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Analytical Methods

Headspace gas chromatography–mass spectrometry determination of alkylpyrazines in cocoa liquor samples

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ABSTRACT

This paper proposes a fast, accurate, and quantitative method for alkylpyrazines determination in cocoa liquors samples using gas chromatography with ion trap mass spectrometry detection (GC/IT-MS) and on-line headspace (HS) sample introduction. The optimization of the experimental conditions of the headspace gas chromatography–mass spectrometry (HS–GC–MS) system was carried out using the univariate method, aiming to find a compromise between time of the analysis, sensitivity and pyrazine resolution in a Carbowax (AV-WAX) fused silica capillary column. The procedure was validated through accuracy and precision studies. The results showed a highly satisfactory accuracy rate for the method with a relative standard deviation (RSD) during 1 day and between days of <5.0% ($n = 5$). On the other hand, the recovery percentages (94–99%) were quantitative in all cases, with a RSD of <4.0% ($n = 5$). Finally, the use the on-line headspace extraction step simultaneously with the analytes separation and detection of the previous sample injection increased the analysis frequency to 3 samples/h. A total of 120 cocoa liquor samples were analyzed.

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1. Introduction

During the last few years the conditions to produce cocoa with a strong aromatic potential have been tried to be optimized, and this is why some researchers point out that the optimization of the cocoa's roasting conditions will allow the maximum use of the bean's aromatic potential. It is well known that aroma precursors in cocoa beans developed in fermentation are converted to cocoa specific aroma during the roasting via the Maillardbrowning reaction (Barel, Leon, & Vincent, 1985). Barel, Gallois and Silwar (Barel et al., 1985; Gallois, 1984; Silwar, 1988), among others, have shown that the principal cocoa aroma compounds are formed after a thermal treatment by non-enzymatic browning reactions between reducing sugars and amino acids. These reactions produce, particularly the pyrazines (Hashim & Chaveron, 1994; Serra Bonvehi & Ventura Coll, 2002). There are many pyrazines in the cocoa aroma, whose concentrations may be variable depending on the time and temperature of the thermal treatment. In order to use these compounds as an indicator of the degree of cocoa roasting it is necessary to count with selective and sensible methods that allow evaluating the pyrazines in cocoa liquor samples. In that sense, most identification and determination methods of pyrazines in foods, for example, cocoa, have been carried out by gas or liquid chromatography (Holm, 1991). In most of them, a sample processing was carried out included a series of extraction steps, where vapor dragging or distillation systems followed by liquid–liquid extractions and more recently solid-phase micro extraction were generally used (Blanch, Tabera, Herraiz, & Reglero, 1993; Counet, Callemien, Ouwerx, & Collin, 2002; Pawliszyn, 1997; Schermann & Schieberle, 1997; Stevenson, Chen, & Mills, 1996). Supercritical fluid extraction (SFE) has proved to be suitable for the extraction of pyrazines and has been used as an experimental set-up for extracting cocoa beans from Ghana and Malaysian cocoa beans (Marsin Sanagi, Hung, & Yasir, 1997). However, in general, the whole sample processing may be tedious, it takes a lot of time and provides low recovery percentages from some of the analytes of interest.

The HS manipulation procedure may be indicated for the isolation and pre-concentration of volatile analytes (Steinhart, Stephan, & Bucking, 2000) from different matrixes. Compared to solvent extraction, equilibrium HS–GC reduces significantly sample preparation time for the analysis of volatile flavor components.

This is why, in order to carry out the alkylpyrazine analysis, in this work a quantitative method using a GC-MS system introducing an on-line sample processing using the HS technique was

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developed. This method offer an interesting alternative for isolation of pyrazines from cocoa samples where the sample manipulation was reduced compared to other related techniques.

2. Experimental

2.1. Materials, chemicals and reagents

HPLC-grade methanol was purchased from J.T. Baker (Phillipsburg, N.J. USA). Double deionized and distilled water was processed through a Milli-Q water system (Millipore, Bedford, MA, USA). Stock methanolic solutions of methylpyrazine; 2,3-, 2,5- and 2,6-dimethylpyrazine; ethylpyrazine; 2,3,5-trimethylpyrazine and 2,3,5,6-tetramethylpyrazine (Aldrich, St. Louis, MO, USA) were used for the quantitative studies. The glass beads were provided by Alltech and they were used to simulate the solid space into the headspace vial when the pyrazines standard solutions were analyzed.

