Labdanum resin from *Cistus ladanifer* L.: evaluation of residual water *vs.* extraction yield

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Abstract

Cistus ladanifer L. (*Cistaceae*) is an endemic and abundant resource in the Iberian Peninsula and North Africa. This plant exudes an aromatic resin nowadays valued in the perfumery and fragrance industry. Traditional processes for the extraction and isolation of such resin use boiling water or alkaline water followed by acidic precipitation. However, a concern arises about the effluents resulting from these extraction processes. To overcome this concern, labdanum resin was extracted with Na_2CO_3 solution (25 g/L) at 60 ºC and precipitated with sulphuric acid (5 M). The residual water was evaluated regarding total phenolic content, suspended solids, electric conductivity, and sulphate, sodium, magnesium, and calcium content. The effluent was characterized by a total phenolic content of 1245 ± 455 mgGAeq/L, 1338 ± 101 mg/L of suspended solids, pH of approximately 2, electric conductivity of 34.8 ± 0.7 mS/cm, 22284 ± 710 mg/L of sulphate, 9696 ± 1072 mg/L of sodium, 3.97 ± 0.24 mg/L of magnesium, 3.52 ± 0.80 mg/L of calcium, and a Sodium Adsorption Ratio of 876 ± 112. Because the values were far from the limit values set by Portugal decree-law 236/98 for residual waters discharged and irrigation waters, it was concluded that efforts should be made to optimize the extraction process. In that regard, a factorial designed experiment was done to evaluate the effect of Na_2CO_3 concentration (0; 2.5; and 25 g/L), extraction temperature (60 and 100 ºC) and acidification extent (pH 2, neutralization, and no acidification) on the residual water quality and on the yield of labdanum resin extraction. Alkalinization and acidification are important to obtain high resin extraction yields (Andalusian *vs.* Zamorean process), but mostly alkalinization may be reduced to meet sulphate criteria for discharge without significantly affecting resin extraction yields. Despite that, to meet salinity criteria for irrigation waters a higher reduction in alkalinization is needed for Andalusian processes. Phenolic content, although lower for extractions done at 60 ºC, was far from the limit values for discharge, regardless experimental conditions. Given the high phenolic content the residual water from labdanum extraction by both traditional processes must be treated before discharge. If separated, phenolic compounds may be valorized as a by-product.

Keywords: Acidic precipitation, Alkaline extraction, By-product, Effluent, Rockrose, Sustainability

Introduction

Cistus ladanifer (Cistaceae) is an evergreen shrub abundantly distributed in the western Mediterranean: south of France, Iberian Peninsula, North Marrocos and Argelia (Demoly and Montserrat 1993; Godinho-Ferreira et al. 2005; Montero et al. 2020). Like many *Cistus* species (Gülz et al. 1996), this plant exudes an aromatic terpenoid and phenolic resin called labdanum. This resin is commercially traded by the name of "labdanum gum" mainly for the perfumery and fragrance industry sectors (Raimundo et al. 2018).

Traditionally, labdanum resin is extracted, using water, by two different processes: the Zamorean process and the Andalusian process (Lawrence 1999; Morgado et al. 2005; Burguer 2016). The Zamorean process consists of a physical extraction where labdanum resin is removed from the surface of *C. ladanifer* plant material using boiling water and then the resin at the surface of the water is continuously separated using a skimmer. The Andalusian process is a chemical extraction where the labdanum resin is extracted from the plant surface with warm alkaline water and afterwards precipitated by lowering the pH with acid and finally separated with a skimmer or by decantation. Andalusian process is currently used as the industrial process and annually between 100 and 1000 tons of labdanum gum is extracted from *C. ladanifer*, according to European Chemicals Agency (ECHA) registrations.

Given the abundance of the plant resource and the use of water as solvent, labdanum gum extraction process line up with "green extraction principles" which in turn are derived from the "green chemistry" and "green engineering" principles (Chemat et al. 2019). However, this "green" approach also highlights the importance of the solvent recovery and the production of valuable by-products instead of waste. Recovery of water is facilitated either by nature or, if needed, by residual water treatment plants. Knowledge is scarce, within scientific literature, about the residual water of the labdanum extraction processes.

