

RESEARCH ARTICLE

Assessment of gamma oryzanol variability, an attractive rice bran bioactive compound

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ABSTRACT

Gamma-oryzanol is a compound present on rice bran, which has been studied for its bioactive properties, including its cytotoxic activity. The gamma-oryzanol biosynthesis occurs on the lipidic membrane of the plant and can be influenced by edaphoclimatic grown conditions. Two Portuguese rice varieties (Ceres and Maçarico) from 6 growing environments were analysed in order to explore the influence of the growing conditions on the gamma-oryzanol content, its constituents, and cytotoxic effect against 4 human tumour cell lines (NCI-H460, HeLa, HepG2, and MCF -7). The overall variability of gamma-oryzanol concentration was from 1.56 g/Kg to 3.19 g/Kg, Ceres reveals higher values than Maçarico; those concentrations also varied with the growing environment. The environment was also determinant for the individual concentration of the 3 gamma-oryzanol compounds (cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, and campesteryl ferulate). A cytotoxic effect was observed for all the tested tumour cell lines and also varied with the growing environment.

Keywords: Cytotoxicity; Gamma-oryzanol; HPLC; Rice bran

INTRODUCTION

Rice bran is a by-product of the rice milling process and comprises the outer layer of rice kernel which mainly includes the pericarp, aleurone, sub aleurone layer and germ, accounting for approximately 10 % of the total rice weight. Despite the claimed health benefits (Agarwal et al., 2016), rice bran is under-utilised as human food and commonly used as animal feed.

In recent years, both academy and industry have shown a growing interest in gamma-oryzanol (GO), which is a mixture of bioactive compounds existing in the unsaponifiable part of rice bran (Bhatnagar et al., 2014). GO have been studied for its health properties for decades, showing positive results for its cholesterol lowering effect in hyperlipidemic subjects (Bumrungpert et al., 2018). The cytotoxic properties of GO have also been explored in tumour-bearing mice (Kim et al., 2012), showing positive effects. In a recent study, Castanho et al. (2019) tested rice bran lipid extracts from different origins and pericarp

colours against four human tumour cell lines (NCI-H460, HeLa, HepG2, and MCF -7); the results showed the effectiveness of the extracts and also a significant negative correlation between GO concentration and GI₅₀ values for HeLa, HepG2 and MCF7.

The high availability of GO and its bioactive potential led to several applications in the cosmetic industry, nutrition and medicines (Peanparkdee and Iwamoto, 2019). GO applications in medicines are almost 50 % of the total market application, followed by nutrition and cosmetics uses (Market Reports World, 2018). The GO market demand was 11520 tons in 2014 and is expected to reach 18598 tons by 2022. Japan is the biggest producer, with about 68 % of the total production followed by China with almost 22 %. The GO is commercialised by several companies being the major ones Oryza Oil & Fat Chemical, TSUNO and Henry Lamotte OILS (Market Reports World, 2018).

Chemically, GO is a mixture of ferulic esters of fatty acid or triterpene alcohols. However 95 % of GO is composed

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of only four compounds: 24-methylenecycloartanyl ferulate (24MCAF), cycloartenyl ferulate (CAF), campesteryl ferulate (CampF) and β -sitosteryl ferulate (β SF), by decreasing order of abundance (Rogers et al., 1993).

GO extraction methods and extracting solvents are considered the key factors influencing the extraction efficiency of the bioactive compounds-rich extracts, due to the chemical instability and complexity of ferulates of triterpene alcohols and sterols (Peanparkdee and Iwamoto, 2019) and it may be the cause of the high variability detected in the published data with range spanning from 0.6 g/Kg to 9.1 g/kg of rice bran (Castanho et al., 2019).

Although there is no standardised method for the separation and quantification of GO, the compounds are usually performed by HPLC with a posterior compound identification by HPLC-MS or GC-MS (Shammugasamy et al., 2015). Several other methods have been described for the GO analysis in rice, bran or rice oil, such as ultraviolet (UV) spectrophotometry (Bucci et al., 2003), normal phase high-performance liquid chromatography (NP-HPLC) (Lerma-García et al., 2009), reverse phase (RP-HPLC) (Rogers et al., 1993), and gas chromatography (GC) (Miller and Engel, 2006). GO compounds identification can be performed by spectroscopy (Rogers et al., 1993), mass spectrometry (MS) (Miller and Engel, 2006) or nuclear magnetic resonance (NMR) spectroscopy (Luo et al., 2005).

