DISPERSIVE SOLID PHASE EXTRACTION/FLUORESCENCE ANALYSIS OF RIBOFLAVIN USING SEPIOLITE AS SORBENT.

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ABSTRACT

The use of a natural clay, sepiolite (Sep), and graphene (G)/Sep mixtures as sorbents for the solid phase extraction in dispersive mode (dSPE) with fluorescence detection of a biologically interesting molecule, riboflavin, is reported. The retention of riboflavin by Sep is quantitative with different volumes of the sample and 10-50 mg of the clay. The desorption process has been performed using aqueous solutions of surfactants of different nature: anionic sodium dodecylsulphate (SDS), cationic hexadecyltrimethylammonium chloride (CTAC) and non-ionic polyoxyethylene-23-lauryl eter (Brij L23). The non-ionic surfactant provides the highest riboflavin recoveries; upon optimization of the process, that takes less than half an hour, an extraction yield of 93% was attained using 40 mM Brij L23. The developed method has been applied to the analysis of beer, soya drink and infant milk samples with excellent accuracy (recoveries of 100%) and very good precision, avoiding the interferences of the matrix components that hinder the direct fluorescence analysis of riboflavin.

1. INTRODUCTION

Riboflavin (vitamin B_2) is an important biological molecule since it is a water-soluble vitamin that can be straightforwardly absorbed into eukaryotic cells (Foraker et al., 2003). Most of the riboflavin is transformed into its cofactor forms, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), with biological activity in enzymes and photoreceptors. Vitamin B_2 is also essential for a wide variety of cellular processes including the metabolism of fats, ketone bodies, carbohydrates, and proteins, hence plays a key role in the human diet. Its uptake is exclusively by food ingestion; thus, its quantification is required for assessment of nutrient content, particularly in vitamin-fortified foods. The determination of riboflavin is also important for food quality evaluation in different products, namely in milk, wines and beers, and it is a natural food colorant (Goldbach et al., 2014).

Riboflavin has native fluorescence and its determination has been carried out mainly by fluorescence and high-performance liquid chromatography (HPLC). Fluorescence is very sensitive, although it suffers from matrix interferences that cause enhancement or quenching of riboflavin fluorescence in real samples. Therefore, chromatographic separation aided by a preceding sample treatment that isolates the riboflavin analyte and removes the interferences is frequently needed. Different solvent extractions and enzyme digestions have been used to isolate riboflavin from food samples (Torre et al., 2010). In this regard, solid phase extraction is an effective way to separate riboflavin from complex matrices, offering an enriched extract that can be directly processed by HPLC systems (Segundo et al., 2013). Several methods have been described using commercial silica C18 cartridges (Quirós et al., 2004; Rudenko and Kartsova, 2010). More recently, commercial molecular imprinted polymers (MIP) have become available for riboflavin

60 determination, providing molecular recognition with a better selectivity (Manesiotis
61 et al., 2009; Oliveira et al., 2010). Ossòrio and coworkers (Ossòrio et al., 2016)
62 proposed a fast and cost-effective method for the detection of riboflavin in milk and
63 infant formula.

64 The selection of the sorbent in solid phase extraction is very important to attain
65 good efficiency and selectivity. Consequently, a large amount of materials has been
66 developed for selectivity improvement. In this regard, nanomaterials such as
67 graphene (G) and its derivatives have become popular as sorbents in solid phase
68 extraction (Liu et al, 2012; Sitko et al., 2013; Wang et al., 2014; Fumes et al., 2015;
69 Ye and Shi, 2015; Andrade-Erioa et al., 2016; González-Sálamo et al., 2016; Ibrahim
70 et al., 2016; Plotka-Wasylka et al., 2016; Ahmadi et al, 2017; Mateos et al., 2017a;
71 Toffoli et al, 2018) as well in cartridges, dispersive solid-phase extraction (dSPE)
72 and in modified techniques with solid sorbents such as magnetic solid-phase
73 extraction, solid-phase microextraction (Piri-Moghadam et al., 2017),
74 microextraction by packed sorbent (Moein et al., 2015), stir bar sorptive extraction
75 (Kawaguchi et al., 2013; Aparicio et al., 2017).

76 dSPE is an alternative modality to solid phase extraction that was introduced in
77 2003 (Anastassiades et al., 2003). The procedure is based on the dispersion of the
78 sorbent material into a sample solution that contains the analytes. After the
79 adsorption step, a suitable solvent is used for the desorption of the analytes. The
80 dSPE technique does not need conditioning and washing steps, therefore it is faster
81 and simpler than the SPE method. When using nanomaterials in this technique, a
82 solid support is required to manipulate the nanomaterial and separate the solid phase
83 and the liquid solution (Plotka-Wasylka et al., 2016; Toffoli et al, 2018).

Sepiolite (Sep) is a natural hydrated magnesium silicate ($Si_{12}Mg_8O_{30}(OH)_4(H_2O)_{24} \cdot 8H_2O$) with a fibrous morphology that presents a crystal structure comprising talc-like ribbons set parallel to the fiber direction (Murray, 2007). Sep has high cation-exchange capacity, since the substitution of Mg by trivalent metals like Al causes a lack of charge in the structure that is compensated by extra-framework cations. One of the foremost characteristics of this silicate is its microporosity, usually superior to 0.3 $cm^3\ g^{-1}$ that leads to an alternating distribution of structural blocks, each of them composed of two tetrahedral silica sheets and a central sheet of magnesium hydroxide, resulting in cavities (tunnels) that grow along the c-axis direction. In these tunnels, organic molecules can be retained as well as in the surface of the clay by interaction with the silanol groups (Ruiz-Hitzky, 2001). This irregular arrangement is responsible for the formation of 1D nanoscale fibers with high specific surface area (200-300 $m^2\ g^{-1}$), which makes it an outstanding adsorbent for many applications in several areas like industry, agronomy and environmental remediation (Murray, 2007). Their elongated shape results in exceptional colloidal properties, in particular the resistance to high concentrations of electrolytes.

Further, the high aspect ratio and good mechanical properties of Sep nanofibers make them suitable as reinforcing fillers for polymeric matrices (Ruiz-Hitzky et al., 2013) and hybrid materials (Aranda et al., 2018). Sep has also been hybridized with GO via intercalation and hydrophobic interaction mechanisms with the aid of surfactants such as hexadecyltrimethylammonium chloride (CTAC). The intercalated GO-Sep hybrid complex was then thermally reduced at high temperature to generate a nanomaterial with clay molecules uniformly distributed on the GO sheets (Vengatesan et al, 2017). The growth of G onto the surface of clays has also been

reported, and the resulting clay-supported hybrids exhibited outstanding features like simultaneous conducting behavior, along with chemical reactivity and adsorption characteristics owed to the silicate backbone, which are of interest for high-performance applications (Ruiz-García et al., 2013). Neither Sep nor G/Sep mixtures have been used as solid phases with analytical purposes in extraction and purification of samples. However, they have a huge potential for the design of phases with different polarities depending on the solid composition, and therefore it would be possible to choose one according to the target analytes.

Surfactants are amongst the most versatile chemicals known to play a key role in many processes of interest in fundamental and applied science such as cleaners, paints, cosmetics, pharmaceuticals, food and medicine (Moradi and Yamini, 2012). Surfactant solutions have been employed to improve the sensitivity and selectivity of analytical methods owed to their ability to dissolve hydrophobic compounds in aqueous solutions (Memon et al., 2008). In particular, surfactant micellar media are highly suitable to solubilize vitamins, and therefore, improving the sensitivity in the detection (San Andrés et al., 2010). Thus, riboflavin, pyridoxine and thiamine have been analysed by synchronous fluorescence using surfactants in pharmaceuticals (García et al., 2001) and different water-soluble vitamins including riboflavin have been simultaneously determined in isotonic drinks via a screening method by measuring the fluorescence in the cationic surfactant CTAC (León-Ruiz et al., 2005). The use of surfactants in sample preparation approaches is important to improve the extraction media by ion-pair based extraction, cloud-point extraction, hemmimicelle/admicelle extraction or solid-phase microextraction with micellar desorption (Yazdi, 2011). In vitamin analysis, the elution in solid phase extraction of

retinol using a CTAB/n-BuOH solution gave a good recovery and it was applied to different oil samples (Torre et al, 2008).

