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Investigating the performance of *in situ* quantitative nuclear magnetic resonance analysis and applying the method to determine the distribution of saccharides in various parts of carrot roots (*Daucus carota* L.)

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ABSTRACT

In order to explore the performance of the analytical method called *in situ* quantitative nuclear magnetic resonance spectroscopy – *is* q NMR – the distribution of glucose, fructose and sucrose in various parts of a carrot root (*Daucus carota* L.) – primary xylem, secondary xylem, phloem, cortex; top part and lower part – was determined. The influence on the quality of spectra of drying samples before analysis was studied, as well as the influence of the length of strips of tissue used in analysis. Finally samples as small as 240 mm^3 could be studied directly, with minimum prior treatment (only drying), along with deuterated water for locking and a sealed capillary tube containing a solution of 0.5% of the sodium salt of (trimethylsilyl)propionic-2,2,3,3-d4 acid, used both as an internal reference and for quantification. With optimized parameters, the coefficients of variation for measurements were observed to have an average value of 0.038, with a standard deviation of 0.047.

1. Introduction

During food preparation ("cooking") or food consumption, food ingredients such as plant or animal tissues exchange "bio-active" compounds – *i.e.* compounds with sensory or nutritional receptors for them existing in the human body, bc – with their environment [1]. In order to investigate the phenomena occurring during food preparation, it is useful not only to be able to determine quantitatively the bc content inside food ingredients before and after "culinary" (for example, thermal) treatments, but also to validate the observed changes with a determination of the quantity of bc released in the environment [2]. The latter determination is straightforward, but for the first one, most analytical methods involve extraction steps, which use important masses of samples [3], and this impedes comparisons. Changes in bioactivity during processes such as thermal treatment of plant tissues were studied, but with no validation of measurements performed solely in the liquid phase, which means that only assumptions could be made about possible molecular modifications during processing.

Some authors investigated the compositional changes of plant tissues such as the roots of *Daucus carota* L. ("carrots") or the bulbs of *Allium cepa* L. (onions), because such tissues are widely consumed by human populations, being important food ingredients both for home culinary production or in the food industry [4,5]. These plant tissues are storage systems having complex micro-structure. Their main bc are saccharides and amino acids [6–8]. In carrots for example, the main water soluble compounds are water, pectins, saccharides, amino acids and organic acids, some of these being in a liquid phase, such as inside the cytosol compartments of the parenchyma tissue [9]. Sucrose (Suc), glucose (Glc) and fructose (Fru) are considered to be the common saccharides of carrot roots. In fresh carrot roots, the total saccharide content ranges from 3.46 to 10.74% [10–12]. According to Rodríguez-Sevilla et al. [13], in raw carrots purchased at local market, Suc was the major saccharide and represented 56.9% of total saccharides, followed by Glc (24.6%) and Fru (18.5%).

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Moreover a number of investigations indicated that the concentrations in free saccharides vary considerably among carrot varieties and are influenced by environmental, farming, and storage conditions [14]. The time-course and spatial distribution of saccharides and ions in carrot roots was studied at cell resolution by Korolev et al. [15] by microfluorometric enzymatic assay [16,17], but with high standard deviation. Many chromatographic methods such as GC/MS, LC/UV and LC/MS have also been used recently for metabolomic studies [18–21], but they ask for a long preparation of samples.

Quantitative ^{1}H NMR (q ^{1}H NMR) spectroscopy can be useful for the fast quantification, in a single analysis, of the major saccharides [22,23,18], organic acids, amino acids and phenolic compounds, giving metabolic profiles of complex mixtures [24]. NMR methods have often less sensitivity than chromatographic ones [25–27], but q ^{1}H NMR allows the detection of most proton-containing metabolites above a minimum threshold level [28], detecting a broad range of metabolites in a non-targeted way. Such ^{1}H NMR chemical fingerprinting has been widely developed for biomedical applications [29–31] but also for food analysis and authentication [32,23,33], for the study of the biochemical mode of action of herbicides [34], or for the study of compositional changes during fruit ripening [25] among many applications. For example, in tomato, q ^{1}H NMR spectroscopy, coupled or not with liquid chromatography, was used for cultivar comparison [35], for the demonstration of substantial equivalence of transgenic plants [36] and for the detection of unintended effects following a genetic modification [37].

The preparation of extracts in view of analysis is a difficult question. In many former studies, bc compounds from plant tissues were extracted before analysis. For saccharides, an optimized method for the determination of the saccharide content of plant tissues was devised by Davis et al. [38] after O'Donoghue [3] and others [39]. In this "modified O'Donoghue" (MOD) method, plant tissues are lyophilised, heated under reflux in a mixture of methanol and water (62.5:37.5, w-w) for 15 min at 55 °C; after filtration, solvent evaporation and lyophilization, the resulting product is analyzed using various analytical techniques, among which q ^{1}H NMR [5,40].

