Chapter 2

Review Of Literature
2.1. Introduction

β-N-oxalyl-L, α, β-diaminopropionic acid (β-ODAP) is a neurotoxic amino acid present in the seeds of *Lathyrus sativus* (Khesari dal/grass pea). The excessive consumption of *Lathyrus* seeds by humans in some countries of Asia (China, Afghanistan, India, Bangladesh and Nepal) and Africa (Ethiopia) is known to result in neurolathyrism, an upper motor neuron degenerative disease (Rao *et al.*, 1964; Spencer *et al.*, 1986; Watkins *et al.*, 1966). It is characterized by a spastic paraplegia of the lower limbs that can vary from mild walking difficulties to bedridden state (Fig. 1). In India 0.53% people are suffering from neurolathyrism especially in eastern Uttar Pradesh, Madhya Pradesh, Bihar and Jharkhand. This crippling disease with sudden onset affects preferentially the most active young men in destitute remote rural areas and/or living in a hand-to-mouth economy. Neurolathyrism occurs due to prolonged over-consumption of grass pea seeds over several months as staple food in an unbalanced diet.

![Patients suffering from neurolathyrism](image)

2.2. Chemistry of β-ODAP

As an analogue of glutamate, β-ODAP (Fig. 2) acts on a well defined class of glutamate receptors in the neurons: the alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propanoic acid AMPA-receptors. It also inhibits the reuptake of glutamate during the neuronal...
signalling, thereby prolonging the excitation (Kusama et al., 2000). It can also be recognised by glutamate specific enzyme. The latter property is used to specifically quantitate the beta-isomer of ODAP (Moges et al., 2004). In aqueous solution, the molecular modelling of energy minimised structures of glutamate, ODAP and tyrosine indicate that the structure of ODAP is conformationally cognate more with tyrosine than with glutamate (Rao, 2001) This is corroborated by the fact that ODAP does not affect any of the enzymes of glutamate biosynthesis but that the enzyme tyrosine aminotransferase is inhibited by β-ODAP in a noncompetitive manner.

![Chemical structure of β-ODAP](image)

**Fig. 2.** Chemical structure of β-ODAP

### 2.3. Biosynthesis of β-ODAP

The biosynthesis of β-ODAP was proposed as an oxalation of 2, 3-diaminopropanoic acid (DAPRO) with oxalyl-coenzyme A. The natural occurrence of DAPRO was never proven and later it was established that this was only a short-lived intermediate between β (isoazolin-5-on-2-yl)-alanine (BIA) and β-ODAP. BIA is a non-protein amino acid present at high concentration in the seedlings of grass pea, garden pea (*Pisum sativum*) and lentil (*Lens culinaris*) up to 2% of the dry weight of 2-4 days old seedlings with cotyledons removed. Only grass pea has ever been implicated in human toxicity. This biosynthetic pathway was confirmed in various tissues, including cultured cell tissues and developing pod tissues where BIA is under the limit of detection (Kuo et al., 1991; Kuo et al., 1994) (Fig. 3). BIA is precursor for the in vivo biosynthesis of the neurotoxin ODAP responsible for human lathyrism and the cotyledons are the major site for this biosynthetic step in the seedling stage. The young leaves and ripening seeds of mature green plants contain high concentrations of ODAP. Labelling experiments with both unstable [14C] and stable carbon [13C] and nitrogen [15N] isotopes proved that the carbon atoms and the 2-amino group are derived from the alanyl side chain of BIA, while the 3-amino nitrogen is derived from the isoazolinone ring (Kuo et al., 1995).
Fig. 3. Biosynthetic pathway for β-ODAP in *Lathyrus sativus* (Lambein et al., 1990)

A H2O-addition to the double bond was proposed as the first step in the opening of the isoxazolinone ring. This water addition also seems to occur under UV-light irradiation (λ=254 nm) in which DAPRO is also released (De Bruyn et al., 1992). These isoxazolinone metabolites are among the most UV-light sensitive natural products having a quantum yield of about 0.5, meaning that on average one molecule isoxazolinone is broken down per two photons absorbed by the solution. Labelling experiments with both unstable [14C] and stable carbon [13C] and nitrogen [15N] isotopes proved glutamate transport into neuronal astrocyte cells. It also exerts some toxicity against those cells that are the ‘power house’ producing the energy-rich ATP for the neurons (Bridges et al., 1991). The mitochondria, the intracellular power houses of the cell, seem to be specifically affected (Ravindranath, 2002). The astrocyte cells normally absorb the glutamate released into the synaptic cleft during neuronal signalling and these glutamate molecules are the normal agents for the excitation at the AMPA receptors that involves the release of short-lived molecules of nitric oxide (NO). Glutamate absorbed by the astrocytes is metabolised into the less excitatory glutamine. Because of this dual effect of β-ODAP to trigger the AMPA-receptor excitation with the release of the signalling molecule glutamate into the synaptic cleft and to inhibit reuptake of glutamate, the excitation is prolonged and enhanced and the production of NO is much increased and so are the reactive oxygen...
species produced by this very unstable NO. Specific scavengers for such free radicals protect against the neuronal damage inflicted by β-ODAP (Willis et al., 1994). The prolonged presence of higher numbers of glutamate molecules that continue to ‘excite’ the neuronal receptors is the primary effect of β-ODAP that contributes to oxidative stress. The reasons for the very low incidence of neurolathyrism in cohorts subsisting on khesari dal have never been explained. Even within a family not everyone is susceptible to neurolathyrism. The fate of orally ingested ODAP from L. sativus had not been examined in detail. Since ODAP was not metabolized in experimental animals including primates, the same had been extended to humans also without any validation. A study established that healthy volunteers and subjects humans can almost quantitatively detoxify/metabolize orally ingested ODAP in sharp contrast to animals. This established for the first time that humans have a unique ability to metabolize ODAP. This raises the question as to how humans can be susceptible to ODAP toxicity when it is being detoxified while all animals examined so far are not susceptible even when they lack the ability to metabolize/detoxify ODAP. This paradox raises the possibility that may be a few individuals in the population are unable to detoxify ODAP and they may be susceptible to neurolathyrism. However, in a small group of individuals no individual has been identified so far who exhibits this trait and calls for a study involving a larger group. The precise biochemical mechanism of metabolism of ODAP in humans however, remains to be elucidated. Oxalate has been shown to be one of the end products in humans. It is likely that humans alone have a unique ability to completely oxidise ingested ODAP. This could also be the reason for not finding cases of neurolathyrism when L. sativus was being used by vast populations as part of their daily diet. A deficiency of sulphur amino acids has been proposed as a contributing factor in precipitating neurolathyrism.

