Comparison of protein quantification and extraction methods suitable for *E. coli* cultures

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Abstract

Many different extraction and analysis methods exist to determine the protein fraction of microbial cells. For metabolic engineering purposes it is important to have precise and accurate measurements. Therefore six different protein extraction protocols and seven protein quantification methods were tested and compared. Comparison was based on the reliability of the methods and boxplots of the normalized residuals.

Some extraction techniques (SDS/chloroform and toluene) should never be used: the measurements are neither precise nor accurate. Bugbuster extraction combined with UV280 quantification gives the best results, followed by the combinations sonication-UV280 and EasyLyse-UV280. However, if one does not want to use the quantification method UV280, one can opt to use Bugbuster, EasyLyse or sonication extraction combined with any quantification method with exception of the EasyLyse-BCA_P and sonication-BCA_P combinations.

Keywords: protein extraction, protein quantification, metabolic modeling, *Escherichia coli*

1 Introduction

Whereas in the past genetic engineering aimed at the massive overexpression and inactivation of microbial genes, metabolic modeling is nowadays increasingly used to identify specific targets, liable for genetic engineering. In most cases the construction of a producer strain was a long and tedious process of trial and error. Indeed, in complex metabolic networks, it is often a difficult task to ad hoc predict the impact, both qualitatively and quantitatively, of a genetic modification [1]. The use of a metabolic model helps to predict the effect of a genetic modification and hence facilitates the process of constructing a desirable producer strain. A central element of metabolic models is the biomass, mainly composed of proteins, lipids, DNA and RNA. The accurate quantification of these components for large numbers of samples at the highest possible sensitivity is required in order to obtain accurate data for the identification of those models. For the components DNA and RNA athorough analysis of the , quantification methods has recently been reported [2].

For protein extraction and quantification many different methods exist as well.

The initial step of any purification procedure involves recovery of the protein from its source. The complexity of this step depends largely upon whether the protein of interest is intracellular or extracellular. Whereas for many applications such as heterologous protein production or industrial enzyme production, where one specific protein has to be purified and quantified, in metabolic modeling, the total protein content of the biomass has to be quantified. For this cell harvesting from the culture medium will be followed by resuspension of the cells in physiological buffer with subsequent cell disruption. Disruption of microbial cells is difficult due to the presence of the microbial complex cell wall.

In the literature, proteins are mostly extracted using cell lysis and sonication but also other extraction methods exist. In this contribution, different analysis and extraction methods for proteins have been assessed.

2 Materials and Methods

2.1 Bacterial strain and culture conditions

Escherichia coli MG1655 [λ^-, F^-, *rph*-1, *rfb*-50, *ilvG*$^-$, *fnr*$^-$] was obtained from the Netherlands Culture Collection of Bacteria (NCCB). Biomass was obtained by running a chemostat culture as described by De Mey et al. [3].

2.2 Protein extraction methods

Preceding each extraction methods, cells were harvested from 40 ml culture broth by centrifugation (4 °C, 15 min, 5000 g) in a Sorvall centrifuge (Goffin-Meyvis, Hoeilaert, Belgium). The pellet was resuspended in 40 ml physiological solution (0.9 % NaCl) and diluted until the optical density at 600 nm was 2. This is referred as the culture solution.

2.2.1 Cell lysis using sonication (Sonication)

Cell lysis using sonication was done as described by Deutcher [4] with some modifications. Sonication of 5 ml of the culture solution was done in a Branson Sonifier® 250 (VWR, Leuven, Belgium) (hold, 50%, strength 5). During sonication, the culture solution was kept on ice. The sonicated culture solution was centrifuged at 5000 g and 4 °C during 5 min in a Sorvall centrifuge (Goffin-Meyvis, Hoeilaert, Belgium) to decrease foaming. Subsequently, 5 ml 2 N NaOH was added to the culture solution and boiled for 10 min, immediately followed by cooling on ice.

2.2.2 Cell lysis using EasyLyse™-kit (Easylyse)

Cell lysis using the EasyLyse™-kit (Epicentre® Biotechnologies, BIOzymTC, Landgraaf, Netherlands) was done as recommended by the supplier in the EasyLyse Bacterial Protein Extraction Solution manual.

2.2.3 Cell lysis using BugBuster® Protein Extraction Reagent (Bugbuster)

Cell lysis using BugBuster® Protein Extraction Reagent (Novagen®, Leuven, Belgium) was done as recommended by the supplier.

