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New method for isolating β-sitosterol from bleaching effluent of sulphite pulp mill

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ABSTRACT

β-sitosterol (BS) is a phytosterol with wide potential in cosmeceutical, biomedical and food applications. In acid sulphite pulp cooking, BS and its derivatives are considered an underutilized natural source of bioactive compounds, which are adsorbed on the resulting pulp or dispersed in the spent liquor. In this work, a new method for the isolation of BS from alkaline extract (AE) of unbleached sulphite pulp purification stage is proposed. BS was recovered by two-step acidification of AE up to pH 3 under pre-selected conditions, followed by fractionation of the formed precipitate with water-miscible organic solvents, having obtained best results (> 90% of BS purity; yield of 70–210 mg/dm^3 of AE) with methanol fractionation followed by BS crystallization induced *via* addition of water (*ca.* 10% vol.). When ethanol was used instead, BS was detected in low amounts in the isolate, being fatty acid sterol esters and fatty acid glycerides the major components, as revealed from analyses by wet chemistry coupled with gas chromatography-mass spectrometry and thin layer chromatography (TLC). The method is adaptable for large-scale industrial production of BS and may represent an important source of sterol-based products.

1. Introduction

β-sitosterol (BS, M_r= 414.71) belongs to the class of plant Δ^5-desmethylsterols (phytosterols), which in turn include sterols and stanols, the latter referring to the saturated structures of the former (Fig. 1). Regardless of saturation degree, phytosterols can be found in their free or conjugated form, *e.g.*, esterified to fatty acids. They are widely distributed in products of plant origin, which have a variety of sterol mixtures, within which BS, campesterol and stigmasterol are the most predominant (Moreau, 2015). In plant cells, these molecules perform various functions at the cell membrane level and are related to plant adaptations to biotic and abiotic stress.

With a structure similar to that of cholesterol, β-sitosterol and its derivatives have the characteristic tetracyclic nucleus common to steroids, cyclopentaneperhydro-phenanthrene

Abbreviations: BS, β-sitosterol; PS, phytosterols; SE, sterol esters; AE, Alkaline extract

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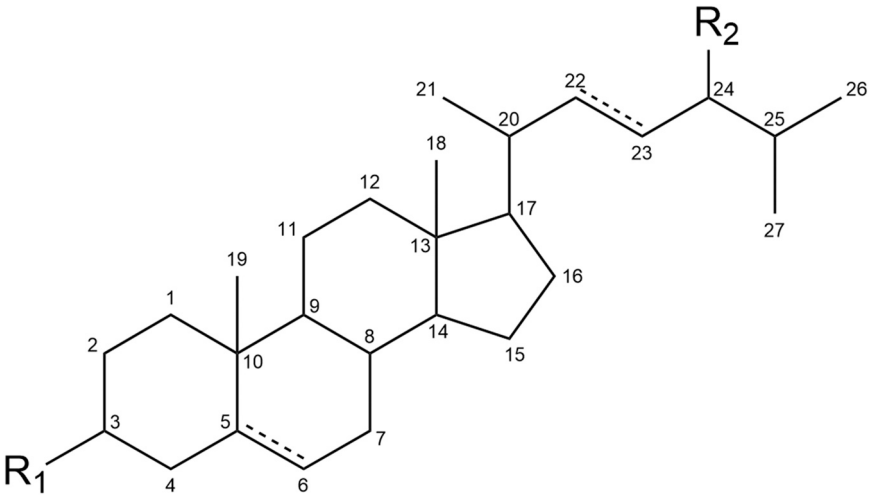

Fig. 1 – General Δ^5-desmethylsterol carbon skeleton with the respective carbon atom numbering. Most common free sterols examples, being R_1-OH and C5 =C6, include cholesterol: R_2-H, campesterol: R_2-CH_3, sitosterol: R_2-CH_2CH_3, and stigmasterol: R_2-CH_2CH_3 and C22 =C23. Sterol conjugates *e.g.*, sitosterol linoleate: R_2-CH_2CH_3 and R_2-$OOC(CH_2)_7$-CH=CH-CH_2-CH=CH-$(CH_2)_4$-CH_3 (linoleic acid). The absence of double bounds will result in the corresponding stanol counterpart *e.g.*, sitostanol: R_1-OH and R_2-CH_2CH_3 with C5-C6.

(Fig. 1). These structural characteristics give paradigmatic properties to these biologically active compounds and refer to several possible clinical applications (Salehi et al., 2021) and to their utility as raw material in pharmaceutical (*e.g.*, production of therapeutic steroids), cosmetic (*e.g.*, constituents in topical formulations) (Chen et al., 2020) or food (*e.g.*, hypocholesterolemic additives in functional foods or substitution of saturated fats in edible matrices) industries (Matheson et al., 2018; Ying et al., 2019). In the context of food applications, formulations based on these compounds are legally regulated possessing recognized health benefits, in dozens of countries (Zawistowski and Jones, 2015). Among phytosterols, β-sitosterol is considered especially interesting (Saeidnia et al., 2014; Bao et al., 2022). However, because high-quality products are practically non-existent on the market, the use of this sterol and the extent of clinical trials have been seriously limited (Ulbricht, 2016).

