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Simultaneous quantitation of volatile compounds in citrus beverage through stir bar sorptive extraction coupled with thermal desorption-programmed temperature vaporization

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ABSTRACT

Due to disparate concentrations and physiochemical properties of analytes, difficulties in terms of sensitivity and reproducibility are commonly encountered in flavour analysis. In this study, we attempted to improve the performance of stir bar sorptive extraction coupled with thermal desorption and programmed temperature vaporization (SBSE–TD–PTV) based on a model citrus beverage. Through response surface methodology, thermal desorption conditions (i.e. desorption flow, thermal desorption time and cryofocusing temperature) were optimised based on constrained optimisation. Solute discrimination during injection was alleviated by normalising the variability of peak responses of different analytes. In addition, the effects of extraction conditions (i.e. ionic strength, stirring speed, extraction time, temperature and pH) were also investigated using partial factorial design. The obtained method showed high precision and good linearity over the concentration ranged from 0.10 to 20.00 $\mu g\,L^{-1}$ with the correlation coefficients higher than 0.991 for most of the selected chemicals, except indole. The limit of detection ranged from 0.03 to 3.89 $\mu g\,L^{-1}$. Hence, our results indicated that through the systematic study, SBSE–TD–PTV method became much less solute discriminative and more reliable to quantitate complex analytes.

1. Introduction

Recent developments in sample preparation have attracted much attention to sorptive extraction techniques, e.g. open-tubular trapping (OTT), solid phase microextraction (SPME) and stir bar sorptive extraction (SBSE) [1,2]. With larger volume of sorbent materials used, the sensitivity of SBSE and sample capacity could be remarkably increased [2–4]. To date, it has been widely applied in environmental analysis [2–10] and biomedical analysis [11–13]. Apart from these extensive applications, SBSE is gaining acceptance in flavour analysis, for example flavour profiling of aroma-active volatiles in wines [14–18], beers [19], fruit juices [20,21] and vinegars [22] as well as elucidation of the changes of volatile metabolites in an intra-oral odour investigation [23]. However, the quantitation of flavour compounds in food samples still remains a challenging task due to their highly diverse physicochemical properties (i.e. volatility and polarity) and disparate concentrations. For instance, distinctive flavours of citrus beverages are contributed to aroma-active volatile compounds that range from ppm to ppb levels while some potent polar oxygenated compounds are present at low ppt levels [24,25]. Moreover, the various soluble solids (e.g. acids, sugars, and pectins) that are usually found in citrus beverages give rise to matrix effects that would further complicate the extraction process [26]. Hence, this has led to the need to develop a more effective and versatile SBSE method for flavour analysis.

SBSE could be generally viewed as a two-step process—the first step involves partitioning of analytes from aqueous phase into sorbent materials and the second step is to desorb the extracted analytes through thermal desorption or solvent dissolution, with the former being more commonly employed. The thermally desorbed analytes can be transferred into a gas chromatograph through a programmed-temperature-vaporization (PTV) inlet, which could focus the compounds in a cryofocusing trap before transferring them into the column [27]. The combination of SBSE and TD–PTV injection is a sensitive yet complicated technique. To improve the performance of SBSE–TD–PTV method, different approaches were attempted in previous studies with one-variable-at-a-time univariate approach [9,15,17,19]. However, response surface methodology

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would be more appropriate in optimising multiple experimental factors whether extraction conditions only were optimised [19] or both important extraction and GC conditions were treated together in a set of sequential experimental designs [22,28]. In our preliminary experiments, we found that the effects of SBSE extraction conditions and TD–PTV injection parameters were basically unrelated (data not shown). In fact in the process of experimental design, all variables should be interrelated/correlated among themselves. Otherwise, the interpretation on the responses could disregard certain unfavourable conditions/discrimination towards certain group of analytes [22,28]. Thus, these factors should be separately optimised by examining the response of each compound and taking advantage of multi-responses optimisation approach to maximize these responses. Through understanding the influence of TD–PTV factors (i.e. thermal desorption time, flow and cryofocusing temperature) and extraction parameters (i.e. extraction time, temperature stirring speed, electrolyte concentration and pH) on the performance of each compound, which was significantly different in physiochemical properties (e.g. boiling point, solubility, etc.), analyte discrimination could be alleviated.

Therefore, our objective was to develop a SBSE–TD–PTV method for simultaneous determination of a wide range of volatile compounds using model citrus beverage. RSM was applied to understand the interactive parameters in the TD–PTV process, while partial factorial was used to prescreen extraction condition. Furthermore, the optimised method was evaluated and validated through various performance parameters (i.e. linearity, repeatability, precision and limit of detection).

2. Experimental section

2.1. Materials and sample preparation

Milli-Q water was generated from a Millipore water system (Milford, MA, USA). Analtyical grade ethanol 96% was obtained from Gadot-Lab, Hezlia, Israel, and methanol from VWR International Ltd., Poole, UK; HPLC grade dichloromethane was purchased from Tedia, Fairfield, OH, USA.

A group of 36 common food flavourings was obtained from Firmenich Asia, Singapore (Table 1). Then, these compounds were diluted with ethanol (10 mg mL^{-1}) as flavouring for further analysis. For each SBSE extraction, 10 μL of this flavouring was spiked into 10.00 mL of Milli-Q water.

