VALERIA DE OLIVEIRA BRITO

ADIÇÃO DE ENZIMAS NO PROCESSAMENTO DE CACAU (THEOBROMA CACAO L.) FORASTERO E TRINITÁRIO

Londrina
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Tese apresentada ao Programa de Pos-Graduacao em Ciencia de Alimentos da Universidade Estadual de Londrina como requisito parcial a obtencao do titulo de Doutora em Ciencia de Alimentos.

Orientadora: Profa. Dra. Adelaide del Pino Beleia

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Londrina, 16 de marco de 2018.

Dedico

Aos meus pais, Francisca e Luiz (in memorian),
Por toda dedicação e carinho.

Ao meu marido, Evangelos, por todo amor e apoio.

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BRITO, Valeria de Oliveira. **Adição de enzimas no processamento de cacau (Theobroma cacao L.) Forastero e Trinitario**. 2018. 146 f. Tese (Doutorado em Ciencia de Alimentos) – Universidade Estadual de Londrina, Londrina, 2018.

RESUMO

A fermentacao e uma etapa essencial para a formacao de compostos precursores de sabor e aroma em massa de cacau (liquor) e chocolate, principais produtos do processamento de cacau, que serao transformados posteriormente nas etapas de secagem, torrefacao e conchagem. A selecao do melhor clone de cacau ou variedade, associada a uma fermentacao controlada por meio de aplicacao de enzimas, podem ser utilizadas para o aumento da quantidade destes precursores e resultar em melhoria dos atributos sensoriais dos produtos finais. O objetivo deste trabalho foi aplicar uma mistura de extratos enzimaticos as amendoas frescas de cacau Forastero e uma mistura de Trinitarios, cultivados em Linhares – Espirito Santo, como uma tentativa de melhoria do sabor e aroma no chocolate amargo 70%; quantificar os compostos de interesse no cacau cru e nas etapas de fermentacao, secagem, producao de liquor e chocolate amargo 70% em escala industrial, alem de submeter os produtos finais, convencionais e tratados enzimaticamente, a uma avaliação sensorial por equipe treinada e por consumidores, que os compararam a dois chocolates comerciais. A composicao de acucares, acidos nao volateis, alcoois, metilxantinas e compostos polifenolicos foram avaliados em sementes frescas de cacau Forastero (Para) e 8 clones de Trinitario. Fermentacao convencional e com adicao de extratos enzimaticos foram realizadas para a variedade Forastero e para uma mistura de clones de cacau Trinitario, seguido de processamento industrial das amendoas. Compostos nao-volateis foram quantificados por CLAE durante a fermentacao e secagem, e os compostos volateis, por SPME-HS-CG-MS ao final da fermentacao e secagem, no liquor e chocolate amargo. Amostras destes produtos provenientes dos quatro testes foram avaliadas pelo nariz eletronico Heracles II baseado em CG-FID, por avaliadores treinados (ADQ) e por consumidores, que os compararam a dois chocolates comerciais. Os resultados foram correlacionados por Partial Least Squares (PLS). Uma mistura ideal de clones e variedades para produção de chocolate que atenda aos requisitos de sabor e produtividade da industria pode ser otimizada, combinando clones ou variedades com alto teor de acucar e o desejado balanceamento de acidos, capaz de fornecer substrato suficiente durante a fermentacao e secagem para a formacao de sabor. A utilizacao de extratos enzimaticos resultou em uma potencial reducao de um dia na fermentacao de cacau Forastero, maior efetividade do desenvolvimento de sabor e aroma desejados em Trinitario, como esteres e pirazinas, e reducao no conteudo de sabores indesejados. O melhor modelo foi obtido pela correlacao entre o termo descritor aroma de cacau, da Analise Descritiva Quantitativa (ADQ), e os dados do nariz eletronico, usando as colunas MXT-5 e MX1701. O chocolate de cacau Forastero com enzimas foi o mais aceito pelos consumidores entre os quatro testes. A correlacao entre o perfil instrumental e sensorial pode ser usado para controle de qualidade para aroma de cacau.

Palavras-chave: Enzimas. voláteis. SPME. Sabor. ADQ. Cromatografia ultrarrápida.

BRITO, Valeria de Oliveira. **Enzyme addition in the processing of cacao (Theobroma cacao L.) Forastero and Trinitario.** 2018. 146 pp. Thesis (Doctorare in Food Science) – Universidade Estadual de Londrina, Londrina, 2018.

ABSTRACT

Fermentation is essential for the flavor and aroma precursors' formation in cocoa mass (liquor) and chocolate production, the main products of cocoa processing, which are subsequently transformed during drying, roasting and conching. The selection of the best cocoa clone or variety, coupled with a controlled fermentation by the enzyme application, can be applied to increase the amount of these precursor and result in sensory attributes enhancement in the final products. The objective of this work was to add a mixture of enzymatic extracts to the fresh beans of Forastero cocoa and a mixture of Trinitarios, grown in Linhares - Espirito Santo, as an attempt to improve the flavor and aroma in 70% dark chocolate; to quantify the target compounds in raw cocoa , fermentation, drying, production of liquor and 70% dark chocolate, on an industrial scale, besides subjecting the final products, conventional and enzymatically treated, to sensory evaluation by trained judges and consumers, that compared the tests to two commercial chocolates. The composition of sugars, non-volatile acids, alcohols, methylxantines and polyphenol compounds was assessed in fresh beans of Forastero (Para) cocoa and in 8 Trinitario cocoa clones. Conventional fermentation and with addition of enzymatic extracts were conducted for Forastero variety and for a mixture of Trinitario clones, followed by the industrial processing of the beans. Non-volatile compounds were quantified by HPLC during fermentation and drying, and volatile compounds, by SPME-HS-GC-MS at the end of fermentation and drying, in the liquor and in dark chocolate. Samples from those two products from the four testes were assessed by Heracles II electronic nose based on GC-FID, trained judges (QDA) and by consumers, that compared the samples to two commercial chocolates. The results were correlated by Partial Least Squares (PLS). An ideal mixture of clones and varieties for chocolate production that fulfills the flavor quality and productivity requirements of the industry may be optimized, combining high sugar content clones or variety with the desired balance of acids, able to provide enough substrate during fermentation and drying for flavor formation. The usage of enzymatic extracts resulted in a potential one day reduction in conventional Forastero fermentation, more effectiveness enhancement in desired flavor and aroma notes in Trinitario, such as esters and pyrazines, and reduction of off-flavor content. The best model was obtained by correlating cocoa aroma descriptor term, from Quantitative Descriptive Analysis (QDA), to the electronic nose data using columns MXT-5 and MX1701. Forastero chocolate with enzymes was the most accepted by the consumers among the four tests. The correlation between instrumental and sensory profile can be used for quality control purposes for cocoa flavor.

Key Words: Enzymes. Volatiles. SPME. Flavor. QDA. Ultrafast chromatography.

SUMÁRIO

1 INTRODUÇÃO

Cacau (*Theobroma cacao L.*), fruto do cacaueiro, originário das Américas Central e Sul, é o principal ingrediente na fabricação de chocolate (BECKETT, 2011; LOPES; PIRES, 2015), cuja importância econômica é atribuída aos diversos derivados de seu processamento industrial, como massa de cacau (liquor), manteiga de cacau, torta de cacau e cacau em pó. A produção industrial de extratos de cacau ricos em compostos polifenólicos, obtidos a partir sementes cruas, fermentadas ou outros derivados, foi destacada como uma nova alternativa (SCHINELLA, 2010; ORTEGA et al., 2010).

A produção de cacau em amêndoas no estado do Espirito Santo, corresponde a 2,58% do total nacional (CONAB, 2016; IBGE, 2016, 2017). O Município de Linhares, que contribui com 90% desta parcela, destaca-se por premiações de diversas fazendas em competições internacionais como cacau de excelência, e pela Indicação Geográfica, concedida pelo Instituto Nacional de Propriedade Industrial (INPI), desde 2012 (ANDRADE, 2015; CEPLAC, 2011; GLOBO, 2017). O Brasil possui todas as etapas da cadeia produtiva de chocolate, que inclui fazendas produtoras de cacau, empresas beneficiadoras das amêndoas fermentadas e secas, produtoras de liquor, chocolate e derivados, além de fabricantes de equipamentos e centros de pesquisa para melhoramento genético do cacaueiro. Apresenta, portanto, elevado potencial para inovações tecnológicas no setor.

Existem dois principais grupos de cacau: *Forastero*, originário da região Amazônica e o *Criollo*, originário da América Central e do norte da América do Sul. O cruzamento entre estes dois grupos originou o híbrido Trinitario (LOPES; PIRES, 2015). Entretanto, há pesquisadores que defendem a existência de um terceiro grupo ou cultivar, cacau *Nacional*, originário do Equador, de características únicas de aroma e sabor (AFOAKWA et al., 2008, APROTOSOAIE; LUCA; MIRON 2016, GIACOMETTI; JOLIĆ; JOSIĆ, 2015), e outros, o consideram como uma variedade tradicional formada por polinização aberta (LOPES; PIRES, 2015).

Atualmente, a utilização de variedades clonais, obtidas por meio de enxertos em árvores, ou a utilização de sementes híbridas, produzidas por agências de pesquisas e resultantes do cruzamento entre clones, são as técnicas mais empregadas no Brasil (LOPES; PIRES, 2015).

A seleção do melhor clone ou variedade pelo produtor é essencial, uma vez que as diferenças em composição química do cacau relacionadas à quantidade de compostos precursores de sabor e polifenólicos, associada às transformações essenciais do pós-colheita de fermentação, secagem e torrefação, podem impactar na intensidade do sabor e aroma do chocolate (AFOAKWA, 2010; COUNET et al.,2004, LUNA et al., 2002, REINECCIUS, 2006).

A fermentação é uma etapa essencial para a fabricação de chocolate, pela formação dos precursores de sabor e aroma (AFOAKWA et al.,2008; APROTOSOAIE; LUCA; MIRON 2016), apesar das limitadas modificações feitas nesta etapa ao longo do tempo em comparação ao restante da cadeia produtiva.

Atualmente, existem propostas para promover a melhoria de sabor e aroma por meio de inoculação de culturas starter ou coquetéis microbianos (AMORIM et al.,2008; BATISTA et al., 2016; CRAFACK et al., 2013, 2014; LEFEBER et al., 2012; MENEZES et al., 2016; SANDHYA et al., 2016; SCHWAN, 1998), ou adição de enzima no cacau (DE BRITO et al., 2004; DOROTEA et al., 2001 ; JINAP ; NAZAMID ; JAMILAH, 2002 ; OLIVEIRA et al., 2011).

Sistemas multi-enzimáticos foram utilizados em outras matrizes alimentares complexas para melhoria na eficiência do processo, extração ou produção de compostos de interesse (MEKASHA et al., 2017; WAGLAY; KARBOURNE, 2017). Em cacau, Oliveira *et al* (2011) aplicaram misturas de proteases e peptidases em cacau fermentado e fresco. Entretanto, não há registros de tal aplicação em cacau fresco, antes do processo de fermentação.

A eficácia das etapas de fermentação, secagem, fabricação de liquor e chocolate pode ser avaliada pela quantificação de compostos de interesse como açúcares, ácidos não-voláteis e compostos voláteis como ácidos, álcoois, acetonas, aldeídos e ésteres (RODRIGUEZ-CAMPOS et al., 2011, 2012, BRITO et al 2016), assim como a investigação de modificações efetuadas nos referidos processos.

Desta forma, propõe-se o uso de uma mistura de extratos enzimáticos, aplicados às amêndoas frescas de cacau Forastero e uma mistura de Trinitarios, cultivados em Linhares – Espírito Santo, como uma tentativa de melhoria do sabor e aroma no chocolate amargo 70%, quantificação de compostos de interesse nas etapas de fermentação, secagem, produção de liquor e chocolate amargo 70% em escala industrial, além de avaliação por uma equipe sensorial treinada e consumidores.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar as modificações provocadas pela aplicação da mistura de extratos enzimáticos sobre as amêndoas frescas, durante a fermentação e secagem, e sobre o liquor de cacau e chocolate 70% obtidos, em comparação com o método de fermentação tradicional.

2.2 OBJETIVOS ESPECÍFICOS

- Avaliar o potencial industrial de clones de cacau para a fabricação industrial de chocolate e extratos polifenólicos, com base na composição química dos cotilédones.
- Avaliar as variações em compostos voláteis e não voláteis em cacau Forastero e Trinitario, com adicao de extratos enzimáticos nas sementes frescas em comparação ao processo tradicional, durante a fermentação, secagem, produção de liquor e chocolate amargo 70%.
- Avaliar os atributos sensoriais em liquor de cacau e chocolate 70% obtidos a partir de cacau fermentado pelos métodos convencional e com adição de extratos enzimáticos e comparar com a quantificação relativa dos compostos voláteis realizada em nariz eletrônico
- Avaliar a aceitabilidade de chocolate 70% obtido a partir de cacau fermentado pelos métodos tradicional e com adição de extratos enzimáticos em comparação a dois chocolates comerciais.

3 REVISÃO BIBLIOGRÁFICA

3.1 CLASSIFICAÇÃO BOTÂNICA DO CACAU E MERCADO

O cacaueiro é uma planta da família Sterculiaceae, cujo fruto, cacau, foi citado pela primeira vez na literatura botânica como *Cacao fructus*, modificado posteriormente pelo botânico Linneu para *Theobroma frutus* e reclassificado em 1753 para *Theobroma cacao*, designação que permanece até hoje. Nativo da floresta tropical úmida americana, seu centro de origem foi, provavelmente, às nascentes dos rios Amazonas e Orinoco (LOPES; PIRES, 2015). Os frutos são ovais e variam em tamanho, formato, textura da superfície e cor externa. As sementes são envolvidas por polpa e estão ligadas à placenta (cibirra). Como a polpa mucilaginosa contem um inibidor de germinação, este processo da semente é retardado. Entretanto, uma vez que o fruto é aberto, a polpa se decompõe rapidamente e a germinação inicia quando em temperatura e umidade apropriadas. É uma prática comum em muitas regiões produtoras de cacau que os frutos sejam empilhados na área de plantio por 3 a 4 dias após a colheita para a abertura no mesmo local ou para o transporte e posterior abertura em local diferenciado (VUYST et al. ,2010).

Theobroma cacao, a espécie mais estudada do gênero *Theobroma*, estende-se das florestas úmidas tropicais da Bacia Amazônica até o Sul do México. A classificação tradicional do cacau contempla dois grupos genéticos: Criollo, originário da América Central e Forastero, da região Amazônica ou América do Sul. Alternativamente pode ser classificado pelas respectivas subespécies, como *T. cacao subsp. cacao* e suas formas cultivadas representantes do grupo dos Criollos, e o cacau proveniente da América do Sul, como *T. cacao subsp. sphaerocarpum* e suas cultivares, representantes do grupo Forastero (LOPES; PIRES, 2015; MOTAMAYOR et al., 2008).

O cacau Criollo apresenta aroma fino e suave. Responsável por menos de 1% de todo o cacau produzido mundialmente, em parte por ser mais sensível às adversidades climáticas, mais vulnerável a insetos e doenças. É cultivado na Venezuela, Madagascar e alguns países da América Central. Seus frutos são grandes, geralmente apresentam a casca fina e rugosa, e resulta em produto de superior qualidade, conhecido comercialmente como “cacau fino”. O cacau Forastero é responsável por mais de 90% da produção mundial, a base para a indústria de cacau

e chocolate. Seus frutos, que variam da forma de cabaça ao amelonado, possuem sementes achatadas de cor violeta-intenso, produzindo um cacau conhecido como tipo “básico”. O cacau Trinitário é híbrido biológico natural entre Criollo e Forastero, originada em Trinidad, onde os colonizadores espanhóis estabeleceram plantações. Esta variedade produz amêndoas “finas” ou “aromáticas” com sabores frutais, de melaço ou de uva passa. Este grupo apresenta um produto de qualidade comercial intermediária. Seus principais representantes possuem características distintas, por serem variedades obtidas por cruzamento. (GRAMACHO et al., 1992).

LOOR et al. (2009) e MOTAMAYOR et al. (2008), consideram um terceiro cultivar de cacau, proveniente de uma população primitiva de *Theobroma cacao*, conhecida como Nacional, existente na região da costa do Pacífico, no Equador. Embora sua origem exata seja desconhecida, é considerada propriedade deste país. Ao longo dos anos, sua classificação variou, considerado inicialmente como Forastero e, posteriormente, como Criollo. Recentemente, tem sido classificado como um grupo diferente do Criollo e Forastero, mas geneticamente mais próximo ao último. O cacau Nacional pode ser derivado de uma população selvagem, que pode ter desaparecido completamente junto com a floresta original que encobria a costa da região. Sugeriu-se que o cacau Nacional tenha sua origem nas montanhas dos Andes, na região Amazônica do Equador. No início de 1600, pequenas plantações de cacau existentes ao longo das margens do Rio Guayas, localizado em uma região chamada “Arriba”, espalharam-se até a área superior dos rios Daule e Babahoyo. As árvores desenvolvem um aroma floral muito intenso conhecido como aroma “arriba” no mercado internacional, muito apreciado para a produção de produtos de chocolate diferenciados, exclusivamente provenientes do Equador. Com exceção da província de Esmeraldas, o cacau Nacional era o único cultivado na costa ocidental do Equador até 1890 quando, pela primeira vez, mudas de cultivares chamados “Venezuela”, provenientes de Trinidad foram introduzidos.

O Brasil, que ocupava a sexta posição no ranking mundial em produção de amêndoas de cacau na safra 2014/2015, ocupou o sétimo lugar para a safra de 2017, atrás de Costa do Marfim, Gana, Indonésia, Camarões, Equador e Nigéria. A produção nacional atualmente é liderada pelos estados da Bahia e Pará, seguido de Espírito Santo, Rondônia, Amazonas, e Minas Gerais (CONAB, 2016; IBGE, 2016, 2017).

A produção de cacau no Brasil sofreu alterações nos últimos anos. Anteriormente liderada pelo estado da Bahia, seguido de Pará, Espírito Santo, Rondônia, Amazonas e Minas Gerais, atualmente, apresenta Pará como líder, e o Espírito Santo, como quarto maior produtor nacional de cacau em amêndoas, atrás de Pará, Bahia e Rondônia, em uma área aproximada de 23 mil hectares, e produção de 5,5 mil toneladas em 2016. Linhares é o maior produtor do Estado do Espírito Santo, com mais de 87% da área total cultivada e responsável por aproximadamente 90% da produção (CONAB, 2017; IBGE, 2017; INCAPER, 2017). Os municípios de São Mateus, Colatina e João Neiva também são produtores de cacau que empregam o sistema de cabruca no estado do Espírito Santo.

Inicialmente, o cacaueiro foi cultivado para a utilização das sementes, matéria prima da indústria do chocolate. As amêndoas, sementes do cacau após as etapas de fermentação e secagem, representam no máximo 10% em relação ao peso do fruto. Posteriormente, iniciou-se o aproveitamento dos 90% restantes do fruto (CEPLAC, 2001). As tabelas 1 e 2 contém informações referentes ao rendimento do fruto e subprodutos.

Tabela 1 - Características físicas do fruto do cacaueiro, a partir de 500g de fruto.

Componentes	Peso (g)	% em relação ao peso do fruto
Fruto	500	100
Casca do fruto	400	80
Sementes frescas	100	20
Sementes secas (amêndoas)	50	10
Nibs (cotilédones limpos)	40	8

Fonte: MORORÓ (2012)

Tabela 2 - Subprodutos do fruto do cacaueiro.

Subprodutos	Rendimento
Semente fresca	1875kg
Cacau seco	750kg
Mel de cacau (suco celular)	200 litros
Polpa	300 a 400 Litros

Fonte: adaptado de CEPLAC (2001)

Produtos diferenciados obtidos a partir do cacau foram desenvolvidos para atender a demanda crescente por alimentos propriedades antioxidantes, entre eles extratos ricos em polifenólicos produzidos industrialmente a partir de amêndoas cruas, como descrito por SCHINELLA et al. (2010), com potencial aplicação como ingrediente. ORTEGA et al. (2008), também avaliaram extratos de cacau como potenciais ingredientes para a indústria de alimentos, porém, obtidos de outros derivados de cacau, como amêndoas fermentadas e secas, nibs (amêndoas torradas), liquor e cacau em pó.

3.2 Composição Química

A polpa do cacau, principal substrato durante a fermentação, apresenta composição química média de 82 - 87% de água, 10 – 15% de açúcares, 1 – 5% de pectina, 1 – 3% de ácido cítrico, 0,1 – 0,4% de ácidos orgânicos não voláteis, como o ácido málico, 0,5 – 0,7% de proteínas e de 8 – 10% de minerais e oligoelementos. Entre os açúcares presentes, a sacarose é o mais abundante, com cerca de 60%, seguida por 39% de uma mistura entre glicose e frutose. (VUYST et al. ,2010). A polpa mucilaginosa que envolve as amêndoas contém, em média, 14% de açúcares simples, 1,5% de pectina e apresenta pH médio de 3,5 (SCHWAN, 1998). PENHA; DA MATTA (1998) estudaram as características físico-químicas e microbiológicas da polpa de cacau comercializada na região sudeste da Bahia (Tabela 3). O período de safra abrange os meses de novembro à fevereiro, enquanto o temporão, de abril a agosto.

Tabela 3 - Características físico-químicas da polpa de cacau fresco.

Determinações	Cacau da safra[1]		Cacau temporão[2]	
	1	2	1	2
Pectina (mg de pectato de cálcio/100 g)	0,06	0,16	2,34	2,27
Amido (g/100 g)	4,58	3,65	ND	ND[3]
Proteína (g/100 g)	1,13	1,12	0,73	0,94
Fibra (g/100 g)	0,29	0,35	0,34	0,35
Extrato etéreo (g/100 g)	0,17	0,12	0,65	0,45
Sacarose (g/100 g)	6,63	8,19	8,22	6,62
Frutose (g/100 g)	4,41	4,54	5,95	5,66
Glicose (g/100 g)	3,72	3,87	5,29	4,84
pH	3,16	3,36	3,61	3,64
Umidade (g/100 g)	75,88	75,33	79,52	80,06
Atividade de água	0,94	0,94	0,90	0,94

[1] Cacau não lavado; [2] Cacau lavado; [3] Não detectado.
Fonte: Adaptado de PENHA; DA MATTA (1998)

Segundo PENHA; DA MATTA (1998), as diferenças no teor de pectina e na presença de amido deve-se a diferenças no regime de chuvas e insolação entre os períodos de safra e temporão. O termo geral pectina designa ácidos pectínicos solúveis em água, com grau variável de grupos metil éster e um grau de metilação capaz de formar gel com açúcares e ácidos em condições adequadas. (UENOJO; PASTORE, 2007). São polissacarídeos complexos que contribuem para a firmeza e a estrutura de tecidos de plantas como parte da lamela média (GUMMADI, MANOJ, KUMAR, 2007)

Pectina consiste em uma estrutura de ligações axiais de unidades de ácido α-1,4-D-galacturônico e contém moléculas de L-ramnose, arabinose, galactose e xilose como cadeias laterais. Ácido péctico é uma designação aplicada a substâncias pécticas compostas de ácido poligalacturônico coloidal, onde os grupos carboxila estão essencialmente livres de grupos metil-éster e seus sais são pectatos neutros ou ácidos. Ácido pectínico é um grupo de compostos que contém ácido poligalacturônico coloidal com poucos grupos metil éster. As pectinas podem ser

degradadas por enzimas, as pectinases, que são produzidas por diversos microorganismos. Entretanto, aquelas de origem fúngica são as mais utilizadas em escala industrial, uma vez que cerca de 90% desta enzima pode ser secretada em meio de cultura. (UENOJO; PASTORE, 2007)

Os carboidratos constituintes da polpa mulcilaginosa foram avaliados por BERBERT apud DIAS (1987), desde o momento da coheita até o partido dos frutos, após 6 dias.

Tabela 4 - Constituintes da polpa da semente de cacau de frutos colhidos no Centro de Pesquisas do Cacau (BA) (% em relação à matéria úmida) (DIAS, 1987)

Açúcar	**Intervalo entre a colheita e a quebra do fruto**	
	Início (%b.u*)	6 dias (%b.u*)
Frutose	2,94	3,62
Glicose	2,67	4,50
Sacarose	8,50	6,56

* b.u: base úmida

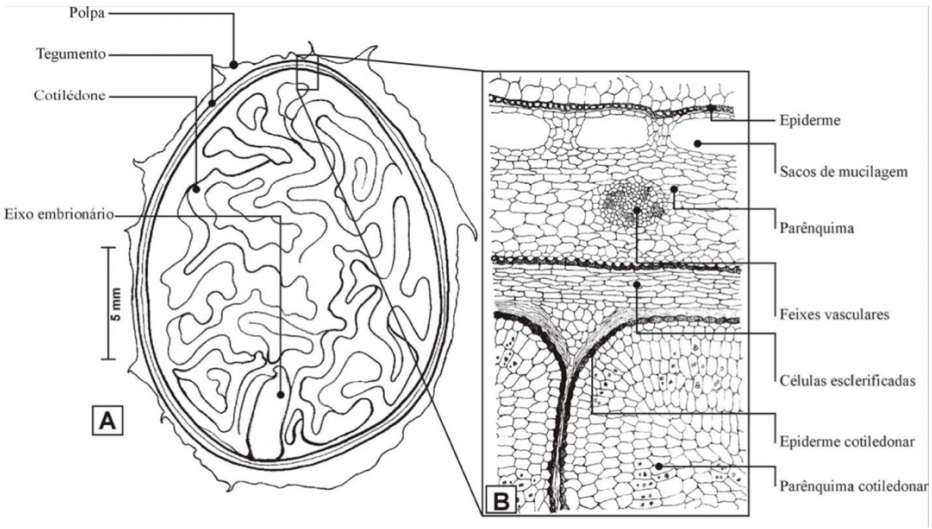
Fonte: Adaptado de DIAS, 1987.

Os percentuais em relação à matéria úmida de frutose e glicose aumentam após 6 dias de colheita sem abertura do fruto, há redução no percentual de sacarose (Tabela 4), o que evidencia a hidrólise da sacarose em dois monossacarídeos. Parte da sacarose da polpa aderida às sementes é transformada em glicose e frutose pela enzima invertase (ou beta-frutofuranosidase) presente no tegumento ou testa, que acelera o processo fermentativo (LOPEZ, DIMICK,1995; SCHWAN, 1998; SCHWAN; WHEALS, 2004; De VUYST et al., 2010). LOPEZ; LEHRIAN; LEHRIAN (1978) testaram preparações em pó com a polpa, testa com polpa e com o cotilédone do cacau para investigar a presença de invertase. Verificam que somente ocorreu inversão de sacarose em sementes frescas quando foi utilizada enzima em pó preparada a partir de sementes, na qual a casca foi incluída. HANSEN; OLMO; BURRI (1998) confirmaram atividade enzimática de invertase na polpa e no cotilédone de cacau genótipo ICS-95 antes de fermentar e até um dia de fermentação.

Segundo De VUYST et al (2010), a concentração de sacarose, glicose e frutose é uma função do cultivar e idade do fruto; unidades verdes contém maior proporção de sacarose, e frutos maduros contém principalmente frutose e glicose. O pH da polpa é relativamente baixo (pH 3,0 – 4,0) principalmente devido ao conteúdo de ácido cítrico. Elevado conteúdo de pectina e outros polissacarídeos (celulose, hemicelulose e lignina) conferem viscosidade, adesividade e coesividade à polpa.

A amêndoa de cacau, envolvida por polpa, possui uma película denominada testa ou tegumento, que reveste os dois cotilédones e o germe ou eixo embrionário (Figura 1). Oferece barreira à penetração de ácidos no interior da amêndoa e saída de componentes indesejáveis como teobromina, cafeína e polifenóis (SCHWAN; WHEALS, 2004). Segundo De VUYST et al (2010) o tegumento do cacau é permeável a moléculas voláteis pequenas, como ácido acético e etanol, e impermeável a moléculas grandes como ácido cítrico, polifenóis e alcalóides.

Figura 1- Estrutura anatômica da semente madura de *Theobroma cacao L.,*



Fonte: SANTOS (2003)

A: Semente em corte longitudinal; B: detalhe da semente em corte longitudinal

O tegumento permanece intacto durante o processo de fermentação, em que apresenta textura levemente elástica e maior resistência à ruptura. Sua aderência ao cotilédone durante as etapas de secagem, armazenamento, transporte e torrefação das amêndoas evita perda dos nibs na indústria de chocolates. Durante o beneficiamento das amêndoas, é mecanicamente removido e triturado, comercializado na forma de farelo de cacau como subproduto da indústria para

produção de ração animal, devido ao elevado teor de proteína bruta quando comparado a outros subprodutos destinados à mesma finalidade (Tabela 5). Em sua constituição, também estão presentes a celulose, hemicelulose e lignina (Tabela 6). Estima-se que tonelada de amêndoas com 7% de umidade pode gerar de 80 a 120kg de casca de cacau após o processamento (AZÊVEDO et al., 2011).

Tabela 5 - Composição química de tegumento de cacau em comparação à silagem de milho como sub-produto para ração animal.

Item	Silagem de milho	Cacau[1]
Matéria Seca	24,10	89,34
Matéria Orgânica	92,96	92,58
Proteína Bruta	6,09	14,33
Extrato Etéreo	2,07	5,07
Lignina	5,17	18,54

Fonte: Adaptado de Azêvedo et al., 2011.

[1]Tegumento com pequenos pedaços de semente

As sementes de *Theobroma* são ricas em amido (15%), proteína (15%), lipídeos (~50%), possuem cerca de 1,5 à 3% de teobromina, um alcalóide estimulante do sistema nervoso central com propriedades semelhantes a outro alcalóide presente nas sementes de Theobroma, a cafeína. As sementes de T. cacao, são geralmente pigmentadas por tanino (vermelho a roxo), com pequenas quantidades de ácido málico e tartárico (SANTOS, 2003). A composição química das sementes encontra-se na Tabela 7.

Tabela 6 - Composição química de farelo de cacau em percentual de matéria seca

Item	Farelo de Cacau %
Matéria Seca	86,0
Matéria Orgânica	91,2
Matéria Mineral	8,8
Nitrogênio Total	2,4
Extrato Etéreo	2,6
Hemicelulose	11,9
Celulose	17,8
Lignina	15,6
Carboidratos Totais	73,6

Fonte: Adaptado de Pires et al. (2009).

