UNIVERSIDADE FEEVALE

MESTRADO ACADÊMICO EM TOXICOLOGIA E ANÁLISES TOXICOLÓGICAS

DESENVOLVIMENTO E VALIDAÇÃO DE MÉTODOS PARA DETERMINAÇÃO DE ETIL PALMITATO E ETIL GLICURONÍDEO EM CABELO HUMANO COMO BIOMARCADORES DO USO CRÔNICO DE ETANOL

MARCOS FRANK BASTIANI

Linha de Pesquisa: Toxicologia Humana e Análises Toxicológicas

Orientador: Prof. Dr. Rafael Linden

Novo Hamburgo, agosto de 2019

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Dissertação apresentada para obtenção do GRAU DE MESTRE em Toxicologia e Análises Toxicológicas da Universidade Feevale.

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Dissertação intitulada "*Desenvolvimento e validação de métodos para determinação de etil palmitato e etil glicuronídeo em cabelo humano como biomarcadores do uso crônico de etanol"*, apresentada ao Programa de Pós-Graduação em Toxicologia e Análises Toxicológicas da Universidade Feevale, como requisito necessário para obtenção do grau de Mestre.

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DEDICATÓRIA

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RESUMO

O consumo crônico de etanol é considerado um problema mundial, estando associado a vários efeitos deletérios no organismo humano e com a ocorrência de diversas patologias, incluindo cirrose hepática, distúrbios neurológicos, patologias gastrointestinais e cardiovasculares. Parâmetros bioquímicos, como as atividades das enzimas aspartato aminotransferase (AST), alanina aminotransferase (ALT) e gama-glutamiltransferase (GGT), são usados para avaliar o comportamento de consumo de álcool, porém com baixa especificidade. A avaliação de biomarcadores mais específicos do consumo de etanol no cabelo é uma alternativa para avaliar a exposição crônica ao etanol. Os biomarcadores de exposição ao etanol em cabelo mais relevantes são o etil glucuronídeo (EtG) e os ésteres etílicos de ácidos graxos (EEAG), particularmente o etil palmitato (EtP), que são metabólitos minoritários do etanol. Neste trabalho, métodos para análise de EtP e EtG em cabelo humano foram otimizados e validados para a avaliação do consumo crônico de etanol. O método para determinação de EtP empregou micro extração em fase sólida por *headspace* e cromatografia gasosa associada a espectrometria de massas (*HS*-MEFS CG-EM), sendo que as condições operacionais da MEFS foram otimizadas empregando experimentos estatisticamente desenhados. Adicionalmente, um método sensível para determinação de EtG foi desenvolvido e validado empregando cromatografia liquida de ultra eficiência acoplada a detector de massas sequencial (CLUE-EM/EM), com melhorias incrementais sobre ensaios previamente descritos. EtP e EtG foram determinados em amostras de cabelo provenientes de 46 voluntários, os quais também responderam a um questionário padronizado (AUDIT-C) para avaliação dos riscos à saúde relacionados ao uso de etanol. As concentrações de EtG em cabelo foram correlacionadas com os escores no AUDIT-C, o que não ocorre com as concentrações de EtP. As concentrações de EtG e EtP em cabelo não foram correlacionadas. A determinação de EtG em cabelo com o ensaio desenvolvido é uma estratégia útil para avaliar o uso crônico de etanol.

Palavras chave: Etil palmitato, Etil Glucuronídeo, Cromatografia Gasosa, Cromatografia Liquida, Espectrometria de Massas.

ABSTRACT

Chronic ethanol consumption is considered a worldwide problem, being associated with several deleterious effects in the human organism, and with the occurrence of several pathologies, including liver cirrhosis, neurological disorders, gastrointestinal and cardiovascular pathologies. Biochemical parameters, such as the activities of the enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyltransferase (GGT), are used to evaluate alcohol consumption behavior, but with low specificity. The evaluation of more specific biomarkers of ethanol consumption in hair is an alternative to assess chronic exposure to ethanol. The most relevant biomarkers of exposure to ethanol in hair are ethyl glucuronide (EtG) and fatty acid ethyl esters (EEAG), particularly ethyl palmitate (EtP), which are the minor metabolites of ethanol. In this work, methods for analysis of EtP and EtG in human hair were optimized and validated for the evaluation of the chronic consumption of ethanol. The method for the determination of EtP employed headspace solid-phase microextraction (SPME) coupled to gas chromatography associated-mass spectrometry (GC-MS). The operational conditions of SPME were optimized using a statistically designed experiment. In addition, a sensitive method for EtG determination was developed and validated using ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS), with incremental improvements over previously described assays. EtP and EtG were measured in hair samples from 46 volunteers, who also answered a standardized questionnaire (AUDIT-C) to evaluate health risks related to the use of ethanol. Concentrations of EtG in hair were correlated with the scores in AUDIT-C, which did not occur with EtP concentrations. Hair concentrations of EtG and EtP were not correlated. The determination of EtG in hair with the developed assay is an useful strategy to evaluate the chronic use of ethanol.

Keywords: Ethyl Palmitate, Ethyl Glucuronide, Gas Chromatography, Liquid Chromatography, Mass Spectrometry**.**

LISTA DE ABREVIATURAS E SIGLAS

ALT – Alanine aminotransferase
AST – Aspartate aminotransferase
AS - Auto Sampler
AUDIT - Alcohol Use Disorder Identification Test
CN - Cone Energy
ColE - Collision Energy
CV – Coeficiente de variação
EtG – Ethyl Glucuronide
EtM – Ethyl Myristate
EtO – Ethyl Oleate
EtP – Ethyl Palmitate
EtS – Ethyl Stereate
EY – Extraction yield
GC-MS – Gas chromatography coupled to mass spectrometry (Cromatografia gasosa associada a detector de massas)
GGT – Gama glutamyltransferase
LC-MS/MS – Liquid chromatography coupled to tandem mass spectrometry (Cromatografia líquida associada a detector de massas sequencial)
LLOQ – Lowest limit of quantification
LOD – Limit of detection
ME – Matrix effect
MRM - Multiple reaction monitoring
MVC – Mean corpuscular volume of red blood cells
ND – Not detected
QC - Quality control
QCH - Quality control at high concentration
QCL - Quality control at low concentration
QCLLOQ - Quality control at the lowest limit of quantification
QCM - Quality control at medium concentration
r - Coefficient of correlation
S/N – Signal to noise ratio
SNC – Sistema nervoso central
SOHT – Society of Hair Test
SPE – Solid-phase extraction
SPME – Solid phase micro extraction
UPLC-MS/MS – Ultra performance liquid chromatography coupled to tandem mass spectrometry
Σ%RE - Cumulative percentage relative error

SUMÁRIO

1 APRESENTAÇÃO GERAL

O presente trabalho tem como objetivo apresentar formas otimizadas para avaliação do consumo excessivo crônico de etanol em amostras de cabelo humano. Este trabalho inicia com uma breve revisão bibliográfica sobre a pesquisa desenvolvida, seguida da apresentação de dois capítulos compostos de artigos científicos encaminhados para publicação em revistas distintas, como relatado a seguir:

CAPÍTULO 1: Artigo aceito para publicação pelo *Journal of Analytical Toxicology,* intitulado "*An optimized solid-phase microextraction and gas chromatography-mass spectrometry assay for the determination of ethyl palmitate in hair"*.

CAPÍTULO 2: Artigo submetido à revista *Forensic Science International,* intitulado "*Improved measurement of ethyl glucuronide concentrations in hair using UPLCMS/MS for the evaluation of chronic ethanol consumption"*.

Durante a realização desta pesquisa, trabalhos foram apresentados em eventos científicos, a saber:

- "*Método otimizado para avaliação de etil palmitato em cabelo como biomarcador do uso crônico de etanol utilizando CG-EM-MEFS-HS*". Apresentado na forma de resumo expandido no Seminário de Pós-Graduação-Inovamundi 2018, da Universidade Feevale, realizado entre os dias 22 a 27 de outubro de 2018.
- "*Optimized method for the determination of ethyl palmitate in hair using automated headspace solid-phase microextraction and gas chromatography-mass spectrometry (HS-SPME-GC-MS)*". Apresentado em forma de resumo/pôster, no 6º Encontro Nacional de Química Forense e 3º Encontro da Sociedade Brasileira de Ciências Forenses, realizado entre os dias 04 a 08 de novembro de 2018.

2 INTRODUÇÃO GERAL

O consumo crônico de etanol é considerado um problema mundial e está associado a vários efeitos deletérios no organismo humano (INGALL, 2012; WORLD HEALTH ORGANIZATION, 2014). O consumo excessivo de etanol tem relação com diversas patologias, incluindo cirrose hepática, distúrbios neurológicos, problemas gastrointestinais e problemas cardiovasculares. Quando consumido por gestantes, pode estar associado à ocorrência de danos físicos e neurológicos no feto (INGALL, 2012; SANTANA; ALMEIDA; MONTEIRO, 2014; WORLD HEALTH ORGANIZATION, 2014, 2017). Além de apresentar efeitos deletérios ao organismo o uso abusivo de etanol está relacionado direta ou indiretamente a 5,9% das mortes mundiais, podendo ainda ter relação com efeitos socioeconômicos negativos como ocorrência de infrações penais, acidentes de trânsito e diminuição da produtividade de trabalho (REHM et al., 2017; WORLD HEALTH ORGANIZATION, 2017).

Marcadores bioquímicos como gama-glutamiltransferase (GGT), alanina aminotransferase (ALT), aspartato aminotransferase (AST) e volume corpuscular médio das hemácias (VCM) são usados para avaliar o comportamento de consumo de álcool (HASTEDT et al., 2012; INGALL, 2012; NEELS et al., 2014). Esses marcadores, porém, apresentam limitações para estimar o uso excessivo e crônico de etanol devido à sua inerente falta de especificidade. São marcadores inespecíficos, principalmente de lesão hepática e fortemente afetados pela idade e pela ocorrência de outras patologias (HASTEDT et al., 2012; INGALL, 2012; NEELS et al., 2014; WURST et al., 2015).

Apesar da dificuldade em se estabelecer um comportamento do consumo de etanol utilizando marcadores clássicos, tal conhecimento é útil no contexto clínico e forense, particularmente nos casos de cirrose hepática, acidentes de trânsito, mortes violentas, transplante de órgãos, guarda de crianças, entre outros (REHM et al., 2017; WURST et al., 2015). A avaliação de biomarcadores mais específicos de etanol, como por exemplo no cabelo, é uma alternativa mais segura e confiável para mensurar a exposição crônica e/ou excessiva ao etanol (CRUNELLE et al., 2014; SUESSE et al., 2012). O uso do cabelo como matriz para esse tipo de avaliação comportamental apresenta vantagens como maior tempo de detecção e maior especificidade quando comparado aos marcadores bioquímicos clássicos (JURADO et al., 2004; WADA et al., 2010; NEELS et al., 2014; ORFANIDIS et al., 2017).

A utilização do cabelo como matriz biológica em análises toxicológicas e forenses tem crescido ao longo dos anos por diversos motivos. É considerada uma amostra de fácil

coleta, não invasiva e pode ser realizada sob supervisão diminuindo a possibilidade de adulteração ou troca do material, além disso, o manuseio, o transporte e o armazenamento das amostras não requerem embalagens especiais e nem mesmo a adição de conservantes ou refrigeração (SCHAFFER; HILL, 2005; PRAGST; BALIKOVA, 2006; PRAGST 2015).

A incorporação de substâncias químicas no cabelo ocorre de várias maneiras, tais como, difusão passiva dos capilares sanguíneos para células da matriz até o fim da zona de queratinização do folículo piloso, por difusão de secreções contidas no suor ou na gordura em toda a extensão do fio e ainda a partir do ambiente externo pelo uso de produtos cosméticos ou deposição de sujidades (COOPER, 2015; PRAGST; BALIKOVA, 2006). Em cabelos não tratados quimicamente, as substâncias incorporadas na matriz podem ser detectadas por vários meses. Tratamentos com substâncias químicas para tingimento e clareamento são geralmente as principais causas de deterioração dos analitos, pois podem causar a degradação da matriz capilar (COOPER, 2015; PRAGST; BALIKOVA, 2006, PRAGST, 2015).

Os biomarcadores capilares mais comuns para avaliação do consumo de etanol são o etil glucuronídeo (EtG) e os ésteres etílicos de ácidos graxos (EEAG) (PRAGST et al., 2001; SUESSE et al., 2012; CRUNELLE et al., 2014).

EEAG são metabólitos secundários do etanol, formados enzimaticamente a partir de ácidos graxos, triglicérides, lipoproteínas ou fosfolipídios na presença de etanol (PRAGST et al., 2001; KINTZ; NICHOLSON, 2014; PRAGST, 2015). Os EEAG mais comuns medidos no cabelo nesse tipo de avaliação são o etil miristato (EtM), etil palmitato (EtP), etil oleato (EtO) e etil stearato (EtS) (PRAGST et al., 2001; KINTZ, 2015). A soma da concentração desses quatro EEAG em cabelos foi proposta como um limiar para classificar o comportamento de consumo de etanol (KINTZ, 2015). Uma concentração de 0,5 ng/mg para a soma dos quatro ésteres em amostra capilar é fortemente sugestiva de consumo excessivo crônico de álcool quando medido no segmento proximal de 0-3 cm. Se o segmento proximal 0-6 cm for usado para teste, a concentração de corte proposta é de 1,0 ng/mg (KINTZ, 2015). A análise de EEAG em cabelo isoladamente não é recomendada para determinar a abstinência do etanol (SOHT, 2016; PRAGST et al., 2017). Recentemente, foi demonstrada uma forte correlação entre a soma das concentrações de EtM, EtP, EtO e EtS no cabelo e a concentração de EtP isoladamente como um marcador complementar de exposição ao etanol (SOHT, 2016; PRAGST et al., 2017). O uso de concentrações de EtP em cabelo, em vez da soma dos quatro EEAG, tem vantagens práticas, como menor custo para aquisição de padrões internos adicionais e processamento de dados mais simples, sem diferença essencial na interpretação dos resultados (SOHT, 2016; PRAGST et al., 2017).

