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Short communication

Underestimation of carbohydrates by sugar alcohols in classical anthrone-based colorimetric techniques compromises insect metabolic and energetic studies

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Short title
Biased carbohydrates estimates in colorimetric assays due to sugar alcohols

Abstract
Physiologically based metabolic studies usually search for easy, sensitive, and cheap techniques to rapidly estimate biological parameters such as nutrient content. Colorimetric methods to estimate carbohydrates have been extensively used (over 120,000 references). However, sugar alcohols are underestimated under conventionally used analytical conditions, in particular if using the popular van Handel method. This may lead to misinterpretations of sugar implications in biological systems. We determined the anthrone reaction with various sugar alcohols and non-alcohols individually under standard conditions (Van Handel 1985). We then manipulated the proportion of either sugar alcohols or non-alcohols in three different sugar mixtures in order to estimate the impact on the total sugar estimation. In the case of a mixture with over 50% of sugar alcohols, total sugars are underestimated by 50% when using glucose as standard.

Key words: nutritional composition of insects, colorimetric assays, anthrone reagent, carbohydrate estimation, sugar alcohols.

Conflict of interest
The authors declare that there is no conflict of interest.

Author contribution
MB designed the overall study, carried out the experiment, and analysed the data, with help from JPC. MB, JC, and DG wrote the manuscript.

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51 **1. Introduction**
52
53 The need for appropriate quantification of metabolites and energy content of insects is
54 recently growing vigorously due to the emerging field of Insects as Food and Feed (Van Huis
55 and Tomberlin 2017). Examples range from the need to maximize proteins contents and
56 energy levels. Also, the need to have standardized product of constant composition is a
57 challenge of this new market. Other fields of scientific investigations requiring nutrient and
58 energy budgets are behavioural ecology questions, multitrophic interactions and conservation
59 biology in the prospect of climate change. Because insects can trade one source of energy for
60 another one only (see Casas et al. 2015), complete energy budgets are needed. A
61 comprehensive if complex framework has been recently proposed, based on dynamic energy
62 budgets (DEB, see Llandres et al. 2015). In this context, quantifying carbohydrates is key, as
63 it is both a large source of energy and also because it comes and is stored in different forms.
64
65 Since its introduction for the determination of carbohydrates (Dreywood 1946),
66 anthrone in sulfuric acid has been extensively employed as a convenient and specific reagent
67 for colorimetric estimation of a variety of carbohydrates in different materials such as food,
68 biological fluids, wastewater, pharmaceutical products, plant or animal extracts (Body et al.
69 2013, 2018a in prep; Cui 2005; Foray et al. 2012; Genest and Chapman 1962; Giron et al.
70 2002; Green and Wade 1952; Handelsam and Sass 1956; Le and Stuckey 2016; Louis et al.
71 2014; Prokhovnik and Nelson 1953; Van Handel 1985; Yemm and Willis 1954). Indeed,
72 "anthrone" and "carbohydrates" (or "sugars") appear in more than 120,000 articles in the web
73 of science database. This easy, sensitive, and cheap analytical method allows to produce a
74 green colour in a quantitative manner when sugars are heated with anthrone under acidic
75 conditions (Bailey 1958; Van Handel 1985).
76
77 Usually, glucose is used as a unique standard for carbohydrate estimation, hence the
78 results of these assays are often presented in terms of glucose-equivalent concentrations.
79 However, while the different reactions of anthrone with the various carbohydrates have long
80 been known by chemists (Cui 2005), there has been no systematic investigation of the
81 consequences for the sugar quantification in complex mixtures which may be encountered in
82 biological materials. Indeed, different sugars analysed under the same conditions could lead to
83 different colour intensities. For example, Yemm and Willis (1954) showed that the colour
84 produced by the disaccharide sucrose and its constituent monosaccharides glucose and
85 fructose – which are the most common carbohydrates – varies widely after a 2 min heating
86 time. Indeed, when the fructose absorbance is 100 %, the sucrose absorbance is 60 % and
87 only 20 % for glucose. This difference between the intensity of colour produced decreases
88 when the heating time increases and this difference is nearly zero after 15 min (Yemm and
89 Willis 1954). That is why numerous analytical methods such as the Van Handel's technique
90 (1985) developed to determine sugar content in mosquitoes with anthrone have a heating time
91 of about 15-20 min to minimize the difference of absorbance between different sugars. Over
92 this limit, the colour quickly disappears and the absorption drastically decreases. This requires
93 scientists to choose the better trade-off between inter-sugar differential reaction and loss of
94 coloration.
95
96 Although sugar alcohols such as glycerol, sorbitol and inositol are prevalent in many
97 biological systems (see below), little attention has been paid to the reaction of this class of
98 compounds with the anthrone reagent (Graham 1963). Contrary to other sugars, sugar
99 alcohols require specific conditions to be analysed. Appropriate conditions of acid
100 concentrations, heating time, heating temperature and wavelength detection are thus necessary

