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A Versatile Targeted Metabolomics Method for the Rapid Quantification of Multiple Classes of Phenolics in Fruits and Beverages

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Supporting Information

ABSTRACT: Compelling evidence of the health benefits of phenolic compounds and their impact on food quality have stimulated the development of analytical methods for the identification and quantification of these compounds in different matrices in recent years. A targeted metabolomics method has been developed for the quantification of 135 phenolics, such as benzoates, phenylpropanoids, coumarins, stilbenes, dihydrochalcones, and flavonoids, in fruit and tea extracts and wine using UPLC/QqQ-MS/MS. Chromatography was optimized to achieve separation of the compounds over a period of 15 min, and MRM transitions were selected for accurate quantification. The method was validated by studying the detection and quantification limits, the linearity ranges, and the intraday and interday repeatability of the analysis. The validated method was applied to the analysis of apples, berries, green tea, and red wine, providing a valuable tool for food quality evaluation and breeding studies.

KEYWORDS: *metabolite profiling, polyphenols, food analysis, UPLC/QqQ-MS/MS, mass spectrometry*

INTRODUCTION

Phenolics represent one of the most chemically diverse and ubiquitous classes of secondary metabolites in plants. Phenolics can be classified into different classes on the basis of their chemical structure and can also occur in polymeric forms such as hydrolyzable tannins, condensed tannins, and lignins and in glycosylated and acylated forms.

Phenolics biosynthesis would seem to be an early acquisition of plants deriving from the primary metabolism during their adaptation to life on land.[1,2] It has been suggested that the ability to produce secondary metabolites with antimicrobial or UV-protection properties, such as those possessed by phenolic compounds, could have given plants some advantages in facing the numerous challenges associated with the water-to-land transition.[3]

Phenolics have been credited with a variety of key functions important for plant growth, development, and survival. Some compounds are common mediators of plant responses to biotic and abiotic stresses.[4,5] Some are part of complex species-specific bouquets emitted as floral attractants to pollinators[6] or contribute to the color of flowers and fruits.[7] Several phenolics act as inducers of plant−microbe symbioses,[8] whereas others exhibit broad-spectrum antimicrobial activity and are therefore thought to help plants fight microbial diseases.[9] They also possess phytoalexin properties, and their synthesis can be induced in response to wounding, feeding by herbivores,[10] or infection by pathogens.[11] Thanks to their structure, containing aromatic rings and hydroxyl groups, phenolic compounds are good protecting agents against UV radiation and potent antioxidants.[12,13]

In recent years, interest in phenolic compounds has been increasing due to compelling evidence of their health benefits and their impact on food quality. Indeed, due to their widespread presence in plants, phenolics enter the human diet from a variety of edible plants and plant products, such as fresh and cooked vegetables, fruit juices, tea, wine, and infusions. For instance, a comprehensive analysis of food composition data revealed that 502 polyphenols, including flavonoids, phenolic acids, lignans, and stilbenes, have been reported in 452 foods so far.[14] Many systematic molecular, in vitro, and epidemiological studies have confirmed their effect on various pathological situations, and their mechanisms of action are under investigation.[15] Recently published papers have reviewed the activity of food polyphenols in decreasing the risk of cancer[16,17] and in preventing allergic diseases,[18] atherosclerosis,[19] obesity,[20] bone resorption,[21] aging,[22] neurodegeneration and dementia,[23] hypertension,[24] and dental caries.[25]

Recently, a few specialist sources, such as Phenol Explorer (www.phenol-explorer.eu)[26] and the USDA database (http://www.ars.usda.gov/Services/docs.htm?docid=8964) have significantly improved the possibility of correctly estimating the nutritional intake of the main classes of phenolics in common

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food.[27] However, the presence of phenolics in most food databases worldwide is still limited, and we are convinced that there is a general need to improve coverage by inserting data on a larger number of phenolic compounds and food sources.

Recent advances in understanding the importance of the compositional quality of plant-derived food for human health are also changing the focus of crop producers and breeders from the traditional improvement of pest resistance and yield to their chemical composition and nutritional value.

As a consequence of this widespread interest in phenolic compounds as food constituents, numerous separation and detection methods for their identification and quantification in different matrices have been developed, these having been recently reviewed by Kalili and de Villiers.[28] However, the number of compounds that have been quantified in a single run with existing methodologies is still relatively modest when compared to the potential coverage of metabolites that could be achieved using the powerful technologies available on the market for chromatography and mass spectrometry, which are widely used in other fields, such as multiresidue pesticide analysis in food[29] and hormones.[30] For instance, a rapid LC-MS/MS method for the quantification of phenols and polyphenols in authentic wine samples, developed by Jaitz et al.[31] targeted only 11 compounds, and the method developed by Guillarme et al. for catechins quantification in tea extracts targeted only 8 metabolites.[32]

Targeted metabolomics represents an attractive strategy for food analysis. This methodology aims to quantify a predefined set of metabolites, typically dozens or hundreds of known compounds, based on metabolite-specific signals.[33,34] In particular, in targeted metabolomics approaches, using triple-quadrupole mass spectrometers, a precursor ion and a fragment of the precursor ion, producing a molecular weight and structure-specific measurement for a single metabolite (referred to as transition), are used for the sensitive and accurate determination of the compound concentration over a wide dynamic range. Simultaneous analysis of multiple transitions results in multiple reaction monitoring (MRM).

The aim of this study was to develop a rapid and versatile targeted metabolomics method for the quantification of multiple classes of phenolics that could be used for high-throughput analysis of fruits and beverages. This would have applications for food quality evaluation but could also assist plant breeders to select a chemical phenotype or "chemotype".

MATERIALS AND METHODS

Chemicals. Methanol and acetonitrile were of LC-MS grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chloroform anhydrous stabilized with 0.5−1% ethanol and formic acid were also purchased from Sigma-Aldrich. The majority of the chemical standards are commercially available and were obtained from different suppliers (Table S1 of the Supporting Information). Viniferins were isolated from leaves of a hybrid *Vitis vinifera* as described by Mattivi et al.[11] The hydroxycinnamoyltartaric acids (*trans*-caftaric acid, *trans*-coutaric acid, and *trans*-fertaric acid) were extracted and purified according to the method described by Vrhovsek.[35] *cis*-Resveratrol and *cis*-piceid were produced by photochemical isomerization of the *trans* forms, as described by Mattivi et al.[36] Milli-Q water was used for the chromatography.

