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RESEARCH PAPER

Application of UHPLC for the determination of free amino acids in different cheese varieties

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Abstract A rapid ultra-high performance liquid chromatography (UHPLC) protocol for the determination of amino acids as their respective 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) derivatives was successfully applied for assessing free amino acid levels in commercial cheese samples representing typical product groups (ripening protocols) in cheesemaking. Based on the *Waters* AccQ.Tag™ method as a high performance liquid chromatography (HPLC) amino acid solution designed for hydrolyzate analyses, method adaptation onto UHPLC was performed, and detection of AQC derivatives was changed from former fluorescence (λ_{Ex} 250 nm/λ_{Em} 395 nm) to UV (254 nm). Compared to the original HPLC method, UHPLC proved to be superior by facilitating excellent separations of 18 amino acids within 12 min only, thus demonstrating significantly shortened runtimes (>35 min for HPLC) while retaining the original separation chemistry and amino acid elution pattern. Free amino acid levels of the analyzed cheese samples showed a high extent of variability depending on the cheese type, with highest total amounts found for original Italian extra-hard cheeses (up to 9,000 mg/100 g) and lowest for surface mold- or bacterial smear-ripened soft cheeses (200–600 mg/100 g). Despite the intrinsic variability in both total and specific concentrations, the established UHPLC method enabled reliable and interference-free amino acid profiling throughout all cheese types, thus demonstrating a valuable tool to generate high quality data for the characterization of cheese ripening.

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Introduction

Along with lipolysis and "glycolysis", casein proteolysis represents the major and by far the most complex biochemical process occurring during cheese ripening. Given the huge variety of different influencing factors intrinsic to cheesemaking, the major accountable agents for catalyzing proteolytic reactions in cheese can be classified in (1) residual coagulant, (2) indigenous milk enzymes, enzymes from (3) starter and (4) non-starter lactic acid bacteria or (5) secondary cultures, and (6) exogenous proteinases and peptidases used for accelerated ripening. As cheese maturation proceeds, the ongoing casein breakdown leads to the formation of large or intermediate-sized peptides, followed by hydrolysis to shorter peptides and eventually to free amino acids (FAAs) that are further catabolized into typical flavor compounds [1–3].

In reference to the multiple approaches (e.g., nitrogen indices after cheese fractionation, electrophoreses of caseins, peptide profiling, etc.) used to assess proteolytic changes [3, 4], analyses of FAAs (total as well as specific amounts) may be considered valuable within the scheme of characterizing cheese maturation.

Since most amino acids lack natural UV or fluorescence response, LC-dedicated methods generally require a derivatization procedure using UV chromophore or fluorophore reagents prior to detection. Besides the "classical" amino acid analyzer with post-column ninhydrin derivatization [5], nowadays analytics also offers a variety of different pre-column techniques suitable for amino acid analyses each with their own advantages and drawbacks [6]. However, as no "universal" reagent has been

commercialized so far, the choice which one to use may ultimately be influenced by the preferences of the chromatographers' themselves. Some of the most common reagents are, e.g., 5-dimethylaminonaphthalene-1-sulfonyl chloride, 4-dimethylaminoazobenzene-4-sulfonyl chloride, phenyl isothiocyanate, *o*-phthalaldehyde or 9-fluorenylmethyl chloroformate. In addition, pre-column derivatizations using 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) [7] might also represent an interesting alternative due to the advantageous method performance such as the fast reaction with primary and secondary amino acids (as well as with biogenic amines), stable derivatives, a facile derivatization protocol (a buffering and heating step) using a commercial kit (AccQ.Fluor™) and the possibility for fluorescence or UV detection [8]. Emphasizing on these method characteristics, the AQC reagent was already successfully used for amino acid analyses in various applications, e.g., [9–12] including the profiling of FAAs in cheese [13–15].

Considering the variety of different amino acid protocols, commercialized HPLC-designed amino acid solutions may offer some benefits as to tailor-made *ready-for-use*/*dilute* eluents, customized derivatization procedures and already optimized gradient elution (e.g., *Waters* AccQ.Tag™ method with AccQ.Fluor™ pre-column AQC derivatization [16]). However, regarding the increasing demand for optimizing method productivity and sample throughput, the use of conventional HPLC (as proposed by the AccQ.Tag™ protocol) may lack some efficiency especially respective faster runtimes (>35 min net separation time for this HPLC method), enhanced resolution or peak capacity.

In an effort to maximize productivity/efficiency of such HPLC methods, one of several approaches made available for the chromatographic community [17], addresses the use of columns packed with sub-2 μm particles allowing to improve overall chromatographic performance and ultimately providing an opportunity for high-throughput separations [18, 19]. Since working with such fine particles is generally associated with higher backpressures (up to 1,000 bar), the term ultra-high pressure (or performance) liquid chromatography (UHPLC or often UPLC™) was accordingly introduced as an equivalent for dedicated LC instruments and applications using sub-2 μm particles. However, aside from such small particles, also the use of other innovative chromatographic supports capable of enhancing separation efficiency (like monolithic column packings or core-shell particles) offers the possibility for more rapid separations, e.g., as in the application for fast amino acid analyses [20, 21].

