Food and Nutrition Sciences, **2012, 3, 959-969**
http://dx.doi.org/10.4236/fns.2012.37127 Published Online July 2012 (http://www.SciRP.org/journal/fns)

Application of Alternative Technologies for Elimination of Artificial Colorings in Alcoholic Beverages Produced by *Citrus medica* and Potential Impact on Human Health

Kolona-Dimitra Prompona[1], Panagiotis Kandylis[1,2], Argiris Tsakiris[3], Maria Kanellaki[1], Yiannis Kourkoutas[2*]

[1]Food Biotechnology Group, Section of Analytical Environmental and Applied Chemistry, Department of Chemistry, University of Patras, Patras, Greece; [2]Applied Microbiology and Molecular Biotechnology Research Group, Department of Molecular Biology & Genetics, Democritus University of Thrace, Alexandroupolis, Greece; [3]Department of Enology & Beverage Technology, Faculty of Food Technology and Nutrition, Technological Educational Institute (T.E.I.) of Athens, Athens, Greece.
Email: *ikourkou@mbg.duth.gr

Received July 20th, 2011; revised May 28th, 2012; accepted June 5th, 2012

ABSTRACT

In the present study elimination of chemical colorings in the alcoholic beverages produced by extraction of citron (*Citrus medica*) fruits and leaves as an alternative to the distillation method, which is currently used in industrial practice and requires addition of synthetic additives, was examined. Additionally, the volatile compounds of citron alcoholic beverages produced by both methods in association with potential antioxidant and anticancer properties were investigated. The results showed that no addition of artificial pigments was required in the novel citron beverages, as color intensity was similar to the commercial products. The most important compounds identified by solid phase microextraction and gas chromatography/mass spectrometry analysis were esters, alcohols, carbonyl compounds, acetals and terpenes. Although all products contained significant amounts of terpenes, which have been proposed as efficient antioxidant and anticancer agents, the highest concentration was determined in the alcoholic beverage produced by 4-fold extraction of citron leaves. Principal component analysis indicated that the production method affected significantly the volatile composition of the products. Finally, the potential of novel functional alcoholic beverages in promotion of human health is highlighted and assessed.

Keywords: *Citrus medica*; Volatiles; SPME GC/MS; Antioxidant; Anticancer; Functional Beverages

1. Introduction

The last decades there is an upsurge of interest in health promoting foods and beverages. Hence, there are numerous studies available in literature concerning the positive effects associated with moderate consumption of certain alcoholic beverages, mainly wine [1-3] and beer [4], due to the action of antioxidant compounds.

However, it is well-known that in alcoholic beverages production, chemicals such as colorings, flavor enhancers, etc, are widely used to improve sensory characteristics. Thus, a number of artificial additives are available and used to adjust or correct food color. However, nowadays, there is a strong debate and interest regarding the safety aspects of synthetic compounds added widely in many food products, as they are considered responsible for many carcinogenic and teratogenic attributes and residual toxicity. To remedy the aforementioned problems, consumers and the European authorities have recently increased the pressure on food manufacturers to substitute the harmful substances with alternative, non toxic, health-promoting, natural ingredients, aiming at the improvement of human health and food quality.

Citron (*Citrus medica*) was probably the only citrus fruit known in ancient times in Europe, since similar species, such as lemon, lime, pomelo and bitter oranges, were all introduced into Europe by the Muslims via the Iberian Peninsula and Sicily [5]. The taxonomy of citrus has been a challenge, since the different species have great ability to produce hybrids. During transferring for Far East, crossing between species became possible, blurring the taxonomic limits previously maintained by geographic isolation [6].

Many previous studies have documented the beneficial effects of extracts derived by various parts of citron fruits. The most important health benefits concerned remarkable antioxidant activity [7] and anticancer properties [5,

*Corresponding author.

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8]. For example, limonene, the major component in the essential oils of citrus fruits, has demonstrated promising cancer preventive and therapeutic effects in preclinical model systems [9,10].

In the Greek island of Naxos, citron fruits and leaves are used for the production of the commercial alcoholic beverage named "Citron". The fruits and leaves are separately distilled with the addition of potable alcohol and the distillates are used for the production of beverages after dilution with water to the desired alcoholic degree. Sugar and artificial pigments addition is usually followed.

The use of the extraction technique using potable alcohol instead of distillation for beverage production was an obvious alternative, as it would result in improved profile of volatiles and potent extraction of pigments, avoiding, thus, the use of synthetic additives. Therefore, the aim of the present work was 1) to examine potential elimination of chemical colorings by producing novel alcoholic beverages based on ethanolic extraction of citron components present in fruits and leaves, instead of the distillation method, which is currently used in Industrial practice and requires addition of chemical colorings, and 2) to investigate the volatile components of citron alcoholic beverages in association with potential antioxidant and anticancer properties, since recently a number of studies linked health benefits with citrus components [5,7,8].

2. Materials & Methods

2.1. Commercial Citron Beverages

The following three commercial samples were used: 1) "Fruit-Citron" (FC) produced as follows: equal amounts of chopped citron fruits (after juice removal) and potable alcohol (96% vol) were introduced into the distillation unit, then water was added until 40% (vol) was obtained. The mixture was periodically agitated for 48 h prior distillation. Distillation was carried out in traditional copper alembics of 100 L, which were filled up to $\frac{3}{4}$ of their capacity. The first 0.5 - 1.0 L of the distilled product corresponding to the beginning of the distillation procedure (known as "heads") was removed. Then, the pure spirit was collected for a time period of about 2.5 h. The distillation product was sequentially obtained until ≈5% (vol). The "tails" (distilled spirit below 5% vol) were re-used in the next distillation process. The distillate was finally diluted to 26% vol with water, then sugar up to 18%, and commercial colourings were added prior filtering and bottling. 2) "Leaves-Citron" (LC) produced by the same procedure as fruit-citron, but using citron leaves instead of fruits. It was of 30% vol. 3) "Special Leaves-Citron" (SLC), produced by double distillation product of citron leaves, as described above. It was of 40% vol, containing no colourings.