Preparation of Standard Solutions. About 145 phenolic compounds were initially selected for the assay. The choice of the metabolites was mainly based on their importance and/or relevance for food quality, covering the major classes. In particular, benzoates, phenylpropanoids, coumarins, stilbenes, dihydrochalcones, and flavonoids commonly occurring in plants were included, together with metabolites specific to a single species or family. High molecular weight polymers such as tannins were not included in this study. Because good separation of the positively charged anthocyanidins requires chromatography to be carried out in particular conditions, that is, with a very low pH, they were not included in the assay.

The compound names, chemical formulas, and CAS Registry No. are listed in Table S1 of the Supporting Information. Furthermore, the METLIN ID of the compounds, which links the compounds to the Metabolite and Tandem MS Database (http://metlin.scripps.edu/), and the KEGG ID, which can help to visualize the metabolites on the pathways (KEGG Pathway Database, http://www.genome.jp/kegg/pathway.html), are provided.

Stock solutions of each individual standard were prepared in pure methanol with the following exceptions: salicin, 4-hydroxybenzoic acid, dihydroxybenzoic acids, and vanillic acid were prepared in methanol/water (1:1). These starting solutions were used to prepare 16 standard mixtures including 6−10 compounds each. Serial dilutions were prepared to obtain 24 lower concentrations (dilution factors of 1−60000) for linear dynamic range assessment. The composition of each mixture and the starting concentrations of the analytes are reported in Table S1 of the Supporting Information.

Sample Preparation. *V. vinifera* cv. Sangiovese grape berries (2009 harvest, Tuscany, Italy), *Malus domestica* cv. Golden Delicious apples, *Fragaria* × *ananassa* cv. Elsanta strawberries, *Rubus idaeus* cv. Tulameen raspberries, and *Prunus avium* cv. Kordia cherries, all purchased from a local market and grown in the Trentino region (Italy), were ground under liquid nitrogen using an IKA analytical mill (Staufen, Germany) to obtain a frozen powder. The protocol used for the extraction of the phenolic metabolites from these matrices was adapted from that of Theodoridis et al.[37] Briefly, 2 g of powder from each sample was extracted in sealed glass vials using 5 mL of a mixture of water/methanol/chloroform (20:40:40). After vortexing for 1 min, the samples were put in an orbital shaker for 15 min at room temperature. Samples were centrifuged at 1000*g* and 4 °C for 10 min, and the upper phases constituted of aqueous methanol extract were collected. Extraction was repeated by adding another 3 mL of water/methanol (1:2) to the pellet and chloroform fractions and shaking for 15 min. After centrifugation, the upper phases from the two extractions were combined, brought to 10 mL, and filtered through a 0.2 μm PTFE filter prior to analysis.

Ground dry green tea leaves (*Camellia sinensis*, Vivi Verde COOP, organic farming) purchased from a local store were extracted with aqueous 80% methanol in a ratio of 1 g to 10 mL of solvent (w/v) using a sonicator for 30 min.[38] After centrifugation at 1000*g* and 4 °C for 10 min, the supernatant was collected and filtered through a 0.2 μm PTFE filter prior to analysis.

Sangiovese wine (red table wine, Tavernello, traceability code LB0097WO, Caviro, Italy) was analyzed after filtration on 0.2 μm PTFE filters.

Liquid Chromatography. Ultraperformance liquid chromatography was performed on a Waters Acquity UPLC system (Milford, MA) consisting of a binary pump, an online vacuum degasser, an autosampler, and a column compartment. Separation of the phenolic compounds was achieved on a Waters Acquity HSS T3 column 1.8 μm, 100 mm × 2.1 mm (Milford, MA, USA), kept at 40 °C. Mobile phase A was water containing 0.1% formic acid; mobile phase B was acetonitrile containing 0.1% formic acid. The flow was 0.4 mL/min, and the gradient profile was 0 min, 5% B; from 0 to 3 min, linear gradient to 20% B; from 3 to 4.3 min, isocratic 20% B; from 4.3 to 9 min, linear gradient to 45% B; from 9 to 11 min, linear gradient to 100% B; from 11 to 13 min, wash at 100% B; from 13.01 to 15 min, back to the initial conditions of 5% B. The injection volume of both the standard solutions and the samples was 2 μL. After each injection, the needle was rinsed with 600 μL of weak wash solution (water/methanol, 90:10) and 200 μL of strong wash solution (methanol/water, 90:10). Samples were kept at 6 °C during the analysis.

Mass Spectrometry. Mass spectrometry detection was performed on a Waters Xevo TQMS (Milford, MA, USA) instrument equipped with an electrospray (ESI) source. Capillary voltage was 3.5 kV in positive mode and −2.5 kV in negative mode; the source was kept at 150 °C;

