IDENTIFICATION AND DETECTION OF PHOSPHORYLATED PROTEINS BY LASER INDUCED BREAKDOWN SPECTROSCOPY

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Nadir ARAS

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We approve the thesis of **Nadir ARAS**

_____
Assoc. Prof. Dr. Şerife YALÇIN
Supervisor

_____
Assist. Prof. Dr. Gülşah ŞANLI
Co-Supervisor

_____
Prof. Dr. Orhan ÖZTÜRK
Committee Member

_____
Assoc. Prof. Dr. Talat YALÇIN
Committee Member

_____
Assist. Prof. Dr. Çağlar KARAKAYA
Committee Member

16 December 2011

_____
Prof. Dr. Durmuş ÖZDEMİR
Head of the Department of Chemisry

_____
Prof. Dr. R. Tuğrul SENGER
Dean of the Graduate School of Engineering and Sciences

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ABSTRACT

IDENTIFICATION AND DETECTION OF PHOSPHORYLATED PROTEINS BY LASER INDUCED BREAKDOWN SPECTROSCOPY

Laser-Induced Breakdown Spectroscopy (LIBS) is an optical atomic emission spectroscopic technique that uses an energetic laser source to generate a luminous plasma. Spectrochemical analysis of the light emitted from the plasma reveals information about the elemental composition of the sample.

Phosphorylation is an important regulatory mechanism that activates or deactivates many proteins and enzymes in a wide range of cellular process. Identification and detection of phosphoproteins have a crucial importance in phosphopeptide mapping.

This study is based on the assessment of the capabilities and limitations of LIBS as a quick and simple method for in-gel identification and determination of phosphorylated proteins, specifically casein and ovalbumin before mass spectrometric analysis for the elucidation of phosporylation sites. For this purpose, an optical LIBS set-up was constructed from its commercially available parts and the system was optimized for LIBS analysis of polyacrylamide gels. Nd:YAG laser operating at 532 nm wavelength and at 10 Hz frequency was used to create plasma on dry gel surfaces. Emitted light from a luminous plasma was analyzed and detected by an Echelle type spectrograph containing Intensified CCD, detector.

With this study, LIBS detection of phosphorous proteins after electrophoretic separation of phosphorylated proteins has been shown, for the first time. After SDS-PAGE gel separation process, phosphoproteins were recognized from prominent P(I) lines (at 253.5 nm and 255.3 nm) in a plasma formed by the focused laser pulses on the gel, just in the center or in the vicinity of the electrophoretic spot. Spectral emission intensity of P(I) lines from LIBS data has been optimized with respect to laser energy and detector timing parameters by using standard Na_2HPO_4. It has been shown that phosphorylated proteins (casein and ovalbumin in mixture) can be identified by LIBS after both coomassie brilliant blue and silver staining procedures. Technique shows a great promise in microlocal spotting of phosphorylated proteins in gel before MS analysis for the determination of the phosphorylation sites.

ÖZET

FOSFORLANMIŞ PROTEİNLERİN LAZER OLUŞTURMALI PLAZMA SPEKTROSKOPİSİ İLE BELİRLENMESİ VE TAYİNİ

Lazer-Oluşturmalı Plazma Spektroskopisi, (LIBS), yüksek enerjili lazer kaynağı kullanılarak ışıklı plazma oluşumuna dayalı bir atomik emisyon spektroskopi tekniğidir. Oluşan plazmadan yayılan optik emisyonların spektrokimyasal analizi, örneğin elemental bileşimi hakkında bilgi verir.

Protein fosforilizasyonu, enzimlerin ve proteinlerin çeşitli hücre içi reaksiyonlarda aktivasyon ve deaktivasyonunu sağlayan düzenleyici mekanizma olarak görev görür. Fosforlanmış proteinlerin belirlenmesi ve tayini, fosforlu peptidlerin haritalanmasında büyük bir öneme sahiptir.

Bu çalışma, fosforlanmış proteinlerin (kazein ve ovalbumin) kütle spektrometrisi ile analizi öncesinde jel içinde belirlenmesi ve tayininde, LIBS'in hızlı ve basit bir metod olarak kullanılabilirliğinin, kapasite ve sınırlarının belirlenmesine dayanmaktadır. Bu amaçla, optiksel bir LIBS düzeneği ticari olarak elde edilen parçaların bir araya gelmesi ile kurulmuş, poliakrilamid jellerin analizi için optimize edilmiştir. 532 nm dalga boyunda, 10 Hz frekansda ışıma yapan, Nd:YAG lazer kaynağı, kurutulmuş SDS-jel yüzeyinde plazma oluşturmak için kullanılmıştır. Plazmadan saçılan ışınlar eşel tip spektrograf ve şiddetlendirilmiş CCD ile kombine edilmiş dedektör sisteminde tayin edilmiştir.

Bu çalışma SDS-jeldeki fosforlanmış proteinlerin tespit ve tayinine dayalı ilk LIBS çalışmasıdır. 1-boyutlu jel ayırma işleminde ayrılan fosforlanmış proteinler, üzerine odaklanan lazer pulsu ile oluşan plazmada açığa çıkan 253.5 nm ve 255.3 nm deki fosforun atomik emisyon çizgileri ile tanınırlar. Fosfor, P(I) spektrumlarındaki emisyon şiddetinin lazer enerjisi ve dedektör zamanlama parametreleri açısından en uygun olduğu koşulların belirlenebilmesi için inorganik fosfor içeren Na_2HPO_4 tuzu pelet haline getirilip analiz edilmiştir. Deneyler sonucunda hem coomassie brilliant blue ile hem de gümüş boyama yöntemiyle boyanmış SDS-jellerin analizi sonucunda protein karışımı içindeki ovalbumin ve kazein gibi fosforlanmış proteinler tayin edilmiştir. Teknik fosforlanmış proteinlerin jel içersinde lokal olarak tayin ve teşhisinde LIBS'in kullanılabilirliği açısından büyük bir gelecek vaad etmektedir.

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CHAPTER 1

INTRODUCTION

1.1. Laser Induced Breakdown Spectroscopy (LIBS)

Laser-Induced Breakdown Spectroscopy, LIBS also known as Laser Induced Plasma Spectroscopy, (LIPS) and Laser Ablation Spectroscopy, (LAS) is a relatively new atomic emission spectroscopic technique that has started to emerge after 1980's with Radziemski and Cremers's pioneering work (Cremers and Radziemski, 1985).

In this technique, highly energetic pulsed laser source is focussed on a target (solid, liquid or gas) to form a luminous plasma. The light emitted from the plasma is collected by suitable optics and directed onto the entrance slit of the spectrograph. Spectrochemical analysis of the light emitted from the plasma provides information about the chemical composition of the sample under investigation (Singh, 2007).

In laser-induced plasmas typical temperatures are around 20.000 K and electron densities may vary between 10^{16}-10^{18} cm^{-3} provided that local thermodynamic equilibrium (LTE) is valid (Sneddon et al., 1997).

There has been a growing interest in LIBS technique within the last three decades and it has been used in a variety of areas including: aerosol analysis (Hahn et al., 2008), industrial applications (Gallou et al., 2011), analysis of pharmaceutical materials (de Carvalho et al., 2010), cultural heritage applications (Gaudiuso et al., 2010), civilian and military applications (Miziolek et al., 2008) environmental contamination studies (Chen et al., 2010), geochemical applications (Harmon et al., 2009) and many more. In the last decade, LIBS has been used in the analysis of biological samples inluding: characterization of malignant tissues (Kumar et al., 2004), detection and identification of bacteria (Baudelet et al., 2006), identification of urinary calculus (Anzano and Lasheras, 2009) and analysis of teeth and dental materials (Samek et al., 2000).

1.2. Advantages and Disadvantages of LIBS

LIBS has many advantages over other types of atomic emission spectroscopic techniques. First of all, it is a multi-elemental analysis technique in which simultaneous detection of all elements present in the sample is possible. This technique provides spectral signatures characteristics of all chemical species, in all environments of solid, liquid or gas. LIBS technique can be used to analyze extremely hard materials such as ceramics and semiconductors. It requires little or no sample preparation steps that eliminates undesired contamination of the sample during its transport.

The amount of sample consumed during LIBS analysis is so small therefore LIBS can be considered as nearly non-destructive process. Analysis of surfaces with craters as small as a few micrometers, in diameter and micrometers range of spatial and depth resolution is possible.

Using fiber optic cables laser pulse and emitted light from the plasma are transported to long distances of hundreds of meters and a remote sensing is obtained. Besides, in-situ analysis by LIBS can also be performed. This technique can also be used for direct analysis, compact probe and stand–off analyses (Miziolek et al., 2006).

Besides many advantages of the LIBS process, there are some deficiencies. Plasmas formed from the homogenous samples such as liquid and gases may represent the actual concentrations of the species present in the sample hovewer this may not always apply for solid samples. Surface composition can be different from the bulk composition and it causes an error in the result of the analyzed sample.

Due to fluctuations in laser energy, shot to shot reproducibility may change and hence precison levels can get decrease.

During LIBS analysis of solid samples, geometric position of the material surface with respect to laser beam incidence is one of the factors that affect the results. Changing the lens to sample distance (LTSD) affects the ablated mass from the surface (Cremers et al., 2006) and hence the temperature of the plasma, electron density and emitted light intensity may vary.

Compared to other atomic spectroscopic techniques, analytical figures of merits like sensitivity, accuracy and detection limits obtained by LIBS are relatively poor. Detection limits obtained for most of the elements are in low ppm range.

1.3. LIBS Instrumentation

In general, an experimental LIBS set-up consists of three main parts. A high energy *pulsed* laser source for plasma formation, focussing and collection optics to direct the laser beam and plasma emission, and a spectrograph equipped with a time resolved detector for spectrochemical analysis and material identification.

Figure 1.1 shows a typical LIBS set-up (Sun et al., 2000). This system consists of a Nd:YAG laser, a motorized XYZ translational stage, focusing-collimating lenses and pierced mirror (optic system), a spectrometer, an intensified charged coupled device (ICCD) detector, detector gating and control electronics and a computer for the control and data acquisition.

In this LIBS set-up, system can be modified according to the physical state of the sample and environmental conditions.

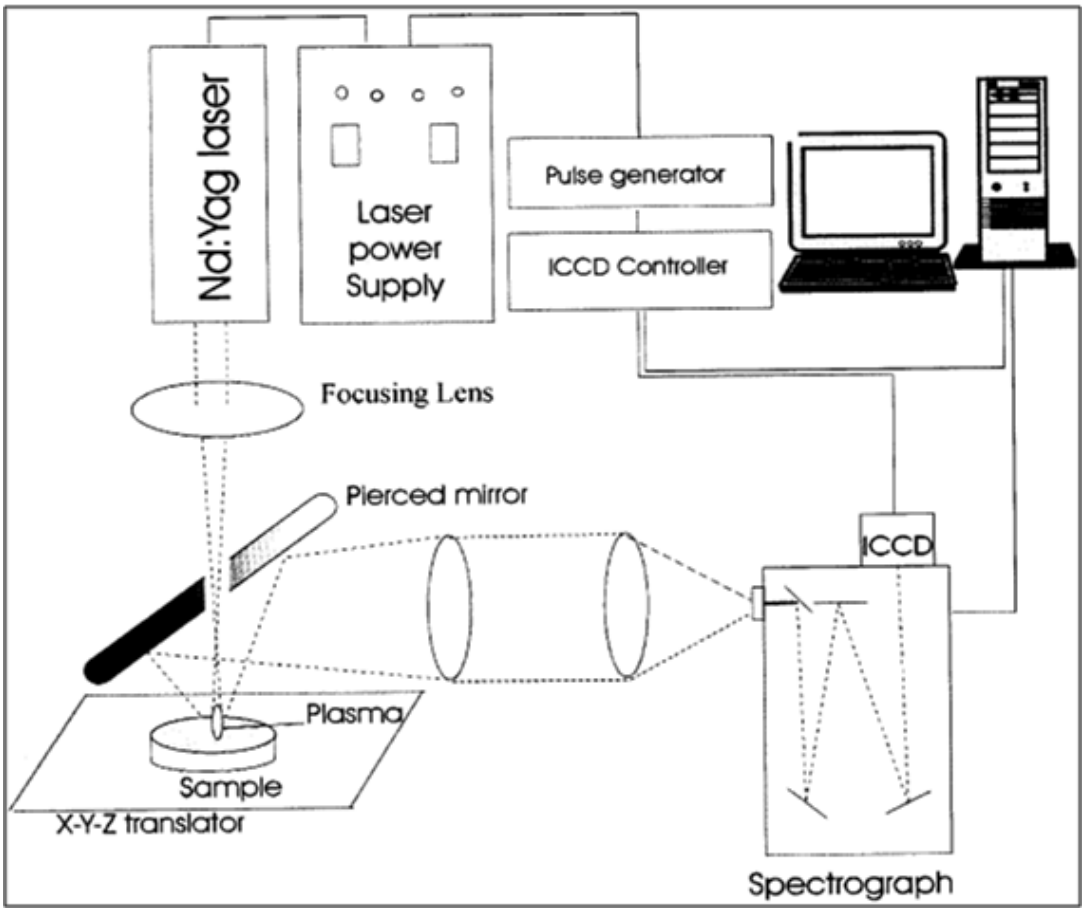

Figure 1.1. A typical LIBS set-up.

1.3.1. Lasers

By the invention of the lasers they have been used in many discipline, especially in the field of atomic spectroscopy. The word laser is acronym for Light Amplification by Stimulated Emission of Radiation.

The basic components of a typical laser system shown in Figure 1.2 are: the *gain medium* that is the basic part of the laser system in which amplification of the light by stimulated emission occurs, a *pumping source* to supply the energy required for the amplification and a *high reflector mirrors* in between the light is reflected many hundred times before exiting the optical cavity (Skoog et al., 2007).

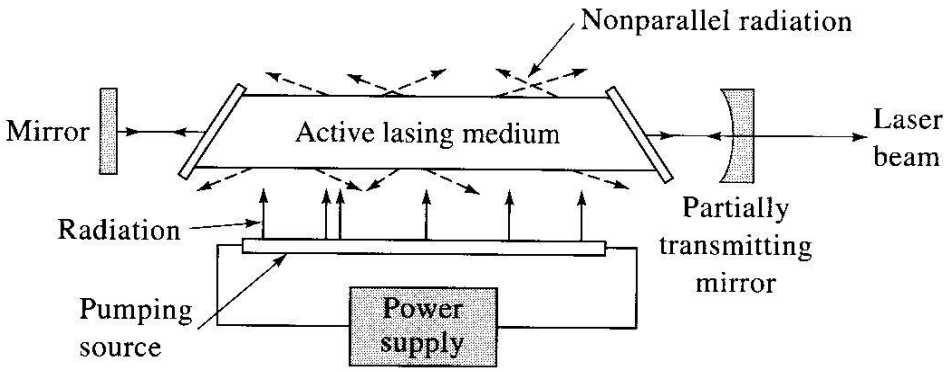

Figure 1.2. Schematical viewing of laser system.

In the gain medium, absorption, spontaneous emission and stimulated emission of photons take place. When an atom in lover energy level, E_1, is irradiated with a photon, excitation to the upper energy level, E_2, occurs through the process called **absorption** (Figure 1.3)**.**

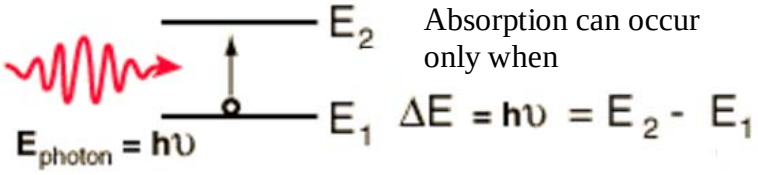

Figure 1.3. Absorption.

Atoms remain at the higher energy state, E_2, for a brief period of time and then decay quickly to a lower energy level by emitting a photon. Photon emission takes place an equal amount of the difference between the two energy levels. If this process occurs

without any external stimulus, it is called **spontaneous emission** (Figure 1.4). The emitted photons are in random directions and have no common phase relationship.

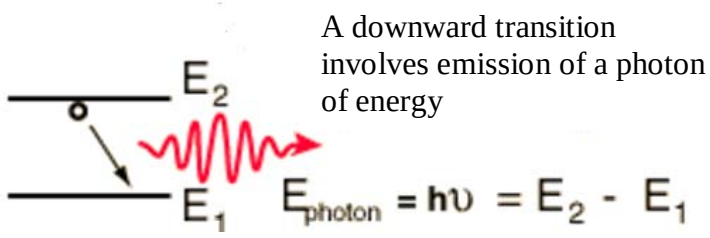

Figure 1.4. Spontaneous emission.

Hovewer, if the excited state atom interacts with the photon of energy equal to the difference between the two energy levels, transition from the higher to a lower energy state occurs by producing an additional photon of the same energy. This process is called as **stimulated emission** (Figure 1.5), (Sneddon et al., 1997). Photons created by stimulated emission have the same phase, frequency, polarization and a direction of travel. Stimulated emission is a process discovered on theoretical grounds by Einstein.

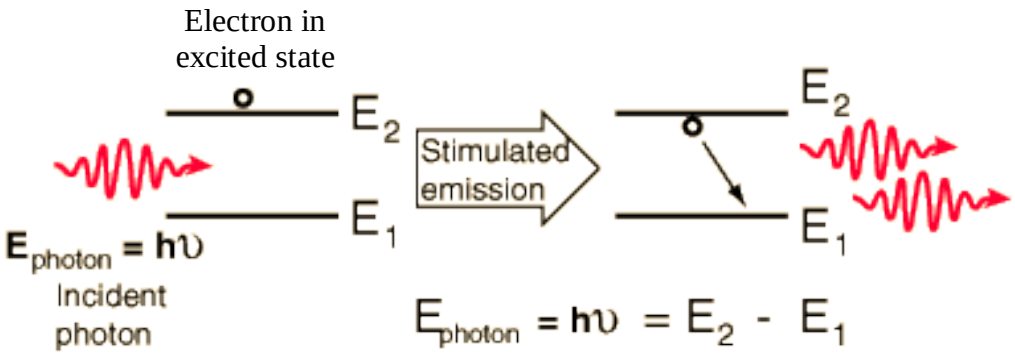

Figure 1.5. Stimulated emission.