In general, large-scale commercial isolation of phytosterols is based on by-products of vegetable oil processing (*e.g.*, oils from vitamin E production or vegetable oils from biodiesel production) or from coniferous wood chemical processing (kraft pulping process), such as the case of "tall oil" (Fernandes and Cabral, 2007; Ying et al., 2019). Most raw materials will have phytosterols that are partially in their ester form and thus it is generally required the hydrolysis of sterol esters *via* temperature and/or chemical saponification. The unsaponifiable fraction is then separated and concentrated on the basis of the difference in solubility between unsaponifiables and the soap matrices in chosen solvents/mixtures of solvents, or the volatility differences between unsaponifiables and non-volatile soaps/salts. Each process will require modifications based on common features that include: phytosterol and fatty acid content, pH value, crystallization conditions, vaporization temperatures of phytosterol mixtures, possible thermal degradation during prolonged heat programs, and close boiling points of phytosterols and other compounds, such as tocopherols (Fernandes and Cabral, 2007). Some of the most notorious examples of patented processes, capable of achieving high purities and yields, are the WO2004111073 (Charlemagne et al., 2004) and WO2004000979 (Czuppon et al., 2003) for vegetable oil by-products, and WO2003022865A1 (Hamunen, 2003) and WO2000015652 (Huibers et al., 2000) for tall oil products. At the same time, β-sitosterol and its derivatives are a relatively abundant part of the lipophilic extracts of such angiosperms as *Eucalyptus*, *Quercus*, *Ulmus*, *Fagus* and *Betula* (Fengel and Wegener, 2003). These wood sources are commonly used in cooking processes for the production of cellulosic pulp.

Among *Eucalyptus* species, *E. globulus* has a moderate sterol content, especially when compared to *E. nitens* (Rencoret et al., 2007). Nonetheless, in *E. globulus*, phytosterols are present in the order of 645.0 ± 10.0 mg/kg and 516.7 ± 17.0 mg/kg for free and conjugated compounds, respectively, with BS being the predominant specie (Gutiérrez et al., 1999). Consequently, this is one of the world's largest industrial sectors that can also be considered as an untapped source of BS and derivatives. In fact, phytosterols are present in different industrial streams from the production of kraft (Costa et al., 2014) and sulphite (Rodrigues et al., 2018) pulps. In the acid sulphite pulping, wood is delignified by SO_2 dissolved in a suitable base solution (Ca^{2+}, Mg^{2+}, Na^+ or NH_4^+) at 130–145°C under acidic conditions (pH < 2) (Evtuguin, 2016). Sulphonated lignin is dissolved in sulphite spent liquor (SSL) along with polysaccharide degradation products and extractives. The obtained cellulosic pulp is separated from SSL and then bleached to achieve the desired brightness by a set of bleaching steps. In the case of regenerable pulping bases (Mg^{2+} and Na^+), the SSL separated from the cellulosic pulp is concentrated by evaporation and burned for energy and reagent recovery. BS, along with other lipophilic components, is present in the main effluent streams of acid sulphite process: SSL and bleaching effluents (Rodrigues et al., 2018). Despite the industrial pulp sector being a prospective source of BS derivatives, viable solutions for their recovery, directly from the industrial effluents, have not yet been proposed.

BS isolation is hampered by its coexistence in plants in a complex mixture of similar sterol structures and other lipophilic compounds. Therefore, it is difficult to obtain high purity products and those commercially available have normally significant amounts of campesterol. In summary, three possible BS production strategies can be considered,

targeting gram quantities: conversion of stigmasterol (considered the most accessible and least expensive phytosterol) *via* selective hydrogenation of Δ^{22-23} alkene with simultaneous protection of Δ^{5-6} double bond (McCarthy et al., 2005); isolation of BS from vegetable oils with a series of crystallizations (very expensive processes with purities rarely exceeding 70%); and purification with silica gel chromatography or Na-Y zeolite (with lengthy column purification cycles) (Srividya et al., 2014). However, these are examples that are difficult to adapt to the industrial context.

In this study, for the first time, a relatively simple methodology was developed for the recovery of BS and its derivatives from the bleaching effluent of the alkaline extraction bleaching stage in the acid sulphite pulp line production of dissolving pulp produced from *E. globulus* wood. The method consists in a selective methanol-based BS extraction of the precipitate obtained from acidified alkaline extracts under predetermined conditions. BS is isolated from the methanol extract by crystallization with addition of small amounts of water as co-solvent. Alternatively, predominantly BS derivatives can be obtained when using ethanol as the extraction solvent. The obtained BS and BS derivatives were thoroughly characterized by wet chemistry, mass-spectrometry and nuclear magnetic resonance spectroscopy techniques. The proposed approach is easily applicable on an industrial scale without the need for expensive equipment, reaching final purities > 90%.