The aim of this work is to test for the first time the suitability of Sep, either by itself or mixed with G, as a new nanometric sorbent in dSPE for extracting riboflavin in food samples using surfactant aqueous solutions as low toxic and cheap extracting agents.

2. EXPERIMENTAL

2.1 Reagents

High purity sepiolite (> 95%), with composition of SiO_2 60.2%; Al_2O_3 1.7%; Fe_2O_3 0.7%; CaO 0.4%; MgO 26.1%; Na_2O 0.1%; K_2O 0.3%, particle size smaller than 75 μm and specific surface of 290 m^2/g, was supplied by Sepiol SA (Azuqueca de Henares, Spain). AvanGRAPHENE, G powder with lamellar structural morphology comprising less than 6 layers with a thickness ≤ 2 nm, was provided by Avanzare Innovación Tecnológica, SL (Logroño, Spain). Riboflavin ($C_{17}H_{20}N_4O_6$, Mw = 376.36 g mol^{-1}), hexadecyltrimethylammonium chloride (CTAC, $C_{19}H_{42}ClN$, Mw = 320 g mol^{-1}) and polyoxyethylene-23-lauryl ether (Brij L23, $C_{12}H_{25}(OCH_2CH_2)_{23}OH$, Mw= 1198.56 g mol^{-1}) were purchased from Sigma (Madrid, Spain). Sodium dodecylsulphate (SDS, $NaC_{12}H_{25}SO_4$, Mw = 288.38 g mol^{-1}) was provide by Merck. All the reagents were of analytical grade and were used without further purification. All the aqueous solutions were prepared using ultrapure water obtained from a Milli-Q system (Millipore, Milford, USA). Different beer brands (with alcohol contents of 0.0%, 1.0% and 5.3% v/v), soya drink and infant milk were purchased from a local market. Four samples of each type were tested to perform a statistical analysis.

157 A stock solution of riboflavin (250 mg L^{-1}) was prepared by weighing the
158 appropriate amount and filling up to 25 mL with ultrapure water containing 28% v/v
159 H_3PO_4. The standard solutions were prepared by diluting the stock solution in
160 ultrapure water. All riboflavin solutions were stored at 4ºC under dark conditions.

161 **2.2 Instruments**

162 Fluorescence spectra were recorded at 25±1ºC with a PerkinElmer LS-50B
163 luminescence spectrophotometer (Perkin-Elmer, USA) equipped with a Xe flash
164 lamp and quartz cuvettes of 1 cm path length thermostatised with a Thermomix BU
165 bath. The excitation and emission slit widths were 5 nm, and the scan speed was
166 1000 nm min^{-1}. The acquisition and data analysis were carried out using the Perkin-
167 Elmer FLWin Lab software.

168 A mechanical stirrer (Selecta, Barcelona, Spain) was used for shaking the
169 mixtures of the sorbent and the riboflavin solutions.

170 An ultrasonic bath (Selecta, Barcelona, Spain) was used to prepare the
171 G/sepiolite mixtures. The solutions were centrifuged using an Orto Alresa Digicen
172 refrigerated centrifuge (Madrid, Spain).

173 Scanning Electron Microscopy (SEM) micrographs were obtained with a Zeiss
174 DSM-950 SEM (Carl Zeiss, Oberkochen, Germany), operating at an acceleration
175 voltage of 15.0 kV, with a magnification of 30,000 x.

176 **2.3 Procedure**

177 *2.3.1 Riboflavin fluorescence in water and in surfactant solutions.*

178 Fluorometric spectra of riboflavin were obtained from the three-dimensional
179 spectra (contour graphs), which were acquired with an initial excitation wavelength

(λ_{exc}) set at 280 nm, and 25 spectra were registered with a λ increment of 10 nm. The optima λ_{exc} and λ_{em} were determined from the maximum intensity observed in the graph.

2.3.2 Analytical characteristics of the fluorescence analysis

The analytical features of the fluorescence method were determined by the external standard method, using standards dissolved in ultrapure water and in the surfactants. The riboflavin concentration was ranged from 0.05 to 15 mg L^{-1} in order to determine the sensitivity, linear range, limit of detection (LOD), limit of quantification (LOQ), robustness, reproducibility and repeatability. The sensitivity was expressed as the slope of the calibration curve. The LOD was calculated as the concentration corresponding to the intercept signal plus three times the standard deviation of the intercept. The LOQ was calculated in the same way considering 10 times the standard deviation of the intercept. The robustness was determined as the relative standard deviation of the slopes obtained in 4 different days. The repeatability, intra-day precision, and reproducibility, inter-day precision, were calculated as the relative standard deviation of 4 measurements performed in the same day and in different days, respectively.

2.3.3 Synthesis of G/Sep mixtures

Several graphene/sepiolite (G/Sep) mixtures with G weight percentages of 2, 5 and 10 were prepared in order to obtain materials with different polarity. For such purpose, dispersions containing 50 mg of G in 100 mL of water were mixed with different amounts of Sep, subsequently placed in an ultrasonic bath for 1 h, centrifuged at 4000 rpm for 15 min and finally filtered with a 0.45 μm cellulose filter. Upon filtration, the resulting mixtures were films that could be bent without

204 breaking. Finally, the G/Sep films were ground to obtain a fine powder (see the
205 different G/Sep mixtures obtained in **Fig.S1** in the ESM).

206 *2.3.4 Dispersive solid phase extraction procedure (dSPE)*

207 Sep or the G/Sep mixture was weighed in a 50 mL centrifuge tube, and 25 mL of
208 a solution containing 0.25 mg L^{-1} of vitamin B_2 was added. The mixture was shaken
209 by mechanical agitation for 5 min and subsequently centrifuged for 5 min at 4000
210 rpm. Once the supernatant was eliminated, a surfactant solution was added in order
211 to desorb the vitamin. Aqueous solutions of surfactants of different nature (anionic
212 SDS, cationic CTAC and non-ionic Brij L23) were tested as extracting solvents. The
213 tube containing the solid and the surfactant solution were stirred and centrifuged for
214 5 min at 4000 rpm. Subsequently, the vitamin was determined in the supernatant.

215 *2.3.5 Analysis of real samples*

216 All the samples analysed were prepared by dilution (1:50) in ultrapure water
217 before the extraction process. This dilution step is compulsory since the direct
218 fluorescence measurement of the samples is not possible due to the opacity to light of
219 the matrix components that dispersed the radiation. 50 mL of the solution was then
220 subjected to the dSPE process using 10 mg of Sep, under the same conditions as the
221 standards. Then, the vitamin was desorbed with 5 mL of 40 mM Brij L23 aqueous
222 solution and measured by fluorescence. Riboflavin was determined in four samples
223 of each beer, soya drink and infant milk.

224 In order to evaluate the accuracy of the developed method, the 1.0% alcohol beer
225 was spiked with a known amount of riboflavin (0.5 mg L^{-1}) prior to the extraction
226 process and the recovery was calculated.

227

3. RESULTS AND DISCUSSION

3.1 Fluorescence of riboflavin in water and in the micellar media

The analysis of riboflavin was carried out by measuring the fluorescence intensity of the molecule in water and in the different micellar media that were subsequently used as extracting media. The optima wavelengths (λ_{exc} = 450 nm and λ_{em} = 520 nm) were obtained from the three-dimensional contour graphs in water (see **Fig.S2** in the ESM) and in the three surfactants studied. No wavelength shifts were found in the different micellar solutions of 40 mM SDS, 40 mM CTAC and 40 mM Brij L23; hence all the fluorescence measurements were performed at these wavelengths.

