ANALYTIC EVALUATION OF OILSEEDS

Doctoral (PhD) Theses

Andrea GUBICSKÓNÉ KISBENEDEK

Head of the Doctoral School of Health Sciences:

Professor József BÓDIS Ph.D., D.Sc.

Doctoral Program: (P-4/1) Oncology-Health Sciences
Gastroenterologic diseases (PR 6/3) subprogram
Program leader:
Professor Zoltán DÖBRÖNTE M.D., Ph.D.,
Supervisor:
Professor Mária FIGLER M.D., Ph.D.,

University of Pécs, Faculty of Health Sciences
Health Sciences Doctoral School

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INTRODUCTION

In the past decade, a great deal of intensive research focusing on free radicals' damage and antioxidant compounds has led to the conclusion that dietary antioxidants play a fundamental role in the prevention of various diseases, including cardiovascular, tumour-related, and several other age-dependent diseases as well. Based on the already existing findings, we may claim that health-maintenance and disease-prevention is possible through the intake of antioxidants or antioxidant-mixtures, which can be found in nature, in animals and plants alike. It would favourably influenc the society's health status if decision makers, health care practitioners and media advocates could channel evidence-based, authentic information into the population. There is evidence that prevention is more cost-effective both for the individual and the national economy than the treatment of developed disease. Of all dietary antioxidants, the role of vitamins in prevention and therapy of diseases caused by increased oxidative stress has become well-known by now. However, despite extensive research, we have little knowledge on the role of non-vitamin-like antioxidants in fighting lifestyle diseases. Plenty of further chemical and biochemical research is required to determine the exact amount of flavonoid contained by comestibles and nutrients, and to map their exact physiological effect.

The use of oilseeds in Hungary is fairly common. It is used in the baking industry to increase fiber-content, and other types are used as snacks, even in salted or roasted forms. Mustard seeds are often used in the kitchen and also in industrial preserving as a spice, and black mustard seed, a special kindvof them, is used in pharmacy as raw material. Furthermore, its use both in human nutrition and animal feeding is advisable, as it has favourable aminoacid-consistence and it is also a source of biologically active compounds. These antioxidative substances play a major protective role in the prevention of cardiovascular- and tumor-related diseases, thus the consumption of oilseeds can be health-protective. The only drawback of the mustard seed is its high erucic acid content, which can be reduced through sublimation, a practice often used in Hungary.

OBJECTIVES

The main goal of the present study was to examine the composition of oilseeds, focusing on detecting resveratrol. Dietary intake of oilseeds may be versatile: some seeds are consumed

roasted, others without roasting. The aim of the study described in this thesis is twofold: to understand the factors influencing the composition of oilseeds, and to find out if the processing heat affects the amount of resveratrol, the most favourable form of oilseeds to consume. The difference in the amounts of of exposable compounds in roasted and unroasted types was examined.

Furthermore, the study also covered the question whether or not the cultivation technology influences the composition of oilseeds; wehter or not bio- and regular mustard seeds have different composition?

In terms of nutrients, protein levels, protein- and amino acid composition, fat levels and fat- and carbohydrate composition of oilseeds were analysed. Microbiological examination determied the total number of living germs; and the microbiological biochemical tests identified the microorganisms on mustard seeds. As there is a string of data on bactericidal and fungicidal features of mustard, the present study focused on the influence mustard seeds have on the best known pathogens, a result of the mustard seeds' volatile oil content and composition. Thus another aim of the study was to define the quantity and composition of volatile oil.

As the present study was performed using the cultivated kinds of individual oilseeds obtained with multiple sampling, the results are not representative for the oilseeds, they only allow for making recommendations in general..

MATERIAL AND METHODS

Time-span and sample

The research presented in this thesis was carried out at the Medical School and Faculty of Health Sciences at the University of Pécs, between 2006 and 2011. Non-randomized, convenience sampling was used. Each test was carried out five times on each sample, and the average of the five measurements was taken for one result. Sampling was carried out twice during the time-span of the research, thus the results describe only the seeds grown and examined in the given year and provide no general information about the types of seeds examined. Statistical analysis conducted in this work included t-tests and analysis of variance (ANOVA), with the help of Microsoft Excel 2010 and SPSS 20. Results were considered significant in case $p \leq 0,05$.

