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Lipase hydration state in the gas phase: Sorption isotherm measurements and inverse gas chromatography

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6 **Evaluation of lipase hydration state in the gas phase, by sorption isotherm**
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8 **measurements and inverse gas chromatography; Relation with activity of**
9
10 **the lipase in solid/gas reactor.**
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17

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44 Abbreviations: **IGC**, Inverse Gas Chromatography **a_w**, water thermodynamic activity, **CALB**,
45 *Candida antarctica* Lipase B, **BET**, Brunauer-Emmet-Teller.
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Abstract

The adsorption of water and substrate on immobilized *Candida antarctica* lipase B was studied by performing adsorption isotherm measurements and using Inverse Gas Chromatography (IGC). Water adsorption isotherm of the immobilized enzyme showed singular profile absorption incompatible with the BET model, probably due to the hydrophobic nature of the support, leading to very low interactions with water. IGC allowed determining the evolution with a_w of both dispersive surface energies and acidity and basicity constants of immobilized enzyme. These results showed that water molecules progressively covered immobilized enzyme, when increasing a_w , leading to a saturation of polar groups above a_w 0.1 and full coverage of the surface above a_w 0.25. IGC also enabled relevant experiments to be performed to investigate the behavior of substrates under a_w that they will experience, in a competitive situation with water. Results indicated that substrates had to displace water molecules in order to adsorb on the enzyme from a_w values between 0.1 to 0.2, depending on the substrate. As the conditions used for these adsorption studies resemble the ones of the continuous enzymatic solid/gas reactor, in which activity and selectivity of the lipase were extensively studied, it was possible to link adsorption results with particular effects of water on enzyme properties.

1 Introduction

Contrary to expectations that enzymes are only active in aqueous solutions, enzyme activity in almost anhydrous media was demonstrated in the 1930s and has been extensively explored for the last decades [1, 2]. These media include among others organic liquid, supercritical fluids, solvent-free, ionic liquids and gas phase systems.

These non-conventional almost anhydrous reaction media possess great advantages over classical aqueous ones, such as shifting of thermodynamic equilibrium in favor of synthesis over hydrolysis and allowing easier recovery of products. However a small amount of water is required for catalytic action of enzyme, as some residual bound water are absolutely essential for maintaining the enzyme active conformational state and for promoting flexibility of enzyme, by acting as a lubricant [3, 4].

The quantity of required water varies depending on each individual case. The optimal amount depends on several parameters, including, polarity of enzyme active site, solvent hydrophobicity, substrate and reaction conditions. In any case, the activity, stability and specificity of enzymes in non-conventional media are greatly affected by the level of water present in the medium.

The best way to quantify the availability of water to the biocatalyst in non-conventional media is to use the parameter “thermodynamic water activity” (a_w), which describes the effect of mass action of water on the equilibrium and is therefore a better measure of the effect of water than the water content [5].

Many studies were performed in organic solvents at varying a_w . Nevertheless, the influence of water on biocatalysis, remains unclear and needs to be further elucidated. In particular, no general trend has been found for the dependence of selectivity, especially enantioselectivity, on a_w [6, 7].

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5 For several years, we have been working with continuous gas phase reactors for performing
6 enzymatic reactions. In this type of reaction medium, a solid packed enzymatic sample is
7 percolated by a carrier gas, which simultaneously carries gaseous substrates to the enzyme
8 and removes gaseous products. In this system the a_w in the reaction medium can be perfectly
9 controlled and fixed independently of the other reaction medium components, by
10 incorporating water in the inlet gas at the correct molar fraction. If the gas phase is assumed
11 as ideal, a_w is then obtained by dividing the partial pressure of water by its saturation pressure
12 at the operating temperature. In case of organic media however, the calculation of a_w requires
13 the knowledge of activity coefficients, whose estimation is far from being straightforward.
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15 The solid/gas reactor is therefore a powerful tool for studying accurately the sole influence of
16 a_w on biocatalysis [8, 9].

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The effect of a_w on different types of reactions catalyzed by lipase B from *Candida antarctica* has been studied extensively in our laboratory, by using solid/gas reactor. It was observed that water had a very complex role, and could simultaneously act as a lubricant, by change in enzyme flexibility and increase of enzyme activity, as a competitive and enantioselective inhibitor, as a competitive substrate, and by interacting with all reactants, free enzyme, transition state and products, like solvent molecules do in liquid media [10-13].

In our different studies performed in solid/gas reactors, all these different effects of water have been assessed as a function of a_w in the reaction medium, but could not be relied to real hydration state of the enzyme. This is unfortunate as such knowledge is essential to gain a fundamental understanding on the effect of water on enzyme catalysis. At which a_w protein charged groups are they likely to be surrounded by clusters of water molecules? Above which a_w is a water monolayer retained on the enzyme surface, surrounding both charged and polar groups of the enzyme? Above which a_w has the substrate to displace water molecules adsorbed to the enzyme to interact with it? The answer to this last question is not only

1
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3 dependent on a_w level allowing water monolayer formation, but also on the ability of
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5 substrate to compete with water for adsorption on the immobilized enzyme.
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8 In the following study, we are trying to answer all these questions, by studying adsorption of
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10 both gaseous water and substrates on enzyme, thanks to adsorption isotherm measurements
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12 and the use of Inverse Gas Chromatography (IGC), which is a dynamic and very accurate
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14 vapor adsorption technique, allowing accurate characterization of surface properties [14-16].
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17 In IGC the roles of stationary (solid) and mobile (carrier gas and vapor probe molecule)
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19 phases are inverted from traditional analytical Gas Chromatography (GC). Indeed, in GC, a
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21 standard column is used to separate and characterize several vapor molecules, whereas in
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23 IGC, known vapor probe molecules are injected into a column packed with the solid sample
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25 under investigation, which is immobilized CALB in our case, and whose surface
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27 characterization is performed.
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30

31 There have been very few experimental studies addressing both water and substrates
32
33 adsorption on enzymes [17, 18], although these adsorption phenomena might be a way to
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35 better elucidate enzyme kinetics and specificity. Recently we have studied molecular
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37 mechanism of hydration of free, deglycosylated, and desalted pure CALB in the gas phase.
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39 We showed that hydration occurs in two phases. For water activities below 0.5, water initially
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41 binds to well-defined water-binding sites at the protein surface and then above 0.5, gradually
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43 a spanning water network is formed [19].
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48 Nevertheless these results cannot be directly used to explain the reaction kinetics we
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50 measured in solid/gas reactor, for which we had to use an immobilized CALB preparation on
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52 silanized Chromosorb. Indeed, this porous diatomite support allows a uniform carrier gas and
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54 probe vapour flow through the enzymatic fix bed, and a better access of gas to the enzyme
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56 surface. Moreover, it avoids large pressure drop across the column containing the enzyme
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58 sample.
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3 In the present paper, we show adsorption studies performed with the same immobilized
4 enzymatic sample, in order to be able to overlay these adsorption phenomena and CALB-
5 catalyzed reaction kinetics in continuous solid/gas reactors.
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10 11 12 **2 Materials and methods**

