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# Aplicação de dried saliva spots na determinação de marcadores de consumo de tabaco

**(Versão final após defesa)**

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# Dedicatória

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“The mystery of human existence lies not in just staying alive, but in finding something to live for” - Fyodor Dostoevsky

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# Abstract

Exposure to tobacco smoke is one of the most common causes of premature death worldwide and is the cause of 8 million deaths annually. For this purpose, it was developed, optimized, and validated a procedure for the detection of nicotine, cotinine and trans-3-hydroxycotinine (biomarkers of tobacco exposure) in oral fluid using the dried saliva spots sampling approach and gas chromatography coupled to tandem mass spectrometry, thus allowing the distinction between active and passive smokers. For optimization, 4 parameters were evaluated, namely extraction solvent, extraction solvent volume, extraction time and spots drying time. During method validation, the parameters selectivity, linearity, precision and accuracy, recovery, stability, and dilution factor were assessed.

Linearity was obtained for all target analytes in the concentration range of 10-200 ng/mL allowing the quantification of compounds up to 1000 ng/mL considering the dilution factor. The method recoveries ranged from 29.2% to 43.30% for nicotine, 66.60% to 89.10% for cotinine and 80.30% to 92.80% for trans-3-hydroxycotinine, while achieving intra-day, inter-day and intermediate precision and accuracy values never greater than 10.37% and ± 6.50% respectively for all compounds. The herein described analytical method is the first that allows the determination of tobacco biomarkers in oral fluid using dried saliva spots, being considered a sensitive, simple, and low-cost alternative to conventional methods.

# Keywords

Tobacco; Nicotine; Oral fluid; Biomarkers of tobacco; Dried saliva spots; Gas chromatography coupled to tandem mass spectrometry

# Resumo Alargado

O consumo de tabaco é um dos principais problemas de saúde pública relacionado com consumo legal de substâncias. Existem mais de 1,1 mil milhões de consumidores em todo o mundo, sendo a exposição ao fumo produzido pelo consumo considerada a causa de mais de 8 milhões de mortes anuais, das quais 1 milhão se deve à exposição passiva frequente, nomeadamente ao fumo de tabaco ambiental. Este consumo pode originar doenças do foro oncológico, respiratório e cardíaco. Atualmente existem diversos formatos para o consumo, variando do mais regular cigarro até aos dispositivos eletrónicos de substituição. De forma a contornar esta problemática, têm sido implementadas diversas políticas que visam controlar o consumo em todo o mundo. O consumo de tabaco provoca a exposição do organismo a mais de 4800 compostos, sendo a nicotina um dos principais compostos e biomarcadores de interesse. Tal se deve à sua elevada percentagem na planta do tabaco e efeito aditivo ao nível do sistema nervoso central, sendo que é um agonista dos recetores colinérgicos nicotínicos. Do metabolismo de nicotina, através da ação das enzimas CYP2A6, FMO3 e UGT, resultam diversos metabolitos dos quais no presente trabalho foram selecionados dois, a cotinina e a trans-3-hidroxicotinina. Estes dois metabolitos são considerados biomarcadores de maior utilidade devido aos seus tempos de meia vida (6-22 e 5-8 horas, respetivamente). Tempos de semi-vida superiores aos observados para nicotina (1,5 a-3,5 horas).

A monitorização deste tipo de compostos torna-se então vital para a área clínica e toxicológica, e por esse motivo o desenvolvimento de técnicas para a sua identificação e determinação tem sido impulsionada.

Para determinar estes biomarcadores, a amostra de fluido oral tem-se revelado ser uma matriz biológica de elevado interesse analítico. As aplicações de métodos de extração miniaturizados têm aumentado ao longo dos últimos anos, sendo disso exemplo os *dried saliva spots* (DSS), os quais têm vindo a ser aplicados como método extrativo para diferentes compostos devido ao baixo volume de amostra necessário e ao facto de possibilitar uma execução rápida e simples.

Tendo como base estas premissas, o presente trabalho descreve o processo de desenvolvimento, otimização e validação de um novo método analítico para a determinação de três biomarcadores de tabaco (nicotina, cotinina e trans-3-hidroxicotinina) em fluido oral com recurso a DSS precedido de precipitação proteica, recorrendo a cromatografia gasosa acoplada a espetrometria de massa em tandem (GC-MS/MS). Foram utilizados como padrões internos os análogos deuterados de cada um dos biomarcadores em estudo (nicotina-d4, cotinina-d3 e trans-3-hidroxicotinina-d3), os quais apresentam comportamento similar aos compostos em estudo. O estudo de

otimização processou-se em várias etapas, sendo avaliado o tipo de solvente utilizado (9 solventes e/ou misturas), o volume de solvente (1-3mL), o tempo de extração (5-30 min) e o tempo de secagem (1h-overnight) para DSS, sendo ainda avaliados outros parâmetros complementares.

A validação do método seguiu os critérios definidos pelo Scientific Working Group for Forensic Toxicology (SWGTOX), onde foram a avaliados a seletividade, linearidade, limites, precisão e exatidão, recuperações, estabilidade e fator de diluição. O método demonstrou ser linear no intervalo de concentração compreendido entre 10 e 200ng/mL, permitindo ainda a quantificação até 1000 ng/mL considerando o fator de diluição estudado. A precisão e exatidão foram avaliadas através dos coeficientes de variação e erro relativo respetivamente, não observando valores superiores a 10,37% e ±6,50% respetivamente. As recuperações obtidas alcançaram valores entre 29,2 a 43,30%para nicotina; 66,60% a 89,10% para cotinina e 80,30% a 92.,80% para trans-3-hidroxicotinina. Posteriormente o método foi aplicado a diversas amostras de fluido oral provenientes de estudantes da Universidade da Beira Interior. O método desenvolvido cumpriu todos os critérios de validação, permitindo alcançar valores de corte (*cut-off*) de 10 ng/mL, valor consensual entre a comunidade científica como concentração limite para a distinção entre consumidores ativos e passivos. Este método demonstrou ser um método simples e de rápida execução, permitindo realizar múltiplas extrações em simultâneo, tendo como principal vantagem a utilização de valores reduzidos de amostra de fluido oral (100 μL). Esta metodologia demonstra ser uma excelente alternativa a outros métodos de extração utilizados na área da toxicologia, contornando assim as limitações recorrentes de obtenção de volumes significativos de fluido oral. Para além disso, este método permite ainda fazer a monitorização da exposição ao fumo de tabaco, podendo ser aplicado em diversos estudos clínicos para os quais a avaliação destes biomarcadores seja pertinente. O método desenvolvido é o primeiro que utiliza fluido oral recorrendo a DSS e GC-MS/MS para a identificação de biomarcadores de consumo e exposição de tabaco.

# Palavras-chave

Tabaco; Nicotina; Biomarcadores de tabaco; Fluido oral; *Dried saliva spots*; Cromatografia gasosa acoplado a espetrometria de massa em tandem

# Index

# Index of figures

## Chapter I: Introduction



## Chapter III: Experimental procedure

# Index of tables

## Chapter I: Introduction



## Chapter III: Experimental procedure

# Index of abbreviations

| | |
|---|---|
| ACN | Acetonitrile |
| AET | Ethyl acetate |
| CI | Chemical ionization |
| CNS | Central nervous system |
| COT | Cotinine |
| CV | Coefficient of variation |
| CYP2A6 | Cytochrome P450 2A6 |
| DBS | Dried blood spot |
| DCM | Dichloromethane |
| DMS | Dried matrix spots |
| DSS | Dried saliva spots |
| ECD | Electron capture detector |
| EI | Electronic impact |
| EI+ | Electron positive ionization |
| ETS | Environmental tobacco smoke |
| FID | Flame ionization detector |
| FMO3 | Flavin-containing monooxygenase 3 |
| GABA | γ-aminobutyric acid |
| GC-MS | Gas chromatography coupled to mass spectrometry |
| GC-MS/MS | Gas chromatography coupled to tandem mass spectrometry |
| HPLC-DAD | High performance liquid chromatography coupled to diode-array detector |
| IONSCAN ® -LS IMS | IONSCAN®-LS ion mass spectrometry |
| IS | Internal standard |
| ISOP | Isopropanol |
| LC-MS | Liquid chromatography coupled to mass spectrometry |
| LC-MS/MS | Liquid chromatography coupled to tandem mass spectrometry |
| LLE | Liquid-liquid extraction |
| LLME | Liquid-liquid microextraction |
| LLOQ | Lower limit of quantification |
| LOD | Limit of detection |
| LTM | Long-term monitoring |
| MeOH | Methanol |
| MRM | Multiple reaction monitoring |
| MSTFA | N-Methyl-N-(trimethylsilyl) trifluoroacetamide |
| nAChRs | Nicotinic cholinergic receptors |
| NIC | Nicotine |
| NPPD | Phosphorus/nitrogen detector |
| OF | Oral fluid |
| OH-COT | Trans-3-hydroxycotinine |
| PAHs | Polycyclic aromatic hydrocarbons |
| PI | Product ion |
| PID | Photoionization detector |
| PP | Protein precipitation |

| | |
|---|---|
| QCs | Quality controls |
| RE | Relative error |
| RTM | Real-time monitoring |
| SLE | Supported liquid extraction |
| SPE | Solid phase extraction |
| SPME | Solid phase microextraction |
| TCD | Thermal conductivity detector |
| TMCS | Trimethylchlorosilane |
| TSNA | Tobacco-specific N-nitrosamines |
| UBI | Universidade da Beira Interior |
| UGT | UDP-glucuronosyltransferase |
| ULOQ | Upper limit of quantification |
| WADA | World Anti-Doping Agency |
| WHO | World Health Organization |
| μ-SPE-BI-LOV | Micro solid phase extraction bead injection lab-on-valve |

# Chapter I: Introduction

## 1 Smoking: A society problematic

Tobacco is the only legal substance on the market whose effects cause severe damage to the human organism, not being possible to define a so-called safe level of exposure (1,2). Although a global decrease in tobacco consumption has been observed, approximately 1.1 billion smokers have been accounted in 195 countries, according to 2017 data, killing 8 million people every year, with 7 million deaths resulting from active smoking and the remaining from frequent environmental smoke exposure (ETS) (2,3). An exhaustive study conducted in Portugal, between 1987 to 2014, points to a decline in the percentage of smokers but it was possible to verify an increase in smoking prevalence among women. In fact, it was the only factor that suffers a significant increase over the years covered by the surveys (4).

Tobacco is a product that can be consumed as a regular cigarette and through other consumption formats such as pipes, shisha, cigarillos, cigars, and recently by new devices such as electronic cigarettes and vapers (1).

Tobacco and its substitutes consumption habits are heavily related to nicotine (NIC) addiction and to fragile socio-economic and environmental indicators such as psychological factors, low educational attainment, unemployment status and civil status, frequency and intensity of exposure (4). The synergy of the mentioned inputs is being responsible for high rates of morbidity related to cardiovascular, oncological and respiratory diseases leading to an increase in worldwide premature mortality, whose consumption patterns are more pronounced in non-developed countries according to World Health Organization (WHO) (1,4). In parallel, the continuous growth of the consumer market in developed countries is pointing to the emergence and globalisation of tobacco substitutes as a probable cause of this consumption increase (1,4). Regarding human health, several diseases, namely carcinogenic diseases, and non-carcinogenic diseases, caused by tobacco exposure and consumption, have been exhaustively described in several studies. The most common type of cancer in smokers and those with a high frequency of passive exposure are typically associated with the respiratory tract, however, pancreatic, liver, breast, stomach and cervical cancers are also related to this human behaviour (1). Some of the most prevalent non-carcinogenic diseases include thrombocytic activity, myocardial infarction, Burger´s disease, pulmonary emphysema and chronical bronchitis, infertility, estrogen deficit and modification of lipoproteins through free radicals, being also responsible for several fetus disorders (1,5).

Nonetheless, in recent years we have witnessed a trend towards the establishment of prevention and awareness policies regarding the use of tobacco and its substitutes, as well as ETS exposure, given the inherent risks to public health and the development of increasingly sensitive detection methods to evaluate the exposure levels.

## 1.1 History of tobacco

It was after Christopher Columbus' arrival in North America that the first records about the *Nicotiana* tobacco plant's origin began to appear (6,7). Due to tobacco medicinal properties described by the indigenous communities, tobacco seeds were exported to countries such as Portugal and Spain, and later to the rest of Europe (6).

Prior to the dissemination of tobacco consumption by colonisers arriving on the American continent, it was common to observe the use of tobacco leaf by indigenous communities as treatments due to its analgesic and antiseptic properties. Also, it was used for several other purposes, ranging from its use as a pesticide in agriculture to its use in rituals (6). For many years, tobacco consumption was associated with a certain social status, subsequently spreading to the rest of society, triggering an increase in tobacco demand and consumption. Nevertheless, in scientific community many critical voices have arisen with a certain level of doubt about its consumption benefits being greater than the damages (6,7).

From the 19th century, large-scale tobacco-production machinery began to be introduced in England and later around the world, causing a major increase in cigarettes consumption rather than pipes due to their practical convenience during the course of World War I (6). Nowadays, more than 6 tobacco species are identified, and its use is widely spread across all continents, even after all the scientific studies indicating its harmful effects (7).

As more information becomes available on the health problems associated with regular tobacco consumption, several companies have been creating alternative solutions to tobacco, such as heated tobacco namely the Philip Morris® brand, with the creation of the IQOS device, which works by heating a cigarette specific to this device (8,9) and electronic cigarette or vapers, which are based on a system that creates an aerosol produced by heating a liquid, with or without NIC (10). During the 21st century the market of these devices has been sharply increased, but there is no evidence of clear benefits to smokers' health, so it should be considered the application of prevention strategies and control measures as those formulated for regular tobacco consumption (8).

## 1.2 Environmental tobacco smoke exposure

The ETS, commonly referred to as passive smoking or second-hand smoke exposure, results from the inhalation of contaminated air resulting from the consumption of tobacco or tobacco substitutes, by active smokers, present in enclosed environments by non-smokers (11). The composition of ETS is defined as the mixture between the exhaled smoke from the cigarette named mainstream smoke and the smoke from the combustion of the cigarette named called sidestream smoke, whose molecular composition changes after diffusion into the air with the "aging" of the mixture (1,12,13). These two streams possess different characteristics, which are defined in Table 1, with sidestream smoke being responsible for a relevant part of the tobacco molecules present in ETS composition (11–13).

**Table 1:** Typical chemical and physical characteristics of cigarette smoke in the mainstream smoke and sidestream smoke. Adapted from (11–13).

| Characteristics | Mainstream smoke | Sidestream smoke |
|---|---|---|
| Temperature | 850-900 ºC | 500-650 ºC |
| Mean particle size | 0.35-0.45 µm | 0.15-0.25 µm |
| Particle concentration | $10^9 – 10^{10}$ $cm^3$ | ≈ $10^5$ $cm^3$ |
| Tobacco burned (%) | 30-40% | 50-60% |
| ETS particulate matter | 15-43% | 57-85% |
| ETS vapor matter | 1-13% | 87-99% |
| pH | Slightly acidic | Slightly alkaline |
| NIC | >99% in particulate phase | >95% vapor phase |
| Sidestream/Mainstream ratio (NIC) | 2.6-3.3 | |

The extent of ETS exposure is influenced by 5 factors: the size of the room where the person is, the type of ventilation, the number of smokers, the amount of cigarettes smoked and the exposure time (1). Following the increase in the number of consumers of tobacco substitutes such as electronic cigarettes, some studies focus on the risks of passive exposure to smoke emanating from electronic cigarette devices compared to smokers of cigarettes (9,10). Here, it was possible to notice an overall mitigation of mostcompounds present in environments where only cigarette consumption exists (10).

