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The relationship between volatile compounds, metabolites and sensory attributes: A case study using lamb and sheep meat

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ABSTRACT

The aim of this study was to use a flavoromics approach to identify key compounds responsible for sensory flavor of lamb and sheep meat. The investigation was confined to volatile compounds from adipose tissue and metabolites in lean meat using headspace-gas chromatography/mass spectrometry (HS-GC/MS) and solvent extraction-GC/MS, respectively. Partial least square regression analysis supported with variable selection were used to correlate identified compounds to sensory attributes. Several metabolites involved in energy production via Krebs cycle and Embden-Meyerhof-Parnas pathway contributed to gamy and grass flavor. Gamy flavor was strongly and positively correlated with aspartic acid, cyclo-leucine, gluconic, citric and pyruvic acid. Gluconic and pyruvic acid together with formic acid, β-caryophyllene, 3-methylphenol, 2-ethylfuran showed strong positive correlation with grass flavor. Sugars (glucose, mannose-6-phosphate and myo-inositol) were negatively correlated with gamy and grass flavor, suggesting a role in suppression of off-flavors in lamb and sheep meat. Bitter flavor was strongly correlated with hypotaurine and (*E*)-2-pentenal. Metallic flavor and bitterness were influenced by almost the same compounds. Acidic flavor was not explained by any compound identified, while rancidity was not detected by panelists. Finally, the flavor components describing grass and bitter flavor could be used to discriminate animals from different production systems.

1. Introduction

Meat flavor is an important quality criterion with a key role in the overall lamb/sheep meat acceptability (Wood et al., 1999). Significant attention has been given to the characteristic mutton and pastoral flavor that negatively affects consumers' acceptance of lamb/sheep products (Sink and Caporaso, 1977; Young et al., 2003). Mutton flavor is described by Wong (1975) as sweaty, sour, urinary, fecal, barnyard, oily, sharp and acrid. This flavor note was associated with branched chain fatty acids (BCFA; C_8 – C_{10}), specifically 4-methyloctanoic, 4-ethyloctanoic and 4-methylnonanoic acids that are more abundant in adipose tissue of aged animals (Wong et al., 1975a,1975b; Watkins et al., 2013). However, it is noteworthy that discrimination of lamb from sheep meat according to BCFA concentration has never been reported as possible (Watkins et al., 2010).

Pastoral flavor described as sheepy, gamy, barnyard, animal, fecal, is related with pasture-fed animals (Schreurs et al., 2008). This flavor note has also been associated with higher concentrations of 3-methylindole (skatole) and 4-methylphenol in lamb adipose tissue (Young et al., 2003).

The chemistry of flavor is very complex and depends of interaction between volatile (aroma) and non-volatile (taste) compounds. A number of studies have been carried out to identify and define key volatile compounds associated with the characteristic flavor in cooked sheep meat (Almela et al., 2010; Bueno et al., 2014; Caporaso et al., 1977; Elmore et al., 2000; Hornstein and Crowe, 1963; Resconi et al., 2010; Young et al., 1997). Generally, limited work has been done on non-volatile (metabolites) compounds and their role in lamb/sheep flavor (Watkins et al., 2013). In addition, the complex nature of meat flavor requires understanding of the essential flavor-active compounds

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isolated both from adipose tissue and lean meat and their joint contribution to perceived flavor.

To understand flavor properties of lamb/sheep meat, in the present study an untargeted approach called flavoromics was applied (Ronningen, 2016). This novel approach in flavor research combines three phases: characterization of volatile and non-volatile (metabolites) compounds, model development and validation of compounds. The analytical information, as an outcome of these three steps, is correlated with sensory properties in order to define compounds responsible for specific attributes. Using this approach, the aim was to: 1) Identify and quantify volatiles and metabolites as constituents of lamb/sheep meat flavor; 2) Evaluate sensory properties of lamb/sheep meat; 3) Elucidate how volatiles and metabolites from different metabolic pathways correlate with sensory attributes using a flavoromics approach.

2. Materials and methods

2.1. Experimental design

Ninety-two female animals were used in the study. In order to get high variability in flavor profiles the following animals were chosen: lambs (5 − 6 months), young sheep (~2 years) and old sheep (4 − 5 years) belonging to two different breed representative for the production system of three country of origin (Bosnia and Herzegovina – BH, Montenegro – MN, and Norway – NW). Lamb (18 animals; NW lamb), young (15 animals; NW 2y) and old sheep (14 animals, NW 4y) belonging to the Norwegian White Sheep breed were selected. Furthermore, lamb (BH lamb) and old sheep (BH 4y), 15 animals each, belonged to Vlašićka Pramenka, being the most common phenotype of Pramenka breed in BH. Thus, fifteen old sheep of Pivska Pramenka from Montenegro (MN 4y), as a second Pramenka phenotype, were included in this experiment. Six months old lambs of Pivska Pramenka from the same herd could not be obtained.

2.2. Tissue sampling

All animals were slaughtered in the country of origin (for more details see Bjelanović et al., 2015). The *M. longissimus thoracis et lumborum* (LTL) from left side of carcass was removed and adipose tissue available on the surface of the muscle was excised within 24 h *post mortem*, wrapped in aluminum foil, vacuum-packed and stored at -80 °C. A slice of LTL was vacuum packed and stored at -80 °C for intramuscular fatty acid analysis. The rest of LTL was vacuum-packed, refrigerated for 7 days (at 4 °C), divided into slices of 2.5 cm thickness, vacuum-packed and stored at -80 °C for sensory and GC/MS analysis. All samples were analyzed in the same laboratory.

2.3. Fatty acid composition

Intramuscular fat was extracted according to AOAC Official Method (AOAC 991.36, 1996). Fatty acid methyl esters (FAME) synthesis was performed according to modified method by Yi et al. (2013). The fatty acids were analyzed by accredited laboratory (http://vitas.no/) according to the O'Fallon method (2007).

