Chapter 3

A protocol for the investigation of the intracellular *Staphylococcus aureus* metabolome

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This report was conceived and planned by HM, MaLi and MiLa. HM made all measurements and statistical analysis with assistance from MaLi and MiLa. All the authors interpreted the data and wrote the paper.

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A protocol for the investigation of the intracellular *Staphylococcus aureus* metabolome

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ABSTRACT

Systems biology studies assume the acquisition of reliable and reproducible data sets. Metabolomics, in particular, requires comprehensive evaluated workflows to enable the analysis of hundreds of different compounds. Therefore, a protocol to elucidate the metabolome of the gram-positive pathogen, *Staphylococcus aureus* COL strain, grown in a chemically defined medium is introduced here. Different standard operating procedures in the field of metabolome experiments were tested for common pitfalls. These included suitable and fast sampling processes, efficient metabolite extraction, quenching effectiveness (energy charge), and estimation of leakage and recovery of metabolites. Moreover, a cell disruption protocol for *S. aureus* was developed and optimized for metabolome analyses, for the express purpose of obtaining reproducible data. We used complementary methods (e.g., gas chromatography and/or liquid chromatography coupled with mass spectrometry) to detect the highly chemically diverse groups of metabolites for a global insight into the intracellular metabolism of *S. aureus*.

In this work, we aimed to develop a protocol for the investigation of the *Staphylococcus aureus* metabolome. *S. aureus* is a versatile human pathogen, which causes a wide range of diseases, including wound infections, endocarditis, osteomyelitis, toxic shock syndrome (TSS) [1] and sepsis. This gram-positive bacterium survives under hostile environmental conditions and colonizes mucous membranes and skin. Beside the regulation, structure, and function of various virulence factors of *S. aureus*, the basic cell physiology contributes in part to its pathogenicity [2]. It was recently shown that tricarboxylic acid cycle activity is linked to virulence; in addition there are more connections between staphylococcal metabolism and virulence factor synthesis [3,4]. Enhancing the knowledge of *S. aureus* in this postgenomic era by a metabolomic study will improve these crossroads and their interplay.

Metabolomics is defined as the analysis of changes and regulations in the complete set of metabolites (small organic molecules, MW < 1000) [5,6]. The term metabolomics was first coined in 1998 in analogy to genomics, transcriptomics, and proteomics and is the most recent of the "omic" sciences [7]. Established methods in this field are the GC/MS, LC/MS, and $^{1}H/^{13}C$ NMR. Identification and quantification of intracellular metabolites have recently been seen as having a necessary role in systems biology. In combination with genomics, transcriptomics, and proteomics, the metabolomics approach promises to deliver a global view and a better understanding of regulatory systems, dynamic ranges, and the control of metabolic pathways.

Microbial metabolomics, in particular, requires efficient and reliable methods for sample preparation, thereby enabling the design of an optimal sampling protocol for the analysis of intracellular metabolites. In numerous studies, sampling protocols are adopted from the literature without critically optimizing the protocol for a given case and the investigated organism, as stated recently [8].

During the sampling procedure, no changes in the metabolite levels should be allowed to occur, thereby ensuring that the sample is a true representation of the biological status of interest. A rapid sampling and quenching technique, which considers the high turnover rate of a vast number of intracellular metabolites, e.g., ATP, PEP, and glucose-6-phosphate, is important [9,10]. A common quenching method is to transfer the cell suspension into cold methanol or another organic solution, which stops the metabolism instantly [10–12]. The latter procedure has a critical disadvantage, where a significant loss of intracellular metabolites during quenching can occur due to cell lysis in the presence of the organic solvent [8,13,14]. Another established method involves quenching with liquid nitrogen [15] before or after the separation of cells from growth medium. If the given situation assumes a separation of intra- and extracellular metabolites, it is therefore crucial to avoid

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the loss of significant amounts of intracellular metabolites. Thus, it is advisable to develop a sampling protocol, which separates the cells from the medium quickly and allows immediate metabolic arrest. A separation of cells and growth medium can be achieved by centrifugation or via a vacuum-dependent sterile filtration system. The rapid filtration method promises to fulfill the aforementioned requirements. Subsequently, after sampling, the cell metabolism is quenched by liquid nitrogen as described previously [8,14,16]. By comparison, the centrifugation method demands too much time, which makes these methods unsuitable for the study of *S. aureus*.

The calculation of energy charge is an established approach that can be used to validate the sample workup procedure, including effective metabolic quenching and the verification of the analytical protocols [13,17–20]. In the cases presented, energy charge can be considered as an indicator for cell maintenance. It was already shown in 1968 that exponentially growing cells have a high concentration of the energy-rich ATP as compared to lower concentrations of ADP and AMP. For nonlimited growing cells the energy charge is generally stable at values around 0.80–0.95 [21,22].

Stress conditions associated with the sampling procedure are considered to be under control, if the analyzed cells show an energy charge in the aforementioned range. In this case it can be concluded that the cells were harvested under nonstressed conditions, for example, without residual enzyme activity.

However, metabolite concentrations can be influenced by matrix or other protocol effects like the extraction solution used. Due to this, several extraction solutions were investigated in this study. These include 60% methanol, 60% methanol containing 0.5/1/2 mM formic acid, 4/2/4 chloroform/water/ethanol, cold and boiling 60% ethanol, and 100% boiling water extraction.

In this study, we present a reliable protocol for metabolome analysis, which includes the sampling and quenching procedure, the washing of the cells to remove all medium components and extracellular compounds, cellular disruption, and the extraction of metabolites. Furthermore, the recovery of metabolites was examined for *S. aureus* through the use of spiking experiments. Under the tested conditions, the fast filtration and subsequent quenching by liquid nitrogen, followed by metabolite extraction with cold 60% ethanol and glass bead cell disruption, were found to be the most appropriate method for *S. aureus*.

Materials and methods

Strain and cultivation

Staphylococcus aureus COL [23] was grown in 100 ml chemically defined medium [24] (all amino acid concentrations were normalized to 1 mM; glycine and MOPS were removed from the medium as the latter interferes with ^{1}H NMR signal assignment and glycine was not essential for growth (data not shown)) under aerobic conditions in 500 ml flasks with vigorous agitation (124 rpm) at 37 °C. Depending on the experiment, samples were taken in the exponential ($OD_{500} \sim 0.5$) or in the stationary ($OD_{500} \sim 3.0$) growth phase. Growth was monitored by measuring the optical density (OD) at 500 nm with a photometer (Novaspec II). All steps were carried out under sterile conditions.

