

# Using one-dimensional (1D) and two-dimensional (2D) quantitative proton ( $^1\text{H}$ ) nuclear magnetic resonance spectroscopy (q NMR) for the identification and quantification of taste compounds in raw onion (*Allium cepa* L.) bulbs and in aqueous solutions where onion tissues are soaked

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**Abstract** Solutions obtained by soaking onion (*Allium cepa* L.) bulbs samples in water are frequently consumed, either directly or as part of dishes, both at home or in the food industry. However, little information is available regarding the extracted metabolites and the extraction mechanisms. In this article, the composition of raw onion extracts and of aqueous solutions where raw onion tissues were soaked was investigated directly by quantitative proton nuclear magnetic resonance spectroscopy (q  $^1\text{H}$  NMR). The assignment of NMR signals was performed, with less than 3% (in area) of unidentified peaks. Analyses of one-dimensional  $^1\text{H}$  NMR spectra with additional two-dimensional NMR studies showed 20 regions of interest where 3 saccharides, 17 amino acids, and 5 organic acids were detected and quantified. Resonance assignment with chemical shift was done for each saccharide, as well as for each amino acid and organic acid, with additional work on spin–spin coupling pattern and on observed and not observed correlations from correlation spectroscopy studies.

Quantification of saccharides was performed and qualified by works on peak decomposition algorithms. Complementary studies by high-performance liquid chromatography, mass spectroscopy and tandem mass spectroscopy, and thin layer chromatography and preparative layer chromatography were carried out in order to validate the NMR results on identification.

**Keywords** Taste · Quantitative  $^1\text{H}$  NMR · *Allium cepa* L. · Saccharides · Amino acids · Organic acids

## Introduction

Most human food is based on plant tissues, animal tissues and liquids prepared from them, including aqueous solutions obtained by thermal processing of plant or animal tissues in water (“stocks”, “sauces”...). Of course, food is consumed for energy, but the selection of food products is also based on flavor, i.e., the synthetic sensation including taste as one component [1]. Molecules contribute to taste by linking to taste receptors from cells in taste buds [2] but before reaching taste buds, they have to dissolve in water, as they must first migrate from food to saliva.

Onions (*Allium cepa* L.) being among the most widely distributed Liliaceous plants in the world [3] and one of the main vegetables consumed in Europe [4], their content in taste compounds was investigated by many authors [3, 5–9]. They are used either directly as vegetable, or as a seasoning for a wide variety of food preparations [10]. In both cases, only the bulbous roots (also called the bulb) are used [11].

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Among water-soluble molecules from plant tissues, saccharides, amino acids, and organic acids are the most abundant [12]. Onion bulbs are also rich in flavonols and flavonol glycosides (mainly quercetin and kaempferol) that contribute to their significant antioxidant properties [13, 14]. Being storage organs, saccharides account for a major part of their dry matter, contributing to as much as 64 to 80 % of the mass of dry matter [15]. Non-structural saccharides are mainly glucose (Glc), fructose (Fru), sucrose (Suc), and fructo-oligosaccharides (FOS), i.e., fructosyl polymers (GF<sub>x</sub>) with degrees of polymerization *x* up to 12 [8, 16–19], the main FOS being GF<sub>2</sub> and GF<sub>3</sub>. Indeed, it was determined that 1-kestose (1<sup>F</sup>-β-D-fructofuranosyl (2*R*,3*R*,4*S*,5*S*,6*R*)-2-((2*S*,3*S*,4*S*,5*R*)-3,4-dihydroxy-2,5-bis(hydroxymethyl)tetrahydrofuran-2-yloxy)-6-(hydroxymethyl)tetrahydro-2H-pyran-3,4,5-triol), Kes, one GF<sub>2</sub>), neokestose (6*G*-β-D-fructofuranosyl (2*R*,3*R*,4*S*,5*S*,6*R*)-2-((2*S*,3*S*,4*S*,5*R*)-3,4-dihydroxy-2,5-bis(hydroxymethyl)tetrahydrofuran-2-yloxy)-6-(hydroxymethyl)tetrahydro-2H-pyran-3,4,5-triol, one GF<sub>2</sub>), nystose (1<sup>F</sup>(1-β-D-fructofuranosyl)<sub>2</sub> (2*R*,3*R*,4*S*,5*S*,6*R*)-2-((2*S*,3*S*,4*S*,5*R*)-3,4-dihydroxy-2,5-bis(hydroxymethyl)tetrahydrofuran-2-yloxy)-6-(hydroxymethyl)tetrahydro-2H-pyran-3,4,5-triol), Nys, one GF<sub>3</sub>), and total fructans represent 0.33, 0.99, 0.16, and 3.43 g/100 g of fresh tissue, respectively [3]. However, the non-structural saccharide composition vary significantly among different cultivars: the mean concentration of fructans was found to be equal to 49.1 and 6.9 mg/g of fresh weight for Pukekohe Longkeeper and Houston Grano varieties, respectively [6].

The free amino acid content in onion tissues varies also considerably in onion bulbs [19], arginine and glutamic acid being the most abundant, with 0.56 and 0.30 g/100 g of fresh tissue for arginine and glutamic acid, respectively; threonine can also be detected in significant amounts [20].

Many analytical methods [21–23] can detect saccharides, amino acids, and other taste compounds, but some of these methods allow only indirect identification (retention factor *R<sub>f</sub>* in chromatographic methods), and this is why they are frequently associated with identification methods such as mass spectrometry (MS). Another interesting method for identification of water-soluble compounds is high-resolution nuclear magnetic resonance (NMR) spectroscopy [24], as all compounds are detected in one single analysis with minimal sample preparation. It is a powerful, non-destructive, and selective tool for plant metabolite analysis [25].

For example, identification of synthetic and onion flavonoids through their silylation and <sup>29</sup>Si NMR spectroscopy—line assignment through <sup>29</sup>Si–<sup>13</sup>C spin–spin couplings was evaluated [26]. Also, one-dimensional (1D) NMR spectroscopy has been used extensively to profile metabolites in mango [27], lettuce leaves [28], tomato [29], tomato juice [30], beer [31], wine

[32], carrot stocks [12]. However, <sup>1</sup>H NMR spectra of extracts of plants often contain overlapping signals of some of the metabolites present in the solution. Two-dimensional (2D) NMR spectroscopy is an easy complementary method for the analysis of the components with overlapped peaks and for validation of the results of the 1D NMR studies [22].

In our general framework of the development of fast analytical methods of complex systems, we wanted to use both techniques in order to see if it was possible to identify and quantify simultaneously the major taste compounds in crushed raw onion and also in aqueous solutions where onion bulb dice were soaked, as is common practice in kitchens [33]. In order to validate our results, comparisons were performed with others techniques such as high-performance liquid chromatography (HPLC), mass spectrometry (MS and MS/MS), and thin layer chromatography (TLC and PLC).

## Materials and methods

All products, samples and hardware used were precisely (± 0.0001 g) weighed at each analytical step. All experiments were replicated three times.

### Chemicals for NMR studies

D<sub>2</sub>O: deuterium oxide, 99.9 atom % D, *Sigma Aldrich*. DCl: deuterium chloride 20 % in deuterium oxide, isotope enrichment of 100 %, *d*=1.189, *SDS*. Sucrose, for biochemistry and microbiology, *Merck*. Glucose: D(+)-glucose, anhydrous for biochemistry, *Merck*. Fructose: D(–)-fructose, *Sigma Aldrich*. 1-Kestose (> 98.0 %), *Fluka*. Nystose (> 98.0 %), *Fluka*. TSP: 3-(Trimethylsilyl) propionic-2,2,3,3-d<sub>4</sub> acid, sodium salt, 98 atom % D, *Sigma Aldrich*. NaN<sub>3</sub>: sodium azide, *Janssen Chimica*, potassium hydrogen phthalate, A.C.S. primary standard, 99.95–100.05 %, *Sigma Aldrich*.

### Preparation of soaking solutions

Onion bulbs (Armstrong variety) were grown in Poland in 2007 (no data on weather available) and stored until experiments at 6 °C (for 6 months). After cutting about 1 cm of the top and the bottom of bulbs due to their different chemical composition [3, 4] and also to reproduce culinary processes [33], the first one or two dry and brown scales, known as papery scales, were removed. In order to ensure that homogeneous material was compared, onion bulbs were cut lengthwise into two halves; one was kept for the experiment on ground raw onion (experiment A), the other was kept for the study on soaking of raw onion dice

in aqueous solution (experiment B); any possible sprouts were removed.