2.2. SBSE procedure

Stir bars coated with 24 μL of polydimethylsiloxane (PDMS, 10 mm length × 0.5 mm thickness) were purchased from Gerstel GmbH & Co.KG (Mülheim an der Ruhr, Germany). Prior to use, stir bars were conditioned for 1 h at 300 °C in a flow of helium at 80 mL min^{-1}. Reconditioning of stir bars was done after use by soaking in Milli-Q water and a mixture of dichloromethane–methanol (1:1) for 2 h; as described elsewhere [19]. SBSE was performed using a multiple position magnetic stirrer (Variomag Poly15, Thermo Fisher Scientific, MA, USA). Prior to optimisation, the extraction time profile was examined by stirring solutions spiked with the flavouring (10 mg mL^{-1}) at room temperature and 800 rpm for durations between 10 min and 24 h. After extraction, the stir bars were dried with a lint-free tissue and placed in a glass thermal desorption tube.

2.3. Analytical procedure

TD–PTV–GC–MS/FID analysis was performed using a thermal desorption unit (TDU) coupled with an Agilent 7890C gas chromatograph with a 5975C mass-selective detector and a flame ionization detector with two-way splitter kit (Agilent Technologies, Santa Clara, CA, USA). The Thermal desorption unit (TDU) was mounted on top of a cooled injection system (CIS-4), a programmed-temperature-vaporization (PTV) type universal GC inlet (Gerstel). The entire system was operated under Maestro (Gerstel) integrated with Chemstation (Agilent Technologies).

Initially, the default condition for TD–PTV was set based on the recommendation by Gerstel, where stir bar was thermally desorbed from 40 °C (held for 1 min) to 250 °C (held for 5 min) at 720 °C min^{-1} with the desorption flow of 60 mL min^{-1}. Using a glass wool liner (ID 2.0 mm), the desorbed compounds were cryofocused inside the CIS-4 at −100 °C. After desorption, CIS-4 was programed from −100 to 250 °C (held for 5 min) at 12 °C s^{-1} to transfer the trapped compounds into the analytical column. Splitless transfer of analytes was performed through solvent vent mode, and the effect of splitless time on the peak areas obtained was predetermined by varying opening time of split valve between 1 min and 7 min.

The separations were carried out on a DB-FFAP fused-silica capillary column of dimensions 60 m × 320 μm and 0.25 μm film thickness (Agilent Technologies). The oven temperature was programmed from 40 °C (held for 5 min) to 145 °C at 5 °C min^{-1}, then to 178 °C at 3 °C min^{-1}, and finally to 230 °C (held for 23 min) at 5 °C min^{-1}. Helium was used as the carrier gas at a flow rate of 1.3 mL min^{-1}. The mass spectrometer was operated in the scan mode with electron ionization of 70 eV.

2.4. Optimisation of TD–PTV injection process

As shown in Table 2, three interactive parameters were desorption flow (40–80 mL min^{-1}), thermal desorption time (5–15 min) and cryofocusing temperature in the PTV injection system (−120 to 40 °C). Central composite design (CCD) was applied in this work, where a total of 20 experimental runs were constructed with 6 central points, 8 cubic points and 6 axial points at α value = 1.68 using Design Expert Version 6.0.10 software (Stat-Ease, MN, USA) [29,30].

The experimental data were fitted by a multiple regression equation including up to the second-order polynomial terms and interaction terms [29]. The adequacy of the model was determined by evaluating the coefficient of determination (R^2) and lack-of-fit tests obtained from the analysis of variance (ANOVA), while statistical significances of the model and model terms were determined at 95% confidence level. The terms found to be non-significant ($p > 0.05$) were dropped from the initial model and refitted with the significant ($p < 0.05$) independent variables in order to obtain the final reduced model. However, some insignificant linear terms were retained in the model if a quadratic or interaction term containing these variables was significant. Three dimensional response surface plots were used to visualize the modelled region and to determine the optimal experimental conditions.

Simultaneous optimisation was carried out through an objective function in the Design Expert software. With the overall objective function, individual desirabilities of all the estimated response variables were combined using geometric mean to give an overall desirability D to achieve desirable response goals.

2.5. Partial factorial design for SBSE extraction

A partial factorial experimental design (2^{5-1}) was used to evaluate the significance of the extraction conditions, as well as the interactions between them. The factors investigated were ionic strength (sodium chloride concentration), stirring speed (rpm), extraction time (h), temperature (°C) and pH. Extraction was carried out in a temperature controlled water bath. All variables were evaluated at two levels, low (denoted as −1) and high (denoted as +1). The significant factors were indicated

Table 1
RSM model and method validation for all volatile compounds.

