METABOLIC MODELLING OF WINE-MAKING : A STATE OF THE ART

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Abstract. The yeast *Saccharomyces cerevisiae* is one of the most studied micro-organism, due to its numerous applications (beer, wine, bakery, bioethanol). This good knowledge of the species was notably considered in modelling approaches where the intracellular behaviour is taken into account. Among them Metabolic Flux Analysis and Elementary Flux Modes are tools of interest to develop a mathematical model of the fermentation process including some characteristic flavour compounds. The present paper describes what has been done in the restrained field of wine-making/fermentation conditions, and underlines the potential of such approaches.

1 Introduction

As in most food processes, there is a need to increase the development of efficient control tools in order to improve the quality of food products. This is one of the issue of the EC CAFE project (www.cafe-project.org). In the context of wine-making, an a priori attractive path may be to consider microbiological knowledge about the production of the important organoleptic properties of the wine in order to develop new appropriate control approaches that can help to guarantee the production of wine of defined quality.

In the nineties, as technical progresses in biotechnology were decisive, *Saccharomyces cerevisiae* has become the first eukaryotic organism[1] whose genome was fully sequenced, denoting the interest for its better understanding. The macroscopic behaviour of the alcoholic fermentation has already been widely studied at this time, resulting in classical macroscopic models where the bioconversion is represented by macro-reactions linking substrates and products. However, the need for engineering micro-organisms in a specific use led researchers to take advantage of the physiological informations coming from microbiology, such as the metabolic behaviour of a cell. Applied mathematics concepts such as steady-state behaviour were involved, and new modelling approaches were introduced:

- Metabolic Flux Analysis (MFA) [31] and Flux Balance Analysis (FBA) [10];
- Elementary Flux Modes (EFMs) [26] and Extreme Pathways (EPs) [24].

Reaction schemes resulting from these approaches can be derived into macro-reactions that accurately describe what takes place in the yeast and are the starting point for developing dynamical models of the process.

For the wine fermentation, the focus was made on main metabolic processes: sugar and nitrogen consumption, ethanol production, and biomass synthesis. Marginal compounds such as flavour markers were not yet involved in this field (due to measurement difficulties) whereas they could be optimized to improve the wine quality.

In this paper, classical kinetic models for wine-making are briefly described before presenting flux analyses approaches. Section 4 relates the notions between EFMs and EPs, and Section 5 concentrates on flavour markers. Conclusion and future prospects are presented in Section 6.

2 Kinetic Models

The first comprehensive kinetic models [2, 6, 29] were describing the influence of sugar and ethanol levels, and of temperature on sugar utilization, capturing the general macroscopic trends found in practice.

Between seven and ten days are typically necessary to achieve a complete fermentation, but if the nitrogen is depleted, the process can take significantly longer to complete (sluggish fermentation) or can leave an important residual sugar level (stuck fermentation). Models have been developed to predict the transition from normal to sluggish or stuck fermentation, with kinetics based on nitrogen as a growth-limiting nutrient, in isothermal conditions [9], and later by including the temperature dependency of some parameters [7].

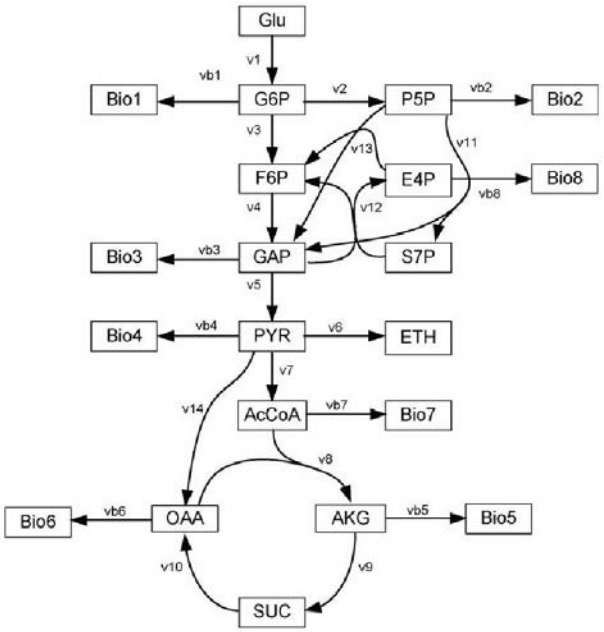
Several more empirical or non-parametric models have also been published. Among them, the group of Malherbe et al. [15] developed a model based on physiological considerations including nitrogen additions, temperature influence, transport of sugar and ethanol inhibition. More than 100 fermentations have been carried out to validate qualitatively and quantitatively the model [8].