This study aims to assess the effluent by evaluating some water quality parameters relevant not only for residual water discharged in the environment but also for its use as irrigation water, according to the guidelines defined by the Portuguese Decree-Law 236/98 which regulates quality criteria and rules for various uses of water, including human consumption, irrigation and residual water discharged in natural waters and soils.

Materials and Methods

Plant material and labdanum extraction (reference method)

Herbaceous and semi-woody plant material was collected from a 5-year-old *C. ladanifer*, located in Penha Garcia, Portugal (GPS coordinates in DMS:40º1'43.4''N 6º59'34.8''W), in August 2018. Labdanum resin was extracted by the method described in Burguer (2016), simulating the Andalusian process, with some modifications. Briefly, 25 g of plant material were immersed in 200 mL of Na_2CO_3 solution (25 g/L) for 1 h at 60 ºC. The solution was cleared from the plant material through filtration and left to rest overnight. Then, H_2SO_4 (5 M) was added slowly to the stirring solution at room temperature to decrease its pH until it reached 2. The precipitated resin was separated from the supernatant by centrifugation (3030 x*g* for 15 min, Mega Star 600R, VWR). The supernatant is the residual water, which was kept for analysis. The solution initially showed a strong black color and after precipitation of the resin it became transparent with a yellow/orange color. The precipitated resin was freeze-dried, and labdanum absolute was obtained by dissolving 1 g of the resin in 20 mL of methanol under an ultrasonic bath, waxes precipitation at -20 ºC overnight and separation by centrifugation (3030 x*g* at -5 ºC for 15 minutes). The procedure was repeated three times and the three methanolic solutions were joined as the absolute extract, which finally was dried using a rotatory evaporator operating at 30 ºC.

Experimental design of method variations

To evaluate the effect of extraction conditions in extraction yields and residual water quality parameters, using the reference method (section 2.1) three independent factors were studied: i) Na_2CO_3 concentration: 0 (simulating Zamorean process), and 2.5 and 25 g/L (simulating Andalusian process); ii) Acidification extent with H_2SO_4 5 M: no acidification (s/), neutralization of carbonates i.e. until CO_2 stops to be release (N), and until pH = 2; iii) temperature of extraction: 60 and 100 ºC. All methods were done in triplicate (n = 3). Resin and resin absolute yields were recorded, and the supernatants were kept for analysis.

Residual water analytical methods

The residual water obtained by the reference extraction method was characterized by standard methods for the examination of water and wastewater (APHA-AWWA, 2012) for the following parameters: total suspended solids were determined by filtration through a 45 µm filter and drying at 105 ± 2 ºC; total sulphate concentration was determined using a gravimetric method based on sulphate precipitation as barium sulphate by the addition of barium chloride and drying the residue at 105 ± 2 ºC; total sodium (Na^+), total calcium (Ca^{2+}), and total magnesium (Mg^{2+}) concentrations were determined by flame atomic absorption spectrometry (Thermo Scientific ice 3500); electrical conductivity was measured using a conductivity meter (WTW inoLabCond Level 1); pH was monitored using a pH meter (Consort C3060).

The potential degree of sodification of soils, due to the use of residual water for irrigation, was evaluated by the sodium absorption ratio (SAR): ionic strength ratio of Na^+ concentration to the Ca^{2+} and Mg^{2+} concentration, calculated according to equation 1:

$$SAR = \frac{[Na^+]}{\sqrt{\frac{[Ca^{2+}] + [Mg^{2+}]}{2}}} \qquad (1)$$

Sulphate and sodium content of the residual waters obtained with other experimental conditions was estimated directly from the volume of H_2SO_4 5 M solution and quantity of Na_2CO_3 spent for the extractions.