The levels of GO may vary according to the genetic diversity and also by the edaphoclimatic conditions of the growing environment. Bergman and Xu (2003) analysed the GO content of 7 rice cultivars grown on 4 different locations along 2 crop years and concluded that year \times location interaction is the main factor of GO content variation, followed by cultivar and crop year. Stress conditions as temperature or drought may also be a factor affecting the GO content, and 4.5 °C temperature increase resulted in higher GO values, mainly on the 24MCAF fraction (Britz et al., 2007); the sterol content and steryl ester fractions of two cultivars seemed to increase when subjected to water stress through 3, 6, 9 and 12 days (Kumar and Krishna, 2015). Genetics also plays an important role regarding the GO content, as some specific alleles may be responsible for GO concentration (Kato et al., 2017; Nakano et al., 2018). Nakano et al. (2018) studied the progenies from the cross of *indica* and *japonica* germplasm, by analysing 80 lines concluded that alleles from the *japonica* type may improve GO, 24MCAF and CAF concentrations in *indica* types, while *indica* type alleles may be used to improve CAF concentrations in *japonica* types.

This study aims to optimise the rice bran GO extraction and quantification on samples of two Portuguese rice varieties

(Ceres and Maçarico) taking in account the environmental influence, the distribution of individual GO compounds and also its cytotoxicity.

MATERIALS AND METHODS

Materials

Two Portuguese rice varieties (Ceres and Maçarico) were grown in field trials by INIAV/Cotarroz, in 6 different growing environments in field plots with the same cultivation methods. The two varieties were recently released by the Portuguese Rice Breeding Program, Ceres (*Japonica* type) and Maçarico (*Indica* type), were sown in a randomised block design with three replicate plots. The plots were 8 m length by 1.2 m width, resulting in 9.6 m² of area. The environments are located in the three main Portuguese rice regions (Tagus (T), Sado (S) and Mondego (M) valleys), and the trials were conducted during 2016 (T), 2017 (S, M and T) and 2018 (T and M) seasons.

The three field replicates were pooled, and the paddy samples were dehusked in a Satake mill (THU, Satake, Taito, Japan). Rice bran samples were obtained by polishing the husked grains in a rice polishing mill (Takayama TM-05 mini testing mill, Taiwan) and after sieving the seed coat fraction in order to retain the particles between 250 μ m and 90 μ m. The bran samples were vacuum sealed until analysis.

Chemicals and reagents

All solvents (methanol, acetonitrile, ethanol, isopropanol, dichloromethane, hexane) were purchased from Sigma (Europe) and GO from TCI (Europe). The mobile phase was composed of HPLC gradient grade solvents (acetonitrile and methanol), filtered with 0.22 μ m nylon membrane (Filter Lab, Barcelona, Spain) and ultrasound degassed (for not less than 30 minutes) before use. Type I water (Milli-Q) with a resistivity of 18.2 M Ω ·cm at 25 °C was utilised for all solutions. There was used fetal bovine serum (FBS), L-glutamine, Hank's balanced salt solution (HBSS), trypsin-EDTA (ethylenediaminetetraacetic acid), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively), RPMI-1640 and DMEM media Hyclone (Logan, UT, USA). Acetic acid, formic acid, ellipticine, sulforhodamine B (SRB), trypan blue, trichloroacetic acid (TCA) and Tris were acquired from Sigma Chemical Co. (St. Louis, MO, USA).

GO Extraction from rice bran

Preliminary studies

The optimisation of the gamma-oryzanol bran extraction procedure was tested with different types of extraction solvents (individual or combined), such as ethanol (EtOH), methanol (MeOH), acetonitrile (ACN), dichloromethane (DCM), hexane (Hex), isopropanol (IsoP), with different

ratios of sample concentration/solvent volume (Extraction 1 (E1) 1 of 80 and E2 of 20) and with different extraction times (E1 of 22min and E2 of 36min) at room temperature, based on studies of Chen and Bergman, 2005. A Synergi Hydro RP column (250 mm x 4.6 mm, 4 μ m, 80 Å, Phenomenex) was previously tested. The GO standard yielded 4 peaks that were better resolved than with the other tested C18 column (150 mm x 4.6 mm, 5 μ m particle size, Waters ODS2). The Synergi Hydro RP column was not adopted as it needed a higher flow (2.0 mL/min) and run time (50 minutes). Fig 1 shows the obtained chromatograms.

Extraction method

GO was extracted from bran with the isopropanol solvent (Castanho et al., 2019). To determine the recovery of the GO, spiking experiments were performed with known standard content before extraction. All rice bran extractions were carried out in duplicate.

GO quantification by HPLC

GO quantification was performed by RP-HPLC coupled to a photodiode array detector (PDA) (Castanho et al., 2019).

Validation of the HPLC method

GO quantification in samples was made based on the correlation obtained by an external calibration curve, and

validation was performed by European Medicines Agency guideline (European Medicines Agency, 2015).

Five to eight standard solutions of GO were prepared according to Castanho et al. (2019), using an injection volume of 20 μ L from each solution (in triplicate) to obtain the 4 peaks chromatograms.

