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Effect of sample pretreatment on the extraction of lemon (*Citrus limon*) components

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ABSTRACT

A study on the key role of lemon sample pretreatment on the analytical results is here presented. The objective of the study was to analyze the differences between extracts obtained from lyophilized and air-dried samples—the most common sample pretreatment in citrus studies—in comparison to extracts from fresh samples. All the extracts were obtained with ultrasound assistance and analyzed by LC–QTOF MS/MS. The dataset, constituted by 74 tentative identified metabolites, was first evaluated by ANOVA, which showed significant differences in the concentration of 44 out of 74 metabolites ($p \leq 0.01$). Also, the pairwise mean comparison (Tukey HSD; $p \leq 0.01$) revealed that the concentration of metabolites in the extracts from fresh and air-dried samples was quite similar and differed from that in lyophilized samples. On the other hand, application of principal component analysis (PCA) showed a clear discrimination between pretreatments, explaining 86.20% of the total variability. The results of this study suggest that the main differences between extracts could be attributed to the effect of freezing or heating on metabolic pathways, and not only to thermolability of the compounds.

1. Introduction

Citrus represent one of the most important crops of fruit trees in the world, with an estimated annual production of 115.5 million tons, distributed in more than 100 countries, mainly in tropical regions [1]. These fruits are commonly recognized by their high content in vitamin C and the refreshing scent of their essential oils, but also because their great number of bioactive compounds—*e.g.* flavonoids, coumarins, phenolic acids, limonoids and vitamins—which play a key role as nutraceuticals [2]. These are the reasons why citrus components are highly appreciated for both maintenance of human health and industrial applications, mainly in the pharmaceuticals, food and cosmetics industries [3,4].

Most studies on bioactive compounds in citrus have focused on few metabolites or on a specific class of compounds. Nowadays, special attention has been paid to flavonoids thanks to their antioxidant, anti-inflammatory and radical-scavenging properties, associated to lowering the risks for different types of cancer and cardiovascular diseases [5]; however, other compounds found in citrus fruits such as coumarins have also demonstrated interesting bioactive properties like antibacterial and anti-inflammatory activity, and as hepatoprotective and anticancer agents [6–8]. The interest in these compounds has promoted research on extraction, identification, purification and on their bioactive properties.

Despite the large number of publications on the extraction of citrus components, only few of them have evaluated the effect of sample pretreatment on the characterization of this sample. Thus, sample conditioning is usually carried out by dehydration, being lyophilization and air-drying the most used methods. Lyophilization protects thermolabile compounds and minimizes exposure to oxygen, which can produce undesirable oxidation reactions; however, lyophilization can lead to volatiles losses and it is also a relatively expensive drying process. Air-drying is cheaper, but the sample is exposed to heat and air for long time intervals [9]. Most of the studies on sample pretreatment have focused on the effect of different air-drying intervals and temperatures on the extracts—particularly on phenolic compounds—and the results have been typically compared whit fresh or lyophilized samples [10,11].

In an attempt to deeper understanding the effect of sample pretreatment on the extraction of natural products, the present

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research was devoted to evaluate the effect of sample pretreatment (lyophilized, air-dried and fresh samples) on the extraction of polar compounds from citrus lemon. The resulting dataset was composed of 74 tentatively identified metabolites, which were globally, grouped in families, or individually analyzed.

2. Experimental

2.1. Samples

Edible lemons (*Citrus lemon*) were purchased in a local market in Córdoba, Spain (January, 2014). Specifications of the product: place of cultivation, Murcia, Spain; size, 53–67 mm; preservative, imazalil. The fruits (1 kg) were washed, cut in slices, lyophilized or air-dried at 45 °C to constant weight and finally grinded (particle diameter ≤ 0.5 mm). The powder was stored in the dark at -20 °C until use. Fresh lemons were ground in a blender until obtaining a homogenous sample, which was immediately subjected to extraction. The remaining moisture content in the samples after application of each dehydration method was considered negligible, and all experiments were carried out considering the sample weight on a dry basis (*i.e.* the total solid content was always the same). The initial moisture content of the sample pool was 85.6 ± 3.8 g water/100 g product (mean $\pm$ SD).

2.2. Reagents

All solvents were LC grade or higher when required. Ethanol and formic acid were from Scharlab (Barcelona, Spain); acetonitrile and methanol from Fluka (Buches, Switzerland). Deionized water (18 MΩ cm) from a Millipore Milli-Q water purification system (Bedford, MA, USA) was used to prepare the mobile chromatographic phases and extractant mixtures.