Compound	Default extraction concentration ($\mu g\ L^{-1}$)	MP[a] (°C)	BP[b] (°C)	log $K_{o/w}$[c]	RSM model	Precision (%RSD)		Linear range ($\mu g\ L^{-1}$)	R^2	LOD ($\mu g\ L^{-1}$)	LOQ ($\mu g\ L^{-1sss}$)
						Repeatability (Intra-day; $n=6$)	Intermediate (Inter-day; $n=5$)				
Hydrocarbons											
Limonene	100	−74	176	4.38	Quadratic with positive interaction	4.96	13.20	1.00–10.00	0.996	0.80	2.67
Ocimene	40	50	100	4.80 (est.)	Quadratic	4.00	16.32	0.50–4.00	0.991	0.50	1.65
beta-Myrcene	20	< −10	166–168	4.17	Quadratic with positive interaction	4.22	15.80	0.20–2.00	0.997	0.15	0.50
para-Cymene	20	−68	177	4.1	Quadratic with positive interaction	4.01	15.45	1.00–10.00	0.998	0.61	2.02
alpha-Pinene	10	−64	155	4.44	Quadratic with positive interaction	5.10	17.06	0.10–1.00	0.999	0.03	0.09
Terpinolene	10	n.a.	183–185	4.47	Quadratic with positive interaction	4.57	20.67	1.00–10.00	0.996	0.81	2.71
beta-Caryophyllene	10	n.a.	262–264	6.30 (est.)	Constant	4.23	13.23	0.20–2.00	0.998	0.12	0.40
Valencene	10	n.a.	271	6.3 (est.)	Constant	5.48	12.87	0.25–5.00	0.999	0.19	0.23
Aldehydes											
Octanal	40	12–15	171	2.78 (est.)	Quadratic with positive interaction	1.98	13.60	2.00–20.00	0.998	1.16	3.87
Citral	40	n.a.	229	3.45	Constant	2.40	6.42	0.50–5.00	0.997	0.37	1.24
Nonanal	20	−18	195	3.27 (est.)	Quadratic with positive interaction	2.37	4.56	0.50–10.00	0.999	0.36	1.19
Decanal	20	n.a.	207–209	3.76 (est.)	Constant	2.67	4.86	0.20–2.00	0.995	0.18	0.60
Dodecanal	20	12	184–186	4.75 (est.)	Constant	4.09	7.08	1.00–10.00	0.998	0.57	1.91
Perillic aldehyde	10	n.a.	237	3.13	Constant	2.24	5.25	0.10–2.50	0.999	0.07	0.25
Decatrienal	10	n.a.	252	3.12 (est.)	Quadratic with negative interaction	2.44	3.35	0.19–1.00	0.979	0.19	0.62
Alcohols											
Ethanol	259	−114	78	−0.31	Linear	13.90	20.53	2.59–25.90	0.997	1.76	5.88
Borneol	40	208	213	2.69	Quadratic with positive interaction	7.58	21.09	3.00–20.00	0.999	1.07	3.58
alpha-Terpineol	40	18	219	3.28	Constant	3.17	3.63	0.40–4.00	0.996	0.31	1.03
1,4-Cineole	20	n.a.	172–174	2.97	Quadratic with positive interaction	1.89	5.11	0.20–2.00	0.994	0.20	0.66
Eucalyptol	20	1.5	176–177	2.74	Quadratic with positive interaction	2.60	12.30	0.20–2.00	0.999	0.08	0.27
Linalool	20	< −20	198–199	2.97	Constant	3.25	4.59	0.50–5.00	0.998	0.26	0.85
Citronellol	20	n.a.	225	3.91	Quadratic with negative interaction	3.80	2.93	0.20–4.00	0.996	0.31	1.03
Geraniol	20	15	229	3.47	Quadratic with negative interaction	2.87	4.58	0.20–2.00	0.998	0.12	0.41
Nerol	10	n.a.	224–245	3.47	Quadratic with negative interaction	3.56	4.39	0.25–2.50	0.999	0.09	0.29
Nerolidol	10	n.a.	121 (at 3 mm Hg)	5.68 (est.)	Linear	4.43	3.35	0.25–5.00	0.999	0.18	0.60
Esters											
Ethyl butyrate	40	−93	120–121	1.85 (est.)	Quadratic with positive interaction	9.16	10.56	0.42–10.00	0.999	0.42	1.38
Citronellyl acetate	20	n.a.	240	4.56 (est.)	Quadratic with negative interaction	2.70	3.27	0.20–2.00	0.996	0.16	0.52

Table 1 (*continued*)

Compound	Default extraction concentration ($\mu g\ L^{-1}$)	MP[a] (°C)	BP[b] (°C)	log $K_{o/w}$[c]	RSM model	Precision (%RSD)		Linear range ($\mu g\ L^{-1}$)	R^2	LOD ($\mu g\ L^{-1}$)	LOQ ($\mu g\ L^{-1sss}$)
						Repeatability (Intra-day; $n=6$)	Intermediate (Inter-day; $n=5$)				
Linalyl acetate	10	85	220	3.93	Quadratic with negative interaction	4.80	21.35	0.10–1.00	0.999	0.02	0.07
Decyl acetate	10	n.a.	244	4.79 (est.)	Quadratic with negative interaction	4.96	6.45	0.25–2.50	0.999	0.12	0.39
Styrallyl acetate	10	n.a.	357	2.50 (est.)	Quadratic with negative interaction	2.78	5.66	0.10–0.75	0.993	0.09	0.29
Geranyl acetate	10	<25	240–245	3.98	Quadratic with negative interaction	1.48	3.76	0.25–2.50	0.996	0.20	0.66
Methyl jasmonate	10	<25	88–90 (at 0.1 mmHg)	2.76 (est.)	Linear	2.44	4.70	0.25–2.50	0.998	0.15	0.51
Others											
Nootkatone	20	36	170	4.88 (est.)	Quadratic with negative interaction	2.99	3.57	1.83–20.00	0.998	1.83	6.09
beta-Ionone	10	−49	126–128	4.42 (est.)	Quadratic with negative interaction	1.62	6.42	0.25–5.00	0.999	0.19	0.63
Methyl-N-methyl anthranilate	10	17–19	255–256	2.81 (est.)	Quadratic with negative interaction	2.28	5.46	0.25–5.00	0.999	0.13	0.45
Indole	1	52–54	253–254	2.14	Quadratic with positive interaction	6.65	9.89	3.89–10.00	0.971	3.89	12.98

[a] Melting point at 760 mm Hg.
[b] Boiling point at 760 mm Hg.
[c] Expressed by the estimated logarithm of the n-octanol/water partition coefficient (from KOWIN v.1.67).