2. Materials and methods

2.1. Raw materials

The industrial effluents from Mg-based acid sulphite dissolving pulp production were supplied by Caima - Indústria de Celulose S.A. (Constância, Portugal). The typical industrial cooking of *Eucalyptus globulus* wood was carried out at *ca.* 142°C for *ca.* 3 h (time-at-temperature) using the total SO_2 charge of *ca.* 23% and the combined SO_2 charge of *ca.* 4.6%. The obtained pulp was bleached employing E-O-P sequence (alkaline extraction (*E*)-oxygen delignification (O)-hydrogen peroxide (P) bleaching). The extract after the alkaline purification step (alkaline extract, AE) was collected from the washing press and immediately cooled down to 30–40°C and neutralized by 20% (w/w) H_2SO_4 to pH 7. Totally, 11 AE samples were collected in the period between 2018 and 2021, when the pulp mill certainly used similar pulping and bleaching conditions and the same wood source. All reagents used in this study were high quality commercial products purchased by chemicals suppliers: H_2SO_4, 98% (w/w), methanol and ethanol PA; diethyl ether, hexane and chloroform HPLC (Aldrich-Chemie, Steinheim, Germany); β-sitosterol ≥ 95% (w/w), tetracosane 99% (w/w), anhydrous pyridine, 99.8% (w/w), and *bis*-(trimethylsilyl) trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) were supplied by Sigma-Aldrich Chem. Comp. (Steinheim, Germany); other lipid standards (cholesterol, cholesterol oleate and triolein) were from Avanti® Polar Lipids, Inc. (Alabaster, AL, USA); Dimethylacetamide (DMA) 99%+ of HPLC grade was supplied by Acros Organics Chem. Comp. (Geel, Belgium). TLC plates were used from different sources (Merck, Macherey&Nagel, Germany; Sigma Chemicals, USA).

2.2. Recovery of β-sitosterol from AE

2000 cm^3 of industrial alkaline extract (AE) after the first stage of the E-O-P bleaching, previously cooled down to 30°C and adjusted to pH 7, was acidified to pH 5 using 20% sulfuric acid (w/w). After 24 h of exposure at room temperature (25°C), in glass containers (surface-to-volume (S/V) ratio of 0.67) sealed using glass or polypropylene stoppers or polymeric film (Parafilm®), the formed precipitate (*ca.* 0.30 g) was separated from the extract by decantation followed by centrifugation (Fig. 2). In a typical trial, the entire effluent (2000 cm^3) was distributed among four glass cylinders of 500 mL capacity each. The precipitate content (g/dm^3) was calculated as an average value from four respective

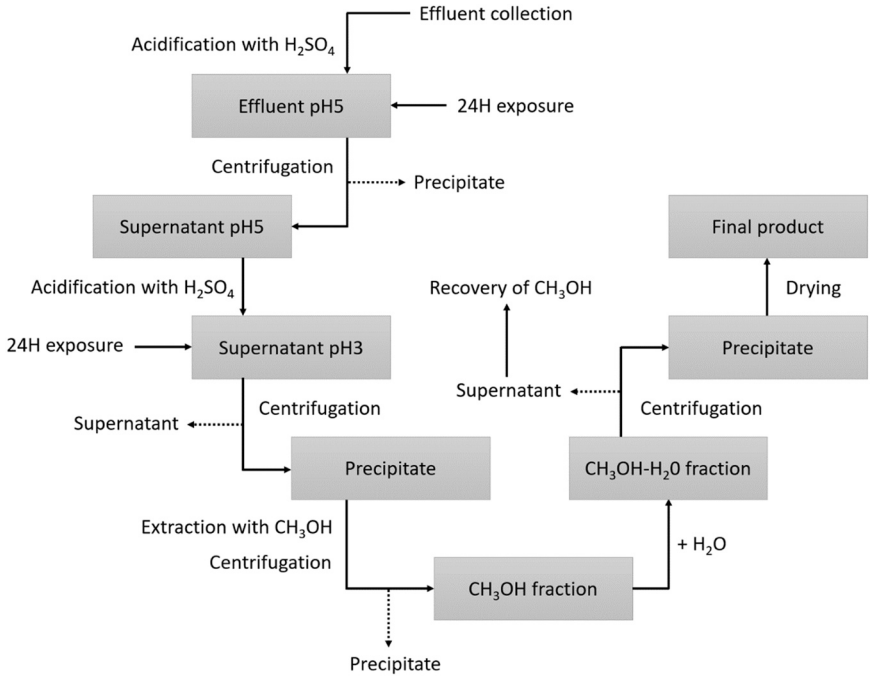

Fig. 2 – Summarized scheme of the steps comprising the developed process for the recovery of BS from alkaline extract after the first stage of the E-O-P bleaching line.

containers. AE with similar composition from two collection campaigns were used in these trials. When the same exposure step (pH 5) was carried out at 60°C for 24 h, a higher amount of precipitate was obtained (*ca.* 0.85 g). Subsequently, the supernatant was further adjusted to pH 3 using 20% (w/w) sulfuric acid. After a second 24 h of exposure at room temperature (25°C) in the same glass container, the formed precipitate was once again separated from the extract by decantation followed by centrifugation. The obtained residue (*ca.* 4.0 g) was successively fractionated under stirring with methanol (3 ×20 cm^3), centrifuging the sample after each extraction step and collecting the supernatant. The addition of 10% v/v of water to the combined methanolic extract obtained induces the precipitation of β-sitosterol, which was separated from the supernatant by centrifugation. After washing with water at room temperature, in order to remove traces of methanol, the precipitate was dried at 30 °C in a vacuum oven VacuTherm™ (Thermo Fisher Scientific, Waltham, Massachusetts, USA), obtaining in the end a dry precipitate of 0.41 g with a BS content of *ca.* 91%. Final yield for all methanolic samples reaching at least 90% in BS content, being expressed in grams of products per 1 liter of AE, was in the range of 0.07–0.21 g/dm^3. This variation in the BS yield was essentially due to variations in the BS content in AE received in the different collection campaigns at the pulp mill. However, for each series of trials, the AE of the same collection campaign was used.

