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Brief report

Rapid approach to determine propionic and sorbic acid contents in bread and bakery products using ^{1}H NMR spectroscopy

Marwa Scharinger [1], Marcel Kuntz [2], Andreas Scharinger [2], Jan Teipel [2], Thomas Kuballa [2], Stephan G. Walch [2] and Dirk W. Lachenmeier [2,*]

[1] Unit of Research of Plant Ecology, Faculty of Sciences, University of Tunis El-Manar II, Campus Academia, 2092 Tunis, Tunisia; marwa.scharinger@outlook.com

[2] Chemisches und Veterinäruntersuchungsamt (CVUA) Karlsruhe, D-76187 Karlsruhe; marcel.kuntz@cvuaka.bwl.de, andreas.scharinger@cvuaka.bwl.de, jan.teipel@cvuaka.bwl.de, thomas.kuballa@cvuaka.bwl.de, stephan.walch@cvuaka.bwl.de.

* Correspondence: Lachenmeier@web.de; Tel.: +49 721 926 5434

Abstract: The food additive sorbic acid is considered as an effective preservative for certain cereal products, and propionic acid is commonly added in bakery wares, e.g. bread and fine bakery wares. The aim of this study is to develop and validate a new nuclear magnetic resonance spectroscopy (^{1}H NMR) method for routine screening and quantification of sorbic and propionic acids in bread and several bakery products for quality control purposes. Results showed that none of the screened samples contained higher concentrations than regulatory maximum limits. However, for some samples, labelling of preservatives was lacking or they were used in food categories, for which the use is not approved. It can be concluded that the developed NMR method can be used for routine screening of bakery products**.**

Keywords: Bread; NMR spectroscopy; propionic acid; quality control; sorbic acid

1. Introduction

Bread has been considered as one of the most important human staple foods. The use of food additives to enhance shelf life also dates back to ancient history [1]. The WHO and FAO have defined food additives as substances 'which are added intentionally to food, generally in small quantities, to improve its appearance, flavor, texture, or storage properties' [2, 3].

It has been postulated that sorbic acid and propionic acid may be safely used as antimicrobial agents (Annex II to EC regulation number 1333/2008) [4, 5]. Both food additives may be used in prepackaged sliced bread and rye bread. In addition, its use is also allowed in pre-baked and packaged bakery products, as well as in energy-reduced bread, partially baked, prepacked bread and prepacked rolls, tortilla and pitta, boller and dansk flutes [4].

Sorbic acid (2,4-hexadienoic acid, E 200) is a straight-chain monocarboxylic acid while potassium sorbate (E202) is its potassium salt. Sorbic acid and potassium sorbate are white and crystalline powders. Potassium sorbate is soluble in alcohol and freely soluble in water while sorbic acid is less soluble in alcohol and ether and only slightly soluble in water [6].

. The safety of Propionic acid (E280) and its salts (E281-283)as a food additive has been re-evaluated in 2019 by the EFSA Panel. No safety concerns for consumers were identified [7].

In the EU, propionic acid has maximum permitted levels for use in bread and rolls ranging from 1000 to 3000 mg/kg [4]. Both the EU Scientific Committee for Food and the Joint FAO/WHO Expert Committee on Food Additives have evaluated propionic acid as food additive on several occasions in 1974, 1973 and in 1990. The latter defined the acceptable daily intake as “not limited” for propionic acid and its sodium, potassium and calcium salts [5, 8]. Nevertheless, EU legislation allows the use only in certain food categories when specified maximum levels are uphold (Table 1).

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Table 1. Maximum permitted levels (MPLs) of propionic acid/propionates (E280–283) and sorbic acid/sorbates (E200-202) in bread and bakery products according to the Annex II of Regulation (EC) No 1333/2008 – levels are expressed as free acid[4].

