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Short communication

An improved method for the isolation of amarogentin, the bitter principle of yellow gentian roots

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ABSTRACT

Amarogentin is well known to be among the most bitter naturally occurring compound. Either as an individual one or extracts, amarogentin is used as a food additive and as a dietary supplement. The aim of the present investigation is to set-up a convenient process to selectively isolate amarogentin from the ethanolic roots extract of *Gentiana lutea*. The process consisted in the treatment of an aqueous suspension of such an extract with a panel of 21 solid inorganic / organic sorbents followed by filtration, desorption, and high performance liquid chromatography (HPLC) analyses. Among the solid materials tested, those containing Mg^{+2} in the frame of a lamellar structure provided very good adsorption yields in the range 86.4% − 99.9% ($p < 0.05$ at Student's *t*-test). The method we set up could be in principle useful to obtain a pure nature-derived food additive to provide bitter taste to foods and beverages.

1. Introduction

Gentiana lutea L. (Fam. Gentianaceae) is a perennial herb typically growing in mountainous regions of Central and Southern Europe and Western Asia (Prakash et al. 2017). Roots and rhizomes of this plant constitute the crude phytotherapeutic encoded as "Gentianae Radix" in numerous national and international Pharmacopeias, traditional Chinese and Ayurvedic medicines, and which is used as an herbal stomachic worldwide (Niiho et al., 2006). While a plethora of literature data mostly focus on pharmacological and healthy properties of extracts, phytopreparations, and individual components from *G. lutea* (Jiang et al. 2021), much less is reported about their properties as a food additive. Although yellow gentian root is the basic ingredient of an alcohol beverage widely consumed in Central and Northern Italy and in general in several other regions of the Alps, very few examples of how water or ethanolic root and rhizome extracts of *G. lutea* are used to impart a bitter taste to food preparations (e.g. liqueurs like vermouth wines and bitter spirits) have been cited in the literature. To the best of our knowledge, the only example found in the literature refers to the use of *G. lutea* root powder as an additive to enhance the bitterness of Chardonnay wines from central France (Biehlmann et al. 2020).

The most of food additives, which are officially registered in numerous countries worldwide, are represented by natural extracts and contain various ingredients. These latter are not always properly defined due to an inaccurate analysis of ingredients in the crude material. Thus, the use of well specified phytochemicals to this aim is still desirable and a field of research of current interest. Referring to yellow gentian root extract, it is nowadays well known how its sensorial and organoleptic properties are mainly due to two components, namely gentiopicroside (**1**) and amarogentin (**2**) (Fig. 1), both having secoiridoid glycoside structures (Ariño et al. 1997).

These two secondary metabolites are among the most bitter naturally occurring compounds with bitter indexes of 58×10^6 for amarogentin (**2**), and 12×10^3 for gentiopicroside (**1**). Both are capable to maintain their bitter taste even diluted 1 : 20,000 in water (Ariño et al. 1997).

In this short communication, we wish to report the effectiveness of a panel of 21 biocompatible solid sorbents to selectively concentrate and isolate, by means of a solid phase extraction process, gentiopicroside (**1**) and amarogentin (**2**) from raw yellow gentian dry ethanolic extracts followed by desorption with the same solvent. The overall aim of this work was to obtain pure active principles and / or enriched blends with a concrete potential for their use as nature-derived food additives to

