

Volatile Organic Compound Based Markers for the Aroma Trait of Rice Grain

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Abstract

A study was conducted to determine the volatile organic compounds (VOCs) associated with rice grain aroma in 37 commonly grown lines within Uganda, as well as elites. The aim of the study was to identify potential volatile biochemical markers, if any, for the rice grain aroma trait. Certified rice seeds were obtained from the Uganda National Crops Resources Research Institute germplasm collection. The seeds were sown into experimental plots, under field conditions and the mature paddy harvested. Polished rice grains were heated to 80 °C and the liberated VOCs subjected to untargeted metabolite analysis using gas chromatography-time-of-flight mass spectrometry. In total, nine functional groups were present; hydrocarbons, alcohols, ketones, aldehydes, N-containing compounds, S-containing compounds, esters, oxygen heterocycles and carboxylic acids. More specifically, 148 VOCs were identified across the 37 rice lines, of which 48 (32.4%) including 2-acetyl-1-pyrroline (2-AP) appeared to elucidate the difference between non-aromatic and aromatic rice. Furthermore, 41 (27.7%) VOCs were found to be significantly correlated with 2-AP abundance, the principle rice aroma compound. Amongst the 41 VOCs, only ten compounds were found to contribute highly towards variation in 2-AP abundance, indicative of their possible modulation roles in regard to rice aroma. Within the ten influential volatiles, three aroma active compounds; toluene, 1-hexanol, 2-ethyl and heptane, 2,2,4,6,6-pentamethyl- were established as the most reliable biochemical surrogates to the rice aroma trait. Thus, the aforementioned compounds may be used in rice breeding programme for enhancing development of the grain aroma trait.

Keywords: 2-acetyl-1-pyrroline, aroma, rice, volatile organic compounds

1. Introduction

Rice (*Oryza sativa* L.) is among the most important staple food crops for approximately two billion persons worldwide and a substantial source of income for several rural households (Sharma et al., 2018). Globally, several rice species exist including *O. sativa* thought to have originated in Asia (Kovach et al., 2009) and *Oryza glaberrima* of West African origin (Linares, 2002). The origin of a rice species is known to influence the physio-chemical attributes of the grain such as aroma and taste, primarily due to genetic inheritance (Rai et al., 2015). Considering aroma, the responsible gene betaine aldehyde dehydrogenase 2 (*badh2*) is believed to have different alleles (Bindusree et al., 2017), with specific allele configurations dependent upon species origin (Pachauri et al., 2010). Consequently, substantial variations in rice grain aroma has been observed in relation to differences in genetic backgrounds (Rai et al., 2015).

Rice cultivars comprise non-aromatic and aromatic categories, of which aromatic cultivars exhibit a sweet popcorn flavour primarily attributed to 2-acetyl-1-pyrroline (2-AP) (Buttery et al., 1983). Aromatic rice is highly desirable amongst consumers, thus, commands high sensory preference and premium prices (Laizer et al., 2018). In spite the consensus that 2-AP contributes substantially towards rice aroma (Daygon et al., 2017), over 71 volatile compounds are thought to influence the perception of rice aroma (He et al., 2018). To date, more than 300 volatile compounds have been reported as constituting the multifaceted rice aroma trait (Champagne, 2008; Hashemi et al., 2013; Wakte et al., 2016; Hinge et al., 2019). Thus, rice aroma is most likely influenced by the comprehensive interactions of many different volatile compounds with relatively low odour thresholds (Hu et al., 2020). Recent evidence appears to support that 2-AP producing rice varieties may have different sensory properties (Daygon et al., 2016), implying that other volatile compounds contribute towards rice aroma or distinguish between non-aromatic and aromatic varieties (Hu et al., 2020). Therefore, earlier works by several authors intended to identify key volatile organic compounds responsible for the rice aroma are most likely unexhausted (Hu et al., 2020). For example, working with 14 Thai rice cultivars, Sansenya et al. (2018) identified 140 volatile organic compounds of which 18 key compounds exhibited substantial influence towards the rice aroma. The key volatiles were namely; hexanal, 1-pentanol, octanal, (*E*)-2-heptenal, 6-methyl-5-hepten-2-one, 1-hexanol, nonanal, 2-butoxy-ethanol, (*E*)-2-octenal, 1-tetradecene, 1-octen-3-ol, decanal, benzaldehyde, (*E*)-2-nonenal, 1-nonanol, benzyl alcohol, isovanillin and vanillin. In addition, the literature appears to suggest that substantial variations in the key rice aroma constituents is widespread and primarily dependent on genetic considerations (Ajarayasiri & Chaiseri, 2008; Stefano & Agronomia, 2011; Mahattanatawee & Rouseff, 2014). Within genetic homologues, variations in rice aroma constituents is reportedly influenced by differences in cultivation environments, agro-cultural practices and post-harvest handling practices (Calingacion et al., 2015; Wakte et al., 2016; Xu et al., 2018). This state of affairs consequentially complicates efforts by national rice breeding programs in development of varieties with enhanced aroma traits.

With regard to Uganda, several rice germplasm were found to exhibit substantial variations in 2-AP abundance and most significantly, within similar aromatic categories (Ocan et al., 2019). The implication being that the substantial intra-group variations are most likely due to other VOCs, besides 2-AP, substantially contributing towards the rice aroma (Cho et al., 2013; Sansenya et al., 2018). However, the key volatiles thought to be responsible for the aromatic profile of commonly grown rice lines in Uganda are not well understood. Thus, the present study aimed to profile the volatile compounds in non-aromatic and aromatic rice lines commonly grown in Uganda to aid establishment of key volatile compounds as potential biochemical markers for the aroma trait.

2. Method

2.1 Selection of Rice Germplasm

Thirty seven commonly grown rice germplasm, 300 g each, were collected from the Rice Research Unit at the National Crops Resources Research Institute (NaCRRI), Uganda. The selected rice germplasm comprised of common and elite lines with different parental lineage and aroma classification (Table 1).

