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Research article

Metabolite profiles of commercial colored Thai corn hybrids (*Zea mays* L.)

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Abstract

Corn hybrids (*Zea mays* L.) of various kernel colorizations are continually being developed due to increased demand, acceptance and utilization among consumers. A broad range gas chromatography-flame ionization detector-based metabolite profiling method was used to investigate the low molecular weight constituents of five colored Thai corn hybrids grown simultaneously at the same location and in the same season. The results showed that the majority of lipid metabolites was higher in the variety 7566 than the others as well as fraction IV (acids) metabolites. Notably, this variety also contained a high amount of nutritionally important phytosterols (sitostanol, campesterol, stigmasterol, Δ7-campestenol). Principal component analysis and hierarchical cluster analysis separated the varieties into three distinct groups based on metabolite abundance. Importantly, there was no separation based on kernel color. No previous research regarding differentiating colored Thai corn hybrids using metabolite profiling currently exists; thus, this method can provide valuable information for crop improvement, nutritional analysis and functional quality assessment within the food industry and nutritional sciences.

Introduction

Corn hybrids (*Zea mays* L.) of certain color and phytochemical composition currently offer unrealized potential both commercially and nutritionally (Urias-Peraldí et al., 2013). Remarkable progress in screening a broad spectrum of crop constituents in a single run has resulted in metabolite profiling becoming the method of choice for food crop metabolite analysis (Frank and Engel, 2013) especially in characterizing and differentiating crop genotypes and phenotypes (De and Nag, 2014; Frank et al., 2007). Extensive applications of this method in rice (De and Nag, 2014; Frank et al., 2007; Frank et al., 2012a), black gram (Na Jom et al., 2015) and mung beans (Na Jom et al., 2011) have demonstrated its utility in food crop analysis. Gas chromatography-mass spectrometry (GC-MS)-based metabolite profiling can evaluate the chemical constituents of corn with regard to genetic and environmental factors (Frank et al., 2012b), agronomic practices (Röhlig and Engel, 2010) and breeding strategies for crops (Zeng et al., 2014). Comparably, the cost-effective gas chromatography-flame ionization detection (GC-FID) based metabolite profiling approach has also been applied in food crops (Frank et al., 2007; Na Jom et al., 2015).

Corn provides nutritional security for millions throughout the world (Shiferaw et al., 2011) based on the calories, protein, vitamins, minerals, and lipids required for survival (Menkir et al., 2008). Corn is one of five major crops grown in Thailand, along with rice, cassava, sugarcane and rubber (Ekasingh et al., 2004). The ever-increasing demand for corn, in Thailand and abroad, as a food and livestock-feed crop (Shiferaw et al., 2011) has resulted in continual maize hybrid diversification and improvement for both the fresh and processing markets (Ketthaisong et al., 2014). Nowadays, maize hybrids offer a wide variety of kernel colors such as white, red, black, purple and yellow (Harakotr et al., 2015), reflecting species diversification

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and metabolite concentration variability (Keurentjes, 2009) and difference in amylose and amylopectin composition (Almeida et al., 2010). Phenotypically, corn kernels can be easily but inaccurately differentiated by explicit visual color identification (Rodríguez et al., 2013) and morphology (Fiehn, 2002). However, consistency in color-based maize selection/sorting is problematic due to poor color-memory in humans (Hasing et al., 2012). Additionally, the detailed information required for species differentiation is unattainable using mere visual selection as contrasted with highly-precise metabolite content analysis (Fiehn, 2002). Metabolites play a significant role in the nutritional quality, color, taste and scent of food crops (Hounsome et al., 2008) and are therefore an appropriate measure of the biological status of a food crop (Asiago et al., 2012). A comparative and comprehensive investigation of metabolite compositional variation, especially in colored Thai corn hybrids, is lacking. Therefore, this study investigated the metabolic profiles of five colored Thai corn hybrids, grown at the same location and in the same season, using GC-FID-based metabolite profiling. Additionally, the amylose and total anthocyanin contents of each hybrid were determined.

Materials and Methods

Corn samples

Five commercial, Thai, colored corn hybrids (*Zea mays* L.)—M80 (creamy white), 7566 (creamy yellow), 8003 (purplish purple), CP09 (purplish purple) and LB-50-LB (light purple)—were graciously donated by the Chai Nat Field Crops Research Centre, Ministry of Agriculture and Cooperatives, Chai Nat, Thailand. These hybrids were grown during the Thai dry season (October–December 2007) in onsite cornfields at the research center.

Sample preparation

Dried corn kernels (10–15% moisture content) were ground and passed through a sieve mesh no. 100 (Retsch; Haan, Germany). The flour was freeze-dried (Gamma 216 LSC, Christ; Osterode am Harz, Germany) for 48 hr after being kept at -60°C for overnight. Freeze-dried corn flour was stored at -20°C until analysis.

Chemicals

Unless otherwise stated, all solvents were high-performance liquid chromatography grade and all other chemicals were analytical grade from Sigma-Aldrich (St. Louis, MO, USA).

