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Published in:
International Journal of Biological Macromolecules

DOI:
10.1016/j.ijbiomac.2014.03.036

Published: 01/06/2014

Document Version:
Peer reviewed version

Structural diversity requires individual optimization of ethanol concentration in polysaccharides precipitation

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Abstract:

Ethanol precipitation is one of the most widely used methods for preparing natural polysaccharides, in which ethanol concentration significant affects the precipitate yield, however, is usually set at 70-80%. Whether the standardization of ethanol concentration is appropriate has not been investigated. In the present study, the precipitation yields produced in varied ethanol concentrations (10-90%) were qualitatively and quantitatively evaluated by HPGPC (high-performance gel-permeation chromatography), using two series of standard glucans, namely dextrans and pullulans, as reference samples. The results indicated that the response of a polysaccharide's chemical structure, with diversity in structural features and molecular sizes, to ethanol concentration is the decisive factor in precipitation of these glucans. Polysaccharides with different structural features, even though they have similar molecular weights, exhibit significantly different precipitation behaviors. For a specific glucan, the lower its molecular size, the higher the ethanol concentration needed for complete precipitation. The precipitate yield varied from 10% to 100% in 80% ethanol as the molecular size increases from 1 kDa to 270 kDa. Our trials, using different ethanol concentrations to extract the polysaccharides from water extracts of eight natural materials, demonstrate and confirm that natural polysaccharides respond differently to different concentrations of ethanol in ethanol precipitation extractions. This paper aims to draw scientists' attention to the fact that, in extracting natural polysaccharides by ethanol precipitation, the ethanol concentration must be individually optimized for each type of material.

Keywords: Ethanol precipitation; Natural polysaccharides; Polysaccharide structures; Ethanol concentration

1. Introduction

Natural polysaccharides are important biologically active components of many medicinal herbs, and have thus been attracting increasing multidisciplinary research interest [1]. Several challenges exist in this research field: crystal structure determination, quality control, *in vivo* detection, and molecular target determination [2-5]. However, before these challenges can be effectively tackled, a more fundamental problem must be addressed, namely, accurate, consistent sample preparation [6-8].

As the commonly-used sample pretreatment operation, ethanol precipitation is generally the first step in preparing crude polysaccharides from water extracts [9-11]. To some extent, the methodology of ethanol precipitation for some specific samples, e.g. *Citrus* pectins [12], inulinases [13], water extracts of Danshen (*Salvia miltiorrhiza* Bge.) and Chuanxiong (*Ligusticum chuanxiong* Hort.) [14], was investigated in terms of ethanol concentration, supernatant pH value, and refrigeration temperature. The results indicated that the yield of total saccharides increases as ethanol concentration increases. The effect of supernatant pH value is not very significant. Although temperature decrease from 25-5 ℃ also leads to an increase of saccharide yield, as the precipitation is usually performed at around 4 ℃ in a laboratory refrigerator, ethanol concentration seems to be the most important variable in ethanol precipitation.

We found and reviewed a total of 171 publications in *ScienceDirect* from Jan 1 to May 30, 2013 (Figure 1) in which ethanol precipitation was used for the preparation of natural polysaccharide. In more than 70% of these publications—i.e., an

overwhelming majority--the ethanol concentration used was 70-80%, which seems a standardized condition. Approximately 15% of these papers did not mention the ethanol concentration they used, suggesting that ethanol concentration was not considered important. And none of these publications include an optimization of the ethanol concentration. Some questions naturally arise: Will a fixed ethanol concentration (e.g., 70-80%) completely precipitate all polysaccharides in every type of natural product? Will varied ethanol concentrations extract different polysaccharides from the same sample? Will different polysaccharides within similar molecular sizes share the same optimal ethanol concentration? Should ethanol concentration be optimized for each natural product?