Total phenolic content was done on filtered residual waters from all experimental conditions by the Folin-Ciocalteu method. Briefly, 2.5 mL of diluted sample (1:10) was mixed with 0.5 mL of Na_2CO_3 solution (7.5% w/v), 1.75 mL of distilled water and 0.25 mL of Folin-Ciocalteu reagent. The reactive mixture was incubated at room temperature, in the dark, for 2 h and the absorbance was read at 725 nm. Sample blanks were prepared by changing the Folin-Ciocalteu reagent for distilled water and their absorbances were subtracted. Standard solutions were prepared with gallic acid at concentrations between 0.1 and 0.003mg/mL. Results are expressed as gallic acid equivalents (mgGAeq/mL).

Statistical analysis

Independent t-tests (Heteroscedasticity: Levene's test $\rho < 0.05$) or Welch's t-tests (Homoscedasticity Levene's test $\rho \geq 0.05$) were used to evaluate effluent quality parameter mean difference between each method variation in relation to the reference method. Data normality was confirmed by performing the Shapiro-Wilk's test ($\rho \geq 0.05$). Statistical analysis was done for a 95% confidence level ($\alpha = 0.05$) using the IBM SPSS Statistics 25 software.

Results and Discussion

Labdanum resin and absolute yields, as dry weight in relation to plant material fresh weight, obtained with all combinations of extraction conditions are shown in Figure 1. The extraction conditions described by Burguer (2016) were considered the reference method because: i) 25 g/L Na_2CO_3 solution (pH ≈ 11.00) maintained higher pH than 2.5 g/L solution or distilled water, after extraction; ii) A temperature of 60 °C is usually less harmful for phytochemical compounds than 100 °C; iii) Acidification until pH 2 resulted in a transparent supernatant/residual water in contrast to turve supernatants resulted from neutralization.

In Figure 1, statistical differences are reported in relation to the reference method. Differences in resin and absolute extraction yields were not found between extraction with 2.5 or 25 g/L Na_2CO_3, 60 and 100 °C, and acidification until pH = 2 or until CO_3^{2-} neutralization (N). Extraction with distilled water resulted in lower resin and absolute yields compared to the reference method regardless of other conditions., and the same happened when acidification was not done (s/), except for labdanum resin extracted at 100 °C with 25 g/L Na_2CO_3. Extraction with water alkalinized with Na_2CO_3 at least at 2.5 g/L and acidification at least until neutralization of CO_3^{2-} are thus important to obtain high yields of labdanum resin and absolute, like the reference method yields. Temperature of extraction (60 or 100 °C) did not show to have influence on extraction yields as well.

Burguer (2016) obtained labdanum resin by a process similar to the reference method of this study with a yield between 7.79-8.86 % (dw/dw) in relation to the plant material which is slightly lower given the fact that in this study results are reported in relation to the fresh weight of plant material. The author used filtration to recover the precipitated resin instead of centrifugation. In fact, filtration was attempted in preliminary experiments but excluded because of the difficulty to recover the resin from the filter. Morgado et al. (2005) obtained a labdanum extraction yield of between 7-18 % (dw/dw) in relation to usable biomass. Authors extracted the resin with a solution of Na_2CO_3 (10 g/L) at 50 °C, precipitating the gum with H_2SO_4 93% (v/v) which was left to rest for 24 hours and separated by decantation and dried at room temperature. Greche et al. (2009) extracted 750 g of plant material with 10 L of an aqueous solution of $NaHCO_3$ at 8.4 g/L for 10 min at 60-70 °C. Then the authors neutralized the solution with HCl at 0.2 M and removed the resin with a perforated skimmer, but extraction yield was not reported.

Labdanum absolute yields in relation to the resin weight are presented in Table 1. In contrast to both resin and absolute yields in relation to the plant material, temperature was the only factor affecting variation of absolute yields in relation to the resin, decreasing at 100 °C. Except for extraction at 100 °C with 25 g/L Na_2CO_3 and without acidification, the decrease in absolute yield in relation to the resin weight compared to the reference method was not enough to produce a statistical difference in the absolute yield in relation to plant material compared to the reference method. Several authors extracted labdanum absolute from labdanum resin by dissolving the dried extracts or resin in warm methanol or ethanol and then precipitating and removing waxes using negative temperatures (de Pascual et al. 1984; Vogt et al. 1987; Chaves et al. 1997; Sosa et al. 2005; Greche et al. 2009; Alías et al. 2012; Burguer 2016). According to Burguer (2016) methanol extracts more waxes (38 % dw/dw) by this process than ethanol (8.5 % dw/dw). In fact, Greche et al. (2009) precipitated waxes from the labdanum absolute prepared with ethanol and even so, wax compounds appeared as significant compounds in the ethanol extract.

