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ARTICLE

Miscanthus x giganteus Extractives: A Source of Valuable Phenolic Compounds and Sterols

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The chemical composition of the lipophilic extracts of bark and core, of the *Miscanthus x giganteus* stalk, was studied by gas chromatography−mass spectrometry (GC−MS). Aromatic compounds, sterols, and fatty acids, followed by long-chain fatty alcohols, were the major families of components present in the *M. x giganteus* stalk. Aromatic compounds are more abundant in the *M. x giganteus* bark (521 mg/kg of bark), with vanillic acid, vanillin, and *p*-hydroxybenzaldehyde as the major compounds of this family. In the *M. x giganteus* core, sterols represent about 949 mg/kg of dry core with β-sitosterol, 7-oxo-β-sitosterol, stigmasterol, and campesterol as the major components. The detection of small amounts of esters in the GC−MS analysis with short columns explains the small increase in the abundance of the identified families after alkaline hydrolysis. The high content of valuable sterols and aromatic compounds in *M. x giganteus* and, particularly, in the core, which is considered a residue in most applications, can open new perspectives for the integrated upgrading of this grass within the biorefinery perspective.

KEYWORDS: *Miscanthus x giganteus*; bark; core; fatty acids; sterols; aromatic compounds; GC−MS analysis

INTRODUCTION

The genus *Miscanthus* is originally from the tropics and subtropics, but different species are found throughout wide climatic regions in East Asia and, nowadays, also in Europe and North America, where they were introduced first as ornamental plants (from Japan in the 1930s) and then as candidate energy crops, because of their excellent productivity, rapid growth, and high resistance to disease (*1*). *Miscanthus* are tall perennial rhizomatous grasses, belonging to the Poaceae family, with C_4 photosynthesis (*1*). Because of their perennial nature and high productivity, these species are very attractive biomass sources. Among these grasses, *Miscanthus x giganteus* Greef and Deuter ex Hodkinson and Renvoize (*2*), a sterile hybrid horticultural genotype, inadequately mentioned in some publications (*1*, *3*−*7*) as *Miscanthus sinensis giganteus*, *Miscanthus sinensis*, *M. sinensis* var. 'Giganteus', *Miscanthus x ogiformis* Honda 'Giganteus', or *Miscanthus* 'Giganteus', among others, has attracted the interest of the European Union (EU) authorities as a biomass source.

In 1993, an European Research Project (*Miscanthus* Productivity Network, AIR1-CT92-0294) (*1*) was implemented to evaluate the potential of *M. x giganteus* as a biomass source in Europe, as well as its use in a wide range of applications. The results obtained have shown that it has a high productivity, reaching yields of 30 tons ha^{-1} $year^{-1}$ (dry matter) in plantations in southern Europe (*1*), and the possibility to be a good source of biomass for solid fuel and construction materials as particle board (*3*). Also, chemical upgrading by organosolv fractionation (*4*) of its main components (cellulose, hemicelluloses, and lignin) opened the possibility of exploiting some of them in the production of methylcellulose (*5*), carboxymethylcellulose (*6*), adhesives (*7*), ethanol (*8*), and activated carbon (*9*), among other products (*10*). Those results open promising perspectives for the integrated exploitation of *M. x giganteus*, within the emerging concept of the biorefinery (*11*), as a biomass source for fuels, chemicals, and materials. Although, in a first stage, the search for new sources of large volume chemicals and materials is the primary aim of a biorefinery, the exploitation of low-volume−high-value chemicals (such as plant extractives) can give an important contribution to the global valorization of plant biomass (*11*). To our knowledge, no studies about the chemical composition of *M. x giganteus* extractives have been published until now. Therefore, to fulfill this gap, a detailed study of the lipophilic fraction of the bark and core of *M. x giganteus* extractives, by gas chromatography−mass spectrometry (GC−MS), was performed.

This work shows that the studied fractions and the core in particular are rich in aromatic compounds, sterols, and fatty acids. Considering that the first two families have useful nutraceutical and pharmacological applications and that core is normally rejected in most applications, these results might open new opportunities for the integrated exploitation of *M. x giganteus* within the biorefinery concept. Furthermore, this knowledge can also contribute to prevent eventual pitch problems and chemical consumption/costs, caused by the accumulation of lipophilic compounds in pulping and bleaching processes (*12*, *13*), whenever this hybrid becomes used as a fiber source.

