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Accumulation of Phytosterols, Triterpene Alcohols and Phytostanols in Developing *Zea mays* L. Kernels

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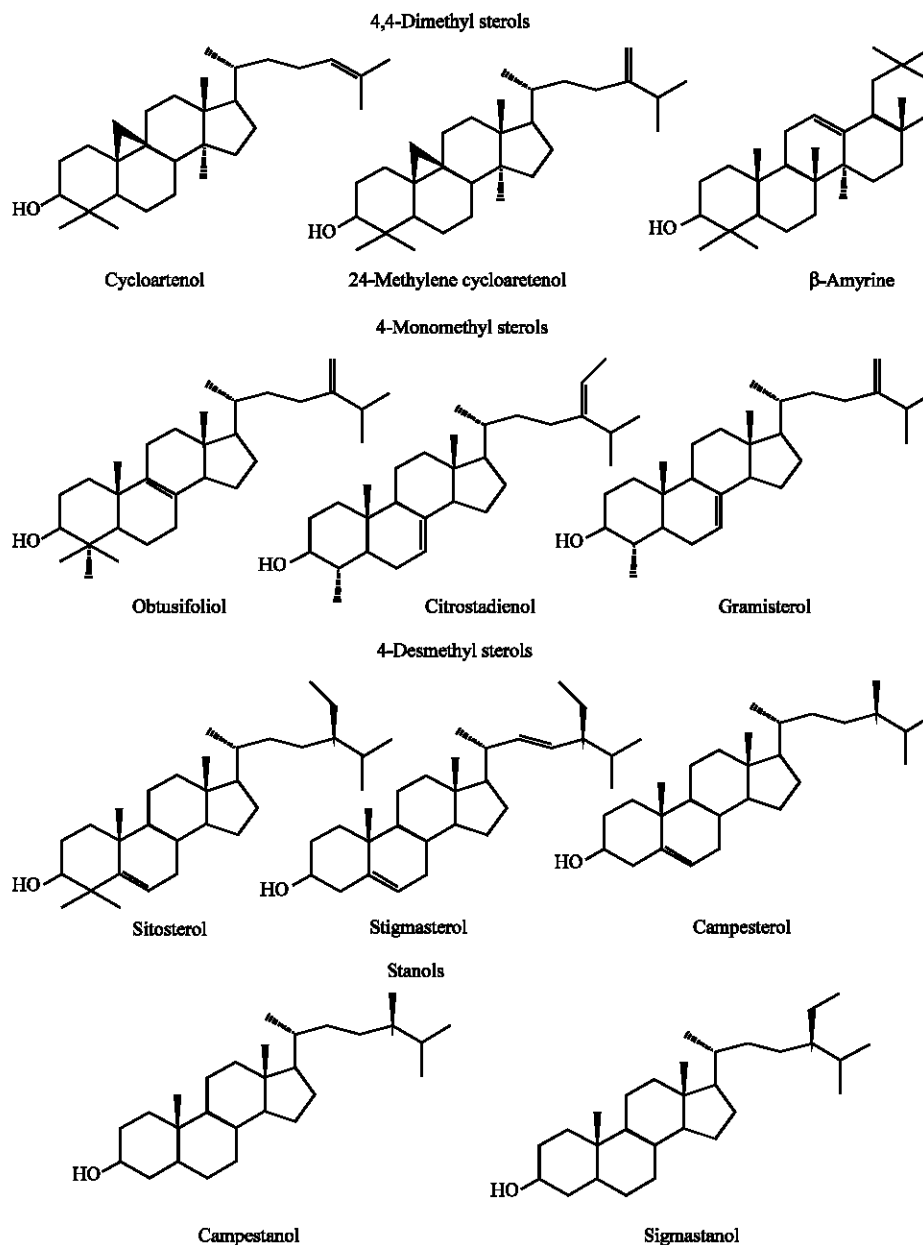
Abstract: Free sterol and free stanol components were determined in two varieties of sweet maize (Bonus and GH2547) and one variety of dent maize (Astro) during kernel maturation. The greatest change in these compounds occurred in the early development period. At maturity the free sterols were mainly composed of 4-desmethyl sterols (64-85%), followed by 4,4-dimethyl sterols (14-33%) and 4-monomethyl sterols (0.5-3%). 4,4-Dimethyl sterols and 4-monomethyl sterols followed the same accumulation pattern (when expressed as a function of total lipid content) during seed maturation. Both of these free sterol forms reached a maximum at 30 days after pollination (DAP). 4-Desmethyl sterol concentrations exhibited a decrease during kernel development. The free stanols showed different trends as compared to the free sterols. Based on Fujika and Acadia's results it is speculated that the decrease in total free stanol content observed at 30 DAP may be due to their potential conversion to brassinosteroids. The qualitative and quantitative differences observed in 4,4-dimethyl sterols and 4-monomethyl sterols suggested that these two minor fractions may be used as markers for the detection of oil adulteration.