Segundo BECKETT (2009), no cacau Forasteiro, os cotilédones são de cor púrpura e no Criollo, brancos. A cor é provenientes antocianinas, grupo de substâncias químicas que conferem a maior parte das cores azuis e roxas das flores e resultam na produção do sabor característico do cacau. As antocianinas de cor púrpura estão associadas a sabores mais fortes e adstringentes. O Criollo, sem as antocianinas, apresenta pigmentação mais suave. O cacau Trinitário, híbrido dos dois tipos principais, apresenta sementes quase brancas ou totalmente púrpuras.

O tecido dos cotilédones é composto por dois tipos de células: aquelas que contém pigmentos compostos de polifenóis (taninos, catequinas, antocianinas e proantocianidinas) e metilxantinas (teobrominas e cafeína); e as células de reserva, contem amido, lipídeos, proteínas e enzimas. Os polifenóis do cacau são armazenados em grandes vacúolos de células e folhas do cacaueiro (AFOAKWA et al. 2008; EFRAIM, 2004; De Vuyst et al., 2010).

Tabela 7 - Composição química de testa, embrião e cotilédones de sementes fermentadas e secas

Compostos	Testa (%)	Embrião (%)	Cotilédones (%)
Lipídeos	4,69	7,70	47,53
Nitrogênio total	3,42	4,67	1,87
Açúcares totais	5,04	5,76	2,93
Amido	11,13	23,76	11,63
Fibras	19,93	n.d.	12,20
Cinzas	10,73	n.d.	2,71
Teobromina	0,48	0,70	0,35
Cafeína	0,15	0,16	0,17
Umidade residual	9,65	9,37	4,44

Fonte: Adaptado de MiNIFIE (1989)

As sementes de cacau contem as proteínas albumina, globulina, prolamina e glutelina, com a albumina presente em maior fração. As proteínas de reserva do cacau podem estar relacionadas com a formação de aroma específico do cacau. Durante a fermentação, essencial para a formação de aroma e sabor, há degradação seletiva das proteínas vacuolares. A seletividade, o grau de proteólise que ocorrem nas sementes durante a fermentação, e o potencial de sabor e aroma da amêndoa de cacau cru, são influenciados pelas condições de fermentação, especialmente o tempo de acidificação oriundo da ação de microrganismos da polpa. Possivelmente, os peptídeos relacionados ao sabor e aroma são derivados de proteínas específicas das sementes de cacau (VOIGT; BIEHL,1993).

3.3 Fermentação do Cacau

A origem do processo de fermentação perdeu-se na antiguidade, mas acredita-se que inicialmente esta etapa era realizada unicamente para a remoção da

polpa mucilaginosa e facilitar a secagem e armazenamento das amêndoas (THOMPSON, MILLER, LOPEZ, 2001).

A amêndoa de cacau crua deve ser fermentada, seca e torrada antes do processamento para produção de chocolate (SCHWAN, 1998). Antes do processo fermentativo, as sementes possuem sabor adstringente, desagradável e amargo e deve ser processada após a colheita, antes de se transformar em um chocolate com sabor e aroma característicos (VUYST et al., 2010).

O processo fermentativo facilita a remoção da polpa em torno das sementes, já que esta inibiria a secagem das amêndoas, o que causaria aumento na umidade e multiplicação microbiana, que resulta em contaminação por bolores. Os componentes da polpa mucilaginosa são utilizados como nutrientes para microrganismos fermentativos (VUYST et al., 2010). Segundo GHEHI et al (2010), apenas a polpa mucilaginosa em torno da semente de cacau passa por fermentação microbiana. O principal motivo para fermentar o cacau é induzir transformações bioquímicas no interior das amêndoas que conduzem formação de cor, precursores de aroma e sabor de chocolate (THOMPSON, MILLER, LOPEZ, 2001).

A fermentação ocorre em duas etapas. A primeira fase é conhecida genericamente como anaeróbica. Na prática, apenas cria-se um ambiente de microaerofilia, já que os cochos de madeira são fechados com tampas de madeira, com folhas de bananeira ou sacos de ráfia. As perfurações na base dos cochos de fermentação ou os espaços existentes nas laterais e entre as ripas de madeiras nas tampas formam este ambiente de microaerofilia. Nesta fase há uma germinação incipiente, necessária para a hidratação das sementes por meio da absorção de água e mobilização de enzimas para preparar o crescimento do gérmen (plântula). Embora a fase seguinte da germinação seja indesejada para amêndoas de cacau destinadas à produção de chocolate, a atividade de enzimas endógenas é necessária para o desenvolvimento do sabor de chocolate. Uma vez que não há evidências de que as enzimas provenientes dos microrganismos fermentativos na polpa penetrem o cotilédone para formar compostos aromáticos, a fermentação adequada depende do calor, etanol e penetração de ácido acético nas amêndoas. Isto é necessário para inibir a germinação da semente, inativar o embrião (no segundo dia de fermentação) e ativar as enzimas hidrolíticas dentro das sementes para o desenvolvimento de aroma e coloração (De VUYST et al., 2010; DOMINGUES, 2010).

Durante as 24 a 48 horas desta primeira etapa ocorre a multiplicação de leveduras que convertem os açúcares da polpa (sacarose, glicose e frutose) em álcool (etanol) (DOMINGUES, 2010). O pH neste momento será baixo (aproximadamente 4,0) pelo conteúdo de ácido cítrico da polpa de cacau. A sacarose remanescente é convertida em glicose e frutose pela enzima invertase das leveduras. A reação de obtenção de etanol é exotérmica, o que provoca aumento de temperatura de 25 a 30ºC, e acordo com as condições ambientais, para 35 a 40ºC em um período de 48 horas. Após 24 horas do início da fermentação, sob pH baixo (4,0), temperatura em torno de 40ºC, elevada concentração de etanol e contínua liquefação da polpa, cria-se um ambiente favorável para bactérias lácticas (LAB), que se multiplicam rapidamente de 24 a 72horas após o início do processo. Este pico de crescimento das LAB coincide com o declínio das leveduras. Estas bactérias fermentam vários tipos de açúcares (especialmente glicose e frutose) e convertem a ácidos orgânicos, como ácido cítrico e ácido málico, em ácido lático (LAB homofermentativas). De acordo com a cepa de LAB (heterofermentativas) pode haver produção de ácido acético, etanol e dióxido de carbono (De VUYST et al., 2010; DOMINGUES, 2010). O etanol é oxidado a ácido acético pelas bactérias acéticas durante o processo fermentativo. Após as 48 horas, inicia-se a segunda fase, conhecida como aeróbica, em que são feitos revolvimentos para aumentar o contato com o oxigênio, o que torna o ambiente favorável para bactérias acéticas (AAB). Na prática, a ocorrência de AAB inicia-se no primeiro estágio da fermentação, em torno de 24 horas após seu início. A cada viragem, usualmente com intervalos de 24 horas, promove-se o contato com o ar e reduz-se a temperatura rapidamente. Neste momento, mais polpa é metabolizada e drenada, há aumento da aeração e quando a temperatura sobe para 37ºC, AAB termotolerantes multiplicam-se na polpa de cacau. A principal atividade das AAB é a oxidação do etanol, formado pelas leveduras, a ácido acético. Esta reação exotérmica é a principal responsável pelo aumento de temperatura, que alcança aproximadamente 50ºC ou mais. O declínio da concentração de etanol coincide com o declínio da concentração de ácido lático, que indica simultâneas oxidação do etanol a ácido acético pelas AAB e transformação de ácido lático em dióxido de carbono e água. O declínio de ácido acético ocorre pela evaporação em alta temperatura. A difusão do ácido acético pela abertura do cotilédone ou micrópilo e não por meio da penetração pela testa, é confirmada por DE BRITO et al (2000) e ROHSIUS et al (2006), quando menciona que substâncias adicionais, produzidas durante a

fermentação e absorvidas pelas sementes incluem ácido acético que, em grandes quantidades, pode ser prejudicial para a qualidade do produto de cacau.

Os precursores específicos de aroma de cacau são formados por processos enzimáticos que ocorrem durante a fermentação das sementes. Neste processo, sementes frescas passam por complexas transformações: os açucares da polpa mucilaginosa em torno das sementes são rapidamente metabolizados e produzem ácidos orgânicos voláteis e não voláteis; posteriormente ocorre a degradação de proteínas em peptídeos e aminoácidos livres; oxidação de polifenóis que formam compostos insolúveis, principalmente ortoquinonas e, hidrólise de glicosídeos, principalmente antocianinas (BONVEHÍ, 2005; ROHSIUS et al., 2007). Na fermentação aeróbica, os pigmentos marrons são formados a partir de polifenóis. Epicatequina e catequina são os polifenóis oxidados a ortoquinonas, e a condensação de proteínas e polifenóis resulta na redução da adstringência e sabor amargo (NIEMENAK et al., 2006).

A sucessão de microrganismos contribui para o desenvolvimento do aroma do cacau, e sua formação depende do grau e tempo de acidificação das amêndoas de cacau, que está relacionada com a liberação de aminoácidos hidrofóbicos específicos das amêndoas (BONVEHÍ, 2005; VUYST et al., 2010).

Durante a fermentação ocorrem alterações nas proteínas (do cotilédone) relacionadas ao aumento de temperatura e de acidez, fatores que causam a morte do embrião e a ruptura das paredes celulares, permitindo que as enzimas entrem em contato com seus respectivos substratos. A ação da endoproteinase aspártica e da carbopeptidase sobre as globulinas do tipo vicilinas origina oligopeptídeos e aminoácidos livres, compostos considerados precursores do aroma específico de cacau fermentado. Entre os peptídeos formados, foram identificados um nonapeptídeo e um hexapeptídeo, cuja sequencia análoga demonstra, em parte, que é formado a partir do nonapeptídeo durante o processo fermentativo (BERTORELLI et al., 2006; VOIGT; BIEHL,1993)

ROHSIUS et al. (2006) também afirmam que os precursores do aroma do chocolate são derivados durante o decorrer dos processos de fermentação e secagem. Estes autores inicialmente descreveram que durante a fermentação de cacau, ácido acético é produzido devido à degradação de açúcares da polpa por bactérias láticas e acéticas. O ácido acético penetra no cotilédone das sementes pelo micrópilo e reduz o pH de 6,4 para 4,5. Sob temperaturas elevadas (acima de 45ºC)

esta acidificação causa desintegração de compartimentos da célula. Isto também ativa a digestão proteolítica, em duas etapas, das maiores proteínas de reserva, resultando em uma mistura de aminoácidos e oligopeptídeos, representando precursores essenciais do aroma. O aroma final é diretamente influenciado pelo processo de acidificação dos cotilédones da amêndoa de cacau, que conduz a uma mudança na coloração, de ardósia a violeta, pela liberação de antocianinas dos vacúolos de armazenamento dos idioblastos contidos nos cotilédones. As antocianinas são vermelhas a um pH ácido e estão ligadas a componentes estruturais das sementes. Finalmente depois do contato com o oxigênio do ar, a coloração violeta transforma-se em marrom. Sementes de cacau retiradas de estágios da fermentação revelam estágios de cor de ardósia (não fermentado) à violeta (sub fermentado), e à marrom (completamente oxidado e fermentado). Os estágios de transição de ardósia a violeta e violeta a marrom revelam padrões reproduzíveis de distribuição de cor. Os autores verificaram que a transição de cor geralmente inicia-se a partir do micrópilo (pequeno orifício) por meio do eixo principal da semente para as regiões exteriores até as margens dos cotilédones. Isto não ocorre a partir de regiões externas da semente até as internas (centro). Desta forma, estes autores deduziram que a acidificação pela entrada do ácido acético não se inicia pela testa em volta do cotilédone como se acreditava anteriormente (difusão), mas inicia-se pela micrópilo. Estes autores propõem então um novo conceito de que a velocidade e a quantidade de troca de compostos solúveis são governadas pela abertura da testa.

O tempo de fermentação ideal varia de 6 a 8 dias, de acordo com o proposto por RODRIGUEZ-CAMPOS et al (2011), já que há formação de ácidos, ésteres e álcoois, que representam os grupos de compostos aromáticos voláteis mais importantes ao final da fermentação.

Durante o processo fermentativo, Bacillus spp, bactérias produtoras de ácidos graxos livres C3-C5, podem atuar de forma a causar off flavour no chocolate (RODRIGUEZ-CAMPOS et al., 2012).

3.3.1 Fermentação Natural

O método tradicional de colheita, quebra do fruto e fermentação natural das sementes de cacau promove manipulação excessiva e expõe o produto a contaminantes presentes na área de colheita, partido dos frutos, nas fibras da madeira

utilizadas na fabricação dos cochos de fermentação ou caixas, ou nos encerados em plástico e folhas de bananeira utilizados para forrar e cobrir as rumas de cacau, respectivamente. Este contato com a microbiota dos utensílios e ambiente foi evidenciado por GRIMALDI (1978) apud GUEHI et al (2010). Nos países em que há cultivo de cacau, os produtores utilizam, em sua grande maioria, o processo fermentativo em caixas de madeira (shallow boxes) ou em rumas (heaps), montes de cacau apoiados em folhas de bananeira ou em plástico. Existem ainda os sistemas que utilizam cestos, bandejas e, em alguns países, que cultivam o cacau Criollo, cujo tempo de fermentação é inferior ao Forastero e Trinitário, fermenta-se o cacau em sacos de juta.

No Brasil, a CEPLAC, Comissão Executiva do Plano de Lavoura Cacaueira, recomenda o uso de caixas de madeira, conhecidas como cochos de fermentação, com dimensões 0,90m a 1,20m de largura por 0,90m a 1,00m de altura e comprimento variável de 2,00 a 6,00m. As caixas são dotadas de paredes divisórias removíveis no sentido da maior dimensão para facilitar o revolvimento da massa em fermentação. O fundo deve conter orifícios com 0,6 a 1,0cm de diâmetro espaçados de 15 em 15 cm, para a drenagem dos líquidos liberados durante o processo e aeração da massa (DIAS, 1987; LOPES; GARCÍA, VASCONCELOS, 2003).

Usualmente o cultivo de cacau no Brasil segue o sistema cabruca de plantio das árvores dentro da região de mata nativa fechada, com sombreamento de cacau por árvores nativas, o que dificulta ou até impossibilita a quebra do fruto em área externa, uma vez que não possui fácil acesso. Desta forma, a abertura do cacau é feita de maneira adaptada, próximo à área de colheita, com facas e lonas sem higienização prévia, o que aumenta o contato do fruto, após a abertura, com a microbiota existente no local, proveniente de folhas secas no solo, da parte externa da casca do fruto, de troncos e folhas das árvores. A região de cultivo do cacaueiro abrange toda a faixa de 20º ao Norte e ao Sul da linha do Equador, caracterizada por clima quente e úmido. Estes fatores associados favorecem a fixação de fungos.

Com o intuito de permitir adequada higienização do fruto antes do partido em área afastada do cacaueiro, alguns produtores buscam sistemas de plantio mais ordenados para substituir o sistema de cabruca, citado por De VUYST et al. (2010) como cultivo à pleno sol (“f*ull sun cultivation*”). PEREIRA et al. (2013) propuseram a fermentação em cocho de inox como uma alternativa à fermentação em

superfície de madeira, para permitir adequada higienização e evitar a presença de microbiota indesejável.

3.3.2 Fermentação com Enzimas ou Extratos Enzimáticos

Recentemente, trabalhos foram publicados com propostas de controle durante o processo fermentativo com o intuito de obter melhorias nas características sensoriais do chocolate, reduzir o tempo de processamento ou adequar as condições de fermentação do cacau às exigências sanitárias vigentes. O controle do processo fermentativo pode ser feito por meio de inoculação de cepas microbianas, utilização de equipamentos, aplicação de enzimas purificadas ou coquetéis de extratos enzimáticos (BATISTA et al., 2016; CRAFACK et al., 2013, 2014; LEAL et al., 2008; MENEZES et al., 2016; SANDHYA et al., 2016; SCHWAN, 1998).

Enzimas são catalizadores de processos biológicos. De acordo com o primeiro relatório da Comissão de Enzimas (EC), formada pela *International Union of Purê and applied Chemistry* (IUPAC) em 1961, as enzimas são divididas em seis classes principais de acordo com o tipo de reação catalisada. São representadas por códigos numéricos, antecedidos pelo prefixo E.C, os quais contém quatro elementos separados por pontos, de acordo com os significados: o primeiro número indica a qual das 6 classes a enzima pertence; o segundo número representa a subclasse; o terceiro número indica a sub-subclasse; o quarto é um número serial da enzima em sua subclasse. As seis classes principais são: oxidoredutases (1), transferases (2), hidrolases (3), liases (4), isomerases (5) e ligases (6) (AEHLE, 2004).

Há várias enzimas, proveniente da microbiota natural do cacaueiro, que participam do processo de fermentação do cacau (Tabela 8).

Tabela 8 - Enzimas ativas durante a fermentação natural de sementes de cacau

Enzima	Localização	Substrato	Produto	pH	T (ºC)
Invertase	Semente	Sacarose	Glicose	4,0-5,25	37
	Testa		Frutose		
Glicosidades	Semente	Glicosídeos	Cianidina	3,8–4,4	45

β-galactosidase	Cotilédone	3-β-galactosidilcianidina 3-α-arabinosidilcianidina	Açúcares		
Proteinases	Semente Cotilédone	Proteínas	Peptídeos Aminoácidos	4,7	55
PFO	Semente Cotilédone	Polifenóis (-)-epicatequina	O-quinona O-diquinona	6,0	31,5-34,5

Fonte: EFRAIM, 2004.

Apesar da comprovação de intensa atividade enzimática proveniente da microbiota local, desde a etapa de colheita até a secagem (HANSEN, DEL OLMO, BURRI,1998; MISNAWI et al., 2002) vários autores abordam a possibilidade de aplicação de um tipo de enzima ou extrato enzimático durante a fermentação do cacau com o intuito de melhorar os atributos sensoriais das amêndoas e/ou reduzir o tempo de processamento (BINH et al., 2012 (a, b)). OLIVEIRA et al. (2011) aplicaram diferentes proteases e carboxipeptidases em sementes de cacau fermentadas e secas consideradas de baixa qualidade para a fabricação de chocolate, sob várias condições, com o intuito de melhorar a formação dos compostos aromáticos. Os resultados obtidos foram comparados àqueles referentes a amêndoas de cacau consideradas de boa qualidade. Os pesquisadores concluíram que é possível obter os aromas característicos de chocolate após a torrefação, em sementes de baixa qualidade, por meio de enzimas microbianas. SOARES (2001) utilizou a protease/peptidase Protezyn Flavour®, obtida de cepas selecionadas do fungo Aspergillus orizae, associada à polpa de pinha (Annona squamosa L.), fonte de polifenoloxidase. A adição de 5,0% do complexo após 96 horas de fermentação aumentou em 43,8% a quantidade de aminoácidos livres nas amêndoas, quando comparadas com a amostra sem o produto.

Há pesquisas em que a aplicação de enzimas foi realizada após o processo fermentativo, como a utilização de enzimas proteolíticas (Flavourzyme MG tipo A©) após o processo de fermentação, secagem e autoclavagem das amêndoas com o intuito de verificar a formação de precursores e avaliar seu efeito sobre aromas

em nibs de cacau (DE BRITO; GARCIA; AMANCIO, 2004). DIAS (1987) utilizou celulases imediatamente após a fermentação.

Apesar de vários trabalhos com utilização de enzimas em cacau, não foram relatadas tentativas de aplicação, em sementes frescas, de uma combinação de peptidases e carboidrases ou seus extratos com diferentes funções de acordo com a etapa de processo.

As hidrolases pertencentes à subclasse 2 (EC 3.2) são aquelas que hidrolisam ligações glicosídicas (C-O-R) ou com nitrogênio (N) ou enxofre (S) em substituição ao oxigênio (O) (COPELAND, 2000). Invertase, beta-glicosidases (celulases) e pectinases, são algumas das hidrolases que podem ser utilizadas na fermentação de cacau.

A enzima invertase ou β–D frutofuranosidase (EC 3.2.1.26) está naturalmente presente na polpa e no cotilédone do cacau (HANSEN, OLMO, BURRI (1998). O estudo da invertase da amêndoa de cacau é de extrema importância, já que sua atividade durante a fermentação resulta em açúcares redutores os quais, juntamente com proteínas e peptídeos, são os precursores das reações de Strecker, responsáveis pelo sabor e aroma de chocolate LOPEZ, LEHRIAN, LEHRIAN (1978). Frutose e glicose são os principais açúcares redutores em amêndoas de cacau, entretanto, açúcares redutores podem também ser formados pela hidrólise enzimática de antocianinas resultando em arabinose e galactose pela ação da glicosidase (MISNAWI, 2008). LOPEZ, LEHRIAN, LEHRIAN (1978) acreditavam que a enzima invertase estava presente apenas no tegumento. Entretanto outros pesquisadores a encontraram também na polpa e no cotilédone (HANSEN, OLMO, BURRI (1998). Uma tentativa de utilização de invertase foi feita, com o intuito de acelerar a fermentação das sementes de cacau ou melhorar os atributos sensoriais das amêndoas de cacau para a produção de chocolate. BINH, et al. (2012b) realizaram testes com a enzima invertase, fornecida pela empresa Novozymes, com o intuito de melhorar as características sensoriais das amêndoas de cacau fermentadas e secas. O objetivo do trabalho foi aumentar a quantidade de açúcares redutores, por meio da inversão da sacarose, para que estes fossem os precursores de aroma e sabor. A quantidade ideal de enzimas obtida nos testes foi de 60mg/Kg de sementes frescas adicionadas antes da fermentação, e resultou em melhores notas na prova de corte, menor acidez titulável e melhores notas para os atributos aroma de cacau e adstringência.

Beta-glicosidase (beta-D-glicosideoglicohidrolase, EC 3.2.1.21) é uma enzima largamente estudada devido a ampla variedade de substratos e ensaios enzimáticos simples (SINGHANIA et al 2013). As beta-glicosidases podem ser classificadas com base na especificidade do substrato em celobiases (alta especificidade para celobiose), aril-beta-glicosidases (alta especificidade para substratos como p-nitrophenil-beta-D-glucopiranosideo (pNPG)), ou beta-glicosidases de larga especificidade. A maioria encaixa-se na última categoria (SØRENSEN et al., 2013). Genericamente, atuam na clivagem da celobiose em glicose e na remoção da glicose de terminais não redutores de pequenas celodextrinas (CORREIA, 2010). As beta-glicosidases podem participar da hidrólise da celulose, em conjunto com outras duas enzimas. Segundo ZHANG, HIMMEL, MIELENZ (2006), o mecanismo largamente aceito para a hidrólise enzimática da celulose envolve ações sinergísticas da endoglucanase (EC 3.2.1.4), exoglucanase or celobiohidrolase (EC 3.2.1.91), e β-glucosidase (EC3.2.1.21). As endoglucanases hidrolizam as ligações glicosídicas intramoleculares mais acessíveis β-1,4 das cadeias de celulose aleatoriamente para produzir novas terminações de cadeia; em seguida, as exoglucanases clivam os terminais das cadeias de celulose para liberar celobiose solúvel ou glicose; e β-glucosidases hidroliza a celobiose para produzir glicose. Esses três processos hidroliticos ocorrem simultaneamente. Segundo PALMIERI; SPAGNA (2007) as beta-glicosidases podem ser extraídas de fungos filamentosos, leveduras e bactérias malolácticas. Há histórico da utilização de beta-glicosidases na produção de vinho, especialmente as glicosidases exógenas provenientes de fungos filamentosos e leveduras para desenvolvimento de aroma. Entre as glicosidases a beta-glicosidase é a mais estudada. Em sucos de frutas vermelhas e alguns vinhos, a beta-glicosidase pode induzir à perda de cor. Antocianinas, responsáveis pela cor das frutas vermelhas, são formadas pela adição de um mono ou dissacarídeo às antocianidinas. O principal açúcar residual das antocianinas é a glicose, e a descoloração é devida à quebra da ligação entre o açúcar residual e a antocianidina, que se degrada espontaneamente em compostos sem cor. Em sucos de uva e vinhos, considerando que as principais antocianinas são monoglicosídicas, a beta-glicosidase induz à perda de cor.

A classificação das enzimas pécticas está baseada no ataque ao esqueleto galacturônico, pela preferência de substrato (pectina, ácido péctico ou protopectina), ação por transeliminação ou hidrólise e por clivagem randômica (enzima endo-, liquidificante ou despolimerizante) ou terminal (enzima exo- ou

sacarificante). Existem basicamente três tipos de pectinases: pectina esterase remove os grupos metil éster; as despolimerizantes (incluem as enzimas hidrolíticas e as liases) catalisam a clivagem das ligações glicosídicas das substâncias pécticas e, as protopectinases que solubilizam protopectina para formar pectina. Estas enzimas foram classificadas e nomeadas de acordo com a “Enzyme Comission” (EC), segundo as recomendações da IUPAC-IUB. As enzimas despolimerizantes são classificadas de acordo com sua atuação sobre as ligações glicosídicas ou pelo mecanismo de ação e preferência por ácido péctico ou pectina como substrato: hidrolases quando catalisam a hidrólise de ligações α-1,4; liases catalisam a β-eliminação. São conhecidas como endo quando utilizam o mecanismo randômico ou aleatório, e como exo, quando atuam a partir do final da molécula. (UENOJO; PASTORE, 2007)

As poligalacturonases (PG) hidrolisam ligações glicosídicas α-1,4 entre dois resíduos de ácido galacturônico. Pode apresentar ação endo- (hidrólise randômica) ou exo- (hidrólise sequencial) do ácido péctico. As poligalacturonases fúngicas são úteis pela alta atividade enzimática e possuírem pH ótimo de atividade na região levemente ácida e temperatura ótima entre 30 e 50 °C. Durante o tratamento enzimático há extensa degradação da lamela média e da pectina das paredes celulares por ação de poligalacturonase, pectina metil esterase e pectina liase. (UENOJO; PASTORE, 2007). Segundo SCHWAN; WHEALS (2004), a atividade desta enzima é crucial para o processo fermentativo durante as 24 horas iniciais, uma vez que promove a liquefação da polpa e permite a penetração de oxigênio na massa de cacau em fermentação, além do crescimento de bactérias acéticas. No processo fermentativo natural, leveduras são os principais microrganismos responsáveis pela produção de enzimas pectinolíticas. Haverá a ruptura do cimento entre as paredes das células da polpa, e o suco resultante, conhecido popularmente como “mel de cacau”, é drenado (também chamado de “suor”).

Figura 3 - Classificação das pectinases

Pectinas	Protopectinases	protopectinases tipo A		
		protopectinases tipo B		
	Desmetoxilantes (pectinaestearase)			
	Despolimerizantes	hidrolases	polimetilgalacturonases (PMG)	
			poligalacturonases (PG)	Endo-
				Exo-
		liases	pectina liases (PMGL)	
			poligalaturonase liases (PGL)	

Fonte: Adaptado do texto de CORREIA (2010)

BINH, et al. (2012a) utilizaram a enzima Ultrazym® 100G, com atividade declarada de poligalacturonase pelo fabricante, para aumentar a velocidade de ruptura da pectina, presente em grande quantidade na polpa aderida à semente, reduzir o tempo da fase anaeróbica da fermentação e, consequentemente, reduzir os teores de etanol e ácido lático na amêndoa. Obtiveram maior percentual de amêndoas completamente fermentadas. Eles concluíram que o aumento da velocidade de liquefação da polpa e a redução do tempo da fase anaeróbica são formas de reduzir o teor de álcool e ácido lático nas amêndoas. A quantidade ideal de enzima por quilograma de sementes frescas foi de 80mg, adicionada no início do processo, para obtenção de maior número de amêndoas bem fermentadas. Com esta proporção, o aumento de temperatura foi maior e mais rápido quando comparado aos demais testes realizados, o que pode favorecer a evaporação do ácido acético e diminuir a acidez do produto final.

Uma enzima proteolítica hidrolisa um peptídeo ou proteína pela clivagem de ligações peptídicas (RAWLINGS, BARRETT, BATEMAN, 2012). O Comitê de Nomenclatura da União Internacional de Bioquímica e Biologia Molecular classifica as proteases (ou peptidases) em 4 subgrupos (hidrolases). Entretanto,

devido à sua enorme diversidade de ação e estrutura, atualmente são classificadas com base em 3 critérios principais: (i) tipo de reação catalisada, (ii) natureza química do sítio catalítico e (iii) relação evolucionária relativa à estrutura. Proteases são subdivididas, de modo simplificado, em dois grupos principais, exopeptidades e endopeptidases, dependendo de seu sítio de ação. As exopeptidases clivam ligações peptídicas próximas aos terminais amino ou carboxila do substrato, enquanto as endopeptidases clivam ligações peptídicas distantes dos terminais do substrato. Baseadas no grupo funcional presente no sítio ativo, as proteases são ainda classificadas em quatro grupos proeminentes: serino proteases, proteases aspárticas, cisteína proteases e metaloproteases (RAO et al., 1998).

A enzima papaína (EC 3.4.22.2) é uma protease classificada como uma cisteína endopeptidase, proteína de massa molecular entre 21 e 30kDa, que catalisa a hidrólise de peptídeos, amidas, ésteres, tióis ésteres e ligações tiono éster. (GRZONKA, KASPRZYKOWSKI, WICZK, 2007). É uma endopeptidase que hidrolisa ligações internas α-peptídicas em cadeias de polipeptídeos, distantes de terminais –N e –C (POLAINA, McCABE, 2007). A papaína é uma protease tradicional proveniente de plantas cujo histórico de utilização é longo. É extraída do látex de frutos Carica papaya, os quais são cultivados em áreas de clima subtropical. É ativada em pH 5 e 9 e mantém sua estabilidade até 80 – 90ºC, na presença de substratos. É utilizada largamente em escala industrial para a preparação de hidrolizados protéicos altamente solúveis e aromáticos (RAO et al., 1998). O látex bruto seco de mamão papaya contém uma mistura de, no mínimo, quatro cisteína proteases (papaína, quimopapaína, caricaína e glicil-endopeptidase) e outras enzimas (Baines and Brocklehurst, 1979 apud GRZONKA, KASPRZYKOWSKI, WICZK, 2007). É uma proteína relativamente básica, com ponto isoelétrico 8,75.

Portanto, o objetivo desta tese foi avaliar o potencial industrial dos clones de cacau utilizados para a produção de chocolate, além das variações em compostos voláteis e não-voláteis em todas as etapas do processamento, após a aplicação de uma mistura de extratos enzimáticos nas sementes frescas. Finalmente, avaliar a aceitabilidade dos chocolates obtidos em comparação a dois produtos comerciais.

