Liquid-Liquid Extraction (LLE) vs. Novum™ Simplified Liquid Extraction (SLE) Case Study: Analysis of Monomeric Phenolic Fermentation Inhibitors in Dilute-Acid Plant Hydrolysate

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Introduction

Pretreatment of lignocellulosic biomass is a prerequisite in order to maximize glucose release from cellulose for subsequent fermentations or chemical conversions. A leading pretreatment involves dilute acid (e.g., 0.3 - 2 % sulfuric acid) at elevated temperatures (140 to 200 °C) with different holding times and biomass/acid loadings[1]. A drawback of this procedure is the partial degradation of lignin, a polyphenolic macromolecule, to smaller phenolics that are released together with the sugars into the so-called hydrolysate. These phenolic compounds are toxic to microorganisms and are potential fermentation inhibitors [2-5].

Analyzing this phenolic profile is important when optimizing pretreatment or detoxification methods aiming to minimize inhibitor levels or to engineer more resistant microorganisms. In a widely employed sample preparation and cleanup step, the phenolic compounds undergo a liquid-liquid extraction using an organic solvent (e.g., ethyl acetate) and are then analyzed via GC/MS[6]. While effective, the liquid-liquid extraction can be time consuming, requiring 4 separate extractions and multiple centrifugation steps. In an effort to reduce time and sample handling steps, a simplified liquid extraction (SLE) using Novum SLE was employed, saving a significant amount of time and reducing the amount of required pipetting and vial handling steps.

Materials and Methods

Lignocellulosic biomass was prepared using two different methods, a traditional liquid-liquid extraction and simplified liquid extraction (SLE) using a Novum SLE MAX 96-well plate (part no. 8E-S138-5GA). Prior to each extraction method, the samples were pretreated as follows:

Pretreatment

A dilute-acid pretreatment hydrolysate (1.5 % sulfuric acid, 190 °C for 1 minute, 25 % biomass loading) from *Miscanthus X giganteus* was filtered using a PES (polyethersulfone) 0.45 µm filter and was spiked with 20 µg/mL iso-propylphenol as an internal standard (IS). The pretreated samples were then extracted by either liquid-liquid extraction or SLE (below).

Liquid-Liquid Extraction Procedure

1 mL aliquots of the spiked hydrolysate from the pretreatment step were extracted 4x each with 0.5 mL of ethyl acetate. After each extraction, a centrifugation step (1500 g for 1 minute) was applied for phase separation. The combined extracts of each sample were dried over sodium sulfate[6] (samples A).

Simplified Liquid Extraction (SLE) Procedure

400 µL aliquots of the spiked hydrolysate from the pretreatment step were loaded into the wells of a Novum SLE MAX 96-well plate. The sample was allowed to soak into the sorbent for 5 minutes and then the ethyl acetate was applied to elute the analytes of interest. Three different volumes were applied to determine the most effective elution volume. Either 1x 0.4 mL (samples B), 1x 0.8 mL (samples C), or 2x 0.4 mL (samples D) of ethyl acetate was applied using a short pulse of vacuum. No further drying of ethyl acetate was performed after the elution.

GC/MS Conditions

Prior to GC analysis, 100 µL aliquots of the extracts (samples A - D) were incubated with 50 µL BSTFA at 70 °C for 30 minutes and then analyzed by GC/MS[6]. All extractions were performed in triplicate and replicates (1-3) were run in the following order: samples A1 - D1, then A2 - D2, then A3 - D3 to avoid any bias because of potential changes in sample composition of unprocessed samples due to the long run time (67 minutes). 1 µL was injected in splitless mode onto a 5-ms capillary GC column (30 m x 0.25 mm x 0.25 µm). An Agilent® 7890A gas chromatograph coupled to an Agilent 5975C single quadrupole mass spectrometer was used for analysis with the following settings: Injector and transfer line temperature 280 °C, carrier gas: helium at 1 mL/min, temperature program: 75 °C for 3 min isocratic, 5 °C/min to 150 °C, 0.5 °C/min to 160 °C, 2 °C/min to 190 °C, 5 °C/min to 240 °C, 70 °C/min to 325 °C, 3 min isocratic. Ions were detected in full scan mode m/z 35-500.