2.2. Citron Beverages Produced by the Extraction Technique

Four novel citron beverages were produced by the extraction technique using the soxhlet apparatus, as follows: 30 g of chopped citron fruit (or ≈26 - 28 g of grated citron leaves) along with 80 mL of potable alcohol (96% vol) were placed in the soxhlet apparatus. After double or four fold extraction, the extracts were diluted to the desired alcoholic degree and sugar was also added, in order to resemble the commercial products. Thus, the following beverages were produced: 1) "EFC1" of 26% vol produced by double extraction of citron fruits, 2) "EFC2" of 26% vol produced by 4-fold extraction of citron fruits, 3) "ELC1" of 30% vol produced by double extraction of citron leaves, and 4) "ELC2" of 40% vol produced by 4-fold extraction of citron leaves.

2.3. Color Intensity

Color intensity was measured by absorbance at 420, 520 and 620 nm using a VIS-7220G spectrophotometer (Biotech Engineering, Management co Ltd, UK).

2.4. Solid Phase Microextraction (SPME) Gas Chromatography/Mass Spectrometry (GC/MS) Analysis

Citron samples were studied for volatile by-products composition using SPME GC/MS analysis. Ten millilitres of the sample and 2.2 g NaCl were introduced into a 20 mL headspace vial fitted with a teflon-lined septum sealed with an aluminum crimp seal, through which the SPME syringe needle (bearing a 2-cm fibre coated with 50/30 mm Divinylbenzene/Carboxen on poly-dimethylsiloxane bonded to a flexible fused silica core, Supelco, Bellefonte, PA, USA) was introduced. The container was then thermostatted at 60˚C for 45 min. The absorbed volatile analytes were then analyzed by GC/MS (Shimadzu GC-17A, MS QP5050, capillary column Supelco CO Wax-10 60 m, 0.32 mm i.d., 0.25 μm film thickness). Helium was used as carrier gas at a flow rate of 1.8 mL/min. Oven temperature was set at 35˚C for 6 min, followed by a temperature gradient of 2˚C/min to 60˚C, held constant for 5 min, raised to 200˚C at 5˚C/min, and then to 250˚C at 25˚C/min with a final isothermal period of 6 min. The injector was operated in splitless mode. Injector and detector temperatures were 280˚C and 250˚C, respectively. The mass spectrometer was operated in the electron impact mode with the electron energy set at 70

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eV and mass range *m/z* 29-400. The identification was effected by comparing the retention times with those of authentic compounds, by mass spectra of these authentic compounds generated in the laboratory, by mass spectra obtained from NIST107, NIST21 and SZTERP libraries, and by determining kovats' retention indexes and comparing with those reported in the literature [11-13]. Kovats' retention indexes were determined by injection of a standard mixture containing the homologous series of normal alkanes (C_8-C_{22}) in pure hexane under exactly the same experimental conditions, as described above. Quantification of volatile compounds was carried out as described recently [14-17] with some modifications. Specifically, 4-methyl-2-pentanol (Sigma-Aldrich, Poole, UK) diluted in pure ethanol was used as an internal standard (IS) at various concentrations (1.62, 16.2 and 162 mg/L). The volatile compounds were quantified by dividing the peak areas of the compounds of interest by the peak area of the IS and multiplying this ratio by the initial concentration of the IS (expressed as mg/L). The peak areas were measured from the full scan chromatograph using total ion current (TIC). Each determination was carried out in triplicate and the mean data are presented (standard deviation for all values was less than ±5% in most cases).

2.5. Preliminary Sensory Evaluation

Sensory evaluation of citron alcoholic beverages was carried out by 15 well-trained tasters. The panel was chosen from an initial group of 20 tasters based on the repetition of their scores and on the agreement of their scores with the average score, as described previously [18]. Panellists were unaware of the samples they tasted (samples were labelled with codes for identification). The sensory evaluation was based on a 0 - 10 scale (0: unacceptable, 10: excellent) and was carried out in triplicate. The samples were stored in a special freezer (Eurocave, France) at 16˚C prior to organoleptic testing and were presented in standardized type INAO glasses.

2.6. Statistical Analysis

The experiments were designed and analyzed statistically by ANOVA. Duncan's multiple range test was used to determine significant differences among results [coefficients, ANOVA tables and significance ($P < 0.05$) were computed using Statistica v.5.0]. Principal component analysis (PCA) of data was computed using SPSS (v. 15.0).

3. Results & Discussion

In the present study, the evaluation of a novel method for citron alcoholic beverages production based on extraction of citron fruits and leaves instead of distillation was of interest. The strategy adopted aimed at investigation of production of new types of citron beverages with improved nutritional value by avoiding addition of chemical colorings. Additionally, a comparative study of the profile of volatiles was carried out. Fruits and leaves were collected by trees grown under the same conditions of soil, climate, and cultural practices, as variations in composition of fruits and leaves mainly depend on genetics and type of environment (soil, cultural practices, weather, etc.).

3.1. Color Intensity

The results of the color intensity are presented in **Table 1**. No addition of chemical pigments was required in the experimental citron beverages, as they resembled the commercial products. More specifically, the color intensity of ELC2 sample was identical to LC and SLC citron.

3.2. SPME GC/MS Analysis

In order to evaluate the aromatic profile, data obtained with SPME GC/MS technique from citron distillates produced by the extraction technique were compared to commercial samples. Quantitative results of the volatile compounds are presented in **Table 2**. In total, 103 compounds were detected, 58 in FC, 51 in LC, 43 in SLC, 36 in EFC1, 34 in EFC2, 44 in ELC1, and 51 in ELC2. The most important compounds identified were esters, alcohols, carbonyl compounds, acetals and terpenes.

