ACOUSTIC SCREENING METHOD FOR THE DETERMINATION OF DEOXYNIVALENOL (DON) IN WHEAT

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Summary. Mycotoxins in food and feed are considered important safety issues of growing concern. Most scientific developments have occurred in the last decades in the area of mycotoxins. Limits and regulations for mycotoxins in food and feed have been established in many countries. Quantitative methods of analysis for regulatory purposes for mycotoxins often make use of laborious and complicated procedures such as of immuno affinity cleanup with Liquid Chromatography (LC) or Gas Chromatography (GC) separation techniques in combination with various types of detectors, including Mass Spectrometry (MS). Therefore, there exist the drive for newer developments in mycotoxin methodology and analysis for screening purposes. These methods include (bio-) sensor-based techniques and non-invasive methods based on infrared and other techniques. Very important mycotoxin from an economic point of view and less from a health point of view is deoxynivalenol (DON) or vomitoxin. DON is a toxin formed by fungi of the genus *Fusarium* and occurs mainly on grains and corn. The aim of the present study was to use such a screening technique as an existing acoustic method to determine the specifically structural changes of grains by *Fusarium graminearum* resulting in shriveled grains and determine via correlations DON in the contaminated grain. The structural properties of wheat were evaluated according to the value of the amplitude of the acoustic signal penetrated through the tested sample of wheat and the amplitude of the short impulse reflected from the tested sample. High correlations confirmed by ELISA (RIDASCREEN) in a calibration experiment between the with DON contaminated wheat sample and the different amounts of shriveled kernels in mixtures determined by the acoustic method were found. The investigations show that the acoustic method was sufficient precise, non-invasive and could be successfully used for an effective control- and screening- of cereals contaminated with *Fusarium* species.

Key words: deoxynivalenol, contaminated grains, acoustic method.

GREITAS AKUSTINIS METODAS DEOKSINIVALENOLIUI (DON) KVIEČIUOSE NUSTATYTI

Santrauka. Mikotoksinais užterštų maisto produktų ir pašarų problema maisto saugos požiūriu aktuali visame pasaulyje. Todėl pastaruoju metu vis didesnis dėmesys skiriamas šių toksiškų medžiagų tyrimui. Daugelyje šalių mikotoksinų kiekis maisto produktuose ir pašaruose reglamentuojamas norminiais dokumentais. Mikotoksinų kiekybinei analizei dažniausiai taikomi imuniniai, dujų ir skysčių chromatografijos metodai, naudojami įvairūs detektoriai bei masių spektrometras. Tačiau iškyla greitų tyrimo metodų poreikis pirminei grūdų patikrai ir produktų užterštumo mikotoksinais kontrolei. Atliekami moksliniai tyrimai siekiant šiems tikslams pritaikyti biosensorius ir infraraudonosios spinduliuotės spektrometrijos ir kitus analitinius metodus.

Tiek ekonominiu, tiek sveikatos požiūriu vienas iš svarbiausių mikotoksinų yra deoksinivalenolis (DON), arba vomitoksinas. Deoksinivalenolis – *Fusarium* genties pelėsinių grybų gaminamas toksinas, dažniausiai aptinkamas grūduose ir kukurūzuose. Šis darbas skirtas akustinio metodo pritaikymui *Fusarium* pažeistų kviečių grūdų struktūrinių savybių pokyčiams įvertinti ir deoksinivalenoliu užterštų grūdų kiekiui nustatyti. Grūdų struktūrinės savybės įvertintos pagal akustinio signalo, praėjusio per tiriamąjį grūdų mėginį, amplitudės bei pagal atspindėto nuo tiriamojo mėginio akustinio impulso amplitudės verčių pokyčius. Nustatyta didelė koreliacinė priklausomybė tarp deoksinivalenoliu užterštų kviečių kiekio grūdų mišiniuose ir akustinio signalo amplitudės verčių. Preliminarūs rezultatai rodo stiprią koreliaciją tarp vystomo akustinio ir ELISA metodų. Tyrimai parodė, kad akustinis metodas yra pakankamai tikslus, bekontaktis ir gali būti sėkmingai taikomas pirminei grūdų patikrai ir produktų užteršimo mikotoksinais kontrolei.

Raktažodžiai: deoksinivalenolis, pažeisti grūdai, akustinis metodas.