Table 2
Central composite design for three factors.

	Factor	Experimental levels				
		$-\alpha$	−1	0	1	α
A	Desorption flow (mL/min)	26.36	40.00	60.00	80.00	93.64
B	Thermal desorption time (min)	1.59	5.00	10.00	15.00	18.41
C	Cryofocusing temperature (°C)	−147.27	−120.00	−80.00	−40.00	−12.73

Table 3
Experimental domain for screening significant factors affecting extraction of SBSE.

Factor	Low (−)	High (+)
A: Ionic strength (% w/v NaCl)	0	30
B: Stirring speed (rpm)	300	900
C: Extraction time (h)	2	6
D: Temperature (°C)	24	60
E: pH	2	7

by the Pareto chart, which was obtained after multiple linear regression and analysis of variance (see Table 3).

2.6. Model evaluation and validation on model citrus beverage

Linearity was determined over an 11-point calibration with citrus flavouring spiked Milli-Q water ranging from 10.3 to 515 $\mu g\ L^{-1}$. The calibration curves prepared by calculating FID absolute peak areas against concentrations were obtained for individual compounds, with a correlation coefficient R^2 of at least 0.99. Based on the calibration curves obtained, figures of merits such as linearity, limit of detection (LOD) and limit of quantification (LOQ) were also determined for each compound. The LOD and LOQ for each compound were established by using the equations $LOD=3s_y/b$ and $LOQ=10s_y/b$, where s_y is the SD of the peak areas obtained from at least five different concentrations within the linear range and b is the slope of the calibration curve.

Model citrus beverage was prepared by spiking 0.10 g of the citrus flavouring (10 mg mL^{-1}) into a 100.00 g synthetic juice matrix, which was made of Milli-Q water (88.82 g), sucrose (10.80 g, SAFC, St. Louis, MO, USA), anhydrous citric acid (0.26 g, SAFC), pectin (0.05 g, Grindsted® AMD783, Danisco, Lakeland, FL, USA), sodium citrate dihydrate (0.04 g, SAFC), and ascorbic acid (0.03 g, SAFC). Ten millilitre of model citrus beverage was used for SBSE extraction.

All experiments were carried out in triplicate and the results were reported as mean values together with standard deviations.

3. Results and discussion

3.1. Optimisation of TD–PTV injection process

Through allowing compounds to be injected under temperature controlled conditions, the PTV technique alleviates the problems of compound discrimination and decomposition, which always occur when analytes are flash vaporized in a hot split/splitless injector [31]. In this work, PTV solvent vent mode was chosen to allow a suitable high desorption flow rate for desorption of the analytes into PTV and consecutively maximize the transfer of desorbed analytes into the GC column through splitless injection. Moreover, the split valve of CIS was closed during desorption, and remained close after desorption until the transfer of analytes was complete. We defined the time period before the split vent valve opens as splitless time. The duration of splitless time should be carefully set to ensure the complete transfer of analytes into the column. An insufficient splitless time could lead to an incomplete transfer of analytes and resulted in poor reproducibility of peak areas, as well as the discrimination for high boiling compounds. Fig. 1 shows the effect of splitless time on the peak areas of the various classes of volatile compounds. Most classes of compounds were maximally transferred into the column after opening the split vent at time 3 min, except for aldehydes and esters, which required a splitless time of 5 min. On the other hand, a progressive loss of the compounds with lower boiling points was observed after a longer splitless time of 5 min, suggesting that prolonged vent time could be a disadvantage or discrimination against these compounds. So, a vent time of 3 min was set as the default to ensure the quantitative transfer of a good majority of the compounds, while minimizing the loss of more volatile compounds.

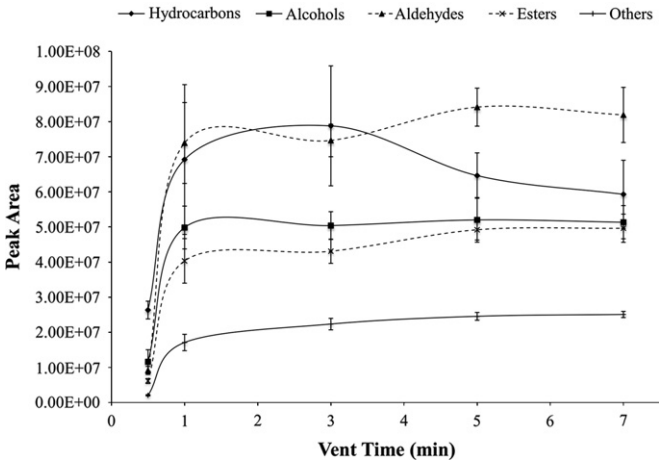

Fig. 1. Effect of splitless time on the quantitation of each class of volatile compounds.

