OPTIMIZED MICROWAVE DIGESTION METHOD FOR IRON AND ZINC DETERMINATION BY FLAME ABSORPTION SPECTROMETRY IN FODDER YEASTS OBTAINED FROM PARAFFIN, METHANOL AND ETHANOL

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abstract: In this paper a simple and rapid acid digestion method by microwave heating in high-pressure Teflon-TFM bombs is reported for the determination of total iron and zinc content in fodder yeasts, by flame atomic absorption spectrometry (FAAS). The analytical results were agreed well, as confirmed by the statistical evaluation, with those obtained after conventional wet digestion method. This procedure was applied to the determination of Fe and Zn in some Romanian fodder yeasts growth on *n*-paraffines, methanol and ethanol.

keywords: Fodder yeasts, microwave digestion, iron, zinc, FAAS.

Introduction

Fodder yeast is the name given to the total dry mater of yeast biomass when is used as an animal feed supplement. Yeast biomass is microbiologically synthesized proteins, usually termed single cell proteins (SCP) due to the unicellular nature of its parent microorganisms (*i.e.* yeasts). Growing microorganisms for animal feed has two main attractions: first, the growth rate of microorganisms is very much faster that those of plants, thus potentially shortening the time needed to produce a given mass of feed; second, a range of raw materials can be used to produce SCP, depending on microorganisms chosen. The main raw materials considered for SCP are oil fractions (hydrocarbons, *n*-paraffins, *n*-alkanes), methanol, ethanol, carbohydrates and cellulosic materials from various sources. Another advantage of SCP as animal feed supplements is its higher nutritional values compared to vegetable proteins [1].

Fodder yeasts could enrich with high quality protein, and other physiologically active substances, the animal feedstuffs reducing the requirements for such materials as soybean meal and fish meal. The nutritional value of fodder yeasts is especially due by the percentages of essential amino acids in proteins, but also by the contents in essential metallic elements. Therefore, the accurate determination of concentrations of metallic elements in fodder yeasts is necessary and may help the specialists in animal nutrition to elaborate an efficient diet for animal feeding [2].

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A broad variety of analytical techniques have been used to determine trace and essential elements in organic matrices materials: atomic absorption spectrometry with flame (FAAS) and graphite furnace (GFAAS), inductively coupled plasma optical emission spectrometry (ICP-OES) and mass spectrometry (ICP-MS), etc. [3]. Most analytical methods for trace element determination in organic matrix materials require total decomposition of the sample. Hence, sample decomposition procedures play an important role and the efficiency of the mineralization procedure is of great importance [4, 5]. Sample preparation is still the major factor contributing to the uncertainty of the final analytical results [6].

Pretreatment of organic matrices materials for atomic spectrometry usually involves ashing of the sample (dry ashing method) and subsequent dissolution of the ash in an acid medium (usually nitric) or, alternatively, direct acid treatment (wet acid digestion method). Sample dry ashing is lengthy and prone to losses of the more volatile elements. Conventional wet acid digestion method using a hot plate requires high boiling point oxidizing acid mixtures and long reaction times. After digestion, filtration is commonly needed, which increases the risk of sample loss or contamination. Although these classical digestion methods are still well accepted, they generally are time-consuming and require a great deal of analyst attention, skills and experience in order to gain accurate and precise analytical results [5, 7].

Based on the consideration of shortening working time and minimizing volatile loss of analytes, microwave digestion technique is regarded as a more convenient and precise sample pretreatment method. The use of microwave energy as a means to rapidly and complete wet acid digestion of samples is increasing, as shown by the large number of reviews and books dealing with the principles and applications of microwave digestion techniques for elemental analysis for a wide range of sample matrices. Open-vessel, closed-vessel, and focused techniques are all well documented in the literature. Although open-vessel microwave digestion is popular because of its simplicity, such methods suffer from the same drawbacks as conventional methods: loss of volatile elements, risk of sample contamination, release of acid gases, and maximum digestion temperatures limited by the boiling point of the acid mixture. In contrast, samples digested in closed-vessel environments are protected from the atmosphere and undergo accelerated decomposition reactions as a consequence of increased pressure and temperature. In addition, the elevated temperature in a closed-vessel procedure increases the oxidizing power of mineral acid and achieves decomposition of matrices components that would not be possible in an open-vessel or other conventional environments. Closed-vessel microwave digestion has been successfully implemented for many different types of organic matrices samples [8 – 25]

