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Enantiomeric distribution and $^{13}C/^{12}C$ isotope ratio determination of γ-lactones: appropriate methods for the differentiation between natural and non-natural flavours?

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ABSTRACT

The quantitative and enantiomeric distribution of γ-lactones in certain fruits (strawberry, raspberry, pineapple, passion fruit, plum and coconut) compared with corresponding fruit concentrates and beverages was determined by multi-dimensional gas chromatography–mass spectrometry with an achiral chiral column combination. It was found that significant variations in enantiomeric excess values of γ-lactones due to varieties and processing influences can occur. On-line multi-dimensional gas chromatographic–isotope ratio mass spectrometric $^{13}C/^{12}C$ isotope ratio measurements of γ-decalactone in vinous beverages and references (synthetic and microbial) were performed, in order to establish how far enantiomeric data in combination with additional $\delta^{13}C$ values can serve as a basis for the validation of the genuineness of flavours and flavoured products.

INTRODUCTION

The current trend toward natural foods and beverages and the differentiation of natural and non-natural flavouring substances required by legislation have increased the demand for appropriate analytical methods to ensure proper labelling and to identify the fraudulent use of synthetic compounds. Especially gas chromatographic (GC) methods such as enantioresolution on chiral capillary columns [1,2] and multi-dimensional GC (MDGC) with achiral–chiral column combinations [3–5] were developed for the determination to the fruit-specific distribution of optically active compounds, mainly γ-lactones. Because of their widespread occurrence in fruits and flavour compositions, they are believed to be useful indicators for the quality evaluation of flavours. Previously the chirality evaluation of natural γ-lactones revealed that especially in the case of deca- and dodecalactone, one enantiomeric from predominates [5–9]. Therefore, it was postulated that the occurrence of racemic lactones in processed foodstuffs and flavour formulations has to be associated with the addition of synthetic flavourings [4]. Consequently, the enantiomeric distribution is widely used as a criterion of genuineness.

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The exclusive use of the "typical" enantiomeric excess values corresponding to the different fruits without considering their natural range due to different varieties [9] and possible changes caused by processing influences may lead to erroneous conclusions. Therefore, in addition to the aforementioned aspects, the quantitative lactone distribution [10] can be regarded as a further criterion.

A particular problem arises when the enantiomeric ratio has been "tuned" using pure (*R*)- γ-decalactone of microbial origin. In this context, the determination of the natural abundance of stable isotopes by means of stable isotope ratio mass spectrometry (IRMS) offers a method for the assignment of the origin and treatment of foods and food ingredients [11–14]. Especially the recent developments in "on-line" GC–IRMS systems [15,16] and MDGC–IRMS [17] for measuring $^{13}C/^{12}C$ isotope ratios have extended its application to the analysis of complex mixtures such as flavour extracts.

The aim of this work was to delineate the limitations arising in the determination of naturalness, based on experimental data obtained by enantioresolution and/or GC–IRMS measurements.

EXPERIMENTAL

Materials

Fresh strawberries (var. Red Gauntlet); deep-frozen raspberries, freeze-dried raspberries (prepared in our laboratory), two commercial raspberry concentrates, fresh pineapples (from the Ivory Coast) [8], five pineapple concentrates [8], fresh yellow passion fruits (from Brazil) [6], fresh purple passion fruits (from Kenya) [6], five passion fruit concentrates (from Brazil) [6], fresh coconut, commercial coconut flakes, fresh plums (from northern Bavaria, frozen for 2 months), two plum wines (made in our laboratory from frozen plums by mash and juice fermentation, respectively), a sparkling strawberry wine, a vinous beverage ("weinhaltiges Getränk") with peach flavour and a vinous beverage named "piña colada" (labelled as a mixture of white wine, pineapple juice, flavour of coconut and other tropical fruits) were obtained from the local market or directly from manufacturers.

Sample preparation

Fruits (100–1000 g) were homogenized with methanol and the homogenates were centrifugated and diluted with water. Fruit concentrates (200–500 ml) were diluted with water. Plum wines, sparkling strawberry wine and alcoholic mixed-beverages (0.5–1 l) were used undiluted. After addition of a suitable proportion (1:5, v/v) of saturated potassium fluoride solution, all samples were extracted with pentane–dichloromethane (2:1).

