SINGLE LABORATORY VALIDATION OF ACOUSTIC METHOD FOR SCREENING *FUSARIUM* MYCOTOXINS IN CEREALS

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DON (deoxynivalenol) is a naturally worldwide occurring toxic metabolite produced by several species of filamentus fungi of the genus Fusarium. It is estimated that about a quarter of the world's food crops (mainly cereals) are affected by this mycotoxin. A lack of awareness about the quality, health and safety risks related to this mycotoxin and effective control has led to numerous health problems worldwide. Since at least 60 % of the food and feed produced in the world originates from cereal crops one can imagine the magnitude of the problem. In the framework of EUREKA ITEA2 project ACOUSTICS the first portable acoustic spectrometer to detect DON in cereal grains has been developed at Kaunas University of Technology in Lithuania. This technique is completely different from currently applied wet chemistry techniques and is based on acoustic wave's penetrating through and/or reflected by air-filled porous materials such as unconsolidated solid beads of grain. To determine the performance criteria for the acoustic method, a single laboratory validation was carried out. For determining the reliability characteristics different wheat model system (DON concentration range 0-4300 µg/kg) were used. To increase the accuracy of the analysis and optimize the acoustic signal an optimal frequency has been determined. High correlations between DON concentration in wheat samples and the different amounts of shriveled grains in mixtures determined by the acoustic technique have been obtained. Good performance characteristics for repeatability have been found. It can be confirmed that the acoustic method is precise and can be used to detect quantitatively direct DON in cereals.

Cereals are an important source of *Fusarium* mycotoxins such as trichothecenes (T-2, deoxynivalenol (DON) and others). They attract worldwide attention because of the significant economic losses associated with there impact on human health, animal productivity and trade [1]. *Fusarium* fungi on cereals are hard to master because it's occurrence is mainly the result of weather conditions. The combination of moisture, temperature and relative humidity at time of flowering plays an important role by the occurrence of head blight or scab on e.g. wheat. Although DON is not as toxic as T-2, HT-2 toxin and other type A trichothecenes its occurrence and exposure is much bigger and not seldom, the Total Daily Intake (TDI) is exceeded [2]. Especially children and the elderly are at risk. Cereal derived foods and drinks are the main contributors to this risk. Regulatory limits for DON have been established in many countries worldwide, also in the EU [3, 4].

Scab is one of the conditions that can result from the action of *Fusarium* mould on several cereals. *Fusarium graminearum* is the most frequent occurring fungus in grain, also *Fusarium culmorum* is occasionally involved. The occurrence of these fungi results in the low yield of grains and the reduction of the technological quality of cereal-based products. The deterioration is triggered by the formation of a group of toxins called type B trichothecenes from which DON is the marker and the most occurring. When the mold strikes, the plant produces an antidote to get rid of the fungus and the fungus on its turn produces the mycotoxin. The result of this event of fungal invasion is that the attacked grains shrivel and become more porous. In wheat, it appears that DON production is necessary for the fungus to produce the scab disease [5]. The results of the dependence of DON, accumulated in grain, on a degree of its damage (prematurely ripening and shriveling) by *Fusarium* was studied extensively by the Russians with wet chemistry methods [6].

They proved the existence of a direct correlative dependence between DON concentration and the percentage of scabby grains in wheat.

The difference between a sample with wholesome and a mixture of wholesome and shriveled / porous grains can be measured with acoustic. It is widely known that the sound propagating through a porous structure is attenuated, and its velocity depends on pore size and the porosity coefficient. It is easy to measure the propagation of short acoustic pulses. As the acoustic wave propagates through or is reflected by the material, any changes to the characteristics of the propagation path affect the velocity and/or amplitude of the wave [7–9].

Changes in velocity and /or amplitude can be monitored. Unconsolidated solid beads of wheat and other cereal grains are such porous structures. The Fast Fourier Transformation (FFT) translates the time- domain results into the frequency-domain and the results become comparable with spectroscopic measurements.

The Americans were among the first to try to determine non-invasive DON directly on grain by near infrared spectroscopy (NIR) [10]. According to the authors the results demonstrated that the combination of a neural network and near infrared spectra could conveniently be used to determine DON levels in barley. JRC (Joined Research Center) tried the Fourier Transformed-Infrared-Attenuated Total Reflection (FT-IR-ATR) method with subsequent evaluation of the spectra by Principle-Component Analysis (PCA) also to determine DON [11]. Only highly contaminated material could be identified to a certain degree.

