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(54) PROCESS TO INCREASE ANTHOCYANINS AND TOTAL POLYPHENOL CONTENT IN RED WINE

(57) The present application is related to a process aiming to increase the content of anthocyanins and polyphenol in red wine.

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Description

Technical field

[0001] The present application related to a process aiming to increase the content of anthocyanins and polyphenol in red wine.

Background art

- [0002] Secondary metabolites are organic compounds produced by plants in response to external stimuli and environmental factors [1]. These metabolites may have an important role in different activities such as pharmaceutical, food and chemical industries, food industry for the organoleptic and sensory characteristics and may also play a bioactive role with beneficial effects on human health [2]. These compounds may have antioxidant [3], anti-cancer [4], antiinflammatory activity or may even improve the sensory properties of food [3].
- Phenolic compounds belong to the group of compounds called secondary metabolites and play an essential role in wine quality. Oenologists try to create products that are more appealing to the consumer, with unique characteristics and properties. Phenolic compounds are important in the quality of grapes and wine by modulating the color, sensory properties, stabilization and aging [5]. Due to these characteristics, the wine sector has been conducting more and more studies related to these metabolites [1]. In the grape, the production of some flavonoids (tannins, anthocyanins) will allow an improvement in the phenolic composition of the grape and, consequently, improve the quality of the wine [1]. Bio-stimulants application as an elicitor molecule has been very popular among researchers to achieve various objectives; may be of biotic/abiotic origin and endogenous or exogenous in action [6, 7].
 - [0003] Chitosan is an example of an exogenous biotic bio-stimulant compound. Chemically, chitosan is a polysaccharide, produced by the deacetylation of chitin [8] in an alkaline medium. This polysaccharide is one of the major constituents of the exoskeleton of insects, crustaceans and the cell wall of fungi [5]. It has antimicrobial properties and is able to stimulate plant defenses against pathogenic organisms [5]. In the case of the agri-food industry, more precisely in the grape, the application of chitosan has been studied in the treatment against gray rot and powdery mildew. Additionally, it was observed that the application of chitosan in the grape, from the beginning of its maturation until its harvest, may play a role in increasing the total polyphenol content and in the antioxidant activity of the wine in relation to other types of fungicide treatments [5, 9-11]. Chitosan was previously reported to modulate wine aromatic and sensorial attributes [12]; however, chitosan in nanoparticles form have not been explored yet in this context.

Summary

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[0004] The present application related to a process to increase anthocyanins and total polyphenol content in red wine by applying an aqueous solution comprising chitosan nanoparticles to grapevines, wherein the concentration of the solution was maintained from 0.1% to 0.0001% (w/v); however the best results were recorded with 0.001% chitosan nanoparticles solution,

wherein the process comprises at least two applications of the solution.

- 40 [0005] In on embodiment the chitosan nanoparticles are morphologically spherical.
 - [0006] In another embodiment the chitosan nanoparticles have a diameter between 30 to 76 nm.
 - **[0007]** In yet another embodiment the first application is done at the beginning of the veraison stage when the grape berries start changing color.
 - [0008] In another embodiment the second application is done 15 days after the first application.
- [0009] In one embodiment the applications are performed mechanically by hand, using a sprayer pump.

General description

[0010] The present application related to a process aiming to increase the content of anthocyanins and polyphenol in red wine.

Chitosan nanoparticles application in grapevine showed potential to elicit the total polyphenols in derived red wines. Anthocyanin content and total tannins improved significantly as well. Additionally, sensorial analysis suggested qualitative enhancement. The overall observation suggested the stimulatory qualitative effect of chitosan nanoparticles application in grapevine.

Brief description of drawings

[0011] For easier understanding of this application, figures are attached in the annex that represent the preferred

forms of implementation which nevertheless are not intended to limit the technique disclosed herein.

Figure 1 shows Table 1 with pH, density, volatile acidity, titrabale acidity, probable alcohol, total anthocyanin, total polyphenols and total tannin content in wines obtained from control, chitosan and chitosan nanoparticles application in vineyards.