2.4. Extraction, derivatization, and GC/MS analysis ($GC/MS_{extraction}$) of meat metabolites

One gram of lean meat was transferred into a 15 mL tube, and 5 mL of a water: methanol: chloroform (1: 2.5: 1) mixture with internal standard ribitol (66 µg/mL) was added. The sample was incubated at 60 °C for 60 min in sonication bath and centrifuged for 10 min at 3 000 rpm at 4 °C. An aliquot of 1 mL was transferred into a 1.5 mL Eppendorf tube, dried in a SpeedVac (Thermo Scientific, Waltham, MA, USA) overnight and stored at -80 °C. The dried residues were re-suspended in 80 µL methoxyamine hydrochloride with pyridine (20 mg/mL) at 30 °C for 60 min and sonicated at 30 °C for 30 min. Finally, samples were treated with 80 µL of N-methyl-N-(trimethylsilyl) trifluoroacetamide at 37 °C for 30 min.

GC/MS analyses were performed according to Sissener et al. (2011). Derivatized samples (1 µL) were analyzed on an Agilent 6890 GC connected with an Agilent 5975 MS detector. A HP-5MS capillary column (i.d. 30 m × 0.25 mm, film thickness 0.25 µm) was used. The carrier gas (He) flow rate through the column was 1 mL/min. The GC temperature program: 70 °C for 5 min, ramped at 5 °C/min until 310 °C. Analysis time was 60 min. The MS was operated at 230 °C, and the recorded mass range was m/z 50 − 700.

MS files from Agilent ChemStation (Agilent Technologies, Waldbronn, Germany) were exported in the netCDF format (OPENChrom, Eclipse Public License 1.0) to MetAlign (version 041012, RIKILT Wageningen UR, Plant Research International) for data preprocessing and alignment. Metabolites were identified with the AMDIS software (version 2.71, National Institute of Standards and Technology, Boulder, CO, USA) in combination with NIST05 (National Institute of Standards and Technology/Gaithersburg, MD, USA) and GOLM metabolome database (Max-Planck Institute for Molecular Plant Physiology, Golm, Germany). Normalization of the peak area was performed on the internal standard ribitol and expressed as mg/kg of meat. Samples were run randomized. Metabolites are presented in Table S-2.

2.5. Headspace gas chromatography/mass spectrometry (HS-GC/MS) analysis of volatile compounds

Frozen adipose tissue was homogenized with a crushing machine (IKA® A11 Basic Analytical Mill, Staufen, Germany) to a fine powder. Four grams of homogeneous tissue were placed in a glass vial (50 mL) and stored at -80 °C until the next preparation step. In order to increase the volatile compounds extraction and generate representative volatile profiles, the homogenized sample was heated at 75 °C in water bath for 30 min on the day of analysis. This treatment improved extraction of volatile compounds from adipose tissue in agreement with Sivadier et al. (2008).

The liquid fat phase (1 g) was transferred to a clean glass vial and kept at 4 °C for ~ 4 h before measured. All samples were analyzed in two replicates.

A mixture of five compounds in Mygliol (AXO INDUSTRY, Warve, Belgium) was used as a control sample throughout the measurement period, at the beginning and end of sequences. These compounds were: butanal (99%), *cis*-2-penten-1-ol (95%), 2-undecanone (99%), and dimethyl sulfone (98%) (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) and acetic acid (100%, VWR, Fontenay-saus-Bois, France).

HS-GC/MS analysis was performed according to a method modified from Volden et al. (2011). The extraction of volatile compounds from 1 g of liquid fat was performed on dynamic headspace analyzer Teledyne Tekmar HT3 (Teledyne Tekmar, Ohio, USA). The sample temperature (75 °C) during headspace extraction step resulted in an unsatisfactory analytical signal. Therefore the temperature was increased to 150°C to improve the extraction and signal quality of volatiles from fat samples. This procedure may unintentionally introduce some reaction products due to heating in addition to the volatiles present at lower temperatures.

The compounds were analyzed by Agilent gas chromatograph 6890 N (Agilent Technologies, Santa Clara, CA, USA). The DB - WAXetr fused silica capillary column (30 m × 0.25 mm i.d., 0.50 µm film thickness; J&W Scientific, USA) was connected to the ion source (230 °C) of a Agilent 5975 (Agilent Technologies, SantaClara, USA) quadrupole mass spectrometer (interface line 250 °C). The carrier gas was He with a flow rate of 1.0 mL/min. The temperature program for GC was: 35 °C for 10 min, ramped 1.5 °C/min up to 40 °C, ramped 4.0 °C/min up to 70 °C, ramped 7.5 °C/min up to 230 °C, and 1 min at 230 °C. Analysis time was 54.62 min, and recorded mass range was m/z 33 − 300. Volatiles were identified by: (i) computer-matching of

generated mass spectra with NIST05 database (National Institute of Standards and Technology/Gaithersburg, MD, USA) and (ii) comparison of retention indices (RIs) with published RI values. Identified compounds (Table S-1) were used for statistics (see below). All compounds referred to below except butyrolactone that we failed to acquire, have been re-identified using pure compounds. The standard solutions run during measurement period were run at four different concentrations (R^2 = 0.996–0.999 for regression line). The concentration for all volatiles was standardized to the calibration curve for most relevant chemical compound present in the standard mix described above.

The two GC methods (GC/$MS_{extraction}$ and HS-GC/MS) were selected with priority on identification of odor and taste related compounds in lamb/sheep.

2.6. Sensory analysis

For sensory testing meat samples were defrosted at 4 °C overnight. The 2.5 cm slices of lean meat were heated in water bath set to 80 °C until internal temperature of 71 °C was achieved (AMSA, 1995) and served as 1 × 1 × 1 cm pieces to each assessor. A panel consisting of 8 trained (ISO 8586–1:1993) assessors (4 females and 4 males 30 − 59 years old) was selected for the sensory analysis. The laboratory for sensory analysis at Faculty of Technology in Novi Sad was designed according to ISO 8589:2007. During the evaluation, water and bread were served to assessors to cleanse their palate between samples. Animal group was randomly selected, and then the whole group was analyzed. Three samples were served per session and two sessions were performed. Sensory traits of lamb/sheep meat were evaluated by the quantitative-descriptive analysis (Lawless and Heymann, 2010), using a scale from one (none) to nine (very intense) according to ISO 4121:2003. Assessors were asked to evaluate the following odor (gamy, grass, rancid) and taste (acidic, bitter, metallic) attributes. Gamy was defined like leather/ horse saddle and grass odor like cut grass. Ferrous sulfate ($FeSO_4 \times 7H_2O$) was used as reference standard for training of assessors for sensory evaluation of metallic taste (ISO 3972-1991). These attributes were selected as they have been observed to distinguish different Norwegian lamb samples earlier (Lind et al., 2011). The other tastes were defined as in basic taste tests (see below). All samples were analyzed in the same sensory laboratory.

