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Analysis of sorghum wax and carnauba wax by reversed phase liquid chromatography mass spectrometry

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ABSTRACT

Sorghum is a genus of plant in the grass family, which is used for both grain and forage production throughout the world. In the United States, sorghum grain is predominantly used as livestock feed, and in ethanol production. In recent years however, sorghum grain has been investigated for other industrial applications, including gluten free food sources for the US food market, and waxes. The United States is the world's largest producer of grain sorghum, which is grown in the arid regions of the southern Great Plains, Arizona and California.

Carnauba wax is used in a variety of products; including cosmetics, industrial polishes, food products, and paper products. The United States has no domestic source of carnauba wax, and imports 100% of its carnauba wax supply. Sorghum wax has demonstrated similar physical properties to carnauba wax, and could potentially be a viable substitute for carnauba wax.

In this paper we present the first successful reversed phase HPLC method, via a C30 column, for the analysis and characterization of waxes, without the need for specialized columns or sample derivation. Sorghum wax is composed of a heterogeneous mixture of compounds, dominated by C28 and C30 saturated and unsaturated species, while carnauba is more homogeneous in nature, and composed primarily of C56-C60 saturated wax esters.

1. Introduction

Sorghum (*Sorghum bicolor* (L.) Moench) is a highly adaptive, drought tolerant crop which is primarily found in the arid climates of Africa, Asia, Australia, Central America, and the United States. Sorghum is used primarily as a food source in Asia and Africa, but in the United States sorghum grain is used primarily for livestock feed for the poultry, beef, dairy, and pork industries; and in ethanol production (Clark et al., 1981; Weller et al., 2006). Sorghum has also been investigated for its phytochemical properties, and was reported to contain several types of health-promoting antioxidants, including tannins, anthocyanins, and other phenolics (Awika and Rooney, 2004; Dlamini et al., 2007; Taylor and Duodu, 2014). The United States is the largest exporter of grain sorghum in the world, consisting of approximately 70–80% of the total domestically produced crop (US Grains Council 2015)

In addition to use as a biofuel feedstock, sorghum kernels have also been shown to contain unique oils and waxes which are being investigated for their industrial applications. (Weller et al., 2006; Hwang et al., 2002; Singh et al., 2003). Previous analysis of sorghum grain wax has revealed minimal amounts of wax esters (WE), but large amounts of fatty acids, fatty aldehydes and fatty alcohols (Bianchi et al., 1979; Avato et al., 1990). Sorghum wax has demonstrated similar physical properties to carnauba wax, and has been suggested as a domestically grown, viable substitute for carnauba. (Weller et al., 2006).

Carnauba wax is derived from the leaves of the *Copernicia prunifera* tree found exclusively in Brazil, and is composed mainly of long chain WE (80%), with the remaining 20% comprised of fatty acids, fatty alcohols and hydrocarbons (Steinle 1936; Valmalle and Karleskind, 1977; Lawrence et al., 1982). Unlike other natural waxes such as beeswax, carnauba wax contains many more branched methyl groups, and a higher percentage of double and triple-bonded carbon atoms (Basson and Reynhardt, 1988). Carnauba wax has the highest melting point of all vegetable waxes, and has been used in a variety of products, including cosmetics, polishes, food products, and the paper industry (United States Tariff

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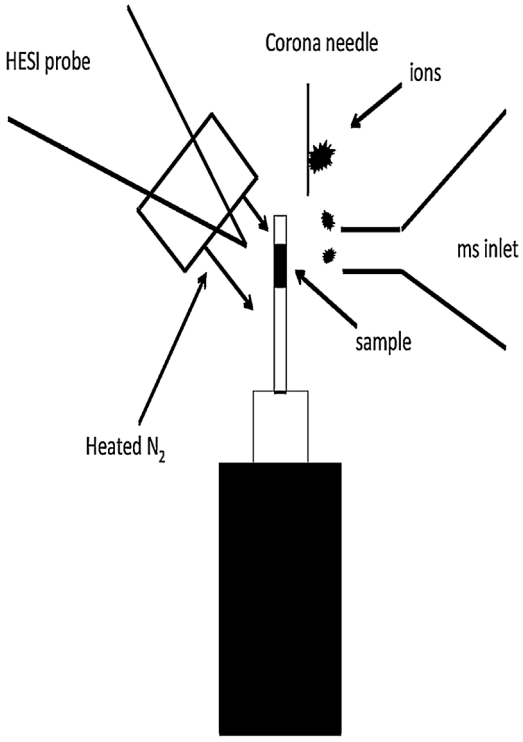

Fig. 1. Schematic of ASAP probe.

Comission, 1968; Lawrence et al., 1982). In addition, carnauba wax is used as an additive to other waxes such as beeswax to increase the melting point, allowing for increased application of these waxes (United States Tariff Comission, 1968). The bulk of carnauba wax imports come from Brazil, producing cost volatility and creating the need for a domestically grown alternative (United States Tariff Comission, 1968).

