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Simultatenous determination of diacetyl and acetoin in traditional turkish butter stored in sheep's rumen (Karinyagi)

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SUMMARY: Commercial Karinyagi (traditionally named karin) is made of cows' milk cream and is produced by filling butter in cleaned sheep's rumen. The effect of butter storage in sheep's rumen on the production of diacetyl and acetoin was investigated. These compounds were determined by GC-MS and they are the typical butter flavor commonly found in fermented dairy products. The modified method for the simultaneous extraction of diacetly and acetoin from butter samples was accurate and precise. The recoveries of diacetyl and acetoin were 94.7 and 110.8%, respectively, while the detection limits were 1.83 and 0.51 $mg \cdot L^{-1}$, respectively. The proposed method was applied for the monitoring of aroma compounds in Karin butter samples during different time intervals. The concentration of acetoin remained stable through 0–50 days while the concentration of diacetyl increased to 33.0 $\mu g \cdot g^{-1}$ up to 40 days and remained constant through 40–50 days.

KEYWORDS: *Acetoin; Butter; Diacetyl; GC/MS*

RESUMEN: ***Determinación simultánea de diacetilo y acetoína en mantequilla tradicional turca (Karin) almacenada en el rumen de oveja.*** El Karinyagi comercial (nombre tradicional Karin) está hecho de crema de leche de vaca, y producido llenando con mantequilla el rumen limpio de ovejas. Se ha investigado el efecto del almacenamiento de la mantequilla en el rumen de ovejas sobre la formación de diacetilo y acetoína. Estos compuestos son el típico flavor a mantequilla que se detecta comúnmente en los productos lácteos fermentados y han sido determinados mediante GC-MS. El método modificado para la extracción simultánea de diacetilo y acetoína en mantequilla resultó ser exacto y preciso y las recuperaciones de 94,7 y 110,8 % respectivamente, mientras que los límites de detección fueron 1.83 y 0,51 $mg \cdot L^{-1}$, respectivamente. El método propuesto se aplicó al control de compuestos aromáticos en Karin y muestras de mantequilla, durante diferentes intervalos de tiempo. La concentración de acetoína se mantuvo estable entre 0–50 días mientras que la concentración de diacetilo aumentó a 33,0 $mg \cdot g^{-1}$ hasta 40 días y se mantuvo constante entre 40–50 días.

PALABRAS CLAVE: *Acetoína; Diacetil; GC/MS; Mantequilla*

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1. INTRODUCTION

The production and consumption of fermented milk products have been increasing throughout the world (Macciola *et al*., 2008). In Turkey, butter is manufactured from two different fermented milk products, cream and yoghurt. Butters have been produced industrially or traditionally by two different methods ("yayik butter" and "karin butter") for centuries in Turkey. Industrial butter is packed with aluminum foil, plastic or paper wrap and stored, whereas Karin butter is stored in a goat or sheep's rumen as a traditional way in the Mediterranean and Aegean region of Turkey. The use of the rumen of sheep and goat allows air and water vapor permeability during the storage of dairy products such as butter and cheese, and is one of the oldest traditional preservation methods.

Karin butter is produced from the cream obtained from the milk cream of cows. For this aim, creams containing 70 to 80% fat are kneaded in a vessel with handles to remove any components except for fat. During the kneading process, the brine formed is discharged from the system by a discharging canal at the bottom of the vessel. After the brine is removed from the structure to a certain ratio, the butter in the vessel is washed with tap water at 18–20 °C. The butter is salted so that the final product contains 2% salt after washed at least three times until the brine becomes clear. Kneading is continued for some time to obtain a homogenized dispersion of the salt. When the kneading operation is complete, the butter is left in cold storage for a night in blocks of 10 kg. The next day, the butter is kneaded again to separate the water remaining in the structure as far as possible, to homogenize the added salt and to obtain a certain consistency for the filling operation. Water in the structure is removed by passing the kneaded butter through a spiral press before filling. The butter separated from its water is carefully filled into the moistened karin without allowing any air pockets to form. The butter filled into karin (rumen) is put on the market after it is stored in cold storage (6–10 °C) for at least 15 days. The storage period of karın butter does not usually exceed 3 months (Gökçe *et al*., 2010).