Highlighting the potential of UHPLC to increase performance, the major objective of the present study was thus to adapt the HPLC-designed *Waters* AccQ.Tag™ amino acid solution onto UHPLC in order to improve runtimes, while retaining the same elution pattern as for the original HPLC protocol. Additionally, detection of AQC amino acid derivatives was to be changed from fluorescence (applied in AccQ.Tag™ method) to more versatile UV detection. To further demonstrate the applicability for food analysis, different commercial cheese samples were to be characterized on their intrinsic FAA levels using the established method.

Experimental

Reagents and standards

Amino acid mix standard (17 hydrolyzate amino acids at 2.5 mM in 0.1 M HCl; L-cystine at 1.25 mM) was obtained from Pierce (Rockford, IL, USA). Additional amino acids L-tryptophan and the internal standard DL-alpha-*n*-amino butyric acid (AABA), both at ≥99 % purity, were purchased from Sigma (St. Louis, MO, USA). AccQ.Tag™ Eluent A concentrate for gradient elution as well as AccQ.Fluor™ pre-column AQC derivatization kit were from Waters (Milford, MA, USA). All additional chemicals and solvents exhibited either analytical or HPLC-grade. Ultrapure water from a SG Ultra Clear UV system (Siemens Water Technologies, Warrendale, PA, USA) was used throughout all experiments.

Amino acid working standards were prepared by merging the mix standard with internal standard solution (AABA at 2.5 mM in 0.1 M HCl) and further dilution in ultrapure water, thus yielding amino acid concentrations ranging from 5 to 80 μM and constant 40 μM for AABA. Since the commercial mix standard is primarily intended for protein hydrolyzate analyses only, tryptophan was additionally integrated within the standard mixes used for UHPLC.

After derivatization (equal to an additional 1:10 dilution), *on-column* amounts for calibration for each of the 18 AQC-derivatized amino acids ranged from 2.5 to 40 pmol per injection for HPLC, and 2 to 32 pmol for UHPLC, respectively (including 20 or 16 pmol per injection for the internal standard AABA, and halved amounts for L-cystine). Linear regressions were calculated using *Waters* Empower 2/Millennium^{32} chromatography software.

Preparation of cheese samples

Commercial cheeses (either as pre-packed slices or already grated) representing typical product groups (ripening protocols) in cheesemaking were purchased at local supermarkets in Vienna, Austria and kept frozen until analysis.

Extraction of FAAs from cheese was performed as described in [13], with some minor modifications. Briefly, 3 g grated cheese sample was suspended in 27 g 0.1 M citrate buffer (pH 2.2), homogenized (7,000 rpm for 2 min) utilizing an Ultra Turrax (IKA, Staufen, Germany) and further stirred at room temperature for 30 min before being filtrated

through a folded filter (Schleicher & Schuell 520 and 595½). One gram of these cheese filtrates was then mixed with 5 g of 3 % (*w*/*v*) 5-sulfosalicylic acid, again incubated at room temperature for another 30 min and finally clarified through a folded filter (Schleicher & Schuell 595½). The acidic cheese extracts were then diluted and neutralized with 0.05 M borate buffer (pH 9.0, dilution 1:10–1:20 depending on the expected FAA concentrations) and directly used for derivatization.

Pre-column AQC derivatization of FAAs was accomplished using *Waters* AccQ.Fluor™ reagent kit. Amino acid standards were derivatized directly by mixing 5 μL with 35 μL AccQ.Fluor™ borate buffer, while for cheese samples, 5 μL neutralized extract was admixed with 5 μL internal standard (AABA at 40 μM) and 30 μL borate buffer. After adding 10 μL derivatizing reagent (~10 mM AccQ.Fluor™ reagent), the mixtures were immediately vortexed, left to rest for 1 min at room temperature and finally heated for 10 min at 55 °C to complete derivatization. Derivatized sample solutions were then filtered through a 0.20 μm syringe filter (Sartorius, Goettingen, Germany) and applied to chromatographic analysis.

HPLC-FL

Chromatographic analyses of AQC amino acid derivatives were performed on a *Waters* HPLC system (Milford, MA, USA) consisting of a model 600E multisolvent delivery system, a Rheodyne 7725i injector, guard column and an AccQ.Tag™ amino acid analysis column (Nova-Pak™ C_{18}, 150 mm×3.9 mm ID, 4 μm). Solvents for gradient elution were mobile phase (A) *Waters* AccQ.Tag™ Eluent A concentrate diluted 1:11 with ultrapure water, (B) HPLC-grade acetonitrile (AcN), and (C) ultrapure water. Column temperature was set to 37 °C, and gradient elution was performed at a flow rate of 1 mL/min according to the *Waters* AccQ.Tag™ protocol [16] for a single-pump gradient delivery system: Initial (A100%/B0%/C0%); 0.5 min (99 %/1 %/0 %); 18.0 min (95 %/5 %/0 %); 19.0 min (91 %/9 %/0 %); 29.5 min (83 %/17 %/0 %); 33 min (0 %/60 %/40 %); 36 min (100 %/0 %/0 %); and further re-equilibration at initial conditions for another 9 min resulting in a total cycle time of 45 min until the next injection. Injection volume was 5 μL, and column eluates were monitored at 395 nm (excitation at 250 nm) using a *Waters* 474 fluorescence detector, while data acquisition was achieved via the *Waters* Millennium[32] software package.