During the lasing action all three processes described above take place: absorption, spontaneous emission and stimulated emission. The occurance rate of the stimulated emission is proportional to the number of excited state atoms. However, the probability of a photon causing stimulated emission is exactly equal to the probability of a photon being absorbed by the ground state atoms. If the number of photons that goes through stimulated emission process is higher than the number of photons being absorbed, then the lasing action occurs through the population inversion in which a higher energy state has a greater population than the lower energy state. In order to have

a lasing action to occur population inversion is the necessary condition to be met.There are some features that affect the quality of the laser beam including, power density, monochromaticity, directionality, pulse-to-pulse stability (for pulsed lasers) and beam shape. These all are important parameters in obtaining a homogenous plasma and smooth craters on the target surface. Typical laser pulse energy used in LIBS measurements range from 10's of microjoules to 100's of millijoules. By using high power density lasers, one can enhance the signal to noise ratio that is also important for reduction of the noise or background in the spectra.

There are various types of lasers according to the their pump source and gain medium and can be listed as dye lasers, gas lasers, semiconductor lasers and solid state lasers.

1.3.1.1. Dye Lasers

The working principle of dye lasers is based on the fluorescent transitions of organic molecules in solution. By changing of laser dyes the wavelength range can tune over a 20 to 50 nm. The active materials in dye laser are organic molecules in solution capable of fluoresce and the bandwidth of dye lasers is about low level of nanometers (Sneddon et al., 1997).

1.3.1.2. Gas Lasers

Gas lasers active materials are gas or mixture of different gases. There are different types of gas lasers available including neutral atom lasers such as Helium-Neon laser (He-Ne), ions lasers, molecular lasers such as CO_2 and excimer lasers. Gas lasers operating range is between ultraviolet and far infrared regions. They can operate both in pulsed and continious mode. He-Ne laser is the most commonly used gas lasers due to their low maintenance costs, being highly reliable and lower power consumption (Skoog et al., 2007).

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1.3.1.3. Semiconductor Lasers

The working principle of semiconductor laser is based on semiconductor gain media in which the optical gain is obtain by stimulated emission. Its operating range covers the visible, near ifrared and mid-infrared spectral region. Most commonly used semiconductor lasers as laser diodes are gallium arsenide (GaAs), Aluminum gallium arsenide (AlGaAs), gallium phosphide (GaP), gallium nitride (GaN) and indium gallium phosphide (InGaP). Over the other types of conventional lasers, semiconductor lasers have some advantages as being small, less expensive and longer lifetimes.

1.3.1.4. Solid State Lasers

Active medium in solid state lasers generally consists of a transparent crystal or a glass containing less than %1 transitional metal (mostly chromium and neodymium). Types of the solid state lasers are ruby lasers, neodymium lasers (Nd:YAG) and tunable solid state lasers (Sneddon et al., 1997). The most commonly preferred laser in LIBS studies is Nd:YAG and will be examined in detail.

1.3.1.4.1. Nd:YAG Lasers

Nd:YAG solid state lasers are the most widely used lasers in LIBS measurements because of its high power density, high repetion rate, ease of operation and maintanence. The active medium of Nd:YAG lasers consist of neodymium ions. Fundamental wavelength of Nd:YAG lasers can be shifted easily to obtain 532 nm, 355 nm, and 266 nm by harmonic generation.

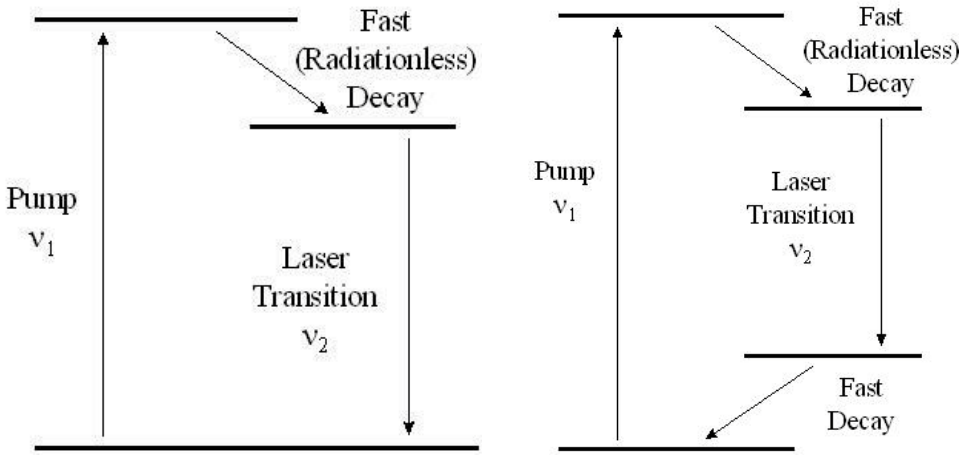


Figure 1.6. Three and four level laser schemes.

Figure 1.6 shows energy diagram of three and four level laser systems. Four level systems also have some advantages than two or three level systems. For three level systems, transition of laser radiation occurs between excited state, E_y and ground state, Eo. Although for the four level systems the transition of radiation is from E_y to E_x, it has more energy than ground state. Also it makes easy the population inversion that is important for laser action. It means that population inversion occurs with smaller pumping energy in four level system (Skoog et al., 2007).

1.3.2. Optical Systems

In LIBS systems in order to focus the laser beam onto the sample to be analyzed lenses are utilized. There are some points to be considered when focusing the laser beam by a lens. These are the focal length, the diameter and the construction material of the lens. Lenses used should match with the laser wavelength with a maximum transmission and be appropriate in type (plano-convex, double convex, etc.).

Lenses are also used to collect emitted light from the plasma and direct through the detector. Therefore, collimating lenses need to be optically transparent to the light emitted from the plasma.

In LIBS systems, highly reflective mirrors that are used to direct and steer the laser beam onto the optical components should be coated and suitable with the laser wavelength.

Fiber optic cables (FOC) are also widely used optical components in LIBS systems, where plasma emission is easily carried onto the entrance slit of the

spectrograph. Fiber optic cables can also be used to transmit the laser beam at longer distances (Cremers et al., 2006).

1.3.3. Spectrograph and Detection Systems in LIBS

Although single channel LIBS systems that contain a monochromator and a photomultiplier tube (PMT) can be used in the detection of plasma emission at a specific wavelength, the trend in recent years has been towards multichannel spectrometers in which a monochromator/polychromator is coupled to a series of PMT's or a time gated, array type detectors.

Among different types of spectrographs, the most common design is Czerny-Turner design, however, use of echelle type of spectrograph in LIBS measurements is ever increasing. Czerney-Turner spectrometers provide higher resolution compared to Echelle type spectrographs, however, it works for a small wavelength range for one set of measurement. One needs to make several sequential measurements to cover the whole spectral range between 200-800 nm. On the other hand, Echelle spectrographs provide an orthogonal dual dispersion in which prism is for order sortering and echelle grating is for wavelength resolution. The echelle spectrograph characteristics are 25 cm focal length with a high number of numerical aperture, providing a maximum resolution between 200-800 nm and carrying out a large dynamic range of measurement. As shown in Figure 1.7 the echelle grating is used at a high angle and it provides a high dispersion in spectra. In order to prevent overlapping of different orders in grating, a low dispersion grating or prism is assembled perpendicular to the echelle grating.

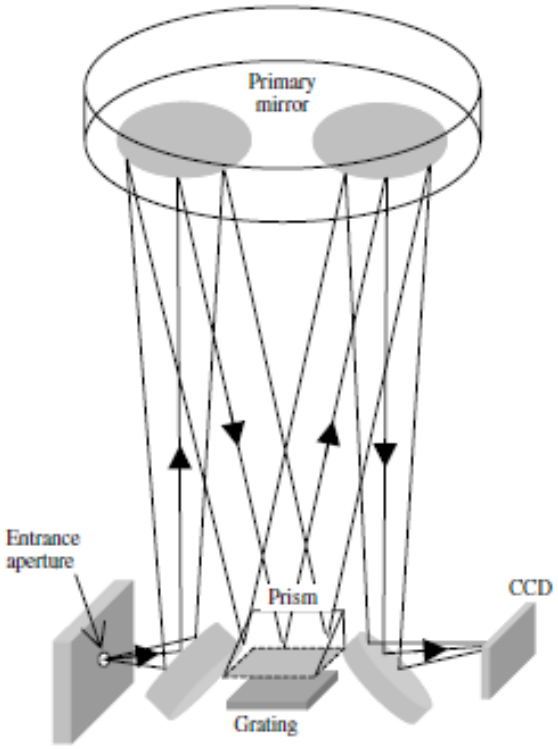


Figure 1.7. Schematical viewing of Echelle Spectrograph.

The multielement capability is one of the major attributes of LIBS and ideal experimental LIBS system should be able to make simultaneous multielement measurements. An ICCD array detector coupled to an Echelle spectrograph or compact Czerney Turner spectrometer serves this purpose for numerious applications. A schematical representation of an ICCD detector is given in Figure 1.8.

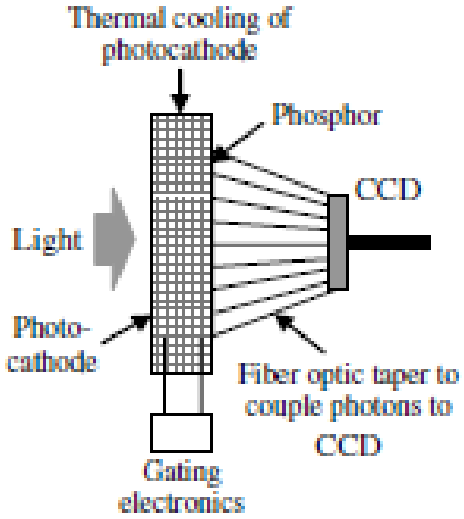


Figure 1.8. Schematical viewing of ICCD.

ICCD is a detector that consists of an optical connection with CCD and image intensifier. It is composed of three parts; a photocathode, a micro channel plate (MCP) and a phosphor screen.

The photons from light source are converted to the electrons when photons fall onto photo-cathode. By the applying electrical potential electrons are accerelated through the MCP. Incoming electrons are multiplied in this part (MCP). Multiplied electrons are accerelated towards the phosphor screen. Here, these electrons are converted into photons in phosphor screen and then intensified after it is sent through the CCD camera by a fiber optic or a lens.

Intensified CCD detectors are the preferred choice in LIBS systems due to providing two-dimensional spatial information, ability to make simultaneous multi element analysis and time resolved detection capability.

CHAPTER 2

BIO-LIBS

Over the decades, LIBS technique has been actively used in variety of applications, including environmental, industrial, geochemical, safety monitoring, archeological pharmaceutical and many more. Recently, biological and medical applications of LIBS has started to emerge. Some of these applications include: analysis of calcified tissue materials like teeth, bones, nail, see shells, soft tissue materials like human skin, hair, plant leaves, wood and some types of bio-fluids like blood and urine. In all of these applications, Laser Induced Breakdown Spectroscopy has been used to provide information on the identification of health problems with a high degree of spatial and depth resolution.

Due to its ability to analyze samples *in situ* and *remotely* without any sample preparation, LIBS technique offers fast, simple and easy method of analysis when dealing with biological specimens. Under certain cases, even *in vivo* analysis may be possible with living organisms. Although the number of publications in this area is actively growing, biological applications of LIBS is still at its infancy stage. Some of the inherent disadvantages of the technique like low reproducibility, high limit of detection and surface to bulk compositional differences due to nonhomogeneity of the sample decreases the diagnostic potential and the ability to accurately determine relative concentrations of the biological samples. However, LIBS still has a great potential for the analysis of biological samples as a real-time multi-elemental analysis technique.

2.1. Literature Works

Publications about Bio-LIBS are not many. One of the pionering research on biological application of LIBS to the analysis of teeth and dental materials is performed by Samek et al. (2000). The utilization of pulsed lasers in dentistry (Walsh, 2003) or drilling has provided many benefits such as controllable laser drilling (preventing a damage to healthy tissues), simultaneous analysis of the tooth sample both *in vivo* or *in vitro* and also obtaining spatial distribution of the elements throughout the tooth sample

is possible. In their earlier study, Samek et al. (1999) have performed quantitative analysis of trace metal accumulation in teeth samples. They have partially quantified including Ag, Al, Ca, Cr, Hg, K, Mg, Mn, Na, Ni, P, Sn, Ti and Zn.

In their recent work Samek et.al have performed clinical applications of LIBS on different dental samples including teeth, dental amalgam, composite restorative material and toothpaste. They have showed that LIBS spectra can be utilized to distinguish a healthy enamel and caries infected teeth by monitoring Ca and P concentrations. They have also observed that aluminum, Al, from tooth paste exposure, migrates only within the enamel layer.

Zn concentrations in the stratum corneum of human skin was investigated by LIBS, for the first time by Sun et al. (2000). They have optimized some instrumental parameters including sample movement, laser energy, detector timing; for obtaining accurate and sensitive results. The results indicated that Zn was absorbed through the skin at differing depths based on the skin type.

The determination of toxic elements in calcified tissue is important to determine the influence of environmental exposure on biological samples. Samek et al. (2001) have performed laser-induced breakdown spectroscopic analysis of calcified tissue samples. They quantitatively determined Al, Pb and Sr concentrations by one and two dimensional mapping approaches. Spatial information for the distribution of trace elements in teeth and bones has been obtained.

Metal toxicity can occur in humans with a variety of reasons. Hair tissue mineral analysis (HTMA) is often used to determine that incidence. Amount of the mineral content can provide information on the general health status of hair. In one of these study, Corsi et al. (2003) has performed mineral analysis in hair tissue by laser-induced breakdown spectroscopy. They have measured relative Na/K and Na/Mg ratio of hair samples from 11 different individuals of differing age, gender and hair color. Samples analyzed with the calibration free (CF)-LIBS technique and relative concentration for relevant elements detected a few mg/% levels have been obtained without any pretreatment. A few milimeters long hair is sufficient for analysis and this will allow the utilization from LIBS for forensic cases.

Especially after September 11, 2001, there have been an increase in LIBS studies based on security issues. Samuels et al. (2003) have investigated discrimination potential of LIBS on bacterial spores, molds, pollens and proteins. Obtained data from the LIBS process was utilized for statistical analysis called principle component

analysis (PCA). It has been shown that LIBS has the capability to distinguish bacterial spores, molds and pollens by PCA. In this study, bundled fiber optic cables were used to collect the emitted light more effectively. and with a higher signal intensity.

Rapid determination of microorganisms is important for the military, agriculture and medical applications. The determination of the chemical compositions of some materials such as protein, nucleic acid and cell provide information about fingerprinting of bacterial strain. Kim et al. (2004) have investigated the spectral fingerprints of bacterial strains by laser induced breakdown spectroscopy. In their study they could be able to distinguish five different nonpathogenic bacterial strains.

One interesting biological application of LIBS in literature that belongs to Kumar et al. (2004) is about characterization of malignant tissue cells by LIBS. This is the first study on differentiation of malignant tissue from the normal tissue by LIBS. LIBS has provided an automated and real time analysis for diagnosing the cancer. They have found differences in various elemental concentrations of cancerous and normal tissue samples. In this study, the LIBS results were also compared with inductively coupled plasma emission spectroscopy (ICP-OES) data and results were found consistent.

Fast and secure analysis of microbiological samples such as molds, pollens, bacteria are important for civil defence or environmental surveillance. Baudelet et al. (2006) in their study, compared femtosecond and nanosecond time-resolved laser induced breakdown spectroscopy for detection and identification of bacteria. Femtosecond lasers have several advantages in analysis of biological samples. Lower and faster decaying temperatures obtained in femtosecond plasmas eliminates the emission of oxygen and nitrogen from the ambient air by this way prevents any interference. Femtosecond laser-induced plasma have large concentrations of molecular fragments, in particular, native CN bonds released by a bacterial samples can be characterized by a short decay time. They have conluded that molecular and atomic spectra provide together valuable data for a specific bacterium to be either detected from a natural environment or distinguished from other biological species.

Martin et al. (2007) have performed high resolution applications of laser induced breakdown spectroscopy for environmental and forensic applications. They have determined carbon content from different soil sources and also in their another work they have analyzed annual tree growth rings to get some information about in fire scar

determinations. There was good agreement between ICP-MS and LIBS results for forensic applications.

LIBS have also been used for the discrimination of different strains of bacteria by Rehse et al. (2007). They have investigated the identification and discrimination of Pseudomanas aeruginosa bacteria grown in blood and bile by laser induced breakdown spectroscopy. In this study, by the determination of inorganic elements such as calcium, magnesium and sodium, classification of bacteria grown in different media is achieved. In order to diagnose some diseases such as dysentery, hemolytic uremic syndrome and pneumonia, determination of E. coli is important. Pseudomoas aeruginosa may also cause various problems in human body like E.coli. In order to explain relationship between two or more distinct groups, discriminant function analysis (DFA) is a data analysis technique like analysis of variance. Different types of bacteria were discriminated by applying DFA to the LIBS results.

In another study by Rehse et al. (2009), they have investigated bacterial identification and discrimination using laser induced breakdown spectroscopy, on a membrane basis. Two different types of bacteria Pseudomonas aeruginosa and Escherichia coli in different nutrient media (a standard TS agar, a MacConkey agar containing 0.01% bile salts and a TS agar with a higher 0.4% concentration of deoxycholate) have been analyzed and discriminated by applying DFA. LIBS provided 90% accuracy regardless of the nutrient medium.

Another study performed by Rehse and Mohaidat (2009), discusses the effect of ambient gas on laser induced breakdown spectroscopic determination of brass samples. This group has also applied the technique for the discrimination of bacterial strains. Four brass alloys with different composition and stoichiometries have been analyzed by LIBS under the two different noble gas conditions (argon and helium). DFA was applied, 15 emission lines from copper, zinc, lead, carbon and aliminium were used to categorize the samples. The results obtained from data analysis showed 100 % accuracy for the categorization of samples.

It is important that the determination of the essential elements in plants for evaluating the nutrional status of crops of economical interest. Trevizan et al. (2009) investigated the evaluation of laser induced breakdown spectroscopy for the determination of micronutrients in plant materials. In this study, analysis of plant materials in the pellet forms were carried out to determine their B, Cu, Fe, Mn and Zn content for evaluating this content with biological certified reference materials.