A high number of esters were identified. Acetates are usually responsible for a pleasant fruit-like aroma [19]. Ethyl octanoate present in all samples except LC and

Table 1. Color intensity in citron alcoholic beverages.

Samples	420 nm	520 nm	620 nm	Sum (420 nm + 520 nm + 620 nm)
FC[a]	-	-	-	-
LC	0.837	0.023	0.185	1.045
SLC	0.494	0.756	0.000	1.250
EFC1	0.138	0.092	0.000	0.230
EFC2	0.201	0.212	0.000	0.413
ELC1	0.380	0.016	0.083	0.479
ELC2	0.660	0.392	0.091	1.143

FC: commercial alcoholic beverage produced by distillation of citron fruits; LC: commercial alcoholic beverage produced by single distillation of citron leaves; SLC: commercial alcoholic beverage produced by double distillation of citron leaves; EFC1: alcoholic beverage produced by double extraction of citron fruits; EFC2: alcoholic beverage produced by 4-fold extraction of citron fruits; ELC1: alcoholic beverage produced by double extraction of citron leaves; ELC2: alcoholic beverage produced by 4-fold extraction of citron leaves. a: No colourings were added in FC alcoholic beverage.

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Table 2. Effect of distillation and extraction method on the volatile compounds (mg/L) isolated by citron alcoholic beverages using the SPME technique.

Compound	Identification method	KI	KIref	FC	LC	SLC	EFC1	EFC2	ELC1	ELC2
Esters										
ethyl formate	KI, MS	920	925[c]	Nd	Nd	Nd	Nd	Nd	Nd	Tr
methyl butanoate	RT, MS			Nd	Nd	Nd	Nd	Nd	Tr	Nd
1-methyl-4-[1-methylethenyl]-cyclohexanol, acetate	KI, MS	1178	N.A.	Nd	63.7 ± 3.4	567.3 ± 28.1	1.4 ± 0.1	0.8 ± 0.1	92.3 ± 5.2	Nd
hexyl acetate	RT, KI, MS	1275	1281[a], 1272[c]	Nd	Nd	Nd	Tr	Nd	Nd	Nd
pentyl 2-methyl-propanoate	MS	1357	N.A.	Nd	Nd	Nd	Nd	Nd	Nd	Tr
ethyl octanoate	RT, KI, MS	1441	1441[c], 1451[b]	0.7 ± 0.1	Nd	Nd	Tr	Tr	0.6 ± 0.1	1.0 ± 0.1
octyl acetate	RT, KI, MS	1482	1479[c], 1497[b]	Nd	Nd	Nd	Nd	Nd	Nd	Tr
ethyl thiocyanoate	MS	1617	N.A.	Nd	Tr	Nd	Nd	Nd	Nd	Nd
ethyl decanoate	RT, KI, MS	1635	1645[c], 1652[b]	1.0 ± 0.1	Tr	Tr	Tr	Tr	5.5 ± 0.4	7.9 ± 0.7
3,7-dimethyl-6-octen-1-ol acetate (citronellol acetate)	RT, KI, MS	1660	1679[b]	8.9 ± 0.9	2.8 ± 0.3	Nd	2.0 ± 0.2	1.1 ± 0.1	Nd	3.9 ± 0.4
ethyl benzoate	RT, MS	1671	N.A.	Nd	0.5 ± 0.1	Nd	Nd	Nd	Nd	Nd
diethyl succinate	RT, MS	1679	N.A.	Nd	1.8 ± 0.2	0.9 ± 0.1	Nd	Nd	1.5 ± 0.1	5.0 ± 0.2
ethyl 9-decenoate	RT, KI, MS	1692	1697[c], 1709[b]	Nd	Nd	Nd	Nd	Tr	1.9 ± 0.1	3.4 ± 0.3
[E]-3,7-dimethyl-2,6-octadien-1-ol formate (trans-geraniol formate)	MS	1710	N.A.	Nd	Nd	Nd	Tr	Tr	Nd	Nd
[Z]-3,7-dimethyl-2,6-octadien-1-ol acetate (cis-geraniol acetate)	RT, MS	1728	N.A.	27.3 ± 2.6	4.6 ± 0.5	Nd	3.5 ± 0.4	2.9 ± 0.3	2.1 ± 0.2	Nd
[E]-3,7-dimethyl-2,6-octadien-1-ol acetate (trans-geraniol acetate)	RT, MS	1758	N.A.	37.6 ± 1.8	4.6 ± 0.5	2.5 ± 0.3	2.4 ± 0.2	1.9 ± 0.2	2.7 ± 0.3	30.8 ± 3.0
2-phenylethyl acetate	RT, KI, MS	1830	1847[b]	Nd	1.1 ± 0.1	Tr	Tr	Tr	Nd	Nd
ethyl dodecanoate	RT, KI, MS	1850	1849[c], 1850[b]	Nd	Nd	Nd	Tr	Tr	1.2 ± 0.1	Nd
ethyl tetradecanoate	RT, KI, MS	2070	2054[c], 2094[b]	Nd	Nd	Nd	Tr	Tr	Nd	Nd
ethyl pentadecanoate	MS	2163	N.A.	Nd	Nd	Nd	Tr	Tr	Nd	Nd
1-methylethyl tetradecanoate	MS	2237	N.A.	1.3 ± 0.1	2.4 ± 0.2	Nd	Tr	Nd	Nd	Nd
ethyl hexadecanoate	KI, MS	2249	2271[b]	2.6 ± 0.3	1.9 ± 0.2	2.4 ± 0.2	1.0 ± 0.1	0.5 ± 0.1	4.7 ± 0.5	18.5 ± 0.9
ethyl-9-hexadecenoate	RT, KI, MS	2269	2288[c], 2292[b]	2.0 ± 0.2	2.1 ± 0.2	3.3 ± 0.3	Tr	Tr	1.8 ± 0.2	7.1 ± 0.7
ethyl octadecanoate	RT, KI, MS	2409	2416[b]	Nd	Nd	Nd	Tr	Tr	Nd	Nd
ethyl-9-octadecenoate	RT, KI, MS	2420	2435[b]	Nd	Nd	nd	Tr	Tr	Nd	Nd