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101 (Graham 1963). These optimal conditions for sugar alcohols are 60 min heating time, at 99
102 °C, with 0.15 % anthrone in 100 % sulfuric acid, and detection at 500 nm. For other sugars,
103 15-17 min heating time, at 90-92 °C, in 0.15 % anthrone in 70 % sulfuric acid, and detection
104 at 630 nm are the optimal conditions needed (Graham 1963; Van Handel 1985).
105

106 Due to drastically different experimental conditions for sugar alcohols and other
107 sugars, biological sample analysis is a real problem. Indeed, the comparison between two
108 samples may be difficult if the sugar alcohols concentration varies between treatments. In that
109 case, overall quantities of sugars may be measured similar while they differ between
110 experimental conditions due to changes in sugar alcohol contents. This is more common than
111 might, at first sight, be supposed.

112 As sugars act both as nutrients and signalling molecules for both plants and animals,
113 the carbohydrate content could vary in composition according to numerous factors such as
114 species, age, nutritional status, organ, location, seasonality or time of the day. In plants,
115 sugars are involved in diverse functions like major transporter for photoassimilated carbon
116 (Lemoine 2000), senescence triggering processes (Wingler and Roitsch 2008), anti-freezing
117 properties (Smallwood and Bowles 2002), salt stress response (Pommerrenig et al. 2007), and
118 biotic and abiotic stress response (Wingler and Roitsch 2008). Sugars also have crucial roles
119 in insects as haemolymph component and structural component of exoskeleton chitin (Wyatt
120 1967), phagostimulant (Bernays and Simpson 1982; Glendinning et al. 2000; Hansen 1969;
121 Schoonhoven et al. 2005), control of diapause (Pullin 1992; Pullin and Wolda 1993), heat
122 stress response (Salvucci et al. 2000), or cryoprotectant (Miller and Smith 1975; Wolfe et al.
123 1998). Because sugar alcohols have key roles in insect phagostimulation (Bernays and
124 Simpson 1982; Glendinning et al. 2000; Hansen 1969), diapause (Pullin and Wolda 1993),
125 and thermal tolerance (Miller and Smith 1975; Wolfe et al. 1998), they are expected to vary
126 according to a large range of factors, including changes occurring in plants as herbivores'
127 food source.
128

129 A recent study reported that carbohydrates present in wastewater measured using these
130 colorimetric methods could be under- or over-estimated due to frequent interference with
131 coexisting compounds found in wastewater (Le and Stuckey 2016). Sugars (8 non-alcohols),
132 quantified in glucose-equivalent, were highly variable depending on colorimetric methods
133 used, while the 3 sugar alcohols remained undetected with all methods. However, Le and
134 Stuckey (2016) did not investigate the reason why all sugar alcohols failed to produce any
135 chromogen, nor the impact of variable proportions of those sugars could affect the accuracy
136 of these colorimetric methods.

137 To highlight the possible bias induced, we therefore determined the specific anthrone
138 reaction with various sugar alcohols and non-alcohols individually under standard conditions
139 (Van Handel 1985). We then manipulated the proportion of either sugar alcohols or non-
140 alcohols in three different sugar mixtures in order to estimate the impact on the total sugar
141 estimation.
142

143 **2. Materials and methods**

144

145 *Chemicals* — Sugars (xylitol, sorbitol, myo-inositol, mannitol, glycerol, trehalose,
146 sucrose, lactose, glucose and fructose) and anthrone reagent were purchased from Sigma
147 Aldrich (Sigma Aldrich, France). The anthrone reagent consisted of 1.0 g of anthrone (Sigma
148 Aldrich, France) dissolved in 500 mL of concentrated sulfuric acid added to 200 mL of
149 MilliQ water (Millipore corporation).
150