Calculated limit of detection values are 2.2 mg kg^{-1} B, 3.0 mg kg^{1} Cu, 3.6 mg kg^{1} Fe, 1.8 mg kg^{1} Mn and 1.2 mg kg^{1} Zn. The results obtained by LIBS compared with the ICP OES.

Another biological application of LIBS was performed by Kaiser et al. (2009). It is about mapping of lead and copper accumulation in plant tissues by laser induced breakdown spectroscopy and laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS). They compared the results obtained from LIBS and LA-ICP-MS with atomic absorption spectrometry (AAS) and thin layer chromatography (TLC). Plants can be used to keep toxic metals through phytoremediation studies. In this study, lead, magnesium and copper content of sunflower leaves analyzed up to 200 µm resolution. This study shows that LIBS is a rapid and practical method for the analysis of large area (cm x cm) samples with high spatial resolution.

Another literature work carried out by Singh et al. (2009) in biomedical science is about cross-sectional study of kidney stones by laser induced breakdown spectroscopy. By the analysis of kidney stone calcium, magnesium, manganese, copper, iron, zinc, strontium, sodium, potassium, sulfur, chlorine, phosphorus, hydrogen, nitrogen and oxygen elements are detected. Having LIBS an advantage of making in situ analysis it may provide information about the spatial distribution without damaging the stones. Results obtained from LIBS were in a good agreement with the ICP spectrometry results.

Another study was performed by Anzano and Lasheras (2009). It is about identification of urinary calculus by LIBS. It is important for preventing of calculi recurrence. So then it requires estimating the composition of urinary calculi. In order to identify urinary calculus, they have applied 2 different methods, the first one is correlation methods by using µ-LIBS system and the second one is the elemental ratios of reference materials with samples. Obtained data for both correlation and ratios method show an ability for identification of organic (Calcium phosphate) and non-organic (Uric acid) form of urinary calculi.

Another interesting study performed by El-Hussein et al. (2010) was about diagnosis of some types of human malignancies. Cancerous tissues could be distinguished from non-cancerous tissues by looking at calcium and magnesium amounts. In order to obtain more sensitive results, LIBS analysis have been performed under vacuum conditions (10^{-2} Torr). For calcium 373,6 nm and 422,6 nm, for magnesium 280,2 nm and 285,2 nm lines were chosen due to the presence of no

interference from other spectral lines and lower self-absorption than other spectral lines. This study presents a great promise on the in vivo measurement of cancer cells for diagnosis by LIBS, in the future.

2.2. Phosphoproteins

Proteins are natural polymer molecules composed of aminoacids bound together. The addition of PO_4 molecule to the amino acid residues of the proteins is known as phosphorylation. Phosphorylation occurs on several amino acid residues however, serine (Ser), tyrosine (Tyr) and threonine (Thr) are the most stable phosphorylated amino acids (Figure 2.1).

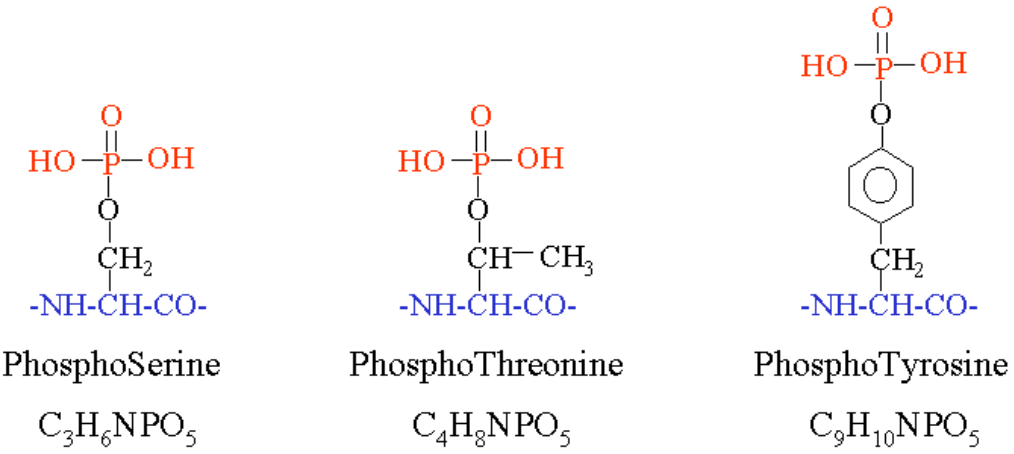


Figure 2.1. Amino acid phosphorylation.

Protein phosphorylation is one of the most frequently occurring post translational modifications that regulate many biological processes in living organisms. This reversible reaction causes changes in the 3D structure of the protein and its functions. As a result of these changes some processes such as gene expression, cell division, signal transduction, cytoskeletal regulation and apoptosis (D'Ambrosio et al., 2007) are observed.

2.2.1. Casein

Casein is one of the most widely known phosphoprotein. In cow milk at around 80 % and in human milk at around 60% of the proteins contain casein. Casein is used

for various purposes like making cheese or as food additive material. It contains amino acids, carbohydrates, calcium and phosphorus elements. The phosphorylation sites in the subunits of casein α, β and κ may vary 8-10, 4-5 and 1 phosphorylation sites were listed, respectively. Casein constitutes an average of 0.7 % phosphorous (Kunz and Lonnerdal, 1990).

2.2.2. Ovalbumin

Ovalbumin is another phosphorylated protein that constitutes almost 60 % of all protein in egg white. Ovalbumin is often used as a reference protein in immunization and biochemical studies. Ovalbumin is a monomer with molecular weight of around 45 kDa. It has two phosphorylation sites available (phosphorylated serines are at residues 68 and 344) (Nisbet et al., 1981).

2.2.3. Identification Detection and Characterization

There are several techniques developed for identification and detection of phosphorylated proteins (Reinders and Sickmann, 2005) however analysis of phosphoproteins is still one of the most challenging task in proteomics research.

Among several methods used for phosphoprotein analysis, due to the ability to detect low levels of concentration, mass spectrometry after several biochemical procedures has been the most widely used one within the last decade. These biochemical procedures include separation and staining-destaining processes.

Phosphorylated proteins usually require tedious and complex separation processes before detection. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis, **SDS-PAGE**, is a technique used to separate proteins according to their electrophoretic mobility. Proteins are separated based on their molecular weight and electrical properties as they migrate through a polyacrylamide gel matrix. Acrylamide gel preparation is a process that involves the crosslinking of acrylamide monomers with the use of catalysts. Once the gel has set, protein samples can be loaded into the gel and separated along an electric field.

After separation, different types of stains (Westermeier, 2006) and Silver Staining (Chevallet et al., 2006) or phosphate specific antibodies Western Blotting (Burnette, 1981) can be used for the visualization of phosphoproteins. Each staining dye produces a colored pattern proportional to the protein concentration for a given sample. Densitometry measurements may be performed by comparing two samples (reference and real sample) staining intensities for defined spots. Figure 2.2 pictures SDS-PAGE separation according to electrophoretic mobility and various number of protein bands separated on gel.

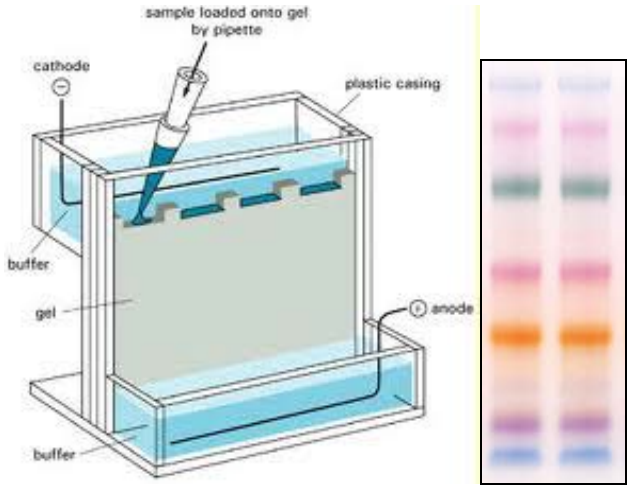


Figure 2.2. SDS-PAGE separation according to electrophoretic mobility and various number of protein spots separated on gel.

In order to produce peptide mass fingerprinting of these electrophoretically separated proteins by using matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) MS, stained spots of interest on gel slices are excised and the proteins are in-gel digested (normally using trypsin) to produce peptides that are extracted from the gel matrix. Figure 2.3. explains the route to be followed for phosphoproteins identification and detection before MS analysis.

Considering the fact that the amount of phosphoproteins in total proteins is within 20%, and excision of hundereds of spots for MS analysis is laborous and time consuming. Besides, especially for low concentration samples preconcentration procedures are required. This may result with the loss of the analyte and contamination of the sample.

In this thesis study, for the identification of phosphorylated proteins from the non-phosphorylated ones, microlocal spotting of phosphorous in gel by Laser Induced

Breakdown spectroscopic detection before MS analysis for the determination of phosphorylation sites is proposed.

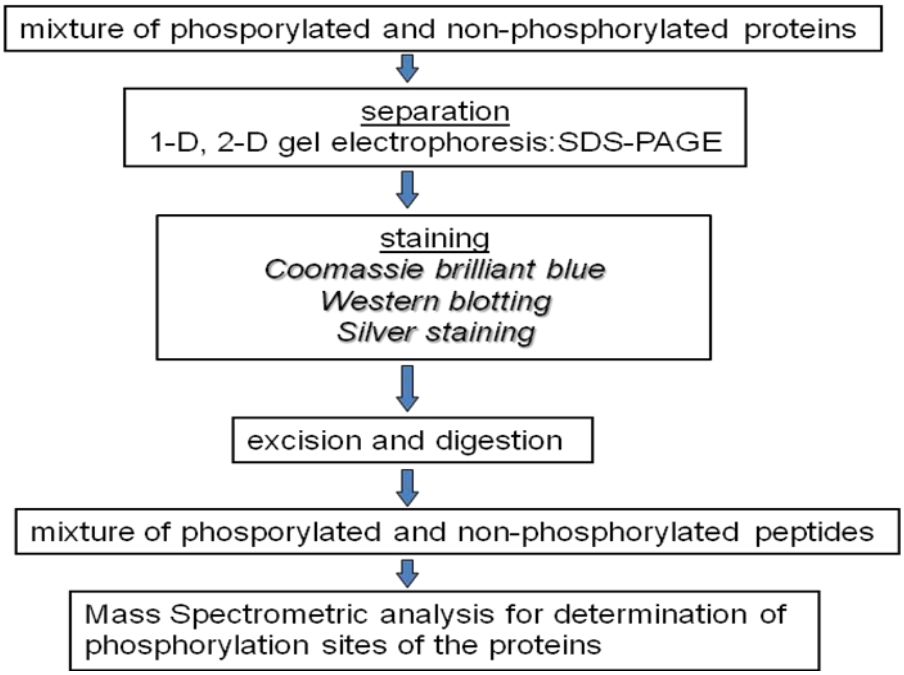


Figure 2.3. Flow chart for phosphoprotein analysis.

The purpose of this thesis is to investigate the capabilities and limitations of Laser-Induced Breakdown Spectroscopy in identification and detection of phosphorylated proteins in-situ on SDS gel spots before MS analysis for the elucidation of the phosphorylation sites. Phosphoproteins were recognized from prominent P(I) lines (at 253.5 nm and 255.3 nm) in a plasma formed by the focused laser pulses on the gel, just in the center or in the vicinity of the electrophoretic spot. It has been shown that phosphorylated proteins (casein and ovalbumin in mixture) can be identified by LIBS after both coomassie brilliant blue and silver staining procedures. Technique shows a great promise in microlocal spotting of phosphorylated proteins in gel before MS analysis for the determination of the phosphorylation sites.

2.2.4. Literature Work in Atomic Spectroscopy for the Detection of Phosphoproteins

There are several studies carried out for the analysis of protein phosphorylation by inductively coupled plasma mass spectrometry (ICP-MS). Venkatachalam et al.

(2007) have analyzed proteins separated by SDS-PAGE and then determined the phosphorous content by coupling laser ablation (LA) system with an ICP-MS detector. According to results, phosphorus content in proteins showed a good linearity between 20 to 500 pmol concentrations and 1.5 pmol limit of detection was obtained for β-casein. They have achieved 6.1 % standard deviation in reproducibility.

Besant and Attwood (2009) have studied histidine phosphorylation. Histidine phosphorylation is important for signal transferring in bacteria, fungi and plants and also some evidences show that it has a role in mammalian cell biology. In that study, analysis of phosphohistidine was performed by mass spectrometry and two types of ionization techniques electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) were chosen.

In another study, Zhang et al. (1998) have separated proteins by polyacrylamide gel electropohoresis and then identified them by mass spectrometry. For this purpose, combination of matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI/TOF) and online capillary liquid chromatography electrospray tandem ion trap mass spectrometry (LC/ESI/MS/MS) have been used. They detected at low nanogram levels of protein in phosphorylation sites.

This study is based on the assessment of the capabilities and limitations of Laser-Induced Breakdown Spectroscopy as a quick and simple method for in-gel identification and quantification of phosphorylated proteins, specifically casein and ovalbumin before mass spectrometric analysis for the elucidation of phosporylation sites. For this purpose, an optical LIBS set-up was constructed from its commercially available parts and the system was optimized for laser-induced breakdown spectroscopic analysis of polyacrylamide gels. Nd:YAG laser operating at 532 nm wavelength and at 10 Hz frequency was used to create plasma on dry gel surfaces. Emitted light from a luminous plasma was analyzed and detected by an Echelle type spectrograph containing Intensified Charged Coupled Device, (ICCD), detector.

CHAPTER 3

EXPERIMENTAL

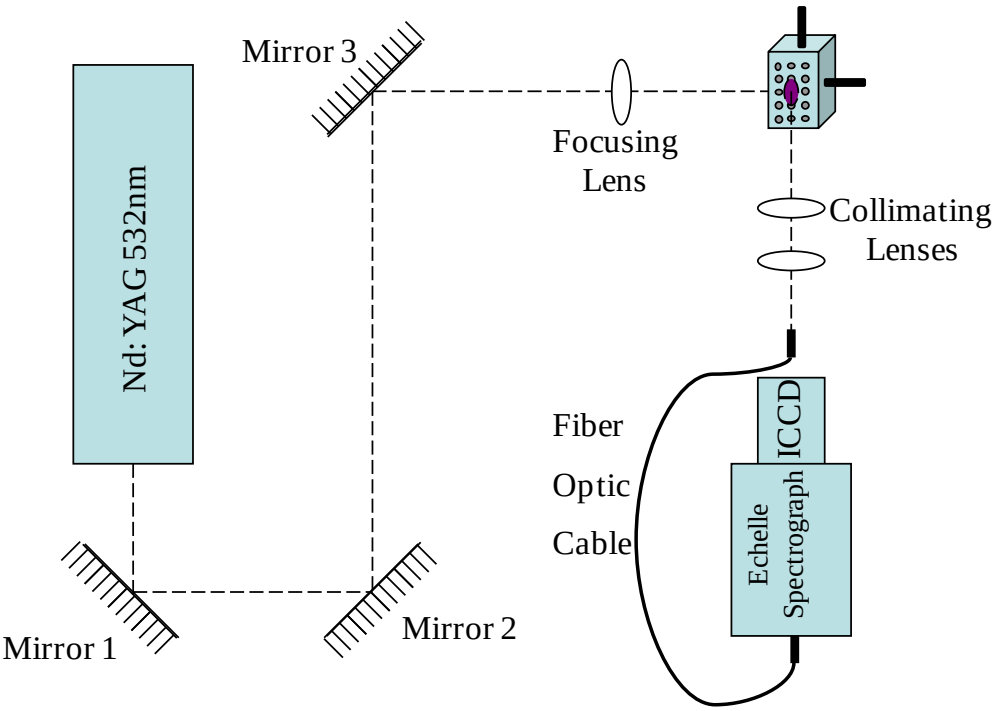
3.1. LIBS Experimental Set-Up

In this study, an experimental LIBS set-up was designed, constructed and optimized for the determination of phosphorous content in SDS-PAGE separated phosphoproteins. Optimization of the instrumental parameters such as laser energy and detector timing parameters, were carried out in pelletized solid disodium hydrogen phosphate (Na_2HPO_4) and casein samples. Phosphoproteins in standard protein samples were seperated by SDS-PAGE and stained by two different staining techniques: Commasiie Brillant Blue and Silver staining. After destaining procedure, analysis of SDS-PAGE separated phosphoproteins were performed under optimum experimental conditions.

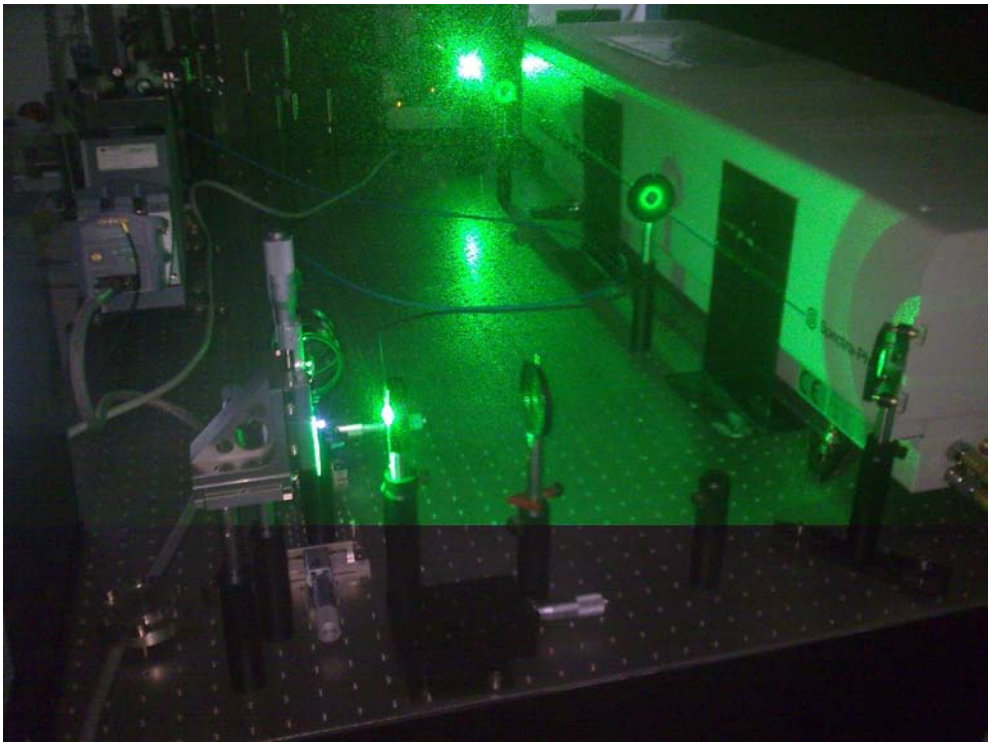
LIBS system used in this work, schematically and pictorally shown in Figure 3.1, was constructed from its commercially available parts. A Q-switched Nd:YAG laser source (Quanta-Ray Lab-170, Spectra Physics, California-USA) has been operated at its second harmonic, 532 nm, with 10 ns duration time. The laser beam was directed on the target by using 532 nm reflective mirrors (1" OD, coated, 532 nm reflective, New Focus, Darmstad-Germany), and was focused onto the sample surface via 20 cm focal length planoconvex lens. Laser pulse energy was measured by a power meter (PE50BB-DIF-V2, Nova II, Ophir, Israel).