Alternatively, 2000 cm^3 of industrial alkaline extract after the first stage of the E-O-P bleaching was stepwise acidified using the same procedure as described above (Fig. 2), but the obtained precipitate formed from AE at pH 3 (*ca.* 4.0 g) was fractionated 3 times with ethanol (3 ×20.0 cm^3) instead of methanol. The addition of 40% v/v of water to the collected ethanolic extract induced the precipitation of an insoluble residue, which was separated from the supernatant by centrifugation, with a dry weight of 0.650 g. The alkaline hydrolysis of this residue was carried out in methanol with sodium methoxide under the conditions described previously (Rodrigues et al., 2018). The reaction products were analyzed by gas chromatography coupled with mass spectrometry (GC-MS) as mixed methylated esters of fatty acids arisen in transesterification reactions and TMS derivatives of compounds possessing hydroxyl groups (Rodrigues et al., 2018).

2.3. *Analyses*

GC-MS analyses were carried out using a Trace Gas Chromatograph 2000 series (Thermo Fisher Scientific, Waltham, Massachusetts, USA) equipped with a Finnigan Trace MS mass spectrometer, using a DB-1 J & W capillary column (30 m x 0.32 mm i.d., 0.25 µm film thickness), and helium as carrier gas (35 cm/s). The chromatographic conditions were as follows: initial temperature: 80°C for 5 min; temperature rate 4 °C/min; final temperature 285°C for 10 min; injector temperature 290°C; transfer-line temperature 290°C; split ratio 1:65. Compounds were analyzed and identified as TMS derivatives (25°C, 24 h, in pyridine with BSTFA + 1% TMCS (2:1)) by comparing their mass spectra with the ones from Wiley® and NIST® libraries, when applicable. Additionally, a comparison with our own injected standards (*e.g.*, TMS-BS derivative) was made. The quantitative analysis was carried out using tetracosane as internal standard and GC-MS calibration was performed *via* injection of authentic derivatized standard, β-sitosterol ≥ 95% Sigma-Aldrich Chem. Comp. (Steinheim, Germany).

Solid-state Cross Polarization - Magic Angle Spinning ^{13}C Nuclear Magnetic Resonance (CP-MAS ^{13}C NMR) spectra were registered on a Bruker Advance 400 spectrometer (magnetic field of 9.4 T). Samples were packed into a zirconia's rotor sealed with Kel-F™ caps and spun at 12 kHz. Acquisition parameters were as follows: *ca.* 8000 scans with a 90° proton pulse, contact time of 1 ms and a recovery delay of 3 s. Glycine was used for the Hartman–Hahn matching procedure and as external standard for the calibration of the chemical shift scale relative to tetramethylsilane ($(CH_3)_4Si$). Proton and carbon NMR spectra were registered in $CDCl_3$ (298 K) on a Bruker ASCEND™ 500 spectrometer (Bruker, Wissembourg, France) operating at 500.16 MHz for proton and at 125.77 MHz for carbon. Proton spectrum was acquired using 60° pulse, 2 s relaxation delay and collecting 300 scans. Carbon spectrum was acquired using 60° pulse, 3 s relaxation delay and collecting 1500 scans. The cellulose, isolated from alkaline extract, was analyzed by size exclusion chromatography (SEC) using a PL-GPC 110 apparatus (Polymer Laboratories, Shropshire, UK), equipped with a pre-column, 10 µm, Plgel and two 300 mm × 7.5 mm columns, 10 µm, Plgel MIXED D (Polymer Laboratories, Shropshire, UK) and a refraction index detector. Columns and the injection system were maintained at 70°C. The eluent flow (0.1 M LiCl in DMA) was 0.9 cm^3/min. The cellulose sample (*ca.* 6 mg) was first dissolved in 50 µl of 8% LiCl DMA solution at 90 °C and then diluted 10 times by DMA. The column calibration was carried out using pullulan standards with Mp= 5.8–380.0 kDa (Polymer Laboratories, Shropshire, UK). Sugars analysis of the cellulose-containing residue was carried out using hydrolysate obtained by Saeman hydrolysis (residue was hydrolyzed for 2.5 h with 72% H_2SO_4 followed by 2 h at 100°C with 4% H_2SO_4; internal standard - 1 mg/mL of 2-deoxyglucose) on a Dionex™ Integrion™ HPIC™ system (Thermo Fisher Scientific, Massachusetts, USA). The sample was analyzed on a Dionex CarboPAC™ SA 10 µm (2 ×250 mm) column protected by Dionex CarboPAC™ SA 10 G-4 µm (2 ×50 mm) guard column and the chromatograms were processed with Thermo Scientific Dionex Chromeleon® software. The eluent was potassium hydroxide (KOH) 1 mM (0.38 cm^3/min).

Thin layer chromatography (TLC) separation was carried out on silica gel TLC plates pre-washed with chloroform/methanol (1:1, v/v) the day before the analysis, dried in the fume hood and put in the oven (100 °C) for 15 min. For TLC analysis, the samples were resuspended in chloroform and 10 µl of the solution containing 30–60 µg were applied on the TLC plate. Well known lipid standards, namely, cholesterol, triolein, and cholesteryl oleate were also applied in the same plate for identification. The elution was made in a pre-saturated TLC chamber with hexane: diethyl ether: acetic acid (8:2:1, v/v) solvent system. After drying, TLC spot localization was made by splaying with primulin solution (50 µg/mL, in acetone: water 8:2, v/v) and the fluorescent bands were observed at UV light (λ = 254 nm).