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151 *Calibration curves* — Colorimetric quantification of carbohydrates with anthrone
152 reagent determines both reducing and non-reducing sugars because of the presence of the
153 strongly oxidizing sulfuric acid. Like the other methods, it is non-stoichiometric and therefore
154 it is necessary to prepare a calibration curve using a series of standards of known
155 concentrations.
156 Stock solution of individual sugars were prepared at a concentration of 1 mg/mL in
157 ethanol 25 %. Three sugar mixtures were also prepared with the same total sugar
158 concentration (1 mg/mL), but using different individual sugar proportions. These artificial
159 sugar mixtures were used compare how different sugar composition impact the accuracy of
160 the quantification. Compositions of the three sugar mixtures were as follow: *solution 1*, 10 %
161 sorbitol, 30 % sucrose, 30 % glucose, 30 % fructose; *solution 2*, 55 % sorbitol, 35 % sucrose,
162 5 % glucose, 5 % fructose; *solution 3*, 55 % sorbitol, 15 % sucrose, 15 % glucose, 15 %
163 fructose.
164
165 *Colorimetric assay protocol* — Calibration curves were carried out for each sugar and
166 sugar mixture following the commonly used protocol in ecology (Foray et al. 2012; Van
167 Handel 1985). Increasing volumes (2, 5, 7.5, 10, 15, 20, 25, 30, 40, and 50 µg/µL) of 1.0
168 mg/mL stock sugar solution were transferred into a borosilicate tube (16 x 100 mm; Fisher
169 Scientific, France) and placed in a water bath at 90 °C to evaporate the solvent down to a few
170 microliters. After adding 1 mL anthrone reagent, the tubes were placed in a water bath at 90
171 °C for 15 min, cooled at 0 °C for 5 min, vortexed and then read in a spectrophotometer
172 (DU®-64 spectrophotometer; Beckman, Villepinte, France) at 630 nm.
173
174 *Concentration calculation* — Sugar concentrations are usually determined using
175 glucose standards of known concentrations. To determine the error of estimation due to the
176 use of a single sugar calibration curve, we estimated sugar concentrations using both generic
177 glucose standard (glucose-equivalent concentrations), and specific sugar standards (real
178 concentrations).
179
180 **3. Results and Discussion**
181
182 The aim of this study was to characterize the anthrone reaction with sugar alcohols and
183 other sugars and then to determine how sugar alcohols affect the colorimetric quantification
184 of total carbohydrates in biological samples. Our results clearly show that calibration curves
185 differ between sugars (Figure 1A). At the same concentration, all sugars do not have the same
186 absorbance. Indeed, reaction between anthrone reagent and sugar alcohols results in lower
187 colour intensity than for other sugars, as well as higher limit of detection (less than 2 µg/µL
188 for non-alcohols vs. 7.5-20 µg/µL for alcohols). This observation suggests that anthrone
189 reaction is affected by carbohydrate structure, and might be the source of some of the
190 problems highlighted by Le and Stuckey (2016). Indeed, it could be due to the steric
191 hindrance of sugar alcohols. The specific conformation of sugar alcohols could disturb the
192 reagent access to some functional groups and so decrease the colorimetric reaction with
193 anthrone.
194
195 As a consequence, we stress that the concentration of sugar alcohols has a high impact
196 on the accuracy of the total sugar content quantification (about 80 % in the case of single
197 sugar, Table 1). Indeed, the sugar alcohols concentration in a mixture profoundly influences
198 the intensity of the developed colour (Figure 1B), and can lead to an underestimation of the
199 sugar pool up to about 50 % in the case of two of our sugar mixtures (Table 1). As the sugar
200 alcohols concentration increased, the optical density of the resulting colour decreased under