Table 1. MRM Parameters of the Selected Metabolites

					quantifier ion		qualifier ion 1			qualifier ion 2	
compound	RT (min)	ES	cone voltage (V)	Q1 *m/z*	collision energy (V)	Q2 *m/z*	collision energy (V)	Q2 *m/z*	expected % ratio ion 1/quantifier	collision energy (V)	Q2 *m/z*
Benzoic Acid Derivatives											
catechol	2.89	−	36	109	12	81	16	53	13		
benzoic acid	5.61	−	22	121	12	77					
3-hydroxybenzaldehyde	3.97	+	20	123	10	95	16	77	13		
salicylic acid	6.06	+	22	139	12	121					
4-hydroxybenzoic acid	2.84	+	20	139	18	77	26	65	9		
2,4-dihydroxybenzoic acid	3.31	+	24	153	14	109	16	65	34		
2,5-dihydroxybenzoic acid	2.88	+	26	153	16	109	16	81	5		
2,6-dihydroxybenzoic acid	3.61	+	26	153	14	109	18	135	11	20	91
3,4-dihydroxybenzoic acid	2.12	+	24	153	20	81	22	91	64		
3,5-dihydroxybenzoic acid	2.10	+	26	153	14	109	14	65	9		
gallic acid	1.41	+	28	169	22	79	22	97	42	18	78
anthranilic acid	3.92	+	14	138	26	65	34	92	11	30	39
vanillin	4.07	+	20	153	16	93	10	125	61	22	65
vanillic acid	3.23	+	18	169	14	93	18	110	25	22	95
methyl gallate	2.90	+	32	183	22	124	16	168	5	28	78
cinnamic acid	7.55	+	16	149	12	131	10	103	1		
acetovanillone	4.56	+	20	167	12	43	12	125	7	18	110
syringaldehyde	4.35	+	20	183	12	123	10	155	51	18	95
syringic acid	3.40	+	22	197	14	182					
salicin	2.21	+	38	309	16	185	18	147	67		
ellagic acid	4.38	−	52	301	34	145	30	185	15		
Coumarins											
4-hydroxycoumarin	6.06	+	42	163	18	91	20	69	99		
umbelliferone	4.40	+	32	163	20	107	20	91	34		
4-methylumbelliferone	5.87	+	34	177	20	121	18	105	84		
daphnetin	3.17	+	36	179	22	123	24	105	16		
esculetin	3.49	+	34	179	22	133	22	123	38		
esculin	2.41	+	24	341	18	179	40	133	9		
scopoletin	4.53	+	34	193	20	133	22	178	32		
fraxin	3.07	+	32	369	20	207	32	192	38		
Phenylpropanoids											
p-coumaric acid	4.04	+	16	165	24	91	26	119	21		
m-coumaric acid	4.76	+	18	165	12	147	16	119	8	22	91
o-coumaric acid	5.70	+	18	165	16	103	22	91	15		
caffeic acid	3.19	+	16	181	16	145	22	117	26		
ferulic acid	4.52	+	12	195	16	145	24	117	40		
sinapic acid	4.55	−	30	223	14	208	18	164	45	20	149
caftaric acid	2.31	−	18	311	10	149	14	179	62	28	135
neochlorogenic acid	2.18	−	26	353	18	191	18	179	52	34	135
cryptochlorogenic acid	2.87	−	34	353	14	173	16	179	19	30	135
chlorogenic acid	2.76	−	36	353	20	191					
1,3-dicaffeoylquinic acid	3.43	−	34	515	20	353	34	191	33	32	179
1,5-dicaffeoylquinic acid	5.20	−	26	515	28	191	16	353	11		
rosmarinic acid	6.04	−	30	359	18	161	18	197	14		
coniferyl aldehyde	5.70	−	22	179	14	147	18	119	23		
coniferyl alcohol	4.04	+	12	163	10	131	18	103	25		
sinapyl alcohol	4.07	−	14	193	10	161	16	133	31	20	105
fertaric acid	3.13	−	20	325	16	193					
trans-coutaric acid	2.85	−	20	295	12	163					
raspberry ketone	5.52	−	36	107	18	77					
Stilbenes											
4-hydroxystilbene	10.67	−	44	195	26	93					
trans-resveratrol	6.56	−	38	227	18	185	26	143	128		
cis-resveratrol	7.55	−	40	227	26	143	22	185	39	18	159
piceatannol	5.10	−	40	243	28	159	22	201	24	20	175
pterostilbene	10.58	−	40	255	18	240	32	197	44	38	169
trans-piceid	4.27	−	28	389	18	227	36	185	7		

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Table 1. continued

compound	RT (min)	ES	cone voltage (V)	Q1 *m/z*	quantifier ion collision energy (V)	quantifier ion Q2 *m/z*	qualifier ion 1 collision energy (V)	qualifier ion 1 Q2 *m/z*	qualifier ion 1 expected % ratio ion 1/quantifier	qualifier ion 2 collision energy (V)	qualifier ion 2 Q2 *m/z*
Stilbenes											
cis-piceid	5.86	−	28	389	20	227	38	185	7		
astringin	3.53	−	36	405	20	243	36	201	6		
isorhapontin	4.69	−	30	419	18	257	36	241	46		
cis-ε-viniferin	7.93	−	48	453	22	347	22	359	80	36	225
trans-ε-viniferin	8.11	−	52	453	22	347	20	359	27	34	225
cis-ω-viniferin	8.50	−	46	453	20	347	20	359	31	30	225
trans-ω-viniferin	8.62	−	48	453	22	347	22	359	21	30	225
caffeic acid + catechin condensation product	5.56	−	34	451	18	341	34	177	10		
pallidol	6.00	−	32	453	30	265	14	359	20		
ampelopsin D + quadrangularin A	6.65	−	42	453	18	359	26	343	47	28	289
α-viniferin	8.59	−	48	677	30	437	26	571	19		
E-cis-miyabenol	8.17	−	54	679	30	345	26	573	15		
Z-miyabenol C	8.85	−	54	679	36	345	24	573	21		
isohopeaphenol	7.57	−	48	905	38	359	30	811	8		
ampelopsin H + vaticanol C-like isomer	8.01	−	52	905	30	811	40	475	3	42	335
Dihydrochalcones											
phloretin	8.23	−	32	273	18	167	24	123	11		
phloridzin	6.22	−	32	435	16	273	30	167	17		
trilobatin	6.72	−	36	435	18	273	18	297	21	32	167
Isoflavones											
daidzein	6.98	+	30	255	24	199	26	137	132		
genistein	8.67	+	44	271	24	215					
Flavones											
6-methoxyflavone	10.62	+	16	253	38	108	26	238	44	40	153
chrysin	10.16	−	44	253	30	143	26	107	58	32	63
apigenin	8.28	−	42	269	34	117	20	151	1	24	148
apigenin-7-*O*-glucoside	5.71	+	24	433	20	271	52	153	6		
apiin	5.44	+	26	565	30	271	14	433	3		
sinensetin	9.84	+	8	373	28	343	26	312	88	48	153
morin	6.73	+	38	303	32	153	26	229	75	28	137
eupatorin-5-methylether	8.87	+	12	359	18	329	26	298	62	46	153
luteolin	7.37	+	52	287	32	153	30	135	40		
luteolin-7-*O*-glucoside	4.56	−	46	447	24	285	48	151	2		
luteolin-8-*C*-glucoside	3.84	+	36	449	30	329	22	413	25	34	299
baicalein	8.82	+	46	271	32	123	32	103	9		
hesperetin	8.60	+	32	303	26	153					
hesperidin	5.84	+	18	611	24	303	10	449			
galangin	6.09	+	48	271	32	153					
Flavanones											
naringenin	8.19	−	32	271	18	151	24	119	20	26	107
naringenin-7-*O*-glucoside	6.20	−	16	435	14	273					
sakuranetin	10.20	−	32	287	30	119	24	167	31	22	147
eriodictyol	7.18	−	30	289	24	153					
Flavan-3-ols											
catechin	2.80	−	32	289	20	203	32	123	33		
epicatechin	3.32	−	34	289	20	203	30	123	46	28	109
gallocatechin	1.89	−	32	305	26	125	18	179	28		
epigallocatechin	2.50	−	32	305	22	125	16	179	34		
catechin gallate	4.45	−	34	441	18	289	20	169	27	40	125
epicatechin gallate	4.36	−	34	441	18	289	20	169	26	38	125
gallocatechin gallate	3.56	−	26	457	20	169	18	305	6	38	125
epigallocatechin gallate	3.37	−	32	457	16	169	20	305	6	38	125
procyanidin A2	4.62	−	42	575	30	285	22	449	15	24	289
procyanidin B1	2.40	−	32	577	26	289	16	425	7	22	407
procyanidin B2 + B4	3.01	−	30	577	24	289	16	425	15	24	407