2.7. Flavor threshold determination

Flavor threshold values were obtained from VCF database (Volatile Compounds in Food 16.6.1; https://www.vcf-online.nl/VcfCompounds.cfm) unless otherwise stated (see Appendix A. Supplementary Material - Table 1 and 2). Potentially interesting compounds had concentrations higher than reported flavor thresholds in air or water/ fat/ oil. External standards from different chemical groups and applied at different concentrations were used for quantification. The analysis is semi-quantitative.

There were no literature data for the flavor thresholds of some relevant compounds dissolved in water. Flavor thresholds for six compounds (2-heptadecanone, gluconic acid, dimethyl sulfone, hypotaurine, mannose-6-phosphate and uridine) that correlated with sensory attributes (gamy, grass, bitter) were identified using 2-AFC method. In order to define minimum and maximum concentration of each compounds for threshold study, preliminary survey was performed based on Maximized Survey-derived Daily Intakes value (MSDI-EU; http://www.thegoodscentscompany.com; Perfumer and Flavorist, 2017a, 2017b, 2017c).

The four basic tastes sweet (sucrose), salty (sodium chloride), sour (citric acid monohydrate) and bitter (caffeine) were prepared as solutions in deionized water and stored at 4 °C in screw glass bottles. Six sensory experienced persons (31–43 yrs old) were assembled at the Norwegian University of Life Sciences. Prior to flavor threshold analysis panelists were re-trained by tasting easily recognizable solutions of sucrose (90 mM), sodium chloride (340 mM), citric acid monohydrate (3260 μM) and caffeine (1.75 mM) as suggested by Gomez et al. (2004) and Torrico et al. (2015). For "blanks" deionized water was used.

On the day of analysis, participants were invited at 11.00 o'clock and instructed to have a light breakfast and avoid smoking, drinking coffee, tea, refreshments or chewing gum for at least 2 h before the test (Gomez et al., 2004). Five solutions of each compound, from 4.7 to 75 mg/kg for 2-heptadecanone, gluconic acid and dimethyl sulfone, from 6.25 to 100 mg/kg for hypotaurine and mannose-6-phosphate, and from 0.625 to 10 mg/kg for uridine were prepared in 250 mL graduated closed flasks using deionized water and stored at 4 °C. The samples for flavor threshold analysis (10 mL) were presented in 15 mL plastic tubes labeled with a 3-digit random code.

For the series of trial, participants were presented with five different concentrations of each chemical in order of increasing concentration until they report difference between chemical solution and distilled water. Upon comparing the samples, being two distilled water samples and one chemical solution sample, the subjects expressed freely their impressions about flavor profiles for chemical solutions using their own expressions. Subjects were informed about chemical safety information for all compounds and the purpose of the test. Threshold concentration was defined as a concentration of compound at which panelists could detect a difference from deionized water 50% of the time. Some compounds (see below) remained without flavor thresholds; one because no pure compound was available and the rest were excluded since they were described as hazardous or there were no available data about their toxic effect.

2.8. Statistical analysis

Sensory scores and fatty acid composition were analyzed using Microsoft Excel 2016, considering the animal group as a single unit. The sensory data have only been used for regression analysis where analysis of variance of sensory data were not relevant. In order to explore the relationship between chemical compounds and meat flavor, Partial least squares regression (PLS) and Principal component analysis (PCA) were carried out using Unscrambler, version X10.1 software (Camo, Trondheim, Norway). The PLS routine was used and the calculations were made in 3 manners. First, the data were kept as is, secondly only the volatiles were multiplied by 100 so that the dimension was changed to (μg/kg meat × 10). The third data set was generated by multiplying only the volatiles by 1000 so that data originally calculated with dimension mg/kg now appeared with the dimension μg/kg meat. This dimension weighting was done to keep the compounds at comparable magnitudes and thereby increase the possibility that a compound present in low quantities could be included in the explanatory model. In addition, the concentrations defining the 3 concentration weighted matrices above were used for modelling both with and without weighting according to standard deviation (SD) of a specific variable/ compound (mean/SD). The latter was an additional principle to changing dimension (from mg to μg or μg × 10) to secure that both compounds in low and large quantities could enter the explanatory models. No compound was selected as influencing a sensory attributes unless the compound's regression coefficient β (w) significantly ($P < 0.05$) differed from zero (equal to zero defined no influence) for all 3 different weighting (×1, ×100, ×1000) principles. The above procedure was selected to reduce the prevalence of false positive associations. It should be pointed out that the procedure to a large extent eliminated the need for correct absolute concentrations. Only the flavor threshold are strictly dependent on absolute concentrations.

The PLS model was set up with random validation using segments of 7 samples; that is approximately 50% of an animal group. As an example, one group would be lamb from a specific region. It was, however, not critical for the results whether the segment number for validation was higher (*e.g.* 10) or lower (*e.g.* 4) than 7.

Principal component analysis (PCA) were performed to visualize flavor (local/global) markers significant for different animal groups in a reduced dimension plot. The PCA models included volatile compounds isolated from adipose tissue and metabolites from lean lamb and sheep meat that were significantly different ($P < 0.001$) between animal groups. Sample names were coded as described in the Experimental design section.