2.4. Neurotoxicity of β-ODAP

One of the very early studies by Watkins et al., (1966) had identified ODAP as one of the most excitatory substances in spinal inter neurons and Betz cells of cats. Several in vitro cell culture studies have established that L-ODAP is an α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor agonist (glutamate receptor agonist) and this has been investigated extensively (Nunn et al., 1987; Ross et al., 1989; Sawutze et al., 1995; Ikegami et al., 1995). This excitotoxic theory has been widely held responsible for all the neurotoxic properties of ODAP and has been overhyped to some extent. Although this theory is a highly attractive proposition, it cannot explain the species differences in
suscetibility to ODAP. The non N-methyl-D-aspartate (NMDA) receptors being universal and common to all systems cannot be different between the black mice and white mice or between the chick and the Wistar rat. Also any receptor interaction has to exhibit significant specific binding but L-ODAP fails to show any significant specific binding to glutamate receptors (Jain et al., 1998). Omelchenko et al., 1999 also reported that compounds which are much more potent than ODAP in electrophysiological studies are in fact nontoxic. The excitotoxic theory however, explains the neurotoxicity of several other compounds such as β-N-methyl-amino alanine (BMAA) the cycad neurotoxin (Karamyan & Speth, 2008). Some studies suggest a free radical oxygen species (ROS) generation as a mechanism of ODAP toxicity in rats following its focal hippocampal application (Van Moorhem et al., 2011). Also, in vitro studies with mouse brain slices treated with ODAP suggest an inhibition of mitochondrial complex 1 (NADH dehydrogenase) (Sriram et al., 1998). Although oxidative damage from ROS is a generally accepted mechanism of toxicity, some of the studies with ODAP have not been confirmed by other workers (Sabri et al., 1995). Most mechanisms suggested for ODAP toxicity fall short of explaining the species differences in susceptibility to ODAP. In this context, the reported inhibition of tyrosine aminotransferase (TAT) by ODAP both in vivo and in vitro suggests an alternative interesting mechanism of neurotoxicity. TAT is the only enzyme identified so far that is substantially inhibited by L-ODAP. C57BL/6J black mice following ODAP treatment show a significant increase in brain DOPA and other chatecholamines (Shasi et al., 1997). BALB/c mice which normally do not respond to L-ODAP, become susceptible when predosed with tyrosine. It is likely that excessive generation of catecholamine metabolites like 6-OH DOPA which are well known neurotoxins might be the cause for neurodegeneration (Olney et al., 1976). Further, 6-OH DOPA is also a non NMDA receptor agonist (Cha et al., 1991). TAT inhibition by ODAP can thus explain the species difference in susceptibility to ODAP at least between black and white mice.

2.5. Nutritional aspect of β-ODAP

Medical and physiological research into the role and the mode of action of β-ODAP has produced important breakthroughs in the understanding of multiple and intricate effects this glutamate analogue can produce in the cascade of biochemical and physico-chemical reactions governing neuronal signalling (Kusama et al., 2000). Botanical and biochemical research has discovered several physiological functions of β-ODAP in the plant as carrier molecule for zinc-ions (Lambein et al., 1994), as scavenger for hydroxyl ions (Zhou et al.,
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2001) and as protector of photosynthesis at high light intensity (Zhang et al., 2003). The relative tolerance of the plant against biotic and abiotic stress may be related to the presence of β-ODAP, but convincing proof has not yet been given by the ongoing research. Environmental factors such as drought, zinc deficiency, iron oversupply and the presence of heavy metals in the soil can considerably increase the level of β-ODAP in the seeds grown in farmers’ fields as compared to more optimal experimental fields (Campbell et al., 1997). The deficiency of zinc in Bangladesh and the high concentration of iron in Ethiopian soils both have an increasing effect on the β-ODAP level in the seeds. The work of Lambein et al., 1994 first shed some light on the relationship between zinc availability and the seed β-ODAP content by using radioactive $^{65}\text{Zn}$. They found that Zn deficiency and oversupply of Fe induced increase in the content of β-ODAP in the ripe seeds. These effects of Zn and Fe on the accumulation of β-ODAP were also confirmed in the callus tissue of grass pea (Haque et al., 1997), and supported by Hussain et al., 1997, who found that application of phosphorus as KH$_2$PO$_4$ both in field plots and in hydroponic cultures inhibited considerably the uptake of Zn, leading to a sharp decrease in Zn level in the shoots and seeds of both high and low-toxin variety, and subsequently β-ODAP accumulation. In a broader investigation (Berger et al., 1999) by growing species at up to 31 sites throughout south-western Australia, the increasing effect of phosphate fertiliser was confirmed on toxin in the seeds. Moreover, the toxin-increasing effect of Zn deficiency (-Zn) was also observed in shoots and roots from short period-treated grass pea seedlings (-Zn treatment for 7 days). The presence of cadmium in the soil can increase the β-ODAP level up to six-fold (Hussain et al., 1995). Misra and Barat (1981) first reported the effects of micronutrients on seed β-ODAP level, and found that both low- and high toxin cultivars exhibited decreased levels of β-ODAP in response to Co (cobalt) or Mo (molybdenum) when sprayed with 0.5 ppm Co (nitrate) or 20 ppm Mo (ammonium molybdate) salts at the maximum flowering stage. In grass pea seedlings, Jiao et al., 2006 found the influence of Mo on β-ODAP levels in shoots and root, in which Mo deficiency (-Mo) resulted in obvious increase in β-ODAP levels in shoots at both 7 and 15 days, but in the roots only at 7 days after treatments.

