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Supercritical Fluid Chromatography development of a predictive analytical tool to selectively extract bioactive compounds by Supercritical Fluid Extraction and Pressurised Liquid Extraction

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## Abstract

Selective extraction is a great concern in the field of natural products. The interest is to apply specific conditions favouring the solubility of targeted secondary metabolites and avoiding the simultaneous extraction of unwanted ones. Different ways exist to reach selective extractions with suited conditions. These conditions can be determined from experimental studies through experimental design, but a full experimental design takes time, energy, and uses plant samples. Prediction from varied solubility models can also be applied allowing a better understanding of the final selected conditions and eventually less experiments.

The aim of this work was to develop and use a chromatographic model to determine optimal extraction conditions without the need for numerous extraction experiments. This model would be applied on the selective extraction of the desired antioxidant compounds in rosemary leaves (rosmarinic and carnosic acids) vs chlorophyll pigments to limit the green colour in extracts. This model was achieved with Supercritical Fluid Chromatography (SFC) and then applied to Supercritical Fluid Extraction (SFE) and Pressurized Liquid Extraction (PLE) assays.

SFC models predicted low solubility of chlorophylls for low (5%) and high (100%) percentage of solvent in carbon dioxide. Also, low solubility was predicted with acetonitrile solvent compared to methanol or ethanol. This was confirmed with different extractions performed using SFE with different percentages of solvent (5, 30, and 70%) and with the three solvents used in the SFC models (acetonitrile, methanol and ethanol). Also extractions using PLE were carried out using the same

neat solvents in order to confirm the SFC models obtained for 100% of solvent. Globally, extractions validated the SFC models. Only some differences were observed between ethanol and methanol showing the complexity of plant extraction due to matrix effect. For all these extracts, the content of carnosic acid and rosmarinic acid was also monitored and selective extraction conditions of bioactive compounds could be determined.

## 1 Introduction

The need for selectivity during plant extraction has different reasons. One of them, maybe the simplest and most logical one, is to enrich the final extract in particular bioactive metabolites, without the need for additional purification steps by preparative chromatography or counter-current chromatography. Numerous articles were published about enrichment of extracts. For example, it has been studied in order to extract more bioactive compounds such as hydroxytyrosol from olive waste [1], or chlorophyll pigments extraction in order to valorise and use them as dye [2]. On the contrary, another interest can be the necessity to avoid particular compounds. Some compounds considered as interferences can be toxic compounds coming from the plant or from the soil or the water used during the plant growth [3–6].

Among many extraction approaches, supercritical fluid extraction with carbon dioxide as extracting fluid has shown interesting selectivity properties, which result from the carbon dioxide itself, and from the possibility to extract sequentially different family of compounds when applying a dynamic and continuous method [7,8]. When the fluid is in the supercritical state, different parameters such as pressure, temperature and the addition of co-solvents can modify carbon dioxide behavior. This change of parameters can then be used to selectively extract the target compounds, due to their varied solubilities depending on the fluid properties.

The classical approach to determine extraction conditions is based on the selection of some parameters and their variation in a defined range. All the extractions are carried out, to determine the best. A more efficient way to obtain optimal conditions with a minimum of experiments exists with design of experiments (DoE).

Different parameters are screened simultaneously then, based on a pre-defined response (concentration of target analytes, yield...), optimum conditions are determined [9–11]. This strategy also helps understanding the influence of the different parameters tested and their interactions. Even if a DoE reduces the number of experiments, it still remains long. Applied to plant extraction, the number of extracts increases exponentially with the number of parameters to be tested.

The aim of this study was to develop a model which would allow identifying the best extraction conditions without the need for a DoE with numerous extractions. This work was focused on chlorophyll pigments. On the one hand, there is an interest for the extraction of chlorophyll pigments to use these compounds as natural dyes. On another hand, there is also an interest for not extracting chlorophyll pigments. For instance, for cosmetic raw materials, plant extracts must contain the lowest possible amount of chlorophyll pigments (with the highest possible amount of bioactive compounds), to obtain white final products. Also, chlorophyll compounds are very sensitive and transform themselves into degradation products such as pheophytin which change the color from green to gray [12] inducing possible colour change of the final products. Rosemary (*Rosmarinus officinalis*) is a well known plant widely used in cosmetic or food applications. Rosemary extracts can be used as conservative agents for example for milk and dairy products [13]. The aerial part of this plant from the *Lamiaceae* family, contains chlorophyll but also interesting bioactive molecules such as carnosic or rosmarinic acids known for their antioxidant or their antiproliferative properties [14,15].

In this paper, the behavior of chlorophyll compounds is first studied by Supercritical Fluid Chromatography and Liquid Chromatography with pure organic solvents as mobile phases. The retention observed in function of the different conditions is then correlated to real extraction processes, and then to SFE or pressurized liquid extraction (PLE). Finally, the different conditions favoring the extraction or non-extraction of chlorophyll will also be studied regarding the extraction of the main bioactive compounds of rosemary. Thus the behavior of carnosic acid and rosmarinic acid will be also investigated in this work because they have different polarities ( $\text{Log } P_{\text{Carnosic acid}}=5.14$ ,  $\text{Log } P_{\text{Rosmarinic acid}}=1.82$ ) [16].

## 2 Material and methods

### 2.1 Solvents and chemicals

For all this study, the solvents used were methanol, ethanol and 2-methyltetrahydrofuran, gradient grade (VWR International, Fontenay-sous-Bois, France), and acetonitrile, gradient grade (Carlo Erba, Val-de-Reuil, France). Propylene carbonate used was for synthesis (Merck KGaA, Darmstadt, Germany). Carbon dioxide (>99.5%) was from Air Liquide (Fleury-les-Aubrais, France). Different additives were tested: water was supplied by an Elga Purelab Flex station from Veolia (Antony, France), and citric acid was obtained from Sigma Aldrich (Saint-Quentin Fallavier, France). In order to develop the analytical method, different standards were used. Carnosic and rosmarinic acid standards were supplied by Extrasynthese (Genay, France). Chlorophyll *a* and *b* were obtained from ivy extracts (protocol described in extraction method part). The dispersant was diatomaceous earth (DE) from Fisher Scientific (Illkirch- France), general purpose grade.

### 2.2 Analytical SFC method development

The chromatographic system used for the retention model or the quantification of extract was a Jasco instrument (see details in [12]).

During the analytical method development in order to analyse rosemary extracts, different columns were tested: HSS C18 (5  $\mu$ m, 4.6 x 150 mm), Torus DEA (1.7  $\mu$ m, 3.0 x 100 mm) (diethylamine group), Torus 2-Pic (1.7  $\mu$ m 3.0 x 100 mm) (picolylamine group), Torus DIOL (1.7  $\mu$ m 3.0 x 100 mm), all from Waters (Saint Quentin en Yvelines). Two elution gradients were used. "Gradient 1": 5% of methanol during 1 min, then from 5 to 50% until 15 min. The following conditions with "Gradient 1" were used for the initial column screening: flow rate: 1.0 mL/min, column oven temperature: 35°C, injection volume: 5  $\mu$ L. The columns packed with small diameter particles and the elution gradient up to 50% methanol both induce a high pressure drop along the column. As a result, the back-pressure was set at 10 MPa, to avoid reaching the upper pressure limit of the pumping system (40 MPa).

Once the stationary phase was selected, and depending on the retention times observed for the target compounds, the gradient was optimized and shortened into "Gradient 2": 5% methanol during 1 min, then increase to 50% in 4 min and maintain at 50% during 4 min, to finally come back to initial condition during 3 min at 5%.

The final SFC method developed used coupled columns: HSS C18 coupled with Torus DIOL.

## 2.3 Plants and extraction methods

Two plants were used in this study. Ivy leaves (*Hedera canariensis* Willd) used for the production of chlorophyll-rich extract were collected in Orleans (France). Rosemary leaves (*Rosmarinus officinalis*) for the study of selective extraction of carnosic acid, rosmarinic acid and chlorophyll *a* were provided by one of our industrial partners, Alban Muller (Fontenay-sur-Eure, France) specialised in plant extraction.

Ivy was harvested and then dried using a Memmert model 500 dryer, at 50°C for 24 h. Then the dried leaves were grinded using Polymix PX-MFC 90 D Kinematica from Grosseron (Coueron, France).

Plant extraction for "standard" extracts used in the two first parts was carried out with Ultrasonic bath Branson 3510. Ivy and Rosemary extracts were prepared from 1 g of plant material and 15 mL ethanol. The mixture was then sonicated for 60 min. The extracts were centrifuged at 7000 rpm at 20°C for 10 min using BR4i Multifunction Centrifuge (Thermo-electron, Nantes, France). The extracts were used for no longer than one week, in order to maintain low amounts of chlorophyll degradation products.

A Waters MV-10 ASFE was used for supercritical fluid extraction. In addition, a UV detector was added between the oven and back-pressure regulator (BPR) modules in order to monitor extraction kinetics. The detector was a Gilson 151 UV / VIS Multiwavelength Detector (GILSON, Villiers-le-Bel, France). The 5-mL extraction cell was prepared using 1 g of plant material mixed with 1 g of dispersant. Cotton served to fill the cell completely and was placed at the top and bottom of the cell.