For each experiment, halves were cut into parallelepipeds (1 cm × 1 cm × thickness of the scale). Samples were frozen in liquid nitrogen and ground using a mortar and pestle. B parallelepipeds were introduced in a 500 mL glass flask with a mass of MilliQ water equal to 3 times the total mass of onion samples and 0.1 % (w/w) sodium azide (to inhibit bacterial growth [34]). The soaking solution was magnetically stirred at room temperature (around 23 °C) and about 25 g of it was sampled after 1 day of soaking. The paste of experiment A and the sample of experiment B were centrifuged (10,000 rpm for 10 min at 20 °C); then the liquid part was filtered through 45 µm (6.2 cm<sup>2</sup>, surfactant-free cellulose acetate membrane and depth filter, Minisart Plus, Sartorius). The limpid liquid was frozen at -24 °C. All experiments were repeated three times.

### <sup>1</sup>H NMR

A Superconducting Ultrashield 300 MHz (7.05 T) 54 mm magnet system NMR spectrometer BZH 30/300/70 E Bruker Biospin (Germany) was used in this study. <sup>1</sup>H NMR spectra were recorded at 21.0 °C in order to avoid different mutarotation equilibria [35] for the Glc and Fru present in the sample. The analysis of each sample was performed using D<sub>2</sub>O as an internal lock. Spectra were acquired under an automation procedure (automatic shimming and automatic sample loading) requiring about 33 min per sample for 1D acquisition. Spectra were Fourier-transformed with 0.3 Hz line broadening, automatically and manually phased and baseline corrected using XWIN NMR software (Bruker Biospin, Karlsruhe, Germany).

- 1D NMR—each spectrum consisted of 64 scans of 32 k data points with a spectral width of 6172.8 Hz and an acquisition time of 5.3 s, a recycle delay of 25 s per scan and a pulse angle of 90 °, in order to allow complete relaxation and absolute quantification [36].
- 2D NMR—COSY sequence was done with 64 scans with a spectral width of 4006.410 Hz and an acquisition time of 0.25 s, and a recycle delay of 2 s.

### NMR sample preparation

The frozen solutions of each sample were freeze-dried (Lyolab A, LSL Secfroid SA, Aclens, Switzerland,—48.8 °C, 1.1 mbar) to eliminate water which may cause NMR signal saturation. Resulting solids were dissolved in D<sub>2</sub>O and freeze-dried again to eliminate residual water. About 15 mg of the resulting solids were re-dissolved in 1 g of D<sub>2</sub>O and the pH was adjusted to 7.0 using known amounts of DCl solution in D<sub>2</sub>O at different appropriate

concentrations in order to avoid chemical shift drift. About 700 µL of each sample were introduced in NMR tubes (5 mm glass, Wilmad-Aldrich, USA) and weighed. A home-made capillary tube with sodium salt of (trimethyl) propionic-2,2,3,3-d<sub>4</sub> acid (TSP) dissolved in D<sub>2</sub>O (0.2 %, w/w) was introduced inside the tube, for chemical shift calibration and for quantification [12].

### Identification

Each major sugar was identified by peak assignment of 1D <sup>1</sup>H NMR spectra using comparisons with 1D <sup>1</sup>H NMR spectra from pure compounds and with published data [12, 24, 36, 37].

As many peaks overlapped in the amino acids and organic acid region (between 1 and 3.5 ppm), identification was performed using 2D <sup>1</sup>H NMR spectra. As COSY acquisitions showed the couplings within three links, couplings between α and β positions (or between β and γ positions, etc.) were visible. Then, to identify the different amino acids and organic acids, one proton was located by the theoretical value given in former publications and other peaks were identified by correlations peaks.

### Quantification

As indicated above, TSP was used for quantification. As previously published [12], the same capillary tube of TSP was introduced in each NMR tube. As TSP has a long relaxation time (over 25 s) [38], the number of protons that are used for quantification was calculated by quantifying TSP with a known concentration solution of potassium hydrogen phthalate, which is a stable and fast relaxing molecule.

Main saccharides quantification was performed with calibration curves for each compound, as each compound relaxes differently. Calibration curves for Glc, Fru, and Suc were previously established using 1D <sup>1</sup>H NMR spectra of solutions at different concentrations of a mixture of the three saccharides in order to take into account possible interactions in complex solutions [39].

Previous studies of spectral interpretation (results not shown) showed that the best results were obtained using: the doublet at 5.24 ppm (H<sub>1α</sub>) for Glc, the multiplet at 4.10–4.11 ppm (H<sub>4β</sub>, H<sub>3β</sub>, H<sub>3α</sub>) for Fru, and the doublet at 5.42 ppm (H<sub>1</sub>) for Suc. Saccharide concentrations in each sample were calculated from concentrations in the NMR tube and soaking solution volume by quantifying the peaks of interest by peak decomposition using the *Igor Pro 5.03* software. For amino acids and organic acids, many overlapping peaks prevent the quantification of every compound, but some amino acids and organic acids concentrations were obtained by direct integration (NMR

*Notebook 2.50* software) of the corresponding peak or complex of peaks (cop).

In order to get a quantitative determination of the various compounds, standard solutions at different concentrations were prepared. For each saccharide considered (Glc, Fru, Suc), a mother solution  $S_0$  in  $D_2O$  was first prepared with a concentration of 75 mg/g. Then five solutions ( $S_1$  to  $S_5$ ) were made for calibration, each  $S_i$  solution being prepared from the solution  $S_{i-1}$  with successive dilution factors of 1/10, 4/10, 1/4, 1/5, 1/10. For each solution, the pH was set to 7 by using solutions of DCI diluted in  $D_2O$  (at different percentages). NMR tubes were loaded with 700  $\mu L$  (weighed) of the solutions. For each solution, three spectra were recorded, the full experiment and procedure being repeated entirely for each spectrum (tubes removed of the spectrometer, then the locking, shimming, and acquisition process being done again).

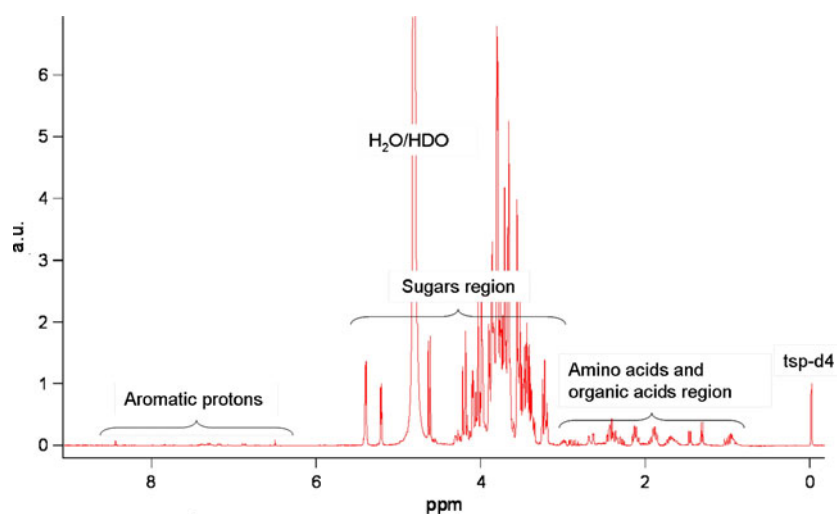
### Validation

Three techniques were used for the validation of the identification of the components (especially the main FOS, Kes and Nys) of onion extracts by NMR, such as HPLC, MS, TLC, and PLC.

### HPLC

Samples of solutions where raw onion dice were soaked were analyzed using a *Phenomenex* RHM column for monosaccharides. The solvent was MilliQ water (flow rate 600  $\mu L/min$ ), injected in the column by a pump working around 100 psi. The detector was a refractometer. Xylitol ((2*R*,3*R*,4*S*)-pentane-1,2,3,4,5-pentanol, Xyl) was chosen as internal standard as its retention time was different from those of the saccharides of interest.

**Fig. 1** Typical 1D  $^1H$  NMR spectrum of an aqueous solution where onion dice were soaked for about 1 day. The reference is the sodium salt of (trimethyl)propionic-2,2,3,3- $d_4$  acid (TSP) dissolved in  $D_2O$  (0.2%, w/w)



Standard solutions made of a mix of Glc, Fru, Suc, and Xyl were prepared at different concentrations, i.e., 5, 2, 1, 0.5, and 0.1 mg/g. Samples used were the limpid liquids obtained after centrifugation and filtration of the soaking solutions that were defrosted. These samples were diluted by 10 (w/w) after 10 min of homogenization in an ultrasonic bath.

