Study of sample preparation influence on bacterial lipids profile in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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ABSTRACT

Lipids are one of the cell components therefore it is important to be able to accurately assess them. One of the analytical techniques used to study lipid profiles is matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS). The present study attempted to select optimal conditions for sample preparation and MALDI MS analysis of bacterial lipidome in both positive and negative ion modes using different extraction protocols - Folch, Matyash and Bligh & Dyer, solvents used to apply samples, and matrices such as 9-aminoacridine (9-AA), α-cyano-4-hydroxycinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (DHB), 2-mercaptobenzothiazole (MBT) and 2,4,6-trihydroxyacetophenone (THAP). The obtained results allowed concluding that DHB or CHCA matrices are suitable for lipid analysis in the positive mode, and in the negative mode THAP or 9-AA. The most appropriate protocol for extracting lipids from bacterial cells was the Bligh & Dyer method in both ionization modes. The use of the solvent TA30, which was a mixture of acetonitrile and 0.1% trifluoroacetic acid in water, provided on the spectra a significant number of signals from lipids in all groups analyzed, such as fatty acyls, glycerolipids and glycerophospholipids.

Keywords: extracts; lipids; MALDI; mass spectrometry; matrix; microorganisms

1. INTRODUCTION

Lipids are cellular components which play an important role in the metabolism of living organisms. They are the building blocks of cell membranes and perform valid cellular functions such as subcellular compartmentalization, signaling and energy storage [1]. According to the classification recommended by the International Lipid Classification and Nomenclature Committee (ILCNC), lipids are divided into eight groups: (a) fatty acids, (b) glycerolipids, (c) glycerophospholipids, (d) sphingolipids, (e) sterol lipids, (f) prenol lipids,

(g) saccharolipids and (h) polyketides [2]. Recognizing changes in lipid composition can provide important information about cellular homeostasis, disease pathogenesis [3], and in the case of microorganisms such as bacteria, allow their identification [4],[5].

Most omics analyses are based on the use of mass spectrometry (MS) techniques, particularly soft ionization methods such as electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) or matrix-assisted laser desorption/ionization (MALDI) [6]. MALDI is an analytical technique that is commonly used in proteomic analysis, however, due to its advantages such as soft and efficient ionization with relatively low fragmentation, high tolerance to buffer salts and detergents, uncomplicated spectra because most ions are singly charged, high detection sensitivity over a wide mass range, rapid analysis, and relatively simple instrumentation [7] is also increasingly being used for lipid analysis [8]. Prior to measurement with the MALDI technique, the sample is mixed with a low-molecular-weight organic acid called a matrix, which is used to assist in the ionization process [9]. Therefore, crucial to the success of the experiment is the selection of the right matrix, since different matrices have different ionization properties and are variously suitable for the ionization of certain classes of substances [10]. Due to the matrix-derived chemical background present in the spectra, some researchers are focusing on the development of matrix-free methods based on different types of nanoparticles and nanostructures, also for applications in lipid analysis [11]. The compounds most commonly used as matrices in lipid analysis are: α-cyano-4-hydroxycinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (DHB), 9-aminoacridine (9-AA), 2-mercaptobenzothiazole (MBT) and 2,4,6-trihydroxyacetophenone (THAP) [6].

The second key aspect of lipid research is the preparation of samples for analysis. Liquid-liquid extraction methods such as the protocols developed by Folch, Bligh & Dyer or Matyash are commonly used to extract lipids from biological material [12]. There are many papers on lipid analysis using MALDI MS [13],[14],[15], comparing available matrices [16] or extraction protocols [12], but there is a paucity of articles containing an experimentally selected set of parameters to effectively analyze bacterial lipid profiles.

The premise of this work was to test different lipid extraction protocols, matrices, and solvents to optimize sample preparation for the analysis of bacterial lipids from Gram-negative and positive strains using the MALDI MS technique in both positive and negative ion modes.

2. MATERIALS AND METHODS

2.1. Reagents and materials

All MALDI matrices used in this study were of the highest commercially available purity: α-cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB) were purchased from Bruker Daltonik GmbH, 2′,4′,6′-trihydroxyacetophenone monohydrate (THAP) was purchased from Sigma-Aldrich, 9-aminoacridine (9-AA) and 2-mercaptobenzothiazole (MBT) were purchased from Tokyo Chemical Industry. Further details on the matrices can be found in the Supplementary Material, labeled SM1. Cesium triiodide used for calibration of mass spectra was of 99.9% purity (Sigma-Aldrich). Trifluoroacetic acid (analytical standard), methanol, ethanol, chloroform, methyl-tert-butyl

ether and tetrahydrofuran (HPLC grade), acetonitrile and water (LC-MS grade) were purchased from Sigma-Aldrich.

2.2. Bacterial cultures

The studies used bacterial strains isolated from the urine of patients diagnosed with prostate cancer. Urine samples were collected from prostate cancer patients and immediately frozen at -80°C. In order to isolate bacteria, urine was inoculated directly onto various culture media (Tryptic Soy Agar, Schaedler Agar, CLED Agar) and incubated overnight at 37°C [17]. Grown bacterial colonies were identified by the MALDI technique using a Bruker microflex mass spectrometer with Biotyper database (Bruker Daltonics, Bremen, Germany). In this study, 4 different bacterial isolates were selected: *Staphylococcus epidermidis*, *Enterococcus faecalis* (Gram-positive strains), and *Escherichia coli*, *Proteus mirabilis* (Gram-negative strains). Before lipid extraction, all strains were incubated for 24 hours on a universal medium, Tryptic Soy Agar (Sigma Aldrich, Steinheim, Germany).

2.3. Lipid extraction

Lipids were extracted from fresh bacterial cultures using three extraction methods:

Folch extraction method [18]: 1 µl of bacterial biomass was suspended in 0.4 mL of methanol. 0.8 mL of chloroform (TCM) was added to the samples and placed in an ultrasonic bath for 10 minutes. Phase separation was induced by adding 0.3 mL of H_2O and vortexing for 10 minutes. The samples were centrifuged at 2,000 rcf for 10 minutes. The organic phase (bottom) was collected into a separate centrifuge tube, and 1 mL of chloroform/methanol mixture (2/1; vol/vol) was added to the upper phase. The samples were vortexed again, centrifuged and the bottom phase was collected. Combined organic phases were dried in a benchtop vacuum concentrator (CentriVap, Labconco).

Bligh & Dyer extraction method [19]: 1 µl of bacterial biomass was suspended in 0.5 mL of methanol. 0.5 mL of TCM was added to the samples and placed in an ultrasonic bath for 10 minutes. 0.45 mL of water was added to the samples and votexed for 10 minutes. The samples were then centrifuged at 2,000 rcf for 10 minutes. The organic (lower) phase was collected in a separate centrifuge tube, and 1 mL of chloroform/methanol mixture (1/1; vol/vol) was added to the upper phase. The samples were vortexed again, centrifuged and the bottom phase was collected. Combined organic phases were dried in a benchtop vacuum concentrator (CentriVap, Labconco).