2.3. Apparatus and instruments

The ultrasound assisted extracts were obtained by a Branson 450 digital sonifier (20 kHz, 450 W) equipped with a cylindrical titanium-alloy probe (12.70 mm diameter). The analytical equipment consisted of an Agilent 1200 series LC coupled to an electrospray ionization source and a quadrupole-time of flight detector 6540 Agilent QTOF (LC–QTOF MS/MS).

2.4. Metabolites extraction

Lemon samples (1 g dry weight each) were extracted in duplicate by 20 mL of 53% of ethanol in water for 5 min with ultrasound assistance (70% amplitude and 0.9 s/s duty cycle). The extraction method was previously developed by the authors using a desirability model to maximize the concentration of ten major compounds in the extracts from lemon [12].

2.5. LC–QTOF MS/MS analysis

Chromatographic separation of the extract components was performed by using an Inertsil ODS-2 C18 analytical column (250×4.6 mm i.d. 5 μm particle) from Análisis Vínicos (Tomelloso, Ciudad Real, Spain). The injection volume was 10 μL, and the mobile phases were 0.1% of formic acid in deionized water (phase A) and acetonitrile (phase B) at a constant flow rate of 1 mL/min. The gradient was as follows: 4% to 10% B in 5 min; change from 10% to 25% B in 30 min; from 25% to 100% B in 15 min and constant 100% B for 5 min more. After analysis, the column was equilibrated to the initial conditions within 5 min. The dual ESI source operated in both positive and negative ionization modes under the following conditions: nebulizer gas at 40 psi, drying gas flow rate and temperature at 12 L/min and 325 °C, respectively. The capillary voltage was set at 3500 V, while the fragmentor, skimmer, and octapole voltages were fixed at 130, 65, and 750 V, respectively. The data were acquired in centroid mode in the extended dynamic range (2 GHz). Full scan was carried out at 6 spectra/s within the *m/z* range of 40–1700, with subsequent activation of the three most intense precursor ions (allowed charge: single or double) by MS/MS using a collision energy of 20 eV and 40 eV at 3 spectra s^{-1} within the *m/z* range 30–1700. An active exclusion window was programmed after the first spectrum and released after 0.75 min to avoid repetitive fragmentation of the most intense precursor ions, thus increasing the detection coverage. To assure the desired mass accuracy of recorded ions, continuous internal calibration was performed during analyses with the use of signals at *m/z* 121.0509 (protonated purine) and *m/z* 922.0098 [protonated hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazine or HP-921] in the positive ion mode; and *m/z* 112.9856 (trifluoroacetic acid anion) and *m/z* 1033.9881 (HP-921) in the negative ion mode.

2.6. Data processing

MassHunter Workstation software (version B6.00 Profinder, Agilent Technologies, Santa Clara, CA, USA) was used to process the data obtained by LC–QTOF in auto MS/MS mode. Treatment of the raw data file started by extraction of potential molecular features (MFs) with the applicable algorithm included in the software. The recursive extraction algorithm considered all ions exceeding 5000 and 10000 counts as cut-off in both positive and negative modes, respectively. Additionally, the isotopic distribution to consider a molecular feature as valid should be defined by two or more ions (with a peak spacing tolerance of 0.0025 *m/z*, plus 10.0 ppm in mass accuracy). Apart from $[M+H]^+$ and $[M-H]^-$ ions, adducts formation in the positive (Na^+) and negative ionization ($HCOO^-$, Cl^-) modes, as well as neutral loss by dehydration were included to identify features corresponding to the same potential metabolite. Thus, ions with identical elution profiles and related *m/z* values (representing different adducts or isotopes of the same compound) were extracted as entities characterized by their retention time (RT), intensity in the apex of the chromatographic peaks and accurate mass. Background contribution was removed by subtraction of MFs linked to the blank. Then, the recursion step assured correct integration of the entities in all analyses. Raw data files containing the area for each entity characterized by *m/z* and RT were created in compound exchange format (.cef files) for each analysis and exported into the Mass Profiler Professional (MPP) software package (version 2.0, Agilent Technologies, Santa Clara, CA, USA) for further processing. Normalization by logarithmic transformation (log2) was used as preprocessing step. Statistical analysis included the ANOVA test applied to find the number of significant flavonoids ($p \leq 0.01$), and pairwise combinations (Tukey HSD) to identify equal concentration of flavonoids between extraction methods. Also, unsupervised analysis by Principal Component Analysis (PCA) was used to find out the main source of variability in the data set and detect clusters.

Once all MFs were extracted and aligned, the software MassHunter Qualitative was used for the targeted extraction of MS/MS information associated to the monitored MFs in the whole set of analyses. This information was used for tentative identification of metabolites by searching in the METLIN MS and MS/MS (http://metlin.scripps.edu), MassBank MS/MS (http://www.massbank.jp) and ReSpect MS/MS (http://spectra.psc.riken.jp) databases.