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Analytical assays to evaluate enzymatic activity and screening of inhibitors for ornithine decarboxylase

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Ornithine decarboxylase (ODC) catalyzes the decarboxylation of ornithine to produce putrescine, the first step in the metabolism of polyamines (putrescine, spermidine, and spermine), which are essential growth factors in eukaryotic cells. ODC is active as a homodimer and depends on pyridoxal 5'-phosphate (PLP) as a cofactor. An increase in the concentration of polyamines has been associated with carcinogenesis. Therefore, there is much interest in identifying inhibitors of this pathway as potential chemotherapeutic and chemopreventive agents. The best-known inhibitor of mammalian ODC is α -difluoromethylornithine (DFMO), a highly selective compound that alkylates Cys-360 (a residue of the ODC active site). Although DFMO was initially developed for the treatment of cancer, the World Health Organization recommends its use in combination with nifurtimox for the treatment of human African trypanosomiasis. Considering the importance of ODC as a promising target for the treatment of various types of cancer and other infectious diseases, choosing the right method for screening potential inhibitors can help to accelerate the discovery of new drugs. Several methods for the determination of ODC activity are found in the literature. Among these, we can mention analysis with radioactive markers, colorimetric assays using auxiliary enzymes to detect CO₂ or H₂O₂ release, chromatographic separations with putrescine derivatization, mass spectrometry, and spectroscopic techniques. In this review, the main analysis methods used will be described, highlighting their advantages and disadvantages, as well as identifying the most promising methods for high-throughput screening.

KEYWORDS

radiolabeling, CO₂ release, putrescine oxidation, TNBS colorimetric assay, HPLC, mass spectrometry, spectroscopic assays

1 Introduction

Ornithine decarboxylase (ODC, EC 4.1.1.17) is an enzyme that limits the rate of the polyamine biosynthetic pathway. Polyamines play an important role in the cellular homeostasis of most living organisms, and ODC has been reported in several species (Miller-Fleming et al., 2015; Bateman et al., 2021). ODC, a pyridoxal 5'-phosphate (PLP) dependent enzyme, catalyzes the decarboxylation of L-ornithine to putrescine *via* Schiff base formation (Figure 1). Putrescine is the precursor of spermidine and spermine in the first step of polyamine biosynthesis (Michael, 2016). Elevated expression of ODC, as well as abnormally high levels of polyamines, are correlated with the occurrence of many diseases, including cancer and Alzheimer's (Gerner and Meyskens, 2004; Mäkitie et al., 2010; Miller-Fleming et al., 2015; Somani et al., 2017; Igarashi and Kashiwagi, 2019; Holbert et al., 2022; Ivanov and Khomutov, 2022). Consequently, ODC is considered an oncogenic enzyme and a target for the treatment of several diseases (McCann and

Pegg, 1992; Marton and Pegg, 1995; Mukhopadhyay and Madhubala, 1995; Casero and Marton, 2007; Smithson et al., 2010a; Colotti and Ilari, 2011; Chakraborty et al., 2013; Somani et al., 2017; Perdeh et al., 2020; Sweeney, 2020; Khan et al., 2021). ODC is a homodimer in a dynamic equilibrium between the active dimeric form and the inactive monomeric form (Solano et al., 1985; Mitchell et al., 1988; Mitchell and Chen, 1990; Coleman et al., 1994; Chai et al., 2020). The two identical active sites in the homodimer are formed at the dimer interface by residues from the N-terminal domain (Lys69) of one subunit and the C-terminal domain (Cys360) of the other (Tobias and Kahana, 1994; Chai et al., 2020). Lys69 was biochemically identified as the covalent binding site for PLP, while Cys360 is involved in covalent adduct formation with DFMO (α -difluoromethylornithine) (Poulin et al., 1992; Coleman et al., 1994; Grishin et al., 1999). In addition, other important interactions at the binding site have also been described, such as Glu274, which stabilizes the positive charge on the pyridine nitrogen of the PLP, increasing the ability to