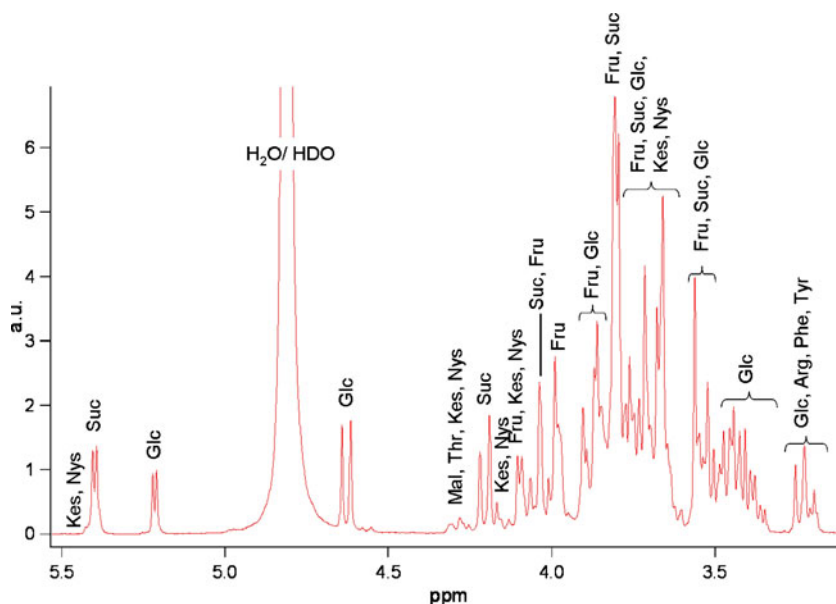
### MS

ESI-QqTOF mass spectra were obtained using a *QStar* mass spectrometer (*Applied Biosystems*, Courtaboeuf, France). Data were recorded using the *Analyst QS* software and processed with *IgorPro*. Samples were analyzed in positive ion mode. Sodium chloride (NaCl) was added to each sample in order to enhance the ionization efficiency [40]. Ten  $\mu L$  of sample were injected in a 100  $\mu L$  injection loop in the flow injection mode at rate of 20  $\mu L/min$  of solvent, i.e., methanol/water, 50:50 (v/v). Standard solutions for the identification of [Glc+Fru], Suc, GF<sub>2</sub> and GF<sub>3</sub> were established using solutions containing Glc, Suc, Kes, and Nys at 5 different concentrations, with addition of a fixed amount of an internal standard, i.e., arabinose (Ara) as its  $m/z$  ( $[Ara+Na]^+$ )=172 does not interfere with the value of the  $m/z$  of sugars. The centrifuged and filtrated samples were defrosted and appropriate dilutions with the solvent were performed, in order to get the best response and minimize matrix effects. MS/MS was used on standards (Suc, Kes, and Nys) in order to obtain reference spectra for the identification of the  $[M+Na]^+$  ions produced during the analysis of onion samples. These reference spectra were compared to MS/MS studies on onion samples.

### TLC and PLC

The TLC plates used were 20×20 cm TLC aluminum sheets precoated silica gel 60 from *Merck*. The size of the

**Fig. 2** The  $\delta$  5.5–3.2 ppm region of the  $^1\text{H}$  NMR spectrum (300 MHz, from internal d-TSP) of an aqueous solution where onion dice were soaked. This region includes peaks from main saccharides: Glc, Fru, Suc; fructo-oligosaccharides: Kes and Nys; some amino acids: arginine (Arg), phenylalanine (Phe), threonine (Thr), and tyrosine (Tyr); and malic acid/malate (Mal)



TLC sheets was  $10 \times 10$  cm. Plates were pretreated by immersion in a 0.02 M solution of sodium acetate [41]. After plates drying at  $55^\circ\text{C}$  in an oven for 5 min, samples were applied at  $1 \text{ cm} \pm 0.1 \text{ cm}$  from the edge of the plate. Each spot was performed by touching a  $20 \mu\text{L}$  capillary tube containing the sample to the plate (the tube was weighed before and after application in order to make quantitative determination of the separated products, using picture analysis of plates after development). The atmosphere of the developing chamber ( $20 \times 20$  cm vertical twin-through glass development chambers) was pre-equilibrated with the developing solvents at least 30 min before the sheets were inserted.

As saccharides are colorless and do not absorb UV radiation, plates were plunged (on the silica side) for 5 s, in a mixture of diphenylamine, aniline, and phosphoric acid in acetone [42]. Baron and Economidis described the prepa-

ration of this saccharide location reagent as a freshly made mixture of 10 mL of a solution A made of 1 g of diphenylamine, 1 g of aniline and acetone to 100 mL with 1 mL of a solution B that is phosphoric acid at 85% [43].

The PLC plates used were  $20 \times 20$  cm PLC plates silica gel 60 F254, 2 mm, from Merck. The sample was applied with a mechanical dispenser, using a syringe Hamilton  $500 \mu\text{L} \pm 10 \mu\text{L}$ . Deposition of samples was performed on a line of  $19 \pm 0.1 \text{ cm}$  at  $2.5 \pm 0.1 \text{ cm}$  from the plate edge, by emptying the syringe several times until a total deposit of  $500 \mu\text{L}$ , with a drying step at room temperature between each application. As the bands have to be revealed by the location agent, a chemical reaction that modify the molecules of interest, after the consecutive developments,  $1.5 \pm 0.1 \text{ cm}$  on the left and on the right of the plate were sprayed with the location agent, keeping the middle part of

**Table 1** Observed resonance assignments with chemical shifts and spin–spin coupling patterns (d: doublet, t: triplet, n. d.: not determined) of Glc identified in 1D  $^1\text{H}$  NMR spectrum of solutions where onions dice were soaked for about 1 day

Compound	Assignment	$^1\text{H}$ multiplicity	$^1\text{H}$ amount	$\delta^1\text{H}$ (ppm)
$\alpha$ -D-Glucopyranose	$\text{H}_{1\alpha}$	d	1	5.22
	$\text{H}_{2\alpha}$	n.d.	1	3.52
	$\text{H}_{3\alpha}$	n.d.	1	3.69
	$\text{H}_{4\alpha}$	n.d.	1	3.37
	$\text{H}_{5\alpha}$	n.d.	1	3.81
	$\text{H}'_{6\alpha}$	n.d.	1	3.75
	$\text{H}''_{6\alpha}$	n.d.	1	3.81
$\beta$ -D-Glucopyranose	$\text{H}_{1\beta}$	d	1	4.64
	$\text{H}_{2\beta}$	t	1	3.23
	$\text{H}_{3\beta}$	n.d.	1	3.48
	$\text{H}_{4\beta}$	n.d.	1	3.37
	$\text{H}_{5\beta}$	n.d.	1	3.44
	$\text{H}'_{6\beta}$	n.d.	1	3.75
	$\text{H}''_{6\beta}$	n.d.	1	3.88

**Table 2** Observed resonance assignments with chemical shifts and spin–spin coupling patterns (n.d.: not determined) of Fru identified in 1D  $^1\text{H}$  NMR spectra of solutions where onion dice were soaked for about 1 day

Compound	Assignment	$^1\text{H}$ multiplicity	$^1\text{H}$ amount	$\delta^1\text{H}$ (ppm)
$\alpha$ -D-Fructofuranose	H <sub>1<math>\alpha</math></sub>	n.d.	2	3.67
	H <sub>3<math>\alpha</math></sub>	n.d.	1	4.10
	H <sub>4<math>\alpha</math></sub>	n.d.	1	3.99
	H <sub>5<math>\alpha</math></sub>	n.d.	1	4.04
	H' <sub>6<math>\alpha</math></sub>	n.d.	1	3.79
	H <sub>6<math>\alpha</math></sub> "	n.d.	1	3.89
$\beta$ -D-Fructofuranose	H <sub>1<math>\beta</math></sub>	n.d.	2	3.54
	H <sub>3<math>\beta</math></sub> , H <sub>4<math>\beta</math></sub>	n.d.	2	4.10
	H <sub>5<math>\beta</math></sub>	n.d.	1	3.79
	H' <sub>6<math>\beta</math></sub>	n.d.	1	3.79
	H <sub>6<math>\beta</math></sub> "	n.d.	1	3.79
$\beta$ -D-Fructopyranose	H' <sub>1<math>\beta</math></sub>	n.d.	1	3.54
	H'' <sub>1<math>\beta</math></sub>	n.d.	1	3.67
	H <sub>3<math>\beta</math></sub>	n.d.	1	3.79
	H <sub>4<math>\beta</math></sub>	n.d.	1	3.89
	H <sub>5<math>\beta</math></sub>	n.d.	1	3.99
	H' <sub>6<math>\beta</math></sub>	n.d.	1	3.67
	H <sub>6<math>\beta</math></sub> "	n.d.	1	4.04

interest free from the spray with an aluminum fold. Assuming that the elution was vertical and homogenous, the “ghost” spot of each saccharide was removed by scratching. After the immersion in the location agent, bands were revealed by heating the plates for 5 min in an oven at 120 °C.