2.6. Significance of ODAP determination

β-ODAP is of particular interest, because this potent non protein neuroexcitatory amino acid has been reported as a major causative agent in the pathogenesis of human neurolathyrism (MacDonald and Morris, 1984; Chase et al., 1985). This disease is characterized by an irreversible, non-progressive spastic paralysis of the lower limbs and
is directly associated with the excessive consumption of seeds containing the neurotoxin (e.g., *L. sativus*, *L. clyrnenum*, and *L. Cicera*) (Spencer and Schaumburg, 1983; Ludolph et al., 1987). Toxicological investigations have demonstrated that \( \beta \)-ODAP: (1) can cross the blood-brain barrier, (2) is accumulated in the central nervous system (CNS) following intraperitoneal or intravenous administration; and (3) produces severe convulsions and neurological signs that mimic neurolathyrism (Rao, 1978; Mehta et al., 1979; Mehta et al., 1980; Parker et al., 1979). Injection of \( \beta \)-L-ODAP into the lumbar cerebral spinal fluid of the rat results in permanent bilateral spasticity and striking histological damage to both gray and white matter of the spinal cord (Chase et al., 1985). Extensive neuronal damage has also been observed in the retina, hypothalamus, and area postremia of immature mice given \( \beta \)-ODAP (Olney et al., 1976).

### 2.7. Various methods for determination of ODAP

Various methods for ODAP detection are available which include colorimetric method (Rao, 1978), flow injection analysis (Moges & Johansson, 1994), reversed phase high pressure liquid chromatography (HPLC) with precolumn derivatization with 1-fluoro 2,4-dinitrobenzene (Wang et al., 2000) and 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) (Chen et al., 2000), capillary zone electrophoresis (Arentoft & Greirson, 1995) and thin layer chromatography (TLC) (Paradkar et al., 2003).

#### 2.7.1. Colorimetric method

**Principle:** Colorimetric analysis is a method of determining the concentration of a chemical element or chemical compound in a solution with the aid of a color reagent. The most widely used method for determining the \( \beta \)-ODAP is a spectrophotometric method that involves alkaline hydrolysis of the toxin to \( \text{L-\( \alpha \)} \), \( \beta \)-diaminopropanoic acid (DAP) followed by reaction with orthopthalaldehyde (OPA), which, in the presence of ethanethiol gives a coloured adduct, that can be measured at 476 nm. The method, however, does not discriminate between the non-toxic \( \alpha \) form and the toxic \( \beta \) isomeric form as both of them are hydrolysed to DAP. Obviously this method is limited (Rao, 1978).

**Merits:** It is a direct quantitative assay and requires small sample volume.

**Demerits:** It has low sensitivity and specificity, hence interfered by several other substances with the chromogen.
2.7.2. Flow injection analysis

**Principle:** Flow injection analysis (FIA) is an approach to chemical analysis that is accomplished by injecting a plug of sample into a flowing carrier stream. FIA is an automated method in which sample is injected into a continuous flow of a carrier solution that mixes with other continuously flowing solutions before reaching a detector. Precision is dramatically increased when FIA was used instead of manual injections and as a result, very specific FIA systems have been developed for a wide array of analytical techniques.

Flow injection (FI) setup was developed consisting of four packed-bed enzyme reactors containing GluOx (20 pL), catalase (20 pL), GluOx (250 pL), and peroxidase (50 pL) in series. The neurotoxic amino acid, 8-Noxalyl-L-<sub>c</sub>-cr, B-diaminopropionic acid (ODAP) was oxidized by immobilized glutamate oxidase (GluOx) to produce hydrogen peroxide. The peroxide reacts with Trinder reagent in a reactor with immobilized horseradish peroxidase to form a red-colored quinoneimine dye, which was detected spectrophotometrically at 512 nm. Glutamate is oxidized quantitatively in the first reactor, but the hydrogen peroxide is destroyed in the second so that interferences from this substrate are removed. This step destroys only a few percent of the ODAP in the sample. Most of the remaining ODAP is oxidized in the third reactor. Injections of 20-pL ODAP standards gave a response curve which was linear within the range 10-650 pM. Phosphate buffer extracts of grass peas were purified by centrifugation and membrane filtration. Samples were injected into the FI setup to assay the toxin at a rate of 20 samples per hour. The β-ODAP content of a batch of dry seed corresponded to 0.74% (w/w) with a relative standard deviation of 2.8% (Moges & Johansson 1994; Wodajo et al., 1997).