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Alcohols										
2-methyl-1-butanol	RT, KI, MS	1244	1218[c]	Nd	1.1 ± 0.1	Nd	Nd	Nd	Nd	Nd
3,7-dimethyl-1-octanol	MS	1283	N.A.	Tr	Nd	Nd	Nd	Nd	Nd	Nd
4-methyl-1-pentanol	RT, KI, MS	1325	1322[c], 1329[b]	Nd	Nd	Nd	Nd	Nd	Nd	Tr
5-methyl-2-[1-methylethyl]-[1S-(1.alpha., 2.beta., 5.beta)]-cyclohexanol (isomenthol)	MS	1302	N.A.	Tr	Nd	Nd	Nd	Nd	Nd	Nd
4,8-dimethyl-3,7-nonadien-2-ol	MS	1311	N.A.	Nd	Nd	Tr	Nd	Nd	Nd	Nd
cis-1-methyl-4-[1-methylethyl]-2-cyclohexen-1-ol	MS	1382	N.A.	Nd	Nd	Nd	Nd	Nd	Nd	1.3 ± 0.1
trans-1-methyl-4-[1-methylethenyl]-2-cyclohexen-1-ol	MS	1412	N.A.	0.6 ± 0.1	Tr	0.9 ± 0.1	Nd	Nd	Nd	Nd
6-methyl-5-hepten-2-ol	MS	1471	N.A.	Tr	Nd	Nd	Nd	Nd	Nd	Nd
{Z]-3,7-dimethyl-2,6-octadien-1-ol (nerol)	MS	1510	N.A.	1.6 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	Tr	Tr	Nd	Nd
2-ethyl-1-hexanol	RT, KI, MS	1515	1520[b]	Nd	Nd	Nd	Tr	Tr	Nd	Nd
3,7-dimethyl-1,6-octadien-3-ol (linalool)	RT, KI, MS	1547	1554[a], 1555[c], 1556[b]	16.9 ± 1.3	7.6 ± 0.5	6.6 ± 0.4	31.7 ± 3.1	10.7 ± 1.0	0.5 ± 0.1	3.1 ± 0.3
1-octanol	KI, MS	1555	1563[a], 1567[c], 1570[b]	Tr	Nd	Tr	Tr	Tr	Tr	Nd
5-methyl-2-[1-methylethenyl]-cyclohexanol (isopregol)	MS	1567	N.A.	3.3 ± 0.3	1.1 ± 0.1	0.7 ± 0.1	Nd	Nd	Nd	Nd
1-ethenyl-1-methyl-2,4-bis-[1-methylethenyl]-[1S-(1.alpha., 2.beta., 4.alpha)]-cyclohexanol	MS	1580	N.A.	Nd	Nd	1.4 ± 0.1	Nd	Nd	Nd	Nd
nonanol	RT, KI, MS	1660	1665[a]	1.9 ± 0.2	Nd	Nd	Nd	Nd	Nd	Nd
cis-carveol	MS	1694	N.A.	1.9 ± 0.2	0.7 ± 0.1	0.6 ± 0.1	Nd	0.5 ± 0.1	Nd	Nd
alpha terpineol	RT, KI, MS	1701	1705[c], 1715[b]	3.4 ± 0.3	1.5 ± 0.1	1.0 ± 0.1	9.1 ± 0.9	3.1 ± 0.3	Nd	Nd
3,7-dimethyl-6-octen-1-ol (beta.citronellol)	RT, KI, MS	1767	1790[b]	18.1 ± 1.8	Nd	Nd	Tr	Nd	Nd	10.5 ± 1.0
[Z]-3,7-dimethyl-2,6-octadien-1-ol (cis-geraniol)	RT, MS	1804	N.A.	18.4 ± 1.8	9.9 ± 1.0	5.6 ± 0.5	3.2 ± 0.3	0.5 ± 0.1	1.9 ± 0.2	24.4 ± 1.4
[E]-3,7-dimethyl-2,6-octadien-1-ol (trans-geraniol)	RT, MS	1852	N.A.	18.2 ± 1.8	11.1 ± 1.1	7.2 ± 0.7	10.5 ± 1.0	7.2 ± 0.7	Nd	39.8 ± 4.0
phenylethyl alcohol	RT, KI, MS	1928	1919[c], 1933[b]	2.5 ± 0.2	2.4 ± 0.2	2.0 ± 0.2	0.5 ± 0.1	0.5 ± 0.1	1.3 ± 0.1	5.8 ± 0.5
3,7,11-trimethyl-1,6,10-dodecatrien-3-ol	MS	2065	N.A.	0.8 ± 0.1	Tr	Nd	Nd	Nd	Nd	1.6 ± 0.2
Carbonyl compounds										
dihydro-5-methyl-2[3H]-furanone	MS	1280	N.A.	Tr	Tr	Nd	Nd	Nd	Nd	Nd