Key words: Sweet corn oil, dent corn oil, 4-desmethyl sterols, 4,4-dimethyl sterols, 4-monomethyl sterols, phytostanols

INTRODUCTION

Plant sterols are very important to human health as they reduce dietary and biliary cholesterol absorption in the intestine, there by increasing fecal excretion of cholesterol (Conchillo *et al.*, 2005). The average dietary consumption of phytosterols and phytostanols is approximately 250 and 25 mg day⁻¹, respectively (Nair *et al.*, 2006). Phytosterols are only produced by plants. Physiologically, phytosterols are essential components of cellular membranes; they regulate membrane fluidity and permeability and interact with lipids and proteins within the membranes (Darnet and Rahier, 2004). Additionally phytosterols serve as intermediates for the synthesis of hormonal sterols and other related pharmaceuticals (Hamama and Bhardwaj, 2004). Sterols are present in three forms: free sterols (the major form with a free 3 β -hydroxyl group), steryl esters and steryl glucosides (Benveniste, 2004). In vegetable oils phytosterols mainly occur as free sterols or steryl esters of fatty acids (Verleyen *et al.*, 2002). According to the number of methyl groups at the C-4 position (scheme 1), sterols are divided into three groups: the 4-desmethyl sterols or simply sterols, the 4,4-dimethyl sterols and the 4-monomethyl sterols (Grunwald, 1975). These later two groups are generally considered to be intermediates in the formation of 4-desmethyl sterols. Therefore, demethylation at the C-4 position is an important step in the metabolism of sterols.

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Scheme1: Chemical structure of phytosterols and phytostanols

Phytostanols (completely saturated phytosterols) are less abundant in nature than phytosterols (Nair *et al.*, 2006). Plant stanols play many important roles and are particularly linked to brassinosteroid synthesis. Campestanol is a biosynthetic precursor of brassinosteroids which are made from campesterol by 5α -reduction at the A ring (Jang *et al.*, 2000).

Previous work on sterol biosynthesis in kernel *Zea mays* has been concerned with the accumulation of 4-desmethyl sterols. From the time of flowering to seed maturity, an accumulation of 4-desmethyl sterols was observed in corn kernels (Davis and Poneleit, 1974). Thus, sitosterol,

campesterol and stigmasterol are the major free sterols during kernel development (Davis and Poneleit, 1975). If the sterol content is expressed on the basis of dry matter or total lipids, accumulation of sterols preceded accumulation of dry matter and lipids (Weber, 1969). However, very little information is available on 4-monomethyl sterol and 4,4-dimethyl sterol accumulation during corn kernel maturation. Despite the fact that phytosterols have the same human health effects as phytosterols (Normén *et al.*, 2006), they have been not studied during corn kernel development. So, quantifying their contents in corn may be of help to the consumer to avoid intakes above the recommended limits, particularly from sweet corn which is the most common corn sold for human consumption.

The main aims of this study were to carry out a qualitative and quantitative characterization of 4,4-dimethyl sterols, 4-monomethyl sterols, 4-desmethyl sterols and phytosterols during sweet and dent corn development. The sweet corn (*Zea mays saccharata*) differs from dent corn (*Zea mays indentata*) by a single recessive gene which prevents some of the sugar from being converted to starch (Bland, 1971). To this end, we employed a TLC/GC-MS method similar those described previously by Conchillo *et al.* (2005), Damirchi *et al.* (2005), Moreau *et al.* (1998) and Jiang *et al.* (2000).

MATERIALS AND METHODS

Plant Material

Three varieties of maize (*Zea mays* L.), Astro (dent maize), Bonus and GH2547 (sweet maize), were grown on the Agronomy farm of the INRAT (Institut National Recherche Agronomie Tunis). Ear samples were collected at intervals after the dates of hand-pollination. Moisture and kernel weight were determined by weighing 100 kernels before and after drying to constant weight in a vacuum oven at 60°C.

Lipid Extraction

The total lipids were extracted by the method of Folch *et al.* (1957) modified by Bligh and Dyer (1959). Seeds (2.5 g) were washed with boiling water for 5 min to denature the phospholipases (Douce, 1964) and then crushed in a mortar with a mixture of CHCl₃-MeOH (2:1, V/V). The fixing water was added and the homogenate was centrifuged at 3000 rpm for 15 min. The lower chloroformic phase containing the total lipids was kept and dried in a rotary evaporator at 40°C.

Saponification

Unsaponifiable lipids were determined by saponifying 5 g of lipid extracts with 50 mL ethanolic KOH 12% (w/v) and heating at 60°C for 1.30 h. After cooling, 50 mL of H₂O was added and the unsaponifiable matter was extracted four times with 50 mL of petroleum ether. The combined ether extract was washed with 50 mL of EtOH-H₂O (1:1). The extracted ether was dried over anhydrous Na₂SO₄ and evaporated to dryness using N₂. The dry residues were dissolved in CHCl₃ for TLC analysis.