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4 MATERIAL E MÉTODOS

Este item **4 MATERIAL E METODOS** foi contemplado com as três adaptações dos artigos científicos abaixo relacionadas e serão apresentados nesta Tese no item **5 RESULTADOS E DISCUSSÃO.**

5.1 ADAPTAÇÃO DO ARTIGO CIENTÍFICO 1

Industrial potential of cocoa (*Theobroma cacao L.*) varieties from Espírito Santo, based on the chemical composition of the cotyledons. Será submetido para publicação – **Journal of Food Science.**

5.2 ADAPTAÇÃO DO ARTIGO CIENTÍFICO 2

Trinitario and Forastero cocoa (*Theobroma cacao L.*) fermentations with enzymatic extracts: assessment of chemical transformations from bean to bar. Submetido para publicação - **Food Research International.**

5.3 ADAPTAÇÃO DO ARTIGO CIENTÍFICO 3

Electronic nose assessment as industrial quality control of liquor and dark chocolate obtained from cocoa (*Theobroma cacao L.*) beans fermented with multi-enzymatic extracts. Submetido para publicação – **Journal of Food Engineering.**

5 RESULTADOS E DISCUSSÃO

Este item **5 RESULTADOS E DISCUSSÃO** foi contemplado com as adaptações dos três artigos científicos supracitados.

5.1 ADAPTAÇÃO DO ARTIGO CIENTÍFICO 1

Industrial potential of cocoa (*Theobroma cacao L.*) varieties from Espírito Santo - BRAZIL, based on the chemical composition of the cotyledons

Abstract

Genotype is related to differences in chemical composition of cocoa (*Theobroma cacao L.*) beans, which play a role in the chocolate final flavor quality and intensity, and in the profile of procyanidins of cocoa extracts. The objective of this work was to evaluate and compare the chemical composition in fresh beans of eight Trinitario cocoa clones and one Forastero variety, grown in Espírito Santo - Brazil, and discuss their role for(industrial production of chocolate and polyphenolic extracts. Sugars and alcohols were quantified by HPLC-RI, non-volatile acids by HPLC-UV, and methylxantines and polyphenols, by UFLC-Q-TOF-MS. Sucrose, glucose, glycerol and the acids oxalic, citric and malic, could be used to classify and cluster the cocoa clones and varieties according to similarieties. PH16 and Ipiranga Trinitario clones were different from the other samples. Theobromine was the most abundant methylxantines and epicatechin had the highest content in cocoa extracts among the procyanidins.

Key words: chocolate, extract, polyphenolic, methylxantines, non-volatiles, procyanidins

1. Introduction

Cocoa (*Theobroma cacao L.*) beans are the essential ingredient for dark and milk chocolate production. Although it is well established in the literature that fermentation

is the key process for developing flavor precursors, other factors contribute to the final flavor quality and intensity, such as genotype , which is related to differences in the chemical compositions of the beans (Afoakwa et al., 2008; Aprotosoaie et al., 2016). During the fermentation process, as the pulp is liquefied and drained, the most important transformations occur inside the cotyledons.

The “witches’ broom” disease, caused by the fungus *Moniliophthora perniciosa*, affected Brazilian cocoa crops in the early 90’s and local production was considerably reduced. Local farmers invested in disease-resistant and more productive Trinitario cocoa clones, which were grown mixed in the same areas, mostly using the cabruca crop system, shaded with native species, in an attempt to recover the cocoa production (Menezes et al., 2016a). The cocoa beans with different genetic backgrounds have different characteristics, such as pulp content, size and color (Lopes & Pires, 2015). As a result, the standardization of chocolate and cocoa products has been a challenge for the food industry as the batches for fermentation are composed by blends of mature cocoa clones or variety available during the harvest, which may be different in every season. Lopes & Pires (2015) suggest the use of monoclonal plots in farms and fermentations using specific clones to reduce variability. Choosing the best clones or variety for chocolate production, based on the fruit yield and without evaluating the chemical composition of the beans, may affect the quality of the final product.

Production of chocolate from specific cocoa clones and varieties, in order to investigate differences in the final flavor, via natural fermentation or with the addition of starter cultures or enzymes have been reported, indicating that there are significant differences in the final flavor of the chocolate (Bacelar Leite, Silva Bispo, & Regina Radomille Santana, 2013; Menezes et al., 2016a; Moreau Cruz, Bacelar Leite, Eduardo Soares, & da Silva Bispo, 2013).

Among the essential compounds for the final flavor, there are sugars (sucrose, fructose and glucose), non-volatile acids (citric, oxalic, acetic, malic, succinic and acetic), and alcohols (ethanol, methanol and glycerol). Those compounds will undergo transformations during the cocoa processing for chocolate production, but their initial concentration might be an indicator for producers to decide which clones will lead to a higher chocolate quality.

Procyanidins (catechin, epicatechin, procianidin B1, procianidin B2) and methylxantines (theobromine, theophylline and caffeine) have been reported as contributors to astringency and bitterness in dark chocolates, and a lower content of catechins was associated to mild flavor in Criollo chocolate (Elwers et al., 2009). Although higher polyphenols contents may not result in the best flavor profile in chocolate, the health benefits provided by those compounds have risen interest in the production of rich phenol extracts to be used as an ingredient in the food industry (Ortega et al., 2008, 2010; Schinella et al., 2010).

The aim of this work was to evaluate and compare the chemical composition in fresh cotyledons of eight Trinitario cocoa clones and one Forastero variety, grown in Espírito Santo - Brazil, in terms of sugars, non-volatile acids, alcohols, methylxantines and polyphenolic compounds for industrial production of chocolate and polyphenolic extracts.

2. Materials and Methods

2.1 Chemicals and standards

Standards of organic non-volatile acids (oxalic, citric, tartatic, malic, succinic, lactic, acetic, propionic, isobutyric, butyric and isovaleric), sucrose, glucose, fructose,

glycerol, methanol, ethanol, theobromine, caffeine, quercetin, (+)-catechin, (-)-epicatechin, procyanidin B1, procyanidin B2 and methanol MS grade were provided by Sigma Aldrich. Theophylline (anhydrous, 99+%) was obtained from Acrós Organics.

Ultrapure water was obtained from a MilliQ water purification system (Millipore Corp., Bedford, MA, USA).

2.2 Processing

Mature and healthy Forastero and Trinitario cocoa pods were harvested on Ceará farm, in Linhares (Espirito Santo, Brazil). The Trinitario hybrids were Cepec 2002, TSH1188, VB900, PS1319, PH16, SJ02, Ipiranga and CCN51, and the Forastero variety was Pará. Five units of each type from an industrial batch of over 1ton were separated and the fruits were transferred to a laboratory inside insulated boxes. Liquid nitrogen was applied when the pods were opened, 48h after the harvesting. The frozen seeds were stored and subsequently, transported for analysis.

2.3 Sugars, non-volatile acids and alcohols

Extraction and analysis were in duplicates according to Moreira et al. (2013). Ten grams of cotyledons were extracted twice with 10 mL of Milli-Q water, followed by 5 min vortexing, resulting in 20mL homogenate, then transferred to another tube and centrifuged at 7000 rpm, 10 min, 4 °C. The supernatant was separated, and the precipitate was suspended in 5 mL of Milli-Q water, vortexed and centrifuged as before, resulting in final 25mL of cotyledon extract, centrifuged as described. An aliquot of 2mL

of the final supernatant was filtered through a 0.22 µm Millipore membrane for organic non-volatile acids, alcohols and sugars HPLC analysis.

A LC-10Ai Shimadzu chromatography system coupled with an UV–Vis detector (SPD 10Ai) and a refractive index detector (RID-10Ai) was used. Percloric acid 100 (mM) was the mobile phase for elution at 0.6 mL/min flow rate in a SCR-101H Shim-pack ion exclusion column (7.9 mm × 30 cm). Non-volatile acids were separated at 50°C and detected by UV detector at 210nm, whilst alcohols and sugars, at 30°C by a RI detector. Compounds were identified by external standards, retention time and the quantification, by analytical curves, were in g of compound per kg of cotyledons in both, wet weight and dry basis.

2.4 Phenolic Extraction and UFLC-Q-TOF-MS method

Compounds extraction was carried out according to Ortega *et al.* (2010), with minor modifications. The frozen samples were ground to a fine powder in liquid nitrogen to obtain 15g of the homogeneous material, which were Portions of 3g were taken to be defatted four times with proportionally 25mL of hexane, in an orbital shaker at 200rpm and centrifuged for 15min at 15.557 x g (Eppendorf, Germany) and dried with nitrogen gas. Subsequently, the defatted samples were extracted four times with the solvent mixture acetone/MilliQ water/acetic acid (70/29.5/0.5, v/v/v), using the proportion 1:5. After each extraction, the material was vortexed for 3 min and centrifuged. The supernatants obtained were combined, filtered through glass wool to remove residual particles, concentrated in a rotary evaporator at 30°C under partial vacuum to remove the organic solvent and freeze-dried to obtain the phenolic extract. A final stock solution

(1mg/mL) of each lyophilized phenolic extract was obtained after filtration through 0.45 and 0.22μm nylon filters.

A Shimadzu (Shimadzu, Kyoto, Japan) UFLC 20AD Prominence system, with a silica column Phenomenex Kinetex EVO C18 (100 x 2,1 mm, 5μm) at 30°C, was coupled to a quadrupole time of flight detector (ESI-Q-TOF-MS) mass spectrometer (model Compact, Bruker, Bremen, Germany).

The mobile phase was composed by eluent A water/ formic acid (99.9/0.1, v/v) and eluent B was methanol MS grade, at a flow rate of 0.20mL/min. The elution gradient in the positive-ion mode was 0–0.10 min, 85–15% B; 0.10–7 min, 70–30% B; 7–7.10 min, 20–80% B; 7.10–8 min, 80% B isocratic; 8– 12 min, 75–15% B; 12–13 min, 15% B isocratic. The elution gradient in the negative-ion mode was 0–0.10 min, 85–15% B; 0.10–3 min, 70–30% B; 3–5 min, 50–50% B; 5–7.10 min, 20–80% B; 7.10–8 min, 80% B isocratic; 8– 12 min, 85–15% B; 12–13 min, 15% B isocratic. The injected volume was 2.0 μl.

MS operated in positive-ion mode for theobromine, theophylline and caffeine and in negative-ion mode for (+)-catechin, (-)-epicatechin, quercetin, procyanidin B1 and procyanidin B2. The ESI source conditions were capillary voltage at 4500V; end plate voltage at 500V; flow rate at 0.20mL/min, nitrogen as the nebulizer at 3.0 bar and drying gas at 9.0 L/min and temperature 200°C. The monitored mass range was 50 to 2000 m/z.

Analytical curves of the standards catequin, epicatequin, procyanidin B1, procyanidin B2, quercetin, theobromine, theophylline and caffeine were used for the correspondent quantifications in the samples, expressed as mg or μg per gram of phenolic extract.

2.5 pH measurement and dry matter determination

The pH of the beans was determined according to AOAC (2012) method 970.21, and the loss on drying, by the AOAC (2012) method 931.04.

2.6 Statistical Analysis

Statistica 12 Ultimate Academic Bundle software (Dell Inc., USA) and XLSTAT Statistical Analysis system (2015) were used for Hierarchical Cluster Analysis (HCA) and Principal Component Analysis (PCA). Significant differences on the compounds quantifications among clones / varieties were assessed by one-way analysis of variance (ANOVA), followed by Tukey test at a significance level of 0.05 ($p < 0.05$).

3. Results and Discussion

3.1 Sugars and Alcohols

Sucrose, glucose, fructose, ethanol and glycerol were quantified in the eight Trinitario clones and one Forastero variety – Pará (Table 1), and the comparisons were described in wet weight to compare with literature (Ho et al., 2014).

Sucrose was more abundant in clones PH16 (15.3mg/g), Ipiranga (14.26mg/g) and Pará (14.40mg/g), and the lowest amount was found in SJ02 beans (6.7mg/g), compared to the other clones and to the average quantification (10.5mg/g). The average amount of fructose was 7mg/g and the highest content was observed in TSH1188 (10.1mg/g) and VB900 (9.90mg/g), while and the lowest in Pará (4.18mg/g) and Ipiranga (4.45mg/g). Among the sugars, glucose had the lowest average content

(6.1mg/g), TSH1188 had the highest amount (8.3mg/g) and 0,8mg/g was the lowest, found in CCN51.

Ho et al. (2014) evaluated unfermented Trinitario beans from Australia and found an initial sucrose content of 17mg/g, 0.5mg/g of glucose and 1mg/g of fructose. Similar amounts of sucrose were also mentioned by Afoakwa et al. (2008), reviewing other authors (15.8mg/g).

Ramos et al. (2014) evaluated the beans of PH16 and PS1319 clones harvested in Bahia, Brazil, exposed for 4h prior to fermentation. They obtained ~3.5mg/g and 5mg/g of glucose and fructose, respectively, for PH16 and ~1 mg/g and 1.2 mg/g for the same compounds in PS1319. Although the same genotypes were evaluated, differences in quantifications may be explained by the geographic origin, exposure of the beans after opening the pods and transportation conditions.

It is essential to evaluate the sugars content in raw cocoa nibs, as sucrose is expected to be hydrolyzed into glucose and fructose throughout the fermentation process, both reducing sugars involved in Maillard reactions during roasting and conching for chocolate flavor development (Ho, Zhao, & Fleet, 2014). Invertase from the testa of the bean is responsible by the sucrose hydrolysis into fructose and glucose that, together with citric acid from the pulp, are transformed by micro-organisms into lactic acid, ethanol and then oxidized acetic acid (Afoakwa et al., 2008; DE Vuyst et al., 2010; Kyi et al., 2005). Ascrizzi et al.(2017) reported that most of the pyrazines are exclusively formed during roasting process through Maillard reactions, except trimethylpyrazine and tetramethylpyrazine, that might be formed during cocoa fermentation by bacteria enzymes. Varieties can be classified according to quantitative differences in flavor precursors, instead of the qualitative aspect (Afoakwa, 2010; Reineccius, 2006). Zambrano et al. (2010) evaluated the flavor and aroma precursors

in cocoa during fermentations and concluded that reducing sugars' content can be considered as a criterion to evaluate the cocoa fermentation process.

Glycerol was found in four Trinitario clones and Pará. Ipiranga had the lowest content (0.08mg/g), and the highest was quantified in VB900 (0.56mg/g). This compound was not detected in unfermented nibs, and according to Ho et al. (2014), glycerol, a secondary product of yeast metabolism of sugars, contributes to sensory properties of mouth-feel and sweet taste. Ethanol was quantified in the same samples at low concentrations that ranged from 0.3mg/g to 0.98mg/g. Methanol was not detected in the samples.

3.2 Non-volatile acids

The quantification of non-volatile acids in cocoa beans are displayed in Table 2. The most abundant was citric acid, with an average of 9.9mg/g, the highest content was found in PS1319 (16mg/g), while VB900 had the lowest and PH16 (4.5mg/g). Ho et al. (2014) reported that citric acid was the most important acid in unfermented cocoa nibs (16 – 17mg/g). The content of citric acid found in Pará (9.2mg/g), the only sample of Forastero variety, was lower than the average (9.9mg/g), and five Trinitario clones higher than 10mg/g. Ramos et al. (2014) evaluated samples of PH16 and PS1319 and found 2mg/g and 1.1g/kg, respectively. Although this is the most abundant acid in both, cotyledons and pulp, it is usually described in as part of essential transformations in the former and its degradation inside the beans remain unclear (Ho et al., 2014).

The average content of oxalic acid in the samples was 7.8mg/g, the highest found in PS1319 (11.8mg/g) and the lowest in PH16 and VB900 (4.7mg/g). Ho et al. (2014) found 7-8mg/g of oxalic acid in unfermented beans, without significant changes in its content during fermentation. Although oxalic acid may have a positive impact on chocolate taste, as reported by Holm at al., (1993), it is an antinutrient, which may cause nephrolithiasis, as an insoluble salt, calcium oxalate, if it is supersaturated in the urine, or reduce the absorption of calcium, magnesium and iron. In addition to it, chocolate is considered a high oxalate food (Schroder et al., 2011). Although Ho et al. (2014) reported a slightly reduction in its content during fermentation process, some control is still required to the final product. Also, an industrial cocoa polyphenolic extract obtained from raw beans without the separation of the acidic fraction, should consider this anti nutritional component.

Malic acid was found in eight samples, with an average of 2.10 mg/g, the lowest amount was quantified in TSH1188 and VB900 (1.45mg/g), and the highest, in PS1319 (3.6mg/g). Malic acid was not found in Ipiranga. Ho et al. (2014) found 1-2mg/g of malic acid in fresh beans and the variation in its concentration during fermentations was not significantly.

Acetic acid was found in all the samples and the average amount was 3.6mg/g. The highest content was in CEPEC 2002 (5.8mg/g) and Pará (5.4mg/g), and the lowest in TS1188 (1.6mg/g) and SJ02 (1.8mg/g). Ramos et al. (2014) reported lower amounts of acetic acid (0,1 – 0,2mg/g) in unfermented beans of clones PH16 and PS1319, while Ho et al. (2014) reported the first quantification after 48h of fermentation in higher amounts (12.5mg/g), reaching reached its maximum after 96h (16 – 20mg/g). Acetic and lactic acid were considered by Holm et al. (1993) as responsible for acid flavor in Asian and Pacific cocoa.

Schwan & Wheals (2004) reported that acetic and lactic acid from the pulp diffuse and dominate the cocoa cotyledons acidity, depending on the oxygen and substrate (pulp) availability for their production by bacteria during the fermentation. De Vuyst et al. (2010) described the cocoa bean testa or tegument as impermeable to large molecules such as citric acid, polyphenols and alkaloids, but permeable to small volatile ones like acetic acid and ethanol, able to penetrate the cotyledons. Despite the testa permeability characteristics, according to Rohsius et al. (2006), during the fermentation, acetic acid infiltrates the cotyledons by the micropyle, lowering the pH, from 6.5 to 4.5, and causing disintegration of the cells compartments at higher temperatures (45ºC), activating the digestion of storage proteins, which releases oligopeptides and amino acids, known as aroma precursors. During drying, most of the acetic acid stays in the products, and part of the lactic acid might be lost (Schwan & Wheals, 2004).

Succinic acid was quantified in all the samples, with an average of 2.7mg/g, the highest in CEPEC 2002 (5.8mg/g) and the lowest in CCN51(1mg/g). Ho et al. (2014) found 13-14mg/g of succinic acid in unfermented beans and reported a slightly increase in its content at the end of the fermentation. Although succinic acid production by yeasts activity in the pulp was previously described by Schwan & Wheals (2004), its dynamics and role inside the beans during the fermentation is scarcely discussed. Propionic and isobutyric acids were found in two samples. The former was found in Pará and PH16 (0.03mmg/g), and the latter was found in VB900 and PH16 (3.4mg/g). Both acids, together with isovaleric acid, were reported by Rodriguez-Campos et al. (2011) as off-flavor indicators, found during fermentation, and disappearing in the beginning of drying. Propionic acid was characterized as pungent and rancid, isovaleric as sweat, acid and rancid, and isobutyric as rancid, butter and cheese profile. When

the fermentation process is extended, isobutyric and isovaleric acids are formed by enzymatic reactions, resulting in hammy flavor Rodriguez-Campos et al. (2011).

Lactic, tartaric, butyric and isovaleric non-volatile acids were not found in the fresh beans. Similar results for lactic acid were reported by Ramos et al. (2014). Schwan & Wheals (2004) described the acids citric, oxalic, malic, tartaric and phosphoric as endogenous and not important to the fermentation, as they are not essential to the bean acidity. Lactic and butyric may be produced during the fermentation by natural microbiota in spontaneous processes (Rodriguez-Campos et al., 2011; Schwan & Wheals, 2004). Transformations in the content of non-volatile acids such as oxalic, citric, tartaric, succinic and lactic, are not significant during roasting (Afoakwa et al., 2008).

3.3 Comparisons among clones and variety

Hierarquical Cluster Analysis (HCA) and Principal Component Analysis (PCA) were carried out to group the Trinitario clones and Pará Forastero variety according to similarities and based on the cotyledon's chemical composition in dry basis (Fig1 and Fig 2).

PCA scores displays 82% of total variance using 3 components. The active variables were sucrose, glucose, glycerol, and the acids oxalic, citric, malic and succinic. Fructose, ethanol, acetic acid and propionic acid were set as supplementary variables. PC1, mostly influenced by citric (29%) and oxalic acid (23%), absorbed 42% of the total variance, followed by PC2 (26%) and PC3 (14%), The former was mostly

influenced by glycerol (38%) and glucose (27%), and the latter, mostly by malic acid (35%) and glucose (29%).

Trinitario clones CCN51 and PS1319 were grouped in the same cluster, both characterized by higher citric and oxalic acids content, positioned to the positive side of the quadrant. Although Ipiranga presents a high content of both citric and oxalic acid, its high sucrose content differs this clone from the other samples.

Pará Forastero variety was clustered to CEPEC2002 and SJ02 clones, due to the higher succinic and malic acid content. VB900 and TSH1188 clones have similar sugar profile, mainly sucrose and glucose content, both higher than the average. Although they have the same average amount of malic acid, this compound did not contribute significantly to the components.

PH16, commonly used for monoclonal chocolate production (Bacelar Leite et al., 2013) , differs from the other samples due to its high sucrose, fructose and propionic acid content, and glucose higher than the average. Those sugars from the cotyledons are flavor precursors that might be transformed into desired flavor compounds during fermentation, roasting and conching, which demonstrates a high potential for a good quality chocolate.

3.4 Methylxantines and Procyanidins in Cocoa Extracts

Methylxantines and procyanidins in the cocoa extracts obtained from the eight clones and from Pará Forastero variety are displayed in Figure 3, expressed as mg/g of dry weight.

The highest content of theobromine was found in VB900 (119.03mg/g) and Pará (119.2mg/g), and the lowest in Ipiranga (43.07mg/g). Caffeine was more abundant in

VB900 (5.55mg/g) and TSH1188 (5.12mg/g) while, it lower contents were quantified in Ipiranga (0.85mg/g) and CCN51 (1.84mg/g). Ortega et al (2008) evaluated similar extracts obtained from differ cocoa sources. Although all the samples were submitted to heating processes, the closest to our samples are the extracts from cocoa beans, described as fermented and roasted, which contained 43.43mg/g of theobromine and 4.5mg/g of caffeine. Later, in another study, Ortega et al (2010), evaluated the same compounds in cocoa nibs extracts and found 19.41mg/g and 0,09mg/g of theobromine and caffeine, respectively. The results show great disparities among similar samples from the same location, Forastero cocoa from West Africa, obtained in different seasons. Different varieties and clones of cocoa beans from a non-traditional production location, Espirito Santo were evaluated, and, thus, presented great variability. Camu et al (2008) reported decrease in the theobromine content during 144h of fermentation, around 20% of the initial content (0.84 – 1.33mg/g)

Catechin was the least abundant procyanidin in raw cocoa extracts. The highest content was found in CCN51 (1.86mg/g), and the lowest, in Pará (0.10mg/g). Analogous extracts from cocoa nibs were obtained by Ortega et al (2010), containing ~1.90mg/g of catechin. Although we evaluated extracts from fresh samples, differences in the quantities are possible due to natural variations. Ortega et al (2008) evaluated similar extracts obtained from different cocoa sources after fermentation and found higher quantities in the nibs (~2.4mg/g), which undergo a more intense heating process (roasting) than the dried beans (~0.3mg/g). Hurst et al. (2010) reported that catechin was not detected in unripe or ripe beans and, can be formed by heat-induced epimerization during medium level roastings, which could explain that catechin levels in processed products can be higher than in raw cocoa. Therefore, a catechin richer extract could be obtained from slightly roasted cocoa beans instead of the raw seeds.

Schinella et al (2010) obtained industrially processed cocoa extracts, using three different methods and evaluated the correspondent procyanidins in wet basis. The flash steamed extract (sample A), presented similar catechin content (4.87mg/g) to our findings (total average 1.09mg/g), when compared to other authors. Despite the average similarity, it is important to emphasize the variability among the samples, as CEPEC 2002 (0.19mg/g), PARÁ(0.10mg/g) and Ipiranga (0.64mg/g), presented much lower content than the other clones.

Epicatechin was the procyanidin with the greatest variability among the clones and Forastero variety and it is possible to separate the sample in two very distinct groups: epicatechin content higher than 35mg/g, and lower than 1mg/g. TSH1188 had the highest content (52.43mg/g) and PH16, the lowest (0.14mg/g). High contents were also found in CEPEC 2002 (36.63mg/g), VB900 (28.3mg/g), Pará (46.19mg/g) and Ipiranga (39.45mg/g). Ortega et al (2008) evaluated epicatechin content in fermented and roasted cocoa beans, and found an average 3.011mg/g of this compound in a similar extract (CE), which is around 10 times lower than our findings for some our cocoa clones and 20 times higher than others. Ortega et al (2010) found ~ 4mg/g of the same compound in cocoa beans. Schinella et al. (2010) found similar results to some of our samples (TSH1188, Pará, Ipiranga and CEPEC2002) in the dried enriched extract (sample B), ~62mg/g.

Hurst et al. (2010) described loss in epicatechin content due to mild heating treatments, while increase of catechin quantities. Camu et al. (2008) reported that around 70% of the initial epicatechin content was lost after 144h of spontaneous cocoa bean heap fermentation, due to diffusion out of the cotyledons. Schwan and Wheals (2004) described that apart from diffusion, cocoa polyphenols content can also be

reduced by enzymatic oxidation (polyphenol oxidases), which produces insoluble tannins, followed by drying and roasting.

Procyanidin B2 had the highest amount in CCN51 (35.64mg/g) and was less abundant in Ipiranga (16.97mg/g). Similar results were found by Schinella et al (2010) in dried extract B (~ 21 mg/g) and extract A (~ 10mg/g). Dimer B2 was quantified by Ortega et al (2008) and Ortega et al (2008), in beans (8.21mg/g) and nibs (8.51mg /g), respectively.

Procyanidin B1 was not found in our fresh samples, but we found during further processed samples obtained from the same clones or variety (unpublished data).

4. Conclusion

Sugars, non-volatile acids and alcohols content are helpful information for choosing the best cocoa clones or variety based on the potential flavor profile to be developed in fermented and dried cocoa beans for chocolate production.

An ideal mixture of clones and varieties for chocolate production that fulfills the flavor quality and productivity requirements of the industry may be optimized, combining high sugar content clones or variety with a desired balance of acids, that will provide enough precursors during fermentation and drying for flavor formation.

Cocoa is a rich source of phenolic compounds, mainly epicatechin and procyanidin B2, as well as theobromine, and a great variety of applications can be developed based on the composition of each clone or variety extract.

The great variability in the quantities of epicatechin among the clones and variety studied, reinforces the importance of a previous characterization and evaluation before processing.

Cocoa clones or varieties with intermediate potential for cocoa fermentation and chocolate production, meaning lower sugars and higher acidic content, can be used alternatively for industrial cocoa extracts.

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Tables:

Table 1 – Quantification of sugars and alcohols in fresh cocoa beans, expressed in miligrams of compound per gram of cotyledons (wet weight).

CLONES / VARIETIES	SUCROSE	FRUCTOSE	GLUCOSE	ETHANOL	GLYCEROL
CEPEC 2002	$8.02^{c,d*} \pm 0.17$	$6.33^{c} \pm 0.13$	$6.83^{b,c} \pm 0.13$	$0.98^{a} \pm 0.02$	$0.31^{c} \pm 0.01$
TSH1188	$9.60^{b} \pm 0.16$	$10.15^{a} \pm 0.02$	$8.34^{a} \pm 0.05$	$0.96^{a} \pm 0.01$	$0.32^{c} \pm 0.00$
VB900	$9.64^{b} \pm 0.12$	$9.90^{a} \pm 0.04$	$8.12^{a} \pm 0.07$	$0.54^{b} \pm 0.01$	$0.56^{a} \pm 0.01$
PARÁ	$14.40^{a} \pm 0.18$	$4.18^{d} \pm 0.10$	$6.55^{b,c,d} \pm 0.12$	$0.29^{c} \pm 0.01$	$0.50^{b} \pm 0.00$
IPIRANGA	$14.26^{a} \pm 0.19$	$4.45^{d} \pm 0.03$	$7.13^{b} \pm 0.03$	$0.30^{c} \pm 0.00$	$0.08^{d} \pm 0.00$
PH16	$15.31^{a} \pm 0.18$	$8.23^{b} \pm 0.10$	$6.30^{c,d} \pm 0.05$	0.00^{d}	0.00^{e}
SJ02	$6.76^{d} \pm 0.02$	$6.86^{c} \pm 0.02$	$5.93^{d} \pm 0.10$	0.00^{d}	0.00^{e}
CCN51	$8.80^{b,c} \pm 0.12$	$6.53^{c} \pm 0.13$	$0.84^{f} \pm 0.02$	0.00^{d}	0.00^{e}
PS1319	$7.95^{c,d} \pm 0.16$	$6.39^{c} \pm 0.06$	$4.85^{e} \pm 0.04$	0.00^{d}	0.00^{e}

Mean values with the same letter in each column are not significantly different ($p>0.05$)

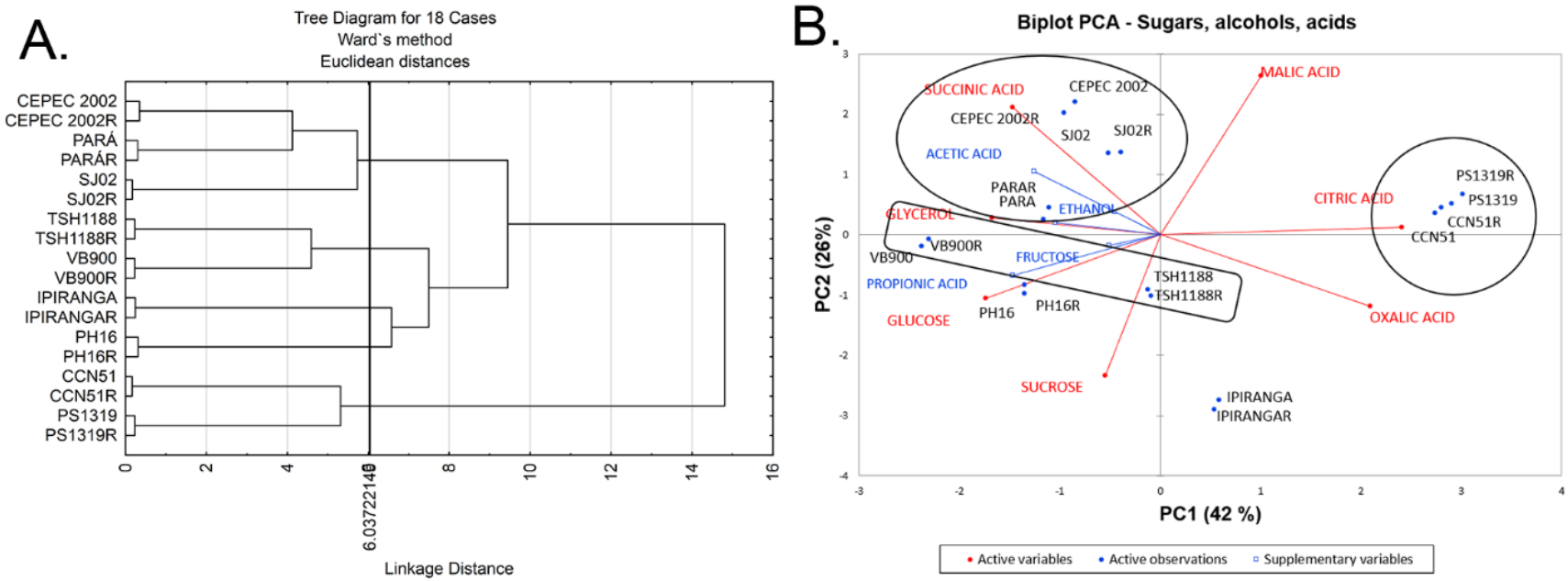
Table 2 – Quantification of non-volatile acids in fresh cocoa beans, expressed in miligrams of compound per gram of cotyledons (wet weight).