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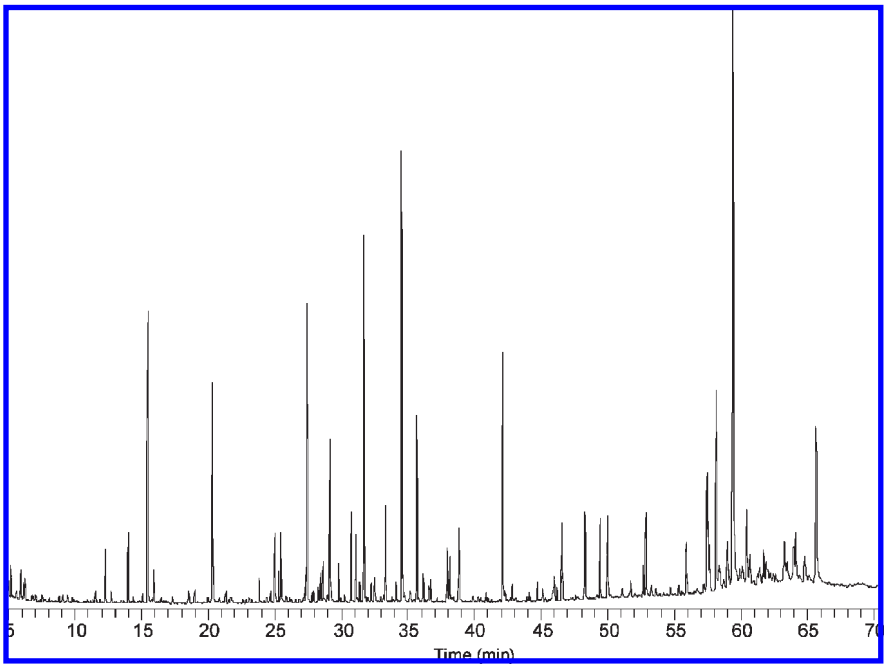

Figure 1. GC−MS chromatogram obtained by a DB-1 30 m column of the derivatized dichloromethane extract of *M. x giganteus* core, before alkaline hydrolysis.

MATERIALS AND METHODS

Chemicals. Potassium hydroxide (puriss, p.a., ≥86%), hydrochloric acid (puriss, p.a., fuming, ≥37%), and methanol (puriss, p.a., absolute, ≥99.8%) were purchased from Fluka Chemie (Madrid, Spain). Dichloromethane (99% purity), pyridine (99% purity), bis(trimethylsilyl)trifluoroacetamide (99% purity), trimethylchlorosilane (99% purity), stigmasterol (95% purity), octadecanoic acid (99% purity), nonadecanol (99% purity), coniferyl alcohol (98% purity), and tetracosane (99% purity) were supplied by Sigma Chemicals Co. (Madrid, Spain).

Samples Preparation. *M. x giganteus*, from an experimental plantation established as part of the European AIR Project (*1*) near Santiago de Compostela (Spain), was used in this study. Stems were harvested, manually separated into bark, core, and leaves, air-dried for 2 weeks, and ground to pass a 40−60 mesh sieve. Then, the ground samples of bark and core were allowed to attain their equilibrium humidity (7.70 and 7.32%, respectively) and finally stored in hermetic polypropylene containers until analysis.

Extraction. Three aliquots (20 g) of each powdered sample (bark and core) were extracted in a Soxhlet with dichloromethane (200 mL) for 8 h. The solvent was evaporated to dryness, and the extracts were weighed. The results were expressed in percent of dry material. Dichloromethane was chosen because it is a fairly specific solvent for lipophilic extractives.

Alkaline Hydrolysis. About 20 mg of each extract was dissolved in 10 mL of 1 M KOH in 10% aqueous methanol. The mixtures were heated at 100 °C, under nitrogen, for 1 h. The reaction mixtures were cooled, acidified with 1 M HCl to pH 2, and then extracted 3 times with dichloromethane. The solvent was evaporated to dryness.