All these developments inspired the group in Kaunas, Lithuania to apply acoustic waves to determine quantitatively DON direct on mixtures of unaffected- and affected grains. It is the speed of the acoustic method to measure porosity and its non-invasive character that one can use the technique in-line to carry out high-throughput analysis like the Americans did with NIR.

The aim of the study was to investigate the possibilities to screen wheat grains for DON with the acoustic method, and to compare the results obtained with those of an ELISA method. Additionally, a single laboratory validation of the acoustic method for the determination of shriveled grains and DON was carried out .

Materials and methods. Wholesome wheat grains (Lithuanian SC “Kauno grudai”) and contaminated wheat grains (French company “Bioplante”, 2011) with a high level of DON (4300 µg/kg) were used for the creation of wheat model systems. Wheat samples were passed through two sieves, one with slotted perforations of 3.5 mm and the other with a slotted perforation of 1.0 mm to eliminate extraneous matter. The model systems were prepared by mixing the wholesome wheat kernels with 5, 10, 15, 20...90 % of shriveled grains (W – wholesome wheat, F_{max} – scabby wheat with DON contaminated with a high level (4300 µg/kg) and their mixtures: W+5 % F_{max}, W+10 % F_{max}, ...W+90 % F_{max}). The wheat model systems were used for the determination of the relationship between the amount of DON in wheat grains against the acoustic signal parameters and to determine the performance criteria of the acoustic method to validate the method.

Additional, wheat grain samples with different DON levels (100-1200 µg/kg) received from French company “Bioplante” and Lithuanian SC “Kauno grudai” were used for the determination of the relationship between the results provided by the acoustic method and DON concentration determined by a ELISA method.

An acoustic spectrometer working in a range of frequencies of 15–45 kHz was used in the experiments. It measures the value in relative units (r.u.) of the amplitude of the acoustic signal penetrated (transmission) through the tested sample (Ap). The schema of this spectrometer is presented in Fig. 1 and works as follows: the generator (2) modulates an electric video pulse produced by the generator (3) (amplitude 9 V and time 200 µs) to continuous produce electric sine signals. The electric signals of the generator (2) are received by the frequency exchange supply (4) and specified by the frequency meter (5). Further, the given electric signals are transferred to the acoustic transmitter (6). The acoustic transmitter radiates signals in the direction of the analogous

receiving acoustic receiver (8), where pulses are detected and controlled by the oscilloscope (10) .The detected pulses are measured by a digital voltmeter (11) and transferred to the computer (1).

