Plant Physiology and Biochemistry 143 (2019) 308–319

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Different forms of nitrogen application affect metabolite patterns in grapevine leaves and the sensory of wine

Carina P. Lang[a], Nikolaus Merkt[a], Iris Klaiber[b], Jens Pfannstiel[b], Christian Zörb[a,*]

[a] University of Hohenheim, Institute of Crop Science, Quality of Plant Products 340e, Emill-Wolff-Str.25, 70599, Stuttgart, Germany
[b] University of Hohenheim, Core Facility Hohenheim, Mass Spectrometry Unit, August-von-Hartmann-Str. 3, 70599, Stuttgart, Germany

ARTICLE INFO

Keywords:
Grapevine
Metabolomics
Nitrogen
Phenolic compounds
Quality

ABSTRACT

The quality of grapevine berries, must and wine is influenced by environmental and viticultural inputs and their complex interactions. Aroma and flavour are decisive for quality and are mainly determined by primary and secondary metabolites. In particular, phenolic compounds contribute to berry and wine quality. The influence of various nitrogen forms on *i)* the composition of phenolic compounds in leaves and wine and; *ii)* the resulting wine quality were studied in a vineyard system. Must and wine quality was evaluated by chemical analysis and sensory testing. Metabolomic profiling was also performed. Aroma and sensory profile were significantly changed by the application of nitrogen in contrast to no nitrogen fertilisation. The levels of 33 metabolites in leaves and 55 metabolites in wine were significantly changed altered by fertilisation with the various nitrogen forms. In leaves, more metabolites were increased by the use of calcium nitrate or ammonium but were decreased by the use of urea. In terms of wine, the used nitrogen forms decreased more metabolites compared with no fertilisation.

1. Introduction

From an economic point of view, the grapevine (*Vitis vinifera* L.) is one of the most important fruit species cultivated worldwide because of their numerous uses in the food production industry, such as grape juice, wine and other beverages (Ali et al., 2009). The quality of the grapevine and its resulting juice and wine is determined by several organoleptic properties, which are attributes of grape variety, fermentation conditions, *terroir,* environmental and viticultural inputs and of the complex interaction of these factors (Jackson and Lombard, 1993; Alañón et al., 2015). Aroma and flavour are decisive quality traits that are mainly determined by primary and secondary metabolites. Primary metabolites, which include sugars, amino acids, biogenic amides, polysaccharides, alcohols and organic acids, are directly involved in the growth, development and reproduction of grapevines. Secondary metabolites comprise a large selection of species-specific chemicals with more than 85 000 compounds. One of the major representatives of these secondary metabolites in grapevines are phenolic compounds (Verpoorte, 2000; Ali et al., 2009). Phenols are the most important contributors to fruit and wine quality, especially for sensory properties astringency and, to a lesser extent, bitterness (Mazerolles et al., 2010). Their range and concentrations are important determinates of flavour and aroma (Jackson and Lombard, 1993). Phenolic compounds also affect flavour, appearance, taste, mouth-feel, fragrance and colour, all off which also define the aroma bouquet of a wine. In addition to their organoleptic properties, phenols provide protection against environmental challenges (Teixeira et al., 2013) and are the main substrates for juice and wine oxidation (Jackson, 2008; Kennedy, 2008; Keller, 2010). Phenols are often present in the leaf epidermis, whereas in the berry, phenolics are mainly produced in the skin and seeds and are influenced by the grape variety, the vinification process, and the degradation and polymerisation that occurs during wine ageing (Winkel-Shirley, 2002; Keller, 2010). The major phenolic compounds in grapevines are the flavonoids such as flavones, flavonols, flavanones, flavan-3-ols and anthocyanins and the non-flavonoids such as phenylpropanoids, volatile phenols and stilbenes (Jackson, 2008; Teixeira et al., 2013).

Nitrogen (N) is an important nutrient both for grapevine growth and for berry quality formation. The N status of a grapevine influences the composition and concentration of the quality components of the berry and, therefore, contributes mainly to wine quality (Bell and Henschke, 2005). Agricultural practices such as N fertilisation can also affect the accumulation of secondary metabolites (Downey et al., 2006; Jezek et al., 2018). Phenolic compounds and aroma precursors are especially influenced by variations in N supply to grapevines (Choné et al., 2006; Portu et al., 2015a). Field trials in vineyards are necessary to evaluate these processes properly and in detail.

* Corresponding author.
E-mail addresses: langca@uni-hohenheim.de (C.P. Lang), Christian.zoerb@uni-hohenheim.de (C. Zörb).

https://doi.org/10.1016/j.plaphy.2019.09.009
Received 28 June 2019; Received in revised form 2 September 2019; Accepted 4 September 2019
Available online 11 September 2019
0981-9428/

Table 1
Oenological parameters of the four experimental musts in response to three different N-forms (CaN, AM, UR) and a control during the two experimental years 2015 and 2016. Capital letters indicate significant differences between the two experimental years 2015 and 2016; lower case letters indicate significant differences between the different N-forms and the control. Data are adjusted means ± SE (n = 4); MIXED MODELS $p \leq 0.05$.

Treatment	pH	Total acid [g L^{-1}]	Tartaric acid [g L^{-1}]	Malic acid [g L^{-1}]	Must weight [° Brix]
2015 A					
CaN	3.39 ± 0.06 a	7.33 ± 0.18	5.87 ± 0.21	4.61 ± 0.13	21.3 ± 2.08
AM	3.26 ± 0.05 ab	7.61 ± 0.18	5.78 ± 0.21	4.89 ± 0.13	21.5 ± 2.10
UR	3.22 ± 0.05 b	7.61 ± 0.17	6.01 ± 0.21	4.81 ± 0.13	21.0 ± 2.17
control	3.24 ± 0.05 ab	7.69 ± 0.18	5.80 ± 0.21	4.86 ± 0.13	21.0 ± 2.11
2016 B					
CaN	3.56 ± 0.06	5.90 ± 0.18	2.07 ± 0.21	2.83 ± 0.13 b	23.8 ± 2.16
AM	3.57 ± 0.06	5.85 ± 0.18	2.01 ± 0.21	3.52 ± 0.13 a	24.0 ± 2.09
UR	3.52 ± 0.06	5.84 ± 0.18	2.20 ± 0.21	3.49 ± 0.13 a	23.8 ± 2.14
control	3.59 ± 0.06	5.84 ± 0.18	2.13 ± 0.21	3.41 ± 0.13 a	23.8 ± 2.06

Table 2
Oenological parameters of the four experimental wines in response to three different N-forms (CaN, AM, UR) and a control during the two experimental years 2015 and 2016. Capital letters indicate significant differences between the two experimental years 2015 and 2016; lower case letters indicate significant differences between the different N-forms and the control. Data are adjusted means ± SE (n = 4); MIXED MODELS $p \leq 0.05$.

Treatment	pH	Total acid [g L^{-1}]	Tartaric acid [g L^{-1}]	Malic acid [g L^{-1}]	Lactic acid [g L^{-1}]	Alcohol [g L^{-1}]
2015 A						
CaN	3.1 ± 0.1	6.5 ± 0.4	2.0 ± 0.04 ab	3.1 ± 0.11	0.88 ± 0.1	83 ± 4.4
AM	3.1 ± 0.1	6.6 ± 0.4	1.9 ± 0.04 b	3.4 ± 0.12	0.85 ± 0.1	87 ± 4.6
UR	3.0 ± 0.1	6.5 ± 0.3	2.1 ± 0.04 a	3.5 ± 0.12	0.79 ± 0.1	81 ± 4.3
control	3.1 ± 0.1	6.6 ± 0.4	2.0 ± 0.04 ab	3.3 ± 0.12	0.81 ± 0.1	85 ± 4.5
2016 B						
CaN	3.6 ± 0.1	5.3 ± 0.2	1.4 ± 0.04 b	2.9 ± 0.12 b	0.86 ± 0.1 a	110 ± 5.8
AM	3.6 ± 0.1	5.4 ± 0.2	1.4 ± 0.04 b	3.1 ± 0.12 a	0.67 ± 0.1 b	106 ± 1.3
UR	3.6 ± 0.1	5.5 ± 0.2	1.6 ± 0.04 a	3.2 ± 0.12 a	0.68 ± 0.1 b	109 ± 5.8
control	3.6 ± 0.1	5.5 ± 0.3	1.5 ± 0.04 a	3.1 ± 0.12 a	0.59 ± 0.1 b	108 ± 5.7

Metabolic profiling is a method for the identification and quantification of as many pre-defined small molecule metabolites as possible occurring within a system, and generally associated with a specific metabolic pathway, whereas metabolic fingerprinting is a high-throughput screening tool for samples each having a different biological status or origin (Dunn and Ellis, 2005; Cozzolino, 2016). Grapevine metabolomic studies can be used to provide insights into a wide domain of flavour and aroma components in berries and wine or in their associated grapevine physiology. Moreover, changes can be identified in phenolic compounds and their related shikimate or phenylpropanoid pathways based on agricultural practices.

The little information that is available, concerning the impact of different N forms in the vineyard system and their effect on the grapevine metabolome of leaves and wine is conflicting. Furthermore, data on phenolic compounds and their effect on aroma and flavour composition are rare. We have studied the influence of various N-forms such as calcium nitrate, ammonium and urea in a vineyard experiment with grafted grapevines, namely *Vitis vinifera* L. *cv.* Regent on rootstock *cv.* SO4. Our metabolomic fingerprint analysis of grapevine leaves and wine provides a first overview of the way in which many phenolic compounds can be detected and influenced by N supply. Not only have must and wine quality been analysed, but also a sensory profile for the resulting wine was conducted. The influences of various N-forms on *i*) general phenolic compounds in leaves and wine and; *ii*) wine quality have been investigated in this study.

2. Materials and methods

2.1. Plant growth conditions and experimental design

Field experiments run from June 2015 to October 2016 in a vineyard located at the campus of the University of Hohenheim, Stuttgart, Germany (Long. 48° 42′ 29.149″N Lat. 9° 12′ 42.25″E). Grapevines with good resistance against powdery and downy mildew, namely *Vitis vinifera* L. *cv.* Regent, were grafted onto the rootstock SO4 (Selection Oppenheim 4). Vines were spaced 1.40 m within the row and 1.80 m between rows. The experimental design of the vineyard was a fully randomised block design with 16 blocks in total; these were divided into four treatments and four biological replications. Each block consisted of four grapevines. The treatments consisted of three different N forms; calcium nitrate ($Ca(NO_3)_2$), ammonium sulfate ($(NH_4)_2SO_4$) and urea (CH_4N_2O). In the cases of ammonium sulfate and urea, nitrification inhibitors (SKW Stickstoffwerke Piesteritz GmbH, Lutherstadt Wittenberg, Germany) were employed. The fourth treatment lacked N and was considered as the control. The following abbreviations are used throughout: calcium nitrate (CaN), ammonium (AM), urea (UR) and control. All N forms were applied in liquid solution, with same amount of water without N being applied to the control. The applied N amount was 60 kg N ha^{-1} and was calculated with regard to the block size (35.3 m^2). Treatment occurred in June 2015 and May 2016, at BBCH stage 53.

2.2. Leaf harvest for metabolite profiling

Samples were collected on 15th October 2016, one day after the grape harvest. In all 16 plots, one whole cane at the two-year-old shoot was chosen from four vines each. At this shoot, the youngest fully expanded leaves in a healthy and green stage where harvested and immediately frozen in liquid nitrogen. For further analysis, samples were ground up under liquid nitrogen and stored at −80 °C.