Figure 2 shows Table 2 with the average of descriptors after sensory analysis of wines of the Sousão variety. Descriptors with the same letter do not present significant differences in the wines evaluated (ANOVA, Duncan's test for $\alpha \le 0.05$).

Best mode for carrying out the invention

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[0012] Now, preferred embodiments of the present application will be described in detail with reference to the annexed drawings. However, they are not intended to limit the scope of this application.

[0013] The present application relates to a process aiming to increase the content of anthocyanins and total polyphenol in red wine. The process consists in applying an aqueous solution comprising chitosan nanoparticles in a concentration from 0.1% to 0.0001% (w/v). However, the best results were recorded with 0.001% chitosan nanoparticles solution.

[0014] In one embodiment the chitosan nanoparticles are morphologically spherical.

[0015] In another embodiment the chitosan nanoparticles have a diameter between 30 to 76 nm.

[0016] The application is preferably done at the beginning of the veraison stage when the grape berries start changing color.

[0017] In one embodiment, at least two applications are performed, one at the beginning of the veraison stage when the grapes start gaining color, and a second application done 15 days after the first application.

[0018] Applications are performed mechanically by hand, using a sprayer pump.

Plant material, Treatment and sample collection

[0019] Vitis vinifera L. cv. Sousão was used as a test, deriving from the field area of casa de mateus, Baixo Corgo subregion of the Demarcated Douro Region, northern Portugal. Three phenotypically similar lines were marked (consisting of 20 plants each) for chitosan treatment, three similar lines for chitosan nanoparticles, while three similar lines were marked as control. The vineyard soil has morainic, grass covered, loamy sand, 15% gravel, and vines are trained according to Guyot system.

[0020] A solution of 0.01% (w/v) chitosan of fungal origin was prepared by dissolving in 0.01 M aqueous acetic acid solution. Chitosan nanoparticles were prepared and characterized by following a previously described method [13]. In brief, chitosan was dissolved (0.5% w/v) in 0.01 M aqueous acetic acid and pH was maintained to 4.8 with 10N NaOH. Aqueous tripolyphosphate solution (0.25%, w/v) was mixed with chitosan solution a ratio of 3:1 (chitosan: tripolyphosphate) drop by drop continuously under magnetic stirring (500 rpm) at a temperature between 20 and 30°C. Nanoparticles were centrifuged at 10,000 rpm/20 min and the supernatant was discarded, the pellet was rinsed with distilled water and dissolved. The final concentration of chitosan nanoparticles was maintained by 0.1% to 0.0001% (w/v) in water for further application in grapevine.

[0021] First applications were performed at the beginning of the veraison stage when the grape berries started to change the color while second application was done after two weeks. All applications were performed manually by using a commercial sprayer. A total of 25kg grapes were collected from each replicate in each application and vinification was performed in lab conditions. Average temperature, humidity, and wind velocity were recorded as 23.4°C, 57.4%, and 6.5 km/h, respectively, during the application until sample collection.

Winemaking

[0022] Winemaking was performed in three sets of experiments, each treatment as well as control in triplicates in 50 It stainless steel tanks containing 25 kg grapes each. The first set belonged to control samples where no application was performed, the second set of wine making represented 0.01% chitosan solution treated grapevines; while the third set represented 0.1% to 0.0001% chitosan nanoparticles treated grapevines. Cluster stems were removed, and grape berries were crushed using a manual crusher/destemmer followed by the addition of 10 g/hL potassium metabisulfite at a temperature between 25 and 30°C. Commercial yeasts were rehydrated according to the manufacturer's instructions; each tank was inoculated with 0.25 g/kg of yeast and allowed to ferment. The fermenting must was pumped over every day; density and temperature were recorded by using a hydrometer and thermometer. Fermentations with maceration were allowed to reach a density of 1.0 g/ml. The wines were extracted by manual pressure and stored in glass bottles.

Determination of pH, density, alcohol content, total titratable acidity and volatile acidity:

[0023] The pH of the wine samples was determined at 20°C adapted from the method [14] using a pH meter by immersing the electrode in the 100 ml wine samples. The temperature and pH values were allowed to stabilize for 2 min and recorded afterwards in triplicates.