Each sample of silica (circa 2–3 g) was then crushed with a pestle in a mortar and the components were extracted by adding milliQ water (circa 100 g). Solutions were stirred for 1 h, centrifugated (10,000 rpm, 10 min, 20 °C), filtrated (Millex 33 mm, 0.45  $\mu\text{m}$ , Millipore), and frozen at –24 °C. Finally, samples were double lyophilized and analyzed by  $^1\text{H}$  NMR spectroscopy (see “ $^1\text{H}$  NMR” section).

For identification by TLC and PLC, standard solutions of Glc, Fru, Suc, Kes, and Nys were prepared at 2 mg/g in milliQ water. For PLC, more concentrated solutions were made with concentrations reaching 300 mg/g.

For TLC experiments, the limpid liquid obtained after centrifugation and filtration was defrost and analyzed straight after 10 min of homogenization in an ultrasonic bath. For PLC studies, a concentrated solution of water where onion dices were soaked during 7 h was prepared by dissolving about 150 mg of freeze-dried matter in 1 g of MilliQ water.

## Software

XWIN NMR 3.5 (Bruker Biospin, Germany) and NMRNotbook 2.60/0.1 (NMRTec SAS, Illkirch-Graffenstaden) software were used for spectrum analysis.

Igor Pro 5.0.3 (Wavemetrics Inc, Oregon, USA) and Maple 13.0 (Maplesoft, Waterloo Maple Inc, USA) software were used for data handling and peak decomposition.

## Results and discussion

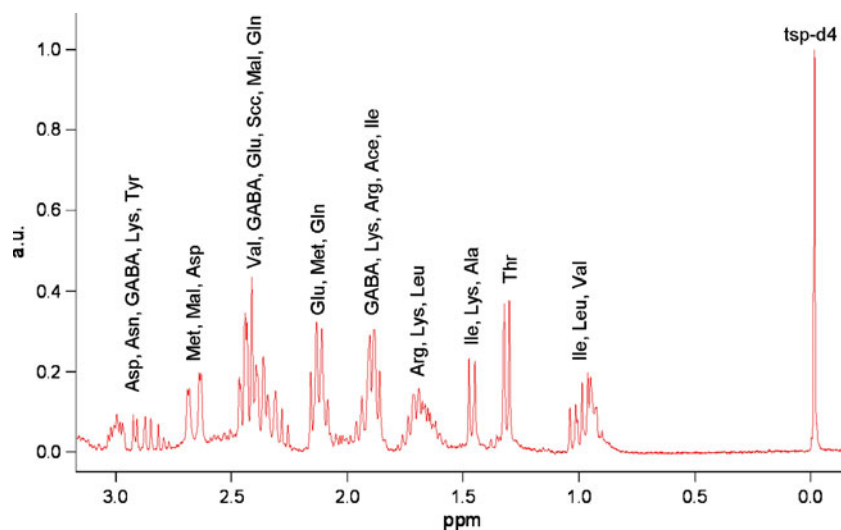
### Identification of major metabolites in raw onion and raw onion soaking solutions

All 1D  $^1\text{H}$  NMR spectra of aqueous solutions where onions tissues were soaked and of raw onion extracts have similar shapes (Fig. 1). Three regions can be recognized: the amino acids and organic acids region (0–3.2 ppm), the saccharide region (3.2–5.5 ppm) and the aromatic protons region (6.0–8.0 ppm).

**Table 3** Observed resonance assignments with chemical shifts and spin–spin coupling patterns (d: doublet, t: triplet, n.d.: not determined) of Suc identified in 1D  $^1\text{H}$  spectrum of water where onion dice were soaked for about 1 day

Compound	Assignment	$^1\text{H}$ multiplicity	$^1\text{H}$ amount	$\delta^1\text{H}$ (ppm)
$\beta$ -D-fructofuranosyl-(2→1)- $\alpha$ -D-glucopyranoside(Sucrose)	H <sub>1</sub>	d	1	5.41
	H <sub>2</sub>	n.d.	1	3.55
	H <sub>3</sub>	n.d.	1	3.74
	H <sub>4</sub>	n.d.	1	3.46
	H <sub>5</sub>	n.d.	1	3.86
	H <sub>6</sub>	n.d.	2	3.80
	H' <sub>2</sub>	n.d.	2	3.67
	H' <sub>3</sub>	d	1	4.21
	H' <sub>4</sub>	t	1	4.04
	H' <sub>5</sub>	n.d.	1	3.90
	H' <sub>6</sub>	n.d.	2	3.80

**Fig. 3** Detail of the  $\delta$  3.2–0 ppm region of the  $^1\text{H}$  NMR spectrum (300 MHz, from internal d-TSP) of water where onion dice were soaked. This region includes peaks from amino acids: alanine (*Ala*), arginine (*Arg*), aspartic acid/aspartate (*Asp*), asparagine (*Asn*), gamma-aminobutyric acid (*GABA*), glutamine (*Gln*), glutamic acid/glutamate (*Glu*), isoleucine (*Ile*), leucine (*Leu*), lysine (*Lys*), methionine (*Met*), threonine (*Thr*), tyrosine (*Tyr*) and valine (*Val*); and organic acids: acetic acid/acetate (*Ace*), malic acid/malate (*Mal*), succinic acid/succinate (*Sec*)



In the saccharide region of 1D  $^1\text{H}$  NMR spectra (Fig. 2), mono- and disaccharides could be detected easily. Glc, Fru, and Suc were identified after peak assignment (chemical shift  $\delta$  and multiplicity) using 1D  $^1\text{H}$  NMR spectra of pure compounds and correlations with published data [12, 24, 28, 30].

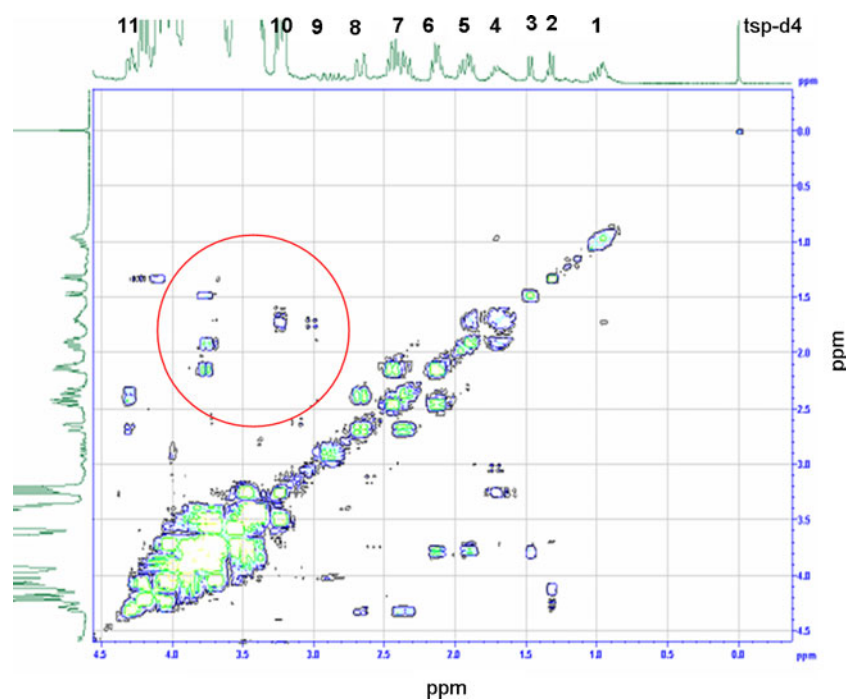
The signals from the three mono- or disaccharides were identified as some of their significant peaks are isolated in the sugar region (Tables 1, 2, and 3).

