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Bioaroma generation: research studies on the sustainable enzyme-catalysed synthesis and advanced gas chromatography analysis of flavour and fragrance compounds

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Contents

I. Abstract

The modern "green economy" places great emphasis on eco-friendliness and sustainability. With increasing awareness of environmental issues, consumers are becoming more selective on how products are sourced and manufactured, favouring companies with environmentally and socially responsible practices. Flavours and fragrances (F&F) are important components in many products, such as food, beverages, cosmetics, and pharmaceuticals. They are traditionally sourced from natural extracts (e.g., plants, animal secretions) or synthetic processes using petrochemicals, but biotechnology has challenged this status quo, offering a natural, selective, and energy-efficient alternative: enzyme-catalysed reactions. As such, the improvement of these processes has become the focus of many studies in the past decade. Thus, different analytical methodologies and a variety of reaction processes are constantly being developed to evaluate and modify a range of products generated by this strategy.

In line with this interest, gas chromatography (GC) and its advanced techniques have been used for qualitative and quantitative analysis of natural, synthetic, and chemically or biologically modified F&F products, including essential oils. GC is a robust, efficient and versatile technique applied to volatile compounds in many types of samples of different complexities. Improved resolution, identification and quantification can be achieved with comprehensive two-dimensional (GC×GC) and multidimensional (MDGC) advanced GC techniques coupled with mass spectrometry, which expand the information collected for the samples, providing important insights into their chemical and biological properties. Thus, there are still opportunities to improve GC methods, especially in identifying variations in a sample's composition and chiral ratio, and correlating chemical profile to the source or processing type.

In this interdisciplinary thesis, analytical and biocatalytic methods were designed to explore the potential of enzyme-catalysed reactions as a more sustainable alternative for the production of aroma compounds. Five lipases and two laccases were applied to standards and essential oil samples for the synthesis of acetyl esters and oxidised olefins, respectively. GC and GC×GC methods were created to assess the changes in all the different stages of the enzyme study, including characterisation, screening, optimisation and application to samples.

The best-performing enzymes were CALA (lipase) and NZ-51003 (laccase), which, under the tested conditions, were not enantioselective and preferably reacted with primary alcohols and phenolic compounds, respectively. The optimum conditions for the lipase (CALA) were 37-40 °C, 3-4 mg/mL of enzyme, and 58-60% (v/v) vinyl acetate, which were effective for a broad range (0.1 – 50% w/v) of substrate concentrations. CALA successfully esterified 53% of the alcohols identified in 35 essential oil samples within 48h, with conversions of 80-100% for most primary and secondary alcohols. Laccases were not optimised due to poor compatibility of the applied enzyme preparation with organic and organic/aqueous medium. The GC methods helped to determine the enzymes' chemo- and stereoselectivity, and the changes in the samples' chemical profile. Especially, GC×GC enabled the identification of 125 target compounds and facilitated the visualisation of the process changes through the 2D plots.

The methods and findings of this research impacts the development of improved and green biocatalytic processes that can generate better, innovative and safer natural products.

II. Publications during enrolment

Published

2019:

- Amaral, M. S. S.; Marriott, P. J. The blossoming of technology for the analysis of complex aroma bouquets — a review on flavour and odorant multidimensional and comprehensive gas chromatography applications. *Molecules.* **2019**, *24*(11), 2080. https://doi.org/10.3390/molecules24112080.

2020:

- Amaral, M. S. S. †; Nolvachai, Y. †; Marriott, P. J. Comprehensive Two-Dimensional Gas Chromatography Advances in Technology and Applications: Biennial Update. *Analytical Chemistry.* **2020**, 92 (1), 85-104. https://doi.org/10.1021/acs.analchem.9b05412.

2022:

- Amaral, M. S. S.; Nolvachai, Y.; Marriott, P. J. Multidimensional gas chromatography platforms for the analysis of flavours and odorants. In: Comprehensive Analytical Chemistry. Cordero, C.E.I. Elsevier, **2022**, Vol. 96, Ch. 5, p.119-153. https://doi.org/10.1016/bs.coac.2021.10.005.
- Amaral, M. S. S.; Hearn, M.; Marriott, P. J. Quantitative assessment of enzymatic processes applied to flavour and fragrance standard compounds using gas chromatography with flame ionisation detection. *Journal of Chromatography B*. **2022**, 1209, 123412. https://doi.org/10.1016/j.jchromb.2022.123412.

2023:

- Nolvachai, Y.; Amaral, M. S. S.; Herron, R.; Marriott, P. J. Solid phase microextraction for quantitative analysis – Expectations beyond design? *Green Analytical Chemistry*. **2023**. 4, 100048. https://doi.org/10.1016/j.greeac.2022.100048.
- Nolvachai, Y.; Amaral, M. S. S.; Marriott, P. J. Foods and contaminants analysis using multidimensional gas chromatography: An update of recent studies, technology, and applications. *Analytical Chemistry.* **2023**, 95(1), 238–263. https://doi.org/10.1021/acs.analchem.2c04680.
- Amaral, M.S.S., Hearn, M.T.W. & Marriott, P.J. Lipase-catalysed changes in essential oils revealed by comprehensive two-dimensional gas chromatography. *Analytical and Bioanalytical Chemistry.* **2023**, 415. https://doi.org/10.1007/s00216-023-04729-0.

† *Equal first co-authorship*

III. Thesis including published works declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes two original papers published in peer reviewed journals. A book chapter and various review papers on GC analysis of flavours and fragrances were published during the course of this PhD program, providing background knowledge for this thesis and helping with the preparation of the introductory Chapter 1.

The core theme of this thesis is the development of analytical and biocatalytic methods to investigate enzyme-catalysed reactions as a more sustainable alternative for the production of compounds of interest to the flavours and fragrances industry. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the School of Chemistry, Faculty of Science under the supervision of Professor Philip J. Marriott and Emeritus Professor Milton T.W. Hearn.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of *Chapters 2 and 3* my contribution to the work involved the following:

Thesis Chapter	Publication Title	Status	Nature and extent (%) of student contribution	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student Y/N
2	Quantitative assessment of enzymatic processes applied to flavour and fragrance standard compounds using gas chromatography with flame ionisation detection	*Published*	90% Conceptualisation, full laboratory experiments design and execution, data analysis, original manuscript and figures preparation, reviewing and editing.	*Philip J. Marriott* (5%), main supervision, resources and editorial assistance. *Milton T.W. Hearn* (5%) supervision, resources and editorial assistance.	*No*
3	Lipase-catalysed changes in essential oils revealed by comprehensive two-dimensional gas chromatography	*Published*	90% Conceptualisation, full laboratory experiments design and execution, data analysis, original manuscript and figures preparation, reviewing and editing.	*Philip J. Marriott* (5%), main supervision, resources and editorial assistance. *Milton T.W. Hearn* (5%) supervision, resources and editorial assistance.	*No*

I have not renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student name: Michelle dos Santos Silva Amaral

Student signature: **Date:** 20 Mar 2023

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main supervisor name: Philip J. Marriott

Main supervisor signature: **Date:** 20 Mar 2023

IV. Acknowledgements

This thesis is a product of my own ideas, hard work and resilience, along with the inspiration, strength and patience given by God in face of many technical challenges and the pandemic times, as well as the valuable collaboration of some amazing people.

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Finally, to my beloved family and friends I could not have made it without your support, encouragement, understanding and love. I also extend my gratefulness to the people that, during the course of this PhD or in the past, have opened doors for me, providing great opportunities, sharing knowledge and giving good advices that contributed to my professional and personal growth.

Muito obrigada à todos vocês!

V. Abbreviations and parameters

A	
α	[chromatography] relative retention ratio or separation / selectivity factor
α	[statistic] significance level
A	peak area
ABTS	2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonate)
Asp	asparagine
Astec CHIRALDEX BPM	non-bonded; 2,3,6-tri-O-methyl derivative of β-cyclodextrin capillary column
a_w	thermodynamic water activity

B	
B.C.	before Christ
BERG	bergamot essential oil sample
BOR	boronia essential oil sample

C	
C%	percent concentration
C%	enzyme conversion percent
CALA	*Candida antarctica* lipase A
CDWA	cedarwood atlas essential oil sample
CDWV	cedarwood Virginian essential oil sample
CINM	cinnamon essential oil sample
CIT	citronella essential oil sample
CLARY	clary sage essential oil sample
CLO	clove essential oil sample
C_M	concentration of the analyte in the mobile phase
COP	copaiba essential oil sample
C_s	concentration of the analyte in the stationary phase
Cu	copper
CV	coefficient of variation
CYP	cypress essential oil sample
Cys	cysteine

D	
^{1}D	first dimension
1D	one-dimensional
^{2}D	second dimension
2D	two-dimensional
3D	three-dimensional
DB-5	5% diphenyl / 95% dimethylpolysiloxane capillary column
d_f	stationary phase film thickness
DL-IIS	DeniLite-IIS
DOE	design of experiments

E

E	enzyme
E°	redox potential
EC	enzyme commission number
ECD	electron capture detection
ee	enantiomeric excess
*e*GC	enantioselective gas chromatography
*e*GC×GC	enantioselective comprehensive two-dimensional gas chromatography
*e*MDGC	enantioselective multidimensional gas chromatography
e-nose	electronic nose detector
EO	essential oil
ES	enzyme-substrate complex
e-tongue	electronic tongue detector
EUCR	eucalyptus essential oil sample

F

F&F	flavour and fragrances
FID	flame ionisation detector
Fig.	figure
FKI	frankincense essential oil sample
FPD	flame photometric detection
FTIR	Fourier transform infrared spectrometer

G

GC	gas chromatography
GC×GC	comprehensive two-dimensional gas chromatography
GER	geranium essential oil sample
GUA	guaiacol

H

h	peak height
H	plate height
H_2	hydrogen gas
H_2O	water
HBT	1-hydroxybenzotriazole
H/C	heart-cut
He	helium gas
His	histidine

I - J

i.d.	capillary column internal diameter
IR	infrared
IRMS	isotope ratio mass spectrometer
JAS	jasmine essential oil sample

K

K	equilibrium constant
k	retention factor or capacity factor
K_c	partition coefficient
K_{cat}	enzyme turnover number
KFL	kaffir lime essential oil sample
K_M	Michaelis constant
KNK	kanuka essential oil sample

L

LAV	lavender essential oil sample
LMG	lemongrass essential oil sample
LZ-CALBL	Lipozyme CALB L
LZ-TLIM	Lipozyme TL IM

M

MDGC	multidimensional gas chromatography
MEGA-DEX DET Beta	diethyl tertbutylsilyl-β-cyclodextrin capillary column
MNK	manuka essential oil sample
MS	mass spectrometer, mass spectrometry or mass spectrum
MSB	masoi bark essential oil sample
MS/MS	tandem mass spectrometry
MYR	myrrh essential oil sample
m/z	mass-to-charge ratio

N

N	number of theoretical plates
n_c	peak capacity
NC-ADL	NovoCor ADL
NC-IUBMB	Nomenclature Committee of the International Union of Biochemistry and Molecular Biology
NMG	nutmeg essential oil sample
NPD	nitrogen-phosphorus detection
NRL	neroli essential oil sample
NZ-435	Novozym 435
NZ-51003	Novozym 51003

O

O	olfactometry detector
O_2	molecular oxygen

P

P	product
PAR	physical-activity relationship
PDMS	polydimethylsiloxane
pH	potential of hydrogen
PINE	pine essential oil sample
p-NP	*p*-nitrophenol
p-NPO	*p*-nitrophenyl octanoate
PPMP	peppermint essential oil sample
PTC	patchouli essential oil sample

Q - R

QSAR	quantitative structure-activity relationship
R^2	coefficient of determination
RI	retention index
ROSE	rose essential oil sample
ROSM	rosemary essential oil sample
R_s	resolution
RSM	response surface methodology

S

S	[enzyme] substrate
S	[statistics] standard deviation
SAR	structure-activity relationship
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SDWA	sandalwood Australian essential oil sample
SDWI	sandalwood Indian essential oil sample
Ser	serine
SLB-IL60	Non-bonded; 1,12-di(tripropylphosphonium) dodecane bis(trifluoromethanesulfonyl) imide capillary column
sn	stereospecific numbering system for glycerol derivatives
SWORG	sweet orange essential oil sample

T

T	temperature
T1, T2, T3	laccase redox sites
TCD	thermal conductivity detector
TMS	toroidal ion trap mass spectrometer
TOFMS	time-of-flight mass spectrometer
t'_R	adjusted retention time
t_R	retention time
${}^1t_R$	retention time in the first dimension
${}^2t_R$	retention time in the second dimension
TTO	tea tree essential oil sample

U

U or IU	enzyme activity units
UV-Vis	ultraviolet-visible spectrophotometry

V

V_0	initial reaction rate or velocity
V_{max}	maximum reaction rate or velocity
V_R	retention volume
VTV0	vetiver essential oil sample (Natoria)
VTV1	vetiver essential oil sample (Australian Botanical Products)
VUV	vacuum ultraviolet spectrophotometry detector

W-Z

$w_{1/2}$	peak width at half-height
w_b	peak width at baseline
YY	ylang-ylang essential oil sample

Units

°C	degree(s) Celsius	M	molar concentration
cm	centimetre(s)	mg/mL	milligram(s) per millilitre
fg	Fenton gram(s)	min	minute(s)
g	gram(s)	mL	millilitre(s)
h	hours	mm	millimetre(s)
kat	katal	mol	mole(s)
kDa	kilo Daltons	pg	picogram(s)
kg	kilogram(s)	ppm	parts per million
L	litre(s)	ppb	parts per billion
µL	microlitre(s)	rpm	rotations per minute
µm	micrometre(s)	s	second(s)
µM	micromolar concentration	V	volts
µmol	micromole(s)	v/v	volume per volume
m	metre(s)	w/v	weight per volume
		w/w	weight per weight

VI. Synopsis of thesis investigation

The research and development of efficient methods to apply and assess the performance of enzyme-catalysed reactions for the sustainable synthesis of high-value compounds is an ongoing and critical effort, which is closely related to the advance and diffusion of biotechnology and green chemistry into different industry sectors. The present work explored lipase and laccase reactions, as well as the high-resolution capacity of GC-based analytical techniques to devise methodology strategies for the synthesis and profiling of compounds relevant to the F&F industry. Thus, the key objectives of this thesis included the creation of GC methods and protocols for the assessment of different enzyme reactions, the investigation of chemo- and enantioselectivity of the biocatalysts studied, and the identification of changes in the composition of complex samples (i.e. essential oils), arising from the bioprocessing.

The background and fundamentals of GC, flavours and fragrances and biocatalysis are presented in **Chapter 1** of this thesis, as an introduction to the theoretical concepts and relevance of this research study. This chapter discusses in more detail the basis of GC and its advanced analytical techniques, their importance to improved separation performance and the steps of the method development process. Subsequently, an overview of F&F is presented, including definitions, importance, sources, essential oil complexity, structure-property relationships, the importance of GC for the study, quality, and safety control of F&F, and the role of green chemistry and biotechnology as superior processes to obtain F&F products. Lastly, lipases and laccases are defined and key factors of enzyme reactions are presented. The preparation of this chapter was supported by the knowledge acquired through the preparation of several review papers on GC analysis of flavours and fragrances published during the course of this PhD program, especially the sections 1.1 and 1.2.4, which contains some of the information and illustrations published.

Chapter 2 covers the development of strategic methods for characterisation, screening and optimisation stages of enzyme reaction studies. In this work, lipases and laccases were used as catalysts, terpene and phenyl standards as aroma-related substrates and GC as the main analytical technique for quantitative assessment and enantiomeric ratio determination to examine the enzymes enantioselectivity. A design of experiments (DOE) surface response methodology was applied to optimise key parameters of lipase reactions. This study sets the stage for the next experiments of this thesis and helps to establish standard parameters to assess the outcomes of similar enzyme studies.

Chapter 3 complements the knowledge from the study in Chapter 2, applying the selected lipase enzyme and optimised reaction conditions to a large set of essential oil samples. The chemical diversity of the targeted (i.e., alcohol substrates) and non-targeted compounds in these samples, helped to expand the knowledge of the enzymes' selectivity and inhibitory effects. Moreover, a GC×GC–MS method was developed to improve the separation and identification of target compounds, and facilitate the visualisation of bioprocessing changes amid essential oil samples complex profiles.

Finally, **Chapter 4** summarises the findings of this thesis, incorporating the research impact, concluding remarks and future directions.

Chapter 1

Background and fundamentals overview

Contents

1.1. Gas Chromatography

1.1.1. Fundamentals and key parameters

Gas chromatography (GC) is a separation technique applied to gases and (semi-)volatile substances that are thermally stable. The analysis of less volatile compounds is also possible, provided they are transformed into volatile derivatives. Conventional GC separations are usually performed on long (10-60 m) and narrow (0.1-0.32 mm i.d.) fused silica capillary columns and are based on the difference of distribution of the analytes between a liquid (or less commonly, a solid) stationary phase and a gas mobile phase (Fig. 1). The stationary phase in the most familiar format of the open tubular GC columns is a non-volatile liquid coating (0.1 – 5 µm d_f), chemically bonded and cross-linked in the column. The general parts of a GC system include the carrier gas line, injection unit, oven, capillary column, detector and a data acquisition/processing computer [1-3].

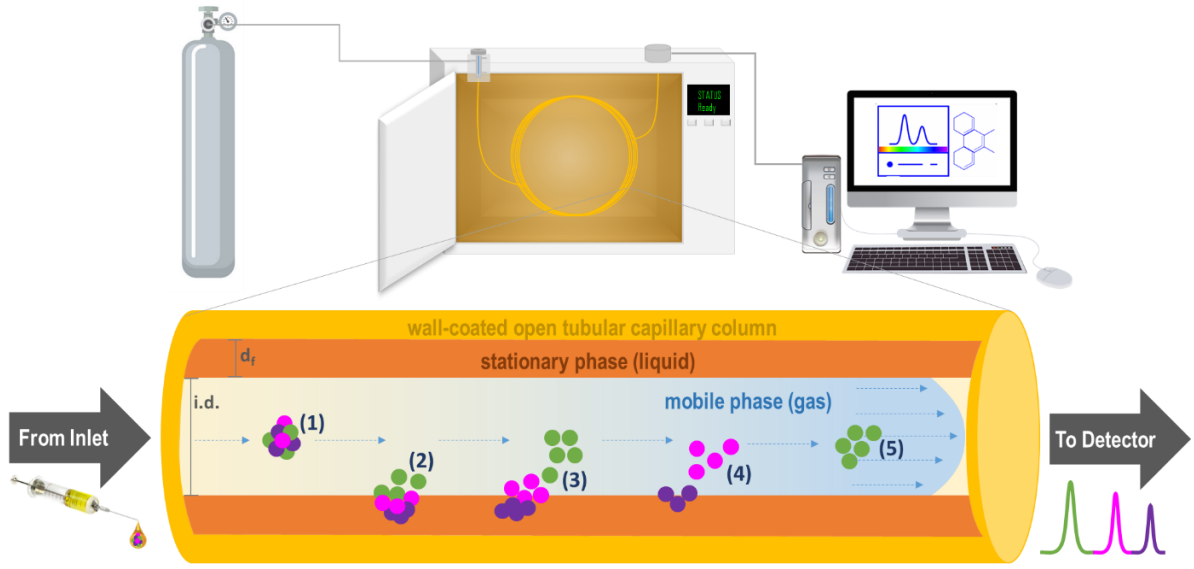

Figure 1. Diagram of the GC system general parts (top) and schematic representation of the GC separation process (bottom), illustrating different stages of equilibrium and separation of analytes between the stationary and mobile phases, from injection (1) to progressively better separation (2 - 5) and finally eluted from the column for detection.

The distribution constant (K_c), also called partition coefficient, is defined by the ratio of distribution of a given analyte between the two phases in a specific GC column, system and separation method ($K_c = C_S / C_M$). The concentrations of the analyte in the stationary and mobile phase are represented by C_S and C_M, respectively. When $K_c > 1$, the analyte is mostly retained in the stationary phase and, therefore, moving slowly through the column and having a longer retention time. If $K_c < 1$, the compound is concentrated predominantly in the mobile

phase, moving faster through the column and having shorter retention time. Other parameters (Table 1), such as the retention time (t_R), peak width, number of theoretical plates, retention factor and resolution can also be good indicators of performance and efficiency in GC, as well as help in the analyte identification and quantification [1-3].

Table 1. Summary of gas chromatography key parameters.

	Parameter	Description / Usefulness	Equation
t_R t'_R	Retention time and adjusted retention time	Time the analyte takes, from the injection, to elute through the column and reach the detector. Peak info / Qualitative	$t'_R = t_R - t_m$
K_c	Partition coefficient	Distribution ratio of analytes between stationary and mobile phase. Performance.	$K_c = C_S/C_M$
k	Retention factor or capacity factor	Indicates how long the analyte is retained in the stationary phase.	$k = \frac{t'_R}{t_M} = \frac{(t_R - t_M)}{t_M}$
V_R	Retention volume	Volume of mobile phase required to elute the analyte.	$V_R = t_R - t_M$
α	Relative retention ratio or separation / selectivity factor	The ratio of retention factors for adjacent peaks. Selectivity.	$\alpha = \frac{t_{R2}}{t_{R1}} = \frac{k_2}{k_1} = \frac{K_{c2}}{K_{c1}}$
N	Number of plates	A theoretical index that expresses the number of equilibrium stages undergone in a separation process. Efficiency.	$N=(16t_R^2)/w_b^2 = (5.54\ t_R^2)/w_{1/2}^2$
H	Plate height	The distance travelled by the analyte during the equilibrium stages in the column. Column efficiency.	H= L/N
R_s	Resolution	The difference in separation between two peaks. Separation efficiency. R_s ≥ 1.5 → peaks are fully separated R_s < 1.5 → partial or full co-elution	For peak width at the base ($w_{b(A)}$ and $w_{b(B)}$): $R_s = 2\left(\frac{t_{R(B)} - t_{R(A)}}{w_{b_{(B)}} + w_{b_{(A)}}}\right)$ For peak width at half-height ($w_{1/2(A)}$ and $w_{1/2(B)}$): $R_s = 1.18\left(\frac{t_{R(B)} - t_{R(A)}}{w_{1/2(B)} + w_{1/2(A)}}\right)$ efficiency, retention, selectivity $R_s = \frac{\sqrt{N}}{4} \cdot \left(\frac{\alpha - 1}{\alpha}\right) \cdot \left(\frac{k_2}{1 + k_2}\right)$
n_c	Peak capacity	Indicates the theoretical number of just resolved peaks that can fit in a chromatogram of total time t_g.	$n_c = \frac{t_g}{w_b}$

1.1.2. Method development and data analysis

The analytical method development in GC is a process of adjusting different parameters (Fig. 2), such as the choice of sampling method, stationary phase, column dimensions and/or use of multiple columns, injection mode, flow rate, oven temperature program and the detector settings.

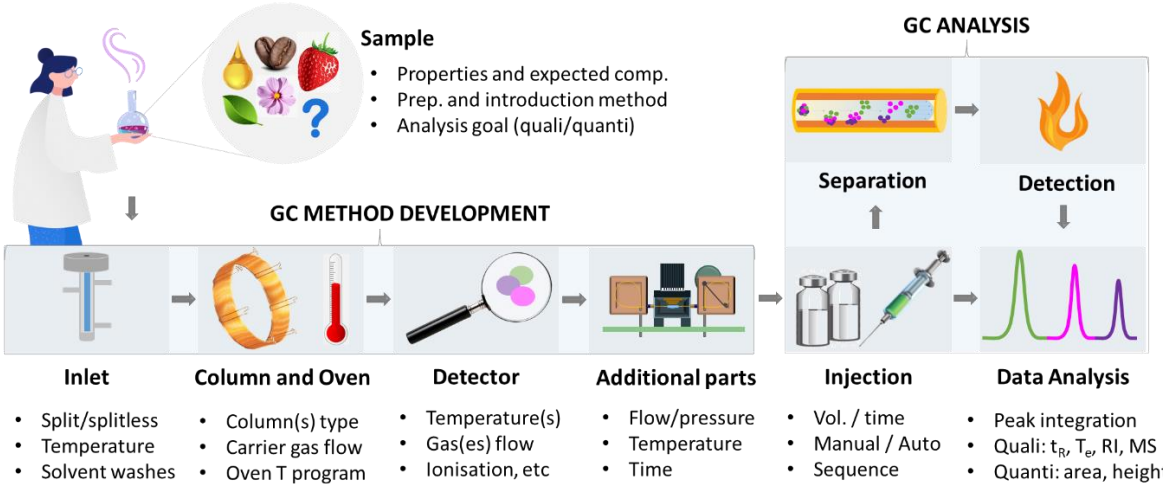

Figure 2. Diagram of the analytical method development process in GC.

Every analytical process actually starts with the sample. General information about the chemistry and physical properties of the sample, such as solubility and expected composition (at least in terms of chemical classes), helps to determine the best approach for sample preparation, injection and the GC analysis. The analysis goals (e.g., targeted/untargeted, qualitative/quantitative) should also be established at this stage. The most commonly applied extraction and sampling techniques for GC analysis are solvent extraction (or dilution), solid phase extraction (SPE) and solid phase microextraction (SPME). Others, such as purge and trap, dynamic headspace, distillation, solvent assisted flavour extraction (SAFE), and variant techniques of SPME and solvent extraction are also frequently applied. Ultrasonic vibration and microwave heating can be used to assist some of these extraction techniques. A chemical derivatisation step may be required prior to the analysis of compounds with low volatility, thermal instability, low detectability or incompatibility with GC analysis [1, 4, 5].

The next step in the GC method development is the choice of stationary phase. The polarity is a key column selection criteria, since it is directly related to the selectivity of the stationary phase, although selectivity cannot be predicted unless further information is available. Thus, depending on the polarity of the most part of the compounds in the sample or the target analytes (i.e., nonpolar, intermediate or high polar), the stationary phases are commonly chosen alike. The columns with 5%-phenyl 95% methylpolysiloxane or polyethylene glycol (also called “wax”) phases are usually a good start point for the analysis of compounds

with low to medium or high polarity, respectively. The challenge arises when a sample is complex and comprises multiple chemical classes spanning a range of polarities [1, 4, 5]. For instance, for essential oils comprising non-polar and polar analytes in approximately equal proportion, any of the above stationary phases can be used. However, more separation steps (e.g., MDGC, GC×GC) may be required to enhance the resolution.

Some capillary columns, such as ionic liquid or cyclodextrin (Fig. 3) stationary phases also have additional mechanisms of separation, such as the interfacial adsorption and trapping processes by size/shape, respectively, which can affect the elution order of analytes [1, 4, 5].

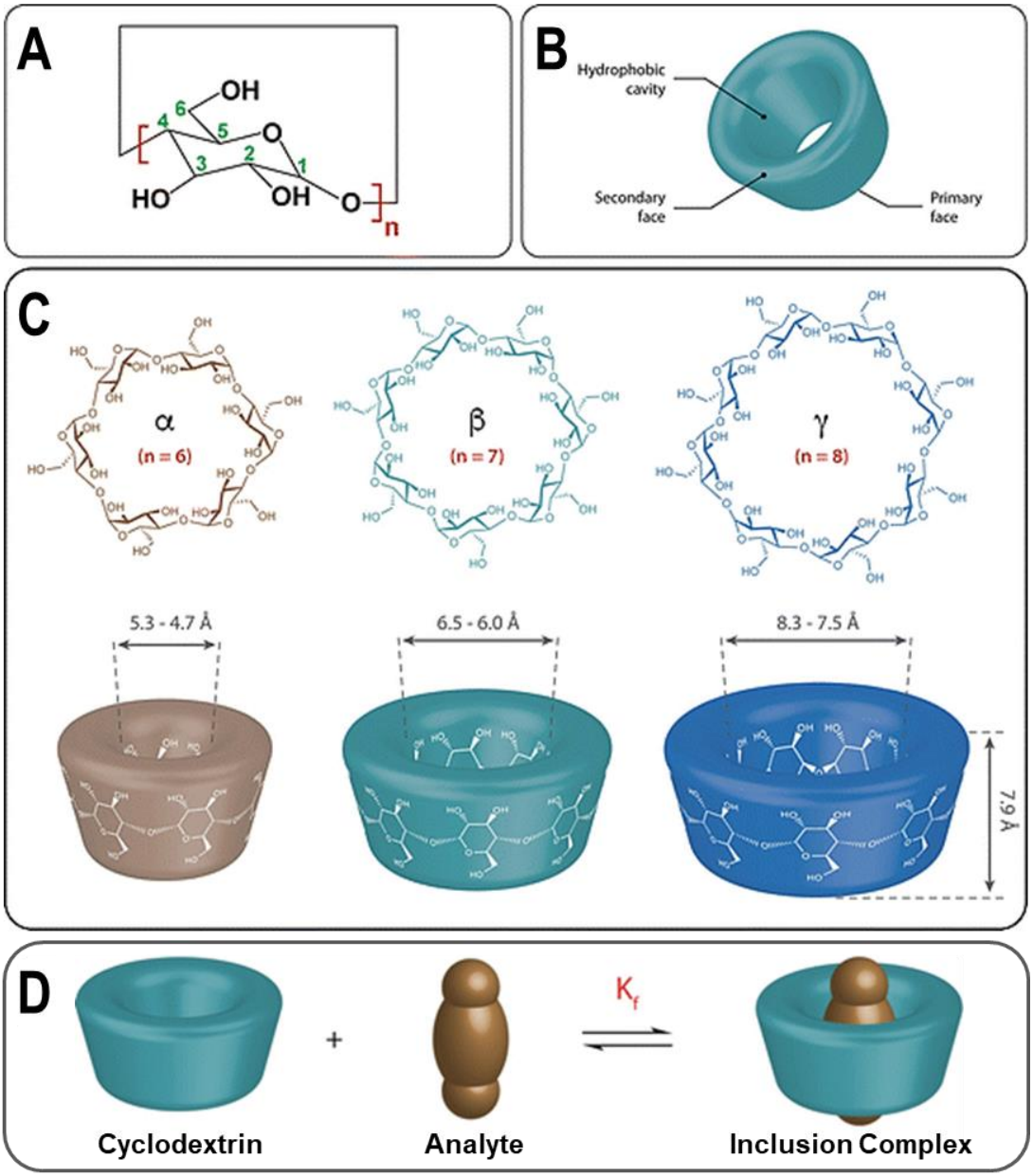

Figure 3. Schematic representation of the cyclodextrin chemical (A) and tridimensional (B) structures and the trapping process towards an analyte to form an inclusion complex (D). The structures of the α-, β- and γ-cyclodextrins (C) are composed by 6, 7 and 8 units of the sugar monomer, respectively, which also increases the cavity size. Adapted with permission from ref. [4]. Copyright 2018 Springer Nature.

Cyclodextrin-based stationary phases are currently a standard approach for general enantioselective separations (*e*GC), which are indispensable for authentication, origin attribution and sensory confirmation of samples, such as essential oils and flavour components. Usually, long (≥ 10 m) and / or narrower (i.e., smaller internal diameter, 0.1 mm) columns are required for a satisfactory chiral separation, hence the use of such columns in fast-GC or as a second dimension (^{2}D) column in GC×GC is difficult. Moreover, the chiral selectivity of these columns is largely affected by temperature, so that the resolution of a given enantiomeric pair may decrease as the temperature increases. Thus, it can be really challenging to achieve full separation of chiral compounds of high molar mass or as a component in complex samples, especially with only one column (i.e., one dimension) [6, 7].

After knowing the sample, defining the analysis goals and the column(s) to be used, it is time to set up the method in the instrument. The GC program is typically optimised by improving a general method found in the literature for the same or similar type of sample. The separations are commonly performed using constant flow rate, using around 1 mL/min, which gives an equivalent linear velocity within the optimum range for the most common carrier gases (He and H_2) according to van Deemter's theory. The inlet and detectors must be kept at a temperature above 100 °C and also above the final temperature of the oven program to allow fast volatilisation and avoid any condensation. Split mode is usually preferred for a fast sample introduction, avoiding peak broadening. A typical oven temperature program can ramp up from around 40 to 250 °C, at 3 or 5 °C/min, varying according to the sample. The injections can be done manually or in an automated sequence, the volume used is around 1 µL, and the sample concentration is usually up to 1 mg/mL (1000 ppm). The injected analyte mass range is limited to around 10^{-11} to 10^{-8} g for the most common capillary columns. Additional valves or modulators, if present, may require setting up temperature, flow and time. It is a good idea taking in consideration the resources available and the approximate number of injections to be made, in order to establish an ideal analysis total time.

Finally, the detector set up typically comprises parameters such as temperature, flow and ionisation voltage. The most commonly employed types of detectors in GC (Fig. 4) are the flame ionisation detector (FID) and the mass spectrometer (MS), due to their universality, mass-sensitivity, quantitative performance and, in the case of the MS, the ability to provide a spectrum that supports the analyte's identification [1, 8, 9].

The FID uses a mixture of purified hydrogen and air to produce an oxygen-rich flame that ionises organic compounds in the gas phase by removing electrons from C–H bonds. These ions are then detected by a collector electrode, generating a current the magnitude of which is proportional to the amount of carbon present in the analytes. Compounds having no C–H bonds or heteroatoms will not have good FID response. Some of the characteristics that make the FID so popular are the reliability, ease of operation, stability, response robustness

and reproducibility, low electronic and flame-based noise, low detection limits (10^{-12} g or 2 pg C/s) and large linear response range (10^7) for most compounds. Moreover, the negligible internal volume, combined with fast transduction of the chemical signal, ensures that the FID imposes minimal detector-based broadening on the chromatographic signal. As a result, the FID produces narrower peaks than other detectors [1, 8, 9] and should closely follow the elution profile of the compound as it elutes from the column.

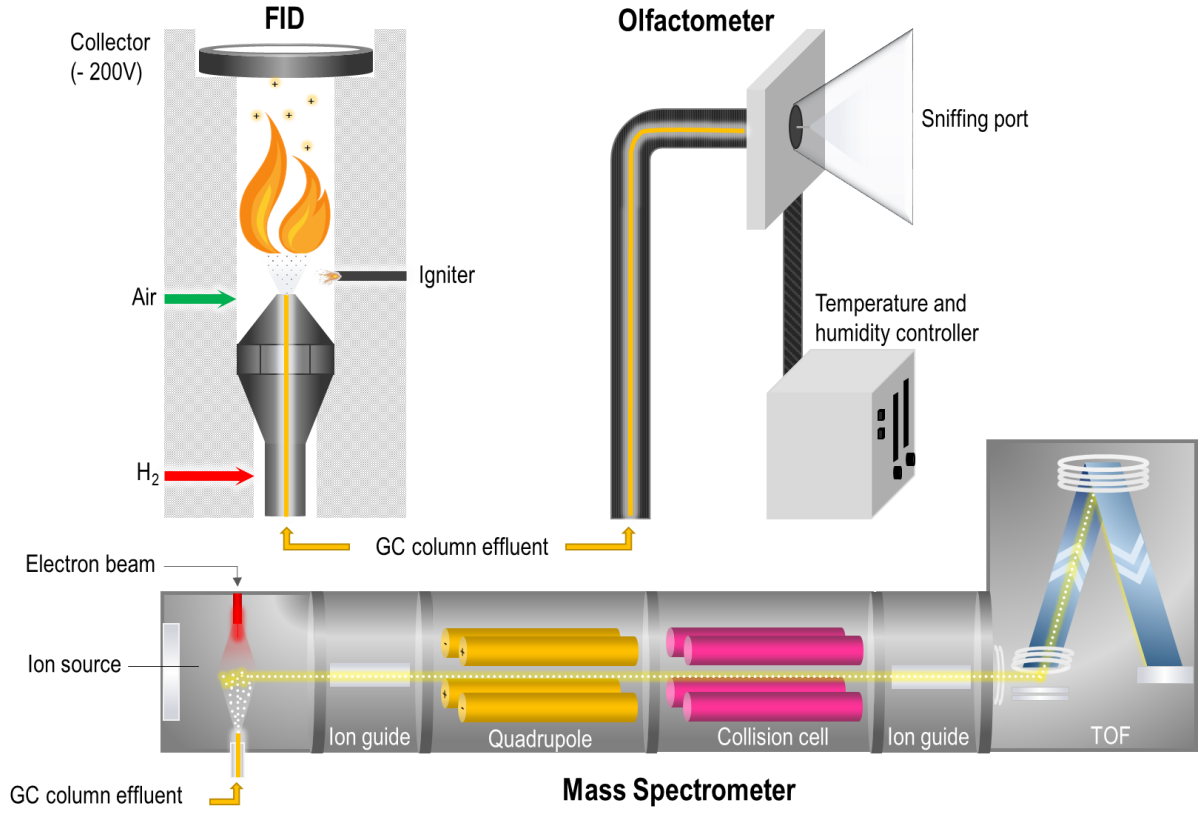

Figure 4. Schematic representation of FID, q-TOFMS and olfactometry detectors.

The MS, in brief, usually uses an electron beam (electron ionisation mode) or chemical reaction to produce ions in a vacuum environment, which are accelerated and deflected through an electromagnetic field, sorted according to their masses, amplified and detected. The energy used for deflection or the time taken for the ions to reach the detector are related to their mass or speed and, consequently to their mass-to-charge ratio (*m/z*). Since the majority of the ions produced by different ionisation methods in mass spectrometry have a charge of ±1, the *m/z* is normally equal to the mass of the molecule or its fragments. The MS also has a good linearity range (10^5) and detection limit (25 fg to 100 pg, depending on analysis mode). The combination of high mass accuracy and resolution enables some MS systems to distinguish ions with very similar *m/z*, improving structure elucidation and compound identification quality [1]. The isotope ratio mass spectrometer (IRMS) is a detector applied to determine stable isotope ratios ($^{13}C/^{12}C$; $^{2}H/^{1}H$; $^{18}O/^{16}O$; $^{15}N/^{14}N$) in a sample and it finds special

usefulness in the authentication of F&F samples, enabling the source-specific discrimination of compounds produced during the biosynthesis process in plants [10]. Other detectors, such as element-specific and olfactometer (Fig. 4) are also important for certain applications. In F&F analysis, olfactometry detection (GC-O) is crucial and it usually consists of transferring the effluent of the GC column to a sniffing device with proper temperature and humidity control, which can then be smelled and described by an expert analyst, thereby, allowing the assessment of the aroma profile and its potency. GC-O is often conducted by splitting part of the GC effluent to the 'O' system, and to a parallel FID or MS detector [11].

The chromatogram is the result taken from the GC analysis and it is a graphic representation of the separation process where the peaks' abundance versus retention times are plotted (Fig. 5). From this result, different types of information can be extracted. The type of data analysis to be performed is closely related to the analysis goals. Quantitative GC analysis aims to determine the concentration of the analytes in the sample. In this case, the peak area (or height) and the response factors for the analytes are crucial. They are obtained with proper chromatogram integration. Internal standards or standard curve analysis are also required in order to comparatively measure the analyte's concentration.