Using validated methods, the residual water from the reference labdanum resin extraction method was evaluated regarding relevant water quality parameters for residual water discharge to the environment and/or use as irrigation water, according to Portuguese decree-law 263/98 (Table 2). Residual water presented values far from the limit values for all the parameters. High suspended solids may be overcome by enhancing the separation process (higher centrifugation or filtration), reducing the parameter to acceptable limits but also recovering more labdanum resin and thus increasing the extraction yield.

Sulfate and sodium ions are present in high amounts in the residual water of the reference method, contributing significantly for the high salinity (total dissolved salts/electric conductivity and SAR). In fact, sulfate content was ten or forty-fold higher than the sulfate limit values for residual water discharge or irrigation water, respectively. Similarly, sodium content alone is more than fifty-fold higher than the total dissolved salts limit for irrigation water, to which sulfate content should also be considered. Those two ions are a direct product of the alkalinization and acidification steps with Na_2CO_3 and H_2SO_4, respectively, during the extraction of the resin. Therefore, their quantity in the residual water is dependent on the quantity of reagents used, i.e, on the Na_2CO_3 concentration during extraction and acidification extent. In addition, the quantity of acid needed to neutralize CO_3^{2-} is directly proportional to Na_2CO_3 concentration. Extractions with 2.5 g/L of Na_2CO_3 needed much less quantity of H_2SO_4 to neutralize carbonates and thus the estimated sulphate content of the residual waters dropped to near or even below the limit values for wastewater discharge (Figure 2). Acidification extent logically affected sulfate content of the residual water and when acidification was not done, sulphate content is expected to be zero. Sodium content in the residual water is also dependent on Na_2CO_3 concentration of the extraction solution. Using a concentration of 2.5 g/L in the extraction solution, sodium content in the residual waters is estimated to be near the maximum recommended value for total dissolved salts in irrigation waters (Figure 3). Both sulfate and sodium ions contribute to water salinity and even at conditions such as 2.5 g/L of Na_2CO_3 and neutralization with H_2SO_4 the residual water would not be recommended for irrigation.

Total phenolic content of the reference method (1245 ± 455 mgGAeq/L) is far from the limit emission value for wastewater discharge (0.5 mg/L, Table 2). In fact, this parameter is far from the limit value regardless of the extraction conditions and showed to be mainly increased by higher extraction temperature since, compared to the reference method, all extractions done at 100 ºC presented a significantly different value, between two and six-fold higher (Figure 4). Folin-Ciocalteu method is not amongst the reference analytical methods to quantify phenols defined by Decree-law 236/98. This method usually indicate higher phenolic content than the Decree-Law reference 4-aminoantipyrine method because it reacts with more types of phenolic compounds but also more with interferents such as ascorbic acid and reducing sugars (Stratil et al. 2007).

Water extracts of *C. ladanifer* milled plant material had shown to be rich in phenolic compounds such as phenolic acids, flavonoids aglycones and glycosylated, and tannins (Barrajón-Catalán et al. 2010; Fernández-Arroyo et al. 2010). Given the high content in phenolic compounds, residual waters from labdanum extraction are not in conditions to be discharged by any means. Phenolic compounds are regarded as toxic pollutants to humans, animals and aquatic life and several methods are available to reduce their content in residual waters by means of separation or degradation (Villegas et al. 2016). If separated, polyphenolic compounds may be regarded as by-products and find applications for food and biological systems (Barrajón-Catalán et al. 2010). In fact, phenolic compounds from olive mill residual waters have been showing potential as food additives and preservatives (Galanakis 2018).