Oilseeds used during examination:

1. Sunflower (Helianthus Annuus L.; Hungarian distributor: Spar Magyarország kereskedelmi Kft)
2. Peanut (Arachis hypogaea L.; Hungarian distributor: Biopont Kft)
3. Almond (Prunus amigdalus; Hungarian distributor: Rapunzel Naturkost GmbH)
4. Linseed (Linum usitatissimum; Hungarian distributor: Biopont Kft)
5. Sesame seed (Sesanum indicum; Hungarian distributor: Biopont Kft)
6. Pumpkin seed (Cucurbita; Hungarian distributor: Rapunzel Naturkost GnbH)
7. White mustard seed (Sinapis alba; foreign white mustard seed: Hungarian distributor: Biopont Kft; bio white mustard seed: Mohács)
8. Black mustard seed (Brassica nigra; bio black mustard seed: collected at Mohács, wild black mustard seed: collected at Mosonmagyaróvár)

In the case of peanut seeds, industrial processing includes a dry roasting procedure with intensive moving at 140-160 ^{o}C for 2-3 minutes. Roasted and unroasted peanut originated from the same sample, thus the examination focused on identical breeds.

In the case of mustard seeds used in laboratory examination, the circumstances and the exact time of cultivation are known. The mustard seeds analysed were grown in 2006. The foreign white mustard seed (Hungarian distributor: Biopont Kft.) originates from Rača, Slovakia, and was cultivated with traditional methods. The bio white and black mustard seeds were grown in a biogarden in Mohács, in accordance with the principles.of ecological agriculture The white mustard seeds in both cases were the ‘Albatros’ breed (type identification was completed in the case of the foreign seed as well, since the sample used in this study was grown in experimental fields); the black mustard seed was the ‘Sámson’ breed. Wild mustard seeds were collected near Mosonmagyaróvár, Hungary, these were not specifically cultivated. All samples used in the present study were cultivated in nearly similar circumstances, as the average hours of sunshine and the quantity of rainwater was equal; the only difference in the quality of soil was a result of the fertilization technique applied: chemical fertilizers were used during regular cultivation and organic cow manure was used as fertilizer during bio cultivation.

Determining resveratrol:

Number of samples: 11 (sunflower seed, roasted peanut, regular peanut, sesame seed, pumpkin seed, almond, linseed, bio white mustard seed, bio black mustard seed, foreign white mustard seed, wild black mustard seed)

The HPLC system consists of Gynkotek M 480 GT pump, Rheodyne 8125 injector (with a 20 µl sample loop) and Gynkotek M 340S UV DAD (diode line) detector. A full 3D visual was produced with the help of the diode line detector (chromatogram + UV spectrum depending on time). The apexes of components essential for the study were located based on the retention time, and identified based on the spectrum. Flow rate was 1.5 ml/min.

Separation was completed with the help of a great surface coverage-, 5 µm average globule diameter C18 (covered with octadec sylanol groups), converse phase pillar, that was developed in the Institute. The pillar was 250 mm long and had a 4.6 mm inside diameter. Evaluation was based on the size of the area under the apexes. Cromatographic separation was completed through a multi-step gradient technique, detecting completed at 306 mm, thus this is the resveratrol's maximum absorption level. Definition of the chromatographic apexes was carried out through comparison of the retention times and the spectra to the standards. Assessment of quantities was completed by calibration curve.

Chemical examination of the mustard seeds:

Number of samples: 4 (bio white mustard seeds, bio black mustard seeds, foreign white mustard seeds, wild black mustard seeds).

Water content was determined with gravimetric method, protein content was determined with Kjeldahl-method.

A BIOTRONIC LC 3000 amino acid analyser, using a BTC2410 cation swapping colophony, separated the amino acids. Quantity assessment was carried out based on comparison, after chromatographing a 2.5 µM/ cm^3 standard amino acid solution. Protein-extraction was completed by gel electrophoresis, using the Laemmle method.