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201 standard analytical conditions (Van Handel 1985). So, in presence of sugar alcohols, standard
202 calibration curves usually done with glucose are inappropriate and do not allow an accurate
203 determination of the sugar content in samples. If the sugar alcohol status is modulated
204 according to one or more factors, comparisons between samples might be biased which may
205 impair ecological interpretations.
206 For example, a study comparing sugar composition of the food source for a moth
207 larva, photosynthetically active (green) leaves and senescing (yellow) leaves on apple-tree,
208 reported a strong alteration of sorbitol content from 50 % in green tissue to 32 % in yellow
209 tissue (Body et al. 2013, 2018b submitted). Such comparison, using colorimetric assays to
210 quantify total soluble sugars, (Body et al. 2018a in prep, 2018b submitted) would be impaired
211 by a change in sugar composition involving sugar alcohols. When quantified using glucose
212 standards (glucose-equivalent concentrations), sugar concentrations were of 60.92 ± 20.90
213 µg/mg DW (average ± S.D.; DW, dry weight) on green leaves, and 43.65 ± 13.26 µg/mg DW
214 on yellow. When a sugar mixture of the main sugars (sorbitol, sucrose, glucose, and fructose),
215 in similar proportions as in the samples, was used for the calibration curve, instead of a single
216 (non-alcohol) sugar, concentrations were of 107.20 ± 31.70 µg/mg DW on green leaves, and
217 50.62 ± 16.59 µg/mg DW on yellow leaves (real concentrations). The sugar pool was thus
218 underestimated by 43 % on green leaves, and 14 % on yellow leaves. Such a difference in the
219 estimation of sugar concentrations in green leaves could impact the conclusion drawn from
220 this study, as sugar concentrations dropped only 28 % during senescing process in the first
221 estimation, compared to a drop of 52 % between green and yellow leaves using an appropriate
222 calibration curve. Worst estimates can be expected with increasing proportion of sugar
223 alcohols in the sample. These changes reported in leaves could impact sugar alcohol
224 composition of herbivores feeding on these different tissues.
225
226 As conclusion, we suggest that metabolic studies must be cautious in their
227 interpretation of physiological data collected with such colorimetric techniques. This is
228 particularly true in biological systems or in specific conditions where sugar alcohols are
229 expected to be involved and we described above a wide range of situations where this
230 happens. Alternatively, one might consider alternative techniques that allow a quantification
231 of individual sugars (Body et al. 2013, 2018b submitted; Cui 2005; Ouchemoukh et al. 2010;
232 Rovio et al. 2007) or, at least, a concomitant analysis of subsamples with methods adapted to
233 either sugar alcohols or non-alcohol ones (Graham 1963; Van Handel 1985).
234
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336 **Table captions**
337
338 **Table 1. Estimation error of sugar concentration.** Equations and R^2 coefficients of
339 determination of all the calibration curves presented in Figure 1 are summarized here. Sugar
340 concentrations are estimated for an absorbance of 0.5 using two different methods: the
341 glucose standard curve (glucose-equivalent concentrations), as classically used in colorimetric
342 assays, and a specific standard curve (real concentrations) for comparison. Specific standard
343 curves, allowing for the calculation of real concentration, are either done using known
344 concentration of the specific sugar to quantify (for example, using sorbitol standard to
345 quantify an unknown concentration of sorbitol), or a mixture of sugars including the main
346 sugars present in the solution to quantify, in similar proportions. The difference between both
347 estimation is calculated for each sugar non-alcohol, alcohol, and mixture.
348
349 **Figure captions**
350
351 **Figure 1. Differential reaction of sugar alcohols and non-alcohols with anthrone reagent.**
352 (A) Calibration curves for sugar non-alcohols (open markers for data points and dotted lines
353 for linear regressions) and alcohols (closed markers for data points and solid lines for linear
354 regressions) under Van Handel (1985) conditions which are commonly used in ecological
355 studies. (B) Calibration curves for three sugar mixtures with the same total sugar
356 concentration but different individual sugar proportions as presented on the chart pies.
357 Equations and R^2 coefficients of determination of all the calibration curves presented in this
358 figure are summarized in Table 1.