3. Results and discussion

3.1. Fatty acid composition

Fatty acid (FA) composition indirectly plays an important role in characteristic meat flavor in various animal species (Kosowska et al., 2017). Fatty acids are directly or indirectly involved in generation of the volatile compounds and flavor constitution (Van Ba et al., 2012). In our study, the total fatty acids of intramuscular fat of *M. longissimus thoracis et lumborum* in 92 female animals was 50 mg/g of meat, with saturated fatty acids (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) presenting 50.5%, 42.1% and 7.4%, respectively (for more details see Bjelanović et al., 2015). PUFAs, namely, α-linolenic acid (2.0%), eicosapentaenoic acid (EPA; 0.4%), docosapentaenoic acid (DPA; 0.5%), docosahexaenoic acid (DHA; 0.2%) were considered. These FA may cause flavor defects as a result of the oxidation induced by cooking (Watkins et al., 2013). Higher levels of oxidation products were previously found for grilled meat from lambs fed supplement rich in EPA and DHA (Elmore et al., 2000, 2005). Campo et al. (2003) found that mixtures of α-linoleic acid, cysteine, ribose and iron were associated with 'grass' flavor and related to meat from grazing animals.

3.2. Sensory attributes

The sensory attributes of all animal groups are presented in Table 1. The assessors used the scale from 1 − 9 to evaluate odor and taste. Gamy and grass odor were clearly identified, bitterness was less well identified, while metallic and acidic taste had limited variation for the examined samples. The assessors did not identify rancidity for the samples and the attribute was therefore excluded.

3.3. Correlation among volatile compounds, metabolites and sensory attributes

In the present study an untargeted approach was used to identify volatile compounds and metabolites in lamb and sheep adipose tissue and lean meat, respectively. Adipose tissues of 92 animals were analyzed. Seventy-five volatile compounds were identified and classified according to their chemical nature (Supplementary Material, Table S-1). They were alkanes (15), alkenes (8), alcohols (11), aldehydes (19), ketones (7), acids (5), lactone, terpene, sulphur compound, phenol, ester and others. Among the selected compounds some were found in adipose tissue of one animal group but not in others, i.e. 3-methylhexane, 3-methylphenol. 3-methylphenol was identified only in adipose tissue of MN 4y sheep. Approximately 50% of all identified volatile compounds were lipid oxidation products. The identified volatiles in our study were in agreement with previously reported volatile profiles of lamb fat and meat heated to lower (< 90°C) temperatures (Osorio et al., 2008; Sivadier et al., 2008; Vasta et al., 2007, 2012). Few cyclic compounds were formed (e.g. cyclic alkenes) that may indicate stronger heat treatment. In addition, 69 metabolites were separated and identified in the lean meat using $GC/MS_{extraction}$ analysis (Table S-2), although unlike volatiles these metabolites were identified in all animals but at different levels.

Table 1
Sensory quality profile (evaluated on a 1 − 9 scale) assessed by trained assessors on lean meat.

Sensory attributes	Mean[a]	SD[b]	Min value	Max value
gamy	4.4	1.0	2.6	6.5
grass	2.6	0.5	1.8	3.8
acidic	2.1	0.3	1.4	2.8
bitter	2.0	0.5	1.1	3.4
metallic	1.6	0.4	1.0	2.5

[a] Mean = average scores for each attribute for 92 animals.
[b] SD = standard deviation.

Interaction between odor (volatiles) and taste (metabolites) compounds is important for meat flavor although taste is dominating response. However, in further discussion for odor and taste attributes we will use term flavor since taste were assessed with open nostrils.

PLS models provided correlations between sensory attributes and all measured chemical compounds. The impact of key volatile and metabolite compounds on sensory flavor properties was determined based on PLS analysis ($P < 0.05$) as variable selection criteria.

Only 17 volatile compounds and 19 metabolites significantly ($P < 0.05$) correlated with sensory attributes in PLS regression analysis (Fig. 1). Chemical compounds that did not pass the selection criteria are listed in Table S-3 (Supplementary Material). Compounds could be left out, i.e. not selected, because: 1) they do not correlate significantly to sensory attributes despite being present above flavor threshold; 2) their measured value varied too much for significance despite having a relevant flavor. Twenty-six of the metabolites and 31 volatile compound were eliminated by first selection criteria. Metabolites presumed above sensory threshold, but left out were: malic acid (acid), sucrose (sweet), fructose (sweet), cysteine (sulpherous) and 4-aminobutyric acid (savory). Volatiles that were above flavor thresholds but left out were pentanal and octanal (fruity note), (*Z*)-4-heptanal (green), dimethyl sulphone (sulfurous/ metallic) and toluene (complex note). To some extent (*Z*)-4-heptanal (green), dimethyl sulphone (sulfurous/ metallic) may seem relevant for grass or metallic flavor but nevertheless these compounds clearly failed the selection criteria.

3.3.1. Gamy flavor

Gamy flavor was well explained (53-51%) and 15 compounds were included in the model (Fig. 1a). The explanation was highest when components present in low quantities were selected in all 3 models (×1, ×100, ×1000) by weighted variables (1/SD).

In principle, there could be 6 independent compounds or cluster of compounds that contributed to gamy flavor. This is because maximum 6 uncorrelated principal components (PC) were identified. Thus, it is possible that more components are associated with gamy flavor since compounds metabolically can correlate with other compounds. Krebs cycle components (*e. g.* citric acid, succinic acid) can be related since these are involved in respiration. In this way malic acid may indirectly contribute to flavor, albeit not being selected, since succinic acid affected flavor. In addition, it is also important to note that the compounds that apparently suppress gamy flavor can also camouflage this sensory attribute and therefore indirectly affect the flavor, i.e. glucose can modify/reduce other flavors (Meinert et al., 2009).

