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## Osmolality of excipients for parenteral formulation measured by freezing point depression and vapor pressure – a comparative analysis

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1 OSMOLALITY OF EXCIPIENTS FOR PARENTERAL  
2 FORMULATION MEASURED BY FREEZING POINT  
3 DEPRESSION AND VAPOR PRESSURE – A COMPARATIVE  
4 ANALYSIS

5  
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25 **Abstract**

26 **Purpose**

27 To investigate the difference in methods to determine the osmolality in solutions of  
28 stabilizers used for long-acting injectable suspensions.

29

30 **Methods**

31 The osmolality was measured by freezing point depression and vapor pressure for 11  
32 different polymers and surfactants (PEG 3350, 4000, 6000, 8000, 20000, PVP K12, K17  
33 and K30, poloxamer 188, 388 and 407, HPMC E5, Na-CMC, polysorbate 20 and 80,  
34 vitamin E-TPGS, phospholipid, DOSS and SDS) in different concentrations.

35

36 **Results**

37 Independently of the measuring method, an increase in osmolality with increasing  
38 concentration was observed for all polymers and surfactants, as would be expected due  
39 to the physicochemical origin of the osmolality. No correlation was found between the  
40 molecular weight of the polymers and the measured osmolality. The osmolality values  
41 were different for PVPs, PEGs, and Na-CMC using the two different measurement  
42 methods. The values obtained by the freezing point depression method tended to be  
43 similar or higher than the ones provided by vapor pressure, overall showing a significant  
44 difference in the osmolality measured by the two investigated methods.

45

46 **Conclusions**

47 For lower osmolality values (e.g. surfactants), the choice of the measuring method was  
48 not critical, both the freezing point depression and vapor pressure could be used.  
49 However, when the formulations contained higher concentrations of excipients and/or

50 thermosensitive excipients, the data suggests that the vapor pressure method would be  
51 more suited.

52

53 **Keywords;** Osmolality, freezing point depression, vapor pressure, suspension,  
54 parenteral vehicles

## 55 **Introduction**

56 Long-acting injectables (LAIs) are a unique drug formulation option that provide a slow,  
57 sustained release of the active pharmaceutical ingredient (API) after administration [1].  
58 LAI formulations present several advantages over traditional oral formulations, including  
59 correct drug usage, reduced frequency of administration, enhanced therapy adherence and  
60 patient compliance as well as mitigation of possible adverse effects by avoiding peak  
61 plasma concentrations. Considering these properties, LAIs offer perspectives of improved  
62 quality of life for patients using these [2-7]. LAIs have attracted special interest in  
63 therapeutic areas such as schizophrenia, hormone replacement therapies,  
64 immunodeficiency virus (HIV), and tuberculosis, where repeated drug administration is  
65 required [6,8-11].

66

67 There are four main formulation classes of LAIs: i) oil solutions, ii) aqueous suspensions,  
68 iii) polymer-based microspheres/implants (including biodegradable and non-  
69 biodegradable), and iv) *in situ* forming gels/implants [12]. For the aqueous suspensions,  
70 stabilizers, polymers and/or surfactants, are added to the formulation to control the  
71 relative kinetics of particle growth of the system [13]. Their addition enables particle size  
72 reduction during milling, prevents particle agglomeration, and particle growth via  
73 Ostwald ripening of particles. This consequently impacts the overall stability, primarily  
74 physical, but also partly chemical, thereby supporting maintenance of the drug release  
75 profile over the shelf-life period [14, 15]. The stabilization mechanism can either be  
76 electrostatic repulsion or steric stabilization, hence the excipients used span a wide range  
77 of biocompatible charged and nonionic surfactants as well as polymers [15]. The selection  
78 of stabilizers is specific and crucial for each individual API [16]. Polysorbate 80,  
79 polysorbate 20, sodium dodecyl sulfate (SDS), and poloxamer 188 are examples of

80 surfactants used as stabilizers in aqueous suspensions[13]. Examples of used polymeric  
81 stabilizers include polyvinylpyrrolidone (PVP), sodium carboxymethylcellulose (Na-  
82 CMC), poloxamer 338, and polyethylene glycol (PEG) 4000 [13, 17]. Some of these  
83 excipients are currently used in commercialized parenteral LAI formulated as aqueous  
84 suspensions, such as Invega Trinza, Aristada, Abilify Maintena, Depo-subQ Provera, see  
85 **Table I.**

86

87 For formulation of parenteral suspensions, a series of parameters should be considered to  
88 ensure formulation stability and safe administration with as little discomfort as possible  
89 for the patient. Such parameters include viscosity, pH, density, osmolality, and  
90 syringeability. Osmolality, as well as pH, is directly related to local irritation, pain, and/or  
91 endothelial damage. Osmolality is an estimation of the osmolar concentration of plasma,  
92 which is proportional to the number of particles per kilogram of solvent and is expressed  
93 as mOsmol/kg [23]. On the other hand, osmolarity (osmotic concentration) is defined as  
94 an estimation of the osmolar concentration of the plasma. This property is proportional to  
95 the number of particles per liter of solvent and its unit is mOsmol/L [24]. As only  
96 osmolality can be measured, a relationship between these two quantities has been  
97 determined through fundamental physical/chemical definitions. These definitions include  
98 the osmotic coefficient, i.e. a conversion factor particular to the solute system and the  
99 partial molal volume(s) of the solute(s) [24].

100

101 According to the United States Pharmacopeia (USP), the osmolality of blood ranges  
102 between 285 and 310 mOsmol/kg [25]. Generally, parenteral formulations should be  
103 isotonic (around 290 mOsm/kg) or moderately hypertonic (up to 500 mOsm/kg) [26],  
104 since hyperosmolality leads to a loss of water from the cells which causes cell shrinkage

105 and an increase in cellular viscosity, which will be associated with pain upon injection  
106 [23].

107

108 Osmometry to measure osmolality is, hence, an essential tool when characterizing the  
109 physicochemical properties of solutions for parenteral application [27]. The most  
110 common osmometers are based on the assessment of three properties of the solution:  
111 freezing point depression, vapor pressure, and osmotic pressure. The freezing point  
112 depression and the vapor pressure are the most commonly applied methods in commercial  
113 available equipment that measures osmolality [28]. Both methods provide a direct  
114 measurement of the osmolality and require a limited amount of sample. Furthermore, both  
115 methods are commonly used in pharmaceutical development as a fast, easy, and accepted  
116 method to determine the osmolality of parenteral formulations [29].

