

# Fractional factorial design and multiple linear regression to optimise extraction of volatiles from a *Lactobacillus plantarum* bacterial suspension using purge and trap

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Received 16 December 1996; revised 17 March 1997; accepted 18 March 1997

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## Abstract

A purge-and-trap technique was used to analyze volatile compounds released by bacteria in a resting cells system. To ensure optimal efficiency of the method and to optimise the extraction of all the compounds comprising the total ion chromatograms, four parameters of the purge and trap were studied simultaneously. A  $2_{IV}^{4-1}$  fractional factorial design consisting of eleven assays including three center points, was performed to study these factors and their interactions. The sum of the areas of the peaks in various sections of the total ion chromatograms were the responses and were studied by multiple linear regression and ANOVA. Depending on the apparatus available, the matrix to be analyzed and the compounds expected to be extracted, the fractional or full factorial design approach may enable optimum use of the variable parameters of the analytical method, and, hence, improve the reliability of the analysis and may allow us to save time and materials. © 1997 Elsevier Science B.V.

**Keywords:** Chemometrics; Factorial design; Multiple linear regression; *Lactobacillus plantarum*; Sample handling

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## 1. Introduction

The purge-and-trap technique, also named dynamic headspace, is widely used in gas chromatography to extract and concentrate volatile compounds. The technique is applied to the analysis of target compounds [1–3] or all volatiles from solid or liquid samples [4–6]. Optimal conditions of extraction can differ depending on the type of apparatus available, the type of matrix analyzed and the nature of the compounds extracted [7,8]. The optimal conditions for efficient recovery of a single compound from a

mixture may be different to those required for the general extraction of a large number of compounds. The optimisation of such extractions has been reported by some authors taking into account different parameters such as volume of purge, drying time, temperature and time of desorption. In most cases the parameters influencing the efficiency of the technique have been studied individually [9] with the consequent risk of neglecting interactions between the factors, thus leading to incomplete or misleading data [10]. Full or fractional factorial designs will permit the study of the importance of each of the parameters, both individually, as well as their interactions [11,12]. This approach would yield optimal

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values for use in order to maximise the relative intensities of the peaks in the chromatograms [7,13]. The aim of this work was to optimise a purge-and-trap apparatus in order to analyze polar compounds released by bacteria in a resting cells system.

## 2. Experimental

### 2.1. Strains and culture conditions

The strain used in order to perform this work has been previously isolated in our laboratory from Spanish high-quality dry sausage and identified as *Lactobacillus plantarum*. For all experiments the strain was grown in 20 ml MRS broth (Oxoid, Unipath, Basingstoke, UK) at 30°C and harvested at early stationary growth phase.

### 2.2. Sample preparation

Samples were prepared as described elsewhere [14] with the following modifications. Cells from a fresh culture were grown in fermentation MRS medium (ADSA-Micro Scharlau, Barcelona, Spain) containing 10 g l<sup>-1</sup> lactose (Panreac, Moutplet and Esteban, Barcelona, Spain). At 15°C at early stationary growth phase, the culture was cooled at the early stationary phase to stop growth and centrifuged (16 000 g, 10 min, 5°C). Cells were washed twice with 50 ml of chilled basal buffer (BB), consisting of 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES) buffer (Sigma, St. Louis, MO, USA) and 35 g l<sup>-1</sup> of sodium chloride (NaCl) (Panreac) adjusted to pH 6 using 1 M NaOH. Pellets were finally resuspended in a minimal volume of BB and absorbance at 550 nm was measured. A volume of the cell suspension in basal buffer supplemented with 10 g l<sup>-1</sup> D-glucose (Panreac) and a 5 g l<sup>-1</sup> mixture of L-amino acids (Gln; Lys; Leu; Ile; Phe and Met) (Sigma), was poured into a 250-ml airtight tube. The final volume of the resting cell suspension was 84 ml contained 3 mg dry mass ml<sup>-1</sup> of cells. After 3 h at 15°C, the tube was cooled, centrifuged (16 000 g, 10 min, 5°C) and the supernatant kept in an airtight sealed vial at 4°C until analysis.