	Bread and bakery type	Restrictions/exceptions	Maximum level (mg/L or mg/kg as appropriate)
Propionic acid	Bread and rolls	only prepacked sliced bread and rye bread	3000
	Bread and rolls	only energy-reduced bread, partially baked prepacked bread and prepacked rolls, tortilla and pitta, prepacked polsebrod, boller and dansk flutes	2000
	Bread and rolls	only prepacked bread	1000
	Fine bakery wares	only prepacked fine bakery wares (including flour confectionery) with a water activity of more than 0.65	2000
Sorbic acid	Bread and rolls	only prepacked sliced bread and rye-bread, partially baked, prepacked bakery wares intended for retail sale and energy-reduced bread intended for retail sale	2 000
	Fine bakery wares	only with a water activity of more than 0.65	2 000

Nuclear magnetic resonance spectroscopy has been developed for routine quality control as well as the detection of harmful substances in various foodstuffs, such as, alcoholic beverages and coffee [9]. The NMR technique provides replicable data and elucidates definite fingerprints that are proven suitable for authenticity testing. Thus far, only high-performance liquid chromatography, gas chromatography and gas chromatography/mass spectrometry are the validated analytical methods for determination preservatives and food contaminants, including cereal products [10, 11, 12]. However, NMR has several advantages, such as, the small amount required for sample preparation and the reduced time of experimentation. It can be regarded as a key method for quantification of specific compounds from different food matrixes. All parameters that are fundamental to calculate a certain concentration out of the spectrum are directly provided by the NMR-experiment, the chemical identity of the molecule investigated and its quantity using an internal or external reference [13, 14].

The aim of this study was to extract sorbic and propionic acids from the complex bread matrix in a short time by automated steam distillation (less than five minutes) and to quantify them precisely and reliably using ^{1}H NMR for quality control and food surveillance purposes.

2. Materials and Methods

2.1. Chemicals

Reagents and standard compounds were of analytical or HPLC grade. Deuterated water (D_2O) was purchased from Deutero GmbH (Kastellaun, Germany). 3-(Trimethylsilyl)propion-2,2,3,3-d_4 acid, Na salt (TSP), sulphuric acid (H_2SO_4), potassium disulphate ($KHSO_4$), chemicals to prepare a buffer solution (mixture of 10 ml of D_2O, 7.5 g of monosodium phosphate (NaH_2PO_4), 1000 mg of phosphoric acid (H_3PO_4 85%) with a pH of 3.2) and propionic and sorbic acids standard solutions were all purchased from Sigma-Aldrich (Steinheim, Germany).

2.2. Sample preparation

Bread and bakery products samples were randomly selected from German markets at several occasions in 2017 and 2019 and homogenously grinded . The sample preparation is based on steam

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distillation specified in the German Official method for the determination of propionic acid in bread (ASU L. 17.00-14; 06-1987) [15]. However, automated instead of manual steam distillation is applied. To achieve this, 10 g of ground sample was filled up into a 750 ml Kjeldahl flask of the automated distillation device (Vapodest 200, Gerhardt GmbH & Co. KG, Königswinter, Germany) set at 75% of steam power. For more details on the automated steam distillation device, see Lachenmeier et al. [16].

Then, a mixture of 10 g of $KHSO_4$, 0.5 ml of sulphuric acid (7.1 M) and 3ml of D_2O were added. Each distillation into a volumetric flask prefilled with some water to encompass the outlet tube lasted four minutes, following that the volumetric flask was adjusted to 100 ml of demineralised water. After shaking of the volumetric flask, 500 µl of the distillate is pipetted into an NMR tube and mixed thereafter with 60 µl of TSP/D_2O solution (10 mg/ml), 60 µl of buffer solution.