The first studies that observed ETS exposure dangers emerged in the 80's of the 20th century, reporting it as a factor responsible for the increase in tobacco smoke-related

diseases. This cause-effect relation was confirmed by several studies (1,6,11,14). One of the groups with most vulnerability to ETS are children, due to their higher respiratory rate, smaller bronchial dimensions and a weaker immune system (1).

In order to mitigate ETS exposure and encourage the cessation of intrinsic smoking habits, several countries have been adopting different control policies in accordance with the MPOWER programme introduced by WHO in 2008 (3). MPOWER stands for "**M**onitor tobacco use and prevention policies; **P**rotect people from tobacco smoke; **O**ffer help to quit tobacco use; **W**arn about the dangers of tobacco; **E**nforce bans on tobacco advertising, promotion and sponsorship; **R**aise taxes on tobacco". In Portugal, laws to control exposure and reduce tobacco smoke and its substitutes have been introduced in accordance with WHO guidelines. The implemented regulation was in compliance with the provisions of the WHO Framework Convention on Tobacco Control, approved by Decree No. 25 -A/2005, of 8 November, resulting in the creation of laws No. 37/2007 and respective updates in laws No. 63/2017 and 88/2019 published in the Portuguese Republic's Diary.

# 2 Tobacco Chemical Compounds

Active tobacco consumption and ETS are currently responsible for human body exposure to more than 4800 different substances, originating from the tobacco leaf or from compounds added during tobacco manufacturing. These including humectants, preservatives and binders or fillers, with a large proportion of the inhaled compounds being potentially hazardous to human health (1,13,15). Although NIC and its metabolites, which are related to smoking addiction, are the main targets studied, all compounds present in tobacco smoke may be linked to the development of several diseases in active or passive smokers (5,11).

Molecules identified as originated from tobacco consumption come from incomplete combustion of cigarettes to which can be added many intermediate reactions such as distillation, pyrolysis, pyrosynthesis, and condensation. Different compounds can be produced from two different regions, exothermic and endothermic, being the first one characterized by combustion reactions and the second one by pyrolysis, pyrosynthesis and distillation reactions (1,12). This distinction can be observed in Figure 1. The inhaled substances level of absorption, in active tobacco smoke, may differ in accordance with several factors related to tobacco use, namely, brand of cigarette, number of cigarettes smoked daily, frequency, duration and number of puffs, lung capacity and flow of inhalation per puff and type of filtering (1). In the combustion region (exothermic zone), gases such as hydrogen, carbon monoxide and carbon dioxide are generated as a result of the reaction between the carbonised tobacco and the available oxygen (13). In the region where the remaining reactions take place (endothermic zone), more than 4700 compounds can be generated at different temperatures (13).

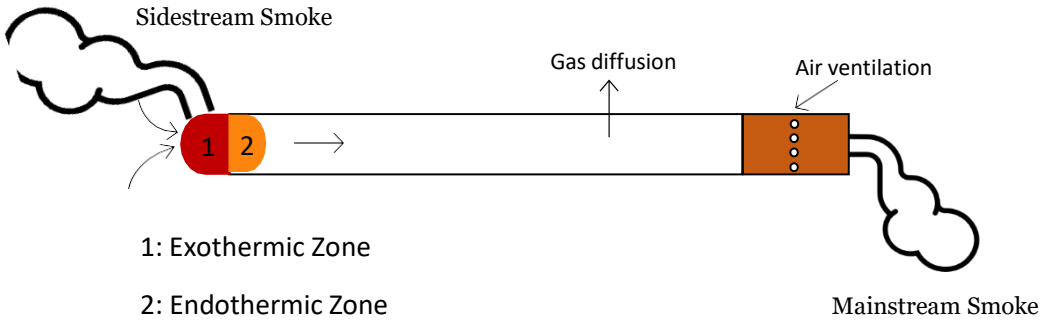


**Figure 1:** Tobacco burning mechanism. Adapted from (13).

Despite a large proportion of the compounds in tobacco smoke are in trace levels, more than 60 compounds in tobacco smoke are toxic for human body. Specifically, 9 of them

can be classified as carcinogenic. So, it is important to monitor the levels to which smokers are exposed (15).

The consumption of regular tobacco implies an exposure to higher concentrations of several toxic compounds when compared to electronic cigarettes, however these new systems show higher levels of metals than those observed in regular tobacco (10). These metals origin from the resistances present in electronic cigarettes equipment, which suffer some degree of deterioration (10).

The compounds in tobacco smoke can be divided in particulate phase and gas phase accounting, respectively, for 4.5% and 1.5% of total smoke, with the remaining constituents coming from the air (13). Typically, the particulate phase contains: pigments, alkanes, miscellaneous, terpenoids, phenols, esters, other alkaloids, NIC, alcohols and humectants, aldehydes and ketones, carboxylic acids, and water (1,13). The gaseous phase consists mainly of gases from combustion such as nitric oxide, carbon dioxide and carbon monoxide, and also includes 1,3-butadiene, formaldehyde, acetaldehyde, acrolein, toluene, acrylonitrile, isoprene, benzene and hydrogen cyanide (1,13).

With the proven cause-effect relation between tobacco smoke exposure and certain diseases, it becomes pertinent to assess the degree of exposure for a clinical evaluation (1,5). Some of the compounds present in tobacco smoke are further classified into 4 groups, based on their carcinogenic properties, according with the directives of the International Agency for Research on Cancer (IARC). Specifically, the Group 1 includes molecules that are carcinogenic to humans, Group 2A includes compounds that are presumably carcinogenic, Group 2B includes compounds that are potentially carcinogenic, Group 3 includes those not classified as carcinogenic, and Group 4 probably not carcinogenic (1).

## 2.1 Nicotine

Among the compounds present in the tobacco leaf (*Nicotiana tabacum*) (Figure 2) is NIC, an alkaloid of natural origin present in high concentrations, highly volatile with insecticidal properties (11,16–19). In each cigarette it is estimated that there are between 7 to 24 mg of NIC, accounting for about 95% of the alkaloid content. About 0.3 to 3 mg of NIC is absorbed through the oral mucosa reaching the lung alveoli, skin, digestive system, and urinary system. In contrast, the amount absorbed through electronic devices still requires exhaustive studies. This is due to the individual variability regarding the mode of consumption and NIC concentration used (2,11,17,18,20). In relation to NIC isomers, S- isomer is the most commonly found in tobacco leaf and usually greater than

99%. The remaining percentage consisting of the R-isomer, yet several studies indicate that 10% of the NIC present in tobacco smoke might be (R)-NIC. These levels can be attributed to possible racemization phenomena upon combustion (17,18).

### 2.1.1 Pharmacokinetics

NIC present in mainstream tobacco smoke is essentially in the particulate phase, with absorption through biological barriers being dependent on NIC's characteristics, the body's pH andthe tobacco's smoke pH produced at the time of consumption (11,17,18). NIC has a pKa of approximately 8, thus being considered as a weak base (17,18,21). When the pH of thesmoke is more acidic (below pH 6), NIC is in its ionized form, becoming more difficult tocross the membranes. Nevertheless, some tobacco products generate smoke with a morealkaline pH (above pH 6.5) in which NIC is in a non-ionized form, thus allowing greaterabsorption through the oral mucosa membrane and the lung alveoli (17,18,22). When it reaches the pulmonary alveoli, a large-scale absorption of NIC into the bloodstream occurs. This is the result of the vast vascularisation of the alveoli, as well as the fact that 31% of NIC is in a non-ionized state at a blood pH of 7.4. Only 5% of total NIC present inthe blood is bound to plasma proteins (17,18,22).

**Figure 2:** *Nicotiana tabacum* (7)

After entering the body, NIC undergoes biotransformation processes that lead to the appearance of several other compounds as can be seen in Figure 3. NIC metabolism is a complex system of two phases, designated as phase I and phase II, carried out mainly in the liver. An inter-individual and racial variability in the metabolism rate has been reported (17,18,21,22).

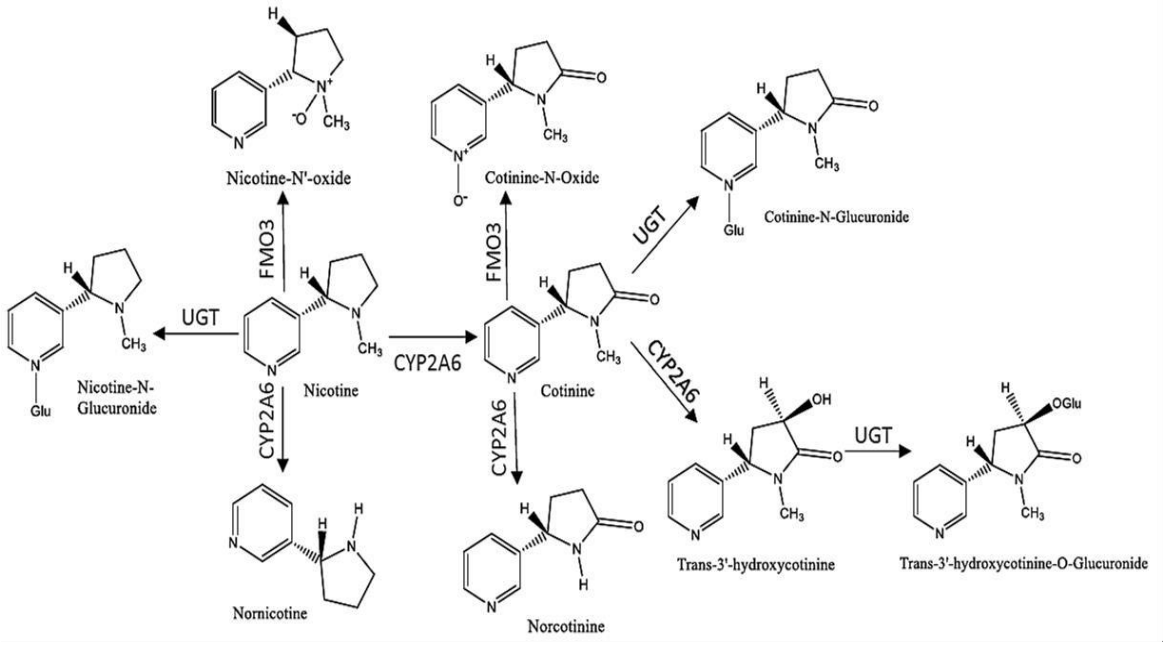


**Figure 3**: Nicotine metabolic pathway (2).

In phase I, NIC is metabolized in cotinine (COT) by cytochrome P450 2A6 (CYP2A6) through C-oxidation, with the conversion rate ranging from 70 to 80% (17,18,22). The metabolization rate depends on the type of polymorphism present in each individual (22). The mechanism of action is performed in two stages. The first step involves hydroxylation by the microsomal enzyme CYP2A6, while the second step consists on the conversion of the aldehyde in COT and is led by the cytoplasmic enzyme aldehyde oxidase (17,18,21,22). Here, minor metabolites are formed in smaller proportions such as Nornicotine and Nicotine-*N*'-oxide by *N*-oxidation, respectively, via CYP2A6 and flavin-containing monooxygenase 3 (FMO3) (17,18,22).

The phase II of NIC metabolism is characterized by *N*-glucuronidation and *O*-glucuronidation performed by the enzyme UDP-glucuronosyltransferase (UGT). These reactions originate more water-soluble compounds, promoting their release through urine, with NIC glucuronide representing between 3 to 5% of the total NIC absorbed by human body (17,18,22). NIC's half-life is approximately 1.5-3.5 hours, reason why NIC is considered a biomarker only for short-term exposure (23).

As previously mentioned, NIC metabolism occurs mainly in the liver, representing 70% of the global metabolization (17,18). Some physiological factors, namely; pathologies, medication, consumption and racial differences, can influence the NIC metabolization rate, either through hepatic blood flow variation or modifications in the enzymes involved in the metabolic pathways (17,18).

NIC excretion from the body occurs through tubular secretion and glomerular filtration, whereby this mechanism is dependent on urinary pH (17,18). In the presence of urine at

an alkaline pH, tubular reabsorption of NIC occurs, due to the increase in its non-ionised fraction (17,18). In acidic pH, NIC is mostly ionized, preventing tubular reabsorption (17,18).

### 2.1.2 Pharmacodynamics

Following tobacco smoke inhalation, NIC is rapidly absorbed in the lung alveoli, passes into the bloodstream and reaches the brain within 10-20 seconds (17,18). It diffuses into the brain and reaches the central nervous system (CNS) bonding stereo-selectively as an agonist to nicotinic cholinergic receptors (nAChRs), typically present in the brain, autonomic ganglia, neuromuscular junctions and adrenal medulla (16,17,21,22,24,25). The nAChRs complex comprises 5 subunits (2α, β, δ, γ or ε). Curiously, neuronal nAChRs are only composed by α and β subunits, with 9 isoforms for α subunit and 3 for β subunit, the most prevalent being the α-4 and β-2 subunits (16,17,21,22,24,25). The β-2 subunit is responsible for the dopamine release and behavioural characteristics of NIC while the α-4 subunit is related to the sensitivity exerted by NIC on nAChRs (21,24). The activation promoted for a continuous exposure to the NIC leads to a upregulation, increasing the expression of nAChRs (25).

Following NIC binding to nAChRs, calcium and sodium channels open allowing the entry of these cations, activating voltage-dependent calcium channels, causing not only the successive entry of more calcium cations but also the release of neurotransmitters such as dopamine, acetylcholine, norepinephrine, beta endorphine, glutamate, serotonin and γ-aminobutyric acid (GABA) (21,24,25).

One of the most complex concepts in pharmacodynamics is the dose-response mechanism. Direct activation in the brain or peripheral stimulation of chemoreceptors leads to sympathetic activation when the organism is exposed to low doses of NIC, thus raising the heart rate and blood pressure (21,25). When exposed to higher doses of NIC, organism initiates a process of catecholamine release through adrenal pathway and ganglionar stimulation proceeding from the direct action of NIC in the peripheral neuronal system (25). When the organism is exposed to higher doses than tolerable, a process of ganglionar blockade begins, leading to the onset of bradycardia and hypotension phenomena (21,25). With continued tobacco exposition, tolerance mechanisms are created resulting from the inactivation of several subtypes of nAChRs, thus reducing the symptoms of consumption (21,25).

### 2.1.3 Effects of nicotine on the Central Nervous System

Although tobacco consumption and consequent NIC use is considered legal, NIC acts as a drug that affects CNS, providing addictive and pleasurable effects when consumed.
A relevant system linked to the addiction mechanism is the dopaminergic system. This fact was confirmed through the ablation of dopaminergic neurons, affecting the stimulating effect on the locomotor system promoted by NIC (22). Another study demonstrating the influence of the dopaminergic system on NIC consumption suggests that after administration of haloperidol, a dopaminergic antagonist, NIC consumption increased, indicating that blocking these receptors led to a decrease in the effect of pleasure and satiety, favouring NIC addiction mechanism (22,24).
The consumption of NIC induces the release of dopamine in the frontal cortex, mesolimbic area and corpus striatum, exerting an effect on the dopaminergic neurons present in the nucleus accumbens and ventral tegmental area, which are essential to the mechanism of addiction (24). Typically, with a prolonged nicotine consumption, a desensitization of nAChRs receptors occurs, leading to a decrease in the inhibitory effect on dopamine release (phenomenon mediated by GABA) but without affecting the stimulatory effect on dopamine release produced by glutamate. The described mechanism increments the responsiveness to NIC consumption (24).

