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# OPTIMIZATION OF SOLID-PHASE MICROEXTRACTION CONDITIONS OF MILK CHOCOLATE

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Oba, S., Toker, O.S., Rasouli Pirouzian, H., Palabiyik, İ., Konar, N. Artik, N., Sagdic, O. (2021). Sütlü çikolata için katı faz mikroekstraksiyon yönteminin optimizasyonu. GIDA (2021) 45 (1) 82-96 doi: 10.15237/gida.GD20055

## ABSTRACT

In the current study, the Central Composite Design was applied to optimize HS-SPME extraction in order to detect 2,3,5,6 tetramethyl pyrazine in isomalt contaning milk chocolate. The optimal conditions for the three experimental responses influencing SPME efficiency were 10 min, 40 min and 57 °C for equilibration time, extraction time and extraction temperature, respectively. SPME fibers coated with 100 m polydimethylsiloxane coating, 65 µm polydimethylsiloxane/divinylbenzene coating, 75 µm carboxen/polydimethylsiloxane coating and 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane on a StableFlex fiber were investigated. The preparation conditions of the chocolate samples were also evaluated by measuring their effects on the coating composition of the head space. The SPME fiber coated with 50/30 µm divinylbenzene/carboxen-polydimethylsiloxane provided the highest extraction efficiency, especially when the samples were extracted at 60 °C for 30 min under dry conditions. Eighty-one blends were extracted and experimentally detected most of which have been formerly stated as odor-active components.

**Keywords:** Chocolate, isomalt, central composite design, optimization, SPME

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## SÜTLÜ ÇİKOLATA İÇİN KATI FAZ MİKROEKSTRAKSIYON YÖNTEMİNİN OPTİMİZASYONU

**ÖZ**

Bu çalışmada, isomalt içeren sütlü çikolatalarda 2,3,5,6 tetra metal pirazin tayini için HS-SPME ekstraksiyonunun optimizasyonunda merkezi kompozit tasarım uygulanmıştır. SPME verimliliği etkileyen dengeleme süresi, ekstraksiyon süresi ve ekstraksiyon sıcaklığı için optimum koşullar olarak sırasıyla 10 dk, 40 dk ve 57 °C olarak belirlenmiştir. Çalışmada StableFlex fibere uygulanmış 100 µm polidimetilsiloksan, 65 µm polidimetilsiloksan/divinilbenzen, 75 µm karboksen/polidimetilsiloksan ve 50/30 µm divinilbenzen/karboksen/polidimetilsiloksan kaplamalar SPME fiberler olarak incelenmiştir. Çikolata örneklerinin hazırlama koşulları ayrıca tepe boşluğunun kaplama bileşimi üzerindeki etkileri ölçülerek de değerlendirilmiştir. Özellikle örneklerin kuru koşullarda 60 °C'de 30 dk örnek ekstraksiyonunda 50/30 µm divinilbenzen/karboksen/polidimetilsiloksan kaplı SPME fiber en yüksek ekstraksiyon verimliliğini sağlamıştır.

**Anahtar kelimeler:** Çikolata, izomalt, merkezi kompozit tasarım, optimizasyon, SPME

## INTRODUCTION

The unique aroma of chocolate and cocoa is the main reason that has made it universal (Ducki et al., 2008; Owusu, 2010; Khairy et al., 2018). This complex and unrivalled aroma is a basic organoleptic attribute of many sweet products (Perego et al., 2004). Also, chocolate is a main portion in several kinds of products and is classified as one of the most popular flavours worldwide (Di Carro et al., 2015).

Isomalt is an ideal sucrose replacer to produce sucrose-free chocolates because it as high bulking capacity, sweetening strength and maintenance the full chocolate flavor (Aidoo et al., 2013). This ingredient contains fewer calories and also induces a very low glycemic effect (Mitchell, 2006). Likewise it can not be dissociated by plaque bacteria and therefore is tooth-friendly (Nabors, 2001). The low hygroscopicity and high temperature stability of isomalt (LM) prevents the moisture absorption and agglomeration during production process, respectively (Radowski, 2006).

Flavour is the most substantial element that identifies the acceptability of chocolate (Afoakwa, 2010). The specific aroma of chocolate is because of a very wealthy volatile fraction, including a blend of hundreds of components that arises from flavour precursors present in cocoa beans, however also via post-harvest processing, being changed into appopriate odour notes in the production process (Di Carro et al., 2015; Braga et al., 2018).

Aroma precursors in cocoa bean created in fermentation are transformed to cocoa particular aroma during the roasting via the Maillard reactions (Brunetto et al., 2009; Kumari et al., 2018). Main elements influencing the aroma of cocoa beans are harvesting time, genotype and geographical origin (Tran et al., 2015; Da Veiga Moreira et al., 2018), fermentation (Rodriguez-Campos et al., 2012; Van Durme et al., 2016; Kone et al., 2016; Da Veiga Moreira et al., 2018; Engeseth and Pangan, 2018; Kumari et al., 2018), drying (Rodriguez-Campos et al., 2011) and roasting (Van Durme et al., 2016; Khairy et al., 2018).

The volatile components that create the chocolate aroma are varied and originated from the chocolate manufacturing processes (Owusu, 2010). In the chocolate manufacturing processes, it can be concluded that apart from the ingredients and compositions, the conching (Owusu, 2010; Hue et al., 2014) and refining (Afaokwa et al., 2008; Beckett, 2008; Afoakwa, 2010) are the important factors in the determination of overall aroma. Conching is considered as fundamental for final flavour formation.