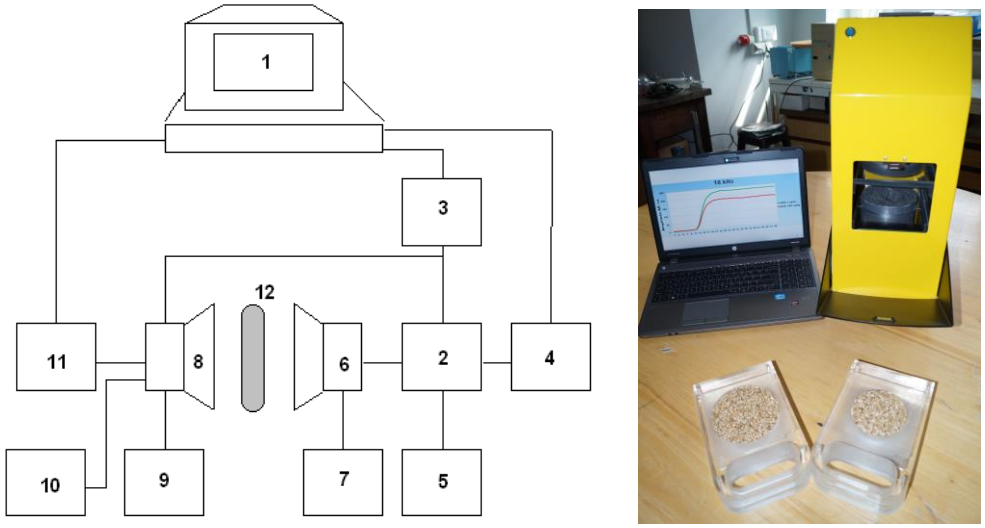

Fig. 1. Scheme of the acoustic spectrometer: 1 – computer; 2 – sine wave generator; 3 – pulse generator; 4 – frequency converter; 5 – frequency meter; 6 – transmitting acoustic aerial; 7 – power supply; 8 –receiving acoustic aerial; 9 – power supply; 10 – oscilloscope; 11 – digitals voltmeter; 12 – wheat sample under test.

For acoustic measurement the grain samples were placed into a plastic cell with a diameter 80 mm, high 45 mm.

As it has been evident from previous experiments the reliability of the acoustic measurements is highly influenced by the frequency of the acoustic signal applied [12, 13]. Taking into account that at very low acoustic wave frequencies (5–9 kHz) various noises are registered, while at frequencies higher than 10–30 kHz the signal propagation is influenced by air eddies, the optimum frequency was accepted within the 10–30 kHz interval. After statistical data evaluation, the optimal frequency was chosen at the point where the standard deviation and variation coefficient are minimal — 19.4 kHz. At this frequency the standard deviation was 0.01774 and the variation — 0.0059. Further investigations were carried out at those optimal frequencies.

To determine the performance criteria for the acoustic method, a single laboratory validation was carried out, since only one instrument is available [14–16]. For determining the reliability characteristics of the acoustic method, 6 replicate analyses of 2 test samples over a period of 2 days for each wheat model system were perform by two analysts that has not been intimately involved in the method development work. The calibration curve was prepared 4 times, at 2 different times on the same day, and on 2 different days. The wheat model systems: W, W+10 % F_{max}, W+20 % F_{max}, W+40 % F_{max}, W+60 % F_{max}, W+80 % F_{max} and F_{max} (DON concentration range 0-4300 µg/kg) were used for calibration purposes. Other wheat model systems (W+5 % F_{max}, W+10 % F_{max}, W+15 % F_{max}, W+30 % F_{max}, W+50 % F_{max}, W+70 % F_{max} and W+90 % F_{max}) were used for the precision estimates. Because no wet chemistry is involved a recovery was omitted.

ELISA method was applied to compare with the acoustic method for wheat model systems and industrial wheat samples. Samples were analyzed for DON using a Veratox®DON 5/5 Quantitative Test Kit (Neogen Corporation, USA) according to the manufacturer's instructions. This test system is a competitive direct enzyme-linked immune-sorbent assay (CD-ELISA) which allows obtaining a DON concentration in mg/kg (ppm). Free DON in the samples and controls is allowed to compete with enzyme-labeled DON (conjugate) for the antibody binding sites. After a wash step, K-Blue Substrate reacts with the bound conjugate to produce a blue color. The optical densities of the controls form the standard curve, and the sample optical densities are plotted against the curve to calculate the concentration of DON. Limits of detection and quantitation for this method were

reported as 0.1 and 0.25 mg/kg, respectively. This test kit does not differentiate between DON and 3-acetyl DON.

The results were obtained by using a Microsoft Excel spreadsheet and a statistical program Analyse-it. The means were compared by one-way analysis of variance (ANOVA). The significance level was $p< 0,05$.

Results and discussion. The experiment has been carried out in two stages: 1) the use of the acoustic method for testing the quality of grains; and 2) the use of the acoustic method for the investigation of the DON levels of the different wheat samples taking into account their specific structural properties.

The results of the analysis of different wheat mixtures contaminated with 10, 20, 30, 40 and 50 % of shriveled grains show that with an increasing percentage of shriveled grains in the model system the amplitude of the penetrated acoustic signal (Ap) decreased (Fig. 2).

Also a high correlation has been found between the different amounts of contaminated (shriveled) kernels in the wheat mixtures and the acoustic signal parameters: correlation coefficients determined by acoustic signal penetration was $R^2 = 0,9381$. It was determined, that the amounts of 10 % of shriveled grains decreased the Ap values on an average by 7–10 %.

