

# Methyl-Branched Flavor Compounds in Fresh and Processed Apples

Katja Schumacher,<sup>†</sup> Sven Asche,<sup>†</sup> Martin Heil,<sup>†</sup> Frank Mittelstädt,<sup>†</sup> Helmut Dietrich,<sup>‡</sup> and Armin Mosandl<sup>\*†</sup>

Institut für Lebensmittelchemie, Johann Wolfgang Goethe-Universität Frankfurt, Marie-Curie-Strasse 9, D-60439 Frankfurt (Main), Germany, and Forschungsanstalt für Weinbau, Gartenbau, Getränketechnologie und Landespflege, Von-Lade-Strasse 1, D-65366 Geisenheim, Germany

Methyl-branched volatiles in apples and processed apples are investigated. The ratio of 3-methyl-branched and 2-methyl-branched flavor compounds was determined and the enantiomeric distribution of the latter investigated. The enantiomeric ratios of 2-methyl-branched volatiles are analyzed, using heptakis(2,3-di-*O*-methyl-6-*O*-*tert*-butyldimethylsilyl)- $\beta$ -cyclodextrin as an efficient enantioselective stationary phase in capillary GC. 2-Methylbutanoic acid and the corresponding esters (methyl-*n*-hexyl ester), 2-methylbutanol, and 2-methylbutyl acetate were detected as genuine flavor compounds favoring (*S*)-configuration and high enantiomeric purity (>99%). 3-Methylbutanoic acid, 3-methylbutanol, and 3-methylbutyl acetate are proved to be not genuine compounds of the apple aroma but fermentation products.

**Keywords:** *Apple flavor; methyl-branched volatiles; enantioselective capillary gas chromatography; enantioselective multidimensional gas chromatography (enantio-MDGC)*

## INTRODUCTION

Apples are of great importance in the German fruit market. Four of five orchards in Germany consist of apple trees (Statistisches Bundesamt, 1997). Thus, there is a strong demand for apple products as food-stuffs. The per capita consumption of apple juice in Germany amounts to ~12.3 L per annum; this is higher than that of any other fruit juice, even orange juice (Verband der Deutschen Fruchtsaft-Industrie e.V., 1997). In the United States, apple juice accounts for about two-thirds of non-citrus juice consumption (National Agricultural Statistics Service, 1997).

In accordance with the strong interest in apple aroma compounds, there are numerous studies on this subject, going back to the beginning of this century (Dimick et al., 1983). In the 1960s, gas chromatography gained more and more importance for the investigation of apple volatiles (Flath et al., 1967; Drawert et al., 1969). The examination included both complete coverage of all constituents and focus on individual compounds; for example, the enantiomeric distributions were investigated (Rettinger et al., 1991; Haase-Aschoff et al., 1991; Karl et al., 1992).

The technological influence on the volatiles was always of interest. In 1965, Drawert et al. showed that there is an obvious difference between the aroma of fresh apples and the aroma of the juice (Drawert et al., 1965). Secondary aroma compounds were identified and investigated (Drawert et al., 1966). Furthermore, detailed studies about the generation and changes of apple flavor during maturation were reported (Drawert et al., 1969). Recently, further important topics were described, such as maturation in controlled atmosphere

(CA) storage and aroma modification by the addition of precursors into the storage atmosphere (Harb et al., 1994).

Methyl-branched components and their importance as flavor impact compounds of apples are the subjects of this investigation.

3-Methyl-branched volatiles are often cited in the literature as apple constituents (Maarse et al., 1989). However, other publications are contradictory, and only small amounts of 3-methylbutanal (Flath et al., 1967) and 2-methyl-branched acid, esters, and alcohol were identified (Karl, 1994).

Furthermore, in view of earlier investigations (Mosandl et al., 1990; Rettinger et al., 1991), the enantiomeric distribution of 2-methylbutanoic acid (**12**), its esters, and the corresponding alcohol 2-methylbutanol (**6**) should be investigated in more detail.

The following products were used: fresh apples, apple juice, German Apfelwein, cider, Calvados, and apple liqueur. Apple juice is produced by pressing healthy fruits, suitable for consumption.

Cider and Apfelwein are produced from apple juice by spontaneous fermentation or by the addition of cultivated yeast. In the literature, the terms cider and Apfelwein are often used synonymously. Nevertheless, on the German market these two products can be clearly distinguished. Generally, Apfelwein contains less residue sugar and has a higher content of total acid.