117

118 Some concerns have been raised regarding both osmometry techniques. Winzor [30]  
119 highlighted a disagreement observed for the osmolality measurements of PEG solutions  
120 by vapor pressure and freezing point depression [30], which was suggested to be due to  
121 the water adsorption by the filter paper disc, inherent to the vapor pressure technique [31].  
122 However, this hypothesis did not consider the temperature effect on the excipients,  
123 specifically those that undergo temperature-dependent changes in hydration, such as  
124 surfactants and polymers used for stabilization of LAI suspensions. One example of these  
125 temperature-dependent polymers is PEG [32, 33]. While some osmolality data for  
126 solutions containing PEGs can be found in the literature [28, 34], there is a general lack  
127 of information about other excipients used to stabilize suspensions. Most of the  
128 publications published do not approach the subject of osmolality and tend to focus on the  
129 effects of different molecular weights of polymers, e.g. as stabilizers of amorphous solid

130 dispersions or in supersaturated drug solutions [35]. Lestari et al. [36] conducted a  
131 systematic screening of different surface modifiers for the production of physically stable  
132 nanosuspensions using wet ball milling. The group concluded that combinations of  
133 anionic surfactant and nonionic surfactant as well as combinations of anionic surfactant  
134 and polymeric stabilizer tend to be more successful for the formation of stable  
135 nanosuspensions. Furthermore, the study stated that the concentration and the principle  
136 of stabilization of surface modifier determines the formation of stable nanosuspensions  
137 [36], but in general no considerations were put on the osmolality. The purpose of the  
138 present study was therefore to study the osmolality of polymers and surfactants relevant  
139 for aqueous suspension-based LAIs at room temperature by two methods, i.e. the freezing  
140 point depression and the vapor pressure. The main goal was to provide general insights  
141 into the potential difference between the two methods across a broader range of  
142 excipients, particularly important for formulation purposes, as discussed above.

143

## 144 **Theoretical approach to osmolality - considerations**

145 Osmolality provides an estimation of the concentration of solutes in a solution, and it can  
146 be assessed through any of the four colligative properties of the solvent [37]. When a  
147 solute dissolve in a pure solvent, specific changes, that are proportional to the solutes  
148 activity/concentration, occur in the solution's colligative properties [38], such as:

- 149 • the freezing point depresses
- 150 • the boiling point raises
- 151 • the osmotic pressure increases
- 152 • vapor pressure lowers [34]

153 In the section below a more detailed description of the freezing point depression and the  
154 vapor pressure are provided.

155

## 156 **Freezing point depression**

157 Freezing point is defined as the temperature at which a solvent/solution will turn from  
158 liquid to solid. When the sample is added to the osmometer, it initially cools according to  
159 Newton's Law of cooling (the rate of cooling is proportional to the difference in  
160 temperature between the sample and its environment). However, for a mixture of solvent  
161 and solute (solution), the solution does not freeze, only the solvent.

162

163 When the freezing point is reached, the sample would remain at a constant temperature  
164 until all mass has been converted to the solid phase. However, solutions tend to supercool,  
165 meaning that the samples may cool below the freezing point temperature of the solvent  
166 until crystallization starts. As more solid is formed, the concentration of the solution  
167 increases at an exponential rate until it reaches a solubility limit – the eutectic point.  
168 Finally, all solvent becomes solid and the mass cools down to the equilibrium temperature  
169 (temperature plateau). During this period, the center of the sample alternates between  
170 thawing and freezing until it completely freezes and the sample slowly turns solid and  
171 cools to the equilibrium temperature [39].

172

173 When a solute is dissolved in a pure solvent, the change in the freezing point is directly  
174 proportional to the molar concentration of the solute and can be determined by the  
175 following equation:

176

$$\Delta T = K_f \cdot m \quad \text{Eq. 1}$$

177

178 where  $\Delta T$  corresponds to the temperature change from the pure solvent's freezing point  
179 to the freezing point of the solution,  $K_f$  is the freezing point constant (for water this is

180 1.86 °C/mol), and  $m$  is the molality of the nonvolatile solute, i.e. the osmolality of the  
181 solution at a particular molality of the solute in the particular solvent [38].

182

183 When measuring the freezing point depression, the sample is vibrated intensely for a  
184 moment, after a fast supercooling of the solvent to a predetermined temperature, which  
185 produces heat of fusion as crystallization occurs. When a plateau of the cooling curve is  
186 reached, its value is measured by a thermistor. This plateau tends to be below the freezing  
187 point of the pure solvent, as explained above, but by relating the unknown with standard  
188 solutions, the osmolality can be determined [38-40].

189

190 This measurement does not provide any information regarding the nature of the particles  
191 (e.g. size, shape or conformation), as the calculation only depends on the number of  
192 particles in solution. However, according to Sweeney and Beuchat [41], Eq. 1 is supported  
193 by a series of assumptions that are often violated, since the relationship between the  
194 freezing point depression and osmolality differs between solutes and solvents [42]. The  
195 freezing point depression constant not only varies between solvents, but also within the  
196 same solvent as a function of solute, i.e. the type and concentration of solute [42].  
197 Furthermore, the value provided by this technique can deviate from the real value for  
198 three different reasons:

- 199 1. violation of thermodynamic assumptions (i.e. solution is very dilute and presents  
200 ideal behavior)
- 201 2. temperature dependence of the solute solubility
- 202 3. mathematical simplifications for osmolality calculations

203

204 **Vapor pressure**

205 In osmolality measurements made using the vapor pressure method, the sample is  
206 inoculated onto a solute-free paper disc in the sample holder, the sample holder is pushed  
207 inside the instrument and the chamber is closed [38]. The sensing element is a  
208 thermocouple hygrometer composed of two thermistors with a sample holder in between.  
209 When the sample is added to the sample holder, it is placed in between these two  
210 thermistors [38]. As vapor pressure equilibrates in the chamber airspace, the  
211 thermocouple senses the ambient air temperature, which will be the reference point for  
212 the measurement. Afterwards, the thermocouple is cooled until a temperature below the  
213 dew point. As a consequence, the solvent condenses in the chamber and forms small  
214 droplets on the surface of the thermocouple. At this point, the temperature of the  
215 thermocouple is controlled by the water condensing onto its surface. As water continues  
216 to condense, the thermocouple temperature tends to increase until the dew point is  
217 reached. At the dew point, water condensation stops and, consequently, the thermocouple  
218 temperature stabilizes giving an output proportional to the differential temperature (dew  
219 point temperature depression) – which is a function of the solution vapor pressure. In this  
220 context, the chemical potential of the solution’s solvent can be compared with the one of  
221 the solvents alone [43].