### 2.3. Purge-and-trap gas chromatography–mass spectrometry

The resting cell suspension was analyzed using 2-ml fractions poured into a 25-ml needle sparger sampler (Supelco, Barcelona, Spain) fitted to a HP 7695 (Hewlett-Packard, Barcelona, Spain) purge-and-trap (PAT) apparatus (equivalent to a Teckmar 3000) equipped with a Vocarb 3000 trap (Supelco) to concentrate the analytes. After a pre-purge using 35 ml of helium, the sample was pre-heated using a pocket-heater (HP) and purged with helium at a flow-rate of 55 ml min<sup>-1</sup>, all transfer lines and valves being maintained at 180°C until automatic injection into the chromatograph. After purging, the trap was dried by flushing with helium and thermally desorbed at 250°C. The analytes reached the injection port of a HP 1800A GCD (Hewlett-Packard) held at 240°C with a split ratio of 5:1. A HP 1800A GCD system was used as a gas chromatograph based on a HP 5890 Series II equipped with an electron ionisation detection (EID) system. To complete the extraction and concentration step, the trap was baked for 20 min at 260°C after desorption. Separation was achieved using a SPB-5 fused-silica capillary column (60 m×0.32 mm I.D., 1 µm film thickness) (Supelco) and helium as the carrier gas at a flow-rate of 0.7 ml min<sup>-1</sup>. After 5 min at 38°C, the column temperature was ramped to 55°C at 3°C min<sup>-1</sup> and to 175°C at 5°C min<sup>-1</sup>. At the end of the run, the column temperature was raised to 240°C at a rate of 30°C min<sup>-1</sup> with a final holding time of 2 min. The eluent passed into the electron ionisation detector through an interface heated at 250°C. Scan mass spectra were obtained scanning from 33 to 250 u (2 scan s<sup>-1</sup>).

### 2.4. Statistical procedure

An orthogonal fractional factorial design 2<sub>IV</sub><sup>4-1</sup> was applied to this study. The experimental domain was set up considering the compatibility with the physical limitations and specifications of the available apparatus. The different levels of the factors are reported in Table 1 in the form of both coded and natural units. Three runs situated at the centre of the experimental domain (coded value 0) were added to estimate the importance of random error and the lack

Table 1  
Boundaries of the experimental domain expressed in coded and natural units

Coded units	Experimental factors			
	Desorption time, <i>D</i> (min)	Dry purge time, <i>P</i> (min)	Purge volume, <i>V</i> (ml)	Sample temperature, <i>S</i> (°C)
-1	2	1	500	30
0	4	2.5	1000	40
1	6	4	1500	50

of fit (LOF) of the model. The relationships between the independent variables and results observed (dependant variables) were evaluated by multiple linear regression. The multiple regression procedure in Statgraphics Version 7.0 was used to model the results and consisted of a simplified first order model  $y = C_0 + \sum_i C_i x_i + \sum_i < \sum_j C_{ij} x_i x_j$  where *y* is the response observed,  $C_0$  is a constant,  $C_i$  and  $C_{ij}$  are the empirical coefficients of regression,  $x_i$  is the variable in coded values and  $x_i$  and  $x_j$  are the second-order interactions between  $x_i$  and  $x_j$  ( $i < j$ ) in coded values. As a first-order model equation was applied, second-order effects and interactions above second order were assumed to be negligible, and, therefore, were not calculated. The statistical significance of the coefficients  $C_i$  and  $C_{ij}$  of the model was determined by the significance level (*P*) for each Student's *t*-test value given by the multiple regression procedure. The goodness of fit (GOF) of the model was checked by the coefficient of determination ( $R^2$ ) and Fisher's

*F* test (Table 2) which was also used to test the LOF of the final model (Table 5). Three responses were studied corresponding to the summed peak areas of different zones (A1, A2 and A3) of the total ion chromatograms recorded (Fig. 1).