### 2.1.4 Psychoactive and physiological effects of nicotine

With the development of NIC addiction, several behavioural and mood changes occur, begin more accentuated in prolonged abstinence scenarios (22,24,26). In regular smokers, after tobacco consumption there is an increase in NIC concentration, which helps to reduce stress and increase pleasure (22,24,26). Once a regular smoker is in abstinence, several symptoms start to appear, being the most common irritableness, anxiety and decreased concentration (22,24,26). Upon NIC consumption, multiple physiological changes occur, for instance, an increase in metabolic activity and a decrease in appetite (22,24,26). These two mechanisms associated with high levels of serotonin release, lead to the loss of body mass (22,24,26). Also is described an enhancement in performance, reaction time and concentration when performing certain tasks such as studying (22,24,26).

## 2.2 Biomarkers

Identification and quantification of biomarkers is a very useful tool for assessing the extent of exposure to different compounds and for understanding the individual pattern of their excretion and metabolism in the organism (1,2,11). Biomarkers enable the assessment of ETS, allowing the distinction between active smokers, passive smokers and non-smokers through analytical methods (1,2,11).

The determination of ETS biomarkers is accomplished by direct quantification of tobacco compounds or respective metabolites, with two different types of monitoring being carried out, long-term monitoring (LTM) or real-time monitoring (RTM). Specifically, the ideal biomarkers for ETS studies should meet the following requirements: extended half-life; highly specific and sensitive; allow the distinction of several degrees of exposure; constant ratio even when the individual is subjected to different degrees of exposure or sources (2,11,27).

The selection of optimal biomarkers for ETS studies is also influenced by their precision, which can be influenced by other sources of exposure, individual variations in metabolism and the analytical methods available for their identification and quantification (1,11,27).

Several classes of compounds are used as biomarkers of tobacco exposure and the main classes are the following: NIC and tobacco alkaloids; carbon monoxide; Tobacco-Specific N-Nitrosamines (TSNA); Polycyclic Aromatic Hydrocarbons (PAHs); volatile organic compounds; heterocyclic and aromatic amines; metals; thiocyanate (2). Among the most sensitive and specific biomarkers to assess the degree of exposure to ETS or active consumption are COT and its major metabolite trans-3-hydroxycotinine (OH-COT), both NIC metabolites (2).

### 2.2.1 Cotinine and Trans-3-hydroxycotinine

COT and OH-COT have proven to be more useful biomarkers to assess tobacco smoke exposure and NIC metabolization rate, due to their longer half-lives, 6-22 and 5-8 hours for COT and OH-COT, respectively (23).

COT undergoes several metabolic processes in the body, originating a variety of metabolites, with only 10-15% of the 70-80% of COT from NIC metabolism being found in its unchanged form in urine (17,18,22). Among the metabolites, the most relevant is the OH-COT, formed by the enzyme CYP2A6, being the trans isomer predominant over the cis due to the high stereo-selectivity of the reaction (17,18,21,22). Both COT and OH-

COT are subjected to glucuronidation processes, with COT undergoing *N*-glucuronidation and OH-COT undergoing *O*-glucuronidation (17,18,22).

The renal clearance of COT is typically low owing to the high rate of tubular reabsorption, with the excretion rate being dependent on urinary flow (17,18). OH-COT is excreted mostly in its unchanged state, appearing in higher concentration in the urine than COT (17,18). On the contrary, COT levels are higher in oral fluid (OF) samples (2,17,18). The ratio between COT and OH-COT in OF is an excellent tool for detection of smoking habits as it allows a distinction to be made between light consumers and heavy consumers (28).

Over the past few years, the literature showed a significant commitment on the development of several methodologies to support the determination of the degree of exposure to tobacco smoke using extractive and/or analytical methods (Table 2).

**Table 2:** Review of analytical methodologies for nicotine and metabolites determination in different biological samples (2).

| Compound | Sample (amount) | Sample preparation | Analytical technique | LOD, ng/mL (pg/mg of hair) | LOQ, ng/mL (pg/mg of hair) | Recovery (%) | Reference |
|---|---|---|---|---|---|---|---|
| NIC, COT | Hair: 20mg | Incubation and LLE | LC-MS/MS | - | 2.5-25 | - | (29) |
| NIC, COT | Hair: 10mg | Incubation and LLE | LC-MS/MS | 0.66-8.6 | 2-26 | >90 | (30) |
| | Urine and Oral fluid: 0.5mL | LLE | | 0.0132-0.158 | 0.04-0.48 | | |
| NIC, COT, OH-COT | Urine: 0.1 mL | Enzymatic hydrolysis and PP | LC-MS/MS | 1.55-3.53 | - | 76-99 | (31) |
| NIC, COT | Oral fluid: 0.5mL | SPE | LC-MS/MS | - | 1-2 | - | (32) |
| NIC | Oral fluid: 0.25mL | LLME | GC-MS | - | - | - | (33) |
| | | | IONSCAN®-LS IMS | 9 | - | 99 | |
| NIC, COT, OH-COT | Oral Fluid and Plasma: 0.5 mL | LLE | LC-MS/MS | - | - | - | (34) |
| NIC, COT, OH-COT | Oral Fluid: 0.5 mL | SPE | GC/MS | 5 | 5 | 67-117.8 | (35) |
| NIC, COT, OH-COT | Oral Fluid: 0.5 mL | SPE | LC-MS/MS | 0.1-0.5 | 0.2-1 | 63.6-95.5 | (36) |
| NIC, COT, OH-COT | Meconium: 500 mg | Enzymatic hydrolysis and SPE | LC-MS/MS | 1.25 | 1.25 | 56.2-95.7 | (37) |
| NIC, COT, OH-COT | Meconium: 250 mg | Hydrolysis and SPE | LC-MS/MS | 2 -10 | 2 -10 | 73.2- 125.4 | (38) |
| NIC, COT | Hair: 1-2mg | Incubation and in-tube SPME | LC-MS/MS | 0.13-0.45 (pg/mL) | 4.4-7.5 | 87-96.1 | (39) |
| NIC, COT | Hair: 20mg | Incubation and LLE | GC-MS | - | - | ≈90 | (40) |
| | Urine: 0.8mL | LLE | | 0.6 | - | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Oral fluid: 0.8mL | LLE | | 0.6 | - | | |
| NIC, COT, OH-COT | Urine: 1mL | SPE | LC-MS/MS | 1 | 2.5 | 87-118 | (41) |
| | Plasma: 1mL | PP and SPE | | 0.25-0.75 | 1 | 63-85 | |
| NIC, COT, OH-COT | Hair: 20 mg | Incubation and SPE | LC-MS/MS | 0.03-0.05 | 0.5-0.10 | 62.9-117.8 | (42) |
| COT | Blood: 5 drops (finger-prick DBS) or 0.05mL (reconstituted DBS) | DBS | LC-MS/MS | - | 0.25 | - | (43) |
| COT | Oral fluid: 1mL | PP and μSPE-BI-LOV | HPLC-DAD | 1.5 | 3 | 95.9 | (44) |
| NIC, COT | Oral fluid: 0.2mL | In-tube SPME | LC-MS | 0.015-0.030 | - | 83-96.5 | (45) |
| | Urine: 0.1mL | | | | | 86.3-93.1 | |
| COT | Urine: 0.02mL | Automated on-line SPE | LC-MS/MS | 0.005 | 0.02 | - | (46) |
| NIC, COT, OH-COT | Liver and Placenta: 0.25g | SLE | LC-MS/MS | 0.7-3.5 | 1-5 | 80.3-107 | (47) |
| NIC, COT, OH-COT | Oral fluid: 0.2mL | SPE | GC-MS/MS | 0.5 | 0.5 | 84.6-99.8 | (48) |

**Legend:** DBS: dried blood spots; GC-MS: gas chromatography coupled to mass spectrometry; GC-MS/MS: gas chromatography coupled to tandem mass spectrometry; HPLC-DAD: high performance liquid chromatography coupled to diode-array detector; IONSCAN ® -LS IMS: IONSCAN LS ion mass spectrometry; LC: liquid chromatography; LC-MS: liquid chromatography coupled to mass spectrometry; LC-MS/MS: liquid chromatography coupled to tandem mass spectrometry; LLE: liquid-liquid extraction; LLME: liquid-liquid microextraction; PP: protein precipitation; SLE: supported liquid extraction; SPE: solid phase extraction; SPME: solid phase microextraction; μ-SPE-BI-LOV: micro-solid phase extraction bead injection lab-on-valve.

# 3 Biological matrix: Oral fluid

Alternative biological matrices have assumed an increasingly important role in toxicology, enabling the development of new analytical techniques for pharmacovigilance and drug exposure monitoring through the determination of biomarkers, as well as for the determination of metabolic and physiological activity levels of the compounds (2,49–51).

OF sample is considered a mixture of saliva, produced from the major salivary glands (sublingual, parotid and submandibular) and other minor glands along with food debris present in the oral cavity, enzymes, mucins, minerals, and other compounds (49–51).

OF collection is performed in a non-invasive way, under medical supervision and without any intimate part being observed, unlike the more common samples such as urine, blood or plasma, establishing this type of sample as less susceptible to adulteration. (2,48–52). This biological matrix also allows for an easy and fast collection, at low cost, and can be performed with or without stimulation (2,48–52). Meanwhile, for the analysis of OF samples, one must consider the recent use of substances through oral route or inhalation, since high concentrations can be observed due to the recent use or residues remaining in the oral cavity. In addition, OF pH can have an influence on the values of some compounds under study (2,49–51). This type of biological matrix has a relatively short detection window, roughly the same as plasma, allowing only recent exposures to be measured, up to a maximum of 36 hours (2,49–51).

One of the most common ways for OF collection is by spitting, a procedure that involves the rejection of OF directly into a sterile tube, requiring fewer resources (50,51). However, this type of collection presents some drawbacks, such as for children sample collection and the significant increase in the number of residues present, which makes it necessary to use a set of clean-up techniques such as protein precipitation (PP) (50,51). Other types of non-stimulated collections can be performed using specific collection equipment, such as draining, which allows to obtain a larger volume than spitting or through swabs, followed by the removal of the sample using diluents (49–51). Nonetheless, the volume of saliva available is not always sufficient due to several constraints such as lack of hydration, stress related to collection and the consumption of certain substances that reduce the flow and consequently the volume available (50,51). To obtain an adequate volume, it might be necessary to use stimulatory measures, such as mouth movements without external stimulants, mechanical stimulants such as teflon, rubber bands among others and gustatory stimulants such as citric acid or some candies (49–52). However, stimulatory measures may contribute to impairments in the accuracy of the analytical levels of some compounds (49–51).

The main mechanism for several compounds to reach OF is by passive diffusion, and depending on several physico-chemical factors such as blood and OF pH, pKa of the compounds, ionization state, percentage of protein binding and size of the molecules, one might observe an increase or decrease of the availability of these compounds in OF (49–51). OF has an average pH of 6.8, however the value can fluctuate in response to factors such as increased flow, circadian rhythm, stress, consumption of other substances and hormonal changes (49–51). Within OF sample it is assumed that only the free non-ionised fraction of several compounds can be found as a result of the low protein levels, fraction which is known to have a pharmacological activity (2,49–51).

The use of OF samples provides, then, a viable alternative for the determination of tobacco exposure, providing an easier way to collect samples while maintaining analytical accuracy and the ability to assess the level of exposure.

# 4 Dried Saliva Spots (DSS): an extraction technique

An increasing number of miniaturised extraction procedures have been developed in response towards to the development of more user and environmentally friendly techniques to monitor and detect several compounds.

In 2014, Abdel-Rehim et al. (53) developed for the first time an extraction procedure using dried saliva spot (DSS) to monitor lidocaine. DSS is one of the subtypes of dried matrix spot (DMS), representing one of the latest miniaturised methodologies to be applied for compounds' extraction (54,55).

Compared to the most common extraction techniques, SPE or LLE, the key advantages of using DSS include an easier and faster extraction procedure and the use of lower volumes of organic solvents, reduced volume of OF required, and reduced transport and storage conditions, thus reducing the costs per analysis (53,54,56–58).

The extraction process comprises two essential steps, the first being the application of a volume of 50 to 100 µL of OF followed by a drying period (56–58). Subsequently, each spot is transferred individually to a tube to which is added the necessary volume of extraction solvent and internal standard (IS). The sample undergoes a process of agitation at a certain speed and for a determined period of time to facilitate the extraction of the target analytes present in the spot (56–58). The final step involves the transfer of the supernatant to a new tube for further analysis, and depending on the requirements, a concentration or derivatization step might be adopted (56–58).

The literature shows a wide range of different studies using DSS as an extraction technique, namely microbiological studies (59), pharmacokinetic studies (60), drugs (57,58) and medication monitoring (56), establishing DSS as a versatile alternative for extraction.

Figure 4 shows the cards used in the extraction procedure. The developed work constitutes a novelty, by using DSS as an extraction method to monitor tobacco smoke exposure.

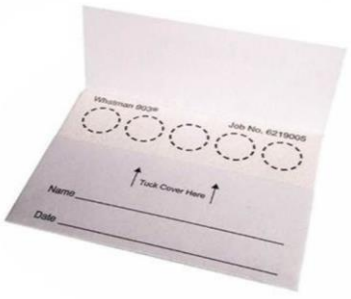


**Figure 4:** Dried Saliva Spot card (61)

# 5 Gas Chromatography coupled to Tandem Mass Spectrometry

Over the years, the demand for more accurate and sensitive analytical equipment has been observed, and these support the analytical procedures applied in the several toxicology fields, such as forensics and clinical. One of the most widespread analytical instruments for the determination and quantification of compounds in the routine of analytical laboratories is gas chromatography coupled with several types of detectors (62). This type of equipment is ideal for volatile compounds that do not decompose at the working temperatures, or stable compounds that, after a derivatisation process, become sufficiently volatile to make their analysis possible (63–66). The compounds' separation process in GC involves the dragging and volatilisation of a sample injected through the sample inlet, passing through a coated capillary column which is subsequently connected to the detector (64). The interaction of the compounds with the column depends on the temperature of the oven (which can range from 40ºC to 400ºC) and the flow rate of the chosen carrier gas (helium or nitrogen) which are adjusted to provide optimum separation (63–65).

Attached to the GC it is common to find several detectors, namely flame ionization detector (FID), photoionization detector (PID), electron capture detector (ECD), phosphorus/nitrogen detector (NPD), thermal conductivity detector (TCD), infrared detector (IR) among others, yet the most common detector coupled to GC is the mass spectrometry (MS), a class of detectors of which tandem mass spectrometry (MS/MS) belongs (64,65).

Mass spectrometry enables the detection and quantification of compounds via mass-to-charge ratio (m/z) upon ionisation by a current of electrons in the ion source (64,67). In tandem mass spectrometry, the identification of compounds is based on multiple reaction monitoring (MRM). MRM analysis involves the selection of a precursor ion in the first quadrupole resulting from compound fragmentation in the ionisation source, followed by further fragmentation in the collision cell, from which several product ions are generated. The most intense transitions (precursor ion – product ion) are selected (64,67).

The ionization source can be of two different types: electronic impact (EI) and chemical ionization (CI), with EI being the most common due to the library of spectra available for it (62,64,66). This component allows the fragmentation of the compounds enabling the identification of each signal through their individual ionization products (64). The ionization parameters are controlled by specific software (64).

The main components of a gas chromatography equipment coupled with mass spectrometry are the carrier gas, injector, column, oven, ion source, detector and data processor as can be seen in Figure 5 (64,65).

Considering the high sensitivity and selectivity required to monitor the extent of exposure to tobacco smoke, the features of this type of equipment provide a substantial advantage over other equipment, justifying its selection for this work.