Thin-layer Chromatography

TLC of unsaponifiable fraction: the unsaponifiable matter was separated into subfractions on preparative silica gel thin-layer plates (silica gel 60G F254) using 1-dimensional TLC with hexane-Et₂O (9:1 by volume) as the developing solvent. The unsaponifiable fraction (4 mg in 100 µL CHCl₃) containing 1% (w/w) each of 5- α -cholestanol and lanosterol as the internal standard for 4-desmethyl sterols and dimethyl sterols, respectively, was applied on the silica gel plates in 3 cm bands. To correctly identify the sterols bands, a reference sample of purified sterol (5- α -cholestanol and lanosterol) were applied on the left and the right sides of the TLC plates. After development the plate was sprayed with 2,7-dichlorofluorescein and viewed under UV light. On the basis of the

Table 1: Retention time and mass spectrometric data for trimethylsilyl derivatives of sterols identified by GC-MS

Compounds	RT ¹	M ²	Major ions ³
Campesterol	19.16	472	457, 382, 367, 343, 129
Campestanol	19.23	474	459, 369, 384, 215
Stigmasterol	19.34	484	469, 394, 255, 129
Sitosterol	19.80	486	471, 396, 381, 357, 129
Stigmastanol	19.87	488	473, 398, 383, 215
Δ^5 avenosterol	20.04	484	469, 386, 296, 281, 129
Δ^7 avenosterol	20.27	484	469, 343, 281, 213
Δ^7 stigmastanol	20.17	486	471, 396, 381, 255, 213
Obtusifoliol	32.43	498	483, 459, 393, 227
β -amyryne	33.34	498	483, 393, 218
Gramisterol	33.63	484	469, 394, 379, 357, 253
Cycloartenol	33.82	498	483, 408, 393, 365, 339, 255
24-methylene cycloartenol	35.22	512	497, 422, 407, 383
Citrostadienol	36.50	498	483, 400, 357, 228

¹Retention times in minutes, ²Molecular weight of the trimethylsilyl derivatised sterol, ³M/z values for major characteristic ions

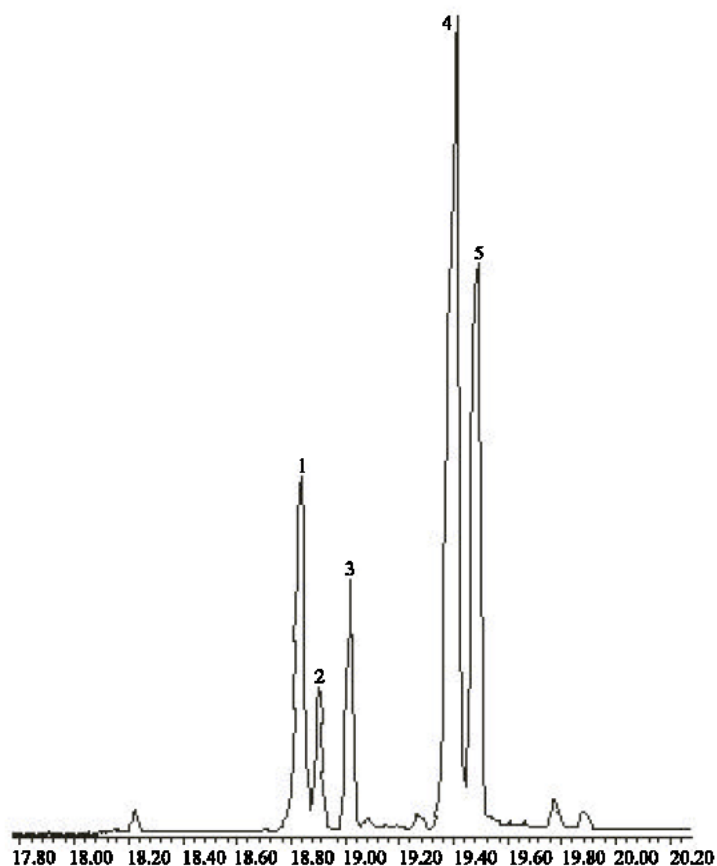


Fig. 1: GC-MS chromatograms of trimethyl silyl ether derivatives of 4-desmethyl sterol and stanols: (1) campesterol, (2) campestanol, (3) stigmasterol (4) sitosterol and (5) stigmastanol

reference spots, the sterols bands were identified. The bands corresponding to 4-desmethyl sterols and triterpene alcohol (4,4-dimethyl sterols) were scraped off separately and extracted three times with $\text{CHCl}_3\text{-Et}_2\text{O}$ (1:1), filtered to remove the residual silica, dried in a rotary evaporator and stored at -10°C for further analysis.