CLONES / VARIETIES	OXALIC ACID	CITRIC ACID	MALIC ACID	SUCCINIC ACID	ACETIC ACID	PROPIONIC ACID	ISOBUTYRIC ACID
CEPEC 2002	$6.27^{e} \pm 0.00$	$10.30^{d} \pm 0.09$	$3.29^{b} \pm 0.02$	$5.48^{a} \pm 0.07$	$5.84^{a} \pm 0.04$	0.00^{c}	0.00^{c}
TSH1188	$9.45^{c} \pm 0.19$	$10.46^{d} \pm 0.06$	$1.45^{g} \pm 0.01$	$1.50^{d} \pm 0.02$	$1.63^{d} \pm 0.01$	0.00^{c}	0.00^{c}
VB900	$4.71^{g} \pm 0.11$	$4.48^{g} \pm 0.08$	$1.45^{g} \pm 0.01$	$2.38^{c} \pm 0.03$	$5.79^{a} \pm 0.08$	$3.38^{b} \pm 0.0$	0.00^{c}
PARÁ	$7.35^{d} \pm 0.08$	$9.20^{e} \pm 0.09$	$2.73^{c} \pm 0.01$	$4.43^{b} \pm 0.05$	$5.44^{a} \pm 0.14$	0.00^{c}	$0.03^{b} \pm 0.0$
IPIRANGA	$11.16^{b} \pm 0.12$	$11.92^{c} \pm 0.08$	0.00^{h}	$1.33^{d,e} \pm 0.01$	$2.47^{c} \pm 0.02$	0.00^{c}	0.00^{c}
PH16	$4.70^{g} \pm 0.13$	$4.52^{g} \pm 0.09$	$1.63^{f} \pm 0.01$	$2.75^{c} \pm 0.08$	$3.88^{b} \pm 0.04$	$3.42^{a} \pm 0.0$	$0.03^{a} \pm 0.0$
SJ02	$5.22^{f} \pm 0.07$	$8.12^{f} \pm 0.04$	$2.22^{e} \pm 0.01$	$4.15^{b} \pm 0.03$	$1.81^{d} \pm 0.02$	0.00^{c}	0.00^{c}
CCN51	$9.47^{c} \pm 0.08$	$13.86^{b} \pm 0.07$	$2.58^{d} \pm 0.01$	$1.02^{e} \pm 0.01$	$1.91^{c,d} \pm 0.02$	0.00^{c}	0.00^{c}
PS1319	$11.76^{a} \pm 0.11$	$16.28^{a} \pm 0.05$	$3.58^{a} \pm 0.01$	$1.41^{d,e} \pm 0.01$	$3.57^{b} \pm 0.04$	0.00^{c}	0.00^{c}

*Mean values with the same letter in each column are not significantly different ($p<0.05$)

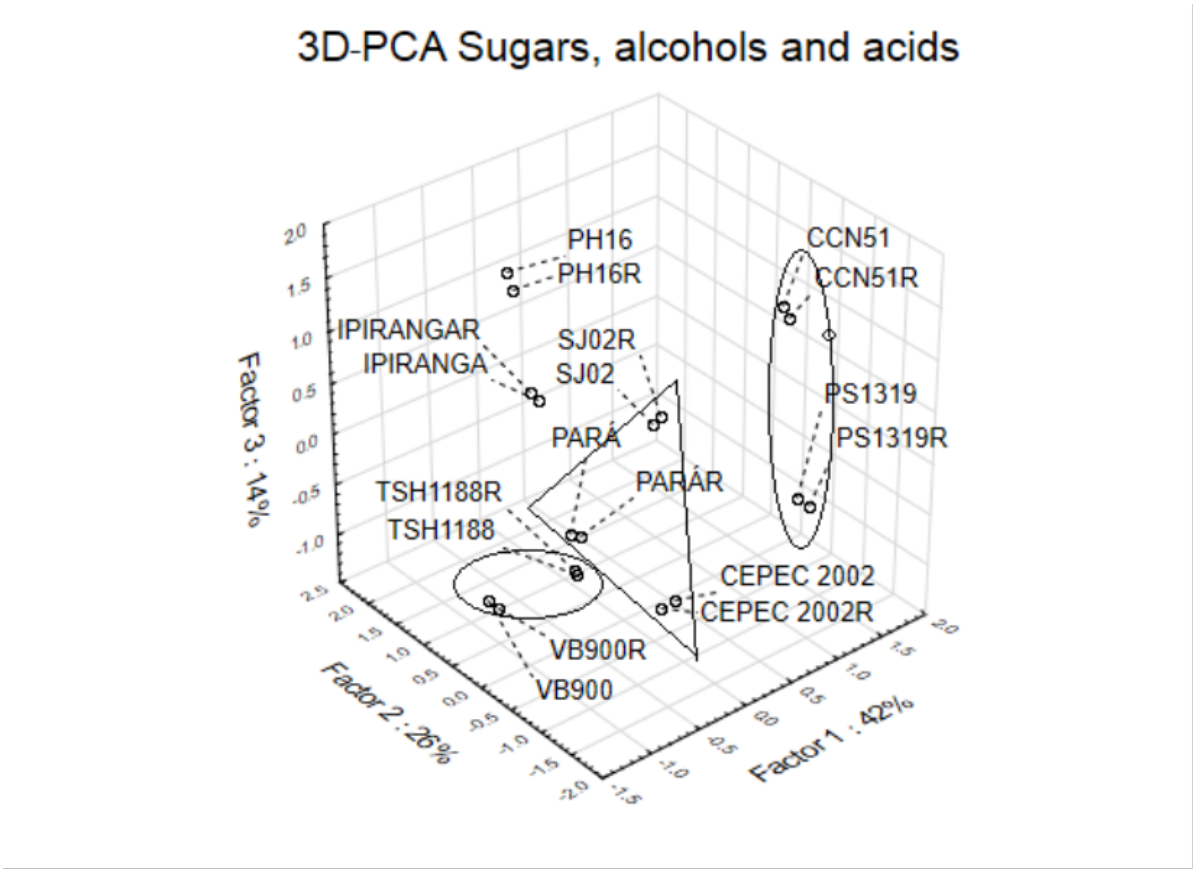
Figures:

Figure 1 - Cluster analysis of the sugars, non-volatile acids and alcohols profile in raw cocoa beans.



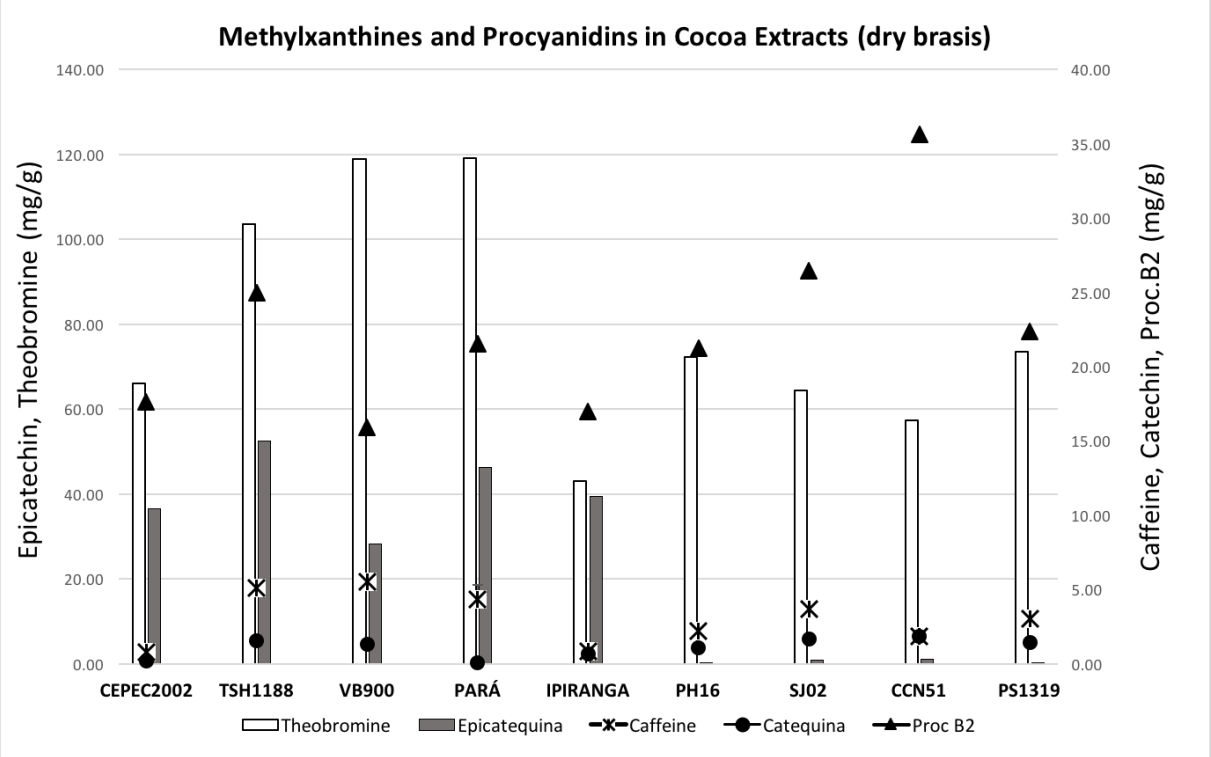
*Samples presenting the letter R after the name, are identified the repetitions.

Figure 2 – Scatterplot of the Principal Components Analysis representing 3D projection of Trinitario clones and Forastero variety, associated to the Figure 1: sugars, alcohols and acids.



*Samples presenting the letter R after the name, are identified as repetitions.

Figure 3 – Methylxanthines and procyanidins profile in cocoa extracts of Trinitario clones and Forastero variety, expressed as dry basis.



Supplementary Material for the Manuscript Entitled:

Industrial potential of cocoa (Theobroma cacao L.) varieties from Espírito Santo, based on the chemical composition of the cotyledons

Supplementary Material

Table 1 – Quantification of sugars and alcohols in fresh cocoa beans. expressed in grams of compound per Kg of dry cotyledons.

CLONES / VARIETIES	SUCROSE	GLUCOSE	FRUCTOSE	GLYCEROL	ETHANOL
CEPEC 2002	11.81	10.12	10.05	0.47	1.55
CEPEC 2002R	12.75	10.80	9.33	0.50	1.45
TSH1188	14.15	12.53	15.35	0.50	1.48
TSH1188R	15.02	12.80	15.48	0.48	1.44
VB900	15.01	12.58	15.19	0.84	0.85
VB900R	14.38	12.18	14.99	0.87	0.78
PARÁ	21.09	9.71	5.75	0.71	0.43
PARÁR	20.20	9.08	6.24	0.73	0.40
IPIRANGA	22.26	11.31	7.02	0.12	0.49
IPIRANGAR	23.33	11.50	7.20	0.12	0.47
PH16	25.10	9.99	13.50	0.00	0.00
PH16R	24.09	10.25	12.94	0.00	0.00
SJ02	9.67	8.76	9.91	0.00	0.00
SJ02R	9.75	8.27	9.79	0.00	0.00
CCN51	14.52	1.30	10.15	0.00	0.00
CCN51R	13.84	1.42	10.90	0.00	0.00
PS1319	14.08	8.41	11.10	0.00	0.00
PS1319R	13.09	8.18	10.75	0.00	0.00

*Samples presenting the letter R after the name, are identified as repetitions.

Table 2 – Quantification of non-volatile acids in fresh cocoa beans, expressed in grams of compound per Kg of dry cotyledons.

CLONES / VARIETIES	OXALIC ACID	CITRIC ACID	MALIC ACID	SUCCINIC ACID	ACETIC ACID	PROPIONIC ACID	ISOBUTYRIC ACID
CEPEC 2002	19.07	16.01	4.98	8.56	9.05	0.00	0.00
CEPEC 2002r	18.94	15.53	5.10	8.21	8.84	0.00	0.00
TSH1188	27.13	15.72	2.17	2.23	2.44	0.00	0.00
TSH1188r	27.88	16.04	2.23	2.33	2.50	0.00	0.00
VB900	10.36	6.61	2.24	3.54	8.63	5.16	0.00
VB900r	10.99	7.06	2.19	3.71	9.03	5.16	0.00
PARÁ	18.76	12.97	3.94	6.22	7.43	0.00	0.04
PARÁr	19.24	13.41	3.89	6.47	8.16	0.00	0.04
IPIRANGA	49.04	19.30	0.00	2.15	4.01	0.00	0.00
IPIRANGAr	48.26	18.82	0.00	2.10	3.88	0.00	0.00
PH16	10.50	7.02	2.65	4.20	6.11	5.49	0.05
PH16r	11.06	7.51	2.59	4.64	6.35	5.49	0.05
SJ02	15.44	11.56	3.17	6.03	2.67	0.00	0.00
SJ02r	15.23	11.78	3.22	5.89	2.54	0.00	0.00
CCN51	33.35	22.13	4.12	1.62	3.02	0.00	0.00
CCN51r	34.07	22.54	4.18	1.67	3.13	0.00	0.00
PS1319	41.79	27.67	6.08	2.38	5.99	0.00	0.00
PS1319r	42.41	27.97	6.15	2.45	6.23	0.00	0.00

*Samples presenting the letter R after the name, are identified as repetitions.

5.2 ADAPTAÇÃO DO ARTIGO CIENTÍFICO 2

Trinitario and Forastero cocoa (*Theobroma cacao L.*) fermentations with enzymatic extracts: assessment of chemical transformations from bean to bar

Abstract

Enzymatic extracts were applied to fresh Trinitario and Forastero cocoa beans in an attempt to increase flavor precursors formation during fermentation and drying for dark chocolate industrial production. Non-volatile compounds were quantified by HPLC during fermentation and drying, and volatile compounds, by SPME-HS-GC-MS at the end of fermentation, and drying, in the liquor and in the dark chocolate. A potential one day reduction in conventional Forastero fermentation was observed. Monosaccharides yield was higher in conventional Trinitario than in Forastero, and were the highest when the enzymes were applied, resulting in higher tetramethylpyrazine content in its liquor. The enzymes were more effective in Trinitario by enhancing desired flavor and aroma notes, as esters and pyrazines, and reducing off-flavor content. Enzymatic extracts are a promising tool to increase cocoa's aroma and productivity.

Key words: aroma, chocolate, enzymes, volatiles, SPME, flavor, HPLC

5. Introduction

Cocoa (*Theobroma cacao L.*), the main ingredient for dark chocolate, is divided into 3 varieties of unique flavor attributes: Nacional, Forastero and Criollo. Trinitario is a natural hybrid between the former two (Afoakwa *et al.,* 2008).

Fermentation is an essential process for chocolate production, when the specific cocoa flavor precursors, the reducing sugars, amino acids and peptides are formed, process that starts in the pulp, composed of water, sugars and polysaccharides (pectin, hemicelluloses and cellulose) (Aprotosoaie *et al.,* 2016; Gutiérrez, 2017) . Acidification of cotyledon is the main transformation for cocoa quality, and pH of well fermented beans vary between 4.75 and 5.19 (Afoakwa et al., 2008).

Pectinolic enzymes, such as arabinase (E.C. 3.2.1.99), transform the pulp, removing arabinose from the homogalacturonan chain, pectin methylesterase (E.C. 3.1.1.11) removes methoxyl groups from pectin producing methanol or polygalacturonase (E.C. 3.2.1.15), which reduces the pectin viscosity and hydrolyse the pulp. Those enzymes may be industrially obtained from fungi (Aehle, 2007). As the pulp is drained, the seeds are exposed to oxygen and acetic acid production starts (Schwan, 1998).

Endogenous or exogenous enzymes form aroma precursors (Jinap et al., 2008). Microbial cocktails have been applied to modulate the cocoa flavor (Leal et al., 2008; Batista et al., 2016; Crafack et al., 2013, 2014; Lefeber et al., 2012; Menezes et al., 2016; Sandhya et al., 2016; Schwan, 1998).

As microbial enzymes transform the substrates, the usage of their isolated enzymes is a promising solution for industrial purposes, for the convenience to use in a farm. Single purified enzymes to improve cocoa quality were used (de Brito et al. 2004; Lima et al., 2001; Jinap et al., 2002; Oliveira et al., 2011; T, 2012; Tr, 2012). However, their cost is still a drawback for the industry.

Multi-enzymatic systems have been applied to complex matrices (Mekasha et al., 2017; Waglay & Karboune, 2017) , and to cocoa after 48 to 96 hours of

fermentation (Oliveira et al., 2011), but they have not been reported for fresh cocoa, prior to fermentation. A combination of enzymatic extracts applied to fresh cocoa beans may modulate the transformation of substrates into desired aroma and flavor compounds.

Invertase or β-fructofuranosidase catalyzes the hydrolysis of sucrose into fructose and glucose (Bhalla et al., 2017). Papain, (EC 3.4.22.2) an endopeptidase, catalyzes the liberation of amides and esters, and hydrolysis of macromolecules (Llerena-suster et al., 2012). Glycoside hydrolases play an important role, considering the content of oligosaccharides in cell walls of cocoa nibs and testa (Redgwell et al., 2003). Endo-1,3-β-glucanase (EC 3.2.1.6) and endo-1,4-β-glucanase (EC 3.2.1.4), cleave the links β-1,3 and β-1,4 between glucose units of β-glucan, to produce glucose and oligosaccharides. Endo-1,4-β-xylanase (EC 3.2.1.8) targets xylan, consisted of a β-1,4-linked D-xylose backbone with side chains of α-arabinose, to yield short-chain sugars. Glucan-endo-1,3-β-D-glucosidase (EC 3.2.1.39) catalyzes the hydrolysis of 1,3-β-D-glucoside linkages in carbohydrates. Those are commercial enzymes of fungal origin, mainly from *Trichoderma* or *Aspergillus* species, or bacterial, from *Bacillus* species (Aehle, 2007).

The aim of this work was to assess changes in volatile and non-volatile compounds in fermented and dried Forastero and Trinitario cocoa beans, using multi-enzymatic extracts during the fermentation, in comparison to the conventional process and their impact on the liquor and dark chocolate flavor.

6. MATERIALS AND METHODS

6.1 Chemicals and standards

Standards for HPLC and HS-SPME-GC-MS techniques were supplied by Sigma Aldrich: acetoin, acetophenone, 2,3-butanedione, 2-pentanone, 1-propanol, 2-methyl-1-propanol, methyl acetate, ethyl acetate, isobutyl acetate, pyrazine, ethylpyrazine, 2,3,5,6-tetramethylpyrazine, sucrose, glucose, fructose, glycerol, methanol, ethanol and the acids acetic, propionic, isobutyric, butyric, pentanoic, hexanoic, octanoic, oxalic, citric, tartatic, malic, succinic, lactic, isobutyric, butyric and isovaleric. The commercial enzymatic extracts mixture, supplied by PROZYN, is described in Table1.

6.2 Samples production

Cocoa pods of Forastero and a mixture of seven Trinitario hybrids, evenly balanced, were harvested for 3 days on Ceará farm, located in Linhares (Espírito Santo, Brazil). Fruits were opened separately and the seeds with pulp were transported to the fermentation boxes.

Fermentation took place from initial day to day6. Wet beans of Forastero (F) and Trinitario (T) were divided in two batches for fermentation at room temperature, with enzymatic extracts (FWE and TWE) and without it (F and T), the conventional, simultaneously until the liquor production.

According to the procedure described by Thompson et al. (2001), the four batches of 600kg beans were placed into $1m^3$ capacity wooden boxes and a water solution containing 50g of extracts was applied over FWE and TWE seeds, with vigorous mixing. Wooden lids closed the boxes for 48 hours, and daily turns were made from day2 to day6. The temperature was measured daily, 20cm deep in the center of

the wooden box. The pulp sweatings were drained through openings at the bottom of the boxes.

Drying was executed from day7 to day10, by spreading the beans in perforated trays inside a greenhouse with fresh air flow, and daily mixing. The four experiments were carried out simultaneously.

Cocoa beans were transported to the factory (Espírito Cacau, Vitória Brazil) and processed in an industrial system (Meller, Vila Velha, Brazil). The nibs were rosted at 125°C for 15 minutes, milled to obtain cocoa liquor, which was mixed with sugar, cocoa butter and emulsifiers in a conche-refiner, for 8 hours of conching at 75°C and 3 hours of refining. The 70% dark chocolate was tempered, crystallized and packed for storage until analysis.

6.3 Analysis

6.3.1 Sampling

Samples from fermentation were collected approximately 40 cm deep from the surface in the center of the boxes, placed into plastic bags, frozen with liquid nitrogen and kept at -20°C until analysis. Pulp and testa were carefully removed with a scalpel to obtain the cotyledons, prior to extractions.

6.3.2 Volatile compounds

SPME-HS-GC-MS analysis were performed according to Rodriguez-Campos *et al.,* (2011) using a Supelco fiber 50/30µm divinylbenzene/carboxene/polydimethylsiloxane (DVB/CAR/PDMS) desorbed for 3 minutes into the capillary column Restek Rtx®-Wax (60 m, 0.25 mm id, 0.25 µm film thickness) in a Shimadzu QP2010SE GC-MS (Shimadzu, Kyoto, Japan) for compounds identification. Targeted compounds were identified by external standards retention time and comparison to the mass spectral library NIST 11 for untargeted compounds. The mean peak areas were used for relative quantification (Crafack et al., 2014; Afoakwa et al., 2009), for samples of days 0, 3, 5 and 6 of fermentation, day7 and day10 of drying, cocoa liquor and 70% dark chocolate.

6.3.3 Non-volatile compounds and pH

Extraction and analysis were in duplicates according to Moreira et al. (2013), for organic acids, alcohols and sugars in a LC-10Ai Shimadzu chromatography system, using a Shim-pack SCR- 101H column (7.9 mm × 30 cm). Compounds were identified by retention time of external standards. The quantification by analytical curve was expressed in g of compound per kg of dried cotyledons of samples from days initial, 3, 5 and 6 of fermentation and day10 of drying. The correspondent pH was determined (AOAC, 2012).

6.3.4 Statistical Analysis

Statistica 12 Ultimate Academic Bundle software (Dell Inc., USA) was used for analyzing data. The number of variables was reduced using PCA (Principal Component Analysis) and differences on the non-volatile compounds quantifications

141 between days were assessed by one-way ANOVA, followed by Tukey test at a
142 significance level of 0.05.

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144 **7. Results and Discussion**

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146 **7.1 pH and temperature**

147 Evaluations were made from initial day, when the seeds were placed in the boxes,
148 to the end of the drying (day10) (see supplementary). A pH measurement made when
149 the pods were harvested showed pH 6.3 for Forastero and pH 6.17 for Trinitario, but
150 after the 3-day pod storage in the field, the pH dropped to 5.2 and 5.1, respectively,
151 due to sugars consumption and acids production. This effect was described by
152 Emmanuel et al. (2012) and Kongor et al. (2016).

153 Temperature increase until day3 of fermentation is associated to the pH reduction in
154 samples, due to the ethanol and acetic acid production, which are exothermic
155 reactions, also reported by Schwan & Wheals (2004). The pH increased from day3 to
156 5 due to citric acid consumption, fact mentioned by Schwan & Wheals (2004). Although
157 Kongor et al. (2016) associated higher citric acid content to lower pH, in F it dropped
158 until day5 (4.37), probably due to the highest total acids content in day5 (44g/kg) (see
159 supplementary).

160 At the end of drying process, all the samples showed pH from 4.75 to 5.19,
161 correspondent to well fermented beans. This ideal pH range was reported by Afoakwa
162 et al. (2008).

163

164 **7.2 Differences between Forastero variety and Trinitario hybrid**

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166 A comparison between Forastero and Trinitario conventional fermentation and
167 drying was made. Dynamics in non-volatile acids, sugars and alcohols are displayed
168 in the Fig.1 and the quantification table, in supplementary.

169 Sucrose was transformed into glucose and fructose in F and T , also reported by
170 Schwan & Wheals (2004) . Monosaccharides yield was higher in T (glucose 6.8,
171 fructose: 9.6g/kg), as its initial sucrose content was higher (1.75fold) than in F, with no
172 significant difference in day5 ($p < 0.05$). Although sucrose was found in the cotyledons,
173 most of the monosaccharides come from the sucrose in the pulp. According to
174 Andersson et al. (2006), the uptake of those compounds is affected by the opening of
175 the testa, as a result of an initial germination of the beans during the first two days of
176 fermentation. Hansen et al. (1998) quantified invertase in the testa and in cotyledons
177 as a proof that sucrose hydrolysis in the beans starts after harvesting. Rodriguez-
178 Campos et al. (2011) analyzed Forastero samples but found ~14g/kg of fructose,
179 similar to our Trinitario test (12.6g/kg), highest in day3. Initial sucrose content found by
180 the same authors (20g/kg) was much higher than ours (3.6g/kg), as well as fructose
181 content in day1 (7.4g/kg), probably because of our pod storage time in the field and
182 transportation time. Sugars contents when the pods were first opened were similar to
183 their results for Forastero (sucrose: 20.6, glucose: 9.4, fructose: 6g/kg) and Trinitario
184 (sucrose: 15.8, glucose: 9 and fructose 10.9g/kg). According to Zambrano et al. (2010)
185 reducing sugar content is a criterion to evaluate the cocoa fermentation as precursors
186 of aromatic substances.

187 Ethanol content immediately after harvesting was close to zero (data not shown).
188 However, its initial day concentration was high because of the storage time in the field

and transportation time after the pods opening (4.6g/kg for Forasteros and 8.0g/kg for Trinitario), indicating the alcoholic fermentation had started and sugars were consumed. Schwan & Wheals, (2004) reported ethanol oxidation to acetic acid, which explains its concentration increase in day3 (F: 7.3 to 16.1g/kg; T from 5.4 to 26.22g/kg) and ethanol depletion in the same period (F: 4.6 to 0.3g/kg; T from 8.0 to 7.4g/kg).

Higher glycerol concentration in F was found in day5 (7.9g/kg), and day6 in T (6.1g/kg), which is probably related to desired volatiles formation. Reineccius (2006) described volatile compounds formation mechanisms that combine alcohols and natural acids, usually acetic or other short chain acid, to yield esters, and the oxidation of alcohols to their correspondent aldehyde, catalyzed by microbial alcohol oxidase. Despite anaerobic conditions, lactic acid was not found during the first 48 hours in F or in T, probably due to a fast pulp drainage and aeration increase.

Cocoa turnings started after 48 hours of the fermentation, affecting acetic acid concentration higher in day6 in T(22.4g/kg), and day5 (12.4g/kg) in F, indicating a possible microbial activity still occurring at the end of fermentation. Acetic acid is beneficial during fermentation for enzymatic activation and cellular protein degradation to form flavor precursors (Voigt & Biehl, 1995), but even volatilized further in the process, it interferes with the chocolate flavor (Schwan & Wheals, 2004).

Reduction of citric acid content in F and T was observed until day5 (F: 3.8; T: 5.3g/kg), and increase in day6 (F: 5.7; T: 8.5g/kg). Significant differences to day6 in both tests were found ($p>0.05$), possibly related to lactic acid bacteria activity. Citric acid consumption by lactic acid bacteria was described by Schwan & Wheals (2004) and its increase after 96h of fermentation was reported by Aprotosoaie et al. (2016). Those results are in accordance to the highest acid lactic content found in F in day5 (8.7 g/kg) and day6 (1.1 g/kg) for Trinitario. Thompson et al. (2001) described the

214 transformation of citric acid into acetic acid, carbon dioxide and lactic acid. According
215 to Andersson et al. (2006), acids do not diffuse into the beans through the testa but
216 through the micropyle opening, allowing them to be quantified inside cotyledons even
217 after some days.

218 Butyric and isovaleric acids, off-flavors indicators, were not found in the samples.
219 Rodriguez-Campos et al. (2012), found those compounds and reported their significant
220 increase in fermentations longer than 6 days. Volatile compounds representation is
221 displayed in Fig2. and the relative quantification is in supplementary.

222 Predominant volatile compounds at the end of fermentation in F were 2-pentanone,
223 with a fruit profile; acetophenone, flowery and sweet odor quality and isobutyric acid,
224 an off-flavor. Desired volatile compounds in F showed higher concentrations when
225 glycerol and acetic acid contents were higher. Those aroma formation reactions were
226 described by Reineccius (2006) and those compounds were also found by Rodriguez-
227 Campos et al. (2011) in the same cocoa variety. Maximum concentration of 2-
228 pentanone was reached in day6, around 4.5 -fold the amount of day5. Isobutyric acid
229 reached its maximum in day6, 3.2-fold the concentration found in day5.
230 Transformations into volatile compounds start after the bean death by acidification in
231 day3, as described by Voigt & Lieberei (2015).

232 In T, isobutyric acid, acetophenone and 2-pentanone were the majority of
233 compounds; 2-3-butanedione, with buttery profile, acetoin, 2-methyl-1-propanol and
234 pentanoic acid were also found. 1-Propanol, with pungent odor, was not found in the
235 samples. Odor profile of the compounds were described by Rodriguez-Campos et al.
236 (2012), Rodriguez-Campos et al. (2011) and Afoakwa et al. (2009).