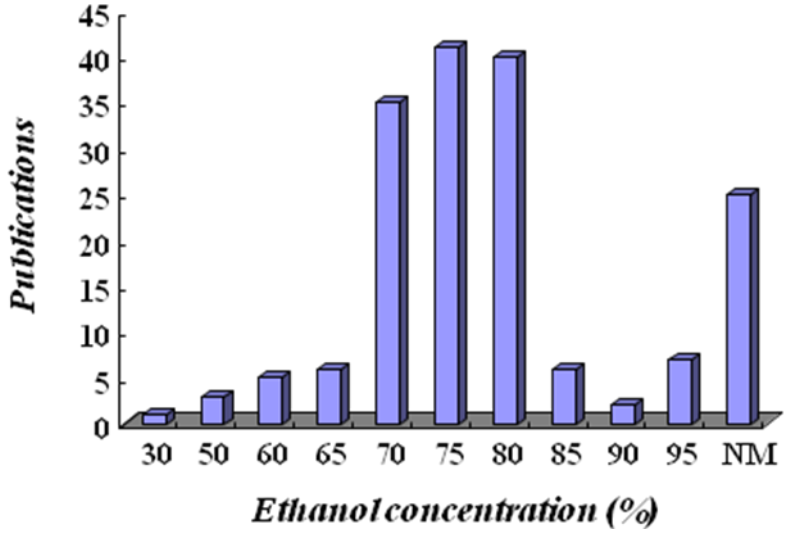


Fig. 1 The statistical results of the ethanol concentration used for polysaccharide precipitation from natural products in the published paper in *Science Direct* Database since Jan, 2013 (data was processed on May 30, 2013) (NM: not mentioned)

In order to answer these questions, in the current study we first used two series of reference glucans, branched dextrans and unbranched pullulans, to qualitatively and quantitatively evaluate the effect of ethanol concentration on the precipitation of polysaccharide by HPGPC (high performance gel permeation chromatography). Multiple parameters that could affect the ethanol precipitation results, such as structural features, molecular size, and ethanol concentration, were systematically investigated. Eight commonly-used polysaccharide-rich herbal/fungi materials were then used as natural samples to determine if and how variation in ethanol concentration affected polysaccharide precipitation.

2. Experimental

2.1 Materials and chemicals

Eight commonly used medicinal herbs/fungi, namely *Angelica sinensis*, *Codonopsis pilosula*, *Dendrobium officinale*, *Ligusticum wallichii*, *Panax ginseng*, *Panax notoginseng*, *Ganoderma lucidum* and *Ganoderma sinensis*, were selected as representative polysaccharide-rich natural samples. The herbal/fungi materials were purchased from herb markets in mainland China and were authenticated by Dr. Chen Hubiao. The voucher specimens were deposited at School of Chinese Medicine, Hong Kong Baptist University, Hong Kong, China.

Deionized water was prepared by Millipore Milli Q-Plus system (Millipore, Bedford, MA, USA) and ethanol was purchased from RCI Labscan Ltd. (Bangkok,

Thailand). The reference glucan substances, dextrans and pullulans (Figure 2) with known molecular sizes (1-270 kDa for dextrans, 6-805 kDa for pullulans), and glucose were bought from Sigma (St. Louis, MO, USA).

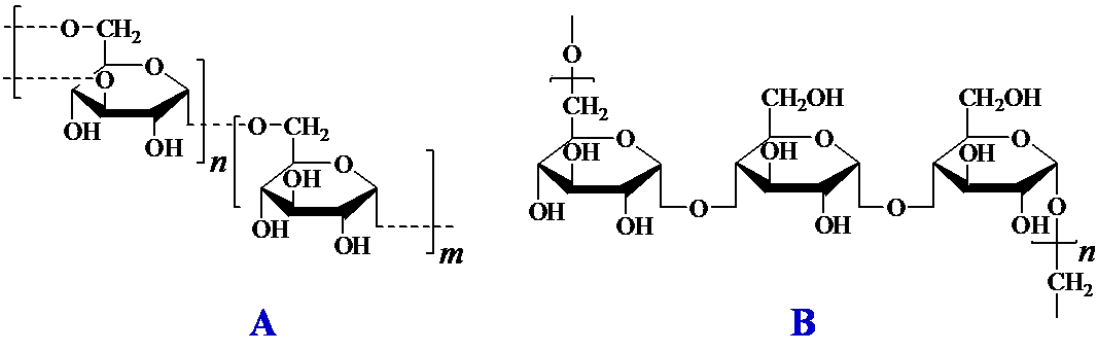


Fig. 2 Chemical structures of reference polysaccharides, branched dextrans (A) and unbranched pullulans (B).

2.2 Preparation of water extracts

Herbal material was dried and powdered. For each sample, 10 g of powder was first ultrasonically extracted with 100 mL of acetone for 1 h to remove liposoluble substances, and then reflux-extracted with water at 100 °C (100 mL) for one hour, twice. The decoctions were combined and centrifuged at 3500 rpm for 10 min. The total sugar content in the solution, calculated as glucose, was adjusted to about 2.0 mg/mL for further analysis [15].