Qualitative GC analysis is made when the aim is to discover the identity of the compounds in a sample. Therefore, a mass spectrometer (MS) or a Fourier transform infrared spectrometer (FTIR) can be used as detectors, as well as performing analysis of authentic standards of the targeted analytes. Key parameters for peak identification are the retention times (t_R), retention indices (RI) and the data/library spectrum comparison (MS or FTIR). Note that, RI values are especially useful (and often required) for essential oil analysis, and so are included here as a standard protocol. After proper chromatogram and spectrum treatment (i.e., integration, deconvolution, baseline subtraction and others as required), the identification process is performed according to the following steps:

- First, the retention times of the samples' components are obtained, as well as for the authentic standards or a mixture of homologous reference compounds. The retention time represents the time an analyte takes to elute through the system and reach the detector.
- Next, the retention indices are calculated. This step is only crucial if no authentic standards are available to confirm the compound's identity. The retention index is a measurement based on the comparison between the t_R of a certain analyte and the t_R of an homologous series of reference compounds (e.g., *n*-alkanes, methyl esters, alcohols, fatty acids). The advantages of this approach are the normalisation of the t_R across different GC systems, the accessibility of the reference chemicals required and the wide range of boiling points covered, but it also suffers variations according to the stationary phase and the separation program used. The most used and widely known retention indices systems were introduced by Kováts (1958; isothermal separations) and van den Dool and Kratz (1963; temperature

programed separations; Equation 1). The difference between the calculations of these two indices is regarding the logarithmic scale used for the adjusted retention times, which are used only by Kováts [1].

$$RI = 100n + 100\left(\frac{t_{R(x)} - t_{R(Cn)}}{t_{R(Cn+1)} - t_{R(Cn)}}\right) \quad \textbf{(Equation 1)}$$

- If an authentic standard was analysed, then its t_R and mass spectrum should match the analyte's result. If the standard is not available, the RIs and mass spectra obtained are compared with the reference library. The library search generates a list of possible compounds and their match factors and probability, which represent how closely the queried compound and the library spectra match. An excellent library match factor and probability are ideally above 900 and 90%, respectively and the experimental RI should be no more than ±20 units from the reference value. However, even when having good library match scores, the analyst should pay close attention to the mass spectrum similarity and investigate prior published works in order to increase the probability of a correct identification. Thus, match quality alone does not guarantee correct structure assignment.

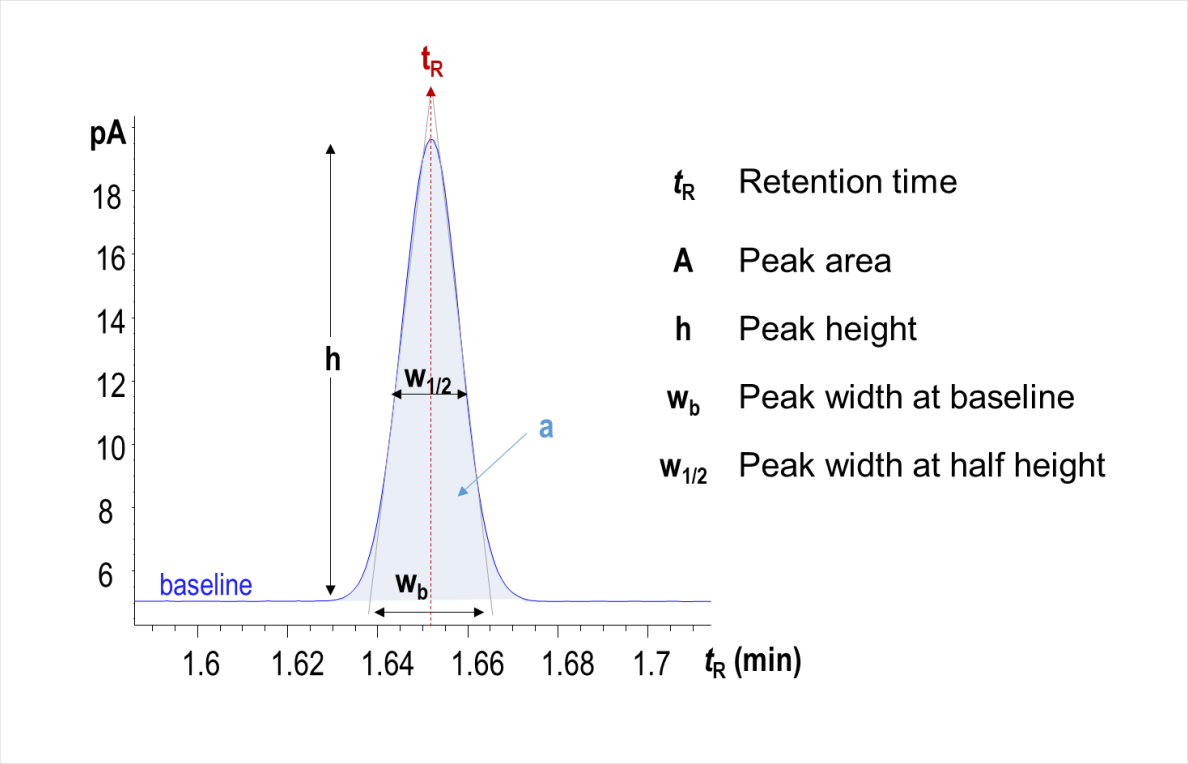

Figure 5. Diagram of the key measurements taken from a chromatographic peak.

The presence of unresolved (overlapping) peaks can make the identification task very difficult, to the point that the identity confirmation is not straightforward even when having an authentic standard. In these cases, deconvolution procedures, MS/MS analysis and multidimensional separation techniques may be required to improve the identification accuracy.

1.1.3. Multidimensional and comprehensive two-dimensional GC

Since its introduction between the 1940s and 1950s, GC has been in constant development (Fig. 6), with the evolution of equipment (e.g., portable and hyphenated systems), accessories (e.g., injection and detection devices, cryogenic traps, switching valves), columns (e.g., packed columns, then capillary and ultra-narrow bore) and stationary phases (e.g., ionic liquid and cyclodextrin phases) [2, 12].

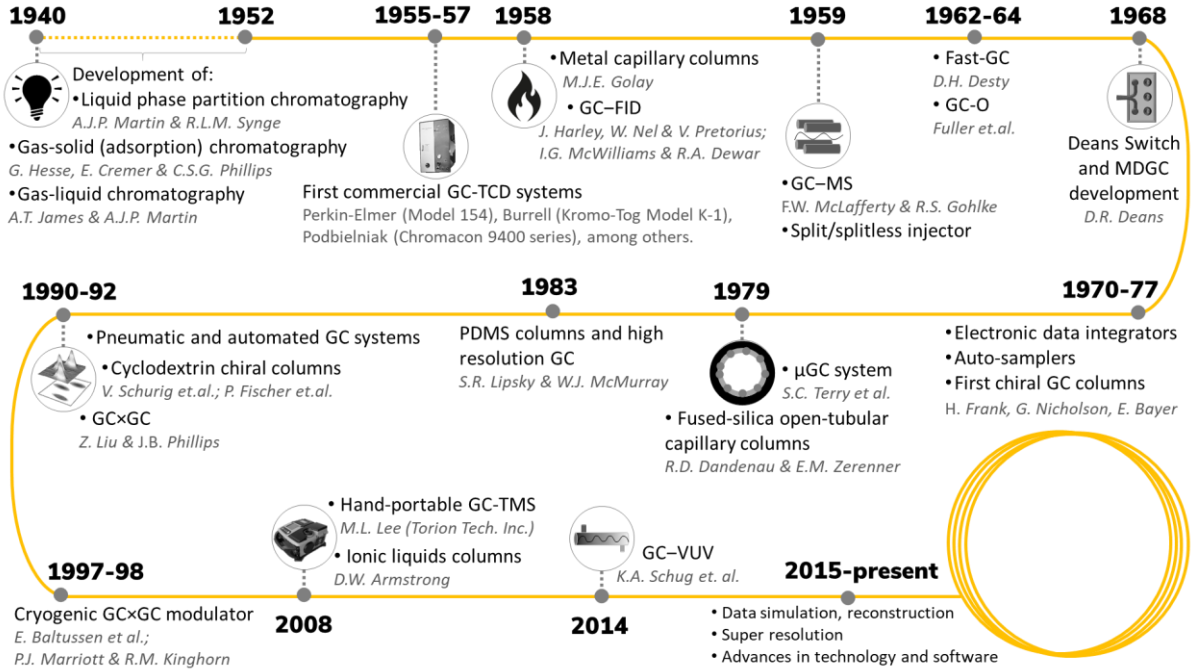

Figure 6. Highlights of the GC history timeline.

Multidimensional (MDGC) and comprehensive two-dimensional gas chromatography (GC×GC) are hyphenated advanced techniques, consisting of two or more consecutive and coupled separation stages that are performed using columns of different polarities and selectivities providing much greater 'peak capacity'. The main objective of these techniques is to improve resolution of target analytes in complex sample analyses. However, they can also offer faster well-resolved results for less complex samples, especially when there is an important region to be better resolved; thus, saving time, resources and energy in some cases [2, 13].

While MDGC is composed of two (or more) separation steps that progressively 'deconvolute' target analytes or a fraction of the primary column chromatographic elution, GC×GC is a two-dimensional modulated untargeted separation method, which is used to obtain improved resolution of the whole sample. The separation columns are referred to as dimensions—first column or dimension ^{1}D and second column ^{2}D—which highlights that they

operate as independent elution stages. Both techniques use substantively different stationary phases in each dimension, in order to obtain improved resolution by means of changing the separation mechanism (orthogonality). Whilst the term 'orthogonal configuration' has been applied to such column selectivities, true orthogonality cannot be obtained with GC separations, since the 'boiling point' parameter is a common 'partial' mechanism to all GC separations. Although multidimensional GC separations have been applied since the early 1960s and the comprehensive technique was introduced in the early 1990s, the number of publications in this field was boosted in the past 20 years by the continuous technological advances in instrumentation, software and data processing [2, 14, 15].

The general advantages of GC over other techniques are well-recognised and include: application to a wide range of samples, low sample volume required, non-destructive separation, capillary column reusability and diversity of stationary phases, great analysis speed and resolution, less preparation and conditioning steps of the instrument before the analysis, improved software capabilities. In addition, advanced GC separation techniques, such as MDGC and GC×GC, have extra features, which are compared on Table 2.

Table 2. Multidimensional (MDGC) and comprehensive two-dimensional gas chromatography (GC×GC) general features and counterpoints. Reproduced from our review paper ref. [16]. Copyright 2019 Creative Commons Attribution (CC BY) licensed by MDPI.

Technique	Features	Pros	Cons
MDGC	- Target analysis (heart-cut; H/C) - Target peaks/regions are transferred according to retention times in ^{1}D, and analysed in ^{2}D - ^{2}D column usually has similar dimensions to the ^{1}D - A cryotrap can be used to focus the transferred analyte peaks/regions	- Allows enrichment of target analytes or fractions through multiple injections - Provides increased resolution on the ^{2}D column and can improve detection specificity and sensitivity - Stereoisomer resolution uses a chiral ^{2}D column and improves resolution from interfering peaks	- Requires a cryogenic trap to reduce ^{1}D dispersion - Requires a switching valve for on-line H/C programing - Not designed to resolve the full sample; - Requires extra GC program to elute the ^{2}D column, unless on-the-fly operation is used
GC×GC	- Non-target analysis; applied to all sample compounds - Modulator sub-samples peaks as small slices to ^{2}D, giving a 2D plot - ^{2}D separates overlapped ^{1}D peaks - ^{2}D column is shorter, to separate transferred compounds before the next modulation	- Full 2D sample resolution can be achieved - Stereoisomer resolution uses a ^{1}D chiral column - The 2D image generated provides excellent profiling / differentiation of samples. - Cryogenic modulation leads to response increase	- Requires a modulator; some can be costly - Method set-up can be more complex - Software and interpretation can be more convoluted - Personnel need special training

Different design configurations are continually being explored for multidimensional GC techniques, using extended or integrated MDGC and GC×GC systems (Fig. 7), coupled with different detectors, to address the complexity of the samples and accomplish greater separation. The overriding aim is to expand the separation capacity of the system.

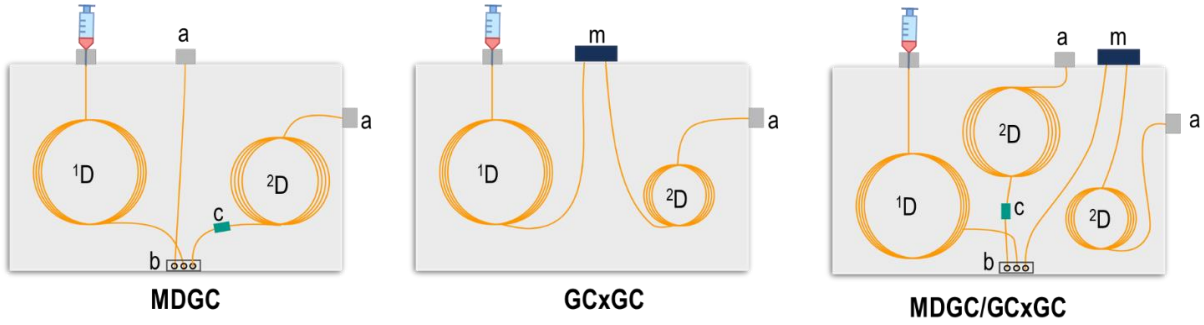

Figure 7. Simplified schematic examples of different multidimensional systems: MDGC, GC×GC and integrated MDGC/GC×GC. Dean switches (b) or modulators (m) connect the first (^{1}D) and second dimension columns (^{2}D). The ^{2}D columns are connected to the detectors (a) and cryogenic traps (c). Adapted from our review paper ref. [16]. Copyright 2019 Creative Commons Attribution (CC BY) licensed by MDPI.

MDGC (Fig. 8) does this by taking heart-cuts of specific regions on a first column and passes them to a conventional length ^{2}D column for greater separation, which is of particular value in enantioselective analysis where the ^{2}D column comprises the enantioselective phase. A cryogenic trap assists in reducing the dispersion of compounds that naturally occurs on the ^{1}D column, by applying low temperature to provide a refocusing step (re-condensation of the analytes) at the start of the ^{2}D column.

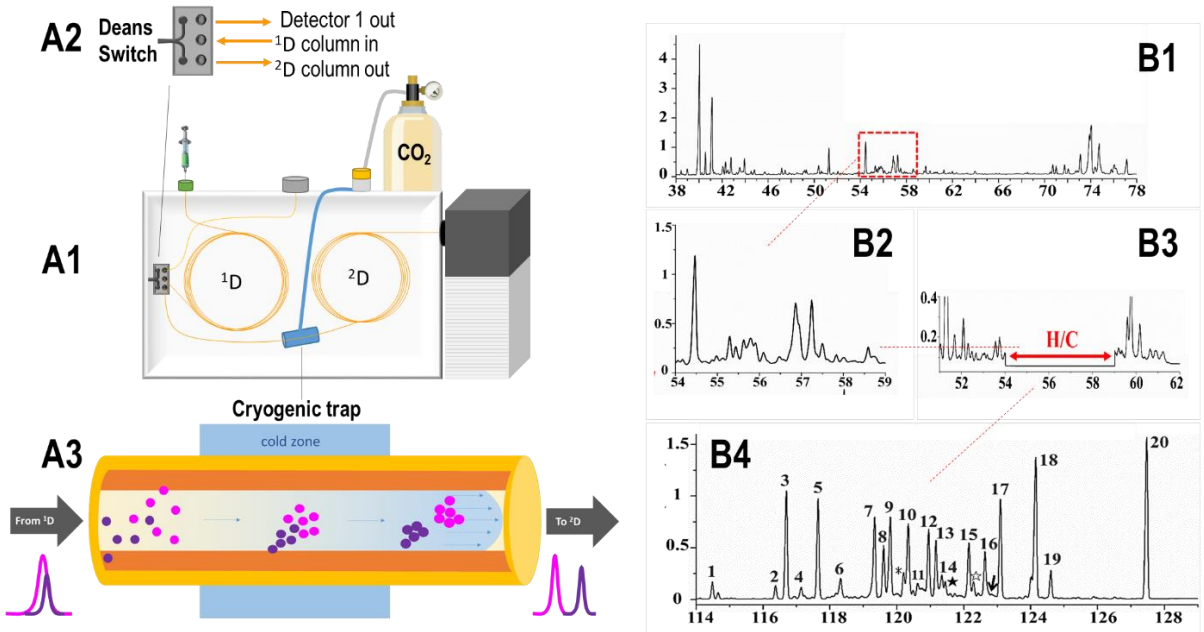

Figure 8. Diagram of the MDGC instrumentation (A1), with schematic representation of the Deans Switch (A2) and cryogenic trapping process (A3), and chromatograms illustrating the steps of the heart-

cutting (H/C) technique (B1-B4). All chromatograms in this figure (part B) were adapted with permission from ref. [17] Copyright 2018 American Chemical Society.

By contrast, GC×GC (Fig. 9) uses a modulator device in conjunction with a short ^{2}D column to rapidly and sequentially transfer solute between the two columns, according to the modulation period, to produce a significantly increased overall capacity for the total sample. Presentation is conveniently expressed as a 2D plot, with axes x and y representing the first and second dimension retention times ($^{1}t_{R}$ and $^{2}t_{R}$), respectively, and individual compounds located throughout the plot according to their chemical/physical properties and interaction with the stationary phases. A further option of hybrid nature (MDGC/GC×GC) allows operations such as select target zones, improve their separation, and subject them to GC×GC.

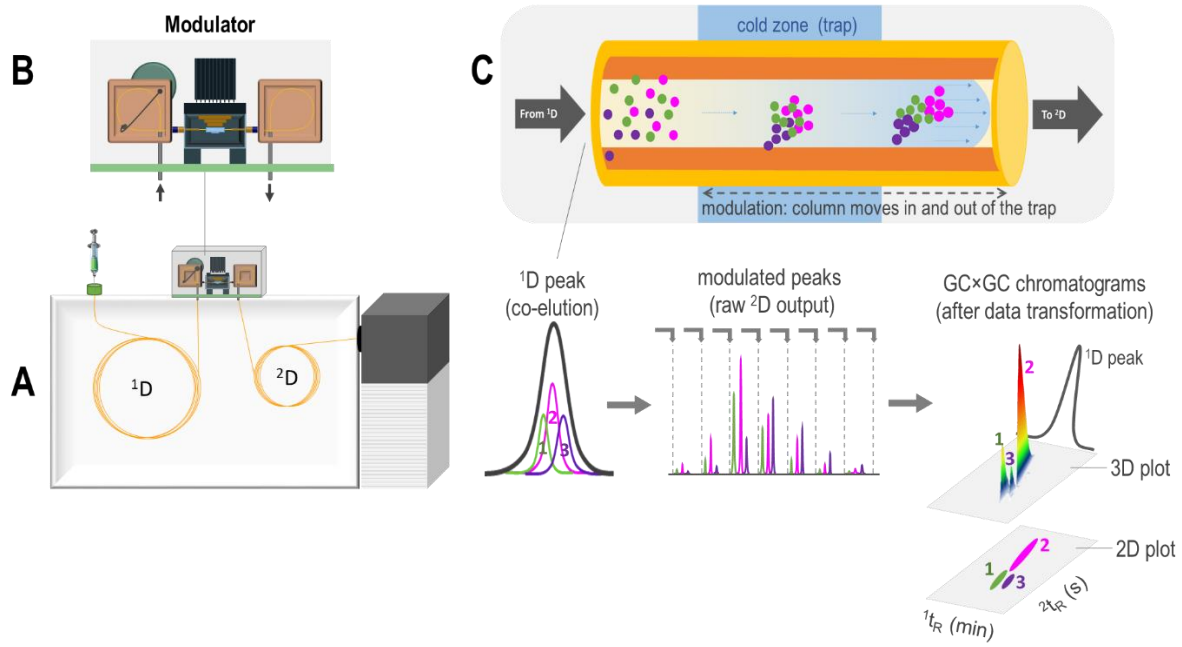

Figure 9. Diagram of the GC×GC instrumentation (A) with a solid state modulator (B), and simulated ^{1}D and ^{2}D chromatographic outputs for three co-eluting peaks (in green, pink and purple), including a schematic representation of the cold trapping process (C).

The analyst may rely on different chromatographic systems and detectors to achieve improved sample characterisation. This is usually performed by matching the chromatographic information (e.g., retention times, retention indices) of sample analytes and authentic standards with the response of different detectors. The detectors can be general/universal (e.g., flame ionisation detection—FID, mass spectrometry—MS, Fourier transform infrared spectroscopy —FTIR, vacuum ultra-violet spectrophotometry —VUV), element-selective (e.g., flame photometric detection—FPD, nitrogen-phosphorus detection—NPD, electron capture detection—ECD) or sensorial (e.g., olfactory port—O, electronic nose—e-nose, electronic tongue—e-tongue) [18]. Important detectors for determination of aroma-active compounds are the mass spectrometer and the olfactory port, which provide a measure of chemical and

sensorial identification, respectively. Other spectroscopic detection techniques, such as Fourier transform infrared spectroscopy (FTIR), provides additional specificity and unique spectra for isomers with similar fragmentation patterns on the mass spectrometer [19]. The continuous development and commercial introduction of new or improved stationary phases, such as ionic liquids and derivatised cyclodextrins, may address new mechanisms of separation, but in complex samples, they may do little to expand the separation capacity in 1D GC. Thus, the problem of incomplete separation of components remains, and for the MS and 'O' detectors, generally the complete resolution provides best information of chemical properties or identification. For instance, the correct description of two different aroma compounds is highly compromised by coelution. The importance and role of GC and various advanced techniques applied to F&F analysis will be expanded in the next section.

1.2. Flavours & Fragrances

1.2.1. Definitions, history and importance

Flavour is a multisensory experience, involving taste (gustation), smell (orthonasal and retronasal olfaction), and trigeminal reception (e.g., cooling, burning, spiciness, fizziness, stinging, tingling, pungency, and astringency), but it is also influenced by texture and other sensorial stimuli. Fragrance, however, usually refers to a pleasant sensation arising mostly from orthonasal and trigeminal nerve stimulation [20, 21].

Olfaction is a chemical sense and a primitive way of communication between living species on Earth [22]. The use of fragrance materials is an ancient heritage and started to be deeply explored by the great civilisations in Egypt, Mesopotamia, China and Indus, between 4000 and 2000 B.C. From a religious practice to a luxury hedonic product, it was only after the Industrial Revolution, especially in the 20th century, that these products became popular and were used in various consumer goods applications [23, 24]. Various trends have emerged in F&F between the years of 1950 and 2000, and they evolved from simple extracts to products with integrated technology, functionality and health aspects [25]. Nowadays, F&F is a world market valued at more than US$ 20 billion, with an annual growth rate between 3-5%. Swiss companies Givaudan and Firmenich hold the largest share of the sector, accounting for 19.5 and 13.9% in 2017, respectively.

Smell and taste are important quality markers that boost the consumers' choice of food, drinks and beverages, fragrances, cosmetics and other products in various industrial segments. Thus, this market demands constant innovation, high volume production, high quality, reproducible formulation, and safety, which can be difficult to achieve in an environmentally friendly way, even for naturally sourced products. Although more than 3000

fragrance ingredients are available, there is still a need for new compounds to extend the perfumer's palette, due to problems of stability, costs, formulation compatibility and performance, plus the production of new fragrance / flavour formats [26]. The production volumes can vary from 0.5 up to 15000 tonnes per annum, depending of the potency, source or purpose of the odorants. Actually, for some products like menthol, natural farming sources have higher carbon footprints than related synthetic routes, although a 'natural' fragrance has important connotations for market acceptance. Thus, the greatest challenge for the fragrance chemists is to design new products that can be sustainably provided in the required scale and at a competitive cost. Therefore, they generally take advantage of interesting feedstocks, intermediates or by-products and technology to achieve this goal [22, 27].

1.2.2. Essential Oils

Essential oils (EO) are among the most important ingredients to create F&F products, having also a remarkable economic value and applications to food, perfumes, cosmetics and pharmaceutics. They are complex mixtures of secondary metabolites biosynthesised by some living organisms, especially plants, where they can be found in oil cells, secretory ducts or cavities, or in glandular hairs in various parts, such as leaves, flowers, buds, fruits, seeds, rhizomes, trunk and bark. Some mosses, liverworts, seaweeds, sponges, fungi and microorganisms, insects and terrestrial and marine animals can also produce EO or volatile aroma compounds. The occurrence and function of EO in nature is usually related to defence, signalling and attraction/reproduction [28, 29].

These natural oils are commonly extracted by distillation or expression techniques and are usually a liquid, with low solubility in water and soluble in the various organic solvents. However, extracts obtained by solvents, fluidised gases or other techniques (e.g., concretes, absolutes, oleoresins) are not classified as EO. Their chemical composition generally include (mono- and sesqui-) terpenoids and phenylpropanoids, but can also contain diterpenes, fatty acids and their esters, or decomposition products of these compounds. In some cases, these molecules can be bound with carbohydrates in the form of glycosides [28, 29]. The oxygenated monoterpenoids are a highlighted class, as they are frequently among the character / impact odorants (i.e., compounds whose smell is very potent or instantly associated with the sample) and, therefore, they have a remarkable contribution to the aroma of the oils. When the sesqui- and higher terpenoids have odours, they may be used as a good base or fixative fragrance ingredient, as their lower volatility (among other attributes) increases their tenacity or persistence. Different factors can influence the chemical profile of these oils, such as species, chemotype, plant origin and age, environmental conditions, extraction method and processing (e.g., plant drying, oil deterpenation).

EO are often used in products that interact directly with the human body and, due to its diverse composition, they can have several interesting biological activities, beyond their odour and flavour properties, such as antioxidant, antibacterial, antifungal, anticancer, insecticidal activities, and as a repellent. For instance, tea tree oil is widely known for its antiseptic and curative properties. Furthermore, some studies are also concentrated in the psychological benefits of the aromas. The so-called aromatherapy and aromachology, are areas that are gaining prominence in aroma science and are directly connected to human behaviour and health. Indeed, some EO such as lavender, are effectively used to aid the treatment of mental disorders like anxiety [30].

Among several EO of commercial importance, some crops, such as orange, lemon, lime, grapefruit, cornmint, peppermint, spearmint, camphor, eucalyptus, cedar, pine, anise, clove, cinnamon, coriander, rosemary, lavender, patchouli, citronella, tea tree and others, are produced in hundreds or thousands of tonnes per year and account for 80% of the world market, while the remaining 20% comprises over 150 crops. The major EO producers are found in developing or emerging economies, such as Brazil, China, Egypt, India, Mexico, Guatemala and Indonesia, while the major consumers are the industrialised countries, such as USA, Western Europe and Japan. The large volumes of EO produced and the limited number of species traded worldwide shows the economic potential of EO plants as new crops [22, 31-33]. Among other high-valued fragrance ingredients, it is quite common to have floral and woody scents, such as rose, jasmine, orris, oud (agarwood) and sandalwood, not to mention the animal-derived odorants, such as musk, ambergris, castoreum and civet, which nowadays are obtained by synthetic processes [22, 27].

Some drawbacks of EO production chain for applications as F&F, refer to the difficulty of cultivation and harvesting of some plants (which sometimes endangers rare species, such as Agarwood [34]), low yield of EO, variations in the chemical composition (quality) of each EO per season, degradation during processing or storage, etc. For example, to produce 1 kg of jasmine oil it is necessary to extract about 7 million flowers, which have to be manually harvested in the early morning, when it is at the peak of EO production [22, 30].

Moreover, toxic, allergenic or undesirable substances of EO composition may hinder their application in products for human use, or became a pollutant in the environment, such as atranol (allergenic), eugenol (allergenic), estragole (hepatotoxic), methyl eugenol, safrole and isosafrole (carcinogenic), musk ambrette (neurotoxicity, photosensitisation, bioaccumulative pollutant) [35]. The presence of allergenic compounds requires all commercial products to be certified to contain less than certain concentrations of these compounds, and this depends on whether the product use is as a leave-on (e.g., perfume), or wash-off (e.g., soap) product. For these reasons, the International Fragrance Association established it is mandatory to keep the concentration of these substances below maximum allowed levels in the final product [35].

Companies may apply methods such as successive fractionation and distillation techniques, laser photolysis, molecular trapping, among others in the post-treatment of EO to remove these unwanted products, however, these processes involve the use of high amounts of solvents, consume time and energy, and often alter the quality of the product due to lack of selectivity. Chemical derivatisation is also unsuitable, due to the complexity of these oils and the lack of selectivity of the process. Thus, enzymatic processing represents an interesting alternative to remove such undesired compounds [30].

1.2.3. Chemical structure and odour activity of F&F

The naturally-derived materials used as F&F are mostly secondary metabolites obtained by biosynthetic processes in plants, although some can be produced by degradative processes of primary metabolites (i.e., proteins, carbohydrates, lipids and nucleic acids). It all starts with the glucose molecule produced during the photosynthesis, which is bioconverted to other metabolites such as acetyl coenzyme-A and shikimic acid, triggering other specific metabolic pathways that will end up producing the key classes of aroma compounds, namely: terpenoids, phenolic compounds, polyketides and alkaloids [22].

High-impact aroma compounds are potent odorants, i.e., of low odour threshold (≤10 ppb), and desirably pleasant characteristics that are instantly associated with a given sample. Other important features of these F&F are: substantivity, stability, safety and commercial applicability [36]. Various studies tried to decode the physical-chemical mechanisms of receptor-odour binding process behind the olfaction, establishing some empirical rules to quantitatively determine the structure-activity relationship (SAR or QSAR) or the physical-activity relationship (PAR). These theories can be based on different chemical or physical descriptors, such as molecular weight, number of atoms, polarity, 3D shape, steric properties, electronic effects, IR vibration patterns, solubility, boiling point, vapour pressure and many others. The structural similarities between organic compounds can be used to predict some of the properties of new synthetic molecules, such as chemical reactivity, physical properties or biological activity. This useful tool is widely used in the design of novel products, such as pharmacological agents, herbicides, pesticides, as well as in the investigation of toxicity and environmental risk of several chemicals. Meanwhile, some of the physical correlations are used in the perfumery industry to predict the substantivity and retention of F&F ingredients, which is the duration a compound remains bound to surfaces such as hair, skin or cloth [26, 37].

In the F&F field, however, the SAR methods are very difficult to apply. Some of the major reasons are: (1) Compounds with similar structures can have completely different odours (e.g., carvacrol enantiomers and thymol), as well as structurally different molecules can have similar smells (e.g., the musk-like odorants Helvetolide®, Muscone and Tonalid®). In addition,

any small change in the structure, such as the addition of a methyl group, can cause complete loss or change of the smell. (2) The difficulty associated with odour measurement, which is subjected to many factors, such as purity, concentration, solvent medium, temperature, humidity, and the analyst's perception and expertise [21, 26, 36]. The "touch" component of the smell sense, which is mostly related to the trigeminal nerve stimulation and can be perceived even by anosmic people, is also very impactful to the odour recognition, especially for small molecules. Some examples of chemicals with strong trigeminal responses are: *L*-menthol (cooling), acetic acid, ethanol, 1,8-cineole, acetone, propanol. In this context, the SAR approach can be usefully applied to larger molecules with complex structures, since they seem to have lower trigeminal distribution. [21].

The three most important SAR techniques to F&F are pattern recognition, Hansch analysis and the olfactophore approach. Pattern recognition deals with the problem of dividing compounds into two or more classes and, therefore, is suitable to large qualitative datasets of structurally diverse compounds. Thus, the generation and use of descriptor systems is widely adopted by many companies and is also the method behind the electronic noses and tongues technology, which use neural networks to match the aroma's identity and sensorial information. A GC–O analysis of original and pure standards, performed in controlled conditions, by an expert panel and using standard odour descriptors lexicon is recommended to avoid discrepancies and obtain precise and reproducible odour data. The Hansch method is widely used when quantitative data are available and suggests that the biological activity of a molecule is a function of its electronic, steric and hydrophobic properties, the latter most often being represented by the partition coefficient between water and octanol. The olfactophore approach assumes that the similarity in the smell of a group of active analogue molecules is based on a common 3D conformation in which key atoms or functional groups are placed at certain relative distances from each other. This model is best suited to qualitative sets of data and for compounds with a limited amount of conformational flexibility. Although all these SAR models are helpful, there is always a degree of uncertainty in directly correlating a chemical structure to a sensorial attribute [21, 26, 36].

1.2.4. GC importance and applications to F&F analysis

Among countless applications of GC and its advanced techniques, F&F analysis is one of the topics most explored in the literature [2]. Historically, GC has provided crucial information to perfumers and flavourists to accurately identify and reconstitute key natural aroma compounds at a lower cost. The use of headspace extraction techniques (e.g., SPME), GC–MS and GC–O has also enabled the *in-vivo* analysis of high-impact odorants emitted by plants, which gives a more true representation of the aroma [36, 38]. The study of F&F and their natural products matrices is usually carried out to determine: (1) the qualitative/quantitative

composition, (2) the authenticity/adulteration/contamination, (3) processing effects, (4) stability, and (5) the product compliance with quality and safety parameters, which are indispensable, since they are present in a wide range of products destined for human use and, therefore, must be free of, or have very low concentration of allergens, pesticides, additives and preservatives. Moreover, GC enables the understanding of the different degrees of complexity of EO, which can vary according to the species, chemotype, geographical origin, cultivation and processing (e.g., plant drying, extraction method, oil deterpenation), as well as different F&F formulations comprising natural and synthetic ingredients in various concentrations [16].

Besides the complexity, the common presence of unsaturated, cyclic and oxygenated compounds with similar chemical structures in EO (and therefore, close retention times), as well as stereoisomers and on-column isomerisation (e.g., tautomerisation, interconversion) are other factors that can complicate the analysis [2, 39, 40]. The molecules' chirality is a very important aspect in aroma science, since different enantiomers of a given compound can have different sensorial and biological activities. For example, (*R*)-(-)-carvone smells like spearmint, while (*S*)-(+)-carvone smells like caraway. Thus, enantioselective separations with mass spectrometry and olfactometry detection are crucial to an accurate F&F analysis. However, the olfactory perception of a single aroma compound can also be affected by other co-eluting odour-active compounds. Therefore, MDGC and GC×GC are frequently required to achieve complete resolution of these samples. The diverse range of component polarities in EO, allows the use of either a less polar ^{1}D/more polar ^{2}D column set or the opposite configuration for GC×GC and MDGC analysis. For enantioselective separations, however, the chiral column should be the first dimension of an *e*GC×GC method and the second dimension for *e*MDGC in order to achieve proper resolution of the components [16].

Many studies have been focusing on the application of GC×GC and MDGC methods to analyse EO and F&F compounds [13, 39, 41-53]. Vetiver essential oil and vetiveryl acetate, an improved odorant product derived from the acetylation of vetiver essential oil alcohols, were targeted by different authors, which used GC×GC analysis to obtain the qualitative and quantitative profile of these samples. The technique has enabled the identification of hundreds of compounds in these samples, and the generated datasets can be used to assess the impact of the manufacturing process on essential oil composition and odour properties [41-43].

Filippi et al. [41] conducted a qualitative and quantitative study of vetiver essential oil components, using a GC×GC–FID/MS system. The method allowed the identification of 135 compounds in four different samples by dosing in internal calibration standards, as well as observation of differences in composition of each sample related to their origins and ages. Tissandié et al. [42] studied the composition of different samples of a perfume ingredient called "vetiveryl acetate," an improved odorant product derived from vetiver essential oil processing.

The GC×GC–MS/FID analysis allowed identification of more than 200 compounds in the samples, generating a new dataset that can be used to assess the impact of the manufacturing process on essential oil composition and odour properties. Francesco et al. [43] used GC×GC to assess the composition of natural vetiver oil with transformed products—a vetiveryl acetate biocatalysed product, obtained from a lipase-catalysed acetylation of vetiver essential oil. The esterification process was highly chemoselective towards primary sesquiterpene alcohols, and had more sustainable processing features (i.e., mild reaction conditions, reusable catalyst, etc.). The use of the comprehensive technique was indispensable to deeply understand and compare the differences of the crude and the modified oils, since the matrix is highly complex and not suitable for discovery purposes when analysis is based on the poor resolution of a 1D GC method.

Fidelis et al. [44], studied the essential oil obtained from the leaves of rosewood (*Aniba rosaeodora* Ducke), from plants of different ages, as a potential, more sustainable, alternative to extraction from the chipped wood. The chemical profile of the leaf oil samples from plants of different ages was similar and was examined by GC×GC, enabling a three-fold increase in the number of compounds reported than a conventional separation. The characterisation of the chemical and odour profile of guaiacwood oil, was obtained by Tissandié et al. [46] from the heartwood of *Bulnesia sarmientoi*. GC×GC–FID/MS was applied for identification and quantification of the substances, which was mainly composed of sesquiterpene hydrocarbons and oxides, alcohols, phenols, ethers, aldehydes and ketones. The main odorants were identified by GC–O, highlighting the compounds bulnesol (rosy) and β-ionone (fruity), which had the most odour impact contribution. Typical notes of leather, clove and vanilla were also detected and related to phenolic compounds.

Flower stereoisomer odorants, linalool and lilac aldehydes/alcohols, from 15 different plant species were assessed by Dötterl et al. [45], using *e*MDGC separations and electrophysiological tests in a noctuid moth. Eight lilac aldehydes and their alcohol isomers were identified, and found to induce the insect response. The *S*-configured isomers were predominant in almost all the plant species studied. The technique was indispensable as a route to identify and collect the individual stereoisomers. He et al. [52] used *e*MDGC–MS to evaluate the stability of chiral flavour compounds in lemon-flavoured hard tea beverages over 8 weeks of storage. The heart-cut analysis showed that (*R*)-(-)-linalool, (*S*)-(-)-α-terpineol and (*S*)-(+)-4-terpineol were the predominant enantiomers in the samples at the beginning; however, the opposite antipode was observed after the storage. Only (*R*)-(+)-limonene was not affected by the storage conditions. In recent years, enantioselective analysis has also been combined with other techniques, such as combustion/pyrolysis GC or stable isotope ratio mass spectrometry (IRMS) to determine the original isotopic abundance of key components in natural products samples and compare it with commercial products in order to track the

geographical origin or assess the authenticity, as performed by Sciarrone et al. [53]. The authors monitored the key-aroma compound bis(methylthio)-methane to assess the authenticity of truffle (*Tuber magnatum* Pico) and related products, one of the most expensive food flavouring products. It is especially important that quantitative separation is achieved when using IRMS, otherwise the appropriate isotopic ratios will be in error.

Marriott and co-workers have contributed a number of technical solutions and separation strategies to achieve better resolution of aroma compounds [13, 17, 34, 39, 49-51, 54, 55]. The successful implementation of many hybrid GC approaches in a single instrument represents a convenient qualitative and quantitative solution to overcome uncertainty of data correlation across multiple hyphenated systems. The use of cryogenic trapping and modulators allows the focusing and/or concentration of the volatile analytes in various modes of operation, which improves separation peak capacity and enhance the quality of analyte characterisation.

