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High performance liquid chromatography-electrospray ionization tandem mass spectrometry (HPLC-ESI-MS-MS) for the quantification of L-kynurenine and indole-3-acetic acid in grape must by isotope dilution assay

by

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S u m m a r y : Isotope dilution assay was used for the qualitative and quantitative analysis of L-kynurenine and indole-3-acetic acid in grape must. After solid phase extraction, highest selectivity and sensitivity was achieved by high performance liquid chromatography-electrospray ionization tandem mass spectrometry (HPLC-ESI-MS-MS) using selected reaction monitoring (SRM). In the 24 samples under study, the amounts of L-kynurenine and indole-3-acetic acid ranged from 0 to 94 $\mu g \cdot l^{-1}$ and from 20 to 380 $\mu g \cdot l^{-1}$, respectively. These compounds are considered as potential precursors of 2-aminoacetophenone, causing the "untypical aging off-flavour" in *Vitis vinifera* white wines.

Hochdruckflüssigchromatographie-Elektrospray-Tandem-Massenspektrometrie (HPLC-ESI-MS-MS) zur Bestimmung von Kynurenin und 3-Indolylessigsäure in Traubenmost mittels Isotopenverdünnungsanalyse

Z u s a m m e n f a s s u n g : Zur qualitativen und quantitativen Analyse von L-Kynurenin und 3-Indolylessigsäure in Traubenmost wurde die Isotopenverdünnungsanalyse eingesetzt. Nach Probenaufarbeitung durch Festphasenextraktion wurde höchste Selektivität und Empfindlichkeit bei Anwendung der Hochdruckflüssigchromatographie-Elektrospray-Tandem-Massenspektrometrie im "selected reaction monitoring" (SRM)-Modus erreicht. In den 24 untersuchten Proben sind Gehalte von 0 bis 94 $\mu g \cdot l^{-1}$ L-Kynurenin und 20 bis 380 $\mu g \cdot l^{-1}$ 3-Indolylessigsäure ermittelt worden. Beide Verbindungen werden als potentielle Vorläufer von 2-Aminoacetophenon, der für den "untypischen Alterston" in Weißwein aus *V. vinifera*-Sorten verantwortlichen Fehlaromakomponente, diskutiert.

K e y w o r d s : electrospray ionization, grape must, indole-3-acetic acid, isotope dilution assay, L-kynurenine, tandem mass spectrometry, "untypical aging off-flavour".

Introduction

L-kynurenine and indole-3-acetic acid are considered as potential precursors of 2-aminoacetophenone (Christoph *et al.* 1996; Dollmann *et al.* 1996; Gessner *et al.* 1996), an aroma compound known to be responsible for the "untypical aging off-flavour" in *Vitis vinifera* white wines (Rapp *et al.* 1993). Thus, information is required about the amounts of the precursors in grape must, from which they can be transformed by fermentation to the "off-flavour" compound. While indole-3-acetic acid is an ubiquitous plant hormone (Arteca 1996), L-kynurenine has not been reported in grapes or wines to date.

Several methods have been developed to determine these compounds in various matrices. Indole-3-acetic acid can be analysed as methyl ester derivative by GC-MS (Badenoch-Jones *et al.* 1995); an isotope dilution assay has also been established (Cohen *et al.* 1986). Recently an HPLC-ESI-MS-MS quantification method with isotope dilution was published for the indole-3-acetic acid methyl ester in bacteria (Prinsen *et al.* 1997). For kynurenine analysis HPLC is a common technique, but also electron capture negative ion chemical ionization mass spectrometry using the pentafluorobenzyl bromide derivatives has been used to study the tryptophan metabolism in cultured cells and physiological fluids (Boni *et al.* 1994). Recently, capillary electrophoresis has been employed to determine tryptophane and indole-3-acetic acid in serum (Petucci *et al.* 1995) as well as tryptophane and kynurenine in brain (Malone *et al.* 1995). As far as we know, a method for the simultaneous qualitative and quantitative trace analysis of both compounds without derivatization is not available to date. In this paper, a new method is described combining HPLC with tandem mass spectrometry under electrospray conditions (HPLC-ESI-MS-MS) to analyze simultaneously L-kynurenine and indole-3-acetic acid in grape must by isotope dilution assay.