3. Results and discussion

3.1. *β-sitosterol and derivatives in industrial streams from pulping of wood*

β-sitosterol (BS) in its free and conjugated form (linked to fatty acids by ester linkage) is present in wood subjected to a

delignification (cooking) process in the production of pulp for paper or other cellulose-based products. In the production of dissolving pulp, after the acid sulphite cooking of *E. globulus* wood, the unbleached pulp is washed and submitted to three-stage bleaching (E-O-P) including the alkali extraction (E), oxygen delignification (O) and hydrogen peroxide bleaching (P) stages (Rodrigues et al., 2018). The obtained bleached dissolving pulp of high brightness (*ca.* 91% ISO) contains low amounts of cellulose contaminants and is widely used for viscose or cellulose derivatives production (Mendes et al., 2021). The first bleaching E stage aims to purify the obtained pulp from the residual lignin, excess of hemicelluloses and extractives (Rodrigues et al., 2018). Stage E is normally running with an alkaline load of about 3–5% on the pulps weight and high temperature (95–110°C). BS derivatives (free and conjugated) adsorbed on the unbleached pulp are extracted in an alkaline reaction medium (pH > 11) and remain mostly in the effluent (Rodrigues et al., 2018). Since this stage does not use any oxidizing agent, it is possible to preserve most of BS derivatives against degradation and isolate them from the alkaline effluent in higher amounts. Surprisingly, in the E stage not all of sterols are saponified and intact conjugated BS coexists in the alkaline extract (AE) despite the drastic conditions of the process in question (Rodrigues et al., 2018). Accordingly, the alkaline extract from E stage contains most of leached BS and its derivatives.

3.2. *Factors affecting the method for isolation of β-sitosterol and derivatives from AE*

The effluent from the E stage has a pH of *ca.* 10 and must be acidified to convert dissolved compounds into protonated form. More often than not, in the analytical laboratory practice, this step is followed by liquid-liquid extraction with a suitable water-immiscible organic solvent to dissolve lipophilic extractives. Instead, in this study, a stepwise precipitation of extract components was carried out at pH 5 and pH 3 to fractionate the major classes of AE constituents (Fig. 2). Noteworthy that direct adjustments of pH to values below 4 lead to uncontrolled co-precipitation of the various constituents, which makes selective BS isolation extremely challenging. According to the results of a previous study on the fractionation of AE, the acidification to pH 5 leads to protonation of ellagic acid dissolved in AE with some other polyphenolics and their selective precipitation without significant contamination from other lipophilic constituents (Evtyugin et al., 2023). Ellagic acid is considered a valuable co-product that has numerous biomedical and food applications (Evtyugin et al., 2020). The cellulose dissolved in AE, of a relatively high molecular weight, can also be precipitated under similar conditions. Accordingly, this primary acidification of AE to pH 5 allows not only to isolate valuable polyphenolics (*e.g.*, ellagic acid), but also pre-purify the effluent (AE) to avoid excessive contamination of BS-containing precipitate obtained at pH 3.

Hence, in the first step, the pH of the alkaline extract from stage E was adjusted to 5 in order to eliminate the dissolved carbohydrates and polyphenolics. This precipitation step at pH 5 is temperature dependent (Evtyugin et al., 2023) and when carried out in a glass container with surface-to-volume ratio (S/V ratio) of 0.67 at 60°C for 24 h, causes the precipitation of the polysaccharides dissolved in the alkaline extract (*ca.* 0.43 g/dm^3), which was identified as β-cellulose by solid-state ^{13}C NMR (Fig. S1, Supplementary materials). According to sugars analysis (glucose content), the purity of obtained cellulose was *ca.* 94%. The molecular weight of obtained cellulose was of 220 kDa as determined by SEC. According to previous results on the isolation of ellagic acid from AE, the temperature range of 40–60°C is also preferable from the point of view of ellagic acid yield (Evtyugin et al., 2023). The need for this temperature range is associated with the peculiarities of the crystallization of ellagic acid. The same temperature interval is favorable for the precipitation of cellulosic material. No significant increase in the amount of precipitate under these conditions was registered for periods of time greater than 24 h. However, if the same operation on the precipitation at pH 5 is carried out in the glass container with the same S/V ratio and the same exposure time (24 h) but at lower temperatures (*ca.* 25°C), much less amount of precipitate was detected (*ca.* 0.15 g/dm^3). This was mainly attributed to the lower amount of precipitated cellulosic material. The increase in precipitate amount, registered for the longer exposure times at 25°C (up to 120 h), was much less significant (Fig. 3). Precipitation at pH 5 had minimal influence on the quantity of β-sitosterol-based final product recovered from the extract. At the same time, the elimination of polyphenolic compounds and polysaccharides from the effluent at pH 5 reduces the concomitants in the second precipitate at pH 3 and promotes purity of the target product (β-sitosterol).