The small quantities of FOS made peak assignment more complex, but TLC and MS analyses with pure compounds and with the same samples used here showed that they are indeed present (results below, section “Validation”).  $\text{GF}_2$  and  $\text{GF}_3$  peaks could be observed in raw onion extracts and

in aqueous solutions where raw onion dice were soaked (only for extraction times longer than 24 h) but their resonances overlap with those from other saccharides [37]. The best resolution was for doublets at about 5.44 ppm and at about 4.27 ppm, corresponding to the proton  $\text{H}_1$  of the Glc unit and to the proton  $\text{H}_3'$  of the Fru unit, respectively. COSY experiments did not make the identification easier as all the correlations for saccharides were broad.

The amino acids and organic acids region of 1D  $^1\text{H}$  NMR spectra (Fig. 3) shows complex multiplicities of the peaks, leading to different cop, containing several amino acids and organic acids together. Peak assignment through 1D  $^1\text{H}$  NMR could not give an accurate determination of the possible chemical content. The unassigned peaks from

**Fig. 4** Part of the 2D  $^1\text{H}$  NMR spectrum of an aqueous solution where onion dice were soaked for about 1 day. Included in the red circle are some of the correlation peaks of amino acids/organic acids that are present in the saccharide region



**Table 4** Resonance assignments with chemical shifts and spin–spin coupling patterns of amino acids identified in 2D  $^1\text{H}$  spectrum of water where onion dice were soaked for about 1 day (a: theoretical values from [24])

Region of interest	$\delta^1\text{H}$ (ppm) <sup>a</sup>	Compound	Assignment	$^1\text{H}$ amount	$^1\text{H}$ multiplicity <sup>a</sup>	
1	0.93	Ile	H $\delta$	3	t	
	0.94	Leu	H $\delta$	3	d	
	0.96	Leu	H' $\delta$	3	d	
	0.99	Val	H $\gamma$	3	d	
	1.04	Ile	H $\gamma$	3	d	
	1.04	Val	H $\gamma$	3	d	
2	1.32	Thr	H $\gamma$	3	d	
	3	1.45	Ile	H $\gamma'$	1	m
1.45		Lys	H $\gamma$	2	m	
1.47		Ala	H $\beta$	3	d	
4	1.68	Arg	H $\gamma$	2	m	
	1.69	Lys	H $\delta$	2	m	
	1.69	Leu	H $\gamma$	1	m	
	1.72	Leu	H $\beta$	2	m	
5	1.88	GABA	H $\beta$	2	q	
	1.89	Lys	H $\beta$	2	m	
	1.92	Arg	H $\beta$	2	m	
	1.92	Ace	H $\beta$	3	s	
	1.96	Ile	H $\beta$	1	c	
	6	2.09	Glu	H $\beta$	2	dt
2.13		Met	S-CH $_3$	3	s	
2.14		Gln	H $\beta$	2	c	
2.15		Met	H $\beta$	2	m	
7		2.27	Val	H $\beta$	1	m
	2.28	GABA	H $\alpha$	2	t	
	2.34	Glu	H $\gamma$	2	dt	
	2.39	Scs	2 H $\alpha$ +2 H $\beta$	4	s	
	2.40	Mal	H $\beta$	1	dd	
	2.45	Gln	H $\gamma$	2	c	
	8	2.63	Met	H $\gamma$	2	t
		2.67	Mal	H $\beta'$	1	dd
2.68		Asp	H $\beta$	1	dd	
9		2.79	Asp	H $\beta'$	1	dd
	2.87	Asn	H $\beta$	1	dd	
	2.95	Asn	H $\beta'$	1	dd	
	3.00	GABA	H $\gamma$	2	t	
	3.01	Lys	$\epsilon\text{CH}_2$	2	t	
	3.05	Tyr	H $\beta$	1	dd	
10	3.13	Phe	H $\beta$	1	dd	
	3.15	Tyr	H $\beta'$	1	dd	
	3.23	Arg	H $\alpha$	2	t	
	3.26	Phe	H $\beta'$	1	dd	
Under saccharide peaks (red circle on the graph)	3.31	Trp	H $\beta$	1	dd	
	3.48	Trp	H $\beta'$	1	dd	
	3.58	Thr	H $\alpha$	1	d	
	3.61	Val	H $\alpha$	1	d	
	3.65	Ile	H $\alpha$	1	m	

**Table 4** (continued)

Region of interest	$\delta^1\text{H}$ (ppm) <sup>a</sup>	Compound	Assignment	$^1\text{H}$ amount	$^1\text{H}$ multiplicity <sup>a</sup>
11	3.69	Leu	H $\alpha$	1	m
	3.74	Lys	H $\alpha$	1	t
	3.75	Glu	H $\gamma$	1	t
	3.77	Gln	H $\alpha$	1	t
	3.77	Ala	H $\alpha$	1	q
	3.78	Arg	H $\alpha$	1	t
	3.85	Met	H $\alpha$	1	t
	3.89	Asp	H $\alpha$	1	dd
	3.93	Tyr	H $\alpha$	1	dd
	3.98	Phe	H $\alpha$	1	dd
	12	4.00	Asn	H $\alpha$	1
		His	H $\alpha$	1	dd
4.05		Trp	H $\alpha$	1	dd
4.25		Thr	H $\beta$	1	m
13	4.28	Mal	H $\alpha$	1	dd
	6.52	Fum	2 H $\alpha$ +2 H $\beta$	4	s
14	6.88	Tyr	ring: H $_3$ , H $_5$	2	d
	7.05	His	ring: H $_2$	1	s
15	7.18	Tyr	ring: H $_2$ , H $_6$	2	d
	7.20	Trp	ring: H $_5$	1	t
	7.28	Trp	ring: H $_6$	1	t
	7.31	Trp	ring: H $_2$	1	s
		Phe	ring: H $_4$	1	m
16	7.40	Phe	ring: H $_3$ , H $_5$	2	m
	7.54	Trp	ring: H $_7$	1	d
17	7.73	Trp	ring: H $_4$	1	d
18	7.78	His	ring: H $_4$	1	s
19	8.46	For	H	1	s

these chemical compounds were then examined by 2D COSY  $^1\text{H}$  NMR spectroscopy (Fig. 4).

Using 1D spectra, 20 regions of interest were identified, in which different amino acids and organic acids were present. 2D analyses showed that 16 alpha amino acids—including alanine (Ala), arginine (Arg), aspartic acid (Asp), asparagine (Asn), glutamic acid/ glutamate (Glu), glutamine (Gln), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), threonine (Thr), tryptophan (Trp), tyrosine (Tyr), and valine (Val)—could be distinguished, as well as gamma amino butyric acid (GABA) and 5 organic acids, including acetic acid/acetate (Ace), fumaric acid/fumarate (Fum), malic acid/malate (Mal), succinic acid/succinate (Scs) and formic acid/formate (For).

Assignments obtained by 2D correlation experiments are listed in Table 4 for each amino acid and organic acid, sorted by region of interest. In COSY experiments, depend-



**Table 5** Correlation of protons of amino acids—obs: observed, n. obs.: not observed

Amino acid	Correlation	
Asp	H <sub>β</sub> and H <sub>β'</sub>	obs.
	H <sub>α</sub> and H <sub>β</sub>	n.obs.
	H <sub>α</sub> and H <sub>β'</sub>	n.obs.
His	H <sub>α</sub> and H <sub>β</sub>	n. obs.
	H <sub>β</sub> and H <sub>β'</sub>	n. obs.
Ile	H <sub>α</sub> and H <sub>β</sub>	obs.
	H <sub>β</sub> and H <sub>γ'</sub>	obs.
	H <sub>β</sub> and H <sub>γ</sub>	n. obs.
	H <sub>β</sub> and H <sub>γ''</sub>	n. obs.
	H <sub>δ</sub> and H <sub>γ'</sub>	n. obs.
Leu	H <sub>δ</sub> and H <sub>γ''</sub>	n. obs.
	H <sub>β</sub> and H <sub>γ</sub>	obs.
	H <sub>γ</sub> and H <sub>δ</sub>	obs.
	H <sub>γ</sub> and H <sub>δ'</sub>	obs.
Met	H <sub>β</sub> and H <sub>α</sub>	n. obs.
	H <sub>β</sub> and H <sub>α</sub>	obs.
	H <sub>β</sub> and H <sub>γ</sub>	n. obs.
Phe	H <sub>α</sub> and H <sub>β</sub>	n. obs.
	H <sub>α</sub> and H <sub>β'</sub>	n. obs.
Tyr	H <sub>α</sub> and H <sub>β</sub>	n. obs.
	H <sub>α</sub> and H <sub>β'</sub>	n. obs.
Thr	H <sub>β</sub> and H <sub>γ</sub>	obs.
	H <sub>β</sub> and H <sub>α</sub>	n. obs.
Val	H <sub>γ</sub> and H <sub>γ'</sub>	obs.
	H <sub>β</sub> and H <sub>γ</sub>	obs.
	H <sub>β</sub> and H <sub>γ'</sub>	obs.
	H <sub>β</sub> and H <sub>α</sub>	n. obs.

ing on the sensitivity threshold, either all peaks and correlation peaks are visible, or some correlation peaks are missing, or peaks and correlation peaks are not detected. In this last case, the reason could be that peaks may be included within bigger peaks (from saccharides by example), or that their quantity is lower than the detection level, or that they are not present in the samples.