However, analyses done by using this MOD method are long, destructive and they need hazardous manipulations with toxic solvent. Moreover This et al. [41] showed that the results obtained can be statistically different from results obtained by *in situ* quantitative ^{1}H NMR (*is* q NMR) methods, where plant tissues are directly analyzed by liquid high resolution NMR in the frequency domain [42]. Such methods are based on the fact that the water content of plant tissues is high. For carrot roots, it is about 88% of fresh weight [43]: in these tissues, as in most plant tissues, the cytoplasm of plant cells can be considered to be a jellified system [26], where metabolites and ions are in an aqueous, liquid environment that can be described by the DSF formula $D_0(W)/D_3(S)$ [1,31]. Indeed, in the cytoplasm, the cytoskeleton forms a network which includes an aqueous solution of metabolites (cytosol) where organelles are dispersed [44]. Moreover, some free aqueous solution makes up the sap which fills the vessels called xylem and phloem; saccharides are dissolved in the elaborated sap [9]. This observation was at the foundation of low magnetic field (time-domain NMR) NMR spectroscopy applied to whole plant tissues, and indeed carrot tissues were formerly studied by time domain NMR spectroscopy [45–47], but these studies intended to determine the hydration state of compounds rather than the concentration of metabolites.

On the other hand, gels such as silica gel were analyzed by high-field (frequency domain) NMR spectroscopy [48]. In previous studies, frequency-domain NMR analysis were performed in order to compare the quantity of saccharides extracted using the MOD method and the quantity of saccharides as determined by the *in situ* method, where non-treated samples of plant tissues are simply analyzed after being introduced in a 5 mm glass NMR tube with enough D_2O for locking.

In other tests, the plant samples were put in the NMR tubes along with a sealed capillary tube containing enough D_2O for locking and a reference compound such as the sodium salt of (trimethylsilyl)propionic-2,2,3,3-d4 acid (TSP), this tube being formerly calibrated using a precisely known solution of potassium phtalate; TSP dissolved in D_2O was used for chemical shift calibration and as a standard. In this way, the analysis of the content of plant tissues (for compounds in the liquid phase) is fast, as there is no preparation, except some drying of samples. Indeed the beneficial effect of this drying was observed but not determined quantitatively, and it was shown that the quantities of saccharides determined by the *is* q NMR methods are higher than with extraction. The study then revealed that the *is* q method is also useful because it is more environmentally friendly than previously used methods, as it does not require the use D_2O nor organic solvents.

One goal of this new work was to investigate the performance of the *is* q NMR method, while applying it to the analysis of the distribution of saccharides inside plant tissues. The aim was to determine the minimum quantity of plant tissue necessary for the quantitative determination of saccharides. It was considered a secondary goal to study the influence of the duration of drying of samples before NMR acquisition. Finally a question was to know whether the distribution of saccharides in a carrot root could be determined.

2. Material and methods

2.1. Chemicals

D_2O (99.9%) and 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid, sodium salt (TSP; 98%) were from Aldrich (Steinheim, Germany). D(+)- Glucose was from Prolabo, Sucrose from Merck, D(−)-Fructose, minimum 99% from Sigma. The purity of all chemicals was checked by q ^{1}H NMR. All samples, hardware and solutions, at each step of the analytical process, were weighed three times, using a 0.1 mg precision balance (Mettler Toledo AG 153).

2.2. Sample preparation for the calibration curves

Because the main saccharides contained in carrot roots are Suc, Glc, Fru, calibration curves were made for each saccharide as all of them relax differently [49]. Calibrations curves were also made using a NMR analysis of a mixture of the three saccharides with different proportions in order to allow for possible interactions [50]. The solvent used to make those solutions was D_2O. For a better dissolution of the saccharides in D_2O, solutions were sonicated during 15 min without heating. Some of the solutions were made several times in order to validate the results. During this step, it was measured that a gradient of concentration exists in a saccharide solution [51] and that the viscosity can affect NMR spectra [52]. These two factors were taken into account for analysis of saccharides in carrot roots. The NMR uncertainties have been estimated differently for the various studied saccharides.

2.3. Sample preparation for the study of carrots

Carrots (variety "Nantaise améliorée", produced in France and Spain, cat 1, season 2011) were obtained from a local supermarket on the day of each experiment. The samples were prepared as