Materials and methods

G r a p e m u s t s : Twenty-four must samples (vintage 1996), taken from the usual winemaking process (Willmes pneumatic press) were immediately frozen at -18 °C and continuously analyzed after thawing (Table).

C h e m i c a l s : L-kynurenine and indole-3-acetic acid (Fluka, Neu-Ulm, Germany), D_5-L-tryptophan and $^{13}C_6$-indole-3-acetic acid (Promochem, Weser, Germany;

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Cambridge Isotope Laboratories, Andover, MA, USA) were used. Furthermore water, methanol, acetonitrile, all of HPLC gradient grade, and trifluoroacetic acid (spectroscopic grade) as well as LiChroprep® RP-18 material for liquid column chromatography (Merck, Darmstadt, Germany), Dowex® WX 8 ion exchange resin (Serva, Heidelberg, Germany) and Isolute® C_{18} (3 ml, 500 mg) solid phase extraction (SPE) columns (ICT, Bad Homburg, Germany) were applied.

Synthesis of d_4-L-kynurenine by photooxygenation of d_5-L-tryptophan: According to NAKAGAWA *et al.* (1985) 100 mg (0.48 mM) d_5-L-tryptophane, 4 mg methylene blue and 1.5 ml ethanol were given into 40 ml 5 % Na_2CO_3-acetic acid buffer (pH 7.0). The mixture was irradiated at 0 °C with 500 W 589 nm under a stream of oxygen. The reaction was stopped after 30 min, 400 µl dimethylsulfide were added and the mixture stirred again for 1 h. Solvents were evaporated and the pH adjusted to 2.5 with acetic acid. The product was separated from dye by ion exchange on Dowex® 50 WX 8 and purified on LiChroprep® RP-18 (40-63 µm) using H_2O (pH 2.5, acetic acid) containing 3 % acetonitrile. ^{1}H-NMR (D_2O): δ = 3.67 (d, J = 5.48 Hz, 2 H [CH_2]), 4.11 ppm (t, J = 5.48 Hz, 1 H [CH]). ^{13}C-NMR (D_2O, d_4-methanol): δ = 39.87 [CH_2], 51.84 [CH], 117.58 [$C_{arom.}$], 118.34 [$C_{arom.}$], 118.85 [$C_{arom.}$], 132.12 [$C_{arom.}$], 136.43 [$C_{arom.}$], 151.38 [$C_{arom.}$], 174.59 [COOH], 201.04 ppm [CO].

Sample preparation: Isotope labeled internal standards were added to 10 ml of grape must in amounts ranging from 70 to 120 µg·l^{-1}. The sample was centrifuged at 3000 *g* for 5 min and the supernatant filtered using a 0.2 µm membrane. The filtrate was applied to a C_{18}-SPE cartridge (3 ml, 500 mg), which had been conditioned with 1 ml methanol followed by 1 ml of 0.2 M acetic acid. The SPE column was washed with 1 ml water and subsequently eluted with 1.5 ml methanol/ammonia (25 % in water) 9:1 (v/v). The eluate was evaporated to dryness in a stream of nitrogen and the residue redissolved in 100 µl of water. The sample was filtered through a 0.2 µm-membrane prior to HPLC analysis.