[1]A single-celled or multicellular organism whose cells contain a distinct membrane-bound nucleus

Nevertheless none of the cited models successfully describes other caracteristic metabolites of yeast such as glycerol, or succinic, acetic or pyruvic acids. In this context, fluxes-oriented- and pathways-oriented-analyses are useful tools that can provide an accurate description of these compounds biosyntheses since it allows to account for the intracellular behaviour.

3 Stoichiometric Model and Metabolic Flux Analysis

A cell can be considered as a microscopic factory where some entering matters (substrates) are transformed by different processes, passing from a state to another (internal metabolites), and finally giving end-products. All these reaction schemes define the metabolic network of the cell, and each of them is characterized by a reaction rate called metabolic flux (example in Fig. 1).



v_1: Glu $\longrightarrow$ G6P
v_2: G6P $\longrightarrow$ P5P
v_3: G6P $\longrightarrow$ F6P
v_{b1}: G6P $\longrightarrow$ Bio1
v_4: F6P $\longrightarrow$ GAP
v_5: GAP $\longrightarrow$ PYR
v_6: PYR $\longrightarrow$ ETH
v_7: PYR $\longrightarrow$ AcCoA
v_8: AcCoA + OAA $\longrightarrow$ AKG
v_9: AKG $\longrightarrow$ SUC
v_{10}: SUC $\longrightarrow$ OAA
v_{11}: P5P $\longrightarrow$ S7P+GAP
...

Figure 1: Schematic diagram of the simplified central yeast metabolic network [34], and some metabolic fluxes (v_i) associated with reaction schemes. Glucose (Glu) is the substrate, while biomass (Bio1-8) and ethanol (ETH) are end-products; other components are internal metabolites.

This reaction network can be synthesized into a stoichiometric matrix $S\ (m \times n)$ where m is the number of metabolites and n the number of metabolic fluxes (v_i).

Using the MFA approach, the fluxes through the pathways of the bioreaction network are estimated from measurements of substrate uptake and product formation rates (via stoichiometric balances). In other words, the consistency of the metabolic network is checked throughout experimental data.

The MFA theory is based on the assumption that the intracellular metabolites are under steady state compared to external metabolites (substrates and products), see [31]. For instance, the changes in the wine fermentation are slow compared to the high pool turnover of most metabolites. These can be rapidly adjusted while an increase of 10 g/l of ethanol in the medium takes more than 15 h [23].

Consequently, the mass balance equation of the intracellular metabolites is expressed as follows:

$$Sv = 0 \tag{1}$$

where $v = (v_1, v_2, \ldots, v_n)$ is the vector of metabolic fluxes.

The resulting system of equations is in general underdetermined (S is singular) because the number of measurements is not large enough to determine all the unknown values (v_i). Some equations originated from operational restrictions or observations may then be added, or objective functions (linear optimization) are created as additional contraints [1]. Besides, the sensitivity of the stoichiometric model should be tested to detect if the calculated rates are sensitive to errors in the measurements [16, 31].

Some stoichiometric models developed for *Saccharomyces cerevisiae* are dedicated to wine-making conditions. These metabolic networks are limited to the main products synthesis as ethanol, glycerol, and some amino acids. Moreover the biomass synthesis is simplified. The performed MFA is mostly used to acquire a better knowledge of the yeast behaviour under different operational conditions, as it is described in the following paragraphs.

In the fundamental work of Nissen et al. [16], the metabolic fluxes are computed at different dilution rates in an anaerobic, glucose-limited continuous cultures, in order to analyze the differences of metabolism between wild-type and genetically modified strains. The metabolic flux analysis revealed its efficiency concerning the assessment of intracellular fluxes distributions and the test for the presence or absence of single reactions or whole pathways of the metabolim, showing its usefulness to decide a strategy for metabolic engineering of a given micro-organism. It has to be noted that the measurements were sufficient to solve Equ.(1) and that extra measurements validated fluxes deduced from the network.