1D peaks and correlation peaks were assigned for Ala, Arg, Asn, Glu, Gln, and Lys (Table 4). For Asp, His, Ile, Leu, Met, Phe, Thr, Trp, Tyr, and Val, some correlation peaks were not detected (Table 5). For cysteine (Cys), glycine (Gly), proline (Pro), and serine (Ser) peaks were not identified. For Gly and Ser, the chemical shifts of the protons would have been comprised in the saccharide region and possible correlation would have been mixed with sugar peaks correlation. For Cys, the H<sub>α</sub> (4.00 ppm) would have been in the sugar region and the H<sub>β</sub> and H<sub>β'</sub> (3.05 and 3.08 ppm respectively) were not identified and for Pro, the biggest peak in γ position (the only peak corresponding to two protons) was not identified at 2.01 ppm and all the other peaks would be in the saccharide region.

Quantification of the main saccharides by 1D <sup>1</sup>H NMR spectroscopy

*Quantification of Glc, Fru, and Suc*

For quantification of Glc, Fru, and Suc we avoided peaks which could include signals from other molecules (the methodology for such quantification is discussed below in a separate paper). Indeed, as can be seen in the selected zone of Fig. 4 and with the data of Table 4, some correlation peaks for amino acids appear in the saccharide region. These correlations could be attributed to protons of 16 amino acids (Ala, Apn, Arg, Asp, Gln, Glu, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Tyr, Val).

Table 6 shows the quantities of the three major saccharides in experiments A and B.

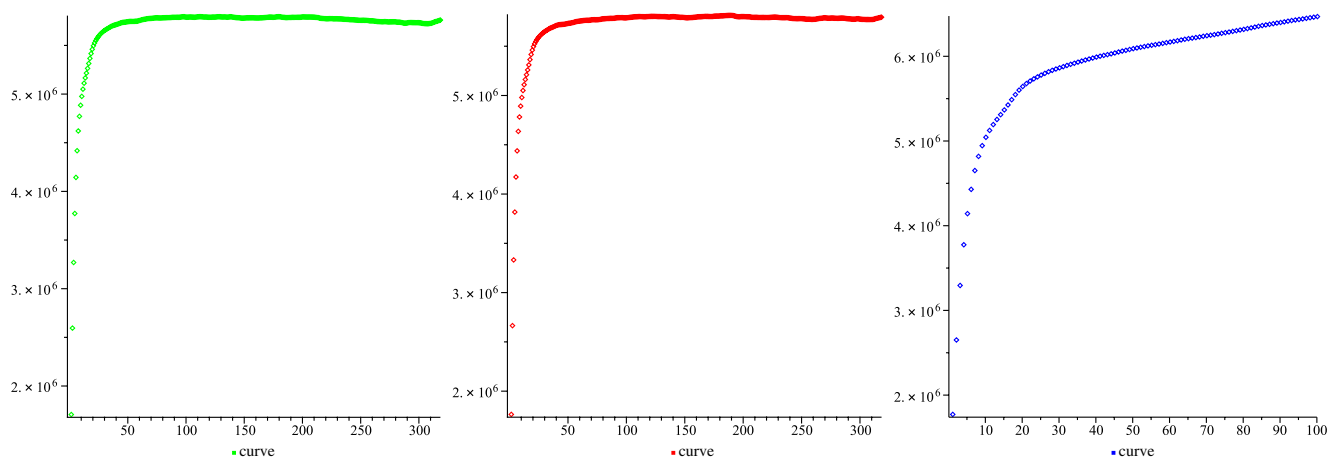
The saccharide content appears to be lower in fresh tissue than in soaking solutions. Of course, the quantification method (including extraction and sample preparation) has to be investigated further, but it should be noted that even with the simple extraction method that was used, the analysis of variance between experiment A and B showed that the Glc, Fru, and Suc contents (in mg/g of fresh weight) are not significantly different at the 10 % level.

For identified FOS (GF2 and GF3), the absolute quantification was not possible, as no single specific peak

**Table 6** Quantification of Glc, Fru, Suc by 1D <sup>1</sup>H NMR for plant tissues and aqueous solutions where these tissues were soaked for about 1 day

Compound	Experiment A		Experiment B		C.V. (%)
	Mass (mg/g f.w.)	s.d. (mg/g f.w.)	Mass (mg/g f.w.)	s.d. (mg/g f.w.)	
Glc	7.74	2.12	10.02	1.87	17
Fru	9.57	2.25	12.00	1.99	7
Suc	7.36	2.65	11.76	4.37	5

Masses (means of three experiments) are expressed in mg.g<sup>-1</sup> of fresh weight of onion, as well as their standard deviation (s.d.). Coefficients of variation (C.V.) are given by the method of quantification (NMR peaks integration by *Igor Pro* software and calibration curves)



**Fig. 5** Integral values of the TSP peak as calculated using various integration intervals, for three spectra acquired from the same D<sub>2</sub>O solution of TSP

could be integrated. However, multi-Lorentzian peak fitting showed that the area under the H<sub>1</sub> doublet of the glucose unit of GF2 or GF3 represented less than 5% of the area under the Suc peak.

#### Comparison of quantification methods

For each peak of interest, two pairs of methods were compared: direct integration, using *Igor Pro* or *NMRNotebook* integration command, integration after fitting, using either a multi-Lorentzian decomposition of a part of the spectrum and integration of the separate peaks, or a multi-Lorentzian decomposition of a part of the spectra and integration of the fitted curve. As very different results are

obtained, we first observed that the noise and baseline were responsible for differences; in these particular conditions, tangent skimming [44, 45] is inappropriate.

In order to get rid of the effect of the noise and the baseline, an automatic algorithm for finding the integral value of peaks was based on the idea that the calculated integral value  $y$  of a resonance belonging to a signal can be expressed as:

$$y = \int_{-x}^x [p(t) + b(t) + n(t)] dt$$

where  $p(t)$  is the peak,  $b(t)$  and  $n(t)$  represent respectively the baseline and the noise, and  $x$  the value of the half interval of integration around the peak maximum value (it is assumed that the noise has a null mean over an interval large enough, and that  $y$  does not change if  $x$  is chosen large enough).

Indeed for Lorentzian peaks such as NMR ones, the integration interval should be infinite, but one can easily determine the proportion lost in the so-called “dead band”: for a peak represented by the equation:

$$y = \frac{A}{x^2 + B},$$

with a noise  $h$ , the proportion of signal lost in the dead band is:

$$P = \frac{\left[ \frac{\pi A}{\sqrt{B}} - 2 \arctan \left( \frac{\sqrt{h(A-hB)}}{h\sqrt{B}} \right) \right] A \sqrt{B}}{\pi A}$$

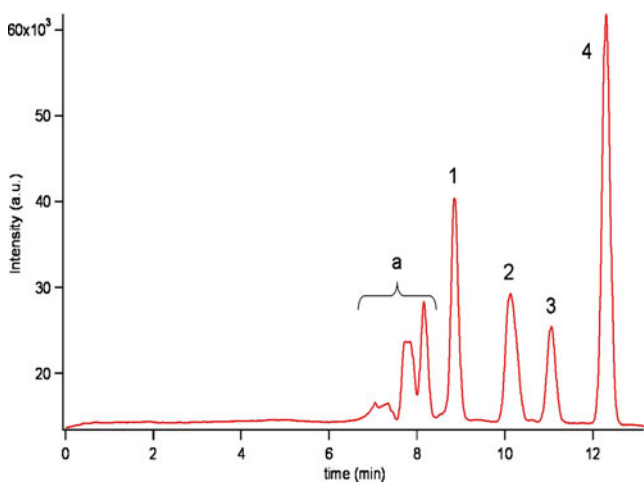
With the assumption that the noise has a constant integral value over any interval larger than a certain period  $T$  and small enough so that it does not include the peak values, one can write:

$$y = \int_{-x}^x p(t) dt + \int_{-x}^x b(t) dt + \int_{-x}^x n(t) dt = \int_{-x}^x p(t) + \frac{2x}{T} k$$