2.3 Ethanol precipitation

Aqueous stock solutions of dextrans and pullulans with different molecular weights (2 mg/mL, 5 mL) were precipitated by adding ethanol to make a final concentration of 10-90% (v/v), respectively, and left overnight (12 h) at 4 °C. After

centrifugation (3500 rpm) for 10 min, the precipitate was collected, washed with ethanol, dried (water bath, 70 °C) to remove any residual ethanol, and then was completely re-dissolved in 5 mL hot water (60 °C) by drastic mechanical vibration for 2 hours. Finally, each solution was filtered through a 0.22 μm syringe filter (Agilent Technologies, USA) for HPGPC analysis [16]. Solutions of the herbal samples were prepared using the same method.

2.4 HPGPC analysis

HPGPC analyses were performed on an Agilent 1100 series (Agilent Technologies, Palo Alto, CA) equipped with DAD and ELSD and two tandem TSK $GMPW_{XL}$ columns (300 mm×7.8 mm i.d., 10 μm) at 40 ℃. Ammonium acetate aqueous solution (20 mM) was used as mobile phase at a flow rate of 0.6 mL/min. DAD was set at 260nm and 280 nm. The parameters of ELSD were set as: the drift tube temperature was 120 °C, and nebulizer nitrogen gas flow rate was at 3.2 L/min, impact-off mode. An aliquot of 20 μL solution was injected for analysis. Because polysaccharides have no UV absorption, UV detector was set at 260 nm and 280 nm in order to monitor the existence of nucleic acid and/or peptide in this study.

Aqueous stock solutions of dextrans and pullulans with different molecular weights were diluted to appropriate concentrations for the construction of calibration curves. At least five concentrations of each solution were analyzed in duplicate, and

then the calibration curves were constructed by plotting the logarithm of the peak area versus concentration of each analyte.

3. Results and discussion

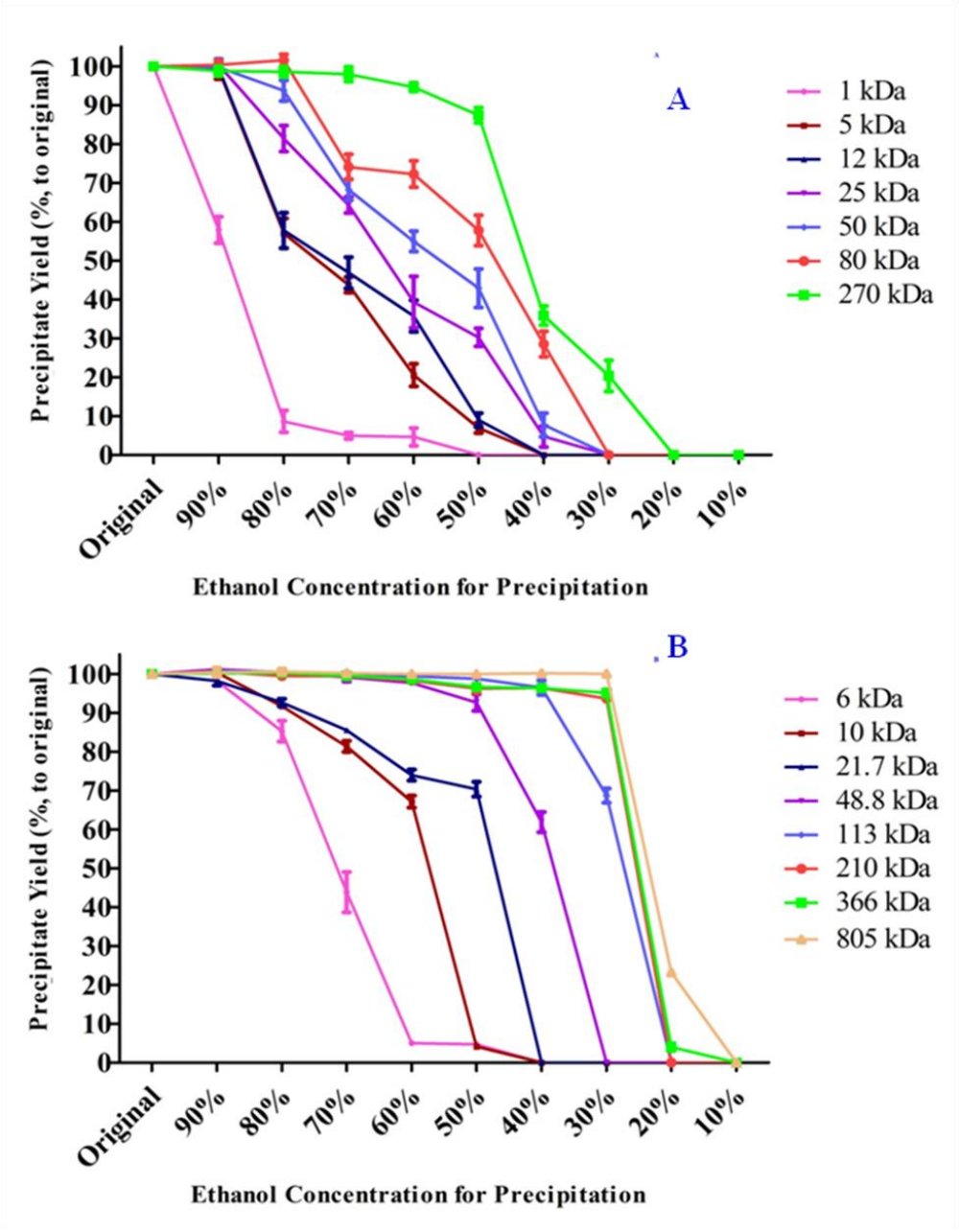
3.1 Impact of ethanol concentration, molecular size and structural features