Extraction parameters for all rosemary extracts were using the percentage of modifier with a total flow of 3.0 mL/min for 30 min at 25°C, 10 MPa. For 5% modifier, a make-up solvent (ethanol) was used at 0.15 mL/min introduced before the BPR to avoid compound precipitation in the transfer capillary. Final volume obtained was 24 mL when using 5% modifier (plus make-up solvent) and 23 mL when using 30% modifier.

Pressurised liquid extraction (PLE) was performed with a Dionex ASE 150 from Thermo (Thermo, Villebon sur Yvette, France): 1 g of plant material mixed with 1 g of dispersant (DE) were extracted in a 22-mL extraction cell.

All extractions were carried out at ambient temperature (Temperature regulation off), 10 MPa, and 3 cycles of 10 min static extraction with 60% flush. Final volume was 32 mL.

Different fluid compositions were selected from varied points of retention values and were applied in extraction by supercritical fluid, with ACN, MeOH, EtOH at 5, 30, 70% in CO<sub>2</sub> (SFE), and 100% liquid (PLE). All extractions of Rosemary leaves were carried out three times in order to evaluate repeatability. The latter is then expressed as standard deviation on the different figures.

Peak areas were measured with the SFC method developed in section 3.1.3. The results are expressed in terms of the area of the peak of chlorophyll *a* normalized by taking into account the final collected volume.

The volumes collected at the end of each extraction being different, it was necessary to find a way to be able to compare these areas with each other. A simple way is to evaporate the sample dissolve it again to a specific volume. With the mass of dry extract and the volume, it is possible to use the concentration. However, since chlorophylls are very sensitive to evaporation (possible degradation), another way was implemented. The final volume of each extract was measured, and then normalization by volumes was carried out. For example, if a normalized volume of 15 mL is selected, the area of an extract of 60 mL is then multiplied by 60 (volume of the extract) and divided by 15 (reference volume).

## 2.4 Retention Model

To establish the model  $\log k = f(\text{solvent percentage})$ , a HSS C18 column (5  $\mu\text{m}$ , 4.6 x 150 mm) was used (Waters, Guyancourt, France). The flow rate was 1.5 mL/min, the outlet pressure set at 10 MPa, the oven temperature at 25°C, and UV detection monitored at 650 nm.

Retention factors ( $k$ ) were calculated using the following equation.

$$k = \frac{t_r - t_0}{t_0}$$

with retention times ( $t_r$ ) and dead time ( $t_0$ ). The dead time was measured at each analytical condition, because it could vary with the compressibility of carbon dioxide.

The latter can change depending on the inlet pressure of the column, which is related, at constant flow rate, to the mobile phase viscosity, i.e. to the modifier (co-solvent) percentage and nature [17,18].

An Ivy extract was used to examine the retention variation of chlorophyll *a* and *b*. The characterisation of this extract was carried out with a Nexera UC On-line SFE-SFC System with Mass-spectrometer in a previous study [12].

## 2.5 Correlation with Hansen solubility model

In order to correlate our retention model with Hansen solubility parameters, other experiments were done with an HPLC instrument LC20-Series (two LC-20AD pumps; autosampler SIL-20AC; degassing unit DGU-20A; Column oven CTO-20AC; Diode array detector SPD-M20A) from Shimadzu (Kyoto, Japan) with the same flow rate, temperature and detection conditions as the SFC experiments.

XLSTAT 2018.1.1 software (Addinsoft, Paris, France) was used for data analysis.



### 3 Results and discussion

In this study, two different analytical SFC methods were used. Chlorophyll retention to serve for extraction models was monitored using conditions summarized in section 2.2. For this purpose, an isocratic SFC method using a C18 stationary phase allowed the analysis of chlorophylls from ivy extracts. However, for other compounds with different polarities, this method was limited. Another method was needed to analyse chlorophylls together with carnosic and rosmarinic acid in rosemary extracts (details in section 2.4). The following part presents the development of this second method.

#### 3.1 Analytical method development for quantification

The objective was to develop an analytical method to separate the phenolic acids of interest in rosemary: carnosic and rosmarinic acid, as well as chlorophylls *a* and *b*. During the development of an analytical method, many parameters influence the separation quality. The use of carbon dioxide in sub- or supercritical conditions makes the development of such a method even more complex. However, even if this can be seen as an additional difficulty, it also allows us to have more parameters to play on in order to separate the compounds and thus refine the method [19]. A diode array UV-visible detector was used for selective detection, and different wavelengths were selected depending on the absorbance of the compounds analysed: carnosic acid: 221 nm, rosmarinic acid: 332 nm, chlorophylls: 650 nm.

One of the major parameters influencing SFC separations is the stationary phase. In order to find the most suitable, a so-called "initial" analysis method was used (described in section 2.2). Unlike RPLC or NPLC, chromatography in supercritical phase allows a wide choice of columns to be used, in particular with different polarity. Different columns were selected (section 2.2).

From the first column screening, the polar Torus DEA column was rejected because chlorophylls eluted in the dead volume. On another hand, the Torus 2-PIC column was unable to retain the two acidic compounds (rosmarinic and carnosic acids), which may be surprising for a basic ligand. As expected, the HSS C18 column was not able to retain rosmarinic acid but provided a satisfactory separation between carnosic acid and chlorophylls. Finally, the Torus DIOL column was selected because all compounds were sufficiently retained. In order to save time, the method was shortened by programming the elution with Gradient 2 (described in section 2.2). It was then possible to analyse all target compounds within 10 minutes.

Despite this change in the mobile phase gradient, the chromatogram obtained with the Torus DIOL column showed broad peak shape for rosmarinic acid. The mobile phase composition was varied to improve peak efficiency. Different additives such as water or acid were added to the modifier in order to improve the peak shape of rosmarinic acid. Finally, citric acid was selected, a polyacid which was reported in other SFC methods [20].

The first noticeable thing is the change in retention time between the different modifiers (Figure S1a). Rosmarinic acid is eluted faster using methanol with water than methanol alone, and even faster using methanol with acidified water. The addition of 5% of water in the modifier slightly decreases the retention of rosmarinic acid.

Adding citric acid to water induced both a greater decrease in retention time of the acidic compound and a strong peak width decrease. The modification of the ionization state of rosmarinic acid (from anionic to neutral), and by consequence, its change in solubility into the mobile phase can explain this change of retention. Besides, as the column ligands carry hydroxyl groups, the addition of an acid in the mobile phase could also change the apparent chemical nature of stationary phase [21–23]. Citric acid may adsorb onto the polar groups, which will affect the nature of the interactions between the analyte and the stationary phase as in HILIC mode [24–26]. The adsorption of citric acid may repulse other acidic compounds from the stationary phase and thus, reduce interactions between analytes and stationary phase.

Despite the best improvement of peak shape of rosmarinic acid obtained with citric acid, the use of this acid leads to visible degradation of the chlorophylls which are therefore no longer detected (Figure S1b). Chlorophyll compounds are sensitive to pH changes and degrade into other forms such as pheophytin (magnesium-free chlorophyll).

Consequently, as water also refined the rosmarinic acid peak, the modifier methanol /water 95/5 v/v was selected for the rest of the development. Thanks to previous optimization steps, a method allowing the analysis of carnosic, rosmarinic acid and chlorophyll *a* and *b* in less than 10 minutes was developed on the Torus DIOL stationary phase. Figure S2 shows chromatograms obtained with the new optimized conditions.

However, chlorophylls also absorb at 221 nm, the wavelength which is used to study carnosic acid. Because it elutes close to the chlorophylls, the peak integration could be biased. For this purpose, another approach with coupled columns was developed.

From the previous column screening, the HSS C18 column seemed to be suitable for carnosic acid and chlorophyll separation. However, a reversal of elution order between chlorophyll *a* and *b* occurred, compared to the one obtained with the Torus DIOL stationary phase. Chlorophyll *b* being more polar than chlorophyll *a*, it was more retained on the polar diol stationary phase and less retained on the non-polar C18 stationary phase. Whatever this drawback, the HSS C18 column was coupled with the Torus DIOL column in order to combine the different effects. Besides, due to the low viscosity of the mobile phase used in SFC, the column coupling does not induce a great pressure drop increase, around 3 MPa more.

However, when coupling column with an elution gradient, placing the most retentive column first is not advised, because the compounds leave the first column and enter the second one with a high modifier amount. Therefore, the most retained analytes in the first column will have very few interactions with the stationary phase of the second column.

For these reasons, the first column was HSS C18 and the second was Torus DIOL. As expected, this coupling made it possible to increase the difference in

retention times between chlorophylls and carnosic acid of about one minute (fig. 1). Moreover, the separation of carnosic acid from impurities was enhanced. The chlorophylls were well separated, with chlorophyll *b* eluting first, showing for these two pigments the predominance of HSS C18 phase on their selectivity. To conclude, the coupled columns are used with the elution conditions developed previously in order to quantify carnosic acid, rosmarinic acid and chlorophylls *a* and *b* from extracts obtained with SFE or PFE.

### 3.2 Solubility predictive model

To achieve a solubility predictive model, we monitored the retention of chlorophyll *a* in different analytical SFC and LC conditions. Plotting the logarithm of the retention factor according to the nature and the percentage of modifier is usually used to improve the understanding of the retention of one compound compared to another in order to develop an analytical method [12,27,28]. Another interest, related to extraction field, is to study the solubility of an analyte in a fluid that is either a mobile phase or an extraction phase. Consequently, specific extraction conditions, extrapolated from the retention curves, may be applied to extraction techniques such as supercritical fluid extraction (SFE) for CO<sub>2</sub>/modifier eluents, or pressurized fluid extraction (PFE) for neat solvent eluents.

