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Method of Determination of Antioxidants, Phthalates and Ultraviolet Absorbers in *Attiéké* "Couscous from Fermented Cassava" Before Its Storage

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Abstract: Antioxidants, phthalates and ultraviolet absorbers are substances used in several domains (cosmetics, plastics, painting...). These substances are known to be endocrine disruptors as for human beings and animals. They are also suspected as carcinogen. It is essential, considering the dangers these products represent, to know their levels of contamination in *attiéké* (a couscous from fermented cassava); which is a staple food in Côte-d'Ivoire. The aim of this work is to validate an assay method for BHT, Irgafos 168, DEP, DIBP, Chimassorb 81 and Chimassorb 944 in *attiéké*. These above-mentioned substances have been extracted from *attiéké* and their quantification was done by a high performance liquid chromatography with a UV detector. The variation coefficients of reproducibility as well as those of repeatability were below 5% for the whole tested data. The limits of quantification were 1, 2.1, 1.1, 0.18, 1.3 and 2.5 mg/Kg respectively for BHT, Irgafos 168, DEP, DIBP, Chimassorb 81 and Chimassorb 944. The average yields of extraction were above 85%. This method deserves a special attention since the BHT, Chimassorb 944 and Chimassorb 81 limits of quantification are clearly below the maximum authorized content. Although the limits of quantification

of Irgafos 168, DEP and DIBP are higher than the accepted limit values, their values are acceptable as compared with those of previous studies.

The analyses done on *attiéké* have detected phthalates (DEP and DIBP) at levels ranging respectively between 1.185 and 2.444 mg/Kg and between 0.18 and 2.1 mg/Kg. The mentioned-above antioxidants and the UV absorbers have not been detected there.

Keywords: Antioxidants, phthalates, UV stabilizers, *attiéké*, validation method, HPLC.

INTRODUCTION

Antioxidants, BHT (2,6-di-tert-butyl-p-cresol) and Irgafos 168 (phosphorous acid, tris (2,4-di-tert-butylphenyl) ester), phthalates, DEP (diethyl phthalate) and DIBP (disobutyl phthalate) and Ultraviolet stabilizers, Chimassorb 81 (2-hydroxy-4-n-octyloxybenzophenone) and Chimassorb 944 (Poly- {6-[1,1,3,3-tetramethylbutyl] -amino] -1,3, 5-triazine-2,4-diyl} {2- (2,2,6,6-tetramethylpiperidyl) -amine}) are endocrine disruptors[1-3] used in several fields such as the industry of cosmetics, plastics, painting... They can unfortunately contaminate *attiéké* during its production process.

Indeed, *attiéké* is a fermented cassava couscous; but it is also a staple food in Côte-d'Ivoire whose annual production is estimated between 18965 and 40000 tons[4]. Its contamination by these substances could therefore have an impact on human and animal health. According to the Directive 10/2011/EC[5], the maximum levels of BHT, Irgafos 168 and Chimassorb 944 are, respectively, 3 mg/Kg, 0.005 mg/Kg and 3 mg/Kg. The maximum levels of DEP and DIBP are 0.01 mg/Kg. The content of Chimassorb 81 in foodstuffs set by the ordinance of the Federal Department of home affairs[6] is 6 mg/Kg. However, most of the dosages of these substances have been validated in the following matrices: plastic polymer, wine and milk[7-9]; but not in *attiéké*. The aim of this work is to validate an assay method for antioxidants, phthalates and UV stabilizers in *attiéké* from the high performance liquid chromatography (HPLC), with an ultraviolet detector in order to know the level of contamination of these substances in *attiéké*.

MATERIAL AND METHODS

The experimental procedures in this work are carried out on the basis of the ASTM D 6953[10], Chengfa *and al*.[8] and Lahimer *and al*.[11] as for the sampling and determination of antioxidants, phthalates and UV stabilizers in *attiéké*.

Study material: The studied material is *attiéké* (couscous from fermented cassava).