3.2 Dispersive solid phase extraction (dSPE)

With the aim to carry out the extraction of riboflavin in dSPE mode, Sep and G/Sep mixtures were tested as sorbents. However, G itself cannot be used as sorbent in this modality, given that it is a very light material that cannot be separated from the supernatant after extraction.

3.2.1 Preparation of graphene/sepiolite (G/Sep) nanomaterials

In dispersive mode, the solid sorbent has to be shaken with the sample, centrifugated and separated from the sample matrix during the first extraction step, while the solid is separated from the extract in the final step. As mentioned above, G is a low-dense material; hence the solid phase extraction in dispersive mode using G requires a solid support that allows the separation of the solid from the sample solution and the extract after centrifugation. For such purpose, Sep was used as a clay support, incorporating G within its structure; the clay was mixed with G in different weight ratios, and depending on the G percentage, supports with different

252 polarities were obtained. The solid support was chosen based on the polarity of the
253 analytes and the sample matrix. The possibilities to obtain different carbon-clay
254 hybrid nanostructured materials have been described in the literature by Ruiz-Hitzky
255 et al. by means of different methods like the synthesis of an intermediate caramel-
256 Sep nanocomposite that is heated at 800 ^{0}C (Ruiz-García et al., 2013) or via the use
257 of ultrasounds (Ruiz-Hitzky et al., 2016).

258 The characterization of the resulting nanomaterials was carried out by SEM, and
259 typical micrographs of neat G, Sep and the G/Sep mixtures are shown in **Fig.1**. Neat
260 G is composed of stacked and wrinkled 2D sheets with a mean thickness of 36 nm,
261 whilst Sep comprises 1D nanometric fibers arranged as large bundles with an
262 average thickness of 78 nm. The images of the G/Sep mixtures reveal a random and
263 homogenous dispersion of G within the Sep nanofibers, with very few agglomerates.
264 The G sheets appear well integrated within the silicate structure, and both
265 components seem to be organized as a stacking of planar sheets in a 3D arrangement,
266 giving rise to homogeneous materials at the micrometer scale.

267 *3.2.2 Extraction of riboflavin with sepiolite*

268 Riboflavin is a water-soluble vitamin with a structure comprising two different
269 parts, a hydrophilic ribose side chain as well as a hydrophobic isoalloxazine ring.
270 This fact enables the extraction of the vitamin molecule with different solid phases
271 that exhibit an intermediate polarity; hence, different G/Sep mixtures were tested.

272 Firstly, a dSPE extraction study of riboflavin was carried out using only Sep as
273 sorbent. For such purpose, 50 mg of Sep and 25 mL of a 0.25 mg L^{-1} riboflavin
274 solution were shaken for 10 min and subsequently centrifuged at 4000 rpm for 5 min.
275 The supernatant was then measured by fluorescence, and no riboflavin was detected,

indicative that the vitamin was quantitatively retained in the Sep sorbent. Clay-riboflavin interaction can occur via formation of H-bonds between the hydroxyl groups of the ribose chain and the silanol moieties of Sep, leading to the inclusion of the riboflavin molecule within the tunnels of the Sep structure.

After the efficient retention process, aqueous solutions of SDS, CTAC and Brij L23, at a concentration of 40 mM, concentration higher than the critical micelle concentration (CMC) in all cases, were tested as extracting solvents.

Calibration curves by the external standard method for riboflavin were carried out in all the solutions used as extractants, since the fluorescence intensity of riboflavin changes in presence of different surfactant solutions as it was studied previously (Mateos et al., 2017b). In 40 mM SDS solution, the average slope of two calibration curves is significantly lower than in 40 mM CTAC and 40 mM Brij L23, Therefore, all the extracts were analysed using the corresponding calibration curve obtained for the standards in the surfactant aqueous solution used as extractant.

In order to study the time required for riboflavin desorption, 10 mL of each surfactant solution were shaken with the solid for different periods ranging between 10 and 50 min, and then the mixture was centrifugated for 5 min at 4000 rpm.

The extraction yields obtained using SDS, CTAC and Brij L23 solutions are shown in **Fig.2a**. In SDS medium, the signal of riboflavin does not appear in the extract; therefore, no vitamin is recovered. The interaction of riboflavin with the silanol groups of Sep or its location within the tunnels of the clay structure seem to be more favourable than the interaction with the anionic micelles and location within their apolar core. CTAC is a better extractant than SDS, leading to a 25% recovery, and the highest yield (about 60%) is attained with the solution of the non-ionic

surfactant Brij L23. As mentioned earlier, this is the only surfactant that does not decrease the fluorescence of riboflavin in water; even more, it induces a slight fluorescence increase (see the change in the normalized fluorescence of the vitamin vs. Brij L23 concentration in **Fig.S3** in the ESM). Therefore, this surfactant provides the highest sensitivity and the best extraction yields. Brij L23 is clearly the best medium to desorb the vitamin retained in the sorbent, favouring the detachment of the riboflavin molecule from the hydroxyl groups of Sep due to competition for H-bond formation with the ether groups of the surfactant.

The dependence of the extraction yield on the agitation time when extracting with 10 mL of 40 mM Brij L23 is shown in **Fig.2b** for a shorter period of time than in **Fig.2a**. Taking into account these results, an agitation time of 5 min was selected, which is enough to reach the maximum extraction yield.

Once the extraction medium was chosen (40 mM Brij L23), the analytical characteristics of riboflavin analysis by fluorescence in this medium and in water were determined from four calibration curves performed in different days in both media, and the results are gathered in **Table 1**.

The best results were obtained with 40 mM Brij L23, but the maximum extraction yield obtained for the vitamin under these initial conditions was only 60%, hence it is necessary to optimize the method in order to improve this value. In this regard, the following parameters were optimized using this surfactant as extraction medium: Brij L23 concentration, agitation type and Sep mass.

The dependence of the extraction yield on Brij L23 concentration is shown in **Fig.3a**. The extraction yield increases with increasing surfactant concentration up to 25 mM, while for higher concentrations the extraction yield remains almost constant

at a maximum value of 60%. Therefore, Brij L23 concentrations higher than 25 mM are the most favourable for the extraction process.

The agitation energy applied during the dSPE process was also optimized to improve the extraction yield. Two types of agitation, mechanic and magnetic, were tested (see **Fig.3b)**, and the former one led to the highest yield. Moreover, the influence of the Sep mass on the extraction process was studied; the amount of Sep used for the extraction of 25 mL of a riboflavin solution (0.25 mg L^{-1}) was varied between 5 and 50 mg (**Fig.3c**). **A**n increase in the clay mass resulted in a rise in the extraction yield up to a maximum value close to 90% for a mass of 10 mg, while for higher Sep amounts the yield decreased again.

Up to this point, the best extraction conditions were mechanic agitation for 10 min followed by centrifugation at 4000 rpm for 5 min, and subsequent desorption with 10 mL of 40 mM Brij L23. In the low mass range, the significant increase in the extraction yield with increasing Sep mass is likely due to a stronger retention of the riboflavin molecule by the Sep surface in the presence of a larger mass of solid; however, for very high amounts of Sep compared to the riboflavin concentration, the desorption step becomes more difficult due to the excess of solid in the mixture. Thus, the highest extraction yield (93.3%) was obtained with 10 mg of Sep.