Sample mounted on XYZ-translational stage (New Focus, Darmstad-Germany) to provide fresh spots during sampling. Luminous plasma emission is collimated and imaged onto the core of a fiber optic cable (Ocean Optics,P600-2-UV-VIS,600 µm diameter) by two plano-convex lenses of 5 cm and 10 cm focal lengths. This fiber optic is used to carry plasma emission onto the entance slit of the echelle spectrograph (Mechelle 5000, Andor Inc., f/7) equipped with an intensified charged coupled device (ICCD) (iStar DH734, Andor Inc.). The spectrograph and detection system spectral range is between 200-850 nm with 0.08 nm resolution at 400 nm. Wavelength

calibration of the spectrograph was done by using Hg-Ar spectral calibration lamp. All specifications of the experimental set-up is listed in Table 3.1.



a



b

Figure 3.1. Experimental LIBS set-up (a) and actual view of the set-up (b).

Table 3.1. LIBS system specifications.

Q-Switched ND: YAG laser	Quanta-Ray Lab 170, Spectra Physics
Power meter	PE50BB-DIF-V2, Nova II, Ophir (Israel)
532 nm reflective mirrors	1" OD, coated, 532 nm reflective, New Focus, Darmstad-Germany
Focusing Lense	20 cm
XYZ translational stage	New Focus-9067 (Darmstad-Germany)
Collecting Lenses	10 cm
	5 cm
Fiber Optic Cable	P600-2-UV-VIS, 600 µm diameter
Echelle spectrograph	Mechelle 5000, Andor, f/7
ICCD detector	iStar DH734, Andor Inc.

3.2. Detection System

Echelle type spectrograph compacted with the ICCD detector system was used during the experiments. Time resolved spectroscopic measurements were performed in order to find optimal time window for P(I) signal intensity at 253.5 nm and 255.3 nm. Experiments were performed at an optimum delay time of 1200 ns and gate time of 0.2 ms where maximum P(I) line emission was observed. Detector gain level was set to 150 throughout the measurements.

3.3. Pellet Analysis

Before direct analysis of the phosphorylated proteins in gel, phosphor containing samples (casein powder and inorganic Na_2HPO_4) were pressed in the form of 1cm diameter pellets by mixing with powdered KBr in differing stoichometric proportions. Instrumental parameters of the LIBS system like laser energy, delay time and gate width were optimized by using these pellets to obtain maximum P(I) signal. Analysis of in-gel phosphorylated proteins were performed under these optimum conditions.

3.4. SDS-PAGE Analysis

3.4.1. Loading and Running Samples

Phosphorylated proteins were separated by SDS-PAGE before identification and detection of their phosphorous content by LIBS. SDS-PAGE analysis was carried out according to Laemmli SDS-Polyacrylamide Gel Electrophoresis procedure (Laemmli, 1970) by using BIO-RAD SDS-PAGE apparatus, shown in Figure 3.2. Each sample, Casein (Sigma-Aldrich C7078 and C6780) and Ovalbumin (Sigma-Aldrich A2512) were boiled at 100 ºC for 30 minutes, before loading into the gel in order to denature the protein molecules further. After the preparation of the separating gel (monomer concentration % 12) and stacking gel (monomer concentration % 4) different quantities and different concentrations of protein samples were loaded into the wells of the SDS-PAGE and then the electric field was applied as 100 V for 2 hours.

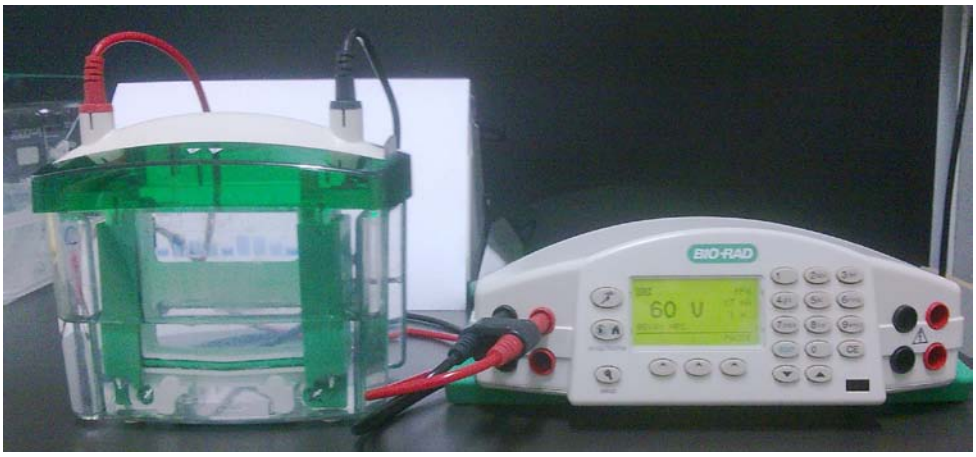


Figure 3.2. SDS-PAGE set-up.

3.4.2. Staining and Destaining of SDS-PAGE

3.4.2.1. Coomasie Brillant Blue Staining And Destaining

Coomasie Brillant Blue is the most commonly used dye for staining proteins in biochemistry. After running the proteins in SDS-gel for a specific period of time, the gel sample containing separated proteins was removed from BIO-RAD SDS-PAGE module

and left in fixation solution, (20% (w/v) TCA), by shaking at 20 rpm speed for 30 minutes. After this step, the gel was treated with enough staining solution, (50% methanol, 10 % acetic acid, 0.05% Coomassie Brillant Blue R250 and 40% dH_2O, on a shaker for 60 minutes. Then the gel was placed into destaining solution, (5% methanol, 10% acetic acid and 85% dH_2O), for about 60 minutes until the gel become transparent. This gel was then left for drying on a flat surface at room temperature for LIBS analysis. Figure 3.3 shows a picture of the Commasie Brillant Blue stained SDS-PAGE.

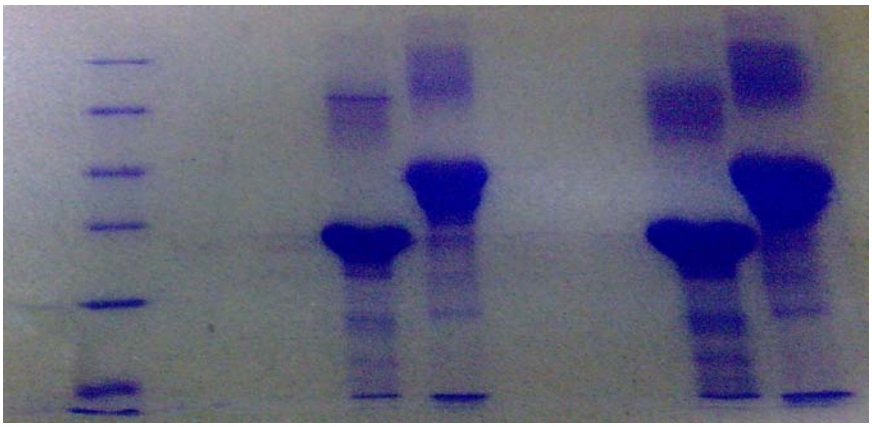


Figure 3.3. Commasiie Brillant Blue stained SDS-PAGE.

In LIBS analysis, each stained bands were exposed to sequential high energy laser pulses for ablation and plasma formation. Spectral emission from the gel plasma was analyzed for its phosphorus content for identification of phosphorylated proteins. During all these processes no phosphorous containing buffers were used.

3.4.2.2. Silver Staining

Silver staining is another commonly used staining technique for detecting proteins after electrophoretic separation. In this study after development of the SDS-PAGE, the gel was removed from BIO-RAD SDS-PAGE module for silver staing. Gel was left in fixing solution (MeOH 50 %, acetic acid 5 %) for 60 minutes in shaking with 20 rpm then washed 3 times with 50% ethanol solutions for 20 minutes. Gel was washed by pretreatment solution(0.02 % (w/v) $Na_2S_2O_3$) for sensitization exactly 1 minute and rinsed with deionized water 3 times for 20 seconds. After this step gel was impregnated with silver nitrate solutions for 20 minutes on shaker with 20rpm speed.

Then, the gel was rinsed with deionized water two times exactly for 20 seconds. Until the protein bands begin to get darken, gel was treated with developing solution. Bands starts to appear as brown by the addition of water. Finally, the gel was left in stopping solution (50 % MeOH, 12 % acetic acid) on a shaker for 10 minutes. This silver stained gel was left to dry on a flat surface at room temperature for LIBS analysis. Figure 3.4. shows a picture silver of stained SDS-PAGE gel.

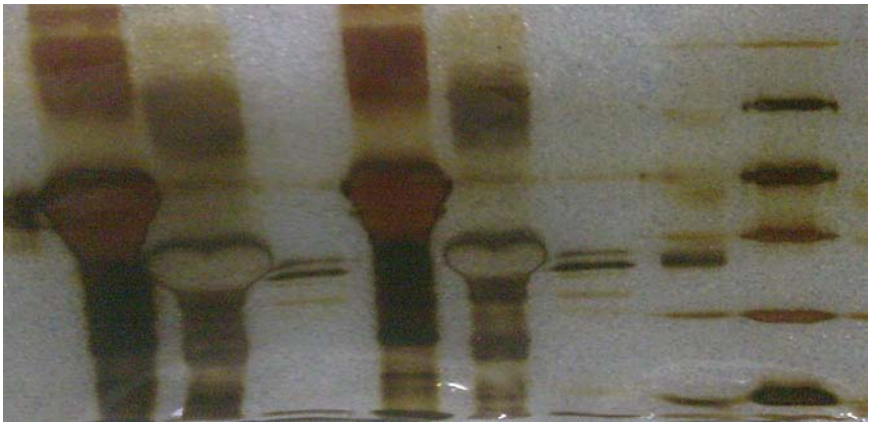


Figure 3.4. Silver stained SDS-PAGE.

3.5. Reagents

During the experiments most of the samples were prepared from analytical grade solid inorganic and organic compounds. In the first part of the experiments, LIBS analyses on pellet samples were performed. To avoid accumulation of the mositure on pelletized samples, Casein hydrolysate (Fluka), casein from bovine milk (Sigma Aldrich), disodium hydrogen phosphate (Sigma Aldrich), pellets were prepared fresh just before the analysis or kept in the desiccator when not in use for a short period of time. Pellets were prepared in stochiometric amounts by mixing with powder potassium bromide (Sigma Aldrich).

For the SDS-PAGE experiments, enough solutions were prepared for a one time except ammonium persulfat solution, it was prepared for each SDS-PAGE study freshly. Acrylamide / bis (30% T, 2,67% C Stock), distilled water, Tris-HCl(1.5 M ,pH:8.8 and 0.5M, pH:6.8), 10% (w/v) SDS, 10% ammonium persulfate and tetramethylethylenediamine (TEMED) solutions were used in each SDS-PAGE work. Protein solutions including α-casein(Sigma Aldrich), albumin (Sigma Aldrich,

Phosdecor (Sigma Aldrich) and unstained protein molecular weight marker (Fermentas) were prepared for a once then kept under -20 ºC temperature.

Commasiie Brillant Blue stain fixation and staining solutions were prepared once and used throughout. Destaining solution were prepared freshly for each new gel. In silver staining technique, all solutions were prepared for once and used when needed.

CHAPTER 4

RESULTS AND DISCUSSION

In this thesis study, LIBS system has been designed, constructed and optimized for identification and determination of phosphorus content in pressed pellets and in SDS-PAGE gels containing varying amounts casein and ovalbumin.

4.1. Instrumental Parameters

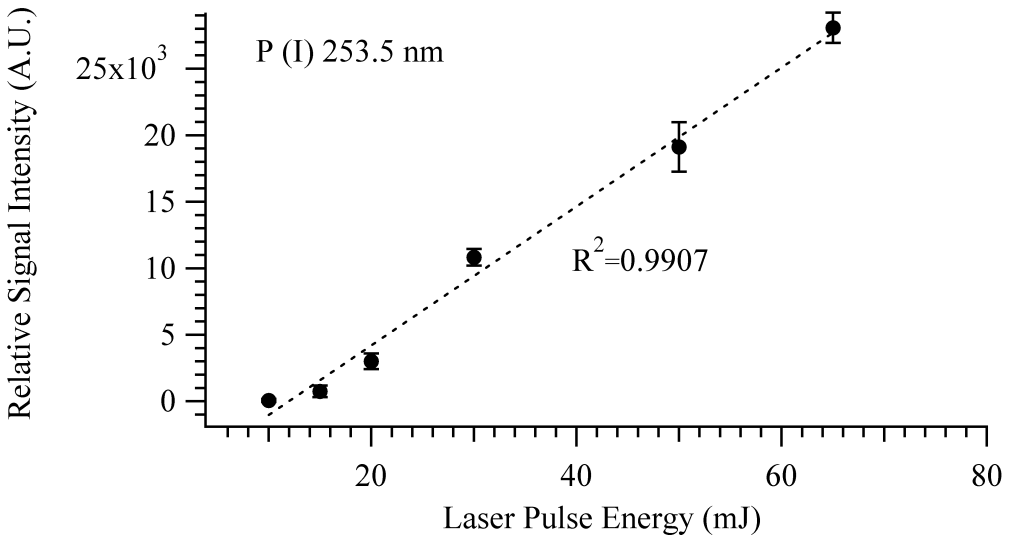
Before starting the analysis of phosphorylated proteins in gel, optimum instrumental conditions for the LIBS system like laser energy and detector timing parameters were determined on pelletized samples. Analysis of gel samples was performed under these optimum conditions.

4.1.1. Effect of Laser Pulse Energy on Signal Intensity

In LIBS measurements, laser pulse energy is one of the key parameter for plasma formation. The magnitude of the laser pulse energy effects plasma temperature, ablated amount from the surface and extent of ionization. By focusing the laser pulse on the sample surface, evaporation, atomization and ionization processes occur. With the use of low energy laser pulses, plasmas with low analyte signals are formed and analyte signal from the background noise it can be hardly differentiate. Therefore sensitivity decreases. At high laser fluences, **plasma shielding** is the most predominant mechanism which results with non-stoichiometric ablation of the target surfaces and hence deviation in growth curves is observed. Therefore, LIBS experiments require laser pulse energy to be optimized for each analyte emission signal with a high S/N.

Figures 4.1. (a) and (b) show LIBS signal intensity variation of P(I) lines with respect to laser energy at 253.5 nm and 255.3 nm emission wavelengths from Na_2HPO_4 samples, respectively. Similar experiment was also performed for Casein samples in the form of pellets and results are given in Figures 4.2.(a) and (b). For Na_2HPO_4, steady

increase in P(I) signals has been observed up to 70 mJ/pulse laser energy however, linearity extends upto 170 mJ/pulse energies for casein samples.