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Table 1. continued

compound	RT (min)	ES	cone voltage (V)	Q1 *m/z*	quantifier ion collision energy (V)	quantifier ion Q2 *m/z*	qualifier ion 1 collision energy (V)	qualifier ion 1 Q2 *m/z*	qualifier ion 1 expected % ratio ion 1/quantifier	qualifier ion 2 collision energy (V)	qualifier ion 2 Q2 *m/z*
Flavan-3-ols											
procyanidin B3	2.72	−	34	577	22	289					
Flavonols											
quercetin	8.40	+	50	303	34	153	28	229	81	30	137
quercetin-3-sulfate	4.50	-	24	381	18	301	28	179	3	30	151
quercetin-3-*O*-rhamnoside	5.55	+	16	449	10	303	14	129	1	22	85
quercetin-3-*O*-glucoside	4.50	+	18	465	12	303	48	229	5	52	153
quercetin-4′-*O*-glucoside	5.69	+	18	465	12	303	48	229	20	56	153
quercetin-3-*O*-galactoside	4.37	+	18	465	12	303	44	229	8	48	153
quercetin-3-*O*-glucose-6′-acetate	5.71	+	20	507	16	303	18	187	8	30	109
quercetin-3-*O*-glucuronide	4.45	+	20	479	18	303	48	229	6	50	153
rutin (quercetin-Glc-Rha)	4.18	+	18	611	22	303	12	465	8	34	85
quercetin-3-Glc-Ara	3.90	+	20	597	22	303	14	465	4	72	153
quercetin-3,4′-diglucoside	3.57	+	18	627	32	303	16	465	39	78	153
taxifolin	4.72	+	20	305	14	259	14	153	30	26	149
kaempferol	8.45	+	50	287	32	153	26	165	26	30	121
kaempferol-3-*O*-glucoside	5.45	+	16	449	14	287	48	153	5		
kaempferol-3-*O*-glucuronide	5.45	+	18	463	16	287	44	165	2	56	153
kaempferol-3-*O*-rutinoside	5.00	+	18	595	20	287	12	449	9	34	85
robinin	3.80	+	22	741	38	287	14	595	6	20	433
dihydrokaempferol	6.07	+	18	289	18	153	16	243	64	22	149
myricetin	6.07	+	44	319	32	153	28	245	23	26	165
myricitrin	4.30	+	14	465	10	319					
laricitrin	7.46	+	44	333	26	318	42	219	79	34	153
syringetin	8.58	+	40	347	28	153	24	287	60	30	258
syringetin-3-*O*-glucoside + syringetin-3-*O*-galactoside	5.71	+	16	509	14	347	40	287	6	42	153
rhamnetin	9.53	+	48	317	42	123	28	243	73	32	123
isorhamnetin	8.67	+	44	317	34	153	26	302	50	32	229
isorhamnetin-3-*O*-glucoside	5.69	+	16	479	14	317	30	302	13	50	153
isorhamnetin-3-rutinoside	5.30	+	18	625	20	317	12	479	7	52	302

desolvation temperature was 500 °C; cone gas flow, 50 L/h; and desolvation gas flow, 800 L/h. Unit resolution was applied to each quadrupole. Flow injections of each individual metabolite were used to optimize the MRM conditions. For the majority of the metabolites, this was done automatically by the Waters Intellistart software, whereas for some compounds the optimal cone voltages and collision energies were identified during collision-induced dissociation (CID) experiments and manually set. A dwell time of at least 25 ms was applied to each MRM transition.

Data Analysis. Data processing was done using Waters MassLynx 4.1 and TargetLynx software. Data visualization and annotation of high-resolution spectra were done using the R software suite (http://www.R-project.org),[39] with specific use of the Gplot library for heat map graphics.

Method Validation Study. Method validation was performed by studying the linear dynamic range, precision of the analysis, and limit of quantification (LOQ) for the standard compounds. The linear dynamic range was evaluated using standard solutions, prepared as described above, in a concentration range spanning >5 orders of magnitude for most of the compounds. The intraday (n = 5) and interday (n = 5) precision of the analyses was evaluated at two intermediate dilutions (100- and 1000-fold dilutions of the starting mixture). The limit of quantification (LOQ) for each compound was evaluated as the concentration at which the quantifier transition presented a signal-to-noise (S/N) ratio of >10.

Application to Fruit, Tea Extracts, and Wine. The method developed was applied to the analysis of several fruit extracts (apple, strawberry, raspberry, and cherry), tea leaves, and red wine. The precision of the instrumental analysis was evaluated by repeatedly injecting the samples (n = 5) and evaluating the RSD% of the peak areas and retention times of the detected metabolites.