3.2.3 Riboflavin extraction using G/Sep mixtures

G/Sep mixtures were tested as sorbents for the extraction of riboflavin. For such purpose, 10 mg of the nanomaterial sorbent were mixed with 25 mL of a stock vitamin solution (0.25 mg L^{-1}) in water, and the results obtained for pure Sep and the three G/Sep mixtures prepared in this work are compared in **Fig.4**. The mixtures lead to lower extraction yields than neat Sep. The higher the G percentage, the lower the

extraction yield is. The extraction with Brij L23 is more effective when neat Sep is used as sorbent due to the more polar character of this silicate in comparison with G. These results can be rationalized as follows: the polarity of the G/Sep mixtures depends on the composition: the higher the G content, the lower the polarity is. The riboflavin-Sep interaction mainly occurs via H-bonding between the hydroxyl groups of the ribose chain and the silanol groups of Sep, but in the presence of G, π-π interactions between the aromatic rings of the flavin moiety and the π cloud of G can also take place. Thus, the interaction between riboflavin and the Sep structure is strengthened in the presence of G, although in all cases the retention is close to 100%. Accordingly, the desorption process is easier when the interaction only takes place with the hydroxyl groups of the ribose chain, since the π-π stacking interactions between the flavin moiety and G are harder to be broken by the nonionic surfactant solution; therefore, the recoveries decrease as the G percentage increases. Nonetheless, the mixtures with a G content of 2 and 5 wt% show recoveries for the whole dSPE process higher than 80%, hence they could be used as sorbents if there were interferences when using Sep alone, thus modifying the solid characteristics. The experimental results reveal that Sep itself, without any G, is the best solid sorbent for riboflavin extraction. This is also the simplest system and the easiest to be applied for the extraction process.

3.2.4 Influence of riboflavin concentration, sample volume and Brij L23 volume on the extraction yield

For the best conditions obtained previously in this study, the influence of other parameters such as the sample volume, the vitamin initial concentration and the volume of the extractant solution (40 mM Brij L23) were investigated, and the results are shown in **Fig.5**. The change in the extraction yield with the surfactant

volume (**Fig.5a**) reveals an increasing trend up to a volume of 5 mL. For higher volumes, the extraction yield remains constant with a maximum value of 92%. Consequently, 5 mL was chosen as the extraction volume.

The study of the influence of the riboflavin concentration on the extraction yield is very important to obtain information about the applicability of the extraction method. The extraction yield should not depend on the analyte concentration. In such case, a calibration curve can be obtained independently of the extraction yield. In this study, the extraction yield remains constant for riboflavin initial concentrations ranging between 0.02 and 0.25 mg L^{-1} (**Fig.5b**). After the extraction process, the concentration measured is five times higher due to the preconcentration process. Thus, it is possible to measure extract concentrations of 1.25 mg L^{-1} with a constant extraction yield of 93%.

On the other hand, the use of 50 mL of the initial sample instead of 25 mL provides the same extraction yields (see **Fig.5c)**. This results in a preconcentration factor of 10 instead of 5, which would be interesting if higher preconcentration is desired for some samples. **Table 2** compares the limits of detection reported in different works with a solid phase extraction step and that obtained in this work by dSPE using Sep and fluorescence in aqueous 40 mM Brij L23 solution. The limit of detection and limit of quantification of the whole dSPE/fluorescence method for riboflavin with a preconcentration factor of 10 are 3 and 11 μg L^{-1}, respectively. These values are lower than those obtained using C18 cartridges in SPE with detection by HPLC (Chatzimichalakis et al., 2004) (limit of detection of 0.17 mg L^{-1}), and with the disadvantage that the extraction process is longer since requires evaporation and reconstitution. The limit of detection using solid phase extraction with molecularly imprinted polymers and fluorescence is 34 μg L^{-1} (Osòrio et al,

2016). The use of resins as sorbents for riboflavin extraction in HPLC with mass detection (Wirkus et al., 2017) provides lower detection limits, but the method requires the use of organic solvents and an expensive instrumentation that it is not cost effective just for riboflavin determination.

3.2.5 Analysis of beer, soya drink and milk samples. Simple elimination of interferences

The proposed extraction and analysis method was applied to the determination of riboflavin in food samples with different matrices. These matrices have many components that can be fluorescent such as amino acids, proteins and other compounds; hence, it was tested the possibility of analysing them successfully using a combination of dSPE and fluorescence techniques.

Firstly, different beers with alcohol contents of 0%, 1.0% and 5.4% (v/v) were analyzed. A known amount of a stock riboflavin solution (0.5 mg L^{-1}) was added to the beer with 1.0% alcohol content in order to determine the accuracy of the developed analytical method. The fluorescence spectra of this beer sample, the sample with the spiked amount of riboflavin, the supernatant after extraction and the extract in 40 mM Brij L23 are shown in **Fig.6**. **Table 3** summarizes the concentration of riboflavin found in the three beers analysed, and **Table 4** compares the results obtained for the 1.0% alcohol beer without and with a spiked amount of riboflavin standard. The recovery values found for the spiked sample are very close to 100% (**Table 4**); further, the accuracy and the precision of the analysis are also excellent.

It is very important to highlight the appearance of interferences in the spectra of the samples that make impossible the differentiation between the riboflavin spectrum and that of the matrix of the sample. After the extraction process, which only took 30

422 min including the retention and desorption steps, the analyte was separated of the
423 matrix, desorbed with 5 mL of 40 mM Brij L23 solution, and the spectrum was free
424 from interferences, thus providing a very accurate and selective analysis.

425 The method was also applied to soya drink and infant milk samples, and their
426 spectra are shown in **Figs.7** and **8,** respectively. In these samples, the riboflavin
427 amount is labelled, and the recoveries obtained by the developed method are close to
428 100% (see **Table 5**). High interference signals are present in the samples that appear
429 identical in the supernatant of the extraction step (**Figs.7** and **8)**, indicating that the
430 interferent molecules remain in the liquid phase during the extraction step. Finally,
431 the riboflavin molecule is recovered with 5 mL of 40 mM Brij L23 aqueous solution,
432 and its spectrum can be clearly found in the extract.

433 **4. CONCLUSIONS**

434 Sepiolite is a very good solid sorbent for the retention of riboflavin when it is
435 mechanically shaken with the sample solution in dSPE. The use of pure sepiolite as
436 sorbent leads to higher retention than graphene/sepiolite mixtures. The highest
437 extraction yield, close to 93%, is obtained using 10 mg of sepiolite and 25 mL of the
438 sample.

439 The desorption of the riboflavin molecule from sepiolite clay using surfactant
440 solutions has been tested for the first time. The developed method provides a very
441 good extraction yield using as extracting medium a non-ionic surfactant such as Brij
442 L23, which is cheap and has very low toxicity. Therefore, the developed extraction
443 method is inexpensive, environmentally-friendly, fast and very simple to apply using
444 only aqueous media. Replacing traditional organic solvents used as extraction agents

by aqueous surfactant solutions offers great benefits to health, safety and sustainability.

The extraction method combined with fluorescence measurements has been successfully applied for the determination of riboflavin in several complex food samples: beers with different alcohol content, soya drink and infant milk. During the extraction process, the matrix interferences were eliminated, thus providing a very accurate and selective analysis. More interestingly, recoveries of 100% were attained by spiking the samples with a known amount of the stock riboflavin solution. The accuracy compared to the labelled amount of riboflavin in soya drink and infant milk was excellent.

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Coflicts of Interest

All authors declare that they have no conflicts of interest.

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616

617 **Table 1.** Analytical characteristics of riboflavin determination by fluorescence in
618 water and in 40 mM Brij L23 aqueous solution.

		Water	**40 mM Brij L23**
Linear range, mg L^{-1}		0.08-0.7	0.11-0.6
Correlation coefficient, r		0.9991	0.9973
Sensitivity, L μg^{-1}		0.534±0.001	0.60 ± 0.01
Limit of detection, mg L^{-1}		0.025 ± 0.006	0.03 ± 0.01
Limit of quantification, mg L^{-1}		0.08 ± 0.01	0.11 ± 0.06
Robustness, %RSD (n=4)		0.2	2.0
Repeatability, %RSD (n=4)		1.5 (0.3 mg L^{-1})	1.2 (0.3 mg L^{-1})
Reproducibility, % RSD(n=4)		11 (0.15 mg L^{-1})	2.2 (0.15 mg L^{-1})
		3.3 (0.3 mg L^{-1})	4.3 (0.3 mg L^{-1})
		1.5 (0.6 mg L^{-1})	1.6 (0.6 mg L^{-1})

619

620

621 **Table 2.** Comparison of the limits of detection obtained by different methods using
622 solid phase extraction and that obtained in this work.