2.2. Samples

The cocoa liquor samples were furnished by the National Institute of Agricultural Research, Experimental Station of Lagunillas, Mérida Venezuela.

The preparation of cocoa liquor involved three processing stages, roasting beans, removing their shells and grinding the nibs into a liquor. All entire procedure was based on the cocoa liquor preparation stages of International Office of Cocoa, Chocolate and Sugar Confectionery (IOCCC) Analytical Method No. 13, 1971.

2.3. Apparatus

Chromatographic analysis on the cocoa extracts and standard pyrazines compounds were carried out with a Varian 3800 gas chromatograph equipped with a Saturn 2000 ion trap mass spectrometer (IT-MS) detector (Varian, California, USA) and a HS-40 headspace sampler (Perkin–Elmer, Norwalk, CT, USA). The headspace (HS) sampler was connected to the gas chromatograph via a heated fused silica transfer line through the split-splitless injector operated in the splitless mode for 0.40 min. Chromatographic separation of analytes was performed on a fused silica capillary column AV-WAX-MS (Carbowax 20 M) (J&W Scientific, 30 m length, 0.25 mm internal diameter I.D. with 0.25 μm film thickness, Folsom, CA, USA). The carrier gas was helium of high purity, 99.9999% supplied by AGA, Maracay, Venezuela. The system was computer-controlled using the Varian Saturn Workstation software.

2.4. Procedure

The operating HS–GC–MS conditions were optimized and are given below. The optimization of these experimental conditions for the determination of alkylpyrazines was carried out with the aim of finding a suitable compromise between peak area, sample throughput, reproducibility and sample amount used per analysis. The extractions were performed inside 22 ml HS sample vials filled with 0.3 g of powdered and fat-reduced cocoa samples and 0.600 ml of methanol, or with 0.3 g of silanized glass bead with 0.600 ml of methanol standard solutions of alkylpirazines. The vials were rapidly sealed with a silicone septum cap, vigorously shaken for 1 min and then thermostated for 15 min at 105 °C. Once equilibrium was reached between the matrix and the gaseous phase, the HS sampling needle descended, pierced the septum cap of the sample vial and it was pressurized with nitrogen at 45 psi for 3 min. Then, the overpressured gas sample in the HS sample vial was applied for 0.08 min (sampling time) onto the GC column through the transfer line maintained at 120 °C. Splitless injection mode and a helium column flow rate of 1 mL min^{-1} were used. Thereafter the GC oven was programmed in order to provide a good separation of the alkylpyrazines. The manifold, trap and transfer line temperatures of the mass spectrometry detector were set at 50, 220 and 220 °C, respectively. The mass spectra were obtained by electronic impact at 70 eV with an electron multiplier voltage established by auto tune at around 1950V (±150 V offset above the adjusted value). The data acquisition in full scan mode was carried out over a mass range of m/z between 40 and 400 at a rate of 1 scan s^{-1}. The emission current of the ionization filament was set at 20 μA generating electrons with