The reference standards dextrans and pullulans were precipitated at different concentrations of ethanol (10-90%). The obtained precipitates were quantitatively determined using the established HPGPC calibration curves (Table 1). The recovered yields of these glucans are shown in Figure 3, and their individual HPGPC chromatogram can be found in Supplementary Figures 1 and 2.

Table 1 Calibration curves of the HPGPC quantitative assay of dextrans and pullulans.

	Mw (kDa)	Range (mg/mL)	Equation	R^2
Dextrans	1	0.13~4.23	y=1.8387x+3.8191 [a]	0.9981
	5	0.23~3.68	y=2.0166x+3.7503	0.9975
	12	0.22~3.59	y=1.9444x+3.7895	0.9997
	25	0.11~3.51	y=1.749x+3.7965	0.9971
	50	0.12~3.72	y=1.8751x+3.7307	0.9989
	80	0.21~3.30	y=1.9263x+3.6545	0.9991
	270	0.23~3.67	y=1.9301x+3.671	0.9973
Pullulans	6	0.12~3.69	y=1.812x+3.8354	0.9991
	10	0.21~3.41	y=1.7843x+3.8979	0.9994
	21.7	0.15~4.89	y=1.797x+3.7683	0.9987
	48.8	0.11~3.48	y=1.8902x+3.8747	0.9974
	113	0.10~3.32	y=1.8518x+3.7968	0.9963
	210	0.13~4.14	y=1.81x+3.7918	0.9955
	366	0.12~3.84	y=1.7973x+3.8851	0.9955
	805	0.11~3.66	y=1.8666x+3.7908	0.9967

[a] X and Y means the logarithms of corresponding saccharide concentration and HPGPC peak area.



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165 Fig. 3 Effects of ethanol concentration (10-90%) on the precipitation of dextrans (A)

166 and pullulans (B) from aqueous solution (n=3)

167

168 As demonstrated in Figure 3, both series of glucan standards exhibit a consistent

169 trend in that the precipitate yield steadily increases as ethanol concentration increases,

170 as investigated, from 10% to 90%. This is consistent with the findings in the

171 published report [14].

More importantly, results suggest that molecular size affects yield: those with larger molecular size could be easily precipitated at a lower ethanol concentration (Figure 3). For example, when ethanol concentration is set at 50%, the precipitate yield reaches 90% for 270 kDa of dextran, while it decreases to zero for 1 kDa. At the most commonly used ethanol concentration of 80%, precipitate yield from dextrans increased from 10% to 100% as molecular size increased from 1 kDa to 270 kDa. Among them, dextrans of both 5.0 kDa and 1.2 kDa generated close yields around 60%. In other words, not all polysaccharides could be completely precipitated in 80% ethanol.