Cell dry weight

For quantitative metabolome analyses the cell dry weight (CDW),[2] as measured by gravimetry, was used. The CDW was determined by weighing dried cells from 20 ml of culture broth. Cells were separated from culture broth by centrifugation for 5 min at 10,015*g* and 4 °C, including a washing step with 99% ethanol to conserve the cells and remove medium compounds. The cell suspension was transferred into glass vials and dried at 60 °C until a constant weight was achieved (modified after [25]). Finally, the mass difference between dried vials with and without cells was used for CDW determination. For the correlation factor between OD and CDW, see Fig. 5.

Sampling of intracellular metabolites—centrifugation

A 20 ml aliquot of cell culture broth was taken from the main culture in exponential growth phase and the cells were separated from the supernatant by centrifugation (Heraeus Instruments) for 5 min at 10,015*g* and 4 °C in 50 ml Falcon tubes. The pellet was washed twice with cold (⩽4 °C) 0.6% NaCl solution and centrifuged for 5 min at 10,015*g* and 4 °C. Metabolic quenching, via inactivation of all enzymes, was achieved by the addition of 5 ml 60% ethanol (w/v, ⩽4 °C) extraction solution to the cell pellet, vortexing for a few seconds, and then transferring the cell suspension into liquid nitrogen. After 15 s in liquid nitrogen the cell suspension was completely frozen (about −70 °C). The extraction solution included 20 nmol each of norvaline and ribitol as internal standards for the described analytical procedure.

Sampling of intracellular metabolites—filtration

A 20 ml aliquot of cell culture broth was taken from the main culture sampled exponentially for energy charge (EC), quenching, and cell disruption studies and in stationary growth phase for leakage and metabolite extraction studies. The cells were rapidly separated from the culture medium by filtration (⩽15 s for cells in exponential growth). Microfil V filtration system and 0.45 μm pore size sterile filters were used (Millipore) [16]. The cells on the sterile filter were washed twice with cold (⩽4 °C) 0.6% NaCl solution. The filter, including the cells, was rapidly transferred into 5 ml ice-cold extraction solution (previously described) in a Falcon tube, which included the internal standards. After the filter was added to the extraction solution, the Falcon tube was subsequently immersed into liquid nitrogen. For cells in exponential growth, the sampling took less than 1 min.

Quenching of metabolism

Following filtration (⩽1 min) or centrifugation (~20 min), metabolism was rapidly quenched by liquid nitrogen. To evaluate the sampling and quenching procedure, 20 ml cell culture in the exponential growth phase at $OD_{500} \sim 0.5$ was sampled rapidly using the filtration or centrifugation method as described above. After extraction with 60% (w/v) ethanol (⩽4 °C) the levels of ATP, ADP, and AMP were measured by LC/MS.

Energy charge calculation

The analyzed concentrations of the adenosine nucleotides ([ATP], [ADP], [AMP]) were used to estimate the energy charge by using the formula in Scheme 1.

$$\text{energy charge} = \frac{[\text{ATP}]+\frac{1}{2}[\text{ADP}]}{[\text{ATP}]+[\text{ADP}]+[\text{AMP}]}$$

Scheme 1.

[2] *Abbreviations used:* CDW, cell dry weight, EC, energy charge; MOPS, 4-morpholinepropanesulfonic acid.

Cell disruption—glass bead method

Strain tubes (SARSTEDT) were filled with 0.5 ml glass beads (Sartorius AG, diameter 0.10–0.11 mm). The cell samples from exponential growth phase, already in organic extraction solution, were thawed on ice (⩽6 °C), while being rigorously mixed and shaken 10 times alternately. Aliquots of 1 ml cell suspension were transferred into an appropriate number of strain tubes containing the beads. Cell disruption of *S. aureus* COL was tested for different cell disruption cycle counts (1–3 cycles for 30 s at 6800 rpm) in a homogenizer (Precellys 24). After cell disruption the glass beads and the cell debris were separated from the supernatant by centrifugation for 5 min at 4 °C and 10,015*g*. The aliquoted samples were combined, and the glass beads were washed once with a. bidest and washing solutions were added to the combined sample. The supernatant including extracted metabolites was restocked with a. bidest to a final organic solution concentration of 10% and stored at −80 °C prior to lyophilization. All steps were carried out on ice (⩽6 °C).

Cell disruption—bead mill method

For the bead mill cell disruption, cell samples harvested in exponential growth phase were thawed on ice (⩽6 °C), vortexed, and shaken 10 times alternately, as described for the glass bead method. The suspension was released as individual drops directly into liquid nitrogen kept in a Teflon vessel of a micro-dismembrator including a 7 mm bead made of tungsten carbide (Braun Melsungen, Germany) [26]. The cells were mechanically broken for 2 min at maximum speed and the frozen sample was rapidly transferred into a Falcon tube. The Teflon vessel was washed once with a. bidest to obtain all metabolites and the collected samples were stored at −80 °C until lyophilization. This method is only feasible if the extraction solution used consists mainly of water as the sample must be frozen during the whole procedure, which appears impractical with organic solvents being subjected to mechanically based heating in the vessel.

Extraction of metabolites

In this work different extraction protocols from the literature were tested. For comparison of the extraction methods, the methanol extraction was carried out in parallel in all experiments. The cells were harvested in stationary growth phase by the filtration method described previously. Every extraction method was performed in triplicate from one cell culture. After filtration and washing, as described above, the cells on the filter were transferred into 5 ml extraction solution in a Falcon tube, which also included the internal standards (20 nmol norvaline, 20 nmol ribitol). The cells were disrupted mechanically as described above with 2 cycles in the homogenizer. The following extraction solutions were tested; cold solutions were precooled at −20 °C and for experiments held on ice (⩽6 °C).

- 60% cold methanol (w/v);
- 60% cold ethanol (w/v);
- 60% cold methanol (w/v) containing, 0.5, 1, or 2 mM formic acid;
- cold chloroform/water/ethanol (4/2/4);
- 60% boiling (70 °C) ethanol: The 60% ethanol was incubated in a water bath at 100 °C for 10 min. The cells on the filter were added to the boiling ethanol and further boiled in a water bath at 100 °C for 5 min;
- 100% boiling (100 °C) water: Carried out as described for boiling ethanol [27].

Recovery of metabolites

Verifying matrix effects from the analytical platform is important in order to validate the acquired metabolomic data [28]. For spiking experiments, it is suggested that chemically different metabolite classes must be represented. Therefore, two standard mixtures were selected for spiking into the quenching solution from cell samples yielded by filtration. Mixture A containing 2-oxoglutarate, fumaric acid, isoleucine, leucine, malic acid, methionine, phenylalanine, serine, succinic acid, threonine, tyrosine, valine, and *cis*-aconitate was used for determining the metabolite recovery by GC/MS, and mixture B consisting of IMP, CMP, CoA, ATP, Frc-1,6-P, Glc-6-P, GDP, and UMP was used for analogous LC/MS measurements.