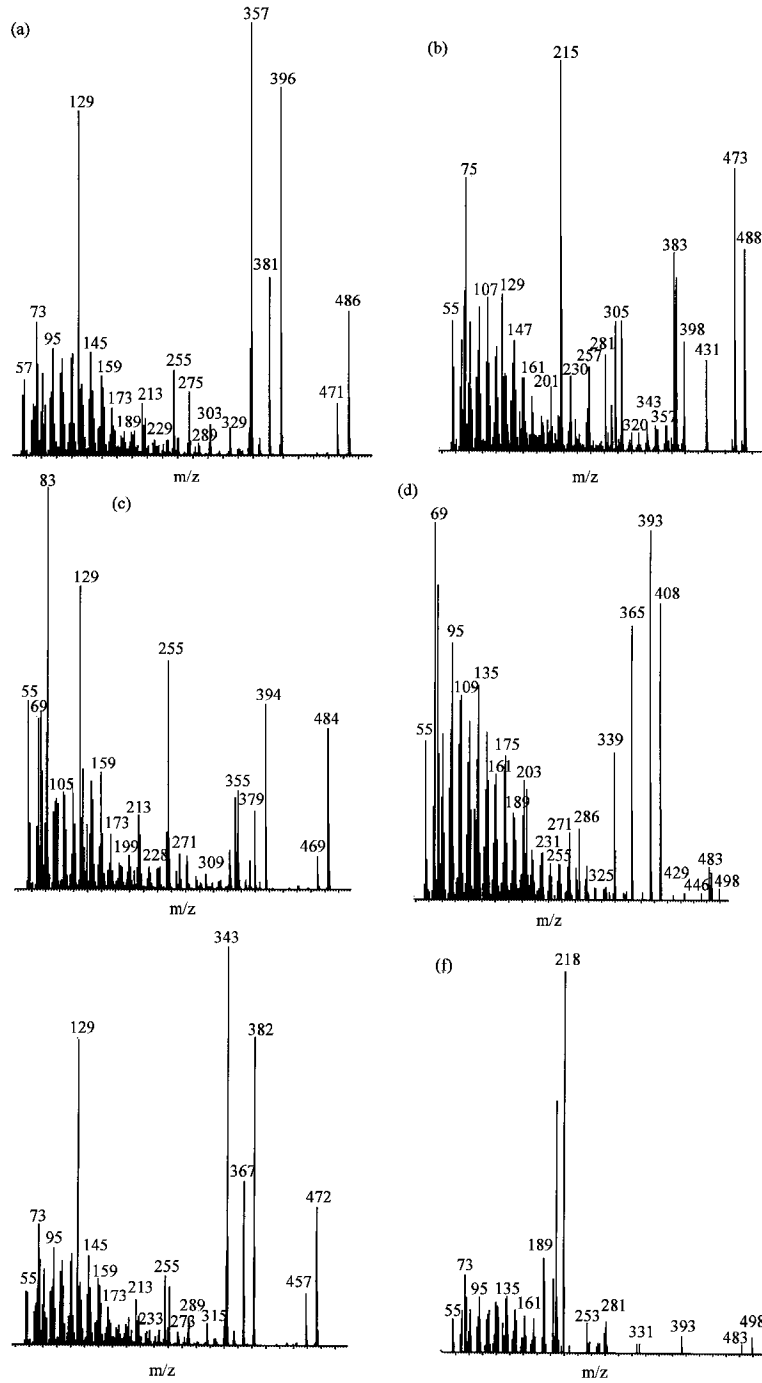


Fig. 2: GC-MS spectra of sterols: (A) sitosterol, (B) stigmasterol, (C) stigmasterol, (D) cycloartenol, (E) campesterol, (F) β -amyrine

GC-MS Analysis

Sterol fractions were silylated and injected into a GC (Hewlett Packard 6890) coupled to mass selective detector set to scan from 50 to 550 m/z. The system was fitted with a capillary HP-5

column (5% Phenyl Methyl Siloxane; 30×0.25 mm, 0.25 µm film thickness) and Helium was used as the carrier gas at 1 mL min⁻¹. The oven temperature was programmed from 150 to 320°C at 10°C min⁻¹. Manual injection of 1 µL of the solution of sterol was performed in the split mode at a 1:50 split ratio.

The sterols were identified by comparing the relative retention times (to a campesterol standard) and mass spectra with those previously published. Likewise, identification of stanols was done by comparing retention time with a stigmastanol standard and their reported mass spectra. Compounds were quantified by directly comparing their respective total ion chromatogram peak areas with that of an internal standard. For 4-desmethyl sterols the internal standard was 5- α -cholestanol while for triterpene alcohols and 4-monomethyl sterols it was lanosterol. Since response factors were not obtained for each sterol/stanol, the reported trends in compound concentrations will be more reliable than their absolute concentrations. Table 1 lists the identified compounds, their retention times and characteristic ions. A typical GC-MS chromatogram of the trimethyl silyl ether derivatives of 4-desmethyl sterols and stanols is shown in Fig. 1 and representative mass spectra for several sterols are presented in Fig. 2.