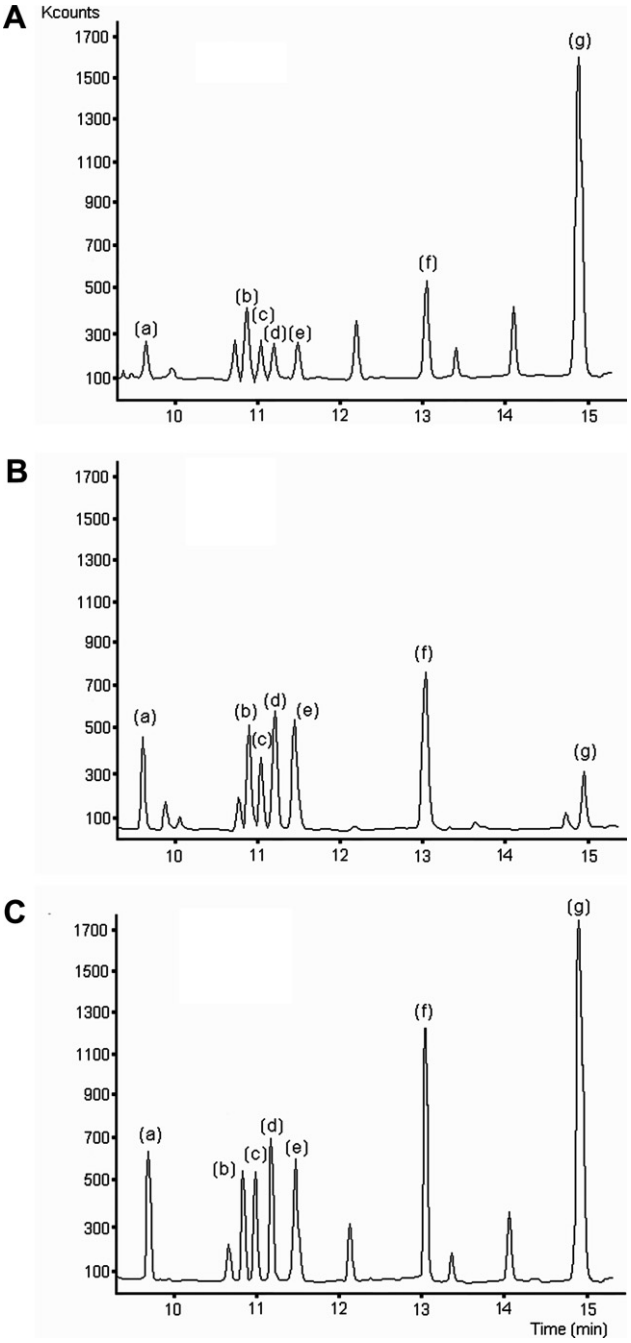

Fig. 1. Chromatograms obtained for: (A) cocoa liquor sample; (B) standard solution of 2-methylpyrazine (a), 2.5-dimethylpyrazine (b), 2.6-dimethylpyrazine (c), ethylpyrazine (d), 2.3-dimethylpyrazine (e), trimethylpyrazine (f), tetramethylpyrazine (g); retention time: 9.57, 10.89, 11.05, 11.34, 11.48, 13.02, 14.93, respectively, at 1.50 μg/mL for each analyte; (C) cocoa liquor sample spiked with alkylpyrazines at 1.50 μg/mL^{-1} for each analyte. Optimal experimental conditions Table 1.

high energy and the axial modulation amplitude voltage was 4.0 V. Peak identification in the GC–IT-MS chromatograms was done by comparing their mass spectra with those contained in the NIST (National Institute of Standards and Technology) library. Confirmation of peak identity was performed after extractions of samples spiked with pure standards. All results from volatile analysis are provided on total area counts.

3. Results and discussion

The optimized variables of the instrumental system were split into three areas: (i) HS extraction; (ii) GC separation and (iii) MS detection. The one-factor-at-a-time method was used to optimize the experimental parameters, while the rest were kept constant. Unless otherwise stated, all the optimization studies were performed in cocoa samples spiked with all alkylpyrazines compounds.

On the other hand, it is important to point out that for the analysis of the methanol standard solutions of alkylpyrazines, in all the cases, we simulate the solid cocoa sample in the HS vial with silanized glass beads of very small diameter. Vials of 22 ml were also used for the whole study because they are those that are part of the HS-40 instrument.

3.1. Optimization of the HS procedure

Static headspace (SHS)–GC is a technique suitable for determining volatile compounds in food samples. The operation of the SHS implies that the solid sample was placed in a sealed vial, heated (thermostating temperature) in an oven during a time period (thermostating time) and the volatile compounds in the solid phase are allowed to reach the equilibrium with the gas phase. The relative concentrations of the analytes in the two phases are determined by the distribution coefficient (K_g), defined as the ratio of the concentration of the analyte in the solid phase to that in the gas phase. An aliquot of the gas is removed and injected into GC.

In that sense, the use of SHS enhances the precision and sensitivity of the analysis when the optimum conditions for the particular analysis have been selected.