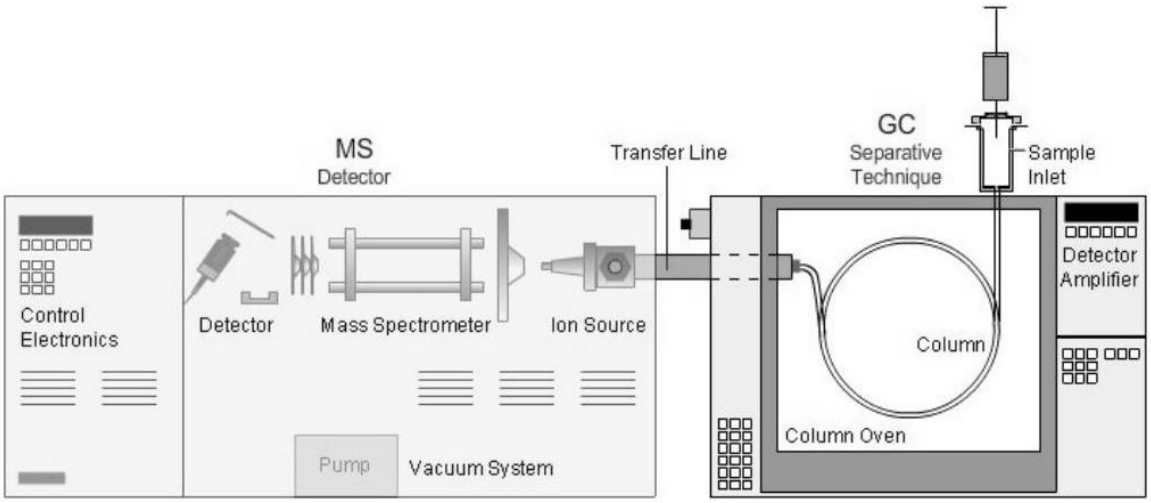


**Figure 5:** Schematic diagram of a GC-MS equipment type (68)

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# Chapter II: Aims

This work describes the entire process of optimization of extraction conditions for both NIC and its major metabolites COT and OH-COT in OF using DSS and their subsequent quantification by GC-MS/MS.

# Chapter III: Experimental procedure

## 1 Material and Methods

### 1.1 Reagents and standards solutions

Standard solutions of NIC, COT and respective deuterated analogues (NIC-d4 and COT-d3) were acquired from LGC Promochem (Barcelona, Spain) at a concentration of 1 mg/mL, except for NIC-d4 (100 µg/mL). OH-COT and its respective internal standard (OH-COT-d3) were purchased from Toronto Research Chemicals (York North, Canada) at a concentration of 1 mg/mL. Trimethylchlorosilane (TMCS) and N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) were supplied by Macherey-Nagel (Düren, Germany), from which a derivatisation solution of MSTFA with 5% TMCS was prepared. Whatman™ 903 protein saver cards were obtained from Sigma-Aldrich (Sintra, Portugal). Working solutions were prepared by diluting the respective analytical standards in methanol (MeOH), thus preparing a mix of NIC, COT and OH-COT at concentrations of 10 and 100 µg/mL. A mix of deuterated standards was prepared at 1 µg/mL in MeOH, and an individual solution of NIC-d4 at 0.5 µg/mL was also prepared inthe same solvent. All solutions were stored protected from light at 4 °C.

### 1.2 Biological samples

The blank oral fluid samples used throughout the experimental procedures were obtained from laboratory staff members who are neither smokers nor exposed to tobacco in their routine.

The authentic samples were obtained from students of Universidade da Beira Interior (UBI) after reading and accepting informed consent (Ethical Committee project: CE-UBI-Pj-2021-046:ID1005). The specimens were collected by spitting without the use of stimulating devices or specific collection devices for this purpose. All samples after collection were frozen at -20ºC.

### 1.3 GC-MS/MS conditions

Chromatographic analysis was accomplished using an HP 7890A gas chromatographic system, coupled with a triple quadrupole mass spectrometer model 7000B (both from Agilent Technologies, Waldbronn, Germany), an MPS2 autosampler and a PTV injector from Gerstel (Mülheim an der Ruhr, Germany). A capillary column (30 m × 0.25-mm I.D., 0.25-µm film thickness) with 5% phenylmethylsiloxane (HP-5MS), purchased from J & W Scientific (Folsom, CA, USA) was employed.

The oven temperature started at 90 ºC, maintained for 2 min, followed by a steady increase of 30 ºC/min until 190 ºC, and a second temperature ramp is performed with an increase of 25 ºC/min until a temperature of 250 ºC is reached. Three µL of the derivatized extract was injected in the splitless mode using helium as carrier gas at a constant flow rate of 0.9 mL/min. The mass spectrometer was set with a filament current of 35 µA and electron energy 70 eV in electron positive ionisation (EI+) mode. Data were acquired in the multiple reaction monitoring (MRM) mode using the MassHunter WorkStation Acquisition Software rev. B.02.01 (Agilent Technologies).

## 2 Sample Preparation

The extraction process designed for the studied compounds was carried out in two steps. Once the samples were thawed, a clean-up process by protein precipitation (PP) by addition of 5 µL of ice-cold acetonitrile (ACN) to 100 µL of sample is performed, and afterwards the samples are homogenized for 10 seconds in vortex and centrifuged for 10 minutes at3500 rpm. Subsequently, extraction by DSS is performed following these steps: application of 50 µL of oral fluid into Whatman™ 903 protein saver cards, followed by a drying step for 1 hour at a temperature of 25 ºC; then, an additional application of 50 µL of sample is performed, after which it will dry for 1 hour at the same temperature. The whole spots are then cut with scissors around the defined circle and placed individually in a falcon tube where 1 mL of acidified methanol (MeOH pH 5.0) and 50 µL of each of the internal standard solutions (internal standard mix NIC-d4, COT-d3 and OH-COT-d3 at 1 µg/mL; NIC-d4 at 0.5 µg/mL) is added. The compounds were then extracted for 5 min at 70 rpm in a roller mixer and then transferred to assay tubes to which 50 µL of a 1% HCl in MeOH is subsequently added. The extracts were evaporated to dryness under a gentle nitrogen stream, after which 20 µL of the derivatization solution (MSTFA+5%TMCS) was added followed by a microwave derivatization process for 2 min at 800W, despite the fact that OH-COT is the only compound that reacts with the derivatization reagents. Finally, a 3 µL aliquot of the derivatized sample is injected into the GC-MS/MS.

# 3 Validation procedure

The developed method was fully validated according to the SWGTOX guidelines (1). The validation process was carried out over 5 days, and the studied parameters were linearity, limit of detection (LOD), lower limit of quantification (LLOQ), selectivity, inter-day, intra-day and intermediate precision and accuracy, recovery, stability, and dilution factor.

# 4 Results and discussion

## 4.1 Identification criteria

An unequivocal identification of the compounds is one of the most important steps in developing analytical monitoring methods. Each compound when subjected to ionization, such as EI, generates a specific mass spectrum that allows its identification. By injecting the compounds certificated analytical standards individually in scan mode, the spectra obtained are compared with those in the library and literature. In this sense, all compounds were analysed in scan mode (Figure 6, 7 and 8) at a concentration of 200 µg/mL where the respective retention times were defined. After appropriate selection of ions according to the mass spectrum obtained in scan mode, their subsequent fragmentation was carried out in product ion (PI) mode (Figure 9, 10, 11) at the same concentration, using 4 different collision energies (5, 10, 15 and 20 eV).

After obtaining the mass spectrum in PI mode, two transitions and their respective collision energies were chosen for each compound, one quantifier and the other qualifier. The criteria for this selection were: ion intensity, m/Z ratio, number of interferents, contribution from other compounds under study. Upon selection of the transitions that best fulfilled the predefined criteria, the parameters for MRM mode were defined. This analytical mode provides a greater sensitivity and selectivity to detect for the target compounds due to the specific features of the MS/MS detector. In addition, the MRM parameters were optimized to improve the sensitivity of the method. Among the latter, the dwell time is very important, corresponding to the time that the detector spends searching for each of the chosen transitions. Table 3 indicates the detection conditions used in MRM for each compound, indicating the quantifier and qualifier transitions, retention time, collision energy and dwell time.