RESULTS AND DISCUSSION

Dry Weight

The patterns of dry weight accumulation for the three maize varieties were the same (Fig. 3). Each of the three samples rapidly accumulated dry weight from 10 to 40 DAP (days after pollination), in agreement with the results of Davis and Poneleit (1975). Synthesis of organic matter such as lipids, protein bodies and carbohydrates occurred during this period leading to an increase in the kernel weight. From 40 DAP until maturity more dry weight was accumulated by dent corn (Astro) than sweet corn (Bonus and GH2547).

Lipid Accumulation

As the corn developed, the amount of total lipid increased to a maximum at 40 DAP for sweet corn and 50 DAP for Astro (Fig. 4). This increase paralleled the observed increase in dry matter. As in a previous study (Weber, 1969) the rate of lipid synthesis was found to be greatest between 15 and 45 DAP. More oil was synthesized by sweet corn than dent corn at all sampling dates. Bonus and GH2547 can be described as intermediate oil-producing samples (having oil contents of 4.2 and 4.0% of total dry weight, respectively, at maturity) while Astro (at 1.7%) is a low-oil seed. The oil content was well within the range reported for kernel corn (Tan and Morrison, 1979). In the mature seed, oil was stored in the form of oil bodies (Voelker, 2001).

Phytostanol Accumulation

Phytostanols were found to be minor components of the lipid fraction. The change in free phytostanol content during corn kernel development has never previously been studied. Figure 5a shows that the greatest change in free phytostanol content (expressed as mg (100 g)⁻¹ of oil) occurred during the early stages of seed development. From 10 to 20 DAP there is an increase in the total amount of free stanols, but from 20 to 30 DAP a dramatic decrease is observed. For the remainder of the period the total amount is relatively constant. Thus, the highest levels of free stanols in each sample were detected at 20 DAP. At maturity the three varieties Astro, Bonus and GH2547 had phytostanol concentrations of 262, 157 and 123 mg (100 g)⁻¹ of oil, respectively. Dent corn oil consistently had more phytostanol content than sweet corn at all stages of development. Two free stanol compounds were detected (Fig. 1) at all stages of kernel development: stigmastanol (or sitostanol) and campestanol. The same trends are exhibited for these two compounds (Fig. 5b). In

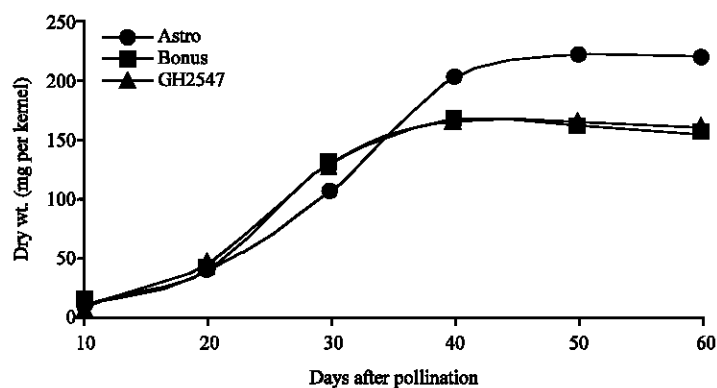


Fig. 3: Accumulation of dry weight during kernel maturation (mg per kernel) for Astro (●), Bonus (■) and GH2547 (▲)

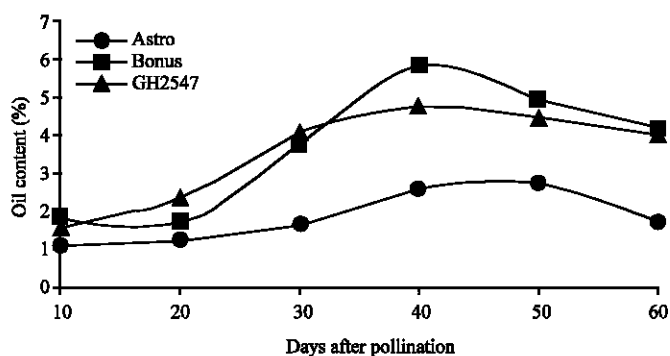


Fig. 4: Evolution of oil content (in % of dry weight) during maturation of three varieties: Astro (●), Bonus (■) and GH2547 (▲)

dent corn, stigmastanol and campestanol accounted for 332 and 94 mg (100 g)⁻¹ of oil, respectively at 20 DAP. But they are reduced to 288 and 48 mg (100 g)⁻¹ of oil, respectively at 30 DAP. This result suggested that the period from 10 to 20 DAP is the most active for stanol synthesis. The decrease of free stanols at 30 DAP may be due to their conversion to brassinosteroids which are steroidal hormones that regulate the growth of immature tissue. Fujika and Acadia (2003) reported that campestanol is the precursor of C₂₈ brassinosteroids whereas stigmastanol is the precursor of C₂₉ brassinosteroids. Additionally, it has been reported that immature seeds were the richest sources of brassinosteroids (Clouse and Sasse, 1998; Kim *et al.*, 2005). During kernel development stigmastanol was expressed at higher levels than campestanol, presumably because of differences in the concentrations of their precursors. During maturation, sitosterol was converted to stigmastanol and it was found at higher levels than the campestanol precursor campesterol.