Analytical procedure: L-kynurenine, d_4-L-kynurenine, d_5-L-tryptophan and indole-3-acetic acid, $^{13}C_6$-indole-3-acetic acid reference solutions were prepared by dissolving 1 mg standard in 1 ml water and 1 ml ethanol, respectively. The stock solutions were diluted 1:100 for using as standard solutions. Analysis was performed on a triple stage quadrupole TSQ 7000 LC-MS-MS system with ESI interface (Finnigan MAT, Bremen, Germany). Data acquisition and mass spectrometric evaluation were conducted on a Personal DECstation 5000/33 (Digital Equipment, Unterföhring, Germany) and ICIS 8.1 software (Finnigan MAT, Bremen, Germany). For HPLC an Applied Biosystems dual syringe pump model 140 B (bai, Bensheim, Germany) was used. Separations were performed on an Eurospher 100-C18 column (100 x 2.0 mm

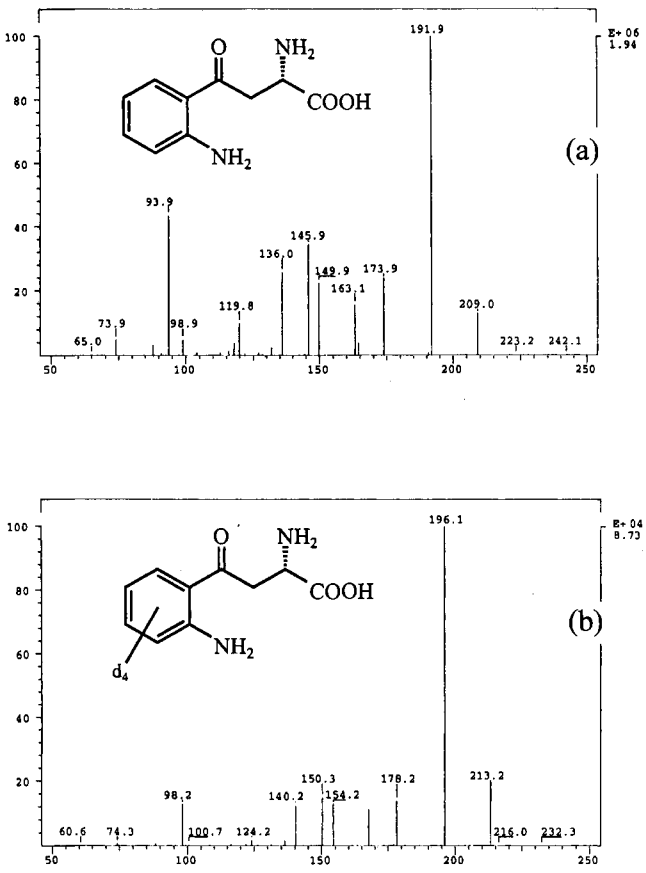

Fig. 1: Product ion mass spectrum of L-kynurenine (**a**) and d_4-L-kynurenine (**b**) obtained by collision-induced dissociation (CID) (argon pressure 0.234 Pa, offset voltage 15 V).