Solid phase extraction sorbent	Analysis technique	Limit of detection	Samples	Reference
C18	HPLC UV	170 µg L^{-1}	Pharmaceuticals Serum	Chatzimichalakis et al, 2004
C18	HPLC-UV (DAD)	0.2 mg L^{-1}	Tarhana (Turkish cereal)	Ekinci and Kadakal, 2005
C18	HPLC UV	0.83 mg L^{-1}	Fruit Vegetables	Plonka et al, 2012
Molecular imprinted polymer (on-line)	HPLC-UV (DAD)	0.05 mg L^{-1}	Energy drink Infant formula Pig liver	Oliveira et al, 2010
Molecular imprinted polymer	Fluorescence	34 µg L^{-1}	Milk, Infant formula	Ossorio et al, 2016
Nanoparticles	Colorimetric	167 nM (62 µg L^{-1})	Tablet	Ma et al., 2016
Functionalized nanoparticles	HPLC-UV	1-10 mg L^{-1} B complex	Plasma	Ali et al., 2016
Graphite carbon nitride	FRET	170 nM (64 µg L^{-1})	Milk Vitamin drink	Han et al., 2016
Ion Exchange resins	HPLC-MS/MS	0.17 µg L^{-1}	Renal ultrafiltrates	Wirkus et al., 2017
Sepiolite	Fluorescence	3 µg L^{-1}	Beer, Milk, Soya drink	This work

623

624 **Table 3.** Analysis of riboflavin in beer samples containing different alcohol percentages.

% alcohol v/v	**5.4**		**1.0**		**0.0**	
[riboflavin], mg L^{-1}	**Extract**	**Beer**	**Extract**	**Beer**	**Extract**	**Beer**
Sample 1	0.434	2.17	0.256	1.28	0.443	2.21
Sample 2	0.430	2.16	0.264	1.32	0.394	1.97
Sample 3	0.434	2.18	0.263	1.31	0.443	2.21
Sample 4	0.437	2.17	0.255	1.27	0.430	2.15
Mean ± SD	0.434 ± 0.003	2.17 ± 0.01	0.259±0.005	1.30 ±0.02	0.43 ± 0.02	2.1 ± 0.1

625 SD: Standard Deviation

626

627

628 **Table 4.** Analysis of riboflavin in a beer (1.0% alcohol content) without and with a spiked amount of a standard riboflavin.

629

Spiked amount = 0.5 mg L^{-1}

[riboflavin] mg L^{-1}	Extract	Difference	Beer	Difference	% Recovery
Sample 1	0.355	0.099	1.77	0.49	99
Sample 2	0.362	0.098	1.81	0.49	99
Sample 3	0.358	0.095	1.79	0.48	95
Sample 4	0.359	0.104	1.79	0.52	104
Mean ± SD	0.358±0.003	0.099±0.004	1.79 ±0.02	0.49±0.02	99 ± 4

SD: Standard deviation

630

631

Table 5. Analysis of riboflavin in soya drink and infant milk samples.

SOYA DRINK, [Riboflavin] labelled = 2.1 mg L^{-1}

[riboflavin], mg L^{-1}	**Extract**	**Infant Milk found**	**% Recovery**
Sample 1	0.405	2.02	96.4
Sample 2	0.418	2.09	99.6
Sample 3	0.424	2.12	101.0
Sample 4	0.409	2.05	97.5
Mean ± SD	0.414 ± 0.009	2.07 ± 0.04	98 ± 2

INFANT MILK, [riboflavin] labelled = 1.2 mg L^{-1}

[riboflavin], mg L^{-1}	**Extract**	**Milk Infant found**	**% Recovery**
Sample 1	0.236	1.18	98.4
Sample 2	0.235	1.17	97.8
Sample 3	0.240	1.20	99.8
Sample 4	0.240	1.20	99.8
Mean ± SD	0.238 ± 0.002	1.19 ± 0.01	99 ± 1

SD: Standard deviation

632 **Figure Captions**

633 **Fig.1** SEM micrographs of neat G, Sep and G/Sep mixtures with 2, 5 and 10 wt% G
634 content, at a magnification of x20000.

635 **Fig.2** Extraction yield of riboflavin vs. agitation time using 50 mg of Sep and with
636 extraction medium 10 mL of 40 mM (a) SDS, CTAC and Brij L23 aqueous
637 solutions; (b) Brij L23 for shorter agitation times.

638 **Fig.3** Extraction yield of riboflavin using a) 50 mg of Sep and with extraction medium
639 10 mL of different concentrations of Brij L23; (b) 50 mg of Sep, 10 mL of 40
640 mM Brij L23 as extracting medium and different agitation types; (c) different
641 mass amounts of Sep and 10 mL of 40 mM Brij L23 as extraction medium.

642 **Fig.4** Extraction yield of riboflavin using 10 mg of different G/Sep mixtures as sorbent
643 and 10 mL of 40 mM Brij L23 as extraction medium.

644 **Fig.5** Extraction yields of riboflavin as a function of a) volume of 40 mM Brij L23; b)
645 concentration of riboflavin in the 25 mL of the initial solution; c) initial volume
646 of the riboflavin 0.25 mg L^{-1} solution.

647 **Fig.6** Fluorescence contour graphs of the beer sample with 1.0% alcohol content, the
648 same sample spiked with a riboflavin stock solution 0.5 mg L^{-1}, the supernatants
649 obtained after extraction for both the spiked and unspiked samples and the
650 extract using 5 mL of 40 mM Brij L23 as extraction medium without the
651 standard riboflavin solution.

652 **Fig.7** Fluorescence contour graphs of the soya drink sample, the supernatant after
653 extraction and the extract using 5 mL of 40 mM Brij L23 as extraction medium.

Fig.8 Fluorescence contour graphs of the infant milk sample, the supernatant solution after extraction and the extract using 5 mL of 40 mM Brij L23 as extraction medium.