Microwave digestion has clear advantages over more traditional procedures: a shorter acid digestion time; a supposed better recovery of volatile elements; lower contamination levels; minimal volumes of reagents are required, more reproducible procedures; and a better working environment. Furthermore, the closed vessel microwave digestion has a unique advantage over other closed vessel technologies. Microwaves only heat the liquid phase, while vapors do not absorb microwave energy. The temperature of the vapor phase is therefore lower than the temperature of the liquid phase and vapor condensation on cool vessel walls takes place. As a result, the actual vapor pressure is lower than the predicted vapor pressure. This sort of sustained dynamic, thermal non-equilibrium is a key advantage of microwave technology, as very high temperatures (and in turn short digestion times) can

be reached at relatively low pressures. These days several microwave sample preparation methods have been accepted by international organizations [21, 26].

The aim of this work was to develop a rapid, reproducible and reliable analytical procedure for the sequential determination of Fe and Zn (which are elements of our main interest) in some Romanian fodder yeast using FAAS following closed-vessel microwave acid digestion. To evaluate the microwave digestion method, the results obtained with this method were compared, from statistical point of view [27] (*F*-test, *t*-test), with those obtained by FAAS after conventional wet digestion in open vessel heated on hot plate.

Experimental

Instrumentation

For conventional wet digestion, all samples were digested in 150 ml Pyrex-glass beakers covered with watch-glass and heated on a hot plate.

The microwave digestion system used for sample preparation was a Milestone MLS 1200 MEGA. The power range of the microwave oven may be set in 10 W increments up to a maximum of 1000 W. The system is equipped with an microwave digestion rotor (MDR 1000/6/100/110) containing six high pressure digestion vessels (each one capacity of 100 mL) model HPS 100/110, and with a laboratory-made cooling station with circulating water. The vessels are constructed of Teflon-TFM (tetrafluormethaxil; Registered Trademark of Hoechst) and are capable of withstanding temperatures of up to 260 °C and a maximum pressure of 110 bars. A detailed description of Milestone MLS 1200 MEGA microwave system was presented elsewhere [2].

Atomic absorption signals were measured with a Zeiss flame atomic absorption spectrometer (model AAS 3, Germany). The instrumental conditions used for the Fe and Zn FAAS measurements are given in Table 1. A single slot 10 cm burner with an air – acetylene flame was used. The burner position, flame condition and aspiration rates were optimized for maximum absorption. The integration period was set as five seconds and the average of five replicate readings was taken.

Table 1. FAAS operating parameters

Instrumental parameters	Fe	Zn
Lamp current (mA)	4	4
Wavelength (nm)	248.3	213.9
Slit (nm)	0.2	0.2
Air / Acetylene ratio	60 / 85	60 / 90
Burner position	Normal	Normal
Observation height (mm)	7	7
Background corrector	NO	NO

Reagents

Triply distilled water (TDW) was used for sample and solutions preparation. All reagents

used (*i.e.* concentrated HCl, HNO_3, $HClO_4$, H_2O_2) were p.a. quality (Merck). Monoelemental Fe and Zn standard solutions (1000 mg element /L), each in Merk Titrisol ampoules, were also used for daily preparation of working standard solution, by serial immediate dilution with TDW, and used freshly prepared.

Labware cleaning

The glass bakers, watch glass, pipettes and calibrated flasks were rinsed with water and cleaned by soaking in 10% (v/v) nitric acid overnight, followed by thoroughly rinsed with TDW. Prior to their use, the Teflon-TFM vessels were first soaked in HNO_3 + HCl + H_2O (1:2:9) solution overnight. Then the following microwave cleaning procedure was carried out: a 30 ml aliquot of 10% HNO_3 was added to each vessel; the digestion vessels were sealed and heated with microwave energy (5 min at 250 W and 10 min at 400 W); after cooling, the contents were discarded and the vessels were thoroughly rinsed with TDW.

Results and Discussion

The aim of the present work was to evaluate the benefits offered by microwave digestion procedure and to create a routine digestion method of fodder yeasts prior to determination of iron and zinc (or other metallic elements) by FAAS. The samples analyzed were tree Romanian powdered fodder yeasts obtained from *n*-paraffins (sample S1, from RONIPROD; Curtea de Arges), methanol (sample S2, from Institute of Chemical and Pharmaceutical Research – ICCF; Bucharest) or ethanol (sample S3, from Institute of Chemical and Pharmaceutical Research – ICCF; Bucharest) as source of carbon substrate in growth medium for microorganisms (*i.e.* yeasts). All samples were dried to constant weight at 60 °C in a oven (about one day). The dried materials then were ground in an agate mortar with an agate pestle, before weighting and carrying out the analysis.