The fact that the butter packaged in this way is more aromatic than butters packed in a commercial way has been confirmed by consumers (Adam, 1971; Sağdiç *et al*., 2002; Seçkin *et al*., 2005; Gökçe *et al*., 2010; Gun and Simsek, 2011). There have been numerous articles dealing with the function of lactic acid bacteria in the improvement of flavor compounds in dairy products. In cultured butter, the main flavor compounds are usually assumed to be diacetyl and acetaldehyde with an acidic background provided largely by lactic acid (Green and Manning, 1982; Seitz, 1990; Brito, 1990; Urbach, 1995; Escamilla-Hurtado *et al.*, 2005).

Diacetyl is a major flavor component of several fermented dairy products including cultured butter which is the final product of citrate metabolism by certain lactic acid bacteria such as *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* and *Leuconostoc*. Diacetyl is produced from the spontaneous oxidative decarboxylation of α-acetolactate which can also be transformed into acetoin by α-acetolactate decarboxylase or by spontaneous non-oxidative decarboxylation (Aymes *et al.*, 1999; de Vos and Hugenholtz, 2004; Leroy and De Vuyst, 2004; Singh *et al.*, 2006; Quintans *et al*., 2008; Mallia *et al*., 2008).

The determination of diacetyl and acetoin by standard methods typically involves the use of preconcentration by vacuum distillation, liquid–liquid extraction, solid phase extraction and more recently, purge and trap techniques (Frank *et al.*, 2004). Recently, diacetyl in milk products has often been determined by a headspace technique coupled with gas chromatography, using flame-ionization or mass spectrometer detectors (Adahchour *et al.*, 1999; Frank *et al*., 2004; Bartowsky and Henschke, 2004; Panseri *et al.*, 2011).

Several techniques such high-pressure liquid chromatography (HPLC), spectrophotometric, fluorometric and capillary zone electrophoresis have been reported for the determination of diacetyl and acetoin (Bartowsky and Henschke, 2004; Ligon *et al*., 2008).

To the best of our knowledge, there are no reports on diacetyl and acetoin concentration in karin butter produced in a traditional way. Sheep rumen is used as packing material for karin butter protection which probably has some affects on the chemical and biochemical properties of the product such as the synthesis of diacetyl processes and increases in the formation of diacetyl.

In this study, the effect of butter storage in sheep's rumen on the productions of diacetyl and acetoin was investigated. These compounds were determined and monitored by GC-MS. The effect of storage time on their concentrations was also investigated.

2. EXPERIMENTAL

2.1. Chemicals and samples

Ultra-pure quality water (resistivity 18.2 $M\Omega \cdot cm^{-1}$) obtained with a reverse osmosis system (Human Corp., Seoul, Korea) was used for cleaning and other related solutions in all experiments. Aceton and n-hexane for the GC-MS analysis were obtain from Sigma-Aldrich (France, >99.9% HPLC grade) for the extraction and dilution solvent. Diacetyl (2,3-butanedione), acetoin (3-hydroxy-2-butanone) and 2,3-pentadione as internal Standard (IS) were purchased from Alfa aesar (Karlsruhe, Germany) and Merck (Holenbrunn, Germany), respectively. Stock standard solutions of 1 $mg \cdot mL^{-1}$ were prepared by diluting aceton:n-hexane (1:1, v/v) and then storing at 4 °C. Fresh working solutions were prepared by appropriate dilution with aceton:n-hexane.

Five butter samples were purchased from traditional local markets in Denizli-Turkey. All the samples were used in the wet form without any treatment.

2.2. Instrument and chromatographic conditions

Chromatographic analyses of aromatic compounds were performed using a split/splitless injector system (AOC 20s auto sampler with AOC 20i auto injector) gas chromatograph (Shimadzu GC 2010) coupled with a mass spectrometer (Shimadzu QP 2010). Ultra-pure helium was used as the carrier gas with a flow rate of 1.00 mL·min^{-1}. The injection port was worked at 250 °C in the splitless mode with 1 min splitless time. A 1 µL injection volume was used for each analysis and the syringe was washed with hexane after each injection. Separation was carried out by DB-WAX (60 m x 0.25 mm I.D. capillary column with a 0.15 µm stationary film thickness) purchased from Agilent J&W (USA). The oven temperature programme was as follows: initial temperature 40 °C, increased by 7 °C·min^{-1} to 200 °C and held at this temperature for 1 min.

Mass spectrometric parameters were set as follows: electron impact ionization with 70 eV energy and 250 °C ion source and interface temperature. The MS system was routinely set in selective ion monitoring (SIM) mode with a solvent delay of 6.5 min. All aromatic compounds were identified by their retention times on the chromatogram and their specific m/z on the mass spectrum (Table 1). The quantification was based on peak area using one target and one at three qualifier ion (s). The dwell times were set depending on the number of ions per group and the peak widths of the analytes.