Figure 1

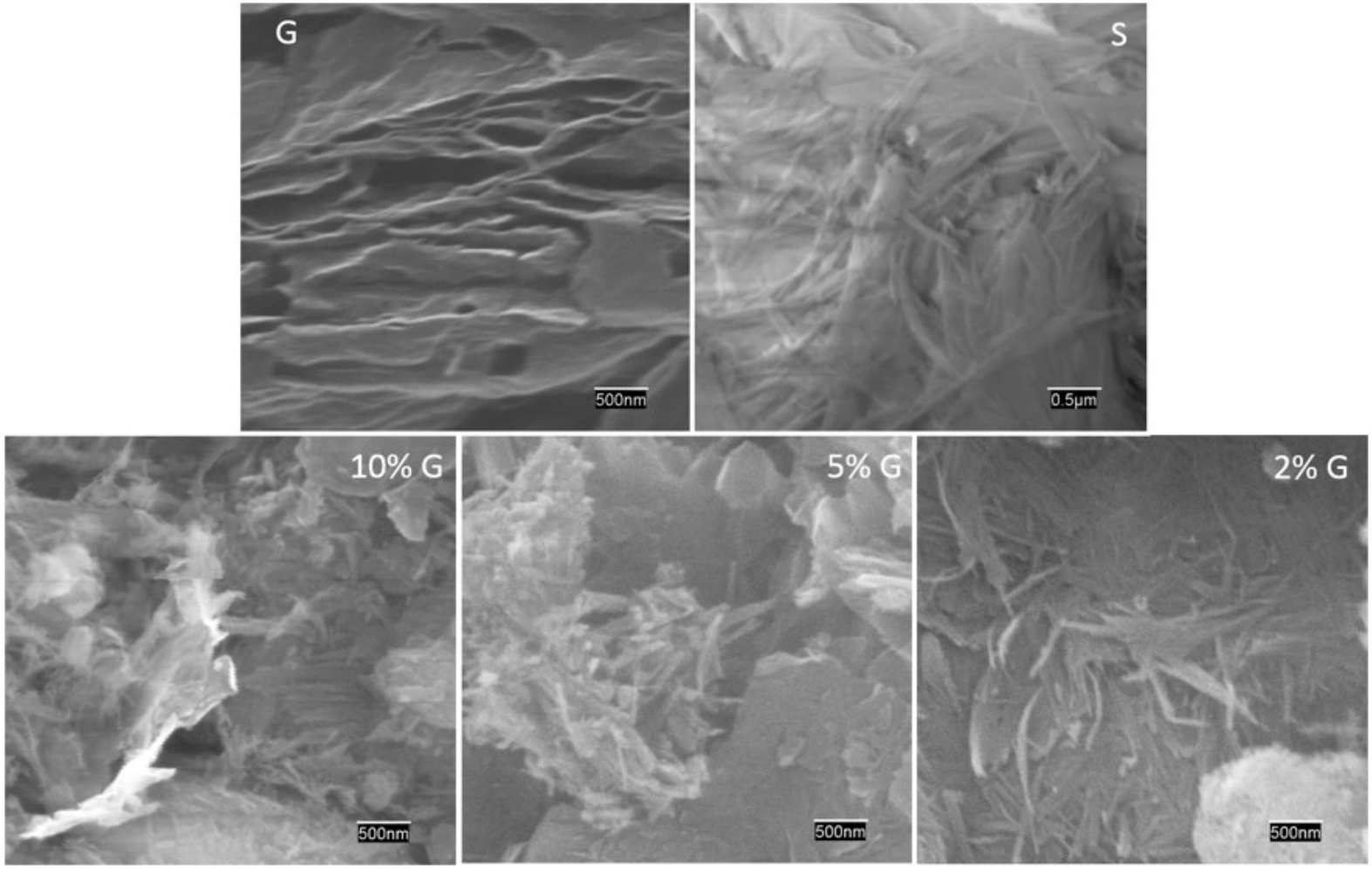

Figure 2

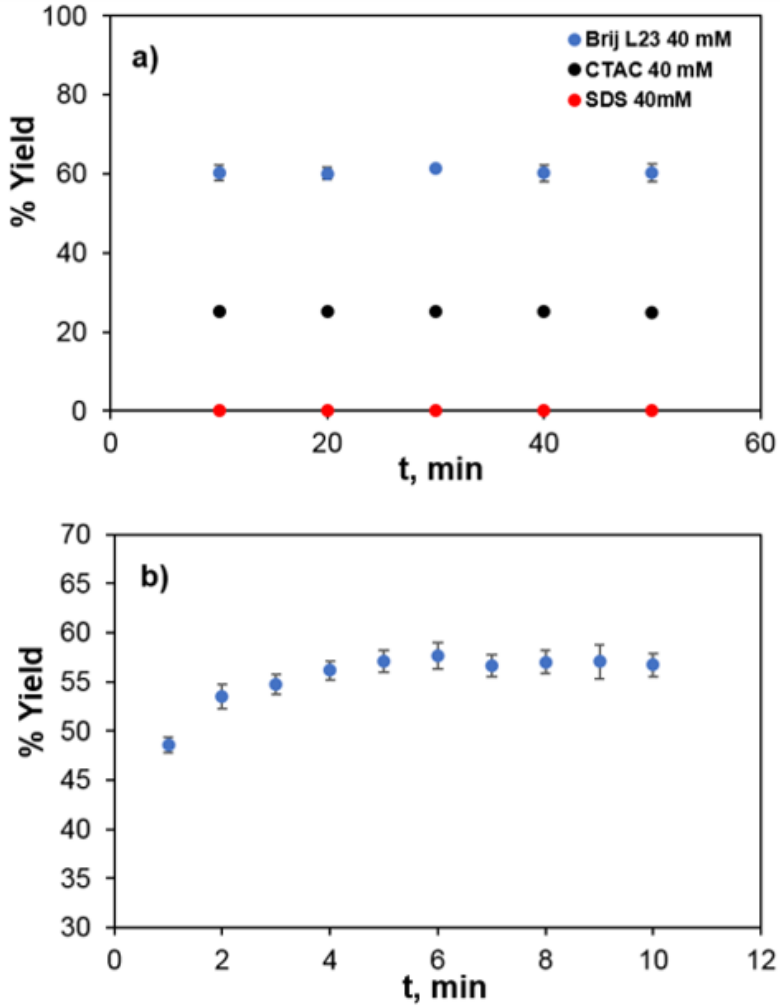

Figure 3

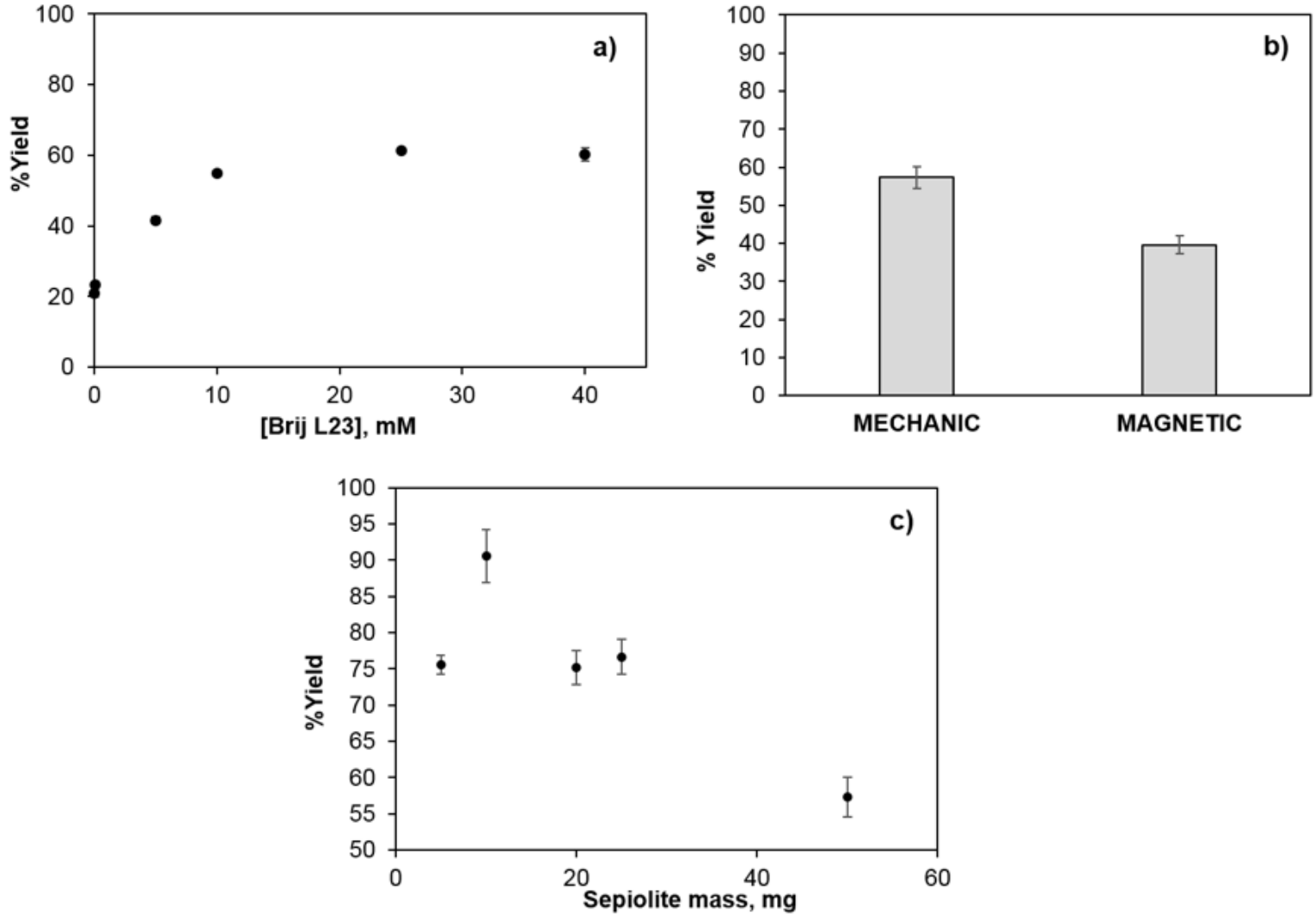
a)
%Yield
[Brij L23], mM
b)
% Yield
MECHANIC
MAGNETIC
c)
%Yield
Sepiolite mass, mg