13 **2.1 Enzyme and chemicals**

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15 *Candida antarctica* lipase B was a lyophilized preparation (Chirazyme L2) kindly supplied by
16 Roche Industrie (Penzberg, Germany). All probes used in IGC experiments were of the
17 highest purity (99% minimum) and checked by gas chromatography before use.
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26 **2.2 Adsorption of lipase onto a solid support**

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28 CALB adsorption was performed onto 60-80 mesh Chromosorb P AW DMCS, (Acid Washed
29 DiMethylChloroSilanized; Varian, France), as previously described [20], by using 30 mg of
30 lyophilized Chirazyme L2 per 6 g of support.
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38 **2.3 Measurement of biocatalyst water adsorption isotherm**

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40 Water adsorption isotherm of Chromosorb alone and with immobilized lipase were measured
41 at 25°C by using a DVS Advantage automated moisture sorption analyzer (Surface
42 Measurement Systems Ltd., London, UK) between 0 and 95% relative humidity. The samples
43 were initially dried for 10 h at 25°C under totally dried flowing air in the DVS moisture
44 sorption analyzer to obtain the starting dry sample mass. The required humidity is generated
45 by mixing dry and water saturated vapor gas flows in the correct proportions using two mass
46 flow controllers and one vapor humidifier. The instrument was run with a dm/dt (m , mass; t ,
47 time) set at $0.002\% \text{ min}^{-1}$ to reach the equilibrium. This means that the relative humidity will
48 remain constant until the mass variation falls consistently below this threshold. Once this
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condition has been reached, the relative humidity of the system will be raised or lowered to the next level.

The water adsorption curve was modeled using the Brunauer-Emmet-Teller (BET) [21] gas adsorption equation (Eq 1).

$$\frac{a_w}{(1-a_w) \times m} = \frac{1}{m_0 \times C} + \frac{a_w \times (C-1)}{m_0 \times C} \quad (1)$$

where m is the mass of bound water (in mg per mg of dry enzyme), m_0 is the monolayer water coverage, a_w the water activity and C a constant. From the linear regression of the plot of

$\frac{a_w}{(1-a_w) \times m}$ versus a_w , the theoretical monolayer water coverage can be calculated:

$$m_0 = \frac{1}{S + I}, \text{ where } S \text{ is the slope and } I \text{ the y-intercept [22].}$$

2.4 Inverse Gas Chromatography (IGC) measurements

Theory of IGC

IGC consists in measuring the retention times of vapor probes of known properties in a column packed with the solid material of interest, which is immobilized CALB in our case. The retention time of these probes is related to the affinity of the probes for the support. Measuring how this retention time changes as a function of probe molecule chemistry, column temperature and ambient relative humidity, allows obtaining a wide range of physical properties of the solid under investigation.

In the present study, IGC was operated in the infinite dilution regime, which means that a very small amount of vapor probe was injected into the carrier gas stream through the column, leading to a very low surface coverage by the adsorbates. The retention volume of the probe is thus independent of its concentration and the result is a linear adsorption isotherm, which can be described by Henry's Law.

The specific retention volume V_G of vapor probes, normalized to a temperature of 273.15K and 1g of supported enzyme was calculated using Eq 2, where t (min) is the retention time of the probe, t_0 (min) is the dead-time obtained with methane, F (mL.min⁻¹) the carrier gas flow rate, m (g) the mass of immobilized CALB and T (K) the temperature of the column. The James and Martin compressibility factor, j , was calculated using Eq 3, where P_i and P_0 are the inlet and outlet pressures, respectively.

$$V_G = j \cdot \frac{(t - t_0)}{m} \cdot \frac{F \times 273.15}{T} \quad (2)$$

$$j = \frac{3}{2} \cdot \frac{(P_i / P_0)^2 - 1}{(P_i / P_0)^3 - 1} \quad (3)$$

The specific retention volume V_G of vapor probes is related to the variation of the free energy of adsorption ΔG_{ads} in the following way:

$$\Delta G_{ads} = -(R \times T \times \ln V_G) + C$$

where C is a constant depending on the choice of a reference state of the adsorbed probe and also on the total area of the solid accessible to the probe.

ΔG_{ads} takes into account two kinds of interactions: dispersive interactions (corresponding to London forces) and specific interactions, which consists mostly out of Lewis acid-base contributions.

$$\Delta G_{ads} = \Delta G_{ads}^d + \Delta G_{ads}^{sp}$$

To obtain the dispersive as well as the specific parts of ΔG_{ads} , different apolar and polar vapor phase probe molecules are respectively injected and their specific retention volume V_G determined.

In case of apolar probes, like alkanes, which can only interact by dispersive interactions, V_G is related to ΔG_{ads}^d by Eq 4 :

$$\Delta G_{ads}^d = -RT \ln V_G + C = -2N(\gamma_S^d)^{\frac{1}{2}} a(\gamma_L^d)^{\frac{1}{2}} + C' \quad (4)$$

Where N is the Avogadro constant, R the gas constant, a the probe surface area, γ_L^d the dispersive component of the probe surface energy, γ_S^d the dispersive component of the solid, and C' a constant. If a series of alkanes is injected, γ_S^d can be derived from the slope of the alkane line in a plot of $RT \ln V_G$ versus $a(\gamma_L^d)^{\frac{1}{2}}$.

If amphoteric polar probe molecules are also injected, then specific interactions can be measured. The experimental points for the polar probe molecules are located above the alkane line in the surface energy plot. The vertical distance between each point and the alkane straight line represents the specific contribution of the interaction, which is expressed as the specific free energy ΔG_{ads}^{sp} (Figure 1). In the present study, the specific free enthalpy of adsorption and the dispersive component of the surface energy of supported enzyme were calculated using the constants given in Table 1.

The enthalpic part of ΔG_{ads}^{sp} (ΔH_{ads}^{sp}) can be calculated by performing the experiment at different temperatures, and calculating the slope of the plot $\Delta G_{ads}^{sp}/T$ versus $1/T$, according to Eq 5, derived from the Gibbs equation:

$$\Delta G_{ads}^{sp}/T = \Delta H_{ads}^{sp}/T - \Delta S_{ads}^{sp} \quad (5)$$

These ΔH_{ads}^{sp} values can then be converted into acid-base parameters if acid-base concepts are applied, which were developed by several authors, including Gutmann [23]. The Gutmann approach involves a simple four-parameter equation, with an acid and base term for each amphoteric polar probe according to Eq 6:

$$\Delta H_{ads}^{sp} = K_A \times DN + K_B \times AN^* \quad (6)$$

K_A is the acceptor (acid) number and K_B is the donor (base) number of the solid surface. DN and AN^* are the donor and acceptor numbers of the amphoteric vapor probe molecules, which are available from literature [24] and given in Table 1.

K_A and K_B can then be obtained by plotting $\Delta H_{ads}^{sp} / AN^*$ versus DN / AN^* , with ΔH_{ads}^{sp} obtained for several amphoteric probes.