For that reason, various conditions for the HS extraction of pyrazines from cocoa samples were tested. We heated the vials from 80 to 110 °C. The results showed that higher thermostating temperature of the sample increases the sensitivity of the analysis. The optimal extraction of the analytes into the headspace was attained at 105 °C. This effect is likely to be due to an enhanced concentration of pyrazines in the gas phase (kinetic effect) owing to a higher partition coefficient of these compounds (Penton, 1992). Nevertheless, the temperature of 105 °C was selected with the purpose of avoiding a cocoa sample toasting process happening during the thermostating time. At higher extraction temperatures Maillard reaction can be performed into the vial, resulting in overestimated values for the concentration of alkylpyrazines (De Oliveira, Pereira, Marsaiolo, & Augusto, 2004; Pini, Brito, García, Valente, & Augusto, 2004). Therefore, for further studies, the vials were thermostated at 105 °C.

The analyte concentration into the HS depends upon the equilibrium occurring between the solid and the gas phases. In order to obtain information on the time required to reach equilibrium, we studied the extraction time between 5 and 25 min at the optimized thermostating temperature. The results indicated that the peak area for all analytes reached a plateau after 15 min which was considered to be optimum time needed to attain equilibrium.

The ratio of the amount of the sample and the gaseous phases in the headspace vial can affect the sensitivity of the headspace determination (Penton, 1992). The effect of different amounts of sample in the HS vial was examined. The results showed that the sensitivity of the analysis improved until an amount of 0.3 g of the cocoa sample. This behavior is due to a concomitant increase of the concentration of the analytes into the HS and a decrease of the HS volume. However for values of samples amount higher than 0.3 g a decrease of the peak areas of analytes was observed. It is possible that great quantities of sample require longer times of extraction to arrive at the extraction equilibrium. Therefore, a 0.3 g of sample was found to be a good compromise between sensitivity, precision and time of analysis.

Addition of NaCl to the extraction media usually augments the extraction efficiency, since for most analytes it causes an increase in their activity coefficients and shifts the extraction equilibrium (Penton, 1992). The magnitude of this effect depends on the salt added and the partition coefficient of the compounds studied. The investigations were performed with vials containing 0.3–1.0 g of sodium chloride. In this case, the responses of most compounds studied in this work to salting out effect are not significantly affected. Beside that for the case of 2-methylpyrazine, trimethylpyrazine and tetramethylpyrazine the peak area decreased with the increase of the amount of NaCl. However, the observed influence was small and because the method's sensitivity for all compounds was very high it was not necessary to add salt, and even without salt addition the method ensures their determination in cocoa matrices

Table 1
Optimized experimental conditions for the determination of alkylpyrazines in cocoa samples

Equipment	Parameters	Value
Headspace	Thermostating temperature	105 °C
	Thermostating time	15.0 min
	Pressurization time	3.0 min
	Injection time	0.08 min
Gas Chromatographer	Injection port temperature	220 °C
	Colum (Carbowax)	30 m × 0.25 mm ID × film 0.25 μm
	Temperature program	50 °C (2.0 min) to 70 °C (10.0 °C/min) to 120 °C (1.0 min) (4.0 °C/min) to 200 °C (1.0 min) to 220 (20 °C/ min) (10 min)
	Column flow	1.0 mL min^{-1}
	Injection mode	Split less (0.40) min
Mass Spectrometer	Ionisation mode	Electrical ionisation
	Ion trap temperature	220 °C
	Manifold temperature	50 °C
	Transferline temperature	220 °C
	Filament current	20 μA

at the desired levels. Therefore in further experiments salts were not added.

In order to transfer the alkylpyrazines present in the HS, the vapor pressure into the vial was not sufficient to permit the analyte to reach the column head and to produce a measurable signal. The best signals were obtained when the vial was pressurized with nitrogen at 45 psi during 3 min.

Finally, the sampling time of the analytes from the HS to the column was varied from 0.04 to 0.1 min. The results indicated that the peak areas of pyrazines increased as the sampling time increased, however, non-reproducible and irregular signals with tailing were obtained for sampling time values higher than 0.08 min. In order to avoid this inconvenience, the capillary head trapping technique could be employed. However, in this case this technique