Subsequently, after separating the supernatant from the precipitate (pH 5), the former is further acidified to pH 3 giving rise to a sediment that contains β-sitosterol derivatives in its constitution (Fig. 2). The preferred temperatures of this precipitation at pH 3 are between 20 and 30°C, while at higher temperatures the amount of precipitate decreased substantially. Thus, a solid obtained at 25°C (*ca.* 2.1 g/dm^3) was separated from the acidified extract and subjected to successive fractionation with a pre-selected organic solvent. The analysis of this precipitate by solid-state ^{13}C NMR (Fig. 4) clearly showed that besides lipophilic substances, showing intensive resonances at 10–40 ppm, it contained remarkable proportion of lignin (resonance of OCH_3 at *ca* 56 ppm and aromatic carbons at 103–160 ppm) and of cellulose (resonance of C-6 at 63–66 ppm and C-2,3,5 at 70–78 ppm) (Gil and Pascoal Neto, 1999).

The choice of organic solvent to dissolve the precipitate obtained at pH 3 determines the amount and purity of

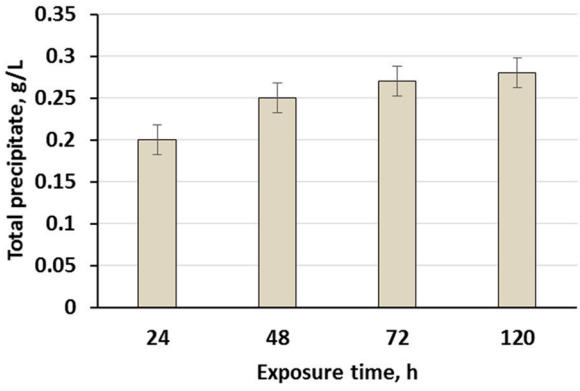

Fig. 3 – Dynamics of precipitate accumulation from acidified AE (pH 5) at 25°C in a glass container (S/V ratio of 0.67) for 24–120 h. Relative error of the determinations did not exceed 5%.

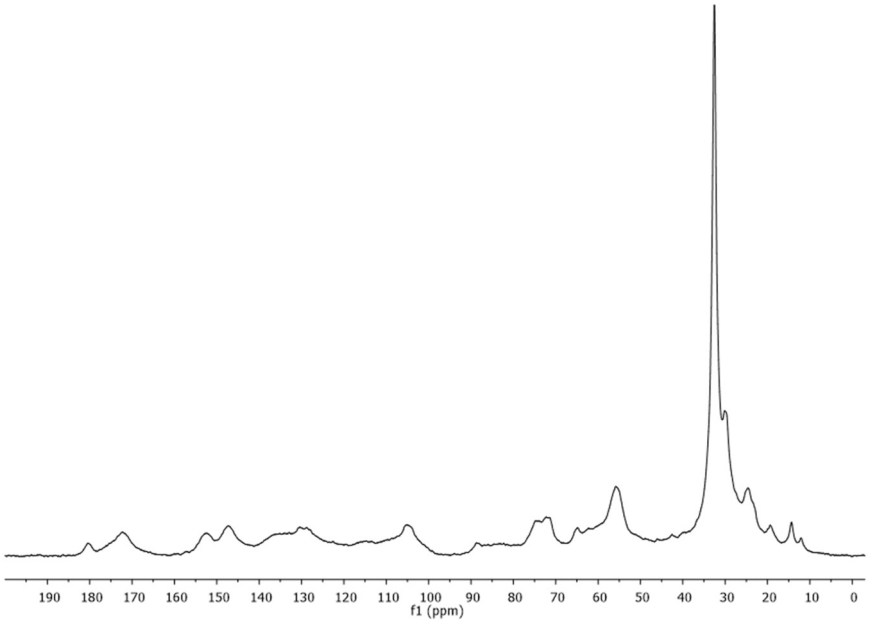

Fig. 4 – CP-MAS ^{13}C NMR spectrum of the precipitate obtained from AE after its acidification to pH 3.

β-sitosterol present in the final product. The amount of solvent used in the fractionation must be minimal, in order to guarantee the saturation of the dissolved product and to avoid an evaporation step to remove the solvent excess. In this study, three successive fractionations of the precipitate were used with an organic solvent (liquid-to-residue ratio of *ca.* 5), combining the obtained fractions. The addition of small percentages of water as co-solvent (up to *ca.* 10%, which may be higher if the precipitation does not occur) to the extract obtained with the organic solvent induced sterol precipitation. In fact, lipids are generally included in the category of non-polar molecules, sparingly soluble or insoluble in water (Moreau, 2005). In the case of BS, it was already documented that water addition to organic solvents can result in a drastic drop in solubility (Bar et al., 1984), which is demonstrated by data in Table 1.

Among examined water-miscible solvents (methanol, ethanol and acetone), the fractionation of the precipitate with methanol followed by crystallization with addition of water (*ca.* 10 vol%) gave the best BS purity (> 90%) with yield of *ca.* 0.21 g/dm^3, quantitatively confirmed by GC- MS (Fig. S2, supporting materials), with the main contaminant being a saturated analogue of the target compound, the sitostanol (*ca.* 4%). In addition, trace amounts of stigmasta-3,5-diene, a product of sitosterol dehydration and the most abundant steroid hydrocarbon present in several *Eucalyptus* species (Rencoret et al., 2007) have been detected. Among non-steroidal lipophilic structures, only trace amounts of n-hexadecanoic (palmitic), 9,12-octadecadienoic (linoleic) and hexacosanoic (cerotic) fatty acids were identified. The obtained crystallized product also showed characteristic patterns for BS in carbon and proton NMR spectra (Fig. S3, Supporting materials and Table 2). Some other classes of extractives, undetected by GC-MS, could also be present. The resulting products with higher BS content (yields up to 0.21 g/dm^3 of AE) were obtained during the extraction of the precipitate with methanol at temperatures between 20 and 30°C. The increase of the extraction temperature, as high as 30°C, led to higher percentage of contaminants due to a less selective solid-liquid extraction.

