIP-it samplers before and after application showed no significant differences. However, DIP-it sampling can be seen as a novel method for non-invasive extraction of wide-ranged analytes, replacing the use of irritant and harmful solvents.

3.2. DART-MS

With its special design, the DART ion source offers the possibility to measure DIP-it samplers with adherent skin material without any further sample pretreatment. Thus, the analysis time is reduced to a minimum. High-resolution mass spectra were obtained, showing signals of various skin surface compounds with $m/z < 1000$. An overview of all detectable compounds is given in Table 1.

Interestingly, FAs and amino acids generally provided signals with high intensities in both positive and negative ion mode. In negative ion mode, analyte ions generated by the abstraction of hydrogen were detected ($[M–H]^-$), which is the most frequently observed ionization form for organic acids. On the other hand, in positive ion mode, protonated molecular ions were detected. Thus, the proton affinity of all identified acids was obviously sufficient to form $[M+H]^+$-ions, resulting in intensive signals in the positive DART ionization mode. Since some of the other skin compounds were not detectable in the negative ion mode, the positive mode was adopted for the analysis of all skin samples. A mass spectrum acquired in positive ion mode showing amino acid and FA signals is presented in Fig. S1a (Supplementary material).

Two of the most prominent signals in all skin spectra were observed at m/z 369.35 and m/z 411.40, respectively. These signals correspond to the $[M–H_2O+H]^+$-ion of cholesterol and the protonated molecular ion of squalene. Due to fragmentation by splitting the ester bond, cholesterol esters also produce the signal at m/z 369.35 and are, thus, not distinguishable from cholesterol. Hence, the signal at m/z 369.35 represents both cholesterol and its esters. It should be emphasized that, in spite of its hydrocarbon character and the absence of heteroatoms, squalene was easily protonated and detected, and provided intensive signals during DART-MS analyses.

TAGs were determinable by their fragments, which were formed by splitting a FA off the glycerol backbone. In addition, fragmentation of diacylglycerides (DAGs) occurred under the same experimental DART conditions, yielding in similar types of fragments through loss of water. As a result, TAG and DAG fragments carrying the same FAs were not distinguishable. By heating the DART ion source up to 300 °C, and using ammonia vapor as a dopant, the intensity of intact TAG ammonium adduct ions was increased. However, due to a growing extent of thermally induced fragmentation under these conditions, a reduction of signal intensity of lower molecular mass analytes, especially NMF analytes, was observed. Therefore, DART conditions were selected providing good sensitivity and detectability for most of the skin surface compounds, resulting in a method where only TAG fragments were observed. A corresponding representive mass spectrum showing signals of cholesterol and its esters, squalene, wax esters, and TAG/DAG fragments is depicted in Fig. S1b (Supplementary material).

Identification of compounds was achieved by elemental composition calculation, and verification by analysis of reference substances, and by use of the data extractor to select or display search results from the human metabolome database (HMDB, http://www.hmdb.ca/search/extractor), which is freely available.

DART-MS spectra of two different skin areas – forearm and forehead – are shown in Fig. 2. Apparently, the relative intensities of signals in both spectra differ from each other. This finding can be correlated with the varying skin surface composition. While the forehead is an area rich in sebaceous glands, which are relevant for sebum lipids secretion, the forearm shows a lower distribution of sebaceous glands and, thus, a decreased relative amount of sebum lipids. As shown in Fig. 2, relative intensities of squalene (m/z 411.40), wax ester (m/z ~450–600), and TAG fragment (m/z ~450–600) signals in the forehead spectrum

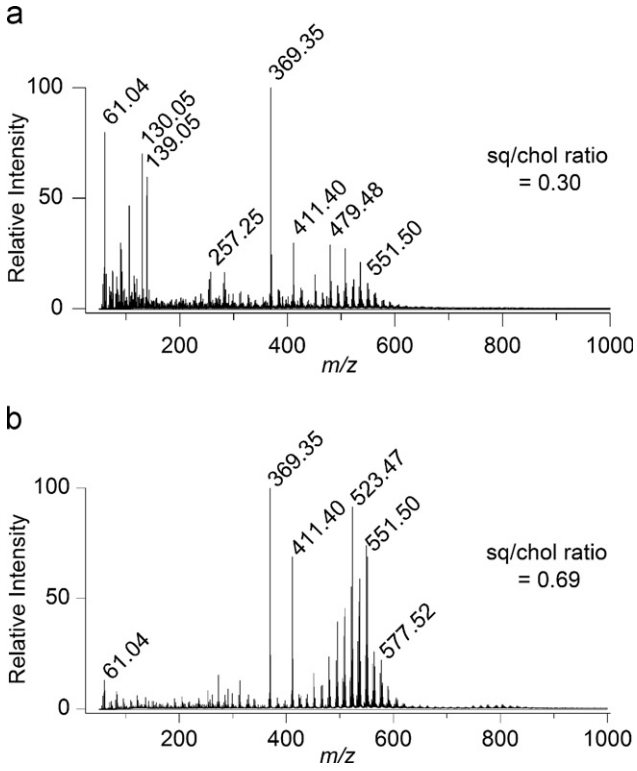

Fig. 2. DART-MS spectra of forearm (a) and forehead skin (b), showing various skin compounds with differences in relative intensities according to the skin surface composition. For each spectrum, the squalene/cholesterol fragment ratio (sq/chol ratio) is depicted. m/z 61.04=urea; m/z 130.05=pyrrolidone carboxylic acid; m/z 139.05=urocanic acid; m/z 257.25=FA C16:0; m/z 369.35=cholesterol fragment; m/z 411.40=squalene; m/z 479.48=wax ester C32:1; m/z 523.47=TAG fragment C30:0; m/z 551.50=TAG fragment C32:0; m/z 577.52=TAG fragment C34:1.