Table 1. Geographic origin, genetic backgrounds (crosses, pedigree) and aroma classification of the 37 selected rice lines

Lines	Crosses	Pedigree	Origin	Aroma classification
AGRA 41	<i>O. sativa indica</i> × <i>O. sativa indica</i>	AGRA-CRI-UPL-3-4	AfricaRice, Benin	Aromatic
AGRA 55	<i>O. sativa indica</i> × <i>O. sativa indica</i>	AGRA-CRI-UPL-4-4	CRI, Ghana	Aromatic
AGRA 60	<i>O. sativa indica</i> × <i>O. sativa indica</i>	AGRA-CRI-UPL-4-13	CRI, Ghana	Aromatic
AGRA 78	<i>O. sativa indica</i> × <i>O. sativa indica</i>	AGRA-CRI-UPL-2-1	CRI, Ghana	Aromatic
ART 4	<i>O. sativa indica</i> × <i>O. sativa indica</i>	ART15-22-10-8-1-B-2-2	AfricaRice, Nigeria	Aromatic
ART 7	<i>O. sativa indica</i> × <i>O. sativa indica</i>	ART15-17-7-8-1-1-1-B-1-1	AfricaRice, Nigeria	Aromatic
ART 10	<i>O. sativa indica</i> × <i>O. sativa indica</i>	ART15-21-2-4-1-B-1-B-1-1	AfricaRice, Nigeria	Aromatic
ARU 1190	<i>O. sativa japonica</i> × <i>O. glaberrima</i>	IR77454-22-B-20-2-2-B-TGR2	AfricaRice, Nigeria	Non-aromatic
Basmati 370	-	-	-	Aromatic
E 20	<i>O. sativa indica</i> × (<i>O. sativa</i> × <i>O. glaberrima</i>)	IRAT325/WAB365-B-1H1-HB	NaCRRI, Uganda	Non-aromatic
Kafaci	-	-	AfricaRice	Non-aromatic
Komboka	<i>O. sativa indica</i> × <i>O. sativa indica</i>	IR05N221	IRRI, Philippines	Aromatic
MET 3	<i>O. sativa indica</i> × <i>O. barthi</i>	ART35-114-1-6N-2	AfricaRice, Nigeria	Aromatic
MET 4	<i>O. sativa indica</i> × <i>O. barthi</i>	ART34-146-1-8N-1	AfricaRice, Nigeria	Aromatic
MET 6	<i>O. sativa indica</i> × <i>O. barthi</i>	ART35-49-1-4N-1	AfricaRice, Nigeria	Aromatic
MET 12	<i>O. sativa indica</i> × <i>O. barthi</i>	ART34-88-1-2-B-1	AfricaRice, Nigeria	Aromatic
MET 13	<i>O. sativa indica</i> × <i>O. barthi</i>	ART34-113-3-2-B-1	AfricaRice, Nigeria	Aromatic
MET 14	<i>O. sativa indica</i> × <i>O. barthi</i>	ART34-256-3-1-B-2	AfricaRice, Nigeria	Aromatic
MET 16	<i>O. sativa indica</i> × <i>O. barthi</i>	ART35-272-1-2-B-1	AfricaRice, Nigeria	Aromatic
MET 25	<i>O. barthi</i> interspecific lines	ART27-58-6-2-1-1-3-2	AfricaRice, Nigeria	Non-aromatic
MET 28	<i>O. barthi</i> interspecific lines	ART27-58-6-2-2-2	AfricaRice, Nigeria	Non-aromatic
MET 33	<i>O. sativa indica</i> × <i>O. barthi</i>	ART27-58-6-2-1-1-3-1	AfricaRice, Nigeria	Non-aromatic
MET 38	<i>O. barthi</i> interspecific lines	ART16-5-9-22-3-B-B-2	AfricaRice, Nigeria	Non-aromatic
MET 40	<i>O. sativa indica</i> × <i>O. barthi</i>	ART27-190-1-4-2-1-1-3	AfricaRice, Nigeria	Aromatic
Namche 1	-	-	NaCRRI, Uganda	Non-aromatic
Namche 2	<i>O. sativa japonica</i> × (<i>O. sativa japonica</i> × <i>O. glaberrima</i>)	NM7-8-2-B-P-11-6	NaCRRI, Uganda	Non-aromatic
Namche 4	<i>O. sativa indica</i> × <i>O. sativa indica</i>	ART3-11L1P1-B-B-2	AfricaRice, Nigeria	Non-aromatic
Namche 5	<i>O. sativa indica</i> × <i>O. sativa indica</i>	NM7-27-1- B-P-77-6	NaCRRI, Uganda	Non-aromatic
Namche 6	<i>O. sativa indica</i> × <i>O. sativa indica</i>	NM7-5-2- B-P-79-7	NaCRRI, Uganda	Non-aromatic
Nerica 4	<i>O. sativa japonica</i> × <i>O. glaberrima</i>	WAB 450-1-B-P-91-HB	AfricaRice, Côte d'Ivoire	Non-aromatic
Nerica 10	-	WAB 450-11-1-1-P41-HB	WARDA/Africa Rice	Aromatic
Sande	<i>O. sativa indica</i> × <i>O. barthi</i>	<i>O. barthi</i> interspecific lines	AfricaRice, Nigeria	Aromatic
Supa 3	<i>O. sativa indica</i> × <i>O. sativa indica</i>	IR 97011-7-7-3-1-B	IRRI, Philippines	Aromatic
Supa 5	<i>O. sativa indica</i> × <i>O. sativa indica</i>	-	IRRI, Philippines	Aromatic
Supa 6	<i>O. sativa indica</i> × <i>O. sativa indica</i>	IR 9712-4-1-2-1-1	IRRI, Philippines	Aromatic
Supa 1052	<i>O. sativa indica</i> × <i>O. sativa indica</i>	-	AfricaRice, Nigeria	Aromatic
Yasmin aromatic	<i>O. sativa indica</i> × <i>O. sativa japonica</i>	-	Egypt	Aromatic

Source: Kanaabi et al. (2018).

2.2 Preparation of the Polished Rice Grains

Paddy rice lines were processed according to the method by He et al. (2018) with slight modifications. Rice lines (300 g each) were submerged in 1 L distilled water and the floated kernels immediately discarded. The settled rice kernels were briefly washed and then sun dried for 48 h, final moisture content approximately 14%. The dry paddy rice were milled and polished using a bench top milling machine (Satake, Tokyo, Japan). Fifty (50) grams of polished rice grains were vacuum packed into air-tight polyethylene bags and delivered to the School of Agriculture and Food Sciences laboratory, University of Queensland, Australia for biochemical analyses.