The microscopic analysis of the contaminated grains by *Fusarium* and the wholesome wheat grains shows the visible damages on the surface of the contaminated wheat kernel. In *Fig. 3* is shown what happens with the structure of the wheat kernel when it has been attacked by *Fusarium*. In *Fig. 3a* the structure of wheat kernel is healthy and wholesome, in *Fig. 3b* the starch granules have been "consumed" by the fungus and a more skeleton type of landscape appears. These changes of surface structure are related to the porosity of the grain matrix. The experiment results also showed that by increasing the amount of scabby wheat in the matrix, grain bulk density decreased (*Fig. 2*). Jackowiak *et al*. [5], Nightingale *et al*. [17] have examined under scanning electron microscopy the endosperm of *Fusarium* infected wheat kernels and have found some characteristic structural changes in many of its region, such as the partial or complete lack of the protein matrix and damage of starch granules caused by fungal proteolytic- and amylolytic enzymes.

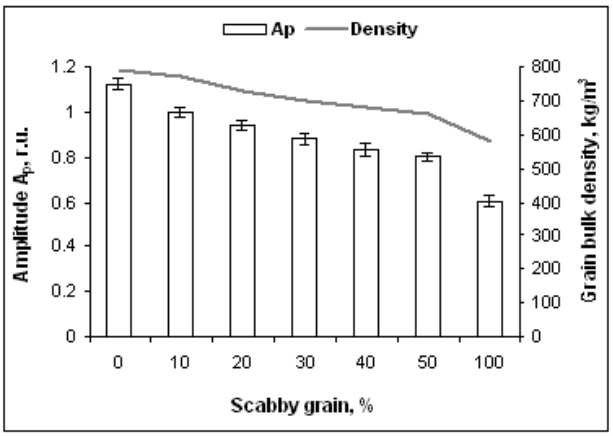

Fig. 2. The influence of scabby wheat content on grain bulk density and on the amplitude of the penetrated (Ap) acoustic signal analyzed by the acoustic spectrometer.

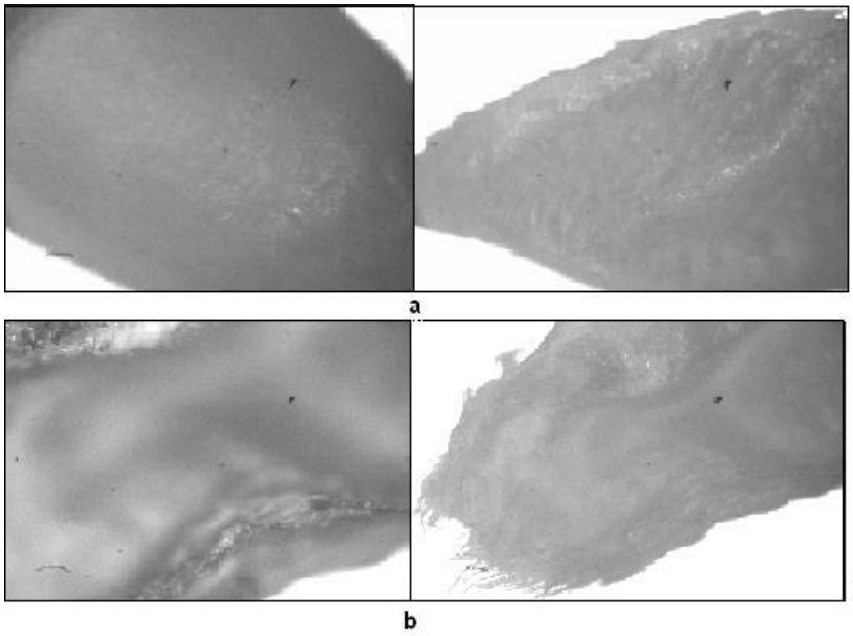

Fig. 3. An optical microscopic view of the surface of wheat grain: a – healthy kernel and b – DON contaminated kernel (4300 μg/kg).