Metabolite profiling

Extraction and fractionation of the freeze-dried corn flour samples were performed as described by Röhlig et al. (2009). This protocol detected a wide range of lipids (fatty acid methyl esters, hydrocarbons, fatty alcohols, sterols) and polar low-molecular weight compounds (for example sugars, sugar alcohols, acids, amino acids and amines). Lipids and polar compounds were consecutively extracted from 400 mg of corn flour. After adding the internal standards I (Triacontane) and II (5α-Cholestane-3β-ol), lipids were transesterified in methanol in the presence of a sodium methylate catalyst. These were then separated by solid phase extraction into fraction I (fatty acid methyl esters (FAME) and hydrocarbons) and fraction II (minor lipids, including sterols and free fatty acids). After adding the internal standards III (phenyl-β-D-glucopyranoside) and IV (4-chloro-L-phenylalanine) and subsequent silylation and selective hydrolysis procedures, the polar extract was separated into one fraction containing silylated sugars and sugar alcohols (fraction III) and another fraction containing organic acids and amino acids (fraction IV). Gas chromatography-flame ionization detection (GC-FID; HP 6890; Agilent Technologies; Palo Alto, CA, USA) was used for the analysis of the four fractions. The gas chromatography (GC) conditions were in accordance with Na Jom et al. (2011). The total runtime of gradient elution by GC was 60 min per fraction per sample. The metabolites were identified by comparing the retention times of the chromatographic analysis peaks to those of reference standards. Biological triplicates were used in the sampling procedure. Injection was also in triplicate. The normalized data were averaged on the basis of peak alignment with the reference sample, reference standards and the internal standard. The GC-FID data were acquired and integrated using a ChemStation A.06.03 (Hewlett Packard; Palo Alto, CA, USA) for further analysis.

Statistical analysis

Principal component analysis (PCA) and analysis of variance were performed using the XLSTAT version 2011.1.01 software package (Addinsoft; New York, NY, USA). Pairwise comparisons were analyzed for statistical differences using the Duncan multiple comparison test ($p < 0.05$).

Results

Metabolite profiling of the corns

Profiling began with consecutively extracted metabolites of freeze-dried corn flour samples via GC-FID analysis. The profiles of the Thai corn hybrids produced a total of 608 peaks, with 113 major compounds identified on the basis of the reference standards, database and spectral library (Fig. 1, Tables 1 and 2). These metabolites were then sub-fractionated into: (I) major lipids, (II) minor lipids, such as free fatty acids and sterols, (III) sugars and sugar alcohols and (IV) organic acids, amino acids and amines. These were subjected to PCA for each fraction (I–IV).

Combined fractions also underwent statistical analysis (Fig. 1). For the score plot of all combined polar and nonpolar metabolite fractions I through IV, a total of 63.19% from PC1 and PC2 could explain the PCA plot variance (Fig. 1A). There was a distinct differentiation of 7566 on PC1 (43.47% of total variation) and of CP09 on PC2 (19.72% of total variation) from other varieties. Although clearly clustered, the remaining three varieties displayed poor separation and no clear separation was exhibited based on coloration.

Based on the individual fraction metabolite datasets, 7566 and 8003 displayed strong PC1 and PC2 separation compared with the other varieties regarding non-polar fractions I and II (Figs. 2B and C). Fraction II clustering was more pronounced than fraction I among the varieties. Fraction II separation was more pronounced than fraction I between LB-50-LB, M80 and CP09. Additionally, with the exception of 7566, all varieties displayed an inverse pattern along PC 2 between fraction I and II. Polar fractions III and IV showed distinct separation in 7566 and CP09 compared with other varieties (Figs. 2D and E). PC1 and PC2 of fraction III explained 36.72% and 42.01% of the total variance, respectively, but could not separate M80 and LB-50-LB from each other. PC1 could separate CP09 from the others, while PC2 separated 7566 and 8003 from the rest. Fraction IV showed clear clustering and pronounced separation of individual varieties. Pronounced separation between each of the four varieties was observed on PC1 (58.65% of total variation), while PC2 explained 58.65% of total variation, clearly separating 7566 from the rest.