The GO content was expressed in g/kg of bran, and the GO compounds content was expressed in % of total GO content.

Cytotoxicity tests

The extracts were tested To evaluate its cytotoxic effect, against four human tumour cell lines: MCF-7 (breast adenocarcinoma), NCI-H460, HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma) obtained from DSMZ (Leibniz-Institut DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). A non-tumour cell line (PLP2), prepared from a freshly harvested porcine liver obtained from a local slaughterhouse, was also tested for hepatotoxicity evaluation. Cells were routinely maintained and analysed, as described in a previous study (Abreu et al. 2011; Castanho et al., 2019). Two independent experiments were performed for each compound; each one carried out in duplicate and the results were expressed as GI₅₀ values in μ g/mL.

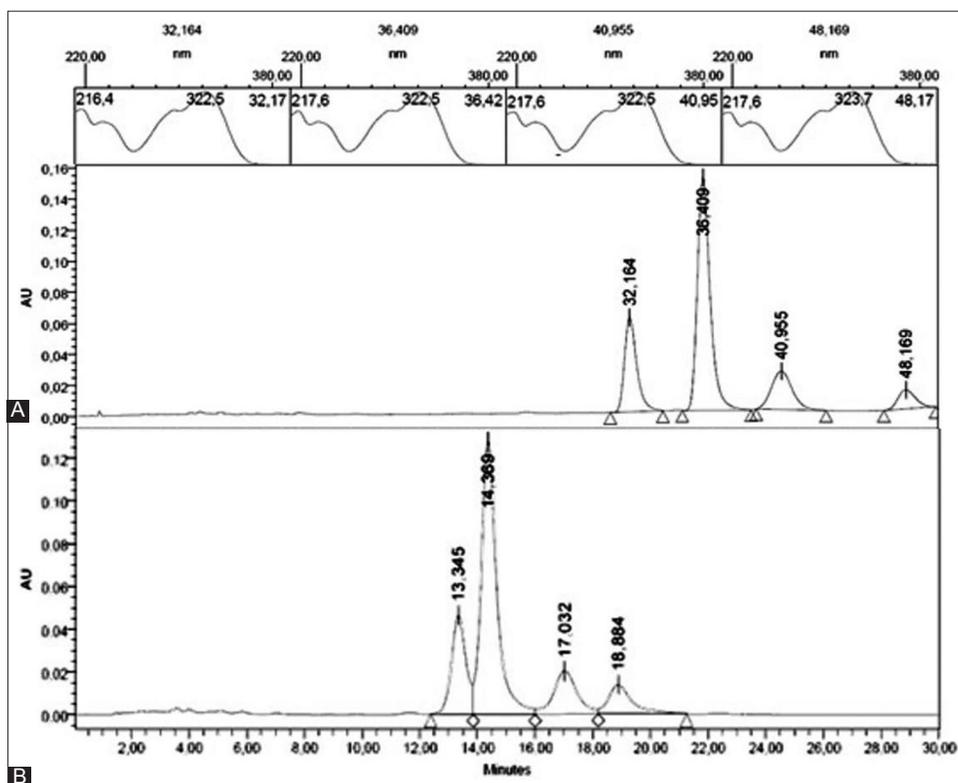


Fig 1. A - Standard GO chromatogram (190 μ g/mL) with Synergi Hydro RP column, CAN/MeOH mobile phase (85: 15), flow of 2 mL/min, injected volume of 100 μ L and a run time of 50 minutes, at the wavelength of 325 nm. Above the chromatogram are the spectra of the 4 obtained peaks used for compounds identification by exit order (CAF, 24MCAF, CampF and β SF). B - Standard GO chromatogram (400 μ g/mL) with ODS2 RP column, ACN/MeOH mobile phase (50:50), flow of 1.2 mL/min, injected volume of 20 μ L and a run time of 30 minutes, at the wavelength of 325 nm.

Statistical analysis

Duncan's comparison tests were performed to identify significant differences at $p < 0.05$. Data are reported as mean \pm standard deviation (SD). All statistical analyses were performed using IBM-SPSS.

RESULTS AND DISCUSSION

Preliminary extraction tests

As GO is soluble in conventional organic solvents like acetone or chloroform and moderately soluble in others (ethanol and n-heptane), hexane has classically been employed in GO extraction. However, GO is an unstable compound that may decompose during saponification since the ester bond between ferulic acid and the triterpene component of GO can be hydrolysed under alkali conditions (Bhatnagar et al., 2014). The extraction rate of GO can be significantly affected by the polarity of the solvent. GO compounds have an alcoholic group in the ferulate part, which makes the molecule highly polar. Some polar solvents like ethyl acetate and isopropanol and some nonpolar solvents like heptane and hexane may also solubilise these compounds.