Sample digestion methods

Conventional wet digestion at normal pressure (reference method).

About 500 mg of dried sample were weighed into Pyrex-glass beaker, covered with a watch glass and 10 mL of HNO_3 were added. The sample was allowed to stand for about two hours at room temperature and then was boiled on hot plate, first gently to oxidize all easily oxidizable matter, then with greater intensity. After cooling, 5 mL of 30% H_2O_2 were added and the content was heated again, until the brown fumes of nitrogen dioxides disappeared. To eliminate the excess of H_2O_2, the solution was boiled once more. Finally, after cooling, 2 mL of $HClO_4$ were carefully added and the content was heated again, very gently, until a clear, colorless solution resulted and a white fume appears. After cooling, the solution was quantitatively transferred into a 25 mL volumetric flask and diluted to volume with TDW. The blank were prepared with the same reagents, without the samples, undergoing a similar digestion procedure. This digestion is considered and accepted as a reference method.

Microwave acid digestion in closed-vessels (proposed method).

All the microwave digestions were performed with a Milestone MLS 1200 MEGA computer controlled microwave system (6 closed Teflon-TFM vessels). Dried sample

(about 200 mg) was weighed directly into digestion vessel and was soaked with 5 ml concentrated HNO_3. The mixture was allowed to stand overnight at room temperature to oxidize all easily oxidizable matter (predigestion step) and then the vessel was closed. By predigesting samples overnight, the organic matrices materials were partially decomposed. The first digestions were performed using microwave oven at 250 W ("unpulsed" mod) for 3 min. After cooling (in a cooling station with circulating water) vessels were opened and 2 mL HNO_3 and 3 mL H_2O_2 (30%) were added. Samples were allowed to react with the peroxide for 1 h before closing, and then heated again with the next digestion program. Step 1: power 250 W, time 1 min; Step 2: power 0 W, time 2 min, Step 3: power 250 W, time 5 min, Step 4: power 400 W, time 5 min, Step 5: power 600 W, time 5 min; Step 6: power 250 W, time 5 min: Step 7 (ventilation): power 0 W, time 2 min. After cooling (about half an hour, in order to reduce the pressure and temperature inside the vessels to ambient values), each "bomb" was opened carefully in a fume cupboard. The sample solutions were transferred to 25 ml calibrated flasks and diluted up to mark with TDW. The blank solution, with identical regents, subjected to the same treatment as the fodder yeast samples, was prepared as well.

Unfortunately, in our microwave digestion system no monitoring accessories for temperature and pressure were available. Because there is no way of monitoring the pressure and temperature inside the high-pressure vessels, the effect of these parameters on the dissolution could not be evaluated. For this reason, our objective was only to obtain conditions that resulted in clear and colorless, or slightly colored solutions, after dilution with TDW. Therefore, as a criterion for the completeness of digestion, the clearness of the sample solutions by visual inspection was used. After conventional wet digestion and after dilution with TDW, clear and colorless solutions without residue resulted for all types of fodder yeast samples.

After microwave digestion, the diluted sample had a light-yellow colour, indicating that, probably, the organic materials in these samples were not completely decomposed. The use of H_2O_2 indeed reduced the carbon content in the digestate as reported by other studies [28]. But a colourless diluted digestate is not an indicator of good analyte recovery as determined by FAAS. Previous tests showed that using HNO_3 + H_2O_2 digestion in which H_2O_2 was added after the overnight predigestion with HNO_3 but 1–2 h prior to the microwave digestion, the analytical precision was improved, especially for the elements with low recoveries. The major concern in the use of H_2O_2 for digestion is the contamination from its impurities. However, if high purity H_2O_2 reagent is available, then the addition of H_2O_2 to samples several hours after the addition of HNO_3 is recommended.

FAAS determination of Fe and Zn in fodder yeasts samples

Iron and zinc (the elements of our main interest) were determined in the digested solutions obtained after both digestion procedures by FAAS, well-known and recognized as a reference technique, directly or after suitable dilutions. For all samples (S1 – S3) three replicates (n = 3) were made, for both digestion methods. Quantification of metals content was based on direct calibration graphs obtained from standard working solutions containing the same acid concentrations as the digested sample solutions. The experimental conditions used for the determination of each element are given in Table 1. All absorbance of standard and sample solutions were corrected for the blank value, which was obtained before

carrying out the quantification.