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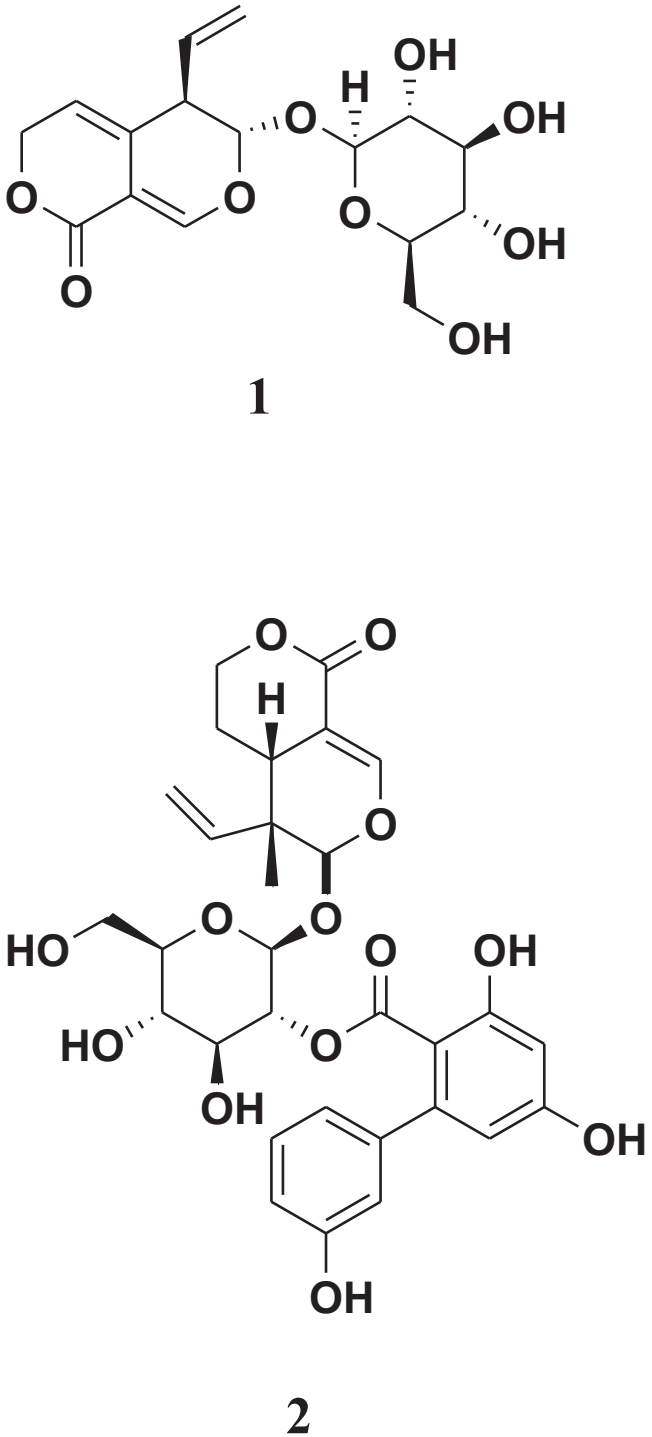

Fig. 1. Structure of gentiopicroside (A) and amarogentin (B).

impart a bitter taste to selected foods and beverages. The contents of the two selected secondary metabolites have been quantified by high performance liquid chromatography (HPLC) coupled to UV/Vis detection.

2. Materials and methods

2.1. Chemistry, plant material, extraction, and solid phase adsorption experiments

Solvents (CH_3CN, H_2O both HPLC grade, EtOH, MeOH, and HCOOH) were purchased from Honeywell Research Chemicals (Charlotte, North Carolina, USA) and Merck Sigma-Aldrich (Milan, Italy). HPLC standards, gentiopicroside (**1**) and amarogentin (**2**) (purity ≥ 99%) were purchased from PhytoLab (Vestenbergsgreuth, Germany). All solid materials were supplied by Prolabin & Tefarm Srl (Perugia, Italy) and are listed in Table 2. Recorded chemico-physical properties were in full agreement with those already reported for the same sorbents (Genovese et al., 2020).

Yellow gentian roots were collected in Maiella mountain (altitude > 1500 m, Abruzzo region, Italy) with the permission obtained from local government authorities. Plant samples were taxonomically properly identified by Authors. A voucher specimen (GL-2021-1) has been stored in the deposit of the laboratory of Chemistry of Natural Compounds at the Department of Pharmacy of the University "G. d'Annunzio" of Chieti-Pescara. The vegetable material was first dried, ground, and finely triturated by an Ultra-Turrax® homogenizer. Ethanolic root extracts were prepared by maceration (room temperature for 24 h). To this aim 33.4 g roots powder were extracted with 150 mL of EtOH followed by filtration, evaporation to complete dryness of this solvent. The weighted crude solid extract was re-dissolved with MeOH or EtOH to reach a concentration of 1000 ppm. The resulting solution was divided into 21 aliquots, each with a volume of 1 mL and poured into a 5 mL round bottom flask, finally evaporating again the solvent to complete dryness under vacuum. The solid material obtained was suspended into H_2O (5 mL) and treated with 200 mg of sorbents **A-Z.** In the case of Mg Al hydroxyl chloride (entry F) we repeated the experiments adopting the same conditions, but decreasing the quantity of the solid material to 100 mg, 50 mg, 25 mg, and 10 mg. Each suspension was magnetically stirred for 24 h at room temperature and, after filtration and centrifugation (13000×g), the supernatant was analysed by HPLC to determine the adsorption capacity of each sorbent herein under study and comparison with the blank sample.