The metabolite recoveries were calculated as

$$\text{recovery}: \frac{(\text{sample incl. standard}) - (\text{sample excl. standard})}{\text{only standard}} * 100\%$$

Analysis of intracellular metabolites

Intracellular metabolite samples were measured by GC/MS and LC/MS using standardized protocols. Samples were lyophilized to complete dryness at 0.5 mbar and −52 °C in a Christ, alpha 1–4 lyophilization apparatus prior to analysis. GC/MS analysis was performed as described previously [29]. Briefly, samples were derivatized for 90 min at 37 °C with MeOX and 30 min at 37 °C with MSTFA. Before extraction or any other protocol development step 20 nmol each of norvaline and ribitol solution was added to the samples and used as internal standards for relative quantifications. For the LC/MS analysis an ion pairing reagent method using a RP-18 column was performed as described by Donat et al. [30]. Herein 100 nmol Br-ATP solution was used as an internal standard for quantification.

Results

Metabolome analyses were performed on *S. aureus* COL grown in a chemically defined medium to ensure a well-defined and reproducible cell physiology. In this study several important steps during the protocol investigation were systematically tested: (i) the filtration and the centrifugation sampling method, (ii) the washing step, including estimation of possible leakage of metabolites while washing, (iii) the efficiency of the quenching, (iv) the extraction with cell disruption, and (v) recovery of metabolites under final conditions.

Sampling of cells including washing

Sampling for metabolite analysis is one of the most important steps in metabolomics. A rapid and reproducible sampling process is the prerequisite for an exact metabolome analysis and further physiological conclusions. Moreover, a selective splitting of intracellular and extracellular metabolites assumes the separation of cells and culture medium, as well as total removal of extracellular metabolites and residues of the medium from the cells. The fast filtration [16], the centrifugation [31], and the direct quenching method [12,32] are commonly described sampling procedures in the field of cellular metabolomics. Even though the direct transfer of sample into a quenching solution is the fastest method for arresting metabolism, this method can have a distinct disadvantage, which is the leakage of metabolites while quenching [8,13,14,33]. Further, this problem makes it necessary to separate cells and culture medium. Since the sampling and separation must

be as fast as possible, the time range of the centrifugation method is a considerable disadvantage. To create a cell pellet of *S. aureus* COL cells, a centrifugation step of 5 min is necessary. The inclusion of two washing steps extends the centrifugation method up to 15–20 min. In contrast, for cells in the exponential growth phase, the filtration method, including the washing step, takes only about 30–60 s. In the stationary phase, depending on optical density, the filtration method, including the washing steps, can take up to 2 min, which is still much faster compared to the centrifugation method.

In addition, the leakage of intracellular metabolites also must be avoided while washing [8]. To decrease the leakage problem during washing steps, a cold (⩽4 °C, isotonic to the cultivation medium) 0.6% NaCl solution was used for washing. The generated washing solutions and the supernatant after incubation of cells (from stationary growth phase) for 5 min with 5 ml NaCl (60%, ⩽4 °C) solution were studied for (i) washing efficiency and (ii) the presence of intracellular metabolites (as an indicator of leakage). To assess the capability of the washing solution, the medium and the resultant washing solutions after each of the three steps were measured by GC/MS. After the two washing steps all extracellular metabolites and medium elements had been removed (Fig. 1). Leakage during washing was determined by the attempted detection of intracellular metabolites in the washing solutions and supernatant following cell incubation (for 5 min). No intracellular metabolites, such as those found in the glycolytic pathway, could be detected. Additionally, we found that no glutamate was detectable in the incubation solution, which was reassuring, as glutamate is a highly abundant intracellular metabolite [8]. Hence, it has been determined that there is no detectable lysis of cells during filtration and washing, resulting in negligible leakage of intracellular metabolites. Comparable results were shown by Bolten and Wittmann [34]. The authors found no leakage of intracellular metabolites after vacuum filtration followed by washing of the cells on the filter with sterile medium [34].

An attempt was also made to elucidate which method was the most suitable and reproducible. To do this, the energy charges were compared for the filtration and centrifugation sampling method. Cells were sampled in exponential growth phase and metabolite extraction was carried out by 60% (w/v) ethanol. The filtration (including washing) method results in an EC ~ 0.89 ± 0.01, whereas the centrifugation method (including washing) results in an EC ~ 0.75 ± 0.023 (Table 1). The EC for the filtration method fell into the range for unstressed cells, while the EC after centrifugation indicated that the cells appeared to be stressed during the centrifugation procedure. Further the relative error (as mean value of the metabolites) indicates that the filtration method (24%) is more reproducible than the centrifugation method (47%) (Table 1). A possible cause for the decrease in EC could be due to the slow adaptation of the temperature of the sample, going from 37 °C to 4 °C, while in the centrifuge. It is possible that during this slow temperature equilibration, enzymatic conversions in the cells will still continue (possibility of cold stress metabolites). Whereas quenching of metabolism normally occurs when cells are dropped into the extraction solution, followed immediately by immersion into liquid nitrogen. It is for these reasons that we deem the centrifugation method to be unsuitable for metabolome analysis of *S. aureus*. Since the filtration method is much faster and results in a better energy charge, this sampling method is appropriate and more suited for *S. aureus*. To further confirm the positive results obtained by using fast filtration, an additional time delay was applied during the filtration procedure. In this case, an energy charge decrease under 0.8 was noted after 3 min. This demonstrates that the described sampling method is fast enough for the gram-positive *S. aureus*, albeit this step takes longer compared to commonly used protocols for fast growing gram-negative bacteria [8]. It is possible that this effect is due to the tough cell wall of this

Table 1
Energy charge (EC) for different sampling methods and further treatment with different extraction solutions (calculation of EC, see Scheme 1).