Fructose was predominant during drying (F: 5.5 and T: 5.3g/kg), followed by glucose (F: 2.4 and T: 2.8g/kg) and sucrose (F: 0.6 and T: 0.7g/kg). Similar results were found by Rodriguez-Campos et al. (2011) (fructose ~7g/kg), despite differences in concentrations. In processes, sucrose content reduction and monosaccharides increase indicates a possible invertase activity during drying.

Oxalic acid was the most abundant acid in F (10g/kg), while acetic acid was in T (6.1g/kg). Schwan & Wheals (2004) described oxalic acid as a significant contributor to flavor. Rodriguez-Campos et al. (2011) found lactic acid predominant in drying, which is not beneficial to chocolate (Thompson, 2001).

Acid lactic after drying reduced in F to 0.4g/kg in day10, and was not found in T, which contributes to chocolate quality, as mentioned by Rodriguez-Campos et al. (2011).

Acetic acid content was reduced during drying, in F (from 6.9 to 5.7g/kg) and in T (from 22.4 to 6.1 g/kg), due to the air flow inside the greenhouse. The removal continues during roasting and conching (Afoakwa et al., 2008).

Some compounds quantified during fermentation in F like 2-pentanone, 2-methyl-1-propanol and acetoin, were not found during drying. Isobutyric acid reduced 4 times from day6 to day7 and 12 times until day10.

Pentanoic and hexanoic acids were predominant in F. Acetophenone, methyl acetate, ethyl acetate, propionic, isobutyric, octanoic, nonanoic acids were also identified. Isobutyric acid content in day7 was 2.7-fold than day10 in T. Acetophenone content reduced 4 times during drying.

Similar to F, most volatiles in T were reduced or not found during drying. Acetoin, a Maillard reaction intermediate mentioned by Reineccius (2006), was the predominant

compound during drying. Rodriguez-Campos et al. (2011) found acetoin to be the predominant aldehyde during fermentation and its content increased in drying.

Comparisons were made using Principal Component Analysis (PCA) to evaluate the effect of the cocoa variety F and hybrid T. Non-volatile compounds PCA scores (fig.3a) shows 84.8% as total variance. PC1 (49.8%) was mostly influenced by non-volatile fraction of ethanol, PC2 (18.5%), by acetic acid and PC3 (16.5%), by propionic acid. Samples taken at the end of fermentation and at drying were on the opposite quadrant. F5 and F6 were grouped in the same cluster, indicating that there is a potential to reduce one day in Forastero fermentation.

7.3 Effect of the enzymes in Forastero variety

Enzymatic extracts effect in Forastero was evaluated by comparing F and FWE. FWE showed a higher yield of glucose and fructose (7.6 and 8g/kg) in day3, probably due to the action of β-fructofuranosidase (temperature 46°C; pH 4.3). Optimum condition for glycoside hydrolases activity in FWE was reached with pH 5.1 in day5. This may explain the increase in sugars production.

Glycerol, was higher in F (7.9g/kg) on day5, and lower in FWE. De Vuyst & Weckx (2016) described glycerol as a side product of ethanol production, together with carbon dioxide, under anaerobic conditions.

Pulp degradation in FWE was faster than in F, due to the polysaccharides degrading enzymes in the commercial extract Powercell® and β-fructofuranosidase, which promoted aeration, more favorable to acetic fermentation instead of lactic. Those

effects were observed by the yield of ethanol, higher in FWE until day3, and lower glycerol production, probably because part of the ethanol was oxidized to acetic acid, resulting in higher content until day5 (20.4g/kg). Related to that, lactic acid was not found in FWE and citric acid production until day3, was higher than in F. Succinic acid content has higher in FWE. Schwan & Wheals (2004) reported its production by yeasts, and Afoakwa et al. (2008) mentioned it is not removed in later production stages.

Desired volatiles methyl-acetate, ethyl acetate, 2-pentanone and 2-methyl-1-propanol were found in F day5 and 6, but were not found in FWE during the same period. Those results may be explained by the higher content of glycerol (7.9g/kg) and acetic acid (12.4g/kg) in F in day5. This ester formation reaction was described by Reineccius (2006).

Off-flavors production occurred in F and FWE, and isobutyric acid had the highest concentration increasing from day5 to day6, 3.2-fold in F and 1.8 times in FWE.

Fructose content increased and was the most abundant sugar during drying in F (5.6g/kg) and FWE (6.2g/kg), indicating invertase activity.

Glycerol had the highest concentration among alcohols (F: 2.3g/kg), oxalic acid, by the end of drying in F (10.1g/kg), and succinic, in FWE. Although endogenous oxalic acid content reduced during fermentation, the final amount in drying (9.8g/kg) was higher than the other acids.

Esters were first quantified in FWE at the end of drying, in its liquor and chocolate, probably due to a possible papain activity, that might have increased due to the higher temperatures during drying, roasting and conching. Papain activity related to esters production was reported by Llerena-suster et al. (2012).

2-pentanone, identified during fermentation, was reduced 27-times from its initial content during drying in FWE, which is negative due to the fruit aroma contribution to the chocolate. Its odor quality was reported by Rodriguez-Campos et al. (2011) , who found in this compound in early fermentation stages.

Acetophenone was found in F and FWE during drying, with content reduction from day7 to day10, 13 times for F and 2.5-fold for FWE. Rodriguez-Campos et al. (2011) found it at the end of drying.

Propionic acid content reduced in fermentation, but increased during drying. This compound was not found in drying by Rodriguez-Campos et al. (2011).

PCA scores (fig.3b) displays 81.7% of total variance. PC1 (44%), influenced by glucose, fructose and ethanol; PC2 (23.9%), by succinic acid and PC3 (13.8%), by acetic acid. Transformations until day3 related to PC2, the increasing of succinic acid in both treatments. FWE5 and FWE6 were in the same cluster, reinforcing a potential to reduce TWE fermentation in one day. Those samples were similar to F6.

GC-MS PCA scores (fig.3f), shows 80.8% as total variance (PC1:41.1%, PC2:26%, PC3:13.7%). PC1 is predominantly influenced by 2-methyl-1-propanol and isobutyric acid, PC2, by ethyl acetate and PC3, by octanoic acid. FWE5 and FWE6 were in the same cluster, proving FWE fermentation may be reduced in one day.

7.4 Effect of the enzymes in Trinitario hybrids

Sucrose dynamics in T and TWE were similar. However, fructose and glucose were different. Their higher contents after day 5 were probably because the temperature ~ 40°C was reached in day 2, when fructose content increased, reaching its maximum

in day 3, the optimum conditions for β-fructofuranosidase and Powercell® hydrolases (46°C, pH ~ 5.3). In T, the temperature rise was faster than in TWE and transformations started earlier.

Acetic acid production is an exothermic reaction and increases the temperature, as in TWE day 3 and 5. Production of this acid was stimulated by vigorous aeration in daily turnings. When the acids production increased between day5 to day6, the temperature reached its maximum (46°C), and the pH dropped to 4.3.

Isobutyric acid, acetoin and acetophenone were predominant during fermentation in T and their contents in TWE, were reduced until day 6: isobutyric acid 25 times, acetoin 5 times and acetophenone 15 times lower than in T. Rodriguez-Campos et al. (2012) found acetoin as predominant aldehyde in Forastero fermentation.

Fructose content increased in T (from 2.1 to 5.3g/kg), but in TWE (from 11.5 to 8.5g/kg), even decreasing, the amount was higher than in T during drying. Higher amounts of reducing sugars at the end of fermentation and drying, like TWE treatment, increases the formation of Maillard flavor compounds, the pyrazines, during roasting (Jinap et al., 2008).

Acetic acid content dropped in T and TWE during drying and the final contents were similar (T:6.1; TWE 10.9g/kg). Citric acid content increase in TWE during drying was significant, from 3.8 to 5.9g/kg ($p<0.05$), the opposite found in T and by Rodriguez-Campos et al. (2011), that attributed their findings in citric acid depletion during fermentation and drying to its transformation into acetic acid, carbon dioxide and lactic acid.

Acetoin was predominant in both tests and its content in T increased 46 times from day 7 to day 10 of drying, when it reached the maximum; in TWE, it increased

355 2.28 times. In both treatments, the content of acetoin in liquor and chocolate production
356 decreased (see supplementary).

357 Fruit flavored compounds like ethyl acetate, isobutyl acetate, 2-pentanone, and
358 methyl acetate were identified in TWE, and later in the correspondent chocolate, even
359 though in lower content compared to day 7, probably because of papain activity related
360 to esters production, which was reported by Llerena-suster et al. (2012).

361 In T, 2-methyl-1-propanol (wine) and 2-3-butanedione (buttery) were found in
362 day 10. The pungent propionic acid content in TWE increased in 1.3-fold during drying.
363 It is produced by *Bacillus* spp., like isobutyric acid, at the end of fermentation
364 (Rodriguez-Campos *et al.,* 2012). Propionic acid was not found in detectable amounts
365 in T during fermentation and drying, but it was in liquor and chocolate, probably due to
366 the difficulty of standardize a solid state fermentation in an industrial scale, as
367 mentioned by Soccol et al. (2017), although the samples were carefully collected and
368 prepared.

369 PCA scores (fig4C) displays 81% as total variance, PC1 (40.7%), influenced by
370 sucrose; PC2, (23.7%), by succinic acid and PC3 (16.6%), by lactic acid. T day 5 and
371 T day 6 were in the same cluster, indicating a potential reduction in fermentation time.
372 However, when the enzymes were added to Trinitario, we noticed differences related
373 to PC2 and PC3, although they are similar in PC1.

374 Volatile's PCA scores (fig.3g) shows 80.6% as total variance. PC1 (41%)
375 influenced by methyl acetate and isobutyl acetate, PC2 (27.4%), by isobutyric acid,
376 and PC3 (12.2%), by ethyl acetate. Again, samples T5 and T6 were in the same
377 cluster, reinforcing similarities and the potential for reducing 24h in fermentation. When

the enzymatic extracts were applied to the seeds, although TWE5 and TWE6 are similar in PC1, differences appeared in PC2 and PC3.

7.5 Forastero and Trinitario with enzymatic extracts

Flavor precursors glucose and fructose content in day6 were higher in TWE (5.8 and 11.7g/kg), as well as total acids content, 71.4 g/kg in TWE, and 53.8 g/kg in, mainly because of acetic acid.

Intense acetic acid production and citric acid consumption were observed in day 3 to day 5 for TWE, indicating that citric acid might have been used as another substrate for acetic acid production, as described by Thompson et al. (2011).

Fruit volatiles like ethyl acetate and 2-pentanone were more abundant in TWE. In FWE, isobutyric acid and acetophenone were predominant at the end of fermentation, while in TWE were pentanoic acid and acetoin.

Reducing sugars content increased in FWE and decreased in TWE during drying. Despite similar contents, glucose and fructose were statistically different ($p>0.05$) and higher in TWE (5.7 and 8.5g/kg).

Total acid content was higher in FWE (40.2 g/kg) than in TWE (33.5g/kg). Acetic acid (10.9g/kg) was the highest contributor in the former, and succinic acid (11.7g/kg), in the later.

Isobutyric and propionic acids were the most abundant during drying in FWE, and in TWE, acetoin and propionic acid. In FWE, the dominant acids were propionic, isobutyric, pentanoic and octanoic; in TWE, besides those acids, esters and ketones,

like methyl acetate, ethyl acetate, isobutyl acetate, 2,3-butanedione and 2-pentanone were found.

PCA scores (fig4D) displays similarities between FWE6 and FWE10, indicating minor changes during drying concerning non-volatiles.

7.6 Cocoa liquors' volatile compounds: F, FWE, T and TWE

Although some authors have reported production of pyrazines during fermentation and drying by *Bacillus* spp. (Reineccius, 2016; Schwan & Wheals, 2004; Selamat et al., 1994), we only found those compounds in our liquors and chocolates. Rodriguez-Campos et al. (2012) found the best temperature to obtain tetramethylpyrazines at 70°C or 80°C. In our process, this temperature was only reached during roasting, which is ideal to transform fermentation flavor precursors through Maillard reactions, especially into pyrazines and aldehydes (Afoakwa et al., 2009).

Pyrazine was quantified in F, while ethylpyrazine and tetramethylpyrazine, were found in all liquors (F, FWE, T, TWE). Tetramethylpyrazine content was higher in TWE and T. The amount in TWE was 1.3-fold higher than in T, and 25-fold in FWE. In F, tetramethylpyrazines content was around 14 times higher than FWE. Tran et al. (2015) and Beckett (2011) mentioned maximum formation of tetramethylpyrazine under medium temperatures (~130°C).

7.7 Dark chocolates' volatile compounds: F, FWE, T and TWE

Compared to drying, acetoin content decreased in all the samples. Reduction from liquor to chocolate was higher in T (5.8-fold), despite the highest final content (2.8-fold

426 TWE). Acetoin was reported by Reineccius (2006) as an intermediate in the
427 biosynthesis of the aminoacids leucine, valine and panthothenate, and part of Maillard
428 reaction, which yields tetramethylpyrazines as final product. The correlation between
429 acetoin and tetramethylpyrazine (r:0.87) indicate the higher the reduction in acetoin
430 content in liquor, the higher the content of tetramethylpyrazine in chocolate. It is not
431 possible to correlate acetoin production to papain enzyme activity, by increasing
432 aminoacids content, as it was not evident an increase of this compound in FWE and
433 TWE compared to their conventionals.

434 Enzymes in Trinitario changed significantly its flavor, compared to the conventional
435 process. TWE was the only sample containing 18 out of 19 targeted volatiles, which
436 indicates the positive enzymatic effect in increasing the volatiles.

437

438 **8. Conclusion**

439

440 Differences between conventional Forastero and Trinitario fermentation process
441 were mainly related to higher monosaccharides, ethanol and acetic acid formation in
442 the former. Forastero, presented higher glycerol content.

443 Enzymatic extracts were more effective in Trinitario for non-volatile flavor
444 precursors formation, enhancement of desired volatiles and reduction of off-flavors. In
445 Forastero, the multi-enzymatic system could reduce undesirable flavors during
446 fermentation, and the fermentation process in 24hours. The former property is innated
447 to conventional Trinitario for volatiles and non-volatiles. In both cases, there is still
448 much potential for other enzymes applications and chocolate flavor modulation.

Application of enzymatic extracts in cocoa fermentation for industrial scale chocolate production is a promising technique to improve quality and productivity through flavor enhancement and process time reduction.

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608

Table 1 - Composition of the enzymatic extracts.

Enzyme Preparation	EC	Declared major activity	Reported side activities	pH range	Temp range ºC	Source
Sweetmax 200®	3.2.1.26 β-fructofuranosidase	200.000 [1]SU/g	none	3,0 to 5,0	40-70	*Saccharomyces cerevisiae*
Powercell®	EC 3.2.1.6 Endo-β-1,3-glucanase EC 3.2.1.4 Endo-β-1,4-glucanase EC 3.2.1.8 Endo-β-1,4-xylanase EC 3.2.1.39 Glucan-endo-1,3-β-D-glucosidase	1180U/g	arabinase	4,0 to 6,5	30-60	*Trichoderma reesei*
Brauzyn®	3.4.22.2 Papain	850 [2]TU/g	none	5,0 to 7,0	30-80	*Carica Papaya L.*

[1]Enzyme activity expressed as summer units (SU) per gram

[2]Enzyme activity expressed as tyrosine units (TU) per gram.

Figures

Figure 1 - Non-volatile compounds in cocoa beans, during fermentation and drying: conventional Forastero (a) sugars, alcohols; b) acids), Forastero with enzymes (c) sugars, alcohols; d) acids), conventional Trinitario (e) sugars, alcohols; f) acids), Trinitario with enzymes (g) sugars, alcohols; H: acids).

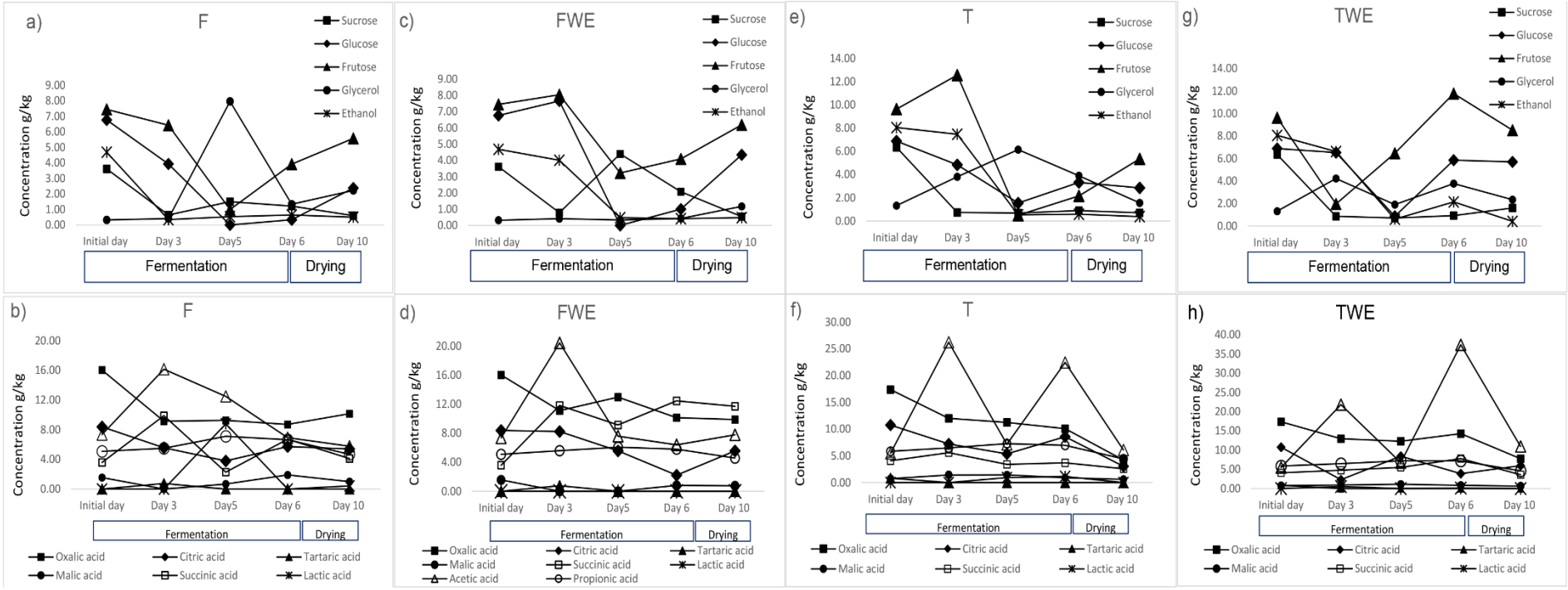


Figure 2 - Volatile compounds in fermentation (day5 and 6) and drying (day7 and 10). A. Forastero (F); B. Trinitario (T); C. Forastero with enzymatic extracts (FWE); D. Trinitario with enzymatic extracts (TWE).

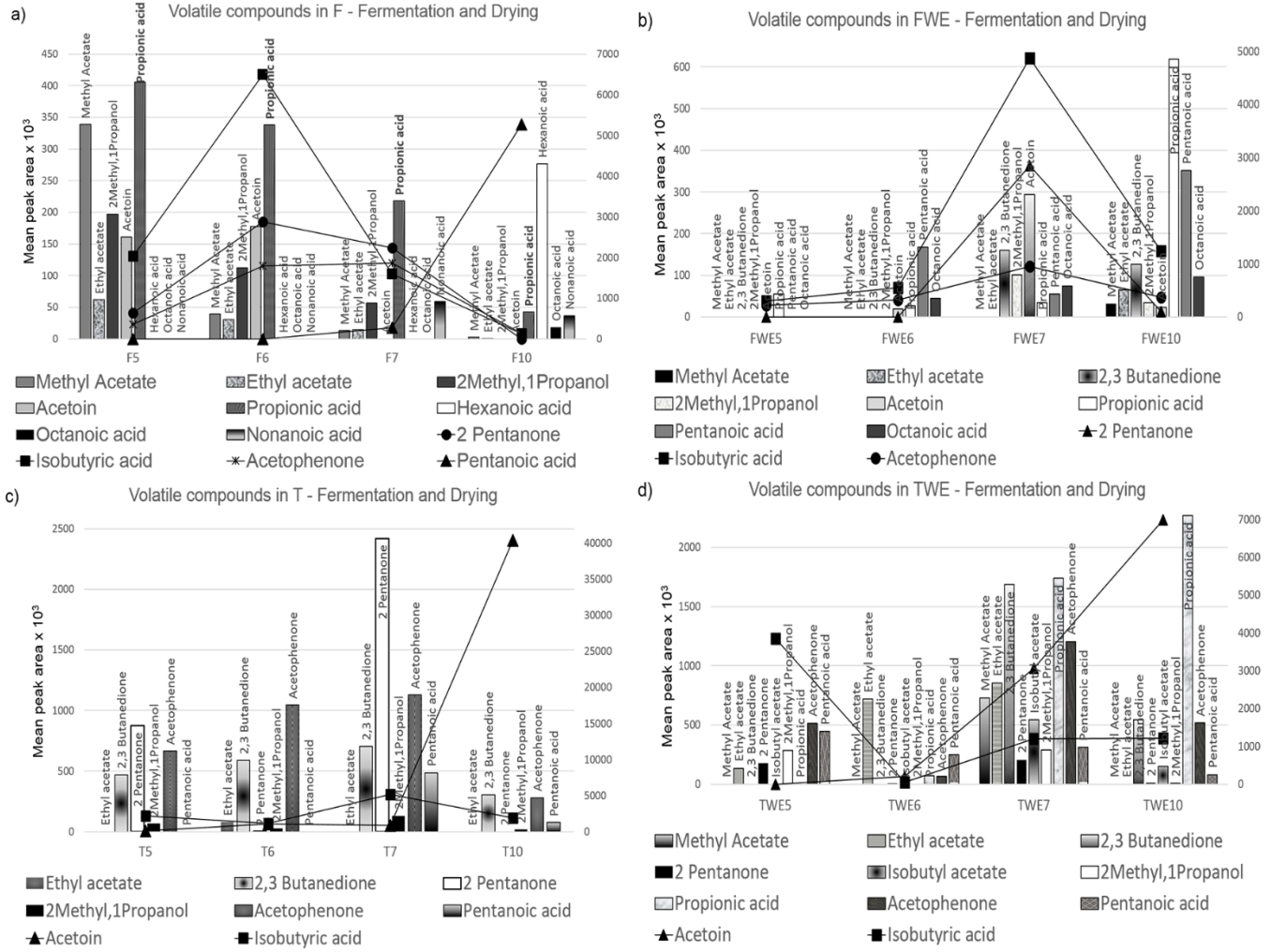


Figure 3 - 3D-PCA of comparisons between treatments, using compounds dynamics for non-volatiles during fermentation and drying: a) F and T; b) F and FWE; c) T and TWE; d) FWE and TWE; volatiles during fermentation and drying: e) F and T; f) F and FWE; g) T and TWE; h) FWE and TWE; volatiles in liquors and chocolates: i) F, FWE, T, TWE.

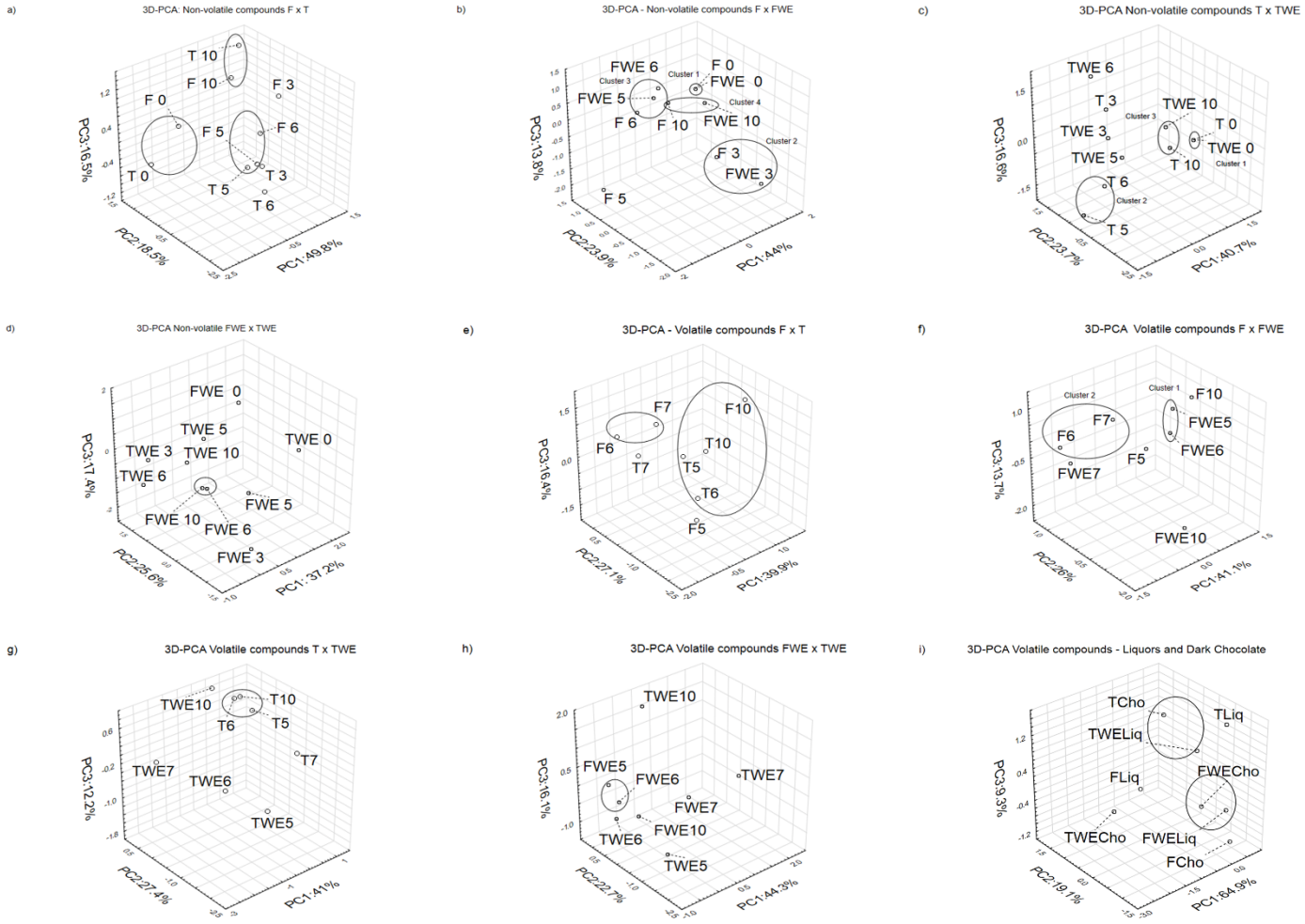
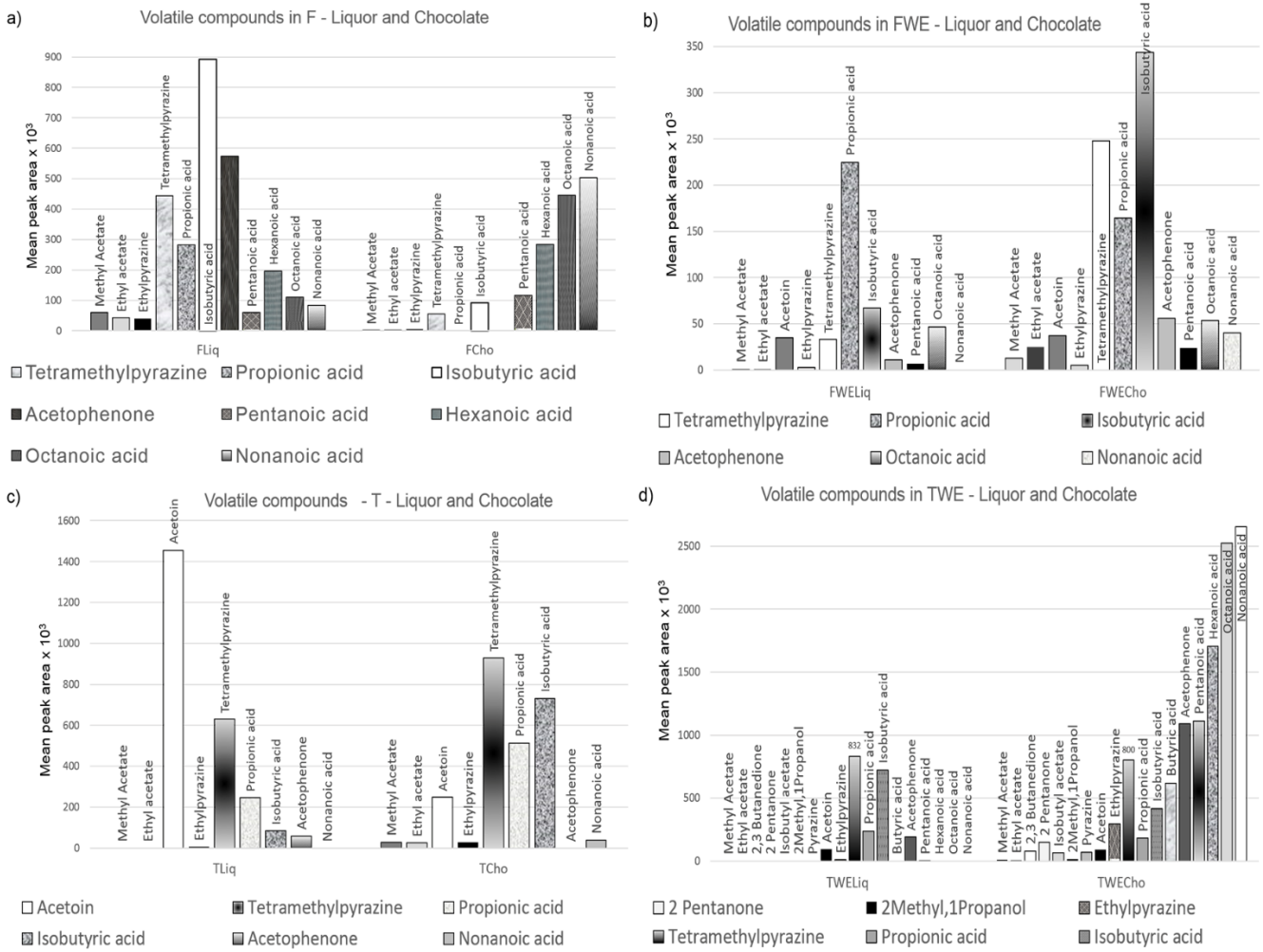


Figure 4 - Volatile compounds in liquor and chocolate. A. Forastero (F); B. Trinitario (T); C. Forastero with enzymatic extracts (FWE); D. Trinitario with enzymatic extracts (TWE).