Equipment and chemicals : The additives qualitative and quantitative determination is made by a SCHIMADZU high performance liquid chromatography chain, composed of a TRAY tank, a DGU-20A5 degasser, a SIL-20A automatic sampler, a LC-20AT pump, a CTO-20A furnace and a SPD-20A UV-VIS detector. The data collection is done using a computer equipped with LC solution software. The six (6) substances were completely separated using a C18 column (Supelcosil LC 18, 15cm x 4.6mm, 5µm). BHT, DEP and DIBP were obtained from Sigma-Aldrich. The Irgafos 168 and the Chimassorb 944 were supplied by Ciba Specialty Chemicals. The n-hexane was obtained from Merck. The Chimassorb 81 was purchased from TCI America and the acetonitrile for HPLC (Rathbum) and the methanol for HPLC from BDH Laboratory Supplies.

Method

Sampling: The method used for this work is the three-stage cluster sampling with, at the primary level, the three southern cities of Côte-D'ivoire known to be the major producers of *attiéké* (Dabou, Jacqueville and Abidjan). At the secondary level, nine districts of Abidjan (Abobo, Adjamé, Attécoubé, Cocody, Koumassi, Marcory, Port-Bouet, Treichville and Yopougon), three villages in Dabou and three villages in Jacqueville; and at the tertiary level, the production sites. The type 1 *attiéké* (small grains *attiéké*) was collected in three production sites: Dabou, Jacqueville and Aboboté (in the district of Abobo). The type 2 a*ttiéké* (large grain *attiéké*) called *Agbodjama*, was collected in Anono in the district of Cocody, in Anoumambo (in the district of Marcory) and in Abobodoumé (in the district of Yopougon). At each site, about 200 g of *attiéké*, taken at 70°C were packaged in brown glass vials.

Determination of BHT, Irgafos 168, DEP, DIBP, Chimassorb 81 and Chimassorb 944

Preparation of the standard solutions: Ten milligrams of each compound were introduced into 10 mL of methanol to obtain concentration solutions of 1000 mg/L. The standard solutions of each substance were prepared by series of dilution in acetonitrile at 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0, 50.0 and 100.0 mg/L. All of these solutions were stored in brown glass vials at 4°C[7].

Mobile phase: The following table shows the percentages of the A mobile phase (acetonitrile) and the B mobile phase (water) depending on the analyses duration[12].

Table 1: The mobile phases according to the duration of the analyses

Duration (minutes)	acetonitrile	Water
0	65	35
3	65	35
20	95	5
30	95	5
31	65	35
35	65	35

Extraction protocol of BHT, Irgafos 168, DEP, DIBP, Chimassorb 81 and Chimassorb 944 from *attiéké*: The sample preparation is done according to ASTM D 6953[10] with some modifications. Five (5) grams of *attiéké* were weighed into a 125 mL flat bottomed vial in which 50 mL of n-hexane were then added using a pipette. A stirring bar was added to homogenize the mixture. Two grams of sodium chloride were also added to improve the extraction[8]. The mixture was boiled on a combined hot-plate magnetic-stirrer device for one hour in a reflux apparatus. The cooling was done at a room temperature by removing the heating plate and maintaining the reflux device. The mixture was filtered (with a Whatman grade 5 filter paper) under vacuum using a buchner funnel. Ten milliliters of n-hexane were added to the solid residue and then, they were filtered (this operation was repeated three (3) times). The filtrate was concentrated using a rotary evaporator (70°C to 150 rpm)[11]. The volume was reduced to the last drop and this drop was then diluted with 1 mL of acetonitrile and analyzed by HPLC[8].

Chromatographic conditions: The detection was carried with a UV detector of 224 nm wavelength, a flow rate of 1.00 ml/min and the analysis duration was 40 minutes. The whole analyses were performed at 30°C under isocratic conditions and the injection volume was 20 µL.

Method validation

The method validation was done in determining parameters such as the linearity, the precision, the limit of detection, the limit of quantification and the accuracy.

Linearity: It is the ability to have, within an interval, results which are directly proportional to the analyzed substance concentration in a sample. It was tested through a linear regression using the 2018.5 version XLSTAT statistics software.