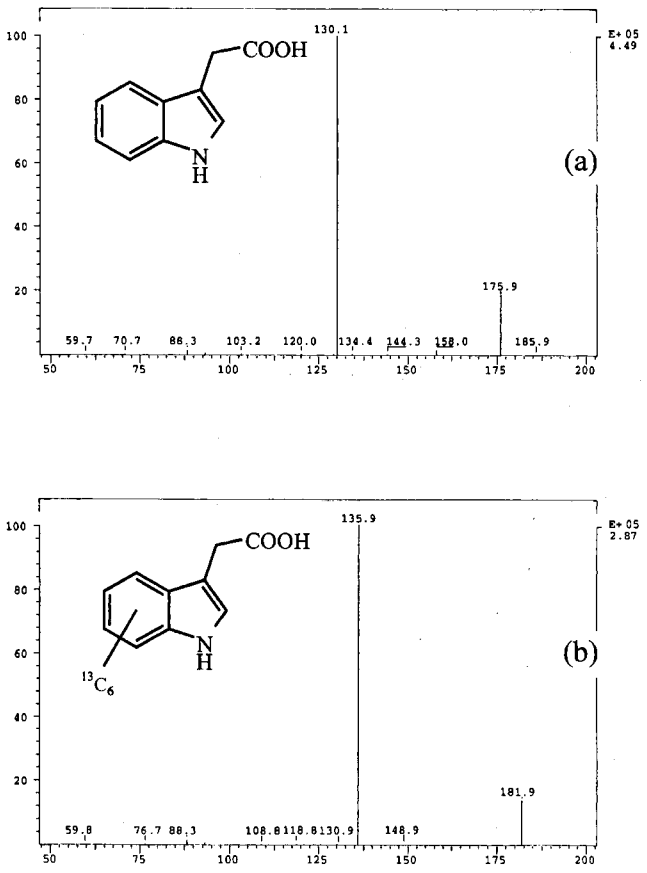

Fig. 2: Product ion mass spectrum of 3-indole-acetic acid (**a**) and $^{13}C_6$-3-indole-acetic acid (**b**) obtained by collision-induced dissociation (CID) (argon pressure 0.234 Pa, offset voltage 15 V).

i.d., 5 µm; Knauer, Berlin, Germany) using a linear gradient. Solvent A was water containing 0.05 % trifluoroacetic acid, solvent B acetonitrile. The gradient program was as follows: 5 % B to 35 % B in 20 min. Solvent flow was 200 µl min^{-1}. A Spark Holland Triathlon autosampler (SunChrom, Friedrichsdorf, Germany) was used to inject 10 µl of the sample by µl-pick-up mode. For the mass spectrometer the following parameters were used in all experiments: temperature of the heated capillary serving simultaneously as repeller electrode (20 V) 220 °C; electrospray capillary voltage 3.5 kV; electron multiplier voltage 2.0 kV; nitrogen served as sheath gas (336 MPa) and auxiliary gas (10 l·min^{-1}).

For MS-MS analysis in scan mode the mass spectra of the single reference compounds were almost exclusively dominated by their protonated molecular ion $[M+H]^+$. The product ion spectra of the compounds were available by collision-induced dissociation (CID) with 0.234 Pa argon as collision gas and 15 V offset voltage.

Time-dependent selected reaction monitoring (SRM) experiment was chosen; positive ions were detected at a total scan duration of 1 s and dwell time of 0.002 s; argon served as collision gas at a pressure of 0.234 Pa; offset voltage was 15 V. Selected ion pairs were m/z 209, 192 for L-kynurenine, *m/z* 213, 196 for d_4-L-kynurenine, *m/z* 176, 130 for indole-3-acetic acid, *m/z* 182, 136 for $^{13}C_6$-indole-3-acetic acid. The experiment list for the time-dependent SRM mode was: 0 to 10 min *m/z* 209, 192 and *m/z* 213, 196; 10 to 25 min *m/z* 176, 130 and *m/z* 182, 136.

Results and Discussion

Initial experiments revealed that the electrospray process effectively transformed the analytes from solution to protonated ions in the gas phase. As a result, abundant protonated molecular ions were observed. Low energy CID produced characteristic product ion spectra (Fig. 1, 2). The fragmentation of L-kynurenine and d_4-L-kynurenine yielded abundant product ions $[M+H-NH_3]+$ at *m/z* 192 and 196, respectively (Fig. 1). The formation of minor fragment ions can be explained by the subsequent loss of NH_3, H_2O and/or CO, i.e. $[M+H-NH_3-H_2O]+$ at *m/z* 174 (and 178), $[M+H-NH_3-CO]^+$ at *m/z* 164 (and 168), $[M+H-NH_3-CO-CH_2]^+$ at *m/z* 150 (and 154), $[M+H-NH_3-H_2O-CO]^+$ at *m/z* 146 (and 150). The fragmentation of indole-3-acetic acid and $^{13}C_6$-indole-3-acetic acid yielded only the abundant product ions $[M+H-H_2O-CO]^+$ at *m/z* 130 and 136, respectively (Fig. 2).