2.3. NMR analysis at 400 MHz

All NMR measurements were performed on a Bruker-Avance 400 Ultra-shield spectrometer (Bruker Biospin, Rheinstetten, Germany) equipped with a 5-mm selective inverse probe (SEI) with Z-gradient coils, using a Bruker Automatic Sample Changer (B-ACS 60). ^{1}H NMR spectra were acquired using the noesygppr1d_d7 pulse program with a time domain (TD) of 131072, 4 dummy scans (DS), 32 scans (NS), sweep width of 20.5617 ppm (SW), the spectral width in Hertz is 8223.685 Hz (SWH), additional delay (D7) of 50 seconds and an acquisition time of 7.696 seconds. Receiver gain (RG) was set to 32 and a relaxation delay (D1) of 4 seconds. A pulse calibration for optimisation was performed before every measurement. The pulse calibration program for the optimisation of pulse lengths, the size of the processed data (SI) is of 262144, the window multiplication mode was exponential (WDW EM) and the Lorentzian broadening factor for exponential window multiplication (LB) is 0.3 Hz. The spectra were automatically phased and base-line corrected using Topspin version 3.2 and 3.5 (Bruker Biospin, Rheinstetten, Germany).

2.4. Validation

Propionic acid and sorbic acid were added to 10 g of bread. The final spiking levels were 250, 500, 1000, 2000 and 3000 mg/kg. The spiked samples were prepared as described above in section 2.2. For the determination of linearity, limits of detection, quantification and recoveries, four concentration levels ranging from 200 to 2500 mg/kg were used. Each of them was five times prepared, measured and evaluated.

2.5. Data Analysis and Quality Control

Peak areas in the ^{1}H-NMR spectra were evaluated using a MatLab script. The peak areas were set on using trapezoidal integration. Quantification was performed using the eretic factor, as previously described [9].

3. Results

3.1. Validation results

The detection limits of propionic and sorbic acids together with the concentration ranges, the expected and obtained concentrations expressed in mg/kg of bread are summarized in Table 2 and Table 3, respectively. Linearity for propionic acid was verified between 210 and 2519.7 mg/kg bread, for sorbic acid between 195.7 and 2348.1 mg/kg bread. The limits of detection and quantification were 94 mg/kg and 314 mg/kg for propionic acid and 258 mg/kg and 849 mg/kg for sorbic acid. The mean recovery rate for propionic acid is 76.1 %. For sorbic acid it is 57.5 %. Table 2 shows that there has been a loss of propionic acid during the analytical process, e.g. during the extraction and measurement steps. Table 3 shows that admixtures 1 to 4 have a very low recovery values (less than 70 %).

The use of propionic and sorbic acid reference standards enabled precise assignment of chemical shifts. The information used for identification and quantification of both acids is shown in Tables 4

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and 5. The characteristic signals can be assigned to the corresponding analytes unambiguously and without overlapping (Figure 1).

Table 2. The theoretical and experimental concentration expressed in mg/kg as well as the recovery values (%) of propionic acid in bread samples

Type	Concentration (in mg/kg)		Recovery (%)
	Experimental	Theoretical	
Bread without admixture	0	0	0
Admixture 1	205.6	260.4	79.0
Admixture 2	398	520.7	76.4
Admixture 3	777.8	1041.5	74.7
Admixture 4	2325.6	3124.4	74.4

Table 3. The theoretical and experimental concentration expressed in mg/kg as well as the recovery values (%) of sorbic acid in bread samples

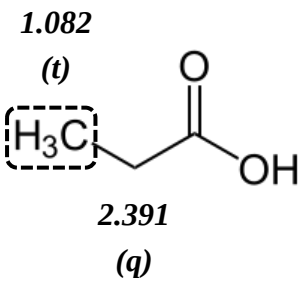
Type	Concentration (in mg/kg)		Recovery (%)
	Experimental	Theoretical	
Bread without admixture	0	0	0
Admixture 1	157.2	242.6	64.8
Admixture 2	289.2	485.3	59.6
Admixture 3	486.2	970.5	50.1
Admixture 4	1618.2	2911.6	55.6

Table 4. Chemical shifts, signal type, number of protons for NMR identification of sorbic and propionic acid.