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octanal	RT, KI, MS	1306	1296[a]	Tr	Nd	Nd	Nd	Nd	Tr	Tr
.alpha.-methyl benzeneacetaldehyde	MS	1314	N.A.	Nd	Nd	Nd	Nd	Nd	Tr	Nd
6-methyl-5-hepten-2-one	KI, MS	1348	1347[a]	15.5 ± 1.5	7.7 ± 0.7	9.2 ± 0.6	Nd	Nd	Tr	2.1 ± 0.2
2-heptenal [E]	RT, KI, MS	1334	1334[a]	Nd	Nd	Nd	Tr	Tr	Nd	Nd
2-nonanone	RT, MS	1389	N.A.	Nd	Nd	Nd	Nd	Nd	Tr	Nd
nonanal	RT, KI, MS	1392	1390[b], 1392[c], 1402[a]	3.9 ± 0.4	1.2 ± 0.1	1.8 ± 0.2	0.6 ± 0.1	Tr	0.7 ± 0.1	3.3 ± 0.3
2-pinen-4-one	RT, MS	1402	N.A.	Nd	Tr	Nd	Nd	Nd	Nd	Nd
[E]-2-octenal	RT, MS	1440	N.A.	Nd	Nd	Nd	Nd	Tr	Nd	Nd
furfural	RT, KI, MS	1477	1486[b]	Nd	Tr	Tr	Nd	Nd	Nd	Nd
3,7-dimethyl-6-octenal (citronellal)	RT, MS	1483	N.A.	2.1 ± 0.2	1.2 ± 0.1	1.7 ± 0.2	Nd	Nd	Nd	0.7 ± 0.1
decanal	RT, KI, MS	1501	1500[c], 1509[b]	4.6 ± 0.4	1.9 ± 0.2	1.6 ± 0.2	Nd	Nd	0.8 ± 0.1	6.7 ± 0.7
benzaldehyde	RT, KI, MS	1528	1533[b], 1540[a]	Nd	0.4 ± 0.1	Tr	Tr	Tr	Nd	Nd
undecanal	RT, MS	1599	N.A.	7.1 ± 0.5	2.7 ± 0.3	2.4 ± 0.2	Nd	Nd	0.6 ± 0.1	9.3 ± 0.9
[Z]-3,7-dimethyl-2,6-octadienal (cis-geranial)	RT, MS	1686	N.A.	17.4 ± 0.7	7.2 ± 0.6	3.3 ± 0.3	2.4 ± 0.2	0.9 ± 0.1	4.6 ± 0.4	17.5 ± 1.1
tetradecanal (myristaldehyde)	RT, MS	1713	N.A.	2.2 ± 0.2	0.8 ± 0.1	0.6 ± 0.1	Nd	Nd	0.8 ± 0.1	6.2 ± 0.6
[E]-3,7-dimethyl-2,6-octadienal (trans-geranial)	RT, KI, MS	1738	1741[b]	23.8 ± 2.4	10.5 ± 1.0	5.1 ± 0.5	4.3 ± 0.4	1.6 ± 0.2	7.6 ± 0.8	31.3 ± 1.2
4-methoxy benzaldehyde (*p*-anisaldehyde)	MS	2069	N.A.	Tr	Nd	Tr	Nd	Nd	Nd	Nd
Acetals										
1,1-diethoxy ethane	RT, KI, MS	925	930[b]	19.5 ± 2.0	Nd	Nd	Tr	Tr	Nd	3.8 ± 0.4
1,1-diethoxy-3-methyl butane (isovaleraldehyde diethyl acetal)	MS	1058	N.A.	1.1 ± 0.1	0.9 ± 0.1	Nd	Nd	Nd	Tr	Tr
1,1-diethoxy-pentane (varelaldehyde diethyl acetal)	RT, MS	1128	N.A.	Tr	Tr	Nd	Nd	Nd	Nd	Nd
1,1-diethoxy-heptane	MS	1340	N.A.	Tr	Tr	Nd	Nd	Nd	Nd	Nd
1,1-diethoxy-octane	MS	1431	N.A.	2.2 ± 0.2	1.4 ± 0.1	Tr	0.8 ± 0.1	Tr	0.5 ± 0.1	1.2 ± 0.1
1,1-diethoxy-nonane	MS	1520	N.A.	11.2 ± 1.1	6.9 ± 0.7	1.4 ± 0.1	5.1 ± 0.5	2.9 ± 0.3	1.5 ± 0.2	10.5 ± 1.0
1,1-diethyl decane	MS	1622	N.A.	4.4 ± 0.4	3.9 ± 0.4	1.1 ± 0.1	Tr	Nd	1.0 ± 0.1	10.8 ± 1.1
Miscellaneous compounds										
alpha.pinene	RT, KI, MS	1015	1020[a]	1.6 ± 0.2	Nd	Nd	Nd	Nd	Nd	11.7 ± 1.0
toluene	RT, KI, MS	1039	1043[a]	Tr	Nd	Nd	Nd	Nd	Nd	2.0 ± 0.2
beta.pinene	RT, MS	1070	N.A.	Tr	Nd	Nd	Nd	Nd	Nd	1.8 ± 0.2
delta-3-carene	KI, MS	1114	1143[a]	Tr	Nd	Nd	Nd	Nd	Nd	Nd