GC−MS Analysis. Before GC−MS analysis, approximately 20 mg of each dried sample, with a measured amount (0.25−0.50 mg) of internal standard (tetracosane), was dissolved in 200 μL of pyridine and the compounds containing hydroxyl and carboxyl groups were converted into trimethylsilyl (TMS) ethers and esters, respectively, by adding 250 μL of bis(trimethylsilyl)trifluoroacetamide and 50 μL of trimethylchlorosilane. After the mixture had remained at 70 °C for 30 min, the derivatized extracts were analyzed by GC−MS following previously described methodologies (*14*, *15*). The GC−MS analysis was performed on a trace gas chromatograph 2000 series, equipped with a Finnigan Trace MS mass spectrometer. Analysis of samples, before and after alkaline hydrolysis, were carried out using a DB-1 J&W capillary column (30 m × 0.32 mm inner diameter, 0.25 μm film thickness); the chromatographic conditions were as follows: initial temperature, 80 °C for 5 min; temperature gradient, 4 °C/min; final temperature, 260 °C; temperature gradient, 2 °C/min; final temperature, 285 °C for 8 min; injector temperature, 250 °C; transfer-line temperature, 290 °C; and split ratio, 1:33 (*14*, *15*).

To check the presence of esterified structures, samples were also analyzed in a DB-1 J&W capillary column (15 m × 0.32 mm inner diameter, 0.25 μm film thickness); the chromatographic conditions were as follows: initial temperature, 100 °C for 3 min; temperature gradient, 5 °C/min; final temperature, 340 °C for 12 min; injector temperature, 290 °C; transfer-line temperature, 290 °C; and split ratio, 1:33 (*14*, *15*).

Chromatographic peaks were identified on the basis of the comparison of: i) their mass spectra with the equipment mass spectral library (Wiley−NIST Mass Spectral Library 1999); ii) their characteristic retention times, obtained under the described experimental conditions (*14*, *15*); and of iii) their fragmentation profiles with published data (refs *14* and *15* and references therein).

For quantitative analysis, GC−MS was calibrated with pure reference compounds, representative of the major lipophilic extractive components (namely, coniferyl alcohol, octadecanoic acid, nonadecanol, and stigmasterol), relative to tetracosane. The respective response factors were calculated as an average of six GC−MS runs. Two aliquots of each extract were analyzed before alkaline hydrolysis, and another two aliquots were analyzed after alkaline hydrolysis. Each aliquot was injected in triplicate. The presented results are the average of the concordant values obtained for each part (less than 5% variation between injections of the same aliquot and between aliquots of the same sample).

RESULTS AND DISCUSSION

The yields of lipophilic extractives from *M. x giganteus* bark and core were very similar, accounting for 0.53% (±0.04) and 0.63% (±0.01) of dry material, respectively. These values are in agreement with those reported for other nonwood materials, used as potential sources of biomass, such as flax (*16*), kenaf (*17*), abaca (*18*), hemp (*19*), giant reed (*20*), or unripe dwarf cavendish pulp (*21*).

The GC−MS analysis revealed that lipophilic extractives from bark and core have quite similar compositions before and after alkaline hydrolysis. **Figure 1** shows the typical GC−MS chromatogram of *M. x giganteus* core, before alkaline hydrolysis, used to identify the main compounds and to carry out the quantitative analysis. These extracts are mainly composed by aromatic

Table 1. Compounds Identified in the Dichloromethane Extracts of *M. x giganteus* Bark before (BH) and after (AH) Alkaline Hydrolysis Expressed in Milligrams of Compound per Kilogram of Dry Material[a]