For the determination of ΔH_{ads} of pentan-2-ol and hexan-3-ol, it was assumed that the adsorption enthalpy was independent on temperature on the range studied. ΔH_{ads} was then obtained from a plot of $\ln(V_G/T)$ versus $1/T$, according to Eq 7, derived from the Clausius-

Clapeyron equation $\left(\frac{\partial \ln p}{\partial (1/T)} \right)_v = -\frac{\Delta H_{st}}{R}$, giving the differential isosteric heat of adsorption

ΔH_{st} , by plotting $\ln p$ versus $1/T$, applied to chromatographic data at infinite dilution, which are necessarily isosteric (constant adsorbate coverage of zero) [25, 26].

$$\ln(V_G / T) = -\Delta H_{ads} / RT + C \quad (7)$$

Conditions for IGC measurements

Experiments were performed using an SMS-IGC2000 apparatus (Surface Measurement Systems Ltd., London, U.K.), allowing fully automated determination of interactions of ten different gas probe molecules with solid column packing material under investigation (Chromosorb alone or with immobilized CALB in our case), under a wide range of humidity and temperature conditions. A complete explanation of the design of this apparatus can be found in reference [27].

For each IGC experiment, DMCS treated 4 mm ID glass column packed with 500 mg of support alone or immobilized CALB was used. Before measurements, column packed was pre-conditioned for 10 hours at the initial temperature and a_w studied.

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3 To obtain the dispersive as well as the specific surface energies, different polar and non-polar
4 vapor phase probe molecules were injected and their specific retention volume V_G determined.
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6 250 μL of each probe were injected with p/p° was fixed to 0.025, where p and p° being
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8 respectively equal to partial pressure of probe in the gas and saturated vapour pressure of
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10 probe, calculated by assuming an ideal gas type behavior by $p^\circ(T)=e^{(-\Delta H_{\text{vap}}/RT)}$, at temperatures
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12 comprised between 313 and 338K and a_w ranging from 0 to 0.4. The carrier gas (helium) flow
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14 rate was 10 mL/min. All measures of retention time were triplicated.
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20 To determine the enthalpies of adsorption (ΔH_{ads}) of pentan-2-ol and hexan-3-ol on
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22 immobilized CALB, 250 μL of pentan-2-ol or hexan-3-ol at $p/p^\circ = 0.05$ were injected at
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24 varying temperature: between 308 and 324K for $0 < a_w < 0.15$ and between 314 and 322K for
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26 higher a_w values with pentan-2-ol; between 314 and 322 for all a_w values with hexan-3-ol.
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32 **3 Results**

34 **3.1 Water adsorption by immobilized CALB, studied by water adsorption isotherm**

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36 Water adsorption experiments were carried out with both silanized Chromosorb support alone
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38 and the same support with immobilized CALB (Figure 2). Although the ratio of enzyme
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40 CALB to support was only 1/200 (m/m), the presence of enzyme on the support induced
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42 significant changes: Support alone presented a slight uptake of water in the total range of a_w ,
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44 with an overall uptake of around 0.15% mass increase at $a_w = 0.9$. Support with immobilized
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46 enzyme adsorption isotherm showed an uptake of water between 2 and 3 higher than support
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48 alone, in the first part of the curve (a_w 0 to 0.35) and a water sorption exponential and much
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50 higher for a_w values above 0.35, reaching 0.92% mass increase at $a_w = 0.9$.
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56 The water adsorption isotherm shape of Chromosorb with immobilized CALB differs from
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58 the sigmoid shape of curves obtained with CALB alone [19], and more generally with isolated
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60 proteins [28], which can be modeled by the BET equation.

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3 The BET model assumes the gradual recovery of surface by homogenous layers, characterized
4 by a constant C , associated to the energy of hydration of the surface. It allows in particular
5 revealing the formation of a water monolayer and its weight, and the occurrence of free water
6 for higher a_w [17]. Yet, BET model is considered as a good mathematical tool to characterize
7 the water adsorption isotherm and to determine the size of the monolayer for proteins, and this
8 for a_w up to 0.4. Beyond this value, the BET model is known to gradually sweep from
9 experimental observations [21].

10
11 In our case, the curve which presents $a_w/(1+a_w)m$ as a function of a_w (Figure 3) does not fit
12 the BET model. This assumption is highlighted at $a_w = 0.2$, where the curve displays a break
13 point whereas a linear curve is expected for enzymes following the BET model [19].

14
15 These results suggest that Chromosorb support prevents the possibility to evaluate the CALB
16 hydration with BET model. The water adsorption isotherm we obtained, resembles type III
17 isotherm, according to IUPAC classification proposed by Brunauer, Deming and Teller [29].
18 This uncommon isotherm suggests that interactions between water and solid are very low,
19 which is actually the case with silanized Chromosorb of hydrophobic nature.

20
21 Thus, we used an alternative method, i. e. IGC, to analyze the feature of CALB adsorbed on
22 support. IGC allows determining many parameters of solid surface, such as dispersive and
23 specific surface energy. By following the evolution of these parameters with varying a_w , the
24 hydration state of CALB can be estimated indirectly.

25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 **3.2 Determination of a_w allowing saturation of water binding sites on immobilized** 54 **CALB, by studying surface properties of immobilized lipase by IGC**

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56 The saturation of water binding sites at the surface of immobilized enzyme CALB, when
57 increasing a_w , can be estimated by determining the surface properties, like propensity to
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3 adsorb apolar molecules or surface Lewis acid-base properties, as a function of a_w . These
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5 investigations can be performed by IGC, which has become a widely used technique to
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7 characterize the surface properties of solid organic compounds like natural fibers [30] and
8
9 food materials [31].
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12 13 14 15 *Influence of a_w on the adsorption of apolar molecules on immobilized CALB*

16
17 Figure 4 presents the evolution of the specific retention volumes V_G of five apolar probes *n*-
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19 undecane (C11), *n*-decane (C10), *n*-nonane (C9), *n*-octane (C8) and *n*-heptane (C7) with the
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21 a_w of the carrier gas, through the column filled with immobilized CALB, at 313K. For the
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23 longest alkanes, *n*-undecane and *n*-decane, it appears that the retention volumes decrease and
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25 then reach a plateau near $a_w = 0.2$. This same observation has been reported by Comte *et al.*
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27 on glass beads for $a_w \geq 0.15$, and was interpreted as coverage of the surface of the support by
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29 water molecules [32].
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34 Indeed water molecules progressively hide interaction sites from alkane probes and their
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36 retention time decrease. When the specific retention volume becomes constant it may be
37
38 assumed that the surface of the immobilized CALB is covered by a layer of water molecules.
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40 Then the alkane probes adsorb on a film of water rather than on supported CALB.
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44 The value of γ_S^d for the immobilized enzyme followed the same trend with increasing
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46 humidity (Table 2), thus confirming the hypothesis of water coverage of the surface at this
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48 water activity level.
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51 52 53 *Influence of a_w on the adsorption of polar molecules*