Amino acids reflect several physiological situations in the animal species: fatigue, stress, postprandial time etc., possibly reducing flavor acceptability (Warner et al., 2007) and alter the experience of a gamy flavor. Maruri and Larick (1992) reported a positive correlation between diterpenoids and off-flavor of grass-fed beef described by sensory panelists as gamy/stale. The actual gamy flavor is so far not well explained in terms of chemical compounds. Aspartic acid and cyclo-leucine showed positive, while glycine negative correlation with gamy flavor. Aspartic acid and glycine were identified as having concentrations above flavor thresholds, where glycine with its slightly sweet note (Drauz et al., 2007) may reduce gamy flavor. Cyclo-leucine is a non proteinogenic amino acid that may be a product of ruminal bacteria. Cyclo-leucine can be related to the level of methionine that correlated

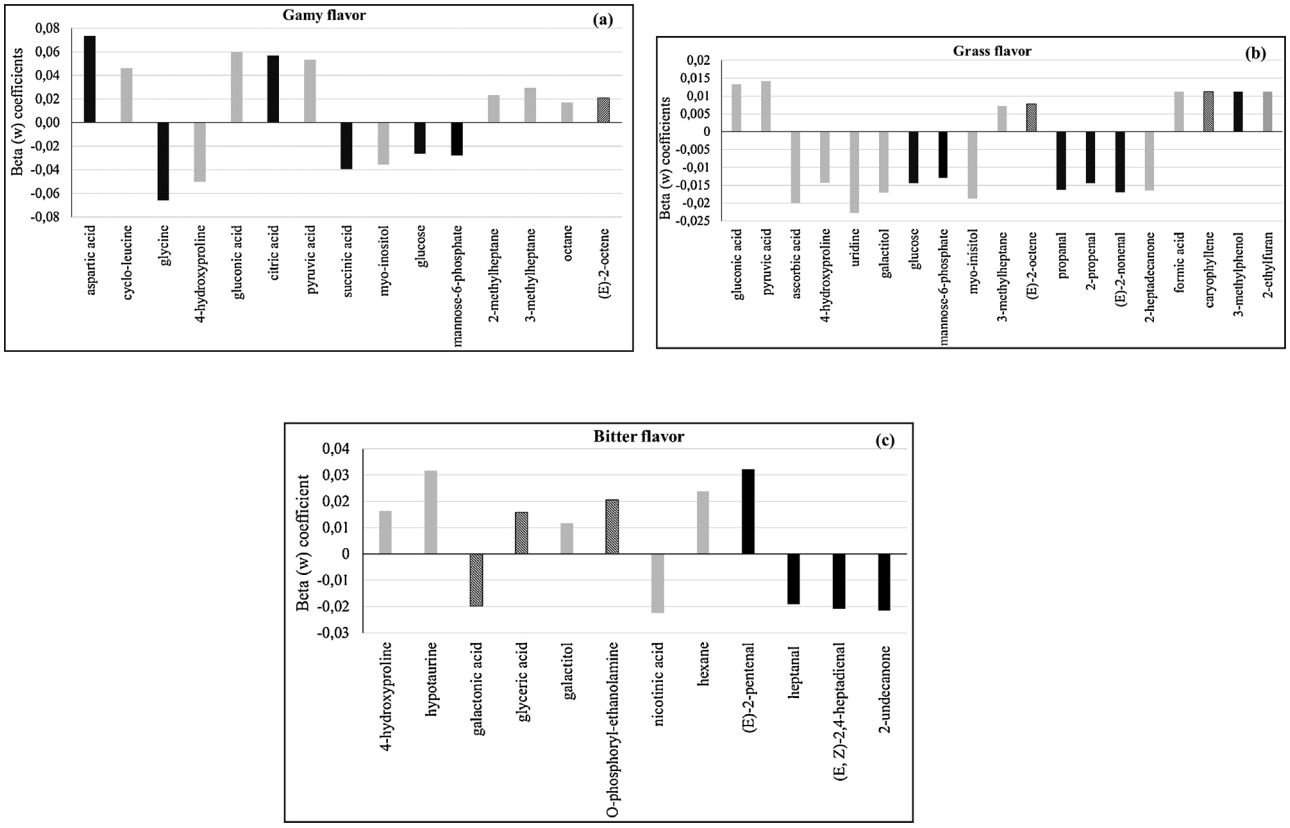

Fig. 1. Estimated regression coefficients (β_w; dimension (1/(mg/kg) for VOC and metabolites) obtained from Partial least squares regression (PLS) analysis of chemical compounds (X) and sensory attributes (Y). Beta (w) coefficients were used for relation between sensory attributes and volatiles and between sensory attributes and metabolites, respectively. Black bars in a plot represent compounds with concentrations higher than flavor threshold (odor, taste or both), grey bars are compounds with concentrations lower than flavor threshold, and bars with pattern are compounds with unknown flavor thresholds.

positively to gamy flavor but did not pass the selection criteria. In addition, cyclo-leucine correlated significantly ($P < 0.001$, linear regression) to aspartic acid, glycine, homocysteine, leucine, lysine, phenylalanine, tryptophan and tyrosine. Therefore, cyclo-leucine together with aspartic acid possibly presents a marker not only for gamy flavor but also for the general catabolic/anabolic status in lamb/sheep that may affect flavor. Nishimura et al. (1988) reported that higher content of free amino acids corresponded to higher intensity of brothy taste of pork and chicken meat. Our results suggest that bouillon-like taste of aspartic acid was probably reduced by bitter taste amino acids, i.e. leucine, lysine, phenylalanine, tyrosine (Schlichtherle-Cerny and Grosch, 1998). The concentration of 4-hydroxyproline, as sweet taste metabolite (Wieser et al., 1977), was below flavor threshold (Fig. 1a) but had a positive correlation to compounds like hypotaurine (r = 0.60, $P < 0.001$), gluconic acid (r = 0.68, $P < 0.001$), ascorbic acid (r = 0.55, $P < 0.001$), uridine (r = 0.61, $P < 0.001$) and hexanal (r = 0.57, $P < 0.001$). In addition, 4-hydroxyproline showed positive correlation to only one amino acid, β-alanine (r = 0.51, $P < 0.001$) but this amino acid is not important in protein biosynthesis. This cluster of compounds is discussed below.