Method	Energy charge
Filtration method, boiling water extraction	0.43 ± 0.132
Centrifugation method, cold 60% EtOH extraction	0.75 ± 0.026
Filtration method, cold 60% EtOH extraction	0.89 ± 0.014
Filtration method with 3 min time delay, cold 60% EtOH extraction	<0.8

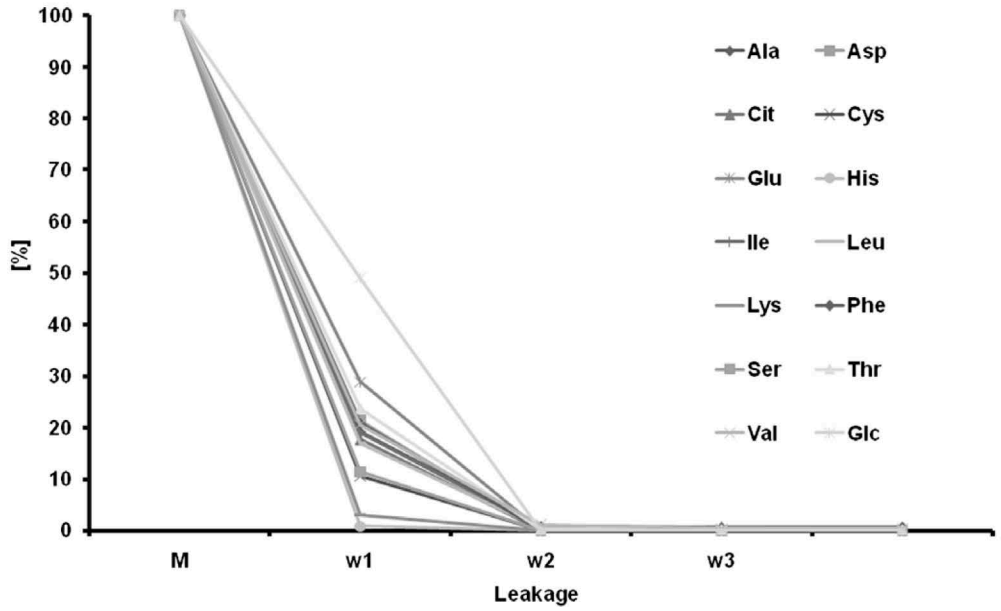

Fig. 1. Washing efficiency and metabolite leakage while washing; the relative concentration of extracellular metabolites and medium elements were calculated by results from GC/MS measurements of the growth medium (M) and for three sequential washing steps (w1, w2, w3). For calculation of the washing efficiency and leakage the medium itself was measured and the percentile amount of metabolites in the medium was calculated to the amount of metabolites in the medium.

gram-positive microorganism [35] which protects the bacterium against fast changing environmental conditions, such as those encountered in the fast filtration protocol.

Quenching

Cells from exponential growth phase were used for quenching experiments. Quenching of the metabolism was achieved using liquid nitrogen (−196 °C) and in addition with the chosen cold extraction solution present in the Falcon tube. As the cells were already separated from the medium by the described filtration method, leakage of metabolites while quenching is no longer of significance. To evaluate the effectiveness of quenching, the energy charges were compared between 10 min boiling H_2O (100 °C) (modified after [27]) and cold 60% ethanol (⩽6 °C) with subsequent liquid nitrogen quenching. The obtained results show that the extraction solution has a considerable effect on quenching (Table 1). We found that metabolism is not stopped immediately and irreversibly while using the boiling process (EC ~ 0.43 ± 0.13). Even though good extraction results could be obtained after extraction with boiling water, as also described by Hiller et al. [27] for *Escherichia coli*, we would not suggest this method for *S. aureus* metabolome analysis due to the low energy charge. In contrast, cold 60% ethanol and liquid nitrogen were found to be highly effective for quenching (EC ~ 0.89 ± 0.01).

Cell disruption

For the gram-positive bacterium *S. aureus* COL a mechanical cell disruption is necessary, since the cell wall will not break up sufficiently using only an organic solution (Fig. 2, 100% line). For the cell wall disruption a glass bead as well as a bead mill cell disruption method was tested and compared (Fig. 3). An extraction with boiling water is a very efficient extraction (Fig. 3A); nevertheless the energy charge is decreased during the quenching procedure, as described previously (see Table 1). The comparison of the bead mill and the glass bead cell disruption method, using water as an extraction solution, showed that the glass bead method is more efficient. The coefficient of determination of 0.96 indicates that both methods correlate well over all tested metabolites, but the gradient of 0.64 argues explicitly for the glass bead method in terms of relative quantified concentrations (Fig. 3B). In addition, the glass bead method after metabolite extraction by 60% (w/v) ethanol was optimized by the addition of several cycles in the homogenizer. Two cycles at 6800 rpm for 30 s were adopted for the cell disruption part of the protocol for *S. aureus* metabolome analysis (Fig. 2), since a higher number of cycles could not obtain higher metabolite concentrations.

Extraction

During the extraction procedures, intracellular metabolites are exposed to various organic solvents. For an optimal metabolome analysis, this solvent should neither chemically nor physically modify or degrade the metabolites. Furthermore, the extraction solution must extract as many metabolites as possible [36]. For this reasons several extraction solutions were tested and compared to find the most efficient. For the comparison of the extraction potential, a commonly used cold (⩽6 °C) 60% (w/v) methanol extraction was carried out in parallel for every extraction method [37]. The boiling (100 °C) water extraction was carried out only for energy charge calculations. It was assumed that even for better extraction efficiency by water, the energy charge will decrease. Probably caused by a reversible inactivation of the enzymes in water or a not fast enough quenching process during the boiling procedure.

In this work cold (⩽6 °C) 60% methanol (w/v) [37,38], cold (⩽6 °C) 0.5 mM formic acid/60% methanol (w/v), cold (⩽6 °C) 4/2/4 chloroform/water/ethanol (modified by [38]), 60% cold (⩽6 °C) ethanol [32], boiling (78 °C) 60% ethanol [37–39], and extraction with boiling (100 °C) water [27] were investigated for cells in the stationary phase, where higher amounts of intracellular metabolites and cell density are present compared to the exponential growth.

To prove that a broad range of chemically and chromatographically different metabolites were being extracted, the relative amounts of 3-phosphoglycerate, adenine, alanine, citric acid, fumaric acid, glutamic acid, histidine, lactic acid, leucine, phosphoenolpyruvate, succinic acid, tryptophan, urea, and valine were determined after the diverse extraction procedures. The different extraction solutions, tested in this study, result in highly varying relative metabolite concentrations (Fig. 4). The observed variances could be attributed to the different effects of the extraction solu-