Chlorophyll retention curves were obtained in SFC and LC, on a C18-bonded phase. Chlorophylls have high log *P* values (superior to 8) [29]. Thus a non-polar C18 stationary phase is able to provide some retention for such compounds. In addition, only limited changes in the stationary phase properties due to adsorption of the mobile phase components are expected. On the opposite, a polar stationary phase would not provide satisfactory retention of the non-polar chlorophylls, and the strong mobile phase adsorption on the polar stationary phase surface when changing the modifier percentage would induce some retention modification that would not be solely related to changes in the analyte solubility.

Three solvents were selected in this study: acetonitrile, ethanol, and methanol. Acetonitrile and methanol are quite different. Their log *P* may look similar (log *P*<sub>acetonitrile</sub>=−0.34 [30] Log *P*<sub>methanol</sub>=−0.77 [31]), but acetonitrile is a non-protic solvent,

which can interact with the analytes through dipole-dipole interactions, whereas methanol is protic and interacts through hydrogen bonding interactions, which leads to different chromatographic behaviours. Ethanol, parent of methanol, is quite close in terms of its polarity ( $\text{Log } P_{\text{ethanol}} = -0.31$  [32]) but has an additional methylene group. In the field of plant extraction, this solvent is widely used due to its polarity, its origin (from agricultural resources), its biodegradability, its low toxicity, its moderate cost, and favourable regulations allowing its use in regulated fields such as cosmetics.

An ivy extract used as chlorophyll standard (preparation method described in section 2.3) was therefore injected on the HSS C18 column in SFC and LC with different percentages of solvent in  $\text{CO}_2$ , ranging from 5 to 100%. The other parameters such as temperature, flow-rate and back-pressure are described in section 2.2. The results are presented in Figure 2. Only the plots for chlorophyll *a* are presented because the plots for chlorophyll *b* had a very similar shape [12]. For acetonitrile, between 5 and 20%, retention was too high and was thus not measured.

The first noticeable point is the curve shape. Conventionally in RP-HPLC, when using C18-bonded stationary phase, the retention is expressed with the logarithm of the retention factor in order to have a straight line with a negative slope as a function of the percentage of organic solvent. In Figure 3, two areas are identifiable. The first one is a decrease in retention until about 40% for acetonitrile and methanol, and 50% for ethanol. Because carbon dioxide is considered as a nonpolar solvent similar to pentane, this decrease was fairly predictable. The more modifier there is, the more the polarity of the mobile phase increases, approaching the polarity of chlorophyll *a*, which favours the analyte solubility in the mobile phase. However, beyond these percentages, the retention increases showing that the polarity of the mobile phase continues to increase above the polarity of the analyte. This behavior was described before with carotenoids [33] and triacylglycerols [19] on C18 stationary phase in SFC.

The shape of this curve also has other consequences. It shows that it is possible to have equal  $\log k$  values at different percentages of modifier. Identical  $\log k$  indicates conditions of iso-solubility. For example, similar  $\log k$  values are measured for the methanol curve around 20-30% and 50-60%. A similar trend is observed with acetonitrile. For ethanol, we observe equal  $\log k$  values between 40-50% and 80%. These conditions applied to extraction could then yield similar results taking into account this property of iso-solubility.

From these curves, it is possible to identify different zones which may be useful in the extraction or non-extraction of chlorophyll, on the basis of the compound solubility expected from the retention models. At low percentage of modifier, chlorophyll *a* has a high retention which means low solubility in the mobile phase. For example, it is the case with CO<sub>2</sub>/EtOH 95/5 v/v. This fluid composition can therefore be used in SFE in order to obtain extracts with low chlorophyll content.

Conversely, the minimum retention represents a high solubility in the mobile phase. Lowest retention was obtained between 30-50% MeOH or 50-80% EtOH. Similar compositions (i.e 30% and 70% modifier) can be applied in SFE in order to obtain chlorophyll-rich extracts. The retention curve obtained with acetonitrile is interpretable in the same manner, but because this curve is located higher than the methanol and ethanol ones, it indicates lower solubility of chlorophylls in these CO<sub>2</sub>/acetonitrile mobile phases.

A final area is observed with 100% modifier, thus in near-HPLC conditions. The only difference is the back-pressure of 10 MPa applied to the SFC system. As a result, this specific point in the curve can be related to pressurised liquid extraction (PLE).

We can see that at 100% modifier, acetonitrile is not a suited solvent for the extraction of chlorophylls, whereas pure methanol is more efficient, and pure ethanol should solubilise chlorophylls well.

In order to validate the expected compound solubility from this chromatographic model, different extractions were done with these three solvents. But first, we attempted to correlate this retention models with the Hansen solubility prediction model.

### 3.3 Correlation with HANSEN solubility prediction model

Varied solubility models exist in order to theoretically determine if compounds are soluble in a given environment, based on chemical interactions. From these varied models [34], the Hansen solubility parameters (HSP), derived from the Hildebrand solubility parameters, are widely used in plant extraction [35]. This model

uses three different parameters to describe the different interactions that can take place between the compounds and the extraction solvent:  $\delta D$  for dispersion (van der Waals forces),  $\delta P$  for polarity (related to dipole moment) and  $\delta H$  for hydrogen bonding. Based on these parameters it is possible to organize all chemicals into a 3-dimensions space ( $\delta P$ ;  $\delta D$ ;  $\delta H$ ). Mixtures of liquid solvents can also have their own HSP. It is simply calculated using the HSP values of the different components, and then pondered according to composition. Equation (1) was used to calculate HSP values for different solvent compositions:

$$\delta_{ab} = \frac{(\delta a * \%a + \delta b * \%b)}{\%tot} \quad (1)$$

With  $\delta_{ab}$  the HSP of the mixture,  $\delta a$  and  $\delta b$  the HSP values of solvent a and solvent b respectively,  $\%a$  and  $\%b$  the percentages of solvent a and solvent b.

From these parameters, solubility can be determined by calculating a distance ( $Ra$ ) between a solvent (neat or mixed) and the compound to solubilize (here chlorophyll *a*). This distance is calculated using equation (2):

$$Ra = \sqrt{4(\delta D2 - \delta D1)^2 + (\delta P2 - \delta P1)^2 + (\delta H2 - \delta H1)^2} \quad (2)$$

With  $\delta D$ ,  $\delta P$  and  $\delta H$  the HSP for component number 1 (solvent) and 2 (analyte). Considering that the lowest distance should reflect the highest compatibility between the solvent and analyte, and *vice versa*, it is then possible to predict the compound solubility.

In this study, we carried out a statistical study in order to correlate the solubility parameter of solvents (for the three solvents previously used as modifier in SFC model ACN, MeOH and EtOH and their mixtures at different compositions) to the log  $k$  values of chlorophyll *a* measured in SFC and LC conditions. Moreover, to improve the relevance of this study, two more solvents (2-methyltetrahydrofuran and propylene carbonate) were added in the study. These solvents, recognized as green solvents, were chosen to extend the HSP range of tested solvents (i.e. lower  $\delta P$  with 2-methyltetrahydrofuran and higher  $\delta D$  with propylene carbonate). By including these solvents, the population of three Hansen parameters is well scattered, improving the applicability domain of the model. The values of log  $k_{\text{chlorophyll } a}$  for different solvent compositions were measured by HPLC and retention curves are presented in Figure 3. For the three solvents previously studied (MeOH, EtOH and ACN), the retention

with pure solvent is close to the ones obtained with 100% modifier in SFC. The curves for the mixtures between these solvents evolve quite linearly. For the two other solvents (Propylene carbonate - PC and 2-methyltetrahydrofuran – MeTHF), mixtures with PC show high retention with high PC content. It is even impossible to elute chlorophyll *a* with PC percentage above 80%. For MeTHF, the contrary is observed as this solvent yields the lowest retention of all the solvents tested.

In addition, different mixtures of methanol and acetonitrile (5/95; 10/90; 20/80 and 50/50) as modifier were used in SFC to extend HSP range.

Population distribution is plotted in supplementary figures S3 and S4 (respectively for liquid-liquid and liquid-CO<sub>2</sub> mixtures) with  $\delta P$  in function of  $\delta H$ .  $\delta D$  was not plotted because this parameter was less dispersed than the others, varying only between 15.1 and 19.1, while  $\delta P$  varied between 4.8 and 18, and  $\delta H$  varied between 4.6 and 22.3. Due to very high retention, some curves are not complete, mainly when using propylene carbonate (PC). All the HSP for the different composition are summarized in supplementary data. Data of calculated solubility parameters for liquid mixtures are summarized in Tables S5-S11 and for liquid-CO<sub>2</sub> mixtures in Tables S12-S18.

A multilinear regression was computed using  $\delta P$ ,  $\delta D$ ,  $\delta H$  as independent variables and  $\log k$  values as dependent variable.

In the first place, only data using liquid mixtures are studied.

The regression on liquid mixtures yielded equation (3) with  $R^2=0.905$ :

$$\log k_{\text{chlorophyll } a} = -4.02 + 0.13 * \delta D + 0.17 * \delta P + 0.03 * \delta H \quad (3)$$

The high regression coefficient demonstrates that  $\log k$  values obtained chromatographically can be correlated with theoretical solubility parameters, in this case, HSP. This result first shows that the calculation of the solubility parameters of liquid mixtures in proportion of the volume fraction seems relevant, and secondly, that the retention of chlorophyll *a* should be predictable from the HSP values. In our point of view, this validates the use of chromatographic retention to study the solubility changes of this compound in liquids. A linear regression between experimental and calculated values of  $\log k$  is represented in supplementary material Figure S19.