UHPLC-UV

UHPLC separations of AQC-derivatized amino acids were performed on a *Waters* Acquity™ ultra-performance LC (UPLC™) system (including binary solvent and sample manager) equipped with an Acquity™ tunable UV (TUV) detector. Maintaining the eluent system of the AccQ.Tag™ method and 37 °C elution temperature, the original gradient profile (HPLC AccQ.Tag™ for a multi-pump solvent delivery system) was adapted to UHPLC operating an Acquity UPLC™ column (BEH C_{18}, 50 mm×2.1 mm ID, 1.7 μm). Mobile phase (A) was again AccQ.Tag™ Eluent A concentrate diluted 1:11 with ultrapure water while (B) was 60 % (*v*/*v*) AcN. The flow rate was set to 0.4 mL/min, and elution was accomplished as follows: Initial (A100%/B0%); 0.4 min (98 %/2 %); 5.0 min (94 %/6 %); 6.3 min (90 %/10 %); 11.0 min (67 %/33 %); 11.5 min (67 %/33 %); 12.0 min (0 %/100 %); 13.0 min (0 %/100 %); 14.0 min (100 %/0 %); and final re-equilibration at initial conditions (0%B) for another 4 min, thus indicating a net separation time of 12 min and 18 min total cycle time until the next injection. Injection volume was 4 μL and UV detection of AQC amino acid derivatives was set to 254 nm at a sampling rate of 40 points/s. Data acquisition was archived using *Waters* Empower 2 chromatography software package.

Results and discussion

UHPLC analysis of AQC amino acid derivatives

Combining AQC pre-column derivatization with dedicated separation chemistry (*ready-to-dilute* solvents, optimized gradient elution), the *Waters* AccQ.Tag™ method represents a well-established "complete" HPLC solution for (acid-) hydrolyzate amino acid analyses [16]. However, the use of HPLC enables rather long net separations (35 min) only with total cycle times until next injection of more than 45 min (Fig. 1a) which might be considered a drawback, especially when facing a higher number of samples. Hence, the objectives of this study were to adapt this very HPLC separation onto UHPLC in order to increase the method throughput as well as to change detection of AQC derivatives from fluorescence to more versatile (and more available) UV.

Since the original AccQ.Tag™ method also facilitates separations using a multi-pump gradient solvent delivery system (eluent (A) AccQ.Tag™ concentrate; (B) 60 % AcN), the effective segments of this gradient elution (without modifying the separation chemistry itself) were adapted from the former 4 μm (150 mm×3.9 mm) onto a 1.7 μm (50 mm×2.1 mm) UHPLC column. Final flow rate was set to 0.4 mL/min to yield a maximum backpressure of around 600 bar, which is below the pressure threshold of the used UHPLC system (1,000 bar for UPLC™) but may still be within the operable limits of new *state-of-the-art* HPLC instruments.

Compared to the HPLC conditions proposed by AccQ.Tag™ protocol (Fig. 1a), the hereby established UHPLC method demonstrated a significantly increased performance by facilitating separations of all 18 targeted amino acids within 12 min only