222

223 The relationship between sample osmolality and the reading obtained by the osmometer  
224 is governed by fundamental considerations. Vapor pressure depression is a linear function  
225 of osmolality, since it is one of the colligative properties of a solution. The relationship  
226 between vapor pressure depression and the dew point temperature is given by Equation 2  
227 [43].

228

$$\Delta T = \frac{\Delta e}{S} \quad \text{Eq. 2}$$

229 where the osmotic pressure,  $\Delta T$ , is the dew point temperature depression in degrees  
230 Celsius,  $\Delta e$  is the difference between saturation and chamber vapor pressure, and  $S$   
231 corresponds to the slope of the vapor pressure temperature function at ambient  
232 temperature [43].

233

234  $S$  is determined by the Clausius-Clapeyron equation (Equation 3), as a function of  
235 temperature ( $T$ ), saturation vapor pressure ( $e_0$ ) and latent heat of vaporization ( $\lambda$ ) [43].

$$236 \quad S = \frac{e_0 \lambda}{RT^2} \quad \text{Eq. 3}$$

237

238 where  $R$  is the universal gas constant. The dew point temperature depression,  $\Delta T$ , is  
239 measured as a voltage signal from the thermocouple and the signal is processed to display  
240 the reading. This voltage is equal to  $\Delta T$  multiplied by the thermocouple responsivity,  
241 which is approximately 62 microvolts per degree Celsius. After voltage amplification by  
242 a preamplifier, the microprocessor processes the voltage signal to provide calibration and  
243 compensation functions and then displays the reading in mmol/kg [43].

244

245 When comparing the freezing point depression and the vapor pressure method, one clear  
246 advantage of the latter is that it does not involve a change in the physical state of the  
247 solution. Additionally, this technique can be performed in a wide temperature range, and  
248 it is not affected by temperature-sensitive changes in solute solubility. Furthermore,  
249 viscosity and/or presence of suspended particles does not influence the measurement.  
250 Nonetheless, it is important to mention that the vapor pressure technique is less suitable  
251 when volatile or organic solvents are present in the solution, as it will influence the  
252 equilibrium reached. Overall, theoretically, the vapor pressure method seems to have a

253 much broader range of minimal error applications when compared to freezing point  
254 depression [38].

255

## 256 **Materials and methods**

### 257 **Materials**

258 Hydroxypropylmethylcellulose 2910, 5 mPas (HPMC E5) was purchased from DDP  
259 Specialty Electronic Materials (DDP Specialty Electronic Materials Plaquemine, LA,  
260 USA). Poloxamer 188, poloxamer 338 parenteral, poloxamer 407, Polyvinylpyrrolidone  
261 (PVP) K12 parenteral, PVP K17 parenteral, PVP K30, and sodium dodecyl sulfate (SDS)  
262 were acquired from BASF (BASF Chemtrade MBH, Germany). Polyethylene glycol  
263 (PEG) 4000 parenteral was sourced from Clariant (Clariant International Ltd,  
264 Switzerland). PEG 3350 was bought from Spectrum (Spectrum Chemical MFG Corp,  
265 CA, USA), PEG 6000 Flake was purchased from Dow Chemical (The Dow Chemical  
266 Company, MI, USA), and PEG 8000 was sourced from Sigma-Aldrich (Sigma Aldrich  
267 corporation, MO, USA). PEG 20000 was bought from Merck (Merck KGaA, Germany).  
268 D- $\alpha$ -tocopheryl polyethylene glycol 1000 succinate (Vitamin E-TPGS) was acquired  
269 from Isochem (France). Polysorbate 20 parenteral and polysorbate 80 were purchased  
270 from Croda Inc. (NL, USA). Docusate sodium was bought from Cytotec (Cytotec Industries,  
271 Netherlands). Sodium carboxymethylcellulose (Na-CMC) was acquired from Ashland  
272 Inc. (France). Lipoid E PG was sourced from Lipoid GmbH (Lipoid GmbH, Germany).  
273 Purified water was freshly prepared using a Milli-Q®integral water purification system  
274 (Milli-Q Advantage A10; MerckMillipore, Merck A/S, Denmark).

275

276 The chemical structures and key physicochemical information regarding the polymers  
277 and surfactants used in this study can be found in **Supplemental information - Tables**  
278 **S1** and **S2**, respectively.

279

## 280 **Methods**

### 281 **Preparation of excipient solutions**

282 All solutions were initially prepared in glass beakers with approximately 80% of the total  
283 volume (100 mL) (**Table II**). The samples were magnetically stirred overnight (300 rpm,  
284 21 °C), protected from light by wrapping beakers in aluminum foil. The volumes were  
285 adjusted to 100 mL with deionized (DI) water on the next day after the complete  
286 dissolution of the excipient.

287

### 288 **Osmolality by Freezing Point Depression**

289 The osmolality was measured using OsmoPRO (Advanced® Instruments 3250,  
290 Norwood, MA, USA), an osmometer based on the freezing point depression principle.  
291 The accuracy of the osmometer was confirmed at the start and completion of each testing  
292 session by assaying a reference solution of known osmolality provided by the  
293 manufacturer, 290 mOsm/kg (Advanced® Instruments 3250, Norwood, MA, USA). All  
294 samples were equilibrated to standard laboratory temperatures (20-21 °C) before  
295 assessment. Osmometry was performed in triplicate using a 20 µL sample.

296

### 297 **Osmolality by Vapor Pressure**

298 The osmolality measurements based on vapor pressure were performed using VAPRO  
299 (Wescor, Inc 370 West, Utah, USA). The osmometer was calibrated with three  
300 concentrations of standard salt solutions (100, 290, and 1000 mOsm/kg) before starting

301 the measurements. Also, for all measurements, solutions were equilibrated to standard  
302 laboratory temperatures (20-21 °C) before assessment. Osmometry was performed in  
303 triplicate using a 10 µL sample.