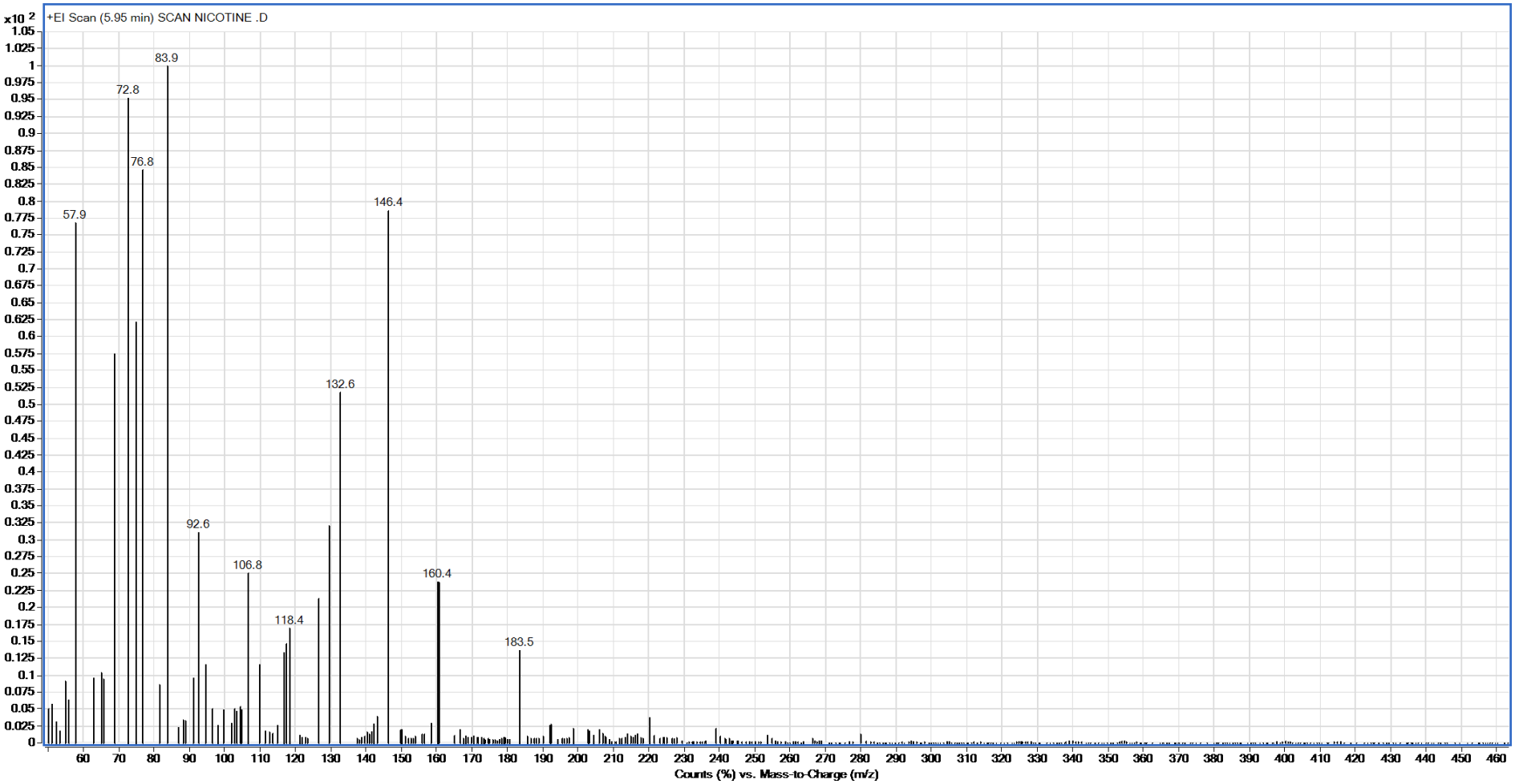


**Figure 6:** Scan mass spectrum for NIC.

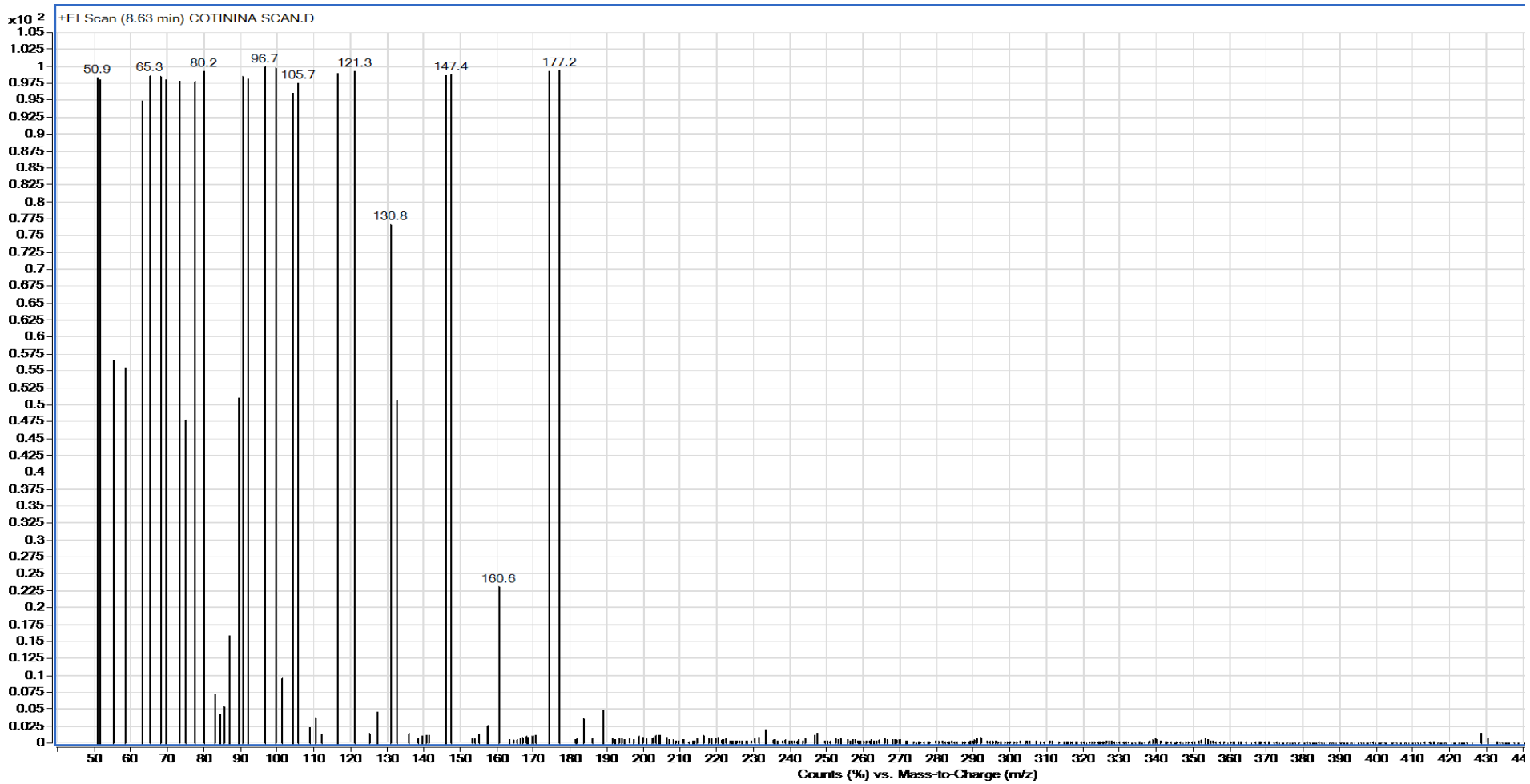


**Figure 7:** Scan mass spectrum for COT

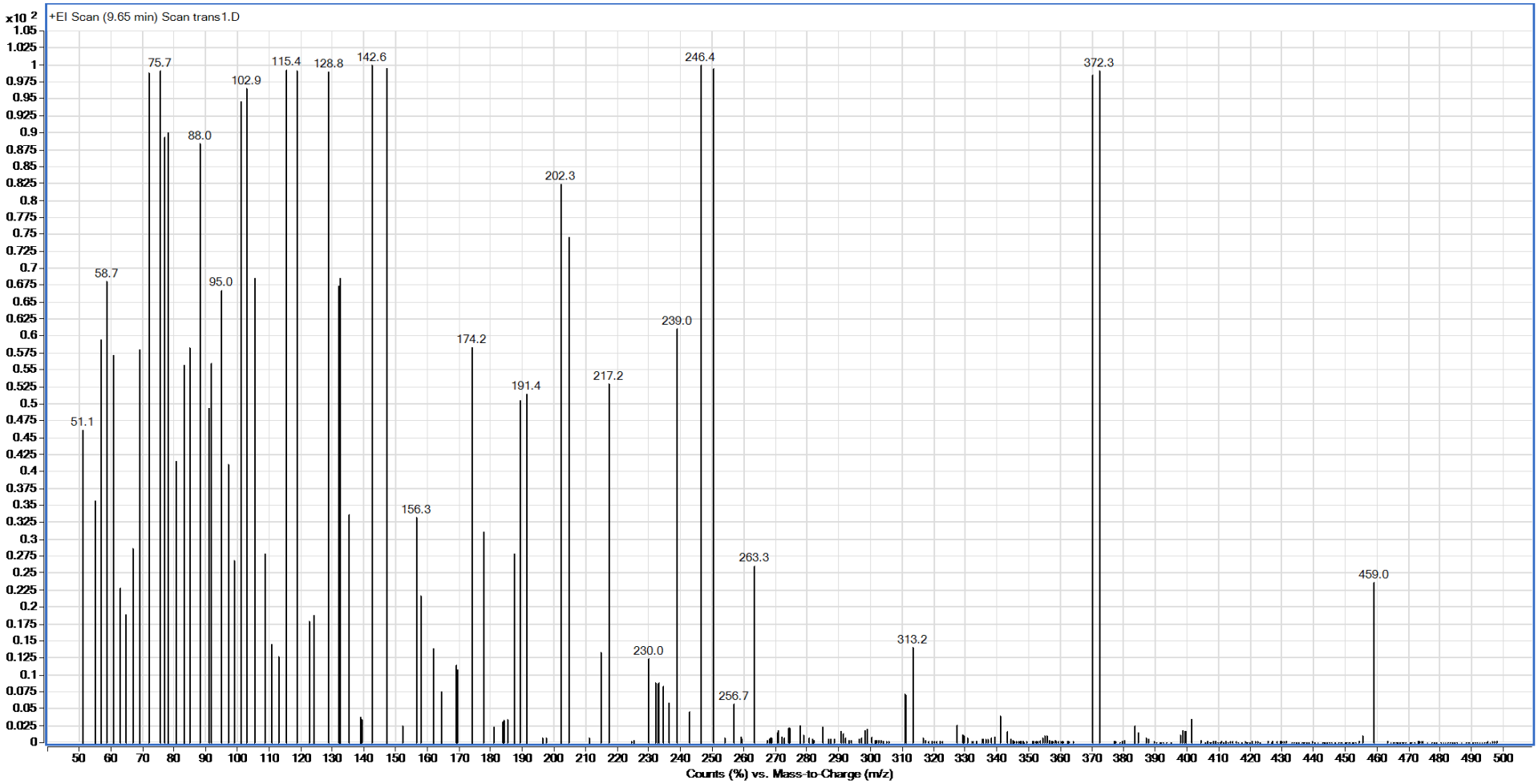


**Figure 8:** Scan mass spectrum for OH-COT

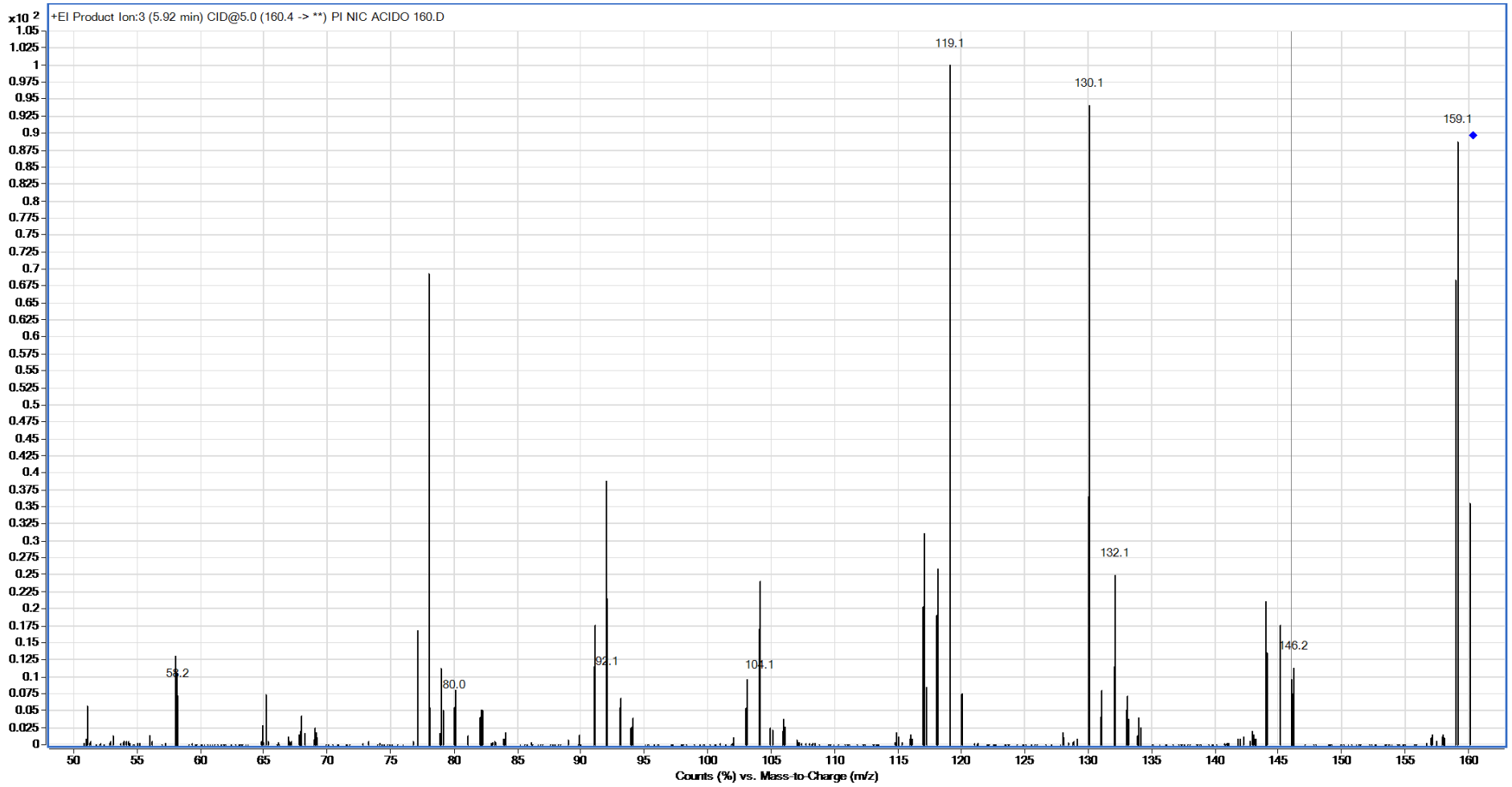


**Figure 9:** PI mass spectrum for NIC

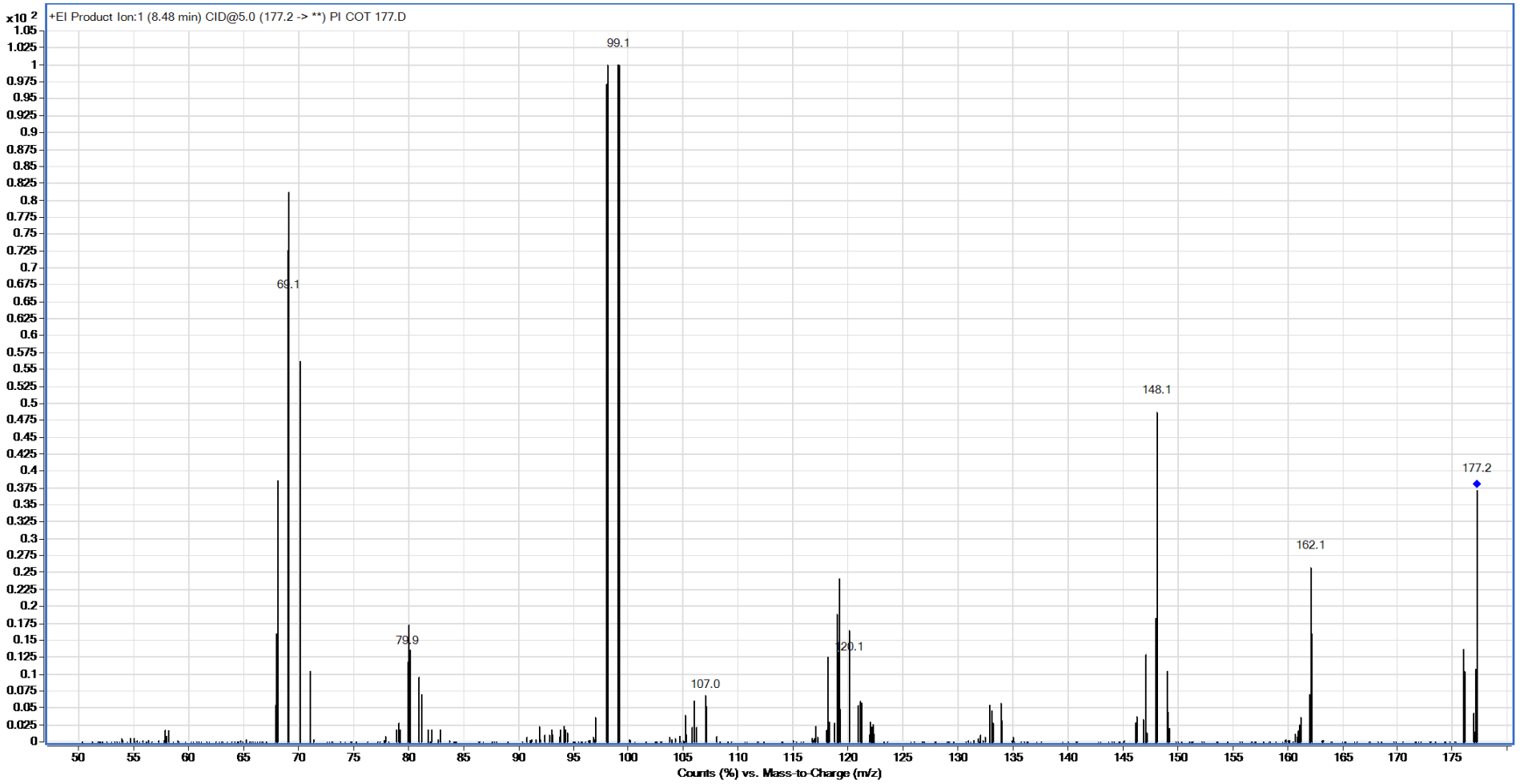


**Figure 10:** PI mass spectrum for COT

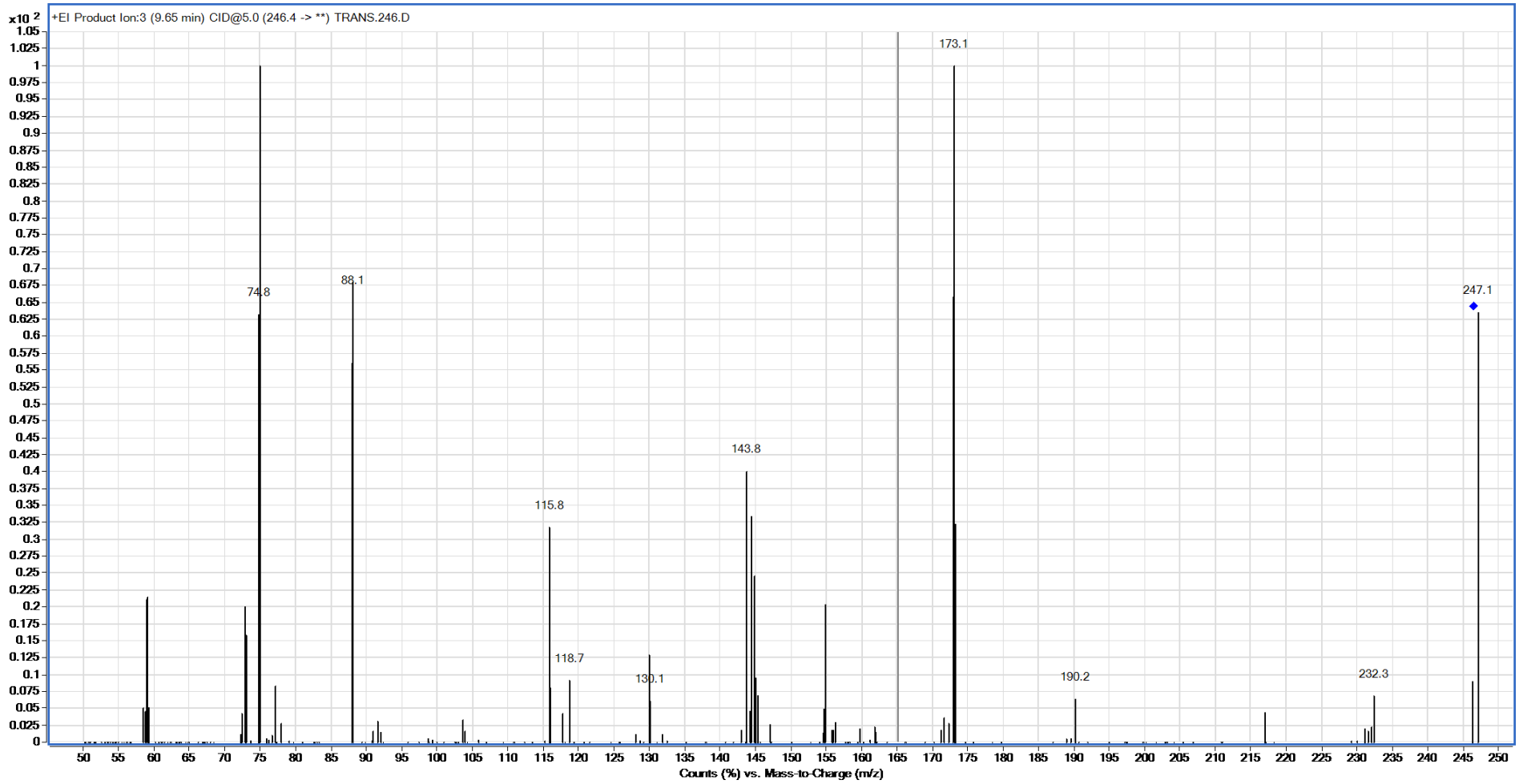


**Figure 11:** PI mass spectrum for OH-COT

**Table 3**. Retention time and GC–MS/MS parameters (quantifier transitions underlined).

| Analyte | Retention time (minutes) | Transitions (m/z) | Collision energy (eV) | Dwell time (µs) |
|---|---|---|---|---|
| NIC | 5.91 | <u>160.4-130.0</u><br>160.4-119.0 | 5<br>5 | 100<br>100 |
| NIC-d4* | 5.90 | 164.8-123.1 | 10 | 100 |
| COT | 8.59 | <u>177.2-69.1</u><br>177.2-99.1 | 20<br>5 | 100<br>100 |
| COT-d3* | 8.60 | 180.1-101.3 | 10 | 100 |
| OH-COT | 9.68 | <u>246.4-116.9</u><br>246.4-173.1 | 5<br>5 | 100<br>100 |
| OH-COT-d3* | 9.67 | 233.2-201.2 | 20 | 100 |

*Internal standard

## 4.2 Optimization of the extraction procedure

The DSS extraction procedure is a manual extraction procedure involving the application of OF on a filter paper spot, letting it dry for a period of time, and subsequent transfer of each spot to a tube where the extraction will occur with a given volume of organic solvent and IS added. The extraction is performed under agitation in a roller mixer, after which a centrifugation step is added, and the supernatant is transferred to a new tube to proceed with the analysis. For this extraction method, 4 conditions were optimised: (*i*) selection of the extraction solvent; (*ii*) volume of extraction solvent; (iii) spot drying time; and (iv) agitation time. Other conditions associated with the extraction step were also optimized to assess their influence on the signal obtained. For optimization, the IS was added only after the extraction.