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55 The use of polar probes allows evaluation of Lewis acid-base interactions between these
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57 probes and a solid support. First, the acid-base character of the immobilization support alone,
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59 silanized Chromosorb, was determined by IGC at $a_w = 0$. Monopolar probes were used:
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3 chloroform and 1,4-dioxane, being respectively Lewis acid and basic probes, in order to
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5 obtain respectively a measure of the Lewis basicity and acidity of the support. It needs to be
6
7 emphasized that ΔG_{ads}^{sp} values measured are not intrinsic properties of the support but depend
8
9 on the properties of the adsorbate as well. Nevertheless results allowed to conclude that the
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11 support presented predominantly an acidic or electron-accepting character, as it presented a
12
13 much higher affinity for 1,4-dioxane ($\Delta G_{ads}^{sp} = 9.5$ kJ/mol/mg) compared to chloroform
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15 ($\Delta G_{ads}^{sp} = 5$ kJ/mol/mg). This could be attributed to the presence of residual silanol groups at
16
17 the surface of the support after silanization treatment.
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22 Moreover, the Lewis basicity and acidity of the support with the immobilized CALB were
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24 determined in the same conditions with both monopolar probes. It was found that support with
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26 the enzyme and buffer salts has an acidic character even more pronounced than the
27
28 Chromosorb alone: the immobilized CALB affinity for 1,4-dioxane is higher ($\Delta G_{ads}^{sp} = 12.4$
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30 kJ/mol.mg) and its affinity for chloroform even lower ($\Delta G_{ads}^{sp} = 2.1$ kJ/mol.mg). These
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32 variations cannot be attributed to the immobilization buffer salts (pH 7.5), they are probably
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34 due to the presence of the enzyme. We assume that amino acids rather basic nature, such as
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36 arginine, lysine and histidine, are in contact with the support (including residual silanol
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38 groups) and become thus hidden. As a result, only the amino acid with acidic side chain may
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40 interact with the polar probes, resulting in a decrease in basicity.
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49 To determine the effect of a_w on Lewis constants for the immobilized CALB, ΔG_{ads}^{sp} of
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51 different polar amphoteric probes (ethanol, acetonitrile and ethyl acetate) were determined at
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53 different humidity. Results obtained at 313K are shown on Figure 5. It appears that values of
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55 ΔG_{ads}^{sp} for the different probes strongly decrease when increasing a_w from 0 to 0.1 and then
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57 stabilize. Such a drop in the specific free energy of sorption with increasing a_w at low
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59 humidity level, followed by a stabilisation at higher a_w level, has been observed by Comte *et*
60

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3 *al.* for the adsorption of gaseous probes on mineral solids [32]. This evolution was interpreted
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5 as a progressive coverage of the surface by water molecules until polar sites were saturated.
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8 The enthalpic part of ΔG_{ads}^{sp} , ΔH_{ads}^{sp} , was calculated for each of these probes by performing
9
10 these experiments at different temperatures for each a_w . Figure 6 shows an example of the
11
12 curves obtained for $a_w = 0.2$, between 313 and 338K.
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14

15 Then, from the donor or acceptor character of electron of injected probes and from
16
17 ΔH_{ads}^{sp} values, K_A acceptor (acidity) and K_B the donor (basicity) properties of the immobilized
18
19 CALB, were evaluated. The Lewis acidity (K_A) and basicity (K_B) constants decrease when a_w
20
21 increase (Table 2), following the same trend as ΔG_{ads}^{sp} values. These results indicate that water
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23 molecules are able to progressively mask polar surface groups of immobilized enzyme and
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25 that these groups are completely covered by water at a_w equal to 0.1.
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30 In accordance with the result obtained with monopolar acidic and basic probes, the supported
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32 enzyme presented a higher Lewis acidity constant K_A than Lewis basicity constant K_B .
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38 **3.3 Determination of competitive adsorption of substrates and water at the surface of** 39 40 **immobilized lipase by IGC**

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42 In our experiments with enzymes in gas phase, different polar substrates were used, including
43
44 primary or secondary alcohols. These compounds may compete with water for adsorption
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46 sites on the immobilized enzyme. A way to investigate this competition is to measure the
47
48 enthalpy of adsorption (ΔH_{ads}) of polar substrates, on immobilized enzyme at different a_w .
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51 This parameter is a direct measure of the strength of interaction between substrate and
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53 supported enzyme and the evolution of this parameter with a_w can be ascribed as site blocking
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55 by water in a competitive adsorption situation.
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3 The influence of a_w on ΔH_{ads} of pentan-2-ol and hexan-3-ol on immobilized CALB is shown
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5 on figure 7.
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8 First, it can be observed that on the total range of a_w studied, for both pentan-2-ol and hexan-
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10 3-ol adsorption, $|\Delta H_{ads}|$ values are higher than the enthalpy of condensation, being
11
12 respectively equal to -52.6 kJ/mol and -43.4 kJ/mol. This implies that the enthalpies of
13
14 adsorption are not only due to the heat of condensation of the compound onto the solid, but
15
16 also to physic-chemical interactions between compound and immobilized enzyme.
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19 Secondly, it appears that the enthalpy of adsorption of pentan-2-ol and hexan-3-ol vary in two
20
21 stages with a_w . In the first stage, $|\Delta H_{ads}|$ decreases rapidly with increasing a_w . In the second
22
23 stage, it continues to decrease but with a very low slope. This stabilisation of the $|\Delta H_{ads}|$
24
25 values corresponds to a decrease of alcohol retention times, and to their lower variation with
26
27 temperature. This could be attributed to the coverage of the immobilized lipase by adsorbed
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29 water molecules, which hides binding sites from alcohols, in a competitive adsorption
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31 situation. The a_w corresponding to the breaking point between these two parts of the
32
33 adsorption curve decreases from 0.2 to 0.1, when changing from pentan-2-ol to hexan-3-ol.
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42 **3.3 Relation between hydration state and CALB activity and selectivity**

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44 The IGC study allows finding some correlation between CALB catalytic activity at different
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46 a_w in gas phase, and water status (strongly or weakly bound). In our solid/gas enzymatic
47
48 reactor experiments, the optimal a_w with respect to alcoholysis reaction was found to be in
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50 range 0.05 to 0.1 [33]. This corresponds to a water activity level at which only stronger
51
52 adsorptive interactions with water occurs, and polar groups of immobilized enzyme are not
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54 totally covered by water. This high catalytic activity of CALB at very low a_w was also
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56 observed by other authors in liquid media [34]. It could be assigned to the ability of the
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58 dehydrated enzyme to keep essential water molecules in its structure by tight bonding. For
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3 many other enzymes, it was shown that they begin to regain their catalytic activity at
4 hydration levels comprised between 0.12 and 0.2 mg H₂O/mg protein, corresponding to a_w
5 values much higher than 0.1 [35].
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10 In our solid/gas experiments, we have extensively studied the ability of CALB to resolve
11 racemic mixtures of secondary alcohols, and found that the influence of a_w on CALB
12 enantioselectivity was very significant. For example in case of the CALB-catalyzed resolution
13 of pentan-2-ol or hexan-3-ol, through the esterification with methyl propanoate, the
14 enantiomeric ratio (E value) varied respectively from 101 and 67 at a_w close to 0, to a
15 maximum of 320 around $a_w = 0.2$ and 120 at $a_w = 0.1$ [12, 13]. Above these optimal a_w values
16 for enantioselectivity, E values decreased for both secondary alcohols.
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26 As far as pentan-2-ol is concerned, the point $a_w = 0.2$ appears as a “turning point” where the
27 CALB enantioselectivity towards this alcohol starts to decrease with increasing a_w . We have
28 shown that the point $a_w = 0.2$ corresponds also to a breaking point for the enthalpy of
29 adsorption of pentan-2-ol on immobilized CALB. Then above $a_w 0.2$, water molecules that
30 were trapped at the enzyme have to leave in order to make room for the adsorption of alcohol,
31 and then gain motional freedom. It can be suggested that above this point, water acts more as
32 a “solvent”, with high entropic effects, than as an enantioselective inhibitor, through very
33 specific steric effects, as it was shown for very low a_w values [12]. This can also be connected
34 to the large entropic term seen for enantioselectivity at high water activity. As CALB
35 enantioselectivity is disfavoured by entropy, the result is a decrease of E with a_w , for $a_w >$
36 0.2, and an increase of E at low a_w where water predominantly acts through steric effects, by
37 binding in the stereospecificity pocket.
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54 Concerning hexan-3-ol, enantioselectivity starts to decrease at a lower a_w value equal to 0.1,
55 than for pentan-2-ol [13]. This gap between pentan-2-ol and hexan-3-ol for “ a_w turning point”
56 may be explained by the bigger size and less polar character of hexan-ol, for which difficulty
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3 in gaining access to non-wetted part of the surface of the immobilized biocatalyst is higher.
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5 Indeed the change in a_w dependence for enantioselectivity coincides with that of the
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7 stabilization for the enthalpy of adsorption of hexan-3-ol on immobilized CALB.
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10 11 12 **4 Concluding remarks**