Matyash extraction method [20]: 1 µl of bacterial biomass was suspended in 0.3 mL of methanol. 1.0 mL of methyl-tert-butyl ether (MTBE) was added to the samples and placed in an ultrasonic bath for 10 minutes. For phase separation, 0.25 mL of water was added to the samples and stirred for 10 minutes. The samples were then centrifuged at 2,000 rcf for 10 minutes. The organic layer (upper) was collected in a separate centrifuge tube, and 1 mL MTBE/methanol mixture (10/3; vol/vol) was added to the lower phase. The samples were vortexed again, centrifuged, and the upper layer was collected. Combined organic phases were dried in a benchtop vacuum concentrator (CentriVap, Labconco).

2.4. Matrix and sample preparation

TA30 solvent is a mixture of acetonitrile and 0.1% trifluoroacetic acid in water in the ratio of 30 to 70 (v/v). The CHCA matrix was prepared as a saturated solution in TA30. 20 mg of DHB was dissolved in 1 mL TA30. A 10 mg/mL solution was prepared for 9-AA matrix in a 9 to 1 mixture of methanol and water, for MBT in a 1:1:1 ethanol:tetrahydrofuran:water mixture. For the THAP matrix, a 14 mg/mL solution was prepared in a 7 to 3 mixture of acetonitrile and water. Lipid samples extracted from bacteria were dissolved in 200 μL of chloroform (trichloromethane, TCM) or TA30. The calibrant was prepared by dissolving 10 mg of CsI in 1 mL of methanol and adding 1 mL of a 20 mg/mL solution of DHB in methanol.

2.5. MALDI mass spectrometry analysis

Analysis was carried out using the Bruker ultrafleXtreme time-of-flight mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a SmartBeam II laser operating at 355 nm and a frequency of 2 kHz. Data processing was performed using FlexControl and FlexAnalysis 3.3 software (Bruker Daltonics, Bremen, Germany). A 0.5 μL sample was spotted onto the MTP 384 target plate ground steel (Bruker Daltonics, Bremen, Germany), and after drying, 0.5 μL of matrix was applied. Each sample was measured in triplicate. At each point, 2000 (4×500) laser shots were made with default random walk applied. Measurement range was *m/z* 100 to 2000. Suppression was turned on typically for ions of *m/z* lower than 95. Mass calibration was performed using external standard (cesium triiodide, CsI_3) [21] with a cubic enhanced calibration strategy. For positive ion mode reflector voltages were 26.64 and 13.59 kV, while the first accelerating voltage was 25.07 kV, and the value for the second ion source voltage was 22.41 kV. For negative ion mode the reflector voltages were 21.30 and 10.82 kV, while the first accelerating voltage was 20.07 kV, and the value for the second ion source voltage was 17.97 kV. The value of detector gain for the reflector was 4× (2010 V). The value of the global attenuator offset was 30%, attenuator offset - 27%, attenuator range - 23%, focus offset - 0%, focus range - 100% and focus position - 36%. The centroid peak detection algorithm was used to create a list of *m/z* values. Signals selected for putative identification had *S/N* values ≥ 3, and propositions for lipids were based on the mass error, which was Δ ± 0.01 *m/z*. The LIPID MAPS Structure Database with "Bulk" Structure Searches tool was used to identify lipids [22]. Microsoft Excel 2010 program was used to create the bar charts. The spectra of the matrices and calibrant in the positive mode are presented in Supplementary Materials SM2, and in the negative mode in Supplementary Materials SM3.

3. RESULTS AND DISCUSSION

The goal of the study was to find the optimal sample preparation and MALDI MS measurement conditions for lipids isolated from bacterial cells. Four bacteria were selected for this purpose: two gram-positive: *Staphylococcus epidermidis* and *Enterococcus faecalis*, and two gram-negative: *Escherichia coli* and *Proteus mirabilis*. Lipids were extracted from the bacteria using the Folch [18], Matyash [20] and Bligh & Dyer [19] methods described in paragraph 2.3. Two-phase extraction protocols were used in the experiment, providing purer lipid mixtures compared to single-phase extractions [23]. The dried lipid fraction was redissolved in TCM or TA30 solution and applied onto the MALDI target along with one of

the 5 matrices. Three measurements were taken for each sample, and the results were averaged. To identify lipids on individual spectra, the LIPID MAPS Structure Database with "Bulk" Structure Searches online tool was used, which contains 47834 unique lipid structures, but molecules belonging to the prenol lipids, saccharolipids and polyketides groups are not available in this search tool [24]. Sphingolipids and sterol lipids were not taken into account in the search for structures due to the lack of their presence in the cells of the analyzed bacteria [25],[26].

3.1. Positive ion mode

In the matrix-assisted laser desorption/ionization mass spectrometry technique with a time-of-flight (TOF) analyzer, the positive ion mode with a reflectron is commonly used to analyze low-molecular-weight compounds, including lipids [6]. It was therefore decided to check the effect of the matrix and the solvent used to redissolve the sample on the obtained results. Most lipid-derived signals were obtained for the DHB matrix for all variants of the extraction methods and solvents used, except for the Matyash method and TA30, where the largest mean number of lipids was identified for the CHCA matrix (Figure 1A). The highest mean number of *m/z* values assigned to lipids (521) was obtained for Folch extracts dissolved in TA30 using DHB the matrix, and the lowest (107 signals) for Bligh & Dyer extracts dissolved in chloroform using MBT matrix. 2,5-Dihydroxybenzoic acid is the most commonly recommended matrix for the analysis of lipids in the positive ion mode [27,28]. Therefore it is not surprising that the largest number of lipid-derived signals were obtained using this matrix. However, there are differences between the spectra recorded for different extracts and solvents using the DHB matrix (Supplementary materials SM4). The mean number of lipids associated with the signals is usually lower when chloroform is used as the solvent (Figure 1).

For MALDI MS analysis, the sample drying prerequisite necessitates careful consideration of analyte solubility, as well as its co-crystallization potential with the matrix [29]. Solvents that facilitate broad solute dissolution and exhibit rapid evaporation rates, such as chloroform, emerge as logical choices for lipid sample preparation. Nonetheless, the method by which the sample is deposited onto the MALDI steel target cannot be overlooked. Due to its reduced contact angle, chloroform disperses across the target, occupying a more expansive area. This dispersion can compromise the sensitivity of the MS analysis, especially when contrasted with samples dissolved in a water and acetonitrile mixture, which concentrate over a limited target zone [30]. Additionally, TA30 encompasses water—a protic solvent—and trifluoroacetic acid, which is capable of donating protons (H^+), thereby enhancing analyte ionization in the positive ion mode. This assertion is corroborated by the heightened signal count in the spectra procured using TA30 as the solvent, compared to those using TCM, with the exception of extracts derived via the Matyash method (refer to Supplementary Material SM4).