Precision: The precision of the method was evaluated by calculating the repeatability and the reproducibility. The repeatability was performed with *attiéké* containing 20 mg/Kg of each compound. This concentration was successively injected 10 times on the same day. The concentrations given by HPLC and the corresponding surfaces were noticed. The coefficients of variation for the repeatability, after injection on HPLC for these substances, should be less than 5%. The reproducibility was also performed with *attiéké* containing 20 mg/kg of each compound. The operation was carried out over 10 days. The coefficient of variation (CV) of each parameter was calculated according to Kouakou *and al.*[13].

$CV = \sigma_{n-1} / \overline{X}$; σ_{n-1} is the standard deviation and $\overline{X}$ is the average.

Limits of detection / Limits of quantification: The limit of detection and the limit of quantification of the method are obtained by the signal/noise ratio of 3 and 10 times, respectively from the base line of the blank matrix[14]. The blank matrix was prepared from the peeled cassava tubers, washed with distilled water and ground with a pestle and porcelain mortar. This matrix was treated according to the compounds extraction protocol from *attiéké* as decribed above.

Accuracy: Accuracy consists in calculating the recovery rate in percentage between the resulted and the introduced quantity[15]. This operation permits, for a given type of matrix and a given level of concentration, to identify the presence of potential interference in the analysis process[14]. In the quantifiable area of the method, 10 real samples are analyzed.

Recovery rate (%) = $(C_f - C)x100/C_a$

C_f is the measured concentration of a fortified sample, C is the measured concentration of a non-fortified sample and C_a the concentration of the added substance. In order to determine the recovery rate, each compound was added to ten grams of *attiéké* so as to obtain concentrations of 10, 20 and 40 mg/Kg. The above-illustrated extraction procedure was applied and the concentration of the compound was determined by HPLC. These analyses were repeated 10 times.

Statistical analyses: The descriptive statistics and statistical analyses were performed using the 2018 version XLSTAT statistics software. A linear regression test was used on a Fisher test with 5% of significance level.

2. RESULTS AND DISCUSSION

Results Of Method Validation

Retention time: The chromatogram which is obtained according to the described operating conditions is represented in **figure 1.** This chromatogram clearly distinguishes the retention time of the various compounds. In addition, the injection of n-hexane did not give any peak to the retention time of the desired compounds; it consequently shows the absence of memory effect.

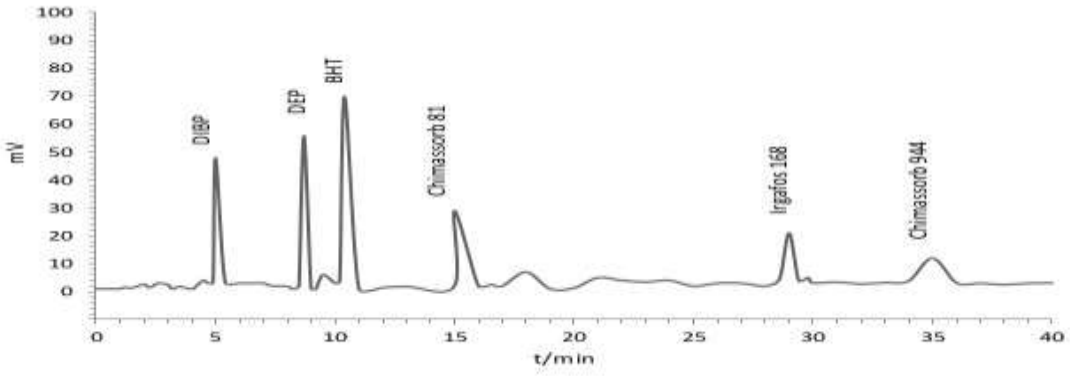


Figure: 1 Chromatogram of the compounds at 10 mg/L

Linearity: The tests of linear regression between the concentrations of the standard solutions and the peak areas, have given the **Table 2** adjustment coefficients. The coefficients of determination (R^2) of these regressions are higher than 0.99 for all the studied compounds. In addition, the assessment of the amount of information supplied by the explanatory variable (peak area) to the models, by the Fischer F test has given 0.0001 as a probability value **(Table 3**). The equations of the model for each compound are shown in **Table 4** and their graphic representations are given in **figures 2, 3, 4, 5, 6 and 7.**