### 3. Results and discussion

As can be seen in Table 2, the  $R^2$  value for the total peak areas A1 and A2 indicates that less than 3% of the total variations observed in the chromatograms is not explained. Fisher's *F* test demonstrates a high significance for the regression models concerning these two areas. The modelling for the total peak area A3 was not so good, the unexplained total variation being 9%. Table 2 shows that the purge volume, *V*, and the sample temperature, *S*, have significant positive effects on the levels of volatile components recovered from the matrix. For all

Table 2  
Model coefficients,  $C_i$ , estimated by multiple linear regression for the three chromatographic zones and model adequacy checks

Response	A1 (6–10 min)		A2 (10–15 min)		A3 (18–32 min)	
	Coefficient $C_i$	Student's <i>t</i> -test	Coefficient $C_i$	Student's <i>t</i> -test	Coefficient $C_i$	Student's <i>t</i> -test
Constant	2463.92	59.29	130.09	48.03	5.14	17.50
$D = P \times V \times S$	-58.75	-1.21	8.57	2.70	0.16	0.48
$P = D \times V \times S$	-45.84	-0.94	-1.46	-0.46	0.13	0.37
$V = D \times P \times S$	1130.37	23.20	48.81	15.37	2.90	8.43
$S = D \times P \times V$	597.49	12.26	37.09	11.68	2.03	5.89
$D \times P = V \times S$	231.24	4.75	14.55	4.58	1.30	3.77
$D \times V = P \times S$	-97.04	-1.99	-4.43	-1.40	-0.16	-0.46
$P \times V = D \times S$	-37.42	-0.77	-2.47	-0.78	0.01	0.03
<i>R</i> -squared (Adj. For d.f. <sup>b</sup> )	$R^2$	0.9861		0.9754		0.9190
Fisher's <i>F</i> test (GOF) <sup>c</sup>	$F_{(7 \text{ and } 3)}^b$ $P_{\text{GOF}}^a$	102.55 0.0014		57.66 0.0034		17.20 0.02

<sup>a</sup> significance level; <sup>b</sup> degrees of freedom; <sup>c</sup> goodness of fit.