Figure 1 shows large differences in the ability to ionize lipids for individual matrices. Also, the spectra made for the same extract using various matrices present a miscellaneous set of signals (Figure 2). There are some differences between the number of lipids found for gram-positive bacteria (*S. epidemidis* and *E. faecalis*) and gram-negative bacteria (*E. coli* and *P. mirabilis*) (Supplementary materials SM5). For both types of bacteria, the DHB matrix and

TA30 as a solvent turned out to be the best choice. However, in the case of gram-positive bacteria, the Folch method was a better extraction protocol, and Bligh & Dyer method for gram-negative bacteria. This may be related to the structure of the bacterial cell wall, which is thicker in gram-positive bacteria, and thus the extraction of lipids is more difficult. A higher proportion of chloroform in the Folch extraction protocol may have a beneficial effect on the efficiency of lipid extraction from gram-positive bacteria. In the case of studies focused on a specific group of lipids, the appropriate isolation protocol and selected matrix should be followed [12]. Target analysis of mass spectra towards fatty acyls (Figure 1B) showed that most lipids from this group are visible in the spectra of Matyash and Bligh & Dyer extracts dissolved in TA30 using the CHCA matrix. A similar result was found for extracts from each of the analyzed bacteria. This result was unexpected because this matrix is not usually recommended for the analysis of this group of lipids [16],[31]. However, the authors of the papers refer in their considerations only to free fatty acids, which are the most important but not the only group of fatty acyls. The high number of fatty acyls detected in the extracts made by the Matyash and Bligh-Dyer methods is understandable given that these two protocols are recommended for the analysis of this group of lipids [12].

Another analyzed group are glycerolipids, among which the largest subgroup are triacylglycerols (TAG) and diacylglycerols (DAG). Triacylglycerols are a storage material in eukaryotic organisms, while their role in prokaryotes has not been fully understood [32], but they were detected using MALDI MS in lipid extracts of lactic acid bacteria [33]. For glycerolipids, the most signals were noted in the spectra of Bligh & Dyer and Folch extracts redissolved in TA30 using the DHB matrix (Figure 1C), however, their number is low (about 40). This may be due to fragmentation taking place in the ion source, whereby TAG and DAG fragments may be visible in the spectrum as free fatty acids [16]. 2,5-dihydroxybenzoic acid, for which the largest number of signals related to glycerolipids were obtained, is the matrix recommended for their analysis in the positive mode [6], however, it is worth paying attention to the MBT matrix, which in some cases (Folch-TCM and Matyash-TA30) proved to be as effective as DHB in ionizing this group of lipids. Mono-, di- and triacylglycerols dissolve well in apolar solvents, which include both chloroform used in the Folch and Bligh-Dyer methods and MTBE used in the Matyash protocol, but glycosylglycerols, which are another important subgroup, were more often extracted using the Folch and Bligh-Dyer techniques [34], which may explain the differences in the number of assigned signals between the methods.

In the case of glycerophospholipids, again the highest number of signals was obtained for the DHB matrix for the extracts made by Folch and Bligh & Dyer protocols redissolved in TA30 solution (Figure 1D). Glycerophospholipids are the main building component of cell membranes [35], therefore it is not surprising that they accounted for nearly half of all detected lipids in MALDI MS spectra. Some authors point out that glycerophospholipids are relatively easily extracted with the protocols used in this study, while lipids belonging to other classes are lost during extraction. This would explain the significant difference in the number of glycerophospholipids detected compared to other lipids [31]. In the positive ion mode, phospholipids can appear in the spectra as both proton and sodium or potassium adducts, and DHB is referred to as the first choice matrix for their analysis, providing optimal results in terms of achievable sensitivity [36]. According to some authors, most glycerophospholipids