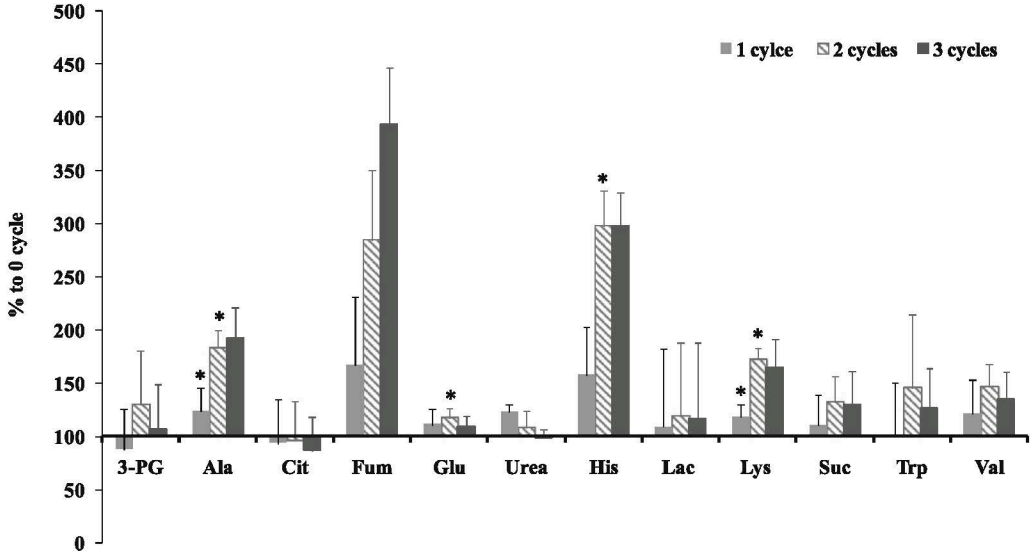

Fig. 2. Cell disruption effectiveness dependent on the cycle number of the glass bead method (each cycle is 30 s at 6800 rpm in a Precellys homogenizator). Percentage of relative metabolite concentrations is shown for no cycle (defined as 100%) (*x*-axis) and compared to 1, 2, and 3 cycles for each metabolite (*y*-axis). *Significant difference by two-tailed *t* test, $P < 0.1$. Cells were harvested in exponential growth phase and metabolites were extracted with 60% (w/v) ethanol.

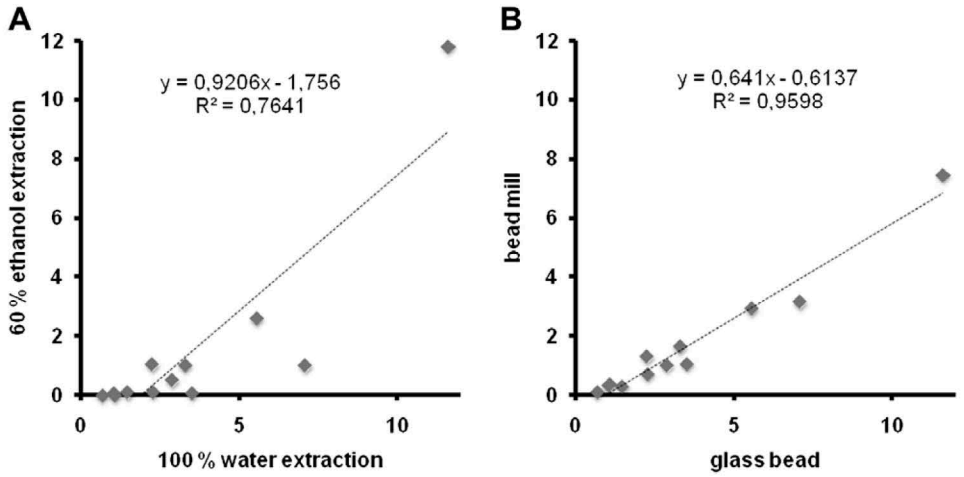

Fig. 3. Correlation plots of relative metabolite concentrations (A) after 100% (v/v) water (*x*-axis) and 60% (w/v) ethanol (*y*-axis) extraction and (B) after bead mill (*y*-axis) and glass bead (*x*-axis) cell disruption using water as extraction solution.

tions on the metabolites, the varying extraction effectiveness, or the effect from the solution on cell lysis. Furthermore it could be noted that only few metabolites were detectable by only one of the extraction procedures (Table 3). Extraction using cold 1 M formic acid results in the lowest relative metabolite concentration for all shown metabolites on average, possibly due to a degradation of the metabolites. Some metabolites from the tricarbonic acid cycle were not found in the cell extract (citrate, malate, and fumarate). Further, 3-PG was not detectable after the acid extraction procedure. In this regard, our results are almost contradictory to results shown by Rabinowitz and Kimball for *E. coli* [40]. They recommend a 0.1 M formic acid/acetonitrile/water extraction as the most useful one. Especially for *E. coli* several different extraction procedures are described in the literature; e.g., perchloric acid was used for monitoring of intracellular metabolites [41,42], while Maharjan and Ferenci reported that perchloric acid extraction exhibited lower extraction efficiencies [37]. Furthermore 40% ethanol/0.8% NaCl [43], hot water [27], 100% methanol [38], and hot ethanol extraction [13] were suggested as the most suitable extraction solution. In our study extraction with boiling ethanol extracted only 42% of the above-noted metabolites, which include adenine, 3-phosphoglycerate, phosphoenolpyruvate, succinic acid, histidine, and tryptophan (1.03 to 1.42-fold), with greater efficiency than cold 60% (w/v) methanol extraction. The remaining metabolites were degraded during the procedure with boiling solvent and AMP could not at all be detected after boiling ethanol extraction. In contrast to the other extraction solutions lactate was detected (see Table 3). In this regard, our results for boiling ethanol extraction were contradictory to results shown by, e.g., Gonzalez et al. [11] and Hajjaj et al. [15] who indicate boiling ethanol as the optimal extraction