Table 2: Regression coefficients of the linear regression between the concentrations of the standard solutions and the surface associated peak

	Values					
Parameters	**BHT**	**Irgafos198**	**DEP**	**DIBP**	**Chimassorb81**	**Chimassorb944**
R (correlation coefficient)	0.9987	0.9995	0.9996	0.9996	0.9993	0.9992
R ^{2} (Coefficient of determination)	0.9975	0.999	0.9992	0.9992	0.9985	0.9984
R ^{2}aj. (Adjusted coefficient of determination)	0.9972	0.9988	0.9991	0.9991	0.9983	0.9981

Table 3: Tests F realized for the linear regressions

Compounds	**Source**	**DDL**	**Sum of squares**	**Average Square**	**F of Fischer**	**Pr > F**
BHT	Model	1	8567.820	8567.820	2459.831	**<0.0001**
	Margin of error	6	20.899	3.483		
	Total adjusted	7	8588.719			
Irgafos 168	Model	1	8579.940	8579.940	5864.140	**<0.0001**
	Margin of error	6	8.779	1.463		
	Total adjusted	7	8588.719			
DEP	Model	1	8582.243	8582.243	7951.699	**<0.0001**
	Margin of error	6	6.476	1.079		
	Total adjusted	7	8588.719			
	Model	1	8581.795	8581.795	7436.388	**<0.0001**

DIBP	Margin of error	6	6.924	1.154		
	Total adjusted	7	8588.719			
Chimassorb81	Model	1	8576.223	8576.223	4118.013	**<0.0001**
	Margin of error	6	12,496	2.083		
	Total adjusted	7	8588.719			
Chimassorb944	Model	1	8575.009	8575.009	3752.893	**<0.0001**
	Margin of error	6	13.709	2.285		
	Total adjusted	7	8588.719			

Table 4: The model equation and the linearity domain of the compounds

Compounds	Equation of the model (y is the concentration in mg/L and x the peak area in area unit)	Range of linearity (mg/L)
BHT	y = 0.822699596011649 + 1.19592678718014E-02 * x	1.0 - 100.0
Irgafos 168	y = -0.490609398053458 + 1.81395998477025E-02 * x	2.0 - 100.0
DEP	y = 0,377747863732544 + 2,46646299321994E-02 * x	0.2 - 100.0
DIBP	y = -0.963896971408552 + 1.65718898455463E-02 * x	0.1 - 100.0
Chimassorb 81	y = -0.242388605780558 + 1.40142106737983E-02 * x	1.0 - 100.0
Chimassorb 944	y = -0,241252050458893 + 5.27974981711409E-02 * x	2.0 - 100.0

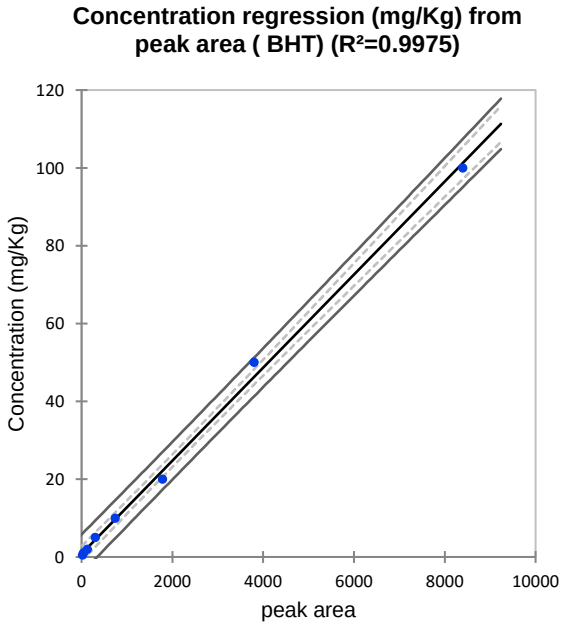


Figure 2: Linear calibration curve of regression, detector result according to the peak area (BHT)