Separation of proteins by molecular weight was carried out in a SDS-PAGE Bis-Tris gel containing 12% acrylamide, followed by the sample analysis in MALDI TOF MS.

Analysis of the spectra resulting from our experiments was carried out by FlexAnalysis 2.4 software. Processed spectra were evaluated by the Mascot (Matrix Science, London) programme, and the result of the MS/MS-based peptide- and protein evaluation was compared to the BLAST database. In the case of peptides and proteins, those were accepted, which had an identification probability above 95% ($p<0,05$).

Fat content analysis was completed by Soxhlet extraction.

Separation of fatty acid methyl esters was completed by Carbo-Erba Fractovap gaschromatography.

Evaluation of total carbohydrate content was carried out by cholhydric acid hydrolysis and Schoorl-method.

Volatile oil content was determined by direct capacity measurement, while separation and determination of the volatile oil components were carried out using thin layer chromatography.

Microbiological examination

Definition of total number of living germs: The total number of living germs was calculated by the number of colonies developed at firm breeding ground from an evident measure of food delution, among specified incubation circumstances.

Microbiological-biochemical identification: based on the taxonomy unit features in connection with a given bacteria's metabolism, that may be identified easily and quickly with various tests.

During examination of the antibacterial effects of mustard, disks containing the hexane- and ethanol-based mustard seed meal was set on the surface of the blood agar base of Salmonella typhimurium, Escherichia Coli, Pseudomonas aeruginosa, and Bacillus cereus cultures. Incubation of the disks was carried out for 24 hours at 37 ˚C temperature, the inhibitive zones around the blotting paper was analysed afterwards.

RESULTS

Results of the resveratrol-analysis:

Highest level of resveratrol was demonstrated in the sunflower seed (0,0398±0,01mg/100g), and smaller amounts were demonstrated in the almond (0,0176±0,021mg/100g), the roasted peanut (0,022±0,013mg/100g) and the wild clack mustard seed (0,023±0,007 mg/100g). The resveratrol-content of the other oilseeds examined was insignificant. Oilseeds can be considered as a good source of resveratrol, but it is also important to mention, that sunflower seed (which contains the highest level of resveratrol of all oilseeds examined in the present study) contains an amount of the beneficial compound equal to that of the traditionally good resveratrol-source red wine, especially merlot and pinot noir types. Results of the current examination also show that the amount of this chemical compound can be increased through roasting, since the analysis of regular and roasted peanuts proved that the latter contained significantly more resveratrol. Thus we may claim, that buying the roasted type is more worthwhile, although the present study provided evidence only for peanuts. Further research is necessary. to clarify whether roasting or other heat-processing increase the resveratrol-level of other oilseeds,

Results of chemical analysis of the mustards seed:

Our analysis confirmed that, based on the nutritional value of mustard seed, it is a highly valuable spice. No significant difference was found between the cultivated and wild breed types, and the data found in the literature. Only small differences were found in the nutrient-content of the individual mustard seeds in terms of protein and fat content. No significant difference was found among the samples in terms of water content – this level was highest in the foreign white mustard seed (7,32±1.12%). Some previous research provides data for 5.16±1.22% moisture, which is significantly lower than what was found in the presenta nalysis ($p \leq 0.05$). Values of the rest of the samples were also higher than those found in the literature, but no significant difference was detected ($p \geq 0.05$). The highest level of moisture was found in the commercially available wild black mustard seed (6.7±1.16%), the lowest level of moisture was found in the bio white mustard seed cultivated in Mohács (6.15±1.31%).

Somewhat greater differences were found in fat-content: the highest level was found in the wild black mustard seed (39.78±0.46%), and the lowest in the bio white mustards seed (20.5

±0.79%). Difference between the other two values is insignificant: foreign white mustard seed's rate was 25.10±0.63%, the bio black mustard seed's was 26.08±0.59%. The two means were also somewhat lower compared to the data reported in previous research, where the average fat-content of the mustard seed is 28%.

Concerning the fatty acid content we may claim that mustard seeds contain a substantial amount of linoleic acid, which is essential for the human body. A smaller amount of saturated fatty acids were found during the analysis, for example palmitic acid and stearic acid. Erucic acid content of the mustard seed in the present study was 32.78g±1.19/100g, which is lower than the data found in the literature (35.13g/100g). No difference was detected in the fatty acid content on the basis of the various types of mustard seeds or the technology of cultivation.