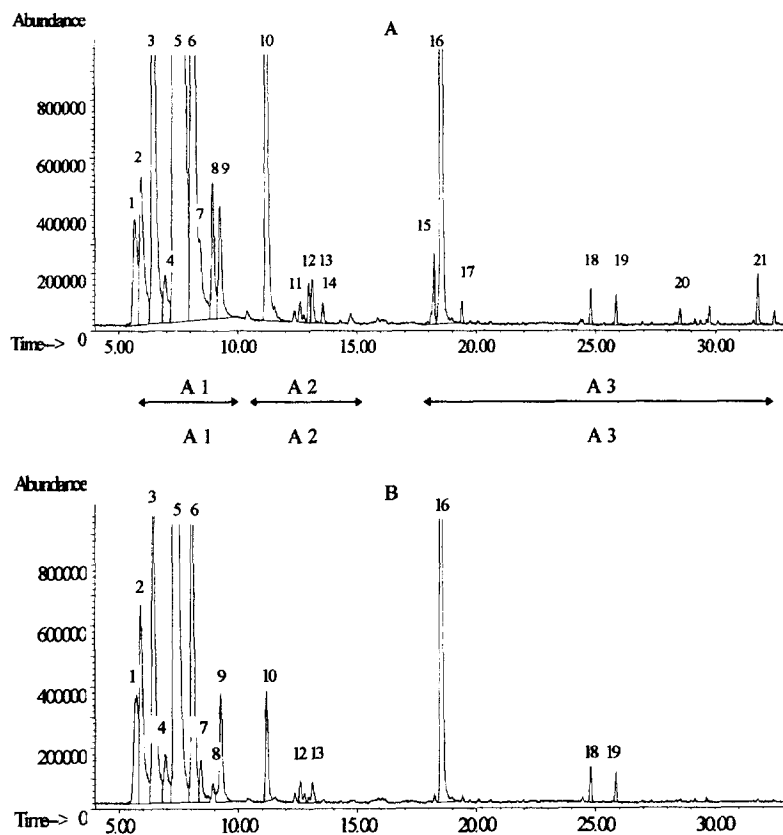


Fig. 1. Total ion chromatograms. (A) Purge volume,  $V$ , and sample temperature,  $S$ , at coded level +1. (B) Purge volume,  $V$ , and sample temperature,  $S$ , at coded level -1. (All other factors being set up at coded level -1.)

chromatographic regions, the responses were influenced by these factors in the same way. No breakthrough was observed in the experimental domain explored. A sample temperature of 50°C increased the amount of volatiles recovered. This suggests that the influence of the increase in solubility of the compounds in solution, due to the temperature, is almost negligible when compared with the rise in vapor pressure of the volatile components, purge efficiency being dependent upon vapor pressure in the purge-and-trap technique. It was of considerable interest to observe the influence of the dryness of the trap after the purge step on the recovery of the volatiles prior to desorption. Since the compounds extracted are polar (Table 3), water removal could result in a loss of those compounds. In this study, the dry purge time,  $P$ , was found to be non-significant, meaning that drying the trap for a short or a long

period did not change the amount of compounds introduced into the GCD. However, previous assays showed that at the very least a short dry purge time must be applied to avoid dramatic loss of resolution of the light volatiles such as ethanal, ethanol and propan-2-one. Due to the type of trap, the conditions of desorption and the nature of the compounds extracted, the desorption time,  $D$ , was found to have non-significant influence on the responses. A desorption time shorter than 2 min was insufficient to transfer all volatiles to the injection port of the chromatograph. However, this parameter must be taken into account as it has been demonstrated to be important when the matrix under study contains heavy compounds such as terpenes [7]. In the three chromatographic regions, a positive effect of the factor  $D \times P$  was found to be significant while the effects of the two main factors  $D$  and  $P$  were

Table 3

Results of the identification of the volatile compounds by mass spectroscopy

Peak	Compound
1	Carbone dioxyde
2	Chlorodifluoromethane
3	Ethanal
4	Methoxyethane
5	Ethanol
6	Propan-2-one
7	1,1'-Oxybis-ethane
8	2-Methylpropan-2-ol
9	Dichloromethane
10	Butane-2,3-dione
11	Trichloromethane
12	Butan-2-ol
13	Tetrahydrofurane
14	2-Methylbutan-2-ol
15	3-Methylbutanol
16	4-Methylpentan-2-one
17	4-Methylpentan-2-ol
18	Dimethylbenzene
19	Dimethylbenzene
20	Benzaldehyde
21	2-Hydroxybenzaldehyde

insignificant. In fact, from the generator of aliases used (Table 2), the interaction  $D \times P$  is aliased with the interaction  $V \times S$  leading to  $(D \times P) = D \times P + V \times S$ ,  $(D \times P)$  being a contrast. Assuming that the interaction of two non-significant factors is negligible [15], the value of the positive effect  $(D \times P)$  can be attributed to the interaction  $V \times S$  of the two significant factors  $V$  and  $S$ . Hence, no complementary assay was performed to separate the aliases. Simplified first-order models were created using only variables found to be statistically significant:

$$y_{A1} = 2463.92 + 1130.37 \times V + 597.49 \times S + 231.24 \times V \times S$$

$$y_{A2} = 130.09 + 48.81 \times V + 37.09 \times S + 14.55 \times V \times S$$

$$y_{A3} = 5.14 + 2.90 \times V + 2.03 \times S + 1.30 \times V \times S$$

with  $y_{A1}$ ,  $y_{A2}$  and  $y_{A3}$  were the total peak areas for the three zones A1, A2 and A3; other variables were not included in the model. The significant variables of the model,  $V$ ,  $S$  and  $V \times S$  act in the same way in