Among the applications explored by the group, an integrated 3D GC-GC×GC system was used to improve the resolution of oxygenated sesquiterpenes of Agarwood (*Aquilaria malaccensis*). In the first multidimensional step a 4 min heart-cut was taken using a Deans switch and cryogenic trap device that gives improved separation, with the number of detected compounds increased by approximately two-fold compared with 1D GC. However, overlapping peaks were still observed and a subsequent separation was necessary. The application of a cryogenic modulation GC×GC step further extended the peak capacity of the heart-cut region by three-fold or more [17]. In another work, Dunn et al. [13, 56] used GC×GC and MDGC to analyse suspected allergens in fragrance products. They found that different column set approaches can be complementary, providing different separations in 2D space. The authors also highlighted the importance of cryogenic focusing to improve peak shapes and separation in the ^{2}D column by reducing the effect of peak dispersion (broadening) on the ^{1}D column. In another study, the authors applied principles of the comprehensive GC×GC technique to develop a rapid repetitive modulation MDGC method to analyse sequential heart-cuts of peppermint essential oil volatiles. Using a relatively short (~ 6 m long), narrow bore (0.1 mm ID) ^{2}D column and a cryogenic modulation system, heart-cuts of 60 s were sampled and delivered to the ^{2}D column to greatly expand separation with each heart-cut analysis shown from the time of delivery to the ^{2}D column; the authors obtained 2D separations in 30 s and with up to a 40-fold increase in signal response.

With the introduction and diffusing of biotechnology processes to F&F, new challenges on methodology and applications of GC arise. Whether this is due to the extra layer of complexity added by the biological matrices/reagents, the need for identification and quantification of biomarkers, metabolites and target analytes, or the need for better understanding of reaction mechanisms and selectivity, GC and its advanced techniques can, precisely and reliably, provide further information about the studied subject, usually with

relatively fewer sample preparation steps [34, 57]. Some of these challenges will be addressed in the next chapters of the present study, with the development of GC and GC×GC methods for a more comprehensive understanding of the reaction process and potential applications to the F&F industry and other areas.

1.2.5. Enzymes and F&F biotechnology

From more than 25000 enzymes existing in nature, about 400 have been applied to stereoselective organic synthesis and F&F production, with the main classes being hydrolases, transferases, oxidoreductases and lyases. Microorganisms are the main source of enzymes for this application, which can be extracted and expressed in recombinant species [58].

Biotechnological processes, such as biocatalysis and fermentation, have been applied in the development of new molecules, transformation of raw materials and are an excellent alternative to obtain commercial amounts of the high-valued compounds that are present in trace amounts in natural sources [59, 60]. The chemical synthesis can sometimes be cheaper and quicker, but they are usually less selective and have a negative impact on the environment, due to the greater number of steps of the reaction and purification and the presence of hazardous reagents and by-products. For instance, the enzymatic reaction of lipases with vetiver alcohol fraction has exhibited a high chemoselectivity towards the primary sesquiterpene alcohols, whereas in the chemical modification, the total conversion of the secondary alcohols into acetyl esters was observed and few tertiary alcohols were also acetylated [43]. However, the most common chemical esterification process relies on the use of carboxylic anhydrides and other additives or catalysts, which are used in some excess and must be distilled at the end.

The stereoselectivity of these reactions is of great advantage to obtain desired chiral compounds, which ideally could enrich a product with the compounds with the most desirable properties. For instance, (2*S*,4*R*)-(-)-cis-rose oxide is the stereoisomer with the lowest odour threshold and exhibits more biodegradability [61, 62], so if more of this potent odorant is preferentially synthesised by an enzyme, fewer steps of purification will be necessary and the final product will be more environmentally-friendly and require less amount of the chemical in the formulation to achieve the same sensorial impact of the mixture of isomers. Accordingly, the modification of target molecules present in the EO can improve their odour, stability, safety, biological activity and biodegradability, enabling a safer use in products for human use and increasing their commercial value [30].

Among the most recent and successful applications of biotechnology to F&F synthesis in industry, the biocatalysis processes involving purified enzymes, such as lipases and laccases, or fermentation using modified *E. coli* that express different enzymes are highlighted. Symrise (2002), used a lipase-catalysed enantioselective hydrolysis of a mixture of menthyl

esters to obtain pure *L*-menthol (*ee*>>99%), which is the enantiomer with stronger cooling effect [27, 58]. Givaudan researchers (2012) obtained a new class of 1-hydroxy-octahydroazulene derivatives with a strong and pleasant woody-floral odour by the application of a laccase-mediated system for the oxidation of sesquiterpenes in an olefinic fraction derived from patchouli [59, 63]. Givaudan also uses microalgae and fermentation processes from plant materials, such as sugar cane, to produce other cosmetic ingredients and aromas, such as BisaboLife® (2017), Ambrofix® (2019) and Sensityl® (2019). Ambrofix® is obtained from the diastero- and enantioselective conversion of β-farnesene, using squalene-hopene cyclases extracted from *Alicyclobacillus acidocaldarius* and expressed in *Escherichia coli* [64]. These bioprocesses require hundreds of times less land to produce a kilo of the product. Firmenich uses a similar fermentation processes to obtain the products Clearwood® (2014) and Dreamwood® (2020), which are biodegradable aromas related to patchouli and sandalwood, respectively. Furthermore, Firmenich researchers, headed by Dr. Laurent Daviet, reproduced in the laboratory the biosynthetic pathway of sclareol, a diterpene alcohol produced by clary sage (*Salvia sclarea*) and used as a precursor to obtain Ambrox®, which was first discovered in the 1950s and started to be bioproduced in 2016. The genetic engineering study resulted in the isolation, functional characterisation and cloning of specific diterpene synthase enzymes from clary sage DNA and introduction of the genes in *E. Coli.* The bacteria was then able to reproduce the plant's enzymatic reactions to produce sclareol in larger amounts in a cost-effective and more sustainable process [65]. The perfumery industry has adopted synthetic ambroxides as a replacement for ambergris, which is a highly priced and valued ingredient with a musky, woody, sweet and earthy odour and fixative properties that was originally obtained from a waxy excretion produced by sperm whales, now an endangered species. Alternative sources were deemed both necessary and acceptable and, thus, plant labdane diterpenoids, like sclareol, are now the major precursors for ambroxide synthesis in industry [66].

The lipase-catalysed large-scale synthesis of (*Z*)-3-hexenyl acetate, with (*Z*)-3-hexenol and acetic acid in hexane was described in the literature. This ester has a fruity odour and shows a significant green note flavour. Lipases are key industrially applied biocatalysts for the stereoselective production of esters and lactones, kinetic resolutions of racemic alcohols and hydrolysis of fatty acids and derivatives. Esters are usually related to fruity notes, especially the low molecular weight compounds, and due to their lower polarity and absence of hydroxyl groups, alkyl esters are usually more volatile (which can favour the odour detection threshold) and less sensitising than their corresponding alcohols when in contact with human skin [58, 67]. As discussed above, the enantioselectivity is important in F&F, since chiral molecules often have different odour perceptions (see Section 1.2.4). Meanwhile, laccases are used in the synthesis of the key grapefruit flavour (nootkatone), to remove undesirable phenolic

compounds that can impact upon the sensory properties of products like wine, beer and fruit juices, and in the repurposing of waste products, such as those containing limonene, pinene and other terpene hydrocarbons, which are discarded in large quantities every year due to their low commercial and aroma value and chemical instability, while many of their oxyfunctionalised metabolites have higher aroma impact and value [58, 68-71]. Further information about these enzymes and biocatalytic reactions will be covered in the next section.

1.3. Biocatalysis

1.3.1. From green chemistry to white biotechnology

Green chemistry can be understood as a set of practices and principles adopted by academia and industries to eliminate the use and generation of hazardous substances and to reduce their release to or impact on the environment and human health during the study, development and application of chemical products and processes [72, 73].

The overview of the history and development of green chemistry can be found in review publications [72-74]. According to this historical record, the idea was introduced in the United States and Europe in the second half of the 20th century, through the work of many scientists and environmentalists such as Rachel Carson, whose book “Silent Spring” (1962) arose awareness about the impact of the massive use of pesticides. In response, governments created environmental protection agencies and more strict laws, forcing companies to amend their practices. By the end of the century (1998), Anastas and Warner introduced the “twelve principles of green chemistry”, a summarised guideline in the quest for the reduction of waste, material and energy use, risk, hazard and cost of chemical processes. Those principles were widely disseminated in order to guide research and innovations to reduce negative impacts from industrial activity.

Subsequently, a new set of 12 principles was published by Winterton (2001), focusing especially on the academic environment in order to raise awareness and direct efforts for the development of green processes that are viable at the industrial scale. The adoption of this second set of principles facilitates evaluation of the potential of chemical reactions studied in the laboratory so that their scale-up can be transposed safely, that good technical solutions are implemented, and without loss of their green characteristics [72-74].

The dissemination of these principles, coupled with the increased rigidity of environmental regulation, competitiveness, social conscience and the consumers’ responsible consumption, has led a large number of companies to invest more in environmentally-friendly technological innovations for their products and processes. The concepts were also adopted

by other fields, such as the agricultural and biotechnology sectors, which have taken benefit of the new green chemical processes and products to improve their practices [72-75]. In biotechnology, the differentiation of the main areas of application has earned its own colour code: white (industrial), green (agricultural), blue (marine and fresh-water), red (pharmaceutical), brown (desert biotechnology), purple (patents and inventions), and yellow (applied entomology) [76].

Biocatalysis is a key tool to white biotechnology and consists in the use of enzymes or whole cell microorganisms for the conversion of specific substrates into compounds of commercial interest. This practice has flourished as one of the most selective and efficient biosynthesis and bioremediation processes, representing a green alternative to traditional synthesis, since it has a lower energy cost, ideally lower content of toxic by-products, reduction or non-use of organic solvents, avoidance of heavy metal reagents, less waste, and with the possibility of reuse of the biocatalysts used. In addition, enzyme reactions are chemo-, regio- and/or stereo-selective and are conducted in mild conditions (such as low temperature and pressure). Finally, the products obtained through this process can be considered natural, if all the substrates and chemicals used are of a natural source. This is a very important aspect of great economic interest for industries, as consumers are increasingly interested in acquiring and using natural products, even though this attribute is not always synonymous with safety [59-62, 77, 78].

Despite the remarkable advantages and the various possibilities of application, at present, the potential of biocatalysis has not been fully exploited. There is still much to be discovered about the enzymes and the reactions they can catalyse. Some challenges related to the application of these processes are: the susceptibility to inhibition of the reaction by the substrate or product; reduction of the activity after immobilisation; reaction yield; the limitation in the operating conditions (e.g., temperature, pH, solubility); and the cost of some processes that is related to the type of enzymes, use of co-factors, co-enzymes, etc. With the advancement of biotechnology and genetic engineering, some of these difficulties are being overcome and an increasing number of enzymes with catalytic potential are being identified, improved and made available commercially, which contributes to the increase in the number of research studies for industrial application. Indeed, quite a number of companies currently adopt, and are increasing their investments in, biotechnology innovations. The main industry sectors that have been accessing these processes are pharmaceutical, food, flavour & fragrances, agrochemical, cosmetics and polymers [59, 60, 77].

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB), enzymes are divided into six major classes according to the types of reactions they catalyse: oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases. The classes of enzymes with the highest industrial application are

hydrolases and oxidoreductases, which may be related to the general accessibility and characteristics of enzymes that are favourable to the process (e.g., stability, efficiency, selectivity, etc.), as well as the commercial importance of the products obtained [59, 60]. Within these two main classes, the two types of enzymes highlighted for F&F applications, are lipases and laccases, which will be discussed in more detail in the next sections.

1.3.2. Lipases

Lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) are among the most popular hydrolases and are an industrially-established group of enzymes with multiple applications, since they can catalyse different reactions (e.g., esterification and transesterification, hydrolysis, epoxidation), accept a wide range of substrates (e.g., alcohols, esters, fatty acids, amines), and remain active in different media (e.g., aqueous, organic, ionic liquids, supercritical fluids, deep eutectic solvents) and over a wide temperature range. Another factor that contributes to their successful application in industrial scale processes is the development of immobilisation techniques, which can increase the performance, stability and allow the reuse of the biocatalyst, besides facilitating the separation of the product [59, 62, 67, 77].

Lipases can be obtained from different sources, such as fungi (e.g., *Candida, Penicillium, Mucor, Rhizopus* and *Aspergillus* sp.), bacteria (e.g., *Pseudomonas, Acinetobacter, Bacillus* and *Chromobacterium* sp.), eukaryotes (e.g., porcine pancreatic lipase), plants (e.g., from papaya, pineapple, castor bean, oil palm and oilseed rape) and expressed in recombinant organisms for industrial applications. Such diversity in origin reflects in their different biochemistry and function. Enzymes from bacteria and fungi are generally more robust, easy to produce and recover from microbial cell culture and, therefore, have the greatest potential for industrial applications [79].

Despite their variability, most lipases display a common catalytic mechanism and structural architecture (i.e., the α/β hydrolase fold), with an active site composed by the catalytic triad: serine, aspartate or glutamate and histidine. This active site is located in a “pocket-like” cavity in the enzyme’s 3D structure (Fig. 10), which can also contain a “lid” that remains closed, unless the enzyme is in a lipophilic media or in aqueous-organic interface. This lid is an amphipathic structure (i.e., hydrophilic outside face and lipophilic inside face) that has not only the opening/closing function, but also helps regulating the enzyme’s activity and selectivity. In several lipases, the lid opening is also responsible for the formation of the so-called “oxyanion hole” which is involved in the stabilisation of the reaction intermediates [79-82].

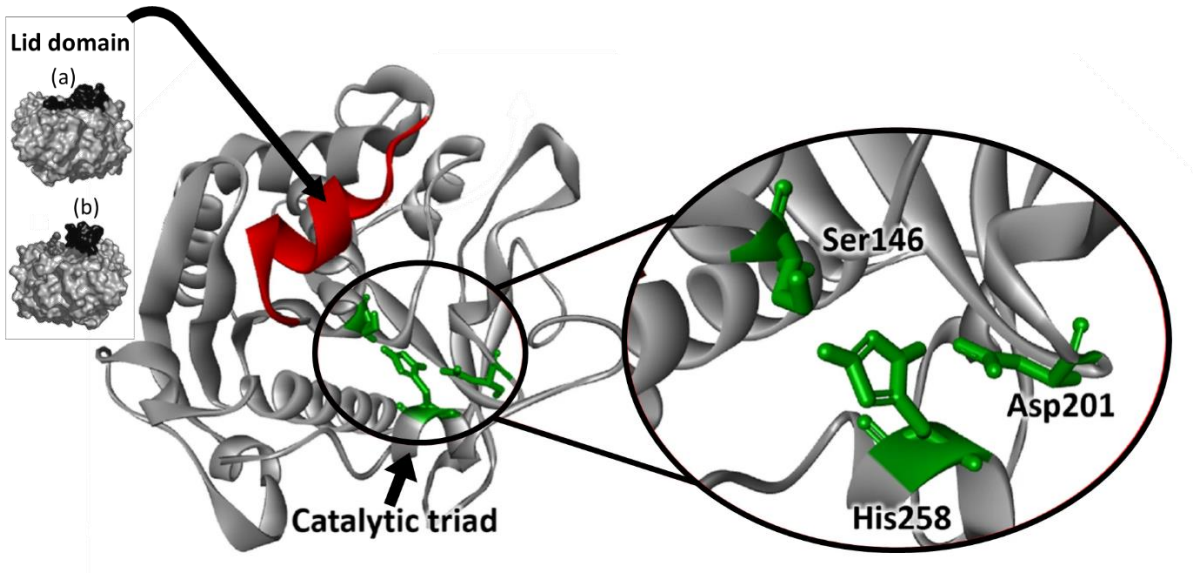

Figure 10. *Thermomyces lanuginosus* lipase 3D ribbon diagram, showing the structure of the catalytic triad in the enzyme's active site (in green), and the lid domain (in red). A space-filling molecular model representation of the *Candida rugosa* lipase (top, left side) shows the closed (a) and open (b) conformations of the enzyme's lid (in black). Adapted with permission from ref. [79, 82]. Copyright 2007 and 2021 Springer Nature, respectively.

Although most lipases share the same structural fold, their substrate binding regions differ greatly in size, structure and physico-chemical properties, which results in their distinct selectivities. The substrate access to the active site is achieved through a binding site located in this pocket, on the top of the central β-sheet. The length, shape and hydrophobicity of the binding pocket has been related to the substrate's chain length preference. Moreover, crystallography studies with the *Burkholderia cepacia* lipase identified multiple binding pockets: one oxyanion hole and three other pockets lined by amino acids with different hydrophobicities, that accommodate the *sn*-1, *sn*-2 and *sn*-3 fatty acid chains as substrates. The *sn*-2 fatty acid chain pocket was found to be a major determinant for this enzyme's stereoselectivity. The hydrogen bonding between the histidine of the active site and the substrates (esters) oxygen atom was influenced by the substituents at the *sn*-2 position of the substrate. However, general rules for lipases chiral selectivity are difficult to establish, since it seems to be dependent on both, the enzymes' and the substrates' structures. The study of these lipase structure-activity relationships allows site-directed mutagenesis, which generates improved activities for target substrates [79].

Lipases generally have two main operation modes: In aqueous media, they favour hydrolysis reactions, acting in the cleavage of carboxyl ester bonds of substrates, such as triacylglycerols, in the first step and using water molecules as co-substrate in the second step. In low-water conditions, the reverse synthetic reaction is favoured with a similar mechanism, leading to esterification, transesterification (Fig. 11), or other types of reaction [79, 83].

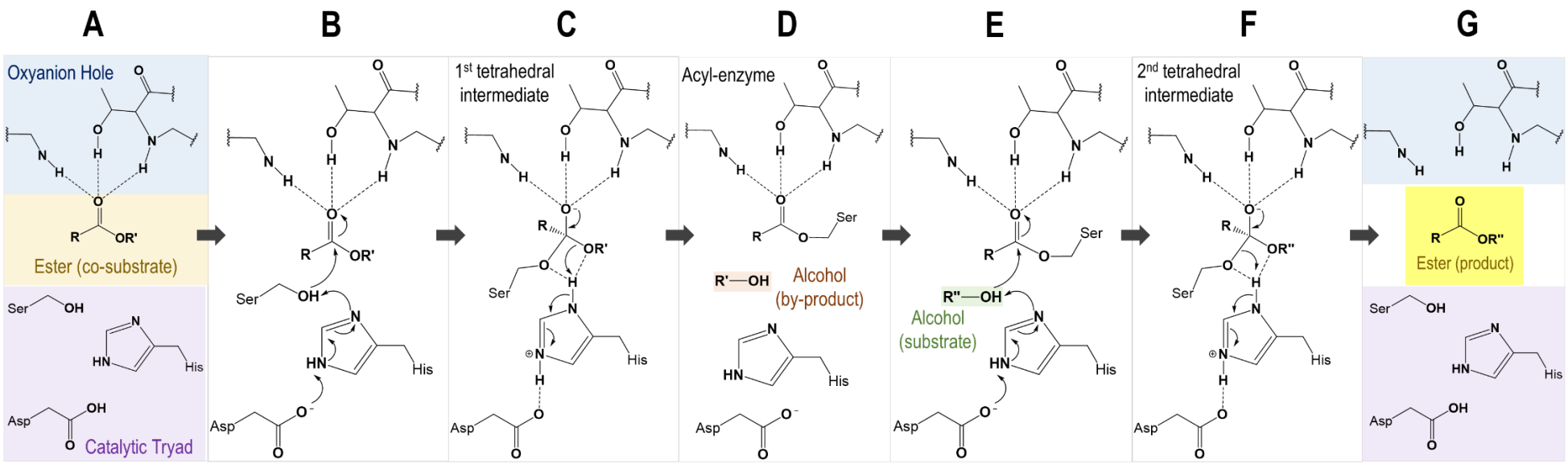

Figure 11. Schematic representation of lipases general transesterification reaction mechanism. The reaction steps include: (A) The formation of the oxyanion hole, which conducts the co-substrate ester to the enzyme calalytic centre; (B) The electron transfer from catalytic centre to the co-substrate-oxyanion hole complex; (C) The formation of the first tetrahedral intermediate; (D) The formation of the covalent acyl-enzyme intermediate and release of an alcohol by-product; (E) The attack of the enzyme to the main substrate alcohol; (F) The formation of the second tetrahedral intermediate; (G) The formation and release of the ester product, with the regeneration of the enzyme's catalytic centre.

In this case, a carboxylic molecule (e.g., ester reagent or “acyl donor”) acts as a co-substrate in the first step, and the main substrate (e.g., alcohol) is attacked by the enzyme in the second step. Therefore, the main steps in lipases reaction mechanism process are: (I) The attack of the enzyme to the carbonyl carbon of the main substrate (or co-substrate) and the formation of a first acyl-enzyme intermediate. (II) The attack of the enzyme to the hydroxyl (or amyl) group of the co-substrate (or main substrate), leading to the hydrolysis of the acyl-enzyme intermediate and formation of the product.

In more detail, the reaction starts by the hydrogen bonding of the carbonyl oxygen of the substrate (or co-substrate) to main-chain NH and OH groups. Such residues build up the so-called “oxyanion hole” that in some lipases is pre-formed in the correct orientation, whereas in others it is positioned upon the opening of the lid structure (Fig. 11A). Consecutively, the ionisation of asparagine and electronic transfer to the catalytic histidine and serine, enhances the nucleophilicity of the active site, which leads to the attack of the carbonyl carbon of the susceptible ester bond (Fig. 11B). A tetrahedral first intermediate is formed (Fig. 11C), carrying a negative charge on the carbonyl oxygen atom that is stabilised through hydrogen bonding. The electronic rearrangement and transfer of a proton from histidine to the ester oxygen results in the bond cleavage and formation of a by-product (e.g., alcohol, water). A covalent acyl-enzyme intermediate is then formed with the substrate (or co-substrate) esterified with the serine residue (Fig 11D). A similar process happens in the second part of the reaction, but this time with the acyl-enzyme and a water molecule (hydrolysis) as a co-substrate or an organic molecule, such as an alcohol (transesterification), as the main substrate (Fig 11E). In this step, the electronic transfer from asparagine and histidine to the main substrate (organic molecule) or co-substrate (water) produces an hydroxide ion that attacks the carbonyl carbon atom in the substrate–enzyme covalent intermediate, generating a negatively charged tetrahedral second intermediate (Fig 11F), which is then stabilised by hydrogen bonds to the oxyanion hole. Finally, the main reaction product and regeneration of the enzyme’s catalytic centre (Fig 11G) are achieved after electronic rearrangements and transfer of a proton from substrate (or co-substrate) to the active site serine [79, 83].

1.3.3. Laccases

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2), are multi-copper containing enzymes that catalyse the oxidation of various types of phenolic compounds (with tyrosine as the main exception) by reduction of molecular oxygen (O_2), with formation of a free cationic radical and water (H_2O) as the only by-product. For this reason, laccases are commonly considered as the ultimate “green catalysts”. The substrate range is further expanded when used along with mediator molecules [84, 85].

Firstly identified in 1883 in exudates of a lacquer tree (*Rhus vernicifera*), laccases are ancient enzymes that can be found in plants, insects, bacteria, but are predominantly obtained from fungi, such as Basidiomycetes, Ascomycetes and Deuteromycetes phyla. Most commercial laccases are expressed in heterologous hosts, such as *Aspergillus* or *Pichia sp.*, which enable the overproduction of the enzyme in industrial scale. Currently, the main applications of laccases in industry include: delignification, dye or stain bleaching, bioremediation, plant fibre modification, ethanol production, oxidative coupling reactions, biosensors and biofuel cells, in various sectors such as pulp and paper, textile, construction and furniture, paint, energy, food, cosmetics, pharmaceutical and nanobiotechnology [84, 85].

Crystallographic and phylogenetic studies indicate that the active site and its environment are structurally highly conserved in all laccases (Fig. 12), even when the rest of the protein molecule varies. Their catalytic centre is composed of four copper atoms in three different redox sites (T1, T2 and T3) and twelve amino acid residues that serve as the copper ligands. The centres T1 and T2 contain one copper atom each, while T3 centre contains two atoms. These copper atoms are classified according to their different spectroscopic and paramagnetic properties, and their location, such as: Cu1 (type I, paramagnetic “blue”, T1 site), Cu2 (type II, paramagnetic “non-blue”, T2 site), Cu3-Cu4 pair (both type III, diamagnetic spin-coupled, T3 site). The T1 copper is bound as a mononuclear cluster and its oxidised resting state, resulted from the covalent bonding with cysteine (Cys-Cu^{2+}), is responsible for the intense greenish-blue colour of laccases. Together, T2 and T3 coppers form a trinuclear cluster, with a triangular geometry, where the reduction of O_2 and release of water takes place. Finally, a tripeptide (His-Cys-His) form the channels that are involved in the electron transfer pathway between the T1 copper and the trinuclear cluster [84-86].

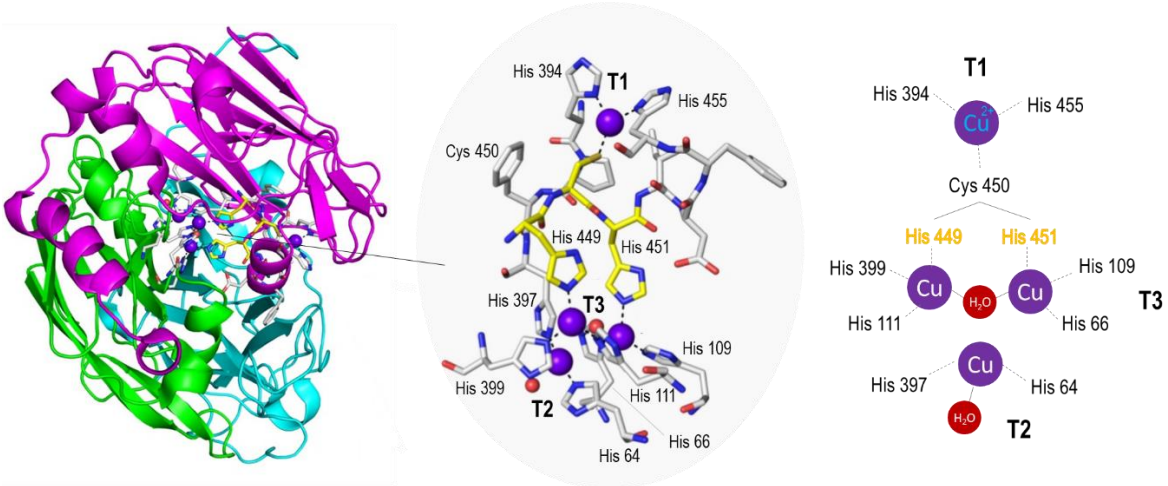

Figure 12. *Trametes trogii* laccase 3D ribbon diagram, showing the 3D and simplified structures of the active site where the trinuclear clusters with copper ions (T1, T2 and T3) are located. Adapted with permission from ref. [85]. Copyright 2015 Elsevier.

The structural conservation reflects in a common electron transfer mechanism from substrate oxidation to molecular O_2 reduction in these enzymes (Fig. 13). Although still not fully understood, the main stages of this mechanism are: (1) the enzyme oxidises the substrate by the abstraction of one electron mediated by the T1 Cu^{2+}, forming a free (cationic) radical. This radical can further undergo laccase-catalysed oxidation or non-enzymatic reactions, such as dimerisation or cleavage of polymers and aromatic rings; (2) Each electron extracted from different substrate molecules at T1 site is transferred to the trinuclear cluster where O_2 is bound; (3) Laccase stores electrons from four substrate molecules monooxidation to completely reduce O_2 to H_2O.

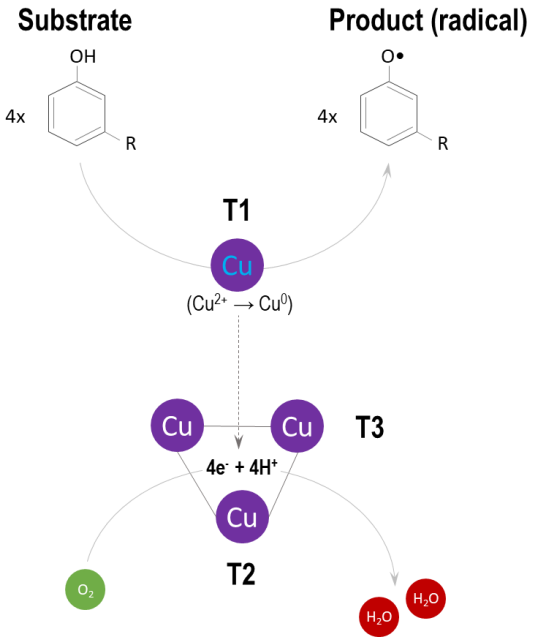

Figure 13. Simplified representation of laccases general oxidation reaction mechanism.

Two solvent channels connect the copper atoms in the T1 site to the T2 and T3 sites, facilitating the fast access to the trinuclear cluster and O_2 molecule, and subsequent easy release of water. The energy required to capture one electron from the substrate with the corresponding formation of a cation radical is given by the T1 site redox potential (E°), which usually varies between +0.4 to +0.8V for fungal laccases. A combination of an enzyme with high E° and a substrate with low E° can result in high reaction rate [84].

Laccase-mediator systems are a combination of the enzymes and a highly diffusible, easily oxidisable and low-molecular weight co-substrate (mediator). The mediator molecules will be oxidised by the enzyme, producing an unstable and reactive cationic radical that will then oxidise target compounds that are not directly converted by the enzymes, either due to steric hindrance or high redox potential. Compounds such as 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonate) (ABTS), 1-hydroxybenzotriazole (HBT), syringaldazine are some of the most known mediators, which can be used alone or in a mixture when a synergistic

effect is observed. The downside of the mediators use is that some of these molecules are toxic, unstable or expensive and can lead to the formation of undesirable by-products and the enzyme inactivation [84].

Laccases are usually unstable, denaturised or inhibited in organic solvent media, where many potential substrates and mediators are better solubilised. Thus, the use of water-solvent (mono or biphasic) systems have been applied in some processes with up to 50% v/v solvent. Maximum reaction rates were found to remain more or less the same when using aqueous solutions, water-miscible solvents (~20-30% v/v) or reverse micelle systems (surfactant + hydrophobic organic solvent, containing a suitable amount of water). However, the presence of these organic co-solvents can significantly affect the structure, the stability and the activity of the enzymes, through direct interactions with the protein molecules or through modification of the thermodynamic water activity (a_w). Other compounds such as chelating agents, ammonium detergents, fatty acids, kojic acid, halogens and some metal ions can also inhibit laccase activity by interrupting the electron transfer, removing or displacing the copper atoms or changing in the amino acids residues conformation. Thus, strategies such as immobilisation and directed molecular evolution are being implemented to overcome these limitations [84, 86].

1.3.4. Factors affecting enzyme reactions

Enzymes are proteins that work as organic catalysts in specific reactions, usually involving the cleavage of bonds in a single substrate or the formation of bonds between two or more substrates, forming one or more products. These specialised proteins are produced by living organisms, but are capable of acting inside or outside the cells. Enzyme-catalysed reactions generally happen in different stages (Equation 2), including the binding of enzyme (E) and substrate (S), the formation of the enzyme-substrate complex (ES) and the formation and release of the product (P). Generally, the fast stage (K_1 and K_{-1}) is a second order and reversible reaction, while the slow step (K_2) is a first order and irreversible reaction, and it is the one who determines the reaction rate (catalytic step).

$$E + S \underset{K_{-1}}{\overset{(\text{fast})\ K_1}{\rightleftharpoons}} \boxed{ES \xrightarrow[\text{catalytic step}]{(\text{slow})\ K_2 \text{ or } K_{cat}} E + P} \qquad \textbf{(Equation 2)}$$

These reactions are susceptible to multiple chemical and physical factors, such as pH, temperature, enzyme type and preparation form (e.g., immobilisation), and concentrations of the substrate and enzyme, that may impact upon their performance in different ways. The selection of the appropriate enzyme is also fundamental when planning the application,

because yield and selectivity of the enzymes can vary greatly. For instance, *Candida rugosa* lipase will give high yields but has a low selectivity, whereas *Aspergillus niger* lipase is highly selective [58]. Thus, finding the best working conditions for a specific enzyme with interesting features is the key to obtaining efficient processes.

Enzyme kinetics studies help to determine the activity (i.e., maximum reaction rate or velocity, V_{max}) and other important parameters that characterise these biocatalysed reactions (Table 3), which ultimately helps to understand the impact of the aforementioned factors on the enzymes' performance (Fig. 14). It is common to use the enzyme activity as a unit of measurement of the enzymes, rather than their mass. The reason is that the function is more important than the purity of the protein, since even a pure enzyme can be totally inactive [87, 88].

Table 3. Summary of key kinetic parameters of enzyme reactions.

Parameter	Abbreviation (common units)	Definition
Maximum enzyme velocity or Enzyme activity units	V_{max} (μmol/min; mol/s) U or IU (μmol/min; kat)	A measure of enzyme activity, which indicates the turnover per time unit at saturating conditions of substrates and cofactors under standard conditions. It is commonly used to express the enzyme amount converting 1 μmol substrate/min. Conversion: 1 kat=60,000,000 U 1 nkat=0.06 U 1 U=0.0000000167 kat
Enzyme volume activity	U/mL; kat/L	Enzyme units per volume unit.
Specific enzyme activity	(U/mg; kat/kg)	Enzyme activity units per protein mass or volume activity/protein concentration.
Enzyme velocity	V (mol/s; μmol/min)	Enzyme turnover per time unit.
Turnover number	k_{cat} (s^{-1})	Catalytic constant given by the enzyme maximum velocity divided by the enzyme concentration ($k_{cat}=V_{max}/[E]_0$).
Michaelis constant	K_M (M)	Substrate concentration for half-maximal velocity ($K_m=(k_{-1}+k_{cat})/k_1$).

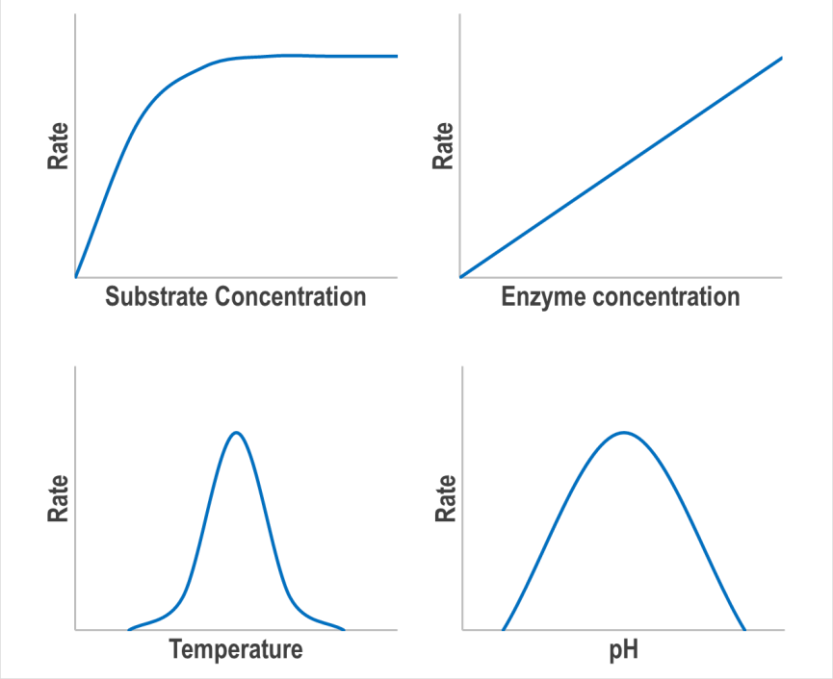

Figure 14. Diagrams exemplifying the effects of different factors on the rate of an enzyme reaction.

Michaelis-Menten and Lineweaver-Burk equations are the main kinetic models used in the calculation of these parameters. The Michaelis-Menten equation (Equation 3) is derived from the rate law of the catalytic step and it is based on the theory of the ES complex formation, which basically assumes that $K_2 << K_{-1}$.

$$V_0 = \frac{V_{max} \cdot [S]}{K_m + [S]} \qquad \textbf{(Equation 3)}$$

The Lineweaver-Burk equation or double-reciprocal is a linearisation approach given by the inverse form of both sides of the Michaelis-Menten equation. This approach makes it easier to determine the kinetics of multisubstrate enzymes and the interaction between enzymes and inhibitors, and is the most used method to determine the enzyme activity (V_{max}). It involves the reaction of the enzyme in a fixed concentration and the substrate in various saturating and evenly spaced concentrations. The initial rate (V_0) is given by the slope of the linear part of each “time vs product (or substrate) concentration” plot. The Lineweaver-Burk plot (Fig. 15) is then obtained by the inverse values of each initial rate ($1/V_0$) and substrate concentration (1/[S]). The y-intercept of this graph gives the enzyme activity (V_{max}) and the slope is used to calculate the Michaelis-Menten constant (K_m).

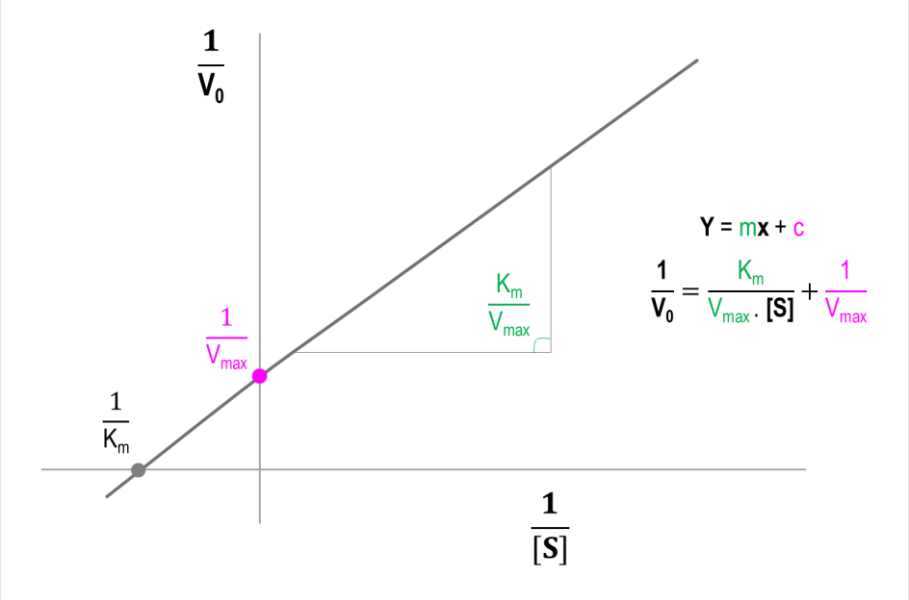

Figure 15. General representation of the Linewaver-Burk plot and equation.

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Chapter 2

Quantitative assessment of enzymatic processes applied to flavour and fragrance standard compounds using gas chromatography with flame ionisation detection

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Contents

2.1. Article

This study investigated the efficacy of different enzymes in the bioprocessing of aroma-related compounds. Methods based on GC-FID analysis were developed to facilitate the evaluation of the stages of characterisation, screening and optimisation, including chiral ratio determination for enzyme enantioselectivity assessment. The characterisation process included activity assays using UV-Vis and GC-FID, as well as protein quantification through NanoDrop spectrophotometry and molar mass estimation via SDS-PAGE electrophoresis. Screening experiments evaluated a range of enzymes, substrates, solvents, acyl donors, and mediators. The aroma-related substrates included terpene and phenolic compounds. Five lipases (CALA, NZ-435, LZ-TLIM, NC-ADL, LZ-CALBL) and two laccases (NZ-51003 and DL-IIS) were investigated. Among these, NZ-435, CALA and NZ-51003 were the best performers in the characterisation and screening stages, achieving conversions above 70% for citronellol (lipases) or 50% for eugenol (laccase). Although, enantioselectivity was not observed under the tested conditions, lipases and laccases demonstrated a chemoselectivity towards primary alcohols and phenolic compounds, respectively, among tested substrates. The optimisation stage was demonstrated through the lipase-catalysed (CALA) transesterification of a standard alcohol mixture (citronellol, menthol, linalool), wherein the best conditions of temperature, enzyme and acyl donor concentrations were investigated using a design of experiments (DOE) approach. Optimal conditions were found to be 37-40°C, 3-4 mg/mL of enzyme, and 58-60% (v/v) vinyl acetate. Confirmation experiments using the same standards and citronella oil sample showed good conversions for citronellol (> 95%/ h) and menthol (20%/ h and 74%/ day). The proposed GC-FID approach was suitable for determining reaction profiles and chiral ratio variations for biocatalysed reactions involving low complexity aroma samples. This study set the foundation to future applications of the enzymes to complex essential oil samples, where it is anticipated that advanced GC separations will be required.