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14 We have shown that water adsorption and IGC studies can be used successfully to monitor
15
16 hydration state of immobilized CALB, and also to investigate the adsorption of substrates in a
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18 competitive situation with water. As the conditions used for these studies resemble the ones of
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20 the continuous enzymatic solid/gas reactor, in which we have extensively studied activity and
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22 selectivity of CALB, it was possible to link adsorption results with particular effects of water
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24 on enzyme properties. As a result, this study allows a further insight into the complex and as
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26 yet not fully understood role of water on biocatalysis.
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5 References

[1] Bell, G., Halling, P.J., Moore, B.D., Partridge, J., Rees, D.G., Biocatalyst behaviour in low-water systems. *Trends Biotechnol.* 1995, 13, 468-473.

[2] Dordick, J.S., Principles and selected applications of non-aqueous enzymology, in: Blanch H.W., Clark D.S. (Eds.), *Applied Biocatalysis*, Marcel Dekker, New York, 1991, pp. 1-51.

[3] Helms, V., Protein Dynamics Tightly Connected to the Dynamics of Surrounding and Internal Water Molecules. *ChemPhysChem* 2007, 8, 23-33.

[4] Trodler, P., Pleiss, J., Modeling structure and flexibility of *Candida antarctica* lipase B in organic solvents. *BMC Struct. Biol.*, 2008, 8, art. n° 9.

[5] Halling, P.J., Biocatalysis in low-water media: understanding effects of reaction conditions. *Curr. Opin. Chem. Biol.*, 2000, 4, 74-80.

[6] Jonsson, A., Wehtje, E., Adlercreutz, P., Mattiasson, B., Thermodynamic and kinetic aspects on water vs. organic solvent as reaction media in the enzyme-catalysed reduction of ketones. *Biochim. Biophys. Acta*, 1999, 1430, 313-322.

[7] Persson, M., Wehtje, E., Adlercreutz, P., Factors Governing the Activity of Lyophilised and Immobilised Lipase Preparations in Organic Solvents. *ChemBioChem*, 2002, 3, 566-571.

1
2
3 [8] Lamare, S., Legoy, M.D., Graber, M. Solid/gas bioreactors: powerful tools for
4 fundamental research and efficient technology for industrial applications. *Green Chem.*, 2004,
5 6, 445-458.
6
7

8
9
10
11
12 [9] Létisse, F., Lamare, S., Legoy, M.D., Graber, M. Solid/gas biocatalysis : an appropriate
13 tool to study the influence of organic compounds on kinetics of lipase catalyzed alcoholysis.
14
15 *Biochim. Biophys. Acta - Proteins & Proteomics*, 2003, 1652, 27-34.
16
17

18
19
20
21
22 [10] Graber, M., Bousquet-Dubouch, M.P., Sousa, N., Lamare, S., Legoy, M.D., Water plays
23 a different role on activation thermodynamic parameters of alcoholysis reaction catalyzed by
24 lipase in gaseous and organic media. *Biochim. Biophys. Acta - Proteins & Proteomics*, 2003,
25 1645, 56-62.
26
27

28
29
30
31
32 [11] Graber, M., Bousquet-Dubouch, M.P., Lamare, S., Legoy, M.D., Alcoholysis catalyzed
33 by *Candida antarctica* lipase B in a gas/solid system: effects of water on kinetic parameters.
34
35 *Biochim. Biophys. Acta - Proteins & Proteomics*, 2003, 1648, 24-32.
36
37

38
39
40
41
42 [12] Léonard, V., Fransson, L., Lamare, S., Hult, K., Graber, M. A water molecule in the
43 stereospecificity pocket of *Candida antarctica* lipase B enhances the enantioselectivity
44 towards 2-pentanol. *ChemBiochem*, 2007, 8, 662-667.
45
46

47
48
49
50
51 [13] Léonard-Nevers, V., Marton, Z., Lamare, S. Hult, K., Graber, M. Understanding water
52 effect on *Candida antarctica* lipase B activity and enantioselectivity towards secondary
53 alcohols. *J. Mol. Catal. B: Enzym.*, 2009, 59, 90-95.
54
55
56
57
58
59
60

1
2
3 [14] Greene, S., Pust, H., The determination of heats of adsorption by gas-solid
4 chromatography. *J. Phys. Chem.*, 1958, 62, 55-58.
5
6
7

8
9
10 [15] Kiselev, A., Yashin, Y. Gas-chromatographic determination of adsorption and specific
11 surface for solids. In: Kiselev A., Yashin Y., (Eds.), *Gas Adsorption Chromatography*,
12 Plenum Press, New York, 1969, pp. 104-145.
13
14
15

16
17
18 [16] Gray, D., Guillet, J., A gas chromatographic method for the study of sorption on
19 polymers. *Macromolecules*, 1972, 5, 316-211.
20
21
22

23
24
25 [17] Dimoula, K, Pohl, M, Büchs, J, Spiess, A. C., Substrates and water adsorption
26 phenomena in a gas/solid enzymatic reactor. *Biotechnol. J.*, 2009, 4, 1-10.
27
28
29

30
31
32 [18] Hidaka, N., Matsumoto, T., Gaseous ethanol oxidation by immobilized enzyme in a
33 packed bed reactor. *Ind. Eng. Chem. Res.*, 2000, 39, 909-915.
34
35
36

37
38
39 [19] Branco, J. F., Graber, M., Denis, V., Pleiss, J., Molecular mechanism of the hydration of
40 *Candida antarctica* lipase B in gas phase: water adsorption isotherms and molecular
41 dynamics simulations. *ChemBioChem*, 2009, 10, 2913-2919.
42
43
44

45
46
47 [20] Léonard, V., Lamare, S., Legoy, M.D., Graber, M., Enantioselective acylation of R-2-
48 pentanol in a solid/gas reactor catalysed by lipase B from *Candida antarctica*. *J. Mol. Catal.*
49 *B: Enzym.*, 2004, 32, 53-59.
50
51
52
53
54
55
56
57
58
59
60

1
2
3 [21] Brunauer, S., Emmett, P. H., Teller, E., Adsorption of gases in multimolecular layers. *J.*
4
5
6 *Am. Chem. Soc.* 1938, *60*, 309-319.