Results and Discussion

Figure 1 shows the relative recoveries of 25 selected phenolic compounds from hydrolysate eluted from the Novum SLE MAX 96-well plate using the 3 elution procedures (0.4 mL, 0.8 mL and 2x 0.4 mL) compared to the values obtained by the conventional liquid-liquid extraction (=100 % on the representative chart). Overall the Novum SLE extraction produced recoveries that were comparable to the traditional liquid-liquid extraction procedure in a fraction of the time (a five-fold decrease in the amount of sample preparation time). The 0.4 mL ethyl acetate elution resulted in a general lower recovery of the analytes compared to the 0.8 mL and 2x 0.4 mL volume extractions. Exceptions were 4-hydroxy-3-methoxy-cinnamaldehyde (**Figure 1**, analyte 9) with almost identical recovery levels observed (95 % and 93 %, respectively) for all elution volumes and sinapaldehyde (**Figure 1**, analyte 12) with a higher recovery for the 0.4 mL extraction volume (93 %) compared to the other extraction volumes applied (89 % and 85 %, respectively). However, a 0.4 mL extraction volume was generally deemed to be suboptimal. Increasing the elution volume to 0.8 mL improved the recovery with most recoveries reaching >90 %. Slightly lower yields were observed for 4-hydroxybenzaldehyde (87 %) (**Figure 1**, analyte 1) and vanillin (89 %) (**Figure 1**, analyte 2). Only the homologs 2-guaiacylacetaldehyde (**Figure 1**, analyte 14) and 2-syringacetaldehyde (**Figure 1**, analyte 16) exhibited lower recovery levels of 75-76 % and 73-75 %, respectively. Applying the 0.8 mL elution volume in two steps (2x 0.4 mL) did not improve the recovery as compared to a single aliquot of 0.8 mL.

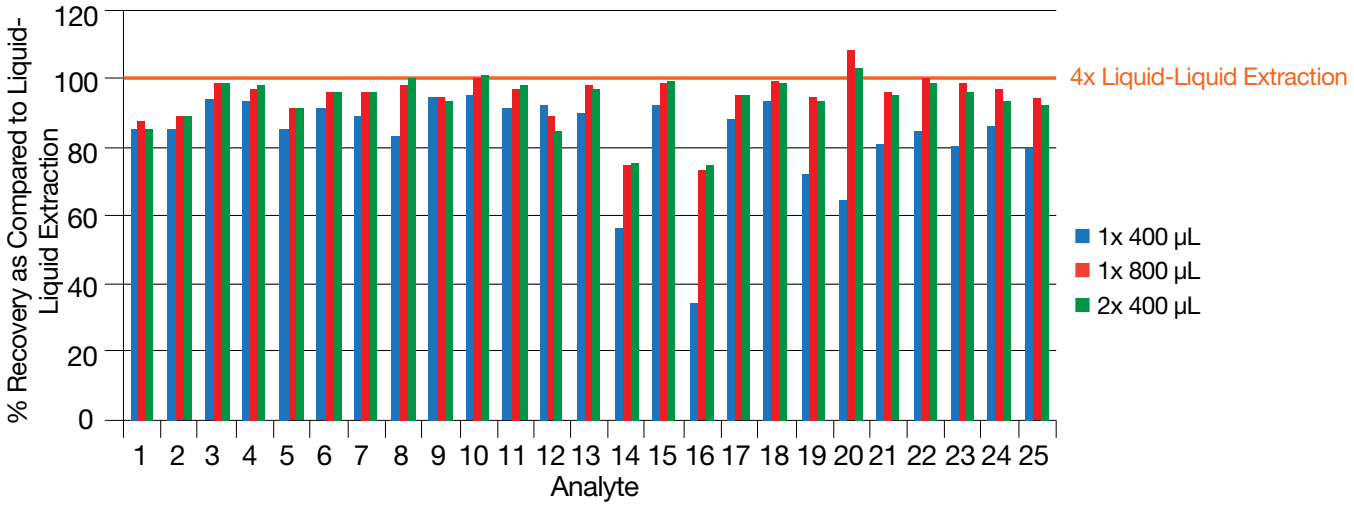
Conclusion

When extracting 0.4 mL of a dilute-acid plant hydrolysate using a Novum™ SLE MAX 96-well plate, a 0.8 mL elution volume of ethyl acetate resulted in high recoveries of >90 % for most phenolic analytes. No further drying of the ethyl acetate phase was necessary to ensure successful derivatization for subsequent GC/MS analysis. Compared to the conventional liquid-liquid extraction, we estimate an up to five-fold decrease in sample preparation time. Furthermore, the Novum SLE extraction also greatly simplifies sample preparation by reducing the amount of required pipetting and vial handling steps. Therefore, sample preparation using the Novum SLE resulted in a phenolic inhibitor profile that was very similar and comparable to the conventional liquid-liquid extraction but at a fraction of the time and labor.