2.2. HPLC, TLC, and total polyphenols content analyses and HPLC method validation

HPLC analyses were performed using an Agilent 1100 (Santa Clara, CA, USA) series instrument equipped with an autosampler, a binary solvent pump, and a diode-array detector (DAD). The separation was achieved by means of a Kromasil RP C18 (4.6 mm ∅ x 150 mm, 5 μm particle size). The mobile phase consisted of a H_2O-HCOOH (99.6–0.4%) (solvent A) and CH_3CN-HCOOH (99.6–0.4%) (solvent B) mixture working in a gradient mode at a flow rate of 1.0 mL min^{-1}. The gradient was changed over time as the following: 0.0–3.0 min, from 2% to 30% B, 3.01–9.0 min. 30% B, 9.01–12.0 min. from 30% to 2% B, 12.01 – 15.0 min. 2% B. The column temperature was set at 25 °C and the injection volume was 20 μL. The wavelength value for the qualitative and quantitative analysis was 254 nm for both gentiopicroside (**1**) and amarogentin (**2**). Each sample solution was filtered through a 0.22 μm pore size Durapore® membrane (Merck Sigma-Aldrich, Milan, Italy) before

Table 1
HPLC method validation more relevant parameters.

	Compounds	
	1	**2**
Slope	114112	112397
Intercept	1650	1501
r^2	0.9997	0.9999
LOD (μg/mL)	0.15	0.05
LOQ (μg/mL)	0.30	0.10
Precision		
Intra-day (n = 6)	2.1–3.9	2.0 – 3.8
Inter-day (n = 6)	2.3–4.0	2.4 – 4.1
Accuracy		
Intra-day (n = 6)	2.1–3.9	2.0–3.7
Inter-day (n = 6)	1.2–2.1	1.0–1.9

LOD = limit of detection; LOQ = limit of quantification.

Table 2
Quantitative determination (concentration expressed as μg/ml and percentages) of gentiopicroside and amarogentin in EtOH extract of *G. lutea* L. absorbed onto solid sorbents **A-Z.**

Entry	LDHs*	Compounds 1 μg/mL ± SD	1 % ± SD	2 μg/mL ± SD	2 % ± SD
A	Zn Al oleate	27.1 ± 0.07	25.2 ± 0.02	11.7 ± 0.06	76.1 ± 0.05
B	Zn Al nitrate	30.8 ± 0.11	28.7 ± 0.03	14.4 ± 0.07	93.9 ± 0.03
C	Zn Al chloride	32.8 ± 0.12	30.5 ± 0.03	12.7 ± 0.07	82.6 ± 0.04
D	Mg Al nitrate	26.9 ± 0.08	25.1 ± 0.02	14.9 ± 0.05	97.1 ± 0.02
E	Mg Al azelate	41.0 ± 0.15	38.2 ± 0.04	15.8 ± 0.06	99.2 ± 0.01
F	Mg Al hydroxide chloride	55.2 ± 0.18	51.4 ± 0.08	16.0 ± 0.04	99.9 ± 0.07
G	Mg Al hydroxide acetate	49.5 ± 0.18	46.0 ± 0.07	15.8 ± 0.06	99.7 ± 0.01
H	Mg Al hydroxide carbonate	47.3 ± 0.07	44.0 ± 0.06	13.3 ± 0.06	86.4 ± 0.03
I	Mg Al acetate	44.3 ± 0.06	41.3 ± 0.05	14.7 ± 0.01	96.1 ± 0.02
L	Zn hydroxy chloride	45.9 ± 0.11	42.7 ± 0.04	13.0 ± 0.01	84.8 ± 0.02
Lamellar solids					
M	$Zr(HPO_4)_2$ **	53.2 ± 0.19	49.5 ± 0.07	7.9 ± 0.02	51.1 ± 0.06
N	$Zr(HPO_4)_2$** + stearamine	36.4 ± 0.11	33.9 ± 0.06	9.6 ± 0.03	62.6 ± 0.06
Oxides / Hydroxides					
O	MgO	25.2 ± 0.04	23.4 ± 0.07	7.6 ± 0.01	49.8 ± 0.05
P	$Mg(OH)_2$	23.5 ± 0.04	21.7 ± 0.06	6.3 ± 0.02	44.0 ± 0.07
Phyllosilicates					
Q	Bentonite	15.5 ± 0.08	14.4 ± 0.03	14.2 ± 0.06	92.4 ± 0.04
R	Talc	15.2 ± 0.02	14.2 ± 0.03	15.0 ± 0.07	98.0 ± 0.03
S	Mica L	47.4 ± 0.13	44.1 ± 0.08	9.5 ± 0.04	61.8 ± 0.04
T	Mica F	42.7 ± 0.13	39.8 ± 0.09	10.7 ± 0.07	69.4 ± 0.05
U	Mica SFG20	46.1 ± 0.11	43.0 ± 0.03	10.5 ± 0.03	68.2 ± 0.05
V	Mg Al benzensulfonate	49.5 ± 0.12	46.1 ± 0.05	16.0 ± 0.03	99.9 ± 0.02
Z	Zn Al benzensulfonate	66.4 ± 0.11	61.8 ± 0.04	15.0 ± 0.03	98.0 ± 0.01