7
8
9
10 [22] Costantino, H.R., Curley, J.G., Hsu, C.C., Determining the water sorption monolayer of
11
12 lyophilized pharmaceutical proteins. *J. Pharm. Sci.* 1997, *86*, 1390-1393.

13
14
15
16
17 [23] Gutman, V., *Donor-Acceptor approach to Molecular Interactions*, Plenum Press, New
18
19
20
21
22
23
24
25
26
27
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48
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54
55
56
57
58
59
60
York, 1978.

[24] Mukhopadhyay, P., Schreiber, H.P., Review: Aspects of acid-base interactions and use of
inverse gas chromatography. *Colloids Surfaces A: Physicochem. Eng. Aspects*, *100*, 1995, 47-
71.

[25] Condor, J.R., Young, C.L., *Physicochemical Measurement by Gas Chromatography*,
John Wiley and Sons, Chichester, 1979.

[26] Kiselev, A.V., Yashin, I., *Gas Adsorption Chromatography*, Plenum Press, New York,
1969.

[27] Newell, H.E., Buckton, G., Butler, D.A., Thielman, F., Williams, D.R., The use of
inverse phase gas chromatography to study the change of surface energy of amorphous lactose
as a function of relative humidity and the processes of collapse and crystallisation. *Int. J.*
Pharm., 2001, *217*, 45-56.

1
2
3 [28] Lee, S.B., Kim, K.-J., Effect of water activity on enzyme hydration and enzyme
4 reaction rate in organic solvents. *J. Ferment. Bioeng.*, 1995, 79, 473-478.
5
6
7

8
9
10 [29] Brunauer, S., Deming, L.S., Deming, W.E., Teller, E., On a theory on the Van der Waals
11 adsorption of gases. *J. Amer. Chem. Soc.*, 1940, 62, 1723-1732.
12
13
14

15
16
17 [30] Heng, J.Y.Y., Pearse, D.F., Thielmann, F., Lampke, T., Bismarck, A., Methods to
18 Determine Surface Energies of Natural Fibres: A Review. *Compos. Interface*, 2007, 14, 581-
19 604.
20
21
22

23
24
25 [31] Zhou, Q., Cadwallader, K.R., Effects of flavor compound chemical structure and
26 environmental relative humidity on the binding of volatile flavor compounds to dehydrated
27 soy protein isolates. *J. Agr. Food Chem.*, 2006, 54, 1838-1843.
28
29
30
31

32
33
34 [32] Comte, S., Calvet, R., Dodds, J.A., Balard, H., Surface properties of low specific surface
35 powders using inverse gas chromatography, *Powder Technol.*, 2005, 157, 39-47.
36
37
38
39

40
41
42 [33] Bousquet-Dubouch, M.P., Graber, M., Sousa, N., Lamare, S., Legoy, M.D., Alcoholysis
43 catalyzed by *Candida antarctica* lipase B in a gas/solid system obeys a Ping Pong Bi Bi
44 mechanism with competitive inhibition by the alcohol substrate and water. *Biochim. Biophys.*
45 *Acta*, 2001, 1550, 90-99.
46
47
48
49

50
51
52 [34] Valivety, R.H., Halling, P.J., Peilow, A.D., Macrae, A.R., Lipases from different sources
53 vary widely in dependence of catalytic activity on water activity. *Biochim. Biophys. Acta.*
54 *Protein structure and molecular enzymology*. 1992, 1122, 143-146.
55
56
57
58
59
60

1
2
3 [35] Roger, B.G. (Ed.), *Protein-Solvent Interaction*, Marcel Dekker, New York, 1994.
4
5
6

7
8 [36] Riddle, F.L., Fowkes, F.M., Spectral shifts in acid-base chemistry. 1. Van der Waals
9 contributions to acceptor numbers. *J. Amer. Chem. Soc.*, 1990, *112*, 3259-3264.
10
11
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Table 1: Characteristics of *n*-alkane and polar probes used in IGC: molecule surface area (*a*), surface tension (γ_L^d) at 20°C, Fowke's acceptor number (*AN**) and Gutman's electron donor numbers (*DN*).

The two parameters *AN* (acceptor number) and *DN* (donor number) define the Lewis acidic and basic nature of the probes. *DN* (kcal/mol) measures the energy of a coordinate bond between a donor atom and the Lewis reference acid SbCl_5 , in a solution of 1,2-dichloromethane [23]. *AN* (dimensionless number) is derived from relative ^{31}P NMR chemical shifts of the basic reference Et_3PO_4 dissolved in the working acid. *AN** (kcal/mol) corresponds to *AN* corrected values, considering *AN* value of Et_3PO_4 equal to 40 kcal/mol [36].

	<i>a</i> (m ²)	γ_L^d (J/m ²)	<i>AN</i> * (kcal/mol)	<i>DN</i> (kcal/mol)
acetonitrile	2.15 E-19	0.0275	4.7	14.1
1,4-dioxane	3.14 E-19	0.0250	0	14.8
ethyl acetate	3.3 E-19	0.0196	1.5	17.1
ethanol	3.53 E-19	0.0211	10.3	19
chloroform	4.4 E-19	0.0332	5.4	0
<i>n</i> -heptane	5.73 E-19	0.0203	-	-
<i>n</i> -octane	6.3 E-19	0.0213	-	-
<i>n</i> -nonane	6.9 E-19	0.0227	-	-
<i>n</i> -decane	7.5 E-19	0.0234	-	-
<i>n</i> -undecane	8.1 E-19	0.0246	-	-

Table 2: Evolution of the dispersive component of surface energy, γ_S^d , at 313K and acid-base properties of immobilized CALB with a_w . (n.d. for not determined)

a_w	0	0.05	0.1	0.15	0.2	0.25	0.3	0.35	0.4
γ_S^d	26.5 ±0.3	25.5 ±0.4	25.3 ±0.6	25.3 ±0.5	25.0 ±0.6	24.5 ±0.6	24.4 ±0.4	24.7 ±0.5	24.1 ±0.5
K_A	1.46	1.02	0.86	0.88	0.84	0.78	1.00	n.d.	n.d.
K_B	0.70	0.89	0.25	0.10	0	0.22	0.37	n.d.	n.d.

Figure legends

Figure 1: Determination by IGC of the dispersive component of the surface energy of the solid (immobilized CALB) γ_S^d and the specific free energy of adsorption ΔG_{ads}^{sp} of polar probes with the solid.

Figure 2: Water adsorption isotherm at 298K of Chromosorb alone (Δ) and with immobilized *Candida antarctica* lipase B (\bullet) (5mg of lyophilized Chirazyme L2 per gram of Chromosorb) (results from [13]).

Figure 3: BET plot for water adsorption at 298K on immobilized *Candida antarctica* lipase B (5mg of lyophilized Chirazyme L2 per gram of Chromosorb).

Figure 4: Evolution of specific retention volume V_G of apolar molecules with a_w on immobilized CALB at 313K : undecane (\blacklozenge), decane (\square), nonane (\blacktriangle), octane (\diamond) and heptane (\circ).