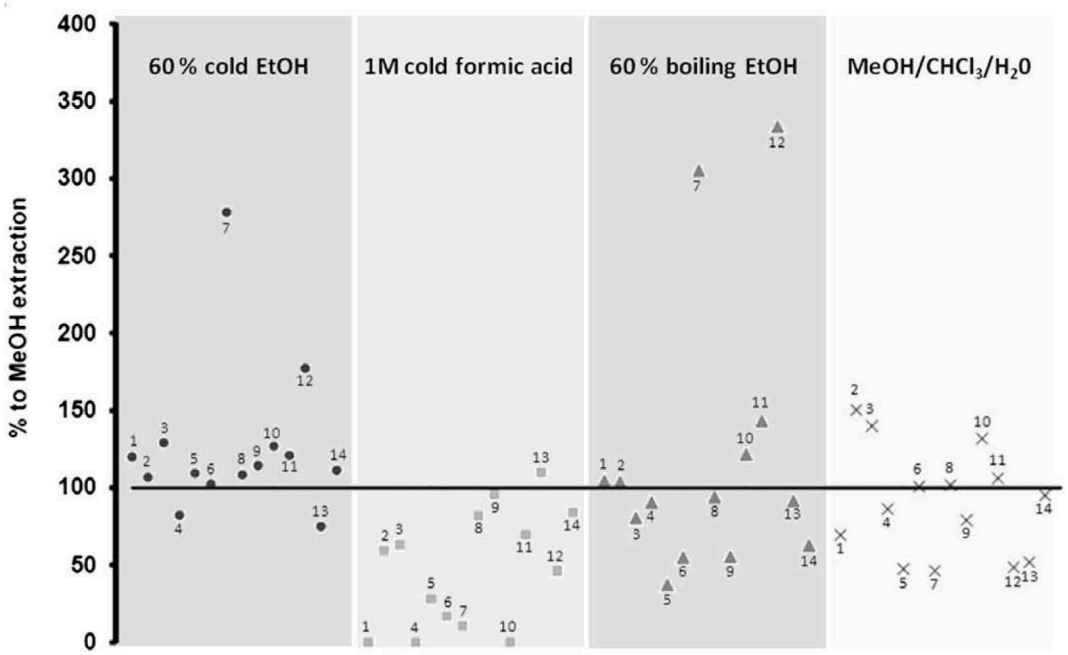

Fig. 4. Extraction efficiency of different tested extraction solutions. For comparisons of extraction potential, 60% (w/v) methanol extractions were carried out in parallel for each tested extraction method and the relative amount of metabolites after methanol extraction was set to 100% (black line). Selected metabolite amounts were presented as filled circles for 60% EtOH; filled quarters for 1 M formic acid; filled triangles for boiling 60% EtOH; and crosses for EtOH/H_2O/$CHCl_3$ extraction. Metabolite list: 1, 3-PG; 2, adenine; 3, ala; 4, cit; 5, fum; 6, glu; 7, his; 8, lac; 9, leu; 10, PEP; 11, suc; 12, trp; 13, urea; and 14, val.

solution. But it must be considered that these experiments were based on the eukaryotic *Saccharomyces cerevisiae* and the fungus *Monascus ruber*. After extraction with a cold chloroform–water–methanol mixture, glutamic acid, lactate, succinic acid, phosphoenolpyruvate, alanine, and adenine were detected in higher amounts than with 60% (w/v) cold methanol extraction, while other metabolites were detected in lower amounts (see Fig. 4). Furthermore a large number of fatty acids (e.g. decanoic acid and pentanoic acid) could be detected after extraction with these lipophilic solvents, as expected and also described for *E. coli* [38]. With regard to the lower extraction efficiency of ~60% for the tested metabolites in comparison to methanol, we would not recommend the use of this lipophilic extraction for a global metabolome analysis unless the focus is a specific lipid study. Cold 60% (w/v) ethanol extraction seems to be the most suitable extraction method. All metabolites, apart from urea and citrate, are detected in higher concentrations than by extraction with cold 60% (w/v) methanol. These results lead us to conclude that cold (⩽6 °C) 60% (w/v) ethanol yields the most useful outcome for a global metabolome analysis (Fig. 4). This extraction method is able to obtain the highest amounts of metabolites, while no disadvantages could be noted in comparison to common extraction methods, as also described by Letisse and Lindley [32] for *Xanthomonas campestris*.

Furthermore, it was observed that for *S. aureus*, cold extraction procedures seem to be more suitable than a hot extraction. This could be attributed to the degradation of some metabolites during the boiling procedure.

By applying the protocol described here, followed by measurement of samples with GC/MS and LC/MS analytics, a high number of intracellular metabolites could be detected. Fig. 5 shows a gas chromatogram of intracellular *S. aureus* metabolites sampled in stationary growth in which numerous amino acids, organic acids, sugars, and nucleotide bases could be detected. As can be observed in Fig. 5, glutamate is an intracellular metabolite with a high concentration and other amino acids, such as alanine, valine, and lysine (especially in stationary growth phase), are also present in high intracellular concentrations. Metabolites from the glycolysis and citrate cycle could only be detected in low concentrations. Metabolites like nucleosides, nucleotides, and cofactors could be detected by LC/MS (Fig. 6), whereas sugar phosphates and fatty acids can be measured by both LC/MS and GC/MS.

Recovery of metabolites from biological samples

Metabolite recovery from spiked cell samples was investigated (i) to calculate the effects of the protocol steps on metabolites, (ii) to investigate the effect of proteins and cell debris on the metabolite levels, and (iii) to estimate irreversible inactivation of enzymes.

Matrix effects are often described as critical in metabolome analysis and the avoidance of these effects is a great challenges [6,44,45]. To study the effect of proteins and cell debris on the metabolites, a standard mixture of amino acids, organic acids, sugar phosphates, nucleotides, and coenzyme A was spiked into biological samples. The median of the recovery was calculated to 95%, and was in the range from 35% to 150% (Table 2). A similar median recovery rate was found for *E. coli* after boiling ethanol metabolite extraction [28].

An excellent recovery rate was achieved for nearly all amino acids, except threonine (recovery <80%) and less low for tyrosine and serine. This observation could be attributed to the interaction of the hydroxyl group on this amino acid with the proteins in the sample.

There is a noticeably lower recovery rate for polar phosphate containing metabolites. As for threonine, it can be assumed that this is a matrix effect, which seems to be unavoidable, since polar metabolites will interact with the proteins in cells.

To estimate irreversible inactivation of enzymes, ATP was spiked into biological samples. ATP is necessary for several reac-