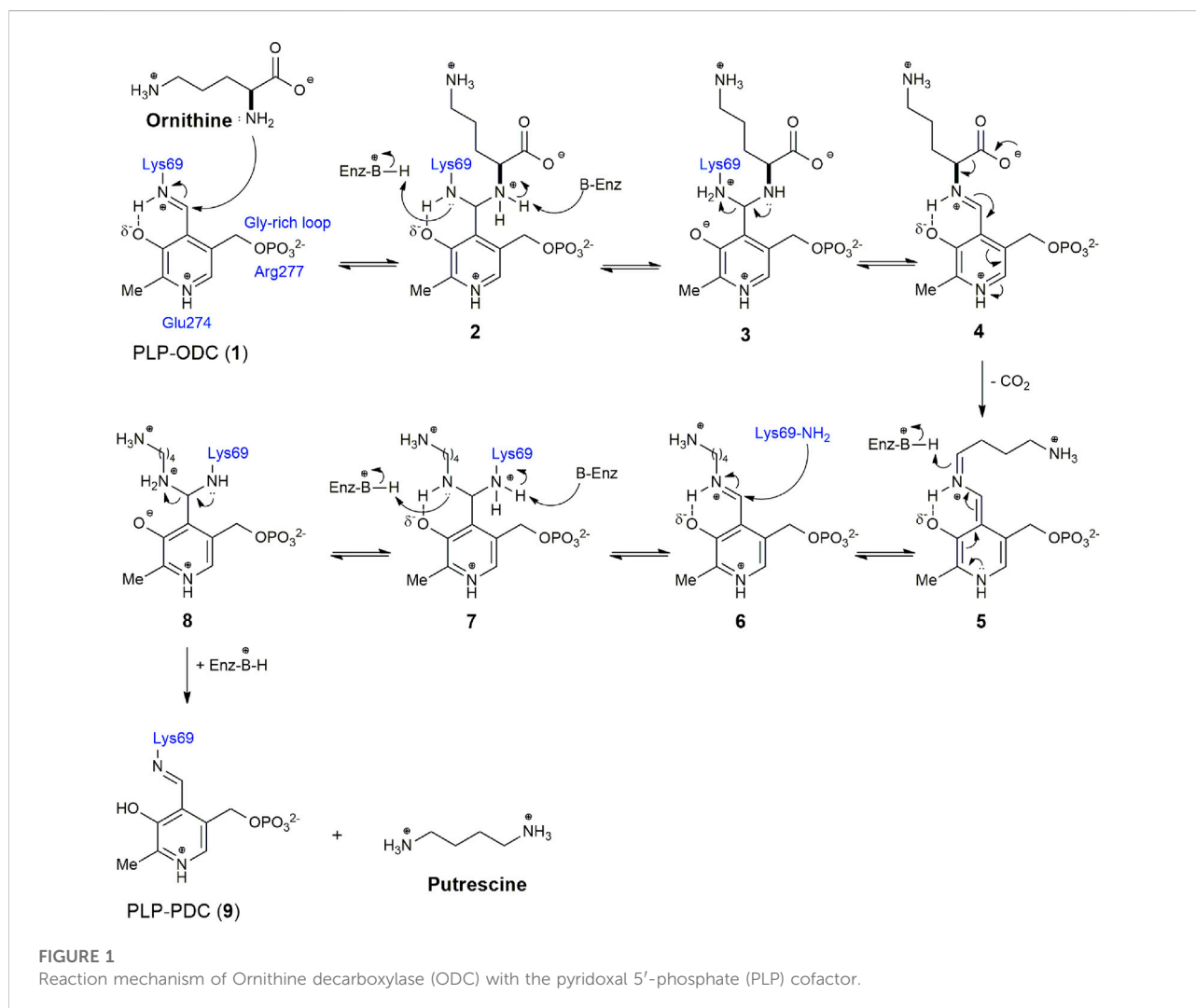


TABLE 1 Methods to assess Ornithine Decarboxylase (ODC) activity and Inhibition.