There have been tested different types of extraction solvents such as ethanol (EtOH), methanol (MeOH), acetonitrile (ACN), dichloromethane (DCM), hexane (Hex), isopropanol (IsoP), different ratios of sample concentration/solvent volume and different extraction times at room temperature (Chen and Bergman, 2005). The isopropanol with extraction E2 (ratio of sample concentration/solvent volume of 20 and extraction time of 36 min), was selected as the chromatograms presented a better resolution, higher yield (as in Peanparkdee et al., 2019), lower RSD (Fig. 2) and better stability.

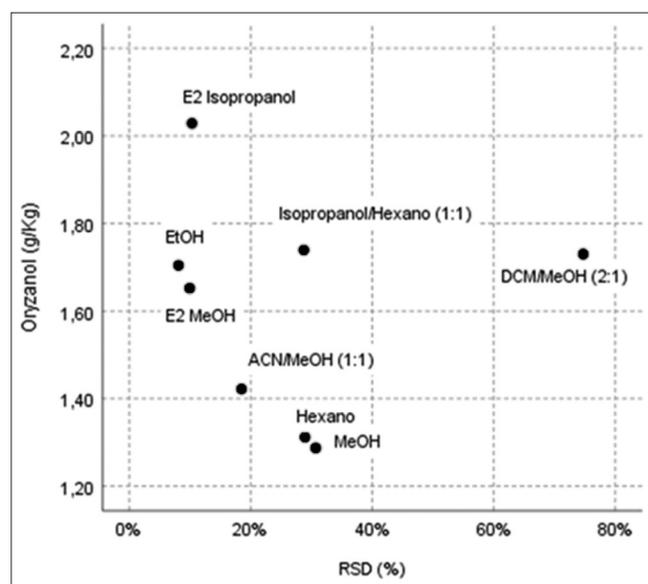


Fig 2. Relation between GO content and the RSD (%) ($n=4$) for the different extraction solvents tested.

HPLC method validation

The identification of the GO peaks (Fig. 1) by exit order was CAF, 24MCAF, CampF and β SF, has been confirmed by typical compounds spectra at the 325 nm wavelength and comparison with the external standard retention times. The chromatograms of GO profile were similar to those of Rogers et al. (1993), Chen and Bergman (2005) and Sakunpak et al. (2014), who mentioned to use the GO standard. The steryl ferulates have a typical spectrum at the maximum wavelength (Fig. 1), which is similar to the ferulic acid spectrum.

A linear regression between the sum of GO peaks areas (average of the 3 injections) and the standard solution concentration (0,32 g/Kg to 3.8 g/Kg) was obtained with R^2 of 0.9997 (Equation 1).

$$\sum_{i=0}^4 (\text{Peak area } i) = (42.5 \pm 0.4) \times 10^3 [\text{GO (g/kg)}] - (35 \pm 1) \times 10^3 \quad (\text{Equation 1})$$

The detection limit (LOD, signal- to-noise ratio of 3) and the quantification limit (LQD, signal-to-noise ratio of 10) were of 0.18 g/Kg and of 0.54 g/Kg, respectively. The method linearity was from 0.31 g/Kg to 3.75 g/Kg of bran with relative standard deviation (RSD) of $99 \% \pm 4 \%$ and recovery was of $95 \% \pm 12 \%$.

GO quantification and compounds separation

GO is mostly quantified as the sum of the 4 (or 5) more abundant constituents by the sum of the HPLC peaks area obtained and not by the individual compounds quantification. A major problem to face in the quantification of individual steryl ferulates is the lack of commercially available pure standards. In fact, the different purity grade of standards obtained through synthesis or by purification from natural sources in the authors' laboratories could lead to a misinterpretation in the quantification of individual steryl ferulates in rice bran.

Different methods for separation of GO constituents were reported (Rogers et al., 1993; Miller and Engel, 2006; Sakunpak et al., 2014; Peanparkdee et al., 2019). The UV spectrophotometry method yields higher determination contents than those obtained by normal phase HPLC (Bucci et al., 2003). The difference between these methods is related to the existence of other substances with maximum absorbance at the GO used maximum wavelength; while in the chromatography the GO is separated from the other compounds before determination, by normal phase HPLC determinations few peaks (minor compounds separation) can be detected and run times are larger than for reverse phase HPLC. For separation of more GO compounds (up to 25 were reported) reverse phase HPLC-MS or GC-MS must be used (Luo et al., 2005; Agarwal et al., 2016).

Rice bran GO content

As shown in Fig. 3:A, the content of GO in rice bran reaches its maximum value (3.19 ± 1.0 g/Kg) in the Maçarico variety grown in the 2018T environment and for Ceres in 2017S (2.92 ± 3.6 g/Kg).