Analytical reseraches have determined more than 600 volatile components in cocoa and chocolate (Afoakwa, 2010). Most of the components that

donate cocoa and chocolate their aroma are N- and O-containing molecules for example pyrazines, pyrroles, furans, aldehydes, amides, alcohols, ethers and esters (Ducki et al., 2008; Owusu, 2010; Di Carro et al., 2015). Pyrazine a class of organic components, which are nitrogenous heterocyclic components specified by low molecular weight and high volatility, are the dominant substances in cocoa (Perego et al., 2004) and chocolate, as well as being the most studied volatile compound group (Brito and Narain, 2003; Di Carro et al., 2015; Braga et al., 2018; Da Veiga Moreira et al., 2018; Engeseth and Pangan, 2018; Khairy et al., 2018).

Due to consumers growing appeal for high quality cocoa products, the chocolate professions is extremely eagered in a more rapid analytical method for cocoa aroma quality assortment (Tran et al., 2015). Solid Phase Microextraction (SPME) method has been applied for quantitative aims in volatile components for several products such as alcoholic beverages, coffee, sausages, juice, and cocoa bean (Rodriguez-Campos et al., 2012). This technique has been applied for the quantitative specification of pyrazines in cocoa origin products in various studies (De Brito et al., 2001; Perego et al., 2004; Pini, De Brito et al., 2004; Rodriguez-Campos et al., 2012; Nicolotti et al., 2013; Di Carro et al., 2015; Toker et al., 2016). The use of HS-SPME-GC/MS has become extendly popular in cocoa flavour researches because of some advantages for example high sensitivity, selectivity and reproducibility (Tran et al., 2015). Also, SPME method has been introduced to be inexpensive, solventless and rapid (Rodriguez-Campos et al., 2012). However, this method is very sensitive to the tentative situations; therefore various extraction situations should be developed to investigate the volatile ingredients of cocoa and cocoa-containing products. The most selected fiber was divinylbenzene/carboxene/ polydimethylsiloxane (DVB/CAR/PDMS) however PDMS/DVB and PDMS were used in different studies. Also two basic parameters, namely temperature and time of exposure could be specified. Several extraction times (ranging from 1-90 min) and temperature conditions (ranging from 4-80 °C) were tested in the previous studies.

Different release rates of essential odorants are reported which indicates various matrix effects because of their particular compositions (Nicollotti et al., 2013). Also, the head-space method may be demonstrated for the isolation and pre-concentration of volatile analytes from different matrixes (Brunetto et al., 2009). The volatile fraction of foods of plant source is frequently a complex blend of chemicals formerly present in the raw matrix, and components whose creation is principally due to a number of reactions (Nicolotti et al., 2013). Therefore, it is necessary to determine the optimum SPME conditions for different types of chocolate. The purpose of the present study was to select SPME fiber coating material and optimum extraction conditions (equilibrium time, extraction temperature, extraction time) using Response Surface Method (RSM) in milk chocolate samples made with isomalt a polyol that is widely used for sucrose substitution in sucrose-free chocolates.

## MATERIAL AND METHODS

### Materials

For the production of milk chocolates, cocoa butter, cocoa mass (Altinmarka, Turkey), sugar (SMS Kopuz, Turkey), milk powder (Besel, Turkey), soy lecithin (Brenntag Chemistry, Turkey), polyglycerol polyricinalate (PGPR) (Palsgaard, Netherlands), vanillin (Ekin Chemistry, Turkey), cocoa butter originated $\beta_V$ seed crystal (SEED100, Uelzena, Germany) and isomalt (Beneo Palatinit, Germany) were used.

### Sample preparation

Chocolates were produced by using isomalt (39.0 g/100 g), cocoa butter (24.0 g/100 g), cocoa mass (14.0 g/100 g), whole milk powder (22.45 g/100 g), soy lecithin (0.30 g/100), PGPR (0.22 g/100 g), vanilla flavor (0.03 g/100 g) and $\beta_V$ seed crystal (0.60 g/100 g). Chocolates were produced by using a pilot system (ChocoEasy 50, Netzsch, Selb, Germany) which is using agitator bead mills instead of five roller mills for refining of the chocolate, the refining and liquid conching processes run at the same time. First the cocoa

mass was melted at 50 °C and the other ingredients were added and mixed for 40 min. In the next step of the process, dry conching, the chocolate mass was conhced at 50 °C for 60 min. At the end of the dry conching, cocoa butter, lecithin and PGPR were added and conching process was performed at 50 °C for 120 min. The mixed ingredients were refined until to mean particle size ($D_{[3,4]}$) ranged between 19 and 25 μ m using refiner.

The conched samples for each model system were tempered using temper machine in the Yıldız Technical University Laboratory (Istanbul, Turkey). The milk chocolates were heated to 47 °C for 10 min in temper machine to melt the whole crystals. The molten chocolate was continuously mixed by connecting to a wall-scraping mixer and was then quenched to 32 °C before (0.5%) $\beta_V$ seed crystals were added. The isomalt milk produced milk chocolate mass were mixed for 10 minutes at 32 °C. The mixture was stirred completely automatically to ensure the $\beta_V$ seeds completely dispersed in the fat phase. Finally, freshly tempered chocolate (32 °C) was deposited in a plastic mould and then were allowed to solidify for 50 min in a temperature-controlled cooling chamber at 12 °C and 45% RH.