The wine density was determined by hydrometer according to the method [14]. The CO2 present in the wine samples was removed by shaking 250 mL for 10 min. The samples were transferred to a 250 mL beaker. The hydrometer and a thermometer were introduced and allowed to stabilize the temperature (approximately 1 min); the sample density was read in triplicate.

[0024] The alcohol content of wines was determined by using an ebuliometer, whose scale was previously calibrated with water. Wine boiling point determinations were performed in triplicates.

[0025] The total (titratable) acidity was determined in 10 mL decarbonized wine samples. Subsequently, titration was performed with 0.1 M NaOH, using an assay potentiometer coupled to an electrode. The volume of NaOH required to neutralize the solution was recorded [14] and the total acidity calculated according to equation 1; expressed in g/L of tartaric acid.

$$Totalacidity\left(\frac{g}{L}tartaricacid\right) = \frac{N*75*V}{v}$$

[0026] Equation 1, Where N represents the normality of the NaOH solution (0.1 N), 75 correspond to the equivalent per gram of tartaric acid, V is the volume of NaOH spent and v the volume of sample studied.

[0027] The volatile acidity was adapted from OIV method [14], to determine the concentration of volatile acids in wine samples. Wine samples (50ml) were shaken in a vacuum flask to remove CO_2 by vacuum pump. Distilled water (200 ml) was placed in the steam generator of the Cazenave-Ferré device and 10 ml of wine sample was transferred to the bubbler where 0.25 g of tartaric acid and 2 drops of H_2O_2 was added. The water flow was switched on to cool the device and condensate the vapors. To the sample volume obtained by distillation, 2 drops of phenolphthalein was added, followed by titrating the solution with 0.1 M NaOH. All tests were performed in triplicates. Volatile acidity was determined and expressed in g/L of acetic acid, calculated according to equation 2.

$$Volatileacidity\left(\frac{g}{L}aceticacid\right) = \frac{N*60*V}{v}$$

[0028] Equation 2, where N represents the normality of the NaOH solution (0.1 N), 60 corresponds to the equivalent per gram of acetic acid, V is the volume of NaOH used to change the color to magenta pink and v is the sample volume.

Determination of Total anthocyanins, total polyphenol and total tannin content:

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40 [0029] Total anthocyanins were determined by mixing the wine samples (50 μL) with 1950 μL of 1 M HCL solution in a 5ml glass tube gently. The tubes were allowed to incubate at 28°C/3hr and absorbance was recorded at 280nm and 520 nm respectively using a spectrophotometer. The total phenolic content was calculated (A280) and expressed as mg of epicatechin equivalents per liter, using a calibration curve for epicatechin. A520 values were converted into malvidin-3-O-glucoside contents based on a generic extinction coefficient of malvidin-3-O-glucoside [15] and the total anthocyanin content was expressed as mg of malvidin-3-O-glucoside equivalents per liter. All determinations were performed in triplicates for each sample.

[0030] The total tannins in wine samples were determined by the methylcellulose precipitation method [16]. In brief, 50 μ L of wine was mixed with 600 μ L of methylcellulose in the presence of 400 μ L of (NH₄)2SO₄. The samples were mixed rigorously by using a vortex and incubated for 10 minutes at room temperature followed by centrifugation at 10000rpm/5min and supernatant was collected. A control containing (NH₄)2SO₄ and distilled water was performed for each sample. The absorbance at 280 nm of the samples was recorded and tannin content was determined using equations 3 and 4.

(Equation 3)
$$A280(tannins) = A280(control) - A280(sample with treatment)$$

(Equation 4)
$$[tannins] = \left(\frac{A280(tannins) - b}{a}\right) \times DF$$

[0031] Where: A280 (tannins) corresponds to the difference in absorbance between the control and the sample with treatment; A280 (control) is the absorbance of the control solution; A280 (sample with treatment) is the absorbance of the sample with treatment; a is the slope and b is the y-intercept of the epicatechin calibration curve (0.01204 and 0.00619, respectively); DF corresponds to the sample dilution factor.