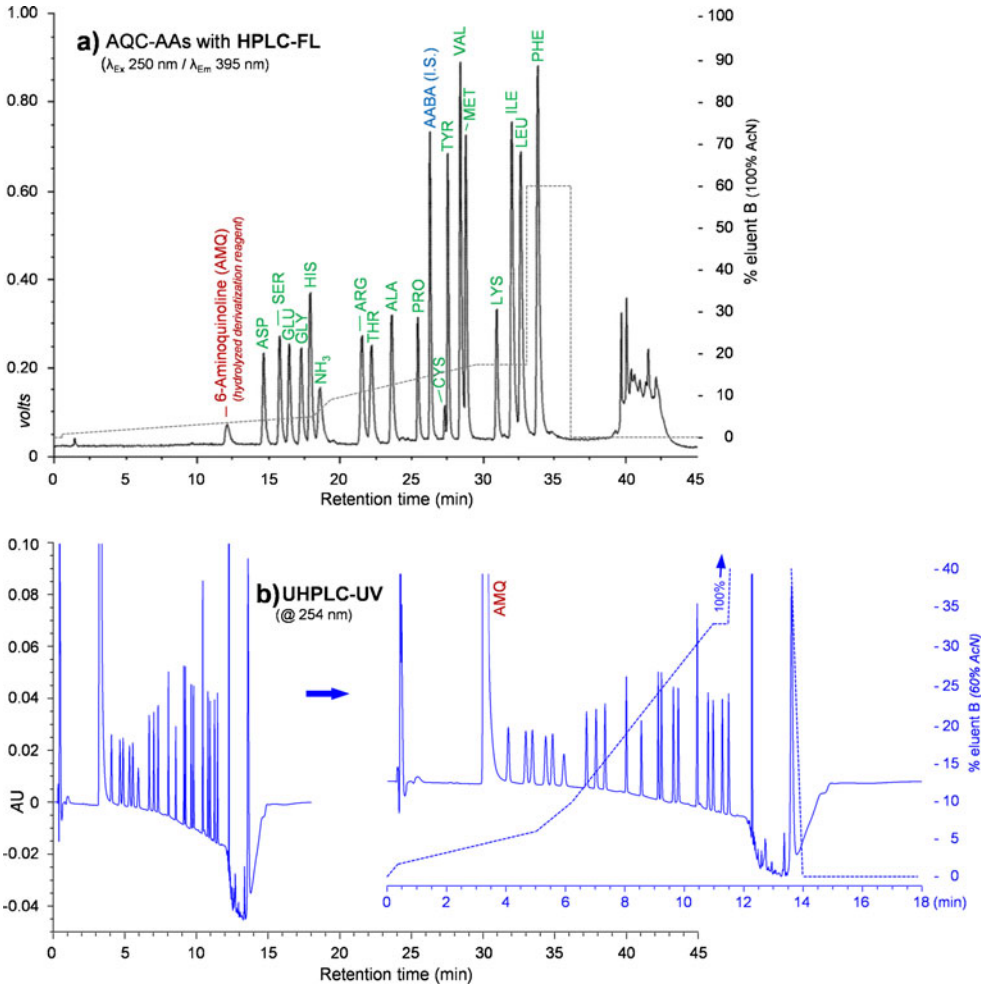

Fig. 1 Side-by-side comparison of HPLC and UHPLC separation performance of 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (*AQC*) derivatized amino acids: **a** HPLC separation according to the *Waters* AccQ.Tag™ method for protein hydrolyzates using a single-pump gradient delivery system, and **b** the adapted UHPLC method with UV detection. Abbreviations: *AMQ* 6-aminoquinoline (derivatization side product), *ASP* aspartic acid, *SER* serine, *GLU* glutamic acid, *GLY* glycine, *HIS* histidine, *ARG* arginine, *THR* threonine, *ALA* alanine, *PRO* proline, *AABA* alpha-amino butyric acid (internal standard), *CYS* cystine, *TYR* tyrosine, *VAL* valine, *MET* methionine, *LYS* lysine, *ILE* isoleucine, *LEU* leucine, *PHE* phenylalanine, *TRP* tryptophan

(Fig. 1b) and a total cycle time of 18 min (including purging the column and re-equilibration). Besides the shorter runtime, the elution pattern of AQC-derivatized amino acids remained the same as for the original separation. Additionally, peak shapes and chromatographic resolution was improved, thus eliminating partly insufficient separations within the former HPLC elution pattern (e.g., valine/methionine, isoleucine/leucine). Moreover, with UHPLC-dedicated peak widths varying from 5–10 s, the estimated theoretical peak capacity for the effective gradient elution (void time until 12 min) ranged up to 130 resolvable peaks per gradient time (68–134 depending on the peak width of the referenced amino acid).

As the adaptation to UHPLC also included the change from fluorescence (λ_{Ex} 250 nm/λ_{Em} 395 nm) to UV (254 nm) detection, the AQC excess reagent had to be taken into account as well. In the course of derivatization, though at a much slower rate, the excess of AQC reagent is hydrolyzed to form 6-aminoquinoline (AMQ), a derivatization side product that shows hardly any fluorescence response indeed (λ_{Em} maximum at 520 nm), but forms a major peak at early retention times when UV at 254 nm is used (Fig. 1a and b) [8, 22]. Applying the UHPLC-adapted gradient profile, the retention of the massive AMQ peak at the onset of the elution window implied, however, only minor interferences with the separation (as to a not fully returned baseline prior to aspartic acid); hence showing no impact on amino acid quantification.

Using their UV response at 254 nm for detection, all analyzed AQC derivatives indicated appropriate linearity ($R^2 \geq 0.999$) within the applied calibration range (2–32 pmol per 4 μL injection volume, respectively). Intra-day instrumentation precision of model mixtures (amino acid standards injected six times within 1 day) varied <1 %, whereas inter-day RSD for multiple sample derivatizations (derivatized six times within 2 weeks) was <4 % for all analytes. Detection (LOD) and quantification limits (LOQ) were calculated at the respective signal-to-noise ratio of 3 and 10, with *on-column* amounts of AQC derivatives ranging between 0.03–0.16 pmol and 0.09–0.54 pmol (each per 4 μL injection volume), respectively. By converting these analytical amounts to milligram FAAs per 100 g cheese, and also including all steps for sample work-up and derivatization, the hereby derived sensitivity (LODs 0.8–3.7 mg/100 g and LOQs 2.7–12.4 mg/100 g) was found to be sufficient for FAA profiling in cheese samples.