As concentrações capilares de EtP são geralmente medidas por cromatografia a gás associada a espectrometria de massas (CG-EM), após micro extração em fase sólida por *head space* (*HS*-MEFS), que permite a separação seletiva do analito dos compostos da matriz (PRAGST et al., 2001; SCHAFFER; HILL, 2005; PRAGST; BALIKOVA, 2006). A maioria dos ensaios descritos baseia-se nas condições de extração capilar originalmente descritas por Pragst et al. (2001), com pequenas modificações (PRAGST et al., 2001). As recomendações mais recentes da *Society of Hair Test* (SOHT) indicam que concentrações de EtP em amostras de cabelo inferiores a 0,12 ng/mg para amostras com 0-3 cm de comprimento e 0,15 ng/mg para amostras de 0-6 cm de cabelo são sugestivas de abstinência de etanol (SOHT, 2016). O mesmo documento preconiza que níveis de EtP em cabelo superiores a 0,35 ng/mg para comprimentos de 0-3 cm e 0,45 ng/mg para comprimentos de 0-6 cm são sugestivos de consumo excessivo crônico de etanol. Sendo considerado consumo excessivo crônico o a ingestão diária de 60g de etanol por vários meses (SOHT, 2016).

O EtG, é um metabólito formado pela biotransformação de uma pequena fração de etanol circulante (<0,1%) pela enzima UDP-glucuroniltransferase para formar um conjugado com ácido glicurônico (INGALL, 2012; KINTZ; NICHOLSON, 2014). A presença de EtG no cabelo fornece uma forte indicação do consumo de etanol (PIRRO et al., 2013). No entanto, devido à possibilidade de resultados falso-negativos, como em casos de pouca ingestão de etanol ou lavagem excessiva de cabelo (KINTZ; NICHOLSON, 2014; PRAGST, 2015), a medição combinada de EtG com EtP pode ser útil (PRAGST, et al., 2017)

A medição de EtG no cabelo é um desafio, devido às baixas concentrações e os níveis recomendados para discriminação entre bebedores e abstinentes, ficando em 7 pg/mg de cabelo, de acordo com a SOHT (SOHT, 2016). Diversos ensaios para a medição de EtG no cabelo foram descritos usando cromatografia gasosa acoplado a detector seletivo de massas (CG-EM/EM), com derivatização química (YEGLES et al., 2004; KHARBOUCHE et al., 2009; SHI et al., 2010; PAUL et al., 2011; CAPPELLE et al., 2015), e cromatografia líquida acoplada a espectrometria de massas sequencial (CLAE-EM/EM) (MORINI et al., 2006; KINTZ et al., 2008; CONCHEIRO et al., 2009; TARCOMNICU et al., 2010; ALBERMANN; MUSSHOFF; MADEA, 2010; KRONSTRAND; BRINKHAGEN; NYSTRÖM, 2012; ALBERMANN et al., 2012; CABARCOS et al., 2013; PIRRO et al., 2013; BINZ; BAUMGARTNER; KRAEMER, 2014; KUMMER et al., 2015; OPPOLZER et al., 2016;). A prevalência de ensaios de CLAE-EM/EM pode ser atribuída à preparação mais simples da amostra e a maior sensibilidade intrínseca do método, sem a necessidade de derivatização química. Os processos de extração de EtG do cabelo são descritos utilizando diferentes misturas de solventes, tempos de incubação

e temperaturas, com ou sem emprego de uma etapa de extração em fase sólida (SPE) (KINTZ et al., 2008; BINZ; KRONSTRAND; BRINKHAGEN; NYSTRÖM, 2012; CABARCOS et al., 2013; BAUMGARTNER; KRAEMER, 2014; KUMMER et al., 2015; OPPOLZER et al., 2016).

Diante do exposto, o objetivo deste estudo foi otimizar e validar métodos analíticos para determinação de EtP e EtG em cabelo, considerando diretrizes e guias internacionais. Além disso, também foi avaliado o desempenho de ambos os ensaios em amostras clínicas, conjuntamente com informações complementares sobre o hábito de consumo de etanol obtido através da aplicação de um questionário padronizado (AUDIT-C).

3 OBJETIVOS

Apresentam-se, a seguir, os objetivos propostos para o estudo.

OBJETIVO GERAL

Desenvolver e validar métodos analíticos para determinação de EtP e EtG em amostras de cabelo humano, avaliando a sua capacidade de identificação do uso crônico de etanol.

OBJETIVOS ESPECÍFICOS

- Otimizar as condições de *HS*-MEFS para extração de EtP a partir de amostras de cabelo humano.
- Validar um método analítico para determinação de EtP em cabelo empregando *HS*-MEFS CG-EM.
- Otimizar as condições de extração de EtG a partir de amostras de cabelo humano.
- Validar um método analítico para determinação de EtG em cabelo empregando *CLUE*-EM/EM.
- Determinar concentrações de EtP e EtG em amostras de voluntários e correlacionar as concentrações encontradas com os resultados de um questionário validado para avaliação do risco à saúde devido ao consumo de etanol.

4 CAPÍTULO 1

AN OPTIMIZED SOLID-PHASE MICROEXTRACTION AND GAS CROMATOGRAPHY-MASS SPECTROMETRY ASSAY FOR THE DETERMINATION OF ETHYL PALMITATE IN HAIR

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Abstract

The use of hair as a matrix for the evaluation of chronic ethanol drinking behavior presents the advantage of a longer window of detection and higher specificity when compared to classical biochemical markers. The most recent recommendations the Society of Hair Testing (SOHT) indicate that ethyl palmitate (EtP) hair levels can be used to estimate the ethanol drinking behavior, alternatively to the combined measurement of four main FAEE. In this study, solid-phase microextraction (SPME) condition for the extraction of EtP from hair were optimized using response surface analysis, after a Box-Behnken experiment. Analysis were performed by GC-MS. The optimized HS-SPME conditions, using a PDMS-DVB (65 μm) fiber, were pre-adsorption time of 6 min, extraction time of 60 min and incubation temperature of 94 °C. Linear range was 0.05 to 3 ng mg^{-1}, with accuracy within 95.15-109.91%. Between-assay and within-assay precision were 8.58-12.53% and 6.12-6.82%, respectively. The extraction yield was 61.3-71.9%. The assay was applied to hair specimens obtained from 46 volunteers, all presenting EtP levels within the linear range of the assay. Using a statistically designed experiment, a sensitive SPME-GC-MS assay for the measurement of EtP in hair was developed and validated, requiring only 20 mg of hair.

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Keywords: Ethyl Palmitate, Hair, Optimization, solid-phase microextraction, GC-MS.

Introduction

Chronic ethanol consumption is associated with several deleterious effects on the human body, being related to liver, gastrointestinal, and cardiovascular pathologies (1). Moreover, ethanol consumption by pregnant women is associated with the occurrence of physical and neurological damage in the fetus (1). Biochemical markers of chronic ethanol use, such as gamma-glutamyl transferase (GGT), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and mean corpuscular volume (MCV) cannot reliably estimate the level of ethanol use due to their inherent lack of specificity (1–3). Despite the difficulties to establish the ethanol consumption behavior using classical markers, the knowledge of an individual's drinking behavior is useful in the clinical and forensic context, particularly in cases of liver cirrhosis, traffic accidents, violent deaths, organ transplantation, child custody, among others (4, 5).

A more specific alternative to assess ethanol drinking behavior is the measurement of ethanol consumption biomarkers in hair. The use of hair as a matrix for the evaluation of chronic ethanol drinking behavior presents the advantage of a longer window of detection and higher specificity when compared to classical biochemical markers (3, 6–8). The most common hair biomarkers are ethyl glucuronide (EtG) and fatty acid ethyl esters (FAEE) (9). EtG measurement is challenging due to its low concentration in hair, requiring the use of sensitive analytical technologies such as liquid chromatography coupled to tandem mass spectrometer (LC-MS/MS) (10). In the other hand, the measurement of FAEE concentration is hair is achieved with common benchtop gas chromatography-single quadrupole mass spectrometry (GC-MS) systems (2, 9, 11).

FAEE are secondary metabolites of ethanol, formed enzymatically from fatty acids, triglycerides, lipoproteins or phospholipids in the presence of ethanol (9, 12, 13). The most common FAEE measured in hair for the evaluation of ethanol consumption are ethyl myristate (EtM), ethyl palmitate (EtP), ethyl oleate (EtO) and ethyl stearate (EtS) (9, 14, 15). The sum of the concentration of these four FAEE in hair was proposed as a threshold to classify ethanol-drinking behavior (14). A cut-off concentration of 0.5 ng mg^{-1} for the sum of the four esters in scalp hair is strongly suggestive of chronic excessive alcohol consumption when measured in the 0–3 cm proximal segment. If the proximal 0-6 cm segment is used for testing, the proposed cut-off concentration is 1.0 ng mg^{-1}. Hair analysis of FAEEs alone is not recommended to determine abstinence from ethanol but may be used in cases of suspected false-negative EtG results. In this cases, the FAEEs cut-off concentration of 0.2 ng mg^{-1} for a 0-3 cm proximal

scalp hair segment, or 0.4 ng mg^{-1} for a 0-6 cm segment, is indicative of ethanol abstinence (14). If other lengths of scalp hair or hair from other body sites are used, there is no consensual cut off (14). Recently, a strong correlation between the sum of the concentrations of EtM, EtP, EtO, and EtS in hair and the concentration of EtP alone was demonstrated. The use of EtP hair concentrations, instead of the sum of the four FAEE, has practical advantages such as lower cost due to the acquisition of additional internal standards and simpler data processing, without no essential difference in the interpretation of the results concerning excessive alcohol consumption (16, 17). In this context, the most recent recommendations the Society of Hair Testing (SOHT) indicate that EtP hair concentrations lower than 0.12 ng mg^{-1} for scalp hair lengths of 0-3 cm and 0.15 ng mg^{-1} for hair lengths of 0-6 cm are suggestive of ethanol abstinence (16, 17). The same document indicates that EtP hair levels higher than 0.35 ng mg^{-1} for hair lengths of 0-3 cm and 0.45 ng mg^{-1} for hair lengths of 0-6 cm are suggestive of excessive ethanol consumption, which is equivalent to 60 g of daily intake for several months (16).

Hair concentrations of EtP are usually measured by GC-MS, after solid-phase microextraction (SPME), which allows the selective separation of the analyte from matrix compounds (9, 15, 16, 18). The majority of the reported assays are based on the hair extraction conditions originally described by Pragst *et al*. (9), with minor modifications. Usually, the main variables influencing the yield of an SPME extraction are evaluated separately, once at a time. This approach does not take into consideration the existence of interactions between the variables of the process, which can be assessed when performing statistically designed experiments. These experiments allow the selection of the optimal conditions of a process, after just a few planned tests (19).

The aim of this study was to optimize the conditions for extraction of EtP from hair, in order to increase the sensitivity of the assay, validate the resulting SPME-GC-MS assay according to bioanalytical validation guidelines, and apply the optimized assay using clinical specimens.

Materials and methods

Reagents and materials

EtP, sodium dodecyl sulphate (SDS) and *n*-heptane were purchased from Sigma-Aldrich (USA). Deuterated EtP (EtP-D5) was purchased from Toronto Research Chemicals (Canada). Dimethyl sulfoxide (DMSO), dichloromethane, monobasic sodium phosphate and acetone were

supplied by Honeywell (Germany). Sodium chloride and dibasic sodium phosphate were supplied by Merck (Germany). SPME was performed using a PDMS-DVB (65 µm) fiber, from Supelco (USA).

Solutions

Stock solution of EtP and EtP-D5, at 1 mg mL^{-1}, were prepared by dissolution of the esters in *n*-heptane. An intermediate solution of EtP, at the concentration of 100 µg mL^{-1}, was obtained diluting the stock with *n*-heptane. Working solutions were produced by dilution of the intermediate with *n*-heptane, obtaining solutions at the concentration levels of 50, 100, 150, 500, 750, 1.000, 2.000, 2.500 and 3.000 ng mL^{-1}. The working solution of EtP-D5, at the concentration of 1.000 ng mL^{-1}, was prepared by dilution the stock solution with *n*-heptane. All EtP and EtP-D5 solutions were prepared and stored in glassware. Phosphate buffer 0.1 M, pH 7.6, was prepared by mixing appropriate amounts of monobasic sodium phosphate and dibasic sodium phosphate stock solutions. SDS 0.1% solution were prepared by dissolution of SDS in ultra-pure water. Ultrapure water was produced by an Elga Purelab Ultra (Elga LabWater, United Kingdom). Calibration and quality control (QC) samples were prepared using human hair obtained from children, previously tested and with no detected amounts of EtP. Extraction optimization assays were performed using hair obtained from an adult volunteer with reported heavy consumption of ethanol.

Instrumentation

Automated SPME and analyte measurements were performed using a Trace GC Ultra gas chromatograph coupled to an ISQ single quadrupole mass spectrometer, equipped with a multi-purpose Triplus autosampler (Thermo Scientific, USA). SPME was automatically performed with the Triplus autosampler. Hair samples were milled with a MM400 ball mill (Restsch, Germany). Liquid extracts were agitated using a Vibramax 100 orbital shaker (Heidolph, Germany).

Chromatographic conditions

Chromatographic separation was performed in a DB5-MS (30 m x 0.25 mm x 0.25 µm) capillary column (Agilent, USA). Helium was the carrier gas, at the constant flow rate of 1mL min^{-1}. Injector temperature was 260 °C. The initial oven temperature was 70 °C (held for 2 min), followed by a 10 °C min^{-1} increase up to 200 °C. This first ramp was followed by a second, of 20 °C min^{-1}, up to 300 °C. The final temperature was maintained for 5 min. Run time was 26 min. Transfer line temperature was 280 °C and ion source temperature was 150 °C. MS

detection was in single ion monitoring (SIM) mode. Monitored *m/z* were 101 (quantification), 157 and 284 for EtP, and 106 (quantification), 162 and 289 for EtP-D5.