2.3 Identification and Quantification of Volatile Organic Compounds

Polished rice grains were ground using a cryogrinder (Qiagen, Hilden, Germany) to particle size < 25 µm, and the flours weighed (1 g) into autosampler tubes. The tightly sealed tubes, in triplicates, were stored at -80 °C until required. Pre-analysis, the frozen flours were left overnight at room temperature to equilibrate (Daygon et al., 2017). Thawed flours were then randomised and analysed in batches of 50. Blank samples were run before analysis of the rice flours to equilibrate the machine and quality control (QC) standards were placed at every 10th queue position. Rice flours were assayed following the method by Daygon et al. (2017). In summary, rice flours were

heated to 80 °C with agitation for 10 min on a CombiPal Autosampler (Agilent, CA, USA) to release the VOCs. Liberated VOCs within the tube headspace (1.5 ml) were collected using 2.5 ml headspace syringes at 80 °C and injected in splitless mode (Pegasus 4D GC×GC-TOF-MS Leco; St. Joseph, MI, USA). Temperature of the gas chromatographer (GC) inlet and transfer lines were maintained at 250 °C. Separation was performed first on a primary column (Agilent DB-624UI midpolar, 30 m × 250 µm × 1.4 µm; Agilent, CA, USA) and then on a secondary column, Stabilwax (polar, 0.9 m × 250 µm × 0.50 µm; Restek, Bellefont, USA). The primary column was initially set to 45 °C for 1 min and then ramped at a rate of 10 °C/min to 235 °C. The secondary column and the modulator were set at 15 °C and 25 °C higher than the primary column respectively, during the entire run. The modulation period was set at 2.5 s, with 0.4 s hot pulse time and 0.85 s cool time between stages. The carrier gas (helium) was maintained at a constant flow rate of 1 ml/min. Data were acquired using a TOF-MS after a 200 s delay with an acquisition rate of 200 spectra/s. The MS scanned analytes within the mass range of 35 to 500 m/z and the ion source was held at 240 °C.

Data pre-processing, alignment and noise correction were done using ChromaTof v4.50. Signal to noise ratio was set at 25. The absence of instrument drift and batch effects was verified using the QC standards and technical replicates. Identification of VOCs was achieved through comparison of retention time and electron ionization (EI) fragmentation patterns of rice flours to an in-house mass spectral library created by running authentic analytical standards (Daygon et al., 2017). The relative amounts of VOCs were calculated by measuring the area under the curve of the VOC peak.

2.4 Data Analysis

Overall mean abundance of VOCs, representative of previously established non-aromatic and aromatic rice lines (Ocan et al., 2019), were subjected to the Student's T-test at 5% significance level using Genstat software 18th Ed. (VSN International, Hemel Hempstead, UK). Correlation analysis between and within VOCs significantly ($p < 0.05$) associated to 2-AP abundance was conducted. Consequentially, VOCs significantly correlated with 2-AP abundance were subjected to simple regression analysis in a bid to determine the key volatiles, if any, as potential biochemical markers for the rice aroma trait.

3. Results

3.1 Identification and Classification of the Rice Volatile Organic Compounds

Aliquots of the volatile organic compounds collected from the 37 rice lines were individually subjected to GC×GC-TOF-MS analysis. In totality, 148 VOCs were identified and classified using ChemDraw software ver. 12.0 (PerkinElmer, Waltham, MA, USA) into their respective functional groups (Table 2).

Table 2. Organic compounds detected in the volatile fraction of the 37 rice lines at 80 °C using gas chromatography-time-of-flight mass spectrometry

Class	Volatile organic compounds (VOCs)
Hydrocarbons	1,3-Pentadiene, (Z)-; 10-Methylnonadecane; 1-Dodecene; Heptylcyclohexane; 1-Iodo-2-methylundecane; 2,3-Dioxabicyclo[2.2.1]heptane, 1-methyl-; 3-Dodecene, (Z)-; 4-Tridecene, (Z)-; 7-Tetradecene; α -Phellandrene; α -Pinene; Benzene, 1,2,3-trimethyl-; Benzene, 1,2,4,5-tetramethyl-; Benzene, 1,2,4-trimethyl-; Benzene, 1-ethyl-2-methyl-; Benzene, 1-ethyl-3-methyl-; Benzene, 1-ethyl-4-methyl-; Benzene, 1-methyl-3-(1-methylethyl)-; Benzene, 2-ethyl-1,4-dimethyl-; Benzene, propyl-; Bicyclo[3.1.0]hex-2-ene, 4-methyl-1-(1-methylethyl)-; Borane, ethyldispropyl-; Camphene; ζ -Terpinene; Cyclohexane; Cyclohexane, hexyl-; Cyclohexene, 1-methyl-4-(1-methylethylidene)-; Cyclopentane, methyl-; Decane, 2,3,5,8-tetramethyl-; Decane, 4-methyl-; Dodecane, 2,6,10-trimethyl-; Ethylbenzene; Furan, 2-pentyl-; Heptadecane; Heptane, 2,2,4,6,6-pentamethyl-; Hexadecane; Hexane, 2,2,3-trimethyl-; Hexane, 2,3-dimethyl-; Hexane, 2,4-dimethyl-; Hexane, 3-methyl-; Longifolene; Longipinane, (E)-; n-Hexane; Nonadecane; Octane, 4-ethyl-; o-cymene; Pentane, 2,2,3-trimethyl-; Pentane; Propylene oxide; p-Xylene; Styrene; Syn-Tricyclo[5.1.0.0(2,4)]oct-5-ene, 3,3,5,6,8,8-hexamethyl-; Tetradecane; Toluene; Tridecane, 3-methyl-; Undecane and Undecane, 2,6-dimethyl-
Alcohols	1-Butanol, 3-methyl-; 1-Butanol; 1-dodecanol, 3,7,11-trimethyl-; 1-Hexanol; 1-hexanol, 2-ethyl-; 1-Nonanol; 1-Octanol, 2-butyl-; 1-Octen-3-ol; 1-Pentanol; 1-propanol; 1-Propanol, 2-methyl-; 2-Hexyl-1-octanol, (Z)-; 2-Pentanol; 2-Penten-1-ol, (Z)-; 2-Propanol, 1-methoxy-; 2-Propanol, 2-methyl-; 3-Methyl-3-buten-1-ol; 4,6-Heptadien-1-ol, (Z)-; 4-Hexen-1-ol, (Z)-; Benzyl alcohol; Cyclohexanol; Ethanol; Ethanol, 2-butoxy-; Isopropyl Alcohol and Z-10-Pentadecen-1-ol
Ketones	1-Penten-3-one, 2-methyl-; 2,3-Butanedione; 2,3-Octanedione; 2-Butanone; 2-Heptanone; 2-Hexanone; 2-Octanone; 2-Pentanone; 3-Octen-2-one; 3-Octen-2-one, (E)-; 3-Pentanone, 2-methyl-; 5-Hepten-2-one, 6-methyl-; Acetoin and Methyl Isobutyl Ketone
Aldehydes	2-Heptenal, (Z)-; 2-Nonenal, (E)-; 2-Octenal, (E)-; 2-Propanal, 2-methyl-; Benzaldehyde; Butanal, 2-methyl-; Butanal, 3-methyl-; Decanal; Heptanal; Hexanal; Methyl acetoacetate; Nonanal; Octanal; Pentanal and Propanal, 2-methyl-
N-containing compounds	2-Propanamine; 3-Butenamide; acetic acid, [(aminocarbonyl)amino]oxo-; acetic acid, cyno-; Ala-Gly; Furmaronitrile; Hydroxylamine, O-decyl-; L-Ala-L-Ala-L-Ala; N,N Dimethyl-3,4-methylenedioxyamphetamine; N- α -(tert-Butoxycarbonyl)-L-Alanine; N-Acetylglycine and Oxime, methoxy-phenyl-
S-containing compounds	2-Mercaptoethanol; 2-Undecanethiol, 2-methyl-; Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-; Cyclohexene, 1-methyl-4-(1-methylethenyl)-, (S)-; Dimethyl sulfide; Dimethyl trisulfides; Disulfide, dimethyl-; Methanesulfonic anhydride; Methanethiol and Sulfur dioxide
Esters	1-Methoxy-2-propyl acetate; 2-Propenoic acid, butyl ester; Dodecanoic acid, 11-amino-, methyl ester; Hexanoic acid, 2-propenyl ester and Sulphuric acid dibutyl ester
Oxygen heterocycles	2(3H)-Furanone,dihydro-3,5-dimethyl-; 2-Acetyl-1-pyrroline; 2-n-Butyl furan; 7-Oxabicyclo[4.1.0]heptane, 3-oxiranyl-; Creatinine; Cyclopentanone, 2-methyl-; Furan, 2,5-dihydro-; Furan, 2-ethyl- and Oxepine, 2,7-dimethyl-
Carboxylic acid	Formic acid