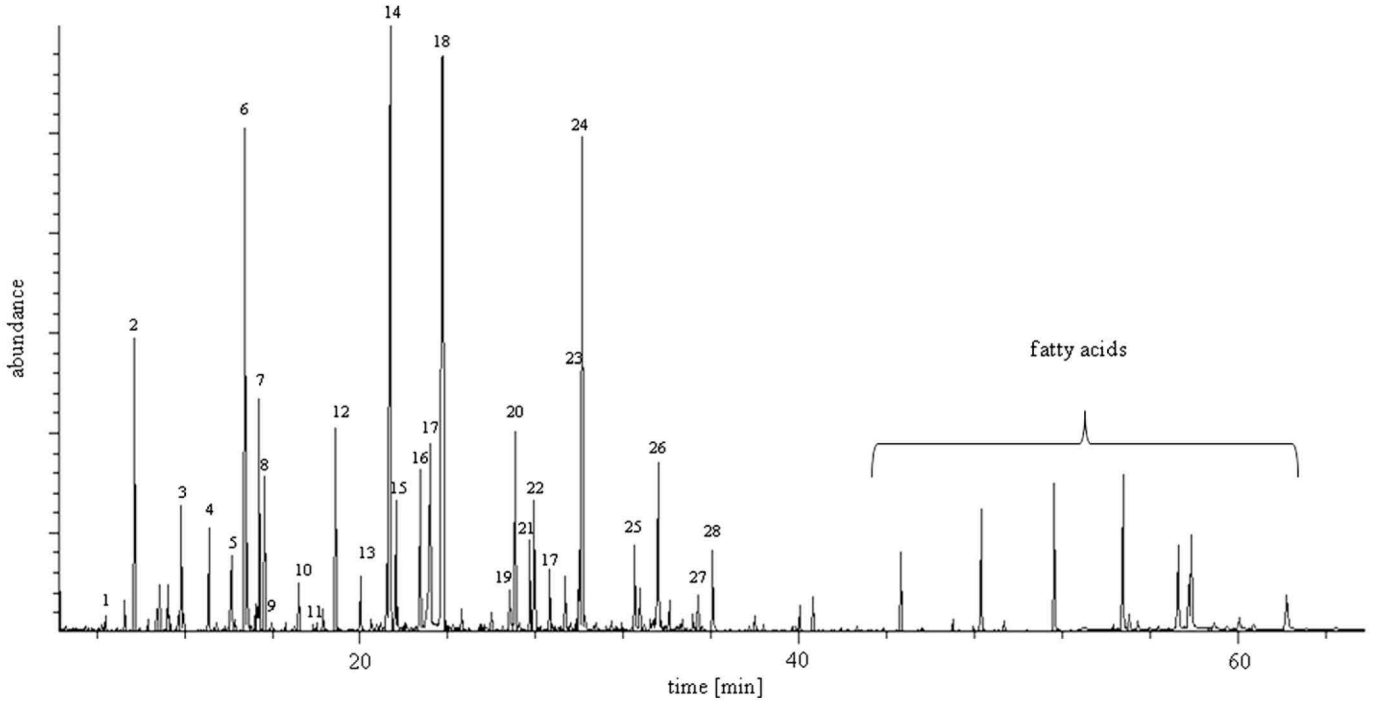

Fig. 5. GC/MS total ion chromatogram of intracellular metabolites from *S. aureus* in stationary growth phase (OD_{500nm} = 3.08, equal to a CDW of 0.641 g cells calculated by x [g/L] = 0.208 * OD_{500}.) and after 60% (w/v) ethanol extraction. 1, pyr; 2, ala*; 3, pro*; 4, val; 5, urea; 6, Pi; 7, pro*; 8, gly*; 9, suc; 10, ser; 11, thr; 12, unknown; 13, unknown; 14, asp; 15, gly*; 16, val*; 17, ala*; 18, glu; 19, PEP; 20, unknown; 21, 3-PG; 22, orn; 23, his; 24, lys; 25, hexadecanoic acid; 26, unknown; 27, trp; 28, octadecanoic acid. (* different derivates after MSTFA/MeOx derivatization.)

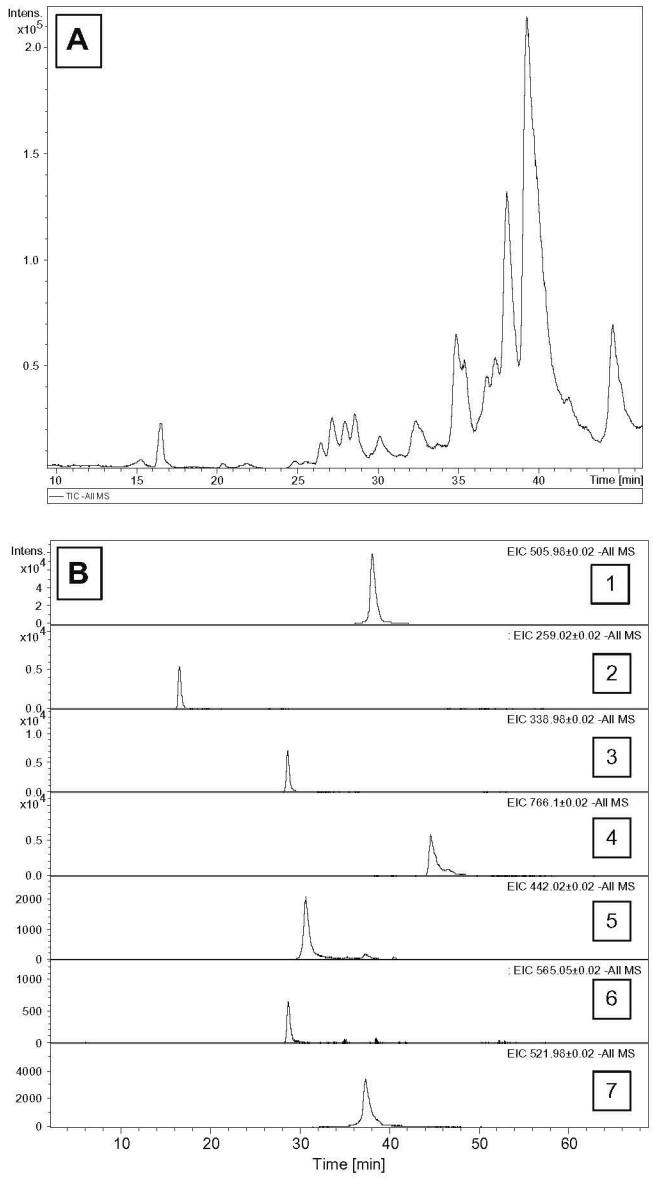

Fig. 6. (A) LC MS total ion chromatogram (TIC) of a representative *S. aureus* sample (exponential growth phase; 60% (w/v) ethanol extraction). (B) Extracted ion chromatograms (EICs) of selected chemically different intracellular metabolites; 1, ATP; 2, glc-6-P; 3, frc-1,6-bp; 4, CoA; 5, GDP; 6, UDP-glc; 7, GTP.

tions and thus ATP has a high turnover and will be quickly degraded to less phosphorylated derivatives (AMP/ADP). Spiking the cell pellet with ATP should show that enzymes were completely inhibited during the whole sample preparation up until the point of measurement, if no AMP/ADP level rises.

The ATP concentration decreases by approximately 17%, with no conspicuous increase of ADP nor AMP, after the spiking of ATP into a biological sample. Enzyme inactivation seems irreversible. Furthermore, it can be assumed that the observed decrease of ATP can be attributed to a direct protocol or matrix effect.

Conclusions

In recent years a significant progression in microbial metabolomics has taken place. By reviewing the accessible literature, it is obvious that there exists nearly no universal methodology in microbial metabolomics that can be applied for all bacterial species

Table 2
Metabolite recovery after spiking and subsequent ethanol (60% w/v) extraction and further glass bead cell disruption of *Staphylococcus aureus* cells.