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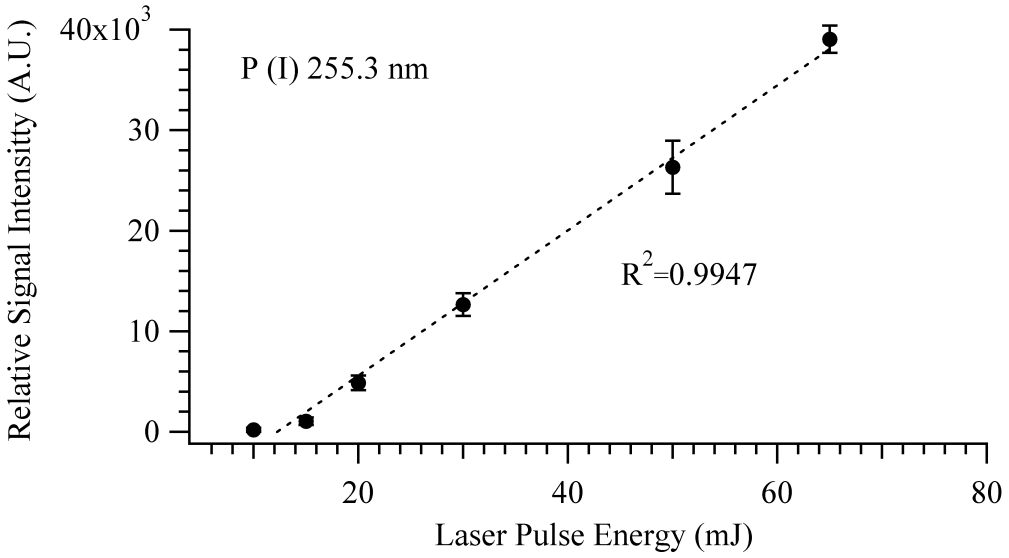
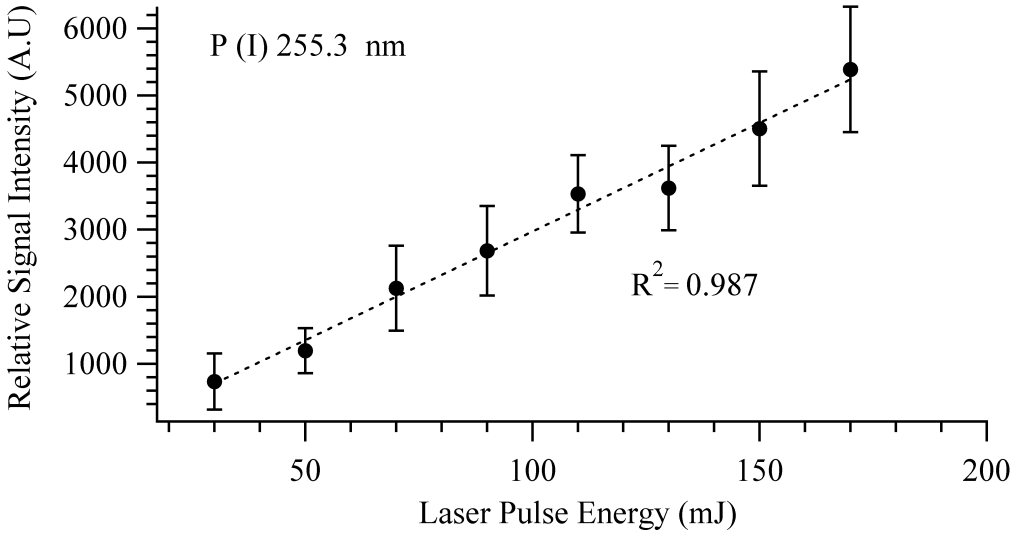
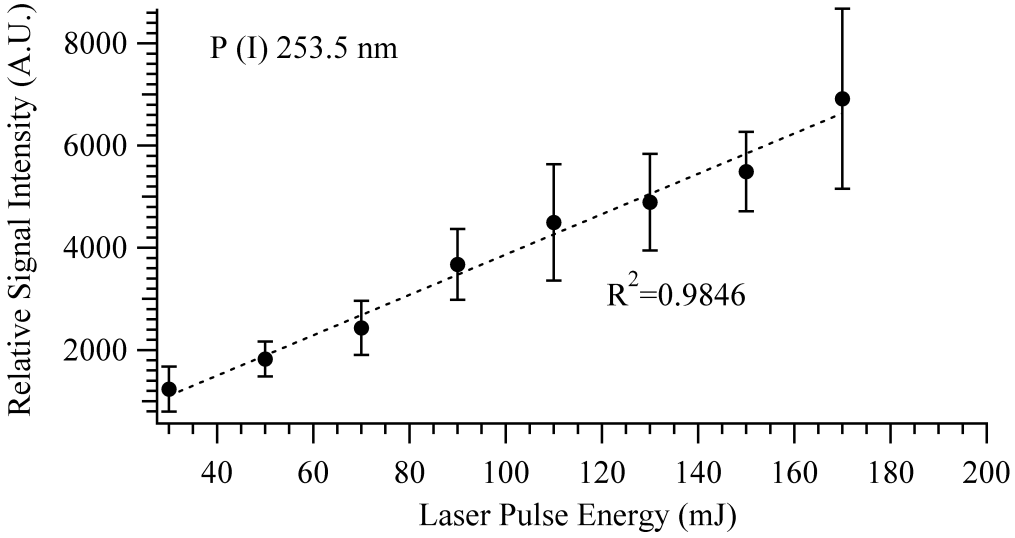


b)

Figure 4.1. Effect of the laser energy to Na_2HPO_4 analysis. LIBS signal intensity variation of (a) 253.5 nn and (b) 255.3 nm P (I) lines with respect to laser energy for inorganic phosphorus containing.



a)



b)

Figure 4.2. Effect of the laser energy to casein analysis. LIBS signal intensity variation of P (I) lines (a) 253.5 nn and (b) 255.3 nm with respect to laser energy for Casein sample.

Optimization studies for laser pulse energy were carried out by the changing the laser pulse energy at certain detector timing parameters for both inorganic (Na_2HPO_4) and organic (casein) molecules in the form of pellets. Detector timing parameters selected for inorganic phosphorus containing sample with delay time of 1000 ns and gate width of 1 ms and for casein sample, delay time of 1200ns and gate widthof 0.2 ms was used. Detector gain level was set as 100.

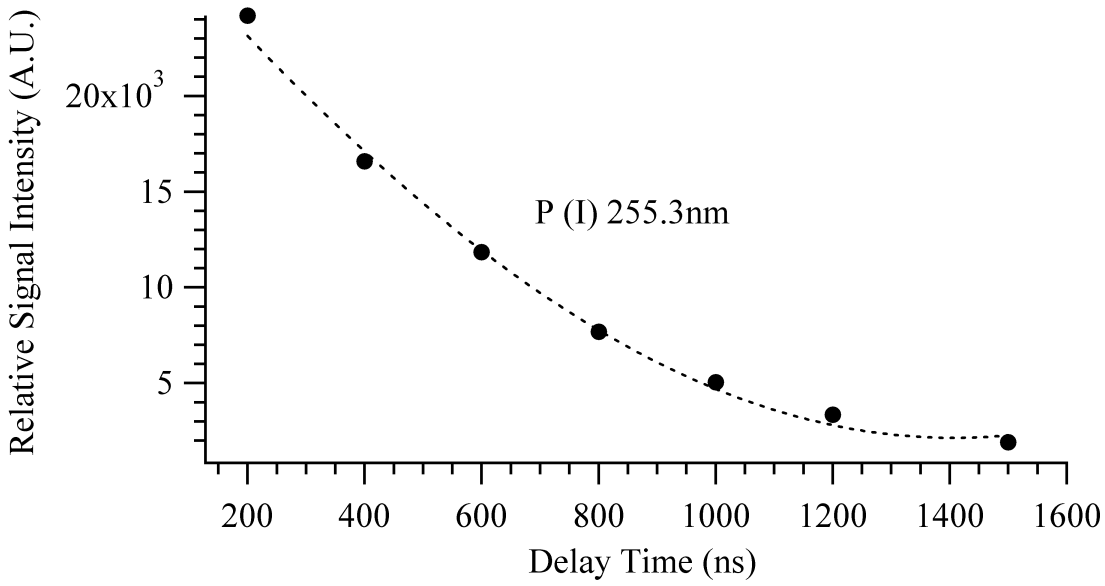
Each data was obtained from the accumulation of signals from five consecutive laser pulses.

120 mj laser pulse energy was chosen as optimum laser energy for the rest of our measurements to eliminate higher noise in thespectra.

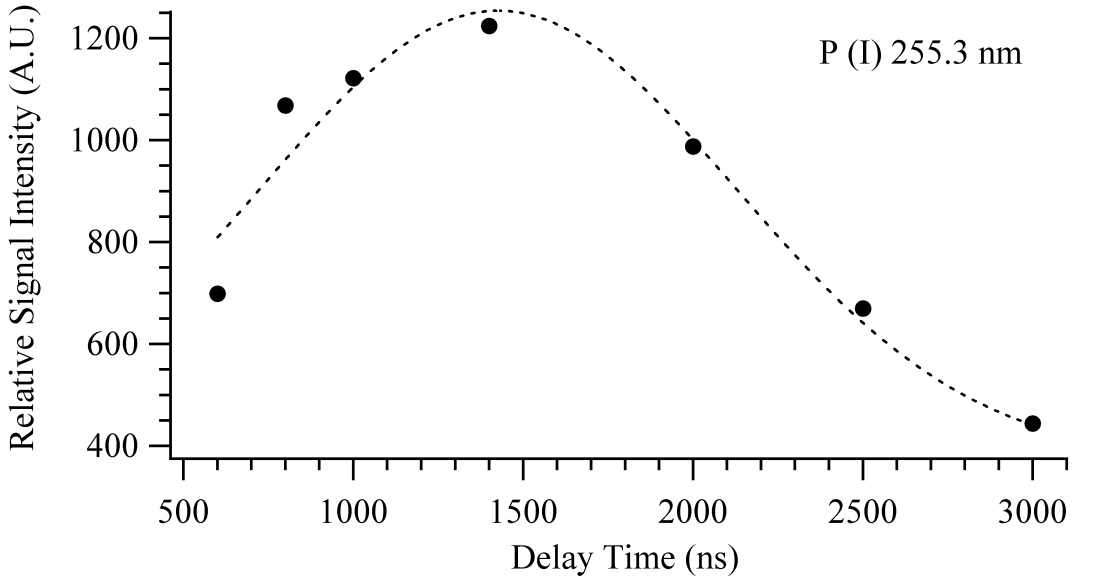
4.1.2. Time Resolution

Time resolution is essential to get more intense signals and decreased background levels in spectra. Time resolved measurements were carried out in order to find optimal time window in terms of delay time and gate time of the detector.

Figure 4.3 (a) and (b) shows variation of P (I) 255.3 nm signal intensity with respect to delay time for Na_2HPO_4 and casein molecules. For inorganic phosphorus containing sample, Na_2HPO_4, relative signal intensity is higher at early times and gradually decreses. However, it is observed that the range between 1000 ns to 1500 ns is suitable for P(I) signal intensity from Casein molecule. It is shown that, optimum timing parameters can be quite different for elements in different matrices, due to differing plasma conditions in the presence of different species.



a)



b)

Figure 4.3. Time resolution for phosphorus containing sample. LIBS signal intensity variation of 255.3 nm P (I) lines with respect to detector delay time for Na_2HPO_4 (a) and casein (b) containing sample.

Figure 4.4, shows the relative signal and background intensities variation in phosphorous emission lines. In general increasing the delay time, the intensity of P lines decrease however an observable decrease in background level increases the signal to noise ratio of the measurements. Unresolved phosphorous lines at 200 and 400 ns delay times start to be baseline separated at later delay times of 1 microsecond and 1200

microseconds. Therefore, not too early nor too late times are preferrable in LIBS measurements.

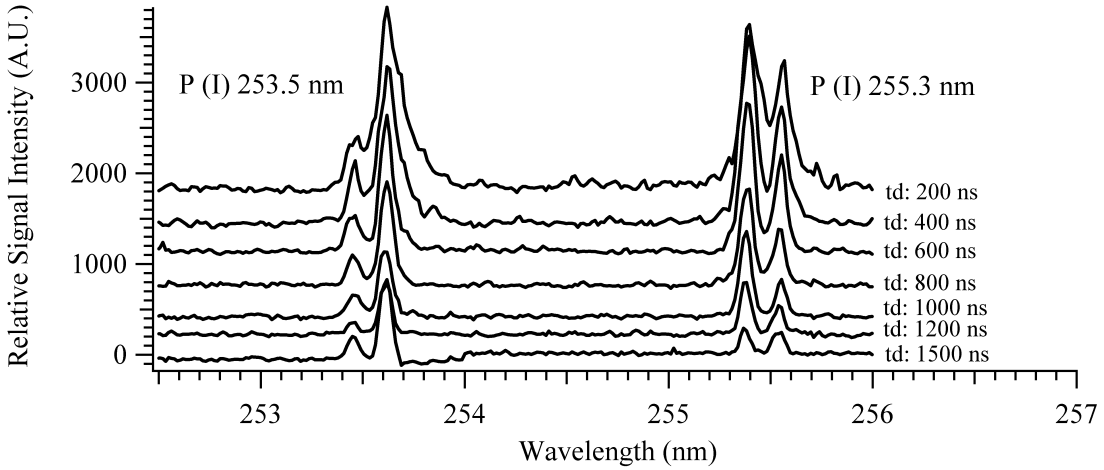


Figure 4.4. Time resolved spectra of Phosphorus containing sample. tg: 1 ms, laser pulse energy 120mJ, detector gain 100 and 3 single shot accumulation.

Optimization studies for gate width measurements were also performed with casein sample. As shown in figure 4.5, relative signal intensity for P (I) 255.3 nm emission increases up to 200 µs and after that point it drastically decreases. Optimum gate with value was selected as 200 µs.

Experiments were performed under 1200 ns detector delay time, 200 µs gate width and 150mj laser pulse energy of.

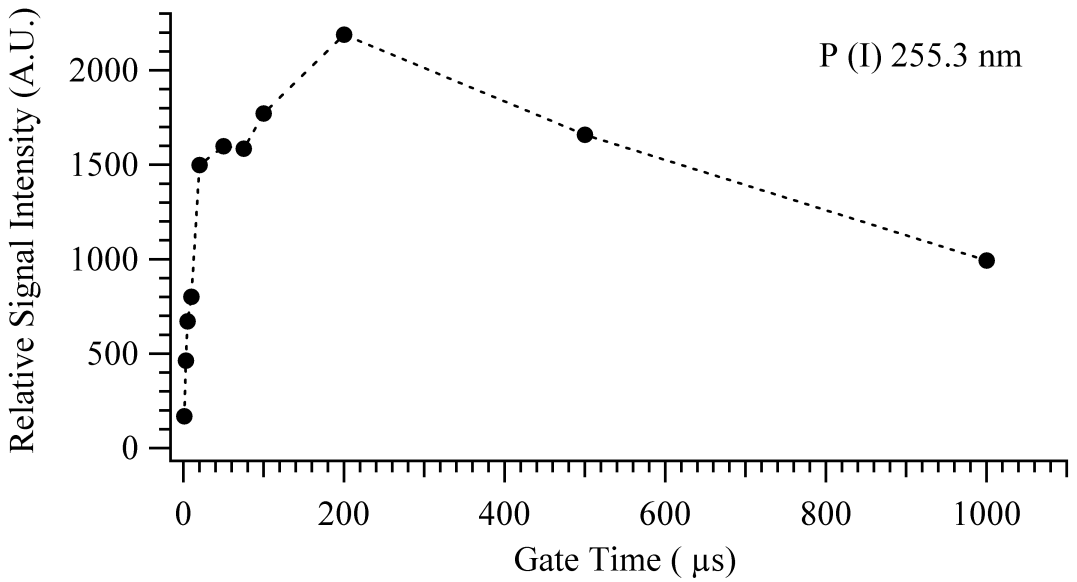


Figure 4.5. Effect of the detector gate time. LIBS signal intensity variation of 255.3 nm P (I) lines with respect to detector gate width for casein. Data was obtained from the accumulation of 5 single laser shots.

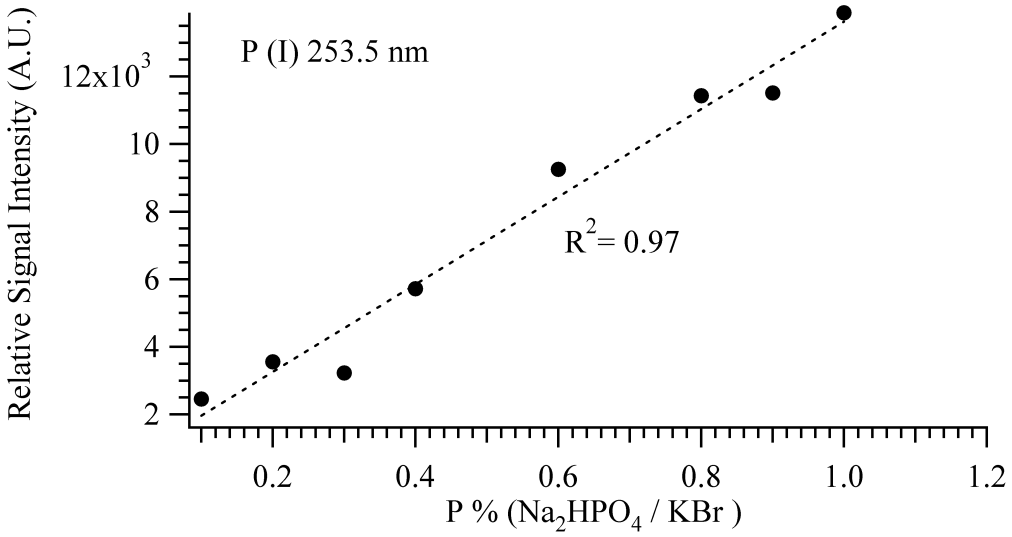
4.1.3. Growth Curves

In order to test the applicability of the LIBS measurements for quantitative analysis of phosphorus in both Na_2HPO_4 and Casein samples, the most intense lines of phosphorus at 253.5 and 255.3 nm have been monitored.

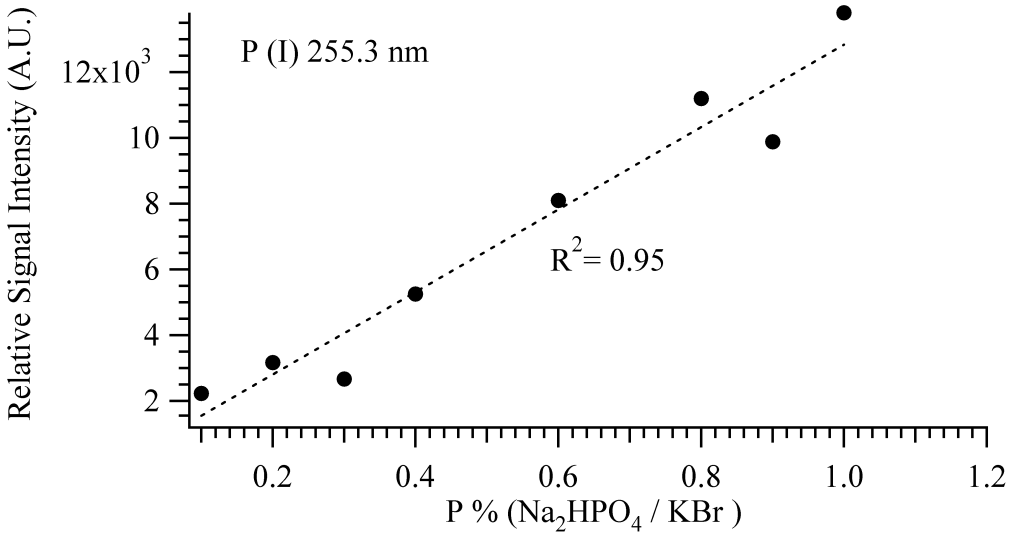
For this purpose, 0.5 grams of pellets with varying phosphorous concentrations in powdered KBr have been prepared and LIBS spectra were measured.