For the volatile analysis of milk chocolate, samples containing isomalt stored at room temperature. Chocolate samples were divided into pieces with the help of a knife. The samples (1.0 g) were placed in a vial (dry conditions) and remained under agitation with a magnetic stir bar during all isolation procedures. Difference in optimization conditions was extracted samples. The ranges of extraction temperature ($X_1$), extraction time ($X_2$) and equilibrium time ($X_3$) for the methodology was based on experimental designs.

**Gas chromatography-mass spectrometry**

HS-SPME-GC-MS technique was applied in order to determine the aromatic/volatile components of the chocolate in the mouth cavity and temperature (Mexis Badeka et al., 2010). The GC/MS analysis was performed with a Shimadzu System. RTX-5MS column (30 m×0.25 mm, 0.25 μm film thickness) was applied with a helium carrier gas at 0.7 mL/min. GC oven temperature was kept at 30 °C for 5 min and heated to 180 ºC for 5 min and programmed to 240 ºC at a rate of 4 °C/min, kept steady for 10 min at 240 °C. The injector temperature was 250 °C. Mass spectrums were taken at 70 eV and the mass range was from m/z 35 to 450.

The volatiles were analyzed by capillary GC using a Shimadzu QP2010 GC system. Flame ionization detector (FID) temperature was set at 200 °C in order to obtain the same elution order with GC/MS. Peak identification was performed by adaptation of the volatile sample mass spectra with spectra in the NIST/WILEY/NIH Mass Spectral Database (National Institute of Standards and Technology, Gaithersburg, MD, Version 2.0a, 2002, USA).

**Selection of fiber coatings**

Volatile components were extracted applying three diverse fibers; 65 μm PDMS/DVB coating, 75 μm CAR/PDMS coating and 50/30 μm DVB/CAR/PDMS on a StableFlex fiber. These fibers were obtained from Supelco (Bellefonte, PA, USA). The SPME fiber was preconditioned before extraction at 250 °C for 10 min. In the primary choice, three of the fibers were examined to choose the one nominating the highest capacity to extract the isomalt milk chocolate. In this stage, all the fibers were subjected to the sample headspace under the procedure SPME models. HS-SPME fibers were exposed to the top cavity of 1.0 g of dry sample placed in vials under the following situations; equilibrium time of 15 min, extraction time of 30 min, extraction temperature of 60 °C. The SPME fibers were introduced into the gas chromatograph injector for desorption of the analytes at a temperature of 250 °C in the split 1:2 mode for a period of 5.0 min. All fibers were tested 3 times and the average value of the results was calculated.

**Selection of sample conditions**

Sample conditions were determined in GC-MS using 50/30 m DVB/CAR/PDMS StableFlex fibers. Milk chocolate containing isomalt was cut

into pieces by means of a knife and placed (1.0 g) into the vials. For sample conditions selection; (A) 1.0 g dry chocolate and (B) 1.0 g dry chocolate and 4 ml of water was used in 20 ml vials closed with cap and Teflon-faced silicone rubber septa (Supelco, Inc., Bellefonte, PA). During all operations, samples were incubated in a magnetic stirrer at 60 °C for 30 min. At this stage, the (DVB / CAR/PDMS) fiber was subjected to the sample upper cavity for 15 min under extraction conditions. The SPME fibers were exposed into the gas chromatograph injector for desorption of the analytes at a temperature of 250 °C in the split 1:2 mode for a period of 5.0 min.

**Optimization of SPME conditions**

In the preliminary, one of the suitable coating fibers to be selected for the identification of the chocolate flavour compounds was HS-SPME extraction efficiency impact factor. The influence of variables on the volatile components of milk chocolates using SPME extraction conditions is technically part of the project that has been examined. For this purpose, optimization of the HS-SPME conditions was carried out with the 3 factors and 3 levels Central Composition Design (CCD) based on the RSM. The effect of three SPME extraction variables includes, extraction temperature ($X_1$: 40–80 °C), extraction time ($X_2$: 20–60 min) and equilibrium time ($X_3$: 5–15 min) choice of experimental levels was preliminary tests. Each variable coded at its three levels (-1, 0, 1) represents lower, middle and higher value. These uncoded and coded independent variables are listed in Table 1.

Table 1. Uncoded and coded independent variables in RSM

| Coded variables | Uncoded variables | Coded levels | | |
|---|---|---|---|---|
| | | -1 | 0 | 1 |
| $X_1$ | Extraction Temperature (°C) | 40 | 60 | 80 |
| $X_2$ | Extraction Time (min) | 20 | 40 | 60 |
| $X_3$ | Equilibrium Time (min) | 5 | 10 | 15 |

Terms of chocolate extracts were to be achieved and the effects of different extraction conditions were determined. Chocolate extracts of different extraction situations and effects were determined. CCD used 16 experimental runs including 2 at central point. The optimization of the HS-SPME experimental conditions created as shown in Table 2.

Therefore, the combined effects of these variables on the format value responses Y assessed during all experiments was the total sum of peak areas, obtained in the GC-MS analysis. Response surface models were worked out by means of least squares using the following equation (Eq. 1);
Eq. (1).

$$Y= \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_{i2} + \Sigma\beta_{ij} X_i X_j + e_i$$

Where Y is the dependent variable by the model; $\beta_0$ is a constant coefficient; and $\beta_i$, $\beta_{ii}$ and $\beta_{ij}$ are the linear, quadratic and interaction coefficients, respectively. In this model, $X_1$, $X_2$ and $X_3$ are the independent variables. The significance of all the terms of polynomial equation was specificed applying model analysis, lack-of-fit tests and coefficients of determination ($R^2$). JMP Statistical Analysis for Optimization Program (Version 12.0.1 version) was determined using experimental design and data analysis.