Introduction. Deoxynivalenol (DON) or vomitoxin is a type B trichothecene mycotoxin, an epoxy-sesquiterpenoid. Surveys have shown that deoxynivalenol occurs predominantly in grains in mean concentrations such as in wheat (1-5700 µg/kg), barley (4-9000 µg/kg), oats (4-760 µg/kg), rye (13-240 µg/kg), corn (3-3700 µg/kg) and rice (6-5100 µg/kg) and less often in sorghum and triticale (FAO FNP 74, 2001). The occurrence is associated primarily with *Fusarium graminearum, F. avenaceum* and *F. culmorum*, all of which are important plant pathogens, causing *Fusarium* head blight or scab[1] in wheat and ear rot in corn (CAST, 2003). The with deoxynivalenol contaminated shrivelled grains that are smaller and lighter then the wholesome and sound kernels are usually separated by sifting or otherwise by the elevators and millers. Before however, manufacturers and traders want to know if the grain is contaminated with deoxynivalenol and want to have some idea about the degree of contamination, because it can effect the quality of these products. Deoxynivalenol has not only been implicated in incidents of acute mycotoxicoses in both humans and farm animals, but has also two characteristic toxicological effects: decreased feed consumption (anorexia) and emesis (vomiting) by i.e. pigs, and brewers are scared to death because of its foaming properties in beer (gushing!). The high contamination levels found in The Netherlands in breakfast cereals (Schothorst, Jekel, 2001) triggered risk assessment procedures and analytical activity in the EU that might lead to improved methodology and firm and strict regulation for trichothecenes including deoxynivalenol in line with the recent Food Safety regulation 178/2002 in the EU and may be even stricter regulation for infant- and baby food. Activity in the analytical field for deoxynivalenol at the moment is worked out in close cooperation with the European Commission of Standardization (CEN).

What is scab?

Head scab or "scabby wheat" is one of the conditions that can result from the action of *Fusarium* mould on wheat kernels. After *Fusarium* infection, diseased spike lets turn to be a bleached out straw color and ripen prematurely.

The diseased wheat kernels are grayish-brown in color (pink-tipped kernels are also another indicators of *Fusarium* mould damage). Scab is a visual sign of mould damage and is one of the factors utilized in determination the quality of wheat. Scab generally becomes visible approximately 10 days to two weeks after initial infection (infection usually occurs during the flowering stage). Scab does not necessarily indicate mycotoxin contamination. The most probable mycotoxins to be produced by *Fusarium* moulds are DON (vomitoxin) and the acetyl forms of DON. "Scab" can be found in wheat, rye, barley and oats.

Official methods and other validated methods have been developed for the analysis of deoxynivalenol in cereals and foodstuffs. The introduction of improved clean-up columns based on charcoal, alumina, and modified diatomaceous earth before determination by Thin Layer Chromatography (TLC), Gas Chromatography (GC), or Liquid Chromatography (LC) has simplified and accelerated the analysis of deoxynivalenol.