Considering the 3 three environments of the 2017 year, there were observed significant differences for the GO content of Maçarico with higher levels in the samples from Sado followed by Mondego and finally from Tejo valleys. Concerning environmental conditions in the three experimental sites, the average daily temperature during the stage of rice grain filling was 3.1 °C higher in Sado valley compared to other regions (IPMA, 2017).

Britz et al. (2007) reported increased GO concentration on 5 varieties studied when subjected to a 4.5 °C temperature increase. In another study, Kumar et al. (2018) observed that rice subjected to water deficit stress increased its phytosterol concentration. Both studies suggested that the increase of GO can be a response to environmental stress.

The GO contents also varied with the rice variety ($p < 0.05$). As shown in Fig. 3:B, the variety Ceres (*Japonica*) exhibit higher levels of GO (except for 2018T environment) when compared with Maçarico (*Indica*). These results corroborate the findings of Kato et al. (2017) who compared the GO concentration between *Indica* and *Japonica* varieties, reporting there are quantitative trait loci (QTL's) responsible for GO concentrations; the authors also referred a significantly higher GO concentration in *Japonica* types compared with *Indica* types (Kato et al., 2017). Nakano et al. (2018) studied the genetic differences between *Japonica* and *Indica* types regarding the major compounds of GO (24-MCAF, and CAF), reporting there are alleles from *Japonica* type varieties that can be used to improve GO concentration on *Indica* types. Those findings can contribute to the genetic improvement of rice regarding its GO content.

The sterol biosynthesis pathway of plants is a sequence of enzyme-catalysed reactions. GO is a mixture of sterols esterified to ferulic acids (Hernandez, 2016), and this process is reported to the action of acyltransferases enzymes (Kumar and Krishna, 2015). Plant sterols can be found with a free 3b-hydroxyl group, but also conjugated by esterification (Piironen et al., 2000). In the case of rice, the major sterols are found as esters of phenolic acids, mainly as sterols esterified to ferulic acids (Hernandez, 2016). During the maturation of rice grain, GO acts as the other phytosterols which play an essential role in maintaining the integrity and fluidity of the lipidic membrane of plant cells as they are responsible for different regulatory functions, properties and structure, maintaining the homeostasis of membrane lipids, the integrity of the membrane during stress, regulation of

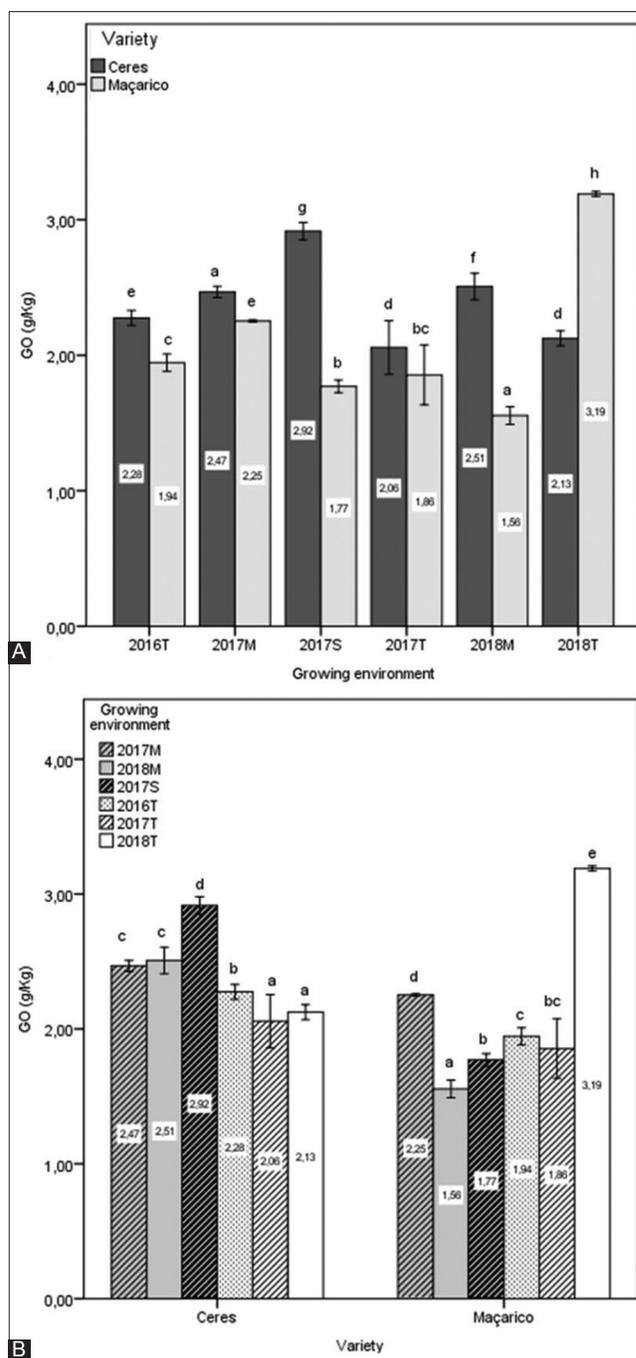


Fig 3. Values of GO per Environment (Year and Location) expressed as mean \pm standard deviation ($n=4$). A - Different letters show statistically significant differences at $p < 0.05$. B - Different letters in each variety show statistically significant differences at $p < 0.05$. Locations: T: Tagus valley; S: Sado valley; M: Mondego valley.

membrane permeability, fluidity, signal transduction events for cell division and the activity of membrane-bound enzymes (Kumar et al., 2015; Valitova et al., 2016).