After publication of this paper, additional screening experiments were conducted for laccases NZ-51003 and DL-IIS, using different water/solvent media and various standards as mediators and substrates. However, these enzyme products underperformed in the applied conditions, mainly due to their incompatibility with organic solvents and substrates (i.e., aroma standards and essential oils). In other words, using a predominantly organic medium or emulsifiers is unfavourable to the enzyme activity, but is necessary for substrates' solubilisation. Additionally, the liquid-liquid extractions were laborious, wasteful and introduced variation in the recovery of target analytes and general sample composition. Enzyme immobilisation could have been a solution to these problems, but it was unfeasible due to agreement restrictions with the enzymes provider, as well as time and resource constraints. Thus, the progress of the studies with laccase optimisation and application to complex essential oil samples was hindered.

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ABSTRACT

The performance of different enzymes towards the bioprocessing of aroma-related compounds was investigated and a strategy based on GC-FID analysis was developed to facilitate assessment of the stages of characterisation, screening and optimisation, including chiral ratio determination. Characterisation included activity assays (UV–Vis and GC-FID), protein quantification (NanoDrop spectrophotometry) and molar mass estimation (SDS-PAGE electrophoresis). Screening experiments assessed different enzymes, substrates, solvents, acyl donors or mediators. Aroma-related substrates comprised terpene and phenolic compounds. The enzymes tested included the lipases CALA (Sigma-Aldrich), NZ-435, LZ-TLIM, NC-ADL, LZ-CALBL and the laccases NZ-51003 and DL-IIS (all from Novozymes). Among those, NZ-435 and NZ-51003 had the highest activities in the characterisation stage and, along with CALA, achieved conversions above 70% for citronellol (lipases) or 50% for eugenol (laccases) at the screening stage. The lipases had preference for the primary alcohol and laccases for phenolic compounds, among the tested substrates. The transesterification reaction between the lipase CALA and the standards mixture (citronellol, menthol, linalool) was used to demonstrate the optimisation stage, where the best levels of temperature, enzyme and acyl donor concentrations were investigated. Optimum conditions were found to be 37–40 °C, 3–4 mg/mL of enzyme and 58–60% (v/v) vinyl acetate. Additional confirmation experiments using the same terpene standards mixture and citronella oil sample, gave a conversion of > 95% for citronellol after 1 h (for both, standards mixture and sample), and 20% or 74% for menthol after 1 h or 24 h, respectively. None of the tested enzymes demonstrated significant enantioselectivity under the tested conditions. The GC-FID approach demonstrated here was suitable to determine the reaction profiles and chiral ratio variations for biocatalysed reactions with aroma compounds in low complexity samples. Advanced separations will be applied to more complex samples in the future.

1. Introduction

As the potential of green chemistry transcends from academia to industry, with different manufacturing sectors working towards a circular economy, the application of innovative biotechnological processes has increasingly become the focus for the development of sustainable synthetic routes to valuable products. Enzyme catalysed reactions are well-known for their general (chemo-, regio- and stereo-) selectivity under mild conditions (in terms of low temperature and pressure requirements), as well as the reusability and biodegradability of the catalyst. Moreover, the potential labelling of the final product as "naturally derived" is often attractive or even crucial to many consumers. Ideally, the use of industrial biocatalysts that efficiently convert abundant, low-value and renewable raw materials into higher-value molecules is a desirable goal [1–4].

Flavours and fragrances (F&F) constitute a multi-billion dollar, global scale market. The design of biosynthetic routes for the sustainable conversion of abundant and lower cost precursors into fine chemicals has broad scope in this field, since some naturally derived F&F products are often susceptible to limited supply, high harvesting costs and numerous environmental and processing variations [2,5]. The chiral selectivity of the enzymatic processes is also of interest for the synthesis of valuable aromas, especially when the target enantiomers possess different odour perceptions and activities, as for example is the case with

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(±)-limonene with the enantiomers exhibiting orange *vs* lemon odour. Similarly, subtle changes in the composition of complex aroma mixtures, such as essential oils, can impact upon their scent and potentially increase the value of the bio-transformed product. Therefore, targeting and changing key attributes of precursor components is highly desirable for aroma synthesis or enhancement. Among these changes, the esterification and oxidation of terpenic compounds catalysed by enzymes are promising pathways to produce unique and strong odorants. Thus, lipases and laccases represent two key groups of enzymes with application to F&F synthesis [2–8].

Lipases are an industrially-established group of enzymes with multiple applications, since they can catalyse different reactions, accept a wide range of substrates, and remain active in different media (e.g., aqueous, organic, ionic liquids, supercritical fluids) and over a wide temperature range. For the F&F industry, esterification and transesterification reactions are the most pertinent application of lipases, with a focus on the acylation of compounds, such as terpene or phenyl alcohols and essential oils. Due to their lower polarity and absence of hydroxyl groups, alkyl esters are usually more volatile (which can favour the odour detection threshold) and less sensitising than their corresponding alcohols when in contact with human skin [6,9–14].

Laccases are multi-copper containing enzymes that catalyse oxidative reactions by transferring an electron from the substrate to molecular oxygen (O_2) and forming water as the only by-product. They accept a broad range of substrates, such as phenols, anilines and benzenethiols. Laccase-mediated systems are of interest to F&F chemistry in many ways. A relevant application is the repurposing of waste products, such as those containing limonene, pinene and other terpene hydrocarbons, which are currently discarded in large quantities every year due to their low commercial and aroma value and chemical instability. However, many of their oxyfunctionalised metabolites have higher aroma impact and value. Indeed, a patented synthetic process involving the oxidation of sesquiterpenes by specific laccase-mediator system has originated a novel class of octahydroazulenol derivatives with strong floral/woody odour [15]. Moreover, laccases can be used to remove undesirable phenolic compounds that can impact upon the sensory properties of products like wine, beer and fruit juices [2,5,16,17].

Enzyme reactions are susceptible to multiple chemical and physical factors that may impact upon their performance in different ways. Thus, finding the best working conditions for a specific enzyme with interesting features is the key to obtaining efficient processes. In order to develop a screening and optimisation strategy (Fig. 1), it is important to pre-select the parameters and scope of the study, since testing all possible factors is usually not feasible. Gas chromatography (GC), often with flame ionisation (GC-FID) and mass spectrometry (GC–MS) detection, is currently the prime technique for the analysis and quality control of volatile compounds. Consequently, it can be used to assess the profiles of enzyme-catalysed synthesis of aroma-related compounds, since many of these compounds are highly volatile, thermally-stable, chiral, lipophilic and do not contain a chromophore, which makes them difficult to directly analyse with other techniques such as UV–Vis. Moreover, GC offers the possibility of direct quantification, identification (*via* GC–MS), and olfactometry detection (GC-O) of products and substrates and chiral separations when using enantioselective columns [18–20].

In the present work, investigations were carried out on the performance of seven commercial enzyme products (5 lipases and 2 laccases), including free (liquid, powdered) or immobilised preparations, for application in the biotransformation of target standard compounds relevant to F&F. To this end, a general GC-based methodology was established for screening of the enzymes' efficiency and selectivity in different biocatalysed reactions, addressing some details beyond those other similar application studies considered, such as: (1) Providing more inclusive and detailed reaction, sample preparation and GC-FID analysis methods, with strategies covering different types of enzyme preparations (e.g. liquid, powdered, immobilised), enzyme classes (e.g. lipases, laccases), and reaction media (e.g. aqueous, organic); (2) Performing the screening step with mixtures of different standard compounds as substrates (and also varying other parameters, such as the co-substrate or solvent) for a better understanding of the enzyme's chemo- and enantioselectivity and inhibitory effects, before optimisation and application to complex samples. With lipases for example, it is important to have a mixture of primary, secondary and tertiary alcohols to successfully select the enzymes able to fully esterify complex samples, such as essential oils; (3) Performing and reporting enzyme activities in buffered aqueous media and also checking this in organic media, which is the one intended for use. This is an important initial step since the activity of

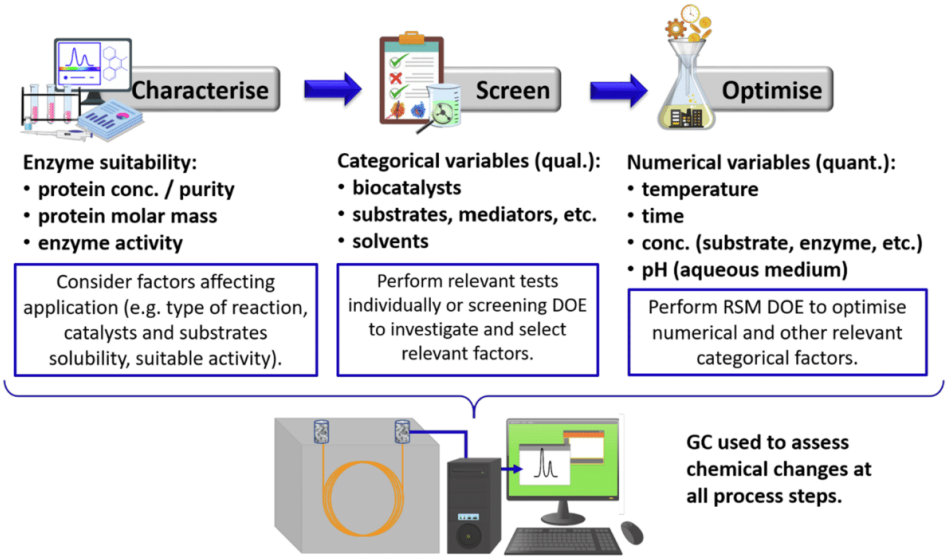


Fig. 1. Diagram of the biocatalysis analytical strategy, illustrating the three key stages of process development, including (i) enzyme characterisation, (ii) enzyme and substrate screening and (iii) system optimisation, before upscaling the application with target F&F samples.

different enzymes from the same class (and in different preparations) can vary significantly, being also affected by the composition of the reaction medium used. Additionally, the molecular mass and concentration of all enzyme products were also determined, generating comparative characterisation data for reference in future studies.

The analytical methods described are focused on quantitative aspects of the first stages of enzyme studies, being mostly based on the use of standards and no additional identification was required (e.g. as would be the case for multi-component essential oils). Thus, GC-FID was preferable for a more reliable and sensitive quantification analysis and proved to be a suitable technique for multistep assessment. Moreover, since more literature information is available for lipases, they served as the main model to develop the generalised approach to incorporate GC strategies for all the steps of enzyme reaction studies, including optimisation, which was demonstrated for lipases reaction with terpene alcohol standards. Laccases were included in the present study in order to provide preliminary data on an alternative class of enzymes, however a more complete evaluation will be reported subsequently. Finally, advanced separation techniques, such as GC×GC analysis and MS detection are important tools for improved chemical separation, profiling and identification, and will be used in the next steps of this project, which will explore the application of enzymes to complex samples.

2. Experimental

2.1. Chemicals and enzymes

The list of chemicals used in this study includes: sodium phosphate dibasic heptahydrate ($Na_2HPO_4{\cdot}7H_2O$, <98%), sodium phosphate dibasic dihydrate ($Na_2HPO_4{\cdot}2H_2O$, 99%), monobasic sodium phosphate (NaH_2PO_4, 99%), triton X-100, 4-nitrophenyl octanoate (90%), 4-nitrophenol (99%), guaiacol (98%), vinyl acetate (99%), (±)-menthol (98%), (*R*)(-)-linalool (98%), (*R*)(+)-β-citronellol (98%), (*R*)(+)-1-phenylethanol (99.5%), (*S*)(-)-1-phenylethanol (99.5%), (*R*)(+)-limonene (97%), (*S*)(-)-limonene (96%), vanillin (99%), sinapic acid (99%), acetone (99%), ethanol (99%) and *n*-decane (99%), all supplied by Sigma-Aldrich (Castle Hill, NSW, Australia); (±)-linalool (97%) and *n*-hexane (99%), obtained from Merck (Bayswater, VIC, Australia); (-)-β-citronellol (95%) from TCI (Tokyo, Japan); anhydrous sodium sulfate (Na_2SO_4, > 99%, from AnalaR, Kilsyth, VIC, Australia) and ethyl octanoate (98%), (1*R*,2*S*,5*R*)(-)-menthol (98%), (±)-citronellol (98%), thymol (98%), eugenol (98%), which were kindly donated by Australian Botanical Products (Hallam, VIC, Australia). The enzymes employed in this investigation are listed in Table 1.

2.2. Characterisation

2.2.1. Protein concentration (NanoDrop UV–Vis) and molecular weight (SDS-PAGE electrophoresis) determination

NanoDrop One UV–Vis spectrophotometry (Thermo Fisher Scientific, DE, USA) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, were performed by the Monash University Biomedical Proteomics Facility (Clayton, VIC, Australia) and used to determine the concentration, purity and molecular weight of the proteins in the enzyme products. Aqueous solutions or suspensions of the enzyme products (~2 mg/mL) were prepared and used to perform both analyses.

NanoDrop spectrophotometry was performed by directly measuring the absorbance of the aqueous sample solution at 280 nm, which gives the estimated concentration of protein (1 Abs = 1 mg/mL protein) by correlation with its amino acid residues and the Lambert-Beer law [28].

The SDS-PAGE experiment was set up and performed according to the manufacturer's instructions for NuPAGE™ gels (Invitrogen, MA, USA), using a 7 cm gel type NuPAGE 4–12% Bis Tris, 1% MES buffer and 10 μL of the Novex® Sharp pre-stained protein standards as gel markers. Based on the concentrations measured by NanoDrop, 160 μg of each sample was added to the NuPAGE™ LDS Buffer containing 4% β-mercaptoethanol, heated at 95 °C for 2 min and loaded into the gel lanes. Electrophoresis running buffer was prepared by dilution (20x) of NuPAGE™ MES SDS Running Buffer and the XCell SureLock Mini-Cell gel tank. The samples were run in the electrophoresis gel for 40 min at 1200 V set on an EPS601 power supply (GE Healthsciences, Upsala, Sweden). The gel was then removed from the cassette and stained with InstantBlue™ Protein Stain (Expedeon, CA, USA) to reveal the protein bands (Supplementary Information, Fig. S1).

Table 1
Enzyme product specifications according to the manufacturer [21,22] and literature [23–27]. *Candida antarctica* A (CALA) was purchased from Sigma-Aldrich and all the others were kindly donated by Novozymes Australia Pty ltd (North Rocks, NSW, Australia).

Enzyme Product, Type (biological source)	Supplier (Batch#)	Product form	*pH	*T (°C)	Substrate specificity	Specific activity**, substrate
CALA, Lipase (*Candida antarctica* A)	Sigma-Aldrich (BCBV6128)	Immobilised (Immobead 150)	6–9	40–90	Sterically hindered alcohols	1590 U/g tributyrin [22]
Novozym 435 (NZ-435), Lipase (*Candida antarctica*)	Novozymes (LC200315)	Immobilised (macroporous acrylic resin)	5–9	30–60	Nonspecific; Esters and alcohols	7922–10000 PLU/g lauric acid [21,23]
Lipozyme TL IM (LZ-TLIM), Lipase (*Thermomyces lanuginosa*)	Novozymes (LA331810)	Immobilised (granulated silica)	6–8	50–75	1,3-Specific; Esters	250 IUN/g-693 PLU/g lauric acid [21,23]
NovoCor ADL (NC-ADL), Lipase (*Candida antarctica* A)	Novozymes (LDN00072)	Liquid	5–9	30–60	Sterically hindered esters	6 KLU/g tributyrin [21]
Lipozyme CALB L (LZ-CALBL), Lipase (*Candida antarctica* B)	Novozymes (LCN02134)	Liquid	5–9	30–60	Esters and alcohols	5 KLU/g tributyrin [21]
Novozym 51003 (NZ-51003), Laccase (*M. thermophile* expressed in *Aspergillus oryzea*)	Novozymes (OMN07030)	Liquid	4.5–7.5	25–40	Nonspecific; Phenols, anilines, thiols and others.	1000 LAMU/g-1220 IU/mL ABTS [21,24]
DeniLite-IIS (DL-IIS), Laccase (*M. thermophile* expressed in *Aspergillus oryzea*)	Novozymes (OM710237)	Powder (non-immobilised)	4–6	40–70	Nonspecific; Phenols, anilines, thiols, and others.	120 U/g –33 U/ mg ABTS [26,27]

* Optimum conditions for the indicated substrates.
** One unit (U) is defined as the amount of enzyme activity which liberates 1 μmol of product from the substrate per minute under defined standard conditions. 1 U/g = 1 PLU/g, 1 IUN/g, 0.001 KLU/g, 1 LAMU/g, 1000 U/mg. KLU (kilo lipase unit), PLU (propyl laurate unit), IU or IUN (interesterification unit), LAMU (laccase unit).

2.2.2. Enzyme activity determination in buffered aqueous medium using UV–Vis continuous assays

The activities of the enzymes in buffered aqueous medium were determined by spectrophotometric (UV–Vis) continuous monitoring assays through the lipase-catalysed hydrolysis of *p*-nitrophenyl octanoate (*p*-NPO) into *p*-nitrophenol (*p*-NP) and octanoic acid [29–32] or the laccase-catalysed oxidative coupling of guaiacol (GUA) [33,34].

The UV–Vis analyses were performed directly in 1 or 4 mL, 1 cm lightpath polystyrene cuvettes (Sarstedt, Mawson Lakes, SA, Australia), using an Agilent Cary 100 UV–Vis system (Agilent, Mt Waverley, Australia) with a thermostatted 12-cell holder and electromagnetic stirring control. The assay final volume was 1 mL for liquid enzyme products and 3 mL for immobilised or powdered enzymes. Stock solutions (50000 μM) of the substrates in ethanol or acetone were prepared in volumetric flasks and used to prepare the calibration curves and reaction solutions at different concentrations. The blanks were composed of a solution containing only the enzyme or the substrate in the reaction medium, which were used to zero the instrument. The assay mixtures at the desired concentrations were allowed to equilibrate for 30 min at the assay temperature before starting the measurements.

The lipases reaction medium was composed of phosphate buffer solution (0.02 M; pH 7.5), Triton X-100 (0.5% v/v), the substrate solutions (*p*-NPO 10, 25, 50, 75, 100, 150, 200 μM) and the enzymes (0.3 mg/mL). A calibration curve was prepared in the same conditions described above, but substituting *p*-NPO (i.e., substrate) for *p*-NP (i.e., product) and excluding the enzymes.

The laccases reaction medium was composed of phosphate buffer solution (0.02 M; pH 5), substrate solutions (GUA 50, 100, 150, 200, 250, 300, 350, 400 μM) and the enzymes (0.5 mg/mL). The calibration curves were constructed by taking the average absorbance values of the last 5 min of the reactions at each concentration, since GUA by itself is colourless.

The assays (triplicate) were commenced by adding the reaction solutions to the cuvette with solid enzymes or by adding 6 μL lipase or 10 μL laccase stock solution (50 mg/mL in water) to the cuvette with the mixture, then quickly and gently mixing by inversion and placing the cuvette in the instrument. Both lipase and laccase reactions were held at 25 °C, under minimum stirring (~50 rpm) with a magnetic stirring bar (5 × 2 mm) and monitored in intervals of 10–30 s until constant absorbance was reached (5–40 min).

The production of *p*-NP (yellow colour) by lipases was monitored at 400 nm and the production of guaiacol dimer (orange colour) by laccases was monitored at 470 nm. Wavelength scans over the range 350–650 nm were carried out before the assay, using the calibration curve solution p-NP 50 μM or the post-reaction solution GUA 200 μM, and allowed the selection of the best wavelength for the assay (Fig. S2).

The average absorbance values (from triplicate measurements) and standard deviations were calculated, and a scatter data plot generated. The initial reaction rate (V_0; abs/min) was obtained from the slope of each reaction plot within the linear range and then converted (V_0; μM/min) using the calibration curves (Fig. S3A and S4A). The inverse values of V_0 and substrate concentrations were calculated and used to construct the Lineweaver-Burk plots (Fig. S5A and S6A), which allowed the determination of the enzyme activities and other kinetic parameters. The aqueous activity values were taken as standard values for the subsequent assays.

2.2.3. Enzyme activity determination in organic medium using GC-FID discontinuous (multi-sampling) assay

The enzymes with the greatest activity in the aqueous assay (lipase NZ-435 and laccase NZ-51003) were reassessed in organic or aqueous/organic medium. The lipase-catalysed transesterification reaction of *p*-NP and ethyl octanoate and laccase-catalysed oxidative coupling of guaiacol were performed in 2 mL glass vials, at room temperature (~25 °C) and under gentle magnetic stirring (~350 rpm); activity values were compared with the aqueous assay.

Lipase reaction solutions (2 mL) were composed of ethyl octanoate (100, 150 or 200 μM), p-NP (1000 μM), internal standard (*n*-decane, 175 μM) diluted in hexane and the enzyme (0.3 mg/mL) was the starting reagent. Stock solutions (50000 μM) of the substrates were previously prepared in acetone, using volumetric flasks. The assay mixture (2 mL) was transferred to the vials containing the enzyme (NZ-435; 0.9 mg) to start the reaction. The final enzyme concentration was fixed, and it was the same as the previous aqueous assay. An ethyl octanoate calibration curve (10, 25, 50, 100, 150, 200, 250, 300 μM) with internal standard (*n*-C10, 175 μM) was also prepared in hexane and analysed using the same GC conditions as the sample.

The non-continuous assay was performed (in triplicate) by monitoring the decrease in ethyl octanoate concentration at different time points 0, 0.5, 1, 1.5, 2, 3, 4 and 5 h after the reaction commencement. Aliquots (50 μL) of the reaction mixture were transferred to a glass micro-insert and centrifuged in a mini centrifuge (6000 rpm; 2 min). Since the NZ-435 enzyme is immobilised and formed a compact pellet and all other components and solvents of the reaction mixture are suitable for GC analysis, no further sample preparation steps were required.

A similar procedure was adopted for the laccase NZ-51003, with some differences in the composition of the reaction mixture, monitoring and sampling. Laccase reaction solutions (2 mL) were composed of guaiacol (300, 350 or 400 μM), internal standard (methyl eugenol 250 μM) diluted in acetone/water 50% (v/v) and the enzyme (0.5 mg/mL) as the starting reagent. The decrease in guaiacol concentration was monitored after 0, 0.5, 1, 1.5, 2, 4, 6 and 24 h of reaction commencement by taking aliquots (100 μL) and performing a liquid–liquid extraction; Guaiacol calibration curve (10, 25, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500 μM) solutions plus internal standard (methyl eugenol 250 μM) were diluted in hexane and analysed using the same GC conditions as the sample.

The liquid–liquid extraction procedure used for all the liquid enzyme experiments, in this and all the subsequent steps, consisted in a water/hexane (1:1) partition of the reaction aliquots (100 μL) in a glass micro-insert, which were mixed by vortex (30 s), centrifuged (6000 rpm; 2 min) and the organic phase transferred to another glass micro-insert in a GC vial for the analysis. The salt-assisted method, using glass micro-insert containing anhydrous Na_2SO_4 (~50 mg) to collect the aliquots, was applied only for laccases reactions to help the extraction of the polar substrates (phenols) from the reaction mixture to the non-polar solvent (hexane). None of the enzyme preparations used in this study were soluble in hexane.

Quantitative analysis was performed by GC-FID, using an Agilent 7890A system with automatic injector G4513A and a DB-5 (30 m × 0.25 mm × 0.25 μm; Agilent J&W) capillary column. Injections (1 μL) were made at 220 °C, split (20:1), with hydrogen (grade 99.99%) as carrier gas, flow 1.5 mL/min, oven program 60 °C, 10 °C/min to 200 °C, and detector 220 °C, hydrogen 40 mL/min, and air 400 mL/min settings. The average corrected chromatographic areas (substrate/internal standard) of the standard calibration curves (Fig. S3B and S4B) and reaction aliquots were obtained using Agilent OpenLab CDS ChemStation Edition Rev. Version C.01.10 [201] software and correlated to calculate the substrates' decrease in concentration per time (V_0) and the enzyme activity (V_{max}) was given by the Lineweaver-Burk plots (Fig. S5B and S6B).

2.3. Screening of enzyme reactions with aroma-related standards in organic medium (GC-FID)

The chemo- and stereoselectivity of the different lipase products towards aroma compounds in organic medium was evaluated from the reaction of the enzymes with a racemic mixture containing (±)-citronellol, (±)-menthol and (±)-linalool, which are primary, secondary and tertiary chiral terpene alcohols, respectively.

The concentration of each racemic pair in the mixture was 200 μM. The reaction solutions (2 mL) were composed of the terpene alcohols

(200 μM) and internal standard (*n*-decane, 175 μM) diluted in hexane/vinyl acetate (1:1). The enzyme products (CALA, NZ-345, LZ-TLIM, LZ-CALBL and NC-ADL) were weighed (0.9 mg) directly in the glass vials and 2 mL of the assay solution was added to start the reaction, so the final enzyme concentration was the same as previously employed (0.3 mg/mL). The same procedure was repeated using triacetin/ethyl acetate (1:1) as acyl donor/solvent system, only for CALA enzyme.

A calibration curve of the chiral alcohol mixture (10–300 μM) plus internal standard (*n*-C10, 175 μM) in hexane was prepared and analysed using the same conditions of the samples (Fig. S3). All the reactions were performed in triplicate, with the same method as previously described for ethyl octanoate reactions, including the liquid–liquid sample preparation for liquid lipases.

Laccase reactions were performed and analysed as previously described for guaiacol in an aqueous/organic medium composed of acetone/water 50% (v/v), but using a mixture containing the aroma standards limonene, thymol, menthol, phenylethanol and eugenol (400 μM each), the enzymes NZ-51003 or DL-IIS and the internal standard (methyl eugenol 250 μM). The same reactions were repeated with the laccase NZ-51003, including guaiacol, vanillin or sinapic acid (4000 μM) as mediators. A calibration curve of this standards mixture (10–500 μM) plus internal standard (methyl eugenol 250 μM) in hexane was prepared and analysed in the same conditions of the samples (Fig. S4).

Limonene, menthol and thymol are monoterpene compounds with similar structures, but different degrees of unsaturation or substitution, whereas phenylethanol and eugenol contain phenyl groups. Moreover, limonene, menthol and phenylethanol were chiral racemic mixtures and, thus interesting for enantioselectivity assessment. Natural substrates derived from lignin, such as syringaldehyde, vanillin and their derivatives, have shown potential application as mediators in laccase-mediator systems (LMS) [5,16,17,35].

The consumption of the substrates during the reaction was quantified by GC-FID, using the same Agilent GC-FID system, software and method as aforementioned for chemo-selectivity and efficiency evaluation studies.

Qualitative analysis for chiral ratio determination was performed in the same instrument, only for relevant samples, using three aliquots from different time points of the reactions (0, 2 and 5 h) and with a different column configuration comprising a DB-5 (1 m × 0.18 mm × 0.18 μm) guard column coupled with a glass press-fit to a MEGA-DEX DET Beta Fast (10 m × 0.1 mm × 0.1 μm) capillary column. The method was as follows: Injection 1 μL, 220 °C, split (20:1), hydrogen (grade 99.99%) as carrier gas, flow 0.5 mL/min, and the oven program was 50 °C, 5 °C/min to 125 °C. Detector 220 °C, hydrogen 40 mL/min, air 400 mL/min. The average enantiomeric ratios (area *R*/ area *S*) were determined and compared.

Chromatographic methods were adapted from previous studies [36,37], varying key parameters, such as flow rate and temperature program to achieve good separation of the targeted standards with the shortest analysis time. The chiral column selection was made by using the above method to analyse a mixture containing (±)-limonene, (±)-linalool, (±)-menthol, (±)-phenylethanol and (±)-citronellol in three different enantioselective capillary columns: Astec CHIRALDEX B-PM (30 m × 0.25 mm × 0.12 μm), MEGA-DEX DET Beta (30 m × 0.25 mm × 0.25 μm) and MEGA-DEX DET Beta FAST (10 m × 0.1 mm × 0.1 μm). Only the flow was changed to 1 mL/min for the analysis with 30 m columns.

2.4. Optimisation of CALA enzyme reaction with aroma standards (GC-FID)

The lipase (CALA) reaction with the terpene alcohols mixture (citronellol, menthol and linalool) was used to demonstrate the optimisation strategy. Laccase reactions were not optimised in the present study, since they were active towards a limited range of substrates in the applied conditions and further investigations will be required before optimisation. The design of experiments (DOE) was based on the Box–Behnken response surface methodology (RSM). In this DOE model, each factor or independent variable is placed at one of three equally spaced values, usually coded as −1, 0 and + 1.

The factors most frequently optimised in enzyme reactions include: temperature, substrate and mediator concentrations, pH and reaction time [1,12,15,16,23,38–45]. Thus, a combination of temperature, enzyme concentration and mediator concentration were used as variables for optimisation through the model (Fig. 2). The reaction time was investigated separate to the DOE, by monitoring the reactions for 24 h. The concentration of the terpene alcohols was kept the same as in the previous step (200 μM), but the concentration of the acyl donor (vinyl acetate) was varied (0.2–99.8% v/v), which means that the solvent (hexane) concentration also varied from > 99% to 0% (i.e., "solvent free"; vinyl acetate 99%). It also means that the substrate/acyl donor ratios varied 1:10, 1:2505 or 1:5000 μM, respectively. The quantitative analysis was performed by GC-FID using the same method previously described. The response was given by the conversion (%) of each alcohol in the most relevant time points. Chiral assessment was performed only for relevant samples. The DOE planning and statistical analysis were performed using the software Minitab® 19.2020.1 (64-bit).

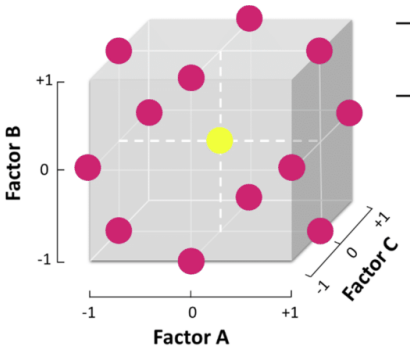
After obtaining the DOE results and predicted optimum conditions, a confirmation experiment was performed using the same terpene alcohols mixture and also a citronella oil sample (assay concentration: 0.3 mg/mL).

3. Results and discussion

In agreement with established practices in enzyme studies, characterisation steps were performed before commencing the targeted applications to confirm the enzymes' integrity and collect standard performance values to compare with further results. The substrates *p*-nitrophenyl octanoate or ethyl octanoate (lipases) and guaiacol (laccases) were selected for the activity assays, due to their suitability to both UV–Vis and GC analyses and also because they are F&F related compounds, with methods adapted from different works [29–34]. The commercial enzyme products investigated exhibited acceptable performance attributes (i.e., activities) under the conditions applied in this study, as demonstrated by the characterisation experiments (NanoDrop, SDS-PAGE and aqueous UV–Vis activity; Table 2). The NanoDrop quantification analysis indicates that the commercial products contain up to 59.6% w/w of protein. The molecular masses of the enzymes (ranging from 35 to 80 kDa) were estimated by the relative position of the bands of the samples and standards in the SDS-PAGE (Fig. S1), except for the immobilised lipase CALA for which no bands were observed, due to poor compatibility with the applied SDS-PAGE conditions. Moreover, the presence of minor bands in some enzyme samples, as evident from the SDS-PAGE results, indicates that the protein preparations contained low levels of oligomers or minor impurities, which did not necessarily present a problem as the enzyme activity of the samples were confirmed.

According to the slope of the UV–Vis calibration curves (Fig. S3A and S4A), the molar extinction coefficient of *p*-NP (pH 7.5, TX-100 0.5%) was found to be $1.15 \times 10^4\ M^{-1}cm^{-1}$ and for guaiacol (pH 5) was $\varepsilon = 4.9 \times 10^3\ M^{-1}cm^{-1}$. The surfactant TX-100 has an impact on the *p*-NP absorbance as clearly demonstrated by the wavelength scan results (Fig. S2), but its use in the lipases aqueous activity assays was necessary to disperse fatty acids released due to enzymatic hydrolysis of *p*-NP esters, avoiding precipitation or turbidity in the UV–Vis assay.

The enzymes' specific activities (U/mg) obtained in aqueous buffered medium were generally higher than the values reported by the manufacturers and literature (Table 1), which were obtained with other substrates and conditions. The lipase NZ-435 and the laccase NZ-51003 showed the highest activity values (U) in the aqueous assay and, therefore, were used to perform the activity reassessments in organic or organic/aqueous medium. A decrease of ≥ 94% in activity was observed



	Factors	Coded Values		
		-1	0	1
A	Temperature (°C)	25	45	65
B	Enzyme (mg/mL)	0.5	2.75	5
C	Acyl Donor (C%)*	0.2	50	99.8

Fig. 2. Diagram of the Box-Behnken DOE experiment for optimisation of the lipase CALA reaction with the terpene alcohols mixture, using vinyl acetate as acyl donor and hexane as solvent. *The variation of the factor C levels (-1, 0 and + 1) also changed the solvent (hexane) concentration (C%) to > 99%, 50 % and ~ 0% ("solvent free"), respectively.

Table 2
Enzyme characterisation results, including aqueous activity data (UV–Vis).

Parameters	Lipases					Laccases	
	CALA	NZ-435	LZ-TLIM	NC-ADL	LZ-CALBL	DL-IIS	NZ-51003
Enzyme activity (U) or Vmax (μmol/min)	56 ± 32	156 ± 30	78 ± 18	108 ± 19	11 ± 1	35 ± 21	286 ± 88
Specific activity* (U/mg)	62 ± 35	174 ± 33	87 ± 20	358 ± 64	38 ± 3	23 ± 14	286 ± 88
Reference specific activity (U/mg)**	1.59	7.92–10	0.25–0.69	6.0	5.0	0.12–33	1
Michaelis constant Km (μM)	219 ± 92	179 ± 51	1019 ± 206	79 ± 12	115 ± 15	715 ± 639	1308 ± 348
Turnover number or Kcat*** (min)	–	159	134	350	18	1644	19
Enzyme product amount in the assay (mg)	0.9	0.9	0.9	0.3	0.3	1.5	1
Assay volume (mL)	3	3	3	1	1	3	2
Activity assay nominal concentration (mg/mL)	0.30	0.30	0.30	0.30	0.30	0.5	0.5
SDS-PAGE molecular mass (kDa)	–	38	35	53	38	80	80
NanoDrop corrected enzyme amount used (mg)	0.02	0.11	0.09	0.02	0.02	0.89	0.07
NanoDrop protein concentration per enzyme product (% w/w)	2.4 ± 0.1	12.5 ± 0.1	9.7 ± 0.7	5.4 ± 0.02	7.7 ± 0.2	59.6 ± 0.2	6.9 ± 0.1

Values ± standard error of the mean (n = 3).
* Calculated using the enzyme product amount in the assay (mg).
** According to Table 1 data, obtained with different substrates. Specific activity values in U/g were converted to U/mg.
*** Calculated using the SDS-PAGE molecular mass (g/mol) and the corrected enzyme assay concentration as per NanoDrop results.

for both enzymes in organic medium (Fig. 3), with specific activities equal to 15 and 7 U/mg for the lipase NZ-435 and laccase NZ-51003, respectively, which were also higher than the reference data (Table 2).

The variability in enzyme activity data, compared to the literature values is expected when the assays are performed under different circumstances, as observed by the difference in reference activity values (Table 2) obtained from different sources [21–27] and other works using alternative media [47,48]. Moreover, as reviewed by Baltierra-Trejo et al. [49], there is a noticeable lack of consistency in the literature regarding the methods used for enzyme activity calculation, with final values ranging from 3.5 to 484,000 U/L for laccase, for example, which makes it difficult to determine accuracy. Differently from the present study, many works in the literature do not clarify how activity was calculated, sometimes not even properly detailing the experimental conditions.

The average percent coefficient of variation (%CV) for the UV–Vis and GC-FID measurements was ~ 11%. The main sources of variation observed for the UV–Vis analysis were due to small delays in starting the absorbance acquisition while pipetting the starting reagent to all the cuvettes (replicates), and the minor interferences of the immobilised enzymes particles with the absorbance measurements. For the GC-FID analysis, on the other hand, the main source of variation would be the sample preparation step, which for immobilised enzymes is minimal compared to liquid preparations in this case. Additionally, variations during the injection time can also occur due to loss of volatile analytes. These variations were minimised by using an automatic injection system, using internal standards, running the replicates consecutively in a sequence, and replacing the caps of the sample vials as soon as possible.

Historically, UV–Vis spectrophotometry has been the mainstay as an analytical method for determination of enzyme activity and in kinetics studies. Although this method often provides good sensitivity, precision, and continuous measurement (real-time monitoring of the reaction progress curve), there are some key limitations when performing procedures specifically applied to different classes of analytes. Some of these limitations can relate to the substrates' low solubilities in water, absence of a chromophore or maximum absorbance being in same region as that of the enzymes (usually in the near UV range) under the applied conditions. Lipase-catalysed reactions are a good example of these limitations as the transesterifications need to be performed in organic or other alternative media (e.g., ionic liquids), since hydrolysis is the enzymes' preferential reaction in aqueous medium.

Thus, the GC-based procedures applied here, although being a non-continuous measurement system and sometimes requiring extra sample preparation steps, are useful alternatives to overcome these UV–Vis limitations, especially for aroma compound studies where sensitivity and specificity of the assay is important. Moreover, the collection of aliquots and post-reaction GC analysis are more time effective, enabling the conclusion of several experiments and replicates at the same time, which is difficult to perform with UV–Vis assays where an immediate start of acquisition is required. In addition, GC analysis offers the possibility of acquiring more information from the single analytical

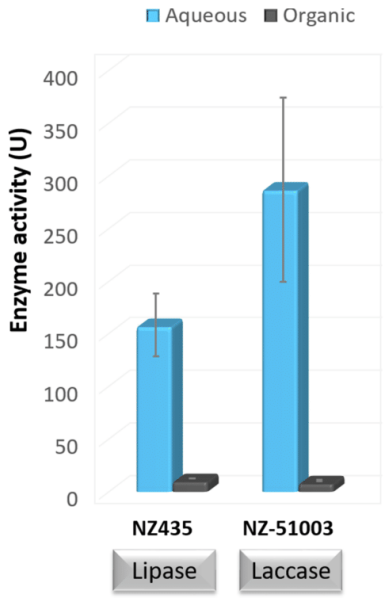


Fig. 3. Comparison between enzyme activity (U) results in buffered aqueous (in blue) and organic media (in black), with UV–Vis and GC-FID assessments respectively. Lipase (NZ-435) buffered aqueous activity was based on the hydrolysis of p-NPO and the organic medium (hexane) activity was the transesterification of p-NP and ethyl octanoate (producing p-NPO). Laccase (NZ-51003) activity in both media was based on guaiacol oxidative coupling, with the organic medium being acetone/water 50%. A significant drop in activity was observed for both the enzymes in the organic medium experiment.

technique (Fig. 4), such as the determination of enzyme activity, reaction yield, product identities and enantiomeric ratios.