**Table 7** Comparison of amino acids (Thr, Tyr, His, Phe and Trp) content (in mg/g f.w.) in onions by Selby (1) and in experiment A (2) and B (3)

Compound	1	2		3	
		Mean	s.d.	Mean	s.d.
Thr	0.32	0.43	0.05	0.07	0.02
Tyr	0.08	0.13	0.04	0.06	0.01
His	0.05	0.10	0.04	0.05	0.01
		0.10	0.02	0.04	0.02
Phe	0.05	0.21	0.04	n.d.	n.d.
		0.19	0.04	0.11	0.03
Trp	n.d.	0.12	0.04	n.d.	n.d.
		0.15	0.04	0.07	0.02
For	-	0.05	0.01	0.01	0.01
Fum	-	0.04	0.01	0.01	0.001
Mal	-	1.24	0.13	1.31	0.29

Three organic acids are also considered: For, Fum and Mal



**Fig. 6** Chromatogram of sample of water where diced onions were soaked diluted by 10. *Peak 1* Suc, *peak 2* Glc, *peak 3* Fru, *peak 4* Xyl and cop “a”: unknown compounds that could be fructans

with:

$$\int_{-x}^{x+T} b(t)dt = k$$

for any  $z$  in the spectrum part considered,  $k$  being a constant, and, as assumed before:

$$\int_{-x}^{x+T} n(t)dt = 0$$

Then  $y$  is can be written:

$$y = \left[ \int_{-x_0}^{x_0} p(t)dt + \frac{2x_0}{T} \right] + \left[ \int_{-x}^{x_0} p(t)dt + \int_{x_0}^x p(t)dt + \frac{2(x - x_0)}{T}k \right]$$

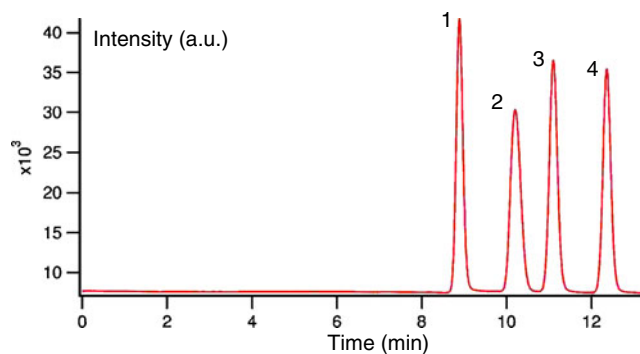
where  $x_0$  is chosen so that the peak value is becoming of the same order of magnitude as the noise.

In this case, the main influence is due to the peak in the first parenthesis and to the noise and baseline in the second parenthesis; in other words, if the noise is random and the baseline is a regular function, as seen in this case, the proposed integration leads to an increase, due to the signal, plus a constant variation (including a null variation) where the signal is low compared to the noise. The formula can be simplified as:

$$y = \int_{-x_0}^{x_0} p(t)dt + \frac{2x}{T}k$$

This is exactly what was observed in our spectra and it explains the variations of signals.

In order to find the value of  $x_0$ , a non arbitrary algorithm has to be found. Using the assumption that the variation of the integral value of the signal is low compared to the integral value of the noise+baseline after a certain value, this



**Fig. 7** Chromatogram of a standard solution made of Suc (*peak 1*), Glc (*peak 2*), Fru (*peak 3*), and Xyl (*peak 4*)

algorithm can look for “constant” evolution of the integral value. Integrating the constant variation gives the equation of a line that can be back-interpolated to the abscissa axis where the real integral value of the peak itself is directly found (interpolating back this line eliminates the second term of the first parenthesis in the equation of the previous formula).

This means that the question is to find when the integral value of the signal is linear. There are many possibilities, such as looking to the derivative of the signal and finding when it is constant. However, we tested another algorithm based on the idea that the signal is not noisy when it is integrated over an interval large enough. In the first part of the integrated signal, the intersection of the tangent to the integral curve cuts the ordinate axis at an increasing value, but when the peak integral is of the same order of magnitude as the noise, the ordinate value of this intersection stabilizes. If noise+baseline is now added, there will be an abscissa when the ordinate value decreases, before oscillating. This means that this abscissa can be considered as the beginning of a tangent line, the second extremity being chosen as when the tangent stabilizes (Fig. 5).

Using this algorithm led to a confirmation of what was known, i.e., that fitting a peak with a Lorentzian using the maximum height and the width at half height is not precise enough, because for narrow peaks a small error on the position of the abscissa used for modeling can have a large influence on the result. It also confirmed that modeling should be cautiously done, in particular because of the noise and baseline.

**Table 8** Retention times (*RT*) of Suc, Glc, Fru and Xyl in standard solutions and in sample of soaking water

Compounds	<i>RT</i> in standard solutions (min)	<i>RT</i> in samples(min)
Suc	8.92±0.04	8.87±0.05
Glc	10.23±0.04	10.16±0.05
Fru	11.13±0.04	11.07±0.05
Xyl	12.39±0.04	12.32±0.05