The main advantage of using methanol, despite much lower solubility of BS than for other examined solvents (Table 1), is that lipids with little or no polar groups (*e.g.*, triacylglycerides and sterol esters), which are highly soluble in hexane, benzene or cyclohexane, or in more polar solvents such as chloroform and diethyl ether, are sparingly soluble in methanol (Moreau, 2005). However, the solubility of these lipids increases in alcoholic solvents with increasing carbon chain length of the alcohol, being more soluble in ethanol and n-butanol; the same is also applicable to BS (Flynn et al., 1979). Thus, extraction with ethanol, acetone and ethyl acetate is also feasible, increasing, however, the possibility of contamination by other lipophilic extractives.

One example of such differences, in the composition of extracted compounds from the precipitate, was seen in extractions employing ethanol instead of methanol. The same extraction procedure of AE precipitate at pH 3 was used to

Table 1 – Solubility of β-sitosterol in different solvents (g/100 g of solvent).*

Methanol		Ethanol		Acetone		95% Ethanol 5% Water		80% Ethanol 20% Water		80% Acetone 20% Water	
T °C	c	T °C	c	T °C	c	T °C	c	T °C	c	T °C	c
30.4	0.492	30.4	2.901	30.7	4.583	30.4	1.735	29.5	0.160	29.5	0.211
33.9	0.571	33.9	3.504	33.9	4.753	34.0	1.825	34.5	0.215	34.5	0.255
38.0	0.680	38.0	3.901	38.0	5.267	38.0	2.244	38.6	0.318	38.0	0.365
45.3	0.824	42.1	4.628	42.1	6.126	41.2	2.572	44.2	0.440	44.2	0.525

* adapted from Bar et al. (1984). The same trends are also corroborated by Wei et al. (2010).

Table 2 – ^{13}C and ^{1}H NMR spectral data of β-sitosterol methanol fraction *vs* reference values.*.

Position	Bond	Chemical shift, δ (ppm)			
		^{13}C NMR		^{1}H NMR	
		Sample	Ref.	Sample	Ref.
1	CH_2	37.25	37.27	-	-
2	CH_2	31.65	31.64	-	-
3	CH(OH)	71.84	71.77	3.527 m	3.525 m
4	CH_2	42.28	42.33	-	-
5	QC (=)	140.74	140.76	-	-
6	CH (=)	121.75	121.69	5.363 t	5.353 t
7	CH_2	31.92	31.92	-	-
8	CH	31.92	31.92	-	-
9	CH	50.12	50.15	-	-
10	QC	36.51	36.51	-	-
11	CH_2	21.09	21.10	-	-
12	CH_2	39.77	39.80	-	-
13	QC	42.32	42.33	-	-
14	CH	56.76	56.78	-	-
15	CH_2	24.31	24.31	-	-
16	CH_2	28.26	28.25	-	-
17	CH	56.04	56.08	-	-
18	CH_3	11.87	11.89	0.680 s	0.680 s
19	CH_3	19.41	19.39	1.009 s	1.009 s
20	CH	36.15	36.16	-	-
21	CH_3	18.78	18.80	0.921d	0.921d
22	CH_2	33.94	33.96	-	-
23	CH_2	26.04	26.11	-	-
24	CH	45.83	45.85	-	-
25	CH	29.13	29.18	-	-
26	CH_3	19.83	19.82	0.813d	0.813d
27	CH_3	19.03	19.05	0.835d	0.835d
28	CH_2	23.06	23.08		
29	CH_3	11.99	11.99	0.846 t	0.844 t

*(Goad and Akihisa, 1997; Nes et al.,1992).

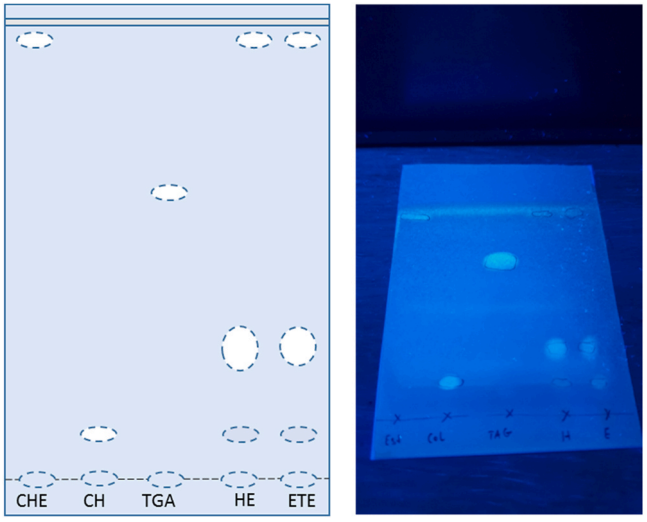

Fig. 5 – Schematic representation of TLC results (left image) of the analysis of ethanol (ETE) and hexane (HE) extracts. The eluent system was composed of hexane/diethyl ether/acetic acid (8:2:1, v/v). The standards were as follows: TGA is triglyceride of oleic acid (triolein); CH is cholesterol and CHE is cholesteryl ester (cholesteryl oleate). The original TLC is presented in the right image.