Çakir et al. [3] used a genetically modified *Saccharomyces cerevisiae* strain which directly converts starch into ethanol, and checked if the ethanol production was optimal by applying Flux Balance Analysis (FBA). In this method, internal *and* external metabolites are considered: regarding Equ.(1), the right term is not equal to zero, as accumulation is considered for some external metabolites. The flux distributions were then calculated with ethanol production rate as maximization criteria. The calculated rates of ethanol were in agreement with experimental measurements, showing that the micro-organism really optimized its production.

Frick and Wittmann [12] compared flux analysis in the yeast under different aerobic growth conditions to describe the Crabtree effect, i.e. the shift from purely oxydative mode to respiro-fermentative and predominantly fermentative modes. Flux redirections and variations allowed for explaining the mechanism of this phenomenon. Within the same trend, Velagapudi et al. [34] explored some gene functions in yeast by using glucose or galactose as carbone source, which respectively gave a growth predominantly fermentative and another one more efficient in aerobic mode. The cultivation method in microtiter plates was underlined, facing continuous or controlled batch cultivations.

The influence of the initial nitrogen concentration on the process (sluggish and stuck fermentations, see Section 2) has been enlightened in the work of Varela et al. [33]. The metabolic network, adapted from the model of Nissen et al. [16], included fructose uptake, transport reactions and amino acid biosynthesis pathways. By using MFA and appropriate measurements, it has been found that nitrogen deficiency has a negative impact on the kinetics of sugar entry into the network, providing a lower viable cells concentration (with reference to a normal fermentation). It was observed that the rate of fermentation was a linear function of biomass, while fermentation time was an exponential function of biomass. To achieve a normal fermentation, early nitrogen supplementation or viable biomass addition is thus needed. Nevertheless, it is mentioned that effects on wine aroma and flavour would need further investigation.

From the same research group, Sainz et al. [23] initiated wine fermentation modelling where kinetic expressions are coupled to metabolic network. Five ordinary differential equations, related to main external metabolites as glucose, ammonia, viable cells, ethanol and glycerol, were fed with metabolic fluxes and rates calculated by an optimization routine. The used metabolic network coming from Nissen et al. [16] contains more reactions than metabolites providing multiple solutions. This was compensated by appropriate measurements in the original work [16] and here linear optimization was applied. The required objective function includes mass balances of the stoichiometric matrix, lower and upper bounds set for metabolix fluxes, and an optimization criteria assuming that the cell maximizes its growth or the activity of the glucose uptake. However, the resulting model had some difficulties to reproduce experimental data with rich and poor nitrogen musts.

Pizarro et al. [20] improved the model from the results of Varela et al. The stoichiometric model was completed with the fructose consumption rate, and the reductive branch of the TCA cycle was included [16, 5]. Seven differential equations related to glucose, fructose, nitrogen source, biomass, ethanol, glycerol, acetate and succinate were implemented. The glucose and fructose uptake rates were redefined and an efficiency factor has been introduced that depends on the fermentation temperature and on the initial assimilable nitrogen concentration. Finally the model accurately predicted glucose, fructose and ethanol concentrations at various temperatures and with different initial assimilable nitrogen concentrations both in laboratory and industrial fermentations. However, predictions of other metabolites were representative but not accurate.

The group of Zhang et al. [36] studied how *Saccharomyces cerevisiae* adapts its metabolism to different carbone sources such as glucose, glycerol and acetate. The reference to metabolic flux analysis in their work is rather confusing. As a matter of fact, 'atom mapping matrices' are used, combined with nuclear magnetic resonance (NMR) spectroscopy [25], and the intracellular fluxes of their metabolic network were estimated with a hybrid-type of computer program combining a local search algorithm with global search algorithm, to minimize a quadratic error function. These isotopic-tracer techniques (as NMR) are not considered in this work because they are laborious and expensive to conduct and cannot be used on an industrial scale [1].

The consistency of flux analysis validates the designed metabolic network, and pathway-oriented approaches as EFMs and EPs allow for linking substrates and end-products. The macro-reactions that are deduced give a better view of the interplay between metabolites and are a first step for macroscopic dynamical modelling.

4 Elementary Flux Modes and Extreme Pathways

Convex analysis has been introduced in the biochemical field to assess the properties that emerge from metabolic networks. This mathematical tool enables the production of a convex set of vectors that can characterize all of the steady-state flux distributions of the considered network, i.e. the whole set of solutions of Equation (1). For this purpose, inequality constraints are applied on the flux values of the irreversible reactions:

$$v_i \geq 0 \tag{2}$$

where v_i is the flux from reaction i.