Extraction optimization

Authentic human hair samples from one volunteer with reported chronic use of ethanol was used for the extraction optimization. Firstly, SPME was optimized, using the hair washing and liquid-extraction procedures previously described (9). The SPME extraction was optimized by response-surface analysis, using a Box-Behnken experimental design. The selection of optimization process variables and values was based on previously published methods (9, 20). The experimental factors evaluated in the experiment were: incubation time (5, 10, and 15 min), adsorption time (15, 37.5 and 60 min), and pre-incubation and incubation temperatures (75, 85 and 95 °C). The SPME experiments are summarized at table 1. The optimized condition was calculated based on the desirability function, aiming to maximize the EtP peak area. Evaluation of experimental data was performed using Design Expert 11® (Stat-Ease, USA).

Table 1. Box-Behnken experimental design used to optimize the SPME extraction of EtP from hair.

Experiment	Incubation time (min)	Extraction time (min)	Incubation and extraction temperature (°C)
1	15	37.5	75
2	10	60	75
3	5	37.5	75
4	10	15	75
5	15	60	85
6	10	37.5	85
7	10	37.5	85
8	5	15	85
9	10	37.5	85
10	10	37.5	85
11	5	60	85
12	10	37.5	85
13	15	15	85
14	15	37.5	95
15	10	15	95
16	5	37.5	95
17	10	60	95

The liquid extraction of EtP from hair, before SPME, was also evaluated. Aliquots of the same authentic hair was extracted with acetone, a mixture of methanol and dichloromethane (1:1, v/v), and a mixture of DMSO and *n*-heptane (1:4, v/v). The extraction with these solvents

was evaluated at extraction times of 30 min, 4 h, and 15 h, in triplicate. The liquid extraction was performed at room temperature and under homogenization (800 rpm). The liquid extraction producing the higher EtP peak area, measured after performing the optimized SPME, was selected for the validation experiments.

Optimized sample preparation

The hair washing procedure was done according to a previous report (9). Briefly, hair was sequentially washed with SDS 0.1% solution, followed by two washing steps with *n*-heptane. The washing steps were performed for 15 min, at 800 rpm. The washed hair was dried under a gentle stream of air, and cut in 1-3 mm segments with scissors. The cut hair was transferred to 2 mL polypropylene tubes and cooled in a freezer at -20 ºC for 5 min. Immediately after removing from the freezer, the hair was milled in a ball mill for 6 minutes at 30 Hz, and cooled again for another 5 min. After, another milling step of 6 min was performed. Comparative tests on the extraction of milled and cut hair samples were carried out in quintuplicates with a specimen from a volunteer known to be user of ethanol. After the washing procedure for external decontamination, the hair was cut with scissors and kept for 10 min in a homogenizer at 30 Hz. After the homogeneization, one aliquot of the hair was milled and both milled and cut hair were submitted to the extraction procedure. EtP peak areas obtained with milled and cut hair were compared by Student's t test (95% confidence level).

Milled hair was selected for extraction. Aliquots of 20 mg of powdered hair were transferred to glass vials and added with 2.5 mL of a mixture of DMSO and *n*-heptane (1:4, v/v) and 20 µL of the internal standard working solution. The vials were agitated at 800 rpm for 15 h, at room temperature. After, the vials were kept at -20 °C for 30 min, allowing the separation of DMSO by freezing, and the n-heptane layer was transferred to a 20 mL headspace vial. The organic extract was dried under a gentle stream of air, at room temperature. The resulting dried extract was added with 1 mL of phosphate buffer pH 7.6 and 0.5 g of sodium chloride. The vials were sealed and submitted to automated SPME using a PDMS-DVB (65 µm) fiber. The optimized SPME conditions were: pre-incubation time 6 min, adsorption time 60 min, and pre-incubation and incubation temperatures of 94 °C.

Method validation

Linearity

Calibration samples were prepared by adding 20 µL of EtP working solutions to weighted and powdered hair before the liquid extraction. Linearity was evaluated by quintuplicate analysis of calibration samples at the concentrations of 0.05; 0.1; 0.2; 0.5; 1; 2 and 3 ng mg^{-1}. Calibration curves were obtained by relating peak area ratios from EtP to EtP-D5 to nominal concentrations of calibrators. Homoscedasticity of calibration data was evaluated using the *F* test at the confidence level of 95%. Weighted regression models were evaluated based on their correlation coefficients (*r*) and percentual cumulative errors (Σ% RE) (21). Additionally, linearity was considered as acceptable if back-calculated concentrations of calibrators were within ±15% of their nominal concentrations (22, 23).

Accuracy and precision

Accuracy and precision were evaluated by testing quality control (QC) samples at three concentration levels. QC samples were prepared by adding 20 µL of EtP working solutions to weighted and powdered hair before the liquid extraction. The concentration of the QC samples were 0.15 ng mg^{-1} (QC at low concentration, QCL), 0.75 ng mg^{-1} (QC at medium concentration, QCM), and 2.5 ng mg^{-1} (QC at high concentration, QCH). QC samples were analyzed in triplicate, at three different days. Accuracy was calculated as the percentage of the nominal concentration represented by the measured concentration of the QC. Precision was calculated by analysis of variance and expressed as CV%. Acceptance criteria for accuracy was values within ±15% of nominal concentrations of the QC samples. Precision was considered acceptable if CV% of the QC was lower than 15 (23, 24).

Lowest limit of quantification

The accuracy and precision of a QC sample at the concentration of the lowest calibrator (QCLLOQ) was evaluated by testing this QC in triplicate, at three different days. Precision and accuracy were determined as previously described. The acceptance criteria for the QCLLO were precision presenting a maximum CV% of 20 and accuracy of 80-120% (23, 24).

Extraction yield

The extraction yield was calculated by comparing EtP peak areas obtained after injecting liquid solutions with concentrations correspondent to complete recovery to peak areas obtained after the complete extraction procedure. The extraction yield was evaluated at the

concentration levels of QCL, QCM, and QCH, in quintuplicate, and calculated as the percentage of the peak area of the liquid injection peak area represented by the peak area obtained in the analysis of the QC sample.

Autosampler stability

The stability in the autosampler was evaluated by processing a batch of QC samples at low and high concentrations (QCL and QCH, respectively), in an alternating sequence, during 24 h. This batch had 8 QCL and 8 QCH samples. Peak area ratios of EtP to EtP-D5 in each control level were compared among tested samples. A variation of ±15% from the values obtained at the first analysis of the QC was considered as acceptable (23, 24).

Study participants and hair collection

Adult patients of both sexes admitted to a drug rehabilitation clinic in the Brazilian state of Rio Grande do Sul were invited to participate in the study. Before hair specimen and data collection, all volunteers signed a consent form. Specimens were collected in the first day of admission. The Institutional Review Board of Universidade Feevale approved the study. Hair was collected from the vertex area of the scalp, following usual procedures (25). Collected hair specimens were wrapped in aluminum foils and stored in paper envelopes, at room temperature (17, 25). The analysis was performed with hair lengths lower than 6 cm. Study volunteers answered the Alcohol Use Disorder Identification Test (AUDIT). The AUDIT is a screening tool developed by the World Health |Organization (WHO) to assess alcohol consumption, drinking behaviors, and alcohol-related problems. (26, 27). In this study, we used AUDIT C, a simplified version of the complete AUDIT, used to evaluate the probable frequency and quantity of ethanol intake. This test was properly validated in the Portuguese language (28). AUDIT C score has a scale of 0 to 12 points, with responders being classified with low, moderate, high, and severe risk of ethanol use disorder (4).

Results and discussion

Chromatography and sample preparation

The chromatographic run had a duration of 26 min, with EtP and EtP-D5 presenting retention time of 15.2 min. Previous reports of EtP analysis in hair by GC-MS present retention times in the range of 8.6 min to 17.8 min (2, 9, 29, 30). Once the automated SPME requires sequential processing of the samples, the long chromatographic run did not affect the sample

processing time once the time limiting step is SPME extraction, with a duration of 60 min. The employed chromatographic conditions were selective for EtP and EtP-D5, without any interfering peak in blank samples. Figure 1 presents representative chromatograms of the assay.

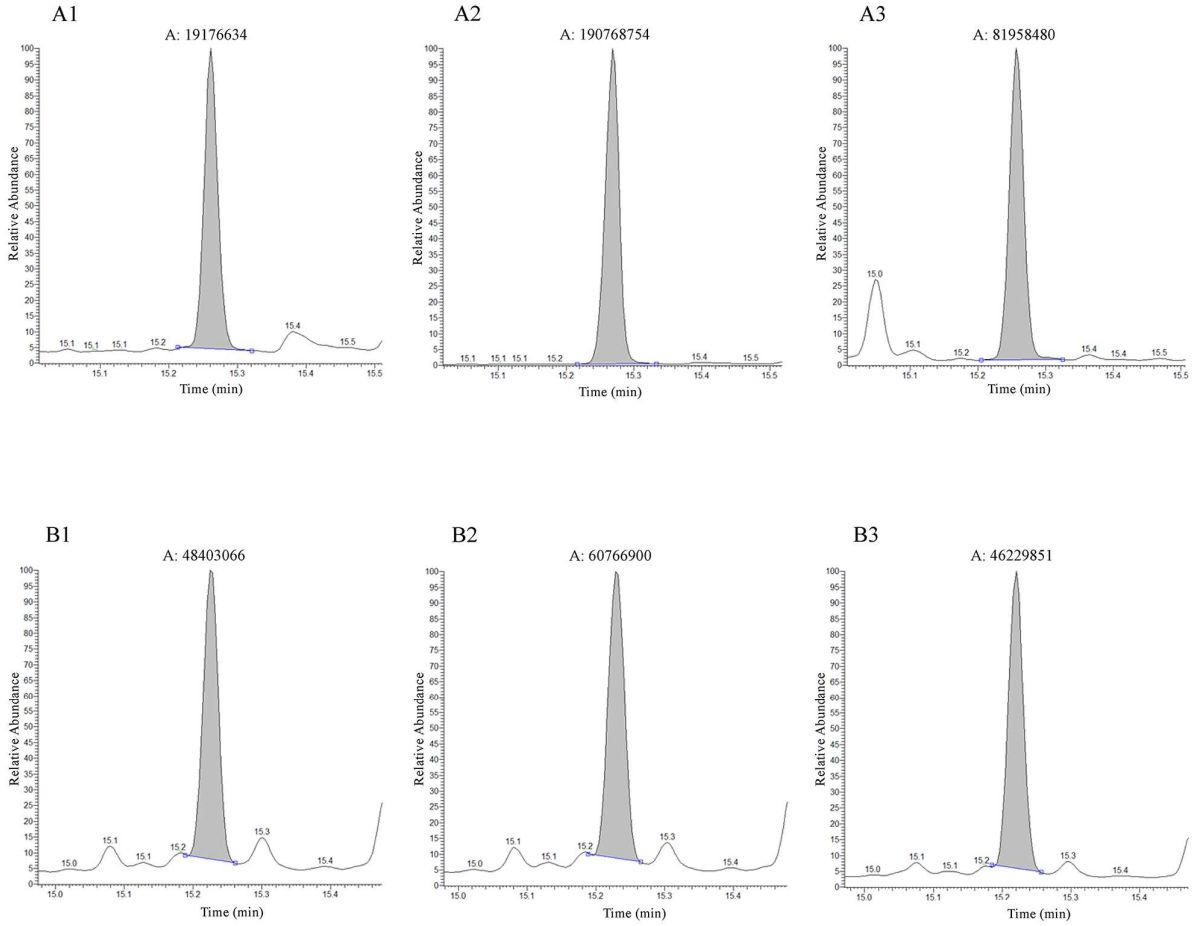

Figure 1. Ion chromatograms presenting EtP extracted from hair (m/z 101 for EtP and m/z 106 for EtP-D5). A1: QCL sample (0.15 ng mg^{-1}); A2: QCH sample (2.5 ng mg^{-1}); A3: Volunteer specimen containing EtP at 1.68 ng mg^{-1}. B1, B2 and B3: EtP-D5 in the QCL, QCH and volunteer sample, respectively.

SPME is the sample preparation method most frequently used for FAEE analysis in hair (2, 9, 29, 31–35). SPME assays usually used PDMS-DVB (65 µm) fibers, which were also used in our study. SPME is a typically multi-factorial process, prone to the application of statistically designed optimization experiments. We used a Box-Behnken experimental design to evaluate the most important process variables in the EtP extraction using SPME, which are usually evaluated one-by-one. The seventeen experiments of the Box-Behnken design are described in table 1.

A linear model best described the relationship between the response, EtP peak area, and the evaluated process variables (pre-incubation time, extraction time and incubation temperature), presenting an adjusted r^2 of 0.7371. There were no significant interactions between the variables. The model describing EtP peak area from the process variables was highly significant (p=0.0001), with no significant lack-of-fit (p=0.1577). The desirability function was used to numerically selected the best values of the variables targeting to maximize EtP peak area. The optimal conditions, within the investigated ranges, were pre-incubation time of 6 min, adsorption time of 60 min, and pre-incubation and incubation temperatures of 94 °C. Already described SPME conditions differ from our optimal values, possibly due to the employed experimental approach and to the need to find a comprise for the response of the several FAEE simultaneously measured. The main differences were in extraction time and temperatures of pre-incubation and extraction, which had reported values of 30 min and 90 °C, respectively (2, 9, 20). The EtP peak areas obtained from milled hair were considerably higher (> 40%) when compared to cut hair (p<0.05in Student's t test). The majority of previous studies used cut hair for FAEE measurements, but the used of milled hair was already described (30, 36). The liquid extraction with a mixture of DMSO and *n*-heptane (1:4, v/v) for 15 h produced the higher EtP peak areas, being 20 and 7% higher than peak areas obtained with the extraction with acetone and the mixture of methanol and dichloromethane (1:1, v/v), respectively, in the same extraction time. This result corroborate previous reports of FAEE extraction from hair, which also used the DMSO : *n*-heptane mixture for 15 h (2, 9, 20, 37).