The mode of the injection could affect the quantification of volatiles where thermal desorption process could be the most critical part in enhancing quantification proficiency. Previous studies that focused on the optimisation of PTV operating parameters have identified that injection temperature, desorption flow, vaporization temperature, vaporization time and cryofocusing temperature could significantly affect the efficiency of thermal desorption [32–34]. Our preliminary screening yielded three interrelated factors (i.e. desorption flow, thermal desorption time and cryofocusing temperature) that were selected for further optimisation using RSM (data not shown). The common practice to optimise an extraction process using RSM is to observe total responses or total peak areas [29–32]. However, such an approach does not account for differences among peak areas in contributing towards extraction efficiency of different compounds. In contrast, we studied these compounds by establishing the relationship between the response of each compound in terms of peak area and three operating parameters.

Fig. 2 illustrates the response surface plots of three representative responses—constant, linear and quadratic, where the nature of the response surface system depends on the signs and

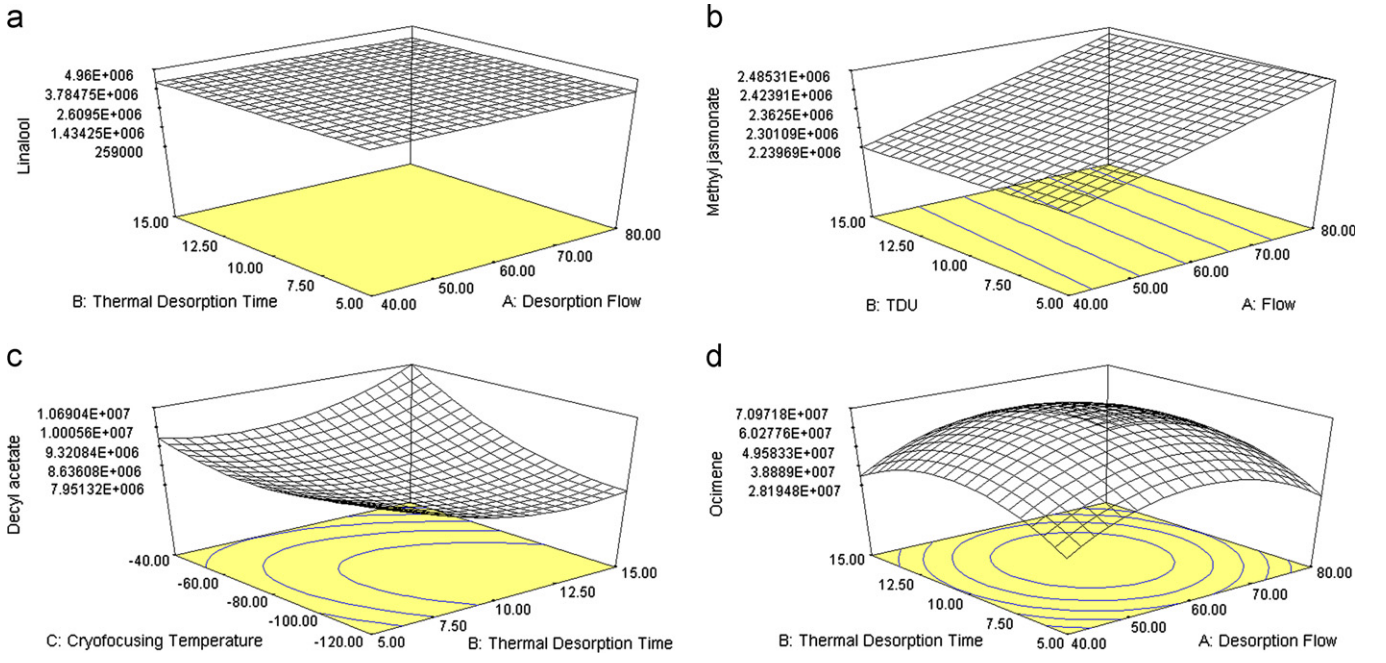

Fig. 2. Typical profiles of surface response generated from a quadratic model in the optimisation of three variables (thermal desportion time, desportion flow and cryofocusing terperature): (a) constant—exemplified by linalool; (b) linear—exemplified by methyl jasmonate; (c) quadratic with minimum response—exemplified by decyl acetate and (d) quadratic with maximum response—exemplified by ocimene.

magnitudes of the coefficients in the model terms. The estimated responses for all compounds are also listed in Table 1. In order to increase the model's predictive accuracy, a stepwise approach was applied to fit the full response surface and eliminate the terms not significant at the $p=0.05$ level.

Although no clear-cut relationship between the models obtained for each compound and its physicochemical properties could be identified, it was observed that those compounds with constant models generally had lower $K_{o/w}$ values and higher solubility in water (e.g. citral, *alpha*-terpineol and linalool), suggesting that they were poorly extracted by SBSE. Therefore, a constant response reflected that the corresponding compound was non-responsive towards the parameters, while a linear response was obtained when the detected peak area was in proportion to the main parameters. The significant first-order terms of ethanol, nerolidol and methyl jasmonate reflected the fact that the low cryofocusing temperature was favourable due to their low boiling points. However, extremely low cryofocusing temperature (i.e. $-150\,^{\circ}C$) could possibly crystallize other higher boiling compounds and the glasswool in the TDU inlet liner. Thus, those less volatile compounds would be trapped before they reached the column. In addition, as suggested by Tredoux et al. [14] a trapping temperature of $-100\,^{\circ}C$ instead of $-150\,^{\circ}C$ greatly improved the peak shapes for early eluting compounds. It is believed that faster heating of the liner to the injection temperature leads to reduced injection times and therefore less band broadening.