Rice bran GO compounds

The GO chromatograms revealed the presence of 4 peaks identified as CAF, 24MCAF, CampF and β SF and confirmed by compounds spectra at 325 nm (Fig. 1).

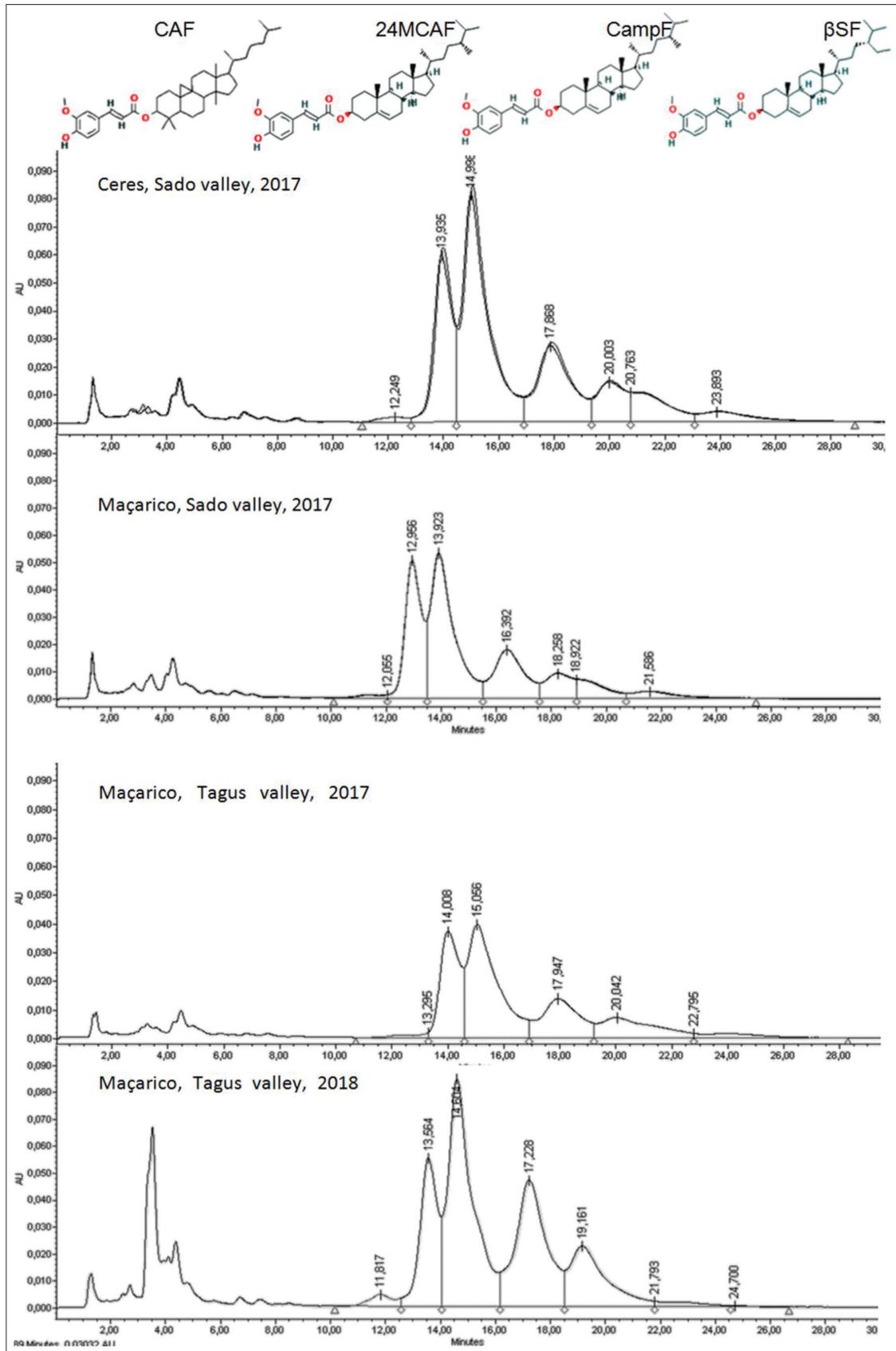


Fig 4. A - GO chromatograms from Ceres and Maçarico from Sado valley in 2017. B - GO chromatograms from Maçarico from Tagus valley in 2017 and 2018. The 4 peaks represent CAF (cycloartenyl ferulate), 24MCAF (24-methylenecycloartenyl ferulate), CampF (Campesteryl ferulate) and βSF (beta-sitosteryl ferulate).