Since the used HPLC AccQ.Tag™ protocol was primarily designed for hydrolyzate analyses, and the original separation chemistry of this method was accordingly adapted onto UHPLC, the acid amides asparagine and glutamine (normally converted to their respective acid forms during acid hydrolysis) are not included in the adapted separation; however, they may result in co-elutions when FAAs are analyzed (Fig. 2). Moreover, given that for fermented foods like cheese also reasonable levels of biogenic amines can be anticipated [23] (formed by microbial decarboxylation of precursor FAAs), and since these amines can be derivatized with AQC [24, 25], the separation selectivity was also evaluated for co-elutions regarding major food relevant biogenic amines. Only colamine and histamine were found to elute within the amino acid pattern (as "isolated" peaks between alanine and proline), while tyramine, putrescine, cadaverine, and tryptamine eluted in the cleaning purge after the amino acids (at a higher AcN ratio); hence no interference with amino acid quantification was observed (Fig. 2).

Determination of FAAs in different cheese varieties

During cheese ripening, casein breakdown occurs leading to the accumulation of FAAs in the maturing product. Moreover, applying chemometric methods, these intrinsic amino acid profiles may contribute significant information enabling cheese authentication regarding relevant topics, e.g., cheese type, ripening time, maturity, or micro flora, etc. [26–29]. Using the established UHPLC method, 28 commercial cheese samples representing typical cheese types (e.g., extra-hard, hard, semi-hard, mold-ripened or smear-ripened, and acid curd cheese) were analyzed on their FAA levels. Although the FAA profiles varied to a great extent for the different cheeses (in both total and specific amounts) the obtained chromatograms proved to be of excellent quality enabling interference-free amino acid profiling and quantification throughout all samples irrespective the cheese type or ripening protocol (e.g., propionic acid fermentation, blue-veined or surface-mold ripening, Fig. 3a–c).

Overall repeatability for multiple (n=8) independent preparations of high (Parmesan ~7,000 mg FAAs/100 g) and medium level (Bergkäse ~2,000 mg FAAs/100 g) cheeses was <1 and <3 % for total FAAs, respectively. Additionally, extraction efficiency from the cheese matrix was assessed by re-extracting the residue after sample preparation, and related to the first, the second extract exhibited 6.2 and 7.3 % of total FAAs for medium and high level cheese (at comparable individual FAA ratios); hence extraction was considered suitable for profiling FAAs in cheese.

The individual FAA concentrations of each cheese sample are summarized in Table 1. Regarding the analyzed extra-hard cheeses (Fig. 4), the samples consisted of original Italian Parmesan cheese retailed either as pre-packed slices or as already grated *ready-for-use* convenience products. Due to the prolonged maturation time (Parmigiano Reggiano 20–24 months, Grana Padano 14–16 months), the highest FAA levels were found for "original" Parmesan reaching up to 9 % of the whole weight (e.g., *Virgilio* Parmigiano Reggiano DOP 8,892 mg/100 g; Parmigiano Reggiano 24 months 8,918 mg/100 g). For Grana Padano, total concentrations were lower ranging from 6,000–7,000 mg/100 g. A comparison of commercial Grana Padano samples (retailed either as pre-packed slices or in already grated form, Fig. 4a–b) implied lower amounts for the latter ones, thus highlighting a potential adulteration with cheese rind, or the use of younger cheeses showing a

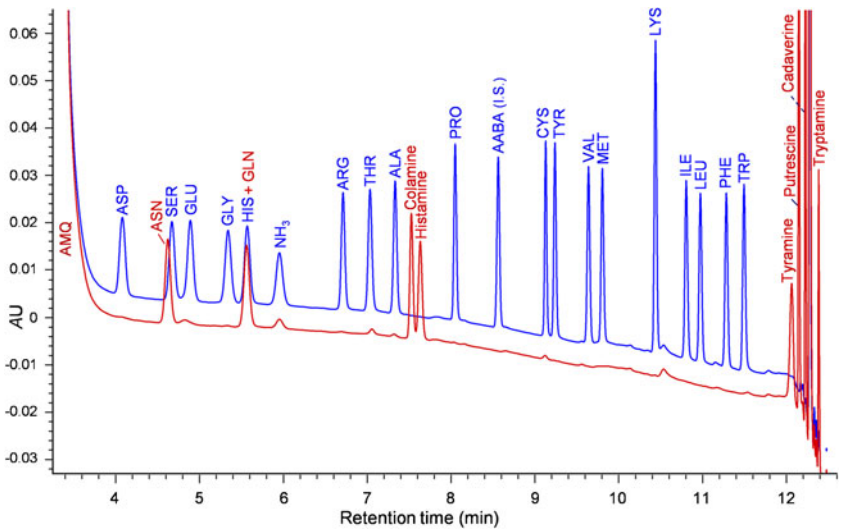

Fig. 2 Evaluation of the established UHPLC separation for co-elutions with the acid amides asparagine (*ASN*) and glutamine (*GLN*), and AQC-derivatized food relevant biogenic amines