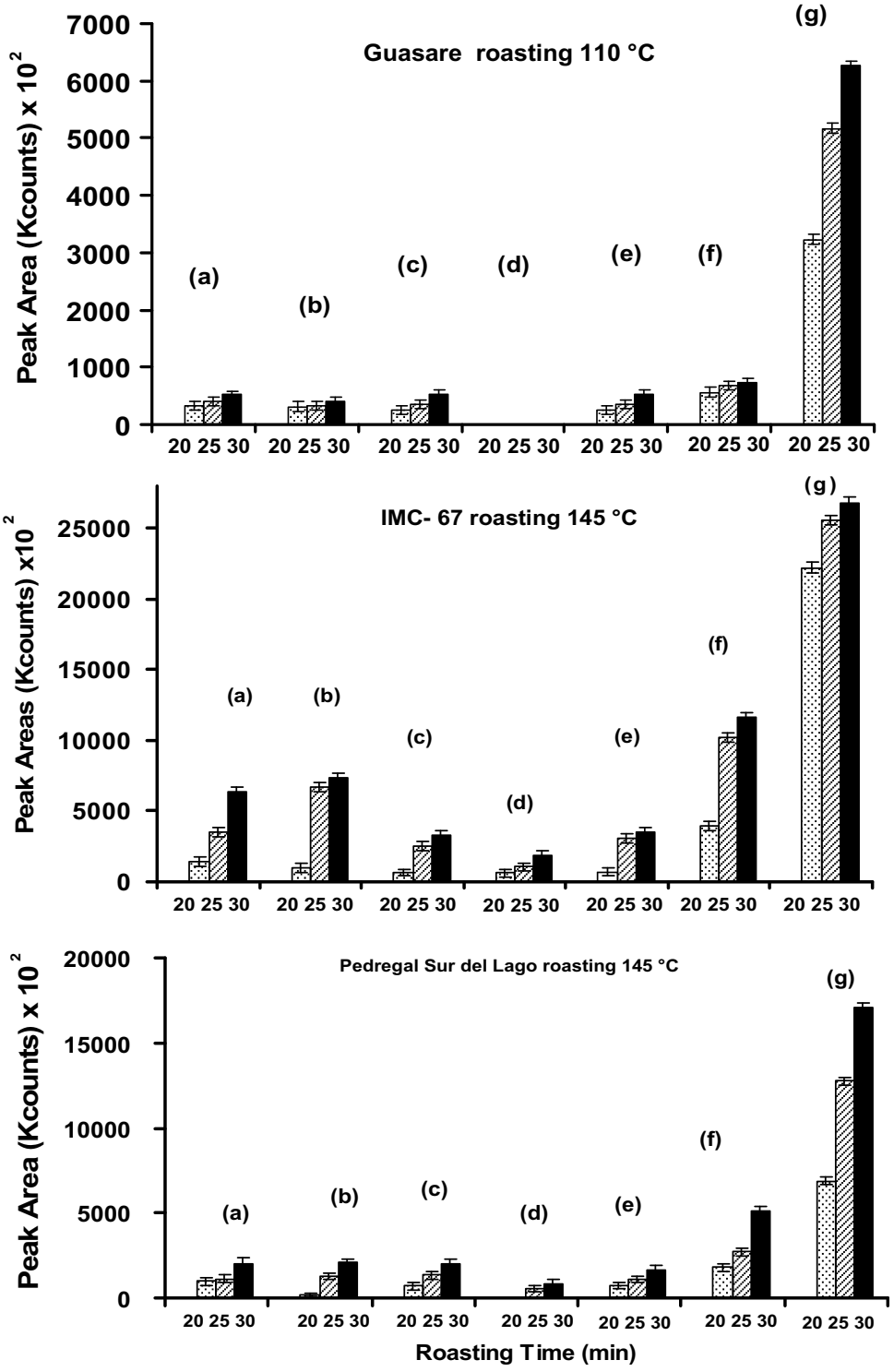

Fig. 2. Graphic representation of peak area as a function of the roasting time of each species of cocoa samples: 2-methylpyrazine (a), 2.5-dimethylpyrazine (b), 2.6-dimethylpyrazine (c) ethylpyrazine (d), 2.3-dimethylpyrazine (e), trimethylpyrazine (f), tetramethylpyrazine (g).

is not necessary due to high levels of the pyrazines in the cocoa samples. Thus, 0.08 min as sampling time was set for all further analysis.