With regard to relative proportion, hydrocarbons constituted the most abundant fraction, 31.1% (56 VOCs). In descending order; alcohols constituted, 16.9% (25 VOCs), followed by ketones and aldehydes each at 9.5% (14 VOCs) then N-containing compounds, 8.8% (13 VOCs) and S-containing compounds, 6.6% (10 VOCs). At the tail end, carboxylic acid comprised 0.7% (1 VOC), esters 4.1% (6 VOCs) and O-heterocycles 6.1% (9 VOCs) (Table 2).

3.2 Overall Mean Abundance of the Key VOCs within Non-aromatic and Aromatic Rice Lines

A comparison between the overall mean abundance of the 148 VOCs across non-aromatic and aromatic lines revealed 48 VOCs that appeared to differentiate the 37 rice lines into two aroma categories (Table 3).

Table 3. Comparison between the overall means of non-aromatic and aromatic rice lines with regards to VOCs that registered significant ($p < 0.05$) differences

Volatile organic compounds (VOCs)	Mass ion abundance		<i>p</i> -value	Aroma description
	Non-aromatic	Aromatic		
<i>Hydrocarbons</i>				
10-Methylnonadecane	240 859	318 805	0.023	
Benzene, 1,2,3-trimethyl-	249 123	378 067	0.003	
Benzene, 1,2,4,5-tetramethyl-	124 163	204 082	0.003	
Benzene, 1,2,4-trimethyl-	43 679	65 154	0.039	
Benzene, 1-ethyl-2-methyl-	202 450	268 955	0.060	
Benzene, 1-ethyl-3-methyl-	83 546	161 805	0.002	
Benzene, 1-ethyl-4-methyl-	46 762	91 274	0.006	
Benzene, 2-ethyl-1,4-dimethyl-	40 910	81 014	0.002	
Decane, 4-methyl-	524 809	848 046	0.002	
Heptane, 2,2,4,6,6-pentamethyl-	442 186	1 012 860	0.002	
Hexadecane	168 298	209 576	0.001	
Hexane, 2,2,3-trimethyl-	325 421	489 209	0.014	
Hexane, 2,3-dimethyl-	16 592	23 325	0.026	
Hexane, 2,4-dimethyl-	532 162	641 542	0.001	
Hexane, 3-methyl-	565 827	681 090	0.033	
Longipinane, (E)-	11 278	21 693	0.039	
n-Hexane	36 349 229	35 497 155	0.033	
Nonadecane	416 779	496 701	0.011	Sweet, rosy ⁵
Octane, 4-ethyl-	194 228	326 142	<0.001	
o-cymene	46 860	101 667	0.003	
Tetradecane	2 499 182	3 273 103	0.001	Gasoline-like, alkane ⁵
Toluene	3 513 759	4 210 663	0.006	Sweet, pungent, ethereal ⁵
Tridecane, 3-methyl-	408 801	468 244	0.035	
Undecane, 2,6-dimethyl	266 999	389 806	0.003	
<i>Alcohols</i>				
1-Hexanol, 2-ethyl-	692 538	924 883	0.029	Sweet, green odour ¹
1-Octanol, 2-butyl-	487 391	620 564	0.013	
1-Octen-3-ol	1 485 477	1 897 820	0.012	Raw mushroom, straw ⁵
1-Pentanol	1 166 224	1 811 653	0.009	Plastic, green, fusel oil-like ⁵
2-Penten-1-ol, (Z)-	566 821	676 221	0.018	Sweet strong odour ¹
Cyclohexanol	1 235 725	1 911 857	0.001	Camphor-like ⁶
<i>Ketones</i>				
1-Penten-3-one, 2-methyl-	123 502	329 569	0.002	
2-Heptanone	701 518	1 110 088	0.011	Fruit, spicy odour ¹
2-Octanone	94 051	146 259	0.016	
5-Hepten-2-one, 6-methyl-	63 794	78 788	0.041	
Cyclopentanone, 2-methyl-	389 186	564 195	0.003	
<i>Aldehydes</i>				
2-Heptenal, (Z)-	260 866	307 491	0.039	Herbaceous, fatty, green ⁵
Benzaldehyde	4 625 220	5 986 177	0.001	Nutty, sweet, bitter odour ^{2,4}
Heptanal	497 022	586 418	0.045	Fatty, rancid ²
Pentanal	2 399 867	3 147 404	0.018	Woody, fruity odour ²
<i>N-containing compounds</i>				
Acetic acid, cyano-	1 283 377	1 689 341	0.038	
Hydroxylamine, O-decyl-	191 618	347 120	<0.001	
<i>S-containing compound</i>				
Disulfide, dimethyl	3 285 490	3 640 312	0.002	
<i>Esters</i>				
Hexanoic acid, 2-propenyl ester	74 118	130 270	0.022	
Methyl acetoacetate	179 467	291 531	<0.001	
<i>O-heterocycles</i>				
2-Acetyl-1-pyrroline	92 964	480 846	0.002	Sweet popcorn ³
2,3-Dioxabicyclo[2.2.1]heptane, 1-methyl-	19 206	54 043	<0.001	
7-Oxabicyclo[4.1.0]heptane, 3-oxiranyl-	58 948	97 305	0.04	
Furan, 2-pentyl-	2 019 058	2 337 019	0.043	Floral, fruity, nutty, almond, beany, green, buttery ⁵