RESULTS AND DISCUSSION

UPLC/QqQ-MS/MS Optimization. UPLC conditions were optimized to achieve good separation of the compounds in a short chromatographic run. Although acetonitrile and methanol were both found to perform well in terms of separation power, the choice of acetonitrile as mobile phase was mainly determined by the lower operational pressure on the column. Addition of 0.1% formic acid to the mobile phases improved chromatographic separation and ionization efficiency for most of the metabolites. Chromatography was performed on an Acquity HSS T3 column packed with a trifunctional C18 alkyl phase, which improves retention of the more polar metabolites. The gradient was optimized to provide separation of isomeric compounds, although in some cases this could not be achieved. For instance, the method allowed separation of all five dihydroxybenzoic acid isomers (2,4-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, 2,6-dihydroxybenzoic acid, 3,5-dihydroxybenzoic acid, and 3,4-dihydroxybenzoic acid). The *ortho*, *meta*, and *para* isomers of coumaric acid could also be separated, as could chlorogenic acid isomers, to mention just a few. Unfortunately, some isomeric forms could not be well separated and were quantified as a single compound. These compounds included glucoside and galactoside forms of

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syringetin, procyanidins B2 and B4, ampelopsin H, and vaticanol C-like isomer. Under the optimized chromatographic conditions, all of the metabolites eluted within 11 min. The total run time was 15 min, including washing and conditioning steps.

MRM conditions were either manually or automatically optimized for each compound, in both positive and negative ESI ionization modes, during infusion studies. All tuning data acquired automatically through the IntelliStart software were manually examined to ensure proper selection of product ions and collision energy. In general, the MS conditions were first optimized in quadrupole 1 (Q1), which transmits only an ion of specific m/z. The ion selected for fragmentation was in most cases the protonated or deprotonated quasi-molecular ion. After CID studies, the conditions were adjusted for the third quadrupole (Q3) to provide optimal signals from the daughter ions. For a few metabolites extensive in-source fragmentation of the molecule was observed. Thus, the main fragment ion was selected as the parent ion. This was the case of coniferyl alcohol, sinapyl alcohol, and raspberry ketone. The two alcohols had in-source fragmentation, and the main ions in the MS spectra were m/z 163 and 193, respectively, generated by the loss of the hydroxyl group, whereas raspberry ketone gave an intense ion at m/z 107. Another particular case was salicin, or 2-(hydroxymethyl)phenyl-β-D-glucopyranoside, occurring mainly as the Na^+ adduct, for which the adduct was selected as parent ion.

During optimization of the methodology, nine compounds were excluded from the study because they displayed ionization problems resulting in an inconsistent response. These were cuminaldehyde, 2-methyl-4-vinylphenol, 1,3-dihydroxybenzene, 1,3,5-trihydroxybenzene, guaiacol, tyrosol, methyl salicylate, 1-phenylethyl acetate, and genistein-4,7-dimethyl ether. Shikimic acid was also excluded from the study because it was not well retained on the column in the chosen conditions.

In total, the MRM signals for 135 metabolites were optimized and a unique acquisition channel was created for each compound. The precursor and product ions, quantifier and qualifiers (when present), collision energies, and cone voltages for the single metabolites are listed in Table 1, as well as the expected ratio between the area of qualifier 1 and the quantifier. This ratio was computed as the average of the experimental values included in the calibration curve of each standard compound. Using the reference standard of each compound to obtain the RT, the quantifier and one or two qualifier ions are accepted as confirmation of the compound.[40] All compounds that achieved this requirement are presented in boldface in Tables S3 of the Supporting Information, whereas all others remain tentatively identified.

Method Validation. One of the challenges that must be faced in the analysis of complex matrices such as fruit extracts, tea, or wine is the fact that the variety of analytes to quantify can occur at very different concentration levels. The measurement sensitivity for each compound can also vary greatly in the assay. Therefore, in this study the linear dynamic range of the instrument for each metabolite was explored, in addition to limits of quantification (LOQs). Dilutions of the 16 mixtures of metabolites were injected to assess the linearity of the response over >4 orders of magnitude. The ranges of linearity, parameters of the curves, and LOQs are reported in Table S2 of the Supporting Information. The distribution of the linearity ranges for the phenolic compounds is shown in Figure 1. The response of the detector was linear over 3−4 orders of magnitude for all of the compounds included in the assay, with correlation coefficients (R^2) >0.990 for >88% of the metabolites. The instrumental LOQs were defined as the concentration at which the quantifier transition presented a signal-to-noise (S/N) ratio >10, and they were calculated using the peak-to-peak algorithm from the closest injected concentration. About 11% of the compounds displayed a LOQ between 0.5 and 5 pg; 58% had an LOQ in the 5−50 pg range, and 31% had an LOQ of >50 pg. The distribution of the LOQs for the metabolites included in the study is shown in Figure 2.

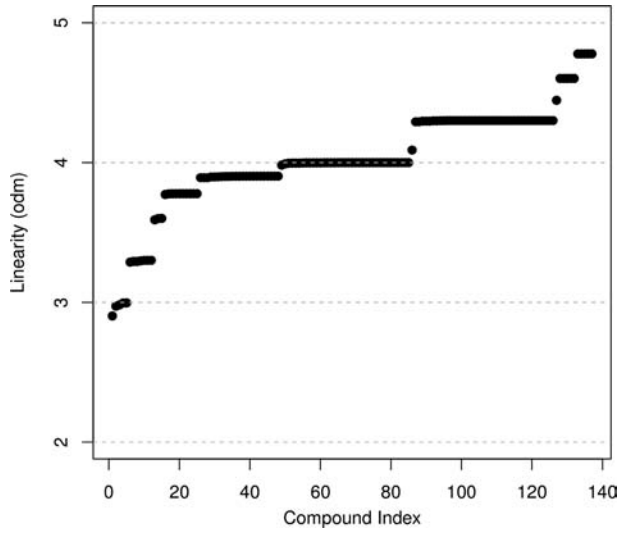

Figure 1. Distribution of the linearity ranges of the assayed metabolites. The y axis indicates the orders of magnitude.