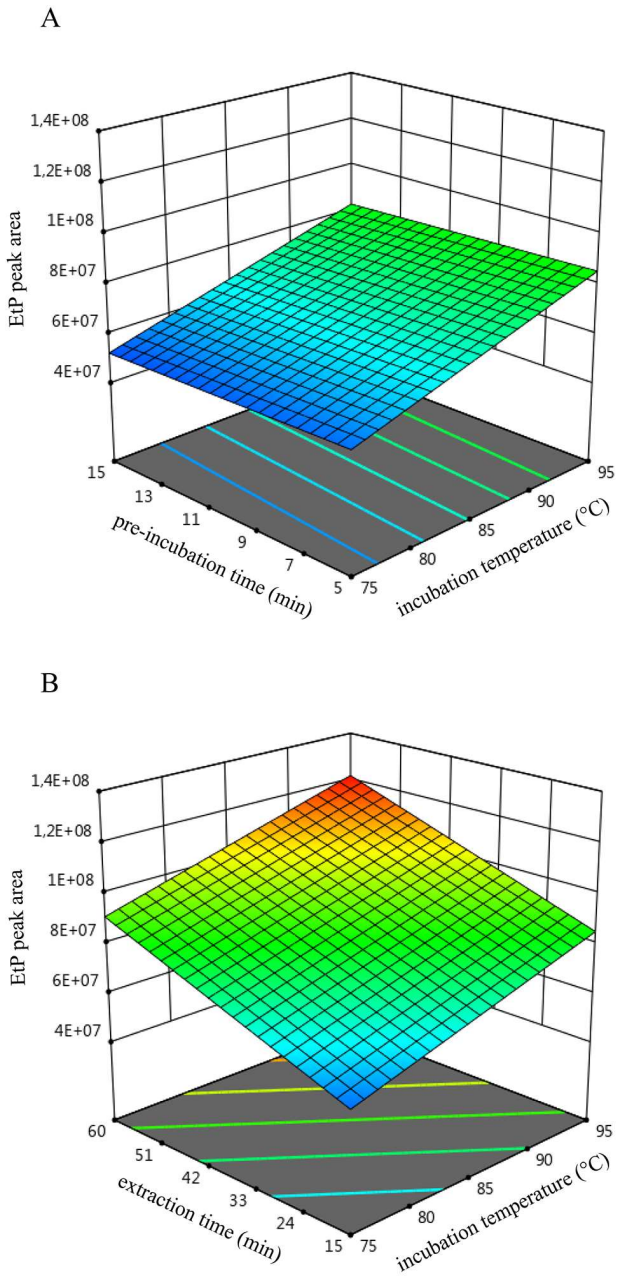

Figure 2. Response surfaces presenting the relationship between evaluated SPME variables and EtP peak areas. A: Influence of pre-incubation time and incubation temperature on EtP peak area. B: Influence of extraction time and incubation temperature on EtP peak area.

The combined use of the optimized SPME extraction along with the selected chromatographic conditions allowed the sensitive determination of EtP, with responses 33% higher than those obtained with conditions in the reference method (9). The improved EtP response also allowed the use of only 20 mg of hair for testing, differently from previous authors that reported the use of 30 to 100 mg hair for FAEE analysis using GC-MS (9, 30, 32, 34, 35, 38)

Assay validation

The assay was linear in the range of 0.05 to 3 ng mg^{-1}. This linear range is adequate to the intended use of the assay, allowing the identification of abstinent and abusive use of ethanol, according to reference values. Calibration presented signification heteroscedasticity, with the weighting factor of 1/x presenting the lower cumulative error, of 1.35 x 10^{-13}. Calibration curves presented values of *r* higher than 0.99, with back-calculated values within ±15% of their nominal concentrations.

The results of the validation assays are summarized in table 2. Accuracy and precision of the assay were acceptable, according to the used criteria (22, 24, 39). Inter-assay and intra-assay precisions were 8.58-12.53% and 6.12-6.82%, respectively. Accuracy was in the range of 95.15 to 109.91%. The QC sample at the lower limit of quantification had intra-assay and inter-assay precision of 19.24-18.01% and accuracy of 109.33%, also fulfilling the acceptance criteria. The EtP extraction yields in the optimized SPME produce described in this study were in the range of 61.3 to 71.9%, higher than the approximately 30% yield reported in the original assay of Pragst *et al.*(9). Additionally, considering the long processing times in the automated SPME, we evaluated the autosampler stability for 24 h. At the end of the 24 h running batch, EtP peak are ratios presented -2.14% (QCH) and 8.83% (QCL) differences from the first analysis of the sequence.

Table 2. Validation parameters for SPME-GC-MS measurement of EtP in hair: precision, accuracy, extraction yield, and autosampler stability.

Control level	Concentration (ng mg^{-1})	Precision (CV %)		Accuracy (%)	Extraction yield (%)	Autosampler stability (% difference)
		Inter-assay	Intra-assay			
QCLLOQ	0.05	18.01	19.24	109.33	-	-
CQL	0.15	12.53	6.13	109.91	71.9	8.83
CQM	0.75	10.10	6.12	102.55	66.9	-
CQH	2.5	8.58	6.82	95.15	61.3	-2.14

QCLLOQ: quality control at the lowest limit of quantification; CQL: quality control at low concentration; CQM: quality control at medium concentration; CQH: quality control at high concentration**.**

Assay application

Hair specimens were collected from 46 volunteers. Hair segments with 0-3 cm were obtained from 18 (39.1%) volunteers, and 0-6 cm segments were obtained from the remaining 28 (60.9%). EtP was quantified within the linear range of the assay in all tested specimens. Only 8 (17.4%) specimens presented EtP values lower than the cut off value for classification of chronic excessive alcohol consumption. The remaining 38 (82.6%) had EtP concentrations compatible with excessive alcohol consumption. According to the AUDIT-C, 27 volunteers (58.7%) presented severe risk of ethanol use disorder, with 3 (6.5%), 4 (8.7%) and 12 (26.1%) being classified as high, moderate and low risk, respectively. Only 22 (47.8%) volunteers presented simultaneously EtP hair concentrations compatible with excessive chronic ethanol consumption and AUDIT-C classification as severe risk of ethanol use disorder. There was no correlation between hair EtP concentrations and AUDIT-C score. This lack of concordance of hair concentration of EtP and AUDIT-C ethanol drinking risk could be attributed to several factors. Volunteers of the study were multi-drug users, with variable degrees of cognitive disorders, which can contribute for non-reliable answers to questionnaires. Social, family and legal issues could also reduce the reliability of questionnaires as tools of evaluating the drug use behavior. These factors reinforce the utility of more objective strategies, such as analytical measurements, to evaluate the ethanol drinking behavior. However, it is also important to consider that some volunteers of the study had poor hygiene habits, which can contribute to increased EtP values in hair (9, 31, 37). This factor, along with the possible formation of EtP from the use of cosmetic products containing ethanol (9, 12, 32), strongly supports the combined used of EtP or FAEE hair levels combined with EtG concentration in hair in order to evaluate the ethanol drinking behavior.

Table 3. AUDIT-C scores and EtP hair levels from the study volunteers.

Patient	Age (sex)	AUDIT C					Hair EtP (ng mg^{-1})	*Consumption behavior according to EtP*	*Analyzed hair length (cm)*
		Q1	Q2	Q3	Total score	Risk level			
1	21 (M)	2	4	2	8	Severe	0.71	Chronic	*0-3*
2	28 (M)	4	4	4	12	Severe	0.72	Chronic	*0-3*
3	36 (M)	0	0	0	0	Low	0.73	Chronic	*0-3*
4	37 (M)	4	4	4	12	Severe	0.74	Chronic	*0-3*
5	21 (M)	2	1	1	4	Moderate	0.94	Chronic	*0-3*
6	53 (M)	4	4	4	12	Severe	0.65	Chronic	*0-6*
7	25 (M)	0	0	0	0	Low	0.65	Chronic	*0-6*
8	35 (F)	4	4	4	12	Severe	0.73	Chronic	*0-6*
9	37 (M)	4	2	4	10	Severe	0.76	Chronic	*0-6*
10	43 (F)	4	3	4	11	Severe	0.79	Chronic	*0-6*
11	68 (M)	4	2	0	6	High	0.97	Chronic	*0-6*
12	32 (M)	4	4	4	12	Severe	0.99	Chronic	*0-6*
13	54 (F)	4	2	4	10	Severe	0.99	Chronic	*0-6*
14	41 (M)	4	2	1	7	High	1.62	Chronic	*0-6*
15	47 (M)	4	4	4	12	Severe	1.68	Chronic	*0-6*
16	62 (M)	4	2	4	10	Severe	1.68	Chronic	*0-6*
17	24 (M)	1	1	0	2	Low	1.69	Chronic	*0-3*
18	39 (M)	4	4	4	12	Severe	1.72	Chronic	*0-6*
19	35 (M)	4	4	4	12	Severe	1.89	Chronic	*0-6*
20	33 (F)	3	1	0	4	Moderate	1.92	Chronic	*0-6*
21	27 (F)	0	0	0	0	Low	1.96	Chronic	*0-6*
22	39 (F)	4	4	3	11	Severe	0.22	Moderate	*0-6*
23	21 (M)	4	4	4	12	Severe	0.29	Moderate	*0-6*
24	47 (F)	4	4	4	12	Severe	0.29	Moderate	*0-6*
25	29 (M)	0	0	0	0	Low	0.32	Moderate	*0-3*
26	31 (M)	4	3	4	11	Severe	0.33	Moderate	*0-3*
27	50 (F)	0	0	0	0	Low	0.35	Moderate	*0-6*
28	44 (M)	0	0	0	0	Low	0.40	Chronic	*0-3*
29	37 (F)	1	1	1	3	Moderate	0.40	Moderate	*0-6*
30	39 (F)	2	3	3	8	Severe	0.41	Moderate	*0-6*
31	43 (M)	4	4	4	12	Severe	0.49	Chronic	*0-6*
32	33 (M)	2	1	2	5	Moderate	0.51	Chronic	*0-3*
33	58 (M)	4	4	4	12	Severe	0.51	Chronic	*0-6*
34	30 (M)	0	0	0	0	Low	0.53	Chronic	*0-3*
35	58 (M)	4	4	4	12	Severe	1.02	Chronic	*0-3*
36	38 (M)	4	4	4	12	Severe	1.03	Chronic	*0-3*
37	65 (M)	4	4	3	11	Severe	1.19	Chronic	*0-3*
38	48 (F)	0	0	0	0	Low	1.19	Chronic	*0-6*
39	26 (M)	1	1	0	2	Low	1.29	Chronic	*0-6*
40	52 (M)	4	4	4	12	Severe	1.30	Chronic	*0-3*
41	23 (M)	1	1	0	2	Low	1.37	Chronic	*0-6*
42	47 (F)	4	4	4	12	Severe	1.38	Chronic	*0-6*
43	28 (M)	1	1	0	2	Low	2.07	Chronic	*0-3*
44	39 (M)	4	4	4	12	Severe	2.23	Chronic	*0-3*
45	39 (M)	4	1	1	6	High	2.29	Chronic	*0-6*
46	48 (M)	4	4	3	11	Severe	2.42	Chronic	*0-3*

Conclusions

The automated SPME extraction of EtP from hair was optimized using a Box-Behnken experiment followed by response surface analysis, resulting in operational conditions slightly different from the previously described. The optimized SPME procedure resulted in EtP peak areas approximately 33% higher than areas obtained with a reference method. The optimized method was validated and applied to clinical samples. Using a statistically designed experiment, a sensitive SPME-GC-MS assay for the measurement of EtP in hair was developed, with incremental improvements over previous reported assays.

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5 CAPITULO 2

IMPROVED MEASUREMENT OF ETHYL GLUCURONIDE CONCENTRATIONS IN HAIR USING ULTRA-PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED TO TANDEM MASS SPECTROMETRY

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ABSTRACT

The presence of ethyl glucuronide (EtG) in hair provides a strong indication of ethanol consumption and its investigation is of interest in both clinical and forensic contexts because of the wide window of detection. However, due to the possibility of false negative results in cases of small ethanol intake or excessive hair washing, the combined measurement of ethyl palmitate (EtP) with EtG could be useful. In this study, a sensitive UPLC-MS/MS procedure for the measurement of EtG in hair was developed and validated, using optimized sample preparation and chromatographic separation. Milled hair was extracted with water for 24 h at room temperature, followed by clean-up of the extract by ion-exchange solid phase extraction (SPE). Extraction was highly efficient, with yield of 96.93-101.06%. Chromatographic separation was performed with a Fluoro-Phenyl stationary phase. The assay was linear from 4 to 500 pg mg^{-1}, with accuracy in the range of 100.30 to 106.16%. Matrix effects (-0.87-5.89%) were adequately compensated by the use of deuterated EtG as internal standard. EtG was measured in hair samples of 46 volunteers, and results were compared with hair concentrations of ethyl palmitate (EtP) and the score in the AUDIT-C questionnaire. EtG hair concentrations were significantly correlated to the AUDIT-C classification (r_S=0.365, $p<0.05$), but not to EtP hair levels. The diagnostic performance of EtG hair concentrations to identify excessive or moderate ethanol use was similar to the capability of AUDIT-C to identify severe and high health risk (Kappa, p=0.013). The developed assay is suitable for clinical use, providing a useful tool to evaluate chronic ethanol consumption.

Keywords: Ethyl Glucuronide, Hair, Optimization, ethanol, LC-MS/MS.

1 INTRODUCTION

Abusive ethanol consumption is a worldwide problem [1], being related to the development of several pathologies including hepatic cirrhosis, neurological disorders, and cardiovascular problems, among others [2]. In addition to its deleterious effects on health, excessive ethanol use has a direct relation to negative socioeconomic effects, like the occurrence of criminal offenses, traffic accidents and decreased productivity at work [2,3].

Indirect ethanol exposure biomarkers such as serum activities of enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyltransferase (GGT) were used to evaluate alcohol consumption behavior [4–6]. However, the measurements of these non-specific markers of liver damage are strongly affected by age and the presence of several pathologies, which limits their use to estimate the abusive use of ethanol [6,7]. The measurement of more specific biomarkers of ethanol consumption in hair is a more specific and reliable alternative to evaluate excessive exposure [8,9]. In this context, ethyl glucuronide (EtG) was proposed as the more informative biomarker of ethanol use in hair [8,9]. EtG is formed by the biotransformation of a small fraction of circulating ethanol (<0.1%) by the enzyme UDP-glucuronyltransferase enzyme to form a conjugate with glucuronic acid [4,10]. The presence of EtG in hair provide a strong indication of ethanol consumption [11]. However, due to the possibility of false negative results in cases of small ethanol intake or excessive hair washing [10,12], the combined measurement of fatty acid ethyl esters (FAEE) with EtG could be useful [13]. FAEE are secondary metabolites of ethanol, formed enzymatically from fatty acids, triglycerides, lipoproteins or phospholipids in the presence of ethanol [10,12,14]. Recently, a strong correlation between the sum of the concentrations of the main FAEE in hair and the concentration of ethyl palmitate (EtP) was demonstrated, which supports the use of EtP hair concentrations as a complementary marker of ethanol exposure [15,16].