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beta.myrcene	RT, MS	1149	N.A.	5.4 ± 0.5	0.8 ± 0.1	Nd	Nd	Nd	2.0 ± 0.2	40.5 ± 3.9
4-carene	MS	1157	N.A.	Nd	Nd	Nd	Nd	Nd	Nd	1.3 ± 0.1
d-limonene	RT, KI, MS	1191	1188[c], 1189[b], 1198[a]	315.9 ± 11.6	63.7 ± 3.4	567.3 ± 50.8	66.6 ± 5.8	41.4 ± 3.8	92.3 ± 8.8	1460.5 ± 112.2
beta.phellandrene	MS	1197	N.A.	0.5 ± 0.1	Nd	Nd	Nd	Nd	Nd	2.3 ± 0.2
eucalyptol	MS	1200	N.A.	1.1 ± 0.1	1.1 ± 0.1	Nd	Nd	Nd	Tr	1.1 ± 0.1
2-[1,1-dimethylethyl]-3-methyl-oxirene	MS	1246	N.A.	Nd	2.5 ± 0.2	2.8 ± 0.3	Nd	Nd	Tr	Nd
1-methyl-4-[1-methylethyl]-1, 4-cyclohexadiene	MS	1251	N.A.	Nd	Nd	Nd	14.8 ± 1.3	Nd	Nd	4.8 ± 0.4
1-dodecene	MS	1255	N.A.	Tr	Nd	Nd	Nd	Nd	Nd	0.9 ± 0.1
3,7-trimethyl-1,3,6-octatriene	MS	1259	N.A.	2.2 ± 0.2	1.0 ± 0.1	8.3 ± 0.5	Nd	Nd	1.8 ± 0.1	22.6 ± 2.2
1-methyl-3-[1-methylethyl]-benzene	MS	1282	N.A.	Tr	Nd	Nd	12.6 ± 1.1	Nd	Nd	1.2 ± 0.1
1,3,5-trimethyl benzene	MS	1291	N.A.	Nd	Nd	Nd	Nd	Tr	Nd	Nd
tetradecane	RT, MS	1400	N.A.	Nd	Nd	Nd	Nd	Nd	Tr	Nd
methoxy-phenyl oxime	MS	1404	N.A.	Nd	Nd	Nd	Nd	Nd	1.4 ± 0.1	4.2 ± 0.4
3-[4-methyl-3-pentenyl]-furan	MS	1421	N.A.	Tr	Tr	0.7 ± 0.1	Nd	Nd	Nd	Nd
1,3-bis-[1,1-dimethylethyl]-benzene	MS	1426	N.A.	Nd	Tr	Tr	Nd	Nd	Nd	Nd
[E]-7-tetradecene	MS	1464	N.A.	Tr	Nd	Nd	Nd	Nd	Tr	Nd
2,2-dimethyl-4,5-dipropenyl-1, 3-dioxolane	MS	1474	N.A.	1.0 ± 0.1	Tr	Tr	Nd	Nd	Nd	Nd
caryophylene	MS	1585	N.A.	14.5 ± 1.5	4.7 ± 0.4	55.7 ± 5.2	Nd	Nd	2.1 ± 0.2	53.7 ± 5.2
hexadecane	RT, MS	1595	N.A.	Nd	Nd	Nd	Nd	Nd	0.7 ± 0.1	Nd
1-hexadecene	MS	1645	N.A.	Nd	Nd	Nd	Nd	Nd	2.6 ± 0.2	6.3 ± 0.4
[Z]-beta.farnesene	RT, KI, MS	1665	1654[a]	Nd	Nd	0.7 ± 0.1	Nd	Nd	Nd	Nd
[E,E]-alpha.farnesene	RT, KI, MS	1749	1738[b], 1757[a]	Nd	Nd	0.8 ± 0.1	Nd	Nd	Tr	6.8 ± 0.5
2,6,10,14-tetramethyl hexadecane	MS	1772	N.A.	Nd	Nd	Nd	Nd	Nd	Tr	Nd
anethole	RT, KI, MS	1842	1827[c], 1843[b]	191.8 ± 10.2	97.6 ± 5.8	147.3 ± 10.8	Nd	Nd	21.5 ± 2.0	49.1 ± 2.9
	MS									
nonadecane	RT, KI, MS	1906	N.A.	Nd	Nd	Nd	Nd	Nd	0.7 ± 0.1	Nd
eicosane	RT, KI, MS	2016	N.A.	Nd	Nd	Nd	Nd	Nd	0.5 ± 0.1	0.8 ± 0.1
caryophyllene oxide		2020	N.A.	2.5 ± 0.2	0.8 ± 0.1	1.0 ± 0.1	Nd	Nd	Nd	2.1 ± 0.2

RT: positive identification by retention times that agree with authentic compounds and by mass spectra of authentic compounds generated in the laboratory; KI: tentative identification by kovats' retention index; MS: positive identification by mass spectra obtained from NIST107, NIST21 and SZTERP libraries; Tr: <0.5 mg/L; N.A.: not available; Nd: not detected; FC: commercial alcoholic beverage produced by distillation of citron fruits; LC: commercial alcoholic beverage produced by single distillation of citron leaves; SLC: commercial alcoholic beverage produced by double distillation of citron leaves; EFC1: alcoholic beverage produced by double extraction of citron fruits; EFC2: alcoholic beverage produced by 4-fold extraction of citron fruits; ELC1: alcoholic beverage produced by double extraction of citron leaves; ELC2: alcoholic beverage produced by 4-fold extraction of citron leaves. [a]: Vichi *et al.*, 2007; [b]: Kandylis *et al.*, 2010; [c]: Mallouchos *et al.*, 2007.

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SLC samples has a floral, fruity, musty impact [20]. Likewise, 2-phenylethyl acetate, detected in all cases except in FC, ELC1 and ELC2 contributes to the aromatic complexity providing a banana-apple aroma [21].

A total of 24 alcohols were identified in citron beverages. Fusel alcohols are generally considered to have rather unpleasant odors. Therefore, it is believed that they contribute more to the intensity of the odor of the wine that to its quality [22]. Distinctive was the peak for 2-phenyl-ethanol in all samples, which is a compound with a characteristic rose aroma [22]. Octanol present only in commercial samples was never outlined as a potential contributor to beverages aroma [22]. It is probably a by-product of the biosynthesis of long-chain fatty acids.

Terpene alcohols were among the principal components of citron beverages. Geraniol detected in all samples provides floral notes [22]. Likewise, linalool gives a flowery odor [23] and was detected at levels little higher than its perception threshold (0.05 mg/L) [21]. Alpha.terpineol was also present in all samples except in ELC1 and ELC2 in amounts higher than its perception threshold (0.4 mg/L) [21]. Beta.citronellol was detected in FC and ELC2 at levels much higher than its perception threshold (0.018 mg/L) [21] and is considered to give a citrus, sweet, floral note [23,24].