## RESULTS AND DISCUSSION

**Selection of sample conditions**

HS-SPME-GC-MS analytical method was created for sugar-free chocolate samples. Approxiametly 1.0 g of chocolate containing isomalt with 0.5% seed crystal that were prepared as dry and aqueous (1:4 v/w) conditions were absorbed on fiber under extraction conditions of 60 °C and 30 min. The temperature of the injector and of the detector was 250 ºC and the SPME fiber desorption time was 5 min (Pini et al., 2004).

It was observed that in dry chocolate samples containing isomalt, the level of components extracted by DVB/CAR/PDMS fiber was higher

than wet situations ($P$ <0.01). In these samples, the effect of major components extracted by CAR/DVB/PDMS fiber on the peak area of dry and aqueous samples are presented in Figure 1. Comparing the volatile fractions the largest differences were observed in the "acid and alcohol" groups. These findings can be clarified by the reality that alcohols and acids are soluble in water, and this decreases their attendance in the volatile phase (Ducki et al., 2008). In addition, some compounds such as benzaldehyde, hexanoic acid, caprylic acid, decane, 2,2-dimethyl could not be detected in wet samples. Finally the dry condition was selected as the most suitable sample preparation method.

Table 2. The optimization of the HS-SPME experimental conditions

| Run order | Extraction temperature (°C) | Extraction time (min) | Equilibrium time (min) |
|---|---|---|---|
| 1 | 80 | 40 | 10 |
| 2 | 80 | 60 | 15 |
| 3 | 80 | 20 | 5 |
| 4 | 60 | 20 | 10 |
| 5 | 60 | 40 | 15 |
| 6 | 60 | 40 | 5 |
| 7 | 80 | 60 | 5 |
| 8 | 40 | 40 | 10 |
| 9 | 60 | 40 | 10 |
| 10 | 80 | 20 | 15 |
| 11 | 60 | 40 | 10 |
| 12 | 40 | 20 | 15 |
| 13 | 40 | 60 | 5 |
| 14 | 60 | 60 | 10 |
| 15 | 40 | 20 | 5 |
| 16 | 40 | 60 | 15 |

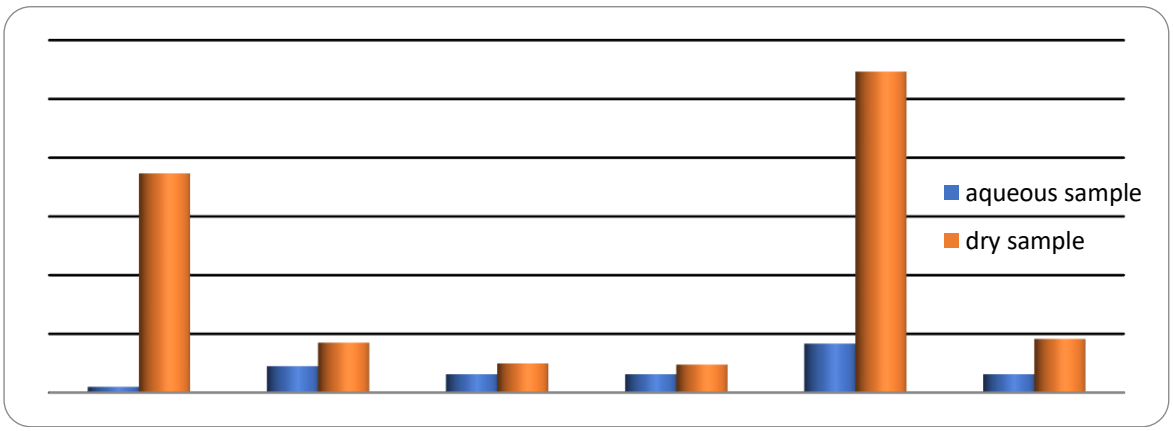



Figure 1. Peak areas of key compounds identified with two types of preparation method

**Selection of fiber coatings**

Methods for extracting aroma compounds from food products were investigated by considering the volatile nature of compounds (Kataoka et al., 2000). SPME is an effective adsorption/desorption technique. This technique is relied on the adsorption of the sample on a stationary phase film coated onto a silica-fused. It has successfully applied for the extraction of volatile and semi-volatile components from environmental, biological and food products.

The partitioning of analytes between sample/air/fiber depends upon several factors such as; the sample matrix, the lipid content of sample, salt concentration, pH, presence of unwanted ingredients, choice of standards, sample and headspace volumes, agitation, temperature and fiber selectivity (Matich, 1999).

For successful separations of analytes in SPME, fiber coated with a appropriate polymeric stationary phase is necessary. The selectivity of extraction in SPME often depends on the polymeric coating of the fiber. Based on different selectivity, different thermal stability and different polarity of analytes, different coatings on the SPME fiber will be used. In SPME, the level of analyte extracted onto the fiber relies on the polarity and thickness of the stationary phase, the molecular weight and molecular size, polarity, boiling point and vapor pressure of analyte, the presence of functional groups in analyte and fiber and also the mechanism of the extraction. For example, polar fibers are more effective for extraction of polar analytes such as hydrocarbons, acids, alcohols, phenols and aldehydes (Mani, 1999; Matich, 1999).

Various kinds of coating fibers, with differend polarities (polar, bipolar and non-polar) and mechanisms (adsorbent or absorbent), are commercially available for the analysis of volatiles of food products. In this study, three kinds of coating fibers with diverse polarities were used for the analysis of volatiles of chocolate. SPME fibers coated with 50/30 μm DVB/CAR/PDMS; 75 μm CAR/PDMS and 65 μm PDMS/DVB, and the extraction efficiency of fibers were investigated.