*LDH = layered double hydroxide; ** type B; SD = standard deviation, $p < 0.05$ at Student's *t*-test.

injection into the HPLC apparatus. All samples were stored in a refrigerator at 4 °C before analysis. Open Labs software (Agilent Technologies, Santa Clara, CA, USA) was used for statistical analysis and data management. The HPLC method was validated according to the ICH guidelines (Bhavyasri et al. 2019) in terms of precision, accuracy, linearity, limits of detection (LOD), and limits of quantification (LOQ). The intra-day precision was determined by the injection of the standard mixture solution five times a day. For the inter-day precision, measurements were conducted once a day on three consecutive days. All these were expressed as relative standard deviations (RSDs). Precision was calculated at three concentration levels for quality control (QC) samples, namely QC_{Low} = 1.0 μg/mL, QC_{Medium} = 25.0 μg/mL, and QC_{High} = 100.0 μg/mL and ascertained in line with the criteria already reported (Taddeo et al., 2017). Accuracy was determined by spiking samples deriving from *G. lutea* extract treated with the hydrotalcite magnesium aluminium azelate (entry E) with three concentrations of the two standard compounds (low, medium, and high spikes). Calibration curves were drawn by injecting the gentiopicroside (**1**) and amarogentin (**2**) stock solutions at the following 9 concentrations values (expressed as μg/mL): 0.5, 1.0, 5.0, 10.0, 25.0, 50.0, 75.0, 100.0 and 200.0. LODs and LOQs were obtained by injecting serial dilutions of the corresponding standard solutions, having a signal-to-noise (S/N) ratio of 3 and 10 as the reference, respectively. Finally, the amounts of gentiopicroside (**1**) and amarogentin (**2**) absorbed onto the sorbents listed in Table 1 have been obtained by subtracting the concentration of each secondary metabolite in the filtrate from the content of the same in the blank original solution of plant extract. Desorption has been accomplished by washing the solid collected on the filter with EtOH (5 × 10 mL) and HPLC analyses of the resulting filtrate solutions carried out under the same experimental conditions as described above. To exclude the co-elution of other phytochemicals applying the above mentioned pre-concentration procedure, thin layer chromatography (TLC) analyses of the filtrate and desorbed solutions were carried out. To this aim SiO_2 gel 60 F_{254} pre-coated aluminium plates were purchased from Merck Millipore (Burlington, MA; USA) and a mobile phase consisting of CH_2Cl_2 / MeOH /H_2O 65 / 25 /: 10 was employed coupled to UV light and I_2 spraying detection (Kumari et al. 2019). Further assays for the same purpose consisted in the total polyphenol content determination that was accomplished using the same general procedure (e.g. Fiolin-Ciocalteau method) as described in the literature (Pavun et al. 2018).

2.3. Statistical analysis

The differences between the means were analysed for statistical significance using the Student's *t*-test. A Bonferroni protection for multiple comparisons was applied to a significance value of 0.05 resulting in an adjusted p value of 0.0167.

3. Results and discussion

The first experimental step consisted in the extraction by a conventional overnight maceration with EtOH of a sample of finely powdered yellow gentian root. After evaporation of the solvent to complete dryness under vacuum, the extractive yield was 6.3%. The filtrate was then analyzed by HPLC to get the original concentration values of gentipicroside (**1**) and amarogentin (**2**) in the parent extract. Such values were 108.2 ± 0.12 μg/mL and 16.0 ± 0.08 μg/mL, respectively. To this concern, even if numerous mobile phase mixture compositions with different ratios of H_2O, CH_3CN, and MeOH with or without acidification with small amounts of some organic acids, (HCOOH, CH_3COOH, and CF_3COOH), in the isocratic and gradient modes, the one indicated in the Materials and Methods section was chosen as the best one providing excellent peak shaping and separation. Retention times of standard gentiopicroside (**1**) and amarogentin (**2**) were 5.3 ± 0.02 min. and 7.9 ± 0.05 min., respectively. A representative HPLC chromatogram of the crude *G. lutea* extract is shown Fig. 2.