304

### 305 **Statistical analysis**

306 All tests were conducted at least in triplicate. The results are reported as the average value  
307 with standard deviation for each solution. Statistical analysis was performed using a  
308 Bayesian bivariate mixed model, since it was verified that the concentration effect on the  
309 osmolality values differences could not be seen as a random variation. The Bayesian  
310 bivariate mixed model is a multivariate linear mixed effect model, which combines the  
311 strength of the paired t-test, by matching the measurements on samples, and on the other  
312 hand, similar to classical ANOVA. This approach allowed us to model the paired  
313 differences by accounting for the concentration effects. A detailed description of the  
314 model can be found in the **Supplemental Information**.

315

316

### 317 **Results and discussion**

318 Parenteral LAIs can be injected via different administration routes depending on a  
319 combination of anatomical, physiological, and physical factors. Most commercially  
320 available LAI are injected intramuscular (IM), but other administrations routes may also  
321 be relevant, e.g. subcutaneous (SC) and intravitreal (as shown in **Table I**). The  
322 administration route is chosen according to the intended therapeutic action [44,45].

323

324 Adjustment of the osmolality of parenteral products is critical for patient comfort and  
325 safety when these drug products are administered. In this work, the focus is on surfactants  
326 and polymers used to stabilize suspensions.

327

## 328 **Osmolality of different aqueous solutions of polymers used as stabilizers** 329 **in suspensions**

330 The polymers investigated in this study were PEG 3350, 4000, 6000, 8000, and 20000;  
331 PVP K12, K17, and K30; poloxamer 188, 338, 407; HPMC E5, and Na-CMC. In general,  
332 PEG and Na-CMC are known suspending agents, while PVP, poloxamers and HPMC are  
333 classified as wetting agents. Therefore, the functionalities of the investigated excipients  
334 differ. However, no matter their function in the formulation, it is important to understand  
335 their contribution to the final obtained osmolality in the solution/suspension they may be  
336 a part of, in order to design the best formulation composition.

337

338 The data obtained for the osmolality of the investigated polymers, measured by both the  
339 freezing point depression and the vapor pressure method, are presented in **Fig. 1**.  
340 Independently of the measuring method, an increase in osmolality with increasing  
341 concentration was observed for all polymers, as would be expected due to the  
342 physicochemical origin of the osmolality (see **Section 2**). Furthermore, no increase in  
343 osmolality with molecular weight for the same excipient concentration was observed. For  
344 example, by taking the 10.5% w/v concentration for PEGs, the average osmolality values  
345 were 124.0, 114.3, 110.0, 85.0 and 99.3 for PEG 3350, PEG 4000, PEG 6000, PEG 8000  
346 and PEG 20000, respectively (see **Fig. 1** and **Supplemental information - Table S3**).  
347 This finding contradicts the trend presented in already published data on PEGs [28, 30,  
348 31]. When the osmolality measured was plotted as a function of the four concentrations,

349 an exponential increase in osmolality was observed with increasing concentration for all  
350 polymers (see **Fig. 1**). This non-linearity for osmolality may be a reflection of the limited  
351 connection between the chemical potential in the solutions and the molar concentration,  
352 but also other important factors such as size, shape, and hydrophobicity of the polymer  
353 chain may explain the observed discrepancy. Another possible explanation can be the  
354 change from dilute to semi-dilute regimes. For polymers in a dilute regime, it is  
355 considered that each polymer coil/particle is independent and not in contact with each  
356 other. However, when the same polymer enters its semi-dilute regime, individual coils  
357 may be in contact with each other, i.e. it is not anymore possible to detect individual coil  
358 particles. Furthermore, when the solutions enters into a concentrated regime, coils  
359 entangle with each other, leading to the destruction of individual particles [46-48]. Having  
360 said this, from the collected data in this work it may be suggested the PVP was always in  
361 a dilute regime (i.e. individual particles) in the concentration range studied, while PEG  
362 was in a semi-dilute regime (i.e. entangled polymer chain, no individual particles),  
363 leading to different behavior as result of concentration between these polymers.

364

365 By comparing the osmolality values of the different polymers, it was observed that the  
366 PVPs, i.e. PVP-K17, and PVP-K30, had osmolality values similar to those of Na-CMC,  
367 although Na-CMC has a much higher molecular weight than PVPs (see **Table S1**). On  
368 the other hand, it was noticed that the different PEGs had osmolality values comparable  
369 to the investigated poloxamers and HPMC E5. The comparable values observed for the  
370 poloxamers were probably due to the similarity in composition of these polymers with  
371 PEG, though the molecular weight of the poloxamers tends to be lower. HPMC E5 has a  
372 higher molecular weight (i.e. 20000 g/mol) when compared to the other two polymers,  
373 PEGs and poloxamers (between 3000 and 18000 g/mol) and presents osmolality values  
374 similar to the PEGs with higher molecular weight (i.e. PEG 20000). Additionally,

375 comparison between Na-CMC and HPMC was performed, since both are cellulose-based  
376 polymers, that differ in charge, anionic versus non-ionic nature, respectively, which may  
377 be a possible and more probable explanation for the difference in osmolality observed  
378 (e.g. at 4 % w/v: 10-20 mOsm/kg for HPMC vs. 95-100 mOsm/kg for Na-CMC). At this  
379 stage, it is important to state and draw attention to the fact that the charge of all substances,  
380 whether anionic, cationic or nonionic, will have some effect on the surface tension when  
381 added to the aqueous solvent. This could contribute to the physicochemical properties of  
382 the system under investigation and, consequently, affect the measured value for  
383 osmolality.