On the other hand, quadratic models with significant second-order coefficients (pure quadratic and interaction terms) played a vital role in estimating the responses and could shed some light on the thermal desorption behaviour of these analytes. For instance, decyl acetate with a high boiling point decreased in its peak area as cryofocusing temperature decreased, while peak area of ocimene with a low boiling point showed an increase in response to a decrease in cryofocusing temperature. Among the compounds with quadratic models, most of them did not have strong interaction effects, but did have strong quadratic terms on main effects. Among these three main effects, cryofocusing temperature had the greatest influence on the analytical responses. Nevertheless, a sufficient thermal desorption time and a high purge flow rate were important to maximize the transfer of analytes (e.g. terpinolene). The response models also revealed that the dissimilarity of responses was mainly due to the physicochemical properties. Compounds with low boiling point (e.g. limonene, ocimene, *beta*-myrcene, octanal and ethyl butyrate) resulted in a maximum response, so they favored a moderate desorption temperature and a sufficiently low cryofocusing temperature. In contrast, a minimum value indicated that the thermal desorption was operated under a condition remote from the optimum (e.g. decatrienal, citronellol, nootkatone). Hence, due to their low volatilities, these analytes required a longer desorption time.

Several studies have been done on wine whether focused on volatile phenols [15] or major wine volatiles [14,16,17,35]; however, to the best of our knowledge, there was no reported study that optimised the operating condition based on all target analytes. In addition, it is often necessary to use constrained optimisation to attain the best operating condition. This is particularly true in our present work to avoid the optimum point falling outside the operating parameters. The optimised factors were determined based on maximizing the desirability of the responses for the flavouring as a whole, which combined the individual desirability of each response into the objective function. The desirability of the response for each compound ranged from 0.00 to 1.00, corresponding to the increase from lowest to the highest in the response values obtained by the experiments. The optimised values of the three factors were $A=74.00\,mL\,min^{-1}$, $B=5.00\,min$ and $C=-120.00\,^{\circ}C$, with an overall desirability of 0.54. Compared to the default thermal desorption method prior to the optimisation with overall desirability of 0.32, some of the peak responses were suppressed while some were enhanced; thereby the peak responses of each target analytes varied from -35% to 100%, with an average enhancement of 32%. This reflected that under the optimised set parameters the variability of peak responses among the analytes was less discriminated.

3.2. Understanding of SBSE extraction

From the extensive reviews on SBSE, the recovery of an analyte could be approximated by its partition coefficient between PDMS and water ($K_{PDMS/W}$) and the phase ratio of PDMS phase/sample volumes [2,19,36]. Moreover, previous studies revealed that some other experimental factors (e.g. extraction temperature, rate of agitation and salt content) could also affect the efficiency of SBSE [9,15,22]. From a preliminary study on extraction–time profile, most of the compounds were recovered substantially after first 2 h of extraction and then gradually increased up to about 4 h. The increase in the amount extracted became less pronounced beyond 4 h and equilibrium was reached after about 6 h, except for hydrocarbons (data not shown). The differences in equilibration time for different classes of analytes thus suggested that kinetic aspect is also important to achieve equilibrium though the uptake rate is mainly determined by diffusion constants, stirring conditions, sample volume, etc. [3]. Even though an extraction time of 12 h could ensure the maximum recovery of all the compounds, such a long extraction time would simply be unrealistic for routine extraction. In addition, equilibration during the extraction process was not necessary in practice since calibration could be carried out for any consistent extraction time [19]. Hence, extraction time of 2 h was chosen as the default extraction time in this study.

Subsequently, a partial factorial experimental design was chosen to investigate the effects of the extraction factors on the efficiency of SBSE. The level of extraction time, temperature and stirring speed took into account the consideration on the sample throughput and minimal evaporation of the analytes for a combination of the extremes of these variables (at 900 rpm, 60 °C for 6 h). The sample was studied in pH range of 2–7 referring to the pH range of different food beverages. The sodium chloride in the concentration range studied involved no addition to saturation. Under the present condition, according to the Pareto charts shown in Fig. 3, extraction time, temperature and stirring speed had no significant effects on enhancing the extraction of SBSE. Nonetheless, pH had a positive effect, especially alcohol group and the other compounds like nootkatone and indole, which was in contrast to the insignificant enhancement on the extraction of volatile phenols from wine [35]. On the other hand, highly positive effect of ionic strength was observed for alcohol group, since the addition of sodium chloride reduces the water solubility of polar analytes. Thus, this resulted in the increased partitioning coefficient between the PDMS and analytes [3]. Finally, esters and hydrocarbons were negatively affected by ionic strength. This could be because the higher solute concentration increased viscosity of the solution and hindered the diffusion efficiency of analytes. During extraction, the solutes should migrate from the sample into the PDMS coating [3]. In this case, sufficient convection or stirring speed is important, so that diffusion efficiency of analytes will not be affected.