Terpene alcohols have been proposed as potential anticancer agents [25]. For example, geraniol present in all samples in our study, may induce apoptosis and increase expression of the proapoptotic protein Bak in cultured pancreatic tumor cells [25]. It also showed chemopreventive activities on hepatocarcinogenesis during the initial phases of the RH model by inhibition of cell proliferation, DNA damage and increasing hepatic placental glutathione S-transferase positive preneoplastic lesions (PNLs) apoptosis and antiproliferative effects on hepatoma and melanoma cell growth [25].

Carbonyl compounds identified included mainly aldehydes and ketones. Benzaldehyde identified in all cases except in FC, ELC1 and ELC samples is described as having an aromatic note of bitter almond [26]. Furfural is characterized by a toasted almond odor [19], however it was detected in very low levels in LC and SLC beverages. Aldehydes that are responsible for green-leaf off-flavors, like butanal, pentanal and hexanal were not detected.

Of note, some acetals were detected, which are generally considered positive contributors to the aroma. However, due to their low concentration, their impact is rather unlikely. Acetals are mainly formed by chemical reactions between aldehydes and alcohols.

In addition, a number of miscellaneous compounds were identified, some groups of which are known to contribute to the complexity of aroma. For example, d-limonene, detected in all samples in large amounts, provides a citrus-like note.

Terpenes, such as limonene, have been also shown to prevent mammary, liver, lung, gastric and other cancers [8-10]. These compounds have also been used to treat a variety of rodent cancers, including breast and pancreatic carcinomas. In addition, *in vitro* data suggested that they may be effective in treating neuroblastomas and leukemias [9].

From a quantitative point of view, the highest ($P < 0.05$) concentration of esters was observed in SLC beverage (**Table 3**), while the lowest ($P < 0.05$) content of alcohols in ELC1 beverage. Single distillation of fruits and 4-fold extraction of leaves resulted in significant ($P < 0.05$) increase of carbonyl compounds. Likewise, FC beverages contained the highest ($P < 0.05$) amount of acetals, whereas 4-fold extraction of leaves resulted in higher ($P < 0.05$) concentration of acetals compared to single or double distillation of leaves. Interestingly, the

Table 3. Sum of volatile compounds (mg/L) determined by SPME GC/MS analysis in citron alcoholic beverages.

Compounds	FC	LC	SLC	EFC1	EFC2	ELC1	ELC2
Esters	81.4 ± 8.6	85.5 ± 8.1	576.4 ± 41.0	10.3 ± 1.4	7.2 ± 1.1	114.3 ± 10.2	77.6 ± 8.9
Alcohols	87.6 ± 12.0	36.4 ± 4.8	26.9 ± 3.7	55.0 ± 7.6	22.5 ± 3.3	3.7 ± 0.6	86.5 ± 10.6
Carbonyl compounds	76.6 ± 8.9	33.6 ± 4.5	25.7 ± 3.3	7.3 ± 1.0	2.5 ± 0.4	15.1 ± 2.3	77.1 ± 7.2
Acetals	38.4 ± 5.4	13.1 ± 1.8	2.5 ± 0.3	5.9 ± 0.8	2.9 ± 0.4	3.0 ± 0.6	26.3 ± 3.7
Miscellaneous compounds	536.5 ± 34.9	172.2 ± 14.4	784.6 ± 96.2	94.0 ± 11.6	41.4 ± 5.4	125.6 ± 16.8	1673.7 ± 184.4
Terpenes[a]	462.2 ± 34.9	120.7 ± 12.2	656.7 ± 83.6	127.8 ± 16.5	65.9 ± 8.9	111.0 ± 15.1	1701.2 ± 186.8
Total volatiles	820.5 ± 69.9	340.8 ± 33.7	1416.1 ± 144.4	172.5 ± 22.5	76.5 ± 10.6	261.7 ± 30.4	1941.2 ± 214.8

FC: Commercial alcoholic beverage produced by distillation of citron fruits; LC: Commercial alcoholic beverage produced by single distillation of citron leaves; SLC: Commercial alcoholic beverage produced by double distillation of citron leaves; EFC1: Alcoholic beverage produced by double extraction of citron fruits; EFC2: Alcoholic beverage produced by 4-fold extraction of citron fruits; ELC1: Alcoholic beverage produced by double extraction of citron leaves; ELC2: Alcoholic beverage produced by 4-fold extraction of citron leaves. a: sum of nerol, linalool, cis-carveol, alpha.terpineol, beta.citronellol, cis-geraniol, trans-geraniol, 2-pinen-4-one, citronellal, cis-geranial, trans-geranial, alpha.pinene, beta.pinene, delta.3-carene, beta.myrcene, 4-carene, d-limonene, beta.phellandrene, caryophyllene, beta.farnesene, alpha.farnesene, and caryophyllene oxide.

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highest ($P < 0.05$) content of miscellaneous compounds, terpenes and total volatiles was detected in ELC2 beverage, due to the high concentration of d-limonene which was the dominant compound (**Table 2**).

3.3. Chemometrics

Principal component analysis is used in exploratory analysis, as it gives graphical representations of inter-sample and inter-variable relationships and provides a way to reduce the complexity of the data.

The application of the PCA algorithm to data showed three distinctive groups (**Figure 1**). The first group is composed by the commercial alcoholic beverages produced by distillation of citron fruits and leaves, while the second group by the novel alcoholic beverages produced by extraction of citron fruits and leaves. A third group consisting of a single sample (ELC2) produced by 4-fold extraction of citron leaves was evident. Of note, this sample contained the highest amount of d-limonene. The results indicated that the production method seems to affect significantly the volatile composition of the products.