2.3. Extraction of diacetyl and acetoin from butter samples

The determination of diacetyl using the liquid-liquid extraction procedure with acetone as a solvent was reported by Macciola *et al.*, (2008). The same procedure for the simultaneous extraction of diacetyl and acetoin from butter samples was used in this study. 2.0 g of butter sample were transferred in eppendorf (15 mL) polyethylene tube. The butter was melted for 5.0 min at 40 °C. Then, 0.1 g $MgSO_4$ was added to the melted butter sample, and spiked with 20 µg of internal standard (2,3-pentadione). The tube was shaken for 30 s at 1600 rpm by vortex (ZX Classic; Velp Scientifica, Usmate, Italy) for homogenization. For the extraction, 2 mL of acetone were added. The mixture solution was rapidly shaken for 30 s at 1600 rpm by vortex. After centrifugation (EBA 20 centrifuge; Hettich, Tuttlingen, Germany) of the mixture solution for 5 min at 5000 rpm the upper phase was filtered through a 0.20 µm disposable syringe membrane filter (Sartorius AG, Göttingen, Gremany). The upper phase was transferred to a small sample vial using a micro-syringe. From the auto-sampler, 1.0 µL of the upper phase was injected into the GC. The final concentrations of diacetyl and acetoin were calculated by the calibration method (Table 2).

3. RESULTS AND DISCUSSION

3.1 Analysis of analytes by GC-MS

Acetone of reagent grade quality was used as a solvent to dissolve diacetyl and acetoin, together. Acetone showed itself to be an optimal solvent

Table 1. Retention times and m/z values of diacetyl, acetoin and internal standard compounds

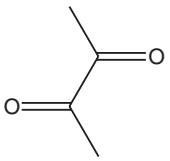
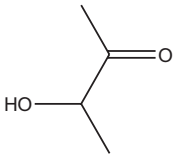
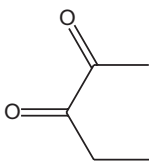
Molecular name	MW	Structure	Retention time (min)	Fragment ions (m/z) for SIM
Diacetyl (2,3-butanedione) ($C_4H_6O_2$)	86.09		7.13	44, 86, 87
Acetoin (3-hydroxy-2-butanone) ($C_4H_8O_2$)	88.11		10.78	43, 45, 88
2,3-pentadione (I.S) ($C_5H_8O_2$)	100.12		12.24	29, 57, 100

Table 2. Figure of merits and validation of the method

Compound	Regression equations (R2)	Regression equations with internal standard (R2)	LOD µg·g^{-1}	LOQ µg·g^{-1}	Added µg·g^{-1}	Founded µg·g^{-1}	RSD %	Recovery (R%)
					–	7.2±0.7	9.7	–
Diacetyl	Y=39003.3x–40369.5	Y=0.1415x+1.38	1.83	5.55	30	35.6±3.6	10.1	94.7
	(R^2=0.9983)	(R^2=0.9998)			60	63.8±8.7	13.6	94.3
					90	98.7±9.6	9.7	101.7
					–	3.2±0.2	6.2	–
Acetoin	Y=23183.5x–4711.9	Y=0.1020x+0.99	0.51	1.53	30	36.4±2.5	7.0	110.7
	(R^2=0.9983)	(R^2=0.9966)			60	57.6±7.4	12.8	90.7
					90	91.2±6.4	7.0	97.8

LOD: Limit of detection, LOQ: Limit of quantification.

for each compound, because the three compounds have a similar chemical structure, being ketones. According to Macciola *et al*., (2008) acetone also has a significant advantage in instantly precipitating milk proteins and colloids.

We employed the method of Macciola for the extraction from karin butter samples. We modified the method for diacetyl and acetion extraction. Butter contains of maximum 16% water and 90% milk-butter. Because of the presence of water in butter samples, we added $MgSO_4$ in range of 0.1–0.5 g for the adsorption of water and the supernatant became clearer. The acetone phase was decreased with the increase in the amount of $MgSO_4$. The optimum amount of $MgSO_4$ was found to be 0.1 g. After the centrifugation of the acetone-butter-$MgSO_4$, fat, water phase and $MgSO_4$ were well separated on the bottom of the tube and the acetone (upper phase) became clear. After the filtration with 0.20-µm disposable syringe membrane filter, it was directly injected into GC-MS for analysis.