10 Sensory analysis

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[0032] Sensorial evaluation of red wines was performed according to standard procedure (ISO 3591: 1977). The evaluation panel consisted of 10 people; 9 women and one man, aged between 30 and 50 years old and with previous training in sensory evaluation of food. A Quantitative Descriptive Analysis (ADQ) was performed [17] to evaluate the intensity of the sensory attributes and aimed to identify and quantify the sensory characteristics [18]. Evaluations were performed on the following descriptors / attributes: Appearance (violet color, clarity); Aroma (intensity, fruity, floral, vegetable, spices, smoked, persistence); Flavor (sweet, acid, bitter, astringency, body, persistence); 'Flavor' (floral, fruity, vegetable, spices, smoked) with a structured scale of 1 to 10 points (ISO 4121: 2003). The tests took place from 15:00 to 17:00 h, in a room with a controlled environment and the necessary material for each task, according to the ISO 8589: 2007 standard.

Statistical analysis

[0033] The statistical analysis of the chemical parameters was carried out in the R Core Team program (2018) and completed with a Tukey test in order to assess the significant differences between control and treatment samples. A significance level of 5% was used. In the sensorial analysis of the wines, the software Statistica® was used. The analysis was completed with the Duncan test in order to assess significant differences at significance level of 5%.

Results and discussion

[0034] The general parameters were analyzed for all the wines obtained from chitosan and chitosan nanoparticles application in vineyard. However, pH, density, titrabale acidity, volatile acidity and alcoholic strength by volume did not change significantly between treatments and control (Figure 1 table 1). Total anthocyanin content was recorded little bit higher in chitosan treatment in comparison to control. Formulation of 0.001% Chitosan nanoparticles application recorded best results with a 34.28% increase in anthocyanin content in wines in comparison to control and 33.37% higher than chitosan treatment (Figure 1 table 1). The nanoparticle form of chitosan application (0.001%) resulted in a significant increase in the anthocyanin content of wines. Total polyphenols were investigated in both the treatments and chitosan treatment recorded 4.02% higher of total polyphenol content in comparison to control. Solution of 0.001% chitosan nanoparticle application showed best results with a significant increase of 30.89% of polyphenols in comparison to control while 25.83% higher than chitosan treatment (Figure 1 table 1). Total tannins were observed to be higher in chitosan treatment by a significant 26.14% and 25.17% in nanoparticles (0.001%) application (Figure 1 table 1). Tannins were observed the highest with chitosan treatment in comparison to control while no significant difference was observed between chitosan and nanoparticles application in this parameter. The overall findings suggested that chitosan application has improved the wine composition; while 0.001% chitosan nanoparticles showed highest potential for increased accumulation of secondary metabolites in respective wines.

[0035] Chitosan application has been tested before in grapevines for their elicitation potential towards qualitatively improvement. However, nanoparticle form has not been applied for this objective. Previously; higher total anthocyanins were recorded in derived wines upon chitosan application while no difference was recorded in total polyphenol content [5]. Another study also reported improvement in color, tannins, aromatic profiles and sensory attributes in red wine upon chitosan application [12]. However, there is no report yet for chitosan nanoparticles application in vineyards and their effect on wine content. Findings suggested (Figure 1 table 1) the higher elicitation potential of chitosan in nanoparticles form towards increased secondary metabolites in grapevine.

[0036] Sensory analysis indicated that wines treated with chitosan have greater astringency compared to the control wine. Additionally, they have less floral aroma, less persistence and acidity and more intense fruity, vegetable aroma, smoky aroma, sweet flavor and vegetable flavor than control. The wines obtained upon chitosan nanoparticles treatments in the vineyard have greater aromatic persistence, more intense body and sweet taste compared to the control wine.

[0037] The intensity of the aroma descriptor of spices and the persistence of flavor decreased with the nanoparticles treatment. Additionally, decreased bitterness, acidic taste and less persistence of taste was recorded in wines treated

with chitosan nanoparticles (Figure 2 table 2). Chitosan application has been investigated earlier for the elicitation potential in grapevine and aromatic/sensorial characters were studied [12]. Results suggested a more pleasant sensorial profile with a somewhat higher aroma intensity and overall acceptance upon chitosan treatment in vineyards.