3.2. GC–MS determination

A satisfactory separation of the pyrazine compounds for standard solutions and cocoa samples was achieved on the carbowax fused silica capillary column. The optimized program temperature yielded sharp and symmetrical peaks for all the compounds. Fig. 1 shows the representative chromatograms obtained when the optimized proposed method was used for the analysis of standard methanol solutions and a cocoa sample spiked with the analytes. Comparing these figures, it can be concluded that the separation of alkylpyrazines was completed without the interference of cacao liquor's endogenous compounds and with a good resolution in a reasonable assay time. The absence of the interfering signals reveals the specificity of the method. On the other hand, it is important to point out that the presence of each one of the pyrazines in the cacao samples was confirmed with the obtained mass spectrum for each chromatographic peak. In order to improve peak identification, the fragment ions were monitored for each analyte by selected ion monitoring (SIM) as specified in the following: m/z 39, 67, 94 (ethylpyrazine), 42, 108 (2-methyl and 2,3-dimethylpyrazine), 80,107 (2,5-dimethylpyrazine) 67,108 (2,6-dimethylpyrazine) 42, 61, 122 (trimethylpyrazine) and 54, 136 (tetramethylpyrazine).

The optimal experimental parameters for the determination of alkylpyrazines in cocoa samples are summarized in Table 1.

3.3. Validation of the method

The accuracy of the proposed method was evaluated by means of recovery experiments carried out using spiked samples at different analyte concentrations levels and also a blank constituted by the unspiked sample. In all cases, the recovery percentage values ranged between 89% and 101.6% with a relative standard deviation <3.52% ($n = 5$). These values demonstrate the excellent headspace extraction efficiency just as all the studied analytes were recovered quantitatively from the cocoa matrix.

Calibration curves of alkylpyrazines were obtained for standard pyrazine solutions prepared in methanol and for spiked cocoa liquor samples. Each calibration set included seven data points and each point was run at least three times. The calibration curves were linear over concentration range studied, indicating no significant deviation from linearity (r values > 0.9951). The matrix effects of the samples were determined by comparing the slopes of the calibration graphs obtained with the standard solutions with those of the standard additions. A paired Student t-test indicated that the slopes of the calibration (using standard solutions) and the addition standard graphs were not statistically different ($P < 0.05$). Hence, the standard calibration technique with pyrazines standard solutions could be used for the determination of pyrazines in cocoa liquor samples.

The precision of the method expressed as the relative standard deviation (R.S.D.%) was computed by analyzing five replicates on the same day and between five different days of standard solutions, cocoa liquor samples and cocoa liquor samples spiked with each analyte at four concentration levels (low, middle and high). The R.S.D.% of within-day and between-day values were <4 for all cases confirming the excellent precision of this procedure, and also that by automatic performance of all SHS-GC steps with the purpose sampler temperatures and times can be much better controlled and held constant than by manual performance.

The limits of detection (LOD) and quantification (LOQ) for alkylpyrazines were determined with the 3 s and 10 s criteria, respectively, using 10 injections of a blank. Values less than 0.02 $\mu g\,L^{-1}$ for all pyrazines were obtained.

3.4. Applications

Using the developed method, we analyzed 120 samples of "Criollos", "Trinitarios" and "Forasteros" cocoa liquor samples roasting at different times and temperatures of 110, 130 and 145 °C for the "Criollos", "Trinitarios" and "Forasteros", respectively. The roasting time was varied between 20 and 30 min. Fig. 2, shows the results obtained from each one of the species of cocoa samples where peak area as a function of the roasting time was graphed. As can be seen, the concentration of all methylpyrazines increased in relation with the roasting time for all cases. The principal methylpyrazines were 2,5-dimethyl, trimethyl and tetramethylpyrazines for the three species. On the other hand, the ethylpyrazine is in smaller proportion or is absent as in the case of the Guasare "Criollo" specie. Finally, it is important to signal that these results are in good agreement with those reported by Marsin Sanagi et al. (1997), Jinap, Wan Rosli, Russly and Nordin (1998), Hashim and Chaveron (1994) and Pini et al. (2004) where the tetramethylpirazine is the one that is in greater proportion.

4. Conclusion

In this work a fast, accurate and sensitive method was developed, allowing for the determination of the methylpyrazines in cocoa liquor samples. The method has shown that headspace used for sample preparation technique may be conveniently applied to the cocoa samples to assess pyrazinic concentration useful for the evaluation of sample quality. The detection limits are compatible with the amounts found in the analyzed samples and the precision was adequate.

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