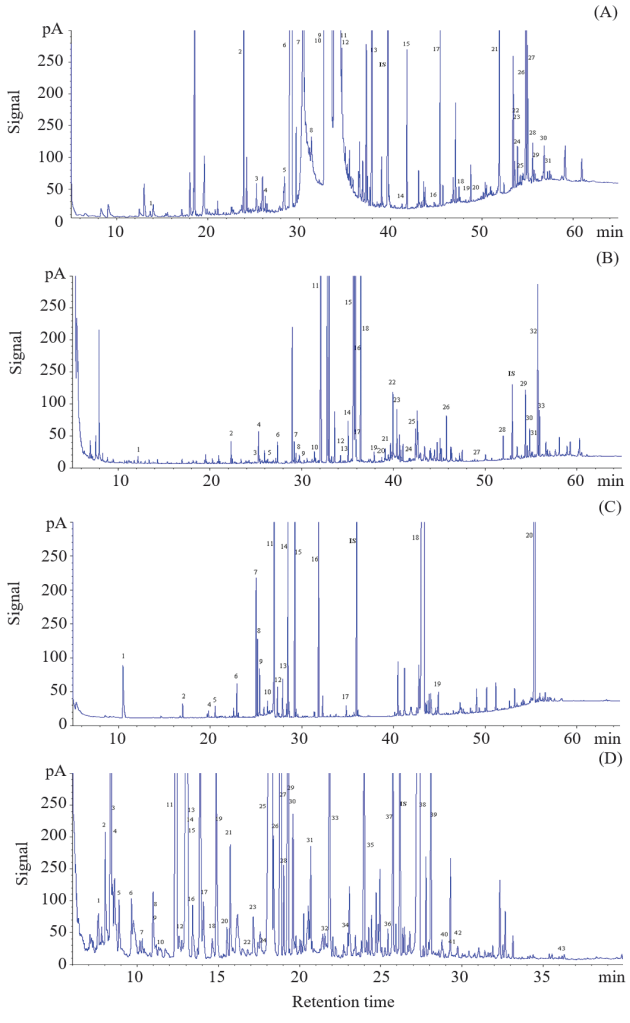

Fig. 1 Gas chromatography-flame ionization detection chromatographs of fraction I (A), fraction II (B), fraction III (C) and fraction IV (D) obtained from colored Thai corns. IS = internal standards tetradecane (A), 5α-cholestane-3*β*-ol (B), phenyl-*β*-D-glucopyranoside (C), *p*-chloro-L-phenylalanine (D), where identification of peaks is given in Tables 1 and 2

Table 1 Compounds identified in fraction I (major lipids) and fraction II (minor lipids)

No.	Compound	Identification code[a]
Saturated fatty acid methyl esters		
1	10:0	A
2	14:0	A
4	15:0	A
6	16:0	A
8	17:0	A
12	18:0	A
13	20:0	A
15	22:0	A
16	23:0	A
17	24:0	A
20	26:0	B
21	28:0	A
Free fatty acids[c]		
1	9:0	A
2	12:0	A
3	13:0	A
6	14:0	A
8	15:0	A
10	16:1	A
11	16:0	A
12	17:0	C
15	18:3 (9Z,12Z,15Z)	A
16	18:2 (9Z,12Z)	A
17	18:1(9Z)	A
18	18:0	A
20	19:0	A
22	20:1 (11Z)	A
23	20:0	A
Unsaturated fatty acid methyl esters		
3	15:1	A
5	16:1	A
7	17:1	A
11	18:1 (9Z)	A
10	18:2 (9Z, 12Z)	A
9	18:3 (9Z,12Z, 15Z)	A
Fatty alcohols[c]		
9	16:0	A
13	18:0	A
14	Phytol	A
21	20:0	A
25	22:0	A
26	24:0	A
27	26:0	A
28	28:0	A
30	32:0	D
Hydroxy fatty acid methyl esters[b]		
19	12-OH 18:1 (9Z)	A
24	9,12-OH 18:0	E
Hydrocarbon		
18	Squalene	A
Sterols/Stanols[b]		
19	Cholesterol	A
22	Campestenol	A
23	Campestarol	A
24	Stigmasterol	A
25	Δ7-Campestenol	A
26	*β*-Sitosterol	A
27	Sitostanol	A
28	Gramisterol	A
29	Δ7-Avenasterol	D, F
31	Citrostradienol	A
Others		
4	Methyl-p-hydroxy cinnamate	A
5	Methyl-3-methoxy cinnamate	A
7	Methyl ferulate	A

[a] identification codes: A = mass spectral data and retention times of reference compound; B = mass spectral data and retention index of Golm Metabolome Database (Kopka et al. 2005); C = mass spectral data of NIST02 mass spectral library; D = mass spectral data; E = according to Xu and Godber (1999); F = according to Kamal-Eldin et al. (1992).
[b] trimethylsilyl derivatives of respective compound.

Table 2 Trimethylsilyl derivatives of compounds identified in fraction III (sugars, sugar alcohols) and fraction IV (acids, amino acids, amines)