| Method | Advantages | Drawbacks | References |
|---|---|---|---|
| Radiolabeling | Allows monitoring of reaction course. No sample pre-treatment required | Requires labeled ¹⁴ C substrate or DFMO and a liquid scintillation spectrometer. Expensive for HTS. | Kobayashi (1963), Jones et al. (1972), Beaven et al. (1978), Smith and Marshall (1988), Henley et al. (1996), Vanisree and Shyamaladevi (2006), Schultz et al. (2020) |
| CO ₂ consumption by coupled enzymes (PEPC-MDH) | Allows monitoring of reaction course. It can be performed in an automated system with 384-well microplates. It has already been used to analyze more than 3,000 compounds | Need a commercial system for bicarbonate detection and N ₂ atmosphere for analysis The cost of the kit can make analysis by HTS unfeasible | Scriven et al. (1988), Smithson et al. (2010b), Liao et al. (2015), Chai et al. (2020), Smithson et al. (2010a) |
| Putrescine oxidation: H ₂ O ₂ released - Colorimetric | Produces a colored complex that absorbs at 505 nm, which can be analyzed in a plate reader. Allows monitoring of reaction course | Use of the soy amine oxidase enzyme from the purified soy plant (purification step) or directly from the crude extract (possibility of interference by other enzymes) | Badolo et al. (1999), Das et al. (2015) |
| Putrescine oxidation: H ₂ O ₂ released - Luminescence | Enables detection of putrescine on a picomolar scale. It can be adapted for luminescence detection in a microplate reader. Allows monitoring of reaction course | It requires an initial step of extracting the phosphocellulose paper. May be interfered with by other polyamines and other enzymes when used with cell extracts | Fagerström et al. (1984), Wang and Bachrach (2000) |
| Adducts of putrescine with trinitrophenyl groups | Use of cheap and affordable reagents | It takes several steps to prepare the sample, with many sources of errors that depend heavily on the analyst's skills. It only assesses the end point of the enzymatic reaction. The cost of 2,4,6-trinitrobenzenesulfonic acid (TNBS) is high for HTS and not widely available | Luqman et al. (2013), Nilam et al. (2017), El-Sayed et al. (2019), Villa-Ruano et al. (2019), Chai et al. (2020) |
| HPLC assays | HPLC resolution, specificity and sensitivity | Cost, long analysis time, and the need for laborious sample pre-treatment. It only assesses the end point of the enzymatic reaction | Kabra et al. (1986), Legaz et al. (2001), Kvannes and Flatmark (1987), Bito et al. (2019), Beeman and Rossomando (1989), del Rio et al. (2018), Xiao et al. (2009) |
| MS assays | MS analysis is fast and reproducible. Detection in the fmol range | Laborious sample pre-treatment. It only assesses the end point of the enzymatic reaction. Expensive equipment | Ducros et al. (2009), Liu et al. (2011), Liu et al. (2011), Gaboriau et al. (2003), Gaboriau et al. (2005) |
| Circular Dichroism | Allows monitoring of reaction course. No need for special reagents. No sample pre-treatment required | As many organic compounds absorb in the 201 nm region, it cannot be used with mixtures of compounds, nor for screening inhibitors. It cannot be performed on microplates. Expensive equipment and low sensitivity | Brooks and Phillips (1996) |
| Fluorescence Assays | Allows monitoring of reaction course. Can be analyzed on a microplate reader. No sample pre-treatment required | Low solubility of cucurbit [6]uril (CB6) | Nilam et al. (2017) |

withdraw electrons from the ring, and Arg277, which is necessary for the binding of the high affinity of PLP through interaction with the cofactor 5'-phosphate (Figure 1) (Osterman et al., 1995, 1997; Brooks and Phillips, 1997).

Due to its importance in several cellular functions and wide distribution in several species, since the 1970s there has been a great therapeutic interest in the inhibition of ODC, with the consequent depletion of polyamine biosynthesis (Mamont et al., 1978; Metcalf et al., 1978). According to the mechanism of the ODC reaction shown in Figure 1, the enzyme and its reaction can be inhibited by cofactor (PLP), substrate (ornithine), or product (putrescine) analogues. α -difluoromethylornithine (DFMO), an ornithine analogue, is the most important and best-known inhibitor of ODC. DFMO, marketed as Eflornithine, belongs to the WHO Model List of Essential Medicines to treat sleeping sickness (WHO 2022). Although it is not effective for the treatment of cancer alone, it has been widely used in combination with other

drugs. It is in several clinical trials as an adjuvant or chemopreventive agent and appears to be highly useful in the treatment of neuroblastoma (Kim et al., 2017; Alhosin et al., 2020; Schultz et al., 2021). In addition to the cofactor, substrate, and ODC reaction product analogues, other classes of inhibitors (benzothiazoles, indoles, bisbiguanide, and dithioamidine) with different binding modes were identified, indicating allosteric sites in the enzyme. The identification of these new classes of inhibitors was made from a high-throughput enzymatic screening with 316,114 different molecules, demonstrating the importance of this methodology for the identification of more selective and potent inhibitors for ODC, with improved pharmacokinetic properties (Smithson et al., 2010a). There are several methods in the literature to assess ODC activity. The most used methods are based on the detection of radiolabeled CO₂ or radiolabeled DMFO linked to ODC, on the capture of CO₂ for the conversion of NADH into NAD with the use of Phosphoenolpyruvate Carboxylase