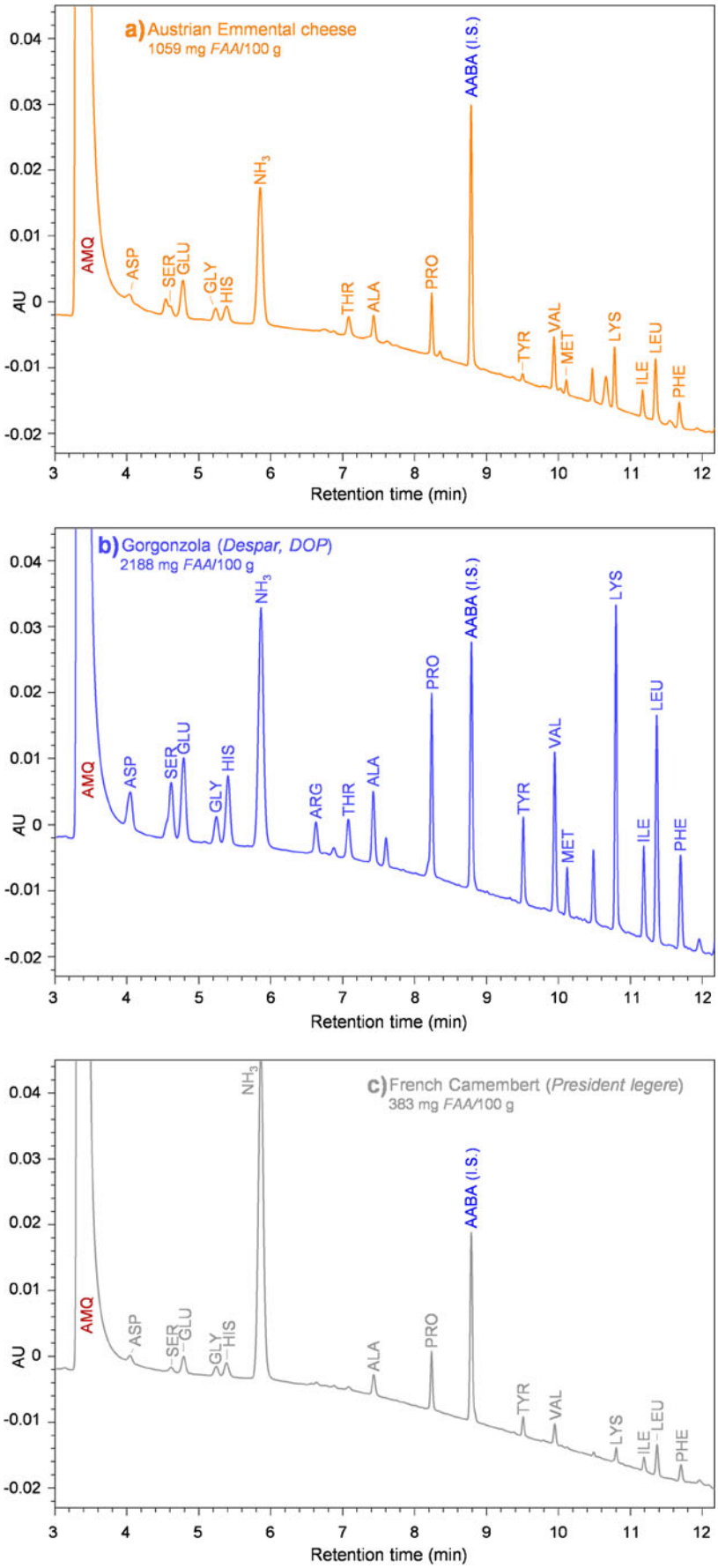

Fig. 3 UHPLC chromatograms of FAA levels of differently ripened cheeses **a** Austrian Emmental cheese—*propionic acid fermentation*, **b** Italian Gorgonzola—*blue-veined ripened*, and **c** French Camembert—*surface mold-ripened*

Table 1 Free amino acid (FAA) contents in commercial cheese samples of different cheese types as retailed in Austria (n=28)