No.	Compound[a]	Identification code[b]
Sugars and sugar alcohols		
1	Glycerol	A
2	Erythritol	A
3,4,5	Arabinose	A
6	Ribitol	A
7,8,9	Fractose	A
10,12	Galactose	A
11,15	Glucose	A
13	Mannitol	A
14	Sorbitol	A
16,17	*myo*-Inositol	A
18	Sucrose	A
19	Trehalose	A
20	Raffinose	A
Acids		
1	Lactic acid	A
2	Hydroxyacetic acid	A
10	4-Hydroxybutyric acid	A
11	Phosphoric acid	A
15	Succinic acid	A
17	Fumaric acid	A
18	Pyrole-2-carboxylic acid (2TMS)	A
20	Glutaric acid	A
26	Malic acid	A
38	Citric acid	A
Amino acids and amines		
3	Alanine	A
4,16	Glycine	A
5	Hydroxyalamine	A
6	Ethanolamine	A
7,22	*β*-Alanine	A
8	Valine	A
9	Norvaline	A
12	Leucine	A
13	Isoleucine	A
14	Proline	A
19	Serine	A
21	Threonine	A
23	Homoserine	A
24	2-Piperidinecarboxylic acid	A
25	*β*-Aminoisobutyric acid	A
27	Pyroglutamic acid	A
28	Metheonine	A
29	Aspartic acid	A
30	γ-Aminobutric acid	A
31	Threonic acid	A
32	Glutamic acid	A
33	Phenylalanine	A
34	Asparagine	A
35	2-Aminoadipic acid	A
36	Putresine	A
37	Glutamine	A
40	Histidine	A
41	Lysine	A
42	Tyrosine	A
43	Tryptophan	A
Others		
39	Adenine	A

[a] metabolites identified as persilylated derivatives, number of trimethylsilyl groups in parentheses.

[b] identification codes: A = mass spectral data and retention times of reference compound.

In identifying the primary metabolites responsible for separating the hybrids, the first two principal components of PCA loading scores were analyzed for the combined four fractions, as well as for the individual fractions (I–IV) (Fig. 3). The results indicated that only 40 metabolites played an important role in differentiation of the samples.

To further confirm the separation of varieties observed using PCA (Fig. 2A), metabolite datasets were subjected to hierarchical cluster analysis (HCA), which generated three distinct clusters (Fig. 4). The first and second clusters consisted of only single varieties, specifically 7566 and CP09, respectively. The third cluster consisted of varieties M80, LB-50-LB and 8003. The dendrogram revealed that 7566 was clearly distinguished from the other varieties. This hybrid had a high fraction I, II and IV metabolite content.

Semi-quantitative analysis of each identified metabolite was also calculated based on the peak area ratio relative to the reference standard. Fig. 5 shows the relative amount of selected metabolite signals of each fraction. The five corn grains displayed significant diversity in metabolite abundance. Saturated and unsaturated fatty acid methyl ester (FAME) content in fraction I and cholesterol in fraction II were significantly higher in 7566 compared to the other varieties (Fig. 5A). In contrast, oleic (C18:1) and arachidic (C20:0) acid methyl esters were highest in 8003. In fraction II, the contents of palmitic acid (C16:0), oleic acid (C18:1), 9,12-OH (18:0), methyl-*p*-hydroxy cinnamate, and phytosterols (sitostanol, campesterol, stigmasterol, Δ7-campestenol) were most abundant in 7566, while hybrid 8003 had the highest concentration of heptadecadoic acid (C17) and pentadecanoic acid (C15:0) (Fig. 5B). Fig. 5C shows the graphs of the selected sugars and sugar alcohols content in fraction III. Monosaccharides (fructose, galactose, glucose) and sugar alcohols (mannitol, erythritol, *myo*-inositol) were notably highest in CP09. Variety 7566 contained the highest content of the disaccharide sucrose, the sugar alcohol glycerol and the trisaccharide raffinose. Pronounced differences ($p < 0.05$) in fraction IV metabolites containing amino acids, organic acids and amines were observed in variety 7566 (Fig. 5D). Hybrid 7566 also contained the highest levels of valine, proline, serine, threonine, methionine, homoserine, phenylalanine glutamic acid, citric acid, pyroglutamic acid, aspartic acid and β-aminoisobutyric acid compared with the other varieties.

Discussion

Metabolite profiling of the corns

Maize varieties were effectively and accurately differentiated via PCA based on the metabolite content of each variety. PCA has been used to contrast different maize hybrids and explain metabolite content cluster separation (Kim et al., 2013; Röhlig et al., 2009). Röhlig et al. (2009) implemented metabolite profiling to differentiate four genetically close maize species grown at one location in the same season (in Frankendorf, Bavaria). Profiling of the full metabolite composition of each species revealed clear differentiation along PC1.

Core metabolites play an important role in variety discrimination (Tarpley et al., 2005). The significant PC1 metabolites which

differentiated 7566 from other varieties were primarily polar: adenine, β-aminoisobutyric acid, citric acid, homoserine, phenylalanine, pyroglutamic acid, serine, succinic acid, 2-piperidinecarboxylic acid and the secondary metabolite Δ7-campestenol and were all significantly higher for this variety. Similarly, since the polar metabolites fructose, galactose, glucose, erythritol, mannitol, ϒ-aminobutyric acid, putrescine and malic acid are most abundant in CP09, these can be used to separate this variety from others along PC2. In addition, Total anthocyanin contentcan also be used to separate CP09 from other varieties. These results might suggest that the genetic backgrounds within the five hybrids influenced metabolite profile differences.