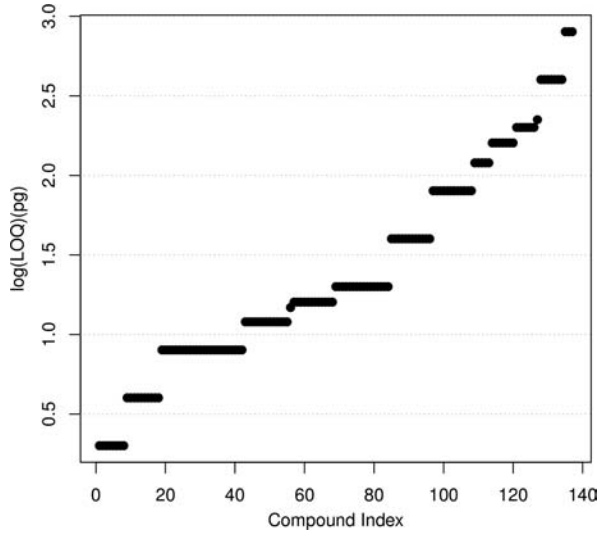

Figure 2. Distribution of the limits of quantification (LOQ) of the assayed metabolites. Note that the y axis sets on a log scale.

Instrumental precision was determined by injecting the 16 standard mixtures at two intermediate concentration values in the linear range, both intraday (n = 5) and interday (n = 5). The samples were analyzed by the same operator, and the relative standard deviation (RSD%) of the peak areas and retention time were calculated. In the intraday precision experiment, the majority of the metabolites (98.6% at the higher concentration and 88.5% at the lower concentration)

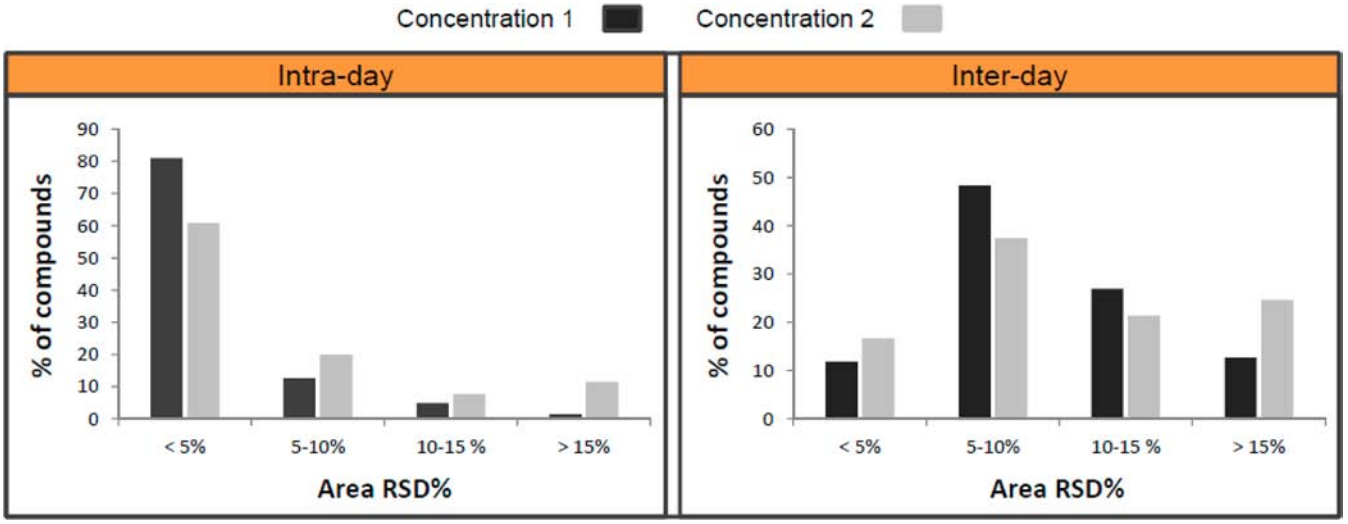

Figure 3. Inter- and intraday instrumental precision. The RSD of the areas of all the metabolites was measured at two different concentrations in the linearity range.

