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Combined use of isotopic fingerprint and metabolomics analysis for the authentication of saw palmetto (*Serenoa repens*) extracts

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ABSTRACT

Saw palmetto (*Serenoa repens, SP) is* the most expensive oil source of the pharmaceutical and healthfood market, and its high cost and recurrent shortages have spurred the development of designer blends of fatty acids to mimic its phytochemical profile and fraudulently comply with the current authentication assays. To detect this adulteration, the combined use of isotopic fingerprint and omic analysis has been investigated, using Principal Component Analysis (PCA) to handle the complex databases generated by these techniques and to identify the possible source of the adulterants. Surprisingly, the presence of fatty acids of animal origin turned out to be widespread in commercial samples of saw palmetto oil.

1. Introduction

Saw palmetto [*Serenoa repens* (W. Bartram)Small, SP, Arecaceae] is a popular herbal supplement for the management of urinary problems associated to benign prostate hyperplasia (BPH) [1]. In sharp contrast to the global relevance of SP for the pharmaceutical and the healthfood market, the plant has a geographically narrow growing range, limited to the swampy areas of the Southeastern coast of United States [2]. Wild harvesting of the berries, in principle a sustainable process, has long been able to meet the market request, but a combination of adverse climatic conditions (heavy rains and hurricanes) during the flowering and harvesting season, and the ensuing diffusion of fungal infections, have put severe stress on the supply chain of SP, further aggravating the plague of adulteration [2]. This was already rampant because of the high manufacturing cost of the harvest, still done manually in often scarcely populated areas difficult to reach [2]. As a result, saw palmetto extracts are more expensive than any other dietary food oil, with price in the range of 170–200 US $/Kg as to March 2016 [2]. The most common adulteration of SP extracts is their replacement, or dilution, with cheaper food-based oils (canola, coconut, olive, palm, sunflower) [2,3]. This procedure is easily detected by evaluating the fatty acids profile and the concentration of specific fatty acids relative to lauric acid, the major fatty acid (> 20%) of SP [2,3]. However, the development of special designer blends of lipids that mimic the analytical profile of SP has made the fatty acid phytochemical profiling insufficient for authentication purposes [2,3], while DNA barcode, a growingly popular authentication technique, could not always be applied due to the lipid nature of SP extracts [4].

To address the issue of adulteration, an authentication method based on principal component analysis (PCA) of the proton NMR spectrum of SPE was developed, evaluating in an omics perspective the spectroscopic fingerprint of the extracts in terms not only of fatty acids but also of minor constituents like phytosterols and fatty alcohols [3]. In alternative to this phytochemical method based on the chemical signature of the plant, stable isotope ratio analysis (SIRA) has also been evaluated, capitalizing on the limited geographical distribution and the peculiar climatic conditions of the plant growing area, that is characterized by a highly dynamic water cycle [5]. Thus, the combined analysis of $\delta^{13}C$, $\delta^{2}H$ and $\delta^{18}O$ of SP extracts is able to differentiate them from various animal and plant fat sources [5]. Of particular relevance was the consistent detection of very high $\delta^{18}O$ values (from +27.2 to +40.7‰), significantly higher than those of plant oils, whose range is from +9 to +31‰, and of animal fats as well. This value of $\delta^{18}O$ is unusual, and cannot be explained solely in terms of the isotopic composition of the local meteoric water [6]. Nevertheless, it provides an interesting isotopic marker for SP. To validate the isotopic approach in a real-world setting, we have comparatively applied omic and isotopic analysis to investigate nine commercial SP extracts and thirty

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authenticated extracts of SP as reference. All the commercial samples were labelled as originating from outside the EU, where most SP processing plants are located, or outside US, where SP grow naturally, while the reference samples were from authenticated collections spanning 2009–2017.

2. Results and discussion

2.1. Isotopic fingerprinting

Isotope Ratio analysis has a tremendous potential to authenticate dietary ingredients and medicinal plant products not only in terms of natural vs synthetic- or plant vs animal origin, but also in terms of association to a precise plant species and geographical area of origin. Thus, depending on the atom analyzed, the isotopic signature of a sample can provide information on its biogenic origin (^{14}C) as well as on the nature of the soil ($^{87}Sr/^{86}Sr$ ratio) and the geo-climatic properties of the area from which it derived ($\delta^{18}O$ and $\delta^{2}H$) [6,7]. Values of $\delta^{13}C$ provide information on the mechanism of photosynthetic carbon assimilation (C3 or C4/CAM plants) and the climatic conditions of an area, and can discriminate between samples of plant- and animal origin [7].

In practice, a depletion of ^{14}C compared to the averaged current biological samples contents of this radiogenic isotope is indicative of a fossil origin, and therefore a synthetic nature, while the ratio between radiogenic (^{87}Sr) and non-radiogenic (^{86}Sr) isotopes of strontium, an element vicariant with calcium and not significantly fractionated by biological processes, is related to the nature of the soil and to agronomic practices like the use of fertilizers [6,7]. On the other hand, the depletion/enrichment on heavy atoms (^{18}O and ^{2}H) is related to hydrogeological parameters, depending mostly on the composition of the local meteoric water, with the depletion of 13C compared to atmospheric carbon dioxide largely depending on the type of photosynthetic mechanism used by a plant [6,7]. It is therefore unsurprising that IRMS is increasingly used to control high-quality plant oils to establish their authenticity as well as its terroir of origin [8–10]. Given the reliability of IRMS with plant oils, we were confident that it could also be successfully applied to the authentication of Serenoa extracts. A databases of thirty samples of SPE obtained with various solvents (supercritical carbon dioxide and ethanol) during the past decade (2009–2017) was used as reference. All these samples came from a controlled supply chain, and showed a consisted profile of stable isotopic signature, making it possible to generate an isotopic authentication fingerprint [5]. The natural origin of all the commercial samples, as well as of the authentic ones, was first confirmed by evaluating their overall contents of ^{14}C, that was not reduced in any sample (data not shown). No significant difference was also found between authentic and commercial samples in terms of isotopic strontium ratios (data not shown), a simple measurement that is therefore non-discriminating for the authentication of SPE.