The measurement of EtG concentrations in hair is challenging due to the low concentrations recommended to discriminate between abstinent and moderate drinkers of ethanol, of 7 pg mg^{-1} in scalp hair, according to the Society of Hair Testing [15]. Several assays for the EtG measurement in hair were described, using gas-chromatography coupled to tandem mass spectrometry (GC-MS/MS), usually employing with chemical ionization [17–21], and liquid-chromatography coupled to tandem mass spectrometry (LC-MS/MS) [11,22–32]. The prevalence of LC-MS/MS assays could be attributed to the simpler sample preparation, without the need for derivatization, and higher sensitivity. However, even considering the existence of

a proposed cut-off value for the classification of ethanol consumption behavior based on EtG concentrations in hair, there is no reference procedure for sample preparation or measurement. EtG has been extracted from hair with different solvent mixtures, incubation times and temperatures, and with or without a clean-up step with solid-phase extraction (SPE) [23,27,29–32].

The aim of this study was to evaluate the several variables influencing the extraction of EtG from hair, in order to obtain an improved sample preparation procedure, and to validate an assay for its measurement using ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). Additionally, the assay performance was evaluated in clinical samples, along with complimentary information of EtP hair levels and a standardized questionnaire.

2 MATERIALS AND METHODS

2.1 Reagents and materials

Standard solutions EtG and EtG-D5, both at 100 µg mL^{-1} in methanol, were purchased from Cerillant (USA). Dichloromethane was supplied by Honeywell (Germany). Acetonitrile, methanol and formic acid were acquired from Merck (Germany). Ammonia hydroxide was purchased from Nuclear (Brazil). Purified water used was produced by an Elga Purelab Ultra system from Elga Labwater (United Kingdom). Solid-phase extraction cartridges Oasis MAX (60 mg, 3 mL) were obtained from Waters (USA).

2.2 Solutions

EtG stock solution was diluted with methanol to obtain an intermediate solution at the concentration of 2.400 ng mL^{-1}. This intermediate solution was diluted with methanol to obtain working solutions at 4.8, 9, 18, 36, 72, 150, 300, and 600 ng mL^{-1} for the preparation of calibration samples, and at 6.6, 96 and 540 ng mL^{-1} for the preparation of quality control (QC) samples. An intermediate solution of EtG-D5 at 1.000 ng mL^{-1} was prepared by dilution of the stock with methanol. The intermediate solution was diluted with methanol to obtain the working internal standard solution, at the concentration of 120 ng mL^{-1}. Ammonia 5% and formic acid 2 % solutions were obtained by mixing appropriate amounts of ammonia hydroxide and formic acid, respectively, with purified water. Calibration and QC samples were prepared using human hair obtained from children, previously tested and with no detected amounts of EtG.

2.3 Hair washing and milling

The hair washing procedure was done according to a previous report [5]. Briefly, hair specimens were washed with dichloromethane for 30 min, followed by a second washing with methanol for 1 min. Washings were performed with agitation at 800 rpm on a Vibramax 100 orbital shaker (Heidolph, Germany). After, the hair was dried under a gentle stream of air, cut in 1-3 mm segments with scissors, and milled with a MM400 ball mill (Retsch, Germany) for 12 min at 30 Hz.

2.4 EtG hair extraction optimization

Authentic human hair samples from volunteers which reported excessive use of ethanol were used for the extraction optimization. The liquid extraction of EtG from hair was optimized by response-surface analysis, using a Box-Behnken experimental design. The selection of optimization process variables and values was based on previously published methods [11,22,33–35]. The experimental factors were: extraction solvent composition (composed of water with 0-10 % of methanol); extraction time (6-24 h) and extraction solvent volume (350-1,500 µL). The experimental data was evaluated using Design Expert 11® (Stat-Ease, USA). After liquid extraction, EtG was separated from the extraction solution using ion-exchange SPE, as already described [29,36]. Additionally, the extraction temperatures of 25, 40 and 55 °C were evaluated, in triplicate, and the average EtG peak area ratios in each temperature were evaluated in using Student's *t*-test (95% confidence).

2.5 Solid-phase extraction of EtG from aqueous extracts

Aliquots of 50 mg of powdered hair were transferred to polypropylene microtubes and added with 1.5 mL of ultra-purified water and 25 µL of the internal standard working solution. The microtubes were homogenized for 24 h at 25 °C and 900 rpm in a Thermomixer C (Eppendorf, Germany). The resulting extract was sonicated for 1.5 h in an ultrasonic bath at 30 °C, followed by centrifugation at 14,200 rpm for 10 min. The supernatant was used for SPE, using an ion-exchange OASIS MAX cartridge (60 mg, 3 mL). SPE cartridges were conditioned sequentially with 2 mL of methanol and 2 mL of ultra-purified water. The aqueous hair extract was loaded to the conditioned cartridges at an approximate flow rate of 0.5 mL min^{-1}. Cartridges were washed with 2 mL of 5% ammonia in water (v/v), followed by a second wash with 2 mL of methanol. After washing, cartridges were dried with vacuum for 10 min. EtG was eluted

with 2 mL of 2% formic acid in methanol (v/v). Liquid flow during all stages of the SPE procedure was approximately 0.5 mL min^{-1}. The extract was evaporated to dryness in a Concentrator Plus vacuum centrifuge (Eppendorf, Germany), at 60 °C. The resulting dried extract was recovered with 100 µL of the initial mobile phase, vortexed for 1 min, and then centrifuged at 14,200 rpm for 10 min. The supernatant was transferred to an autosampler vial and 10 µL were injected into the chromatograph [29,32,36].

2.6 Chromatographic and mass spectrometric conditions

Analysis were performed with an UPLC-MS/MS system composed of an Acquity I-Class chromatograph coupled to a Xevo TQS-Micro triple quadrupole mass spectrometer (Waters, USA), in negative electrospray mode. The chromatographic separation was obtained with an Acquity CSH Fluoro-Phenyl column (2,1 x 100 mm, particle diameter of 1.7 µm), supplied by Waters. The column was kept at 40 °C during the analysis. The autosampler temperature was set to 10 °C. The mobile phase A was water containing 0,1% formic acid and mobile phase B was acetonitrile containing 0.1% formic acid. Elution flow rate was 0.3 mL min^{-1}. The mobile phase gradient started at 95% A, maintained for 0.5 min, followed by a linear gradient to 50% A in 3 min, followed by another gradient to 10% A in 3.5 min. This condition was held for 0.5 min, returning to the initial composition at 4 min, followed by 2 min of stabilization at initial conditions. Total run time was 6 min. The mass spectrometer operational parameters were: ion source temperature of 550 °C; capillary energy of 3.0 kV, dessolvatation gas flow of 1000 L h^{-1} and source gas flow of 50 L h^{-1}. The analyses were performed in multiple reaction monitoring (MRM) mode. Acquisition parameters were selected after an infusion of working solutions of EtG and EtG-D5 (1.000 ng mg^{-1} in methanol). The optimized conditions of cone energy (CN), collision energy (ColE), ion transitions and retention times for EtG and EtG-D5 are presented in table 1.

Table 1. Optimized mass spectrometric acquisition parameters for the analysis of EtG and EtG-D5 and chromatographic retention times.

Compound	MRM transitions (m/z)[a]	Cone energy (V)	Collision energy (V)	Retention time (min)
EtG	221.0→74.9	20	14	2.09
	<u>221.0→84.9</u>		16	
EtG-D5	<u>226.0→85</u>	20	17	2.08

[a] Quantifier transitions were underline. *MRM*: multiple reaction monitoring.

2.7 Linearity

Linearity was evaluated by quintuplicate analysis of calibration samples at the concentrations of 4; 7,5; 15; 30; 60; 125; 250 e 500 pg mg^{-1}. Calibration samples were obtained by adding 25 µL of the appropriate working solutions of EtG to 50 mg of powdered hair. Calibration curves were obtained by relating peak area ratios from EtG to EtG-D5 to nominal concentrations of calibrators. Homoscedasticity of calibration data was evaluated using the *F* test, at the confidence level of 95%. Weighted regression models were evaluated based on their correlation coefficients (*r*) and percentual cumulative errors (Σ% RE) [37]. Additionally, linearity was considered as acceptable if back-calculated concentrations of calibrators were within ±15% of their nominal concentrations [38,39].

2.8 Accuracy and precision

Accuracy and precision were evaluated by testing QC samples at the concentration levels of 5.5 pg mg^{-1} (quality control at low concentration, QCL), 80 pg mg^{-1} (quality control at medium concentration, QCM), and 450 pg mg^{-1} (quality control at high concentration, QCH), extracted as previously described. QC samples were obtained by adding 25 µL of the appropriate working solutions of EtG to 50 mg of powdered hair. QC samples were analyzed in triplicate, at three different days, along with a calibration curve. Accuracy was calculated as the percentage of the nominal concentration represented by the measured concentration of the QC. Precision was calculated by analysis of variance and expressed as CV%. The acceptance criterion for accuracy was values within ± 15% of nominal concentrations of the QC samples. Precision was considered acceptable if CV% of the QC was lower than 15 [39,40].

2.9 Lowest limit of quantification

Precision and accuracy of an additional QC sample at the concentration of 4 pg mg^{-1} (quality control at the lowest limit of quantification, QCLLOQ), was tested in triplicate, at three different days. Precision and accuracy were determined as described above. The acceptance criteria for the QCLLOQ were precision presenting a maximum CV% of 20 and accuracy of 80-120% [39,40].

2.10 Matrix effect and extraction yield

Matrix effect (ME) and extraction yield (EY) were calculated by the post-extraction spike method [41]. In this method, ME and EY are determined from the analysis of three sets of samples. The set A was composed of solutions of EtG and EtG-D5 in the initial mobile phase at concentrations correspondent to an EY of 100% for the concentration levels of QCL, QCM, and QCH. The sample set B was composed of fifteen extracts of blank hair specimens, recovered with initial mobile phase containing EtG and EtG-D5 in concentrations equivalent to EY of 100% also from the concentration levels of QCL, QCM and QCH. The sample set C was composed of QCL, QCM and QCH samples, prepared and analyzed as described above. The evaluated response in each set of samples was the EtG to EtG-D5 area ratios. ME was calculated as ME (%) = (B/A x 100)-100, and EY was calculated as EY (%) = C/B x 100.

2.11 Autosampler stability

Stability of EtG in extracts was assessed at the concentration levels of QCL and QCH at autosampler conditions. These QC samples were extracted as described above and injected into the chromatograph at time intervals of 1 h, over 12 h. Peak areas from EtG and peak area ratios of EtG and EtG-D5 obtained at the first injection was compared with those achieved at the end of the series and a linear regression between peak responses and injection time was obtained to evaluate tendencies. A decrease or an increase lower than 15% in peak area ratios of EtG and EtG-D5 was considered as acceptable [40].

2.12 Determination of EtP concentrations in hair

Hair samples were washed with SDS 0.1% solution, followed by two washing steps with *n*-heptane. The washing steps were performed for 15 min, at 800 rpm. After the hair was dried under a gentle stream of air, cut in 1-3 mm segments with scissors, and milled with a ball mill at 30 Hz, for 12 min. Aliquots of 20 mg of powdered hair were transferred to glass vials and added with 2.5 mL of a mixture of DMSO and n-heptane (1:4, v/v) and 20 µL of the internal standard working solution (EtP-D5 at 1.000 ng mL^{-1} in *n*-heptane), agitated at 800 rpm for 15 h, at room temperature. After, the vials were kept at -20 °C for 30 min, allowing the separation of DMSO by freezing, and the *n*-heptane layer was transferred to a 20 mL headspace vial. The organic extract was dried under a gentle stream of air, at room temperature. The resulting dried

extract was resuspended with 1 mL of phosphate buffer pH 7.6 and 0.5 g of sodium chloride. The vials were sealed and submitted to automated SPME using a PDMS-DVB (65 µm) fiber, in a TriPlus autosampler (Thermo Scientific, USA). The SPME conditions were: pre-incubation time 6 min, adsorption time 60 min, and pre-incubation and incubation temperatures of 94 °C. Chromatographic separation was performed in a DB5-MS (30 m x 0.25 mm x 0.25 µm) capillary column (Agilent, USA) in a Trace GC Ultra gas chromatograph (Thermo Scientific, USA). Helium was the carrier gas, at the constant flow rate of 1 mL min^{-1}. Injector temperature was 260 °C. The initial oven temperature was 70 °C (held for 2 min), followed by a 10 °C min^{-1} increase up to 200 °C. This first ramp was followed by a second, of 20 °C min^{-1}, up to 300 °C. The final temperature was maintained for 5 min. Run time was 26 min, with EtP retention time of 15.2 min. Detection was performed in a ISQ single quadrupole mass spectrometer (Thermo Scientific), operating in single ion monitoring (SIM) mode. Transfer line temperature was 280 °C and ion source temperature was 150 °C. EtP monitored *m/z* were 101 (quantification), 157 and 284. EtP-D5 monitored *m/z* were 106 (quantification), 162 and 289. The assay was linear between 0.05 and 300 ng mg^{-1}, with precision in the range of 6.12 to 12.53% and accuracy in the range of 95.15 to 109.91%.

2.13 Study participants and specimen collection

Adult volunteers of both sexes admitted to a drug rehabilitation clinic in the Brazilian state of Rio Grande do Sul were invited to participate in the study. Before hair specimen and data collection, all volunteers signed a consent form. Specimens were collected at the day of admission. The institutional review board of Universidade Feevale approved the study. Hair was collected from the vertex area of the scalp, following usual procedures [42]. Collected hair specimens were wrapped in aluminum foils and stored in paper envelopes, at room temperature [12,42]. Analysis was performed with hair lengths lower than 6 cm. EtG and EtP concentrations were measured in the collected specimens. Specimens presenting EtG concentrations higher than 500 pg mg^{-1} were re-analyzed using 10 mg of hair.