261 can be extracted by each of the three protocols with similar yields [12], but this does not
262 explain the lower number of identified glycerophospholipids when extracted using the
263 Matyash method in our study. The reason for this noticeable difference may be the lower
264 recovery for the Matyash method [37].
265 The percentage of individual classes of lipids is very similar for different extracts and
266 solvents (Figure 3A-E). However, the differences are evident between the matrices used. The
267 highest percentage of fatty acyls (about 40% of all lipids) was obtained for the 9-AA matrix
268 (Figure 3A), for glycerolipids the highest share (from 13 to 30%) was recorded for MBT
269 (Figure 3D), and for glycerophospholipids the highest percentage (about 60%) was recorded
270 for DHB (Figure 3C) and THAP (Figure 3E). The Venn diagram in Figure 3F shows the
271 average numbers of unique and common lipids between the three extraction methods for
272 TA30 solvent and DHB matrix. The most common lipids were recorded for the Folch and
273 Bligh-Dyer methods. This may be due to the similarity of these two methods of lipid
274 extraction, where the same reagents are used only in different proportions. A similar effect of
275 the extraction method on the lipid profiles was observed for human urine extracts [38]. The
276 markedly lower number of lipids identified in extracts made by the Matyash method seen in
277 Figures 3F and 1A is probably due to lower recoveries compared to other extraction methods
278 [37]. Figure 3G presents the Venn diagram for the three matrices with the highest number of
279 lipid-derived signals for the extract made by the Bligh-Dyer method dissolved in TA30. A
280 large number of common lipids between DHB and CHCA matrices, as well as DHB and
281 THAP, are noticeable. However, the vast majority of the identified lipids are unique to each
282 matrix, suggesting that several matrices should be used to study the entire bacterial lipidome
283 with MALDI TOF MS. A figure containing MALDI MS spectra in positive mode for lipid
284 extracts from *P. mirabilis*, *S. epidermidis* and *E. faecalis* is presented in Supplementary
285 Materials SM6.
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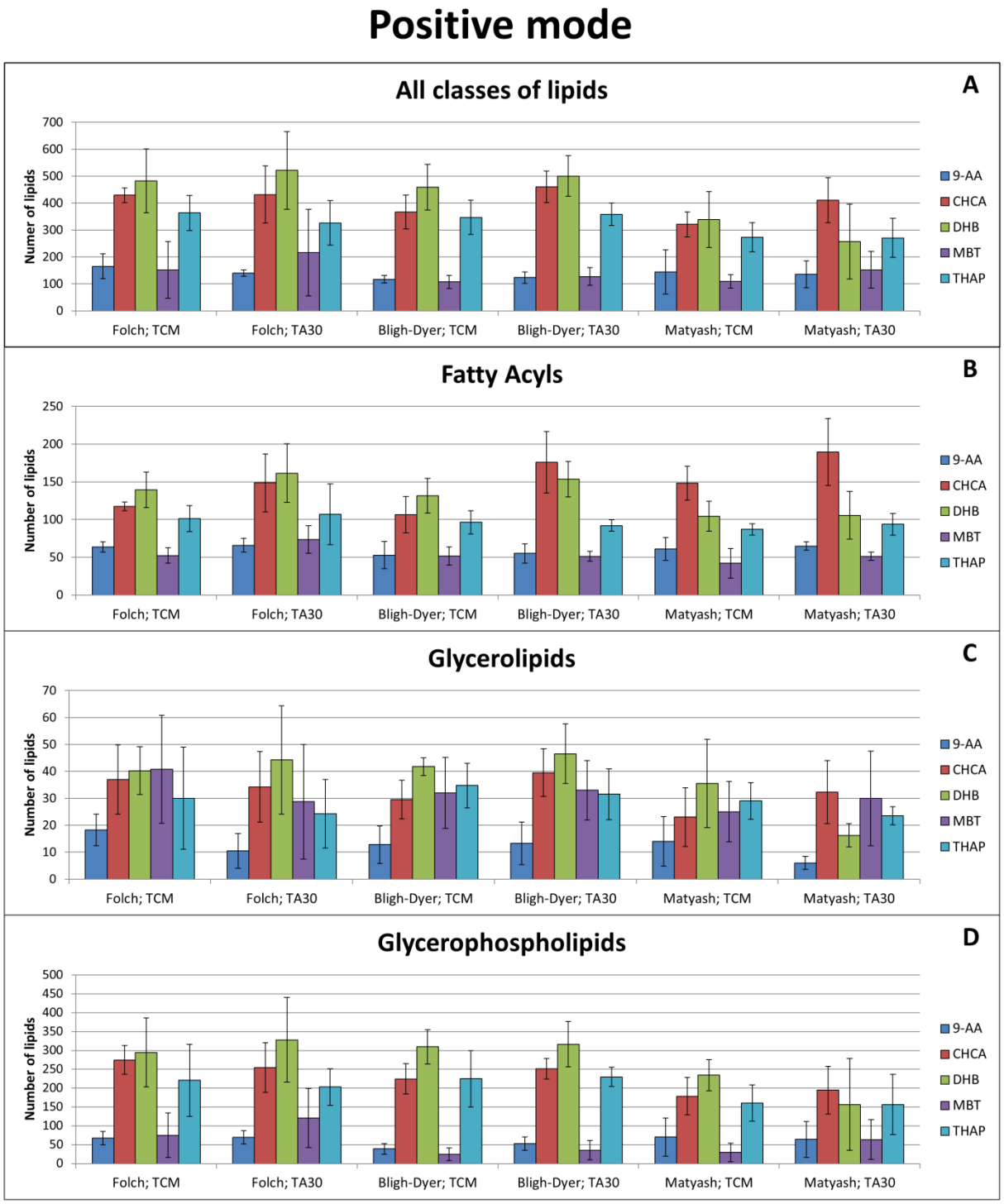

Figure 1. Average number of lipids detected on MALDI MS spectra taken in positive ion mode for different extraction methods, solvents and matrices used, for: all lipid classes (A), fatty acyls (B), glycerolipids (C) and glycerophospholipids (D) for all analyzed lipid extracts from bacteria.

