Trends in analytical methods for analysis of tobacco products: An Overview

Tendências em métodos analíticos para análise de produtos do tabaco: uma visão geral

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Resumo

Atualmente, devido uma forte tendência nas políticas de redução do tabagismo, várias preocupações relacionadas ao controle de qualidade e a regulamentação dos produtos do tabaco têm sido levantadas, uma vez que uma grande variedade de produtos está disponível no mercado para os consumidores. O desenvolvimento de métodos analíticos robustos e seletivos para o tabaco e os produtos derivados tem se mostrado uma tarefa extremamente necessária, porém laboriosa, devido à complexidade das matrizes. Este trabalho propõe uma revisão geral no que se refere aos avanços, inovações e tendências no desenvolvimento de métodos analíticos, com foco na análise por cromatografia dos principais analitos do tabaco, dentre os quais, a nicotina, nitrosaminas específicas do tabaco (TSNAs), umectantes, pesticidas, hidrocarbonetos policíclicos aromáticos (HPAs) e açúcares. A cromatografia gasosa (GC) acoplada a diferentes detectores é amplamente usada para analisar nicotina, umectantes e TSNAs em folhas de tabaco e em diversos produtos derivados, enquanto a cromatografia líquida com detecção por espectrometria de massas em tandem (LC-MS/MS) é frequentemente usada para analisar TSNAs em cigarros convencionais, na fumaça e nos produtos sem combustão. Os pesticidas são analisados no tabaco usando tanto GC ou LC com detector de massas (MS), enquanto os HPAs e os açúcares são analisados na fumaça e no tabaco usando GC-MS e LC-MS, respectivamente. Além dos métodos cromatográficos já estabelecidos e, apesar da falta de padronização a nível mundial, esforços significativos foram feitos nos últimos anos visando o desenvolvimento de procedimentos analíticos para uma ampla variedade de produtos do tabaco, com uma ampla gama de métodos cromatográficos inovadores disponíveis. Neste sentido, uma potencial tendência é a possibilidade de determinação simultânea de múltiplos componentes, a fim de se reduzir, principalmente, o tempo de análise. O presente estudo examinou os principais artigos que desenvolveram ou aprimoraram métodos analíticos para identificação e quantificação de compostos prioritários em diferentes produtos do tabaco, e visa contribuir para pesquisas futuras com esse objetivo, além de promover a padronização dos termos técnicos usados nesta área analítica. Ressalta-se que alguns dos métodos citados não foram validados sendo necessários estudos com maior aprofundamento a fim de se obter métodos analíticos reprodutíveis para fins regulatórios.

Palavras-Chave: Análises cromatográficas; folhas de tabaco; cigarros; fumaça de cigarros; produtos sem combustão.

Abstract

Currently, due to the strong trends in policies to reduce smoking, several concerns related to quality control and regulation of tobacco products have been raised, as a great variety of products are available to consumers in the market. Considering that development of robust and selective analytical methods for tobacco and derivative products has been an important and necessary task over the years, however laborious, due to the complexity of the matrices, this work proposes a general review regarding advances, innovation, and trends in analytical methods development, focused on chromatographic analysis of nicotine, tobacco-specific nitrosamines (TSNAs), humectants, pesticides, polycyclic aromatic hydrocarbons (PAHs) and sugars in different tobacco products. Gas chromatography (GC) coupled with different detectors is widely used to analyze nicotine, humectants and TSNAs in tobacco leaves and derivative products, while liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been frequently used to analyze TSNAs in mainstream cigarette smoke and smokeless tobacco products. Pesticides were mainly analyzed in tobacco using GC or LC coupled with mass detector (MS), while PAHs and sugars were generally analyzed in tobacco and smoke using LC-MS and GC-MS techniques, respectively. In addition to already established methods and despite the lack of a worldwide standardization of methods, significant efforts have been made to develop analytical procedures for a wide variety of tobacco products, with a broad range of innovative chromatographic methods available. In this sense, a potential trend is the possibility of simultaneous determination of multiple components aiming to reduce the analysis time. The present study examined the main works that developed or improved analytical methods for identifying and quantifying priority compounds in different tobacco matrices and aims to contribute to future research with this objective, in addition, to promoting standardization of technical terms used in this analytical area. It is noteworthy that some of the methods mentioned here have not been validated and further studies are needed in order to obtain reproducible analytical methods for regulatory purposes.

Keywords: chromatography analyses; tobacco leaves; cigarette; cigarette smoke; smokeless tobacco products.

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Introduction

Tobacco (*Nicotiana tabacum* L.) is a highly cultivated non-food agricultural crop and has great economic and social importance worldwide (Banožić, Babić & Jokić, 2020; Drope *et al.* 2018). Tobacco products consist of a great diversity of types and formulations, entirely or partially made from tobacco leaves and manufactured for smoking, sucking, chewing or snuffing (WHO, 2003). In most products, dry tobacco is used in complex mixtures with additional ingredients that can modify its composition and expose even more users to highly harmful health compounds, in addition to stimulating consumption of these products. Among the substances commonly added to tobacco products are flavorings, humectants and sugars (Pefetti & Rodgman, 2011; Stepanov & Hatsukami, 2020). It is estimated that tobacco smoke contains over 7,000 chemical compounds, which may vary according to ingredients added during processing or formulation of the products and depending on new compounds generated during tobacco pyrolysis and combustion (Pefetti & Rodgman, 2011; USDHHS, 2010).

Major tobacco products include cigarettes, cigars, cigarillos, waterpipe tobacco, rolling tobacco, pipe tobacco, bidis, kreteks and a wide range of STPs including diverse products generally used in the oral cavity (Stepanov & Hatsukami, 2020; WHO, 2018). Cigarette smoking is the most common form of tobacco use, and it is known that tobacco filler and cigarette smoke contain toxic, mutagenic and carcinogenic substances (Ding *et al.* 2008; Ishizaki & Kataoka, 2010; Edwards *et al.* 2017). Currently, tobacco smoking is the major cause of death in the world, killing about 8 million people every year (WHO, 2020). Epidemiological studies evidence that smoking is the leading risk factor for lung cancer and related preventable diseases, such as cardiovascular ailments and chronic pulmonary disease (Hecht, 2003; Teo *et al.* 2006; Jindal *et al.* 2006). Furthermore, it is evident that smoking can impair lung function, consequently making it difficult to combat serious infectious diseases such as COVID-19 pneumonia, and is associated with the disease progression (Vardavas, Nikitara, 2020; Berlin, Thomas, Le Faou & Cornuz, 2020).

Although the negative effects of cigarette smoking on human health have been clarified, global cigarette consumption continues to grow, mainly as a result of the increasing uptake of smoking by young people in developing regions (Drope *et al.* 2018 ; WHO, 2019). About 6 trillion cigarettes are produced annually worldwide, being consumed by more than one billion smokers. Another worrisome issue is the emergence of new tobacco products, such as STPs, that despite not undergoing combustion, can be as highly harmful to health as smoked tobacco products and also lead to nicotine addiction (WHO, 2020; Novotny *et* al. 2015).

Globally, there is already an eminent policy for tighter control of tobacco products, especially when it comes to smoke quitting interventions and public health issues related to nicotine and tobacco use and dependence (Gottlieb & Zeller, 2017; Benowitz & Henningfield, 2018; Stanton & Hatsukami, 2019). As a tobacco smoking prevention measure, some of the largest world producer countries of tobacco have banned the use of substances that provide aroma and flavor to tobacco-based smoking products, since they stimulate their consumption and, mainly, attract young smokers (ANVISA, 2012). Given the wide range of constituents present in tobacco products and disease risks associated with their consumption, the appropriate quality control of these products is urgently necessary.