**Merits:** It is applicable to a broad range of compounds using a variety of simple photometric and electrochemical detectors. It provides accurate and precise sample manipulation with high precision and low detection limits.

**Demerits:** It requires higher reagent consumption and more waste due to continuously flowing reagent. It demands trained and experienced personal for setup, operation, and troubleshooting, increasing capital costs.

2.7.3. Capillary zone electrophoresis

**Principle:** Capillary electrophoresis (CE), also known as capillary zone electrophoresis (CZE), can be used to separate ionic species by their charge and frictional forces and...
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hydrodynamic radius. CE encompasses a family of related separation techniques that use narrow-bore fused-silica capillaries to separate a complex array of large and small molecules. High electric field strengths are used to separate molecules based on differences in charge, size and hydrophobicity. Sample introduction is accomplished by immersing the end of the capillary into a sample vial and applying pressure, vacuum or voltage. Depending on the types of capillary and electrolytes used, the technology of CE can be segmented into several separation techniques. Separation by CE can be detected by several detection devices. The majority of commercial systems use UV or UV-Vis absorbance as their primary mode of detection. Fluorescence detection can also be used in capillary electrophoresis for samples that naturally fluoresce or are chemically modified to contain fluorescent tags. In order to obtain the identity of sample components, capillary electrophoresis can be directly coupled with mass spectrometers or Surface Enhanced Raman Spectroscopy (SERS). In most systems, the capillary outlet is introduced into an ion source that utilizes electrospray ionization (ESI). The resulting ions are then analyzed by the mass spectrometer. Micellar electro kinetic capillary chromatography (MECC) allows the resolution of uncharged molecules by adding surfactants to modify the conditions to extend the application of CE.

A method of CZE has been developed for the simultaneous quantitative determination of neurotoxic nonprotein amino acid β-N-oxalyl-l-α,β-diaminopropionic acid (β-ODAP) in the seeds of grass pea. Samples of 0.5 g of seed flour were extracted in 20 ml of ethanol-water (6:4) by tumbling for 45 min. A linear response was recorded in the concentration range of 0.015-1.8 mM at pH 7.8 and 40°C. The detection limit was 0.1g/Kg. Comparison between CZE method and a standard colorimetric method for measuring total ODAP in 38 lines of grass pea gave a coefficient of correlation of 0.994 (Arentoft & Greirson, 1995).

CZE method was also developed for the simultaneous quantitative determination of β-N-oxalyl-l-α,β-diaminopropionic acid (β-ODAP) and homoarginine in grass pea. A new Na2B4O7-Na2SO4 run buffer was used and the pH was 9.20, contents of β-ODAP and homoarginine in crude extracts of LS plant material were determined with this method, the RSDs of peak areas of β-ODAP and homoarginine were 2.62% and 3.61%, respectively. The equilibrium concentration ratio of α- and β-ODAP decreased from 34.5/65.5 to 28.6/71.4 when the pH of the solution increased from pH 3.0 to pH 11.0 (Zhao et al., 1999).
Merits: The main advantage comes from efficient heat dissipation compared to traditional electrophoresis—due to large internal surface area to volume. This enables the use of high field strengths, which decreases analysis time and minimizes band diffusion. Detection occurs as the separation progresses with resolved zones producing an electronic signal as they migrate past a UV absorbance or fluorescence detector for example. This configuration increases efficiency. It requires no staining or destaining. Small sample volume—minimizes the distortion in the applied field caused by the presence of sample.

Demerits: Single sample injected—limited throughput, multiple samples are analysed serially. Larger sample volumes required for auto sampler - dead volume. Inner diameter of the capillary tube is also the path length of the cuvette which leads to limited detection limit and lower sensitivity.

2.7.4. Reversed phase high pressure liquid chromatography

Principle: Reversed-phase high-performance liquid chromatography (RP-HPLC) involves the separation of molecules on the basis of hydrophobicity. The separation depends on the hydrophobic binding of the solute molecule from the mobile phase to the immobilized hydrophobic ligands attached to the stationary phase, i.e., the sorbent. The solute mixture is initially applied to the sorbent in the presence of aqueous buffers, and the solutes are eluted by the addition of organic solvent to the mobile phase. Elution can proceed either by isocratic conditions where the concentration of organic solvent is constant, or by gradient elution where the amount of organic solvent is increased over a period of time. The solutes are, therefore, eluted in order of increasing molecular hydrophobicity.

A rapid and simple method is presented for determining neuroexcitatory non protein amino acid 3-N-oxalyl-2, 3-diaminopropionic acid (β-ODAP) and non-protein amino acids in *Lathyrus sativus*. Seed and foliage extracts of *Lathyrus sativus* were treated with 1-fluoro-2, 4-dinitrobenzene (FDNB) and a reversed-phase high-performance liquid chromatography method (RP-HPLC) for the separation of the derivatives in the pmol range is reported. The RP-HPLC method and a colorimetric method were compared for measuring ODAP (Wang et al., 2000).