Residual water pH is a direct consequence of the acidification extent: compared to the reference method, pH of the residual waters is higher for methods with none or less acidification (Figure 5). Neutralization rendered residual waters with a pH within the upper and lower limit emission values for residual water discharge and admissible values for irrigation waters. Extraction with 2.5 g/L of Na_2CO_3 also rendered residual waters with pH within the upper and lower limits. Compared to the reference method, both conditions did not significantly affect labdanum resin and absolute extraction yields but when done together those conditions significantly decrease them (Figure 1). As discussed above, neutralization produces a very turve residual water which should mean higher suspended solids and thus less resin recovery, however not clearly showed by this study.

Conclusion

Regarding aqueous labdanum resin extraction, alkalinization of the water followed by acidification (Andalusian process) renders higher resin and resin absolute yields in comparison to the simulated Zamorean process. However, overall salinity, sulphate content, and phenolic content do not allow the residual water of the Andalusian process to be used as irrigation water or to be discharged as a wastewater before any treatment, according to the Portuguese Decree-Law 263/98. Salinity and sulphate content of the residual water are significantly reduced using 2.5 g/L instead of 25 g/L of Na_2CO_3 for alkaline extraction without significantly reducing yields, however not enough for the residual water to be recommended for irrigation. Acidification until neutralization reduced directly sulphate content compared to acidification until pH = 2 but, when together with Na_2CO_3 concentration reduction, it significantly reduced extraction yields. Total phenolic content of the residual water is far above limits regardless the extraction conditions, significantly lower when using 60 ºC instead of 100 ºC, limiting its discharge to the environment. Extraction parameters may still be optimized to render less pollutant residual waters, mainly in what regards salinity for Andalusian processes, but a pre-treatment to remove phenolic compounds from the residual water, regardless the traditional process, will still be needed before discharging in the environment. If those phenolic compounds are recovered, they may be considered a by-product worth to valorise.

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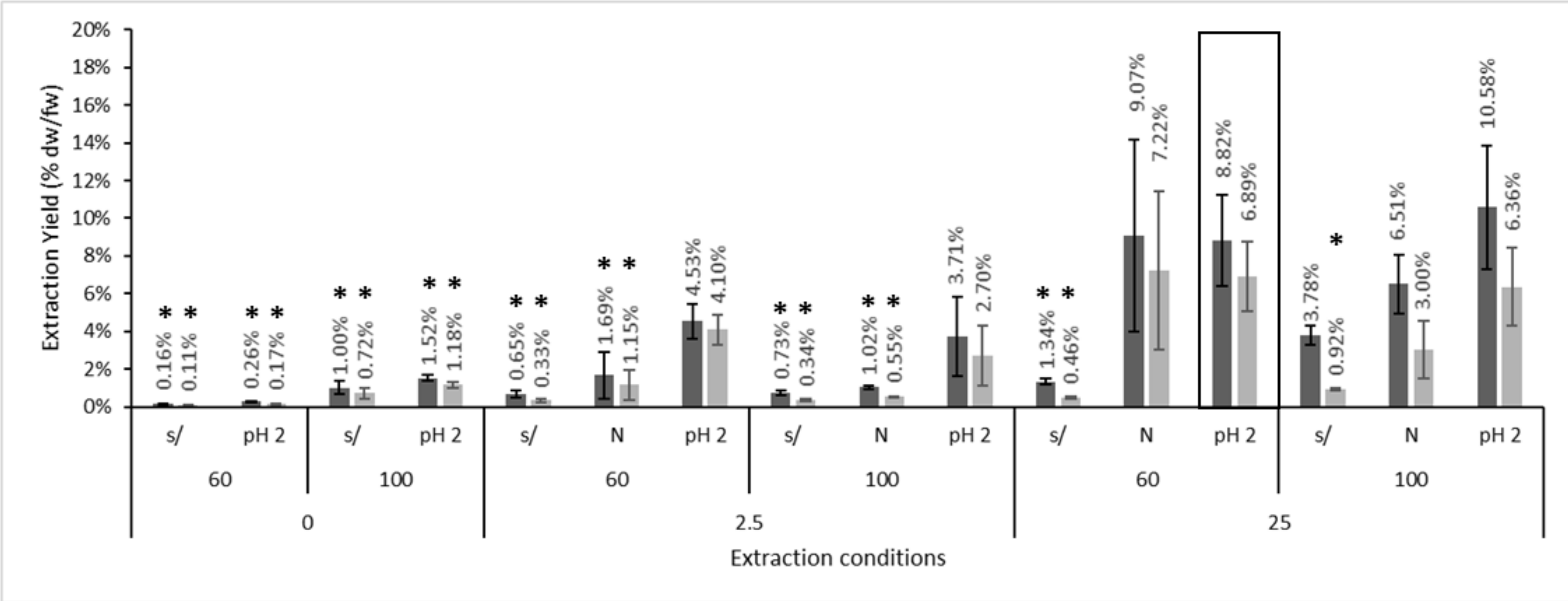