The development of analytical methods is fundamental to strengthen preventive measures and can make great contributions to standardization and regulation of diverse tobacco products. Moreover, chemical analysis of tobacco-specific compounds is highly necessary to characterize product components, monitor their levels and estimate potential effects on consumers' health. Thus, it is essential that accurate and robust analytical methods be constantly developed or improved to ensure accuracy of the results even at trace levels. This work aims to survey the main analytical methods developed in the last 20 years to analyze compounds that directly impact the quality and safety of tobacco products, including nicotine, TSNAs, humectants, pesticides, PAHs and sugars, focusing on the innovation and future trends for the analysis of these compounds in tobacco leaves, cigarette filler, smoke and STPs (Figure 1).

Methodology

A bibliographic review was carried out through scientific articles related to the topic addressed. The searches were carried out primarily on the Periodicals Capes Portal, on the Web of Science, PubMed, Scopus, ACS Publications databases, and through the Google Scholar tool. The search for free terms was performed based on the following combined keywords: "chromatography", "methods development" "tobacco analysis", "tobacco products analysis", "smokeless tobacco products", "nicotine", "TSNAs", "humectants", "pesticides", "PHAs" and "sugars". Repeated articles were excluded and only those published in the period comprised the years 2000 to 2020 and focused on the development and/or improvement of analytical methods by chromatography for the analysis of tobacco and derived products were selected. The search results were organized in the database form using the Excel software (Microsoft®, USA) to elaborate the schematic figures. In general, the main reported techniques for analysis of priority compounds from tobacco products are
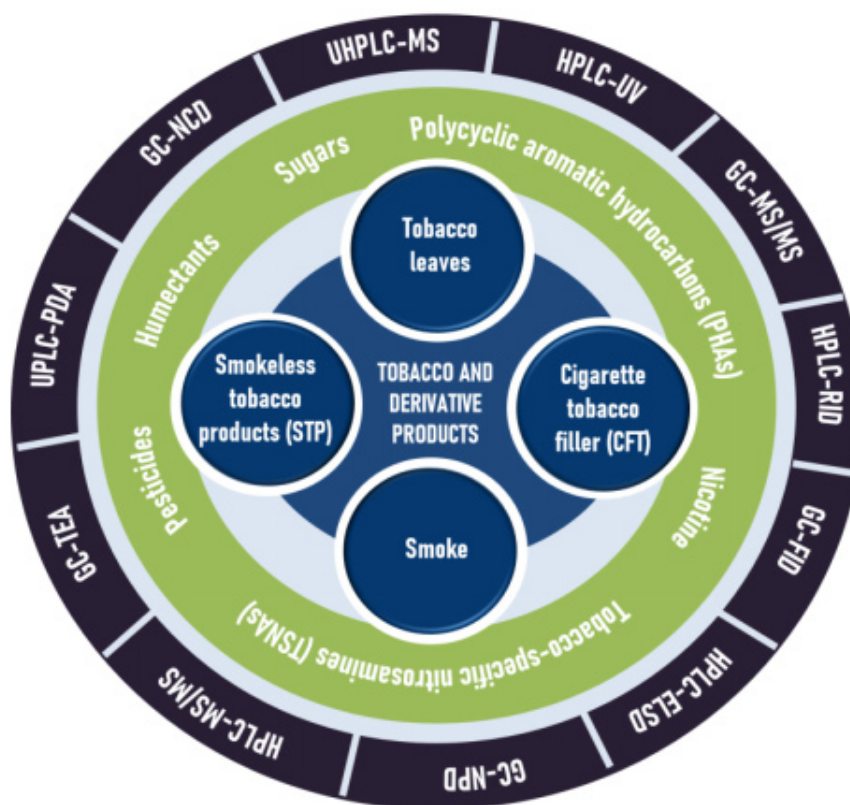
Figure 1. Main reported techniques for analysis of priority compounds from tobacco products.

summarized in Figure 1 and the developed or improved methods for different tobacco products analysis were summarized in Table 1. The scope of this work is structured according to the following topics: Nicotine, tobacco specific N-nitrosamines (TSNAs), humectants, pesticides, polycyclic aromatic hydrocarbons (PAHs), sugars and concluding remarks.

Nicotine

Nicotine is the main alkaloid present in tobacco products and in the smoke, whose levels depend on the tobacco blend and type of product, among other factors (Stepanov & Hatsukami, 2020; Cai, Liu, Lin & Su, 2003; Paillat, Périchet, Lavoine, Meierhenrich & Fernandez, 2012). A growing number of studies shown that nicotine exposure poses potential risks to human health, since it is the main agent accountable for continued tobacco consumption, in addition to reacting during the burning of tobacco to form toxic compounds (Stepanov & Hatsukami, 2020; Gottlieb & Zeller, 2017; Benowitz & Henningfield, 2018; Wu, Ashley & Watson, 2003). Nowadays, a proposal to reduce and standardize nicotine contents within non-addictive levels in tobacco products, as well as for rulemaking, is already in place (Benowitz & Henningfield, 2018; Stanton & Hatsukami, 2019). Although there is a well-established method for nicotine analysis (CORESTA, 2005), development of chromatographic methods for determination

of nicotine in tobacco products, such as tobacco leaves and STPs, has been often reported (Table 1).

Many analytical approaches focused on nicotine quantification have been reported. Among them, Wu, Ashley & Watson (2002) seem to have presented the first use of headspace analysis using solid-phase micro extraction (SPME) combined with GC-MS for detection of nicotine and other minor tobacco alkaloids. This method is completely automated and combines the advantages of SPME, such as high throughput and minimal use of solvent, with a high chromatographic resolution of capillary column and high specificity and sensitivity afforded by mass spectral detection. In the same direction, fast analysis of nicotine in tobacco using double-shot pyrolysis-gas chromatography-mass spectrometry (DSP-GC-MS) has been proposed by Lee *et al.* (2007). The DSP method requires less than half the time of solvent extraction methods and requires less sample, in addition to being almost solvent-free and less labor-intensive. Cai et al. (2003) developed a fast method involving a brief extraction step and fast analysis of 7 nicotine-related alkaloids using megabore capillary column and GC coupled with flame ionization detector (FID). The method was applied to both tobacco and cigarette smoke samples and the extraction and analysis time was decreased allowing the analysis of about 100 samples per day.

Table 1. Developed or improved methods for different tobacco products analysis.

Matrix	Analytes	Analytical technique	LOD	LOQ	Reference
Cigarette tobacco filler	Nicotine	GC-MS	0.209-1.80 µg	NR	Wu et al. (2002)
Cured and green tobacco	Nicotine	GC-NPD	NR	NR	Yang et al. (2002)
Flue-cured tobacco and cigarette smoke	Nicotine	GC-FID	0.01 µg/mg	NR	Cai et al. (2003)
Tobacco leaves	Nicotine	DSP-GC-MS	NR	NR	Lee et al. (2007)
Tobacco leaves	Nicotine	GC-MS	2.5 ng	NR	Hossain et al. (2013)
Fermented extracts of tobacco fresh leaves	Nicotine	GC-MS GC-FID	NR	5.0 µg/mL	Millet et al. (2009)
Moist snuff and smokeless tobacco products	Nicotine	GC-FID GC-MS	0.16 mg/g	NR	Stanfill et al. (2009)
Swedish-style snus pouch; American-style loose moist snuff; looseleaf chewing tobacco; chopped loose-leaf chewing tobacco	Nicotine	UPLC-PDA	0.130 µg/mL	5.0 µg/mL	Miller et al. (2020)
Hookah tobacco products	Nicotine	GC-MS	0.025 ng/mL	0.083 ng/mL	Ali et al. (2020)
Cigarette tobacco filler	TSNAs: NNN; NAT; NAB; NNK	GC-NCD	50.8-205.8 ng/cig	153.8-623.8 ng/cig	Soares et al. (2017)
Mainstream cigarette smoke	TSNA: NNN; NAT; NAB; NNAL	HPLC-ESI-MS/MS	0.05-1.23 ng/mL	NR	Wu et al. (2003)
Mainstream cigarette smoke	TSNAs: NNN; NAT; NAB; NNK	LC-MS/MS	0.04-0.1 ng/mL	NR	Wagner et al. (2005)
Mainstream cigarette smoke	TSNAs: NNN; NAT; NAB; NNK	GC-IT-MS/MS	0.1-0.6 ng/mL	NR	Zhou et al. (2007)
Mainstream cigarette smoke	TSNAs: NNN; NAT; NAB; NNK	LC-MS/MS	0.003-0.021 ng/mL	0.005-0.069 ng/mL	Xiong et al. (2010)

Table 1 (cont.). Developed or improved methods for different tobacco products analysis.