Free Sterol Accumulation

Sterols are a minor component of the lipid fraction in most vegetable oils. It has been reported that the free sterol content changes during plant development (Huang and Grunwald, 1988; Izzo and Navari-Izzo, 1993; Moreau *et al.*, 1998). There are three forms of free sterols: 4-desmethyl sterols, 4,4-dimethyl sterols and 4-monomethyl sterols and all were found in the oil of the three strains of corn

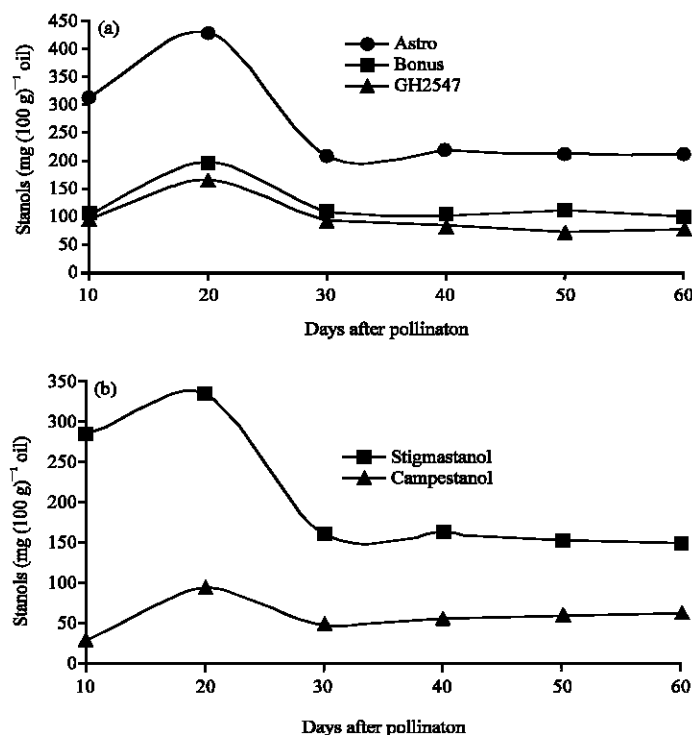


Fig. 5: Change in phytostanol concentration (mg (100 g oil)⁻¹): (a) total stanols during maturation of the three varieties. (b) Stigmastanol and campestanol amounts during maturation of dent corn kernel

investigated here, at all stages of development. During the development of the kernel, the amount of total free sterol decreased relative to other lipid fractions, in agreement with the results of Weber (1969), with 4-desmethyl sterols accounting for the major portion of this decrease (Fig. 6). The decrease in the total free sterols may be due either to their conversion to steryl esters or by the conversion of the most abundant 4-desmethyl sterols (sitosterol and campesterol) to stanols. Davis and Poneleit (1974) suggested that free sterols expressed as mg g⁻¹ dry weight decreased rapidly between 10 and 26 DAP, while steryl esters increased during corn kernel development. At maturity, Astro, Bonus and GH2547 had total free phytosterol contents of 1452, 1188 and 985 mg (100 g)⁻¹ of oil, respectively. The corn oil was very rich in phytosterols in agreement with the literature (Verleyen *et al.*, 2002; Mottram *et al.*, 2003; Ferrari *et al.*, 1996). At all stages of development, the dent corn oil had much higher quantities of 4-desmethyl sterols than did the sweet corn oil.

β -Sitosterol, campesterol and stigmasterol were the major 4-desmethyl sterols in the developing maize kernels, accounting for over 90% of sterols at all stages. Δ 5-Avenasterol (3%), Δ 7-avenasterol (4%) and Δ 7-stigmasterol (2%) amounted to less than 10% of the total 4-desmethyl sterol content. The components of the 4-desmethyl sterol fraction and their percentages were similar to those reported previously for oil corn (Davis and Poneleit, 1974; Davis and Poneleit, 1975). The Δ 5-sterols were mainly accumulated in the plasma membrane, where they are believed to regulate the membrane fluidity (Grandmougin *et al.*, 1989).

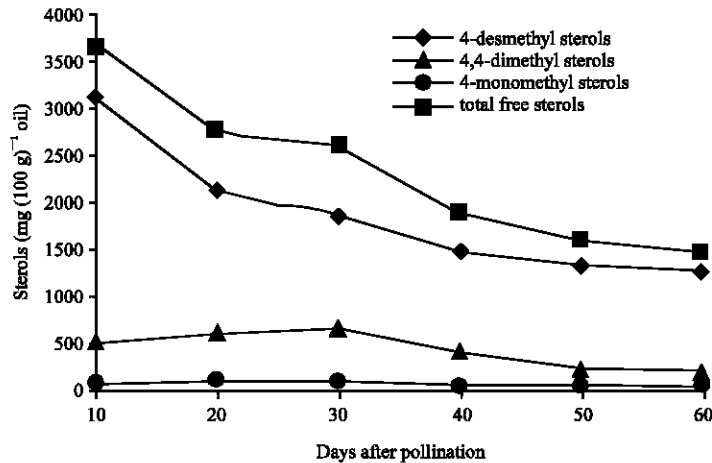


Fig. 6: Evolution of sterols (mg 100g/oil) in developing seeds of dent corn. Total free sterol concentration is represented by (■), together with that of 4-desmethyl sterols (◆), 4,4-dimethyl sterols (▲) and 4-monomethyl sterols (●)