As shown in the GC-MS chart (Figure 1), diacetyl, acetoin and 2,3-pentanodione (I.S) were detected at the retention times of 7.13, 10.78 and 12.24 min, respectively. Diacetyl and acetoin were identified by their specific charge to mass ratio (m/z) values in MS (Table 1). The quantification was based on the peak area using one target and two qualifier ions.

3.2. Validation of the GC-MS method

The corresponding calibration equation, correlation of determination (R^2) value, limits of detection (LODs), limits of quantification (LOQs) and recovery were calculated under the optimum conditions and the results are discussed in this section.

The liquid-liquid extraction (LLE) method allowed for the determination of the examined two compounds in the concentration range of 0.2–100 mg·L^{-1} by GC-MS with and without internal standard. An internal standard is a chemical substance that is added in a constant amount to

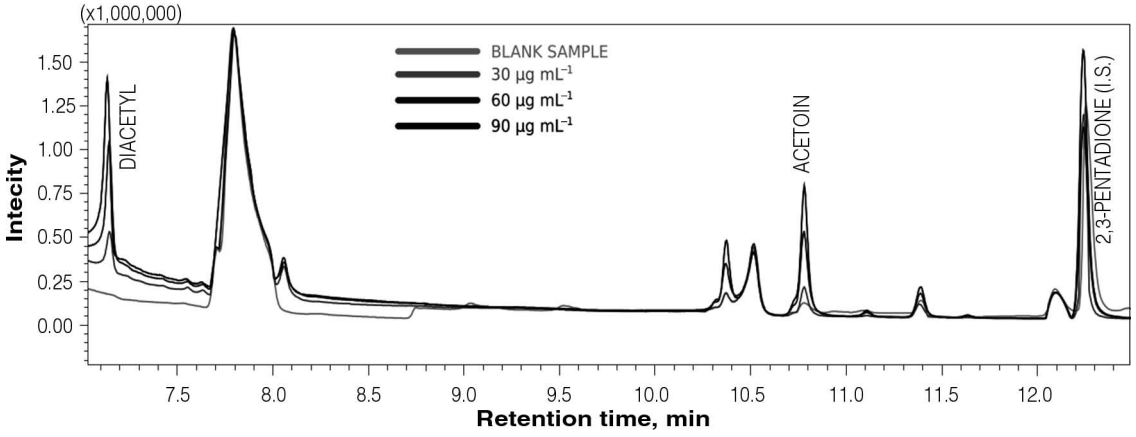


Figure 1. GC-MS chromatograms of diacetyl and acetoin in karin butter: without standard added (blank sample) and with standard added.

samples, the blank and calibration standards in a chemical analysis. This substance can then be used for calibration by plotting the ratio of the analyte signal to the internal standard signal as a function of the analyte concentration of the standards. The calibration equations with and without internal standard are given in Table 2.

The reproducibility was studied in 16 replicate experiments. The good precision data for the determination of all the aromatic compounds without IS and with IS (RSD <13.6%) were obtained by extracting the butter samples spiked at 30, 60 and 90 $\mu g \cdot g^{-1}$ levels of each of compound (Table 2). The LODs, based on S/N of 3, were in 1.83 and 0.51 $\mu g \cdot g^{-1}$ for diacetyl and acetoin, respectively. To validate the method procedure and investigate possible matrix effects, spiking recovery tests of two the compounds at concentrations of 30, 60 and 90 $\mu g \cdot g^{-1}$ from the butter samples were performed. The recoveries of diacetyl and acetoin from butter samples were achieved in the range of 90.7–110.7%, which indicates that the procedure was free from matrix effects (Table 2). The chromatograms of the butter sample after performing the LLE method are displayed in Figure 1, prior and after the spiking of samples at 30, 60 and 90 $\mu g \cdot g^{-1}$ levels of each compounds and IS. Thus, the precision and accuracy of the GC-MS method was found to be satisfactory for the analysis of the karin butter samples.

3.3. Evaluation of diacetyl and acetoin concentration in the karin butter samples

In the final part of the research, the GC-MS method was used to determine diacetyl and acetoin in the karin butter samples. Diacetyl and acetoin determinations were carried out with four independent replicates. Mean, standard deviation (SD), analysis of variance (ANOVA), surface plot and the Tukey test were statistically analyzed by MINITAB 16.0 software (Minitab, State College, PA) at a significant level of 0.05.

