Influence of Extraction Solvent and Temperature on the Quantitative Determination of Oligosaccharides from Plant Materials by High-Performance Liquid Chromatography

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The effect of extraction solvents and temperatures on extraction yields of monosaccharides, sucrose, and raffinose oligosaccharides from plant materials was investigated. Toasted soybean meal, cotton seed meal, field peas, and a feed mixture were extracted in either water, 50% (v/v), or 80% (v/v) aqueous methanol or ethanol at 20 or 50 °C or at the boiling point of the solvent. Extraction in 80% (v/v) alcohol was strongly influenced by the extraction temperature and maximum extraction was only achieved at the boiling point. Extraction in water and 50% (v/v) methanol or ethanol was less heat sensitive and gave comparable results. Aqueous ethanol (50%, v/v) was as effective as 50% (v/v) methanol, whereas lower yields were seen at higher alcohol strength. There was no consistent difference in the extraction yield when comparing reflux with constant stirring and water bath with occasional mixing for any of the extraction solvents used.

Keywords: *Oligosaccharides; α-galactosides; HPLC; extraction solvent; extraction temperature*

INTRODUCTION

Seeds of legumes, mallow, composite, and mustard are rich in α-galactosides of sucrose (raffinose, stachyose, verbascose, and ajugose)—often termed as raffinose oligosaccharides. The concentrations and compositions of raffinose oligosaccharides differ among plant species and varieties and have different potency for creating flatulence (Saini and Gladstones, 1986). This naturally leads to an interest in detection and quantification of the individual raffinose oligosaccharides in food- and feedstuffs. A number of analytical procedures—mostly HPLC or GLC—are described in the literature. Much emphasis has been put on the separation and detection principles, whereas only a few studies deal with the influence of extraction media, temperature, and time.

Water is the optimal extraction solvent for the low molecular weight (LMW) sugars. Unfortunately it is also an excellent solvent for interfering hydrophilic components such as polysaccharides, proteins, etc. Precipitation of noncarbohydrate components prior to HPLC was common in previous methods to prevent fouling of the column and interference with the analytes, especially when aqueous acetonitrile is used as eluent. Furthermore, α-amylases (EC 3.2.1.1) and α-galactosidases (EC 3.2.1.22) present in the plant material may degrade starch and the raffinose oligosaccharides if not inactivated during or prior to extraction. Extraction in aqueous alcohols minimizes these problems, but alcohol strength, extraction temperature, and method vary considerably among the methods described. Eighty percent ethanol or methanol (v/v) is most commonly used, but there are indications that these solvents in some cases lead to incomplete extraction. Increasing the alcohol strength in the range of 50−90% (v/v) has previously been shown to strongly reduce the amount of raffinose oligosaccharides extracted from plant material (Shukla, 1987; Cegla and Bell, 1977; Bach Knudsen and Li, 1991). Furthermore, marginally higher extraction yields have been noted with methanol compared to ethanol (Shukla, 1987). This is in contrast to other studies showing no difference between 80% methanol and water in extraction values of LMW sugars except for products with a high maltose content (Li and Schuhmann, 1980; Li et al., 1985). Concordant, Knudsen (1986) found that water extraction at 60 °C and boiling in aqueous ethanol (80%, v/v) gave comparable results. The discrepancies in the literature are in part due to the extraction conditions used and the material analyzed, as the extractability of oligosaccharides may vary between products. A routine method for the analysis of raffinose oligosaccharides must be a compromise between optimal extraction of a range of different products, safety, and convenience. The objective of the present study was therefore to compare the extractability of oligosaccharides in water and two concentrations of aqueous methanol and ethanol at different temperatures. Furthermore we compared two extraction methods (reflux with constant stirring and water bath with occasional mixing) to see how this affected the extractability.

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EXPERIMENTAL PROCEDURES

Samples and Chemicals. Toasted soybean meal (SBM), cotton seed meal (CSM), field peas, and a feed mixture consisting of toasted soybean meal (20%), field peas (24%), rape seed cake (12%), barley (17.1%), wheat (17.2%), animal fat (5%), and a vitamin−mineral mixture (4.7%) were used for the study. The samples were milled to pass a 0.5 mm screen. Pure standards of L-arabinose, D-glucose, D-fructose, and raffinose were obtained from Merck (Darmstadt, Germany), sucrose and stachyose were from Sigma (St. Louis, MO), and verbascose was from Megazyme (Warriewood, NSW, Australia). Other reagents were of analytical grade.