Spiked metabolites	Recovery [%]	RSD [%]
2-Oxoglutarate	102.3	5.1
cis-Aconitate	94.3	4.0
Fumaric acid	93.8	3.3
Isoleucine	92.2	6.1
Leucine	88.1	6.0
Malic acid	108.6	3.6
Methionine	84.9	2.9
Phenylalanine	97.8	6.1
Serine	86.6	24.6
Succinic acid	104.8	4.9
Threonine	72.7	7.4
Tyrosine	84.1	2.5
Valine	94.0	5.6
ATP	83.2	23.2
CMP	74.3	34.4
CoA	71.4	41.9
frc-1,6-bP	72.1	32.1
glc-6-P	110.1	94.9
GDP	35.4	2.8
IMP	144.2	14.1
UMP	150.3	8.6

[34]. A global protocol for all organisms would be preferable, as previously shown in a first approach for gram-positive prokaryotes, gram-negative prokaryotes, and eukaryotic cells [43]. With regard to the high heterogeneousness of microbial organisms, protocols developed for a specific group of microorganisms cannot be simply adopted. Some reasons also stated by others are differences in the cell wall structure, cell membrane, and growth behavior [34,36,38]. This study aimed to develop a method specifically for the analysis of intracellular metabolites specific from *S. aureus*. The methods and results described above show that paving the way to an optimal protocol is extremely complex.

A guided metabolomic approach was performed here to determine optimal metabolomic workup conditions. We have ascertained that the presented filtration method was the fastest sampling procedure for *S. aureus*. Further pitfalls, such as the leakage of metabolites while quenching, can be overcome by this method, since the cells are separated from the medium at the time of quenching. In addition, it was shown that a complete splitting of intra- and extracellular metabolites could be attained by washing the cells twice with 0.6% NaCl solution, without a detectable leakage of intracellular metabolites. Finally, extraction with 60% (w/v) ethanol is most suitable for high recovery values of intracellular compounds. The complete protocol workflow is summarized in Fig. 7. Further, the study of the recovery of metabolites from biological samples implies that matrix effects and the workup steps induce a negligible decrease of amino acids and organic acids. The combination of energy charge calculation and spiking of ATP into biological samples showed that ATP degrading enzymes are irreversibly inhibited during the work flow.

This approach now allows us to decipher the *S. aureus* metabolome by all available techniques. The obtained metabolite data can be combined with transcriptome and proteome data in a holistic way to give a complete systems biology approach. This would allow a global insight into the cell physiology of the versatile human pathogen, *S. aureus*.

In future, different *S. aureus* strains or important regulatory mutants can be compared [30] and the effect of antibiotics or other agents on the physiology of *S. aureus* can be analyzed on the metabolome level. The next step is to prove and adopt the protocol to related important gram-positive pathogens like *Staphylococcus epidermidis* or *Streptococcus pneumonia*.

Table 3
Metabolite classes extracted by the different extraction solutions.

	60% (w/v) methanol	60% (w/v) ethanol	Boiling 60% (w/v) ethanol	0.5 mM formic acid	Chloroform–water–ethanol–mix (4:2:4)
Amino acids	+	+	+	+	+
Organic acids	+	+	+	– Fumarate,	+
			+ Lactate	– Malate,	
Monosaccharides	+	+	+	+	+
Monosaccharide–phosphates	+	+	+	+	+
Nucleobases/nucleosides/ nucleotides	+	+	+ – AMP	+	+
Organic phosphoric acids	+	+	+	+ – 3PG	+
Fatty acids	+	+	+	+	+ + Decanoic acid, + Pentanoic acid, + Heptanoic acid, + 20 not identifiable, additional peaks of fatty acids
Other	+	+	+	+	+

Metabolites, only extracted by one method are listed: (+) detectable, (–) not detectable. Selected classes were defined as organic acids (citrate, fumarate, malate, succinate, *cis*-aconitate, pyruvate, lactate); amino acids (alanine, aspartate, cysteine, glutamate, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, ornithine); monosaccharides (fructose, glucose); monosaccharide–phosphates (glucose-6-phosphate, erythrose-4-phosphate, fructose-6-phosphate); nucleobases (uracil, adenine); nucleosides (adenosine, uridine); nucleotides (AMP); fatty acids (C-10 to C-25); organic phosphoric acid derivatives (3-phosphoglycerate, phosphoenolpyruvate), and other (*N*-acetylglucosamine, *N*-acetylglutamate, urea, glycerol-3-phosphate).

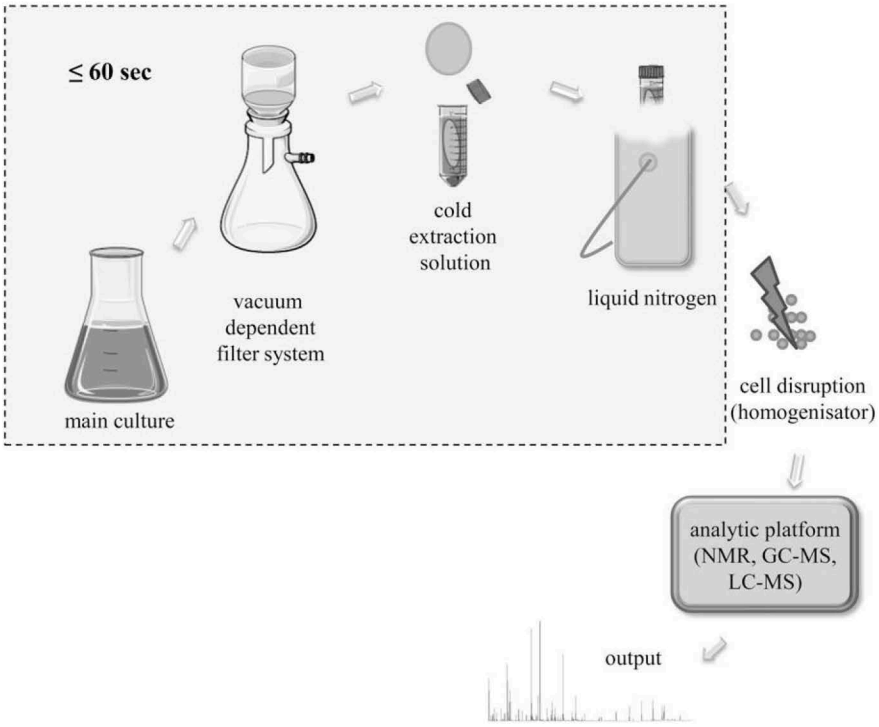

Fig. 7. Work flow for cell sampling, medium separation, quenching, cell disruption, extraction, and analysis of the metabolites occurring from *Staphylococcus aureus* cell cultures.

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