		bark		core	
tr (min)	compound	BH	AH	BH	AH
fatty acids		393	450	453	551
saturated		351	404	363	438
6.2	hexanoic acid	5	3	5	4
9.4	heptanoic acid	3	2	3	2
12.7	octanoic acid	5	5	4	4
15.9	nonanoic acid	8	8	10	11
19.0	decanoic acid	3	2	5	5
24.7	dodecanoic acid	4	7	4	6
27.3	tridecanoic acid	3	3	9	10
29.8	tetradecanoic acid	7	11	13	18
32.2	pentadecanoic acid	3	4	8	9
34.6	hexadecanoic acid	89	112	133	175
36.7	heptadecanoic acid	4	4	10	9
38.8	octadecanoic acid	19	20	26	33
40.9	nonadecanoic acid	1	1	3	3
42.8	eicosanoic acid	11	17	6	7
44.7	heneicosanoic acid	2	3	7	8
46.6	docosanoic acid	8	11	31	32
48.3	tricosanoic acid	8	9	29	32
50.0	tetracosanoic acid	13	17	29	34
51.8	pentacosanoic acid	4	4	6	5
53.6	hexacosanoic acid	12	15	4	5
55.6	heptacosanoic acid	7	7	1	1
57.7	octacosanoic acid	109	116	14	22
59.8	nonacosanoic acid	5	4	ND[b]	ND
62.0	triacontanoic acid	18	19	3	3
unsaturated		16	19	17	23
33.9	hexadecenoic acid	1	1	1	1
38.2	*cis*-9-octadecenoic acid	15	18	16	22
α-hydroxy acids		26	27	73	90
46.0	2-hydroxyeicosanoic acid	3	2	7	10
49.4	2-hydroxydocosanoic acid	7	10	28	35
51.1	2-hydroxytricosanoic acid	4	3	2	4
52.9	2-hydroxytetracosanoic acid	12	12	36	41
aromatic compounds		521	313	829	509
11.5	benzoic acid	3	2	6	5
15.4	*p*-hydroxybenzaldehyde	64	49	115	78
17.3	resorcinol	3	TR[c]	3	1
20.3	vanillin	93	99	105	133
23.8	4-hydroxybenzoic acid	7	TR	14	TR
25.0	syringaldehyde	22	34	28	54
27.4	vanillic acid	108	68	115	61
28.6	4-hydroxy-3-methoxycinnamaldehyde	17	2	24	4
29.0	3-vanillylpropanol	3	4	5	4
30.8	syringic acid	18	17	41	38
31.1	1-guaiacyl-2-hydroxyethanone	21	TR	34	1
31.7	*p*-coumaric acid	50	18	160	27
32.5	3,5-dimethoxy-4-hydroxy cinnamaldehyde	7	TR	14	2
33.3	guaiacyl glyoxylic acid	39	6	55	8
34.1	2-hydroxy-1-syringylethanone	5	ND	13	1
35.2	ferulic acid	2	13	8	91
35.7	aromatic non-identified	54	ND	83	1
46.2	vanillylethanediol	5	1	6	ND
sterols		275	292	949	1006
57.5	campesterol	33	31	84	97
58.2	stigmasterol	45	50	137	156
59.4	β-sitosterol	98	110	408	432
59.6	stigmasta-3,5-dien-7-one	15	18	41	55
60.5	stigmast-4-en-3-one	29	19	64	86
60.7	stigmast-6-en-3,5-diol	10	11	19	13
61.8	7-hydroxy-β-sitosterol	7	10	29	ND
65.7	7-oxo-β-sitosterol	38	43	167	167
fatty alcohols		93	160	32	33
52.1	hexacosan-1-ol	6	15	2	2
54.0	heptacosan-1-ol	3	4	1	1
54.8	pentacosan-1,2-diol	3	2	4	4
56.0	octacosan-1-ol	81	139	25	26
others		88	35	40	36
25.5	pentadecan-2-one	19	16	38	34
47.7	heptacosane	11	9	2	2
53.3	octacosanal	58	10	ND	ND
non-identifiedcompounds		33	41	76	69

[a] Results are the average of the concordant values obtained (less than 5% variation between injections) for the two aliquots of each sample injected in triplicate. [b] ND = not detected. [c] TR = traces.

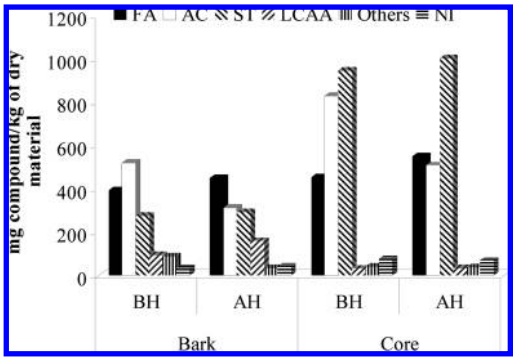

Figure 2. Major families of the components identified in the dichloromethane extracts of *M. x giganteus* bark and core before (BH) and after (AH) alkaline hydrolysis. FA, fatty acids; LCAA, long-chain aliphatic alcohols; ST, sterols; AC, aromatic compounds.

compounds, sterols, and fatty acids, followed by smaller amounts of long-chain aliphatic alcohols. However, the relative abundance of the identified compounds and their families differ considerably between the two substrates, either before or after alkaline hydrolysis (**Table 1** and **Figure 2**).