Besides, from the coefficient values of the three solubility parameters, it is difficult to deeply understand which interactions, dispersion, dipole-dipole or hydrogen bonding play a preponderant role in the retention changes, i.e. in the solubility changes. However, the dispersion and dipole-dipole interactions, which have the highest coefficient values in Eq. (3), seem mainly involved in the retention changes of chlorophyll *a*.

Based on the chromatographic retention model in Eq. (3), solvents with high  $\delta P$  and  $\delta D$  would induce a poor solubility of chlorophyll *a*. It is the case for PC ( $\delta P = 18$ ,  $\delta D = 20$ ) which shows poor solubility, so high retention in chromatographic model.

The contrary is observed with MeTHF ( $\delta P = 4.8$ ;  $\delta D = 16.8$ ): low retention indicating high solubility.

The same data processing has been applied on CO<sub>2</sub>-solvent data (log *k* values measured in SFC conditions). However, these parameters depend on the fluid density, they can change in function of pressure and temperature [36]. Due to the complexity of the determination of its HSP, CO<sub>2</sub> HSP were selected for the closest condition possible (40°C and 100 bar) [37].

Figure 4 shows the change in the  $R^2$  regression coefficient related to the calculation of multilinear regression starting with the data of neat liquids (Eq. (3), on the right of the figure) and including progressively the data obtained with increasing CO<sub>2</sub> percentage in the mobile phase, up to 95% CO<sub>2</sub> (5% of modifier, on the left of the figure). For instance, it means that to obtain the  $R^2$  value for the point 90% of liquid, we added to the numerous values of retention obtained for all the pure liquids studied (data of Figure 3), the retention values of the mobile phases composed by the mixtures 90/10 solvent/carbon dioxide. Then, each following point of fig. 4 is obtained by adding to the previous retention data at each CO<sub>2</sub>/modifier percentage, the retention values at the studied mixtures. Figure 4 shows that reasonably good correlation coefficients are obtained for modifier percentage above 30% ( $R^2 > 0.8$ ). However, the correlation coefficient decreases dramatically for the lowest amounts of modifier, from 30 to 5%

This result highlights the difficulty to calculate the HSP values for mixtures with lower amounts of modifier. This difficulty is either related to the higher compressibility of such fluids, or/and to the solubility changes in this area, which may be due to the

formation of solvent clusters around the analyte. It was shown in supercritical fluid chromatography, that with low modifier percentages in carbon dioxide, a solvating sphere of modifier can surround the compounds. Thus in the immediate vicinity of the analyte (called the cybotactic region), the concentration of modifier is higher than in the bulk of the fluid [19]. Obviously, this must have a strong effect on the real solubility of the compounds, but this local environment is not easy to predict and describe with macromolecular data, such as the HSP, or any other type or polarity parameters.

Figure 5 shows the changes in the coefficient values for the three computed Hansen parameters when the proportions of CO<sub>2</sub> and solvents are varied. All these coefficients decrease when adding carbon dioxide to the modifier.

This was expected from the respective values of the HSP parameters of acetonitrile, methanol, ethanol and carbon dioxide. The dispersion coefficient  $\delta D$  decreases rapidly upon addition of CO<sub>2</sub>-solvent data, as the decrease is observed from 80% of liquid downwards. The other two are rather constant up to 40 % of liquid and decrease only when the proportion of solvent is further reduced. This confirms the apparent changes in the compound solubility model below 40% of liquid mixed to carbon dioxide.

For the lowest modifier percentages, i.e. the highest carbon dioxide ones, the main Hansen parameter acting on retention/solubility is the dipole-dipole parameter. However, we have to keep in mind the very low regression coefficients obtained for these mixtures, suggesting limited interpretability of these models, which moderates this statement.

Nevertheless, almost of these results indicate reasonably good correlation between the chromatographic retention (measured from reverse phase supercritical and liquid chromatography) and the calculated HSP values either for liquid mixtures, or for rich liquid/carbon dioxide ones. It is then possible to predict solubility by choosing the right HSP: high  $\delta P$  and  $\delta D$  values should yield poor solubility, and *vice versa*.

Further developments could be done in the future, to back-calculate the accurate HSP values for low liquid content in carbon dioxide, based on retention values, and including more analytes with varied chemical structures.

#### 4 Extraction

First, we can notice the small amount of chlorophyll *a* extracted with acetonitrile, compared to methanol and ethanol extractions (fig. 6). According to the retention model (fig. 3), this solvent (mixed to carbon dioxide or used as pure liquid) yielded high chromatographic retention of chlorophyll *a*, and this is related to the low amount of chlorophyll extracted, whether in SFE or in PLE.

Between SFE points at 5, 30, and 70%, the worst extraction conditions are obtained at 5% modifier in CO<sub>2</sub>, regardless of the modifier used. These experimental values of extracted amounts are in accordance with log *k* curves in Figure 2. Indeed, log *k* at 5% was the highest retention point for each solvent (no measurement was possible with acetonitrile due to very high retention of chlorophylls). Conversely, SFE experiments using 30% and 70% modifier extracted a higher chlorophyll content for all modifiers. However, between these two points, the content extracted at 70% modifier is higher than the chlorophyll content extracted at 30% modifier for ACN, MeOH and slightly higher for EtOH. These results do not follow the retention model for acetonitrile and methanol (Figure 2) since the measured chromatographic retention at 70% modifier was higher than the one at 30%. Differences at intermediate points could come from a lack of accuracy of the model. This may be because the model uses pure analyte molecules and then cannot predict all the intraparticle diffusional resistances (for the dried plant particles), which may occur into organelles (especially chloroplast) of plant cells. It could be some accessibility phenomena for chlorophyll compounds stored in chloroplast and the use of 70% of modifier would still be better to reach and solubilise them [38].

Besides, all PLE extracts all show lower content of extracted chlorophyll compared to SFE at both 30% and 70% for all three solvents. In that case, and whatever the intraparticle diffusivity, these results are in accordance with the retention model.

However, a difference is observed between the retention model and extraction content for methanol and ethanol. For percentages above 20% of modifier, log *k*<sub>ethanol</sub> was lower than log *k*<sub>methanol</sub>. This result, when related to the compound solubility,

would mean that chlorophyll content would be lower for extractions with methanol than ethanol at 30, 70 and 100% of modifier but the contrary was observed. However, the retention model does not take into account the matrix effect, i.e. the ability to the fluid to penetrate the matrix. Figure 7 shows the inlet pressure changes from the chromatographic measurements. For acetonitrile and methanol, the increase in pressure is regular and almost linear whereas for ethanol, inlet pressure strongly increases to reach very high values. Obviously, because the inlet pressure is related to the fluid viscosity, it shows the dramatic increase of viscosity with the addition of ethanol, and consequently, the decrease of both the diffusivity of the fluid, meaning a probable limitation of the fluid penetration in the dried plant, and of the diffusion coefficient of analytes, that also could limit the recovery of compounds when viscous solvent is mixed to carbon dioxide. Such behaviour, a decrease in the diffusion coefficient reducing the penetration capacity of the solvent and consequently the diminution in the extraction yield have been reported for chlorophyll in microalga [39,40].

However, these diffusivity changes related to the viscosity variations do not explain the increased extraction recovery of chlorophylls between 30 and 70% of modifier that are observed whatever the modifier, acetonitrile, methanol or ethanol. An additional phenomenon should take place, which is neither related to the modifier nature, nor to the viscosity of the extraction solvent.

To conclude, the extraction experiments allowed us to correlate them with predicted conditions obtained by chromatographic technique. Nevertheless, even if most of the extraction results could be correlated to chromatographic observations, this study demonstrated the complexity of plant extraction and all the matrix effects that could impact the extraction. It is especially the case for conditions with low retention meaning high extraction capacity.

After focusing on model validation for chlorophyll extraction, the study was focused on the extraction of bioactive compounds of rosemary, in function of different conditions tested previously. The results are shown in Figure 8. For any of the selective conditions for chlorophyll extraction (70% of modifier) or non-extraction (5% of modifier), carnolic acid was extracted. For the most selective SFE conditions, for instance low percentage of any modifier or with acetonitrile, the amounts of extracted carnolic acid were lower with regards to extraction conditions using higher modifier

contents and/or alcohol modifiers. With SFE, extraction of carnosic acid was favoured by the use of ethanol compared to acetonitrile and methanol. However, in PLE extraction, the amount of extracted carnosic acid was similar with the three solvents. For rosmarinic acid, which is more polar compared to carnosic acid, the extraction selectivity was much higher. With the lowest modifier percentage up to 30%, no or small amounts of this compound were extracted. High methanol or ethanol proportions were required to achieve satisfactory extraction of rosmarinic acid. Whatever the ethanol amount in SFE and the extraction method (SFE and PLE), the amounts of rosmarinic acid extracted with ethanol remained lower than with methanol, which is in accordance with the higher polarity of methanol. Finally, the yield obtained for both acids from PLE remained below the best yields obtained from SFE. In that case, one can claim that it could be due to the difference of the extraction mode between PLE and SFE, as the first one was done in static conditions, whereas the second one was done in dynamic conditions.