2.2.4 Cell lysis by SDS/chloroform treatment (SDS_Chl)

Cell lysis by SDS/chloroform treatment was done following the protocol described by Miller [5] with some modifications. The culture solution was diluted with physiological solution to a 2x diluted culture solution (1:1 v:v). To 1 ml diluted culture solution 50 µl 0.1 % SDS and 100 µl chloroform was added and this was vortexed during 10 s followed by centrifugation at 18000 g during 5 min (Heraeus Biofuge Stratos, Goffin-Meyvis, Hoeilaert, Belgium). The supernatant was used for analysis.

2.2.5 Cell lysis by toluene treatment (toluene)

Cell lysis by toluene treatment was performed following the protocol described by Miller [5] with some modifications. The culture solution was diluted with physiological solution to a 2x diluted culture solution (1:1 v:v). To 1980 µl diluted culture solution 20 µl toluene was added and this was vortexed during 10 s followed by centrifugation at 18000 g during 5 min (Heraeus Biofuge Stratos, Goffin-Meyvis, Hoeilaert, Belgium). The supernatant was used for analysis.

2.2.6 Cell lysis by cooking in KOH (KOH)

Cell lysis by cooking in KOH was done as described by Verduyn *et al.* [6].

2.3 Protein quantification methods

2.3.1 UV absorbance at 280 nm (UV280: 0.1-100 mg/ml)

Protein measurements were performed as described by Sambrook & Russell [7]. Absorbance data ($A = \log I/I_0$) were collected using a nanodrop ND-1000 spectrophotometer (Isogen Life Science, Sint-Pieters-Leeuw, Belgium). The absorption of proteins is maximal at 280 nm.

2.3.2 Absorbance at 595 nm using Bradford reagent (Bradford: 20-2000 µg/ml)

Protein measurements were performed as recommended by Ohnishi & Barr [8]. 3 ml Bradford-reagent was added to 100 µl sample and mixed. After an incubation period of 15 min at room temperature, the absorbance at 595 nm was measured using an UVIKOM 922 spectrophotometer (BRS, Brussel, Belgium).

2.3.3 Absorbance at 725 nm using Biuret reagent and Folin reagent (Biuret: 150-1000 µg/ml)

Protein measurements were done as recommended by the supplier (Sigma, Bornem, Belgium). 2.2 ml Biuret-reagent was added to 0.2 ml sample and mixed. After 10 min incubation at room temperature, 100 µl Folin-reagent was added immediately followed by vortexing. After incubation of 30 min at room temperature, the absorbance at 725 nm was measured using an UVIKOM 922 spectrophotometer (BRS, Brussel, Belgium).

2.3.4 Bicinchoninic acid method (BCA: 20-2000 µg/ml)

Protein measurements were done as described by Deutscher *et al.* [4].

2.3.5 Bicinchoninic acid method using commercial kit of Pierce (BCA_P: 20-2000 µg/ml)

Protein measurements were done as described by the supplier (Perbio Science, Erembodegem, Belgium).

2.3.6 Advanced protein assay (AdvProt: 1-40 µg/ml)

Protein measurements were done as described by the supplier (Fluka, Bornem, Belgium).

2.3.7 Absorbance at 555 nm using $CuSO_4$ (CuSO4: 0.2-8 mg/ml)

Protein measurements were done as described by Schultze [9] with some modifications. 1 ml sample was added to 3 ml 2.5 % (w/v) $CuSO_4$. After 5 min incubation at 4 °C and centrifugation (Sorvall, Goffin-Meyvis, Hoeilaert, Belgium), the absorbance at 555 nm was measured.

2.4 Statistical analysis

The quality of the different protein acid extraction methods is mainly determined by the slope of the response variable (absorbance) to concentration of dilution series (linear regression). The lower this slope (less difference in absorbance for the same concentration range), the less sensitive the method. However, the slope is not the only important parameter. Also the variance on the slope should be taken into account. Moreover, the different detection methods can not be compared directly, as they do not give the same response and are not applicable to the same range of concentrations. Therefore each slope is divided by its standard deviation. This value represents the reliability of the method and is independent of any scale.

After performing the linear regression, the residuals were calculated. To compare the different combinations of quantification methods and extraction methods, a boxplot of the residuals was generated.