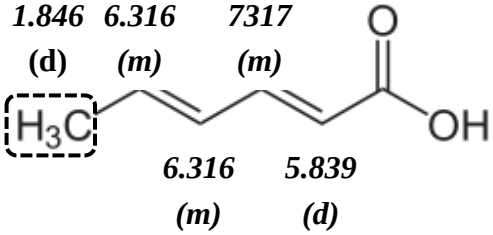
Substance	Propionic acid		Sorbic acid			
ppm	1.08	2.39	1.84	5.82	6.31	7.33
Signal type	Triplet	Quartet	Doublet	Doublet	Multiplate	
Number of protons	3	2	3	1	2	1

Table 5. Information for NMR quantification of sorbic and propionic acid

Analyte	CAS-No	*M* /(g/mol)	*δ* /ppm	Multiplet	*J* /Hz	Integration from /ppm	Integration to /ppm	*N*(H)
Propionic acid	79-09-4	74.08	1.082	t	7.5	1.117	1.045	3
Sorbic acid	110-44-1	112.13	1.846	d	5.0	1.875	1.820	3



a)



b)

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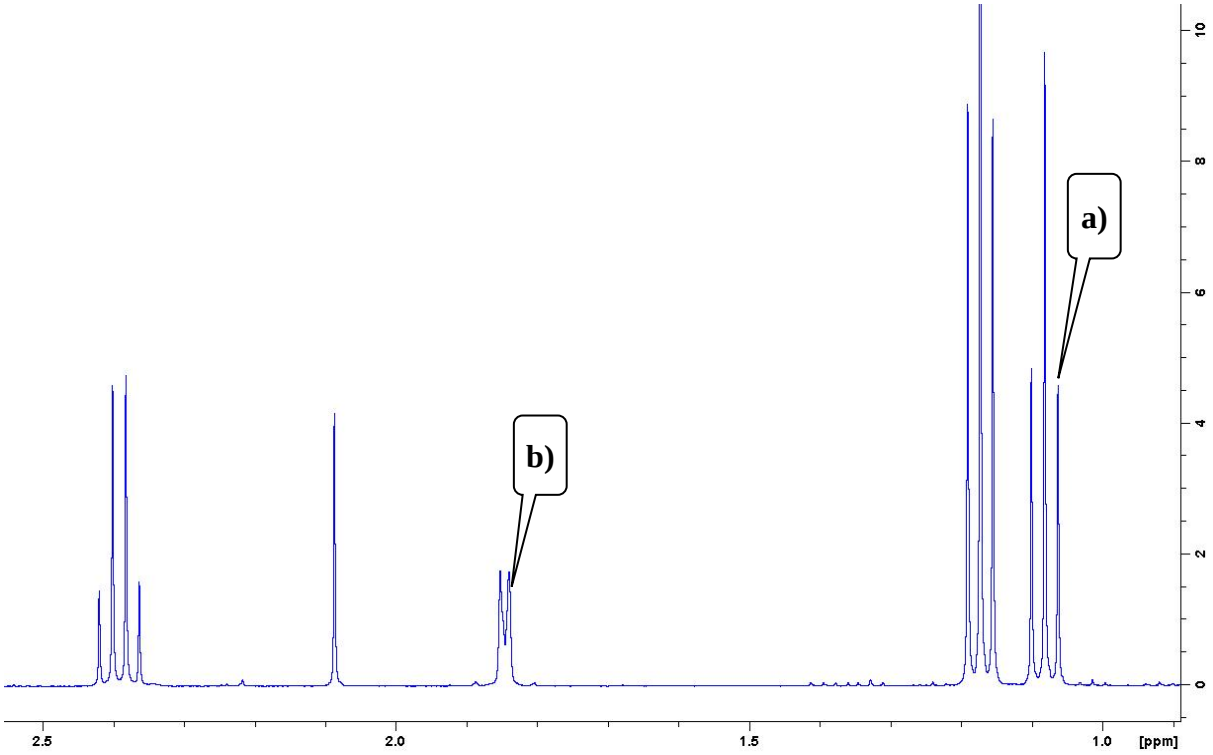

Figure 1. Assignment of ^{1}H-NMR signals of propionic acid (a) and sorbic acid (b)

3.2. Propionic and sorbic acids contents in bread and bakery samples

A typical NMR spectrum of a positive sample including magnifications of target resonances is shown in Figure 2. Both compounds were observed in the low frequency region δ 1-1.3 ppm. The green spectrum represents an admixture with added standard substances while the red spectra represents the sample.