In summary, by applying acoustics the structural properties of the porous cereal matrix can be determined by measuring the amplitude of the acoustic signal penetrated through / reflected by the tested sample. The given data confirm that the acoustic method detects the changes of kernel structure and that there is a strong correlation with the quantity of by *Fusarium* infected grains. It is known that DON is the most widespread mycotoxin of the fusariotoxins group. In the CAST report [1] it says that studies of wheat and corn infected with *F. graminearum* in the field indicate that wheat can have high levels of DON but usually has little or no zearalenone (ZEN). According to the results of DON and ZEN investigations in cereal harvested in 1986–1988 in Russia, the occurrence of DON in wheat varies between 60–100 % in the *Fusarium* natural habitat, the presence of the ZEN was determined only in 7 % of samples. Also the Russians based on extensive research revealed a strong correlation of 0.80-0.96 between the DON concentration determined with wet chemistry methods and the percentage of scabby wheat [6]. This confirms our idea to apply the acoustic method as screening method not only for detection of shriveled grains but also for the determination of DON in wheat. The specificity of the acoustic method for DON and other trichothecenes and its application for screening other cereals must be confirmed by additional investigations.

The results show that the acoustic method gives adequate and encouraging quantitative information of DON in wheat. As yet only calibration curves have been made using conventional analytical methods (ELISA) for DON, in order to try to get information if the acoustic method was capable and precise enough to characterize the quality of wheat contaminated with DON. The relationship between the penetrated acoustic signal amplitude (Ap) and the DON content in the wheat model systems is shown in *Fig. 4*. A same pattern has been noticed by using the acoustic impulse spectrometer, where also with an increasing concentration of DON the amplitude of the reflected acoustic signal (Ar) decreases.

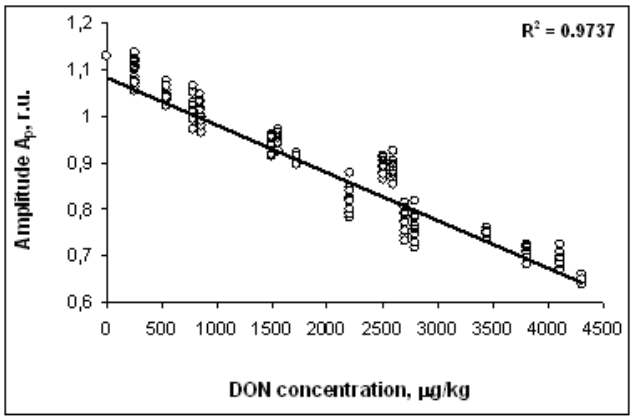

Fig. 4. The relationships between the penetrated acoustic signal amplitude (Ap) analyzed by the acoustic spectrometer and the DON concentration in the wheat model systems.

The results of the additional study of the wheat samples from French company "Bioplante" and Lithuanian SC "Kauno grudai" are shown in *Fig. 5 and Fig. 6*. The collection of soft wheat contaminated with DON was analyzed using the acoustic spectrometer. It was determined, that by the increase of the DON concentration in the wheat samples, the values of the acoustic signal amplitude Ap decreased (*Fig. 5*).

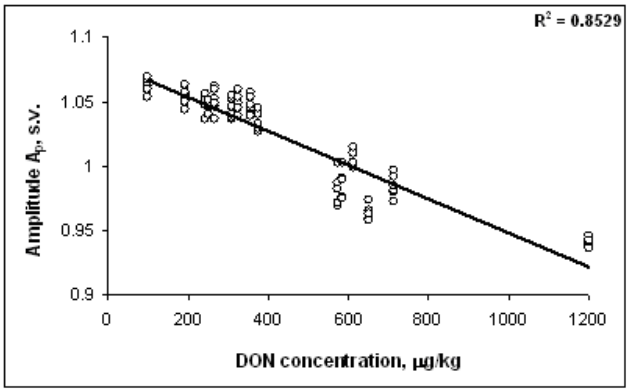

Fig. 5. Relationship between the penetrated acoustic signal amplitude (Ap) and DON concentration in wheat samples from French company "Bioplante" and Lithuanian SC "Kauno grudai".

The ANOVA showed significant differences in Ap values between the samples with different DON concentrations ($p<<0.05$). From the relationship curve between the penetrated acoustic signal amplitude (Ap) and the DON concentration, the DON content in the tested wheat samples was calculated. The strong correlation (R^2 = 0,8822) was found between the DON concentrations determined by acoustic and ELISA methods (*Fig. 6*). The relative standard deviation of repeatability for the acoustic method was less than or equal to 24,0 % if the DON concentration was 245–500 µg/kg and less than or equal to 14,0 % if the DON concentration was more than 500 µg/kg. These results of the investigation of the industrial wheat samples from Germany confirm that the acoustic method gives quantitative information for this mycotoxin.