Figure 1- Labdanum resin (dark grey) and labdanum methanol absolute (light grey) extraction yields in relation to plant material weight (mean, n = 3, % dw/fw), for the various combinations of extraction conditions (base to top: Na_2CO_3 (g/L), T (ºC), Acidification extent). "s/" and "N" means no acidification and carbonates neutralization, respectively. Error bars correspond to the 95 % confidence interval. (*) statistical difference in relation to the reference (marked with rectangle), found by performing independent t-tests for equal variances not assumed (α = 0.05).

Table 1- Labdanum absolute yield, in relation to resin (% dw/dw), for the various combinations of extraction conditions (n = 3, mean ± 95 % confidence interval). "s/" and "N" means no acidification and carbonates neutralization, respectively. (*) statistical difference absolute yields and the yields obtained with the conditions (marked with rectangle), found by performing independent t-tests for equal variances not assumed (α = 0.05).

Na_2CO_3 (g/L)	**0**				**2.5**						**25**					
T (ºC)	**60**		**100**		**60**			**100**			**60**			**100**		
Acidification extent	**s/**	**pH 2**	**s/**	**pH 2**	**s/**	**N**	**pH 2**	**s/**	**N**	**pH 2**	**s/**	**N**	**pH 2**	**s/**	**N**	**pH 2**
Yield (% dw/dw)	69.7 ± 6.8	65.0 ± 5.8	71.2 ± 2.1	77.2 ± 3.2	50.1 ± 1.7*	70.8 ± 6.5	90.7 ± 0.8	46.2 ± 0.9*	53.4 ± 3.3*	71.9 ± 3.0	34.4 ± 1.8*	79.0 ± 1.7	78.2 ± 5.3	24.6 ± 3.5*	44.5 ± 14.2*	59.8 ± 1.1*

Table 2- Water quality parameters of the residual water from the reference method used to extract labdanum resin, assessed by analytical methods or by estimation considering the quantity of reagents used (mean values ± estimative error at 95 % confidence interval). Emission limit value (VLE) for residual water discharge and allowable maximum limit (VMA) for irrigation waters as regulated by the Portuguese decree-law 263/98.

Parameter	Residual Water		Discharge (VLE)	Irrigation water (VMA)
	Method	Estimated		
Suspended solids (mg/L)	1338 ± 101		60	60
Sulfate (SO_4^{2-}, mg/L)	22284 ± 710	24019 ± 959	2000	575
Sodium (Na^+, mg/L)	9696 ± 1072	11709 ± 261		
Magnesium (Mg^{2+}, mg/L)	3.97 ± 0.24	-	-	640 (total dissolved salts)
Calcium (Ca^{2+}, mg/L)	3.52 ± 0.80	-		
SAR	876 ± 112	-	-	8
Electric conductivity (mS/cm)	34.8 ± 0.7	-	-	1
pH	2.02 ± 0.01	-	6.0-9.0	4.5-9.0
Phenols (mgGAeq/L)	1245 ± 455	-	0.5	-