For every HPLC runs we observed a complete baseline separation of peaks corresponding to gentipoicroside (**1**) and amarogentin (**2**) without any appreciable interferences from the vegetable matrices. Calibration curves were drawn on 9 concentration points and plotted using weighted ($1/x^2$) linear least-squares regression analyses. All curves were linear over all the concentration range tested (0.5 μg/mL – 200.0 μg/mL), furnishing r^2values of 0.9997 for gentiopicroside (**1**) and 0.9999 for amarogentin (**2**). LOD and LOQ values were 0.15 μg/mL and 0.30 μg/mL, and 0.05 μg/mL and 0.10 μg/mL for these two compounds, respectively. A survey of all relevant HPLC parameters for the two standards is reported in Table 1.

For gentiopicroside (**1**) and amarogentin (**2**), RSD values for intra- and interday precisions were not higher than 3.4%, while BIAS % ones of accuracies related to the same phytochemicals ranged from −1.7% to 2.9%. Considered as a whole, such data confirm that the analytical method we set up is in full agreement with the contents provided by the

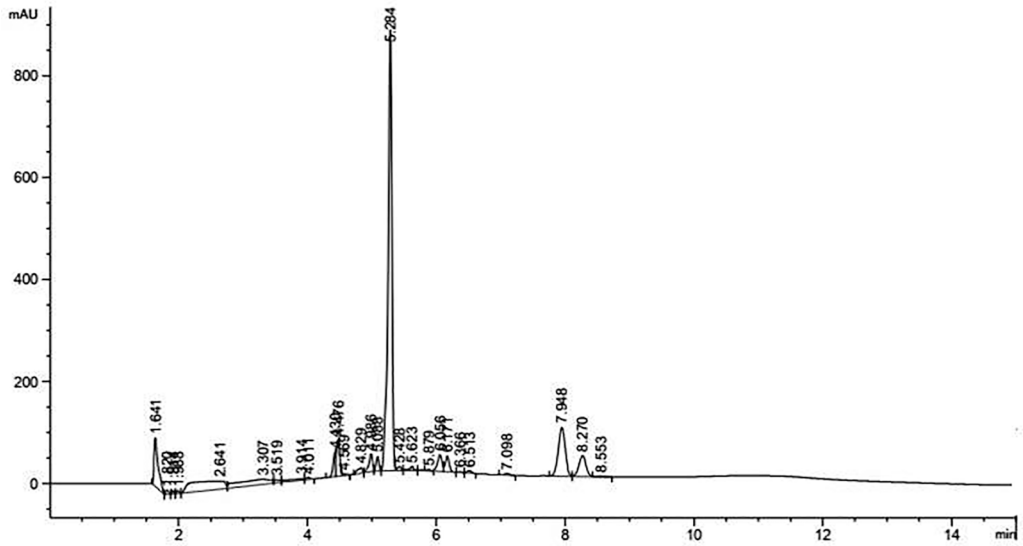

Fig. 2. HPLC chromatogram of *G. lutea* raw extract.