The solutions provided by Equation (1) combined with Equation (2) define a high-dimensional cone located in a space where each axis corresponds to a reaction flux, and every valid flux distribution is represented by a nonnegative combination of the convex basis vectors.

Recently, two different uses of these vectors have been considered: elementary flux modes (EFMs) [26] and extreme pathways (EPs) [24]. Subtle differences exist between both and they have been underlined in some comparison publications [14, 18], especially concerning the internal reversible reactions. EPs algorithm decouples reversible reactions into two separate irreversible reactions (forward and backward directions) and then calculates the pathways, while EFMs algorithm accounts for reaction directionality through a series of rules in the corresponding calculations of the modes. As conclusions:

- EFMs and EPs are equivalent when all exchange fluxes are irreversible, and constitute the edges of the high-dimensional cone;
- if there are reversible reactions, the set of EPs constitutes the convex basis vectors and EFMs are a superset of the extreme pathways, including additional network pathways that meet specific criteria, i.e. that the elementary modes are the set of all routes through the metabolic network [18] (example in Fig. 2).

Consequently EFMs can be computationally more expensive but seems to be more suitable for network analysis as the set of EPs may miss genetically independent pathways [14]. Macro-reaction schemes can be deduced and corresponding differential equations can be derived to obtain a dynamical model of the process [22]. As the

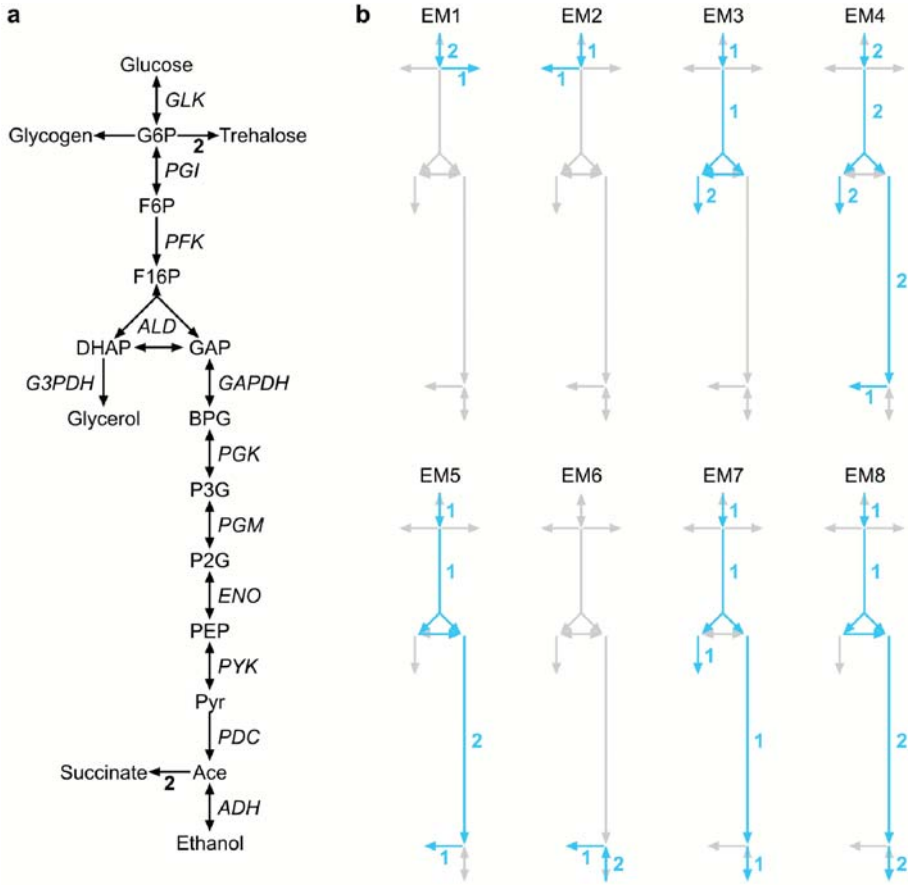


Figure 2: Model of yeast glycolysis [28] and its eight associated elementary modes. Numbers indicate stoichiometries of reactions when different from one. External metabolites: glucose, glycogen, trehalose, glycerol, succinate, and ethanol.

number of EFMs can be very high, softwares have been developed to compute them easily: Metatool [19] and FluxAnalyzer [13].