Another factor apparently affecting precipitate yield is the physical structure of polysaccharides. Of these two kinds of reference polysaccharides, which have the same sugar composition and similar molecular size, one is branched and the other is unbranched (Figure 2), and they exhibited distinctively different ethanol precipitation behaviors. Pullulan, the branched glucan, seems to be precipitated more easily. As demonstrated in Figure 3, in 70% ethanol, pullulan with a molecular size of 48.8 kDa was completely precipitated, while dextran with a similar molecular size of 50 kDa was only 70% extracted. Moreover, in 90% ethanol, pullulan of 6 kDa was fully precipitated, while dextran of 5 kDa was only 50% extracted. If we want to ensure a high yield above 90%, the minimum ethanol concentration needs to be set at 50% for pullulan with molecular sizes of 48.8 kDa and above, while for dextran with similar molecular size, it needs to be 80%.

In summary, our results indicate that molecular size and structure influence polysaccharide precipitation in ethanol. Different concentrations of ethanol precipitate different polysaccharides to greater and lesser extents. Thus, for maximum yield of any given polysaccharide, the ethanol concentration must be individually optimized.

3.2 Further tests on natural samples

Our tentative conclusion was confirmed in further tests on natural materials that are rich in polysaccharides. First, high diversity in the molecule distribution pattern of these investigated natural materials was revealed by HPGPC-ELSD-UV analysis. As shown in Figure 4 (the originals), small molecules below 1 kDa are dominant in *Angelica sinensis* and *Panax ginseng*; molecules in the range of 1-22 kDa are in the majority in both *Ganoderma* species, yet in the minority in *Dendrobium officinale*; macromolecules beyond 22 kDa were dominant in *Dendrobium officinale*, but hardly found in *Ganoderma* samples. Any influence from nucleic acids or peptides was excluded because the major ELSD peaks had no obvious UV absorbance under the investigated conditions (data not shown).