Table 4

Model coefficients,  $C_i$ , estimated by multiple linear regression for the total peak area of the chromatogram and model adequacy checks (all factors)

Independent variable	Coefficient $C_i$	Student's $t$ -test
Constant	2630.75	65.72
$D = P \times V \times S$	-39.73	-0.85
$P = D \times V \times S$	-46.27	-0.98
$V = D \times P \times S$	1189.23	25.34
$S = D \times P \times V$	640.70	13.65
$D \times P = V \times S$	249.41	5.31
$D \times V = P \times S$	-97.82	-2.08
$P \times V = D \times S$	-37.52	-0.80
$R$ -squared	$R^2$	0.9884
(Adj. for d.f. <sup>b</sup> )		
Fisher's $F$ test	$F_{(7 \text{ and } 3)}^b$	123.30
(GOF) <sup>c</sup>	$P_{\text{GOF}}^a$	0.0011

<sup>a</sup> significance level; <sup>b</sup> degrees of freedom; <sup>c</sup> goodness of fit.

the three responses; therefore, we considered the total peak area of the chromatogram (Table 6) and a new multiple linear regression was performed taking into account all the factors studied (Table 4). A second calculation was performed using only the factors found to be significant in the previous regression (Table 5). (Table 6 shows the total peak area of the chromatogram.) A new simplified first-order model was created:

$$y = 2630.75 + 1189.23 \times V + 640.70 \times S + 249.41 \times V \times S$$

Table 5

Model coefficients,  $C_i$ , estimated by multiple linear regression for the total peak area of the chromatogram and model adequacy checks (significant factors)

Independent variable	Coefficient $C_i$	Student's $t$ -test
Constant	2630.75	55.91
$V = D \times P \times S$	1189.23	21.55
$S = D \times P \times V$	640.70	11.61
$D \times P = V \times S$	249.41	4.52
$R$ -squared	$R^2$	0.9840
(Adj. for d.f. <sup>b</sup> )		
Fisher's $F$ test	$F_{(3 \text{ and } 7)}^b$	206.64
(GOF) <sup>c</sup>	$P_{\text{GOF}}^a$	0.0000
(LOF) <sup>d</sup>	$F_{(5 \text{ and } 2)}^b$	8.58
	$P_{\text{LOF}}^a$	<0.3

<sup>a</sup> significance level; <sup>b</sup> degrees of freedom; <sup>c</sup> goodness of fit; <sup>d</sup> lack of fit.

Table 6  
Observed values for the total peak area of the chromatogram

Assay no.	Observed values
1	1040.16
2	1938.93
3	1805.25
4	1138.87
5	4970.73
6	2915.41
7	3022.92
8	4528.03
Central points	
9	2465.49
10	2588.69
11	2523.74

y being the total peak area of the chromatogram, other factors not being included in the model. The goodness of fit, GOF, and the lack of fit, LOF, suggest that this final model (Table 5) is able to provide useful predictions concerning the optimal conditions for the extraction of volatiles from the cell suspension.

#### 4. Conclusion

A fractional factorial design was used to optimise the extraction of volatiles from a cell suspension involving the use of a purge-and-trap device coupled to a GCD system. The results show that this method facilitates the simultaneous study of the effects of the various headspace parameters of extraction and their interactions on the efficiency of the analysis. The resulting models may be used to predict the shape of the chromatograms. The influencing parameters can be optimised to maximise the summed peak surface areas for different regions of the chromatograms. Thus, the non-influential parameters can be set up in a manner allowing the greatest saving of time and materials during the analyses. For future analyses of

this specific type of samples, 1500 ml of helium will be applied to the cell suspension heated at 50°C and to save gas and time, the trap will be dried for 1 min and finally desorbed for 2 min.

#### Acknowledgments

The author thanks Dr. L. Izquierdo and Dr. A.P. MacCabe for helpful discussions. This research was financially supported by the European union fellowship contract AIR1-CT94-6413 and project AIR2-CT94-1517.

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