From 76 fresh bread samples (i.e. not prepacked bread), none of the samples contained either sorbic or propionic acid. On the other hand, from 21 prepacked bakery wares 10 samples contained sorbic acid, but none of them propionic acid (Table 6). It must be noted that the recorded sorbic acid concentrations apart from one sample were lower than the maximum permitted levels (Table 6). A yufka sample contained both acids. The yufka sample was also exceptional, as it did not provide labelling of the contained acids, which is an infringement of the EU food information laws.

Table 6. Propionic acid and sorbic acid concentration expressed in mg/kg in all analyzed samples (n.d. not detectable (propionic acid: < 94; sorbic acid: < 258 mg/kg)

Concentration (mg/kg)				
Bread, not prepackaged (n=76)	**Yufka, pasts (n=2)**		**Rye-bread, partially baked, prepacked bakery wares (n=21)**	
	Sorbic acid	**Propionic acid**	**Sorbic acid**	**Propionic acid**
n.d. (all samples)	1183	1288	1005	n.d.
			1872	n.d.
			1453	n.d.
			1502	n.d.
			1770	n.d.
			2173	n.d.
			1399	n.d.
			1885	n.d.

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	1235	n.d.
	1300	n.d.

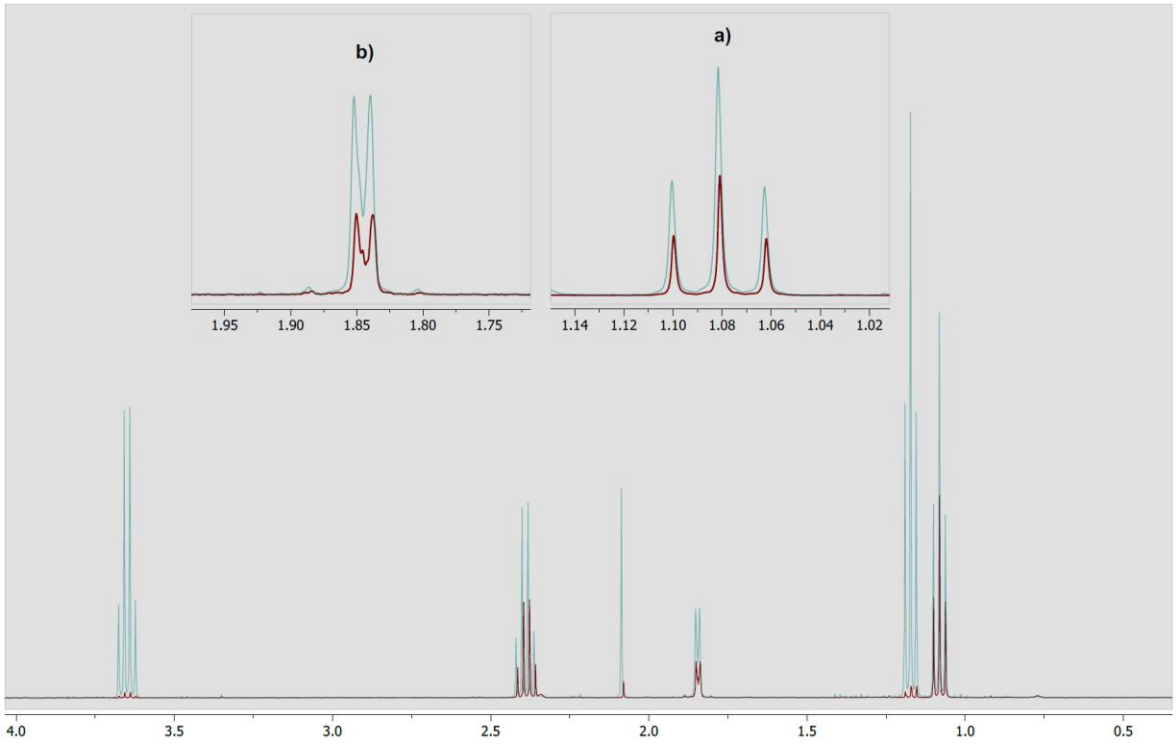

Figure 2. Representative ^{1}H NMR spectra of target additives in yufka bread sample: (a) propionic acid; (b) sorbic acid (x-axis: ppm, y-axis arbitrary units).