are more intensive than in the forearm spectrum. Furthermore, signals caused by NMF components (m/z ~60–160) are more abundant in the spectrum obtained by analysis of forearm skin. In accordance with recently published results [20], the squalene/cholesterol fragment ratio of both skin areas reflects the strong distribution of sebaceous glands in the forehead region (ratio=0.69) in comparison to forearm skin (ratio=0.30). Thus, differentiation of two varying skin areas could be easily achieved by the described method.

3.3. MALDI-MS

According to MALDI-MS, DIP-it samplers with adherent skin surface material cannot be used directly. Hence, a dissolving step is required. In our work, skin surface compounds were dissolved by the use of 2-propanol (2-propanol solution) followed by extraction with deionized water (aqueous solution). Various matrixes (DHB, DMAN, 9-AA) and the commercially available NALDI-target (Bruker Daltonics) were applied for MALDI-MS measurements. A summary of the used matrixes and the measurable compounds is depicted in Table 1.

By use of the DMAN matrix, also called proton sponge, which based upon the matrix-assisted ionization laser desorption (MAILD) principle, where ions are generated already in the co-crystallization layer and not in the gas phase [21], FAs are measurable sensitively. Characteristic FAs of the skin surface, e.g. FA C18:0, are determinable accurately in the negative ion mode due to their ability to split the proton of the carboxyl group. In comparison to the DART-MS results, longer FA, e.g. FA C20:0,

were detectable (Table 1). A representive mass spectrum is presented in Fig. S2a (Supplementary material).

Furthermore, 9-AA is a MALDI matrix typically used in the negative ion mode for measuring substances which contain sulfate or phosphate groups. Applying 9-AA to the aqueous solution, FAs were not detectable. However, a mass signal for cholesterol sulfate existed in all samples (Fig. S2b). Formed in the basal and spinous layer of the epidermis, its concentration decreases in the *stratum corneum* as a result of steroid-sulfate sulfatase acitivity [22]. The distribution of cholesterol sulfate within the epidermis using the MALDI mass spectrometric imaging (MALDI-MSI) technique and the 9-AA matrix was recently published by our group [23]. Coming back to the swabs from the forehead and the 9-AA matrix, all samples represented a distribution with a shift of *m/z* 44 in the lower mass range (Fig. S2b), deriving from lauryl ether sulfate (LES), a typical ingredient in shampoos and other cleansing products. Meaning, detectable LES is caused by matutinal showering of the volunteers. Myristyl ether sulfate as a secondary component in LES raw material was also detected (Fig. S2b).

DHB matrix and NALDI target are usually applied in positive ion mode. By use of DHB matrix and the NALDI target, respectively, TAGs were detectable in their intact form (see Table 1 and (Fig. S2c). TAG fragments, derived from a FA loss, were observed as a function of the laser beam energy. Additionally, DAGs were identified (data not shown). Moreover, ceramides Cer(d18:0/23:0) and Cer(d18:0/25:0) were determinable, but only in two forehead samples. This can be attributed to a relatively small sample size and to normal variability in biological samples.

As described in the DART-MS section above, cholesterol and squalene are easily detectable in skin surface swabs. In contrary, detection of the mentioned molecules by use of the DHB matrix was not successful due to the molecule structure and the difficulty to protonate or deprotonate, resulting in a low sensitivity for cholesterol and squalene. However, using the DHB matrix in combination with the auxiliary reagent AgTFA, cholesterol and squalene were detectable as ^{107}Ag and ^{109}Ag adducts (Fig. S2d). Due to the apolarity of cholesterol and squalene, AgTFA is often added to the DHB matrix in order to improve the ion yield.

According to accurate identification of skin surface compounds by use of MALDI-MS, calculated monoisotopic masses were entered into the human metabolome database (HMDB) data extractor (http://www.hmdb.ca/search/extractor) to select or display search results. Furthermore, verification was achieved by analysis of reference substances.

Due to matrix signals and interference, MALDI-MS cannot be seen as the method of choice for measuring low molecular weight compounds, especially in the mass range below 300 Da. Using the NALDI approach, these drawbacks can be overcome. Many pure NMF compounds, e.g. amino acids, were detectable in this way. Hence, aqueous solutions of the swabs were deposited on the NALDI target and mass spectra were generated in a range of 50–400 Da. Unfortunately, identification of the resulting mass signals is not trivial, due to the mass accuracy of MALDI-MS. In summary, DART-MS can be seen as the method of choice for detecting NMFs.

3.4. DART-MS and MALDI-MS as complementary techniques

The introduced method allows simultaneous detection of various skin surface compounds using a novel sampling procedure. The use of irritant and harmful solvents can be avoided. While DART-MS was used to determine wax esters and NMF compounds, MALDI-MS was applied to analyze cholesterol sulfate and ceramides. Both techniques, MALDI-MS and DART-MS, were suitable to detect FAs, TAGs, cholesterol, and squalene. Relative intensities of signals in DART-MS spectra were shown to reflect different compositions of skin surface compounds of forearm and forehead skin.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.10.073.

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