The GC-FID methods developed in the present work have served the purpose of having a satisfactory separation for all the standards analysed in the shortest time, enabling the time course assessment of the substrate's conversion and determination of reaction profiles. Further optimisations may be required for more complex samples applications. The MEGA-DEX DET Beta FAST column coupled with the guard column (DB-5) provided the best results among the tested chiral columns (Fig. S7), separating all the enantiomers in the test mixture using a lower flow rate and shorter analysis time, therefore was the one selected for this study. The *e*GC-FID (enantioselective GC-FID separation) method was successful in providing the chiral ratio profile of the reaction for each time aliquots (Fig. 5), although the lipases did not show significant chiral selectivity and the laccases did not react with the chiral substrates under any of the conditions applied.

Among the aroma standards tested with each type of enzyme in the screening phase (GC-FID), the most significant results were observed for citronellol acylation (lipases; Fig. 6A) and eugenol oxidation (laccases;

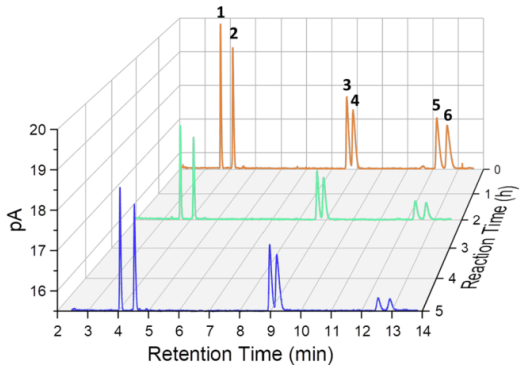


Fig. 5. Chromatograms (*e*GC-FID) of different time points of the reaction between the lipase CALA and the aroma standards mixture containing (*R*)-(-)-linalool (**1**), (*S*)(+)-linalool (**2**), (1*R*,2*S*,5*R*)(-)-menthol (**3**), (1*S*,2*R*,5*S*)(+)-menthol (**4**), (*R*)(+)-citronellol (**5**) and (*S*)(-)-citronellol (**6**). The results indicate that citronellol enantiomers (**5** and **6**) are converted in equal ratios, demonstrating this enzyme is not enantioselective. The internal standard (*n*-C10) retention time was 1.08 min in the MEGA DEX DET Beta FAST column and the coefficient of variation (%CV) of the peak area was ~ 7%. Reaction and analysis conditions according to the screening experiments.

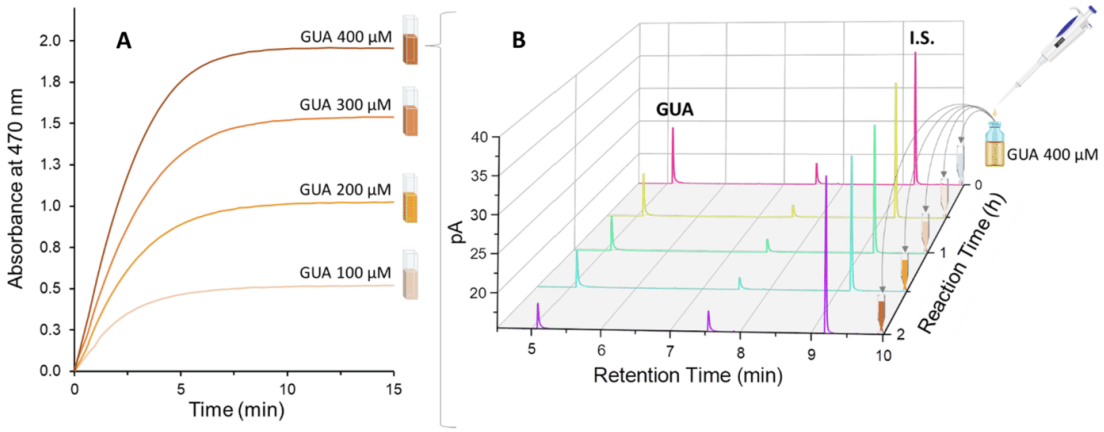


Fig. 4. Comparison between (A) UV–Vis, and (B) GC-FID profiles for the reaction of guaiacol (GUA) and the laccase (NZ-51003) in aqueous and semi-organic medium, respectively. The chromatograms (DB-5 column) provide more information for each time point aliquot, enabling to monitor the conversion of the substrate(s), the appearance of product(s) and the variation of the internal standard (I.S.; methyl eugenol). The UV–Vis line chart (A) essentially plots the average data points ($\overline{CV}\% = 3.7$) as smooth profiles of the absorbance readings for the reactions at different concentrations. The illustrations to the right of plots (A) and (B) show cuvettes and GC glass micro-inserts with a representation of the colours of the solutions.

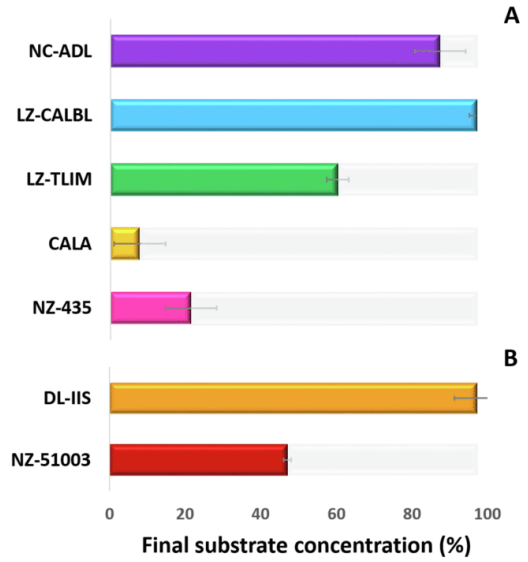


Fig. 6. Maximum conversion of the substrates (A) citronellol by lipases after 5 h reaction, and (B) eugenol by laccases after 24 h reaction at 25 °C in organic medium. The lipases CALA and NZ-435, and the laccase NZ-51003 were the most efficient in converting the substrates in the applied conditions. Reaction and analysis conditions according to the screening experiments.

Fig. 6B).

The tested laccases demonstrated activity only towards eugenol among the selected aroma standards under the evaluated conditions, with NZ-51003 and DL-IIS converting 50% and 3% of eugenol after 24 h reaction, respectively. The compounds investigated as potential mediators apparently did not improve the efficiency of the oxidation reactions towards the aroma standards in the applied settings. However, the enzymes were active towards the tested mediators with emphasis on vanillin, for which a total conversion (100%) was achieved after 4 h reaction time with NZ-51003 in acetone/water 50% medium.

As evident from these results, the laccases performances vary considerably with different substrates and reaction media. Further experiments will be necessary before the optimisation stage to confirm the suitability of the selected parameters for a specific substrate. Although performing laccase catalysed reactions in an organic medium remains challenging, the use of organic solvents/water systems was demonstrated and reviewed by different authors with a pre-incubation of some laccases in acetone having a positive effect on the activity [46–48]. A broader screening of substrates and mediators in different organic solvent systems, as well as the immobilisation of the enzymes could help overcoming the current application challenges of employing organic medium.

The evaluated lipases demonstrated a preference for the primary alcohol (citronellol) and no conversion of the tertiary alcohol (linalool) was observed in any of the conditions tested in the screening or optimisation stage. The immobilised enzymes CALA and NZ-435 had the most significant performance, converting 92% and 78% of citronellol after 5 h of reaction in the screening phase, respectively. CALA was also active towards the secondary alcohol (menthol), with a conversion of 17% after 5 h of reaction. It is interesting to observe that, even though CALA was not the most active enzyme in the characterisation stage, it outperformed NZ-435 in the application to aroma-related compounds under the conditions applied here. This fact illustrates, once again, how different substrates and conditions can provide different outcomes in these biocatalytic reactions.

The acyl donor/solvent experiment with CALA and the same terpene alcohols mixture revealed that vinyl acetate/hexane (1:1) outperformed the triacetin/ethyl acetate (1:1) system (Fig. 7) and therefore, it was chosen to perform the optimisation stage. In this experiment, vinyl acetate enabled the conversion of 100% of citronellol and 40% menthol in 24 h. Triacetin is still a good (and greener) alternative to vinyl acetate if only primary alcohols are targeted, since 82% conversion was achieved for citronellol in 24 h reaction.

In the optimisation stage, the statistical analysis of the DOE data reveals that it is a normal distribution, as indicated in the residuals plots (Appendix S8B and S9B) by the bell-shaped overall pattern in the histograms and the points in the normal probability plots all following the fitted regression line very closely. This is confirmed by the model summary tables (Appendix S8A and S9A), which indicates the model fits the data adequately (R^2 = 96.29% citronellol; 90.54% menthol) and reasonably describes the response (S = 11.14 citronellol; 5.19 menthol). Moreover, the residuals versus fit plots show the points are randomly distributed on both sides of the zero line with no recognisable patterns, which means the variance of the residuals is constant. The residuals versus order plots indicate that the points fall randomly around the centre line and, therefore, are independent from one another.

The Pareto charts (Appendix S8C and S9C) display the absolute values of standardised effects (from the largest to the smallest) and a reference line (in red) which delimits the significance level for the variables. The results indicate that the factor B (enzyme concentration), as well as the interactions between factors AC, AA, CC have a statistically significant effect in the response (conversion %) for both analytes (citronellol and menthol) to different extents, since the bars cross the reference line. The significance level is given by p-value $\leq \alpha$ (0.05). This is confirmed by the interaction plots (Appendix S8D and S9D) in which the relationship between factors is demonstrated by the crossing of lines. It is possible to observe that factor C (acyl donor concentration) has the most significant interaction with factor A (temperature), followed by factor B (enzyme concentration). The main effects plots (Appendix S8E and S9E) and the response surface plots (Fig. 8) curvatures indicate the model contains statistically significant quadratic term coefficients, which are the temperature and the acyl donor concentration.

The conversion (%) of citronellol and menthol obtained from each of the DOE reaction aliquots, taken at different times, revealed that the maximum consumption of citronellol (100%) was achieved in 1 h of reaction. For menthol, a maximum conversion of 25% was observed after 1 h and 60% after 24 h. The response surface plots (Fig. 8) and contour plots (Supplementary Information, Appendix. S8F and S9F) demonstrate that these maxima were obtained when the reaction was performed at around 40 °C, using 4 to 5 mg/mL of the enzyme and 60% of acyl donor in the system. The response optimisation model (Appendix S10) predicted that the optimal conditions for the reaction with both substrates, would be 37 °C, 3 mg/mL enzyme and 58.5% (v/v) vinyl acetate (acyl donor/solvent ratio around 1:1), which would give a maximum conversion of 100% for citronellol and 25% or 55% for menthol in a 1 h or 24 h reaction time, respectively.

The additional experiment using the model predicted optimised conditions yielded a conversion of > 95% for citronellol after 1 h (in both, the standards mixture and citronella oil), and 20% or 74% for menthol in the standards mixture after 1 h or 24 h, respectively. The other major alcohol in citronella oil (geraniol) was also completely converted (100%) after 1 h reaction. These results, therefore, confirm that the optimised conditions fall within 37–40 °C, 3–4 mg/mL of the enzyme and 58–60% of acyl donor and suit both the analytes (primary and secondary monoterpene alcohols). Applications to more complex essential oil samples may be tested using a similar single experimental condition for likewise conversion of chemical constituents.

4. Conclusion

The development of efficient enzyme biocatalysed methods has the

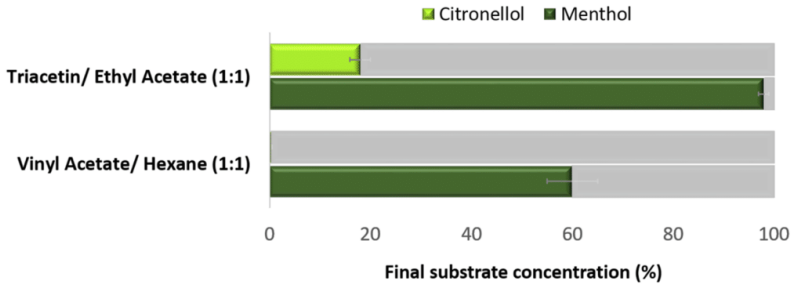


Fig. 7. Lipase (CALA) conversion of citronellol and menthol with the different acyl donor/solvent systems after 24 h reaction at 25 °C. The reaction with vinyl acetate/hexane (1:1) had the highest conversion for the both substrates in the applied conditions. Reaction and analysis conditions according to the screening experiments.

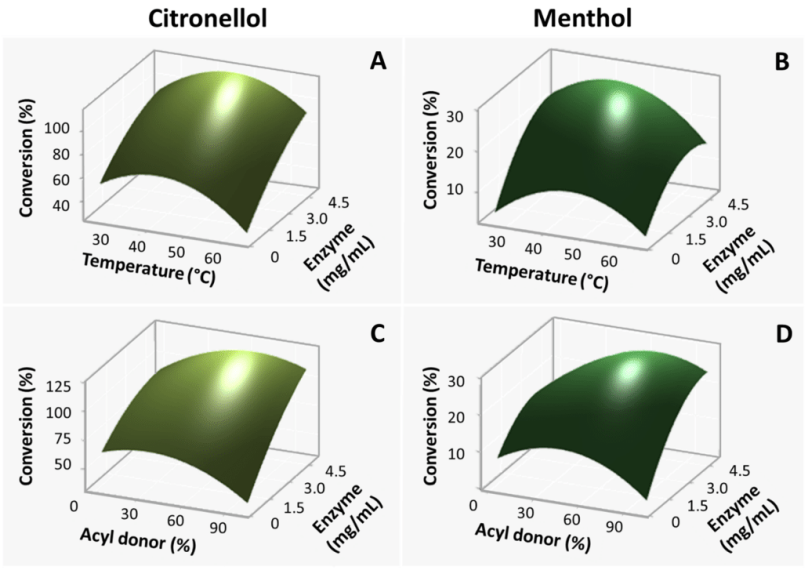


Fig. 8. Response surface plots of the DOE optimisation of the reactions with the lipase CALA and the substrates citronellol and menthol. The graphs (A and B) demonstrate the interaction between the pairs of variables enzyme concentration *vs* temperature for each substrate, and the graphs (C and D) demonstrate the interaction between enzyme concentration *vs* acyl donor concentration for each substrate. The hold values for the third variables were acyl donor concentration (50%) and temperature (45 °C), respectively.

potential to boost the applications and discovery of novel odour-active molecules and the production of high-value compounds from more environmentally-friendly processes. The selection of relevant variables for the above described GC-based characterisation, screening and optimisation strategies was achieved by combining the knowledge from precedent studies, the specificities of each biocatalyst and the nature of the desired application, such as the chemical properties of the targeted substrates and products (e.g., chemical class, polarity, solubility) and taking green chemistry principles into consideration.

UV–Vis spectrophotometric determination of the enzyme activities in aqueous medium enabled the confirmation of enzyme activity and protein concentration, setting the ground for the development of GC-based methods. Different to the most common analytical methods used in enzyme reactions and activity studies (titration or UV–Vis spectrophotometry), GC provides the direct quantification of substrates and products without the use of chemical indicators. Moreover, GC analysis offers the possibility for structural identification of reaction products, when using mass spectrometry as an on-line detection technique (GC–MS), and the assessment of the enantioselectivity of the reactions, which is not possible to achieve with such precision and specificity when non-chromatographic methods are used. Enantioselective GC and isotope ratio mass spectrometry (IRMS) are additional techniques applied for authentication of F&F samples, enabling the source-specific discrimination of compounds produced in bioprocesses either *in vivo* (e.g. plants, microorganisms) or *in vitro* (e.g. isolated enzyme reactions). Moreover, the use of stable isotope analogues of selected compounds in enzyme assays offers both quantification and insights into kinetic effects in the mechanisms of enzyme-catalysed reactions and their metabolic pathways [50].

Aroma compounds are volatile odour-active substances, usually containing up to 20 carbon atoms and with limited solubility in water, which means organic medium and mild temperatures (to avoid evaporation) may be more suitable for their biocatalytic transformations. The results obtained for the lipases in the optimisation phase confirm this hypothesis. These findings also agree with precedent studies which indicated the use of vinyl acetate/ hexane as the most efficient system [6,9–14] and the enzymes' preference for primary alcohols. Although secondary and tertiary alcohols are challenging substrates (i.e. less reactive) in lipase-catalysed transesterifications, in the present study we have successfully demonstrated the conversion (up to 74%) of a secondary terpene alcohol (menthol) and 100% conversion of a primary terpene alcohol (citronellol). The selected laccases reacted directly with

phenolic compounds only, including aroma standards and mediators. Among the enzymes tested, the best ones for application to the selected aroma compounds in the conditions investigated were the lipases CALA and NZ435, and the laccase NZ-51003. Further optimisations and applications of this approach to more complex samples, such as essential oils, will be explored in future studies with advanced GC separations.

CRediT authorship contribution statement

Michelle S.S. Amaral: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Milton Hearn:** Conceptualization, Writing – review & editing, Supervision, Resources. **Philip J. Marriott:** Conceptualization, Writing – review & editing, Supervision, Resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2022.123412.

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2.2. Supplementary Information

Quantitative assessment of enzymatic processes applied to flavour and fragrance standard compounds using gas chromatography–flame ionisation detection

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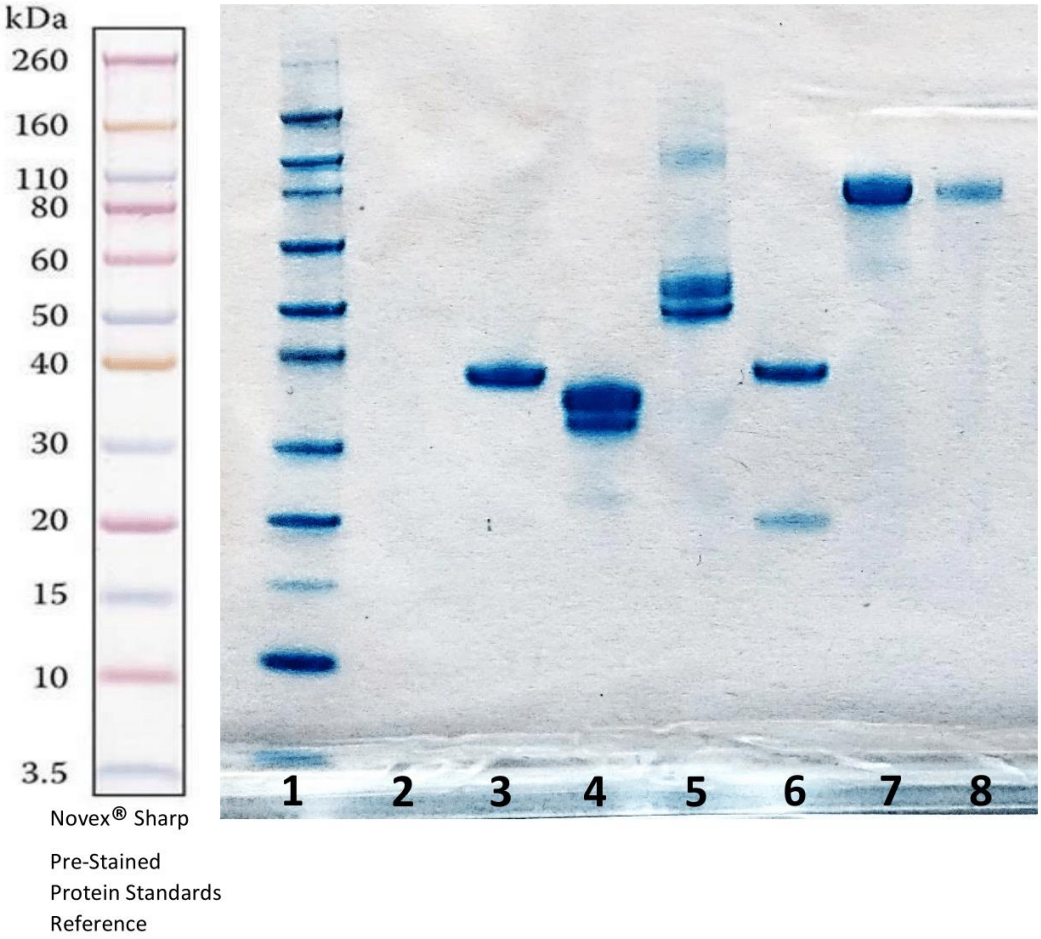


Figure S1. SDS-PAGE analysis of the protein molecular weight reference compounds (lane 1; 3.5 to 260 kDa;) and enzyme products tested CALA (lane 2; not observed), NZ-435 (lane 3; ~38 kDa), LZ-TLIM (lane 4; ~35 kDa), NC-ADL (lane 5; ~53 kDa), LZ-CALBL (lane 6; ~38 kDa), DL-IIS (lane 7; ~80 kDa) and NZ-51003 (lane 8; ~80 kDa).

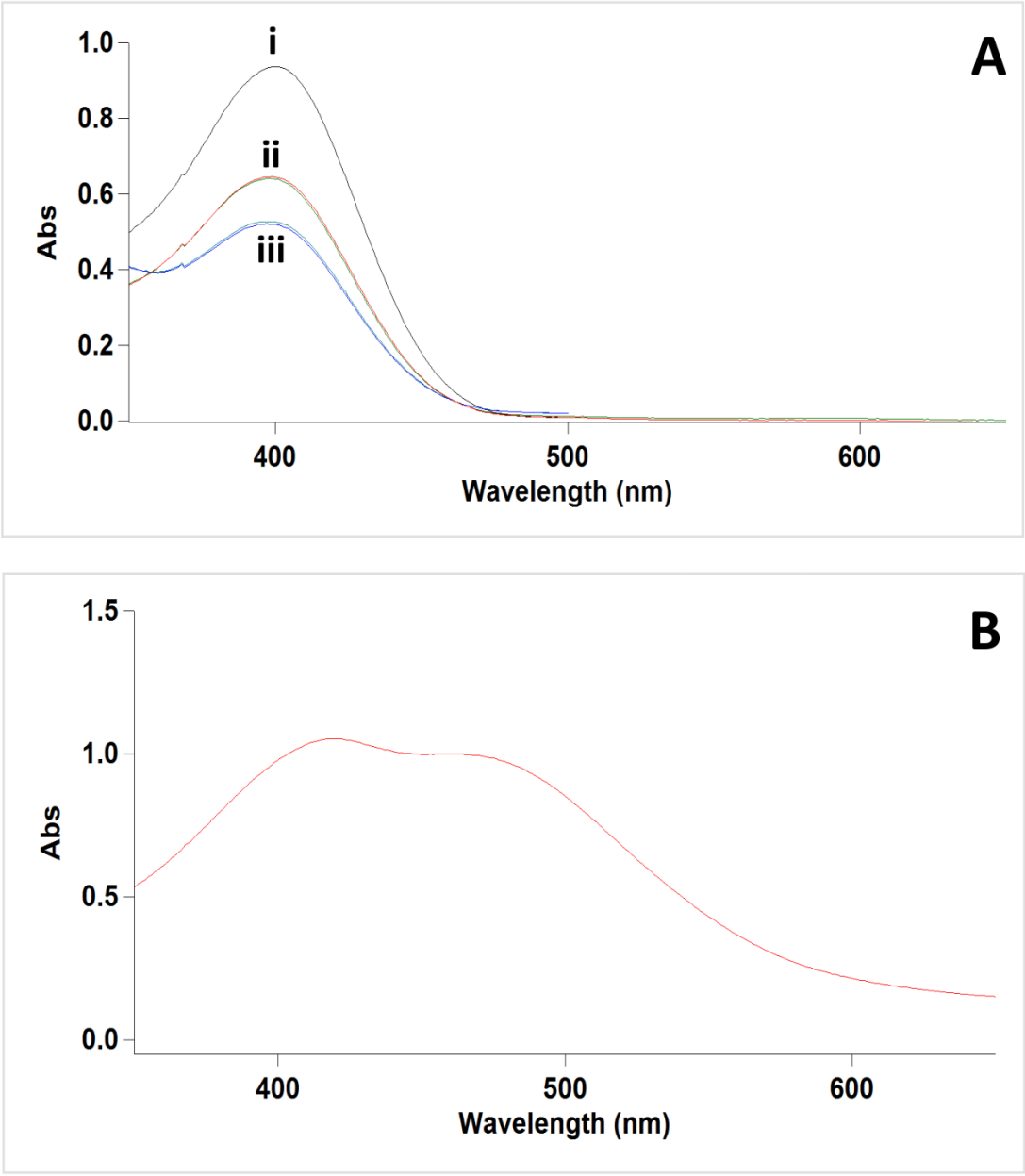


Figure S2. UV-vis wavelength scans (350-650 nm) for (A): the substrate *para*-nitrophenol (*p*-NP; λ_{max} 400 nm), including solutions (i) without the surfactant TX-100, and with (ii) 0.5% TX-100, and (iii) 1% TX-100. (B): the scan for guaiacol (GUA; λ_{max} 470 nm).

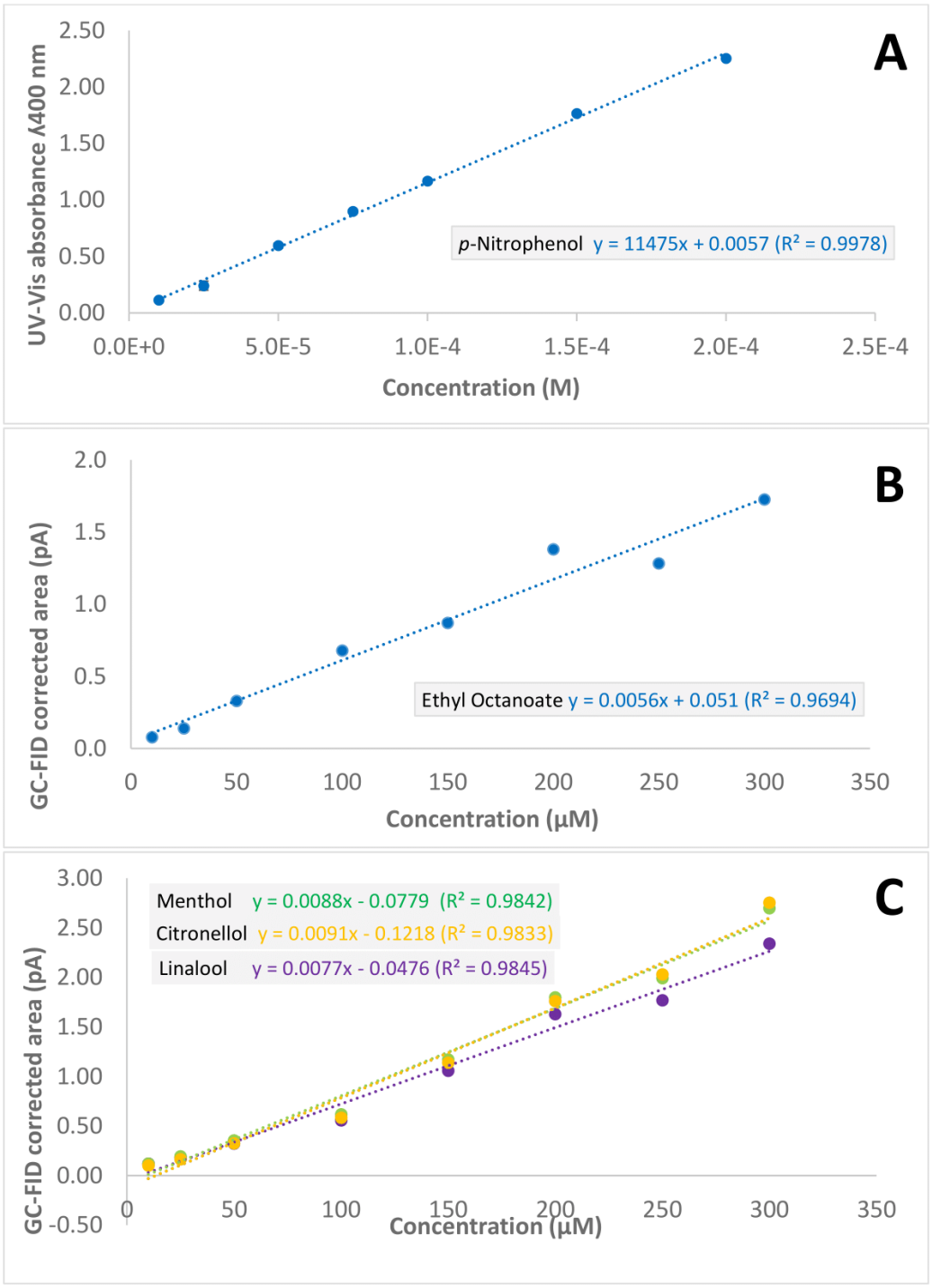


Figure S3. Calibration curves for lipase activity assays using (A): UV-Vis (*p*-NP in buffer pH 7.5), and (B): GC-FID (ethyl octanoate in hexane/vinyl acetate 50%) and (C): aroma standards screening mixture (citronellol, menthol, linalool in hexane/vinyl acetate 50%). Concentrations 10, 25, 50, 100, 150, 200, 250, 300 µM.

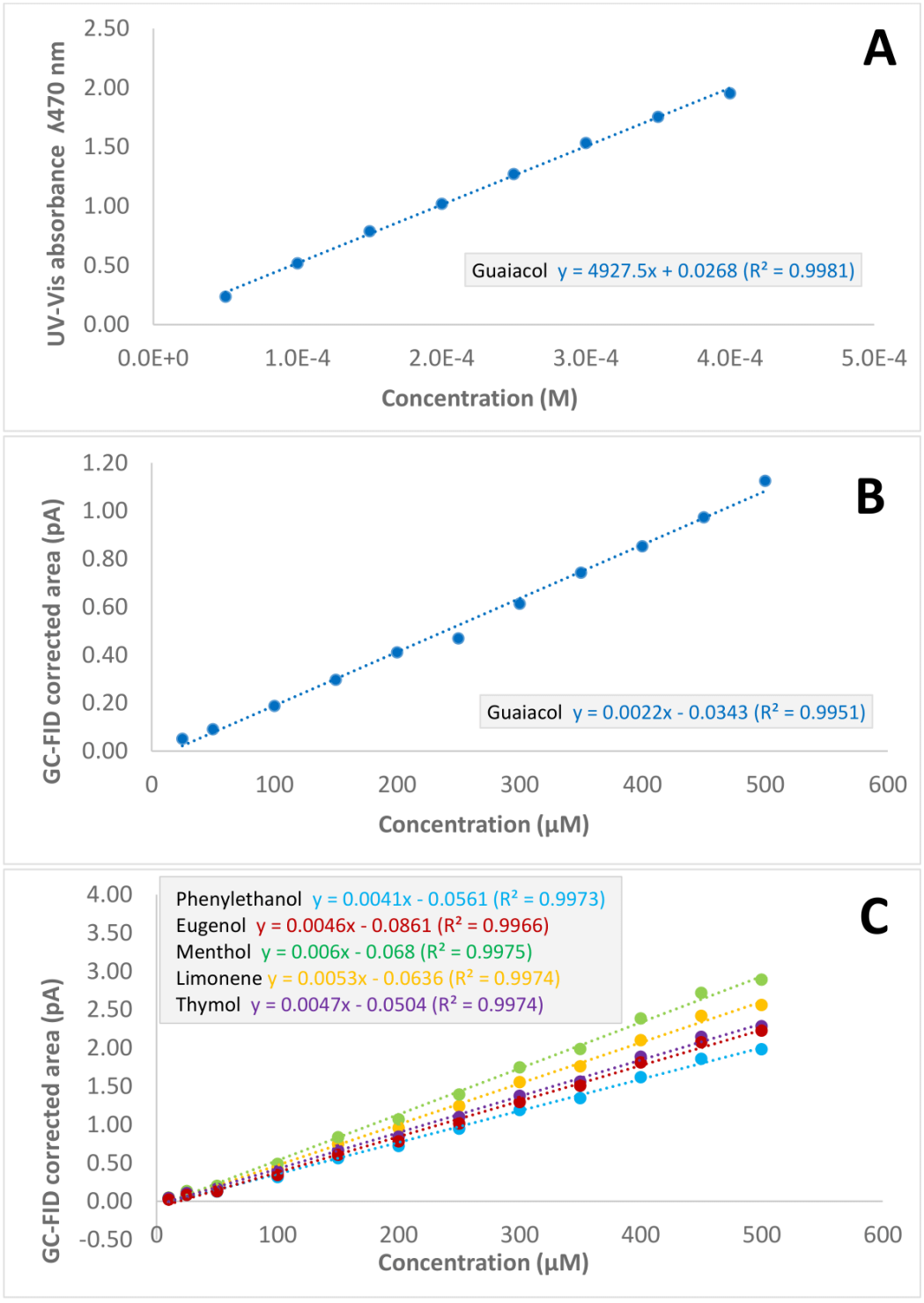


Figure S4. Calibration curves for laccase activity assays using (A): UV-Vis (GUA in buffer pH 5), and (B): GC-FID (GUA in acetone/water 50%) and (C): aroma standards screening mixture (phenylethanol, eugenol, menthol, limonene and thymol in acetone/water 50%). Concentrations 10, 25, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500 µM.

5

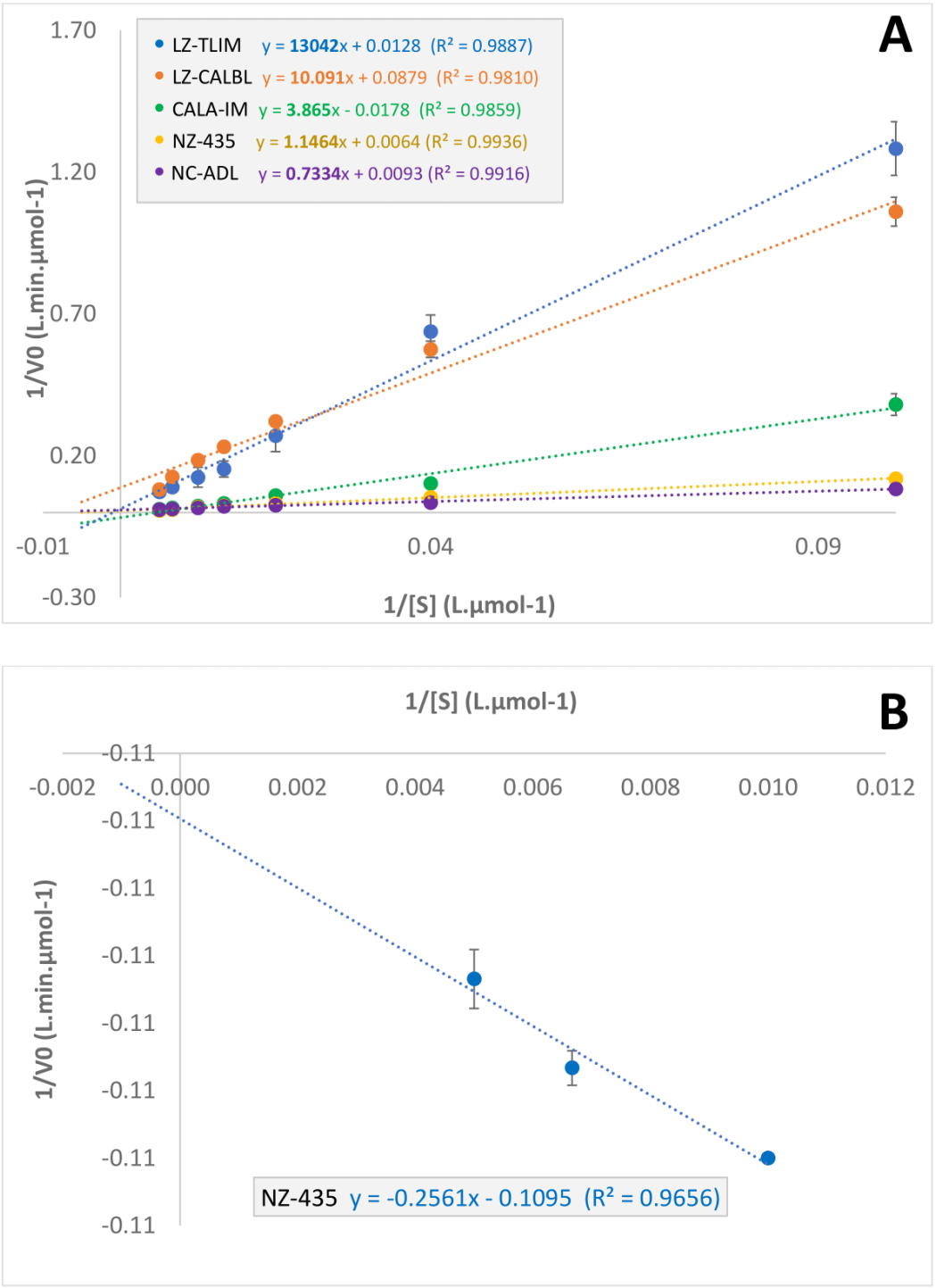
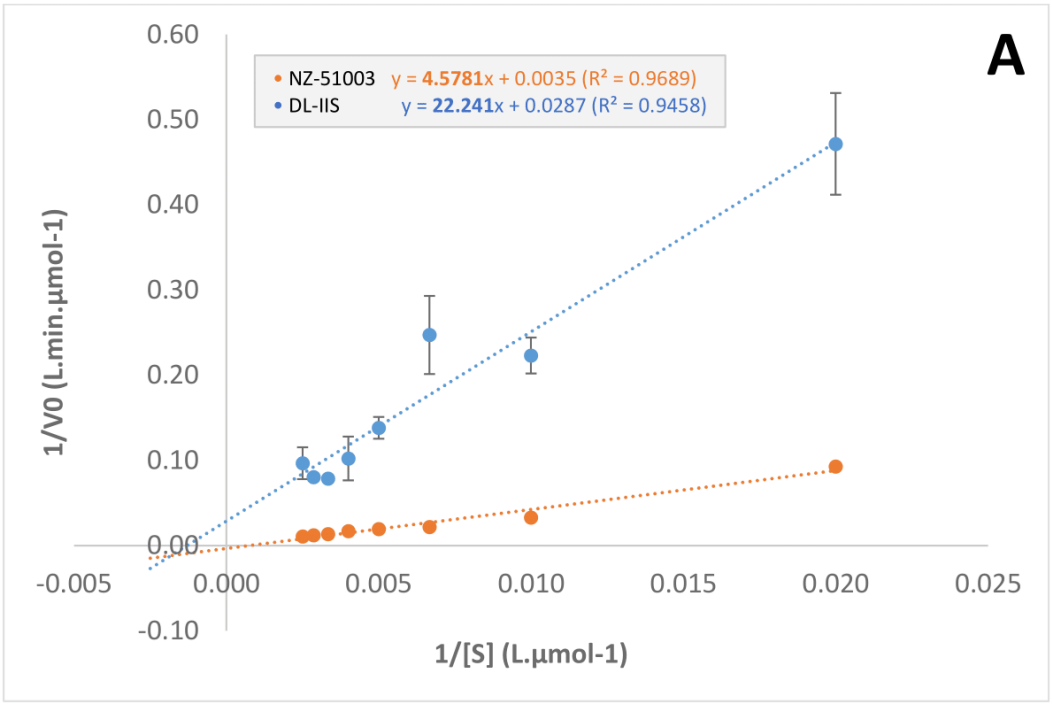


Figure S5. Lineweaver-Burk plots for the lipase-catalysed reactions with (A): *p*-NPO; UV-Vis in aqueous medium at pH 7.5, and (B): for the reactions with *p*-NP and ethyl octanoate using GC-FID, in organic medium (hexane/vinyl acetate 50%).