The milk of one coconut (30 ml) was applied to an Extrelut column (Merck, Darmstadt, Germany) containing 14 g of Extrelut, and extracted with 60 ml of pentane–dichloromethane (2:1). One portion of the flesh (100 g) was homogenized in methanol, extracted with pentane–dichloromethane and the organic supernatant was decanted after freezing. Another 100-g portion was extracted by means of simultaneous distillation extraction (SDE) with diethyl ether. Commercial coconut flakes were extracted in the same way. Coconut fat obtained from flakes by Soxhlet extraction (46 g of fat from 100 g of flakes) was heated at 100–150°C for 3 h under a continuous

stream of oxygen, and flavour compounds were subsequently extracted by SDE with diethyl ether.

All extracts were dried over anhydrous sodium sulphate and concentrated to a final volume of 0.3–0.5 ml on a Vigreux column (40°C).

Enantioresolution of γ-lactones (MDGC–MS)

A Siemens SiChromat 2 dual-over gas chromatograph, equipped with a liquid injector (220°C, splitting ratio 1:11) and a total transfer device, was directly coupled with a Finnigan 4021 C quadrupole mass spectrometer. Preseparation of 2–5 μl of the samples was achieved on a wide-bore CW20M precolumn (G. Leupold, Weihenstephan, Germany) (30 m × 0.53 mm I.D.; film thickness 1.5 μm), programmed from 100 to 220°C at 5°C/min. The carrier gas was helium at 6 ml/min (100°C). At the corresponding retention ranges of γ-lactone reference substances, quantitative cuts into a precooled intermediate trap (liquid nitrogen; −150°C) were performed. On-line transfer of the enriched lactones into the chiral main column was achieved by heating the trap to 200°C, whilst the precolumn was backflushed. The enantiomers were separated on a chiral Lipodex B main column (Macherey, Nagel & Co., Düren, Germany) (50 m × 0.25 mm I.D.), programmed from 135°C (30 min) to 200°C at 3°C/min. The carrier gas was helium at 0.5 ml/min (135°C). Mass spectra were recorded at 70 eV in the mass range 50–150 u.

$^{13}C/^{12}C$ isotope ratio determination (MDGC IRMS)

A Siemens SiChromat 2 dual-oven gaschromatograph, equipped with a liquid injector (220°C) and a "live-T" switching device, directly coupled with a VG Model Isochrom II GC–IRMS system [15,17], was used. Preseparation of 2–3 μl (splitless injection) of the samples was achieved on an SPB-5 precolumn (Supelco, Bellefonte, PA, USA) (30 m × 0.25 mm I.D.; film thickness 1.0 μm), programmed from 80 to 200°C (5 min) at 4°C/min and from 200 to 225°C at 2°C/min. The carrier gas was helium at 0.3 ml/min. After selective transfer of the γ-decalactone (cut: 37.3–41 min), the enantiomers were separated on a chiral Lipodex B fused-silica main column (Macherey, Nagel & Co.) (50 m × 0.25 mm I.D.), programmed from 140°C (60 min) to 150°C (20 min) at 1°C/min. Selective cuts of the chiral column effluent were introduced (transfer line temperature 180°C) into the combustion furnace filled with copper (II) oxide (operated at 800°C) by means of a Deans-type switching device (SGE, Ringwood, Australia). Water of reaction was removed continuously by a cryogenic trap (−100°C). The $^{13}C/^{12}C$ isotopic ratios are given in δ‰ relative to the values of PDB.

RESULTS

In the following some selected results of our recent studies on fruits and processed fruit products are presented. In additon to the determination of the total flavour composition, the quantitative distribution and enantiomeric ratios of γ-lactones were of particular interest. The data obtained were taken as a basis in order to establish their usefulness for the differentiation between natural and non-natural flavours.

Strawberry

As summarized in Table I, most of the lactones are predominantly in the *R* configuration. Small amounts of γ-heptalactone occur as a racemate, and for γ-hexalactone the *S* enantiomer preponderates. A similar enantiomeric distribution was observed in a commercially available sparkling strawberry wine.