Positive mode

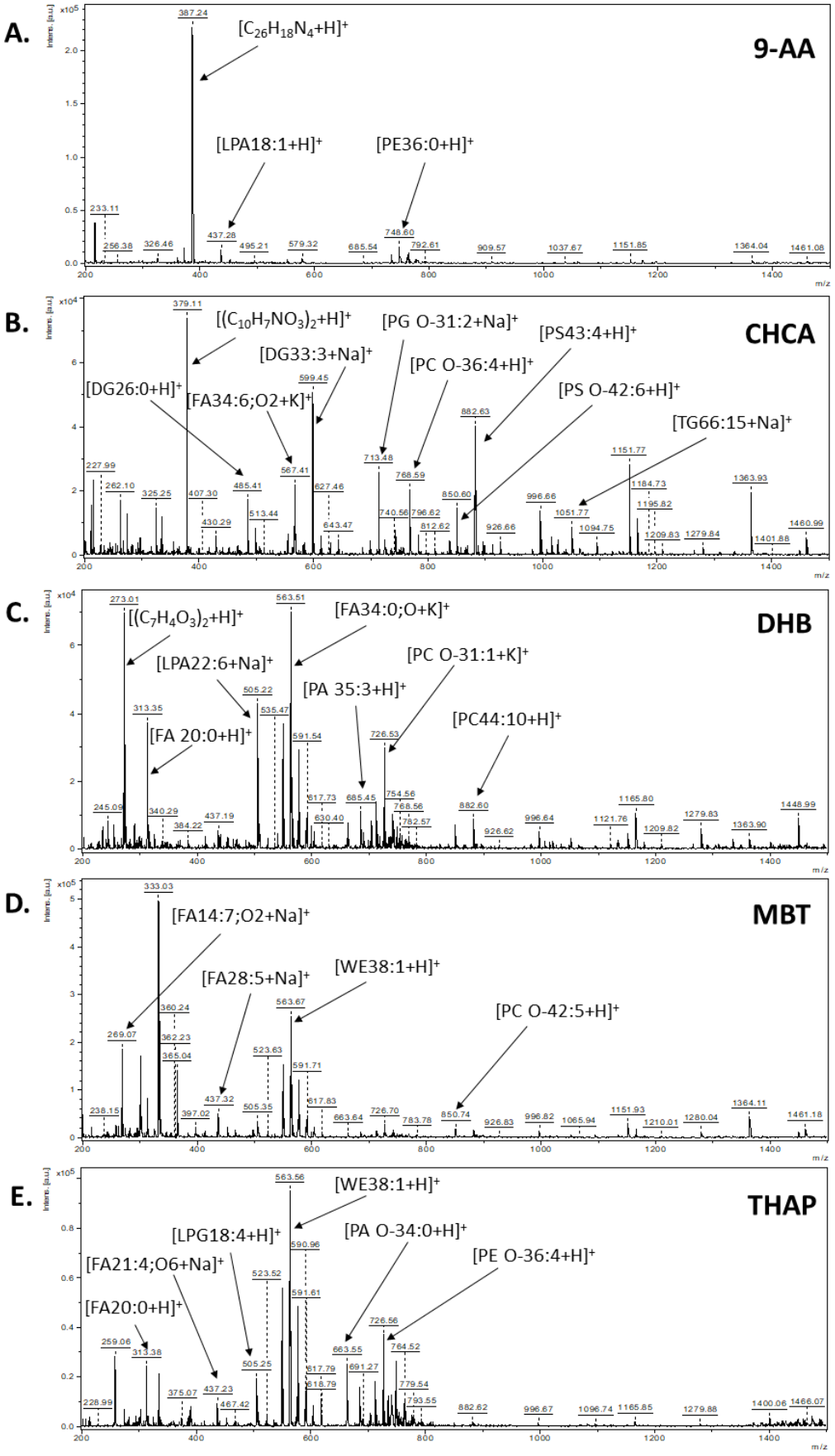

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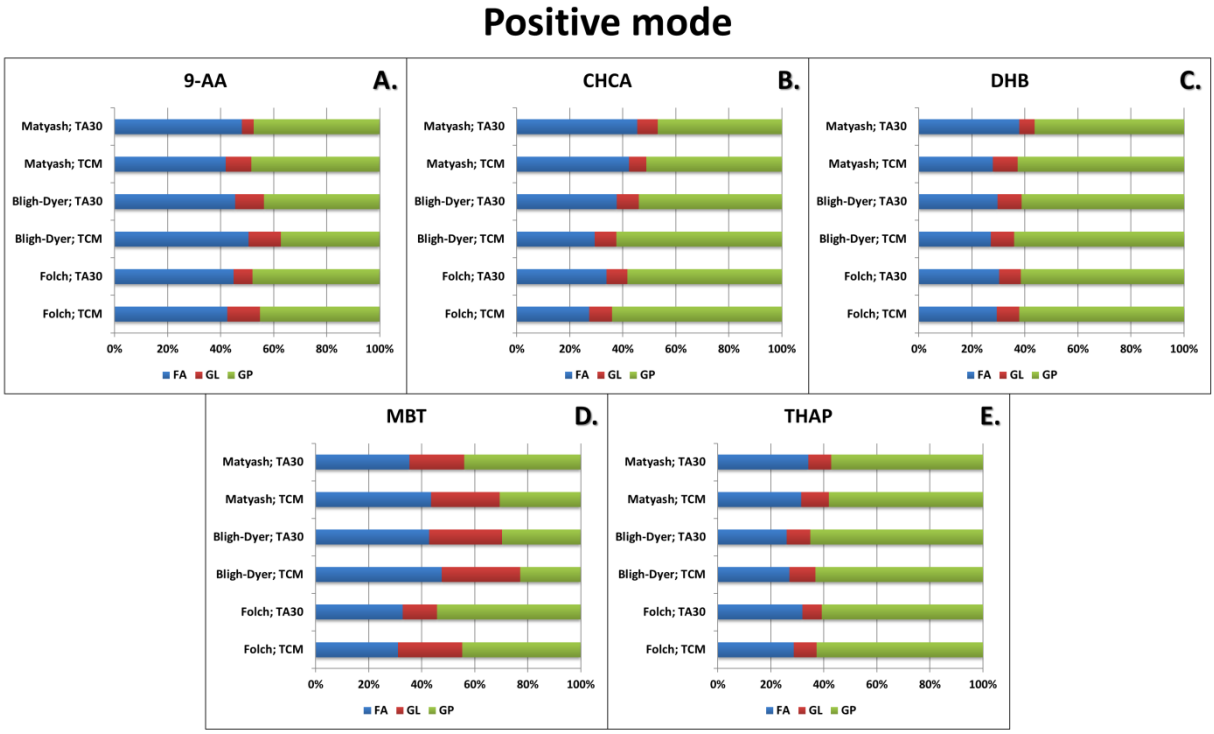
Figure 2. MALDI mass spectra of lipid extract from *E. coli* obtained by Folch method, redissolved in TA30 in positive ion mode using 9-AA (A), CHCA (B), DHB (C), MBT (D) or THAP (E) matrix.



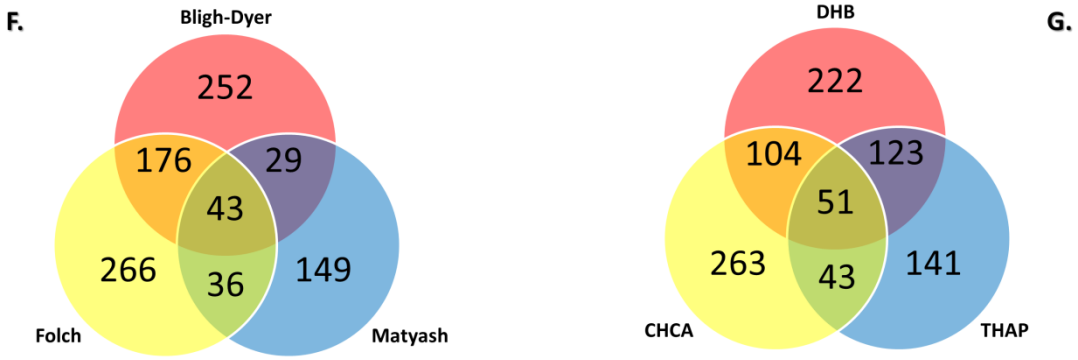


Figure 3. Percentage of individual lipid classes in MALDI MS spectra obtained with the following matrices: 9-AA (A), CHCA (B), DHB (C), MBT (D), THAP (E), and Venn diagrams showing average numbers of unique and common lipids between the three extraction methods for TA30 and DHB (F) or the three best matrices for Bligh-Dyer and TA30 (G) in positive ion mode.

3.2. Negative ion mode

Some classes of lipids ionize more easily in the negative mode [39]. To check which matrix and solvent are most suitable for the analysis of bacterial lipid profiles in the negative ion mode in MALDI TOF MS, similar tests were performed as for the positive ion mode. For the four extract-solvent variants, i.e. Folch-TCM; Matyash-TCM and Bligh & Dyer TCM and TA30, the highest mean number of lipid-derived signals was obtained for the THAP matrix (Figure 4A). The second, and in two cases, the first (Folch-TA30 and Matyash-TA30) having the most lipid signals in the negative mode, was the 9-AA matrix. However, it should be

noted that even for the highest lipid count recorded for the Bligh & Dyer extract dissolved in TA30 using the THAP matrix (264 signals), it is half the best result in the positive ion mode. This result is consistent with other studies [38],[40], as most types of lipids are easier to ionize in the positive than negative mode [41]. This result is consistent with other studies, as most types of lipids are easier to ionize in the positive than negative mode. In the negative mode, the lowest average number of *m/z* values assigned to lipids (62 signals) was obtained for the Matyash extract dissolved in TA30 using MBT matrix. The greater number of signals assigned to lipids in the spectra using THAP was surprising. Although this matrix is recommended by some authors for the analysis of lipids, in the positive mode [42], and for the negative mode, the commonly recognized matrix is 9-AA [16],[43].

Mass spectra for different extracts and different solvents made using the THAP matrix presented in Supplementary materials SM7 are quite similar due to the intense signal at *m/z* 333, only changing the range from 400 to 1500 Da allowed to see many signals with lower intensities (Supplementary materials SM8). In the negative ion mode, there are no significant differences between the spectra recorded for different solvents.