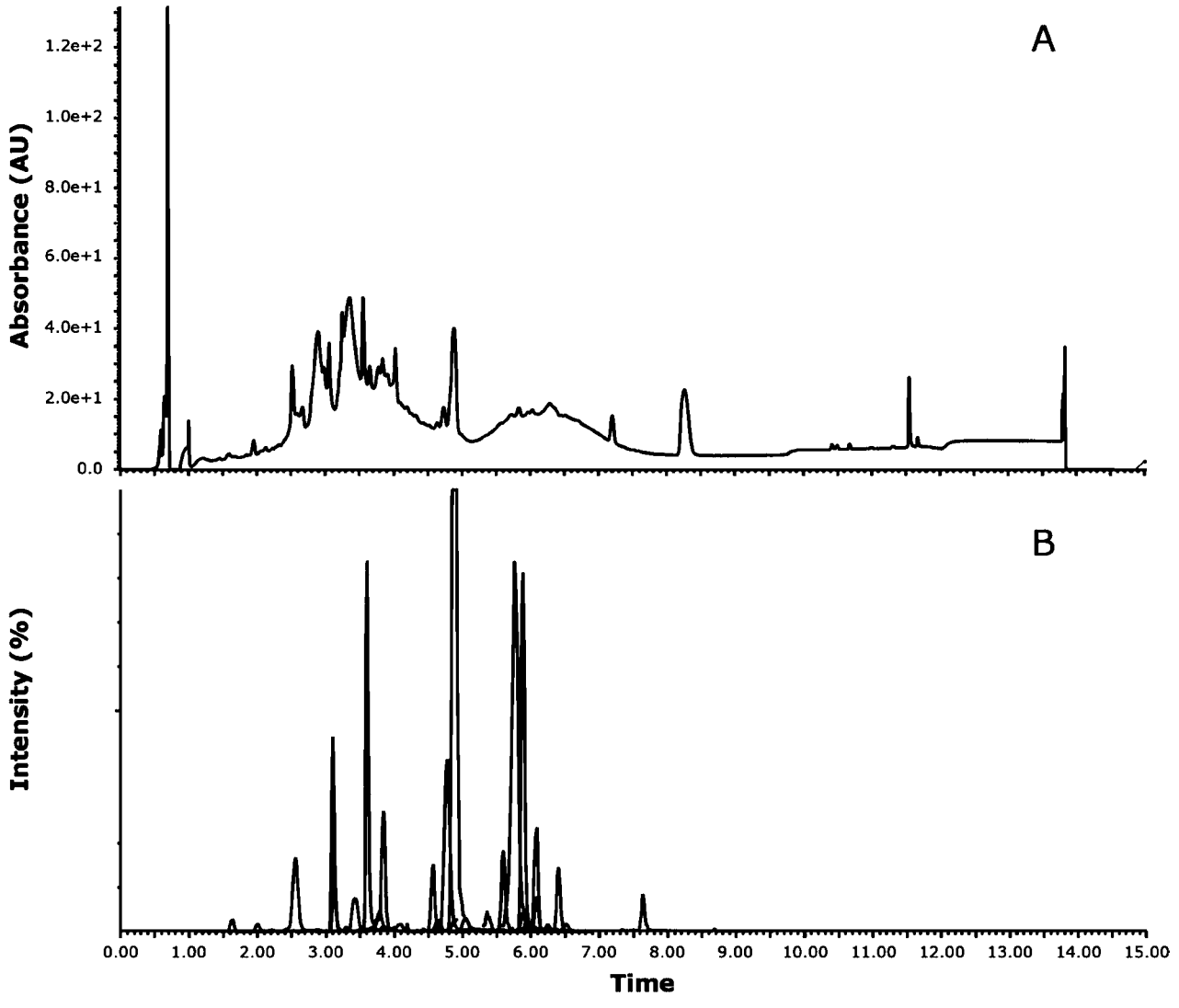

Figure 4. UPLC-DAD (A) and UPLC/QqQ-MS/MS (B) chromatograms obtained from grape extract. Whereas in the DAD chromatogram the separation is incomplete and does not allow quantification of the metabolites, the MRM ion chromatograms display clean peaks with baseline resolution that allow unambiguous quantification.

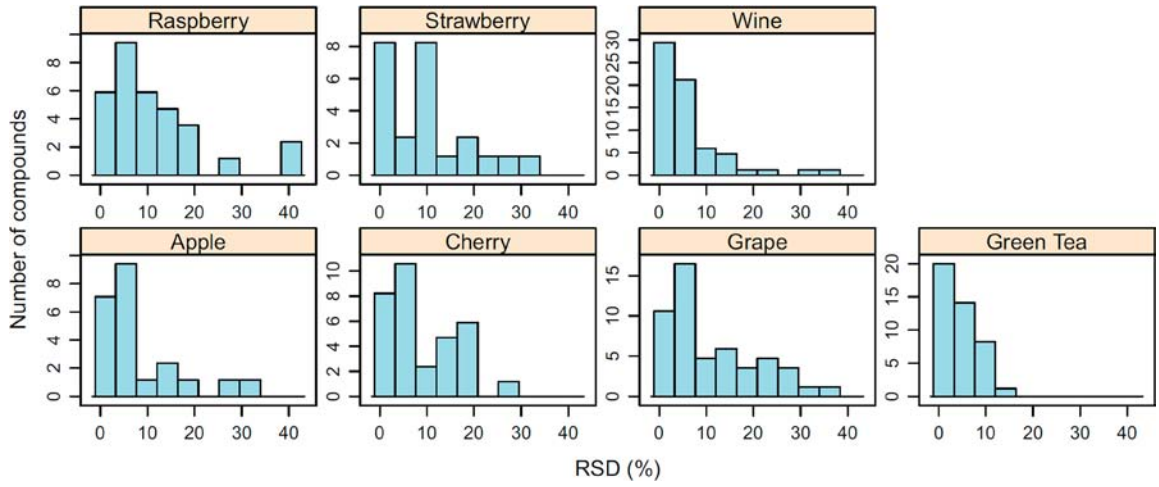

Figure 5. Instrumental precision of the matrix analysis. The RSD% of the areas of the metabolites identified in the different matrices is displayed as histograms.

displayed a RSD of the area lower than 15% (Figure 3). It is worth noting that 81% of the compounds displayed an area RSD lower than 5% at concentration 1 (100-fold dilution of the standard mixtures) and 60.8% at concentration 2 (1000-fold dilution). In the interday experiment the number of metabolites with a RSD% lower than 15% was 87.3% at concentration 1 and 75.4% at concentration 2, respectively, indicating that the instrumental response was stable over a period of 5 days (Figure 3). The retention time of the standard compounds was also very stable. In fact, with very few exceptions, its RDS was lower than 0.01 min (as displayed in Figure S1A of the Supporting Information).

Occurrence of Phenolic Compounds in Fruit, Green Tea, and Red Wine. The validated method was applied to quantitative analysis of several fruit extracts, green tea, and red wine. In particular, apples, grapes, strawberries, raspberries, and cherries were included in this preliminary study. The number of metabolites detected at a concentration above the LOQ varied in the different matrices: it was possible to quantify 17 compounds in apples, 49 in grapes, 22 in strawberries, 29 in raspberries, 33 in cherries, 58 in green tea, and 57 in red wine. Furthermore, some metabolites were detected in these samples at concentrations lower than the LOQ and could not be quantified. In total, 87 metabolites were identified and quantified in at least one extract, representing a detection rate of about 64%. Figure 4 shows the MRM traces of the phenolics present in the grape extract, as an example.

The precision of the analysis was evaluated after repeated injection of the samples (n = 5) by calculating the RSD of the detected peak area and the standard deviation of the retention time. The results showed that for the majority of the hits the RSD% was lower than 10, and values above 20 were exceptions indicating that the reproducibility of the analysis was good, as shown in Figure 5. The RSD of retention time of the detected compounds was found to be lower than 0.02 min for the majority of the compounds (Supporting Information, Figure S1B).

The results of the analysis are shown in the form of a heat map in which the concentration value logarithms are within a color scale (Figure 6). The concentrations of the detected metabolites (provided in Table S3 of the Supporting Information) spanned several orders of magnitude, from nanograms to milligrams per gram of extracted material. For about 90% of the 236 hits the concentration values occurred in the linearity range, whereas 25 measurements were above the upper limit. These data suggest that the method represents a promising approach for the quantification of phenolic compounds in a wide dynamic range, although in some cases sample dilution could be required.