The lower overall content of major lipids (triglycerides) and the higher content of minor lipids (free fatty acids) in 8003, in contrast to the higher overall content of major lipids (triglycerides) and the lower content of minor lipids (free fatty acids) in LB-50-LB, M80 and CP09, apparently resulted in the inverse pattern observed between fractions I and II. A negative correlation between the triglyceride content and free fatty acids was also reported by Röhlig et al. (2009) where the pattern of fractions I and II mirrored each other along PC1 for four corn varieties grown in one season and at one location. The negative correlation between the triglyceride content and free fatty acids has been ascribed to the interplay between triglycerides synthesis and their potential precursor (Voelker and Kinney, 2001).

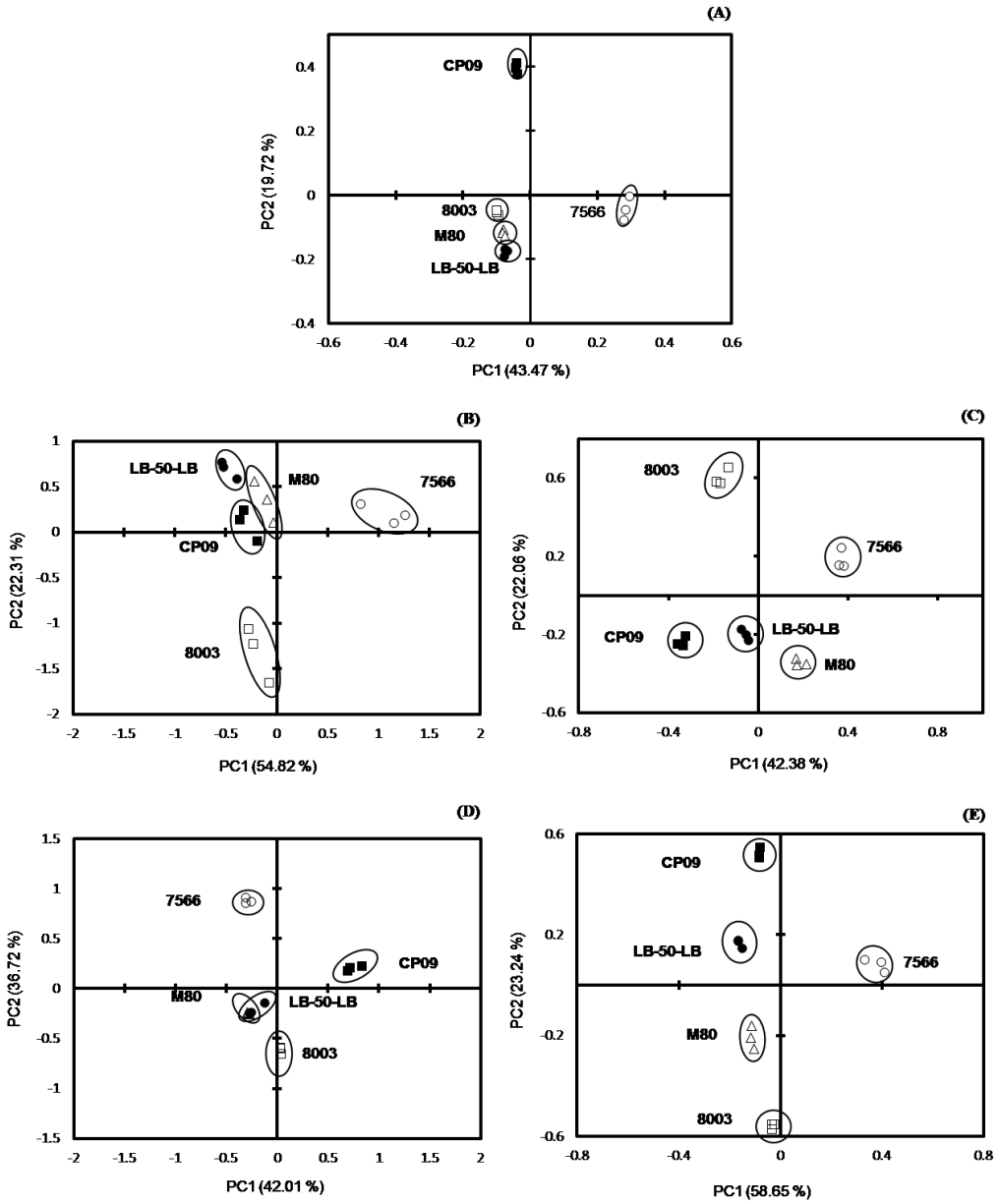

Fig. 2 Principal component analysis of metabolite profiling data from four fractions: (A) combined fractions I–V (I: fatty acid methyl esters, hydrocarbons; II: minor lipids; III: sugars, sugar alcohols, IV: acids, amino acids, amines); (B) fraction I; (C) fraction II; (D) fraction III; (E) fraction IV, where PC1 = principal component 1 and PC2 = principal component 2