[0038] However, chitosan nanoparticles formulation has not been tested yet for present objective. Present study is first demonstration of wine phenolics, anthocyanin and sensorial attributes improvement by chitosan nanoparticles application in field condition; to the best of our knowledge. Results suggested that nanoparticles form exerted higher elicitation potential, leading to higher phenolics and anthocyanins accumulation in grapes and thus in the derived wines.

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[0039]

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Claims

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- 1. Process to increase anthocyanins and total polyphenol content in red wine **characterized by** applying an aqueous solution comprising chitosan nanoparticles to grapevines, wherein the concentration of the solution varies from 0.1% to 0.0001% (w/v); and wherein the process comprises at least two applications of the solution.
 - 2. Process according to the previous claim, wherein the chitosan nanoparticles are morphologically spherical.
- **3.** Process according to any of the previous claims, wherein the chitosan nanoparticles have a diameter between 30 to 76 nm.
 - **4.** Process according to any of the previous claims, wherein the first application is done at the beginning of the veraison stage when the grape berries start changing color.
 - **5.** Process according to any of the previous claims, wherein the second application is done 15 days after the first application.
 - **6.** Process according to any of the previous claims, wherein the applications are performed mechanically by hand, using a sprayer pump.

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	Control	Chitosan	Chitosan nanoparticles
рН	3.61± 0.13 a	3.64± 0.08 a	3.62± 0.09 a
Density $(d_2O g/ml)$	0.9932± 0.17 ª	0.9952± 0.21 ª	0.9937± 0.11 ª
Volatile acidity (g/L of acetic acid)	0.58± 0.23 a	0.46± 0.28 ª	0.52± 0.17 ª
Titrabale acidity (g/l tartaric acid)	6.96± 0.11 ª	6.28± 0.14 ª	6.43± 0.09 a
Alcoholic strength by volume (%v/v)	12.27±0.35 ¢	11.82±1.66 a	12.39± 0.13 ª
Total anthocyanin (mg/l)	735.23 ± 0.13 ª	740.67 ± 0.08 ª	987.64 ± 0.09 b
Total polyphenols (mg/l)	4301.04± 0.17 ª	4474.09± 0.21 ª	5630.69± 0.11 b
Total tannins (mg/l)	911.69 ± 0.11 ª	1149.98± 0.23 b	1141.12± 0.27 b

Figure 1

	Control	Chitosan	Chitosan nanoparticles
Aroma			
descriptors			
Color tint	10ª	10ª	9.8ª
Color intensity	5.0ª	8.0b	8.0b
Clarity	8.0b	6.2ª	5.2ª
Aroma Fruity	4.0ª	4.1ª	4.2ª
Aroma Floral	5.0b	4.0a	4.0ª
Aroma Vegetal	4.0b	3.0ª	3.6ª
Aroma spicy	4.0 ^b	3.6ª	3.5ª
Aroma smoked	3.0ª	3.9b	3.4ªb
Aroma	2.0ª	3.9b	3.3bc
persistence	2 • 0**	J • 9"	3.3~
Taste			
descriptors			
Acidic taste	8.0 ^b	5.5ª	6.3ª
Sweetness	2.0ª	3.2b	3.6 ^b
Bitterness	5.0 ^b	4.1ª	4.2ª
Body	3.0a	3.1ª	3.6 ^b
Adstringency	5.0ª	5.5ª	4.8a
Persistence	8, Ob	6,9ª	6,7ª
Flavor Floral	3.0ª	3.3ª	3.6ª

Flavor Fruity	3.0ª	3.1ª	3.4 ^b
Flavor Vegetal	3.0ª	2.8 ^{ab}	3.2 ^{ab}
Flavor spicy	3.0ª	3.2ª	3.0ª
Flavor smoked	3.0ª	3.0ª	3.0ª