To verify the presence of esterified structures, the dichloromethane extracts before hydrolysis were also analyzed by GC−MS with a 15 m column (**Figure 3**), using chromatographic conditions that allow for the elution and detection of such low-volatile lipophilic compounds (*14*, *15*). In the GC−MS chromatogram of the studied samples (**Figure 3**), such esterified structures (mainly steryl glucosides and steryl esters) can be found in small amounts for retention times above 37 min.

Aromatic compounds represent (**Table 1** and **Figures 2** and **4**) about 521 and 829 mg/kg of dry *M. x giganteus* bark and core, respectively, before alkaline hydrolysis (36 and 34% of the lipophilic components of bark and core, respectively), with vanillic acid, vanillin, syringaldehyde, *p*-hydroxybenzaldehyde, and *p*-coumaric acid as the major components (**Figure 4**). After alkaline hydrolysis, the total amount of aromatic compounds detected has decreased substantially (**Table 1** and **Figure 2**), probably because some of them are lost during the alkaline treatment, either by degradation or by solubilization in the aqueous media, particularly, in the case of aromatic acids. Only a small increase is observed in the amount of some aromatic compounds, namely, vanillin, syringaldehyde, and ferulic acid. This increase might be related to the fact that hydroxycinnamic-type acids might appear esterified with lignin and polysaccharides in wood and suberized tissues (*14*, *22*−*26*), as well as esterified with fatty alcohols and hydroxy fatty acids (*14*). However, in the present study, these types of compounds were not detected in

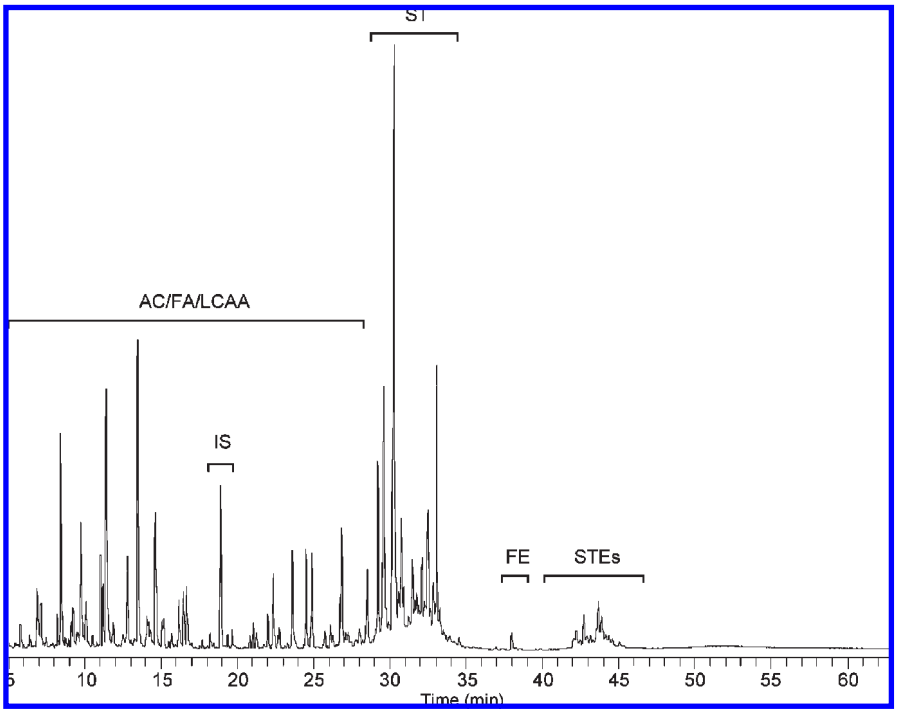

Figure 3. GC−MS chromatogram obtained by a DB-1 15 m column of the derivatized dichloromethane extract of *M. x giganteus* core, before alkaline hydrolysis. IS, internal standard; AC, aromatic compounds; FA, fatty acids; LCAA, long-chain aliphatic alcohols; ST, sterols; FE, ferulate; STEs, steryl esters.