Figure 5: Variation of specific free energy of adsorption of ethanol (\diamond), acetonitrile (Δ) and ethyl acetate (\blacksquare) with a_w on immobilized CALB at 313K. Standard deviations were too small to be represented on the figure.

Figure 6: Determination of enthalpy of adsorption of ethanol (\diamond), acetonitrile (Δ) and ethyl acetate (\blacksquare) at a_w equal to 0.2, by plotting $\Delta G_{ads}^{sp}/T$ versus $1/T$ for T comprised between 313 and 338 K.

Standard deviations were too small to be represented on the figure.

1
2
3 Figure 7: Evolution of enthalpy of adsorption of pentan-2-ol (Δ) (results from [13]) and
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5 hexan-3-ol (\blacklozenge) on immobilized CALB as a function of water activity.
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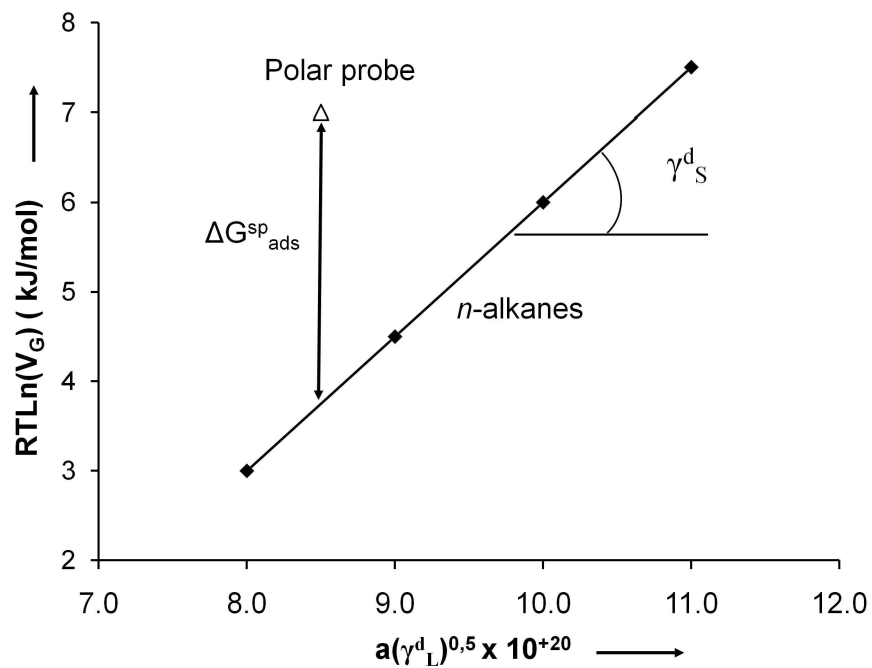


Figure 1: Determination by IGC of the dispersive component of the surface energy of the solid (immobilized CALB) and the specific free energy of adsorption of polar probes with the solid.
120x80mm (600 x 600 DPI)

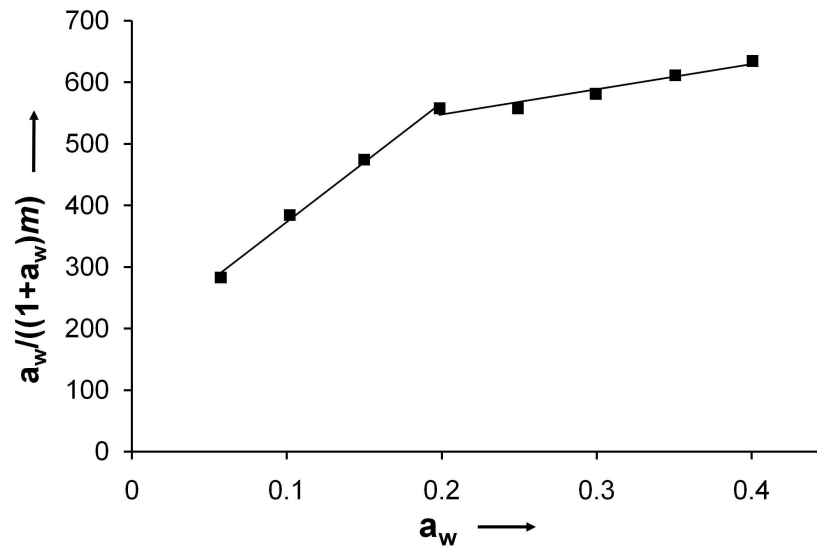


Figure 2: Water adsorption isotherm at 298K of Chromosorb alone (Δ) and with immobilized *Candida antarctica* lipase B (\bullet) (5mg of lyophilized Chirazyme L2 per gram of Chromosorb) (results from [13]).
120x80mm (600 x 600 DPI)

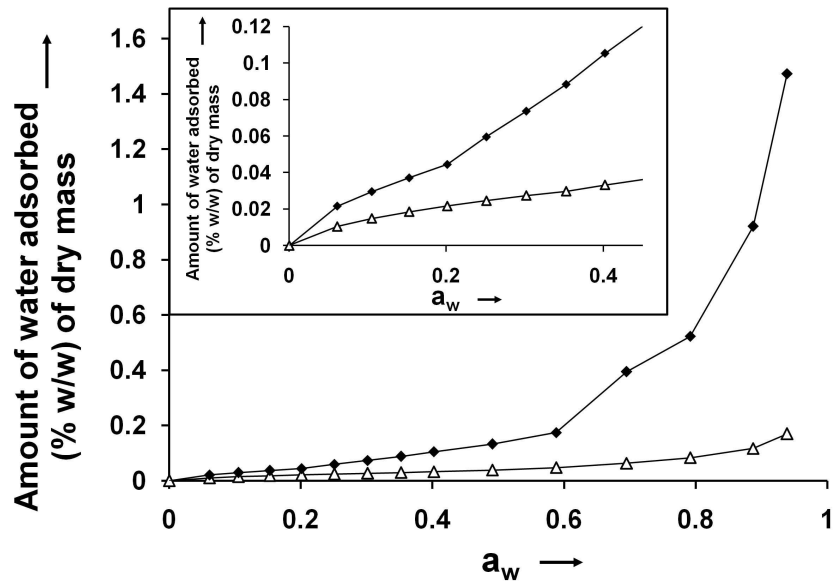


Figure 3: BET plot for water adsorption at 298K on immobilized *Candida antarctica* lipase B (5mg of lyophilized Chirazyme L2 per gram of Chromosorb).
120x80mm (600 x 600 DPI)