The overall $\delta^{13}C$ of SPE (−30.4/000 ± 0.4) was typical of C3 plants, as expected from the wet habitat of the plant. The corresponding value was significantly lower in the commercial samples (−29.2/000 ± 0.4), and their fraudulent origin was further supported by $\delta^{18}O$ enrichment and $\delta^{2}H$ depletion data (Table 1 of Supporting information) [11]. The stable isotope ratios of C, H and O of the samples [5] was analyzed by chemometric methods, using Principal Component Analysis (PCA), an unsupervised statistical tool that represents a simple and non-parametric method to extract relevant information from complex data [12]. As Fig. 1 shows, PCA clearly set all the commercial samples outside the variance area of the authentic SP extracts. Therefore, none of the commercial samples was derived from *Serenoa*, rather being fat mixtures skillfully blended to mimic the fatty acid composition of SPE. Comparison with a database of $\delta^{18}O$ and $\delta^{2}H$ for various oil sources, suggested that the commercial products analyzed were mostly not plant-derived, but rather of animal origin, possibly obtained from the

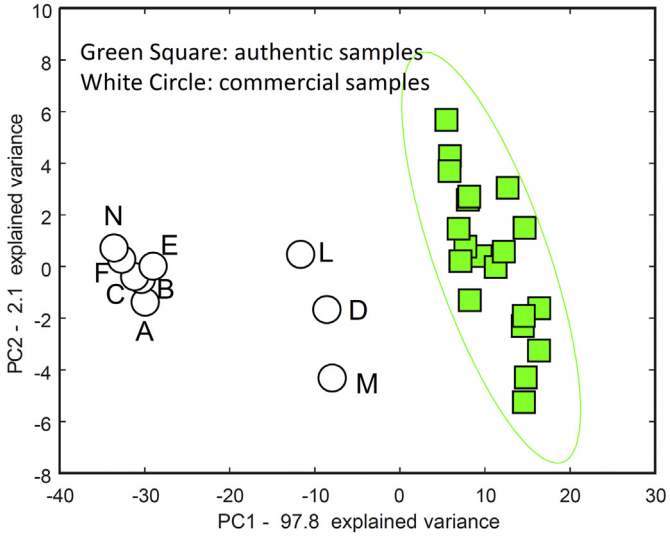

Fig. 1. PCA of the $\delta^{18}O$ and $\delta^{2}H$ data for the authentic extracts (SPE) and the commercial samples (A-F, L-N).

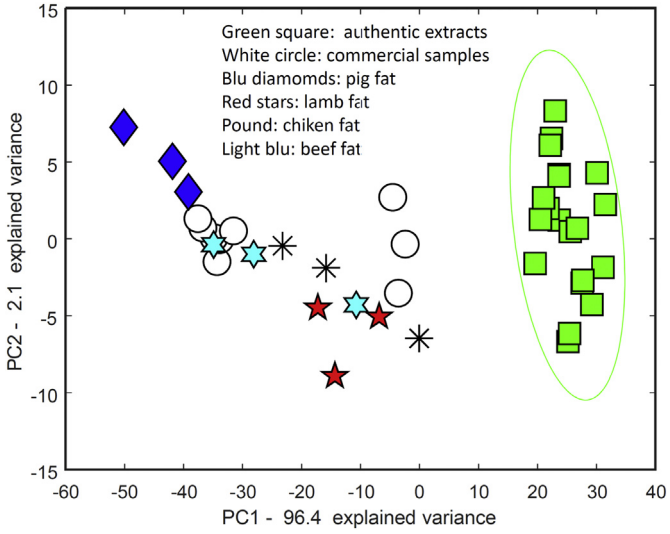

Fig. 2. PCA of the $\delta^{18}O$ and $\delta^{2}H$ data for SP extracts and a series of fat sources (blue = pig fat, light blue = chicken fat, red = lamb fat). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

combination of pig fats with fats from chicken and lamb (Fig. 2).

2. Chromatographic and spectroscopic fingerprint.

Analysis of the fatty acids profile, of the contents of lauric acid (not < 20%), and of the ratio between the concentration of the major fatty acids and the one of lauric acid was done by GLC according to the USP/NF method [13]. As a results, three commercial samples did not comply with the USP/NF fatty acids ratios for SP, while the other seven commercial samples as well as all the references complied with the specifications (Supporting information, Tables 4 and 5). The ratios were also elaborated by PCA, mean-centering each variable and then projecting in the maximum variance bi-dimensional space the data for the two components giving a maximum contribution to total variance (Fig. 3). Three samples (L, D, M) were clearly located outside the multivariate space of the authentic SP extracts, supporting the view that they were adulterated.

PCA was then applied to identify the possible adulterant(s) contained in the three samples that did not comply with the USP/NF specifications. Comparison with the fatty acid profile of a series of oils