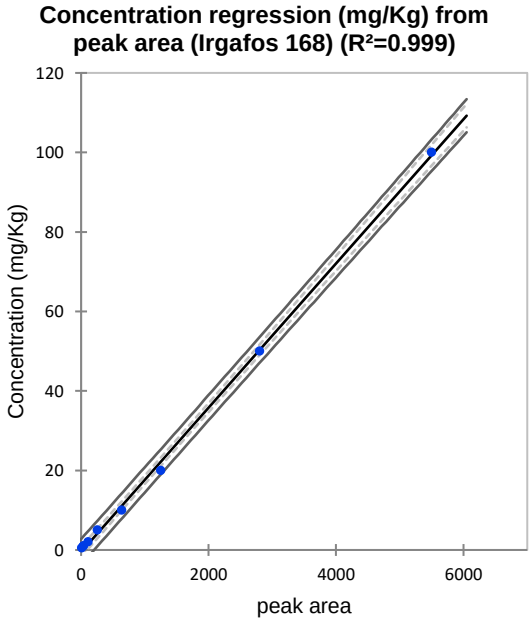


Figure 3: Linear calibration curve of regression, detector result according to the peak area (Irgafos 168)

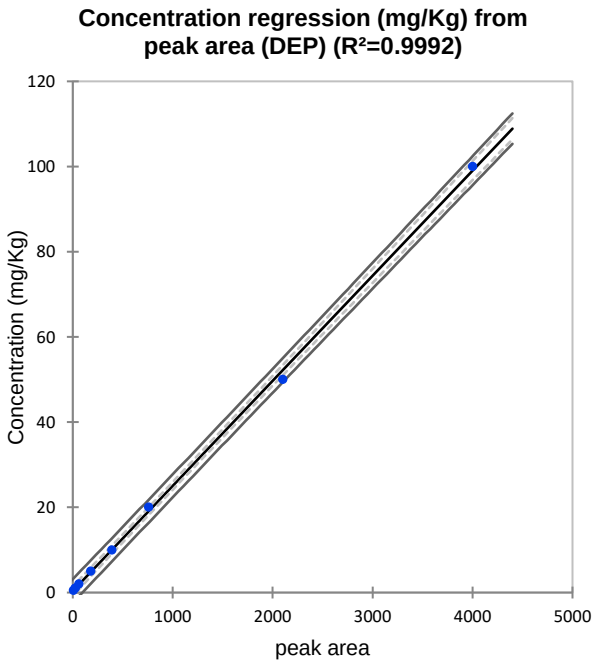


Figure 4: Linear calibration curve of regression, detector result according to the peak area (DEP)

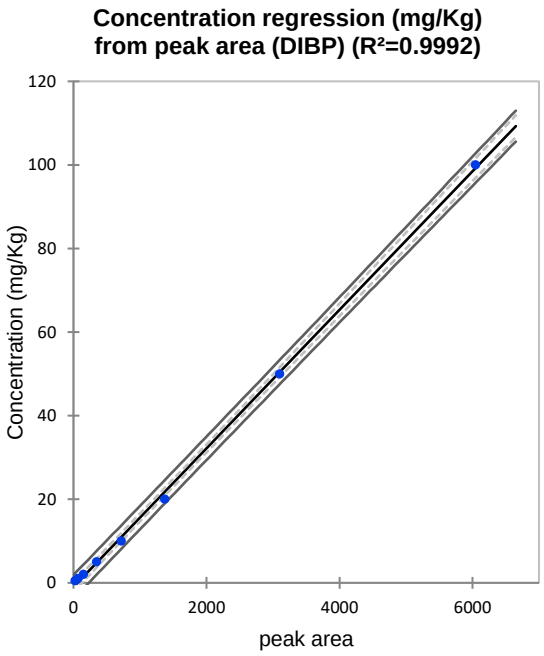


Figure 5: Linear calibration curve of regression, detector result according to the peak area (DIBP)