Matrix	Analytes	Analytical technique	LOD	LOQ	Reference
Mainstream cigarette smoke	TSNAs: NNN; NAT; NAB; NNK	GC-MS/MS	0.023-0.028 ng/cig	0.077-0.093 ng/cig	Wu et al. (2013)
Tobacco	Pesticides (11)	HPLC-ESI-MS/MS	NR	0.01-0.75 µg/g	Mayer-Helm et al. (2008)
Tobacco	Pesticides (52)	HPLC-ESI-MS/MS	NR	near or below 10 ng/g	Mayer-Helm et al. (2009)
Tobacco	Pesticides (13)	GC-ECD	0.3-1.6 µg/kg	1.0-4.5 µg/kg	Zhou et al. (2012)
Tobacco	Pesticides (2)	HPLC-DAD	5-19.5 µg/kg	16.5 - 64.3 µg/kg	Ge et al. (2014)
Tobacco	Pesticides (47)	GC-MS/SIM MDGC-MS	3-5 ng/g NR	7.5-15 ng/g NR	Khan et al. (2014)
Tobacco leaves and cut tobacco (cigarettes)	Pesticides (18)	On-line coupled LC-GC-ECD	1.5-3.3 µg/kg	4.5-10.0 µg/kg	Qi et al. (2014)
Tobacco	Pesticides (259)	LPGC-MS/MS GC-MS/MS	NR NR	0.2-1.95 ng/g 0.94-2.24 ng/g	Khan et al. (2015)
Tobacco	Pesticides (26)	Online GPC-GC-MS/MS	0.01275-3.150 ng/g	0.04125-10.50 ng/g	Luo et al. (2015b)
Tobacco	Pesticides (10)	Online GPC-GC-MS/MS	0.940-100 ng/L	3.10-340 ng/L	Luo et al. (2015a)
Tobacco	Pesticides (55)	UHPLC/MS GC-MS/MS	8-23µg/kg	25-75µg/kg	Bernardi et al. (2016)
Fresh and flue-cured tobacco leaf	Pesticides (25)	HPLC-MS/MS	0.024-0.30 mg/kg	0.08-1.00 mg/kg	Chen et al. (2020)
Mainstream cigarette smoke	PAHs (15)	GC-MS	NR	NR	Forehand et al. (2000)

Table 1 (cont.). Developed or improved methods for different tobacco products analysis.

Matrix	Analytes	Analytical technique	LOD	LOQ	Reference
Mainstream cigarette smoke	PAHs (5)	GC-MS	NR	NR	Li et al. (2003)
Mainstream cigarette smoke	PAHs (14)	GC-MS	3-155 ng	NR	Ding et al. (2005)
Mainstream cigarette smoke	PAHs (14)	GC-MS	NR	NR	Ding et al. (2006)
Mainstream cigarette smoke	PAHs (10)	HPLC-APPI-MS/MS	11-166 pg	NR	Ding et al. (2007)
Mainstream cigarette smoke	PAHs (15)	GC(HR)-MS	0.01 ng/cig	NR	Zha et al. (2002)
Smokeless tobacco: Conventional moist snuff; Smokeless spit-free tobacco	PAHs (23)	GC-MS	0.1-3.8 ng/g	0.3-10.9 ng/g	Stepanov et al. (2010)
Mainstream cigarette smoke	PAHs (14)	GC-MS	0.05-0.36 ng/cig	0.17-1.19 ng/cig	Shi et al. (2015)
Mainstream cigarette smoke	PAHs (16)	LC–APPI-MS/MS	0.04-1.35 ng/cig	0.12-4.51 ng/cig	Zhang et al. (2015)
Mainstream cigarette smoke	PAHs (16)	GC-MS	0.02-0.07 ng/cig	0.07-0.22 ng/cig	Wang et al. (2015)
Tobacco	Sugars: Fructose; Glucose; Sucrose; Maltose	HPLC-ELSD	1.6-2.0 mg/L	NR	Sun et al. (2004)
Tobacco leaf	Sugars: Fructose; Glucose; Maltose; Raffinose; Sucrose; Xylose	HPLC-ELSD	2-4 µg/mL	NR	Pang et al. (2006)
Cigars Chewing tobacco Snuff	Sugars: Fructose; Glucose; Sucrose	LC-MS/MS	0.05-0.025 µg/mL	0.5 µg/mL	Clarke et al. (2006)

Table 1 (cont.). Developed or improved methods for different tobacco products analysis.

Matrix	Analytes	Analytical technique	LOD	LOQ	Reference
Virginia type cigarettes Blended type cigarettes Cigars	Sugars: Fructose; G-glucose Galactose; Mannose; Sucrose	HPAEC-PAD	11.1-204.5 ng/mL	NR	Tang et al. (2007)
Tobacco filler	Sugars: Fructose; Glucose; Sucrose	HPLC-ELSD	0.3-0.5 mg/g	NR	Li et al. (2003)
Tobacco leaves	Sugars: Xylose; Arabinose; Ribose; Fructose; Glucose; Sucrose; Furanose; Maltose; Raffinose	GC-MS	NR	NR	Jansen et al. (2014)
Tobacco leaves; Cigarettes; Pipe tobacco cigars; Cigars; Chewing tobacco	Sugars: Fructose; Glucose; Sucrose	IC-PAD SFA	NR	NR	Talhout et al. (2006)
Tobacco and casing	Simultaneous analysis Sugars: Fructose; Glucose; Sucrose Humectants: Glycerol; Propylene glycol	HPLC-RID	Sugars: 1.5-2.0 mg/L Humectants: 1.8-2.5 mg/L	NR	Lan et al. (2006)
Mainstream cigarette smoke	Simultaneous analysis: PAHs (3) TSNAs	Online GPC-GC-MS/MS	PAHs : 0.01-0.04 ng/cig TSNAs : 0.03-0.23 ng/cig	0.02-0.12 ng/cig 0.09-0.76 ng/cig	Luo et al. (2016)

Table 1 (cont.). Developed or improved methods for different tobacco products analysis.

Matrix	Analytes	Analytical technique	LOD	LOQ	Reference
Tobacco products: Cigarillo Little cigar; Cigarette	Simultaneous analysis: Sugars: Fructose; Glucose; Sucrose; Maltose; Mannose Humectants: Glycerol; Propylene glycol; Triethylene glycol	UHPLC-MS/MS	Sugars: 0.2-1.2 ng/mL Humectants: 3.6-4.5 ng/mL	NR	Wang et al. (2017)
Tobacco filler	Simultaneous analysis: Nicotine, alkaloids, carbohydrates, organic acids, humectants	DART-MS	NA	NR	Jiang et al. (2019)

Detection limit (LOD); Quantification limit (LOQ); Not reported (NR); Not applicable (NA); Cigarette (Cig); Polycyclic aromatic hydrocarbons (PAHs); Multi-dimensional Gas Chromatography (MDGC); Low Pressure Gas Chromatography (LPGC); Double-shot pyrolyzer (DSP); Flame Ionization Detection (FID); Electron Capture Detector (ECD); Real time mass spectrometry (DART-MS); Chemiluminescence detector (NCD); Gel Permeation Chromatography (GPC). Micro flame ionization dettector (μFID). Single Ion Monitoring (SIM); High Resolution (HR); Gas Chrmotography (GC); Liquid Chromatography (LC); Mass spectometry (MS); Ultra high performance liquid chromatography (UHPLC); Electrospray ionization (ESI); Chemical Ionization (CI); Atmospheric Pressure Photoionozation (APPI); High Performance Liquid Chromatography (HPLC); Diode Array Detector (DAD); Time of Flight (TOF); Fluorescence (FL); Refractive Index (RI); Evaporative Light Scattering Detector (ELSD); Pulsed Amperometric Detector (PAD); High-Performance Anion-Exchange Chromatography (HPAEC); N-nitrosonornicotine (NNN); 4-methyl-N-nitrosamino-1-(3-pyridyl)-1-butanone (NNK); N-nitrosoanabasine (NAB); N-nitrosoanatabine (NAT); 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL); Hydrophilic interaction liquid chromatography (HILIC); Thermal Energy Analyzer (TEA); Slected ion monitoring (SIM); Segmented Flow Analysis (SFA).