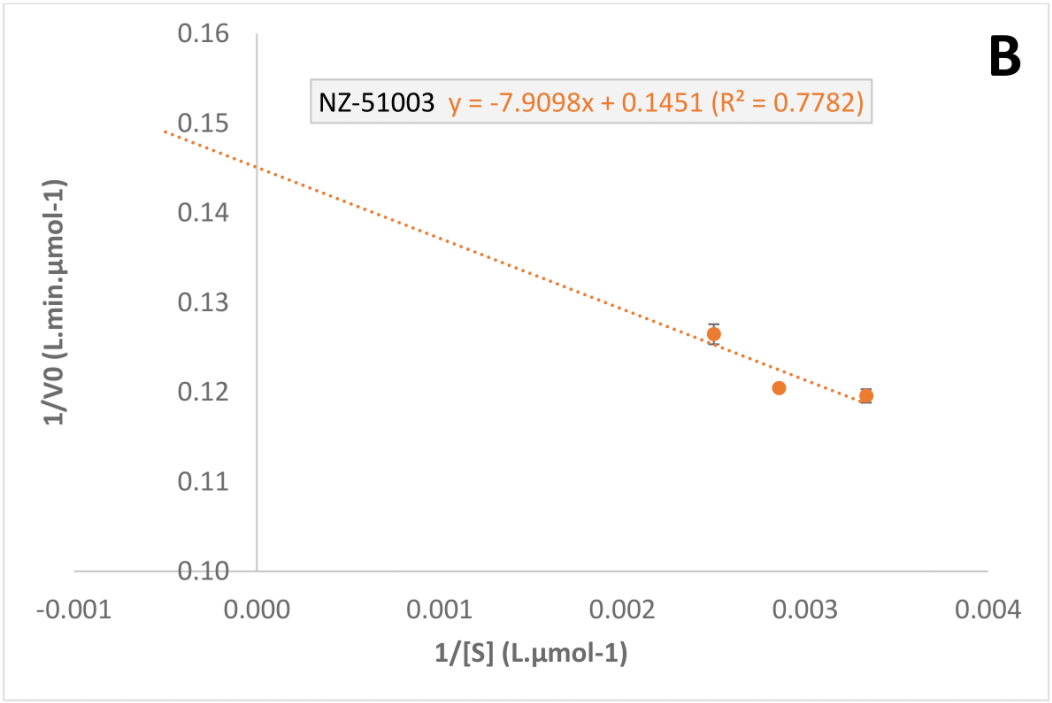


Figure S6. Lineweaver-Burk plots for the laccase-catalysed reactions with GUA in (A): aqueous medium, UV-Vis; pH 5, and (B): in organic medium, hexane/vinyl acetate 50%; GC-FID.

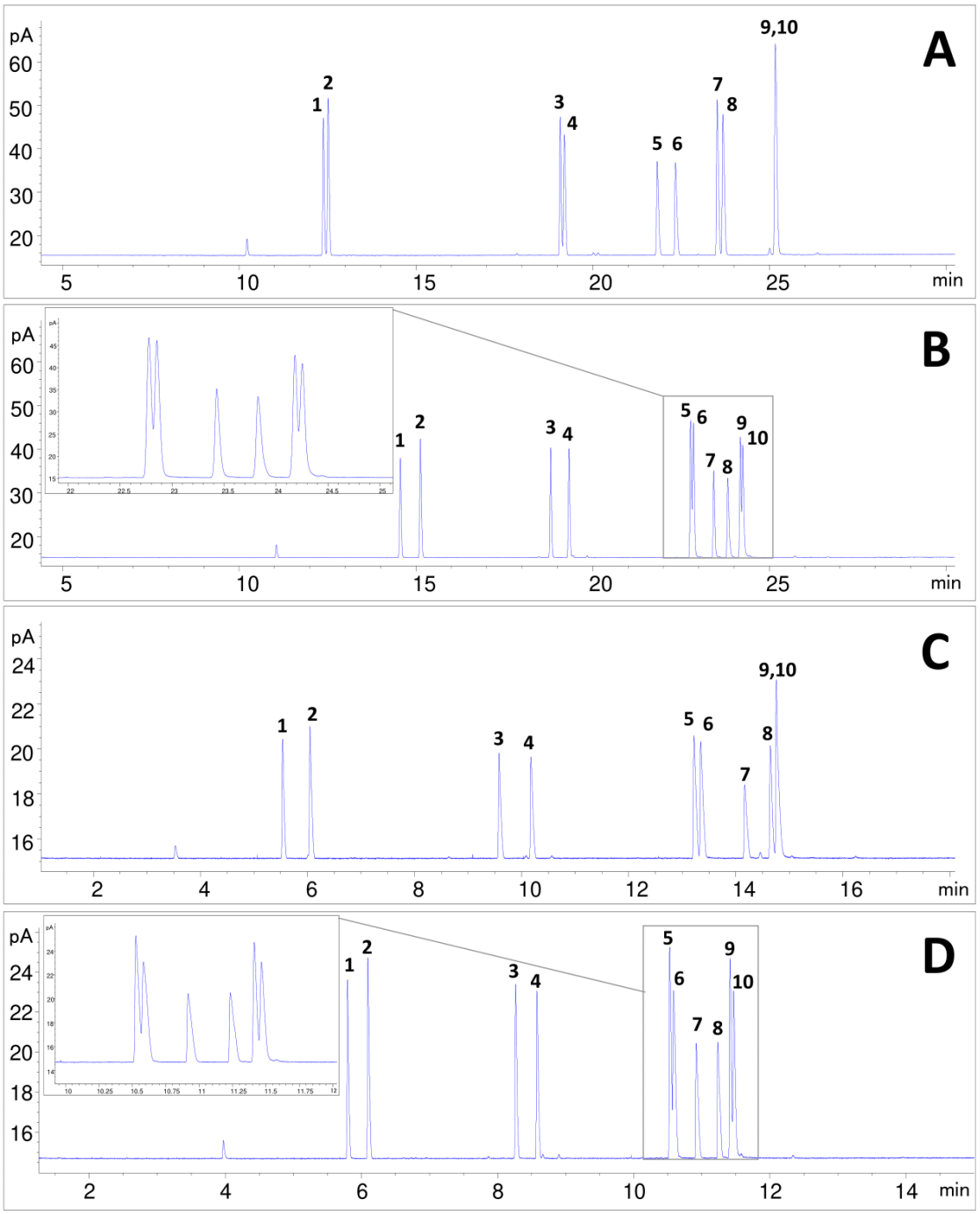


Figure S7. GC-FID chiral chromatogram separations of the chiral mixture (-)-Limonene (1), (+)-Limonene (2), (-)-Linalool (3), (+)-Linalool (4), (-)-Menthol (5), (+)-Menthol (6), (+)-Phenylethanol (7), (-)-Phenylethanol (8), (+)-citronellol (9), (-)-citronellol (10) using different columns: (A): Astec CHIRALDEX B-PM, (B): MEGA-DEX DET Beta, (C): MEGA-DEX DET Beta FAST without guard column,and (D): MEGA-DEX DET Beta FAST with guard column DB-5. Column dimensions and analysis conditions are presented in the Experimental section.

Appendix S8. DOE report for citronellol conversion (%) after 1 h reaction with CALA

Response Surface Regression

A

Coded Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	97.00	6.43	15.08	0.000	
Temperature	-5.38	3.94	-1.36	0.230	1.00
Enzyme	25.00	3.94	6.35	0.001	1.00
Acyl donor	-1.13	3.94	-0.29	0.787	1.00
Temperature*Temperature	-24.62	5.80	-4.25	0.008	1.01
Enzyme*Enzyme	-7.37	5.80	-1.27	0.259	1.01
Acyl donor*Acyl donor	-16.63	5.80	-2.87	0.035	1.01
Temperature*Enzyme	5.50	5.57	0.99	0.369	1.00
Temperature*Acyl donor	-42.25	5.57	-7.59	0.001	1.00
Enzyme*Acyl donor	11.00	5.57	1.98	0.105	1.00

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
11.1378	96.29%	89.62%	42.14%

Regression Equation in Uncoded Units

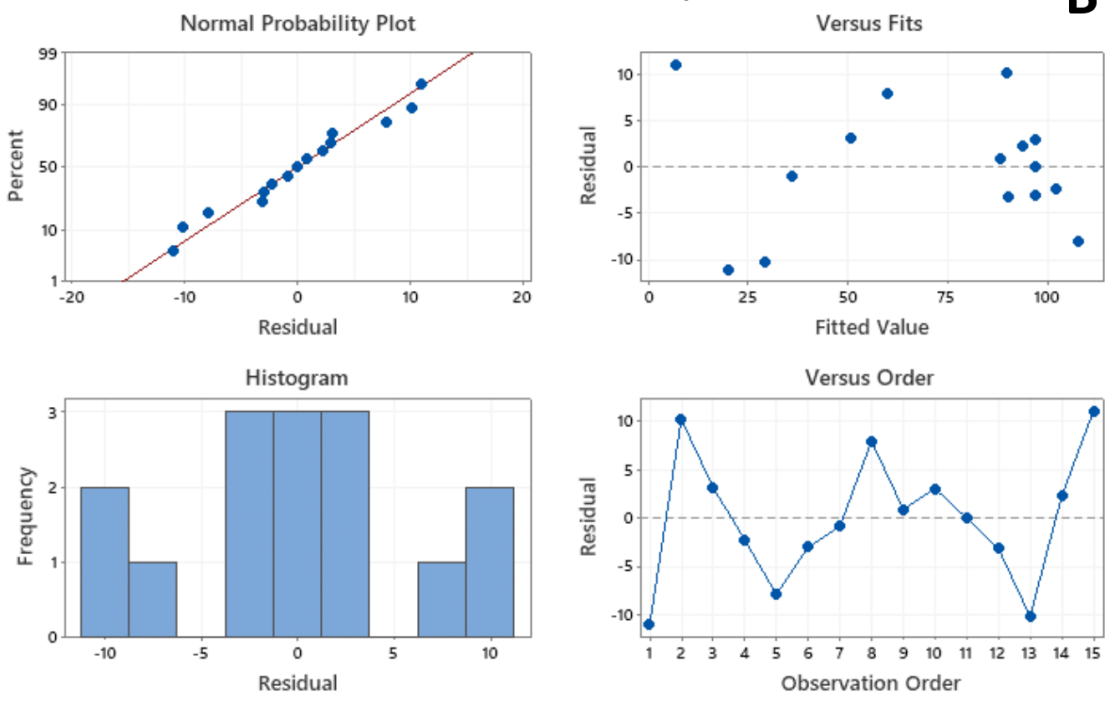
Citronellol Conversion (%) = -139.6 + 7.06 Temperature + 8.71 Enzyme + 2.287 Acyl donor - 0.0616 Temperature*Temperature - 1.46 Enzyme*Enzyme - 0.00670 Acyl donor*Acyl donor + 0.122 Temperature*Enzyme - 0.04242 Temperature*Acyl donor + 0.0982 Enzyme*Acyl donor

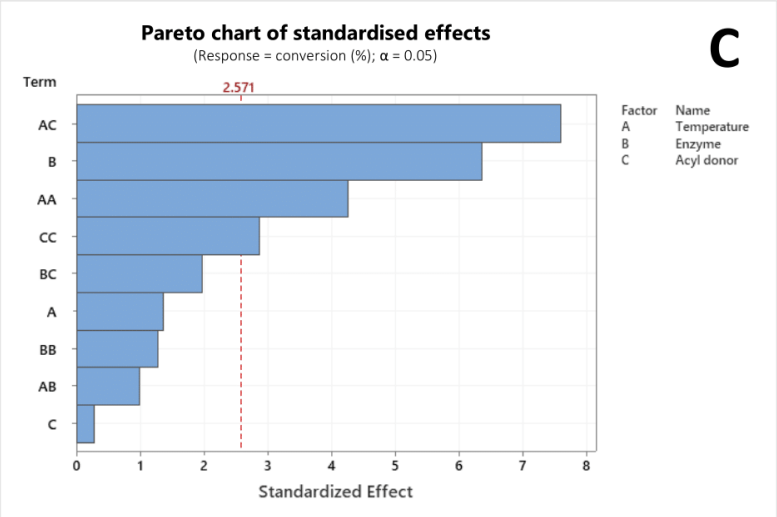
Analysis of Variance

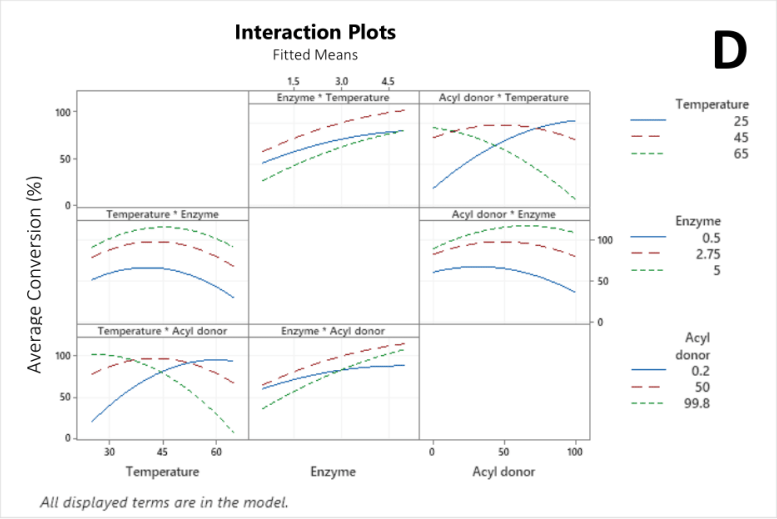
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	16104.7	1789.41	14.42	0.004
Linear	3	5241.3	1747.08	14.08	0.007
Temperature	1	231.1	231.12	1.86	0.230
Enzyme	1	5000.0	5000.00	40.31	0.001
Acyl donor	1	10.1	10.12	0.08	0.787
Square	3	3118.2	1039.39	8.38	0.021
Temperature*Temperature	1	2239.0	2238.98	18.05	0.008
Enzyme*Enzyme	1	200.8	200.83	1.62	0.259
Acyl donor*Acyl donor	1	1020.5	1020.52	8.23	0.035
2-Way Interaction	3	7745.2	2581.75	20.81	0.003
Temperature*Enzyme	1	121.0	121.00	0.98	0.369
Temperature*Acyl donor	1	7140.2	7140.25	57.56	0.001
Enzyme*Acyl donor	1	484.0	484.00	3.90	0.105
Error	5	620.3	124.05		
Lack-of-Fit	3	602.3	200.75	22.31	0.043
Pure Error	2	18.0	9.00		
Total	14	16724.9			

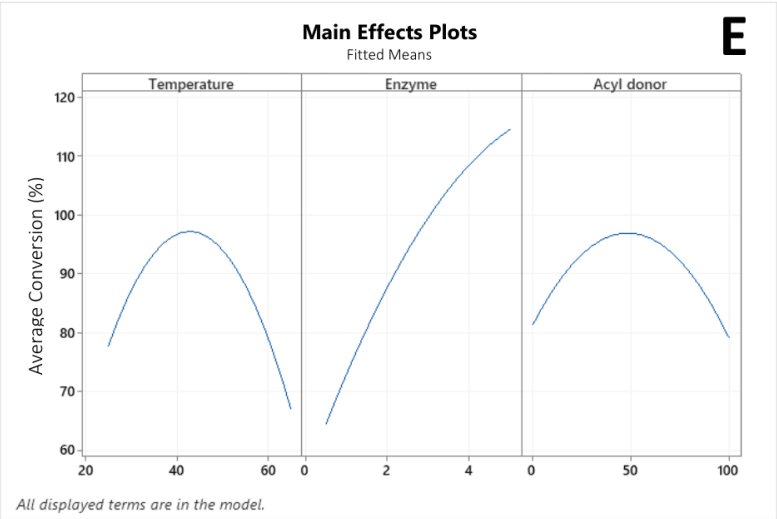
Residual Plots

B

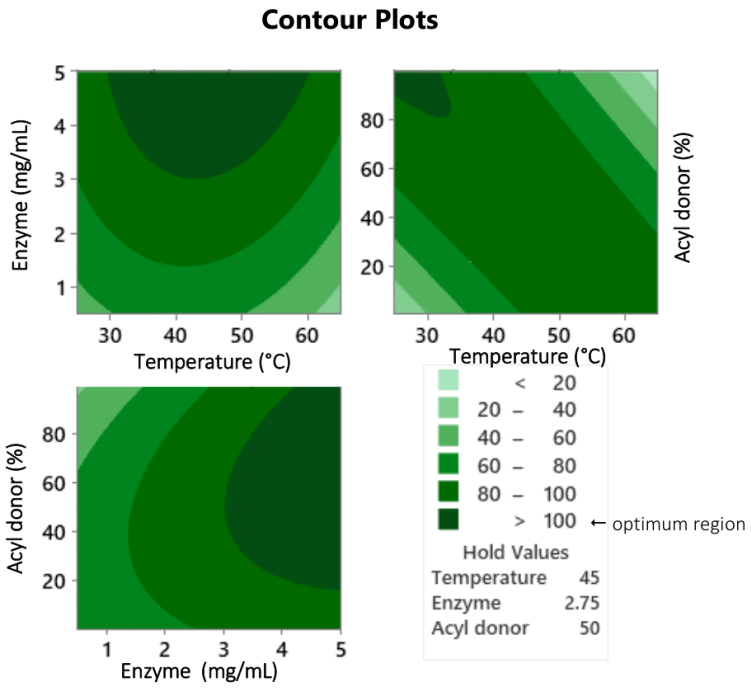



Pareto chart of standardised effects
(Response = conversion (%); α = 0.05)
C
Term
2.571
AC
B
AA
CC
BC
A
BB
AB
C
0
1
2
3
4
5
6
7
8
Standardized Effect
Factor Name
A Temperature
B Enzyme
C Acyl donor


Interaction Plots
Fitted Means
D
1.5
3.0
4.5
Enzyme * Temperature
Acyl donor * Temperature
Temperature * Enzyme
Acyl donor * Enzyme
Temperature * Acyl donor
Enzyme * Acyl donor
Average Conversion (%)
Temperature
Enzyme
Acyl donor
Temperature
25
45
65
Enzyme
0.5
2.75
5
Acyl donor
0.2
50
99.8
All displayed terms are in the model.


Main Effects Plots
Fitted Means
E
Temperature
Enzyme
Acyl donor
Average Conversion (%)
All displayed terms are in the model.

F


Contour Plots
Enzyme (mg/mL)
Temperature (°C)
Acyl donor (%)
Temperature (°C)
Acyl donor (%)
Enzyme (mg/mL)
< 20
20 – 40
40 – 60
60 – 80
80 – 100
> 100
← optimum region
Hold Values
Temperature 45
Enzyme 2.75
Acyl donor 50

11

Appendix S9. DOE report for menthol conversion (%) after 1 h reaction with CALA

Response Surface Regression

A

Coded Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	24.33	3.00	8.11	0.000	
Temperature	-1.75	1.84	-0.95	0.385	1.00
Enzyme	7.00	1.84	3.81	0.013	1.00
Acyl donor	1.25	1.84	0.68	0.527	1.00
Temperature*Temperature	-8.04	2.71	-2.97	0.031	1.01
Enzyme*Enzyme	-5.54	2.71	-2.05	0.096	1.01
Acyl donor*Acyl donor	-7.54	2.71	-2.79	0.039	1.01
Temperature*Enzyme	-1.75	2.60	-0.67	0.531	1.00
Temperature*Acyl donor	-8.75	2.60	-3.37	0.020	1.00
Enzyme*Acyl donor	3.75	2.60	1.44	0.209	1.00

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
5.19936	90.54%	73.51%	0.00%

Regression Equation in Uncoded Units

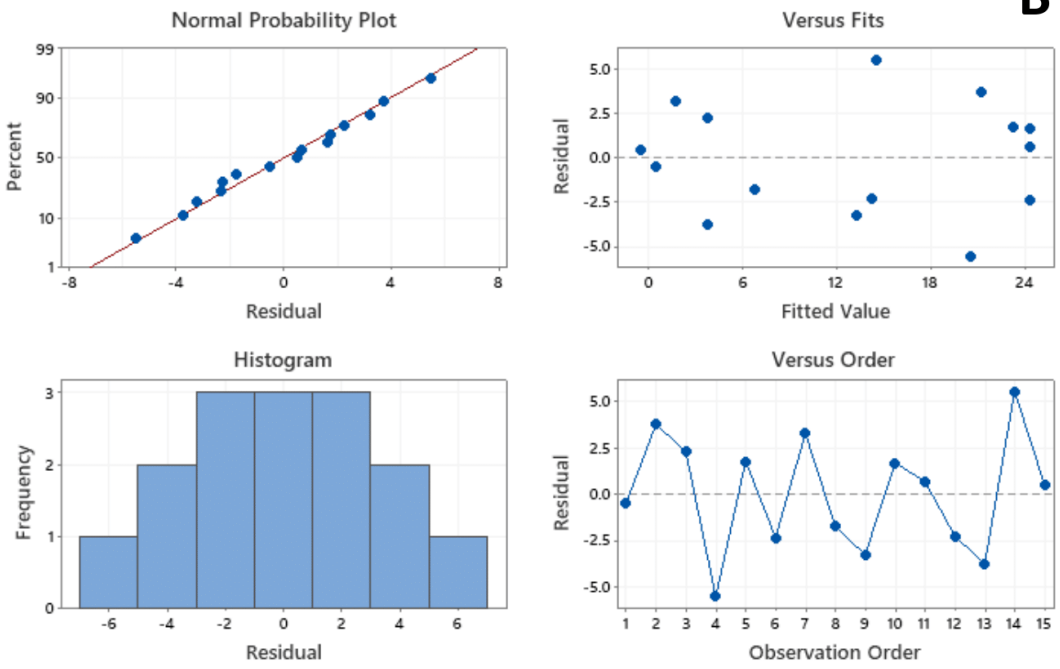
Menthol Conversion (%) = -58.1 + 2.268 Temperature + 9.21 Enzyme
+ 0.632 Acyl donor
- 0.02010 Temperature*Temperature
- 1.095 Enzyme*Enzyme
- 0.00304 Acyl donor*Acyl donor
- 0.0389 Temperature*Enzyme
- 0.00879 Temperature*Acyl donor
+ 0.0335 Enzyme*Acyl donor

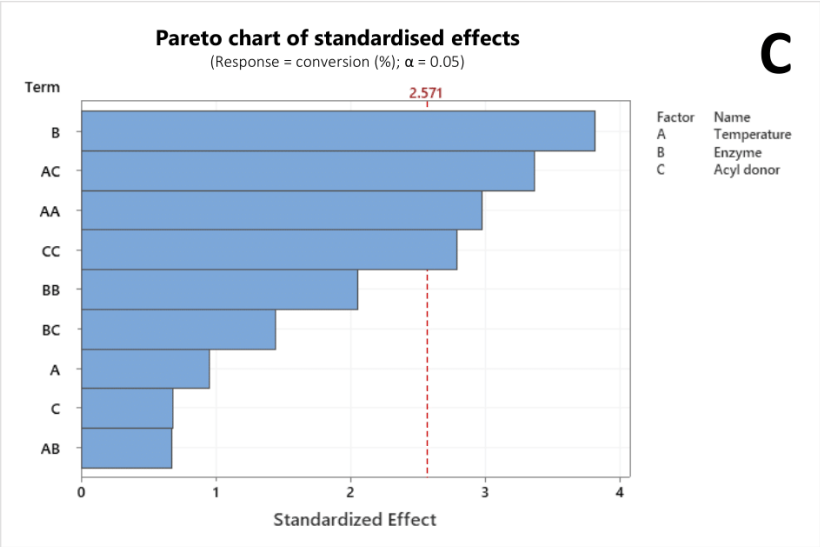
Analysis of Variance

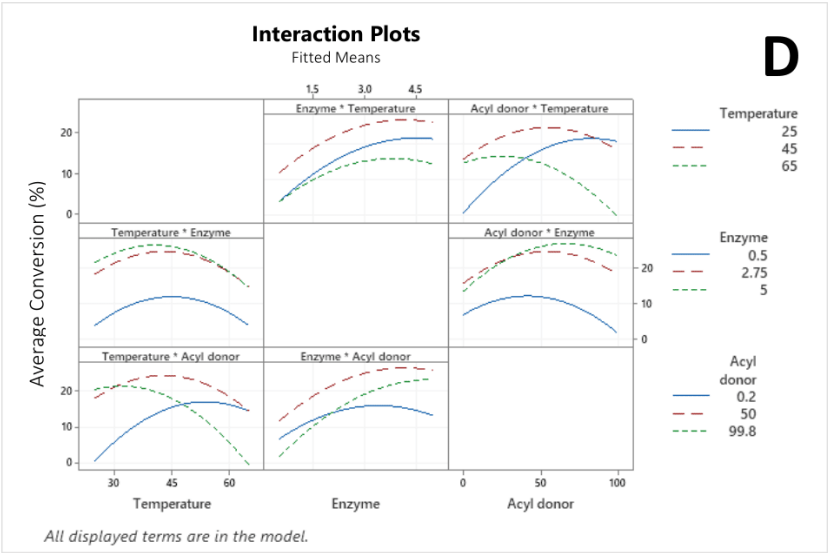
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	1293.77	143.752	5.32	0.040
Linear	3	429.00	143.000	5.29	0.052
Temperature	1	24.50	24.500	0.91	0.385
Enzyme	1	392.00	392.000	14.50	0.013
Acyl donor	1	12.50	12.500	0.46	0.527
Square	3	490.02	163.339	6.04	0.041
Temperature*Temperature	1	238.78	238.776	8.83	0.031
Enzyme*Enzyme	1	113.39	113.391	4.19	0.096
Acyl donor*Acyl donor	1	210.01	210.006	7.77	0.039
2-Way Interaction	3	374.75	124.917	4.62	0.066
Temperature*Enzyme	1	12.25	12.250	0.45	0.531
Temperature*Acyl donor	1	306.25	306.250	11.33	0.020
Enzyme*Acyl donor	1	56.25	56.250	2.08	0.209
Error	5	135.17	27.033		
Lack-of-Fit	3	126.50	42.167	9.73	0.095
Pure Error	2	8.67	4.333		
Total	14	1428.93			

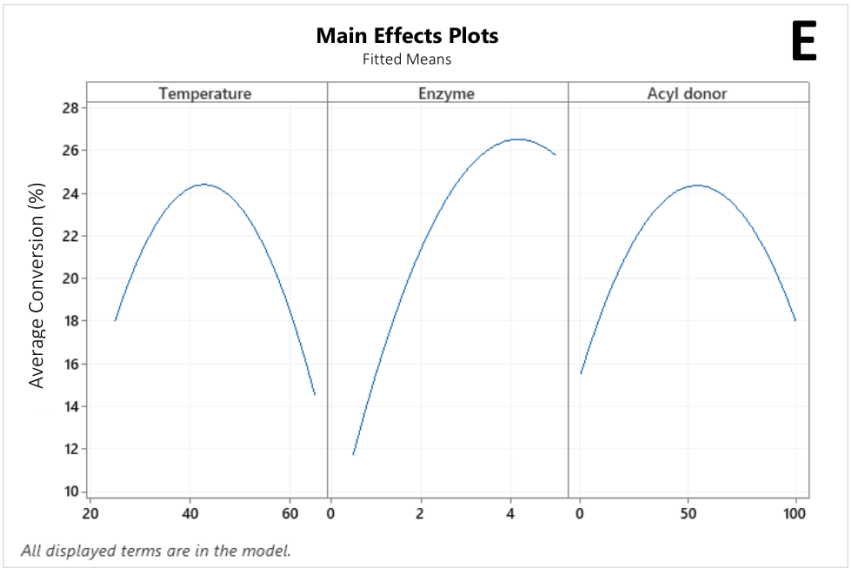
Residual Plots

B



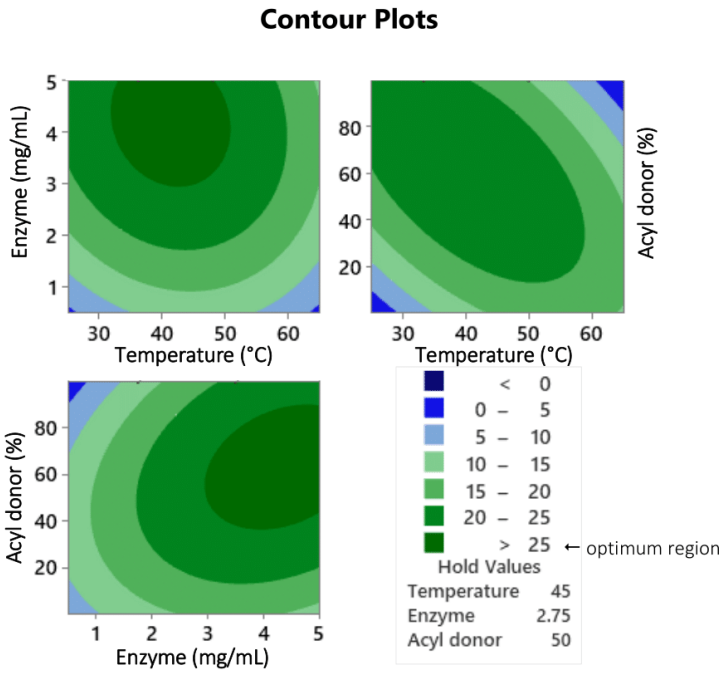

Pareto chart of standardised effects
(Response = conversion (%); α = 0.05)
C
Term
2.571
B
AC
AA
CC
BB
BC
A
C
AB
0
1
2
3
4
Standardized Effect
Factor Name
A Temperature
B Enzyme
C Acyl donor


Interaction Plots
Fitted Means
D
Enzyme * Temperature
Acyl donor * Temperature
Temperature * Enzyme
Acyl donor * Enzyme
Temperature * Acyl donor
Enzyme * Acyl donor
Average Conversion (%)
Temperature
Enzyme
Acyl donor
Temperature
25
45
65
Enzyme
0.5
2.75
5
Acyl donor
0.2
50
99.8
All displayed terms are in the model.


Main Effects Plots
Fitted Means
E
Temperature
Enzyme
Acyl donor
Average Conversion (%)
All displayed terms are in the model.

13

F


Contour Plots
Enzyme (mg/mL)
Temperature (°C)
Acyl donor (%)
Temperature (°C)
Acyl donor (%)
Enzyme (mg/mL)
< 0
0 – 5
5 – 10
10 – 15
15 – 20
20 – 25
> 25
← optimum region
Hold Values
Temperature 45
Enzyme 2.75
Acyl donor 50

Appendix S10. DOE response optimisation for the maximum conversion (%) of the substrates menthol and citronellol with the lipase CALA:

Parameters

Response	Goal	Lower	Target	Upper	Weight	Importance
Menthol Conversion (%) 24h	Maximum	3	60		1	1
Menthol Conversion (%) 1h	Maximum	0	26		1	1
Citronellol Conversion (%) 1h	Target	9	100	110	1	1

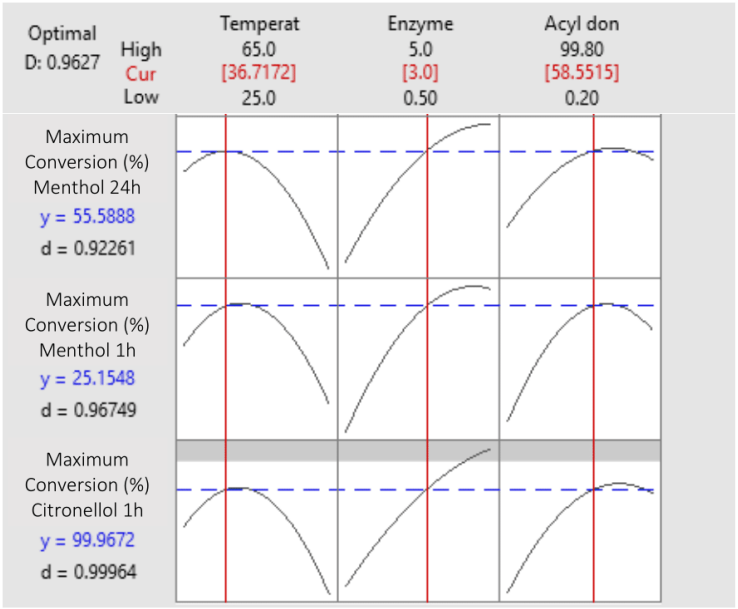
Solution

Solution	Temperature	Enzyme	Acyl donor	Ment Conv. (%) 24h Fit	Ment Conv.(%)1h Fit	Cit Conv. (%) 1h Fit	Composite Desirability
1	36.7172	3	58.5515	55.5888	25.1548	99.9672	0.962727

Multiple Response Prediction

Variable	Setting
Temperature	36.7172
Enzyme	3
Acyl donor	58.5515

Response	Fit	SE Fit	95% CI	95% PI
Menthol Conversion (%) 24h	55.59	4.92	(42.94, 68.24)	(29.23, 81.95)
Menthol Conversion (%) 1h	25.15	2.84	(17.84, 32.47)	(9.92, 40.39)
Citronellol Conversion (%) 1h	99.97	6.09	(84.30, 115.63)	(67.33, 132.60)



Chapter 3

Lipase-catalysed changes in essential oils revealed by comprehensive two-dimensional gas chromatography

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Contents

3.1. Article

This Chapter builds on the characterisation of enzyme activity, and initial studies outlining preferred sampling approaches introduced in Chapter 2. The enzymatic transesterification of terpene and phenyl alcohols in 35 essential oil samples was carried out using Candida antarctica lipase A (CALA), identified as the best performing commercial biocatalyst in the previous stage of this study (Chapter 2). Comprehensive two-dimensional gas chromatography with mass spectrometry (GC×GC–MS) analysis was selected as the most appropriate analytical technique for analysis of the essential oils (EO), and enabled the separation and identification of 125 compounds, allowing the instant visualisation of the reaction process changes, amid the complex chemical background of the samples. Preliminary selection of a range of capillary GC phases for use for ^{1}D and ^{2}D separation was undertaken, in order to provide the most appropriate use of the 2D separation space in the GC×GC experiment. Thus a DB-5ms / SLB-IL60 column set of appropriate dimensions was selected. The use of such large sample set with different degrees of complexity allowed detailed investigation of the enzyme's performance towards an extensive number of alcohol substrates, which helped to determine the selectivity and apparent lack of inhibition effects for the enzyme in the tested conditions. The results indicate that 42 out of 79 alcohols identified were fully or partially esterified within 48 h of reaction, with primary alcohols showing the highest conversions (90-100%), and were clearly the enzyme's preferred substrates. Secondary alcohols had mostly ~80-100% conversion, while no significant conversion was observed for tertiary alcohols and phenols under the tested conditions. The enzyme performed consistently well for primary alcohol substrates in various samples. The observed selectivity, efficiency, robustness, scalability (enzyme/substrate working concentration ratio >1:160), potential reusability, mild reaction conditions, and other factors make this process a greener and more sustainable alternative for industry applications, particularly for the manufacture of novel flavours and fragrances.

The development of MDGC-O/MS methods for sensory analysis of the enzymatic reaction products was originally planned. This kind of analysis often requires a trained panel of analysts and, in the case of complex samples, it is necessary to do multiple injections of the same sample with aroma assessment being performed in short blocks, in order to avoid olfactory fatigue. However, the project timeline was compromised, due to the COVID-19 pandemic, and so the chemical analysis was prioritised.

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RESEARCH PAPER

Lipase-catalysed changes in essential oils revealed by comprehensive two-dimensional gas chromatography

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Abstract
Candida antarctica lipase A (CALA) was applied for the chemo-selective enzymatic transesterification of terpene and phenyl alcohols in 35 different essential oil samples. Comprehensive two-dimensional gas chromatography with mass spectrometry (GC×GC−MS) analysis enabled the separation and tentative identification of a cohort of 125 compounds, allowing the instant visualisation of the reaction process changes, amid the complex chemical background of the samples. The results indicate that 42 out of 79 alcohols so-identified were fully or partially esterified within 48 h of reaction, with primary alcohols being the substrates of preference of the enzyme (90–100% conversion), followed by secondary alcohols (mostly ~ 80–100% conversion). No significant conversion of tertiary alcohols and phenols was observed using the tested conditions. Overall, the enzyme's performance was consistent for primary alcohol substrates identified in multiple samples of different compositions. The observed selectivity, efficiency, robustness, scalability (enzyme/substrate working concentration ratio > 1:160), potential reusability, mild reaction conditions, and other factors make this process a greener and more sustainable alternative for industry applications, particularly for the manufacture of novel flavours and fragrances.

Keywords Enzymes · GC×GC−MS · Biocatalysis · Esterification · Natural products

Introduction

In the past two decades, green and biotechnological processes have quickly evolved and many have been adopted in different industrial applications. The use of enzymes as biocatalysts for the synthesis of fine chemicals, such as flavours and fragrances (F&F), is an increasing trend in the market. This processing option has potential to produce high-value compounds by the conversion of abundant, less expensive, and renewable raw materials, and using a more chemically and energy efficient, chemo-, regio-, and stereo-selective, reusable, biodegradable, and natural catalyst. It can also improve the safety, sensory quality, and biological activity of F&F products, while keeping their "naturally derived" labelling. These appealing advantages, as opposed to the often harsher synthetic chemical processes or extraction/isolation methods from scarce natural sources, are some of the reasons for the growing adoption of biocatalysis [1–5].

In this context, hydrolytic enzymes, such as lipases, have been established and increasingly dominate the industrial enzyme market. Versatile and robust, lipases essentially act on carboxylic groups, being able to catalyse different reactions, accept a wide range of substrates, and remain active in different media (e.g. aqueous, organic, ionic liquids, supercritical fluids) and over a wide temperature and pH range. These attributes, along with their successful immobilisation and bioengineering improvements, favour their compatibility with numerous applications [4–6].

Lipase-catalysed [trans]esterification and chiral resolution of carboxylic, terpenoid, and phenyl alcohols are some of the most important processes of interest for the F&F, food, pharmaceutical, and personal care industries. Similarly, the esterification of fatty acids is in demand in the same fields. Esters generally exhibit strong and distinctive aromas, lower polarity, lower boiling point (higher volatility), and fewer skin-sensitising properties than their respective alcohols or acids, due to the derivatisation of the hydroxyl group by

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alkyl groups. These physical and sensory aspects favour their incorporation into different formulations as emulsifiers, thickening agents, emollients, aroma enhancers, fragrances, or flavours.