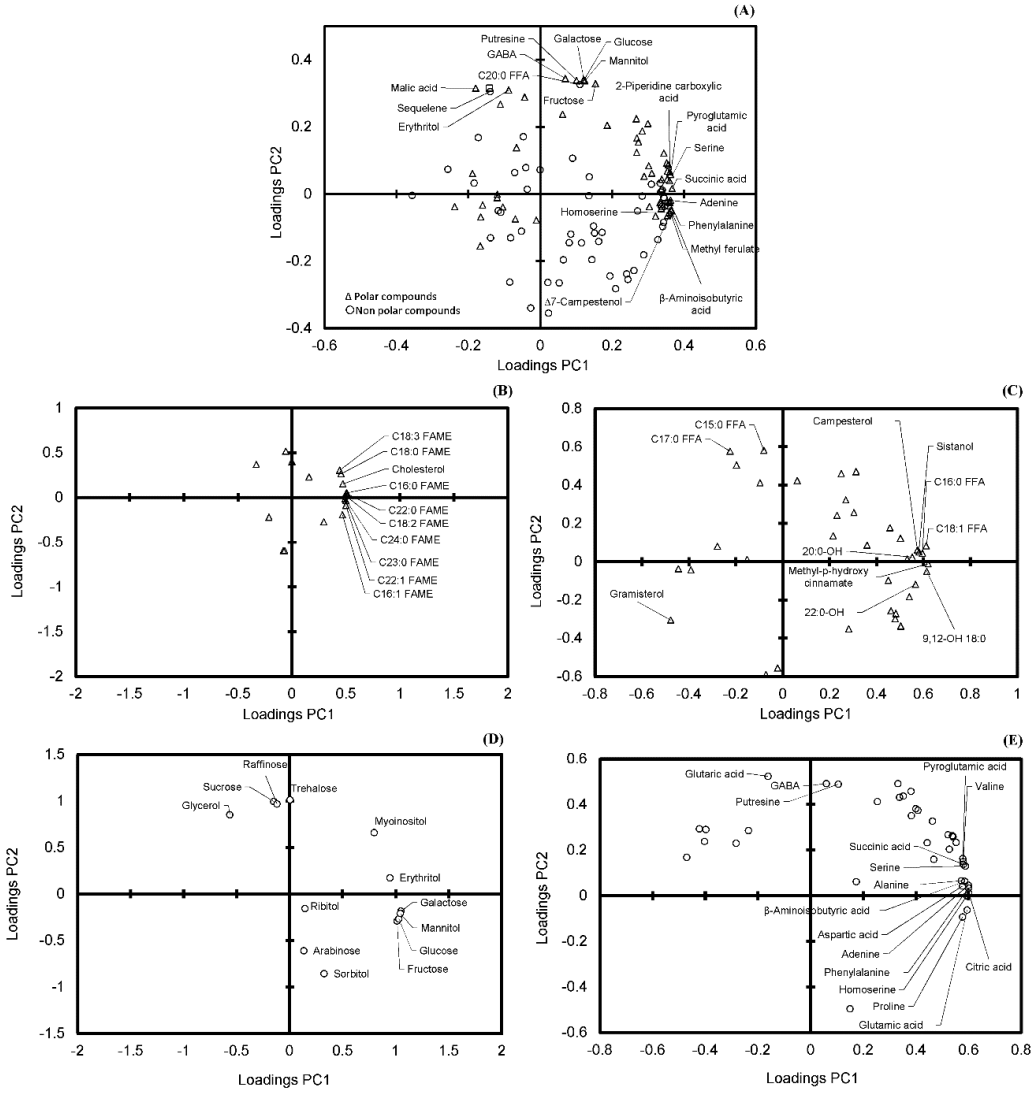

Fig. 3 Loading plots of metabolite profiling data from four fractions: (A) combined fractions I–V (I: fatty acid methyl esters, hydrocarbons; II: minor lipids; III: sugars, sugar alcohols; IV: acids, amino acids, amines); (B) fraction I; (C) fraction II; (D) fraction III; (E) fraction IV, where FAME = fatty acid methyl esters, FFA = free fatty acids, GABA = ϒ-aminobutyric acid, PC1 = principal component 1 and PC2 = principal component 2

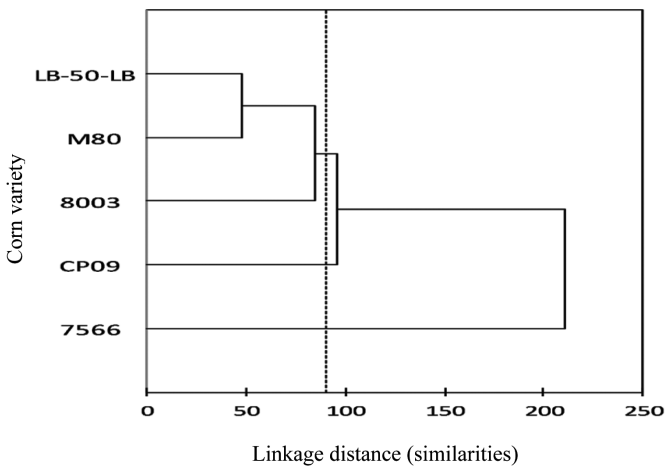

Fig. 4 Hierarchical cluster analysis of five colored corn hybrids (8003, CP09, 7566, M80, LB-50-LB)