Extraction. Triplicate samples (500 mg) were extracted with 10 mL of extraction solvent containing 1 $mg \cdot mL^{-1}$ arabinose as internal standard. The extraction media were deionized water or aqueous alcohols: 50% or 80% (v/v) methanol or ethanol. The extraction in water took place with or without pretreatment of samples with aqueous ethanol as

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follows: The sample was suspended in 2 mL of 99% (v/v) ethanol and heated in a boiling water bath for 5 min. The sample was left standing in the fume cupboard until all ethanol had evaporated. The samples were reflux-extracted for 60 min with constant stirring at either 20 or 50 °C or at the boiling point of the extraction solvent. Boiling point was determined to be 78.5 °C (SD 0.56) for 50% methanol, 73.8 °C (SD 1.17) for 80% methanol, and 83.2 °C (SD 0.25) and 81.7 °C (SD 0.82) for 50% and 80% ethanol, respectively.

In a following experiment we compared reflux extraction at 50 °C with constant stirring (as described above) to extraction in a 50 °C water bath with occasional mixing using the same samples and extraction solvents as previously. This was done to see whether the latter more convenient procedure would be acceptable for routine analysis of oligosaccharides.

Sample Cleanup. An aliquot of 5−7 mL of supernatant was filtered through a Sep-Pak C_{18} cartridge, which was prewetted with 1 volume of methanol, 2 volumes of deionized water, and 1 volume of the extraction solvent. The filtration took place under vacuum using a Vac Elut SPS 24 instrument (Analytichem International, Harbor City, CA). An aliquot of the filtrate (1.5 mL) was evaporated to dryness at 50 °C in a HBI vortex-evaporator (HaakeBuchler Instruments Inc., Saddl Brook, NJ) fitted with a Savant VP 100 refrigerated vapor trap (Savant Instruments Inc., Farmingdale, NY) and finally redissolved in 1.5 mL of deionized water.

Separation and Quantification by HPLC. The sugars were separated on a Shodex Ionpak KS-801 resin-based column in the sodium form (Waters, Milford, MA) with deionized water as eluent (0.6 mL·min^{-1}) using a Waters LC Module I integrated injector, solvent pump, and autosampler system. The detector was a Waters 410 differential refractometer (Waters, Milford, MA); 20 μL of the water extract was injected. The column was kept at 85 °C and the temperature in the RI detector was set at 45 °C, the sensitivity at 32, and the time constant at 1 s. Data were collected and processed on a Baseline 825 chromatography workstation (Waters, Milford, MA).

Calculation and Statistical Analysis. The concentration of sugars was calculated from the peak height of detector response as:

$$\text{sugars, \% of dry matter} = \frac{H_s R_s W_{Is}}{H_{Is} R_s W_s} \times 100 \qquad (1)$$

H_s and H_{Is} are peak heights, and W_s and W_{Is} are dry weights of sample and internal standard (Is), respectively. R_s and R_{Is} are response factors (amount/height) for sugars and internal standard in a solution containing a known amount of each component.

For each sample, data were analyzed using a two-way analysis of variance model (Snedecor and Cochran, 1973):

$$X_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \epsilon_{ijk} \qquad (2)$$

where X_{ijk} is a dependent variable (content of sugar), μ the overall mean, α_i the effect of extraction medium, β_j the effect of temperature or extraction procedure, and $\epsilon_{i,j,k}$ a normal distributed measurement error, $N(0,\delta^2)$.

RESULTS AND DISCUSSION

Chromatographic Conditions. The detector response of raffinose and stachyose was linear in the range 0.05−9.0 mg/mL extract corresponding to an injected amount of 1−180 μg. The correlation coefficients (r) of detector response vs concentration were 0.9991 and 0.9940 of raffinose and stachyose, respectively, both when calibrated on basis of height and area. In the range tested (0.06−6.0 mg/mL), verbascose gave a linear response (r = 0.999 97). For the monosaccharides and sucrose correlation coefficients of 0.9999 were obtained when calibrating on both height and area in the range 0.05−9.0 mg/mL.

Table 1. Mean Values (g × 100 g^{-1} of Dry Matter), Standard Error, and Coefficient of Variation (CV) for Determination of Monosaccharides, Sucrose, and Raffinose Oligosaccharides in the Feedstuff Samples Extracted in Various Solvents at Temperatures 20 and 50 °C and Boiling Point

component	mean	SE	CV, %
fructose	0.15	0.032	20.9
glucose	0.13	0.022	16.5
sucrose	3.53	0.089	2.5
raffinose	1.66	0.032	1.9
stachyose	2.45	0.055	2.2
verbascose[a]	1.09	0.032	2.9
total	8.46	0.179	2.1

[a] Only pea and the feed mixure are included.