The statistical package R [10] was used for all the statistical analyses.

3 Results and Discussion

In the first part of this study, seven different protein analysis methods were tested and compared: 1) UV absorbance at 280 nm (UV280), 2) absorbance at 595 nm using Bradford reagent (Bradford), 3) absorbance at 725 nm using Biuret reagent and Folin reagent (Biuret), 4) Bicinchoninic acid method (BCA), 5) BCA assay of Pierce® (BCA_P), 6) Advanced protein assay (AdvProt), and 7) absorbance at 555 nm using $CuSO_4$ (CuSO4).

To be able to compare the different quantification methods, they were applied on series of standard dilutions (0, 0.25, 0.5, 0.75, and 1 g/l) of bovine serum albumin (BSA, Sigma®, Belgium). After normalizing the data between zero and one, linear regression was performed for each method and the residuals were calculated. The boxplot of these residuals can be found in Figure 1. Each entry represents an analysis method.

Figure 1

From Figure 1, it can be concluded that the UV absorbance at 280 nm is the best analysis method. However, due to possible interfering absorbance of contaminating molecules in culture broths, it was decided to retain the four best methods: 1) UV absorbance at 280 nm, 2) absorbance at 595 nm using Bradford reagent, 3) absorbance at 725 nm using Biuret reagent and Folin reagent, and 4) BCA assay of Pierce®.

In the second part of this study, six different protein extraction protocols were tested with *Escherichia coli* cells, harvested from a chemostat experiment: 1) cell lysis using sonication (sonication), 2) cell lysis using EasyLyse™-kit (Easylyse), 3) cell lysis using BugBuster® Proteïn Extraction Reagent (Bugbuster), 4) cell lysis by SDS/chloroform treatment (SDS_Chl), 5) cell lysis by toluene treatment (toluene), and 6) cell lysis by cooking in KOH.

Subsequently dilution series (0, 0.25, 0.5, 0.75 and 1x) were made from the *E. coli* cells, cell lysis was performed followed by protein quantification via the four analysis methods selected above. Processing of the data was done as described in section 2.4.

For each combination of extraction/analysis method, a linear regression was performed using the statistical package R (data not shown). Common sense dictates that the steeper the slope, the more sensitive the measurements are. However, the error on the slope should also be taken into account: a steep slope with a high confidence interval is less desirable than a somewhat more horizontal slope with a small confidence interval. Thus to compare the reliability of the different combinations of quantification and extraction methods, the slopes were divided by their variance (figure 2a). To assess the precision of the measurement/extraction combinations, boxplots of the residuals were generated, after rescaling the response of each extraction/quantification combination between zero and one, so that the residuals have the same scale and are thus comparable (Figure 2b).

Figure 2

From figure 2a it can be concluded that the toluene extraction method should never be used. In all four quantification methods it performs poorly. SDS_Chl is also a relatively poor quantification technique for the four extraction methods. It has a somewhat higher reliability in combination with UV280, but it is still lower than Sonication, Easylyse or Bugbuster (figure 2a). Furthermore the boxplot reveals the presence of outliers. KOH extraction should only be used in combination with the BCA_P quantification method. Although the reliability of KOH in combination with Bradford and UV280 is relatively high, the boxplots show outliers (KOH.Bradford) or non standard distributed errors (KOH.UV280).

The extraction methods Bugbuster, EasyLyse and sonication are the most reliable. Moreover, the combination Bugbuster-UV280 is the best combination, followed by sonication-UV280 and EasyLyse-UV280. However, if one does not want to use the UV280 quantification method, one can also opt to use Bugbuster, EasyLyse or sonication combination except for EasyLyse-BCA_P and sonication-BCA_P.

This is also confirmed by the % recovery for the different protein extraction methods (data not shown). For a chemostat at D=0,1 h^{-1} the protein content of the *E. coli* biomass is estimated to be around 70% [11]. For sonication-UV a maximum protein content of 69,13% was obtained and this was referred to as 100% recovery. Whereas the % recovery for the extraction methods Sonication, Easylyse or Bugbuster is above 90 %, the % recovery for KOH is 80 % and for Toluene and SDS_Chl is it even less than 40 %.