2.3. Juice and wine samples

Vitis vinifera L. *cv.* Regent grapes were harvested by hand-picking on 25th September in the experimental year 2015 and on 14th October in the experimental year 2016. Because of the technical requirements of

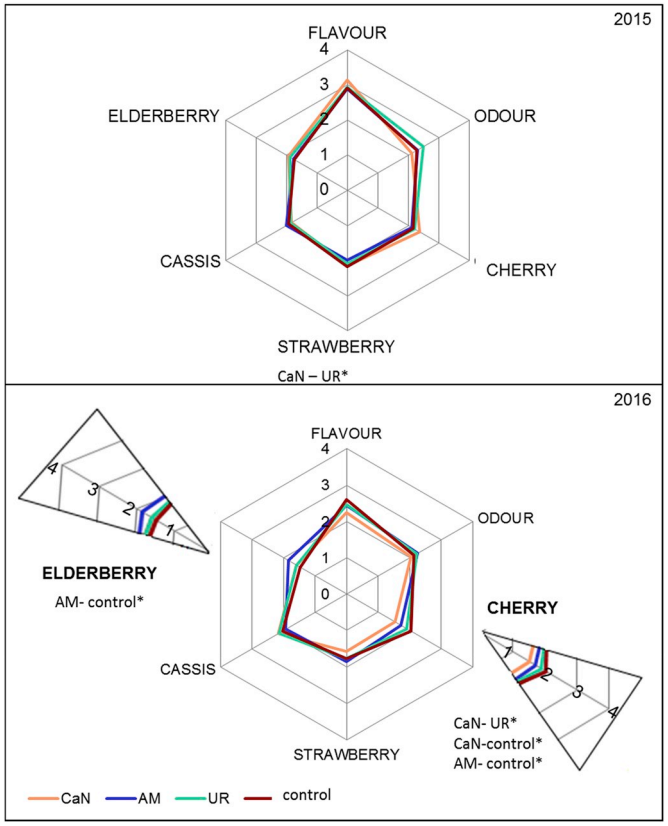

Fig. 1. Spider plots of the sensory profile of the experimental wines from *Vitis vinifera* L. *cv.* Regent in response to three different N-forms (CaN ; AM ; UR) and a control ; during the two experimental years 2015 and 2016. Bold aroma attributes indicate significant differences between these aroma attributes in the two years 2015 and 2016. Asterisks indicate significant differences between different N-forms within one aroma attribute and within one year. Inverse-transformed adjusted means are shown (n = 4); MIXED MODELS $p \leq 0.05$.

the following vinification process, each of the 16 blocks was harvested separately. Four vines from each block were harvested and the same vines as those for leaf sampling were used. Grapes were processed separately. 16 different wines were each produced in the two experimental years giving a total of 32 wines. Must weight (total soluble solids in °Brix), pH and total acid (TA) of the must were analysed. Vilification was performed at the viticulture unit of the Department of Quality of Plant Products, Institute of Crop Science at the University of Hohenheim, Stuttgart, Germany. Before the berries were pressed by using a hydraulic press, they were squeezed to scratch the skin. Must samples of approximately 40 mL were taken and immediately frozen for further analyses. Particulates (sedimented skin residues and pulp), were removed from the must samples by centrifugation for 5 min at 14.000 × *g*. After pressing, 2 g L^{-1} bentonite was added and the must was inoculated with 0.3 g L^{-1} yeast (Anchor Vin, 2000; *S. cerevisiae*). At the end of fermentation, wines were separated from the yeast and sulfured with 200 mg L^{-1} potassium disulfite ($K_2S_2O_5$). All wines were stored in bottles in the wine cellar of the institute at 12 °C ambient temperature before being tasted.

2.3.1. Oenological parameters in must and wine

The must and the wine were analysed for both experimental years. The total soluble solids (°Brix) were measured with a refractometer (Opton, Zeiss, Germany). Total acid (TA) and pH were measured by means of a titrator (TiroLine easy, Schott, Mainz). The parameters lactic acid (LA), tartaric acid (TTA) and malic acid (MA) (only for wine) and the alcohol content were determined by high performance liquid chromatography (HPLC) (Merck-Hitachi, Darmstadt, Germany; column oven: Knauer Berlin, Germany). For analysis of the organic acids in the must and wine, potassium phosphate (20 mM, pH 1.5) at a flow rate of 1 mL min^{-1} and detection at 210 nm UV was used as the mobile phase. The utilised separation column was a Synergi™ 4 µm Hydro-RP 80 Å, LC column 250 × 4.6 mm, Ea, (Phenomenex, Aschaffenburg, Germany). For the analysis of the alcohol content in the wine, sulfuric acid (isocratic, 0.05 N) at a flow rate of 0.5 mL min^{-1} and detection at 210 nm UV was used as the mobile phase. The utilised separation column was a Phenomenex Rezex™ ROA-Organic Acid H^+ (8%), LC column 300 × 7.8 mm, Ea, (Phenomenex, Aschaffenburg, Germany) and the utilised precolumn was a Phenomenex SecurityGuard Cartridge, Carbo-H 4 × 3.0 mm (Phenomenex, Aschaffenburg, Germany).

2.3.2. Wine sensory analysis

Sensory analysis of the wine was conducted from both experimental years 2015 and 2016. The wines were evaluated twice by a trained tasting panel during four sessions at the institute. During the first two sessions on different days, participants evaluated the wine of the experimental year 2015 (14 participants at the 1st session and 13 participants at the 2nd session). During the last two sessions (again on different days), participants evaluated the wine of the experimental year 2016 (12 participants at the 3rd session and 10 participants at the 4th session). In total, 32 wines were tested for intensity (four different wines in four replications) in random order. Wines were served at ambient temperature and in clear glasses. Water was provided. The panellists were given defined aroma attributes for evaluation. In total, six attributes, namely four for aroma (cherry, strawberry, cassis and elderberries) and two for flavour/odour were used. These aroma attributes were scored on a five-point scale, with '0' representing non-characteristic or non-existent intensity and '5' representing a high or extreme intensity. During the whole sensory session, the panellists had access to commercial aroma attributes, for comparison purposes.

2.4. Metabolite extraction of the leaf samples

Fresh, ground leaf material (a pooled sample of all biological replicates) from the vintage 2016, (approximately 500 mg) was mixed with 1 mL pre-cooled methanol:water solution (80:20 v/v). Three technical replicates per N-form of treatment were conducted. The samples were homogenised by being shaken vigorously for 5 min and were then stored at −20 °C overnight for protein precipitation. Each sample was centrifuged (Heraeus Pico 21 centrifuge, Thermo Fisher Scientific, Waltham, MA, USA) for 10 min at 14 0.000 × *g* at 4 °C. The supernatant was filtered through a 0.22 µm sterile filter (Rotilabo syringe filter PVDF sterile, pore size 0.22 µm, Carl Roth GmbH, Karlsruhe, Germany) and stored at −20 °C until LC-MS analysis.

2.4.1. Metabolite extraction of wine samples

Wine samples from the vintage 2016 were taken from bottles one day before measurement. From each of the 16 wines, three technical replicates were taken and filtered through a 0.2 µm sterile filter (Rotilabo syringe filter PVPF sterile, pore size 0.22 µm, Carl Roth GmbH, Karlsruhe, Germany). Samples were stored at 6 °C until LC-MS analysis.

2.5. UHPLC-MS analysis

UHPLC-MS analysis was performed by using an Agilent 1290 Ultra Performance Liquid Chromatography system coupled to a Q-Exactive Plus Mass Spectrometer (Thermo Fischer Scientific, Waltham, MA, USA). Analyte separation was achieved by a Waters ACQUITY CSH C18 column (1.7 µm, 2.1 µm × 150 mm); the mobile phases were acetonitrile and water each with 0.2% formic acid for leaf samples and methanol and water each with 0.2% formic acid for wine samples. The column temperature was set to 40 °C and the auto sampler temperature was set to 10 °C. The injection volumes used were 3 µL for leaf samples

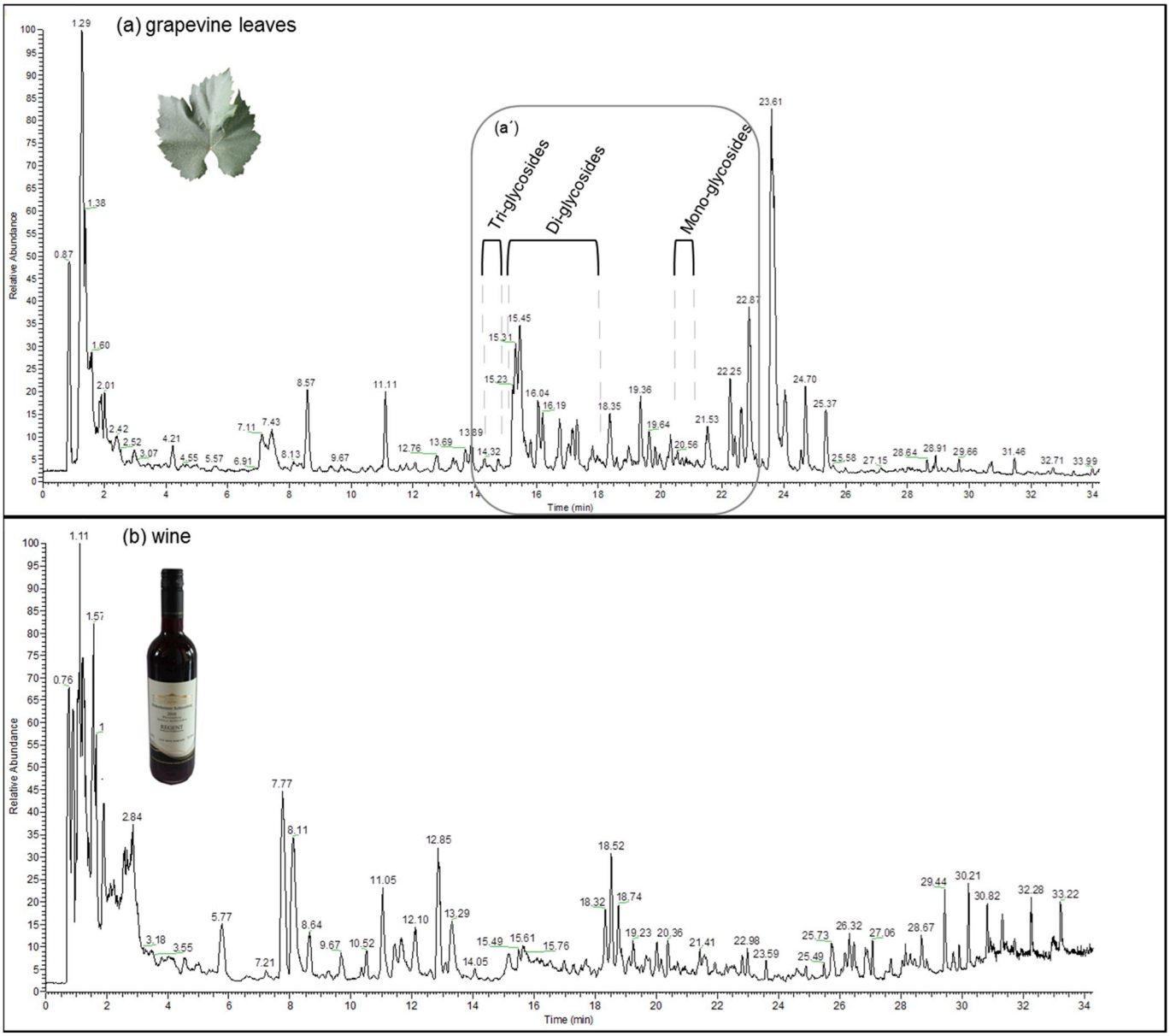

Fig. 2. UHPLC-ESI-MS chromatograms under ESI + ionisation mode of the metabolic extracts from (a) grapevine leaves, control as example and (b) wine, UR as example. The insert (a') highlights the parts of the chromatogram where different kaempferol and quercetin glycosides (mono-, di-, or tri-glycosylation) are detected.

and 4 µL for wine samples. The flow rate for the separation mode of the leaf samples was set to 0.30 mL min^{-1} and the separation mode for the wine samples was set to 0.35 mL min^{-1}. Mass spectrometry analysis was performed in ESI+ and ESI- ionisation modes by using a Xcalibur version 4.0.27.42. Data of the ESI + mode are shown.