TABLE I

ENANTIOMERIC COMPOSITION AND QUANTITATIVE DISTRIBUTION OF γ-LACTONES IN FRESH STRAWBERRIES AND A COMMERCIALLY AVAILABLE SPARKLING STRAWBERRY WINE

γ-Lactone	Strawberries			Sparkling strawberry wine		
	R (%)	*S* (%)	ppb	*R* (%)	*S* (%)	ppb
Hexa-	36	64	150	46	54	40
Hepta-	55	45	10	55	45	1
Octa-	85	15	120	68	32	15
Nona-	88	12	30	73	27	15
Deca-	99	1	10150	98	2	190
Dodeca-	100	0	1850	97	3	30

Raspberry

In addition to the small amounts of γ-lactones determined in raspberries, it is noticeable that the main component γ-octalactone and the higher homologous lactones may occur as racemates (Table II). One of the processed raspberry samples showed an obvious excess of (*R*)-γ-deca- and -dodecalactone, which might be caused by contamination during processing or due to varietal differences.

TABLE II

ENANTIOMERIC COMPOSITION AND QUANTITATIVE DISTRIBUTION OF γ-LACTONES IN DEEP-FROZEN AND PROCESSED RASPBERRY SAMPLES.

γ-Lactone	Deep-frozen raspberry			Processed raspberries	
	R (%)	*S* (%)	ppb	*R* (%)	Share (%)[a]
Hexa-	34	66	30	26–38	100
Hepta-	25	75	1	26–30	0–5
Octa-	44	56	65	40–55	50–150
Nona-	28	72	3	32–53	10–20
Deca-	49	51	2	54–68	5–10
Undeca-	55	45	1	51–55	0–5
Dodeca-	50	50	5	51–84	5–20

[a] Relative to γ-hexalactone as 100%.

TABLE III

ENANTIOMERIC COMPOSITION AND QUANTITATIVE DISTRIBUTION OF γ-LACTONES IN PINEAPPLE AND PINEAPPLE CONCENTRATES

γ-Lactone	Pineapple (9)[a]		Pineapple (5)[a] concentrates	
	R (%)	ppb	*R* (%)	Share (%)[b]
Hexa-	55–75	90–280	36–46	100
Hepta-	60–73	0–5		0
Octa-	67–75	40–190	52–76	6–20
Nona-	61–77	0–5		0
Deca-	63–91	1–10	65–84	1–4
Dodeca-	82–100	0–5	88	0–1

[a] No. of samples in parentheses.
[b] Relative to γ-hexalactone as 100%.

Pineapple

In contrast to the distribution in raspberries, the higher γ-lactones in fresh pineapple fruits exhibit a predominance of the *R* enantiomer, whereas for the major γ-hexalactone nearly equal amounts of *R* and *S* enantiomers could be determined (Table III). The shift towards higher *S* enantiomer contents for γ-hexa- and -octalactone in concentrates is assumed to be caused by cleavage of pineapple-typical acetoxy esters, mainly with *S* configuration [18]. In contrast to fresh fruits, only traces of the acetoxy esters were found in the concentrates [8].

The quantitative dominance of γ-hexalactone in the concentrates may be attributed to the concentration process. More water-soluble, short-chain γ-lactones remain in the fruit pulp, whereas less water-soluble, long-chain γ-lactones are released into the condensate [19].

Passion fruit

A preferred *R* configuration of the higher C_{10}- and C_{12}-lactones is also observed in yellow and purple passion fruits (Table IV). In yellow passion fruits γ-hep-

TABLE IV

ENANTIOMERIC COMPOSITION AND QUANTITATIVE DISTRIBUTION OF γ-LACTONES IN RED AND YELLOW PASSION FRUITS AND PASSION FRUIT CONCENTRATES

γ-Lactone	Yellow (3)[a]		Red (1)[a]		Passion fruit (5)[a] concentrates	
	R (%)	Share (%)[b]	*R* (%)	Share (%)[b]	*R* (%)	Share (%)[b]
Hexa-	79–89	100	73	100	79–87	100
Hepta-	56–65	0–3	75	3		0
Octa-	55–78	33–66	72	23	69–82	3–12
Nona-	50–56	13–66	66	3	53	0–1
Deca-	87–93	39–127	93	102	84–94	14–36
Dodeca-	99–100	73–151	100	270	100	10–27

[a] No. of samples in parentheses.
[b] Relative to γ-hexalactone as 100%.

ta-, γ-octa- and especially γ-nonalactone can also occur as racemic mixtures [6]. No significant differences in enantiomeric distribution could be detected on comparing fresh fruits with concentrates. The significant increase in the relative amount of γ-hexalactone is in accordance with our findings for pineapple concentrates.