+TOF MS: 1.333 min from Sample 1 (01) of 080710\_H1.wiff

Max: 801.0 counts

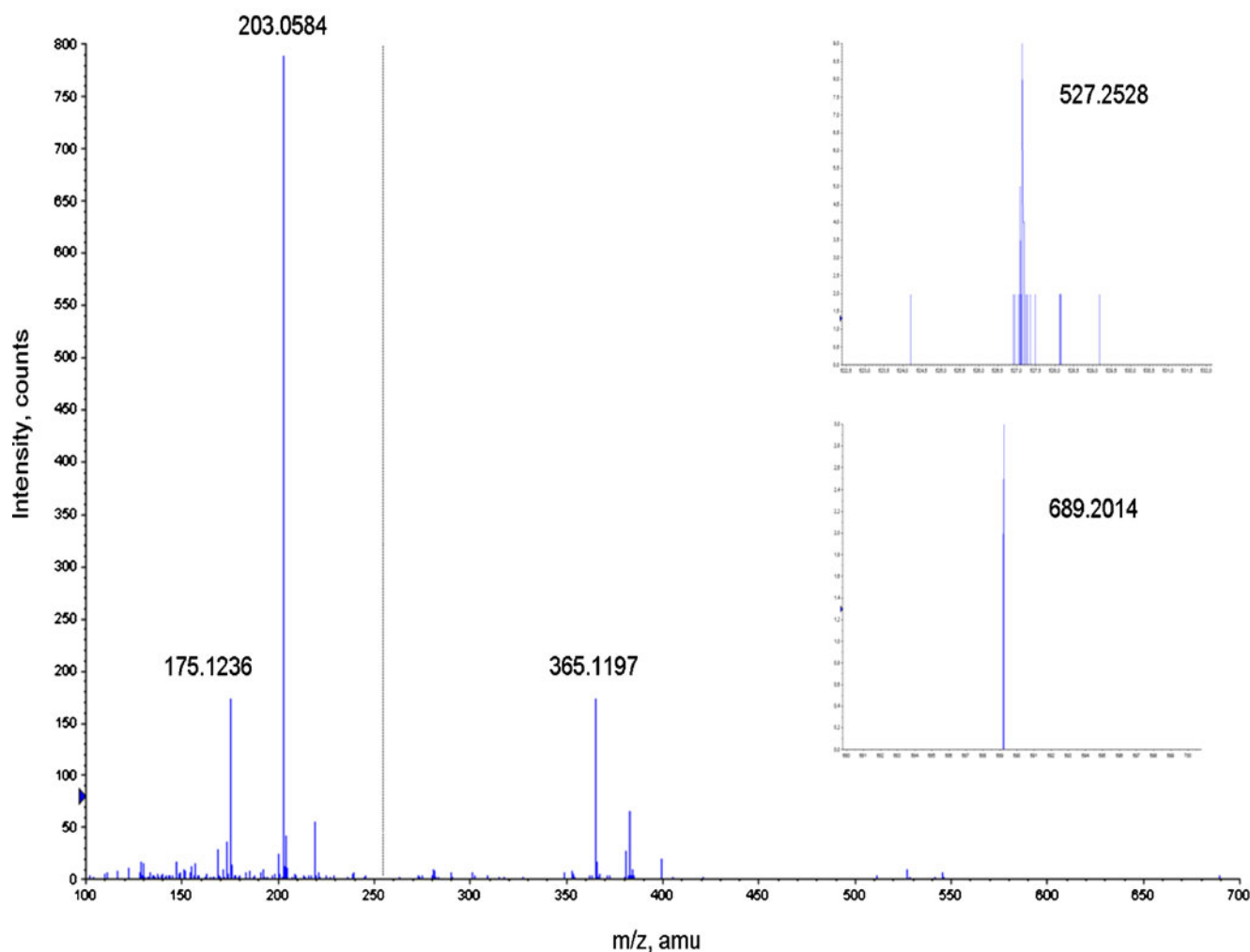


Fig. 8 Mass spectrum of onion sample at  $t_0$  of raw onion soaking

Our spectra analysis also led to calculate a coefficient of variation of 0.007. Using it, we tested our quantitative methods with a mixture of three saccharides, and calibration curves with  $R^2$  higher than 0.99 were found.

Quantification of some amino acids and organic acids by 1D  $^1\text{H}$  NMR spectroscopy

In NMR spectra, signals from amino acids and organic acids overlapped in 20 cop (Table 4). Integrating them led to 20 equations with 22 unknowns (amino acid and organic acid content). Some cop correspond to a single product, as for For, Fum, His (2 cop), Thr, Trp (2 cop) and Tyr. Some equations could also be solved independently, which allowed the quantification of Mal and Phe. Arg appears in a cop where its signal (doublet) overlapped with Glc, Phe, and Tyr (Fig. 2); as Glc, Phe and Tyr can be quantified, it was first hoped that Arg could be determined; however Glc quantification by calibration curve comprises all the isomeric forms

whereas the peak of Arg is included in the  $\text{H}_{2\beta}$  peak of Glc (Table 1). Finally, 9 irreducible equations with 14 unknowns could not be solved for quantification of other compounds. Table 7 shows the results of the quantification of For, Fum, His, Thr, Trp, and Tyr for experiment A and B, with comparison with the data of Selby [20].

The same chemical shifts were observed in this study as in previous studies with carrots stocks [12] and pure compounds [24, 28, 30]. This was expected as the pH was adjusted to the same values and the samples diluted as in previous studies. The same mono- and disaccharides as for carrots stocks could be observed. Our results confirm previous studies of onions bulbs with other analytical techniques.

For amino acids, our results were in general accordance with chromatographic analysis by Selby [20]. However, whereas Trp could not be detected by that author, we observed its signal in our spectra. On the other hand, NMR spectroscopy failed to detect Gly, and Pro in spite of published concentrations (0.076 and 0.068  $\mu\text{mol g}^{-1}$  of fresh weight,



**Fig. 9** TLC of an aqueous solution where onion dice were soaked. *G* Glc; *F* Fru; *S* Suc; *K* Kes; *N* Nys; *M* mix of *K+N*; *X* onion. Red circle “unknown” compound

respectively [20]) which could allow detection. No ethanol was identified, contrary to published data on carrot stocks [12].

Quantification of amino acids probably needs improvement of the method as, except for Mal and Thr, the quantity of amino acids in the NMR tube was calculated to be below 0.1 mg. Of course, amino acid signals could be increased using bigger amounts of lyophilisate, but saccharide cop would prevent detection of amino signals. However, for the amino acids that could be quantified, we obtained results of the same order of magnitude as Selby. Techniques for peaks suppression are to be considered.

**Validation**

*Identification by HPLC of Glc, Fru, and Suc of onion soaking samples*

Identification of Glc, Fru and Suc in aqueous solutions where onion dice were soaked (Fig. 6) was performed by comparison of retention times and retention factors ( $R_f$ ) with those obtained by the analyses of standard solutions (Fig. 7, Table 8). Other peaks (noted “a” in Fig. 6) appeared as they elute before Suc, they could be representative of FOS, for example.

*Identification by MS and MS/MS*

From standard solutions, Glc, Fru, Suc, Kes, and Nys were all detected as  $[M+Na]^+$  whereas no  $[M+H]^+$  ions were

detected. Fru was detected with the same  $m/z$  than Glc:  $m/z$  ( $[M+Na]^+$ )=203=180+23, because Glc and Fru have the same molecular weight (180 g.mol<sup>-1</sup>). The  $m/z$  value of  $[Suc+Na]^+$ ,  $[Kes+Na]^+$  and  $[Nys+Na]^+$  were, respectively, 365 (342+23), 527 (504+23) and 689 (666+23).

All MS analyses of onion samples gave similar spectra, such as shown in Fig. 8. Ions  $[M+Na]^+$  of  $m/z$  equal to 172, 203, 356, 526, and 689, were identified. They were therefore assumed to correspond to Ara (internal standard), Glc and Fru, Suc, GF<sub>2</sub> and GF<sub>3</sub>, respectively.

MS/MS analyses of ions  $[M+Na]^+$  of  $m/z$  equal to 356, 526 and 689 were also performed and results corroborated with MS/MS analyses of standard solutions of Suc, Kes, and Nys, respectively. As a conclusion, MS and MS/MS analyses not only confirmed NMR studies on the presence of monosaccharides (Glc+Fru) and Suc, but they were also helpful to identify GF<sub>2</sub> and GF<sub>3</sub>.

*Identification by TLC and PLC*

Quantitative TLC studies [46] confirmed that Glc, Fru and Suc were actually present in samples of water where onions were soaked (Fig. 9). This study also highlighted the presence of Kes and Nys, with dots having the same retention factor ( $R_f$ ) of standard solutions (Table 9).

Also, as clearly shown in Fig. 9 (circled in red), another spot between Suc and Kes spots was revealed by the sugar specific dyeing agent. In order to investigate this “unknown” compound, preparative chromatography was carried out. The silica surface corresponding to the unknown compound was scratched and analyzed by NMR spectroscopy. Surprisingly, the intensity of the NMR peaks of the unknown compound in the onion spectrum was not big enough to identify this compound through NMR spectroscopy.

MS analyses were also conducted to go further in the analysis of the unknown compound as well as MS/MS studies. The MS spectrum of the unknown compound showed the presence of Suc ( $m/z$ =365.1211) and GF<sub>2</sub> ( $m/z$ =527.1920) but no molecular peak was found with a  $m/z$  between 365 and 527. The MS/MS spectrum on the ion of  $m/z$ =365 showed similarities with the MS/MS spectrum of Suc. However, the MS/MS spectrum on the ion of  $m/z$ =527 shows a peak at  $m/z$ =365 but the relative intensities are

**Table 9** Retention factor ( $R_f$ ) of Glc, Fru, Suc, Kes and Nys

Compound	$R_f$	$\Delta R_f$
Glc	0.53	0.2
Fru	0.53	0.2
Suc	0.47	0.2
Kes	0.37	0.2
Nys	0.30	0.2

different from those of the MS/MS spectrum of Kes. Different conformations of molecule can lead to different MS/MS spectra [47]. Then, the unknown compound could have been the neoform of kestose (that is more present in onion tissues than Kes). Unfortunately, as neokestose is not a product that is commercially available, the MS/MS spectrum of this pure compound could not have been performed to test this hypothesis.

## Conclusion

In this study, complete assignment of 3 main saccharides (Glc, Fru, Suc), 17 amino acids and 5 organic acids was performed for onion tissues and for soaking solutions using 1D and 2D  $^1\text{H}$  (COSY sequences) NMR spectroscopy. These results were validated using other analytical methods, such as HPLC, MS, and MS/MS, and TLC and PLC. Quantification of Glc, Fru, Suc, as well as of some amino acids and organic acids was achieved, with sometimes large coefficients of variation due to the very small amounts of compounds. The quantification of saccharides and amino acids from raw tissues could be improved by better extractions methods. Davis [48] compared various methods, but further studies (to be published) showed that saccharides were not fully recovered by methanol extraction as proposed by O'Donoghue [6].

As a whole, this study confirms that one of the big advantages of NMR spectroscopy is the simultaneous and direct detection of all compounds of low molecular weights.

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