2.6. Metabolite profiling and data processing

Data for the metabolomics workflow were analysed by using Compound Discoverer software 3.0 (Thermo Fischer Scientific, Waltham, MA, USA). The metabolic annotation was based on the defined four levels of metabolic identification given by Sumner et al. (2007). Individual compounds were assigned based on exact mass, isotope pattern or either by ChemSpider (formula or exact mass) or by an in-house mass list of common grapevine compounds based on peer-reviewed research articles. A tentative annotation of metabolites without a standard was based on spectral features (mass deviation < 5 ppm of the theoretical value, on isotopic pattern fit and at least one indicative fragment ion in the MS/MS spectrum), on literature information concerning chromatographic properties and mass spectra records from external databases such as HMDB, KEGG and MassBank and on an internal database including wine metabolites based on peer reviewed literature. MS/MS data were also used for the further support of the annotation of a few tentative marker metabolites. Thereafter, two filter steps were carried out: the first filter settings were specified in the program itself (Suppl. Table. 1) and the second filter settings were based on experience (review of molecular formula, molecular mass, retention time and peak formation) and on peer-reviewed literature. Two additional filter criteria were added for the annotation of tentative phenolic compounds; the molecular formula had to have a C-H-O backbone, and the structural formula proposed by the software required an aromatic ring (C6 carbon structure).

To obtain an overview about all regulated compounds, the results from the grapevine leaves and wine were depicted in volcano plots (Suppl. Fig. 1.1 for grapevine leaves and Suppl. Fig. 1.2 for wine).

Table. 3a

Regulated tentative compounds in grapevine leaves of *Vitis vinifera* L. *cv.* Regent during the experimental year 2016 by using UHPLC-ESI-MS in positive ionisation mode. Relative ratios of the generated Log^2 fold changes and *p*-values of the three different N - forms (CaN, AM, UR) and a control are shown. Significance is indicated by colour coding of the Log^2 fold changes (significance = ≥ +1/≥ −1) and the *p*-values (p ≤ 0.05); ANOVA (pooled samples n = 3).

-2.01 → -3.00	1.01 → -2.00	0.00 → 0.99	1.00 → 2.00	2.01 → 3.00

1.0-0.05	0.05-0.01	0.01-0.005	0.005-0.001	0.001-0.0005	0.0005-0

Metabolite annotation	Formula	Molecular Mass [*m/z*]	RT [min]	Log^2 Fold						*p*-value					
				CaN / control	AM / control	UR / control	CaN / AM	CaN /UR	AM / UR	CaN / control	AM / control	UR / control	CaN / AM	CaN /UR	AM / UR
Acetophenone	$C_8 H_8 O$	120.0578	1.39	-1.35	-0.93	0.36	-0.42	-1.72	-1.30	0.00019	0.00152	0.24122	0.23247	0.00004	0.00020
Tyramine	$C_8 H_{11} N O$	137.0842	1.40	-1.34	-0.93	0.36	-0.41	-1.71	-1.30	0.00019	0.00149	0.23788	0.24170	0.00004	0.00019
UNKNOWN*		170.0192	3.69	1.15	0.50	0.31	0.66	0.85	0.19	0.00057	0.05463	0.18980	0.02290	0.00709	0.81014
Tentative compound	$C_{13} H_{22} O_2$	210.1621	17.78	1.30	0.82	0.60	0.48	0.70	0.22	0.00044	0.00892	0.09229	0.10330	0.00989	0.37972
Tentative compound	$C_{18} H_{28} O$	260.2140	36.63	0.88	1.02	0.44	-0.14	0.44	0.59	0.00151	0.00157	0.14732	0.99998	0.03146	0.03312
Tentative compound	$C_{18} H_{30} O$	278.2246	35.07	1.15	0.94	0.47	0.20	0.67	0.47	0.00011	0.00028	0.02983	0.72549	0.00401	0.01527
UNKNOWN*		295.1634	3.58	-1.05	-1.54	-1.69	0.48	0.63	0.15	0.00154	0.00025	0.00007	0.34454	0.03959	0.43953
Tentative compound	$C_{21} H_{34} O_4$	350.2458	32.86	1.20	0.95	0.92	0.24	0.27	0.03	0.03000	0.00043	0.00129	0.00152	0.71101	0.62688
Tentative compound	$C_{21} H_{36} O_4$	352.2615	35.47	1.38	1.10	0.42	0.28	0.96	0.68	0.00004	0.00018	0.03909	0.29993	0.00073	0.00630
Tentative compound	$C_{21} H_{36} O_4$	352.2615	36.98	1.38	1.22	0.64	0.16	0.74	0.58	0.00004	0.00013	0.00816	0.46336	0.00259	0.01768
Tentative compound	$C_{19} H_{32} O_7$	372.2149	17.78	1.20	0.76	0.47	0.44	0.73	0.29	0.00024	0.00705	0.07312	0.05419	0.00541	0.37090
UNKNOWN*		377.1588	21.44	0.61	-1.62	-1.77	2.22	2.37	0.15	0.05085	0.00007	0.00005	0.00001	0.00001	0.94722
UNKNOWN*		413.2263	8.65	1.11	0.66	0.41	0.45	0.70	0.25	0.00020	0.00895	0.14255	0.03123	0.00240	0.25906
UNKNOWN*		435.2836	17.78	1.36	0.82	0.59	0.54	0.77	0.24	0.00003	0.00111	0.02248	0.01226	0.00069	0.13930
Tentative phenolic compound	$C_{25} H_{26} O_7$	438.1683	29.24	1.93	1.23	0.74	0.70	1.19	0.49	0.00000	0.00005	0.00305	0.00381	0.00006	0.01204
UNKNOWN*		454.1632	27.44	1.54	1.33	0.58	0.21	0.96	0.75	0.00003	0.00010	0.00655	0.31827	0.00162	0.01554
UNKNOWN*		479.2005	1.39	-1.22	-0.75	0.41	-0.48	-1.63	-1.16	0.00024	0.00290	0.15842	0.14799	0.00003	0.00025
ASN-LYS-SER-TYR	$C_{22} H_{34} N_6 O_8$	510.2443	36.63	0.89	1.07	0.43	-0.17	0.46	0.64	0.00262	0.00124	0.20784	0.90647	0.04377	0.01747
Tentative compound	$C_{27} H_{46} O_9$	514.3146	36.63	0.95	1.10	0.46	-0.15	0.49	0.63	0.00125	0.00111	0.13701	0.99943	0.02678	0.02292
Tentative phenolic compound	$C_{31} H_{28} O_{13}$	608.1533	24.07	-1.69	-0.93	-1.29	-0.76	-0.39	0.36	0.00003	0.00127	0.00022	0.01037	0.11975	0.34983
Kaempferol-di-hexoside	$C_{27} H_{30} O_{16}$	610.1540	16.64	-1.65	-1.50	-1.75	-0.15	0.09	0.24	0.00017	0.00028	0.00009	0.92972	0.87082	0.55652
Kaempferol-di-glucoside	$C_{27} H_{30} O_{16}$	610.1541	17.48	-1.49	-1.36	-1.61	-0.13	0.12	0.25	0.00057	0.00085	0.00030	0.97819	0.90266	0.71614
Quercetin- di-hexoside	$C_{27} H_{30} O_{17}$	626.1483	15.43	-1.00	-0.69	-1.27	-0.31	0.27	0.58	0.00529	0.02779	0.00144	0.60674	0.68989	0.15803
Quercetin- di-glucoside-galctoside	$C_{27} H_{30} O_{17}$	626.1486	16.03	-0.75	-0.62	-1.19	-0.13	0.44	0.57	0.00711	0.01716	0.00046	0.90339	0.14208	0.05491
UNKNOWN*		630.2692	30.99	1.79	1.35	0.45	0.44	1.34	0.90	0.00010	0.00085	0.05895	0.17949	0.00200	0.03669
Quercetin-hexoside-glucuronide	$C_{27} H_{28} O_{18}$	640.1281	20.71	-0.75	-0.75	-1.24	0.00	0.50	0.50	0.00068	0.00036	0.00003	0.91902	0.03465	0.08424
UNKNOWN*		652.1981	8.62	1.11	0.81	0.20	0.30	0.91	0.61	0.00625	0.02762	0.71678	0.68414	0.02546	0.12370
UNKNOWN*		676.3672	35.07	1.02	1.03	0.32	-0.01	0.70	0.71	0.00031	0.00036	0.08037	0.99813	0.00708	0.00878
UNKNOWN*		693.3936	35.47	1.70	1.48	0.74	0.22	0.96	0.74	0.00003	0.00009	0.00793	0.43326	0.00160	0.01086
UNKNOWN*		721.4248	35.07	1.01	1.10	0.46	-0.09	0.55	0.64	0.00071	0.00037	0.02434	0.90519	0.07050	0.02756
Kaempferol-tri-glucoside	$C_{33} H_{40} O_{21}$	772.2065	14.28	-1.79	-1.04	-1.68	-0.74	-0.11	0.63	0.00001	0.00079	0.00002	0.00371	0.76711	0.01265
UNKNOWN*		791.3216	25.76	1.13	0.77	0.38	0.36	0.75	0.39	0.00122	0.01677	0.16082	0.21172	0.02206	0.41822
UNKNOWN*		833.0926	19.21	-1.15	-0.44	-0.09	-0.71	-1.06	-0.35	0.00037	0.02974	0.59024	0.02271	0.00138	0.18223

*UNKNOWN metabolites – not identified, tentative molecular mass indicated, tentative phenolic compounds were annotated based on two more filter criteria: C-H-O backbone and aromatic ring (C6 carbon structure).

Table. 3b

Regulated tentative phenolic compounds in grapevine leaves of *Vitis vinifera* L. *cv.* Regent during the experimental year 2016 by using UHPLC-ESI-MS in positive ionisation mode. Relative ratios of the generated Log^2 fold changes and as *p*-values of the three different N - forms (CaN, AM, UR) and a control are indicated. Significance is shown by colour coding of the Log^2 fold changes (significance = ≥ +1/≥ −1) and the *p*-values (p ≤ 0.05); ANOVA (pooled samples n = 3).