384

385 The data collected by the vapor pressure technique showed similar trends to the freezing  
386 point depression technique with respect to the molar dependency between the polymers,  
387 though with some differences, as can be seen by analyzing the different graphs presented  
388 in **Fig. 1**. The osmolality values were different for PVPs, PEGs, and Na-CMC using the  
389 different measurement methods. It could be seen that the values given by the freezing  
390 point depression method tended to be similar or higher than the ones provided by vapor  
391 pressure. However, the differences observed were statistically significant, so before  
392 defining the technique intended to be used for the osmolality analysis, a careful analysis  
393 should be performed with respect to which method that would be most suitable for  
394 determining the osmolality of LAIs suspensions that contain those specific polymers. For  
395 suspensions it is common use to add more than one excipient, but since the surfactant  
396 contribution to the osmolality tends to be relatively low as is discussed below, the main  
397 contributors towards osmolality would be a potentially added polymer. In a suspension,  
398 the API would also have a limited contribution to the osmolality.

399

400 Na-CMC was present in a much lower concentration compared to the other polymers  
401 investigated. The osmolality values obtained for the poloxamers were different from the  
402 osmolarities measured for HPMC E5 (see **Fig. 1** and **Supplemental information - Table**  
403 **S3, Fig. S1**). For example, for HPMC solutions osmolality was zero up to 10 % w/v, while  
404 some poloxamers show osmolality of about 20-30 mOsm/kg at 7 % w/v (see **Fig. 1** –  
405 HPMC compared to poloxamer 188 or 338) [49,50].

406 Based upon the reports from literature there was a reason to assume that there would be  
407 a correlation between the molecular weight of PEG and osmolality value, which was not  
408 observed in the present study nor for any of the other investigated polymers, i.e. PVP and  
409 poloxamer. As can be seen in **Fig. 2**, there was no clear correlation between molecular  
410 weight of PEG and osmolality value, and the osmolality values in between PEGs did not  
411 vary much at the same concentration, in the present study.

412

413 Overall, when comparing the osmolality values obtained by both techniques, it was clear  
414 that the values determined by vapor pressure were in general lower than those determined  
415 by freezing point depression. The difference in the values observed for both techniques  
416 was most pronounced for PEG 20000, PVPs, poloxamers, and HPMC E5. Possible  
417 explanations for the differences might be due to micelle formation for poloxamers.  
418 Thermoresponsive properties or viscosity changes influence the osmolality measurement  
419 as discussed above, which can also be influenced by the molecular weight of the polymer  
420 and the concentration of excipient in solution [30]. According to Ashland's product  
421 properties sheets the dynamic viscosity of a 1 % w/v solution in water of PVP-K12 and  
422 PVP-K30 is 10-14 mPa.s and 27-33 mPa.s, respectively [51]. As shown in **Fig. 1** and  
423 **Supplemental information - Table S3**, the osmolality measured by the freezing point  
424 depression and the vapor pressure methods was, at the concentration of 3.5 % w/v, 29.3

425  $\pm 0.6$  mOsm/kg and  $20.7 \pm 2.3$  mOsm/kg for PVP K12, respectively; and  $14.3 \pm 1.2$   
426 mOsm/kg and  $3.7 \pm 1.2$  mOsm/kg for PVP K30, respectively, which does not support  
427 viscosity as an important factor for the difference observed. Sweeney and Beuchat [41]  
428 have discussed the same hypothesis from a theoretical perspective and claimed that the  
429 sample dynamic viscosity differences in principle violates the thermodynamical  
430 assumptions of osmolality determination. This means that for the freezing point  
431 depression method, the cryoscopy constant ( $K_f$ ) may deviate from the  $1.86$  K/(mol/kg)  
432 often used (see **Section 2**) [41]. Additionally, Michel and Kauffmann [52] demonstrated  
433 a temperature dependency of the osmolality of PEG 6000, supporting the theoretical  
434 analysis made by Sweeney and Beuchat [41], and in accordance with the differences  
435 observed between the freezing point depression and the vapor pressure methods in the  
436 present work. These inconsistencies might be extrapolated to other molecules with  
437 temperature-dependent behaviors, such as poloxamers and HPMC.

438

439 The difference in osmolality found between the two measurement methods tended to be  
440 approximately constant across all the PEGs, with a slight increase across the different  
441 molecular weights, with increasing polymer concentration (see **Supplemental**  
442 **information - Table S3**). Comparable observations were seen for the PVPs, Poloxamers,  
443 and HPMC E5, whereas less difference was seen for Na-CMC. It can be concluded that  
444 molecular weight plays a critical role when it comes to osmolality determination for most  
445 of the polymers. However, the information provided by the supplier on the molecular  
446 weight are the average of the polymer composition which then could generate deviances  
447 in the value osmolality between suppliers.

448

449 **Osmolality of different solutions of surfactants used as stabilizers in**  
450 **suspensions**

451 The osmolality values for solutions of surfactants measured by both the freezing point  
452 depression and the vapor pressure method are presented in **Fig. 3**. As observed for the  
453 polymers, the osmolality for the surfactant solutions also increased with increasing  
454 excipient concentrations. For the freezing point depression technique, a higher osmolality  
455 was measured for polysorbate 20 than for polysorbate 80, which can be due to the  
456 saturated chains in polysorbate 20 relative to the unsaturated double bond in polysorbate  
457 80 (see **Table S2**), producing a lower chemical activity [53]. Additionally, polysorbates  
458 present similar values of osmolality for similar concentrations of PEG (i.e. 4% w/v  
459 polysorbate vs 3.5% w/v PEG), which might be explained by the fact that polysorbates  
460 contain PEG as a part of their molecular structure (**Support Information – Table S1-**  
461 **S2**). Lipoid E PG and Vitamin E-TPGS are surfactants with none to marginal  
462 contributions to the osmolality of a solution, but also the contribution of the two  
463 investigated polysorbates was very limited. In parenteral formulations antioxidants (e.g.  
464 ascorbic acid, citric acid), preservatives (e.g. benzoic acid, phenol), and potentially  
465 chelating agents (e.g. disodium edetate, detate calcium disodium) may be used. As  
466 presented in the work published by Rayaprolu et al. [54] the concentrations used of these  
467 agents, 0.001-2% w/v, is so low that their impact on the osmolality is not considered  
468 significant [54,55].

469

470 DOSS and SDS contributed to the osmolality, as shown in **Fig. 3** and osmolality values  
471 are also presented in the supplemental information (**Table S4**). While the other surfactants  
472 are non-ionic, DOSS and SDS are anionic surfactants, and ionize when in water which  
473 may explain why these two surfactants behaved differently than the others investigated.