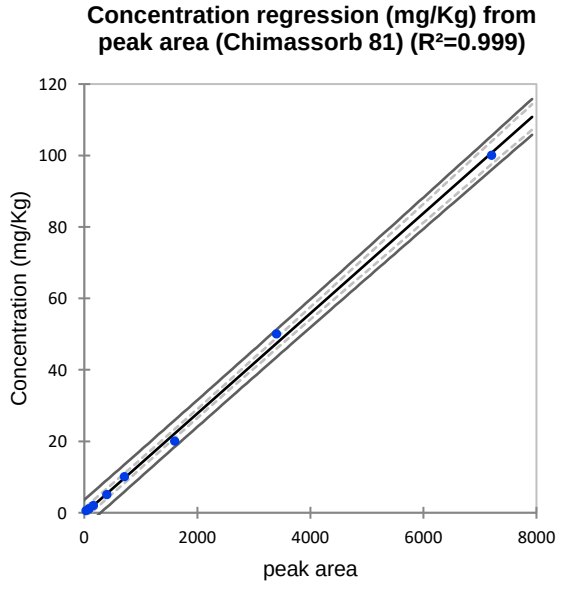


Figure 6: Linear calibration curve of regression, detector result according to the peak area (Chimassorb 81)

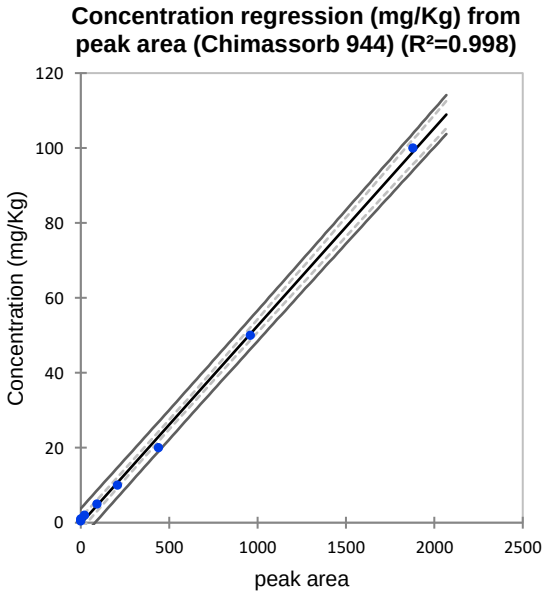
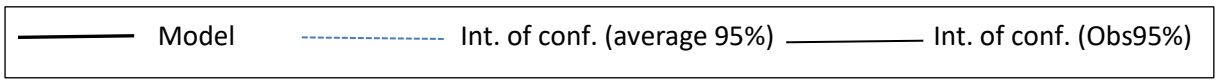


Figure 7: Linear calibration curve of regression, detector result according to the peak area (Chimassorb 944)



Precision, limit of detection / limit of quantification: The coefficients of variation of repeatability vary from 2.73 to 4.23% and those of the reproducibility from 2.24 to 4.93% (see **Tables 5** and **6**).

The detection limits are between 0.06 and 0.82 mg/Kg and the quantification limits between 0.18 and 2.5 mg/Kg. These results are shown in **Table 7**.

Table 5: Repeatability of the same sample of *attiéké* containing 20 mg/Kg of each compound

Compounds	BHT	Irgafos 168	DEP	DIBP	Chimassorb 81	Chimassorb 944
Concentration (mg/Kg)	19.75	20.69	19.69	19.57	21.06	18.96
	21.3	21.36	19.89	20.13	20.37	18.97
	19.45	19.67	20.88	21.54	19.94	19.68
	18.97	20.33	21.1	19.21	19.35	20.19
	20.47	19.57	19.21	19.87	20.67	21.34
	19.87	19.67	19.66	19.37	19.22	19.89
	19.67	20.31	19.97	20.68	18.99	20.13
	20.97	20.06	20.1	19.47	20.9	21.55
	21.3	19.88	21.34	19.86	19.36	19.88
	20.14	19.96	19.98	20.12	20.11	20.03
Coefficient of Variation (%)	3.96	2.73	3.42	3.49	3.72	4.23

Table 6: Reproducibility of the same sample of *attiéké* containing 20 mg/Kg of each compound