-2.01 → -3.00	1.01 → -2.00	0.00 → 0.99	1.00 → 2.00	2.01 → 3.00

1.0-0.05	0.05-0.01	0.01-0.005	0.005-0.001	0.001-0.0005	0.0005-0

Metabolite annotation	Formula	Molecular Mass [*m/z*]	RT [min]	Log^2 Fold						*p*-value					
				CaN / control	AM / control	UR / control	CaN / AM	CaN / UR	AM / UR	CaN / control	AM / control	UR / control	CaN / AM	CaN / UR	AM / UR
Tentative phenolic compound	$C_{25} H_{26} O_7$	438.1683	29.24	1.93	1.23	0.74	0.70	1.19	0.49	0.00000	0.00005	0.00305	0.00381	0.00006	0.01204
Tentative phenolic compound	$C_{31} H_{28} O_{13}$	608.1533	24.07	-1.69	-0.93	-1.29	-0.76	-0.39	0.36	0.00003	0.00127	0.00022	0.01037	0.11975	0.34983
FLAVONOLS															
Kaempferol-di-hexoside	$C_{27} H_{30} O_{16}$	610.1540	16.64	-1.65	-1.50	-1.75	-0.15	0.09	0.24	0.00017	0.00028	0.00009	0.92972	0.87082	0.55652
Kaempferol-diglucoside	$C_{27} H_{30} O_{16}$	610.1541	17.48	-1.49	-1.36	-1.61	-0.13	0.12	0.25	0.00057	0.00085	0.00030	0.97819	0.90266	0.71614
Quercetin- di-galactoside	$C_{27} H_{30} O_{17}$	626.1483	15.43	-1.00	-0.69	-1.27	-0.31	0.27	0.58	0.00529	0.02779	0.00144	0.60674	0.68989	0.15803
Quercetin- di-glucoside-galctoside	$C_{27} H_{30} O_{17}$	626.1486	16.03	-0.75	-0.62	-1.19	-0.13	0.44	0.57	0.00711	0.01716	0.00046	0.90339	0.14208	0.05491
Quercetin-hexoside-glucuronide	$C_{27} H_{28} O_{18}$	640.1281	20.71	-0.75	-0.75	-1.24	0.00	0.50	0.50	0.00068	0.00036	0.00003	0.91902	0.03465	0.08424
Kaempferol-tri-glucoside	$C_{33\ 2}H_{40}\ O_1$	772.2065	14.28	-1.79	-1.04	-1.68	-0.74	-0.11	0.63	0.00001	0.00079	0.00002	0.00371	0.76711	0.01265

Tentative phenolic compounds were annotated based on two more filter criteria: C-H-O backbone and aromatic ring (C6 carbon structure).

Table. 4a

Regulated tentative compounds in wine of *Vitis vinifera* L. *cv.* Regent during the experimental year 2016 by using UHPLC-ESI-MS in positive ionisation mode. Relative ratios of the generated Log^2 fold changes and *p*-values of the three different N - forms (CaN, AM, UR) and a control are shown. Significance is indicated by colour coding of the Log^2 fold changes (significance = ≥ +1/≥ −1) and the *p*-values (p ≤ 0.05); ANOVA (pooled samples n = 3).

-4.01 → -5.00 | -3.01 → -4.00 | -2.01 → -3.00 | -1.01 → -2.00 | 0.00 → 0.99 | 1.00 → 2.00 | 2.01 → 3.00 | 3.01 → 4.00 | 4.01 → 6.00

1.0-0.05 | 0.05-0.01 | 0.01-0.005 | 0.005-0.001 | 0.001-0.0005 | 0.0005-0

Metabolite annotation	Formula	Molecular Mass [m/z]	RT [min]	Log^2 Fold						*p*-value					
				CaN / control	AM / control	UR / control	CaN / AM	CaN / UR	AM / UR	CaN / control	AM / control	UR / control	CaN / AM	CaN / UR	AM / UR
Tentative compound	$C_{10}H_9N$	143.0945	1.23	0.24	-0.7	-1.01	0.94	1.25	0.31	0.07439	0.00014	0.00001	0.00002	2.2805E-06	0.04561
Tentative compound	$C_{10}H_7NO$	157.0526	18.61	0.3	-0.76	0.45	1.05	-0.15	-1.21	0.00133	2.1393E-06	0.00004	1.1191E-07	0.01694	2.8272E-08
Tentative compound	$C_7H_{11}NO_3$	157.0737	11.01	-1.2	-0.41	-0.67	-0.79	-0.53	0.26	9.14E-10	2.6049E-06	3.9946E-08	1.4965E-08	2.7269E-07	0.00007
Tentative compound	$C_4H_{12}NO_4P$	169.0498	9.70	-1.64	-0.64	0.6	-0.99	-2.24	-1.24	9.9925E-09	0.00002	0.00003	2.4477E-07	1.2986E-09	7.4271E-08
Tentative compound	$C_8H_{10}O_4$	170.0576	9.70	-1.87	-0.74	0.68	-1.13	-2.55	-1.42	2.2651E-09	2.0404E-06	2.0734E-06	3.9757E-08	5.324E-11	1.1581E-08
Tentative compound	$C_5H_5N_7O$	179.0555	10.99	-1.2	-0.39	-0.62	-0.81	-0.58	0.23	1.3423E-06	0.00494	0.00012	0.00002	0.00050	0.02586
UNKNOWN*		186.0638	6.26	-0.72	-1.21	-0.64	0.49	-0.08	-0.57	0.00893	0.00015	0.01696	0.02010	0.95963	0.01051
Tentative compound	$C_8H_{16}N_2O_3$	188.1159	2.97	0.92	1.09	-0.50	-0.17	1.42	1.59	9.044E-07	2.3281E-07	0.00016	0.08993	2.9039E-08	1.6578E-08
Tentative compound	$C_7H_{19}P_3$	196.0709	20.54	-0.17	-0.59	0.79	0.42	-0.96	-1.38	0.46406	0.00146	0.00006	0.00907	0.00002	1.4145E-06
Tentative compound	$C_8H_{14}N_2O_4$	202.0949	12.82	-0.23	-0.87	0.29	0.64	-0.52	-1.16	0.15654	0.00015	0.01577	0.00156	0.00092	8.0276E-06
Tentative compound	$C_{11}H_{14}O_4$	210.0889	20.98	1.64	0.41	-1.51	1.22	3.15	1.92	1.0537E-07	0.00181	2.0071E-07	1.7306E-06	1.4258E-09	2.4929E-08
Tentative compound	$C_{10}H_7N_7$	225.0762	24.40	0.29	-0.54	-0.84	0.83	1.13	0.3	0.06397	0.00052	0.00008	0.00004	9.1481E-06	0.22193
Tentative compound	$C_{14}H_{22}O_3$	238.1565	27.09	-0.3	-0.79	0.24	0.49	-0.54	-1.03	0.00292	1.1583E-06	0.00987	0.00003	0.00005	1.3821E-07
Tentative compound	$C_{12}H_{24}N_2O_3$	244.1784	14.87	0.82	0.46	-0.38	0.37	1.20	0.84	2.9053E-06	0.00029	0.00256	0.00080	1.91E-07	5.2279E-06
Tentative compound	$C_{13}H_{27}PS$	246.1576	10.59	1.03	0.76	-0.37	0.27	1.40	1.13	9.9556E-07	9.5723E-06	0.00276	0.01330	7.7117E-08	5.3552E-07
Tentative compound	$C_{13}H_{26}N_2O_3$	258.1939	18.36	0.74	0.36	-0.44	0.38	1.18	0.80	2.3549E-07	0.00007	0.00001	0.00004	9.2797E-09	1.0252E-07
UNKNOWN*		261.0421	9.76	0.95	-0.28	-0.81	1.24	1.76	0.53	1.7519E-06	0.02056	0.00001	2.6479E-07	1.9524E-08	0.00029
UNKNOWN*		268.9679	11.24	-0.2	0.55	-0.6	-0.76	0.4	1.15	0.00549	2.3947E-06	1.9124E-06	2.1743E-07	0.00004	1.1875E-08
UNKNOWN*		274.0008	11.00	-1.2	-0.47	-0.73	-0.73	-0.47	0.26	0.00002	0.01900	0.00039	0.00057	0.03202	0.03924
UNKNOWN*		276.0775	7.20	-0.35	0.29	-0.95	-0.64	0.60	1.24	0.00252	0.00327	2.5731E-06	0.00002	0.00010	1.8932E-07
UNKNOWN*		288.0164	11.00	-1.16	-0.33	-0.58	-0.84	-0.58	0.25	6.608E-07	0.00184	0.00006	0.00002	0.00029	0.02490
UNKNOWN*		288.0411	11.24	-0.23	0.47	-0.66	-0.71	0.43	1.13	0.00707	0.00008	0.00002	3.498E-06	0.00069	1.282E-07
Tentative compound	$C_{15}H_{12}O_6$	288.0629	17.85	0.93	1.14	-0.17	-0.21	1.10	1.31	2.4608E-07	4.2641E-08	0.01554	0.01278	4.4708E-08	1.6888E-08
Tentative compound	$C_9H_{11}N_3O_6S$	289.0369	9.76	1.37	-0.18	-0.62	1.55	1.99	0.44	5.2904E-07	0.03647	0.00004	1.044E-07	1.6585E-08	0.00085
(±) Epicatechin	$C_{15}H_{14}O_6$	290.0785	17.85	1.04	1.3	-0.18	-0.26	1.22	1.48	6.6609E-09	1.2797E-09	0.00139	0.00018	2.0289E-09	2.933E-10
Tentative compound	$C_{16}H_{12}O_7$	316.0580	18.71	-0.04	-0.7	0.81	0.66	-0.85	-1.51	0.92616	0.00476	0.00880	0.01025	0.00412	0.00006
UNKNOWN*		316.0725	17.59	-0.08	0.67	-0.57	-0.75	0.49	1.24	0.15623	2.1938E-07	7.0402E-07	7.5159E-08	2.3332E-06	3.4526E-09
Tentative compound	$C_{14}H_{27}N_3O_5$	317.1946	4.74	0.69	-0.36	0.19	1.04	0.50	-0.55	8.4952E-08	0.00001	0.00195	6.6385E-09	1.3011E-06	6.4114E-07
Tentative compound	$C_{15}H_{14}O_8$	322.0685	14.39	1.01	0.32	-1.7	0.69	2.71	2.02	1.2006E-07	0.00076	5.3588E-09	3.0858E-06	3.3751E-11	1.3767E-09
Tentative compound	$C_{17}H_{26}N_2O_4$	322.1888	19.44	0.79	0.23	-0.35	0.56	1.14	0.58	1.7757E-06	0.01986	0.00108	0.00002	8.774E-08	0.00004
Tentative compound	$C_{16}H_{31}N_3O_4$	329.2308	15.84	0.96	0.48	1.09	0.49	-0.13	-0.61	1.4306E-06	0.00040	8.096E-07	0.00016	0.67204	0.00006
UNKNOWN*		331.2101	15.92	0.72	-0.32	0.3	1.04	0.42	-0.62	1.5177E-08	9.2658E-06	0.00001	1.5468E-09	8.4778E-07	3.9678E-08
Tentative compound	$C_{15}H_{29}N_3O_5$	331.2101	11.72	0.84	-0.2	0.27	1.04	0.57	-0.47	6.3227E-08	0.00292	0.00055	1.6294E-08	1.6406E-06	8.2437E-06
UNKNOWN*		349.1367	9.70	-2.14	-0.75	0.74	-1.39	-2.88	-1.49	1.613E-09	3.3829E-06	2.9998E-06	2.1455E-08	4.114E-11	1.5262E-08
Tentative compound	$C_{12}H_{21}N_3O_8$	349.1370	1.46	0.65	0.51	-0.89	0.15	1.54	1.40	0.00011	0.00107	8.3186E-06	0.15110	1.0274E-07	3.1749E-07
UNKNOWN*		354.0921	9.70	-1.90	-0.74	0.67	-1.16	-2.57	-1.41	1.432E-09	1.6163E-06	2.0802E-06	2.5797E-08	2.836E-11	1.0746E-08
UNKNOWN*		356.1254	25.90	-0.52	-0.81	0.31	0.29	-0.83	-1.12	0.00441	0.00014	0.05485	0.04115	0.00017	0.00001
Tentative compound	$C_{18}H_{35}N_3O_4$	357.2622	19.72	0.66	-0.41	0.16	1.07	0.5	-0.57	6.1874E-06	0.00008	0.03022	6.903E-08	0.00007	6.4798E-06
Tentative compound	$C_{20}H_{21}N_3O_4$	367.1526	15.49	0.99	0.28	-0.23	0.71	1.22	0.51	3.3675E-07	0.00581	0.00266	3.8044E-06	3.5455E-08	0.00003
UNKNOWN*		370.0661	9.70	-2.11	-0.77	0.69	-1.34	-2.8	-1.46	5.8756E-10	9.3067E-07	2.7873E-06	1.5465E-08	1.0273E-11	9.7997E-09
UNKNOWN*		385.0215	9.70	-2.09	-0.82	0.86	-1.26	-2.95	-1.68	3.41E-09	2.4084E-06	2.8648E-06	7.1551E-08	1.1255E-10	1.3524E-08
Tentative compound	$C_{20}H_{39}N_3O_4$	385.2935	22.90	0.74	-0.44	0.24	1.18	0.5	-0.68	0.00007	0.00443	0.04471	2.7147E-06	0.00152	0.00015
UNKNOWN*		407.1723	17.22	0.23	1.02	0.91	-0.79	-0.68	0.11	0.00046	8.9237E-09	1.717E-08	3.4328E-08	1.5089E-07	0.01919
UNKNOWN*		434.0354	9.70	-2.51	-0.83	0.82	-1.68	-3.33	-1.65	1.529E-08	0.00004	0.00005	4.5901E-07	2.6422E-09	1.7801E-07
UNKNOWN*		466.1041	14.13	-0.02	0.47	-1.3	-0.49	1.28	1.77	0.99631	0.00004	1.7063E-08	0.00004	1.6444E-08	2.7939E-09
UNKNOWN*		468.1239	18.15	-1.25	-0.71	0.29	-0.54	-1.54	-1.00	9.7472E-09	5.1E-07	0.00066	5.4086E-06	2.7684E-09	3.1459E-08
Quercetin-glucoronide	$C_{21}H_{18}O_{13}$	478.0743	22.21	-0.75	0.39	0.81	-1.14	-1.56	-0.42	0.00016	0.00202	0.00006	3.1874E-06	4.6557E-07	0.02109
UNKNOWN*		501.2792	16.10	0.08	-0.57	0.45	0.65	-0.37	-1.02	0.62183	0.00001	0.00010	6.6569E-06	0.00029	1.4259E-07
UNKNOWN*		566.1568	19.92	-0.04	0.44	-0.91	-0.48	0.87	1.35	0.93862	0.00093	3.0022E-06	0.00052	4.0985E-06	1.2853E-07
UNKNOWN*		623.1777	18.43	-0.24	0.39	-0.62	-0.62	0.38	1.01	0.01930	0.00038	0.00001	0.00002	0.00031	2.9982E-07
Isorhamnetin-caffeoyl-conjugate	$C_{31}H_{28}O_{14}$	624.1472	22.07	0.29	0.5	-0.93	-0.21	1.22	1.43	0.10079	0.00460	0.00023	0.17739	0.00003	6.0664E-06
UNKNOWN*		682.1697	22.49	0.26	0.37	-0.76	-0.11	1.02	1.13	0.04783	0.00403	0.00002	0.30801	2.3623E-06	9.1598E-07
UNKNOWN*		686.2024	9.70	-4.3	-2.12	1.66	-2.18	-5.96	-3.78	4.1063E-06	0.00072	0.00249	0.00059	2.5573E-07	8.9853E-06
UNKNOWN*		692.1707	22.49	0.18	0.33	-0.75	-0.15	0.93	1.08	0.05696	0.00180	0.00001	0.10171	1.9848E-06	5.1994E-07
UNKNOWN*		788.1697	18.79	-0.35	-0.46	-1.13	0.12	0.78	0.67	0.19325	0.03054	0.00006	0.57682	0.00045	0.00176