Using Na_2SO_4 buffer as eluent, Bach Knudsen and Li (1991) found a better separation between verbascose and stachyose on a Ca-loaded resin column, whereas better separation between stachyose and raffinose was found on a Na-based resin column. The column we used had the advantage over the columns used by Bach Knudsen and Li (1991) in the ability to use water as eluent instead of buffer without rapid deterioration of the column. Although complete base-line separation between raffinose, stachyose, and verbascose was not achieved, there was no evidence of a poorer determination of one raffinose oligosaccharide than the other. The coefficients of variation (CV) of all sugars but the monosaccharides were constant, suggesting a higher SE with larger amounts in the sample (Table 1). The high CV of the monosaccharides was due to a very low content in the samples, as the SE was in the same order as those for the other sugars.

Choice of Extraction Solvent and Temperature. For all sugars in the four samples, there was a statistically strong interaction between the extraction solvent and temperature ($p < 0.001$). This suggests that the solvent and temperature had divergent influence on the amount of oligosaccharides extracted in the different extraction solvents. The influence was also dependent on the sample analyzed. These results stress the importance of analyzing a range of materials, when a general method of analysis is proposed. The amount of LMW sugars obtained from CSM (Figure 1) was consistently higher with increasing temperature in both water and aqueous alcohol (50% and 80%). Also SBM (Figure 2) had the tendency for higher extraction yields with increasing temperature, except for water extraction of inactivated material, which gave lower values at boiling point. Peas (Figure 3) and the feed mixture (Figure 4) were hardly affected by temperature when extractions were performed in water or 50% ethanol. Extraction in 80% alcohol was strongly influenced by the temperature for all samples, whereas extraction in water or 50% alcohol was less heat sensitive.

Extraction of LMW sugars from SBM, peas, and the feed mixture at 20 and 50 °C led to the same recovery as at boiling point (92−114%) when water or 50% alcohol was used. An exception was a slightly lower recovery (87%) from peas in 50% ethanol at 50 °C. Extraction of CSM in water at 50 °C gave almost similar recovery (94−98%) to that of boiling, but reducing the temperature to 20 °C or using 50% alcohol reduced the recovery to 83−86% as compared to the amount extracted at boiling point in the respective solvents. Reducing the extraction temperature had a dramatic effect on the extraction yield, when 80% alcohol was used. Extraction of the feed mixture in 80% methanol at 50 °C led to a recovery of LMW of 95% compared to boiling point, whereas the proteinous feedstuffs gave

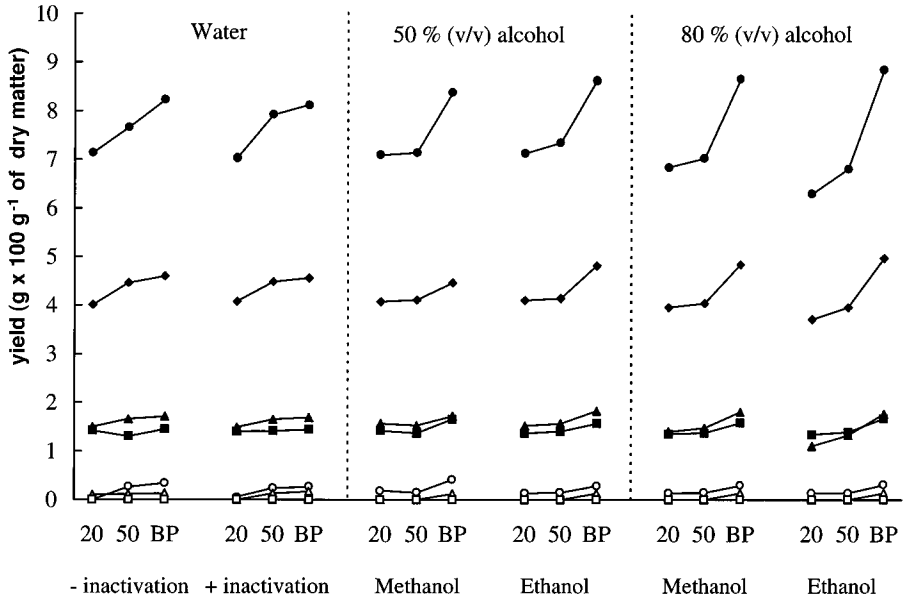

Figure 1. Effect of extraction solvent and temperature on amount of oligosaccharides in CSM: (○) fructose, (△) glucose, (■) sucrose, (◆) raffinose, (▲) stachyose, (□) verbascose, and (●) sum of LMW sugars.