The chemical analysis of waxes is very challenging, due to a multitude of reasons, including hydrophobicity and low polarity (Hamilton 1995). Waxes have typically been analyzed by high temperature gas chromatography coupled with mass spectrometry (GC–MS), but many higher mass WE have low volatility which limits the effectiveness of GC–MS (Lawrence et al., 1982; Stránský et al., 2006; Urbanová et al., 2012). The hydrophobic nature of waxes, lack of chromophores, and low solubility, in most organic solvents, makes analysis by high performance liquid chromatography (HPLC) challenging; however the use of specialized detectors such as an evaporative light scattering detector (ELSD) and charged aerosol detectors (CAD), and HPLC columns with specialized packing such as silver ions, has allowed for detection and quantification of some wax components (Hwang et al., 2002; Adlof and List 2004; Megoulas and Koupparis, 2005; Moreau et al., 2002).

A major advantage that HPLC offers is the utility of coupling to softer forms of ionization such as electrospray ionization mass spectrometry (ESI–MS) and atmospheric pressure chemical ionization mass spectrometry (APCI-MS), limiting fragmentation of the analyte. Many groups have reported LCMS wax ester analysis by these methods (Vrkoslav et al., 2011; Vrkoslav et al., 2010; Fitzgerald and Murphy, 2007).

Despite these advancements, the development of a reversed phase LCMS method for saturated WE analysis has been slow. The hydrophobic and nonpolar nature of the waxes severely limits the mobile phase solvent selection and the effectiveness of ESI for ionization and analysis, but this situation can be mitigated somewhat by using APCI with additives to achieve ionization (Vrkoslav et al., 2013). Traditional APCI-MS can be used to analyze smaller, thermally stable polar and non-polar compounds, but this method has limitations as well, as solvent effects and flow rate can limit the ionization efficiency (Rauha et al., 2001).

The atmospheric solids analysis probe (ASAP) is an APCI technique that has been widely used in drug screening and polymer analysis, as well as in raw tissue and crude oil analysis (McEwen et al., 2005; Harris et al., 2011). In ASAP (Fig. 1), the sample is placed on the tip of a melting point (mp) tube and heated gas is directed at the sample surface vaporizing volatile compounds ionized by the corona discharge needle. This allows low polarity compounds not amenable to ESI or APCI, to be ionized with a high degree of sensitivity (McEwen et al., 2005; Harris et al., 2011).

A very attractive feature of the ASAP probe is that it can be adapted to multiple mass spectrometers, including those with ultra-high mass resolution, such as the Orbitrap Exactive, enabling exact mass measurements to be determined (McEwen et al., 2005; Lloyd et al., 2009). By using the ASAP method in concert with LCMS, we were able to validate our LCMS method's effectiveness at separating and ionizing the structures of the waxes. In this paper we report a new reversed phase LCMS method for the separation and characterization of carnauba wax and sorghum wax, and correlate this data with exact mass measurements and molecular formula determinations.

2. Materials and methods

2.1. Materials

HPLC grade methanol (MEOH), chloroform ($CHCl_3$), tetrahydrofuran (THF), Methyl Tertiary Butyl Ether (MTBE), acetonitrile (ACN), and LC–MS grade formic acid were purchased from Fisher Scientific Inc. (Pittsburgh, PA). Carnauba wax was purchased from Sigma Aldrich (St. Louis, MO). Sorghum (Macia) kernels were obtained from the Center for Grain and Animal Health Research (CGAHR) USDA-ARS-PA in Manhattan, KS. Sorghum wax was extracted from intact sorghum kernels by both soxhlet extraction, as well as automated solvent extraction (Dionex). For soxhlet extraction, 100.4 g of intact, non-milled sorghum kernels were extracted with 500 ml of dichloromethane. The sorghum kernels were subjected to six washings and upon evaporation of dichloromethane 0.266 g of wax was recovered. In addition, extractions were performed in a Dionex Accelerated Solvent Extractor (ASE) Model 200 (Dionex, Sunnyvale, CA) as previously described (Moreau et al., 2003; Moreau et al., 2007).

For ASE extraction, 7 g samples of sorghum kernels were placed in 14 individual 11 cc extraction vessels (99.756 g total). Extractions were conducted at 100 °C and 1000 psi with dichloromethane. The extractor was programmed to extract each sample with 3, 7.5 ml portions of solvent, for 10 min each. The entire extract (21.5 ml) from each sample was pooled, the solvent was evaporated under a stream of N2, and the mass of residue recovered was 0.262 g. The residue was then dissolved in warm chloroform, and HPLC analyses were conducted.

Samples of carnauba wax, sorghum wax, and jojoba wax were prepared in chloroform at a concentration of 5 mg/ml. The solutions were placed in mini-vials then put in a warm water bath to facilitate the dissolution of the waxes. Upon dissolution the samples were cooled and 2 μl were injected into the LCMS. In addition, jojoba wax was used as a standard to determine the effectiveness of the method for analysis of wax esters.

2.2. Atmospheric solids analysis probe analysis

Mass spectra were acquired using a Thermo Scientific Orbitrap Exactive mass spectrometer (Bremen, Germany) using a heated