*UNKNOWN metabolites – not identified, tentative molecular mass indicated; tentative phenolic compounds were annotated based on two more filter criteria: C-H-O backbone and aromatic ring (C6 carbon structure).

2.7. Statistical analysis

Data were analysed by using SAS software (version 9.4, Cary, NC, U.S.A.). A MIXED MODEL with a Kenward-Roger test and a significance level of $p \leq 0.05$ were employed. Log-transformed and thus inverse-transformed data were used for the parameters pH_{must} and $alcohol_{wine}$. For the sensory analysis of the wine, every aroma compound was analysed separately and compared between the N-forms within one year. Log-transformed and thus inverse-transformed data were used for the aroma attributes odour, cherry, strawberry, cassis and elderberry.

Table. 4b

Regulated tentative phenolic compounds in wine of *Vitis vinifera* L. *cv.* Regent during the experimental year 2016 by using UHPLC-ESI-MS in positive ionisation mode. Relative ratios of the generated Log^2 fold changes and *p*-values of the three different N - forms (CaN, AM, UR) and a control are shown. Significance is indicated by colour coding of the Log^2 fold changes (significance = ≥ +1/≥ −1) and the *p*-values ($p \leq 0.05$); ANOVA (pooled samples n = 3).

-3.01 → -4.00	-2.01 → -3.00	-1.01 → -2.00	0.00 → 0.99	1.00 → 2.00	2.01 → 3.00	3.01 → 4.00

1.0-0.05	0.05-0.01	0.01-0.005	0.005-0.001	0.001-0.0005	0.0005-0

Metabolite annotation	Formula	Molecular Mass [*m/z*]	RT [min]	Log^2 Fold CaN /control	AM / control	UR / control	CaN / AM	CaN / UR	AM / UR	*p*-value CaN /control	AM / control	UR / control	CaN / AM	CaN /UR	AM / UR
Tentative phenolic compound	$C_{11}H_{14}O_4$	210.0889	20.98	1.64	0.41	-1.51	1.22	3.15	1.92	1.0537E-07	0.00181	2.0071E-07	1.731E-06	1.426E-09	2.493E-08
Tentative phenolic compound	$C_{15}H_{12}O_6$	288.0629	17.85	0.93	1.14	-0.17	-0.21	1.10	1.31	2.4608E-07	4.2641E-08	0.01554	0.01278	4.471E-08	1.689E-08
Tentative phenolic compound	$C_{16}H_{12}O_7$	316.0580	18.71	-0.04	-0.7	0.81	0.66	-0.85	-1.51	0.92616	0.00476	0.00880	0.01025	0.00412	0.00006
Tentative phenolic compound	$C_{15}H_{14}O_8$	322.0685	14.39	1.01	0.32	-1.7	0.69	2.71	2.02	1.2006E-07	0.00076	5.3588E-09	3.086E-06	3.375E-11	1.377E-09
FLAVANOLS (FLAVAN -3-OLS)															
(±) Epicatechin	$C_{15}H_{14}O_6$	290.0785	17.85	1.04	1.3	-0.18	-0.26	1.22	1.48	6.6609E-09	1.2797E-09	0.00139	0.00018	2.029E-09	2.933E-10
FLAVONOLS															
Quercetin-glucoronide	$C_{21}H_{18}O_{13}$	478.0743	22.21	-0.75	0.39	0.81	-1.14	-1.56	-0.42	0.00016	0.00202	0.00006	3.187E-06	4.656E-07	0.02109
Isorhamnetin-caffeoyl-conjugate	$C_{31}H_{28}O_{14}$	624.1472	22.07	0.29	0.5	-0.93	-0.21	1.22	1.43	0.10079	0.00460	0.00023	0.17739	0.00003	6.066E-06

Tentative phenolic compounds were annotated based on two more filter criteria: C-H-O backbone and aromatic ring (C6 carbon structure).

For the data analysis of leaf and wine metabolomics, an analysis of variance (ANOVA) and a Tukey HSD test (posthoc test) with a *p*-value of $p \leq 0.05$ were carried out by using Compound Discoverer 3.0 Software (Thermo Fischer Scientific, Waltham, MA, USA). The corresponding principal component analyses (PCA; PC1 *vs* PC2), the volcano plots (Log^2-FoldChange: 0.5) and the heatmaps were performed with the same Software; $p \leq 0.05$. The heatmaps were based on a Pearson distance function and the hierarchical trees were calculated by the average linkage method the normalised data were scaled before clustering.

3. Results

3.1. Oenological parameters of must and wine in response to different N-forms

No significant differences were detected in the oenological parameters of the must, except for pH (Table 1). The N-form CaN led to significantly higher pH values (mean: 3.4) and the N-form UR led to significantly lower pH values (mean: 3.2). In total the values of the chemical attributes were higher in 2015 compared with 2016, except for pH and must weight. Only a few significant changes were detectable in the oenological parameters of the wine (Table 2). In the wine of 2015, the acid concentration was influenced by the N-forms. UR treatment led to significantly higher concentrations (mean. 2.1) and AM led to significantly lower concentrations (mean: 1.9) in TTA. In the experimental year 2016 the chemical attributes TTA, MA and LA were significantly influenced by the different N-forms (Table 2); UR lead to significantly higher acid concentrations (mean: 1.6 TTA; 3.2 MA) and CaN resulted in significantly lower concentrations (mean: 1.4 TTA; 2.9 MA). The N-form CaN (mean: 0.86) was associated with significantly higher concentrations of LA in comparison with the other N-forms. In total, the concentrations of the oenological parameters in 2015 were higher compared with 2016, except for pH and alcohol.

The spider plots of the aroma profile of the various wines in 2015 (Fig. 1) showed a significant change between the N-forms CaN and UR in the expression of the aroma attribute strawberry. The aroma profile of the wine 2016 exhibited significant changes between the N-forms AM and the control with regards to the expression of the aroma attribute elderberry as well as between the N-forms CaN-UR, CaN-control and AM-control with respect to the aroma attribute cherry.

3.2. Metabolic responses in grapevine leaves and wine of the different N-forms

Fig. 2 shows the UHPLC-ESI-MS analyses of extracted compounds (metabolites) from grapevine leaves (Fig. 2a) and wine (Fig. 2b).

In total, 5166 features in grapevine leaves were detected by the Compound Discoverer software, of which 37 were significantly changed in abundance (Log^2 fold changes ≥ 1 and p-value ≤ 0.05, Suppl. Table. 1). Eight of these compounds belonged to the group of polyphenols (Tables 3a and 3b). In addition, 355 unregulated phenolic compounds were detected (see supplemental data Suppl. Table. 2.1). In wine, 5521 features were detected in total, 55 of which were changed in abundance (Suppl. Table. 1). Seven of these compounds belonged to the group of polyphenols (Tables 4a and b) and 125 unregulated phenolic compounds were detected (data see supplemental Suppl. Table. 1.2). UHPLC-ESI-MS data are summarised in Tables 3a and b and , Tables 4a and b and in the supplementary material (Suppl. Tables 2.1 and 2.2).

A principal component analysis (PCA, scores plot) of the grapevine leaves and of the wine was performed to detect the impact of the different N-forms on the leaves and the wine metabolome (Fig. 4a andb and). The purpose was to classify the data into their variability by means of so-called principal components and thereby identify patterns in the dataset (Ringnér, 2008; Næs et al., 2010). The technical replicates (triplicates of each N-form, pooled QC samples) were clustered together in a shared colour plot (heatmap, Fig. 4a andb and) or in a one-point cloud (PCA, Fig. 3ab and), indicating high reproducibility of the analytical method applied. The PCA of the grapevine leaves (Fig. 3a) revealed that all N-forms could be separated from the control by principal component one. Principal component two clearly separated UR from the other N-forms and the control. The PCA of the wine (Fig. 3b) revealed that all N-forms were separated from the control. However, principal component two clearly separated the N-forms CaN and AM. The Venn diagrams (Fig. 5a and b) also illustrated the pattern indicated by the PCA. CaN showed the highest change of metabolite profile compared with the control, both in leaves and in wine. In the order of the highest number of regulated tentative metabolites was: CaN - control > AM - control > UR – control. However, when CaN was compared with other N-forms, this pattern for leaves slightly changed to; CaN - UR > AM - UR > CaN - UR and for wine to; AM - UR > CaN - UR > CaN - UR. This indicates that the N-forms CaN and AM had similar effects on the metabolic profile of leaves and wine.