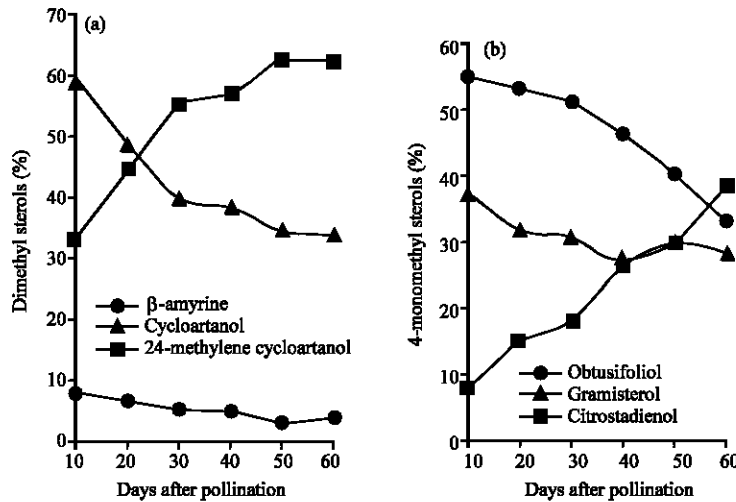


Fig. 7: Changes in 4,4-dimethyl sterol and 4-monomethyl sterol composition during maturation of corn kernels (variety Astro): (a) β-amyrine, cycloartenol and 24-methylene cycloartenol in % of total 4,4-dimethyl sterols. (b) Obtusifoliol, gramisterol and citrostadienol in % of 4-monomethyl sterols

The patterns of 4,4-dimethyl sterol and 4-monomethyl sterol accumulation were the same in the three samples. As the corn kernel developed, the abundance of 4,4-dimethyl sterols and 4-monomethyl sterols increased to a maximum at 30 DAP and then decreased during the last stages of the developing kernel.

The 4,4-dimethyl sterols were mostly cycloartenol and 24-methylene cycloartenol with only small amount of β-amyrine present. The evolution of these three components in the dent corn oil during kernel maturation is presented in Fig. 7a. Expressed as per cent of total 4,4-dimethyl sterols, the proportion of cycloartenol gradually decreased and reached a constant value of 34% at 50 DAP.

On the other hand the proportion of 24-methylene cycloartenol increased to a constant value of about 62% at 50 DAP. This result is likely due to the conversion of cycloartenol into 24-methylene cycloartenol. Cycloartenol is formed as the first cyclic triterpenoid precursor of sterols (Goodwin, 1979) and is the substrate for the first methylation reaction, resulting in 24-methylene cycloartenol. Furthermore, the percentage of β -amyryne dropped from its initial value of 7.8 to 3.1% as the grain matured, so at all stages the cycloartenol concentration was higher than that of β -amyryne. Considering the fact that cycloartenol and β -amyryne have the same precursor, 2,3(S)-oxidosqualene (Benveniste, 2002), we suggested that this latter compound was mainly cyclized into cycloartenol by 2,3(S)-oxidosqualene-cycloartenol cyclase. High plants contain several 2,3(S)-oxidosqualenes, a family of biocatalysts that convert 2,3(S)-oxidosqualene (OS) to polycyclic triterpenes (amyryne, lupeol, etc). The formation of cycloartenol is initiated in the chair-boat-chair-like conformation of OS while the formation of amyryns and related pentacyclic triterpenes is initiated in the chair-chair-chair-like conformation of OS (Benveniste, 2002). At maturity, 24-methylene cycloartenol was the most predominant (60.0 to 67.3% of the total 4,4-dimethyl sterol fraction) and cycloartenol (29.13 to 37.0%) was the second most abundant triterpene alcohol, while β -amyryne constituted less than 5%. This contrasts with the composition of lupin oil where cycloartenol and 24-methylene cycloartenol are the minor components of the 4,4-dimethyl sterol fraction and lupeol and β -amyryne accounted for more than 90% of total dimethyl sterols (Hamama and Bhardwaj, 2004). Additionally, Damirichi *et al.* (2005) reported that in olive oil 24-methylene cycloartenol was the major component with the proportion of 36 to 53%, followed by cycloartenol (22-25%) and β -amyryne (6-10%). These quantitative and qualitative differences observed in the components of the 4,4-dimethyl sterol fraction of vegetable oils suggested that this fraction may be used as a marker for the detection of oil adulteration.