Calvados, coming from the Normandy region, is distilled from cider. Apple liqueur is a sweet spirit; its apple-like taste is due to fruit essences, natural or nature-identical flavor compounds.

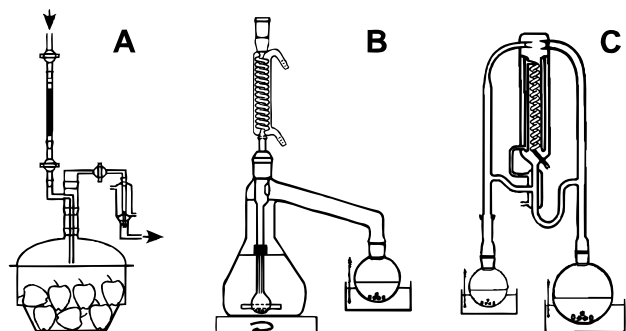
## MATERIALS AND METHODS

**Material.** The apple spirit (Calvados) and liqueur as well as the various ciders were obtained from the market. Fresh apples, apple juices, and the German Apfelwein were provided

\* Author to whom correspondence should be addressed.

<sup>†</sup> Johann Wolfgang Goethe-Universität Frankfurt.

<sup>‡</sup> Forschungsanstalt Geisenheim.



**Figure 1.** Apparatus used for sample preparation: dynamic headspace (A); liquid-liquid extractor according to Ludwig (B); simultaneous distillation-extraction, Likens-Nikerson, as modified by Schultz et al. (C).

by the Forschungsanstalt für Weinbau, Gartenbau, Getränke-technologie und Landespflege, Geisenheim.

**Sample Preparation.** The flavors of apples and their products were extracted by the following methods.

**Dynamic Headspace.** A desiccator was filled with ~2 kg of fresh apples. Their volatiles were absorbed with Tenax GC (2,6-diphenylparaphenylene oxide polymer; AKZO, Fibers and Polymers, Wuppertal, Germany). Desorption was performed by elution with 5 mL of distilled diethyl ether. Apparatus (Figure 1A) and protocol were as described by Karl et al. (1993).

**Solvent Extraction.** This procedure was used for juices and other liquid apple products. The extraction was started with 0.75 L of liquid samples, which were filled into a 3 L liquid-liquid extractor (Ludwig, 1972) (Figure 1B). The extractor was topped up with deionized water and solution extracted with pentane/diethyl ether (1 + 1; v + v) during 14 h.

**Simultaneous Distillation-Extraction.** This method was used for both the study of apples of different ripeness and for the investigation of apple juice produced from definite varieties, such as Melrouge and Granny Smith, respectively. The apples were either crushed or treated by a juice extractor. The mash and juice were treated by a Likens-Nikerson apparatus, as modified by Schultz et al. (1977) (Figure 1C). The volatiles were extracted within 2 h, using diethyl ether as a solvent.

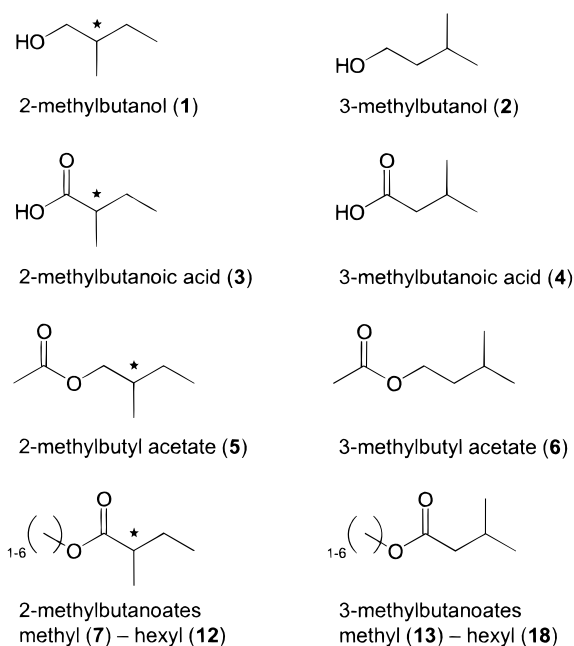
Subsequently, aroma extracts were dried over  $\text{Na}_2\text{SO}_4$  and concentrated to a volume of ~1 mL. These concentrated extracts were ready for chromatographic analysis.