3.3 Measures accompanying the analysis

The method is suitable for clearly identifying propionic and sorbic acid in bread. The signals are specific. The method is suitable for the quantitative determination of propionic acid in bread at the usual concentrations for this matrix (undetectable up to the currently accepted maximum value of 3000 mg/kg). For sorbic acid, the method provides only guidance values due to low recoveries. The determined limits of quantification are sufficient for routine tests. Each measurement series was accompanied by a quality control samples. The quality control charts (Figure 3) show sufficient long term stability of the assay. The slight downward trend was judged as being due to potential loss of the acids due to evaporation or chemical degredation during storage rather than due to problems with measurement of spectrometers.

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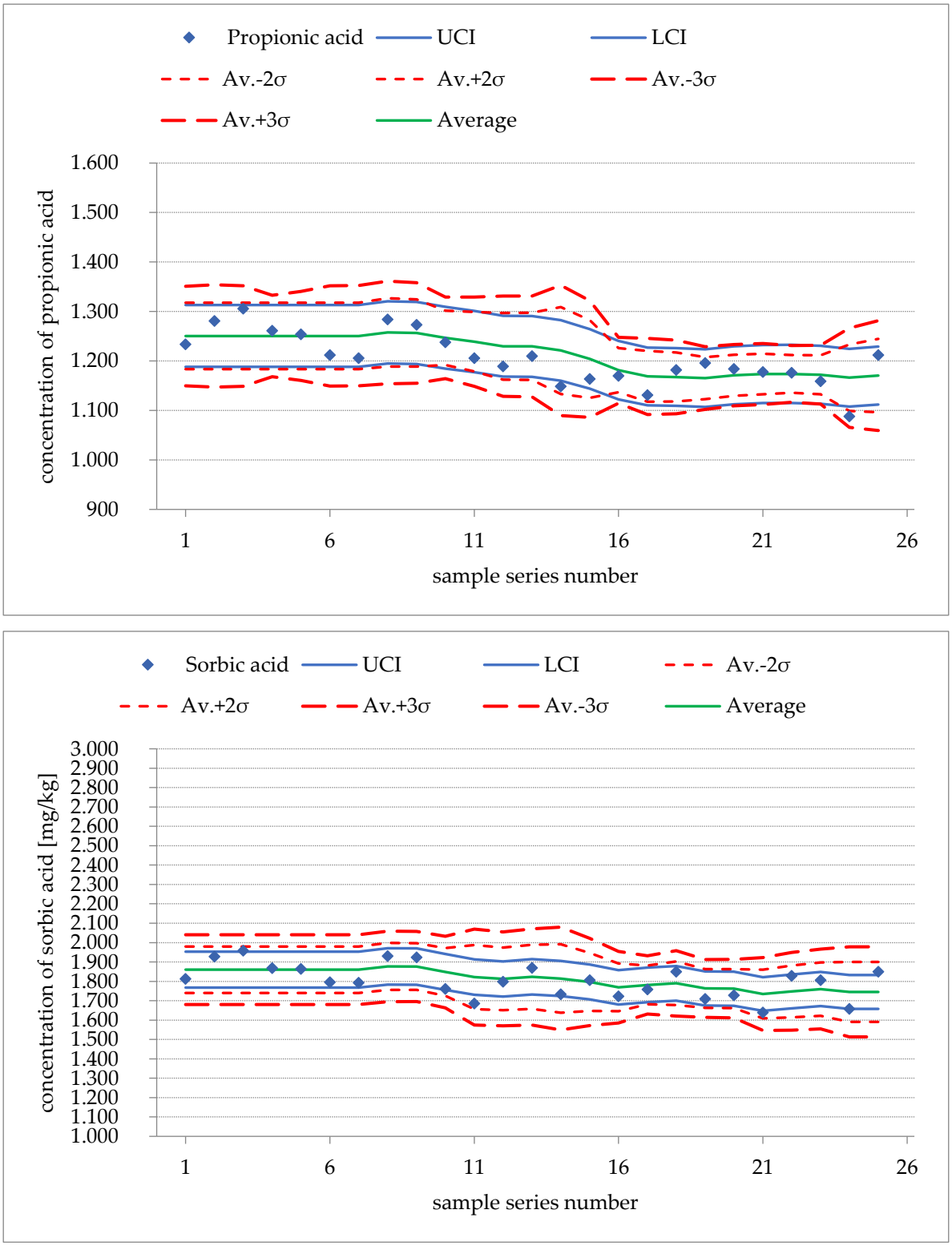