474 It is important to refer that although this observation was clear for SDS and DOSS, almost  
475 all substances ionize to some extent, which can partly also influence the determined  
476 osmolality values. The osmolality values recorded for the surfactants by the vapor  
477 pressure method were lower than the measurements obtained by the freezing point  
478 depression method for all investigated surfactants, as was observed with the polymers.  
479 Nonionic surfactants are in general known to have temperature dependent CMC values  
480 [56], i.e. this may at least partly explain the difference between the two methods (see **Fig.**  
481 **3** and **Supplemental information - Table S4**) [56]. The observations were in accordance  
482 with data published by Kiyosawa [28] and Windsor [30] with respect to PEG. Both  
483 authors suggested that the observed discrepancies were caused by the different  
484 temperatures applied in the two measurement methods, i.e. referring to the temperature  
485 dependency of the measurement. As explained above, osmolality is a measurement of the  
486 number of solute molecules dissolved in solution. The vapor pressure method is based on  
487 the temperature difference recorded to achieve a stable vapor pressure inside the chamber,  
488 hence the method may be less reliable at the lower concentration range, i.e. lower  
489 osmolality range (see **Section 2**). However, when it comes to defining an osmotic  
490 parenteral formulation, this would be less critical, as the concentrations of the excipients  
491 used in the formulations tend to be higher and osmotic agents may be added. These  
492 osmotic agents can be water-soluble salts of inorganic acids (e.g. magnesium chloride or  
493 sulfate; sodium, or potassium), water-soluble salts of organic acids (e.g. sodium and  
494 potassium acetate, sodium benzoate, sodium citrate, sodium ascorbate), carbohydrates  
495 (e.g. xylose, glucose, mannose, sucrose, maltose), water-soluble aminoacids (e.g. glycine,  
496 leucine, alanine, methionine, etc.) or organic polymers (e.g. hydroxyethyl,  
497 methylcellulose, cross-linked PVP, polyethylene oxide, polyacrylamides, etc.) [54].  
498

## 499 **Comparison of osmolality values of excipients**

500 The data presented in **Figs. 1** and **3** (see also **Supplemental information - Table S3-S4**)  
501 demonstrated that the osmolality values obtained for the surfactants were generally lower  
502 than those obtained for polymers, except for SDS which gave osmolality values  
503 comparable to the lower polymer concentrations. Also, the different polysorbates,  
504 poloxamers and PEGs had almost the same osmolality at the same concentration. The  
505 difference observed in the osmotic contribution was most likely a reflection of the  
506 different interaction with the aqueous phase for the two classes of excipients, i.e.  
507 hydration of molecules (polymers, some surfactants) versus hydrophobic surfactants.  
508 Furthermore, ionic versus non-ionic substances, the small ions (e.g. Na<sup>+</sup>) tend to have a  
509 significant impact on increasing osmolality, as shown by comparing osmolality values to  
510 HPMC and Na-CMC in this work (see **Fig. 1**). The same was applicable to SDS and  
511 DOSS, which showed the highest osmolality, for a given concentration, among the  
512 surfactants tested.

513

514 As discussed above (see **Supplemental information - Table S4**) for the lower  
515 concentrations of polysorbate 20, polysorbate 80, and all concentrations of Vitamine E-  
516 TPGS and Lipoid E PG, the differences between the osmolality values estimated by the  
517 two techniques can be overlooked, simply because the values were almost zero for the  
518 majority of the concentrations of surfactants studied. For the higher concentrations of  
519 polysorbate 20 and 80 as well as DOSS and SDS some tendencies towards a difference  
520 were observed (see **Supplemental information – Fig. S2**).

521

522 Furthermore, a greater difference was observed between the two measurement methods  
523 for the polymers. To better understand the difference between the two osmometry

524 methods a statistical analysis was made for the following groups of polymers investigated  
525 in this study, namely PEG (3500, 4000, 6000, 8000, 20000), poloxamer (188, 338, 407),  
526 and PVP (K-12, K-17, K-30). The results are presented in **Fig. 4**.

527

528 As it can be seen in **Fig. 4**, the difference in osmolality measured by freezing point  
529 depression and vapor pressure for these three groups of polymers was significant for  
530 PEGs and poloxamers, since the confidence intervals for the two different osmometry  
531 methods do not overlap. The difference was greatest for PEG, followed by poloxamer,  
532 i.e. the findings for PEG reported here and in the literature [30-32] may also be  
533 extrapolated to other polymer classes.

534

535 When evaluating the data presented in this study, it was clear that for lower osmolality  
536 values that the freezing point depression method seemed most accurate for systems  
537 without thermosensitive excipients. However, as the osmolality became higher and  
538 reached the relevant range for injectable formulations, large differences were observed  
539 depending on the method applied. When working with formulations with thermosensitive  
540 excipients it therefore would be recommended to use the vapor pressure method. For very  
541 dilute formulations, the method used may be less critical, however, it is in general  
542 recommended to consider carefully which method to use.

543

## 544 **Conclusion**

545 The present study showed a dataset of osmolality values for a range of excipients, e.g.  
546 including polymers and surfactants that may be used in parenteral formulations. When  
547 designing a formulation comprising of drug nano/microsuspensions, the contribution of

548 the excipients on the osmolality should be taken into account with respect to obtaining an  
549 isotonic drug product or the targeted tonicity.

550

551 The non-linearity of osmolality and difference between the two osmometry methods  
552 investigated was most probably a reflection of the limited relationship between the  
553 chemical potential in the solutions and the activity of the polymers, micellar formation  
554 for surfactants, thermoresponsive properties influencing the measurement, viscosity of  
555 the solution, molecular weight of the excipients, the ionic charge of the excipients and the  
556 concentration of excipient in solution.

557

558 The osmolality values obtained for the surfactants were generally lower than those  
559 obtained for polymers, which reflects the different interactions with the aqueous phase  
560 for the two classes of excipients, e.g. micelle formation versus solubilization.

561

562 The data presented in the present study shows that for lower osmolality values, the  
563 freezing point depression method seemed more accurate for systems without  
564 thermosensitive excipients than the vapor pressure method. However, as the osmolality  
565 became higher and in the relevant range for injectable formulations large differences  
566 between the two methods were observed. When working with formulations containing  
567 thermosensitive excipients it is recommended to use the vapor pressure method.