Further methods using GC-MS and GC-FID were tested for analysis of nicotine in tobacco leaves. GC-MS combined with classic methylpolysiloxane column was only successful for qualitative analysis, as the nicotine content data showed great variability. Quantification was possible through development of a validated method that used GC-FID with an amine-deactivated column employing anabasine as internal standard (IS) (Millet, Stintzing & Merfort, 2009). A preliminary study was carried out for determination of nicotine in tobacco leaves and stems by GC-MS after extraction with methanol, clean-up through a column with anhydrous sodium sulfate and silica gel, besides prior dilution of extract (Hossain & Salehuddin, 2013). Despite being a modification of a previously reported method, this procedure proved to be simple and showed linearity ranging from 5.0 to 1000 ng, good precision (5-100 µg/mL) and recoveries in the range of 83-96%, besides detection limits (LODs) at ppm levels for non-selective monitoring and at nanogram level for selective detection. It should highlight the advantages of GC-MS, such as high sensitivity, nicotine specific-detection and low instrumentation cost.

A modification of a nicotine standard method that uses GC-FID was proposed by Stanfill, Jia, Ashley and Watson (2009), where a rapid and chemically selective nicotine quantification method was developed for STPs using GC-MS in selective ion-monitoring mode to reduce signal interferences that can influence nicotine values. The proposed changes made it possible to reduce the analysis time from 26 to 3.7 min. Moreover, the GC-MS method provided confirmation of chromatographic peak purity in highly flavored products and calibration curves spanning a concentration range of 0.05-65.62 mg/g. Recently, the efficiency of a GC-MS method for nicotine quantification in hookah (Water Pipe) tobacco products has been improved and a single-step extraction procedure has been developed (Ali, 2020). The method presented a low LOD and a wide linear range, in addition to good accuracy, precision and recovery values.

An analytical procedure using GC with nitrogen phosphorus detector (GC-NPD) using previously optimized experimental conditions was modified and improved to determine nicotine and other alkaloids in green and cured tobacco (Yang, Smetena & Huang, 2002). Briefly, tobacco samples were treated with an aqueous ammonia solution to loosen tobacco tissue and adjust pH, extracted with methanol–dichloromethane using 2,4'-dipyridyl as IS and then analyzed. According to the authors, composition of the extraction solution affected recovery of the alkaloids and contributed to carry-over in the injection liner and quenching of the NPD. This problem was eliminated reducing injection volume by using a packed injection liner besides reducing the amount of pretreatment with aqueous ammonia. A narrow bore capillary column was used to

improve sensitivity and resolution and to increase the speed of analysis, resulting in an appropriate procedure for routine analysis of alkaloid in tobacco samples.

Recent studies regarding the development of methods using LC for nicotine analysis have not been found. This can be justified by pH restrictions, the nature of LC columns and complexity of the tobacco matrix that hinders analysis using this technique. In this sense, only a sensitive ultra-performance liquid chromatography coupled with photodiode array detector (UPLC-PDA) method was tested for quantitation of nicotine released from a variety of STPs, using a smart flow-through system and artificial saliva as dissolution medium (Miller *et* al. 2020). Although significant advances have taken place in the last few years, many studies related to development of methods for nicotine analysis in tobacco and derived products had been established before the last 20 years. Additionally, recent works that aimed to determine nicotine content in tobacco products applied previously established methods. Overall, several analytical approaches for analysis of tobacco alkaloids are available and the latest advances have demonstrated a suitable procedure for *in vivo* monitoring of nicotine biosynthesis in tobacco leaves by low-temperature plasma mass spectrometry with a 3D printed probe (Martínez-Jarquín, Herrera-Ubaldo, de Folter & Winkler, 2018). A portable device was developed for on-site evaluation of nicotine in tobacco and environmental tobacco smoke based on surface-enhanced Raman scattering (SERS) and the use of a miniaturized platform based on screen-printed gold electrode to achieve specific detection of nicotine in natural tobacco within 30s was recently proposed (Lin *et* al. 2019; Yu *et* al. 2020).

Tobacco specific N-nitrosamines (TSNAs)

Cigarette tobacco filler (CTF), smoke and STPs contain a complex mixture of chemicals compounds, including TSNAs. Most TSNAs are formed by nitrosation of tobacco alkaloids in the post-harvest period, while others are produced during cigarette burning (Wu et al., 2003; Konstantinou *et* al. 2018). TSNAs levels in tobacco vary according to blend, storage conditions and form of processing, while in smoke and STPs these compounds are present in extremely low concentrations (Stepanov & Hatsukami, 2020; Edwards *et* al. 2017; Wu, Lu, Lin, Zhou & Gu, 2013), requiring different sample preparation processes besides specific analytical methodologies for each matrix. Due to concerns with the health of smokers, there is a longstanding interest in developing analytical methods for these compounds (Brunnemann & Hoffmann, 1991; Stepanov & Hatsukami, 2020), and N-nitrosonornicotine (NNN), N′-nitrosoanatabine (NAT), N-nitrosoanabasine (NAB) and nicotine-derived

nitrosamine ketone (NNK) are frequently analyzed in tobacco products and smoke (Stepanov & Hatsukami, 2020; Ishizaki & Kataoka, 2019; Edwards *et* al. 2017). Overall, chromatographic methods for tobacco and CTF analysis mainly include GC and LC techniques coupled with nitrogen chemiluminescence (NCD) or MS detectors, respectively (Edwards *et* al. 2017; Rickert, Joza, Sharifi & Wu, 2008; Wu, Joza, Sharifi, Rickert & Lauterbach, 2008; CORESTA, 2017). Thus, a recent report determined all TSNAs in CFT simultaneously using GC with NCD and extraction based on only two steps. The method proved promising, besides presenting high accuracy, precision in the range of 4.7-6.8%, working range from 50 to 700 ng/mL and LODs and LOQs from 50.8-205.8 ng/cig and 198.3-623.8 ng/cig, respectively (Soares, Chiapetta & Pacheco, 2017).

Nowadays, although tobacco and CTF have been analyzed, smoke seems to be the main matrix of analytical interest to TSNAs, since burning tobacco generates these compounds and trace level determination is required (Wagner, Finkel, Fossett & Gillman, 2005; Zhou, Bai & Zhu, 2007; Xiong, Hou, Jiang, Tang & Hu, 2010; Sleiman, Maddalena, Gundel & Destaillats, 2009). When it comes to mainstream cigarette smoke (MSS), a specific and sensitive method was developed and validated to simultaneously analyze TSNAs in the particulate phase by two-step sample preparation and SPE clean-up, followed by isotope dilution liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) analysis. Although a clean-up step was necessary, the method achieved excellent reproducibility and accuracy with LODs from 0.05 to 1.23 ng/mL, in addition to a high-throughput of analysis (8 min/sample) (Wu, Ashley & Watson, 2003). Based on this, methods using LC-MS/MS have been improved for TSNAs analysis in MSS, allowing for decreased sample preparation and analysis time, besides being significantly more sensitive than traditional methods (Zhou et al., 2007; Soares et al., 2017).