(PEPC) and Malate Dehydrogenase (MDH) enzymes, on the chemiluminescence or colorimetric detection of H₂O₂ produced by putrescine oxidation by amine oxidases, and in derivatization for analysis by high-performance liquid chromatography (HPLC) or mass spectrometry (MS) and fluorescence. However, we did not find a compilation of existing methods in the literature, which makes it difficult to choose the most appropriate method to assess ODC activity and screening for inhibitors. Therefore, we will make a brief description of the main methods found (Table 1), seeking, whenever possible, to present their advantages and disadvantages and the possibility of application in high-throughput screening (HTS).

2 Methods to assess ODC activity and inhibition

2.1 Radiolabeling

The measurement of ¹⁴CO₂ release from [¹⁴C]carboxyl-labeled amino acids is widely used as a method of assay for decarboxylase activity (Kobayashi, 1963). For *in vitro* ODC activity, the usual procedure is to incubate the enzyme sample in a medium with EDTA, sodium/potassium phosphate buffer, pyridoxal phosphate, and the [1-¹⁴C]-L-ornithine in a vial sealed with the cap containing the paper discs impregnated with hyamine/sodium hydroxide to capture the release of radiolabeled carbon dioxide. The enzymatic reaction is stopped by the addition of sulfuric/citric acid. After an additional incubation period to complete ¹⁴CO₂ absorption, the caps are removed and transferred to a scintillation vial containing scintillation fluid. Measurements of disintegrations per minute are made in a liquid scintillation spectrometer. The specific ODC activity is expressed as nmol CO₂ released/min/mg protein (Jones et al., 1972; Beaven et al., 1978; Smith and Marshall, 1988; Henley et al., 1996; Vanisree and Shyamaladevi, 2006; Schultz et al., 2020).

Another method to monitor the ODC activity is by using α -[5-¹⁴C]Difluoromethylornithine, an irreversible ODC inhibitor that forms a covalent bond with the enzyme. For *in vitro* ODC activity, mouse tissues are treated with α -[5-¹⁴C]DFMO and incubated in the enzymatic reaction medium. After precipitation of protein by addition of acid and centrifugation, the pellet is dissolved in hydroxide sodium solution, mixed with scintillation fluid, and has the radioactivity counted in a liquid scintillation spectrometer (Seely et al., 1982). For *in vivo* controlling, the labeled ODC can be monitored by autoradiographic examination of mouse kidneys or livers after administration of α -[5-¹⁴C]DFMO (Pegg et al., 1982; Pösö and Pegg, 1983; Zagon et al., 1984).

2.2 CO₂ consumption by coupled enzymes (PEPC-MDH)

The most used method for the detection of CO₂ produced by decarboxylases, such as ODC, makes use of CO₂ consumption

through reactions with the enzymes Phosphoenolpyruvate Decarboxylase (PEPC) and Malate Dehydrogenase (MDH), known as PEPC-MDH (Figure 2). From the CO₂ produced by the decarboxylase reaction, PEPC catalyzes the condensation of bicarbonate with phosphoenol pyruvate to form oxaloacetate. In the next step, MDH (using NADH as a cofactor) reduces oxaloacetate to form malate and NAD⁺. NADH absorbs light at 340 nm, but NAD⁺ does not. The presence of CO₂ in the reaction system leads to a decrease in light absorbance, used to assess the production of CO₂ (Scriven et al., 1988). These reactions can be performed using commercial kits, standardized for CO₂ detection (Smithson et al., 2010b; Liao et al., 2015; Chai et al., 2020). This assay allows the monitoring of the reaction in real-time and is considered by some authors as a good candidate for adaptation to HTS (Smithson et al., 2010b; Chai et al., 2020). Smithson et al. (Smithson et al., 2010b), optimized this assay using a commercial system for the detection of bicarbonate, carried out the experiments in N₂ atmosphere, performed the analyzes in an automated system in 384-well microplates, and optimized all the conditions reactions to evaluate 3,600 compounds. This method was used to screen a library of 316,114 compounds as inhibitors of *T. brucei* ODC. New classes of ODC inhibitors were identified that probably bind to new non-active sites of the enzyme, demonstrating the importance of this type of screening for the identification and development of new inhibitors (Smithson et al., 2010a).