568

## 569 **Supplementary information**

570 The online version contains supplementary material.

571

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575

## 576 **Conflict of Interest statement**

577 The Authors declare that they have no conflict of interests to disclose.

578

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## 731 **Figures Legends**

732 **Fig. 1** Overview of the osmolality values of polymer solutions prepared with respective  
733 concentrations in percentage weight per volume (% w/v) and the difference between the  
734 two measuring principles based on the average values and respective standard deviations:  
735 freezing point depression (empty square) and vapor pressure (full circle).

736

737 **Fig. 2** Plots of osmolality by vapor pressure (left) and freezing point depression (right)  
738 against concentration for the different molecular weights of the same polymers on one  
739 graph – a) PEGs, b) PVPs, and c) poloxamers.

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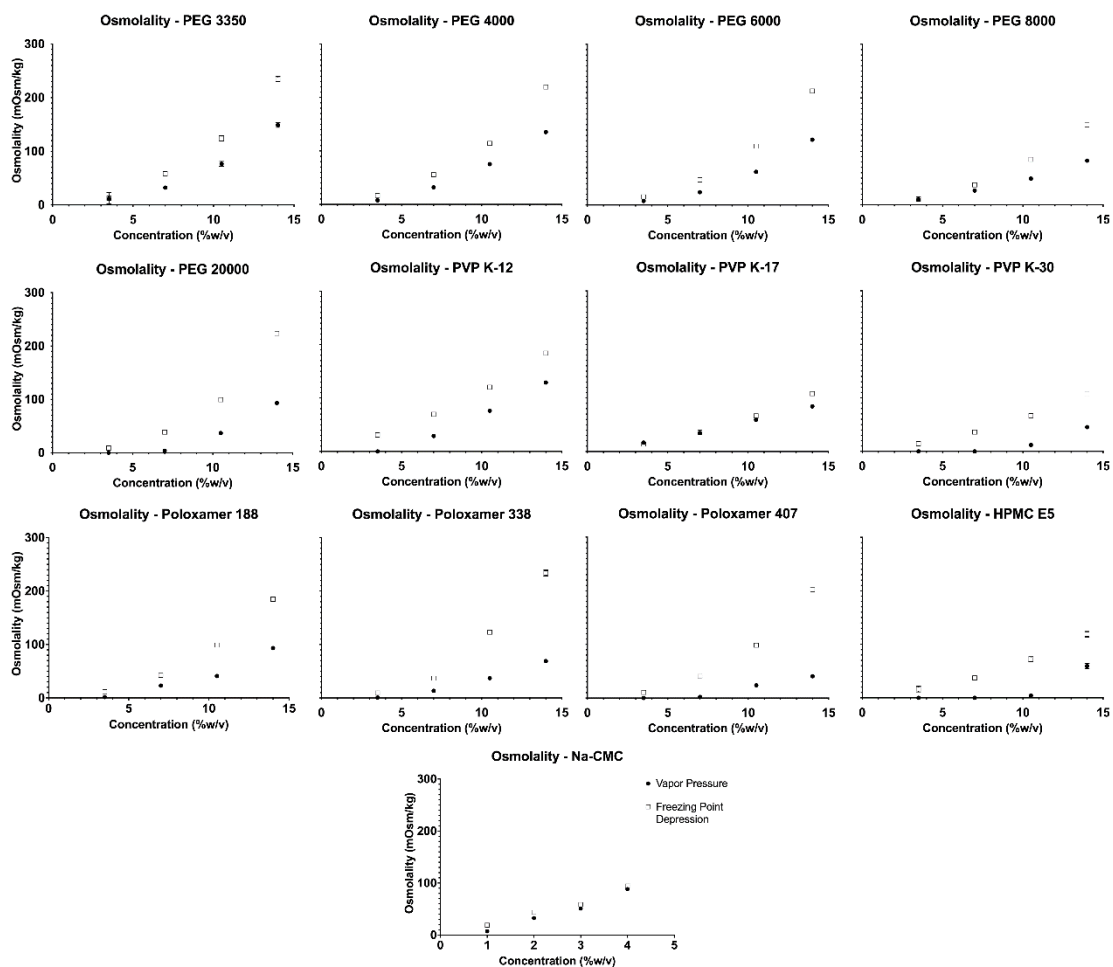
741 **Fig. 3** Overview of the osmolality values for surfactant solutions prepared with  
742 respective concentrations in percentage weight per volume (% w/v) and the difference  
743 between the two measuring principles based on the average values and respective  
744 standard deviations: freezing point depression (empty square) and vapor pressure (full  
745 circle).

746

747 **Fig. 4** Graphs show the estimated osmolality and respective 95% confidence interval for  
748 the vapor pressure or freezing point depression method grouped by concentration, for  
749 three different excipients: a) PEGs, b) PVPs, and c) poloxamers.