Note. ¹ = Wilkie et al. (2007); ² = Givianrad (2012); ³ = Buttery et al. (1983); ⁴ = He et al. (2018); ⁵ = Hinge et al. (2016); ⁶ = Xia et al. (2017).

Considering the 48 VOCs that revealed significantly ($p < 0.05$) different quantities between non-aromatic and aromatic rice categories; hydrocarbons constituted the majority at 50.0% (24 VOCs), followed by alcohols 12.5% (6 VOCs), ketones 10.4% (5 VOCs), aldehydes 8.3% (4 VOCs), O-heterocycles 8.3% (4 VOCs), N-containing compounds 4.2% (2 VOCs), esters 4.2% (2 VOCs) and S-containing compounds 2.1% (1 VOC) (Table 3). With the exception of the carboxylic acid group, the total number of functional groups present within both the non-aromatic and aromatic rice categories remained unchanged.

However, a substantial reduction in the number of VOCs established as possible discriminators between the rice aroma categories (Table 3) and the total number of VOCs that constitute rice aroma (Table 2) was observed. Amongst the 148 VOCs identified as constituting rice aroma, only 48 compounds seem to discriminate between non-aromatic and aromatic rice lines, a 67.6% reduction. Specifically, the percentage decrease within the respective functional groups were dissimilar, *i.e.*, O-heterocycles (55.6% reduction), had the lowest decrease followed by hydrocarbons (57.1% reduction), ketones (64.3% reduction), Esters (60% reduction), aldehydes (73.3% reduction), alcohols (76.9% reduction), N-containing compounds (83.3% reduction) and S-containing compounds (90% reduction). Out of the nine functional groups earlier determined (Table 2), only five namely; hydrocarbons, alcohols, ketones, aldehydes and O-heterocycles, had compounds with specific rice aroma descriptions (Table 3). For all 15 VOCs with known rice aroma descriptions, the non-aromatic category registered significantly ($p < 0.05$) lower abundance compared to the aromatic. Intriguingly, the observation was also true for compounds with undesirable aroma descriptions, *i.e.*, tetradecane (gasoline-like), 1-pentanol (plastic, fusel oil-like) and heptanal (rancid).

3.3 Relationships, between and within, 2-Acetyl-1-pyrroline and Associated Volatile Organic Compounds

A correlation analysis of the 148 VOCs (Table 2) revealed 12 (8.1%) compounds to be highly associated to 2-AP, while 13 (8.7%) compounds were moderately associated and 16 (10.8%) compounds weakly associated (Tables 4a, 4b and 4c). Among the highly associated compounds to 2-AP, five functional groups were represented (Table 4a), while for the moderately associated compounds three functional groups were present (Table 4b). On the other hand, several VOCs were determined as weakly associated to 2-AP (Table 4c), thus, of limited importance in the present study.

Furthermore, with the exception of methyl acetoacetate and undecane, 2,6-dimethyl-, all compounds found to be highly associated to 2-AP were also highly or moderately associated amongst themselves (Table 4a). For moderately associated compounds to 2-AP, it was noted that all compounds were either highly or moderately associated amongst each other with the exception of: cyclopentane, methyl; cyclohexane; 1-penten-3-one, 2-methyl and hexane, 2,3-dimethyl (Table 4b).

Comparison of the compounds in Table 4a to Table 3 revealed that 9 out of 12 (91.7%) VOCs were common to both analyses. In addition, a similar comparison between compounds in Table 4b to Table 3 revealed that 12 out of 13 (92.3%) VOCs were common in both sets of results. Expectedly, out of the 16 VOCs that were determined as weakly associated to 2-AP, only five compounds namely; benzaldehyde; 7-Oxabicyclo[4.1.0]heptane, 3-oxiranyl-; 1-Octanol, 2-butyl-; tridecane, 3-methyl- and nonedecane were common to both sets of results (Tables 4c and 3).