Determination of TSNAs in MSS by LC-MS/MS and validation under ISO and CI smoking regimes were performed by Xiong et al. (2010). The method has low LODs (0.003-0.021 ng/mL), with a linear calibration range from 1 to 200 ng/mL, and takes advantage of the resolving power and selectivity of atmospheric pressure ionization (API) tandem MS. Few studies used two smoking regimes to obtain smoke and further comparison, which is important, since a single smoking regime cannot imitate the true behavior involved in human smoking and the potential exposure to harmful substances. A similar procedure was performed by Edwards et al. (2017) using HPLC-MS/MS. Recently, Ishizaki and Kataoka (2019) developed a new, simple and sensitive method for simultaneous analysis of TSNAs in mainstream and sidestream smoke using automated online in-tube solid-phase microextraction

coupled with LC tandem mass spectrometry (SPME-LC-MS/MS) and a laboratory-made smoking machine. This procedure facilitated extraction, concentration and sample analysis. Additionally, the obtained LOD showed 20-fold higher sensitivity than the direct injection method and 50-times higher sensitivity than the previously described LC-MS/MS method (Xiong et al., 2010). An interesting approach in this same context was the use of LC-MS/MS to determine the main TSNAs both in CTF and MSS, changing only the sample preparation procedure (Brunnemann & Hoffmann, 1991). Instead, Wu et al. (2008) used two independent techniques, LC-MS/MS and GC with thermal energy analyzer (GC-TEA), respectively, to analyze CTF and MSS.

Zhou et al. (2007) developed a procedure using an acetic ether extraction and clean-up by SPE followed by GC/ion trap MS (GC-IT-MS/MS) analysis. Four TSNAs from MSS were analyzed by a sensitive and selective technique that provides excellent reproducibility and accuracy. According to the authors, this approach has several additional advantages over previous methods (HPLC-MS/MS and GC-TEA) in terms of lower cost and easier maintenance. A similar method using GC-IT-MS/MS for determination of NNN, NNA, NNK and NNAL in secondhand smoke was developed by Sleiman et al. (2009). In this work, MS parameters were optimized for each TSNA and the major fragmentation pathways were elucidated. The method showed excellent performance, with a linear dynamic range from 2 to 1000 ng/mL, low LODs (30-300 pg/mL) and precision with experimental errors below 10% for all compounds. Furthermore, no interfering peaks were observed, indicating a high selectivity of the MS/MS and sample preparation involved only extraction with methanol followed by centrifugation, without the need for a sample clean-up step. Wu et al. (2013) also performed a rapid and selective determination of TSNAs in MSS by GC coupled with a positive chemical ionization triple quadrupole MS (GC-CI-MS/MS), using isotopically labeled analogs as IS. The method proved fast, reproducible, consistent with traditional procedures and effective for routine analysis of MSS.

TSNAs have been analyzed in a variety of STPs, including traditional moist snuff, snus, strips and other oral tobacco products. Moist snuff samples were generally analyzed using GC-TEA, GC-FID, GC-MSD and LC-MS/MS. Analysis by GC-TEA allowed the quantification of NNN, NNK, NAT and NNAL, despite requiring a derivatization step prior to analysis, due to the need of volatilization of analytes, which makes the analysis method complex and highly time-consuming. Although GC-TEA is not currently the most used technique, the possibility of determining TSNAs in several STPs has been demonstrated (Djordjevic, Fan, Bush, Brunnemann, & Hoffann, 1993; Stepanov, Jensen, Hatsukami & Hecht, 2006). In contrast, fast analysis

using LC-MS/MS allowed determination of the TSNAs in moist snuff without the need of a derivatization step (Jansson, Paccou & Österdahl, 2003; Österdahl, Jansson & Paccou, 2004), as proposed in a similar method (Richter, Hodge, Stanfill, Zhang & Watson, 2008). Recently, a high breakthrough in research related to TSNAs analysis in tobacco products is noticeable, and there is a clear trend towards development of fast and simpler methodologies. We highlight the determination of TSNAs in tobacco using high-throughput screening by flow injection analysis coupled with high-resolution mass spectrometry (HTS-FIA-HRMS), besides the prediction of matrix-bound TSNAs using regression models (Kaiser *et al.* 2018). Another interesting trend is the analysis of TSNAs in different samples using the same analytical technique (Ishizaki & Kataoka, 2019).

Humectants

Humectants, mainly glycerol and propylene glycol, are tobacco additives commonly used to facilitate the processing of tobacco leaves and maintain the flavor and moisture content in final tobacco products (Geiss & Kotzias, 2007; Carmines & Gaworski, 2005; Klus, Scherer & Müller, 2012). While glycerol also occurs naturally in tobacco leaves, propylene glycol (PG) and tri-ethylene glycol (TEG) do not. The concentrations of humectants can vary greatly among different tobacco products, and heating of these substances generate potentially toxic compounds (Rodgman, 2002; Rainey, Shifflett, Goodpaster & Bezabeh, 2013; Jansen, Ramlal, Cremers & Talhout, 2017; Gaworski, Oldham & Coggins, 2010). Few methods have been reported for analysis of humectants in tobacco products, usually applying GC coupled to MS and, FID detectors, HPLC-RID or HPLC-MS, after a pre-analytical conditioning and solid-liquid extraction (Rainey et al., 2013; Jansen et al., 2017). Thus, it is important to improve and develop methods that can provide chromatographic separation and selective detection of both humectants in different tobacco matrices.

Most of the reported analytical methodologies and data on humectants in tobacco and MSS have been provided over the last decades. From them, changes and improvements were made, so that methods could be applied to various tobacco products. The CORESTA (2015) and CORESTA (2019), Health Canada T-304 (Health Canada, 1999) and SOP06 (WHO, 2016) procedures are commonly used for quantification of humectants in tobacco, CTF and tobacco products, and served as a basis for the currently developed methods. Overall, these methods were based on a high-speed mechanical extraction with water or methanol followed by GC-FID, GC-MS or HPLC-RID analysis. In order to improve Health Canada method T-304, Rainey et al. (2013) performed quantitative analysis of humectants

in tobacco products using GC simultaneously with MS and FID. They describe a modification to incorporate simultaneous MS and FID into analysis of humectants in different tobacco matrices. Briefly, tobacco was extracted in methanol containing 1,3-butanediol as IS, filtered and separated in a megabore DB-Wax column. Post-column flow was distributed using a microfluidic splitter between MS and FID for simultaneous detection. While a high degree of correlation was obtained between the two techniques used, a minimal chromatographic problem was observed between glycerol and triethylene glycol, which restricts applicability of FID to samples containing low levels of both humectants.

Likewise, and considering that during the conditioning step of tobacco and cigarettes significant decreases occur, especially of propylene glycol levels, Jansen et al. (2017) proposed a method with no conditioning that minimizes this loss. The method is similar to previously described besides showing LOQs between 0.094 and 0.30 mg/g, and omitting the conditioning step in the analytical procedure and adding a method to measure water content before and after complete drying of tobacco samples. Another analytical method for the quantification of 3 humectants in tobacco filler using a sample clean-up method with a two-step process consisting of mechanical extraction, followed by solid-phase extraction and HPLC-MS analysis, was developed and validated (Xizheng, Valentín-Blasini, Watson, Cardenas, 2018). This work seems to be the first to develop a method to analyze these compounds in tobacco filler using isotopically labeled IS for a selective and accurate quantitation. The method is characterized by its straightforward sample preparation, good sensitivity, selectivity, suitability and precision, with LODs from 0.039 to 0.575 mg/cig. In recent work, Wang, Stanfill, Valentin-Blasini, Watson and Cardenas (2019) determined humectants in STPs with a previously developed and validated method using HPLC-MS/MS (Wang, Cardenas & Watson, 2017). Samples were ionized using ESI and introduced into the triple quad MS operated under multiple-reaction monitoring (MRM) mode. This method is an interesting and potentially efficient alternative, since it allows simultaneous determination of humectants and sugars in tobacco products.