4 Conclusion

Nowadays, metabolic engineering uses metabolic models as tool for the construction of high producer strains, instead of the trial and error approach of massive overexpressing and inactivating of genes. A crucial element of those metabolic models is biomass, consisting mainly of DNA, RNA and proteins. The accurate quantification of proteins for large numbers of samples at the highest possible sensitivity is required in order to obtain accurate data for the identification of metabolic models. In this contribution, different extraction and quantification methods for proteins were compared. The combination Bugbuster-UV280 is the best combination followed by sonication-UV280 and EasyLyse-UV280. However, if one does not want to use the quantification method UV280, one can opt to use Bugbuster, EasyLyse or sonication extraction combined with Biuret or Bradford quantification methodology or Bugbuster extraction in with BCA_P quantification.

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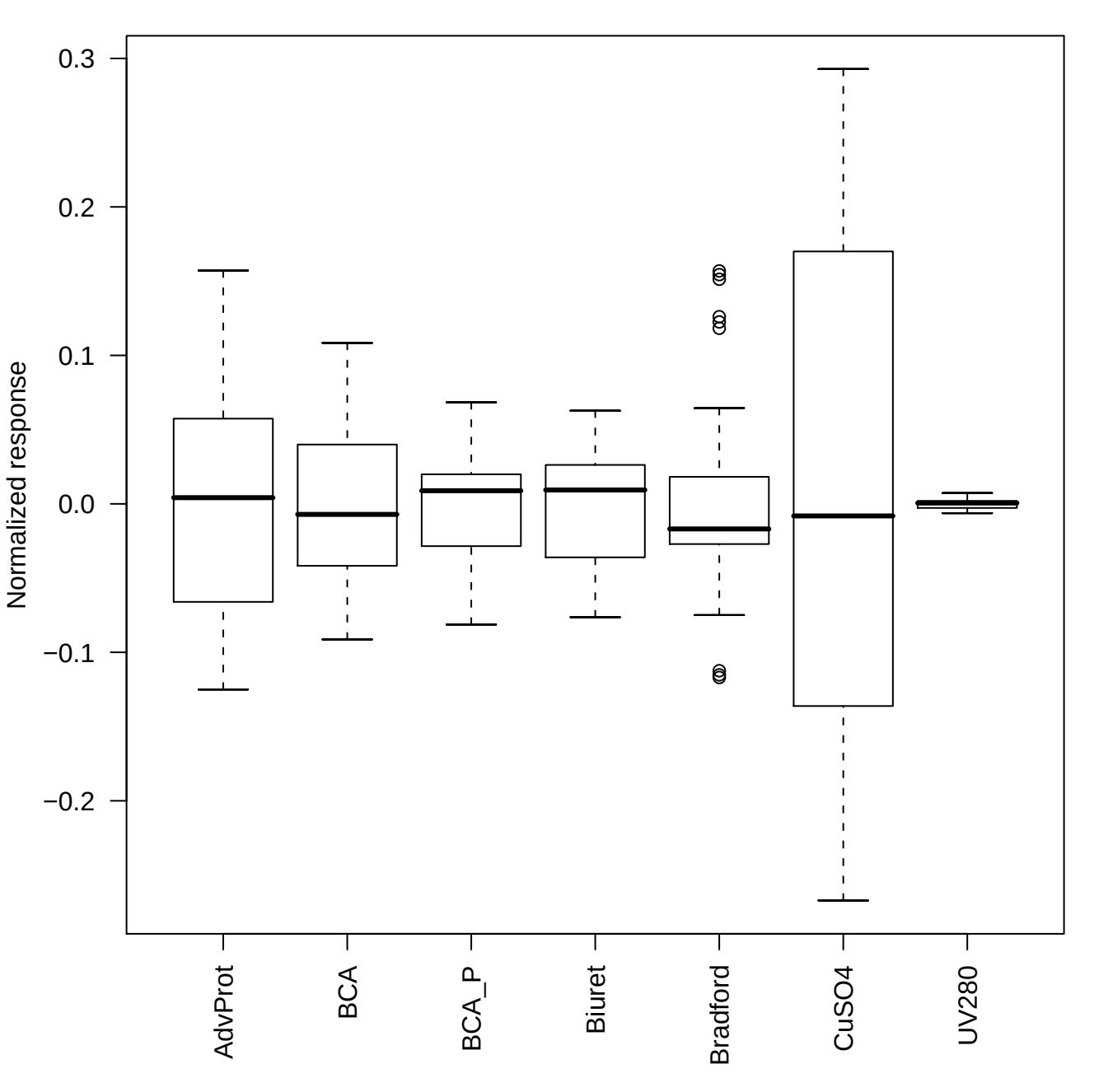
Figures Legends

Figure 1.