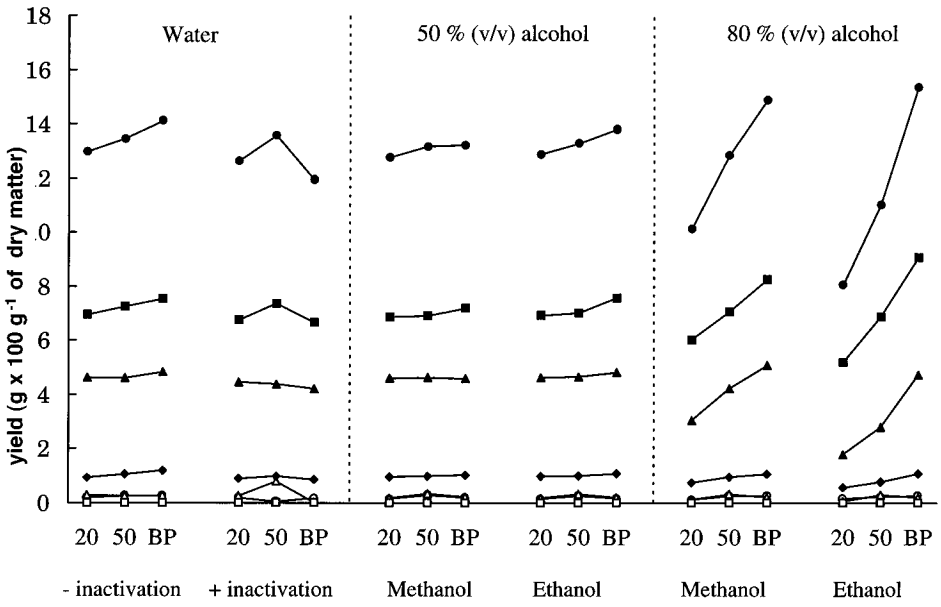

Figure 2. Effect of extraction solvent and temperature on amount of oligosaccharides in SBM: (○) fructose, (△) glucose, (■) sucrose, (◆) raffinose, (▲) stachyose, (□) verbascose, and (●) sum of LMW sugars.

recoveries of 81−87%. Corresponding values for extraction in 80% ethanol were 89% for the feed mixure and 71−76% for the other materials. Reducing the extraction temperature to 20 °C led to an even poorer recovery. Especially for peas, there was a drastic reduction compared to the yield at boiling point, with 46% and 55% recovery in 80% ethanol and 80% methanol, respectively.

Ethanol gave slightly lower yields than methanol at 20 and 50 °C at the high alcohol strength (80%), whereas identical amounts were obtained at boiling point. This supports previous findings of higher extraction yields in methanol compared to ethanol with enlarged difference at higher alcohol strength (Shukla, 1987). Soybean meal gave higher yields in 80% alcohol at boiling point compared to any other extraction medium or temperature, which was mainly due to a higher extraction of sucrose. Extraction at 20 and 50 °C, on the other hand, gave very low yields of sucrose, stachyose, and raffinose. As previously noted by Bach Knudsen and Li (1991), this may be a result of complex formation of sucrose to other components, e.g., stachyose (Conkerton et al., 1983), which render the individual sugars less extractable.

The observed differences between extraction media are in agreement with those of Bach Knudsen and Li (1991). They found incomplete extraction of sucrose and the raffinose oligosaccharides from protein rich feedstuffs using 80% ethanol or methanol at ambient temperature, whereas 50% alcohol gave results comparable to water (Bach Knudsen and Li, 1991). Consistently, Shukla (1987) showed that the amount of stachyose extracted from soybean declined with increasing alcohol strength from 50% to 90% (v/v) at ambient temperature. An increase in alcohol strength from 0% to 50% (ethanol or methanol) did not affect extraction yields. Sucrose was severely affected only when the concentration of ethanol was raised from 85% to 90%. If 80% alcohol is used for extraction of oligosaccharides