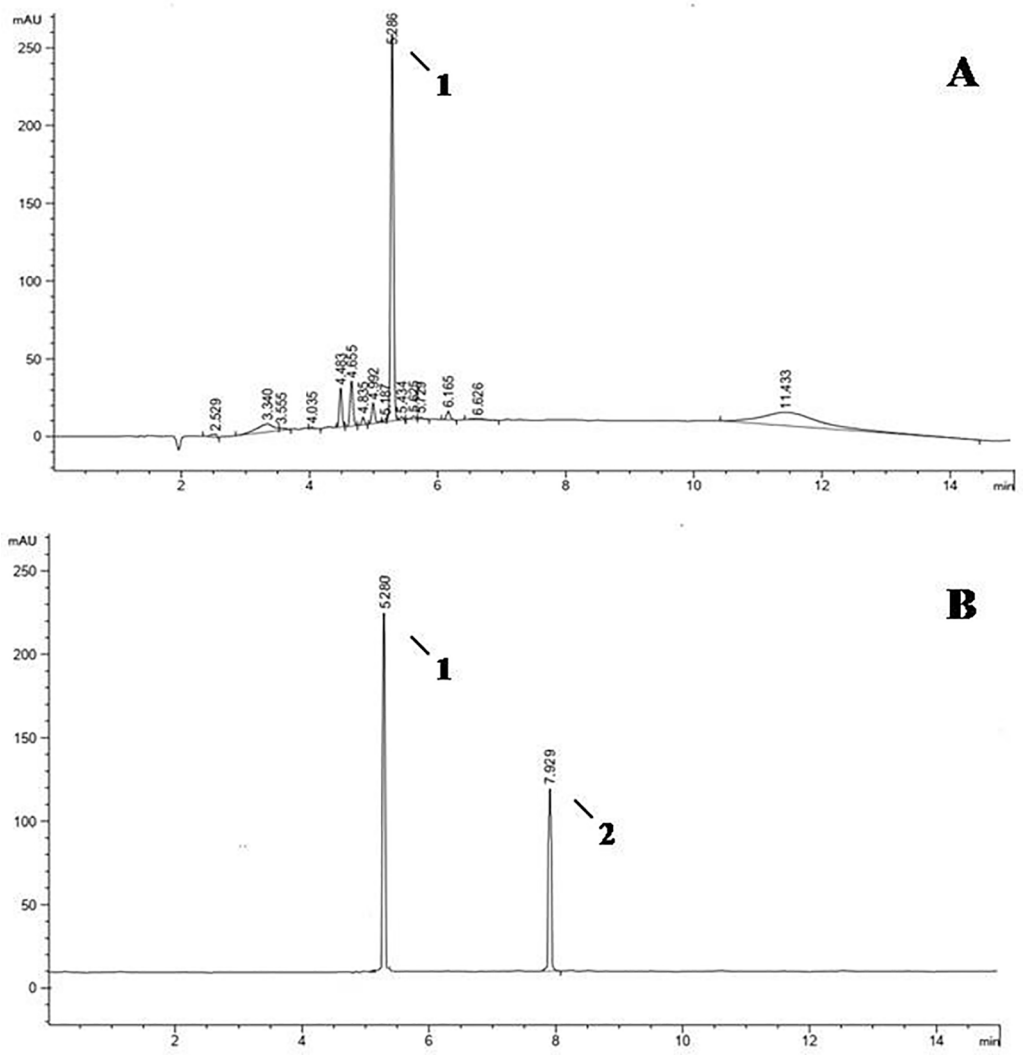

Fig. 3. HPLC chromatogram of the filtrate solution obtained after treatment of *G. lutea* extract with Mg Al hydroxide chloride (entry F) (A) and after desorption with EtOH (B).

ICH guidelines. The carry over effect (<0.12%), quantified following the general procedure recently reported in the literature (Zhou et al., 2017), was not observed. Recoveries of gentipicroside (**1**) and amarogentin (**2**) were > 99.6% with a good precision (RSD < 1.1%).

Going on with experimental procedure after the above mentioned step, the parent extract was re-dissolved in MeOH (otherwise EtOH can also be used as an alternative providing very similar final quantification data) and the resulting solution divided into 21 aliquots of the same volume, that were subsequently re-evaporated, suspended in distilled H_2O, and finally treated with solid supports listed in Table 1 to assess their adsorption yields and capacities. All mixtures were kept under magnetic stirring at room temperature for 24 h, followed by filtration under vacuum and extensive washing with distilled H_2O (3 × 10 mL) of the solid material collected on the filter. Residual contents of gentipicroside (**1**) and amarogentin (**2**) in the filtrates were determined by HPLC analyses. Recorded values were then compared to the concentrations of each secoiridoid glycoside in the parent yellow gentian root extract to preliminarily calculate by difference the amount adsorbed on each solid support. Results of these quantifications are reported in the Table 2.

Data outlined in Table 2 provide quite a clear picture about the pattern of adsorption capacities and efficiencies by the selected inorganic / organic solids. First, all sorbents displayed a marked greater preference for retaining amarogentin (**2**) respect to gentiopicroside (**1**). In fact, for this latter secoiridoid, the best percentage of adsorption did not exceed 61.8% (Zn Al benzensulfonate, entry Z) with poor to modest values for all the other samples (14.2% − 51.4%). For amarogentin (**2**), with the exception of Zr-based lamellar solids (entries M and N), MgO (entry O), $Mg(OH)_2$ (entry P) and synthetic micas L, F, and SFG20 (entries S-U), nearly a quantitative adsorption was recorded, especially for Mg-based materials like the layered double hydroxides (LDHs) Mg Al nitrate (entry D), Mg Al azelate (entry E), Mg Al hydroxide chloride (entry F), Mg Al hydroxide acetate (entry G), Mg Al acetate (entry I), the natural phyllosilicate talc (entry R) and synthetic Mg Al benzensulfonate (entry V). An explicative and sample HPLC chromatogram of the filtrate solution obtained after treatment with the LDH Mg Al hydroxide chloride (entry F) is shown in Fig. 3A.

The best performance of Mg-based clays may be explained on the basis of data already acquired in the recent literature (Adeyemo et al., 2017; Zhang et al., 2019) indicating a strong preference of such materials for the adsorption and / or inclusion of natural compounds incorporating a polyphenolic portion, pending the presence of a lamellar structure [this hypothesis may explain why both MgO and $Mg(OH)_2$ are not effective like other Mg-containing material as they are not featured by such a structural arrangement]. The polyphenolic portion of amarogentin (**2**) respect to gentipicroside (**1**) may also explain the large differences in percentages recorded between these two secondary metabolites.

