Journal of Chromatography A, 1439 (2016) 54–64

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Lipidomics of tobacco leaf and cigarette smoke

Melissa N. Dunkle [a,1], Yuta Yoshimura [b,1], Ruben t'Kindt [a], Alexia Ortiz [a], Eri Masugi [b], Kazuhisa Mitsui [b], Frank David [a], Pat Sandra [a], Koen Sandra [a,*]

[a] Research Institute for Chromatography (RIC), President Kennedypark 26, 8500 Kortrijk, Belgium
[b] Japan Tobacco Inc., 6-2 Umegaoka Aoba-ku Yokohama-shi, Kanagawa 227-8512, Japan

ARTICLE INFO

Article history:
Received 13 July 2015
Received in revised form 4 October 2015
Accepted 29 October 2015
Available online 1 November 2015

Keywords:
Lipidomics
Proteomics
Liquid chromatography
Mass spectrometry
Tobacco leaf/smoke

ABSTRACT

Detailed lipidomics experiments were performed on the extracts of cured tobacco leaf and of cigarette smoke condensate (CSC) using high-resolution liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (LC-Q-TOF MS). Following automated solid-phase extraction (SPE) fractionation of the lipid extracts, over 350 lipids could be annotated. From a large-scale study on 22 different leaf samples, it was determined that differentiation based on curing type was possible for both the tobacco leaf and the CSC extracts. Lipids responsible for the classification were identified and the findings were correlated to proteomics data acquired from the same tobacco leaf samples. Prediction models were constructed based on the lipid profiles observed in the 22 leaf samples and successfully allowed for curing type classification of new tobacco leaves. A comparison of the leaf and CSC data provided insight into the lipidome changes that occur during the smoking process. It was determined that lipids which survive the smoking process retain the same curing type trends in both the tobacco leaf and CSC data.

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1. Introduction

The lipidome refers to the total composition of the different types of lipids in a material (e.g. tobacco leaf/smoke), and lipidomics refers to the study of the lipidome of a material, eventually for comparative purposes. Most often, lipidomics uses an LC–MS approach, and with the advances in mass spectrometry, has become an emerging field of study [1–7].

In terms of studying lipids in plants, much of the information found is from *Arabidopsis thaliana*, which is a small flowering plant native to Europe, Asia, and northwestern Africa, and was the first plant to have its genome sequenced [8]. Several lipid classes have been identified in *Arabidopsis* leaf, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidic acid (PA), phosphatidylserine (PS), lysophosphatidylcholine (lysoPC), lysophosphatidylethanolamine (lysoPE), lysophosphatidylglycerol (lysoPG), phosphatidylglycerol (PG), monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol (SQDG) [9–11].

To date, much of the work on tobacco revolves around the analysis of freshly harvested leaf or tobacco seeds. Several lipid classes common to *Arabidopsis*, among others, were reported in fresh tobacco leaf: PE, PC, PA, PI, phosphatidylinositol 4-phosphate (PI4P), phosphatidylinositol 4,5-bisphosphate (PI(4,5)P_2), DGDG, glucosylceramides (GluCER), steryl glucosides (SG), acylated steryl glucoside (ASG), and sterols, in addition to more exotic compounds such as lipid peroxides (e.g. trioxidized fatty acids and trioxidized phospholipids) [12,13]. Finding lipid information on cured tobacco leaf is more challenging. Curing is a process that green tobacco leaf undergoes in order to enhance the flavors and reduce the harshness of the leaf prior to becoming a cigarette. The three most commonly used curing types include flue-curing, air-curing, and sun-curing; each of which affords different flavors (i.e. sweet, smoky, roasted, etc.) to the leaf. No information was able to be found on the influence of air-curing or sun-curing on the lipid content, and only a few reports from the 1970s were found concerning flue-cured tobacco leaf and lipid content [14–16]. One paper in particular [14] reported that the amount of total polar lipids decreased from 13.0 to 5.6% (of crude lipids) after flue-curing tobacco leaf. It was also reported that PI, PS, PE and PC were not degraded during flue-curing; however, MGDG, DGDG, SQDG, and PG were all degraded up to more than 70%. Lipid content in flue-cured tobacco leaf ranges from 5 to 15% of dry leaf [14], and 1.7 to 4% of the dry leaf is made up of fatty acids [17]. With respect to tobacco smoke, some lipid classes have been found to survive the smoking process, while others are degraded. For example, solanesol is an important prenol lipid that has been studied extensively in both tobacco leaf and