**Acetylation.** One milliliter of diethyl ether extract of the samples was added with 100  $\mu\text{L}$  of glacial acetic acid and 10 mg of *p*-toluenesulfonic acid. After 12 h, the mixture was washed one time with 2%  $\text{NaHCO}_3$  solution and two times with saturated NaCl. The organic phase was dried over  $\text{NaSO}_4$ .

**Gas Chromatography (GC).** *System 1.* A Carlo Erba Mega HRGC 5160 was used, equipped with a split injector and a flame ionization detector (FID). A capillary column, 30 m  $\times$  0.23 mm i.d. ( $d_f = 0.25 \mu\text{m}$ ), was coated with 25% heptakis-(2,3-di-*O*-methyl-6-*O*-*tert*-butyldimethylsilyl)- $\beta$ -cyclodextrin (DIME- $\beta$ -CD) in PS 268. Conditions: carrier gas,  $\text{H}_2$  at 1.25 mL/min; injector temperature, 240 °C; detector temperature, 240 °C; split, 30 mL/min; temperature program, 40 °C held for 10 min and then increased by 1.0 °C/min to 100 °C.

*System 2.* A Carlo Erba Mega HRGC 5160 was used, equipped with a split injector and an FID. A capillary column, 50 m  $\times$  0.32 mm i.d. ( $d_f = 0.20 \mu\text{m}$ ), was coated with CP-Carbowax 400 (Chrompack, Middelburg, The Netherlands). Conditions: carrier gas,  $\text{H}_2$  at 2.4 mL/min; injector temperature, 240 °C; detector temperature, 240 °C; split, 30 mL/min; temperature program, 55 °C isothermal.

**Moving Capillary Switching System (MCSS).** *System 3.* A DB-5 fused silica column (30 m  $\times$  0.32 mm i.d., film thickness = 0.25  $\mu\text{m}$ ) was used as the precolumn and a fused silica column, coated with DIME- $\beta$ -CD (30 m  $\times$  0.32 mm i.d., film thickness = 0.32  $\mu\text{m}$ ) as the main column. Both columns were installed into a Fisons GC 8000 gas chromatograph and coupled via an MCSS (Fisons Instruments, Egelsbach, Ger-



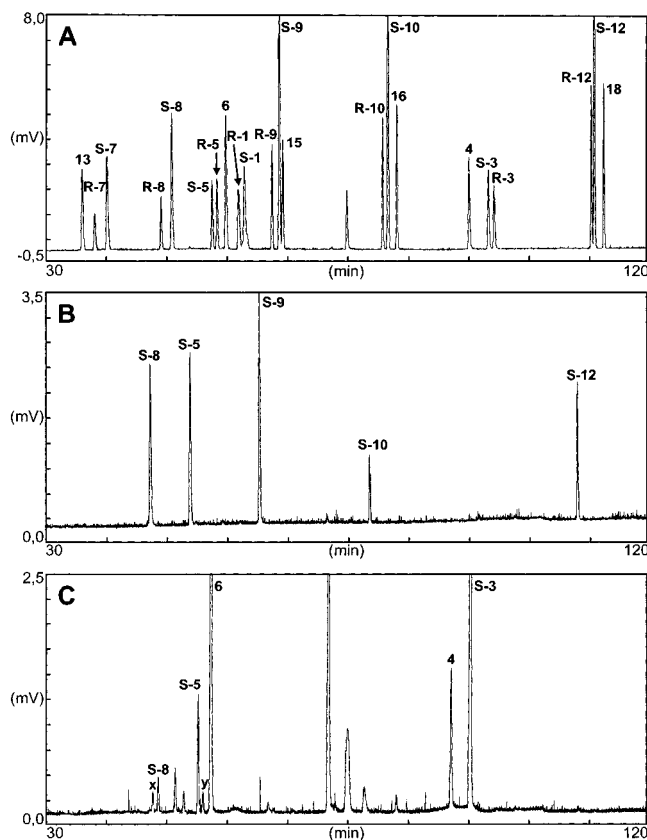
**Figure 2.** Examined compounds.

many). The MCSS was equipped with a split injector and two FIDs on the outlet of the precolumn and the main column. Conditions: carrier gas precolumn, He at 0.89 mL/min; carrier gas main column, He at 0.98 mL/min; injector temperature, 240 °C; detector temperature each, 240 °C; split, 30 mL/min; temperature program, 40 °C held for 10 min, then increased by 1.0 °C/min to 50 °C, held for 10 min, then increased by 1.0 °C/min to 140 °C, held for 0 min, then increased by 2.0 °C/min to 200 °C, and held for 20 min.