In the current study, the milk chocolate samples containing isomalt (1.0 g) were located in the vial (dry conditions) and conditioned for 10 min (without fiber) and 30 min (with fiber) at the temperature 60 °C. Three kinds of fibers were applied to assay their extraction performances. Several components that have been extracted and characterized are; acids, alcohols, esters, hydrocarbons, aldehydes, ketones, furan and pyrazine derivatives, phenols and others (Burbank and Qian, 2005). The SPME fiber coated with DVB/CAR/PDMS obtained the highest extraction performance. As a result of HS-SPME analysis, the key odor-active compounds of chocolate that were extracted and identified were; tetramethylpyrazine, dihyro-2-(3H)-furanone, benzaldehyde, 3-methylbutanal, 2-phenylethanol, octadien-3-ol, d-limonene, iso amyl acetate and phenethyl acetate.

By using this method, more than 50 volatile aromatic components are made through different metabolic pathways and their mass spectra and retention indexes (RI) are shown in the Table 3. The CAR/PDMS (75 μm) and PDMS/DVB (65 μm) fibers seemed to have the similar retention index (RI) however the DVB/CAR/PDMS (50/30 μm) indicated different RI. Using the mentioned fibers, several flavour compounds including; 16 aldehyde, 15 alcohols, 6 ester, 9 acid and 34 other compounds were extracted. Inspection of the total peak area for each fiber indicated that the most efficient fiber is CAR/PDMS, extracting around 3 times more than the PDMS/DVB and 5 times more than the DVB/CAR/PDMS fiber. The CAR/PDMS fiber presented the best and highest extraction efficiency (total peak area <8,000,000) presenting wide peaks (RI <1526) at the beginning of the chromatogram. However some chocolate volatile compounds were not detected on the chromatogram by CAR/PDMS fiber making this fiber inappropriate for the current study.

In our study, a split injection (split 1:1) lead to sharper peaks. The extraction efficiency of the PDMS/DVB fiber (total peak area >2,000,000) was lower than the CAR/PDMS fiber, but the peaks were sharper and a wide chromatogram was obtained. The results from analysis showed that 23 volatile compound were recognized applying the CAR/PDMS fiber and 40 flavour components were extracted by PDMS/DVB fiber.

CAR/PDMS fiber was efficient for the extraction of non-polar or semi-non-polar analytes for example volatile flavour components of chocolate. Different cmponents have been

noticed by SPME extraction as key odorant components in milk chocolate; 2-phenylethanol, 2-ethylhexanol, 2-phenylethyl acetate, isovaleric acid, 2H-Pyran-2-one, 2-decanone, hexadecane, 2,6-dimethyl-3-propylpyrazine and 9 different compounds, including isocoumarin. These compounds were exclusively extracted by PDMS/DVB fiber.

Table 3. Key odorant compounds identified by CAR/PDMS, PDMS/DVB and DVB/CAR/PDMS fiber

| Compound | RI | Peak area | | |
|---|---|---|---|---|
| | | CAR/PDMS | PDMS/DVB | DVB / CAR-PDMS |
| Acetic Acid | 712 | 29.61 | 3.53 | 20.45 |
| 2-Butanone, 3-hydroxy- | 738 | 2.08 | - | - |
| Propanoic acid | 788 | 1.36 | 1.19 | - |
| 2,3-Butanediol | 800 | 7.53 | 5.91 | 4.16 |
| Butanoic acid | 855 | 10.86 | 1.71 | 5.12 |
| Pentanoic acid | 930 | 1.61 | - | |
| Methane, sulfonylbis- | 956 | 3.56 | 1.79 | 1.52 |
| Benzaldehyde | 998 | - | - | 1 |
| Decane, 2,2-dimethyl- | 1030 | - | 2.71 | 4.14 |
| Undecane | 1042 | - | 4.47 | 0.41 |
| Hexanoik asit | 1047 | 6.50 | 1.42 | 3.43 |
| 2,2,4,4,6,8,8 heptamethyl- | 1070 | - | - | 0.89 |
| dl-Limonene | 1072 | - | - | 0.80 |
| 1-Hexanol, 2-ethyl- | 1076 | 4.58 | 2.12 | 2.36 |
| Nonane, 5-(2-methylpropyl)- | 1103 | - | - | 1.16 |
| Ethanone, 1-(1H-pyrrol-2yl)- | 1112 | 2.30 | - | - |
| Methyl heptyl keton | 1141 | - | - | 0.80 |
| 2,3,5,6 tetramethyl pyrazine | 1135 | - | 1.64 | 1.51 |
| Nonanal | 1155 | - | 1.76 | 2.03 |
| Phenethyl alcohol | 1164 | | 1.12 | 1.01 |
| Benzoic acid | 1226 | - | 1.55 | - |
| Caprilic acid | 1236 | 3.17 | 2.83 | 1.85 |
| Phenethyl acetate | 1356 | - | 1.55 | 1.27 |
| Benzaldehyde | 1527 | 2.20 | 31.88 | 30.45 |

The botanical and geographical origin of chocolates and also the technological processing has been associated with chocolate flavour. They can provide useful information in analyzing the volatile compounds. However, the results may vary depending on the extraction procedure. The volatile compounds such as; 3-methylbutanal, 2-phenylethylacetate, amyl acetate which are obtained from the cocoa, tetramethylpyrazine, n-hexanal, n-nonane and benzyl alcohol, phenethyl acetate isoamyl benzoate, methyl nonyl ketone which are obtained and identified from chocolate are extracted using only DVB/CAR/PDMS fiber. Also in the latest studies, various cocoa products and conventional chocolate volatile compounds were investigated by using DVB/CAR/PDMS fiber with different extraction conditions (Braga et al., 2018; Da Viega Moreira et al., 2018; Khairy et al., 2018). Moreover, the DVB/CAR/PDMS fiber presented higher resolution chromatograms in comparison to the other fibers chromatograms (Figure 2).