The central metabolism of yeast has been studied with these tools to get physiological informations, yet not specifically in fermentation conditions. In Förster et al. [11], convex analysis is used to identify essential genes, EFMs providing optimal yield, and characteristics of the active pathway structure in order to elucidate the function of orphan genes. Çakir et al. [4] extended the reaction set of Förster et al. [11] and applied EFMs for gene deletion phenotype analysis: if a gene is deleted, the impact can be observed experimentally, and EFMs resulting from this deletion can hopefully provide the same information. A typical example is the determination of lethal gene deletions [4]. Schwartz and Kanehisa [28] associated structural (EFMs) and kinetic modelling, deducing a significant reduction of the range of possible behaviours of the metabolic system. Different carbon sources have been tested for bioethanol production in the work of Xu et al. [35], and EFMS combined with linear optimization allowed to maximize the yield.

In the work of Nookaew et al. [17], a new technique for the determination of fluxes has been developed. The convex properties of EFMs are used to calculate a weighing factor for each EFM corresponding to an appropriate fractional operation of this mode within the complete set of EFMs. Briefly, each flux can be specified in terms of the relative flux

$$\begin{aligned} \overline{Y} &= \mathrm{FRC}_1\,\overline{E_1} + \mathrm{FRC}_2\,\overline{E_2} + \mathrm{FRC}_3\,\overline{E_3} + \ldots + \mathrm{FRC}_m\,\overline{E_m} \qquad (3)\\ \text{with}\quad \overline{Y} &= [1 \;\; v_2/v_1 \;\; v_3/v_1 \;\; v_4/v_1 \;\; \ldots \;\; v_n/v_1]\\ \text{and}\quad \overline{E_{\mathrm{i}}} &= [1 \;\; e_{2,\mathrm{i}}/e_{1,\mathrm{i}} \;\; e_{3,\mathrm{i}}/e_{1,\mathrm{i}} \;\; e_{4,\mathrm{i}}/e_{1,\mathrm{i}} \;\; \ldots \;\; e_{\mathrm{n},\mathrm{i}}/e_{1,\mathrm{i}}] \end{aligned}$$

where FRC_i ($i = 1$ to m) are non-negative entries of a weighting vector called the flux regulation coefficients (FRC) to reflect their biological meaning. An objective function is associated, maximizing the number of EFMs ($\overline{E_\mathrm{i}}$).

This approach has been considered for growth on glucose, glycerol or acetate as the sole carbon source, and with respiratory, respiro-fermentative and fermentation data. The calculated fluxes were compared to those obtained with MFA, FBA, and PSI (a method to determine the fluxes using elementary modes based on the Moore-Penrose generalized inverse (or pseudo-inverse) matrix [21]). The results from FBA and MFA are close to each other, but the PSI approach (also based on EFMs) provides a weight vector containing negative elements that is not consistent with the convex combination property of EFMs. Besides the macro-reactions that can be deduced from EFMs, the FRC technique could be used to validate results from metabolic flux analysis and to corroborate the accuracy of this approach.

Flavour compounds originated from wine-making are never considered in all these studies whereas such tools would certainly provide consistent information about the quality improvement of wine. To fill these gaps, future work will focus on the organoleptic properties transferred to the wine during the fermentation under the action of *Saccharomyces cerevisiae*.

5 Flavour Markers

Flavour markers, as higher alcohols, esters, and sulfur compounds result from complex biochemical reactions and their modelling requires a good knowledge of the yeast metabolism. Figure 3 illustrates schematically how these compounds are originated and that these really are by-products of main processes such as pyruvate synthesis, tricarboxylic acid cycle, etc.

It is important to note that these compounds represent less than 5% of the yeast production and correspond to (very) low concentrations and that their measurements involve specific and expensive measuring devices.

The first step in this research is the (cautious) design of the metabolic network on the basis of the available measurements:

- only the pathways describing the synthesis of the main products and of the flavour markers that can be measured have to be considered;
- in these pathways only the intermediate metabolites that are important or involved in different reactions have to be kept.