Coconut

Based on the assumption that autoxidation of fatty acids leads to the formation of racemic γ-lactones, the effect of heating coconut fat was investigated. Neither in fresh coconut flesh and milk nor in commercially available coconut flakes could any γ-lactones be detected. As expected, considerable amounts of racemic γ-lactones were formed on heating extracted coconut fat under a continous stream of oxygen (see Fig. 1). The predominant formation of γ-octalactone corresponds with the fact that octanoic acid was found to be the major fatty acid in the extracted fat. This is in agreement with the findings of Pai *et al.* [20], who observed the preponderant production of γ-dodecalactone in autoxidized coconut fat consisting mainly of dodecanoic acid.

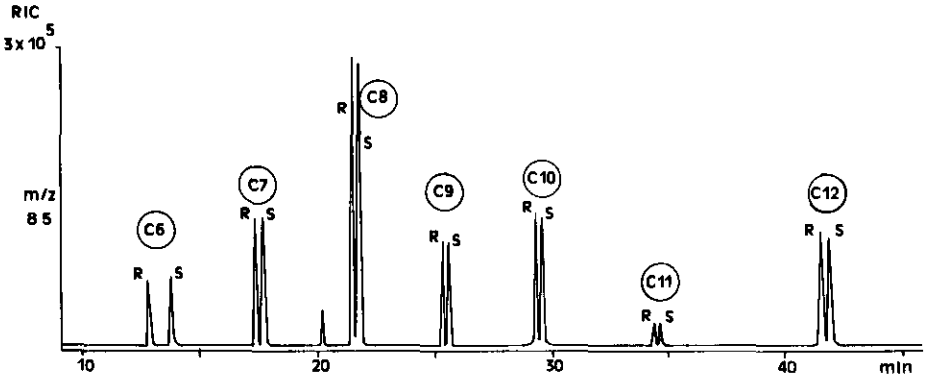

Fig. 1. Enantiomeric distribution of γ-lactones in heated coconut fat. Reconstructed ion chromatogram (*m/z* 85) of selected cuts from the precolumn on a Lipodex B column; see Experimental for details.

Plum

To evaluate the influence of fermentation processes on the lactone distribution, two plum wines fermented in different ways were investigated. As summarized in Table V, fermentation has no significant effect on the enantiomeric distribution, except for γ-nonalactone (Table V). As the content of the *R* enantiomer and the concentration of γ-nonalactone, relative to other γ-lactones, increased during fermentation, it is believed to be a fermentation product.

Similar results were achieved on investigating gooseberry wine and beer wort. The gooseberry concentrate exhibits only very small amounts of γ-decalactone (*R*:*S* = 78:22), which remain unchanged during fermentation. The fermented product additionally contains γ-nonalactone (*R*:*S* = 89:11).

In beer wort small amounts of racemic γ-hexa- and γ-nonalactone were identified. During fermentation γ-hexalactone remains racemic, whereas a twofold increase in the γ-nonalactone concentration, mainly with *R* configuration (γ-nonalactone in

TABLE V

ENANTIOMERIC COMPOSITION AND QUANTITATIVE DISTRIBUTION OF γ-LACTONES IN PLUMS AND TWO DIFFERENT PLUM WINES

γ-Lactone	Plum			Mash-fermented wine			Juice-fermented wine		
	R (%)	*S* (%)	ppb	*R* (%)	*S* (%)	ppb	*R* (%)	*S* (%)	ppb
Hexa-	88	12	100	86	14	10	89	11	8
Octa-	86	14	70	87	13	4	88	12	3
Nona-	70	30	20	84	16	4	81	19	4
Deca-	91	9	700	92	8	28	92	8	28
Dodeca-	100	0	1600	99	1	12	100	0	14

beer wort, *R*:*S* = 53:47; in beer, *R*:*S* = 74:26; see Fig. 2), indicates that (*R*)-nonalactone has to be regarded as a by-product of fermentation.

Vinous beverages (weinhaltige Getränke)

In Table VI the enantiomeric distribution of two beverages, containing wine and a mixture of fruit products (according to German legislation called "weinhaltige Getränke"), is shown. In both "vinous beverages" large amounts of (*R*)-γ-decalactone were found.