### 4.2.1 Extraction solvent selection

The first optimization step was the study of the proper extraction solvent that would result in greater analyte recoveries. A total of 9 different solvents were tested (n=3): hexane, methanol:acetonitrile (MeOH:ACN) (50:50; v/v), acidified acetonitrile (ACN pH 5), methanol (MeOH), acidified methanol (MeOH pH 5), acetonitrile (ACN), ethyl acetate (AET), isopropanol (ISOP), and dichloromethane (DCM). For this study, the following preliminary conditions were defined: 2 mL volume of extraction solvent, overnight drying at room temperature and 15-minute extraction in the roller mixer. Using the SPSS software (version 27) it was possible to compare the obtained relative peak areas obtained for NIC, COT and OH-COT when the different solvents were used. A non-parametric test (Friedman's test) with a significance value <0.05 was the chosen test. No significant differences were observed when the 9 different solvents were evaluated, however MeOH pH 5 appeared as the most suitable (Fig. 12), resulting in higher relative peak areas for all compounds, in particular COT (the most important biomarker). Moreover, this solvent also resulted in cleaner chromatograms and fewer interferences.

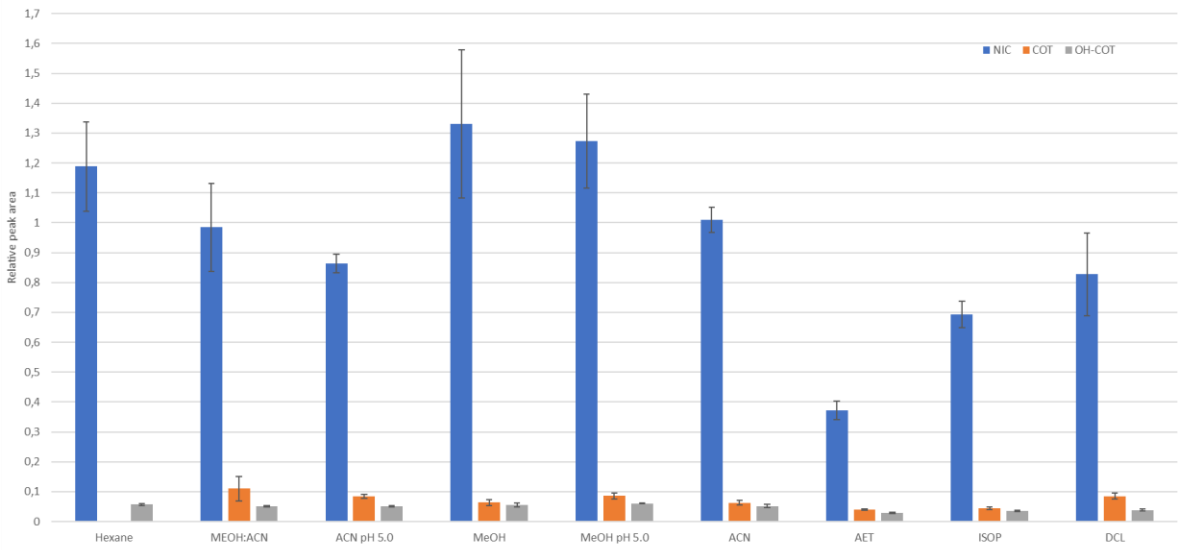


**Figure 12:** Evaluation of extraction solvent

Legend: ACN (acetonitrile); AET (ethyl acetate); DCL (dichloromethane); ISOP (isopropanol); MeOH (methanol)

### 4.2.2 Solvent volume selection

Keeping the initial extraction conditions, and now establishing MeOH pH 5 as extraction solvent, the next step of optimization was carried out in order to evaluate the volume of MeOH pH 5 that would result in greater analytes' recoveries. Three different volumes were tested (n=3): 1, 2 and 3 mL. After performing Friedman's test with the obtained relative peak areas, no significant differences were observed again (p= 0.457, 0.097 and 0.896 for NIC, COT and OH-COT respectively). Therefore, the lowest volume of solvent (1 mL) was selected for the extraction procedure. This is in accordance with the green chemistry statements which involve the development of more sustainable methods, and subsequently reduces costs per analysis (Fig. 13).

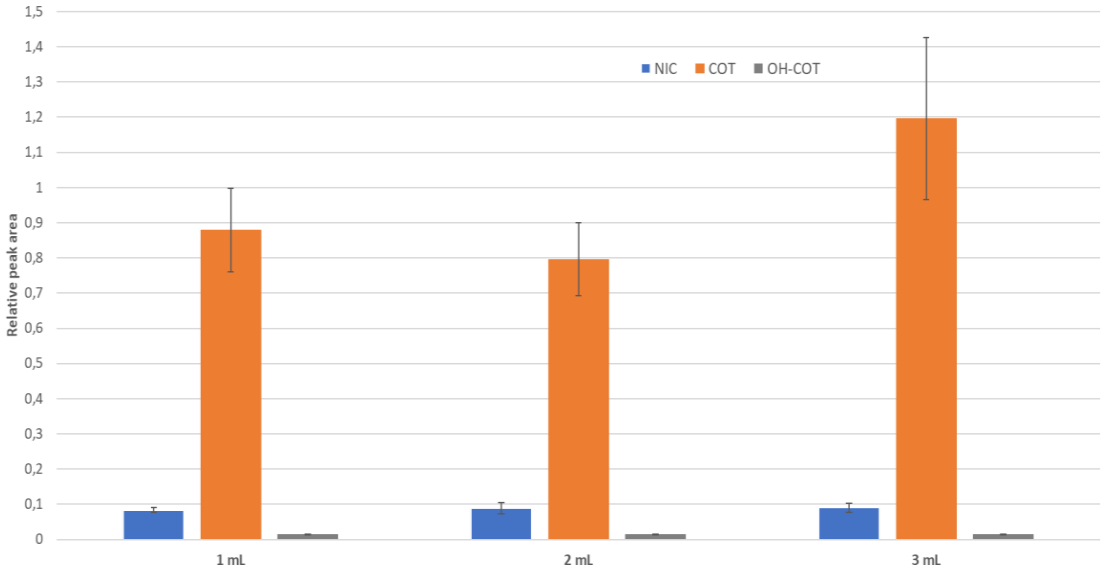


**Figure 13:** Evaluation of extraction solvent volume

### 4.2.3 Extraction time selection

The extraction time is an important optimization step, allowing not only to define the most efficient time to extract the compounds from each spot, but also to define the shortest possible time for the analysis of each sample. For that, 3 different extraction times were evaluated (n=3): 5, 15 and 30 minutes. Statistical results of these extraction assays indicate that there was no significant difference between the different extraction times for COT and OH-COT, with p-values of 0.097 and 0.717 respectively; however, a significant difference was observed for NIC between 5 and 30 minutes (p-value = 0.043, value adjusted by Bonferroni correction), and as such, the shortest extraction time (5 minutes) was selected (Fig. 14).

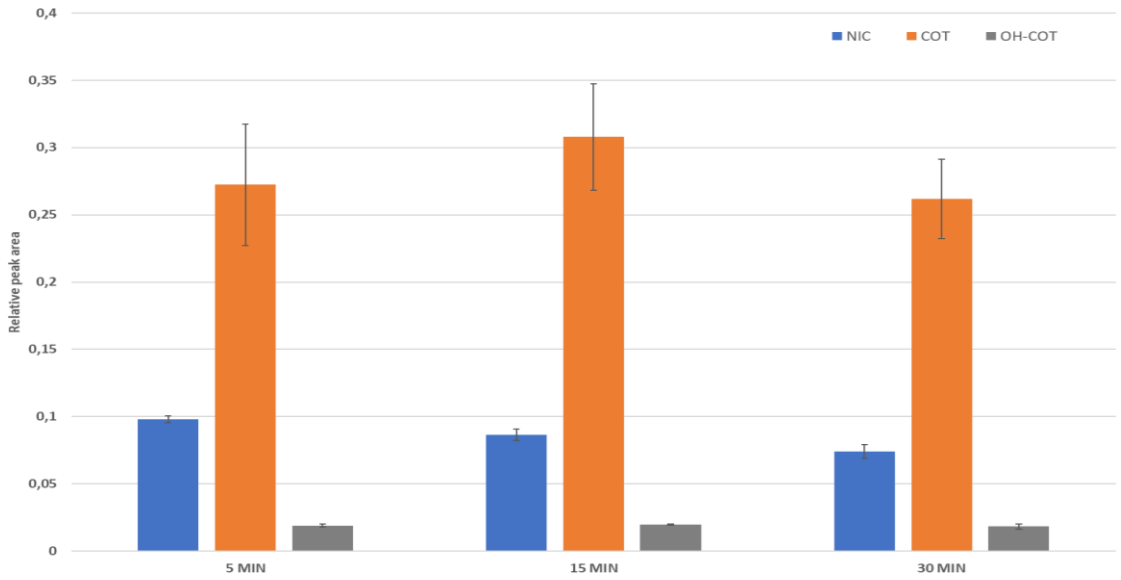


**Figure 14:** Evaluation of extraction time

### 4.2.4 Drying time selection

Lastly, the DSS drying time after sample application was evaluated. To this end, three drying times were evaluated (n=3): 1 and 3 hours, and overnight. No significant differences were observed between different drying times (p-value of 0.097, 0.717, 0.368 for NIC, COT and OH-COT respectively). As such, the shortest drying time was chosen (1 hour) (Fig.15), thus allowing faster analysis.

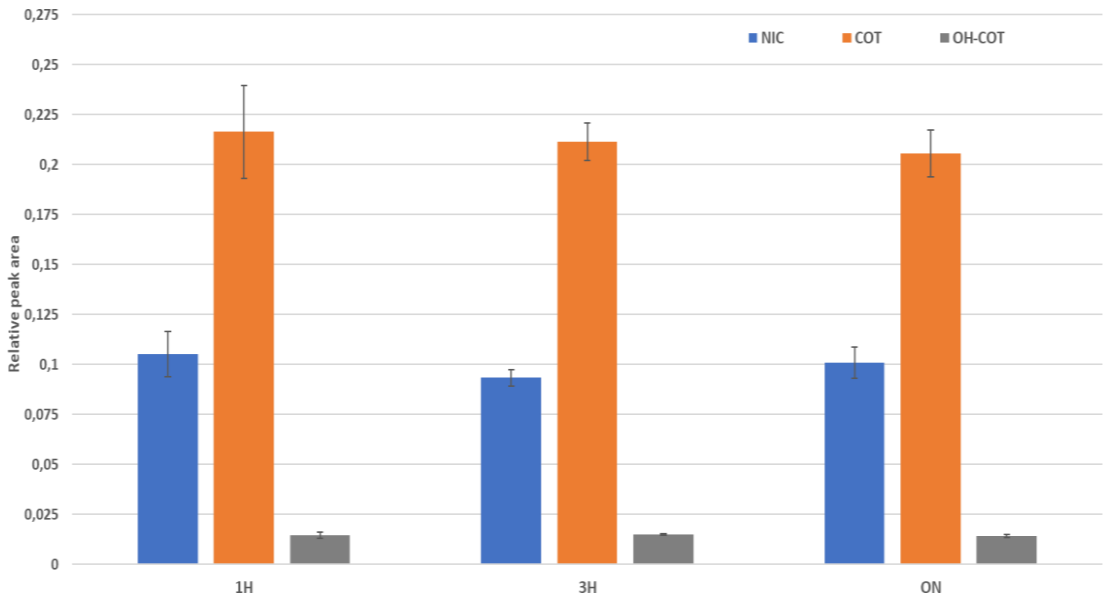


**Figure 15:** Evaluation of drying time

### 4.2.5 Additional optimization conditions

To further optimize the extraction procedure, the importance of a previous clean-up step was evaluated in order to obtain chromatograms with fewer interferents. To this end, a PP procedure was adopted in a sample spiked prior the DSS extraction procedure (5 µL of frozen ACN added for each 100 µL of OF sample), and compared with a sample spiked at the same concentration without PP. After analysis of the relative peak areas, it was possible to observe a decrease in the obtained signal, however, the chromatograms obtained from samples subjected to the PP procedure presented fewer interferences and cleaner chromatograms. The latter allowed a more unequivocal analysis of each compound, without affecting the sensitivity required for the method, thus the PP clean-up step described above was adopted for the extraction procedure. Additionally, 50 µL of a 1% HCl solution in MeOH (v/v) was added after the extraction procedure to increaseNIC absolute peak area as a result of a hydrochloride salt formation of this analyte, thereby decreasing its loss in the evaporation process (2).

## 4.3 Method validation

### 4.3.1 Selectivity

The method's selectivity indicates the ability to detect the compounds under study even in the presence of other interferences present in the biological sample used (3). Several interferences may be found in OF sample, such as bacteria, food residues, mineral salts, enzymes, among others. Extraction eliminates most of these, but some may remain in the final extract. In order to verify if the method is selective, it is necessary to check, considering the retention time and transitions defined, if it is not affected by matrix components. The selectivity study was performed through the analysis of 10 oral fluid samples of different origin, belonging to individuals not exposed to tobacco smoke, which were compared to blank samples spiked at the lowest limit of quantification (LLOQ) and analysed by the developed methodology. This comparison may be seen in Figure 16. The World Anti-Doping Agency (WADA) technical document for separation by GC-MS/MS (4) was used.