Supplementary Material for the Manuscript Entitled:

"Trinitario and Forastero cocoa (Theobroma cacao L.) fermentations with enzymatic extracts: assessment of chemical transformations from bean to bar"

Table 1. Quantification of non-volatile acids, sugars and alcohols in samples in dry basis), in grams of compound per Kg of dry cotyledons.

	Sucrose	Glucose	Fructose	Glycerol	Methanol	Ethanol
F 0	3.61 ± 0.29^{b}	6.77 ± 0.20^{b}	7.45 ± 0.42^{c,d,e}	0.32 ± 0.02^{i}	0^{b}	4.69 ± 0.30^{c}
F 3	0.64 ± 0.01^{d}	3.94 ± 0.12^{d,e}	6.43 ± 0.25^{d,e,f}	0.41 ± 0.05^{i}	0.44 ± 0.01^{a}	0.35 ± 0.01^{e}
F 5	1.50 ± 0.01^{c,d}	0^{j}	0.99 ± 0.03^{j}	7.96 ± 0.28^{a}	0^{b}	0.53 ± 0.02^{e}
F 6	1.21 ± 0.04^{c,d}	0.34 ± 0.01^{i,j}	3.92 ± 0.29^{g,h}	1.34 ± 0.06^{h}	0^{b}	0.64 ± 0.02^{e}
F 10	0.60 ± 0.01^{d}	2.38 ± 0.18^{g}	5.58 ± 0.30^{f,g}	2.23 ± 0.08^{e,f}	0^{b}	0.50 ± 0.01^{e}
FWE 0	3.61 ± 0.29^{b}	6.77 ± 0.20^{b}	7.45 ± 0.42^{c,d,e}	0.32 ± 0.02^{i}	0^{b}	4.69 ± 0.30^{c}
FWE 3	0.79± 0.02^{c,d}	7.65 ± 0.28^{a}	8.04 ± 0.47^{b,c,d}	0.42 ± 0.01^{i}	0^{b}	4.01 ± 0.13^{c}
FWE 5	4.40± 0.34^{b}	0^{j}	3.23 ± 0.13^{h,i}	0.34 ± 0.00^{i}	0^{b}	0.49 ± 0.03^{e}
FWE 6	2.08 ± 0.04^{c}	1.01 ± 0.04^{h,i}	4.10 ± 0.16^{g,h}	0.43 ± 0.02^{i}	0^{b}	0.42 ± 0.01^{e}
FWE 10	0.55 ± 0.01^{d}	4.34 ± 0.28^{c,d}	6.19 ± 0.19^{e,f}	1.17 ± 0.08^{h}	0^{b}	0.48 ± 0.02^{e}
T 0	6.35 ± 1.03^{a}	6.88 ± 0.35^{a,b}	9.64 ± 0.76^{b}	1.32 ± 0.07^{h}	0^{b}	8.06 ± 0.31^{a}
T 3	0.74 ± 0.02^{c,d}	4.84 ± 0.16^{c}	12.58 ± 0.34^{a}	3.81 ± 0.03^{c,d}	0^{b}	7.47 ± 0.29^{a}
T 5	0.68 ± 0.00^{c,d}	1.54 ± 0.04^{h}	0.49 ± 0.00^{j}	6.15 ± 0.10^{b}	0^{b}	0.52 ± 0.00^{e}
T 6	0.89 ± 0.01^{c,d}	3.33 ± 0.01^{e,f}	2.15 ± 0.12^{i,j}	3.89 ± 0.05^{c,d}	0^{b}	0.58 ± 0.01^{e}
T 10	0.70 ± 0.03^{c,d}	2.85 ± 0.11^{f,g}	5.36 ± 0.24^{f,g}	1.55 ± 0.07^{g,h}	0^{b}	0.37 ± 0.00^{e}
TWE 0	6.35 ± 1.03^{a}	6.88 ± 0.35^{a,b}	9.64 ± 0.76^{b}	1.32 ± 0.07^{h}	0^{b}	8.06 ± 0.31^{a}
TWE 3	0.87 ± 0.01^{c,d}	6.52 ± 0.41^{b}	1.96 ± 0.09^{i,j}	4.22 ± 0.16^{c}	0^{b}	6.63 ± 0.32^{b}
TWE 5	0.71 ± 0.01^{c,d}	0.87 ± 0.01^{h,i}	6.47 ± 0.32^{d,e,f}	1.91 ± 0.12^{f,g}	0^{b}	0.60 ± 0.01^{e}
TWE 6	0.93 ± 0.02^{c,d}	5.85 ± 0.17^{b}	11.77 ± 1.03^{a}	3.77 ± 0.20^{d}	0^{b}	2.14 ± 0.08^{d}
TWE 10	1.60 ± 0.01^{c,d}	5.71 ± 0.25^{b}	8.52 ± 0.43^{b,c}	2.34 ± 0.03^{e}	0^{b}	0.42 ± 0.02^{e}

*Mean value ± standard deviation; results having the same letter were not significantly different ($p < 0.05$)

Table 1. Quantification of non-volatile acids, sugars and alcohols in samples in dry basis), in grams of compound per Kg of dry cotyledons.

	Oxalic acid	Citric acid	Tartaric acid	Malic acid	Succinic acid	Lactic acid	Acetic acid	Propionic acid
F 0	16.03 ± 0.19^{a,b}	8.38 ± 0.28^{b}	0^{d}	1.56 ± 0.04^{b}	3.58 ± 0.10^{g,h}	0^{d}	7.35 ± 0.33^{f,g,h}	5.08 ± 0.00^{g}
F 3	9.13 ± 0.42^{h,i}	5.57 ± 0.36^{d}	0.75 ± 0.00^{c}	0^{i}	9.89 ± 0.21^{b}	0^{d}	16.15 ± 0.23^{d}	5.50 ± 0.05^{f}
F 5	9.27 ± 0.51^{g,h,i}	3.80 ± 0.04^{e}	0^{d}	0.67 ± 0.13^{f,g,h}	2.28 ± 0.15^{i}	8.79 ± 0.28^{a}	12.48 ± 0.22^{e}	7.11 ± 0.04^{a,b}
F 6	8.71 ± 0.33^{h,i}	5.73 ± 0.25^{d}	0^{d}	1.91 ± 0.03^{a}	6.72 ± 0.14^{d}	0^{d}	6.93 ± 0.27^{f,g,h}	6.65 ± 0.01^{c}
F 10	10.16 ± 0.25^{e,f,g,h}	5.40 ± 0.07^{d}	0^{d}	0.99 ± 0.02^{c,d}	4.11 ± 0.11^{f,g}	0.42 ± 0.01^{c}	5.77 ± 0.16^{g,h}	4.68 ± 0.03^{h}
FWE 0	16.03 ± 0.19^{a,b}	8.38 ± 0.28^{b}	0^{d}	1.56 ± 0.04^{b}	3.58 ± 0.10^{g,h}	0^{d}	7.35 ± 0.33^{f,g,h}	5.08 ± 0.00^{g}
FWE 3	11.12 ± 0.40^{d,e,f,g}	8.23 ± 0.09^{b}	0.76 ± 0.01^{b}	0^{i}	11.82 ± 0.69^{a}	0^{d}	20.45 ± 1.49^{c}	5.58 ± 0.03^{f}
FWE 5	12.97 ± 0.45^{c,d}	5.56 ± 0.36^{d}	0^{d}	0^{i}	9.14 ± 0.30^{b}	0^{d}	7.60 ± 0.44^{f,g}	6.07 ± 0.04^{d}
FWE 6	10.13 ± 0.50	2.22 ± 0.06^{f}	0^{d}	0.80 ± 0.03^{e,f,g}	12.46 ± 0.32^{a}	0^{d}	6.40 ± 0.10^{f,g,h}	5.80 ± 0.03^{e}
FWE 10	9.89 ± 0.13^{f,g,h}	5.56 ± 0.20^{d}	0^{d}	0.76 ± 0.02^{e,f,g,h}	11.70 ± 0.18^{a}	0^{d}	7.79 ± 0.40^{f}	4.58 ± 0.04^{h,i}
T 0	17.37 ± 1.09^{a}	10.74 ± 0.27^{a}	0.83 ± 0.00^{a}	0.73 ± 0.02^{f,g,h}	4.08 ± 0.10^{f,g}	0^{d}	5.47 ± 0.10^{h}	5.85 ± 0.02^{e}
T 3	11.99 ± 0.50^{d,e}	7.21 ± 0.39^{c}	0^{d}	1.43 ± 0.03^{b}	5.59 ± 0.33^{e}	0^{d}	26.22 ± 0.01^{b}	6.49 ± 0.05^{c}
T 5	11.24 ± 0.02^{d,e,f}	5.37 ± 0.06^{d}	0^{d}	1.44 ± 0.05^{b}	3.40 ± 0.03^{g,h}	0.94 ± 0.00^{b}	7.17 ± 0.01^{f,g,h}	7.28 ± 0.03^{a}
T 6	10.07 ± 0.33^{e,f,g,h}	8.57 ± 0.04^{b}	0^{d}	0.92 ± 0.00^{d,e}	3.70 ± 0.00^{g}	1.14 ± 0.03^{b}	22.42 ± 0.44^{c}	6.97 ± 0.06^{b}

	Oxalic acid	Citric acid	Tartaric acid	Malic acid	Succinic acid	Lactic acid	Acetic acid	Propionic acid
T 10	4.21 ± 0.12^{i}	3.05 ± 0.00^{e,f}	0^{d}	0.60 ± 0.00^{h}	2.58 ± 0.01^{h,i}	0^{d}	6.11 ± 0.23^{f,g,h}	4.43 ± 0.06^{i}
TWE 0	17.37 ± 1.09^{a}	10.74 ± 0.27^{a}	0.83 ± 0.00^{a}	0.73 ± 0.02^{f,g,h}	4.08 ± 0.10^{f,g}	0^{d}	5.47 ± 0.10^{h}	5.85 ± 0.02^{e}
TWE 3	12.97 ± 0.60^{c,d}	2.31 ± 0.08^{f}	0^{d}	0.90 ± 0.02^{d,e}	4.76 ± 0.42^{e,f}	0.53 ± 0.01^{c}	21.82 ± 0.49^{c}	6.49 ± 0.07^{c}
TWE 5	12.29 ± 0.38^{d}	8.40 ± 0.22^{b}	0^{d}	1.12 ± 0.04^{c}	5.49 ± 0.25^{e}	0^{d}	6.73 ± 0.36^{f,g,h}	7.21 ± 0.07^{a}
TWE 6	14.29 ± 0.31^{b,c}	3.86 ± 0.10^{e}	0^{d}	0.83 ± 0.03^{e,f}	7.78 ± 0.36^{c}	0.14 ± 0.00	37.39 ± 1.05^{a}	7.19 ± 0.10^{a}
TWE 10	7.76 ± 0.34^{i}	5.97 ± 0.09^{d}	0^{d}	0.64 ± 0.02^{g,h}	3.60 ± 0.21^{g,h}	0^{d}	10.93 ± 0.41^{e}	4.60 ± 0.06^{h,i}

*Mean value ± standard deviation; results having the same letter were not significantly different ($p < 0.05$)

Butyric and isovaleric acids were not found in the samples.

Table 2. Targeted volatile compounds in days 5 and 6 of fermentation, days 7 and 10 of drying, liquor and 70% dark chocolates production in Forastero and Trinitario hybrids, with and without addition of enzymatic extracts during fermentation. Mean GC-MS peak areas x 10^3.

Samples	Methyl Acetate	Ethyl acetate	2,3 Butanedione	2 Pentanone	Isobutyl acetate	1 Propanol	2Methyl, 1Propanol	Pyrazine	Acetoin
F5	**339**	**62**	**0**	**639**	**0**	**0**	**197**	**0**	**161**
F6	**39**	30	0	2873	0	0	113	0	178
F7	**13**	14	0	2232	0	0	57	0	0
F10	**3**	1	0	0	0	0	0	0	0
FLiq	**60**	43	0	0	0	0	0	36	0
FCho	**2**	2	0	0	0	0	0	0	0
FWE5	**0**	0	0	0	0	0	0	0	0
FWE6	**0**	0	0	0	0	0	0	0	19
FWE7	**0**	0	159	2849	0	0	101	0	294
FWE10	**32**	65	126	103	0	0	35	0	23
FWELiq	**1**	1	0	0	0	0	0	0	35
FWECho	**13**	25	0	0	0	0	0	0	37
T5	**0**	0	467	873	0	0	67	0	103
T6	**0**	81	588	3	0	0	29	0	1108
T7	**0**	0	702	2420	0	0	132	0	862
T10	**0**	0	302	0	0	0	18	0	4037 3
TLiq	**0**	0	0	0	0	0	0	0	1455
TCho	**29**	26	0	0	0	0	0	0	248
TWE5	**0**	133	0	177	0	0	283	0	0
TWE6	**0**	718	0	5	0	0	0	0	212
TWE7	**730**	855	1686	205	544	0	290	0	3059
TWE10	**0**	0	544	10	153	0	6	0	6991
TWELiq	**0**	0	0	0	0	0	0	0	94
TWECho	**10**	9	78	147	64	0	14	69	89

*Semi-quantification was by SPME-HS-GC-MS, expressed as mean peak area x 10^3

*F= Forastero cocoa; FEW = Forastero cocoa with enzymes; T= Trinitario cocoa; TWE: Trinitario cocoa with enzymes; Liq= cocoa liquor or mass; Cho: 70% dark chocolate

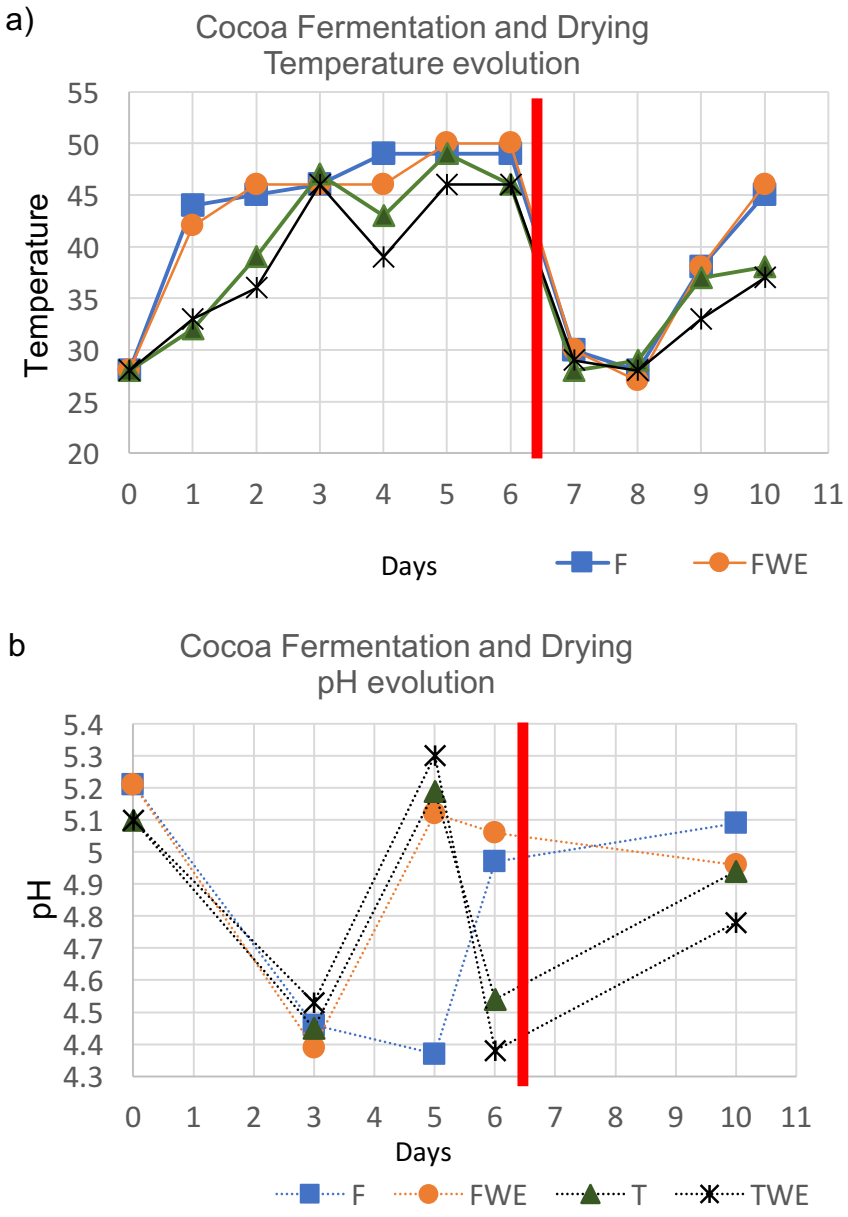
Table 2. Targeted volatile compounds in days 5 and 6 of fermentation, days 7 and 10 of drying, liquor and 70% dark chocolates production in Forastero and Trinitario hybrids, with and without addition of enzymatic extracts during fermentation. Mean GC-MS peak areas x 10^3.

Samples	Ethylpyrazine	Tetramethylpyrazine	Propionic acid	Isobutyric acid	Butyric acid	Acetophenone	Pentanoic acid	Hexanoic acid	Octanoic acid	Nonanoic acid
F5	**0**	**0**	**406**	**2031**	**0**	**354**	**0**	**0**	**0**	**0**
F6	0	0	338	6501	0	1796	0	0	0	0
F7	0	0	218	1595	0	1866	277	0	0	59
F10	0	0	42	125	0	136	5276	277	19	37
FLiq	41	444	283	892	0	573	60	197	110	84
FCho	5	56	0	92	0	0	117	284	445	503
FWE5	0	0	55	297	0	215	0	0	0	0
FWE6	0	0	27	549	0	325	168	0	44	0
FWE7	0	0	34	4871	0	950	55	0	75	0
FWE10	0	0	619	1247	0	374	351	0	96	0
FWELiq	3	33	225	67	0	11	7	0	47	0
FWECho	5	248	164	344	0	56	24	0	54	40
T5	0	0	0	2218	0	666	0	0	0	0
T6	0	0	0	1123	0	1046	0	0	0	0
T7	0	0	0	5162	0	1129	485	0	0	0
T10	0	0	0	1883	0	280	76	0	0	0
TLiq	6	629	245	84	0	59	0	0	0	0
TCho	28	929	513	731	0	0	0	0	0	39
TWE5	0	0	0	3850	0	515	445	0	0	0
TWE6	0	0	69	46	0	67	248	0	0	0
TWE7	0	0	1743	1207	0	1205	310	0	0	0
TWE10	0	0	2269	1215	0	516	79	0	0	0
TWELiq	9	833	239	722	0	189	2	0	0	0
TWECho	294	800	181	419	620	1089	1112	1706	2525	2652

*Semi-quantification was by SPME-HS-GC-MS, expressed as mean peak area x 10^3

*F= Forastero cocoa; FEW = Forastero cocoa with enzymes; T= Trinitario cocoa; TWE: Trinitario cocoa with enzymes; Liq= cocoa liquor or mass; Cho: 70% dark chocolate

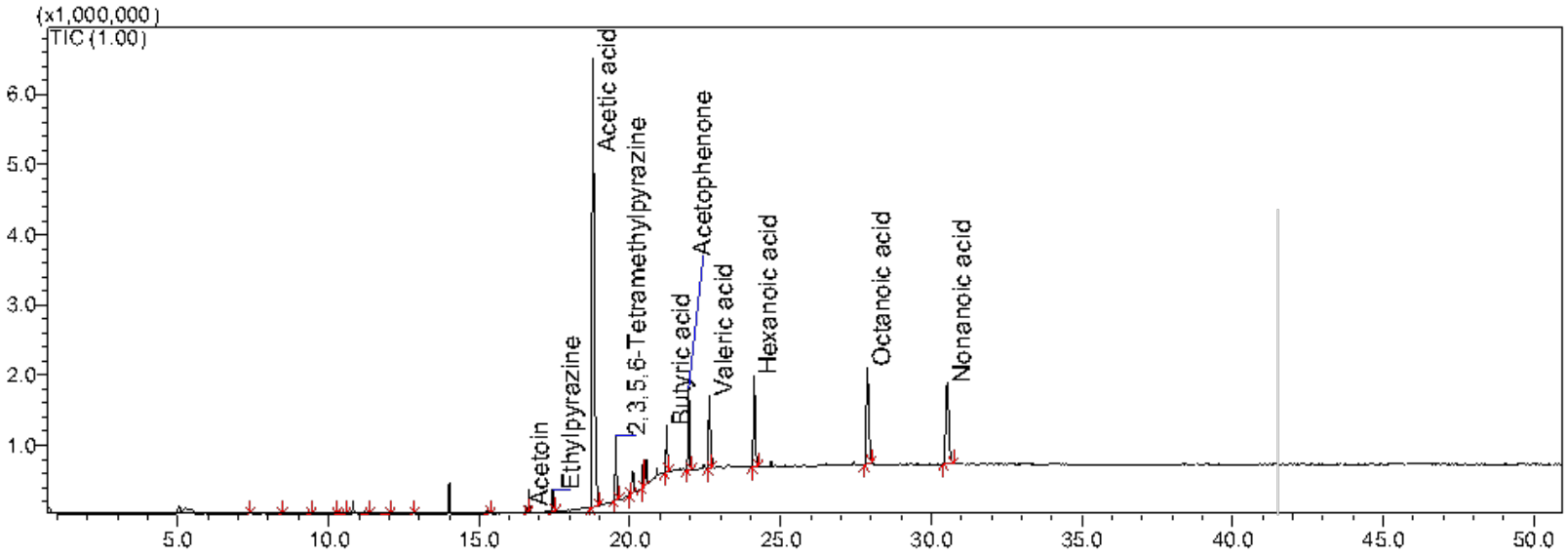
Figure1. Evolution throughout fermentation and drying process of F*, FWE, T, TWE: a) Temperature ; b) pH.



Days 1 – 6 : Fermentation
Days 7 – 10: Drying

*F= Forastero cocoa; FEW = Forastero cocoa with enzymes; T= Trinitario cocoa; TWE: Trinitario cocoa with enzymes

Figure 2. GC-MS chromatogram of the Trinitario Chocolate with the identification of the target compounds peaks.



5.3 ADAPTAÇÃO DO ARTIGO CIENTÍFICO 3

Electronic nose assessment as industrial quality control of liquor and dark chocolate obtained from cocoa (*Theobroma cacao L.*) beans fermented with multi-enzymatic extracts

Abstract

The effect that a multi-enzymatic system added to Forastero and Trinitario cocoa prior to the fermentation had on the liquor and dark chocolate were assessed by Heracles II electronic nose and trained judges, and correlated by Partial Least Squares (PLS). Chocolate notes as 2/3-methylbutanal, 2-methylfuran were associated to Trinitario chocolate and the acidic, pungent notes, to the Trinitario with enzymes. No differences were identified in liquors and chocolates between Forasteros with enzymes and conventional, but differences between Trinitario chocolates with enzymes and conventional were perceived by both, instrumental and trained judges, related to cocoa flavor. The best model was obtained by correlating cocoa aroma to the electronic nose data using columns MXT-5 (Q^2=0.992) and MX1701 (Q^2=0.917). The models obtained for liquors did not have a good quality index. Forastero chocolate with enzymes was the most accepted by flavor among the tests. The correlation between instrumental and sensory profile can be used for quality control purposes for cocoa flavor.

Keywords: QDA, ultrafast chromatography, enzymes, Forastero, Trinitario

1. Introduction

Chocolate flavor is essential for its overall quality and acceptance by consumers. Liquor, a cocoa mass obtained from the cocoa beans, is the main ingredient for dark chocolate production and its sensory profile evaluation by professionals are decisive for industrial purposes. Some flavor precursors as reducing sugars, amino acids and peptides, as well as aromatic compounds as esters and ketones, are associated to desired sensory descriptors in liquor and chocolate, and are produced during cocoa fermentation and drying. The final desired compounds, as pyrazines, are developed during roasting for liquor production, and conching to obtain the chocolate. (Aprotosoaie et al 2016; Kongor et al., 2016; Misnawi et al., 2004)

Modifications in the process have been applied to improve aroma and flavor in liquors and chocolates, including the addition of enzymes or starter cultures during fermentation, drying or prior to roasting (Crafack et al., 2013; 2014; T, 2012; Tr, 2012).

Traditional gas chromatography, coupled to mass spectrometer or FID detector can identify and quantify volatile compounds related to aroma and flavor, as well as sensory evaluation but the high cost and time-consuming are disadvantages for the food industry (Afoakwa et al., 2009; Crafack et al., 2014; Diab et al., 2014),

Electronic nose based on ultrafast gas chromatography is a faster, lower cost and reliable alternative to measure volatile compounds like human olfactory

perception, suitable to the food industry quality control (Loutfi et al.,, 2015; Mildner-szkudlarz et al.,, 2007; Tran et al., 2015; Wojtasik-Kalinowska et al., 2016).

Correlations between instrumental aroma measurement and sensory analysis in chocolate to investigate an instrumental technique similar to the human perception, were reported (Owusu et al., 2013; Queiroz & Garcia, 1999), but a precise relationship has not been found.

The aim of this work was to correlate the volatiles' profile, obtained by the electronic nose, of Trinitario and Forastero cocoa liquors and dark chocolates produced from conventional and enzymatic fermented beans, to the QDA descriptor terms established by trained judges, and compare the tested chocolates acceptance to commercial products.

2. Material and Methods

2.1 Material

Chemical standards acetoin, acetophenone, 2,3-butanedione, 2-pentanone, 1-propanol, 2-methyl-1-propanol, methyl acetate, ethyl acetate, isobutyl acetate, pyrazine, ethylpyrazine, 2,3,5,6-tetramethylpyrazine, ethanol, the acids acetic, propionic, isobutyric, butyric, pentanoic, hexanoic, octanoic, nonanoic (Sigma-

Aldrich®), alkane standard mixture (C6-C16) (Restek®), Trinitario and Forastero cocoa (*Theobroma cacao L.*) beans (Fazenda Ceará), and the Prozyn® multi-enzymatic extract system combining by Sweetmax 200® (3.2.1.26 β-fructofuranosidase), Brauzyn® (3.4.22.2 Papain) and Powercell® (EC 3.2.1.6 Endo-β-1,3-glucanase, EC 3.2.1.4 Endo-β-1,4-glucanase, EC 3.2.1.8 Endo-β-1,4-xylanase, EC 3.2.1.39 Glucan-endo-1,3-β-D-glucosidase) were used in this study.

2.2 Methods

2.2.1 Samples production

Trinitario (T) and Forastero (F) pods were harvested and opened on Ceará farm, in Linhares (Espírito Santo, Brazil). The seeds of each variety were divided into 2 batches of 600kg for the fermentation in 1m^3 wooden boxes: naturally fermented as control (F and T), and mixed with 50g of the multi-enzymatic extracts (FE and TE). The four boxes (F, T, FE, TE) were closed with lids for 48 hours, when the daily turnings started, up to day 6 (144h). Subsequently, the beans were dried in perforated trays inside a greenhouse for four days, up to day 10 (240h).

Dried beans were transported to the factory (Espírito Cacau, Vitória, Brazil), pre-roasted, deshelled, cleaned, roasted at 125°C for 15 minutes and milled to obtain the cocoa liquor, in a semi-continuous industrial system (Meller, Espírito Santo, Brazil). The chocolates were conched for 8 hours in a 50 Kg mixer-conche-refiner (Meller, Espírito Santo, Brazil), where sugar, cocoa butter and were added to the

liquor. Two commercial 70% dark chocolates, manufactured in France (ISTD) and a Brazilian chocolate (NSTD) were purchased in a local market for the acceptability test. Portions of liquor, commercial and teste chocolates were melted, manually tempered, molded in 5g tablets, and packed until analysis.

2.2.2 Electronic nose

Volatile compounds analysis was carried out in the electronic nose HERACLES II (Alpha M.O.S, Toulouse, France), an ultrafast gas chromatography system with a low-polarity MXT-5 and medium-polarity MXT-1701 capillary columns (10 m, 0.18mm id, 0.40 µm film thickness), coupled to two flame ionization detectors (FID), using hydrogen as carrier gas, resulting in two simultaneous chromatograms after a 120 seconds run. Triplicates of 20mL vials containing 2.0 g of ground samples were incubated for 15 min under agitation (500 rpm) at 60°C, and 5mL from the headspace were injected into the Tenax trap where the injector temperature was 200°C. The trapping duration was 30s, at initial temperature 40°C and final 240°C, at 80kPa and split 10mL/min. The oven temperature program was 40°C for 5s, increased to 270°C at a rate of 4°C. s^{-1}, and the detectors were kept at 280°C. The carrier gas flow rate was 0.41 $mL.min^{-1}$ for each column. External standards were analyzed under the same conditions as the samples. Alkanes standard mix (20 µL; n-hexane to n-hexadecane) was used to convert retention time in Kovats index and identify the compounds with the AroChemBase database. The results were expressed as relative peak areas.

2.2.3 Cocoa liquor sensory profile

Liquors from naturally fermented control (TLiq and FLiq) and from the batches treated with enzymes (TELiq and FELiq) were prepared according to Queiroz & Garcia (1999). A suspension of boiling water containing 5% (m/m) of cocoa liquor was served at 50°C to establish the sensory profile using Quantitative Descriptive Analysis (QDA®) (Stone and Sidel, 1993).

Twenty volunteers were recruited for a pre-selection of evaluators, using triangular analysis to test their ability to discriminate the basic tastes, and the results were evaluated by WALD's sequential analysis (Amerine *et al.,* 1965).

Twelve pre-selected judges listed the descriptive terms during preliminary training sessions, after tasting all the chocolates, 3 samples at a time, and comparing them in pairs, according to the Grid's Repertory Method (Moskowitz, 1983). The final list of sensory descriptors, definitions, references and intensity was developed after the group consensus (Table 1 supplementary).