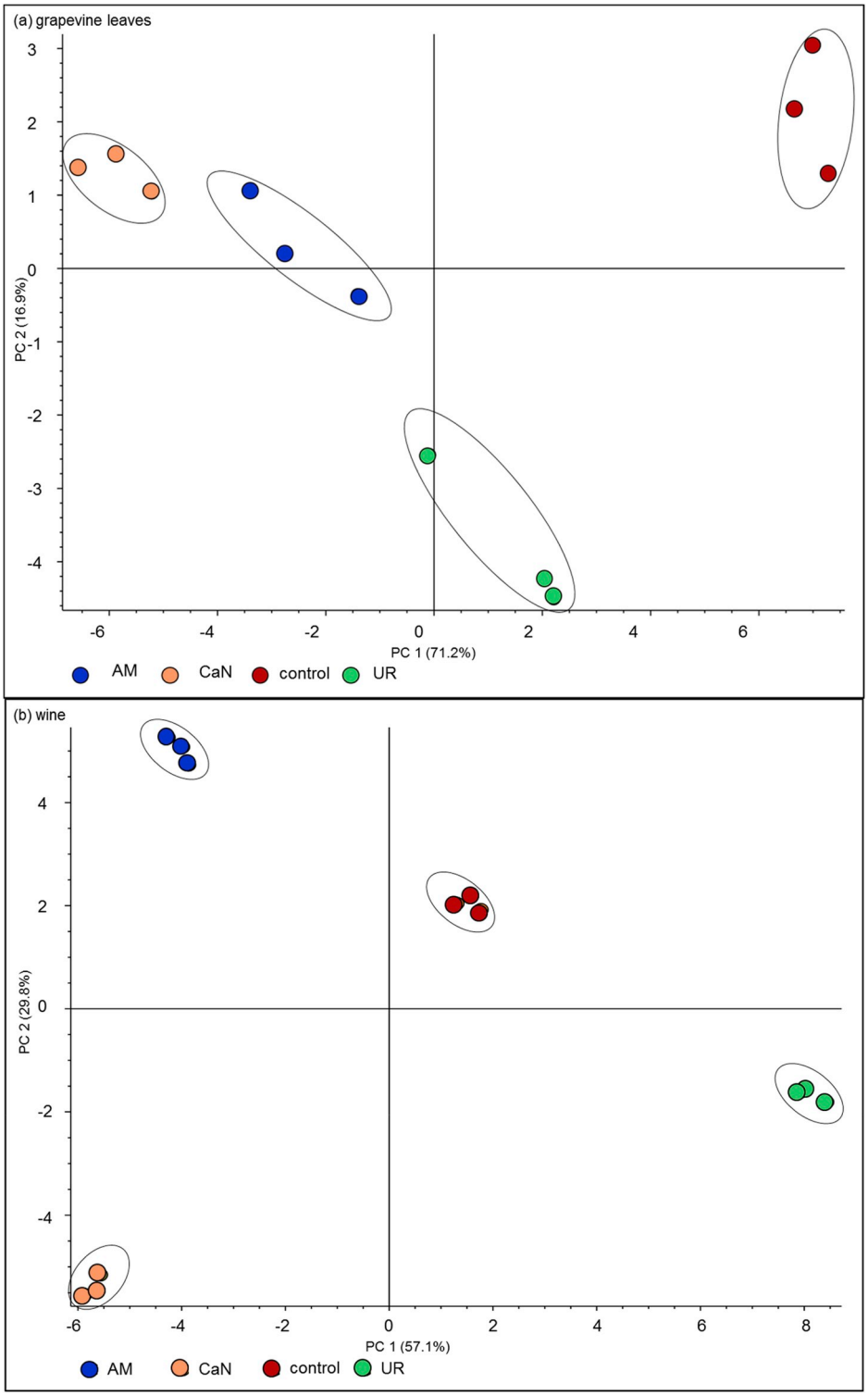

Fig. 3. Principal component analysis (PCA; PC1 vs. PC2) in (a) leaves and (b) wine were generated from normalised data of regulated compounds of the three different forms CaN ( ); AM ( ) and UR ( ) and a control ( ); nitrogen patterns are marked with circles, (pooled samples n = 3); $p \leq 0.05$.

3.3. Metabolic profiling of phenolic compounds

The peak detection data of all differentially abundant compounds are summarised in Table 3a for grapevine leaves and in Table 4a for wine. However, since we focused on phenolic compounds, these were listed in separate tables (Tables 3b and 4b). We detected 8 significantly changed phenolic components in the leaves, accounting for 24% of all regulated metabolites (Table 3b). Six phenolic compounds could be assigned to the group of flavonols, including three kaempferol and three quercetin derivatives based on the precise *m/z* value, the deduced sum formula and the fragmentation spectrum. For two phenolic compounds (tentative phenolic compounds, based on the filter criteria) only the molecular formula and the molecular mass [*m/z*] is provided, since the fragmentation spectrum did not provide sufficient information for unambiguous identification. The glycosylation of the detected kaempferol and quercetin derivatives decreased with increasing retention time (insert Fig. 2 a') and the glycosides eluted in the order Tri > Di > Mono. Notably, only one phenolic compound increased in abundance compared to the control based on the N treatment. In contrast, all 6 flavonols were significantly decreased in abundance by N treatment,

especially when the vines were treated with UR. Kaempferol derivatives decreased under all forms of N fertilisation compared with the control (Table 3b).

In the wine, we to detected 7 phenolic compounds that changed in abundance accounting for 13% of all changed metabolites. Three of them were identified and assigned, namely the flavanol (±) epicatechin and the flavonols quercetin and isorhamnetin. A highly significant increase in the abundance of the tentative phenolic compounds, and of (±) epicatechin could be seen when vines were treated with CaN and AM in comparison with the control. On the other hand, the N-form UR caused a highly significant decrease in the abundance of the phenolic compounds in comparison with the control. The flavonols showed no change after any N fertilisation compared with the control. On evaluating the differences between the various N-forms, we observed that, under CaN treatment, most of the identified phenolic compounds increased in abundance compared with treatment with other N-forms.

4. Discussion

4.1. Quality influences on must and wine in response to different N-forms

Acid and pH are two of the most important quality factors in grapes and wine. In addition to wine stability and microbiological control, both parameters have an influence on organoleptic parameters (Torija et al., 2003; Ali et al., 2009). The typical pH range for white wines and red wines are 3.0–3.4 and 3.3–3.7, respectively. Lower values within this range in musts are preferred because pH increases during or after fermentation (Waterhouse et al., 2016). The UR treatment increased the concentration of TTA (Table 2), which might have a positive effect on the fermentation process. Similar results have been reported by Portu et al. (2015a). Our results indicate that CaN increases the concentration of LA. During alcoholic fermentation, malolactic fermentation conducted by lactic acid bacteria produces LA (Cappello et al., 2017). We have studied the influence of fertilised versus non-fertilised (control) vines and of the N-forms CaN and UR on wine (Fig. 1). The two mentioned N-forms showed the highest difference in the evaluation of the aroma attributes. The results of the oenological parameters and the sensory evaluation seem to support the controversially discussed hypothesis that N fertilisation does indeed influence the sensory attributes as reviewed by Bell and Henschke, 2005. However, the N-forms CaN and AM also appear to influence the wine sensory profile, leading to individual fruity tasting nuances. Furthermore, a difference can be seen between the experimental years. The vintage effect and thus the unique climate conditions are known significantly to influence fruity aroma composition (Robinson et al., 2014).

4.2. Metabolic changes in grapevine leaves and wine

The metabolite profiling of grapevine leaves and wine, suggests that

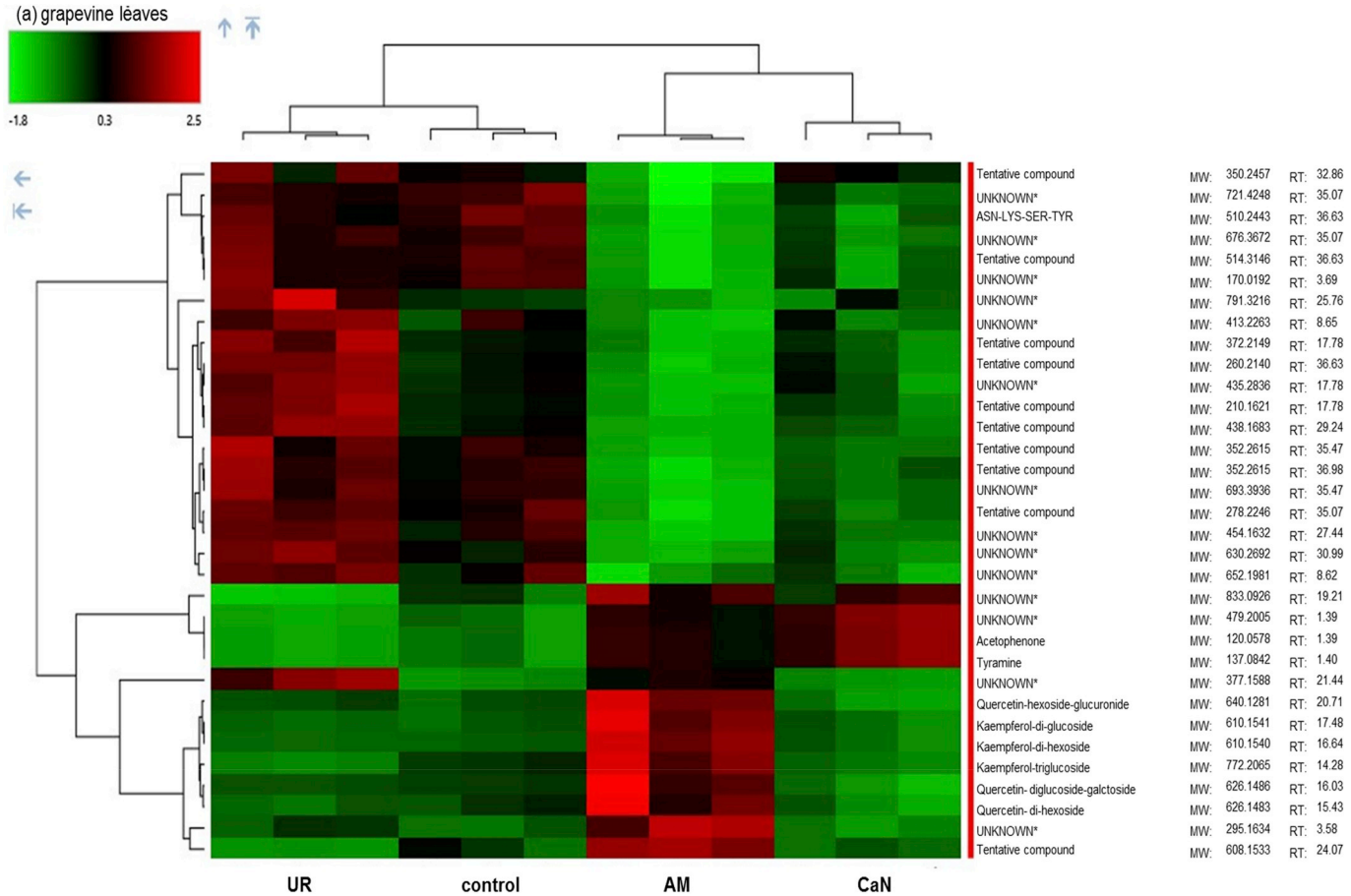

Fig. 4. Clustered heatmaps of the regulated compounds of (a) grapevine leaves and (b) wine of *Vitis vinifera* L. *cv.* Regent during the experimental year 2016. Individual metabolites are represented by rows and N treatments are represented by columns (CaN, AM, UR and a control). Heatmap visualisation of metabolomic differences is based on the relative amount (by area) in a given N sample. Green: downregulation of the metabolite; red: upregulation of the metabolite. Hierarchial clustering was formed by Pearson's distance function. Hierarchial trees were calculated by the average linkage method (pooled samples n = 3); $p \leq 0.05$. Each rectangle represents the relative amount (by area) of a particular compound in a given N sample (CaN, AM, UR and control). The dendogram, represents the distance or similarity between samples. Clusters, define a site characteristic reaction pattern between the N-forms or between the compounds. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