Box plots of the residuals for each protein quantification method after rescaling the response for each method between zero and one

Figure 2.

(a) The slope divided by its standard deviation for each extraction (lower horizontal axis)/ quantification (upper horizontal axis) method. (b) Box plots for each extraction/ quantification method combination after rescaling the responses between zero and one.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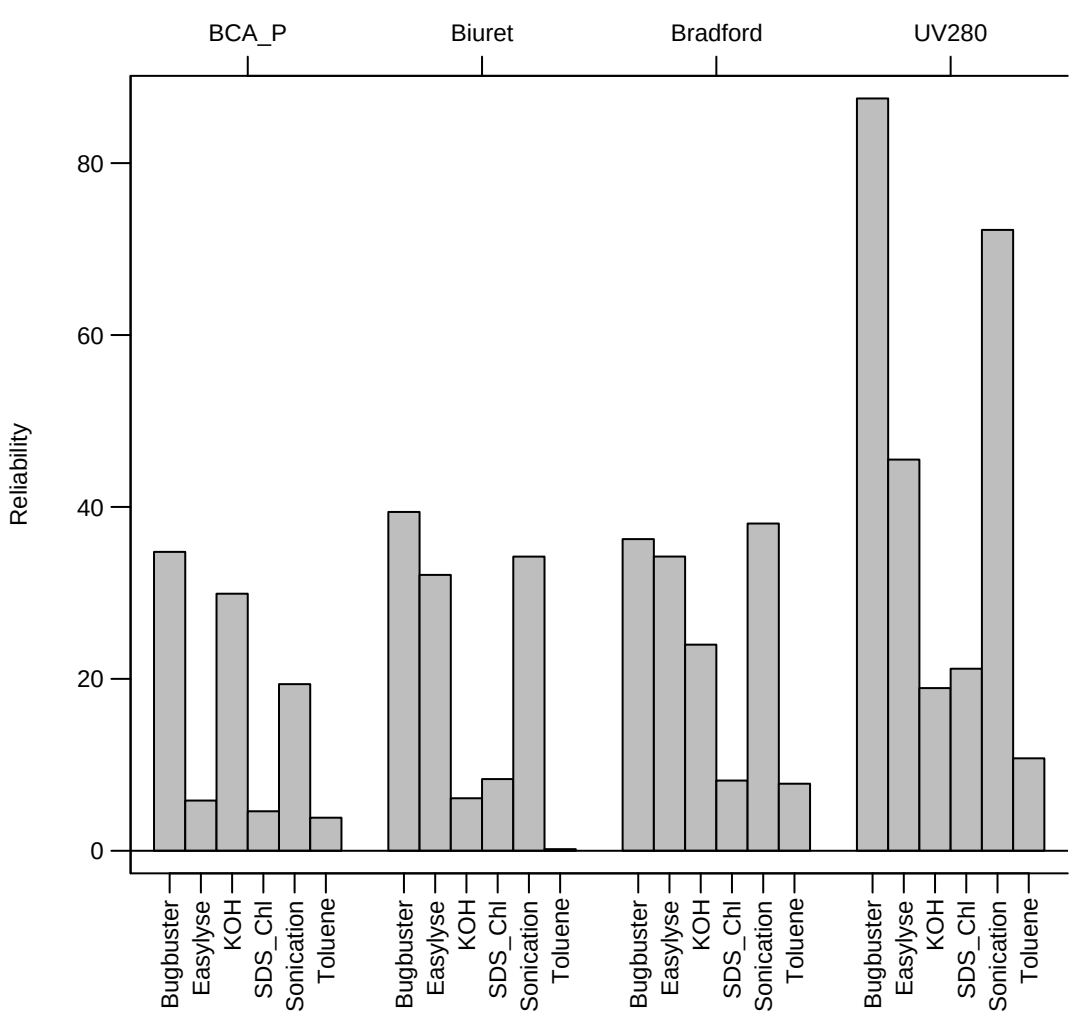