Figure 4.6. (a) and (b) show the growth curves at two different emission lines of neutral phosphorous from Na_2HPO_4 with P content ranging from 0.1 to 1.0 %. Calibration curves obtained were linear with regression constants of 0.97 and 0.95 for 253.5 and 255.3 nm wavelength emissions, respectively. In order to prevent saturation of the P(I) lines due to self absorption effect, concentration range was kept low, upto 1%.

Measurements were performed with laser pulses of 100 mJ energy, 1.2 microsecond delay time and 200 microsecond gate time. Detector gain setting was 100 and 5 single shot accumulation was performed.



a)

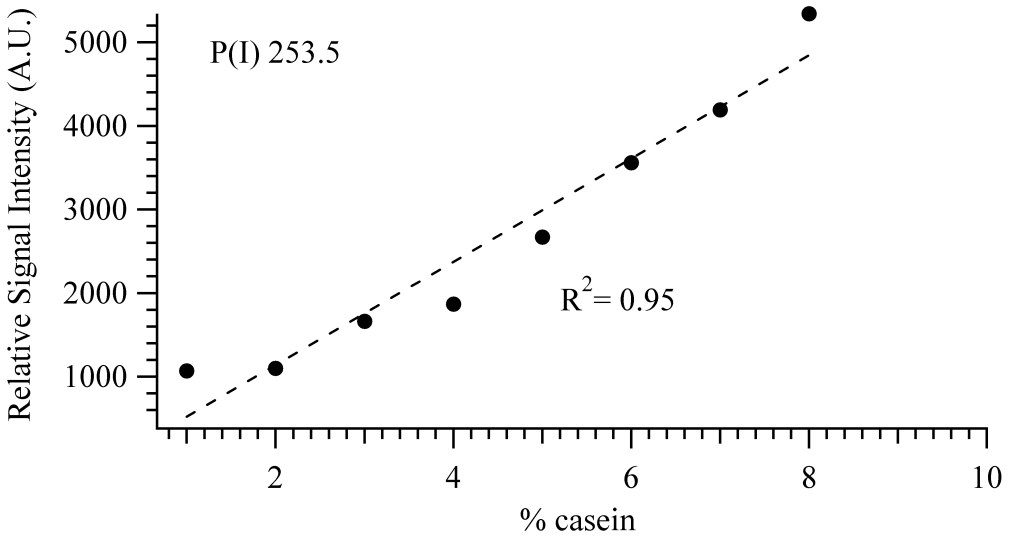


b)

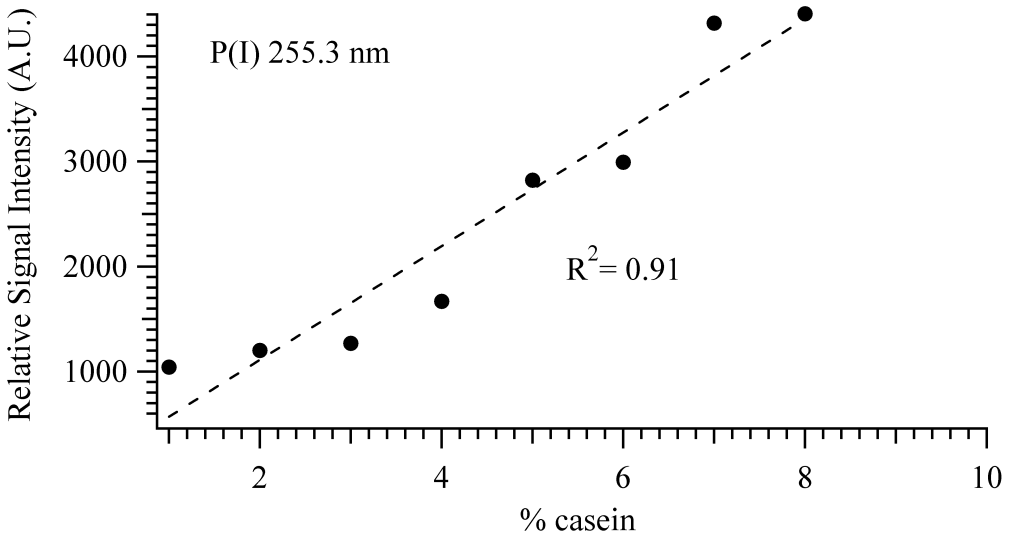
Figure 4.6. Growth curves for Na_2HPO_4. LIBS signal intensity variation of 253.5 and 255.3 nm P (I) lines with respect to % P content in Na_2HPO_4 + KBr mixture, Energy 100mJ/pulse, td:1200ns, tg:0.2 ms, gain:100, 5 single shot accumulation.

The growth curves at two different emission lines of neutral phosphorous from Casein samples with P content ranging up to 8.0 % have been presented in Figure 4.7. (a) and (b). It is apparent that, phosphorous signal from the organic matrix, Casein, is relatively low compared to inorganic phosphorous signal, especially at low concentrations, even higher laser pulse energy was used for the analyses. LIBS sytem appears to be insensitive below 3% phosphorous concentrations within Casein matrix.

Calibration curves are linear after 3% concentrations with regression constants of 0.95 and 0.91 at 253.5 nm and 255.3 nm, respectively.



a)



b)

Figure 4.7. Growth curves for casein. LIBS signal intensity variation of 253.5 and 255.3 nm P (I) lines with respect to % P content in casein + KBr mixture, Energy 150mJ/pulse, td:1200ns, tg:0.2 ms, gain:100, 5 single shot accumulation.

4.2. Qualitative Analysis

The amount of phosphorous in phosphorylated proteins, e.g. in casein has been a matter of some dispute for years. In this study, ICP-MS results has been utilized to provide information about the phosphorus content of casein and ovalbumin used. Results indicate 0.8 ± 0,05 % P from casein and 0.24±0.06 % P from ovalbumin. Typical phosphorous emmision lines at 253.5 nm and 255.3 nm from casein and ovalbumin samples are presented in Figure 4.8. Relative to its phosporous content ovalbumin sample presents lower emission signal compared to casein sample.

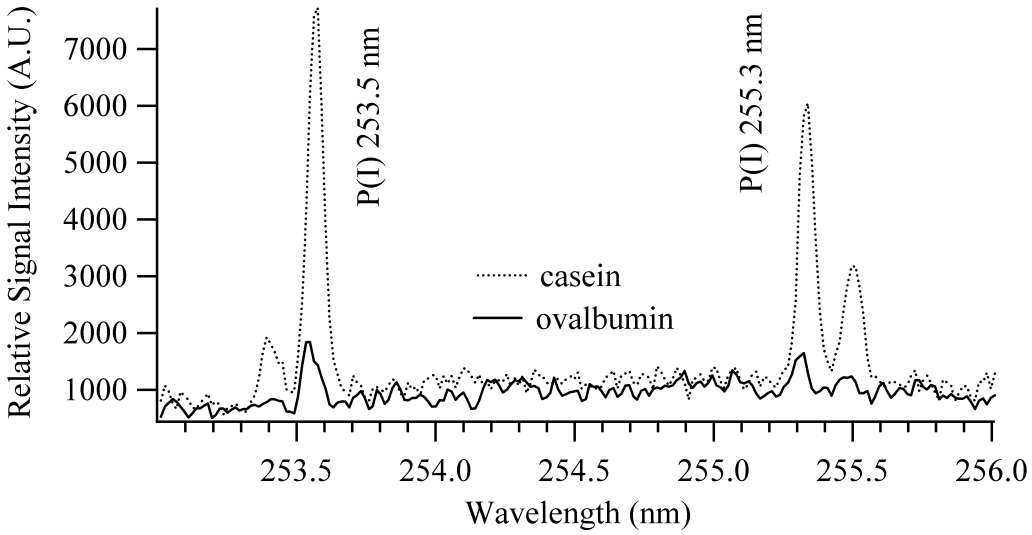


Figure 4.8. P lines for casein and ovalbumin.

Representative LIBS spectra obtained from 1% P containing Na_2HPO_4, casein and casein + SDS + acrylamide mixture, under optimum experimental conditions are given in Figure 4.9, 4.10 and 4.11, respectively. All elements present in the matrix like Ca, Mg, Na, C, H, O and N were determined simultaneously. Also Figure 4.11 shows the analysis of a pellet with similar composition of the gel medium.

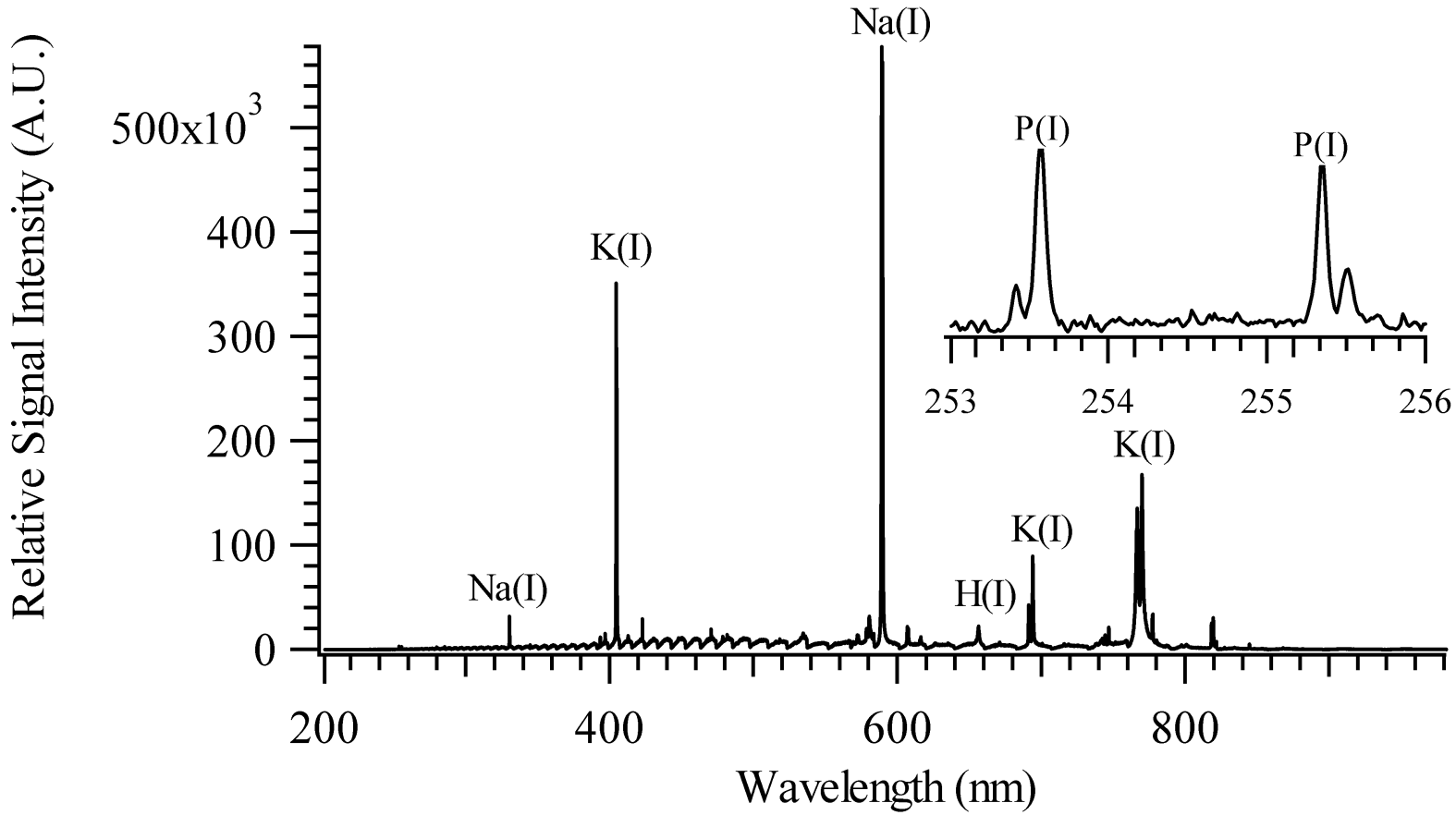


Figure 4.9. Representative LIBS spectra for Na_2HPO_4 + KBr mixture (%1 P) at t_d=1200ns, t_g= 0.2ms, laser energy 100mJ (5 single shot accumulation).

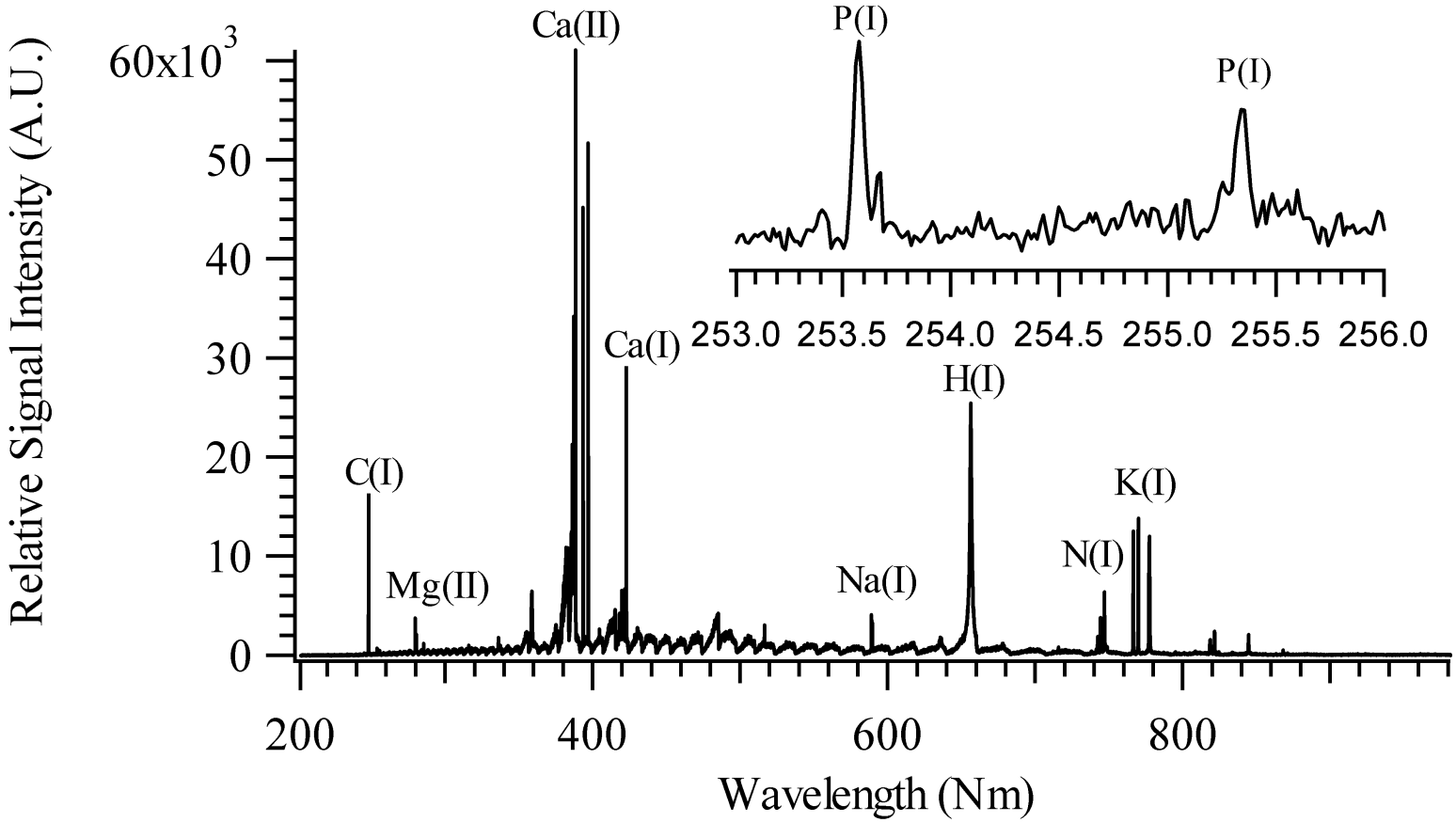


Figure 4.10. Representative LIBS spectra for casein at t_d=1200ns, t_g= 0.2ms, laser energy 100mJ (5 single shot accumulation).

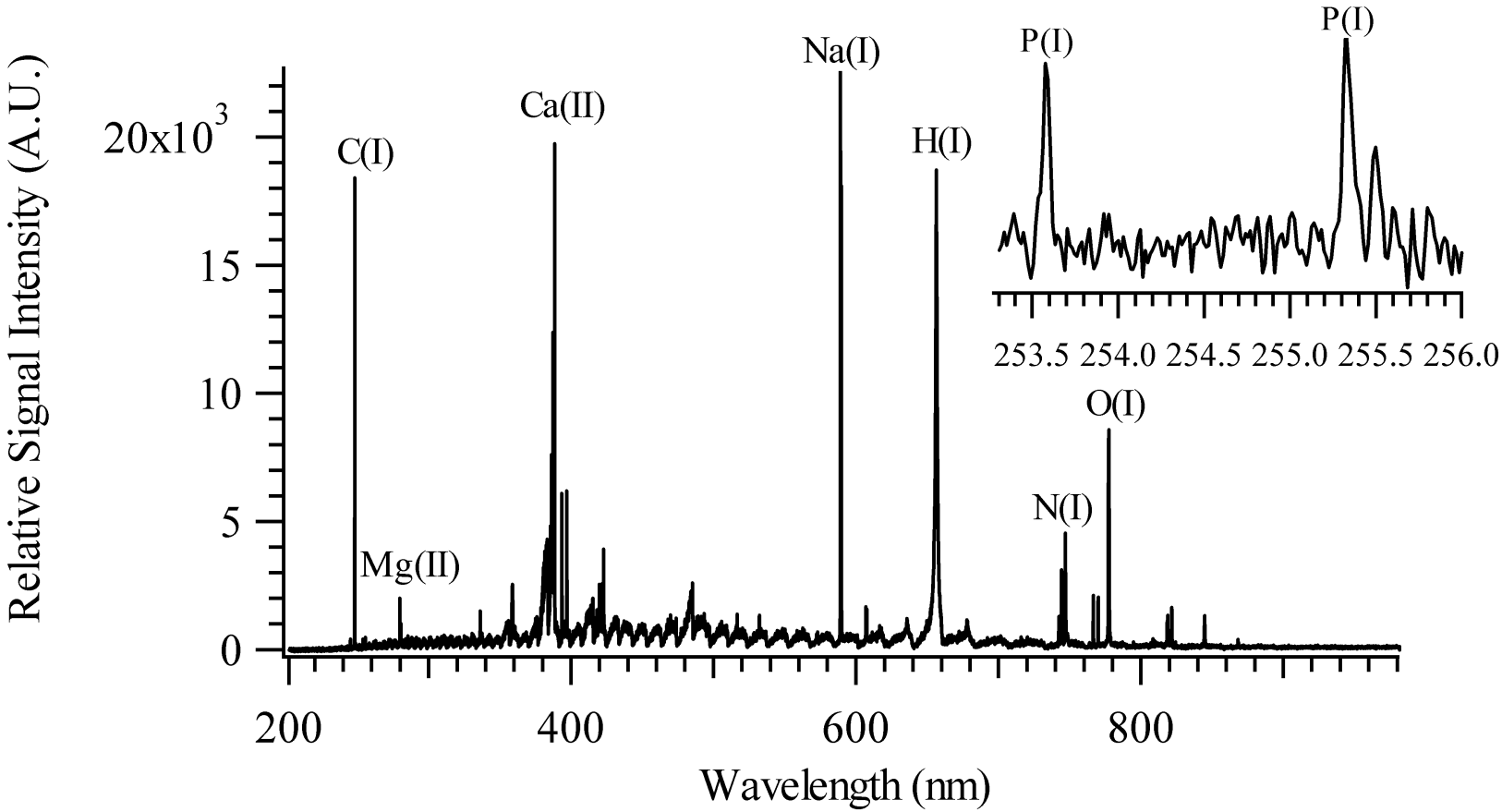


Figure 4.11. Representative LIBS spectra for casein + SDS + acrylamide mixture (%80 casein) at t_d=1200ns, t_g= 0.2ms, laser energy 150mJ (10 single shot accumulation).