Following a recent screening and optimisation study [7], the present work focuses on the application of the immobilised *Candida antarctica* lipase A (CALA)—identified as the best-performing commercial biocatalyst in previous stages of this study—to the biotransformation of terpenoid and phenyl alcohols in essential oil samples. Unlike other similar studies targeting only one or a few samples or standards [5, 8–12], the performance of the aforementioned biocatalyst towards an extensive number of alcohol substrates in 35 natural samples of different complexities is investigated here, in order to better understand the selectivity and inhibition effects (if any) involved in such processes. Comprehensive two-dimensional gas chromatography with mass spectrometry detection (GC×GC−MS) was used for its superior resolution and improved compound identification capacity, enabling the qualitative assessment of reaction changes in samples of different complexities. The comparison between the position of the peaks in the 2D chromatograms of the same sample before and after the enzymatic processing reaction can readily display the absence or reduction in abundance of the alcohols (substrates), and corresponding appearance of the esters (products).

Experimental

Chemicals, enzyme, and samples

The list of chemicals used in this study includes vinyl acetate (99%), acetone (99%), *n*-alkanes C_8-C_{26} (99%), all supplied by Sigma-Aldrich (Castle Hill, NSW, Australia), and *n*-hexane (99%), obtained from Merck (Bayswater, VIC, Australia). The enzyme lipase CALA (*Candida antarctica* A, Batch# BCBV6128) immobilised in Immobead 150 was purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). The commercial essential oils (E.O.) and their sample codes and suppliers are as follows: bergamot (BERG), boronia (BOR), cedarwood atlas (CDWA), cedarwood Virginian (CDWV), cinnamon (CINM), citronella (CIT), clary sage (CLARY), clove (CLO), cypress (CYP), geranium (GER), jasmine (JAS), kanuka (KNK), lavender (LAV), lemongrass (LMG), myrrh (MYR), pine (PINE), peppermint (PPMP), patchouli (PTC), rose absolute (ROSE), rosemary (ROSM), sandalwood Australian (SDWA), sandalwood Indian (SDWI), tea tree (TTO), and vetiver (VTV1) were kindly donated by Australian Botanical Products (Hallam, VIC, Australia). Additionally, copaiba (COP), eucalyptus (EUCR), frankincense (FKI), kaffir lime (KFL), manuka (MNK), masoi bark (MSB), nutmeg (NMG), neroli (NRL), sweet orange (SWORG), ylang-ylang (YY), and another vetiver (VTV0) samples were obtained from other research partners or purchased online from Amazon Australia. More specifications about the samples are provided in the Appendix S1 of the Supplementary Information.

Lipase enzyme transesterifications

Lipase reactions were performed according to our previous optimisation study [7], in 2 mL vials, at 40 °C, with 350 rpm magnetic stirring, using 3 mg/mL of CALA enzyme product, 100 mg/mL (10% w/v) of the essential oil samples as the substrate, and 1 mL of a vinyl acetate/hexane solution (6:4) as acyl donor/solvent system. Citronella oil was initially used as a general test sample in different concentrations (1, 10, 100, 500, and 900 mg/mL, which is equivalent to 0.1, 1, 10, 50, and 90% w/v). Based on this initial test, a 10% w/v concentration was selected to perform the reactions with all other essential oils. The reactions were terminated after 48 h by decanting the reaction fluid from the immobilised enzyme and transferring the recovered solution to a new vial. The samples were then diluted with hexane (1:10) for the GC×GC−MS analysis.

GC×GC−MS analysis

The chromatographic analyses were performed on an Agilent 7890A GC system, with automatic injector G4513A, coupled with a 5975C single quadrupole mass spectrometer detector (MSD; Agilent Technologies, Mulgrave, Australia), and a J&X SSM1800 solid-state modulator system (J&X Technologies, Nanjing, China). The system was equipped with a DB-5 ms UI (30 m × 0.25 mm × 0.25 µm) as the first dimension (^{1}D) column, and a SLB-IL60 (1.5 m × 0.1 mm × 0.1 µm) as the second dimension (^{2}D) column. Fused silica capillaries were used as the modulator column (1 m × 0.15 mm) and the transfer line to the MSD (0.45 m × 0.1 mm). All columns were connected by glass press fits.

The chromatographic method was adapted from previous studies [7, 13], in order to achieve a satisfactory separation for most analytes in all the different samples. Injections (0.6 µL of a ~ 10 mg/mL sample solution) were made at 250 °C, in split mode (20:1), with helium (grade 99.99%) as carrier gas, flow 1 mL/min. The oven temperature program commenced at 60 °C (1 min), with heating at 5 °C/min to 150 °C, then at 3 °C/min to 210 °C, and finally at 15 °C/min to 285 °C (1 or 16 min hold time, depending on the sample); a post-run cooling to 60 °C and oven equilibration was set at 12 min. Other settings were as follows: transfer line to the MSD was held at 285 °C, with the MSD set to scan mode, 12,500 u/s speed, 23.5 scans/s, 80 threshold, 50–350 *m/z* range, 4.6 min solvent delay, 70 eV electron

ionisation energy, source and quadrupole temperatures at 230 °C and 150 °C, respectively.

The solid-state modulator entry oven was set according to the GC oven, while the exit oven was set to 80 °C (1 min), 5 °C/min to 170 °C, 3 °C/min to 230 °C, 15 °C/min to 305 °C (1 or 16 min hold time, depending on the sample). The modulator's cold trap was set to 10 °C (3 min), – 50 °C/min to – 50 °C (5 min), 2 °C/min to 20 or 50 °C (0.80 min). The modulation period was 6 s, desorption time was 1 s, and no delay was used. The final hold time for the GC and modulator methods was increased for the essential oil samples containing a high number of sesquiterpenes.

Agilent MassHunter workstation Qualitative Analysis Version 10.0 and J&X Canvas Version W1.5.14.30115 were the main software used for data processing and 2D chromatogram generation, respectively. The chromatographic peaks were integrated and tentatively identified, according to NIST 11 mass spectrometry library match. Alternatively, for the peaks of sesquiterpene alcohols and esters in vetiver that were not found in the NIST library, the similarity with the mass spectrum and retention indices was compared with those reported by Tissandié et al. [14] and Notar Francesco et al. [9]. The van den Dool & Kratz retention indices were calculated through correlation with the analysis of C_8-C_{25} *n*-alkanes using the same conditions and based on ^{1}D column retention (see Appendix S3 of the Supplementary Information for *n*-alkanes data). Although the quantitative analysis was not the aim of this work, the percentage difference between the chromatographic areas of the alcohol peaks before and after the reaction was used to assess the conversion percentage (C%) of the substrates, which was related to the process efficiency.

Results and discussion

The initial assessment of the bioreaction with citronella oil at different concentrations demonstrated that the total conversion of the major alcohols (citronellol and geraniol) was completed within 24 h for the test solutions containing 0.1% to 10% w/v of the oil. When 50% oil was used, the same analytes had an average conversion of about 95% after 24 h and 100% after 48 h reaction. No significant changes were observed for the 90% w/v solution within 48 h of reaction, suggesting that the enzyme efficiency is much reduced at such a high sample concentration. Thus, a 10% w/v essential oil sample concentration and 48 h reaction time were chosen as the most suitable working condition for further experiments, taking into consideration that there are differences in the composition and analyte abundance in the other samples, such as the presence of secondary and tertiary alcohols, which may take longer to react.

Additionally, these results indicate that the previously optimised reaction conditions [7] are similarly efficient for an essential oil sample at a concentration (mg/mL) around 167 times higher than that of the enzyme, and it may continue to produce the desired reaction products slightly above this level, provided the reaction is left to proceed for a longer duration. This is important information for future scale-up studies, even though the essential oil sample concentrations are not directly equivalent to the concentration of the total substrate alcohols within each sample.

The GC×GC−MS method enabled the tentative identification of 125 target analytes, including 79 alcohols and 46 esters (Table 1), within the 35 essential oil samples studied, before and after enzyme bioprocessing. The compounds that did not satisfactorily match the reference mass spectrum and retention indices were not included in the table.

Thus, the occurrence of a given substrate alcohol in multiple samples was frequently observed, which means that many of these specific analytes were tested multiple times within samples of different compositions and complexities. In general, a consistent efficiency was observed for the conversion of primary alcohols identified in multiple samples, such as citronellol and geraniol, indicating that neither the number of analytes, their chemical diversity, nor their abundance in the samples seems to adversely affect or inhibit the enzyme activity towards these substrates.

A scatter diagram of the GC×GC−MS chromatogram (Fig. 1) illustrates the retention time coordinates of the target analytes (i.e. alcohols and esters) and demonstrates the superior separation capacity through additional separation on the ^{2}D column, and easy visual assessment of chemical changes that this technique offers. Generally, the relative position of a given analyte peak in the 2D space does not significantly vary from one sample to another, provided that the analytical conditions are kept the same for all the sample sets. This attribute allows an analyst to instantly locate the target analytes across the sample set once they are identified in a sample, to recognise clustering groups with similar chemical class and/or molecular features (e.g. monoterpene alcohols and monoterpene esters), and to determine any differences in the overall chemical profile of the samples, such as the changes resulting from the enzymatic process.

A total of 42 out of the 79 alcohols identified were successfully converted into their respective acyl esters. The lipase-catalysed esterification reactions were generally quite efficient for primary and secondary alcohols, such as β-citronellol, geraniol, menthol, (*Z*)-carveol, β-santalol, (*E*)-farnesol, (*E*)-nuciferol, 1-octen-3-ol, and benzyl alcohol (Fig. 2), with conversions (C%) of 80 to 100% within 48 h reaction time. Some secondary alcohols, such as fenchol, isopulegol, isoborneol, and borneol (Fig. 2), were only partially converted (C% = 30–60) within the same time. No significant conversion was observed in the tested conditions for

Table 1 Alcohols and their corresponding esters identified using GC×GC−MS analysis in the suite of essential oil samples investigated

Peak #	Alcohols	Type	$^{1}t_R$ (min)	$^{2}t_R$ (s)	RI (calc)	RI (lib)	C %	Peak #	Esters	$^{1}t_R$ (min)	$^{2}t_R$ (s)	RI (calc)	RI (lib)	Samples
Monoterpenoids														
1	(*Z*)-linalool oxide (furanoid)	3°	14.07	1.62	1073	1074	-	-	-	-	-	-	-	BERG, CYP, CLARY, LAV, GER
2	sabinene hydrate	3°	14.17	1.26	1076	1070	-	-	-	-	-	-	-	PPMP, FKI, ROSM, CIT, NMG
3	*β*-linalool	3°	14.89	1.35	1101	1099	-	25	linalyl acetate*	19.27	1.20	1252	1257	LAV, CLARY, BERG, NRL, GER
4	fenchol	2°	15.67	1.30	1128	1113	28	26	fenchyl acetate	18.47	1.15	1224	1223	KNK, ROSM, PINE, LAV, CYP
5	trans-p-mentha-2,8-dien-1-ol	3°	15.67	1.51	1128	1123	-	-	-	-	-	-	-	BERG, FKI, LMG, ROSM, SWORG
6	cis-p-mentha-2,8-dien-1-ol	3°	16.07	1.79	1141	1140	-	-	-	-	-	-	-	BERG, FKI, LMG, PINE
7	trans-pinocarveol	2°	16.37	1.57	1152	1139	100	27	trans-pinocarvyl acetate	20.67	1.58	1300	1297	KNK, CYP, FKI, NMG, ROSM
8	*β*-terpineol	3°	16.47	1.54	1155	1153	-	28	*β*-terpinyl acetate*	20.17	1.30	1283	1317	NRL, ROSM, BOR, BERG, EUCR
9	cis-verbenol	2°	16.47	2.01	1155	1142	100	29	cis-verbenyl acetate	20.47	1.55	1293	1279	FKI, PINE, ROSM, KNK, MNK
10	*α*-phellandren-8-ol	3°	16.57	1.66	1159	1167	-	-	-	-	-	-	-	FKI, LAV, CYP, GER, PINE
11	lavandulol	1°	16.77	1.64	1166	1170	100	30	lavandulyl acetate	20.17	1.30	1283	1289	LAV
12	isopulegol	2°	16.87	1.25	1169	1163	49	31	isopulegol acetate	19.87	1.19	1273	1285	PPMP, BERG, CLO, JAS, CIT
13	isoborneol	2°	17.07	1.43	1176	1157	32	32	isobornyl acetate	20.57	1.40	1297	1286	ROSM
14	borneol	2°	17.27	1.66	1183	1166	61	33	borneyl acetate	20.47	1.38	1293	1285	ROSM, PINE, LAV, LMG, FKI
15	menthol	2°	17.37	1.60	1186	1166	87	34	menthyl acetate	20.47	1.27	1293	1295	PPMP, GER, ROSM
16	terpinen-4-ol	3°	17.47	1.34	1190	1182	-	35	4-terpinenyl acetate*	21.57	1.41	1330	1301	NRL, TTO, NMG, LAV, EUCR
17	*α*-terpineol	3°	17.87	1.60	1204	1189	-	36	*α*-terpinyl acetate*	22.17	1.46	1350	1350	EUCR, BOR, NRL, TTO, CLARY
18	myrtenol	1°	17.87	2.04	1204	1213	100	37	myrtenyl acetate	21.47	1.41	1327	1327	FKI, KNK, ROSM, BERG, CYP
19	trans-piperitol	2°	18.27	1.68	1217	1208	100	38	piperitol acetate	20.67	1.26	1300	1303	NMG, TTO, EUCR, FKI, PPMP
20	(*Z*)-carveol	2°	18.47	1.87	1224	1219	96	39	carvyl acetate	21.67	1.56	1333	1336	FKI, BERG, KNK, PPMP, SWORG

Table 1 (continued)

Peak #	Alcohols	Type	^1t_R (min)	^2t_R (s)	RI (calc)	RI (lib)	C %	Peak #	Esters	^1t_R (min)	^2t_R (s)	RI (calc)	RI (lib)	Samples
21	*β*-citronellol	1°	18.57	1.83	1228	1228	100	40	*β*-citronellyl acetate	22.07	1.35	1347	1354	CIT, GER, BOR
22	nerol	1°	18.57	1.84	1228	1228	100	41	neryl acetate	22.37	1.40	1357	1342	CLARY, LAV, NRL, EUCR, LMG
23	geraniol	1°	19.27	1.97	1252	1255	100	42	geranyl acetate	22.97	1.46	1377	1382	CIT, GER, LMG, NRL, ROSE
24	perillyl alcohol	1°	20.87	2.42	1307	1296	100	43	perillyl acetate	24.77	1.98	1434	1436	BERG, FKI, LMG,PPMP
Sesquiterpenoids														
44	hedycaryol	3°	28.57	2.02	1553	1559	-		-					CIT, SDWA, FKI, YY, VTV1
45	elemol	3°	28.57	2.04	1553	1549	-		-					CIT, VTV1, VTV0, MYR, YY
46	nerolidol	3°	28.77	1.18	1560	1564	-	-	-	-	-	-	-	YY, SDWA, CINM, ROSM, NRL
47	spathulenol	3°	29.67	2.01	1588	1577	-	-	-	-	-	-	-	PTC, KNK, GER, CLARY, FKI
48	globulol	3°	30.07	1.95	1600	1580	-	-	-	-	-	-	-	PTC, TTO, CDWV, YY, VTV1
49	guaiol	3°	30.27	1.76	1606	1596	-	-	-	-	-	-	-	YY, GER, SDWA, COP, MYR
50	viridiflorol	3°	30.37	1.87	1609	1591	-	-	-	-	-	-	-	KNK, COP, CDWV, VTV0, MSB
51	widdrol	3°	30.77	2.05	1621	1610	-	-	-	-	-	-	-	CDWV, VTV0, VTV1
52	cedrol	3°	30.97	1.84	1627	1598	-	-	-	-	-	-	-	CDWV, CDWA, CYP, KNK
53	selin-6-en-4α-ol	3°	31.17	1.80	1634	1636	-	-	-	-	-	-	-	VTV0, VTV1
54	cubenol	3°	31.27	1.70	1636	1642	-	-	-	-	-	-	-	VTV0, VTV1, YY, CDWA, MNK
55	*γ*-eudesmol	3°	31.27	1.85	1636	1631	-	-	-	-	-	-	-	GER, VTV1, VTV0, YY, SDWA
56	*α*-acorenol	3°	31.57	1.51	1646	1649	-	-	-	-	-	-	-	CDWV, SDWA, SDWI, VTV0,VTV1
57	*τ*-cadinol	3°	31.77	1.71	1652	1640	-	-	-	-	-	-	-	FKI, VTV1, YY, MYR, CIT
58	*τ*-muurolol	3°	31.77	1.98	1652	1642	-	-	-	-	-	-	-	YY, VTV0, CIT, MNK, MSB
59	palustrol	3°	31.97	1.30	1658	1568	-	-	-	-	-	-	-	PTC, KNK, TTO, GER, YY

Table 1 (continued)

Peak #	Alcohols	Type	$^{1}t_R$ (min)	$^{2}t_R$ (s)	RI (calc)	RI (lib)	C %	Peak #	Esters	$^{1}t_R$ (min)	$^{2}t_R$ (s)	RI (calc)	RI (lib)	Samples
60	*α*-cadinol	3°	32.17	2.09	1664	1653	-	-	-	-	-	-	-	YY, CIT, MSB, COP, CYP
61	*α*-eudesmol	3°	32.27	1.74	1667	1653	-	-	-	-	-	-	-	VTV0, VTV1, SDWA, MSB,COP
62	*β*-eudesmol	3°	32.27	2.06	1667	1649	-	-	-	-	-	-	-	VTV0, VTV1, SDWA, CIT, TTO
63	eudesma-4,11-dien-2-ol	2°	32.28	2.21	1667	1690	98	85	eudesma-4,11-dien-2-ol acetate	36.68	1.87	1803	1830	VTV0, VTV1
64	cyclocopacamphenol	1°	32.47	2.41	1673	1646	100	86	cyclocopacamphenyl acetate	35.58	1.70	1769	1759	VTV0, VTV1
65	*β*-bisabolol	3°	32.58	1.24	1676	1671	-	-	-	-	-	-	-	VTV0, VTV1
66	*α*-santalol	1°	32.77	2.18	1682	1681	92	87	*α*-santalyl acetate	35.97	1.74	1781	1773	SDWA, CDWV
67	patchouli alcohol	3°	32.97	2.13	1688	1660	-	-	-	-	-	-	-	PTC, BOR
68	*α*-bisabolol	3°	33.07	1.46	1691	1684	-	-	-	-	-	-	-	SDWA, CDWA, VTV1, COP, YY
69	ziza-6(13)-en-3-α-ol	2°	33.07	2.74	1691	1677	100	88	ziza-6(13)-en-3-*α*-yl acetate	36.68	1.87	1803	1775	VTV0, VTV1
70	(*Z*,*Z*)-farnesol	1°	33.09	1.24	1692	1713	100	89	(*Z*,*Z*)-farnesyl acetate	36.78	0.74	1807	1817	SDWA, SDWI, YY, CIT
71	(*Z*)-α-bergamotol	1°	33.17	2.18	1694	1700	99	90	(*Z*)-*α*-bergamotol acetate	36.37	1.45	1794	1790	SDWI, SDWA, CDWV
72	khusian-2-ol	2°	33.57	2.22	1706	1694	-	91	khusian-2-yl acetate	36.28	1.83	1791	1761	VTV0, VTV1
73	juniper camphor	3°	33.67	1.82	1710	1692	-	-	-	-	-	-	-	VTV0, VTV1, COP, MSB, GER
74	*β*-costol	1°	33.87	2.59	1716	1778	100	92	*β*-costol acetate	39.18	1.78	1882	1882	VTV0, VTV1
75	(*E*,*E*)-farnesol	1°	33.89	1.28	1716	1722	100	93	(*E*,*E*)-farnesyl acetate	37.58	0.76	1832	1843	SDWA, SDWI, VTV0, VTV1
76	isokhusimol	1°	34.17	2.51	1725	-	97	94	isokhusimyl acetate	37.78	1.88	1838	1843	VTV0, VTV1
77	*β*-santalol	1°	34.27	2.21	1728	1715	91	95	*β*-santalyl acetate	37.17	1.45	1819	1813	SDWA, SDWI
78	(*E*)-nuciferol	1°	34.37	3.44	1732	1766	95	96	(*E*)-nuciferyl acetate	37.47	2.02	1828	1837	SDWA, SDWI
79	vetiselinenol	1°	34.47	2.42	1735	1723	100	97	vetiselinenyl acetate	37.98	1.82	1844	1852	VTV0, VTV1
80	(*Z*)-*β*-curcumen-12-ol	1°	35.27	2.27	1760	1746	100	98	(*Z*)-*β*-curcumen-12-yl acetate	37.47	1.74	1828	-	SDWA, SDWI
81	valerenol	1°	35.07	2.63	1753	1736	100	99	valerenyl acetate	39.48	1.44	1891	1832	VTV0, VTV1
82	cis-lanceol	1°	35.47	2.19	1766	1763	100	100	cis-lanceol acetate	38.57	1.58	1863	1860	SDWA, SDWI, PTC
83	khusimol	1°	35.37	2.94	1763	1740	97	101	khusimyl acetate	38.68	2.15	1866	1875	VTV0, VTV1
84	(*E*)-isovalencenol	1°	36.57	2.44	1800	1788	91	102	(*E*)-isovalencenyl acetate	39.78	1.36	1901	1906	VTV0, VTV1

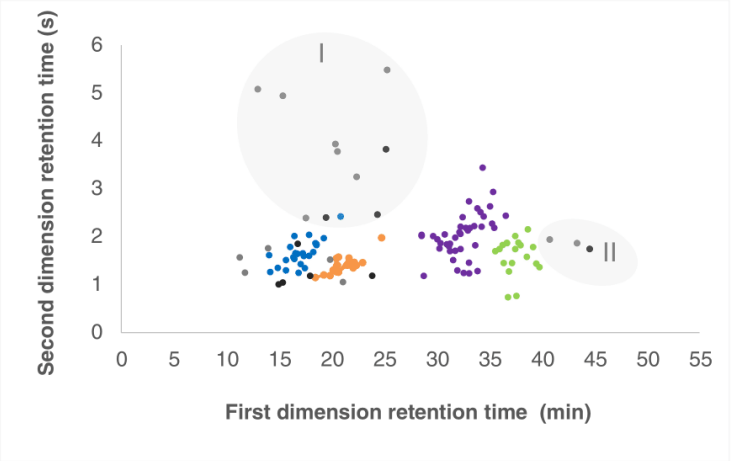
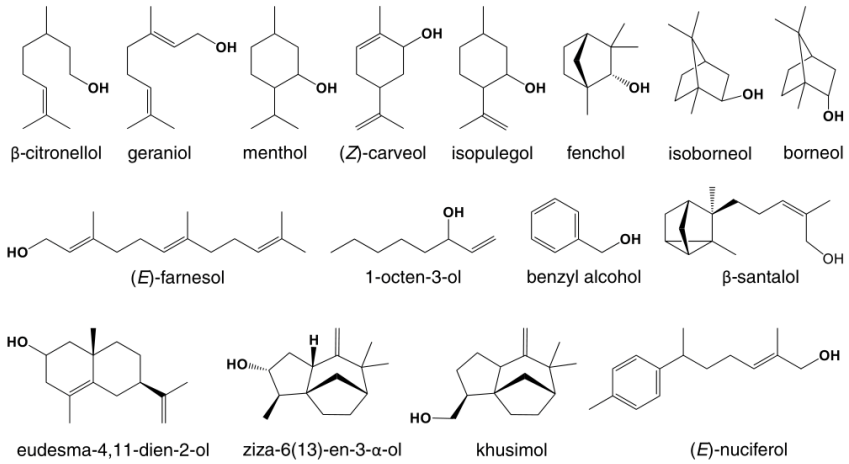


Fig. 1 Scatter diagram of the GC×GC−MS results, demonstrating the relative position of the target analyte peaks in the 2D space (^1t_R and ^2t_R according to Table 1) and clustering of groups of monoterpene alcohols (blue), monoterpene esters (orange), sesquiterpene alcohols (purple), and sequiterpene esters (green). Other alcohols (grey) and esters (black) are also included, with clustering of groups of phenyl (area I) and diterpenoid (area II) compounds highlighted. GC×GC−MS analysis was performed using a DB-5/SLB-IL60 (non-polar/polar) column set (see details in the "Experimental" section); hence, generally more polar analytes elute at later retention times in the second dimension

Fig. 2 Chemical structures of some of the alcohols esterified by CALA lipase



tertiary alcohols and phenols, which are major components in many of the samples investigated (e.g. linalool in lavender and eugenol in clove). Despite that, for completeness, the GC×GC−MS chromatograms of all the tested E.O. have been included in the Appendix S2 of the Supplementary Information.

As demonstrated in our previous study [7], large conversions (C% ≥ 90) can be quickly achieved (within ~ 1 h) for primary alcohol reaction with CALA enzyme, in the tested conditions. Secondary alcohols had around 20% conversion within the same time and around 70% within 24 h in the same study. Thus, in the present work, the reaction was allowed to proceed for 48 h to obtain the highest possible C% for all types of alcohol substrates across all the samples tested. This also seems to have contributed to less variations for most part of the compounds with high C% and identified in multiple samples, such as (*Z*)-carveol (C% = 90–96), as well as citronellol and geraniol which were 100% converted in all of the samples containing them. The highest variations were observed for a few compounds, such as isopulegol (C% = 29–49) and borneol (C% = 34–61). However, it is important to reiterate that these C% are estimated from the difference in the indicative chromatographic areas of these alcohols before and after the reaction, but this was not a quantitative measurement, which was not the aim of this study.

By comparison of all the E.O. investigated, the most significant bioprocessing changes were observed for vetiver and sandalwood, which contain a large number of sesquiterpene alcohols that were successfully esterified. GC×GC−MS enabled the tentative identification of 18 of those alcohols and their respective esters, as well as other alcohols that were not esterified or are from other groups. The samples showing fewer changes contained lower amounts (in concentration and number) of the enzyme's preferred substrates (i.e. primary and secondary alcohols).

The comparison of the 2D chromatograms of the original and bioprocessed vetiver (Fig. 3A and B, respectively) and sandalwood (Fig. 3C and D) samples allows the location of clustering areas in the 2D space corresponding to alcohols (Fig. 3, area I) and esters (Fig. 3, area II), and to clearly observe the chemical changes in the samples obtained with enzymatic processing of the original oil.

Sandalwood samples had conversions of 92% and 91% for the major alcohols α- and β-santalol (Fig. 2) within 48 h, respectively. Other sesquiterpene alcohols, such as (*E*)-farnesol and (*E*)-nuciferol (Fig. 2), were also present in high abundance and were efficiently esterified (90–100%). A number of high-abundance compounds can be observed in sandalwood chromatograms, indicated by large peaks in the contour plot, with some apparent extended tailing. However, to adequately study the effects of lower abundance peaks, some overloading of high-abundance peaks is required (as opposed to diluting the sample).

Specifically for vetiver samples, the enzymatic esterification of secondary terpene alcohols, such as eudesma-4,11-dien-2-ol, and ziza-6(13)-en-3-α-ol, as well as the full conversion of the major alcohol khusimol (Fig. 2), outperforms the biocatalysis results reported by Notar Francesco et al. [9], being closely comparable to their observations for chemical esterification of vetiver alcohols. According to these authors, the acetylation processing can introduce grapefruit, sandalwood, and cedarwood undertones to vetiver oil, as opposed to the usual earthy notes, which can increase the value of this fine fragrance ingredient to almost double the price of the original oil [9].

The use of such chemically diverse natural samples as lipase substrates, aligned with the resolution power of GC×GC−MS analysis, has enabled the assessment of the enzyme's performance towards specific compounds, such as the aforementioned vetiver alcohols, for which the pure standards are very expensive or not commercially available.

It is important to highlight that a positive overall odour change arising from the esterification of such complex samples could be related to either the pleasant aroma of the new esters produced, or the suppression of the off-flavour character of the initial alcohols. A lower odour activity of these new esters in comparison to their respective alcohols could also bring other types of odorants present in the original oil to the spotlight as the new key aroma impact compounds in the processed sample. In the case of vetiver, for example, there are different compounds (unrelated to the esterification process) with grapefruit notes present in the raw oil, such as nootkatone, β-vetivone, and valencene, which could also play a more significant role to the overall aroma of the sample after processing. In fact, Tissandié et al. [14] observed in

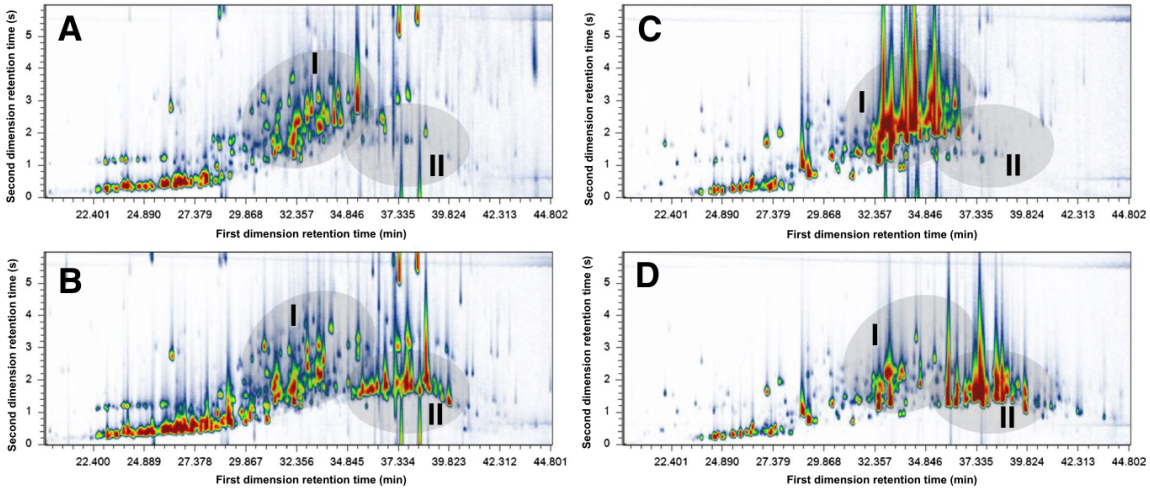


Fig. 3 GC×GC−MS chromatograms of vetiver (**A** and **B**; sample VTV1) and sandalwood (**C** and **D**; sample SDWA) essential oils, illustrating the chemical changes achieved after the lipase-catalysed esterification processing (**B** and **D**), in comparison with the original samples (**A** and **C**). The highlighted areas I and II represent the location in the 2D space where most of the alcohols and esters are found, respectively

the olfactory assessment of the esterified vetiver oil that the main esters produced were generally odourless or with low odour impact to the overall sample, except for 12-norziza-6(13)-en-2α-yl acetate, which apparently has a stronger vetiver-like note. Thus, the accurate aroma assessment of the overall esterified samples and the identification of their key odorants are complex tasks that require additional analytical steps, which are not covered in the present study.

Conclusion

The biocatalysed transesterification of a large set of alcohol substrates in 35 E.O. samples was successfully performed in the present study by applying a pre-optimised enzyme reaction with a selected immobilised lipase (CALA). Remarkable results were achieved with the production of esters from primary and secondary alcohols, surpassing previous investigations.

The immobilised CALA enzyme is fully compatible with non-aqueous systems and E.O. samples with different levels of complexity (i.e. number, concentration, and chemical diversity of components), as well as being robust, efficient, easy to apply, and readily removed from the reaction media. Our results also indicate that the scalability of the method should be relatively facile, since a considerable increase in the substrate's concentration did not compromise the efficiency of the process. Although the present study has not investigated the reusability of this specific catalyst, studies elsewhere have found that other immobilised lipase products can be reused multiple times or in continuous flow systems without significant loss in activity [5, 9].

A panel sensory assessment was originally planned, in order to draw specific conclusions about the odour changes in the samples. However, this is a laborious task that requires a certain number of volunteers and training, which was not possible to do, due to COVID-19 and other issues. For this reason, a more in-depth discussion on the odour changes of the samples was not included in the present study. The method developed here aimed to demonstrate that the molecular changes accompanying enzyme treatment could be followed by using GC×GC analysis, with appropriate conversion of chemical classes. A more complete study with odour assessment is a future objective.

The combination of low-cost natural raw materials, such as some essential oils, with the selectivity, efficiency, and robustness of more environmentally friendly synthetic processes, such as biocatalysis, offers a benign avenue to manufacture novel flavour and fragrance products with improved properties and safety, without compromising the branding associated with "natural" labelling.

As a tool for the discovery of specific composition changes in complex samples, GC×GC−MS has proven to be a promising technique with superior resolution and identification capabilities, able to generate 2D plots that facilitate the visualisation of the changes achieved with the enzymatic processing, as well as any other differences in the samples' chemical profiles. The comparison of the GC×GC–MS data of the original oils with their enzymatically transformed products is a straightforward process that generates data with a high information content and outstanding results in comparison with what would be available from a single dimension GC–MS analysis where the target chemical classes are not as readily displayed chromatographically. However, data analysis can still be a laborious and time-consuming task in the absence of efficient, integrated, and user-friendly software. A desirable interface would allow quick, precise, and simultaneous analysis of multiple samples, providing all the main peak parameters and 2D chromatograms (with overlay function) as well as connecting with updated MS databases to provide more accurate compound identification lists.

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Author contribution Michelle S. S. Amaral: conceptualisation; methodology; investigation; formal analysis; visualisation; writing—original draft preparation; reviewing and editing. Philip Marriott and Milton Hearn: conceptualisation; writing—review and editing; supervision; and resources.

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Declarations

Competing interests The authors declare no competing interests.

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3.2. Supplementary Information

Supplementary Information

Lipase-catalysed changes in essential oils revealed by comprehensive two-dimensional gas chromatography

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Analytical and Bioanalytical Chemistry

Table of contents

Appendix S1. Essential oil samples table

Code	Essential oil	Scientific name	Plant part	Manufacturer / Supplier
BERG	Bergamot	*Citrus bergamia*	Fruit peel	Auroma / ABP
BOR	Boronia	*Boronia megastigma*	Flower	Auroma / ABP
CDWA	Cedarwood Atlas (*Morocco wild*)	*Cedrus atlantica*	Stem	Essential Therapeutics/ ABP
CDWV	Cedarwood Virginian	*Juniperus virginiana*	Stem	Auroma / ABP
CINM	Cinnamon	*Cinnamomum cassia*	Bark	ABP
CIT	Citronella Java	*Cymbopogon winteranus*	Leaves	ABP
CLARY	Clary Sage	*Salvia sclarea*	Leaves	Auroma / ABP
CLO	Clove	*Syzygium aromaticum*	Flower buds	Auroma / ABP
COP	Copaíba	*Copaifera martii* H.	Gum resin	Pacific Scents
CYP	Cypress	*Cupressus sempervirens*	Stems/ Leaves	Essential Therapeutics / ABP
EUCR	Eucalyptus	*Eucalyptus radiata*	Leaves	Australian Lab
FKI	Frankincense	*Boswellia serrata*	Gum resin	Primavera
GER	Geranium	*Geranium spp.*	Flower	Essential Therapeutics / ABP
JAS	Jasmine	*Jasminum spp.*	Flower	Essential Therapeutics / ABP
KFL	Kaffir Lime	*Citrus hystrix*	Leaves	Natoria
KNK	Kanuka	*Kunzea ericoides*	Leaves	Essential Therapeutics / ABP
LAV	Lavender	*Lavandula angustifolia*	Leaves/ Flower	Auroma / ABP
LMG	Lemongrass *(Nepal, Organic)*	*Cymbopogon flexuosus*	Leaves	Essential Therapeutics / ABP
MNK	Manuka	*Leptospermum scoparium*	Leaves	Gya labs
MSB	Masoi Bark	*Cryptocarya massoy*	Bark	Natoria
MYR	Myrrh	*Commiphora myrrha*	Gum resin	Essential Therapeutics / ABP
NMG	Nutmeg	*Myristica fragans*	Seeds	Natoria
NRL	Neroli	*Citrus aurantium*L.	Flower	Gya labs
PINE	Dwarf Pine	*Pinus pumila*	Leaves	ABP
PPMP	Peppermint	*Mentha piperita*	Leaves	Essential Therapeutics / ABP
PTC	Patchouli *(Indonesia)*	*Pogostemon cablin*	Leaves	Essential Therapeutics / ABP
ROSE	Rose absolute	*Rosa damascena*	Flower	Auroma / ABP
ROSM	Rosemary	*Rosmarinus officinalis*	Leaves	Essential Therapeutics / ABP
SDWA	Sandalwood Australian	*Santalum spicatum*	Root Heartwood	Auroma / ABP
SDWI	Sandalwood East Indian	*Santalum album*	Heartwood	ABP
SWORG	Sweet Orange	*Citrus sinensis*	Fruit peel	Gya labs
TTO	Tea Tree	*Melaleuca alternifolia*	Branch / Leaves	Auroma / ABP
VTV0	Vetiver *(Indonesia)*	*Chrysopogon zizanioides*	Roots	Natoria
VTV1	Vetiver *(Indonesia)*	*Chrysopogon zizanioides*	Roots	Essential Therapeutics / ABP
YY	Ylang-Ylang	*Cananga odorata*	Flower	Natoria

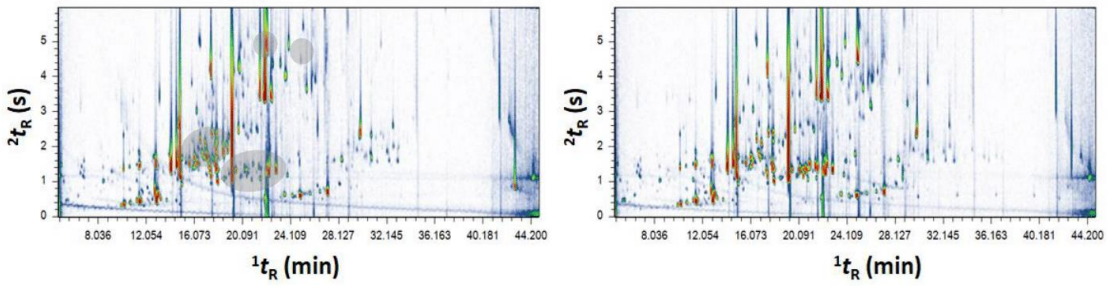
The sources of the essential oil samples are provided in the manuscript "Experimental" Section 2.1.

Appendix S2. GC×GC–MS chromatograms for the essential oil samples.

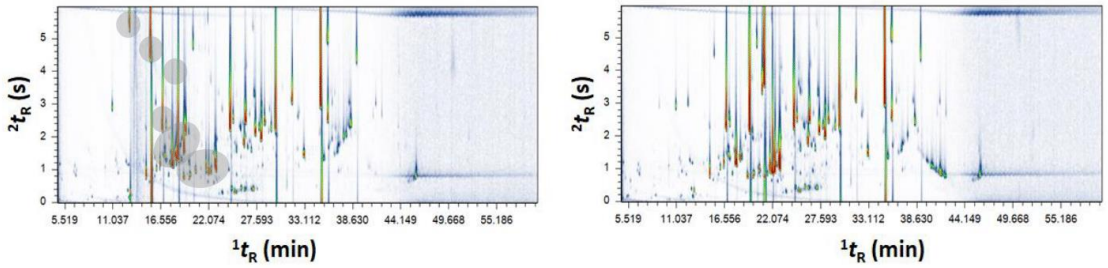
The GC×GC–MS procedures employed in this study are described in the manuscript Experimental Section 2.3. The First dimension retention time and Second dimension retention time are abbreviated as $^{1}t_{R}$ and $^{2}t_{R}$ here. Some of the main areas in the 2D plots of compounds undergoing changes (that either are reduced in the original oil, or are generated in the product) are highlighted in the chromatograms of the "before enzyme reaction".