* Corresponding author.
E-mail address: koen.sandra@richrom.com (K. Sandra).
[1] These authors contributed equally.

http://dx.doi.org/10.1016/j.chroma.2015.10.090
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Table 1
Sample information.

Abbrev. leaf	Abbrev. smoke	Average CSC (mg)	Curing type	Region
L01	S01	44.0	Flue-Cured	Region 1
L02	S02	23.5	Flue-Cured	Region 6
L03	S03	39.9	Flue-Cured	Region 2
L04	S04	36.7	Air-Cured	Region 1
L05	S05	32.6	Air-Cured	Region 3
L06	S06	14.5	Air-Cured	Region 2
L07	S07	16.6	Air-Cured	Region 6
L08	S08	42.8	Sun-Cured	Region 4
L09	S09	39.0	Sun-Cured	Region 5
L10	S10	41.0	Sun-Cured	Region 5
L11	S11	41.5	Flue-Cured	Region 3
L12	S12	38.1	Flue-Cured	Region 3
L13	S13	29.2	Flue-Cured	Region 3
L14	S14	28.0	Flue-Cured	Region 6
L15	S15	25.7	Flue-Cured	Region 6
L16	S16	51.2	Flue-Cured	Region 2
L17	S17	20.7	Air-Cured	Region 1
L18	S18	29.1	Air-Cured	Region 3
L19	S19	19.5	Air-Cured	Region 3
L20	S20	29.0	Air-Cured	Region 6
L21	S21	30.3	Air-Cured	Region 2
L22	S22	35.5	Sun-Cured	Region 4
L23[a]			Flue-Cured	Region 3
L24[a]			Flue-Cured	Region 3
L25[a]			Flue-Cured	Region 3
L26[a]			Flue-Cured	Region 3

[a] Only tobacco leaf analyzed; used to test prediction models generated from L01–L22 data.

tobacco smoke [18–21]. Additionally, oxidation products are produced during the smoking process, and the oxidation products of solanesol from tobacco smoke have been well described [22].

It is well established that lipids are related to taste and aroma [23–26]; however, it is yet unknown if it is possible to have curing type (flue-cured, air-cured, sun-cured) differentiation based on the observed lipids. This would provide valuable information that could eventually be used to control the taste of a cigarette. In order to better understand the taste and aroma of tobacco, the present manuscript describes the comprehensive measurement of lipids in cured tobacco leaf and cigarette smoke condensate (CSC). Lipids were extracted, according to the modified Folch method [27] and fractionated by solid-phase extraction (SPE). A previously reported SPE procedure [28,29] was simplified and optimized for use with the tobacco leaf and CSC extracts. To afford better reproducibility, the SPE procedure was automated using a dedicated autosampler. All extracts were analyzed using a previously developed lipidomics LC–MS method [30,31], which allows for the separation of different lipid classes.

This article furthermore details the results obtained from the large-scale comparative lipidomics study of tobacco leaf extracts and CSC extracts, including multivariate data processing that divulged the differentiation of curing types within the leaf and CSC data based solely on lipid information. In addition, a description of the generation of prediction models based on the curing type differentiation for the leaf samples is given. Lastly, a correlation observed between the leaf lipidomics data and proteomics data acquired from the same tobacco leaf samples is detailed.