Use of these columns in combination with GC and Electron - Capture Detection (ECD), Flame - Ionization Detection (FID) or Mass Spectrometry (MS) detection after derivatization of deoxynivalenol are the common techniques for quantification. These techniques allow simultaneous determination of deoxynivalenol and other trichothecenes at concentrations of a few nano grams per gram, even in complex food matrices. However, matrix problems may occur in GC analysis. LC-MS or high performance TLC and/or Enzyme Linked Immuno Sorbent Assays (ELISA) are considered for screening purposes. LC with MS detection can be used for direct, simultaneous determination of several trichothecenes, but its high cost prohibits its routine use. TLC, particularly high performance, is still a convenient method for quantifying deoxynivalenol. However, interlaboratory comparisons clearly showed that further improvements are needed in analytical methodology for deoxynivalenol with respect to recovery, accuracy, and precision of measurements. A very sensitive newer method, is the GC-MS method in combination with a rigorous clean up procedure recently developed at the National Institute of Public Health and Environment (RIVM) in The Netherlands for the determination of deoxynivalenol and other trichothecenes in baby food. The CGC-FID method developed by the same institute for wheat with CH_3CN/H_2O extraction and with a Mycosep clean – up procedure scored a promising recovery of 96 % and a CV of 4.8% and a limit of quantification of 75 µg/kg for deoxynivalenol in wheat (Van Egmond, Jonker, 2003; Schothorst et. al., 2003). However for rapid screening of cereals for DON other, much faster methods are to be preferred, e.g. based on biosensors. For example, recently a biosensor-based method was developed by Tüdos *et al* (6, 7) for the selective and quantitative determination of DON. This biosensor assay is of the Surface Plasmon Resonance (SPR) type. DON conjugate is immobilized on the sensor. The assay is based on the competition for antibody binding between the immobilized DON conjugate and the free molecules in the sample extract. The assay was claimed to be robust, reproducible, fast, and inexpensive and suited for multi-component assay. The analysis results of the optimized biosensor assay and an (in-house validated) LC-MS-MS method, practiced by the Netherlands Food Inspection Service (Amsterdam) were compared for naturally contaminated wheat samples with DON levels ranging from approx. 200-1400 mg/kg. The results indicate good agreement between the two techniques, especially for wet-ground samples. Wider availability of (certified) reference materials for deoxynivalenol and regular international comparative studies such as proficiency testing schemes (e.g. Food Analysis Performance Assessment Scheme (FAPAS) or the American Oil Chemists' Society (AOCS) are needed to improve internal and external quality assurance (validation). All this methods might be validated one day and used as reference method, but none of them will be fast, effective, simple and non-invasive etc. and capable to be used in- and on-line. Non-destructive (screening) methods have recently gained great interest in international practice, as they do not destroy the structure of the products analysed and are fast, effective, simple and reliable. Based on the papers and discussions of the EU/ICC Cereal Conference 2002 great attention is paid to developing end-product quality related tests. The methods should preferably also be suitable for in- and on-line process control. So the aim of this study is to apply an available acoustic method to determine the specifically structural changes in consignments of grain by *Fusarium* resulting in shrivelled grains (called "scab") and confirm with an analytical technique such as an ELISA (RIDASCREEN) or CGC-FID via correlations deoxynivalenol in the possible contaminated grains. The present work is focused on further development of this acoustic method for Food Safety purposes such as the determination of deoxynivalenol in wheat because of the encouraging correlations and the unique possibilities of this technology. The occurrence of rather specific structure of shrivelled grain needs special requirements for this analysis. This work has been carried out to develop and create an effective screening- and control-method in cereal processing and marketing. The application of the physical acoustic method for this product relates to new technical and methodological solutions.

Material, methods and equipments. In the experiment samples of scabby wheat with different known levels of DON: 100 ppb (F 100), 420 ppb (F 420), and a high amount of ppb's ($F_{max)}$ provided by the Lelystad branch of the Plant Research Institute (PRI) in Wageningen, The Netherlands have been used. All the samples were sieved into fractions and the most homogeneous (biggest) fraction in grain size was used for analysis. In an additional experiment the mixtures of wheat with shrivelled grain have been investigated. For preparing these samples scab wheat was collected (every shrivelled grain) and mixed with wholesome wheat kernels in the following percentages: 10, 20, 30, 40 and 50%. (K – control, F_{max} – wheat contaminated with a high amount of DON and their mixtures: K+10% F_{max}, K+20% F_{max}, K+30% F_{max}, K+40% F_{max}, K+50% F_{max}).

For the analysis of wheat contaminated with DON two different acoustic methods and equipment were used:

1. An acoustic spectrometer for contact free measurement working in a range of frequencies of 5-36 kHz investigating wheat according to the value of the amplitude of the acoustic signal (Ap) penetrating through the tested sample (transmission). The schema is presented in *Figure 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 electric sine signals. The electric signals of generator (2) are received by the frequency exchange supply (4) and specified by frequency meter (5). Further, the given electric signals are transferred to the acoustic transmitter (6). Acoustic transmitter radiate signals in the direction of 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).