To conclude, it is possible to extract selectively carnosic acid without chlorophyll with SFE using low modifier percentage or acetonitrile. However, due to the high polarity of rosmarinic acid, its extraction requires the use of high modifier percentage and polar solvent, also yielding the extraction of chlorophylls.

## 5. Conclusion

During this study, a SFC method was developed to predict extraction behaviour of chlorophyll *a*, and to easily find selective extraction conditions between pigments and bioactive compounds, rosmarinic and carnosic acids. This approach can be used either for liquid extraction by PLE with neat organic solvent or by SFE with mixtures of carbon dioxide and solvent in supercritical state.

Besides, correlation between the Hansen solubility parameters and the retention for chlorophyll *a* was studied. Rather satisfactory correlations were reported when using pure liquids or mixed CO<sub>2</sub>/liquid mobile phases with modifier percentages higher than 30%. For the lower modifier contents, the use of the HSP model seems inadequate to describe the retention values, either due to erroneous calculation of the HSP values based on the volume fractions, or to inhomogeneity of such mobile phases, with significant clustering of the modifier around the analyte.

This retention model can be helpful to determine the fluid compositions allowing to reach selective extractions. For instance, the solubility of chlorophyll *a* was strongly reduced with acetonitrile, whether employed as neat solvent in PLE or mixed to CO<sub>2</sub> in different proportions in SFE. Moreover, low modifier (5%) in carbon dioxide reduced chlorophyll solubility with regards to intermediate values of modifier (30%). However, we also observed, based on triplicate extractions with real samples, that some matrix effects can induce different results to the ones predicted from the chromatographic model, maybe due to different diffusivity of the solvents in the plant matrix. Nonetheless, selective conditions to extract carnosic acid without chlorophyll were identified thanks to this method (using 5% acetonitrile as modifier). However, it was harder to obtain selective conditions for rosmarinic acid due to its higher polarity. In order to extract it, higher modifier percentages or more polar solvents are required, which are also extracting significant amounts of chlorophyll. In addition, it was possible to extract most of the chlorophyll using 30% of ethanol as modifier in CO<sub>2</sub>, showing that SFE can be a good alternative for more ecological extraction requiring low amounts of green solvents. The latter (CO<sub>2</sub> and ethanol) are compatible with cosmetic regulations.

Finally, once different conditions are identified that can extract selectively the studied compounds, one of the solutions could be to use these different conditions successively on the same plant sample. Supercritical fluid extraction techniques use a dynamic extraction system which means that extraction conditions can be changed at any time during the extraction process. This will be investigated in the future. However, this study also shown that the modelling from the chromatographic behaviour, could not take into account the various matrix effects, i.e. the fluid diffusivity into the complex samples, and the presence of other compounds in the plant.

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Figure captions

Figure 1. SFC chromatograms of carnosic acid, rosmarinic acid and chlorophylls with final gradient conditions (gradient 2) and the coupled column system (HSS C18 and Torus DIOL).

Figure 2. Logarithm of retention factor of chlorophyll *a* vs modifier percentage. Column: HSS C18, Flow rate: 1.5 mL/min; T = 25°C; P = 10 MPa.

Figure 3. Logarithm of retention factor of chlorophyll *a* vs different percentage of solvent B for different mixtures noted A – B with the solvents identified on the figure. Column: HSS C18, Flow rate: 1.5 mL/min; T = 25°C.

Figure 4. Variation of the regression coefficient  $R^2$  from cumulative retention values of chlorophyll *a* measured either with pure mobile phases (100%) or added with values from mixed CO<sub>2</sub>/liquid mobile phases.

Figure 5. Variations of the (a,b,c) HSP coefficients from the computed multilinear regression  $\log k = f(a\delta P + b\delta D + c\delta H)$ . Each point was computed from cumulative retention values of chlorophyll *a* measured with pure liquid mobile phases (100%) or progressively added with values from mixed CO<sub>2</sub>/liquid mobile phases (see text for details).

Figure 6. Chlorophyll *a* content in rosemary extracts vs the extraction conditions both for SFE (left side) and PFE (right side), with different solvent compositions of extracting solvent.

Figure 7. Variation of the inlet pressure vs the modifier percentage in SFC system with the HSS C18 column.

Figure 8: Peak areas of Carnosic acid (solid color) measured in SFC at 221 nm and rosmarinic acid (white dots) measured at 332 nm in function of extraction conditions for SFE (left side) and PFE (right side).

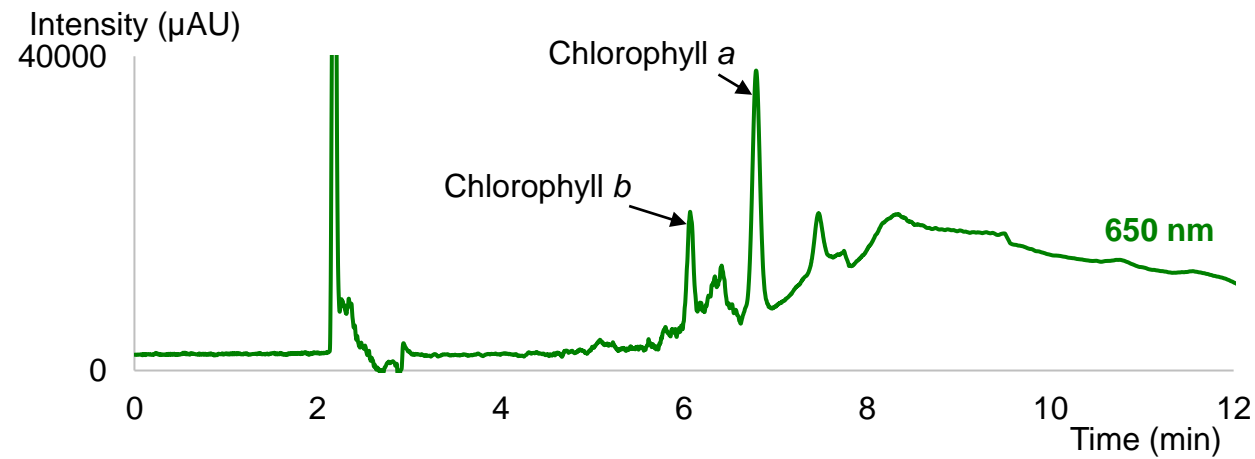
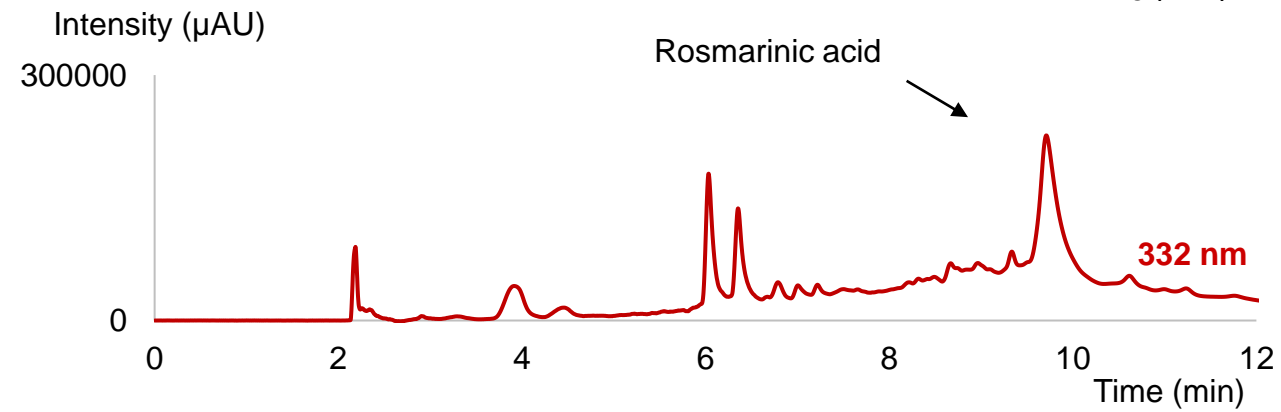
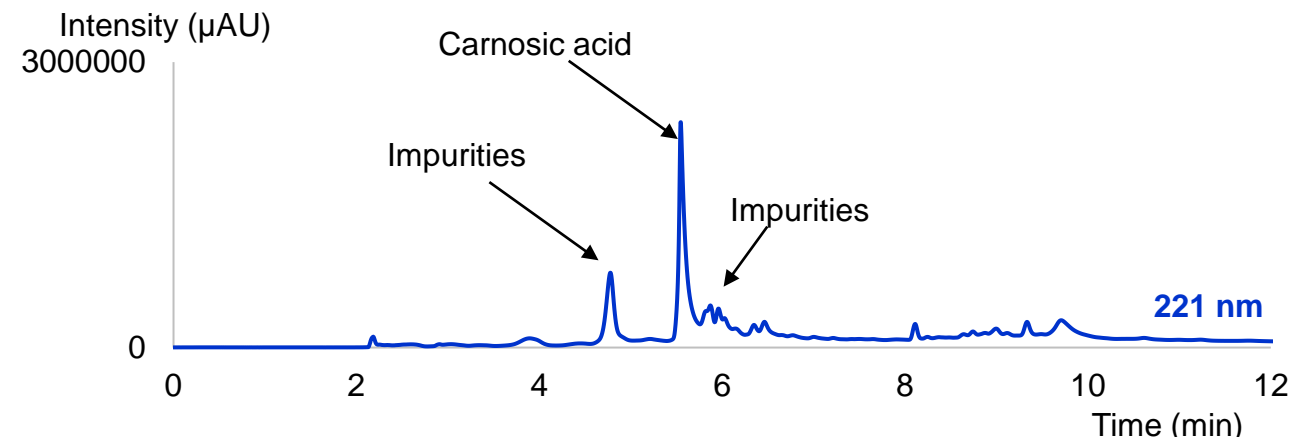