2.3 Putrescine oxidation: H₂O₂ released

2.3.1 Colorimetric

This method is based on the oxidation of putrescine by soybean amine oxidase (SAO), does not require polyamine purification, and can be performed in a 96-well plate for analysis in a plate reader. In the ODC reaction, the concentration of putrescine formed is determined by the amount of H₂O₂ generated by the reaction with SAO. Oxidation of 1 mol of putrescine by SAO generates 1 mol of H₂O₂ which reacts with 4-aminoantipyrine and phenol catalyzed by horseradish peroxidase to produce a colored complex that absorbs at 505 nm (Figure 2). Negative control can be done by replacing protein or substrate with buffer in the ODC enzyme reaction mix (Badolo et al., 1999; Das et al., 2015). In these assays, the soy amine oxidase enzyme was isolated from a soy plant and used in crude (Das et al., 2015) or purified form (Badolo et al., 1999), which increases the test performance by one more step. Furthermore, the possibility of test compounds acting as inhibitors of one of the additional enzymes should always be considered in HTS assays, which could lead to false positives for ODC.

2.3.2 Luminescence

The luminescence-based method was developed for the determination of total polyamines in biological samples (Fagerström et al., 1984) and adapted to determine ODC activity in cell extracts (Wang and Bachrach, 2000). This method consists in

for the detection of polyamines. The major drawbacks are the cost, long analysis time, and the need for a laborious pretreatment of the sample. Several methods describe the detection and analysis of polyamines through HPLC analysis, but only a few have been applied to monitor ODC activity. In general, the enzymatic reaction is terminated by the addition of HClO_4 , before centrifugation for protein precipitation. The amine-containing supernatant is then used for derivatization and analysis.

This pretreatment and a classical derivatization procedure using NaOH and benzoyl chloride was recently performed by Liu and coworkers for the screening of ODC inhibitors (Chai et al., 2020). The dansylation of the diamines can also be performed using the amine-containing supernatant, saturated sodium carbonate solution, and dansyl chloride. After derivatization, a pre-purification of the mixture using a Bond-Elut C18 cartridge was performed before HPLC analysis (Kabra et al., 1986; Legaz et al., 2001). *o*-Phthalaldehyde (OPA) can also be used for amine derivatization from the mixture of the neutralized supernatant, OPA, a borate buffer, and mercaptoethanol for reagent stabilization. A fluorescence detector with standard filters for OPA was used for detection (Kvannes and Flatmark, 1987; Bito et al., 2019). Tritium-labeled ornithine was used for the detection of ODC activity, obtained directly from the murine embryonic submandibular gland. After complete disruption of the sample in phosphate buffer (pH 7.3), followed by centrifugation, analysis was performed by HPLC equipped with a scintillation counter (Beeman and Rossomando, 1989). Other recent derivatizations include carbamoylation with isobutyl chloroformate (Ah Byun et al., 2008), derivatization with diethyl ethoxymethylenemalonate (del Rio et al., 2018), and the use of commercially available derivatization kits (Xiao et al., 2009).

2.6 MS assays

Given the importance of detecting very low concentrations (traces) of polyamines in biological fluids, many chromatographic methods have been used, mainly based on HPLC separation with fluorometric detection (Yoshida et al., 2004), GC separation (Li et al., 2008), and HPLC with MS detection (Ducros et al., 2009; Liu et al., 2011). The detection by HPLC/Q-TOF MS of benzoylated polyamines allowed the determination of very low concentrations of polyamines in urine (Liu et al., 2011). Mass spectrometry without chromatographic separation was used by Gaboriou and colleagues to detect and quantify biologically relevant polyamines after dansylation. In the Atmospheric Pressure Chemical Ionization (APCI) mass spectra, the positive ions for each dansylated polyamine were generated after optimization by flow injection analysis (FIA). FIA together with MS detection by monitoring selected ions has greatly increased the sensitivity of polyamine detection. The method proved to be linear over a wide range of polyamine