4.3. Lateral Variation of Pellet

In order to investigate homogenous distribution of the phosphorous content throughout the pellet, lateral variation of the P/K line was measured in casein and KBr mixture containing 10 % casein. The data obtained from 50 different parts of pellet by LIBS provided about lateral variation of pellet. The aim here is to get information about the pellet homogeneity as well as to determine the effect of weighing errors if coccurred any. As shown in Figure 4.12., P(I) 253.5 nm /K(I) 404.5 nm line ratio is quite stable with relative standard deviation of 10.6 % over the entire region sampled on the pellet.

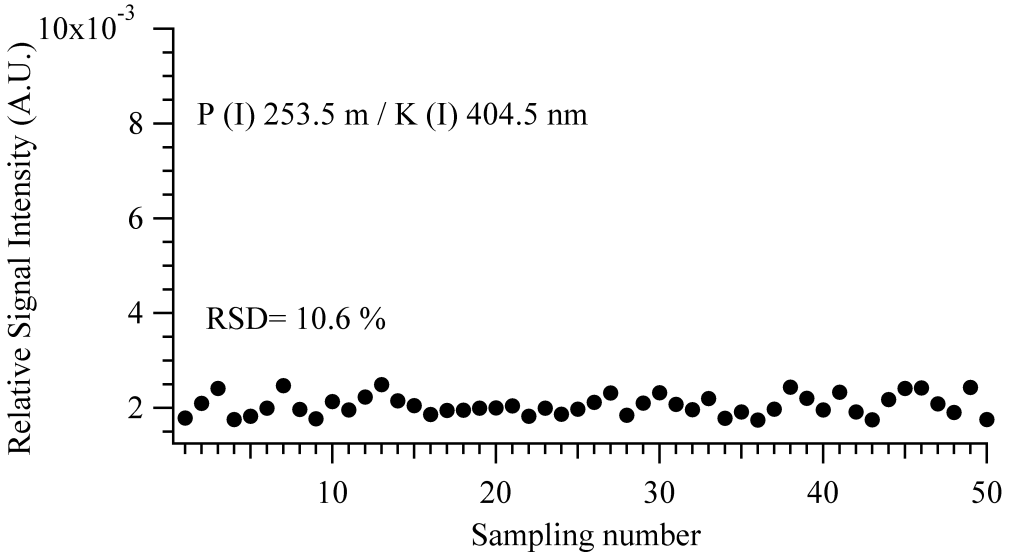


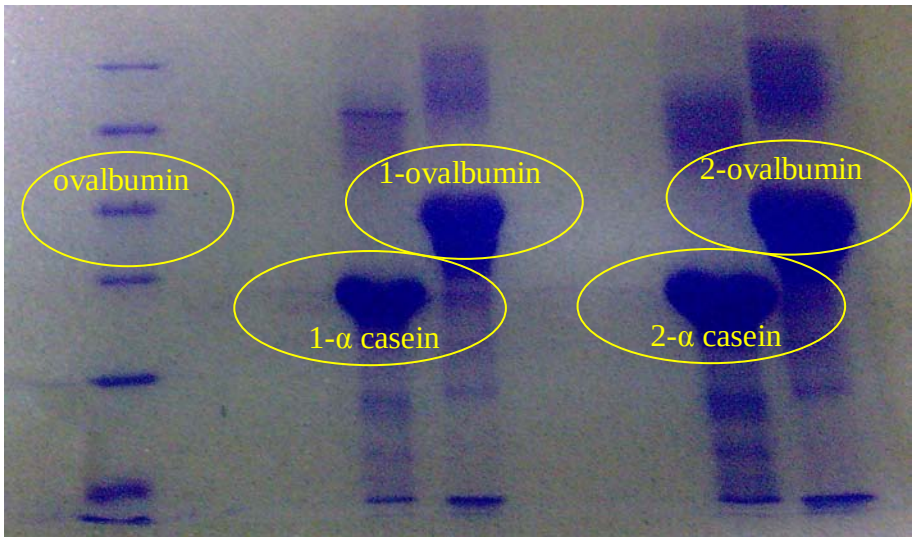
Figure 4.12. Lateral variation of P signal in powdered casein+ KBr mixture. Pellet contains 10 % casein. Laser energy:150 mJ, t_d:1200 ns, t_g:0.2 ms, 5 single shot accumulation.

4.4. Gel Analysis

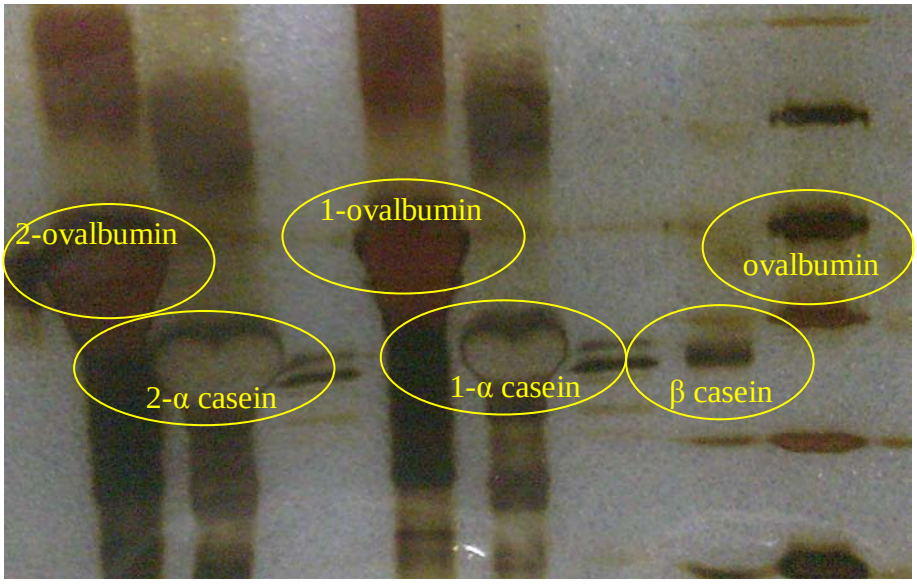
After determining the optimum instrumental parameters from the analysis of phosphorus containing samples in pellet forms, gel analysis have been performed. Analysis of different concentrations of phosphorylated proteins and the phosphorylated proteins in mixture were carried out.

4.4.1. SDS-PAGE Studies

Laemmli protocol was applied to prepare SDS-PAGE. Different concentrations of samples were loaded onto the gel and run by BIO-RAD SDS-PAGE system. Separated proteins by SDS-PAGE were stained with both coomassie brilliant blue and silver staining techniques. Stained gels with two different techniques destained and were dried for LIBS analysis.



a)



b)

Figure 4.13. a) Gels stained with Coomassie Brilliant Blue and b) silver staining technique.

Stained gels by two different techniques are shown in figure 4.13. (a) and (b). For the first figure, it was stained by coomassie brilliant blue and it is indicated that the bands of phosphorylated proteins casein and ovalbumin. The first ovalbumin band belongs to Fermentas unstained protein molecular weight marker. Marker consists of the following proteins from top to bottom: β-galactosidase, bovine serum albumin, ovalbumin, lactate dehydrogenase, Rease Bsp98I, β-lactoglobulin and lysozyme. For the α-casein, first one has lower total protein concentration (4.40 mg/ml) while second α-casein has higher total protein concentration (39,62 mg/ml). For the ovalbumin samples, first ovalbumin has lower total protein concentration (3.34 mg/ml) while second ovalbumin has higher total protein concentration (29.78 mg/ml). For the silver stained gel the ovalbumin and α-casein total protein concentrations are the same as coomassie stained gel. β-casein bands belongs to Sigma phosdecor control protein mixture. Phosdecor control consists of β-galactosidase, phosphorylase b, albumin, ovalbumin, β-casein, lysozyme. Both gels were dried smoothly for LIBS analysis at room temperature.

4.4.2. Analysis of Phosphorylated Proteins In-Gel by LIBS

Separated protein bands by SDS-PAGE were analyzed by LIBS. Laser beam was directly focused on the target that was placed on the translational stage. Emitted lights from the plasma were sent through the detector to identify phosphorus in proteins.

Full spectrum belonging coomassie and silver stained gels are shown in figure 4.14. and 4.15., respectively. The differences between two gels arise from two different staining techniques and reagents. While many elements are the same for both staining techniques in gels, cobalt is presented just in the coomassie stained gel and silver is presented only in silver stained gel.

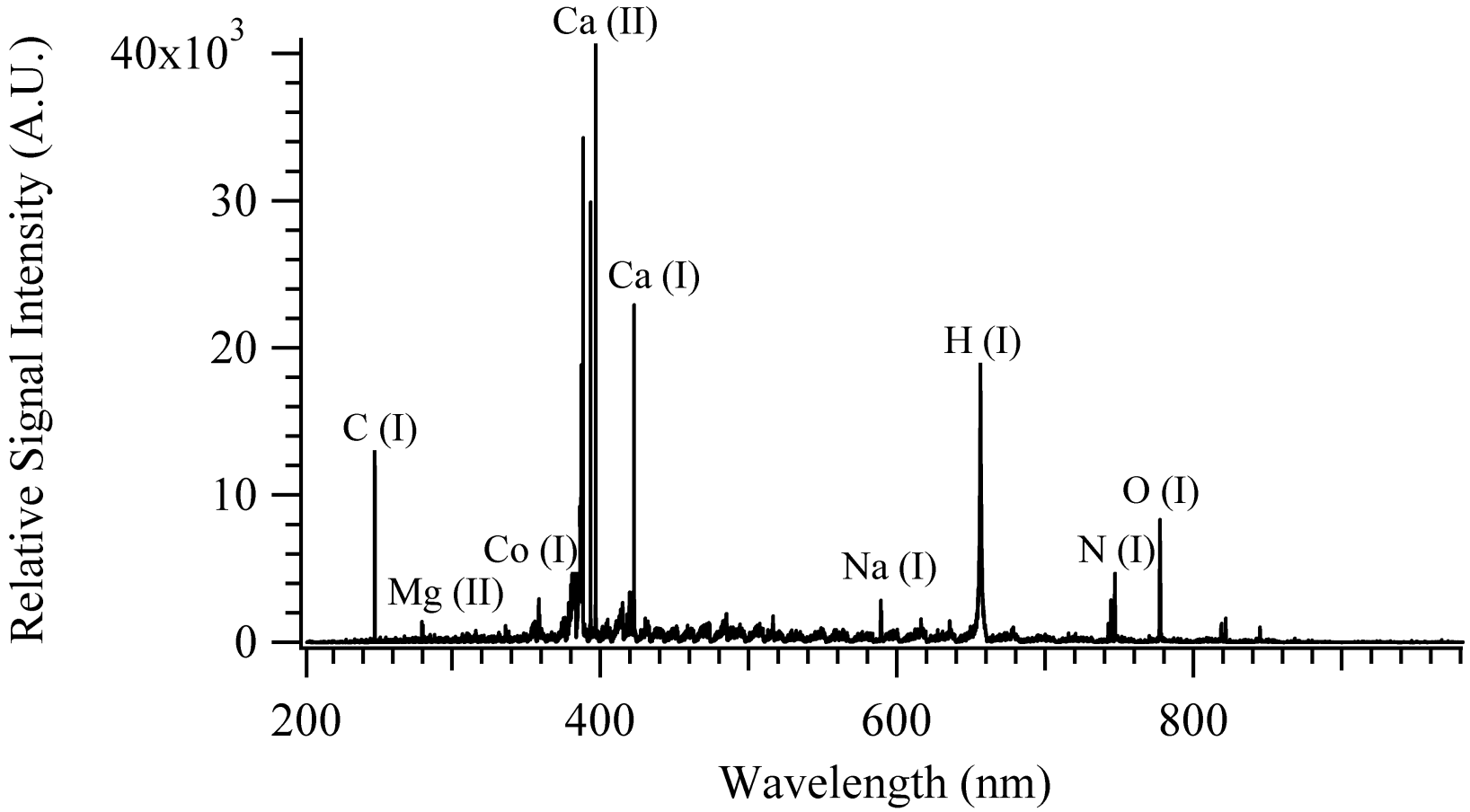


Figure 4.14. Typical LIBS spectrum of the coomassie stained gel. (Laser pulse energy: 120mJ, t_d:200 ns, t_g:0.2 ms, gain.150,single shot).

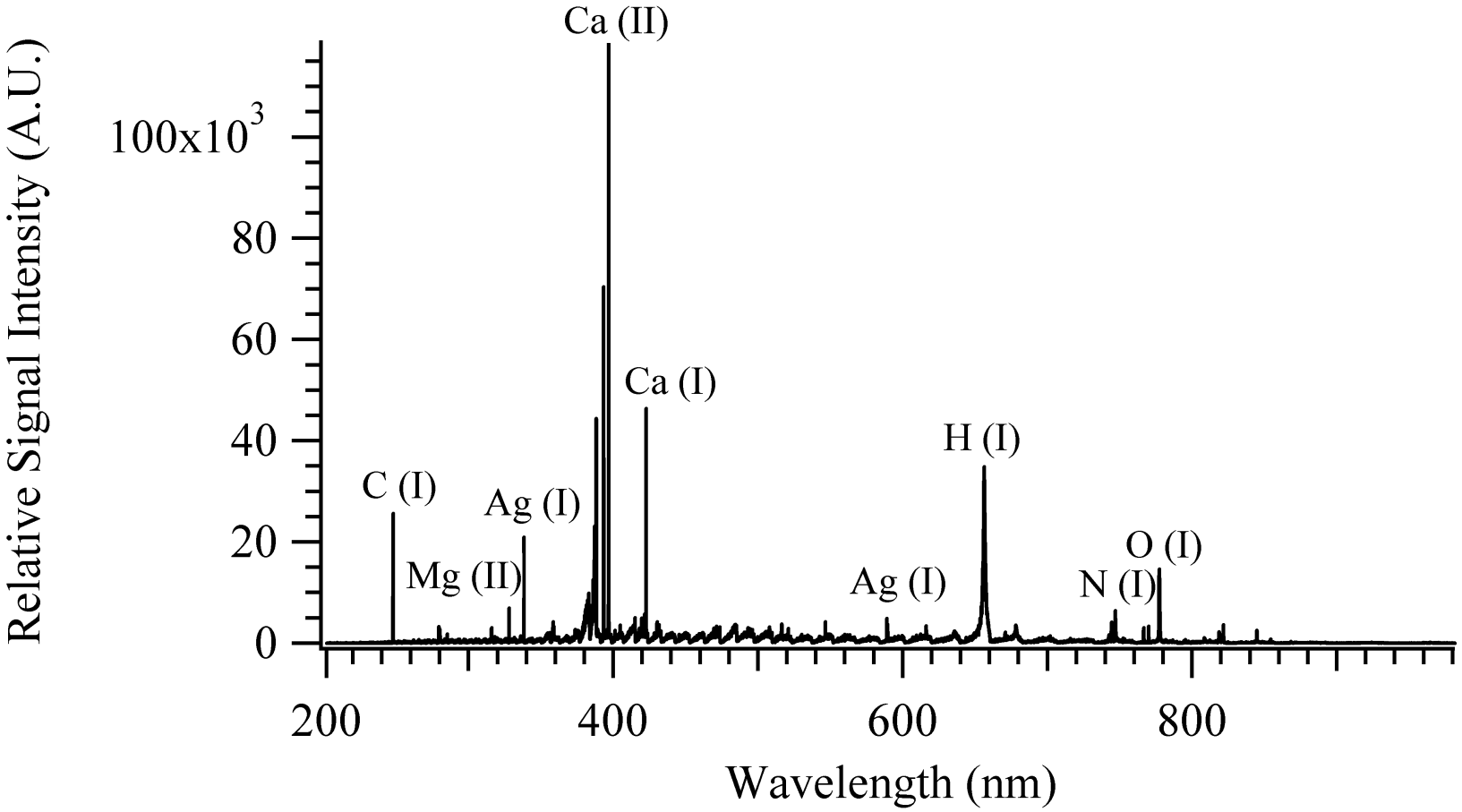


Figure 4.15. Typical LIBS spectrum of silver stained gel. (Laser pulse energy: 120mJ, t_d:200 ns, t_g:0.2 ms, gain.150,single shot).