Three organic acids (citric acid, pyruvic acid and succinic acid) were correlated with gamy flavor, where citric and pyruvic acid were positively correlated. Pyruvic acid was below flavor threshold, but correlated to several compounds in the citric acid cycle including a positive correlation to malic acid (r = 0.30, $P = 0.004$). Lugaz et al. (2005) reported that both citric and malic acids evoke stronger sour sensation than lactic or acetic acid at equal concentration. In addition, pyruvic acid correlated to sucrose (r = 0.61; related to pyruvate production through glycolysis, $P < 0.001$) that was present in concentration above flavor threshold. The sugar acids (gluconic, glyceric and ribonic acid) also correlated to pyruvic acid suggesting that the metabolic status of sugar polymer degradation may be involved in defining gamy flavor. Succinic acid, as metabolite that contributes to umami taste of Swiss cheese (Cadwallader and Singh, 2009) and chicken broth (Dunkel and Hofmann, 2009), showed negative correlation (r = 0.4, $P < 0.001$) with gamy flavor. Pyruvic acid, as a key compound in several metabolic pathways, and other Krebs cycle substrates are likely involved in the development of gamy flavor as many wild animals have oxidative muscles (Curry et al., 2012) that need to be furnished by the Krebs cycle to produce ATP for extensive movements.

Glucose and mannose-6-phosphate can be regarded as one variable due to their correlation (r = 0.57, $P < 0.001$). Glucose was present in a far higher concentration than the other metabolites and above flavor threshold, possibly it reduced the intensity of gamy flavor. Mannose-6-phosphate positively correlated to two metabolites from Embden-Meyerhof-Parnas pathway, fructose-6-phosphate and glucose-6-phosphate (r = 0.80 and r = 0.90, respectively, $P < 0.001$), but also to many other components like fructose. Strangely enough sucrose tended to enhance gamy flavor (r = 0.39, $P < 0.001$). Myo-inositol is a carbohydrate/sugar alcohol with half the sweetness of sucrose and it was negatively correlated with gamy flavor. Koutsidis et al. (2008) suggested that higher concentration of sugars and sugar-related compounds in beef meat is related with higher glycogen level *pre-mortem* and/or intensified glycolytic activity *post-mortem*.

The volatiles 2-methylheptane, 3-methylheptane, octane and (*E*)-2 octene can actually be looked upon as one variable due to positive correlation between the 4 selected compounds (r = 0.89 – 0.94, $P < 0.001$, Fig. 1a). Only (*E*)-2-octene may be above threshold since the other volatiles were below. However, (*E*)-2-octene is classified as dangerous (European Chemicals Agency - ECHA) organic compound and its threshold was therefore not determined. In addition, this volatile compound may not be a universal marker for gamy flavor since the

compound was not identified in all animals, but was most typical for sheep from Montenegro. (*E*)-2-octene has previously been detected in beef fat obtained from the renal periphery of beef carcasses (Umano and Shibamoto, 1987), in grilled lamb meat (Madruga et al., 2013) but also in fermented meat (Hui and Evranuz, 2012). The selected volatiles (2-methylheptane, 3-methylheptane and octane) had a positive correlation to citric and pyruvic acid (r = 0.67 – 0.78, $P < 0.001$) in addition to several other lipid degradation products for example octane (r = 0.88, $P < 0.001$).

Metabolites that correlated with gamy flavor were identified in all animals at various levels (Table S-2) and can therefore be universal markers of gamy flavor, while important volatiles were commonly only found in a fraction of the samples and cannot be equally suitable markers.

3.3.2. Grass flavor

As for gamy flavor, maximum 6 compounds or clusters of correlated compounds were suggested for grass flavor by the PLS analysis. Nineteen compounds, volatiles and metabolites (Fig. 1b), explained grass flavor up to 50% depending on weighting. Grass flavor positively correlated (r = 0.62, $P < 0.001$) with previously described gamy flavor. Therefore it is expected that some compounds that contributed to gamy flavor will also be relevant for grass flavor. It should be noted here that no amino acid was positively correlated to grass flavor or above its threshold. However, 4-hydroxyproline was included as relevant based on its correlation to other compounds.

Only two acids, gluconic and pyruvic acid, were positively correlated to grass flavor. Gluconic acid is an oxidation product of glucose in animal tissue (Salmony and Whitehead, 1954). This metabolite has complex taste (acid, bitter, metallic). Gluconic acid was negatively correlated to pyruvic acid, 3-methylheptane and (*E*)-2-octene, with the two latter compounds positively correlated to grass flavor. The acid was found in all animals and can possibly be a universal marker of grass flavor.

Volatiles, formic acid, β-carophyllene, 3-methylphenol, 2-ethylfuran, were positively correlated to grass flavor. 3-methylphenol was above threshold in this group and actually had a flavor that makes it likely to influence grass flavor (see Table S-1), but not as a universal marker. In addition, four volatiles were identified as negatively affecting grass flavor. Propanal (r = -0.39, $P < 0.001$) and 2-propenal (r = -0.33, $P = 0.002$) were negatively correlated with grass flavor, although both compounds were above threshold and described as pungent. (*E*)-2-nonenal and 2-heptadecanone also showed negative correlation (r = -0.33, $P = 0.024$) with grass flavor. (*E*)-2-nonenal was above threshold and has a flavor that would normally be associated with lipid oxidation (Kosowska et al., 2017). Although the trained panelists could not identify rancidity as a discriminator this cannot exclude lipid oxidation as an undesirable process that ultimately leads to development of off-flavors.

Group of antioxidants composed of 4-hydroxyproline, ascorbic acid, galactitol and uridine where negatively correlated with grass flavor. 4-hydroxyproline as a sweet taste compound was identified in stewed beef juice (Schlichtherle-Cerny and Grosch, 1998). Sweet taste properties of 4-hydroxyproline and antioxidant function of ascorbic acid (Howes et al., 2015) could suppress grassy flavor. Antioxidant compounds were below thresholds and therefore had no direct influence on flavor. In addition, antioxidant capacity of muscle is associated with vitamin E as a fat soluble vitamin (Howes et al., 2015). Hopkins et al. (2013) found that vitamin E can prevent lipid oxidation, when PUFA was present at high levels, i.e. at concentration above 2.95 mg/kg of muscle. The old sheep studied here had high content of vitamin E (2.5 mg/kg; Bjelanović et al., 2015), close to reported threshold. This can possibly explain the relatively low number of lipid-oxidation products that correlated with grass flavor and the absence of rancidity.