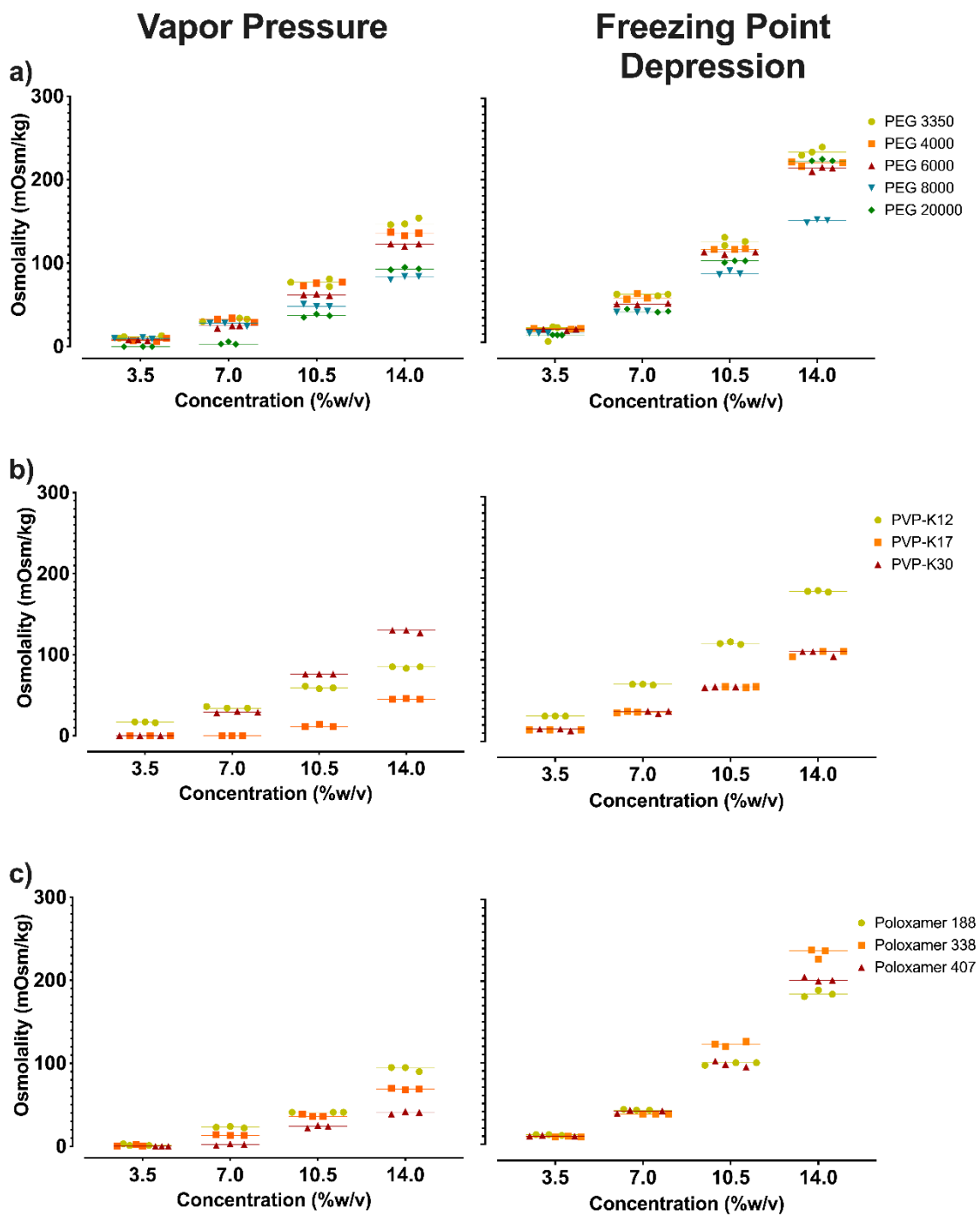
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751 **Figure 1**



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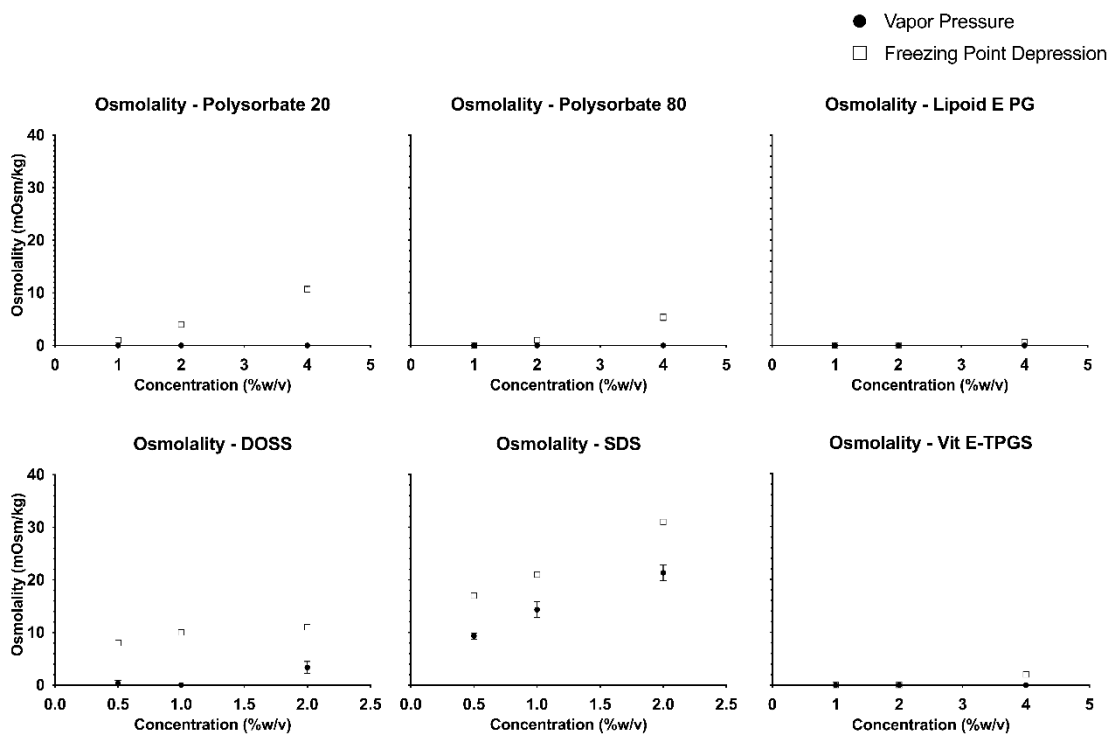
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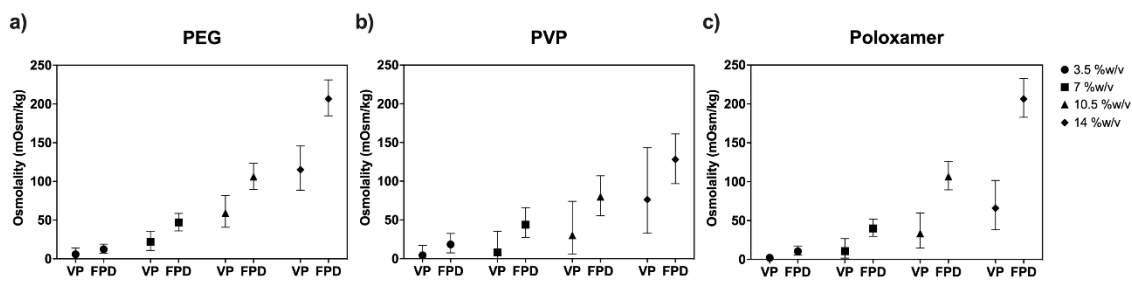
757 **Figure 3**



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759

760 **Figure 4**



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