Figure 1

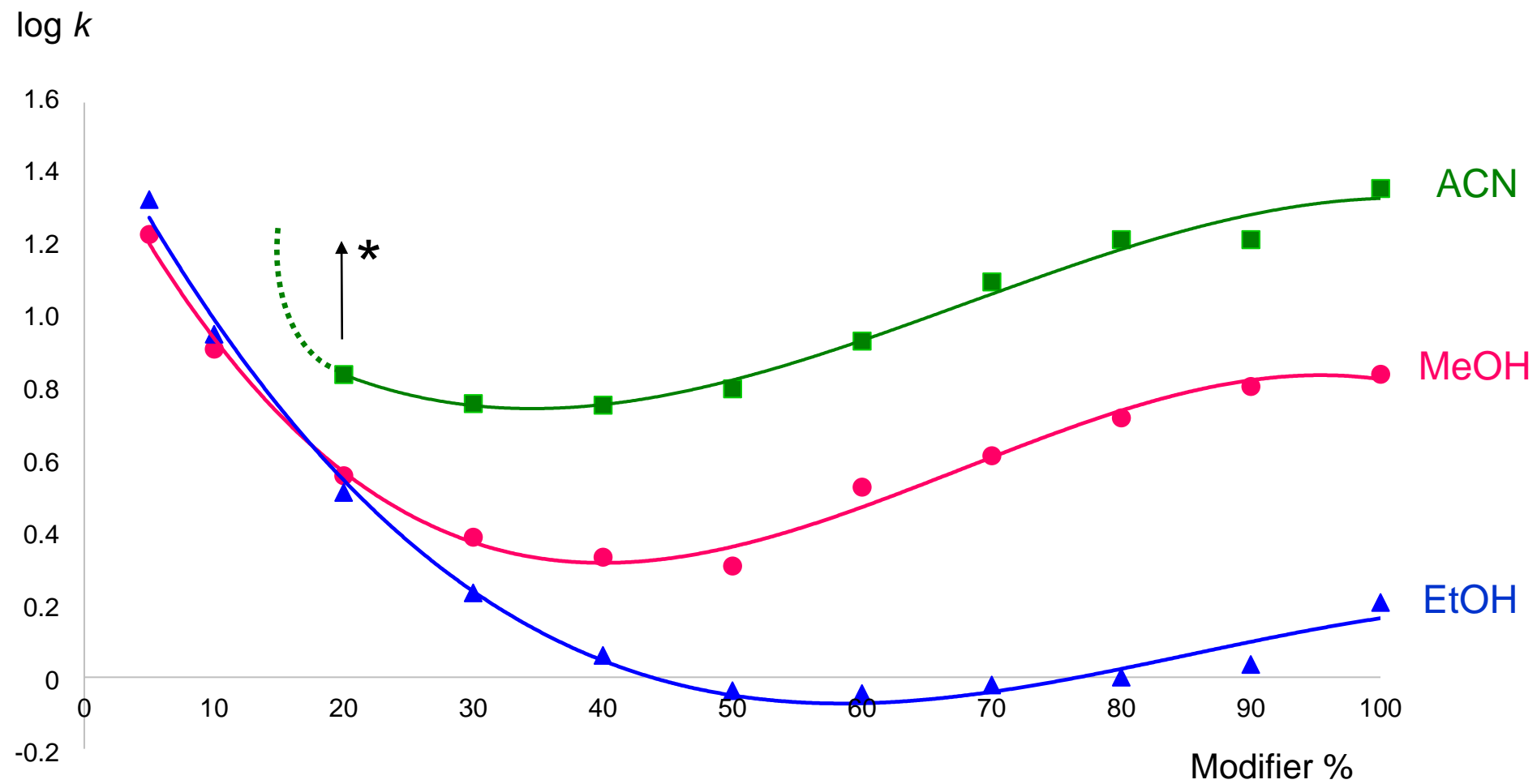


Figure 2

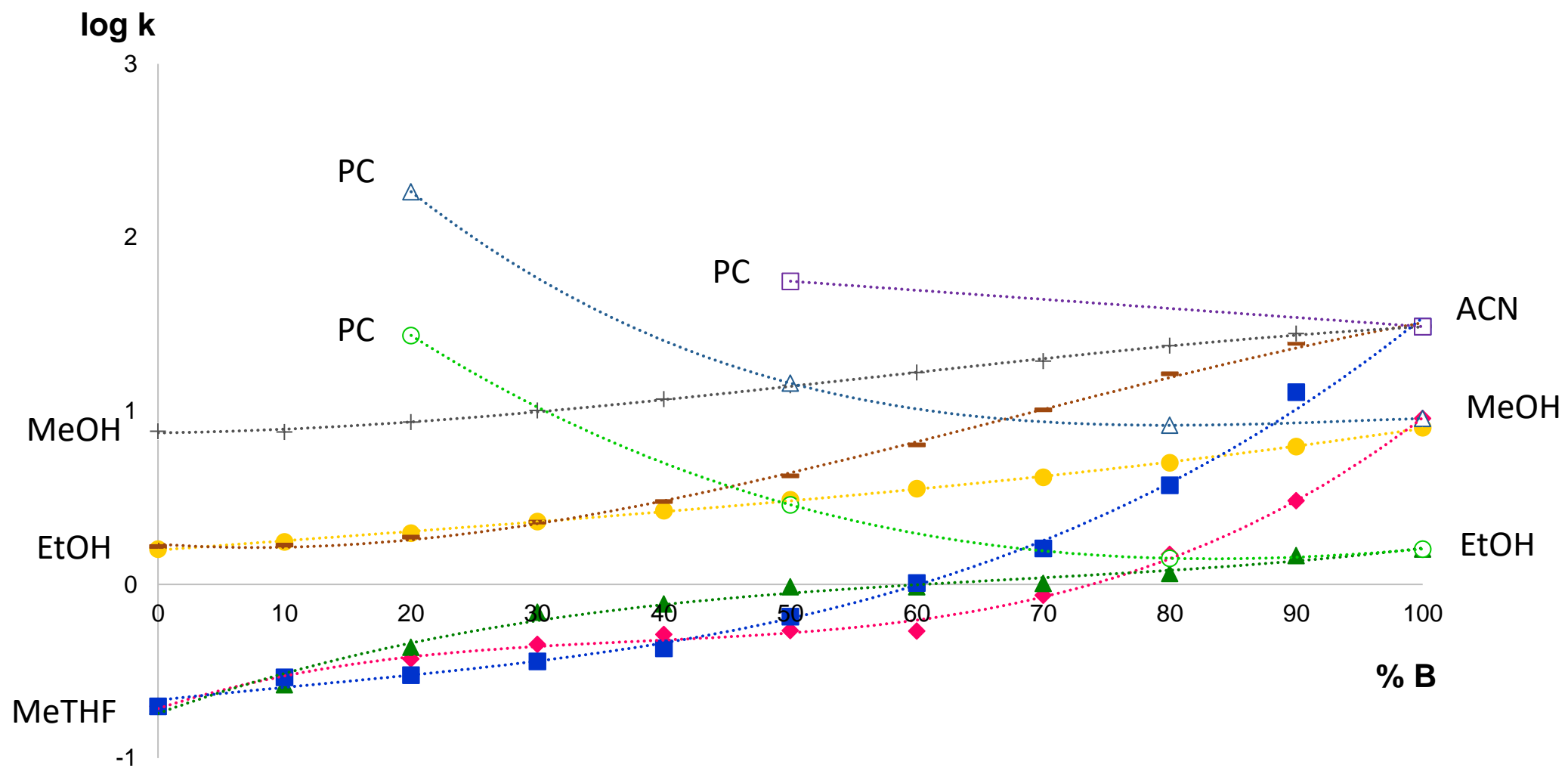


Figure 3

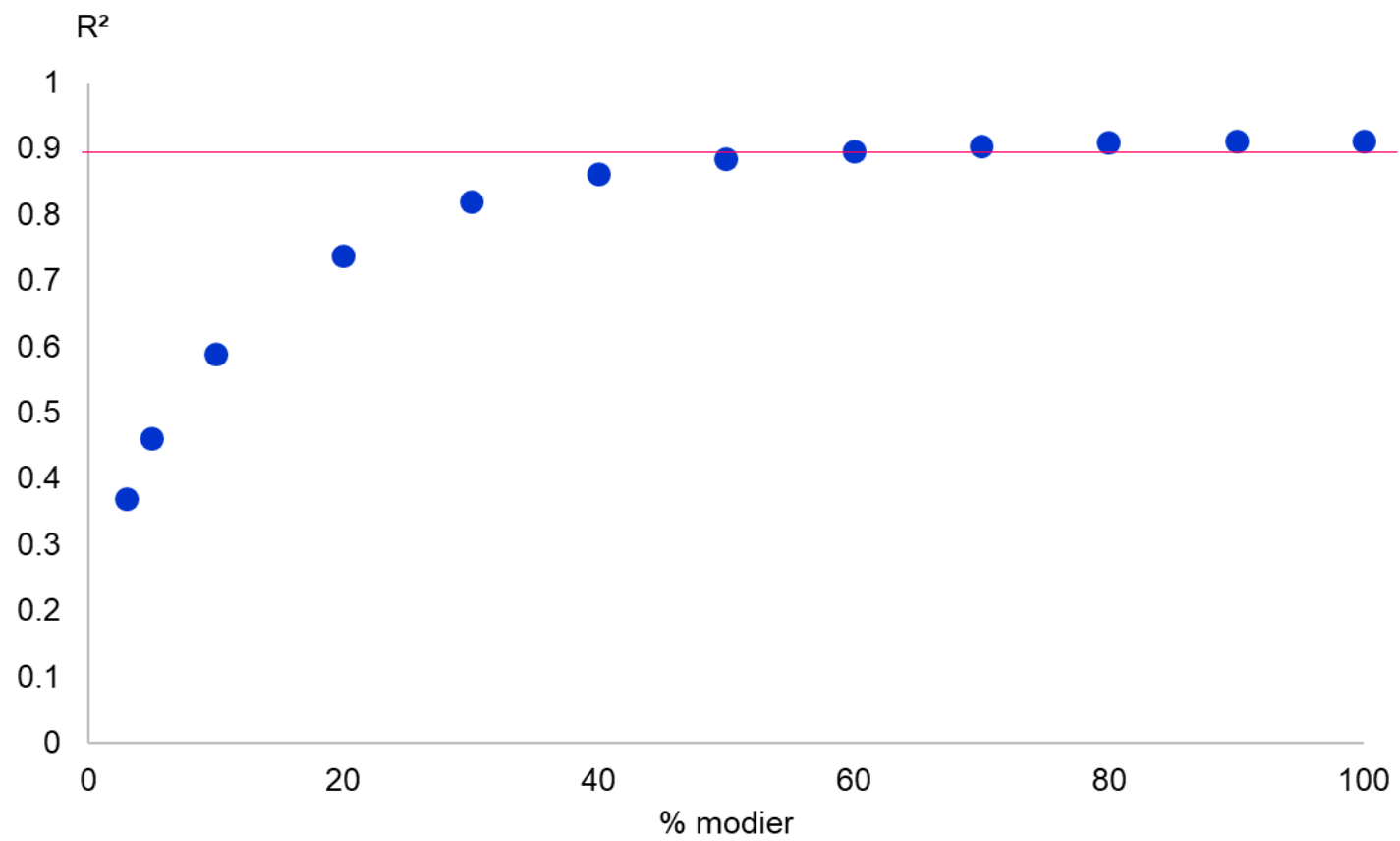