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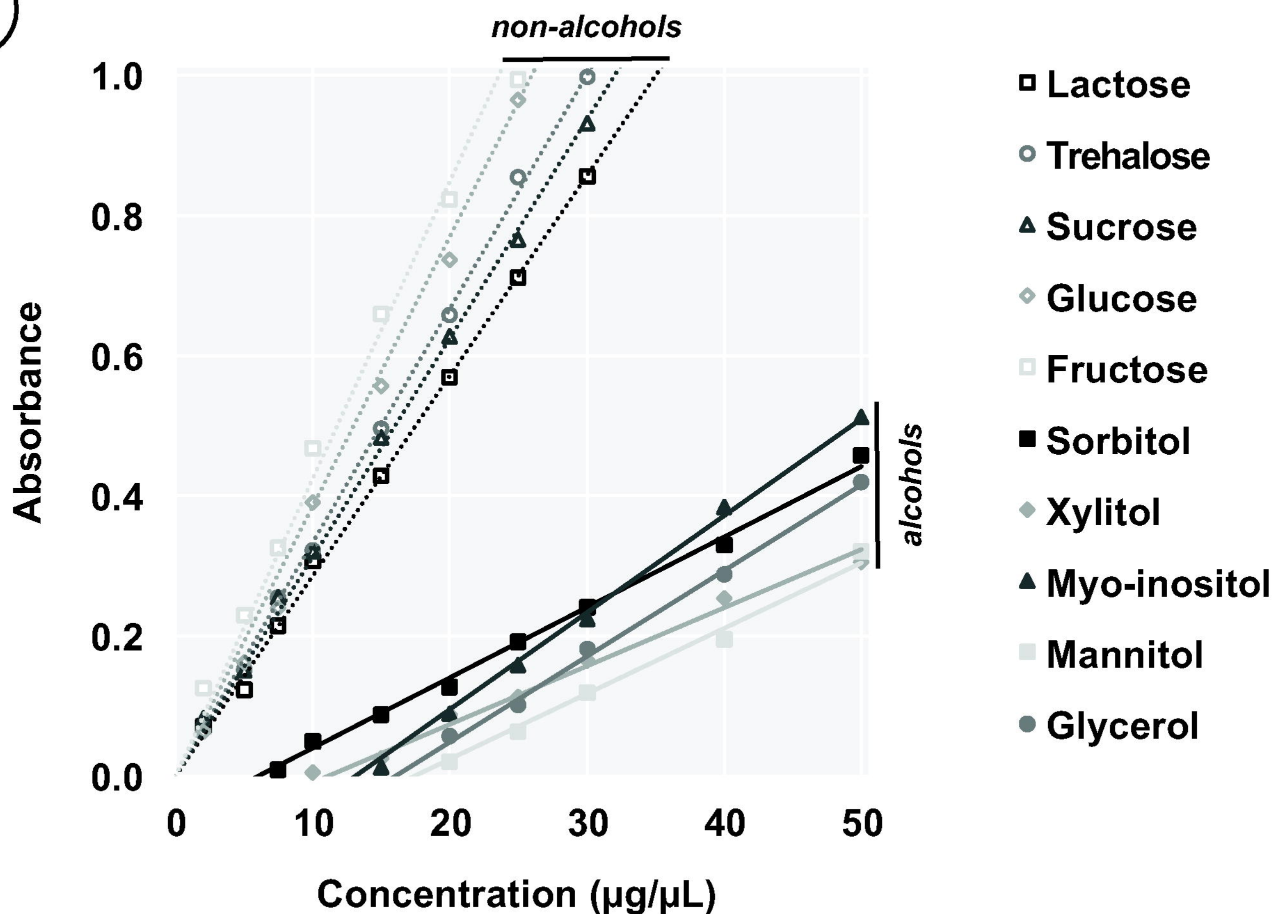

B

Absorbance

Concentration (µg/µL)

Solution 1

Solution 2

Solution 3

Sorbitol 10 %

Fructose 30 %

Sucrose 30 %

Glucose 30 %

Fructose 5 %

Glucose 5 %

Sucrose 35 %

Sorbitol 55 %

Fructose 15 %

Glucose 15 %

Sorbitol 55 %

Sucrose 15 %

1 **Table 1.**
2

Sugar	Equation	R^2	Glucose-equivalent concentration (µg/µL)	Real concentration (µg/µL)	Difference
Non-alcohol					
Trehalose	y = 0.0334x	0.9989	12.99	14.97	-13 %
Sucrose	y = 0.0312x	0.9978	12.99	16.03	-19 %
Lactose	y = 0.0286x	0.9981	12.99	17.48	-26 %
Glucose	y = 0.0385x	0.9936	12.99	12.99	0 %
Fructose	y = 0.0424x	0.9906	12.99	11.79	+10 %
Alcohol					
Xylitol	y = 0.0083x - 0.0922	0.9921	12.99	71.35	-82 %
Sorbitol	y = 0.0100x - 0.0602	0.9956	12.99	56.02	-77 %
Myo-inositol	y = 0.0138x - 0.1808	0.9986	12.99	49.33	-74 %
Mannitol	y = 0.0093x - 0.1622	0.9923	12.99	71.20	-82 %
Glycerol	y = 0.0122x - 0.1959	0.9894	12.99	57.04	-77 %
Sugar mixture					
Solution 1 *(10 % sorbitol, 30 % sucrose, 30 % glucose, 30 % fructose)*	y = 0.0391x	0.9931	12.99	12.79	+2 %
Solution 2 *(55 % sorbitol, 35 % sucrose, 5 % glucose, 5 % fructose)*	y = 0.0202x	0.9985	12.99	24.75	-48 %
Solution 3 *(55 % sorbitol, 15 % sucrose, 15 % glucose, 15 % fructose)*	y = 0.0183x	0.9971	12.99	27.32	-52 %

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