Specific chromatograms profiles for the 4 GO compounds can be achieved by each variety as shows in Fig. 4:A in 2017S and also for the environment, as shown in Fig. 4:B for Maçarico 2017T and 2018T.

The percentage of GO compounds (Table 1) reveals the predominance of 24MCAF followed by CAF, which amounts exceed 60 % of the whole compounds.

The environmental means distribution of GO compounds for Ceres and Maçarico varieties are in the range of the previous data (Castanho et al., 2019), only noticed a slight increase in β SF.

Although the results show similar means for Ceres and Maçarico regarding GO composition, Ceres show lower variability range between growing environments. The growing conditions seem to be a factor of variation to the composition profile in both varieties, as the results show statistically significant differences between the values of all the compounds.

Rice bran cytotoxic activity by variety and environment

All the extracts were tested against four human tumour cell lines (NCI-H460, HeLa, HepG2, and MCF-7) and in a non-tumour primary culture (PLP2). The results are expressed in Table 2, where lower GI_{50} values indicate more effectiveness in the cell lines growth inhibition.

Table 1: Percentage of peak area of GO compounds as CAF (cycloartenyl ferulate), 24MCAF (24-methylenecycloartenyl ferulate), CampF (Campesteryl ferulate) and β SF (beta-sitosteryl ferulate) for Ceres and Maçarico Varieties on the 6 environments. Different letters in the same column show statistically significant differences at $p < 0.05$. Locations: T: Tagus valley; S: Sado valley; M: Mondego valley

Variety	Growing environment (year and location)	CAF	24MCAF	CampF	β SF
Ceres	2017M	25.10 ^c ±0.5	36.16 ^a ±0.5	18.65 ^c ±0.2	15.90 ^c ±0.1
	2018M	26.91 ^c ±0.5	36.35 ^a ±0.5	17.43 ^a ±0.2	14.92 ^a ±0.3
	2017S	21.88 ^b ±0.8	39.81 ^b ±0.8	18.40 ^{bc} ±0.1	15.42 ^{ab} ±0.1
	2016T	25.65 ^c ±0.7	41.15 ^c ±0.9	18.22 ^b ±0.2	14.98 ^a ±0.4
	2017T	22.37 ^b ±0.8	40.07 ^b ±0.6	17.45 ^a ±0.1	15.47 ^{bc} ±0.4
	2018T	19.28 ^a ±0.7	40.26 ^b ±0.2	20.75 ^d ±0.4	15.34 ^{ab} ±0.1
	Mean	25.5±2.7	38.9±2.0	18.4±1.1	15.3±0.4
	Range	19.2 - 26.9	36.1 – 41.1	17.4 – 20.75	14.9 – 15.9
Maçarico	2017M	28.76 ^c ±0.1	36.28 ^a ±0.2	17.06 ^a ±0.1	13.68 ^a ±0.1
	2018M	27.55 ^c ±0.3	37.69 ^b ±0.5	18.87 ^b ±0.2	12.93 ^b ±0.3
	2017S	25.76 ^b ±1.2	39.99 ^c ±1.5	17.00 ^a ±0.2	13.20 ^{bc} ±0.4
	2016T	28.74 ^c ±1.0	40.80 ^c ±0.3	18.41 ^b ±0.2	12.05 ^a ±0.8
	2017T	25.83 ^b ±1.2	39.92 ^c ±0.5	16.78 ^a ±0.8	13.34 ^{bc} ±0.1
	2018T	18.48 ^a ±1.0	37.44 ^b ±0.3	26.36 ^c ±0.1	15.65 ^d ±0.6
	Mean	25.8±2.8	38.6±1.6	18.9±2.5	13.47±0.8
	Range	18.4 – 28.7	36.2 – 40.8	16.7 – 26.3	12.0 – 15.6
Overall mean		24.6±2.8	38.8±1.7	18.7±1.6	14.4±1.1
Overall range		18.4–28.7	36.1–41.1	16.7–26.3	12.0–15.9

Table 2: Cytotoxic effect of rice bran extracts grown in different environments against different human tumour cell lines (NCIH460, MCF7, HepG2 and HeLa) and a normal cell line PLP2, expressed in GI_{50} values (μ g / mL). Locations: T: Tagus valley; S: Sado valley; M: Mondego valley