3.3. Method evaluation and validation

With the aid of response surface methodology, solute discrimination during thermal desorption and cryofocusing was reduced. Furthermore, SBSE extraction was found to be more

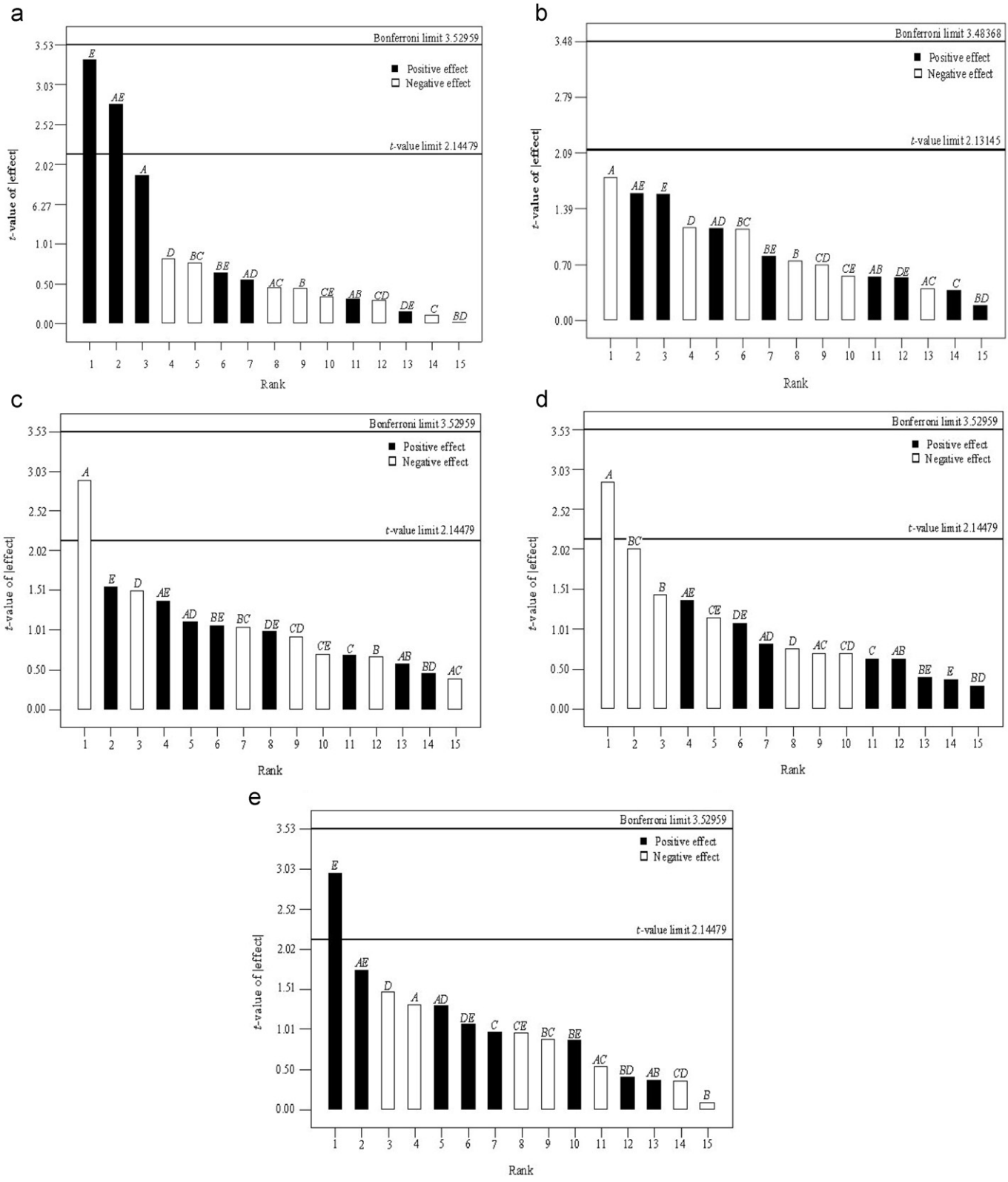

Fig. 3. Pareto chart of the statistical analysis of the screening of factors for the extraction step of (a) alcohols; (b) aldehydes; (c) esters; (d) hydrocarbons; and (e) others. The vertical line indicates the threshold value for proclaiming the statistical significant terms on the effect of (A) ionic strength; (B) stirring speed; (C) extraction time; (D) temperature and (E) pH.

favourable in neutral pH, while temperature and stirring speed were not the major factors as long as there was sufficient extraction time. In addition, extraction efficiency of polar organic analytes can be improved by increasing ionic strength. In the following experiment, sample matrix was not altered in order to maintain the true ratio in the flavouring.

In order to validate the multiple regression equations obtained, FID peak responses of each compound were examined

under two combinations within the experimental range: (1) $A=74.00$ mL min^{-1}, $B=15.00$ min and $C=-120.00$ °C and (2) at $A=48.00$ mL min^{-1}, $B=5.00$ min and $C=-54.00$ °C. Our results indicated that the values of the peak areas obtained from the actual experiments fall within 95% confidence interval of the predicted range of the regression model.