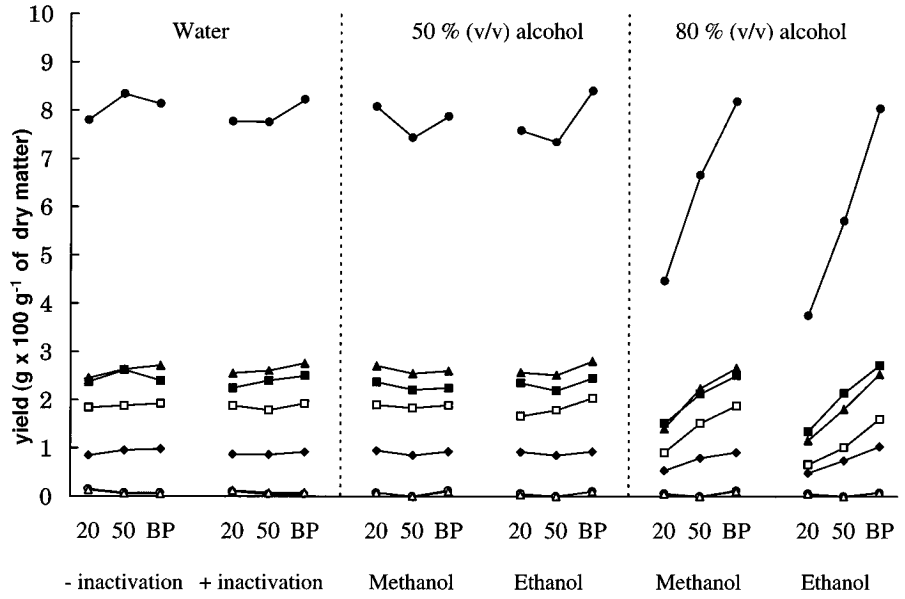

Figure 3. Effect of extraction solvent and temperature on amount of oligosaccharides in peas: (○) fructose, (△) glucose, (■) sucrose, (◆) raffinose, (▲) stachyose, (□) verbascose, and (●) sum of LMW sugars.

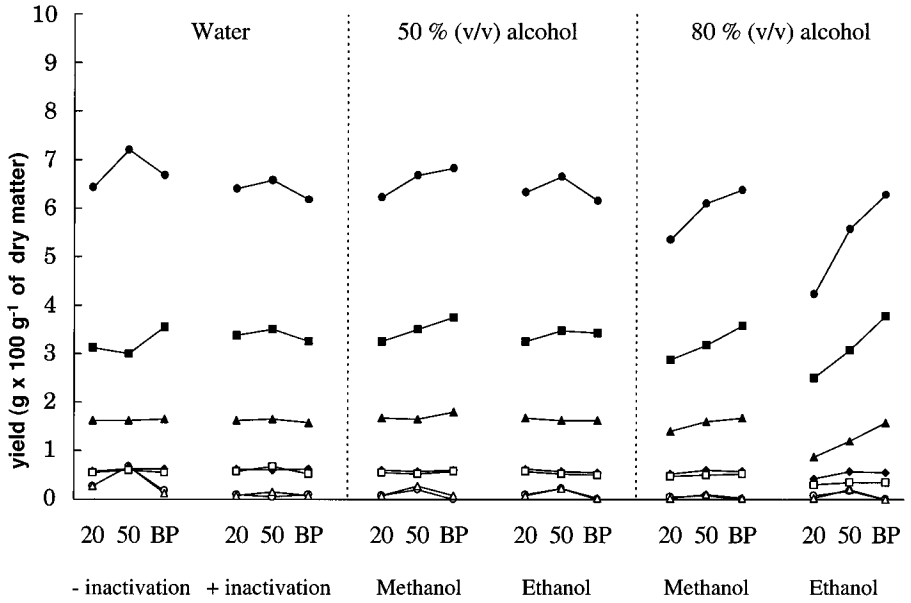

Figure 4. Effect of extraction solvent and temperature on amount of oligosaccharides in feed mixture: (○) fructose, (△) glucose, (■) sucrose, (◆) raffinose, (▲) stachyose, (□) verbascose, and (●) sum of LMW sugars.

as common practice, it is important to extract at boiling point in order to obtain maximum yield.