There seems to have little progress in the development of analytical methods for humectants analysis in tobacco matrices. Overall, results of the latest research showed substitution of TCD and FID for MS and the insertion of the HPLC technique, which provided improvements in precision for determination of the main humectants when compared to previous studies. Although the FID is a sensitive detector and has been used, noteworthy it is not selective and relies on chromatographic retention time to differentiate analytes, which limits the application of this detector for humectants analysis (Rainey et al., 2013). In comparison

with initially proposed studies, where humectants were analyzed individually, it is now possible to analyze different humectants in a single and rapid analytical run.

Pesticides

Pesticides are chemical substances used to control pests, insects, fungi, rodents and weeds. Extensive use of these substances in agriculture exposes the environment and humans to residues of these agrochemicals through their diet and habits, for instance, smoking (WHO, 2019). Due to their toxicity, pesticides must be monitored in order to comply with regulations (WHO, 2016). Thus, development of analytical methodologies capable of detecting and quantifying residues of these substances in several matrices, including tobacco, has been widely sought over the past few years. GC-MS is the most widespread technique for determination of pesticides (Table 1), due to the volatility of most of these analytes and the sensitivity of mass spectrometry. When it comes to pesticides analysis in tobacco by GC, sample preparation is one of the most important steps, due to the need of eliminating matrix interferents and obtaining clean extracts to prevent contamination of GC components. Therefore, many pesticide sample extraction strategies and clean-up procedures have been studied throughout the last decades (Figure 2), including Pressurized

Liquid Extraction (PLE), Solid Phase Extraction (SPE), Dispersive Solid Phase Extraction (d-SPE), Liquid-Liquid Extraction (LLE), Low-Temperature Precipitation (LTP) and QuEChERS (Haib, Hofer & Renaud, 2003; Lee, Park, Jang & Hwang, 2008; Khan *et al.* 2014; Luo *et al.* 2015a; Luo *et al.* 2015b; Bernardi *et al.* 2016).

PLE, followed by a clean-up step using 3 different SPE procedures, has been reported as a suitable method for extraction of pesticides in tobacco, providing satisfactory recoveries (75-125%) as well as absence of matrix effect (ME) when samples were analyzed by a triple quadrupole MS coupled to a GC (Haib et al., 2003). In order to determine the most accurate and rapid methodology, Lee et al. (2008) also used a triple quadrupole GC-MS to quantify and compare pesticide residue levels obtained from 3 different extraction methods (PLE, LLE, QuEChERS). They also tested the effect of 3 different sorbents, primary secondary amine (PSA), octadecylsilane (C_{18}) and graphitized carbon black (GCB), in the QuEChERS method, to investigate their influence on recovery rate and ME. PSA alone provided good recoveries and relative standard deviation (RSD) values, while the QuEChERS method yielded better recovery of pesticides when compared to LLE and PLE, with no need of complex clean-up procedures. QuEChERS was also compared by Khan et al. (2014) with their proposed extraction methodology, which added $MgSO_4$ in the dispersive clean-up step in addition
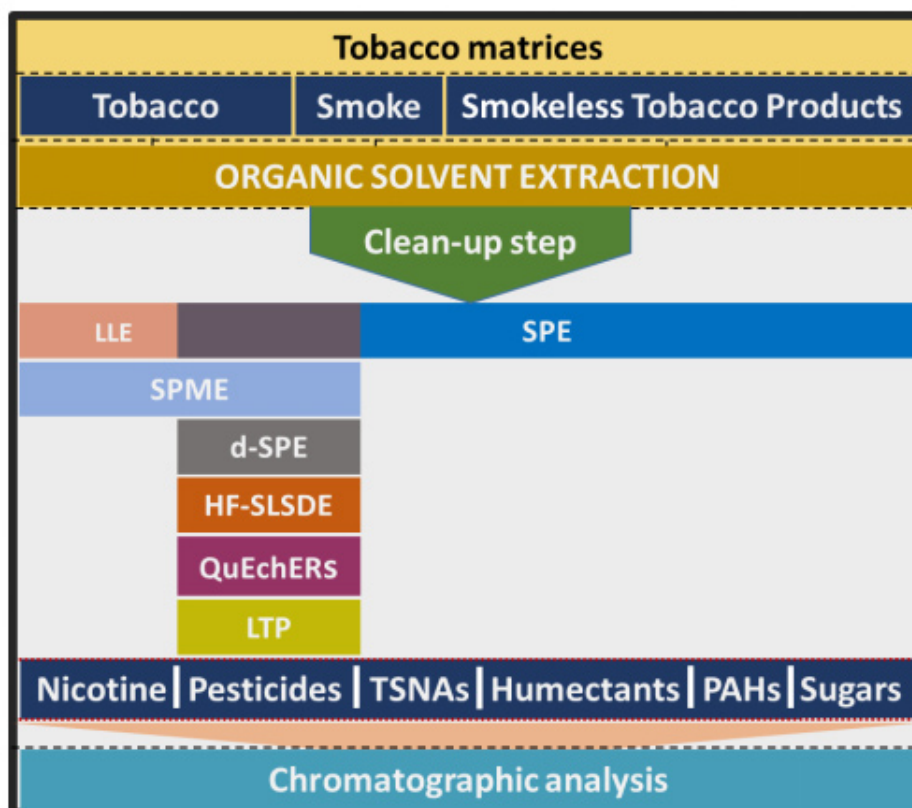
Figure 2. Overview of sample preparation processes for different tobacco matrices and analytes.

to PSA, C18 and GCB. To reduce interferences and nullified co-elution related to false detections, a multi-dimensional gas chromatography heart-cut (MDGC) was applied, with two capillary columns connected in series, one in a GC oven with Electron Capture Detector (ECD) and another in a second GC oven connected to MS. Compared to traditional QuEChERS, the proposed method succeeded to minimize ME from some major tobacco compounds, such as nicotine, by using a combination of 75 mg GCB with 150 mg PSA, 150 mg C18 and 300 mg $MgSO_4$. Another QuEChERS modification was proposed by Luo et al. (2015a,b) in two different works. Firstly, GCB, PSA and magnetite (Fe_3O_4) were used as sorbents, while in a later work they synthesized magnetic graphene to use as sorbent. The optimized procedures were coupled with on-line gel permeation chromatography–gas chromatography–tandem mass spectrometry (GPC–GC–MS/MS). The proposed methods showed a better clean-up efficiency when compared to the traditional QuEChERS, successfully removing pigments in tobacco**.** However, when applied to real samples, the magnetic graphene method was affected by interferences to some extent, once recoveries ranged between 43 and 85%. Bernardi et al. (2016) also applied a sample preparation procedure based on the QuEChERS citrate method to tobacco samples. In this work, for the first time LTP was reported to be used prior to d-SPE clean-up, which was crucial to obtain a clean extract, suitable for LC and GC analysis.

As pesticide analysis and extraction can high time-consuming, one-step sample preparation and automated techniques have been developed. Extraction and clean-up were integrated into a single step in a method named hybrid field-assisted solid-liquid-solid dispersive extraction (HF-SLSDE), and 13 organochlorine pesticides were analyzed using GC-ECD, achieving low LODs (0.3-1.6 µg/kg) and LOQs (1.0-4.5 µg/kg) (Zhou, Xiao & Li, 2012). Qi et al. (2014) introduced a totally automated methodology, coupling liquid and gas chromatography (LC-GC-ECD), aiming to provide higher sample capacity, faster sample evaporation and better retention of volatile compounds. Employment of a switching valve between the pre-column and separation column during solvent evaporation allowed the LC solvent to be completely removed, preventing it from reaching the GC column and detector, besides reducing loss of volatile components, permitting large volume of LC eluent and preventing decomposition of labile compounds, which was not achieved with other interfaces previously developed. Afterwards, Khan et al. (2015) employed low-pressure gas chromatography technique tandem triple quadrupole mass spectrometry (LPGC-MS/MS) for the determination of 259 multi-pesticide residues in tobacco. The optimized LPGC-MS/MS methodology was three-times faster than the usual GC-MS/MS and provided low LOQs (<2 µg/L) for all compounds, which could be achieved due to an average 2-3-times enhancement in signal/noise. To increase performance of the methods and improve separation of analytes in a complex matrix as tobacco, comprehensive two-dimensional gas chromatography (GCxGC) has been used coupled with MS or ECD, the latter limited to organochlorine pesticide class. In this sense, a methodologies based on GCxGC were proposed by Cochran (2008) to identify 14 pesticides in tobacco and by Khan et al. (2014) as previously mentioned.