Glucose was above threshold and its sweetness suppressed grass flavor. This sugar had negative correlation to grass flavor (r = -0.50, $P < 0.001$). In addition, glucose is metabolically related to many compounds; positive correlation to fructose, galactose and mannose-6-phosphate (r = 0.83, 0.84, 0.50, respectively, $P < 0.001$) was found. Sugars seems to play a significant role in suppression of off-flavors in lamb/sheep and this is most obvious for gamy and grass flavor. Myo-inositol possibly has the same role as suggested for the gamy flavor.

3.3.3. Bitter flavor

Bitter flavor was the third best explained flavor attribute (27%) that did not show high correlation to gamy or grass flavor. Maximum 6 clusters were suggested by PLS analysis. If variables with no correlation to bitter flavor were removed, i.e. the data set of volatiles was reduced, 40% of the variation in bitter flavor was explained. Fig. 1c shows positive correlation of hypotaurine (r = 0.52, $P < 0.001$) with bitter flavor. Although, the threshold concentration for hypotaurine was by the criteria used, higher than identified concentration in lamb and sheep, participants recognized bitter flavor at the lowest tested concentration (6.25 mg/kg). Thus, since the lowest tested concentration was close to highest identified concentration in some animals (4.7 mg/kg) it is plausible that hypotaurine contributed to bitterness in sheep.

The metabolites 4-hydroxyproline, hypotaurine, O-phosphoryl-ethanolamine, were positively correlated with bitter flavor, while galactonic acid showed a negative correlation (r = -0.46, $P < 0.001$) with this attribute. Additionally, galactonic acid positively correlated with several sugar phosphates (e.g. glucose-6-phosphate, r = 0.53, $P < 0.001$), known as sweet compounds, and that may explain its negative contribution to bitter flavor. O-phosphoryl-ethanolamine, positively correlated (r = 0.36, $P < 0.05$) with bitter flavor, having a direct or indirect effect. Mabuchi et al. (2018) reported positive correlation of O-phosphoryl-ethanolamine with umami taste and hypotaurine with acidic bitterness taste of fish.

Glyceric acid and galactitol showed positive correlation (r = 0.31, $P = 0.003$) to bitter flavor. Glyceric acid positively correlated with gluconic acid and pyruvic acid (r = 0.60 and r = 0.54, $P < 0.001$, respectively). Galactitol was below threshold and positively correlated with glucuronic acid (r = 0.73) together with cysteine (r = 0.54), arabitol (r = 0.53) and inosine (r = 0.55), all with $P < 0.001$. Cysteine was above threshold in all samples and showed positive correlation (r = 0.15, $P = 0.004$) to bitterness, without being picked out directly as important for describing bitter flavor. Negative correlation (r = -0.28, $P = 0.01$) of nicotinic acid with bitter flavor was surprising since this compound has a bitter flavor. Possible explanation for this phenomenon is the presence of nicotinic acid in the concentration far below flavor threshold. In addition, positive correlation of nicotinic acid to glycine (r = 0.49, $P < 0.001$), as slightly sweet compound, modulated bitter perception of this metabolite.

Other positive correlation was observed for hexane with hypotaurine (r = 0.39, $P < 0.05$) and bitter flavor (r = 0.36, $P < 0.05$). However, the contribution of hexane to bitter flavor in this study was small since this volatile compound was present in concentration below flavor threshold. In addition, (*E*)-2-pentanal showed positive correlation to bitter flavor (r = 0.51, $P < 0.05$) and it was present in concentration above flavor threshold, but could not directly contribute to bitter flavor as it was described with fruity/green flavor. Three volatile compounds, heptanal, (*E,Z*)-2,4-heptadienal (r = -0.34 and r = -0.36, respectively, $P < 0.05$) and 2-undecanone (r = -0.34, $P < 0.01$), were above threshold level and negatively correlated with bitter flavor. Fatty perception of these compounds possibly had negative effect on bitter attribute.

3.3.4. Metallic flavor

Metallic flavor, including taste and olfactory sensation, may results from iron compounds (Mitterer-Daltoé et al., 2012). In our study metallic flavor was the 4th best explained flavor attribute (19% explained, validated). There were max 3 independent factors. The issue with metallic flavor was that it correlated significantly to bitter (R-

square = 0.63, $P < 0.001$, not validated). This was apparent from the compound listed below. There were no really new compounds to explain metallic flavor but arabitol was selected as indirectly involved in bitter flavor. By selecting a subset of compounds it was possible to explain 30% of the variation in metallic flavor; this means that it was not a well explained attribute. Among volatiles hexane may have a small positive (r = 0.33, $P = 0.002$) influence on metallic flavor. Most lipid volatiles had negative effects on metallic flavor.

Acidic flavor was not explained when model validation was used. The nominally lowest P value was to galactonic acid (r = 0.1, $P = 0.37$).

3.4. Relationship between identified flavor compounds and meat origin

Tracing the origin of products is important for authentication of meat from different production systems. Therefore additional exploration of marker of origin was therefore carried out.

Principal component analysis (PCA) was used to differentiate the animal population based on identified volatile compounds isolated from adipose tissues and metabolites from lean meat of lamb and sheep.

PCA, the first two components, carried out on the volatile compounds isolated from lamb, young and old sheep from BH, MN, and NW, are shown in Fig. 2a. A clear differentiation between volatile profiles of 4y old sheep that belonged to two phenotypes of the Pramenka breed was observed. MN 4y sheep volatile profile was determined by 14 compounds, including gamy and grass-related (2-methylheptane, 3-methylheptane, (*E*)-2-octene and 3-methyphenol) flavor compounds. The meat from MN 4y was also significantly more gamy ($P < 0.05$) and nominally more grassy than meat from BH 4y and NW 4 y. Butanol and 3-methylphenol (latter clearly above threshold) were only identified in MN animals presenting potential biomarkers of this production system. Furthermore, β-caryophyllene, almost exclusively synthesized in plant tissue, was identified only in BH 4y sheep. The role of sesquiterpenes in lamb and sheep flavor profiles needs further investigation regarding seasonal changes.