Compounds	BHT	Irgafos 168	DEP	DIBP	Chimassorb 81	Chimassorb 944
Concentration (mg/Kg)	20.3	20.1	19.67	19.64	20.03	19.8
	19.75	19.4	21.3	21.34	19.7	19.57
	19.23	20.9	18.97	22.1	19.62	19.687
	20.38	18.76	21.34	19.37	20.13	18.67
	21.1	19.7	21.5	21.1	21.1	19.92
	19.01	20.4	19.42	19.24	19.2	19.64
	18.9	21.5	19.75	19.3	20.3	18.76
	20.3	19.34	20.14	20.01	19.62	19.36
	19.51	20.87	18.86	19.8	19.75	19.33
	22.1	20.2	20.76	20.2	20.45	19
Coefficient of Variation (%)	4.98	4.16	4.93	4.85	2.68	2.24

Table 7: Limit of detection (LOD) and limit of detection (LOQ) of the compounds in *attiéké*

Compounds	LOD (mg/Kg)	LOQ (mg/Kg)
BHT	0.32	1
Irgafos 168	0.7	2.1
DEP	0.35	1.1
DIBP	0.06	0.18
Chimassorb 81	0.43	1.3
Chimassorb 944	0.82	2.5

Accuracy: The average recovery rate of each compound in *attiéké* as well as their coefficient of variation (expressed in percentage) are shown in the table below. The recovery rates are generally high as for the concentrations of 40 mg/Kg and low for the concentrations of 10 mg/Kg. They generally vary between 85 and 96% and the coefficients of variation between 0.9 and 8.25% (see **Table 8).**

Table 8: Recovery rate (%) of the compounds and coefficient of variation (%)

	Added concentration	10 mg/Kg	20 mg/Kg	40 mg/Kg
Recovery rate (Coefficient of variation) (%)	BHT	90.7 (7.3)	89.3 (4.1)	92.1 (3.8)
	Irgafos 168	92.5 (8.25)	93.4 (7.21)	94.2 (6.75)
	DEP	96.1 (1.4)	93.4 (5.7)	95.3 (3.4)
	DIBP	93.7 (2.2)	94.6 (0.9)	95.3 (1.6)
	Chimassorb 81	89.6 (5.4)	91.7 (6.7)	92.9 (3.4)
	Chimassorb 944	87.2 (2.1)	89.8 (3.7)	91.8 (2.8)

Analyses of Antioxidants, Phthalates and UV Absorbers in *Attiéké*: The DEP content is higher in Aboboté and Abobodoumé while, in the four (4) other sites, their contents are substantially identical. High levels of DIBP were observed in the *attiéké* from Abobodoumé, Aboboté and Anoumambo and low levels from those of Jacqueville, Dabou and Anono. The analysed antioxidants and UV stabilizers were not detected. All these results are reported in **Table 9**.

Table 9: Content of the analyzed compounds in *attiéké* **(**nd = not detected)

	Concentration of compound ± standard deviation (mg/Kg)					
Additives	**DABOU**	**JACQUEVILLE**	**ABOBOTE**	**ANONO**	**ABOBODOUME**	**ANOUMAMBO**
BHT	nd	nd	nd	nd	nd	nd
Irgafos 168	nd	nd	nd	nd	nd	nd
DEP	1.185±0.167	1.265 ± 0.146	2.444±0.466	1.5±0.209	1.79±0.147	1.4 ± 0.221
DIBP	0.97 ± 0.217	0.18±0.03	1.678 ± 0.2	0.766 ± 0.092	2.1 ± 0.61	1.92 ± 0.32
Chimassorb 81	nd	nd	nd	nd	nd	nd
Chimassorb944	nd	nd	nd	nd	nd	nd

DISCUSSION

Retention time: The compounds were determined by comparison with the retention time corresponding to the peak of the standard solutions. This method is selective because the chromatogram in **figure 1** clearly distinguishes the retention times of each compound. Several authors have proved the selectivity of these substances by the HPLC method[7,8,11].

Linearity: The coefficient of determination (R^2), higher than 0.99, means that more than 99% of the concentration variability of the studied compounds is explained by the area of the peak. This is a good coefficient of determination since it is very close to one (1). The coefficient of determination R^2 with a value higher than 0.99 complies with the values of Bo *and al.*[7]; determined in plastic packaging, Basma *and al.*[9] in infant milk and Grinbaum[15] in wine. Since the probability associated with the test F (0.0001) for all the compounds is less than 5% (tolerance threshold), we draw the conclusion that the peak area brings a significant amount of information to the model. The probability of being wrong using

this model is only 0.01%. The linearity range found is from 1 to 100 mg/L for BHT and Chimassorb 81 and from 2 to 100 mg/L for Irgafos 168. On the other, Chengfa *and al*.[8] have found a linearity range from 1 to 50.15mg/L for BHT, 0.99 to 98.78 mg/L for irgafos 168 and 1 to 49.78 mg/L for the Chimassorb 81. Thus, the interval of linearity is large and gives more possibilities of analysis.