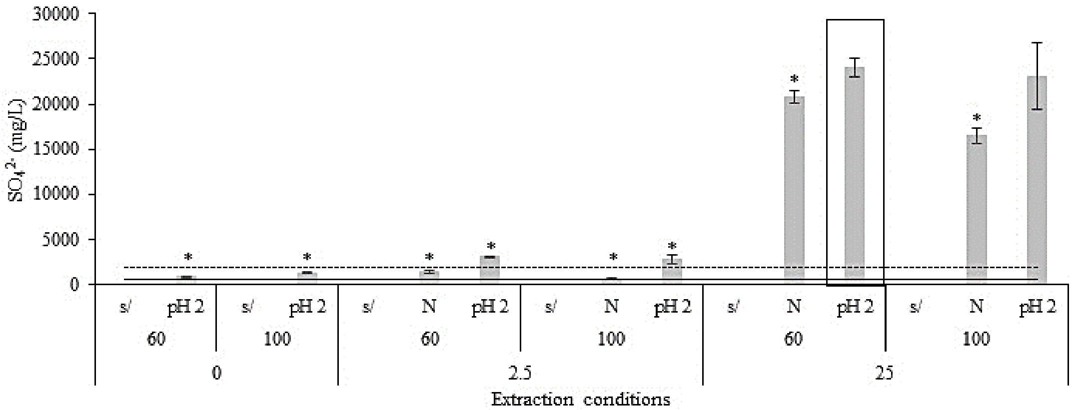

Figure 2- Sulphate (SO_4^{2-}) estimated content of the residual waters resulted from labdanum resin extraction with different extraction conditions (base to top: Na_2CO_3 (g/L), T (ºC), Acidification extent). Error bars correspond to the 95 % confidence interval. "s/" and "N" means no acidification and carbonates neutralization, respectively. (*) statistical difference in relation to the reference (marked with rectangle), found by performing independent t-tests for equal variances not assumed (α = 0.05). Limit emission value for residual water discharge (2000 mg/L, dot line) and maximum recommended value for irrigation water (575 mg/L, straight line) defined in Portuguese decree-law nº 236/98.

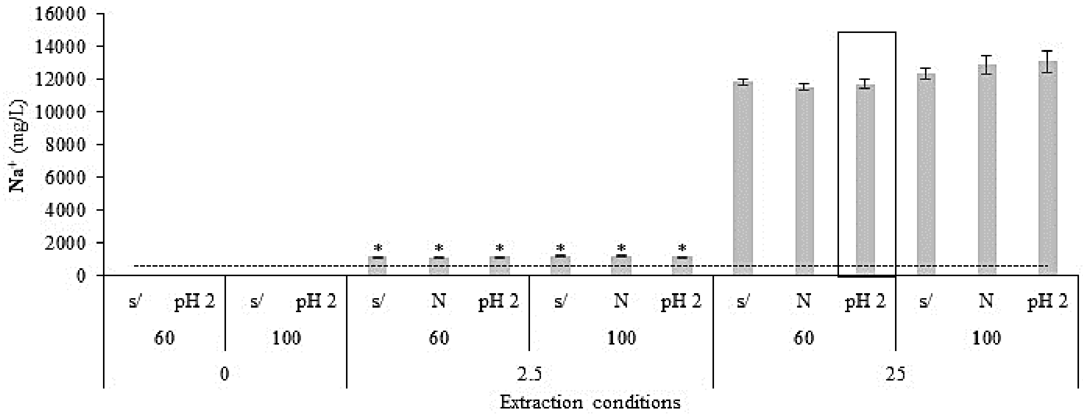

Figure 3- Sodium (Na^+) estimated content of the residual waters resulted from labdanum resin extraction with different extraction conditions (base to top: Na_2CO_3 (g/L), T (ºC), Acidification extent). Error bars correspond to the 95 % confidence interval. "s/" and "N" means no acidification and carbonates neutralization, respectively. (*) statistical difference in relation to the reference (marked with rectangle), found by performing independent t-tests for equal variances not assumed (α = 0.05). Maximum recommended value for total dissolved salts in irrigation water (645 mg/L, dot line) defined in Portuguese decree-law nº 236/98.