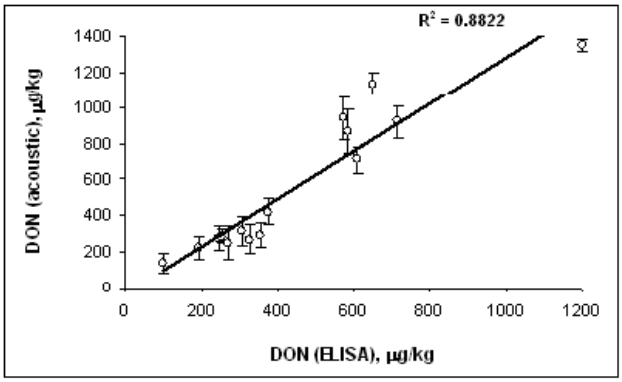

Fig. 6. Relationship between DON concentration in wheat samples from French company "Bioplante" and Lithuanian SC "Kauno grudai" determined by acoustic and ELISA methods.

Results of the mathematical statistical evaluation (ANOVA) showed that reliable results were obtained of the acoustical evaluation ($p<0.05$). As shown in Table 1 a strong linear relationship between the quantity of scabby wheat in model samples and the amplitude of the acoustic signal ($R^2 = 0.9513–0.9786$) as well as between the DON concentration and the amplitude of the acoustic signal ($R^2 = 0.9173–0.9480$) was obtained and the calibration (analytical) curves prepared at different times on the same day and on two different days were replicated and stable.

Table 1

Stability characteristics of the calibration curves

Calibration		For scabby wheat content		For DON concentration	
		Equation	R^2	Equation	R^2
Day 1	1	y = -0.0045x + 1.086	0.9786	y = -0.0001x + 1.098	0.9173
	2	y = -0.0044x + 1.102	0.9595	y = -0.0001x + 1.068	0.9480
Day 2	1	y = -0.0044x + 1.085	0.9774	y = -0.0001x + 1.088	0.9343
	2	y = -0.0045x + 1.103	0.9513	y = -0.0001x + 1.104	0.9333
x – Concentration of analyte (scabby grain or DON), y – amplitude of the acoustic signal.					

A successful single laboratory validation has been carried out and performance characteristics for repeatability (RSDr) have been found (*Table 2*). The actual RSD(r) values for the most tested samples (with DON concentration range 550–3890 µg/kg) were better than target "best case" values for RSD(r) ([1/2]×PRSD(R)), and the HORRAT(r) values were falling well within the prescribed acceptable HORRAT(r) region of 0.3–1.3 [16, 18]. No recovery % could be reported because of the non-invasive character of the method. According to the results of the investigation it can be confirmed that the acoustic method is precise and can be used to detect the quantity of DON in fusarioses grain.

A multi laboratory validation based on the harmonized protocol could not been carried out because only one instrument is available. Besides, the availability of a sufficient amount of contaminated samples slows down developments. It needs also additional support and/or funds to carry out a multi laboratory validation. A satisfactory full validation will open a new avenue for monitoring and high throughput analysis of DON in grains.

Non-invasive procedures like acoustic and infrared techniques have many advantages over invasive wet chemistry methods, because they are not only fast but go around the complicated and costly mycotoxin testing procedures as is shown in *Fig. 7*.