Figure 4 shows the differences in lipid ionization capacity for individual matrices in the negative mode. Also, the spectra taken for the same extract using different matrices show different sets of signals (Figure 5). As in the case of the positive test, when examining a specific group of lipids, one should follow the appropriate isolation protocol and the selected matrix [12]. The results divided into gram type of bacteria are consistent with the results for all analyzed lipid extracts, however, the average number of lipids detected for gram-positive bacteria is lower than for gram-negative bacteria (Supplementary materials SM9). Similarly to what was described for the positive MS mode, this may be related to the thickness of the cell wall, which affects the extraction efficiency.

The putative identification of fatty acyls gave the best result for the 9-AA matrix for all samples, and the highest number of lipids was identified in the spectra of the Bligh and Dyer extract after re-dissolving in TA30 solution (Figure 4B). 9-Aminoacridine is a moderately strong base with a pKa ≈ 10, so 9-AA readily accepts protons leading to the formation of $[M-H]^-$ ions [44], especially for acidic compounds such as fatty acids [45]. In addition, the use of 9-AA as a matrix gives a small background in the low *m/z* region, thus enabling the analysis of low molecular weight compounds, which is why this matrix is often recommended for fatty acid analysis using the MALDI MS method [6]. The Bligh & Dyer method is one of the two techniques recommended for the extraction of fatty acyls [12], but the results obtained for the other extract-solvent systems are very similar.

For glycerolipids, the highest average number of lipid-derived signals was obtained for the Folch extract using the 9-AA matrix and the Bligh & Dyer extract using the THAP matrix (Figure 4C). In both cases, chloroform was used as the solvent. In the case of glycerolipids, better results were obtained for TCM than for TA30, but the differences between the number of lipids obtained using 9-AA and THAP matrices for individual extracts and solvents are not large and amount to ±10. As described in section 3.1, all three protocols are suitable for the extraction of mono-, di- and triglycerols, however, using the methods of Folch and Bligh-Dyer, it is possible to isolate other subgroups of lipids belonging to the glycerolipids [34].

The last analyzed group, are glycerophospholipids, which are the main component of bacterial cell membranes [35]. Within the lipidomic landscape under investigation, a

particular class of lipids warrants distinct attention: cardiolipins. These phospholipids, characterized by their unique dimeric structure, occupy a crucial role in the structural and functional dynamics of bacterial membranes. Notably, their presence is not limited to one bacterial type; they have been identified in the membranes of both gram-negative [46] and gram-positive bacteria [47], underscoring their ubiquitous nature and functional importance across diverse bacterial taxa. Our analytical approach, utilizing advanced mass spectrometry techniques, predominantly revealed the presence of cardiolipins in the negative ion mode, as depicted in Figure 5. Such an observation aligns with the properties of cardiolipins, which facilitate their detection in this specific mode. A comprehensive elucidation of the methodologies, spectral data, and intricate interpretations related to cardiolipins and affiliated lipids is available in Supplementary Material SM10. Glycerophospholipids account for more than half of all identified lipids in negative ion mode. For all analyzed samples the highest average number of assigned glycerophospholipids was achieved using the THAP matrix, but the most signals were noted in the spectra of Matyash extract redissolved in TCM and Bligh & Dyer redissolved in TA30 (Figure 4D). 2,4,6-Trihydroxyacetophenone is the matrix recommended by Stübiger and Belgacem for the MALDI MS analysis of glycerophospholipids [42], however in their studies it was used in the positive mode. Comparable results were observed for all the extract-solvent systems, which is consistent with the literature data that glycerophospholipids are extracted with similar efficiency for each of the extraction methods [12].

The percentages of the analyzed lipid groups in the total number of lipids identified in the individual spectra are shown in Figure 6A-E. The results are similar to the results from the positive mode, except for the CHCA matrix (Figure 6B), for which a significant predominance of the share of fatty acyls was observed (52-66% of all lipids). The highest percentage of fatty acyls was recorded for the MBT matrix and ranged from 52% to even 74% (Figure 6D). As in the case of the positive mode, also in the negative mode, the largest share of glycerolipids was obtained using the MBT matrix, but their share decreased to about 12% of all lipids. The percentage of glycerophospholipids above 60% was visible in the MALDI MS spectra made using the THAP matrix (Figure 6E). The average number of common signals for the 3 extraction methods using TA30 as a solvent and THAP as a matrix (Figure 6F) is lower compared to the positive mode, but consistent considering the smaller number of signals in the spectra. There is a very amall average number of common signals for the three matrices with the most numerous lipid-derived signal groups for the Bligh & Dyer extract and the use of TA30 as solvent, as shown in the Venn diagram in Figure 6G. This gives information about the high variability of lipid profiles using different extraction protocols and matrices. A figure containing MALDI MS spectra in negative mode for lipid extracts from *P. mirabilis*, *S. epidermidis* and *E. faecalis* is presented in Supplementary Materials SM10.