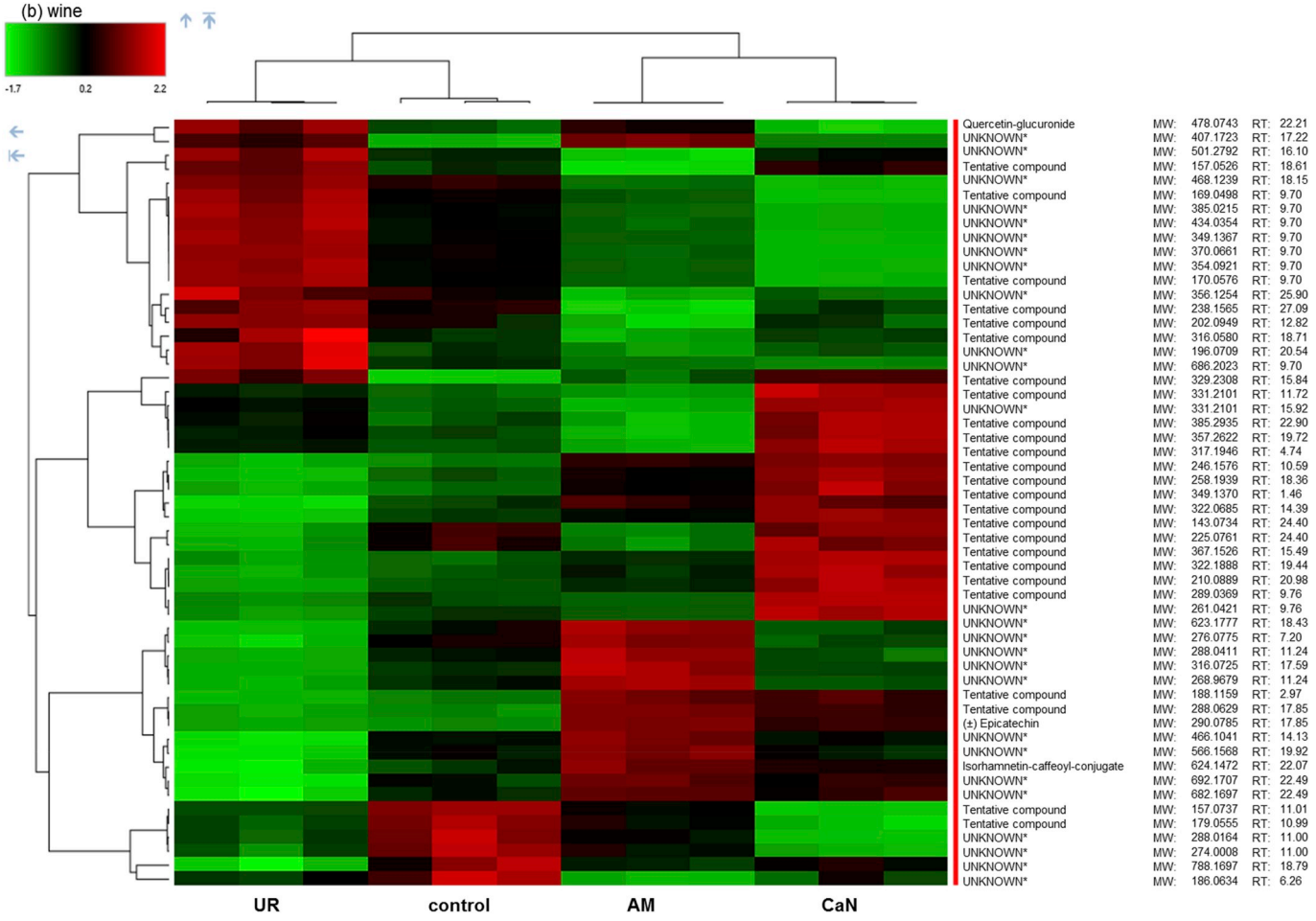

Fig. 4. (*continued*)

fertilisation with CaN, AM or UR has a significant influence on the grapevine metabolome. In general, CaN had the greatest impact on the metabolite profile in leaves and in wine (Fig. 5a and b), followed by AM and UR. After AM and UR treatment, the tentative phenolic compounds, show a comparable pattern (Tables 3b and 4b). As mentioned above, the N-forms CaN and AM are similar in their regulation pattern. Notably, UR treatment mainly leads to a decreased abundance of metabolites in leaves and wine. In contrast, CaN and AM mainly increase the abundance of metabolites in leaves but, decrease the metabolites in wine, especially the tentative phenolic compounds. Nevertheless, the extent of the abundance change was dependent on the compound itself. Leser and Treutter (2005) have measured a significant reduction of phenolic compounds upon treatment with increasing N amounts and conclude a reduction in flavonoid biosynthesis because of high N supply in apple leaves. Portu et al. (2015b) have found similar results in wine. The annotated polyphenols in the leaves and wine (Tables 3b and 4b) are mainly flavonoids and not only belong to a subgroup of flavonols, involved in the co-pigmentation process of anthocyanins in red berries, but also have a high antioxidant and free radical scavenging activity. Flavonols in leaves and berries can also serve as UV protectors (Castillo-Muñoz et al., 2007; De Rosso et al., 2014) that can be increased by the N fertilisation rate. (±) Epicatechin belongs to the flavanols (flavan -3-ols), also named condensed tannins; they are important contributors to wine stability and organoleptic properties such as body and mouth-feel (Downey et al., 2006; Teixeira et al., 2013).

Nitrogen also increases vegetative growth, which in turn has an influence on the ripening of the grapes and thus has an effect on quality. Delayed maturity affects the biochemical composition of the berry (Lang et al., 2018) and thus affects the formation of aroma compounds and taste. These effects are based on changes in flavonoid metabolism attributable to a possible imbalance or a competition for sugar between leaf and berry (Braidot et al., 2008). This implies that a higher supply of N might shift the sink: source ratio in favour of plant biomass and that therefore less sugar is available for the berry. As a result, less aroma precursors accumulate in the berries. Since, in the present experiment, the same amount of total N was applied in all N treatments, we consider that the differences in the metabolic profile, especially the phenolic compounds, are based on the different uptake or utilisation capacity of the different N-forms. Nitrate and ammonium are major N sources for the grapevine (Lang et al., 2018; Loulakakis and Roubelakis-Angelakis, 2001). Their uptake is an active process driven by root-based membrane transporters such as NRT for nitrate or AMT for ammonium (Goel and Singh, 2015). In contrast to nitrate and ammonium, the uptake process for urea is still under debate; it can be absorbed both actively and passively by the plant (Witte, 2011). In their study on the effects of the growth conditions of grapevines, including defoliation, Rossouw et al. (2018) showed that polyphenols and other products of the shikimate pathway are affected through N availability. Therefore, we suggest that UR might be available to a lower extent for the vine root compared with CaN and AM. In addition, we assume that the different N-forms may trigger the induction of specific phenolic biosynthesis such as the shikimate and the phenylpropanoid pathways, to a different metabolic extent.

To our surprise, a higher number of metabolites in wine were affected by the different N-forms than in the leaves. We think that this is because large part of the N that occur in must and, that is used for fermentation, the so-called yeast-assimilable nitrogen (YAN), is metabolised by the yeast. Therefore, more significant differences are

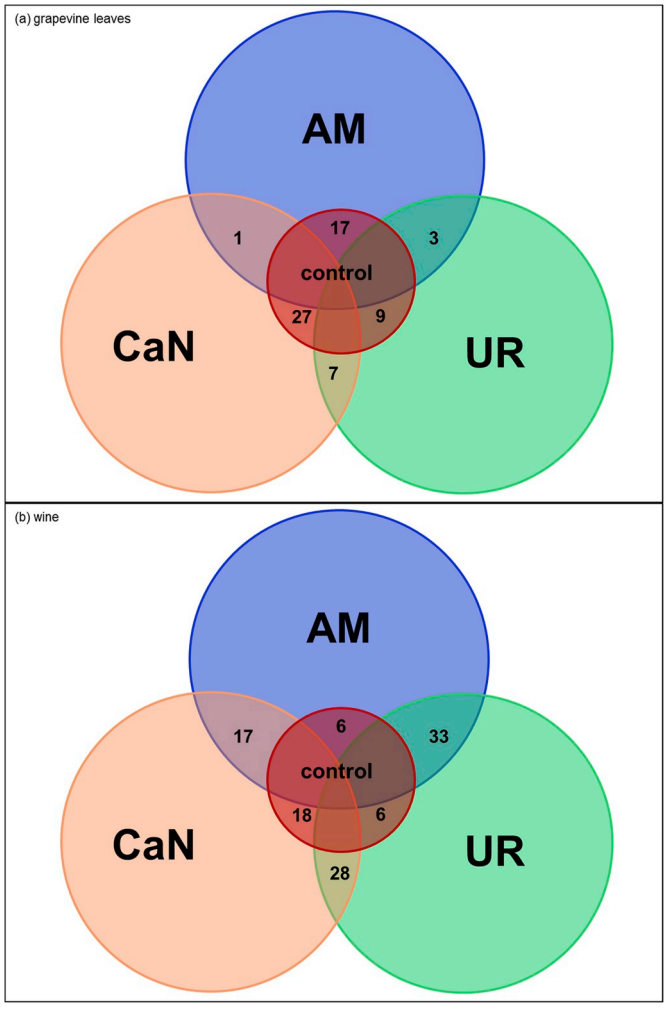

Fig. 5. Venn diagram of regulated compounds of (a) leaves and (b) wine in response to measured significant differences of various N-forms. Numbers include the distribution of all regulated compounds detected in all N comparisons CaN/control, AM/control, UR/control, CaN/AM, CaN/UR, AM/UR.

detectable, especially in the N forms CaN and AM, because of their better plant availability. We assume that CaN and AM provide significantly more N for the yeast cultures than UR. However, as rosé wines were used in the present work, the number of detectable phenolic compounds is lower compared with those in red wine but is higher than those in white wine (Sun et al., 2007). Furthermore, the maceration stage was shortened and thus less polyphenols were able to diffuse from the berry skin into the must. In general, a comparison between leaf and wine might be difficult as the wine is berry juice, which has gone through several processes. Nevertheless, in our experiment, we have been able to detect an influence of fertilisation with different the N-forms on the vines with respect to the phenol composition of the wine and thus their influence on the organoleptic and sensory properties of the wine.