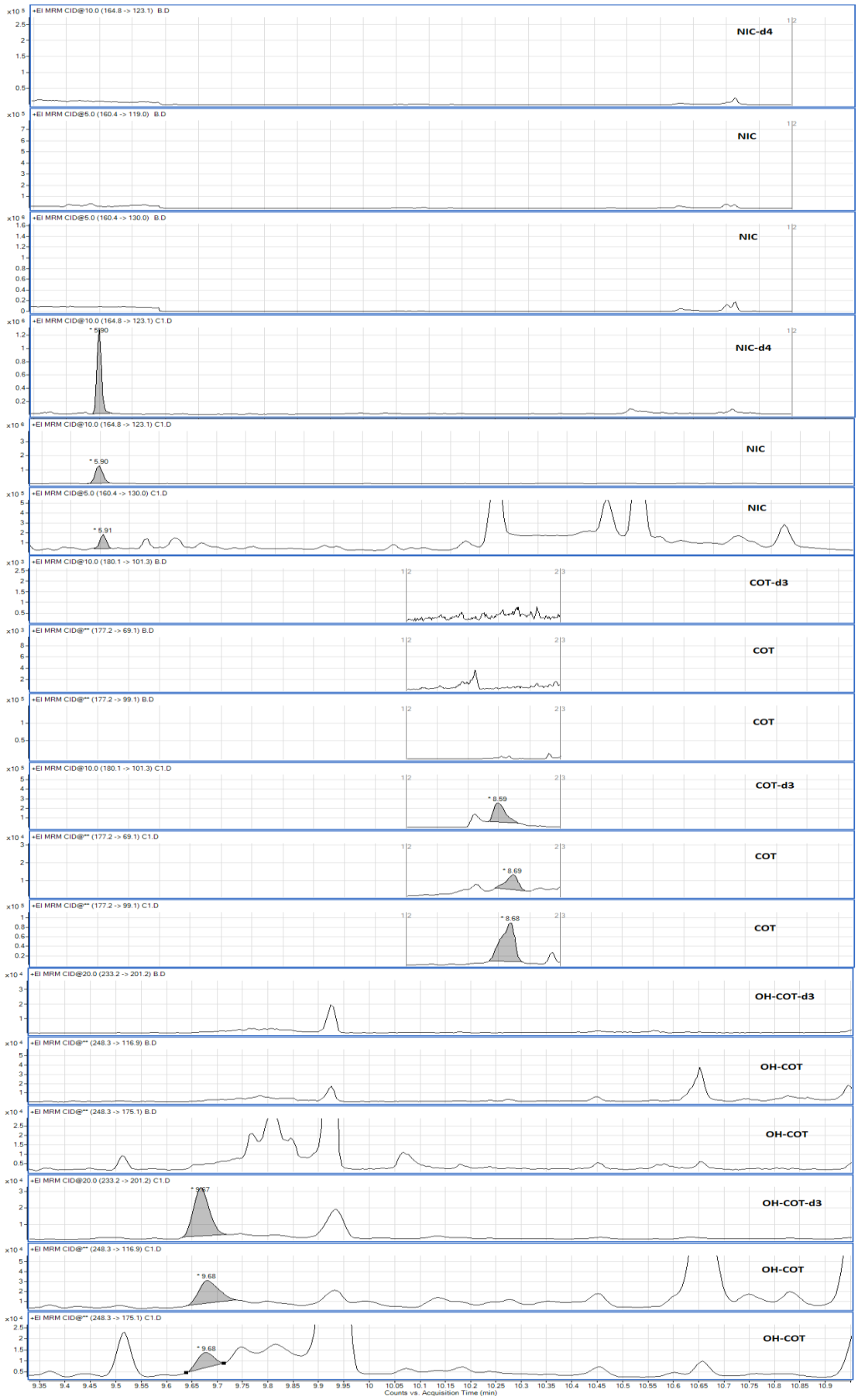


**Figure 16:** Chromatogram of a blank sample and a spiked sample at the LLOQ (10 ng/mL) for all three compounds

### 4.3.2 Calibration curves and limits

Blank samples were spiked in a concentration range from 10 to 200 ng/mL for all compounds. In order to evaluate the linearity of the method, 7 calibrators were evenly distributed within this range (n=5). In order to establish a calibration curve for each of the compounds under study, the relationship between the peak area of each compound and the respective internal standard (IS) was evaluated, and subsequently related to the concentration of each of the calibrators. The acceptance criteria for the calibration line were as follows: calibrator's accuracy within ±15% (except at the LLOQ, where ±20% was accepted) and a coefficient of determination ($R^2$) of at least 0.99. To fulfil this criterion, weighted least squares regression (1/x) was adopted. Calibration data is presented in Table 4. The method's LOD was defined as the lowest analyte concentration with acceptable chromatography, in which all transitions were present with signal-to-noise ratios of at least 3 and retention time within ±0.2 min of the average retention time of the calibrator (n=10). The LOD was 5 ng/mL for all compounds.

OF is an uncommon sample for the detection of tobacco biomarkers in general; however, when compared to other samples, it presents some advantages. According to a study with multiple biological matrices, Pérez-Ortuño et al. (5) obtained lower LLOQ values for NICand COT, although using a 5 times larger volume (0.5 mL) of biological sample aftersalivary stimulation with a sweet (Smint®), LLE as extraction technique and a moresensitive analytical equipment (LC-MS/MS). Another study performed by Feng et al. (6)for NIC and COT analysed distinct levels of exposure to tobacco smoke, using aQuantisal™ device for sampling, saving it in a buffer, and using 0.5 mL of OF, obtaininglower LLOQ using SPE (Phenomenex Trace B) as extraction technique. Also using SPE(Clean Screen® ZSDAU020) and 0.5 mL of OF mixed with 2 mL of sodium acetate buffer,Kim et al. (2) obtained LLOQ of 5 ng/mL for NIC, COT and OH-COT by GC-MS. Nevertheless, the obtained limits allow, considering an established consensus amongseveral authors, to differentiate between active and passive consumption, using lowervolumes of OF, which is the main advantage of the herein presented study (7).

**Table 4**. Linearity data (n=5).

| Analyte | Linear range (ng/mL) | Linearity* | | $R^{2*}$ | LOD (ng/mL) | LLOQ (ng/mL) |
|---|---|---|---|---|---|---|
| | | Slope | Intercept | | | |
| **NIC** | 10-200 | 0.0011 ± 0.0001 | 0.0103 ± 0.0042 | 0.9973 ± 0.0012 | 5 | 10 |
| **COT** | 10-200 | 0.0012 ± 0.0002 | 0.0137 ± 0.0112 | 0.9964 ± 0.0015 | 5 | 10 |
| **OH-COT** | 10-200 | 0.0553 ± 0.0076 | 0.7745 ± 0.3621 | 0.9942 ± 0.0021 | 5 | 10 |

*Mean values ± standard deviation. The weighting factor was 1/x for all analytes

### 4.3.3 Intra-day, inter-day and intermediate precision and accuracy

Precision and accuracy are two essential requirements to ensure that the values obtained from the analytical method of quantification are as close as possible to the real values and that its performance remains stable. The parameters of precision and accuracy were evaluated using the coefficient of variation (CV) and relative error (RE) respectively. The acceptance criterion for RE is ±15% for each concentration, and for CV the defined criterion is that it cannot exceed 15% for each concentration (34,35).

For the assess of intra-day precision and accuracy, 3 different quality control samples (QCs) (low, intermediate, and high) (n=6) were analysed on the same day, obtaining CVs of 5.10%, 5.44% and 6.15% and REs of ±3.20%, ±4.13% and ±6.62% for NIC, COT and OH-COT respectively (Table 5).

In a set of 5 different runs (n=5), inter-day precision and accuracy were assessed for each of the calibrators used to define the calibration curve, obtaining CV values of 8.99%, 10.37% and 9.60% and RE of ±4.23%, ±6.50% and ±2.93% for NIC, COT and OH-COT respectively (Table 6).

To assess the precision and accuracy parameters for intermediate quantification values of the calibration curve, 3 QCs with concentrations of 20, 100 and 180 ng/mL were evaluated during a period of 5 days in triplicate for each one of the concentrations (n=15), obtaining CV values below 7.40%, 7.69% and 8.32% and RE of ±4.51%, ±4.80% and ±1.54% for NIC, COT and OH-COT respectively (Table 7).

**Table 5.** Intra-day precision and accuracy

| Analyte | Concentration (ng/mL) | Intra-day (n=6) | | |
|---|---|---|---|---|
| | | Measured* | CV (%) | RE (%) |
| NIC | 40 | 40.08 ± 1.92 | 4.80 | 0.21 |
| | 110 | 106.48 ± 3.53 | 3.31 | -3.20 |
| | 200 | 194.47 ± 9.92 | 5.10 | -2.76 |
| COT | 40 | 41.65 ± 2.27 | 5.44 | 4.13 |
| | 110 | 106.94 ± 5.01 | 4.69 | -2.78 |
| | 200 | 194.15 ± 7.93 | 4.09 | -2.93 |
| OH-COT | 40 | 41.74 ± 2.12 | 5.07 | 4.35 |
| | 110 | 117.28 ± 7.21 | 6.15 | 6.62 |
| | 200 | 201.56 ± 10.68 | 5.30 | 0.78 |

*Mean values ± standard deviation; CV - coefficient of variation; RE - relative error.

**Table 6.** Inter-day precision and accuracy

| Analyte | Concentration (ng/mL) | Inter-day (n=5) | | |
|---|---|---|---|---|
| | | Measured* | CV (%) | RE (%) |
| NIC | 10 | 10.00 ± 0.54 | 5.40 | 0.00 |
| | 40 | 40.20 ± 2.85 | 7.08 | 0.50 |
| | 70 | 69.03 ± 6.20 | 8.99 | -1.38 |
| | 110 | 105.35 ± 6.47 | 6.14 | -4.23 |
| | 140 | 134.18 ± 7.13 | 5.32 | -4.16 |
| | 170 | 175.93 ± 4.25 | 2.42 | 3.49 |
| | 200 | 206.38 ± 3.87 | 1.87 | 3.19 |
| COT | 10 | 10.65 ± 0.50 | 4.65 | 6.50 |
| | 40 | 38.08 ± 3.95 | 10.37 | -4.79 |
| | 70 | 67.01 ± 4.42 | 6.60 | -4.28 |
| | 110 | 106.86 ± 6.18 | 5.78 | -2.85 |
| | 140 | 137.97 ± 4.76 | 3.45 | -1.45 |
| | 170 | 172.57 ± 6.50 | 3.77 | 1.48 |
| | 200 | 205.91 ± 1.18 | 0.57 | 2.96 |
| OH-COT | 10 | 10.00 ± 0.60 | 5.96 | 0.01 |
| | 40 | 39.53 ± 2.42 | 6.13 | -1.18 |
| | 70 | 70.16 ± 6.74 | 9.60 | 0.23 |
| | 110 | 108.31 ± 9.62 | 8.89 | -1.54 |
| | 140 | 138.17 ± 4.60 | 3.33 | -1.31 |
| | 170 | 166.98 ± 2.07 | 1.24 | -1.78 |
| | 200 | 205.86 ± 12.78 | 6.21 | 2.93 |

*Mean values ± standard deviation; CV - coefficient of variation; RE - relative error.

**Table 7.** Intermediate precision and accuracy

| Analyte | Concentration (ng/mL) | Intermediate (n=15) | | |
|---|---|---|---|---|
| | | Measured* | CV (%) | RE (%) |
| NIC | 20 | 19.88 ± 1.47 | 7.40 | -0.62 |
| | 100 | 95.49 ± 4.71 | 4.93 | -4.51 |
| | 180 | 178.44 ± 11.63 | 6.52 | -0.87 |
| COT | 20 | 19.80 ± 1.52 | 7.69 | -1.02 |
| | 100 | 95.20 ± 5.73 | 6.01 | -4.80 |
| | 180 | 177.90 ± 11.76 | 6.61 | -1.17 |
| OH-COT | 20 | 20.31 ± 1.40 | 6.92 | 1.54 |
| | 100 | 98.91 ± 8.23 | 8.32 | -1.09 |
| | 180 | 181.88 ± 14.45 | 7.94 | 1.04 |

*Mean values ± standard deviation; CV - coefficient of variation; RE - relative error.

### 4.3.4 Recoveries

The evaluation of the recovery of the developed extraction methodology for all compounds was performed at the concentrations of 25, 75 and 150 ng/mL, and the results are presented in Table 8. The obtained recoveries ranged between 29.2% and 43.3% for NIC, 66.6% and 89.1% for COT and between 80.3% and 92.8% for OH-COT. In the methodology developed by Kim et al. (2) obtained recoveries that ranged from 67.0% to 117.8% for NIC, COT and OH-COT, using 0.5 mL of OF and SPE procedure, which employs higher volumes of organic solvent than the method developed here. In another study performed on OF with a volume of 0.5mL Shakleya et al.(8) used a

Quantisal™ device to collect saliva sample obtaining recoveries between 63.6% and 96.5% using an SPE column like the one used by Kim et al. (2), resorting however to LC-MS/MS as analytical procedure for the detection of NIC, COT and OH-COT compounds. In another study by Perez-Ortuño et al. (5), 0.5 mL of OF was used, and the samples were collected by stimulation using Smint® followed by spitting and LLE with dichloromethane, obtaining recoveries for NIC and COT greater than 90%. Furthermore, Da Fonseca et al. (9) have obtained recoveries higher than 89.2%, 84.6% and 86.7% for NIC, COT and OH-COT respectively, using 0.2 mL of OF sample diluted in sodium hidroxide (0.5 M) and a SPE procedure.

Although the recoveries obtained for NIC are relatively low compared to those obtained for COT and OH-COT and to those obtained by other authors, our recoveries were sufficient to reach the desired LLOQ, and therefore we can say that the recoveries proved to be acceptable for the developed method, bearing in mind that this is the first method developed for multiple compounds using this sampling approach.

**Table 8** Absolute recoveries (n=3)

| Analyte | Concentration (ng/mL) | Recovery* (%) |
|---|---|---|
| **NIC** | 25 | 29.20 ± 13.60 |
| | 75 | 43.30 ± 3.73 |
| | 150 | 39.20 ± 2.22 |
| **COT** | 25 | 73.40 ± 8.00 |
| | 75 | 89.10 ± 10.47 |
| | 150 | 66.60 ± 4.16 |
| **OH-COT** | 25 | 81.70 ± 1.40 |
| | 75 | 80.30 ± 8.22 |
| | 150 | 92.80 ± 2.01 |

*Mean values ± standard deviation

### 4.3.5 Stability parameters

Stability studies are an important parameter when developing analytical procedures, since it allows to deal with several problems that arise in the routine of a laboratory, which may affect the reliability of the results (1). Stability was studied under four different parameters: processed sample stability, room temperature stability, freeze-thaw stability, and long-term stability.

Two acceptance criteria were used to evaluate stability: CV of less than 15% and RE ±15%. To evaluate the different stability parameters, 3 QCs at different concentrations (20, 100 and 180 ng/mL) were used (1,3). The assessment of stability in processed samples (Table 9) was performed after the analysis of the 3 QCs under normal conditions, making a re-

injection of the same vials (n=3) after 24 hours at room temperature, obtaining values below 3.76% for CV and ±12.56% for RE. Room temperature stability (Table 10) was determined by analysing samples of OF that had been previously spiked (n=3) and left at room temperature for 24 hours, followed by the standard DSS extraction process. When comparing the values obtained with fresh QCs at the same concentration, it was observed that the CV did not exceed 8.63%, obtaining a RE always below ±10.87%. The freeze-thaw stability (Table 11) was performed after 4 cycles of freezing and thawing of QC samples, after which the compounds under study were extracted using DSS (n=3), obtaining CV of less than 6.76% and RE below ±9.98% when compared to fresh QCs analysed on the same day. Long-term stability (Table 12) was evaluated based on the application of spiked QCs (n=3) in the spots, which were left at room temperature during the period of 1, 2, 3 and 4 weeks; after each of these periods, samples were extracted and analysed. The CV and RE obtained for the data resulting from the analysis after a period of 1 to 4 weeks were under 10.16% and ±12.00% for NIC, 16.06% and ±56.43% for COT and 13.26% and ±12.24% for OH-COT respectively; this shows that extracting simultaneously the three compounds after the same period of sample application time is not feasible without affecting the results. However, evaluating individually the CV and RE values for each of the weeks after sample application, it is possible to verify that only the values obtained in the 3rd and 4th week for COT exceeded the values indicated in the used guidelines. Thus, it was concluded that NIC and OH-COT remain stable after application in DSS for 4 weeks, while COT only remains stable for a period of 2 weeks after application in DSS.