These precautions allow to avoid a too large network complexity and consequently:

- to make easier the solution of the equations system involved in the metabolic flux analysis. The number of additional constraints that are necessary to determine all the fluxes values depends on this and must be kept reasonable;
- to reduce the risk of combinatorial explosion during the EFMs computation because a complex network involves many different pathways from substrates to end-products, strongly increasing the number of EFMs.

The first version of this metabolic network, based on sources like the SGD project database [30] for instance, explores reaction pathways corresponding to the synthesis of many flavour-active compounds. When the available measurements will be identified, this network will be refined to describe the corresponding reaction schemes.

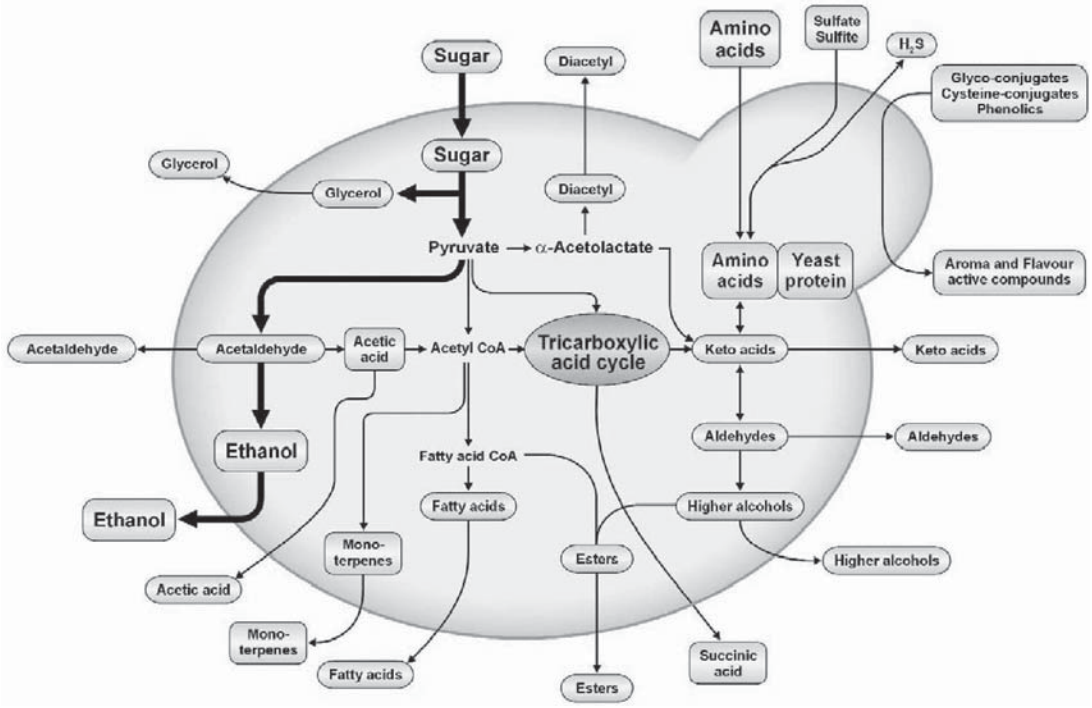


Figure 3: Schematic representation of derivation and synthesis of flavour-active compounds from sugar, amino acids and sulfur metabolism by wine yeast, [32].

The next step will involve the metabolic fluxes analysis and the need for additional equations to solve the system. EFMs will be finally computed thanks to Metatool [19] and the deduced macro-reactions will be derived into differential equations [22]. FRCs [17] could also be computed for a cross-validation of the MFA.

The possibility to work with subnetworks [27] or to couple a classical macroscopic model with the flavour-oriented network will be considered as well.

6 Conclusion and Future Prospects

The objective of the EC CAFE project is to provide new paradigms for the smart control of food processes as wine-making, for instance. Metabolic modelling strategies assuming that internal metabolites are under steady state have been used to explore the physiology of yeast and to engineer a strain in order to optimize the production of a particular compound (e.g. ethanol). Flux analyses (MFA/FBA) and pathway analyses (EFMs/EPs) have been widely proved successful and efficient to represent intracellular behaviour and to manage changes of operational conditions. Therefore these methods will be applied to improve the wine quality by considering the marginal reaction schemes leading to flavour markers.

Further development in this research will involve the development of a metabolic network oriented to the synthesis of flavour-active compounds, and MFA-EFMs analyses will hopefully provide enough information to derive a reliable dynamical model.

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