Consequently, tandem mass spectrometry enabled us to apply multiple selected reaction monitoring (SRM) mode experiments, resulting in high sensitivity and selectivity as well as effective reduction of background noise. The general procedure for SRM experiment is: quadrupole 1 is set to a defined *m/z* ratio; in quadrupole 2 ("collision cell") this ion is fragmented by collision with argon gas molecules (CID). Finally, only one characteristic product ion can pass through quadrupole 3, because it is also set to a defined *m/z* ratio. Besides its HPLC retention time, the

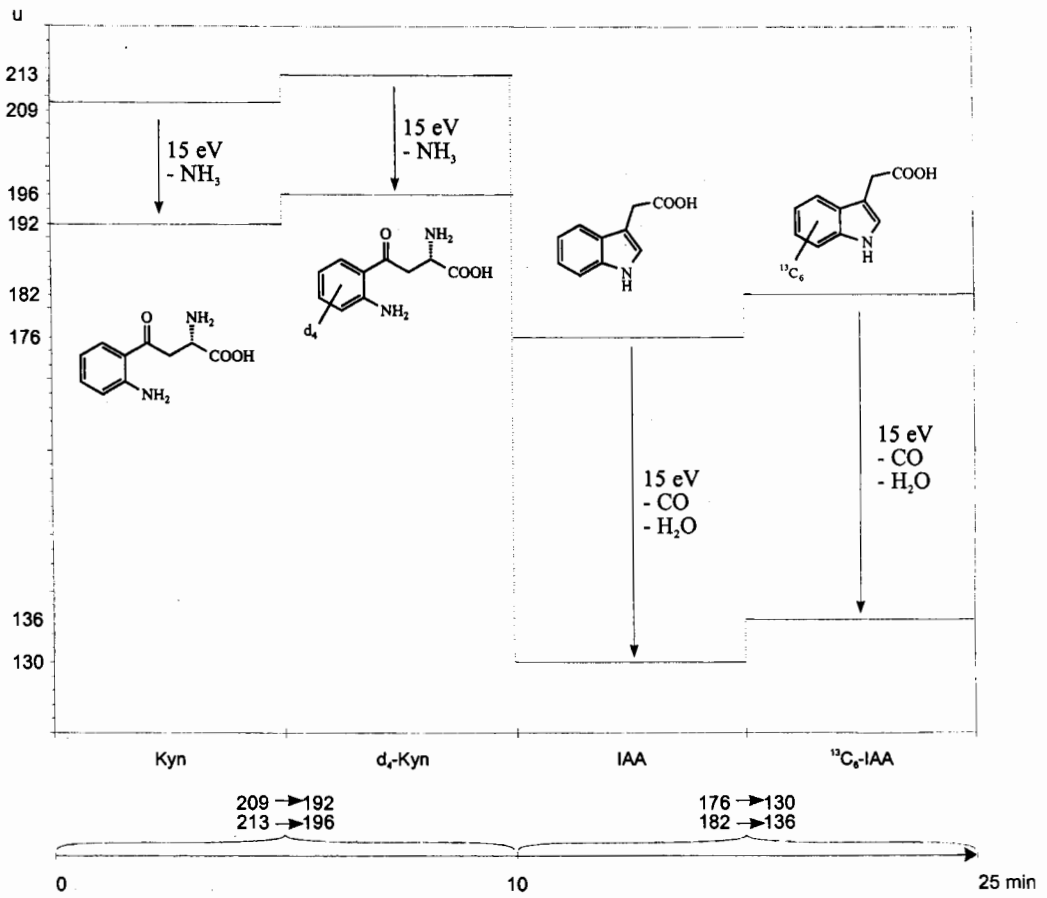

Fig. 3: Scheme of the selected reaction monitoring (SRM) scan.

analyte is determined by its protonated molecular ion (selected in quadrupole 1), representing a spectral filter on molecular mass information together with its specific product ion (selected in quadrupole 3) as structure-specific information. This procedure guarantees the high selectivity of the SRM experiment; coeluting and therefore interfering matrix components are excluded from detection. Combining two different SRM experiments within one HPLC run we were able to analyze L-kynurenine and indole-3-acetic acid simultaneously (Fig. 3). The ion pairs selected for SRM represent the protonated molecular ion $[M+H]^+$ and the most abundant product ion for each compound.