2.14 Alcohol Use Disorder Identification Test (AUDIT)

Study volunteers answered the Alcohol Use Disorder Identification Test (AUDIT). The AUDIT is a screening tool developed by the World Health Organization (WHO) to assess alcohol consumption, drinking behaviors, and alcohol related problems [43,44]. In this study,

we used AUDIT C, a simplified version of the complete AUDIT, used to evaluate the probable frequency and quantity of ethanol intake. This test was properly validated in the Portuguese language [45]. The score of the AUDIT-C allowed the classification of the volunteers as presenting low (score 0-3), moderate (score 4-5), high (score 6-7), or severe risk (score 8-12) of ethanol use disorder [43].

2.15 Evaluation of the ethanol consumption behavior using biomarkers and AUDIT-C

The ethanol consumption behavior of the volunteers was evaluated using the hair concentrations of EtG and EtP, as well as the AUDIT-C score. Ethanol consumption using EtG and EtP hair levels was classified based on SOHT consensus. For statistical evaluation, the concentration of the biomarkers was evaluated in isolation. EtG hair concentrations lower than 7 pg mg^{-1} was considered as indicative of ethanol abstinence, whereas concentrations higher than 7 pg mg^{-1} were indicative of repeated alcohol consumption. EtG hair levels higher than 30 pg mg^{-1} was considered as indicative of chronic excessive ethanol consumption [15,16]. EtP concentrations lower than 0.12 or 0.15 ng mg^{-1}, in hair specimens with 0-3 or 0-6 cm of length, respectively, were suggestive of ethanol abstinence. EtP concentrations higher than the values indicative of abstinence and lower than 0.35 or 0.45 ng mg^{-1} in hair specimens with 0-3 or 0-6 cm of length, respectively, were suggestive of repeated alcohol consumption. EtP concentrations higher than 0.35 or 0.45 ng mg^{-1} in hair specimens with 0-3 or 0-6 cm of length, respectively, were indicative of chronic excessive ethanol consumption [15,16].

Descriptive analysis was performed for all variables, including the Shapiro-Wilk test for normal distribution. The concordance among the ethanol drinking behavior classification using EtG, EtP and AUDIT-C were evaluated by Kappa test and Spearman correlation coefficient. Statistical evaluation of data was carried out using SPSS v.22.0 (IBM, United States).

3. RESULTS AND DISCUSSION

3.1 Extraction of EtG from hair and analytical conditions

Hair samples were washed using previously evaluated procedures, which did not affect EtG concentration [5,13,22,46]. After washing, the hair was milled at a ball mill. Hair milling was used because it was demonstrated to improve extraction of EtG when compared to the

extraction of cut hair [31]. Previous reports presented a wide variability of sample preparation strategies for the determination of EtG in hair. Sample preparation details and analytical characteristics of previous studies describing the measurement of EtG in hair are summarized in table 2.

Response surface analysis was used to evaluate the results of the Box-Behnken experiment. The model describing EtG peak area ratio from the process variables was not significant (P=0.1991), showing little impact of the investigated variables. This lack of significance of the model could be attributed to the small difference in the measurements obtained at the different evaluated conditions, which were confounded with the experimental noise due to the experimental variability. The only factor presenting statistical significance was incubation time. Even considering that presence of methanol in water did not influenced the magnitude of the measurements, we observed an increased number of interferences in the chromatogram when methanol was presented in the extraction solvent. Based on these observations, as we intend to have the highest possible analytical signal, we selected to use water for extraction of EtG from hair, with an extraction time of 24 h. The majority of previous reports employed extraction with water, with extraction times of 2 to 18 h [23,24,27–32]. Extraction temperature did not show influence in the extraction efficiency of EtG from hair, as no significant difference was found among the tested temperatures (25, 40 e 55°C), as previously reported [11,22–30,32]. Solvent volume did not affect the efficiency of the extraction. Considering that the liquid extract was further processed with SPE, we selected the volume of 1.5 mL for easier handling and to avoid losses during transfer of the extract for SPE cartridges. SPE was used because the injection of aqueous extracts, after evaporation under a gentle stream of air and recovery with mobile phase, produced chromatograms with many interferences,

retention of the highly polar EtG, even without the use of 100% aqueous mobile phase negatively influencing the sensitivity of the assay. Anion-exchange SPE has been described in several reports of EtG measurement in hair, after liquid extraction of hair, with few variations [23]. A range of specimen amounts were reported for EtG measurement in hair, from 25 to 100 mg (table 2). Using 50 mg of hair, we were able to obtain acceptable sensitivity, as presented below, allowing the detection of the relevant concentrations to evaluate ethanol consumption, as proposed in SOHT consensus [11,22–32].

Total run time was 6 min, with retention times for EtG and EtG-D5 of 2.09 and 2.08 min, respectively (Figure 1). Previous reports, using octadecyl, HILIC or carbon-based stationary phases, reported run times of 5 to 21 min (table 2). In this study, we describe the first

application of a CSH Fluoro-Phenyl stationary phase for EtG analysis, which provided significant retention of EtG considered the flow rate of 0.3 mL and the UPLC column dimensions (capacity factor of approximately 10). This stationary phase allowed considerable conditions at any time, which is usually required when using octadecyl silica columns [8,30]. The used separation conditions allowed sensitive quantification of EtG, with minimal matrix effects, as presented below.

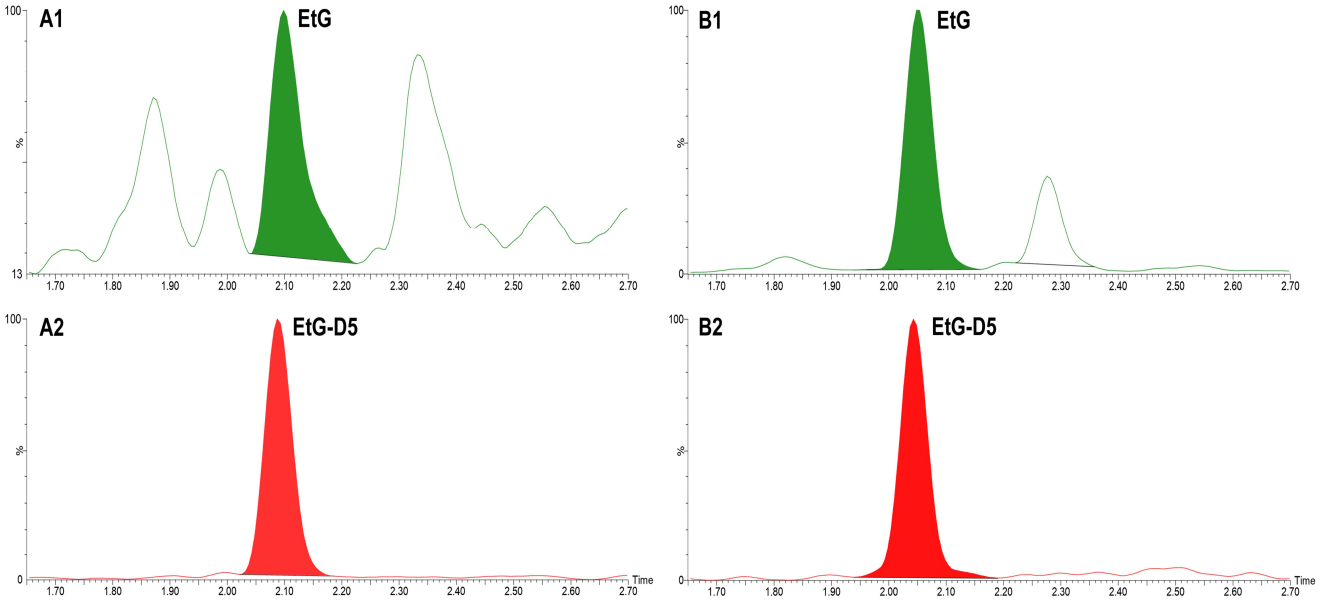

Figure 1. Ion chromatograms presenting EtG extracted from hair. A1: QCLOQ sample (4 pg mg^{-1}); B1: Volunteer specimen containing EtG at 84.3 pg mg^{-1}. A2 and B2: EtG-D5 (internal standard) in the QCLOQ and volunteer sample, respectively.

Table 2. Short overview of the available LC-MS methods for the determination of EtG.

Hair amount (mg)	Extraction solvent	Incubation time	Sonication time	Clean up	Extraction yield	Column	LOQ (pg mg^{-1})	Run time (min)	Retention time (min)	Matrix Effect (%)	Article (ref.)
100	700µL H_2O + 20µL MeOH	Overnight	2h	None	_	Chrompack Inertsil ODS-3	3	21	4	7.11-19.86	[22]
50	2mL H_2O	np	2 h	Oasis MAX SPE	80%	Acquity BEH HILIC	10	14	3.7	20-50	[23]
100	2mL H_2O	Overnight	2 h	None	> 55%	Hypercarb	50	15	5.5	25.4	[24]
25	1mL H_2O, 1mL ACN, 25µL MeOH	16 h	1.5 h	None	QCL 111 %, QCH 72%	Luna HILIC	20	19	3.5	49-65	[25]
100	700µL H_2O + 20µL MeOH	Overnight	2 h	None	QCL 97.5%, QCH 102.1%	Sinergy Polar RP	4	20	3.3	< 15	[26]
30	1.5mL H_2O	18 h	np	Clean Screen EtG	115%	Acquity UPLC HSS T3	2	3	1.4	34	[27]
75	500µL H_2O	Overnight	2 h	None	QCL 97.5%, QCH 102.1%	Hypercarb	2.3	35	3.3	0.6-4.9	[28]
50	500µL H2O / MeOH (35:1, v/v)	15 h	1.5 h	None	_	Acquity BEH C18	1	21	1	9	[11]
100	2mL H_2O	np	15 min	Oasis MAX SPE	QCL 94.56%, QCH 127.2%	Inertsil ODS-3	20	18	7.9	14.39	[29]
25	1.5mL H_2O	15 min	np	Oasis MAX SPE	QCL 105.1%, QCH 103.7%	Hypercarb	2	16	6.6	4-19	[30]
50	1.5mL H_2O	np	2 h (40°C)	BondElut SAX	> 53%	Acquity UPLC HSS T3	10	5	-	34	[31]
30	2mL H_2O	np	2 h	Oasis MAX SPE	82.5%	Synergy 4u fusion RP	3	6	1.86	Non-existent	[32]

np: not performed.

3.2 Assay validation

The developed assay presented linearity in the range of 4 to 500 pg mg^{-1}, with calibration curves always presenting $r \geq 0.99$. Due to the heteroscedasticy of the calibration data, weighted linear regression was using to obtain the calibration curves. The weighting factor presenting the lowest Σ% RE (-4.13×10^{-14}) was 1/x, which was used throughout the study. Back-calculated concentrations of the calibrators were within ±15% of nominal values.

Precision and accuracy presented acceptable results according to bioanalytical validation guidelines [40,41]. Table 3 presents a summary of the method validation results. Intra and inter-assay precision ranged between 5.14% to 10.42% and 3.99% to 6.92%, respectively. Assay accuracy was 100.30% to 106.16%. The lowest limit of quantification was 4 pg mg^{-1}, with control samples containing EtG at this concentration levels presenting intra-assay precision of 8.12%, inter-assay precision of 3.02% and accuracy of 100.91%.

Matrix effects were completely compensated by the use of a deuterated analog of EtG as internal standard. Matrix effects evaluated at the concentration levels of CQL, CQM e CQH, were 5.89%, -0.87%, and -0.47%, respectively (-28.71 to -38.78% without correction by the internal standard). The selected extraction conditions allowed a complete recovery of EtG, with extraction yields ranging from 96.93 to 101.06% (Table 3). The recovered SPE extracts were stable for 12 h at the chromatograph's autosampler, with peak area ratios variations of 0.16% and -0.86% for QCB and QCH, respectively.

The sample preparation and analysis employed in this study allowed high extraction yields and negligible matrix effects, representing an incremental advance over previous assays presenting either lower extraction efficiency or higher matrix effects [11,22,26,27,31]. The minimization of matrix effects could also be attributed to the selective separation from matrix components obtained with the used chromatographic conditions.

Table 3. Validation parameters for the EtG determination in hair: precision, accuracy, recovery extraction yield, matrix effect and stability.

Compound	Concentration (ng mg^{-1})	Precision (CV%)		Accuracy (%)	Extraction yield (%)	Matrix effect (%)	Autosampler stability 12 h (≠ in %)
		Inter-assay	Intra-assay				
QCLLOQ	4	3.02	8.12	100.91	-	-	-
CQL	5.5	5.14	5.18	103.43	96.93	5.89	0.16
CQM	80	5.60	6.92	100.30	101.06	- 0,87	-
CQH	450	10.42	3.99	106.16	100.48	- 0.47	-0.86

QCLLOQ: quality control at the lowest limit of quantification; CQL: low quality control; CQM: medium quality control; CQH: high quality control.

3.3 Application of the assay to volunteer specimens

The assay was applied to scalp hair specimens of 46 volunteers, collected in the day of admission to a drug rehabilitation clinic. Among the collected specimens, 18 (39.1%) had 0-3 cm of length, whereas 28 (60.9%) had 0-6 cm. Using SOHT criteria, the ethanol use behavior was classified using EtG and EtP hair concentrations. Results of the AUDIT-C and hair concentrations of EtG and EtP are presented in table 4. EtG hair concentrations from volunteers ranged from 4 to 1798.3 pg mg^{-1}, while EtP concentrations were in the range of 0.22 to 2.42 ng mg^{-1}. Using EtG concentrations, 20 (43.5%) volunteers were classified as excessive abusive user of ethanol, 16 (34.8%) were classified as moderate chronic consumers of ethanol, and 10 (21.7%) presented results compatible with ethanol abstinence. Using EtP hair concentrations to evaluate the consumption of ethanol, 38 (82.6%) volunteers were considered as chronic abusive users of ethanol, while 8 volunteers (17.4%) were not. not being considered chronic users of ethanol.

Table 4. AUDIT-C scores and EtG and EtP hair levels from the study volunteers.