The LC, although to a less extent than GC, has also been used for analysis of pesticides in tobacco, such as carbamates, which, in some cases, need derivatization processes to be analyzed by GC. Three multi-residue methodologies were proposed in different works to analyze pesticides in tobacco by HPLC coupled with positive electrospray ionization tandem triple quadrupole mass spectrometry (HPLC-ESI-MS/MS) (Mayer-Helm, Hofbauer & Müller, 2006; Mayer-Helm, Hofbauer & Müller, 2008; Mayer-Helm, 2009). The methods differed mainly regarding sample preparation, where some purification steps, which consisted of the use of a Chem Elut cartridge containing diatomaceous earth, d-SPE and SPE, were added in order to fulfill recovery and sensitivity requirements (Mayer-Helm et al., 2008; Mayer-Helm, 2009). All validated methods provided LOQs in compliance with the Guidance Residues Levels (GRLs) from Coresta, with clean elutes obtained after improvements in the clean-up steps. HPLC was also used in a simple method for simultaneous determination of Chlorantraniliprole (CAP) and Spirodiclofen (SDF) residues in tobacco by liquid chromatography with diode-array detection (HPLC-DAD) (Ge, Wu, Qi, Qin & Sun, 2014). Pesticides were extracted by accelerated solvent extraction (ASE) and the validated method provided LOQs of 16.5 µg/kg for CAP and 64.3 µg/kg for SDF, which is lower than their maximum residue limits (MRLs). Recently, HPLC-MS/MS was used to determine 25 herbicides in soil and fresh and flue-cured tobacco leaf, after a simple sample preparation step consisting of extraction with acetonitrile followed for a d-SPE clean-up. The method proved suitable for trace analysis, providing satisfactory linearity ($R^2 \geq 0.9904$), LOQs (0.08-1.00 mg/kg), LODs (0.024-0.30 mg/kg) and recovery rates (72.32-116.83%) (Chen *et* al. 2020). Overall, GC-MS/MS is the most widespread technique for pesticide analysis, although good results have been achieved with the use of HPLC-MS/MS. Sample preparation is a relevant aspect in terms of pesticides analysis, with trend on automation of methods in order to reduce steps and make extractions more efficient and faster (Luo et al., 2015a,b; Khan et al., 2015).

Polycyclic aromatic hydrocarbons (PAHs)

PAHs are a class of environmental contaminants comprising two or more fused aromatic rings. They are generated by the incomplete combustion of organic materials, including tobacco, whose smoke contains considerable levels of these compounds. Since several PAHs, such as benzo[a]pyrene (BaP), are carcinogenic and have human health implications, they have been the subject of numerous studies aiming to detect their presence in tobacco matrices (IARC, 2010; Klingbeil, Hew, Nygaard & Nadeau (2014). Although the physical and chemical properties of PAHs allow them to be analyzed both by GC and HPLC, most studies reported the use of CG coupled to MS to determine their presence in tobacco matrices. As they are formed during the tobacco combustion process, the main object of study of PAHs is the MSS. Therefore, many extraction methods have been developed to extract PAHs from the total particulate matter (TPM) of smoke so they could be identified and quantified.

The simultaneous distillation and extraction (SDE) technique was reported as efficient for extracting PAHs and other analytes from the smoke particulate phase (Forehand, Dooly & Moldoveanu, 2000). The extract was analyzed by GC-MS without further treatment and the results obtained for benzo[a]pyrene were in good agreement with the literature. A procedure using SPE was applied for PAHs extraction from the smoke particulate phase (Zha & Qian, 2002). A Bond Elut CH cartridge was used and its clean-up efficiency allowed GC analysis using either high- or low-resolution MS detection. Despite the SPE procedure being very time-consuming, the method proved robust and provided a high recovery rate (97%) and low LOD for benzo[a]pyrene (0.01 ng/cig). SPE was also employed in two different works to determine 14 PAHs in MSS (Ding, Trommel, Yan, Ashley & Watson, 2005; Ding *et al.* 2006). In both studies, cigarettes were smoked under the ISO smoking regime and the Cambridge filter pads (CFPs) were extracted with methanol. The methods differed mainly in the clean-up step, where automated SPE was used instead of the traditional SPE previously proposed. Samples were analyzed using GC-MS, which presented limitations in detecting higher molecular weight PAHs and those at trace levels.

To improve MSS purification, a novel SPE procedure was developed (Shi *et al.* 2015). A graphene oxide was synthesized and bonded to silica to be used as sorbent during the clean-up step. The new sorbent succeeded in removing polar substances from the matrix, providing a colorless extract as well as a simple chromatogram due to the reduced background and matrix interferences. The validated method was applied to Kentucky reference cigarette and commercial cigarette samples, where 14 PAHs were analyzed by GC-MS, providing LODs and LOQs in the range of 0.05-0.36 ng/cig and 0.17-1.19 ng/cig, respectively. Following this trend, a graphene-coated SPME fiber was employed for PAHs extraction from MSS prior to GC-MS analysis (Wang, Wang, Qin, Ding & Xie, 2015). Compared to commercial fibers, the homemade graphene-coated fiber performed better in terms of replicates use, and the total ion chromatogram exhibited a resolved baseline and nearly no interference. After validation, the method was also applied to commercial and reference cigarette samples and proved to be sensitive and selective, besides user friendly.

A technique developed to overcome the need of smoking several cigarettes to collect TPM to analyze PAHs was presented by Li et al. (2003). The method allowed PAHs analysis from a single puff of MSS, offering the capability of detection at trace levels, as well as providing information on formation of PAHs from each puff. An impaction trap was used to collect TPM from a single puff of smoke and 5 PAHs, including naphthalene, phenanthrene, fluoranthene, pyrene, and benzo[a]pyrene were analyzed using GC-MS. Since no sample treatment was necessary, the sample loss associated to extraction and clean-up procedures was eliminated, reducing analysis time. Despite the analysis of PAHs being still strongly related to cigarette smoke, other matrices, such as STP, have also been the subject of studies. Thus, a GC-MS methodology to determine 23 PAHs in moist snuff and smokeless spit-free tobacco products was developed (Stepanov *et al.* 2010). The sample preparation was based on a simple two-step extraction and purification procedure, which consisted on a cyclohexane extraction followed by a SPE clean-up step. The method provided satisfactory linearity ($R^2 > 0.99$), 87% average recovery and the LOQs and LODs ranged between 0.3-0.9 ng/g and 0.1-3.8 ng/g, respectively.

HPLC-MS has been applied to higher molecular weight PAHs, as they are thermally labile and, consequently, less amenable to analysis by GC. A very sensitive method based on high performance liquid chromatography/atmospheric pressure photoionization tandem mass spectrometry (HPLC/APPI-MS/MS) was developed to determine 10 PAHs on MSS (Ding, Ashley & Watson, 2007). Briefly, samples were extracted from CFPs with cyclohexane, following a SPE clean-up procedure. LODs ranged from 11 to 166 pg and mean accuracy numbers for all analytes ranged from 83 to 108%. Another method using HPLC-APPI-MS/MS for determination of 16 PAHs in cigarette smoke condensate (CSC) was posteriorly reported (Zhang *et al.* 2015). This method had some similarities with that developed by Ding et al. (2007), in which the sample preparation procedure was based. However, it presented the

advantage of analyzing all PAHs using just one analytical column. Besides, the proposed method was sensitive enough to quantify PAHs from a single cigarette, unlike other previously developed procedures that needed to collect CSC from multiple cigarettes. To date, it should be noted that PAHs extraction is usually performed using SPE, and attempts to improve this technique by introducing new sorbents during the clean-up step, such as graphene, appear to be relevant (Shi *et al.* 2015; Wang et al., 2015). In addition, there is a trend in the development of simpler and faster methodologies, such as the possibility of collecting smoke TPM from a single puff, elimination of purification steps and automation of extraction (Ding et al., 2006; Li et al., 2003).