Analytical results of iron and zinc determination, obtained after both conventional wet digestion and microwave digestion, corresponding to the fodder yeast analyzed, are shown in Table 2 and Table 3, respectively. Analytical results are expressed as mean concentration ± standard deviation (SD), expressed in µg/g. In general, the analytical results for iron and zinc appear to be slightly higher after conventional wet digestion. A comparison of methods precision, expressed as relative standard deviation (RSD%), shows the microwave digestion method being more precise that conventional wet digestion method.

Moreover, in order to evaluate the precision and accuracy of the microwave digestion method, the analytical results obtained after this digestion method were compared, by means of the *F*-test and Student's *t*-test [27], with those obtained after conventional wet digestion. The calculated values of statistical parameters *F* and *t* (F_{calc} and $|t_{calc}|$) are also presented in Table 2 and 3. These values are smaller than the theoretical (tabulated) values of *F* (39.0) and *t* (2.776) at the 95% confidence level. This indicates that the two methods have comparable standard deviations and there are no statistically significant differences between mean results for iron and zinc determination in all fodder yeast samples analyzed. The variance values (squares of the standard deviations) of both methods are within the limits of random errors. The proposed method can be considered free of systematic errors.

Table 2. Analytical results (µg/g) for FAAS determination of Fe in fodder yeast

Sample	Conventional wet digestion		Microwave digestion		Statistical parameters	
	Mean ± SD	RSD%	Mean ± SD	RSD%	F_{calc}	$\|t_{calc}\|$
S1	355 ± 6.6	1.86	374 ± 13.0	3.48	3.867	2.260
S2	1034 ± 37.0	3.58	986 ± 20.7	2.10	3.199	1.962
S3	278 ± 15.1	5.44	250 ± 9.2	3.68	2.695	2.745

Table 3. Analytical results (µg/g) for FAAS determination of Zn in fodder yeast

Sample	Conventional wet digestion		Microwave digestion		Statistical parameters	
	Mean ± SD	RSD%	Mean ± SD	RSD%	F_{calc}	$\|t_{calc}\|$
S1	142 ± 8.5	5.99	130 ± 3.6	2.77	5.562	2.187
S2	543 ± 24.5	4.52	581 ± 35.8	6.27	2.135	1.118
S3	169 ± 13.7	8.11	156 ± 9.6	6.16	2.048	1.308

Conclusions

The main goal of this work was to demonstrate the feasibility of high-pressure digestion procedure with microwave energy of various fodder yeast samples, obtained from *n*-paraffins, methanol or ethanol that allows determining sequentially the two elements in the same solution in order to make the FAAS method less time consuming and laborious. Statistical evaluation of analytical results obtained fallowing the procedure described indicate that this digestion method is accurate, and sufficiently precise.

Closed-vessel microwave digestion method by MLS 1200 MEGA system has a number of advantages compared to the open wet method. It takes less time (without cooling time, 28 min compared to almost 2 h) and requires less reagents quantities. It this way, low blank value and reliable results are ensured. The system is easy to control and almost does not require supervision. In addition, the use of closed-vessels prevents contamination from the laboratory environment or cross-contamination from other samples.

Safety is an important consideration, because this completely closed system does not expose laboratory personnel to undesirable or potentially dangerous acid fumes. In addition, productivity increases, because, on the one hand, six simultaneous digestions can by carried out in one run and, one the other hand, the analysts can accomplish other tasks while the microprocessor-controlled unit runs through the digestion routine unattended.

Looking at the limitations of the current closed-vessel microwave digestion technologies, perhaps the most limiting factor at present is the amount of sample that can be digested. This is particularly true for organic matrices. The maximum working pressure allowed in the closed-vessel microwave digestion system used in this study limits the maximum amount of the fodder yeast sample to be efficiently digested.

During a microwave closed-vessel acid digestion, the heating raises the temperature and the vapor pressure of the solution. Furthermore, the chemical reaction itself may also generate gases further increasing the pressure inside the closed vessel. A simplified reaction between a generic organic sample and nitric acid can be summarized in this form:

$$ORG + HNO_3 \Rightarrow MetalsNO_3 + CO_2(gas) + NO_X(gas) + H_2O$$

Obviously, the larger sample amount, the higher the pressure generated by the reaction. It can be noted that a larger sample amount not only produces a higher pressure, but also a more exothermal reaction. Although microwave technology has entered thousands of laboratories worldwide, it is still a relatively new and expensive technology.

Taking the above into account, the explored closed-vessel microwave digestion method is simple, rapid, and perfectly suitable for routine decomposition of fodder yeast prior to determination of iron and zinc (and other metallic elements) by FAAS.

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