4-Monomethyl sterols were not major plant sterols, but were interesting because they are intermediates in the biosynthesis of 4-desmethyl sterols. In the present study, they were found to comprise less than 5% of the total free sterol content, at all stages of kernel development. Fig. 7b shows the changes in the abundance of three 4-monomethyl sterols: obtusifoliol, gramisterol and citrostadienol for maturing Astro corn kernels. As the grain developed the percentages of obtusifoliol and gramisterol (or 24-methylene lophenol) decreased, while those of citrostadienol (or 24-ethylidene lophenol) increased. Obtusifoliol and gramisterol together accounted for more 90% of the 4-monomethyl sterol composition at 10 DAP but decreased to 62% at maturity, while citrostadienol increased from 8.0 to 38.3% from 10 DAP to maturity. These results were in agreement with literature data indicating that gramisterol was the preferred substrate for the second methylation reaction leading to citrostadienol during the biosynthesis of phytosterols (Goodwin, 1979; Benveniste, 2002). According to Sims *et al.* (1972), citrostadienol was detected in wheat germ oil as the main component of the 4-monomethylsterol fraction. Also Malecka (2002) has identified citrostadienol in tomato seed oil and suggested that this compound may exhibit antioxidant activity. Our results show that at maturity 4-monomethyl sterols were a mixture of obtusifoliol (30 to 33.5% of total 4-monomethyl sterols), gramisterol (23- 28.39%) and citrostadienol (38.33-43.47%). The qualitative and quantitative composition of corn oils was different from olive oil which had citrostadienol as the most abundant monomethylsterol followed by obtusifoliol and cycloeucaenol. Gramisterol was less than 7% of the total (Damirichi *et al.*, 2005). Therefore, the monomethyl sterol fraction may also be useful for detecting the adulteration of oil. The free sterol composition of dent corn was different from sweet corn. In dent corn oil, 4-desmethyl sterols make up 85.5% of the total free sterols, while 4,4-dimethyl sterols and 4-monomethyl sterols constituted 14 and 0.5%, respectively. Sweet corn oil contained a much lower 4-desmethyl sterols (64-67%) and higher levels of 4,4-dimethyl sterols (31-33%). So, the relative proportions of the components of the free sterol fractions can be used for chemo taxonomical investigations.

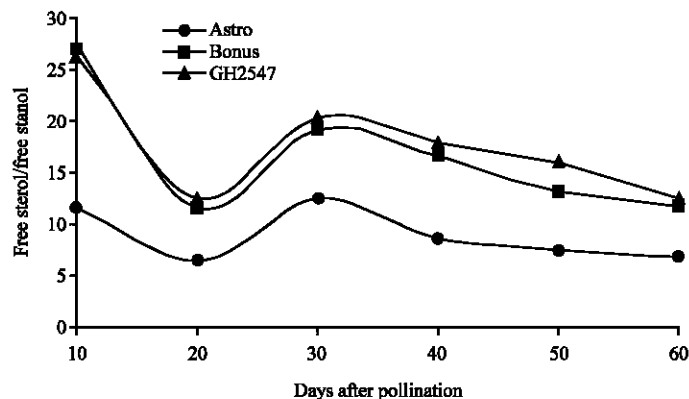


Fig. 8: Variation in the free sterol to free stanol ratio during maturation of corn kernels of Astro (●), Bonus (■) and GH2547 (▲)

Hydrogenation of the $\Delta 5$ double bond of $\Delta 5$ - sterols yields, stanols. The ratio of free phytosterols to free phytostanols (Fig. 8) was consistent with the trends observed in Fig. 5 and 6. At maturity this ratio was about 12 for sweet corn oil and 6.8 for dent corn oil. Moureau *et al.* (2000) suggested that in corn kernels, phytostanols were localized mainly in the aleurone cells and that the germ oil contained phytosterols but no phytostanols. Additionally, it has been reported that the distribution of kernel lipids was 76-83% in germ and 13-15% in aleurone (Tan and Morrison, 1979). Thus, it would be reasonable to attribute the low phytostanol content of corn oil to their accumulation mainly in the aleurone lipid.

CONCLUSIONS

In summary, this survey indicates that free phytostanols, as well as three forms of free phytosterols, were present in corn oil at all stages of kernel development. However, phytostanols and sterols exhibited different trends in accumulation during kernel maturation. At all stages of kernel development the 4-desmethyl sterols were the major free sterol fraction, while 4,4-dimethyl sterols and 4-monomethyl sterols were present in much lower amounts. We can conclude that the conversion of 4,4-dimethyl sterols to 4-monomethyl sterols (which in turn are converted to 4-desmethyl sterols) occurred principally before 10 DAP or prior there is a rapid increase in oil content. The qualitative and quantitative characterization of 4,4-dimethyl sterols and 4-monomethyl sterols seem to be useful for many aspects of vegetable oil production, including detection of adulteration, quality control and product development.

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