Four animal groups (BH lamb, NW lamb, NW 2y sheep, NW 4y sheep) that showed poor separation in first PCA, was used to develop a second PCA model. Fig. 2b shows that the volatile profile of BH lamb did not clustered together with NW animals. Two volatiles were associated with BH lamb profile, among them (*E*)-2-pentenal which has been proposed as a potential, indirect biomarker of bitter flavor in our data. BH lambs had the most bitter tasting meat and was significantly ($P < 0.05$) more bitter than all other meats.

In order to obtain more information about relationship between metabolites and animal population, a third PCA analysis were performed. The PCA plot in Fig. 2c showed a clear differentiation for all animal groups, and the animals from the same production system clustered together. Although NW animals clustered, no characteristic

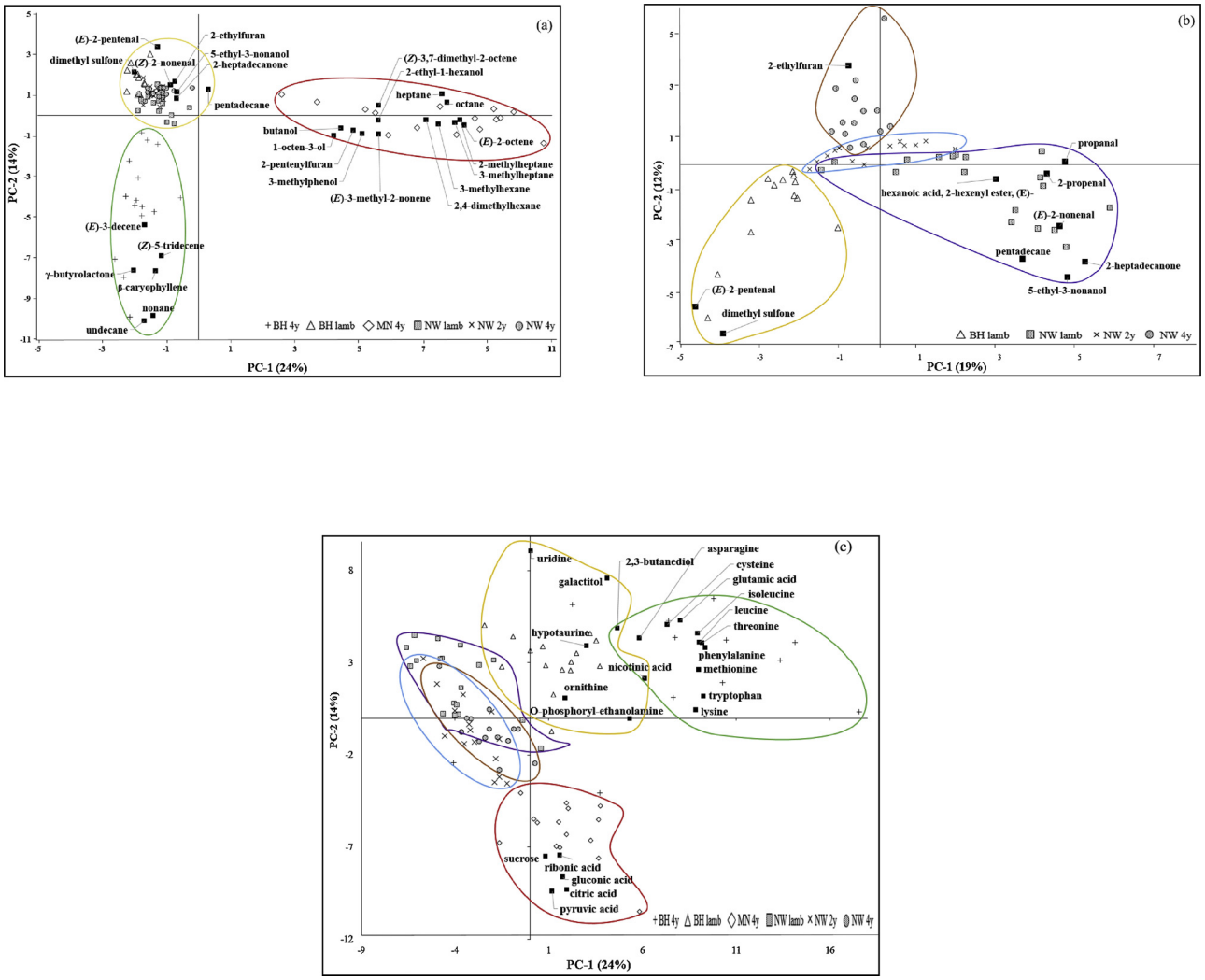

Fig. 2. Differentiation of animal groups ($P < 0.001$) based on: (a) volatile compounds isolated from heated adipose tissue of all animal groups (BH lamb, BH 4y, MN 4y, NW lamb, NW 2y, and NW 4y); (b) volatile compounds isolated from heated adipose tissue of four animal groups (BH lamb, NW lamb, NW 2y and NW 4y) that showed poor separation when all animal groups were included in PCA; (c) metabolite compounds isolated from lean meat of all animal groups.

metabolite was identified. Gluconic and pyruvic acid related to MN animals' meat and supports the gamy and grass flavor note of the meat. BH 4y meat pattern was defined by high concentration of essential amino acids that clustered with bitter flavor and these amino acids seem to support bitterness in the more bitter meats; NW lambs and NW 4y being the least bitter meats. These two meats were also the least metallic. Furthermore, antioxidant compounds that suppressed off-flavor properties may contribute to the unique BH lamb metabolite pattern.

Our results indicate that PCA plots offer an interesting approach regarding discrimination of animals from different production system using flavor markers. However, some of the markers might change depending of pasture season.

Declaration of Competing Interest

Per Berg is employed in the Research Unit of the cooperative meat production in Norway. He has provided the Norwegian animals, but not influenced the data treatment and conclusions. All authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.smallrumres.2019.09.022.

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