Patient	Sex	Age	AUDIT C					Hair EtG (pg mg^{-1})	*Consumption behavior according to EtG*	Hair EtP (ng mg^{-1})	*Consumption behavior according to EtP*
			Q1	Q2	Q3	Total Score	Risk level				
1	M	36	0	0	0	0	Low	295.37	Excessive	0.73	Excessive
2	M	25	0	0	0	0	Low	7.42	Moderate	0.65	Excessive
3	M	24	1	1	0	2	Low	<LQ	Abstinent	1.69	Excessive
4	F	27	0	0	0	0	Low	14.33	Moderate	1.96	Excessive
5	M	29	0	0	0	0	Low	4.84	Abstinent	0.32	Moderate
6	F	50	0	0	0	0	Low	73.36	Excessive	0.35	Moderate
7	M	44	0	0	0	0	Low	246.98	Excessive	0.4	Excessive
8	M	30	0	0	0	0	Low	5.41	Abstinent	0.53	Excessive
9	F	48	0	0	0	0	Low	9.54	Moderate	1.19	Excessive
10	M	26	1	1	0	2	Low	6.07	Abstinent	1.29	Excessive
11	M	23	1	1	0	2	Low	9.33	Moderate	1.37	Excessive
12	M	28	1	1	0	2	Low	6.85	Abstinent	2.07	Excessive
13	M	21	2	1	1	4	Moderate	11.44	Moderate	0.94	Excessive
14	F	33	3	1	0	4	Moderate	<LQ	Abstinent	1.92	Excessive
15	F	37	1	1	1	3	Moderate	15.96	Moderate	0.4	Moderate
16	M	33	2	1	2	5	Moderate	5.92	Abstinent	0.51	Excessive
17	M	68	4	2	0	6	High	178.33	Excessive	0.97	Excessive
18	M	41	4	2	1	7	High	335.94	Excessive	1.62	Excessive
19	M	39	4	1	1	6	High	21.57	Moderate	2.29	Excessive
20	M	21	2	4	2	8	Severe	16.79	Moderate	0.71	Excessive
21	M	28	4	4	4	12	Severe	90.99	Excessive	0.72	Excessive
22	M	37	4	4	4	12	Severe	94.49	Excessive	0.74	Excessive
23	M	53	4	4	4	12	Severe	19.73	Moderate	0.65	Excessive
24	F	35	4	4	4	12	Severe	20.19	Moderate	0.73	Excessive
25	M	37	4	2	4	10	Severe	31.42	Excessive	0.76	Excessive
26	F	43	4	3	4	11	Severe	13.53	Moderate	0.79	Excessive
27	M	32	4	4	4	12	Severe	258.32	Excessive	0.99	Excessive
28	F	54	4	2	4	10	Severe	14.29	Moderate	0.99	Excessive
29	M	47	4	4	4	12	Severe	596.4	Excessive	1.68	Excessive
30	M	62	4	2	4	10	Severe	819.5	Excessive	1.68	Excessive
31	M	39	4	4	4	12	Severe	596.4	Excessive	1.72	Excessive
32	M	35	4	4	4	12	Severe	16.23	Moderate	1.89	Excessive
33	F	39	4	4	3	11	Severe	19.19	Moderate	0.22	Moderate
34	M	21	4	4	4	12	Severe	30.73	Excessive	0.29	Moderate
35	F	47	4	4	4	12	Severe	4.77	Abstinent	0.29	Moderate
36	M	31	4	3	4	11	Severe	22.31	Moderate	0.33	Moderate
37	F	39	2	3	3	8	Severe	30.98	Excessive	0.41	Moderate
38	M	43	4	4	4	12	Severe	46.05	Excessive	0.49	Excessive
39	M	58	4	4	4	12	Severe	<LQ	Abstinent	0.51	Excessive
40	M	58	4	4	4	12	Severe	1798.3	Excessive	1.02	Excessive
41	M	38	4	4	4	12	Severe	6.14	Abstinent	1.03	Excessive
42	M	65	4	4	3	11	Severe	466.58	Excessive	1.19	Excessive
43	M	52	4	4	4	12	Severe	200.98	Excessive	1.3	Excessive
44	F	47	4	4	4	12	Severe	84.91	Excessive	1.38	Excessive
45	M	39	4	4	4	12	Severe	975.65	Excessive	2.23	Excessive
46	M	48	4	4	3	11	Severe	7.72	Moderate	2.42	Excessive

The volunteers also answered to the AUDIT-C questionnaire, where 58.7% had severe, 6.5% high, 8.7% moderate, and 26.1% low health risks related to ethanol consumption. EtG and EtP hair concentrations measured in volunteers' specimens were not normally distributed (SW 0.92, $p< 0.01$ for EtP; SW 0.54, $p<0,001$ for EtG). EtG and EtG hair levels were not significantly correlated, which could be attributed to the low specificity of FAAE as markers of ethanol intake. EtP can be formed by the use of ethanol containing cosmetic products, or accumulated due to poor hygiene habits of the specimen donor, which is a limitation for its clinical and forensic use [9,23,32]. Currently, FAEE or EtP hair concentrations are recommended only in cases of suspected false-negative for EtG [10,12]. These false negative results can occur in cases of small ethanol intake or excessive hair washing [10,12]

We stratified the ethanol consumption behavior of the volunteers based on the measured EtG concentrations on three groups: abstinent (n=10), moderate (n=16), and excessive drinker (n=20), and compared this classification with AUDIT-C. In the group of ethanol abstinent volunteers, 5 had AUDIT-C presenting low health risk (50% of concordance). EtG and AUDIT-C classification as moderate consumption of the ethanol and moderate health risk were concordant in 6,5% of the volunteers. Among the 20 volunteers with excessive consumption of ethanol according to EtG hair concentrations, 17 had high or severe risk according to AUDIT-C (85% of concordance).

Ethanol consumption classification categories defined with EtG hair levels were significantly correlated to the AUDIT-C classification ($r_S=0.365$, $p<0.05$). The diagnostic capability of EtG hair concentrations to identify excessive or moderate ethanol use was similar to the capability of AUDIT-C to identify severe and high health risk (kappa, P=0.013) (Table 5). Figure 2 presents the distribution of EtG concentrations according to the risk profile classified by AUDIT-C. Even considering the high level of concordance between EtG hair levels and AUDIT-C health risk classification due to ethanol consumption, the use of an objective indicator instead of a questionnaire, which can be biased for individual and social motivations, is highly recommended for the evaluation of chronic use of ethanol.

Table 5. AUDIT-C risk classification with ethanol consumption classification for EtG and EtP.

AUDIT-C risk	EtG		*P* value	EtP		*P* value
	Moderate (n)	Excessive (n)		Moderate (n)	Excessive (n)	
Low/Moderate	13	3	0.013	3	13	0.859
High/Severe	13	17		5	25	

P values assessed as Kappa test.

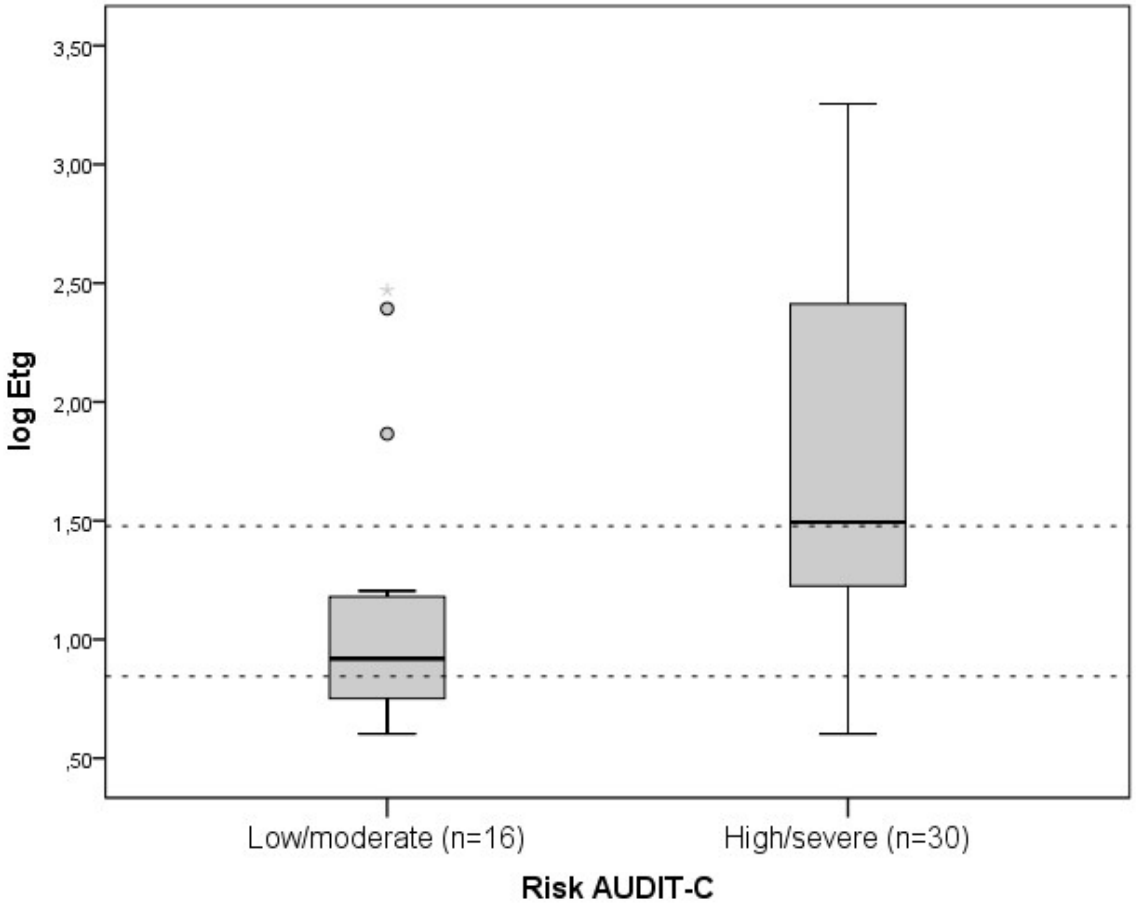

Figure 2. Distribution of EtG concentrations according to the risk profile classified by AUDIT-C. Dashed lines represent the cut-off value of 7 to 30 pg mg -1 for EtG on a logarithmic scale.

4. CONCLUSIONS

An improved UPLC-MS/MS assay for the determination of EtG in hair was developed, based on the aqueous extraction for 24 h, followed by ion-exchange solid phase extraction. EtG was efficiently separated from other matrix components using a Fluoro-Phenyl stationary phase, in only 6 min. The selected sample preparation and chromatographic conditions allowed minimal matrix effects. The assay was sensitive, presenting a lower limit of quantification of 4 pg mg^{-1}, also fulfilling additional validation criteria. Ethanol consumption classification categories defined with EtG hair levels were significantly correlated to the AUDIT-C classification, but EtG and EtP hair concentrations were not correlated. The diagnostic capability of EtG hair concentrations to identify excessive or moderate ethanol use was similar to the capability of AUDIT-C to identify severe and high health risk. The developed assay is

suitable for the routine analysis of EtG in hair, providing an useful tool to objectively evaluate chronic ethanol consumption behavior.

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6 CONSIDERAÇÕES FINAIS

Considerando os inúmeros problemas relacionados ao consumo abusivo do etanol, o diagnóstico do uso crônico dessa substância é importante do ponto de vista clínico e forense (KINTZ; NICHOLSON, 2014; PRAGST, 2015). Isso torna imprescindível o aprimoramento de testes laboratoriais capazes de estabelecer parâmetros objetivos para uma mensuração correta e efetiva da exposição direta ao etanol (OPOLZER et al., 2016).

Neste estudo, um método para determinação de EtP em cabelo foi otimizado e validado, empregando HS-MEFS CG-EM, de acordo com as guias internacionais **(capítulo 1)**. As condições experimentais para a MEFS automatizada foram otimizadas através de análise de superfície de resposta, após um experimento estatisticamente planejado, abordagem que ainda não havia sido aplicada neste problema analítico. As condições otimizadas para MEFS do EtP diferiram daquelas inicialmente propostas por Pragst, et al. (2001), as quais foram utilizadas em numerosos trabalhos posteriores (SCHAFFER; HILL, 2005; PRAGST; BALIKOVA, 2006; CABARCOS et al., 2009; ALBERMANN; MUSSHOFF; MADEA, 2011; HASTEDT et al., 2009, 2012). As condições diferenciadas, selecionadas neste estudo, foram tempo de pré-incubação de 6 min e temperatura de pré-incubação e adsorção de 94 °C, diferentemente dos 30 min e 90 °C previamente relatados. A otimização do método possibilitou um incremento na resposta do EtP e com isso pôde-se obter áreas de picos até 33% maiores do que com o método de referência, o que viabilizou a utilização de uma massa reduzida de amostra, de 20 mg. O método foi validado e aplicado em amostras clínicas, entretanto apresentou baixa correlação com a classificação de risco à saúde devido ao uso de etanol estimado pelo questionário AUDIT-C. Essa falta de concordância da concentração capilar do EtP e do risco de consumo de etanol no AUDIT-C pode ser atribuída a vários fatores. Os voluntários do estudo eram usuários de múltiplas drogas e com variáveis graus de distúrbios cognitivos, o que pode contribuir para respostas não confiáveis aos questionários. Questões sociais, familiares e legais também poderiam reduzir a confiabilidade dos questionários como ferramentas de avaliação do comportamento do uso de drogas. E ainda, cabe ressaltar a possibilidade de formação de EtP por uso de produtos cosméticos com álcool em sua formulação.

Adicionalmente, foi otimizado e validado um método para detecção de EtG em amostras de cabelo humano por CLUE-EM/EM **(capítulo 2).** A presença de EtG no cabelo fornece uma forte indicação do consumo de etanol, pois tem relação direta com as dosagens no cabelo, além disso, sofre menor influência de produtos cosméticos do que o EtP (PIRRO et al., 2013; SOHT,

2016; PRAGST et al., 2017). A determinação de EtG em cabelo é desafiadora devido às baixas concentrações encontradas, particularmente no nível de corte para avaliação de abstinência, de 7 pg/mg (SOHT, 2016). Para tanto, foi empregada a CLUE-EM/EM, precedida de extração aquosa por 24 h e limpeza do extrato por extração em fase sólida. Diversas variáveis da etapa de extração aquosa foram investigadas, resultando em um procedimento com melhorias incrementais com relação a estudos anteriores, particularmente no rendimento da extração e nos efeitos de matriz. O ensaio foi validado de acordo com guias internacionais e foi aplicado em amostras clínicas. Diferentemente do EtP, as concentrações de EtG em cabelo foram correlacionadas com os escores do teste AUDIT-C. Além disto, a estratificação do consumo de etanol pelas concentrações de EtG e pelos níveis de risco do AUDIT-C foram altamente concordantes.