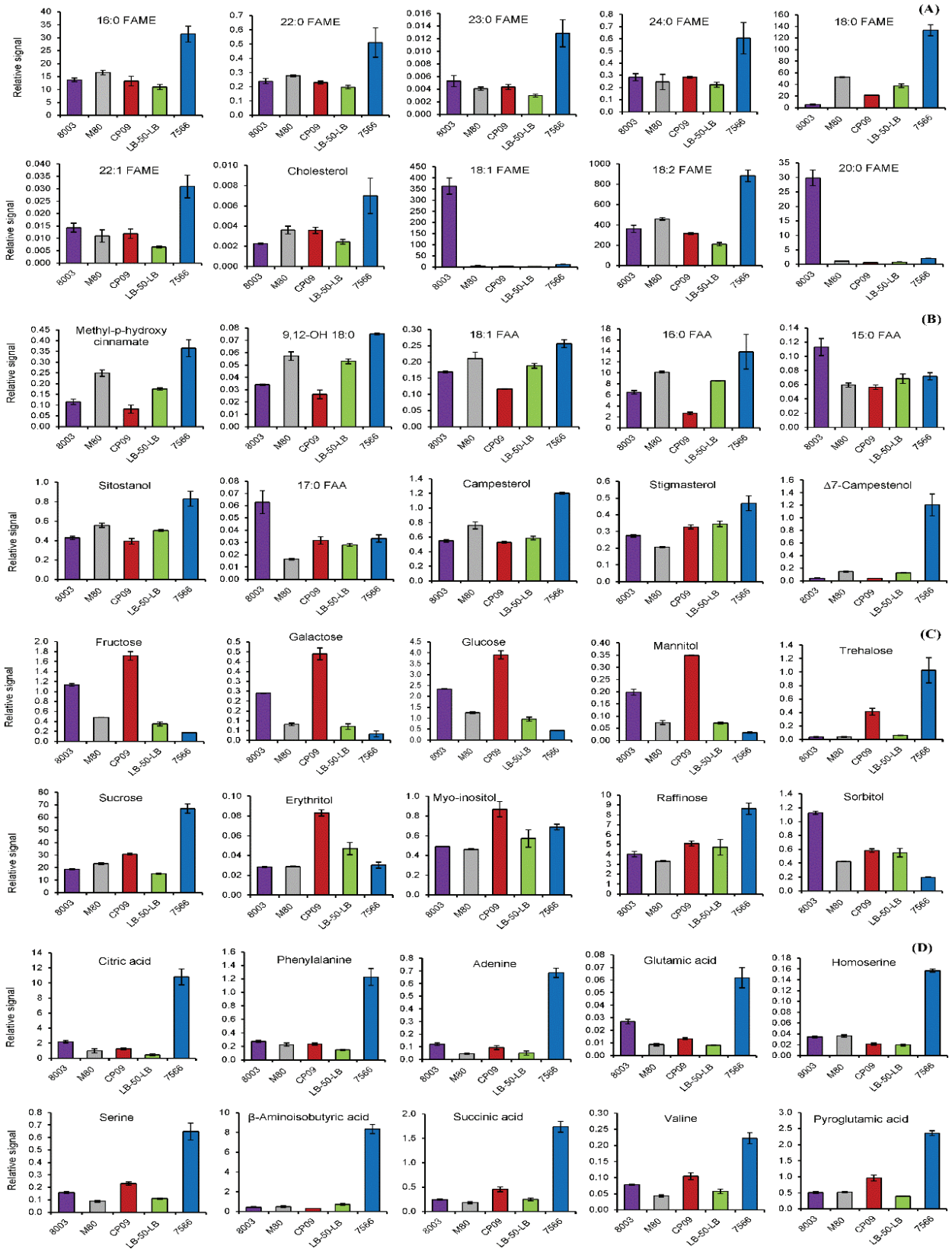

Fig. 5 Comparison of metabolite levels of five commercial colored Thai corn hybrids (8003, CP09, 7566, M80, LB-50-LB) using semi-quantitative levels of identified compounds from fractions I–IV: (A) faction I: fatty acid methyl esters/hydrocarbons; (B) fraction II: free fatty acids/ sterols; (C) fraction III: sugars and sugar alcohols (D) fraction IV: acids/amino acids/amines, where FAME = fatty acid methyl esters, FFA = free fatty acids and error bars = SE

Polar fractions III and IV could be used to separate 7566 and CP09 from the other varieties. The metabolites responsible for 7566 separation were raffinose, sucrose, and trehalose due to their relatively high content in this variety. For the same reason, fructose, galactose, glucose, erythritol and mannitol were metabolites used for differentiating CP09. Conversely, 7566 can be PC2 differentiated using raffinose, sucrose and trehalose as the separating metabolites. Fraction IV could clearly differentiate the four non-yellow colored varieties from yellow 7566 along PC 2.