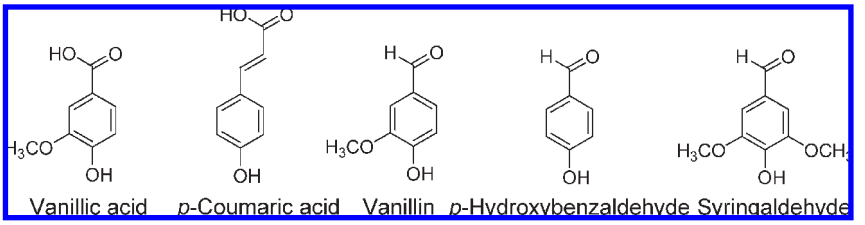

Figure 4. Structures of the main five aromatic compounds identified in the dichloromethane extracts of *M. x giganteus* bark and core.

measurable amounts in the analysis with short-length columns (**Figure 3**). In fact, only a small peak of a ferulate-type structure was detected at 38.1 min. This peak was identified as 1,3-diferuloylglycerol, based on its characteristic fragmentations at *m*/*z* 73, 249, 645, and 660 (*27*).

Fatty acids represent about 393 and 453 mg/kg of *M. x giganteus* bark and core, respectively, before alkaline hydrolysis (**Table 1** and **Figure 2**). After hydrolysis, only a slight increase in the amount of fatty acids (up to 450 and 551 mg/kg of dry bark and core, respectively) is observed. This observation is in agreement with the fact that the analysis of the extracts before hydrolysis, with the 15 m column, revealed small amounts of esterified structures that, once hydrolyzed, contributed to a slight increase in the fatty acids proportion (**Figure 3**). The fatty acids identified in *M. x giganteus* bark and core ranged from hexanoic to triacontanoic acids, including two unsaturated structures (C_{16} and C_{18}) and four α-hydroxy fatty acids (**Table 1**). The most abundant compounds identified in bark substrate were octacosanoic and hexadecanoic acids, followed by smaller amounts of octadecanoic and triacontanoic acids. In the core extracts, hexadecanoic acid was the most abundant compound, followed by similar amounts of docosanoic, tetracosanoic, tricosanoic, and two α-hydroxy acids (α-hydroxytetracosanoic and α-hydroxydocosanoic acids). The presence of α-hydroxy fatty acids has been previously reported in other plants (*18*, *28*−*30*). A considerable number of odd-chain fatty acids ranging from heptanoic to nonacosanoic acids were also identified in bark and core extracts. Tricosanoic acid, in particular, was found among the major components in the core extracts. Although these compounds are not very common in trees (*14*), they are frequently found in other plants belonging to the Musaceae (*18*, *28*) and Poaceae families (*20*) or in the straw of different cereals (*31*, *32*).

According to its abundance, sterols (**Table 1** and **Figures 2** and **5**) were the third class of compounds present in bark extracts, accounting for 275 mg/kg of dry bark material (19.6% of the lipophilic estracts in bark), whereas in the core, they are the most important family, accounting for 949 mg/kg of dry material (39.9% of the lipophilic estracts in the core). β-Sitosterol followed by stigmasterol and campesterol are the dominant components representing 64.0 and 66.3% of total sterols in the bark and core, respectively (**Table 1**). These components are common in tissues and fruits of tropical plants (*18*, *28*) and other rhizomatous grasses (*20*). Several oxidized sterol derivatives were also identified (**Figure 5**), and whereas stigmasta-3,5-dien-7-one has already been detected, for example, in giant reed (*20*) and sisal (*30*), the oxidized β-sitosterol derivatives (**Figure 5**) have been frequently referred as degradation products related to air exposure (or industrial processing) (*33*, *34*), which suggests that, in this case, some oxidative degradation of β-sitosterol during sample storage might have occurred. After alkaline hydrolysis, only a small increase in the amount of sterols was observed, which, once more, is in agreement with the low amount of steryl ester-type structures detected in the GC−MS analysis with short columns (**Figure 3**). Moreover, it is also worth mentioning that the variation in the amount of sterols, with hydrolysis, is not related to the presence of steryl glucosides because they are only detected