3.4. Preliminary Sensory Evaluation

The results of the preliminary sensory evaluation are presented in **Table 4**. The novel citron beverages produced in the laboratory were accepted by the panel and were characterized as high quality products when compared to the commercial products, although the ELC2 scored the statistically lowest value ($P < 0.05$).

4. Conclusion

As a final consideration, it was concluded that extraction

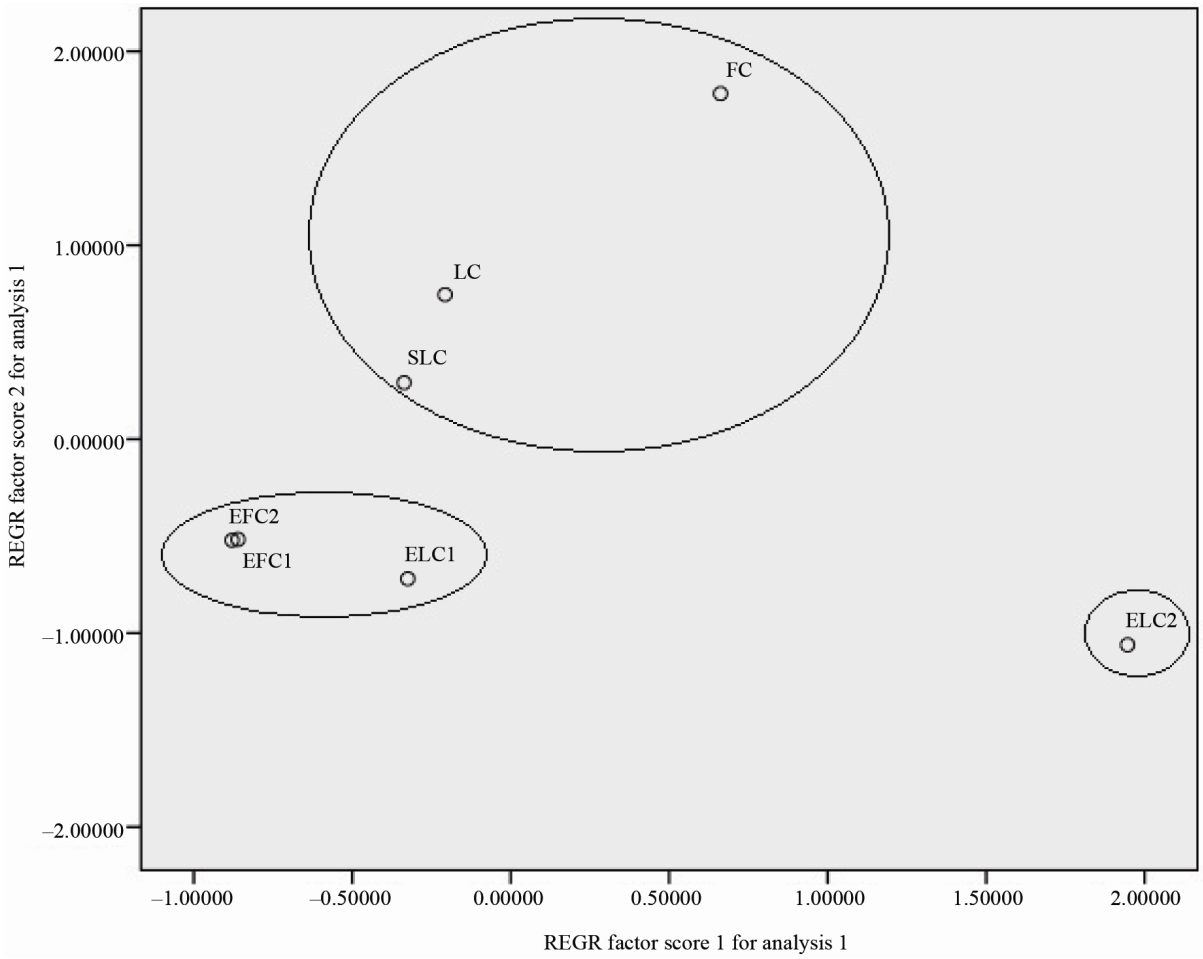

Figure 1. PCA plot of citron alcoholic beverages. FC: commercial alcoholic beverage produced by distillation of citron fruits; LC: commercial alcoholic beverage produced by single distillation of citron leaves; SLC: commercial alcoholic beverage produced by double distillation of citron leaves; EFC1: alcoholic beverage produced by double extraction of citron fruits; EFC2: alcoholic beverage produced by 4-fold extraction of citron fruits; ELC1: alcoholic beverage produced by double extraction of citron leaves; ELC2: alcoholic beverage produced by 4-fold extraction of citron leaves.

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Table 4. Preliminary sensory evaluation in citron alcoholic beverages.

Samples	Overall Quality
FC	7.23 ± 0.50
LC	7.47 ± 0.64
SLC	7.67 ± 0.41
EFC1	7.63 ± 0.49
EFC2	7.23 ± 0.42
ELC1	7.26 ± 0.62
ELC2	6.67 ± 0.45

FC: commercial alcoholic beverage produced by distillation of citron fruits; LC: commercial alcoholic beverage produced by single distillation of citron leaves; SLC: commercial alcoholic beverage produced by double distillation of citron leaves; EFC1: alcoholic beverage produced by double extraction of citron fruits; EFC2: alcoholic beverage produced by 4-fold extraction of citron fruits; ELC1: alcoholic beverage produced by double extraction of citron leaves; ELC2: alcoholic beverage produced by 4-fold extraction of citron leaves.

of fruits or leaves could substitute distillation during alcoholic beverages production and it may lead to improved quality, by avoiding addition of chemical colorings. Although the adverse effects of ethanol, future clinical tests will give more insight into the positive role of functional alcoholic beverages in human health associated with moderate consumption. However, more research is still required in the field.

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