A significant difference was detected among the samples in terms of protein-content: the highest level of protein was found in the foreign white mustard seed (38,17± 2,34 %), and the lowest level was found in the bio white mustard seed (27,20 ± 2,16%); the difference among them is significant ($p \leq 0,05$). The level detected in the bio black mustard seed (34.8 ± 3.004%) is in accordance with previous findings, and also there is only a slight difference between previously published data and the outcome of the present study (34.49± 2.57%) in the protein level of the wild mustard seed.

During amino-acid-analysis we have found that the mustard seed also contains essential amino-acids, while it lacks tryptophan and isoleucine. Like all proteins of plant origin, mustard-protein is also rich in glutamic acid and asparagine acid. High levels of sulphur-based amino-acid- and lysine-content proves that mustard-protein possesses positive amino-acid compound. Amount of the essential amino acid in case of 100 grams mustard protein is 36.30± 1.97g. Limiting amino acid of the mustard-protein is valine. In the case of the mustard seed, only one difference in the compound of the amino-acid was found: in the amount of glutamine acid. The levels in the wild mustard seed (21,2±0,78g), were slightly higher than those of the cultivated types, but the difference here was not significant.

Based on the results of the current protein-analysis we may claim, that we succeeded in demonstrating proteins in the mustard seed that prove the so-far known and assumed positive physiological effects of this oilseed-breed. During our analysis we have detected enzymes splitting in the seeds, enzyme inhibitors and structural proteins. The protease inhibitor proteins can also be found in the pumpkin seed. In the mustard seed this inhibitor protein was

detectable in all samples examined, just like the proteins playing a role in the synthesis of the flavonoids, to which previously published data can be compared in terms of the mustard's phenol-content. RanBP1-protein was identified in the present study as well, which plays a role in the synthesis of the phenol-compounds in the body of the plant and was previously identified from the pulse-type plant resembling the clover, that lives near the Mediterranean Sea. Furthermore, the allergen *Sin a 2* protein was detected from all of our samples, although the primary cause of the allergic reactions, the *Sin a 1* protein was not detected in our samples. This may influence the severity of the evolved allergic reaction, since the development of the anaphylaxic shock is justified by previous findings in the case of *Sin a 1* protein, and only less severe reactions are described in the case of the *Sin a 2* proteins. Based on the above, we may assume that not all types of mustard seeds contain *Sin a 1* proteins, thus in the case of particular types the evolving allergic reactions may cause less severe skin irritation or asthmatic complains.

Based on the result of the preswent study, we may claim that except for one case, the oil-content of the protein-containing mustard varieties was generally lower. A statistically significant (confidence level: 95%) negative correlation was found between the protein- and oil content. All this is in line with previously published data describing negative correlation between the protein- and oil-content of several plants. For example, Wilcox (1998) described a similar interdependence in the case of soy.

Regarding carbohydrate-content, our analysis proved that the various samples examined contain approximately similar amounts, and there is only a small difference compared to the data found in the literature. The highest carbohydrate-content was found in the bio white mustard (21,06±0,73%), and the lowest in the bio black mustard. This is in between the amount found in the other two samples (20,44-21,06%). Previous research reported 18±0,58%. Thus we may claim that the differences between the various samples are not significant ($p \geq 0,05$). This is also the case when we compare them to previously published data.

During the **determination of volatile oil**, content figures were found higher than preeviously published data (0,16 ±0,07ml/100g) in all cases. The highest data was found in the case of the wild black mustard seed (0,62 ±0,31 ml/100g), the rest of the figures were approximately similar to one another 0,22-0,28 ml/100g). During the volatile oil-component identification

process, the following compounds were detected: piperidine, limonene, dipiperine, eucalyptol, and the terpinen-4-ol.

Results of the **microbiological study** prove that the examined samples' germ count on all substrates was lower than the limit value defined by the Ministry of Health in the decree nr. 4/ 1998 (XI. 11). The results of the various germ-identification processes show that they do not contain enteral pathogens.