Figure 2



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Category	Citation of document with indication, of relevant passages	where appropriate,	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)	
Y,D	RUPESH KUMAR SINGH ET AL Application in Vineyards L. cv. Tinto Cão) Induce Anthocyanins and Other P Berries, Mediated by Mod Transcription of Seconda Genes", INTERNATIONAL JOURNAL OF SCIENCES, vol. 21, no. 1, 2 January 2020 (2020-01-XP055721843, DOI: 10.3390/ijms2101030 * page 3/10, line 1 - li * page 8/10, line 4 - li * figure 1 * abstract *	(Vitis vinifera s Accumulation of henolics in ifications in the ry Metabolism MOLECULAR 02), pages 306-1,	1-6	INV. A01N43/16 A01P21/00 A23L33/00 C12G1/00	
Y,D	SINGH ET AL: "Chitosan Genes of the ROS Pathway Antioxidant Potential of vinifera L. 'Touriga Fra Cāo') Tissues", ANTIOXIDANTS, vol. 8, no. 11, 3 November 2019 (2019-11 XP055721846, DOI: 10.3390/antiox81105 * page 3/17, line 10 - 1 * page 11/17, line 23 - * figure 1 * * tables 2, 3 *	and Enhances the Grape (Vitis nca' and 'Tinto -03), pages 525-1, 25 ine 23 *	1-6	TECHNICAL FIELDS SEARCHED (IPC) A01N A23L C12G	
	The present search report has been draw	·			
		Date of completion of the search 12 August 2020	Hab	Examiner abermann, Jörg	
X : part Y : part docu A : tech	ATEGORY OF CITED DOCUMENTS icularly relevant if taken alone icularly relevant if combined with another iment of the same category inological background -written disclosure	T : theory or principle u E : earlier patent docur after the filing date D : document cited in t L : document cited for	underlying the i ment, but publishe application other reasons	nvention shed on, or	

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	DOCUMENTS CONSIDERED TO BE RELEVANT					
	Category	Citation of document with in of relevant pass	ndication, where appropriate, ages		lelevant o claim	CLASSIFICATION OF THE APPLICATION (IPC)
10	Y,D	upon Chitosan Solut	cation on the Pheno nt and Antimicrobia vidual Grape Compone	olic	6	
20		vol. 9, no. 2, 21 February 2020 (2 178-1, XP055721849, DOI: 10.3390/antiox * paragraphs 2.1, 2 * tables 1, 2 *	9020178			
25	Y,D	and free radical so grape and wine", AUSTRALIAN JOURNAL	powdery mildew oves polyphenol cont cavenging activity o	tent	6	
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35		* abstract *	-/			
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45		The present search report has	been drawn up for all claims			
		Place of search	Date of completion of the s	search		Examiner
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PO FORM 1503 03.82 (P04C01)	X : par Y : par doc A : tecl O : nor	ATEGORY OF CITED DOCUMENTS icularly relevant if taken alone icularly relevant if combined with anot ument of the same category incological background invitten disclosure rmediate document	E : earlier p after the her D : docume L : docume	r of the same p	at, but publis application er reasons	

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EUROPEAN SEARCH REPORT

Application Number EP 20 16 7287

CLASSIFICATION OF THE APPLICATION (IPC)

Relevant to claim

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5		DOCUMENTS CONSID	EDED TO BE BEI EVANT	
			ndication, where appropriate,	Rele
	Category	of relevant pass		to cl
10	Y,D	and wine phenolic of application to grap different elicitors chitosan, and yeast	pevine of three s: Methyl jasmonate, c extract",	1-6
15		FOOD CHEMISTRY, ELS vol. 201, 21 Januar pages 213-221, XPO2 ISSN: 0308-8146, DO 10.1016/J.FOODCHEM. * paragraphs 2.2, 2	ry 2016 (2016-01-21), 29413721, DI: .2016.01.086	
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	1	The present search report has	<u> </u>	<u> </u>
	93)	Place of search	Date of completion of the search	
	P04C	Munich	12 August 2020	
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hs 2.2, 2, column, column, column, column, -3 *	1, lines 6-7 * 2, line 5 - line 7 * 1, line 55 - line 58 * 1, line 60 - line 61 *		TECHNICAL FIELDS SEARCHED (IPC)		
rch report has	been drawn up for all claims				
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REFERENCES CITED IN THE DESCRIPTION

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