**Multidimensional Gas Chromatography/Mass Spectrometry (MDGC/MS).** *System 4.* The MDGC/MS analysis was performed by a Siemens Sichromat 2-8, equipped with two independent column ovens and a live-T-switching. The precolumn was of duran glass (30 m  $\times$  0.25 mm i.d.), coated with PS268 (film thickness = 0.4  $\mu\text{m}$ ) and fronted with a deactivated fused silica column (2 m  $\times$  0.25 mm i.d.). The main column was of duran glass (30 m  $\times$  0.32 mm i.d.), coated with 30% DIME- $\beta$ -CD in SE 52 (film thickness = 0.6  $\mu\text{m}$ ). The Sichromat was equipped with a split injector and an FID on the outlet of the precolumn. The main column was coupled to the transfer line of a Finnigan MAT ITD 800, using an open split interface. Conditions: carrier gas precolumn, 1.2 bar of  $\text{H}_2$ ; carrier gas main column, 0.7 bar of  $\text{H}_2$ ; injector temperature, 220 °C; split, 20 mL/min; detector temperature (FID), 280 °C; temperature program precolumn, 40 °C increased by 3.0 °C/min to 250 °C and held for 20 min; temperature program main column, 40 °C held for 5 min, then increased by 1.5 °C/min to 80 °C, and then increased by 2.0 °C/min to 200 °C. ITD 800: transfer line, 250 °C; source temperature, 250 °C; EI, 70 eV.

## RESULTS AND DISCUSSION

DIME- $\beta$ -CD has proved to be a highly efficient chiral stationary GC phase for the simultaneous analysis of 2-methylbutanoic acid (3), the corresponding esters (7-12), 2-methylbutanol (1), and 2-methylbutyl acetate (5). Furthermore, nearly all enantiomers were successfully separated from their 3-methyl-branched constitutional isomers (compounds given in Figure 2). In this way 22 stereoisomers of methyl-branched flavor compounds were successfully analyzed (Figure 3A). [Normally, the enantiomers of pentyl 2-methylbutanoate (11) and the isomer pentyl 3-methylbutanoate (17) were separated by system 3; however, they coeluted with the enantiomers of the 2-methylbutanoic acid (3). For this reason



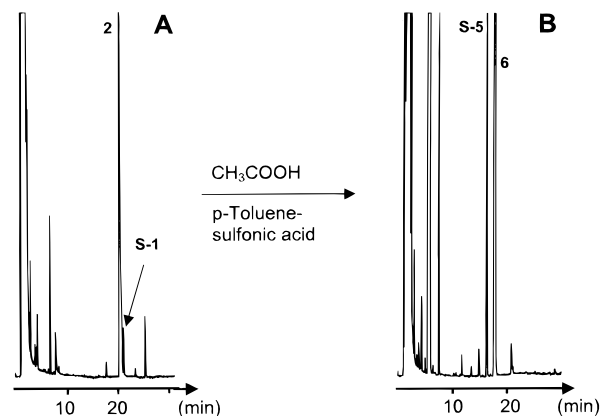
**Figure 3.** Chromatogram system 3: mixture of reference substances (A); headspace aroma of McIntosh apples (B); liquid-liquid extract of British cider (C). 2-Methylbutanol (1); 2-methylbutanoic acid (3); 3-methylbutanoic acid (4); 2-methylbutyl acetate (5); 3-methylbutyl acetate (6); 2-methylbutanoates (7–10, 12); 3-methylbutanoates (13, 15, 16, 18); *R*, *S* corresponding (*R*)- and (*S*)-enantiomers; *x* and *y* unknown compounds.

these compounds were not included in the standard mixture. Anyway, as we know from earlier investigations (Schumacher, 1998), pentyl 2-methylbutanoate (11) occurs only in very small amounts in the flavor of apples.]

The 3-methyl-branched isomers of 2-methylbutanol (1) and ethyl 2-methylbutanoate (8) eluted in the above-described MCSS, a "one-oven MDGC" system (system 3) between the enantiomers. For this reason the "cut" times (transfer of substances from the precolumn onto the main column via the MCSS) were set in such a way that the 3-methyl-branched isomers were left on the precolumn, to improve baseline separation of the 2-methyl-branched enantiomers.