Table 2

Reliability characteristics of the acoustic method

	Samples						
	1	2	3	4	5	6	7
Scabby grain, %	5	10	15	30	50	70	90
DON (ELISA), µg/kg [a]	220±20	550±40	815±25	1520±60	2550±110	2750±90	3980±160
A_p, r.u.[b]	1.09	1.05	1.02	0.94	0.89	0.78	0.70
SD	0.04	0.04	0.05	0.04	0.04	0.04	0.03
For scabby wheat content determined from calibration curve							
Scabbygrain, % [c]	1.11	10.85	16.39	33.30	46.50	70.83	87.66
SD	0.12	0.15	0.21	0.44	0.57	0.79	0.92
RSD(r), %	10.81	1.38	1.28	1.32	1.23	1.12	1.05
PRSD(R), %	3.13	2.83	2.66	2.40	2.22	2.11	2.03
HORRAT(r)	3.45	0.49	0.48	0.55	0.55	0.53	0.52
For DON concentration determined from calibration curve							
DON, µg/kg [c]	50	488	738	1498	2093	3187	3945
SD	8.28	29.05	41.91	81.96	96.61	138.74	159.09
RSD(r), %	16.48	5.95	5.68	5.47	4.62	4.35	4.03
PRSD(R), %	19.94	17.38	16.38	14.92	13.81	13.65	12.93
HORRAT(r)	0.83	0.34	0.35	0.37	0.33	0.32	0.31

[a] DON concentration determined by ELISA method (mean values of dublicate measurements).
[b] Ap – amplitude of penetrated acoustic signal (mean values of 24 measurements: 6 replicate analyses of 2 test samples over a period of 2 days).
[c] Scabby grain content and DON concentration determined by acoustic method (mean values of 24 measurements: 6 replicate analyses of 2 test samples over a period of 2 days).

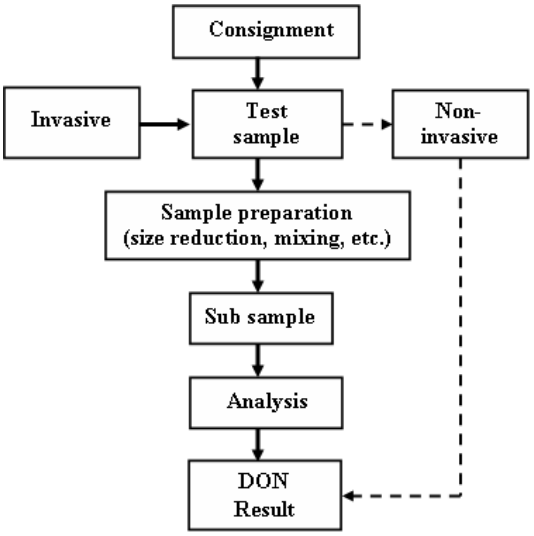

Fig. 7. Invasive mycotoxin testing procedure versus a non-invasive procedure.

The mycotoxin testing procedure generally consists of three steps: (1) a sample is taken from a lot, (2) the sample is ground and a sub-sample is removed from the comminute sample, and (3) the mycotoxin is extracted from the comminute sub-sample and quantified. Both procedures include the first step (1). The invasive procedure however includes subsequently step two (2) and includes usually steps such as solvent extraction, centrifugation, filtration, drying, and dilution before quantification. By every additional manipulation one can makes mistakes. As a result, replicated analyses can already vary considerably. In the non-invasive procedure quantization is straight determined, so goes by the laborious and costly wet chemistry method. It is also well

known that sampling is an important aspect of obtaining reliable analytical results. For both procedures apply step (1), but in the case of the invasive procedure a sub-sample procedure follows the initial sampling procedure, while for the non-invasive procedure this might not be the case. Since an intuitive "feeling" of where and how to obtain "representative samples" is the typical scenario for sampling, almost always leading to incorrect results and considerable economical losses and one adds to it the variability's of the above mentioned steps in the invasive procedure. A non-invasive technique that only needs step (1) may provide fast results, so that much larger amounts of samples could be screened. Wich could lead to a more representative picture of the lot investigated and in- and on line monitoring.

C O N C L U S I O N S

1. To increase the accuracy of the analysis and optimize the acoustic signal an optimal frequency has been determined. High correlations between DON concentration in wheat samples and the different amounts of shriveled grains in mixtures determined by the acoustic technique have been found.

2. The acoustic method gives reliable results in the quantitative determination of shriveled grains and DON in wheat and is sufficiently precise. Good performance characteristics for repeatability have been found.

3. Because of the speed of the acoustic method and its non-invasive character one can use the technique in-line to carry out high-throughput analysis of consignments of cereal grains and can quantitatively direct determine DON. Monitoring of cereals becomes now possible in-line, which will increase food safety at lower cost.

Acknowledgements. The research was funded by a grant (EUREKA ITEA 2 project 09041 ACOUSTICS) from Lithuanian Agency for Science, Innovation and Technology.