The final step of our study consisted in the desorption of amarogentin (**2**) [and obviously of the part of gentipicroside (**1**) retained] from the most effective solids in order to quantify its recovery from these matrices and assay its chemical stability. Thus, powder of sorbents providing adsorption values > 86.4%, were extensively washed with EtOH (5 × 10 mL) and the content of amarogentin (**2**) in the filtrate quantified by HPLC analyses under the same experimental conditions as above. Results are reported in Table 3.

All tested sorbents showed a very good recovery indicating that treatment with EtOH was a very good mean for an effective desorption of both secondary metabolites. To this regard, percentages were > 96.6% with the only exception of Mg Al hydroxide carbonate (entry H), for which a slighter less value was recorded for both amarogentin (**2**) and gentipicroside (**1**). This experimental result, coupled also to the value indicated in Table 2 for the same solid, may indicate a partial chemical decomposition of both bitter principles (e.g. cleavage of the lactone ring and / or hydrolysis of the glycoside) hypothetically due to the extreme alkaline environment featuring the surface and interlayer spaces of this clay (Kim et al. 2016). An explicative HPLC chromatogram of the filtrate solution obtained after desorption from the LDH Mg Al hydroxide chloride (entry F) is shown in Fig. 3B.

Table 3
Quantification of amarogentin and gentipicroside after desorption with EtOH from solid sorbents.

Entry	μg/mL ± SD	Recovery (%)	μg/mL ± SD	Recovery (%)
B	30.2 ± 0.05	98.0 ± 0.01	14.2 ± 0.03	98.6 ± 0.02
D	14.4 ± 0.06	97.4 ± 0.04	14.4 ± 0.07	96.6 ± 0.03
E	39.9 ± 0.04	97.3 ± 0.01	15.7 ± 0.04	99.3 ± 0.02
F	53.1 ± 0.11	96.3 ± 0.07	15.9 ± 0.02	99.3 ± 0.04
G	49.0 ± 0.05	98.9 ± 0.05	15.6 ± 0.02	98.7 ± 0.03
H	46.3 ± 0.08	97.8 ± 0.04	11.6 ± 0.04	87.2 ± 0.02
I	43.1 ± 0.09	97.2 ± 0.05	14.6 ± 0.02	99.3 ± 0.01
Q	14.6 ± 0.05	94.2 ± 0.01	14.0 ± 0.03	98.5 ± 0.02
R	14.0 ± 0.02	92.1 ± 0.04	14.8 ± 0.07	98.6 ± 0.01
V	48.1 ± 0.05	97.1 ± 0.02	15.9 ± 0.03	99.3 ± 0.02
Z	65.2 ± 0.09	98.2 ± 0.03	14.6 ± 0.05	97.3 ± 0.04

SD = standard deviation, $p < 0.05$ at Student's *t*-test.

Finally, to exclude the co-elution of other phytochemicals (e.g. polyphenols) contained in the yellow gentian root raw extract during the solid phase adsorption, TLC analyses of the filtrate and desorbed solutions were carried out. Both UV light and I_2 spraying detection of eluted plates showed the presence of only spots related to gentiopicroside (**1**) and amarogentin (**2**). The same pure standards employed for HPLC analyses were used also to this aim. To confirm this finding, we also compared the quantities of amarogentin (**2**) in the desorbed solutions from Mg-based solids as determined by HPLC analyses with values obtained by the Folin-Ciocalteau assay applied to the same solutions to quantifity the total polyphenols content. Differences between the two quantification method differed by percentages values <0.35%, thus indicating that amarogentin (**2**) was virtually the only phenolic compound retained on all solids without co-elution of other structurally related secondary metabolites.

Discussing about the optimization of the experimental conditions, the quantities and ratios indicated in the Materials and Methods section represent the optimal protocol to get the best extractive yields for both gentipicroside (**1**) and amarogentin (**2**). Stating the high bitter index of both phytochemicals, the easiness and low-cost by which all solids can be synthesized, the fact that all materials can be recycled without loss of their adsorption capacities, as stated below, 1 mg to 200 mg ratio between the extract and sorbents cannot be considered a drawback from an economical point of view, especially when thinking about an industrial application of the process described herein in the next future. However, for a greater completeness of the present study, we repeated the same experiments decreasing the amount of solid sorbent [e.g. Mg Al hydroxide chloride (entry F) 100 mg, 50, mg, 25 mg, and 10 mg] with the same quantity of extract. Gentipicroside (**1**) have been retained on the solid in percentages ranging from 32.4% to 45.2% (from 10 mg to 100 mg), while amarogentin (**2**) in percentages ranging from 61.7% to 86.1%. For what concerns the less performing sorbents, increasing operational times up to 72 h and / or sorbent loading did not lead to appreciable improvements in yields, while raising temperature up top 80 °C resulted in a large loss of the two secondary metabolites under investigation probably due to thermal degradation, as previously observed (Mehta et al., 2016; Mudrić et al., 2020).