Prior to work-up by solid phase extraction on C-18 material (cf. experimental) the samples were spiked with isotope labeled internal standards. In Fig. 4 and 5 the time-dependent SRM chromatograms of the reference compounds and a grape must spiked with d_4-L-kynurenine and $^{13}C_6$-indole-3-acetic acid, respectively, are represented. Amounts were calculated from peak areas and the added isotopic internal standard concentration, considering background-subtracted mass spectra. For the sample shown in Fig. 5 an amount of 237 $\mu g \cdot l^{-1}$ L-kynurenine and 19 $\mu g \cdot l^{-1}$ indole-3-acetic acid was calculated. Artefactual origin of the analysed kynurenine by the sample preparation was excluded by spiking a sample with deuterated tryptophane. No deuterated kynurenine was detected after work-up.

The quantitative data obtained from the 24 samples under study are summarized in the Table. Amounts of 0-94 $\mu g \cdot l^{-1}$ and 20-379 $\mu g \cdot l^{-1}$ were determined for L-kynurenine and indole-3-acetic acid, respectively. The method allowed routine quantification limits of 4 $\mu g \cdot l^{-1}$ and 15 $\mu g \cdot l^{-1}$ for L-kynurenine and indole-3-acetic acid, respectively, in grape must (signal-to-noise ratio 4:1) at relative standard deviations of 10.1 % for kynurenine and 12.1 % for 3-indole-acetic acid as well as recoveries ranging from 96 to 106 %.

It has to be pointed out that the method can also be applied to the analysis of wines. Preliminary results revealed even improved quantification limits due to the simple wine matrix, i.e. values of 1 $\mu g \cdot l^{-1}$ and 5 $\mu g \cdot l^{-1}$ were evaluated for L-kynurenine and indole-3-acetic acid, respectively.

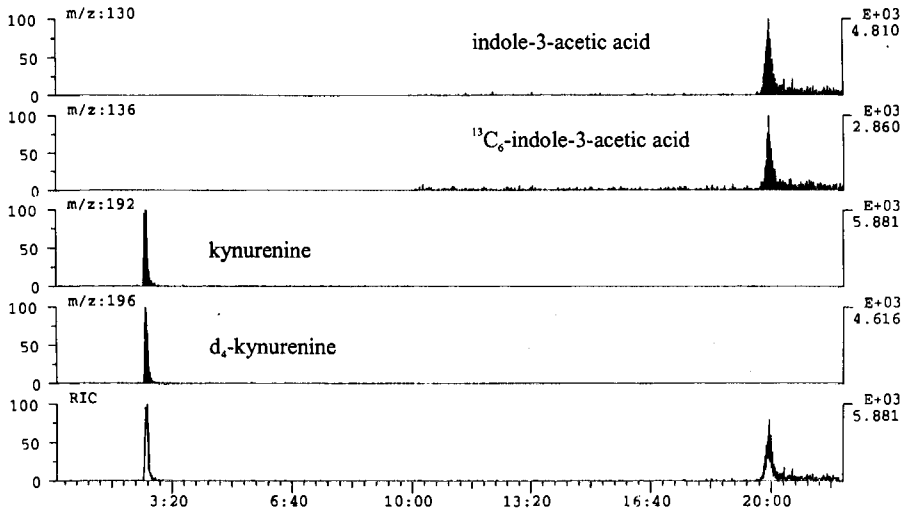

Fig. 4: Chromatogram of the compounds obtained by time-dependent SRM experiment.