A method of RP-HPLC was developed for the quantitative determination of the neurotoxic nonprotein amino acid, 3-N-oxalyl-1-2,3-diaminopropionic acid (β-ODAP), and its nontoxic α-isomer, 2-N-oxalyl-1-2,3-diaminopropionic acid (α-ODAP), in the plant samples of *Lathyrus sativus* after derivatization with 6-aminoquinolyl-N-hydroxy succinimidyl carbamate (AQC). Hippuric acid was used as an internal standard. A linear
response was recorded in the concentration range 0.32–32 nmol with \( r > 0.999 \). The RP-HPLC detection limit for both isomers was 1.8 ng. A ternary gradient system was used to determine toxin and other non-protein amino acids. The RP-HPLC method and a colorimetric method were compared for measuring ODAP (Chen et al., 2000).

**Merits:** It is an effective separation technique when all appropriate parameters and equipment are used. This method is especially effective, when the compounds within the mixture are colored.

**Demerits:** Shortcomings of the method are the long runtime, the instability of ninhydrin, the necessity of protein precipitation, which impedes complete automation and crosstalk by analytes other than AAs and related compounds that may react with ninhydrin in complex biological samples and prevent accurate quantitation.

### 2.7.5. Thin layer chromatography

**Principle:** Thin-layer chromatography (TLC) is a chromatographic technique used to separate non-volatile mixtures. Thin-layer chromatography is performed on a sheet of glass, plastic, or aluminium foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide, or cellulose. This layer of adsorbent is known as the stationary phase. After the sample has been applied on the plate, a solvent or solvent mixture (known as the mobile phase) is drawn up the plate via capillary action. Because different analytes ascend the TLC plate at different rates, separation is achieved.

Thin layer chromatography method was developed to study adulteration of chickpea and red gram with *Lathyrus sativus* in pressure-cooked batters and found to be 100 and 200 g/kg respectively. When processed as a curried liquid dal, *L. sativus* could be detected at 200 g/kg in chickpea and 100 g/kg in red gram. Processing into fried bhajiyas resulted in a detection limit of 200 g/kg in both red gram and chickpea (Paradkar et al., 2003).

**Merits:** Minimum equipment and little time for separation are required. It is more sensitive and its detection limit is lower than that of paper chromatography. A small quantity of sample is sufficient for analysis.

**Demerit:** Spots are often faint, and results are difficult to reproduce.
2.7.6. Biosensing methods

The history of biosensors started in 1962, with the development of enzyme electrodes by scientist Leland C. Clark (Clark and Lyons, 1962), who is known as the father of Biosensors. He carried out this by using an enzyme transducer. Clark’s oxygen electrode was the glucose oxidase, entrapped in dialysis membrane. The method of detection was based on the decrease in soluble O$_2$ concentration which was directly proportional to glucose concentration. Clark’s ideas became commercial reality in 1975 with the successful re-launch (first launch 1973) of the Yellow Springs Instrument Company (Ohio) glucose analyser based on the amperometric detection of hydrogen peroxide. Since then, the designs and applications of biosensors in analytical chemistry have developed rapidly during past four decades and communities from various fields such as very large scale integration (VLSI), physics, chemistry, and material science have come together to develop more sophisticated, reliable, and mature biosensing device.

2.7.7. Basic Principle of biosensors

A biosensor can be defined as an analytic device incorporating a biological sensing element connected to a transducer to convert an observed response into a measurable signal, whose magnitude is proportional to the concentration of a specific chemical or set of chemicals (Eggins, 1996) (Fig. 4). Biosensors are based on enzymes, either consumption of oxygen e.g. all the oxidases, or production of hydrogen peroxide or generation of (indirectly) the reduced form of β-nicotinamide adenine dinucleotide (phosphate), NAD(P)H, e.g., dehydrogenases. According to the receptor type, biosensors can be classified as enzymatic biosensors, genosensors, immunosensors, etc. Biosensors can be also divided into several categories based on the transduction process, such as electrochemical, optical, piezoelectric, and thermal/calorimetric response (Fig. 5). Among all these various kinds of biosensors, electrochemical biosensors are a class of the most widespread, numerous and successfully commercialized devices of biomolecular electronics (Dzyadevych et al., 2008). According to a recently proposed IUPAC definition, “A biosensor is a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor), which is in direct spatial contact with a transducer element.
The biosensors have been employed in the areas of clinical, diagnostic, medical applications, process control, bioreactors, quality control, agriculture, veterinary medicine, bacterial and viral diagnostic, drug production, control of industrial waste water, mining and military defence industry (Liu & Lin, 2005; Sadana, 2006). The application of biosensors have improved the performance of the conventional analytical tools and eliminated slow preparation and the use of expensive reagents, thus provided low cost analytical tools. Biosensors are specific due to their immobilized enzyme/protein systems. Biosensors can measure non polar molecules which do not respond to most measurement devices. Further biosensors are simple, rapid and portable in minitaurized form. The present review focuses on various types of ODAP biosensors.

2.8. ODAP biosensors

Principle: As β-ODAP is the structural analog of glutamate. Glutamate oxidase catalyses the oxidation of β-ODAP to produce α-keto acid and H₂O₂. On applying potential, H₂O₂ gets split into 2 H⁺, 2e⁻ and O₂. These electrons generate the signal in the form of current, which is amplified and displayed.