Ethyl 3-methylbutanoate (14) is detected in the precolumn chromatogram. Several methods were available for quantifying the ratio of the 3-methyl- (2) and 2-methylbutanol (1), depending on kind of samples analyzed.

In the case of spirits, the alcohols (1, 2) were derivatized (acetic acid/*p*-toluenesulfonic acid) and analyzed as the corresponding acetates (5, 6) using system 1 (Figure 4). In these samples the content of the fusel alcohols, 2-methylbutanol (1) and 3-methylbutanol (2), was 100-fold higher than the content of 2-methylbutyl acetate (5) and 3-methylbutyl acetate (6), so the latter did not affect the results of the quantification significantly. However, this method was not suitable for apple juices because of their lower content of these alcohols.



**Figure 4.** Acetylation of the British cider (chromatograms using system 1) sample before (A) and after acetylation (B). 2-Methylbutanol (1); 3-methylbutanol (2); 2-methylbutyl acetate (5); 3-methylbutyl acetate (6); *S* corresponding (*S*)-enantiomer.

**Table 1.** Enantiomeric Distribution of the Headspace Volatiles from Fresh Apples, Evaluated by MCSS (System 3) or by MDGC/MS Analysis (System 4)<sup>a</sup>

headspace sample	5		8		9		10		12	
	% <i>S</i>	% <i>R</i>	% <i>S</i>	% <i>R</i>	% <i>S</i>	% <i>R</i>	% <i>S</i>	% <i>R</i>	% <i>S</i>	% <i>R</i>
McIntosh	>98	nd <sup>b</sup>	>98	nd	>99	nd	>96	nd	>98	nd
Idared	>98	nd	nd	nd	>75	nd	>92	nd	>98	nd
Jonica	>99	nd	nd	nd	>97	nd	>99	nd	>97	nd
Melrouge	– <sup>c</sup>	–	99.8 <sup>a</sup>	0.2 <sup>a</sup>	–	–	–	–	–	–
Granny Smith	–	–	99.5 <sup>a</sup>	0.5 <sup>a</sup>	–	–	–	–	–	–

<sup>a</sup> Evaluated by system 4. 2-Methylbutyl acetate (5); 2-methylbutanoic acid ethyl ester (8), propyl ester (9), butyl ester (10), hexyl ester (12). <sup>b</sup> nd, not detectable. <sup>c</sup> –, not examined.

Underivatized isomeric alcohols (1, 2) were separated on Carbowax 400 (system 2).

The main column chromatograms (system 3) of apple samples investigated are outlined in Figure 3B,C. Changing retention times during a longer sequence of analysis are compensated by co-injection of reference substance to the samples in an additional run. The headspace analysis of McIntosh apples exclusively detects 2-methylbutanoic acid esters (8–10, 12) and the 2-methylbutyl acetate (5), as can be seen from Figure 3B. In Figure 3C the main column chromatogram of British cider liquid extract is shown. The cider is dominated by the alcohols (1, 2), acids (3, 4), and acetates (5, 6). In the presented chromatographic run the alcohols (1, 2) were left on the precolumn, to improve the separation of the other compounds. However, Figure 3C also demonstrates the difficulties in separating so many components in one chromatographic run. Peak *y* simulates the presence of (*R*)-2-methylbutyl acetate (*R*-5) in this sample. By transferring only 2-methylbutyl acetate (5) from the precolumn onto the main column, compound *y* is proved to be not identical with (*R*)-2-methylbutyl acetate (*R*-5). Peak *x* might be mixed with (*R*)-ethyl 2-methylbutanoate (*R*-8), but the co-injection of the reference substance confirmed that (*R*)-ethyl 2-methylbutanoate (*R*-8) is eluted at a slightly different retention time.