Figure 4

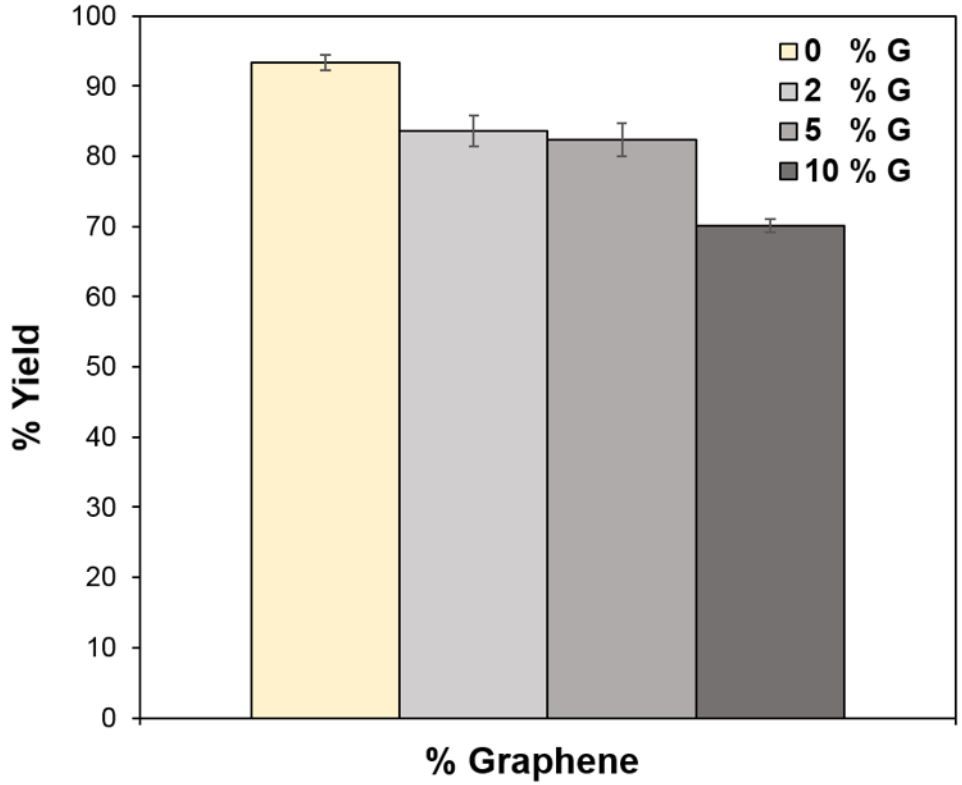

Figure 5

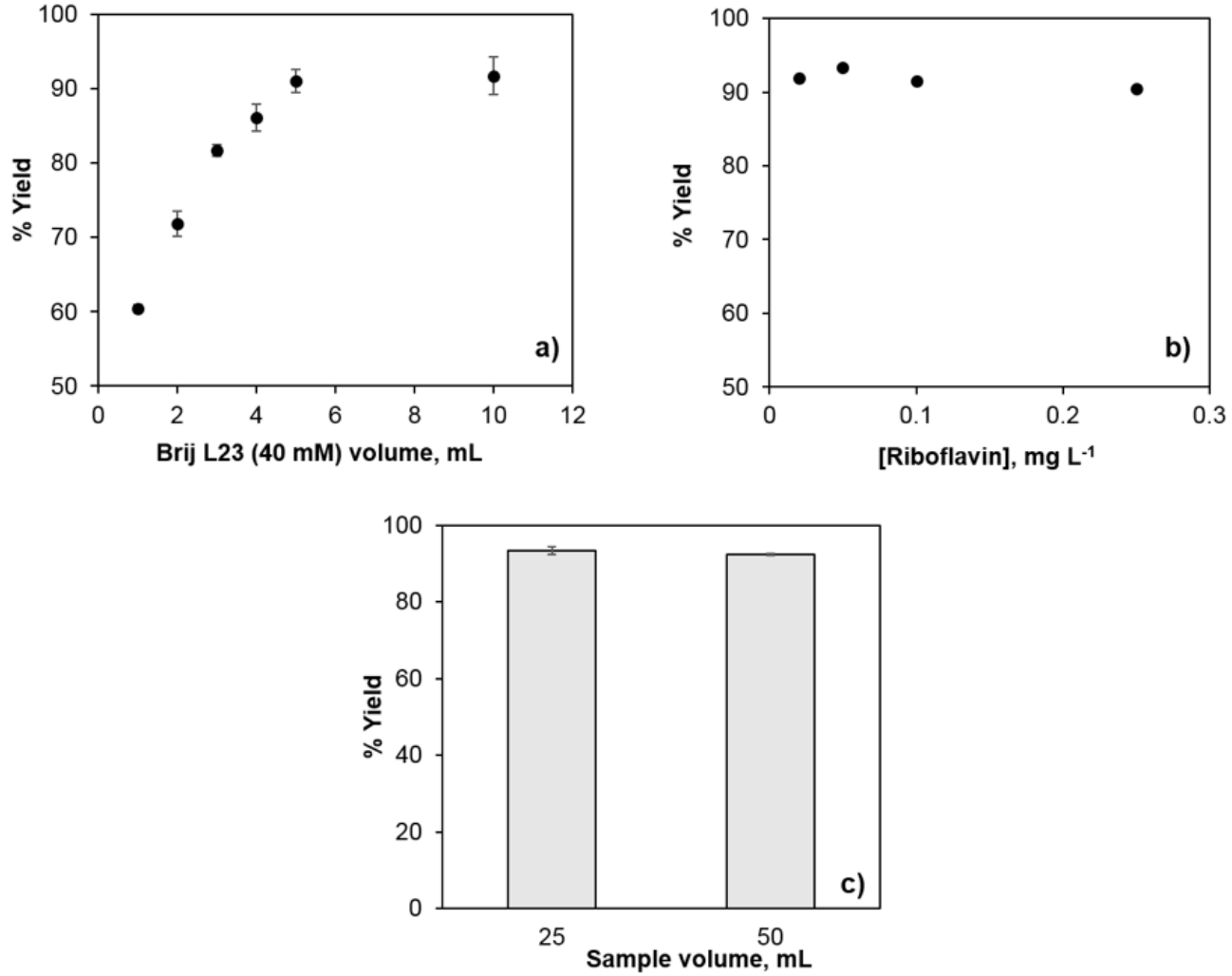
100
90
80
70
60
50
% Yield
0
2
4
6
8
10
12
Brij L23 (40 mM) volume, mL
a)
100
90
80
70
60
50
% Yield
0
0.1
0.2
0.3
[Riboflavin], mg L-1
b)
100
80
60
40
20
0
% Yield
25
50
Sample volume, mL
c)