2.8.1. Classification of ODAP biosensors

2.8.1.1. Amperometric ODAP biosensors

Amperometry is a method of electrochemical analysis in which the signal of interest is a current that is linearly dependent upon the concentration of the analyte. In contrast to the
logarithmic relationship in potentiometric systems, amperometric systems possess a linear concentration dependence and measure change in the current by the working electrode. In amperometric technique, certain electroactive species are oxidized or reduced (redox reactions) at inert metal electrodes (Ding et al., 2008), under a constant applied potential. As a result electrons are transferred from the analyte to the working electrode (WE) or to the analyte from the electrode. The direction of flow of electrons depends upon the properties of the analyte and can be controlled by the electric potential applied to the WE. Typically, the current is measured at a constant potential and this is referred to as amperometry. The electrochemical cell, where the amperometric experiment is carried out consists of a WE, a reference electrode (RE) and usually a counter (auxiliary) electrode (CE). The WE is the one, where the reaction or transfer of interest takes place and constructed generally from a metal wire such as platinum or gold. The RE provides a known and stable potential, against which the potential of WE is compared. The most common RE systems used in aqueous medium are the silver-silver chloride (Ag/AgCl) and saturated calomel (Hg/HgCl₂), which have electrode potential independent of the composition of electrolyte. The measured cell current (diffusion current) is a quantitative measure of the analyte of interest (Wang, 2008). Amperometry is an electrochemical technique that measure as a function of an independent variable that is, typically, time or electrode potential, when the current recorded at a fixed potential as a function of time, the technique is called chronoamperometry and when potential varies with time in a predetermined manner and the current is measured as a function of potential is called voltammetry or voltamperometry. At voltammetry, an increasing (decreasing) potential is applied to cell until the oxidation (reduction) of the substance to be analyzed occurs and there is sharp rise (fall) in the current to give a peak current. The height of the peak current in directly proportional to concentration of electroactive material the appropriate oxidation (reduction) potential is known, one may step the potential directly to the value and observe the current. It involves probing a small region of a solution containing, for example, metal ions, by performing small-scale electrolysis between an indicator microelectrode and a RE. A RE, such as the saturated calomel electrode (SCE), is by definition nonpolarizable.
Fig. 5. Various elements and selected components of a typical biosensor

That is, its potential remains the same regardless of the potential difference imposed between it and the indicator electrode. The latter is described as polarizable, because it faithfully adopts any potential imposed on it relative to the reference. If the potential
difference between indicator and reference electrode can be controlled accurately and 
varied uniformly, criteria which modern potentiostatic devices ensure, the corresponding 
currents that flow reflect the nature and concentration of oxidizable or reducible solutes in 
solution. Currents flow because of the exchange of electrons between the indicator 
electrode and electroactive solutes. The term voltammetry encompasses a broad area of 
electroanalytical chemistry that includes polarography, linear scan voltammetry (LSV), 
cyclic voltammetry (CV), pulsed voltammetry (PV) and stripping voltammetry (SV). 
Overall voltammetry is very versatile and can be used for the analysis of many redox 
active species. Amperometric biosensors also have the advantages of being more highly 
sensitive and rapid as compared to conductometric and potentiometric biosensors 
(Chaubey and Malhotra, 2002; Dzyadevyeh et al., 2002). In case of ODAP biosensors, the 
following electrochemical reactions occur:

\[
\begin{align*}
\text{L-glutamate} + \text{O}_2 + \text{H}_2\text{O} &\xrightarrow{\text{GluOx}} \text{2-oxoglutarate} + \text{NH}_3 + \text{H}_2\text{O}_2 \\
\beta-\text{ODAP} + \text{O}_2 + \text{H}_2\text{O} &\xrightarrow{\text{GluOx}} \alpha-\text{keto acid} + \text{NH}_3 + \text{H}_2\text{O}_2 \\
\text{H}_2\text{O}_2 &\xrightarrow{0.135 \text{ V}} \text{O}_2 + 2\text{H}^+ + 2\text{e}^- \\
\text{GluOx} &= \text{L-glutamate oxidase (EC 1.4.3.11)}
\end{align*}
\]

This enzyme belongs to the family of oxidoreductases, specifically those acting on the 
CH-NH$_2$ group of donors with oxygen as acceptor. The systematic name of this enzyme 
class is L-glutamate:oxygen oxidoreductase (deaminating).

A number of amperometric ODAP biosensors have been developed (Belay et al., 1997; 
Yigzaw et al., 2001; Yigzaw et al., 2002; Beyene et al., 2003; Beyene et al., 2004; 
Marichamy et al., 2005; Varma et al., 2006; Isildak et al., 2014).

2.8.1.2. Classification of amperometric ODAP biosensors on the basis of type of 
electrode

(i) graphite electrode based ODAP biosensor

A graphite rod modified with an Os$^{2+/3+}$ redox polymer cross-linked with L-glutamate 
oxidase and horseradish peroxidase has been developed for the determination of the 
neurotoxin $\beta$-N-oxalyl-$L$-$\alpha,\beta$-diaminopropionic acid ($\beta$-ODAP) after liquid chromato-
-graphic (LC) separation of β-ODAP from interfering amino acids on an anion exchange column coupled. This LC-bienzyme electrode analytical system enabled monitoring of as low as 4 μM β-ODAP (injection volume 100 μL). The β-ODAP content in real grass pea samples was measured to the range, 3.3 to 5.2 g kg⁻¹ in dry grass pea (Belay et al., 1997).