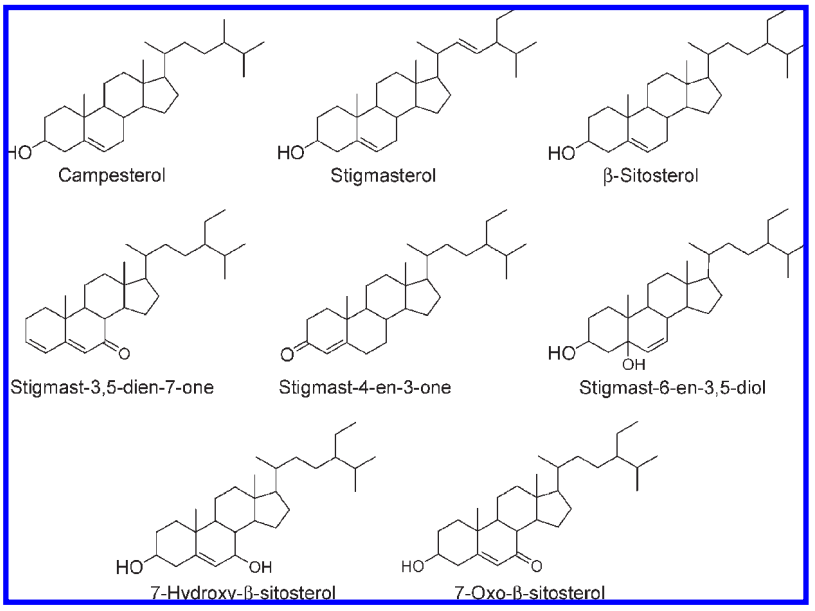

Figure 5. Structures of the sterols identified in the dichloromethane extracts of *M. x giganteus* bark and core.

in trace amounts (**Figure 3**) but also because these acetal-type structures are resistant to alkaline hydrolysis (*21*).

Long-chain aliphatic alcohols are the less abundant family of aliphatic compounds (**Table 1**), representing 93 and 32 mg/kg of dry bark and core, respectively. The main aliphatic alcohol identified in bark extracts was octacosan-1-ol, which accounted for the increase of this chemical group after alkaline hydrolysis. Finally, minor amounts of other compounds, such as octacosanal, heptacosane, and pentadecan-2-one, were also identified.

From the chemical composition described above, these *M. x giganteus* extracts might be seen as an interesting source of simple phenolic compounds and phytosterols, considering that these two families have a wide range of nutraceutical and phytopharmaceutical properties. For example, sterols have properties for reducing blood cholesterol levels (*35*). Phytosterols, as functional ingredients in foods, appear to be a practical and safe option for reducing cholesterol levels in the population (*35*). On the other hand, phenolic compounds have been recognized for many years by their antioxidant properties as food additives and nutraceuticals but also by their wide range of physiological properties, such as anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, anti-thrombotic, cardioprotective, and vasodilatory effects (*36*). These phytochemicals can contribute to the intake of natural antioxidants in the human diets. Obviously, the exploitation of these fractions would only be economically feasible if integrated with the exploitation of the main macromolecular fractions. In this perspective, they could be extracted before the use of biomass as biofuel, before the organosolv fractionation processes studied above for bark, or eventually partially recovered from the liquid effluents of the organosolv process. However, the industrial exploitation of these type of fractions will obviously require the choice of more environmentally friendly extraction systems, which, given their lipophilic nature, could involve, for example, supercritical CO_2 extraction.

In a different perspective, the knowledge of the composition of these lipophilic fractions is also relevant in relation to efficiency of the fractionation processes, especially when they are based on pulping and bleaching technologies, because it is known that these fractions are frequently responsible for the formation of deposits in the machinery. These deposits, known as pitch, increase processing costs and reduce fiber purity and quality. Concerning the processing of this species, particular attention has to be taken in relation to the long-chain fatty acids (higher than C_{16}) as well as the sterols fraction, which are known to cause severe pitch problems (*12*, *13*).

In the case of the core, because it is normally not used in the above-mentioned process and, particularly, because it is even richer in these components than bark, its exploitation would necessarily involve their pre-extraction before the remaining biomass would be used for other applications. Both exploitation approaches mentioned above would obviously represent a contribution to the integrated exploitation of this species biomass, within the biorefinery perspective.

Finally, in view of these promising results, in the future, an analysis of leaf extractives should be the next step in the global use of this grass.

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