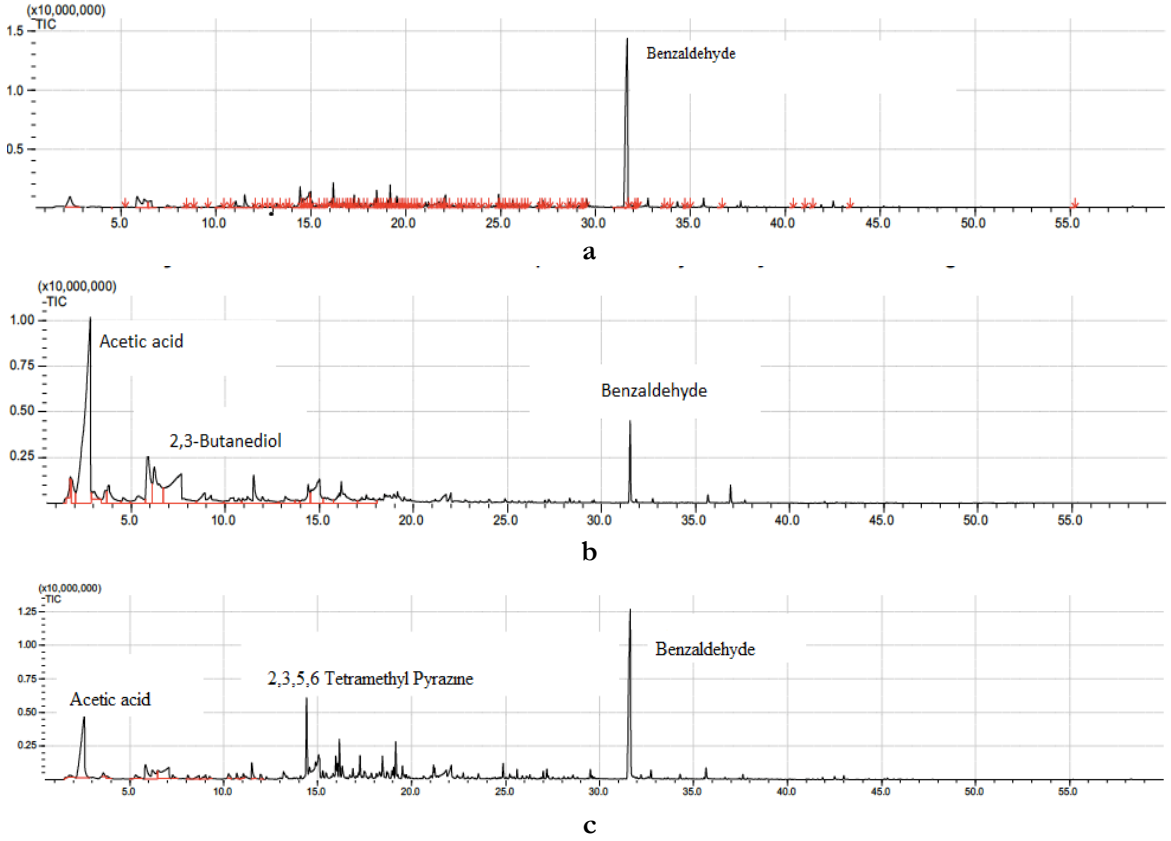



Figure 2. Chromatograms of key compounds extracted from chocolate with (a) PDMS/DVB, (b) CAR/PDMS and (c) Chromatogram of key compounds extracted from chocolate with DVB/CAR/PDMS fiber

**Optimization of HS-SPME parameters**

The extraction efficiency of SPME method can be influenced by temperature, time and equilibrium time as the different experimental conditions as well as the amount and composition of the sample, thickness of the stationary phase etc. (Yang and Perpard, 1994).

In this study, several preliminary methods were performed on condition of SPME to evaluate the influence of one factor whereas others were kept fixed. The criteria for optimization of the isolation of tetramethylpyrazine of milk chocolate containing isomalt were higher number of peaks area of the chromatogram which evaluated possible influence of each parameter by chromatographic analysis. Based on the results of the pre-trial indicated that the optimized SPME analytical method was applied to determine the methylpyrazine levels in the chocolates and the following variables (extraction temperature, extraction time, equilibrium time) were determined.

The experimental data and levels involved in CCD optimization. The responses of tetramethylpyrazine peak area were gained in GC-MS analysis. The degree of freedom, sum of squares; F and *P*-value for lack of fit as well as $R^2$ the coefficients of the model are showed in Table 4. For any of each term in the model was assessed as a function of main, quadratic, interaction effects of independent variables ($X_1$: extraction temperature, $X_2$: extraction Time, $X_3$: equilibrium Time). These effects of a small *P*-value would illustrate a more considerable effect on the respective response variables. The result reported in Table 4 and the model is all statistically meaningful ($P < 0.01$).