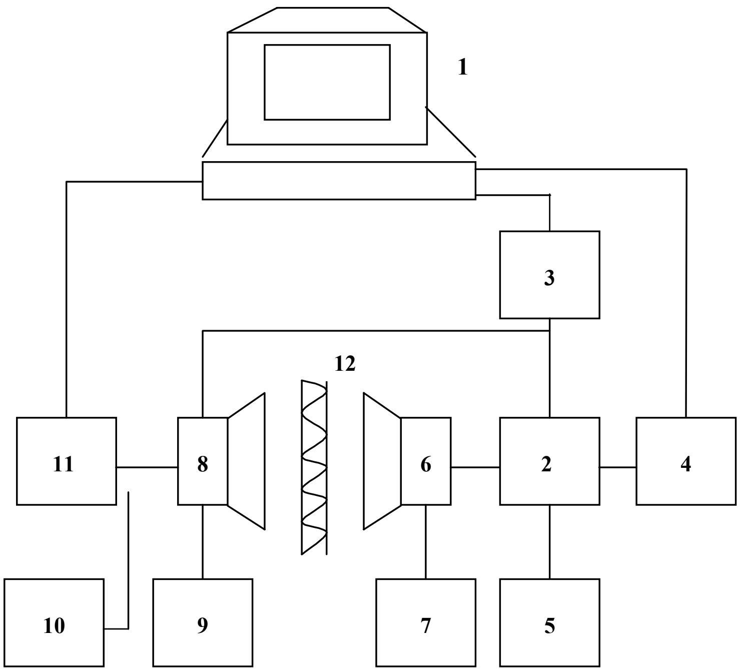

Figure 1. **The principal sheme of the Acoustic Spectrometer**.
1 – computer, 2 – sine-wavegenerator,
3 – videopulse generator, 4 – frequency exchange supply,
5 – frequency meter, 6 – transmitting acoustic transducer,
7 – power supply, 8 – receiving acoustical transducer,
9 – power supply, 10 – oscilloscope, 11 – digital voltmeter

2. An acoustic impulse device working in a frequency range 0 – 125 kHz measures the amplitude of the acoustic signal reflected from the wheat sample is used. It is well known that an electric spark emits an impulsive sound. So an electric microspark was used for the radiation of short acoustic pulses, and ¼'' measuring capacitance microphones MK801 of RFT was used for reception. The block diagram of the experimental arrangement used for the electric-spark based measurement is shown in Figure. The microphone is placed in the front of the air-filled porous specimen close to the sample. The distance from the electric spark to the sample is 80 cm, and from the sample to the measurement microphone 4 cm. The thickness of the sample is 2 cm. For each experiment complete signals are captured: the signal passing directly to the microphone (the reference signal) and a signal reflected from the whole specimen. From these signals the relation between the frequency characteristics of both signals is obtained, and are used to correct the transfer function. A digital oscilloscope with 5 MHz sampling frequency is used for registration, and 2048 points are registered.

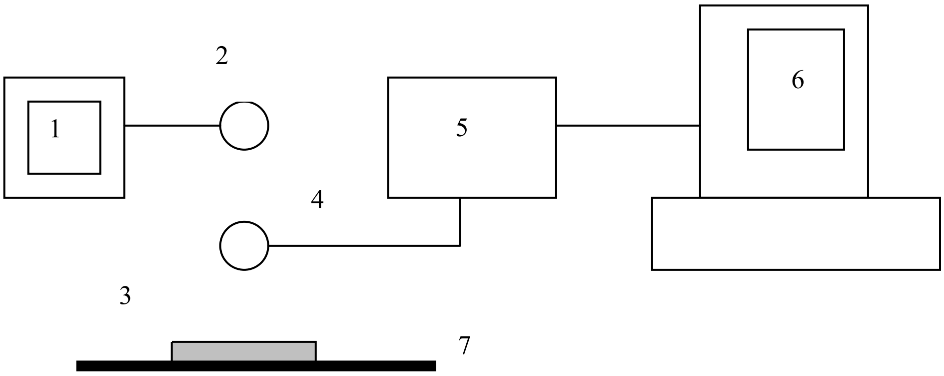

Figure 2. **Block diagram of the experimental arrangement to study sound pulse (generated by the electric spark) reflected from air-filled porous materials.** *1 – high tension source, 2 - electric spark, 3 - sample, 4 – measurement microphone 5 – digital oscilloscope, 6 - personal computer, 7 – solid plate.*

The frequency characteristics of the acoustic waves are evaluated by matematical accounting of the acoustic signal, applying a diskrete *Fourier* transformation.

In the Department of Food Technology of Kaunas University of Technology methods and acoustic devices for contact free and effective measurement have been developed as well as equipment for the application for on-line production (Juodeikiene et. al., 1991; 1993; 1993a; 1993b; 1990; 1995; 1995a; 1994; 2000)

The experiment has been carried out in two stages:

- The selection of the optimum acoustic signal frequency in order to apply the acoustic method for testing the quality of wheat grains;
- The use of the acoustic method for the investigation of the deoxynivalenol (DON) of the different samples of wheat taking into account their specific structural properties.

The test results were mathematically processed by means of the statistical program Sigma Plot for Windows.

Results and discussion.

The selection of the optimum acoustic signal frequency. While applying the acoustic method for a new object of investigation, it is important to check the accuracy and reliability of the measurements obtained. As it has been evident from previous experiments (Juodeikiene, 1995) this is highly influenced by the frequency of the acoustic signal applied.