Figure 4

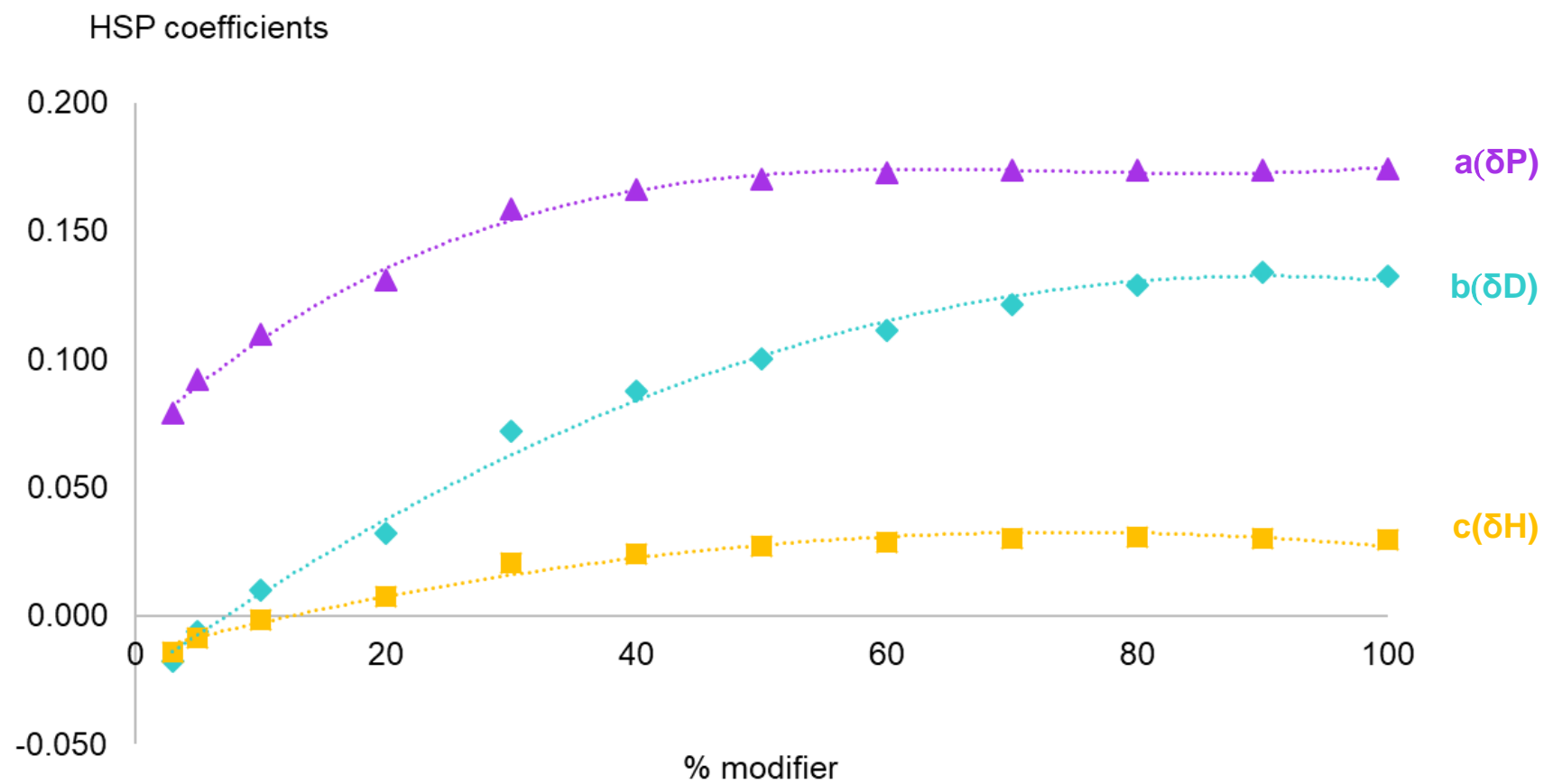


Figure 5

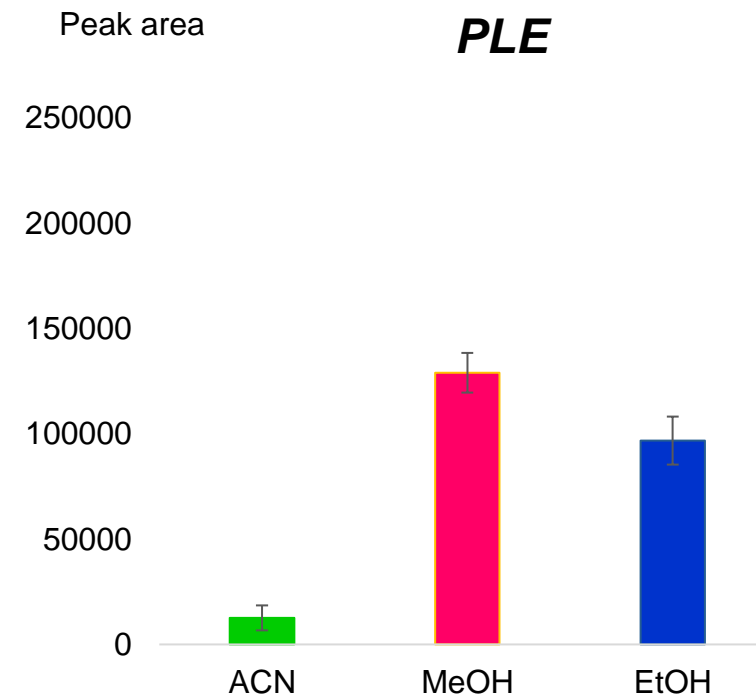
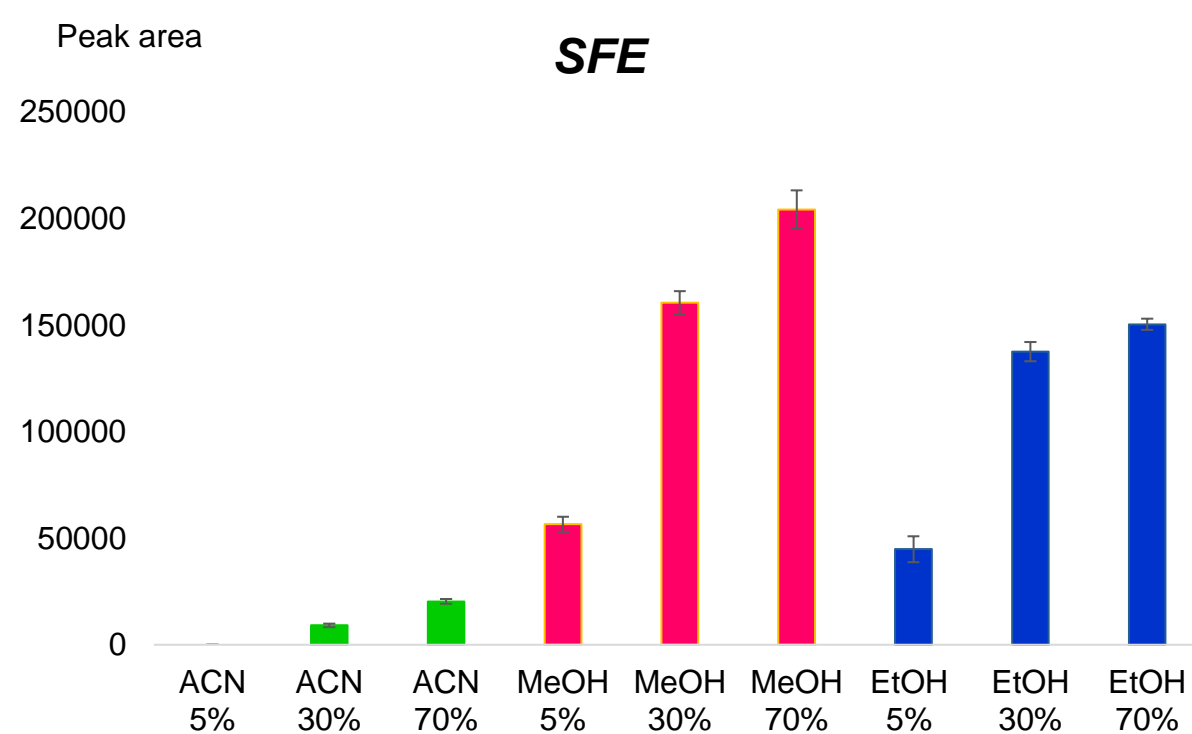