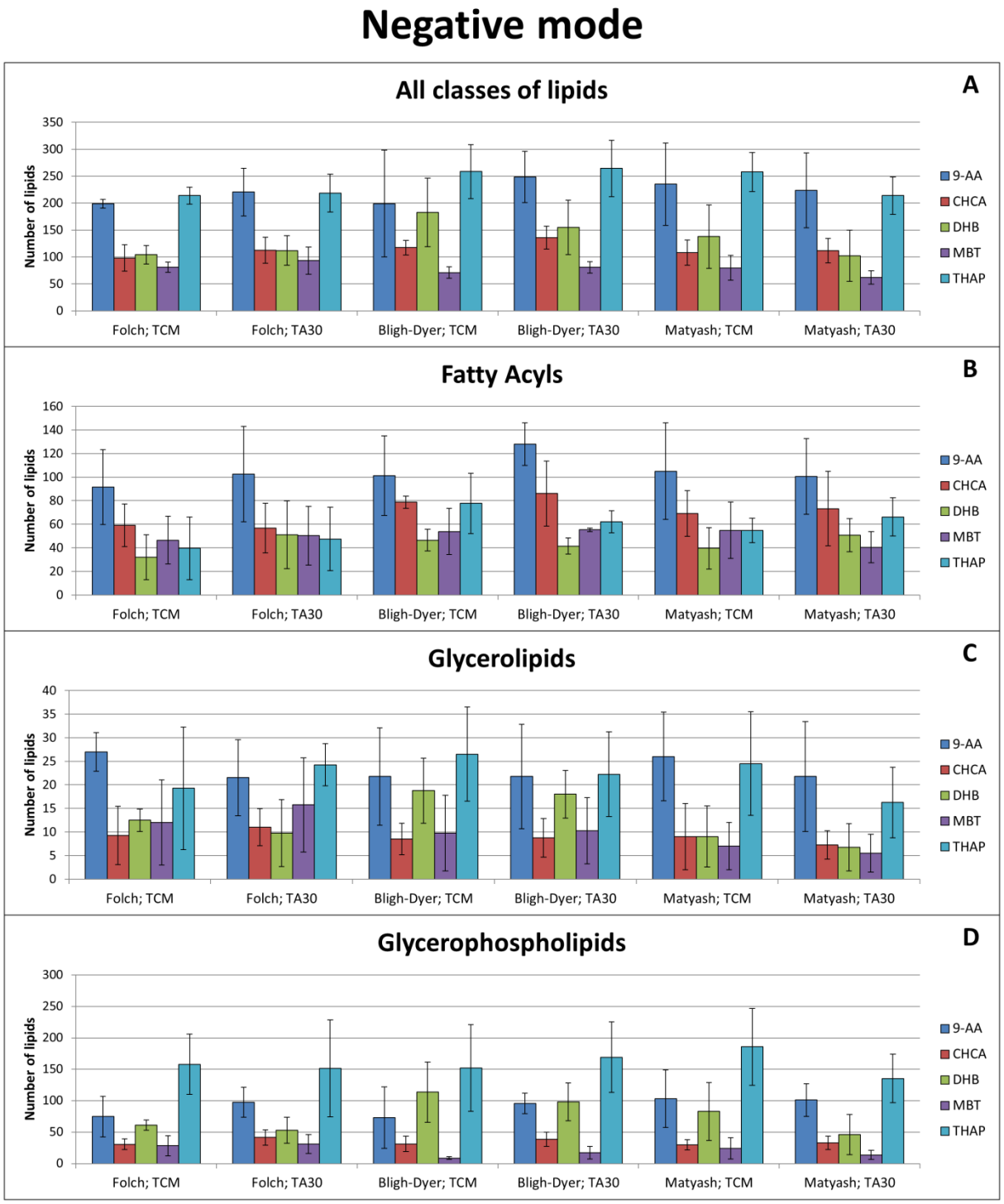


Figure 4. Average number of lipids detected on MALDI MS spectra taken in negative ion mode for different extraction methods, solvents and matrices used, for: all lipid classes (A), fatty acyls (B), glycerolipids (C) and glycerophospholipids (D) for all analyzed lipid extracts from bacteria.

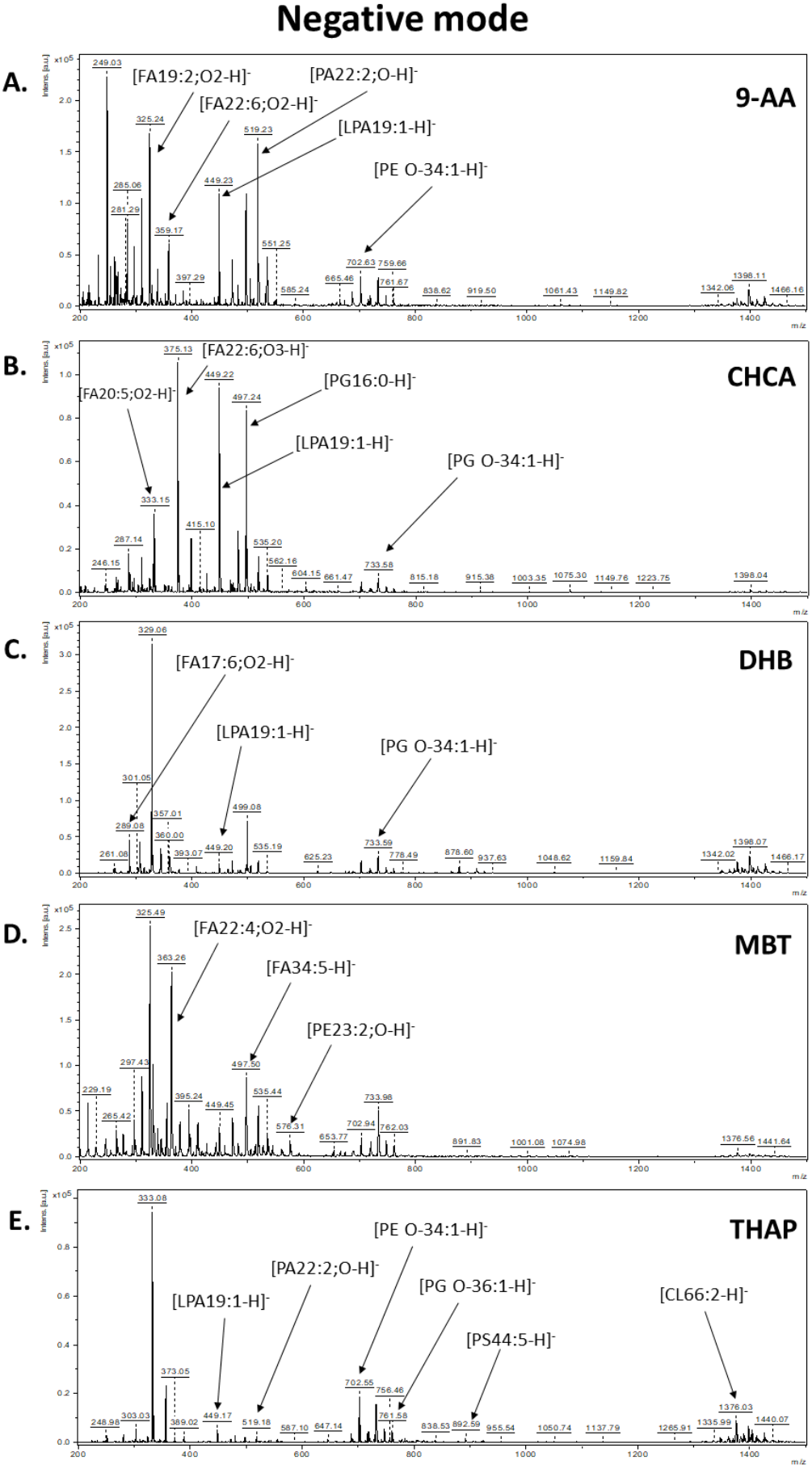

Negative mode
A.
9-AA
[FA19:2;O2-H]-
[FA22:6;O2-H]-
[PA22:2;O-H]-
[LPA19:1-H]-
[PE O-34:1-H]-
B.
CHCA
[FA22:6;O3-H]-
[FA20:5;O2-H]-
[PG16:0-H]-
[LPA19:1-H]-
[PG O-34:1-H]-
C.
DHB
[FA17:6;O2-H]-
[LPA19:1-H]-
[PG O-34:1-H]-
D.
MBT
[FA22:4;O2-H]-
[FA34:5-H]-
[PE23:2;O-H]-
E.
THAP
[PE O-34:1-H]-
[PA22:2;O-H]-
[LPA19:1-H]-
[PG O-36:1-H]-
[PS44:5-H]-
[CL66:2-H]-