Sugars

Sugars are substances that occur naturally in tobacco or are added during its manufacturing process to neutralize its harsh taste, sweetening it and, consequently, making it more pleasant and attractive for smokers (Talhout, Opperhuizen & van Amsterdam, 2006). It is known that when tobacco is burned, sugars are pyrolyzed, giving off toxic substances and carcinogenic degradation products (Roemer *et al.* 2012). Although, to the best of our knowledge, no methodologies have been reported as capable of distinguishing whether the amount of sugar found in tobacco occurs naturally or has been added, many methods have been proposed to identify and quantify these compounds in tobacco and derivative products (Table 1). Main established methods for the analysis of sugars in tobacco include LC coupled to different detectors, and since carbohydrates are usually hydrophilic, neutral and lack satisfactory chromophores for UV detection, detectors such as evaporative light scattering detector (ELSD) and MS have been used (Jansen, Cremers, Borst & Talhout, 2014; Pang *et al.* 2006; Sun *et al.* 2004; Clarke, Bezabeh & Howard, 2006).

ELSD detector has been widely used due to its higher sensitivity, better baseline stability and gradient elution possibility (Jansen et al., 2014; Pang *et al.* 2006). Following this trend, an analytical method using HPLC-ELSD for tobacco water-soluble carbohydrates was proposed (Sun *et al.* 2004). Carbohydrates were leached by water, leaching proteins were deposited by lead acetate and the supernatant was purified by a C18 solid extraction column. The method showed good linearity and the LODs ranged between 1.6-2.0 mg/L. Another method based on SPE-HPLC-ELSD was developed to determine xylose, fructose, glucose, sucrose, maltose and raffinose in tobacco leaves (Pang *et al.* 2006). Ground leaves were extracted with acetic acid, methanol, pentaerythritol and water in ultrasonic bath and then submitted to SPE clean-up using NH_2 as sorbent. Sugar content was determined before and after biocatalysis

of tobacco leaves to guarantee the usefulness of the method. Addition of acetic acid quickened extraction time, making it possible to extract sugars in only 5 minutes. Besides that, the method provided satisfactory linearity and sensitivity, with a recovery rate between 96-117%. Posteriorly, a simple and robust method for analyzing sucrose, glucose and fructose by HPLC-ELSD was developed and applied to 58 samples from different cigarette brands (Jansen et al., 2014). Tobacco samples were extracted with water and centrifuged prior to analysis; D-sorbitol was used as IS and separation of analytes was carried out in a MetaCarb 67C column. The methodology provided good reproducibility, sensitivity and repeatability, enabling fast and accurate determination of sugars in about 80 cigarettes per day, in addition to a simple sample preparation step.

Sugar content in tobacco products such as cigars, chewing tobacco and snuff has also been investigated. For this purpose, a method using LC-MS/MS was developed for the determination of fructose, glucose, and sucrose in these matrices and the results obtained were compared with those obtained by IC-PAD (Clarke et al., 2006). Samples were extracted with water containing D-flucose-d2 as IS, followed by agitation and filtration. The LC-MS/MS method increased the linear range and precision, with RSD≤2%, improved selectivity and specificity, besides providing LODs in the range of 0.025-0.05 μg/mL and LOQ of 0.5 μg/mL for all analytes. Although LC-MS/MS has proven to be as efficient as IC-PDA, the latter has often been used to analyze carbohydrates in tobacco. In this sense, an IC-PAD method was compared to Segmented Flow Analysis (SFA) for quantitative characterization of carbohydrates in tobacco leaves, cigarettes, cigars and chewing tobacco (Shifflett, Jones, Limowski & Bezabeh, 2012). Sample preparation was performed using two different procedures. Briefly, samples were extracted with 1% acetic acid, followed by stirring and filtration, or extracted using deionized water, followed by agitation and two steps of SPE for removal of interferents, such as sugar amines and chlorogenic acid. Although both techniques presented the same tendencies for sugar contents at analyzed matrices, the SFA methodology proved to be simpler, especially on sample preparation. Moreover, 72 samples could be analyzed in 1 h using the SFA method, while the IC-PAD method was limited to 4 samples per hour. IC-PAD method was also more laborious and had low reproducibility of the peak area, leading to significant variation in results, which appeared to be related to detector performance.

Regarding analysis of sugars, other methods besides IC-PDA and HPLC-MS/MS have been reported. Tang et al. (2007) developed and validated a fast and selective methodology to analyze sugars, alditols and alcohols in tobacco in a single run. Analytes were extracted from samples by SLE with water and

analyzed using high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The method proposed presented LODs in the range of 2.2-204.5 ng/mL, recovery between 80.5-116.3%, satisfactory linearity, repeatability and precision, beyond discarding a derivatization step and avoiding use of expensive and toxic reagents.

Although analysis of sugars by GC is not common, since it is necessary to perform derivatization processes, some studies based on this technique have been reported. Li et al. (2011) developed a method for determination of the metabolic profile of tobacco leaves by GC-MS, where about 44 analytes were identified, including organic acids, alcohols, amino acids and saccharides. Samples were extracted by a mixture of water, methanol and acetonitrile, followed by shaking in ultrasonic bath, centrifugation and refrigeration. The extract was derivatized with N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) and O-methylhydroxylamine hydrochloride following methoxyamination and trimethylsilylation processes. Since identification of compounds by derivatization can be very laborious and time-consuming, in addition to the fact that mass spectra of some saccharides may be identical, the authors proposed a combination of methods to solve the problem of identifying compounds, which included deconvolution with Automated Mass Spectral Deconvolution and Identification System (AMDIS), comparison with standard spectra deposited in libraries and subsequent confirmation by linear retention index and commercial compounds. The method showed good reproducibility, with RSD under 20% for most analytes. Overall, few relevant studies regarding the development of methods for sugar analysis in tobacco and derivative products have been reported. A recent and interesting approach is the simultaneous determination of different compounds, such as humectants and sugars, by the same procedure, which significantly reduces analysis time and sample preparation. For this purpose, LC-MS/MS, HPLC-RID and UPLC-MS/MS have been used (Lan, Zhong, Ci-Qing, Shuai & Xiao-Dong, 2006; Wang et al., 2017; Wang et al., 2019).

Concluding remarks

A wide range of studies on the development of analytical methods for nicotine, TSNAs, humectants, pesticides, PAHs and sugar analysis in diverse tobacco products have been reported in the past two decades. A potential trend for tobacco and derivative products analysis is the possibility of simultaneous determination of multiple components. This analytical procedure was previously proposed for analysis of sugars and humectants in tobacco and casing. Posteriorly, PAHs and TSNAs were simultaneously analyzed in smoke using on-line GPC-GC-MS/MS (Luo *et* al. 2016). Multiple component analysis of different tobacco products was also performed using LC-MS/MS, UHPLC-MS/MS and by direct analysis in real-time mass spectrometry (DART-MS) (Wang et al., 2017; Wang et al., 2019; Jiang, Dai, Li & Chen, 2019), with the advantage of reducing analysis time. The present study examined the main papers that developed or improved analytical methods for identification and quantification of priority compounds in different tobacco products, and can contribute to future research with this objective. It should be noted that some of the methods cited here have not been validated and further investigations are necessary in order to provide reproducible analytical methods for regulatory purposes.

Declaration of Interests

No potential conflicts of interest were disclosed.

Acknowledgments

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