Precision, Limit of detection / limit of quantification: The coefficients of variation of repeatability and reproducibility are below 5% for all the studied results. So these results show that the proposed method is reliable. The results are in accordance with some authors who have worked with different matrices. Hadjmohammadi *and al*.[17] have found a coefficient of variation of 4.2% for BHT in edible vegetable oils. Bo *and al*.[7] have identified coefficients of variations lower than 5% for the repeatability and the reproducibility with BHT, DEP, DIBP and Chimassorb 81 in plastic food packaging. The limits of detection determined in wine, milk and vegetable oils are between 0.004 and 2.7 mg/L and the quantification limits between 0.01 and 9 mg/L in studies conducted by Grinbaum[15], Basma *and al*.[9], Hadjimohammadi *and al*.[17] and Bo *and al*.[7].

Accuracy: The recovery rates of the compounds and the coefficients of variation of BHT, Irgafos 168, DEP, DIBP, Chimassorb 81 and Chimassorb 944 are shown in **Table 8**.

Despite the differences in the studied matrices, the recovery rates of the compounds are not far from those of some authors. Chengfa *and al*.[8] have found a recovery rate between 88.43 and 115.28%. As for Hadjmohammadi *and al*.[17], they have got rates higher than 92% in edible vegetable oils of sunflowers, soybeans and olives. The higher recovery rates found by some of these authors are due to the fact that they have worked on oil as a substrate. Indeed, the studied compounds are more soluble in lipids. On the other hand, *attiéké* is a staple food with low lipid levels (0.64%) and very rich in carbohydrates (96.10%)[18].

Quantification of antioxidants, phthalates and UV absorbers in *attiéké*: The HPLC analysis detected neither antioxidants (BHT and Irgafos 168), nor UV stabilizer (Chimassorb 81 and Chimassorb 944) in *attiéké*. These synthetic substances can only be found in *attiéké* if the latter is in contact with these contaminants. However, DEP and DIBP phthalates contents were determined. In fact, although phthalates are synthetic, they are used in several domains (cosmetics, plastics, painting, inks, drugs, school materials ...). As a result, they have become ubiquitous compounds. The contamination was certainly made at some stages of the *attiéké* preparation process such as the two or three days conservation in jute bags, the spinning which is done in nylon bags, the drying on plastic bags...[18]. These materials generally contain phthalates used as plasticizers during their production and are likely to migrate into food[19].

The identified phthalates can be harmful to human beings and animals. Metabolite of DIBP, the mono-isobutyl phthalate (MIBP) was significantly and positively correlated in women who have given birth by caesarean section[20]. Kwack *and al*.[21] have observed a significant decrease in sperm rate or mobility in male rats exposed to MEP (mono-ethyl phthalate) which is a metabolite of DEP.

CONCLUSION

The method used in this work has permitted to identify and quantify antioxidants (BHT and Irgafos 168), phthalates (DEP and DIBP) and UV stabilizers (Chimassorb 81 and Chimassorb 944) with a recovery rate higher than 85 %. The limits of detection and quantification of this method are respectively between 0.06 and 0.82 mg/Kg and between 0.18 and 2.5 mg/Kg. The results reveal that this method can be used to determine these contaminants in *attiéké*. Antioxidants (BHT and Irgafos 168) and UV stabilizer (Chimassorb 81 and Chimassorb 944) were not detected in crude *attiéké* (before packaging) by high performance liquid chromatography. However, phthalates (DEP and DIBP) have been identified and quantified in *attiéké* because they are ubiquitous. The resulted values are far above the maximum

levels allowed by the European Union regulation. Given the high consumption of this food, there could be a public health problem.

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