	Free amino acids (mg/100 g)																
Cheese sample description	ASP	SER[a]	GLU	GLY	HIS[a]	ARG	THR	ALA	PRO	TYR	VAL	MET	LYS	ILE	LEU	PHE	Total
Parmesan-type extra-hard cheeses retailed as pre-packed slices (n=7)																	
Virgilio Parmigiano Reggiano DOP	388	599	1633	247	302		284	443	788	274	696	236	1082	595	843	481	8892
S.Paolo Caseifico Parmigiano R. DOP	311	665	1331	216	260		297	394	902	195	578	150	927	440	538	400	7604
Parmigiano Reggiano, 24 months	377	788	1695	235	298	393	357	417	964	74	632	161	1124	456	519	429	8918
Zarpellon Grana Padano Selezionata	195	278	1138	143	315	361	240	340	654	164	471	146	789	368	627	369	6597
Despar Grana Padano DOP	188	272	1101	138	300	354	232	338	630	149	448	134	756	361	606	347	6354
Grana Padano	218	299	1278	209	322		261	402	786	126	559	166	904	474	664	393	7062
Grana Padano, 14 months	212	317	1132	156	300	319	256	372	666	188	511	156	805	416	640	369	6818
Parmesan-type (extra-)hard cheeses retailed in grated form (n=8)																	
Stabiumi Grana Padano	223	293	1057	164	254		180	334	617	162	430	142	700	394	544	316	5808
Despar Grana Padano	216	356	1084	163	276	56	215	370	631	183	468	154	761	416	610	352	6312
Saviola Grana Padano	167	266	687	119	188	54	187	313	499	169	357	122	484	314	469	276	4670
Casa Italiana Grana Padano	196	295	969	145	298		233	346	618	198	460	144	716	379	601	352	5949
Fallini Realparma[b]	89	98	300	46	86		50	35	216	61	164	56	210	120	232	120	1884
Zanetti Parmonia[b]	192	222	620	110	155	68	95	65	415	120	324	115	446	278	439	240	3903
Apesan Italiana Formaggia[b]	72	81	231	43	70		36	25	188	57	143	48	179	103	202	111	1590
Austrian hard cheese	177	246	929	131	569	356	215	78	705	279	468	169	752	349	680	412	6516
Hard cheeses and semi-hard cheeses (n=7)																	
Austrian Emmental cheese	35	18	177	29	98		40	10	119	13	110	38	86	55	150	81	1059
Pecorino Romano DOP	135	169	234	64	200	56	66	57	310	120	264	102	407	231	343	164	2922
Cheddar Rot, Irish hard cheese	23	25	139	28	60		9	7	38	23	62	15	48	10	163	91	739
Moosbacher		110	333	38	174		53	18	136	72	157	59	201	65	301	147	1863
Bergkäse	24	135	445	54	83		64	30	223	38	152	46	252	88	250	122	2005
Schärdinger Bergbaron	13	49	101	35	198		53	14	119	21	141	50	184	73	307	132	1492
Vergeer Kaas, Dutch Gouda cheese	31	32	202	22	107		21	9	60	48	87	27	85	44	213	99	1087
Mold-ripened soft and blue-veined cheeses (n=4)																	
Sirius, Austrian Camembert	24	11	35	4[c]	51		5	4	12	8	14		32	5	17	9	231
President legere, French Camembert	19	5[c]	43	12	49			8	73	36	27		13	17	46	32	383
Despar Gorgonzola DOP	99	100	223	38	204	86	55	16	198	138	171	66	250	109	286	149	2188
Schärdinger Österkron, green-veined	173	116	555	66	248	143	60	17	128	170	197	106	503	173	290	159	3103
Smear-ripened and acid curd cheeses (n=2)																	
Schlierbacher Klosterkäse			52	20	46	11		4	38	39	62	40	123	30	74	78	618
Ölmützer Quargel	19	5[c]	26	12	54			3[c]	59	9	13	7	18	5	13	17	259

[a] Co-elution with the acid amides asparagine and glutamine

[b] "Grated Italian cheese" retailed as substitute for original grated Parmesan; mostly dried/de-hydrated hard cheeses and cheese mixtures