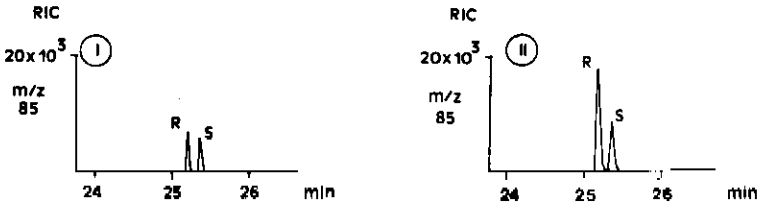

Fig. 2. Enantiomeric distribution of γ-nonalactone in (I) beer wort compared with (II) fermented beer. Reconstructed ion chromatograms (*m*/*z* 85) of selected cuts from the precolumn on a Lipodex B column; see Experimental for details.

TABLE VI

ENANTIOMERIC COMPOSITION AND QUANTITATIVE DISTRIBUTION OF γ-LACTONES IN TWO VINOUS BEVERAGES

γ-Lactone	Beverage I[a]			Beverage II[a]		
	R (%)	*S* (%)	ppb	*R* (%)	*S* (%)	ppb
Hexa-	70	30	10	40	60	15
Octa-			0	70	30	< 5
Nona-	70	30	< 5	70	30	5
Deca-	100	0	3300	98	2	2800

[a] I, with peach flavour; II, "piña colada".

TABLE VII

$^{13}C/^{12}C$ STABLE ISOTOPE RATIOS OF γ-DECALACTONES OF DIFFERENT ORIGIN

γ-Decalactone sample	$\delta^{13}C$ value (by MDGC–IRMS)		$\delta^{13}C$ value (conventional)		$\delta^{13}C$ ranges (literature data)	
	‰ (PDB)	n^a	‰ (PDB)	n^a	‰ (PDB)	Ref.
Synthetic	−28.7[b] ± 0.2	5	−28.7 ± 0.2	3	−24.4 to −28.3	7,21
Microbial	−30.3 ± 0.5	3	−30.2 ± 0.2	3	−30.1 to −31.2	7,21
Beverage I[c]	−30.2 ± 0.3	3				
Beverage II[c]	−30.3 ± 0.2	3				
Peach					−38.5 to −40.9	21
Strawberry					−26.0 to −30.5	16,21

[a] n = Number of samples.

[b] $\delta^{13}C$ values of *R* and *S* enantiomers are within the range of system specification (± 0.3‰) and therefore not distinguishable.

[c] Vinous beverages: I, with peach flavour; II, "piña colada".

$^{13}C/^{12}C$ isotope ratio measurements

Owing to quantitative irregularities in the γ-decalactone content of the above-mentioned vinous beverages, their $\delta^{13}C$ values were determined and compared with data corresponding to γ-decalactone of synthetic and microbial origin (Table VII).

DISCUSSION

A direct chirospecific analysis of γ-lactones on chiral stationary phases is not recommended when dealing with complex flavour extracts, as co-eluting sunstances may interfere with the resolved enantiomers. Additionally, column overloading due to the main components in the sample can affect the column performance. These disadvantages can be avoided by MDGC, as the possibility of heart-cutting allows the selective transfer of the peak group of interest, after preseparation from other interfering constituents of the complex mixture. This is of special importance in the case of isotopic ratio determinations. Despite the high resolving power of MDGC, its application to IRMS measurements requires very careful adjustment of the cut parameters. Because of different elution rates of isotopic compounds (^{13}C compounds precede their ^{12}C counterparts by 10–100 ms), each peak must be integrated over its entire width to obtain the true isotopic ratio [16]. Therefore, partial heart-cutting of a component of interest has to be avoided. Overloading effects, especially with high injection volumes for the determination of trace amounts of lactones, are minimized if a precolumn with high capacity (*e.g.*, wide-bore columns with a 1 2-μm film thickness) is used. MS monitoring allows the control of the heart-cutting and separation efficiency and selective ion monitoring is capable of greater sensitivity for the determination of traces of lactones in enantiomeric ratio determination.

The potential of both methods for the determination of the naturalness of flavours is of particular interest. The legislation that regulates flavouring compounds is not uniform and has led to a differentiation between "natural", "artificial" and in some countries "nature-identical" substances [22].

Generally, the determination of qualitative and quantitative irregularities in the composition of main constituents can be used to detect adulterations due to the addition of single nature-identical and/or artificial flavouring substances. Frequently the determination of the "category" to which an aroma component belongs is difficult, especially if sensorial active traces have been used [22]. If chiral flavour-contributing compounds are detectable, the determination of the enantiomeric distribution is assumed to be an appropriate method to prove the authenticity of samples, as it is expected [4] that fruit-specific distributions with predominances of molecules with *R* or *S* configuration occur.