Growing environment (Year and location)	Variety	NCIH460	MCF7	HepG2	HeLa	PLP2
2016T	Ceres	68.27 ^a ±2.0	106.99 ^b ±2.5	111.32 ^e ±4.4	98.52 ^d ±1.5	>400 ^c
	Maçarico	74.29 ^a ±2.2	225.37 ^c ±6.0	185.02 ^f ±8.9	135.01 ^e ±1.6	>400 ^c
2017S	Ceres	75.13 ^a ±5.1	59.91 ^a ±3.2	40.09 ^{ab} ±19.9	51.47 ^{ab} ±2.0	135.18 ^a ±16.0
	Maçarico	61.16 ^a ±7.0	63.57 ^a ±1.5	21.61 ^a ±2.6	61.8 ^{abc} ±13.1	132.42 ^a ±10.9
2017M	Ceres	76.63 ^a ±12.0	59.38 ^a ±2.4	32.19 ^{ab} ±2.2	47.74 ^a ±5.7	129.38 ^a ±15.9
	Maçarico	220.50 ^c ±13.6	67.10 ^a ±4.1	44.93 ^b ±25.6	61.43 ^{abc} ±26.8	182.77 ^b ±10.0
2017T	Ceres	64.11 ^a ±1.1	64.56 ^a ±0.3	24.09 ^{ab} ±1.2	56.82 ^{abc} ±1.4	137.05 ^b ±7.4
	Maçarico	77.98 ^a ±1.5	123.1 ^b ±1.1	33.14 ^{ab} ±0.7	68.85 ^{cd} ±2.6	138.43 ^a ±2.8
2018T	Ceres	121.53 ^b ±64.7	58.82 ^a ±1.4	29.58 ^{ab} ±9.4	70.91 ^c ±2.8	142.55 ^a ±8.7
	Maçarico	58.77 ^a ±2.5	61.40 ^a ±3.4	40.53 ^a ±17.0	66.74 ^{bc} ±2.5	136.70 ^a ±3.7
2018M	Ceres	69.2 ^a ±0.3	56.04 ^a ±0.2	91.88 ^d ±2.2	60.54 ^{abc} ±1.2	128.90 ^a ±1.0
	Maçarico	69.6 ^a ±1.3	55.03 ^a ±0.0	68.83 ^c ±0.6	62.70 ^{abc} ±0.4	134.84 ^a ±2.6

GI_{50} values correspond to the extract concentration achieving 50 % of growth inhibition in human tumour cell lines or in liver primary culture PLP2. Reference ellipticine GI_{50} values: 1.21 μ g/mL (MCF-7); 1.03 μ g/mL (NCI-H460); 0.91 μ g/mL (HeLa); 1.10 μ g/mL (HepG2) and 2.29 μ g/mL (PLP2)

All the extracts presented positive results against the studied tumour cell lines; however, except the samples from the 2016SM environment, the remaining extracts show hepatotoxicity. Overall, the extracts had better results for HepG2. The cytotoxic effect varied with the growing environment ($p < 0.05$).

In a previous study, Castanho et al. (2019) tested the extracts of rice bran with different pericarp colours against the same tumour cell lines; the authors performed the same experiment with 2016 Ceres and Maçarico extracts. These Portuguese varieties showed stronger cytotoxic activity, only being surpassed by a purple pericarp rice, containing a high concentration of GO (3.20 ± 0.1 g/Kg). The authors also found a negative correlation between GO content and HeLa, HepG2 and MCF7 GI_{50} values. The same correlation was also found by Uttama et al. (2010), who tested a sample of Homali 105 rice bran extract against lung (CORL23), cervical (HeLa), prostate (PC3) and breast (MCF-7) cancer cell lines.

The data obtained in the cytotoxicity assays relate negatively to the GO content with some exceptions. As shown in Fig. 3:A, Ceres presents higher GO content for all the environments except 2018T; that inversion is also shown in NCIH460 and HeLa cytotoxicity values. Although these results are not statistically correlated, which may be related to the low variability of the results (1.56 - 3.18 g/Kg) comparing to other studies (0.59 - 3.30 g/Kg) (Castanho et al., 2019).

CONCLUSION

Gamma-oryzanol (GO) is a relevant bioactive compound present on rice bran and its concentration and composition can vary with the growing conditions. The rice bran from Ceres (*Japonica*) reveals higher levels of GO in five out of six environments analysed when compared with Maçarico (*Indica*) with overall means of 2.39 ± 0.3 g/kg and 2.09 ± 0.5 g/Kg respectively. A cytotoxic effect was observed for all the tested tumour cell lines and also varied with the growing environment. Overall the results show that in a temperate climate scenario the GO concentration in rice bran can be increased with higher temperatures during grain filling. Those findings can contribute to the increase of rice bran GO content and create new value to the release varieties in the breeding programs.

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