In general, the PCA indicated that both polar (III and IV) and non-polar (I) fractions could differentiate 7566 from other varieties. Differentiation of 7566 from other varieties along PC1 for fractions I and II (in addition to the PCA of all four fractions) suggested a richness of fraction I and II metabolites in this variety that set it apart from rest. For non-polar fractions, the majority of both fraction I and II metabolites were prevalent in variety 7566. Not only fractions I and II, but the majority of fraction IV metabolites were abundant in 7566.

HCA is highly effective for clustering food crop grains. In the current study, HCA was conducted to confirm the separation pattern observed in PCA for all combined fractions. HCA classified corn varieties into three distinct clusters. This agreed with the variety separation pattern observed with the PCA for the same metabolite data set for all combined fractions. Both HCA and PCA differentiated 7566 and CP09 from the other varieties, indicating that their metabolites are much different from the others. The large degree of separation of 7566 from the other four varieties in the dendrogram could have been due to the high content of the majority of metabolites in fractions I, II, and IV.

Clustering of M80, LB-50-LB and 8003 into one cluster suggested that the metabolite abundance for each was more or less similar. However, contrary to the current study where no kernel-based coloration clustering was observed, Frank et al. (2012a) used HCA to cluster and separate colored rice grains from non-colored rice. This might have been due to coverage of the metabolite compounds in this study being limited to the samples.

Primary and secondary metabolites play essential roles in plant growth, development, stress adaptation and defense (Hounsome et al., 2008). Besides their importance to the plant itself, such metabolite constituents play a vital role in food crop nutritional quality (Fernie et al., 2006; Hounsome et al., 2008), color, taste and smell (Hounsome et al., 2008). Comparison of the metabolites identified among the five corn grains revealed significant diversity in their abundance.

FAMEs represent triglyceride-derived fatty acids (Frank et al., 2012a). Triglycerides are the storage form of seed oil consisting primarily of the triglycerides from palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2) and linolenic (C18:3) acids (Voelker and Kinney, 2001). In the current study, methyl esters accounted for 98% of the triglyceride fatty acid moieties in 7566. Röhlig et al. (2009) reported that stearic (C18:0), oleic (C18:1) and linoleic acids (C18:2) methyl esters were the most abundant in corn triglycerides. A decrease in the oleic content (C18:1) and an increase in linoleic (C18:2) acid methyl ester in the same variety could have been due to oleic acid (C18:1) being the precursor of linoleic acid synthesis (Beló et al., 2008). Sitostanol, campesterol, stigmasterol and Δ7-campestenol are among the phytosterols that play important roles in food crop grain dietary quality (Anastasi et al., 2010). The plant oil oleic acid (C18:1) content is important for human and animal nutrition, and its presence in higher quantities adds beneficial cooking properties in oil (Beló et al., 2008).

Monosaccharides (fructose, galactose, glucose) and sugar alcohols (mannitol, erythritol, *myo*-inositol) apart from their nutritional importance, are potentially valuable in plant breeding, playing an important part in drought adjustment mechanisms (Lanzinger et al., 2015). The lower sucrose content in the four varieties, and the relatively higher fructose content within the same variety may have been due to fructose being derived primarily from a sucrose breakdown in plant seeds (Chen et al., 2016). Sucrose is a required carbohydrate for the enzymatic hydrolysis of monosaccharides in the human gastrointestinal system which are absorbed directly into the bloodstream (Hounsome et al., 2008). Sucrose, as the major disaccharide, was in all studied maize varieties, ranging from 70% in 8003 to 87% in 7566. Similarly, Frank et al. (2012a) reported sucrose accounting for 90% of total sugars in black rice. Notably, *myo*-inositol and raffinose, key antinutrients in maize (Venkatesh et al., 2016), were highest in CP09 and 7566, respectively. *Myo*-inositol plays an important role in plant metabolism such as seed development (Frank et al., 2012a), mineral nutrient storage, seed desiccation, osmoregulation and stress response (Loewus and Murthy, 2000) and therefore has attracted plant-breeder interest.

In the current study, for the first time, a broad range GC-FID-based metabolite profiling method was applied to investigate low molecular weight constituents of colored Thai corn hybrids grown in the same season and at the same location, thereby enabling differentiation according to metabolite profile abundances. Applied metabolite profiling, in combination with multivariate and univariate analysis, provided valuable information likely to contribute toward crop improvement, nutritional assessment and functional quality within the food industry and nutritional science. Further metabolite profiling studies could help to understand the best geographic location, agronomic practices and growing and harvesting seasons to get the desired nutritional composition, color, taste and scent of a particular hybrid.

Conflict of Interest

The authors declare that there is no conflict of interest.

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