An improved chromatography based ODAP biosensor was based on cross-linking horseradish peroxidase (HRP) and an Os-containing mediating polymer with poly(ethyleneglycol) (400) diglycidyl ether (PEGDGE), forming an inner hydrogel layer and then immobilising L-glutamate oxidase (GluOx) as an outer layer on graphite electrode. Addition of polyethylenimine (PEI) to the hydrogel is believed to have sensitivity and stability enhancing effect on the biosensor. The double-layer approach in the biosensor construction avoided direct electrical wiring of GluOx and resulted in a higher sensitivity of 4.6 mA/M/cm² with respect to β-ODAP and a wider linear range (1-250 μM) for both L-glutamate and β-ODAP when compared with a single-layer approach where GluOx, HRP, and Os-polymer are cross-linked together. The limit of detection for the chromatographic-biosensor system was found to be 2 μM with respect to β-ODAP and 0.7 μM with respect to L-glutamate. The refractive index detection on-line with the biosensor enabled full control of the chromatographic system for the determination of the total amount of ODAP, selectively the amount of β-ODAP and L-glutamate. Ten grass pea samples were collected from Lathyrism prone areas of Ethiopia to test the applicability of the presently developed analytical system for real sample analysis. The toxin levels of grass pea collections were determined in an aqueous extracts and ranged from 0.52 to 0.76%, dry mass basis. Comparison of results of an established spectrophotometric assay and that of the present system showed an extraordinary degree of agreement as revealed by parallel "t" test (90% confidence limit). The present system had operational stability of more than 50 h. Analysis time per sample is 10 min after extraction for 90 min (Yigzaw et al., 2001).

Improvements in the above hydrogel-based amperometric biosensor using glutamate oxidase and horseradish peroxidase were achieved in terms of dynamic linearity range, sensitivity and detection limits. The bilayer approach in the construction of the enzyme modified electrode in this work, together with high enzyme loading, enabled detection limits of 0.7 μM of the primary substrate for the enzyme system, L-glutamate and 2 μM for the secondary substrate β-N-oxalyl-α, β-diaminopropionic acid (β-ODAP). The sensor
response was linear up to 250 µM for both substrates. There was a good reproducibility between equally prepared electrodes and the electrodes showed an operational stability of more than 50 h (Yigzaw et al., 2002).

Another ODAP biosensor was based on co-immobilization of glutamate oxidase (GluOx) and horseradish peroxidase (HRP), on the end of a spectrographic graphite electrode. β-ODAP was oxidised by GluOx to form H₂O₂, which in turn was bioelectrocatalytically reduced by HRP through a mediated reaction using a polymeric mediator incorporating Os²⁺/³⁺. The hydrogen peroxide released during the enzymatic cycle was detected at a low applied potential, ~50 mV versus Ag/AgCl. The sensor response was linear up to 600 µM (Marichamy et al., 2005).

**Merits:** Graphite electrode possesses low electrical resistance, good flexibility, favourable mechanical performance, in almost any size and very low cost.

**Demerits:** The major drawback of graphite is the fine dust it produces during its machining.

**(ii) Screen printed carbon electrode based ODAP biosensor**

An amperometric β-ODAP biosensor was developed based on immobilization of glutamate oxidase in a nafion film mounted on MnO₂ bulk-modified screen printed carbon electrode. The biosensor exhibited a wider linear range (50-500 mg L⁻¹) than earlier biosensors as well as extraordinary stability. The biosensor included/employed glutamate decarboxylase (GluDC) in removing any interference from inherent glutamate that was present in grass pea seeds (Beyene et al., 2003).

β-ODAP biosensor was developed based on immobilization of glutamate oxidase (GluOx) in a Nafion-film on screen printed carbon electrode (SPCE) bulk-modified with manganese dioxide. The performance of the biosensor was optimized using glutamate as an analyte. Optimum parameters were as follow: operational potential 440 mV (vs. Ag/AgCl), flow rate 0.2 mL min⁻¹, and carrier composition 0.1 mol L⁻¹ phosphate buffer (pH 7.75). The same conditions were used for the determination of β-ODAP. The signal was linear within the concentration range 53–855 µM glutamate and 195–1950 µM β-ODAP. Detection limits (as 3σ value) for both analytes were 9.12 and 111.0 µM, respectively, with corresponding relative standard deviations of 3.3 and 4.5%. The
biosensor retained more than 73% of its activity after 40 days of on-line use (Beyene et al., 2004).

**Merits:** Screen printed carbon electrodes are easily obtainable at minimal costs and are especially suitable for preparing an electrode material with desired composition, and hence, with pre-determined properties can be used as highly selective sensors.

**Demerits:** Instability and high cross-sensitivity towards anions.

**(iii) Glassy carbon electrode based**

A sensitive ODAP biosensor was developed based on glutamate oxidase (GluOx) and a Prussian blue (PB) modified glassy carbon (GC) electrode. The configuration of the system was able to detect the hydrogen peroxide released during the enzymatic cycle at a low applied potential, −50 mV versus Ag/AgCl, in the flow injection mode. To achieve it, GluOx was coupled to PB electrodeposited onto a glassy carbon electrode and stabilised by treatment with tetrabutylammonium toluene-4-sulfonate (TTS) during one of the steps in the electrodeposition. GluOx was cross-linked with glutaraldehyde (GA), bovine serum albumin (BSA) and Tween-20 on the surface of the PB modified GC electrodes. Addition of 0.01% and 0.001% polyethyleneimine (PEI) to the immobilisation mixture resulted in an enhancement of the response signal with about 35% and 62% for glutamate and β-ODAP, respectively, when using 0.01% PEI and with 164% and 200% for glutamate and β-ODAP, respectively, when using 0.001% PEI. The linear response range for β-ODAP was extended from 0.05–0.5 mM to 0.01–1 mM, when 0.001% PEI was used. However, a higher concentration of PEI, 0.1%, caused a decrease in the sensitivity of the biosensor (Varma et al., 2006).