Table 4a. Correlation coefficient, between and within, 2-Acetyl-1-pyrroline and other highly associated volatile organic compounds

	2-AP	BZTM2	BZTM3	EDE	ETL	HAD	HNLE	HPTM	HPTM3	MA	MID	Toluene	UDM
2-AP	-												
BZTM2	0.52***	-											
BZTM3	0.55***	0.87***	-										
EDE	0.53***	0.72***	0.82***	-									
ETL	0.58***	0.59***	0.46**	0.47**	-								
HAD	0.56***	0.84***	0.91***	0.87***	0.43**	-							
HNLE	0.56***	0.61***	0.78***	0.75***	0.48***	0.73***	-						
HPTM	0.52***	0.93***	0.83***	0.64***	0.53***	0.84***	0.49**	-					
HPTM3	0.59***	0.83***	0.79***	0.72***	0.68***	0.79***	0.64***	0.86***	-				
MA	0.52***	0.27	3.40	0.34*	0.08	0.37*	0.28	0.28	0.20	-			
MID	0.52***	0.75***	0.83***	0.94***	0.52***	0.87***	0.76***	0.68***	0.78***	0.35*	-		
Toluene	0.63***	0.78***	0.70***	0.69***	0.59***	0.71***	0.58***	0.75***	0.73***	0.44*	0.66***	-	
UDM	0.55***	0.77***	0.87***	0.90***	0.43*	0.94***	0.74***	0.74***	0.70***	0.39*	0.89***	0.65***	-

Note. 2-AP = 2-Acetyl-1-pyrroline; BZTM2 = Benzene, 1,2,3-trimethyl-; ZTM3 = Benzene, 1,2,4,5-tetramethyl-; EDE = Hexadecane; ETL = Ethanol, 2-butoxy; HAD = Hydroxylamine, O-decyl-; HPTM = Heptane, 2,2,4,6,6-pentamethyl-; HPTM3 = 2,3-Dioxabicyclo[2.2.1]heptane, 1-methyl-; MA = Methyl acetoacetate; UDM = Undecane, 2,6-dimethyl-; HNLE = 1-Hexanol, 2-ethyl- and MID = 10-Methylnonadecane.

* = significant ($p < 0.05$); ** = significant ($p < 0.01$); *** = significant ($p < 0.001$).

Table 4b. Correlation coefficient, between and within, 2-Acetyl-1-pyrroline and other moderately associated volatile organic compounds

	2-AP	BE2M	BE3M	BE4M	BEDM	BZTM	CME	CPTM	CXN	CXNL	DCM	PTEM	XNDM	XNTM
2-AP	-													
BE2M	0.47**	-												
BE3M	0.48**	0.86***	-											
BE4M	0.51**	0.82***	0.94***	-										
BEDM	0.49**	0.79***	0.91***	0.98***	-									
BZTM	0.47**	0.74***	0.78***	0.74***	0.69***	-								
CME	0.46**	0.68***	0.72***	0.80***	0.85***	0.64***	-							
CPTM	0.46**	0.27	0.53***	0.54***	0.53***	0.46**	0.39*	-						
CXN	0.45**	0.37*	0.65***	0.65***	0.65***	0.48**	0.46**	0.97***	-					
CXNL	0.45**	0.66***	0.81***	0.83***	0.75***	0.76***	0.62***	0.52***	0.60***	-				
DCM	0.49**	0.64***	0.80***	0.87***	0.85***	0.60***	0.66***	0.57***	0.67***	0.75***	-			
PTEM	0.49**	0.36*	0.42*	0.55***	0.51**	0.44**	0.46**	0.59***	0.58***	0.65***	0.58***	-		
XNDM	0.43**	0.41*	0.53***	0.58***	0.60***	0.53***	0.58***	0.64***	0.60***	0.47**	0.64***	0.43**	-	
XNTM	0.50**	0.87***	0.91***	0.93***	0.92***	0.75***	0.81***	0.49**	0.60**	0.80***	0.76***	0.51**	0.56***	-

Note. BZTM = Benzene, 1,2,4-trimethyl-; BE2M = Benzene, 1-ethyl-2-methyl-; BE3M = Benzene, 1-ethyl-3-methyl-; BE4M = Benzene, 1-ethyl-4-methyl-; BEDM = Benzene, 2-ethyl-1,4-dimethyl-; CME = o-cymene; CPTM = Cyclopentane, methyl-; CXN = Cyclohexane; CXNL = Cyclohexanol; DCM = Decane, 4-methyl-; PTEM = 1-penten-3-one, 2-methyl-; XNDM = Hexane, 2,3-dimethyl- and XNTM = Hexane, 2,2,3-trimethyl-.

* = significant ($p < 0.05$); ** = significant ($p < 0.01$); *** = significant ($p < 0.001$).

Table 4c. Correlation coefficient, between and within, 2-Acetyl-1-pyrroline and other weakly associated volatile organic compounds

	2-AP	ACTG	Ag	BAL	BM	BP	BZDH	DCA2	DMS	HPTO	NA	NAE2	NDCE2	OTLB	PTM	TDCE4	TRDM
2-AP	-																
ACTG	0.39*	-															
Ag	0.37*	-0.08	-														
BAL	0.39*	0.06	-0.24	-													
BM	0.36*	0.30	-0.13	0.23	-												
BP	0.40*	0.23	-0.19	0.51***	0.54***	-											
BZDH	0.40*	0.03	-0.21	0.78***	0.57*	0.66***	-										
DCA2	0.38*	0.16	-0.32	0.28	0.50**	0.34	0.39*	-									
DMS	0.34*	0.40*	0.21	0.19	0.04	0.07	0.22	0.12	-								
HPTO	0.41*	0.05	-0.19	0.45**	0.48**	0.37*	0.62***	0.46**	0.36*	-							
NA	0.39*	-0.19	-0.19	0.40*	0.59***	0.52***	0.49**	0.53***	-0.17	0.43**	-						
NAE2	0.35*	0.18	-0.04	0.16	0.32	0.21	0.19	0.37*	0.08	-0.05	0.28	-					
NDCE2	0.52*	0.38	-0.13	0.43*	0.66***	0.69***	0.50**	0.60***	0.12	0.38*	0.69***	0.51**	-				
OTLB	0.42*	0.10	0.01	0.30	0.32	0.35*	0.51**	0.45**	0.55***	0.61***	0.27	0.15	0.45**	-			
PTM	0.42*	0.17	-0.05	0.44**	0.36*	0.43**	0.59***	0.05	0.23	0.26	0.18	0.16	0.36*	0.50**	-		
TDCE4	0.42*	0.21	0.23	0.38*	0.28	0.55***	0.39*	-0.01	0.25	0.33	0.43**	0.10	0.51***	0.26	0.36*	-	
TRDM	0.42*	0.11	-0.12	0.49**	0.61***	0.65***	0.55***	0.54***	0.10	0.57***	0.68***	0.38*	0.84***	0.47**	0.34*	0.58***	-

Note. ACTG = N-Acetyl glycine; Ag = Ala-Gly; BAL = Benzyl alcohol; BM = Butanal, 2-methyl-; BP = Benzene, propyl; BZDH = Benzaldehyde; DCA2 = Decanal; DMS = Dimethyl sulfide; HPTO = 7-Oxabicyclo[4.1.0]heptane, 3-oxiranyl-; NA = Nonanal; NAE2 = 2-Nonenal; NDCE2 = Nonadecane; OTLB = 1-Octanol, 2-butyl-; PTM = Pentane, 2,2,3-trimethyl-; TDCE4 = 4-Tridecene, (Z) and TRDM = Tridecene, 3-methyl-.