5. Conclusion

Fertilisation with different N-forms such as calcium nitrate, ammonium or urea influences the metabolite profile in grapevine leaves and the sensory attributes of the resulting wine. The quality of must and wine and, the sensory profile of the wine, show greater changes between fertilised and non-fertilised vines and between the N-forms CaN and UR. The metabolic changes found in leaves are less pronounced than those in wine. The N-forms CaN and AM are similar in their regulation pattern. UR mainly decreases the metabolites and phenolic compounds in leaves and in wine, whereas CaN and AM mainly decreases the phenolic compounds in the leaves but increases phenolic compounds and the tentative phenolic compounds of wine. These phenolic components influence the organoleptic and sensory properties of wine. Based on our results, we conclude that CaN and AM fertilisation might have a positive effect on wine quality compared with the application of UR.

Contribution

Conceptualization: Christian Zörb, Jens Pfannstiel, Nikolaus Merkt. Data curation: Carina Paola Lang. Formal analysis: Carina Paola Lang, Christian Zörb. Investigation: Carina Paola Lang. Methodology: Carina Paola Lang, Nikolaus Merkt, Iris Klaiber, Jens Pfannstiel. Software: Carina Paola Lang, Iris Klaiber. Supervision: Christian Zörb, Nikolaus Merkt. Validation: Carina Paola Lang, Christian Zörb. Visualisation: Carina Paola Lang, Iris Klaiber. Writing- original draft: Carina Paola Lang. Writing-review & editing: Christian Zörb, Nikolaus Merkt, Jens Pfannstiel.

Funding

This work was supported by a doctoral scholarship “State Graduate Scholarship” from the Ministry of Science, Research and the Arts of Baden-Württemberg, Germany.

Acknowledgments

We would like to thank Ms. Heike Hahn (SKW Stickstoffwerke Piesteritz GmbH, Lutherstadt Wittenberg) for providing the nitrate inhibitors used in these experiments. We would also like to thank Ms. Johanna Beeck and Mr. Frederic Fürstenberger, who monitored the fermentation and vinification process of the wine for us. We would like to thank the Ministry of Science, Research and the Arts of Baden-Württemberg, Germany, for supporting this work.

Abbreviations used

AM	ammonium
CaN	calcium nitrate
C:N ratio	carbon: nitrogen ratio
ESI	electrospray ionisation
HPLC	high performance liquid chromatography
LA	lactic acid
LC-MS	liquid chromatography mass spectrometry
MA	malic acid
N	nitrogen
PC	principal component
PCA	principal component analysis
SO4	rootstock Selection Oppenheim 4
TA	total acid
TIC	total ion chromatogram
TTA	tartaric acid
UHPLC	ultra high performance liquid chromatography
UR	urea
YAN	yeast-assimilable nitrogen

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.09.009.

References

Alañón, M.E., Pérez-Coello, M.S., Marina, M.L., 2015. Wine science in the metabolomics

era. Trends Anal. Chem. 74, 1–20. https://doi.org/10.1016/j.trac.2015.05.006.
Ali, K., Maltese, F., Choi, Y.H., Verpoorte, R., 2009. Metabolic constituents of grapevine and grape-derived products. Phytochem. Rev. 9 (3), 357–378. https://doi.org/10.1016/j.trac.2015.05.006.
Bell, S.J., Henschke, P.A., 2005. Implications of nitrogen nutrition for grapes, fermentation and wine. Aust. J. Grape Wine Res. 11 (3), 242–295. https://doi.org/10.1111/j.1755-0238.2005.tb00028.x.
Braidot, E., Zancani, M., Petrussa, E., et al., 2008. Transport and accumulation of flavonoids in grapevine *(Vitis vinifera L.)*. Plant Signal. Behav. 3 (9), 626–632. https://doi.org/10.4161/psb.3.9.6686.
Cappello, M.S., Zappardi, G., Logrieco, A., Bartowsky, E.J., 2017. Review - linking wine lactic acid bacteria diversity with wine aroma and flavour. Int. J. Food Microbiol. 243, 16–27. https://doi.org/10.1016/j.ijfoodmicro.2016.11.025.
Castillo-Muñoz, N., Gómez-Alonso, S., García-Romero, E., Hermosín-Gutíerrez, I., 2007. Flavonol profiles of *Vitis vinifera* red grapes and their single-cultivar wines. J. Agric. Food Chem. 55, 992–1002. https://doi.org/10.1021/jf062800k.
Choné, X., Lavigne-Cruège, V., Van Leeuwen, C., et al., 2006. Effect of vine nitrogen status on grape aromatic potential flavour precursors (S-cysteine conjugates), glutathione and phenolic content in Vitis Vinifera L. cv. Sauvignon Blanc grape juice. J. Int. Sci. Vigne Vin 40 (1), 1–6. https://doi.org/10.20870/oeno-one.2006.40.1.880.
Cozzolino, D., 2016. Metabolomics in grape and wine: definition, current status and future prospects. Food Anal. Methods 9 (11), 2986–2997. https://doi.org/10.1007/s12161-016-0502-x.
De Rosso, M., Tonidandel, L., Larcher, R., et al., 2014. Identification of new flavonols in hybrid grapes by combined liquid chromatography-mass spectrometry approaches. Food Chem. 163, 244–251. https://doi.org/10.1016/j.foodchem.2014.04.110.
Downey, M.O., Dokoozlian, N.K., Krstic, M.P., 2006. Cultural practice and environmental impacts on the flavonoid composition of grapes and wine: a review of recent research. Am. J. Enol. Vitic. 57 (3), 257–268.
Dunn, W.B., Ellis, D.I., 2005. Metabolomics: current analytical platforms and methodologies. Trends Anal. Chem. 24 (4), 285–294. https://doi.org/10.1016/j.trac.2004.11.021.
Goel, P., Singh, A.K., 2015. Abiotic stresses downregulate key genes involved in nitrogen uptake and assimilation in *Brassica juncea* L. PLoS One 10 (11), e0143645. https://doi.org/10.1371/journal.pone.0143645.
Jackson, D.I., Lombard, P.B., 1993. Environmental and management practices affecting grape composition and wine quality – a review. Am. J. Enol. Vitic. 44 (4), 409–430.
Jackson, R.S., 2008. Chapter 6 chemical constituents of grapes and wine. In: Jackson, R.S. (Ed.), Wine Science Principles and Applications, 3. Elsevier Inc, Amsterdam, Boston, Heidelberg, London, New York, Oxford, Paris, San Diego, San Francisco, Sydney, Tokyo, pp. 270–331.
Jezek, M., Zörb, C., Merkt, N., Geilfus, C.M., 2018. Anthocyanin management in fruits by fertilisation. J. Agric. Food Chem. 66, 753–764. https://doi.org/10.1021/acs.jafc.7b03813.
Keller, M., 2010. Chapter 6 developmental physiology. In: Keller, M. (Ed.), The Science of Grapevines: Anatomy and Physiology. Elsevier Inc. Burlington, London, San Diego, pp. 169–225. https://doi.org/10.1016/C2009-0-01866-2.
Kennedy, J.A., 2008. Grape and wine phenolics. Observations and recent findings. Cienc. Investig. Agrar. 35 (2), 77–90. https://doi.org/10.4067/S0718-16202008000200001.
Lang, C.P., Merkt, N., Zörb, C., 2018. Different nitrogen (N) forms affect responses to N form and N supply of rootstocks and grafted grapevines. Plant Sci. 277, 311–321. 5. https://doi.org/10.1016/j.plantsci.2018.10.004.
Leser, C., Treutter, D., 2005. Effects of nitrogen supply on growth, contents of phenolic compounds and pathogen (scab) resistance of apple trees. Physiol. Plant. 123, 49–56. https://doi.org/10.1111/j.1399-3054.2004.00427.x.
Loulakakis, K.A., Roubelakis-Angelakis, K.A., 2001. Chapter 3: nitrogen assimilation in grapevine. In: Roubelakis-Angelakis, K.A. (Ed.), Molekular Biology 6 Biotechnology of the Grapevine, Edition 1. Springer science + Business Media, Dordrecht, The Netherlands, pp. 59–85. https://doi.org/10.1007/978-94-017-2308-4_3.
Mazerolles, G., Preys, S., Bouchut, C., et al., 2010. Combination of several mass spectrometry ionization modes: a multiblock analysis for a rapid characterization of the red wine polyphenolic composition. Anal. Chim. Acta 678, 195–202. https://doi.org/10.1016/j.aca.2010.07.034.
Næs, T., Brockhoff, P.B., Tomic, O., 2010. Chapter 14 principal component analysis. In: Næs, T., Brockhoff, P.B., Tomic, O. (Eds.), Statistics for Sensory and Consumer Science. John Wiley & Sons Ltd, Chichester, West Sussex, United Kingdom, pp. 209–225.
Portu, J., González-Arenzana, L., Hermosín-Gutiérrez, I., et al., 2015a. Phenylalanine and urea foliar applications to grapevine: effect on wine phenolic content. Food Chem. 180, 55–63. https://doi.org/10.1016/j.foodchem.2015.02.008.
Portu, J., López-Alfaro, I., Gómez-Alonso, S., et al., 2015b. Changes on grape phenolic composition induced by grapevine foliar applications of phenylalanine and urea. Food Chem. 180, 171–180. https://doi.org/10.1016/j.foodchem.2015.02.042.
Ringnér, M., 2008. What is principal component analysis? Nat. Biotechnol. 26 (3), 303–304.
Robinson, A.L., Boss, P.K., Solomon, P.S., et al., 2014. Origins of grape and wine aroma. Part 1. Chemical components and viticultural impacts. Am. J. Enol. Vitic. 65 (1), 1–13. https://doi.org/10.5344/ajev.2013.12070.
Rossouw, G.C., Šukljea, K., Smitha, J.P., et al., 2018. *Vitis vinifera* berry metabolic composition during maturation: implications of defoliation. Physiol. Plant. 164, 120–133. https://doi.org/10.1111/ppl.12715.
Sumner, L.W., Amberg, A., Barrett, D., et al., 2007. Proposed minimum reporting standards for chemical analysis chemical analysis working group (CAWG) metabolomics standards initiative (MSI). Metabolomics 3 (3), 211–221. https://doi.org/10.1007/s11306-007-0082-2.
Sun, J., Liang, F., Bin, Y., Duan, C., 2007. Screening non-colored phenolics in red wines using liquid chromatography/ultraviolet and mass spectrometry/mass spectrometry libraries. Molecules 12, 679–693. https://doi.org/10.3390/12030679.
Teixeira, A., Eiras-Dias, J., Castellarin, S.D., Gerós, H., 2013. Berry phenolics of grapevine under challenging environments. Int. J. Mol. Sci. 14, 18711–18739. https://doi.org/10.3390/ijms140918711.
Torija, M.J., Beltran, G., Novo, M., et al., 2003. Effect of organic acids and nitrogen source on alcoholic fermentation: study of their buffering capacity. J. Agric. Food Chem. 51, 916–922. https://doi.org/10.1021/jf020094r.
Verpoorte, R., 2000. Chapter 1 secondary metabolism. In: Verpoorte, R., Alfermann, A.W. (Eds.), Metabolic Engineering of Plant Secondary Metabolism. Kluwer Academic Publishers, Dordrecht, Boston, London, pp. 1–29.
Waterhouse, A.L., Sacks, G.L., Jeffery, D.W., 2016. Part A wine components and their reactions chapter 3 acids. In: Waterhouse, A.L., Sacks, G.L., Jeffery, D.W. (Eds.), Understanding Wine Chemistry. 'John Wiley & Sons, Ltd, Chichester, West Sussex, United Kingdom, pp. 19–33.
Winkel-Shirley, B., 2002. Biosynthesis of flavonoids and effects of stress. Curr. Opin. Plant Biol. 5 (3), 218–223. https://doi.org/10.1016/S1369-5266(02)00256-X.
Witte, C.P., 2011. Review – urea metabolism in plants. Plant Sci. 180, 431–438. https://doi.org/10.1016/j.plantsci.2010.11.010.