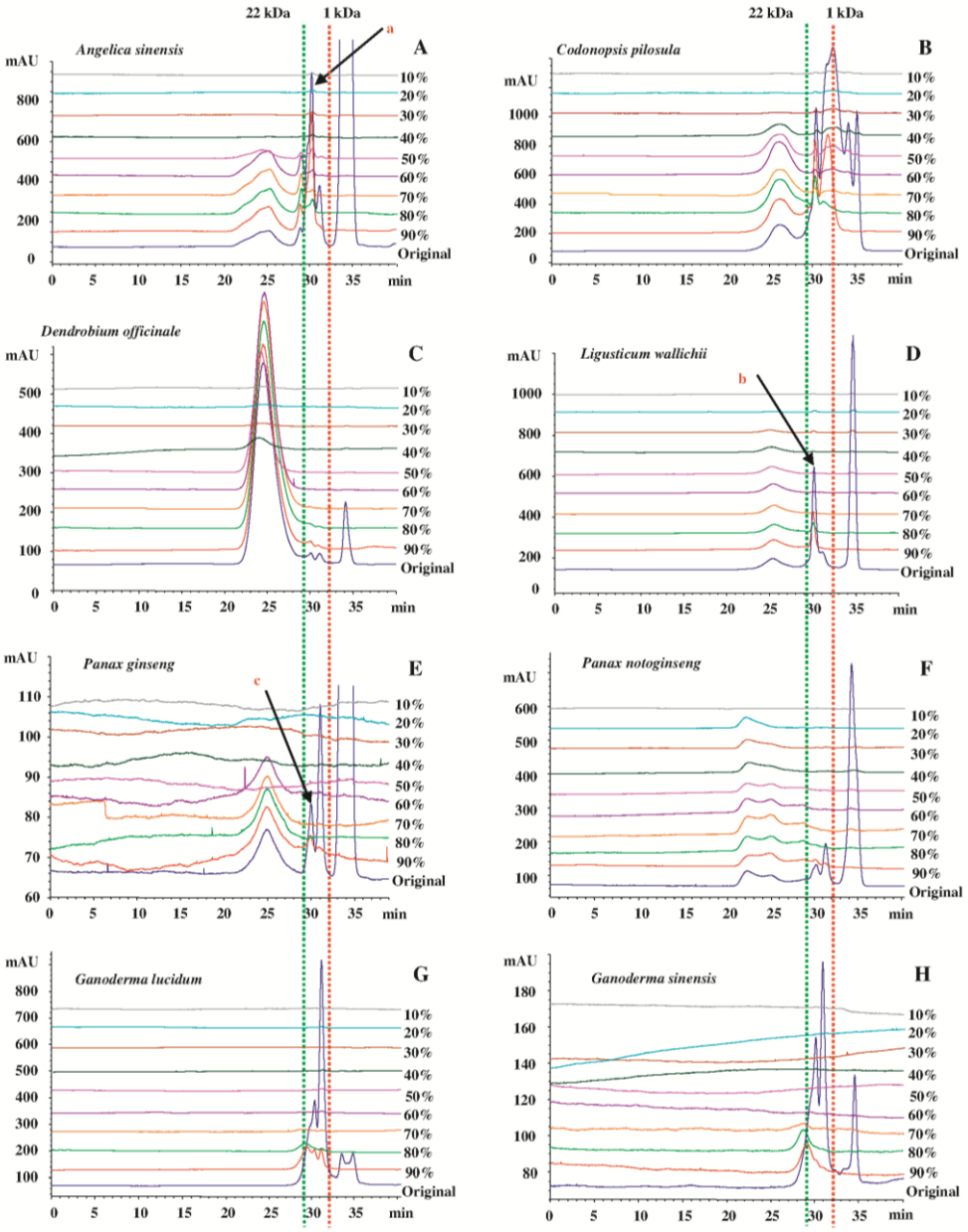

Fig. 4 HPGPC chromatograms of water extracts of investigated herbal materials before (original) and after (10-90%) ethanol precipitation. A. *Angelica sinensis*, B. *Codonopsis pilosula*, C. *Dendrobium officinale*, D. *Ligusticum wallichii*, E. *Panax ginseng*, F. *Panax notoginseng*, G. *Ganoderma lucidum* and H. *Ganoderma sinensis*

These natural polysaccharides responded significantly differently to variations in ethanol concentration. For instance, as shown in Figure 4A, peak a in *Angelica*

sinensis was mostly released into its 90% ethanol precipitate; while for peak b in *Ligusticum wallichii* and peak c in *Panax ginseng*, both possessing molecular size similar to peak a (based on the identical retention time), only around half of them were obtained after 90% ethanol precipitation (Figure 4D and 4E). More significantly, the critical ethanol concentration for macromolecules beyond 22 kDa varied greatly in different cases: it was 60% for *Angelica sinensis* (Figure 4A) and *Panax ginseng* (Figure 4E), 40% for *Codonopsis pilosula* (Figure 4B), 50% for *Dendrobium officinale* (Figure 4C), 30% for *Ligusticum wallichii* (Figure 4D), and 20% for *Panax notoginseng* (Figure 4F).

3.3 Impact of other factors

In this study, other factors like precipitation (a) time, (b) temperature, and the (c) original sample concentration were also evaluated for their impact on ethanol precipitation of polysaccharides. The results indicated that they are not decisive factors. (a) It was found that the solubility of polysaccharides in ethanol is so poor that the precipitation of polysaccharides is always quickly finished within minutes. Hence, time is not a factor. (b) Polysaccharides exhibit relatively stable solubility between 4°C and room temperature (25°C). And the precipitation is usually performed at around 4 °C in a laboratory refrigerator. Hence, temperature in the average laboratory is not a factor. (c) As for the original sample concentration, no significant variation in the precipitate yield was observed during a wide range of concentrations (2-40 mg/ml, Supplementary Figure 3). While, theoretically, too little precipitate for

detection might be produced if the concentration is too low, in practice this is seldom a cause for concern.

4. Concluding remarks

In conclusion, our work demonstrates that chemical diversity in polysaccharides including different structural features and varied molecular sizes is the decisive factor of ethanol precipitation of polysaccharides. Polysaccharides with different structural features and molecular sizes will precipitate to different degrees in different concentrations of ethanol. This has significant implications for researchers. Currently, an overwhelming majority of researchers use 70-80% ethanol for precipitation. This might be appropriate for comparison work, however, in cases where the goal is different, e.g. to extract as much or as many polysaccharides as possible from a given sample, it is strongly recommended that the ethanol concentration used in precipitation of natural polysaccharides should be individually optimized in advance.

Acknowledgements

This study was funded by Hong Kong Baptist University (FRG2/11-12/048, FRG1/12-13/018, FRG2/12-13/006, and RC-start up grant).

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