concentrations allowing detection in the fmols range. The method was considered fast, efficient, economical, reproducible, and simple enough to allow its routine application (Gaboriau et al., 2003). Subsequently, this method was used to detect the putrescine formed *in vitro* during the decarboxylation of L-ornithine by ODC present in cell extracts of a mouse hepatoma cell line, allowing detection of ODC activity as low as 0.05 nmol/mg (Gaboriau et al., 2005).

2.7 Circular dichroism

L-ornithine is chiral and when decarboxylated by ODC it is converted to putrescine, achiral. This difference in chirality makes it possible to use circular dichroism to monitor ODC reactions. As an amino acid, L-ornithine generates a positive signal at 201 nm in the CD spectrum due to the interaction of the carboxylate group with chiral C_α . This signal is dependent on the concentration of L-ornithine, with a linear decay of its intensity along the ODC reaction. The monitoring of ODC reactions by CD is very simple, requiring only buffer (phosphate or tris-less interference in the spectrum), substrate, PLP, and enzyme. However, it has the limitation that several organic compounds absorb around 201 nm and cannot be used with cell extracts or mixtures of compounds (Brooks and Phillips, 1996). In addition, it cannot be analyzed on microplates, which makes it difficult to perform many analyses simultaneously.

2.8 Fluorescence assays

A fluorescence-based assay to continuously monitor ODC activity was developed based on the principle of the tandem supramolecular enzyme assay. Tandem assays rely on pairs of reporter compounds with different affinities for a macrocyclic receptor and a fluorescent dye. This difference in macrocycle affinity causes a change in the spectroscopic properties of the fluorescent dye. In tandem enzymatic assays, the macrocycle receptor interacts with the substrate or enzymatic reaction product, leading to a displacement of the fluorescent dye. The change in fluorescence to be detected will depend on the difference in affinity of the substrate or product for the macrocycle compared to the fluorescent dye (Ghale and Nau, 2014). In the assays carried out by Nilam et al. (Nilam et al., 2017) *trans*-4-[4-(dimethylamino)styryl]-1-methylpyridinium iodide (DSMI) was used as a fluorescent dye and cucurbit [6]uril (CB6) as the macrocyclic receptor (Nau et al., 2009; Li et al., 2012). L-ornithine has little affinity for the macrocycle, so DSMI is preferentially encapsulated by the receptor (CB6) and is strongly fluorescent. With the conversion of L-ornithine into putrescine by the ODC, the putrescine, with greater affinity for the macrocycle, will be encapsulated, continuously releasing the DSMI, with a decrease in the fluorescence intensity with the

course of the reaction. This assay can be adapted for reading on microplates and allowed the determination of the enzymatic kinetic parameters of the ODC, using the known inhibitors DFMO and (–)-epigallocatechin (EGCG) as positive controls (Nilam et al., 2017).

3 Perspective for HTS

Despite the importance of ODC as a therapeutic target, the identification of new inhibitors from the screening of large libraries of compounds is very limited by the difficulty in assaying ODC activity in a high-throughput manner. This has been a major challenge for decarboxylases, as both the substrate and the product are not directly detected by spectroscopic techniques, needing derivatization or conversion processes into spectrophotometrically detectable complexes. Among the assays presented, only the PEPC-MDH and the fluorescence assay proved to be suitable for HTS due to their biological relevance, sensitivity, robustness, and economic viability (Blay et al., 2020). However, just in the PEPC-MDH the experimental and automation conditions were evaluated and optimized to meet the criteria for a high-quality HTS. Given the difficulties of HTS assays for ODC based on enzymatic reactions, a promising alternative would be to perform HTS assays based on ligand-enzyme processes (Bergsdorf and Ottl, 2010), employing libraries of compounds or molecular fragments (Erlanson, 2012) – which have shown to be very promising in the last few decades– developing several compounds that are in clinical phase trials and some drugs on the market (Erlanson et al., 2016).

Author contributions

LT Planned the organization of the manuscript, wrote and revised BS Wrote topic about HPLC JS Wrote topic about

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Conflict of interest

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