Four training sessions with the reference materials were done using the 9-cm unstructured scale with defined extremes for each descriptor term, generated by the judges. The final team had 10 trained judges selected based on significant discrimination (pF_{sample}<0,30) and reproducibility ($pF_{repetitions}$>0,05) (Damasio, Costell, 1991, Luís et al., 2009; Palazzo et al., 2011) . All the samples were evaluated in four repetitions by each selected judge, in monadic presentation, using

132 balanced incomplete block design. Sensory data were collected using Fizz Sensory
133 Network Model H 2:40 (Biosystemes, Couternon, France 2009).

134

135 ### 2.2.4 Dark chocolate sensory profile

136

137 TCho, TECho, FCho and FECho were served as 5g tablets to generate the
138 sensory profile, using QDA® as described for cocoa liquor.

139 Twenty volunteers were recruited, and 14 pre-selected judges listed the
140 descriptive terms and the reference materials for the chocolates, used in five training
141 sessions (Table 2 supplementary). The final team had 13 judges, that work at the
142 sensory analysis laboratory at UNICAMP, and reported previous experience in
143 chocolate sensory evaluation.

144

145 ### 2.2.5 Acceptance

146

147 The consumer group (n = 120) consisted of 65% women, graduate and
148 undergraduate students, between 18 and 62 years old, all regular chocolate
149 consumers, evaluated the four chocolate samples and the 2 commercial 70% dark
150 chocolates. The acceptance test occurred in a single session using monadic
151 presentation, balanced samples and a 9-cm unstructured scale with anchors “dislike
152 extremely” and “like extremely”, to evaluate the descriptors: appearance, aroma,
153 flavor, texture and overall liking (Luís et al., 2009).

154

155 **2.3 Statistical analysis**

156

157 Statistica 12 Ultimate Academic Bundle software (Dell Inc., USA), XLSTAT
158 Statistical Analysis system (2015) and Statistical Analysis System - SAS (2017) were
159 used for statistical analysis.

160 Analysis of variance (ANOVA) was generated to evaluate QDA® and Acceptance
161 test data. The mean ratings were calculated using Tukey´s test (p=5%) (Luís et al.,
162 2009).

163 Principal Component Analysis (PCA) was used to evaluate and compare the
164 effects of the enzymes on the liquors and chocolates' volatile compounds profiles,
165 and to generate the Internal preference map for the 6 chocolates ((Luís et al., 2009).

166 Partial Least Squares (PLS) was applied to generate External preference maps
167 to correlate data from electronic nose (both columns), sensory descriptors and the
168 consumers' preferences. Hierarchical Cluster Analysis (HCA) was applied to group
169 samples according to similarities, using Euclidean distances and Ward linkage
170 method (Owusu et al., 2013, Pimentel et al., 2015).

171

172 **3. Results and Discussion**

173

174 **3.1 Electronic nose**

175

176 The relative mean peak areas of volatile compounds in the liquors and chocolates
177 using columns MXT-5 and MXT1701, and the sensory descriptors are in Table 1. A
178 summary of the HCA samples grouping and PCA are in Table 2.

179 PCA and HCA carried out for liquors, using columns MXT-5 and MXT-1701 (Fig1
180 a, b), grouped samples FLiq and FELiq in the same cluster, indicating the effect of
181 enzymes was not detected, while liquors TLiq and TELiq were grouped in different
182 clusters, demonstrating that the enzymes probably modified the volatiles profile in
183 TELiq. The exception was the third FE replicate, which was clustered with TLiq using
184 column MXT-1701. Samples TLiq were associated with methyl acetate and
185 acetaldehyde, while TELiq were related to acetic acid and propanal, characterized
186 as acidic pungent profile. FLiq and FELiq were correlated to 2-propanone, 2-
187 methylbutanal, 1-propanol, 3-methylbutanal and 2-methylfuran, described as strong
188 chocolate notes (Table 1). Those compounds were found in our previous work (Brito
189 et al., 2016) as untargeted compounds. Diab et al (2014) found 2-methylfuran in
190 roasted beans. Acetaldehyde, a Strecker aldehyde, that play a key role in dark
191 chocolate flavor formation (Afoakwa et al., 2009) , was found during fermentation
192 process by Brito et al (2016) and Ramos et al (2014) using silica WAX polar
193 columns, and in roasted beans by Diab et al. (2014).

194 Chocolates FCho and FECho, were grouped using column MXT-5, and TCho
195 and TECho were separated in different clusters (Fig1 c). Using column MXT-1701,
196 the separation was not clear for FCho samples.

TECho was mostly associated to acetic acid and acetaldehyde, while TCho was correlated to 1-propanol, methyl acetate, 2-propanone, acetaldehyde, 2-methylbutanal and 2-methylfuran. Samples FECho were not connected to specific compounds and FCho could be associated with acetic acid and acetaldehyde using column MXT-1701 (Fig1d). Mexis et al (2010) found 2-propanone in dark chocolates.

The four chocolates and liquors presented high acetic acid concentration, using both columns, characteristic of Brazilian cocoa beans, fermented in boxes (Papalexandratou et al. , 2011).

PCA scores carried out for the 4 liquors and 4 chocolates displays 88.74% as total variance using MXT-5, and 86.06% using MXT-1701 (Fig1 c, d and Table 2), grouping FCho, FECho and TECho in the same cluster, as well as FLiq and FELiq using both columns, despite their polarities. The only difference between the columns was the classification of TCho.

Using column MXT-5 (Table 2), differences between varieties, Forastero and Trinitario, in conventional liquors and chocolates were identified, as well as in enzymatically treated liquors, but not in the enzymatically treated chocolates. Differences between treatments, conventional and enzymatic, for the same variety, were detected for Trinitarios' liquor and chocolate, but not for Forastero. However, differences between Trinitario liquor and chocolate were only found in enzymatically treated beans using MXT-5.

The most important difference comparing to column MXT-1701 is that TLiq and TCho were grouped in different clusters, indicating that using this column, it might

219 be possible to differ conventional Trinitario liquors and chocolates according to their
220 volatile compounds profile, although grouping TCho with FLiq and FELiq might
221 require further investigation.

222 Pyrazines and other volatile acids, detected in liquors and chocolates by
223 traditional SPME-HS-GC-MS using a polar WAX column in our previous work (Brito
224 et al., 2016), were not found in the samples using dynamic headspace. Furthermore,
225 the limited available literature for the columns coupled to FID reinforces the need of
226 complementary standards to identify compounds.

227

228 **3.2 QDA Liquors**

229

230 Differences between treatments and variety were investigated. The mean
231 intensity values of the QDA sensory descriptors in liquors are in Table 3 and the PCA
232 is in Fig1 supplementary, which displayed 72.76% as total variance, using brightness
233 (Bri), sweet aroma (SwA), bitter taste (BiT), bitter aftertaste (BiAft), acid taste (AcT),
234 viscosity (Visco), and the non significative descriptors brown color (BrC),
235 homogeneity (Homo), cocoa aroma (CoA), cocoa butter aroma (CBuA), cocoa flavor
236 (CoF), were set as supplementary variables.

237 Trained judges did not discriminate the samples FELiq, characterized as BiT and
238 aftertaste and TELiq, intense CoA, grouping FLiq and TLiq in the same cluster. They
239 identified similarities ($p \leq 0.05$) related to aroma and flavor between varieties in FLiq
240 and TLiq for BiT, BiAft and differences in AcT, which was higher in conventional

241 Trinitario (Table 3). The enzymes enhanced BiT and AcT in Forastero. Sweet aroma
242 was reduced in TELiq. The AcT increased in FELiq compared to the conventional
243 FLiq.

244 Although cocoa liquors are usually characterized by bitterness and astringency,
245 the former was not identified in the samples, possibly due to a higher alkaloids
246 content when compared to polyphenols, as associations between lower astringency
247 and lower polyphenols content, to higher bitterness was described by Tran et al.,
248 (2015).

249 Negative correlation between SwA and CoA, as well as a positive correlation
250 between BiAft, BiT, CoA, AcT were found (Fig1, Fig2a,b supplementary). SwA and
251 CbuA were associated to 2-propanone and methyl acetate. CoF, AcT, BiT and BiAft
252 were related to 2-methylbutanal and 3-methylbutanal, while CoA was connected to
253 acetic acid and propanal. Correlation between acetic acid and acid flavor in liquors
254 was reported by Luna et al (2002), as well as between CoA and acidity (Misnawi et
255 al., 2004)

256 Although there was no difference in CoA among the samples ($p > 0.05$), this
257 descriptor was in the four liquors, as roasting is the main process to develop desired
258 CoA via Maillard reaction (Tran et al., 2015).

259 The correlation between instrumental and QDA scores by PLS had $Q^2 \leq 0.05$
260 PLS, meaning a low-quality model to correlate the techniques, possibly due to the
261 difficulty to evaluate some descriptors in liquors, as CoA (Luna et al., 2002).

Cocoa liquor is exclusively commercialized as an ingredient for the food industry, and for this reason, the liquors were not subjected to the consumers evaluation.

3.3 Chocolate Acceptance and QDA

Consumers (n=120) evaluated the 4 tested chocolates and 2 commercials samples (Table 4). Concerning overall liking, the samples F, FE, T, TE, ISTD and NSTD were equally preferred by the consumers (Table 4). Samples T and TE was the least preferred by the consumers. Comparing varieties and treatments, F and FE were preferred, when compared to the correspondent Trinitarios T and TE.

There was no preference among samples when evaluating aroma, but there were in appearance and texture, possibly because those descriptors are very related to the manual production of the six samples.

The internal preference map (Fig3 a,b supplementary) was built with the overall liking of each consumer for the 6 samples, using PCA and HCA. The most accepted by the consumers, represented by the greater number of dots close to the samples were NSTD and F, followed by FE. Consumers differed NSTD and ISTD from all the tests, as well as the varieties Forastero (F, FE) from Trinitario. (T, TE). However, consumers could not differ between conventional and enzymatically treated samples for the same variety.

Commercial standards ISTD and NSTD were selected by their higher market share for 70% dark fine chocolate, and were not subjected to chromatographic or

QDA evaluation. Comparing flavor scores between varieties, T (4.19) was lower than F (4.94) and TE (4.31) was lower than FE (5.04). Consumers did not differentiated samples NSTD, F and FE (Table 4).

Trained judges evaluated the chocolates according to the sensory descriptors (Table 3 supplementary) and the QDA mean intensity values are in Table 5. The descriptors evaluated were: brown color (BrC), cocoa aroma (CoA), cocoa butter aroma (CBuA), bitter aroma (BiA), sweet aroma (SwA), acid aroma (AcA), roasted aroma (RoA), bitter taste (BiT), acid taste (AcT), sweet taste (SwT), cocoa flavor (CoF), residual grease flavor (RgF), bitter aftertaste (BiAft) and melting in the mouth (Melt).

Cocoa flavor (CoF) was the only significant descriptor, and the highest scores were ascribed to TE (3.94) and T (3.38). Judges did not perceive differences between varieties Forastero (2.93) and Trinitario (3.38), as well as among T, F and FE. Those results corroborate the electronic nose data, which did not discriminate the enzymatic effect in Forasteros.

Comparing chocolates (Table5) to liquors' QDA scores (Table3), BiT, AcT and sweet aroma differed in the former, but not in the latter, while CoA was different in the chocolates. Possibly the innovative conching process influenced those results, by transforming compounds related to those softened descriptors which were not perceived in the chocolates and enhanced compounds related to CoA (Afoakwa et al., 2008).

305 QDA descriptors (X variable) for the 4 tested chocolates were correlated to
306 consumers' overall liking (Y variable) by PLS (Fig4. Supplementary) and had
307 Q^2=0.947, excellent prediction model coefficient to associate the consumers' and
308 judges' evaluation, explaining 100% of the overall liking mean values and 82.4% of
309 the QDA descriptors average values with 2 components. NSTD and ISTD were not
310 subjected to QDA analysis and thus, were not considered in the PLS.

311 Chocolate T was characterized as RoA and BrC, and TE, one the less accepted
312 samples, presented CoA, BiAft and CoF profile. F was classified as RgF, BiA, SwT
313 and CBuA, and FE, as SwA, AcT.

314 The descriptors that contributed to the consumers acceptance of the 4 tested
315 chocolates were determined by the variable importance in the projection (VIP)
316 greater than 0.8, and the regression coefficients representation (Fig.2) (Pimentel et
317 al., 2015, XLSTAT, 2009). CBuA and BiA contributed positively to the acceptance,
318 and CoF, negatively. Although BiaAft) and RgF presented VIP higher than 0.8, they
319 were not significant based on the coefficients (XLSTAT, 2009).

320 Besides CBuA and BiA, the most accepted FCho and FECho by consumers had
321 sweet taste and aroma profile, respectively, although they were not significant in
322 QDA (Rohm et al., 2009). CoF negative contribution corroborated the low
323 acceptance of TECho, highly CoF, CoA and considered as fine chocolate by
324 experts, probably because BiAft and RoA were also associated to TECho and TCho,
325 respectively.

The high positive correlation between CoF and BiAft, and BrC and RoA possibly indicates the consumers' perception as intense BiAft in high cocoa mass chocolates due to the strong CoA, and a darker chocolate associated to a more intense roasting aroma, although the processing and formulations were the same. (Delwiche, 2004)

3.4 Relationship of Electronic nose and Sensory data

The model to correlate electronic nose mean peak areas (X variable) and the significant QDA descriptor CoF mean values (Y variable) (Owusu, et al 2013) for the 4 samples (TCho, TECho, FCho, FECho) by PLS (Fig. 5 a, b supplementary) had a Q^2=0.992, using the column MXT-5, considered excellent prediction, explaining 93.5% of the electronic nose data and 99.8 % of the CoF scores, with two components. Using the column MXT-1701, the coefficient was Q^2=0.917, explaining 87.4% of the electronic nose data and 97.5% of the CoF values, using two components. Good correlations between instrumental and sensory analysis were previously reported by Owusu et al (2013), that obtained a coefficient 0.83 comparing GC-MS to GC-O) important compounds selected by trained judges in QDA.

Applying PLS using the 14 QDA descriptors (Y variable) and electronic nose data (X variables) using the columns MXT-5 and MXT-1701, resulted in Q^2<0.05, meaning that the descriptor considered not significant by trained judges do not

correlate to the volatile compounds, and the chromatograms represent the descriptor CoF.

Correlating the consumer's overall liking average (Y variable) to the 14 QDA descriptors and electronic nose data (X variables) (Fig. 6 a, b supplementary) resulted in Q^2=0.984 and Q^2=0.961 using columns MXT-5 and MXT-1701, respectively, while Q^2=0.664 and Q^2=0.735 correlating to CoF, and Q^2=0.979 and Q^2=0.967 correlating to aroma and flavor, meaning the consumers associate more sensory descriptors to chromatographic data for chocolate acceptance, as the similarities and differences are not perceived without training.

External preference maps (Fig 5 a, b supplementary) displays the similarities between FCho and FECho, the differences between TCho and TECho, as well as the enzymatic treatment applied to the samples, positioned in different quadrants using column MXT-5.

Acetic acid was positively related to CoA and BiT, also reported by Luna et al (2002), as BiAft, CoF to propanal, 3-methylbutanal, 2-methylbutanal and acetaldehyde (Fig 3 a,b, and supplementary Fig 6) . Afoakwa et al (2008) reported 2-methylbutanal, a degradation product of the amino acid leucine, as CoA.

RgF, BiA and CBuA were not related to compounds using the column MXT-5, but positively correlated to acetaldehyde using MXT-1701. RoA was associated to 1-propanol, methyl acetate, 2-propanone and 2-methylfuran. A compound similar to 2-methylfuran was associated to roasted meat-like by Aprotosoaie et al. (2016), Beckett (2009) and Rodriguez-Campos et al (2011), related 1-propanol to pungent

369 and sweet candy, as well as 2-propanone to malty, chocolate profile, validating our
370 results.

371 The External Preference Map for columns MXT-5 and MXT-1701 (Fig.3 a,b) are
372 similar, except for 1-propanol detected by MXT-5, and acetaldehyde, correlated to
373 RgF, BiA and CBuA.

374

375 **4. Conclusions**

376

377 A high positive relationship of electronic nose and sensory assessments of
378 chocolate was found. The highest correlations were obtained using the non-polar
379 column MXT-5, although a good model was obtained with the intermediate polarity
380 column MXT-1701.

381 Evaluations made by trained judges and the electronic nose identified
382 differences between varieties F and T, and the transformations in volatile
383 compounds in Trinitario chocolates, using enzymes in fresh cocoa. Those results
384 indicate the electronic nose was capable to measure changes in volatile compounds
385 associated to different varieties F and T, and to the enzymatic treatment in Trinitario,
386 and thus can be used in chocolate quality control.

387 Consumers did not differentiated tested and commercial samples. The less
388 preferred samples were the ones with high CoF profile, T and TE.

389

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Table 1a – Relative mean peak areas of volatile compounds tentatively identified in liquor* and chocolate** samples by Kovats index and external standards using columns MXT-5 and MXT-1701.

	Sensory Descriptors	**Kovats**	**RT (s)**	**TLiq**	**TELiq**	**FLiq**	**FELiq**	**TCho**	**TECho**	**FCho**	**FECho**
Column MXT-5											
Acetaldehyde	Strong pungent	379.94	12.11	22561^{a***}	13002^{bc}	14447^{b}	10996^{c}	10406^{c}	6681^{d}	6229^{d}	4590^{d}
Propanal	Pungent	422.57	13.49	9900^{bc}	19083^{a}	6712^{c}	6387^{c}	11620^{b}	11412^{b}	8374^{bc}	7417^{c}
2-Propanone	-		14.59	5685^{ab}	2978^{e}	6143^{a}	5514^{abc}	4411^{cd}	3389de	4676^{bc}	3526de
Methyl acetate	Fruity	500	15.97	6241^{a}	1972^{c}	4221^{b}	3981^{b}	4292^{b}	808^{d}	1258^{cd}	989^{d}
1-Propanol	Pungent, sweet, candy	541.69	17.3	7692^{ab}	4801^{b}	8784^{ab}	13292^{a}	6570^{b}	3072^{b}	3638^{b}	2557^{b}
Acetic acid	Acidic, sour, vinegar	617.02	19.84	64444^{cd}	199146^{a}	45986^{cd}	59139^{cd}	36878^{d}	145589^{b}	76433^{c}	55168^{cd}
3-methylbutanal	Malty, chocolate	657.38	23	11132^{bc}	14174^{abc}	16479^{ab}	21790^{a}	9213^{bc}	8639^{bc}	6802^{c}	5359^{c}
2-methylbutanal	Malty, chocolate	667.02	23.64	8014^{abc}	7364^{abc}	10312^{ab}	12647^{a}	5471^{bc}	4002^{bc}	3444^{c}	2721^{c}

*, ** Liq: cocoa liquor, Cho: dark chocolate, T - Conventional Trinitario. TE- Trinitario with enzymes, F – Conventional Forastero. FE – Forastero with enzymes.

*** Means in a row with different lowercase letters indicate differences between samples ($p \leq 0.05$) for the same compound.

Table 1 (Continuation) – Relative mean peak areas of volatile compounds tentatively identified in liquor* and chocolate** samples by Kovats index and external standards using columns MXT-5 and MXT-1701.

Column MXT-1701	Sensory Descriptors	Kovats	RT (s)	TLiq	TELiq	FLiq	FELiq	TCho	TECho	FCho	FECho
Acetaldehyde	Strong pungent	379.94	13.53	2127^{a}	1859^{abc}	1691^{abc}	1895^{abc}	1424^{c}	1651^{abc}	2011^{ab}	1493^{bc}
2-Propanone	-	476.72	14.23	18994^{a}	10182^{bc}	11349^{b}	8506^{cd}	8047^{cde}	6686^{def}	5643^{ef}	4302^{f}
Propanal	Pungent	555.11	16.6	9084^{ab}	18211^{a}	6301^{c}	6267^{c}	10911^{b}	10553^{b}	7740^{ab}	6763^{c}
Methyl acetate	Fruity	597.99	18.2	9342^{a}	3558^{c}	7990^{ab}	7732^{b}	6825^{b}	0^{d}	0^{d}	0^{d}
2-methylfuran	Chocolate burnt	639	20.39	7639^{abc}	4764^{bc}	8774^{ab}	12639^{a}	5749^{bc}	2613^{c}	3355^{bc}	2294^{c}
2-methylbutanal	Malty, chocolate	744.66	26.91	8556^{bc}	9760^{bc}	13133^{ab}	18218^{a}	7486^{bc}	5925^{bc}	5211^{bc}	4161^{c}
Acetic acid	Acidic, sour, vinegar	769.14	28.59	47233^{cd}	162730^{a}	27067^{d}	37001^{cd}	23782^{d}	115352^{b}	56632^{c}	39688^{cd}

Table 2 – Summary of the HCA and PCA* analysis obtained from both columns MXT-5 and MXT1701 for liquors and chocolates.

Samples	MXT-5	MXT-1701
Liquors	F = FE**	F = FE
		T ≠ TE
	T ≠ TE	Exception: sample FELiq3
Chocolates	F = FE	F = FE
		T ≠ TE
	T ≠ TE	Exception: sample FCho3
Liquors and Chocolates	TELiq	TELiq
	TECho	TECho
	FCho	FCho
	FECho	FECho
	FLiq	FLiq
	FELiq	FELiq
		TCho
	TCho	
	TLiq	TLiq

*The correspondent PCA loadings are displayed in supplementary material.

**Samples grouped in the same cluster were classified as similar

Table 3 – Mean intensity values of the Quantitative Descriptive Analysis (QDA) descriptor terms of cocoa liquors*

Descriptor term	Symbol	Liquors **			
		T	TE	F	FE
Appearance					
Brown color	BrC	7.01[a*]	7.01[a]	6.89[a]	7.11[a]
Homogeneity	Homo	4.93[a]	4.19[b]	4.88[a]	5.10[a]
Brightness	Bri	5.88[a]	4.51[b]	6.14[a]	5.6[a]
Aroma					
Cocoa aroma	CoA	5.98[a]	6.36[a]	5.68[a]	5.89[a]
Cocoa butter aroma	CBuA	3.71[a]	2.95[a]	3.40[a]	3.26[a]
Sweet aroma	SwA	3.00[ab]	2.55[c]	3.73[a]	3.32[ab]
Flavor					
Cocoa flavor	CoF	6.31[a]	6.38[a]	6.11[a]	6.46[a]
Bitter taste	BiT	3.98[ab]	4.12[ab]	3.62[b]	4.69[a]
Bitter aftertaste	BiAft	5.86[a]	6.23[a]	5.75[a]	6.70[a]
Acid taste	AcT	5.44[ab]	5.09[bc]	4.49[c]	6.09[a]
Texture					
Viscosity	Visco	4.63[a]	4.58[a]	3.95[b]	4.20[ab]

*Means in a row with different lowercase letters indicate differences between cocoa liquors ($p \leq 0.05$) for the same sensory descriptor (unstructured 9cm scale).

**Liquors: T – conventional Trinitario, TE- Trinitario with enzymes, F – conventional Forastero, FE- Forastero with enzymes

Table 4 - Acceptability of tested dark chocolates* and commercial samples**

Descriptor terms	Chocolates*					
	T	**TE**	**F**	**FE**	**ISTD**	**NSTD**
Appearance	5.84^{cd}	5.47^{d}	6.45^{ab}	6.15^{bc}	6.68^{ab}	6.81^{a}
Aroma	5.68^{a}	5.70^{a}	5.98^{a}	5.86^{a}	5.54^{a}	6.08^{a}
Flavor	4.19^{d}	4.31^{cd}	4.94^{abc}	5.04^{ab}	4.76^{bcd}	5.45^{a}
Texture	5.02^{c}	5.23^{abc}	5.67^{ab}	5.76^{a}	5.17^{bc}	5.44^{abc}
Overall liking	4.79^{bc}	4.77^{c}	5.37^{a}	5.32^{ab}	5.24^{abc}	5.66^{a}

*Chocolates: T (conventional Trinitario), TE (Trinitario with enzymes), F (conventional Forastero) and FE (Forastero with enzymes)

**Commercial samples: ISTD, NSTD

***Means in the same row with different lowercase letters indicate differences between chocolates ($p \leq 0.5$).

Table 5 – Mean intensity values of the dark chocolates* Quantitative Descriptive Analysis (QDA) descriptor terms

Descriptor term	Symbol	Chocolates **			
		T	TE	F	FE
Brown color	BrC	5.63 [a]	4.55 [a]	4,97 [a]	4,60[a]
Cocoa aroma	CoA	4.44 [a]	4.31 [a]	4.04 [a]	4.65 [a]
Cocoa Butter aroma	CBuA	3.20 [a]	3.05 [a]	3.49 [a]	3.59 [a]
Bitter aroma	BiA	3.59[a]	3.30 [a]	3.69[a]	3.76[a]
Sweet aroma	SwA	2.35[a]	2.31[a]	2.48[a]	2.66[a]
Acid aroma	AcA	1.63[a]	1.65 [a]	1.70[a]	1.38[a]
Roasted aroma	RoA	1.21[a]	1.23[a]	1.18[a]	1.08[a]
Bitter taste	BiT	5.20[a]	5.57[a]	5.15[a]	5.59[a]
Acid taste	AcT	2.10[a]	2.22[a]	2.25[a]	2.63[a]
Sweet taste	SwT	5.10[a]	4.77[a]	4.81[a]	4.91[a]
Cocoa flavor	CoF	3.38[ab]	3.94[a]	2.93[b]	2.89[b]
Residual grease flavor	RgF	2.17[a]	1.85[a]	2.46[a]	2.20[a]
Bitter aftertaste	BiAft	3.57[a]	3.69[a]	3.02[a]	3.38[a]
Melting in the mouth	Melt	4.41[a]	4.75[a]	4.52[a]	5.24[a]

Means in a row with different lowercase letters indicate differences between cocoa liquors ($p \leq 0.05$) for the same sensory descriptor (**unstructured 9cm scale**).

**Chocolates: T – conventional Trinitario, TE- Trinitario with enzymes, F – conventional Forastero, FE- Forastero with enzymes

Fig. 1 – Principal component analysis (PCA) biplot of liquors and chocolates electronic nose data with columns MXT-5 and MXT-1701

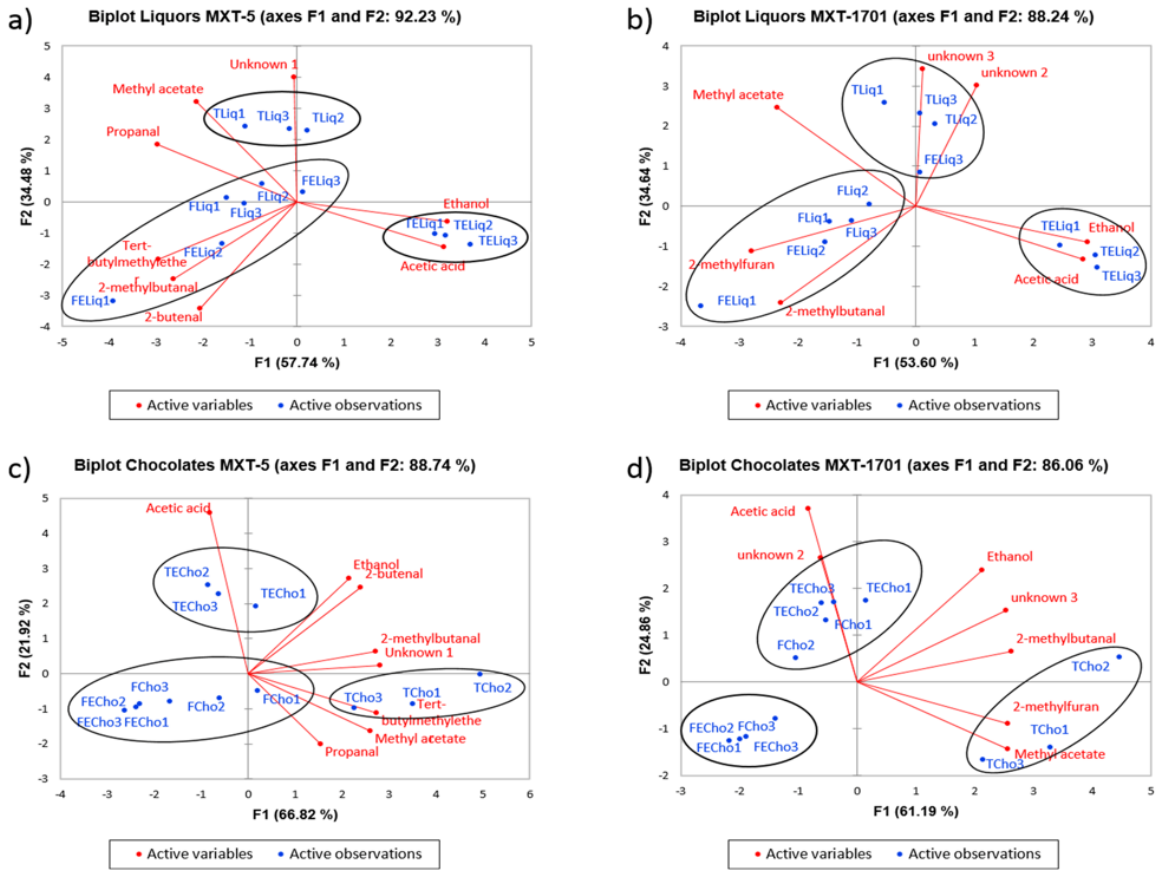
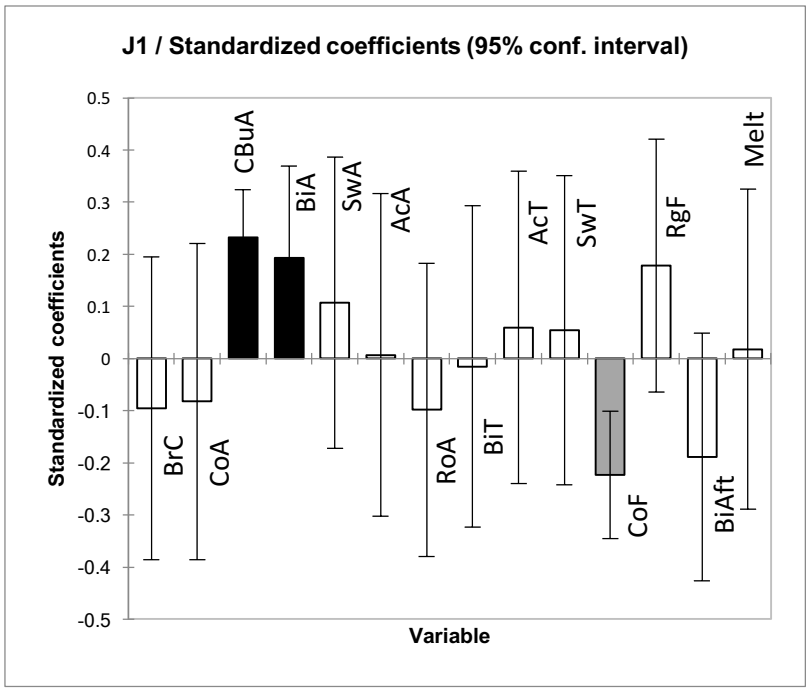


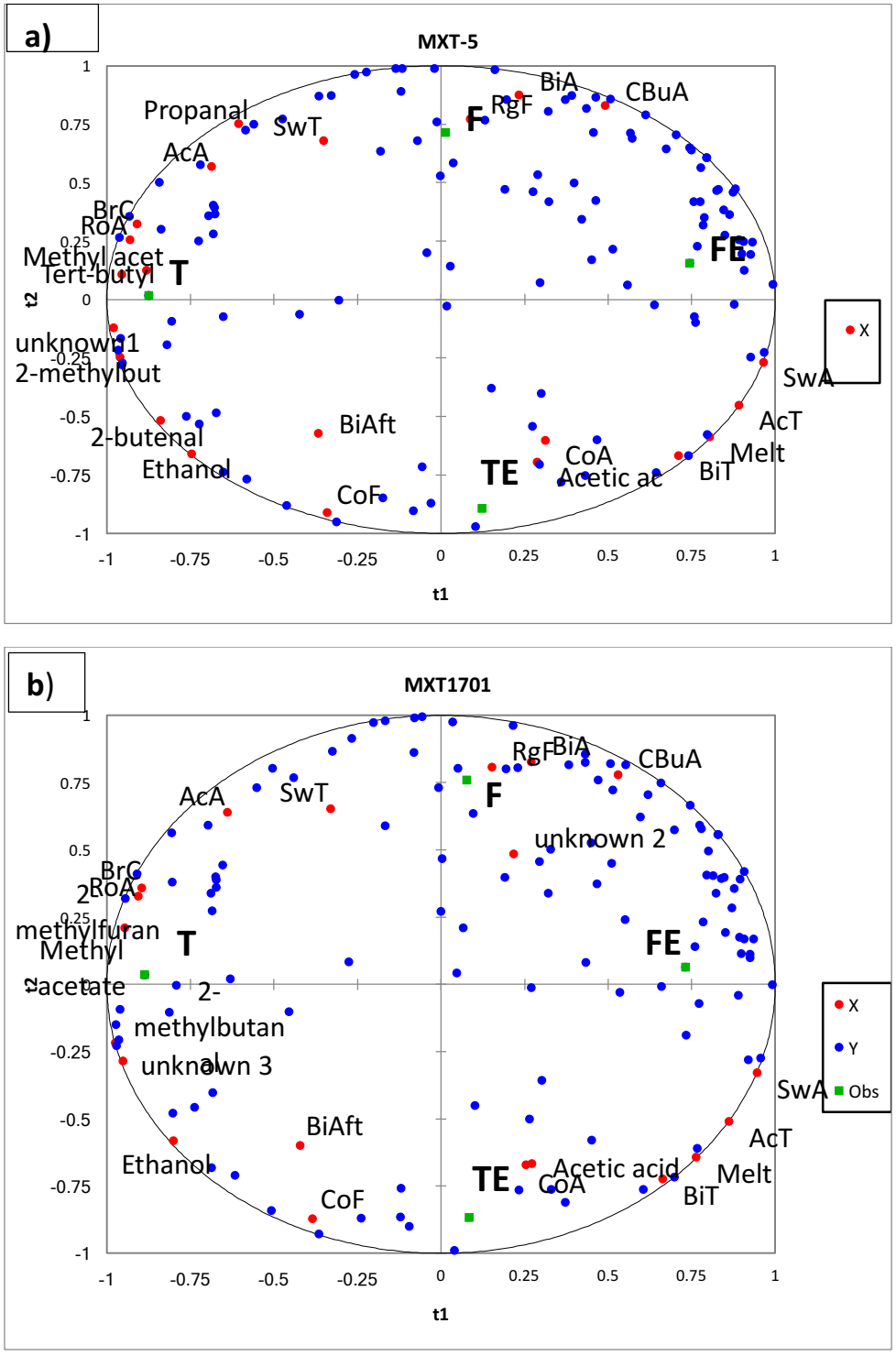
Fig. 2 – PLS regression coefficients, using QDA scores and overall liking for the chocolates F, FE, T and TE.