The precision of each compound was evaluated in terms of its repeatability and reproducibility. Repeatability was reported as the RSD of the peak area obtained from six consecutive analyses within the same day, whereas intermediate precision was reported as the RSD of the peak areas obtained from five consecutive analyses on different days. Table 3 shows the repeatability and intermediate precision for each compound. RSD of 1.7% and 6.3% were obtained for the total peak areas from intra-day and inter-day analyses, respectively.

A linear relationship of absolute peak areas against concentrations was obtained for individual compounds, with a correlation coefficient R^2 of at least 0.99. Based on the calibration curves obtained, the linearity, limit of detection (LOD) and limit of quantification (LOQ) were also determined for each compound (see Table 3). All the compounds had low LOD values from 0.03 to 3.89 μg L^{-1}. This data indicated that the analytical method developed could facilitate the simultaneous quantitation of nearly all of the compounds present when the flavouring was spiked in water.

In contrast to previous studies that semi-quantified extracted aroma compounds using internal standard [20] or estimated the relative levels of target compounds in wine samples from the ratios of their MS areas to that of the relevant internal standards using scan mode [18], all the above discussions were based on the absolute FID peak area. In present study, two internal standards (i.e. *n*-undecane and 6-methyl-5-hepten-2-one) were selected in an attempt to determine the concentration of each compound. However, owing to the diversity of compounds in citrus flavouring, the peak responses obtained for the two selected internal standards were not proportional to those obtained for all compounds (data not shown). Hence, this would have given rise to an over-estimation or bias when the concentrations of all the compounds were calculated based on normalising their responses against the internal standards.

3.4. Matrix effect of model citrus beverage on SBSE extraction

The results presented thus far were obtained from experiments carried out by extracting citrus flavouring from a blank matrix (aqueous only) to develop the analytical method with minimum interferences from the matrix. A recent study determined the presence of 9 synthetic musks using SBSE in four kinds of aqueous matrices (i.e. effluent and influent wastewater treatment plant, effluent of a reverse osmosis treatment plant and river water) which no matrix effect was observed [37]. On the other hand, the presence of ethanol in model wine sample matrix resulted in decreased sensitivity of the SBSE method towards most of the volatile compounds [20]. Beverage products usually contain different types of soluble solids (e.g. acids, sugars, and pectins) which give rise to matrix effects that would further complicate the extraction process [26]. The presence of flavour–hydrocolloid interaction does have an impact on the amount of volatile compounds released from the food matrix [26,38,39]. As there is limited information of the flavour–hydrocolloid interaction on SBSE efficiency, a model citrus beverage was prepared to evaluate the matrix effect on SBSE extraction. To verify the applicability of the developed analytical method in real citrus beverage, the method was applied on a model citrus beverage.

Among food hydrocolloids, high-ester pectin is one the most commonly used hydrocolloids that can be used as an emulsifying

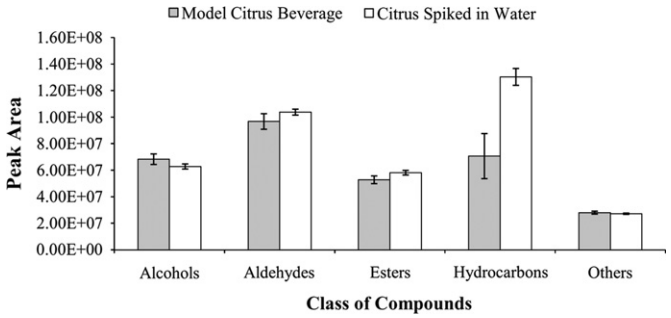

Fig. 4. FID peak areas of SBSE extraction on different matrices.

and stabilizing agent in various applications such as acidified beverages [26]. Sucrose and high-ester pectin were two main ingredients that could alter the rheological properties of the matrix and influence flavour partitioning in an emulsion system. The presence of sucrose and high-ester pectin generally resulted in changes of liquid partition coefficients. The salting-out effect was likely to be the reason for this phenomenon, whereby the sugar interacted with water, enhancing the concentration of flavour compounds. On the other hand, pectin was added in model citrus beverage with a pH value of 3.25; a weak gel network was formed with hydrogen bonds and hydrophobic interactions. This macromolecular network could form flavour–matrix interactions with the volatile compounds and thereby enhance or suppress the release of the compounds to be extracted by SBSE [26].

From our observation, the variance of volatile extractions between the model citrus beverage and blank matrix was insignificant, except for hydrocarbons and alcohols (Fig. 4). This could be explained by the hydrophobic interaction between non-polar terpenes and pectin network, thereby reducing their availability for extraction by SBSE. Previous study has demonstrated that both orthonasal and retronasal odour thresholds were much higher in reconstituted orange juice than in water [24]. On the other hand, the noticeable enhancement of alcohol extraction could be due to the reduced water activity; with the presence of soluble solid, alcohol could be more easily absorbed by PDMS.

4. Conclusion

A systematic approach was applied to understand the factors affecting SBSE–TD–PTV analysis of a complex mixture. Detailing the responses of different flavour compounds, cryofocusing temperature was the most influential factor among three TD–PTV parameters. Consequently, variability of GC peak responses among the analytes was alleviated. Furthermore, through partial factorial design, the results for SBSE extraction indicated the positive influence of ionic strength and neutral pH on extraction of alcohol compounds. Finally, the optimised method was evaluated and validated through measuring linearity, the detection limits and repeatability values. Therefore, this methodology may be approved to be effective in improving the performance of SBSE–TD–PTV analysis.

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