Figure 6

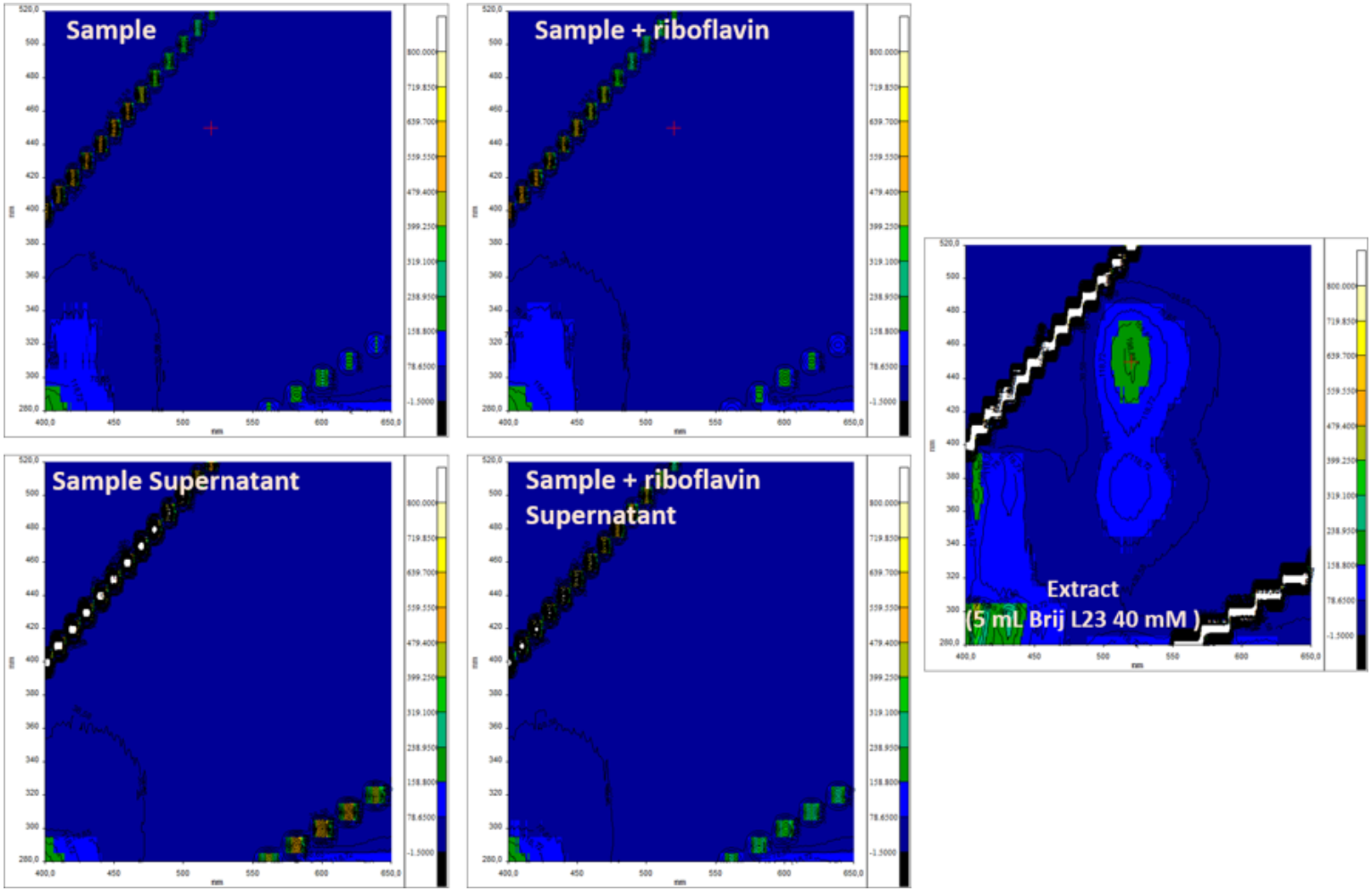
Sample
Sample + riboflavin
Sample Supernatant
Sample + riboflavin
Supernatant
Extract
(5 mL Brij L23 40 mM)

Figure 7

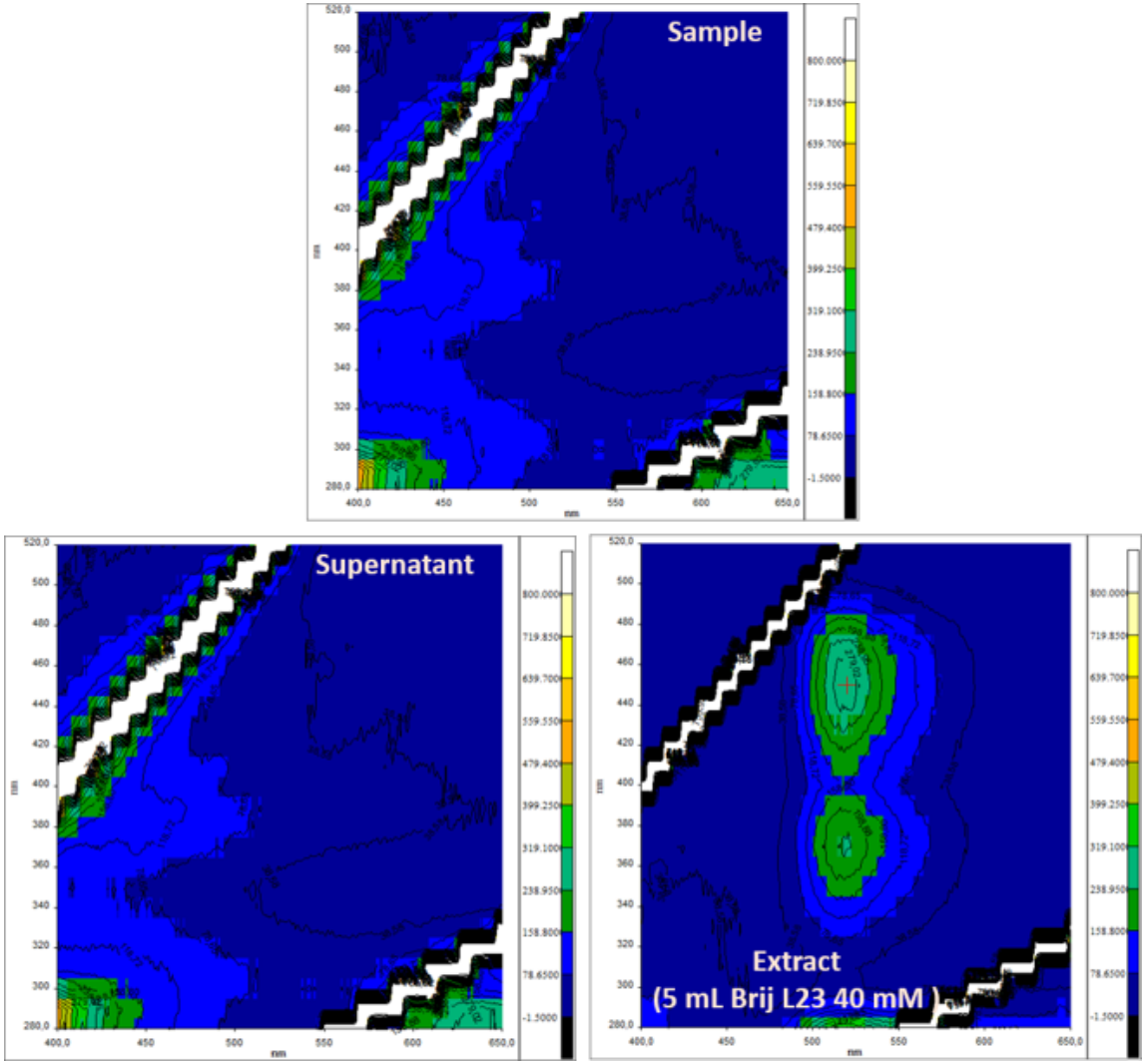

Figure 8