The values of the acoustic signal (A_P) after penetration through the sample were measured in the frequency range 4.95 – 35.71 kHz, graded into 64 measurement points. The values of the acoustic signal (A_R) after reflection from the sample were measured in the frequency range 0 – 125 kHz, graded into 2048 measurement points.

Figure 3 presents the changes in penetrated acoustic signal amplitude values for the wheat grains at different signal frequencies and *Figure 4* – the changes in reflected acoustic signal amplitude values at different signal frequencies.

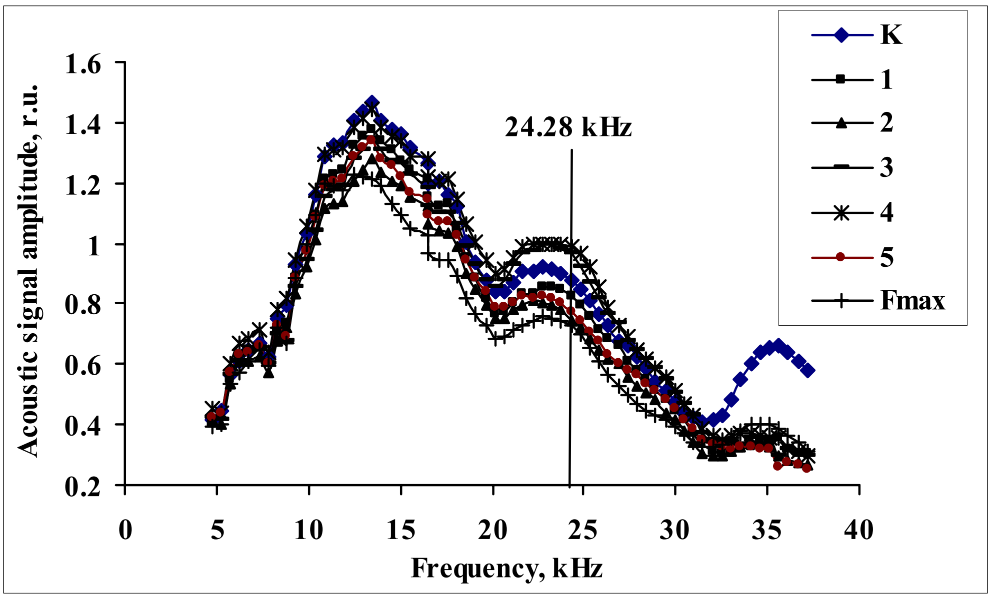

Figure 3. **The penetration acoustic signal at different frequencies of wheat samples (K – control, F_{max} – wheat contaminated with DON and their mixtures:** *1 – K+10% F_{max}, 2 – K+20% F_{max}, 3 – K+30% F_{max}, 4 – K+40% F_{max}, 5 – K+50% F_{max}. (The selected frequency – 24.28 kHz – is optimal).*