Table 4. Analysis of variance and statistical parameters of the model

| Source | DF | Sum of squares | F value | *P* value | $R^2$ |
|---|---|---|---|---|---|
| Model | 9 | 1.17 | 18.26 | 0.0011** | 0.96 |
| $X_1$ | 1 | 0.07 | 2.88 | 0.0281* | |
| $X_2$ | 1 | 0.02 | 0.75 | 0.4829 | |
| $X_3$ | 1 | 0.06 | 2.54 | 0.0439* | |
| $X_1 * X_2$ | 1 | 0.00 | 0.29 | 0.7797 | |
| $X_1 * X_3$ | 1 | 0.20 | 6.81 | 0.0005** | |
| $X_2 * X_3$ | 1 | 0.11 | 3.89 | 0.0081** | |
| $X_1 * X_1$ | 1 | 0.37 | 7.13 | 0.0004** | |
| $X_2 * X_2$ | 1 | 0.63 | 1.22 | 0.2689 | |
| $X_3 * X_3$ | 1 | 0.43 | 7.74 | 0.0002** | |
| Lack of fit | 5 | 0.00 | | 0.3734 | |
| Residual error | 6 | 0.04 | | | |
| C. total | 15 | 1.21 | | | |

$^{**}P < 0.01$ $^{*}P < 0.05$

ANOVA showed that the resulting coefficients of multiple determinations ($R^2$) of 0.96 for the responses of tetramethylpyrazine. The use of $R^2$ showed that the second-order polynomial model makes it beneficial as a measure of success of forecasting the dependent variable from the independent variables (Nagelkerke, 1991). Coefficients of determination for continuous predicted values have been explained that a good fitting regresion model should have $R^2$ higher than 80%. An upper value ($R^2$) indicates that model is appropriate for effects variables (Little and Hills, 1978).

The response surface analysis of data calculated that was obtained from mathematical models was able to describe independent variables is quadratic, linear effect as a function of the three studied variables in the domain of interest. An Eq. (2) polynomial equations show for tetramethylpyrazine, Y, in significant terms is as follow:
Eq. (2).

$$Y = 0.88 - 0.077X_1 + 0.02X_2 - 0.068X_3 - 0.00875\ X_1X_2 - 0.20375\ X_1X_3 - 0.11625\ X_2X_3 - 0.37155\ X_1X_1 - 0.06344\ X_2X_2 + 0.06344\ X_3X_3$$

The influence of independent variables on the tetramethylpyrazine of peak area was then determined by studying the so obtained and validated model (Table 5). Three-dimensional response surface plots indicated how response variable relate to two continious design variables.

**Effect of extraction temperature**

The values illustrated that extraction temperature had a remarkable negative linear effect and negative quadratic effect of extraction temperature ($P < 0.001$: $P < 0.001$). In general, the cause of significantly negative linear affects the extraction temperature of the volatile components for the following reasons:

(a) The vapor pressure of the volatile components and extraction rate can be improved by increasing the extraction temperature. But also, it provides satisfactory sensitivity that should be used. Tetramethylpyrazine showed an increased peak area upon heating to 57°C, above these temperature extraction yields decreased due to its high boiling point. (b) The temperature should not be applied at high temperatures. Extraction efficiency can be increased but may cause undesired reactions and the forming artifacts. In this study, it was determined that the furfural of volatile compounds was formed in a high temperature (80°C). (c) The effect of extraction temperature on the tetramethylpyrazine concentration was determined to have negative effect on the relationship between chocolate and sweetener.

The most significant flavour tetramethylpyrazine was forming in cocoa duruing roasting, drying and fermentation which maillard reactions are the products of heat-induced chemical processes. This compound is precursors in cocoa. The determination of tetramethylpyrazine on chocolate has been carried out using SPME methods. However, in the research heating of the samples, which can produce additional levels of tetramethylpyrazine by interaction between Maillard reactions still present on the matrixes. Therefore, concentrations of this component are reached exaggerated values. In this study, chocolate was manufactured using isomalt and so, no maillard reactions happen during extraction process.

Table 5. Experimental design conditions and results

| Run order | Extraction temperature (°C) | Extraction time (min) | Equilibrium time (min) | Coded value | Tetramethylpyrazine peak area |
|---|---|---|---|---|---|
| 1 | 80 | 40 | 10 | A00 | 0.4 |
| 2 | 80 | 60 | 15 | +++ | 0.45 |
| 3 | 80 | 20 | 5 | +−− | 1 |
| 4 | 60 | 20 | 10 | 0a0 | 0.9 |
| 5 | 60 | 40 | 15 | 00A | 1.25 |
| 6 | 60 | 40 | 5 | 00a | 1.3 |
| 7 | 80 | 60 | 5 | ++− | 1.38 |
| 8 | 40 | 40 | 10 | a00 | 0.6 |
| 9 | 60 | 40 | 10 | 000 | 0.9 |
| 10 | 80 | 20 | 15 | +−+ | 0.8 |
| 11 | 60 | 40 | 10 | 000 | 0.9 |
| 12 | 40 | 20 | 15 | −−+ | 1.2 |
| 13 | 40 | 60 | 5 | −+− | 1 |
| 14 | 60 | 60 | 10 | 0A0 | 0.97 |
| 15 | 40 | 20 | 5 | −−− | 0.85 |
| 16 | 40 | 60 | 15 | −++ | 1.15 |

SPME with GC-MS can be used for analysing tetramethylpyrazine of milk chocolate containing isomalt. Generally, extraction temperature was affected on the matrix composition of raw material. The different chromatographic profiles obtained are related to the chocolate composition, mainly to the cocoa, milk, process and the other ingerdient sources. The similar behaviour was reported by Afoakwa (2008) that the matrix composition and structure of chocolate has an effect on the release of the aroma. The sensory attributes is affected with fat content, tempering process and sweeteners remain unclear.