BEFORE ENZYME REACTION	AFTER ENZYME REACTION

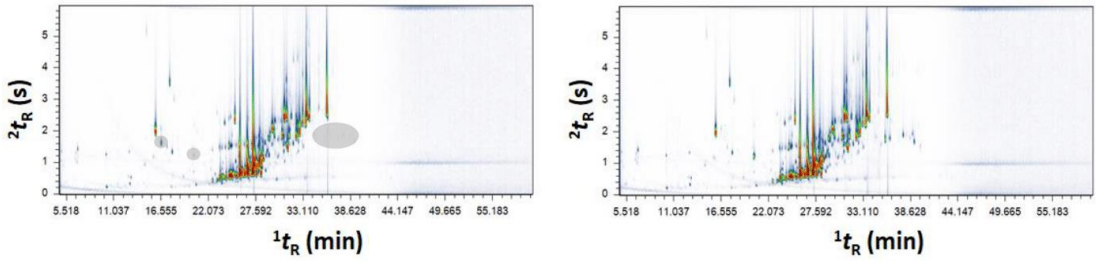
Bergamot (BERG)



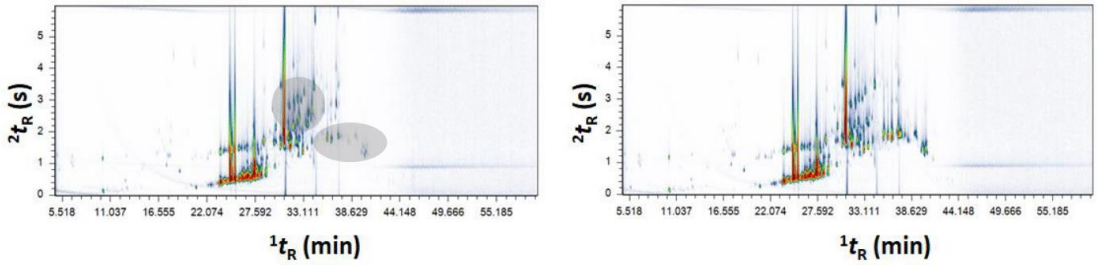
Boronia (BOR)



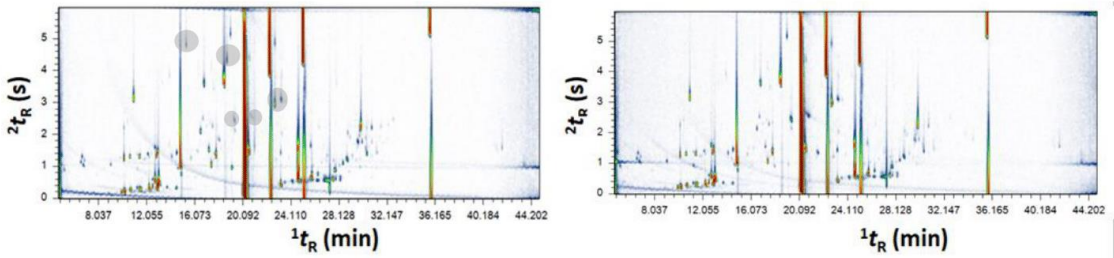
Cedarwood Australian (CDWA)



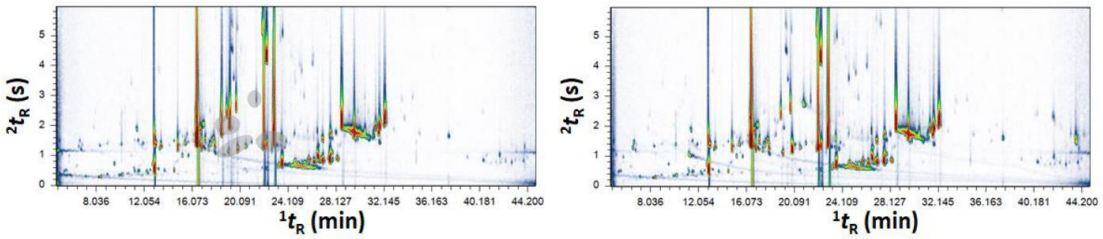
Cedarwood Virginian (CDWV)



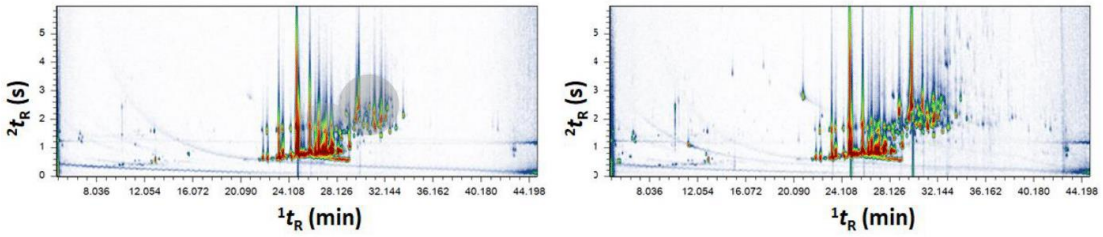
Cinnamon (CINM)



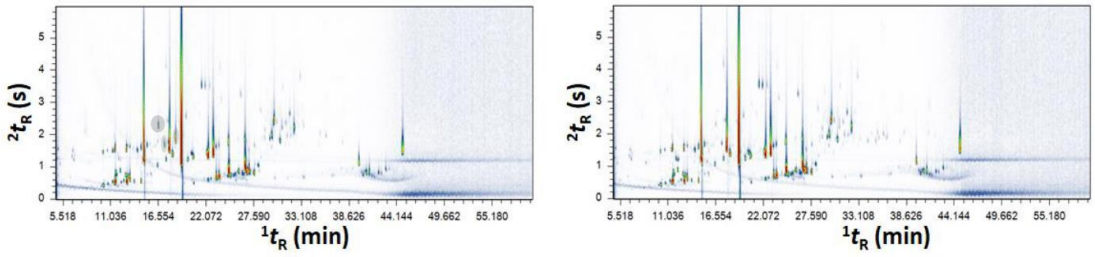
Citronella (CIT)



Copaíba (COP)

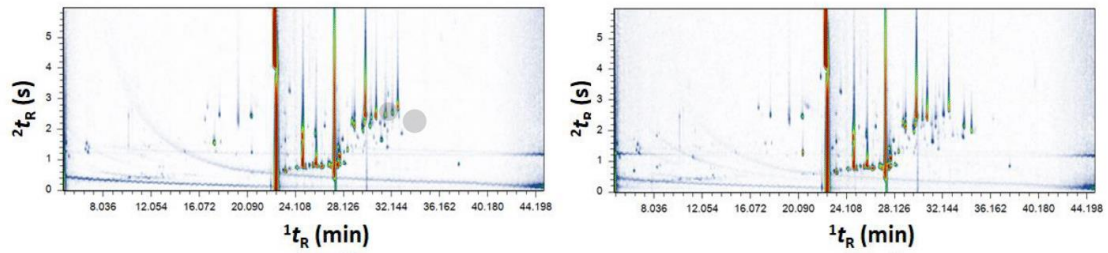


Clary Sage (CLARY)

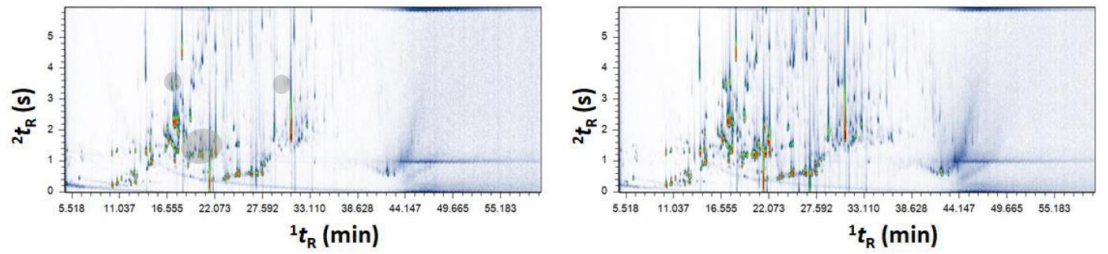


4

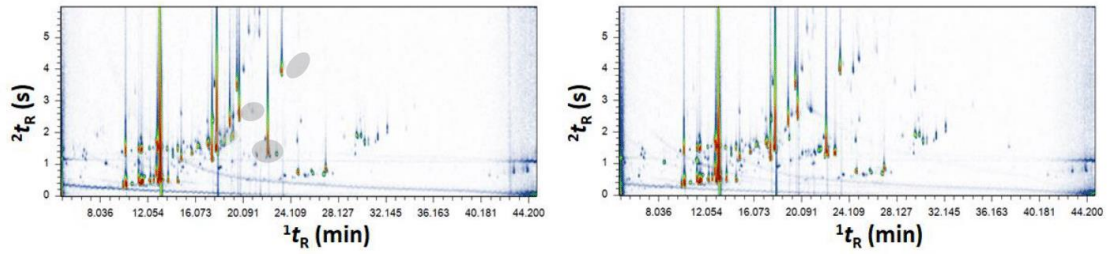
Clove (CLO)



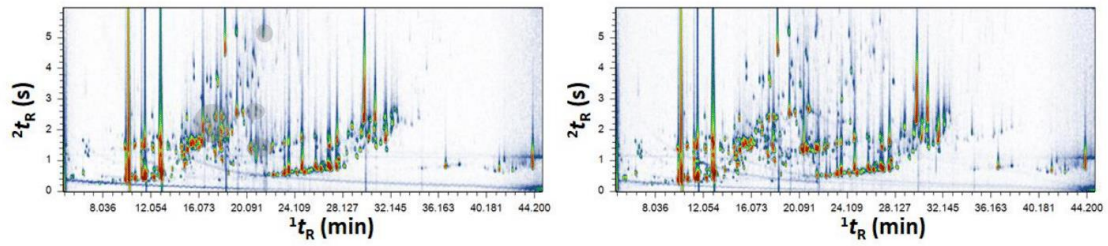
Cypress (CYP)



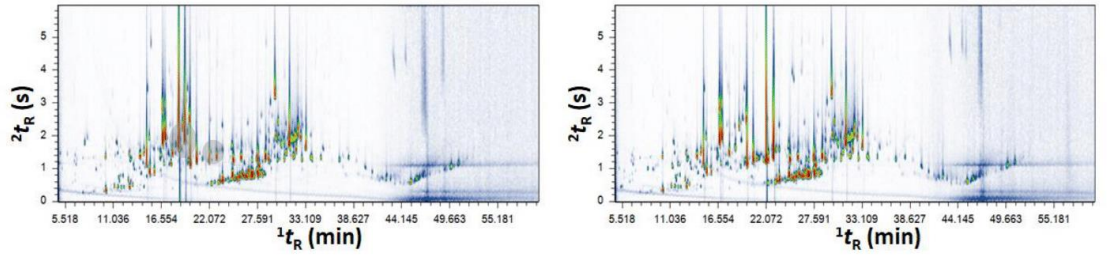
Eucalyptus R. (EUCR)



Frankincense (FKI)

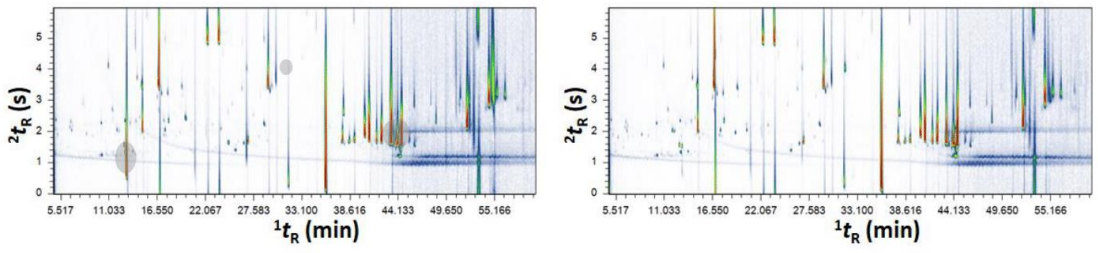


Geranium (GER)

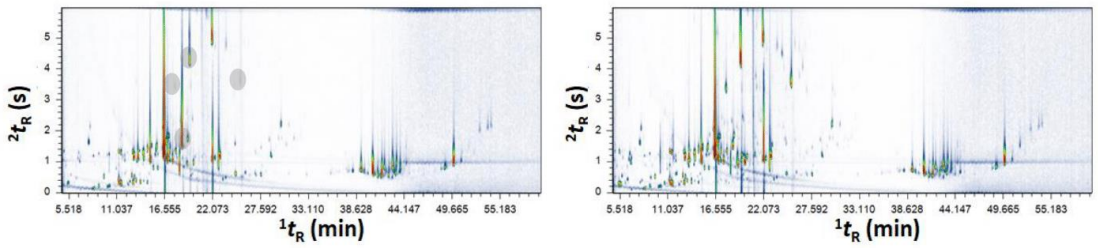


5

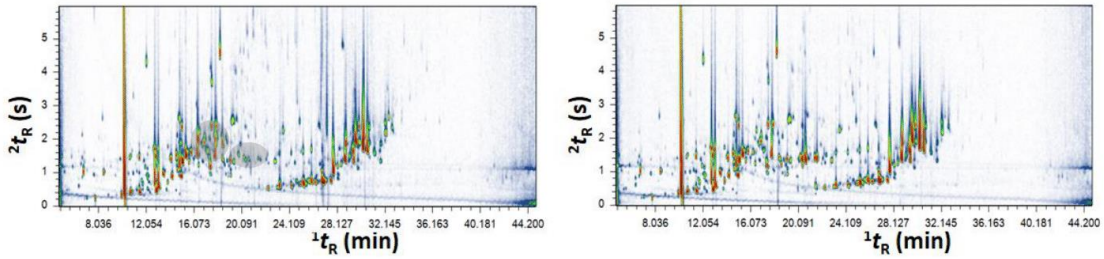
Jasmin (JAS)



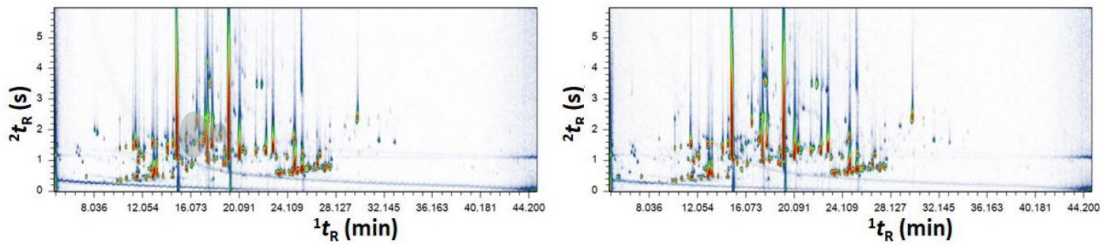
Kaffir Lime (KFL)



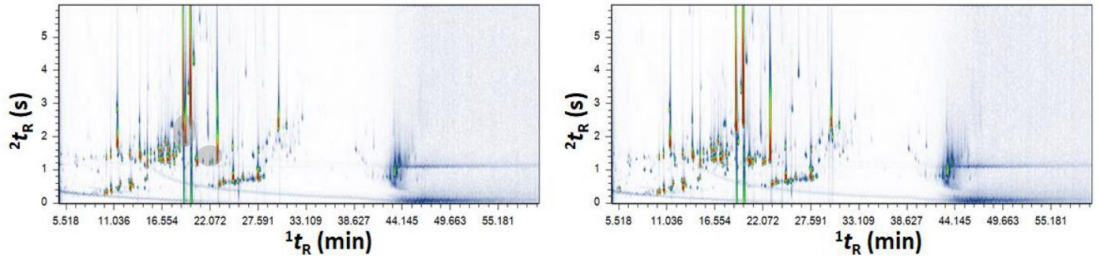
Kanuka (KNK)



Lavender (LAV)

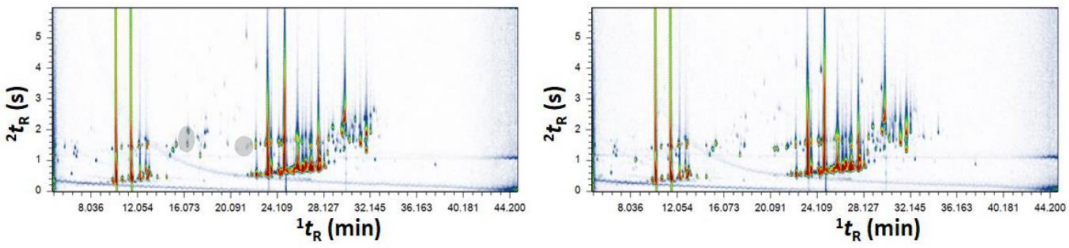


Lemongrass (LMG)

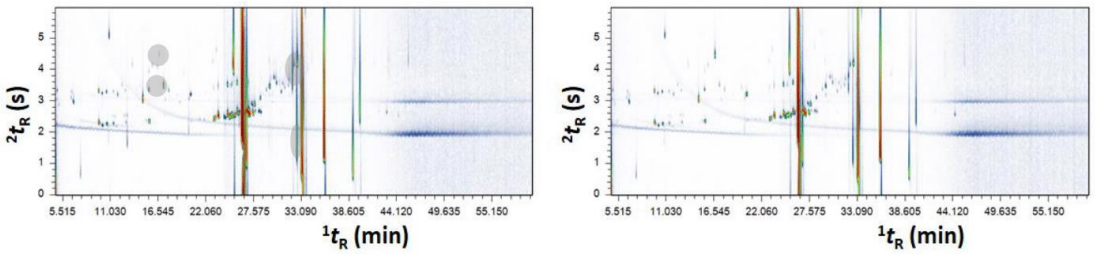


6

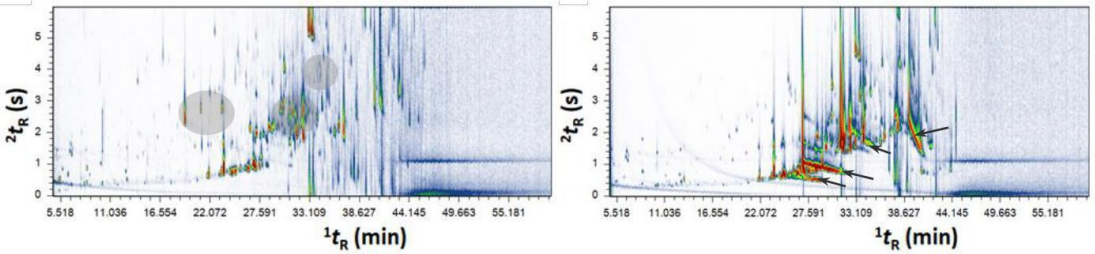
Manuka (MNK)



Masoi Bark (MSB)

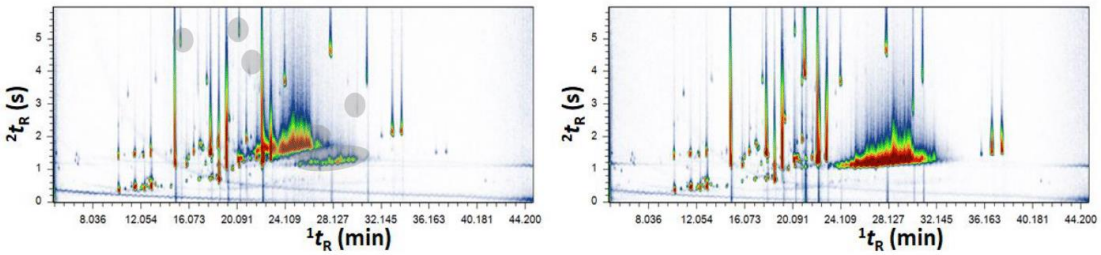


Myrrh (MYR)

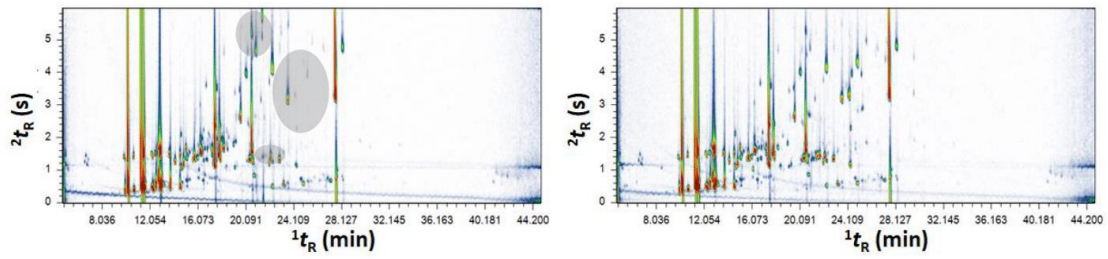


The unusual 'peaks' in the form of bands (indicated here by arrows) observed for MYR in the chromatogram "after enzyme reaction" and other samples, such as CIT and COP, are attributed to an interconversion phenomenon that occurs on the column for certain compounds during the GC separation. The tentative identification of the compounds presenting this elution pattern in the present study indicates they overall belong to the class of terpene hydrocarbons, which is unrelated to the enzyme's targeted substrates (i.e. alcohols or esters).

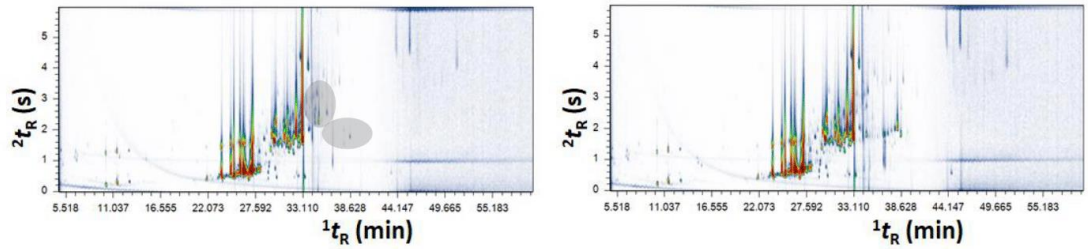
Neroli (NRL)



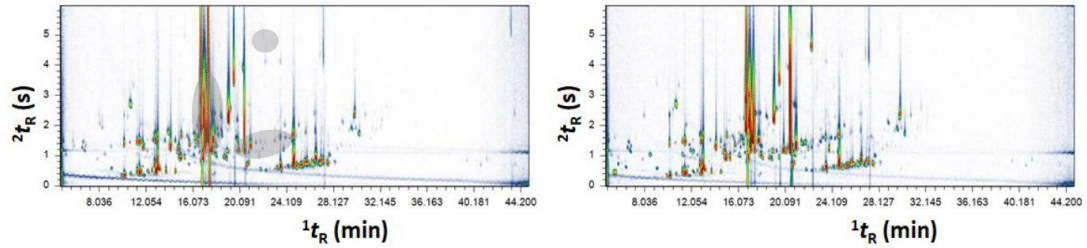
Nutmeg (NMG)



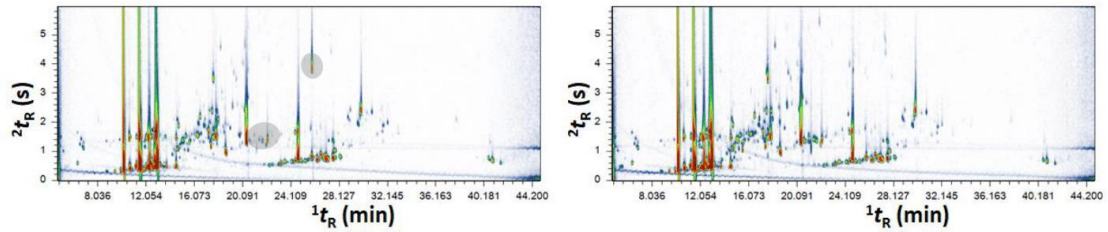
Patchouli (PTC)



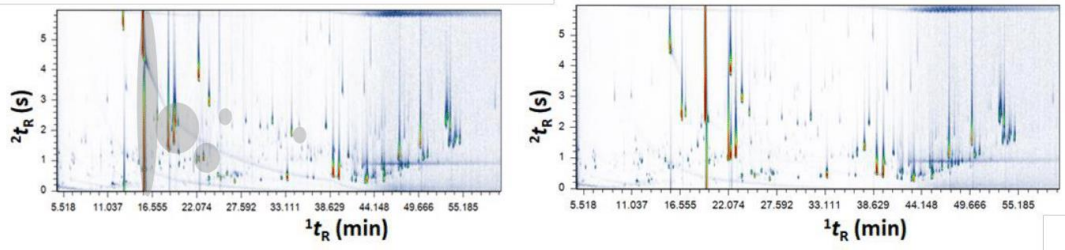
Peppermint (PPMP)



Pine (PINE)

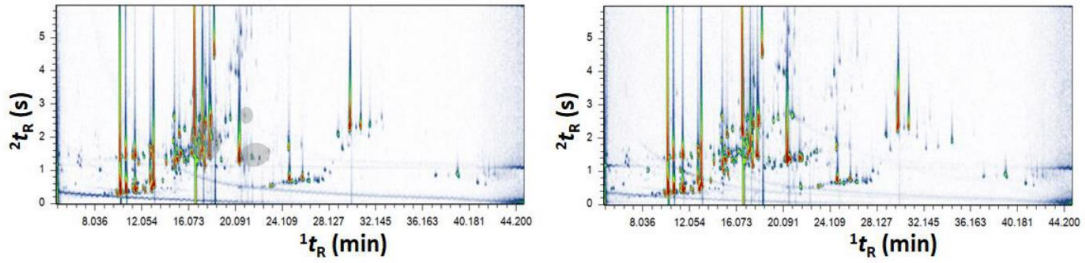


Rose (ROSE)

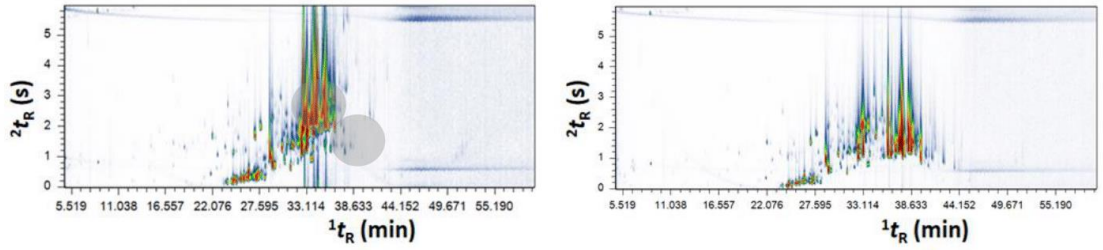


8

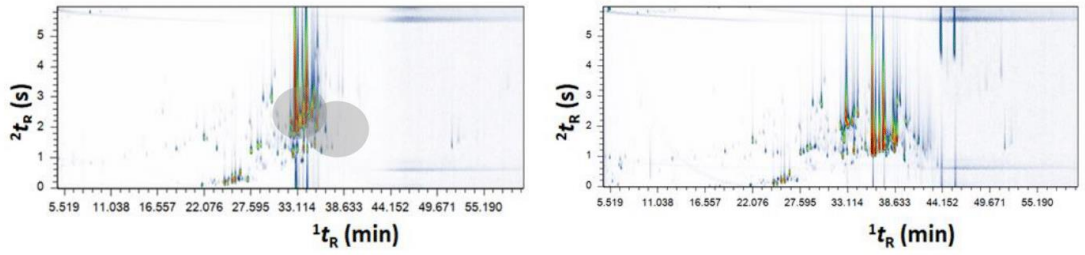
Rosemary (ROSM)



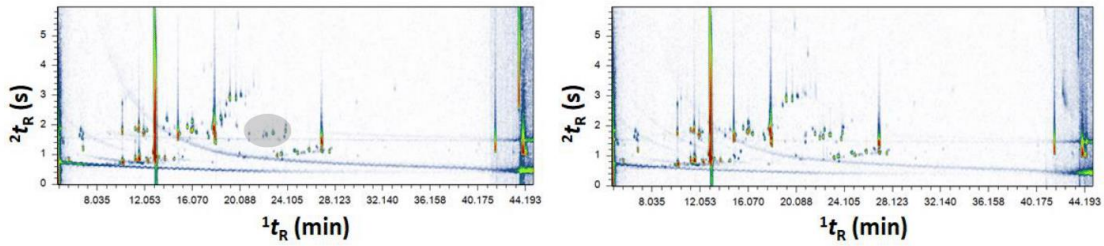
Sandalwood Australian (SDWA)



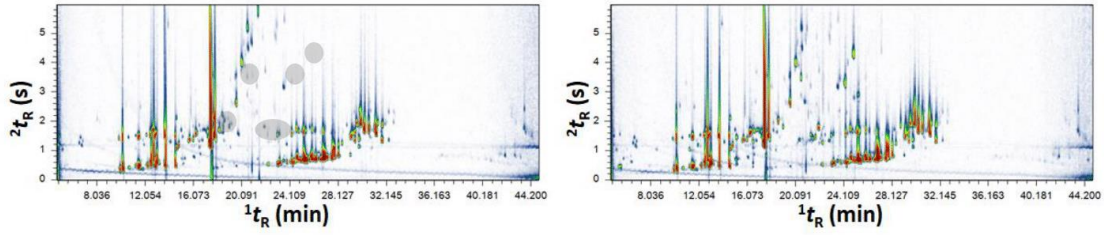
Sandalwood Indian (SDWI)



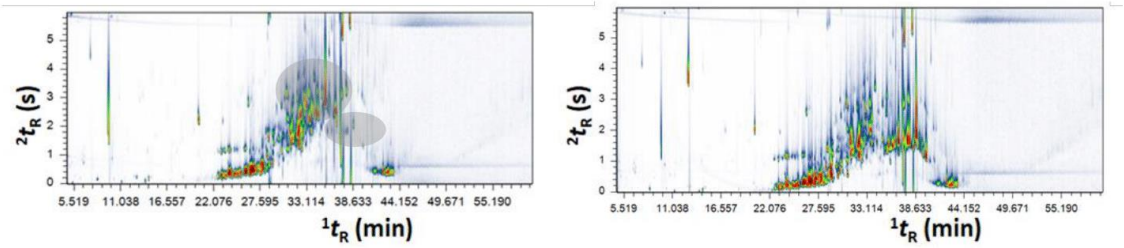
Sweet Orange (SWORG)



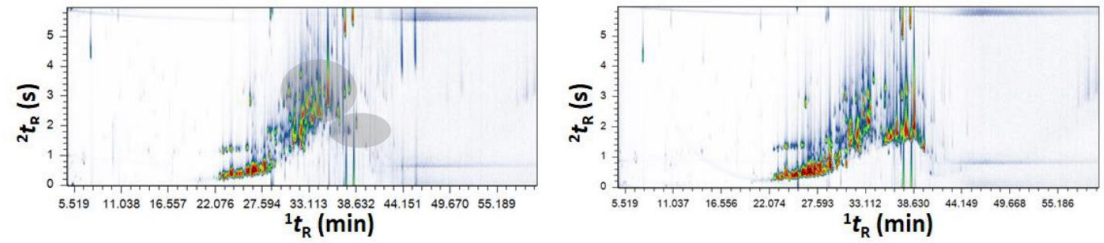
Tea Tree (TTO)



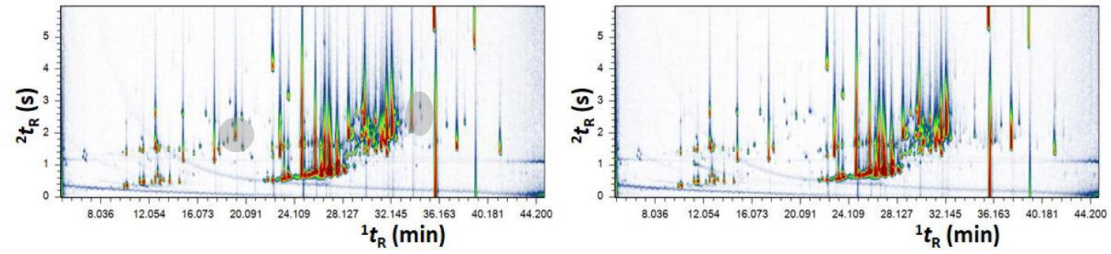
Vetiver (VTV0)



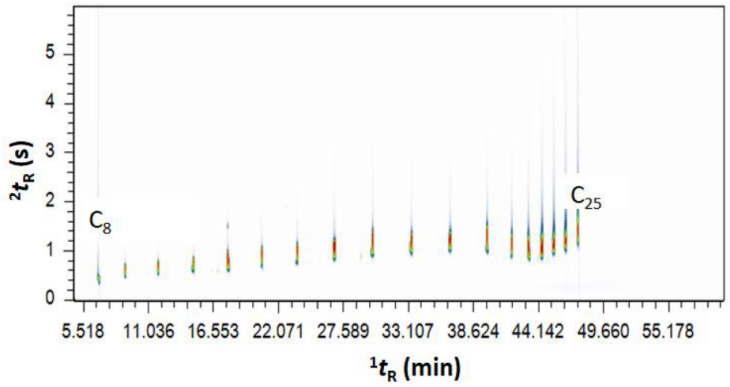
Vetiver (VTV1)



Ylang-Ylang (YY)



Appendix S3. GC×GC–MS chromatogram and table for the *n*-alkanes (C_8-C_{25})



C_n	^1t_R (min)	^2t_R (s)
8	6.87	0.39
9	9.07	0.54
10	11.87	0.60
11	14.87	0.66
12	17.77	0.77
13	20.67	0.85
14	23.67	0.94
15	26.87	1.02
16	30.07	1.11
17	33.37	1.11
18	36.57	1.11
19	39.77	1.19
20	41.87	0.95
21	43.27	0.97
22	44.47	0.98
23	45.47	1.08
24	46.47	1.16
25	47.47	1.31

Chapter 4

Conclusion and future directions

The investigation of enzyme-catalysed alternatives for the sustainable synthesis of aroma compounds and the development of GC analytical strategies are presented in this thesis.

Seven commercially available enzyme products were studied, including five lipases and two laccases. The activity, selectivity and efficiency of the enzymes were determined in different experimental steps, including characterisation, screening, optimisation and application to samples. Characterisation included activity assays (UV-Vis and GC–FID), protein quantification (NanoDrop spectrophotometry) and molar mass estimation (SDS-PAGE electrophoresis). Screening experiments assessed different enzymes, substrates, solvents, acyl donors or mediators. At this stage, the enzyme substrates included aroma-related standards, such as terpene and phenolic compounds. A response surface design of experiment approach was applied to optimise different variables of the enzyme reactions, including temperature, enzyme concentration and co-substrate (acyl donor) concentration, while keeping the substrate concentration constant. Finally, the application step investigated the performance of selected enzymes towards a wide-range of essential oil samples.

CALA, NZ-435 (lipases) and NZ-51003 (laccase) were the best-performing enzymes in the characterisation and screening stages, achieving conversions above 70% for citronellol (lipases) or 50% for eugenol (laccase). CALA lipase was selected for the next steps due to their better performance towards secondary alcohols. Optimised CALA conditions were found to be 37- 40 °C, 3-4 mg/mL of enzyme, and 58-60% (v/v) vinyl acetate. These settings resulted in a conversion of >95% for citronellol after 1 h (for both, standards mixture and citronella oil sample), and 20% or 74% for menthol after 1 h or 24 h, respectively. In the application to samples phase, CALA optimised conditions have demonstrated to be effective in (≥25x) higher substrate concentrations. The enzyme was able to esterify 42 of the 79 alcohols identified in 35 samples within 48 h, with conversions above 90% and 80% for most primary and secondary alcohols, respectively. Overall, lipases have shown a preference for primary alcohols, while laccases preferred phenolic compounds among the tested substrates. No significant conversion of tertiary alcohols and phenols were observed for lipases in the tested conditions. None of the tested enzymes demonstrated significant enantioselectivity under the tested conditions.

The use of organic medium and mild temperatures (to avoid evaporation) were found to be more suitable biocatalytic conditions for most aroma compounds, due to their limited solubility in water and high-volatility. The immobilised lipases were fully compatible with these requirements and were not inhibited by the different levels of complexity (i.e., number, concentration and chemical diversity of components) of essential oil samples, as well as being easy to apply and remove from the reaction media. Meanwhile, both of the laccases

investigated showed low compatibility with organic and organic/aqueous medium, which hindered the study from progressing beyond the screening phase for these enzymes.

The enzyme studies included in this thesis also highlighted the importance of testing the prospective enzymes to a broad range of substrates (e.g., standards and samples) in order to make a better selection and understand the enzymes' selectivity, robustness and inhibitory effects in the desired application settings. This strategy generated remarkable results for CALA lipase, with the production of esters from primary and secondary alcohols, surpassing previous investigations.

In parallel with the above enzyme experiments, different strategies based on GC analysis were developed to facilitate the assessment of all the stages of the biocatalysis study. The analytical methods developed included GC–FID, *e*GC–FID with chiral ratio determination, and GC×GC–MS analysis.

The GC–FID and *e*GC–FID methods were suitable to determine the reaction profiles and chiral ratio variations for biocatalysed reactions with aroma compounds in low complexity samples. This methodology is unique in that it provides direct quantification of substrates and products without the use of chemical indicators, which is usually not possible with non-chromatographic methods. The use of GC analysis also offers the possibility of structural identification of reaction products, when using mass spectrometry as an on-line detection technique (GC–MS), as well as the assessment of the enantioselectivity of the reactions. These are essential for authentication of F&F samples and enable the source-specific discrimination of compounds produced in bioprocesses.

The developed GC×GC–MS method has enabled the separation and identification of 125 target compounds in 35 essential oil samples of different complexities. This advanced chromatographic technique offers superior resolution and improved compound identification capabilities, expanding the analytical information content and enabling the instant qualitative assessment through the 2D plots, which facilitate the visualisation and comparison of the differences in the samples' chemical profiles before and after the enzymatic processing.

The designed methodology and findings of this thesis have significant impact for the analysis and development of more efficient enzyme-catalysed methods that can be used to produce high-value aroma compounds in a more environmentally friendly and cost-effective way. This important area of research has the potential to revolutionise the F&F industry, since the combination of low-cost natural raw materials, such as some essential oils, with the robustness, selectivity, efficiency, scalability, biodegradability, and reusability of the correctly selected biocatalysts, are compliant with the green chemistry principles and have the ability to improve the properties and safety of the final products, without compromising the branding associated with "natural" labelling.

As future directions, the investigation of immobilised laccase preparations could improve their performance towards aroma compounds in organic medium. Additional immobilised enzymes could also be explored to achieve an enantioselective processing, generating products enriched with single enantiomers possessing the most favourable properties (e.g. odour or biological activity). Reusability and scaling up studies would generate interesting insights to support future industrial applications. The investigation of waste products, such as wood, paper, food waste and used cooking oil, as substrates could reduce even more the environmental impact of this process, and so has potential applications beyond the EO / F&F studies considered herein. Additionally, MDGC-O/MS methods could be developed to facilitate the sensory assessment of the bioprocessed essential oil samples by a trained panel. This could help to determine the impact of the enzymatic changes to the odour activity of the target molecules, as well as for the overall sample. Biological activity tests could also be performed to help to determine the allergenicity and toxicity of these new aroma products. Thus, based upon the preliminary studies reported above, there is considerable opportunity to undertake further research in this vast and promising field.