Water is the solvent of choice for extraction of the oligosaccharides. It bears, however, the risk of degradation of oligosaccharides and starches by the action of enzymes during the extraction. In our material there was only a weak indication of such degradation in the feed mixture with elevated levels of fructose and glucose at 50 °C along with a reduced level of sucrose. However, there might be other types of plant materials in which the enzymatic activity is higher. In that case, water extraction is not recommended, unless deactivation is preceding the extraction. This will, however, add another step to the procedure. Instead we recommend the use of 50% alcohol at elevated temperature (50−65 °C). Extraction in 50% alcohol prevents enzymatic degradation and provides complete extraction in most samples.

Mode of Extraction. The relation between time, temperature, and mode of extraction must be acknowledged. Saini (1988) compared four previously described methods using leguminous seeds as material. Boiling of undefatted samples for 5 min in 70% ethanol, extraction of defatted samples in 70% ethanol at 65 °C for 30 min and twice extraction of defatted samples for 1 h in water at 30 °C gave comparable results. Reflux extraction at 92 °C in 40% (v/v) methanol for 2 h, on the other hand, led to incomplete extraction of the α-galactosides (Saini 1988). Shukla (1987) found no difference in the extraction yield of oligosaccharides from soybean concentrate with mechanical mincing in ultraturrax for 2 min, 1 h shaking at room temperature, or 10 min shaking at 80 °C in 50% methanol. In contrast, Knudsen (1986) found that extraction of legumes in a shaking water bath at ambient temperature for 1 h led to incomplete extraction. Raising the temperature to 60 °C was as effective as boiling in 80% ethanol for 1 h. However, due to the risk of enzymatic degradation of starch and oligosaccharides, an initial inactivation (boiling) prior to extraction was suggested. In the present study we used the reflux setup to provide equal conditions when comparing the different solvents and

Table 2. Mean Values (g × 100 g^{-1} of Dry Matter) of Monosaccharides, Sucrose, and Raffinose Oligosaccharides in the Feedstuff Samples Extracted in Various Solvents at 50 °C in Water Bath or Reflux

		fructose	glucose	sucrose	raffinose	stachyose	verbascose	total
water − inactivation	water bath	0.3	0.2	3.4	1.7	2.6	0.5	8.7
	reflux	0.3	0.3	3.5	1.7	2.6	0.6	9.0
water + inactivation	water bath	0.2	0.1	3.5	1.7	2.6	0.6	8.7
	reflux	0.1	0.3	3.6	1.7	2.6	0.6	8.9
methanol, 50% (v/v)	water bath	0.2	0.2	3.7	1.7	2.7	0.6	9.0
	reflux	0.2	0.2	3.5	1.6	2.6	0.6	8.6
ethanol, 80% (v/v)	water bath	0.2	0.2	3.7	1.7	2.7	0.6	9.0
	reflux	0.2	0.1	3.5	1.6	2.6	0.6	8.7
methanol, 80% (v/v)	water bath	0.2	0.1	3.5	1.7	2.2	0.4	8.1
	reflux	0.1	0.1	3.4	1.6	2.4	0.5	8.1
ethanol, 80% (v/v)	water bath	0.2	0.1	3.1	1.4	1.3	0.2	6.4
	reflux	0.1	0.1	3.4	1.5	1.8	0.3	7.3
overall mean	water bath	0.2	0.2	3.5	1.7	2.3	0.5	8.3
	reflux	0.2	0.2	3.5	1.6	2.4	0.5	8.4

extraction temperatures. Extraction at boiling point could otherwise only be performed with great caution (caps not screwed tight) due to volume expansion. This may, however, cause the alcohol to evaporate. At temperatures below boiling point, extraction in water bath is safe and more convenient. At 50 °C, we found only marginal and inconsistent differences in the amounts of sugars extracted from the four samples as a result of the different procedures (Table 2). The amount of sugars extracted in 80% alcohol was lower than that in water and 50% alcohol, and especially water bath extraction in 80% ethanol seemed to give incomplete extraction.

Conclusion. The results of the present study show that extraction for 1 h either in water or in 50% (v/v) alcohol (methanol or ethanol) is sufficient for most applications. Boiling provides the maximum yield, but the loss by reducing the temperature to 50 °C is marginal in most cases. Complete extraction is not obtained when using 80% alcohol unless boiling is included in the extraction procedure. Aqueous ethanol (50%, v/v) is as effective as methanol as an extraction medium, whereas lower yields are observed at higher alcohol strength. Since ethanol is less hazardous compared to methanol, it is recommended to use the former at the strength of 50% (v/v). There was no consistent difference in the use of reflux with constant stirring compared to extraction in water bath with occasional mixing. The latter is more convenient for routine analysis of many samples and can be used as a standard procedure.

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