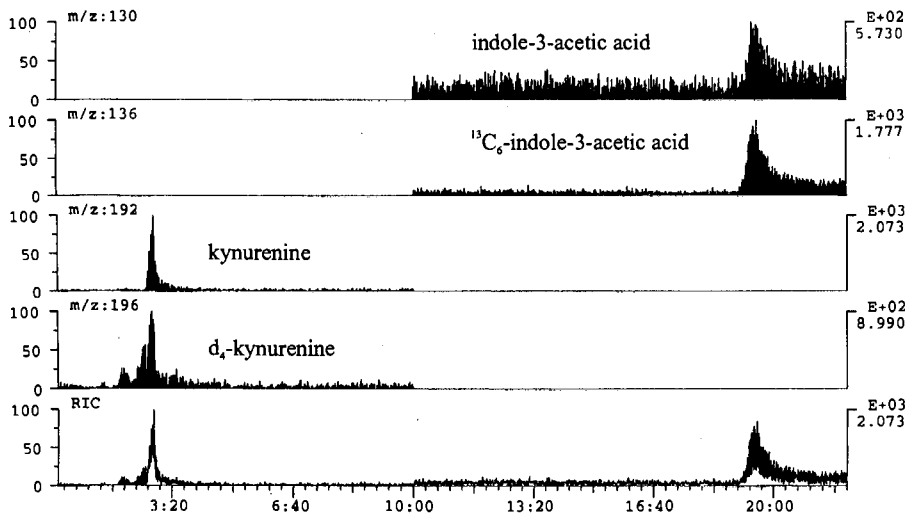

Fig. 5: Chromatogram of a grape must sample.

Table

Analysis of L-kynurenine (KYN) and indole-3-acetic acid (IAA) of different grape musts (vintage 1996) by HPLC-ESI-MS-MS

No.	cultivar	harvest date	KYN ($\mu g \cdot l^{-1}$)	IAA ($\mu g \cdot l^{-1}$)
1	Kerner	25.9.	n.d.	121.7
2	Kerner	1.10.	8.8	19.6
3	Kerner	9.10.	38.4	175.7
4	Kerner	9.10.	55.3	226.7
5	Kerner	9.10.	93.6	312.4
6	Kerner	9.10.	5.2	257.4
7	Kerner	23.10.	39.0	193.2
8	Kerner	23.10.	24.6	240.6
9	Kerner	23.10.	n.d.	325.4
10	Kerner	23.10.	4.4	379.4
11	Müller-Thurgau	25.9.	25.2	84.0
12	Müller-Thurgau	1.10.	9.7	40.4
13	Müller-Thurgau	1.10.	65.4	47.7
14	Müller-Thurgau	1.10.	86.2	20.4
15	Müller-Thurgau	1.10.	79.6	54.5
16	Müller-Thurgau	14.10.	n.d.	221.3
17	Müller-Thurgau	14.10.	84.0	27.7
18	Müller-Thurgau	18.10.	61.8	70.9
19	Müller-Thurgau	23.10.	n.d.	79.8
20	Müller-Thurgau	23.10.	28.6	71.8
21	Müller-Thurgau	23.10.	21.7	36.1
22	Ortega	25.10.	45.4	32.0
23	Müller-Thurgau	25.10.	32.6	226.5
24	Müller-Thurgau	25.10.	24.4	156.5

n.d.: not detectable

Conclusion

The high sensitivity and selectivity of HPLC-ESI-MS-MS analysis offers a new tool for the analysis of non-volatile flavour and off-flavour precursors in various matrices at low quantification limits. Isotopic labeled internal standards assure the accuracy of quantitative analysis as they are identical or closely related in structure, chemical properties and chromatographic behavior to the analytes. With the technique presented the prerequisites are evaluated to subsequently study the precursor-to-product relation (L-kynurenine/indole-3-acetic acid-to-2-aminoacetophenone) in musts and off-flavoured wines produced from them.

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