Based on the results of the present study, we may claim that the mustard seed meal has antimicrobial effect against some bacteria, for example, a rather strong bacteriostatic effect can be observed in the case of Salmonella and Escherichia coli. However, mustard seed meal had no effect on the Pseudomonas aeroginosa which has high resistance against antibiotics, thus this result is not surprising.

Despite the favourable chemical composition, the mustard seed's wide consumption is limited by its bitter taste, and itsoil's high erucic acid ontent. Because of its favourable nutrition physiology content, the use of mustard seed in food- and pharmaceutical industry is more and more approvable – just like its use in animal nutrition, which is mentioned in some previous publications on the subject. Further analysis is required to define in what form, and in exactly what quantity could mustard seeds be applied. Thus oilseeds must play a role in healthy eating. The most important limitation to their intake is their energy-content. Our result show that humans should consume greater quantities from these seeds (around 200-300g) for the proper level of resveratrol intake. Our suggestions primarily consider the consumption of sunflower seeds, as this is the most popular type among Hungarian consumers due to its availability and affordable price. Also, our analysis proved that this seed contains the highest resveratrol level, as well. Furthermore, one should assume that it is not only these plants that cover the resveratrol-intake. Nutrition studies and the recommendations based on them suggest that 30-40g oilseeds per day is sufficient, when it is consumed as part of a balanced diet. This is also supported by the study published in November 2013 where the positive health effects of oil seeds (walnuts, specifically) were analysed. That work consisted of a 30-year follow-up examination of the frequency of oilseed consumption in relation to mortality resulting from chronic cardiovascular disease, cancer, type-II diabetes and respiratory disease. The results showed that those participants who consumed 30 g of these types of seeds per day reduced mortality rate by 20%. Factors blurring the estimation were excluded during the examination process, thus the authors highlight the importance of oilseed- and hard-shell

seeds consumption in a Mediterranean diet. Increasing the sample sizes of the forthcoming examinations should provide a better picture concerning the compunds of the oilseeds. We also aim to define the resveratrol-content in all types of roasted oilseeds. However, an obstacle to carrying out such a study is in purchasing such roasted and unroasted seeds parallel to the identification of the type. Also, in our further examinations we aim to include types of oilseeds not examined in the present study (eg: soy, conola and grapeseed).

NEW RESULTS

1. The resveratrol-content of the examined wild black mustard seed was 0,023±0,007 mg/100g. The bio black mustard seed's resveratrol-content was lower, only 0,017± 0,0116mg/100g. The difference is not significant. Difference between white and black mustard seed of our sample is not significant either.
2. Detectable resveratrol-content of the peanut can be increased through industrial roasting.
3. The highest level of protein-content was found in the case of foreign, traditionally cultivated white mustard seeds, 38,17 ±2,34%; the lowest protein-content in our sample was found in the case of bio white mustard seeds, 27,20±2,16%.
4. The wild black mustard seed examined in the present study has a higher level of glutamic acid (21,2±0,78g/100g) than the other types of mustard seeds of the sample.
5. Ran BP1 domain-containing protein was demonstrated in the analysed mustard seeds, which has a role in the biosynthesis of flavonoids.
6. The types of mustard analysed in this study contain serpin-6 precursor protein, which is the inhibitor protein of proteinase.
7. The mustard seeds analysed in the present study contain glucan synthase enzyme.
8. The types of mustard analysed in the present study contain steroleosin, which play a role in the signalling process and the composition of lipopolysaccharides.
9. The mustard seeds analysed also contained the Mpt5 protein, which is the mutation of the POP2 gene and has anti-carcinogenic effect.
10. The highest fat-content was found in the wild black mustard seed sample (39,78±0,46%), and the lowest in the bio white mustard seed sample (20,5±0,79%). The traditional cultivation technology resulted in fat-content higher than the bio cultivation technique.
11. The black mustard seed contained significantly higher levels of volatile oil than the other samples examined in this study.

12. The mustard seed meal examined in this study has antimicrobial effect. There was a bacteriostatic effect observed in Salmonella and the Escherichia coli. The highest inhibitive zone was observed in the wild black mustard seed.

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