produce an ethanol extract, which then was mixed with water (*ca.* 40 vol%) to obtain a precipitate (*ca.* 0.33 g/dm^3 of AE) presumably containing BS. However, the analysis of this precipitated matter revealed just trace amount of BS (Fig. S4A, Supporting materials). At the same time, alkaline methanolysis followed by TMS-derivatization of obtained products revealed a noticeable amount of BS and fatty acid methyl esters (FAMEs) when analyzed by GC-MS (Fig. S4B, Supporting materials). A major part of β-sitosterol was also identified in the form of stigmasta-3,5-diene, a dehydration product of BS formed during the hydrolysis of sterol conjugates. The approximate amount of BS and its dehydration product was *ca.* 50% of identified compounds. Among fatty acids formed in the alkaline methanolysis, linoleic acid was the predominant one, which agrees with existing information in the literature for conjugated sterols and fats of *E. globulus* (Gutiérrez et al., 1999). Hydrolysis products of fats were also identified, including glycerol and a set of different FAMEs. Among these, FAMEs of hexadecanoic (16:0), 9,12-octadecadienoic (18:2), eicosanoic (20:0), tetracosanoic (24:0) and hexacosanoic (26:0) acid were detected (Fig. S4B, Supporting materials). Thus, the product isolated from the ethanolic extract was mostly composed of fatty acid glycerides and conjugated sterols, with only small amounts of free sterols. These findings corroborate that BS, present in AE, exists in both its free and conjugated form, and that the solvent chosen for the extraction of the AE precipitate at pH 3 plays a decisive role in the composition of the final product.

Given the importance of the selectivity role of organic solvent in the amount and type of BS derivatives isolated from the AE precipitate obtained at pH 3, an additional study was carried out to reveal the composition of the fractions obtained by extraction with ethanol and hexane. The last solvent is commonly used for a targeted extraction of fats and waxes from plant sources and industrial by-products (Hewavitharana et al., 2020). Even though hexane is not a very appropriate solvent to extract and purify sterols from the AE precipitate that still contains significant amounts of water, it was used for comparative reasons. The preliminary qualitative analysis of ethanol (ETE) and hexane (HE) extracts by thin-layer chromatography (TLC) confirmed that the major components of these fractions are esterified BS (presumably mainly β-sitosterol conjugates) and fatty acid mono- and diglycerides (Fig. 5). The last proposition is based on the known relative retention times of different mono-, di- and triglycerides reported for the same eluent system (Hwang et al., 2002). Practically no fatty acid triglycerides were detected in these set of samples. The last fact could be explained by partial alkaline hydrolysis of fats during the E stage of the E-O-P bleaching sequence. In general, visually, the content of free BS in HE was smaller than in ETE (Fig. 5). This was also confirmed by GC-MS analysis of HE, which was practically identical to ETE, but with almost negligible peak corresponding to BS (results not shown).

4. Conclusions

The results of this study clearly demonstrated the possibility of effective isolation of high purity β-sitosterol (BS) from alkaline bleaching effluent in the industrial production of dissolving *Eucalyptus* sulphite pulp. The BS recovery proceeds *via* fractionation of the precipitate formed after alkaline

effluent (AE) stepwise acidification to pH 3, with suitable water-miscible organic solvent. The choice of the organic solvent used in extraction of the obtained precipitate plays a crucial role in selectivity and purity of obtained target product. The dissolved BS is crystallized from organic solution by addition of water as a co-solvent. The best results for the BS recovery were obtained with methanol fractionation of the AE precipitate at nearly room temperature (20–25°C) followed by the crystallization with addition of *ca*. 10% (vol.) of water. This approach allows above 90% in β-sitosterol content, with a yield in the range of 70–210 mg/dm^3 of effluent. However, when using ethanol instead of methanol for the AE precipitate fractionation, only small amounts of BS were isolated by the same procedure, being fatty acid BS esters and fatty acid glycerides the main products. Noteworthy that after the isolation of BS, the effluents can still be reintroduced into the conventional industrial process for energy and reagent recovery, thus creating no additional environmental impacts. Methanol can be easily recovered from an aqueous solution by distillation since it does not form azeotrope with water. The process fits perfectly within circular economy and biorefinery concepts, is adaptable to the large-scale industrial production of sterol-based products from industrial acid sulphite process streams and can represent an important profit for pulp companies.

CRediT authorship contribution statement

Dmitry D. Evtyugin: Methodology, Investigation, Visualization, Writing – original draft. **Antonio Prates:** Project administration; Supervision, Resources, Validation, Writing – review & editing. **M. Rosário Domingues:** Supervision, Validation, Writing – review & editing. **Susana Casal:** Supervision, Validation, Writing – review & editing. **Dmitry V. Evtuguin:** Funding acquisition, Conceptualization, Supervision, Resources, Validation, 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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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/ j.fbp.2022.xx.xxx. The CP-MAS ^{13}C NMR spectrum of the precipitated β-cellulose; total ion chromatogram (TIC) of the precipitate from AE; ^{1}H and ^{13}C NMR spectra of the obtained final product and total ion chromatograms (TIC) of the GC-MS analysis (as TMS derivatives) of the ethanolic extract precipitate from AE, before and after alkaline methanolysis with sodium methoxide.

Appendix B. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fbp.2023.05.007.

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