* = significant ($p < 0.05$); ** = significant ($p < 0.01$); *** = significant ($p < 0.001$).

3.4 Relationships between the Key VOCs and 2-Acetyl-1-pyrroline Abundance

Given the limitations of correlation in revealing cause-effect relationships, a regression analysis was further performed on all 41 VOCs earlier found to be associated to 2-AP (Tables 4a, 4b and 4c).

Table 5. Volatile organic compounds responsible for major variation in 2-AP abundance

No.	Volatile organic compounds (VOCs)	Parameter	Standard error	p-value	R ²
1	Toluene	0.224	0.046	<0.001	38.400
2	2,3-Dioxabicyclo[2.2.1]heptane, 1-methyl-	4.340	1.060	<0.001	30.400
3	Hydroxylamine, O-decyl-	1.414	0.366	<0.001	27.900
4	Ethanol, 2-butoxy	1.547	0.392	<0.001	28.200
5	Undecane, 2,6-dimethyl-	1.587	0.417	<0.001	27.300
6	Methyl acetoacetate	1.427	0.383	<0.001	26.400
7	1-Hexanol, 2-ethyl-	0.782	0.210	<0.001	26.200
8	Benzene, 1,2,3-trimethyl-	1.383	0.382	<0.001	25.200
9	Heptane, 2,2,4,6,6-pentamethyl-	0.263	0.073	<0.001	25.200
10	Benzene, 1,2,4,5-tetramethyl-	2.253	0.635	<0.001	24.300
11	Benzene, 1-ethyl-3-methyl-	2.210	0.644	0.002	23.100
12	Nonadecane	1.653	0.484	0.002	22.900
13	Hexadecane	3.970	1.180	0.002	22.400
14	Hexane, 2,2,3-trimethyl-	0.951	0.284	0.002	22.100
15	10-Methylnonadecane	2.095	0.628	0.002	22.000
16	Hexane, 2,3-dimethyl-	5.000	0.628	0.007	22.000
17	1-Penten-3-one, 2-methyl-	0.557	0.171	0.002	21.100
18	Decane, 4-methyl-	0.453	0.140	0.003	20.900
19	Benzene, 1-ethyl-4-methyl-	3.350	1.040	0.003	20.800
20	Benzene, 2-ethyl-1,4-dimethyl-	4.080	1.260	0.003	20.800
21	Benzene, 1-ethyl-2-methyl-	2.177	0.688	0.003	20.000

Regression analysis revealed two broad categories of compounds namely; highly ($p < 0.001$) and moderately ($p > 0.001 \leq 0.007$) related to the variation in 2-AP abundance (Table 5). In the highly related category, ten compounds were present and comprised five functional groups, *i.e.*, hydrocarbons, alcohols, N-containing compounds, esters and O-heterocycles. Concerning the relative percentage abundance of functional groups within the highly related category, hydrocarbons constituted 50% followed by alcohols at 20% while N-containing compounds, esters and O-heterocycles constituted 10%, each. Relatedly, in descending order of R^2 , VOCs under the highly related category were: toluene; 2,3-dioxabicyclo[2.2.1]heptane, 1-methyl-; hydroxylamine, O-decyl-; ethanol, 2-butoxy; undecane, 2,6-dimethyl-; methyl acetoacetate; 1-hexanol, 2-ethyl-; benzene, 1,2,3-trimethyl-; heptane, 2,2,4,6,6-pentamethyl- and benzene, 1,2,4,5-tetramethyl-. On the other hand, for the moderately related category, eleven compounds were present and two functional groups represented, *i.e.*, hydrocarbons and ketones.

4. Discussion

4.1 Identification and Classification of the Volatile Organic Compounds

Earlier studies have revealed wide variations in the number and chemical nature of VOCs associated with rice grain aroma. For example; 47 volatiles in Lee et al. (2019), 50 volatiles in Shanthine et al. (2019) and 140 compounds in Sansenya et al. (2018). Thus, the number of 148 VOCs in the present study is more closely aligned to 140 VOCs as reported by Sansenya et al. (2018). The wide variation (47 – 148 VOCs) in-between the numbers of volatile organic compounds reported under the different studies appears most likely due to differences in the technologies employed, nature and number of cultivars utilized. Generally, studies involving more advance techniques such as GC×GC-TOF-MS and numerically more rice lines, appeared to report higher numbers of VOCs (Lee et al., 2019; Fatemi et al., 2014; Shanthine et al., 2019; Sansenya et al., 2018). In regards to functional groups, earlier studies revealed slight variations in the nature and number constituting rice aroma. In the present study, nine functional groups were established (Table 2). This number is in agreement with earlier works by Fatemi et al. (2014) and Lee et al. (2019). These authors, reported the rice aroma metabolites to comprise; ketones, aldehydes, alcohols, carboxylic acids, esters, hydrocarbons, oxygen heterocycles, N-containing compounds, benzene and benzene derivatives. Considering the nature of the functional groups involved in rice aroma, the three studies were similar with the exception of S-containing compounds.