The authors thank French company "Bioplante" French company "Bioplante" and Lithuanian SC "Kauno grudai" for collections of wheat samples.

ВАЛІДАЦІЯ В ОДНІЙ ЛАБОРАТОРІЇ АКУСТИЧНОГО МЕТОДУ ДЛЯ СКРИНІНГУ *FUSARIUM* МІКОТОКСИНІВ У ЗЕРНОВИХ

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А Н О Т А Ц І Я

ДОН (деоксиніваленол) є токсичним метаболітом, якого роблять декілька видів мікроскопічних грибів роду *Fusarium*, і поширений по всьому світу. Підраховано, що близько чверті світових продовольчих культур (в основному зернових) заражена цим мікотоксином. недостатня обізнаність з приводу якості, здоров'я і безпеки, пов'язаних з цим мікотоксином, і відсутність ефективних методів контролю привела до численних проблем здоров'я у всьому світі. Оскільки близько 60 % вироблюваних у світі харчових продуктів і кормів походять із зернових культур, легко можна представити масштаби цієї проблеми. У рамках EUREKA ITEA2 проекту АКУСТИКА перший портативний акустичний спектрометр для виявлення ДОН в зернових був розроблений в Каунаському технологічному університеті (Литва). Акустичний метод повністю відрізняється від вживаних нині хімічних методів визначення ДОН і грунтований на вимірі амплітуди акустичного сигналу, що пройшов через і / або відбитого від пористих матеріалів, таких як неконсолідовані тверді намистини зерна.

Для визначення параметрів ефективності акустичного методу зроблена його валідація в одній лабораторії. Для визначення характеристик точності вимірів були використані різні модельні системи пшениці (з ДОН концентрацією в діапазоні 0–4300 мкг/кг). Для збільшення точності аналізу і оптимізації акустичного сигналу оптимальна частота була визначена. Висока кореляція між ДОН концентрацією в зразках пшениці і різною кількістю зморщених зерен в суміші, визначених акустичним методом, була отримана. Були знайдені надійні характеристики повторюваності. Це підтверджує, що акустичний метод є точним і може бути використаний для виявлення ДОН, а також для визначення його кількості в зернових культурах.

ВАЛИДАЦИЯ В ОДНОЙ ЛАБОРАТОРИИ АКУСТИЧЕСКОГО МЕТОДА ДЛЯ СКРИНИНГА *FUSARIUM* МИКОТОКСИНОВ В ЗЕРНОВЫХ

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А Н Н О Т А Ц И Я

ДОН (деоксиниваленол) является токсичным метаболитом, которого производят несколько видов микроскопических грибов рода *Fusarium*, и распространен по всему миру. Подсчитано, что около четверти мировых продовольственных культур (в основном зерновых) заражено этим микотоксином. Недостаточная осведомленность по поводу качества, здоровья и безопасности, связанных с этим микотоксином, и отсутствие эффективных методов контроля привело к многочисленным проблемам здоровья во всем мире. Так как около 60 % производимых в мире пищевых продуктов и кормов происходят из зерновых культур, легко можно представить масштабы этой проблемы. В рамках EUREKA ITEA2 проекта АКУСТИКА первый портативный акустический спектрометр для обнаружения ДОН в зерновых был разработан в Каунасском технологическом университете (Литва). Акустический метод полностью отличается от применяемые в настоящее время химических методов определения ДОН и основан на измерении амплитуды акустического сигнала, прошедшего через и / или отраженного от пористых материалов, таких как неконсолидированные твердые бусины зерна. Для определения параметров эффективности акустического метода произведена его валидация в одной лаборатории. Для определения характеристик точности измерений различные модельные системы пшеницы (с ДОН концентрацией в диапазоне 0-4300 мкг/кг) были использованы. Для увеличения точности анализа и оптимизации акустического сигнала оптимальная частота была определена. Высокая корреляция между ДОН концентрацией в образцах пшеницы и различным количеством сморщенных зерен в смеси, определенных акустическим методом, была получена. Надежные характеристики повторяемости были найдены. Это подтверждает, что акустический метод является точным и может быть использован для обнаружения ДОН, а также определения его количества в зерновых культурах.

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