Our results clearly demonstrate that this assumption has no general validity, and therefore additional information apart from the enantiomeric distribution is necessary. Single-fruit products, *e.g.*, fruit wines, where German legislation does not allow any flavouring additives, are at first glance the easiest to asses. Chirality evaluation is thought to give valuable information with regard to the origin if, in addition to the typical distribution of volatiles due to fermentation, which should be known, the qualitative and quantitative composition shows no irregularities in comparison with data from fresh fruits. Whereas this holds for the strawberry wine analysed, which exhibits typical enantiomeric excesses for the higher γ-lactones (see Table I), it cannot be applied to raspberries, where apart from γ-hexa- and γ-heptalactone, racemic forms are observed (Table II). Similar findings for pineapple concentrates (Table III), yellow passion fruits (Table IV), coconut fat (Fig. 1) and mango [5,23] demonstrate that the presence of racemates cannot *a priori* be interpreted as evidence for adulteration. In such instances, in addition to the typical enantiomeric distribution in fresh fruits, shifts due to processing such as fermentation (Table V), hydrolytic cleavage of precursors and autoxidation of fats have to be considered.

According to the aforementioned considerations it might be stated that for strawberry wine no additives have been used, as data on the enantiomeric ratios and quantitative distribution (taking into account the dilution factor) in fresh fruits and wine are comparable (Table I). However, the dominant (*R*)-γ-decalactone could also be an additive of microbial origin. In this special case, the additional determination of the $\delta^{13}C$ isotopic abundance of γ-decalactone does not definitely enhance the capability to determine the authenticity of the sample. The scarce data available in the literature show that overlapping ranges of $\delta^{13}C$ values can be found for γ-decalactone from strawberries, of synthetic and microbial origin (see Table VII). More definite information for a reliable validation might be obtained if, instead of comparing single isolated $\delta^{13}C$ values, a related set of values in the form of a "fingerprint" is taken into consideration. However, up to now such information is not available. In combination with $\delta^{2}H$ values more possibilities for differentiation could be achieved [24].

A different situation was experienced with the peach-flavoured "vinous beverage" (see Table VI), containing large amounts of nearly exclusively (*R*)-γ-decalactone. Whereas the determination of the enantiomeric distribution gave no evidence for the use of a non-natural lactone, the $\delta^{13}C$ value seems to serve as a useful indicator for the determination of origin. According to literature data [7], γ-decalactone from peach has a $\delta^{13}C$ value of about −40‰. Instead, a value of −30‰ was found (Table VII), which so far on the basis of available data has to be considered as atypical for peach. A microbial origin of this γ-decalactone can be assumed. The large amount of nearly exclusively γ-decalactone supports this finding.

The importance of a comprehensive knowledge of the quantitative and enantiomeric composition of the ingredients of a mixed-fruit product can be illustrated with the results obtained for "piña colada", a vinous beverage labelled as a mixture of wine, pineapple juice and coconut flavour. The data for γ-hexa-, γ-octa- and γ-nonalactone can be attributed to pineapple and wine (see Table III and V), respectively, whereas the large amount of (*R*)-γ-decalactone does not correspond with the raw materials processed. Measurement of the carbon isotopic ratio revealed that this lactone ($\delta^{13}C$ value −30‰, see Table VII) might be of biotechnological origin.

These results are important, as German legislation does not allow the addition of "isolated flavour compounds" [10] or those isolated from natural sources (including microbial origin) to vinous beverages ("weinhaltige Getränke").

CONCLUSIONS

The exclusive use of the enantiomeric distribution of γ-lactones (occurrence of racemates or predominant *R* enantiomers) generally is not suitable for drawing conclusions about the authenticity or adulteration of samples.

Variations in the enantiomeric ratio due to varietal differences and technological influences, and also the quantitative lactone distribution, have to be considered as additional parameters in order to be able to make realiable statements.

Validation of naturalness based solely on isolated $^{13}C/^{12}C$ isotope ratio measurements of γ-lactones is not always reliable, as overlapping ranges of $\delta^{13}C$ values are encountered. The inclusion of additional $\delta^{13}C$ values for fruit typical components in form of "fingerprints" is recommended.

Comprehensive data on carbon isotope ratios of flavour compounds together with the consideration of possible variations due to external influences should be elaborated in order to permit a more definite evaluation of genuiness.

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