Similarities between the samples analyzed in terms of metabolite content are shown, in the form of a dendrogram, in Figure 6. The phenolic profiles of strawberries, apples, and raspberries appeared to be closely related. This hierarchical chemotype clustering information partially reflects the phylogenetical classification of the Rosaceae family. Indeed, raspberries and strawberries belong to the Rosoideae subfamily, whereas cherries and apples belong to the Spiraeoideae subfamily.[41,42] The analysis also emphasized the similarities between grapes and wine in terms of metabolite composition, whereas green tea clustered separately from all of the other samples, as expected.

The high sensitivity offered by the method developed also provided some interesting information about the composition of the fruit extracts analyzed in this study, revealing the presence of compounds in fruits that had never previously been reported, probably due to their low concentration. One of the most interesting observations was the presence of the stilbenes *trans*- and *cis*-piceid (or *trans*- and *cis*-resveratrol-3-*O*-glucosides), which mainly occur in grapes, peanuts, and some berries, such as strawberries and lingonberries, in apples at concentrations above the LOQ (0.04 and 0.02 μg/g, respectively). Furthermore, the dihydrochalcone phloridzin (phloretin-3-*O*-glucoside), normally occurring in apples and plums, was found in grapes at a concentration of 0.08 μg/g.

Further experiments were carried out to validate these hits using a completely different approach. New extracts were prepared and analyzed using a longer chromatography (63 min) and a time-of-flight (TOF) detector according to the method of Theodoridis et al.[37] Peak matching and annotation were performed on the basis of mass values and retention times by comparison with a database developed in-house for plant secondary metabolites. The results fully confirmed the hits with a mass accuracy lower than 5 ppm and a retention time window of 20 s. High-resolution spectra of *trans*- and *cis*-piceid and phloridzin in apples and grapes are provided in Figure S1 of the Supporting Information. Although relatively unusual, these

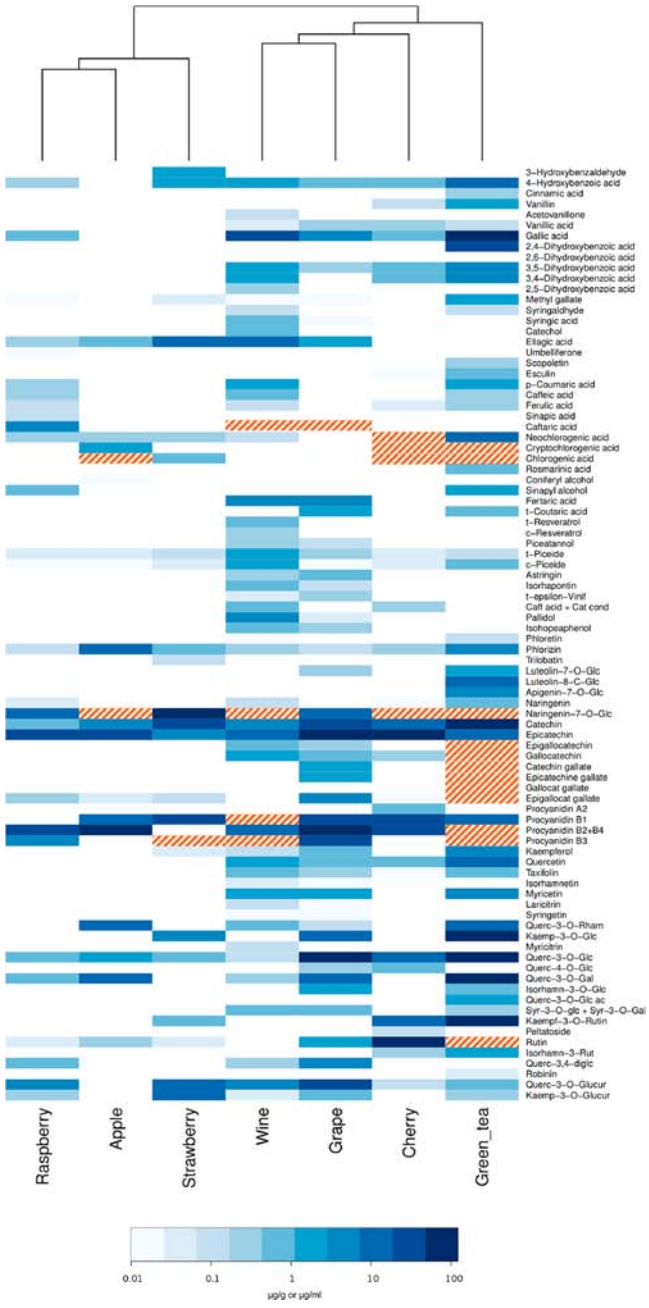

Figure 6. Concentrations of the metabolites in the different matrices visualized as a heat map. The dendrogram represents the hierarchical clustering of the samples. Diagonal slashes indicate compounds in saturation.

results are also supported by evidence that stilbene synthases developed from chalcone synthases during the course of their evolution and that their activity can be directed toward one or another product by substituting a few amino acids.[43] Moreover, minor cross-reactions have been observed, possibly due to conformational flexibility of their active sites.[44] It is therefore possible to speculate that the occurrence of minute amounts of stilbenes in apples and dihydrochalcones in grapes are the result of cross-reactions of the stilbenes and chalcone synthases responsible for their production.

In conclusion, we developed a UPLC/QqQ-MS/MS metabolite profiling method for food analysis that should allow rapid exploration of the presence of polyphenols in different food matrices, with converging opportunities for research applications in plant science and human nutrition. This work shows that targeted metabolomics using UPLC/QqQ-MS/MS represents an attractive and effective strategy for food analysis. Indeed, the high sensitivity of MRM-based mass spectrometry and the wide dynamic range of triple-quadrupole spectrometers provide a valuable tool for the analysis of complex matrices such as fruit, and more in general food, in which analyte concentrations span several orders of magnitude. The method we have developed for the profiling of phenolic compounds is versatile, and it could be successfully applied to the analysis of a range of different matrices, not limited to those chosen for validation. Its sensitivity revealed the presence of compounds that were not reported before in some matrices, suggesting that this approach can also play a role in redesigning metabolic networks, also exploring minor branches of the plant metabolism. It could be easily integrated with the insertion of additional groups of compounds, repeating the simple protocol of optimization here described. The short duration of the analysis and the straightforward sample preparation make the methodology suitable for high-throughput varietal screening studies and for use in assisting plant breeders to select specific chemotypes.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional figures and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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