2. Materials and methods

2.1. Materials

All tobacco samples were provided by Japan Tobacco Inc. (Kanagawa, Japan). Table 1 contains all sample information. For the leaf lipidomics study of 22 cured tobacco leaves, a quality control (QC) was created by weighing equal amounts of leaf samples L01–L22. For the CSC lipidomics study of the same 22 cured tobacco samples, the QC was created by pooling equal volumes of the Folch extract obtained from each of the CSC samples S01–S22. Leaf samples L23–L26 were used to test the prediction models that were generated from the leaf lipidomics study (L01–L22 data).

The solvents used for the modified Folch extraction included chloroform, methanol, and water (HPLC grade) from Biosolve (Valkenswaard, The Netherlands). For the extraction, 2:1 chloroform/methanol was prepared and stored at −20 °C until ready for use.

The solvents used for SPE were hexane, chloroform, isopropanol, diethyl ether, and methanol from Biosolve. The hexane was Pesti-S grade, the diethyl ether was AR grade, and the chloroform, isopropanol, and methanol were all HPLC grade. Acetic acid (99.7%, ACS Reagent) was purchased from Sigma–Aldrich (St. Louis, MO, US). For automated SPE, Machery-Nagel Chromabond NH2-MPS cartridges (3 mL/500 mg) were purchased (Macherey-Nagel GmbH & Co. KG, Düren, Germany).

The solvents used for the LC–MS separation were ULC–MS grade water and ULC–MS grade methanol from Biosolve. HPLC grade ammonium formate and formic acid (>99%) were purchased from Sigma–Aldrich. Mobile phase A consisted of 20 mM ammonium formate, pH 5, and mobile phase B was methanol. The column utilized for the separation was an Acquity UPLC BEH Shield RP18 (2.1 × 100 mm, 1.7 μm) from Waters (Milford, MA, USA).

2.2. Methods

2.2.1. Modified Folch extraction for tobacco leaf

First, the ground leaf (1 g ± 0.05 g) was weighed into a 20 mL headspace vial. Then 6 mL ice cold 2:1 chloroform/MeOH (v/v) was added. The vial was capped and vortex mixed. Next, 4 mL water was added, and the vial was again capped and vortex mixed. The vial was then centrifuged at 1000 × g for 10 min to allow for the liquid phases to separate. The lipid layer (bottom layer) was collected and filtered using a 0.2 μm syringe filter. Two mL of the filtered extract was transferred to a 10 mL headspace vial and evaporated at 60 °C for 40 min using an mVap (Gerstel, Mülheim an der Ruhr, Germany). The evaporated samples were then stored at 4 °C until SPE was performed. All leaf extracts were prepared in triplicate. Randomization of all study and QC samples was performed prior to lipid extraction.

2.2.2. Modified Folch extraction for cigarette smoke

First, the cigarette samples were collected using a smoking machine (Smoking Machine LM-1, Borgwaldt KC, Germany) based on ISO smoking regime (puff volume: 35 mL, puff duration: 2 s, puff interval: 58 s). The cigarette smoke condensate (CSC) from one cigarette was collected on a Cambridge Filter (44 mm diameter glass fiber filter, Borgwaldt KC, Germany) [32]. The averaged CSC amounts are shown in Table 1. Then 6 mL ice cold 2:1 chloroform/MeOH (v/v) was added. The vial was capped and sonicated for 15 min. The entire contents of the vial were filtered into a new 20 mL headspace vial using a 0.2 μm syringe filter. Next, 4 mL water was added. Further on, the samples were treated identically as the tobacco leaf samples described in Section 2.2.1. All CSC extracts were prepared in triplicate. Randomization of all study and QC samples was performed prior to lipid extraction.

2.2.3. Automated SPE protocol

Prior to SPE, 300 μL chloroform was added to reconstitute the extracts. Next, the concentrated extracts were transferred to 1.5 mL high recovery vials and automated SPE was performed. For automated SPE, an MPS-2 Dual Head workstation (Gerstel) was configured with the following add-ons: 500 μL syringe, 2.5 mL syringe, gripper adaptor, tray and holder for 10 mL headspace vials, tray and

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