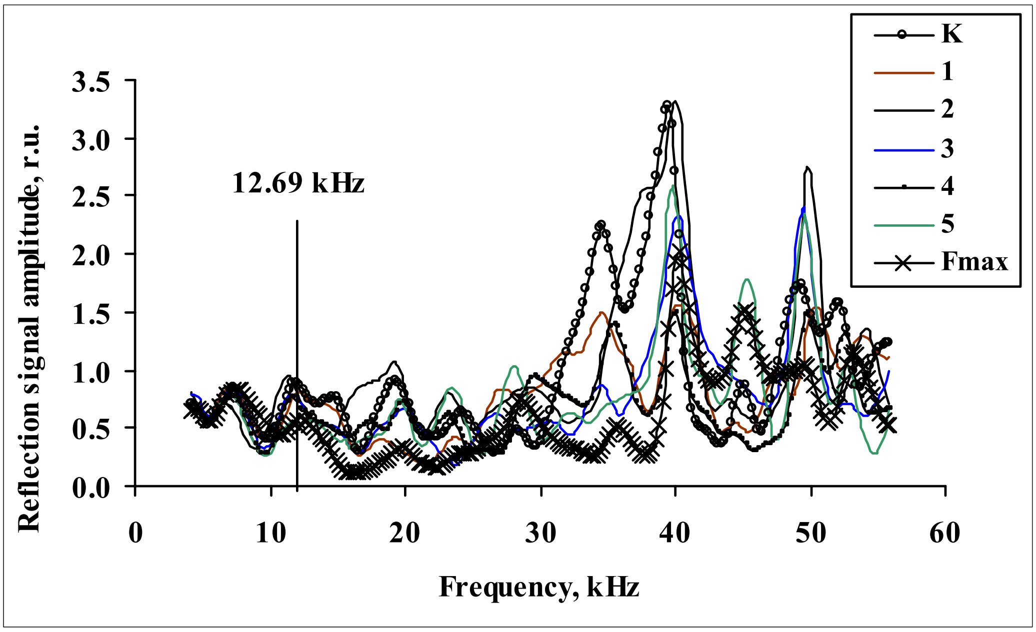

Figure 4. **The reflection signal at different frequencies of wheat samples (K – control, F_{max} – wheat contaminated with a high amount of DON and their mixtures:** *1 – K+10% F_{max}, 2 – K+20% F_{max}, 3 – K+30% F_{max}, 4 – K+40% F_{max}, 5 – K+50% F_{max}. (The selected frequency – 12.69 kHz – is optimal).*

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 interval 10-30 kHz.

The optimal frequency was chosen at the point where the standard deviation and variation coefficient values are minimal.

Samples of wheat have been tested 10 times and the optimal frequencies were determined. In the case of transmission this frequency was – 24.28 kHz and for reflection – 12.69 kHz. At these frequency the standard deviation in case of transmission was 0,105 and the variation – 0.12 and for reflection – 0.089 and 0.13 respectively.

Further investigations were carried out by those optimal frequencies.

Use of ultrasound for the analysis of wheat contaminated with DON. The results between the samples of "scab" contaminated wheat with different levels of DON and penetrated acoustic signal amplitude are presented in *Figure 5*. The results of the investigation show that there exists a dependency between the investigated parameters, e.g. the wheat samples with different levels of DON and the amplitude values of the penetrated signal. It means that by an increase in wheat deoxynivalenol the amplitude values of penetrated acoustic signal decreases.

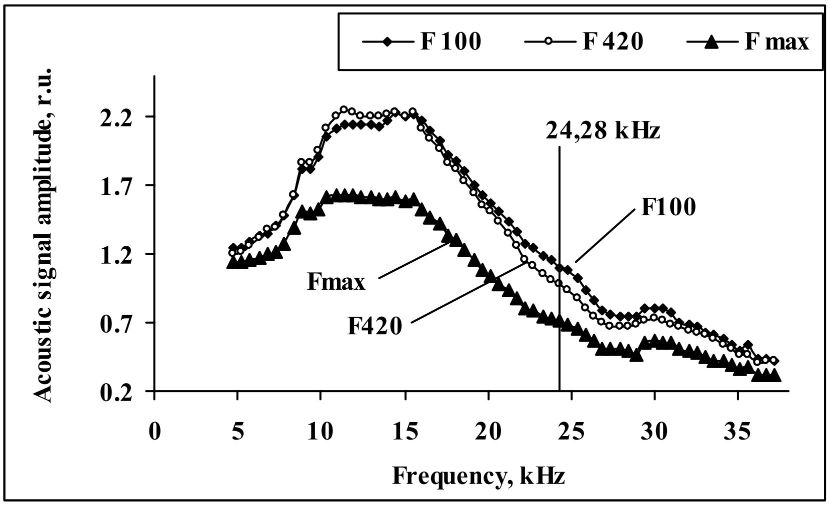

Figure 5. **Acoustic signal amplitude (A_P) at different frequencies by investigation samples of wheat contaminated with different levels of DON:** *F_{100} - 100 ppb, F_{420} - 420 ppb and F_{max} - high amount of ppb's (optimal frequency 24.28 kHz)*

The results of the wheat grain samples after separation (*Figure 6*) showed more differences between the samples as in the previous experiment. The results of the investigations show that carrying out separations of samples could improve the precision of the analysis.