References

1. Humbird D, Davis R, Tao L, Kinchin C, Hsu D, Aden A, Schoen P, Lukas J, Olthof B, Worley M, Sexton D, Dudgeon D (2011) Process Design and Economics for Biochemical Conversion of Lignocellulosic Biomass to Ethanol: Dilute-Acid Pretreatment and Enzymatic Hydrolysis of Corn Stover. Technical Report NREL/TP-5100-47764. United States National Renewable Energy Laboratory, US Department of Energy. May 2011
2. Jönsson LJ, Palmqvist E, Nilvebrant N-O, Hahn-Hägerdal B (1998) Detoxification of wood hydrolysates with laccase and peroxidase from the white-rot fungus Trametes versicolor. Appl Microbiol Biotechnol 49:691-697
3. Luo C, Brink DL, Blanch HW (2002) Identification of potential fermentation inhibitors in conversion of hybrid poplar hydrolyzate to ethanol. Biomass Bioenergy 22:125-138
4. Larsson S, Reimann A, Nilvebrant N-O, Jönsson LJ (1999) Comparison of different methods for the detoxification of lignocellulose hydrolyzates of spruce. App Biochem Biotechnol 77-79:91- 103
5. Buchert J, Niemelä K, Puls J, Poutanen K (1990) Improvement in the fermentability of steamed hemicellulose hydrolysate by ion exclusion. Process Biochem Int 176-180
6. Mitchell VD, Taylor CM, Bauer S (2014) Comprehensive analysis of monomeric phenolics in dilute acid plant hydrolysates. Bioenerg Res 7:654-669

Figure 1. Relative recovery (%) after extraction using Novum SLE under various extraction solvent volumes as compared to a liquid-liquid extraction



Analyte Key:

1	4-hydroxybenzaldehyde
2	vanillin
3	acetovanillone
4	4-hydroxybenzoic acid
5	syringaldehyde
6	vanillic acid
7	homovanillic acid
8	3,4-dihydroxybenzoic acid
9	4-hydroxy-3-methoxy-cinnamaldehyde
10	syringic acid
11	trans-p-coumaric acid
12	sinapaldehyde

Analyte Key *cont'd*:

13	trans-ferulic acid
14	2-guaiacylacetaldehyde
15	guaiacylacetone
16	2-syringacetaldehyde
17	3-guaiacylpropanol
18	2-hydroxy-1-guaiacylpropanone
19	3-guaiacylacetol
20	3-syringylacetol
21	1-guaiacylacetol
22	3-(4-hydroxyphenyl)acetol
23	1-syringylacetol
24	2-hydroxy-1-guaiacylethanone
25	2-hydroxy-1-syringylethanone

APPLICATIONS

Ordering Information

Novum™ Simplified Liquid Extraction (SLE) 96-Well Plates

Part No.	Description	Unit
8E-S138-FGA	Novum SLE MINI 96-Well Plate	1/pk
8E-S138-5GA	Novum SLE MAX 96-Well Plate	1/pk

96-Well Plate Accessories

Part No.	Description	Unit
Collection Plates (deep well, polypropylene)		
AH0-7192	96-Well Collection Plate, 350 µL/well	50/pk
AH0-7193	96-Well Collection Plate, 1 mL/well	50/pk
AH0-7194	96-Well Collection Plate, 2 mL/well	50/pk
AH0-8635	96-Well Collection Plate, 2 mL/well Square/Round-Conical	50/pk
AH0-8636	96-Well Collection Plate, 2 mL/well Round/Round, 8 mm	50/pk
AH0-7279	96-Well Collection Plate, 1 mL/well Round, 7 mm	50/pk
Sealing Mats		
AH0-8597	Sealing Mats, Pierceable, 96-Square Well, Silicone	50/pk
AH0-8598	Sealing Mats, Pre-Slit, 96-Square Well, Silicone	50/pk
AH0-8631	Sealing Mats, Pierceable, 96-Round Well 7 mm, Silicone	50/pk
AH0-8632	Sealing Mats, Pre-Slit, 96-Round Well 7 mm, Silicone	50/pk
AH0-8633	Sealing Mats, Pierceable, 96-Round Well 8 mm, Silicone	50/pk
AH0-8634	Sealing Mats, Pre-Slit, 96-Round Well 8 mm, Silicone	50/pk
AH0-7362	Sealing Tape Pad	10/pk
Vacuum Manifold		
AH0-8950	96-Well Plate Manifold, Universal with Vacuum Gauge	ea

Novum SLE Tubes

Part No.	Description	Unit
8B-S138-FAK	Novum SLE 1 cc tubes	100/pk
8B-S138-5BJ	Novum SLE 3 cc tubes	50/pk
8B-S138-JCH	Novum SLE 6 cc tubes	30/pk
8B-S138-KDG	Novum SLE 12 cc tubes	20/pk

Tube Accessories

Vacuum Manifolds		
Part No.	**Description**	**Unit**
AH0-6023	12-Position Vacuum Manifold Set	ea
AH0-6024	24-Position Vacuum Manifold Set	ea

guarantee

If Novum SLE products do not perform as well or better than your current SLE product, return the product with comparative data within 45 days for a FULL REFUND.

APPLICATIONS

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