Figure 6



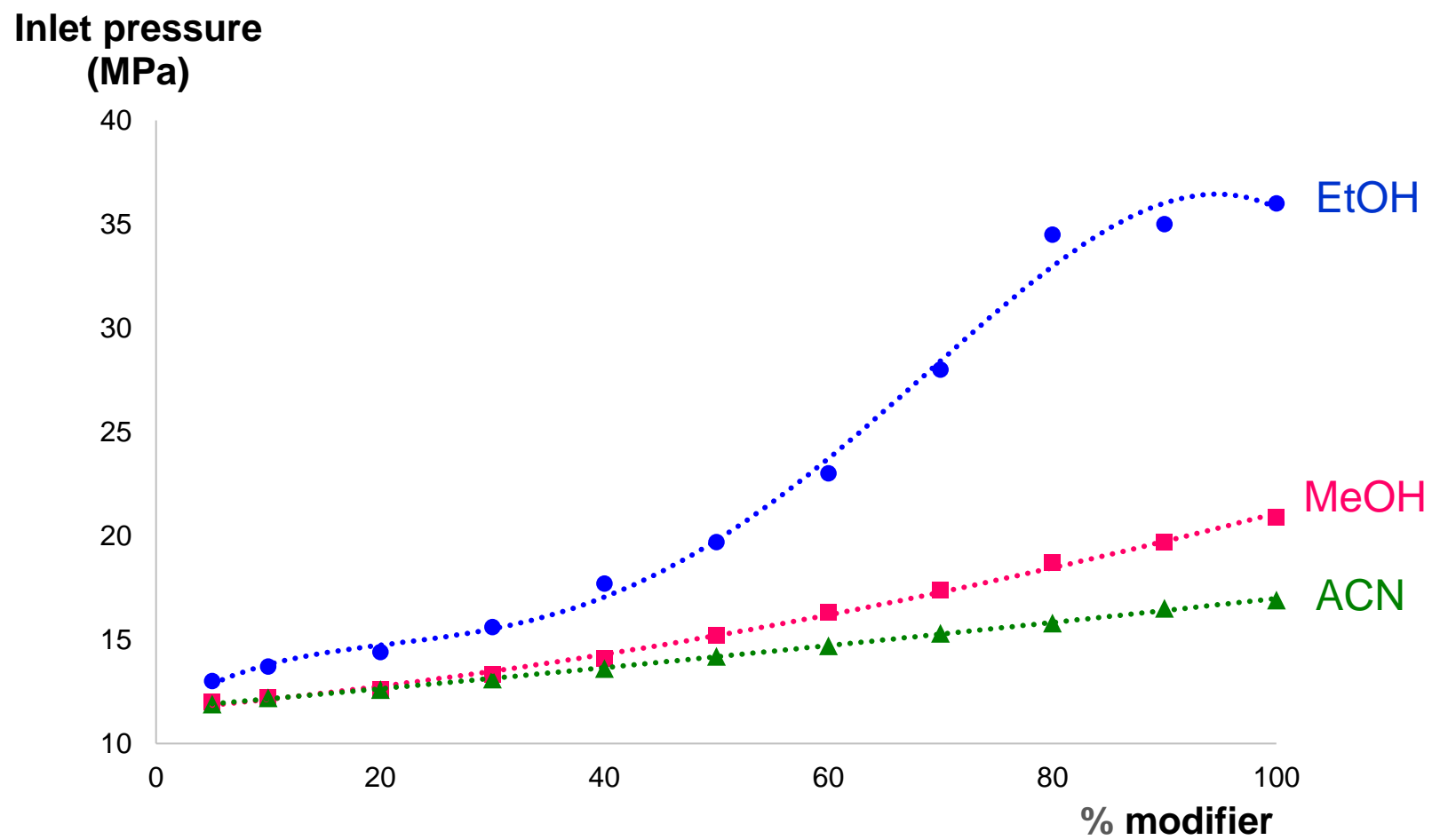


Figure 7

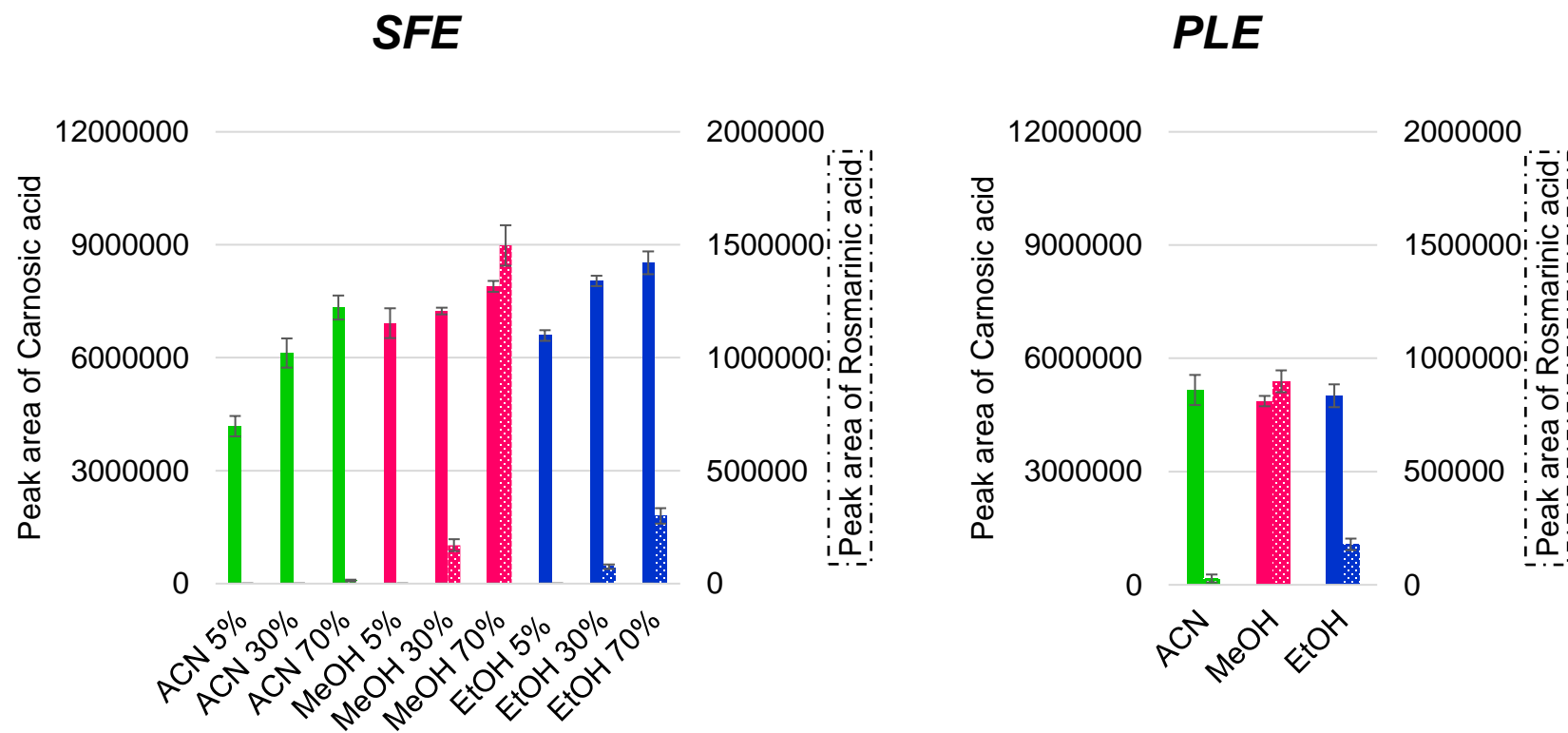


Figure 8