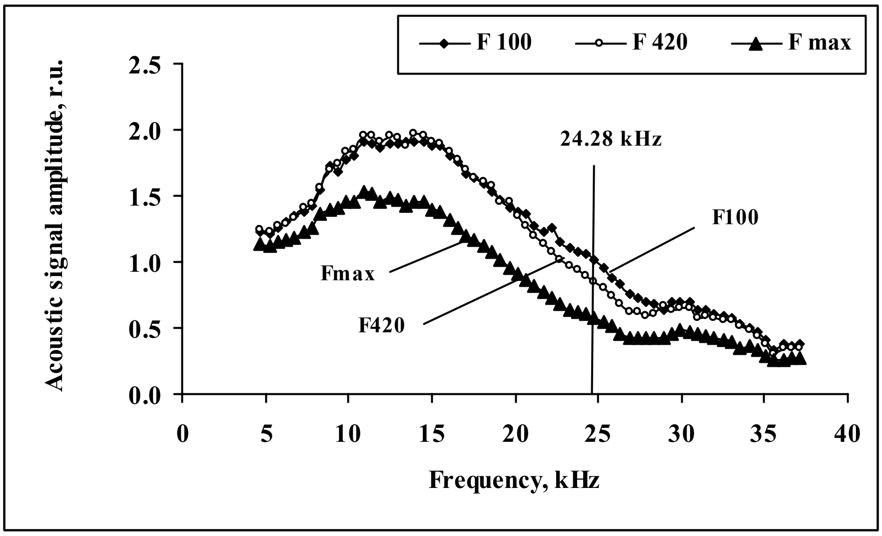

Figure 6. **Acoustic signal amplitude (A_P) at different frequencies after separation through a sieve No. 8 (2x20mm) samples of wheat contaminated with different levels of DON:** *F_{100} - 100 ppb, F_{420} - 420 ppb and F_{max} - high amount of ppb's (optimal frequency 24.28 kHz)*

The results of the different mixtures contaminated with 10; 20; 30; 40 and 50% of shriveled grain determined by the penetrated acoustic signal are shown in *Figure 7*. From this one can see that with an increasing percentage of shriveled grains the amplitude of the penetrated acoustical signal also decreases. So, the given data show that the results of the acoustic measurement using the acoustic spectrometer correspond well with the quantity of shriveled grains in the wheat samples.

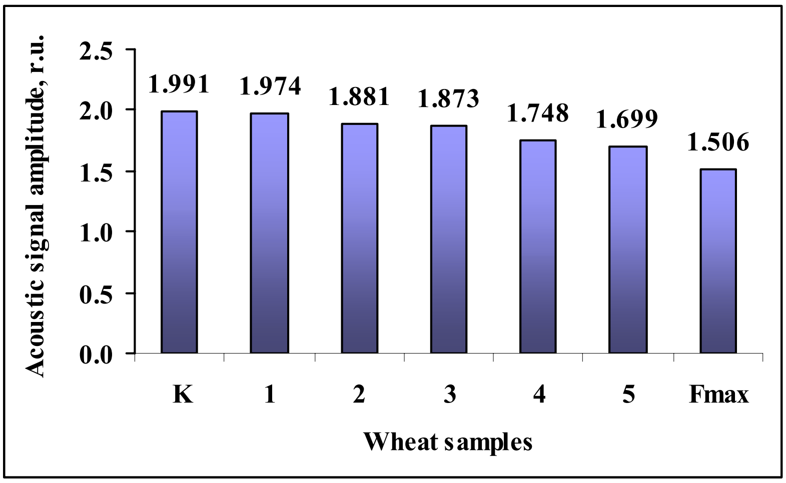

Figure 7. **The penetration acoustic signal amplitude at optimal frequency of wheat samples (K – control, F_{max} – wheat contaminated with a high amount of DON and their mixtures:** *1 – K+10% F_{max}, 2 – K+20% F_{max}, 3 – K+30% F_{max}, 4 – K+40% F_{max}, 5 – K+50% F_{max}.*

The same mixtures were used for the analysis by short acoustic impulse reflection. The results presented in *Figure 8* show that with an increasing percentage of shrivelled grains the amplitude of reflected acoustic signal also decreases. These results show that the acoustic methods (transmission as well as reflection) give adequate and encouraging information for DON investigation in wheat.

Preliminary results of a calibration experiment carried out with an ELISA method (RIDASCREEN) show high correlations (R^2 = 0, 9239) between the acoustic parameter (Ap) and DON quantity as is shown in *Figure 9*.

Fine tuning, correlations and calibrations are now in process to evaluate more precise the results in the ppb range.

At the moment calibration curves are made using conventional analytical methods as discussed above for DON analysis, trying to get information if the acoustic method is capable and precise to characterize the quality of wheat contaminated with DON.