**Merits:** The biosensor has good operational stability, long storage lifetimes, relatively short response time and high sensitivity. In addition, all biosensors achieve the required analytical range.

**Demerits:** Poor coupling of biochemical recognition materials and electrochemical transducers, however, affect selectivity, sensitivity, dynamic range and stability of electrochemical biosensors.

2.8.1.3. **Potentiometric ODAP biosensors**

In this type of biosensor, the measured parameter is oxidation or reduction potential of an electrochemical reaction. In other words, in potentiometric biosensors, the potential difference between the reference electrode and the indicator electrode is measured without polarizing the electrochemical cell, that is, very small current is allowed. The reference electrode is required to provide a constant half-cell potential. The indicator electrode
develops a variable potential depending on the activity or concentration of a specific analyte in solution. The change in potential is related to concentration in a logarithmic manner. The Nernst equation relates the potential difference at the interface to the activities of species in sample phases (s) and in the electrode phase (β):

\[ E = E_0 + \frac{RT}{Z_iF} \ln \frac{a_i^s}{a_i^β} \]

where \( E_0 \) is the standard electrode potential of the sensor electrode; \( a_i \) is the activity of the ion, \( R \) is the universal gas constant; \( T \) is the absolute temperature; \( F \) is the Faraday constant; \( Z_i \) is the valency of the ion. The lowest detection limits for potentiometric devices are currently often achieved with ion-selective electrodes (ISE) (Bobacka et al., 2008). The ion-selective electrode (ISE) for the measurement of electrolytes is a potentiometric technique routinely used in clinical chemistry. In many cases, the potentiometric biosensor comprises a membrane with a unique composition, noting that the membrane can be either a solid (i.e., glass, inorganic crystal) or a plasticized polymer e.g. potentiometric biosensors based on silicon and porous silicon for triglyceride and urea (Basu et al., 2004), and the ISE composition is chosen in order to impart a potential that is primarily associated with the ion of interest via a selective binding process at the membrane-electrolyte interface. These are semiconductor Field Effect Transistor (FETs) having an ion-sensitive surface. The surface electrical potential changes when the ions and the semiconductor interact. This change in the potential can be subsequently measured. The ion sensitive field effect transistor (ISFET) is constructed by covering the sensor electrode with a polymer layer. This polymer layer is selectively permeable to analyte ions. The ions diffuse through the polymer layer and in turn cause a change in the FET surface potential. This type of biosensor is also called an enzyme field effect transistor (ENFET) and used primarily for pH detection e.g. A biosensor based on an H⁺ ion sensitive field effect transistor (H⁺-ISFET) and Penicillin G acylase has been developed for the determination of Penicillin G (Liu et al., 1998). The variety of ions, for which low detection limits are possible, is currently quite limited and missing such important analytes as: nickel, manganese, mercury and arsenate ions. The sensitivity and selectivity of potentiometric biosensor are outstanding due to the species-selective working electrode used in the system. However, a highly stable and accurate reference electrode is always required and challenging to maintain, which may potentially limit the application of potentiometry in microbial biosensors.
A potentiometric sensor based on ionophore (Cd(NH2CH2CH2OCH2CH2OCH2CH2NH2) Ag3(CN)5) for the determination of β-N-oxalyl-l-α, β-diaminopropionic acid (ODAP) was developed. The ODAP-selective membrane sensor demonstrated high sensitivity and short response time. The detection limit of the ODAP-selective membrane sensor was about $2 \times 10^{-6}$ mol L$^{-1}$ and the response time was shorter than 6 s. The linear dynamic range of the ODAP-selective membrane sensor was between ODAP concentrations of $1.0 \times 10^{-2}$ and $1 \times 10^{-6}$ mol L$^{-1}$. The ODAP-selective membrane sensor exhibited good operational stability for at least one week in dry conditions at 4–6°C. It had a reproducible and stable response during continuous work for at least 10 h with a relative standard deviation of 0.28% ($n = 18$) (Isildak et al., 2014).

2.9. Use of nanomaterials in biosensors

During the past one decade, nanomaterials have emerged as powerful and widely applied materials in biosensing methods. They provide a new range of structures (1 to 100 nm in diameter) acquiring a big impact on conception and development of biosensors and rapid diagnostic tests. Nanoscale materials not only used to achieve direct wiring of enzymes to electrode surface to promote electrochemical reaction, but also brings new possibilities for signal amplification of biorecognition event. Nanoparticles have attracted much interest owing to their unique properties including high mechanical strength, oxygen ion conductivity, biocompatibility and retention of biological activities.

Important advances in these aspects has been achieved with the utilization of several kinds of nanomaterials such as metal nanoparticles (Batra et al., 2013), oxide nanoparticles (Batra et al., 2012), magnetic nanomaterials (Chawla et al., 2011) and carbon materials (Batra et al., 2013) to improve electrochemical signal of biocatalytic events occurred at electrode/electrolyte interface (Fig. 6). Recent advances in bionanoelectrochemistry have reported about the enormous impact of nanomaterials when utilized as transducing element in modified electrodes (Batra et al., 2013). In particular, the exploration of gold nanostructured materials has provided new paths for enzymatic biosensors development.
2.10. Nanomaterials based β-ODAP Biosensors

To the best of our knowledge, till date no nanomaterials have been employed in the construction of β-ODAP biosensors.

Fig. 6. Various nanomaterials used in biosensors