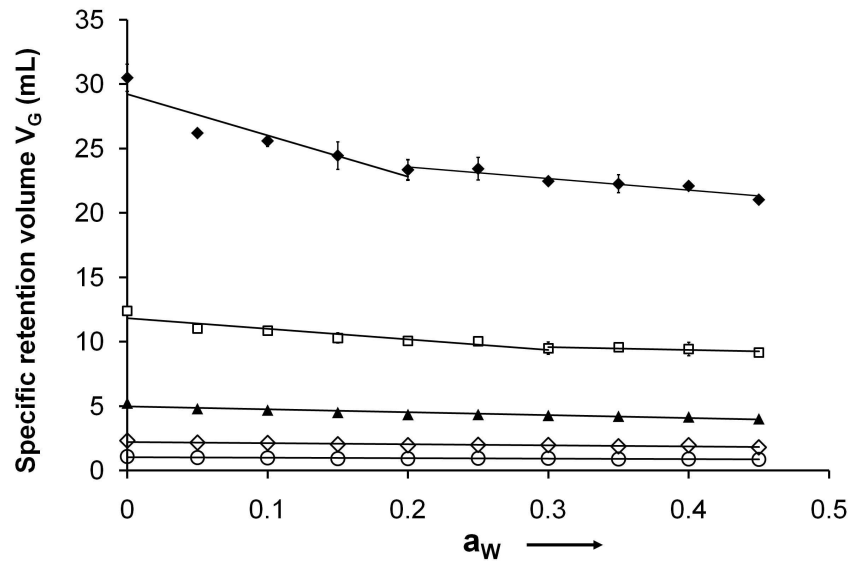


Figure 4: Evolution of specific retention volume V_G of apolar molecules with a_w on immobilized CALB at 313K : undecane (◆), decane (□), nonane (▲), octane (◇) and heptane (○).
120x80mm (600 x 600 DPI)

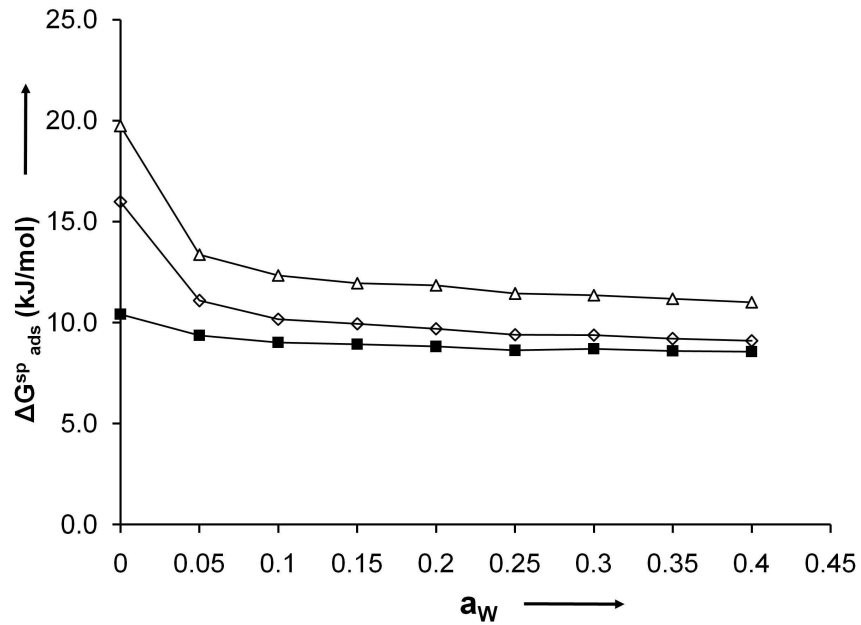


Figure 5: Variation of specific free energy of adsorption of ethanol (\diamond), acetonitrile (Δ) and ethyl acetate (\blacksquare) with a_w on immobilized CALB at 313K. Standard deviations were too small to be represented on the figure.
120x80mm (600 x 600 DPI)

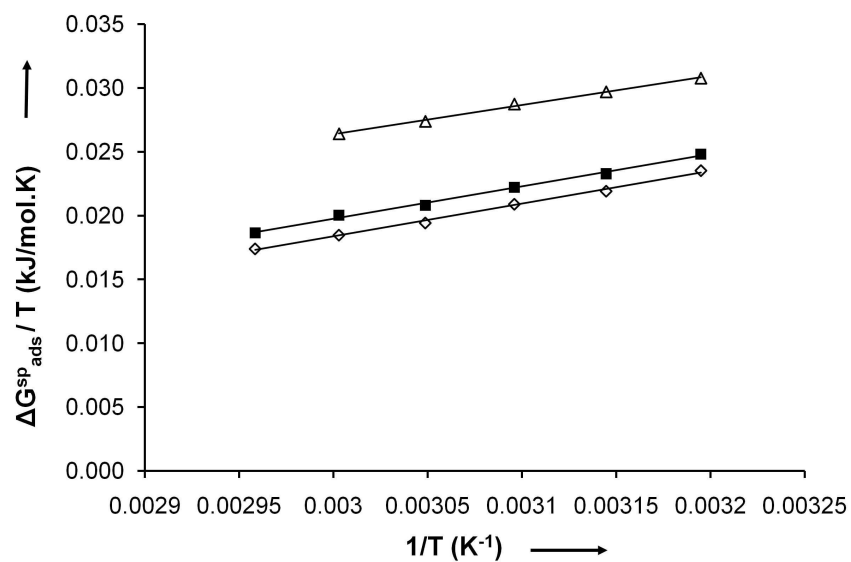


Figure 6: Determination of enthalpy of adsorption of ethanol (◇), acetonitrile (Δ) and ethyl acetate (■) at aW equal to 0.2, by plotting $\Delta G^{\text{SP}}_{\text{ads}}/T$ versus $1/T$ for T comprised between 313 and 338 K.

Standard deviations were too small to be represented on the figure.

120x80mm (600 x 600 DPI)

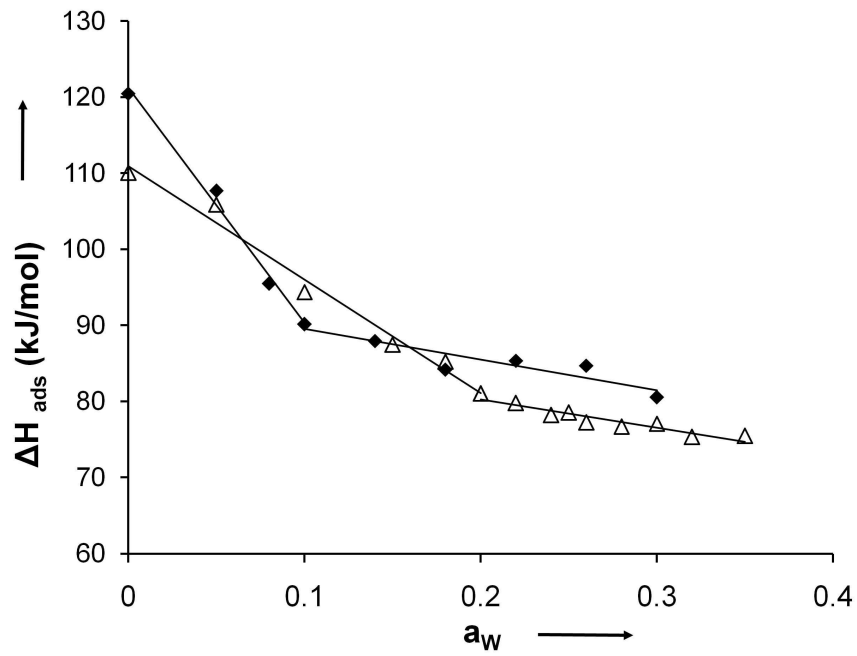


Figure 7: Evolution of enthalpy of adsorption of pentan-2-ol (Δ) (results from [13]) and hexan-3-ol (\blacklozenge) on immobilized CALB as a function of water activity.
120x80mm (600 x 600 DPI)