Figure 3. Quality control charts for propionic acid (upper panel) and sorbic acid (lower panel) (x-axis sample series number, y-axis concentration mg/kg).

4. Discussion

Accompanying the technological development and modernization of food production and processing, the quality control of finished products has received the attention of a large range of scientific researches. The latter have been oriented towards the analysis of the effectiveness of the quality control as well as the quality and integrity of food additive. In spite of that, fraud was

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constantly occurring in terms of wrong labelling of the product or the type and origin of ingredients. Therefore, fully automated NMR spectroscopy, combined with multivariate data analysis techniques, has proven to be an efficient multipurpose tool to cover these challenges [9, 13, 17].

As a matter of principle, recovery measurements are expressed in terms of the addition of a known amount of analyte to a sample and then determining the percent of the added amount. Food samples can therefore be spiked with varying amounts of a pure standard to give concentrations at the upper and lower reference limits [18]. Therefore, the recovery rate is usually given in percent and it describes the benchmarks for evaluating the quality of chemical analytical methods. It is defined as "the ratio of the amount of an analyte that is added to a sample before sample preparation and the amount of this analyte that is found as the measurement result" [16]. According to the same guidelines, if the recovery rate of an analyte is 100%, there has been no loss of this analyte during the entire analytical process (through particular analytical steps such as extraction and measurement). However, if the recovery rate is lower than 70%, this is an indication of substance loss. The percentage of losses can be verified by using additional internal standards or a recovery function [18, 20].

Some evidences in the literature concluded that if the recovery observed for a spiked solution is identical to or iterates around 10 % of the added concentration, the accuracy of the assay is considered as sufficient [19,20]. We have observed similar patterns in some spiked samples, especially in the case of propionic acid. As for sorbic acid, the recorded recovery rates were not fitting the general IUPAC criteria [18].

In this study, the developed NMR method showed efficiency in detecting propionic and sorbic acids amounts in all analyzed bread samples. Nevertheless, we have observed that for both compounds, missing information were observed in the list of ingredients in some packaging (e.g. Yufka samples). Furthermore, according to Article (18), paragraph (4) in connection with Annex VII, Part C of regulation (EU) No. 1169/2011 in regard to the provision of food information to consumers, food additives must be named with the name of their respective class. More attention should be drawn to the labelling and the use of the correct class designation in bread samples.

5. Conclusions

Our study is the first to provide NMR validation results for propionic and sorbic acid contents in bread and bakery products. The validated results showed that the developed method is quick, and reproducible regarding quantitative analysis of both substances. In order to use the developed NMR analysis for quality control of a broad range of cereals, further validation studies for other matrices (e.g. semolina, corn, doughs, dry mixes) is worthwhile. Nevertheless, further investigations of the sample preparation of both acids are necessary to increase the recovery rates, as the loss is expected to occur during the distillation step rather than the NMR measurement.

Supplementary Materials:

Author Contributions: Conceptualization, D.W.L.; methodology, A.S., T.K., J.T. and D.W.L.; formal analysis, A.S.; resources, S.G.W. and D.W.L.; data curation, A.S.; writing original draft preparation, M.S.; writing—review and editing, D.W.L., M.K., J.T., A.S., T.K., S.G.W.; supervision, D.W.L. and S.G.W. All authors have read and agreed to the published version of the manuscript.

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