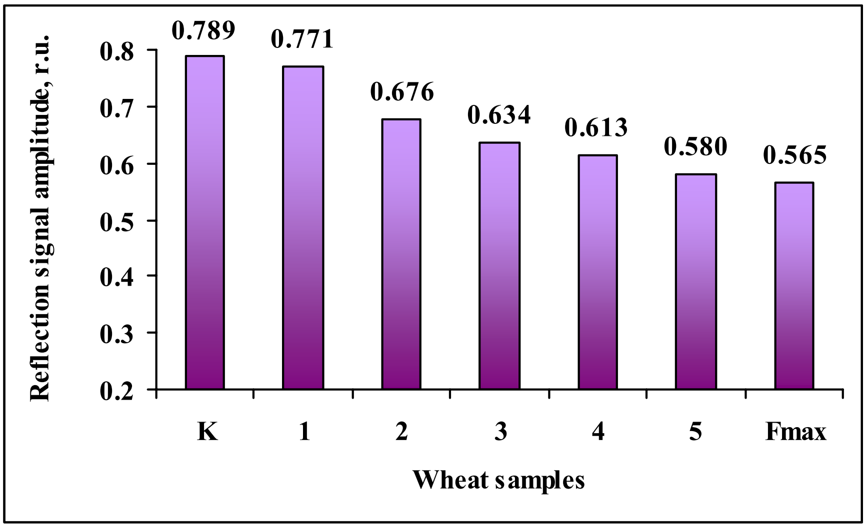

Figure 8. **The reflection signal amplitude at optimal frequency (12.69 kHz) of different wheat samples (K – control, F_{max} – wheat contaminated with a high amount of DON and their mixtures:** *1 – K+10% F_{max}, 2 – K+20% F_{max}, 3 – K+30% F_{max}, 4 – K+40% F_{max}, 5 – K+50% F_{max}.*

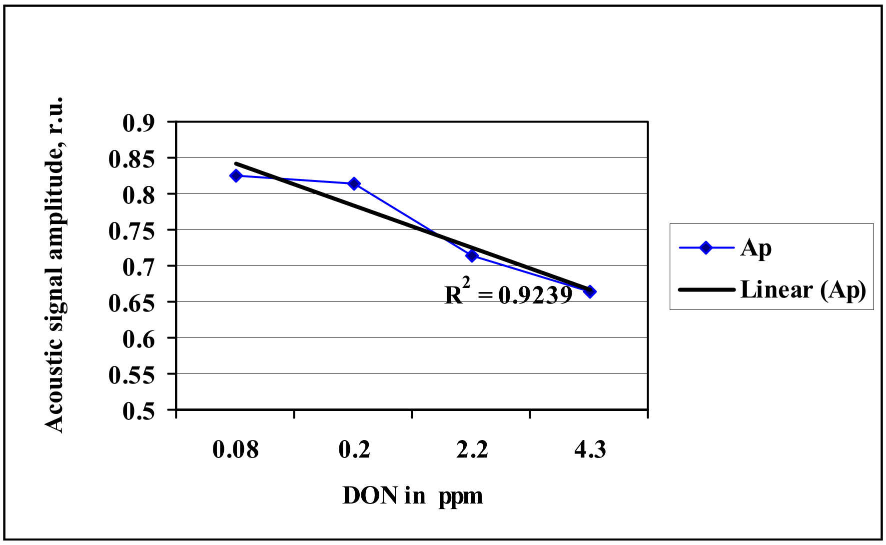

Figure 9. **The relationship between the acoustic signal amplitude (Ap) and the results of DON in ppm by the used ELISA method (RIDASCREEN).**

Conclusions.

- The method originally developed for the structure, texture and extraction of porous food products (bakery and tea) gives promising results in the determination of deoxynivalenol (DON) in wheat.
- To increase the accuracy of the analysis and optimize the acoustic signal an optimal frequency has been determined.
- The acoustic method was improved after investigation in the extended range of frequencies. The precision of the measurements was increased by establishing a proper frequency of the acoustic wave.
- High correlations between the with DON contaminated wheat sample and the different amounts of shriveled kernels in mixtures determined by the acoustic signal penetration and reflection method were found. This means possible application of the given method as screening method for the determination of deoxynivalenol in wheat.
- The investigations show that the acoustic method was sufficient precise. Because the acoustic method is non- invasive it could be successfully used as an effective control- and screening- method for cereals contaminated with *Fusarium* species such as *F. graminearum* producing deoxynivalenol (DON).
- Preliminary results of a calibration experiment carried out with an ELISA method (RIDASCREEN) to confirm the different relationships show high correlations.

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