We also established the recyclability and reusability of the most effective materials as a result of the experiments detailed in Table 1. Thus, we carried out 4 additional steps of solid phase adsorption of amarogentin (**2**) and gentipicroside (**1**) from the parent extract of yellow gentian roots, employing as explicative examples the LDHs Mg Al hydroxide chloride (entry F), Mg Al hydroxide acetate (entry G) and the synthetic Mg Al benzenesulfonate (entry V) under the same experimental conditions indicated above. The percentages of adsorption recorded were 99.8%, 99.8%, 99.6%, and 99.3% for Mg Al hydroxide chloride, 99.9%, 99.7%, 99.6%, and 99.3% for Mg Al hydroxide acetate,

and finally 99.3%, 99.8%, 99.2%, and 99.2% for Mg Al benzenesulfonate. These percentages values clearly indicate that all solids mentioned can be reasonably considered recyclable and could be re-used to run out additional steps without virtually no appreciable loss of efficiency.

The approach we depicted in this short communication, consisting in the selective removal of virtually total amarogentin (**2**) and partial one of gentipicroside (**1**) from raw ethanolic extracts of *G. lutea* by an extraction in the heterogeneous phase followed by desorption with EtOH is unprecedented in the literature, to the best of our knowledge. Other reported techniques include static extraction, continuous shaking extraction, ultrasonic extraction (Kshirsagar et al. 2019), microwave extraction (Kaur et al. 2019), heat-assisted extraction (Mudrić et al., 2020), fast centrifugal partition chromatography (Xynos et al., 2016), high-speed counter-current chromatography (Chen et al. 2017), microwave-assisted ethanol-salt aqueous two-phase systems (Cheng et al. 2020), and others that have been recently reviewed (Xu et al. 2017). In some instances good results in terms of extractive yields and selectivity have been achieved. However, the main drawback of the listed methodologies is represented, in our opinion, by the fact that each needs a specific instrumentations, the cost of which is not easily accessible in most cases. On the other hand, the one described herein represents a valid and substantial alternative green-chemical process that has in principle the potential to get access to a novel category of food additive and could find its use, for example, in liqueurs and spirits industry. To this concern it has to be reminded how the phytochemical and overall organoleptic qualities and properties of yellow gentian root extract, the basic ingredient of gentian liqueur, strictly depend on parameters related to the plant like age, period of collection, climate, geographical factors, storage, overall processing, the intimate method by which the extraction is carried out, and similar (Marković et al. 2019). Due to variations in these factors, often entire batches of gentian-based liqueur production have to be discarded due to malevolent odours and flavours ("herbaceous like" or "earthy-like") due to a low content of bitter principles and / or presence of excess of lignin depolymeraztion derived compounds, diterpenes, xanthones, or volatiles.

4. Conclusions

The methodology we set up and described herein enables to have at disposition a purely nature-derived food additive which can be used as a flavour and odour corrector of the same gentian-based liqueurs and / or other flavoured drinks, and food in general, where a bitter taste is necessary for the compliance of the final consumer. Furthermore, the use of easy to handle solids like those listed in this communication, would in principle allow to obtain amarogentin in very good yields from alternative natural plant sources, which are not protected by law species like *G. lutea*, such as other Gentianaceae (e.g. those encoded by Chinese, Tibetan, Indian, and Ayurvedic medicine) and *Swertia* spp. For both purposes, first tests to quantify the sensorial properties of the amarogentin-blends we obtained and secondly the search for additional plant species to which applying the same or slightly modified experimental schemes, are now ongoing in our laboratories.

CRediT authorship contribution statement

Serena Fiorito: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **Francesco Epifano:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **Lorenzo Marchetti:** Investigation, Methodology, Software, Validation, Visualization. **Lucia Palumbo:** Investigation, Methodology, Software, Validation, Visualization. **Fabrizio Mascioli:** Conceptualization, Investigation, Methodology. **Maria Bastianini:** Investigation, Methodology, Software, Validation, Visualization. **Fabio Cardellini:** Investigation, Methodology, Software, Validation, Visualization. **Roberto Spogli:** Conceptualization, Formal analysis, Investigation, Methodology, Resources, Software, Supervision, Validation, Visualization. **Salvatore Genovese:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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