ANOVA was used to determine the significant differences in the concentrations of karin butter aroma compounds during the storage time of butter samples. Overall, the concentration of diacetyl in karin butter samples increased with the storage time. The concentration of acetoin in the same butter also fluctuated at different storage times, but there was no significant difference. For example, the samples analyzed from the karın butter at different storage times, the diacetyl concentrations at 20, 30, 40 and 50 days were 7.79, 9.82, 18.91 and 20.85 $\mu g \cdot g^{-1}$, respectively.

The results indicate that the two-way interactions of butter samples and storage time for the production of diacetyl and acetoin were statistically significant ($p<0.05$). Tukey's Honestly Significant Difference (HSD) test procedure appears to be more useful than ANOVA in the sense that it specifies which treatment means have a statistically significant difference. According to the Tukey test results, there were two groups for diacetyl and one group for acetoin. There were not differences between group (1) and group (2) for diacetyl ($p>0.05$). The Butter sample and storage time for diacetyl and acetoin were statistically insignificant ($p>0.05$). On the contrary, according to the ANOVA results, the butter sample and storage time for diacetyl and acetoin were statistically significant ($p<0.05$). The experimental and statistical results are summarized in Table 3. The results show that the concentration of acetoin remains stable throughout the 50 days while

Table 3. Summary of statistical analysis for diacetyl and acetoin

	Summary of the results, ($\mu g \cdot g^{-1}$)							Results of ANOVA					Tukey test, (α;0.05)	
Compound	Day	N	Mean	Std. dev.	Std. Error	Min. value	Max. value		Sum of squares	Degree of freedom	Mean squares	F	P	group, (1)	group, (2)
Diacetyl	0	5	0.00	0.00	0.00	0.00	0.00								
	20	5	7.79	8.24	3.69	0.00	19.30	Intergroup	1455.4	4	363.8			7.79	7.79
	30	5	9.82	5.47	2.44	5.74	16.11	Intragroup	1420.3	20	71.1			9.82	9.82
	40	5	18.91	11.42	5.11	5.13	31.20	Total	2875.7	24		5.12	0.005		18.9
	50	5	20.85	10.26	5.03	8.62	33.13								20.8
	Total	25	11.47	10.94	2.19	0.00	33.13							(p: 0.38)	(p: 0.14)
Acetoin	0	5	8.14	0.34	0.15	7.58	8.45							8.14	
	20	5	8.37	0.45	0.20	7.85	9.01	Intergroup	82.5	4	20.6			8.36	
	30	5	8.64	0.55	0.24	7.92	9.42	Intragroup	126.9	20	6.3			8.64	
	40	5	12.66	4.29	1.92	8.85	18.31	Total	209.4	24		3.25	0.033	11.27	
	50	5	11.27	3.57	1.60	9.12	17.36							12.66	
	Total	25	9.82	2.95	0.60	7.58	18.31							(p: 0.068)	

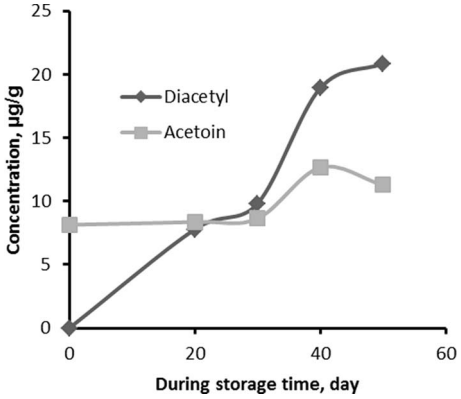


FIGURE 2. Relationship between storage time with concentration of diacetyl and acetoin.

the concentration of diacetyl increased to mean 20.85 $\mu g \cdot g^{-1}$ up to 40 days and remained constant thereafter (Figure 2).

The contents of diacetyl were found to be dependent on storage time but the contents of acetoin rarely changed with storage time.

CONCLUSIONS

In this study, the main aroma compound contents of karin butter were determined and monitored by GC-MS. The recoveries of diacetyl and acetoin from butter samples were achieved in the range of 90.7–110.7% with relative standard deviations which were generally less than 13.6%. This modified method was found to be comparatively more economical, faster, more precise and accurate for the simultaneous extraction of diacetyl and acetoin form butter samples.

The method could be used as a routine technique for the determination of diacetyl and acetoin in butter samples. Furthermore, the concentration of diacetyl in karin butter was increased with storage time, and then remained constant. The method used for the determination of diacetyl and acetoin was sensitive to changes in temperature, vapor pressure and sample matrix composition.

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