4.2 Volatile Organic Compounds Able to Differentiate Non-aromatic and Aromatic Rice Categories

The presence of several VOCs capable of differentiating between non-aromatic and aromatic rice lines is probably due to the genetic diversity of rice lines within Uganda (Table 3). Similar to earlier report by Hinge et al. (2019), most volatiles in both the rice aroma categories were similar, but with different proportions. Therefore, for the 15 VOCs with aroma description, aromatic rice lines on average had more abundance of the compounds. Intriguingly, these included three volatiles with undesirable aroma tints namely; tetradecane (gasoline-like), 1-pentanol (plastic, fusel oil-like) and heptanal (rancid). Among these compounds, the contribution of tetradecane is most likely negligible due to the high odour threshold limitation of most hydrocarbons (Hu et al., 2020). In contrast to Setyaningsih et al. (2019) who found; pentanal, hexanal, 2-pentyl-furan, 2,4-nonadienal, pyridine, 1-octen-3-ol and (E)-2-octenal as volatile markers capable of differentiating between non-aromatic and aromatic rice, the present study found no similarity. Given the similarities in methodology, the absence of commonality in the results was most likely due to genetic differences in the rice lines. This highlights the fact that identified volatile biochemical markers may only be applicable in differentiating between specific rice lines and within similar environs. The present study also revealed that not all key volatile biochemical compounds possess aroma descriptions. This could possibly be construed to imply that not all volatile biochemical markers are aroma active compounds. This would be in contrast to the study by Setyaningsih et al. (2019) in which all the seven volatile biochemical markers were aroma active compounds. Hence, the present study deemed it necessary to further refine the list of possible volatile biochemical markers by subjecting the VOCs to correlation and regression analyses.

4.3 Relationships, between and within, 2-Acetyl-1-Pyrroline and Associated Volatile Organic Compounds

Hinge et al. (2016) reported that 2-AP abundance was not correlated to other aroma active compounds in aromatic rice. The authors postulated that the expression pattern of 2-AP was probably unique and specifically delinked from other volatiles of aroma importance. However, recently Daygon et al. (2017) documented a strong correlation between 2-AP abundance and amine heterocycles, namely; 6-methyl, 5-oxo-2,3,4,5-tetrahydropyridine (6M5OTP), 2-acetylpyrrole, pyrrole and 1-pyrroline. In the present study, a total of 25 VOCs were found to be highly or moderately associated to 2-AP abundance (Tables 4a and 4b). These 25 compounds comprised of six functional groups; hydrocarbons, alcohols, ketones, N-containing compounds,

esters and O-heterocycles. In contrast, the functional groups for the present study did not contain amine heterocycles as earlier reported by Daygon et al. (2017). This is probably due to genetic differences in the constitution of the rice lines studied. Specifically, the present study employed genotypes developed from *O. barthi*, *O. longistaminata*, *O. glaberrima* and *O. sativa* backgrounds (Kitara et al., 2015; Lamo et al., 2017; Kanaabi et al., 2018) in contrast to the *O. sativa* intra-specific crosses used by Daygon et al. (2017).

In the present study, hydrocarbons and alcohols constituted the highest percentage of VOCs correlated to 2-AP, cumulatively 84%. In contrast, ketones, N-containing compounds, esters and O-heterocycles constituted only 16%. This is consistent with the importance of alcohols and hydrocarbons in rice aroma as earlier reported by Stefano and Agronomia (2011) and Lee et al. (2019). The absence of carboxylic acids, S-containing compounds and aldehydes (Table 4a and 4b) may be construed to imply that these compounds are of limited contribution towards 2-AP synthesis. However, this would be in contradiction to the finding by earlier authors concerning the importance of S-containing compounds and aldehydes in rice aroma (Sansenya et al., 2018). Thus, it is important to note that the absence of correlation between a given volatile compound and 2-AP may not indicate limitations in its sensory contribution towards rice aroma. Interestingly, with the exception of; methyl acetoacetate, heptane, 2,2,4,6,6-pentamethyl- and ethanol, 2-butoxy, all compounds highly associated to 2-AP were equally highly associated to each other (Table 4a). This implies that methyl acetoacetate, heptane, 2,2,4,6,6-pentamethyl- and ethanol, 2-butoxy may be important through associated pathways such as those influencing antagonistic effects beneficial to the perception of rice grain aroma (Champagne et al., 2008; Chambers et al., 2013).

4.4 Possible Volatile Biochemical Markers for the Rice Aroma Trait

With regards to identifying possible volatile biochemical markers for the aroma trait in rice, earlier studies have established the reliability of GC-MS techniques coupled with multivariate analyses. Setyaningsih et al. (2019) reported that out of 51 volatile organic compounds subjected to principal component analysis, seven key-marker volatile compounds namely; pentanal, hexanal, 2-pentyl-furan, 2,4-nonadienal, pyridine, 1-octen-3-ol and (E)-2-octenal were able to differentiate between non-aromatic and aromatic rice. The authors postulated that these volatile compounds were key-markers for the rice aroma trait. Griglione et al. (2015) conducted rice aroma fingerprinting and consequentially identified three volatile biochemical markers; heptanal, octanal and 2-ethyl hexanol. In the present study, 21 volatile organic compounds were indicative of 2-AP abundance and differentiated between the non-aromatic and aromatic rice lines. Further multivariate analyses revealed; toluene; 2,3-dioxabicyclo[2.2.1]heptane, 1-methyl-; hydroxylamine, O-decyl-; ethanol, 2-butoxy; undecane, 2,6-dimethyl; methyl acetoacetate; 1-hexanol, 2-ethyl; benzene, 1,2,3-trimethyl-; heptane, 2,2,4,6,6-pentamethyl- and benzene, 1,2,4,5-tetramethyl- as candidates for volatile biochemical markers of the rice aroma trait. With the exception of 2-ethyl hexanol, all identified volatile markers in the three studies were not similar (Griglione et al., 2015; Setyaningsih et al., 2019). Importantly, careful examination of all earlier reported volatile markers indicated that the compounds were aroma active. Thus, toluene, 1-hexanol, 2-ethyl and heptane, 2,2,4,6,6-pentamethyl- are potential volatile biochemical markers for the rice aroma trait.

In conclusion, volatile organic compounds associated with rice grain aroma are quantitatively enormous and of diverse functional group classification. However, it is apparent that not all VOCs associated to rice aroma aid in the segregation of the rice grains into non-aromatic and aromatic types as perceived by consumers. Further, amongst the VOCs associated with the aroma trait in rice grains, not all compounds contribute equally. A few VOCs are responsible for both the discrimination of rice into non-aromatic and aromatic groups in addition to aiding desired sensory perception. These three compounds, namely: toluene, 1-hexanol, 2-ethyl and heptane, 2,2,4,6,6-pentamethyl- are therefore potential volatile biomarkers for the rice aroma trait. Thus, the aforementioned compounds may be used in rice breeding programmes for enhancing development of the aroma trait.

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