O estudo resultou no desenvolvimento e na validação de dois ensaios de elevada sensibilidade e completamente validados para avaliação do consumo crônico de etanol empregando amostras de cabelo. Estes ensaios podem ser utilizados como indicadores específicos e objetivos do comportamento de consumo de etanol, sem os vieses típicos de resposta que ocorrem com o uso de entrevistas, além de apresentarem múltiplas aplicações em toxicologia clínica e forense.

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ANEXO I

Comprovante de envio do artigo "*An optimized solid-phase microextraction and gas chromatography-mass spectrometry assay for the determination of ethyl palmitate in hair*" na revista *Journal of Analytical Toxicology*.

Enc: Journal of Analytical Toxicology submission acknowledgment - Manuscript ID JAT-19-2867

De: Journal of Analytical Toxicology <onbehalfof@manuscriptcentral.com>
Enviado: domingo, 26 de maio de 2019 19:31
Para: Rafael Linden
Assunto: Journal of Analytical Toxicology submission acknowledgment - Manuscript ID JAT-19-2867

26-May-2019

Dear Dr. Linden:

Your manuscript titled "AN OPTIMIZED SOLID-PHASE MICROEXTRACTION AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY ASSAY FOR THE DETERMINATION OF ETHYL PALMITATE IN HAIR" has been successfully submitted online and is presently being given full consideration for publication in the Journal of Analytical Toxicology.

Your manuscript ID is JAT-19-2867.

Please mention the above manuscript ID in all future correspondence or when calling the office for questions. If there are any changes in your street address or e-mail address, please log in to Manuscript Central at https://mc.manuscriptcentral.com/jat and edit your user information as appropriate.

ANEXO II

Comprovante de apresentação de trabalho no Salão de Pós-Graduação da Universidade Feevale.

CERTIFICADO

Concedemos ao trabalho

MÉTODO OTIMIZADO PARA AVALIAÇÃO DE ETIL PALMITATO EM CABELO COMO BIOMARCADOR DO USO CRÔNICO DE ETANOL UTILIZANDO CG-EM-MEFS-HS,

de autoria de

MARCOS FRANK BASTIANI, LILIAM DE LIMA LIZOT FELTRACO, ANNE CAROLINE CEZIMBRA DA SILVA, ROBERTA ZILES HAHN

e orientação de

RAFAEL LINDEN,

este certificado de apresentação no evento

SEMINÁRIO DE PÓS-GRADUAÇÃO (SPG) - INOVAMUNDI 2018,

realizado no período de 22 a 27 de outubro de 2018.

Âmbito: Internacional
Promoção: Universidade Feevale - Pró-Reitoria de Pesquisa, Pós-Graduação e Extensão
Organização: Pró-Reitoria de Pesquisa, Pós-Graduação e Extensão

Novo Hamburgo, 17 de dezembro de 2018.

Dr. João Alcione Sganderla Figueiredo
Pró-Reitor de Pesquisa, Pós-Graduação e Extensão

ANEXO III

Comprovante de apresentação de trabalho no 6º Encontro Nacional de Química Forense e 3º Encontro da Sociedade Brasileira de Ciências Forenses.

.

3º encontro da SBCF Sociedade Brasileira de Ciências Forenses

6º ENQFor Encontro Nacional de Química Forense

04 a 08 de novembro de 2018

CERTIFICADO

Certificamos que

MARCOS FRANK BASTIANI, LILIAN DE LIMA LIZOT FELTRACO, ANNE CAROLINE CEZIMBRA DA SILVA, ROBERTA ZILES HAHN, GIOVANA PIVA PETTEFI, RAFAEL LINDEN.

apresentaram o trabalho

OPTIMIZED METHOD FOR THE DETERMINATION OF ETHYL PALMITATE IN HAIR USING AUTOMATED HEADSPACE SOLID-PHASE MICROEXTRACTION AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY (HS-SPME-GC-MS)

no 6º Encontro Nacional de Química Forense - ENQFor e 3º Encontro da Sociedade Brasileira de Ciências Forenses, realizados de 04 a 08 de novembro de 2018, no Centro de Convenções de Ribeirão Preto - SP, na forma de **pôster**.

Ribeirão Preto, 08 de novembro de 2018.

A autenticidade deste documento pode ser verificada através da URL:
http://www.sbcf.org.br/congresso/certificado/3BA98019

Profa. Dra Aline Thaís Bruni
Presidente da SBCF

Prof. Dr. Celso Teixeira M. Junior
Coordenador do 6º ENQFor e 3º Encontro da SBCF

Promoção e Realização

Patrocínio

Apoio

ANEXO IV

Parecer consubstanciado do Comitê de Ética em Pesquisa da Universidade Feevale.

UNIVERSIDADE FEEVALE/ASSOCIAÇÃO PRÓ-ENSINO SUPERIOR EM NOVO

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Avaliação do estresse oxidativo e de biomarcadores de exposição em pacientes internados para tratamento de dependência por álcool, cocaína e crack

Pesquisador: MAGDA SUSANA PERASSOLO

Área Temática:

Versão: 2

CAAE: 81406617.6.0000.5348

Instituição Proponente: ASSOCIACAO PRO ENSINO SUPERIOR EM NOVO HAMBURGO

Patrocinador Principal: ASSOCIACAO PRO ENSINO SUPERIOR EM NOVO HAMBURGO

DADOS DO PARECER

Número do Parecer: 2.467.042

Apresentação do Projeto:

De acordo.

Objetivo da Pesquisa:

De acordo.

Avaliação dos Riscos e Benefícios:

De acordo.

Comentários e Considerações sobre a Pesquisa:

De acordo.

Considerações sobre os Termos de apresentação obrigatória:

De acordo.

Recomendações:

Não há.

Conclusões ou Pendências e Lista de Inadequações:

De acordo.

Considerações Finais a critério do CEP:

Em conformidade com a Resolução nº 466 de 12 de dezembro de 2012, do Conselho Nacional de Saúde, e com as normas internas do Comitê de Ética em Pesquisa da Universidade Feevale,

Endereço: RS 239, nº 2755
Bairro: Vila Nova **CEP:** 93.525-075
UF: RS **Município:** NOVO HAMBURGO
Telefone: (51)3586-8800 **Fax:** (51)3586-8800 **E-mail:** ranieli@feevale.br

todos os documentos necessários à análise do projeto acima referido por este Comitê foram apresentados.

Este projeto preserva os aspectos éticos dos sujeitos da pesquisa, sendo, portanto, aprovado pelo Comitê de Ética em Pesquisa da Universidade Feevale.

Reiteramos que o Comitê de Ética em Pesquisa da Instituição encontra-se à sua disposição para equacionar eventuais dúvidas e/ou esclarecimentos que se fizerem necessários.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1051762.pdf	11/01/2018 16:09:42		Aceito
Declaração de Instituição e Infraestrutura	DeclaracaoFeliz.pdf	21/12/2017 13:12:21	MAGDA SUSANA PERASSOLO	Aceito
Declaração de Instituição e Infraestrutura	DeclaracaoCai.pdf	21/12/2017 13:12:06	MAGDA SUSANA PERASSOLO	Aceito
Projeto Detalhado / Brochura Investigador	formulario.doc	18/12/2017 09:06:16	MAGDA SUSANA PERASSOLO	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE.doc	18/12/2017 09:06:02	MAGDA SUSANA PERASSOLO	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCUD.pdf	18/12/2017 09:05:50	MAGDA SUSANA PERASSOLO	Aceito
Outros	formulariocep.pdf	18/12/2017 09:05:37	MAGDA SUSANA PERASSOLO	Aceito
Folha de Rosto	folharostoassinada.pdf	18/12/2017 09:05:10	MAGDA SUSANA PERASSOLO	Aceito
Declaração de Pesquisadores	declaracaopesquisador.pdf	18/12/2017 09:05:00	MAGDA SUSANA PERASSOLO	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:

Endereço: RS 239, nº 2755
Bairro: Vila Nova **CEP:** 93.525-075
UF: RS **Município:** NOVO HAMBURGO
Telefone: (51)3586-8800 **Fax:** (51)3586-8800 **E-mail:** ranieli@feevale.br

Não

NOVO HAMBURGO, 15 de Janeiro de 2018

Assinado por:
Ranieli Gehlen Zapelini
(Coordenador)

Endereço: RS 239, nº 2755
Bairro: Vila Nova **CEP:** 93.525-075
UF: RS **Município:** NOVO HAMBURGO
Telefone: (51)3586-8800 **Fax:** (51)3586-8800 **E-mail:** ranieli@feevale.br

ANEXO V

Termo de consentimento livre e esclarecido, apresentados aso voluntários do estudo.

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (TCLE)

Você está sendo convidado a participar do projeto institucional intitulado: Avaliação do estresse oxidativo e de biomarcadores de exposição em pacientes internados para tratamento de dependência por álcool, cocaína e crack, sob responsabilidade da professora Dra. Magda Susana Perassolo. Os objetivos deste estudo são avaliar parâmetros de estresse oxidativo e biomarcadores de exposição em pacientes antes e após a internação hospitalar, para tratamento da dependência por álcool e cocaína/crack, correlacionando com sua evolução clínica.

Sua participação nesta pesquisa será voluntária e consistirá em responder ao questionário de avaliação das características gerais dos pacientes, ao questionário ***AUDIT (***teste para avaliação do consumo de bebidas alcoólicas), realizar uma coleta de sangue para avaliação de parâmetros laboratoriais (função renal, hepática e estresse oxidativo). Além disto, doar uma amostra de cabelo com diâmetro em torno de 3 a 4 mm, que será fixada na sua parte proximal com barbante e cortada rente ao couro cabeludo, preferencialmente na parte posterior da cabeça, onde o crescimento é relativamente mais uniforme. O cabelo coletado será acondicionado, corretamente rotulado e armazenado em local seco e protegido da luz para posterior análise. Não haverá riscos relacionados à sua participação na pesquisa, apenas o desconforto da picada de sangue.

O pesquisador responsável e a Universidade Feevale envolvidas nas diferentes fases da pesquisa proporcionarão assistência imediata e integral aos participantes da pesquisa no que se refere às possíveis complicações e danos decorrentes. Os participantes da pesquisa que vierem a sofrer qualquer tipo de dano resultante de sua participação na pesquisa, previsto ou não neste documento, têm direito à indenização, por parte do pesquisador, do patrocinador e das instituições envolvidas nas diferentes fases da pesquisa.

A sua participação nesta pesquisa estará contribuindo para: melhor conhecer os efeitos do álcool e da cocaína/crack e as formas de detectar marcadores da exposição destas substâncias no organismo.

Garantimos o sigilo de seus dados de identificação primando pela privacidade e por seu anonimato. Manteremos em arquivo, sob nossa guarda, por 5 anos, todos os dados e documentos da pesquisa. Após transcorrido esse período, os mesmos serão destruídos. Os dados

obtidos a partir desta pesquisa não serão usados para outros fins além dos previstos neste documento.

Você tem a liberdade de optar pela participação na pesquisa e retirar o consentimento a qualquer momento, sem a necessidade de comunicar-se com o(s) pesquisador(es).

Este Termo de Consentimento Livre e Esclarecido será rubricado em todas as folhas e assinado em duas vias, permanecendo uma com você e a outra deverá retornar ao pesquisador. Abaixo, você tem acesso ao telefone e endereço eletrônico institucional do pesquisador responsável, podendo esclarecer suas dúvidas sobre o projeto a qualquer momento no decorrer da pesquisa.

Nome do pesquisador responsável: Magda Susana Perassolo
Telefone institucional do pesquisador responsável: 3586-8800 ramal 8938 ou 9040
E-mail institucional do pesquisador responsável: magdaperassolo@feevale.br

_____
Assinatura do pesquisador responsável

Local e data: _____, _____ de _____20_____.

Declaro que li o TCLE: concordo com o que me foi exposto e aceito participar da pesquisa proposta.

_____
Assinatura do participante da pesquisa

APROVADO PELO CEP/FEEVALE – TELEFONE: (51) 3586-8800 Ramal 9000
E-mail: cep@feevale.br

ANEXO VI

Questionário padronizado AUDIT-C, aplicado aos voluntários do estudo.

1. **Com que frequência você toma bebidas alcoólicas?**
   a) Nunca
   b) Mensalmente ou menos
   c) De 2 a 4 vezes por mês
   d) De 2 a 4 vezes por semana
   e) 4 ou mais vezes por semana

   Score_____

2. **Nas ocasiões em que bebe, quantas doses você consome tipicamente ao beber?**
   a) 1 ou 2
   b) 3 ou 4
   c) 5 ou 6
   d) 7, 8, ou 9
   e) 10 ou mais

   Score_____

3. **Com que frequência você toma seis ou mais doses de uma vez?**
   a) Nunca
   b) Menos do que uma vez por mês
   c) Mensalmente
   d) Semanalmente
   e) Todos ou quase todos os dias

   Score_____

A pontuação do AUDIT- C é feita em uma escala de 0 a 12 pontos. Cada pergunta do instrumento tem cinco opções de resposta, possibilitando uma pontuação de 0 a 4 em cada: a = 0 pontos, b = 1 pontos, c = 2 pontos, d = 3 pontos, e = 4 pontos. A pontuação de 0 a 3 é considerada de baixo risco; entre 4 e 5 pontos, risco moderado; entre 6 e 7 pontos, alto risco e de 8 a 12 pontos, risco severo.

Dose padrão equivalente:

a) Pinga, uísque ou vodka – 40mL
b) Vinhos do Porto, vermute ou licores – 85mL
c) Vinhos de mesa – 140 mL
d) Cerveja ou chopp – 340mL