Figure 4.16, represent P lines identified from the gels containing ovalbumin and casein from standards prepared in laboratory and stained with two different staining techniques. For all figures, except number of laser shots used, instrumental parameters were kept the same; laser pulse energy: 120mJ, t_d: 200 ns, t_g: 0.2 ms and gain: 150. From their respective concentrations, 20 microliters of protein samples were loaded onto gels. The amounts of casein that correspond to observed LIBS signal intensities were quantified as 70 µg from Coomassie Blue staining and Silver staining procedures. Similarly, ovalbumin amounts detected from Coomassie Blue and silver staining was 50 and 300 µg.

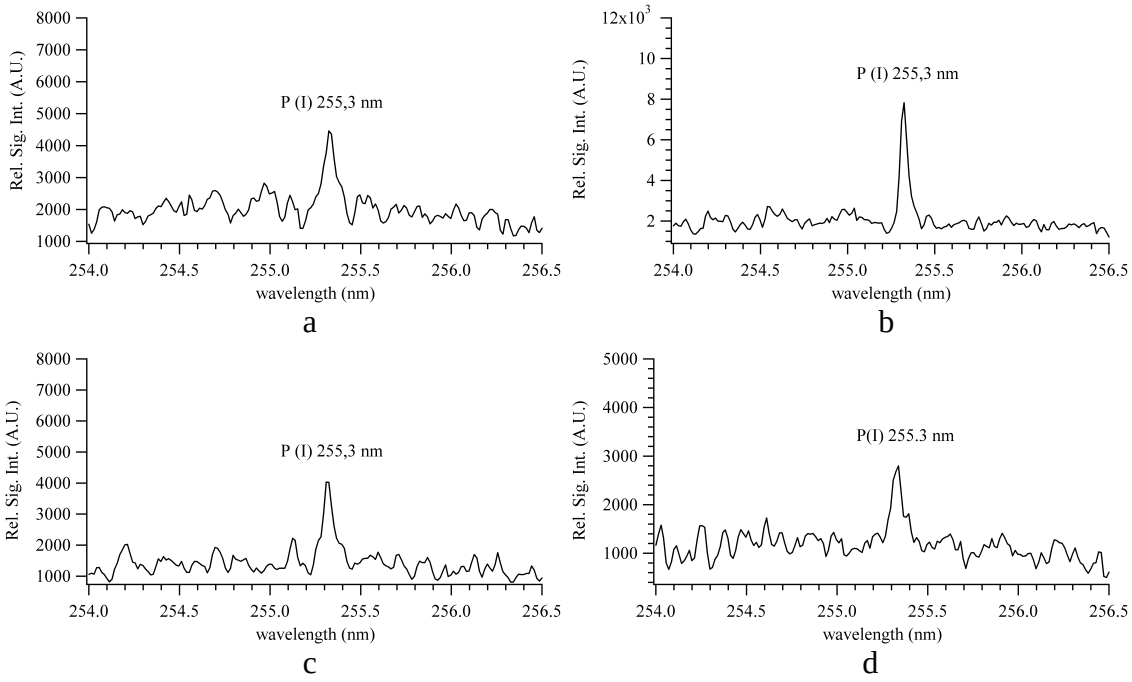


Figure 4.16. Phosphorus signals in gels. (a)silver stained gel containing 2900 ppm ovalbumin, 14 single laser shot (b)coomassie stained gel containing 16667 ppm ovalbumin,41 single laser shot (c) coomassie stained gel containing 3900 ppm α-casein, 16 single laser shot and (d)silver stained gel containing 3900 ppm α-casein, 10 single laser shot.

Figure 4.17 shows spectra from Fermentas protein molecular weight marker that contains 7 different proteins, between 253-258 nm spectral range

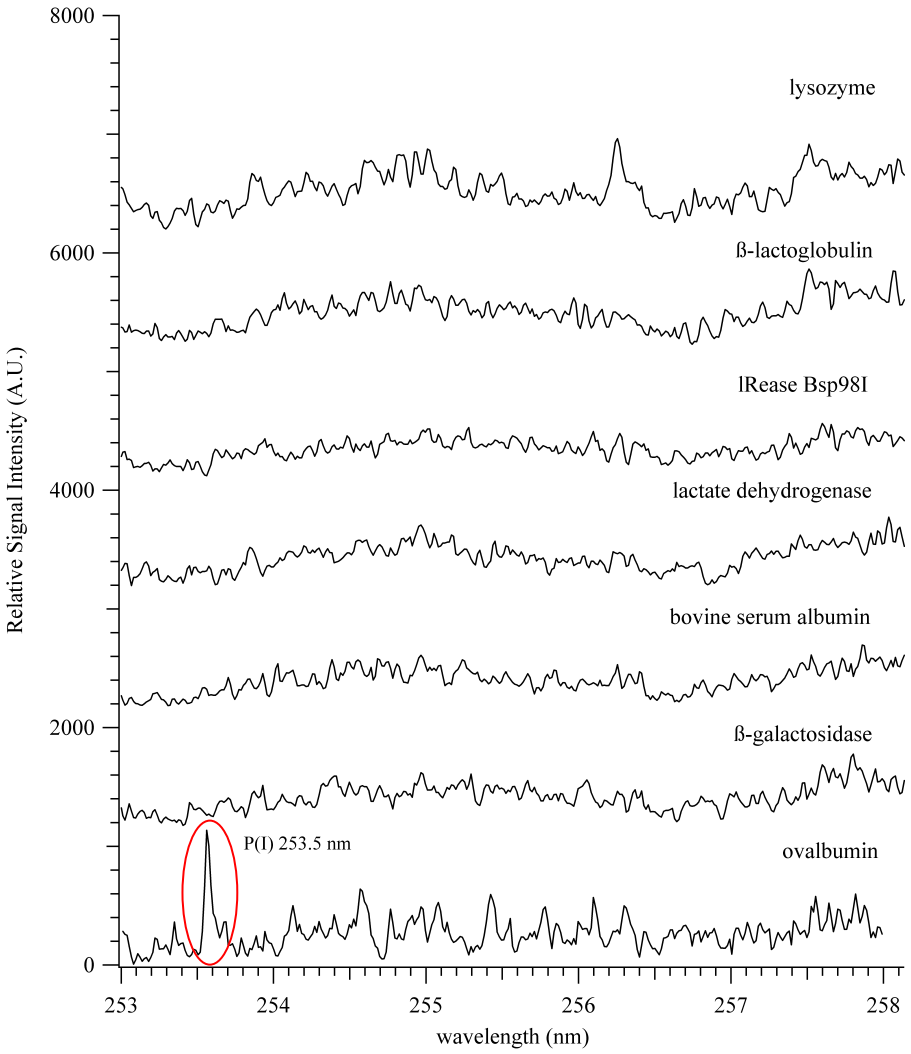
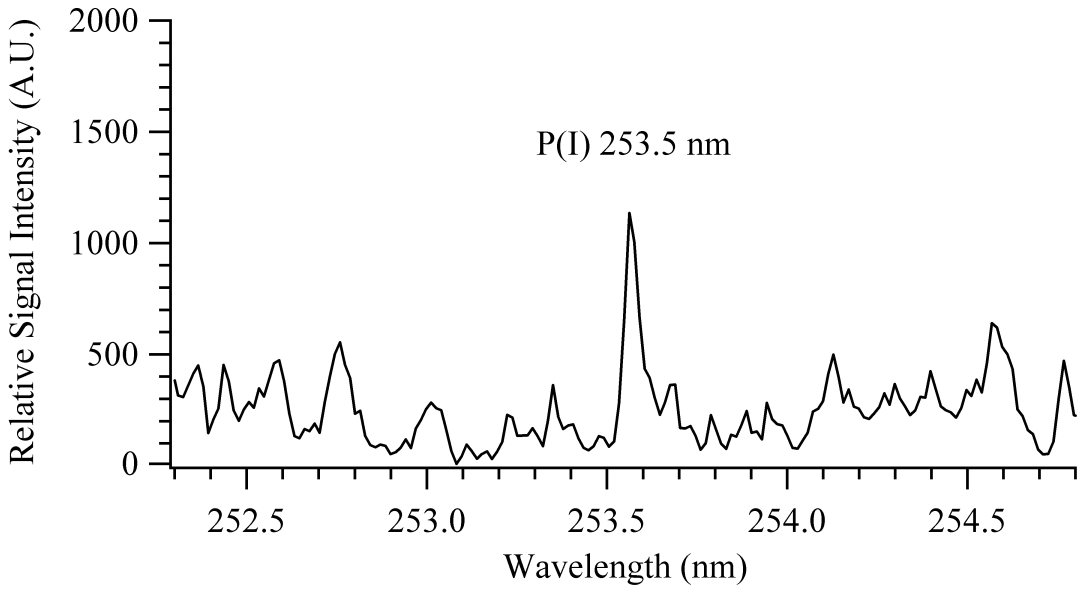


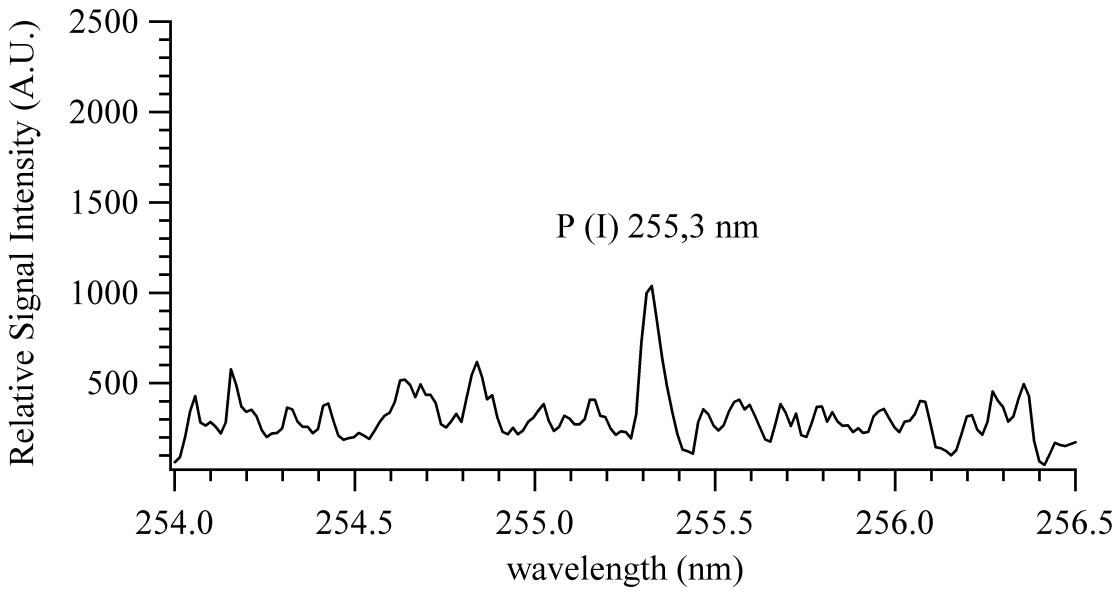
Figure 4.17. Phosphorus signals in coomassie stained gel from Fermentas protein marker.

As shown in the Figure 4.17., phosphorus signal was only observed at 253.5 nm from ovalbumin band which was the only phosphorylated protein. LIBS analysis the analysis of other protein bands separated on the gel, namely β-galactosidase, bovine serum albumin, lactate dehydrogenase, Rease Bsp98I, β-lactoglobulin and lysozyme, did not result with any phosphorus signals.

Figure 4.18 presents phosphorus signals from 200 ppm ovalbumin containing Fermentas protein marker stained by coomassie (a) and silver (b) staining protocols after electrophoretic seperation. It can be clearly observed that, both staining technique presents similar signal intensities at 253.5 nm.



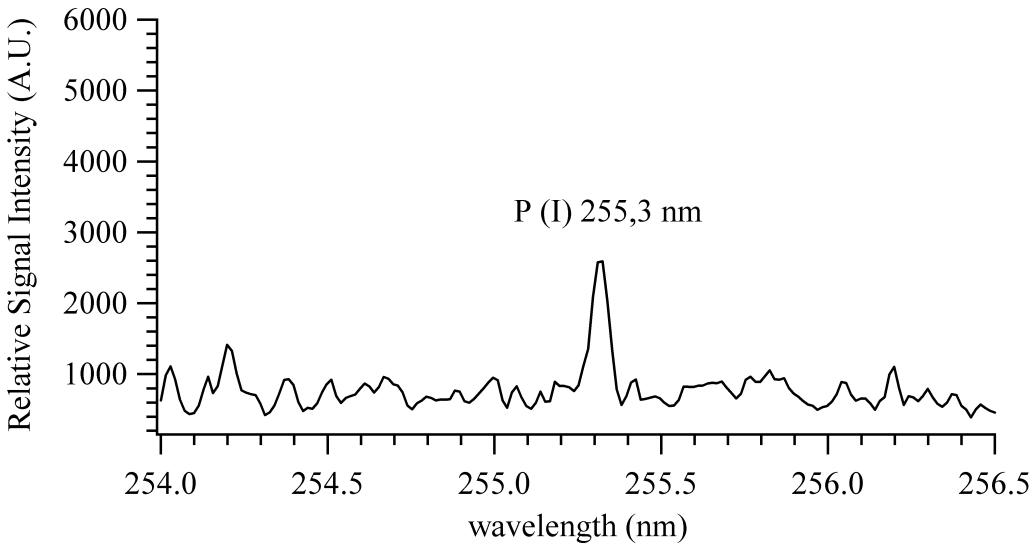
a) The accumulation of 20single laser shot



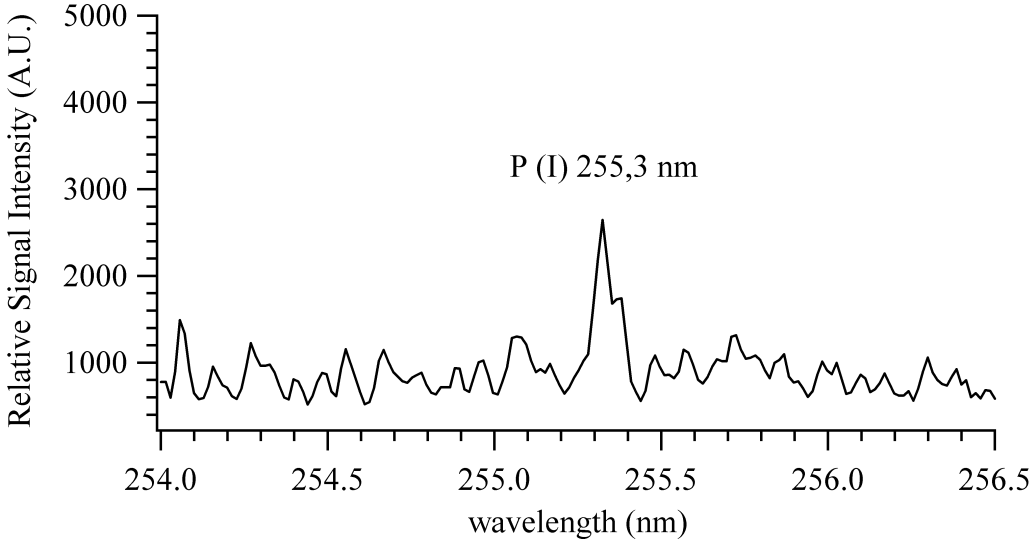
b) The accumulation of 5 single laser shot

Figure 4.18. Fermentas protein marker, phosphorus signals in coomassie (a) and silver (b) stained gel containing 200 ppm ovalbumin.

Another protein standard used for testing the detection capability of LIBS was Phosdecor control that contains 6 different proteins with two of them phosporylated. Protein concentrations were about the same as in Fermantes marker. Phosphorous lines detected by LIBS for two staining technique is given in Figure 4.19. below.



a) The accumulation of 10 single laser shot



b) The accumulation of 9 single laser shot

Figure 4.19. Phosdecor control, phosphorus signals in silver stained gel for β-casein 250 ppm (a) and ovalbumin 250 ppm (b).

In general, identification and detection of phosphorylated proteins directly in-gel with coomassie and silver staining techniques have been evaluated. Due to high amounts of loadings, phosphorus signals detected with more intense signals in gels that contained laboratory prepared standards of ovalbumin and casein. However, with protein markers containing more than one protein (Phosdecor control and Fermentas) nanogram levels of phosphorus in casein and ovalbumin were detected by LIBS.

4.5. SEM Analysis

The morphology and size of the ablated spots on the dry gels were investigated by SEM. The approximate crater size was measured about 100 μm in diameter by ablation with single laser pulse (120 mJ). At this point the calculated focal area was $4x10^{-6}$ cm^2. Ablated sample amount from surface was high nanogram levels and detected phosphorus quantitites from that amounts were detected at low nanagram levels.

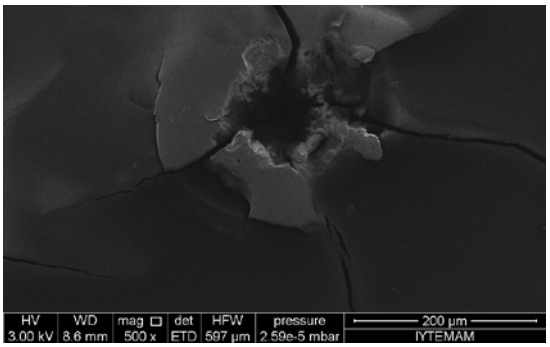


Figure 4.20. SEM image of crater obtained from a single laser shot of 120 mJ energy.

CHAPTER 5

CONCLUSIONS

In this study, a laboratory constructed LIBS set-up has been optimized for identification and detection of phosphorylated proteins in SDS-gel samples. For this purpose, the LIBS system was optimized in terms of instrumental parameters, such as delay time, gate width and laser energy.

Under optimum experimental conditions growth curves for inorganic phosphorous containg Na_2HPO_4 and Casein samples were obtained by their prominent P lines at 253.5 nm and 255.3 nm.

Two commercially avaiable biomarkers (Phosdecor and Fermantes) along with laboratory prepared protein standards (casein and ovalbumin) were run according to SDS-PAGE separation technique and separated proteins were stained by two different staining procedures. It has been demonstrated that LIBS can be used for local spotting of phosphoproteins after SDS-PAGE separation before MS detection. Nanogram quantities of phosphorus were determined in gel by LIBS.

LIBS technique shows a great promise in phosphoprotein spotting before MS analysis and currently far from low levels of quantitative analysis hovewer, suitable enrichment techniques could be helpfull in semi-quantitative analysis.

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