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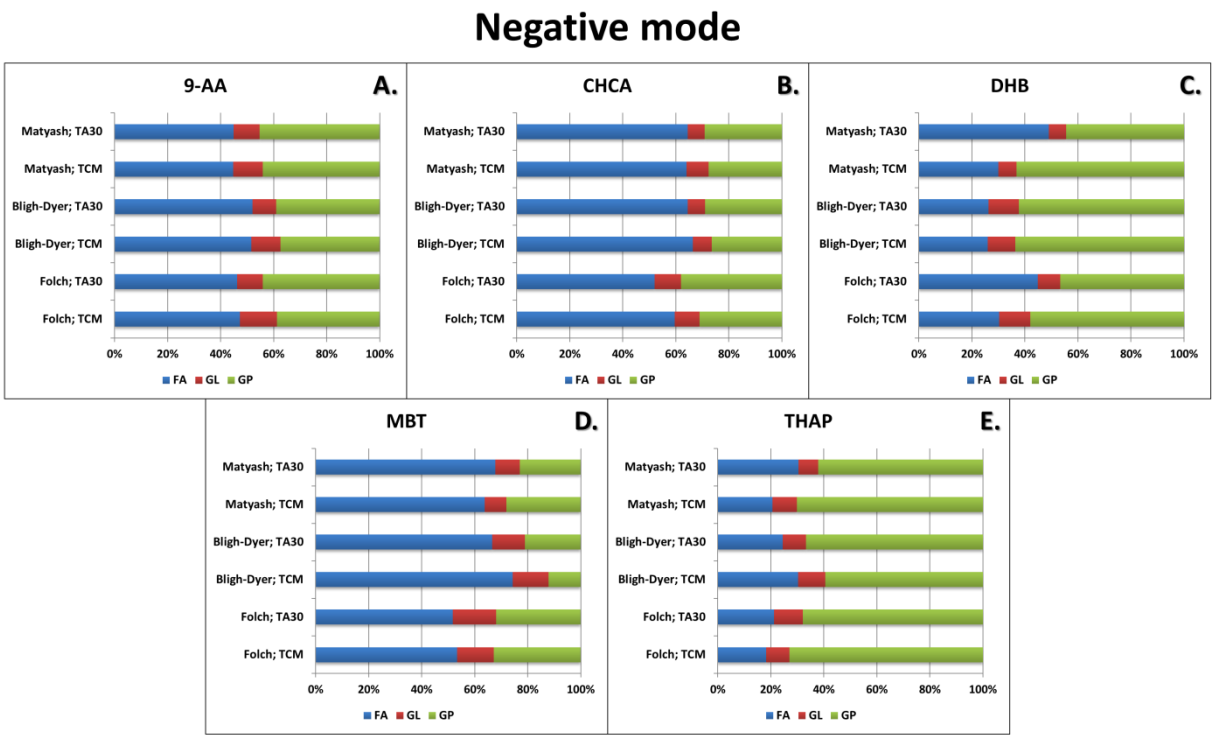
Figure 5. MALDI mass spectra of lipid extract from *E. coli* obtained by Bligh & Dyer method, redissolved in TA30 in negative ion mode using 9-AA (A), CHCA (B), DHB (C), MBT (D) or THAP (E) matrix.



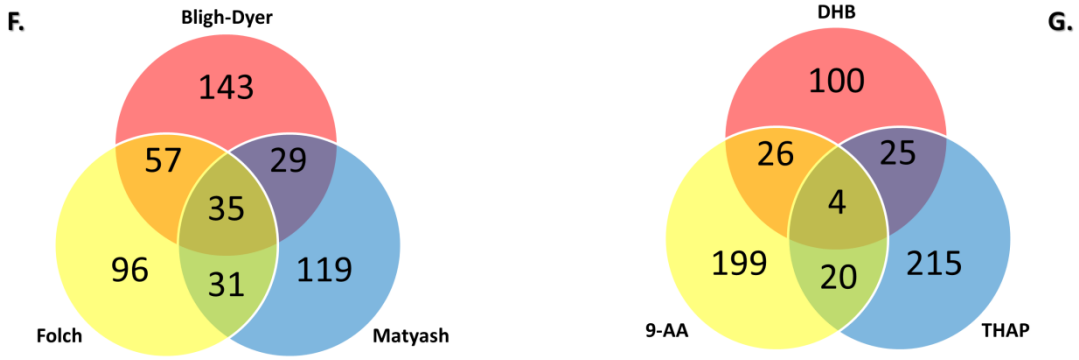


Figure 6. Percentage of individual lipid classes in MALDI MS spectra obtained with the following matrices: 9-AA (A), CHCA (B), DHB (C), MBT (D), THAP (E), and Venn diagrams showing average numbers of unique and common lipids between the three extraction methods for TA30 and THAP (F) or the three best matrices for Bligh-Dyer and TA30 (G) in negative ion mode.

4. CONCLUSION

MALDI TOF MS analyzes of lipid extracts from *E. coli*, *P. mirabilis*, *S. epidermidis* and *E. faecalis* were performed in both positive and negative ion modes. Extracts were made by Folch, Matyash and Bligh & Dyer protocols and then dissolved in chloroform or a mixture of acetonitrile and 0.1% trifluoroacetic acid in water (TA30). Five matrices were used in the measurements: α-cyano-4-hydroxycinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (DHB), 9-aminoacridine (9-AA), 2-mercaptobenzothiazole (MBT) and 2,4,6- trihydroxyacetophenone (THAP).

The obtained MS spectra allow the assignment of lipids to the signals, and this enables the determination which extraction protocol, solvent and matrix are most suitable for the analysis of bacterial lipids by MALDI MS. In the positive ion mode, the DHB or CHCA matrix is most suitable, for both non-targeted and targeted analysis. In the negative mode, the highest average numbers of lipid signals are obtained for THAP and 9-AA matrices. The most appropriate extraction protocol is Bligh & Dyer, and the solvent TA30 enables the detection of a significant number of lipids in each of the analyzed groups in both ionization modes, such as fatty acyls, glycerolipids and glycerophospholipids.

AUTHOR CONTRIBUTIONS

Conceptualization, A.A; methodology, A.A., E.S., M.Z.; resources, A.A., E.S., W.M.; formal analysis, A.A., E.S., P.F.; writing-original draft preparation, A.A. and E.S.; writing-review and editing, A.A., M.Z., D.G., P.P.; visualization, A.A.; project administration, P.P. and D.G.; funding acquisition, D.G. All authors have read and agreed to the published version of the manuscript.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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