Contributions to the consumer acceptance by descriptor term: Black: positive influence on acceptance,

Grey: negative influence on acceptance, White: not significant influence on acceptance.

Fig. 3 – External preference map of the four dark chocolates F, FE, T and TE, correlating QDA scores, electronic nose data and overall acceptance: a) column MXT-5 and b) column MXT-1701



X: descritptive terms obtained from QDA; Y: consumers overall liking; Obs: samples evaluated

Supplementary Material for the Manuscript Entitled: Electronic nose assessment as industrial quality control of liquor and dark chocolate obtained from cocoa (*Theobroma cacao L.*) beans fermented with multi-enzymatic extracts

Table 1 - Composition of the enzymatic extracts.

Enzyme Preparation	Declared major activity	Reported side activities	pH range	Temp range ºC	Source
Sweetmax 200®	200.000 [1]SU/g	none	3,0 to 5,0	40-70	*Saccharomyces cerevisiae*
Powercell®	1180 U/g	arabinase	4,0 to 6,5	30-60	Trichoderma reesei
Brauzyn®	850 [2]TU/g	none	5,0 to 7,0	30-80	*Carica Papaya L*.

[1]Enzyme activity expressed as summer units (SU) per gram

[2]Enzyme activity expressed as tyrosine units (TU) per gram.

Table 2 - Descriptors, definitions and references samples developed by the sensory team for the evaluation of cocoa liquor.

Descriptors	Definition	Reference sample
Appearance		
Brown color (BrC)	Characteristic brown color of milk chocolate	Weak: Nestlé™ instant powdered chocolate Strong: Garoto™ semi-dark chocolate
Brightness (Bri)	Property of the sample to reflect the light	Weak: Barry Callebaut cocoa liquor Strong: Hershey´s milk chocolate
Homogeneity (Homo)	Characteristic of a uniform surface	Low: Barry Callebaut cocoa liquor A lot: Garoto semi-dark chocolate
Aroma		
Cocoa Aroma (CoA)	Characteristic aroma of roasted cocoa	Weak: Baton (Garoto) milk chocolate Strong: Barry Callebaut cocoa liquor
Sweet aroma (SwA)	Characteristic aroma of caramel formed by sugar, that releases a sweet aroma	Weak: 1.0g of Toddy cocoa mix in 200mL of water Strong: Laka (Kraft) white chocolate
Cocoa butter aroma (CBuA)	Characteristic aroma of cocoa butter	Absent: water Strong: Barry Callebaut melted cocoa butter

Flavor

Cocoa flavor (CoF)	Characteristic taste of powdered chocolate	Weak: Chocolápis (Pan) milk chocolate Strong: Nestlé instant powdered chocolate
Bitter taste (BiT)	Characteristic taste of caffeine aqueous solution	Weak: Baton (Garoto) milk chocolate Strong: Nestlé instant powdered chocolate
Acid taste (AcT)	Characteristic taste of acetic acid	Weak: Nothing Strong: acetic acid solution of Belmonti™ white wine vinegar, ratio 1:100mL
Bitter Aftertaste (BiAft)	Permanence of bitter taste in the oral cavity that occurs after tasting the product	Absent: water Strong: 25g of Toddy cocoa mix in 200mL of Líder whole milk and 0.15% stevia (Clariant) with Reb-A at 40%

Table 3 - Descriptors, definitions and references samples developed by the sensory team for the evaluation of 70% dark chocolate.

Descriptors	Definition	Reference sample
Appearance		
Brown color (BrC)	Characteristic brown color of dark chocolate	Weak: Arcor™ 53% dark chocolate Strong: Lindt™ 85% dark chocolate
Aroma		
Cocoa Aroma (CoA)	Characteristic aroma of dark cocoa	Weak: dutched cocoa powder Strong: Cargill™ cocoa liquor
Cocoa butter aroma (CBuA)	Characteristic aroma of cocoa butter	Low: Nothing A lot: Olan™ deodorized cocoa butter
Bitter aroma (BiA)	Characteristic aroma of cocoa liquor	Weak: Arcor™ 53% dark chocolate Strong: Cargill™ cocoa liquor
Sweet aroma (SwA)	Characteristic sweet aroma of dark chocolate	Weak: Lindt™ 85% dark chocolate Strong: Arcor™ 53% dark chocolate

Acid aroma (AcA)	Characteristic aroma of acetic acid	Weak: Nothing Strong: acetic acid solution of Belmonti™ white wine vinegar, ratio 1:100mL
Roasted aroma (RoA)	Characteristic aroma of cocoa liquor	Weak: Nothing Strong: ethylpyrazine ratio ratio 0.1:100mL
Flavor		
Bitter taste (BiT)	Characteristic taste of cocoa	Weak: Arcor™ 53% dark chocolate Strong: Lindt™ 85% dark chocolate
Acid taste (AcT)	Characteristic taste of acetic acid	Weak: Nothing Strong: acetic acid solution of Belmonti™ white wine vinegar, ratio 1:100mL
Cocoa flavor (CoF)	Characteristic taste of cocoa	Weak: dutched cocoa powder Strong: Lindt™ 85% dark chocolate
Residual greasy flavor (RgF)	Permanence of the cocoa butter taste in the oral cavity that occurs after tasting the product	Weak: dutched cocoa powder Strong: Arcor™ 53% dark chocolate
Bitter aftertaste (BiAft)	Permanence of the bitter taste in the oral cavity that occurs after tasting the product	Absent: dutched cocoa powder Strong: Lindt™ 85% dark chocolate
Texture		

Melting in the mouth (Melt)	Melting inside the oral cavity without mastication	Absent: Lindt™ 85% dark chocolate Strong: Arcor™ 53% dark chocolate

Fig. 1 – Principal component analysis (PCA) biplot of liquors' QDA scores

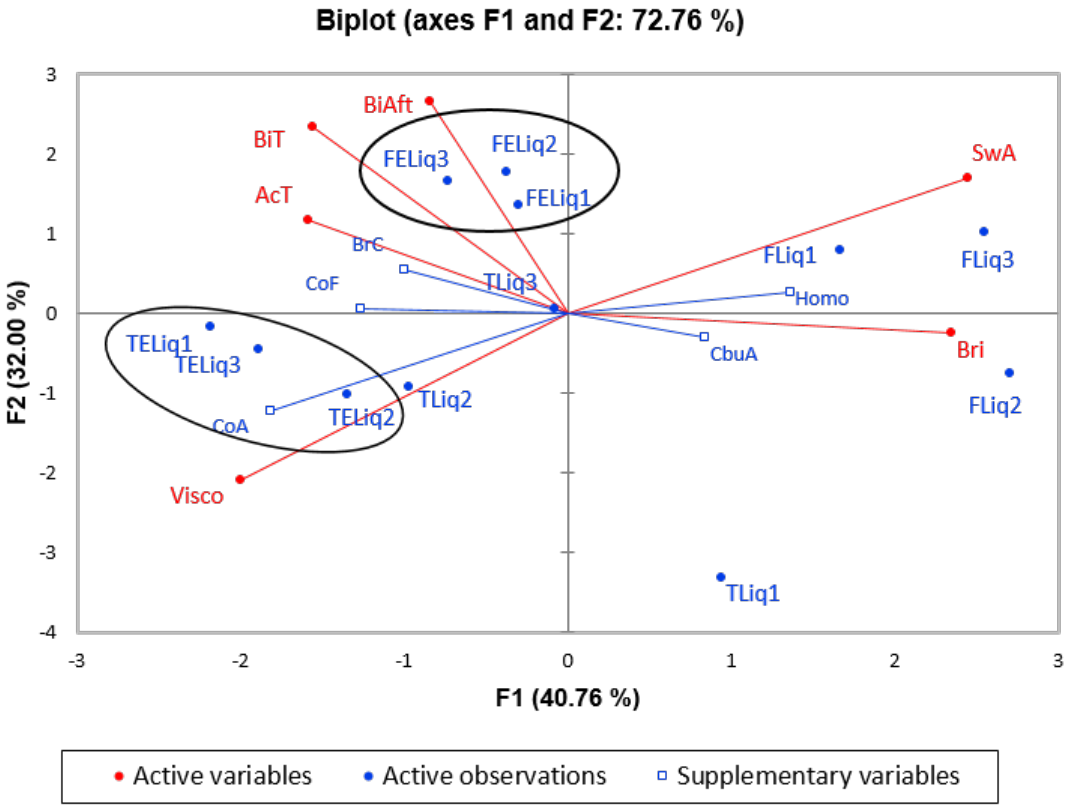
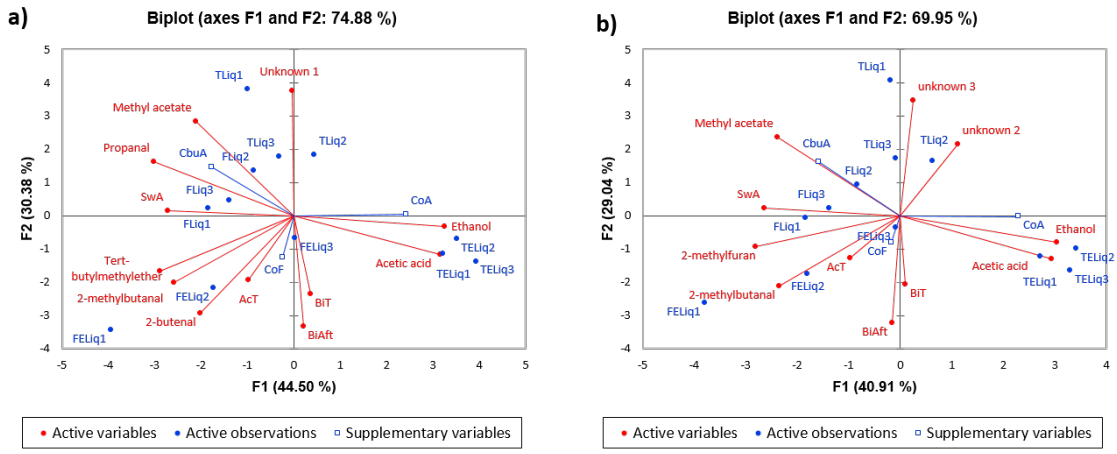


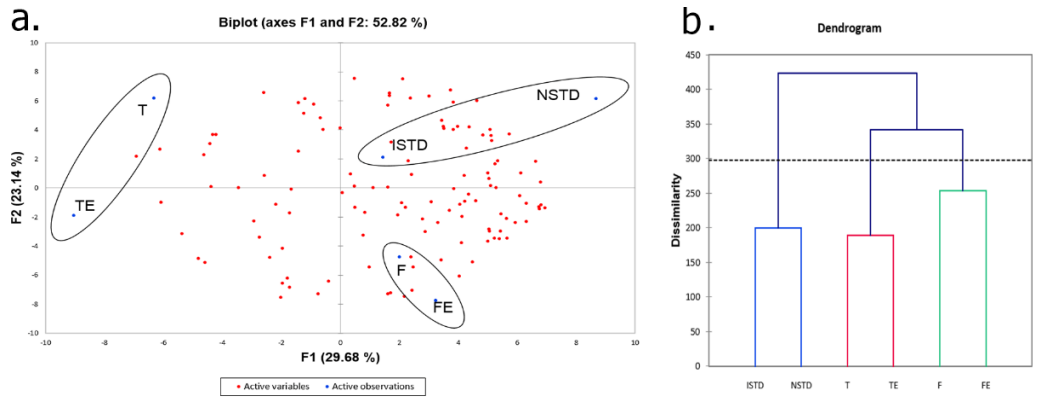
Fig. 2 – Principal component analysis (PCA) biplot of liquors'* QDA aroma and flavor scores and volatile compounds identified by electronic nose: a) column MXT-5 and b) column MXT-1701



*liquors:

F: conventional Forastero; FE: Forastero with enzymes; T: conventional Trinitario ; TE: Trinitario with enzymes

Fig. 3 – Internal preference map for chocolates* F, FE, T, TE, ISTD, NSTD, using biplot PCA (a) and dendogram (b)



*chocolates:

F: conventional Forastero chocolate; FE: Forastero with enzymes chocolate; T: conventionalTrinitario chocolate; TE: Trinitario with enzymes chocolate; ISTD: Imported commercial chocolate; NSTD: local commercial chocolate

Fig. 4 – External preference map of the dark chocolates F, FE, T and TE to correlate QDA and overall acceptance average scores.

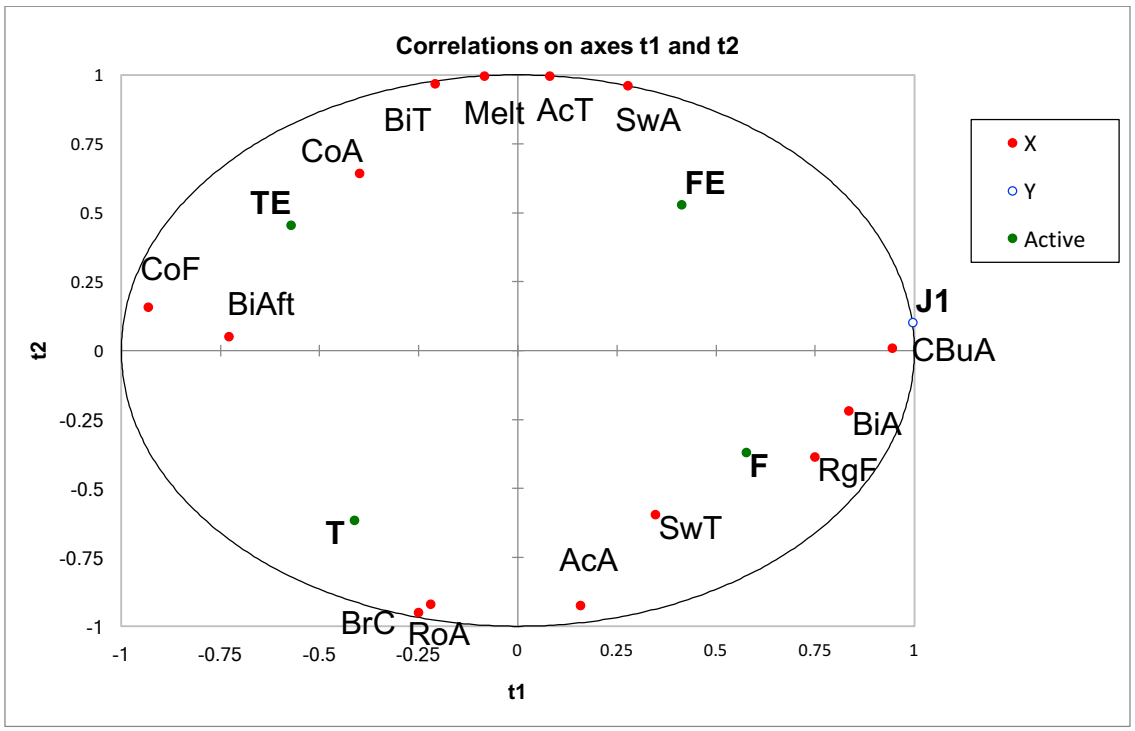


Fig. 5– External preference map of the dark chocolates F, FE, T and TE, correlating cocoa flavor (CoF) ADQ scores and electronic nose data: a) column MXT-5 and b) column MXT-1701

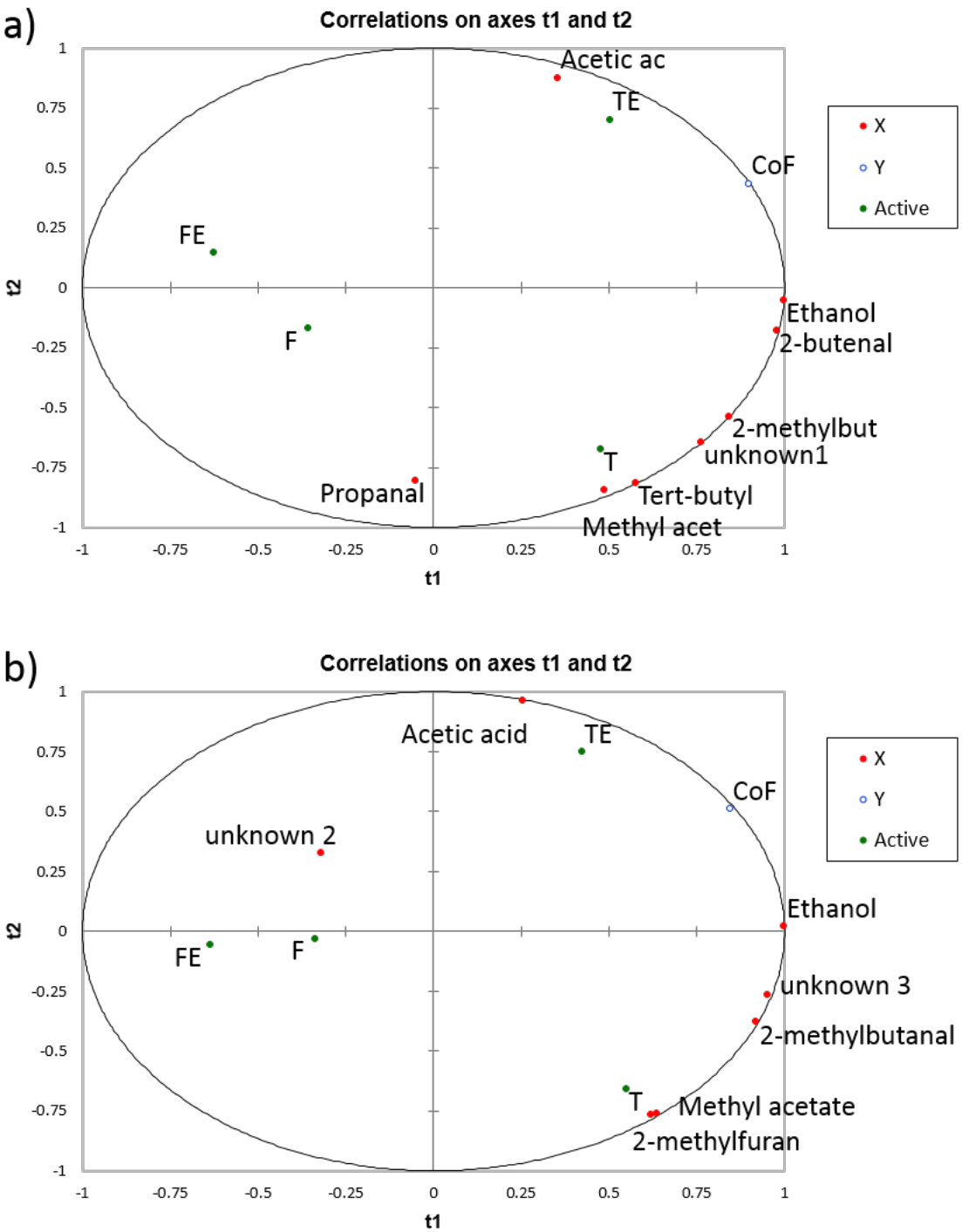
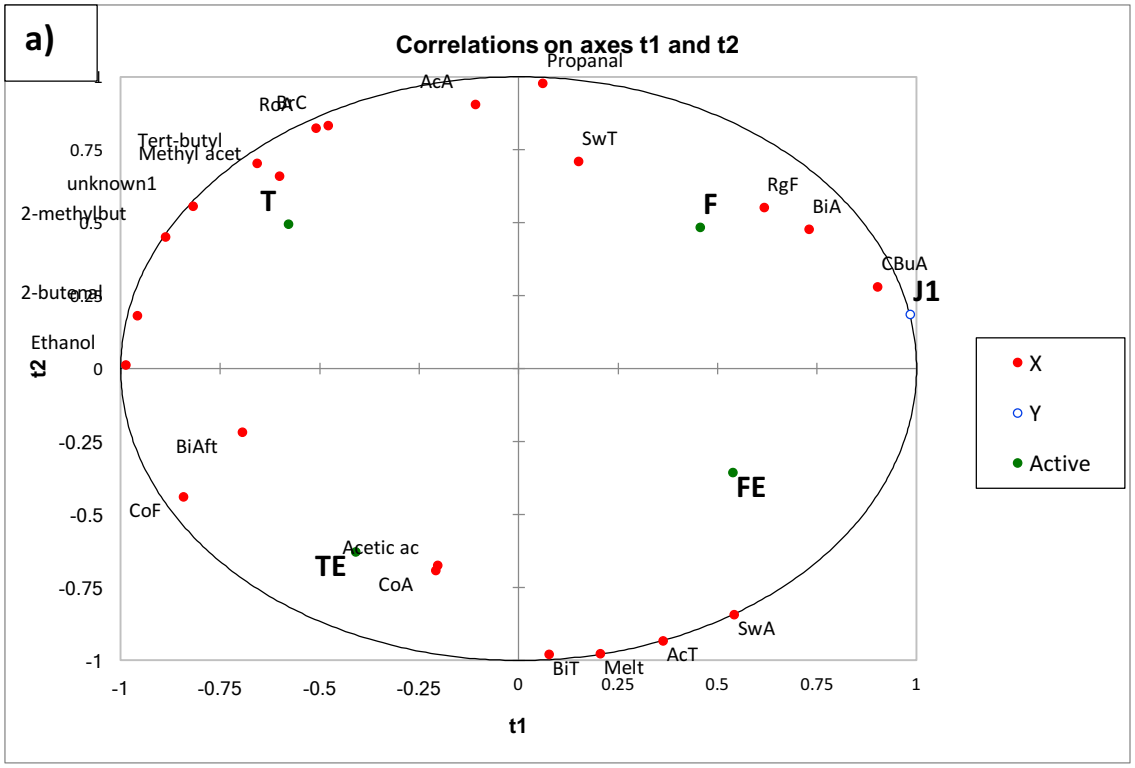
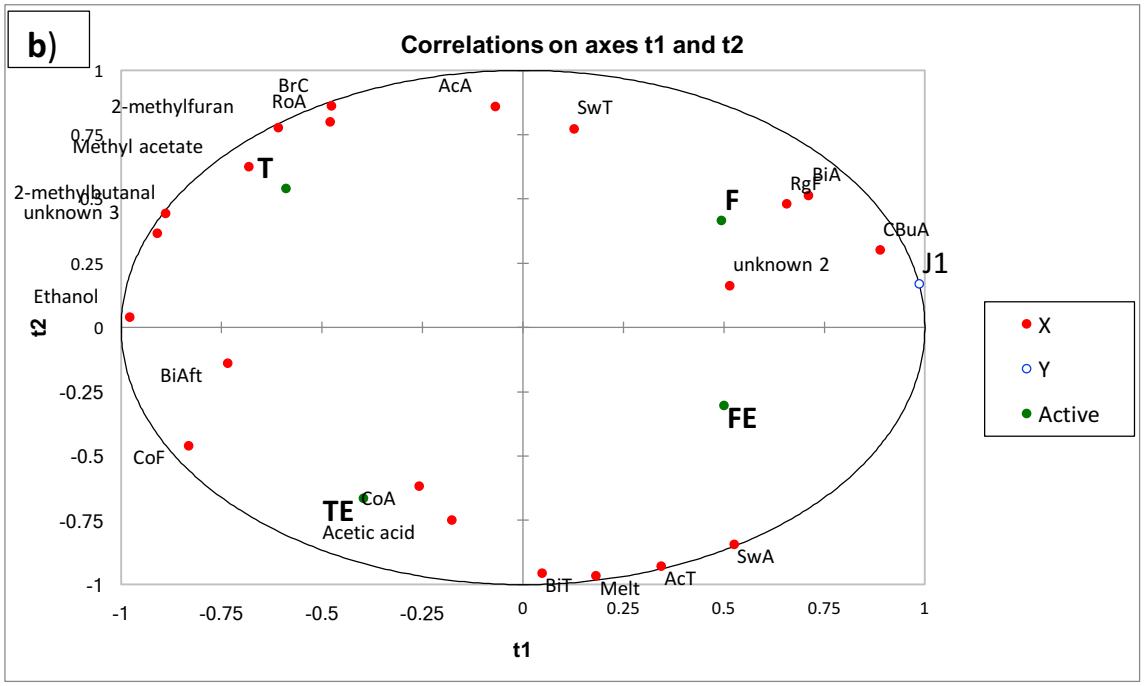


Fig. 6 – External preference map of the dark chocolates F, FE, T and TE, correlating ADQ scores, electronic nose data and consumers acceptance: a) column MXT-5 and b) column MXT-1701





6 CONCLUSÕES

- A avaliação da composição química dos cotilédones de cacau, especialmente o perfil de açúcares, ácidos, álcoois, metilxantinas e procianidinas, em diferentes clones e variedade, contribui para o melhor aproveitamento dos frutos e direcionamento da produção industrial.
- Clones de cacau com menor potencial para a produção de chocolate, podem ser utilizados alternativamente para a produção de extratos fenólicos a ser comercializado como ingrediente.
- A fermentação de cacau Forastero convencional apresentou potencial redução em 24horas (16%) sobre o tempo total de fermentação de 144horas.
- O rendimento na formação de precursores de sabor e aroma foi maior na fermentação convencional de cacau Trinitario, em comparação ao Forastero.
- A aplicação de extratos enzimáticos aumentou a produção de compostos aromáticos em cacau Trinitario, especialmente pirazinas e ésteres, em relação ao equivalente convencional.
- O nariz eletrônico foi capaz de identificar diferenças no perfil de compostos voláteis provenientes do uso das variedades Forastero e Trinitario, e entre tratamentos, com e sem aplicação de extratos enzimáticos.
- Os modelos preditivos de alta correlação entre os resultados obtidos com o uso do nariz eletrônico e avaliação por equipe sensorial treinada para chocolate, confirmam o potencial de aplicação deste instrumento ferramenta para controle de qualidade e suporte para desenvolvimento de novos produtos.
- O uso do nariz eletrônico como ferramenta para controle de qualidade de chocolates, especificamente para sabor de cacau, possibilita a redução do tempo de análise instrumental em 96% (de 51 minutos por GC-MS para 2 minutos)
- Não houve diferença em preferência dos consumidores pelos chocolates de cacau Forastero, Trinitario, e amostras comerciais
- A aplicação de misturas de extratos enzimáticos para a fermentação de cacau apresenta ótimas perspectivas para a melhoria da qualidade do liquor e chocolates.