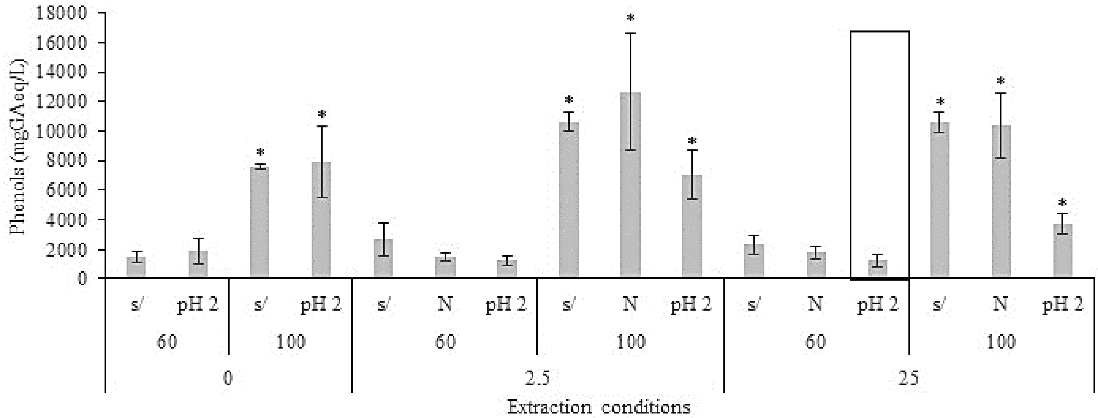

Figure 4- Total phenol content (Gallic acid equivalents) of the residual waters resulted from labdanum resin extraction with different extraction conditions (base to top: Na_2CO_3 (g/L), T (ºC), Acidification extent). Error bars correspond to the 95 % confidence interval. "s/" and "N" means no acidification and carbonates neutralization, respectively. (*) statistical difference in relation to the reference (marked with rectangle), found by performing independent t-tests for equal variances not assumed (α = 0.05). Limit emission value for residual water discharge is defined at 0.5 mg/L in Portuguese decree-law nº 236/98.

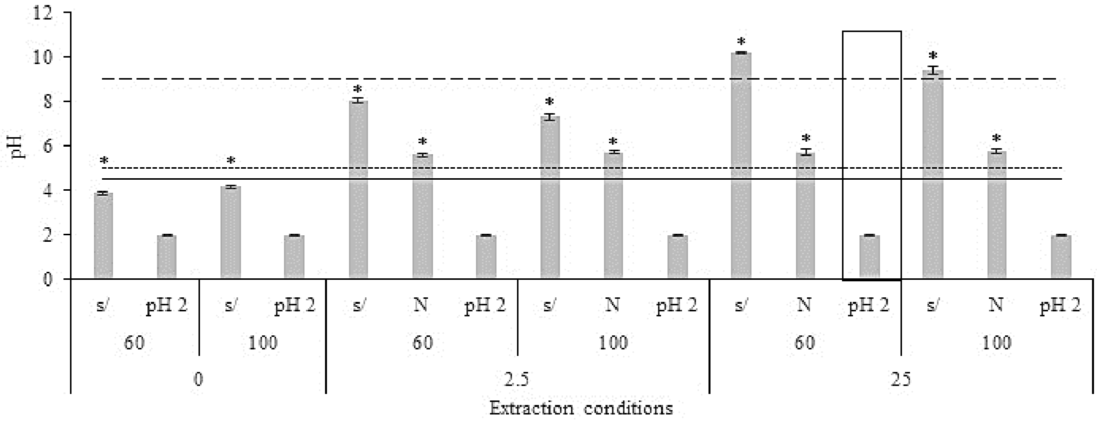

Figure 5- pH (Sorensen scale) of the residual waters resulted from labdanum resin extraction with different extraction conditions (base to top: Na_2CO_3 (g/L), T (ºC), Acidification extent). Error bars correspond to the 95 % confidence interval. "s/" and "N" means no acidification and carbonates neutralization, respectively. (*) statistical difference in relation to the reference (marked with rectangle), found by performing independent t-tests ($\alpha = 0.05$). Lower limit emission value for residual water discharge (pH = 5, dot line), minimum admissible value for irrigation water (pH = 4.5, straight line), and upper limit emission value for residual water discharge and maximum admissible value for irrigation water (pH = 9, trace line), defined in Portuguese decree-law nº 236/98.