**Effect of extraction time**

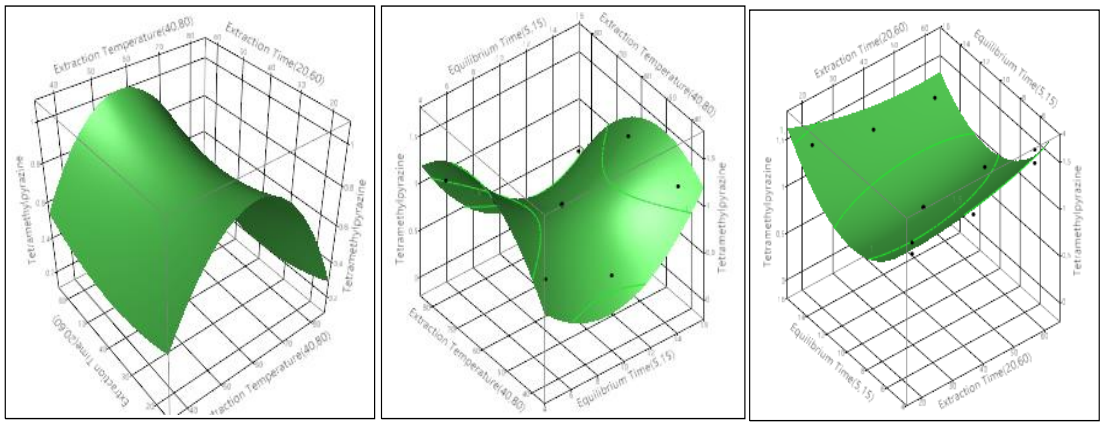
The Responce Surface analysis in Table 4 demostrated that extraction time has no significant effect on extraction of tetramethylpyrazine of milk chocolate containing isomalt. As an independent variable, the equilibration time caused to be insignificant the impact on the extraction time. Because of features such as a porous structure of fiber, the whole level of analytes accumulated on the fiber and this situation limited extraction time. Some research showed that the amount of tetramethylpyrazine extracted depends also on the extraction time, but in some case increasing extraction time would not be affected. The high boiling compounds such as pyrazine overloaded the fiber.

**Effect of equilibrium time**

The extraction of yield of tetramethylpyrazine was found to be function of the negative linear effect and positive quadratic effect of equilibrium time ($P$ <0.05: $P$ <0.001). The equilibrium time for tetramethylpyrazine extraction from chocolate, if convection or agitation or both are stable, the level of analyte extracted is related to time. It is considered finished SPME extraction when the

concentration of volatile components in the headspace reaches the equilibrium distribution between the sample matrix and the fiber coating. Therefore the yield of peak areas were also linearly related to negative interaction between equilibrium time and extraction time ($P$ <0.01) (Table 4). This means that once equilibrium has been reached, the extracted level is stable within the limits of experimental error and it is independent of further increases of extraction time.

The result showed that peak areas were also linearly related to negative interaction between equilibrium time and extraction temperature ($P$ <0.001). The diffusion coefficient increases with increasing temperature and a reduction in the amount extracted and distribution constant for equilibrium times. Figure 3 indicated that the high level of this peak area trend to increase with decreasing extraction time and extraction temperature less than 40 min and 60 ºC. In this study equilibration time of 10 min is sufficient for obtaining higher peak area pyrazine.




(a) (b) (c)

Figure 3. Response surface plots of significant interaction effects of showing the effects (a) extraction temperature and extraction time on tetramethylpyrazine of peak area (b) extraction temperature and equilibrium time on tetramethylpyrazine of peak area (c) extraction time and equilibrium time tetramethylpyrazine of peak area

## CONCLUSION

HS-SPME coupled to GCMS has proven a worth technique for analysing of a large number of volatile and semi-volatile components from chocolate products, present in high and low concentrations. The proposed HS-SPME with GC/MS method may be a useful technique for the optimization of chocolate compounds to better understand the chemical mechanisms of their formation. Differences in the peak area of aromatic compounds are observed due to differences in the composition and production of sugar-free chocolate. Therefore, it is very sensitive to technical and experimental situations; fiber coating, extraction temperature, extraction time, equilibrium time, and sample situations affected the extraction efficiency. As a consequence the DVB/CAR/PDMS fiber afforded the most efficient extraction of both volatile and semi-volatile components from the analyte's headspace.

Isolation of the characteristic tetramethylpyrazine in chocolate samples was determined as the criterion of the peak area and the effect of each variable was evaluated by this component. In this study, the pyrazin equilibrium time of 10 min is sufficient to obtain the highest peak area. In addition, the highest extraction efficiency; It has been determined that the extraction time has reached maximum efficiency with 30 minutes and temperature was 57 ºC. This SPME technique was developed to investigate the extraction conditions of tetramethyl pyrazine which is changed by

chemical reactions in the presence of sweetener. Because there is an increasing interest in determining the chemical alterations in the process leading to the production of chocolate and the standardization of the analysis conditions.

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**CONFLICT OF INTEREST**
The authors express no conflict of interest associated with this work.

**AUTHORS' CONTRIBUTIONS**
SO designed the research. OST, İP, NA, and OS carried out microbiological analyzes of the research and also made statistical analyzes. HRP, NK, OST and SO wrote the paper. All authors contributed to the article and approved the submitted version.

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