Table 1 shows the headspace analysis of whole, fresh apples of different varieties. Exclusively, 2-methyl-branched esters are detectable and easily distinguished from the flavor of processed apple products (see Figure 3B,C). The headspace extracts are free of 2-methylbutanoic acid (3), and only in some cases are small amounts of 2-methylbutanol (1) detectable. The "greater

**Table 2. Ratio of 2- and 3-Methyl-Branched Volatiles and Enantiomeric Distribution of 2-Methyl-Branched Compounds from Liquid Apple Products<sup>a</sup>**

apple product	[%] 2 of T(1, 2)		[%] 4 of T(3, 4)		[%] 6 of T(5, 6)		[%] 14 of T(8, 14)	
Calvados	81.7		21.2		92.3		tr <sup>d</sup>	
apple liqueur	75.4		nd <sup>d</sup>		70.2		nd	
British cider	81.2		18.7		84.8		nd	
French cider	77.2		12.5		82.8		nd	
Hessian cider	80.7		19.2		89.8		nd	
Apfelwein	80.4		32.5		85.3		nd	
apple juice 1	1.4		3.7		10.4		nd	
apple juice 2, <sup>c</sup> pure Gloster	1.0		0.7		9.4		nd	

apple product	1		3		5		8	
	% S	% R	% S	% R	% S	% R	% S	% R
Calvados	>99	nd	>99	nd	>96	nd	>95	nd
apple liqueur	>98	nd	>97.1	2.9	>97	nd	>93.3	6.7
British cider	>99	nd	>99	nd	>98	nd	>80	nd
French cider	>99.5	nd	>98	nd	>97	nd	>76	nd
Hessian cider	>99.7	nd	>99	nd	>99	nd	>80	nd
Apfelwein	>99.8	nd	>99	nd	>98	nd	>90 <sup>b</sup>	nd
apple juice 1	>98	nd	>99	nd	>97	nd	>99.7 <sup>b</sup>	0.3 <sup>b</sup>
apple juice 2, <sup>c</sup> pure Gloster	>99	nd	>99.6	nd	>92	nd	>99.8 <sup>b</sup>	0.2 <sup>b</sup>
apple juice 3	>99	nd	>99	nd	>92	nd	>99.7 <sup>b</sup>	0.3 <sup>b</sup>
apple juice 4, pure Melrouge	–	–	>99	nd	–	–	>99.8 <sup>b</sup>	0.2 <sup>b</sup>
apple juice 5, pure Granny Smith	–	–	>99	nd	–	–	>99.5 <sup>b</sup>	0.5 <sup>b</sup>

<sup>a</sup> [%] Y of T(x, y) = percentage of compound y of the total content of compounds x and y; figures marked by b were results of MDGC/MS analysis (system 4). <sup>c</sup> Supplementary: [%] (S)-2-methylbutanoic acid methyl ester (S-7) >90 and [%] (S)-2-methylbutanoic acid hexyl ester (S-12) >77; 2-methylbutanol (1); 3-methylbutanol (2); 2-methylbutanoic acid (3); 3-methylbutanoic acid (4); 2-methylbutyl acetate (5); 3-methylbutyl acetate (6); ethyl 2-methylbutanoates (8); ethyl 3-methylbutanoic acid (14). <sup>d</sup> nd, not detectable; tr, trace. <sup>e</sup> –, not examined.

than (>)” sign prefixed to the percentages indicates the accuracy of the measurement, depending on the amount of the compound to be analyzed. Thus, percentages of the (S)-enantiomers somewhat smaller than 100% give no conclusive indication of the amount of the corresponding (R)-enantiomers. As their true amounts are far too low, (R)-enantiomers as well as the corresponding 3-methyl-branched compounds are not detectable.

**Table 3. Ratio of 2- and 3-Methyl-Branched Volatiles and Enantiomeric Distribution of 2-Methyl-Branched Compounds from Apples with Different Ripenesses<sup>a</sup>**

study with rotten apples	[%] 2 of T(1, 2)		[%] 4 of T(3, 4)		ratio of 2- and 3-methylbutyl acetates and ratio of 2- and 3-methylbutanoic acid esters												
	% S	% R	% S	% R	1		3		5		7		8		9		12
Gloster I	tr <sup>b</sup>		nd <sup>b</sup>		>96	nd	>98	nd	>98	nd	>98	nd	>98	nd	>97	nd	
Gloster II	5.0		nd		>90	nd	>95	nd	>90	nd	>95	nd	>95	nd	>90	nd	
Gloster III	10.2		nd		nd	nd	nd	nd	nd	nd	>95	nd	nd	nd	nd	nd	
Idared I	nd		nd		nd	nd	>98	nd	nd	nd	nd	nd	nd	nd	>80	nd	
Idared II	tr		nd		nd	nd	>99	nd	nd	nd	nd	nd	nd	nd	>83	nd	
Jonagold I	1.2		nd		nd	nd	>98	nd	nd	nd	nd	nd	nd	nd	>98	nd	
Jonagold III	39.5		nd		nd	nd	>98	nd	nd	nd	nd	nd	nd	nd	>98	nd	