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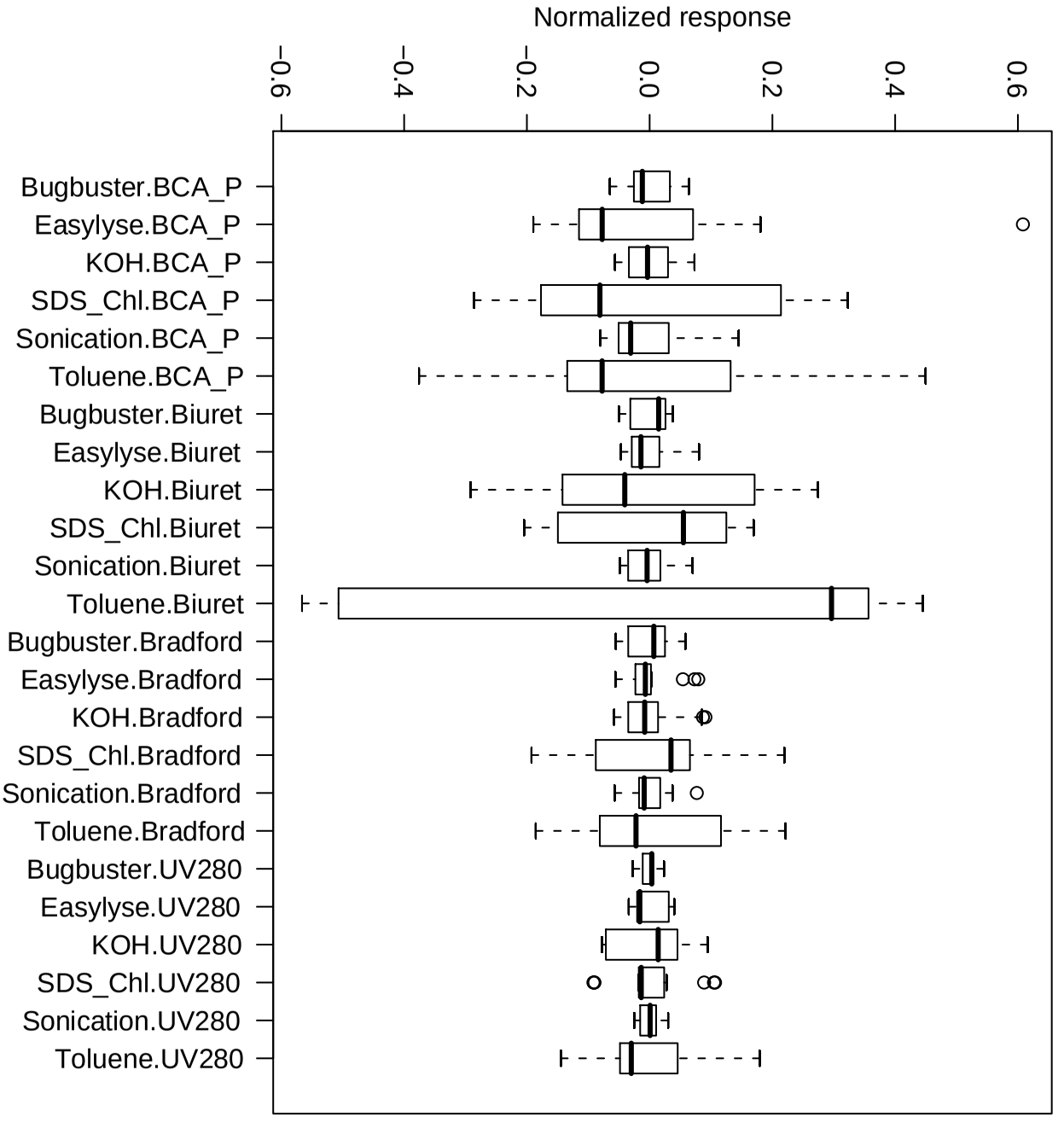
KOH
SDS_Chl
Sonication
Toluene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−0.2
0.0
0.2
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Bugbuster.BCA_P
Easylyse.BCA_P
KOH.BCA_P
SDS_Chl.BCA_P
Sonication.BCA_P
Toluene.BCA_P
Bugbuster.Biuret
Easylyse.Biuret
KOH.Biuret
SDS_Chl.Biuret
Sonication.Biuret
Toluene.Biuret
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KOH.Bradford
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