[c] FAA concentration below LOQ

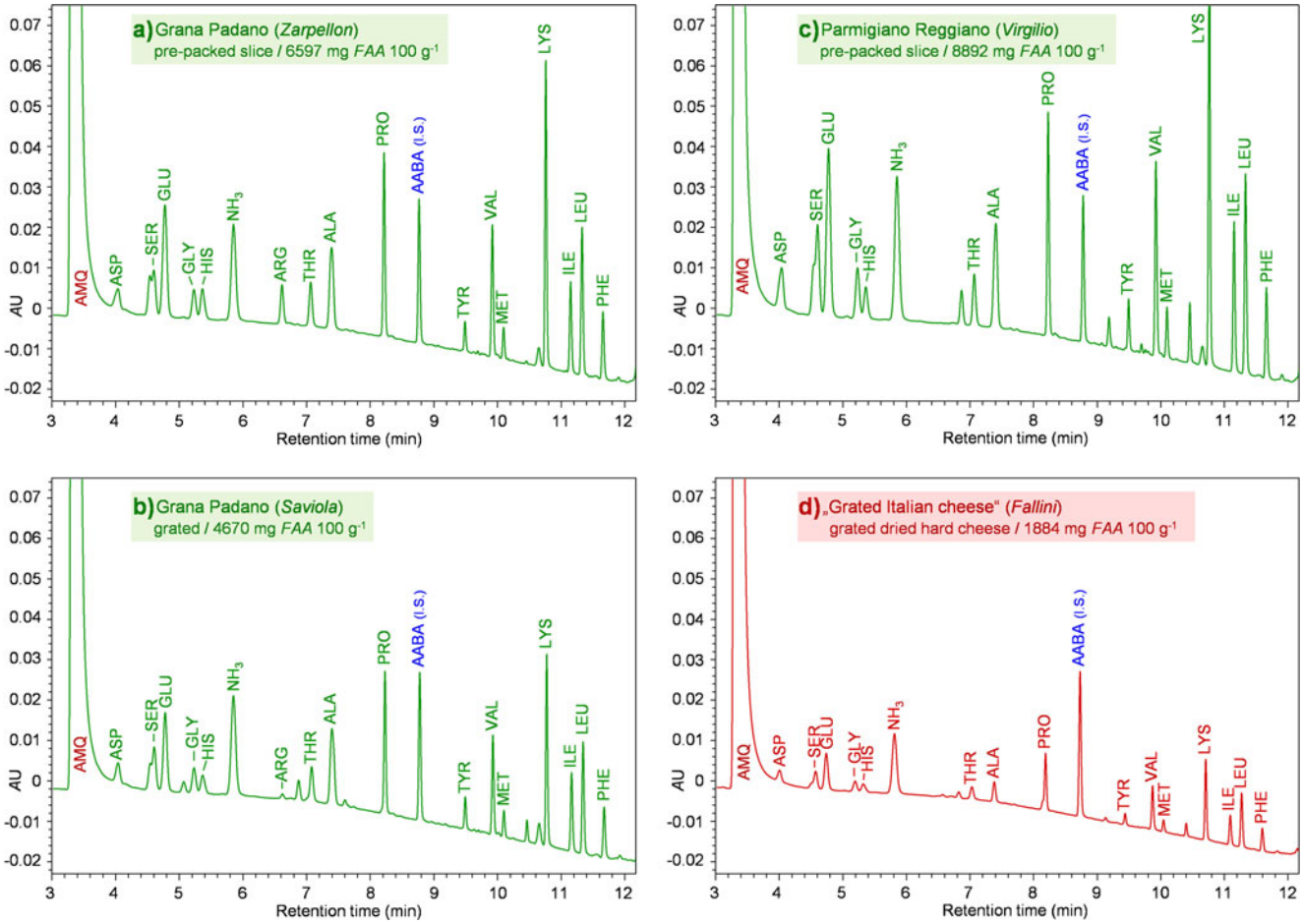

Fig. 4 UHPLC chromatograms of FAA levels of Parmesan-type cheeses: **a** Grana Padano retailed as pre-packed slice, **b** Grana Padano retailed in already grated form, **c** "original" Parmigiano Reggiano, and **d** "grated Italian cheese"

lower degree of proteolysis. However, Parmesan-type samples commonly labeled as "grated Italian cheese" (e.g., *Fallini Realparma*, *Zanetti Parmonia*, *Apesan Italiana Formaggia*) exhibited significantly lower FAA concentrations (e.g., *Fallini Realparma* 1,884 mg/100 g) since they are basically manufactured from dried (hard)cheese mixtures and are therefore most definitely not comparable with "original" Parmesan (Fig. 4c–d).

For the analyzed hard and semi-hard cheeses, total FAAs were found to vary in a rather narrow range between 1,000–2,000 mg/100 g (e.g., Austrian Emmental cheese 1,059 mg/100 g, Fig. 3a). However, considering the differences in cheesemaking (e.g., Dutch-type, Cheddar-type, Swiss-type) as well as the variable extents of ripening protocols, the intrinsic FAA concentrations may indeed differ significantly.

The lowest FAA levels throughout all analyzed samples were detected for surface mold-ripened cheeses (e.g., French Camembert 383 mg/100 g, Fig. 3c). In contrast, the blue-veined, mold-ripened cheeses showed an intensive degree of proteolysis with FAA concentrations ranging up to 3,000 mg/100 g (e.g., Gorgonzola 2,188 mg/100 g, Fig. 3b). For bacterial surface-ripened (smear-ripened) cheeses, low FAA levels were found comparable to those of the analyzed Camembert-type samples.

Conclusions

The AccQ.Tag™ HPLC amino acid solution was adapted to UHPLC conditions, and additionally detection of AQC amino acid derivatives was changed from former fluorescence to UV. The hereby established UHPLC protocol proved to be superior compared to the original HPLC method due to a significantly improved runtime and enhanced overall chromatographic performance. Moreover, this method was successfully applied to monitor FAA concentrations in various commercial cheese samples. Despite the high variability of FAA levels within the different analyzed cheese types, using UHPLC enabled reliable and interference-free FAA profiling throughout all samples, thus demonstrating a valuable tool to generate high quality data for the characterization of proteolysis during cheese ripening.

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