<sup>a</sup> I, fresh uninjured apples; II, intact apples with several brown bruises; III, moldy, completely rotten apples with disintegrated cell tissue; [%] Y of T(x, y) = percentage of compound y of the total content of compounds x and y; 2-methylbutanol (1); 3-methylbutanol (2); 2-methylbutanoic acid (3); 3-methylbutanoic acid (4); 2-methylbutyl acetate (5); 2-methylbutanoates (7–9, 12). <sup>b</sup> nd, not detectable; tr, trace.

In Table 2 apple products are investigated. The upper part of the table shows the evaluation of the isomeric ratios. The analysis is realized by MCSS (system 3) under the condition of complete substance transfer from the precolumn onto the main column (“cut”). Furthermore, all results are verified by system 1.

Obviously, the 3-methyl-branched compounds are characteristics of fermented products. In apple juices only small amounts of 3-methylbutanol (2), 3-methylbutanoic acid (4), and 3-methylbutyl acetate (6) are found. Esters are not detectable, apart from some traces of ethyl 2-methylbutanoate (8) in Calvados. It is interesting to note the differing lower ratio of 3-methylbutanoic acid (4) to the total content of 3-methyl- (4) and 2-methylbutanoic acid (3) in comparison with the ratio of the corresponding alcohols (1, 2) and acetates (5, 6). The higher content of 3-methylbutanoic acid (4) in German Apfelwein significantly differs from the ciders analyzed. The analysis of the liqueur is not comparable with other results because this liqueur does not originate from natural apple essence, as can be seen from enantioselective analysis.

Furthermore, the enantiomeric purity of the 2-methyl-branched constituents is listed in Table 2. The aroma profile of the processed apple products is dominated by 2-methylbutanoic acid (3), 2-methylbutanol (1), and 2-methylbutyl acetate (5). The other esters are hydrolyzed to a large extent. Only small amounts of 2-methylbutanoic acid ethyl ester (8) are detected. This is why a relatively high detection limit of (R)-ethyl 2-methylbutanoate (R-8) is discussed in relation to the total amount of ethyl 2-methylbutanoate (8). Methyl 2-methylbutanoate (7) and hexyl 2-methylbutanoate (12) are found only exceptionally, favoring high-grade surplus of (S)-configuration. Obviously, the content of ~0.3% (R)-enantiomer is a characteristic of apples. Decreased enantiomeric purities of chiral apple liqueur compounds may be explained by the addition of nature-identical flavor compounds.

In the third part of this study apples of varying ripeness were examined. The ripeness is subdivided into three degrees: fresh, uninjured apples (I); intact apples with several brown bruises (II); moldy, com-

pletely rotten apples with disintegrated cell tissue (III). Above all, it should be clarified whether microbiological contamination influences the enantiomeric distribution. In the upper part of Table 3 the isomeric ratios of the alcohols (**1**, **2**), acids (**3**, **4**), and esters (**5** – **18**) are outlined. As expected, fresh apples are nearly free of 3-methyl-branched volatiles; only very small amounts of 3-methylbutanol (**2**) are detectable. During the loss of freshness the content of 3-methylbutanol (**2**) increases.

The evaluation of the enantiomeric distribution of the 2-methyl-branched compounds in the lower half of Table 3 proves that microbiological contamination did not cause racemization.

## CONCLUSIONS

2-Methylbutanoic acid (**3**), the corresponding methyl-hexyl esters (**7**–**12**), 2-methylbutanol (**1**), and 2-methylbutyl acetate (**5**) are impact flavor compounds of apple aroma. They are nearly completely dominated by their (*S*)-enantiomers. Only small amounts of their (*R*)-enantiomers (~0.3%) are detectable. Obviously, this ratio is a characteristic of apples.

3-Methyl-branched volatiles are often reported as constituents of apple aroma. This investigation proves that these compounds are not genuine apple compounds. They derive from the metabolism of microorganisms that infect apples naturally or by purposeful introduction. The small amounts in apple juice might be explained by the beginning of fermentation that cannot be prevented completely. Nevertheless, the ratios are clearly distinguishable from those of fermented products.

## ACKNOWLEDGMENT

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