**Table 9.** Processed sample stability.

| Analytes | Concentration (ng/mL) | Processed samples stability (n=3) | | |
|---|---|---|---|---|
| | | Measured* | CV (%) | RE (%) |
| NIC | 20 | 18.98 ± 0.54 | 2.84 | -5.10 |
| | 100 | 87.94 ± 0.39 | 0.45 | -12.06 |
| | 180 | 157.40± 3.79 | 2.41 | -12.56 |
| COT | 20 | 17.64 ± 0.61 | 3.43 | -11.80 |
| | 100 | 93.49 ± 2.24 | 2.39 | -6.51 |
| | 180 | 161.54 ± 5.28 | 3.27 | -10.26 |
| OH-COT | 20 | 18.29 ± 0.40 | 2.21 | -8.54 |
| | 100 | 94.46 ± 3.55 | 3.76 | -5.54 |
| | 180 | 171.29 ± 2.30 | 1.34 | -4.84 |

*Mean values ± standard deviation; CV - coefficient of variation; RE - relative error

**Table 10.** Room temperature sample stability.

| Analytes | Concentration (ng/mL) | Room temperature stability (n=3) | | |
|---|---|---|---|---|
| | | Measured* | CV (%) | RE (%) |
| NIC | 20 | 18.26 ± 1.02 | 5.57 | -8.72 |
| | 100 | 93.21 ± 4.71 | 5.06 | -6.79 |
| | 180 | 170.29 ± 14.69 | 8.63 | -5.39 |
| COT | 20 | 18.75 ± 1.05 | 5.62 | -6.24 |
| | 100 | 92.66 ± 3.75 | 4.04 | -7.34 |
| | 180 | 160.43 ± 2.08 | 1.30 | -10.87 |
| OH-COT | 20 | 18.29 ± 0.81 | 4.43 | -8.55 |
| | 100 | 92.05 ± 5.49 | 5.97 | -7.95 |
| | 180 | 160.70 ± 6.25 | 3.89 | -10.72 |

*Mean values ± standard deviation; CV - coefficient of variation; RE - relative error.

**Table 11.** Freeze/thaw sample stability.

| Analytes | Concentration (ng/mL) | Freeze/thaw stability (n=3) | | |
|---|---|---|---|---|
| | | Measured* | CV (%) | RE (%) |
| NIC | 20 | 19,58 ± 0.09 | 0.46 | -2.08 |
| | 100 | 93.75 ± 4.48 | 4.78 | -6.25 |
| | 180 | 167.62 ± 1.81 | 1.08 | -6.88 |
| COT | 20 | 18.38 ± 0.57 | 3.12 | -8.10 |
| | 100 | 95.07 ± 2.43 | 2.55 | -4.93 |
| | 180 | 168.61 ± 8.69 | 5.15 | -6.33 |
| OH-COT | 20 | 19.00 ± 0.97 | 5.09 | -5.02 |
| | 100 | 92.94 ± 6.28 | 6.76 | -7.06 |
| | 180 | 162.03 ± 5.70 | 3.52 | -9.98 |

*Mean values ± standard deviation; CV - coefficient of variation; RE - relative error.

**Table 12.** Long-term sample stability.

| Analytes | Concentration (ng/mL) | Long-term stability (n=3) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Week 1 | | | Week 2 | | | Week 3 | | | Week 4 | | |
| | | Measured* | CV (%) | RE (%) | Measured* | CV (%) | RE (%) | Measured* | CV (%) | RE (%) | Measured* | CV (%) | RE (%) |
| NIC | 20 | 19.37 ± 0.96 | 4.96 | -3.14 | 20.10 ± 1.29 | 6.40 | 0.52 | 20.88 ± 1.43 | 6.87 | 4.23 | 19.05 ±1.94 | 10.16 | -4.99 |
| | 100 | 89.25 ± 2.82 | 3.16 | -10.75 | 89.26 ± 2.22 | 2.48 | -12.00 | 92.35 ± 5.77 | 6.25 | -8.28 | 92.65 ± 6.66 | 7.19 | -7.93 |
| | 180 | 168.12 ± 11.49 | 6.83 | -6.60 | 163.39 ± 7.58 | 4.64 | -10.27 | 161.90 ± 7.69 | 4.75 | -11.18 | 163.85 ± 6.16 | 3.76 | -9.86 |
| COT | 20 | 19.94 ± 0.46 | 2.28 | -0.32 | 18.33 ± 1.39 | 7.58 | -9.09 | 16.02 ± 2.57 | 16.06 | -24.82 | 12.79 ±1.37 | 10.70 | -56.43 |
| | 100 | 89.03 ± 2.05 | 2.30 | -10.97 | 89.94 ± 3.51 | 3.91 | -11.19 | 94.47 ± 4.07 | 4.31 | -5.86 | 90.42 ± 4.36 | 4.82 | -10.60 |
| | 180 | 170.05 ± 11.96 | 7.04 | -5.53 | 159.29 ± 2.20 | 1.38 | -13.00 | 159.84± 6.54 | 4.09 | -12.61 | 162.92 ± 8.25 | 5.07 | -10.48 |
| OH-COT | 20 | 20.53 ± 0.97 | 4.73 | 2.64 | 20.24 ± 1.98 | 9.80 | 1.18 | 18.94 ± 1.93 | 10.19 | -5.59 | 19.56 ± 2.60 | 13.26 | -2.11 |
| | 100 | 99.07 ± 8.50 | 8.58 | -0.93 | 98.07 ± 9.51 | 9.69 | -1.97 | 90.53 ± 1.56 | 1.72 | -10.46 | 93.71 ± 7.32 | 7.81 | -6.72 |
| | 180 | 165.25 ± 11.02 | 6.67 | -8.19 | 176.05 ± 0.74 | 0.42 | -2.24 | 164.75 ± 9.31 | 5.65 | -9.26 | 160.23 ± 5.10 | 3.18 | -12.24 |

*Mean values ± standard deviation; CV - coefficient of variation; RE - relative error.

### 4.3.6 Dilution factor

Dilution integrity is an important parameter for the analysis of real samples whose concentration value exceeds the upper limit of quantification (ULOQ) of the calibration curve of the developed method. Therefore, a dilution factor of 1:5 was tested at a concentration of 1000 ng/mL for the three compounds. A blank sample of OF spiked at that concentration was used in order to study this parameter and was then applied to DSS after dilution. The obtained CV and RE values were below 6.84% and ±8.46% respectively for the three compounds. The values are summarized in Table 13.

**Table 13.** Dilution integrity (n=3)

| Analytes | Concentration (ng/mL) | Dilution factor (1:5) | | |
|---|---|---|---|---|
| | | Measured* | CV (%) | RE (%) |
| **NIC** | 1000 | 953.81 ± 65.20 | 6.84 | -4.62 |
| **COT** | 1000 | 915.36 ± 19.98 | 2.18 | -8.46 |
| **OH-COT** | 1000 | 947.3 ± 42.78 | 4.52 | -5.26 |

*Mean values ± standard deviation; CV - coefficient of variation; RE - relative error.

### 4.3.7 Method applicability

After the complete optimization and validation of the extraction method, it was subsequently applied to 8 samples of OF from volunteer students of the Universidade da Beira Interior (Table 14). After analysing the samples, it was found that the samples of three of the volunteers (samples 1, 4 and 8) must have come from active smokers, because the detected COT levels were above the cut-off of 10 ng/mL for OF indicated byFlorescu et al. (10), which was further corroborated by the presence of high concentrations of NIC and OH-COT. The use of OF samples as biological matrix for the determination of the degree of exposure has been growing over the last years mainly dueto the easy availability of this type of matrix; however, the development of methods usinglow sample volumes presents a significant advantage. The study in real samples developed was limited in the number of samples given the current pandemic situation, requiring a larger set of samples of active or passive smokers to determine the accuracy of the method and draw further conclusions. Although other methods reach lower LLOQs than those herein obtained, all methods allow distinguishing between active and passivesmokers. Kim et al. (11) also described in another article the usefulness of the ratio between COT and OH-COT for the identification of active consumers in real samples.

Figure 17 represents the chromatogram obtained from the analysis of one of those samples.

**Table 14.** Analysis of authentic samples.

| Sample number | Concentration (ng/mL) | | |
|---|---|---|---|
| | **NIC** | **COT** | **OH-COT** |
| **1** | 195.29 | 74.12 | 68.43 |
| **2** | Negative | Negative | Negative |
| **3** | Negative | Negative | Negative |
| **4** | 46.03 | 43.45 | 22.51 |
| **5** | Negative | Negative | Negative |
| **6** | Negative | Negative | Negative |
| **7** | 13.71 | Negative | Negative |
| **8** | 179.54 | 42.14 | 38.76 |

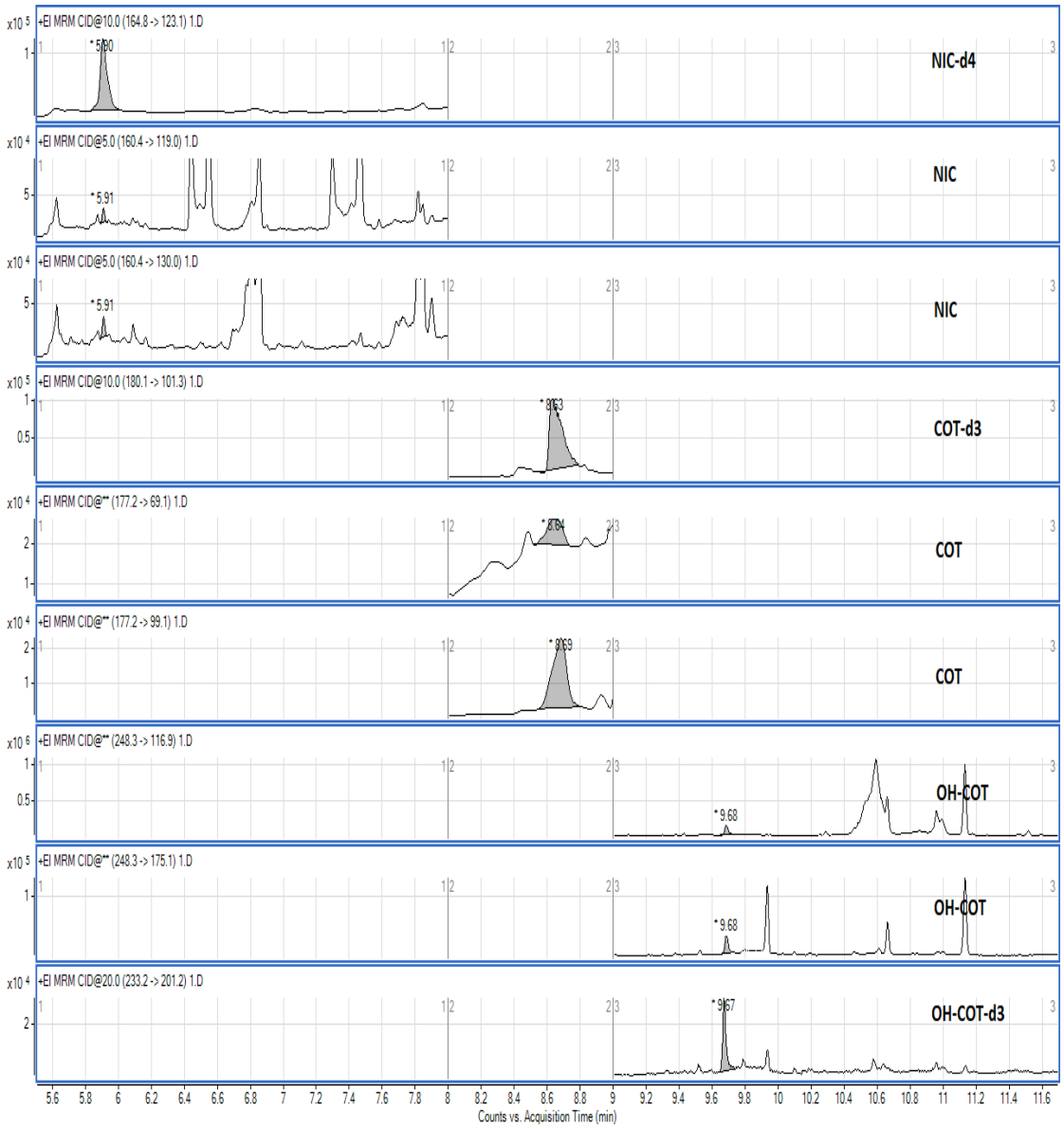


**Figure 17**. Chromatogram obtained by analysis of one positive authentic sample (NIC: 195.29 ng/mL; COT: 74.12 ng/mL and OH-COT: 68.43 ng/mL).

# 5 Conclusion

The method developed for the identification of tobacco biomarkers nicotine, cotinine and trans-3-hydroxycotinine in oral fluid samples using DSS as extraction method by GC-MS/MS analysis was fully optimised and validated. The recovery and clean-up procedure accomplished allowed to achieve satisfactory extraction values ranging from 29.2% to 92.8%, given the sample volume used. The developed method represents a new sampling approach, allowing the use sample volumes as low as 100 µL, which can be useful when sample amount is limited. In addition, the herein described procedure is fast when compared to other methods using the same type of sample, allowing multiple extractions in shorter periods of time, thus constituting an advantage towards its applicability in the routine of an analytical laboratory. Furthermore, since it employs fewer solvent volumes, this methodology is more environmentally friendly, also contributing to a reduction of the costs per analysis. Considering that through the spitting collection methodology the volume obtained is often greater than necessary, this type of methodology can be considered as a first approach in the detection of such compounds, allowing a subsequent analysis with the remaining volume or to perform other complementary tests. Although the sample volume used was small, quantification limits of 10 ng/mL were obtained for all compounds, and it is possible to analyse the studied compounds for a period of 2 weeks after sample application to the spot without affecting the performance and reliability of the methodology. This is the first method developed that combines the extraction of these 3 compounds using DSS and GC-MS/MS. This method allows the distinction between passive and active consumers in real samples, being a very sensitive method useful in cases where there is a limited volume of oral fluid sample. Furthermore, it enables various extraction parameters to be changed to adapt easily to a laboratory's routine.

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## Compliance with Ethical Standards

The present study was approved by the ethics committee Universidade da Beira Interior (CE-UBI-Pj-2021-046) and has been conducted according to ethical standards. The analyzed authentic samples belonged to individuals who provided an informed consent for their use, and all analyses were carried out according to the ethical standards of the institution.

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# Attachments

The present dissertation was disseminated in different events in the scientific area, as it was submitted to the publication.

**Presentations in events:**

DRIED SALIVA SPOTS: OPTIMIZAÇÃO DE UMA TÉCNICA PARA A DETERMINAÇÃO DE MARCADORES DE TABACO EM FLUIDO ORAL (Poster communication)
Marques H., Cruz-Vicente P., Rosado T., Simão A., Soares S., Gonçalves J., L. Passarinha, E.Gallardo
XII Dia da Bioquímica, Universidade de Aveiro, April 2021

DRIED SALIVA SPOTS: OPTIMIZAÇÃO E VALIDAÇÃO DE UMA TÉCNICA PARA A DETERMINAÇÃO DE MARCADORES DE TABACO EM FLUIDO ORAL (Oral communication)
Marques H., Rosado T., L. Passarinha, Simão A., E.Gallardo
III Jornadas Ibéricas da Toxicologia (Desafios da Toxicologia), UBI (Covilhã), June 2021

**Published articles:**

OPTIMIZATION AND VALIDATION OF A PROCEDURE USING THE DRIED SALIVA SPOTS APPROACH FOR THE DETERMINATION OF TOBACCO MARKERS IN ORAL FLUID
Hernâni Marques[1,2], Tiago Rosado[1,2], Mário Barroso[3], Luis Passarinha[1,2,4,5], Eugenia Gallardo[1,2*]
Journal of Pharmaceutical and Biomedical Analysis 2022; volume 212: 114648. doi:10.1016/j.jpba.2022.114648

RECENT DEVELOPMENTS IN THE DETERMINATION OF BIOMARKERS OF TOBACCO SMOKE EXPOSURE IN BIOLOGICAL SPECIMENS: A REVIEW.
Marques, H.; Cruz- Vicente, P.; Rosado, T.; Barroso, M.; Passarinha, L.A.; Gallardo, E.
International Journal of Environmental Research and Public Health 2021; volume 18: 1768. doi: 10.3390/ijerph18041768